Polyphenol oxidase: Activity, properties of its products and inactivation by innovative technologies

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Doctoral Thesis

Polyphenol oxidase: Activity, properties of its products and inactivation by innovative technologies.

Dissertation presented by Víctor Falguera Pascual, Agricultural Engineer, to fulfill the requirements of the degree of Doctor by the University of Lleida.

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Al poble d'Albatàrrec:
bressol, camí i destí.

Take the best
and make it better.

Barry Mann & Cynthia Weil, 1981.

Abstract.

In recent times, concerns about the impact of the food that people consume on their own health, as well as the social and environmental consequences that it entails, have led to major changes in all steps of the food chain including all the agents from the producer to the retailer. These modifications have made the idea of *food quality* to change, considering new factors and adding new parameters to its classical definition.

Among all these new trends that food industries are being forced to meet, two main streams appear. On the one hand, those aspects related to health: providing health benefits and avoiding potential toxicity. On the other hand, issues regarding organoleptic perception of food. And in the case of fruits, vegetables, mushrooms and their derivatives, polyphenol oxidase (PPO) affects both kinds of matters that are nowadays at the forefront of the *quality* concept.

Indeed, polyphenol oxidase, the main enzyme responsible for enzymatic browning, is still the major problem in fruits postharvest handling, storage and processing. In this Thesis, PPO activity has been characterized in different fruits and situations, and mathematical models to describe melanin formation from monophenolic and *o*-diphenolic substrates have been developed and tested. In addition, according to the abovementioned health concerns, potential toxicity of these melanins on the pancreatic proteases carboxypeptidase A, carboxypeptidase B and trypsin has been studied.

The second part of the Thesis covers non-conventional processing methods for improving fruit derivatives organoleptic and nutritional quality. It is devoted to PPO inactivation by innovative technologies, and the side effects that they cause on different parameters. Thus, ultraviolet-visible irradiation in a plane photoreactor with one single lamp has been modeled, and PPO inactivation by this method has been assessed in model solutions and in apple, pear and grape juices. And going one step beyond, the effects of must irradiation on the quality of wine have also been assessed. Furthermore, in a last different approach to innovative technologies, high-hydrostatic pressure effectiveness in inactivating apple PPO was tested, as well as its effects on juices color.

By covering all the mentioned issues, this Thesis tries to provide a thorough discussion of a subject that is currently essential in new products design and in new processes validation. Different mathematical tools have been applied, including models development, kinetic analysis, classical statistics and multivariate techniques. As a result, different kinds of experiments have been performed and very diverse results are reported, all of them related by the same aim: advance in the knowledge of polyphenol oxidase and its properties.

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Main abbreviations.

CPA	carboxypeptidase A
CPB	carboxypeptidase B
GMO	Genetically Modified Organisms
HACCP	Hazard Analysis and Critical Control Points
NEB	non-enzymatic browning
PCA	Principal Component Analysis
PDO	Protected Designation of Origin
PG	polygalacturonase
PME	pectinmethylesterase
POD	peroxidase
PPO	polyphenol oxidase
PVPP	polyvinylpolypyrrolidone
UV	ultraviolet
vis	visible

Main notation and symbols.

A_λ	absorbance at the λ wavelength
C_A	concentration of A
I	radiation intensity
k_0	zero-order kinetic constant
k_1	first-order kinetic constant
k_2	second-order kinetic constant
k_I	kinetic constant for the first inactivation step
k_{II}	kinetic constant for the second inactivation step
k_a	kinetic constant for the first step of melanogenesis from monophenols
k_b	kinetic constant for the second step of melanogenesis from monophenols
K_M	Michaelis-Menten constant
r	reaction rate
r_{max}	maximum reaction rate
RA	relative activity
W	radiation flux
ε_λ	extinction coefficient

1. Introduction.

1.1. Current trends in food consumption: what do people expect from their food?

The increasing complexity of food chains is associated, at least in industrial societies, with a progressive shift during the past 30 years away from systems that were largely nationally-based, supply-oriented and state-regulated and supported (Lowe *et al*., 2008). The growth of cities was matched by the development of a global food industry, which adopted intensive methods of production in order to meet the increasing demands of both consumers for food and shareholders for profit (McGill, 2009). In this way, the agricultural sector was oriented to optimizing yields and producing products with good appearance and shelf-life. Health and other concerns were not at the forefront in selecting crop varieties and animal breeds or production methods (Traill *et al*., 2008).

In recent times, supply chains have had to become more demand-oriented and food production more responsive to feedback signals from the market and from consumers, who are now regarded as active agents in the food chain (Lowe *et al*., 2008). Growing awareness of the environmental and social costs associated with the provenance and processes of food production is associated with wider changes in modern societies attitudes towards what social scientists term *reflexive consumption* (Giddens, 1991). This concept defines how people think of themselves as active, discerning consumers, whose choices contribute to their sense of identity. This is a well-known statement in current marketing strategies, in which the social or community attitude beyond the individual level must be considered in order to understand consumer behavior (Cova, 1997). Moreover, there is an expanding demand for *natural* (free from chemical fertilizers or pesticides) foods and ingredients that are fresh or minimally processed, readily available, reasonably priced and yet safe for consumption, according to local standards (McGill, 2009).

Thus, nowadays several socio-economic factors have a strong influence on diet composition. Some of these factors are culture, personal preference,

price, availability, convenience and environmental, social or health concerns (Gerbens-Leenes *et al*., 2002; McGill, 2009). People consume not only to fulfill their basic biological needs, but also to express a sense of self and improve psychological well-being. Health and fitness preoccupations, cultural and ethical concerns, worries about animal welfare or political and moral standpoints are good examples of some issues that strongly influence people's behavior towards food selection. People consume images and ideas as well as the basic nutritional content of food (Lowe *et al*., 2008; Casey, 2009). Indeed, the social consequences of consumption are becoming more important every day. In publicity companies, this fact leads to different socially-oriented strategies included the so-called *societing or tribal marketing*: *the link is more important than the thing* (Cova, 1997).

These changes in consumers' demand lead, in the short term, to substantial changes throughout the whole food chain, which have technical, social, economic and environmental implications. Regarding the primary production, for example, it is necessary to predict the impact of changing agricultural practices for biodiversity and landscape quality. In order to assess this impact, it is essential to use an approach that can combine the variation in demand with information on the suitability of agricultural land for different production methods. Thus, while technical advances have greatly expanded the potential to produce nutritious food in an efficient and environmentally sustainable manner, social and economic factors will determine the value of this research, as well as its future direction (Lowe *et al*., 2008; Phillipson & Lowe, 2008; Traill *et al*., 2008).

In order to study the causes and the possible consequences of these changes, they must be considered from different points of view, but integrating these different outlooks. Interdisciplinary research, i.e. technical research embedded in a social, economic and environmental framework, helps to overcome fragmented perspectives and the partiality that can arise when natural or social scientists make naive assumptions about their own field (Cova, 1997; Habib & Lescourret, 1999; Phillipson & Lowe, 2008). The technological solutions derived from this research must be responsive to

consumer demands and sensitive to the social and economic contexts in which they are to be applied (Phillipson & Lowe, 2008). In other words, there is a need to provide integrated solutions for problems that are both social and technical in character (Lowe *et al*., 2008). In fact, in their day-to-day decisions, small-scale farmers already integrate biophysical factors with social, economic, cultural and environmental considerations to manage complex farming systems. This approach contrasts with the traditional organization of knowledge, where reductionist approaches prevail, leading to severe deficiencies in the analysis of complex systems (Temu *et al*., 2010).

The idea of linking these different sciences at a broader scale aims to secure a profitable and internationally competitive food and farming sector that respects the environment and improves nutrition and public health (Traill *et al*., 2008). In addition, it should allow faster and broader responses to these complex problems generated by outstanding changes in the socio-economic context (Habib & Lescourret, 1999). In this background, social sciences can bring different perspectives and methodologies to assist in interpreting uncertainties and divergent views. Moreover, they may be useful in understanding the economic and political factors determining the behavior of complex socio-technical and socio-ecological systems. Many natural scientists and technologists working in agri-food R&D, and indeed many funders and users of technical research, acknowledge the need to understand better the social and economic parameters of their work (Lowe *et al*., 2008).

Consumers' mistrust towards the food chain.

From a general point of view, one of the main reasons of the changes in consumers' demand is the fact that people have more information every day about all of these different knowledge areas. However, sometimes this information may be rather disorienting. A good example of food-related confusing information has to do with the lack of harmonised risk communication in situations of scandals or foodborne crisis (Bánáti, 2011), such as the possible contamination of food after the radioactive fallout in Japan (Moy & Lacroix, 2011) or the case lived in Germany in June 2011 with the pathogenic *Escherichia coli* O104:H4 strain (Warriner, 2011). Sometimes,

these confusing situations are the consequence of the conflict between what Shepherd (2008) defines as *scientific language* and *public language*. Nevertheless, these *languages* are usually less distinct than they are presented, with expert knowledge incorporating implicit judgments and social assumptions and public knowledge using empirical evidence and reasoning.

In spite of the fact that food has never been safer, it seems that consumers are considerably uncertain, anxious and increasingly critical about the safety of food (Bánáti, 2011). During the last two decades there have been widespread food scandals and fears of foods that have focused both consumers and authorities attention to food safety, leading to an increased recognition of the potentially negative effects of scientific and technological developments. As Phillipson & Lowe (2008) stated, *farming crises, chronic health risks, food safety scares and resource and habitat depletion have evoked considerable mistrust of the science and technology underpinning food chains and have been associated with an assertion of consumer/public interest not only in what food is produced but also how it is produced*. In addition, these processing systems used in food chains are often portrayed as out-of-touch with public concerns and driven by narrow disciplinary or commercial logics (Food Ethics Council, 2004). As a result, consumers have become more and more interested in and worried about food technologies and certain food substances, and they have started to fear of foods and of new food technologies (Bánáti, 2011; Rollin *et al*., 2011).

Food scandals have undermined consumers' trust in the safety of agri-food systems. These large-scale scares have tended to arise from contamination of food chains at the primary level, and so have fuelled consumers' concerns over the technological transformation of farming (Lowe *et al*., 2008). In addition, consumers have also been sensitized by the initial mismanagement of some of those crisis, where they were sometimes wrongly reassured that food was safe when it was not (Rollin *et al*., 2011). These problems directed the authorities' attention to the lack of the integrated approach of the food chain (taking into account the points of view of consumers and other

stakeholders) and new principles have been established such as risk analysis and communication or traceability (Shepherd, 2008; Bánáti, 2011).

The worries about food safety are the reason for many of those who, in increasing numbers, are turning to organic food and mistrusting new food technologies such as GMO and irradiation, although several studies have concluded that these new technologies are safe (Ibarz, 2008; Mostafavi *et al*., 2010). According to Rollin *et al*. (2011), the factors that influence consumers' acceptance of food innovations are risk-benefit perceptions, socio-demographic attributes and knowledge and information, as well as the level of trust in the source of that information.

Trends in food selection: towards foods that bring health, social and environmental benefits.

The movement towards a more complex, technology-led, globalized, privately regulated and demand-oriented food system has met resistance from different social movements. These movements include the anti-globalization lobby, environmentalists, farmers, the health lobby, advocates of sustainable development, rural and countryside interests and food campaigners (Lowe *et al*., 2008). Moreover, consumers' emphasis has been attached to non-traditional quality attributes of food (Casey, 2009), being every day more concerned about the impact of their decisions on the environment and social equality. Indeed, quality aspects have become more and more linked to an *added value* basket of indirect and invisible food quality criteria vaguely described as *healthy environment*, *animal welfare* and *fair trade*. These consumer criteria may be traduced, at the professional level, into four kinds of quality (Boller *et al*., 2004): product quality (including food safety), production quality (including ecological criteria and animal welfare), ethical quality and social quality.

The changes in consumers' demand have forced the food industry to create and develop new products and marketing campaigns that are able to fulfill all these requirements. In this way, the variety of necessities and the scope for new ones also opens up possibilities for alternative food networks. Growing

demand in both developed and developing countries for diverse and novel foods is creating new markets for underutilized crops, which are cultivated on a relatively small scale and have traditional uses in local areas (Gancel *et al.*, 2008). These new demands are of special economic interest for tropical countries, where a great variety of fruits are cultivated. Nevertheless, the access for bio-diverse products to the European market needs many requirements, specially the knowledge of their composition and nutritional value. Besides their organoleptic quality, some of their real nutritional and physiological properties remain still undetermined (Mertz *et al.*, 2009). Other tendencies include products engineered to fulfill special dietary needs, fair trade foodstuff, or PDO (Protected Designation of Origin), but perhaps the two kinds of products with the greatest commercial impact are functional foods and organic products.

The main current trends. Example 1: functional foods.

As already mentioned, one of the most important reasons why consumers' demand is changing is their concern about healthy and unhealthy products. In this way, if all consumers were to follow recommended healthy eating guidelines there would be major implications for food consumption, land use and international trade. According to Traill *et al*. (2008) this is unlikely to happen, at least in the short term, but it is realistic to anticipate some dietary adjustment towards the recommendations, resulting in an improvement in diet quality. Although consumers are reluctant to make major changes to their diets, they may be prepared to substitute existing foods for healthier alternatives. And functional foods claim to be these alternatives.

The changes in food products to improve their composition in order to make them healthier would bring them into the category of functional food, which Diplock *et al*. (1999) define as food that improves health or wellbeing, or reduces disease risk, through beneficially targeting the body's functions. A functional food is similar in appearance to, or may be, a conventional food; is consumed as part of a usual diet and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions (Walji & Boon, 2008).

From the food industry point of view, functional foods have appeared as a guarantee against a world of saturated demand and increasing global competition (Grunert, 2010). They constitute the perfect marketing strategy by creating differentiated, value-added products, appealing to health (a basic and universal human need) and directed to a premium-price sector. However, many of those innovative products have failed, probably due to a lack of knowledge management between the functional disciplines involved in the new product development process, especially consumers' attitudes knowledge (Jousse, 2008; Betoret *et al*., 2011). Particularly, people in Europe are more reluctant in adopting functional foods than in Asia or North America (Siro *et al*., 2008; Grunert, 2010). As an example, the last survey of the Spanish government revealed that consumers attach more importance to the origin of the product (i.e. those produced in their own region, or with Protected Denomination of Origin) than functionality (OCDA, 2011).

Although functional foods are currently in fashion, the majority of people are unsure of their benefits. Actually, a positive or negative perception of a functional food product is based strongly on the nutrient content of the base product and less so on its health claim (Traill *et al*., 2008). In this way, consumers tend to prefer food products that bring a simple but clear health benefit (Bitzios *et al*., 2011), and even those that are more concerned about health issues perceive products that are intrinsically healthy (such as yogurt, cereals and juice) as preferable and credible carriers of functionality (Annunziata & Vecchio, 2011). In addition, there are strong differences between different population groups. For example, women, people with higher income and married couples have a more positive view of functional food (Verbeke, 2006; Traill *et al*., 2008; Markovina *et al*., 2011). As Barrena & Sánchez (2010) discussed, household structure (e.g. whether there are children) has also a definitive impact on the cognitive process that results in purchase decision. Some studies also show that functional foods are even less accepted if the functionality is achieved by means of genetic modification (Frewer, 2003).

For most consumers, the most important functional food attributes are taste, price/quality ratio and, in third place, functionality (Markovina *et al*., 2011). Indeed, organoleptic features are still at the forefront of most consumers' preferences, being more important than potential health benefits (Verbeke, 2005 & 2006; Urala & Lähteenmäki, 2007; Annunziata & Vecchio, 2011).

As far as economic issues are concerned, developing a new functional food is an expensive process, which requires detailed knowledge of the product to be created or modified and of the consumers to whom it is addressed (Betoret *et al*., 2011). The current difference in price between conventional and functional food is often too large for consumers to change their shopping habits (Asselin, 2005; OCDA, 2011), although they are willing to pay a small amount extra (Traill *et al*., 2008). Even so, it is not clear if this amount is enough to cover the increase in production costs of some functional foods compared with their traditional counterparts. In this way, consumers acknowledge that functional foods should be more expensive than conventional ones because they provide additional benefits, but in most cases they find this difference to be excessive (OCDA, 2011).

The main current trends. Example 2: organic products.

Besides functional foods, another important trend in food consumption is focused on organic products. Both tendencies have in common its main motivation: consuming foods that may provide health benefits. Organic products, though, have been claimed to be also more respectful to the environment than their conventional counterparts. Increasing uses of pesticides and fertilizers have spawned criticism in popular publications, and have led to a growth of organic farming alternative (McGill, 2009; Sheng *et al*., 2009). Concerns focused initially on chemical pesticides, but widened in the 1970s and 1980s to cover the intensification of agricultural production and its consequences for farmland ecology, agricultural pollution and landscape change. Environmentalists have sought to encourage consumers to buy 'environmentally friendly' products, which is known as *green marketing* (Lowe *et al*., 2008). Indeed, consumers perceive foods labeled as *organic* to be healthier (Sheng *et al*., 2009). However, in recent times the profit interests

have undermined the use of the adjective *natural*, as well as the term *organic*, leading consumers to a generalized mistrust understanding that it does not necessarily mean *safe* (Carvalho & Luz, 2011).

On the other hand, objective analyses of environmental benefits of organic farming are not so clear. In this way, while there is evidence that smaller-scale production is more efficient in terms of energy use, it generally involves lower productivity than either large-scale agriculture or non-farm work (Bravin *et al*., 2010; Woodhouse, 2010). Thus, since the yields obtained by organic farming are lower compared with conventional farming, the overall environmental benefits are strongly reduced or even disappear after correcting for these lower produced quantities per hectare (Backer *et al*., 2009; Woodhouse, 2010). Therefore, more research should be done on how the yields in organic farming can be substantially increased without increasing the environmental burden to give objective sense to environmentalists' motives.

Table 1.1.1. *Average prices of organic and conventional food products in the USA.*

Product	Source	Unit	Price ($US)	
			Conventional	Organic
Milk	Retail	Half gallon	2.28	4.43
Eggs	Retail	Dozen	1.35	3.99
Rice	Retail	Pound	0.93	2.35
Carrots	Retail	Pound	1.59	2.21
Salad mix	Retail	Pound	3.85	8.14
Spinach	Retail	Pound	4.45	8.59
Strawberries	Retail	Pound	3.23	5.14
Poultry	First receiver	Pound	0.80	2.37
Broccoli	Wholesale market	16 count bunches	14.06	32.30
Apple	Wholesale market	Carton tray pack	32.61	45.89
Banana	Wholesale market	40 pounds carton	19.57	24.48
Orange	Wholesale market	7/10 bushel cartons	13.87	27.24
Pear	Wholesale market	4/5 bushel cartons	30.13	47.08
Raspberry	Wholesale market	12 6-oz cups	20.75	24.03
Sweet potato	Wholesale market	40 pounds carton	23.57	36.54
Pea	Wholesale market	10 pounds carton	17.32	43.75
Tomato	Wholesale market	10 pounds carton	13.92	24.81

Source: USDA-ERS (2009).

In addition to environmental aspects, price and organoleptic features of organic products must also be considered. In a similar way to what occurs with functional foods, several studies have concluded that consumers would not

sacrifice organoleptic properties for potential health benefits (Traill *et al*., 2008; Markovina *et al*., 2011). Furthermore, consumers are also willing to pay only a small amount extra for products with the *organic* label, and there is no evidence showing that this amount is enough to cover the decrease in productivity, mainly due to the perception of overpricing (Ferjani *et al*., 2011; Mesias-Diaz *et al*., 2011). Indeed, as shown in Table 1.1.1 (USA data), there is a big difference between the prices of both kinds of products at the retailer level. In some cases, such as poultry, this difference may represent nearly a 200% of increase. On the contrary, overpricing is less important in imported foodstuff: organic bananas were only 25% more expensive than conventional ones.

Table 1.1.2. *Product share and value of the organic market in the UK.*

Product	Share (%)	Value (thousand £)	Change 2010/2009 (%)
Dairy	30.5	528	-2.7
Fruit, vegetables and salad	23.2	402	-6.3
Baby food	7.8	135	+10.3
Beverages	7.5	130	-3.2
Fresh meat	4.8	83	-5.8
Confectionery	3.6	62	-8.3
Eggs	2.9	50	-9.4
Chilled convenience foods	2.2	38	-36.0
Fresh poultry	2.0	35	-13.2
Breakfast cereals	1.8	31	-15.2
Bread	1.2	21	-20.6
Fresh fish	0.7	12	-16.0
Other	11.8	204	-13.9

Source: Soil Association (2011).

Moreover, it must be considered that since 2009 the economic recession has strongly affected the demand for organic products, for example in the United Kingdom, where it declined by 13.6% in 2010 and by 5.9% in 2011 (European Commission, 2010; Soil Association, 2011). In this country, the most affected products were chilled convenience foods with a decrease of 36% in 2011, followed by bread (-20.6%), fresh fish (-16.0%) and breakfast cereals (-15.2%) (Table 1.1.2). Meanwhile, organic baby foods are the only exception to this downward trend, growing by 10.3%. This different tendency may be explained by the fact that most consumers think that organic products *may be safer*, but they are only willing to pay for them if these foods are for the children (OCDA, 2011). In this context, experts recommend adjusting the prices of agricultural products in order to ensure that the costs of production are suitable for both

the producers and the great mass of consumers, but taking the minimum possible impact on public finances (Simtion & Luca, 2010).

Implications for rural environment: changes in agricultural practices.

Diet composition can have implications for land use and rural environment of a similar order of magnitude as those related to population growth or changes in agricultural productivity (Gerbens-Leenes *et al*., 2002). Agricultural policy liberalization, concern about unhealthy diets and growing recognition of the importance of sustainable land use have fostered interest in the development of competitive food chains based around products that are beneficial to the rural environment (Traill *et al*., 2008). However, the new trends in consumers' demand are having a global impact on trade and can produce serious negative effects on less developed countries and their basic agriculture (McGill, 2009). In addition, conventional farmers in developed countries may also be affected, since they have to cope with the changing institutional environment created by international governmental and non-governmental organizations. Consequently, these implications for rural environment may modify the old agricultural models, the standards of good practices, traditional crop varieties and the entire agricultural production systems.

These recent changes inevitably lead to the need for an integrated consideration of primary production (far from classical isolated points of view), which may be easily seen in agricultural production models. Such models have been traditionally developed considering this isolated sector, in order to predict changes in agricultural inputs, outputs and prices across continental, national or regional scale. However, these classic models have certain limitations, such as the inability to provide spatially explicit predictions of land use change, but rather probabilities of particular changes in the regional or national distribution of different types of agriculture (Traill *et al*., 2008). Furthermore, modern food chains have several agents that were not considered by those classical models, such as the large amount of intermediaries or the consumers' behavior caused by *reflexive consumption*.

The standards of quality, safety and good practice are likely to vary greatly as the distance between producer and supplier, both geographically and culturally, grows. The demand for safe, fresh, *natural* food is beginning to rely on imports. In most cases, it may paradoxically imply an increase in risks for consumers, since increasing sales demand an expanding range of suppliers and an expanding supply chain, which may be traduced in a relaxed risk assessment towards imported products (McGill, 2009). This situation is also unfair for farmers in developed countries, who in most cases have frequently been paid a minute fraction of the market value of their products. As far as new crop varieties are concerned, advances in biological technologies have opened up a multitude of new substitution possibilities and eroded the dependence of food production on specific geographical areas. Supply chains have become more extended and complex, and its intermediaries have taken on a more prominent role between producers and consumers (Lowe *et al*., 2008).

In fact, the established pattern of *industrial agriculture* in developed countries is in crisis, mainly due to environmental impacts and the rising cost of energy inputs. Thus, alternatives to industrial agriculture (i.e. organic producers) need to recognize more explicitly the need for cross-subsidy of food producers. Otherwise, it will be very difficult for organic-production farms to survive, even more than conventional ones. Indeed, in its last report about the situation of the organic sector, the European Commission (2010) acknowledges that organic farms receive on average higher subsidies in absolute terms ad per hectare than conventional farms.

It seems clear that a balance point must be found between conventional agricultural production systems and organic ones. These balanced practices should ensure a reasonable productivity, but according to current environmental and social concerns and, if possible, minimizing the subsidies that farmers need to receive from public administrations. This middle point seems to have been met by integrated production methods. The integrated production system looks for long-term sustainability, the rational use of natural resources, the application of regulations to ensure the substitution of pollutant

agents, the use of a combination of biological and chemical control methods, the employment of adequate monitoring methodologies and the traceability of the whole process, without sacrificing crop quality and productivity (Chandler *et al.*, 2008; Almeida *et al.*, 2009). Emphasis is placed on a holistic systems approach, involving the entire farm as the basic unit (Boller *et al.*, 2004). Such an approach also meets the integrated consideration of primary production that was not possible with classical agricultural production models. In addition, integrated management also takes advantage of modern technology to improve on the system, resulting in better quality of soil, water and air (European Commission, 2002). Boller *et al.* (2004) carried out a thorough review of integrated production systems principles and technical guidelines.

Indeed, integrated production systems are the scientific answer to combine intensification in farming and low environmental impact practices, meeting market requirements along with economic objectives (Habib & Lescourret, 1999). For consumers, this philosophy would be traduced as providing healthy and environmentally-friendly foodstuff at an affordable price. For the food industry, it would represent meeting the four kinds of quality described by Boller *et al.* (2004) that have been previously mentioned. All aspects of production are formalized in integrated production schedules, recognized by the relevant regional or national authorities and overseen by accredited independent bodies (Patrie, 2006). In Europe, farmers in most countries apply integrated production methods, but in an unharmonized manner due to the absence of a common legal base. This situation has lead to a proliferation of private quality management systems and private standards (Wiegand *et al.*, 2008), which will be discussed later.

Regarding research about integrated production systems, the bulk of scientific reports concerns the independent effects of various factors on crop performance, such as irrigation, fertilization, soil management, growth regulators, planting density, crop protection, and so on (Habib & Lescourret, 1999). Consequently, there is a need for considering all these production inputs together in order to find appropriate ways to combine them in a more efficient way. Due to the heterogeneity of these data, including these inputs,

different crop varieties and the particular properties of each geographic location, the pursuit of these models constitutes a challenging issue for researchers all over the world.

Implications for rural environment: changes in rural population.

Concerning rural population, the traditional small and middle-sized farms scheme in Europe is very likely to change. The globalization of markets has modified the traditional networks of information, production and marketing, which in most developed countries had been largely established and maintained by comprehensive governmental support policies. New institutions have come into the picture establishing the links between small producers and larger markets. Prices and demand patterns fluctuate widely, leaving small producers vulnerable to market forces and raising the level of economic insecurity (Casey, 2009; Keyder & Yenal, 2011). This situation brings about a rapid de-ruralization of the population in most developed countries. Consequently, farmers seek for seasonal employment in other fields, diversifying their economic activity and permitting the rural population to remain in the countryside.

Looking forward, the sustainable development of rural territories and the conservation of small and medium-sized farms network in Europe implies stabilizing populations in rural areas. This should be achieved by raising their living standards, improving the efficiency of the rural economy and ensuring rational utilization of natural resources (Atyukova, 2009). Specific issues concerning this development must include opportunities for development of local authority structures, diversification of agricultural production, adoption of alternative forms of activity in rural areas and the development of rural (and agricultural) institutional activity, providing information and other support. Hence, as Fischer & O'Neill (2005) stated, decisions at the farm level will be strongly affected by off-farm income earning opportunities. This indicates that cross-sectoral linkages and spatially explicit contexts should be considered when modeling and predicting production, consumption and investment decisions of rural households.

Public and private standards and regulatory aspects as a response to changes in the agri-food system.

In traditional societies, if shared resources seemed to be under threat, users often agreed on rules for their management. But in modern societies, the affected people are often unable to take direct action, and must channel their demands through the political and legal systems (Harrison & Pearce, 2000). Food scares and scandals initiated different changes in the European food policy and food legislation, which have substantially been changed in the last decade.

In addition, both public and private standards have also had to be reworked in order to integrate the new properties of today's food-related issues: new trends in food consumption, new processing technologies, rising global trade in food and agricultural products, growing economic concentration, the merging of food and pharmacy, chronic obesity in the midst of hunger and new disease and pest vectors (Busch, 2003; Busch & Bingen, 2006). Several new principles such as risk analysis, traceability and an integrated food chain have been introduced, resulting in a paradigm change in Europe. A risk analysis structure had been built up consisting of science-based risk management, assessment and communication, with the aim of improving food safety in the EU, ensuring a high level of consumer protection and restoring and maintaining confidence in the EU food supply system (Bánáti, 2011). The first important step of this program was the creation of the European Food Safety Authority (EFSA) in 2002. After that, an entire new raft of legislation known as the *hygiene package* was created to merge, harmonize and simplify the very detailed and complex hygiene requirements, which were previously scattered over 17 EU Directives (Rollin *et al*., 2011).

The need for new integrated approaches to minimize food risks has led to several consequences. Food companies must ensure full traceability of food chains, which is known as *farm-to-fork* procedures. From the industry managers' viewpoint, the main reasons that have been reported to be motivating for HACCP system (Hazard Analysis and Critical Control Points) implementation are the likelihood of future regulation, the marketing value of

HACCP certification, the improvement of training schemes for standard operation procedures and the value of HACCP in avoiding potential litigation (Wilcock *et al*., 2011).

Legislations adopted to improve food safety include public and private standards regarding the characteristics of the final product, production practices, traceability and the legal liability of the supply chain. While public standards are mandatory, private ones may confer competitive advantage due to improved control and increased efficiency. These requirements have forced food industries to adapt their infrastructure, equipment, modes of production, processing and quality management and coordination with other stakeholders (Casey, 2009; Hammoudi *et al*., 2009).

But perhaps one of the most important consequences for primary producers has been the proliferation of private standards. These quality guides often stipulate more stringent demands than required by law, setting and monitoring of suppliers, and the advent of such social technologies as supply chain management, risk assessment and logistics (Lowe *et al*., 2008). GlobalGAP is a retailer-led private sector body that sets Good Agricultural Practice (G.A.P.) standards for agricultural products that was originally established as EurepGAP in 1997. GlobalGAP is the most prominent example of the private governance of food safety and quality and represents a critical case in the study of governance beyond the state and even beyond the structures established by the WTO (Busch & Bain, 2004; Henson, 2008; Casey, 2009). GlobalGAP exerts an enormous influence and control over the European food market, as over 85% of all Western European retailers require this certification (Casey, 2009). Other examples of leading private standard certifications include Tesco's *Nature's Choice*, the Global Food Safety Initiative (GFSI, CIES Business Forum), Walmart's *Ethical Standards Program*, the animal welfare standard established and certified by the Society for the Prevention of Cruelty to Animals (SPCA) in Canada or the business-to-business standards of the International Organization for Standarization (ISO).

The rise in these private standards has resulted in a shift in responsibility for this task to third-party certifiers (TPC). This development is reconfiguring social, political, and economic relations throughout the agents of the agrifood chain, reflecting the growing power of supermarkets to regulate the global system (Hatanaka *et al*., 2005; Hatanaka & Busch, 2008). Hobbs (2010) carried out a thorough review about the specifications and potential consequences of all these different private certifications.

Both public and private standards are not only focused on consumers' safety, but also try to embrace ethical and environmental concerns. In this way, public regulations try to protect small producers and low-intensity farming. Small and medium-sized producers and manufacturers may find it more difficult to comply with the product licensing requirements due to their lack of resources associated with the limited number of employees and small facilities (Lowe *et al*., 2008; Walji & Boon, 2008). Thus, farmers that obtain low-productivity crops, such as those dedicated to organic farming (Backer *et al*., 2009; Reddy, 2010; Woodhouse, 2010), must be necessarily protected by legislation. Public subsidies are often aimed at the promotion of rural areas with lower productivity (Adamczyk-ojewska, 2004), especially those with higher environmental and landscape value (Torquati *et al*., 2006). Most of these low-productivity farms, including organic ones (Nieberg *et al*., 2005; Lakner, 2009; Daugbjerg *et al*., 2011) would not survive without this help (Kitsopanidis, 2002; Reddy, 2010; Breustedt *et al*., 2011; Graaff *et al*., 2011). In some cases, these direct payments for organic production compose up to 20% of the final gross production of farm and about 57% of farm profit (Jonczyk & Kopinski, 2009).

As a consequence, public subsidies and public and private standards affect competition, since they are highly relevant in the trade context, often acting as international trade barriers (Henson, 2008; Hammoudi *et al*., 2009; Hobbs, 2010). Analyzing their effects requires the perspectives of both public economics and social choice (provision of a socially desirable level of food) and industrial economics (cost efficiency and competition). The increased use of private standards, and especially collective private standards, has fuelled an ongoing debate on the substitutability/complementarity of private and public

standards. This is an important and complex issue that needs to be analyzed considering both the functioning of markets and safety in the final market, as well as their social and ethical framework. This issue is especially pertinent in the international trade context, as the effects of market distortions resulting from standards may be most severe for primary producers (Busch, 2002; Hammoudi *et al.*, 2009).

From the consumers' point of view, one suggested benefit of the new regulations is that they will be more assured of good quality products and the new product labels will contain more information to allow consumers to make more informed product choices. However, this large amount of information may usually increase consumers' confusion, especially when it is related to health claims. A good example of this situation can be found in the case of natural health products (NHPs). NHPs might be manufactured from plant-based materials or other ingredients also commonly found in foods. The current regulation from Health Canada states that this concept includes *vitamins and minerals; herbal remedies; homeopathic medicines; traditional medicines such as traditional Chinese medicines; probiotics, and other products like amino acids and essential fatty acids* (Yada, 2010). However, the distinctions and interpretations of what is to be classified as a NHP or as a food are often confusing and unclear (Walji & Boon, 2008).

To solve this problem, regulations on health claims are becoming more rigid every day. New laws are being designed to protect consumers from misleading and false marketing campaigns, and also to protect disruption of innovation (Puspa & Kuhl, 2009). Health claims used in functional foods commercialization must be supported by strong scientific evidence. In Europe, health claims process guidelines are described in the *European Regulation 1924/2006 on Nutrition and Health Claims made on Foods*, in effect since January 2007 (Walter, 2008; Grunert *et al*., 2011). On the other hand, organic foods production and labeling practices are described by the *Council Regulation (EC) 834/2007* and *the Commission Regulation (EC) 889/2008* (Signorini *et al*., 2008).

As a result, it can be stated that public and private standards may have either positive or negative outcomes. Due to the number of roles that they play throughout the agri-food chain, these outcomes include a wide range of issues such as economic, social, environmental, technological and ethical ones (Busch & Bingen, 2006). For this reason, there are several kinds of organizations that develop, modify and enforce standards, some of which may be more restrictive than others (Busch, 2002).

Perspectives in the near future: how to deal with these new trends?

To sum up, it can be stated that agri-food systems comprise complex technologic, social, economic and environmental linkages that require integrated research approaches. In recent times, consumers' concerns about the social and environmental consequences of their consumption attitudes, as well as the growing preoccupations about living healthier lifestyles, have led to severe changes throughout the whole food chain, including all the agents from the producer to the retailer.

Consequently, food supply chains have had to give response to the feedback signals of market trends. Among those trends, functional and organic products are currently two of the most in-fashion ones, involving strong modifications in the primary production, food processing industry and food-related scientific research. However, there is some evidence pointing to the fact that those trends are the result of successful socially-oriented marketing campaigns, rather than the consequence of a deep and science-based meditation. In addition, the difference in price between conventional and functional/organic food is often too large for consumers to change their shopping habits, and objective and productivity-corrected analyses of environmental benefits of organic farming are not clear. Moreover, since 2009 the economic recession seems to have affected strongly the demand for these trendy products, which are considerably more expensive than their conventional counterparts.

Furthermore, those changes in consumers' demand are leading to important consequences not only in different kinds of food production, but also in rural environment and population. Land use change plays a key role in global

environmental change, as well as in the future availability of water and other natural resources. Modeling those changes in land use presents several challenges, particularly in integrating biophysical and socioeconomic data and the heterogeneity that these data entail. The success on these challenges will only be possible if future research approaches consider the different aspects such as technical, social, environmental and economic ones as a whole thing, in a similar way to how integrated production systems have met the balance point to optimize food production in farms.

1.2. Polyphenol oxidase: functions, properties and control of its activity.

As it has been introduced in the previous section, consumers' demand has been progressively oriented towards new food products and processing methods that provide, among others, fresh-like quality attributes such as flavor, texture, color, vitamin content, aroma and overall appearance. These attributes, as well as product shelf-life, are closely related to microbial quality and to biochemical and enzymatic reactions (Campus, 2010; Landl *et al*., 2010). In this way, enzymatic browning is considered one of the main causes of post-harvest fruit deterioration, along with pigment degradation and peroxidase activity. In fact, polyphenol oxidase activity, which is the main protein responsible for enzymatic browning, is still the major practical limitation for fruit handling, storage and processing (Jiang *et al.*, 2004). In addition, browning processes affect the nutritional quality and appearance, reduce consumer's acceptability and cause a significant economic impact, both to food producers and to food processing industry.

Polyphenol oxidase (PPO, E.C. 1.14.18.1) is a copper-containing enzyme, widely distributed philogenetically from bacteria to mammals, which catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of a heterogeneous group of melanins (Duckworth & Coleman, 1970; Ziyan & Pekyardimci, 2003; Muñoz-Muñoz *et al.*, 2007). As an example, melanogenesis from L-tyrosine is shown in Figure 1.2.1. PPO converts this monophenol firstly to L-DOPA (an *o*-diphenol) and this to *o*-dopaquinone, which is spontaneouslly cyclated in form of leukodopachrome and quickly converted to dopachrome, which polymerizes forming *melanins* (De Faria *et al.*, 2007). This enzyme can also directly act on any *o*-diphenolic substrate conducting only the catecholase activity, from which the other changes and polimerizations will occur spontaneously (Madani *et al.*,

1999). In this case, there is not a first stage consisting in the synthesis of an *o*-diphenol from a monophenol (cresolase activity), so there is not an enzymatic reaction that depends on the reaction rate of another one. This fact is essential to understand some properties of the kinetic behavior of PPO, whose knowledge is fundamental to easily predict the quantity and properties of the melanin that can be synthesized, as well as the influence of the parameters that can modify the reaction such as the pH or the nature and concentration of the substrate. Currently, there is not any suitable kinetic model in the literature that can easily fulfill these functions, with practical parameters that give useful information to advance in understanding polyphenol oxidase mechanisms. The wide range of polyphenol oxidase substrate specificity has led to many methods being proposed to measure its activity: radiometric, electrometric, chronometric and especially spectrophotometric, which are fast and affordable by most laboratories (García-Molina *et al.*, *2007*).

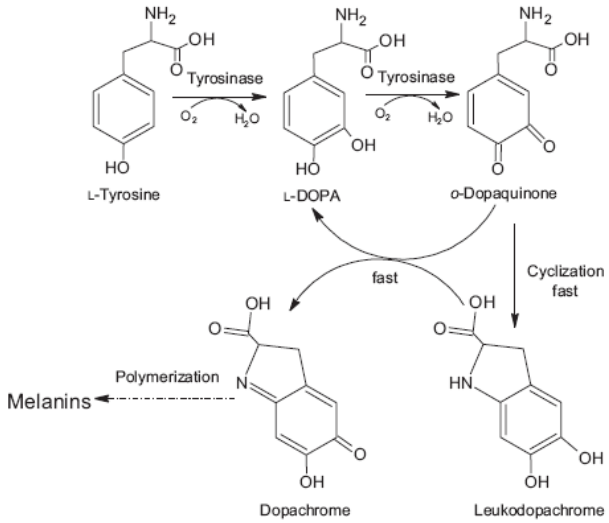

Figure 1.2.1. *Mechanism by which polyphenol oxidase (tyrosinase) converts L-tyrosine firstly to L-DOPA and then to o-dopaquinone, and the following spontaneous steps that lead to melanin synthesis (De Faria et al., 2007).*

Besides melanogenesis, plant PPOs have been reported to intervene in other physiological processes such as oxygen scavenging and defense mechanisms against plant pathogens and herbivore insects. Since these mechanisms act via some products of PPO activity, they will be discussed in the next section.

As mentioned, PPO action mechanism is based on its capacity to oxidize phenolic compounds. In the fruits cells, the enzyme is located inside plastids while potential substrates are stored in vacuoles, and this separation in different organelles limits enzymatic browning. In post-harvest evolution, fruits undergo some physiological changes that lead to a decreased ability to eliminate active oxygen. Thus, membranes become more affected by oxidative activity, which results in enhanced lipid peroxidation, reduced membrane fluidity and increased membrane permeability. After these transformations, or when the tissue is damaged, the rupture of plastids leads to the enzyme coming into contact with these phenolic compounds (Mayer & Harel, 1979; Jiang *et al.*, 2004).

There are several factors that may have an effect on PPO activity in fruits. Among them, fertilization and pesticide application on trees have been proved to have a definitive effect on the ripening process and phenolic expression (Carbonaro & Mattera, 2001; Nicolas *et al.*, 1994). In particular, nitrogen application has been related to maturity and quality factors: adding excessive amounts causes delays in maturation and unbalanced nutrition in calcareous soils, while insufficient quantities reduce tree growth and harvest yields (Falguera *et al.*, 2012; Rufat *et al.*, 2011). However, there is no information in literature about the direct impact of nitrogen fertilization on PPO activity in peach fruits, since all the previous studies refer to its impact on fruit ripening.

The different methods to control PPO activity.

The mechanisms by which enzymatic browning is controlled may be summarized in three main groups:

a) Direct inhibition or inactivation of PPO. The different mechanisms to inhibit PPO activity should act on one or more of the essential components necessary for the reaction: enzyme, oxygen, copper or substrate (Queiroz *et al.*, 2008). Chelating agents, for example, can react with the prosthetic group making it unavailable for the enzyme to accomplish its activity (Pilizota & Subaric, 1998). Lowering the pH of foods below 3 also prevents products from browning, but it also affects its flavor. PPO can also be

irreversibly inactivated by different technologies, some of which will be discussed later.

b) Non-enzymatic reduction of the *o*-quinones that are formed by enzymatic oxidation of *o*-phenols. Some compounds can reacts with the products of the first enzymatic reactions turning them back to its original state, or forming complexes with diphenols and quinones thus removing them from the reaction medium.

c) Chemical modification or removal of the phenolic substrates of PPO.

Several of the compounds commonly used as inhibitors act according to the first two mechanisms. It is the case of sulfites, ascorbic acid and its derivates, cysteine, mercaptanes and 4-hexylresorcinol. On the other hand, β-cyclodextrin act via the third mechanism (Pilizota & Subaric, 1998). Among all of these molecules that are commercially used to avoid enzymatic browning, sulfites are widely used in a great variety of products due to their additional functions in the control of non-enzymatic browning and microbial growth. However, their application is limited by sanitary regulations because of their adverse effects on health. Allergic reactions or asthmatic episodes incurred by sulphite-sensitive individuals are only two examples of these problems (Taylor *et al*., 1986; Pilizota & Subaric, 1998; Threlfall & Morris, 2002; Fredericks *et al*., 2011).

The use of sulfites and the study of its alternatives deserve special interest in the wine industry. Traditionally, sulfur dioxide (SO_2) has been used to control unwanted microorganisms and polyphenol oxidase activity during winemaking, being added to machine-harvested grapes and to wine after malolactic fermentation (Bartowsky, 2009; Oliveira *et al*., 2011). However, in the last times the wine industry is challenged to meet consumers' demands of reducing the amount of SO_2 added to wine, especially since it has been associated with the aforementioned health risks. Moreover, its excessive use may affect the quality of wine, giving unpleasing flavors and aromas and making it turn cloudy during storage (Li *et al*., 2008). So far, the total substitution of SO_2 for another compound or technique that fulfill the same functions without its disadvantages remains unsuccessful. However, some studies have shown that a partial

replacement may be possible if SO_2 is combined with another hurdle (Li *et al*., 2008; Bartowsky, 2009; Fredericks *et al*., 2011; Oliveira *et al*., 2011).

Recent research trends in the field of PPO inhibition also include innovative technologies such as edible films and coatings. An edible coating is a thin layer of edible material formed as a coating on a food product, while an edible film is a preformed, thin layer, made of edible material, which once formed can be placed on or between food components (McHugh, 2000). The main difference between these food systems is that the edible coatings are applied in liquid form on the food, usually by immersing the product in a solution-generating substance formed by the structural matrix (carbohydrate, protein, lipid or multicomponent mixture), and edible films are first molded as solid sheets, which are then applied as a wrapping on the food product. These active envelopes inhibit PPO activity mainly by blocking O_2 transference between the product and the environment.

Some researchers have proved the effectiveness of edible films and coatings on the control of browning processes and polyphenol oxidase activity. Vangnai *et al*. (2006) applied chitosan coatings on "Daw" longan (*Dimocarpus longan* Lour.) fruits, finding that these treatments reduced increasing activities of PPO during the 20 days of storage at 4ºC, reducing pericarp browning. Chitosan coatings were also used by Eissa (2008), who found that they delayed discoloration associated with reduced enzyme activity of PPO and other enzymes, and had a good effect on the evolution of color properties and parameters of fresh-cut mushroom during storage at 4ºC. Ponce *et al*. (2008) applied chitosan films enriched with olive and rosemary oleoresins on pumpkin (*Cucurbita moschata* Duch) slices, which showed a clear antioxidant effect by slowing the action of polyphenol oxidase (PPO) and peroxidase (POD) within five days of storage. In addition, these edible coatings showed no deleterious effects on the sensory acceptability of the pumpkin juice.

Hui-Min *et al.* (2009) investigated the effects of three kinds of edible coatings (carrageenan, carboxymethyl cellulose (CMC) and sodium alginate) and their combinations on browning parameters of fresh-cut peach (*Prunus persica*) fruits

during storage at 5ºC. Sodium alginate coating and the various composite ones reduced the decrease of Hunter *L** value and the increase of Hunter *a** and Hunter *b** values, inhibited PPO activity and reduced the browning degree of peach fruits.

Zhang *et al*. (2004) applied combinations of an ozone water treatment and different coatings on minimally processed cucumber (*Cucumis sativus* L.). The study showed that a concentration of 4.2 $mg \cdot m^{-3}$ ozone and a composite coating made of polyvinyl alcohol (1%), chitosan (1%), lithium chloride (0.5%), glacial acetic acid (2.5%) and sodium benzoate (0.05%) inhibits respiration, chlorophyll breakdown and polyphenol oxidase activity.

1.3. Melanins, the products of polyphenol oxidase activity: for and against.

Initial studies of polyphenol oxidase were motivated by a desire to understand and prevent the enzymatic browning that occurs in the presence of air when mushrooms, fruits or vegetables are cut or bruised. Melanins, formed by these enzymatic reactions in vegetable tissues containing phenolic or polyphenolic molecules, have been considered up to the present substances that cause deterioration in many foods, especially in fruit juices and other fruit derivatives, therefore decreasing its market value. Nevertheless, recent discoveries on beneficial features on health, such as anti-oxydative, anti-inflammatory, immune and anti-tumor properties, have done that not only its elimination should be reconsidered, but also its addition could be proposed to take advantage of these facts.

In this way, the first studies that led to the idea of potential health benefits of melanin were carried out by the middle of the XX century. In 1954, Commoner *et al*. suggested that melanins that were contained in some biological tissues acted as an electron sink that contributed to stabilize free radicals. These results were later confirmed by Mason *et al*. (1960), who attributed this behavior to the electron resonance that can be found in the extensively conjugated aromatic structure of melanins and its semiquinone residues. Thus, melanins have also been proved to have a protective effect on cells against reactive oxygen and nitrogen species (ROS and RNS) (Wang & Casadevall, 1988). The same electrochemical properties give these polymers other practical applications, such as buffering or antioxidant ones (Gan *et al*., 1976; Bell & Wheeler, 1976; Pathak, 1995). In addition, its amfipolar behavior has lead to other applications as a bioemulsifier (Oloke & Glick, 2005).

In the medical area, some researchers have proved the anti-inflammatory and immunomodulatory effects of melanin, as well as the protection that it gives against carcinogenic substances such as hydrazine (Avramidis *et al*., 1998; Hung *et al*., 2002, 2003 & 2004; El-Obeid *et al*., 2006). Attending to its

applications in food industry, melanin properties have been used to achieve color and flavor enhancement in certain products such as tea, cocoa and coffee (Polaina & MacCabe, 2007). And besides melanin properties, some intermediate products of melanogenesis have been used as medicines. That is the case of L-DOPA, which has been the preferred drug for treatment of Parkinson's disease since 1967 (De Faria *et al.,* 2007). These different advantages have opened new fields for polyphenol oxidase and melanin potential applications, including the production of cross-linked protein networks for use as medicines or novel food additives. The wide majority of these fields still require important research efforts to improve the knowledge about the synthesis processes and their consequences on human health.

On the other hand, as mentioned in the previous section, attending to the natural functions of vegetal and fungal melanin, these molecules have been proved to intervene in defense mechanisms against pests and diseases. Indeed, although they are not essential for growth and development, they enhance the survival and competitive abilities of species in certain environments (Bell & Wheeler, 1986). In transgenic tomato plants, for example, the overexpression of PPO activity provides a greater resistance to cauliflower mosaic virus (Li & Steffens, 2002). Phenolic compounds serve as precursors in the formation of physical polyphenolic barriers, limiting pathogen translocation. Moreover, the quinones formed by PPO can bind plant proteins, reducing protein digestibility and their nutritive value to herbivores (Constabel *et al.*, 2000; Queiroz *et al.*, 2008).

Recent studies have also shown that some large polymers can inhibit the action of proteases thus decreasing also the nutritive value to potential predators. In some cases, protein digestibility may be affected by the formation of non-enzymatic browning (NEB) products, as it has been reported by several authors (Öste & Sjödin, 1984; Öste *et al*., 1986; Hirano *et al*., 1994 & 1996; Ibarz *et al*., 2008a & 2009a). Thus, protease inhibitors have been recognized to be significant in protein metabolism and endocrine systems. More than 100 kinds of proteinous and non-proteinous inhibitors have been isolated and

identified from various living beings such as animals, plants and microbes (Hirano *et al.*, 1994).

Some of these proteases that are potential targets to plant defense mechanisms are carboxypeptidases (A and B) and trypsin. Carboxypeptidases A and B are metallopeptidases that contain a zinc atom in their active structure, which acts as an electrophilic catalyst (Walsh, 1979). Carboxypeptidase A (CPA) hydrolyzes the C-terminus of proteins, being specific for hydrophobic side chains of phenylalanine, tyrosine and tryptophan. Carboxypeptidase B (CPB) is specific for positively charged side chains of lysine and arginine. Their structure is closely related, as in the case of trypsin and chymotrypsin, being the 49% of their sequences identical. The differences between their tertiary structures are concentrated on the external regions, being the most important one the presence of an Ile-255 in CPA, which is an Asp-255 in CPB to fix the side chain of basic substrates (Fersht, 1977). The proenzyme procarboxypeptidase A is secreted by the pancreas and is activated by removal of a 60-amino-acid N-terminal piece to yield the 307-residue active enzyme (Walsh, 1979).

CPA and CPB show higher activity in the alkaline pH range (Rathinaraj *et al.*, 2010), with a maximum around 7.5 that depends on the basic form of a group with a pK_a of 6.0 and on the acid form of another group with a pK_a of 9.1 in the non-bounded enzymes (Fersht, 1977). As mentioned, the active CPA contains a zinc atom per active site, with histidines at positions 69 and 196 and the γ-carboxylate of Glu^{72} acting as ligands. The fourth ligand is H_2O in the free enzyme, which is replaced by the carbonyl oxygen of the susceptible peptide bond in the enzyme-substrate complex (Walsh, 1979).

In view of their function releasing free amino acids from dietary protein, carboxypeptidases may play an important role in the digestion process, and probably for that reason plants have developed some molecules targeted at the inhibition of these enzymes as a protective strategy. Such carboxypeptidase inhibitors have been identified in solanaceous species like potato (potato carboxypeptidase inhibitor; PCI) and tomato. So far, all mammalian members of

the A/B subfamily of carboxypeptidases appear to be highly susceptible to inhibition by PCI (Bayés *et al*., 2006). For this reason, it seems feasible that plant defense mechanisms such as melanin synthesis can also have an inhibitory effect on these proteases.

As far as trypsin is concerned, it is synthesized in the pancreas in the inactive form of trypsinogen, and its activity is located in the small intestine where it degrades proteins to polypeptides and amino acids in a medium pH of about 8.0 (Ibarz *et al*., 2009a). Concretely, it catalyses the hydrolysis of the peptidic and ester bonds formed by the carboxyl group and the basic amino acids L-lysine and L-arginine. Trypsin activity is negatively influenced by physical parameters (temperature and pH), by conformational changes (denaturalization), by chemical modifications (substitution of amino acid residues and reduction of disulphite bridges) or by specific interactions with inhibitors. Sometimes, the formation of the catalytically inactive enzyme-inhibitor complex can be useful to understand the formation of the enzyme-substrate complex and the interactions that occur during the catalysis (Schellenberger *et al*., 1994).

Thus, on the one hand, melanin has been reported to be beneficial for health, but on the other hand it has been observed that similar polymers (as non-enzymatic browning products or several ones synthesized as plant defense mechanisms) have an inhibitory effect on proteases activity. With these premises, potential applications of melanin will depend on the evaluation of their toxic effects on proteases and, if that occurs, it will be essential to determine the kind of inhibition and its kinetic parameters.

1.4. Inactivation of polyphenol oxidase by innovative technologies.

Food safety is one of the most important issues that food industries and food service companies have to face. Currently, the growing demand for a greater variety of prepared food and dishes on a menu makes the risk of contamination increases, especially due to the characteristics of this kind of food that gives a big importance to the quickness of meals. The application of HACCP (Hazard Analysis and Critical Control Points) has increased safety guarantees in production systems, thermal treatments and cold chains, helping to reduce the opportunities for pathogens to gain access to food and / or grow to levels that will pose a risk of infection or toxin production. However, the incidence of foodborne diseases continues to rise in most industrialized countries (Bintsis *et al.*, 2000).

The elaboration and processing of liquid food like fruit juices has to be carried out under hygienic conditions that ensure final product safety. Nevertheless, in some cases there can be compounds with adverse effects on health that are not eliminated with conventional processes, such as mycotoxins, which come from mold-contaminated pieces of fruit. In the other hand, depending on the kind of juice, the presence of enzymes may represent important problems in its resulting quality. In this way, as it has been explained, polyphenol oxidase (PPO) and melanin formation are one of the major problems. Moreover, other dark compounds, melanoidins, can appear in fruit juices after non-enzymatic browning by Maillard reaction. Both melanins as melanoidins have a negative effect on final quality of the juice.

Besides PPO, other enzymes with big influence on juice quality are amylases and pectic enzymes. In the production of clarified and depectinated juices it is essential to allow these enzymes act in order to eliminate pectin and starch. Nevertheless, in purees and juices with suspended pulp, it is very important to inactivate them to avoid the cloud loss in the product. If they are not completely inactivated, in the storage stage the residual activity on pectic

fractions may result in sedimentation of the pulp, due to the destabilization of the system. Thus, in this kind of product it is imperative to ensure that there is no enzymatic activity, as it may adversely affect the quality of the juice.

Currently, heat treatment process is the most commonly used hurdle for inactivating microorganisms and enzymes extending products shelf-life, due to its availability, cost and effectiveness (Mújica-Paz *et al*., 2011). However, this process may have adverse effects on certain components of food products, reducing their vitamin content and other nutrients, as well as sensory features that make them less attractive in terms of color and textural properties (Braddock, 1999; Alwazeer *et al*., 2002; Blasco *et al*., 2004). In this context, where consumers demand minimally processed foods with properties as similar as possible to fresh products, non-thermal technologies have received increasing attention in recent years, especially in the preservation of beverages. These processing techniques are an alternative to thermal treatment that is being studied and developed in order to obtain a better final product sensory quality on the subject of flavor, color and nutritional value (Noci *et al*., 2008), but without neglecting microbial safety. Furthermore, recent studies have also shown that products processed by non-thermal methods may be less allergenic than those obtained by conventional thermal ones (Shriver & Yang, 2011).

Along with high intensity pulsed electric fields, ultraviolet (UV) and/or visible (vis) irradiation and high hydrostatic pressure are the most widely investigated non-thermal technologies for liquid food processing. However, most of the studies about UV-vis irradiation of liquid food have been carried out in order to assess its effect in microbial inactivation, but there are few references about its influence on some enzyme activities that are important in fruit derivatives, especially polphenol oxidase. On the contrary, both issues have been more exhaustively studied in high hydrostatic pressure processing (which is already implemented at industrial scale), but there are still some facts related to high-pressure inactivation of PPO that need further investigation. Consequently, in the following sections, a deep review of the former and a brief introduction to the latter will be carried out.

1.4.1. Ultraviolet-visible irradiation.

Ultraviolet irradiation is widely applied in industries to disinfect water. Other common applications include air and surfaces sterilization, especially in food packagers, brewers, bottling and cosmetics (Guerrero-Beltrán & Barbosa-Cánovas, 2004). In water treatment, UV irradiation is used as an alternative to chlorine, which may deliver toxic by-products such as trihalometanes and may be harmful for aquatic ecosystem. In addition, this technology is able to impair refractory molecules, which remain after conventional water processing. In some cases such as in wastewater treatment, the addition of photosensibilizers is essential to achieve the desired effect on water disinfection (Ibarz & Esplugas, 1989; Guerrero-Beltrán & Barbosa-Cánovas, 2004).

Regarding foodstuff processing, UV irradiation is not widely used yet. Currently, in the manufacture of fruit juices, the FDA requires to apply a preservation method capable of reducing pathogens at least 5 logarithmic cycles (FDA, 2000). In this way, this technology has been proved to be effective to achieve this reduction, but its implementation inside industries is very limited. Other possible applications that have been used at laboratory scale but with low industrial presence include dairy industry, wine industry and cheese processing (Guerrero-Beltrán & Barbosa-Cánovas, 2004).

One of the main reasons why UV irradiation is not widely implemented in industries may be found in consumers distrust. As mentioned in the first section, irradiation of foods is a technique that has been given an unfortunate name, as it has been related with nuclear energy (Ibarz, 2008). This has meant that many times irradiated food has been mistaken for being radioactive, which is completely wrong. Despite the fact that irradiated foods and the process of food irradiation have been carefully tested, the quantity of irradiated foods in the global food trade is not significant. Many surveys and market trials have shown that consumers recognize the better quality of

irradiated products but they show a bad attitude towards the idea of consuming irradiated food (Mostafavi *et al*., 2010), in a similar way to what happened in some countries with conventional pasteurization (Satin, 1997).

More than 100 years of research to achieve the accepting of the safe and successful use of irradiation as a food safety method is more than any other technology used in the food industry today, even canning (Satin, 1997; Mostafavi *et al*., 2010). Even though, there are still several scientific tests being carried out in order to corroborate its healthiness. This has resulted in international bodies including the World Health Organisation (WHO), the Food and Agriculture Organisation (FAO), the International Atomic Energy Agency (IAEA) and the Codex Alimentarius commending the process. UV irradiation has been proved to be very successful against living organisms that contain DNA and/or RNA but do not cause any significant loss of macronutrients (Ibarz, 2008; Mostafavi *et al*., 2010).

1.4.1.1. *Fundamental engineering aspects.*

For each desired application, critical decisions about equipment design must be taken. Such decisions include radiation sources, reactor geometries and reaction medium properties. In order to quantify and predict chemical changes produced by ultraviolet radiation in a food system and to infer essential information for reactors design, modeling is required in each case, since the absorbed amount of radiation has a definitive effect on the reaction rate of the process in each point of the reaction medium depending on its location. An important part of the theoretical research in the field of UV irradiation of liquid food in the near future must be focused in the development of these models for different photoreactor configurations. But before, it is essential to review all the engineering aspects that will take part in this design.

a) Radiation sources.

In the electromagnetic spectrum, ultraviolet light occupies a wide band of wavelengths in the non-ionizing region, including the ones between 200 nm (X rays) and 400 nm (visible light). The UV spectrum can be divided into three regions: short-length UV light (UVC) with wavelengths in the range of 200-280 nm, 2) medium-length UV light (UVB) with wavelengths in the range 280 to 320 nm, 3) full-length UV light (UVA) with wavelengths in the range of 320-400 nm.

Unfortunately, there is no source that works efficiently for all photochemical reactions, and the suitable one has to be chosen in each situation. Since the light must be absorbed to induce a photochemical change, it is necessary to use a source that emits radiation in the area of the absorption spectrum of the reactants, which will cause the desired photochemical change. This spectrum can be obtained irradiating a solution with a well-known concentration of the compound at different wavelengths, and measuring its resulting absorbance. This absorption spectrum usually has maximum absorbance peaks, which determine the optimum area of working wavelengths (Ibarz *et al*., 1985a; Ibarz

& Esplugas, 1989). Besides having a great emission power in the desired wavelengths, the main properties that a radiation source for an industrial use must have are: emission stability, long life, good physical dimensions, ease of operation and low cost. The following sub-sections briefly describe the most important radiation sources.

Natural sources.

The Sun is the most important natural radiation source, with a spectrum distribution that includes wavelengths from 250 to 1,200 nm. The most interesting radiation section is the one that reaches Earth's outer layers. Solar radiation includes a wide range of wavelengths, whose spectral distribution varies depending on the considered area (longitude, latitude, height, weather conditions, and so on). For most of the possible photochemical reactions, the energy flow that reaches the sea level is very small (nearly 0.1 W/cm^2) and has a spectral distribution with too much infrared radiation, which only has a heating effect and is not able to produce chemical changes. At sea level, approximately 9% of the radiation corresponds to the ultraviolet region, 42% to the visible one and 49% to the infrared one. Therefore, it may be noted the large amount of infrared radiation (“useless”) that reaches sea level compared with the low ultraviolet one ("useful").

The Sun is the first UV radiation source that can be considered, since it emits over a wide range of wavelengths. Nevertheless, the fraction corresponding to UV radiation that reaches the Earth's surface largely depends on its attenuation through the atmosphere. Figure 1.4.1A shows the difference between the solar spectrum above the atmosphere and the one that reaches sea level. The UVC fraction is completely absorbed in the upper and middle parts of the atmosphere, due to the presence of ozone and molecular oxygen. With the middle wavelength region (UVB) it happens almost the same, although a small fraction of it reaches the Earth’s surface. However, UVA light is hardly affected. Some authors consider that the flux reaching the sea level is in a range form 35 to 50 W/m^2 (Kramer & Ames, 1987; Bintsis *et al*., 2000).

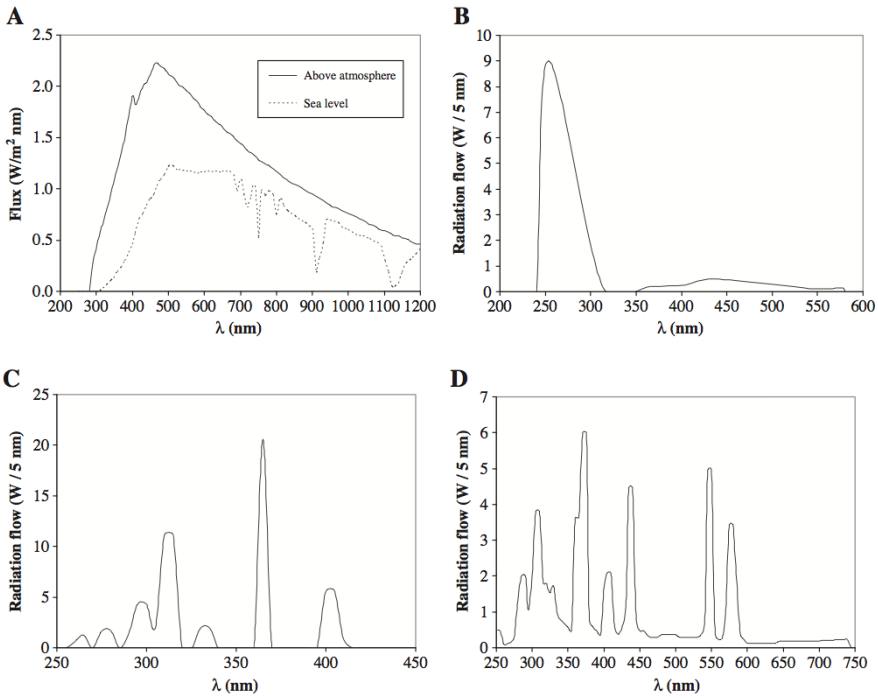

Figure 1.4.1. *Emission spectra from different UV radiation sources. A: the Sun. B: low-pressure mercury lamp. C: medium-pressure mercury lamp. D: high-pressure mercury lamp (adapted from Ibarz & Esplugas, 1989).*

Artificial sources.

Before reviewing artificial sources, it should be mentioned that there are filters of radiation, consisting of special glasses or liquid solutions, which absorb radiation of certain wavelengths and can eliminate some unwanted ones. As artificial sources, there are some kinds of lamps usually with different mercury vapor pressure, which allow different emission spectra. These lamps are classified according to this emission spectrum and if the predominating wavelengths are short, medium or long ones.

Lamps are artificial radiation sources with the greatest interest to be used in photochemical reactions. The use of radioactive waste presents lower interest. The main kinds of lamps are (Rabek, 1982):

- Incandescent lamps, consisting of tungsten filaments (3000-3400 K), which emit radiation in continuous in accordance with the laws of radiation emission for black surfaces. Most of the radiation they emit is infrared one; less than

30% is visible and the little amount of ultraviolet radiation is filtered through the glass envelope.

- Halogen lamps, in which a halogen molecule (I_2 or Br_2) is added in form of gas in the incandescent lamp, causing some chemical reactions between the gas and the tungsten filament. As a result, there is a greater output of visible radiation and some ultraviolet one compared to simple incandescent lamps.

- Arches of carbon and plasma. The need for radiation sources with greater intensity led to the use of other systems such as arches of carbon and plasma. However, these arches have the disadvantage of emitting a lot of infrared radiation. The type of radiation from a plasma arch is determined by the plasma-forming gas. The one with the highest photochemical interest is that made of argon, with a maximum energy peak between 300 and 500 nm.

- Fluorescent lamps, like the ones used for household lighting. There are many different types and shapes in the market, which emit in interesting visible areas, but their radiation level is too low for industrial use.

- High intensity discharge lamps. These lamps produce high intensity light by passing an electric current through metal in a gaseous state. The commercially available ones are made with mercury, sodium and xenon.
* Mercury lamps. The inert properties of mercury, together with its relatively low ionization energy and enough vapor pressure at moderate temperatures, make it the optimum metal for use in gas discharge tubes. To improve the emission spectrum of these lamps, metal halides are sometimes added. Attending to gas pressure, arches are usually classified into low, medium or high pressure (Figure 1.4.1B, C, and D). As pressure increases, the emission spectrum becomes more complex. While for low pressure the radiation emission is almost exclusively at 254 nm, there are more emission lines for medium pressure, and the spectrum is almost continue for high pressure. On the other hand, the power that high-pressure lamps may have (2,500 W) is much greater than the power that low-pressure ones may achieve (30 W), making them more effective. Mercury lamps (high, medium or low-pressure

ones), similarly to fluorescent lamps, require an electrical device for their ignition and continuous operation.

* Sodium lamps. The characteristics in construction, operation and emitted radiation of the high-pressure sodium lamps are quite different from other high-intensity discharge lamps. The tube of these lamps is made of ceramic material (translucent aluminum oxide) to prevent chemical attack of sodium vapor at high pressure and temperature. Its spectrum is very rich in visible wavelengths, making it useful in outdoor lighting (roads, highways, and so on).

* Xenon lamps. It is one of the most interesting sources of radiation due to its spectral distribution, almost continuous, similar than that of the Sun. They can have high power (2,000 W), but they require more care than mercury lamps. They are point sources, must operate in a vertical position and require a good control of the cooling system. They operate at a very high voltage, making the electrical equipment and maintenance to be expensive.

Lamp manufacturers usually provide their emission spectra, as well as their nominal power. However, nominal power does not usually coincide with the real power emitted by the lamp, because as time goes by its power decreases. Therefore, in any quantitative study of photochemical processes it is necessary to know the real power. There are two usual ways to measure this real power. On the one hand, it can be accomplished using UV sensors that provide this magnitude in W/m^2 (Guerrero-Beltrán & Barbosa-Cánovas, 2004). On the other hand, it is also usual to carry out the so-called actinometric reactions or simply actinometries.

An actinometric reaction is a standard photochemical reaction, with well-known absorption and kinetic parameters, which easily allows measuring changes in the concentration of some of the species involved in the reaction. The actinometry is the best method to find the radiation flow that enters the photoreactor due to the presence of a radiation-emitting source, and then making it possible to calibrate this source. The most commonly used actinometric reactions are the photochemical decomposition of oxalic acid in

the presence of uranyl cation and the photodecomposition of ferrioxalate (Calvert & Pitts, 1967; Rabek, 1982).

b) Photochemical reactors.

There are a lot of different reactors that can be used in photochemical processes, but attending to their mode of operation they can be classified in continuous and discontinuous. Some of them are described below.

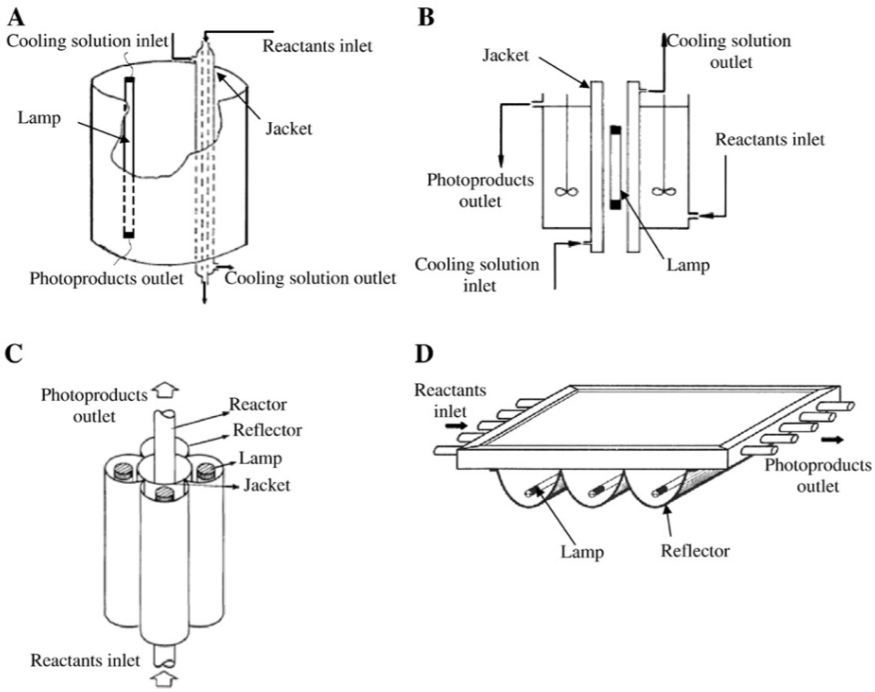

Figure 1.4.2. *Different photoreactor configurations. A: elliptic. B: annular. C: multilamp, tubular. D: multilamp, plane (adapted from Ibarz & Esplugas, 1989).*

Discontinuous reactors.

They are often used to carry out reactions with low quantum yield, which need high irradiation times. This kind of reactors is also used if the reactants have a high viscosity. Among the several existing designs, stirred tank type is the most used one. It essentially consists of a perfect mixing tank with a set of lamps immersed inside it, so that radiation reaches any point. Another kind is the elliptical photoreactor (Figure 1.4.2A), which is used in some quantitative

studies operating in batch. This design consists of a cylindrical shell with elliptical cross section, built with a reflective material to radiation, in which the lamp is placed in a focal axis and the reactor in the other one. With this configuration, all radiant energy emitted by the lamp strikes, theoretically, in the reactor either in a direct way or through the reflection in the cylindrical shell. This will get high radiation intensities in the reaction zone.

Continuous reactors.

Photochemical reactors with a continuous flow system are used in reactions with large quantum yields, achieving small irradiation times. Below, there is a brief description of these continuous photoreactors.

* Tubular annular photoreactor, which consists of a cylinder with annular section, with the lamp placed in this central annulus space.

* Cylindrical stirred tank type photoreactor (Figure 1.4.2B), similar to that operating in a discontinuous mode, consists of a perfectly stirred tank with one or more immersed lamps.

* Elliptical photoreactor, as the one described above, but operating in continuous. Lu *et al.* (2010a&b) developed a special kind of elliptical thin-film photoreactor with enhanced efficiency. It consisted of two UV mercury vapor lamps located inside elliptical reflectors, in which UV rays were reflected in such a way that they could converge at a light point: the focus. At this UV light point, the radiant energy was transmitted into quartz optical fibers bound by the fiber cluster. The reactor body, made of stainless steel, was studded with the optical fibers.

* Parallel flat-plate reactor, which consists of two parallel plates placed very close, with the reactants circulating among them, while the radiant energy comes through one side from the outside. This face is made of a material transparent to radiation, which comes either directly from the exterior lamp or by reflection from a parabolic envelope constructed with a reflective material. This kind of reactor is suitable for reactants with very high optical density, since the distance between the plates is very small and the fluid flows with a very low thickness, making it easier for the radiation to reach every point of the reactant fluid.

* Descendent film photoreactor. It is a tubular reactor in which the lamp is placed in the central axis, and the reactant fluid flows in form of film down the inner face of the tube. Shama *et al*. (1996) developed a thin film photoreactor, based on a nozzle of special design, which generated an unsupported thin liquid film commonly referred to as a liquid 'bell'. The arrangement described allowed liquids to be irradiated without making contact with either the UV sources or any solid walls in the vicinity of the sources. Milly *et al.* (2007) developed a novel photoreactor consisting of an inner rotating axis and a stationary quartz housing, which was used to induce *controlled cavitation* to ensure a homogeneous exposure of the treated product.
* Particle bed photoreactor, consisting of a bed of glass particles in whose surface there is a layer of a radioisotope attached, being covered by a fluorescent material. Thus, high-energy radiation emitted by the radioisotope interacts with the fluorescent material to produce visible or ultraviolet radiation energy.

To achieve high radiation doses, multilamp reactors are often used. They basically consist of a single reactor as the ones described above with several lamps (Figure 1.4.2C and D). In addition to all the reactors exposed, there are other kinds of designs, although it should be emphasized that for photochemical treatment of solid materials, these are usually exposed to radiation in cameras that have artificial radiation sources like the lamps described above.

c) Photoreactor mathematical model.

To elaborate the mathematical model of a conventional reactor it is necessary to carry out the mass and energy balances, as well as the kinetic equation. Furthermore, in photoreactors it is necessary to make an additional radiation balance (Bird *et al*., 1964), which has a great importance in the general model and depends on the geometry of the system. If the process works in isothermal state, it is not necessary to perform the energy balance. In addition, the variation of the photochemical reaction rate with temperature is a minor factor.

Mass balance.

Considering a continuous operation and perfect mixing, the balance for a component B_j when steady state is reached would be:

$$q(C_{j0} - C_j) = -\int_V r_j dV \qquad j = 1, 2, ..., S$$

where q is the volumetric flow of reactants; C_{j0} and C_j component j concentrations at the entrance and exit of the reactor, respectively; r_j the reaction rate for that component; and V the reaction volume.

If the reaction is developed in discontinuous, a different term for accumulation appears, resulting the mass balance:

$$V\frac{dC_j}{dt} = \int_V r_j dV$$

These expressions are similar to the ones from conventional reactors, but it should be noted that the reaction rate r_j is a function, as well as of reactants and products concentrations, of the intensity of radiation at the absorption wavelengths in the reaction chamber.

Radiation balance.

The radiation balance is posed by adopting a model that is able to provide information about light rays, taking certain considerations about the kind of emission and the geometry of the radiation source itself. Light emission models can be radial, spherical or specular and diffuse. For the emission source three models can be set, depending on if the lamp is considered as a line, as a cylindrical surface or as a volumetric region with cylindrical shape. The most common emission models are the radial one and the spherical one, which combined with the different types of emission sources lead to:

- Radial linear model.
- Non-extensive radial cylindrical model.
- Extensive radial cylindrical model.
- Linear spherical model.
- Non-extensive spherical cylindrical model.
- Extensive spherical cylindrical model.

The *extensive* and *non-extensive* term refers to the fact that the emitting source is considered as a volume or as a surface, respectively. Nonlinear models are not widely used because of the big complexity presented in its calculations. Among linear models, the radial one (Harris & Dranoff, 1965) considers the lamp as a line on which every point emits radially rays with the same intensity, which are contained in a plane that is perpendicular to the lamp by that emitting point. This model has an error that is greater than the one obtained with spherical model, so it is only used in cases where an approximate result is desired. Among all models, the one that gives a more accurate result is the extensive spherical cylindrical model (Cerdà *et al.*, 1973: Esplugas, 1975), which considers the lamp as a cylinder whose volume emits spherically. However, the most used model is the linear spherical one, which has an easier resolution, and whose results hardly differ from the extensive spherical cylindrical model.

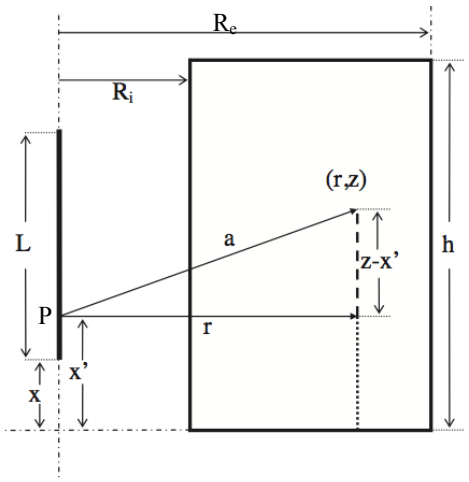

Figure 1.4.3. *Geometric parameters of a cylindrical photoreactor.*

The spherical linear model is described in the literature (Esplugas, 1975; Jacob & Dranoff, 1969 & 1970). It assumes that the lamp is a line that emits spherically, so that the radiation intensity depends on the axial and radial coordinates. One of the most used photochemical reactors in the annular one, either perfect mixing or tubular, in which the radiation source is placed in the center of the ring (Figure 1.4.2B), so that the reaction chamber surrounds the

lamp. Thus, in descending film reactors, the lamp is inside the tube through which the fluid descends and receives the radiation. In these cases, attending to the geometry of the system, it is considered that the closest surface to the lamp is at a distance that corresponds to the radius R_i, with the fluid contained in an annular space thickness (R_e-R_i), being R_e external radius of the system. The radiation balance between a *P* point of the lamp and another one with coordinates (*r, z*) within the reacting fluid (Figure 1.4.3), is given by Lambert's law for spherical emission (Esplugas *et al.*, 1983):

$$I(r,z) = \frac{W_L}{4\pi L} \int_x^{x+L} \frac{\exp(-\mu \cdot B)}{r^2 + (z-x')^2} dx'$$

where

$$B = \left(1 - \frac{R}{r}\right)\left[r^2 + (z-x')^2\right]^{1/2}$$

and where μ is the medium absorbance; I is the intensity corresponding to a point with coordinates (*r, z*); the product ($\mu \cdot B$) is the attenuation of the ray until it reaches the point (*r, z*); W_L is the energy flow emitted by the lamp; and L is the lamp length.

The radiation level on the inner surface of reaction is obtained for $r = R_i$, and depends on the position (*z*) of the considered point. In this case, radiation does not find in its way any reaction medium in $r < R_i$. Consequently, the absorbance is null, and the last equation has an analytical solution:

$$I(R_i,z) = \frac{W_L}{4\pi L R_i}\left[\tan^{-1}\left(\frac{z-x}{R_i}\right) - \tan^{-1}\left(\frac{z-x-L}{R_i}\right)\right]$$

It has been observed that the intensity depends on the axial position, having a maximum value when the lamp is centered over the reactor (Esplugas *et al.*, 1983).

For any photochemical reaction, its rate is proportional to the absorbed radiation intensity. Therefore, the evaluation of the total amount of radiation absorbed by the reaction medium is an interesting parameter to be estimated. In the mentioned reactor kinds, if the medium absorption is very high, or when the thickness of the reaction medium is high (high values of R_e), it can be

assumed that all the radiation that reaches the reaction medium is absorbed. Therefore, the absorbed radiation flow coincides with the one existing in the radial position R_i. To know this radiation flow, an integration of the radiation for the entire height of reactor must be taken, according to the expression (Esplugas *et al*., 1983):

$$W = \frac{W_L R}{2L}\left\{\begin{array}{l}\left[\left(\left(\frac{h-x}{R_i}\right)^2+1\right]^{1/2}-\left[\left(\frac{x}{R_i}\right)^2+1\right]^{1/2}-\right.\\ \left.-\left[\left(\frac{h-L-x}{R_i}\right)^2+1\right]^{1/2}-\left[\left(\frac{x+L}{R_i}\right)^2+1\right]^{1/2}\right.\end{array}\right\}$$

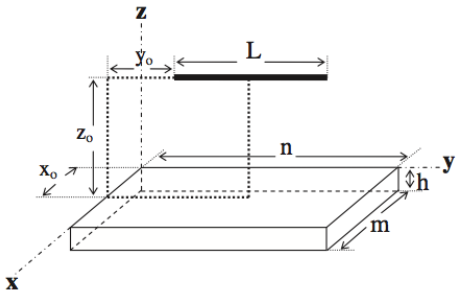

Figure 1.4.4. *Geometric parameters of a plane photoreactor.*

Another kind of reactor is the so-called plane, in which the lamp is parallel and placed at a certain distance from the surface of a fluid in a vessel. This reactor is essentially a device with parallelepiped shape with an external lamp (Figure 1.4.4). As in the previous case, if the absorbance of the reaction chamber is high, all the radiation entering the reactor will be absorbed by the medium. Therefore, the absorption rate has the same value as the flow rate reaching the surface. Assuming that the lamp is a line that emits spherically (Jacob & Dranoff, 1970), the amount of radiation can be obtained in a similar way as described above, integrating for the entire surface the radiation intensity that reaches it. Thus, it is obtained that the radiation flow of on the surface of the reactor can be expressed by (Esplugas & Vicente, 1991):

$$W = \frac{W_L z_o}{4\pi L}\left[E(L+y_o)-E(L+y_o-B)+E(y_o-n)-E(y_o)\right]$$

where

$$E(y) = F(y,0) - F(y,m)$$

$$F(y,X) = \frac{y}{z_o}\tan^{-1}\left\{\frac{y}{z_o}\frac{x_o - x}{\left[(x_o - x)^2 + y^2 + z_o^2\right]^{1/2}}\right\} + 0.5\ln\left\{\frac{\left[(x_o - x)^2 + y^2 + z_o^2\right]^{1/2} + (x_o - x)}{\left[(x_o - x)^2 + y^2 + z_o^2\right]^{1/2} - (x_o - x)}\right\}$$

in which *m* and *n* are the dimensions of width and length of the reaction vessel, which are defined, along with x_0, y_0 and z_0, in Figure 1.4.4. This equation allows evaluating the radiation flow in plane photoreactors only using the spectral distribution of the lamp and the dimensions of the reactor.

There are several additional models with more complex analytical solutions (i.e. elliptical geometry, multilamp reactors, and so on) that can be found in literature (Esplugas, 1983; Alfano *et al.*, 1990).

Quantum yield.

The energy states of molecules are quantized. The lowest energy state in which a molecule or atom can be is called the fundamental or normal state. Generally speaking, these states have paired electrons, which implies that the total spin is zero (singlet state). If an electron in one of these states goes to a higher energy level, it may remain unpaired, resulting in a state of non-zero total spin. If the molecule is in an excited state, its natural tendency is to move towards a minimum energy state deactivating itself. For a given photochemical reaction consisting of a multi-stage reaction mechanism, the *quantum yield* (φ) measures the fraction of deactivated molecules in each mechanism, existing a quantum yield value for each one. For each mechanism:

$$\phi_i = \frac{\text{Number of excited molecules deactivated by the } i \text{ mechanism}}{\text{Total number of excited molecules}}$$

If the final product of each process is P_j:

$$\phi_i = \frac{\text{Total number of } P_j \text{ molecules produced}}{\text{Total number of photons absorbed}}$$

If the expression is referred to the reactants and the global process:

$$\phi_i = \frac{\text{Total number of deactivated molecules}}{\text{Total number of photons absorbed}}$$

or also:

$$\phi_i = \frac{\text{Reactant disappearance molar rate}}{\text{Absorbed photons flow}}$$

According to the last definition, a process in which the value of quantum yield is known, from the absorbed photons flow calculated by the shown equations for each photoreactor geometry it is possible to determine an approximate value of the reaction rate. Only if the absorbance of the medium is infinite it can be ensured that all incident radiation on the reactor surface coincides with the absorbed flow, and in this case the estimated value will be exactly the reaction rate. Typically, industrial processes are often worked with high absorbance values to ensure that the radiation emitted by the lamp is fully exploited. From the definition of quantum yield:

$$\phi_i \leq 1$$

$$\sum_i \phi_i = 1$$

Photodegradation kinetics.

As indicated above, the mathematical expression of the reaction rate is usually a function of reactants and products concentrations, as well as the radiation intensity of each wavelength:

$$r_j = r_j\left(C_1,...,C_S,I_{\lambda 1},...,I_{\lambda n}\right)$$

where reaction rates must be calculated at each point of the reactor and integrated throughout the whole volume.

Since r_j depends on I_λ, and following the radiation models it is a function of μ_λ, r_j will be also a function of μ_λ. The resolution of this mathematical model is complicated, since the r_j integral in the whole reactor volume must be calculated. Generally, r_j can be decomposed in a summatory for all wavelengths:

$$r_j = \sum_\lambda r_{j,\lambda}$$

where:

$$r_{j,\lambda} = r_{j,\lambda}\left(C_1,...,C_S,I_\lambda\right)$$

There are some cases in which the resolution of the mathematical model becomes easier. Thus, in photocatalytic reactions, in which a substance absorbs radiation without being decomposed and catalyzes another reaction, the concentration of the radiation absorbing species remains constant, so that the absorbance μ_λ is also constant.

Although photocatalysis is a special case of photochemical reactions, most of them are not so, since the substance that absorbs radiation breaks down and its concentration changes with time. This makes its absorbance (μ_λ) not constant, but it is usually proportional to its concentration:

$$\mu_\lambda = \varepsilon_\lambda \cdot C$$

being ε_λ the molar extinction coefficient. In some cases this relationship between absorbance and concentration is not linear, but follows a power function.

The kinetic equation depends on the reaction mechanism, which also depends on the energy absorbed by the reactants. A simple case of a photochemical reaction mechanism is the assumption that there are three stages, as shown in the following scheme:

$$A \xrightarrow{h_\upsilon} A^*$$

$$A^* \xrightarrow{k_D} A$$

$$A^* \xrightarrow{k_1} \text{Photoproducts}$$

Following this mechanism, in a first step the A reactant in its fundamental state absorbs radiation and goes into an excited state A*. From this state, it can return to its fundamental state or be decomposed into photoproducts. The variation of every one of the species that take part in the reaction mechanism can be expressed as:

$$\frac{dC_A}{dt} = k_D C_{A^*} - \sum_\lambda \varepsilon_\lambda I_\lambda C_A$$

$$\frac{dC_{A^*}}{dt} = \sum_\lambda \varepsilon_\lambda I_\lambda C_A - k_D C_{A^*} - k_1 C_{A^*}$$

Assuming that for reaction intermediates the pseudo-steady state is applied, an expression for the photochemical degradation rate of the A reactant is obtained:

$$r_A = -\sum_{\lambda} \varepsilon_\lambda I_\lambda k_A C_A$$

where:

$$k_A = \frac{k_1}{k_1 + k_d}$$

Therefore, for batch operation:

$$\frac{dC_A}{dt} = -\frac{1}{V}\int_V \sum_{\lambda} \varepsilon_\lambda I_\lambda k_A C_A dV$$

The radiation flow absorbed by the reaction medium can be defined by the expression:

$$W_{abs,A} = \int_V \sum_{\lambda} \varepsilon_\lambda I_\lambda C_A dV$$

To evaluate this parameter it is necessary to know the distribution of the intensity emitted by the lamp at each wavelength, as well as the molar extinction coefficient of the reaction medium at each wavelength. To solve the integration it is necessary to use computer software that calculates the total absorbed radiation flow. This method allows obtaining this absorbed flow at a fixed concentration of the reaction medium. However, the concentration is changing with reaction time, so the value of this parameter may be changing. If this variation with concentration is mild, a mean value can be taken within the working concentration range ($\overline{W}_{abs,A}$). Once this parameter is known, it is possible to solve the differential equation, obtaining:

$$C_A = C_{0,A} \exp\left(-\frac{\overline{W}_{abs,A} k_A}{V} t\right)$$

In the case of continuous operation, this calculus can be carried out in a similar way, noting that it is also necessary to assess the radiation flow absorbed by the reaction medium.

This is a simple case of evaluating the kinetics of a photodegradation of an A reactant through a simple three-step mechanism. However, there are some cases where it is necessary to assume more complicated mechanisms that

may involve dissolved oxygen or other reactants present in the reaction (Ibarz *et al*., 1985b; Ibarz *et al*. 1996; Panadés *et al*., 1997). Whatever the proposed kinetic equation, it will be necessary to calculate the absorbed flow radiation term by using specific software in each case.

Absorbed radiation dose.

When any product is irradiated, it is important to define the radiation dose that it receives. This dose depends on the incident radiation, expressed as the amount of radiation received per unit of time and area or *intensity flux* (D_r, in W/m^2), so that the dose corresponds to the product of this one by exposure time (Bintsis *et al*., 2000; Guerrero-Beltrán & Barbosa-Cánovas, 2004):

$$D = D_r\, t$$

In continuous processes, exposure time coincides with the time of residence. From this equation it can be deduced that radiation dose has units of J/m^2.

When UV irradiation is used to inactivate microorganisms, some authors (Stermer *et al*., 1987; Guerrero-Beltrán & Barbosa-Cánovas, 2004) consider that the inactivation kinetics is first order, so that the number of organisms that survive photochemical treatment can be expressed by the equation:

$$N = N_0 \exp(-k\, D) = N_0 \exp(-k\, D_r\, t)$$

where N and N_0 are the number of microorganisms for any treatment time and the initial number of microorganisms, respectively; and k the inactivation rate constant.

By analogy with conventional heat treatments, a decimal reduction dose (D_{UV}) can also be defined as the dose that is necessary to reduce to one-tenth the initial number microorganisms, and is equal to 2.303/k. If the experiment is carried out with a constant lamp and geometry, D_{UV} can also be changed into the time of irradiation (expressed in min). Table 1.4.1 shows minimum and maximum doses for a total inhibition of various types of microorganisms, using an UV-C radiation source that emits at 254 nm.

Table 1.4.1. *Low and high UV-C light dosages (254 nm) needed for inhibiting 100% of several types of microorganisms (Guerrero-Beltrán & Barbosa-Cánovas, 2004).*

Organism	Microorganism	Low dose (J/m^2)	Microorganism	High dose (J/m^2)
Algae	*Chloella vulgaris*	220	Blue green algae	4200
Bacteria (vegetative)	*Bacillus megatherium*	25	*Sarcina lutea*	264
Bacteria (spores)	*Bacillus subtilis*	220	*Bacillus anthracis*	462
Molds	*Oospora lactis*	110	*Aspergillus niger*	3300
Viruses	Adeno virus type III	45	Tobacco mosaic	4400
Yeasts	Brewer's yeast	66	*Saccharomyces* sp.	176

1.4.1.2. *Effects on microorganisms and on food components and properties.*

Once the most important engineering aspects of ultraviolet irradiation of liquid food have been reviewed, attention can be moved to the effectiveness of this technology in inactivating microorganisms and its effects on food components. It is essential to keep in mind that, besides safety, organoleptic and nutritional features must be considered in the evaluation of the feasibility of a new processing technology.

a) Effect on microorganisms.

Among other applications, irradiation with ultraviolet light (UV) can be used as a disinfectant treatment to reduce the microbial load in food (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Tran and Farid, 2004). The most effective wavelengths are located between 200 and 280 nm (the so-called UVC), especially at 254 nm, while at 320 nm its efficiency is almost null (Bachmann, 1975; Bintsis *et al.*, 2000). UV radiation affects the DNA of bacteria, viruses, fungi and other microorganisms exposed to it in such a way that prevents them from reproducing (Billmeyer, 1997; Giese, 1997; Hijnen *et al.* 2006). The effect of radiation on microorganisms depends on various factors such as species, strain, culture and growth phase (Bachmann, 1975; Morgan, 1989). In addition, the kind and composition of the food that is irradiated has also a great influence.

The application of UV light with germicidal effects has been used in three areas: air disinfection, liquids sterilization and inhibition of microorganisms in surface (Bintsis *et al*., 2000). In food industry UV-C irradiation has been mainly applied in various processes and products such as air disinfection in meat or vegetables processing, on the water that will be used in some stages of the process, on surfaces of fresh products, chicken, fish, eggs, and various liquid food: milk, fruit juices or cider (Wong *et al*. 1998; Duffy *et al*., 2000; Liltved & Landfald, 2000; Basaran *et al*., 2004; Quintero-Ramos *et al*., 2004; Matak *et al*., 2005; Hadjock *et al*., 2008).

Air disinfection.

The use of UV-C as a germicidal agent for the decontamination of air is a method that has been known for decades. Several studies have been conducted on various microorganisms present in the air, such as bacteria and viruses (Jensen, 1964; Bailey *et al.*, 1996) or bacterial and fungal spores (Xu *et al.*, 2003). Microorganisms are more sensitive to UV-C if they are suspended in the air than in water, and these, in turn, are more sensitive to those found in fruit juices (Bintsis *et al.*, 2000).

Jensen (1964) irradiated aerosolized virus by passing them through an aluminum cylindrical tube with a highly reflective inner surface, in the center of which a UV lamp was placed. In the most favorable conditions, more than 99.9% inactivation for Coxsackie, Influenza, Sindbis and Vaccinia virus was achieved. Xu *et al.* (2003) evaluated the effectiveness of germicidal UV radiation on bacterial spores and vegetative mycobacteria cells. UV treatment reduced between 46 and 80% the concentration of *Bacillus subtilis* spores, and between 83 and 98% *Mycobacterim parafortuitum* ones. Josset *et al*. (2007) designed a new photoreactor to decontaminate high-speed airflow through UVA radiation. A 93% inactivation rate was obtained in a single pass through the photoreactor with airflow of 5 m^3/h in air with a concentration of $1.2 \cdot 10^6$ $CFU \cdot L^{-1}$ of *Legionella pneumophila*.

Liquids sterilization.

One of the main limitations of UV radiation in fluids treatment is its low penetration, which is determined by the properties of the irradiated liquid. Thus, in distilled water, the loss of radiation intensity at 40 cm from the surface is up to 30%, while in a 10% sucrose solution the same intensity loss can be achieved at only 5 cm (Snowball & Hornsey, 1988). In fruit juices, 90% of ultraviolet light is absorbed in the first 1 mm from the surface (Sizer & Balasubramaniam, 1999). Table 1.4.2 shows absorption coefficients of some kinds of liquid food for 254 nm UV-C radiation. The presence of dissolved organic solutes and compounds in liquid foods leads to strong UV attenuation effects. Fan & Geveke (2007) found that the major components of apple juice/cider are sugars including fructose, sucrose, and glucose followed by

organic acids, mainly malic acid and a very low amount of ascorbic acid. The three sugars absorbed little UV in the range of 240–360 nm although the fructose solution had higher UV absorbance at 260–280 nm than glucose and sucrose solutions. All three sugars had high absorbance near 200 nm. Malic acid mainly absorbed UV at wavelengths less than 240 nm while ascorbic acid had a strong absorbance between 220 and 300 nm even at a very low concentration (0.001%).

Table 1.4.2. *Absorption coefficient for some liquid foods for UV-C at 254 nm (Guerrero-Beltrán & Barbosa-Cánovas, 2004).*

Liquid food	α (cm^{-1})
Distilled water	0.007–0.01
Drinking water	0.02–0.1
Clear syrup	2–5
White wine	10
Red wine	30
Beer	10–20
Dark syrup	20–50
Milk	300

The first application of UV radiation for drinking water disinfection was carried out in 1910 in Marseille (Henry *et al.*, 1910). At that time its use was limited by its high cost, the low reliability of the equipment and the advent of chlorination, which was a cheaper, most reliable method and with the ability to measure the residual disinfectant (Wolfe, 1990; Hoyer, 2004). Since then, UV irradiation has been gaining interest and since the 80s, in Europe, it has been widely used to disinfect drinking water, reaching in some cases to replace chlorination (Qualls & Johnson, 1983; Lodge *et al.*, 1996; Downey *et al*., 1998; Gibbs, 2000). The rise in the use of this technology is mainly due to the fact that it hardly produces oxidation products as it happens with chlorination or ozonation. The real breakthrough in the use of UV irradiation as a primary disinfection process came after the discovery of its high efficacy against *Cryptosporidium* and *Giardia*, two of the main pathogens affecting drinking water safety (Clancy *et al.*, 1998).

There are several pieces of work in literature studying the disinfection of both drinking and wastewater by ultraviolet irradiation (Chang *et al*., 1985; Whitby & Palmateer, 1993; Liltved & Cripps, 1999; Sommer *et al*., 2000; Sutton *et al*.,

2000). Hijnen et al. (2006) performed an extensive and selected review on the use of ultraviolet irradiation for disinfection of drinking water. In the case of wastewater disinfection, one of the factors affecting the effectiveness of UV irradiation is the quality of treated wastewater. In this way, UV irradiation has been proved to be effective in treating high quality secondary and tertiary effluents (Blatchley *et al*., 1996; Braunstein *et al*., 1996, Oppenheimer *et al*., 1997). However, the effectiveness is further discussed in the case of primary wastewaters or low quality effluents (Zukovs *et al*., 1986; Whitby & Palmateer, 1993; Sakamoto, 1997). This is due in part to the presence of suspended particles in wastewater, which increase the survival probability of microorganisms exposed to UV radiation. Suspended solids cannot only attenuate the UV dose via light scattering, but may also provide a site for the aggregation of bacteria to particles surface (Koutchma, 2009). Whitby & Palmateer (1993) reported the relationship between the concentration of suspended solids and the survival rate of fecal coliforms in wastewater. Taghipour (2004) concluded that to reduce one log cycle the concentration of *Escherichia coli* in primary and secondary effluents UV radiation doses of 35 and 62 J/m^2, respectively, were necessary.

UV irradiation has also been successfully applied in the pasteurization of liquid foods such as milk and fruit juices (Koutchma *et al*., 2004; Matak *et al*., 2005). Although the treatment of opaque liquid foods by UV irradiation is an additional problem, in the dairy industry this method has been used for different applications (Bintsis *et al*., 2000). Thus, for example, brines used in the production of Mozzarella cheese have been irradiated (Anonymous, 1994). Lodi *et al*. (1996) succeeded in reducing the total colony count between 50 and 60%, and coliforms in 80-90% in goat milk using UV-C radiation. In 1951, Burton carried out a piece of work in which milk pumped at high speed through transparent tubes of 1 cm diameter was irradiated, so that 80% of UV radiation reached the milk, destroying about 99% of bacteria initially present. Matak *et al*., (2005) also proved that UV radiation could be used to reduce the population of *Listeria monocytogenes* in goat milk. This fresh goat milk was inoculated with a concentration of 107 CFU/mL and irradiated with UV light at doses from 0 to 20 mJ/cm^2, managing to reduce the microbial load by more

than 5 log units when the milk received an accumulative dose of 15.8 mJ/cm^2. In a later piece of work, Matak *et al*. (2007) assessed the chemical and sensory effects on goat milk treated for 18 s with a dose of 15.8 mJ/cm^2, concluding that milk irradiated with UV light at a wavelength of 254 nm suffered severe sensory and chemical changes, probably due to lipid oxidation. Ibarz *et al*. (1986) also lowered significantly the microbial load of raw milk by using UV irradiation.

In juices and fruit derivatives many authors have studied the germicidal effect of UV irradiation on different organisms (Anonymous, 1999; Worobo, 1999; Basaran *et al*., 2004; Tran & Farid, 2004; Guerrero-Beltrán & Barbosa-Cánovas, 2005; Keyser *et al*., 2008; Franz *et al*., 2009; Gabriel and Nakano, 2009). Gabriel & Nakano (2009) irradiated different strains of *Escherichia coli* (K-12 and O157:H7), *Salmonella* (*S. enteriditis* and *S. typhimurium*) and *Listeria monocytogenes* (AS-1 and M24-1) in phosphate buffer and in clarified apple juice. *S. typhimurium* was the most sensitive one to ultraviolet irradiation with a *D* value of 0.27 min, while *L. monocytogenes* AS-1 was the most resistant one with a *D* value of 1.26 min. Keyser *et al*. (2008) successfully used UV irradiation to reduce microbial load in different fruit juices and nectars. In clarified apple juice they were able to reduce by more than 7 log units the population of *E. coli* with a radiation dose of 1,377 J·L^{-1}, while a dose of 230 J·L^{-1} was enough to reduce aerobic plate counts by 3.5 log units and by 3 log units other molds and yeasts in the juice. In similar experiments carried out in orange juice containing cells of 7.5-10% by weight, and after applying a higher radiation dose (1,607 J·L^{-1}) reductions of only 0.3 log units were achieved for mesophilic aerobic microorganisms and for molds and yeasts. These disparate results are due to the large amount of suspended matter (such as orange cells and fiber) in orange juice, which act as a protective barrier to microorganisms against UV radiation.

Guerrero-Beltrán & Barbosa-Cánovas (2005) studied the reduction of *Saccharomyces cerevisiae*, *Escherichia coli* and *Listeria innocua* population in apple juice when it was irradiated with ultraviolet light. The results showed that the higher the treatment time and the flow rate were, the greater the probability

that these organisms were damaged or inactivated by the radiation. Walkling-Ribeiro *et al*. (2008) treated apple juice, previously inoculated with *Staphylococcus aureus* TSS 2.4, with a combined method of UV irradiation, preheating and high-intensity pulsed electric fields, managing to reduce the microbial population by 9.5 log units. In the most severe conditions the reduction achieved by using this combined techniques became even greater than that achieved with conventional pasteurization. In a study conducted by Ngadi *et al*. (2003) the count of *E. coli* O157: H7 in apple juice was reduced about 4.5 log units using a dose of 3,000 $mJ \cdot cm^{-2}$ and a liquid depth of 1 mm. This kind of treatment has also been used to reduce the microbial load in apple cider (Harrington & Hills, 1968, Wright *et al*., 2000). Worobo (1999) also managed to reduce more than 5 log units the population of *E. coli* in apple cider irradiated with ultraviolet light in a UV CiderSure 3500. Milly et al. (2007) achieved the inactivation of *E. coli* 25922 in apple juice and skim milk with a reduction of 4.5 and 3 logs, respectively.

Guerrero-Beltrán *et al*. (2009) processed grape, cranberry and grapefruit pasteurized juices inoculated with *S. cerevisae*, using an UV-C disinfection unit and working at different flow rates and dose of UV light (75-450 kJ/m^2). The inactivation of *S. cerevisae* could be described by means of first order kinetics, obtaining times of decimal reduction that ranged from 61.7 to 113.7, 12.2 to 40.7 and 12.5 to 20.7 min for grape, cranberry and grapefruit juices, respectively. The maximum reduction log was 0.53, 2.51 and 2.42 for yeast count in grape, cranberry and grapefruit juices, respectively, for a flow rate of 1.02 L/min after 30 min of recirculation.

In brewing and beverage industry many producers have adopted UV irradiation as a water disinfection system, as it is essential that the treatment does not alter the taste and quality of the final product (Egberts, 1990, Oliver *et al*., 1990; Greig & Warne, 1992). The radiation dose required to treat water in brewing industry is much higher than the dose used in drinking water treatment, since it must guarantee the absence of any microbial alteration during the early stages of beer production. Lu *et al.* (2010) applied a thin film apparatus with quartz optical fibers for UV-radiation delivery to inactivate

bacteria in beer. With this apparatus, a reduction of inoculated *S. cerevisae* and *L. brevis* in beer of around 5 log and from aproximately10^4 CFU·mL^{-1} to non-detectable limits at doses of 16.1 and 9.7 mJ/cm^2, respectively, were achieved. However, the inactivation of *S. cerevisae* was not so efficient. In addition, the beneficial yeasts of beer were hardly inactivated.

In liquid egg derivatives UV-C irradiation may be an alternative treatment to obtain a microbiologically safe and stable product (Bintsis *et al*., 2000; Donahue, 2004), avoiding alterations that other methods such as high hydrostatic pressure, high-intensity pulsed electric fields or thermal pasteurization may have on product properties due to protein denaturation (Unluturk *et al*., 2008). In a study carried out by Ngadi *et al.* (2003) on liquid egg white (pH 9.1) inoculated with *E. coli* O157:H7, a decrease in microorganisms count from 10^8 to $10^{3.8}$ CFU·mL^{-1} after an exposure to UV radiation dose of 300 mJ·cm^{-2} was achieved. Unluturk *et al.* (2008) conducted a study about liquid egg products in which the effect of UV irradiation on non-pathogenic strains of *E. coli* (ATCC 8739) and *S. typhimurium* was investigated. These authors also studied the effect of the liquid medium depth, the UV light intensity and the exposure time. The maximum reduction, more than 2 log units, was obtained on *E. coli* (ATCC 8739) in liquid egg white with a medium depth of 0.153 cm and an UV intensity of 1,314 mW·cm^{-2}. However, under the same conditions, in liquid egg yolk and in liquid whole egg, the maximum reductions achieved were of only 0.675 log units and 0.361 log units, respectively, showing that the fat content of egg yolk had a protective effect for microorganisms.

Application to surfaces disinfection.

Nowadays, the main application of ultraviolet irradiation in industry is the sterilization of packaging materials such as containers or bottle tops (Bintsis *et al*. 2000). In aseptic packaging of products treated by UHT (e.g. milk) UV radiation is used to sterilize the lids of aluminum bottles (Nicolas, 1995) or cartons for liquid products (Kuse, 1982). The materials for aseptic processing and packaging can also be sterilized by combining the treatments with hydrogen peroxide (H_2O_2) and ultraviolet radiation (Marquis & Bladeck, 2007)

taking advantage of their synergistic effect on bacterial spores destruction. The combination of ozone and UV radiation is often used in the treatment of polymer plastics for food packaging (Ozen & Floros, 2001).

UV-C radiation may also be used for the treatment of food surfaces. Thus, there are several papers in literature demonstrating the effectiveness of UV radiation in reducing the surface population of pathogenic microorganisms in red meat, chicken and fish (Huang & Toledo, 1982; Stermer *et al*., 1987; Sumner *et al*., 1995). In a study conducted on pork skin and muscle (Wong *et al*., 1998), the authors demonstrated the effectiveness of UV light to reduce *Escherichia coli* and *Salmonella senftenberg* in these surfaces. In the tested conditions, UV light was more effective on *S. senftenberg* than on *E. coli* and its effects were faster on skin than on pork muscle. Other researchers have found that with this kind of treatment the commercial life of fresh meat may increase from 12 to 28 days (Dejenane *et al*., 2001). The application of UV light reduced by 61% the content of *Salmonella typhimurium* in chicken skeleton, without affecting its color (Wallner-Pendleton *et al*., 1994). Lyon *et al*. (2007) reduced about 2 log units the content of *Listeria monocytogenes* in chicken breast fillets with a treatment with UV light. Chun *et al*. (2010) have recently shown that treatment with UV-C radiation can reduce the microbial load of *Campylobacter jejuni, Listeria monocytogenes* and *Salmonella typhimurium* in chicken breast. Similarly, UV-C radiation can be used as a method for improving microbial safety of ready-to-eat food such as ham slices (Chun *et al*., 2009).

Kuo *et al*. (1997) proved that UV-C radiation is effective in reducing total aerobic count and molds, as well as *Salmonella typhimurium*, on eggshells. Likewise, various studies show the effectiveness of UV-C radiation to reduce surface microbial load on vegetables (Allende & Artés, 2003) and fruits (González-Aguilar *et al*., 2001). UV-C radiation has also been used to reduce post-harvest deterioration of onions (Lu *et al*., 1987), carrots (Mercier & Arul, 1993), tomatoes (Maharaj, 1995) and zucchini (Erkan *et al*. 2001). There are also numerous studies that show the effectiveness of UV-C radiation to reduce

diseases in fruits such as apple and peach (Stevens *et al.*, 1996), table grapes (Nigro *et al*., 1998), grapefruit (Droby *et al*., 1993) or papaya (Cia *et al*., 2007).

In the same way, UV-C light can also be applied on fresh fruits, vegetables and edible roots before storage. The purpose of this treatment is twofold: to reduce the microbial load in surface and to induce resistance to microorganisms. The beneficial effect of this treatment on fresh foods is called *hormesis* (Stevens *et al*., 1997 & 1999). This effect is due to the fact that UV light can stimulate the production of phenylalanine ammonialyase (PAL), which induces the formation of phenolic compounds (phytoalexins) that can improve the resistance of fruits and vegetables to microorganisms (Ben-Yehoshua *et al*. 1992; Stevens *et al*., 1999; D'Hallewin *et al*., 2000). Onursal *et* al. (2010) found that the irradiation of pomegranate fruits (*Punica granatum* L.) with UV-C radiation increased the total phenolics content in juice, peel and seeds. In the treatment of broccoli, UV-C has been shown to delay senescence. In addition, its antioxidant capacity increases, which could be useful from the nutritional point of view (Costa *et al*., 2005).

b) Effect on toxins.

The presence of mycotoxins in food is a severe problem for consumers' safety. Furthermore, in conventional heat processes mycotoxins are not affected, and it would be necessary to find an alternative treatment to eliminate or reduce its content in food. Aflatoxins can be destroyed in some degrees applying UV radiation and sunlight. There are some studies, for example, that show that it is possible to destroy this kind of toxin in peanut (Leeson *et al*., 1995). In the case of citrinin and ochratoxin A, it has been shown that there is a limited decomposition when they are treated with UV light (Neely & West, 1972). Also the use of ionizing radiation achieves this purpose, but so high doses are required that they finally impair the quality of food components and nutrients, losing practical application.

UV radiation has been used to degrade aflatoxin M_1 in raw and heated milk (Yousef & Marth, 1985), observing that in the samples irradiated at 25°C the quantity of aflatoxin M1 was reduced by 32% more than in the ones irradiated

at 5°C; however, when the treatment temperature was 65ºC the reduction was only 25.5%. These data suggest that it is possible to degrade aflatoxin in milk at lower temperature values than pasteurization. The degradation of this toxin in aqueous solution has also been studied (Yousef & Marth, 1987), observing that its elimination was accompanied by an accumulation of aflatoxin M_x, which was also degraded when the treatment temperature was raised to 60ºC (Figure 1.4.5).

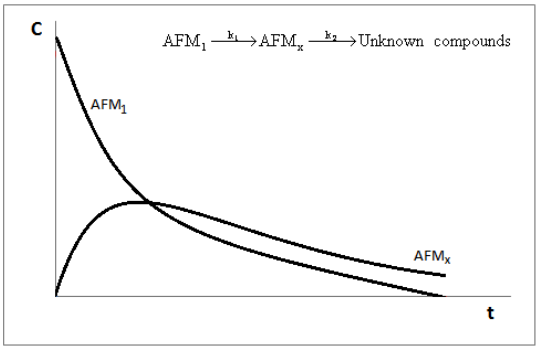

Figure 1.4.5. *Elimination of aflatoxin M_1 and M_x with UV irradiation at 60°C (Adapted from Yousef & Marth, 1987).*

Among edible materials, peanut oil is the most common product that contains aflatoxins. Shantha & Sreenivasa-Murthy (1977) investigated the effect of light of different wavelengths, using lamps that emit in the ultraviolet and solar region. The results showed that with long irradiation times high removal rates were obtained; thus, with UV light 87% destruction was reached, while under sunlight it was 82%. The absorption spectra of these samples containing aflatoxin exposed to ultraviolet and sunlight showed a shift of the absorption maximum, along with a considerable reduction of toxicity (Shantha & Sreenivasa-Murthy, 1977). It has also been observed that aflatoxin B1 contained in rice is degraded under the action of the light emitted by a mercury-tungsten lamp (Nkama & Muller, 1988). In that case, the moisture content of the samples and the intensity of the used light had a high influence on the results. In this way, in rice samples containing approximately 1,000 $mg \cdot kg^{-1}$ it was observed that after 2 h of irradiation with an intensity of 64 $mW \cdot cm^{-2}$ the level of aflatoxin decreased by approximately 70%, whereas with an intensity 43 $mW \cdot cm^{-2}$ its decline was 60%.

Samarajeewa *et al.* (1990) have made a review of physical and chemical methods used in the detoxification of aflatoxin in food and animal feed. One of these techniques is the utilization of ultraviolet and visible light. Thus, aflatoxin B1 has an absorption spectrum with some peaks at wavelengths of 222, 265 and 362 nm, with maximum at 362 nm. Irradiation at these wavelengths activates this aflatoxin and increases its susceptibility to degradation. Aflatoxin B1 is highly sensitive to UV radiation at pH values below 3 or above 10, since its structure is affected in the terminal furan ring, having the active point of link removed (Lillard & Lantin, 1970). It seems that the presence of oxygen increases the degradation of the free radicals caused by UV irradiation of the aflatoxin crystals (Bencze & Kiermeier, 1973).

Kinetic studies of aflatoxin degradation lead to a first-order kinetic process with a possible dimerization of the coumarin molecule (Aibara & Yamagishi, 1968). It appears that the intermediate formed products have some toxicity, and research should be expanded in this regard. On the other hand, a white light source could provide better breakdown of primary compounds derived from aflatoxin decomposition to secondary nontoxic ones (Samarajeewa *et al*., 1990). Solar radiation, which has radiant energy in the ultraviolet and visible spectrum, has shown great efficiency in the degradation of aflatoxins in foods (Samarajeewa *et al*., 1974). Edible oils irradiated with sunlight have shown no toxicity in mice and ducks (Shantha & Sreenivasa-Murthy, 1977 & 1980; Gamage *et al*., 1985; Samarajeewa *et al*., 1987). Aflatoxin B1 degradation by sunlight in foods suggests the presence of aflatoxin in two states, one easily accessible and another one less accessible. The first one is easily degraded, being described as *unbound* in casein and peanut (Shantha & Sreenivasa-Murthy, 1981), following first-order kinetics in its degradation in rice (Nkama *et al*., 1987). Less accessible aflatoxin is described as *linked* and does not follow first order kinetics for its degradation. These differences are probably related to the fact that aflatoxins located in food surface are reachable to solar radiation, while the molecules located inside the food are protected by the limited penetration of this radiation. Studies about solar and ultraviolet

radiation indicate that the double bond in the terminal furan ring of aflatoxin B1 is probably the photodegradation sensitive point (Samarajeewa *et al*., 1990).

Unlike in solid food, solar radiation can penetrate more easily in thin liquid films, providing a decontamination technique. Studies carried out at pilot plant scale have shown the efficiency of solar radiation on the degradation of aflatoxins in coconut oil and peanut oil (Shantha & Sreenivasa-Murthy, 1981; Samarajeewa *et al*., 1985). Considering all the explained evidence, it seems that solar radiation can be effective in the degradation of aflatoxins.

c) Effect on enzymes.

Enzymes play an important role in fruit juices manufacture, both in clarified juices and in purees. As it has been explained in the previous sections, it is essential to inactivate polyphenol oxidase (PPO) in order to avoid the formation of melanins that cause juices color deterioration. In addition, other important enzymes in fruit juices are the pectic ones, especially pectinmethylesterase (PME), endo-polygalacturonase (endo-PG) and exo-polygalacturonase (exo-PG). There are few studies about the effect of UV radiation on enzymes, some of which report slightly contradictory results.

Polyphenol oxidase, ATPase and acid phosphatase molecules may be denatured when they are irradiated with ultraviolet light, with a consequent reduction in its enzymatic activity (Seiji & Iwashita, 1965). The loss of enzyme activity is dependent on radiation intensity, reaching a 75% inactivation in the case of polyphenol oxidase and about 50% for the other two enzymes. In a piece of work about ultraviolet treatment at 254 nm of orange juice carried out by Tran & Farid (2004), besides the effects on disinfection, commercial life and vitamin C, the authors also studied the effect on pectinmethylesterase activity. The juice was treated with 73.8 $mJ \cdot cm^{-2}$, obtaining an inactivation of 5%. However, it should be noted that the lamp used in this treatment only had an emission at 254 nm, so additional studies with lamps with a wider emission spectrum should be developed. In addition, this study was carried out with juices containing suspended pulp, which surely exert a protective effect against UV light.

Ibarz *et al.* (2009b) irradiated aqueous solutions of carboxypeptidase A and trypsin, using a medium-pressure mercury lamp that emits in the range of 250-740 nm. These authors observed that it is possible to completely inactivate both enzymes. The inactivation of the enzymes chymotrypsin, lysozyme, ribonuclease and trypsin by ultraviolet light at 253.7 nm (Luse & McLaren, 1963) can be related to the probability that the light is absorbed by a particular amino acid residue, where the link rupture is not important. McLaren *et al.* (1953) studied the inactivation of ribonuclease and carboxypeptidase using UV light (253.7 nm) with quantum yields of 0.03 and 0.001 to 0.005, respectively. The quantum yields for low molecular weight protein are around 0.03 and are higher than those of peptide bonds. It has been postulated that the primary process of inactivation involves the modification of aromatic residues in proteins.

d) Effect on some components and properties of fruit juices.

As it has been already introduced, irradiation of fruit juices has the problem that the optical path of radiation is small, because it is completely absorbed within a small distance from the surface. This is because most fruit juices are *opaque* to UV radiation due to suspended solids in juices with pulp and to the color of clarified ones. This coloration is due to the pigments extracted from the fruit itself and melanins and melanoidins from the enzymatic and non-enzymatic browning, respectively. The penetration depth of UV-C radiation through liquids is a few millimeters, in which 90% of incident light is absorbed (Sizer & Balasubramaniam, 1999). The penetration of UV light depends on the kind of fluid, on its absorption coefficient, on its soluble solids content and on suspended matter. The higher the soluble solids content, the lower the penetration. Thus, to ensure effective penetration of radiation in fruit juices it is necessary to have good mixing of the reaction medium in the container, and in the case of using a continuous operation, to ensure that the fluid flows with high turbulence (Keyser *et al.* 2008).

There are still few studies conducted on fruit juices irradiation (Farid *et al.*, 2000; Tran & Farid, 2004), although this treatment has been also used for the disinfection of similar fluids such as sugar syrup in beverages processing

(Nakayama & Shinya, 1981; Gibbs, 2000). Heat-treated juices tend to change color and lose some of its aromas and vitamins during the heating process (Choi & Nielsen, 2005), while the juices treated with UV radiation tend to maintain its aroma and color (Tran & Farid, 2004).

Both melanins as melanoidins contained in fruit derivatives are polymeric compounds that provide a brown coloration, which is detrimental to sensory quality. The presence of melanin has a protective effect on UV inactivation of various enzymes (Seiji & Iwashita, 1965), probably due to the fact that melanin absorbs ultraviolet radiation. Nevertheless, melanoidins can be degraded when they are irradiated with UV-vis radiation (Kwak *et al*., 2004). In addition, in the irradiation of clarified apple, peach and lemon juices (Ibarz *et al*., 2005) important changes have been found in different colorimetric parameters, attributed to the destruction of both native pigments as the melanins and melanoidins present. Thus, pigments impairment can be observed both in CIELab parameters variation and in the decreasing of absorbance in the whole visible range of the absorbance spectrum of the samples.

Ibarz & Perez-Teijón (1990) conducted a direct exposure to sunlight of clarified apple juice, noting that there was a decrease in the absorbance at 420 nm value, suggesting a destruction of the juice pigments. In a later piece of work, Ibarz *et al*. (2005) irradiated apple, peach and lemon juices with a lamp that emits in the range of 250-740 nm, studying the effect on CIELab colorimetric parameters. There was an increase of luminosity L^*, while the samples had a significant decrease in the parameters a^* and b^*, which was an indication that the pigments and compounds that give brown coloration were destroyed. In the irradiation of apple juice with ultraviolet light to reduce the microbial load, Guerrero-Beltrán & Barbosa-Cánovas (2005) observed that the treatment influenced color parameters, especially a^* and b^*, decreasing from a value of 2.4 to 1.4 and from 21.3 to 16.3, respectively. This indicates again that there is a photodegradation of apple juice pigments.

As mentioned, it is important to avoid the formation of melanins and melanoidins, both in processing and in storage of the juice. However, once

they are in the juice it would be desirable to seek for methods to eliminate these compounds from the samples. A possible method to be used in their removal is by adsorption with activated carbon or resins (Carabasa *et al*., 1998; Ibarz *et al*., 2008b). An alternative may be found in the use of UV radiation. In this way, Kwak *et al.* (2004) studied the influence of pH on the photodegradation of model melanoidin solutions, using a Xe lamp that emits in the range of 200-1,000 nm, and a halogen-tungsten lamp emitting in the range of 350-1000 nm.

Due to food complexity in terms of the variety of components they contain, UV radiation can affect many of these compounds. An extensive exposure of the effects of such radiation on the multitude of compounds present in food will not be made. However, some representative cases will be cited, trying to give an idea of the potential, both positive and negative, of the photochemical treatment processes.

Fruit juices, depending on the kind of fruit from which they come, contain different types of vitamins. Carotenoids are important constituents in many foods, because in addition to providing a distinctive color they may have potential health benefits (Klaui & Baunernfeind, 1981). Processing and storage conditions can degrade carotenoids, destroying the color and reducing the nutritional value of food. Pesek & Warthesen (1990) studied the kinetics of β-carotene photodegradation in irradiated model solutions and in carrot juice finding first-order kinetics, being the *cis* isomer the most sensitive one to degradation. In another piece of work (Pesek & Warthesen, 1987) the same authors studied the effects of light on lycopene, α-and β-carotene in tomato and carrot juices, obtaining first-order kinetics with values of kinetic constants from 0.1 to 0.3 $days^{-1}$, being lycopene the most resistant molecule to photodegradation. It was also found that light-treated samples had higher values of brightness, while the colorimetric parameters *a** and *b** showed some decrease (lower intensity of red and yellow), which can be related to degradation color of the studied carotenoids. These same researchers (Pesek & Warthesen, 1988) also studied the photodegradation of β-carotene in model systems containing this carotenoid at different temperatures, both in solid or

liquid state, finding higher degradation rate constants at higher temperatures. Tran & Farid (2004) irradiated orange juice with an UV lamp that emits at 254 nm, finding a vitamin C degradation of 12% with a dose of 73.8 $mJ \cdot cm^{-2}$.

Whited *et al*. (2002) studied the effect of light on the vitamin A in skim, semi-skim and whole milk, concluding that fat had a protective effect on the degradation of this vitamin. In all cases it was observed, apart from the loss of this vitamin, a loss of milk quality because in sensory analysis panelists highlighted the flavor of oxidized milk. Riboflavin, known as vitamin B_{12}, is a yellow water-soluble vitamin, consisting of a dimenthyl isoalloxazine ring linked with ribitol, an alcohol derived from ribose. The three rings form the isoalloxazine and ribitol is the 5-carbon chain at the top. Furaya *et al*. (1984) studied the photodegradation of riboflavin in macaroni, skim milk powder, and buffer solutions. In liquid systems these authors found first order photodegradation kinetics, while in solid food systems a two-step mechanism was observed.

Due to potential health problems that artificial food dyes may have, some attention has been paid to the development of natural pigments to be used as colorants. Among the red pigments, betalains and anthocyanins are potential pigment sources. However, natural pigments can be degraded by various agents. As it has been explained, light is one of these factors affecting their stability. In this way, the effect of light on betanine extracted from beet and on cranberry anthocyanins has been studied (Attoe & von Elbe, 1981). For both kinds of pigments it was found that photochemical degradation follows first order kinetics, observing that the presence of molecular oxygen in the medium plays an important role in the degradation of these compounds.

Another important problem that exists in the manufacture of juices are pesticide residues from fruit. In this regard, the effectiveness of ultraviolet irradiation on the decomposition of some of these compounds such as pyridine (Ibarz *et al*., 1985), carbendazim (Ibarz & Perez-Teijón, 1990), benomyl (Ibarz *et al*., 1996), thiabendazole (Panadés *et al*., 1997) and indole (Ibarz *et al*., 1998) has been proved.

1.4.2. High hydrostatic pressure.

Among novel processing alternatives, high-pressure processing (HPP) has been adopted at the fastest rate, experiencing a considerable growth in the last 20 years as reflected by the number of units installed (Mújica-Paz *et al*., 2011; Figure 1.4.6). This fast implementation has been followed by the modification of the corresponding regulatory framework. In 2001, the European Commission authorized the placing on the market of pasteurized fruit-based preparations produced using high-pressure pasteurization (European Commission, 2001). Moreover, in 2009 the US-FDA approved combined pressure-temperature processing (also known as pressure-assisted thermal sterilization, PATS) for commercial sterilization of mashed potato in a specific high-pressure unit (Bermúdez-Aguirre & Barbosa-Cánovas, 2011).

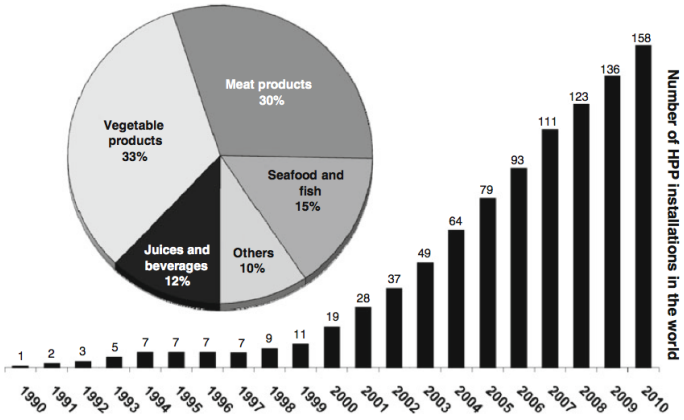

Figure 1.4.6. *World growth of the food industry use of high-pressure processing technology (from Mújica-Paz et al., 2011).*

In HPP, pressures of approximately 300 to 900 MPa for periods from some seconds to a few minutes are used to inactivate pathogenic bacteria such as *Listeria*, *Salmonella*, *Escherichia coli* and *Vibrio*, as well as spoiling bacteria, yeasts and moulds (Bull *et al*., 2004, Buckow *et al*., 2009). The required pressure treatment depends on the target microorganism or enzyme to be inactivated. Bacterial vegetative cells, yeasts and moulds are sensitive to

pressures between 200 and 700 MPa, while some enzymes and some bacterial spores may survive pressurization above 1,000 MPa if the process is carried out at room temperature (Arroyo *et al.*, 1999; Bull *et al*., 2004). In this way, deterioration of food due to the outgrowth of bacterial spores and residual enzyme activities must be controlled by using complementary methods such as refrigeration and/or acidification.

Although juices and beverages only represent 12% of the products commercially available that are treated by high-pressure, several studies are nowadays being conducted on this kind of foodstuff, especially on apple juice, which is one of the most commonly consumed all over the world. Since the beginning of HPP technology development, apple polyphenol oxidase has focused the interest of scientists due to its high-pressure resistance (Bayındırlı *et al*., 2006; Buckow *et al*., 2009; Valdramidis *et al*., 2009). In order to achieve a satisfactory degree of PPO inactivation in apple juice, a combination of high pressure with mild initial temperatures (60-90ºC) may be useful (Buckow *et al*., 2009). This high pressure thermal processing (HPT; or PATS), allows reduction of treatment times taking advantage of the synergistic effect of pressure and temperature (Ramirez *et al*., 2009; Knoerzer *et al*., 2010; Mújica-Paz *et al*., 2011).

In order to optimize these processes, quantitative kinetic data is essential to achieve the development of predictive models (Buckow *et al*., 2009). However, the effect of the complex matrix of apple juice requires specific kinetic research for each variety, since the activity and properties of polyphenol oxidase depend on several composition parameters (Yemenicioglu *et al*., 1997; Soysal, 2008; Ramirez *et al*., 2009).

In addition, fruit juices are one of the food products in which color is more important for their appearance and acceptability (Hartyáni *et al*., 2011). High-pressure processing has been widely reported to keep color properties closer to the fresh-made juice than thermal processes, as well as other organoleptic and nutritional features (Cheftel, 1995; Oey *et al*., 2008; Bull *et al*., 2004; Bermúdez-Aguirre & Barbosa-Cánovas, 2011). However, the effects of HPP

on these properties also depend on each fruit and variety. In addition, not only the color changes caused by the treatment itself are important for the industry, but also the evolution of the juice color after the processing.

From an industrial point of view, the applicability of a new technology is always subjected to prove its effectiveness on each one of the varieties that each company uses for its final product, which always comes from a very specific variety combination that gives it an organoleptic and nutritional profile. Thus, assessment of the effect of high-pressure processing at different temperatures on PPO inactivation and color changes in juices from different varieties still has to be investigated in most fruits.

2. Objectives and Working plan.

Although it is widely accepted as one of the most important enzymes in fruit and vegetables handling and processing, there are several issues concerning polyphenol oxidase that still require further research. This Thesis attempts to improve this knowledge in some of these aspects, by carrying out a progressive and thorough approach to those phenomena related to PPO that have been previously described.

Such progressive approach must consider different matters, from the characterization of PPO activity in underused fruits that could fulfill the consumers' demands for innovative products, to kinetic information about its inactivation that may be useful in the design of innovative fruit juices processing technologies. These techniques include UV-vis irradiation, in which the knowledge about PPO inactivation and its side effects on the product quality is quite limited, and high-pressure processing, in which more detailed studies would be useful from an industrial point of view. In addition, as far as melanin synthesis by PPO activity is concerned, the kinetic behavior of the enzyme and the different variables that may intervene in the process have been barely studied. And moreover, according to their natural functions in vegetable organisms and other related evidence, these melanins may have potential negative effects on health, which have neither been proved.

On account of these reasons, the main aims and the specific objectives to be pursued in this Thesis are listed next.

2.1. Aims & Objectives.

Main aims.

a) Characterization of PPO activity in different media, assessing the effects of different kinds of variables and providing useful information for future applications.

b) Evaluation of the potential toxic effects of melanins on some proteases.

c) Detailed study of UV-vis irradiation as an alternative technology for PPO inactivation in fruit juices, in four steps: mathematical modeling, study on model solutions, study on real fruit juices and study of the side effects on different final products.

d) Study of PPO inactivation and side effects of high-pressure processing at different temperatures of apple juices made from different varieties.

Specific objectives:

a1. To characterize PPO activity in underutilized tropical fruits with high agroindustrial potential, such as lulo, mangosteen, Castilla blackberry, mango, apple guava, tree tomato, papaya, soursop, banana passionfruit, giant granadilla, sweet granadilla and passionfruit.

a2. To evaluate the effect of nitrogen fertilization on PPO activity in peach.

a3. To develop and check the validity of kinetic expressions to describe and predict melanin synthesis from PPO activity using spectrophotometric measurements. One kinetic expression must be developed for melanin synthesis from monophenolic substrates, and another one for PPO activity on *o*-diphenolic ones. Such models must be useful to assess the influence of pH and substrate type and concentration on melanogenesis.

b1. To assay if melanins act as carboxypeptidases and trypsin inhibitors and, in that case, to determine the kind of inhibition and its kinetic parameters.

c1. To develop a mathematical tool to describe UV-vis irradiation processes that provides information about radiation penetration.

c2. To study PPO inactivation by UV-vis irradiation in model solutions containing the enzyme and different polymers that can be found in fruit juices, assessing its potential protective effect.

c3. To study PPO inactivation by UV-vis irradiation in apple, pear and grape juices made from different varieties, assessing the side effects on different properties.

c4. To infer the side effects of UV-vis irradiation of musts from different grape varieties for PPO inactivation in the final wines chemical quality, evaluating this technology as an alternative to replace or reduce SO_2 utilization in winemaking.

d1. To study PPO inactivation in apple juices made from different varieties processed by high hydrostatic pressure at different temperatures.

d2. To assess the side effects on color parameters of apple juices made from different varieties after high hydrostatic pressure processing at different temperatures.

2.2. Working plan.

In the pursuit of the abovementioned objectives, the general working plan was structured in the following stages:

1. Thorough bibliographic review of all the different issues that will be addressed, and determination of the specific samples and variables to be analyzed in each step.

2. Analytical and statistical methods preparation and optimization.

3. Analysis of PPO activity (among other parameters) in the mentioned tropical fruits from the Colombian region of Tolima (at Universidad del Tolima, Ibagué, Colombia).

4. Analysis of PPO activity (among other parameters) in peach samples from trees with different nitrogen fertilization levels, supplied by an UdL-IRTA (Universitat de Lleida – Institut de Recerca i Tecnologia Agroalimentàries, Lleida) project that aims to study the effects of combined irrigation and nitrogen levels on peach quality.

5. Detailed kinetic study of melanogenesis from commercial *Agaricus bisporus* PPO and two kinds of substrate: L-tyrosine (monophenol) and 4-methylcatechol (*o*-diphenol). Development and test of mathematical expressions that give practical information about both melanin synthesis processes and, by means of their kinetic parameters and multivariate statistical techniques, analysis of the most important variables.

6. Synthesis and recovery of melanins from commercial *Agaricus bisporus* PPO and both kinds of substrate, and inhibition assays with carboxypeptidase A, carboxypeptidase B and trypsin. Determination of the inhibition parameters.

7. From the available mathematical expressions, development of UV-vis irradiation modeling of the UdL Food Technology Department reactor. The obtained expressions must allow calculating, among other parameters, the radiation that reaches every point of the reactor as a function of the reaction medium depth and absorbance.

8. Study of commercial *Agaricus bisporus* PPO inactivation by UV-vis irradiation in model solutions containing different kinds of melanins and melanoidins.

9. Study of PPO inactivation by UV-vis irradiation in juices from Golden, Starking, Fuji and King David apples, Abate Fétel, Passa Crassana, Ercolini, Flor de invierno, Blanquilla and Conference pears, and Red Globe, Emperor, Victoria and Dauphine grapes. Assessment of the side effects on some properties such as color, physicochemical parameters and the activity of other enzymes.

10. UV-vis irradiation of musts from different grape varieties commonly used in winemaking (Cabernet Franc, Xarel·lo and Parellada), and assessment of different quality parameters of the obtained wines after vinification. Such parameters should be compared with those of the wines obtained for control samples using no treatment and SO_2.

11. Study of the effect of the variety on apple juice PPO inactivation by high-pressure processing, and its consequences on color parameters. Selected varieties include Braeburn, Fuji, Gala, Golden Delicious, Granny Smith and Red Delicious (at the Center for Nonthermal Processing of Food, Washington State University, Pullman, USA).

12. Thorough discussion of the results, conclusions writing and publication of these studies in form of conference contributions and scientific papers.

3. Materials and Methods.

3.1. Materials.

Juices from tropical fruits for characterization of its PPO activity.

Lulo var. Quitoense (*Solanum quitoense*), mangosteen var. purple (*Garcinia mangostana*), Castilla blackberry (*Rubus glaucus* Benth), mango var. Tommy Atkins (*Mangifera indica*), apple guava var. Klom sali (*Psidium guajava L.*), tree tomato var. Gold (*Cyphomandra betacea*), papaya var. yellow (*Carica papaya*), soursop var. Elita (*Annona muricata*), banana passionfruit var. mollissima (*Passiflora tripartita*), giant granadilla (*Passiflora quadrangularis L.*), sweet granadilla var. Valluna (*Passiflora ligularis Juss*) and passion fruit var. yellow (*Passiflora edulis f. flavicarpa*) were purchased from a local market located in Ibagué (Colombia). These fruits were washed, peeled and kept in water while waiting to be cut and squeezed with a household juicer. The resulting juices were centrifuged using a Hettich EBA 20 centrifuge (Andreas Hettich GmbH & Co., Tuttlingen, Germany) for 20 minutes at 2,500 rpm. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. All these steps were carried out in a refrigerated room (temperature 20±1ºC) in order to slow enzymatic processes in the juices. Fractions of 250 mL of juice were stored in the freezer at -12ºC for a maximum of two days. All the determinations were carried out on portions of the same sample.

Peaches for determination of nitrogen fertilization influence on PPO activity.

Eleven-year-old peach trees (*Prunus persica* (L.) Batsch. cv. Andross) on GF-677 rootstock were used and trained to a free palmeta. The trees were planted on shallow loam soil at 5.0 m by 2.8 m at a commercial peach orchard for processing (preparing processed purées) in the horticultural zone of Lleida (Northeast of Spain). Annual precipitation was 355 mm and annual reference evapotranspiration 1,172 mm. Soil organic matter concentration was 3.2%.

A randomized complete block design with four repetitions was established. Three nitrogen fertilization treatments were evaluated: 0 kgN/ha (N-0), 60

kgN/ha (N-60) and 120 kgN/ha (N-120). An automated drip-fertigation system with auto compensated emitters was used. Each elementary plot consisted of 27 trees distributed in three rows and determinations were done on the 5 central trees. Trees were mechanically harvested on August 5th, 2011, with a continuous trunk shaker. Sampling was done the previous day, and fruit samples consisted of five fruits per individual plot.

***Agaricus bisporus* PPO and substrate solutions for kinetic modeling.**

Agaricus bisporus PPO (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U/mL, distributed in aliquots of 1 mL and frozen at -12ºC until use. This solution was stored at 4ºC since 12 hours before experiments started, and then pre-incubated at room temperature for 1 hour. L-Tyrosine and 4-methylcatechol (Sigma Chemical, St. Louis, MO) were prepared in sodium phosphate buffer (pH of 5.0, 6.0, 7.0 and 8.0) in a concentration range from 0.5 mM up to 5.0 mM (0.5, 1.0, 2.5, 4.0 and 5.0 mM). For polymerization kinetics determination, two melanin samples were prepared with every concentration of 4-methylcatechol. After 24 h of reaction, PPO was inactivated boiling the solution for 2 min. Then, the preparation was ultrafiltrated using a 300 kDa cut-off ceramic membrane in order to separate melanin polymers and obtain a permeate with only colorless intermediate products.

Melanin synthesis and recovery (for inhibition assays and model solutions).

Agaricus bisporus PPO (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U/mL, distributed in aliquots of 1 mL and frozen at -12ºC until use. This solution was stored at 4ºC since 12 hours before experiments started, and then kept at room temperature for 1 hour. L-Tyrosine (monophenol) and 4-methylcatechol (*o*-diphenol) (Sigma Chemical, St. Louis, MO) were prepared in sodium phosphate buffer (pH 6.0 and 8.0 respectively, in a concentration of 4.0 mM. The final enzyme content in the reaction mixture was 10 U/mL.

After 24 h of reaction, melanins were precipitated adjusting the solution pH to 2.0 with HCl. The mixture was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 12 minutes at 12,000 rpm. The supernatant was discarded and the pellet was recovered with distilled water. Melanins were lyophilized and rediluted in DMSO. Preliminary results showed that the obtained polymers were the same if the variables *type of substrate*, *substrate concentration*, *pH* and *time of reaction* (before starting the recovery process) were kept constant. Assays carried out by means of an ultrafiltration device showed that keeping these variables constant the proportion of polymer that was retained by a 300-kDa membrane was also constant.

Melanoidin synthesis, purification, fractionation and recovery (for model solutions).

For melanoidin synthesis, a solution was prepared by dissolving 300 g of fructose (Fru) and 2 g of glutamic acid (Glu) in 1 L of distilled water, which was kept at 95 ºC for 13 days (Ibarz *et al*., 2009a). After the thermal process, the solution was passed through a 5-cm-diameter glass column with a fiberglass plate in the bottom that contained a 10-g layer of activated carbon. The melanoidins were adsorbed to the activated carbon, which was then washed with distilled water until the outgoing solution showed no reaction to reducing sugars (by Fehling's reagent reduction method). After sugar surplus removal, the melanoidins were recovered by passing a 25% pyridine solution through the activated carbon layer. After filtering with a Whatman paper no. 1, the solvent was partially removed by rotovaporation (Resona Technics, Switzerland). Finally, the resulting pre-concentrated melanoidins were lyophilized. Afterwards, half of the obtained melanoidins were dissolved in distilled water, and a fractionation was carried out with a tubular ceramic membrane system of 150-kDa cut-off (Tami Industries, Nyons, France), in order to separate low and high molecular mass polymers. The obtained fractions were lyophilized once again.

Apple, pear and grape juices for UV-vis irradiation.

In a similar way to tropical fruits, apples from the four different varieties (Golden, Starking, Fuji and King David), pears from the six studied varieties

(Abate Fétel, Passa Crassana, Ercolini, Flor de invierno, Blanquilla and Conference) and grapes from the four assessed varieties (Red Globe, Emperor, Victoria and Dauphine) were washed and kept in water while waiting to be squeezed with a household juicer. The resulting juice was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 25 minutes at 13,000 rpm. This process was done at 4°C to make separation of supernatant easier and to reduce enzymatic activities during this stage. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. Fractions of 800 mL of each juice were stored in the freezer.

Must preparation for wine studies.

First study

For the first study, grapes from a red variety (Cabernet Franc) and a white one (Xarel·lo) were provided by local farmers (Manresa and Sant Sadurní d'Anoia respectively, Barcelona, Spain). The fruits were washed, squeezed with a household juicer and pressed. The resulting juice was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 30 min at 13,000 rpm, at 5ºC. As usual, after centrifugation was complete the supernatant was recovered and the pellet was discarded. The must was separated in fractions of 800 mL. Four different batches of grapes from each variety were used. The must from each one of these four batches was split into two different fractions, one of which was irradiated. Therefore, four samples for each combination variety - irradiation/no irradiation were vinified.

Second study

After the preliminary results obtained in the first study, a second one was conducted. In this case, grapes from two white varieties, Xarel·lo and Parellada, were provided by local farmers (Raimat –Lleida- and Sant Sadurní d'Anoia -Barcelona-, respectively). The musts were prepared exactly as described in the first study. From every batch, the juice was split into different fractions of 800 mL, half of which were frozen. The resulting samples were divided in three groups, one of which was vinified without further processing, while another group was vinified after SO_2 addition (50 mg/L of potassium metabisulphite, as suggested by the industry) and the last group was vinified after UV irradiation.

As a result, four samples (from different batches) for each combination variety (Xarel·lo/Parellada) – treatment (no treatment/SO_2/irradiation) – fresh/frozen must were vinified (12 possible factor combinations, as shown in Figure 3.1.1).

Figure 3.1.1. *Variable combinations that describe the assayed samples in the second wine study.*

Apple juices for high-pressure processing.

Apples from six different varieties (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith and Red Delicious) were purchased from a local supermarket of Pullman (WA, USA). All these apples were produced in the state of Washington and were commercially available the last week of July, 2011. The apples were washed, cut in four pieces and kept in water while waiting to be squeezed with a household juicer. The resulting juice was centrifuged in a Sorvall RC6+ centrifuge (Thermo Fisher Scientific Inc., Waltham, USA) for 15 min at 7,500 rpm. As usual, this process was carried out at 4ºC to make separation of supernatant easier and to reduce enzymatic activities during this stage. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. Fractions of 30 mL of juice were placed in 0.1016 mm thickness polyethylene bags (Consolidated Plastics Company, Inc., Twinsburg, USA), thermo-sealed and stored in the freezer at -20ºC.

3.2. Methods.

Physical and chemical analyses carried out on juices and wines.

pH

The pH of the juices from tropical fruits was measured with a Handylab pH 11 pHmeter (Schott Instruments GmbH, Mainz, Germany). In the other cases, pH was assessed by means of a Crison micropH 2000 pHmeter (Crison Instruments, S.A., Alella, Spain).

Soluble solids content

Soluble solids content in juices from tropical fruits was assessed using an HP Handheld Brix 35HP refractometer (Reicherd Instruments, GmbH, Seefeld, Germany). In the other samples, soluble solids content was measured using an Atago RX-1000 digital refractometer (Atago Co. Ltd., Japan).

Color measures

Juices and wines color was monitored using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Japan) in the CIELab color space. For tropical fruit juices, the color measures were carried out immediately after juice extraction and after 90 minutes keeping juices at room temperature (30ºC).

Absorbance spectra

Absorbance spectra of the different samples between 250 and 750 nm were measured with a Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1 cm width optical glass cell. In tropical fruit juices, these measurements were accomplished on 1:10 dilutions of the samples with distilled water and at two different times: on the fresh juices and after 24 h with the samples kept at room temperature (30 ± 1ºC).

Total phenolic content

Total phenolic content was determined as described by Shaghaghi *et al.* (2008), with a spectrophotometric method using the Folin-Ciocalteu reagent.

Formol index

Formol index was measured according to the official method of the International Federation of Fruit Juice Producers (IFFJP, 1984), with a potentiometric titration of the acidity of the compounds formed by the reaction of formaldehyde and amino acids in the juice up to a pH of 8.1.

Sugars

Total and reducing sugars were determined by the reduction of the Fehling reagent, according to the method of the IFFJP (1972).

Titratable acidity

Titratable acidity was also assessed by the official method of the IFFJP (1996), with a potentiometric titration with sodium hydroxide to a pH value of 8.1. Unless otherwise indicated, results are expressed as g of malic acid per L of juice. In peach fruits for the study of nitrogen fertilization effects, titratable acidity was obtained from 10 mL of juice of each sample, adding 10 ml of distilled water and titrated with NaOH 0.1 N.

Vitamin C

Vitamin C was quantified by the dicloroindophenol method, using a metaphosphoric/acetic acid solution as stabilizing agent (AOAC, 2000). Unless otherwise stated, the results were expressed as ascorbic acid mg/100 mL.

Maturity index

Maturity index was calculated as the ratio of total soluble solids to titratable acidity (Ladaniya, 2008).

Viscosity

Viscosity was assessed at 20ºC using a Haake RS-80 RheoStress Rheometer (Gebrüder Haake GmbH, Karlsruhe, Germany) with a Z40-DIN coaxial-cylinder

sensor system (radii ratio 1.0847). For temperature control, a Thermo Haake C25P bath (Gebrüder Haake GmbH) was used, using a glycol–water solution (50% w/w) as coolant fluid, which allows an interval variation of 0.2ºC. The samples were sheared at a constant shear rate of 400 s^{-1} for 3 min, after which a downward ramp to 0 s^{-1} and another upward ramp until 400 s^{-1} were accomplished. The average shear stress values of these two ramps were used to calculate the juice viscosity by means of Newton's equation, since all the samples showed Newtonian behavior (Ibarz *et al*., 2009c).

Additional parameters measured on peach fruits

Mesocarp firmness was determined with a manual penetrometer (Penefel, France). Results are expressed as N. In order to assess consistency, the fruits were crushed and homogenized. Consistency was measured using a Bostwick consistometer (Central Scientific Co., Alexandria, VA) in centimeters of flow per 30 seconds.

Additional parameters measured on wines

In wine samples, tartaric acid content, alcoholic degree and volatile acidity were measured by means of near infrared spectroscopy (NIR) with a Foss WineScan FT120 analyzer (FOSS Analytical, Hillerød, Denmark).

Enzymatic activities.

Polyphenol oxidase

PPO activity in the samples was assayed measuring the increase in absorbance at 420 nm using catechol as a substrate, prepared in McIlvaine buffer solution. The reaction was carried out in a 1 cm light path optical glass cell, containing 2.5 mL L-tyrosine or 4-methylcatechol (as indicated in each case) at different concentrations and 1.0 mL of the sample. A data point was taken every 120 s with VisionLite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). Unless otherwise stated, one unit of PPO was defined as the amount of enzyme that caused the increase of one unit of absorbance at 420 nm in one minute (Ülker-Yerlitürk *et al.*, 2008). In the case of PPO determination in all juices, the substrate solution was 5 mM 4-methylcatechol, except for tropical fruit juices, where it was 5 mM catechol.

For PPO activity assessment in peach fruits, 10 g of crushed peach flesh were mixed with 10 mL of McIlvaine buffer (pH 6.6) and 0.51 g (2.5% w/v) of polyvinylpolypyrrolidone (PVPP) as phenolic scavenger. The mix was homogenized and centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 10 min at 5,500 g (RCF) and 5ºC. After centrifugation was complete, the pellet was discarded and the supernatant was used for PPO analysis.

In order to test the kinetic models developed for PPO activity, the method for its determination was quite different. Three hundred μL of the PPO preparation (150 U) were added to 15 mL of substrate solution (final enzyme content: 10 U/mL), and the absorbance was recorded for 600 min. For polymerization kinetics determination, the absorbance of the ultrafiltration permeate with intermediate reaction products was monitored for 600 min.

Peroxidase

Peroxidase (POD) activity was determined by the method described by Kwak *et al.* (1995) using pyrogallol as a substrate. The standard assay reaction mixture contained, in a total volume of 3 mL: juice sample (2.2 mL), 100 mM K-Pi buffer (pH 6, 0.32 mL), 5% pyrogallol (0.32 mL, w/v) and 0.147 M H_2O_2 (0.16mL). The reaction was initiated by the addition of H_2O_2, and the increase in A_{420} was recorded for 3 min. One unit of POD activity was defined as that forming 1 mg of purpurogallin from pyrogallol in 3 min at pH 6.

Pectinmethylesterase

Pectinmethylesterase (PME) activity was measured by determining free carboxyl groups formed as a result of enzyme action on pectin. The reaction mixture was composed of 40 mL of the 1% pectin solution in 0.15 mol·L^{-1} NaCl and 10 mL of the juice sample. The pH of this mixture was adjusted to 7.7 with 0.05 mol·L^{-1} NaOH. One hundred μL of 0.05 mol·L^{-1} NaOH were added to the reaction mixture, and the time needed to recovery the pH of 7.7 (50 ± 2ºC) was measured. One unit of PME was defined as the amount of enzyme that released 1 μmol of carboxyl groups in 1 min (de Assis *et al*. 2007).

Polygalacturonases (PG)

Exo-polygalacturonase (exo-PG) activity was assayed in a mixture containing 1% pectin solution (CP Kelco, Atlanta, USA) in 0.2 mol·L^{-1} sodium acetate buffer (pH 5.5) at 50 ºC for 10 min. The number of reducing groups, expressed as galacturonic acid released by enzymatic action, was quantified by the DNS method. One unit of enzyme activity was defined as the amount of enzyme releasing 1 mmol of galacturonic acid per minute under these assay conditions (Silva *et al.*, 2007).

Endo-polygalacturonase (endo-PG) was measured viscosimetrically by adding 10 mL of juice sample to 40 mL of 0.2 M citrate–NaOH buffer (pH 5.5) containing 3% of pectin. The reaction mixture was incubated at 50 ºC for 15 min, after which its viscosity was determined with a Haake RS80 rheometer (Gebrüder Haake GmbH, Karlsruhe, Germany). One unit of enzyme activity was defined as the amount of enzyme that reduced the initial viscosity by 50% per minute, under these conditions (Silva *et al.*, 2007).

Carboxypeptidase A (CPA)

The principle of the enzymatic assayed reaction is the action of bovine CPA (Sigma Chemical, St. Louis, MO) on hippuryl-L-phenilalanine (Hip-Phe) in Tris buffer at pH 7.5 with the presence of 0.5 M NaCl at 25 °C, giving hippuric acid and L-alanine (Bergmeyer *et al.*, 1974). This reaction can be followed measuring the solution absorbance at 254 nm with a 1 cm width quartz cell, using an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and monitoring the results with the Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). A data point was taken every 3 s. CPA activity was expressed as the absorbance variation for each second and proteic amount of the enzyme (ΔA_{254} s^{-1} μg^{-1}).

Carboxypeptidase B (CPB)

In the same way, the principle of this enzymatic reaction is the action of porcine CPB (Sigma Chemical, St. Louis, MO) on hippuryl-L-arginine (Hip-Arg) in aqueous solution at pH 7.65 with 0.1 M NaCl and 25 °C, giving hippuric acid and L-arginine (Bergmeyer *et al.*, 1974). This reaction is followed, as in the case

of CPA, measuring the solution absorbance at 254 nm and monitoring the results with the Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). A data point was taken every 3 s. CPB activity was expressed as the absorbance variation for each second and proteic amount of the enzyme (ΔA_{254} s^{-1} μg^{-1}).

Trypsin

In this case, the principle of the enzymatic assayed reaction is the action of bovine trypsin (Sigma Chemical, St. Louis, MO) on Nα-benzoyl-L-arginine ethyl ester (BAEE) in aqueous solution at pH 7.6 and 25 °C, giving Nα-benzoyl-L-arginine (Bz-L-Arg) and ethanol (Bergmeyer *et al*., 1974). This reaction can be followed measuring the solution absorbance at 253 nm with a 1 cm width quartz cell, using a Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and monitoring the results with the Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). A data point was taken every 3 s. Trypsin activity was expressed as the absorbance variation (formation of Bz-L-Arg) for each minute and protein amount of the enzyme (ΔA_{253} s^{-1} μg^{-1}).

Enzyme inhibition assays.

Carboxypeptidases A and B inhibition assays

Experimental series were carried out in a substrate concentration range from 0.1 to 0.5 mM, since higher substrate concentrations led to inhibition by substrate. A CPA standard solution containing 10 U/mL (51.25 U/mg protein) was prepared and used for melanin from L-tyrosine inhibition analysis. Another CPA standard solution containing 10 U/mL (80.00 U/mg protein) was used for melanin from 4-methycatechol inhibition analysis. These differences are due to batch variability in the commercial enzyme purchased from Sigma. In the same way, a CPB standard solution containing 7 U/mL (140 U/mg protein) was used for melanin from L-tyrosine inhibition analysis, while another CPB solution with 7 U/mL (168 U/mg protein) was used for melanin from 4-methylcatechol assays.

Table 3.2.1 shows the concentrations of the standard melanin solutions, which were prepared in DMSO. These melanin concentrations were found to be the optimal ones for quantifying the inhibitory effect after several previous experiments, considering the polymer solubility, the resulting absorbance of the solution and the enzyme-substrate-inhibitor interaction degree. As it was reported by Ibarz *et al*. (2008a) in the case of melanoidins, no clear tendency was seen in the experimental results for higher melanin contents.

Table 3.2.1. *Melanin concentrations in the standard solutions used for inhibition analysis.*

Enzyme	Melanin type	Melanin concentrations (mg/mL)
CPA	L-tyrosine	1, 2, 4
CPA	4-methylcatechol	0.205, 0.41, 0.82
CPB	L-tyrosine	3, 4, 8
CPB	4-methylcatechol	0.205, 0.41, 0.82

The reaction mixture contained 2.9 mL of substrate (with one of the five different concentrations assayed) and 200 μL of a melanin solution (results are expressed as a function of final melanin content in the cell, in mg/mL). One hundred μL of the enzymatic solution were added to the different mixtures (final enzyme content: 6.125 μg/mL in the system CPA-L-tyrosine, 3.85 μg/mL in the system CPA-4-methylcatechol, 1.654 μg/mL in the system CPB-L-tyrosine and 1.254 μg/mL in CPB-4-methylcatechol) and the absorbance evolution was monitored. The differences in final enzyme content in μg/mL are due to differences in the enzymes provided by the manufacturer, since the solutions were prepared to achieve the final equivalent activity (10 U/mL for CPA and 7 U/mL for CPB, also with the data provided by the manufacturer).

Trypsin inhibition assays

Experimental series were carried out in a substrate concentration range from 0.2 to 0.6 mM, since higher BAEE concentrations led to inhibition by substrate (Ibarz *et al*., 2009a). A trypsin standard solution containing 500 U/mL and three melanin solutions with concentrations of 0.82, 0.41 and 0.205 mg/mL in DMSO were prepared. The reaction mixture contained 3 mL of substrate and 300 μL of a melanin solution (final melanin contents: 0.07, 0.035 and 0.0175 mg/mL). Twenty μL of the enzymatic solution (10 U) were added to the different mixtures (final enzyme content: 0.22 μg/mL) and the absorbance at 253 nm evolution

was monitored. Blank experiments were carried out in order to prove that there was not any interference of the different substances (mainly melanin and DMSO) with the absorbance at 253 nm.

UV-vis irradiation.

UV-vis irradiation was carried out in a dark chamber containing the appropriate sample and the lamp. The sample was placed in a methacrylate tank of 22x15x10 cm. 800 mL of liquid were processed, reaching a height of 2.4 cm inside the tank. A refrigeration system consisting of a metallic coil fed with 50% ethylene glycol was used to control temperature and avoid heating. Sample temperature was kept at 25 °C ± 1 °C in all experiments. A magnetic stirrer was used during irradiation to ensure that the entire sample had the same concentration of all its components at any time.

UV-vis radiation was produced with a Philips HPM-12 medium-pressure mercury lamp of 400W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands). The real incident energy, determined as described by Esplugas & Vicente (1991), was $3.88 \cdot 10^{-7}$ Einstein·min^{-1} (Ibarz *et al.*, 2009b). Figure 3.2.1 shows the emission spectrum (data provided by the lamp manufacturer). The distance between the sample surface and the lamp was 22.5 cm.

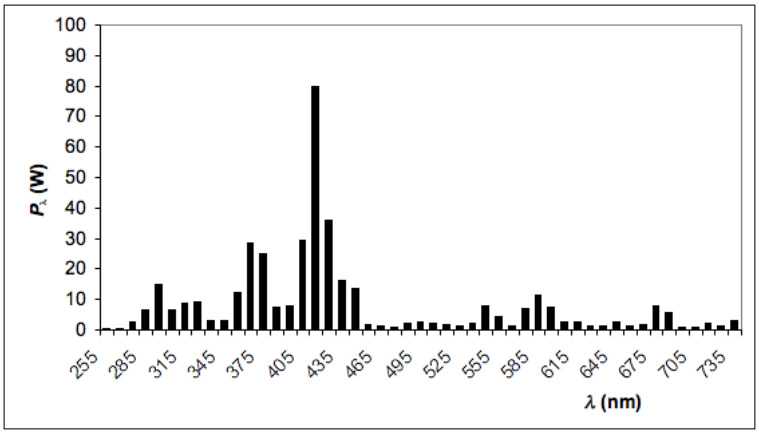

Figure 3.2.1. *Emission spectrum of the medium-pressure mercury lamp used for UV-vis irradiation.*

Irradiated juices were removed from the freezer 2 hours before irradiation started, while model solutions were freshly prepared every day. The lamp was lit 10 minutes before putting the juice in the chamber. Irradiation was carried out for different times in each kind of experiment. In the case of juices, a sample was taken every 20 minutes. Additional samples were taken at 5, 10 and 15 min to analyse peroxidase activity.

For model solutions, each sample contained 12.5 $U \cdot mL^{-1}$ of PPO in sodium phosphate buffer (pH 6.5) and different melanin or melanoidin concentrations. A sample was taken every minute during the first 10, and an additional one every 5 min until the experiment finished. In both wine studies, samples were taken at 0, 60, 120, 180 and 210 min and immediately analyzed.

Vinification.

All processed and unprocessed must samples were fermented in the Raimat winery (Codorníu Group, Lleida, Spain). Five hundred mL of each must were placed into a glass bottle. Once the must had reached 20ºC, it was inoculated with 10 mL (2% v/v) premix of QA-23 commercial yeast (Lallemand Inc., Montréal, Canada), which was prepared with 10 g of active dry wine yeast and 100 mL of water at 40ºC. 0.1 g of diammonium phosphate (DAP) were added as fermentation activator. Each bottle was covered with an S-shape airlock (*bubble*). During the fermentation, the temperature was kept at 20±1ºC. Temperature and density were measured every 24 h. It was considered that the fermentation had finished when density did not change in three consecutive days. The end of the fermentation was also checked with a sugar analysis by means of the method described by Rebelein (1973). Yeast and solid remains were separated by decantation. Then, 375 mL glass bottles were filled with the wine until 55 mm of the top. The remaining space was filled with CO_2-N_2 protective atmosphere and the cork was placed.

High-pressure processing.

Polyethylene bags containing apple juice from the six different varieties were placed into a cylindrical liner made of white polypropylene (internal diameter 75 mm, external diameter 100 mm, height 21.5 mm; McMaster-Carr, Atlanta, USA).

The liner with samples was placed inside the cylindrical chamber vessel (internal height 25 cm, diameter 10 cm) of the high pressure equipment (Engineered Pressure Systems, Inc., Andover, USA). The unit was operated with an electrohydraulic intensifier pump (Hochdruck-Systeme GmbH, AP 10-0670-1116, Sigless, Austria) that pressurized the vessel to operating pressure in a few seconds (average 7 s). A 5% Mobil Hydrasol 78 water solution was used as pressure medium. In order to carry out the treatment, the equipment was preheated to target nominal temperature (25, 60 and 80ºC). Pressure medium temperature was measured by means of three thermocouples placed inside the vessel. Processing pressures were 400, 500 and 600 MPa. Apple juice samples were processed for different times: come-up time (the time necessary to achieve the target pressure followed by immediate pressure release, average 7 s), 4 min, 8 min, 12 min and 16 min. Figure 3.2.2 shows pressure and compression fluid temperature records for an experiment carried out at 500 MPa and 60ºC for 4 min of holding time.

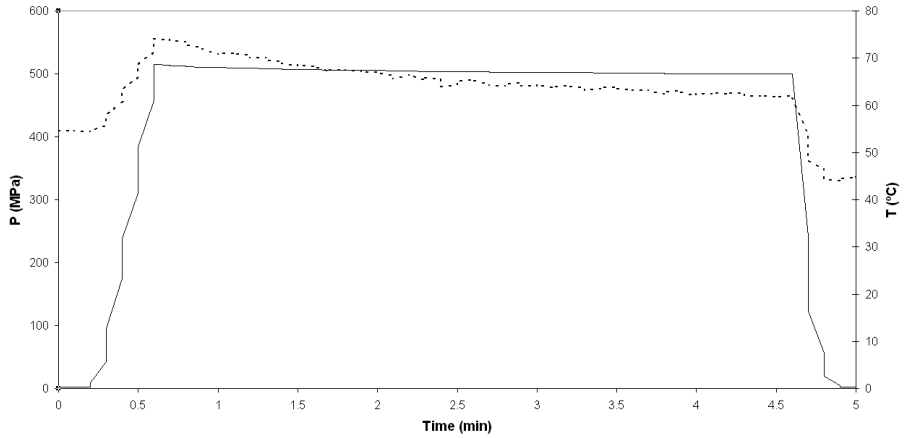

Figure 3.2.2. *Pressure (solid) and compression fluid temperature (dotted) records for an experiment carried out at 500 MPa and 60ºC for 4 min of holding time.*

Mathematical models and statistical processing.

Kinetic models used in enzyme inhibition assays

From the increase of the absorbance with the time of reaction, it is possible to obtain the maximum reaction rates for each substrate and melanin concentration, which is reached at zero time. With this aim, the monitored variation in the absorbance with reaction time can be fitted to exponential

curves:

$$A_\lambda = a - b \cdot \exp(-k \cdot t)$$

From this expression it is possible to obtain the initial reaction rate, since that is the value of its derivative at the initial time (t=0) (Ibarz *et al*., 2008a):

$$r_0 = \left(\frac{dA_\lambda}{dt}\right)_{t=0} = b \cdot k$$

From the data of the initial rate of reaction for the different substrate and melanin values used, the Lineweaver–Burk method allows to calculate the Michaelis-Menten (MM) kinetic type parameters, which are the MM constant (K_M) and the maximum reaction rate (r_{max}) (Segel, 1982). The data from this representation was adjusted to a straight line by means of the least squares method.

Kinetic models used to describe PPO inactivation

During a reaction at constant pressure and temperature, the general inactivation process can be described as:

$$N \xrightarrow{k} D$$

where *N* and *D* are the native and the inactivated form of the enzyme, respectively. These changes in the relative activity of an enzyme (RA) with time (t) can be described by a differential balance (Ramirez *et al*., 2009):

$$-\frac{dRA}{dt} = k \cdot RA^n$$

where *k* is the rate constant for the reaction at a given pressure and temperature, and *n* is the reaction order. Integration of this balance leads to expressions for zero (*n*=0), first (*n*=1) and second (*n*=2) order kinetics:

$$RA = RA_0 - k_0 \cdot t \qquad \text{(n=0)}$$

$$RA = RA_0 \cdot \exp(-k_1 \cdot t) \qquad \text{(n=1)}$$

$$\frac{1}{RA} = \frac{1}{RA_0} + k_2 \cdot t \qquad \text{(n=2)}$$

However, in some cases, enzyme inactivation is supposed to occur in two consecutive irreversible first-order steps with the presence of intermediate active forms of the enzyme (*I*), being the first one faster than the second one (Giner-Seguí *et al*., 2006):

$$N \xrightarrow{k_I} I \xrightarrow{k_{II}} D$$

and leading to the equation:

$$RA = e^{-k_I \cdot t} - \frac{k_I \cdot \Lambda}{\left(k_I - k_{II}\right)}\left(e^{-k_I \cdot t} - e^{-k_{II} \cdot t}\right)$$

being k_I and k_{II} the kinetic constants for the first and the second stages, respectively, and Λ the ratio between the activities of the intermediate (partially inactivated, *I*) and the native (*N*) forms of the enzyme.

Kinetic models used to describe color changes in high-pressure processed apple juices

In the case of color studies in HPP of apple juice, the same expressions previously described were used, changing relative activity (*RA*) into color difference (ΔE), which was calculated as:

$$\Delta E = \sqrt{\left(L^* - L_0^*\right)^2 + \left(a^* - a_0^*\right)^2 + \left(b^* - b_0^*\right)^2}$$

where L_0^*, a_0^* and b_0^* are defined as the reference color parameters measured after the come-up time treatment (the time necessary to achieve the target pressure followed by immediate pressure release) for each apple variety and processing conditions combination.

Data processing for nitrogen fertilization effects on peach PPO activity

In this case, statistical analysis of data was carried out using the SAS-STAT package (SAS®, Version 9.2. SAS Institute Inc., Cary, NC, 1989-2009). Analysis of variance (ANOVA) was carried out for Nitrogen effects. Means were compared using the Tukey HSD and contrast tests. A discriminant analysis was performed with SAS PROC CANDISC.

Non-linear regression procedures

Experimental results were fitted to the different mathematical expressions using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were always calculated at a 95% significance level.

Root Mean Square Error

In some cases, the kinetic expression describing best the activity changes as a function of time was then used to determine k_i values as a function of temperature and pressure for each apple variety. The Root Mean Square Error (RMSE) index was used to decide which model provided the best fit for the experimental data. This index was defined as:

$$RMSE = \sqrt{\frac{1}{n}\sum\left[RA_{\exp} - RA_{\text{pred}}\right]^2}$$

where n is the number of samples of each variety (i.e. considering all the experiments at different pressures and temperatures), RA_{exp} is the experimental relative activity and RA_{pred} is the predicted relative activity corresponding to each sample and fitting model. The same expression was used in the case of color studies in HPP of apple juice, but changing relative activity (RA) into color difference (ΔE).

Principal Component Analysis

Multivariate projection techniques have been used in some studies to determine the most important variables for data characterization and to see if there were any group patterns. In order to eliminate the effects of the different variable units, data autoscaling is usually performed prior to each analysis. These techniques have been widely applied for quality assessment, authentication or even classification of a great variety of food products, including vegetables, fruits, juices, wines, oils and others (Tzouros & Arvanitoyannis, 2001).

In the case of PPO characterization in tropical fruit juices, a Principal Component Analysis (PCA) of all the data obtained in the samples characterization was carried out using the Unscrambler v. 6.11a software (Trondheim, Norway) (Camo Software AS, 2010). Moreover, another group of variables was created from absorbance spectra of the samples, corresponding to the differences between the asorbance of the fresh juice and that measured 24 h later. An additional PCA was carried out with these spectrophotometrical data in order to infer the most important wavelengths to follow the color changes produced by enzymatic browning.

In the study of PPO inactivation by high-pressure processing in apple juices, the data matrix consisted of 276 samples (6 apple varieties x 3 pressures x 3 temperatures x 5 treatment times, plus 6 unprocessed samples) and 25 variables (including those defining the experiment and the ones described in the previous sections). All the values included in the matrix were the average of 6 determinations, carried out on two different sample batches. In the case of color analysis, the data matrix consisted of the same 276 samples and 1,228 variables. These variables included the ones defining the experiment and the ones described in the previous sections. Moreover, two additional groups of variables were created from absorbance spectra of the samples in the visible region, from 400 to 700 nm in intervals of 0.5 nm. The first group (601 variables) corresponded to the differences between the absorbance of the fresh juice and the absorbance measured after high-pressure processing, for each wavelength. The second group (601 variables) belonged to the differences between the absorbance immediately after HPP and 24 h later, so as to assess the evolution of juices' color (as in the case of tropical fruit juices). Additional PCAs were carried out with these spectrophotometrical data in order to infer the most important wavelengths to follow these color changes.

4.Results and Discussion.

4.1. A first approach to polyphenol oxidase activity.

According to the different reasons that have been given in the *Introduction*, polyphenol oxidase characterization is an essential issue to assess the commercial possibilities of every fruit and vegetable. That is the case, for example, of those fruits that have not been widely used for industrial purposes yet. In addition, knowing the most important variables that may be related to this enzyme may also be useful for partially avoiding its negative effects. The following sections are intended to fulfill these functions in some specific cases.

4.1.1. Polyphenol oxidase and other properties characterization in twelve tropical fruits with high agroindustrial potential.

In this first study, PPO activity of twelve fruits cultivated and locally commercialized in the Tolima region (Colombia) was evaluated and related to their composition properties. Searching for a practical application of these data, PPO characterization was carried out directly from squeezed fresh fruits, since extracted and purified enzymes don't usually show real values for enzymatic activity as when PPO is in contact with other substances inside fruits (Bora *et al.*, 2004). Polyphenol oxidase activities of the twelve analyzed fruits appear in Table 4.1.1. Mangosteen and lulo showed the highest activities (0.1435 and 0.1298 $U \cdot mL^{-1}$, respectively), while passion fruit and banana passionfruit had the lowest ones (0.0101 and 0.0093 $U \cdot mL^{-1}$).

Table 4.1.1. *Polyphenol oxidase activity of the twelve tropical fruits.*

Fruit	PPO activity ($U \cdot mL^{-1}$)	Fruit	PPO activity ($U \cdot mL^{-1}$)
Lulo	0.1298 ± 0.0002	Giant granadilla	0.0189 ± 0.0059
Mangosteen	0.1435 ± 0.0037	Tree tomato	0.0124 ± 0.0001
Castilla blackberry	0.0347 ± 0.0005	Papaya	0.0125 ± 0.0017
Mango	0.0159 ± 0.0021	Sweet granadilla	0.0321 ± 0.0006
Apple guava	0.0153 ± 0.0002	Passion fruit	0.0101 ± 0.0054
Banana passionfruit	0.0093 ± 0.0003	Soursop	0.0384 ± 0.0003

Mean value ± Standard deviation

Physicochemical properties.

Table 4.1.2 shows composition and physicochemical properties of the twelve analyzed tropical fruits. The pH of the samples, which has been reported to be one of the main factors that influence polyphenol oxidase activity (Queiroz *et al.*, 2008), had the lowest value in passion fruit (2.89) and the highest one in papaya (4.97). Total phenolics, the molecules that are susceptible to act as PPO substrates, were found to be in a range between 243.7 $mg \cdot L^{-1}$ (papaya) and 908.7 $mg \cdot L^{-1}$ (mango) of gallic acid equivalents. Cano *et al.* (1996) found that PPO activity in papaya is very limited mainly due to this low phenolics

***Table 4.1.2**. Physicochemical properties of the twelve tropical fruits.*

Fruit	pH	Titratable acidity	Formol index	Total phenolics	Soluble solids
	-	$g \cdot L^{-1}$ malic acid	mL NaOH 0.1 M/100 mL	$mg \cdot L^{-1}$ gallic acid	ºBrix
Lulo	3.45 ± 0.01	0.315 ± 0.005	37.5 ± 0.5	433.9 ± 16.5	8.1 ± 0.1
Mangosteen	3.36 ± 0.01	0.056 ± 0.002	13.5 ± 0.5	644.7 ± 42.3	16.1 ± 0.1
Castilla blackberry	3.41 ± 0.01	0.281 ± 0.002	31.0 ± 0.1	757.0 ± 31.3	7.1 ± 0.1
Mango	3.63 ± 0.00	0.061 ± 0.001	5.0 ± 0.1	908.7 ± 24.2	12.3 ± 0.3
Apple guava	3.96 ± 0.02	0.056 ± 0.002	18.5 ± 1.5	854.1 ± 5.4	7.9 ± 0.1
Banana passionfruit	3.21 ± 0.01	0.364 ± 0.001	28.5 ± 0.5	805.8 ± 29.2	7.7 ± 0.1
Giant granadilla	4.35 ± 0.02	0.140 ± 0.002	33.5 ± 0.5	335.5 ± 79.4	15.1 ± 0.1
Tree tomato	4.00 ± 0.00	0.175 ± 0.003	57.5 ± 0.5	468.5 ± 13.5	8.1 ± 0.1
Papaya	4.97 ± 0.02	0.010 ± 0.001	10.0 ± 0.1	243.7 ± 7.9	9.3 ± 0.1
Sweet granadilla	4.73 ± 0.02	0.040 ± 0.001	20.0 ± 0.1	434.9 ± 1.3	13.4 ± 0.1
Passion fruit	2.89 ± 0.02	0.441 ± 0.003	27.5 ± 0.5	433.5 ± 10.2	14.2 ± 0.1
Soursop	3.74 ± 0.01	0.116 ± 0.002	19.0 ± 0.5	285.1 ± 7.3	14.5 ± 0.1

Fruit	Total sugars	Reducing sugars	Non-reducing sugars	Maturity index[1]
	$g \cdot L^{-1}$ glucose	$g \cdot L^{-1}$ glucose	$g \cdot L^{-1}$ glucose	ºBrix·L·g^{-1}
Lulo	33.8 ± 0.9	16.8 ± 0.3	17.1 ± 0.6	26
Mangosteen	96.6 ± 7.1	25.4 ± 0.7	71.2 ± 6.5	288
Castilla blackberry	22.4 ± 0.4	13.6 ± 0.4	8.8 ± 0.1	25
Mango	90.0 ± 4.1	26.8 ± 0.1	63.2 ± 4.1	202
Apple guava	47.1 ± 0.1	45.0 ± 2.0	2.1 ± 2.0	141
Banana passionfruit	110.0 ± 6.1	61.9 ± 3.9	48.1 ± 2.2	21
Giant granadilla	82.5 ± 3.4	56.6 ± 1.6	25.9 ± 1.8	108
Tree tomato	43.5 ± 0.5	32.5 ± 2.7	11.1 ± 2.2	46
Papaya	67.1 ± 5.7	60.0 ± 1.8	7.1 ± 2.2	930
Sweet granadilla	107.0 ± 8.7	45.0 ± 0.1	62.0 ± 8.7	335
Passion fruit	74.7 ± 4.2	50.8 ± 1.3	23.9 ± 5.5	32
Soursop	99.0 ± 10.0	63.9 ± 2.1	35.1 ± 7.9	125

Mean value ± Standard deviation.
[1]Calculated as soluble solids/titratable acidity.

content. In contrast, mango showed to have the highest content, but according to Arogba *et al*. (1998) the presence of endogenous PPO inhibitors in mango tissues can limit enzymatic browning in this fruit. Although mangosteen and lulo had an intermediate total phenolics content (644.7 and 433.9 $mg \cdot L^{-1}$ of gallic acid equivalents, respectively), some of these compounds have been reported to have important antioxidant, free radical scavenging and skin protective activities, especially α-mangostin, epicatechin and tannin in mangosteen and chlorogenic acids, flavonol glycosides and dihydrocaffeoyl spermidines in lulo (Gancel *et al.*, 2008; Ngawhirunpat *et al.*, 2010). On the contrary, apple guava samples contained a higher phenolics concentration (854.1 $mg \cdot L^{-1}$), but its composition does not seem to have significant antioxidant activity (Rojas-Barquera & Narváez-Cuenca, 2009). The assayed physicochemical properties were significantly different within the four fruits from the *Passiflora* genus, which was also observed by Aular *et al.* (2004).

Color changes.

Polyphenol oxidase, as the main responsible factor for enzymatic browning, causes color changes in fruit pulps that can be easily assessed measuring CIELab parameters, especially the decrease of brightness with *L** (Lozano *et al.*, 1994). In this way, Table 4.1.3 shows these values for some of the assayed fruits. *L** decreased more in the fruits that presented a high PPO activity: 10.51 units in lulo and 9.01 units in mangosteen. In addition, lulo showed the greatest variation in *b** (a decrease of 13.12 units) and mangosteen in *a** (an increase of 5.59 units). These two fruits also showed the highest ΔE value. Although papaya had a relatively low PPO activity and consequently a low decrease of *L** was detected (1.62 units), important changes were found in the pulp color during the first 90 min after fruit crushing, leading to a decrease of 5.19 units in *a** and 6.08 units in *b**. The low PPO activity found in papaya could be attributed to the fact that the fruits were in the climacteric phase of the ripening process, and it is known that the enzyme activity significantly decreases during this stage (Cano *et al.*, 1996). In this way, these color changes can be due to other processes like natural pigments degradation or peroxidase activity (Jiang *et al.*, 2004).

Table 4.1.3. *Color parameters of tropical fruits juices: fresh and 90 min after extraction.*

Fruits	*L** 0 min	*L** 90 min	*a** 0 min	*a** 90 min
Lulo	53.64 ± 0.37	43.13 ± 0.01	-3.88 ± 0.02	-1.08 ± 0.03
Mangosteen	61.35 ± 0.13	52.34 ± 0.09	3.18 ± 0.02	8.77 ± 0.04
Castilla blackberry	29.41 ± 0.01	26.78 ± 0.03	4.65 ± 0.04	2.65 ± 0.03
Banana passionfruit	57.00 ± 0.03	55.69 ± 0.01	13.28 ± 0.02	11.92 ± 0.01
Papaya	34.34 ± 0.07	32.72 ± 0.01	16.18 ± 0.06	11.00 ± 0.01
Sweet granadilla	42.28 ± 0.01	39.04 ± 0.11	-2.27 ± 0.02	-2.32 ± 0.01
Passion fruit	45.44 ± 0.13	43.69 ± 0.01	-2.01 ± 0.03	-1.69 ± 0.01
Soursop	74.20 ± 0.01	72.65 ± 0.02	-1.75 ± 0.01	-1.97 ± 0.01

Fruits	*b** 0 min	*b** 90 min	*ΔE*
Lulo	34.34 ± 0.01	21.22 ± 0.09	17.00 ± 0.40
Mangosteen	11.86 ± 0.1	10.87 ± 0.08	10.65 ± 0.05
Castilla blackberry	1.19 ± 0.01	0.59 ± 0.01	3.36 ± 0.02
Banana passionfruit	32.4 ± 0.1	32.32 ± 0.07	1.89 ± 0.04
Papaya	17.99 ± 0.11	11.91 ± 0.03	8.15 ± 0.12
Sweet granadilla	4.72 ± 0.07	4.90 ± 0.10	3.25 ± 0.12
Passion fruit	29.74 ± 0.02	27.29 ± 0.19	3.03 ± 0.21
Soursop	8.26 ± 0.01	7.28 ± 0.02	1.85 ± 0.01

Mean value ± Standard deviation.

Principal Component Analysis.

A PCA was carried out in order to determine the variables that explain the maximum information and consequently show the general tendencies present in the samples. This data processing, which has also been applied to find useful correlations to authenticate different products such as olive oil (Arvanitoyannis & Vlachos, 2007) and honey (Arvanitoyannis *et al*., 2005), was accomplished with the values of physicochemical variables, polyphenol oxidase activity and the change observed in the CIELab parameter *L** in the first 90 min after the juice extraction, since it has been reported to be the main color indicator of fruit browning (Lozano *et al.*, 1994) (total 10 variables). Figure 4.1.1 represents variable loadings in the PC1 vs PC2 space, in which 55% of the global variance is explained. First of all, the analysis revealed that the most important variable that influences fruit variability is total sugars content, which is highly correlated with soluble solids.

This diagram also shows that there are two variables strongly related with PPO activity: total phenolics content and *L** variation. This indicates that, on the one hand, in most of the samples PPO is more active in those fruits with higher

phenolics content, as these molecules are more suitable to contact the enzyme and act as its substrate. On the other hand, PPO catalysis leads to enzymatic browning, which can be easily observed and measured by the decrease of the CIELab parameter *L**.

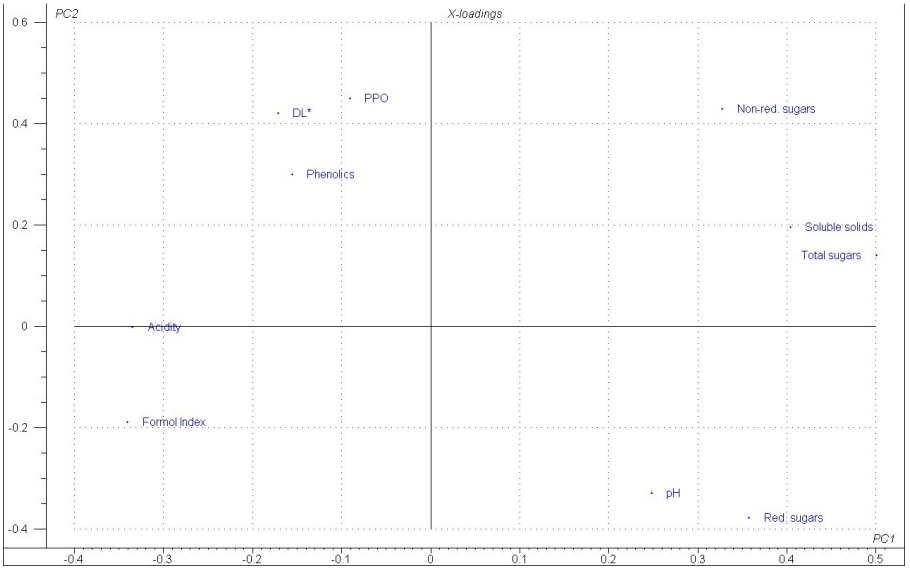

Figure 4.1.1. *Tropical fruits: Composition variable loadings represented in the Principal Component space.*

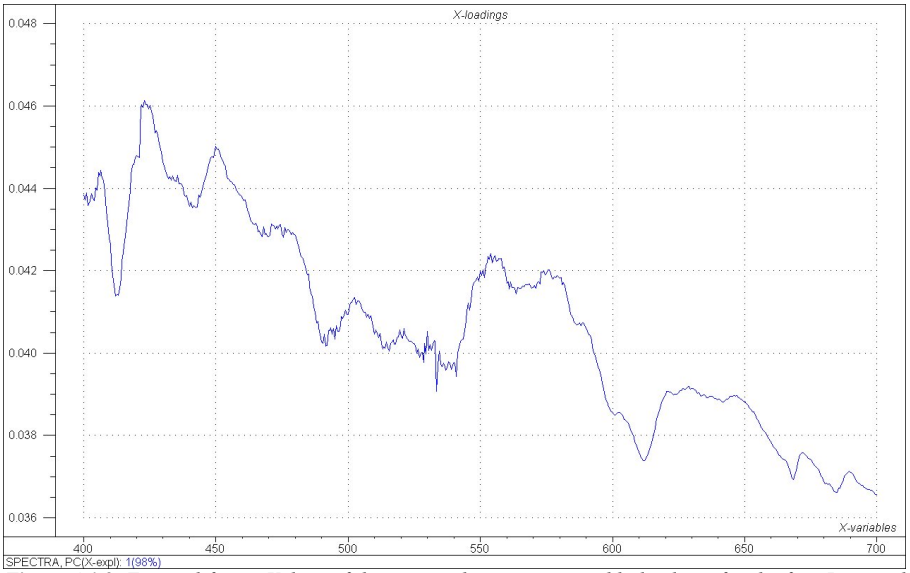

Figure 4.1.2. *Tropical fruits: Values of the spectrophotometric variable loadings for the first Principal Component, which explains 98% of the overall variance.*

The PCA carried out on the spectrophotometrical data revealed that one single PC was enough to describe 98% of the overall variance. This fact means that the existing data are highly correlated, showing that enzymatic browning affects absorbances in a similar way in all samples. Observing variable loadings of this first PC (Figure 4.1.2) an absolute maximum is found in 423.0 nm, so that the colored compounds formed by polyphenol oxidase activity in the studied fruit juices have their maximum absorbance at this wavelength, and therefore this would be the best one to follow these enzymatic changes. Sample scores (not shown) revealed that the most important samples were the ones with highest polyphenol oxidase activities.

4.1.2. Influence of nitrogen fertilization on polyphenol oxidase activity in peach fruits.

Besides the intrinsic properties of each fruit and variety that determine PPO activity, several parameters related to cultivation and growth can also have an effect on the expression of this enzyme. And among these factors, fertilization in general, and nitrogen supplementation in particular, is one of the main ones to be controlled in crop management. Since applying the appropriate nitrogen dose is important to peach production yield and quality, and PPO activity is an essential parameter to assess postharvest quality, this second piece of work aims at the characterization of the effect of nitrogen application on the expression of polyphenol oxidase in the specific case of peach fruits, besides the indirect effect that it can carry out via ripening delay. Moreover, additional maturity parameters were also assessed in relationship to the different nitrogen application levels, so as to guarantee that the used samples were in the same maturity stage.

Table 4.1.4. *ANOVA for peach polyphenol oxidase (PPO) activity ($U \cdot mL^{-1}$), mesocarp firmness (N), flesh color parameters (L*, a*, b*), maturity index ($^{o}Brix \cdot L \cdot g^{-1}$) and Bostwick's index (cm/30 s).*

	PPO	Firmness	L^*	a^*	b^*	Maturity index	Bostwick's index
model	0.0077	0.0174	ns	ns	ns	ns	0.0013
N	0.0049	ns	ns	ns	ns	ns	0.0006
BLOCK	ns	0.0127	ns	0.0483	0.0057	ns	ns
0 kg/ha	0.23^{b}	32.7	63.8	3.0	53.5	2.3	6.7^{a}
60 kg/ha	0.29^{a}	32.8	65.1	3.1	53.7	2.6	5.1^{b}
120 kg/ha	0.28^{a}	36.9	66.5	3.1	54.7	2.3	5.4^{b}

Values with different superscripts are significantly different according to the Tukey HSD test ($p < 0.05$).
ns: non-significant

The analysis of variance (Table 4.1.4) showed that, among the studied parameters, only polyphenol oxidase activity and Bostwick's index were significantly affected by nitrogen application ($p<0.005$ for the former and $p<0.0006$ for the latter). If nitrogen fertilization is applied PPO activity increases, while Bostwick's value decreases. However, in both cases there were no differences between the application of 60 or 120 kg/ha. In other words, PPO activity and consistency of peach fruits are affected by nitrogen supplementation, but an excessive dose provides no differences.

On the other hand, firmness was not found to be affected by nitrogen fertilization, meaning that in the used samples there was not a delay in fruit maturation due to nitrogen. Since all the other important parameters for assessing fruit maturity (color parameters and maturity index) do not show any differences with nitrogen application and dose, the different values for PPO activity are a direct response to nitrogen application and not to fruit maturity stage.

It is generally assumed that as fruit matures, PPO activity increases (Ferrer *et al*., 2005) and Bostwick's parameter also increases (consistency decreases) (Falguera *et al*., 2012). In addition, it was also proved that nitrogen fertilization causes a delay in the ripening process (Rufat *et al*., 2011). Generally, when nitrogen effects on fruit properties are assessed, different samples harvested at the same date are usually assayed, which therefore are in different maturity stages. This method makes it difficult to investigate the direct effects of fertilization on the different quality parameters without the influence of the ripening stage. However, the findings of this study, which was carried out on peach fruits that were statistically at the same maturity stage, showed that nitrogen application (regardless the dose) caused a decrease of Bostwick's index, but also an increase of PPO activity, i.e. as regards to PPO, nitrogen had the opposite effect to a delay of ripening.

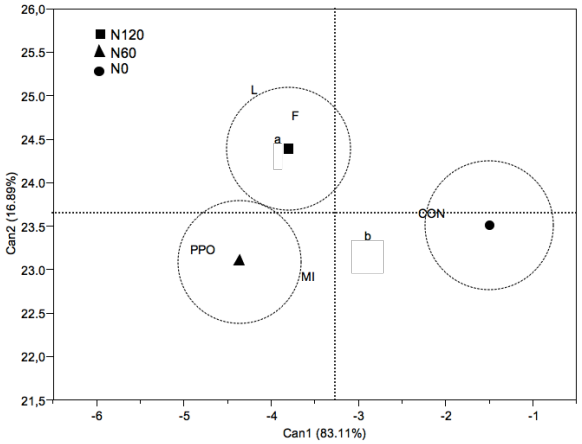

Figure 4.1.3. *Discriminant analysis: Plot of the centroids (mean values) and their 95% confidence ellipses for the first two canonical variables of nitrogen treatments. F: firmness. CON: Bostwick's consistency. MI: maturity index.*

The conclusions about the effect of nitrogen application on the assayed variables can also be reached by means of the canonical discriminant analysis (Figure 4.1.3). In this multivariate statistical technique, 95% confidence ellipses appear separated in the first canonical variable (which represents 83.1% of the overall variance and is represented in the *x* axis) according to nitrogen application (or not), while the ones that belong to the different doses (60 and 120 kg/ha) appear overlapped.

***Table 4.1.5**. Correlation coefficients between PPO activity, CIELab color parameters of fruit mesocarp, maturity index, firmness and Botswick's index.*

	Maturity index	Firmness	Bostwick's index	*b**	*a**	*L**
PPO activity	-0.33	0,14	-0,33*	0,34	-0,10	0,11
*L**	-0.06	-0,01	-0,14	0,42*	-0,45*	
*a**	0.28	-0,07	0,16	-0,03		
*b**	-0.25	0,15	-0,29			
Bostwick's index	-0.15	-0,26				
Firmness	-0.11					

** Indicates significant correlations (P < 0.05)*

As far as the relationship between the different studied variables is concerned, PPO activity and Bostwick's index, the only two variables that were strongly affected by nitrogen fertilization, were found to be negatively correlated ($p<0.05$; Table 4.1.5), which is consistent with the results observed in Table 4.1.4 and previously commented. Neither maturity index nor firmness was significantly correlated with any other variable. Moreover, among CIELab parameters, the most important (negative) correlation was found between *a** and *L**: high redness values indicate high pigments content (Westwood, 1993), which also causes a decrease of brightness. Finally, PPO activity was not found to be significantly correlated with maturity index.

4.2. Modeling of polyphenol oxidase activity.

So far, polyphenol oxidase activity has been indirectly described by the increase in absorbance caused by melanin formation after a certain time of reaction, since spectrophotometric measures are easy, fast and cheap. However, there is not any kinetic model in the literature that describes how this absorbance changes with time, providing additional information about melanogenesis and how the different variables that define the experiment can modify the process. According to the reaction mechanism (an example of which is shown in Figure 1.2.1), such kinetic expressions must be different for the cases in which PPO acts on a monophenolic substrate (where it must fulfill two functions before the reaction intermediates spontaneously lead to melanin formation) and that of its action on an *o*-diphenolic substrate (where only catecholase activity is needed before these spontaneous steps of melanogenesis).

4.2.1. Modeling of polyphenol oxidase activity on monophenolic substrates.

According to the reaction mechanism that leads a monophenolic substrate to melanins (De Faria *et al.*, 2007), an autocatalytical reaction in two steps, each one defined by a kinetic constant, has been assumed:

$$Monophenol \xrightarrow{k_a} Melanin$$
$$Monophenol + Melanin \xrightarrow{k_b} 2\,Melanin$$

If C_A is the monophenol concentration and C_M the melanin concentration, the mass balance for the global reaction in a discontinuous stirred reactor would be (Levenspiel, 1986):

$$\frac{dC_A}{dt} = -\left(k_a \cdot C_A + k_b \cdot C_A \cdot C_M\right) \qquad [4.2.1]$$

For each time of reaction, melanin concentration (C_M) can be expressed as the difference between the initial monophenol concentration (C_A^0) and the concentration at this moment (C_A):

$$C_A^0 - C_A = C_M$$

Then, using this expression in the mass balance and rearranging it:

$$\frac{dC_A}{k_a \cdot C_A + k_b \cdot C_A \cdot \left(C_A^0 - C_A\right)} = -dt$$

Now, the integration can be done with these boundary conditions:

When t = t_0 → $C_A = C_A^0$.

When t = t → $C_A = C_A$.

So then:

$$\int_{t_0}^{t} dt = \int_{C_A}^{C_A^0} \frac{dC_A}{k_b \cdot C_A \left[\left(\frac{k_a + k_b \cdot C_A^0}{k_b}\right) - C_A\right]} \qquad [4.4.2]$$

where t_0 is the induction time. The result of this integration leads to equation [4.2.3]:

$$\ln\left[\frac{C_A^0\left[\left(\frac{k_a + k_b \cdot C_A^0}{k_b}\right) - C_A\right]}{C_A\left[\left(\frac{k_a + k_b \cdot C_A^0}{k_b}\right) - C_A^0\right]}\right] = \left(k_a + k_b \cdot C_A^0\right)\left(t - t_0\right) \qquad [4.2.3]$$

Being X_A the fractional conversion of substrate, the substrate concentration in each moment can be expressed in the following way:

$$C_A = C_A^0\left(1 - X_A\right) = C_A^0 - C_A^0 X_A \qquad [4.2.4]$$

Then, using expression [4.2.4] in [4.2.3]:

$$\ln\left(\frac{1 + \frac{k_b}{k_a} C_A^0 X_A}{1 - X_A}\right) = \left(k_a + k_b C_A^0\right)\left(t - t_0\right) \qquad [4.2.5]$$

Rearranging this expression and using the Lambert-Beer law in order to obtain the absorbance as a function of the reaction time:

$$A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_a \cdot \left[\exp\left[\left(k_a + k_b \cdot C_A^0\right) \cdot \left(t - t_0\right)\right] - 1\right]}{k_b \cdot C_A^0 + k_a \cdot \exp\left[\left(k_a + k_b \cdot C_A^0\right) \cdot \left(t - t_0\right)\right]} \qquad [4.2.6]$$

where ε_λ is the extinction coefficient at a λ wavelength, l is the cell width, C_A^0 the initial substrate concentration and t_0 the induction time.

4.2.2. Modeling of polyphenol oxidase activity on *o*-diphenolic substrates.

In the case of *o*-diphenols, there is not an enzymatic reaction that depends on the reaction rate of another one. Thus, the product formation is expected to follow typical kinetics of systems with a single substrate and a single enzyme (Michaelis-Menten kinetics) (Ibarz *et al.*, 2000). However, these immediate products do not give color to the solution themselves, but the compounds resulting from later changes (melanins) are the ones that will allow the detection of an absorbance increase. Thus, if this magnitude is monitored the detected variations will depend on two factors: on the one hand, the synthesis rate of the enzyme reaction products, on the other hand, the polymerization rate of these intermediates.

If the rate of the second group of reactions (non-enzymatic, or chemical) is greater than the rate of the first one (enzymatic, or biochemical), a typical Michaelis-Menten response will be observed until the substrate is depleted. Otherwise, if the synthesis of intermediate products occurs faster than the polymerization, this characteristic absorbance curve will be concealed by the increase described as a consequence of the later stage.

Attending to the enzymatic step, from the Michaelis-Menten (MM) equation (Stauffer, 1989):

$$r = \frac{r_{\max} C_S}{K_M + C_S} \qquad [4.2.7]$$

where r is the reaction rate, r_{max} the maximum reaction rate, C_S the substrate concentration and K_M the MM constant. In the case of a discontinuous reactor and the condition of a null product concentration at a zero time, the Henri expression can be deduced:

$$C_P = r_{\max} t - K_M \ln\left(\frac{C_S^0}{C_S^0 - C_P}\right) \qquad [4.2.8]$$

where C_P is the product concentration, C_S^0 the initial substrate concentration and t the reaction time. Its linearized form can be obtained dividing all terms by the time of reaction:

$$\frac{C_P}{t} = r_{\max} - \frac{K_M}{t} \ln\left(\frac{C_S^0}{C_S^0 - C_P}\right) \qquad [4.2.9]$$

This form of the Henri equation allows obtaining the MM parameters r_{max} and K_M from experimental series in which the evolution of product concentration with time is measured.

However, in the case of the action of polyphenol oxidase the parameter that is measured is not the melanin concentration directly, but an indirect magnitude: the absorbance. Therefore, as in the case of the previous model, the Lambert-Beer law must be included in expression [4.2.9], resulting:

$$\frac{A_\lambda}{\varepsilon_\lambda\, l\, t} = r_{\max} - \frac{K_M}{t} \ln\left(\frac{C_S^0}{C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda\, l}}\right) \qquad [4.2.10]$$

The inclusion of the Lambert-Beer law introduces a new unknown parameter: the extinction coefficient ε_λ. This coefficient, which will be different for each type of melanin depending on the conditions of the experiment, can be calculated from the law itself:

$$\varepsilon_\lambda = \frac{A}{l\, C_P} \qquad [4.2.11]$$

Obviously, to use this expression it is necessary to know the product concentration, and there are only two moments in which that is possible. Firstly, as already mentioned, it is assumed that at zero time this value is null. In addition, for long reaction times, when the reaction is complete and the substrate is depleted, if the stoichometry is one-to-one the product concentration is the same as the initial substrate concentration ($C_P^\infty = C_S^0$) (Ibarz *et al.*, 2000). In this point, if there is only an enzymatic reaction, the maximum solution absorbance of this step will be reached. Thus, the increase in absorbance is defined by an exponential curve:

$$A_\lambda = A_{\max} - b \cdot \exp(-k \cdot t) \qquad [4.2.12]$$

Knowing this maximum absorbance and the product concentration in the same point, the Lambert-Beer expression that will allow the calculation of the extinction coefficient will be:

$$\varepsilon_\lambda = \frac{A_{\max}}{l\, C_S^0} \qquad [4.2.13]$$

Then, knowing ε_λ and following equation [4.2.10], a non-linear regression can be carried out with $\left[\frac{1}{t}\ln\left(C_S^0 \Big/ \left(C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda\, l}\right)\right)\right]$ vs. $\left[\frac{A_\lambda}{\varepsilon_\lambda\, l\, t}\right]$. The result will be a straight line, in which $-K_M$ will be the slope and r_{max} the intercept.

In addition, it is important to consider that for long times of reaction this enzymatic stage will have finished, and the increase in absorbance will be only due to the polymerization step, which will follow its own kinetic pattern.

4.2.3. Testing the developed models: Kinetic and multivariate analysis of melanogenesis.

In order to test the developed kinetic expressions, different experimental melanogenesis series were carried out from monophenolic (L-tyrosine) and *o*-diphenolic (4-methylcatechol) substrates using *Agaricus bisporus* polyphenol oxidase, as described in the *Materials and methods* section. Then, experimental results were fitted to the appropriate model by non-linear regression procedures, and multivariate analysis techniques were used to reinforce the conclusions and to infer more information about these processes. A step-by-step comment of the results is detailed next.

Results overview.

First of all, a PCA model was carried out over all the data in order to determine the variables that explain the maximum information and consequently show the general tendencies presented by the samples. Studying together the samples scores and the variables loadings in the Principal Component space it can be easily seen that, in the experimental conditions that have been used, the most

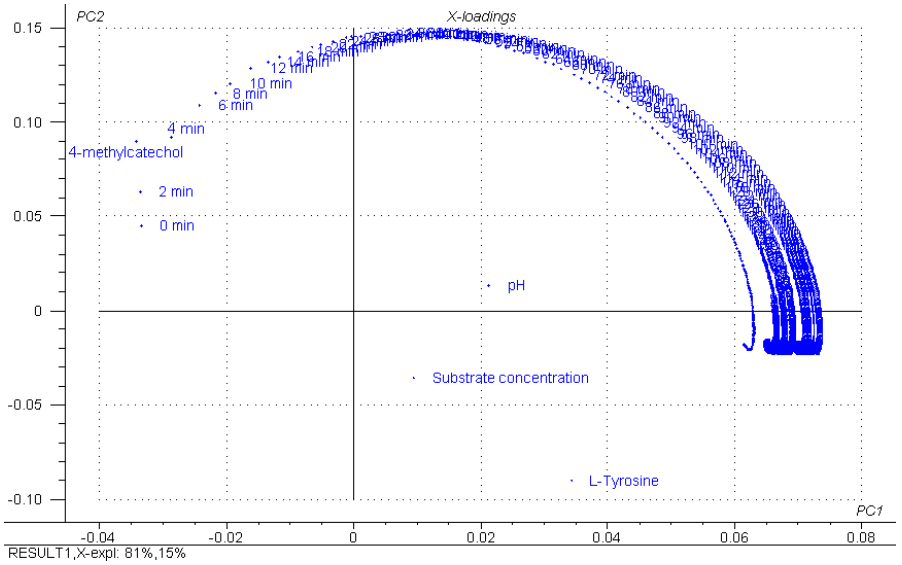

Figure 4.2.1. *Variable loadings represented in the two first Principal Components space.*

important variable that must be considered to determine the absorbance evolution (and consequently the formed melanin properties) is the kind of substrate, followed by the pH and lastly the substrate concentration (Figure 4.2.1). Nevertheless, regardless the other conditions, when the pH value is 5.0 the obtained results are similar in all cases. This can be explained by the lack of enzymatic activity in this pH, which makes that little melanin is produced and therefore high values of absorbance are not achieved. Regarding spectrophotometric variables, the time between 240 and 306 min was the period with the greatest influence on the overall variance of data.

Melanogenesis from L-tyrosine (monophenol).

When acting on a monophenol, PPO exhibits an unusual kinetic behavior due to an autocatalytical process depending on the generation of a dihydric phenol substrate, which in turn acts as an activator of the enzyme (Cooksey *et al.*, 1997). These reactions lead to a sigmoid absorbance curve preceded by a lag period. Figure 4.4.2 shows four examples of this kinetic pattern obtained with different L-tyrosine concentrations at a pH of 6.0. The absorbance data obtained with each experimental serie was fitted to equation [4.2.6]. The parameters of this statistical processing are shown in Table 4.2.1.

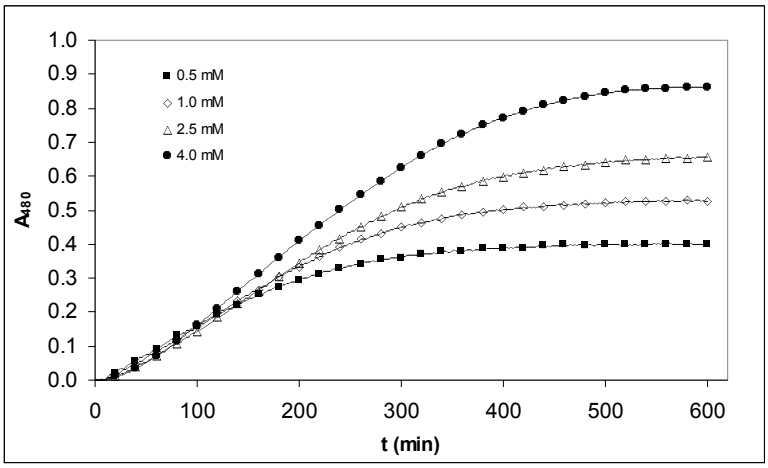

Figure 4.2.2. *Four examples of the sigmoid absorbance curve obtained in melanogenesis using different concentrations of L-tyrosine as PPO substrate in a pH 6.0 solution.*

Table 4.2.1. *Parameters of fitting experimental data from L-tyrosine series to the kinetic model:* $A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_a \cdot \left[\exp\left[\left(k_a + k_b \cdot C_A^0\right) \cdot \left(t - t_0\right)\right] - 1\right]}{k_b \cdot C_A^0 + k_a \cdot \exp\left[\left(k_a + k_b \cdot C_A^0\right) \cdot \left(t - t_0\right)\right]}$

pH (-)	C_A^0 (mM)	ε_{480} (L·mol^{-1}·cm^{-1})			k_a (min^{-1})			k_b (L·mol^{-1}·min^{-1})			t_0 (min)			R^2
5.0	0.5	0.08285	±	0.00016	0.0101	±	0.0004	0.0114	±	0.0016	5.1	±	0.7	0.9979
	1.0	0.06288	±	0.00011	0.0080	±	0.0003	0.0069	±	0.0006	6.5	±	0.7	0.9985
	2.5	0.04387	±	0.00018	0.0043	±	0.0005	0.0056	±	0.0005	8	±	3	0.9933
	4.0	0.03109	±	0.00014	0.0024	±	0.0003	0.0036	±	0.0003	9	±	5	0.9940
	5.0	0.02624	±	0.00014	0.00237	±	0.00007	0.00095	±	0.00005	11.1	±	1.6	0.9991
6.0	0.5	0.8044	±	0.0008	0.00399	±	0.00006	0.0144	±	0.0003	5.8	±	0.5	0.9998
	1.0	0.5318	±	0.0005	0.00259	±	0.00003	0.00823	±	0.00009	7.1	±	0.5	0.9999
	2.5	0.2664	±	0.0003	0.001893	±	0.000024	0.00322	±	0.00003	10.1	±	0.6	0.9999
	4.0	0.2233	±	0.0005	0.00171	±	0.00004	0.00188	±	0.00003	14.8	±	1.2	0.9997
	5.0	0.1229	±	0.0006	0.00160	±	0.00003	0.00110	±	0.00003	20.0	±	1.2	0.9996
7.0	0.5	0.4838	±	0.0005	0.00401	±	0.00013	0.0316	±	0.0007	7.1	±	0.9	0.9995
	1.0	0.2965	±	0.0004	0.00302	±	0.00013	0.0169	±	0.0004	11.7	±	1.3	0.9993
	2.5	0.1361	±	0.0003	0.00199	±	0.00009	0.00498	±	0.00012	22.1	±	1.8	0.9992
	4.0	0.09473	±	0.00018	0.00208	±	0.00007	0.00291	±	0.00006	23.1	±	1.5	0.9994
	5.0	0.07798	±	0.00023	0.00140	±	0.00008	0.00237	±	0.00006	29	±	3	0.9990
8.0	0.5	0.7269	±	0.0022	0.00337	±	0.00003	0.00496	±	0.00024	8.4	±	0.5	0.9998
	1.0	0.483	±	0.005	0.00291	±	0.00009	0.00303	±	0.00035	13.3	±	1.7	0.9984
	2.5	0.188	±	0.003	0.00185	±	0.00010	0.00208	±	0.00018	32	±	3	0.9964
	4.0	0.1134	±	0.0012	0.00206	±	0.00012	0.00153	±	0.00012	35	±	3	0.9963
	5.0	0.0935	±	0.0014	0.00123	±	0.00008	0.00137	±	0.00009	50	±	4	0.9964

Signification level: α=0.05.

The extinction coefficient decreases with the increase of the initial substrate concentration. Its highest values are achieved with pH 6.0, and ranged from 0.8044 to 0.1229 L·mol^{-1}·cm^{-1}. On the contrary, the lowest ones correspond to a solution pH of 5.0. This indicates that melanin chains with different composition will differently affect the global value. In other words, as the extinction coefficient is normalized to the pigment concentration and it decreases as substrate concentration increases, the chromophore molecules should be different. Actually, the maximum absorbances were reached with pH 6.0, and the minimum ones with pH 5.0. This highest PPO activity with pH 6.0 and the lowest one with pH 5.0 were also revealed in the diagrams corresponding to the PCA model calculated with only the data of the experimental series using L-tyrosine as substrate. In addition, the loadings of this analysis (not shown) confirmed that, in the studied intervals of the variables, pH was more important than the substrate concentration in the overall variance. The fact that the extinction coefficient decreases with the increase of the substrate concentration would indicate that, the lower the tyrosine concentration, the later polymerization that leads to insoluble molecules formation.

First kinetic constant (k_a) and second kinetic constant (k_b) decrease as the initial tyrosine concentration increases. This behavior means that for high tyrosine concentrations the initial reactions of intermediate products formation and the later polymerization will be slower, and the stabilization of absorbance will occur later. The maximum values for k_a correspond to a solution pH of 5.0, while the maximum k_b are for pH 7.0. The second kinetic constant is always higher than the first one, what means that PPO has always a lower catalytic effect on L-tyrosine than on L-DOPA. The behavior of all these parameters (extinction coefficient and both kinetic constants) is in agreement with the known reaction pathway.

The lag period (t_0), i.e. the time while only colorless intermediates are produced, increases with increasing substrate concentration, as it was observed by Cooksey *et al.* (1997), and with increasing pH.

Melanogenesis from 4-methylcatechol (*o*-diphenol).

First of all, the most different fact with the results obtained with L-tyrosine is the fast increase of the absorbance in the initial stage of the reaction. Later, this rising tends to become smaller as time elapses, describing a Michaelis-Menten kinetics as it happens with all *o*-diphenolic substrates on which the enzyme only carries out its catecholase activity (Madani *et al*., 1999; Hsu *et al.*, 2007). It is important to remember that, if PPO catalyses both reactions from a monophenolic substrate, the *o*-diphenol synthesized in the first step will act as activator of the enzyme, accelerating the subsequent transformations (Rodríguez-López *et al*., 1992).

Observing the evolution of the absorbance, two stages can be defined in all cases. The first one, as mentioned, a typical Michaelis-Menten curve. The second one, an increase following zero-order kinetics. The transition between these two stages depends on the pH as it can be observed in Figure 4.2.3 (the lower pH values, the later the transition occurs), but not on the substrate concentration. This limit can be established in 400 minutes at pH 5.0, in 300 minutes at pH 6.0, in 200 minutes at pH 7.0 and in 100 minutes at pH 8.0.

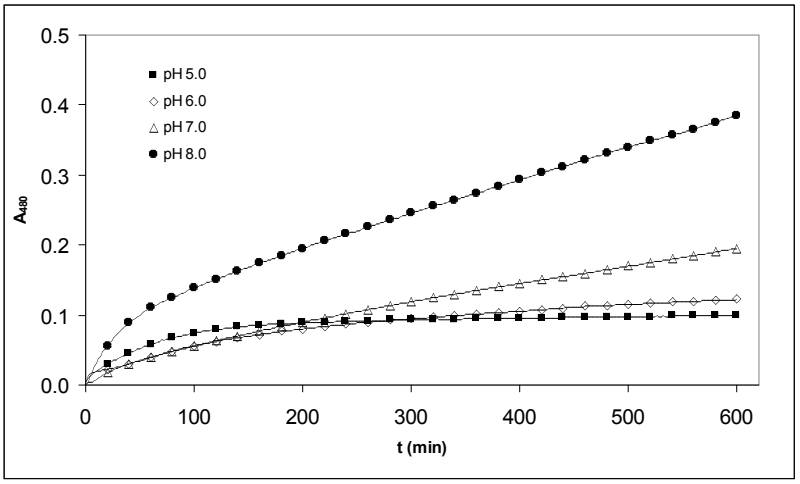

Figure 4.2.3. *Examples of the absorbance curve obtained in melanogenesis using 4.0 mM 4-methylcatechol as PPO substrate in different solution pH.*

Thus, it appears that the higher the pH, the greater enzyme activity, and therefore intermediate compounds that polymerize to melanin are formed more quickly, causing the first stage (enzymatic) to be faster. These observations are consistent with data from the principal component analysis, in which it was concluded that the samples of pH 8.0 were the most important ones for the overall variance, indicating that under these conditions a greater amount of melanin can be synthesized. These results contrast with the ones of Fan & Flurkey (2004), which set the optimal activity of polyphenol oxidase of Portabella mushroom around pH 7.0 using catechol as substrate.

Observing this unusual kinetic behavior, it was necessary to verify that the polymerization step led to an absorbance increase of zero-order. After thermally inactivating PPO and separating melanin polymers by ultrafiltration with a 300 kDa cut-off membrane, a permeate solution with colorless intermediate products was obtained. The absorbance of this solution was monitored for 10 hours. Figure 4.2.4 shows this evolution in the polymerization step from 5.0 mM 4-methylcatechol in a pH 8.0, fitted to zero-order kinetics:

$$A_\lambda = A_0 + k_0 \cdot t \qquad [4.2.14]$$

where A_0 is the absorbance at the beginning of the second stage and k_0 the zero-order kinetic constant.

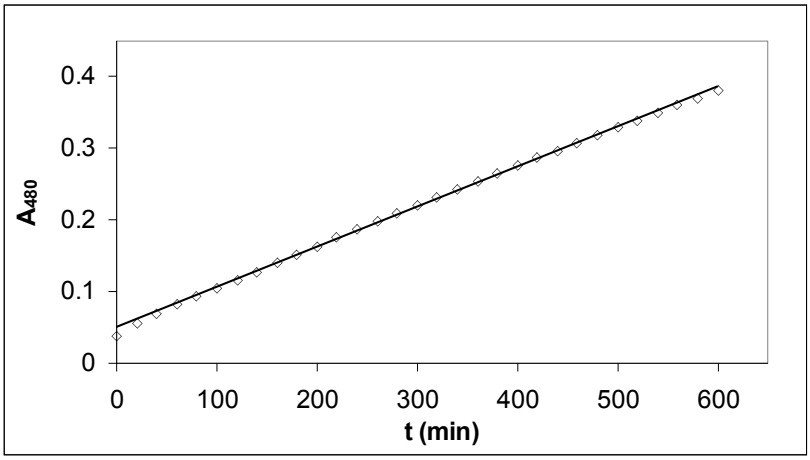

Figure 4.2.4. *Absorbance evolution in the polymerization step of melanins from 5.0 mM 4-methylcatechol in a pH of 8.0, after inactivating tyrosinase and separating melanin polymers previously synthesized. Experimental data fitted to zero order kinetics.*

To perform the kinetic study, experimental series were split into both stages. In the first one, linearized Henri equation including Lambert-Beer law (equation [4.2.10]) allows obtaining Michaelis-Menten parameters and the extinction coefficient. In the second one, the zero-order kinetic constant that determines the increase of absorbance resulting from the later polymerization is obtained.

Table 4.2.2 shows the values for the first stage. Firstly, all the obtained determination coefficients are higher than 0.90. The extinction coefficient tends to decrease with increasing initial substrate concentration, just as happened with L-tyrosine. Thus, although in this case melanogenesis starts from a diphenolic substrate, the lower the initial concentration, the later formation of insoluble strings. And again, it also seems that if the pigment chains are greater their extinction coefficient is smaller.

MM constant (K_M) corresponds to the concentration of substrate in which the reaction rate is half the maximum, and it is an indicative of the inverse of the affinity of the enzyme for the substrate. Thus, low values indicate that the enzyme-substrate complex is strongly linked and it rarely dissociates without the substrate being transformed into product. The results depend on the kind of

substrate and on the conditions of the experiment (Nelson & Cox, 2000). This parameter increases as increasing both the initial concentration of 4-methylcatechol and pH. Therefore, the presence of substrate and the absence of protons favor complex dissociation, and consequently that the enzyme becomes free to react with another molecule.

Table 4.2.2. *Parameters of fitting experimental data from the first (enzymatic) stage of 4-methylcatechol series to the kinetic model:* $\frac{A_\lambda}{\varepsilon_\lambda\, l\, t} = r_{\max} - \frac{K_M}{t}\ln\left(\frac{C_S^0}{C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda\, l}}\right)$.

pH (-)	C_A^0 (mM)	ε_{480} ($L \cdot mmol^{-1} \cdot cm^{-1}$)	K_M ($mmol \cdot L^{-1}$)	r_{max} ($mmol \cdot L^{-1} \cdot min^{-1}$)	R^2
5.0	0.5	0.16180 ± 0.00020	0.53874 ± 0.00018	0.0045 ± 0.0009	0.9934
	1.0	0.0879 ± 0.0003	1.22 ± 0.05	0.0105 ± 0.0008	0.9097
	2.5	0.0372 ± 0.0003	2.69 ± 0.05	0.0181 ± 0.0008	0.9891
	4.0	0.0301 ± 0.0006	4.02 ± 0.07	0.0110 ± 0.0006	0.9844
	5.0	0.0270 ± 0.0005	5.34 ± 0.09	0.0144 ± 0.0007	0.9865
6.0	0.5	0.1156 ± 0.0003	0.530 ± 0.011	0.00312 ± 0.00016	0.9847
	1.0	0.090 ± 0.006	1.030 ± 0.014	0.00409 ± 0.00017	0.9928
	2.5	0.0342 ± 0.0005	2.59 ± 0.04	0.0050 ± 0.0003	0.9908
	4.0	0.0247 ± 0.0007	3.98 ± 0.15	0.0087 ± 0.0018	0.9481
	5.0	0.036 ± 0.003	5.45 ± 0.03	0.01163 ± 0.00018	0.9990
7.0	0.5	0.0927 ± 0.0009	0.489 ± 0.009	0.00385 ± 0.00023	0.9911
	1.0	0.0450 ± 0.0007	1.09 ± 0.03	0.0059 ± 0.0005	0.9788
	2.5	0.0352 ± 0.0005	2.88 ± 0.08	0.0079 ± 0.0022	0.9612
	4.0	0.035 ± 0.003	6.3 ± 0.3	0.0170 ± 0.0018	0.9482
	5.0	0.0296 ± 0.0009	7.8 ± 0.4	0.0212 ± 0.0022	0.9436
8.0	0.5	0.1901 ± 0.0004	0.66 ± 0.03	0.0123 ± 0.0010	0.9781
	1.0	0.1118 ± 0.0003	1.69 ± 0.13	0.0194 ± 0.0023	0.9486
	2.5	0.064 ± 0.006	4.91 ± 0.22	0.058 ± 0.004	0.9847
	4.0	0.0463 ± 0.0005	7.22 ± 0.16	0.069 ± 0.003	0.9948
	5.0	0.0361 ± 0.0006	10.1 ± 0.3	0.118 ± 0.004	0.9951

Signification level: α*=0.05.*

The maximum reaction rate (r_{max}) is the maximum number of reactions per time unit that are catalyzed by the enzyme. This parameter also increases with the increase of initial substrate concentration as it could be expected, because the more molecules of 4-methylcatechol exist in the environment, the easier the complex is formed. In addition, maximum reaction rate also increases with increasing pH, coinciding with the greater dissociation ease of the complex that has been previously discussed and with the conclusions drawn in the principal component analysis that pointed out to a greater PPO activity.

In the second stage of the reaction (non-enzymatic), absorbance data were adjusted to a zero-order model (equation [4.2.14]). The results appear in Table

4.2.3. The constant k_0 keeps a very small and almost constant value for all initial substrate concentrations at pH 5.0. If the absorbances of these series are observed, it can be seen that when time exceeds 400 minutes they are nearly stabilized. In the other cases, k_0 clearly increases with the concentration of substrate and especially with the pH of the solution.

Table 4.2.3. *Parameters of fitting experimental data from the second (non-enzymatic) stage of 4-methylcatechol series to the zero-order kinetic model:* $A_\lambda = A_0 + k_0 \cdot t$.

pH (-)	C_A^0 (mM)	A_0 (-)	$k_0 \cdot 10^4$ (min^{-1})	R^2
5.0	0.5	0.0737 ± 0.0003	0.168 ± 0.005	0.9779
	1.0	0.0812 ± 0.0005	0.151 ± 0.009	0.9178
	2.5	0.08642 ± 0.00024	0.131 ± 0.005	0.9686
	4.0	0.088 ± 0.0003	0.190 ± 0.005	0.9799
	5.0	0.0996 ± 0.0003	0.190 ± 0.005	0.9882
6.0	0.5	0.0455 ± 0.0005	0.365 ± 0.012	0.9617
	1.0	0.0625 ± 0.0004	0.383 ± 0.008	0.9840
	2.5	0.04121 ± 0.0002	0.911 ± 0.004	0.9992
	4.0	0.0684 ± 0.0007	0.920 ± 0.015	0.9896
	5.0	0.1007 ± 0.0006	1.100 ± 0.013	0.9949
7.0	0.5	0.03733 ± 0.00016	0.418 ± 0.004	0.9959
	1.0	0.02966 ± 0.00015	0.593 ± 0.004	0.9981
	2.5	0.03075 ± 0.00022	1.590 ± 0.005	0.9994
	4.0	0.041 ± 0.0004	2.581 ± 0.010	0.9992
	5.0	0.0434 ± 0.0003	2.742 ± 0.008	0.9996
8.0	0.5	0.0856 ± 0.0006	1.150 ± 0.015	0.9891
	1.0	0.077 ± 0.0007	2.301 ± 0.018	0.9961
	2.5	0.0933 ± 0.0004	4.471 ± 0.011	0.9996
	4.0	0.0978 ± 0.0008	4.840 ± 0.021	0.9988
	5.0	0.0926 ± 0.0005	5.851 ± 0.013	0.9997

Signification level: α=0.05.

4.3. Melanin properties: inhibitory effect on pancreatic proteases.

Up to now, a detailed study of polyphenol oxidase activity has been carried out. Melanins, the final products of this activity, have been used as indirect indicators, due to their absorbance properties that make it easy to measure the reactions that occur when the enzyme and its substrates are put together. But, according to different previous conclusions reached by other researchers, the impact of melanin on certain potential negative effects on health still remain unclear. In this section, the results of inhibition assays of different kind of melanins on three pancreatic proteases is reported.

4.3.1. Inhibitory effect of melanins on carboxypeptidases A and B.

In order to study the effect of the melanins synthesized from L-tyrosine and 4-methylcatechol on carboxypeptidases A and B (CPA and CPB) activity, three different concentrations of each melanin were assayed on each enzyme, with 5 different substrate concentrations. For both CPA and CPB and for each substrate concentration, it was observed that the initial reaction rate had a great dependence on the presence of melanin from L-tyrosine: as the concentration of this polymer increased, the reaction rate decreased. On the contrary, for both enzymes little influence was observed in the case of melanin from 4-methylcatechol. To assess and quantify the inhibitory effect, these initial reaction rates were transformed following the Lineweaver-Burk method.

Table 4.3.1 shows the values of MM constant and maximum reaction rates for the experimental series carried out with different melanin concentrations for CPA and CPB. For both enzymes, when the reaction medium contains melanin from L-tyrosine MM constant (K_M) shows an increasing trend, which indicates that this polymer decreases the apparent affinity between these enzymes and

their respective substrates (Nelson & Cox, 2000). However, this fact is not appreciated when melanin from 4-methylcatechol is added. In this case, this kinetic parameter scarcely changes, keeping its value around 0.85 mM for CPA and 0.32 mM for CPB.

Table 4.3.1. *Kinetic parameters for CPA and CPB with different concentrations of the two types of melanin assayed as possible inhibitors in the reaction medium.*

Enzyme	Melanin type*	$C_{Melanin}$ (mg/mL)	K_M (mM)	r_{max} ($\Delta A_{254} \cdot s^{-1} \cdot \mu g^{-1}$)	R^2
CPA	L-tyrosine	0	0.45 ± 0.03	$8.8 \cdot 10^{-4} \pm 3.6 \cdot 10^{-4}$	0.9971
CPA	L-tyrosine	0.0625	0.52 ± 0.05	$8.2 \cdot 10^{-4} \pm 1.6 \cdot 10^{-4}$	0.9538
CPA	L-tyrosine	0.125	0.59 ± 0.05	$7.6 \cdot 10^{-4} \pm 3.6 \cdot 10^{-4}$	0.9956
CPA	L-tyrosine	0.25	0.60 ± 0.05	$7.0 \cdot 10^{-4} \pm 4.3 \cdot 10^{-4}$	0.9963
CPA	4-methylcatechol	0	0.86 ± 0.21	$1.3 \cdot 10^{-3} \pm 2.7 \cdot 10^{-4}$	0.9994
CPA	4-methylcatechol	0.0128	0.82 ± 0.40	$1.2 \cdot 10^{-3} \pm 5.0 \cdot 10^{-4}$	0.9974
CPA	4-methylcatechol	0.0256	0.86 ± 0.62	$1.6 \cdot 10^{-3} \pm 2.1 \cdot 10^{-4}$	0.9906
CPA	4-methylcatechol	0.0513	0.88 ± 0.76	$1.3 \cdot 10^{-3} \pm 9.2 \cdot 10^{-4}$	0.9933
CPB	L-tyrosine	0	0.42 ± 0.21	$4.5 \cdot 10^{-3} \pm 2.1 \cdot 10^{-3}$	0.9916
CPB	L-tyrosine	0.1875	0.51 ± 0.31	$4.0 \cdot 10^{-3} \pm 1.8 \cdot 10^{-3}$	0.9893
CPB	L-tyrosine	0.25	0.56 ± 0.37	$3.9 \cdot 10^{-3} \pm 1.7 \cdot 10^{-3}$	0.9943
CPB	L-tyrosine	0.5	1.00 ± 0.51	$3.2 \cdot 10^{-3} \pm 1.1 \cdot 10^{-3}$	0.9697
CPB	4-methylcatechol	0	0.32 ± 0.03	$3.0 \cdot 10^{-3} \pm 2.1 \cdot 10^{-4}$	0.9858
CPB	4-methylcatechol	0.0128	0.36 ± 0.14	$3.4 \cdot 10^{-3} \pm 9.2 \cdot 10^{-4}$	0.9984
CPB	4-methylcatechol	0.0256	0.29 ± 0.13	$3.0 \cdot 10^{-3} \pm 8.8 \cdot 10^{-4}$	0.9968
CPB	4-methylcatechol	0.0513	0.27 ± 0.07	$2.7 \cdot 10^{-3} \pm 4.3 \cdot 10^{-4}$	0.9989

Signification level: $\alpha=0.05$.
**Indicates the substrate from which the melanin was synthesized by means of* Agaricus bisporus *PPO.*

Regarding the maximum reaction rate, the increase in melanin from L-tyrosine in the reaction medium causes a progressive decrease in its value for both CPA and CPB. In the first case, 0.25 mg/mL lead to a 20.5% decrease of r_{max}, while in the second one this lowering is 13.3% with the same L-tyr melanin content. As far as melanin from 4-methylcatechol is concerned, its addition does not modify the maximum reaction rate in any definite way, as it occurred with the MM constant.

The behavior of these two kinetic parameters (a progressive increase in K_M and a decrease in r_{max} when a higher amount of inhibitor is in the reaction medium) is evidence enough to infer that the presence of melanin from L-tyrosine inhibits CPA and CPB activity in a mixed way (Segel, 1982). Since these melanins are a heterogeneous group of polymers with different chain lengths, the different fractions are expected to act in different moments of the

catalysis, joining both the enzyme alone or the enzyme-substrate complex and leading to mixed inhibition kinetics when the process is observed at a macromolecular level. However, as it has been seen in the previous section, melanin from 4-methylcatechol is generally composed by polymers with lower chain lengths, and this may be one of the reasons why this type of pigment does not cause a strong modification in CPA and CPB kinetics.

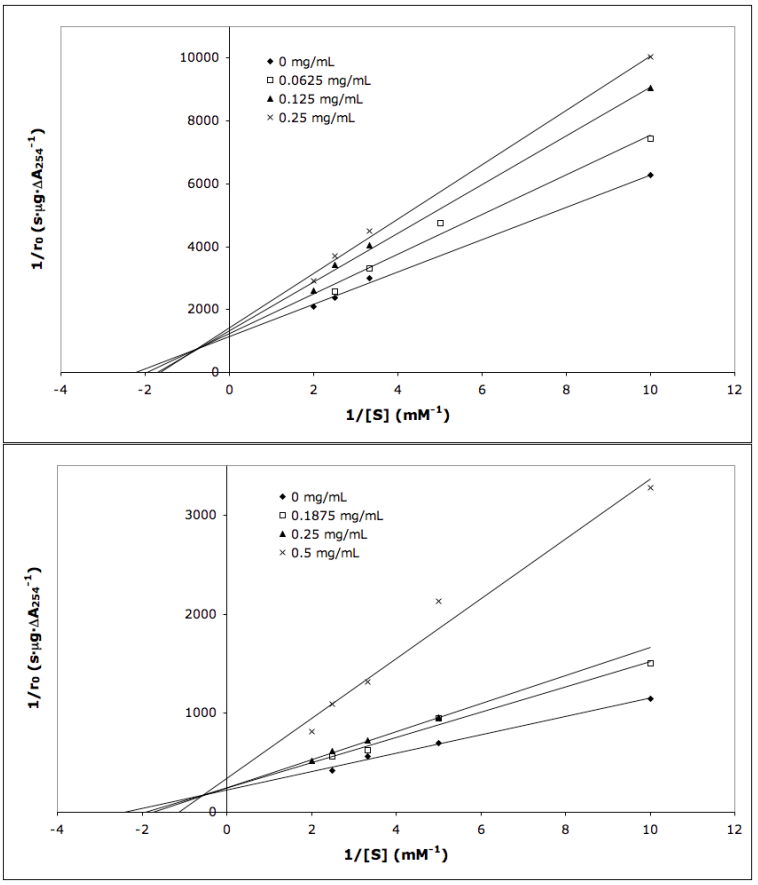

Figure 4.3.1. *Lineweaver-Burk plots of samples with different concentrations of melanin from L-tyrosine acting as carboxypeptidase A (upper) and carboxypeptidase B (lower) inhibitor.*

Figure 4.3.1 shows Lineweaver-Burk plots for CPA and CPB with melanin from L-tyrosine. In these graphs, it can be also seen that this polymer inhibits both enzymes in a mixed way, since the straight regression lines have their intersections in the second quadrant (Segel, 1982) (inhibition scheme for CPA is shown in Figure 4.3.2. Scheme for CPB is analogous, with hippuryl-L-arginine

as substrate, giving hippuric acid and L-arginine). Thus, the inhibition constant αK_i (enzyme-substrate-inhibitor complex) must be higher than the inhibition constant K_i (enzyme-inhibitor complex), and then the value of α must be higher than the unit. To obtain the values of these parameters, the slope and the intercept of the Lineweaver-Burk regressions have been represented in front of the inhibitor concentration (Segel, 1982; Bayés *et al*., 2006). For the system CPA-melanin from L-tyrosine, K_i was found to be 1.01 mg/mL, while αK_i was 2.56 mg/mL and therefore α = 2.52. In the case of the interaction between CPB and the same melanin, K_i = 0.78 mg/mL, αK_i = 6.76 mg/mL and α = 8.64. Indeed, the values of α constant were found to be higher than the unit in both cases, showing that melanin from L-tyrosine has more affinity for enzyme-substrate complex than for the enzyme alone.

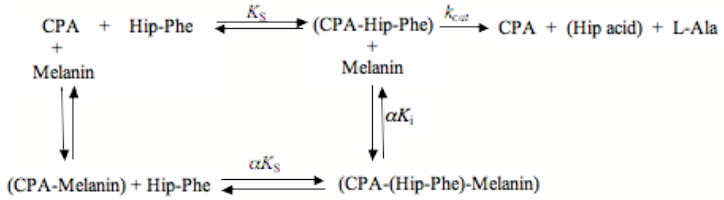

Figure 4.3.2. *Mixed enzymatic inhibition mechanism (adapted from Segel, 1982). CPA: carboxypeptidase A. Hip-Phe: hiippuryl-L-phenilalanine. Hip acid: hippuric acid. L-Ala: L-alanine.*

Considering Lineweaver-Burk plots of the systems in which melanin from 4-methylcatechol was assayed as a possible inhibitor (not shown), these regressions turned out to be coinciding straight lines with the one obtained without melanin. Thus, it can be concluded that this type of polymer does not act as CPA or CPB inhibitor.

As it has been already introduced, those differences between the inhibitory effect of both types of melanin on CPA and CPB may be attributed to differences in their structure. Some evidence found in the previous section pointed to the fact that melanin synthesized from L-tyrosine is formed by larger chains than those integrating melanin from 4-methylcatechol. In other studies performed with non-enzymatic browning products (Hirano *et al*., 1996; Ibarz *et al*., 2008a), the inhibition of carboxypeptidases and trypsin was attributed to an

allosteric effect, as a result of the interaction between long polymer chains with high bonding ability and the globular-shaped enzymes. In this way, the polymer is supposed to trap a number of enzyme molecules. Moreover, the inhibition was also partially attributed to an electrostatic insufficiency in the interaction enzyme-polymer, due to the nature of the side groups of the amino acids that form the enzyme (Jencks, 1969; Hirano *et al*., 1996).

4.3.2. Inhibitory effect of melanins on trypsin.

On the basis of the previous results, it could be concluded that melanin from L-tyrosine acted as CPA and CPB inhibitor, while melanin from 4-methylcatechol did not. Thus, additional experimental series were carried out to investigate if melanin from L-tyrosine also inhibited trypsin. For that purpose, three different melanin concentrations were assayed on BAEE solutions. It was observed that the initial reaction rate had a great dependence on melanin concentration, being lower as it increased. To characterize and quantify this inhibitory effect these initial reaction rates were transformed following the Lineweaver-Burk method (Figure 4.3.3).

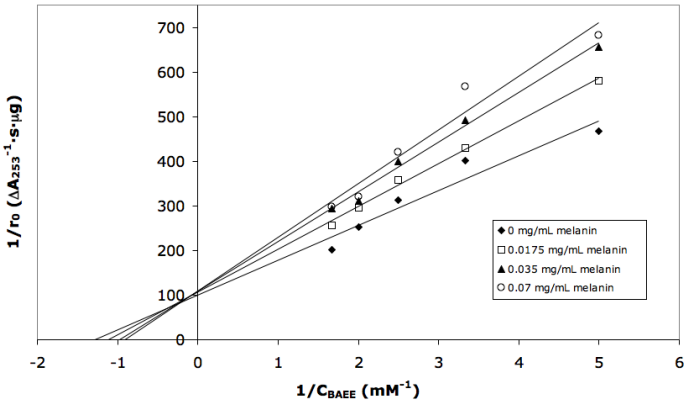

Figure 4.3.3. *Lineweaver-Burk plots of samples with different concentrations of melanin from L-tyrosine acting as trypsin inhibitor.*

Table 4.3.2 shows the values of MM constant and maximum reaction rates for the experimental series carried out with different melanin concentrations. MM constant increases with the melanin concentration in the solution, which indicates that this polymer decreases the apparent affinity between trypsin and its substrate (Nelson & Cox, 2000). In addition, the maximum reaction rate tends to decrease with the increase of melanin content in a linear tendency. The highest obtained inactivation was 32.6% with a BAEE concentration of 0.6

mM and a melanin content of 0.07 mg·mL^{-1}. By extrapolation of this linear tendency, the necessary melanin concentration to inhibit the enzyme completely would be 0.50 mg·mL^{-1}. However, this deduction could not be empirically proved, since melanin solubility made it impossible to work with concentrations higher than 0.25 mg·mL^{-1}. At higher melanin contents, some of the added polymer remained constantly insoluble.

Table 4.3.2. *Kinetic parameters for trypsin inhibition by melanin from L-tyrosine.*

$C_{Melanin}$ (mg/mL)	K_M (mM)	r_{max} ($\Delta A_{253} \cdot s^{-1} \cdot \mu g^{-1}$)	R^2
0	0.86 ± 0.16	0.0108 ± 0.0027	0.9345
0.0175	0.96 ± 0.44	0.00990 ± 0.00039	0.9987
0.035	1.09 ± 0.13	0.00979 ± 0.00095	0.9938
0.07	1.10 ± 0.19	0.0092 ± 0.0015	0.9526

Signification level: α=0.05.

Once again, these facts are evidence enough to determine that the presence of melanin from L-tyrosine inhibits trypsin activity in a mixed way (Segel, 1982). Inhibition ways are represented in Figure 4.3.4, in a scheme that is also valid for the cases of CPA and CPB mixed-way inhibition. Since melanins are a heterogeneous group of polymers with different chain lengths, the different fractions are expected to act in different moments of the catalysis, joining both trypsin alone or trypsin-BAEE complex and leading to mixed inhibition kinetics. Ibarz *et al*. (2009a) found a similar behavior in the interaction between trypsin and melanoidins, which is also a heterogeneous group of polymers with different chain lengths.

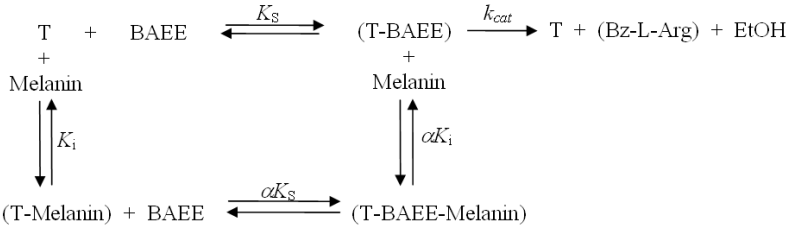

Figure 4.3.4. *Mixed enzymatic inhibition mechanism (adapted from Segel, 1982). T: trypsin. BAEE: Nα-benzoyl-L-arginine ethyl ester. Bz-L-Arg: Nα-benzoyl-L-arginine. EtOH: ethanol.*

As it has been mentioned in the case of carboxypepidases, inhibition mechanisms may partially occur via electrostatic interactions. According to Jencks (1969), the electrostatic and apolar contacts dictate enzyme-substrate complementarity, which is necessary for surmounting the activation energy barrier between the ground and transition states. As substrate binding sites are preformed and relatively rigid, the free energy of substrate binding can be converted to catalysis without a large entropic penalty. Therefore, rate acceleration must also depend on the ability of distal portions to stabilize the binding, so the whole protein architecture must play an important role (Perona *et al.*, 1995). Moreover, it is known that the differences in substrate specificity between trypsin and chymotrypsin are provided by variations in the distal portions that create a particular electrostatic environment, since the structure of the active site is the same in both enzymes (Stroud, 1974; Hedstrom *et al.*, 1992). Then, any molecule present in the reaction medium may have an active effect on these electrostatic and apolar bindings, modifying the local environment that is necessary to create the enzyme-substrate links. Melanin chains with different molecular weight may create bindings with different sites of enzyme molecules, either blocking the active site or modifying these electrostatic forces.

The Lineweaver-Burk plot also reveals that the inhibition is mixed-way. Thus, the inhibition constant αK_i (enzyme-substrate-inhibitor complex) must be higher than the inhibition constant K_i (enzyme-inhibitor complex), and then the value of α will be higher than the unit. To obtain the values of these parameters, the slope and the intercept of the Lineweaver-Burk regressions were represented in front of the inhibitor concentration (Segel, 1982). K_i was found to be 0.148 mg·mL^{-1}, while αK_i was 0.438 mg·mL^{-1}. Thus, α value was 2.95. The fact that α is higher than the unit, but close to it, indicates that, indeed, the inhibition is mixed-type (Copeland, 2000), supporting the evidence observed in Figure 4.3.2. Ibarz *et al.* (2009a) found an α value of 1.88 in the inhibition of trypsin by melanoidins synthesized from glucose and asparagine.

4.4. Polyphenol oxidase inactivation by UV-vis irradiation (1): Modeling and study of model solutions.

Once the main properties of polyphenol oxidase and its products have been assessed, attention can be moved to the inactivation of this enzyme by innovative technologies. In the case of ultraviolet-visible (UV-vis) irradiation, although it has been widely investigated, there are still several issues concerning its application on fruit derivatives that must be studied before its industrial implementation. Such research must include four steps: theoretical study of the behavior of radiation inside fluid food, study of the direct interaction between radiation and the enzyme, study of enzyme inactivation within real fruit derivatives and study of the side effects that the process entails. In this section, specific modeling of radiation absorption in a system formed by a perfect-mix plane photoreactor and a single lamp will be developed. The obtained model must allow seeing, for example, how radiation penetrates inside the reaction medium depending on its absorbance. Later, model solutions containing PPO and some polymers naturally present in fruit derivatives will be prepared and processed.

4.4.1. Modeling of absorbed radiation profiles in a system composed by a plane photoreactor and a single lamp.

In order to quantify and predict chemical changes produced by UV-vis irradiation in a food system, specific modeling is required in each case. As in any chemical reaction, performing the mass and energy balances will be necessary to achieve the kinetic expression. Nevertheless, if it can be considered that the process works in isothermal state, it is not necessary to perform the energy balance, and the variation of the photochemical reaction rate with temperature is a minor factor (Bird *et al*., 1964). But, in addition, in the case of photochemical processes also a radiation balance must be developed, since the absorbed amount of radiation has a definitive effect on the reaction

rate of the process in each point of the reaction medium depending on its location.

System geometry.

The considered system is composed by a parallelepipedal device, in which the sample is placed, and a single lamp without reflectors. Consequently, there is only a direct radiation flow between the lamp and the reactor. The plane photoreactor (whose geometrical parameters were described in Figure 1.4.4) has a width of 15 cm (x axis), a length of 22 cm (y axis) and a depth of 2.4 cm (z axis). The origin of coordinates is located in an upper corner of this device. The lamp, whose length is 4.5 cm, is placed following the *y* axis from a point (x_0, y_0, z_0) to a point (x_0, y_1, z_0), being x_0 7.5 cm, y_0 8.75 cm, y_1 13.25 cm and z_0 (the distance between the lamp and the reactor surface) 22.5 cm.

Radiation balance.

The radiation balance is performed adopting a model that should be able to provide information about the behavior of radiation rays, taking certain considerations about the type of emission and the geometry of the radiation source itself. In this way, as it was explained in the *Introduction*, emission models can be radial, spherical and diffuse. Regarding the emission source, three models can be set, depending on if the lamp can be considered as a line, as a cylindrical surface or as a volumetric region with cylindrical shape.

Among all models, the one that gives a more accurate result is the extensive spherical cylindrical one (Cerdá *et al.*, 1973; Esplugas, 1975), which considers the lamp as a cylinder whose volume emits spherically. However, the most used model is the spherical linear one, which has an easier resolution, and whose results hardly differ from the extensive spherical cylindrical model. The validity of this model is restricted to those installations in which the lamp radius is negligible compared with the distance between the lamp and the reactor surface, so that the differences between the linear model and the extended emission source model will be small. This assumption implies only small displacements from the centre of the lamp, in order to consider only those

points for which the emission source can be considered as a line (Esplugas *et al*., 1987).

The spherical linear model is described in the literature (Jacob & Dranoff, 1970). This model was also used by Esplugas & Vicente (1991) to develop an equation to evaluate the direct radiation between a linear source and a plane photoreactor. These authors considered that if the absorbance of the reaction chamber were high enough, all the radiation entering the reactor would be absorbed by the reaction medium. Therefore, the absorption rate in the whole volume would have the same value as the flow rate reaching the reactor surface, which is easy to be calculated by integrating the radiation intensity that reaches the entire surface.

But, as it has been already introduced, the mathematical expression of the reaction rate is a function of the radiation intensity at each wavelength, as well as of reactants and products concentrations. Thus, although perfect mixing ensures that these concentrations will be homogeneous throughout the volume of the reaction medium, the reaction rate will be different in each point of the reactor since it also depends on the radiation intensity, which in turn is a function of the location and the geometry of the system. Therefore, the radiation balance must be performed considering the attenuation of the radiation rays inside the reactor medium.

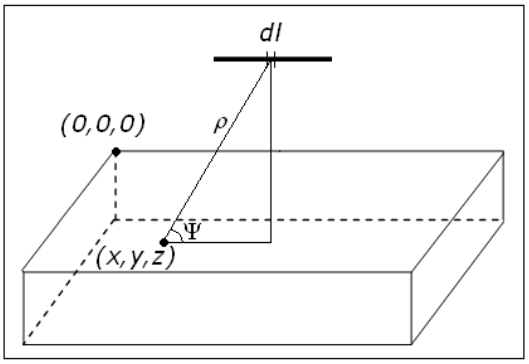

Figure 4.4.1. *The two points between which the radiation balance is considered. The first point constitutes a dl length of the lamp. The second one is a point (x,y,z) located inside the reactor. ρ is the distance between these two points, and Ψ is the angle between the radiation flow density vector and the reactor surface.*

Thus, the radiation balance between a point of the lamp and another one with coordinates (x,y,z) located inside the reacting fluid (Figure 4.4.1), is given by Lambert-Beer law for spherical emission (Esplugas *et al*., 1983; Unluturk *et al*., 2004):

$$I(x,y,z) = \frac{W_L}{4\pi L}\int_{y_0}^{y_0+L} \frac{\exp\left(-\mu z / \sin\Psi\right)}{(x_0-x)^2+(l-y)^2+(z_0+z)^2}\,dl \qquad [4.4.1]$$

where I is the radiation intensity that reaches the point (x,y,z) $\left(Einstein/cm^2\cdot s\right)$, W_L is the energy flow emitted by the lamp $\left(Einstein/s\right)$, L is the lamp length (cm),μ is the medium absorbance (cm^{-1}), Ψ is the angle between the radiation flow density vector and the reactor surface (rad) and the product $\left(\mu z/\sin\Psi\right)$ is the attenuation of the ray until it reaches the point (x,y,z).

Considering the geometry that has been described, the value of the sine of the angle between the radiation flow density vector and the reactor surface is calculated as:

$$\sin\Psi = \frac{z_0+z}{\left((x_0-x)^2+(l-y)^2+(z_0-z)^2\right)^{1/2}} \qquad [4.4.2]$$

Then, the total absorbed radiation flux can be calculated integrating the product ($\mu\cdot I$) in the three dimensions of the reactor:

$$W_{abs} = \int_0^A\int_0^B\int_0^C \mu I(x,y,z)\,dx\,dy\,dz \qquad [4.4.3]$$

where A, B and C are the whole width, length and depth of the reactor, respectively. This equation has no analytical solution, so that a network of ($\mu\cdot I$) values must be created in the entire volume in order to be integrated by numerical methods. If these integrations in the three dimensions are carried out step-by-step, different kinds of absorbed radiation profiles can be achieved, which allows extracting different interesting information that will be detailed next.

Absorbed radiation profiles.

Integrating the ($\mu \cdot I$) values in the *z* axis leads to an absorbed radiation profile in the whole depth depending on the horizontal (x,y) position. Figure 4.4.2 shows two examples of this profile for two different values of the medium absorbance (μ), 0.1 and 0.5 cm^{-1}. If μ=0.1 cm^{-1}, the positions in the four corners of the reactor will absorb only $2.27 \cdot 10^{-5}$ $\left(Einstein/cm^2 \cdot s\right)$, which is the 25.2% of the radiation absorbed in the central position (x=7.5 cm, y=11 cm). If μ=0.5 cm^{-1}, the radiation absorbed in the four corners will be only $7.15 \cdot 10^{-5}$ $\left(Einstein/cm^2 \cdot s\right)$, the 29.1% of that absorbed in the central position.

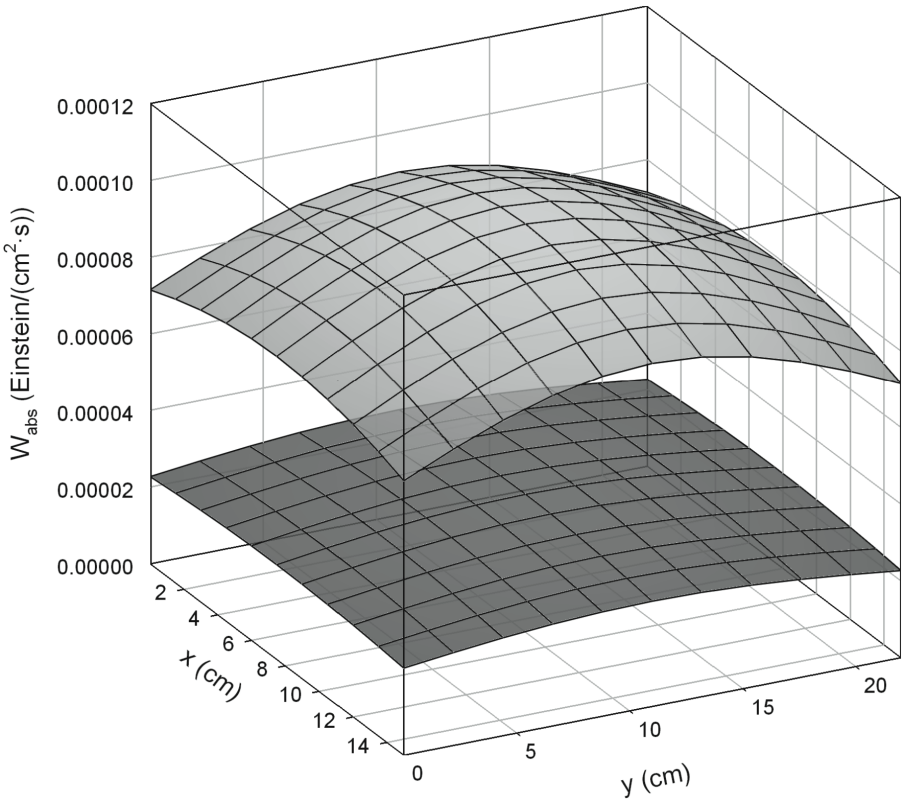

Figure 4.4.2. *Absorbed radiation profiles in the whole reactor depth depending on the (x,y) position for two different values of the medium absorbance. Upper graph: μ=0.5 cm^{-1}. Lower graph: μ=0.1 cm^{-1}.*

If the values of Figure 4.4.2 are integrated in the other two dimensions, the total radiation absorbed in the whole reactor will be found for each μ value. Figure 4.4.3 shows how the total absorbed radiation changes with medium absorbance. This graph tends to stabilization, meaning that as medium absorbance increases the total radiation absorbed will be increasingly closer to the total radiation that reaches the reactor surface, as it was stated by Esplugas & Vicente (1991). For example, when the medium absorbance is 0.1, the total amount of absorbed radiation is $9.05 \cdot 10^{-3}$ (Einstein/s), when μ=0.5 it increases to $2.95 \cdot 10^{-2}$ Einstein/s, and if μ=5, W_{abs}=$4.42 \cdot 10^{-2}$ (Einstein/s).

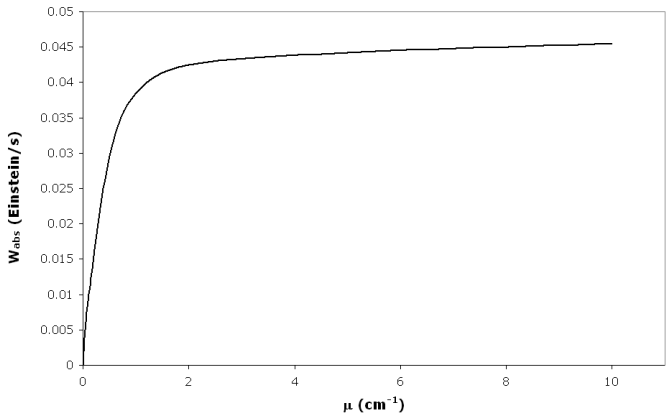

Figure 4.4.3. *Total radiation absorbed in the whole reactor depending on the medium absorbance.*

Low penetration of ultraviolet radiation inside liquid food systems has been stated to be one of the main limitations of this technology. The penetration effect of UV radiation depends on the type of liquid, its UV absorbance, soluble solids content and suspended matter, as well as on the light source, flow profile and geometric configuration of the photoreactor. Since this last parameter is essential to ensure that all the system receives the same radiation dose, some researchers have tested different geometric configurations to produce, for example, thin film throughout pipes, a liquid bell formed by spraying the liquid with nozzles, turbulent flow throughout the pipes or perfect-mixing (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Unluturk *et al.*, 2004).

Knowing this limitation, one of the most interesting distributions to be investigated in each ultraviolet processing photoreactor is the radiation absorption profile as a function of the reaction medium thickness. To obtain this representation, the ($\mu \cdot I$) values network must be integrated in the x and y axes. As it has been seen in the previous figures, the medium absorbance has a big effect on the obtained results. In order to compare the different profiles avoiding these scale problems, relative absorbed radiation has been calculated as:

$$R(z,\mu) = \frac{W_{abs}(z,\mu) / W_L}{\left(W_{abs}(z,\mu) / W_L \right)_{\max}} \qquad [4.4.4]$$

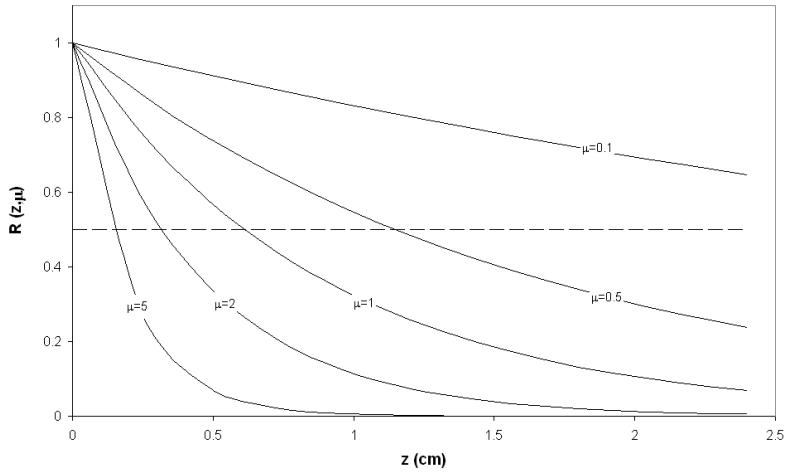

Figure 4.4.4. *Relative absorbed radiation profile as a function of medium depth for different medium absorbances.*

Figure 4.4.4 shows some profiles corresponding to different values of the reaction medium absorbance. For a reaction medium with an absorbance of 0.1, hardly 30% of the emitted radiation is absorbed, while for a μ value of 5 50% of the radiation is absorbed in the first 2 mm, and nearly the whole radiation is absorbed in the first cm. It is important to consider that fruit juices usually have values between 2 and 5 in this scale for most wavelengths. Thus, these profiles constitute the evidence of the fact that although the fluid may have a high absorbance the radiation does penetrate in it.

There are a great variety of profiles that can be achieved from the ($\mu \cdot I$) values network, integrating them with different conditions in one or two axes or just drawing a group of these values in different planes of the reactor. For example, Figure 4.4.5 shows the absorbed radiation in the *xz* plane (perpendicular to the lamp) corresponding to the central position, *y*=11 cm, for a μ value of 1. In this graph it is also easy to see how radiation absorption decreases with the distance from the reactor surface and from the lamp.

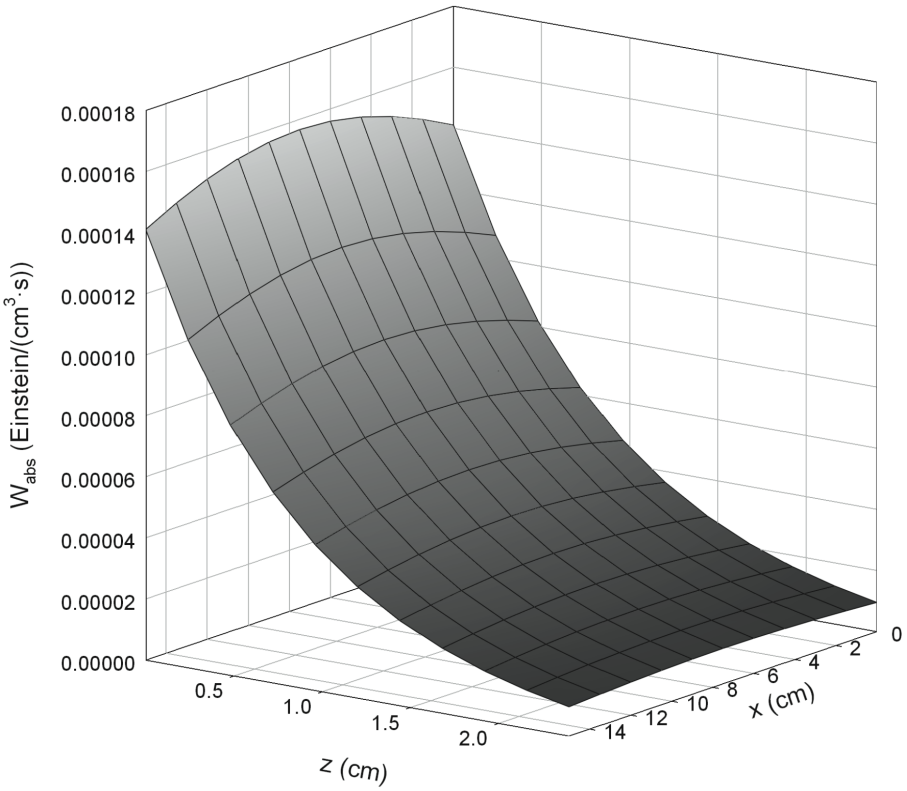

Figure 4.4.5. *Absorbed radiation profile in the xz plane corresponding to y=11 cm, for a μ value of 1 cm^{-1}.*

4.4.2. Inactivation of polyphenol oxidase by UV-vis irradiation in model solutions.

The second step of UV-vis processing analysis aimed at the inactivation of polyphenol oxidase in model solutions containing the enzyme and some polymers that can be found in fruit and vegetable derivatives, in order to assess their potential protective effects when compared with a model solution containing only the enzyme. In a first group of experimental series, melanins from L-tyrosine and 4-methylcatechol (which have been studied in section 4.3) were used, while in a second experimental set the chosen polymers were a certain group of melanoidins synthesized from fructose and glutamic acid.

4.4.2.1. *Inactivation of polyphenol oxidase by UV-vis irradiation in model solutions: protective effect of melanins.*

In this first study, model solutions were prepared with melanins synthesized from PPO activity on L-tyrosine (monophenolic substrate) and 4-methylcatechol (*o*-diphenolic substrate), rediluted in dimethyl sulfoxide (DMSO) so as to increase melanin solubility. As it has been detailed in the *Materials and methods* section, each sample (including those without melanins) contained 10% v/v DMSO.

Inactivation of polyphenol oxidase in model solutions without any melanins.

UV-vis irradiation of the sample containing 12.5 $U \cdot mL^{-1}$ of *Agaricus bisporus* polyphenol oxidase led to the complete inactivation of the enzyme after 35 min of treatment. However, in the first 90 s a big decrease of PPO activity was observed, being reduced to nearly 70% of its original value. After 4 minutes of irradiation, residual activity was only 30% of the initial activity. This experimental series can be observed in the following figures (4.4.6 and 4.4.7), in which the other ones containing melanins are also plotted. It is also important to state that

PPO activity was not recovered after 24 h in any taken sample, so the achieved inactivation was irreversible. In a previous piece of work performed with the same equipment, 20 minutes of irradiation were required to completely inactivate bovine carboxypeptidase-A, while 12 minutes were necessary to denature porcine trypsin (Ibarz *et al.*, 2009b).

As far as the mechanisms by which PPO is inactivated by UV-vis radiation are concerned, there are some possible explanations. Some studies carried out on crystallins have shown that, in those enzymes, UV radiation causes oxidation of SH groups, changes in native conformation of the latter and formation of covalent cross-links between polypeptides (Krivandin *et al*., 2009). Since enzymatic activity depends on its structure, these modifications lead to enzyme inactivation. Such UV light-induced modifications have been reported to occur via two major routes: photo-oxidation arising from the absorption of radiation by the protein structure or a bound chromophore, and indirect protein oxidation mediated by singlet oxygen generated by energy transfer by either protein bound or other chromophores. In spite of this knowledge, the effect of UV light on structure and function of food enzymes is still a matter of speculation (Manzocco *et al*., 2009).

Inactivation of polyphenol oxidase in model solutions containing melanins from L-tyrosine.

Figure 4.4.6 shows the evolution of polyphenol oxidase residual activity with irradiation time depending on melanin concentration. The enzyme was completely inactivated in all experimental series; the time that was necessary to accomplish this denaturalization was higher as pigment concentration increased, showing the protective effect of these polymers. Thus, as it has been stated, with no pigments in the medium polyphenol oxidase was inactivated after 35 min of irradiation. Meanwhile, with 0.200 $mg \cdot mL^{-1}$ of melanin from L-tyrosine 70 min were required. The bigger decrease in enzyme activity in the first 90 s of treatment was especially remarkable in the samples with low melanin content. Again, PPO activity was not recovered after 24 h in any sample, so the achieved inactivation was also irreversible.

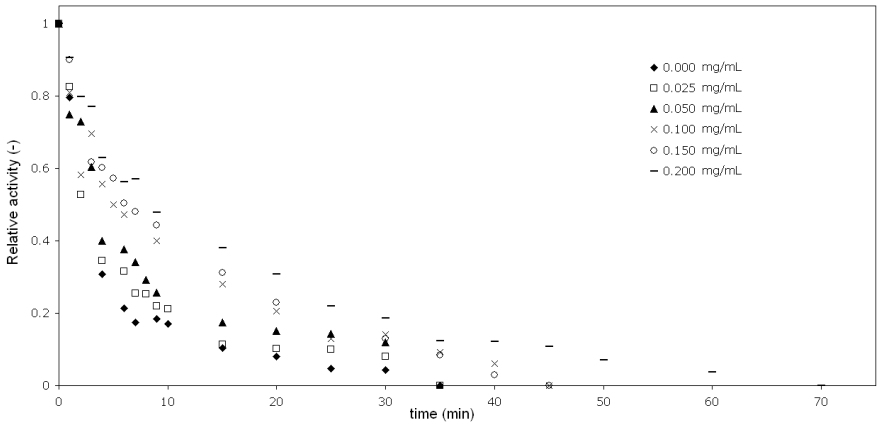

Figure 4.4.6. *Polyphenol oxidase activity decreasing with irradiation time in samples with different content in melanin synthesized from L-tyrosine.*

The determination coefficients obtained with the fittings of experimental data to the first order and the composite kinetic models are shown in Table 4.4.1. The values obtained with the first order model corresponding to the samples with high melanin concentrations (from 0.10 to 0.20 mg·mL^{-1}) are always higher than 0.97, while the ones corresponding to the samples with low melanin content (from 0.00 to 0.05 mg·mL^{-1}) are considerably lower. This is probably due to the fact that the first order model is not able to explain the fast first step of PPO inactivation, which has a bigger effect when the pigment concentration in the reaction medium is low. On the contrary, the composite model is able to explain both steps, so that determination coefficients are remarkably higher in the first group of samples. Nevertheless, it can also be observed that there is little variation with the values corresponding to higher pigment concentrations. Consequently, regarding to these samples the first order model would be more appropriate to describe this process due to its simplicity.

Table 4.4.1. *Determination coefficients of the fittings of data from series with melanin from L-tyrosine to the first order and the composite kinetic models.*

Model	Melanin content (mg·mL^{-1})					
	0.000	0.025	0.050	0.100	0.150	0.200
First order	0.8320	0.8544	0.8365	0.9747	0.9856	0.9895
Composite	0.9661	0.9925	0.9178	0.9766	0.9951	0.9904

Table 4.4.2 shows estimated values of the parameters from the most suitable kinetic model for each sample. In the samples with low melanin content, k_1 tends

to decrease as pigment concentration increases, meaning that the presence of melanins makes the first inactivation step slower. Meanwhile, k_{II} and Λ do not show a defined tendency. In the other samples (with melanin content from 0.10 to 0.20 mg·mL^{-1}), the first order kinetic constant (k) decreases as melanin concentration increases, meaning that the inactivation gets slower due to the protective effect of melanins. In addition, RA_0 gets closer to 1 as melanin concentration increases, showing that the inactivation in the first seconds is smoother because the radiation is absorbed by the pigments in a higher degree. Consequently, the first order kinetic model is able to fit better the data points corresponding to the initial irradiation times as the melanin concentration increases.

Table 4.4.2. *Estimated values of the parameters of the most suitable kinetic model for each sample, in experimental series with melanin from L-tyrosine.*

Melanin content (mg·mL^{-1})	Model	Parameter	Value	Parameter	Value	Parameter	Value
0.000	Composite	k_I (min^{-1})	0.790	k_{II} (min^{-1})	0.057	Λ (-)	0.245
0.025	Composite	k_I (min^{-1})	0.543	k_{II} (min^{-1})	0.065	Λ (-)	0.338
0.050	Composite	k_I (min^{-1})	0.268	k_{II} (min^{-1})	0.027	Λ (-)	0.233
0.100	First order	RA_0 (-)	0.752	k (min^{-1})	0.064		
0.150	First order	RA_0 (-)	0.822	k (min^{-1})	0.062		
0.200	First order	RA_0 (-)	0.840	k (min^{-1})	0.050		

Inactivation of polyphenol oxidase in model solutions containing melanins from 4-methylcatechol.

Residual enzyme activity with irradiation time depending on 4-methylcatechol melanin concentration appears in Figure 4.4.7. As expected, higher treatment times are required as melanin content increases. However, the fast decrease in enzyme activity in the first seconds has not been observed using this kind of melanin. In this case, it was also proved that the inactivation was irreversible, since no activity recovery was observed after 24 h. Table 4.4.3 shows the determination coefficients obtained with the fittings of experimental data to the first order and the composite kinetic models. Contrary to what happened with the melanin from L-tyrosine, these values were higher than 0.99 in all experimental series containing melanin from 4-methylcatechol using both kinetic models. These parameters support the previous statement that in this case the fast first step was not observed, so that the first order equation is also able to fit experimental data.

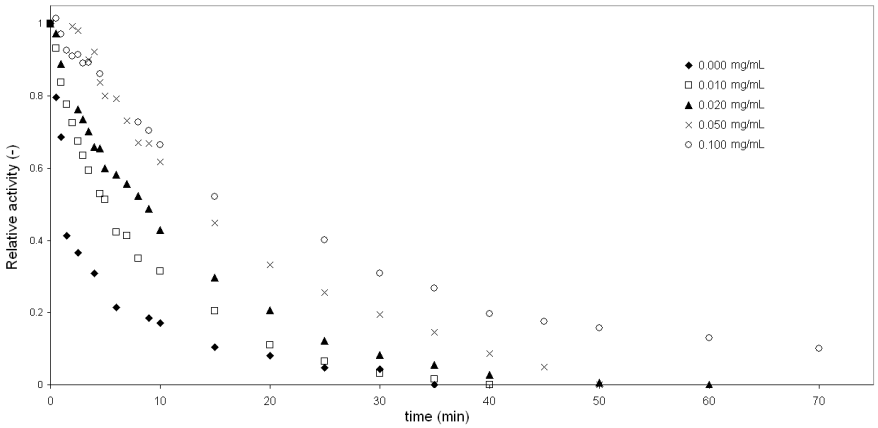

Figure 4.4.7. *Polyphenol oxidase activity decreasing with irradiation time in samples with different content in melanin synthesized from 4-methylcatechol.*

Table 4.4.3. *Determination coefficients of the fittings of data from series with melanin from 4-methylcatechol to the first order and the composite kinetic models.*

Model	Melanin content ($mg \cdot mL^{-1}$)				
	0.000	0.010	0.020	0.050	0.100
First order	0.8320	0.9922	0.9945	0.9969	0.9950
Composite	0.9661	0.9986	0.9974	0.9973	0.9951

Table 4.4.4 corresponds to the kinetic parameters of fitting experimental data from series with melanin from 4-methylcatechol to both models. Firstly, attending to the first order kinetic model, the inactivation constant (k) clearly decreases as melanin concentration increases, being an appropriate parameter to quantify the protective effect of these dark polymers on polyphenol oxidase denaturalization. In this case, RA_0 is close to the unit in all series, reinforcing the fact that the fast inactivation in the first seconds did not occur.

Table 4.4.4. *Estimated values of the parameters of both kinetic models for each sample, in experimental series with melanin from 4-methylcatechol.*

Melanin content ($mg \cdot mL^{-1}$)	Model	Parameter	Value	Parameter	Value	Parameter	Value
0.000	Composite	k_I (min^{-1})	0.790	k_{II} (min^{-1})	0.057	Λ (-)	0.245
0.010	First order	RA_0 (-)	0.95	k (min^{-1})	0.123		
0.020	First order	RA_0 (-)	0.97	k (min^{-1})	0.083		
0.050	First order	RA_0 (-)	1.06	k (min^{-1})	0.072		
0.100	First order	RA_0 (-)	1.06	k (min^{-1})	0.034		
0.010	Composite	k_I (min^{-1})	0.514	k_{II} (min^{-1})	0.096	Λ (-)	0.621
0.020	Composite	k_I (min^{-1})	0.723	k_{II} (min^{-1})	0.075	Λ (-)	0.813
0.050	Composite	k_I (min^{-1})	0.911	k_{II} (min^{-1})	0.074	Λ (-)	0.990
0.100	Composite	k_I (min^{-1})	1.010	k_{II} (min^{-1})	0.034	Λ (-)	1.021

Regarding the composite model, the kinetic constant of the first step (k_I) increases as melanin concentration grows up, meaning that the transformation of the native form of the enzyme (N) into the intermediate one (I) is faster. Nevertheless, as it has been observed, this first stage lasts for a very short time. Inactivation constant (k_{II}), which describes the transformation of the intermediate form (I, still active) into the denatured one (D), significantly decreases as melanin content increases, showing that this step is slower due to the protective effect of melanins, as it was also seen with the k constant of the first order model. Finally, the parameter Λ shows a clear upward trend. Therefore, it was confirmed that the transition from the native form to the intermediate form is faster at higher melanin concentrations.

Comparing both kinds of melanin it can be seen that, at the same concentration, melanin from 4-methylcatechol has a higher protective effect than melanin from L-tyrosine. With a polymer content of 0.1 $mg \cdot mL^{-1}$, the values of the inactivation constant from the first order kinetic model (k) are 0.064 min^{-1} for L-tyrosine melanin (Table 4.4.2), and 0.034 min^{-1} for 4-methylcatechol melanin (Table 4.4.4). Consequently, with this pigment concentration the decrease in enzyme activity with irradiation time will be 47% slower with melanin from 4-methylcatechol.

4.4.2.2. *Inactivation of polyphenol oxidase by UV-vis irradiation in model solutions: protective effect of melanoidins.*

In the last section, it has been proved that chemical differences in the structure and composition of similar colored polymers can lead to strong differences in the protection they provide on PPO inactivation by UV-vis irradiation. In a similar way, the protective effect of other pigments that can be naturally present (endogenous) or formed by postharvest and/or processing reactions should also be studied. Next, the results of analogous research carried out with melanoidins are reported. Since the only commercially available polyphenol oxidase is that of *Agaricus bisporus*, such melanoidins were synthesized from the major sugar and amino acid of this mushroom: fructose and glutamic acid, respectively (Hammond, 1985; Mattila *et al*., 2002). In addition, the effect of the global obtained melanoidins has been compared with the effect caused by lower molecular mass (<150 kDa) and higher molecular mass compounds (>150 kDa).

Inactivation of polyphenol oxidase in model solutions without any melanoidins.

First of all, 12.5 U/mL of polyphenol oxidase were completely inactivated after 85 min of irradiation if the reaction medium contained no melanoidins. In the last section, which was carried out using the same equipment, only 35 min of treatment were necessary to inactivate the enzyme in this model solution at the same pH (6.5). This large difference may be attributed to the fact that, in that case, all the samples contained 10% dimethyl sulfoxide (DMSO) to ease melanin solubilization, which could have played an important role affecting PPO denaturation. This issue should be extensively studied in the future.

In this standard sample, residual activity decreased rapidly in the first 10 min, being reduced to 56% of its original value. After this moment, inactivation pace was slower. Neither in this solution nor in the ones containing melanoidins PPO activity was recovered after 24 h, so the achieved inactivation was irreversible.

Inactivation of polyphenol oxidase in model solutions containing melanoidins.

In order to assess the effect of melanoidins synthesized from Fru-Glu, three different groups of experimental series were performed: in the first one (Figure 4.4.8), non-fractioned melanoidins (NF) were added in concentrations from 0.1 up to 0.4 mg/mL. The other experimental groups were carried out adding melanoidins lower or higher than 150 kDa, in the same concentrations. Quantification of those polymers after ultrafiltration and recovering showed that the obtained Maillard reaction products contained 46.6% of molecules of less than 150 kDa and 53.4% of molecules with a molecular mass higher than this value.

As expected, the higher the melanin content, the slower PPO inactivation. Table 4.4.5 shows the determination coefficients of experimental data fittings to the first order and the composite kinetic models. This coefficient is always higher in the composite model. In many cases, the first-order equation does not fit well the experimental data, mainly due to the rapid decrease of activity that occurs in the first minutes (as explained before) that this model cannot describe. In this way, in the three series the first-order determination coefficient increases with increasing polymer concentration: as melanoidin content increases, the inactivation of PPO is slower, mitigating the effect of this fast inactivation in the first minutes.

Table 4.4.5. *Determination coefficients of experimental data fittings to the first order and the composite kinetic models.*

	Melanoidin content	First order model	Composite model
	0 mg/mL	0.9693	0.9918
Non-fractioned	0.1 mg/mL	0.9275	0.9828
	0.2 mg/mL	0.9423	0.9941
	0.4 mg/mL	0.9638	0.9782
<150 kDa	0.1 mg/mL	0.6787	0.9780
	0.2 mg/mL	0.7047	0.9535
	0.4 mg/mL	0.8838	0.9503
>150 kDa	0.1 mg/mL	0.7232	0.9856
	0.2 mg/mL	0.7421	0.9592
	0.4 mg/mL	0.9599	0.9866

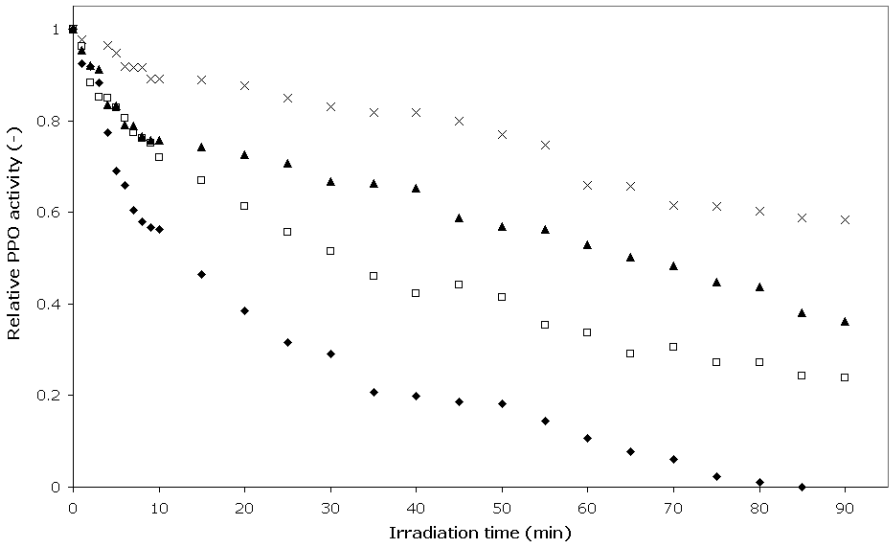

Figure 4.4.8. *Relative activity of polyphenol oxidase as a function of UV-vis irradiation time, in a solution containing different concentrations of non-fractioned melanoidins from glucose and glutamic acid.* ◆: *0 mg/mL (no melanoidin).* □: *0.1 mg/mL.* ▲: *0.2 mg/mL.* ×: *0.4 mg/mL.*

Therefore, the kinetic parameters obtained with the composite model (Table 4.4.6) must be studied to infer the effects of the different melanoidin fractions (kinetic parameters from the first-order model are not shown). Inactivation constant, k_{II}, describes the transformation of the intermediate enzyme form (I, still active) into the inactive one (D). As it has been already commented, this second step of the process lasts for longer than the first one. This kinetic constant decreases as melanoidin content increases in all series, which shows that the polymers prevent the enzyme from being inactivated, making this phase to be slower. Comparing the values obtained with the different fractions, it can be stated that this protective effect is greater in the separated fractions than in the NF. In this way, for each single melanoidin concentration, the polymers lower than 150 kDa exhibited the greatest protection (k_{II} has the lowest value), followed by the fraction of more than 150 kDa and, lastly, the NF. This fact can be explained by agglomeration phenomena between the polymers in the solution, which were more important in the NF (aggregation is favored by the presence of different chain length molecules) leading to a lower absorbance of the solution (Figure 4.4.8).

Table 4.4.6. *Estimated values of the kinetic parameters obtained with the composite kinetic model.*

	Melanoidin content	k_I (min^{-1})	k_{II} (min^{-1})	Λ (-)
	0 mg/mL	0.072 ± 0.034	0.0252 ± 0.0053	0.392 ± 0.025
Non-fractioned	0.1 mg/mL	0.108 ± 0.047	0.0144 ± 0.0006	0.455 ± 0.102
	0.2 mg/mL	0.212 ± 0.045	0.0074 ± 0.0005	0.752 ± 0.029
	0.4 mg/mL	0.404 ± 0.017	0.0038 ± 0.0006	0.831 ± 0.024
<150 kDa	0.1 mg/mL	0.352 ± 0.032	0.0051 ± 0.0003	0.653 ± 0.017
	0.2 mg/mL	0.432 ± 0.075	0.0025 ± 0.0006	0.787 ± 0.006
	0.4 mg/mL	0.788 ± 0.027	0.0010 ± 0.0001	0.816 ± 0.012
>150 kDa	0.1 mg/mL	0.086 ± 0.016	0.0064 ± 0.0008	0.698 ± 0.016
	0.2 mg/mL	0.243 ± 0.036	0.0046 ± 0.0004	0.733 ± 0.005
	0.4 mg/mL	0.408 ± 0.027	0.0013 ± 0.0001	0.757 ± 0.048

Signification level: α=0.05.

As far as the first step of the process (the transformation of the native form, N, into the intermediate one, I, described by k_I) is concerned, it is faster (it lasts shorter) as melanoidin content increases. Moreover, this parameter has its highest values in the series with polymers smaller than 150 kDa. If the kinetic parameters obtained in this piece of work are compared with those found for melanins from 4-methylcatechol at the same concentration, it can be stated that these melanoidins (regardless the considered fraction) have a greater protective effect than that melanins. This fact can be explained by the difference in the solubility between both kinds of polymers, which leads to different absorbance of their aqueous solutions.

As it has been already commented, absorbance spectra of the solutions containing the different fractions of melanoidins (Figure 4.4.9) can explain the different protection degree that these polymers offer against PPO inactivation by UV-vis irradiation. Since the polymers with molecular mass lower than 150 kDa were the most protective ones, followed by the fraction higher than 150 kDa and in the last place the NF, the most important wavelengths to inactivate the enzyme must be those in which the absorbance of the different samples follow the same order. This condition is accomplished between 260 and 310 nm. This fact leads to the conclusion that, although the lamp emitted radiation between 250 and 740 nm, UV radiation contributed more to PPO inactivation than visible radiation, since in that region of the spectrum (between 400 and 700 nm) the absorbance of the different melanoidin solutions did not follow this order. In addition, the absorbance of the different solutions in the entire

spectrum (not shown) decreased as processing time increased (with the most remarkable decrease at wavelengths between 260 and 310 nm), meaning that melanoidin polymers were partially impaired by UV irradiation. In other words, these molecules absorbed radiation and were partially broken, leaving a lower radiation dose to inactivate the enzyme.

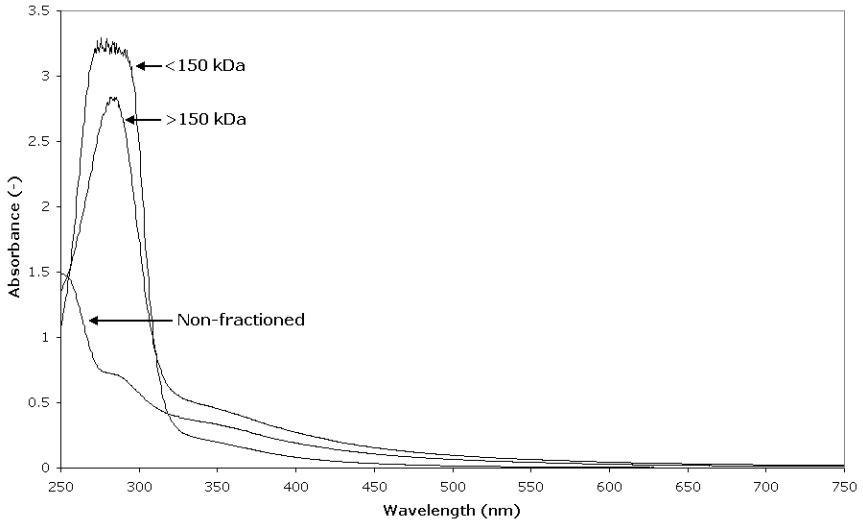

Figure 4.4.9. *Absorbance spectra of the different fresh melanoidin solutions at a concentration of 0.1 mg/mL.*

4.5. Polyphenol oxidase inactivation by UV-vis irradiation (2): Study of fruit juices.

According to the previous results, it is feasible to inactivate polyphenol oxidase with an UV-vis irradiation process, and the time required to achieve this inactivation largely depends on the other compounds that surround the enzyme, which are also affected by the radiation. Therefore, in order to assess the suitability of this technology to process fruit juices, the effects on all the other compounds and quality parameters must be addressed. This section includes the results of UV-vis irradiation studies of apple, pear and grape juices.

4.5.1. UV-vis irradiation of apple juice.

Enzymatic activities.

Polyphenol oxidase (PPO).

The four studied juices had an initial PPO activity of 0.18 U/mL (Golden), 0.45 U/mL (Starking), 0.14 U/mL (Fuji) and 0.06 U/mL (King David). As it will be explained, King David had the lowest PPO activity in part due to its low pH, although it had the highest phenolics content. Figure 4.5.1 shows the change of PPO activity of the four juices with irradiation time. Regardless its initial value, PPO was completely inactivated in all samples after 100 min of treatment.

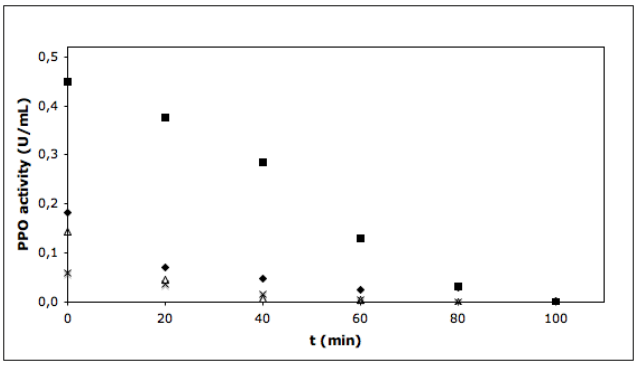

Figure 4.5.1. *Inactivation of polyphenol oxidase in apple juices with UV-vis irradiation time. ◆Golden. ■Starking. △Fuji. ×King David.*

Peroxidase (POD).

Initial activities of peroxidase in fresh-made juices were very small. The assays showed an activity of 0.05 U/mL in Golden, 0.09 U/mL in Starking, 0.1 U/mL in Fuji and 0.03 U/mL in King David. POD was the most sensitive enzyme to UV irradiation, since the null activity was reached after only 15 minutes of treatment in all samples.

Pectinmethylesterase (PME).

No PME activity was found in the juices made from Golden, Fuji and King David. The juice from Starking showed a very small activity of $4.7 \cdot 10^{-5}$ U/mL, which was completely inactivated after 40 min of irradiation. The fact that no activity was found in three of the four samples and in the other one was very small can be related to the juice preparation procedure, since a centrifugation was carried out after squeezing in order to separate the pulp. Once the apple structures are broken, all the enzymes are released and come into contact with its substrate, starting to act quickly. In this way, pectinolytic enzymes will start to act over pulp. Thus, during centrifugation, all those molecules linked to substrate will be separated, remaining in the pellet.

Exo-polygalacturonase and endo-polygalacturonase (exo-PG and endo-PG).

In a similar way to what happened with PME, no activity was found for polygalacturonases in any sample. Since these are pectinolytic enzymes, they also must have remained in the pellet after centrifugation. The variations observed in these two kinds of analysis between the samples and the blanks are very small and can be attributed to measurement error.

Physical and chemical analyses.

pH, soluble solids content, formol index, total phenolics and sugars.

Although there were important initial differences between the juices from the four varieties, these parameters kept their original value constant during the 120 min the experiment lasted. For example, total phenolics content was 2.55 $mg \cdot L^{-1}$ in Starking juice and 10.57 in King David one. The average values of all these composition variables are shown in Table 4.5.1. pH measures showed a

maximum variation of 0.03, while in soluble solids it was 0.2 ºBrix. All the observed variations can be attributed to measurement error. Noci *et al.* (2008) also found no differences in the evolution of pH and soluble solids content in the UV irradiation of apple juice. However, they also found a decrease in total phenolics content during the process that has not been observed in the present work.

***Table 4.5.1**. Physical and chemical properties of the raw juices from the four different apple varieties.*

Sample	Golden	Starking	Fuji	King David
pH (-)	3.89 ± 0.03	4.35 ± 0.03	4.29 ± 0.02	3.48 ± 0.03
Soluble solids (ºBrix)	10.7 ± 0.1	14.4 ± 0.2	14.7 ± 0.2	11.1 ± 0.1
Formol index (mL NaOH/100 mL)	5.4 ± 0.4	6.4 ± 0.4	3.6 ± 0.3	9.4 ± 0.5
Total phenolics (pyrogallol eq., mg/L)	7.84 ± 0.10	2.55 ± 0.06	4.55 ± 0.07	10.57 ± 0.09
Reducing sugars (glucose eq., g/L)	68.6 ± 0.5	47.8 ± 0.5	53.8 ± 0.3	73.8 ± 0.7
Total sugars (glucose eq., g/L)	245.7 ± 0.9	182.2 ± 0.7	201.2 ± 0.6	252.2 ± 0.9

Mean value ± Standard deviation.

Juice color.

CIELab parameters *L**, *a** and *b** were measured during irradiation. Figure 4.5.2 shows the evolution of these parameters in the 120 min. *L**, which is an indicator of the brightness of the juice, increased with the time of irradiation in Golden, Starking and Fuji samples. In addition, Table 4.5.2 shows the differences found in absorbance at different wavelengths in the visible region between the samples taken before starting and after 120 min of irradiation. This indicates that UV radiation impairs some of the pigments present in the juice, either those initially present or the ones formed later by the rapid action of polyphenol oxidase (melanins) as well as the Maillard reaction between sugars and amino acids (melanoidins). This observation is consistent with the findings of other authors such as Ibarz *et al*. (2005) in their UV irradiation of clarified apple, peach and lemon juices. The juice from King David, which had the greatest initial brightness, kept its value almost invariable.

The parameter *a** decreased during irradiation in the samples from Golden, Starking and Fuji, which means that the juices became less red. Again, in the sample from King David, the parameter *a**, which had the lowest initial value, slightly changed during processing. Regarding parameter *b**, its value decreased in the samples from Golden (which had the highest initial one),

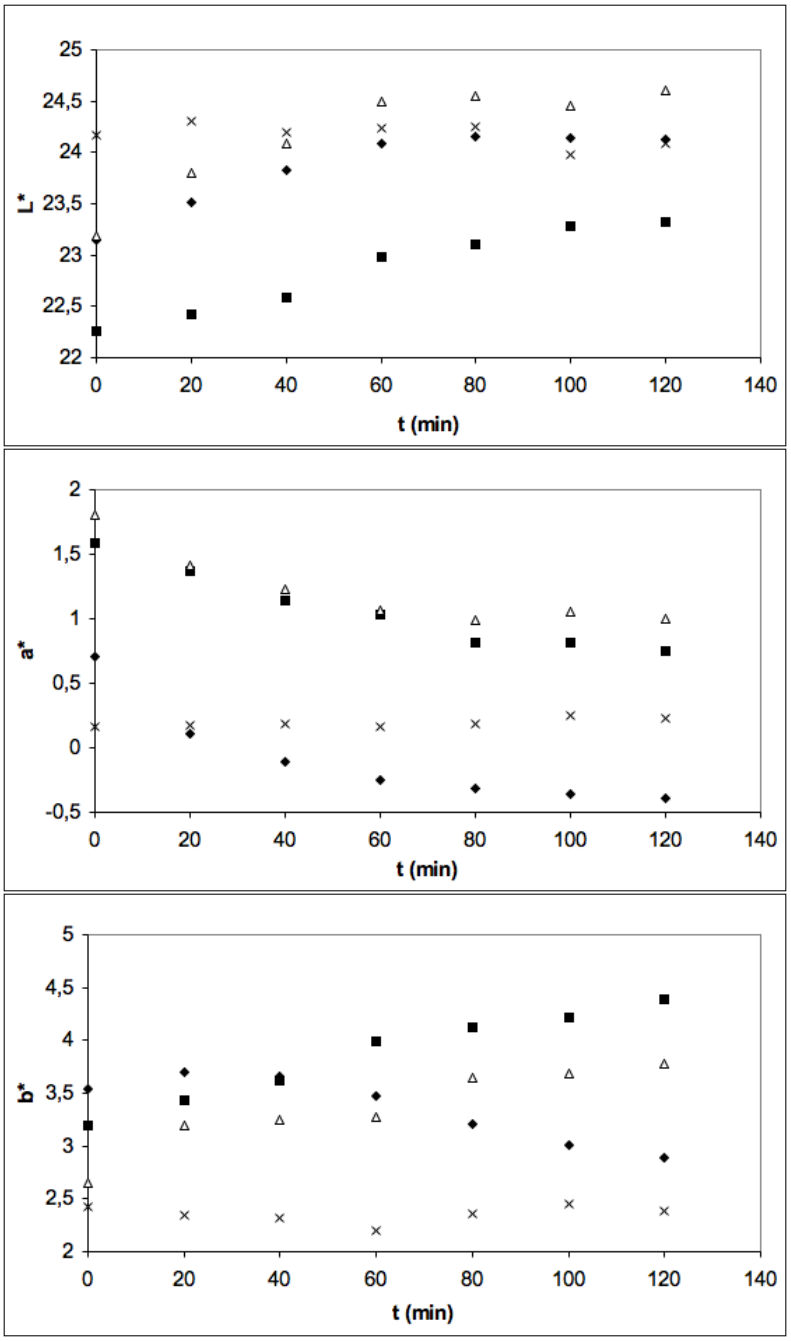

Figure 4.5.2. *Evolution of CIELab color parameters of apple juices during UV-vis irradiation. Upper graph: L*. Middle graph: a*. Lower graph: b*.* ◆*Golden.* ■*Starking.* △*Fuji.* ×*King David.*

slightly increased in the samples from Starking and Fuji and remained almost constant in the juice from King David, which had the lowest initial value. Pesek & Warthensen (1987) working with tomato and carrot juices, and Guerrero-Beltrán & Barbosa-Cánovas (2005) in the UV irradiation of apple juice, observed a decrease in both *a** and *b**, reporting the photodestruction of some pigments of the juice. The samples made from King David apples had the lightest color (as it has been observed with the three CIELab parameters), in part due to its low pH value that entails a slower action of polyphenol oxidase in the fresh-prepared juice.

***Table 4.5.2**. Absorbance at different wavelengths in the visible region of the juices from the four different apple varieties, at zero time and after 120 min of irradiation.*

Apple	*t* (min)	Wavelength (nm)			
		400	500	600	700
Golden	0	1.322	0.586	0.284	0.183
	120	0.964	0.423	0.247	0.178
Starking	0	1.587	1.751	0.891	0.610
	120	0.851	1.674	0.678	0.390
Fuji	0	1.702	0.615	0.202	0.105
	120	1.085	0.315	0.121	0.079
King David	0	0.358	0.118	0.051	0.033
	120	0.323	0.115	0.044	0.024

Absorbance spectrum.

The variation in the juice color that has been described before and the influence of UV radiation on colored compounds can also be observed with the evolution of the absorption spectrum. Each sample has a characteristic curve depending on its pigmentation. For example, in King David juice a very low absorbance is observed in the visible range, reflecting this lack of pigments because of the sharp decrease of PPO activity. Thus, the variations observed during the experiment were very small.

Regarding the other juices, the absorbance decreased as irradiation time increased (Figure 4.5.3) because some pigments of the juice were degraded during the process. The decrease was especially significant between 400 and 450 nm, which corresponds to purple and blue colors and to the largest peak of the emission spectrum of the lamp (shown in Figure 3.2.1). Thus, the molecules

that absorb radiation of these wavelengths are the most affected by irradiation. This fact can explain why the parameter *b** increased in some of the juices.

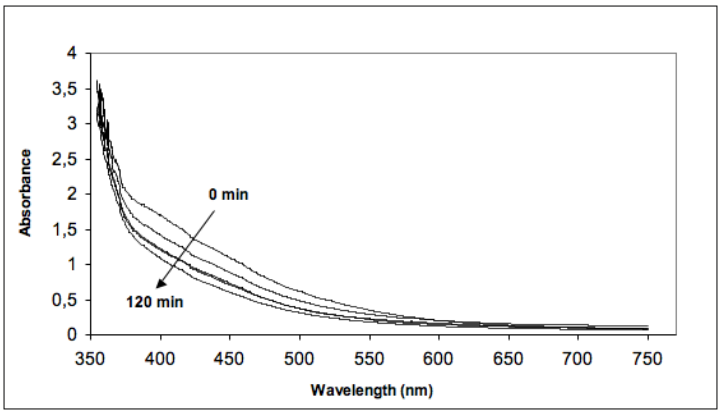

Figure 4.5.3. *Evolution of the absorbance spectrum of the juice made from Fuji apples during UV-vis irradiation.*

Vitamin C.

The content in vitamin C of the juices had very different behaviors depending on the sample. Figure 4.5.4 shows the evolution of vitamin C in apple juices made from the four different varieties. In the fresh-made juices, the one from Golden had an initial content of 826.6 mg/L of ascorbic acid, the one from Starking 788.2 mg/L, the one from Fuji 588.6 mg/L and the one from King David 277.4 mg/L. The content in juices from Golden, Starking and Fuji slightly decreased during the experiment. The loss in Golden juice after 120 min of UV irradiation was 5.7%, while in the Starking one was 5.6%, and in the Fuji one 4.0%.

However, in the juice from King David, which had the lowest initial content, the loss was 70.0%. This significant difference can be attributed to the lack of pigmentation of this juice that has been discussed before. These pigments absorb the energy of the radiation and have a protective effect on the other molecules of the system, as it happened between melanins or melanoidins and PPO. Moreover, in Golden, Starking and Fuji juices the losses after the first hour of treatment were 2.4%, 1.7% and 1.9% of the initial content. Then, in these three cases in the second hour more vitamin C was damaged than in the

first 60 min, meaning that as pigments were degraded (and the juice color was lighter) its protective effect was smaller. In the King David juice, where there were a very fewer concentration of colored compounds, the loss after 20 min was 62.4% of the initial content, and after 60 min it was 69.8%. Ibarz & Pérez-Teijón (1990), in their work with commercial clarified apple juice, found a 43% decrease of the vitamin C initial content in the first 30 min, remaining this value invariable until the end of the experiment (120 min). Tran & Farid (2004) concluded that the loss of vitamin C content of an UV-irradiated orange juice was similar to the loss caused by a thermal treatment.

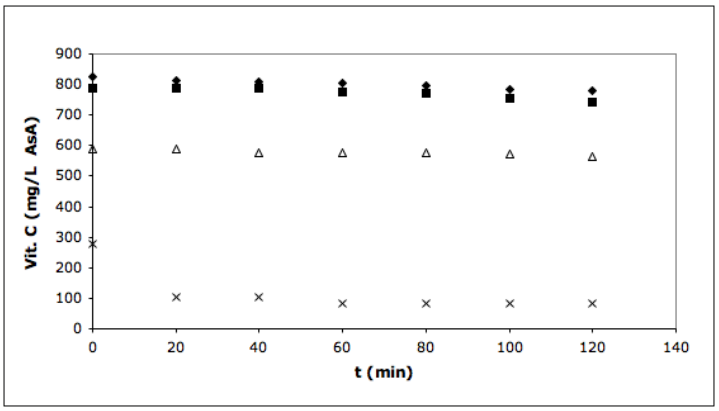

Figure 4.5.4. *Evolution of vitamin C in the apple juices with UV-vis irradiation time (in mg·L^{-1} of ascorbic acid (AsA)).* ◆*Golden.* ■*Starking.* △*Fuji.* ×*King David.*

4.5.2. UV-vis irradiation of pear juice.

Enzymatic activities.

Polyphenol oxidase (PPO).

As observed in Table 4.5.3, Conference is the pear with the highest activity, whereas the Flor de Invierno variety is the one with the lowest. Figure 4.5.5 shows the evolution of the relative PPO activity with irradiation time for the juices of the six investigated varieties. It was observed that after 120 minutes the residual activity percentage was almost null for most of the samples, but in the Ercolini variety it still had 17% of its initial activity. However, when observing the absolute values of the PPO activities, there is barely any enzymatic activity at the end of the process.

Moreover, it is important to remark that after 20 minutes of irradiation PPO activity had decreased in more than 50% for the juices of five varieties. The most different behavior is that of Conference variety, reaching after 120 min 43% of the initial enzymatic activity. Nevertheless, as its initial enzymatic activity is the highest one, the absolute activity variation was also the highest one.

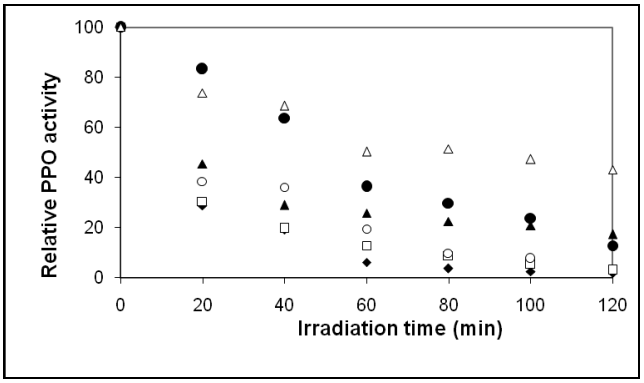

Figure 4.5.5. *Inactivation of polyphenol oxidase in pear juices with UV-vis irradiation time. ♦ Abate Fétel. ▲ Ercolini. • Blanquilla. □ Passa Crassana. ○ Flor de Invierno. △ Conference.*

Table 4.5.3. *Change of physicochemical parameters and enzymatic activities of pear juices with irradiation.*

	Abate Fétel		**Passa Crassana**	
Parameter	Initial	Final	Initial	Final
pH	5.08 ± 0.01	5.02 ± 0.01	3.9 ± 0.1	3.81 ± 0.01
Acidity [a]	0.26 ± 0.01	0.26 ± 0.01	1.54 ± 0.08	1.55 ± 0.05
Sol. solids (ºBrix)	11.0 ± 0.1	11.1 ± 0.1	11.7 ± 0.1	11.9 ± 0.1
*L**	23.61 ± 0.90	34.00 ± 1.41	29.31 ± 0.10	36.35 ± 0.02
*a**	17.11 ± 2.52	12.81 ± 0.12	11.70 ± 0.21	5.14 ± 0.02
*b**	12.29 ± 0.61	20.01 ± 1.61	9.23 ± 0.23	14.25 ± 0.01
PPO (U/mL)	0.246 ± 0.004	0.0044 ± 0.0003	0.097 ± 0.008	0.0029 ± 0.0002
POD (U/mL)	0.09 ± 0.05	0 ± 0	0.114 ± 0.003	0 ± 0
Phenolics [b]	300 ± 19	307 ± 24	405 ± 3	418 ± 6
Formol Index [c]	8.5 ± 0.7	9.0 ± 0.2	4.0 ± 0.1	4.1 ± 0.1
Total sugars (g/L)	75.2 ± 1.6	76.3 ± 0.8	88.0 ± 3.7	90.0 ± 3.9
Red. sugars (g/L)	73.6 ± 0.7	74.1 ± 2.2	73.0 ± 1.4	73.6 ± 0.7
Ascorbic ac. (mg/L)	357 ± 22	169 ± 22	286 ± 10	236 ± 22
	Ercolini		**Flor de Invierno**	
Parameter	Initial	Final	Initial	Final
pH	4.84 ± 0.01	4.80 ± 0.01	4.89 ± 0.02	4.97 ± 0.01
Acidity [a]	0.16 ± 0.02	0.18 ± 0.01	0.13 ± 0.01	0.14 ± 0.01
Sol. solids (ºBrix)	8.3 ± 0.1	8.5 ± 0.1	11.7 ± 0.1	11.8 ± 0.1
*L**	34.69 ± 0.19	42.01 ± 0.41	26.40 ± 0.10	32.74 ± 0.06
*a**	7.70 ± 1.51	-0.50 ± 0.18	15.20 ± 0.20	8.86 ± 0.06
*b**	22.41 ± 1.29	11.51 ± 0.39	5.71 ± 0.41	11.68 ± 0.03
PPO (U/mL)	0.0591 ± 0.0005	0.0102 ± 0.0002	0.038 ± 0.002	0 ± 0
POD (U/mL)	0.20 ± 0.08	0 ± 0	0.034 ± 0	0 ± 0
Phenolics [b]	200 ± 5	218 ± 1	350.4 ± 20.1	382.1 ± 30.4
Formol Index [c]	10.5 ± 0.7	12.5 ± 3.5	7.5 ± 0.7	7.5 ± 0.7
Total sugars (g/L)	46.3 ± 0.6	48.0 ± 1.1	85.4 ± 1.4	88.7 ± 1.1
Red. sugars (g/L)	46.3 ± 0.6	47.8 ± 0.3	85.1 ± 2.0	85.8 ± 1.0
Ascorbic ac. (mg/L)	229 ± 17	106 ± 17	467 ± 12	371 ± 12
	Blanquilla		**Conference**	
Parameter	Initial	Final	Initial	Final
pH	4.35 ± 0.01	4.39 ± 0.03	4.66 ± 0.01	4.64 ± 0.01
Acidity [a]	0.43 ± 0.01	0.44 ± 0.01	0.20 ± 0.01	0.20 ± 0.01
Sol. solids (ºBrix)	13.1 ± 0.1	14.0 ± 0.1	12.6 ± 0.1	13 ± 0.1
*L**	22.79 ± 0.01	28.50 ± 0.02	24.89 ± 0.09	34.96 ± 0.05
*a**	14.10 ± 0.19	8.49 ± 0.07	10.57 ± 0.05	6.46 ± 0.07
*b**	4.09 ± 0.10	6.73 ± 0.07	1.55 ± 0.05	2.68 ± 0.04
PPO (U/mL)	0.041 ± 0.004	0.005 ± 0.001	0.6 ± 0.15	0.247 ± 0.003
POD (U/mL)	0.032 ± 0.008	0 ± 0	0.032 ± 0.001	0 ± 0
Phenolics [b]	154.1 ± 0.6	189.0 ± 0.9	169.0 ± 1.1	194.1 ± 1.3
Formol Index [c]	13.5 ± 0.7	13.0 ± 0.1	13.5 ± 0.7	13.0 ± 0.2
Total sugars (g/L)	118.6 ± 1.0	118.0 ± 1.9	73.4 ± 0.4	73.6 ± 0.2
Red. sugars (g/L)	101.7 ± 1.0	102.8 ± 1.4	73.1 ± 0.1	72.1 ± 0.2
Ascorbic ac. (mg/L)	128 ± 10	93 ± 12	141 ± 25	99 ± 10

Mean value ± Standard deviation
a: expressed as malic acid eq., g/100 mL
b: expressed as pyrogallol eq., g/L
c: expressed as mL NaOH 0.1M/100 mL

Peroxidase (POD).

As far as POD activity is concerned, this enzyme is generally more sensitive to the UV-Visible radiation, and shorter treatment times are necessary than those of PPO to reach a decrease in its activity, as it was seen in the case of apple juices (previous section). For juices of Passa Crassana, Conference and Blanquilla varieties, total inactivation is reached in 30 minutes; meanwhile, for Abate Fétel and Ercolini varieties, 60 minutes are required. The Flor de Invierno variety is a special case, which keeps its initial enzymatic activity during the first 15 minutes, slightly decreasing with longer times.

Physical and chemical analyses.

Table 4.5.3 shows the values for pH, acidity, soluble solids, CIELab colorimetric parameters, PPO and POD enzymatic activity, phenolic content, formol index, total and reducing sugars and vitamin C of the pear juice samples from the six investigated varieties, before and after 120 minutes of photochemical treatment. Depending on the pear variety, the juices show different values for each of the analyzed parameters. This table shows that the 120-minute photochemical treatment of the pear juices barely has an effect in the pH, acidity and formol index. The small differences can be attributed to measurement error. However, a slight increase is observed in soluble solids, which could be attributed to the fact that, in the long irradiation time, agitation of the sample can cause a slight evaporation of water, which would be the cause for this slight increase of ºBrix. In addition, a slight increase is detected in phenolics content, probably due to the breakdown of complex phenolic polymers that leads to the release of simpler phenolic compounds. These effects were not observed on phenolic content in UV-vis-irradiated apple juices.

Juice color.

Figure 4.5.6 shows the brightness (L^*) evolution with the irradiation time for the six pear juice varieties, observing that in all of them this parameter's values have an increasing trend. In addition, this figure also shows the evolution of a^* and b^*, observing that the former tends to decrease, reducing the red tonalities, while b^* parameter tends to more yellow tonalities (except in the Ercolini variety, where the juice behaves in the opposite way). Moreover, in the case of Ercolini,

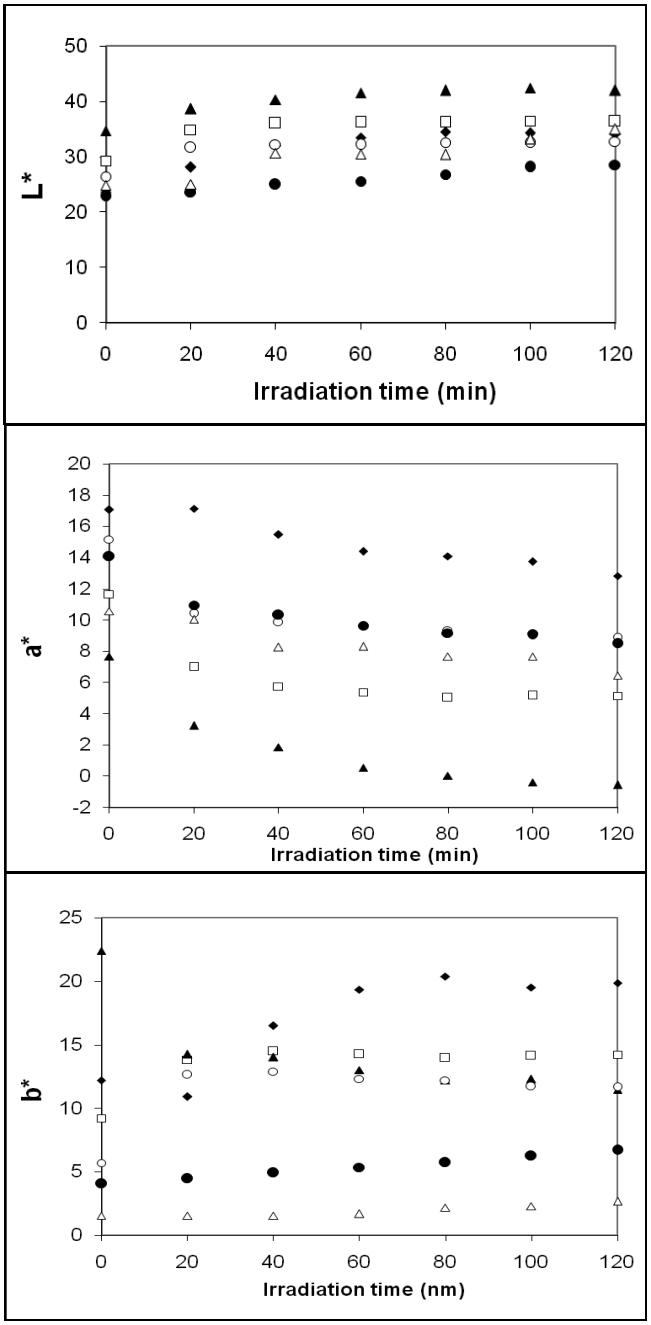

Figure 4.5.6. *Evolution of CIELab color parameters of pear juices during UV-vis irradiation. Upper graph: L*. Middle graph: a*. Lower graph: b*.* ♦ *Abate Fétel.* ▲ *Ercolini.* • *Blanquilla.* □ *Passa Crassana.* ○ *Flor de Invierno.* △ *Conference.*

*L** and *a** values show a globally much less colored juice. Generally speaking, these analyses confirm that irradiated pear juice samples become clearer. The different behavior observed in Ercolini juice proves the need for carrying out a single study for every single variety.

These results are consistent with those obtained by Ibarz *et al.* (2005), where the commercial apple, peach and lemon juices irradiated with a lamp emitting in the UV-vis spectrum suffered an appreciable discoloration. Likewise, these CIELab parameters show a variation that is analogous to that of different variety apple juices (Guerrero-Beltrán & Barbosa-Cánovas, 2005; previous section) and tomato and carrot juices (Pesek & Warthesen, 1987).

Absorbance spectrum.

The absorbance spectrum is another measure of the juice coloration, which depends on its pigment content. Figure 4.5.7 shows the absorption spectrum of the Passa Crassana variety pear juice in the interval between 350 nm and 750 nm. Absorbance spectra for the other varieties are similar, except for the Ercolini variety, the absorption spectrum of which is remarkably lower (not shown). It was observed that irradiation produces a slight decrease in the absorption spectrum of the Ercolini variety.

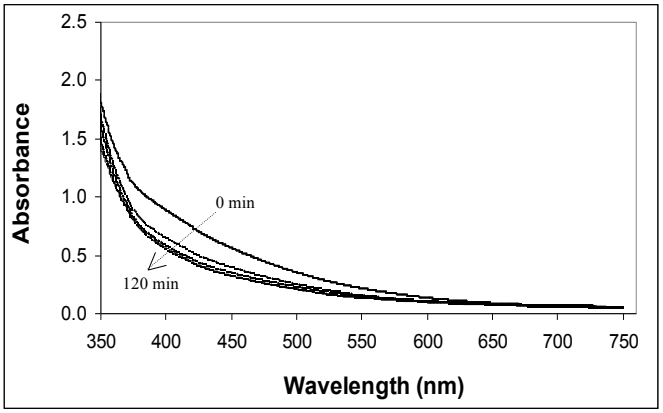

Figure 4.5.7. *Evolution of the absorbance spectrum of the juice made from Passa Crassana pears during UV-vis irradiation.*

For the rest of varieties, in the first twenty minutes of irradiation there was a very important decrease of the absorbance values, being more remarkable for wavelengths between 375 nm and 450 nm. This interval corresponds to purple colorations and to two important peaks of the lamp's emission spectrum. Thus, it was predictable that there would be a degradation of the juices' own pigments in this interval, besides the reddish-brown polymers formed in the browning reactions, both enzymatic and non-enzymatic ones. Likewise, these results are in concordance with the variation of the CIELab colorimetric parameters shown before. Similar results were obtained for apple juices (previous section).

Vitamin C.

Photochemical treatment of pear juices produces a decrease of vitamin C content. Figure 4.5.8 shows the evolution of this vitamin content with irradiation time, being observed that there is a continuous decrease. In the case of Abate Fétel and Ercolini juices, the vitamin C content at the end of the treatment has 60,5% and 50% decrease, respectively, in relation to the initial content. In the other varieties this decrease is found in the 20% to 30% interval.

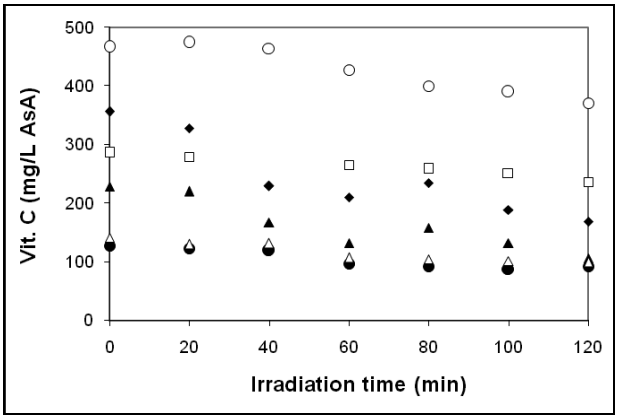

Figure 4.5.8. *Evolution of vitamin C in the pear juices with UV-vis irradiation time (in mg·L^{-1} of ascorbic acid (AsA)). ♦ Abate Fétel. ▲ Ercolini. • Blanquilla. □ Passa Crassana. ○ Flor de Invierno. △ Conference.*

The great decrease observed in the Ercolini variety juices could be explained by the fact that, as mentioned before, the juice presented clearer tonalities, with more luminosity and less coloration and absorbance, which indicate a lower presence of pigments in the juice. As it has been largely discussed, these

pigments can absorb the incident radiation, acting as photoprotectors and therefore avoiding the degradation of other compounds. Due to this fact, the protective effect in the juice from Ercolini is not as high as in other varieties, and this variety will suffer more intense vitamin C degradation by the UV-vis radiation. The results obtained in the present work show a similar behavior to irradiated apple (previous section) and orange juices (Tran & Farid, 2004).

4.5.3. UV-vis irradiation of grape must.

Enzymatic activities.

Polyphenol oxidase (PPO).

Initial PPO activities in the fresh-made grape juices from Emperor and Red Globe (pink varieties) were 0.023 U/mL and 0.0072 U/mL, while from Victoria and Dauphine (white varieties) were, 0.0071 U/mL and 0.0053 U/mL, respectively. Figure 4.5.9 shows the evolution of PPO relative activity of the four grape musts with irradiation time. Juices from white grape varieties were more affected by UV irradiation than the pink ones. There was a decrease higher than 80% in the activity of PPO in these juices after 140 min of irradiation, while for juices of pink grapes the decrease in PPO activity was 50% on average. In fact, multiple range tests showed that there were no significant differences between the values of residual PPO activity of both varieties of the same kind.

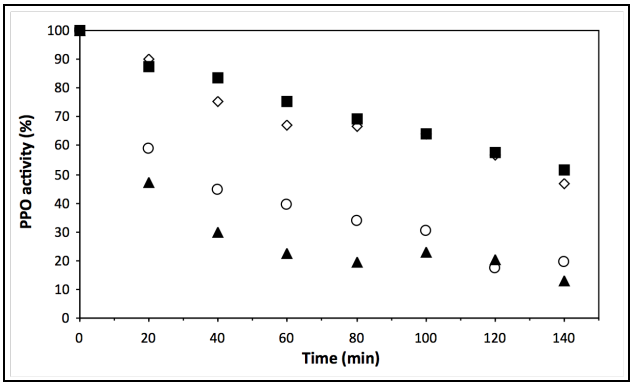

Figure 4.5.9. *Changes in PPO relative activity in grape musts with UV-vis irradiation time.* ■ *Emperor,* ◇ *Red Globe,* ▲ *Victoria,* ○ *Dauphine.*

This difference in stability of PPO could be a result of the different properties of the white and pink grape juices, since the content of pigments in pink grape juices was higher, playing a protective effect on the PPO, as it has been reported for the different model solutions and apple and pear juices. The effects of UV radiation on PPO have been studied by some other researchers. Thus, Guerrero-Beltrán and Barbosa-Cánovas (2006) found that the decrease in

polyphenol oxidase activity in mango nectar, after 30 min of UV treatment, was 81%. In a different way, Noci *et al*. (2008) found that PPO was not affected when fresh apple juices were UV-irradiated. However, in that case apple juice was irradiated with a 30 W UV light source that emitted at only 254 nm for 30 min (lower power, narrower emission spectra and shorter time than in the present work).

Peroxidase (POD).

Initial activities of peroxidase in all grape juices samples were quite low. Initial POD activity in Dauphine was 0.07 $U \cdot mL^{-1}$, in Emperor 0.053 $U \cdot mL^{-1}$, in Victoria 0.029 $U \cdot mL^{-1}$ and in Red Globe 0.009 $U \cdot mL^{-1}$. López-Miranda *et al*. (2011) reported that the average POD activity recorded for three years in Crimson Seedless grape varied from 0.024 to 0.075 $U \cdot g^{-1}$. In all samples, after 30 minutes of UV irradiation the activity of POD was practically null. Thus, POD was the most sensitive enzyme to UV irradiation. Similar results were found in apple juices, where POD was completely inactivated after only 15 minutes of treatment. In contrast, Noci *et al*. (2008) found that POD activity in fresh apple juices was unaffected by UV irradiation at 254 nm with a 30W lamp for 30 min.

Pectinmethylesterase (PME).

No activity of pectinmethylesterase was found in the juices from the four grape varieties tested. However, Lee *et al*. (1978) studied the PME activity in several varieties of grape, and reported values from 42 to 106 $U \cdot ml^{-1}$. They also observed that red grapes showed higher PME activity than white grapes. Large portions of PME and pectin substances in grapes are located in the skin (Lee *et al*. 1979; Lacampagne *et al*., 2010), which could be a reason to explain the lack of PME activity in these samples.

Physical and chemical analyses.

pH, ºBrix, titratable acidity, formol index, total phenolics and sugars.

The properties of grape juices used for UV-vis irradiation processing are listed in Table 4.5.4. During irradiation, no significant changes in pH, °Brix and titratable acidity were observed. These results are consistent with the ones obtained with juices from different apple varieties. Similar results were also

observed for sonicated red grape juice (Tiwari *et al*., 2010) and for ozonated grape juice (Tiwari *et al*., 2009).

Table 4.5.4. *Physicochemical properties of fresh grape musts.*

Sample	Red Globe	Emperor	Victoria	Dauphine
pH (-)	^{a}3.30±0.06	^{c}3.78±0.02	^{b}3.67±0.03	^{a}3.22±0.12
Titratable acidity [v]	^{c}0.81±0.01	^{a}0.60±0.02	^{b}0.73±0.01	^{d}1.59±0.01
Soluble solids (ºBrix)	^{b}14.41±0.30	^{d}17.46±0.10	^{a}12.46±0.04	^{c}17.10±0.19
Formol index [w]	^{b}13.00±0.71	^{a}11.25±0.35	^{c}14.50±0.01	^{d}15.75±0.35
Total phenolics [x]	^{c}350±21	^{a}263±4	^{b}310±7	^{b}317±21
Reducing sugars [y]	^{d}114.04±0.01	^{b}87.95±1.05	^{c}104.36±3.71	^{a}63.86±0.28
Total sugars [y]	^{d}123.56±2.08	^{b}89.07±0.01	^{c}108.11±1.59	^{a}66.73±0.30
*CIELab L**	^{b}31.85±0.14	^{a}29.51±0.49	^{c}40.02±0.32	^{d}41.85±0.02
*CIELab a**	^{d}30.69±0.04	^{c}18.86±0.12	^{a}3.80±0.01	^{b}4.72±0.06
*CIELab b**	^{c}14.18±0.20	^{a}1.97±0.01	^{c}13.93±0.50	^{b}11.42±0.08
Maturity index [z]	^{b}18±1	^{c}29±1	^{b}17±1	^{a}11±1

Mean value ± Standard deviation. Different superscripts in a row indicate significant differences.
v: expressed as tartaric acid eq. (g/100 mL)
w: expressed as mL NaOH 0.1M/100 mL
x: expressed as pyrogallol eq. (mg/L)
y: expressed as glucose eq. (g/L)
z: expressed as (ºBrix·L·g^{-1})

The white varieties had higher formol index values than the pink ones. Thus, formol index was 15.75 mL NaOH/100mL in Dauphine and 11.25 mL NaOH/100mL in Emperor. The content of reducing and total sugars also depended on the variety of grape. These parameters were not affected by UV irradiation. Total phenolics content was influenced, somehow, by the UV irradiation treatment. Whereas in Emperor and Dauphine the content of total phenolics increased during irradiation (from 264 to 303 mg·L^{-1} and from 316 to 372 mg·L^{-1}, respectively), in Red Globe and Victoria it slightly decreased (from 350 to 340 mg·L^{-1} and from 310 to 305 mg·L^{-1}, respectively). This fact would mean that there are different processes during UV irradiation that affect phenolics content, some of which may enhance their extraction, and other ones may result in an impairment of some phenolic molecules.

Juice color.

To determine the effect of the applied UV treatment on the color of grape juices, CIELab parameters (*L**, lightness; *a**, redness-greenness and *b**, yellowness-blueness) were measured. Figure 4.5.10 shows the evolution of these parameters in the 140 min of UV processing. The final values for the

three parameters were found to be significantly different at a 95% confidence level for the four varieties.

In this way, significant changes in the color of grape juice were observed during UV irradiation treatment. In general, pink grapes (Red Globe and Emperor) experimented a greater variation of the studied parameters than the white ones (Victoria and Dauphine). This behavior can be due to the fact that pink grapes contain a higher concentration of pigments than the white ones, some of which were impaired. In pink grape juices, *L** values varied from 31.85 in untreated samples to 39.98 after 140 min of UV irradiation in Red Globe, and from 29.51 to 37.63 in Emperor. These increasing values indicate that grape juices from pink varieties became brighter. Similar increase in *L** was reported in sonicated red grape juice (Tiwari *et al*., 2010) and ozonated grape juice (Tiwari *et al*., 2009). Parameters *a** and *b** decreased during irradiation in samples from Red Globe, which means that the juice became less red and less yellow, respectively. This indicates a destruction of pigments in the juice of Red Globe. However, in the Emperor variety, parameter *a** decreased with the irradiation time, whereas *b** increased, which indicates that there is a destruction of initial pigments and a formation of new ones during irradiation. The color of grape juice is a mixture of various pigments depending on the maturity stage of the grape berry and cultivar; anthocyanins are the main ones, being responsible for colors ranging from pink through red and violet to dark blue (Corrales *et al*., 2009). In this way, observing the decrease in *a** (redness) in all pink varieties, these anthocyanins were severely damaged by UV irradiation.

The studied white varieties showed a slight decrease in *L**, which implies that the juices became a little darker. Parameters *a** and *b** also showed a similar evolution during the irradiation treatment, which indicates that samples became slightly less red and less yellow due to the photodestruction of pigments from the juice.

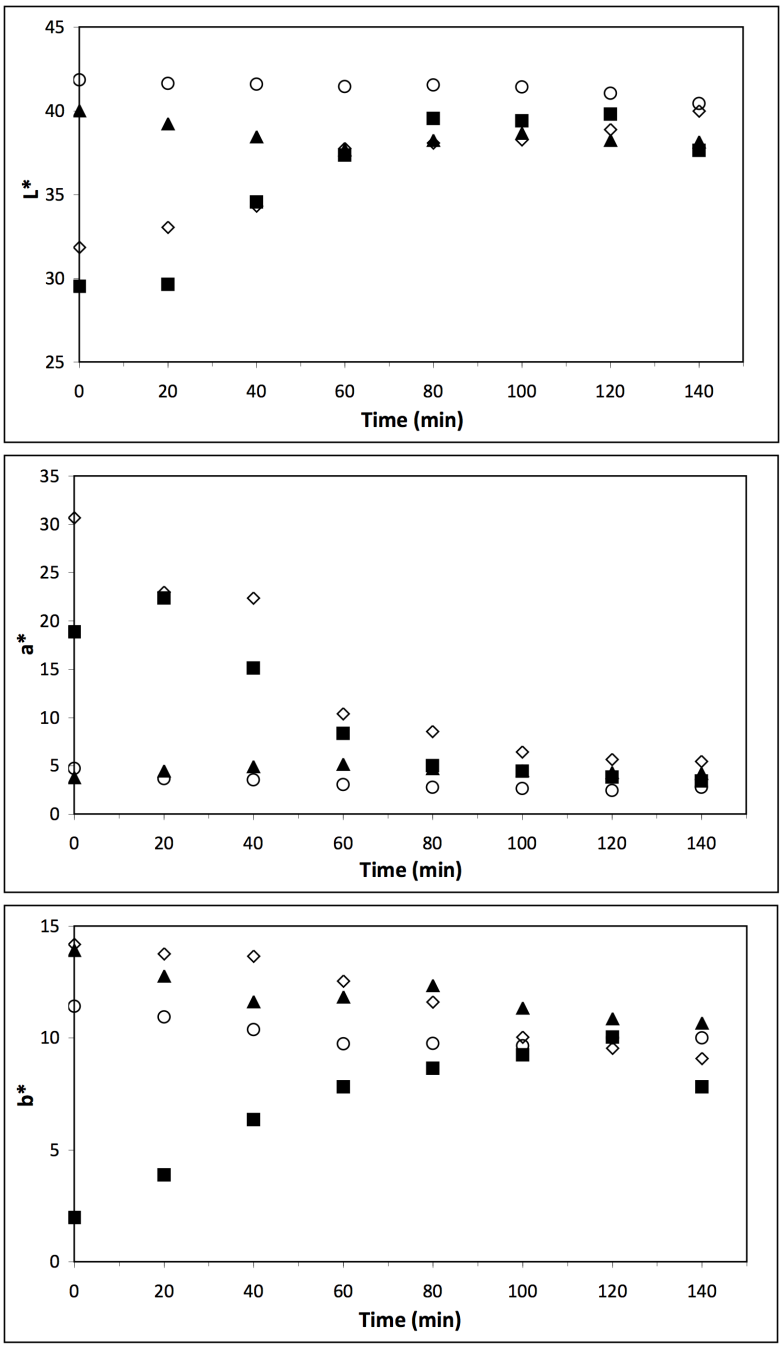

Figure 4.5.10. *Evolution of CIELab color parameters of grape musts during UV-vis irradiation. Upper graph: L*. Middle graph: a*. Lower graph: b*.* ■ *Emperor,* ◇ *Red Globe,* ▲ *Victoria,* ○ *Dauphine.*

Absorbance spectrum.

As usually, the influence of UV radiation on colored compounds of grape juice has also been observed with the evolution of the absorption spectrum. Figure 4.5.11 shows the changes of absorbance spectrum of the juice from pink grape varieties. Generally speaking, the absorbance slightly increased as irradiation time increased; consequently, lower absorbance was observed in unprocessed samples than in those irradiated for 140 minutes. This effect was clearly observed in juices from white grape varieties (spectra not shown) and it is consistent with the results previously discussed.

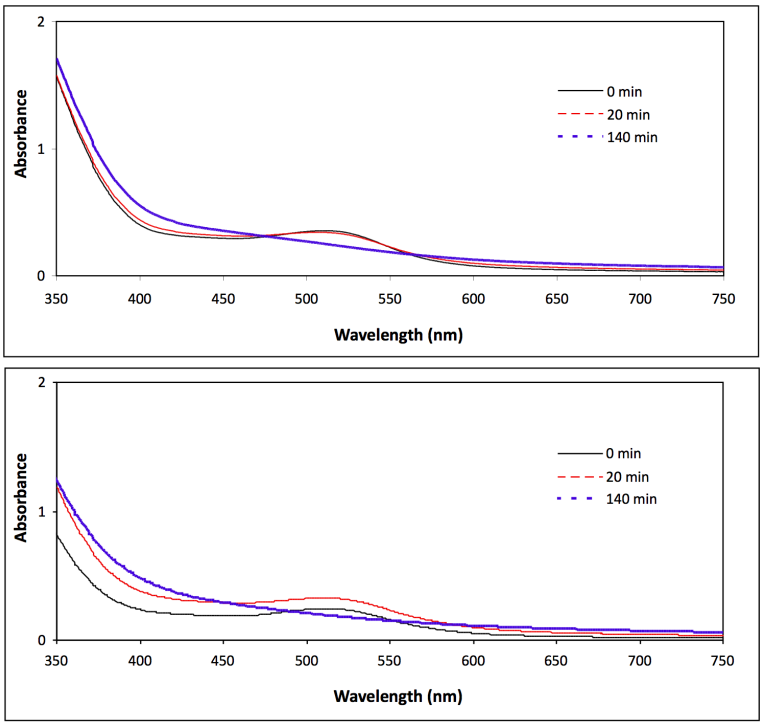

Figure 4.5.11. *Changes in the absorbance spectra of the juice made from pink grape varieties during UV-vis irradiation. Upper graph: Red Globe. Lower graph: Emperor.*

In the same way, absorbance of fresh-made must samples from pink grapes generally increased with irradiation time. However, unprocessed samples from pink grapes showed a characteristic peak between 450-550 nm, with a maximum around 510 nm. This peak disappears after 140 minutes of treatment, confirming the breakdown of some pigments in these juices by the treatment

with UV-vis radiation. The combination of these two factors in pink grapes (a general increase in absorbance below 450 nm and above 550 nm, but the disappearance of the peak between those wavelengths) may explain the results obtained for *L**, which increased with time of irradiation meaning that the juice became lighter.

Vitamin C.

The content in vitamin C of the juices changes in a different way depending on the variety. In the unprocessed musts, the one from Red Globe had an initial content of 452.5 mg/L of ascorbic acid, the one from Victoria 405.5 mg/L, the one from Emperor 273.0 mg/L and the one from Dauphine 136.5 mg/L. In all samples content of vitamin C decreases with UV-vis irradiation time. Red Globe juice, which had the highest initial content, showed, after 140 minutes of irradiation, the highest loss in vitamin C, with 30.10% of the initial content degraded. However, in Victoria juice, which had an initial content of vitamin C quite similar to Red Globe, the loss was 11.96 %, the lowest of the four grape varieties irradiated. Regarding the Emperor and Dauphine juices, the losses of vitamin C were 20.97 and 29.03 %, respectively.

Tran & Farid (2004) reported that the degradation of vitamin C in orange juice was 17% under high UV exposure of 100 mJ/cm^2. Since some of the vitamin C destruction could have been due to air oxidation, these authors measured the effect of air oxidation at 20 ºC, and concluded that the vitamin C loss in orange juice caused by air oxidation was only 1.0 %, compared to 17.0% due to UV irradiation. In the study of apple juices, the significant difference in vitamin C degradation rates was attributed to the high content of pigments in Golden, Staking and Fuji juices, which had a protective effect against UV radiation; in contrast, the lack of pigmentation of King David juice favored vitamin C destruction. In the present work, however, no direct relationship was observed between must pigmentation and the protection against vitamin C degradation.

4.6. Polyphenol oxidase inactivation by UV-vis irradiation (3): Study of side effects in winemaking.

The results obtained in the previous sections have proved that UV-vis irradiation can be useful in the inactivation of polyphenol oxidase in some fruit juices, including grape musts from different varieties. However, the process still needs to be improved in order to achieve higher inactivation degrees and shorter processing times. Moreover, the side effects on physicochemical parameters (mainly color) should be studied in each case in order to assess the suitability of this technology. These side effects may be a problem for UV-vis irradiation implementation in beverage industries that provide higher added value, such as winemaking, where the effects of must irradiation (as an alternative to reduce SO_2 utilization) on the final quality of wine still remain unclear.

This section aims at the characterization of these effects. In a first step, the impact of must treatment on the chemical properties of a wine from a white grape (Xarel·lo) and on a wine from a red one (Cabernet Franc) are studied. And in a second step, different parameters that may have influence on white wines quality are compared: the grape variety (Xarel·lo or Parellada), the use of SO_2 vs. UV-vis irradiation and whether must is frozen before fermentation.

4.6.1. Preliminary study: Effects of UV-vis irradiation of must on the chemical quality of white and red wines.

Table 4.6.1 shows the main properties of fresh Cabernet Franc (red) and Xarel·lo (white) grape musts. Cabernet Franc must had a higher soluble solids content, while Xarel·lo one had a lower pH and a lower polyphenol oxidase (PPO) activity. Regarding initial colorimetric parameters (shown in Table 4.6.2), Cabernet Franc had bigger red (+a^*) and blue (-b^*) values, and this stronger color was also traduced in a smaller brightness (L^*) and a higher absorbance at 420 nm.

Table 4.6.1. *Initial properties of the grape musts from both varieties.*

	Cabernet Franc	Xarel·lo
pH (-)	[a]3.34 ± 0.07	[b]2.93 ± 0.02
Soluble solids (ºBrix)	[a]18.2 ± 0.2	[b]17.1 ± 0.2
Absorbance at 420 nm	[a]0.967 ± 0.069	[b]0.251 ± 0.054
PPO activity (U/mL)	[a]0.019 ± 0.003	[b]0.011 ± 0.002

Mean value ± Standard deviation; different superscripts in a row indicate significant differences.

Polyphenol oxidase inactivation.

In Xarel·lo must, an inactivation higher than 85% was achieved after 210 min, while in Cabernet Franc must only 66% of the initial activity was eliminated. In both cases, the highest inactivation was achieved in the first hour: 53% in Xarel·lo and 25% in Cabernet Franc. The fittings of experimental data to the first-order kinetic model showed that, for Cabernet Franc, RA_0 was (1.01±0.03) and k (5.3±0.4)·10^{-3} min^{-1} (with a determination coefficient R^2 of 0.9986). Meanwhile, the value of RA_0 for Xarel·lo was (0.98±0.05) and the value of k (10.1±0.5)·10^{-3} min^{-1} (with a R^2 of 0.9852). Therefore, the inactivation constant was 91% higher for the white variety.

These differences between the achieved degrees of inactivation suggest that the colored compounds of the red must (mainly anthocyanins, as it will be addressed in the next section) also absorb UV-vis radiation and consequently the dose that reaches the other compounds (such as the enzyme) is lower. Comparing the inactivation degree with those achieved in other fruits, it can be stated that it is more difficult to inactivate grape polyphenol oxidase by means of UV-vis irradiation than in others such as apple or pear.

Color parameters and absorbance spectra.

CIELab parameters (Table 4.6.2) and absorbance spectra were measured in order to assess the changes produced in color by the irradiation process and the vinification. In Cabernet Franc must, UV irradiation produces a significant increase in *L** (it becomes brighter), while *a** and *b** are less affected. Meanwhile, in the case of Xarel·lo, the must becomes redder after irradiation, but brightness (*L**) and yellowness (*b**) are not significantly affected. Absorbance spectra of both grape juices before and after irradiation (shown in

Figure 4.6.1) confirmed the facts observed in CIELab parameters. As an example, the disappearance of a peak between 450 and 600 nm in Cabernet Franc must after irradiation shows this impairment of pigments. This peak is characteristic of anthocyanin-derived pigments that are usually found in red grapes and wines (Oliveira *et al*., 2006; Lopes-Lutz *et al*., 2010). Therefore, these results mean that anthocyanins are strongly affected by UV-vis irradiation, as it was observed in section 4.5.3.

Table 4.6.2. *CIELab color parameters of fresh musts, irradiated musts and wines from both of them.*

Variety	*Product*	*L**	*a**	*b**
Cabernet Franc	Fresh must	^{a}15.39 ± 0.81	^{a}18.99 ± 1.29	^{a}-11.48 ± 0.76
	Must after irradiation	^{c}20.58 ± 3.44	^{a}18.45 ± 4.07	^{ab}-4.79 ± 4.37
	Wine from non-irradiated must	^{ab}17.12 ± 0.26	^{b}12.49 ± 3.75	^{a}-10.50 ± 1.24
	Wine from irradiated must	^{bc}19.28 ± 1.55	^{b}13.64 ± 0.71	^{b}1.47 ± 8.49
Xarel·lo	Fresh must	^{x}39.55 ± 2.60	^{x}2.42 ± 0.24	^{x}8.24 ± 2.18
	Must after irradiation	^{x}36.19 ± 6.25	^{y}3.97 ± 0.93	^{x}8.83 ± 5.04
	Wine from non-irradiated must	^{y}30.13 ± 3.54	^{y}3.86 ± 0.57	^{xy}4.26 ± 2.89
	Wine from irradiated must	^{y}27.32 ± 0.60	^{z}4.87 ± 0.31	^{y}1.27 ± 0.81

Mean value ± Standard deviation; different superscripts in a column (for each variety) indicate significant differences.

Comparing color parameters of the wines obtained from irradiated and non-irradiated Cabernet Franc musts, the only significant difference is found in their *b** parameter. In both cases fermentation caused a decrease of blueness, but in irradiated must this decrease was of nearly 12 units (6.7 of which decreased during the irradiation process), while in non-irradiated must this variation was not found to be significant. This fact may constitute a problem in the application of UV irradiation in some red wines manufacturing, since keeping the blue color is a highly appreciated property. On the contrary, this effect can be beneficial in rosé wines manufacturing, where the industry searches for a purer red color. Meanwhile, the differences between both Cabernet Franc wines in *L** and *a** parameters were not significant.

In wines from Xarel·lo (white) must, fermentation caused a decrease in *b** that was found to be significant only in the wine from irradiated must. However, there were no statistical differences between *b** values of wines from irradiated

or from non-irradiated must. On the contrary, fermentation caused a significant increase in *a** in both kinds of samples, and redness was higher in wines from Xarel·lo musts treated by UV-vis irradiation.

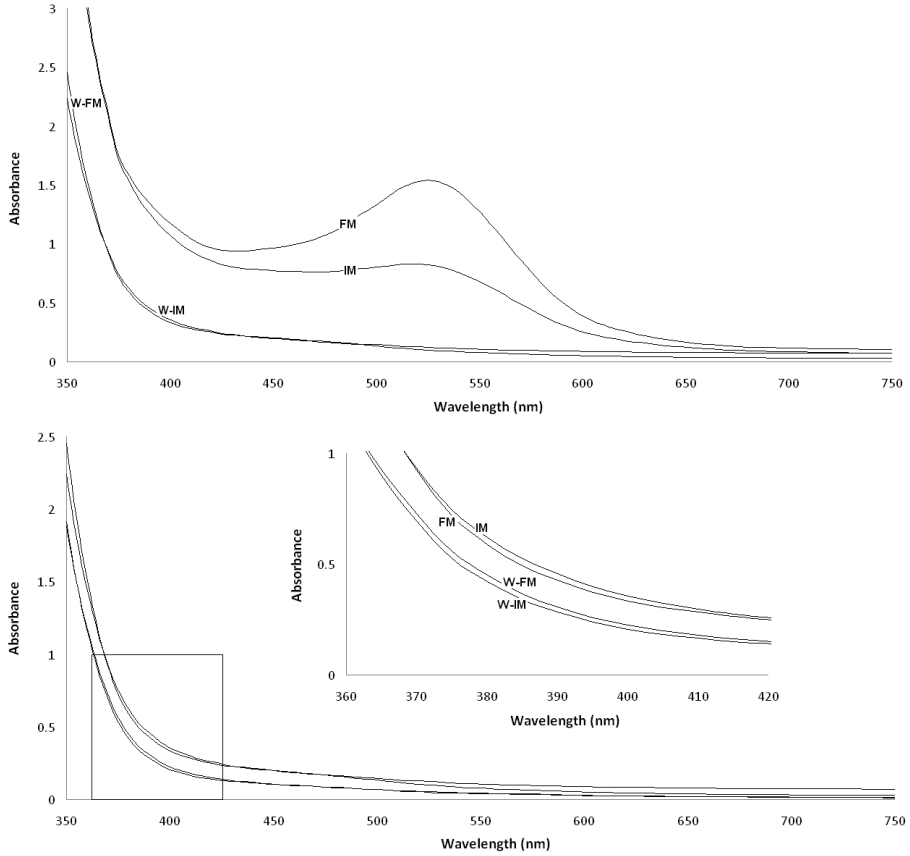

Figure 4.6.1. *Absorbance spectra of irradiated and non-irradiated grape musts and their wines. FM: fresh must. IM: irradiated must. W-FM: wine from fresh must. W-IM: wine from irradiated must. Upper graph: Cabernet Franc. Lower graph: Xarel·lo.*

Observing absorbance spectra (Figure 4.6.1) it can be stated that, comparing both processes undergone by the samples, fermentation has a greater effect than irradiation, being the profiles of wines from irradiated and from non-irradiated musts quite similar. This fact is especially remarkable in the case of Cabernet Franc, since irradiation caused an impairment of anthocyanins that led to a decreased absorbance in the range between 450 and 600 nm, but these differences were not found in the product after fermentation.

Fermentation process and wine chemical quality parameters.

Figure 4.6.2 shows the evolution of density during the fermentation process of the four kinds of vinified musts: irradiated and non-irradiated from Cabernet Franc and from Xarel·lo. The initial values of density were (1082±1) and (1065±1) mg/cm^3 for the red and the white musts, respectively, while the final value was (995±1) mg/cm^3 for all wines after 16 days of fermentation. However, irradiated samples fermented slower than non-irradiated ones, especially after the second day in Cabernet Franc and after the third day in Xarel·lo. The reason for this slower fermentation can be attributed to the elimination of the natural microflora of the musts during the irradiation process. These yeasts can also contribute in a definitive way to fermentation, being able to start and develop a natural vinification (Bartowsky, 2009). Nevertheless, the pasteurization carried out by means of the UV treatment can be beneficial from the point of view of the wine industry, since the added yeast would be the only microorganism responsible for the transformation of must into wine, and therefore standardisation of the process and the final product will be easier. However, confirmation of this aspect would require specific microbiological analysis.

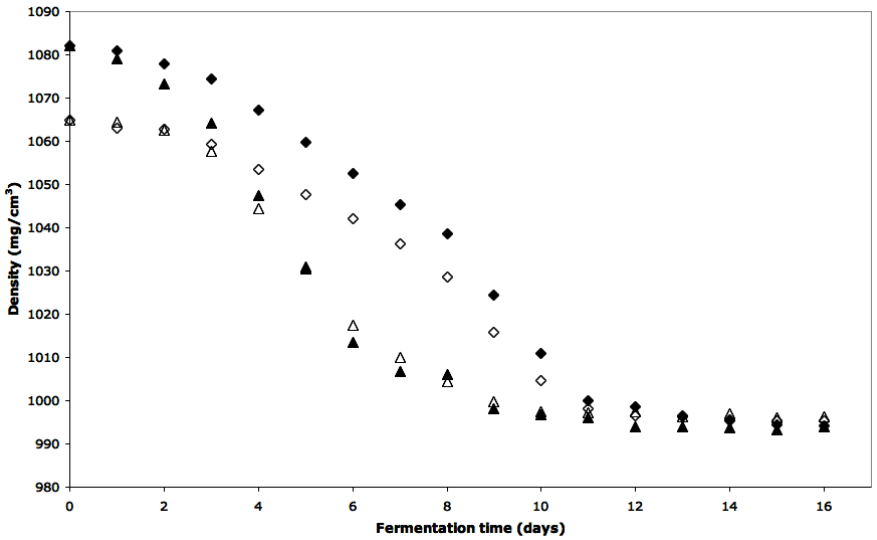

Figure 4.6.2. *Evolution of density during fermentation of irradiated and non-irradiated musts from Cabernet Franc and Xarel·lo. ◆ Cabernet Franc, irradiated. ▲ Cabernet Franc, non-irradiated. ◇ Xarel·lo, irradiated. △ Xarel·lo, non-irradiated.*

Regarding wine quality parameters (shown in Table 4.6.3), no significant differences were found in pH, alcohol degree and tartaric acid content. Volatile acidity, which is an indirect measure of wine spoilage, was much higher in wines from non-irradiated musts in both varieties. In addition, volatile acidity measured in irradiated samples was more homogeneous, which was traduced in lower values of standard deviation. Therefore, these chemical analyses indicate that UV irradiation of must before vinification helps preventing the wine from being damaged by spoiling microorganisms, regardless it is white or red wine. Rossi (1963) already found that the application of UV irradiation at 253 nm prevented wines from acetification without the use of excessive amounts of SO_2 gas, having negligible effects on wine quality even when product exposure was exaggerated.

Table 4.6.3. *Chemical quality parameters of wines from the four Cabernet Franc and Xarel·lo musts.*

	Cabernet Franc		Xarel·lo	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
pH	^{a}4.10 ± 0.38	^{a}3.78 ± 0.24	^{x}4.16 ± 0.39	^{x}3.91 ± 0.43
Tartaric acid (g/L)	^{a}6.37 ± 0.26	^{a}6.83 ± 0.91	^{x}6.94 ± 1.70	^{x}6.74 ± 1.06
Alcohol (% v/v)	^{a}8.37 ± 0.37	^{a}9.87 ± 1.20	^{x}9.59 ± 1.33	^{x}9.87 ± 1.35
Volatile acidity (g/L)	^{a}2.75 ± 0.46	^{b}0.30 ± 0.26	^{x}1.00 ± 0.69	^{y}0.13 ± 0.10

Mean value ± Standard deviation; different superscripts in a row (for each variety) indicate significant differences.

4.6.2. UV-vis irradiation of must: an alternative to reduce SO_2 use in white wines?

Initial must properties.

Table 4.6.4 shows the main properties of the different batches of Xarel·lo and Parellada musts that were processed just after being obtained (in fresh) or after freezing and thawing. The main differences between Xarel·lo and Parellada musts are found in soluble solids content, being higher in the former. In addition, in both varieties freezing and thawing caused an increase in absorbance at 420 nm (which was already higher in Parellada than in Xarel·lo) and a decrease in polyphenol oxidase activity that was only significant in Parellada must. Furthermore, as it can be seen in Table 4.6.6, Xarel·lo must was brighter, while Parellada juice was redder (had a higher *a** value) and more yellow (with a higher *b** value). Nevertheless, freezing and thawing did not cause any significant change in brightness (*L**), but an important increase in colorimetric parameters *a** and *b** in both musts. This darkening of thawed samples, as well as the increase in absorbance at 420 nm, was mainly attributed to the oxidation caused by PPO activity during this process.

Table 4.6.4. *Initial properties of the grape musts from Xarel·lo and Parellada, fresh or frozen and thawed.*

	Xarel·lo (fresh)	Xarel·lo (frozen/thawed)	Parellada (fresh)	Parellada (frozen/thawed)
pH (-)	^{a}3.25 ± 0.14	^{a}3.09 ± 0.19	^{a}3.36 ± 0.11	^{a}3.12 ± 0.20
Sol. solids (ºBrix)	^{a}20.6 ± 0.6	^{a}19.5 ± 1.0	^{b}17.5 ± 0.9	^{b}16.2 ± 1.7
Abs. at 420 nm	^{a}0.090 ± 0.02	^{b}0.200 ± 0.074	^{b}0.269 ± 0.044	^{c}0.430 ± 0.092
PPO (U/mL)	^{a}0.0036 ± 0.0008	^{a}0.0032 ± 0.0001	^{c}0.0084 ± 0.0032	^{b}0.0043 ± 0.0014

Mean value ± Standard deviation; different superscripts in a row indicate significant differences.

Samples listed in Table 4.6.4 (fresh and frozen/thawed) were divided in three groups: the first one vinified without any protective treatment, the second one protected against spoilage and enzymatic activity with SO_2 and the third one processed by UV-vis irradiation. In the samples with added SO_2, polyphenol oxidase was completely inactivated.

Polyphenol oxidase inactivation in the irradiated samples.

PPO was partially inactivated in all the samples that were treated by UV-vis irradiation. The residual activity of the enzyme depended mostly on the grape variety. In this way, Xarel·lo PPO still kept (18±1)% of its original activity after the treatment, while for Parellada samples this residual activity was (30±1)%. These differences can be attributed to the fact that Parellada juice had a higher concentration of colored compounds than Xarel·lo must (leading to a higher absorbance, a lower value of *L** and a higher value of *a** and *b**). These compounds absorb part of the UV-vis radiation, making the available dose to inactivate the enzyme to be lower. No statistical differences were found in a single variety comparing the achieved PPO inactivation degree in fresh or frozen/thawed must.

Regarding PPO inactivation rate, Table 4.6.5 shows the results of fitting experimental data to the first-order kinetic model. Inactivation constants (k) for Xarel·lo were higher than those obtained for Parellada, reinforcing the idea of a faster denaturation of the enzyme. In addition, these values do not show any remarkable difference between fresh or frozen and thawed samples of both varieties. Although in the case of Parellada k is lower for fresh samples, this difference remains inside the 95% confidence interval.

Table 4.6.5. *Kinetic parameters for PPO residual activity data fitted to the first-order model.*

Sample	RA_0 (-)	k (min^{-1})	R^2
Xarel·lo (fresh must)	1.00 ± 0.05	0.0087 ± 0.0009	0.9979
Xarel·lo (frozen/thawed must)	1.00 ± 0.06	0.0088 ± 0.0010	0.9976
Parellada (fresh must)	0.98 ± 0.09	0.0055 ± 0.0010	0.9909
Parellada (frozen/thawed must)	0.97 ± 0.10	0.0060 ± 0.0011	0.9887

Signification level: α=0.05.

Color parameters and absorbance spectra.

First of all, it can be stated that irradiation itself did not cause a brightness decrease neither in Xarel·lo nor in Parellada musts. In Xarel·lo wines, fermentation only caused a decrease of *L** in fresh samples that had been previously irradiated, but not in SO_2-added ones. On the contrary, this decrease of brightness after vinification was found in frozen/thawed samples in untreated, irradiated and SO_2-added samples. In addition, comparing the wines from unfrozen and from frozen must, the latter are significantly less bright regardless

the treatment. In the case of Parellada, fermentation caused a decrease of *L** in fresh irradiated must and in untreated frozen/thawed must. In the samples from this variety, if no hurdle is applied against microorganisms or enzymes wine brightness significantly decreases if the must has been frozen.

Table 4.6.6. *CIELab color parameters of all musts and their resulting wines.*

Variety	*Product*	*L**	*a**	*b**
Xarel·lo (fresh must)	Fresh must	^{a}41.79 ± 2.81	^{ab}0.89 ± 0.25	^{a}4.37 ± 1.98
	Irradiated must	^{a}41.11 ± 4.30	^{abc}1.39 ± 0.76	^{bc}9.26 ± 2.02
	Wine from fresh must	^{a}40.26 ± 0.85	^{a}0.76 ± 0.03	^{a}5.53 ± 0.59
	Wine from must with SO_2	^{a}40.11 ± 3.15	^{abc}1.17 ± 1.67	^{a}4.40 ± 2.56
	Wine from irradiated must	^{b}34.60 ± 2.85	^{de}2.29 ± 0.52	^{ab}6.28 ± 3.07
Xarel·lo (frozen/thawed must)	Frozen/thawed must	^{a}39.25 ± 2.35	^{e}4.95 ± 1.69	^{c}10.85 ± 3.57
	Irradiated must	^{ab}38.16 ± 3.24	^{de}3.06 ± 0.60	^{c}11.10 ± 3.42
	Wine from frozen/thawed must	^{c}32.08 ± 2.21	^{cd}2.12 ± 0.49	^{a}4.42 ± 1.70
	Wine from must with SO_2	^{c}33.90 ± 2.00	^{cd}1.9 ± 0.22	^{a}5.21 ± 1.98
	Wine from irradiated must	^{c}32.84 ± 2.10	^{e}3.44 ± 0.49	^{ab}6.47 ± 2.35
Parellada (fresh must)	Fresh must	^{r}37.78 ± 3.74	^{rst}4.33 ± 1.39	^{s}14.78 ± 2.26
	Irradiated must	^{r}37.34 ± 2.10	^{tu}6.45 ± 1.80	^{s}14.79 ± 1.16
	Wine from fresh must	^{rst}30.63 ± 1.59	st4.74 ± 0.55	^{t}4.63 ± 2.77
	Wine from must with SO_2	^{rs}35.76 ± 3.21	^{rs}4.04 ± 0.60	^{s}13.33 ± 4.07
	Wine from irradiated must	^{stu}29.33 ± 1.52	^{uv}8.55 ± 1.56	^{t}5.15 ± 2.49
Parellada (frozen/thawed must)	Frozen/thawed must	^{rs}35.08 ± 1.98	^{v}10.01 ± 2.28	^{r}17.08 ± 2.27
	Irradiated must	^{stu}29.38 ± 3.30	^{v}10.50 ± 1.91	^{s}10.81 ± 3.47
	Wine from frozen/thawed must	^{u}22.89 ± 15.04	^{v}9.09 ± 1.78	^{t}4.21 ± 2.47
	Wine from must with SO_2	^{rst}31.14 ± 1.44	^{r}2.48 ± 1.06	^{t}3.19 ± 1.94
	Wine from irradiated must	^{tu}26.42 ± 1.04	^{t}6.24 ± 0.69	^{u}-0.15 ± 1.88

Mean value ± Standard deviation; different superscripts in a column (for each variety) indicate significant differences.

When it comes to redness (*a**), freezing and thawing also led to a higher value of this parameter in untreated and irradiated musts. In addition, wines from irradiated musts were redder than those from SO_2-added ones, although irradiation itself did not cause any variation in *a**. Regarding yellowness (*b**), for untreated musts its values were higher in frozen samples, although this increase was not found in irradiated musts. In Xarel·lo wines, no differences were found between the different treatments or between fresh and frozen samples. However, fermentation of frozen/thawed musts caused an important decrease of yellowness that was not found in the vinification of the fresh ones. If the *b** values of the wines from irradiated and SO_2-added musts are compared,

the former are less yellow than the latter in Parellada, but no significant differences were found in the case of Xarel·lo.

No remarkable differences were found between the absorbance spectra of the wines from the musts with SO_2 and the wines from irradiated musts, neither in the case of Xarel·lo nor in Parellada, both in fresh and in frozen and thawed samples.

Fermentation process and wine quality parameters.

Figure 4.6.3 shows the evolution of density during the fermentation of frozen and thawed Xarel·lo musts with the different kinds of treatment. Parellada frozen must and fresh musts from both varieties (data not shown) behaved in the same way, leading to analogous graphs. Irradiated samples fermented slower than non-irradiated and SO_2-added ones, probably due to the elimination of the natural microflora of the musts during this process, as in the previous preliminary study. No remarkable differences were found in density evolution between musts with SO_2 and untreated ones.

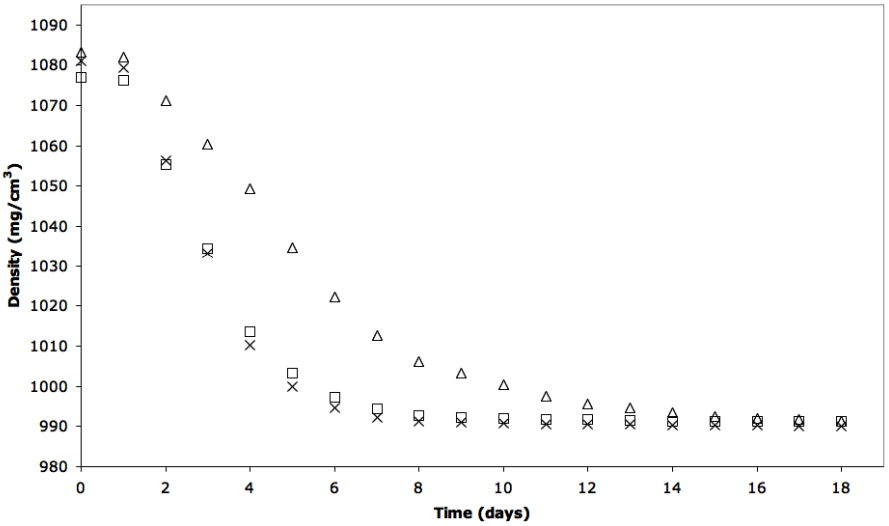

Figure 4.6.3. *Evolution of density during fermentation. Samples from frozen and thawed Xarel·lo musts:* × *untreated.* □ *with added* SO_2. △ *irradiated.*

Quality parameters of the wines obtained from the musts with the different treatments are shown in Table 4.6.7. In the case of Xarel·lo, musts freezing and thawing led to wines with lower pH, higher tartaric acid content and higher volatile acidity, while no significant differences were found in alcohol content. However, none of these general trends were found in Parellada wines. As far as the different treatments are concerned, in most of the studied cases wines from irradiated musts had lower volatile acidity (an indirect measure of wine spoilage) than those from untreated musts, while no significant differences were found for this parameter between using UV-vis irradiation and SO_2.

Table 4.6.7. *Wine quality parametes of samples from Xarel·lo and Parellada.*

Variety	*Must sample*	*pH (-)*	*Tartaric acid (g/L)*	*Alcohol (%v/v)*	*Volatile acidity (g/L)*
Xarel·lo (fresh must)	Fresh must	$^{b}3,70 \pm 0,20$	$^{a}4,82 \pm 0,22$	$^{ab}12,00 \pm 0,15$	$^{a}0,18 \pm 0,05$
	Must with SO_2	$^{bc}3,75 \pm 0,19$	$^{a}5,02 \pm 0,20$	$^{b}12,44 \pm 0,44$	$^{a}0,19 \pm 0,07$
	Irradiated must	$^{c}3,98 \pm 0,14$	$^{a}4,48 \pm 0,16$	$^{ab}12,19 \pm 0,33$	$^{a}0,07 \pm 0,05$
Xarel·lo (frozen/thawed must)	Frozen/thawed must	$^{a}2,96 \pm 0,10$	$^{c}7,23 \pm 1,21$	$^{ab}11,97 \pm 0,52$	$^{c}3,52 \pm 1,11$
	Must with SO_2	$^{a}3,00 \pm 0,06$	$^{b}5,46 \pm 0,36$	$^{ab}12,10 \pm 0,38$	$^{b}1,44 \pm 0,31$
	Irradiated must	$^{a}2,92 \pm 0,02$	$^{c}7,47 \pm 0,40$	$^{a}11,63 \pm 0,47$	$^{b}1,73 \pm 0,84$
Parellada (fresh must)	Fresh must	$^{r}3,07 \pm 0,15$	$^{r}8,19 \pm 1,78$	$^{s}9,65 \pm 0,38$	$^{r}3,71 \pm 1,20$
	Must with SO_2	$^{rs}3,23 \pm 0,13$	$^{s}5,61 \pm 1,02$	$^{t}10,67 \pm 0,58$	$^{s}2,41 \pm 1,01$
	Irradiated must	$^{rs}3,23 \pm 0,04$	$^{st}5,29 \pm 0,26$	$^{st}10,52 \pm 0,53$	$^{s}2,20 \pm 0,26$
Parellada (frozen/thawed must)	Frozen/thawed must	$^{t}3,46 \pm 0,08$	$^{s}5,58 \pm 0,94$	$^{st}9,76 \pm 0,57$	$^{s}2,20 \pm 1,38$
	Must with SO_2	$^{s}3,25 \pm 0,12$	$^{st}5,03 \pm 0,70$	$^{st}9,82 \pm 0,83$	$^{st}1,18 \pm 0,93$
	Irradiated must	$^{t}3,56 \pm 0,09$	$^{t}3,97 \pm 0,24$	$^{r}8,24 \pm 0,71$	$^{t}0,62 \pm 0,14$

Mean value ± Standard deviation; different superscripts in a column (for each variety) indicate significant differences.

4.7. Polyphenol oxidase inactivation by high hydrostatic pressure: Study of the effects of the variety on apple juice processing.

The effectiveness of high pressure processing on the inactivation of apple polyphenol oxidase has been widely investigated. However, from an industrial point of view, there is a need for assessing this effectiveness on each one of the different apple varieties that each company uses for their products. This last part of the study fills in this gap, assessing the effect of the variety on apple juice PPO inactivation and on the side effects caused on color, which is one of the most important organoleptic features. Both effects have been addressed in two different ways: a kinetic study, finding the best model for each case, and a multivariate study consisting of a principal component analysis of data.

Physicochemical properties of the raw juices and initial PPO activity.

The juices from the six apple varieties had several differences in their composition and properties. Table 4.7.1 shows the values obtained for the measured physicochemical parameters. Fuji juice had the highest soluble solids content (12.5 ºBrix) and the lowest titratable acidity (0.215 $g \cdot L^{-1}$) (and therefore the highest maturity index, 58.3), the lowest viscosity (4.61 mPa·s), the highest pH (4.39) and also the highest content of reducing and total sugars (92.3 and 117.3 $g \cdot L^{-1}$) and vitamin C (44.7 mg/100mL). In addition, Fuji juice had the highest initial PPO activity (0.088 $U \cdot mL^{-1}$), this value being much higher than the second one (Braeburn, with 0.032 $U \cdot mL^{-1}$).

At the other side of the scale, Granny Smith juice had the lowest maturity index (13.1), especially due to its high titratable acidity (0.769 $g \cdot L^{-1}$). However, the lowest initial PPO activity was found to be in Golden Delicious juice, with 0.007 $U \cdot mL^{-1}$. Regarding total phenolics, the potential PPO substrates, the highest content was found in Red Delicious juice (347.6 $mg \cdot L^{-1}$), which had an intermediate PPO activity (0.016 $U \cdot mL^{-1}$). According to Cheng & Crisosto (1995), a general correlation between total phenolics and PPO activity cannot be established, since the browning potential depends on each phenolic compound.

Table 4.7.1. *Physicochemical properties of the apple juices from the six studied varieties.*

	pH at 20ºC (-)	Conductivity ($\mu S \cdot cm^{-1}$)	Soluble solids (ºBrix)	Density ($g \cdot mL^{-1}$)	Viscosity (mPa·s)
Braeburn	3.64 ± 0.01	1440 ± 7	10.9 ± 0.1	1.051 ± 0.002	4.66 ± 0.30
Fuji	4.39 ± 0.02	1529 ± 5	12.5 ± 0.1	1.055 ± 0.001	4.61 ± 0.29
Gala	3.89 ± 0.02	1507 ± 2	10.2 ± 0.1	1.051 ± 0.002	4.70 ± 0.29
Golden Delicious	3.69 ± 0.02	1799 ± 3	9.9 ± 0.3	1.054 ± 0.002	4.73 ± 0.29
Granny Smith	3.26 ± 0.02	1413 ± 3	10.1 ± 0.3	1.047 ± 0.001	4.73 ± 0.32
Red Delicious	3.99 ± 0.02	1579 ± 6	12.4 ± 0.1	1.059 ± 0.003	4.78 ± 0.29

	Formol index (mL NaOH/100 mL)	Reducing sugars ($g \cdot L^{-1}$ glucose eq.)	Total sugars ($g \cdot L^{-1}$ glucose eq.)	Titratable acidity ($g \cdot L^{-1}$ malic acid eq.)
Braeburn	5.3 ± 0.6	65.3 ± 1.7	23.0 ± 0.1	0.474 ± 0.015
Fuji	3.7 ± 0.6	92.3 ± 1.7	58.3 ± 0.1	0.215 ± 0.013
Gala	6.7 ± 0.6	78.7 ± 2.5	23.3 ± 0.1	0.438 ± 0.020
Golden Delicious	2.3 ± 0.6	85.4 ± 1.5	17.4 ± 0.3	0.568 ± 0.015
Granny Smith	7.0 ± 1.0	71.3 ± 1.0	13.1 ± 0.3	0.769 ± 0.008
Red Delicious	3.3 ± 0.6	92.3 ± 1.7	32.6 ± 0.1	0.380 ± 0.008

	Total phenolics ($mg \cdot L^{-1}$ gallic acid eq.)	Vitamin C (mg ascorbic acid/100 mL)	Maturity index (ºBrix·L·g^{-1})	PPO activity ($U \cdot mL^{-1}$)
Braeburn	292.3 ± 5.9	35.3 ± 2.0	23.0 ± 0.1	0.032 ± 0.002
Fuji	237.6 ± 3.0	44.7 ± 2.0	58.3 ± 0.1	0.088 ± 0.003
Gala	285.1 ± 5.1	40.0 ± 3.5	23.3 ± 0.1	0.012 ± 0.002
Golden Delicious	317.2 ± 12.7	32.9 ± 3.5	17.4 ± 0.3	0.007 ± 0.001
Granny Smith	298.1 ± 11.3	36.5 ± 3.5	13.1 ± 0.3	0.022 ± 0.002
Red Delicious	347.6 ± 34.7	29.4 ± 3.5	32.6 ± 0.1	0.016 ± 0.001

Mean value ± Standard deviation

Study of polyphenol oxidase inactivation.

PPO activity variation caused by instant exposure to target conditions.

Instant exposure of freshly squeezed apple juice to mild temperatures (25-60ºC) and pressures (followed by immediate pressure release and cooling) caused an increase in PPO activity (Table 4.7.2). This phenomenon has also been reported by several authors (Anese *et al*., 1995; Bayındırlı *et al*., 2006; Soysal, 2008; Buckow *et al*., 2009), and it has been often attributed to the release of latent enzymes (Yemenicioglu *et al*., 1997). In this case, the maximum PPO increase was found during exposure to 400 MPa and 25ºC in all varieties. These conditions caused an increase of 61.5% in Red Delicious PPO, while the lowest increase (36.3%) was found in Braeburn PPO. On the contrary, exposure to 80ºC caused a reduction of PPO activity in almost all varieties and pressures, except for Red Delicious at 400 and 500 MPa, and Golden Delicious at 400 MPa. In this way, the maximum reduction was found in Granny Smith juice at 80ºC and 600 MPa (74.9%). These results suggest that the exposure to pressure/temperature conditions affects apple PPO to a different degree depending on apple variety.

Table 4.7.2. *Change of PPO activity (%) relative to the freshly squeezed apple juice after come-up time (heating and pressure increase to target conditions followed by immediate pressure release and cooling).*

		25ºC	60ºC	80ºC
	P (MPa)	*Change of PPO activity (%)*		
Braeburn	400	36.3	22.9	-9.2
	500	30.4	20.7	-24.6
	600	11.1	14.2	-34.4
Fuji	400	49.9	41.5	-33.5
	500	40.5	0.7	-39.1
	600	28.5	-18.7	-57.2
Gala	400	44.2	22.0	-20.7
	500	33.8	9.4	-25.3
	600	29.4	-3.7	-34.1
Golden Delicious	400	52.9	43.3	11.1
	500	32.4	17.0	-17.2
	600	18.5	18.2	-30.6
Granny Smith	400	41.6	39.7	-51.3
	500	38.4	37.3	-74.0
	600	31.3	32.0	-74.9
Red Delicious	400	61.5	57.6	6.1
	500	51.1	44.5	0.6
	600	33.9	32.0	-4.1

Kinetic study of PPO inactivation.

Experimental values of PPO relative activity as a function of processing time for some combinations apple variety-nominal temperature-pressure are shown in Figure 4.7.1. It can be easily seen that the apple variety did not only affect the initial PPO activity (shown in Table 4.7.1), but also the inactivation pattern and the achieved inactivation degree. In this way, Granny Smith PPO was completely inactivated after 8 min at 600 MPa and 80ºC, while Fuji PPO activity was only reduced to 27.7% after 16 min under the same conditions (however, a 57% loss was already achieved in the compression phase). Observing all experimental results at room temperature (25ºC), the maximum inactivation after 16 min was achieved in Granny Smith at 600 MPa (residual activity 71.5%), while Braeburn and Golden Delicious PPO were the most resistant under these conditions (residual activity 93%).

All the experimental relative values from the 54 processing series (6 varieties x 3 pressures x 3 temperatures) were fitted to zero-order, first order and second order kinetic models by means of non-linear regression procedures, in order to obtain RA_0 and k_i parameters for each one. Then, predicted relative activity

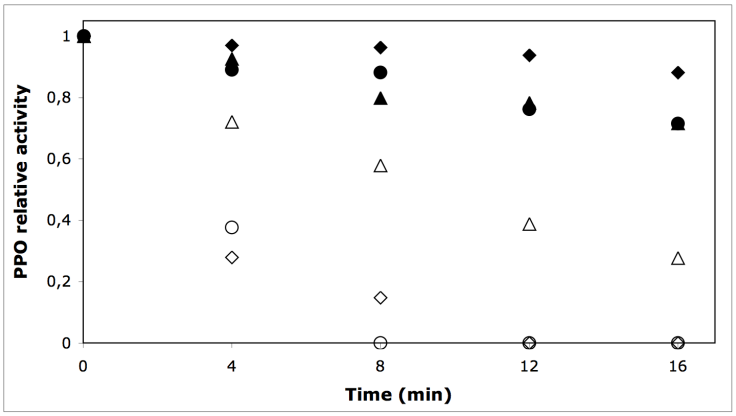

Figure 4.7.1. *Polyphenol oxidase relative activity as a function of high-pressure processing time for some apple varieties and temperature-pressure conditions: Braeburn (◆ 400 MPa 25ºC; ◇ 600MPa 80ºC), Fuji (▲ 400 MPa 25ºC; △ 600MPa 80ºC) and Granny Smith (● 400 MPa 25ºC; ○ 600MPa 80ºC).*

values were calculated from these parameters. Table 4.7.3 shows RMSE values obtained when comparing the 45 (3 pressures x 3 temperatures x 5 processing times) experimental vs. predicted values for each variety and kinetic model. Moreover, an additional RMSE value has been calculated for each kinetic model considering the samples from all apple varieties (270), in order to compare the general goodness of each expression.

Table 4.7.3. *RMSE values for the three kinetic models applied on polyphenol oxidase relative activity data from the six different apple varieties.*

Variety	Zero-order	First-order	Second-order
Braeburn	0.067	0.051	0.092
Fuji	0.050	0.106	0.041
Gala	0.077	0.077	0.140
Golden Delicious	0.059	0.078	0.171
Granny Smith	0.105	0.068	0.184
Red Delicious	0.087	0.078	0.161
All samples	0.076	0.078	0.138

Zero-order kinetic model was the most appropriated one for describing PPO inactivation in Golden Delicious juice, since the lowest RMSE was obtained with this expression (0.059). Likewise, the first order model was the best one for Braeburn (0.051), Granny Smith (0.068) and Red Delicious (0.078), while the second order one was the best for Fuji (0.041). As far as Gala juice is concerned, both zero-order and first order models gave the same RMSE value

Table 4.7.4. *Kinetic parameters obtained fitting the experimental polyphenol oxidase relative activity values to the most appropriated kinetic model for each apple variety.*

Variety	Kinetic model	T (ºC)	P (MPa)	RA_0 (-)	k_i (min^{-1})	R^2
Braeburn	First order	25	400	1.005 ± 0.039	0.004 ± 0.002	0.9087
		25	500	0.993 ± 0.018	0.007 ± 0.004	0.9396
		25	600	0.989 ± 0.050	0.011 ± 0.006	0.9327
		60	400	0.981 ± 0.046	0.008 ± 0.005	0.8890
		60	500	0.983 ± 0.053	0.015 ± 0.006	0.9564
		60	600	1.022 ± 0.091	0.035 ± 0.011	0.9727
		80	400	1.082 ± 0.349	0.056 ± 0.047	0.8575
		80	500	1.046 ± 0.405	0.109 ± 0.084	0.9056
		80	600	1.002 ± 0.125	0.298 ± 0.089	0.9934
Fuji	Second order	25	400	1.003 ± 0.086	0.025 ± 0.009	0.9642
		25	500	0.984 ± 0.048	0.012 ± 0.005	0.9491
		25	600	1.003 ± 0.094	0.047 ± 0.010	0.9879
		60	400	1.038 ± 0.223	0.017 ± 0.017	0.6785
		60	500	0.854 ± 0.418	0.082 ± 0.058	0.8707
		60	600	0.906 ± 0.202	0.029 ± 0.025	0.8211
		80	400	1.006 ± 0.157	0.034 ± 0.016	0.9398
		80	500	1.004 ± 0.217	0.088 ± 0.022	0.9819
		80	600	1.287 ± 1.177	0.160 ± 0.072	0.9436
Gala	Zero-order	25	400	1.018 ± 0.056	0.015 ± 0.006	0.9583
		25	500	1.001 ± 0.035	0.014 ± 0.004	0.9814
		25	600	0.944 ± 0.129	0.016 ± 0.013	0.8231
		60	400	0.983 ± 0.098	0.010 ± 0.010	0.7776
		60	500	0.937 ± 0.146	0.021 ± 0.015	0.8656
		60	600	1.025 ± 0.096	0.029 ± 0.010	0.9682
		80	400	1.111 ± 0.424	0.051 ± 0.043	0.8256
		80	500	1.030 ± 0.190	0.041 ± 0.019	0.9384
		80	600	0.898 ± 0.508	0.053 ± 0.052	0.7817
Golden Delicious	Zero-order	25	400	0.988 ± 0.049	0.014 ± 0.005	0.9648
		25	500	0.989 ± 0.030	0.004 ± 0.003	0.8441
		25	600	1.021 ± 0.059	0.014 ± 0.006	0.9455
		60	400	0.997 ± 0.038	0.011 ± 0.004	0.9670
		60	500	0.986 ± 0.078	0.023 ± 0.008	0.9668
		60	600	1.059 ± 0.206	0.040 ± 0.021	0.9255
		80	400	0.961 ± 0.148	0.039 ± 0.015	0.9582
		80	500	0.995 ± 0.306	0.069 ± 0.031	0.9432
		80	600	0.876 ± 0.451	0.066 ± 0.046	0.8723
Granny Smith	First order	25	400	0.995 ± 0.072	0.010 ± 0.008	0.9544
		25	500	1.009 ± 0.036	0.019 ± 0.004	0.9597
		25	600	0.973 ± 0.107	0.021 ± 0.013	0.8881
		60	400	0.981 ± 0.174	0.054 ± 0.026	0.9455
		60	500	1.014 ± 0.099	0.112 ± 0.022	0.9929
		60	600	1.034 ± 0.205	0.148 ± 0.057	0.9785
		80	400	1.001 ± 0.117	0.180 ± 0.042	0.9930
		80	500	1.048 ± 0.510	0.221 ± 0.221	0.9096
		80	600	1.016 ± 0.221	0.297 ± 0.154	0.9812
Red Delicious	First order	25	400	0.982 ± 0.051	0.010 ± 0.006	0.9166
		25	500	0.978 ± 0.053	0.010 ± 0.006	0.9123
		25	600	0.996 ± 0.027	0.017 ± 0.003	0.9810
		60	400	0.982 ± 0.046	0.013 ± 0.005	0.9565
		60	500	0.992 ± 0.136	0.032 ± 0.017	0.9283
		60	600	1.033 ± 0.173	0.055 ± 0.024	0.9540
		80	400	1.086 ± 0.373	0.072 ± 0.056	0.8820
		80	500	1.091 ± 0.737	0.181 ± 0.181	0.8315
		80	600	0.994 ± 0.264	0.278 ± 0.171	0.9696

Signification level: $\alpha = 0.05$.

(0.077). Thus, these results show that the kinetic pattern in PPO inactivation of apple juice definitively depends on the variety. If all the samples are considered, the global RMSE value revealed that the zero-order model (0.076) was the one that best described the inactivation of PPO in apple juice from these six varieties under the mentioned conditions.

Table 4.7.4 shows the kinetic parameters (RA_0 and k_i) obtained with the best model for each variety. In the case of Gala juice, the mathematically simplest model (zero-order) is considered the most suitable one, and the shown parameters have been calculated by means of this expression. As a general rule, kinetic constants (k_i) become higher as both pressure and temperature increase. In this way, for example, k_1 for Braeburn increases from 0.0041 min^{-1} at 400 MPa and 25ºC to 0.0112 min^{-1} at 600 MPa and 25ºC, and its value becomes 0.2980 min^{-1} at 600 MPa and 80ºC.

Multivariate study of PPO inactivation.

In order to identify the general trends in the relationship between variables and any possible group or patterns in the data, a Principal Component Analysis (PCA) was carried out. First of all, Figure 4.7.2 (upper) corresponds to the scores of the samples in the two first principal components space, which represents 56% of the overall variance of the original matrix (25 variables). This plot clearly shows six different groups of samples, which are distributed mainly along the maximum variance direction (PC1) and can be identified with the six different apple varieties. Therefore, the variety is the variable that contributes the most to the overall variance (i.e. the most important variable in the study). This clear separation of data groups suggests the need for building a separated PCA model for each one of these sample groups.

Observing variable loadings in the PC1-PC2 space for the same global PCA model (Figure 4.7.2, lower), some correlations between variables may be established. In this way, juices density is highly correlated with total and reducing sugars, and acidity is negatively correlated with pH (the higher the acidity, the lower the pH). Likewise, this plot also shows that Fuji is the variety with the highest PPO activity. As discussed before, PPO activity does not show

any direct relationship with phenolics content. Maturity index and vitamin C content are the two physicochemical variables that show the highest (although weak) correlation with PPO activity.

Figure 4.7.2. *Principal Component Analysis of the overall data. Sample scores (upper graph) and variable loadings (lower graph) in the two first principal components space (explained variance: 56%). The scores are clearly distributed in six groups according to apple varieties: BR: Braeburn. FU: Fuji. GA: Gala. GD: Golden Delicious. GS: Granny Smith. RD: Red Delicious.*

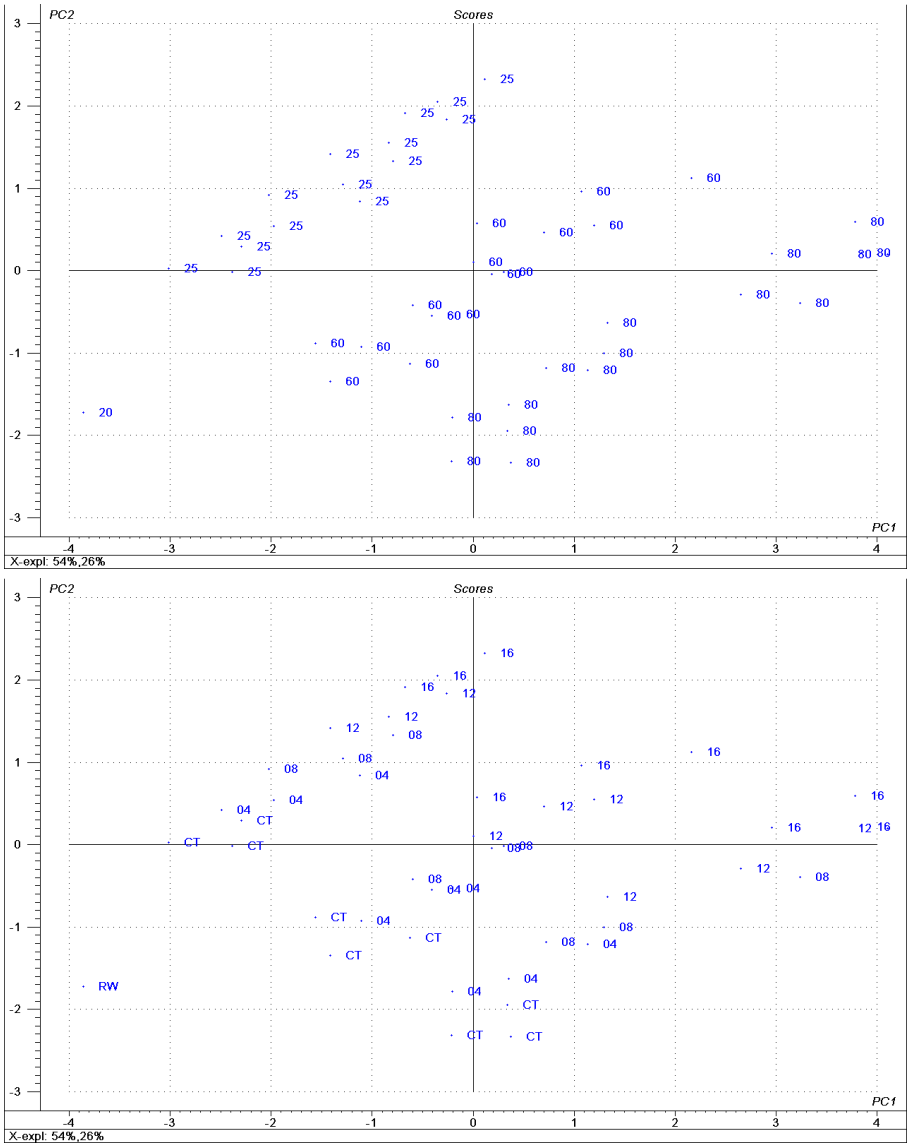

Figure 4.7.3. *Principal Component Analysis of the data corresponding to the Gala apple variety. Sample scores in the two first principal components space (explained variance: 80%). Upper graph: codes correspond to nominal processing temperature. Lower graph: codes correspond to processing time.*

When a particular PCA model is carried out for each one of the six apple varieties, the explained variance of the obtained models increases (80% on average in the two first PCs), mainly due to the loss in variability caused by the matrix dimension reduction. Figure 4.7.3 (upper) shows sample scores from the PCA of Gala variety data, codified according to processing temperature. It can

be easily seen that the samples are grouped following a clear trend along the two first PCs (especially the first one). It is also worth mentioning that, as processing temperature increases, dispersion of the samples becomes greater. This fact reinforces the conclusion that at higher temperatures, the other variables (time, pressure, variety) have more effect on PPO inactivation rate (as it was stated analyzing kinetic constants in the previous section). As far as processing time is concerned (Figure 4.7.3, lower), although a tendency appears especially in the vertical direction (PC2), no clearly defined groups can be identified, not being this effect as marked as in the case of temperature. In both diagrams, the unprocessed sample (20ºC raw; *20* in the upper graph, *RW* in the lower one) appears isolated. Codification for processing pressure is not shown, since no pattern defining any phenomenon has been seen (the information regarding this variable appears randomly distributed in the samples, not only concerning the PC1 - PC2 space, but also when more PCs are taken into account in calculating the model). The same trends were found in the PCA modeling of all the other varieties.

Regarding variable loadings for one single variety, their plot in the two first principal components space showed that the three variables related with PPO (the absolute activity, relative activity and activity increase produced in the come-up time –data from Table 4.7.2-) were the ones that contributed the most to the overall variance. Among the parameters that define the experiment, the temperature is the most important one in the maximum variance directions, followed by the processing time and the pressure. This fact indicates that, in the variable range that has been studied, increasing the temperature has more effect on PPO inactivation than increasing processing time, and this than increasing the pressure. Analyzing kinetic constants from Table 4.7.4, it was also concluded that increasing the temperature has a greater effect on the inactivation rate than increasing the pressure. However, it must be remembered that the studied temperature range was 25-80ºC, while the pressure range was 400-600 MPa.

Study of color changes.

Initial CIELab parameters.

Table 4.7.5 shows the CIELab parameters of the freshly squeezed apple juices from the six studied varieties. Granny Smith juice was the one with the highest initial brightness (*L**, 62.69) and the lowest *a** and *b** values (2.88 and 20.85, respectively). Meanwhile, Fuji juice had the lowest brightness (30.25) and the highest initial redness (*a**, 18.18), and the highest yellowness (b*) was found in Red Delicious juice (40.91). All the juices had a higher yellowness (*b**) than redness (*a**); in Granny Smith juice, *b** was more than 7 times greater than *a**, while in Fuji the *b**/*a** ratio was only 1.2. These initial parameters show the big color differences that exist between the freshly squeezed juices form the different varieties.

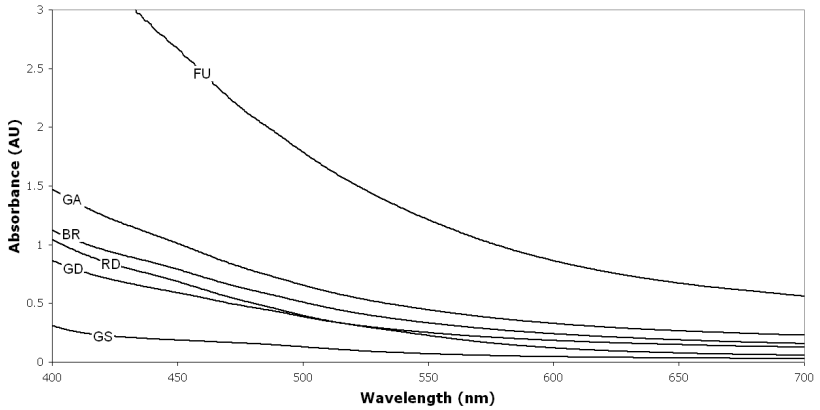

Figure 4.7.4. *Absorbance spectra of the unprocessed (raw) apple juices. BR: Braeburn. FU: Fuji. GA: Gala. GD: Golden Delicious. GS: Granny Smith. RD: Red Delicious.*

Another type of measurement that may be useful to describe these differences is the absorption spectrum in the visible region (between 400 and 700 nm, Figure 4.7.4). It can be clearly observed that Fuji and Granny Smith juices have the highest and the lowest absorbance in the entire visible region, respectively. The other four varieties (Braeburn, Gala, Golden Delicious and Red Delicious) offered closer spectra.

Table 4.7.5. *CIELab parameters of the six unprocessed apple juices.*

Variety	*L** (-)	*a** (-)	*b** (-)
Braeburn	48.37 ± 0.46	23.0 ± 0.1	37.77 ± 0.04
Fuji	30.25 ± 0.23	58.3 ± 0.1	22.36 ± 0.25
Gala	47.11 ± 0.31	23.3 ± 0.1	37.07 ± 0.38
Golden Delicious	51.97 ± 0.19	17.4 ± 0.3	35.45 ± 0.10
Granny Smith	62.69 ± 0.06	13.1 ± 0.3	20.85 ± 0.04
Red Delicious	43.92 ± 0.54	32.6 ± 0.1	40.91 ± 0.31

Mean value ± Standard deviation

Color difference (ΔE) caused by instant exposure to target conditions.

Color of fruit juices is extremely sensitive to processing. In this way, an instant exposure to high pressures and/or mild temperatures is enough to change these parameters. Table 4.7.6 shows color difference (*ΔE,* using unprocessed samples of each variety as reference) caused by this instant exposure to processing conditions followed by immediate pressure release and cooling. First of all, it must be stated that in all cases, exposure for 1 second to processing conditions caused a decrease in *L** value and an increase in *a** and *b**, i.e. the juice became darker and more colored.

Table 4.7.6. *Difference of color (ΔE) relative to the freshly squeezed apple juice after come-up time (heating and pressure increase to target conditions followed by immediate pressure release and cooling).*

		25ºC	60ºC	80ºC
	P (MPa)	Color difference (*ΔE*)		
Braeburn	400	0.92	1.79	1.85
	500	2.04	1.81	1.89
	600	4.34	1.93	3.96
Fuji	400	3.46	3.60	13.91
	500	5.40	9.03	14.89
	600	7.70	15.27	19.33
Gala	400	2.22	6.01	6.57
	500	3.66	6.80	7.28
	600	5.14	7.67	7.69
Golden Delicious	400	1.59	2.36	1.76
	500	2.47	4.47	4.50
	600	3.74	5.03	7.30
Granny Smith	400	1.16	1.58	3.00
	500	1.25	3.07	4.71
	600	3.11	6.27	6.91
Red Delicious	400	3.84	3.31	3.64
	500	3.85	3.46	6.28
	600	4.05	5.88	7.09

The largest values for ΔE were found in Fuji juice, while Braeburn juice was the most stable one. Generally speaking, the produced color change was greater as both temperature and pressure increased. Thus, color of Fuji juice exposed to 400 MPa and 25ºC for 1 second changed 3.46 units, while at 600 MPa and 80ºC a change of 19.33 units was found. Color differences for Braeburn juice under the same conditions were 0.92 and 3.96, respectively.

Kinetic study of color difference (ΔE).

As it has been described, apple juice samples were treated for different times: come-up time, 4, 8, 12 and 16 min. Experimental results showed that, after the initial color variations caused by the come-up time, CIELab parameters behaved in the opposite way: as processing time increased, the juices became brighter (L^* increased) and less colored (a^* and b^* decreased), even at 60 and 80ºC. These results are consistent with those found by other researchers when processing fruit juices with high hydrostatic pressure (Sánchez-Moreno *et al*., 2005; Hartyáni *et al*., 2011), attributing color changes to natural pigments impairment. On the contrary, Landl *et al*. (2010) found that a^* and b^* remained constant (although L^* increased) after high pressure processing of apple purée at 400 and 600 MPa.

In this piece of work, the changes in color have also been assessed by means of the absorbance spectrum in the visible region (400-700 nm). Figure 4.7.5 corresponds to the evolution of Gala juice spectrum after processing at 600 MPa and 80ºC. It can be seen that as processing time increases, absorbance decreases in the whole region. Moreover, in the case of Gala juice, there is a greater shift between the samples corresponding to the come-up time and 4 min than between the other consecutive processing times, which was observed in almost all pressure-temperature combinations. However, this greater shift was found between different processing times depending on the variety (for example, in the case of Fuji juice the greatest shift was found between the samples processed for 4 and 8 min, but was also repeated for almost all pressure-temperature combinations). This greater shift was observed easier as processing temperature was higher.

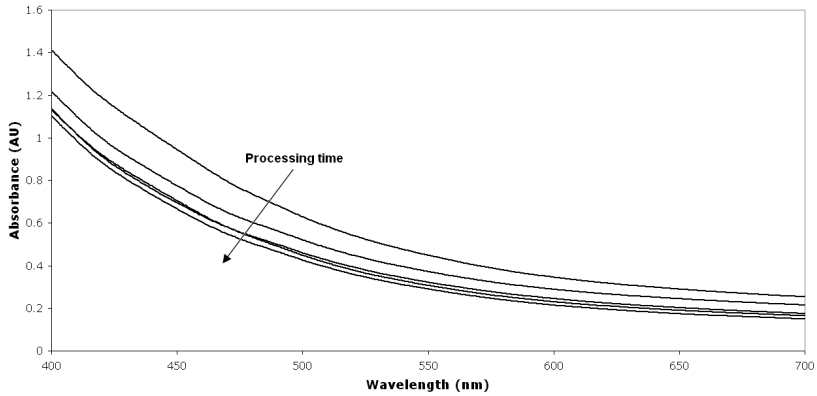

Figure 4.7.5. *Absorbance spectrum evolution of Gala juice processed at 600 MPa and 80ºC with processing time (come-up time, 4, 8, 12 and 16 minutes).*

In order to find the kinetic pattern of color changes for each variety-pressure-temperature combination, color difference (ΔE) was calculated for each one of the 54 series (as described in *Materials and methods* section) and fitted to zero-order, first order and second order models by means of non-linear regression procedures. The used kinetic analysis was the same as in the study of PPO inactivation. These procedures allowed obtaining $(\Delta E)_0$ and k_i parameters for each model, from which predicted color difference values were calculated. Table 4.7.7 shows RMSE values obtained when comparing the 45 (3 pressures x 3 temperatures x 5 processing times) experimental vs. predicted values for each variety and kinetic model. Moreover, an additional RMSE value was calculated for each kinetic model considering the samples from all apple varieties (270).

Table 4.7.7. *RMSE values for the three kinetic models applied on color difference (ΔE) data from the six different apple varieties.*

Variety	Zero-order	First order	Second order
Braeburn	0.771	1.087	1.976
Fuji	0.543	1.001	1.174
Gala	1.711	1.148	1.581
Golden Delicious	0.948	1.288	0.743
Granny Smith	0.577	0.805	0.808
Red Delicious	1.033	1.344	0.914
All samples	1.057	1.143	1.328

Zero-order kinetic model was the most appropriated one for describing color difference evolution in Braeburn and Fuji juices, since the lowest RMSE values

for these varieties were obtained with this expression (0.771 and 0.543, respectively). In the same way, the first order model was the best one for Gala (1.148) and Granny Smith (0.805) juices, while the second order equation was more appropriated for Golden Delicious (0.743) and Red Delicious (0.914) juices. These results show that color changes kinetic pattern of high-pressure processed apple juice highly depends on the variety. If all the varieties are considered together, the RMSE value showed that the zero-order model (1.057) was the best one.

Table 4.7.8 shows the kinetic parameters ($(\Delta E)_0$ and k_i) obtained with the best model for each variety. As a general rule, kinetic constants (k_i) become higher as both pressure and temperature grow. As an example, in the case of Braeburn, k_0 at 25ºC is 0.156 min^{-1} for 400 MPa and 0.630 min^{-1} for 600 MPa, while at 80ºC k_0 is 0.573 min^{-1} for 400 MPa and 0.676 min^{-1} for 600 MPa. Observing directly the kinetic constants, it is not possible to determine whether the pressure or the temperature have the greatest effect on color variation, since the growth of these parameters with both variables follows different trends depending on the variety.

Multivariate study of color difference (ΔE).

Again, a first Principal Component Analysis (PCA) was carried out on the non-spectral data (26 variables) to identify any possible sample groups and variable correlation trends. Observing samples scores in the PC1 vs. PC2 space (not shown), which represented 59% of the overall variance, six different groups of samples could be clearly identified according to varieties, being distributed mainly along the maximum variance direction (PC1) in a similar way to what was observed for PPO inactivation. Therefore, this separation between groups suggested again a particular PCA model for each one of them. Regarding loadings plot, maturity index was positively correlated with *a** and negatively correlated with *L**. Thus, as fruit matures, the juice becomes darker and the concentration of compounds that confer it a redder colour increases (Westwood, 1993). *b** was not found to be correlated with the other CIELab parameters.

Table 4.7.8. *Kinetic parameters obtained fitting the experimental ΔE values to the most appropriated kinetic model for each apple variety.*

Variety	Kinetic model	*T* (ºC)	*P* (MPa)	RA_0 (-)	k_i (min^{-1})	R^2
Braeburn	Zero-order	25	400	0.145 ± 0.065	0.156 ± 0.067	0.9483
		25	500	-0.104 ± 0.096	0.416 ± 0.098	0.9839
		25	600	-0.349 ± 0.140	0.630 ± 0.142	0.9851
		60	400	0.724 ± 0.234	0.404 ± 0.239	0.9643
		60	500	0.202 ± 0.065	0.518 ± 0.474	0.8691
		60	600	0.569 ± 0.134	0.552 ± 0.137	0.9833
		80	400	0.389 ± 0.097	0.573 ± 0.099	0.9906
		80	500	0.932 ± 0.216	0.665 ± 0.322	0.8970
		80	600	1.035 ± 0.234	0.676 ± 0.341	0.8259
Fuji	Zero-order	25	400	-0.334 ± 0.080	0.379 ± 0.081	0.9865
		25	500	0.440 ± 0.266	0.599 ± 0.272	0.9425
		25	600	0.527 ± 0.119	0.701 ± 0.096	0.9185
		60	400	0.315 ± 0.113	0.508 ± 0.115	0.9879
		60	500	1.203 ± 0.271	0.565 ± 0.211	0.9019
		60	600	2.599 ± 0.529	0.852 ± 0.112	0.8514
		80	400	2.883 ± 0.133	0.385 ± 0.070	0.8776
		80	500	3.068 ± 0.733	0.702 ± 0.286	0.7651
		80	600	2.711 ± 0.499	1.078 ± 0.343	0.8627
Gala	First order	25	400	0.921 ± 0.077	0.087 ± 0.056	0.9844
		25	500	2.747 ± 0.275	0.107 ± 0.089	0.8754
		25	600	0.504 ± 0.035	0.145 ± 0.045	0.9952
		60	400	1.800 ± 0.180	0.100 ± 0.107	0.8045
		60	500	0.823 ± 0.082	0.165 ± 0.080	0.9543
		60	600	2.880 ± 0.288	0.215 ± 0.087	0.8317
		80	400	0.423 ± 0.042	0.105 ± 0.151	0.9603
		80	500	0.679 ± 0.068	0.133 ± 0.133	0.7975
		80	600	1.442 ± 0.144	0.243 ± 0.095	0.8732
Golden Delicious	Second order	25	400	1.864 ± 0.069	0.002 ± 0.018	0.9523
		25	500	2.370 ± 0.156	0.010 ± 0.020	0.8534
		25	600	2.544 ± 0.174	0.019 ± 0.018	0.8385
		60	400	1.535 ± 0.033	0.018 ± 0.013	0.9820
		60	500	2.011 ± 0.088	0.020 ± 0.020	0.9107
		60	600	2.524 ± 0.184	0.027 ± 0.016	0.8789
		80	400	3.578 ± 0.104	0.016 ± 0.007	0.9396
		80	500	8.749 ± 0.152	0.021 ± 0.002	0.9396
		80	600	2.334 ± 0.117	0.031 ± 0.019	0.8962
Granny Smith	First order	25	400	1.162 ± 0.116	0.080 ± 0.097	0.8834
		25	500	0.920 ± 0.086	0.110 ± 0.064	0.9660
		25	600	1.364 ± 0.136	0.137 ± 0.080	0.8107
		60	400	1.141 ± 0.114	0.086 ± 0.086	0.8676
		60	500	0.565 ± 0.057	0.113 ± 0.119	0.8432
		60	600	0.982 ± 0.098	0.119 ± 0.101	0.8832
		80	400	0.644 ± 0.064	0.073 ± 0.092	0.9336
		80	500	1.001 ± 0.100	0.082 ± 0.082	0.8539
		80	600	2.333 ± 0.233	0.141 ± 0.073	0.7978
Red Delicious	Second order	25	400	3.250 ± 0.064	0.009 ± 0.005	0.9857
		25	500	2.669 ± 0.076	0.010 ± 0.010	0.9576
		25	600	3.131 ± 0.163	0.011 ± 0.015	0.9216
		60	400	3.368 ± 0.181	0.015 ± 0.013	0.8729
		60	500	1.325 ± 0.109	0.015 ± 0.042	0.8376
		60	600	2.735 ± 0.180	0.017 ± 0.016	0.8338
		80	400	4.534 ± 0.267	0.013 ± 0.010	0.8716
		80	500	3.909 ± 0.270	0.016 ± 0.011	0.8240
		80	600	0.145 ± 0.065	0.156 ± 0.067	0.9483

Signification level: $\alpha = 0.05$.

If additional PCA models are carried out for the samples of each variety (graphs not shown), the variance explained by the two first PCs in each one of them increases (78% on average). In these new plots, samples scores codified according to processing time show a pattern distributed along the first PC, although no separate groups appear. Thus, this maximum variance direction can be identified with the processing time. In the same way, if the samples are codified according to processing temperature, they appear distributed vertically along the second PC and therefore, this second maximum variance direction can be clearly associated with this variable. Regarding processing pressure, no pattern defining any trend was observed, since the information in this variable appeared randomly distributed.

As far as variable loadings for one single variety are concerned (graphs not shown), colorimetric variables are the ones with the strongest influence on the overall variance, since they have the highest values for PC1. In addition, *L** appears again strongly negatively correlated with *a**. As regards to processing conditions, time seems the most important variable and is more identified with the direction that PC1 represents, while temperature appears as the second most important variable and is clearly identified with PC2, as it was stated observing the codes for sample scores.

The colorimetric multivariate study was completed with two additional PCA models on two spectral variable sets. In the first one, 601 variables were calculated as the difference between the absorbance in the visible range of the fresh juice and the absorbance measured after processing for each wavelength. By means of this analysis (Figure 4.7.6) it can be stated that high pressure processing affects in a stronger way the absorbance of colors located near 400 nm, since the loadings for the first PC (that explains 97% of the overall variance) are the highest ones in this region, with a peak in 401.5 nm. These results are consistent with what has been previously discussed, since *a** (redness) was the CIELab parameter with the highest loading values for the first PC. Equivalent results were obtained in the PCAs carried out only on the samples from each variety (not shown), obtaining maximum peaks between 400.5 (Fuji) and 434.0 nm (Braeburn).

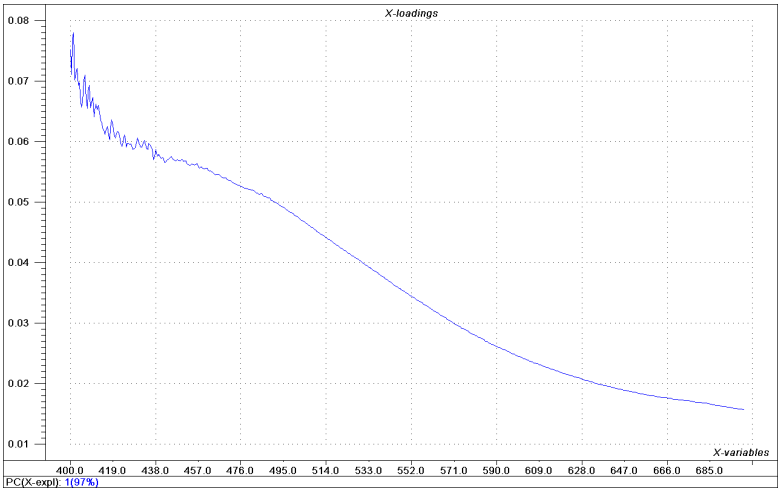

Figure 4.7.6. *Principal Component Analysis of the spectral data for changes produced in the absorbance spectrum by high pressure processing in all samples. Variable loadings for the first principal component (explained variance: 97%).*

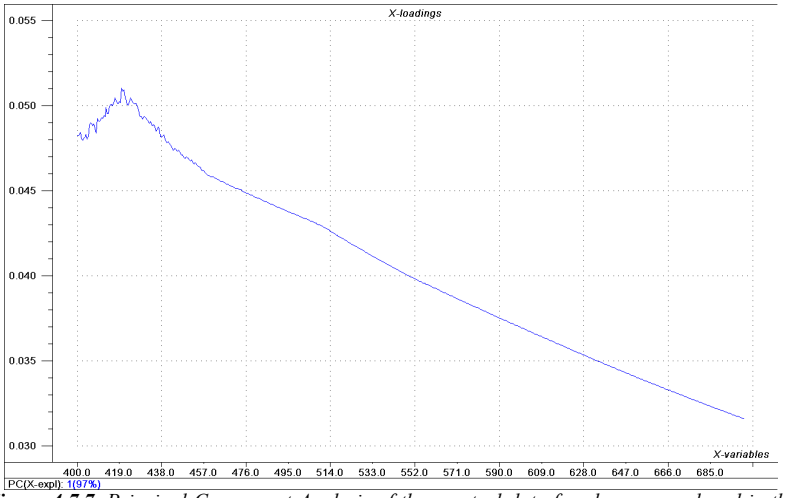

Figure 4.7.7. *Principal Component Analysis of the spectral data for changes produced in the absorbance spectrum by enzymatic browning, in all samples kept at 25ºC for 24 h. Variable loadings for the first principal component (explained variance: 97%).*

For the second spectral variable set, 601 variables were calculated as the difference between the absorbance at each wavelength in the visible region immediately after high pressure processing and after 24 h at room temperature (25±1ºC), in a similar way to the procedure that was carried out for indirect characterization of tropical fruits PPO (in section 4.1.1). As it can be observed in Figure 4.7.7, there is a maximum peak in 419.5 nm. If the PCA is carried out

individually on the samples from each variety (graphs not shown), this peak is found between 416.5 (Gala) and 426.0 nm (Braeburn). These color changes, affecting mostly the wavelengths around 420 nm, can be attributed to the enzymatic browning mainly caused by the residual polyphenol oxidase activity after the treatment. Polyphenol oxidase has been reported to be highly resistant to high pressure processing (Buckow *et al*., 2009), and therefore it remains partially active after the treatment. Since polyphenol oxidase inactivation is a critical point for juice manufacturing, this assessment of color changes by means of PCA can also be a good indicator to test the effectiveness of the inactivation of this enzyme by means of high pressure processing.

5. Conclusions and Future research.

5.1. Conclusions.

According to the different results that have been previously reported and discussed, the following main conclusions have been reached.

a1. Among the twelve studied tropical fruits (lulo, mangosteen, Castilla blackberry, mango, apple guava, tree tomato, papaya, soursop, banana passionfruit, giant granadilla, sweet granadilla and passion fruit), mangosteen and lulo showed the highest polyphenol oxidase activities, while passion fruit and banana passionfruit had the lowest ones. PPO activity in papaya can be very limited mainly due to its low phenolics content. In contrast, mango showed to have the highest phenolics content, but the presence of endogenous PPO inhibitors can limit enzymatic browning in this fruit. Polyphenol oxidase causes color changes in fruit juices that can be easily assessed measuring the decrease of brightness by means of the CIELab parameters. The Principal Component Analysis revealed that total phenolics content and *L** variation are strongly related with PPO activity. Thus, PPO is more active in those fruits with higher phenolics content, and this process leads to a greater decrease of the CIELab brightness.

a2. Nitrogen fertilization directly enhances polyphenol oxidase activity and increases fruit consistency (decreases Bostwick's index) in peach fruits, besides the effect that it can have in delaying the ripening process. Therefore, if nitrogen supplementation is applied, although the fruits may reach their commercial maturity later, at that time their PPO activity will be higher, with the subsequent problems in postharvest handling and processing. However, no significant differences were found between the application of 60 and 120 kg N/ha.

a3. Melanogenesis from monophenolic substrates by means of mushroom polyphenol oxidase is an autocatalytical reaction that can be described by the kinetic model: $A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_1 \cdot \left[\exp\left[\left(k_1 + k_2 \cdot C_A^0\right) \cdot \left(t - t_0\right)\right] - 1\right]}{k_2 \cdot C_A^0 + k_1 \cdot \exp\left[\left(k_1 + k_2 \cdot C_A^0\right) \cdot \left(t - t_0\right)\right]}$. In the same way, melanogenesis from *o*-diphenolic substrates can be divided in two stages; in the first one, the enzymatic process can be described by the kinetic model: $\frac{A_\lambda}{\varepsilon_\lambda \cdot l \cdot t} = r_{\max} - \frac{K_M}{t} \ln\left(\frac{C_S^0}{C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda \cdot l}}\right)$; while the second non-enzymatic stage can be described by a zero-order kinetic expression. Among the studied variables, the factor that has the highest influence on melanogenesis is the nature of substrate. Looking at the other two variables that define experimental conditions, pH is more important than the concentration of substrate. Time between 240 and 306 minutes of reaction are those that provide the greatest variance in data. With L-tyrosine (monophenol), PPO has its optimal activity at pH 6.0. However, with 4-methylcatechol (*o*-diphenol) the optimum pH is 8.0. In melanin formation from L-tyrosine, the extinction coefficient of the resulting product (ε_λ) depends on the initial substrate concentration and the pH. Initially, absorbance does not increase due to the biosynthesis of intermediate colorless substances, leading to a lag period that grows with increasing initial concentration of substrate. In this reaction, polyphenol oxidase has a lower catalytic effect on L-tyrosine (cresolase activity) than on L-DOPA (catecholase activity). On the other hand, the transition between the two stages defined on melanogenesis from 4-methylcatechol depends on the pH, being later as this is lower, but does not depend on the concentration of substrate. The Michaelis constant (K_M) and the maximum reaction rate (r_{max}) increase as both the initial concentration of 4-methylcatechol and pH increase. In the second stage, k_0 kinetic constant keeps a very small and almost constant value for all initial concentrations of substrate at pH 5.0. In the other cases, this parameter clearly increases with the concentration of substrate and the pH of the solution.

b1. Melanins synthesized from L-tyrosine and *Agaricus bisporus* polyphenol oxidase have an inhibitory effect on the pancreatic proteases carboxypeptidase A (CPA), carboxypeptidase B (CPB) and trypsin. In the three cases, the Michaelis-Menten constant (K_M) increased with melanin concentration in the solution, which indicates that this polymer decreases the apparent affinity between the enzymes and their respective substrates. In contrast, the maximum reaction rate (r_{max}) tends to decrease with the increase of melanin content in a lineal tendency. These facts show that melanin from L-tyrosine inhibits the three enzymes in a mixed way. For the system CPA-melanin from L-tyrosine, K_i was found to be 1.01 mg·mL^{-1}, while αK_i was 2.56 mg/mL and therefore α = 2.52. In the case of the interaction between CPB and the same melanin, K_i = 0.78 mg·mL^{-1}, αK_i = 6.76 mg·mL^{-1} and α = 8.64. And, for trypsin and L-tyr melanin, K_i was found to be 0.148 mg·mL^{-1}, αK_i was 0.438 mg·mL^{-1} and α = 2.95. As far as melanin from 4-methylcatechol is concerned, this polymer was not found to inhibit CPA or CPB.

c1. Mathematical modeling of absorbed radiation profiles in a system composed by a plane photoreactor (with the dimensions of the one of Food Technology Department, UdL) and a single lamp has been developed. The application of the linear spherical model leads to an easy equation that allows calculating a network of absorbed radiation values in discrete points of the reactor volume. The step-by-step integration by numerical methods of these values in the three dimensions of the space provides interesting information about the radiation dose that would be used in photochemical reactions. Among these conclusions, for example, the obtained profiles have shown that, with the considered geometry and dimensions, if the absorbance of the reaction medium is 0.1, hardly 30% of the emitted radiation is absorbed, while for a μ value of 5 half of the radiation is absorbed in the first 2 mm, and nearly the whole radiation is absorbed in the first cm.

c2. It is possible to completely and irreversibly inactivate *Agaricus bisporus* polyphenol oxidase with an UV-vis irradiation process with a lamp of 400 W of nominal power that emits in a range between 250 and 740 nm. The presence of melanins or melanoidins in the reaction medium protects the enzyme, making the inactivation process slower as their concentration increases. If the reaction medium contains melanins synthesized from L-tyrosine, when their content is below 0.05 $mg \cdot mL^{-1}$ a composite kinetic model is required to explain the fast inactivation in the early stages. When the pigment content is higher, these molecules also protect PPO from this fast denaturalization, making the simple first order kinetic model able to explain the entire process. With melanins from 4-methylcatechol in the reaction medium, both models fitted experimental data. The first order kinetic model is more appropriate to easily describe and quantify the protective effect due to its mathematical simplicity. However, the use of the composite kinetic model brings additional information about the transformation of the native enzyme to an intermediate form and to the denatured inactive form later. At the same concentration, melanin from 4-methylcatechol has a higher protective effect than melanin from L-tyrosine. When it comes to the protective effect of the melanoidins synthesized from fructose and glutamic acid, the achieved inactivation degrees after 90 min of processing and the kinetic study led to the conclusion that melanoidins with molecular mass lower than 150 kDa have a greater protective effect than those molecules higher than 150 kDa. If the obtained melanoidins are not fractioned by their molecular mass, the protective effect that they exert is lower due to aggregation phenomena favored by the presence of different chain length polymers, which leads to a lower absorbance in the UV region of the spectrum. At the same concentration, these melanoidins (regardless the considered fraction) have a greater protective effect than melanins. The study of the protective effect of the different melanoidin solutions and their absorbance spectra showed that the most effective radiation to inactivate PPO is located between 260 and 310 nm.

c3. The effectiveness of UV-vis irradiation to inactivate polyphenol oxidase in freshly squeezed apple, pear and grape juices depends on each fruit and variety. In apple juices, regardless their initial activity, polyphenol oxidase was completely inactivated after 100 min of treatment. In pear juices, the residual activity after 120 minutes of treatment was almost zero (except for Conference PPO, whose initial activity was the highest one). Besides, after 20 minutes, it was possible to reduce the activity in more than 50%. In grape musts, PPO was not entirely inactivated after 140 minutes of UV-vis irradiation in any of the four assayed varieties. In white grapes (Victoria and Dauphine), an 80% inactivation was achieved, while only 50% of initial PPO activity was reduced in the samples from pink grapes (Red Globe and Emperor). No significant variations were observed in pH, soluble solids content, formol index and sugars in any of the assayed apple, pear or grape juices. In apple juices, the content of vitamin C in those from Golden, Starking and Fuji slightly decreased during the experiment (4.0-5.7%), while in the juice from King David the loss was 70.0% due to the lack of pigmentation of this juice. The same phenomenon, although at a different scale, was observed in pear juices: In the Abate Fétel and Ercolini ones, which had the lowest pigmentations, vitamin C content at the end of the treatment had a 60,5% and 50% decrease, respectively. In the other varieties (Conference, Passa Crassana, Flor de invierno and Blanquilla) this decrease was found in the 20% to 30% interval. However, in grape musts, the content of vitamin C decreased (between 12 and 30%) with processing time, being impossible to find a direct relationship between the pigmentation of the juice and the protection against vitamin C degradation. As far as color is concerned, as a general rule, UV-vis irradiation caused an increase of brightness (L^*) in the juices, meaning that radiation impairs some of the pigments present in the juice, either those initially present or the ones formed later. This impairment is especially remarkable in red pigments, since in all samples (regardless the fruit or variety) a decrease of CIELab parameter a^* was observed, along with the disappearance of the peaks in absorbance spectra that belong to these colored compounds. On the contrary, yellowness (b^*) behavior depends on each fruit and variety.

c4. UV-vis irradiation of musts before fermentation in winemaking is a technology that can help reducing the quantity of SO_2 that is added to wines. A residual use of this product would be necessary to prevent musts from an excessive oxidation, since polyphenol oxidase is not completely inactivated (especially in red musts, where anthocyanins absorb radiation reducing the available dose for inactivating PPO). In addition, chemical quality analyses indicate that UV irradiation can also ensure a greater stability of the final product by means of the destruction of the natural yeasts and bacteria of the musts. As a result, wines from irradiated musts ferment slower and have less volatile acidity than those that have not been treated. Moreover, no significant differences were found in alcohol degree, pH and tartaric acid content between the wines from irradiated samples and those from musts with SO_2. As far as color parameters are concerned, irradiation of white musts (Xarel·lo or Parellada) causes an increase of wine redness, while in red (Cabernet Franc) musts there is an important decrease of blueness. This decrease may represent a problem in red wines production, but the same effect may be interesting in the production of rosé wines. Must freezing before processing caused some variations in quality parameters in Xarel·lo wines, but not in those made from Parellada.

d1. Apple polyphenol oxidase from the six varieties processed by HHP (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith and Red Delicious) is extremely pressure-resistant if the process is carried out at approximately room temperature (25ºC): after 16 min at 600 MPa, the obtained reduction (additional to the loss caused by compression and instant decompression) was found between 7% (Braeburn and Golden Delicious) and 28.5% (Granny Smith). The apple variety does not only affect the initial PPO activity, but also the inactivation pattern and the achieved inactivation degree. Multivariate analysis (after eliminating unit scale effects) showed that, in a process at temperatures between 25 and 80ºC, pressures from 400 to 600 MPa and processing times up to 16 min, among the variables that define the experiment the apple variety is the one with the strongest influence in PPO inactivation achieved degree, followed by the temperature, the processing time and lastly, the pressure.

d2. The color of raw apple juice has very different properties depending on the variety (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith or Red Delicious) from which it is made. Although all the juices had a greater initial yellowness (*b**) than redness (*a**), the latter was more influenced by high pressure processing. Compression to target pressure followed by instant decompression caused a decrease in *L** value and an increase in *a** and *b**, i.e. the juice became darker. For longer processing times, the three parameters behaved in the opposite way, even at 80ºC. In addition, the variety also determines the way in which color is affected after the treatment. In this case, multivariate analysis showed that, in a process at temperatures between 25 and 80ºC, pressures from 400 to 600 MPa and processing times up to 16 min, among the variables that define the experiment the apple variety is the one with the strongest influence in color changes, followed by the processing time, the temperature and the pressure. Moreover, wavelengths between 400.5 and 434.0 nm were the most affected by high pressure processing, while the wavelengths between 416.5 and 426.0 nm were the ones that changed the most in the first 24 h after the treatment due to residual PPO activity. On the other hand, maturity index was found to have a strong positive correlation with *a** and a strong negative correlation with *L**.

5.2. Recommendations for future research.

On account of the conclusions that have been reached within the different studied fields, some final considerations must be stated in order to define the next steps in this research line.

- ✓ The developed kinetic models to describe melanogenesis from monophenolic and *o*-diphenolic substrates should be tested on polyphenol oxidase activity on other substrates and in other conditions, in order to check their validity and find whether the different parameters behave in the same way.

- ✓ Different kinds of melanins synthesized from different substrates should be tested as potential protease inhibitors, and studies should be conducted in a more detailed way in order to find the reasons why some of these polymers behave as inhibitors while others do not.

- ✓ Further investigation is required, at a molecular scale, in order to understand the interaction between polyphenol oxidase and UV-vis radiation that leads to enzyme inactivation, and the interaction of both with the different polymers that can prevent PPO from being inactivated (i.e. pigments that are naturally contained in fruit derivatives).

- ✓ UV-vis irradiation of juices and musts as an alternative to thermal pasteurization has shown promising (although still preliminary) results. However, its implementation at industrial scale raises some difficulties, specially related to the long treatment times that are needed to achieve the desired effects on enzyme inactivation. Thus, the next steps in this research line should focus on two main aims: on the one hand, developing new reactor kinds or configurations that make it possible to reduce processing times; on the other hand, in winemaking, finding the appropriate relation between UV-vis irradiation and the necessary residual SO_2 dose.

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APPENDIXES.

Appendix I.
Scientific publications in ISI-indexed journals.

APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.1.

Title: An integrated approach to current trends in food consumption: moving toward functional and organic products?

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Review

An integrated approach to current trends in food consumption: Moving toward functional and organic products?

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ABSTRACT

In recent times, concerns about the impact of the food that people consume on their own health, as well as the social and environmental consequences that it entails, have led to major changes in all steps of the food chain including all the agents from the producer to the retailer. These phenomena comprise complex technologic, social, economic and environmental linkages that require integrated research approaches, i.e. the changes in consumers' demand and their consequences need to be considered from all these different points of view. This piece of work reviews the main causes and consequences of these changes in consumers' attitude toward foodstuff selection, dealing with different topics as regulation modifications or the impact on rural network in Europe. In addition, some references that cast doubt on the appropriateness of expanding markets for expensive products such as functional and organic ones at a time of economic recession have been provided.

Contents

1. The changes in consumers' demand and the need for an integrated approach

The increasing complexity of food chains is associated, at least in industrial societies, with a progressive shift during the past 30 years away from systems that were largely nationally-based, supply-oriented and state-regulated and supported (Lowe, Phillipson, & Lee, 2008). The growth of cities was matched by the development of a global food industry, which adopted intensive methods of production in order to meet the increasing demands of both consumers for food and shareholders for profit (McGill, 2009). In this way, the agricultural sector was oriented to optimizing yields and producing products with good appearance and shelf-life. Health and other concerns were not at the forefront in selecting crop varieties and animal breeds or production methods (Traill et al., 2008).

In recent times, supply chains have had to become more demand-oriented and food production more responsive to feed-back signals from the market and from consumers, who are now

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regarded as active agents in the food chain (Lowe et al., 2008). Growing awareness of the environmental and social costs associated with the provenance and processes of food production is associated with wider changes in modern societies attitudes toward what social scientists term *reflexive consumption* (Giddens, 1991). This concept defines how people think of themselves as active, discerning consumers, whose choices contribute to their sense of identity. This is a well-known statement in current marketing strategies, in which the social or community attitude beyond the individual level must be considered in order to understand consumer behavior (Cova, 1997). Moreover, there is an expanding demand for *natural* (free from chemical fertilizers or pesticides) foods and ingredients that are fresh or minimally processed, readily available, reasonably priced and yet safe for consumption, according to local standards (McGill, 2009).

Thus, nowadays several socio-economic factors have a strong influence on diet composition. Some of these factors are culture, personal preference, price, availability, convenience and environmental, social or health concerns (Gerbens-Leenes, Nonhebel, & Ivens, 2002; McGill, 2009). People consume not only to fulfill their basic biological needs, but also to express a sense of self and improve psychological wellbeing. Health and fitness preoccupations, cultural and ethical concerns, worries about animal welfare or political and moral standpoints are good examples of some issues that strongly influence people's behavior toward food selection. People consume images and ideas as well as the basic nutritional content of food (Casey, 2009; Lowe et al., 2008). Indeed, the social consequences of consumption are becoming more important every day. In publicity companies, this fact leads to different socially-oriented strategies included the so-called *societing or tribal marketing*: *the link is more important than the thing* (Cova, 1997).

These changes in consumers' demand lead, in the short term, to substantial changes throughout the whole food chain, which have technical, social, economic and environmental implications. Regarding the primary production, for example, it is necessary to predict the impact of changing agricultural practices for biodiversity and landscape quality. In order to assess this impact, it is essential to use an approach that can combine the variation in demand with information on the suitability of agricultural land for different production methods. Thus, while technical advances have greatly expanded the potential to produce nutritious food in an efficient and environmentally sustainable manner, social and economic factors will determine the value of this research, as well as its future direction (Lowe et al., 2008; Phillipson & Lowe, 2008; Traill et al., 2008).

In order to study the causes and the possible consequences of these changes, they must be considered from different points of view, but integrating these different outlooks. Interdisciplinary research, i.e. technical research embedded in a social, economic and environmental framework, helps to overcome fragmented perspectives and the partiality that can arise when natural or social scientists make naïve assumptions about their own field (Cova, 1997; Habib & Lescourret, 1999; Phillipson & Lowe, 2008). The technological solutions derived from this research must be responsive to consumer demands and sensitive to the social and economic contexts in which they are to be applied (Phillipson & Lowe, 2008). In other words, there is a need to provide integrated solutions for problems that are both social and technical in character (Lowe et al., 2008). In fact, in their day-to-day decisions, small-scale farmers already integrate biophysical factors with social, economic, cultural and environmental considerations to manage complex farming systems. This approach contrasts with the traditional organization of knowledge, where reductionist approaches prevail, leading to severe deficiencies in the analysis of complex systems (Temu, Rudebjer, & Chakeredza, 2010).

The idea of linking these different sciences at a broader scale aims to secure a profitable and internationally competitive food and farming sector that respects the environment and improves nutrition and public health (Traill et al., 2008). In addition, it should allow faster and broader responses to these complex problems generated by outstanding changes in the socio-economic context (Habib & Lescourret, 1999). In this background, social sciences can bring different perspectives and methodologies to assist in interpreting uncertainties and divergent views. Moreover, they may be useful in understanding the economic and political factors determining the behavior of complex socio-technical and socio-ecological systems. Many natural scientists and technologists working in agri-food R&D, and indeed many funders and users of technical research, acknowledge the need to understand better the social and economic parameters of their work (Lowe et al., 2008).

2. Consumers' mistrust toward the food chain

From a general point of view, one of the main reasons of the changes in consumers' demand is the fact that people have more information every day about all of these different knowledge areas. However, sometimes this information may be rather disorienting. A good example of food-related confusing information has to do with the lack of harmonized risk communication in situations of scandals or foodborne crisis (Bánáti, 2011), such as the possible contamination of food after the radioactive fallout in Japan (Moy & Lacroix, 2011) or the case lived in Germany in June 2011 with the pathogenic *Escherichia coli* O104:H4 strain (Warriner, 2011). Sometimes, these confusing situations are the consequence of the conflict between what Shepherd (2008) defines as *scientific language* and *public language*. Nevertheless, these *languages* are usually less distinct than they are presented, with expert knowledge incorporating implicit judgments and social assumptions and public knowledge using empirical evidence and reasoning.

In spite of the fact that food has never been safer, it seems that consumers are considerably uncertain, anxious and increasingly critical about the safety of food (Bánáti, 2011). During the last two decades there have been widespread food scandals and fears of foods that have focused both consumers and authorities attention to food safety, leading to an increased recognition of the potentially negative effects of scientific and technological developments. As Phillipson and Lowe (2008) stated, farming crises, chronic health risks, food safety scares and resource and habitat depletion have evoked considerable mistrust of the science and technology underpinning food chains and have been associated with an assertion of consumer/public interest not only in what food is produced but also how it is produced. In addition, these processing systems used in food chains are often portrayed as out-of-touch with public concerns and driven by narrow disciplinary or commercial logics (Food Ethics Council, 2004). As a result, consumers have become more and more interested in and worried about food technologies and certain food substances, and they have started to fear of foods and of new food technologies (Bánáti, 2011; Rollin, Kennedy, & Wills, 2011).

Food scandals have undermined consumers' trust in the safety of agri-food systems. These large-scale scares have tended to arise from contamination of food chains at the primary level, and so have fueled consumers' concerns over the technological transformation of farming (Lowe et al., 2008). In addition, consumers have also been sensitized by the initial mismanagement of some of those crisis, where they were sometimes wrongly reassured that food was safe when it was not (Rollin et al., 2011). These problems directed the authorities' attention to the lack of the integrated approach of the food chain (taking into account the points of view of consumers and other stakeholders) and new principles were

established such as risk analysis and communication or traceability (Bánáti, 2011; Shepherd, 2008).

The worries about food safety are the reason for many of those who, in increasing numbers, are turning to organic food and mistrusting new food technologies such as GMO and irradiation, although several studies have concluded that these new technologies are safe (Ibarz, 2008; Mostafavi, Fathollahi, Motamedi, & Mirmajlessi, 2010). According to Rollin et al. (2011), the factors that influence consumers' acceptance of food innovations are risk-benefit perceptions, socio-demographic attributes and knowledge and information, as well as the level of trust in the source of that information.

3. Trends in food selection: toward foods that bring health, social and environmental benefits

The movement toward a more complex, technology-led, globalized, privately regulated and demand-oriented food system has met resistance from different social movements. These movements include the anti-globalization lobby, environmentalists, farmers, the health lobby, advocates of sustainable development, rural and countryside interests and food campaigners (Lowe et al., 2008). Moreover, consumers' emphasis has been attached to non-traditional quality attributes of food (Casey, 2009), being every day more concerned about the impact of their decisions on the environment and social equity. Indeed, quality aspects have become more and more linked to an *added value* basket of indirect and invisible food quality criteria vaguely described as *healthy environment*, *animal welfare* and *fair trade*. These consumer criteria may be traduced, at the professional level, into four kinds of quality (Boller et al., 2004): product quality (including food safety), production quality (including ecological criteria and animal welfare), ethical quality and social quality.

The changes in consumers' demand have forced the food industry to create and develop new products and marketing campaigns that are able to fulfill all these requirements. In this way, the variety of necessities and the scope for new ones also opens up possibilities for alternative food networks. Two of the main examples of these innovative trends are functional foods and organic products. Other tendencies include products engineered to fulfill special dietary needs, fair trade foodstuff, or PDO (Protected Designation of Origin).

3.1. Functional foods

As already mentioned, one of the most important reasons why consumers' demand is changing is their concern about healthy and unhealthy products. In this way, if all consumers were to follow recommended healthy eating guidelines there would be major implications for food consumption, land use and international trade. According to Traill et al. (2008) this is unlikely to happen, at least in the short term, but it is realistic to anticipate some dietary adjustment toward the recommendations, resulting in an improvement in diet quality. Although consumers are reluctant to make major changes to their diets, they may be prepared to substitute existing foods for healthier alternatives. And functional foods claim to be these alternatives.

The changes in food products to improve their composition in order to make them healthier would bring them into the category of functional food, which Diplock et al. (1999) define as food that improves health or wellbeing, or reduces disease risk, through beneficially targeting the body's functions. A functional food is similar in appearance to, or may be, a conventional food; is consumed as part of a usual diet and is demonstrated to have physiological benefits and/or reduce the risk of chronic disease beyond basic nutritional functions (Walji & Boon, 2008).

From the food industry point of view, functional foods have appeared as a guarantee against a world of saturated demand and increasing global competition (Grunert, 2010). They constitute the perfect marketing strategy by creating differentiated, value-added products, appealing to health (a basic and universal human need) and directed to a premium-price sector. However, many of those innovative products have failed, probably due to a lack of knowledge management between the functional disciplines involved in the new product development process, especially consumers' attitudes knowledge (Betoret, Betoret, Vidal, & Fito, in press; Jousse, 2008). Particularly, people in Europe are more reluctant in adopting functional foods than in Asia or North America (Grunert, 2010; Siro, Kápolna, Kápolna, & Lugasi, 2008). As an example, the last survey of the Spanish government revealed that consumers attach more importance to the origin of the product (i.e. those produced in their own region, or with Protected Denomination of Origin) than functionality (OCDA, 2011).

Although functional foods are currently in fashion, the majority of people are unsure of their benefits. Actually, a positive or negative perception of a functional food product is based strongly on the nutrient content of the base product and less so on its health claim (Traill et al., 2008). In this way, consumers tend to prefer food products that bring a simple but clear health benefit (Bitzios, Fraser, & Haddock-Fraser, 2011), and even those that are more concerned about health issues perceive products that are intrinsically healthy (such as yogurt, cereals and juice) as preferable and credible carriers of functionality (Annunziata & Vecchio, 2011). In addition, there are strong differences between different population groups. For example, women, people with higher income and married couples have a more positive view of functional food (Markovina, Čačić, Kljusurić, & Kovačić, 2011; Traill et al., 2008; Verbeke, 2006). As Barrena and Sánchez (2010) discussed, household structure (e.g. whether there are children) has also a definitive impact on the cognitive process that results in purchase decision. Some studies also show that functional foods are even less accepted if the functionality is achieved by means of genetic modification (Frewer, 2003).

For most consumers, the most important functional food attributes are taste, price/quality ratio and, in third place, functionality (Markovina et al., 2011). Indeed, organoleptic features are still at the forefront of most consumers' preferences, being more important than potential health benefits (Annunziata & Vecchio, 2011; Urala & Lähteenmäki, 2007; Verbeke, 2005). In fact, consumer willingness to compromise on the taste of functional foods for health is decreasing over time and constitutes a highly speculative and risky strategic option (Verbeke, 2006).

As far as economic issues are concerned, developing a new functional food is an expensive process, which requires detailed knowledge of the product to be created or modified and of the consumers to whom it is addressed (Betoret et al., 2011). The current difference in price between conventional and functional food is often too large for consumers to change their shopping habits (Asselin, 2005; OCDA, 2011), although they are willing to pay a small amount extra (Traill et al., 2008). Even so, it is not clear if this amount is enough to cover the increase in production costs of some functional foods compared with their traditional counterparts. In this way, consumers acknowledge that functional foods should be more expensive than conventional ones because they provide additional benefits, but in most cases they find this difference to be excessive (OCDA, 2011).

3.2. Organic products

Apart from functional foods, another important trend in food consumption is focused on organic products. Both tendencies have in common its main motivation: consuming foods that may provide

health benefits. Organic products, though, have been claimed to be also more respectful to the environment than their conventional counterparts. Increasing uses of pesticides and fertilizers have spawned criticism in popular publications, and have led to a growth of organic farming alternative (McGill, 2009; Sheng, Shen, Qiao, Yu, & Fan, 2009). Concerns focused initially on chemical pesticides, but widened in the 1970s and 1980s to cover the intensification of agricultural production and its consequences for farmland ecology, agricultural pollution and landscape change. Environmentalists have sought to encourage consumers to buy 'environmentally-friendly' products, which is known as *green marketing* (Lowe et al., 2008). Indeed, consumers perceive foods labeled as *organic* to be healthier (Sheng et al., 2009). However, in recent times the profit interests have undermined the use of the adjective *natural*, as well as the term *organic*, leading consumers to a generalized mistrust understanding that it does not necessarily mean *safe* (Carvalho & Luz, 2011).

On the other hand, objective analyses of environmental benefits of organic farming are not so clear. In this way, while there is evidence that smaller-scale production is more efficient in terms of energy use, it generally involves lower productivity than either large-scale agriculture or non-farm work (Bravin, Hoffmann, Kockerols, & Weibel, 2010; Woodhouse, 2010). Thus, since the yields obtained by organic farming are lower compared with conventional farming, the overall environmental benefits are strongly reduced or even disappear after correcting for these lower produced quantities per hectare (Backer, Aertsens, Vergucht, & Steurbaut, 2009; Woodhouse, 2010). Therefore, more research should be done on how the yields in organic farming can be substantially increased without increasing the environmental burden to give objective sense to environmentalists' motives.

In addition to environmental aspects, price and organoleptic features of organic products must also be considered. In a similar way to what occurs with functional foods, several studies have concluded that consumers would not sacrifice organoleptic properties for potential health benefits (Markovina et al., 2011; Traill et al., 2008). Furthermore, consumers are also willing to pay only a small amount extra for products with the *organic* label, and there is no evidence showing that this amount is enough to cover the decrease in productivity, mainly due to the perception of overpricing (Ferjani, Reissig, & Mann, 2011; Mesias-Diaz, Pleite, Martinez-Paz, & Gaspar Garcia, 2011). Indeed, as shown in Table 1 (USA data), there is a big difference between the prices of both kinds of products at the retailer level. In some cases, such as poultry, this difference may represent nearly a 200% increase. On the contrary, overpricing is less important in imported foodstuff: organic bananas were only 25% more expensive than conventional ones.

Moreover, it must be considered that since 2009 the economic recession has strongly affected the demand for organic products, for example in the United Kingdom, where it declined by 13.6% in 2010 and by 5.9% in 2011 (European Commission, 2010; Soil Association, 2011). In this country, the most affected products were chilled convenience foods with a decrease of 36% in 2011, followed by bread (−20.6%), fresh fish (−16.0%) and breakfast cereals (−15.2%) (Table 2). Meanwhile, organic baby foods are the only exception to this downward trend, growing by 10.3%. This different tendency may be explained by the fact that most consumers think that organic products *may be safer*, but they are only willing to pay for them if these foods are for the children (OCDA, 2011). In this context, experts recommend adjusting the prices of agricultural products in order to ensure that the costs of production would be suitable for both the producers and the great mass of consumers, but taking the minimum possible impact on public finances (Simtion & Luca, 2010).

Table 1
Average prices of organic and conventional food products in the USA.

Product	Source	Unit	Price ($US)	
			Conventional	Organic
Milk	Retail	Half gallon	2.28	4.43
Eggs	Retail	Dozen	1.35	3.99
Rice	Retail	Pound	0.93	2.35
Carrots	Retail	Pound	1.59	2.21
Salad mix	Retail	Pound	3.85	8.14
Spinach	Retail	Pound	4.45	8.59
Strawberries	Retail	Pound	3.23	5.14
Poultry	First receiver	Pound	0.80	2.37
Broccoli	Wholesale market (Boston)	16 count bunches	14.06	32.30
Apple	Wholesale market (San Francisco)	Carton tray pack	32.61	45.89
Banana (imported)	Wholesale market (San Francisco)	40 pounds carton	19.57	24.48
Orange	Wholesale market (San Francisco)	7/10 bushel cartons	13.87	27.24
Pear	Wholesale market (San Francisco)	4/5 bushel cartons	30.13	47.08
Raspberry	Wholesale market (San Francisco)	12 6-oz cups	20.75	24.03
Sweet potato	Wholesale market (Boston)	40 pounds carton	23.57	36.54
Pea	Wholesale market (San Francisco)	10 pounds carton	17.32	43.75
Tomato	Wholesale market (San Francisco)	10 pounds carton	13.92	24.81

Source: USDA-ERS (2009).

3.2.1. Implications for rural environment: changes in agricultural practices

Diet composition can have implications for land use and rural environment of a similar order of magnitude as those related to population growth or changes in agricultural productivity (Gerbens-Leenes et al., 2002). Agricultural policy liberalization, concern about unhealthy diets and growing recognition of the importance of sustainable land use have fostered interest in the development of competitive food chains based around products that are beneficial to the rural environment (Traill et al., 2008). However, the new trends in consumers' demand are having a global impact on trade and can produce serious negative effects on less developed countries and their basic agriculture (McGill, 2009). In addition, conventional farmers in developed countries may also be affected, since they have to cope with the changing institutional environment created by international governmental and non-governmental organizations. Consequently, these implications for rural environment may modify the old agricultural models, the standards of good practices, traditional crop varieties and the entire agricultural production systems.

Table 2
Product share and value of the organic market in the UK.

Product	Share (%)	Value (thousand £)	Change 2010/2009 (%)
Dairy	30.5	528	−2.7
Fruit, vegetables and salad	23.2	402	−6.3
Baby food	7.8	135	+10.3
Beverages	7.5	130	−3.2
Fresh meat	4.8	83	−5.8
Confectionery	3.6	62	−8.3
Eggs	2.9	50	−9.4
Chilled convenience foods	2.2	38	−36.0
Fresh poultry	2.0	35	−13.2
Breakfast cereals	1.8	31	−15.2
Bread	1.2	21	−20.6
Fresh fish	0.7	12	−16.0
Other	11.8	204	−13.9

Source: Soil Association (2011).

These recent changes inevitably lead to the need for an integrated consideration of primary production (far from classical isolated points of view), which may be easily seen in agricultural production models. Such models have been traditionally developed considering this isolated sector, in order to predict changes in agricultural inputs, outputs and prices across continental, national or regional scale. However, these classic models have certain limitations, such as the inability to provide spatially explicit predictions of land use change, but rather probabilities of particular changes in the regional or national distribution of different types of agriculture (Traill et al., 2008). Furthermore, modern food chains have several agents that were not considered by those classical models, such as the large amount of intermediaries or the consumers' behavior caused by *reflexive consumption*.

The standards of quality, safety and good practice are likely to vary greatly as the distance between producer and supplier, both geographically and culturally, grows. The demand for safe, fresh, *natural* food is beginning to rely on imports. In most cases, it may paradoxically imply an increase in risks for consumers, since increasing sales demand an expanding range of suppliers and an expanding supply chain, which may be traduced in a relaxed risk assessment toward imported products (McGill, 2009). This situation is also unfair for farmers in developed countries, who in most cases have frequently been paid a minute fraction of the market value of their products. As far as new crop varieties are concerned, advances in biological technologies have opened up a multitude of new substitution possibilities and eroded the dependence of food production on specific geographical areas. Supply chains have become more extended and complex, and its intermediaries have taken on a more prominent role between producers and consumers (Lowe et al., 2008).

In fact, the established pattern of *industrial agriculture* in developed countries is in crisis, mainly due to environmental impacts and the rising cost of energy inputs. Thus, alternatives to industrial agriculture (i.e. organic producers) need to recognize more explicitly the need for cross-subsidy of food producers. Otherwise, it will be very difficult for organic-production farms to survive, even more than conventional ones. Indeed, in its last report about the situation of the organic sector, the European Commission (2010) acknowledges that organic farms receive on average higher subsidies in absolute terms ad per hectare than conventional farms.

It seems clear that a balance point must be found between conventional agricultural production systems and organic ones. These balanced practices should ensure a reasonable productivity, but according to current environmental and social concerns and, if possible, minimizing the subsidies that farmers need to receive from public administrations. This middle point seems to have been met by integrated production methods. The integrated production system looks for long-term sustainability, the rational use of natural resources, the application of regulations to ensure the substitution of pollutant agents, the use of a combination of biological and chemical control methods, the employment of adequate monitoring methodologies and the traceability of the whole process, without sacrificing crop quality and productivity (Almeida et al., 2009; Chandler, Davidson, Grant, Greaves, & Tatchell, 2008). Emphasis is placed on a holistic systems approach, involving the entire farm as the basic unit (Boller et al., 2004). Such an approach also meets the integrated consideration of primary production that was not possible with classical agricultural production models. In addition, integrated management also takes advantage of modern technology to improve on the system, resulting in better quality of soil, water and air (European Commission, 2002). Boller et al. (2004) carried out a thorough review of integrated production systems principles and technical guidelines.

Indeed, integrated production systems are the scientific answer to combine intensification in farming and low environmental impact practices, meeting market requirements along with economic objectives (Habib & Lescourret, 1999). For consumers, this philosophy would be traduced as providing healthy and environmentally-friendly foodstuff at an affordable price. For the food industry, it would represent meeting the four kinds of quality described by Boller et al. (2004) that have been previously mentioned. All aspects of production are formalized in integrated production schedules, recognized by the relevant regional or national authorities and overseen by accredited independent bodies (Patrie, 2006). In Europe, farmers in most countries apply integrated production methods, but in an unharmonized manner due to the absence of a common legal base. This situation has lead to a proliferation of private quality management systems and private standards (Wiegand, Sessler, & Becker, 2008), which will be discussed later.

Regarding research about integrated production systems, the bulk of scientific reports concerns the independent effects of various factors on crop performance, such as irrigation, fertilization, soil management, growth regulators, planting density, crop protection, and so on (Habib & Lescourret, 1999). Consequently, there is a need for considering all these production inputs together in order to find appropriate ways to combine them in a more efficient way. Due to the heterogeneity of these data, including these inputs, different crop varieties and the particular properties of each geographic location, the pursuit of these models constitutes a challenging issue for researchers all over the world.

3.2.2. Implications for rural environment: changes in rural population

Concerning rural population, the traditional small and middle-sized farms scheme in Europe is very likely to change. The globalization of markets has modified the traditional networks of information, production and marketing, which in most developed countries had been largely established and maintained by comprehensive governmental support policies. New institutions have come into the picture establishing the links between small producers and larger markets. Prices and demand patterns fluctuate widely, leaving small producers vulnerable to market forces and raising the level of economic insecurity (Casey, 2009; Keyder & Yenal, 2011). This situation brings about a rapid de-ruralization of the population in most developed countries. Consequently, farmers seek for seasonal employment in other fields, diversifying their economic activity and permitting the rural population to remain in the countryside.

Looking forward, the sustainable development of rural territories and the conservation of small and medium-sized farms network in Europe implies stabilizing populations in rural areas. This should be achieved by raising their living standards, improving the efficiency of the rural economy and ensuring rational utilization of natural resources (Atyukova, 2009). Specific issues concerning this development must include opportunities for development of local authority structures, diversification of agricultural production, adoption of alternative forms of activity in rural areas and the development of rural (and agricultural) institutional activity, providing information and other support. Hence, as Fischer and O'Neill (2005) stated, decisions at the farm level will be strongly affected by off-farm income earning opportunities. This indicates that cross-sectoral linkages and spatially explicit contexts should be considered when modeling and predicting production, consumption and investment decisions of rural households.

4. Public and private standards and regulatory aspects as a response to changes in the agri-food system

In traditional societies, if shared resources seemed to be under threat, users often agreed on rules for their management. But in

modern societies, the people affected are often unable to take direct action, and must channel their demands through the political and legal systems (Harrison & Pearce, 2000). Food scares and scandals initiated different changes in the European food policy and food legislation, which have substantially been changed in the last decade.

In addition, both public and private standards also have had to be reworked in order to integrate the new properties of today's food-related issues: new trends in food consumption, new processing technologies, rising global trade in food and agricultural products, growing economic concentration, the merging of food and pharmacy, chronic obesity in the midst of hunger and new disease and pest vectors (Busch, 2003; Busch & Bingen, 2006). Several new principles such as risk analysis, traceability and an integrated food chain have been introduced, resulting in a paradigm change in Europe. A risk analysis structure had been built up consisting of science-based risk management, assessment and communication, with the aim of improving food safety in the EU, ensuring a high level of consumer protection and restoring and maintaining confidence in the EU food supply system (Bánáti, 2011). The first important step of this program was the creation of the European Food Safety Authority (EFSA) in 2002. After that, an entire new raft of legislation known as the *hygiene package* was created to merge, harmonize and simplify the very detailed and complex hygiene requirements, which were previously scattered over 17 EU Directives (Rollin et al., 2011).

The need for new integrated approaches to minimize food risks has led to several consequences. Food companies must ensure full traceability of food chains, which is known as *farm-to-fork* procedures. The application of HACCP (Hazard Analysis and Critical Control Points) has increased safety guarantees in production systems, thermal treatments and cold chains, helping to reduce the opportunities for pathogens to gain access to food and/or grow to levels that would pose a risk of infection or toxin production (Falguera, Pagán, & Ibarz, 2011). From the industry managers' viewpoint, the main reasons that have been reported to be motivating for HACCP implementation are the likelihood of future regulation, the marketing value of HACCP certification, the improvement of training schemes for standard operation procedures and the value of HACCP in avoiding potential litigation (Wilcock, Ball, & Fajumo, 2011).

Legislations adopted to improve food safety include public and private standards regarding the characteristics of the final product, production practices, traceability and the legal liability of the supply chain. While public standards are mandatory, private ones may confer competitive advantage due to improved control and increased efficiency. These requirements have forced food industries to adapt their infrastructure, equipment, modes of production, processing and quality management and coordination with other stakeholders (Casey, 2009; Hammoudi, Hoffmann, & Surry, 2009).

But perhaps one of the most important consequences for primary producers has been the proliferation of private standards. These quality guides often stipulate more stringent demands than required by law, setting and monitoring of suppliers, and the advent of such social technologies as supply chain management, risk assessment and logistics (Lowe et al., 2008). GlobalGAP is a retailer-led private sector body that sets Good Agricultural Practice (G.A.P.) standards for agricultural products that was originally established as EurepGAP in 1997. GlobalGAP is the most prominent example of the private governance of food safety and quality and represents a critical case in the study of governance beyond the state and even beyond the structures established by the WTO (Busch & Bain, 2004; Casey, 2009; Henson, 2008). GlobalGAP exerts an enormous influence and control over the European food market, as over 85% of all Western European retailers require this certification (Casey, 2009). Other examples of leading private standard certifications include Tesco's *Nature's Choice*, the Global Food Safety Initiative (GFSI, CIES Business Forum), Walmart's *Ethical Standards Program*, the animal welfare standard established and certified by the Society for the Prevention of Cruelty to Animals (SPCA) in Canada or the business-to-business standards of the International Organization for Standarization (ISO).

The rise in these private standards has resulted in a shift in responsibility for this task to third-party certifiers (TPC). This development is reconfiguring social, political, and economic relations throughout the agents of the agri-food chain, reflecting the growing power of supermarkets to regulate the global system (Hatanaka, Bain, & Busch, 2005; Hatanaka & Busch, 2008). Hobbs (2010) carried out a thorough review about the specifications and potential consequences of all these different private certifications.

Both public and private standards are not only focused on consumers' safety, but also try to embrace ethical and environmental concerns. In this way, public regulations try to protect small producers and low-intensity farming. Small and medium-sized producers and manufacturers may find it more difficult to comply with the product licensing requirements due to their lack of resources associated with the limited number of employees and small facilities (Lowe et al., 2008; Walji & Boon, 2008). Thus, farmers that obtain low-productivity crops, such as those dedicated to organic farming (Backer et al., 2009; Reddy, 2010; Woodhouse, 2010), must be necessarily protected by legislation. Public subsidies are often aimed at the promotion of rural areas with lower productivity (Adamczyk-ojewska, 2004), especially those with higher environmental and landscape value (Torquati, Boggia, Massei, & Bartolini, 2006). Most of these low-productivity farms, including organic ones (Daugbjerg, Tranter, Hattam, & Holloway, 2011; Lakner, 2009; Nieberg, Offermann, & Zander, 2005) would not survive without this help (Breustedt, Latacz-Lohmann, & Tiedemann, 2011; Graaff, Kessler, & Duarte, 2011; Kitsopanidis, 2002; Reddy, 2010). In some cases, these direct payments for organic-production compose up to 20% of the final gross production of farm and about 57% of farm profit (Jonczyk & Kopinski, 2009).

As a consequence, public subsidies and public and private standards affect competition, since they are highly relevant in the trade context, often acting as international trade barriers (Hammoudi et al., 2009; Henson, 2008; Hobbs, 2010). Analyzing their effects requires the perspectives of both public economics and social choice (provision of a socially desirable level of food) and industrial economics (cost efficiency and competition). The increased use of private standards, and especially collective private standards, has fueled an ongoing debate on the substitutability/complementarity of private and public standard. This is an important and complex issue that needs to be analyzed considering both the functioning of markets and safety in the final market, as well as their social and ethical framework. This issue is especially pertinent in the international trade context, as the effects of market distortions resulting from standards may be most severe for primary producers (Busch, 2002; Hammoudi et al., 2009).

From the consumers' point of view, one suggested benefit of the new regulations is that they will be more assured of good quality products and the new product labels will contain more information to allow consumers to make more informed product choices. However, this large amount of information may usually increase consumers' confusion, especially when it is related to health claims. A good example of this situation can be found in the case of natural health products (NHPs). NHPs might be manufactured from plant-based materials or other ingredients also commonly found in foods. The current regulation from Health Canada states that this concept includes vitamins and minerals; herbal remedies; homeopathic medicines; traditional medicines such as traditional Chinese

medicines; probiotics, and other products like *amino acids and essential fatty acids* (Yada, 2010). However, the distinctions and interpretations of what is to be classified as a NHP or as a food are often confusing and unclear (Walji & Boon, 2008).

To solve this problem, regulations on health claims are becoming more rigid every day. New laws are being designed to protect consumers from misleading and false marketing campaigns, and also to protect disruption of innovation (Puspa & Kuhl, 2009). Health claims used in functional foods commercialization must be supported by strong scientific evidence. In Europe, health claims process guidelines are described in the *European Regulation 1924/2006 on Nutrition and Health Claims made on Foods*, in effect since January 2007 (Grunert, Scholderer, & Rogeaux, 2011; Walter, 2008). On the other hand, organic foods production and labeling practices are described by the *Council Regulation (EC) 834/2007* and *the Commission Regulation (EC) 889/2008* (Signorini, Biagi, Nannipieri, Marzotto, & Pandolfi, 2008).

To sum up, it can be stated that public and private standards may have either positive or negative outcomes. Due to the number of roles that they play throughout the agri-food chain, these outcomes include a wide range of issues such as economic, social, environmental, technological and ethical ones (Busch & Bingen, 2006). For this reason, there are several kinds of organizations that develop, modify and enforce standards, some of which may be more restrictive than others (Busch, 2002).

5. Final remarks

Agri-food systems comprise complex technologic, social, economic and environmental linkages that require integrated research approaches. In recent times, consumers' concerns about the social and environmental consequences of their consumption attitudes, as well as the growing preoccupations about living healthier lifestyles, have led to severe changes throughout the whole food chain, including all the agents from the producer to the retailer.

Consequently, food supply chains have had to give response to the feedback signals of market trends. Among those trends, functional and organic products are currently two of the most in-fashion ones, involving strong modifications in the primary production, food processing industry and food-related scientific research. However, there is some evidence pointing to the fact that those trends are the result of successful socially-oriented marketing campaigns, rather than the consequence of a deep and science-based meditation. In addition, the difference in price between conventional and functional/organic food is often too large for consumers to change their shopping habits, and objective and productivity-corrected analyses of environmental benefits of organic farming are not clear. Moreover, since 2009 the economic recession seems to have affected strongly the demand for these trendy products, which are considerably more expensive than their conventional counterparts.

Furthermore, those changes in consumers' demand are leading to important consequences not only in different kinds of food production, but also in rural environment and population. Land use change plays a key role in global environmental change, as well as in the future availability of water and other natural resources. Modeling those changes in land use presents several challenges, particularly in integrating biophysical and socio-economic data and the heterogeneity that these data entail. The success on these challenges will only be possible if future research approaches consider the different aspects such as technical, social, environmental and economic ones as a whole thing, in a similar way to how integrated production systems have met the balance point to optimize food production in farms.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.2.

Title: Edible films and coatings: Structures, active functions and trends in their use.

Authors: Víctor Falguera, Juan Pablo Quintero, Alberto Jiménez, José Aldemar Muñoz & Albert Ibarz.

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TRENDS IN FOOD SCIENCE & TECHNOLOGY

Review

Edible films and coatings: Structures, active functions and trends in their use

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Edible films and coatings are thin layers of edible materials applied on food products that play an important role on their conservation, distribution and marketing. Some of their functions are to protect the product from mechanical damage, physical, chemical and microbiological activities. Their use in food applications and especially highly perishable products such as horticultural ones, is based on some particular properties such as cost, availability, functional attributes, mechanical properties (flexibility, tension), optical properties (brightness and opacity), the barrier effect against gases flow, structural resistance to water and microorganisms and sensory acceptability. In this piece of work, the lastest advances on their composition (polymers to be used in the structural matrix), including nanoparticles addition, and properties have been reviewed, as well as the trends in the research about their different applications, including oil consumption reduction in deep-fat fried products, their use in combination with bioactive compounds that bring foodstuff additional functions and shelf life extension of highly perishable products.

Introduction

An edible coating (EC) is a thin layer of edible material formed as a coating on a food product, while an edible film (EF) is a preformed, thin layer, made of edible material, which once formed can be placed on or between food components (McHugh, 2000). The main difference between these food systems is that the EC are applied in liquid form on the food, usually by immersing the product in a solution-generating substance formed by the structural matrix (carbohydrate, protein, lipid or multicomponent mixture), and EF are first molded as solid sheets, which are then applied as a wrapping on the food product.

The envelope (packaging, wrapping or coating) plays an important role on the conservation, distribution and marketing of foodstuff. Some of its functions are to protect the product from mechanical damage, physical, chemical and microbiological activities. Some studies have recognized the importance of assessing the preformed matrix of edible films in order to quantify various parameters such as mechanical, optical and antimicrobial properties, since this envelope creates a modified atmosphere (MA) restricting the transfer of gases (O_2, CO_2) and also becoming a barrier for the transfer of aromatic compounds (Miller & Krochta, 1997).

Standard packaging technologies can be improved by the incorporation of EC or EF solutions. In a study about maize starch EC mixed with glycerol as a plasticizer and applied to Brussels sprouts (*Brassica oleracea L.* var. Gemmifera), the buds were treated with the solution, stored in polystyrene trays and covered with polyvinyl chloride (PVC) film, preserving the quality parameters regarding different factors such as weight loss, firmness, surface color of the food, commercial acceptability and nutritional quality, because the ascorbic acid content, total flavonoids and antioxidant activity remained constant during 42 days of storage at a temperature of 0 °C (Viña *et al.*, 2007).

The use of EC or EF in food applications and especially highly perishable products such as horticultural ones, is conditioned by the achievement of diverse characteristics-such as cost, availability, functional attributes, mechanical properties (flexibility, tension), optical properties (brightness and opacity), the barrier effect against gases flow, structural resistance to water and microorganisms and

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sensory acceptability. These characteristics are influenced by parameters such as the kind of material implemented as structural matrix (composition, molecular weight distribution), the conditions under which films are preformed (type of solvent, pH, components concentration and temperature) and the type and concentration of additives (plasticizers, cross-linking agents, antimicrobials, antioxidants or emulsifiers) (Guilbert, Gontard, & Gorris, 1996; Rojas-Grau, Soliva-Fortuny, & Martín-Belloso, 2009a).

In this review, recent trends in edible films and coatings are summarized, with emphasis on applications to the horticultural chain and their effects on fresh and minimally processed products. In addition, some biopolymers implemented in the development of new EC and EF have been reviewed, stating the importance of their optimization regarding various parameters such as mechanical properties, microbiological stability, wettability and their ability to be associated with compounds with nutraceutical properties and with various additives that improve sensory attributes in processed fruits and vegetables.

Structural matrix: carbohydrates, proteins and lipids

Edible coatings and films are usually classified according to their structural material. In this way, films and coatings are based on proteins, lipids, polysaccharides or composite. For example, a composite film may consist of lipids and hydrocolloids combined to form a bilayer or a cluster (Krochta, Baldwin, & Nisperos-Carriedo, 1994). In some recent studies the production of edible and biodegradable films by combining various polysaccharides, proteins and lipids is considered with the aim of taking advantage of the properties of each compound and the synergy between them. The mechanical and barrier properties of these films not only depend on the compounds used in the polymer matrix, but also on their compatibility (Altenhofen, Krause, & Guenter, 2009). Table 1 summarizes the main compounds used in EF and EC structural matrices, whose applications will be explained in this section.

The optimization of edible films composition is in one of the most important steps of the research in this field, since they must be formulated according to the properties of the fruits and vegetables to which they have to be applied (Rojas-Grau *et al*., 2009a). Thus, it is very important to characterize and test different coating solutions on fresh and minimally processed food, since each one of them has different quality attributes to be maintained and enhanced during the storage time (Oms-Oliu, Soliva-Fortuny, & Martin-Belloso, 2008a).

Hydrocolloids (proteins and polysaccharides) are the most widely investigated biopolymers in the field of EC and EF. Some of these are: carboxymethylcellulose, casein (Ponce, Roura, del Valle, & Moreira, 2008) and its derivatives (Fabra, Jiménez, Atarés, Talens, & Chiralt, 2009), locust bean gum, guar gum, ethyl cellulose (Shrestha, Arcot, & Paterson, 2003), mesquite gum (Bosquez-Molina, Tomás, & Rodríguez-Huezo, 2010), gelatin supplemented with glycerol, sorbitol and sucrose as plasticizers (Arvanitoyannis, Psomiadou, Nakayama, Aiba, & Yamamoto, 1997; Sobral, Menegalli, Hubinger, & Roques, 2001), composite EF of gelatin-casein cross-linked with transglutaminase (Chambi & Grosso, 2006), pectin (Maftoonazad, Ramaswamy, Moalemiyan, & Kushalappa, 2007), cassava starch with natural antimicrobial compounds (Kechichian, Ditchfield, Veiga-Santos, & Tadini, 2010), pre-gelatinized standard maize starch (Pagella, Spigno, & De Faveri, 2002), wheat gluten (Tanada-Palmu & Grosso, 2005) and mixtures of sodium alginate and pectin, with the addition of $CaCl_2$ as a crosslinker material affecting mechanical properties, water solubility, moisture content, film thickness and its ability to contain calcium (Altenhofen *et al*., 2009).

In the same way, multicomponent or composite EF have been optimized attending to its mechanical properties and transparency, looking for consumers acceptability and for the ability to withstand mechanical stress and handling during the transport. In the pursuit of these aims, the design of response surface methodology has been implemented, in order to determine the optimal mix of components that allows to take advantage of the features of the added substances (Ozdemir & Floros, 2008). However, when lipids are added for improving moisture barrier properties, other features such as transparency can be affected. As an

Table 1. Summary of different compounds used in EF and EC.

Compounds	Reference
Carboxymethylcellulose, casein	Ponce *et al*., 2008
Casein derivates with beeswax and fatty acids	Fabra *et al*., 2009
Locust bean gum, guar gum, ethyl cellulose	Shrestha *et al*., 2003
Mesquite gum	Bosquez-Molina *et al*., 2010
Gelatin with glycerol, sorbitol and sucrose	Arvanitoyannis *et al*., 1997 Sobral *et al*., 2001
Gelatin-casein cross-linked with transglutaminase	Chambi & Grosso, 2006
Pectin	Maftoonazad *et al*., 2007
Cassava starch	Kechichian *et al*., 2010
Pre-gelatinized maize starch	Pagella *et al*., 2002
Wheat gluten	Tanada-Palmu & Grosso, 2005
Sodium alginate and pectin cross-linked with $CaCl_2$	Altenhofen *et al*., 2009
HPMC with fatty acids	Jiménez *et al*., 2010
Beeswax	Morillon *et al*., 2002
Carnauba wax	Shellhammer & Krochta, 1997
Chitosan	Romanazzi *et al*., 2002 No *et al*., 2002 Devlieghere *et al*., 2004 Martínez-Camacho *et al*., 2010 Aider, 2010
Chitosan-gelatin	Arvanitoyannis *et al*., 1997
Maize starch-chitosan-glycerin	Liu *et al*., 2009
HPMC-tea tree essential oil	Sánchez-González *et al*., 2010
Cashew gum	Carneiro-da-Cunha *et al*., 2009 Souza *et al*., 2010
Galactomannans	Cerqueira *et al*., 2009a
Galactomannans-collagen-glycerol	Lima *et al*., 2010

example, hydroxypropylmethylcellulose (HPMC) has been used in combination with fatty acids to obtain composite films with lower water vapor permeability (WVP) and less transparency in comparison with the same film without lipids (Jiménez, Fabra, Talens, & Chiralt, 2010).

Polysaccharides and proteins are great materials for the formation of EC and EF, as they show excellent mechanical and structural properties, but they have a poor barrier capacity against moisture transfer. This problem is not found in lipids due to their hydrophobic properties, especially those with high melting points such as beeswax and carnauba wax (Morillon, Debeaufort, Bond, Capelle, & Volley, 2002; Shellhammer & Krochta, 1997).

To overcome the poor mechanical strength of lipid compounds, they can be used in combination with hydrophilic materials by means of the formation of an emulsion or through lamination with an hydrocolloid film lipid layer. The efficiency of an edible film against moisture transfer cannot be simply improved with the addition of hydrophobic materials in the formulation, unless the formation of a homogeneous and continuous lipid layer inside the hydrocolloid matrix is achieved (Karbowiak, Debeaufort, & Voilley, 2007; Martin-Polo, Mauguin, & Voilley, 1992). In this way, it has been found that fatty acids can form stable layers in sodium caseinate or HPMC matrices, whose properties depend on their chain length: the lower the chain lenght, the greater the layers (Fabra *et al*., 2009; Jiménez *et al*., 2010).

Emulsion-based films are less efficient in controlling water transfer than bilayer films, as a homogeneous distribution of lipids is not achieved. However, they exhibit good mechanical strength and require a simple process for their manufacture and application, whereas multilayer films require a complex set of operations that depend on the number of coatings. It has been proved, in emulsion-based films, that the smaller the particle size or lipid globules and the more homogeneously distributed, the lower WVP (Debeaufort & Voilley, 1995; McHugh & Krochta, 1994; Pérez-Gago & Krochta, 2001). However, its permeability to water vapor can be similar to the values presented by the films based on proteins or polysaccharides (Morillon *et al*., 2002).

Among polysaccharides, bioactive compounds such as chitosan and its derivatives show a great number of applications focused on active coating systems, in view of the increasing concern about the production of poorly biodegradable plastic materials. Chitosan has a vast potential that can be applied in the food industry because of its particular physicochemical properties such as biodegradability, biocompatibility with human tissues, null toxicity and especially its antimicrobial and antifungal properties (Aider, 2010). In addition to research based on its antimicrobial properties, some aspects such as mechanical and thermal properties and permeability to gases (O_2, CO_2) have been quantified, revealing that chitosan-gelatin films plasticized with water and polyols suffer an increase in permeability as the amount of plasticizers in their formulation is increased (Arvanitoyannis *et al*., 1997).

Chitosan is a polysaccharide obtained by deacetylation of chitin, which is extracted from the exoskeleton of crustaceans and fungal cell walls. It has been extensively used in films and coatings due to its ability to inhibit the growth of various bacteria and fungal pathogens (Romanazzi, Nigro, Ippolito, Di Venere, & Salerno, 2002). Chitosan has also been studied in combination with other biopolymers. Films composed of maize starch-chitosan plasticized with glycerin have shown improved mechanical properties (such as elongation at break) and water vapor permeability in contrast to membranes developed with only one of these structural components, as a result of interactions between the hydroxyl groups of starch and the amino groups of chitosan. Its antibacterial activity has been proved observing inhibition zones by disk diffusion on agar containing *Escherichia coli* O157:H7 (Liu, Qin, He, & Song, 2009). New research and recent reviews on the use of chitosan gather some information on the effect of the deacetylation degree on its antimicrobial activity, its use in active coating and its interaction with other components of the treated food products (Aider, 2010; Devlieghere, Vermeulen, & Debevere, 2004; Martínez-Camacho *et al*., 2010; No, Park, Lee, & Meyers, 2002). Besides, EF have been formulated by mixing chitosan with essential oils. Sánchez-González, González-Martínez, Chiralt, and Cháfer (2010) found that chitosan-tea tree essential oil based films were effective against *Listeria monocytogenes*.

Other very interesting hydrocolloids are the gum exuded from the cashew tree (*Anacardium occidentale L*), known as cashew gum, and galactomannans. First edible films based on cashew gum have been evaluated, testing its mechanical properties, wettability, surface tension, opacity, tensile strength, elongation at break and water vapor permeability, in order to obtain biopolymer structures able to generate edible coatings applied to minimally processed fruits. In addition, properties such as wettability and surface tension were quantified by using it as a coating on Golden apples. As a result, it was found that concentrations below 1.5% w/v create fragile films; the addition of Tween80 reduced cohesive forces and therefore decreased surface tension, increasing wettability of the coating solution and thereby improving the compatibility of the EC with the fruit surface (Carneiro-da-Cunha *et al*., 2009). Edible films based on cashew gum were also tested in mango (*Mangifera indica* var. Tommy Atkins) with the aim of determining its effect on the shelf life of refrigerated fresh product. It was determined that it acts as a barrier to mass transport, reducing weight loss as a result of respiration processes (Souza *et al*., 2010).

Galactomannans are hydrocolloids that deserve some interest due to their contribution to strengthen matrix structures. They are stored as reserve polysaccharides and extracted from seeds. Their polymeric structure is mainly influenced by the proportion of mannose/galactose units and the distribution of galactose residues in the main chain

(Cerqueira *et al*., 2009a). *Adenanthera pavonina* and *Caesalpinia pulcherrima*, two plants belonging to the legume family, were recently used to develop coatings from new sources of galactomannans (Lima *et al*., 2010).

In an exhaustive study carried out by Lima *et al*. (2010), different proportions of galactomannans, collagen and glycerol were prepared and tested in order to design possible mixtures with a high degree of wettability, this is having the ability to be easily adhered and homogeneously distributed in mango and apple fruits. With the assayed products and conditions (the films were maintained at 20 °C and 50% relative humidity), it was determined that the best mixes for mango and apple are: 0.5% of galactomannan from *A. pavonina*, 1.5% collagen and 1.5% glycerol, or 0.5% of galactomannan from *A. pavonina*, 1.5% collagen without the addition of glycerol. A lower use of O_2 (28%) and a lower production of CO_2 (11.0%) was achieved in coated mango compared to the control samples (without coating). In apples, the production and consumption of O_2 and CO_2 was approximately 50% lower in the presence of the coating. These results suggest that the galactomannan-based coatings can reduce gas-transfer and thus become useful tools to extend the shelf life of these fruits.

Edible films and coatings and their role as active packages

The development of coatings based on polysaccharides has brought a significant increase in their applications and in the amount of products that can be treated, extending the shelf life of fruits and vegetables due to the selective permeability of these polymers to O_2 and CO_2. Table 2 summarizes some of these compounds and their effects. These polysaccharide-based coatings can be used to modify the internal atmosphere of fruits, delaying senescence (Rojas-Grau *et al*., 2009a). Edible coatings create a passive modified atmosphere, which can influence various changes in fresh and minimally processed foodstuff in some areas such as: antioxidant properties, color, firmness, sensory quality, microbial growth inhibition, ethylene production and volatile compounds as a result of anaerobic processes (Oms-Oliu, Soliva-Fortuny, & Martin-Belloso, 2008b).

The effectiveness of an edible coating to protect fruits and vegetables depends on the control of wettability (Cerqueira, Lima, Teixeira, Moreira, & Vicente, 2009b), on the film ability to maintain the functionality of some compounds (plasticizers, antimicrobials, antioxidants) within the matrix, as the loss of these molecules affects the thickness of the film (Park, 1999), and the solubility in water as it is necessary to avoid the dissolution of the coating (Ozdemir & Floros, 2008).

Although some EF have been successfully applied to fresh products, other applications adversely affected quality. The modification of the internal atmosphere through the use of edible coatings can increase disorders associated with a high concentration of CO_2 or low O_2 (Ben-Yehoshua, 1969). In fresh-cut melon coated with gellan gum a growing increase of phenolic compounds was quantified in response to stress generated by excessive change in the atmosphere of the minimally processed fruit during storage. Although the generation of these substances (phenols) contributed to the antioxidant power, sensory properties such as odor, color and flavor were affected. Translucent tissue was also observed, which appeared to be a symptom of senescence (Oms-Oliu *et al*., 2008a). When a gas barrier is created, an increase in the presence of some volatiles associated with anaerobic conditions can be induced. This is the case of ethanol and acetaldehyde, which were detected after two weeks of storage in apple slices treated with alginate and gellan gum EC. The production of these substances is related to anaerobic fermentation, to a decrease of sensory quality and especially to the loss of minimally processed fruit flavors (Rojas-Grau, Tapia, & Martín-Belloso, 2008). Therefore, it is clear that the control of gas permeability should be a priority in the development and study of active coatings (Parra, Tadini, Ponce, & Lugão, 2004).

Edible films and coatings with antimicrobial properties have innovated the concept of active packaging, being developed to reduce, inhibit or stop the growth of

Table 2. Summary of different components of EF and EC used as active packages.

Components	Effect	Reference
Gellan gum	Increase of phenolics	Ben-Yehoshua, 1969
Alginate and gellan gum	Gas permeability modification	Rojas-Grau *et al*., 2008
Sorbic acid, benzoic acid, sodium benzoate, citric acid	Antimicrobial	Quintavalla & Vicini, 2002
Potassium sorbate	Antimicrobial	Ozdemir & Floros, 2008
Nicines, pediocin	Antimicrobial	Sebti & Coma, 2002
Natamycin in a chitosan matrix	Antimicrobial	Durango *et al*., 2006 Ribeiro *et al*., 2007 Fajardo *et al*., 2010 Maqbool *et al*., 2010
Tea tree essential oil in HPMC matrix	Antimicrobial	Sánchez-González *et al*., 2009
Chitosan	Antimicrobial	El Ghaouth *et al*., 1992 Coma *et al*., 2002 Ponce *et al*., 2008 Kyu Kyu *et al*., 2007 Maqbool *et al*., 2010
Chitosan	Shelf life extension	Lazaridou & Biliaderis, 2002 Geraldine *et al*., 2008 Márquez *et al*., 2009
Chitosan-oleic acid	Shelf life extension	Vargas *et al*., 2006
Chitosan	Tissue firmness conservation	El Gaouth *et al*., 1997
Chitosan	Respiration rate reduction	Li & Yu, 2000
Chitosan	Fungistatic	Martínez-Camacho *et al*., 2010
Essential oils	Antimicrobial and antioxidant	Atarés *et al*., 2010 Sánchez-González *et al*., 2010

microorganisms on food surfaces (Appendini & Hotchkiss, 2002). In most fresh or processed products microbial contamination is found with the highest intensity on their surface. Therefore, an effective system to control the growth of that biota is required (Padgett, Han, & Dawson, 1998). Traditionally, antimicrobial agents are directly added to foods, but their activity can be inhibited by different components of these products, decreasing its efficiency. In such cases, the implementation of films or coatings can be more efficient that antimicrobial additives used in the foodstuff, since they can migrate selectively and gradually from the wrapping compounds to the surface of the food (Ouattara, Simard, Piette, Bégin, & Holley, 2000).

Antimicrobial EC and EF have been shown to be an efficient alternative in the control of food contamination. Spoilage and pathogens can be reduced by incorporating antimicrobial agents into edible films and coatings (Sorrentino, Gorrasi, & Vittoria, 2007). Some of these compounds included into EF and EC are sorbic acid, benzoic acid, sodium benzoate, citric acid (Quintavalla & Vicini, 2002), potassium sorbate (Ozdemir & Floros, 2008), and bacteriocins such as nicin or pediocin (Sebti & Coma, 2002), or even natamycin in a chitosan EC, which had the ability to release the compound and synergistically prevent the growth of molds and yeasts (Durango, Soares, & Andrade, 2006; Fajardo *et al*., 2010; Maqbool, Ali, Ramachandran, Smith, & Alderson, 2010; Ribeiro, Vicente, Teixeira, & Miranda, 2007). Additionally, hydrophobic compounds such as tea tree essential oil in HPMC based films have also been used (Sánchez-González, Vargas, González-Martínez, Chiralt, & Cháfer, 2009).

As it has been already mentioned, chitosan is a polysaccharide that has been used in films and coatings due to its ability to inhibit the growth of various microbial pathogens. In some fungi, chitosan can cause alterations in membrane function, through its strong interaction with the electronegative surface charge, leading to permeability changes, metabolic disturbances and even death (Fang, Li, & Shih, 1994). According to Muzzarelli *et al*. (1990), the antimicrobial activity of chitosan against bacteria could be due to the polycation nature of the molecule, which enables interaction and formation of polyelectrolyte complexes with acidic polymers produced in the surface of the bacterial cell (as lipopolysaccharides or teichoic acid). Chitosan-based coatings and films tested on *L. monocytogenes* showed inhibitory effect on the growth of this bacteria (Coma *et al*., 2002; Ponce *et al*., 2008). Other studies have shown that chitosan-based coatings have the potential to increase the shelf life of fruits and vegetables by inhibiting the growth of microorganisms, reducing ethylene production, increasing the concentration of carbon dioxide and reducing oxygen levels (Geraldine, Ferreira, Alvarenga, & Almeida, 2008; Lazaridou & Biliaderis, 2002; Márquez, Cartagena, & Pérez-Gago, 2009). Furthermore, chitosan-oleic acid based coatings are able to increase significantly the shelf life of cold-stored strawberries as it have been studied by Vargas, Albors, Chiralt, and González-Martínez (2006).

This hydrocolloid (chitosan) has the ability to slow the growth of certain microorganisms that are deleterious in fruit postharvest such as *Fusarium* spp., *Colletotrichum musae* and *Lasiodiplodia theobromae* in banana (*Musa acuminate* L. Var. Kluai Hom Thong) (Kyu Kyu, Jitareerat, Kanlayanarat, & Sangchote, 2007; Maqbool *et al*., 2010), or *Botrytis cinerea* on pepper (*Capsicum annuum* L. Var. Bellboy). In this case, mold suffered cell damage in invading hyphae and reduced the production of polygalacturonase, which has an effect in maintaining the firmness of the tissues (El Gaouth, Arul, Wilson, & Benhamou, 1997). Its activity has also been reported in fruits of peach (*Prunus persica* L. Batsch.), reducing the respiration rate represented in the production of CO_2 and maintaining the firmness of the fruit covered until the end of 12 days of storage at a temperature of 23 °C (Li & Yu, 2000). Moreover, El Ghaouth, Ponnampalam, Castaigne, and Arul (1992) showed that the coatings with chitosan content between 1% and 2% reduced the incidence of deterioration in tomato mainly caused by *Botrytis cinerea*. In addition, some studies suggest that chitosan shows fungistatic activity even if it is used inside a preformed film matrix. Some factors such as storage temperature and changes in the mechanical and barrier properties influenced by additives and other types of antimicrobials can promote antimicrobial effect of these films (Martínez-Camacho *et al*., 2010).

Nowadays edible films have different applications, and their use is expected to be expanded with the development of Active Coating Systems. This second generation of coating materials can use chemicals, enzymes or microorganisms that prevent, for example, microbial growth or lipids oxidation in coated food products. In this sense essential oils, in combination with structural polymers, can be a promising source since different pieces of work have constituted the evidence of their effectiveness as antimicrobial and antioxidant compounds (Atarés, Bonilla, & Chiralt, 2010; Sánchez-González *et al*., 2010). Coatings of second generation may contain nutrients or other bioactive compounds that have a positive effect on health, especially due to the application of new microencapsulation or nanoencapsulation techniques. In this way, coating materials would act as carriers of these bioactive compounds to be transported to target sites such as the intestine without losing its activity, being within a matrix during its passage through the gastrointestinal tract (Korhonen, 2005).

Effect of edible films and coatings in food browning and polyphenol oxidase activity

In food products, not only microbiological stability plays an indispensable role in its quality, but also sensory aspects are essential to ensure that the application of emerging technologies such as edible films and coatings become successful (Rojas-Grau, Oms-Oliu, Soliva-Fortuny, & Martín-Belloso, 2009b). Thus, color is one of the most

important parameters that must be controlled, and enzymatic browning is the main process that modifies it. Polyphenol oxidase (PPO) is the main enzyme responsible for these changes in vegetable tissues that contain phenolic or polyphenolic molecules. It catalyzes the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of*o*-diphenols to o-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of an heterogeneous group of melanins (Falguera, Gatius, Pagan, & Ibarz, 2010).

Some researchers have proved the effectiveness of edible films and coatings on the control of browning processes and polyphenol oxidase activity. Vangnai, Wongs-Aree, Nimitkeatkai, and Kanlayanarat (2006) applied chitosan coatings on "Daw" longan (*Dimocarpus longan* Lour.) fruits, finding that these treatments reduced increasing activities of PPO during the 20 days of storage at 4 °C, slightly reducing pericarp browning. Chitosan coatings were also used by Eissa (2008), who found that they delayed discoloration associated with reduced enzyme activity of PPO and other enzymes, and had a good effect on the evolution of colour characteristics and parameters of fresh-cut mushroom during storage at 4 °C. Ponce *et al.* (2008) applied chitosan films enriched with olive and rosemary oleoresins on pumpkin (*Cucurbita moschata* Duch) slices, which showed a clear antioxidant effect by slowing the action of polyphenol oxidase (PPO) and peroxidase (POD) within five days of storage. In addition, these edible coatings showed no deleterious effects on the sensory acceptability of the pumpkin juice.

Hui-Min, To, Li-Ping, and Hai-Ying (2009) investigated the effects of three kinds of edible coatings (carrageenan, carboxymethyl cellulose (CMC) and sodium alginate) and their combinations on browning parameters of fresh-cut peach (*Prunus persica*) fruits during storage at 5 °C. Sodium alginate coating and the various composite ones reduced the declines of Hunter L^* value and the increases of Hunter a^* and Hunter b^* values, inhibited PPO activity and reduced the browning degree of peach fruits.

Zhang, Xiao, Luo, Peng, and Salokhe (2004) applied combinations of an ozone water treatment and different coatings on minimally processed cucumber (*Cucumis sativus* L.). The study showed that a concentration of 4.2 mg m^{-3} ozone and a composite coating made of polyvinyl alcohol 134 (1%), chitosan (1%), lithium chloride (0.5%), glacial acetic acid (2.5%) and sodium benzoate (0.05%) inhibits respiration, chlorophyll breakdown and polyphenol oxidase activity.

Edible films as a matrix of nanobiocomposites

Different methods to improve the properties of biopolymer-based films such as lipids or antimicrobial components addition have been mentioned. In addition, a novel technique based on the use of very small particles has become remarkable in food developments recently.

Nowadays nanotechnology is applied with great results in many research areas. One of these fields of application is polymer research. A nanoparticle is an ultrafine particle in the nanometer size order (Hosokawa, Nogi, Makio, & Yokoyama, 2008), which is able to form nanobiocomposite films when it is combined with natural polymers. The research and development of nanobiocomposite materials for food applications is expected to grow with the advent of new polymeric materials with inorganic nanoparticles, although it is not widely widespread yet (Restuccia *et al.*, 2010; Sorrentino *et al.*, 2007). Some of the applications associated with nanotechnology include improved taste, color, flavor, texture and consistency of foodstuffs, increased absorption and bioavailability of food or food ingredients (nutrients), and the development of new food-packaging materials with improved mechanical, barrier and antimicrobial properties (Restuccia *et al.*, 2010).

Traditionally, mineral fillers such as clay, silica and talc have been incorporated in film preparation in the range of 10–50% w/w in order to reduce its cost or to improve its performance in some way (Rhim & Ng, 2007). Thus, the most important nanoparticles that have been used to provide enhanced properties to edible films are clays. According to Rhim and Ng (2007), the nanometer-size dispersion of polymer-clay nanocomposites exhibit the large-scale improvement in the mechanical and physical properties compared with pure polymer or conventional composites. Both proteins (Shotornvit, Rhim, & Hong, 2009) and polysaccharides (Casariego *et al.*, 2009; Tang, Alavi, & Herald, 2008) have given rise to films in combination with nano-clay particles. However, other nanoparticles such as tripolyphosphate-chitosan (De Moura *et al.*, 2009), microcrystalline cellulose (Bilbao-Sáinz, Avena-Bustillos, Wood, Williams, & McHugh, 2010) and silicon dioxide (Tang, Xiong, Tang, & Zou, 2009) have also been added to biopolymers to obtain films. These nanoparticles are able to improve moisture barrier properties (Casariego *et al.*, 2009; De Moura *et al.*, 2009; Shotornvit *et al.*, 2009) and restrict microbial growth (Shotornvit *et al.*, 2009). In this way, Rhim, Hong, Park, and Perry (2006) found that the use of nanoparticles has a potential application in the development of natural biopolymer-based biodegradable packaging materials. In this study different nanoparticles improved the physical properties of chitosan-based films as well as showed promising antimicrobial activity. Regarding optical properties, these were more or less affected depending on the nano-clay type as it has been observed in isolated whey protein based films (Shotornvit *et al.*, 2009). In addition, nanoparticles have also been added to conventional polymers such as EVOH (Cabedo, Giménez, Lagarón, Gavara, & Saura, 2004) or PP/HDPE (Chiu, Yen, & Lee, 2010).

Trends in the use of edible films and coatings

The properties that have been reviewed have given edible films and coatings several uses. Nowadays, some of the

research lines involving these active envelopes include oil consumption reduction in deep-fat fried products, transport of bioactive compounds and shelf life extension of highly perishable products.

Oil consumption reduction in deep-fat fried products

Deep-fat frying is a widely used method in the preparation of tasty food with an attractive appearance. The tenderness and humidity of the inner part of these products combined with a porous crunchy crust provides an increase in palatability that is responsible for their great acceptance. However, fried foods have a significant fat content, reaching, in some cases, 1/3 of the total weight of the product. The development of more acceptable products for consumers, who are increasingly more conscious and concerned about their health, has led to the need to reduce oil incorporation during the frying process (Freitas *et al*., 2009). Some hydrocolloids with thermal gelation or thickening properties, such as proteins and carbohydrates, have been tested on the migration of oil and water (Debeaufort & Voilley, 1997; Williams & Mittal, 1999). Various coating options are being studied for the reduction of oil incorporation during frying, such as alginate, cellulose and its derivatives, soy protein isolate, whey protein, albumin, corn, gluten and pectin (Albert & Mittal, 2002; Khalil, 1999; Mallikarjunan *et al*., 1997; Mellema, 2003; Salvador, Sanz, & Fiszman, 2005).

Research with mashed potato spheres coated with zein, hydroxypropylmethylcellulose (HPMC) and methylcellulose (MC) has reported a decrease in food moisture of 14.9, 21.9 and 31.1% and in fat consumption of 59.0, 61.4, and 83.6%, respectively (Mallikarjuna, Chinnan, Balasubramaniam, & Phillips, 1997). Other studies have also shown that MC films have better barrier properties against fat absorption than hydroxypropylcellulose (HPC) and gellan gum ones (Williams & Mittal, 1999).

García, Ferrero, Bértola, Martino, and Zaritzky (2002) used MC and HPMC in the formulation of coatings applied to potato chips (0.7 × 0.7 × 5.0 cm) and wheat flour discs (3.7 cm diameter × 0.3 cm high), which were submerged in the coating suspension for 10 s and fried immediately. The most effective coatings were 1% MC and 0.75% sorbitol for wheat flour discs and 1% MC and 0.5% sorbitol for potato chips, reducing the oil consumption to 35.2% and 40.6% respectively. The use of coatings did not have a significant impact on the sensory quality, according to the group of panelists.

Albert and Mittal (2002) carried out an extense piece of work comparing eleven hydrocolloid materials including gelatine, gellan gum, k-carrageenan-konjac-blend, locust bean gum, methyl cellulose (MC), microcrystalline cellulose, three types of pectin, sodium caseinate, soy protein isolate (SPI), vital wheat gluten and whey protein isolate (WPI), as well as some composite films made of different combinations of these compounds. Two of them, SPI/MC and SPI/WPI composite coatings, provided the highest index *reduction in fat uptake/decrease of water loss* value, and reduced the fat uptake up to 99.8%.

Singthong and Thonkaew (2009) investigated the influence of sodium alginate, carboxyl methyl cellulose (CMC) and pectin on the oil absorption in banana chips. The uncoated control sample had an oil consumption of 40 g/100 g sample, while lower values were obtained for banana chips blanched in 0.5% $CaCl_2$ and treated with a coating matrix of 1% pectin or 1% CMC. Using these two coatings oil consumption was reduced to 22.89 and 22.90 g/100 g of sample, respectively.

Freitas *et al*. (2009) investigated the effect of edible coatings from pectin, whey protein and soy protein isolate in the deep-fat frying of preformed products made of cassava flour and cassava puree. Whey protein coating was the most effective one regarding fat absorption due to its thickness, achieveing a 27% reduction.

Otherwise, EC can improve the crispness of fried products by reducing the moisture diffusion between fish meat and the crust during reheating in a microwave as it have been observed by Chen *et al*. (2008). This experience was based in the thermal gelation of HPMC which occurs at high temperature.

Transport of bioactive compounds

Consumers require fresh and minimally processed foods that are exempt from chemically synthesized substances, and look for those enriched with natural substances that bring health benefits and maintain nutritional and sensory characteristics (Falguera, Pagan, & Ibarz, 2011). Therefore, in recent times the efforts of researchers have been focused on searching for new naturally occurring substances that act as possible alternative sources of antioxidants and antimicrobials (Ponce *et al*., 2008).

Rojas-Grau, Tapia, Rodríguez, Carmona, and Martin-Belloso (2007) proved the ability of edible coatings based on sodium alginate and gellan gum to transport N-acetylcysteine and glutathione as antibrowning agents, besides the positive effect of the addition of vegetable oils in these edible coatings to increase resistance to water vapor transport in minimally processed fruits of Fuji apple. Moreover, it was also stated that the coatings were able to keep the vegetable oil enriched with essential fatty acids (ω3 and ω6) encapsulated.

Biodegradable films based on cassava starch (*Manihot esculenta* Crantz) have been characterized from some points of view, including mechanical properties, the effect of various plasticizers such as glycerol and polyethylene glycol and cross-linkers as glutaraldehyde or $CaCl_2$ on water vapor transmission, and their possible use in the food industry because this hydrocolloid is abundant and cheap (Parra *et al*., 2004; Ribeiro *et al*., 2007). At present, studies have been guided to the ability of these films to transport natural antimicrobial agents such as chitosan (Vásconez, Flores, Campos, Alvarado, & Gerschenson, 2009).

As it has been already introduced, sensory aspects are very important in the evaluation of films and coatings applications. In order to slow changes in flavor during food storage, the encapsulation of aromatic compounds has

been implemented as a possible strategy to reduce the effect of degrading reactions such as oxidation. Marcuzzo, Sensidoni, Debeaufort, and Voilley (2010) encapsulated 10 different aromatic compounds in carrageenan films, including ethyl acetate, ethyl butirate, ethyl isobutyrate, ethyl hexanoate, ethyl octanoate, 2-pentanone, 2-heptanone, 2-octanone, 2-nonanone and 1-hexanol. Carrageenan films were appropriate to conduct these experiments because they show high affinity for polar volatile compounds. These EF may achieve the aim of gradually releasing aroma compounds and thereby maintain the sensory characteristics such as aroma and taste for certain periods of time.Furthermore, Hambleton, Debeaufort, Bonnotte, and Voilley (2009) proved that matrices made of other polysaccharides such as alginate are able to protect an encapsulated aroma compound (n-hexanal), due to its low oxygen permeability.

On the one hand, according to the mentioned studies it can be concluded that polysaccharide matrices are able to encapsulate aroma compounds in order to maintain the organoleptic quality in food systems. On the other hand, proteins have been less studied as protective polymers for aroma components, maybe due to its minor effectivity for this purpose. In this sense, Monedero *et al*. (2010) found that it was necessary to add beeswax to improve the capacity of soy protein isolate based films to retain n-hexanal.

The transport and release of various active compounds (antioxidants, flavorings, antibrowning and antimicrobial compounds, vitamins or enzymes) is one of the most important aspects within the features of edible films and coatings. Nowadays, trends in research consider the use of nanotechnology solutions, previously reviewed, using encapsulated nanoparticles of functional and bioactive compounds, which can be released from the matrix that contain them in a controlled pace (Rojas-Grau *et al*., 2009a).

Shelf life extension of highly perishable products

One of the most important uses of edible films and coatings is focused on the shelf life extension of horticultural products. Consequently, there are many pieces of work investigating the application of different coatings on different foodstuff, some of which are reviewed in this section.

Ribeiro *et al*. (2007) studied the ability of edible coatings based on polysaccharides (starch, carrageenan and chitosan) to extend the shelf life of strawberry fruits (*Fragaria ananasa* cv. Camarosa) and its possible industrial application. The best wettability was achieved with combinations of 2.0% starch and 2.0% sorbitol, 0.3% carrageenan, 0.75% glycerol and 0.02% Tween 80 or 1.0% chitosan and 0.1% Tween 80. The oxygen permeability of carrageenan films was approximately 40.0% of the value obtained with starch ones. The values of the fruit firmness loss were the lowest ones in carrageenan films with added calcium chloride. The minimum mass loss was achieved in edible coatings based on carrageenan and chitosan with added calcium chloride. The lower microbial growth rate was observed in strawberries coated with chitosan and calcium chloride.

Carrot is one of the most popular vegetables, but its marketing is limited by its rapid deterioration during storage, mainly due to physiological changes that reduce its shelf life. The product suffers a loss of firmness, with the production and release of a characteristic odor generated by anaerobic catabolism, due to high respiration rate and microbial spoilage (Barry-Ryan, Pacussi, & O'Beirne, 2000). Durango *et al*. (2006) developed coatings based on yam (*Dioscorea* sp.) starch and chitosan. The maximum antimicrobial activity was obtained in the EC containing 1.5% of chitosan, which was completely effective on the growth of molds and yeasts reducing the count by 2.5 log units in the carrot sticks that were stored for 15 days. Coating with a chitosan concentration of 0.5% controlled the growth of molds and yeasts for the first 5 days of storage. After this time, tested samples generated a count similar to the one of the control sample. Thus, the use of antimicrobial coatings based on chitosan and yam starch significantly inhibited the growth of lactic acid bacteria, total coliforms, psychrotrophic microorganisms, mesophilic aerobes, molds and yeasts. Subsequently, Pastor, Sánchez-González, Cháfer, Chiralt, and González-Martínez (2010) obtained films based on HPMC and ethanolic extract of propolis which are effectively against *Aspergillus niger* and *Penicillium italicum*. These films appeared yellowish, which can restrict their use on different foodstuff but not in carrots or oranges, where their shade would mask films colour.

Maqbool *et al*. (2010) applied edible coatings based on arabic gum, 95% deacetylated chitosan and arabic gum + chitosan composite films on fresh banana fruits, in order to determine their potential in the control of *Colletotrichum musae*. This fungus causes anthracnose, a disease that affects postharvest quality in transport and storage of bananas. *In vivo* tests determined that composite EC formed by 10% arabic gum and 1% chitosan was the best treatment, because it had the lowest disease incidence (16%). In addition, the composite film reduced the percentage of weight loss, retained fruit firmness during and after storage and marketing conditions compared to control samples, minimizing moisture loss. Arabic gum + chitosan edible coating showed a synergistic behavior that allowed maintaining sensory quality and microbiological parameters, without phytotoxic effects on bananas stored for 33 days.

Edible films and coatings: commercial and regulatory aspects

The commercial use of edible films has been limited due to problems related to their poor mechanical and barrier properties when compared to synthetic polymers (Azeredo *et al*., 2009). As it has been explained, several nanocomposites have been developed by adding reinforcing compounds (nanofillers) to biopolymers, improving their properties and enhancing their cost-price-efficiency (Sorrentino *et al*., 2007). However, there are many safety concerns about

nanomaterials, as their size may allow them to penetrate into cells and eventually remain in the human organism. While the properties and safety of the materials in their bulk form are usually well known, the nano-sized counterparts frequently exhibit different properties from those found at the macro-scale, and there is limited scientific data about their eventual toxicological effects. So the need for accurate information on the effects of nanomaterials on human health following chronic exposure is imperative before any nanostructured food packaging is available for commercialization.

Anyway, several authors have stated that the use of edible films and coatings is expected to grow, in part due to the growing trend for individualized portion size, which has made packaging-per-unit to increase. In addition, their functions fall entirely into "green-packaging" applications, such as the US EPA suggested plan for improved municipal waste management and reduction (Dangaran, Tomasula, & Qi, 2009). In order to reduce the initial amount of packaging, the EPA suggests designing packaging systems that reduce the amount of environmentally toxic materials used in packaging to make it easier to reuse or compost them. They also suggest packaging that reduces the amount of damage or spoilage to food products, increasing their shelf life. Edible films and coatings fit both criteria.

In Europe, the European framework regulation (2004/1935/EC) authorizes the concept of active packaging with intentional active agents' release (Guillart *et al*., 2009). With the formation of the European Union, legislation of all member states was harmonized in order to create a single market and overcome barriers to trade. So far, the EU legislation on materials in contact with food products has protected the health of consumers by ensuring that no material in contact with foodstuffs can bring about a chemical reaction that would change their composition or organoleptic properties. Regulation 1935/2004/EC repeals this legislation in order to allow packaging to benefit from technological innovation. This was necessary in the EU because all packaging materials (including those that intentionally add substances to food) are subject to all requirements for food-contact materials, including the overall migration limits (OMLs) and specific migration limits (SMLs) (Restuccia *et al*., 2010).

Regarding the compounds that can be incorporated into edible coating formulations, these ingredients are majorly regarded as food additives and are listed within the list of additives for general purposes, although pectins, Acacia and karaya gums, beeswax, polysorbates, fatty acids, and lecithin are mentioned apart for coating applications. The use of these coating forming substances is permitted provided that the 'quantum satis' principle is observed (Rojas-Grau *et al*., 2009a). In addition, the Directive 2008/84/EC introduces specific purity criteria for food additives. Since edible coatings could have ingredients with a functional effect, inclusion of these compounds should be mentioned on the label.

Conclusions

Edible films and coatings applied to fresh, minimally processed and processed fruits and vegetables are effective in extending their shelf life, maintaining their microbiological, sensory and nutritional quality. Some formulations have been specifically tested on their ability to inhibit polyphenol oxidase activity and delay browning reactions. In addition, EF and EC are able to transport substances that bring some benefits not only for food itself but also for the consumer, through the encapsulation of bioactive compounds, developing new products with nutraceutical or functional effect.

The most important properties to be evaluated in an edible coating are its microbiological stability, adhesion, cohesion, wettability, solubility, transparency, mechanical properties, sensory and permeability to water vapor and gases. Knowing these properties, their composition and behavior may be predicted and optimized.

Nowadays, trends in the use of these active envelopes include oil consumption reduction in deep-fat fried products, transport of bioactive compounds and shelf life extension of highly perishable products. Thus, research in this field aims at the characterization of new hydrocolloid films based on non-conventional sources, as well as at the determination of the ability of these compounds to release molecules with specific functions such as vitamins, antioxidants, natural colors, flavors, aromatics and assess the interactions that can provide these molecules with the encapsulation matrix.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.3.

Title: Ultraviolet processing of liquid food: A review. Part 1: Fundamental engineering aspects.

Authors: Víctor Falguera, Jordi Pagán, Salvador Garza, Alfonso Garvín & Albert Ibarz.

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Review

Ultraviolet processing of liquid food: A review. Part 1: Fundamental engineering aspects

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ABSTRACT

Ultraviolet irradiation constitutes an alternative to thermal treatment that is being studied and developed to obtain a better final product sensory quality, but without neglecting microbial safety. Critical decisions about the development of ultraviolet irradiation equipment include radiation sources, reactor geometry, reaction medium properties and the relationship between all these parts that will lead to the achievement of the desired effect on food systems and their components. In this piece of work, engineering aspects of ultraviolet irradiation such as radiation sources, different kinds of photochemical reactors and mathematical modeling of the different geometries have been reviewed.

Contents

1. Introduction

1.1. Problems in liquid food manufacturing

Food safety is one of the most important issues that food industries and food service companies have to face. Currently, the growing demand for a greater variety of prepared food and dishes on a menu makes the risk of contamination increase, especially due to the characteristics of this kind of food that gives a big importance to the quickness of meals. The application of HACCP (Hazard Analysis and Critical Control Points) has increased security guarantees in production systems, thermal treatments and cold chains, helping to reduce the opportunities for pathogens to gain access to food and/or grow to levels that will pose a risk of infection or toxin production. However, the incidence of foodborne diseases continues to rise in most industrialized countries (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000; Falguera, Pagán, & Ibarz, 2011).

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The elaboration and processing of liquid food like fruit juices have to be carried out under hygienic conditions that ensure final product safety. Nevertheless, in some cases there can be compounds with adverse effects on health that are not eliminated with conventional processes, such as mycotoxins, which come from mold-contaminated pieces of fruit. In the other hand, depending on the kind of juice, the presence of enzymes may represent important problems in its resulting quality. In this way, polyphenol oxidase (PPO) is the enzyme responsible for enzymatic browning, transforming phenolic compounds into *o*-quinones, which result in melanins after a polymerization stage. Moreover, other dark compounds, melanoidins, can appear in fruit juices after non-enzymatic browning by Maillard reaction. Both melanins as melanoidins have a negative effect on final quality of the juice.

Apart from PPO, other enzymes with a great influence on juice quality are amylases and pectic enzymes. In the production of clarified and depectinated juices it is essential to leave these enzymes to act in order to eliminate pectin and starch. Nevertheless, in purees and juices with suspended pulp, it is very important to inactivate them to avoid the cloud loss in the product. If they are not completely inactivated, in the storage stage they may have a residual activity on pectic fractions resulting in sedimentation of the pulp, due to the destabilization of the system. Thus, in this kind of product it is imperative to ensure that there is no enzymatic activity, as it may adversely affect the quality of the juice.

1.2. Non-thermal technologies and UV irradiation

Treatments on food products are carried out to extend its shelf life, but above all to ensure its safety for consumers. Thermal technologies are the most widely applied to achieve these purposes, but these treatments have a negative effect on certain components of the food itself, reducing its vitamin content and other nutrients, as well as sensory features that make them less attractive in terms of color and textural properties. Non-thermal technologies are an alternative to thermal treatment that is being studied and developed in order to obtain a better final product sensory quality, but without neglecting microbial safety. In this way, these alternatives to thermal technologies can produce food products without enzymes and harmful microorganisms, while maintaining nutritional characteristics and minimizing the loss of quality in terms of flavor, color and nutritional value. One of these innovative technologies is ultraviolet irradiation (Butz & Tauscher, 2002; Noci, Riener, Walking-Ribeiro, Cronin, Morgan, & Lyng, 2008).

The light radiation has to be absorbed to carry out a photochemical change; therefore, the kind of light source to be used is determined by the absorption spectrum of the reactants. To study the effect of radiation on a compound, the specific absorption spectrum at different wavelengths has to be known. This spectrum can be obtained irradiating a solution with a well-known concentration of the compound at different wavelengths, and measuring its resulting absorbance. This absorption spectrum usually presents maximum absorbance peaks, which determine the optimum area of working wavelengths (Ibarz & Esplugas, 1989; Ibarz, Esplugas, & Graell, 1985).

Industrial-scale reactions are often carried out and controlled by means of catalysis or addition of energy. It can be supplied in form of radiant energy, with a special interest in the area of the electromagnetic spectrum that includes the ultraviolet (200–400 nm) and the visible (400–700 nm) regions, in which the addition of this energy produces electronically excited molecules, which can lead to chemical reactions.

2. Radiation sources

In the electromagnetic spectrum, ultraviolet light occupies a wide band of wavelengths in the non-ionizing region, including the ones between 200 nm (X-rays) and 400 nm (visible light). The UV spectrum can be divided into three regions: short-length UV light (UVC) with wavelengths in the range of 200–280 nm, 2) medium-length UV light (UVB) with wavelengths in the range 280 to 320 nm, 3) full-length UV light (UVA) with wavelengths in the range of 320–400 nm.

Unfortunately, there is no source that works efficiently for all photochemical reactions, and the suitable one has to be chosen in each situation. Since the light must be absorbed to induce a photochemical change, it is necessary to use a source that emits radiation in the area of the absorption spectrum of the reactants, which will cause the desired photochemical change. Therefore, it is extremely interesting to know the absorption spectrum of the reactants in the moment of the radiation source election. Besides having a great emission power in the desired wavelengths, the main characteristics which must have a radiation source for an industrial use are: emission stability, long life, good physical dimensions, ease of operation and low cost. The following section briefly describes the most important radiation sources.

2.1. Natural sources

The Sun is the most important natural radiation source, with a spectrum distribution that includes wavelengths from 250 to 1200 nm. The most interesting radiation section is the one that reaches the Earth's outer layers. Solar radiation includes a wide range of wavelengths, whose spectral distribution varies depending on the considered area (longitude, latitude, height, weather conditions, etc.). For most of the possible photochemical reactions, the energy flow that reaches the sea level is very small (nearly 0.1 W/cm^2) and has a spectral distribution with too much infrared radiation, which only has a heating effect and is not able to produce chemical changes. At sea level, approximately 9% of the radiation corresponds to the ultraviolet region, 42% to the visible one and 49% to the infrared one. Therefore, it may be noted that there is a large amount of infrared radiation ("useless") that reaches sea level, in comparison with the low amount of ultraviolet one ("useful").

The Sun is the first UV radiation source that can be considered, since it emits over a wide range of wavelengths. Nevertheless, the fraction corresponding to UV radiation that reaches the Earth's surface depends largely on its attenuation through the atmosphere. Fig. 1A shows the difference between the solar spectrum above the atmosphere and the one that reaches sea level. The UVC fraction is completely absorbed in the upper and middle parts of the atmosphere, due to the presence of ozone and molecular oxygen. With the middle wavelength region (UVB) it happens almost the same, although a small fraction of it reaches the Earth's surface. However, UVA light is hardly affected. Some authors consider that the flux reaching the sea level is in a range from 35 to 50 W/m^2 (Bintsis et al., 2000; Kramer & Ames, 1987).

2.2. Artificial sources

Before of the presentation of artificial sources, it should be mentioned that there are filters of radiation, consisting of special glasses or liquid solutions, which absorb radiation of certain wavelengths and can eliminate some unwanted ones. As artificial sources, there are some kinds of lamps usually with different mercury vapor pressure, which allows obtaining different emission spectra. These lamps are classified according to this emission spectrum and whether the predominating wavelengths are short, medium or long ones.

Lamps are artificial radiation sources with the greatest interest to be used in photochemical reactions. The use of radioactive waste presents lower interest. The main kinds of lamps are (Rabek, 1982):

- Incandescent lamps, consisting of tungsten filaments 3000–3400 K, which emit radiation in continuous in accordance with the laws of radiation emission for black surfaces. Most of the radiation they emit is the infrared one; less than 30% is visible and the little amount of ultraviolet radiation is filtered through the glass envelope.

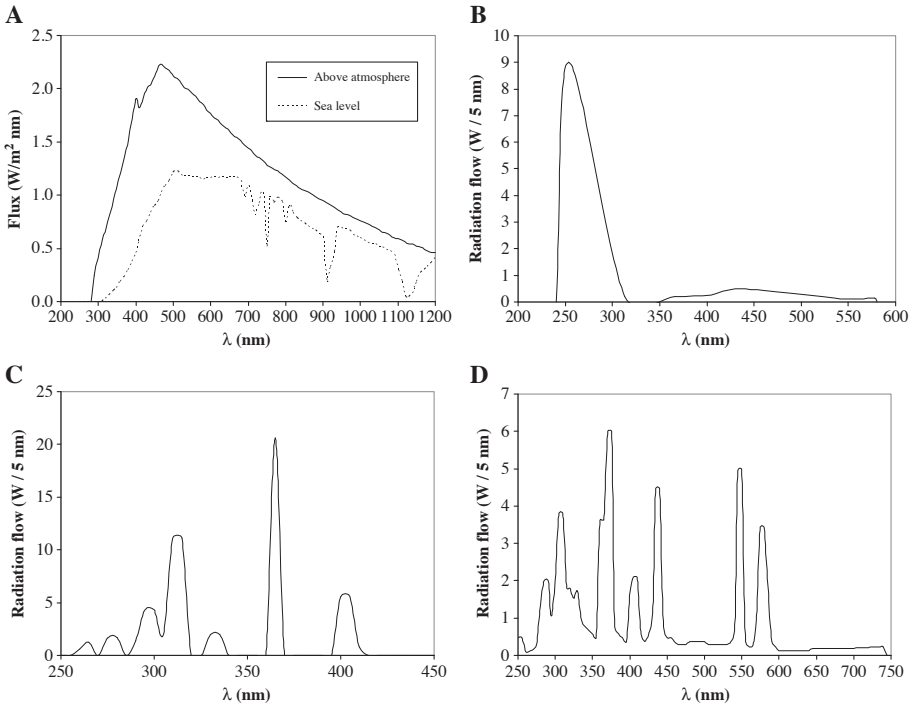

Fig. 1. Emission spectra from different UV radiation sources. A: the Sun. B: low-pressure mercury lamp. C: medium-pressure mercury lamp. D: high-pressure mercury lamp. (Adapted from Ibarz & Esplugas, 1989).

- Halogen lamps, in which a halogen (I_2 or Br_2) is added in form of gas in the incandescent lamp, causing some chemical reactions between the halogen and the tungsten filament. As a result, there is a greater output of visible radiation and some ultraviolet one compared to simple incandescent lamps.
- Arches of carbon and plasma. The need for radiation sources with greater intensity led to the use of other systems such as arches of carbon and plasma. However, these arches have the disadvantage of emitting a lot of infrared radiation. The type of radiation from a plasma arch is determined by the plasma-forming gas. The one with a highest photochemical interest is the one made of argon, with a maximum energy peak between 300 and 500 nm.
- Fluorescent lamps, like the ones used in household lighting. There are many different types and shapes in the market, which emit in interesting visible areas, but the radiation level is too low for industrial use.
- High intensity discharge lamps. These lamps produce high intensity light by passing an electric current through metal in a gaseous state. The commercially available ones are mercury, sodium and xenon.
  * Mercury lamps. The inert characteristics of mercury, together with its relatively low ionization energy and sufficient vapor pressure at moderate temperatures, make it the optimum metal for use in gas discharge tubes. To improve the emission spectrum of these lamps, sometimes metal halides are added. Attending to gas pressure, arches are usually classified into low, medium or high pressure (Fig. 1B–D). As pressure increases, the emission spectrum becomes more complex. While for low pressure the emission of radiation is almost exclusively at 254 nm, there are more emission lines for medium pressure, and the spectrum fairly continues for high pressure. On the other hand, the power that high-pressure lamps may have (2500 W) is much greater than the power that low pressure ones may achieve (30 W), making them more effective. Mercury lamps (high, medium or low pressure ones), similar to fluorescent lamps, require an electrical device for their ignition and continuous operation.
  * Sodium lamps. The characteristics in construction, operation and emitted radiation of the high pressure sodium lamps are quite different from other high intensity discharge lamps. The tube of these lamps is made with ceramic material (translucent aluminum oxide) to prevent chemical attack of sodium vapor at high pressure and temperature. Its spectrum is very rich in visible wavelengths, making it useful in outdoor lighting (roads, highways, etc.).
  * Xenon lamps. It is one of the most interesting sources of radiation because of its spectral distribution, almost continuous, similar to the Sun. They can have high power (2000 W), but they require more care than mercury lamps. They are point sources, must operate in a vertical position and require a good control of the cooling system. They operate at a very high voltage, making the electrical equipment and maintenance expensive.

Lamp manufacturers usually provide their emission spectra, as well as their nominal power. However, nominal power does not usually coincide with the real power emitted by the lamp, because as time goes on its power decreases. Therefore, in any quantitative study of photochemical processes it is necessary to know the real power. There are two usual ways to measure this real power. On the one hand, it can be accomplished using UV sensors that provide this

magnitude in W/m^2 (Guerrero-Beltrán & Barbosa-Cánovas, 2004). On the other hand, it is also usual to carry out the so-called actinometric reactions or simply actinometries.

An actinometric reaction is a standard photochemical reaction, with well-known absorption and kinetic characteristics, which easily allows measuring changes in the concentration of some of the species involved in the reaction. The actinometry is the best method to find the radiation flow that enters the photoreactor due to the presence of a radiation-emitting source, and then making it possible to calibrate this source. The most commonly used actinometric reactions are the photochemical decomposition of oxalic acid in the presence of uranyl cation and the photodecomposition of ferrioxalate (Calvert & Pitts, 1967; Rabek, 1982).

3. Photochemical reactors

There are a lot of different reactors that are used in photochemical processes, but attending to their mode of operation they can be classified as continuous and discontinuous. Some of them are described below.

3.1. Discontinuous reactors

They are often used to carry out reactions with low quantum yield, which need high irradiation times. This kind of reactors is also used if the reactants have a high viscosity.

Among the several existing designs, stirred tank type is the most used one. It essentially consists of a perfect mixing tank with a set of lamps immersed inside it, so that radiation reaches any point. Another kind is the elliptical photoreactor (Fig. 2A), which operated in batch is used in some quantitative studies. This design consists of a cylindrical shell with elliptical cross section, constructed with a reflective material to radiation, in which the lamp is placed in a focal axis and the reactor in the other one. With this configuration, all radiant energy emitted by the lamp strikes, theoretically, in the reactor either in a direct way or through the reflection in the cylindrical shell. This will get high radiation intensities in the reaction zone.

3.2. Continuous reactors

Photochemical reactors, which are a continuous flow system, are used in reactions with large quantum yields, achieving small irradiation time of the reactants. Therefore, in reactions with high rates of radiation absorption reactors operating in continuous are commonly used. Below, there is a brief description of these continuous photoreactors.

Tubular annular photoreactor consists of a cylinder with annular section, with the lamp placed in this central annulus space.

Cylindrical stirred tank type photoreactor (Fig. 2B), similar to that operating in a discontinuous mode, consists of a perfectly stirred tank with one or more immersed lamps.

Elliptical photoreactor, identical to the elliptical one described above, but operating in continuous. Lu, Li, Liu, Cui, Yao, and Zhang (2010), Lu, Li, Liu, Cui, Xia, and Wang (2010) developed a special kind of elliptical thin-film photoreactor with enhanced efficiency. It consisted of two UV mercury vapor lamps located inside elliptical reflectors, in which UV rays were reflected in such a way that they could converge at a light point: the focus. At this UV light point, the radiant energy was transmitted into quartz optical fibers bound by the fiber cluster. The reactor body, made of stainless steel, was studded with the optical fibers.

Parallel flat-plate reactor, which consists of two parallel plates placed very close, with the reactants circulating among them, while the radiant energy comes through one side from the outside. This face is made of a material transparent to radiation, which comes either directly from the exterior lamp or by reflection from a parabolic envelope constructed with a reflective material. This kind of reactor is suitable for reactants with very high optical density, since the distance between the plates is very short and the fluid flows with a very low thickness, making it easier that the radiation reaches every point of the reactant fluid.

Descendent film photoreactor is a tubular reactor in which the lamp is placed in the central axis, and the reactant fluid flows in form of film down the inner face of the tube. Shama, Peppiatt, and Biguzzi (1996) developed a thin film photoreactor, based on a nozzle of special design which generated an unsupported thin liquid film commonly referred to as a liquid 'bell'. The arrangement described permitted liquids to be irradiated without making contact with either the UV sources or any solid walls in the vicinity of the sources. Milly, Toledo, Chen, and Kazem (2007) developed a novel photoreactor consisting of an inner rotating rotor and a stationary quartz housing, which was used to induce *controlled cavitation* to ensure a homogeneous exposure of the treated product.

Particle bed photoreactor, consisting of a bed of glass particles in which in the surface there is a layer of a radioisotope attached, being covered by a fluorescent material. Thus, high-energy radiation emitted by the radioisotope interacts with the fluorescent material to produce visible or ultraviolet radiation energy.

To achieve high radiation doses, multilamp reactors are often used. They basically consist of a single reactor as the ones described above with several lamps (Fig. 2C and D).

In addition to all the reactors exposed, there are other kinds of designs, although it should be emphasized that for photochemical treatment of solid materials, they are usually exposed to radiation in cameras that have installed artificial radiation sources like the lamps described above.

4. Photoreactor mathematical model

To elaborate the mathematical model of a conventional reactor it is necessary to carry out the mass and energy balances, as well as the kinetic equation. Furthermore, in photoreactors it is necessary to make an additional radiation balance (Bird, Stewart & Lightfoot, 1964), which has a great importance in the general model and depends on the geometry of the system. If the process works in isothermal state, it is not necessary to perform the energy balance. In addition, the variation of the photochemical reaction rate with temperature is a minor factor.

4.1. Mass balance

Considering a continuous operation and perfect mixing, the balance for a component B_j when steady state is reached would be:

$$q\left(C_{j0}-C_j\right) = -\int_V r_j dV \quad j = 1, 2, \ldots, S \tag{1}$$

being: q the volumetric flow of reactants, C_{j0} and C_j component j concentrations at the entrance and exit of the reactor, respectively, r_j the rate of disappearance of that component and V the reaction volume.

If the reaction is developed in discontinuous, a new accumulation term appears, resulting in the mass balance:

$$V\frac{dC_j}{dt} = \int_V r_j dV. \tag{2}$$

These expressions are similar to the ones from conventional reactors, but it should be noted that the reaction rate r_j is a function, as well as of reactants and product concentrations, of the intensity of radiation at the absorption wavelengths in the reaction chamber.

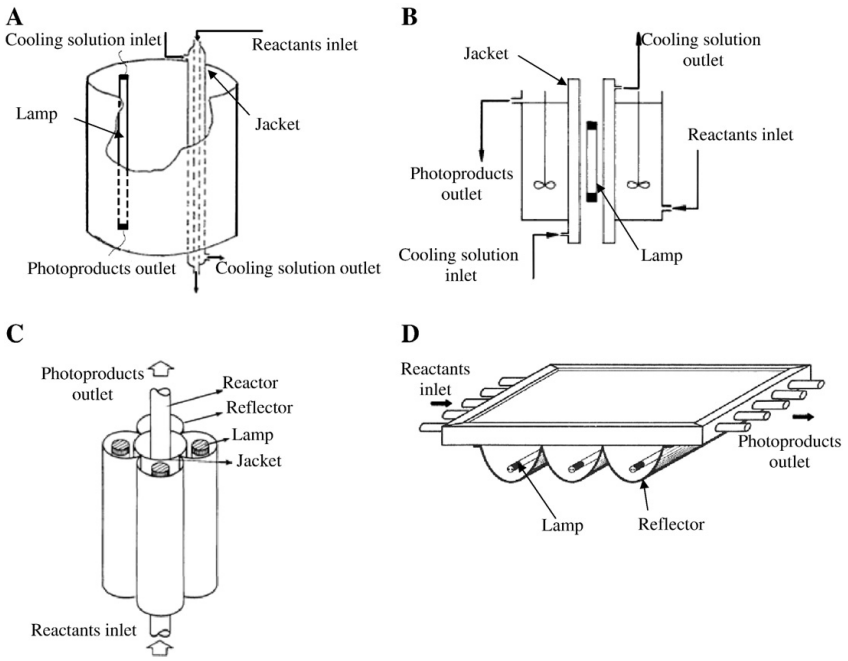

Fig. 2. Photoreactor types: A: elliptic. B: annular. C: multilamp tubular. D: multilamp plane. (Adapted from Ibarz & Esplugas, 1989).

4.2. Radiation balance

The radiation balance is posed by adopting a model that is able to provide information about light rays, taking certain considerations about the kind of emission and the geometry of the radiation source itself. Light emission models can be radial, spherical or specular and diffuse. For emission source three models can be set, depending on if the lamp can be considered as a line, as a cylindrical surface or as a volumetric region with cylindrical shape.

The most common emission models are the radial one and the spherical one, which combined with the different types of emission sources lead to:

- Radial linear model.
- Non-extensive radial cylindrical model.
- Extensive radial cylindrical model.
- Linear spherical model.
- Non-extensive spherical cylindrical model.
- Extensive spherical cylindrical model.

The *extensive* and *non-extensive* terms refer to the fact that the emitting source is considered as a volume or as a surface, respectively.

Nonlinear models are not widely used because of the big complexity presented in its calculations. Among linear models, the radial one (Harris & Dranoff, 1965) considers the lamp as a line on which every point emits radially rays with the same intensity, which are contained in a plane that is perpendicular to the lamp by that emitting point. This model has an error that is greater than the one obtained with spherical model, so it is only used in cases where an approximate result is desired. Among all models, the one which gives a more accurate result is the extensive spherical cylindrical model (Cerdà, Irazoqui, & Cassano, 1973: Esplugas, 1975), which considers the lamp as a cylinder whose volume emits spherically. However, the most used model is the linear spherical one, which has an easier resolution, and whose results hardly differ from the extensive spherical cylindrical model of conventional reactors.

The spherical linear model is described in the literature (Esplugas, 1975; Jacob & Dranoff, 1969, 1970). It assumes that the lamp is a line that emits spherically, so that the radiation intensity depends on the axial and radial coordinates. One of the most used photochemical reactors in the annular one, either perfect mixing or tubular, in which the radiation source is placed in the center of the ring (Fig. 2B), so that the reaction chamber surrounds the lamp. Thus, in descending film reactors, the lamp is inside the tube through which the fluid descends and receives the radiation. In these cases, attending to the geometry of the system, it is considered that the closest surface to the lamp is at a distance that corresponds to the radius R_i, with the fluid contained in an annular space thickness ($R_e - R_i$), being R_e external radius of the system. The radiation balance between a P point of the lamp and another one with coordinates (r, z) within the reacting fluid (Fig. 3), is given by Lambert's law for spherical emission (Esplugas, Ibarz, & Vicente, 1983):

$$I(r,z) = \frac{W_L}{4\pi L} \int_{x}^{x+L} \frac{\exp(-\mu \cdot B)}{r^2 + (z-x')^2}\, dx' \tag{3}$$

where

$$B = \left(1 - \frac{R}{r}\right)\left[r^2 + (z-x')^2\right]^{1/2}$$

where μ is the medium absorbance, I is the intensity corresponding to a point with coordinates (r, z), the product ($\mu \cdot B$) is the attenuation of the ray until it reaches the point (r, z), W_L is the energy flow emitted by the lamp and L is the lamp length.

The radiation level on the inner surface of reaction is obtained for $r = R_i$, and depends on the position z of the considered point. In this case, radiation does not find in its way any reaction medium in $r < R_i$.

Consequently, the absorbance is null, and Eq. (3) has an analytical solution:

$$I(R_i,z) = \frac{W_L}{4\pi L R_i}\left[\ tan^{-1}\left(\frac{z-x}{R_i}\right) - tan^{-1}\left(\frac{z-x-L}{R_i}\right)\right]. \quad (4)$$

It is observed that the intensity depends on the axial position, having a maximum value when the lamp is centered over the reactor (Esplugas et al., 1983).

For any photochemical reaction, its rate is proportional to the radiation intensity absorbed. Therefore, the evaluation of the total amount of radiation absorbed by the reaction medium is an interesting parameter to be estimated. In the mentioned reactor kinds, if the medium absorption is very high, or when the thickness of the reaction medium is high (high values of R_e), it can be assumed that all the radiation that reaches the reaction medium is absorbed. Therefore, the absorbed radiation flow coincides with the one existing in the radial position R_i. To know this radiation flow, an integration of the radiation for the entire height of reactor must be taken, according to expression (5) (Esplugas et al., 1983):

$$W = \frac{W_L R}{2L}\left\{\begin{array}{l}\left[\left(\frac{h-x}{R_i}\right)^2+1\right]^{1/2} - \left[\left(\frac{x}{R_i}\right)^2+1\right]^{1/2} - \\ -\left[\left(\frac{h-L-x}{R_i}\right)^2+1\right]^{1/2} - \left[\left(\frac{x+L}{R_i}\right)^2+1\right]^{1/2}\end{array}\right\}. \quad (5)$$

Another kind of reactor is the so-called plane, in which the lamp is parallel and placed at a certain distance from the surface of a fluid in a vessel. This reactor is essentially a device with parallelepiped shape with an external lamp (Fig. 4). As in the previous case, if the absorbance of the reaction chamber is high, all the radiation entering the reactor will be absorbed by the medium. Therefore, the absorption rate has the same value as the flow rate reaching the surface reaction. Assuming that the lamp is a line that emits spherically (Jacob & Dranoff, 1970), the amount of radiation can be obtained in a similar way as described above, integrating for the entire surface the radiation intensity that reaches it. Thus, it is obtained that the radiation flow of on the surface of the reactor can be expressed by (Esplugas & Vicente, 1991):

$$W = \frac{W_L z_o}{4\pi L}[E(L+y_o) - E(L+y_o-B) + E(y_o-n) - E(y_o)] \quad (6)$$

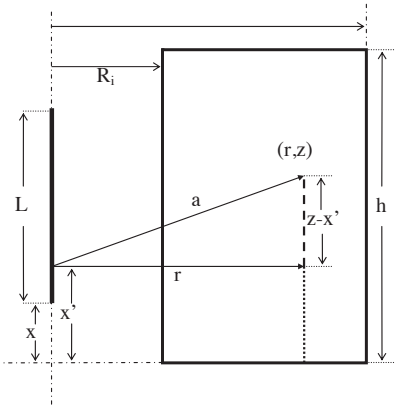

Fig. 3. Geometric parameters of cylindrical photoreactor.

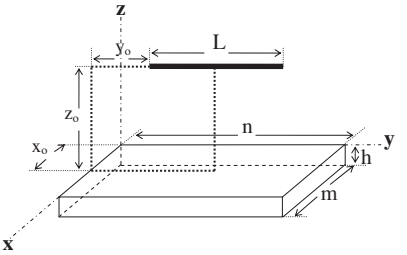

Fig. 4. Geometric parameters of plane photoreactor.

where

$$E(y) = F(y,0) - F(y,m)$$

$$F(y,X) = \frac{y}{z_o}\ tan^{-1}\left\{\frac{y}{z_o}\frac{x_o-x}{\left[(x_o-x)^2+y^2+z_o^2\right]^{1/2}}\right\} + 0.5\ ln\left\{\frac{\left[(x_o-x)^2+y^2+z_o^2\right]^{1/2}+(x_o-x)}{\left[(x_o-x)^2+y^2+z_o^2\right]^{1/2}-(x_o-x)}\right\} \quad (7)$$

in which m and n are the dimensions of width and length of the reaction vessel, which are defined, along with x_0, y_0 and z_0 in Fig. 4. This equation allows evaluating the radiation flow in plane photoreactors only using the spectral distribution of the lamp and the dimensions of the reactor.

There are several additional models with more complex analytical solutions (i.e. elliptical geometry, multilamp reactors, etc.) that can be found in literature (Alfano, Vicente, Esplugas, & Cassano, 1990; Esplugas, 1983).

4.3. Quantum yield

The energy states of molecules are quantized. The lowest energy state in which a molecule or atom can be is called the fundamental or normal state. In general, these states have paired electrons, which implies that the total spin is zero (singlet state). If an electron in one of these states goes to a higher energy level, it may remain unpaired, resulting in a state of non-zero total spin. If the molecule is in an excited state, its natural tendency is to move towards a minimum energy state deactivating itself. For a given photochemical reaction consisting of a multi-stage reaction mechanism, the *quantum yield* (φ) measures the fraction of deactivated molecules in each mechanism, being possible to calculate a quantum yield for each one.

For each mechanism:

$$\phi_i = \frac{\text{Number of excited molecules deactivated by the i mechanism}}{\text{Total number of excited molecules}}.$$

If the final product of each process is P_j:

$$\phi_i = \frac{\text{Total number of } P_j \text{ molecules produced}}{\text{Total number of photons absorbed}}.$$

If the expression is referred to the reactants and the global process:

$$\phi_i = \frac{\text{Total number of deactivated molecules}}{\text{Total number of photons absorbed}}$$

or also:

$$\phi_i = \frac{\text{Reactant disappearance molar rate}}{\text{Absorbed photons flow}}.$$

According to the last definition, a process in which the value of quantum yield is known, from the absorbed photons flow calculated by Eqs. (5) or (6) (depending on the kind of photoreactor) it is possible to determine an approximate value of the reaction rate. Only if the absorbance of the medium is infinite it can be ensured that all incident radiation on the reactor surface coincides with the absorbed flow, and in this case the estimated value will be exactly the reaction rate. Typically, industrial processes are often worked with high absorbance values to ensure that the radiation emitted by the lamp is fully exploited.

From the definition of quantum yield:

$$\phi_i \leq 1$$

$$\sum_i \phi_i = 1.$$

4.4. Photodegradation kinetics equation

As indicated above, the mathematical expression of the reaction rate is usually a function of reactants and product concentrations, as well as the radiation intensity of each wavelength:

$$r_j = r_j(C_1, \ldots, C_S, I_{\lambda 1}, \ldots, I_{\lambda n}) \tag{8}$$

where reaction rates must be calculated at each point of the reactor and integrated throughout the whole volume.

Since r_j depends on I_λ, and following the radiation models it is a function of μ_λ, r_j will be also a function of μ_λ. The resolution of this mathematical model is complex, since the r_j integral in the whole reactor volume has to be calculated. Generally, r_j can be decomposed in a summatory for all wavelengths:

$$r_j = \sum_\lambda r_{j,\lambda} \tag{9}$$

where:

$$r_{j,\lambda} = r_{j,\lambda}\ (C_1, \ldots, C_S, I_\lambda). \tag{10}$$

There are some cases where the resolution of the mathematical model becomes easier. Thus, in photocatalytic reactions, in which a substance absorbs radiation without being decomposed and catalyzes another reaction, the concentration of the radiation absorbing species remains constant, so that the absorbance μ_λ is also constant.

Although photocatalysis is a special case of photochemical reactions, most of them are not so, since the substance that absorbs radiation breaks down and its concentration changes with time. This makes its absorbance (μ_λ) not constant, but it is usually proportional to its concentration:

$$\mu_\lambda = \varepsilon_\lambda \cdot C \tag{11}$$

being ε_λ the molar extinction coefficient. In some cases this relationship between absorbance and concentration is not linear, but follows a power function.

The kinetic equation depends on the reaction mechanism, which depends on the energy absorbed by the reactants. A simple case of a photochemical reaction mechanism is the assumption that there are three stages, as shown in the following scheme:

$$A \longrightarrow A^*$$

$$A^* \xrightarrow{k_D} A$$

$$A^* \xrightarrow{k_1} \text{Photoproducts}$$

Following this mechanism, in a first stage the A reactant in its fundamental state absorbs radiation and goes into an excited state A*. From this state, it can return to its fundamental state or be decomposed into photoproducts. The variation of every one of the species that take part in the reaction mechanism can be expressed as:

$$\frac{dC_A}{dt} = k_D C_{A^*} - \sum_\lambda \varepsilon_\lambda I_\lambda C_A \tag{12}$$

$$\frac{dC_{A^*}}{dt} = \sum_\lambda \varepsilon_\lambda I_\lambda C_A - k_D C_{A^*} - k_1 C_{A^*}. \tag{13}$$

Assuming that for reaction intermediates the pseudo-steady state is applied, an expression for the photochemical degradation rate of the A reactant is obtained:

$$r_A = -\sum_\lambda \varepsilon_\lambda I_\lambda k_A C_A \tag{14}$$

where:

$$k_A = \frac{k_1}{k_1 + k_d}. \tag{15}$$

For batch operation, substituting Eq. (14) in Eq. (2):

$$\frac{dC_A}{dt} = -\frac{1}{V}\int_V \sum_\lambda \varepsilon_\lambda I_\lambda k_A C_A dV. \tag{16}$$

The radiation flow absorbed by the reaction medium can be defined by the expression:

$$W_{abs,A} = \int_V \sum_\lambda \varepsilon_\lambda I_\lambda C_A dV. \tag{17}$$

To evaluate this parameter it is necessary to know the distribution of the intensity emitted by the lamp at each wavelength, as well as the molar extinction coefficient of the reaction medium in each wavelength. To solve the integral term it is necessary to use computer software that calculates the total radiation flow absorbed. This method allows obtaining this absorbed flow in a fixed concentration of the reaction medium. However, the concentration is changing with reaction time, so the value of this parameter may be changing. If this variation with concentration is mild, a mean value can be taken within the working concentration range ($\overline{W}_{abs,A}$). Once this parameter is known, it is possible to solve the differential equation (Eq. (16)), obtaining:

$$C_A = C_{0,A} \exp\left(\frac{-\overline{W}_{abs,A} k_A}{V} t\right). \tag{18}$$

In the case of continuous operation, this calculus can be carried out in a similar way, noting that it is also necessary to assess the radiation flow absorbed by the reaction medium.

This is a simple case of evaluating the kinetics of a photodegradation of an A reactant through a simple three-step mechanism. However, there are some cases where it is necessary to assume more complicated mechanisms that may involve dissolved oxygen or other reactants present in the reaction, developed in an earlier work (Ibarz, Esplugas, & Costa, 1985; Ibarz, Panadés, & Tejero, 1996; Panadés, Alonso, & Ibarz, 1997). Whatever the proposed kinetic equation, it will be necessary to calculate the absorbed flow radiation term by using specific software in each case.

Table 1
Low and high UV-C light dosages (254 nm) needed for inhibiting 100% of several types of microorganisms (Guerrero-Beltrán and Barbosa-Cánovas, 2004).

Organism	Microorganism	Low dose (J/m^2)	Microorganism	High dose (J/m^2)
Algae	*Chloella vulgaris*	220	Blue green algae	4200
Bacteria (vegetative)	*Bacillus megatherium*	25	*Sarcina lutea*	264
Bacteria (spores)	*Bacillus subtilis*	220	*Bacillus anthracis*	462
Molds	*Oospora lactis*	110	*Aspergillus niger*	3300
Viruses	Adeno virus types III	45	Tobacco mosaic	4400
Yeasts	Brewer's yeast	66	*Saccharomyces* sp.	176

4.5. Absorbed radiation dose

When any product is irradiated, it is important to define the radiation dose that it receives. This dose depends on the incident radiation, expressed as the amount of radiation received per unit of time and area or *flux intensity* (D_r, in W/m^2), so that the dose corresponds to the product of this one by exposure time (Bintsis et al., 2000; Guerrero-Beltrán & Barbosa-Cánovas, 2004):

$$D = D_r t. \tag{19}$$

In continuous processes, exposure time coincides with the time of residence. From this equation it can be deduced that radiation dose has units of J/m^2.

When UV irradiation is used to inactivate microorganisms, some authors (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Stermer, Lasater-Smith, & Brasington, 1987) consider that the inactivation kinetics is first order, so that the number of organisms that survive photochemical treatment can be expressed by the equation:

$$N = N_0 \exp(-kD) = N_0 \exp(-kD_r t) \tag{20}$$

where N and N_0 are the number of microorganisms for any treatment time and at the beginning, respectively, and k is the inactivation rate constant. By analogy with conventional heat treatments, a decimal reduction dose (D_{UV}) can also be defined as the dose that is necessary to reduce to one-tenth the initial number of microorganisms, and is equal to 2.303/k. If the experiment is carried out with a constant lamp and geometry, D_{UV} can be also changed into the time of irradiation (expressed in min). Table 1 shows minimum and maximum doses for a total inhibition of various types of microorganisms, using an UV-C radiation source that emits at 254 nm.

5. Industrial applications, safety concerns and consumers acceptability

Ultraviolet irradiation is widely applied in industries to disinfect water. Other common applications include air and surface sterilization, especially in food packagers, brewers, bottling and cosmetics (Guerrero-Beltrán & Barbosa-Cánovas, 2004). In water treatment, UV irradiation is used as an alternative to chlorine, which may deliver toxic by-products such as trihalomethanes and may be harmful for aquatic ecosystem. In addition, this technology is able to impair refractory molecules, which remain after conventional water processing. In some cases such as in wastewater treatment, the addition of photosensibilizers is essential to achieve the desired effect on water disinfection (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Ibarz & Esplugas, 1989).

Regarding foodstuff processing, UV irradiation is not widely used yet. Currently, in the manufacture of fruit juices, the FDA requires to apply a preservation method capable of reducing pathogens at least 5 logarithmic cycles (Falguera et al., 2011). In this way, this technology has been proved to be effective to achieve this reduction, but its implementation inside industries is very limited. Other possible applications that have been proved at laboratory scale but with low industrial presence include dairy industry, wine industry and cheese processing (Guerrero-Beltrán & Barbosa-Cánovas, 2004).

One of the main reasons why UV irradiation is not widely implemented in industries may be found in consumers distrust. According to Ibarz (2008), irradiation of foods is a technique which has been given an unfortunate name, as it has been related with nuclear energy. This has meant that many times irradiated food has been mistaken for being radioactive, which is completely wrong. Despite the fact that irradiated foods and the process of food irradiation have been carefully tested, the quantity of irradiated foods in the global food trade is not significant. Many surveys and market trials have shown that consumers recognize the better quality of irradiated products but they show a bad attitude towards the idea of consuming irradiated food (Mostafavi, Fathollahi, Motamedi, & Mirmajlessi, 2010), in a similar way to what happened in some countries with conventional pasteurization (Satin, 1997).

More than 100 years of research to achieve the accepting of the safe and successful use of irradiation as a food safety method is more than any other technology used in the food industry today, even canning (Mostafavi et al., 2010; Satin, 1997). Even though, there are still several scientific tests being carried out in order to corroborate its healthiness. This has resulted in international bodies including the World Health Organization (WHO), the Food and Agriculture Organization (FAO), the International Atomic Energy Agency (IAEA) a\nd the Codex Alimentarius commending the process. UV irradiation has been proved to be very successful against living organisms which contain DNA and/or RNA but do not cause any significant loss of macronutrients (Ibarz, 2008; Mostafavi et al., 2010).

6. Final considerations

Ultraviolet irradiation constitutes an alternative to thermal treatment that is being studied and developed to obtain a better final product sensory quality, but without neglecting microbial safety. It is very important to consider that for each desired application critical decisions about equipment design must be taken. These decisions include radiation source, reactor geometry and reaction medium properties. In order to quantify and predict chemical changes produced by ultraviolet radiation in a food system and to infer essential information for reactor design, a concrete modeling is required in each case, since the absorbed amount of radiation has a definitive effect on the reaction rate of the process in each point of the reaction medium depending on its location. An important part of the theoretical research in the field of UV irradiation of liquid food must be focused in the near future on the development of these models for different photoreactor configurations.

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Notation

C: concentration ($mol \cdot L^{-1}$)
D_r: flux intensity ($W \cdot m^{-2}$)
D: radiation dose ($J \cdot m^{-2}$)
h: height of the photoreactor (m)
I: intensity of radiation ($einstein \cdot m^{-2} \cdot s^{-1}$)
k: constant reaction rate (s^{-1})
k: inactivation rate constant ($J^{-1} \cdot s^{-1}$)
L: lamp length (m)
m: width of plane photoreactor (m)
n: length of plane photoreactor (m)
N: number of microorganisms
q: volumetric flow rate ($m^3 \cdot s^{-1}$)
r: intensive reaction rate ($mol \cdot m^3 \cdot s^{-1}$)
r: radial coordinate (m)
R: radius of cylindrical photoreactor (m)
t: time (s)
V: volume (m^3)
W: radiation flow rate ($einstein \cdot s^{-1}$)
x: x-coordinate (m)
y: y-coordinate (m)
z: z-coordinate (m)

Greek symbols

ε: molar extinction coefficient ($m^2 \cdot mol^{-1}$)
ϕ: quantum yield ($mol \cdot einstein^{-1}$)
μ: attenuation coefficient or absorbance (m^{-1})

Subscripts

A: A reactant
abs: absorbed
e: external
i: inner
L: lamp
o: lamp position
w: wall
λ: wavelength
0: initial

APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.4.

Title: Ultraviolet processing of liquid food: A review. Part 2: Effects on microorganims and on food components and properties.

Authors: Víctor Falguera, Jordi Pagán, Salvador Garza, Alfonso Garvín & Albert Ibarz.

Journal: *Food Research International*, 44 (2011), 1580-1588.

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Review

Ultraviolet processing of liquid food: A review
Part 2: Effects on microorganisms and on food components and properties

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ABSTRACT

The effectiveness of ultraviolet irradiation in inactivating microorganisms has been known for decades. Nevertheless, in the processing of foodstuff other important issues must be considered, such as organoleptic or nutritional properties preservation. Ultraviolet irradiation constitutes an alternative to thermal treatment that is being studied and developed to obtain a better final product sensory quality, but without neglecting microbial safety. Moreover, it has been proved that this technology can be useful to decompose some toxins that are not affected by thermal processing. In this piece of work, the effect of UV irradiation on all of these contaminants and components of liquid food has been reviewed.

Contents

1. Introduction

Food safety is one of the most important issues that food industries and food service companies have to face. Currently, the growing demand for a greater variety of prepared food and dishes on a menu makes the risk of contamination increase, especially due to the characteristics of this kind of food that gives a big importance to the quickness of meals. The application of HACCP (Hazard Analysis and Critical Control Points) has increased security guarantees in production systems, thermal treatments and cold chains, helping to reduce the opportunities for pathogens to gain access to food and/or grow to levels that will pose a risk of infection or toxin production. However, the incidence of foodborne diseases continues to rise in most industrialized countries (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000; Falguera, Pagán, & Ibarz, 2011).

The elaboration and processing of liquid food like fruit juices have to be carried out under hygienic conditions that ensure final product safety. Nevertheless, in some cases there can be compounds with adverse effects on health that are not eliminated with conventional processes, such as mycotoxins, which come from mold-contaminated pieces of fruit. On the other hand, depending on the kind of juice, the presence of enzymes may represent important problems in its resulting quality. In this way, polyphenol oxidase (PPO) is the enzyme responsible for enzymatic browning, transforming phenolic compounds into *o*-quinones, which result in melanins after a polymerization stage. Moreover, other dark compounds, melanoidins, can appear in fruit juices after non-enzymatic browning by Maillard reaction. Both melanins as melanoidins have a negative effect on final quality of the juice.

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Apart from PPO, other enzymes with a great influence on juice quality are amylases and pectic enzymes. In the production of clarified and depectinated juices it is essential to leave these enzymes to act in order to eliminate pectin and starch. Nevertheless, in purees and juices with suspended pulp, it is very important to inactivate them to avoid the cloud loss in the product. If they are not completely inactivated, in the storage stage they may have a residual activity on pectic fractions resulting in sedimentation of the pulp, due to the destabilization of the system. Thus, in this kind of product it is imperative to ensure that there is no enzymatic activity, as it may adversely affect the quality of the juice.

Treatments on food products are carried out to extend its shelf life, but above all to ensure its safety for consumers. Thermal technologies are the most widely applied to achieve these purposes, but these treatments have a negative effect on certain components of the food itself, reducing its vitamin content and other nutrients, as well as sensory features that make them less attractive in terms of color and textural properties. Non-thermal technologies are an alternative to thermal treatment that are being studied and developed in order to obtain a better final product sensory quality, but without neglecting microbial safety. In this way, these alternatives to thermal technologies can produce food products without enzymes and harmful microorganisms, while maintaining nutritional characteristics and minimizing the loss of quality in terms of flavor, color and nutritional value. One of these innovative technologies is ultraviolet irradiation (Butz & Tauscher, 2002; Noci et al., 2008).

2. Effect of UV irradiation on microorganisms

Among other applications, irradiation with ultraviolet light (UV) can be used as a disinfectant treatment to reduce the microbial load in food (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Tran & Farid, 2004). The most effective wavelengths are located between 200 and 280 nm (the so-called UVC), especially at 254 nm, while at 320 nm its efficiency is almost null (Bachmann, 1975; Bintsis et al., 2000). UV radiation affects the DNA of bacteria, viruses, fungi and other microorganisms exposed to it in such a way that prevents them from reproducing (Billmeyer, 1997; Giese, 1997; Hijnen, Beerendonk, & Medema, 2006). The effect of radiation on microorganisms depends on various factors such as species, strain, culture and growth phase (Bachmann, 1975; Morgan, 1989). In addition, the kind and composition of the food that is irradiated also has a great influence.

The application of UV light with germicidal effects has been used in three areas: air disinfection, liquid sterilization and inhibition of microorganisms in surface (Bintsis et al., 2000). In the food industry UV-C irradiation has been mainly applied in various processes and products such as air disinfection in meat or vegetable processing, on the water that will be used in some stages of the process, on surfaces of fresh products, chicken, fish, eggs, and various liquid food: milk, fruit juice or cider (Basaran, Quintero-Ramos, Moake, Churey, & Worobo, 2004; Duffy, Churey, Worobo, & Schaffner, 2000; Hadjock, Mittal, & Warriner, 2008; Liltved & Landfald, 2000; Matak et al., 2005; Quintero-Ramos, Churey, Hartman, Barnard, & Worobo, 2004; Wong, Linton, & Gerrard, 1998).

2.1. Air disinfection

The use of UV-C as a germicidal agent for the decontamination of the air is a method that has been known for decades. Several studies have been conducted on various microorganisms present in the air, such as bacteria and viruses (Bailey, Buhr, Cox, & Berrang, 1996; Jensen, 1964) or bacterial and fungal spores (Xu et al., 2003.). The microorganisms are more sensitive to UV-C if they are suspended in the air than in water, and these, in turn, are more sensitive to those found in fruit juices (Bintsis et al., 2000).

Jensen (1964) irradiated aerosolized viruses by passing them through an aluminum cylindrical tube with a highly reflective inner surface whose center contained a UV lamp, achieving, in the most favorable conditions, of more than 99.9% inactivation for Coxsackie, Influenza, Sindbis and Vaccinia viruses. Xu et al. (2003) evaluated the effectiveness of germicidal UV radiation on bacterial spores and vegetative mycobacteria cells. UV treatment reduced between 46 and 80% the concentration of *Bacillus subtilis* spores, and between 83 and 98% the *Mycobacterim parafortuitum* ones. Josset et al. (2007) designed a new photoreactor to decontaminate high-speed airflow through UVA radiation. A 93% inactivation rate was obtained in a single pass through the photoreactor with airflow of 5 m^3/h in air with a concentration of $1.2 \cdot 10^6$ CFU·L^{-1} of *Legionella pneumophila*.

2.2. Liquid sterilization

One of the main limitations of UV radiation in fluid treatment is its low penetration, which is determined by the characteristics of the irradiated liquid. Thus, in distilled water, the loss of radiation intensity at 40 cm from the surface is up to 30%, while in a 10% sucrose solution the same intensity loss can be achieved at only 5 cm (Snowball & Hornsey, 1988). In fruit juices, 90% of ultraviolet light is absorbed in the first 1 mm from the surface (Sizer & Balasubramaniam, 1999). Table 1 shows absorption coefficients of some kinds of liquid food for 254 nm UV-C radiation. The presence of dissolved organic solutes and compounds in liquid foods leads to strong UV attenuation effects. Fan and Geveke (2007) found that the major components of apple juice/cider are sugars including fructose, sucrose, and glucose followed by organic acids mainly malic acid and a very low amount of ascorbic acid. The three sugars absorbed little UV in the range of 240–360 nm although the fructose solution had higher UV absorbance at 260–280 nm than glucose and sucrose solutions. All three sugars had high absorbance round 200 nm. Malic acid mainly absorbed UV at wavelengths less than 240 nm while ascorbic acid had a strong absorbance between 220 and 300 nm even at a very low concentration (0.001%).

The first application of UV radiation for drinking water disinfection was carried out in 1910 in Marseille (Henry, Helbronner, & Recklinghausen, 1910). At that time its use was limited by its high cost, the low reliability of the equipment and the advent of chlorination, which was a cheaper, more reliable method and with the ability to measure the residual disinfectant (Hoyer, 2004; Wolfe, 1990). Since then, UV irradiation has been gaining interest and since the 80s, in Europe, it has been widely used to disinfect drinking water, reaching in some cases to replace chlorination (Downey, Giles, Delwiche, & MscDonald, 1998; Gibbs, 2000; Lodge et al., 1996; Qualls & Johnson, 1983). The rise in the use of this technology is mainly due to the fact that it hardly produces oxidation products as it happens with chlorination or ozonation. The real breakthrough in the use of UV irradiation as a primary disinfection process came after the discovery of its high efficacy against *Cryptosporidium* (Clancy, Hargy, Marshall, & Dyksen, 1998) and *Giardia*, two of the main pathogens affecting drinking water safety.

Table 1
Absorption coefficient for liquid food for UV-C at 254 nm (Guerrero-Beltrán & Barbosa-Cánovas, 2004).

Liquid food	α (cm^{-1})
Distilled water	0.007–0.01
Drinking water	0.02–0.1
Clear syrup	2–5
White wine	10
Red wine	30
Beer	10–20
Dark syrup	20–50
Milk	300

There are several works in literature studying the disinfection of both drinking and wastewater by ultraviolet irradiation (Chang et al., 1985; Liltved & Cripps, 1999; Sommer, Lhotsky, Haider, & Cabaj, 2000; Sutton, Yu, Grodzinski, & Johnstone, 2000; Whitby & Palmateer, 1993). Hijnen et al. (2006) performed an extensive and selected review on the use of ultraviolet irradiation for disinfection of drinking water. In the case of wastewater disinfection, one of the factors affecting the effectiveness of UV irradiation is the quality of treated wastewater. In this way, UV radiation has proved to be effective in treating high quality secondary and tertiary effluents (Blatchley et al., 1996; Braunstein, Loge, Tchobanoglous, & Darby, 1996, Oppenheimer, Jacangelo, Lane, & Hoagland, 1997). However, the effectiveness is further discussed in the case of primary wastewaters or low quality effluents (Sakamoto, 1997; Whitby & Palmateer, 1993; Zukovs, Kollar, Monteith, Ho, & Ross, 1986). This is due in part to the presence of suspended particles in wastewater, which increase the survival probability of microorganisms exposed to UV radiation. Suspended solids (SS) cannot only attenuate the UV dose via light scattering, but may also provide a site for the aggregation of bacteria to the particle's surface (Koutchma, 2009). Whitby and Palmateer (1993) reported the relationship between the concentration of suspended solids and the survival rate of fecal coliforms in wastewater. Taghipour (2004) concluded that to reduce one log cycle of the concentration of *Escherichia coli* in primary and secondary effluents UV radiation doses of 35 and 62 J/m^2 were necessary, respectively.

UV irradiation has also been applied successfully in the pasteurization of liquid foods such as milk and fruit juices (Koutchma, Keller, Chirtel, & Parisi, 2004; Matak et al., 2005). Although the treatment of opaque liquid foods by UV irradiation is an additional problem, in the dairy industry this method has been used for different applications (Bintsis et al., 2000). Thus, for example, brines used in the production of Mozzarella cheese have been irradiated (Anonymous, 1994). Lodi, Brasca, Mañaspina, and Nicosia (1996) succeeded in reducing the total colony count between 50 and 60%, and coliforms in 80–90% in goat milk using UV-C radiation. Burton (1951) carried out a piece of work in which milk pumped at high speed through transparent tubes of 1 cm diameter was irradiated, so that 80% of UV radiation reached the milk, destroying about 99% of bacteria initially present. Matak et al. (2005) also demonstrated that UV radiation could be used to reduce the population of *Listeria monocytogenes* in goat milk. This fresh goat milk was inoculated with a concentration of 107 CFU/mL and irradiated with UV light at doses from 0 to 20 mJ/cm^2, managing to reduce the microbial load by more than 5 log units when the milk received an accumulative dose of 15.8 mJ/cm^2. In a later work, Matak et al. (2007) assessed the chemical and sensory effects on goat milk treated for 18 s with a dose of 15.8 mJ/cm^2, concluding that milk irradiated with UV light at a wavelength of 254 nm suffered severe sensory and chemical changes. Ibarz, Pagán, and Vicente (1986) also lowered significantly the microbial load of raw milk by using UV irradiation.

In juices and fruit derivatives many authors have studied the germicidal effect of UV irradiation on different organisms (Anonymous, 1999; Basaran et al., 2004; Franz, Specht, Cho, Graef, & Stahl, 2009; Gabriel & Nakano, 2009; Guerrero-Beltrán & Barbosa-Cánovas, 2005; Keyser, Müller, Cilliers, Nel, & Gouws, 2008; Tran & Farid, 2004; Worobo, 1999).

Gabriel and Nakano (2009) irradiated different strains of *E. coli* (K-12 and O157:H7), *Salmonella* (*enteriditis* and *typhimurium*) and *L. monocytogenes* (AS-1 and M24-1) in phosphate buffer and in clarified apple juice. *S. typhimurium* was the most sensitive one to ultraviolet irradiation with a *D* value of 0.27 min, while *L. monocytogenes* AS-1 was the most resistant one with a *D* value of 1.26 min. Keyser et al. (2008) successfully used UV irradiation to reduce microbial load in different fruit juices and nectars. In clarified apple juice they were able to reduce by more than 7 log units the population of *E. coli* with a radiation dose of 1377 J·L^{-1}, while a dose of 230 J·L^{-1} was enough to reduce aerobic plate counts by 3.5 log units and by 3 log units other molds and yeasts in the juice. In similar experiments carried out in orange juice containing cells of 7.5–10% by weight, and after applying a higher radiation dose (1607 J·L^{-1}) reductions of only 0.3 log units were achieved for mesophilic aerobic microorganisms and for molds and yeasts. These disparate results are due to the large amount of suspended matter (such as orange cells and fiber) in orange juice, which act as a protective barrier to microorganisms against UV radiation.

Guerrero-Beltrán and Barbosa-Cánovas (2005) studied the reduction of *Saccharomyces cerevisiae*, *E. coli* and *Listeria innocua* population in apple juice when it was irradiated with ultraviolet light (Fig. 1). The results showed that the higher the treatment time and the flow rate were, the greater the probability that these organisms were damaged or inactivated by the radiation was. Walkling-Ribeiro et al. (2008) treated apple juice, previously inoculated with *Staphylococcus aureus* TSS 2.4, with a combined method of UV irradiation, preheating and high-intensity pulsed electric fields, managing to reduce the microbial population by 9.5 log units. In the most severe conditions the reduction achieved by using this combined techniques became even greater than that achieved with conventional pasteurization. In a study conducted by Ngadi, Smith, and Cayoutte (2003) the count of *E. coli* O157: H7 in apple juice was reduced about 4.5 log units using a dose of 3000 mJ·cm^{-2} and a liquid depth of 1 mm. This kind of treatment has also been used to reduce the microbial load in apple cider (Harrington & Hills, 1968, Wright, Sumne, Hackney, Pierson, & Zoecklein, 2000). Worobo (1999) also managed to reduce more than 5 log units of the population of *E. coli* in apple cider irradiated with ultraviolet light in a UV CiderSure 3500. Milly, Toledo, Chen, and Kazem (2007) achieved the inactivation of E. coli 25922 in apple juice and skim milk with a reduction of 4.5 and 3 logs, respectively.

Guerrero-Beltrán, Welti-Chanes, and Barbosa-Cánovas (2009) processed grape, cranberry and grapefruit pasteurized juices

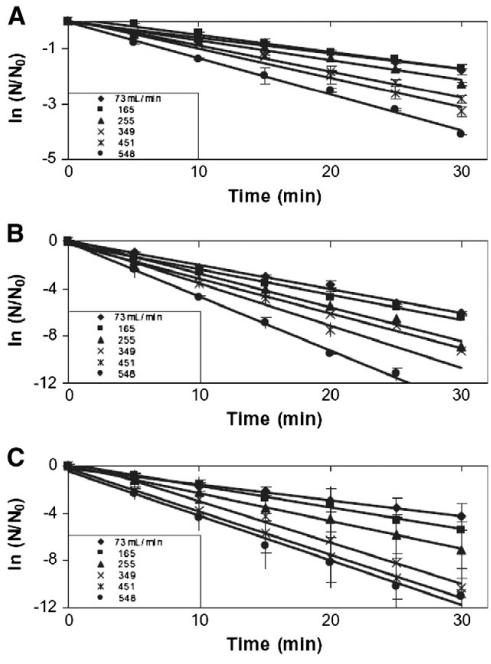

Fig. 1. Inactivation of *Saccharomyces cerevisiae* (A), *Listeria innocua* (B) and *Escherichia coli* (C) in apple juice. Experimental data fitted to a first order kinetic model (Guerrero-Beltrán & Barbosa-Cánovas, 2005).

inoculated with *S. cerevisiae*, using an UV-C disinfection unit and working at different flow rates and dose of UV light (75–450 kJ/m^2). The inactivation of *S. cerevisiae* can be described by means of first order kinetics, obtaining times of decimal reduction ranging from 61.7 to 113.7, 12.2 to 40.7 and 12.5 to 20.7 min for grape, cranberry and grapefruit juices, respectively. The maximum reduction log was 0.53, 2.51 and 2.42 for yeast count in grape, cranberry and grapefruit juices, respectively, at a flow rate of 1.02 L/min after 30 min of treatment.

In brewing and beverage industry many producers have adopted UV irradiation as a water disinfection system, as it is essential that the treatment does not alter the taste and quality of the final product (Egberts, 1990; Greig & Warne, 1992; Oliver, Bach, & Kryschi, 1990). The radiation dose required to treat water in brewing industry is much higher than the dose used in drinking water treatment, since it must guarantee the absence of any microbial alteration during the early stages of beer production. Lu et al. (2010) applied a thin film apparatus with quartz optical fibers for UV-radiation delivery to inactivate bacteria in beer. With this apparatus a reduction of inoculated *S. cerevisiae* and *L. brevis* in beer of around 5 log and from aproximately10^4 CFU/mL to non-detectable limits at doses of 16.1 and 9.7 mJ/cm^2, respectively, was achieved. However, the inactivation of *S. cerevisiae* was not so efficient. In addition, the beneficial yeasts of beer were hardly inactivated.

In liquid egg derivatives UV-C irradiation may be an alternative treatment to obtain a microbiologically safe and stable product (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000; Donahue, Canitez, & Bushway, 2004), avoiding alterations that other methods such as high hydrostatic pressure, high-intensity pulsed electric fields or thermal pasteurization may have on product properties due to protein denaturation (Unluturk, Atilgan, Baysal, & Tari, 2008). In a study carried out by Ngadi et al. (2003) on liquid egg white (pH 9.1) inoculated with *E. coli* O157: H7, there was a decrease in microorganism count from 10^8 to $10^{3.8}$ CFU·mL^{-1} after an exposure to UV radiation dose of 300 mJ·cm^{-2}. Unluturk et al. (2008) conducted a study about liquid egg products in which the effect of UV irradiation on non-pathogenic strains of *E. coli* (ATCC 8739) and *S. typhimurium* was investigated. These authors also studied the effect of the liquid medium depth, the UV light intensity and the exposure time. The maximum reduction, more than 2 log units, was obtained on *E. coli* (ATCC 8739) in liquid egg white with a medium depth of 0.153 cm and an UV intensity of 1314 mW·cm^{-2}. However, under the same conditions, in liquid egg yolk and in liquid whole egg, the maximum reductions achieved were of only 0.675 log units and 0.361 log units, respectively.

2.3. Application to surfaces disinfection

The main application of ultraviolet irradiation in industry is the sterilization of packaging materials such as containers or bottle tops (Bintsis et al., 2000). In aseptic packaging of products treated by UHT (e.g. milk) UV radiation is used to sterilize the lids of aluminum bottles (Nicolas, 1995) or cartons for liquid products (Kuse, 1982). The materials for aseptic processing and packaging can also be sterilized by combining the treatments with hydrogen peroxide (H_2O_2) and ultraviolet radiation (Marquis & Baldeck, 2007) taking advantage of their synergistic effect on bacterial spore destruction. The combination of ozone and UV radiation is often used in the treatment of polymer plastics for food packaging (Ozen & Floros, 2001).

UV-C radiation may also be used for the treatment of food surfaces. Thus, there are several papers in literature demonstrating the effectiveness of UV radiation to reduce the surface population of pathogenic microorganisms in red meat, chicken and fish (Huang & Toledo, 1982; Stermer, Lasater-Smith, & Brasington, 1987; Sumner, Wallner-Pendleton, Froning, & Stetson, 1995). In a study conducted on pork skin and muscle (Wong et al., 1998), the authors demonstrated the effectiveness of UV light to reduce *E. coli* and *S. senftenberg* in pig skin and muscle surfaces. In the tested conditions, UV light was more effective on *S. senftenberg* than on *E. coli* and its effects were faster on skin than on pork muscle. Other researchers have found that with this kind of treatment the commercial life of fresh meat may increase from 12 to 28 days (Dejenane, Sánchez-Escalante, Beltrán, & Roncalés, 2001). The application of UV light reduced by 61% the content of *S. typhimurium* in chicken skeleton, without affecting its color (Wallner-Pendleton, Sumner, Froning, & Stetson, 1994). Lyon, Fletcher, and Berrang (2007) reduced about 2 log units the content of *L. monocytogenes* in chicken breast fillets with a treatment with UV light. Chun, Kim, Lee, Yu, and Song (2010) have recently shown that treatment with UV-C irradiation can reduce the microbial load of *Campylobacter jejuni, L. monocytogenes* and *S. typhimurium* in chicken breast. Similarly, UV-C radiation can be used as a method for improving microbial safety of ready-to-eat food such as ham slices (Chun, Kim, Chung, won, & Song, 2009).

Kuo, Carey, and Ricke (1997) demonstrated that UV-C radiation is effective in reducing total aerobic count and molds, as well as *S. typhimurium*, on eggshells. Likewise, various studies show the effectiveness of UV-C radiation to reduce surface microbial load on vegetables (Allende & Artés, 2003) and fruits (González-Aguilar, Wang, Buta, & Krizek, 2001). UV-C radiation has also been used to reduce post-harvest deterioration of onions (Lu, Stevens, Yakabu, Loretan, & Eakin, 1987), carrots (Mercier & Arul, 1993), tomatoes (Maharaj, 1995) and zucchini (Erkan, Wang, & Krizek, 2001). There are also numerous studies that show the effectiveness of UV-C radiation to reduce diseases in fruits such as apple and peach (Stevens et al., 1996), table grapes (Nigro, Ippolitto, & Lima, 1998), grapefruit (Droby et al., 1993) or papaya (Cia, Pascholati, Benato, Camili, & Santos, 2007).

In the same way, UV-C light can also be applied on fresh fruits, vegetables and edible roots before storage. The purpose of this treatment is twofold: to reduce the microbial load on the surface and to induce resistance to microorganisms. The beneficial effect of this treatment on fresh foods is called "hormesis" (Stevens et al., 1997, 1999). This effect is due to the fact that UV light can stimulate the production of phenylalanine amonialyase (PAL), which induces the formation of phenolic compounds (phytoalexins) that can improve the resistance of fruits and vegetables to the organisms (Ben-Yehoshua, Rodov, Jin, & Carmeli, 1992; D'Hallewin, Schirra, Pala, & Ben-Yehoshua, 2000; Stevens et al., 1999). Onursal, Gozlekci, Erkan, and Yildirim (2010) found that the irradiation of pomegranate (*Punica granatum* L.) fruits with UV-C radiation increased the total phenolics content in juice, peel and seeds. In the treatment of broccoli UV-C has been shown to delay senescence. In addition, its antioxidant capacity increases, which could be useful from the nutritional point of view (Costa, Vicente, Civello, Chaves, & Martínez, 2005).

3. Effect of UV and solar irradiation on toxins

The presence of mycotoxins in food is a serious problem for consumers' safety. Furthermore, in conventional heat processes mycotoxins are not affected, and it would be necessary to find an alternative treatment to eliminate or reduce its content in food. Thus, aflatoxins can be destroyed by applying UV radiation and sunlight in some degree. There are studies, for example, that show that it is possible to destroy this kind of toxin in peanuts (Leeson, Díaz, & Summers, 1995). In the case of citrinin and ochratoxin A, it has been shown that there is a limited decomposition when they are treated with UV light (Neely & West, 1972). Also the use of ionizing radiation achieves this purpose, but high doses are required that they finally impair the quality of food components and nutrients, losing practical application.

UV radiation has been used to degrade aflatoxin M_1 in raw and heated milk (Yousef & Marth, 1985), observing that in the samples irradiated at 25 °C the quantity of aflatoxin M1 was reduced by 32%

more than in the ones irradiated at 5 °C; however, when the treatment temperature was 65 °C the reduction was only 25.5%. These data suggest that it is possible to degrade aflatoxin in milk at lower temperature values than pasteurization. The degradation of this toxin in aqueous solution has also been studied (Yousef & Marth, 1987), observing that its elimination was accompanied by an accumulation of aflatoxin M_x, which was also degradated when the treatment temperature was raised to 60 °C (Fig. 2).

Among the edible materials, peanut oil is the most common product that contains aflatoxins. Shantha and Sreenivasa-Murthy (1977) investigated the effect of light of different wavelengths, using lamps that emit light in the ultraviolet and solar regions. The results showed that with long irradiation times high removal rates were obtained; thus, with UV light 87% destruction was reached, while under sunlight it was 82%. The absorption spectra of these samples containing aflatoxin exposed to ultraviolet and sunlight show a shift of the absorption maximum, accompanied by a considerable reduction of toxicity (Shantha & Sreenivasa-Murthy, 1977). It has also been observed that aflatoxin B1 contained in rice is degraded under the action of the light emitted by a mercury-tungsten lamp (Nkama & Muller, 1988). In this case, the moisture content of the samples and the intensity of the used light had a high influence on the results. In this way, in rice samples containing approximately 1000 $mg \cdot kg^{-1}$ it was observed that after 2 h of irradiation with an intensity of 64 $mW \cdot cm^{-2}$ the level of aflatoxin decreased by approximately 70%, whereas with an intensity 43 $mW \cdot cm^{-2}$ its decline was 60%.

Samarajeewa, Sen, Cohen, and Wei (1990) have made a review of physical and chemical methods used in the detoxification of aflatoxin in food and animal feed. One of these techniques is the utilization of ultraviolet and visible light. Thus, aflatoxin B1 has an absorption spectrum with some peaks at wavelengths 222, 265 and 362 nm, with maximum at 362 nm. Irradiation at this wavelength activates this aflatoxin and increases its susceptibility to degradation. Aflatoxin B1 is highly sensitive to UV radiation at pH values below 3 or above 10, since the structure of aflatoxin is affected in the terminal furan ring, having the active point of link removed (Lillard & Lantin, 1970). It seems that the presence of oxygen increases the degradation of the free radicals caused by UV irradiation of the aflatoxin crystals (Bencze & Kiermeier, 1973).

Kinetic studies of aflatoxin degradation lead to a first order kinetic process with a possible dimerization of the coumarin molecule (Aibara & Yamagishi, 1968). It appears that the intermediate products formed have some toxicity, and research should be expanded in this regard. On the other hand, a white light source could provide better breakdown of primary compounds derived from aflatoxin decomposition to secondary nontoxic ones (Samarajeewa et al., 1990). Solar radiation, which has radiant energy in the ultraviolet and visible spectra, has shown great efficiency in the degradation of aflatoxins in foods (Samarajeewa & Arseculeratne, 1974). Edible oils irradiated with sunlight have shown no toxicity in mice and ducks (Gamage, Samarajeewa, Wettimuny, & Arseculeratne, 1985; Samarajeewa, Gamage, & Arseculeratne, 1987; Shantha & Sreenivasa-Murthy, 1977, 1980). Aflatoxin B1 degradation by sunlight in foods suggests the presence of aflatoxin in two states, one easily accessible and another one less accessible. The first one is easily degraded, being described as "unbound" in casein and peanut (Shantha & Sreenivasa-Murthy, 1981), following a first-order kinetics in its degradation in rice (Nkama, Mobbs, & Muller, 1987). Less accessible aflatoxin is described as "linked" and does not follow a first order kinetics for its degradation. These differences are probably related to the fact that aflatoxin located on the food surface is reachable by solar radiation, while the molecules located inside the food are protected by the limited penetration of this radiation. Studies about solar and ultraviolet radiations indicate that the double bond in the terminal furan ring of aflatoxin B1 is probably the photodegradation sensitive point (Samarajeewa et al., 1990).

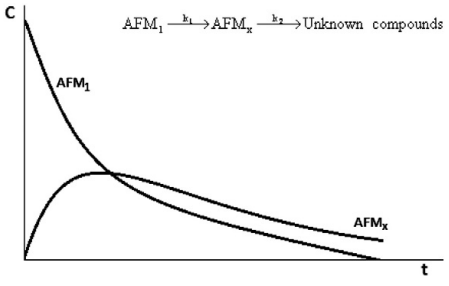

Fig. 2. Elimination of aflatoxin M_1 and M_x with UV irradiation at 60 °C (Adapted from Yousef & Marth, 1987).

Unlike in solid food, solar radiation can penetrate more easily in thin liquid films, providing a decontamination technique. Studies carried out in pilot plant scale have shown the efficiency of solar radiation on the degradation of aflatoxins in coconut oil and peanut oil (Shantha & Sreenivasa-Murthy, 1981; Samarajeewa, Jayatilaka, Ranjithan, Gamage, & Arseculeratne, 1985). Considering all the explained evidence, it seems that solar radiation can be effective in the degradation of aflatoxins.

4. Effect of UV and solar irradiation on enzymes

Enzymes play an important role in fruit juice manufacturing, both in clarified juices and in purees. Polyphenol oxidase (PPO) is the protein that causes the so-called enzymatic browning, so it is important to inactivate it to prevent the formation of melanins that cause juice color deterioration. In addition, other important enzymes in fruit juices are the pectic ones, especially pectinmethylesterase (PME), endo-polygalacturonase (endo-PG) and exo-polygalacturonase (exo-PG). The inactivation of pectic enzymes is essential in fruit juice derivatives in order to avoid losing its "cloud" and the sedimentation of the suspended solid fraction.

There are few studies about the effect of UV radiation on enzymes. Polyphenol oxidase, ATPase and acid phosphatase molecules may be denatured when they are irradiated with ultraviolet light, with a consequent reduction in its enzymatic activity (Seiji & Iwashita, 1965). The loss of enzyme activity is dependent on the intensity of radiation, reaching a 75% inactivation in the case of polyphenol oxidase and about 50% for the other two enzymes. In a work of ultraviolet treatment at 254 nm of orange juice (Tran & Farid, 2004), besides the effects on disinfection, commercial life and vitamin C, the authors also studied the effect on pectinmethylesterase activity. The juice was treated with 73.8 $mJ \cdot cm^{-2}$, obtaining an inactivation of 5%. However, it should be noted that the lamp used in this treatment only had an emission at 254 nm, so additional studies with lamps with a wider emission spectrum should be accomplished. In addition, this work was carried out with juices containing suspended pulp, which surely exert a protective effect against UV light. Falguera et al. (2011) managed to completely inactivate polyphenol oxidase after 100 min (Fig. 3) and peroxidase after 15 min of irradiation in apple juice from four different varieties with a mercury vapor lamp of 400 W of nominal power.

Ibarz, Garvin, Garza, and Pagan (2009) irradiated aqueous solutions of carboxypeptidase A and trypsin, using a medium pressure mercury lamp that emits in the range of 250–650 nm. These authors observed that it is possible to completely inactivate these two enzymes. The inactivation of the enzymes chymotrypsin, lysozyme, ribonuclease and trypsin by ultraviolet light at 253.7 nm (Luse & McLaren, 1963) can be related to the probability that the light is absorbed by a particular amino acid residue, where the link rupture is not important. McLaren, Gentile, Kirk, and Levin (1953) studied the

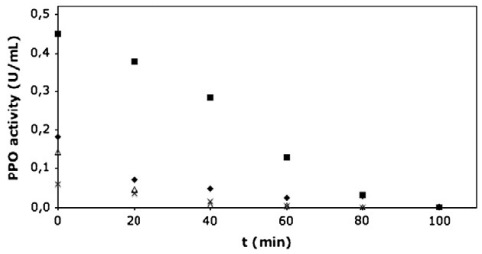

Fig. 3. Inactivation of PPO activity in apple juices from four different varieties with UV irradiation. ◆Golden. ■Starking. ΔFuji. × King David (Falguera et al., 2011).

inactivation of ribonuclease and carboxypeptidase using UV light (253.7 nm) with quantum yields of 0.03 and 0.001 to 0.005, respectively. The quantum yields for low molecular weight protein are around 0.03 and are higher than those of peptide bonds. It is postulated that the primary process of inactivation involves the modification of aromatic residues in proteins.

5. Effect of UV and solar irradiation on some properties and components of fruit juices

As it has been already introduced, the irradiation of fruit juices has a problem in that the optical path of radiation is small, because it is completely absorbed within a small distance from the surface. This is because most fruit juices are "opaque" to UV radiation due to suspended solids in juices with pulp and to the color of clarified ones. This coloration is due to the pigments extracted from the fruit itself and melanins and melanoidins from the enzymatic and non-enzymatic browning, respectively. The penetration depth of UV-C radiation through liquids is a few millimeters, in which 90% of incident light is absorbed (Sizer & Balasubramaniam, 1999). The penetration of UV light depends on the kind of fluid, on its absorption coefficient, on its soluble solids content and on suspended matter. The higher the soluble solid contents are, the lower the penetration. Therefore, UV light treatment of the juice is difficult due to the low transmittance of radiation through the juice. Thus, to ensure effective penetration of radiation in fruit juices it is necessary to have a good mixing of the reaction medium in the container, and in the case of using a continuous operation, to ensure that the fluid flows with high turbulence (Keyser et al., 2008).

There are still few studies conducted on fruit juices irradiation (Falguera et al., 2011; Farid, Chen, & Dost, 2000; Tran & Farid, 2004), although this treatment has been also used for the disinfection of sugar syrup in the treatment of beverages (Nakayama & Shinya, 1981; Gibbs, 2000). The heat-treated juices tend to change color and lose some of its aromas and vitamins during the heating process (Choi & Nielsen, 2005), while the juices treated with UV radiation tend to maintain its aroma and color (Tran & Farid, 2004).

Both melanins as melanoidins contained in fruit derivatives are polymeric compounds that provide a brown coloration, which is detrimental to sensory quality. The presence of melanin has a protective effect on UV inactivation of various enzymes (Seiji & Iwashita, 1965), probably due to the fact that melanin absorbs ultraviolet radiation. Nevertheless, melanoidins can be degraded when they are irradiated with UV–Vis (Kwak, Lee, Murata, & Homma, 2004). In addition, in the irradiation of clarified apple, peach and lemon juices (Ibarz, Pagán, Panadés, & Garza, 2005) important changes have been found in different colorimetric parameters, attributed to the destruction of both native pigments as the melanins and melanoidins present. As Fig. 4 shows, the pigment's impairment can be observed both in CIELab parameter variations and in the decreasing of absorbance in the whole visible range of the absorbance spectrum of the samples.

Ibarz and Pérez-Teijón (1990) conducted a direct exposure to sunlight of clarified apple juice, noting that there was a decrease in the absorbance at 420 nm value, suggesting a destruction of the juice pigments. In a later work, Ibarz et al. (2005) irradiated apple, peach and lemon juices with a lamp that emits in the range of 250–650 nm, studying the effect on CIELab colorimetric parameters. There was an increase of luminosity L^*, while the samples had a significant decrease in the parameters a^* and b^*, which was an indication that the pigments and compounds that give brown coloration were destroyed in this type of treatment. In the irradiation of apple juice with ultraviolet light to reduce the microbial load of the juice, Guerrero-Beltrán and Barbosa-Cánovas (2005) observed that irradiation treatment influenced color parameters, especially a^* and b^*, decreasing from a value of 2.4 to 1.4 and from 21.3 to 16.3, respectively. This indicates again that there is a photodegradation of apple juice pigments.

As mentioned, it is important to avoid the formation of melanins and melanoidins, both in processing and in storage of the juice. However, once they are in the juice it would be desirable to seek methods to eliminate these compounds from the samples. A possible method to be used in their removal is by adsorption with activated carbon or resins (Carabasa, Ibarz, Garza, & Barbosa-Cánovas, 1998; Ibarz, Garza, Garvín, & Pagán, 2008). An alternative may be found in the use of UV radiation. In this way, Kwak et al. (2004) studied the influence of pH on the photodegradation of model melanoidin solutions, using a Xe lamp that emits in the range of 200–1000 nm, and a halogen-tungsten lamp emitting in the range of 350–1000 nm.

Fruit juices, depending on the kind of fruit from which they come, contain different types of vitamins. Carotenoids are important constituents in many foods, because in addition to providing a distinctive color they may have potential health benefits (Klaui & Bauernfeind, 1981). Processing and storage conditions can degrade carotenoids, destroying the color and reducing the nutritional value of food. Pesek and Warthesen (1990) studied the kinetics of β-carotene

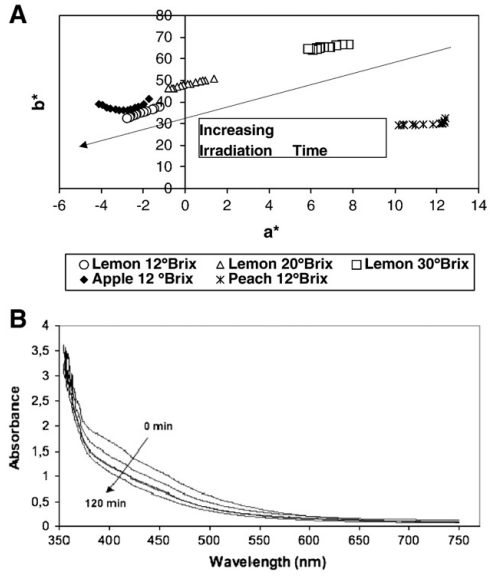

Fig. 4. UV effect on the impairment of fruit juice pigments. Evolution of a* and b* CIELab parameters in lemon, apple and peach juices (A, Ibarz et al., 2005) and the decrease of absorbance in the visible region in apple juice (B, Falguera et al., 2011).

photodegradation in irradiated model solutions and in carrot juice finding a first-order kinetics, isomer being cis the most sensitive to degradation. In another piece of work (Pesek & Warthesen, 1987) the same authors studied the effects of light on lycopene, α-and β-carotene in tomato and carrot juices, obtaining first-order kinetics with values of kinetic constants from 0.1 to 0.3 $days^{-1}$, lycopene being the most resistant molecule to photodegradation. It was also found that light-treated samples had higher values of brightness, while the colorimetric parameters a^* and b^* showed some decline (lower intensity of red and yellow), which can be related to degradation color of the studied carotenoids. These same researchers (Pesek & Warthesen, 1988) also studied the photodegradation of β-carotene in model systems containing this carotenoid at different temperatures, both in solid or liquid state, finding higher degradation rate constants at higher temperatures. Falguera et al. (2011) observed a loss of vitamin C between 4 and 6% in the irradiation of apple juices from the varieties Golden, Starking and Fuji for 120 min, while in the same processing conditions applied on the juice made from the variety King David the loss was 70%. These differences were attributed to the lack of pigmentation of the latter juice. Tran and Farid (2004) irradiated orange juice with an UV lamp that emits at 254 nm, finding a vitamin C degradation of 12% with a dose of 73.8 $mJ \cdot cm^{-2}$.

Another important problem that exists in the manufacture of juices is pesticide residues from fruit. In this regard, the effectiveness of ultraviolet irradiation on the decomposition of some of these compounds such as pyridine (Ibarz, Esplugas, & Costa, 1985), carbendazim (Ibarz & Pérez-Teijón, 1990), benomyl (Ibarz, Panadés, & Tejero, 1996), thiabendazole (Panadés, Alonso, & Ibarz, 1997) and indole (Ibarz, Tejero, Panadés, & Esplugas, 1998) has been proved.

6. Effect of UV and solar irradiation on some food compounds

Due to food complexity in terms of the variety of components they contain, UV radiation can affect many of these compounds. An extensive exposure of the effects of such radiation on the multitude of compounds present in food will not be made. However, some representative cases will be cited, trying to give an idea of the potential, both positive and negative, of the photochemical treatment processes.

Whited, Hammond, Chapman, and Boor (2002) studied the effect of light on the vitamin A in skimmed, semi-skimmed and whole milk, concluding that fat had a protective effect on the degradation of this vitamin. In all cases it was observed, apart from the loss of this vitamin, a loss of milk quality because in sensory analysis panelists highlighted the flavor of oxidized milk.

Riboflavin, known as vitamin B_{12}, is a yellow water-soluble vitamin, consisting of a dimenthyl isoalloxazine ring linked with ribitol, an alcohol derived from ribose. The three rings form the isoalloxazine and ribitol is the 5-carbon chain at the top. Furaya, Warthesen, and Labuza (1984) studied the photodegradation of riboflavin in macaroni, skimmed milk powder, and buffer solutions. In liquid systems these authors found first order photodegradation kinetics, while in solid food systems a two-step mechanism was observed.

Due to potential health problems that artificial food dyes may have, some attention has been paid to the development of natural pigments to be used as colorants. Among the red pigments, betalains and anthocyanins are potential pigment sources. However, natural pigments can be degraded by various agents. Light is one of these factors affecting their stability. In this way, the effect of light on betanine extracted from beet and on cranberry anthocyanins has been studied (Attoe & von Elbe, 1981). For both kinds of pigments it was found that photochemical degradation follows first order kinetics, observing that the presence of molecular oxygen in the medium plays an important role in the degradation of these compounds.

7. Final considerations

Ultraviolet treatments can be considered as a non-thermal processing emerging technology. UV processing can provide food with fresh features. Furthermore, UV-C radiation is lethal to most microorganisms and can be applied to ensure food safety. Currently, this technology is not widely used in food processing, but it could potentially be applied in liquid and solid foods, although in each process the correct type of lamp to use should be taken into account in order to optimize its effects on pathogens and spoilage. It is also very important to conduct studies on the effect that this type of radiation has on food, regarding nutritional and sensory evaluation.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.5.

Title: Characterization of polyphenol oxidase activity in juices from 12 underutilized tropical fruits with high agroindustrial potential.

Authors: Víctor Falguera, Andrea Milena Sánchez-Riaño, Juan Pablo Quintero-Cerón, Carlos Antonio Rivera-Barrero, Jonh Jairo Méndez-Arteaga & Albert Ibarz.

Journal: *Food and Bioprocess Technology*, (in press).

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COMMUNICATION

Characterization of Polyphenol Oxidase Activity in Juices from 12 Underutilized Tropical Fruits with High Agroindustrial Potential

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Abstract Polyphenol oxidase activities of lulo, mangosteen, Castilla blackberry, mango, apple guava, tree tomato, papaya, soursop, banana passionfruit, giant granadilla, sweet granadilla, and passion fruit were determined and related to their physicochemical properties. Mangosteen and lulo showed the highest activities (0.1435 and 0.1298 UmL^{-1}, respectively), while passion fruit and banana passionfruit had the lowest ones (0.0101 and 0.0093 UmL^{-1}). The two fruits with highest PPO activity were also the ones in which highest color differences (ΔE) during the first 90 min after fruit crushing were achieved. The Principal Component Analysis revealed a high correlation between PPO activity and total phenolic content for the compounds that are susceptible to act as enzyme substrate. These two variables were also highly correlated with the decrease produced in brightness (measured with the CIELab parameter L*).

Keywords Polyphenol oxidase · Enzymatic browning · Tropical fruits · Fruit characterization · Multivariate analysis

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Introduction

Polyphenol oxidase (PPO, tyrosinase, E.C. 1.14.18.1) is a copper-containing enzyme, widely distributed philogenetically from bacteria to mammals, which catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of a heterogeneous group of melanins (Falguera et al. 2010a).

Besides melanogenesis, plant PPOs have been reported to intervene in other physiological processes such as oxygen scavenging and defense mechanisms against plant pathogens and herbivore insects. In transgenic tomato plants, for example, the overexpression of PPO activity provides a greater resistance to cauliflower mosaic virus (Li and Steffens 2002). Phenolic compounds serve as precursors in the formation of physical polyphenolic barriers, limiting pathogen translocation. The quinones formed by PPO can bind plant proteins, reducing protein digestibility and their nutritive value to herbivores (Constabel et al. 2000; Queiroz et al. 2008). The techniques to control PPO activity should act on one or more of the essential components necessary for the reaction: oxygen, enzyme, copper, or substrate (Queiroz et al. 2008).

As it has been already introduced, PPO action mechanism is based on its capacity to oxidize phenolic compounds. In the fruits cells, the enzyme is located inside plastids while potential substrates are stored in vacuoles, and this separation in different organelles limits enzymatic

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browning. In post-harvest evolution, fruits undergo some physiological changes that lead to a decreased ability to eliminate active oxygen. Thus, membranes become more affected by oxidative activity, which results in enhanced lipid peroxidation, reduced membrane fluidity, and increased membrane permeability. After these transformations, or when the tissue is damaged, the rupture of plastids leads to the enzyme coming into contact with these phenolic compounds (Mayer and Harel 1979; Jiang et al. 2004). The wide range of polyphenol oxidase substrate specificity has led to many methods being proposed to measure its activity: radiometric, electrometric, chronometric, and especially spectrophotometric, which are fast and affordable by most laboratories (García-Molina et al. 2007; Falguera et al. 2010b).

Enzymatic browning is considered one of the main causes of post-harvest fruit deterioration, along with pigment degradation and peroxidase activity. In fact, PPO activity is still the major practical limitation to fruit handling, storage, and processing (Jiang et al. 2004). In addition, browning processes affect the nutritional quality and appearance, reduce consumer's acceptability, and cause significant economic impact, both to food producers and to food processing industry. In this way, tropical and subtropical fruits and vegetables are the most susceptible to these reactions (Queiroz et al. 2008).

In the other hand, growing demand in both developed and developing countries for diverse and novel foods is creating new markets for underutilized crops, which are cultivated on a relatively small-scale and have traditional uses in local areas (Gancel et al. 2008). These new demands are of special economic interest for tropical countries, where a great variety of fruits are cultivated. Nevertheless, the access for bio-diverse products to the European market needs many requirements, specially the knowledge of their composition and nutritional value. Besides their organoleptic quality, some of their real nutritional and physiological properties remain still undetermined (Mertz et al. 2009).

In the Colombian region of Tolima, there are many different species of these fruits with high agroindustrial potential, some of which are underutilized. Then, one of the main issues to determine their commercial possibilities is their PPO activity, which is the major practical limitation to fruit stability. In this work, PPO activity of 12 fruits cultivated and locally commercialized in the Tolima region has been evaluated and related to their composition properties. Searching for a practical application of these data, PPO characterization has been carried out directly from squeezed fresh fruits, since extracted and purified enzymes do not usually show real values for enzymatic activity as when PPO is in contact with other substances inside fruits (Bora et al. 2004).

Materials and Methods

Raw Material

Lulo var. Quitoense (*Solanum quitoense*), mangosteen var. purple (*Garcinia mangostana*), Castilla blackberry (*Rubus glaucus* Benth), mango var. Tommy Atkins (*Mangifera indica*), apple guava var. Klom sali (*Psidium guajava L.*), tree tomato var. Gold (*Cyphomandra betacea*), papaya var. yellow (*Carica papaya*), soursop var. Elita (*Annona muricata*), banana passionfruit var. mollissima (*Passiflora tripartita*), giant granadilla (*Passiflora quadrangularis L.*), sweet granadilla var. Valluna (*Passiflora ligularis Juss*), and passion fruit var. yellow (*Passiflora edulis f. flavicarpa*) were purchased from a local market located in Ibagué (Colombia). These fruits were washed, peeled, and kept in water while waiting to be cut and squeezed with a household juicer. The resulting juices were centrifuged using a Hettich EBA 20 centrifuge (Andreas Hettich GmbH & Co., Tuttlingen, Germany) for 20 min at 2,500 rpm. After centrifugation was complete, the supernatant was recovered, and the pellet was discarded. All these steps were carried out in a refrigerated room (temperature 20±1 °C) in order to slow enzymatic processes in the juices. Fractions of 250 mL of juice were stored in the freezer at −12 °C for a maximum of 2 days. All the determinations were carried out on portions of the same sample.

Physical and Chemical Analysis

The pH of the samples was measured with a Handylab pH 11 pHmeter (Schott Instruments GmbH, Mainz, Germany). Soluble solids content was assessed using an HP Handheld Brix 35HP refractometer (Reicherd Instruments, GmbH, Seefeld, Germany). Juices color was monitored using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Japan) in the CIELab color space. The color measures were carried out immediately after juice extraction and after 90 min keeping juices at room temperature (30 °C). Absorption spectrum between 400 and 750 nm was measured with a Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1-cm width optical glass cell.

Total phenolic content was determined as described by Shaghaghi et al. (2008), with a spectrophotometric method using the Folin-Ciocalteu reagent. Formol index was measured according to the official method of the International Federation of Fruit Juice Producers (IFFJP 1972), with a potentiometric titration of the acidity of the compounds formed by the reaction of formaldehyde and amino acids in the juice up to a pH of 8.1. Total and

reducing sugars were determined by the reduction of the Fehling reagent, according to the method of the IFFJP (1972). Titratable acidity was also assessed by the official method of the IFFJP (1972), with a potentiometric titration with sodium hydroxide to a pH value of 8.1. All the analyses were carried out by duplicate. Maturity index was calculated as the ratio of total soluble solids to titratable acidity (Ladaniya 2008).

Polyphenol Oxidase Activity

PPO activity in the samples was assayed measuring the increase in absorbance at 420 nm using catechol as a substrate, prepared in a citrate buffer solution with a pH of 4.0. The reaction was carried out in a 1 cm light path optical glass cell, containing 2.5 mL of 5 mM catechol and 1.0 mL of the sample. One unit of PPO was defined as the amount of enzyme that caused the increase of one unit of absorbance at 420 nm in 10 min (Ülker-Yerlitürk et al. 2008). PPO activity was assayed by duplicate.

Absorbance Spectra

Absorption spectra of the visible range (between 400 and 700 nm) was measured with a Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1-cm width optical glass cell. These measurements were accomplished to dilutions 1:10 of the samples with distilled water and at two different times: on the fresh juices and after 24 h with the samples kept at room temperature (30±1 °C).

Principal Component Analysis

Multivariate projection techniques have been used in this study to determine the most important variables for data characterization and to see if there are any group patterns. These techniques have been widely applied for quality assessment, authentication, or even classification of a great variety of food products, including vegetables, fruits, juices, wines, oils, and others (Tzouros and Arvanitoyannis 2001). In this case, a Principal Component Analysis (PCA) of all the data obtained in the samples characterization was carried out using the Unscrambler v. 6.11a software (Trondheim, Norway; Camo 1996).

Moreover, another group of variables was created from absorbance spectra of the samples, corresponding to the differences between the asorbances of the fresh juice and the ones measured 24 h later. An additional PCA was carried out with these spectrophotometrical data in order to infer the most important wavelengths to follow the color changes produced by enzymatic browning.

Results and Discussion

Polyphenol Oxidase Activity

Polyphenol oxidase activities of the 12 analyzed fruits appear in Table 1. Mangosteen and lulo showed the highest activities (0.1435 and 0.1298 UmL^{-1}, respectively), while passion fruit and banana passionfruit had the lowest ones (0.0101 and 0.0093 UmL^{-1}). Generally, except mangosteen

Table 1 Physicochemical properties of the 12 tropical fruits

Fruits	pH	Titratable acidity	Formol index	Total phenolics	Soluble solids	Total sugars	Reducing sugars	Non-reducing sugars	Maturity index[a]
	–	$g{\cdot}L^{-1}$ malic acid	mL NaOH 0.1 M/100 mL	$mg{\cdot}L^{-1}$ gallic acid	ºBrix	$g{\cdot}L^{-1}$ glucose	$g{\cdot}L^{-1}$ glucose	$g{\cdot}L^{-1}$ glucose	$ºBrix{\cdot}L{\cdot}g^{-1}$
Lulo	3.45±0.01	0.315±0.005	37.5±0.5	433.9±16.5	8.1±0.1	33.8±0.9	16.8±0.3	17.1±0.6	26
Mangosteen	3.36±0.01	0.056±0.002	13.5±0.5	644.7±42.3	16.1±0.1	96.6±7.1	25.4±0.7	71.2±6.5	288
Castilla blackberry	3.41±0.01	0.281±0.002	31.0±0.1	757.0±31.3	7.1±0.1	22.4±0.4	13.6±0.4	8.8±0.1	25
Mango	3.63±0.00	0.061±0.001	5.0±0.1	908.7±24.2	12.3±0.3	90.0±4.1	26.8±0.1	63.2±4.1	202
Apple guava	3.96±0.02	0.056±0.002	18.5±1.5	854.1±5.4	7.9±0.1	47.1±0.1	45.0±2.0	2.1±2.0	141
Banana passionfruit	3.21±0.01	0.364±0.001	28.5±0.5	805.8±29.2	7.7±0.1	110.0±6.1	61.9±3.9	48.1±2.2	21
Giant granadilla	4.35±0.02	0.14±0.002	33.5±0.5	335.5±79.4	15.1±0.1	82.5±3.4	56.6±1.6	25.9±1.8	108
Tree tomato	4.00±0.00	0.175±0.003	57.5±0.5	468.5±13.5	8.1±0.1	43.5±0.5	32.5±2.7	11.1±2.2	46
Papaya	4.97±0.02	0.01±0.001	10.0±0.1	243.7±7.9	9.3±0.1	67.1±5.7	60.0±1.8	7.1±7.5	930
Sweet granadilla	4.73±0.02	0.04±0.001	20.0±0.1	434.9±1.3	13.4±0.1	107.0±8.7	45.0±0.1	62.0±8.7	335
Passion fruit	2.89±0.02	0.441±0.003	27.5±0.5	433.5±10.2	14.2±0.1	74.7±4.2	50.8±1.3	23.9±5.5	32
Soursop	3.74±0.01	0.116±0.002	19.0±0.5	285.1±7.3	14.5±0.1	99.0±10.0	63.9±2.1	35.1±7.9	125

[a] Ratio soluble solids/titratable acidity

Table 2 Polyphenol oxidase activity of the 12 tropical fruits

Fruits	Measured in U·mL^{-1}
Lulo	0.1298±0.0002
Mangosteen	0.1435±0.0037
Castilla blackberry	0.0347±0.0005
Mango	0.0159±0.0021
Apple guava	0.0153±0.0002
Banana passionfruit	0.0093±0.0003
Giant granadilla	0.0189±0.0059
Tree tomato	0.0124±0.0001
Papaya	0.0125±0.0017
Sweet granadilla	0.0321±0.0006
Passion fruit	0.0101±0.0054
Soursop	0.0384±0.0003

and lulo, all these tropical fruits showed significantly lower polyphenol oxidase activities than the ones found in Spanish apples from four different varieties in a previous work (Falguera et al. 2011). In that case, the obtained values were 0.4485 UmL^{-1} in Starking apple, 0.1830 UmL^{-1} in Golden, 0.1435 UmL^{-1} in Fuji, and 0.0600 U·mL^{-1} in King David.

Physicochemical Properties

Table 2 shows composition and physicochemical properties of the 12 analyzed tropical fruits. The pH of the samples, which has been reported to be one of the main factors that influence polyphenol oxidase activity (Queiroz et al. 2008), had the lowest value in passion fruit (2.89) and the highest one in papaya (4.97). Total phenolics, the molecules that are susceptible to act as PPO substrates, were found to be in a range between 243.7 mg L^{-1} (papaya) and 908.7 mg L^{-1} (mango) of gallic acid equivalents. Cano et al. (1996) found that PPO activity in papaya is very limited mainly due to the low phenolic content. In contrast, mango showed to have the highest content, but according to Arogba et al. (1998), the presence of endogenous PPO inhibitors in mango tissues can limit enzymatic browning in this fruit. Although mangosteen and lulo showed to have an intermediate total phenolic content (644.7 and 433.9 mg L^{-1} of gallic acid equivalents, respectively), some of these compounds have been reported to have important antioxidant, free radical scavenging, and skin protective activities, especially α-mangostin, epicatechin, and tannin in mangosteen and chlorogenic acids, flavonol glycosides, and dihydrocaffeoyl spermidines in lulo (Gancel et al. 2008; Ngawhirunpat et al. 2010). On the contrary, apple guava samples contained a higher phenolic concentration (854.1 mg L^{-1}), but its composition does not seem to have significant antioxidant activity (Rojas-Barquera and Narváez-Cuenca 2009). The assayed physicochemical properties were significantly different within the four fruits from the *Passiflora* genus, which was also observed by Aular et al. (2004).

Color Changes

Polyphenol oxidase, as the main responsible factor for enzymatic browning, causes color changes in fruit pulps that can be easily assessed measuring CIELab parameters, especially the decrease of brightness with L^* (Lozano et al. 1994). In this way, Table 3 shows these values for some of the assayed fruits. L^* decreased more in the fruits that presented a higher PPO activity: 10.51 units in lulo and 9.01 units in mangosteen. In addition, lulo showed the greatest variation in b^* (a decrease of 13.12 units) and mangosteen in a^* (an increase of 5.59 units). These two fruits also showed the highest ΔE value. Although papaya had a relatively low PPO activity and consequently a low decrease of L^* was detected (1.62 units), important changes were found in the pulp color during the first 90 min after fruit crushing, leading to a decrease of 5.19 units in a^* and 6.08 units in b^*. The low PPO activity found in papaya could be attributed to the fact that the fruits were in the

Table 3 Color parameters of studied tropical fruits juices: fresh and 90 min after extraction

Fruits	L*		a*		b*		ΔE
	0 min	90 min	0 min	90 min	0 min	90 min	
Lulo	53.64±0.37	43.13±0.01	−3.88±0.02	−1.08±0.03	34.34±0.01	21.22±0.09	17.00±0.40
Mangosteen	61.35±0.13	52.34±0.09	3.18±0.02	8.77±0.04	11.86±0.1	10.87±0.08	10.65±0.05
Castilla blackberry	29.41±0.01	26.78±0.03	4.65±0.04	2.65±0.03	1.19±0.01	0.59±0.01	3.36±0.02
Banana passionfruit	57.00±0.03	55.69±0.01	13.28±0.02	11.92±0.01	32.4±0.1	32.32±0.07	1.89±0.04
Papaya	34.34±0.07	32.72±0.01	16.18±0.06	11.00±0.01	17.99±0.11	11.91±0.03	8.15±0.12
Sweet granadilla	42.28±0.01	39.04±0.11	−2.27±0.02	−2.32±0.01	4.72±0.07	4.90±0.10	3.25±0.12
Passion fruit	45.44±0.13	43.69±0.01	−2.01±0.03	−1.69±0.01	29.74±0.02	27.29±0.19	3.03±0.21
Soursop	74.20±0.01	72.65±0.02	−1.75±0.01	−1.97±0.01	8.26±0.01	7.28±0.02	1.85±0.01

Data expressed in the CIELab color space

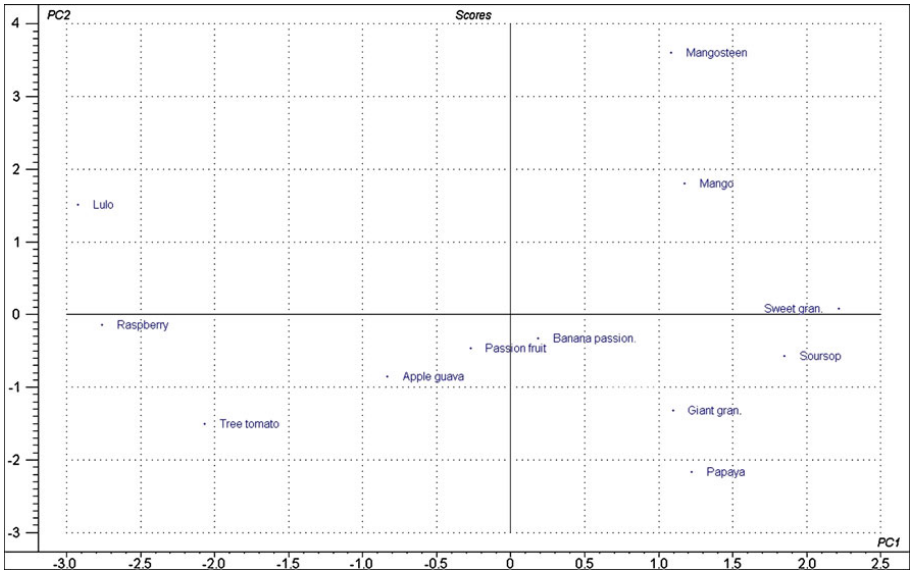

Fig. 1 Composition variable loadings represented in the Principal Component space

climacteric phase of the ripening process, and it is known that the enzyme activity decreases significantly during this stage (Cano et al. 1996). In this way, these color changes can be due to other processes like natural pigments degradation or peroxidase activity (Jiang et al. 2004).

Principal Component Analysis

A PCA was carried out in order to determine the variables that explain the maximum information and consequently show the general tendencies present in the samples. This

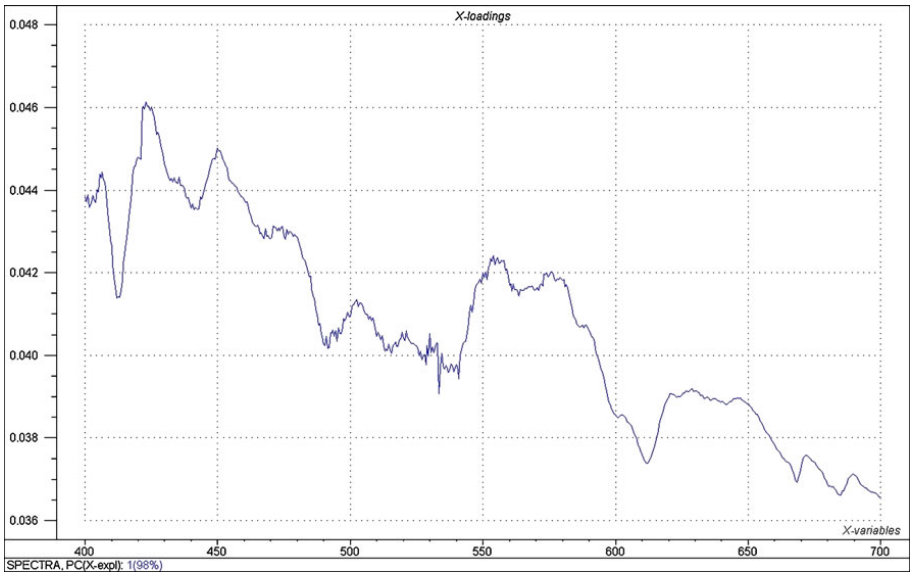

Fig. 2 Values of the spectrophotometric variable loadings for the first Principal Component, which explains 98% of the overall variance

data processing, which has also been applied to find useful correlations to authenticate different products such as olive oil (Arvanitoyannis and Vlachos 2007) and honey (Arvanitoyannis et al. 2005), was accomplished with the values of physicochemical variables, polyphenol oxidase activity and the change observed in the CIELab parameter L^* in the first 90 min after the juice extraction, since it has been reported to be the main color indicator of fruit browning (Lozano et al. 1994; total ten variables). Figure 1 represents variable loadings in the PC1 vs PC2 space, in which 55% of the global variance is explained. First of all, the analysis revealed that the most important variable that influences fruit variability is total sugars content, which is highly correlated with soluble solids.

This diagram also shows that there are two variables strongly related with PPO activity: total phenolic content and L^* variation. This indicates that, on the one hand, in most of the samples PPO is more active in those fruits with higher phenolic content, as these molecules are more suitable to contact the enzyme and act as its substrate. On the other hand, PPO catalysis leads to enzymatic browning, which can be easily observed and measured by the decrease of the CIELab parameter L^*.

The PCA carried out on the spectrophotometrical data revealed that one single PC was enough to describe the 98% of the overall variance. This fact means that the existing data are highly correlated, showing that enzymatic browning affects absorbances in a similar way in all samples. Observing variable loadings of this first PC (Fig. 2) an absolute maximum is found in 423.0 nm, so that the colored compounds formed by polyphenol oxidase activity in the studied fruit juices have their maximum absorbance at this wavelength, and therefore this would be the best one to follow these enzymatic changes. Sample scores (not shown) revealed that the most important samples were the ones with highest polyphenol oxidase activities.

Conclusions

Mangosteen and lulo showed the highest polyphenol oxidase activities (0.1435 and 0.1298 UmL^{-1}, respectively), while passion fruit and banana passionfruit had the lowest ones (0.0101 and 0.0093 UmL^{-1}). Total phenolics, potentially PPO substrates, were found to be in a range between 243.7 (papaya) and 908.7 mg L^{-1} (mango) of gallic acid equivalents. PPO activity in papaya can be very limited mainly due to the low phenolic content. In contrast, mango showed to have the highest content, but the presence of endogenous PPO inhibitors can limit enzymatic browning in this fruit. The assayed physicochemical properties were significantly different within the four fruits from the *Passiflora* genus.

Polyphenol oxidase causes color changes in fruit pulps that can be easily assessed measuring the decrease of brightness by means of the CIELab L^*. This parameter decreased more in the fruits that presented a higher PPO activity: 10.51 units in lulo and 9.01 units in mangosteen. Lulo also showed the greatest variation in b^* (a decrease of 13.12 units) and mangosteen in a^* (an increase of 5.59 units). Although papaya had a relatively low PPO activity and consequently a low decrease of L^* was detected (1.62 units), a* and b* parameters changed significantly, probably due to other processes like natural pigments degradation or peroxidase activity.

The Principal Component Analysis revealed that the most important variables that influence overall variability are total sugars content and soluble solids. In addition, two variables were found to be strongly related with PPO activity: total phenolic content and L^* variation. Thus, PPO is more active in those fruits with higher phenolic content, and this process leads a greater decrease of the CIELab parameter L^*.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.6.

Title: Influence of nitrogen fertilization on polyphenol oxidase activity in peach fruits.

Authors: Víctor Falguera, Joan Lordan, Ferran Gatius, Miquel Pascual, Josep M. Villar, Albert Ibarz & Josep Rufat.

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Short communication

Influence of nitrogen fertilization on polyphenol oxidase activity in peach fruits

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ABSTRACT

Generally, when nitrogen effects on fruit properties are assessed, different samples harvested at different maturity stages are assayed. This method makes it difficult to investigate the direct effects of fertilization on the different quality parameters without the influence of the ripening stage. In this piece of work, the effects of three levels of nitrogen fertilization (0, 60 and 120 kg/ha) on polyphenol oxidase (PPO) activity and consistency of peach fruits at the same maturity stage were studied. The results showed that nitrogen application (regardless the dose) had a direct effect on the studied parameters, causing an increase of PPO activity and also an increase of fruit consistency. However, no significant differences were found between the application of 60 and 120 kgN/ha. This enhanced activity of the enzyme may represent a problem in peach postharvest handling and processing.

1. Introduction

Prevention of enzymatic browning is, along with product safety, one of the major challenges for fruit and vegetable industries. This phenomenon, which is considered the most important practical limitation to fruit handling, storage and processing (Jiang et al., 2004), is mainly caused by the action of polyphenol oxidase (PPO; E.C. 1.14.18.1) (de la Rosa et al., 2010; Falguera et al., in press). PPO is a copper-containing enzyme, widely distributed philogenetically from bacteria to mammals, which catalyzes two different reactions involving molecular oxygen and various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these unstable compounds leads to the formation of a heterogeneous group of dark polymers, called melanins (Falguera et al., in press).

In fruits cells, the enzyme is located inside plastids while its substrates are stored in vacuoles, and therefore this separation limits its enzymatic browning. In post-harvest evolution, fruits undergo some physiological changes that lead to a decreased ability to eliminate active oxygen, which results in enhanced lipid peroxidation, reduced membrane fluidity and increased membrane permeability. After these processes, as well as after tissue physical damage, plastids are broken and PPO comes into contact with its substrates, developing fruit browning.

PPO activity in fruits has been reported to be dependent on several factors such as the own fruit, the variety, the ripening stage or even the extraction and analysis conditions (Carbonaro and Mattera, 2001; Lee et al., 1990). In this way, the presence of endogenous phenolic substrates and quinones in the enzyme extract may interfere in the measured activity, making it impossible to find small differences between samples. In order to avoid this problem, phenolic-binding agents are commonly used, such as polyethylene-glycol or polyvinylpolypyrrolidone (PVPP).

Fertilization and pesticide application on trees have been proved to have a definitive effect on the ripening process and phenolic expression (Carbonaro and Mattera, 2001; Nicolas et al., 1994). In particular, nitrogen application has been related to maturity and quality factors: adding excessive amounts causes delays in maturation and unbalanced nutrition in calcareous soils, while insufficient quantities reduce tree growth and harvest yields (Falguera et al., 2012; Rufat et al., 2011). However, there is no information in literature about the direct impact of nitrogen fertilization on PPO activity in peach fruits, since all the previous studies refer to its impact on fruit ripening.

Therefore, since applying the appropriate nitrogen dose is important to peach production yield and quality, and PPO activity is an essential parameter to assess postharvest quality, this work aims at the characterization of the effect of nitrogen application

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Table 1
Polyphenol oxidase (PPO) activity units; mesocarp firmness (*N*) and color parameters (CIELab L^*, a^*, b^*); maturity index (SS/AC) and Bostwick's index (cm/30 s). Means with different letters are significantly different according to the Tukey HSD test ($p < 0.05$).

	PPO	Firmness	L^* flesh	a^* flesh	b^* flesh	Maturity index	Bostwick's index
Model	0.0077	0.0174	ns	ns	ns	ns	0.0013
N	0.0049	ns	ns	ns	ns	ns	0.0006
BLOCK	ns	0.0127	ns	0.0483	0.0057	ns	ns
N 0 kg/ha	b 0.23 ± 0.03	32.7 ± 8.1	63.8 ± 3.6	3.0 ± 1.7	53.5 ± 4.5	2.3 ± 0.3	a 6.7 ± 1.1
N 60 kg/ha	a 0.29 ± 0.04	32.8 ± 6.0	65.1 ± 2.1	3.1 ± 1.4	53.7 ± 3.1	2.6 ± 0.5	b 5.1 ± 0.9
N 120 kg/ha	a 0.28 ± 0.04	36.9 ± 7.2	66.5 ± 2.9	3.1 ± 2.2	54.7 ± 2.1	2.3 ± 0.3	b 5.4 ± 1.0

on the expression of this enzyme in peach fruits. Moreover, additional maturity parameters have also been assessed in relationship to the different nitrogen application levels, so as to guarantee that the used samples were in the same maturity stage.

2. Materials and methods

2.1. Experimental site and fruit sampling

Eleven-year-old peach trees (*Prunus persica* (L.) Batsch. cv. Andross) on GF-677 rootstock were used and trained to a free palmeta. The trees were planted on shallow loam soil at 5.0 m by 2.8 m at a commercial peach orchard for processing (preparing processed purées) in the horticultural zone of Lleida (Northeast of Spain). Annual precipitation was 355 mm and annual reference evapotranspiration 1172 mm. Soil organic matter concentration was 3.2%.

A randomized complete block design with four repetitions was established. Three nitrogen fertilization treatments were evaluated: 0 kgN/ha (N-0), 60 kgN/ha (N-60) and 120 kgN/ha (N-120). An automated drip-fertigation system with auto compensated emitters was used. Each elementary plot consisted of 27 trees distributed in three rows and determinations were done on the 5 central trees. Trees were mechanically harvested on August 5th, 2011, with a continuous trunk shaker. Sampling was done the previous day. Fruit samples consisted of five fruits per individual plot.

2.2. Physical, chemical and enzymatic analyses

Mesocarp firmness (F) was determined with a manual penetrometer (Penefel, France). Results are expressed as N. Total soluble solids (SS) concentration (°Brix) was measured using a thermocompensated refractometer (Atago Bussan Co., Tokyo, Japan). Titratable acidity (AC) was obtained from 10 ml of juice of each sample, adding 10 ml of distilled water and titrated with NaOH 0.1 N. Results are expressed as g of malic acid per L of juice. Maturity index (MI) was obtained as the ratio between SS and AC.

In order to assess consistency and fruit flesh color, the fruits were crushed and homogenized. Consistency (CON) was measured using a Bostwick consistometer (Central Scientific Co., Alexandria, VA) in centimeters of flow per 30 s. Flesh color was measured with a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Japan) in the CIELab color space. Parameters a^*, b^* and L^* were determined.

For polyphenol oxidase activity determination, 10 g of crushed peach flesh were mixed with 10 ml of McIlvaine buffer (pH 6.6) and 0.51 g (2.5%, w/v) of polyvinylpolypyrrolidone (PVPP) as phenolic scavenger. The mix was homogenized and centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 10 min at 5500 × *g* (RCF) and 5 °C. After centrifugation was complete, the pellet was discarded and the supernatant was used for PPO analysis, using 10 mM 4-methylcatechol (Sigma Chemical, St. Louis, MO, USA) as substrate, prepared in pH 6.6 McIlvaine buffer. The reaction was carried out in a 1 cm light path quartz cell, and the absorbance at 420 nm was recorded for 3 min by means of a Helios Omega spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and its software. One unit (U) of PPO was defined as the amount of enzyme that caused the increase of one absorbance unit (AU) at 420 nm in 1 min (Falguera et al., in press).

2.3. Statistical analysis

Statistical analysis of data was carried out using the SAS-STAT package (SAS®, Version 9.2. SAS Institute Inc., Cary, NC, 1989–2009). Analysis of variance (ANOVA) was carried out for Nitrogen effects. Means were compared using the Tukey HSD and contrast tests. A discriminant analysis was performed with SAS PROC CANDISC.

3. Results and discussion

The analysis of variance (Table 1) showed that, among the studied parameters, only polyphenol oxidase (PPO) activity and Bostwick's parameter (consistency, CON) were significantly affected by nitrogen application ($p < 0.005$ for the former and $p < 0.0006$ for the latter). If nitrogen fertilization is applied PPO activity increases, while Bostwick's value decreases. However, in both cases there were no differences between the application of 60 or 120 kg/ha. In other words, PPO activity and consistency of peach fruits are affected by nitrogen supplementation, but an excessive dose provides no differences.

On the other hand, firmness was not found to be affected by fertilization, meaning that in the used samples there was not a delay in fruit maturation because of nitrogen. Since all the other important parameters for assessing fruit maturity (color parameters and maturity index) do not show any differences with nitrogen application and dose, the different values for PPO activity are a direct response to nitrogen application and not to fruit maturity stage.

It is generally assumed that as fruit matures, PPO activity increases (Ferrer et al., 2005) and Bostwick's parameter also increases (consistency decreases) (Falguera et al., 2012). In addition, it is also proved that nitrogen fertilization causes a delay in the ripening process (Rufat et al., 2011). Generally, when nitrogen effects on fruit properties are assessed, different samples harvested at the same date are usually assayed, which therefore are in different maturity stages. This method makes it difficult to investigate the direct effects of fertilization on the different quality parameters without the influence of the ripening stage. However, the findings of this study, which has been carried out on peach fruits that are statistically at the same maturity stage, show that nitrogen application (regardless the dose) caused a decrease of Bostwick's index, but also an increase of PPO activity, i.e. as regards to PPO, nitrogen had the opposite effect to a delay of ripening.

The conclusions about the effect of nitrogen application on the assayed variables can also be reached by means of the canonical discriminant analysis (Fig. 1). In this multivariate statistical technique, 95% confidence ellipses appear separated in the first canonical variable (which represents 83.1% of the overall variance) according to nitrogen application (or not), while the ones that belong to the different doses (60 and 120 kg/ha) appear overlapped. Regarding the

Table 2
Correlation coefficients between CIELab color parameters of fruit mesocarp, maturity index, firmness and Bostwick's index.

	Maturity index	Firmness	Bostwick's index	b^*	a^*	L^*
PPO activity	−0.33	0.14	−0.33*	0.34	−0.10	0.11
L^*	−0.06	−0.01	−0.14	0.42*	−0.45*	
a^*	0.28	−0.07	0.16	−0.03		
b^*	−0.25	0.15	−0.29			
Bostwick's index	−0.15	−0.26				
Firmness	−0.11					

* denotes a significant correlation ($p < 0.05$).

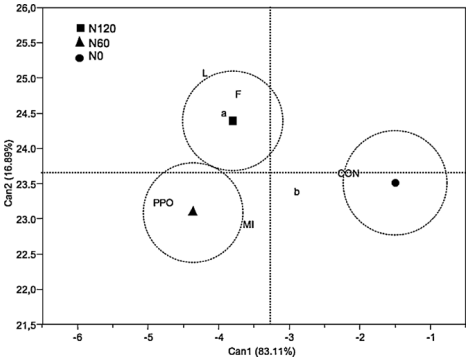

Fig. 1. Plot of the centroids (mean values) and their 95% confidence ellipses for the first two canonical variables of nitrogen treatments. Abbreviations for variables are defined in Section 2.

distribution of the measured parameters along the first canonical variable (*x* axis), Bostwick's index (CON) appears related to the non-application of N (0 kg/ha), while PPO activity (as well as firmness, L^* and a^*) has *x* values similar to those of 60 and 120 kg/ha. Although closer values of this first canonical variable indicate higher values of the parameter, these relationships were only found to be significant in the case of PPO and Bostwick's index (Table 1).

As far as the relationship between the different studied variables is concerned, PPO activity and Bostwick's index, the only two variables that were strongly affected by nitrogen fertilization, were found to be negatively correlated ($p < 0.05$; Table 2), which is consistent with the results observed in Table 1 and previously commented. Neither maturity index nor firmness was significantly correlated with any other variable.

To sum up, it can be stated that nitrogen fertilization directly enhances polyphenol oxidase activity and increases fruit consistency (decreases Bostwick's index) in peach fruits, besides the effect that it can have delaying the ripening process. Therefore, if nitrogen supplementation is applied, although the fruits may reach their commercial maturity later, at that time their PPO activity will be higher, with the subsequent problems in postharvest handling and processing. In other words, it seems that nitrogen fertilization delays some of the processes that lead to peach maturation, but instead of delaying the synthesis of polyphenol oxidase, it enhances its activity. However, no significant differences were found between the application of 60 and 120 kgN/ha.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.7.

Title: A kinetic model describing melanin formation by means of mushroom tyrosinase.

Authors: Víctor Falguera, Jordi Pagán & Albert Ibarz.

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A kinetic model describing melanin formation by means of mushroom tyrosinase

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ABSTRACT

Melanins are an heterogeneous group of polymers formed by enzymatic reactions in vegetable tissues containing phenolic or polyphenolic molecules. Recent studies have discovered some beneficial properties of melanins on health, such as anti-oxidative, anti-inflammatory, immune and anti-tumor properties, so not only its elimination should be reconsidered, but also its addition could be proposed to functional food of new creation. Then, a further knowledge about the kinetic mechanism of melanogenesis is required prior to its possible industrial utilization. In this work, an autocatalytic kinetic model to explain melanin formation from L-tyrosine using mushroom tyrosinase and measuring the absorbance of the solution has been developed and fitted to experimental data. This expression allows to describe melanin formation as a function of time of reaction, including some important parameters such as the extinction coefficient. Absorbance will start growing after a lag period in which colorless intermediates are produced. The extinction coefficient of the resulting products is not a constant value, because it depends on the conditions of each experiment. Tyrosinase seemed to have a lower catalytic effect on L-tyrosine than on L-DOPA.

1. Introduction

Tyrosinase (polyphenoloxidase, PPO, E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of an heterogeneous group of melanins (Duckworth & Coleman, 1970; Muñoz-Muñoz et al., 2007; Ziyan & Pekyardimci, 2003).

Tyrosinase converts L-tyrosine, a monophenol, firstly to L-DOPA (an *o*-diphenol) and this to *o*-dopaquinone, which is spontaneously cyclated in form of leukodopachrome and quickly converted to dopachrome, which polymerizes forming melanins (De Faria, Moure, Lopes, Krieger, & Mitchell, 2007). These reactions are shown in Fig. 1.

Initial studies of tyrosinases were motivated by a desire to understand and prevent the enzymatic browning that occurs in the presence of air when mushrooms, fruits or vegetables are cut or bruised. These melanins, formed by these enzymatic reactions in vegetable tissues containing phenolic or polyphenolic molecules, have been considered up to the present substances that produce deterioration in many foods, specially in fruit juices and other fruit derivatives, and decrease its market value. Nevertheless, recent discoveries on beneficial properties on health, such as anti-oxidative, anti-inflammatory, immune and anti-tumor properties, have done that not only its elimination should be reconsidered, but also its addition could be proposed to functional food of new creation. In addition, some intermediate products have been used as medicines, like L-DOPA which has been the preferred drug for treatment of Parkinson's diseases since 1967 (De Faria et al., 2007).

The wide range of substrate specificity of polyphenoloxidase has led to many methods being proposed to measure its activity: radiometric, electrometric, chronometric and especially spectrophotometric, which are fast and affordable by most laboratories (García-Molina et al., 2007). To implement these benefits that have recently been discovered, it is fundamental to have a mathematical expression to easily predict the quantity and characteristics of the melanin that can be synthesized. The aim of this work has been to develop and check the validity of a kinetic expression that allows describing and predicting melanin formation as a function of the reaction time using a fast and cheap analysis method like spectrophotometric measurements.

2. Kinetic considerations

As it has been explained, the first step to apply this enzymatic process is to obtain a kinetic characterization of melanin formation. In this work, a kinetic model to explain this reaction as a spectrophotometrical function has been developed. According to the reaction mechanism that leads L-tyrosine to melanins (De Faria

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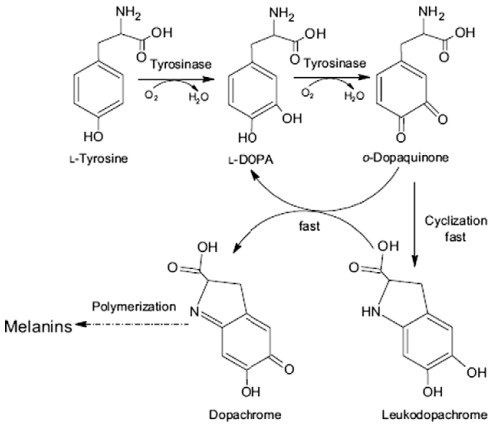

Fig. 1. Mechanism by which tyrosinase converts L-tyrosine firstly to L-DOPA and then to *o*-dopaquinone, and the following steps that lead to melanin formation (Adapted from De Faria et al., 2007).

et al., 2007), an autocatalytical reaction in two-steps, each one defined by a kinetic constant, has been assumed:

$$L\text{-Tyrosine} \xrightarrow{k_1} \text{Intermediate}$$

$$L\text{-Tyrosine} + \text{Intermediate} \xrightarrow{k_2} 2\ \text{Melanin}$$

The mass balance for the global reaction in a discontinuous stirred reactor would be (Levenspiel, 1986):

$$\frac{dC_A}{dt} = -(k_1 \cdot C_A + k_2 \cdot C_A \cdot C_M) \tag{1}$$

where C_A is the tyrosine concentration and C_M the melanin concentration.

For each time of reaction, melanin concentration (C_M) can be expressed as the difference between the initial tyrosine concentration (C_A^0) and the concentration at this moment (C_A):

$$C_A^0 - C_A = C_M$$

Then, using this expression in the mass balance and rearranging it:

$$\frac{dC_A}{k_1 \cdot C_A + k_2 \cdot C_A\ (C_A^0 - C_A)} = -dt$$

Now, the integration can be done with the boundary conditions:

$$\text{For} \quad t = 0 \rightarrow \quad C_A = C_A^0$$
$$\text{For} \quad t = t \rightarrow \quad C_A = C_A$$

So then:

$$\int_0^t dt = \int_{C_A}^{C_A^0} \frac{dC_A}{k_2 \cdot C_A\left[\left(\frac{k_1 + k_2 C_A^0}{k_2}\right) - C_A\right]} \tag{2}$$

The result of this integration leads to:

$$\ln\left[\frac{C_A^0\left[\left(\frac{k_1 + k_2 C_A^0}{k_2}\right) - C_A\right]}{C_A\left[\left(\frac{k_1 + k_2 C_A^0}{k_2}\right) - C_A^0\right]}\right] = (k_1 + k_2 C_A^0)(t - t_0) \tag{3}$$

Being X_A the fractional conversion of tyrosine, the substrate concentration in each moment can be expressed in the following way:

$$C_A = C_A^0(1 - X_A) = C_A^0 - C_A^0 X_A \tag{4}$$

Then, using expression (4) in (3):

$$\ln\left(\frac{1 + \frac{k_2}{k_1} C_A^0 X_A}{1 - X_A}\right) = (k_1 + k_2 C_A^0)(t - t_0) \tag{5}$$

Rearranging this expression and using the Lambert–Beer law in order to obtain the absorbance as a function of the reaction time it is obtaining:

$$A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_1 \cdot [\exp[(k_1 + k_2 \cdot C_A^0)(t - t_0)] - 1]}{k_2 \cdot C_A^0 + k_1 \cdot \exp[(k_1 + k_2 \cdot C_A^0)(t - t_0)]} \tag{6}$$

where ε_λ is the extinction coefficient at a λ wavelength, l is the cell width, C_A^0 the initial tyrosine concentration and t_0 the induction time.

3. Materials and methods

Experimental data was obtained following the absorbance of the solutions containing tyrosinase and L-tyrosine at eight different concentrations in order to obtain a different curve for each one, so the kinetic expression (6) can be tested. *Agaricus bisporus* tyrosinase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U/mL, distributed in aliquots of 1 mL and frozen at −12 °C until use. This solution was stored at 4 °C since 12 h before experiments started, and then pre-incubated at room temperature for 1 h. L-Tyrosine (Sigma Chemical, St. Louis, MO) was prepared in aqueous solution in a concentration range from 0.5 mM up to 5.5 mM (0.5, 1.0, 1.75, 2.5, 3.25, 4.0, 5.0 and 5.5 mM).

Three hundred μL of the tyrosinase preparation (150 U) were added to 15 mL of tyrosine solution of each concentration (final enzyme content: 10 U/mL). The evolution of absorbance at 480 nm was taken with an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1 cm width cell. A data point was taken every 120 s with Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). All experiments were carried out at room temperature by duplicate. The shown results are the average of these two series for each concentration.

The experimental results obtained were fitted to the developed kinetic expression using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95% significance level.

4. Results and discussion

As indicated above, tyrosinase exhibits an unusual kinetic behavior due to its autocatalytical process depending on the generation of a dihydric phenol substrate, which acts an activator of the enzyme (Cooksey et al., 1997; Rodríguez-López, Tudela, Varón, García-Carmona, & García-Cánovas, 1992). These reactions lead to a sigmoid absorbance curve preceded by a lag period, as it is

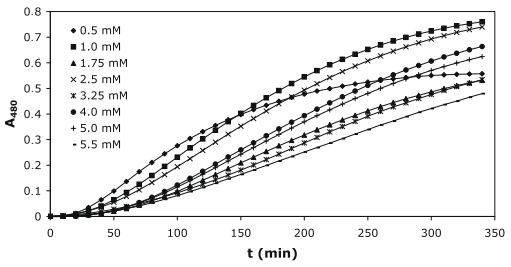

Fig. 2. Experimental data fitted to the developed kinetic model.

Table 1
Parameters of fitting experimental data to the developed kinetic model: $A_i = \frac{C_A^0 \varepsilon_i \cdot l \cdot k_1 \cdot \exp[(k_1 + k_2 \cdot C_A^0)(t - t_0)] - 1]}{k_2 \cdot C_A^0 + k_1 \cdot \exp[(k_1 + k_2 \cdot C_A^0)(t - t_0)]}$.

C_A^0 (mM)	ε_{480} (L mol^{-1} cm^{-1})	k_1 (min^{-1})	k_2 (L mol^{-1} min^{-1})	t_0 (min)	R^2
0.5	1.129 ± 0.006	0.00457 ± 0.00022	0.0239 ± 0.0013	12.3 ± 1.2	0.9987
1.0	0.805 ± 0.003	0.00249 ± 0.00009	0.0114 ± 0.0003	16.2 ± 1.3	0.9995
1.75	0.3455 ± 0.0009	0.00155 ± 0.00007	0.00663 ± 0.00012	23.5 ± 2.1	0.9993
2.5	0.3256 ± 0.0013	0.00206 ± 0.00008	0.00427 ± 0.00011	15.4 ± 1.5	0.9995
3.25	0.2168 ± 0.0008	0.00123 ± 0.00007	0.00282 ± 0.00007	17.9 ± 1.7	0.9997
4.0	0.1904 ± 0.0008	0.00162 ± 0.00007	0.00278 ± 0.00006	25.4 ± 1.8	0.9994
5.0	0.1450 ± 0.0006	0.00158 ± 0.00004	0.00217 ± 0.00005	24.0 ± 1.9	0.9993
5.5	0.1286 ± 0.0008	0.00129 ± 0.00004	0.00133 ± 0.00003	22.9 ± 1.7	0.9996

Signification level: $\alpha = 0.05$.

shown in Fig. 2. The stabilization of the absorbance curve depends on the initial substrate concentration, so the higher the L-tyrosine content is, the later stabilization will be. As can be expected, the reactive concentration is a limitant factor of the reaction, so in the few concentrated solutions the substrate will be depleted earlier. In this way, the only experimental series that reached this state in the time the experiment lasted were the ones with 0.5 mM of substrate. More concentrated samples needed a longer time to exhibit the same behavior.

The evolution of absorbance at 480 nm obtained with each initial tyrosine concentration was fitted to Eq. (6). The parameters of this statistical processing are shown in Table 1.

First of all, it was verified that the developed mathematical model fits well experimental data, because all of the determination coefficients are higher than 0.99. The extinction coefficient at 480 nm, ε_{480}, decreases with the increase of the initial substrate concentration, from 1.129 to 0.1286 L mol^{-1} cm^{-1}. Theoretically, the extinction coefficient is an intrinsic property of each molecule, and should be a constant if the formed product is the same in all experiments. Nevertheless, melanin formation is a complex reaction in which a group of heterogeneous length chain pigments are formed. In this way, chains with different composition will affect differently to the global coefficient. In addition, longer reaction times produce higher polymerization that leads to insoluble molecules formation. Then, these high molecular weight melanins precipitate and the solution absorbance decreases. The fact that the extinction coefficient decreases with the increase of the substrate concentration would indicate that, the lower the tyrosine concentration is, the further polymerization will happen later. Moreover, the extinction coefficient indicates how 1 mol of the molecule contributes to the solution absorbance. Thus, it seems that if the molecules are bigger its extinction coefficient is smaller.

The first kinetic constant (k_1) generally decreases as the tyrosine concentration increases, what means that for high concentrations these first reactions of intermediate products formation are slower and consequently the global reaction in the initial steps will be slower too. The second kinetic constant (k_2) shows the same behavior, decreasing as the initial tyrosine concentration increases, supporting the fact that the polymerization will be slower and the stabilization of absorbance will happen later. Then, the maximum absorbance reached will be higher as the initial substrate concentration (C_A^0) increases.

This second kinetic constant is always higher than the first one, and the ratio between these parameters (k_2/k_1), which is shown in Table 2, gradually diminishes from 5.23 to 1.03 L mol^{-1} with the increase of the initial substrate concentration. These results suggest that the global reaction rate is determined by the first step but, the higher the initial tyrosine concentration is, the differences between the two-steps reaction rate are lower. In addition, it implies that tyrosinase has always a lower catalytic effect on tyrosine than on DOPA, as it was also described by Rodríguez-López et al. (1992).

Table 2
Ratio between first and second kinetic constants.

C_A^0 (mM)	k_2/k_1 (L mol^{-1})
0.5	5.23
1.0	4.58
1.75	4.28
2.5	2.07
3.25	2.29
4.0	1.72
5.0	1.37
5.5	1.03

As shown in Fig. 1, the stoichiometry of the pathway implies that one molecule of tyrosinase must accomplish two turnovers in the hydroxylase cycle for each one in the oxidase cycle (Koval, Gamez, Belle, Selmeczi, & Reedijk, 2006). Then, according to the obtained data, an accumulation of DOPA was produced in the steady-state. This fact was also observed by Rodríguez-López et al. (1992), and could also explain why the second kinetic constant is higher than the first one. Thus, the behavior of all these parameters (extinction coefficient and the two kinetic constants) is in agreement with the known reaction pathway (Fig. 1) and the facts observed previously by the above mentioned authors.

The lag period (t_0), the time while only colorless intermediates are produced, did not show a defined tendency. Nevertheless, Cooksey et al. (1997) found an increase of this lag period with increasing substrate concentration.

5. Conclusions

Melanogenesis from L-tyrosine by means of mushroom tyrosinase is an autocatalytical reaction that can be described monitoring the absorbance at 480 nm. The proposed kinetic model fits experimental data. The extinction coefficient at 480 nm of the resulting products is not a constant value, because it depends on the initial substrate concentration. In the first step, the absorbance does not increase due to biosynthesis of colorless intermediates, and this will be the lag period. First kinetic constant (k_1) and second kinetic constant (k_2) decrease as the initial tyrosine concentration increases. This behavior means that for high concentrations the initial reactions of intermediate products formation and the later polymerization will be slower. The ratio between the two constants suggests that tyrosinase has a lower catalytic effect on L-tyrosine than on L-DOPA.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.8.

Title: Kinetic analysis of melanogenesis by means of *Agaricus bisporus* tyrosinase.

Authors: Víctor Falguera, Ferran Gatius, Jordi Pagán & Albert Ibarz.

Journal: *Food Research International*, 43 (2010), 1174-1179.

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Kinetic analysis of melanogenesis by means of *Agaricus bisporus* tyrosinase

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ABSTRACT

Melanins are a heterogeneous group of polymers formed by enzymatic reactions in vegetable tissues that contain phenolic or polyphenolic molecules. Recent studies have discovered some beneficial properties of melanins on health, making that not only its elimination should be reconsidered, but also its addition could be proposed to functional food of new creation. A further knowledge about the kinetic mechanism of melanogenesis is required prior to its possible industrial utilization. In this work, the kinetics of melanogenesis from 4-methylcatechol using mushroom tyrosinase and measuring the absorbance of the solution has been analyzed. The reaction pathway has been divided in two steps, and a mathematical expression has been developed to describe each one of them. The first one, an enzymatic reaction from the *o*-diphenol to colorless intermediate products. The second one, a polymerization of these intermediates leading to melanin chains. These expressions allow describing melanin formation as a function of reaction time, including some important parameters such as the extinction coefficient. In addition, the effect of pH and substrate concentration has been assayed in melanogenesis from two kinds of tyrosinase substrates: monophenolic (L-tyrosine) and *o*-diphenolic (4-methylcatechol).

1. Introduction

Tyrosinase (polyphenol oxidase, PPO, E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of an heterogeneous group of melanins (Duckworth & Coleman, 1970; Ziyan & Pekyardimci, 2003; Muñoz-Muñoz et al., 2007).

L-Tyrosine and 4-methylcatechol are two of the most important endogenous phenolic substrates for tyrosinase in a lot of vegetables, along with chlorogenic acid, catechol, catechin, cafeic acid, L-DOPA, DHPAA, 4-hydroxyphenylpyruvic acid, *p*-coumaric acid and *m*- and *p*-cresol (Madani, Kermasha, & Bisakowski, 1999). Tyrosinase converts L-tyrosine, a monophenol, firstly to L-DOPA (an *o*-diphenol) and this to *o*-dopaquinone, which is spontaneously cyclated in form of leukodopachrome and quickly converted to dopachrome, which polymerizes forming melanins (De Faria, Moure, Lopes, Krieger, & Mitchell, 2007). This enzyme can also directly act on 4-methylcatechol (an *o*-diphenolic substrate) conducting only the catecholase activity, from which the other changes and polymerizations will occur spontaneously (Madani et al., 1999). In this case, there is not a first stage consisting in the synthesis of an *o*-diphenol from a monophenol (cresolase activity), so there is not an enzymatic reaction that depends on the reaction rate of another one.

Initial studies of tyrosinases were motivated by a desire to understand and prevent the enzymatic browning that occurs in the presence of air when mushrooms, fruits or vegetables are cut or bruised. Nevertheless, recent discoveries on beneficial properties on health, such as anti-oxidative, anti-inflammatory, immune and anti-tumor properties, have done that not only its elimination should be reconsidered, but also its addition could be proposed to functional food of new creation (De Faria et al., 2007).

To implement these benefits that have recently been discovered, it is fundamental to know the kinetics of the process in order to easily predict the quantity and properties of the melanin that can be synthesized, as well as the influence of the parameters that can modify the reaction such as the pH or the nature and concentration of the substrate. In a previous work (Falguera, Pagán, & Ibarz, 2010), a kinetic expression describing the melanogenesis from L-tyrosine measuring the absorbance of the solution (as an indirect measure of product formation) was developed.

The present study has two main aims. The first one is to find a kinetic model to describe spectrophotometrically the melanogenesis from an *o*-diphenol using 4-methylcatechol as a substrate. Then, the second one is to assay the influence of pH and the type and concentration of substrate on the kinetics of tyrosinase.

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2. Kinetic considerations

In monophenols, the evolution of the solution absorbance (A_λ) can be followed by the kinetic model developed in a previous work (Falguera et al., 2010):

$$A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_1 \cdot [\exp[(k_1 + k_2 \cdot C_A^0) \cdot (t - t_0)] - 1]}{k_2 \cdot C_A^0 + k_1 \cdot \exp[(k_1 + k_2 \cdot C_A^0) \cdot (t - t_0)]} \quad (1)$$

where ε_λ is the extinction coefficient at a λ wavelength, l is the cell width, C_A^0 the initial monophenol concentration and t_0 the induction time.

In the case of *o*-diphenols, as it has already been said, there is not an enzymatic reaction that depends on the reaction rate of another one. Thus, the product formation is expected to follow a typical kinetic of systems with a single substrate and a single enzyme (Michaelis–Menten kinetics) (Ibarz, Barbosa, Garza, & Gimeno, 2000). However, these products do not give a coloration to the solution in themselves, but the compounds resulting from the changes later (melanins) are the ones that will allow the detection of an increase in absorbance. Then, if this magnitude is monitored the detected variations will depend on two factors: on one hand, the synthesis rate of the enzyme reaction products, on the other, the polymerization rate of these.

If the rate of the second group of reactions (non-enzymatic, or chemical) is greater than the rate of the first (enzymatic, or biochemical), a typical Michaelis–Menten response will be observed until the substrate is depleted. Otherwise, if the synthesis of intermediates occurs faster than the polymerization, this characteristic absorbance curve will be concealed by the increase described as a consequence of the later stage.

Attending to the enzymatic step, from the Michaelis–Menten (MM) equation (Stauffer, 1989):

$$r = \frac{r_{\max} C_S}{K_M + C_S} \quad (2)$$

where r is the reaction rate, $r_{\max}$ the maximum reaction rate, C_S the substrate concentration and K_M the MM constant. In the case of a discontinuous reactor and the condition of a null product concentration at a zero time, the Henri expression can be deduced:

$$C_P = r_{\max} t - K_M \ln\left(\frac{C_S^0}{C_S^0 - C_P}\right) \quad (3)$$

where C_P is the product concentration, C_S^0 the initial substrate concentration and t the reaction time. Its linearized form can be obtained dividing all terms by the time of reaction:

$$\frac{C_P}{t} = r_{\max} - \frac{K_M}{t} \ln\left(\frac{C_S^0}{C_S^0 - C_P}\right) \quad (4)$$

This form of the Henri equation allows to obtain the MM parameters $r_{\max}$ and K_M from an experimental series in which the evolution of product concentration with time is measured.

However, in the case of the action of polyphenol oxidase the parameter that is measured is not the melanin concentration directly, but an indirect magnitude: the absorbance. Therefore, the Lambert–Beer law must be included in expression (4), resulting:

$$\frac{A_\lambda}{\varepsilon_\lambda l t} = r_{\max} - \frac{K_M}{t} \ln\left(\frac{C_S^0}{C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda l}}\right) \quad (5)$$

The inclusion of the Lambert–Beer law introduces a new unknown parameter: the extinction coefficient ε_λ. This coefficient, which will be different for each type of melanin depending on the conditions of the experiment, can be calculated from the law itself:

$$\varepsilon_\lambda = \frac{A}{l C_P} \quad (6)$$

Obviously, to use this expression is necessary to know the product concentration, and there are only two points on which it is possible. Firstly, as already mentioned, it is assumed that at zero time this value is null. In addition, for long times, when the reaction is complete and the substrate is depleted, if the stoichiometry is one-to-one the product concentration is the same as the initial substrate concentration ($C_P^\infty = C_S^0$) (Ibarz et al., 2000). In this point, if there is only an enzymatic reaction, the maximum solution absorbance of this step will be reached. Thus, the increase in absorbance is defined by an exponential curve:

$$A_\lambda = A_{\max} - b \cdot \exp(-k \cdot t) \quad (7)$$

Knowing this maximum absorbance and the product concentration in the same point, the Lambert–Beer expression that will allow to calculate the extinction coefficient will be:

$$\varepsilon_\lambda = \frac{A_{\max}}{l C_S^0} \quad (8)$$

Then, knowing ε_λ and following Eq. (5), a regression can be carried out with $\left[\frac{1}{t}\ln(C_S^0/(C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda l}))\right]$ vs. $\left[\frac{A_\lambda}{\varepsilon_\lambda l t}\right]$. The result will be a straight line, in which $-K_M$ will be the slope and $r_{\max}$ the intercept.

In addition, it is important to consider that for long times of reaction this enzymatic stage will have finished, and the increase in absorbance will be only due to the polymerization step, which will follow its own kinetic pattern.

3. Materials and methods

Experimental data was obtained following the absorbance of the solutions containing tyrosinase and the substrate (L-tyrosine or 4-methylcatechol) in five different concentrations and four different pH, in order to obtain a different curve for each series. *Agaricus bisporus* tyrosinase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U/mL, distributed in aliquots of 1 mL and frozen at −12 °C until use. This solution was stored at 4 °C since 12 h before experiments started, and then pre-incubated at room temperature for 1 h. L-Tyrosine and 4-methylcatechol (Sigma Chemical, St. Louis, MO) were prepared in sodium phosphate buffer (pH of 5.0, 6.0, 7.0 and 8.0) in a concentration range from 0.5 mM up to 5.0 mM (0.5, 1.0, 2.5, 4.0 and 5.0 mM).

Three hundred microliter of the tyrosinase preparation (150 U) were added to 15 mL of substrate solution (final enzyme content: 10 U/mL). The evolution of absorbance at 480 nm was taken with a Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1 cm width cell. A data point was taken every 120 s with VisionLite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). All experiments were carried out at room temperature by duplicate. The shown results are the average of these two series for each concentration and pH.

For the determination of polymerization kinetics, two melanin samples were prepared with every concentration of 4-methylcatechol. After 24 h of reaction, tyrosinase was inactivated boiling the solution during 2 min. Then, the preparation was ultrafiltrated using a 300 kDa cut-off ceramic membrane in order to separate melanin polymers and obtain a permeate with only colorless intermediate products. The 480 nm absorbance of this permeate was monitored in the following 10 h.

The experimental results obtained in this work were fitted to the kinetic expressions using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, Md, USA). The fittings and the estimates were calculated at a 95% significance level.

Multivariate projection techniques have also been used in this study to determine the most important variables for data characterization and to see if there are any group patterns. For this purpose, a principal component analysis (PCA) of all the data obtained in the experiments (studying both substrates together) was carried out using the Unscrambler v. 6.11a software (Trondheim, Norway) (Camo, 1996). The presence of groups suggested the need of two additional PCA models, calculated separately from the data of each kind of substrate using the same software.

4. Results and discussion

4.1. Results overview

First of all, a global PCA model was carried out over all the data in order to determine the variables that explain the maximum information and consequently show the general tendencies present in the samples. Studying together the samples scores and the variables loadings in the Principal Component space it can be easily seen that, in the experimental conditions that have been described before, the most important variable that has to be considered to determine the absorbance evolution (and consequently the formed melanin properties) is the kind of substrate, followed by the pH and lastly the substrate concentration (Fig. 1). Nevertheless, regardless the other conditions when the pH value is 5.0 the obtained results are similar in all cases. This can be explained by the lack of action that the enzyme has in this pH, which makes that little melanin is produced and therefore high values of absorbance are not achieved. Regarding on spectrophotometric variables, the time between 240 and 306 min was the period with the greatest influence on the overall variance of data.

4.2. Melanogenesis from L-tyrosine

When acting on a monophenol, tyrosinase exhibits an unusual kinetic behavior due to its autocatalytical process depending on the generation of a dihydric phenol substrate, which acts as an activator of the enzyme (Cooksey et al., 1997). These reactions lead to a sigmoid absorbance curve preceded by a lag period (Falguera et al., 2010). Fig. 2 shows four examples of this kinetic pattern

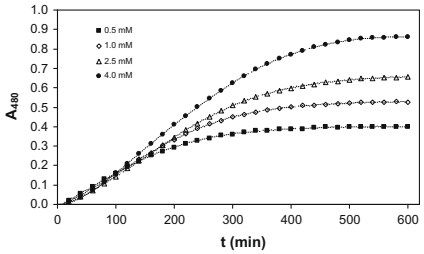

Fig. 2. Four examples of the sigmoid absorbance curve obtained in melanogenesis using different concentrations of L-tyrosine as PPO substrate in a pH 6.0 solution.

obtained with different L-tyrosine concentration at a pH solution of 6.0. The absorbance data obtained with each experimental serie was fitted to Eq. (1). The parameters of this statistical processing are shown in Table 1.

The extinction coefficient at 480 nm, ε_{480}, decreases with the increase of the initial substrate concentration. Its highest values are achieved with pH 6.0, and go from 0.8044 to 0.1229 L mol^{-1} cm^{-1}. On the contrary, the lowest ones correspond to a solution pH of 5.0. This indicates that melanin chains with different composition will affect differently to the global value. In other words, as the extinction coefficient is normalized to the pigment concentration and it decreases as increasing substrate concentration, the chromophores molecules should be different. Actually, the maximum absorbances were reached with pH 6.0, and the minimum with 5.0. This highest PPO activity with pH 6.0 and the lowest one with pH 5.0 were also revealed in the diagrams corresponding to the PCA model calculated with only the data of the experimental series using L-tyrosine as a substrate. In addition, the loadings of this analysis confirmed that, in the studied intervals of the operative variables, the pH was more important that the substrate concentration in the overall variance (diagram not shown). The fact that the extinction coefficient decreases with the increase of the substrate concentration would indicate that, the lower the tyrosine concentration is, the further polymerization that leads to insoluble molecules formation will happen later.

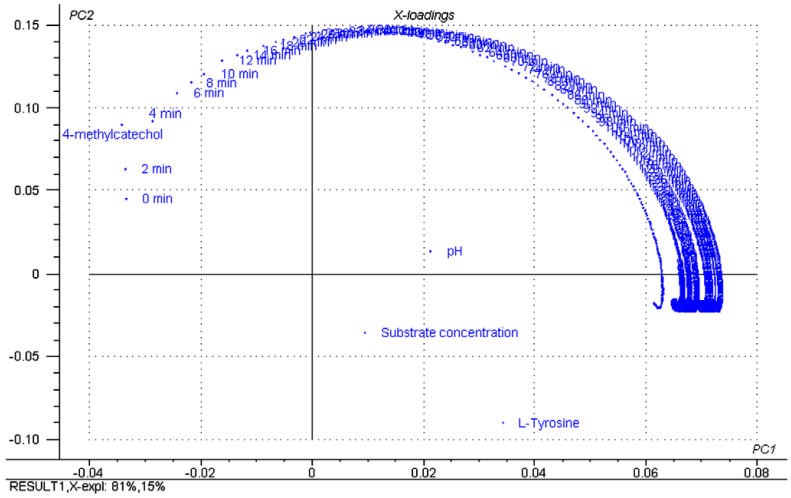

Fig. 1. Variable loadings represented in the Principal Component space.

Table 1
Parameters of fitting experimental data from L-tyrosine series to the kinetic model: $A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_1 \cdot [\exp[(k_1+k_2 \cdot C_A^0)\cdot(t-t_0)]-1]}{k_2 \cdot C_A^0 + k_1 \cdot \exp[(k_1+k_2 \cdot C_A^0)\cdot(t-t_0)]}$.

pH (–)	C_A^0 (mM)	ε_{480} (L mol^{-1} cm^{-1})	k_1 (min^{-1})	k_2 (L mol^{-1} min^{-1})	t_0 (min)	R^2
5.0	0.5	0.08285 ± 0.00016	0.0101 ± 0.0004	0.0114 ± 0.0016	5.1 ± 0.7	0.9979
	1.0	0.06288 ± 0.00011	0.0080 ± 0.0003	0.0069 ± 0.0006	6.5 ± 0.7	0.9985
	2.5	0.04387 ± 0.00018	0.0043 ± 0.0005	0.0056 ± 0.0005	8 ± 3	0.9933
	4.0	0.03109 ± 0.00014	0.0024 ± 0.0003	0.0036 ± 0.0003	9 ± 5	0.9940
	5.0	0.02624 ± 0.00014	0.00237 ± 0.00007	0.00095 ± 0.00005	11.1 ± 1.6	0.9991
6.0	0.5	0.8044 ± 0.0008	0.00399 ± 0.00006	0.0144 ± 0.0003	5.8 ± 0.5	0.9998
	1.0	0.5318 ± 0.0005	0.00259 ± 0.00003	0.00823 ± 0.00009	7.1 ± 0.5	0.9999
	2.5	0.2664 ± 0.0003	0.001893 ± 0.000024	0.00322 ± 0.00003	10.1 ± 0.6	0.9999
	4.0	0.2233 ± 0.0005	0.00171 ± 0.00004	0.00188 ± 0.00003	14.8 ± 1.2	0.9997
	5.0	0.1229 ± 0.0006	0.00160 ± 0.00003	0.00110 ± 0.00003	20.0 ± 1.2	0.9996
7.0	0.5	0.4838 ± 0.0005	0.00401 ± 0.00013	0.0316 ± 0.0007	7.1 ± 0.9	0.9995
	1.0	0.2965 ± 0.0004	0.00302 ± 0.00013	0.0169 ± 0.0004	11.7 ± 1.3	0.9993
	2.5	0.1361 ± 0.0003	0.00199 ± 0.00009	0.00498 ± 0.00012	22.1 ± 1.8	0.9992
	4.0	0.09473 ± 0.00018	0.00208 ± 0.00007	0.00291 ± 0.00006	23.1 ± 1.5	0.9994
	5.0	0.07798 ± 0.00023	0.00140 ± 0.00008	0.00237 ± 0.00006	29 ± 3	0.9990
8.0	0.5	0.7269 ± 0.0022	0.00337 ± 0.00003	0.00496 ± 0.00024	8.4 ± 0.5	0.9998
	1.0	0.483 ± 0.005	0.00291 ± 0.00009	0.00303 ± 0.00035	13.3 ± 1.7	0.9984
	2.5	0.188 ± 0.003	0.00185 ± 0.00010	0.00208 ± 0.00018	32 ± 3	0.9964
	4.0	0.1134 ± 0.0012	0.00206 ± 0.00012	0.00153 ± 0.00012	35 ± 3	0.9963
	5.0	0.0935 ± 0.0014	0.00123 ± 0.00008	0.00137 ± 0.00009	50 ± 4	0.9964

Signification level: $\alpha = 0.05$.

First kinetic constant (k_1) and second kinetic constant (k_2) decrease as the initial tyrosine concentration increases. This behavior means that for high tyrosine concentrations the initial reactions of intermediate products formation and the later polymerization will be slower, and the stabilization of absorbance will occur later. The maximum values of k_1 correspond to a pH solution of 5.0, while the maximum k_2 are from the pH of 7.0. The second kinetic constant is always higher than the first one, what means that tyrosinase has always a lower catalytic effect on tyrosine than on DOPA. The behavior of all these parameters (extinction coefficient and the two kinetic constants) is in agreement with the known reaction pathway and the facts observed in a previous work (Falguera et al., 2010).

The lag period (t_0), the time while only colorless intermediates are produced, grows up with increasing substrate concentration, as it was observed by Cooksey et al. (1997), and with increasing pH.

4.3. Melanogenesis from 4-methylcatechol

First of all, the most different fact with the results obtained with L-tyrosine is the fast increase of the absorbance in the initial stage of the reaction. Later, this rising tends to become smaller as time elapses, describing a Michaelis–Menten kinetics as it happens with all *o*-diphenolic substrates on which the enzyme only carries out its catecholase activity (Madani et al., 1999; Hsu, Chang, Lu, & Chung, 2007). It is important to remember that, if tyrosinase catalyzes the two reactions from a monophenol, the diphenol synthesized in the first step will act as activator of the enzyme, accelerating the subsequent transformations (Rodríguez-López, Tudela, Varón, García-Carmona, & García-Cánovas, 1992).

Noting the evolution of the absorbance two stages can be defined in all cases. The first, as mentioned, a typical Michaelis–Menten curve. The second, an increase following a zero-order kinetics. The transition between these two stages depends on the pH as it can be observed in Fig. 3 (at lower pH values, the later the transition occurs), but does not on the substrate concentration. This limit can be established in 400 min at pH 5.0, in 300 min at pH 6.0, in 200 min at pH 7.0 and in 100 min at pH 8.0.

Thus, it appears that the higher the pH is, the greater enzyme activity is, and therefore intermediate compounds that polymerize to melanin are formed more quickly, causing the first stage (enzymatic) to be faster. These observations are consistent with data from the principal component analysis, in which it was concluded that the samples of pH 8.0 were the most important in the variance, indicating that under these conditions a greater amount of melanin can be synthesized. The results contrast with the ones of Fan and Flurkey (2004), which set the optimal activity of polyphenol oxidase of Portabella mushroom around pH 7.0 using catechol as substrate.

Observing this unusual kinetic behavior, it was necessary to verify that the polymerization step led to an absorbance increase

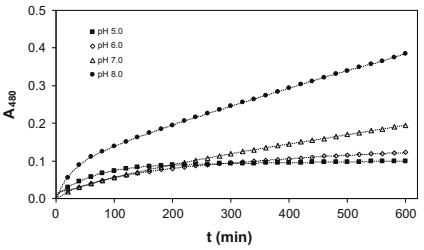

Fig. 3. Examples of the absorbance curve obtained in melanogenesis using 4.0 mM 4-methylcatechol as PPO substrate in different solution pHs.

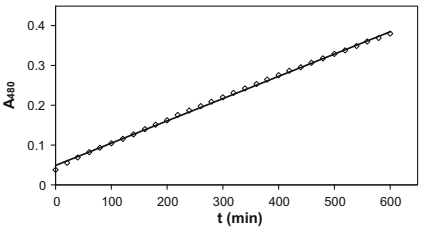

Fig. 4. Absorbance evolution in the polymerization step from 5.0 mM 4-methylcatechol in a pH of 8.0, after inactivating tyrosinase and separating melanin polymers previously synthesized. Experimental data fitted to zero-order kinetics.

Table 2

Parameters of fitting experimental data from the first (enzymatic) stage of 4-methylcatechol series to the kinetic model: $\frac{A_\lambda}{\varepsilon_\lambda \cdot l} = r_{max} - \frac{K_M}{t} \ln\left(\frac{C_A^0}{C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda \cdot l}}\right)$.

pH (−)	C_A^0 (mM)	ε_{480} (L mmol^{-1} cm^{-1})	K_M (mmol L^{-1})	r_{max} (mmol L^{-1} min^{-1})	R^2
5.0	0.5	0.16180 ± 0.00020	0.53874 ± 0.00018	0.0045 ± 0.0009	0.9934
	1.0	0.0879 ± 0.0003	1.22 ± 0.05	0.0105 ± 0.0008	0.9097
	2.5	0.0372 ± 0.0003	2.69 ± 0.05	0.0181 ± 0.0008	0.9891
	4.0	0.0301 ± 0.0006	4.02 ± 0.07	0.0110 ± 0.0006	0.9844
	5.0	0.0270 ± 0.0005	5.34 ± 0.09	0.0144 ± 0.0007	0.9865
6.0	0.5	0.1156 ± 0.0003	0.530 ± 0.011	0.00312 ± 0.00016	0.9847
	1.0	0.090 ± 0.006	1.030 ± 0.014	0.00409 ± 0.00017	0.9928
	2.5	0.0342 ± 0.0005	2.59 ± 0.04	0.0050 ± 0.0003	0.9908
	4.0	0.0247 ± 0.0007	3.98 ± 0.15	0.0087 ± 0.0018	0.9481
	5.0	0.036 ± 0.003	5.45 ± 0.03	0.01163 ± 0.00018	0.9990
7.0	0.5	0.0927 ± 0.0009	0.489 ± 0.009	0.00385 ± 0.00023	0.9911
	1.0	0.0450 ± 0.0007	1.09 ± 0.03	0.0059 ± 0.0005	0.9788
	2.5	0.0352 ± 0.0005	2.88 ± 0.08	0.0079 ± 0.0022	0.9612
	4.0	0.035 ± 0.003	6.3 ± 0.3	0.0170 ± 0.0018	0.9482
	5.0	0.0296 ± 0.0009	7.8 ± 0.4	0.0212 ± 0.0022	0.9436
8.0	0.5	0.1901 ± 0.0004	0.66 ± 0.03	0.0123 ± 0.0010	0.9781
	1.0	0.1118 ± 0.0003	1.69 ± 0.13	0.0194 ± 0.0023	0.9486
	2.5	0.064 ± 0.006	4.91 ± 0.22	0.058 ± 0.004	0.9847
	4.0	0.0463 ± 0.0005	7.22 ± 0.16	0.069 ± 0.003	0.9948
	5.0	0.0361 ± 0.0006	10.1 ± 0.3	0.118 ± 0.004	0.9951

Signification level: $\alpha = 0.05$.

of zero order. After inactivating tyrosinase and separating melanin polymers by ultrafiltration, a permeate with colorless intermediate products was obtained. The absorbance of this solution was monitored during 10 h. Fig. 4 shows this evolution in the polymerization step from 5.0 mM 4-methylcatechol in a pH 8.0, fitted to zero-order kinetics:

$$A_\lambda = A_0 + k_0 \cdot t \quad (9)$$

where A_0 is the absorbance at the start of the second stage and k_0 the zero-order kinetic constant.

To perform the kinetic study, experimental series were divided in the two stages. In the first one, linearized Henri equation including Lambert–Beer law (Eq. (5)) allows to obtain Michaelis–Menten parameters and the extinction coefficient. In the second one, the zero-order kinetic constant that determines the increase of absorbance resulting from the later polymerization is obtained. Table 2 shows the values for the first stage.

Firstly, all the determination coefficients obtained are higher than 0.90. The extinction coefficient at 480 nm (ε_{480}), tends to decrease with increasing initial substrate concentration, just as happened using L-tyrosine. Thus, although in this case the melanogenesis starts from a diphenol, the lower the initial concentration is, the later formation of insoluble strings will occur. And again, it also seems that if the pigment strings are greater its extinction coefficient is smaller.

MM constant (KM) corresponds to the concentration of substrate in which the reaction rate is half the maximum, and is an indicative of the inverse of the affinity of the enzyme for the substrate. Thus, low values indicate that the enzyme–substrate complex is strongly united and it rarely dissociates without the substrate being transformed into product. The results depend on the kind of substrate and on the conditions of the experiment (Nelson & Cox, 2000). This parameter increases as increasing both the initial concentration of 4-methylcatechol and pH. Therefore, the presence of substrate and the absence of protons favor complex dissociation, and consequently that the enzyme becomes free to react with another molecule.

The maximum reaction rate (r_{max}) is the maximum number of reactions per time unit that are catalyzed by the enzyme. This parameter also increases with the increase of initial substrate concentration as it could be expected, because the more molecules of 4-methylcatechol exist in the environment, the easier the formation of the complex will be. In addition, maximum reaction rate also increases with increasing pH, coinciding with the greater dissociation ease of the complex that has been previously discussed and with the conclusions drawn in the principal component analysis that pointed to a greater tyrosinase activity.

In the second stage of the reaction (non-enzymatic), absorbance data were adjusted to a zero-order model (Eq. (9)). The results appear in Table 3. The constant k_0 keeps a very small and almost constant value for all initial concentrations of substrate at pH 5.0. If the absorbances of these series are observed, it can be seen that when time exceeds 400 min they are nearly stabilized. In the other cases,

Table 3

Parameters of fitting experimental data from the second (non-enzymatic) stage of 4-methylcatechol series to the zero-order kinetic model: $A_\lambda = A_0 + k_0 \cdot t$.

pH (−)	C_A^0 (mM)	A_0 (AU)	$k_0 \times 10^4$ (AU min^{-1})	R^2
5.0	0.5	0.0737 ± 0.0003	0.168 ± 0.005	0.9779
	1.0	0.0812 ± 0.0005	0.151 ± 0.009	0.9178
	2.5	0.08642 ± 0.00024	0.131 ± 0.005	0.9686
	4.0	0.088 ± 0.0003	0.190 ± 0.005	0.9799
	5.0	0.0996 ± 0.0003	0.190 ± 0.005	0.9882
6.0	0.5	0.0455 ± 0.0005	0.365 ± 0.012	0.9617
	1.0	0.0625 ± 0.0004	0.383 ± 0.008	0.9840
	2.5	0.04121 ± 0.0002	0.911 ± 0.004	0.9992
	4.0	0.0684 ± 0.0007	0.920 ± 0.015	0.9896
	5.0	0.1007 ± 0.0006	1.100 ± 0.013	0.9949
7.0	0.5	0.03733 ± 0.00016	0.418 ± 0.004	0.9959
	1.0	0.02966 ± 0.00015	0.593 ± 0.004	0.9981
	2.5	0.03075 ± 0.00022	1.590 ± 0.005	0.9994
	4.0	0.041 ± 0.0004	2.581 ± 0.010	0.9992
	5.0	0.0434 ± 0.0003	2.742 ± 0.008	0.9996
8.0	0.5	0.0856 ± 0.0006	1.150 ± 0.015	0.9891
	1.0	0.077 ± 0.0007	2.301 ± 0.018	0.9961
	2.5	0.0933 ± 0.0004	4.471 ± 0.011	0.9996
	4.0	0.0978 ± 0.0008	4.840 ± 0.021	0.9988
	5.0	0.0926 ± 0.0005	5.851 ± 0.013	0.9997

Signification level: $\alpha = 0.05$.

k_0 constant clearly increases with the concentration of substrate and especially the pH of the solution.

5. Conclusions

The factor that has the highest influence on melanogenesis by means of Agaricus bisporus tyrosinase from L-tyrosine and 4-methylcatechol is the nature of substrate. Looking at the other two variables that define experimental conditions, the pH is more important than the concentration of substrate. Time between 240 and 306 min of reaction are those that provide the greatest variance in data. With L-tyrosine, tyrosinase has its optimal activity at pH 6.0. However, with 4-methylcatechol the optimum pH is 8.0. Meanwhile, all experiments carried out at pH 5.0 provided similar results in absorbance, due to the small action that the enzyme has in this pH.

In melanin formation from L-tyrosine, the 480 nm extinction coefficient of the resulting product depends on the initial substrate concentration and the pH. Initially, the absorbance does not increase due to the biosynthesis of intermediate colorless substances, leading to a lag period that grows with increasing initial concentration of substrate. In this reaction, the polyphenol oxidase has a lower catalytic effect on L-tyrosine (cresolase activity) than on L-DOPA (catecholase activity).

The absorbance evolution produced by the diphenolase activity on 4-methylcatechol can be defined in two stages. The first, a typical Michaelis–Menten curve. The second, an increase following a zero-order kinetics due exclusively to intermediates polymerization. The transition between the two stages depends on the pH, being later as this is lower, but does not depend on the concentration of substrate. The first stage can be characterized combining linearized Henri equation and Lambert–Beer law. The Michaelis constant (KM) and the maximum reaction rate (r_{max}) increase as both the initial concentration of 4-methylcatechol and pH increase. In the second stage, k_0 kinetic constant maintains a very small and almost constant value for all initial concentrations of substrate at pH 5.0. In the other cases, this parameter clearly increases with the concentration of substrate and the solution pH.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.9.

Title: Inhibitory effect of melanins from *Agaricus bisporus* polyphenol oxidase and two different substrates on carboxypeptidases A and B activity.

Authors: Víctor Falguera, Oriol Miarnau, Jordi Pagán & Albert Ibarz.

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SHORT COMMUNICATION

Inhibitory effect of melanins from *Agaricus bisporus* polyphenol oxidase and two different substrates on carboxypeptidases A and B activity

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Abstract In recent years, some beneficial effects on health of melanins that are formed as a result of polyphenol oxidase action have been discovered. However, it is also known that similar polymers have a detrimental effect on proteases activity. Thus, the aim of this work was to assay whether melanins from two different substrates act as carboxypeptidase A and carboxypeptidase B inhibitors. It was found that melanin synthesized from L-tyrosine decreases both the apparent affinity between carboxypeptidases and their substrates and the maximum reaction rate. Consequently, it is possible to conclude that these melanins inhibit CPA and CPB activity in a mixed way. On the contrary, no inhibition was found in the case of melanin synthesized from 4-methylcatechol.

Keywords Enzymatic browning · Enzyme inhibition · Carboxypeptidase · Digestion · Melanins

Introduction

Polyphenol oxidase (E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The later polymerization of these compounds leads to the formation of one of the enzymatic browning final products, called melanins [1, 2].

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These melanins have been considered up to the present substances that produce deterioration in many foods, especially in mushrooms, fruit juices and other fruit derivatives, decreasing its market value. Nevertheless, recent discoveries on beneficial properties on health, such as antioxidative, antiinflammatory, immune and antitumor properties, have done that not only its elimination should be reconsidered but also its use could be proposed in order to take advantage of these properties [2].

Carboxypeptidases A and B are metallopeptidases that contain a zinc atom in their active structure, which acts as an electrophilic catalyst [3]. Carboxypeptidase A (CPA) hydrolyzes the C-terminus of proteins, being specific for hydrophobic side chains of phenylalanine, tyrosine and tryptophan. Carboxypeptidase B (CPB) is specific for positively charged side chains of lysine and arginine. Their structure is closely related, as in the case of trypsin and chymotrypsin, being the 49% of their sequences identical [4]. The differences between their tertiary structures are concentrated on the external regions, being the most important one the presence of an Ile-255 in CPA, which is an Asp-255 in CPB to fix the side chain of basic substrates [4]. The proenzyme procarboxypeptidase A is secreted by the pancreas and is activated by the removal of a 60-amino-acid N-terminal piece to yield the 307-residue active enzyme [3].

These proteases show higher activity in the alkaline pH range [5], with a maximum around 7.5 that depends on the basic form of a group with a pK_a of 6.0 and on the acid form of another group with a pK_a of 9.1 in the non-bounded enzymes [4]. As mentioned, the active CPA contains a zinc atom per active site, with histidines at positions 69 and 196 and the γ-carboxylate of Glu^{72} acting as ligands. The fourth ligand is H_2O in the free enzyme, which is replaced by the carbonyl oxygen of the susceptible peptide bond in the enzyme–substrate complex [3].

In view of their function releasing free amino acids from dietary protein, carboxypeptidases may play an important role in the digestion process, and probably for that reason, plants have developed some molecules targeted at the inhibition of these enzymes as a protective strategy. Such carboxypeptidase inhibitors have been identified in solanaceous species like potato (potato carboxypeptidase inhibitor; PCI) and tomato. So far, all mammalian members of the A/B subfamily of carboxypeptidases appear to be highly susceptible to inhibition by PCI [6].

In addition, it is accepted that processing and storing of foodstuffs may cause a reduction in the quality of the constituents, affecting both its biological value and its digestibility [7]. In some cases, protein digestibility may be affected by the formation of non-enzymatic browning (NEB) products, as it has been reported by several authors [7–11]. In this way, protease inhibitors have been recognized to be significant in protein metabolism and endocrine systems. More than 100 kinds of proteinous and non-proteinous inhibitors have been isolated and identified from various living beings such as animals, plants and microbes [9].

Thus, on the one hand, melanins have been reported to be beneficial for health, but on the other hand, it has been observed that similar polymers (as non-enzymatic browning products) have a toxic effect on proteases activity. With these premises, the aim of this work has been to assay whether melanins (enzymatic browning products) synthesized by means of *Agaricus bisporus* tyrosinase from two different substrates (a monophenol and an *o*-diphenol) act as protease inhibitors on CPA and CPB and, in that case, to determine the kind of inhibition and its kinetic parameters.

Materials and methods

Melanin preparation

Agaricus bisporus tyrosinase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U/mL, distributed in aliquots of 1 mL and frozen at −12 °C until use. This solution was stored at 4 °C since 12 h before experiments started and then kept at room temperature for 1 h. L-tyrosine (monophenol) and 4-methylcatechol (*o*-diphenol) (Sigma Chemical, St. Louis, MO) were prepared in sodium phosphate buffer (pH 6.0 and 8.0, respectively, following the optimal pH assessed in a previous piece of work [12]) in a concentration of 4.0 mM. The final enzyme content in the reaction mixture was 10 U/mL.

After 24 h of reaction, melanins were precipitated adjusting the solution pH to 2.0 with HCl. The mixture was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 12 min at 12,000 rpm. The supernatant was discarded, and the pellet was recovered with distilled water. Melanins were lyophilized and rediluted in DMSO. Previous results [12] showed that the obtained polymers were the same if the variables *type of substrate*, *substrate concentration*, *pH* and *time of reaction* (before starting the recovery process) were kept constant. Assays carried out by means of an ultrafiltration device showed that keeping these variables constant the proportion of polymer that was retained by a 300-kDa membrane was also constant.

CPA activity determination

The principle of the enzymatic assayed reaction is the action of bovine CPA (Sigma Chemical, St. Louis, MO) on hippuryl-L-phenylalanine (Hip-Phe) in Tris buffer at pH 7.5 with the presence of 0.5 M NaCl at 25 °C, giving hippuric acid and L-alanine [13]. This reaction can be followed measuring the solution absorbance at 254 nm with a 1 cm width quartz cell, using a Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and monitoring the results with the Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). A data point was taken every 3 s. CPA activity was expressed as the absorbance variation for each second and proteic amount of the enzyme (ΔA_{254} s^{-1} μg^{-1}).

CPB activity determination

In the same way, the principle of this enzymatic reaction is the action of porcine CPB (Sigma Chemical, St. Louis, MO) on hippuryl-L-arginine (Hip-Arg) in aqueous solution at pH 7.65 with 0.1 M NaCl and 25 °C, giving hippuric acid and L-arginine [14]. This reaction is followed, as in the case of CPA, measuring the solution absorbance at 254 nm and monitoring the results with the Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). A data point was taken every 3 s. CPB activity was expressed as the absorbance variation for each second and proteic amount of the enzyme (ΔA_{254} s^{-1} μg^{-1}).

Enzyme inhibition analysis

Experimental series were carried out in a substrate concentration range from 0.1 to 0.5 mM, since higher substrate concentrations led to inhibition by substrate (Ibarz et al. [10] found this inhibition by substrate with concentrations higher than 1 mM for CPA and CPB). A CPA standard solution containing 10 U/mL (51.25 U/mg protein) was prepared and used for melanin from L-tyrosine inhibition analysis. Another CPA standard solution containing

Table 1 Melanin concentrations in the standard solutions used for inhibition analysis

Enzyme	Melanin type	Melanin concentrations (mg/mL)
CPA	L-Tyrosine	1, 2, 4
CPA	4-Methylcatechol	0.205, 0.41, 0.82
CPB	L-Tyrosine	3, 4, 8
CPB	4-Methylcatechol	0.205, 0.41, 0.82

10 U/mL (80.00 U/mg protein) was used for melanin from 4-methylcatechol inhibition analysis. These differences are due to batch variability in the commercial enzyme purchased from Sigma. In the same way, a CPB standard solution containing 7 U/mL (140 U/mg protein) was used for melanin from L-tyrosine inhibition analysis, while another CPB solution with 7 U/mL (168 U/mg protein) was used for melanin from 4-methylcatechol assays.

Table 1 shows the concentrations of the standard melanin solutions, which were prepared in DMSO. These melanin concentrations were found to be the optimal ones for quantifying the inhibitory effect after several previous experiments, considering the polymer solubility, the resulting absorbance of the solution and the enzyme–substrate–inhibitor interaction degree. As it was reported by Ibarz et al. [10] in the case of melanoidins, no clear tendency was seen in the experimental results for higher melanin contents.

The reaction mixture contained 2.9 mL of substrate (with one of the five different concentrations assayed) and 200 μL of a melanin solution (results are expressed as a function of final melanin content in the cell, in mg/mL). One hundred microliters of the enzymatic solution was added to the different mixtures (final enzyme content: 6.125 μg/mL in the system CPA-L-tyrosine, 3.85 μg/mL in the system CPA-4-methylcatechol, 1.654 μg/mL in the system CPB-L-tyrosine and 1.254 μg/mL in CPB-4-methylcatechol), and the absorbance evolution was monitored. The differences in final enzyme content in μg/mL are due to differences in the enzymes provided by the manufacturer, since the solutions were prepared to achieve the final equivalent activity (10 U/mL for CPA and 7 U/mL for CPB, also with the data provided by the manufacturer).

Data processing and statistical analysis

From the increase in the absorbance with the time of reaction, it is possible to obtain the maximum reaction rates for each substrate and melanin concentration, which is reached at zero time. With this aim, the monitored variation in the absorbance at 254 nm with reaction time can be fitted to exponential curves:

$$A_\lambda = a - b \cdot \exp(-k \cdot t) \quad (1)$$

From this expression, it is possible to obtain the initial reaction rate, since that is the value of its derivative at the initial time ($t = 0$) [10]:

$$r_0 = \left(\frac{dA_\lambda}{dt}\right)_{t=0} = b \cdot k \quad (2)$$

From the data of the initial rate of reaction for the different substrate and melanin values used, the Lineweaver–Burk method allows to calculate the Michaelis–Menten (MM) kinetic type parameters, which are the MM constant (K_M) and the maximum reaction rate (r_{max}) [15]. The data from this representation were adjusted to a straight line by means of the least squares method.

The fittings to the different kinetic and mathematical expressions were carried out using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95% significance level. All shown results are the average of three determinations.

Results and discussion

In order to study the effect of the melanins synthesized from L-tyrosine and 4-methylcatechol on CPA and CPB activity, three different concentrations of each melanin were assayed on each enzyme, with 5 different substrate concentrations. For both CPA and CPB and for each substrate concentration, it was observed that the initial reaction rate had a great dependence on the presence of melanin from L-tyrosine: as the concentration of this polymer increased, the reaction rate decreased. On the contrary, for both enzymes, little influence was observed in the case of melanin from 4-methylcatechol. To assess and quantify the inhibitory effect, these initial reaction rates were transformed following the Lineweaver–Burk method.

Table 2 shows the values of MM constant and maximum reaction rates for the experimental series carried out with different melanin concentrations for CPA and CPB. For both enzymes, when the reaction medium contains melanin from L-tyrosine, MM constant (K_M) shows an increasing trend, which indicates that this polymer decreases the apparent affinity between these enzymes and their respective substrates [16]. However, this fact is not appreciated when melanin from 4-methylcatechol is added. In this case, this kinetic parameter scarcely changes, keeping its value around 0.85 mM for CPA and 0.32 mM for CPB.

Regarding the maximum reaction rate, the increase in melanin from L-tyrosine in the reaction medium causes a

Table 2 Kinetic parameters for CPA and CPB with different concentrations of the two types of melanin assayed as possible inhibitors in the reaction medium

Enzyme	Melanin type[a]	$C_{Melanin}$ (mg/mL)	K_M (mM)	r_{max} (ΔA_{254} s^{-1} μg^{-1})	R^2
CPA	L-Tyrosine	0	0.45 ± 0.03	$8.8 \times 10^{-4} \pm 3.6 \times 10^{-4}$	0.9971
CPA	L-Tyrosine	0.0625	0.52 ± 0.05	$8.2 \times 10^{-4} \pm 1.6 \times 10^{-4}$	0.9538
CPA	L-Tyrosine	0.125	0.59 ± 0.05	$7.6 \times 10^{-4} \pm 3.6 \times 10^{-4}$	0.9956
CPA	L-Tyrosine	0.25	0.60 ± 0.05	$7.0 \times 10^{-4} \pm 4.3 \times 10^{-4}$	0.9963
CPA	4-Methylcatechol	0	0.86 ± 0.21	$1.3 \times 10^{-3} \pm 2.7 \times 10^{-4}$	0.9994
CPA	4-Methylcatechol	0.0128	0.82 ± 0.40	$1.2 \times 10^{-3} \pm 5.0 \times 10^{-4}$	0.9974
CPA	4-Methylcatechol	0.0256	0.86 ± 0.62	$1.6 \times 10^{-3} \pm 2.1 \times 10^{-4}$	0.9906
CPA	4-Methylcatechol	0.0513	0.88 ± 0.76	$1.3 \times 10^{-3} \pm 9.2 \times 10^{-4}$	0.9933
CPB	L-Tyrosine	0	0.42 ± 0.21	$4.5 \times 10^{-3} \pm 2.1 \times 10^{-3}$	0.9916
CPB	L-Tyrosine	0.1875	0.51 ± 0.31	$4.0 \times 10^{-3} \pm 1.8 \times 10^{-3}$	0.9893
CPB	L-Tyrosine	0.25	0.56 ± 0.37	$3.9 \times 10^{-3} \pm 1.7 \times 10^{-3}$	0.9943
CPB	L-Tyrosine	0.5	1.00 ± 0.51	$3.2 \times 10^{-3} \pm 1.1 \times 10^{-3}$	0.9697
CPB	4-Methylcatechol	0	0.32 ± 0.03	$3.0 \times 10^{-3} \pm 2.1 \times 10^{-4}$	0.9858
CPB	4-Methylcatechol	0.0128	0.36 ± 0.14	$3.4 \times 10^{-3} \pm 9.2 \times 10^{-4}$	0.9984
CPB	4-Methylcatechol	0.0256	0.29 ± 0.13	$3.0 \times 10^{-3} \pm 8.8 \times 10^{-4}$	0.9968
CPB	4-Methylcatechol	0.0513	0.27 ± 0.07	$2.7 \times 10^{-3} \pm 4.3 \times 10^{-4}$	0.9989

[a] indicates the substrate from which the melanin was synthesized by means of *Agaricus bisporus* polyphenol oxidase

progressive decrease in its value for both CPA and CPB. In the first case, 0.25 mg/mL leads to a 20.5% decrease in r_{max}, while in the second one, this lowering is 13.3% with the same L-tyr melanin content. As far as melanin from 4-methylcatechol is concerned, its addition does not modify the maximum reaction rate in any definite way, as it occurred with the MM constant.

The behavior of these two kinetic parameters (a progressive increase in K_M and a decrease in r_{max} when a higher amount of inhibitor is in the reaction medium) is evidence enough to infer that the presence of melanin from L-tyrosine inhibits CPA and CPB activity in a mixed way [15]. Since these melanins are an heterogeneous group of polymers with different chain lengths, the different fractions are expected to act in different moments of the catalysis, joining both the enzyme alone or the enzyme–substrate complex and leading to mixed inhibition kinetics when the process is observed at a macromolecular level. However, melanin from 4-methylcatechol is generally composed by polymers with lower chain length [12], and this may be one of the reasons why this type of pigment does not cause a strong modification in CPA and CPB kinetics.

Figure 1 shows Lineweaver–Burk plots for CPA and CPB with melanin from L-tyrosine. In these graphs, it can be also seen that this polymer inhibits both enzymes in a mixed way, since the straight regression lines have their intersections in the second quadrant [15]. Thus, the

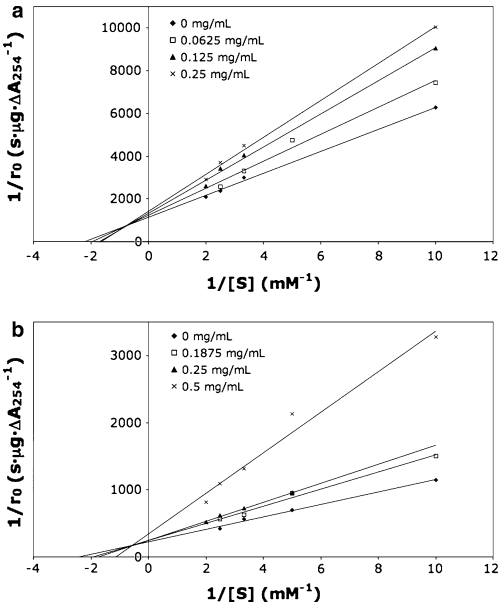

Fig. 1 **a** Lineweaver–Burk plot of samples with different concentrations of melanin from L-tyrosine acting as carboxypeptidase A inhibitor. **b** Lineweaver–Burk plot of samples with different concentrations of melanin from L-tyrosine acting as carboxypeptidase B inhibitor

inhibition constant αK_i (enzyme–substrate–inhibitor complex) must be higher than the inhibition constant K_i (enzyme–inhibitor complex), and then the value of α must be higher than the unit. To obtain the values of these parameters, the slope and the intercept of the Lineweaver–Burk regressions have been represented in front of the inhibitor concentration [6, 15]. For the system CPA–melanin from L-tyrosine, K_i was found to be 1.01 mg/mL, while αK_i was 2.56 mg/mL, and therefore, $\alpha = 2.52$. In the case of the interaction between CPB and the same melanin, $K_i = 0.78$ mg/mL, $\alpha K_i = 6.76$ mg/mL and $\alpha = 8.64$. Indeed, the values of the α constant were found to be higher than the unit in both cases, showing that melanin from L-tyrosine has more affinity for enzyme–substrate complex than for the enzyme alone.

Considering Lineweaver–Burk plots of the systems in which melanin from 4-methylcatechol was assayed as a possible inhibitor (not shown), these regressions turned out to be coinciding straight lines with the one obtained without melanin. Thus, it can be concluded that this type of polymer does not act as CPA nor CPB inhibitor.

As it has been already introduced, those differences between the inhibitory effect of both types of melanin on CPA and CPB may be attributed to differences in their structure. Some evidence found in a previous piece of work [12] pointed to the fact that melanin synthesized from L-tyrosine is formed by larger chains than those integrating melanin from 4-methylcatechol. In other studies performed with non-enzymatic browning products [10, 17], the inhibition of carboxypeptidases and trypsin was attributed to an allosteric effect, as a result of the interaction between long polymer chains with high bonding ability and the globular-shaped enzymes. In this way, the polymer is supposed to trap a number of enzyme molecules. Moreover, the inhibition was also partially attributed to an electrostatic insufficiency in the enzyme–polymer interaction, due to the nature of the side groups of the amino acids that form the enzyme [17, 18].

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.10.

Title: Inhibitory effect of enzymatic browning products on trypsin activity.

Authors: Víctor Falguera & Albert Ibarz.

Journal: *Afinidad*, LXVIII, 556, (2011), 435-438.

DOI: -

Inhibitory effect of enzymatic browning products on trypsin activity

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Efecto inhibitorio de los productos del pardeamiento enzimático sobre la actividad de la tripsina

Efecte inhibitori dels productes d'embruniment enzimàtic sobre l'activitat de la tripsina

Recibido: 24 de octubre de 2011; aceptado: 12 de enero de 2012

RESUMEN

El objetivo de este trabajo fue evaluar si las melaninas sintetizadas a partir de polifenol oxidasa de *Agaricus bisporus* y uno de sus principales sustratos (L-tirosina) actúan como inhibidores de la enzima pancreática tripsina y, por tanto, pueden tener un efecto tóxico en la digestión proteica. Se encontró que estos polímeros disminuyen la afinidad aparente entre la enzima y su sustrato (Nα-benzoyl-L-Arginine ethyl ester, BAEE). Además, la velocidad máxima de reacción (r_{max}) disminuye con el incremento de la concentración de melanina (hasta un 32,6% añadiendo 0,07 mg·mL^{-1}). Se puede concluir que la presencia de melaninas inhibe la actividad de la tripsina de forma mixta. La constante alpha (α) resultó ser 2,95.

Palabras clave: pardeamiento enzimático; inhibición enzimática, digestión, tripsina, melaninas.

SUMMARY

The aim of this piece of work was to assay if the melanins synthesized from *Agaricus bisporus* polyphenol oxidase and one of its main substrates (L-tyrosine) act as trypsin inhibitors, and therefore may have a toxic effect on protein digestion. It was found that these polymers decrease apparent affinity between trypsin and its substrate (Nα-benzoyl-L-arginine ethyl ester, BAEE). In addition, the maximum reaction rate (r_{max}) decreases with the increase of melanin concentration (up to 32.6% adding 0.07 mg·mL^{-1}). It can be concluded that the presence of melanins from L-tyrosine inhibits trypsin activity in a mixed way. Alpha (α) constant was found to be 2.95.

Keywords: enzymatic browning; enzyme inhibition; digestion; trypsin; melanins.

RESUM

L'objectiu d'aquest treball fou avaluar si les melanines sintetitzades a partir de polifenol oxidasa d'*Agaricus bisporus* i un dels seus principals substrats (L-tirosina) actuen com a inhibidors de l'enzim pancreàtic tripsina i, per tant, poden tenir un efecte tòxic en la digestió proteica. Es va trobar que aquests polímers disminueixen l'afinitat aparent entre l'enzim i el seu substrat (Nα-benzoyl-L-arginine ethyl ester, BAEE). A més a més, la velocitat màxima de reacció (r_{max}) disminueix amb l'increment de la concentració de melanina (fins a un 32,6% afegint-ne 0,07 mg·mL^{-1}). Es pot concloure que la presència de melanines inhibeix l'activitat de la tripsina de manera mixta. La constant alpha (α) resultà 2,95.

Paraules clau: embruniment enzimàtic; inhibició enzimàtica; digestió; tripsina; melanines.

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1.INTRODUCTION

Enzymatic browning products are a heterogeneous group of polymers formed by polyphenol oxidase action in vegetable tissues containing phenolic or polyphenolic molecules, which have been considered up to the present substances that produce deterioration in many foods, especially in mushrooms, fruit juices and other fruit derivatives, decreasing its market value. Nevertheless, recent discoveries on beneficial properties on health, such as anti-oxidative, anti-inflammatory, immune and anti-tumour properties, have done that not only their elimination should be reconsidered, but also their addition could be proposed in order to take advantage of these properties (Falguera *et al*., 2010a).

Polyphenol oxidase (E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (catecholase activity). The later polymerization of these compounds leads to the formation of the enzymatic browning final products, called melanins (De Faria *et al*., 2007; Falguera *et al*., 2010a).

It is accepted that processing and storing of foodstuffs may cause a reduction in the quality of the constituents, affecting both its biological value and its digestibility (Öste & Sjödin, 1984). In some cases, protein digestibility may be affected by the formation of non-enzymatic browning (NEB) products, as it has been reported by several authors (Öste & Sjödin, 1984; Öste *et al*., 1986; Hirano *et a*l., 1994; Ibarz *et al.*, 2008; Ibarz *et al*., 2009). Thus, protease inhibitors have been recognized to be significant in protein metabolism and endocrine systems. More than 100 kinds of proteinous and non-proteinous inhibitors have been isolated and identified from various living bodies such as animals, plants and microbes (Hirano *et al*., 1994).

Trypsin (E.C. 3.4.21.4), as pepsin and chymotrypsin, is one of the main digestive proteases. It is synthesized in the pancreas in the inactive form of trypsinogen, and its activity is located in the small intestine where it degrades proteins to polypeptides and amino acids in a medium pH of about 8.0 (Ibarz *et al*., 2009). Concretely, it catalyses the hydrolysis of the peptidic and ester bonds formed by the carboxyl group and the basic amino acids L-lysine and L-arginine.

Trypsin activity is negatively influenced by physical parameters (temperature and pH), by conformational changes (denaturalization), by chemical modifications (substitution of amino acid residues and reduction of disulphite bridges) or by specific interactions with inhibitors. Sometimes, the formation of the catalytically inactive enzyme-inhibitor complex can be useful to understand the formation of the enzyme-substrate complex and the interactions that occur during the catalysis (Schellenberger *et al*., 1994).

Thus, on the one hand, melanins have been reported to be beneficial for health, but on the other hand it has been observed that similar polymers (NEB products) have a toxic effect on proteases activity. The aim of this work has been to assay if enzymatic browning products synthesized by means of *Agaricus bisporus* polyphenol oxidase act as trypsin inhibitors and, in that case, to determine the kind of inhibition and its kinetic parameters.

2. MATERIALS AND METHODS

2.1. Melanin preparation

Agaricus bisporus polyphenol oxidase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U·mL^{-1}, distributed in aliquots of 1 mL and frozen at -12ºC until use. This solution was stored at 4ºC since 12 hours before experiments started, and then pre-incubated at room temperature (22±2ºC) for 1 hour. L-Tyrosine (Sigma Chemical, St. Louis, MO) was prepared in sodium phosphate buffer (pH 6.0, following the optimal pH determined in a previous piece of work (Falguera *et al*., 2010b)) in a concentration of 4.0 mM. The final enzyme content in the reaction mixture was 10 U/mL.

After 24 h of reaction, melanins were precipitated adjusting the solution pH to 2.0 with HCl. The mixture was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 12 minutes at 12,000 rpm. The supernatant was discarded and the pellet was recovered with distilled water. Melanins were lyophilized and rediluted in dimethyl sulfoxide (DMSO) in a concentration of 0.82 mg/mL.

2.2. Trypsin activity determination

The principle of the enzymatic assayed reaction is the action of bovine trypsin (Sigma Chemical, St. Louis, MO) on Nα-benzoyl-L-arginine ethyl ester (BAEE) in aqueous solution at pH 7.6 and 25 °C, giving Nα-benzoyl-L-arginine (Bz-L-Arg) and ethanol (Bergmeyer *et al*., 1974). This reaction can be followed measuring the solution absorbance at 253 nm with a 1 cm width quartz cell, using an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) and monitoring the results with the Vision Lite 1.0 software (Thermo Spectronic, Thermo Fisher Scientific Inc., Waltham, USA). A data point was taken every 3 s. Trypsin activity was expressed as the absorbance variation (formation of Bz-L-Arg) for each minute and protein amount of the enzyme (ΔA_{253} s^{-1} mg^{-1}).

2.3. Trypsin inhibition analysis

Experimental series were carried out in a substrate concentration range from 0.2 to 0.6 mM, since higher BAEE concentrations led to inhibition by substrate (Ibarz *et al*., 2009). A trypsin standard solution containing 500 U/mL and three melanin solutions with concentrations of 0.82, 0.41 and 0.205 mg/mL in DMSO were prepared.

The reaction mixture contained 3 mL of substrate and 300 mL of a melanin solution (final melanin contents: 0.07, 0.035 and 0.0175 mg/mL). 20 mL of the enzymatic solution (10 U) were added to the different mixtures (final enzyme content: 0.22 mg/mL) and the absorbance at 253 nm evolution was monitored. Blank experiments were carried out in order to prove that there was not any interference of the different substances (mainly melanin and DMSO) with the absorbance at 253 nm.

2.4. Data processing and statistical analysis

From the increase of the absorbance with the time of reaction, it is possible to obtain the maximum reaction rates for each substrate and melanin concentration, which is reached at zero time. With this aim, the monitored variation in the absorbance at 253 nm with reaction time can be fitted to exponential curves:

$$A_\lambda = a - b \cdot \exp(-k \cdot t) \qquad (1)$$

From this expression it is possible to obtain the initial reaction rate, as that is the value of its derivative at the initial time (Ibarz *et al*., 2008):

$$r_0 = \frac{dA_\lambda}{dt}\Big)_{t=0} = b \cdot k \qquad (2)$$

From the data of the initial rate of reaction for the different substrate and melanin values used, the Lineweaver–Burk method allows to obtain the Michaelis-Menten (MM) kinetic type parameters, which are the MM constant (K_M) and the maximum reaction rate (r_{max}) (Segel, 1982). The data from this representation was adjusted to a straight line by means of the least squares method.

The fittings to the different kinetic and mathematical expressions were carried out using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95 % significance level. All shown results are the average of three determinations.

3. RESULTS AND DISCUSSION

In order to study the effect of the melanin synthesized from L-tyrosine on trypsin activity, three different melanin concentrations were assayed on BAEE solutions. It was observed that the initial reaction rate had a great dependence on melanin concentration, being lower as it increased. To characterize and quantify this inhibitory effect these initial reaction rates were transformed following the Lineweaver-Burk method (Figure 1).

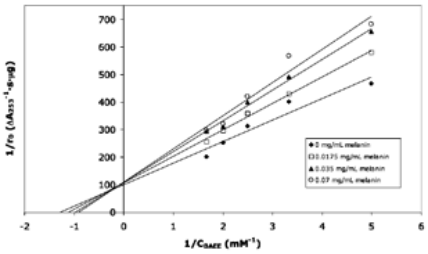

Figure 1. *Lineweaver-Burk representation of samples with different concentrations of melanin from L-tyrosine.*

Table 1. *Kinetic parameters for trypsin inhibition by melanin from L-tyrosine.*

$C_{Melanin}$ (mg/mL)	K_M (mM)			r_{max} ($\Delta A_{253} \cdot s^{-1} \cdot \mu g^{-1}$)			R^2
0	0.86	±	0.16	0.0108	±	0.0027	0.9345
0.0175	0.96	±	0.44	0.00990	±	0.00039	0.9987
0.035	1.09	±	0.13	0.00979	±	0.00095	0.9938
0.07	1.10	±	0.19	0.0092	±	0.0015	0.9526

Table 1 shows the values of MM constant and maximum reaction rates for the experimental series carried out with different melanin concentrations. MM constant increases with the melanin concentration in the solution, which indicates that this polymer decreases the apparent affinity between trypsin and its substrate (Nelson & Cox, 2000). In addition, the maximum reaction rate tends to decrease with the increase of melanin content in a linear tendency.

The highest obtained inactivation was 32.6% with a BAEE concentration of 0.6 mM and a melanin content of 0.07 mg·mL^{-1}. By extrapolation of this linear tendency, the necessary melanin concentration to inhibit the enzyme completely would be 0.50 mg·mL^{-1}. However, this deduction could not be empirically proved, since melanin solubility made it impossible to work with concentrations higher than 0.25 mg·mL^{-1}. At higher melanin contents, some of the added polymer remained constantly insoluble.

These facts are evidence enough to determine that the presence of melanin from L-tyrosine inhibits trypsin activity in a mixed way (Segel, 1982). Inhibition ways are represented in Figure 2. Since melanins are a heterogeneous group of polymers with different chain lengths, the different fractions are expected to act in different moments of the catalysis, joining both trypsin alone or trypsin-BAEE complex and leading to mixed inhibition kinetics. Ibarz *et al*. (2009) found a similar behavior in the interaction between trypsin and melanoidins, which is also a heterogeneous group of polymers with different chain lengths.

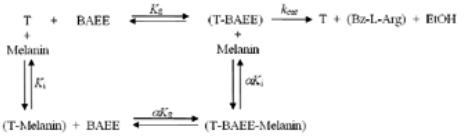

Figure 2. *Mixed enzymatic inhibition mechanism (adapted from Segel, 1982). T: trypsin. BAEE: Nα-benzoyl-L-arginine ethyl ester. Bz-L-Arg: Nα-benzoyl-L-arginine. EtOH: ethanol*

According to Jencks (1969), the electrostatic and apolar contacts dictate enzyme-substrate complementarity, which is necessary for surmounting the activation energy barrier between the ground and transition states. As substrate binding sites are preformed and relatively rigid, the free energy of substrate binding can be converted to catalysis without a large entropic penalty. Therefore, rate acceleration must also depend on the ability of distal portions to stabilize the binding, so the whole protein architecture must play an important role (Perona *et al*., 1995). Moreover, it is known that the differences in substrate specificity between trypsin and chymotrypsin are provided by variations in the distal portions that create a particular electrostatic environment, since the structure of the active site is the same in both enzymes (Stroud, 1974; Hedstrom *et al*., 1992). Then, any molecule present in the reaction medium may have an active effect on these electrostatic and apolar bindings, modifying the local environment that is necessary to create the enzyme-substrate links. Melanin chains with different molecular weight may create bindings with different sites of trypsin molecules, either blocking the active site or modifying these electrostatic forces.

The conclusion that the inhibition is mixed-type can also be stated observing that the intersection of the regression lines in the Lineweaver-Burk plot is found in the second quadrant. Thus, the inhibition constant αK_i (enzyme-substrate-inhibitor complex) must be higher than the inhibition constant K_i (enzyme-inhibitor complex), and then the value of α will be higher than the unit. To obtain the values of these parameters, the slope and the intercept of the Lineweaver-Burk regressions were represented in front of the inhibitor concentration (Segel, 1982). K_i was found to be 0.148 mg·mL^{-1}, while αK_i was 0.438 mg·mL^{-1}. Thus, α

value was 2.95. The fact that α is higher than the unit, but close to it, indicates that, indeed, the inhibition is mixed-type (Copeland, 2000), supporting the evidence observed in Figure 1. Ibarz *et al.* (2009) found an α value of 1.88 in the inhibition of trypsin by melanoidins synthesized from glucose and asparagine.

4. CONCLUSIONS

To sum up, it can be stated that melanins synthesized from L-tyrosine and *Agaricus bisporus* polyphenol oxidase have an inhibitory effect on trypsin activity. The higher melanin concentration was, the more important the inhibition factor was found. The highest obtained inactivation was 32.6%, corresponding to a BAEE concentration of 0.6 mM and a melanin content of 0.07 mg·mL^{-1}. MM constant (K_M) increased with the melanin concentration in the solution, which indicates that this polymer decreases the apparent affinity between trypsin and its substrate. In contrast, the maximum reaction rate (r_{max}) tends to decrease with the increase of melanin content in a lineal tendency. These facts show that this inhibition is mixed-type.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.11.

Title: Modeling of absorbed radiation profiles in a system composed by a plane photoreactor and a single lamp.

Authors: Víctor Falguera, Santiago Esplugas, Manel Vicente & Albert Ibarz.

Journal: *Food Research International*, 44 (2011), 3111-3114.

DOI: 10.1016/j.foodres.2011.06.009

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Short communication

Modeling of absorbed radiation profiles in a system composed by a plane photoreactor and a single lamp

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ABSTRACT

Ultraviolet irradiation constitutes an alternative to thermal treatment that is being studied and developed to obtain a better final product sensory quality, but without neglecting microbial safety. In order to quantify and predict chemical changes produced by ultraviolet radiation in a food system and to infer essential information for reactors design, a concrete modeling is required in each case, since the absorbed amount of radiation has a definitive effect on the reaction rate of the process in each point of the reaction medium depending on its location. Although the basic equations were set decades ago, there are still several concrete cases whose three-dimensional modeling cannot be found in literature. In this piece of work, the linear spherical emission model has been applied to a system composed by a plane photoreactor and a single lamp, obtaining different profiles that show how radiation is absorbed as a function of the position inside the reactor and the absorbance of the reaction medium.

1. Introduction

Food safety is one of the most important issues that food industries and food service companies have to face. Currently, the growing demand for a greater variety of prepared food and dishes on a menu makes the risk of contamination increases, especially due to the characteristics of this kind of food that gives a big importance to the quickness of meals (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000; Falguera, Pagán, & Ibarz, 2011a).

The elaboration and processing of liquid food have to be carried out under hygienic conditions that ensure the final product safety. Thermal technologies are the most widely applied ones to achieve these purposes, but these treatments have a negative effect on certain components of food products, reducing their vitamin content and other nutrients, as well as sensory features that make them less attractive in terms of color and textural properties. In addition, in some cases the product may contain compounds with adverse effects on health that are not eliminated with conventional processes, such as mycotoxins, which come from mold-contaminated pieces of fruit (Butz & Tauscher, 2002; Falguera, Pagán, Garza, Garvín, & Ibarz, 2011b; Guerrero-Beltrán & Barbosa-Cánovas, 2004).

Non-thermal technologies are an alternative to thermal treatment that is being studied and developed in order to obtain a better final product sensory quality, but without neglecting microbial safety. In this way, these alternatives to thermal technologies can produce food products without enzymes and hazardous microorganisms, while maintaining nutritional characteristics and minimizing the loss of quality in terms of flavor, color and nutritional value. One of these innovative technologies is ultraviolet irradiation, which has been proved to be useful in liquid food processing (Butz & Tauscher, 2002; Guerrero-Beltrán & Barbosa-Cánovas, 2004; Noci et al., 2008; Falguera, Pagán, Garza, Garvín, & Ibarz, 2011c.

The use of radiant energy to produce chemical reactions has some interesting properties. In this way, in photochemical processes reaction rate can be increased without increasing the temperature of the system. Nevertheless, among the most important limitations, building and operating the irradiation equipment are more expensive than in the case of a thermal processing installation, and not the whole radiation emitted by the lamp is useful in producing chemical changes (Ibarz & Esplugas, 1989; Falguera et al., 2011b,c).

In order to quantify and predict these chemical changes produced by ultraviolet radiation in a food system, a concrete modeling is required in each case. As in any chemical reaction, performing the mass and energy balances will be necessary to achieve the kinetic expression. But, in addition, in the case of photochemical processes also a radiation balance has to be developed, since the absorbed amount of radiation has a definitive effect on the reaction rate of the process in each point of the reaction medium depending on its location.

In this piece of work, radiation absorption modeling of a system formed by a perfect-mix plane photoreactor and a single lamp has been developed. This modeling allows seeing, for example, how radiation penetrates inside the reaction medium depending on its absorbance and on the system geometry.

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2. Mathematical modeling

As it has been stated, mass, energy and radiation balances have to be performed prior to developing the kinetic expression. Nevertheless, if it can be considered that the process works in isothermal state, it is not necessary to perform the energy balance. In addition, the variation of the photochemical reaction rate with temperature is a minor factor (Bird, Stewart, & Lightfoot, 1964).

2.1. System geometry

The considered system, which was used in a previous piece of work to study the effects of ultraviolet irradiation on apple juice (Falguera et al., 2011a), is composed by a parallelepipedal device, in which the sample is placed, and a single lamp without reflectors. Consequently, there is only a direct radiation flow between the lamp and the reactor. The plane photoreactor (Fig. 1) has a width of 15 cm (x axis), a length of 22 cm (y axis) and a depth of 2.4 cm (z axis). The origin of coordinates is located in an upper corner of this device. The lamp, whose length is 4.5 cm, is placed following the y axis from a point (x_0, y_0, z_0) to a point (x_0, y_1, z_0), being x_0 7.5 cm, y_0 8.75 cm, y_1 13.25 cm and z_0 (the distance between the lamp and the reactor surface) 22.5 cm.

2.2. Mass balance

Considering a continuous operation and perfect mixing, the balance for a j component when steady state is reached would be:

$$q\left(C_{j0}-C_j\right) = -\int_V r_j dV \quad j = 1,\ 2,\ldots,\ S \tag{1}$$

being q the volumetric flow rate of reactants; C_{j0} and C_j component j concentrations at the entrance and exit of the reactor, respectively; r_j the reaction rate of that component and V the reaction medium volume.

If the reaction is developed discontinuously, a new accumulation term appears, resulting in the mass balance:

$$V\frac{dC_j}{dt} = \int_V r_j dV. \tag{2}$$

These expressions are similar to the ones from conventional chemical reactors, but it should be noted that the reaction rate r_j is a function, as well as of reactant and product concentrations, of the radiation intensity at the absorption wavelengths in the reaction chamber.

2.3. Radiation balance

The radiation balance is performed adopting a model that should be able to provide information about the behavior of radiation rays, taking certain considerations about the type of emission and the geometry of the radiation source itself. In this way, emission models can be radial, spherical and diffuse. Regarding the emission source, three models can be set, depending on if the lamp can be considered as a line, as a cylindrical surface or as a volumetric region with cylindrical shape.

Among all models, the one that gives a more accurate result is the extensive spherical cylindrical one (Cerdá, Irazoqui, & Cassano, 1973; Esplugas, 1975), which considers the lamp as a cylinder whose volume emits spherically. However, the most used model is the spherical linear one, which has an easier resolution, and whose results hardly differ from the extensive spherical cylindrical model. The validity of this model is restricted to those installations in which the lamp radius is negligible compared with the distance between the lamp and the reactor surface, so that the differences between the linear model and the extended emission source model will be small. This assumption implies only small displacements from the center of the lamp, in order to consider only those points for which the emission source can be considered as a line (Esplugas, Vicente, Ibarz, Prat, & Costa, 1987).

The spherical linear model is described in the literature (Jacob & Dranoff, 1970). This model was also used by Esplugas and Vicente (1991) to develop an equation to evaluate the direct radiation between a linear source and a plane photoreactor. These authors considered that if the absorbance of the reaction chamber was high enough, all the radiation entering the reactor would be absorbed by the reaction medium. Therefore, the absorption rate in the whole volume would have the same value as the flow rate reaching the reactor surface, which is easy to be calculated by integrating the radiation intensity that reaches the entire surface.

But, as it has been already introduced, the mathematical expression of the reaction rate is a function of the radiation intensity at each wavelength, as well as of reactants and products concentrations. Thus, although the perfect mixing ensures that these concentrations will be homogeneous throughout the whole volume of the reaction medium, the reaction rate will be different in each point of the reactor since it also depends on the radiation intensity, which is a function of the point location and the geometry of the system. Therefore, the radiation balance must be performed taking into consideration the attenuation of the radiation rays inside the reactor medium.

The radiation balance between a point of the lamp and another one with coordinates (x,y,z) located inside the reacting fluid (Fig. 2), is given by Beer–Lambert law for spherical emission (Esplugas, Ibarz, & Vicente, 1983; Unluturk, Arastoopour, & Koutchma, 2004):

$$I(x,y,z) = \frac{W_L}{4\pi L}\int_{y_0}^{y_0+L}\frac{exp(^{-}\mu z/\sin\Psi)}{(x_0-x)^2+(l-y)^2+(z_0+z)^2}\,dl \tag{3}$$

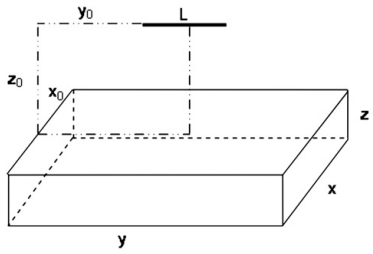

Fig. 1. The plane photoreactor. System geometry and axis denomination.

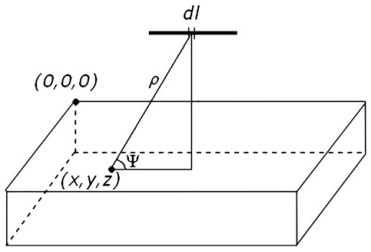

Fig. 2. The two points between which the radiation balance is considered. The first point constitutes a dl length of the lamp. The second one is a point (x,y,z) located inside the reactor. ρ is the distance between these two points, and Ψ is the angle between the radiation flow density vector and the reactor surface.

where I is the radiation intensity that reaches the point (x,y,z) $\left(\text{Einstein}/\text{cm}^2\cdot\text{s}\right)$, W_L is the energy flow emitted by the lamp $\left(\text{Einstein}/\text{s}\right)$, L is the lamp length (cm), μ is the medium absorbance (cm^{-1}), Ψ is the angle between the radiation flow density vector and the reactor surface (rad) and the product $\left(\mu z/\sin\Psi\right)$ is the attenuation of the ray until it reaches the point (x,y,z).

Considering the geometry that has been described in Figs. 1 and 2, the value of the sinus of the angle between the radiation flow density vector and the reactor surface is calculated as:

$$\sin\Psi = \frac{z_0 + z}{\left((x_0-x)^2 + (l-y)^2 + (z_0-z)^2\right)^{1/2}} \quad (4)$$

Then, the total absorbed radiation flux can be calculated integrating the product $(\mu\cdot I)$ in the three dimensions of the reactor:

$$W_{abs} = \int_0^A \int_0^B \int_0^C \mu I(x,y,z)\ dx\ dy\ dz \quad (5)$$

where A, B and C are the whole width, length and depth of the reactor, respectively. This equation has no analytical solution, so that a network of $(\mu\cdot I)$ values must be created in the whole volume in order to be integrated by numerical methods. If these integrations in the three dimensions are carried out step-by-step, different kinds of absorbed radiation profiles can be achieved, which allows extracting different interesting information.

3. Absorbed radiation profiles

Integrating the $(\mu\cdot I)$ values in the z axis leads to an absorbed radiation profile in the whole depth depending on the horizontal (x,y) position. Fig. 3 shows two examples of this profile for two different values of the medium absorbance (μ), 0.1 and 0.5 cm^{-1}. If $\mu = 0.1\ \text{cm}^{-1}$, the positions in the four corners of the reactor will absorb only $2.27\cdot10^{-5}\left(\text{Einstein}/\text{cm}^2\cdot\text{s}\right)$, which is the 25.2% of the radiation absorbed in the central position ($x = 7.5$ cm, $y = 11$ cm). If $\mu = 0.5\ \text{cm}^{-1}$, the radiation absorbed in the four corners will be only $7.15\cdot10^{-5}\left(\text{Einstein}/\text{cm}^2\cdot\text{s}\right)$, the 29.1% of that absorbed in the central position.

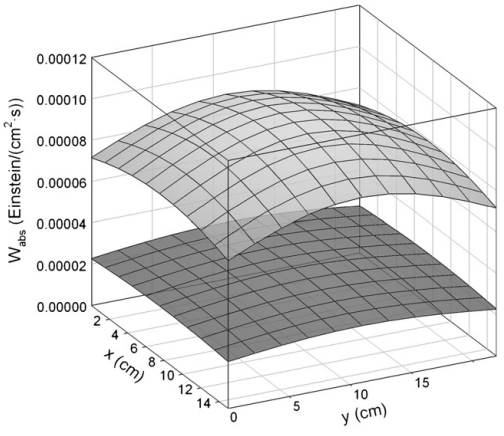

Fig. 3. Absorbed radiation profiles in the whole reactor depth depending on the (x,y) position for two different values of the medium absorbance. Upper graph: $\mu = 0.5\ \text{cm}^{-1}$. Lower graph: $\mu = 0.1\ \text{cm}^{-1}$.

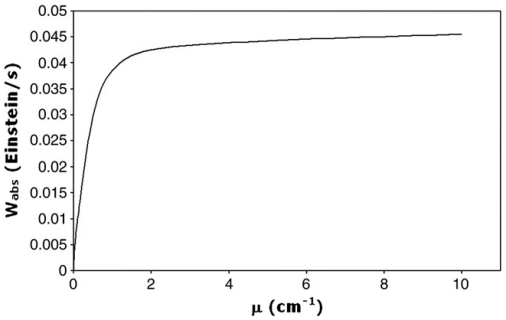

Fig. 4. Total radiation absorbed in the whole reactor depending on the medium absorbance.

If the values of Fig. 3 are integrated in the other two dimensions, the total radiation absorbed in the whole reactor will be found for each μ value. Fig. 4 shows how the total absorbed radiation changes with medium absorbance. This graph tends to stabilization, meaning that as medium absorbance increases the total radiation absorbed will be increasingly closer to the total radiation that reaches the reactor surface, as it was stated by Esplugas and Vicente (1991). For example, when the medium absorbance is 0.1, the total amount of absorbed radiation is $9.05\cdot10^{-3}$ (Einstein/s), when $\mu = 0.5$ it increases to $2.95\cdot10^{-2}$ Einstein/s, and if $\mu = 5$ $W_{abs} = 4.42\cdot10^{-2}$ (Einstein/s).

This mathematical tool to calculate the total amount of absorbed radiation can be used to infer the radiation dose that is necessary to achieve a desired effect. As an example, this procedure has been applied and related to one of the results obtained in a previous piece of work: the inactivation of polyphenol oxidase in fresh juice obtained from Fuji apples (Falguera et al., 2011a). In that case, the irradiation equipment consisted of the reactor that has been described, with a high-pressure mercury lamp of 400 W of nominal power that emits in a range between 250 and 740 nm. Among these wavelengths, the peak of emission was located in the range of 360–370 nm, emitting 7.06% of the lamp power in this interval (28.34 W, data provided by the manufacturer). The performed actinometric reaction showed that the total energy emitted by the lamp was $6{,}47\cdot10^{-7}$ Einstein/s, so the fraction corresponding to the 360–370 peak is $4.57\cdot10^{-10}$ Einstein/s. Regarding the reaction medium (Fuji apple juice), the absorbance spectrum showed that the average absorbance between 360 and 370 nm was 2.835 absorbance units, i.e., 6.530 cm^{-1}. With these data, the application of the proposed mathematical process leads to a total amount of absorbed radiation of $2.06\cdot10^{-11}$ Einstein/s in the reactor. With this radiation dose, 100 min were necessary to completely inactivate polyphenol oxidase in Fuji juice, whose initial activity was 0.14 U/mL.

Low penetration of ultraviolet radiation inside liquid food systems has been stated to be one of the main limitations of this technology. The penetration effect of UV radiation depends on the type of liquid, its UV absorbance, soluble solute content and suspended matter, as well as on the light source, flow profile and geometric configuration of the photoreactor. Since this last parameter is essential to ensure that all the system receives the same radiation dose, some researchers have tested different geometric configurations to produce, for example, a thin film throughout pipes, a liquid bell formed by spraying the liquid with nozzles, turbulent flow throughout the pipes or perfect-mixing (Falguera et al., 2011b,c; Guerrero-Beltrán & Barbosa-Cánovas, 2004; Unluturk et al., 2004).

Knowing this limitation, one of the most interesting distributions to be investigated in each ultraviolet processing photoreactor is the radiation absorption profile as a function of the reaction medium thickness. To obtain this representation, the $(\mu\cdot I)$ values network has to be integrated in the x and y axes. As it has been seen in the previous

figures, the medium absorbance has a big effect on the obtained results. In order to compare the different profiles avoiding these scale problems, relative absorbed radiation has been calculated as:

$$R(z,\mu) = \frac{W_{abs}(z,\mu)/W_L}{\left(W_{abs}(z,\mu)/W_L\right)_{max}}. \quad (6)$$

Fig. 5 shows some profiles corresponding to different values of the reaction medium absorbance. For a reaction medium with an absorbance of 0.1, hardly a 30% of the emitted radiation is absorbed, while for a μ value of 5 the 50% of the radiation is absorbed in the first 2 mm, and nearly the whole radiation is absorbed in the first cm. It is important to consider that fruit juices usually have values between 2 and 5 in this scale for most wavelengths. Thus, these profiles constitute the evidence of the fact that although it may have a high absorbance the radiation does penetrate in the fluid.

There are a great variety of profiles that can be achieved from the ($\mu \cdot I$) values network, integrating them with different conditions in one or two axes or just drawing a group of these values in different planes of the reactor. For example, Fig. 6 shows the absorbed radiation in the *xz* plane (perpendicular to the lamp) corresponding to the central position, $y = 11$ cm, for a μ value of 1. In this graph it is also easy to see how radiation absorption decreases with the distance from the reactor surface and from the lamp.

4. Conclusions

The development of absorbed radiation profiles has to be carried out in order to quantify and predict the chemical changes induced by ultraviolet irradiation in photochemical reactors, since the absorbed amount of radiation has a definitive effect on the reaction rate of the process in each point of the reaction medium depending on its location. In this way, the most important profile that must be considered during the design of irradiation equipment is the evolution of absorbed radiation with the reactor depth depending on the medium absorbance.

The application of the linear spherical model to a plane photoreactor leads to an easy equation that allows calculating a network of absorbed radiation values in discrete points of the reactor volume. The step-by-step integration by numerical methods of these values in the three dimensions of the space provides interesting information about the radiation dose that would be used in photochemical reactions.

Among these interesting conclusions, for example, the obtained profiles have shown that, with the considered geometry and dimensions, if the absorbance of the reaction medium is 0.1, hardly a 30% of the emitted radiation is absorbed, while for a μ value of 5 half

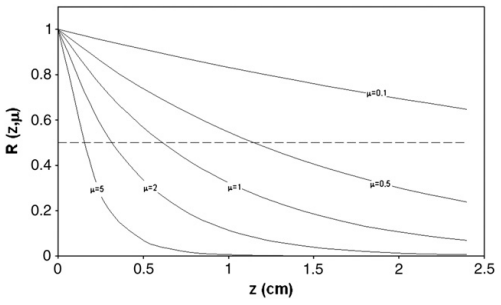

Fig. 5. Relative absorbed radiation profile as a function of medium depth for different medium absorbances.

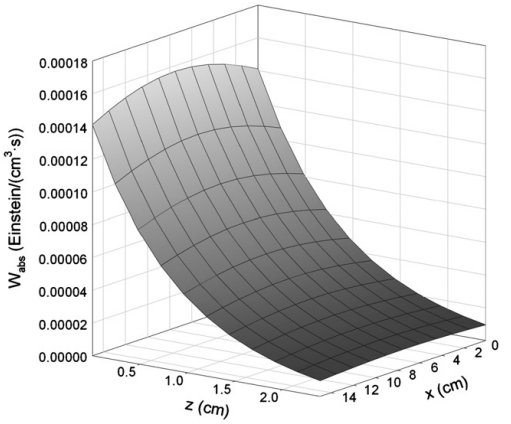

Fig. 6. Absorbed radiation profile in the *xz* plane corresponding to $y = 11$ cm, for a μ value of 1 cm^{-1}.

the radiation is absorbed in the first 2 mm, and nearly the whole radiation is absorbed in the first cm.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.12.

Title: Inactivation of polyphenol oxidase by ultraviolet irradiation. Protective effect of melanins.

Authors: Víctor Falguera, Jordi Pagán, Salvador Garza, Alfonso Garvín & Albert Ibarz.

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Inactivation of polyphenol oxidase by ultraviolet irradiation: Protective effect of melanins

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ABSTRACT

Most of the studies about UV irradiation of fruit and vegetable derivatives have been carried out in order to assess its effect on microbial inactivation. Nevertheless, there are few references about UV influence on some enzyme activities that are important in this kind of food, especially polyphenol oxidase, which is responsible for enzymatic browning in fruit and vegetable tissues containing phenolic or polyphenolic compounds. In this work, the effect of UV–Vis irradiation on polyphenol oxidase from *Agaricus bisporus* was investigated. A reduction of 58.7% in enzyme activity was achieved in the first 90 s, and it was completely inactivated after 35 min of treatment. In addition, the protective effect of melanins synthesized by the action of the same polyphenol oxidase was assessed. These pigments absorbed some of the radiant energy and led to a slower inactivation of polyphenol oxidase.

1. Introduction

Polyphenol oxidase (PPO, tyrosinase, E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen and various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of an heterogeneous group of melanins (Falguera et al., 2010a). The techniques to control PPO activity should act on one or more of the essential components necessary for the reaction: oxygen, enzyme, copper or substrate. PPO is one of the main enzymes that have to be inactivated during fruit derivatives processing (Falguera et al., 2011).

Food safety is one of the most important issues that food industries and food service companies have to face. The growing demand for a greater variety of prepared food and dishes on a menu makes the risk of contamination increases, especially due to the characteristics of this kind of food that gives a great importance to the quickness of meals. The application of HACCP (Hazard Analysis and Critical Control Points) has increased safety guarantees in production systems, thermal treatments and cold chains, helping to reduce the opportunities for pathogens to gain access to food and/or grow to levels that will pose a risk of infection or toxin production. However, the incidence of foodborne diseases continues to rise in most industrialized countries (Bintsis et al., 2000).

Currently, heat treatment process is the most commonly used hurdle for inactivating microorganisms and enzymes, extending products shelf life. However, this process may have adverse effects on sensory and nutritional quality of food (Braddock, 1999). In this context, non-thermal technologies have received increasing attention in recent years, especially in the preservation of beverages, due to its potential for inactivating spoilage and pathogenic microorganisms (Noci et al., 2008).

Most of the studies about UV irradiation of liquid food have been carried out in order to assess its effect in microbial inactivation. Nevertheless, there are few references about UV influence on some enzyme activities that are important in fruit derivatives, especially tyrosinase, which is still the major practical limitation to fruit handling, storage and processing (Jiang et al., 2004). In addition, fruit juices contain some components that may protect these enzymes to be denatured by the irradiation process (Guerrero-Beltrán and Barbosa-Cánovas, 2004; Koutchma, 2009). Among these compounds, the dark polymers formed by enzymatic browning have a special interest.

Some studies carried out on crystallins have shown that, in those enzymes, UV radiation causes oxidation of SH groups, changes in native conformation of the latter and formation of covalent cross-links between polypeptides (Krivandin et al., 2009). Since enzymatic activity depends on its structure, these modifications lead to enzyme inactivation. Such UV light-induced modifications have been reported to occur via two major routes: direct photo-oxidation arising from the absorption of radiation by the protein structure or bound chromophore and indirect protein oxidation mediated by singlet oxygen generated by energy transfer by either protein bound or other chromophores. In spite of this

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knowledge, the effect of UV light on structure and function of food enzymes is still a matter of speculation (Manzocco et al., 2009).

Thus, this piece of work has had two main aims. The first one has been to assay the inactivation of *Agaricus bisporus* polyphenol oxidase by means of UV–Vis irradiation. The second one to assess the protective effect that melanins synthesized from this enzyme and the two kinds of its substrates (L-tyrosine, a monophenol, and 4-methylcatechol, an *o*-diphenol) have on PPO during the irradiation process.

2. Materials and methods

2.1. Melanin synthesis and recovery

Agaricus bisporus polyphenol oxidase (Sigma Chemical, St. Louis, MO) was diluted in a 50 mM sodium phosphate buffer (pH 6.5) to an enzyme solution activity of 500 U mL^{-1} and distributed in aliquots of 1 mL. L-Tyrosine and 4-methylcatechol (Sigma Chemical, St. Louis, MO) were prepared in sodium phosphate buffer (pH 6.0 and 8.0, respectively, following the optimal pH for each kind of substrate determined in a previous work – Falguera et al., 2010b) in a concentration of 4.0 mM. The final enzyme content in each reaction mixture was 10 U mL^{-1}. After 24 h of reaction, melanins were precipitated adjusting the solution pH to 2.0 with HCl. The mixture was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 12 min at 12,000 rpm. The supernatant was discarded; the pellet was recovered with distilled water, lyophilized and rediluted in 50 mL of dimethyl sulfoxide (Sigma Chemical, St. Louis, MO) in different concentrations.

2.2. UV–Vis irradiation process

UV–Vis irradiation was carried out in a dark chamber containing the sample and the lamp. Five hundred milliliters of the sample was placed in a methacrylate tank of 22 × 15 × 10 cm. Each sample contained 12.5 U mL^{-1} of PPO in sodium phosphate buffer (pH 6.5) and different melanin concentrations, from 0.00 to 0.20 mg mL^{-1}. A refrigeration system consisting in a metallic coil fed with cold water was used to control temperature and avoid heating. Temperature was maintained at 25 ± 1 °C in all experiments. A magnetic stirrer was used during irradiation to ensure that the entire sample was subjected to the same UV dose. UV radiation was produced with a Philips HPM-12 high-pressure mercury lamp of 400 W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands), used in previous pieces of work (Ibarz et al., 2009; Falguera et al., 2011). Experimental series were carried out by duplicate.

2.3. PPO activity determination

PPO activity was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol as substrate, prepared in a phosphate buffer solution with pH 6.5. The reaction was carried out in a 1 cm light path optical glass cell. One unit of PPO was defined as the amount of enzyme that caused the increase of one unit of absorbance at 420 nm in one min (Ülker-Yerlitürk et al., 2008). PPO activity was assayed by duplicate.

2.4. Kinetic models

In some cases using different techniques, enzyme inactivation has been reported to follow a first order kinetic mechanism (Giner et al., 2001):

$N \xrightarrow{k} D$

where *N* and *D* are the native and the inactivated form of the enzyme, respectively.

This scheme leads to the following kinetic equation:

$$RA = RA_0 \cdot e^{(-k \cdot t)} \quad (1)$$

where *RA* is enzyme residual activity, RA_0 is the intercept of the curve, *k* is the first order kinetic constant and *t* is the time of treatment. In other cases, enzyme inactivation is supposed to occur in two consecutive irreversible first order steps with the presence of intermediate active forms of the enzyme (*I*), being the first one faster than the second one (Giner-Seguí et al., 2006):

$N \xrightarrow{k_1} I \xrightarrow{k_2} D$

and leading to the equation:

$$RA = e^{-k_1 \cdot t} - \frac{k_1 \cdot \Lambda}{(k_1 - k_2)}(e^{-k_1 \cdot t} - e^{-k_2 \cdot t}) \quad (2)$$

being k_1 and k_2 are the kinetic constants for the first and the second stages, respectively, and Λ *is* the ratio between the activities of the intermediate (partially inactivated, *I*) and the native (*N*) forms of the enzyme.

Experimental data were fitted to the kinetic expressions by non-linear regression procedures using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95% significance level.

3. Results and discussion

3.1. Inactivation of polyphenol oxidase in a model solution without any pigmentation

The irradiation of the sample containing 12 U mL^{-1} of *A. bisporus* polyphenol oxidase led to the complete inactivation of the enzyme after 35 min of treatment. However, in the first 90 s a big decrease in PPO activity was observed, being reduced to nearly the 70% of its original value. After 4 min of irradiation, residual activity was only 30% of the initial activity. It is also important to state that PPO activity was not recovered after 24 h in any taken sample, so the achieved inactivation was irreversible. In a previous piece of work performed with the same equipment, 20 min of irradiation were required to completely inactivate bovine carboxypeptidase-A, and 12 min were necessary to denature porcine trypsin (Ibarz et al., 2009).

3.2. Inactivation of polyphenol oxidase in a model solution containing melanins from L-tyrosine

Fig. 1 shows the evolution of polyphenol oxidase residual activity with irradiation time depending on melanin concentration in the medium. The enzyme was completely inactivated in all experimental series; the time that was necessary to accomplish this denaturalization was higher as the pigment concentration increased, showing the protective effect of these polymers. Thus, as it has been stated in the previous section, with no pigments in the medium polyphenol oxidase was inactivated after 35 min of irradiation. Meanwhile, with 0.200 mg mL^{-1} of melanin from L-tyrosine 70 min were required. The bigger decrease in enzyme activity in the first 90 s of treatment was especially remarkable in the samples with low melanin content. Another time, PPO activity was not recovered after 24 h in any sample, so the achieved inactivation was also irreversible.

In a previous piece of work carried out with the same equipment, 100 min were required to inactivate polyphenol oxidase in apple juices from four different varieties (Falguera et al., 2011). These differences show that the other compounds that juices contain have also a protective effect against the enzyme denaturaliza-

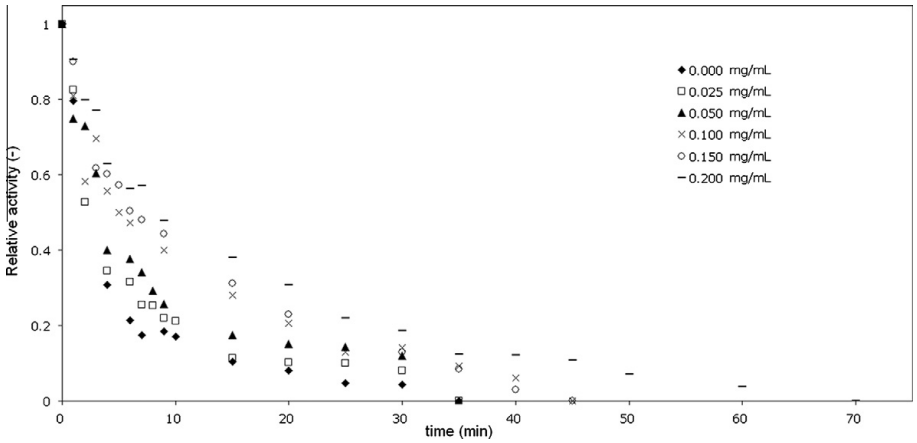

Fig. 1. Polyphenol oxidase activity decreasing with irradiation time in samples with different content in melanin synthesized from L-tyrosine.

tion. In that study, it was also found that the absorbance in the spectrum between 350 and 750 nm decreased as irradiation time increased, meaning that some pigments of the juice were impaired. The most affected ones were the molecules with absorbance peaks between 400 and 450 nm. In this way, the melanins formed by polyphenol oxidase activity have their maximum absorbance around 420 nm (Falguera et al., 2010b), leading to a higher protection than other colored compounds.

The determination coefficients obtained with the fittings of experimental data to the first order and the composite kinetic models are shown in Table 1. The values obtained with the first order model (Eq. (1)) corresponding to the samples with high melanin concentrations (from 0.10 to 0.20 mg mL^{-1}) are always higher than 0.97, while the ones corresponding to the samples with low melanin content (from 0.00 to 0.05 mg mL^{-1}) are considerably lower. This is probably due to the fact that the first order model is not able to explain the fast first step of PPO inactivation, which has a bigger effect when the pigment concentration in the reaction medium is low. On the contrary, the composite model (Eq. (2)) is able to explain both steps, so that determination coefficients are notably higher in the first group of samples. Nevertheless, it can also be observed that there is little variation with the values corresponding to higher pigment concentrations. Consequently, regarding to these samples the first order model would be more appropriate to describe this process due to its simplicity.

Table 2 shows estimated values of the parameters from the most suitable kinetic model for each sample. In the samples with low melanin content, k_1 tends to decrease as pigment concentration increases, meaning that the presence of melanins makes the first inactivation step slower. Meanwhile, k_2 and Λ do not show a defined tendency.

In the other samples (with melanin content from 0.10 to 0.20 mg mL^{-1}), the first order kinetic constant (k) decreases as melanin concentration increases, meaning that the inactivation gets slower due to the protective effect of melanins. In addition, RA_0 gets closer to 1.0 as melanin concentration increases, showing that the inactivation in the first seconds is smoother because the radiation is absorbed by the pigments in a higher degree. Consequently, the first order kinetic model is able to fit better the data points corresponding to the initial irradiation times as the melanin concentration grows up.

3.3. Inactivation of polyphenol oxidase in a model solution containing melanins from 4-methylcatechol

Residual enzyme activity with irradiation time depending on 4-methylcatechol melanin concentration appears in Fig. 2. As expected, higher treatment times are required as the melanin content increases. However, the fast decrease in enzyme activity in the first seconds has not been observed using this kind of melanin. In this case, it was also proved that the inactivation was irreversible, since no activity recovery was observed after 24 h. Table 3 shows the determination coefficients obtained with the fittings of experimental data to the first order and the composite kinetic models. Con-

Table 1
Determination coefficients of the fittings of data from series with melanin from L-tyrosine to the first order (Eq. (1)) and the composite (Eq. (2)) kinetic models.

Model	Melanin content (mg mL^{-1})					
	0.000	0.025	0.050	0.100	0.150	0.200
First order	0.8320	0.8544	0.8365	0.9747	0.9856	0.9895
Composite	0.9661	0.9925	0.9178	0.9766	0.9951	0.9904

Table 2
Estimated values of the parameters from the most suitable kinetic model for each simple, in experimental series with melanin from L-tyrosine.

Melanin content (mg mL^{-1})	Model	Parameter	Value	Parameter	Value	Parameter	Value
0.000	Composite	k_1 (min^{-1})	0.790	k_2 (min^{-1})	0.057	Λ (–)	0.245
0.025	Composite	k_1 (min^{-1})	0.543	k_2 (min^{-1})	0.065	Λ (–)	0.338
0.050	Composite	k_1 (min^{-1})	0.268	k_2 (min^{-1})	0.027	Λ (–)	0.233
0.100	First order	RA_0 (–)	0.752	k (min^{-1})	0.064		
0.150	First order	RA_0 (–)	0.822	k (min^{-1})	0.062		
0.200	First order	RA_0 (–)	0.840	k (min^{-1})	0.050		

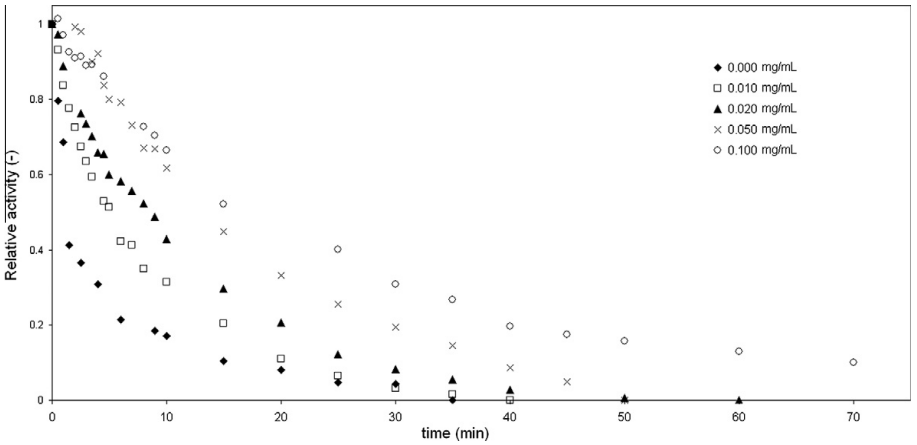

Fig. 2. Polyphenol oxidase activity decreasing with irradiation time in samples with different content in melanin synthesized from 4-methylcatechol.

Table 3
Determination coefficients of the fittings of data from series with melanin from 4-methylcatechol to the first order (Eq. (1)) and the composite (Eq. (2)) kinetic models.

Model	Melanin content (mg mL^{-1})				
	0.000	0.010	0.020	0.050	0.100
First order	0.8320	0.9922	0.9945	0.9969	0.9950
Composite	0.9661	0.9986	0.9974	0.9973	0.9951

trary to what happened with the melanin from L-tyrosine, these values were higher than 0.99 in all experimental series containing melanin from 4-methylcatechol using both kinetic models. These parameters support the previous statement that in this case the fast first step was not observed, so that the first order equation is also able to fit experimental data.

Table 4 corresponds to the kinetic parameters of fitting experimental data from series with melanin from 4-methylcatechol to both models. Firstly, attending to the first order kinetic model, the inactivation constant (k) clearly decreases as melanin concentration increases, being an appropriate parameter to quantify the protective effect of these dark polymers on polyphenol oxidase denaturalization. In this case, RA_0 is close to the unit in all series, reinforcing the fact that the fast inactivation in the first seconds did not occur.

Regarding the composite model, the kinetic constant of the first step (k_1) increases as melanin concentration grows up, meaning that the transformation of the native form of the enzyme (N) into the intermediate one (I) is faster. Nevertheless, as it has been observed, this first stage lasts for a very short time. Inactivation constant (k_2), which describes the transformation of the intermediate form (I, still active) into the denatured one (D), significantly decreases as melanin content increases, showing that this step is slower due to the protective effect of melanins, as it was also seen with the constant k of the first order model. Finally, the parameter A shows a clear upward trend. Therefore, it is confirmed that the transition from the native form to the intermediate form is faster at higher melanin concentrations.

Comparing both kinds of melanin it can be seen that, at the same concentration, melanin from 4-methylcatechol has a higher protective effect than melanin from L-tyrosine. With a polymer content of 0.1 mg mL^{-1}, the values of inactivation constant from the first order kinetic model (k) are 0.064 min^{-1} for L-tyrosine melanin (Table 2), and 0.034 min^{-1} for 4-methylcatechol melanin (Table 4). Consequently, with this pigment concentration the decrease in enzyme activity with irradiation time will be a 47% slower with melanin from 4-methylcatechol.

4. Conclusions

It is possible to completely and irreversibly inactivate *A. bisporus* polyphenol oxidase with an UV–Vis irradiation process. About 12.5 U mL^{-1} were inactivated after 35 min of treatment with a high-pressure mercury lamp of 400 W of nominal power. The presence of melanins in the reaction medium protects the enzyme, making the inactivation process slower as their concentration increases.

If the reaction medium contains melanins synthesized from L-tyrosine, when their content is below 0.05 mg mL^{-1} a composite kinetic model is required to explain the fast inactivation in the

Table 4
Estimated values of the parameters from both kinetic models for each sample, in experimental series with melanin from 4-methylcatechol.

Melanin content (mg mL^{-1})	Model	Parameter	Value	Parameter	Value	Parameter	Value
0.000	Composite	k_1 (min^{-1})	0.790	k_2 (min^{-1})	0.057	A (–)	0.245
0.010	First order	RA_0 (–)	0.95	k (min^{-1})	0.123		
0.020	First order	RA_0 (–)	0.97	k (min^{-1})	0.083		
0.050	First order	RA_0 (–)	1.06	k (min^{-1})	0.072		
0.100	First order	RA_0 (–)	1.06	k (min^{-1})	0.034		
0.010	Composite	k_1 (min^{-1})	0.514	k_2 (min^{-1})	0.096	A (–)	0.621
0.020	Composite	k_1 (min^{-1})	0.723	k_2 (min^{-1})	0.075	A (–)	0.813
0.050	Composite	k_1 (min^{-1})	0.911	k_2 (min^{-1})	0.074	A (–)	0.990
0.100	Composite	k_1 (min^{-1})	1.010	k_2 (min^{-1})	0.034	A (–)	1.021

early stages. When pigment content is higher these molecules also protect PPO from this fast denaturalization, making the simple first order kinetic model able to explain the whole process.

With melanins from 4-methylcatechol in the reaction medium, both models fitted experimental data. The first order kinetic model is more appropriate to easily describe and quantify the protective effect due to its mathematical simplicity. However, the use of the composite kinetic model brings additional information about the transformation of the native enzyme to an intermediate form and to the denatured inactive form later.

At the same concentration, melanin from 4-methylcatechol has a higher protective effect than melanin from L-tyrosine. For example, with a melanin content of 0.1 mg mL^{-1} the decrease in polyphenol oxidase activity with irradiation time will be a 47% slower with melanin from 4-methylcatechol.

Although the obtained results (in model solutions) bring a closer approach to the interaction between ultraviolet radiation and polyphenol oxidase in its quaternary structure, further research at a molecular scale should be conducted in order to understand the mechanism by which this interaction leads to the inactivation of the enzyme.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.13.

Title: Protective effect of melanoidins from fructose-glutamic acid on polyphenol oxidase inactivation by ultraviolet-visible irradiation.

Authors: Víctor Falguera, Aurea Folch, Alfonso Garvín & Albert Ibarz.

Journal: *Food and Bioprocess Technology* (in press).

DOI: 10.1007/s11947-012-0887-5.

Food Bioprocess Technol
DOI 10.1007/s11947-012-0887-5

COMMUNICATION

Protective Effect of Melanoidins from Fructose–Glutamic Acid on Polyphenol Oxidase Inactivation by Ultraviolet–Visible Irradiation

Víctor Falguera · Aurea Folch · Alfonso Garvín · Albert Ibarz

Received: 8 February 2012 / Accepted: 2 May 2012

Abstract Ultraviolet irradiation has been proved to be effective for inactivating polyphenol oxidase in some fruit derivatives. However, some compounds that may be found in these products, such as melanoidins, can protect the enzyme during the irradiation process. In this piece of work, the protective effect of melanoidins synthesized from fructose and glutamic acid on *Agaricus bisporus* polyphenol oxidase inactivation by ultraviolet–visible irradiation has been assessed. The polymers with molecular mass lower than 150 kDa had a greater protective effect than those molecules higher than 150 kDa. If the obtained melanoidins are not fractioned by their molecular mass, the protective effect that they exert is lower. It was found that the most effective radiation to inactivate this polyphenol oxidase is that between 260 and 310 nm.

Keywords Melanoidin · Polyphenol oxidase · UV irradiation · Browning · Mushroom

Introduction

In recent times, consumers' demand has experienced a progressive shift toward new food products and processing methods that provide fresh-like quality attributes such as flavor, texture, color, vitamin content, aroma, and overall appearance. These attributes, as well as product shelf life, are closely related to microbial quality and biochemical and enzymatic reactions (Campus 2010; Landl et al. 2010). In this context, novel technologies constitute an alternative to classic thermal processes that are being studied and developed in order to obtain a better final product sensory quality, without neglecting microbial safety and avoiding the loss of these fresh-like features (Falguera et al. 2011a; Mújica-Paz et al. 2011). In addition, recent studies have shown that nonthermal methods may produce less allergenic products than those obtained by conventional thermal ones (Shriver and Yang 2011).

Among nonthermal food processing technologies, ultraviolet irradiation has been proved to be effective in the inactivation of polyphenol oxidase in some fruit derivatives (Falguera et al. 2011b). Polyphenol oxidase (PPO, E.C. 1.14.18.1) is the main enzyme responsible for enzymatic browning in vegetable tissues, and therefore, it is the major practical limitation to fruit handling, storage, and processing (Jiang et al. 2004). Enzyme inactivation by ultraviolet light has been reported to occur via two major routes that cause structure modifications, showing a clear relationship between structure and function: direct photooxidation arising from the absorption of radiation by the protein itself or a bounded chromophore and indirect protein oxidation mediated by singlet oxygen generated by energy transfer by either protein bound or other chromophores (Manzocco et al. 2009).

However, there are some compounds inside food systems that can prevent the enzyme from being denatured when the product is subjected to ultraviolet processing. In this way, the presence of dark polymers (melanins) formed by polyphenol oxidase activity or even high concentrations of the natural pigments of fruits lead to slower polyphenol oxidase inactivation (Falguera et al. 2011b, 2011c). However, there are no references in literature quantifying the protective effect of the colored compounds formed by the so-called

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Maillard reaction (melanoidins), which are the main products of nonenzymatic browning and represent one of the most important deterioration mechanisms of fruits, vegetables, mushrooms, and their derivatives (Ibarz et al. 2009a). This piece of work fills in this gap, in the specific case of *Agaricus bisporus* and the melanoidins synthesized from its major sugar, fructose (Hammond 1985), and amino acid, glutamic acid (Mattila et al. 2002). In addition, the effect of the global obtained melanoidins has been compared with the effect caused by low molecular mass (<150 kDa) and high molecular mass (>150 kDa) compounds.

Materials and Methods

Melanoidin Synthesis, Purification, and Recovery

For melanoidin synthesis, a solution was prepared by dissolving 300 g of fructose (Fru; Panreac Quimica S.A.U., Castellar del Vallès, Spain) and 2 g of glutamic acid (Glu; Probus S.A., Badalona, Spain) in 1 L of distilled water. This solution was kept at 95 °C for 13 days (Ibarz et al. 2009a). After the thermal process, melanoidin system was passed through a 5-cm-diameter glass column with a fiberglass plate in the bottom that contained a 10-g layer of activated carbon. Melanoidins were adsorbed to the activated carbon, which was then washed with distilled water until the outgoing solution showed no reaction to reducing sugars (by Fehling's reagent reduction method). After sugar surplus removal, melanoidins were recovered by passing a 25 % pyridine solution through the activated carbon layer. After filtering with a Whatman paper no. 1, the solvent was partially removed by rotovaporation (Resona Technics, Switzerland). Finally, the resulting preconcentrated melanoidins were lyophilized.

Afterwards, half of the obtained melanoidins were dissolved in distilled water, and a fractionation was carried out with a tubular ceramic membrane system of 150-kDa cutoff (Tami Industries, Nyons, France). This membrane was selected, based on preliminary results, so as to separate approximately 50 % of the polymers above and below this molecular mass. The obtained fractions were lyophilized once again for quantification.

Ultraviolet–Visible Irradiation Process

UV–vis irradiation was carried out in a dark chamber containing the sample and the lamp. Five hundred milliliters of the sample was placed in a methacrylate tank of 22×15×10 cm. Each sample contained 12.5 $U mL^{-1}$ of *A. bisporus* PPO (Sigma Chemical, Saint Louis, MO, USA) in McIlvaine buffer (pH 6.5) and different melanoidin concentrations, from 0.0 to 0.4 mg mL^{-1}. A refrigeration system consisting of a metallic coil fed with a 50 % ethylene glycol solution was used to control the sample temperature, which was kept at (25±1)°C in all experiments. A magnetic stirrer was used during irradiation to ensure sample homogeneity. UV–vis radiation was produced by means of a Philips HPM-12 medium-pressure mercury lamp of 400 W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands), used in previous pieces of work (the emission spectrum can be found in Ibarz et al. 2009b and Falguera et al. 2011b). The real emission power of the lamp that reached the reactor surface was $3.88 \cdot 10^{-7}$ E min^{-1} (Falguera et al. 2011b). Therefore, UV–vis radiation fluence was $1.18 \cdot 10^{-9}$ E min^{-1} cm^{-2}. Experimental series were carried out by duplicate, and the absorbance spectrum of the irradiated samples was measured every 10 min by means of a Helios Omega spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

PPO Activity Determination

PPO activity was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol (Sigma Chemical, Saint Louis, MO, USA) as substrate, prepared in McIlvaine buffer at pH 6.5. The reaction was carried out in a 1-cm light path optical glass cell. One unit of PPO was defined as the amount of enzyme that caused the increase of one unit of absorbance at 420 nm in 1 min (Falguera et al. 2011c). PPO activity was assayed by duplicate.

Kinetic Models and Statistical Processing

In some cases using different techniques, enzyme inactivation has been reported to follow a first-order kinetic mechanism (Giner et al. 2001):

$$N \xrightarrow{k} D$$

where N and D are the native and the inactivated form of the enzyme, respectively. This scheme leads to the following kinetic equation:

$$\mathrm{RA} = \mathrm{RA}_0 \cdot e^{(-k \cdot t)} \quad (1)$$

where RA is enzyme residual activity, RA_0 is the intercept of the curve, k is the first-order kinetic constant, and t is the time of treatment. In other cases, enzyme inactivation is supposed to occur in two consecutive irreversible first-order steps with the presence of intermediate active forms of the enzyme (I), being the first one faster than the second one (Giner-Seguí et al. 2006):

$$N \xrightarrow{k_1} I \xrightarrow{k_2} D$$

and leading to the equation:

$$RA = e^{-k_1 \cdot t} - \frac{k_1 \cdot \Lambda}{(k_1 - k_2)}\left(e^{-k_1 \cdot t} - e^{-k_2 \cdot t}\right) \quad (2)$$

being k_1 and k_2 the kinetic constants for the first and the second stages, respectively, and Λ the ratio between the activities of the intermediate (partially inactivated, *I*) and the native (*N*) forms of the enzyme.

Experimental data were fitted to the kinetic expressions by nonlinear regression procedures using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The results are expressed by means of the estimated value and the standard error.

Table 1 Determination coefficients of experimental data fittings to the first order (Eq. 1) and the composite (Eq. 2) kinetic models

	Melanoidin content (mg/mL)	First-order model	Composite model
Non-fractioned	0	0.9693	0.9918
	0.1	0.9275	0.9828
	0.2	0.9423	0.9941
	0.4	0.9638	0.9782
<150 kDa	0.1	0.6787	0.9780
	0.2	0.7047	0.9535
	0.4	0.8838	0.9503
>150 kDa	0.1	0.7232	0.9856
	0.2	0.7421	0.9592
	0.4	0.9599	0.9866

Results and Discussion

First of all, it can be stated that 12.5 U/mL of polyphenol oxidase was completely inactivated after 85 min of irradiation when the reaction medium contained no melanoidins. In a previous piece of work carried out using the same equipment (Falguera et al. 2011c), only 35 min of treatment was necessary to inactivate the enzyme in this model solution at the same pH (6.5). This large difference may be attributed to the fact that, in that case, all the samples contained 10 % dimethyl sulfoxide to ease melanin solubilization, which could have played an important role affecting PPO denaturation. This issue should be extensively studied in the future.

In this standard sample, residual activity rapidly decreased in the first 10 min, being reduced to 56 % of its original value. After this moment, inactivation pace was slower. Neither in this solution nor in the ones containing melanoidins PPO activity was recovered after 24 h, so the achieved inactivation was irreversible.

In order to assess the effect of melanoidins synthesized from Fru–Glu, three different groups of experimental series were performed: in the first one, non-fractioned melanoidins (NF) were added in concentrations from 0.1 up to 0.4 mg/mL. The remaining experimental groups were carried out adding melanoidins lower or higher than 150 kDa, in the same concentrations. Quantification of those polymers after ultrafiltration and recovering showed that the obtained Maillard reaction products contained 46.6 % of molecules of less than 150 kDa and 53.4 % of molecules with a molecular mass higher than this value.

As expected, the higher the melanin content, the slower the PPO inactivation. Table 1 shows the determination coefficients of experimental data fittings to the first order (Eq. 1) and the composite (Eq. 2) kinetic models. This coefficient is always higher in the composite model. In many cases, the first-order equation does not fit well the experimental data, mainly due to the rapid decrease of activity that occurs in the first minutes (as explained in the case of the standard solution) that this model cannot describe. In this way, in the three series the first-order determination coefficient increases with increasing polymer concentration: as melanoidin content increases, the inactivation of PPO is slower, mitigating the effect of this fast inactivation in the first minutes.

Therefore, the kinetic parameters obtained with the composite model (Table 2) must be studied to infer the effects of the different melanoidin fractions (kinetic parameters from the first-order model are not shown). Inactivation constant, k_2, describes the transformation of the intermediate enzyme form (*I*, still active) into the inactive one (*D*). Since the application of this technology aims at the complete inactivation of PPO, this second constant will be the most important parameter to be studied. As it has been already commented, this second step of the process lasts for longer than the first one. This kinetic constant decreases as melanoidin content increases in all series, which shows that the polymers prevent the enzyme from being inactivated, making this phase slower. Comparing the values obtained with the different fractions, it can be stated that this protective effect is greater in the separated fractions than in the NF. In this way, for each single melanoidin concentration, the polymers lower than 150 kDa exhibited the greatest protection (k_2 has the lowest value), followed by the fraction of more than 150 kDa and, lastly, the NF. This fact can be explained by agglomeration phenomena between the polymers in the solution, which were more important in the NF (aggregation is favored by the presence of different chain length molecules) leading to a lower absorbance of the solution (Fig. 1).

As far as the first step of the process (the transformation of the native form, *N*, into the intermediate one, *I*, described by k_1) is concerned, it lasts for shorter as melanoidin content

Table 2 Estimated values of the kinetic parameters obtained with the composite kinetic model (Eq. 2)

	Melanoidin content (mg/mL)	k_1 (min^{-1})	k_2 (min^{-1})	Λ (−)
	0	0.072±0.034	0.0252±0.0053	0.392±0.025
Non-fractioned	0.1	0.108±0.047	0.0144±0.0006	0.455±0.102
	0.2	0.212±0.045	0.0074±0.0005	0.752±0.029
	0.4	0.404±0.017	0.0038±0.0006	0.831±0.024
<150 kDa	0.1	0.352±0.032	0.0051±0.0003	0.653±0.017
	0.2	0.432±0.075	0.0025±0.0006	0.787±0.006
	0.4	0.788±0.027	0.0010±0.0001	0.816±0.012
>150 kDa	0.1	0.086±0.016	0.0064±0.0008	0.698±0.016
	0.2	0.243±0.036	0.0046±0.0004	0.733±0.005
	0.4	0.408±0.027	0.0013±0.0001	0.757±0.048

Mean value±standard deviation

increases. Considering the kinetic model from the mathematical point of view, this k_1 is useful to describe the great decrease of activity in the first minutes. In this way, as the melanoidin content (and therefore its protective effect) increases, the effect of this fast early inactivation is minor. This parameter has its highest values in the series with polymers smaller than 150 kDa. If the kinetic parameters obtained in this piece of work are compared with those found for melanins from 4-methylcatechol at the same concentration (Falguera et al. 2011c), it can be stated that these melanoidins (regardless of the considered fraction) have a greater protective effect than those melanins. This fact can be explained by the difference in the solubility between both kinds of polymers, which leads to different absorbance of their aqueous solutions.

As it has been already commented, absorbance spectra of the solutions that contain the different fractions of melanoidins (Fig. 1) can explain the different protection degree that these polymers offer against PPO inactivation by UV–vis irradiation. Since the polymers with molecular mass lower than 150 kDa were the most protective ones, followed by the fraction higher than 150 kDa and in the last place the NF, the most important wavelengths to inactivate the enzyme must be those in which the absorbance of the different samples follows the same order. This condition is accomplished between 260 and 310 nm. This fact would indicate that, although the lamp emitted radiation between 250 and 740 nm, UV radiation would have contributed more to PPO inactivation than visible radiation since in this region of the spectrum (between 400 and 700 nm) the absorbance of the different melanoidin solutions did not follow this order. However, this statement should be verified in the future by using stable filters for the different regions of the emission spectrum.

In addition, the absorbance of the different solutions in the entire spectrum (not shown) decreased as processing time increased (with the most remarkable decrease at wavelengths between 260 and 310 nm), meaning that melanoidin polymers were partially impaired by UV irradiation. In other

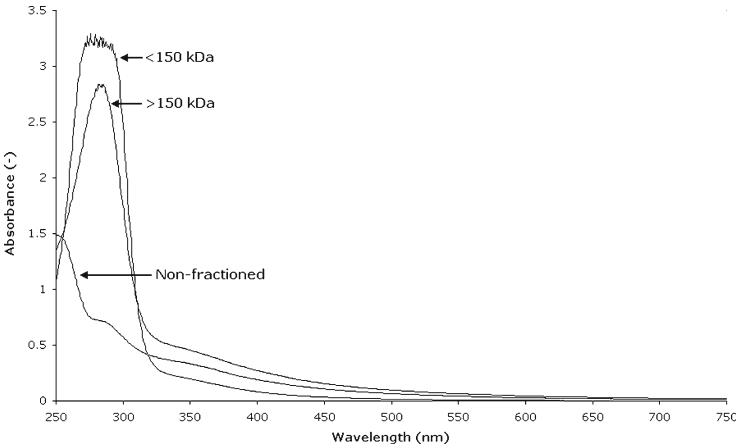

Fig. 1 Absorbance spectra of the different fresh melanoidin solutions at a concentration of 0.1 mg/mL

words, these molecules absorbed radiation and were partially broken, leaving a lower radiation dose to inactivate the enzyme. This observation is consistent with other results that can be found in literature since it has been proved that this process affects the colored compounds of fruit and vegetable derivatives, either those that are naturally present (endogenous pigments) or the ones formed later by the action of PPO (melanins) and by Maillard reaction (melanoidins) (Falguera et al. 2011c).

Conclusions

A. bisporus polyphenol oxidase (12.5 U/mL) was completely and irreversibly inactivated after 85 min of irradiation with a medium-pressure mercury lamp that emits in a range between 250 and 740 nm. However, the study of the protective effect of different melanoidin solutions and their absorbance spectra would indicate that the most effective radiation to inactivate PPO might be that emitted between 260 and 310 nm.

Regarding the effect of the melanoidins synthesized from fructose and glutamic acid on PPO denaturation, the presence of these molecules makes the denaturation process slower, preventing the enzyme from being completely inactivated in the same processing time. The achieved inactivation degrees after 90 min of processing and the kinetic study led to the conclusion that melanoidins with molecular mass lower than 150 kDa have a greater protective effect than those molecules higher than 150 kDa. If the obtained melanoidins are not fractioned by their molecular mass, the protective effect that they exert is lower due to aggregation phenomena favored by the presence of different chain length polymers, which leads to a lower absorbance in the UV region of the spectrum. These results provide new information about how some of the molecules that are contained in fruit derivatives can affect PPO inactivation by UV–vis irradiation. Such information can be useful to predict processing times for different products depending on their absorbance and their content in different pigments.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.14.

Title: Effect of UV irradiation on enzymatic activities and physicochemical properties of apple juices from different varieties.

Authors: Víctor Falguera, Jordi Pagán & Albert Ibarz.

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Effect of UV irradiation on enzymatic activities and physicochemical properties of apple juices from different varieties

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ABSTRACT

The influence of ultraviolet irradiation (UV) on some quality attributes (colour, pH, soluble solids content, formol index, total phenolics, sugars and vitamin C) and enzymatic activities (polyphenol oxidase, peroxidase and pectinolytic enzymes) of fresh apple juice was investigated. Apple juices made from four different varieties (Golden, Starking, Fuji and King David) were irradiated during 120 min with a high-pressure mercury lamp of 400 W that emits in a range between 250 and 740 nm with a resulting incident energy of $3.88 \cdot 10^{-7}$ E·min^{-1}. The treatment was effective in the inactivation of the assayed enzymes. Polyphenol oxidase was inactivated in 100 min, while peroxidase was completely destroyed in only 15 min in all the four varieties. No variations were observed in pH, soluble solids content, formol index, total phenolics and sugars. The colour of the juice made from King David was unvariable during all the experiment. In the ones made from Golden, Starking and Fuji, UV radiation impaired some of the pigments present in the juice, either initially present or the ones formed later. This fact can be especially observed in the increase of the CIELab parameter L*, which increased from 23.2 to 24.6 in Fuji juice. The content of vitamin C in juices from Golden, Starking and Fuji slightly changed during the experiment, decreasing a 4.0% in Fuji juice, 5.7% in Golden one and 5.6% in Starking one. Meanwhile, in the juice from King David the loss was 70.0%. This significant difference can be attributed to the lack of pigmentation of this juice.

1. Introduction

Food safety is one of the most important issues facing food industries and food service companies. Currently, the growing demand for a greater variety of prepared food and dishes on a menu makes the risk of contamination increases, especially due to the characteristics of this kind of food that gives a great importance to the quickness of meals. The application of HACCP (Hazard Analysis and Critical Control Points) has increased security guarantees in production systems, thermal treatments and cold chains, helping to reduce the opportunities for pathogens to gain access to food and/or grow to levels that will pose a risk of infection or toxin production. However, the incidence of foodborne diseases continues to rise in most industrialized countries (Bintsis, Litopoulou-Tzanetaki, & Robinson, 2000).

Moreover, in recent times the demand by consumers for minimally processed foods with characteristics as similar as possible to fresh products, has increased rapidly. Currently, heat treatment process is the most commonly used hurdle for inactivating microorganisms and enzymes, extending products shelf-life. However, this process may have adverse effects on sensory and nutritional quality of food (Braddock, 1999). In this context, non-thermal technologies have received increasing attention in recent years, especially in the preservation of beverages, due to its potential for inactivating spoilage and pathogenic microorganisms. In addition, some of these techniques can help to minimize the loss of quality in terms of flavor, colour and nutritional value (Noci et al., 2008).

UV radiation has been used for decades to disinfect water, being an effective method for microbial decontamination of surfaces and packaging in the food industry (Bintsis et al., 2000). The main limitations of UV application are related to the low penetration and the effect reduction in the presence of suspended solids. In distilled water, UV radiation at 254 nm suffers a loss of 30% of its intensity 40 cm below the surface, while the sea water will cause the same reduction in just 10 cm. A 10% sucrose solution or water with high iron levels cause the same loss in 5 cm (Guerrero-Beltrán & Barbosa-Cánovas, 2004; Snowball & Hornsey, 1988).

Regarding the preservation of the organoleptic and nutritional quality some research has shown promising results in the exposure of apple juice to UV irradiation (Harrington & Hills, 1968). Currently,

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in the manufacture of fruit juices, the FDA requires to apply a preservation method capable of reducing pathogens at least 5 logarithmic cycles (FDA, 2000). They also approved ultraviolet irradiation as a suitable method for the preservation of fruit juices, just if the turbulent flow conditions can be ensured throughout the treatment process. However, this requirement is not necessary when UV radiation is not the only hurdle.

Most of the studies about UV irradiation of fruit juices have been carried out in order to assess its effect in microbial inactivation. Nevertheless, there are few references about UV influence on some enzyme activities that are important in this kind of fruit derivatives, especially tyrosinase, which is responsible of enzymatic browning in vegetable tissues containing phenolic or polyphenolic compounds (Falguera, Pagán, & Ibarz, 2010). In this work, the effect of UV irradiation on apple juices from 4 varieties has been assayed, measuring some physicochemical properties and the activities of some enzymes (including tyrosinase, peroxidase and pectinolytic enzymes).

2. Material and methods

2.1. Juice preparation

Apples from the four different varieties (Golden, Starking, Fuji and King David) were washed, peeled and kept in water while waiting to be cut in four and squeezed with a household juicer. The resulting juice was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 25 min at 13,000 rpm. This process was done at 4 °C to make separation of supernatant easier and to reduce enzymatic activities After centrifugation was complete, the supernatant was recovered and the pellet was discarded. Fractions of 800 mL of juice were stored in the freezer.

2.2. UV processing

UV irradiation was carried out in a dark chamber containing the juice and the lamp. Apple juice was placed in a methacrylate tank of $22 \times 15 \times 10$ cm. 800 mL of juice were processed, reaching a height of 2.4 cm inside the tank. A refrigeration system consisting in a metallic coil fed with cold water was used to control temperature and avoid juice heating. Juice temperature was maintained at $25°C \pm 1$ °C in all experiments. A magnetic stirrer was used during irradiation to ensure that all the juice was subjected to the same UV dose. UV radiation was produced with a Philips HPM-12 high-pressure mercury lamp of 400 W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands), used in previous works (Ibarz, Pagán, Panadés, & Garza, 2005; Ibarz, Garvín, Garza, & Pagán, 2009). The real incident energy, determined as described by Esplugas and Vicente (1991), was $3.88 \cdot 10^{-7}$ E·min^{-1} (Ibarz et al., 2009). Fig. 1 shows the spectrum emitted by the lamp (data provided by the lamp manufacturer). The distance between the juice surface and the lamp was 22.5 cm.

Irradiated juice was removed from the freezer 2 h before irradiation started. The lamp was lit 10 min before putting the juice in the chamber. Irradiation was carried out during 2 h. A sample was taken every 20 min and placed in a refrigerator until its analysis. Additional samples were taken at 5, 10 and 15 min to analyse peroxidase activity. All determinations were carried out in duplicate.

2.3. Physical and chemical analysis

The pH of the juice before and after treatment was measured with a Crison micropH 2000 pHmeter (Crison Instruments, S.A., Alella, Spain). Soluble solids content was assessed using an Atago RX-1000 Digital Refractometer (Atago Co. Ltd., Japan). The juice colour was monitored using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Japan) in the CIELab colour space. Absorption spectrum between 350 and 750 nm was measured with an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA), using a 1 cm width quartz cell.

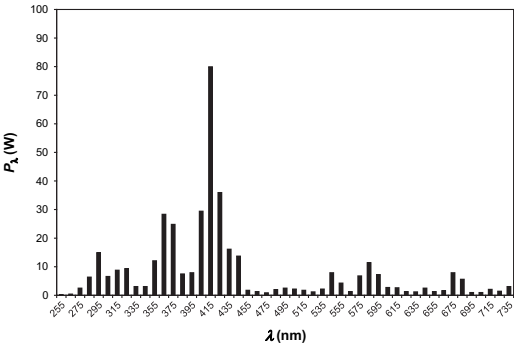

Fig. 1. Emission spectrum of the Philips HPM-12 lamp. P_λ is the power of emission at a λ wavelength.

Total phenolic content was determined as described by Shaghaghi, Manzoori, and Jouybanb (2008), with a spectrophotometric method using the Folin-Ciocalteu reagent. Formol index was measured according to the official method of the International Federation of Fruit Juice Producers (IFFJP, 1984), with a potentiometric titration of the acidity of the compounds formed by the reaction of formaldehyde and amino acids in the juice up to a pH of 8.1. Total and reducing sugars were determined by the reduction of the Fehling reagent, according to the method of the IFFJP (1972). Vitamin C was quantified by iodine titration, as described by Suntornsuk, Gritsanapun, Nilkamhank, and Paochomb (2002).

2.4. Enzymatic activities

Polyphenol oxidase activity in the juice was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol as a substate prepared in a buffer solution with a pH of 3.5. The reaction was carried out in a 1 cm light path quartz cell. One unit (U) of PPO was defined as the amount of enzyme that caused the increase of one absorbance unit (AU) at 420 nm in 10 min (Ülker-Yerlitürk, Arslan, Sinan, Gencer, & Özensoy, 2008).

Peroxidase activity was determined by the method described by Kwak et al. (1995) using pyrogallol as a substrate. The standard assay reaction mixture contained, in a total vol. of 3 mL: juice sample (2.2 mL), 100 mM K-Pi buffer (pH 6, 0.32 mL), 5% pyrogallol (0.32 mL, w/v) and 0.147 M H_2O_2 (0.16 mL). The reaction was initiated by the addition of H_2O_2, and the increase in A_{420} was recorded in 3 min. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 3 min at pH 6.

Pectinmethylesterase (PME) activity was measured by determining free carboxyl groups formed as a result of enzyme action on pectin. The reaction mixture was composed of 40 mL of the 1% pectin solution in 0.15 mol L^{-1} NaCl and 10 mL of the juice sample. The pH of this mixture was adjusted to 7.7 with 0.05 mol L^{-1} NaOH. 100 μL of 0.05 mol L^{-1} NaOH were added to the reaction mixture, and the time needed to recovery the pH of 7.7 (50 ± 2 °C) was measured. One unit of PME was defined as the amount of enzyme that released 1 μmol of carboxyl groups in 1 min (de Assis, Martins, & de Faria, 2007).

Exo-polygalacturonase (exo-PG) activity was assayed in a mixture containing 1% pectin solution (CP Kelco) in 0.2 mol L^{-1} sodium acetate buffer (pH 5.5) at 50 °C for 10 min. The number of reducing groups, expressed as galacturonic acid released by enzymatic action, was quantified by the DNS method. One unit of enzyme activity was defined as the amount of enzyme releasing 1 mmol of galacturonic acid per minute under these assay conditions (Silva et al., 2007).

Endo-polygalacturonase (endo-PG) was measured viscosimetrically by adding 10 mL of juice sample to 40 mL of 0.2 M citrate–NaOH buffer (pH 5.5) containing 3% of pectin (CP Kelco). The reaction mixture was incubated at 50 °C for 15 min, after which its viscosity was determined with a Haake RS80 rheometer (Gebrüder Haake GmbH, Karlsruhe, Germany). One unit of enzyme activity was defined as the amount of enzyme that reduced the initial viscosity by 50% per minute, under these conditions (Silva et al., 2007).

3. Results and discussion

3.1. Physical and chemical analysis

3.1.1. pH, soluble solids content, formol index, total phenolics and sugars

Although there were important initial differences between the juices from the four varieties, these parameters kept their original value constant during the 120 min the experiment lasted. For example, total phenolics content was 2.55 mg L^{-1} in Starking juice and 10.57 in King David one. The average values of all these composition variables are shown in Table 1. pH measures showed a maximum variation of 0.03, while in soluble solids it was 0.2 °Brix. All the observed variations can be attributed to experimental error. Noci et al. (2008) also found no differences in the evolution of pH and soluble solids content in the UV irradiation of apple juice. However, they also found a decrease in total phenolics content during the process that has not been observed in the present work.

3.1.2. Juice colour

CIELab parameters L*, a* and b* were measured during the irradiation process. Fig. 2 shows the evolution of these parameters in the 120 min. L*, which is an indicator of the brightness of the juice, increased with the time of irradiation in Golden, Starking and Fuji samples. In addition, Table 2 shows the differences found in the absorbance at different wavelengths in the visible region between the samples taken before starting and after 120 min of irradiation. This indicates that UV radiation impairs some of the pigments present in the juice, either initially present or the ones formed later by the rapid action of polyphenol oxidase (melanins) as well as the Maillard reaction between sugars and amino acids (melanoidins).

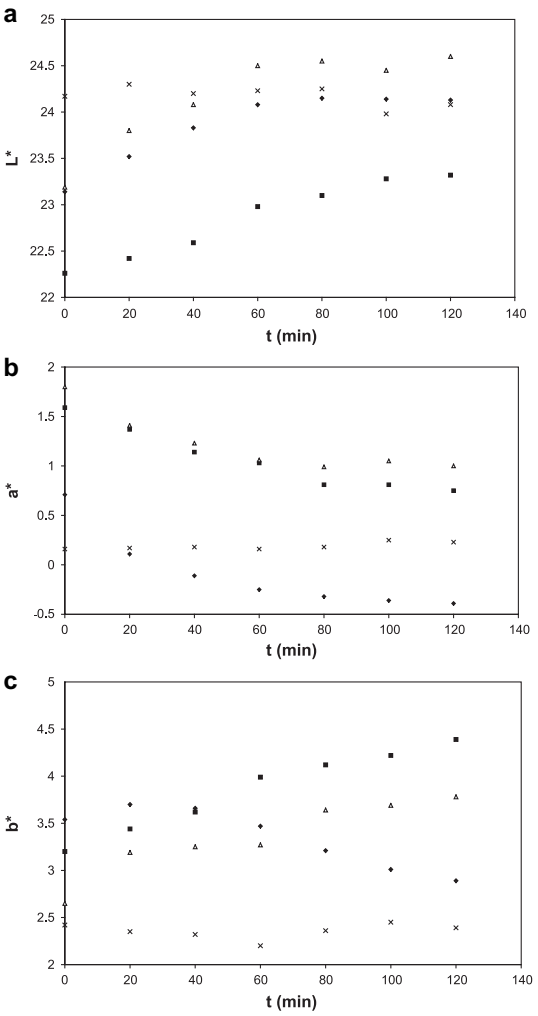

Fig. 2. a). Evolution of the CIELab parameter L* during the UV irradiation. b) Evolution of the CIELab parameter a* during the UV irradiation. c) Evolution of the CIELab parameter b* during the UV irradiation. ◆Golden, ■Starking, △Fuji, ×King David.

Table 1
Physical and chemical properties of the juices from the four different apple varieties.

Sample	Golden	Starking	Fuji	King David
pH(−)	3.89 ± 0.03	4.35 ± 0.03	4.29 ± 0.02	3.48 ± 0.03
Soluble solids (°Brix)	10.7 ± 0.1	14.4 ± 0.2	14.7 ± 0.2	11.1 ± 0.1
Formol index (mL NaOH/100 mL)	5.4 ± 0.4	6.4 ± 0.4	3.6 ± 0.3	9.4 ± 0.5
Total phenolics (pyrogallol eq., mg/L)	7.84 ± 0.10	2.55 ± 0.06	4.55 ± 0.07	10.57 ± 0.09
Reducing sugars (glucose eq., g/L)	68.6 ± 0.5	47.8 ± 0.5	53.8 ± 0.3	73.8 ± 0.7
Total sugars (glucose eq., g/L)	245.7 ± 0.9	182.2 ± 0.7	201.2 ± 0.6	252.2 ± 0.9

(Shown results are the average of 12 determinations.).

Table 2
Absorbance at different wavelengths in the visible region of the juices from the four different apple varieties, at zero time and after 120 min of irradiation.

Juice	t (min)	Wavelength (nm)			
		400	500	600	700
Golden	0	1.322	0.586	0.284	0.183
	120	0.964	0.423	0.247	0.178
Starking	0	1.587	1.751	0.891	0.610
	120	0.851	1.674	0.678	0.390
Fuji	0	1.702	0.615	0.202	0.105
	120	1.085	0.315	0.121	0.079
King David	0	0.358	0.118	0.051	0.033
	120	0.323	0.115	0.044	0.024

This observation is consistent with the findings of other authors such as Ibarz et al. (2005) in their UV irradiation of clarified apple, peach and lemon juices. The juice from King David, which had the greatest initial brightness, kept its value almost unvariable.

The parameter a* decreased during irradiation in the samples from Golden, Starking and Fuji, which means that the juices became less red. Again, in the sample from King David a*, which had the lowest initial value, slightly changed during the processing time. Regarding the parameter b*, its value decreased in the samples from Golden (which had the highest initial one), slightly increased in the samples from Starking and Fuji and remained almost constant in the juice from King David, which had the lowest initial value. Pesek and Warthesen (1987) working with tomato and carrot juices, and Guerrero-Beltrán and Barbosa-Cánovas (2005) in the UV irradiation of apple juice, observed a decrease in both a* and b*, reporting the photodestruction of the pigments of the juice. The samples made from King David apples had the lightest colour (as it has been observed with the three CIELab parameters), in part due to its low pH value that entails a slower action of polyphenol oxidase in the fresh-prepared juice.

3.1.3. Absorption spectrum

The variation in the juice colour that has been described in the previous section and the influence of UV radiation on coloured compounds can also be observed with the evolution of the absorption spectrum. Each sample has a characteristic curve of its own depending on its pigmentation. For example, in King David juice a very low absorbance is observed in the visible range, reflecting this lack of pigments because of the sharp decrease of PPO activity. Then, the variations observed during the experiment were very small.

Regarding the other juices, the absorbance decreased when the irradiation time of the treatment increased (Fig. 3) because some pigments of the juice were degraded during the process. The decrease was specially significant between 400 and 450 nm, which corresponds to colours purple and blue and to the largest peak of the emission spectrum of the lamp (shown in Fig. 1). Thus, the molecules that absorb radiation of these wavelengths are the most affected by the irradiation. This fact can explain why the parameter b* increased in some of the juices.

3.1.4. Vitamin C

The content in vitamin C of the juices had very different behaviors depending on the sample. Fig. 4 shows the evolution of vitamin C in apple juices made from the four different varieties. In the fresh-made juices, the one from Golden had an initial content of 826.6 mg/L of ascorbic acid, the one from Starking 788.2 mg/L, the one from Fuji 588.6 mg/L and the one from King David 277.4 mg/L. The content in juices from Golden, Starking and Fuji slightly decreased during the experiment. The loss in Golden juice after 120 min of UV irradiation was 5.7%, while in Starking one was 5.6%, and in Fuji one 4.0%. However, in the juice from King David, which had the lowest initial content, the loss was 70.0%. This significant difference can be attributed to the lack of pigmentation of this juice that has been explained in the previous sections. These pigments absorb the energy of the radiation and have a protective effect on the other molecules of the system. Seiji and Iwashita (1965) also proved this protective effect of melanins on the inactivation of some enzymes by UV irradiation. Moreover, in Golden, Starking and Fuji juices the losses after the first hour of treatment were 2.4%, 1.7% and 1.9% of the initial content. Then, in these three cases in the second hour more vitamin C was damaged than in the first 60 min, meaning that as pigments were degraded (and the juice colour was lighter) its protective effect was less. In the King David juice, where there are a very fewer concentration of coloured compounds, the loss after 20 min was 62.4% of the initial content, and after 60 min it was 69.8%. Ibarz and Pérez-Teijón (1990), in their work with clarified apple juice, found a decrease of 43% of the vitamin C initial content in the first 30 min, remaining this value unvariable until the end of the experiment (120 min). Tran and Farid (2004) found that the loss of vitamin C content of an UV irradiated orange juice was similar to the loss caused by a thermal treatment.

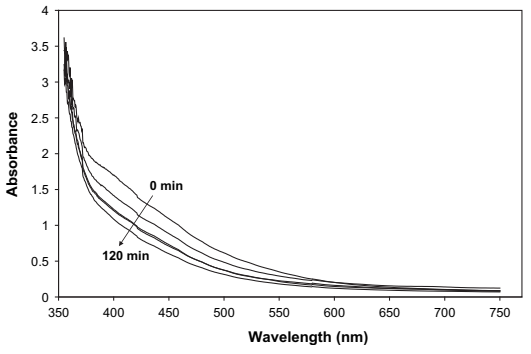

Fig. 3. Evolution of absorbance spectrum of the juice made from Fuji apples during the UV irradiation.

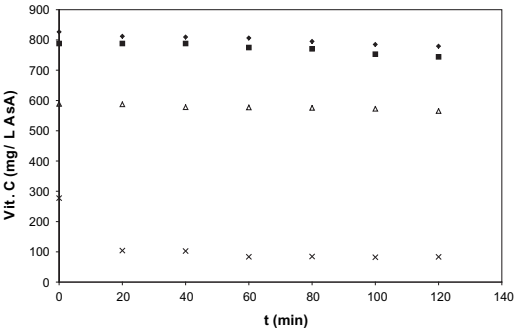

Fig. 4. Evolution of vitamin C in the apple juices with the time of UV irradiation (in mg L^{-1} of ascorbic acid (AsA)). ◆Golden, ■Starking, △Fuji, ×King David.

3.2. Enzymatic activities

3.2.1. Polyphenol oxidase (PPO)

The four juices had an initial PPO activity of 0.18 U/mL (Golden), 0.45 U/mL (Starking), 0.14 U/mL (Fuji) and 0.06 U/mL (King David). As it has been explained, King David had the lowest PPO activity because of its low pH, although it had the highest total phenolics content. Fig. 5 shows the evolution of the PPO activity of the four juices with the irradiation time. Regardless its initial value, the PPO activity was completely inactivated in all samples after 100 min of treatment. Seiji and Iwashita (1965) achieved an inactivation of 74.6% of the PPO activity with a dose of $11.7 \cdot 10^6$ erg cm^{-2} in the UV irradiation of smooth surfaced membranes containing the enzyme.

3.2.2. Peroxidase (POD)

Initial activities of peroxidase in fresh-made juices were very small. The assays showed an activity of 0.05 U/mL in Golden, 0.09 U/mL in Starking, 0.1 U/mL in Fuji and 0.03 U/mL in King David. POD

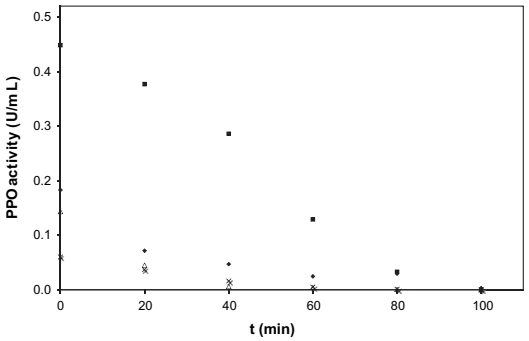

Fig. 5. Inactivation of PPO activity in the apple juices with the time of UV irradiation. ◆Golden, ■Starking, △Fuji, ×King David.

was the most sensitive enzyme to UV irradiation, since the null activity was reached after only 15 min of treatment in all samples.

3.2.3. Pectinmethylesterase (PME)

No PME activity was revealed from the assays in the juices made from Golden, Fuji and King David. The juice from Starking showed a very small activity of $4.7 \cdot 10^{-5}$ U/mL, which was completely inactivated after 40 min of irradiation. The fact that no activity was found in three of the four samples and in the other one was very small can be related with the juice preparation procedure, since a centrifugation was carried out after the squeezing in order to separate the pulp. Once the apple structures are broken, all the enzymes are released and come into contact with its substrate, starting to act quickly. In this way, pectinolytic enzymes will start to act over pulp. Thus, during centrifugation, all those molecules that are forming the enzyme–substrate complex will be separated, remaining in the pellet.

3.2.4. Exo-polygalacturonase and endo-polygalacturonase (exo-PG and endo-PG)

In a similar way that happened with PME, no activity was found for polygalacturonases in any sample. Since these are pectinolytic enzymes, they also must have remained in the pellet after centrifugation. The variations observed in these two kinds of analysis between the samples and the blanks are very small and can be attributed to experimental error.

4. Conclusions

The application of UV irradiation to freshly squeezed apple juice was effective in the inactivation of the assayed enzymes. Regardless its initial activities, polyphenol oxidase was completely inactivated after 100 min of treatment, and peroxidase after only 15 min. No activity of pectinolytic enzymes was found in these apple juices due to the preparation procedure, except a small activity for pectinmethylesterase in the one made from Starking. This activity was inactivated after 40 min of irradiation.

No variations were observed in pH, soluble solids content, formol index, total phenolics and sugars. The colour of the juice made from King David was unvariable during all the experiment. On the contrary, in the ones made from Golden, Starking and Fuji, L* brightness increased with the time of treatment, meaning that UV radiation impairs some of the pigments present in the juice, either initially present or the ones formed later. The parameter a* decreased during irradiation, which means that the juices became less red. b* decreased in the samples from Golden and slightly increased in the samples from Starking and Fuji. These changes in colour could also be observed in the absorption spectrum between 350 and 750 nm, in which the major decrease of absorbance matched with the largest peak of the emission spectrum of the lamp.

The content of vitamin C in juices from Golden, Starking and Fuji slightly decreased during the experiment. The loss in Golden juice after 120 min of UV irradiation was 5.7%, while in Starking one was 5.6%, and in Fuji one 4.0%. In the juice from King David the loss was 70.0%. This significant difference can be attributed to the lack of pigmentation of this juice. In the three first cases, more vitamin C was damaged in the first 60 min than in the second hour, meaning that as pigments were degraded (and the juice colour was lighter) its protective effect was less. In the King David juice the loss after 20 min was 62.4% of the initial content, and after 60 min it was 69.8%.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.15.

Title: Effect of UV-vis photochemical processing on pear juices from six different varieties.

Authors: Víctor Falguera, Alfonso Garvín, Salvador Garza, Jordi Pagán & Albert Ibarz.

Journal: Under third review in *Food and Bioprocess Technology*.

DOI: -

Falguera V, Garvín A, Garza S, Pagán J, Ibarz A. Effect of UV-vis photochemical processing on pear juices from six different varieties. Food Bioprocess Technol. 2013 Feb 23 [Epub ahead of print]. DOI 10.1007/s11947-013-1069-9

Title: *Effect of UV-vis photochemical processing on pear juices from six different varieties.*

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Abstract

The effect of ultraviolet irradiation on the activity of polyphenol oxidase (PPO) and peroxidase (POD) in pear juices from six different varieties (Abate Fétel, Passa Crassana, Ercolini, Flor de Invierno, Blanquilla and Conference) was investigated. The samples were irradiated for 120 minutes with a 400W nominal power medium-pressure mercury lamp, emitting in a range between 250 and 740 nm, with a resulting incident energy of $3{,}88 \cdot 10^{-7}$ $E \cdot min^{-1}$. The temperature was kept at 25 ± 1 ºC with help of a refrigeration system consisting on a metallic coil fed with cold water. Polyphenol oxidase was inactivated in different rates, depending on each variety's properties. Besides, as irradiation time increased, there was a general decrease in the absorption spectrum, which means that some of the juice's pigments could have been damaged. This decrease was especially significant between 400 and 450 nm. No variations in the pH nor the juices soluble solids content were observed.

Keywords: pear juice; ultraviolet irradiation; polyphenol oxidase; peroxidase.

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Introduction

Nowadays, consumers have increased their demand for minimally processed food with similar characteristics to fresh products. With the aim of increasing the lifespan of food, thermal treatments are nowadays the most commonly employed in the inactivation of microorganisms and enzymes. However, heat processing can produce adverse effects in food's sensory and nutritive features (Braddock, 1999). With the aim of avoiding these effects, new treatment technologies allowing the inactivation of pathogen microorganisms and the deterioration, but maintaining the nutritive and quality characteristics of the treated food are being investigated (Noci *et al*., 2008).

Ultraviolet-visible (UV-vis) irradiation has been employed since several decades ago as water tertiary treatment, as well as in the decontamination of surfaces and packing materials in the food industry (Falguera *et al*., 2011a). The main limitation in the UV-vis irradiation application is the low penetration and the effect of suspended solids in the treatment of fluid food. Nevertheless, it was recently shown that in liquid food with similar absorbance to clarified fruit juices, radiation can penetrate up to 1cm inside the juice, still keeping 50% of the incident power (Falguera *et al*., 2011b). Despite the limited penetration inside the fluid, working with a stirred reactor causes an optimal use of the incident radiation on the reacting medium.

Most studies about UV treatment of fruit juices were carried out with the aim of reducing the microbial load. On the contrary, there are very few references about the effect of this kind of treatment on the activity of the main enzymes of fruit juices. However, the feasibility of using this technology for juices processing at industrial scale depends on its effectiveness in inactivating the main enzymes, since it is assumed that enzyme inactivation generally requires higher radiation doses than microbial inactivation (Falguera *et al*., 2011a). In addition, the effect of the complex matrix of pear juices requires specific research for each variety, in order to assess the necessary doses to inactivate each enzyme and the potential side effects. From an industrial point of view, implementation of UV-vis irradiation will be subjected to prove its effectiveness on each one of the varieties that each company uses for its final product, which always comes from a very specific variety combination that gives it an organoleptic and nutritional profile. This work studies the consequences of irradiating with a medium-pressure UV-vis lamp the juices of six pear varieties. The evolution of polyphenol oxidase and peroxidase enzymatic activities and other physical and chemical parameters of juices has been investigated.

Material and methods

Juice preparation

Six different varieties of pears were used: Abate Fétel, Passa Crassana, Ercolini, Flor de Invierno, Blanquilla and Conference. All of these pears were cultivated in the region of Lleida (Spain). The fruits were cleaned, peeled and squeezed with a household juicer. The resulting juice was centrifuged using an Avanti J-26XP centrifuge (Beckman Coulter, USA) for 25 minutes at 26,000 g (RCF). This process was carried out at 4 ºC with the aim of removing the suspended particles and keeping the enzymatic activity. With the fraction of clarified juice, 800 mL aliquots were separated and frozen.

UV radiation treatment, sampling and statistical processing

The photochemical treatment with UV-vis radiation is similar to that described by Falguera *et al.* (2011c), which essentially consists of a dark chamber containing the

reaction tank and the UV lamp. An 800 mL aliquot was taken from the freezer and kept at room temperature until it reached the work temperature. Based on the results of preliminary experiments, it was seen that this volume was enough to avoid the effects that a significant reduction of sample height (due to sampling) would have brought. The juice was placed in a parallelepiped methacrylate tank with a base of 22x15 cm, where it reached a height of 2.4 cm. With the aim of controlling the juice temperature, a refrigeration system consisting of a metallic coil fed with cold water was used, which allowed to maintain the sample temperature at 25 ± 1 ºC. A magnetic stirrer was used to ensure that the tank was perfectly agitated. The irradiation source was a Philips HPM-12 400 W medium-pressure mercury lamp (Philips, Eindhoven, The Netherlands), emitting in a range between 250 and 740 nm (Figure 1). The real emission power of the lamp that reaches the reactor surface, assessed by means of an actinometric reaction consisting of the degradation of oxalic acid in the presence of uranyl nitrate, was $3.88 \cdot 10^{-7}$ E·min^{-1} (Falguera *et al.*, 2011c). Therefore, UV-vis radiation flux was $1{,}18 \cdot 10^{-9}$ E·min^{-1}·cm^{-2}, and the total received radiation during the 120 minutes of treatment was $1{,}41 \cdot 10^{-7}$ E·cm^{-2}. The distance between the lamp and the juice surface was 22.5 cm. The lamp was switched on 10 minutes before the juice was placed inside the camera. The juices were irradiated for 120 minutes, taking samples every 20 minutes (to analyze all the parameters except peroxidase), which were kept under refrigeration until their analysis. For peroxidase analysis, additional samples were taken every 5 minutes. Two series of each juice were irradiated, and all determinations were made in duplicate. Results are expressed as mean value ± standard deviation in all cases.

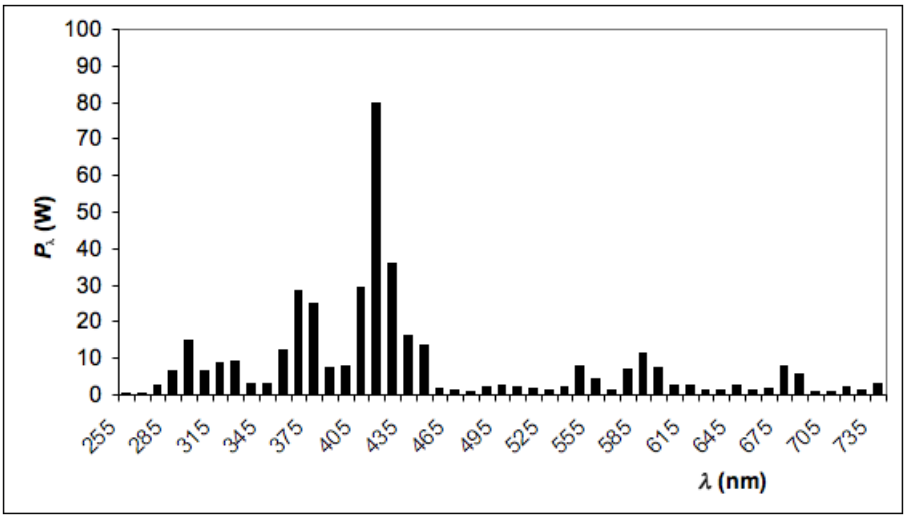

Figure 1. *Emission spectrum of the lamp. P_λ is the power of emission at each (λ) wavelength.*

Enzymatic activities

Polyphenol oxidase (PPO) activity in the juice was assessed measuring the increase in absorbance at 420 nm using 4-methylcatecol as substrate, prepared in a buffer solution with a pH of 3.5. The reaction was carried out in a 1-cm-path quartz cell. One unit (U) of PPO is defined as the quantity of enzyme that causes the increase of one absorbance unit (AU) at 420 nm in 1 minute (Falguera *et al.*, 2011c).

Peroxidase (POD) activity was determined with the method described by Kwak *et al.* (1995), using pyrogallol as substrate. The reaction starts with the addition of H_2O_2, measuring the increase in absorbance at 420 nm for 3 minutes. One unit of POD activity is defined as the enzyme quantity that forms 1 mg of purpurogallin from pyrogallol in 3 minutes at pH 6.

Physical and chemical analyses

The pH of the juices before and after the photochemical treatment was measured with a Crison micropH pH-meter (Crison Instruments, S.A., Alella, Spain). The content in soluble solids of the juices was evaluated using an Atago RX-1000 digital refractometer (Atago Co. Ltd., Japan).

Absorption spectra of the pear juice samples between 350 and 750 nm were obtained with a Helios Gamma spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA) using a 1-cm-path quartz cell. The colour of the juices was determined using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing Inc., Japan) in the CIELab space. The values of brightness (L^*), a^* (red-green) and b^* (yellow-blue) were obtained.

Total phenolic content was determined according to the spectrophotometric method described in the work of Shaghaghi *et al.* (2008), using the Folin-Ciocalteau reagent. Formol index was assessed according to the official method of the International Federation of Fruit Juice Producers (IFFJP, 1984), by means of a potentiometric titration of the acidity of the compounds formed by the reaction of formaldehyde and amino acids in the juice, up to a pH of 8.1. Total and reducing sugars were determined according to the IFFJP method (1984) with the reduction of the Fehling reagent. The content in Vitamin C was determined by iodometric titration, as described by Suntornsuk *et al.* (2002).

Statistical analysis

Data means and standard deviations were calculated from experimental data by means of StatGraphics Plus v.5.1 software (STSC Inc., Rockville, MD, USA). The same software was used to carry out multiple range tests in order to find out significant differences between the means at a 95% confidence level.

Results and discussion

Physicochemical properties

Table 1 shows the values for pH, acidity, soluble solids, CIELab colorimetric parameters, phenolic content, formol index, total and reducing sugars, vitamin C and PPO and POD enzymatic activities of the pear juice samples from the six investigated varieties, before and after 120 minutes of photochemical treatment. Depending on the pear variety, the juices show different values for each of the analyzed parameters. This table shows that the 120-minute photochemical treatment of the pear juices barely has an effect in pH, acidity, formol index and sugars. The small differences can be attributed to experimental error and were not found to be significant. However, a slight increase was observed in soluble solids, which was only significant in Blanquilla and Conference (the two varieties with the highest soluble solids content). This slight increase could be attributed to the fact that, in the long period of irradiation, a slight evaporation of water due to diffusion phenomena from the juice to the surrounding air may occur, which is enhanced by the continuous agitation. In addition, a slight increase

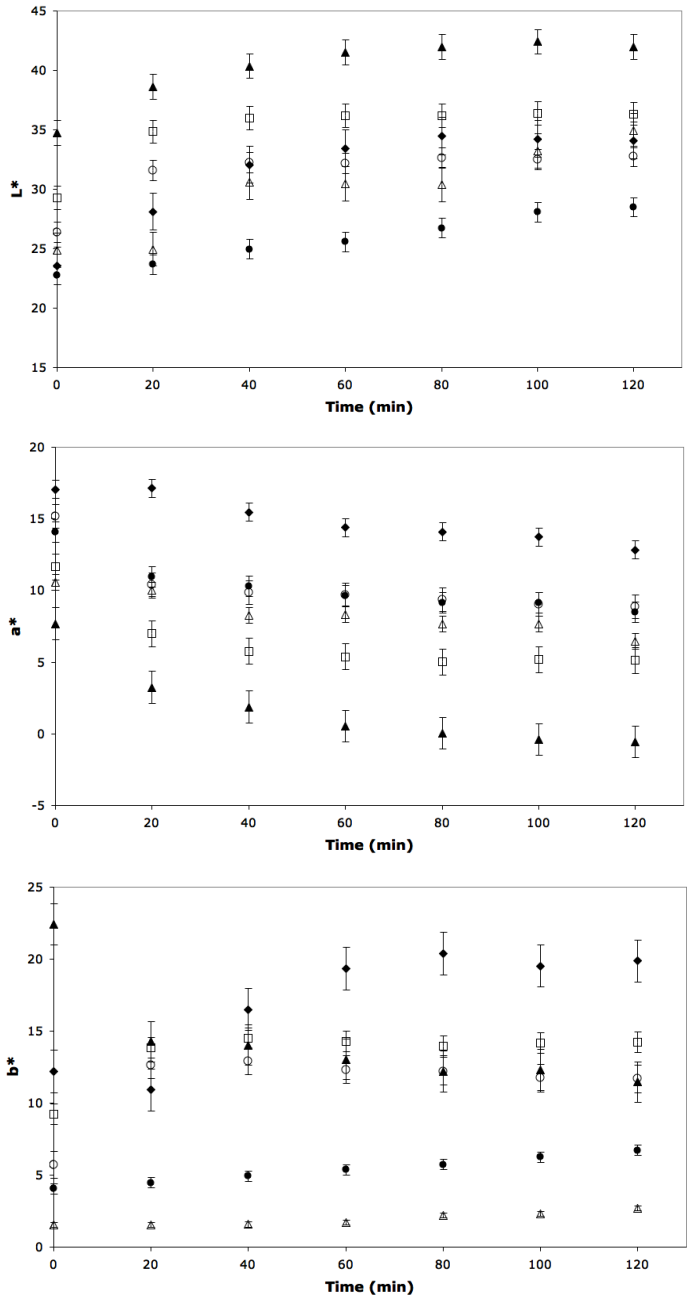

Figure 2. *Change of CIELab parameters with UV-vis irradiation time (received energy $3.88 \cdot 10^{-7}$ E·min^{-1}). a) parameter L*. b) parameter a*. c) parameter b*. ◆ Abate Fétel. ▲ Ercolini. ● Blanquilla. □ Passa Crassana. ○ Flor de Invierno. △ Conference.*

Table 1. *Change of physicochemical parameters and enzymatic activities of pear juices with irradiation.*

	Abate Fétel		**Passa Crassana**	
Parameter	Initial	Final	Initial	Final
pH	^{g}5.08 ± 0.01	^{fg}5.02 ± 0.01	^{a}3.90 ± 0.1	^{a}3.87 ± 0.01
Acidity [x]	^{c}0.26 ± 0.01	^{c}0.26 ± 0.01	^{e}1.54 ± 0.08	^{e}1.55 ± 0.05
Sol. solids (ºBrix)	^{b}11.0 ± 0.1	^{b}11.1 ± 0.1	^{c}11.7 ± 0.1	^{c}11.9 ± 0.1
*L**	^{a}23.61 ± 0.90	^{f}34.00 ± 1.41	^{d}29.31 ± 0.10	^{g}36.35 ± 0.02
*a**	^{j}17.11 ± 2.52	^{gh}12.81 ± 0.12	^{fg}11.70 ± 0.21	^{b}5.14 ± 0.02
*b**	^{e}12.29 ± 0.61	^{g}20.01 ± 1.61	^{d}9.23 ± 0.23	^{f}14.25 ± 0.01
Phenolics [y]	^{d}300 ± 19	^{d}307 ± 24	^{fg}405 ± 3	^{g}418 ± 6
Formol Index [z]	^{bc}8.5 ± 0.7	^{bc}9.0 ± 0.2	^{a}4.0 ± 0.1	^{a}4.1 ± 0.1
Total sugars (g/L)	^{b}75.2 ± 1.6	^{b}76.3 ± 0.8	^{cd}88.0 ± 3.7	^{d}90.0 ± 3.9
Red. sugars (g/L)	^{b}73.6 ± 0.7	^{b}74.1 ± 2.2	^{b}73.0 ± 1.4	^{b}73.6 ± 0.7
Ascorbic ac. (mg/L)	^{f}357 ± 22	^{c}169 ± 22	^{e}286 ± 10	^{d}236 ± 22
PPO (U/mL)	^{b}0.246 ± 0.004	^{a}0.0044 ± 0.0003	^{b}0.097 ± 0.008	^{a}0.0029 ± 0.0002
POD (U/mL)	^{b}0.09 ± 0.05	^{a}0 ± 0	^{b}0.114 ± 0.003	^{a}0 ± 0
	Ercolini		**Flor de invierno**	
Parameter	Initial	Final	Initial	Final
pH	^{de}4.84 ± 0.01	^{d}4.80 ± 0.01	^{e}4.89 ± 0.02	^{f}4.97 ± 0.01
Acidity [x]	^{ab}0.16 ± 0.02	^{ab}0.18 ± 0.01	^{a}0.13 ± 0.01	^{ab}0.14 ± 0.01
Sol. solids (ºBrix)	^{a}8.3 ± 0.1	^{a}8.5 ± 0.1	^{c}11.7 ± 0.1	^{c}11.8 ± 0.1
*L**	^{f}34.69 ± 0.19	^{h}42.01 ± 0.41	^{c}26.40 ± 0.10	^{e}32.74 ± 0.06
*a**	^{cd}7.70 ± 1.51	^{a}-0.50 ± 0.18	^{i}15.20 ± 0.20	^{de}8.86 ± 0.06
*b**	^{h}22.41 ± 1.29	^{e}11.51 ± 0.39	^{c}5.71 ± 0.41	^{e}11.68 ± 0.03
Phenolics [y]	^{c}200 ± 5	^{c}218 ± 1	^{e}350.4 ± 20.1	^{f}382.1 ± 30.4
Formol Index [z]	^{cd}10.5 ± 0.7	^{de}12.5 ± 3.5	^{b}7.5 ± 0.7	^{b}7.5 ± 0.7
Total sugars (g/L)	^{a}46.3 ± 0.6	^{a}48.0 ± 1.1	^{c}85.4 ± 1.4	^{cd}88.7 ± 1.1
Red. sugars (g/L)	^{a}46.3 ± 0.6	^{a}47.8 ± 0.3	^{c}85.1 ± 2.0	^{c}85.8 ± 1.0
Ascorbic ac. (mg/L)	^{d}229 ± 17	^{ab}106 ± 17	^{g}467 ± 12	^{f}371 ± 12
PPO (U/mL)	^{b}0.0591 ± 0.0005	^{a}0.0102 ± 0.0002	^{b}0.038 ± 0.002	^{a}0 ± 0
POD (U/mL)	^{b}0.20 ± 0.08	^{a}0 ± 0	^{b}0.034 ± 0.001	^{a}0 ± 0
	Blanquilla		**Conference**	
Parameter	Initial	Final	Initial	Final
pH	^{b}4.35 ± 0.01	^{b}4.39 ± 0.03	^{c}4.66 ± 0.01	^{c}4.64 ± 0.01
Acidity [x]	^{d}0.43 ± 0.01	^{d}0.44 ± 0.01	^{bc}0.20 ± 0.01	^{bc}0.20 ± 0.01
Sol. solids (ºBrix)	^{e}13.1 ± 0.1	^{f}14.0 ± 0.1	^{d}12.6 ± 0.1	^{e}13.0 ± 0.1
*L**	^{a}22.79 ± 0.01	^{d}28.50 ± 0.02	^{b}24.89 ± 0.09	^{f}34.96 ± 0.05
*a**	^{hi}14.10 ± 0.19	^{d}8.49 ± 0.07	^{ef}10.57 ± 0.05	^{bc}6.46 ± 0.07
*b**	^{b}4.09 ± 0.10	^{c}6.73 ± 0.07	^{a}1.55 ± 0.05	^{ab}2.68 ± 0.04
Phenolics [y]	^{a}154.1 ± 0.6	^{bc}189.0 ± 0.9	^{ab}169.0 ± 1.1	^{bc}194.1 ± 1.3
Formol Index [z]	^{e}13.5 ± 0.7	^{e}13.0 ± 0.1	^{e}13.5 ± 0.7	^{e}13.0 ± 0.2
Total sugars (g/L)	^{e}118.6 ± 1.0	^{e}118.0 ± 1.9	^{b}73.4 ± 0.4	^{b}73.6 ± 0.2
Red. sugars (g/L)	^{d}101.7 ± 1.0	^{d}102.8 ± 1.4	^{b}73.1 ± 0.1	^{b}72.1 ± 0.2
Ascorbic ac. (mg/L)	^{ab}128 ± 10	^{a}93 ± 12	^{bc}141 ± 25	^{a}99 ± 10
PPO (U/mL)	^{b}0.041 ± 0.004	^{a}0.005 ± 0.001	^{b}0.6 ± 0.15	^{a}0.247 ± 0.003
POD (U/mL)	^{b}0.032 ± 0.008	^{a}0 ± 0	^{b}0.032 ± 0.001	^{a}0 ± 0

Mean value ± Standard deviation. Different superscripts (for each parameter) indicate significant differences according to multiple range tests. For enzymes (PPO and POD), different superscripts indicate significant differences between initial and final values within each variety.
x: expressed as malic acid eq., g/100 mL
y: expressed as pyrogallol eq., g/L
z: expressed as mL NaOH 0.1M/100 mL

was detected in phenolics content (which was found to be significant in Flor de Invierno and Blanquilla), probably due to the breakdown of complex phenolic polymers that leads to the release of simpler phenolic compounds. In the analysis of total phenolic content, each one of these simpler molecules reacts with the Folin-Ciocalteau reagent, giving a higher result. In a previous piece of work (Falguera *et al*., 2011c), this behaviour was not observed in UV-vis-irradiated apple juices. However, similar tests performed on red grape musts (Falguera *et al*., 2012) showed that anthocyanins are highly degraded by UV-vis irradiation, and it is accepted that anthocyanin breakdown results in the formation of different phenolic compounds (Adams, 1973; Sadilova *et al*., 2007) that can react with the Folin-Ciocalteau reagent.

Figure 2a shows the brightness (L^*) evolution with the irradiation time for the six pear juice varieties, observing that in all of them this parameter's values have a significant increasing trend, according to the statistical analysis (Table 1). Figures 2b and 2c show the evolution of a^* and b^*, observing that the former tends to decrease, reducing the red tonalities, while b^* parameter tends to more yellow tonalities (except in the Ercolini variety, where the juice behaves in the opposite way). Moreover, in the case of Ercolini, L^* and a^* values show a globally much less coloured juice. Generally speaking, these analyses confirm that irradiated pear juice samples become lighter. These results are consistent with those obtained by Ibarz *et al.* (2005), where the apple, peach and lemon juices irradiated with a lamp emitting in the UV-visible spectrum suffer an appreciable discoloration. Likewise, these CIELab parameters show a variation that is analogous to that of different variety apple juices (Guerrero-Beltrán & Barbosa-Cánovas, 2005; Falguera *et al*., 2011c), tomato and carrot juices (Pesek & Warthesen, 1987) and grape musts (Falguera *et al*., 2012).

Absorbance spectrum

The absorbance spectrum is another measure of the juice coloration, which depends on its pigment content. Figure 3 shows the absorption spectrum of the Passa Crassana variety pear juice in the interval between 350 nm and 750 nm. Absorption spectra for the rest of varieties are similar, except for the Ercolini variety, the absorption spectrum of which is remarkably lower (not shown). For the rest of varieties, it was observed that in the first twenty minutes of irradiation there was a very important decrease of absorbance values, being more remarkable for wavelengths between 375 nm and 450 nm. This interval corresponds to purple colorations and to two important peaks of the lamp's emission spectrum (Figure 1). Thus, there was a degradation of the juices' own pigments in this interval, besides the reddish-brown polymers formed in the enzymatic browning reactions that may have occurred before juice processing. Likewise, these results are consistent with the variation of the CIELab colorimetric parameters shown before and with apple and grape juices behaviour observed in previous pieces of work (Falguera *et al*., 2011c & 2012).

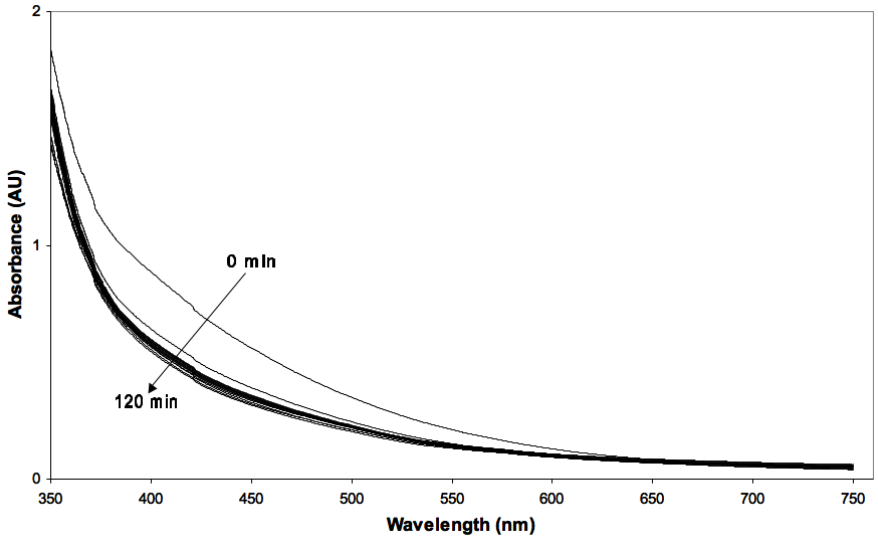

Figure 3. *Evolution of the absorbance spectrum for Passa Crassana pear juice during UV-vis irradiation (received energy* $3.88 \cdot 10^{-7}$ $E \cdot min^{-1}$*).*

Vitamin C

The photochemical treatment of pear juices produces a decrease of vitamin C content. Figure 4 shows the evolution of this vitamin content with the irradiation time, being observed that there is a continuous decrease. In the case of Abate Fétel and Ercolini juices, the vitamin C content at the end of the treatment has a 60,5 % and 50 % decrease, respectively, in relation to the initial content. In the other varieties this decrease is found in the 20 % to 30 % interval. The great decrease observed for the Ercolini variety juices could be explained by the fact that, as stated before, the juice presented clearer tonalities, with more luminosity and less coloration and absorbance, which indicate a lower presence of pigments in the juice. The pigments can absorb the incident radiation, acting as photoprotectors, avoiding the degradation of other compounds. Because of this, the protector effect in the juice from Ercolini is not as high as in other varieties, and this variety will suffer a more intense degradation of the vitamin C by the radiation. The results obtained in the present work show a similar behaviour to irradiated apple (Falguera *et al*., 2011c) and orange juice (Tran & Farid, 2004). As far as the effect of vitamin C degradation on absorbance properties is concerned, Bradshaw *et al*. (2001) proved that ascorbic acid has its maximum absorbance at wavelengths near 245 nm, and the products of its degradation cause an increase in absorbance at 300 nm. Consequently, as vitamin C is degraded the juice may absorb higher radiation doses at this wavelength, but this phenomenon does not cause any increase in the visible region, as it can be observed in Figure 3.

Enzymatic activities

Table 1 shows the initial PPO activities. Conference was the variety in which the highest activity was found, whereas in Flor de Invierno, Blanquilla and Ercolini it was significantly lower. Figure 5 shows the evolution of the activity related to the initial PPO with the irradiation time for the juices of the six varieties investigated. In most varieties, after 120 minutes the residual activity percentage is almost null, but in the

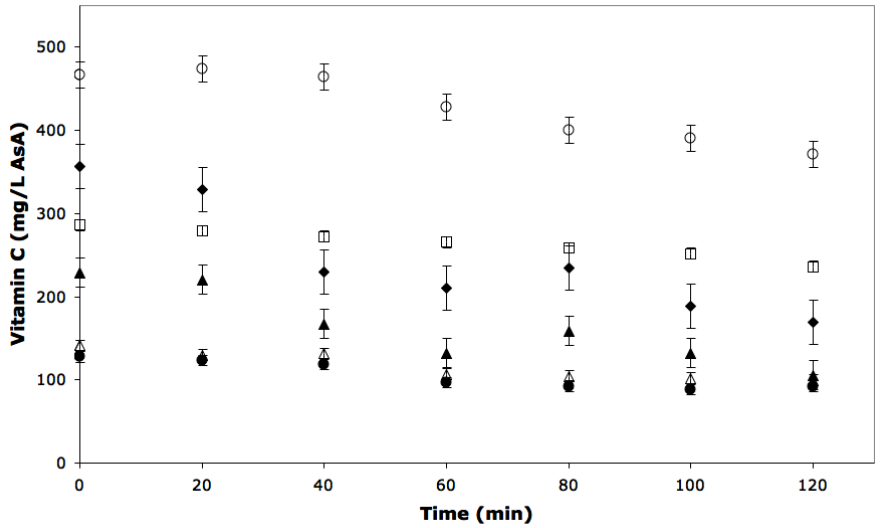

***Figure 4**. Evolution of Vitamin C content in pear juices with UV-vis irradiation time (received energy $3.88 \cdot 10^{-7}$ E·min^{-1}). ♦ Abate Fétel. ▲ Ercolini. ● Blanquilla. □ Passa Crassana. ○ Flor de Invierno. △ Conference.*

Ercolini juice it still has 17% of its initial activity. Moreover, it is important to state that after 20 minutes of irradiation it is observed that the PPO activity decreases in more than 50% for the juices of five varieties. The most different behaviour is for Conference variety, reaching after 120 min 43% of the initial enzymatic activity. Nevertheless, as its initial enzymatic activity is the highest one, the absolute variation is also the highest one.

PPO inactivation constitutes the most important difference between the results obtained in this work and those obtained for apple (Falguera *et al*., 2011c) and grape (Falguera *et al*., 2012) juices using the same equipment. In the case of apple juices, in the four studied varieties PPO was completely inactivated after 100 min of treatment. However, those juices had higher a^* and b^* values, and therefore higher concentration of coloured compounds. As far as grape juices are concerned, PPO was not completely inactivated: after 140 minutes of irradiation, the juices from white grape varieties still kept 20% of their initial activity, while in the case of pink grape juices the inactivation was only of 50%. Thus, if individual results are considered, it can be stated that in apple juices PPO inactivation follow a similar pattern regardless the variety, while in pear juices this inactivation largely depends on the variety. Meanwhile, in the case of grape juices, PPO denaturation depends on the kind of grape (white or pink), but inside each kind, the studied varieties behave in the same way.

As far as POD activity is concerned, this enzyme is generally more sensitive to the UV-vis radiation, and shorter treatment times are necessary than those of PPO to reach a decrease in its activity (Falguera *et al*., 2011c). For juices of the Passa Crassana, Conference and Blanquilla varieties, a total inactivation is reached in 30 minutes; meanwhile, for the Abate Fétel and Ercolini varieties, 60 minutes are necessary to reach their inactivation. The Flor de Invierno variety is a special case, which keeps its initial enzymatic activity during the first 15 minutes, slightly decreasing with longer times.

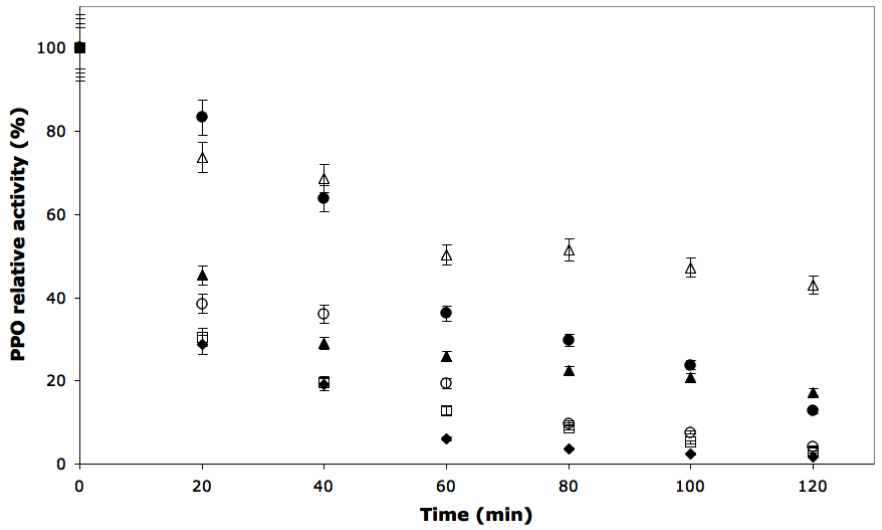

Figure 5. *Variation of the relative PPO activity with the irradiation time (received energy $3.88 \cdot 10^{-7}$ $E \cdot min^{-1}$) for pear juices from the six different varieties. ◆ Abate Fétel. ▲ Ercolini. ● Blanquilla. □ Passa Crassana. ○ Flor de Invierno. △ Conference.*

Considering the obtained results, it can be stated that UV-vis irradiation is a technology that can be useful in inactivating PPO and POD in pear juices. Additionally, it brings a slight discoloration, which may be desirable in the case of the pear varieties that provide dark juices. However, the long treatment times that are needed to achieve the desired effects raises an important handicap for its implementation at industrial scale. Therefore, further optimization of these processes must involve developing new reactor kinds or configurations that make it possible to reduce processing times.

Conclusions

The photochemical treatments using the lamp emitting in the UV-vis spectrum have been proved to be effective for PPO and POD inactivation of pear juices of six different varieties. The residual activity after 120 minutes of treatment is almost null (except Conference PPO, whose initial activity was the highest one). Besides, after 20 minutes, it was possible to reduce the activity in more than 50%. No appreciable variations in the pH, soluble solids content, formol index, total phenolics and sugars values were observed. In all the juices treated, brightness (L^*) increased and a^* colorimetric parameter values (red-green) decreased, meaning that some of the pigments of the juices were destroyed by the radiation. Vitamin C was partially impaired, in a ratio between 20% and 60% depending on the pear variety. These different impairment degrees are mainly related to the pigmentation of the juices: the coloured compounds absorb incident radiation and therefore act as photoprotectors.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.16.

Title: Effect of UV-vis irradiation on enzymatic activities and physicochemical properties of four grape musts from different varieties.

Authors: Víctor Falguera, Salvador Garza, Jordi Pagán, Alfonso Garvín & Albert Ibarz.

Journal: *Food and Bioprocess Technology*, (in press).

DOI: 10.1007/s11947-012-0781-1

Food Bioprocess Technol
DOI 10.1007/s11947-012-0781-1

COMMUNICATION

Effect of UV–Vis Irradiation on Enzymatic Activities and Physicochemical Properties of Four Grape Musts from Different Varieties

Víctor Falguera · Salvador Garza · Jordi Pagán · Alfonso Garvín · Albert Ibarz

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Abstract The influence of ultraviolet–visible irradiation on some quality attributes (pH, soluble solids content, color parameters, absorption spectrum, vitamin C, total acidity, total phenolics, formol index, and total and reducing sugars) and polyphenol oxidase, peroxidase, and pectinmethylesterase activities of fresh grape juices was investigated. Grape juice was made from two varieties of white grapes (Victoria and Dauphine) and two varieties of pink grapes (Emperor and Red Globe). Each juice was irradiated for 140 min with a high-pressure mercury lamp of 400 W that emits in a range between 250 and 740 nm. Polyphenol oxidase was not completely inactivated during irradiation, reducing only 80% of its initial activity in juices from white grapes and only 50% in those from pink grapes. On the contrary, peroxidase was completely inactivated. A slight or nonexistent variation was observed in most of the measured physicochemical parameters, showing high stability during irradiation. Only vitamin C and color had a significant variation, with an average decrease of 30% for vitamin C in all varieties and a more important variation of color parameters in the pink varieties than in the white ones.

Keywords Ultraviolet irradiation · Grape juice · Physicochemical properties · Polyphenol oxidase · Peroxidase

Introduction

Currently, heat treatment process is the most commonly used hurdle for inactivating microorganisms and enzymes, extending products shelf-life. During heat treatment, different percentages of desirable constituents such as nutrients, color, aroma, and texture are destroyed (Alwazeer et al. 2002; Blasco et al. 2004). Due to these unresolved drawbacks of thermal treatments, nonthermal food processing techniques are receiving attention because of their potential for quality and safety improvement of food. Nonthermal processing methods such as high-intensity pulsed electric fields, supercritical carbon dioxide, high hydrostatic pressure, and ultrasound could be applied to reduce the number of pathogenic microorganisms in foods and extend their shelf life. Among them, ultraviolet (UV) processing can be used to pasteurize fresh juices, liquid egg and dairy products (such as milk, cheese milk, and whey–protein concentrates), and sweeteners. With the prevalent negative public reaction to chemical food additives, UV processing could reduce contamination levels because of its broad antimicrobial action, providing effective inactivation of viruses, vegetative bacteria, bacterial spores, yeast, conidia (fungal spores), and parasites (Falguera et al. 2011a). UV light treatment of foods does not use chemicals, generate waste effluents, or produce any byproducts, which makes it ecologically friendly. Moreover, most nutritional components, which are sensitive to heat, are not destroyed by UV light or potentially suffer less destruction compared to heat treatment (Koutchma 2009).

Industrial applications of UV technology, however, are still limited because UV light transmittance through fluid foods, and beverages is low, producing a low penetration and the effect reduction in the presence of suspended solids (Guerrero-Beltrán and Barbosa-Cánovas 2004; Snowball and Hornsey 1988). However, there are engineering alternatives to ensure that all the fluid receives the appropriate amount of radiation (Falguera et al. 2011a). The FDA approved ultraviolet irradiation as a suitable method for the preservation of fruit juices, since this method is capable of

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reducing pathogens at least five logarithmic cycles (FDA 2000), just if the turbulent flow conditions can be ensured throughout the process. However, this requirement is not necessary when UV radiation is not the only hurdle (Falguera et al. 2011b).

There are not too many published pieces of work in the scientific literature that have studied the effect of UV–visible irradiation on enzymes in fruit juices, especially focusing on the differences among different varieties of the same fruit. It is for this reason that this paper examines its effect on grape juices from four different varieties, measuring the enzymatic activities of polyphenol oxidase (PPO, the main cause of enzymatic browning) peroxidase (POD) and pectinmethylesterase (PME). In addition, the effects on different physicochemical parameters have also been studied.

Materials and Methods

Juice Preparation

Grapes from two white varieties (Dauphine and Victoria) and two pink ones (Red Globe and Emperor) were purchased from a local market. The fruits were washed and squeezed with a household juicer. The resulting juice was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 30 min at 26,000×*g* (RCF). This process was done at 5 °C to make separation of supernatant easier and to reduce enzymatic activities. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. Fractions of 800 mL of juice were stored in the freezer.

UV Processing

UV irradiation was carried out in a dark chamber containing the juice and the lamp. Grape juice was placed in a methacrylate tank of 22×15×10 cm. Eight hundred milliliters of juice was processed, reaching a height of 2.4 cm inside the tank. A refrigeration system consisting of a metallic coil fed with cold water was used to control temperature and avoid juice heating. Juice temperature was maintained at 25±1 °C in all experiments. A magnetic stirrer was used during irradiation to ensure that all the juice was subjected to the same UV dose. UV radiation was produced with a Philips HPM-12 high-pressure mercury lamp of 400 W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands), used in previous pieces of work (Ibarz et al. 2005, 2009; Falguera et al. 2011b). The real incident energy, determined as described by Esplugas and Vicente (1991), was 3.88×10^{-7} E·min^{-1} (Ibarz et al. 2009). The distance between the juice surface and the lamp was 22.5 cm.

Grape juice to be irradiated was removed from the freezer 2 h before irradiation started. The lamp was lit 10 min before putting the juice in the chamber. Irradiation was carried out during 2 h and 20 min. A sample was taken every 20 min and placed in a refrigerator until its analysis. Additional samples were taken at 5, 10, and 15 min to analyze peroxidase activity.

Physical and Chemical Analysis

The pH of the juice before and after treatment was measured with a Crison micropH 2000 pH meter (Crison Instruments, S.A., Alella, Spain). Soluble solids content was assessed using an Atago RX-1000 Digital Refractometer (Atago Co. Ltd., Japan). The juice color was monitored using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Japan) in the CIELab color space. Absorption spectrum between 350 and 750 nm was measured with an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc.,Waltham, USA), using a 1-cm-wide quartz cell. Total phenolic content was determined as described by Shaghaghi et al. (2008), with a spectrophotometric method using the Folin–Ciocalteu reagent. Formol index was measured according to the official method of the International Federation of Fruit Juice Producers (IFFJP 1984), with a potentiometric titration of the acidity of the compounds formed by the reaction of formaldehyde and amino acids in the juice up to a pH of 8.1.

Total and reducing sugars were determined by the reduction of the Fehling reagent, according to the method of the IFFJP (1972). Reducing sugars were assessed by means of a direct titration, while an acidic hydrolysis of nonreducing sugars was carried out before the titration in order to measure total sugars content. Vitamin C was quantified by iodine titration, as described by Suntornsuk et al. (2002). Total acidity was carried out by titration of 10 mL of grape juice with 0.1 N NaOH until the final pH reached 8.1. The result is expressed in concentration of tartaric acid (IFFJP 1972). Maturity index was calculated as the ratio of total soluble solids to titratable acidity (Ladaniya 2008).

Enzymatic Activities

PPO activity in the juice was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol as a substrate, prepared in a buffer solution with a pH of 3.5. The reaction was carried out in a 1-cm light path quartz cell. One unit (U) of PPO was defined as the amount of enzyme that caused the increase of one

absorbance unit at 420 nm in 10 min (Ülker-Yerlitürk et al. 2008).

POD activity was determined by the method described by Kwak et al. (1995) using pyrogallol as substrate. The standard assay reaction mixture contained, in a total volume of 3 mL: juice sample (2.2 mL), 100 mM K–Pi buffer (pH 6, 0.32 mL), 5% pyrogallol (0.32 mL, *w*/*v*), and 0.147 M H_2O_2 (0.16 mL). The reaction was initiated by the addition of H_2O_2, and the increase in A_{420} was recorded in 3 min. One unit of POD activity is defined as that forming 1 mg of purpurogallin from pyrogallol in 3 min at pH 6.

PME activity was measured by determining free carboxyl groups formed as a result of enzyme action on pectin. The reaction mixture was composed of 40 mL of the 1% pectin solution in 0.15 mol L^{-1} NaCl and 10 mL of the juice sample. The pH of this mixture was adjusted to 7.7 using 0.05 mol L^{-1} NaOH. One hundred milliliters of 0.05 mol L^{-1} NaOH was added to the reaction mixture, and the time needed to recovery the pH of 7.7 (50± 2 °C) was measured. One unit of PME was defined as the amount of enzyme that released 1 mmol of carboxyl groups in 1 min (de Assis et al. 2007).

Statistical Analysis

Two samples from the same batch were irradiated for each grape variety. In addition, all the determinations that have been described in the previous subsections were carried out in duplicate. Shown data correspond to mean value and standard deviation of the results obtained from these four analyses. Multiple range tests were performed with StatGraphics Plus v. 5.1 software (STSC Inc., Rockville, MD) in order to find out significant differences between the means at a 95% confidence level.

Results and Discussion

Physical and Chemical Properties

pH, Degrees Brix, Titratable Acidity, Formol Index, Total Phenolics, and Sugars

The properties of grape juices used for UV irradiation are listed in Table 1. During UV irradiation no significant changes in pH, degrees Brix, and titratable acidity were observed. These results are consistent with those of Falguera et al. (2011b) for UV-irradiated juices from different apple varieties. Similar results were also observed for sonicated red grape juice (Tiwari et al. 2010) and for ozonated grape juice (Tiwari et al. 2009). Noci et al. (2008) obtained no significant differences in pH and degrees Brix when processing fresh apple juice by UV irradiation and pulsed electric fields.

The white varieties had higher formol index values than the pink ones. Thus, formol index was 15.75 mL NaOH/100 mL in Dauphine and 11.25 mL NaOH/100 mL in Emperor. The content of reducing and total sugars also depended on the variety of grape. These parameters were not affected by UV irradiation. Total phenolics content was influenced, somehow, by the UV irradiation treatment. Whereas in Emperor and Dauphine the content of total phenolics increased during irradiation (from 264 to 303 mg L^{-1} and from 316 to 372 mg L^{-1}, respectively), in Red Globe and Victoria, it slightly decreased (from 350 to 340 mg L^{-1} and from 310 to 305 mg L^{-1}, respectively). This fact would mean that there are different processes during UV irradiation that affect phenolics content, some of which may enhance their extraction, and other ones may result in an impairment of some phenolic molecules.

Table 1 Physical and chemical properties of the musts from the four different grape varieties

Sample	Red Globe	Emperor	Victoria	Dauphine
pH	3.30±0.06a	3.78±0.02c	3.67±0.03b	3.22±0.12a
Titratable acidity (g tartaric acid eq./100 mL)	0.81±0.01c	0.60±0.02a	0.73±0.01b	1.59±0.01d
Soluble solids (°Bx)	14.41±0.30b	17.46±0.10d	12.46±0.04a	17.10±0.19c
Formol index (mL NaOH/100 mL)	13.00±0.71b	11.25±0.35a	14.50±0.01c	15.75±0.35d
Total phenolics (pyrogallol eq., mg/L)	350±21c	263±4a	310±7b	317±21b
Reducing sugars (glucose eq., g/L)	114.04±0.01d	87.95±1.05b	104.36±3.71c	63.86±0.28a
Total sugars (glucose eq., g/L)	123.56±2.08d	89.07±0.01b	108.11±1.59c	66.73±0.30a
CIELab *L**	31.85±0.14b	29.51±0.49a	40.02±0.32c	41.85±0.02d
CIELab *a**	30.69±0.04d	18.86±0.12c	3.80±0.01a	4.72±0.06b
CIELab *b**	14.18±0.20c	1.97±0.01a	13.93±0.50c	11.42±0.08b
Maturity index (°Bx·L·g^{-1})	18±1b	29±1c	17±1b	11±1a

Mean value ± standard deviation. Different lowercase letters in a row indicate significant differences

Juice Color

To determine the effect of the applied UV treatment on the color of grape juices, CIELab parameters (L^*, lightness; a^*, redness-greenness; and b^*, yellowness-blueness) were measured. Figure 1 shows the evolution of these parameters in the 140 min of UV processing. The final values for the three parameters were found to be significantly different at a 95% confidence level for the four varieties.

In this way, significant changes in the color of grape juice were observed during UV irradiation treatment. In general, pink grapes (Red Globe and Emperor) experimented a greater variation of the studied parameters than the white ones (Victoria and Dauphine). This behavior can be due to the fact that pink grapes contain a higher concentration of pigments than the white ones, some of which were impaired. In pink grape juices, L^* values varied from 31.85 in untreated samples to 39.98 after 140 min of UV irradiation in Red Globe, and from 29.51 to 37.63 in Emperor. These increasing values indicate that grape juices from pink varieties became brighter. Similar increase in L^* was reported in sonicated red grape juice (Tiwari et al. 2010) and ozonated grape juice (Tiwari et al. 2009). Parameters a^* and b^* decreased during irradiation in samples from Red Globe, which means that the juice became less red and less yellow, respectively. This indicates a destruction of pigments in the juice of Red Globe. However, in the Emperor variety, parameter a^* decreased with the irradiation time, whereas b^* increased, which indicates that there is a destruction of initial pigments and a formation of new ones during irradiation. The color of grape juice is a mixture of various pigments depending upon the maturity stage of the grape berry and cultivar; anthocyanins are the main ones, being responsible for colors ranging from pink through red and violet to dark blue (Corrales et al. 2009). In this way, observing the decrease in a^* (redness) in all pink varieties, these anthocyanins were severely damaged by UV irradiation.

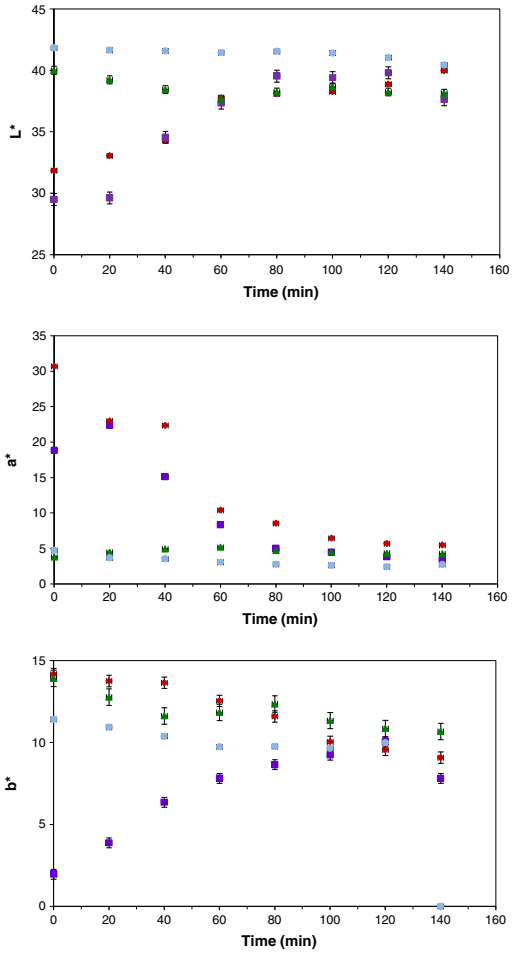

Fig. 1 Plot of color changes in grape juices during UV irradiation: **a** Changes of CIELab parameter lightness (L^*). **b** Changes of CIELab parameter redness (a^*). **c** Changes of CIELab parameter yellowness (b^*). Emperor (*violet squares*), Red Globe (*red diamonds*), Victoria (*green triangles*), Dauphine (*blue circles*)

The studied white varieties showed a slight decrease in L^*, which implies that the juices became a little darker. Parameters a^* and b^* also showed a similar evolution during the irradiation treatment, which indicates that samples became slightly less red and less yellow due to the photodestruction of pigments from the juice.

Absorption Spectrum

The influence of UV radiation on colored compounds of grape juice can also be observed with the evolution of the absorption spectrum. Figure 2 shows the changes of absorbance spectrum of the juice from pink grape varieties. In general, the absorbance slightly increased as the irradiation time of treatment increased; consequently, lower absorbance was observed in unprocessed samples than in those irradiated for 140 min. This effect was clearly observed in juices from white grape varieties (spectra not shown), and it is consistent with the results discussed in the previous section.

In the same way, absorbance of fresh-made must samples from pink grapes generally increased with irradiation time. However, unprocessed samples from pink grapes showed a characteristic peak between 450 and 550 nm, with a maximum around 510 nm. This peak disappears after 140 min of treatment, confirming the breakdown of some pigments in these juices by the treatment with UV–vis radiation. The combination of these two factors in pink grapes (a general

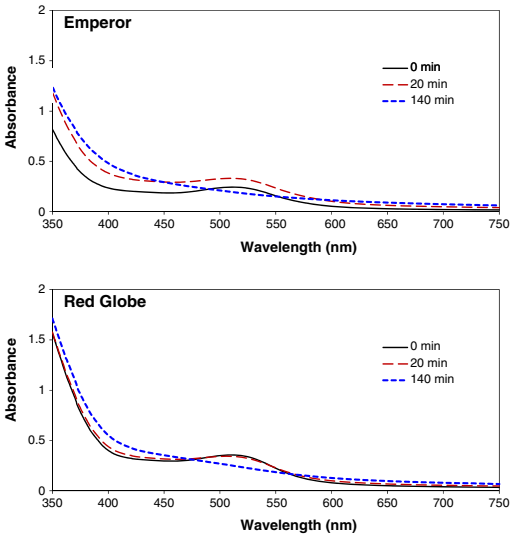

Fig. 2 Evolution of absorbance spectrum of pink grape varieties

increase in absorbance below 450 nm and above 550 nm, but the disappearance of the peak between those wavelengths) may explain the results obtained for L^*, which increased with time of irradiation meaning that the juice became lighter.

Vitamin C

The content in vitamin C of the juices changes differently depending on the variety. In the unprocessed musts, the one from Red Globe had an initial content of 452.5 mg/L of ascorbic acid, the one from Victoria 405.5 mg/L, the one from Emperor 273.0 mg/L, and the one from Dauphine 136.5 mg/L. In all samples content of vitamin C decreases with the time of UV irradiation. Red Globe juice, which had the highest initial content, showed, after 140 min of irradiation, the highest loss in vitamin C, with the 30.10% of the initial content degraded. However, in Victoria juice, which had an initial content of vitamin C quite similar to Red Globe, the loss was 11.96%, the lowest of the four grape varieties irradiated. Regarding the Emperor and Dauphine juices, the losses of vitamin C were 20.97 and 29.03%, respectively. Tran and Farid (2004) reported that the degradation of vitamin C in orange juice was 17% under high UV exposure of 100 mJ/cm^2. Since some of the vitamin C destruction could have been due to air oxidation, these authors measured the effect of air oxidation at 20 °C, and concluded that the vitamin C loss in orange juice caused by air oxidation was only 1.0%, compared to 17.0% due to UV irradiation. Falguera et al. (2011b) reported losses of vitamin C in juices from Golden, Starking, and Fuji apples between 4.0% and 5.7% after 120 min of UV irradiation. However, in samples from King David apples, processed in the same conditions, the loss was 70%. This significant difference was attributed to the high content of pigments in Golden, Staking, and Fuji juices, which had a protective effect against UV radiation; in contrast, the lack of pigmentation of King David juice favored vitamin C destruction. In the present work, however, no direct relationship was observed between the pigmentation of the juice and the protection against vitamin C degradation.

Enzymatic Activities

Polyphenol Oxidase

Initial PPO activities in the four fresh-made grape juices from Emperor, Red Globe, Victoria, and Dauphine grapes were 0.023, 0.0072, 0.0071, and 0.0053 U/mL, respectively. Figure 3 shows the evolution of PPO activity of the four grape juices with the irradiation time.

Juices from white grape varieties were more affected by UV irradiation than the pink ones. There was a decrease higher than 80% in the activity of PPO in these juices after 140 min of irradiation, while for juices of pink grapes the decrease in PPO activity was of 50% on average. In fact, multiple range tests showed that there were no significant differences between the values of residual PPO activity of both varieties of the same kind. This difference in stability of PPO could be a result of the different properties of the white and pink grape juices, since the content of pigments in pink grape juices was higher, playing a protective effect on the PPO. In the same way, Falguera et al. (2011c) suggested a similar protective effect of melanins against the polyphenol oxidase denaturalization by UV radiation in different model solutions. The effects of UV radiation on PPO have been studied by some researchers. Thus, Guerrero-Beltrán

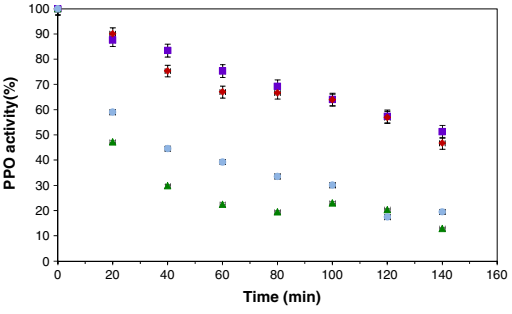

Fig. 3 Changes in PPO activity in grape juices with the time of UV irradiation. Emperor (*violet squares*), Red Globe (*red diamonds*), Victoria (*green triangles*), Dauphine (*blue circles*)

and Barbosa-Cánovas (2006) found that the decrease in polyphenol oxidase activity in mango nectar, after 30 min of UV treatment, was 81%. In a different way, Noci et al. (2008) found that PPO was not affected when they irradiated fresh apple juices by UV. However, in that case, apple juice was irradiated with a 30-W UV light source that emitted at only 254 nm for 30 min (lower power, narrower emission spectra and shorter time than in the present work).

Peroxidase

Initial activities of peroxidase in all grape juices samples were quite low. Initial POD activity in Dauphine was 0.07 U/mL, in Emperor 0.053 U/mL, in Victoria 0.029 U/mL, and in Red Globe 0.009 U/mL. López-Miranda et al. (2011) reported that the average POD activity recorded for 3 years in Crimson Seedless grape varied from 0.024 to 0.075 U g^{-1}. In all samples, after 30 min of UV irradiation, the activity of POD was practically null. Thus, POD was the most sensitive enzyme to UV irradiation. Similar results were found by Falguera et al. (2011b): when apple juice samples were exposed to UV irradiation, they observed that POD activity was completely inactivated after only 15 min of treatment. In contrast, Noci et al. (2008) found that POD activity in fresh apple juices was unaffected by UV irradiation at 254 nm with a 30-W lamp for 30 min.

Pectinmethylesterase

No activity of pectinmethylesterase was found in the juices from the four grape varieties tested. However, Lee et al. (1979) studied the PME activity in several varieties of grape, and reported values from 42 to 106 U mL^{-1}. They also observed that red grapes showed higher PME activity than white grapes. Large portions of PME and pectin substances in grapes are located in the skin (Lee et al. 1979; Lacampagne et al. 2010), which could be a reason to explain the lack of PME activity.

Conclusions

UV–vis irradiation was successfully applied to denaturate peroxidase in freshly squeezed grape juices. POD activity was completely inactivated in all samples after 30 min of irradiation, regardless pigmentation. As far as PPO is concerned, activity of this enzyme was not entirely inactivated after 140 min of UV irradiation in any of the four grape varieties assayed. White grape (Victoria and Dauphine) PPO was more affected by irradiation, being observed an inactivation of 80% of its initial enzyme activity. However, only the 50% of initial PPO activity was reduced in the samples of pink grape (Red Globe and Emperor) juices in the same conditions. Residual PPO activity may cause important changes in color and in phenolic compounds, and therefore, an additional hurdle would be required in order to avoid these changes in the musts during processing. For example, UV–vis-treated grape juices should be stored under refrigeration or, if they were used in winemaking, a residual use of SO_2 would be necessary.

Significant changes in the color of pink grape juices were observed during UV irradiation treatment, which could also be observed in the absorption spectrum. In general, the absorbance grew as the irradiation time of treatment increased. In the case of pink grape juices, it was clearly observed that some pigments, present in the unprocessed juices, were degraded by the effect of UV–vis irradiation. In all samples the content of vitamin C decreased with processing time.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.17.

Title: Effect of UV-vis irradiation of must on Cabernet Franc and Xarel·lo wines chemical quality.

Authors: Víctor Falguera, Maria Forns & Albert Ibarz.

Journal: *International Journal of Food Science & Technology*, (in press).

DOI: 10.1111/j.1365-2621.2012.03059.x

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Short communication

Effect of UV-vis irradiation of must on Cabernet Franc and Xarel·lo wines chemical quality

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Keywords Must, polyphenol oxidase, ultraviolet irradiation, vinification, wine.

Introduction

Nowadays, the techniques used in winemaking are essentially the same as the ones used by ancient civilizations, but the stability of the final product must be guaranteed in such a way that does not affect its organoleptic properties, which usually have to meet high-quality standards. Several secondary metabolites produced by spoiling microorganisms are volatile and potentially affect sensory qualities (Bartowsky, 2009), while many constituents of wine are susceptible to enzymatic or nonenzymatic oxidation during the wine-making process and lead to undesirable products (Li *et al*., 2008).

Many of the microorganisms that may be found in grape musts (especially yeasts) will not be able to grow in wine because of the ethanol content, acidity and limited nutrients, but certain bacteria (such as acetic acid ones) must be controlled to avoid the production of undesirable compounds like acetic acid, acetaldehyde and ethyl acetate (Fredericks *et al*., 2011). As far as enzymatic oxidation is concerned, polyphenol oxidase (PPO) is the most important oxidoreductase responsible for browning during grape processing, followed by laccase and peroxidases (Li *et al*., 2008; Oliveira *et al*., 2011).

In winemaking, some emerging technologies have been studied to inactivate microorganisms and enzymes, being an alternative to partially or totally replace SO_2 (Puértolas *et al*., 2009; Luo *et al*., 2012). In addition, these techniques have been also used to accelerate processes like oxidation or ageing (Bhaskaracharya *et al*., 2009) or to enhance the extraction of some compounds before fermentation (Donsì *et al*., 2010; Vorobiev & Lebovka, 2010). Ultraviolet-visible (UV-vis) irradiation is one of these technologies, because of its proved effect in the pasteurisation of beverages (Falguera *et al*., 2011a). However, the effects of the irradiation of must on the final quality of wine have not been assessed. This piece of work aims at the characterisation of these effects, comparing the consequences of the same treatment (must irradiation) on a wine from a white grape (Xarel·lo) and on a wine from a red one (Cabernet Franc).

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Materials and methods

Must preparation

Grapes from a red variety (Cabernet Franc) and a white one (Xarel·lo) were provided by local farmers (Manresa and Sant Sadurní d'Anoia respectively, Barcelona, Spain). The fruits were washed, squeezed with a household juicer and pressed. The resulting juice was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, Indianapolis, IN, USA) for 30 min at 26 000 g. This process was done at 5 °C to make separation of supernatant easier and to reduce enzymatic activities. After centrifugation was complete, the supernatant was recovered, and the pellet was discarded. The must was separated in fractions of 800 mL.

Four different batches of grapes from each variety were used. The must from each one of these four batches was split into two different fractions, one of which was irradiated. Therefore, four samples for each combination variety – irradiation/no irradiation – were vinified.

UV-vis processing

Half of the fractions of must from each variety were irradiated. UV-vis irradiation was carried out by means of the same equipment used in a previous piece of work (Falguera *et al*., 2011b), which includes a medium-pressure mercury lamp of 400 nm of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, the Netherlands). The lamp was lit 10 min

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before putting the must in the chamber. Irradiation was carried out for 3 h and 30 min. Samples were taken at 0, 60, 120, 180 and 210 min and immediately analysed.

Vinification

Both irradiated and nonirradiated samples were fermented in the Raimat winery (Codorníu Group, Lleida, Spain). Five hundred milli Litre of each must was placed in a glass bottle. Once the must had reached 20 °C, it was inoculated with 10 mL (2% v/v) premix of QA-23 commercial yeast (Lallemand Inc., Montréal, QC, Canada), which was prepared with 10 g of active dry wine yeast and 100 mL of water at 40 °C. 0.1 g of diammonium phosphate was added as fermentation activator. Each bottle was covered with an S-shape airlock (*bubble*). During the fermentation, the temperature was kept at 20 ± 1 °C.

Temperature and density were measured every 24 h. It was considered that the fermentation had finished when density did not change in three consecutive days. The end of the fermentation was also checked with a sugar analysis by means of the method described by Rebelein (1973). Yeast and solid remains were separated by decantation. Then, 375-mL glass bottles were filled with the wine until 55 mm of the top. The remaining space was filled with CO_2-N_2 protective atmosphere, and the cork was placed.

Physical, chemical and enzymatic analyses

The pH of the must (before and after irradiation) and the wine was measured with a Crison micropH 2000 pHmeter (Crison Instruments, S.A., Alella, Spain). Soluble solids content was assessed using an Atago RX-1000 Digital Refractometer (Atago Co. Ltd., Tokyo, Japan). Must and wine colour was monitored using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Sakai, Japan) in the CIELab colour space. Must and wine absorption spectra between 350 and 750 nm were measured with an Helios Gamma Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA), using a 1-cm width quartz cell. In wine samples, tartaric acid content, alcoholic degree and volatile acidity were measured by means of near infrared spectroscopy with a Foss WineScan FT120 Analyzer (FOSS Analytical, Hillerød, Denmark).

Polyphenol oxidase activity in the juice was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol as a substrate, prepared in a McIlvaine buffer solution with a pH of 4.0. The reaction was carried out in a 1-cm light-path quartz cell. One unit (U) of PPO was defined as the amount of enzyme that caused the increase of one absorbance unit at 420 nm in 1 min (Ülker-Yerlitürk *et al.*, 2008). All determinations were carried out in duplicate.

Statistical analysis

Data means and standard deviations were calculated from experimental data by means of StatGraphics Plus v.5.1 software (STSC Inc., Rockville, MD, USA). The same software was used to carry out multiple range tests to find out significant differences between the means at a 95% confidence level. Furthermore, for PPO inactivation, experimental data were fitted to the first-order kinetic model:

$$RA = RA_0 \cdot e^{(-k \cdot t)} \quad (1)$$

where RA is the residual enzyme activity, RA_0 is the intercept of the curve, k is the first-order kinetic constant and t is the irradiation time (Giner *et al.*, 2001).

Results and discussion

Table 1 shows the main properties of Cabernet Franc (red) and Xarel·lo (white) grape musts. Cabernet Franc must has a higher soluble solids content, while Xarel·lo one has a lower pH and a lower PPO activity. Regarding initial colorimetric parameters (shown in Table 2), Cabernet Franc has bigger red ($+a^*$) and blue ($-b^*$) values, and this stronger colour is also traduced in a smaller brightness (L^*) and a higher absorbance at 420 nm.

Polyphenol oxidase inactivation

The evolution of PPO relative activity in both musts during irradiation is shown in Fig. 1. In Xarel·lo must, an inactivation higher than 85% was achieved after 210 min, while in Cabernet Franc must, only 66% of the initial activity was eliminated. In both cases, the highest inactivation was achieved in the first hour: 53% in Xarel·lo and 25% in Cabernet Franc. The fittings of experimental data to the first-order kinetic model (eqn 1) showed that, for Cabernet Franc, RA_0 was (1.01 ± 0.03) and k (5.3 ± 0.4)·10^{-3} min^{-1} (with a determination coefficient R^2 of 0.9986). Meanwhile, the value of RA_0 for Xarel·lo was 0.98 ± 0.05, and the value of k, 10.1 ± 0.5 10^{-3} min^{-1} (with a R^2 of 0.9852). Therefore, the inactivation constant was 91% higher for the white variety.

Table 1 Initial properties of the grape musts from both varieties

	Cabernet Franc	Xarel·lo
pH	3.34 ± 0.07[a]	2.93 ± 0.02[b]
Soluble solids (ºBrix)	18.2 ± 0.2[a]	17.1 ± 0.2[b]
Absorbance at 420 nm	0.967 ± 0.069[a]	0.251 ± 0.054[b]
Polyphenol oxidase activity (U/mL)	0.019 ± 0.003[a]	0.011 ± 0.002[b]

Mean value ± Standard deviation; different superscripts in a row indicate significant differences.

Table 2 CIELab colour parameters of fresh musts, irradiated musts and wines from both of them

Variety	Product	L*	a*	b*
Cabernet Franc	Fresh must	15.39 ± 0.81^{a}	18.99 ± 1.29^{a}	−11.48 ± 0.76^{a}
	Must after irradiation	20.58 ± 3.44^{c}	18.45 ± 4.07^{a}	−4.79 ± 4.37^{ab}
	Wine from non-irradiated must	17.12 ± 0.26^{ab}	12.49 ± 3.75^{b}	−10.50 ± 1.24^{a}
	Wine from irradiated must	19.28 ± 1.55^{bc}	13.64 ± 0.71^{b}	1.47 ± 8.49^{b}
Xarel·lo	Fresh must	39.55 ± 2.60^{x}	2.42 ± 0.24^{x}	8.24 ± 2.18^{x}
	Must after irradiation	36.19 ± 6.25^{x}	3.97 ± 0.93^{y}	8.83 ± 5.04^{x}
	Wine from non-irradiated must	30.13 ± 3.54^{y}	3.86 ± 0.57^{y}	4.26 ± 2.89^{xy}
	Wine from irradiated must	27.32 ± 0.60^{y}	4.87 ± 0.31^{z}	1.27 ± 0.81^{y}

Mean value ± Standard deviation; different superscripts in a column (for each variety) indicate significant differences.

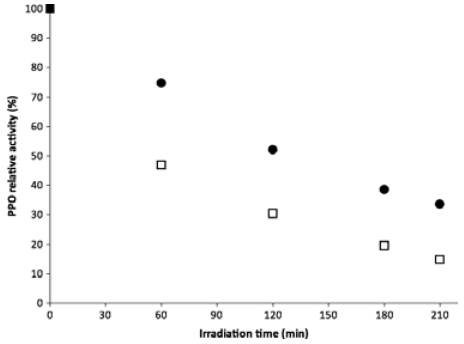

Figure 1 Polyphenol oxidase relative activity as a function of irradiation time, in musts from Xarel·lo (empty squares) and Cabernet Franc (solid circles) grapes.

These differences between the achieved degrees of inactivation suggest that the coloured compounds of the red must (mainly anthocyanins, as it will be addressed in the next section) also absorb UV-vis radiation, and consequently, the dose that reaches the other compounds (such as the enzyme) is lower. In a previous piece of work (Falguera *et al.*, 2011b), it was proved that the presence of coloured compounds in the medium makes the inactivation process slower and, depending on the concentration of these pigments, modifies its kinetic pattern. Comparing the inactivation degree with those achieved in other fruits, it can be stated that it is more difficult to inactivate grape PPO by means of UV-vis irradiation than in others such as apple (100% inactivation in 100 min using the same equipment, Falguera *et al.*, 2011c).

Colour parameters and absorbance spectra

CIELab parameters (Table 2) and absorbance spectra were measured to assess the changes produced in colour by the irradiation process and the vinification. In Cabernet Franc must, UV irradiation produces a significant increase in L^* (it becomes brighter), while a^* and b^* are less affected. Meanwhile, in the case of Xarel·lo, the must becomes redder after irradiation, but brightness (L^*) and yellowness (b^*) are not significantly affected. Absorbance spectra of both grape juices before and after irradiation (shown in Fig. 2) confirmed the facts observed in CIELab parameters. As an example, the disappearance of a peak between 450 and 600 nm in Cabernet Franc must after irradiation shows this impairment of pigments. This peak is a characteristic of anthocyanin-derived pigments that are usually found in red grapes and wines (Oliveira *et al.*, 2006; Lopes-Lutz *et al.*, 2010). Therefore, these results mean that anthocyanins are strongly affected by UV-vis irradiation.

Comparing colour parameters of the wines obtained from irradiated and nonirradiated Cabernet Franc musts, the only significant difference is found in their b^* parameter. In both cases, the fermentation caused a decrease of blueness, but in irradiated must, this decrease was of nearly 12 units (6.7 of which decreased during the irradiation process), while in nonirradiated must, this variation was not found to be significant. This fact may constitute a problem in the application of UV irradiation in some red wines manufacturing, because keeping the blue colour is a highly appreciated property. On the contrary, this effect can be beneficial in rosé wines manufacturing, where the industry searches for a purer red colour. Meanwhile, the differences between both Cabernet Franc wines in L^* and a^* parameters were not significant.

In wines from Xarel·lo (white) must, the fermentation caused a decrease in b^* that was found to be significant only in the wine from irradiated must. However, there were no statistical differences between b^* values of wines from irradiated or from nonirradiated must. On the contrary, fermentation caused a significant increase in a^* in both kinds of samples, and redness was higher in wines from Xarel·lo musts treated by UV-vis irradiation.

Observing absorbance spectra shown in Fig. 2, it can be stated that, comparing the two processes undergone by the samples, fermentation has a greater effect than irradiation, being the profiles of wines from irradiated

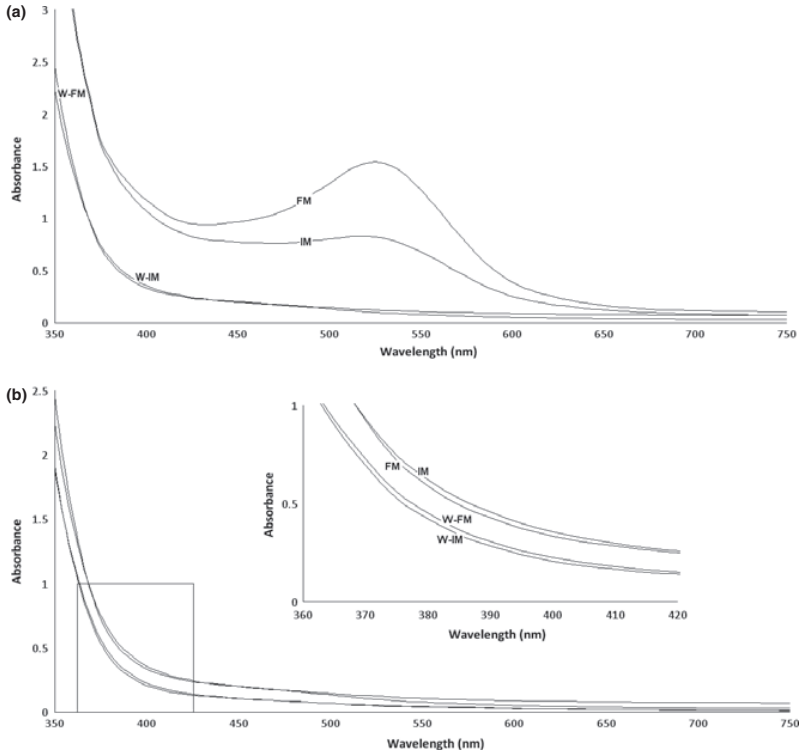

Figure 2 Absorbance spectra of irradiated and nonirradiated grape musts and their wines. FM, fresh must. IM, irradiated must. W-FM, wine from fresh must. W-IM, wine from irradiated must. Upper graph: Cabernet Franc. Lower graph: Xarel·lo.

and from nonirradiated musts similar. This fact is especially remarkable in the case of Cabernet Franc, because irradiation caused an impairment of anthocyanins that led to a decreased absorbance in the range between 450 and 600 nm, but these differences are not found in the product after fermentation.

Fermentation process and wine quality parameters

Figure 3 shows the evolution of density during the fermentation process of the four kinds of vinified musts: irradiated and nonirradiated from Cabernet Franc and from Xarel·lo. The initial values of density were (1082 ± 1) and (1065 ± 1) g cm^{-3} for the red and the white musts, respectively, while the final value was (995 ± 1) g cm^{-3} for all wines after 16 days of fermentation. However, irradiated samples fermented slower than nonirradiated ones, especially after the second day in Cabernet Franc and after the third day in Xarel·lo. The reason for this slower fermentation can be attributed to the elimination of the natural microflora of the musts during the irradiation process. These yeasts can also contribute in a definitive way to the fermentation, being able to start and develop a natural vinification (Bartowsky, 2009). Nevertheless, the pasteurisation carried out by means of the UV treatment can be beneficial from the point of view of the wine industry, because the added yeast would be the only microorganism responsible for the transformation of must into wine, and therefore, the standardisation of the process and the final product will be easier. However, confirmation of this aspect would require specific microbiological analysis.

Regarding wine quality parameters (shown in Table 3), no significant differences were found in pH, alcohol degree and tartaric acid content. Volatile acidity, which is

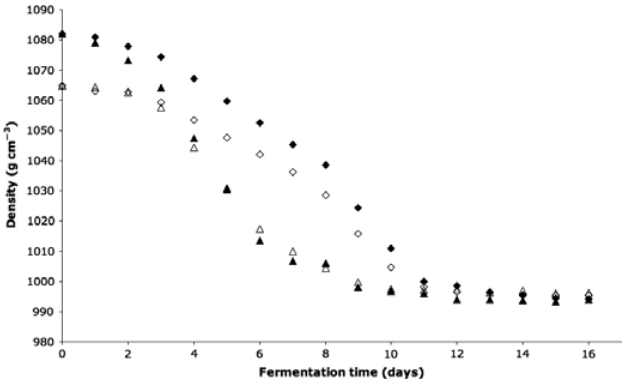

Figure 3 Evolution of density during the fermentation of irradiated and nonirradiated musts from Cabernet Franc and Xarel·lo. ♦ Cabernet Franc, irradiated. ▲ Cabernet Franc, nonirradiated. ◇ Xarel·lo, irradiated. △ Xarel·lo, nonirradiated.

Table 3 Quality parameters of the wines from the four musts

	Cabernet Franc		Xarel·lo	
	Non-irradiated	Irradiated	Non-irradiated	Irradiated
pH	4.10 ± 0.38^{a}	3.78 ± 0.24^{a}	4.16 ± 0.39^{x}	3.91 ± 0.43^{x}
Tartaric acid (g L^{-1})	6.37 ± 0.26^{a}	6.83 ± 0.91^{a}	6.94 ± 1.70^{x}	6.74 ± 1.06^{x}
Alcohol (% v/v)	8.37 ± 0.37^{a}	9.87 ± 1.20^{a}	9.59 ± 1.33^{x}	9.87 ± 1.35^{x}
Volatile acidity (g L^{-1})	2.75 ± 0.46^{a}	0.30 ± 0.26^{b}	1.00 ± 0.69^{x}	0.13 ± 0.10^{y}

Mean value ± Standard deviation; different superscripts in a row (for each variety) indicate significant differences.

an indirect measure of wine spoilage, is much higher in wines from nonirradiated musts in both varieties. In addition, volatile acidity measured in irradiated samples was more homogeneous, which was traduced in lower values of standard deviation. Therefore, these chemical analyses indicate that UV irradiation of must before vinification helps preventing the wine from being damaged by spoiling microorganisms, regardless it is white or red wine. Rossi (1963) already found that the application of UV irradiation at 253 nm prevented wines from acetification without the use of excessive amounts of SO_2 gas, having negligible effects on wine quality even when product exposure was exaggerated.

To sum up, it can be stated that UV-vis irradiation of musts has shown promising (although preliminary) results. However, its implementation at industrial scale raises some difficulties, specially related to the long treatment times that are needed to achieve the desired effects on enzyme inactivation. Thus, the next steps in this research line should focus on two main aims: on the one hand, finding the appropriate relation between UV-vis irradiation and the necessary residual SO_2 dose; on the other hand, developing new reactor kinds or configurations that make it possible to reduce processing times.

Conclusions

Ultraviolet-visible irradiation of musts before fermentation in winemaking is a technology that can help reducing the quantity of SO_2 that is added to wines. A residual use of this product would be necessary to prevent musts from an excessive oxidation, because PPO is not completely inactivated (especially in red musts, where anthocyanins absorb radiation reducing the available dose for inactivating PPO). In addition, chemical quality analyses indicate that UV irradiation can also ensure a greater stability of the final product by means of the destruction of the natural yeasts and bacteria of the musts. As a result, wines from irradiated musts ferment slower and have less volatile acidity. Moreover, no significant differences were found in alcohol degree, pH and tartaric acid content. As far as colour parameters are concerned, the irradiation of white musts (Xarel·lo) causes a significant increase in wine redness, while in red (Cabernet Franc) musts, there is an important decrease of blueness. This decrease may represent a problem in red wines production, but the same effect may be interesting in the production of rosé wines. These results show that UV-vis irradiation may constitute a suitable alternative in winemaking, but some issues as the design of a reactor that allows

reducing treatment times must be solved before it can be implemented at industrial scale.

Acknowledgments

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.18.

Title: UV-vis irradiation: an alternative to reduce SO_2 in white wines?

Authors: Víctor Falguera, Maria Forns & Albert Ibarz.

Journal: Under review in *LWT-Food Science & Technology*.

DOI: -

Falguera V, Forns M, Ibarz A. Uv-vis irradiation: An alternative to reduce SO2 in white wines?. LWT-Food Sci Technol. 2013; 51(1): 59-64. DOI 10.1016/ j.lwt.2012.11.006.

Title: *UV-vis irradiation: an alternative to reduce SO_2 in white wines?*

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Abstract: Ultraviolet-visible irradiation was tested as an innovative technology that can help reducing the amount of sulfur dioxide used in white wine manufacturing. In addition, the effects of must freezing before processing were also investigated. Musts from Xarel·lo and Parellada varieties were vinified without any protective treatment, with a standard amount of SO_2 addition or after an irradiation step. The results showed that UV-vis irradiation of must was able to prevent wine from spoilage in the same degree as SO_2, without changing other quality parameters such as pH, tartaric acid and alcohol content. However, a residual addition of SO_2 would be required in order to completely inhibit polyphenol oxidase activity. Moreover, the obtained changes in wine color parameters led to the conclusion that further optimization of the irradiation process is required before its implementation. Must freezing showed to have some influence on quality parameters of Xarel·lo wines, but not in those from Parellada.

Keywords: must; wine; ultraviolet irradiation; polyphenol oxidase; volatile acidity.

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1. Introduction

Winemaking (or vinification), i.e. the transformation of grape must into wine, has a long history dating back over 8,000 years. Nowadays, the production techniques are essentially the same as the ones used by ancient civilizations, but elaborating a flavorsome and stable wine that does not spoil during storage requires considerable expertise on the part of the winemaker. In addition, this stability must be guaranteed in such a way that does not affect its organoleptic properties, which usually have to meet high quality standards.

A number of factors may affect wine stability. On the one hand, several secondary metabolites produced by spoiling microorganisms are volatile and potentially affect wine sensory qualities (Bartowsky, 2009). On the other hand, many constituents of wine, including phenolics, certain metals, tyrosine and aldehydes, are susceptible to oxidation during the winemaking process and lead to undesirable products that adversely affect its sensory and nutritional value (Li *et al*., 2008). These oxidative processes can be classified in enzymatic and non-enzymatic. The former almost entirely occurs in grape must (having also consequences on wine quality), while the latter can happen both in grape must and wine (Es-Safi *et al*., 2003; Oliveira *et al*., 2011).

Regarding microorganisms, the natural microflora found in grape must includes several dozen species of yeasts (being *Saccharomyces cerevisiae* the main one), four genera of lactic acid bacteria (*Lactobacillus*, *Leuconostoc*, *Oenococcus* and *Pediococcus*) and two genera of acetic acid bacteria (*Acetobacter* and *Gluconobacter*) (Bartowsky, 2009). Many of these microorganisms (especially yeasts) will not be able to grow in wine due to the ethanol content, acidity and limited nutrients, but certain species of bacteria (such as acetic acid ones) must be controlled in order to avoid the production of undesirable compounds like acetic acid, acetaldehyde and ethyl acetate. Spoilage defects are usually recognized by haze formation, increase in acetic acid or volatile acidity, ethanol concentration, volatile phenols, volatile sulphur and viscosity of wine (Fredericks *et al*., 2011).

As far as enzymatic oxidation is concerned, polyphenol oxidase (PPO) is the most important oxidoreductase responsible for browning during grape processing, followed by laccase and peroxidases (Li *et al*., 2008; Oliveira *et al*., 2011). PPO is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen and various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of a heterogeneous group of dark polymers called melanins (Falguera *et al*., 2012b).

Traditionally, sulfur dioxide (SO_2) has been used to control unwanted microorganisms and polyphenol oxidase activity during winemaking, being added to machine-harvested grapes and to wine after malolactic fermentation (Bartowsky, 2009; Oliveira *et al*., 2011). However, in the last times the wine industry is challenged to meet consumers' demands of reducing the amount of SO_2 added to wine, especially since it has been associated with some health risks such as allergic reactions incurred by sulphite-sensitive individuals (Threlfall & Morris, 2002; Fredericks *et al*., 2011). Moreover, its excessive use may affect the quality of wine, giving unpleasing flavors and aromas and making it turn cloudy during storage (Li *et al*., 2008). So far, the total substitution of SO_2 for another compound or technique that fulfill the same functions without its disadvantages remains unsuccessful. However, some studies have shown that a partial replacement may be possible if SO_2 is combined with another hurdle (Li *et al*., 2008; Bartowsky, 2009; Fredericks *et al*., 2011; Oliveira *et al*., 2011).

Some emerging technologies have been used to eliminate microorganisms in liquid food and beverages such as high hydrostatic pressure, pulsed electric fields, ultrasound and ultraviolet irradiation. In the particular case of wine, some of them may also be used to accelerate processes like oxidation or ageing, developing a more rounded taste and flavor (Bhaskaracharya *et al*., 2009), or to enhance the extraction of some compounds before fermentation (Donsì *et al*., 2010; Vorobiev & Lebovka, 2010).
Ultraviolet-visible (UV-vis) irradiation is one of these technologies that can be used for eliminating or reducing the addition of SO_2 in winemaking, due to its proved effect in the pasteurization of beverages (Falguera *et al*., 2011b). However, the effects of the irradiation of must on the final quality of wine still remain unclear. This piece of work aims at the characterization of these effects on white wine production, comparing different parameters that may have influence on the product quality: the grape variety (Xarel·lo or Parellada), the use of SO_2 vs. UV-vis irradiation and must freezing before fermentation.

2. Materials and methods

2.1. Must preparation

Grapes from two white varieties, Xarel·lo and Parellada, were provided by local farmers (Raimat –Lleida- and Sant Sadurní d'Anoia -Barcelona-, respectively). The fruits were washed, squeezed with a household juicer and pressed. The resulting juice was centrifuged in an Avanti J-26XP Centrifuge (Beckman Coulter, USA) for 30 min at 26,000 g. This process was done at 5ºC to make separation of supernatant easier and to reduce enzymatic activities. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. From every batch, the juice was split into different fractions of 800 mL, half of which were frozen.
The resulting samples were divided in three groups, one of which was vinified without further processing, while another group was vinified after SO_2 addition (50 mg/L of potassium metabisulphite, as suggested by the industry) and the last group was vinified after UV irradiation. As a result, four samples (from different batches) for each combination variety (Xarel·lo/Parellada) – treatment (no treatment/SO_2/irradiation) – fresh/frozen must were vinified (12 possible factor combinations, as shown in Figure 1).

Figure 1. *Variable combinations that describe the assayed samples.*

2.2. UV processing

UV irradiation was carried out in a dark chamber containing the juice and the lamp. The must was placed in a methacrylate tank of 22 x 15 x 10 cm. 800 mL of juice were processed, reaching a height of 2.4 cm inside the tank. A refrigeration system consisting of a metallic coil fed with cold water was used to control temperature and avoid must heating. Juice temperature was maintained at 25ºC ± 1ºC in all experiments. A magnetic stirrer was used during irradiation to ensure that all the must was subjected to the same UV dose. UV radiation was produced with a Philips HPM-12 medium-pressure mercury

lamp of 400W of nominal power that emits in a range between 250 and 740 nm (Philips, Eindhoven, The Netherlands), used in previous pieces of work (Ibarz *et al.,* 2009; Falguera *et al*., 2011a). The real incident energy, determined as described by Esplugas and Vicente (1991), was $3.88 \cdot 10^{-7}$ E·min^{-1} (Ibarz *et al*., 2009). The distance between the juice surface and the lamp was 22.5 cm. The lamp was lit 10 min before putting the juice in the chamber. Irradiation was carried out for 3 h and 30 min. Samples were taken at 0, 60, 120, 180 and 210 min and placed in a refrigerator until its analysis.

2.3. Vinification.

Both irradiated and non-irradiated samples were fermented in the Raimat winery (Codorníu Group, Lleida, Spain). Five hundred mL of each must were placed in a glass bottle. Once the must had reached 20ºC, it was inoculated with 10 mL (2% v/v) premix of QA-23 commercial yeast (Lallemand Inc., Montréal, Canada), which was prepared with 10 g of active dry wine yeast and 100 mL of water at 40ºC. 0.1 g of diammonium phosphate (DAP) were added as fermentation activator. Each bottle was covered with an S-shape airlock (*bubble*). During fermentation, temperature was kept at 20±1ºC.

Temperature and density were measured every 24 h. It was considered that the fermentation had finished when density did not change in three consecutive days. The end of the fermentation was also checked with a sugar analysis by means of the method described by Rebelein (1973). Yeast and solid remains were separated by decantation. Then, 375 mL glass bottles were filled with the wine until 55 mm of the top. The remaining space was filled with CO_2-N_2 protective atmosphere and the cork was placed.

2.4. Physical, chemical and enzymatic analyses.

The pH of the must (before and after irradiation) and the wine was measured with a Crison micropH 2000 pHmeter (Crison Instruments, S.A., Alella, Spain). Soluble solids content was assessed using an Atago RX-1000 Digital Refractometer (Atago Co. Ltd., Japan). Must and wine color was monitored using a Chroma Meter CR-400 tristimulus colorimeter (Konica Minolta Sensing, Inc., Japan) in the CIELab color space. Must and wine absorption spectra between 350 and 750 nm were measured with an Helios gamma spectrophotometer (Thermo Fisher Scientific Inc.,Waltham, USA), using a 1 cm width quartz cell. In wine samples, tartaric acid content, alcoholic degree and volatile acidity were measured by means of near infrared spectroscopy (NIR) with a Foss WineScan FT120 analyzer (FOSS Analytical, Hillerød, Denmark).

Polyphenol oxidase (PPO) activity in the juice was assayed measuring the increase in absorbance at 420 nm using 4-methylcatechol as a substrate, prepared in a McIlvaine buffer solution with a pH of 4.0. The reaction was carried out in a 1 cm light path quartz cell. One unit (U) of PPO was defined as the amount of enzyme that caused the increase of one absorbance unit (AU) at 420 nm in 1 min (Ülker-Yerlitürk *et al*., 2008). All determinations were carried out in duplicate.

2.5. Statistical analysis.

Data means and standard deviations were calculated from experimental data by means of StatGraphics Plus v.5.1 software (STSC Inc., Rockville, MD, USA). The same software was used to carry out multiple range tests in order to find out significant differences between the means at a 95% confidence level. Furthermore, for PPO inactivation, experimental data was fitted to the first order kinetic model:

$$RA = RA_0 \cdot e^{(-k \cdot t)} \quad (1)$$

where RA is the residual enzyme activity, RA_0 is the intercept of the curve, k is the first-order kinetic constant and t is the irradiation time (Giner *et al*., 2001).

3. Results and discussion.

3.1. Initial properties.

Table 1 shows the main properties of the different batches of Xarel·lo and Parellada musts that were processed just after being obtained (in fresh) or after freezing and thawing. The main differences between Xarel·lo and Parellada musts are found in soluble solids content, being higher in the former. In addition, in both varieties freezing and thawing caused an increase in the absorbance at 420 nm (which was already higher in Parellada than in Xarel·lo) and a decrease in polyphenol oxidase activity that was only significant in Parellada must. Furthermore, as it can be seen in Table 2, Xarel·lo must is brighter, while Parellada juice is redder (has a higher a^* value) and more yellow (with a higher b^* value). Nevertheless, freezing and thawing do not cause any significant change in brightness (L^*), but an important increase in colorimetric parameters a^* and b^* in both musts. This darkening of thawed samples, as well as the increase in absorbance at 420 nm, was mainly attributed to the oxidation caused by PPO activity during this process.

Samples from Table 1 (fresh and frozen/thawed) were divided in three groups: the first one vinified without any protective treatment, the second one protected against spoilage and enzymatic activity with SO_2 and the third one processed by UV-vis irradiation. In the samples with added SO_2, polyphenol oxidase was completely inactivated.

3.2. Polyphenol oxidase inactivation in the irradiated samples.

PPO was partially inactivated in all the samples that were treated by UV-vis irradiation. The residual activity of the enzyme depended mostly on the grape variety. In this way, Xarel·lo PPO still kept (18±1)% of its original activity after the treatment, while for Parellada samples this residual activity was (30±1)%. Moreover, in a previous piece of work, the average residual PPO activity for two other white grape varieties (Victoria and Dauphine) after UV-vis irradiation was 20% (Falguera *et al*., 2012a). These differences can be attributed to the fact that the must from the varieties with a lower achieved inactivation had a higher concentration of colored compounds (leading to a higher absorbance, a lower value of L^* and a higher value of a^* and b^*). These compounds absorb part of the UV-vis radiation, making the available dose to inactivate the enzyme to be lower (Falguera *et al*., 2012b). Fredericks *et al*. (2011) observed the same phenomenon in the reduction of spoilage in musts and wines with different optical properties (color and turbidity). No statistical differences were found in a single variety comparing the achieved PPO inactivation degree in fresh or frozen/thawed must.

Regarding PPO inactivation rate, Table 3 shows the results of fitting experimental data to the first-order kinetic model. Inactivation constants (k) for Xarel·lo were higher than those obtained for Parellada, reinforcing the idea of a faster denaturation of the enzyme. In addition, these values do not show any remarkable difference between fresh or frozen and thawed samples of both varieties. Although in the case of Parellada k is lower for fresh samples, this difference remains inside the 95% confidence interval.

3.3. Color parameters and absorbance spectra.

First of all, it can be stated that irradiation itself did not cause a brightness decrease neither in Xarel·lo nor in Parellada musts. In Xarel·lo wines, fermentation only caused a decrease of L^* in fresh samples that had been previously irradiated, but not in SO_2-added ones. On the contrary, this decrease of brightness after vinification was found in frozen/thawed samples in untreated, irradiated and SO_2-added samples. In addition, comparing the wines from unfrozen and from frozen must, the later are

significantly less bright regardless the treatment. In the case of Parellada, fermentation caused a decrease of L^* in fresh irradiated must and in untreated frozen/thawed must. In the samples from this variety, if no hurdle is applied against microorganisms or enzymes wine brightness decreases significantly if the must has been frozen.
When it comes to redness (a^*), freezing and thawing also led to a higher value of this parameter in untreated and irradiated musts. In addition, wines from irradiated musts were redder than those from SO_2-added ones, although irradiation itself did not cause any variation in a^*.
Regarding yellowness (b^*), for untreated musts its values were higher in frozen samples, although this increase was not found in irradiated musts. In Xarel·lo wines, no differences were found between the different treatments or between fresh and frozen samples. However, fermentation of frozen/thawed musts caused an important decrease of yellowness that was not found in the vinification of the fresh ones. If the b^* values of the wines from irradiated and SO_2-added musts are compared, the former are less yellow than the later in Parellada, but no significant differences were found in the case of Xarel·lo.
No remarkable differences were found between the absorbance spectra of the wines from the musts with SO_2 and the wines from irradiated musts, neither in the case of Xarel·lo nor in Parellada, both in fresh and in frozen and thawed samples.

3.4. Fermentation process and wine quality parameters.
Figure 2 shows the evolution of density during the fermentation of frozen and thawed Xarel·lo musts with the different kinds of treatment. Parellada frozen must and fresh musts from both varieties (data not shown) behaved in the same way, leading to analogous graphs. Irradiated samples fermented slower than non-irradiated and SO_2-added ones, probably due to the elimination of the natural microflora of the musts during this process. These yeasts can also contribute in a definitive way to the fermentation, being able to start and develop a natural vinification (Bartowsky, 2009). No remarkable differences were found in density evolution between musts with SO_2 and untreated ones.

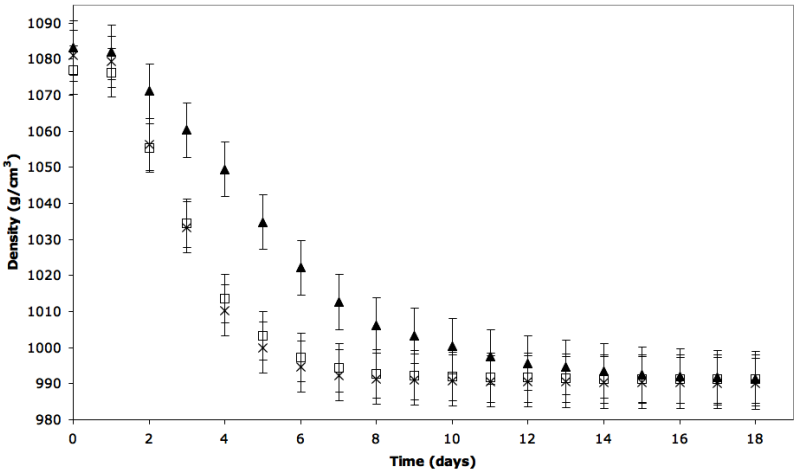

Figure 2. *Evolution of density during fermentation. Samples from frozen and thawed Xarel·lo musts:* × *untreated.* □ *with added* SO_2. ▲ *irradiated.*

Quality parameters of the wines obtained from the musts with the different treatments are shown in Table 4. In the case of Xarel·lo, musts freezing and thawing led to wines with lower pH, higher tartaric acid content and higher volatile acidity, while no significant differences were found in alcohol content. However, none of these general trends were found in Parellada wines. As far as the different treatments are concerned, in most of the studied cases wines from irradiated musts had a lower volatile acidity (an indirect measure of wine spoilage) than those from untreated musts, while no significant differences were found for this parameter between using UV-vis irradiation and SO_2. Although UV light has traditionally been reported to have detrimental effects on wine (Hartley, 2008), Rossi (1963) already found that acetification could be prevented in different kinds of wine through the application of UV irradiation at 253 nm and a residual amount of SO_2 gas, having negligible effects on quality (even when product exposure was exaggerated). However, in those experiments, irradiation was applied on wine (after fermentation), instead of must (before fermentation), and the effects on color parameters were not considered. Even though, this author (as well as Fredericks *et al*., 2011) also acknowledged the need for a residual use of SO_2 to ensure wine preservation.

4. Conclusions.

UV-vis irradiation has been proved to be effective in partially inactivating polyphenol oxidase and reducing volatile acidity (an indirect spoilage measure) in white wines from Xarel·lo and Parellada (when compared with untreated samples). No significant differences were found in quality parameters (pH, tartaric acid, alcohol content and volatile acidity) of wines obtained from musts that have been treated with SO_2 or with UV-vis irradiation. Meanwhile, wines from irradiated musts have a higher redness, a lower brightness and, in the case of Parellada, a lower yellowness than those with added SO_2. Must freezing before processing caused some variations in quality parameters in Xarel·lo wines, but not in those made from Parellada.

Since PPO residual activity is still important after UV-vis irradiation, an additional hurdle would be required to completely inactivate this enzyme and avoid color changes in the must during processing. In other words, this technology may be useful for reducing the amount of SO_2 used in winemaking, but a residual amount would be necessary. In addition, further research should be carried out in order to optimize the UV-vis irradiation process, in terms of time and dose, for each grape variety and used equipment.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.19.

Title: Kinetic and multivariate analysis of polyphenol oxidase inactivation by high pressure and temperature processing in apple juices made from six different varieties.

Authors: Víctor Falguera, Ferran Gatius, Albert Ibarz & Gustavo V. Barbosa-Cánovas.

Journal: *Food and Bioprocess Technology* (in press).

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ORIGINAL PAPER

Kinetic and Multivariate Analysis of Polyphenol Oxidase Inactivation by High Pressure and Temperature Processing in Apple Juices made from Six Different Varieties

Víctor Falguera · Ferran Gatius · Albert Ibarz · Gustavo V. Barbosa-Cánovas

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Abstract The effectiveness of high pressure processing on the inactivation of apple polyphenol oxidase has been widely investigated. However, from an industrial point of view, there is a need for assessing this effectiveness on each one of the different apple varieties that each company uses for their products. This piece of work fills in this gap, studying the effect of the variety on apple juice polyphenol oxidase inactivation. Six varieties were assayed (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith, and Red Delicious), searching for this influence from two different approaches: a kinetic study, finding the best model for each one of them, and a multivariate part consisting of a principal component analysis of data.

Keywords Apple juice · High-pressure processing · Variety · Polyphenol oxidase · Multivariate analysis

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Introduction

In recent times, consumers' demand has experienced a progressive shift towards new food products and processing methods that provide fresh-like quality attributes such as flavor, texture, color, vitamin content, aroma and overall appearance. These attributes, as well as product shelf-life, are closely related to microbial quality and to biochemical and enzymatic reactions (Campus, 2010; Landl et al., 2010). Thermal processing, which negatively affects these fresh-like properties, is still the prevailing method to achieve microbial and enzyme inactivation due to its availability, cost and effectiveness (Mújica-Paz et al., 2011). In this context, novel technologies constitute an alternative to thermal treatment that are being studied and developed in order to obtain a better final product sensory quality, but without neglecting microbial safety (Falguera, Esplugas et al. 2011a). Furthermore, recent studies have also shown that products processed by non-thermal methods may be less allergenic than those obtained by conventional thermal ones (Shriver and Yang, 2011).

Among novel processing alternatives, high pressure processing (HPP) has been adopted at the fastest rate, experiencing a considerable growth in the last 20 years as reflected by the number of units installed (Mújica-Paz et al., 2011). In 2001, the European Commission authorized the placing on the market of pasteurized fruit-based preparations produced using high pressure pasteurization (European Commission, 2001). Moreover, in 2009 the US-FDA approved combined pressure-temperature processing (also known as pressure-assisted thermal sterilization, PATS) for commercial sterilization of mashed potato in a specific high-pressure unit (Bermúdez-Aguirre and Barbosa-Cánovas, 2011).

Fruit juices are one of the main food products in which novel processing techniques are being developed and tested. Among these juices, apple juice is one of the most commonly consumed all over the world. In the USA, the state of Washington produced $2.5 \cdot 10^9$ kg of apples in 2009, which represents 56.5 % of the national production (USDA, 2010). This large production results in a significant availability of surpluses that are processed into juice.

Since the beginning of HPP technology development, apple polyphenol oxidase has focused the interest of scientists due to its high pressure resistance (Buckow et al., 2009; Valdramidis et al., 2009). Polyphenol oxidase (PPO, E.C. 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions involving molecular oxygen with various phenolic substrates: the *o*-hydroxylation of monophenols to *o*-diphenols (monooxygenase or cresolase activity) and the subsequent oxidation of *o*-diphenols to *o*-quinones (diphenolase or catecholase activity). Later polymerization of these compounds leads to the formation of a heterogeneous group of melanins, responsible for product browning (Falguera et al., 2011b). Enzymatic browning caused by the action of PPO has been extensively reported to be the major problem for apple juice (Valdramidis et al., 2009).

In order to achieve a satisfactory degree of PPO inactivation in apple juice, a combination of high pressure with mild initial temperatures (60–90 °C) may be useful (Buckow et al., 2009). This high pressure thermal processing, also called pressure-assisted thermal processing, allows reducing treatment times taking advantage of the synergistic effect of pressure and temperature (Ramirez et al., 2009; Knoerzer et al., 2010; Mújica-Paz et al., 2011).

Therefore, in order to optimize these processes, quantitative kinetic data is essential to achieve the development of predictive models (Buckow et al., 2009). However, the effect of the complex matrix of apple juice requires specific kinetic research for each variety, since the activity and properties of polyphenol oxidase depend on several composition parameters (Yemenicioglu et al., 1997; Soysal, 2008; Ramirez et al., 2009). From an industrial point of view, the applicability of a new technology will be subjected to prove its effectiveness on each one of the varieties that each company uses for its final product, which always comes from a very specific variety combination that gives it an organoleptic and nutritional profile. Thus, this study aims at the characterization of the effect of high pressure processing at different temperatures on polyphenol oxidase inactivation in juices from six different apple varieties. The dependence of PPO inactivation pattern on the variety is assessed from two different approaches: a kinetic study and multivariate analysis.

Material and Methods

Apple Juice Preparation

Apples from six different varieties (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith, and Red Delicious) were purchased from a local supermarket of Pullman (WA). All these apples were produced in the state of Washington (USA) and were commercially available the last week of July, 2011. The apples were washed, cut in four pieces and kept in water while waiting to be squeezed with a household juicer. The resulting juice was centrifuged in a Sorvall RC6+ centrifuge (Thermo Fisher Scientific Inc., Waltham, USA) for 15 min at 7,500 rpm. This process was carried out at 4 °C to make separation of supernatant easier and to reduce enzymatic activities. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. Fractions of 30 mL of juice were placed in 0.1016 mm thickness polyethylene bags (Consolidated Plastics Company, Inc., Twinsburg, USA), thermo-sealed and stored in the freezer at −20 °C. Before processing, samples were thawed by immersing them in a water bath at 20 °C for 1 h.

High-Pressure Processing

Polyethylene bags containing apple juice from the six different varieties were placed into a cylindrical liner made of white polypropylene (internal diameter 75 mm, external diameter 100 mm, height 21.5 mm; McMaster-Carr, Atlanta, USA). The liner with samples was placed inside the cylindrical chamber vessel (internal height 25 cm, diameter 10 cm) of the high pressure equipment (Engineered Pressure Systems, Inc., Andover, USA). The unit was operated with an electrohydraulic intensifier pump (Hochdruck-Systeme GmbH, AP 10-0670-1116, Sigless, Austria) that pressurized the vessel to operating pressure in a few seconds (average 7 s). A 5 % Mobil Hydrasol 78 water solution was used as pressure medium. In order to carry out the treatment, the equipment was preheated to target nominal temperature (25, 60, and 80 °C). Pressure medium temperature was measured by means of three thermocouples placed inside the vessel. Processing pressures were 400, 500, and 600 MPa. Apple juice samples were processed for different holding times: come-up time (the time necessary to achieve the target pressure followed by immediate pressure release, average 7 s), 4 min, 8 min, 12 min and 16 min. Fig. 1 shows pressure and compression fluid temperature records for an experiment carried out at 500 MPa and 60 °C for 4 min of holding time.

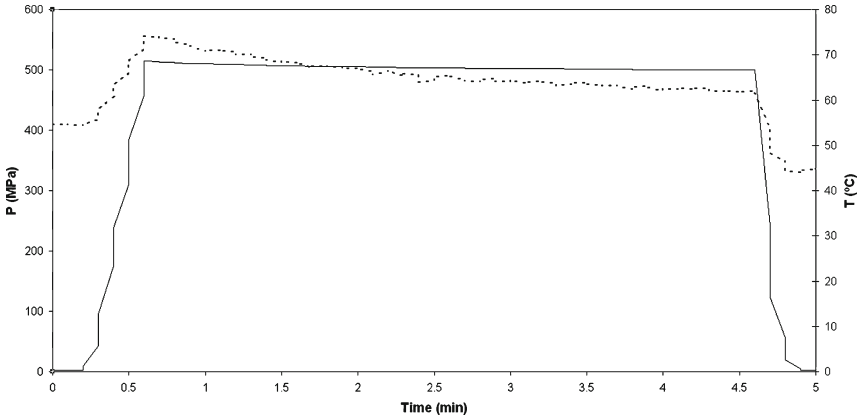

Fig. 1 Pressure (*solid*) and compression fluid temperature (*dotted*) records for an experiment carried out at 500 MPa and 60 °C for 4 min of holding time

Polyphenol Oxidase Activity

Polyphenol oxidase activity in the juice was assayed measuring the increase in absorbance at 420 nm using catechol as substrate, prepared in a citrate buffer solution with a pH of 6.0 (Buckow et al., 2009; Falguera et al., 2011c). The reaction was carried out in a 1-cm light path quartz cell. Absorbance was measured for 3 min with a Shimadzu UV-2550 spectrophotometer and recorded by means of its UV Probe v.2.31 software (Shimadzu Scientific Instruments, Columbia, USA). One unit of PPO activity was defined as the amount of enzyme that caused the increase of one absorbance unit at 420 nm in 1 min.

Physical and Chemical Analyses

pH and conductivity of the juices were measured with a Crison micropH 2000 pHmeter (Crison Instruments, S.A., Alella, Spain). Soluble solids content was assessed using an Atago RX-1000 Digital Refractometer (Atago Co. Ltd., Japan). Total phenolic content was determined as described by Shaghaghi et al. (2008), with a spectrophotometric method using the Folin–Ciocalteu reagent. Formol index was measured according to the official method of the International Federation of Fruit Juice Producers (IFFJP, 1984), with a potentiometric titration of the acidity of the compounds formed by the reaction of formaldehyde and amino acids in the juice up to a pH of 8.1. Total and reducing sugars were determined by the reduction of the Fehling reagent, according to the method of the IFFJP (1972). Titratable acidity was also assessed by the official method of the IFFJP (1972), with a potentiometric titration with sodium hydroxide up to a pH value of 8.1. Results are expressed as malic acid in milligrams per liter. Vitamin C was quantified by the dicloroindophenol method (AOAC, 2000), expressing the results as ascorbic acid in milligrams per 100 mL. Maturity index was calculated as the ratio of total soluble solids to titratable acidity (Ladaniya, 2008).

Viscosity was assessed at 20 °C using a Haake RS-80 RheoStress Rheometer (Gebrüder Haake GmbH, Karlsruhe, Germany) with a Z40-DIN coaxial-cylinder sensor system (radii ratio 1.0847). For temperature control, a Thermo Haake C25P bath (Gebrüder Haake GmbH) was used, using a glycol–water solution (50 % *w*/*w*) as coolant fluid, which allows an interval variation of 0.2 °C. The samples were sheared at a constant shear rate of 400 s^{-1} for 3 min, after which a downward ramp to 0 s^{-1} and another upward ramp until 400 s^{-1} were accomplished. The average shear stress values of these two ramps were used to calculate the juice viscosity by means of Newton's equation, since all the samples showed Newtonian behavior (Ibarz et al., 2009).

Kinetic Models and Statistical Analysis

To carry out the kinetic studies, the initial PPO activity (A_0) was defined as the activity found after the come-up time treatment (the time necessary to achieve the target pressure followed by immediate pressure release) for each apple variety (Buckow et al., 2009). Then, relative activity (RA) was calculated for each treatment time. During a reaction at constant pressure and temperature, changes in the relative activity of an enzyme (RA) with time (t) can be described by a differential balance (Ramirez et al., 2009; Eq. 1):

$$-\frac{dRA}{dt} = k \cdot RA^n \tag{1}$$

where k is the rate constant for the reaction at a given pressure and temperature, and n is the reaction order. Integration of this balance leads to expressions for zero (n=0, Eq. 2), first (n=1, Eq. 3) and second (n=2, Eq. 4) order kinetics:

$$RA = RA_0 - k_0 \cdot t \quad (2)$$

$$RA = RA_0 \cdot \exp(-k_1 \cdot t) \quad (3)$$

$$\frac{1}{RA} = \frac{1}{RA_0} + k_2 \cdot t \quad (4)$$

The kinetic expression describing best the activity changes as a function of time was then used to determine k_i values as a function of temperature and pressure for each apple variety. The root mean square error (RMSE) index was used to decide which model provided the best fit for the experimental data. This index was defined as:

$$RMSE = \sqrt{\frac{1}{n}\sum \left[RA_{exp} - RA_{pred}\right]^2} \quad (5)$$

where n is the number of samples of each variety (i.e., considering all the experiments at different pressures and temperatures), RA_{exp} is the experimental relative activity and RA_{pred} is the predicted relative activity corresponding to each sample and fitting model.

Experimental data were fitted to the kinetic expressions by non-linear regression procedures using Statgraphics Plus 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The same software was used to carry out multiple range tests in order to find out significant differences between the means at a 95 % confidence level.

Multivariate Analysis

Multivariate projection techniques (Martens and Naes, 1989) have been used in this study to determine the most important variables for data characterization and to see if there were any group patterns. These techniques have been widely applied for quality assessment, authentication, or even classification of a great variety of food products, including vegetables, fruits, juices, wines, oils, and others (Tzouros and Arvanitoyannis, 2001). In this case, the data matrix consisted of 276 samples (6 apple varieties×3 pressures×3 temperatures×5 treatment times, plus 6 unprocessed samples) and 25 variables (including those defining the experiment and the ones described in the previous sections). All the values included in the matrix were the average of 6 determinations, carried out on two different sample batches. In order to eliminate the effects of the different variable units, data autoscaling was performed prior to each analysis. Principal Component Analyses (PCA) of the data obtained in the samples characterization were carried out using the Unscrambler v.10.1 software (CAMO Software AS, 2010; Trondheim, Norway).

Results and Discussion

Physicochemical Properties of the Raw Juices and Initial PPO Activity

The juices from the six apple varieties had several differences in their composition and properties. Table 1 shows the values obtained for the measured physicochemical parameters. Fuji and Red Delicious juices had the highest soluble solids content (12.5 and 12.4ºBrix, respectively). Fuji also had the lowest titratable acidity (0.215 $g \cdot L^{-1}$; and therefore the highest maturity index, 58.3), the highest pH (4.39) and also the highest content of reducing and total sugars (92.3 and 117.3 $g \cdot L^{-1}$) and vitamin C (44.7 mg/100 mL). In addition, Fuji juice had the highest initial PPO activity (0.088 $U mL^{-1}$), this value being much higher than the second one (Braeburn, with 0.032 $U mL^{-1}$).

At the other side of the scale, Granny Smith juice had the lowest maturity index (13.1), especially due to its high titratable acidity (0.769 $g L^{-1}$). However, the lowest initial PPO activity was found to be in Golden Delicious juice, with 0.007 $U mL^{-1}$. Regarding total phenolics, the potential PPO substrates, the highest content was found in Red Delicious juice (347.6 mg L^{-1}), which had an intermediate PPO activity (0.016 $U mL^{-1}$). According to Cheng and Crisosto (1995), a general correlation between total phenolics and PPO activity cannot be established, since the browning potential depends on each phenolic compound.

PPO Activity Variation Caused by Instant Exposure to Target Conditions

Instant exposure of freshly squeezed apple juice to mild temperatures (25–60 °C) and pressures (followed by immediate pressure release and cooling) caused an increase in PPO activity (Table 2). This phenomenon has also been reported by several authors (Anese et al., 1995; Bayındırlı et al., 2006; Soysal, 2008; Buckow et al., 2009), and it has been often attributed to the release of latent enzymes (Yemenicioglu et al., 1997). In this case, the maximum PPO increase was found during exposure to 400 MPa and 25 °C in all varieties. These conditions caused an increase of 61.5 % in Red Delicious PPO, while the lowest increase (36.3 %) was found in Braeburn PPO. On the contrary,

Table 1 Physicochemical properties of the raw apple juices from the six used varieties

	pH at 20 °C (–)	Conductivity at 20 °C ($\mu S \cdot cm^{-1}$)	Soluble solids (°Brix)	Density ($g \cdot mL^{-1}$)	Viscosity (mPa·s)	Titratable acidity ($g \cdot L^{-1}$ malic acid eq.)	Formol index (mL NaOH/ 100 mL)	Reducing sugars ($g \cdot L^{-1}$ glucose eq.)	Non-reducing sugars ($g \cdot L^{-1}$ glucose eq.)	Total phenolics ($mg \cdot L^{-1}$ gallic acid eq.)	Vitamin C (mg ascorbic acid/ 100 mL)	Maturity index (°Brix·$L \cdot g^{-1}$)	PPO activity ($U \cdot mL^{-1}$)
Braeburn	3.64±0.01b	1440±7b	10.9±0.1b	1.051±0.002b	4.66±0.30a	0.474±0.015d	5.3±0.6c	65.3±1.7a	23.0±0.1c	292.3±5.9bc	35.3±2.0bc	23.0±0.1c	0.032±0.002e
Fuji	4.39±0.02f	1529±5d	12.5±0.1c	1.055±0.001c	4.61±0.29a	0.215±0.013a	3.7±0.6b	92.3±1.7e	58.3±0.1e	237.6±3.0a	44.7±2.0d	58.3±0.1e	0.088±0.003f
Gala	3.89±0.02d	1507±2c	10.2±0.1a	1.051±0.002b	4.70±0.29a	0.438±0.020c	6.7±0.6d	78.7±2.5c	23.3±0.1c	285.1±5.1b	40.0±3.5c	23.3±0.1c	0.012±0.002b
Golden Delicious	3.69±0.02c	1799±3f	9.9±0.3a	1.054±0.002bc	4.73±0.29a	0.568±0.015e	2.3±0.6a	85.4±1.5d	17.4±0.3b	317.2±12.7c	32.9±3.5ab	17.4±0.3b	0.007±0.001a
Granny Smith	3.26±0.02a	1413±3a	10.1±0.3a	1.047±0.001a	4.73±0.32a	0.769±0.008f	7.0±1.0d	71.3±1.0b	13.1±0.3a	298.1±11.3bc	36.5±3.5bc	13.1±0.3a	0.022±0.002d
Red Delicious	3.99±0.02e	1579±6e	12.4±0.1c	1.059±0.003d	4.78±0.29a	0.380±0.008b	3.3±0.6ab	92.3±1.7e	32.6±0.1d	347.6±34.7d	29.4±3.5a	32.6±0.1d	0.016±0.001c

Mean value±standard deviation. Different letters in a column indicate significant differences

exposure to 80 °C caused a reduction of PPO activity in almost all varieties and pressures, except for Red Delicious at 400 and 500 MPa, and Golden Delicious at 400 MPa. In this way, the maximum reduction was found in Granny Smith juice at 80 °C and 500 or 600 MPa (74.0 % and 74.9 %, respectively). These results suggest that the exposure to pressure/temperature conditions affects apple PPO to a different degree depending on apple variety.

Kinetic Study of PPO Inactivation

Experimental values of PPO relative activity as a function of processing time for some combinations apple variety-nominal temperature–pressure are shown in Fig. 2. It can be easily seen that the apple variety did not only affect the initial PPO activity (shown in Table 1), but also the inactivation pattern and the achieved inactivation degree. In this way, Granny Smith PPO was completely inactivated after 8 min at 600 MPa and 80 °C, while Fuji PPO activity was only reduced to 27.7 % after 16 min under the same conditions (however, a 57 % loss was already achieved in the compression phase). Observing all experimental results at room temperature (25 °C), the maximum inactivation after 16 min was achieved in Granny Smith at 600 MPa (residual activity 71.5 %), while Braeburn and Golden Delicious PPO were the most resistant under these conditions (residual activity 93 %).

All the experimental relative values from the 54 processing series (6 varieties×3 pressures×3 temperatures) were fitted to zero-order, first order and second order kinetic models (Eq. 2, 3 and 4, respectively) by means of non-linear regression procedures, in order to obtain RA_0 and k_i parameters for each one. Then, predicted relative activity values were calculated from these parameters. Table 3 shows RMSE values obtained when comparing the 45 (3 pressures×3 temperatures×5 processing times) experimental vs. predicted values for each variety and kinetic model. Moreover, an additional RMSE value has been calculated for each kinetic model considering the samples from all apple varieties (270), in order to compare the general goodness of each expression.

Zero-order kinetic model was the most appropriated one for describing PPO inactivation in Golden Delicious juice, since the lowest RMSE was obtained with this expression (0.059). Likewise, the first-order model was the best one for Braeburn (0.051), Granny Smith (0.068), and Red Delicious (0.078), while the second-order one was the best for Fuji (0.041). As far as Gala juice is concerned, both zero-order and first order

Table 2 Change of PPO activity (%) relative to the freshly squeezed apple juice after come-up time (heating and pressure increase to target conditions followed by immediate pressure release and cooling)

	P (MPa)	25 °C Change of PPO activity (%)	60 °C	80 °C
Braeburn	400	36.3±0.7wx	22.9±0.5s	−9.2±0.5j
	500	30.4±0.7tu	20.7±0.8rs	−24.6±0.8g
	600	11.1±0.5no	14.2±0.6op	−34.4±1.1e
Fuji	400	49.9±3.5γ	41.5±3.2zαβ	−33.5±3.4ef
	500	40.5±2.6yzα	0.7±0.4l	−39.1±3.0d
	600	28.5±2.9t	−18.7±2.1hi	−57.2±3.1b
Gala	400	44.2±1.4β	22.0±2.4s	−20.7±1.9h
	500	33.8±0.7vw	9.4±1.4mn	−25.3±2.8g
	600	29.4±0.8tu	−3.7±1.2k	−34.1±2.3e
Golden Delicious	400	52.9±1.0γ	43.3±1.1αβ	11.1±0.7no
	500	32.4±1.2uv	17.0±1.5pq	−17.2±1.4i
	600	18.5±0.8qr	18.2±1.0qr	−30.6±1.1f
Granny Smith	400	41.6±0.6zαβ	39.7±0.4yz	−51.3±1.2c
	500	38.4±0.6xyz	37.3±0.1xy	−74.0±1.5a
	600	31.3±0.7tuv	32.0±0.8uv	−74.9±1.9a
Red Delicious	400	61.5±1.4ε	57.6±1.0δ	6.1±0.2m
	500	51.1±0.7γ	44.5±1.1β	0.6±0.2l
	600	33.9±0.9vw	32.0±0.9uv	−4.1±0.1k

Mean value±standard deviation. Different characters after the values indicate significant differences

models gave the same RMSE value (0.077). Thus, these results show that the kinetic pattern in PPO inactivation of apple juice definitively depends on the variety. If all the samples are considered, the global RMSE value revealed that the zero-order model (0.076) was the one that described the best the inactivation of PPO in apple juice from these six varieties under the mentioned conditions.

Table 4 shows the kinetic parameters (RA_0 and k_i) obtained with the best model for each variety. In the case of Gala juice, the mathematically simplest model (zero-order) is considered the most suitable one, and the shown parameters have been calculated by means of this expression. As a general rule, kinetic constants (k_i) become higher as both pressure and temperature grow. In this way, for example, k_1 for Braeburn increases from 0.0041 min^{-1} at 400 MPa and 25 °C to 0.0112 min^{-1} at 600 MPa and 25 °C, and its value becomes 0.2980 min^{-1} at 600 MPa and 80 °C.

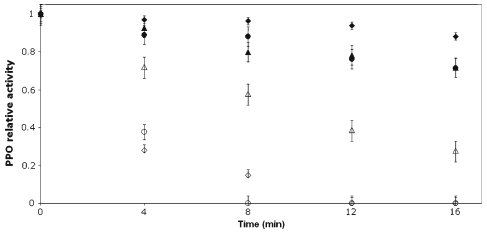

Fig. 2 Polyphenol oxidase relative activity as a function of processing time for some apple varieties and temperature-pressure conditions: Braeburn (*filled diamond* 400 MPa 25 °C; *open diamond* 600 MPa 80 °C), Fuji (*filled upright triangle* 400 MPa 25 °C; *open upright triangle* 600 MPa 80 °C) and Granny Smith (*filled circle* 400 MPa 25 °C; *open circle* 600 MPa 80 °C)

Table 3 RMSE values for the three kinetic models applied on polyphenol oxidase relative activity data from the six different apple varieties

Variety	Zero-order	First-order	Second-order
Braeburn	0.067	0.051	0.092
Fuji	0.050	0.106	0.041
Gala	0.077	0.077	0.140
Golden Delicious	0.059	0.078	0.171
Granny Smith	0.105	0.068	0.184
Red Delicious	0.087	0.078	0.161
All samples	0.076	0.078	0.138

Table 4 Kinetic parameters obtained fitting the experimental polyphenol oxidase relative activity values to the most appropriated kinetic model for each apple variety

	Kinetic model	T (°C)	Pres. (MPa)	RA_0 (−)	k_i (min^{-1})	R^2
Braeburn	First order	25	400	1.005±0.039	0.004±0.002	0.9087
		25	500	0.993±0.018	0.007±0.004	0.9396
		25	600	0.989±0.050	0.011±0.006	0.9327
		60	400	0.981±0.046	0.008±0.005	0.8890
		60	500	0.983±0.053	0.015±0.006	0.9564
		60	600	1.022±0.091	0.035±0.011	0.9727
		80	400	1.082±0.349	0.056±0.047	0.8575
		80	500	1.046±0.405	0.109±0.084	0.9056
		80	600	1.002±0.125	0.298±0.089	0.9934
Fuji	Second order	25	400	1.003±0.086	0.025±0.009	0.9642
		25	500	0.984±0.048	0.012±0.005	0.9491
		25	600	1.003±0.094	0.047±0.010	0.9879
		60	400	1.038±0.223	0.017±0.017	0.6785
		60	500	0.854±0.418	0.082±0.058	0.8707
		60	600	0.906±0.202	0.029±0.025	0.8211
		80	400	1.006±0.157	0.034±0.016	0.9398
		80	500	1.004±0.217	0.088±0.022	0.9819
		80	600	1.287±1.177	0.160±0.072	0.9436
Gala	Zero-order	25	400	1.018±0.056	0.015±0.006	0.9583
		25	500	1.001±0.035	0.014±0.004	0.9814
		25	600	0.944±0.129	0.016±0.013	0.8231
		60	400	0.983±0.098	0.010±0.010	0.7776
		60	500	0.937±0.146	0.021±0.015	0.8656
		60	600	1.025±0.096	0.029±0.010	0.9682
		80	400	1.111±0.424	0.051±0.043	0.8256
		80	500	1.030±0.190	0.041±0.019	0.9384
		80	600	0.898±0.508	0.053±0.052	0.7817
Golden Delicious	Zero-order	25	400	0.988±0.049	0.014±0.005	0.9648
		25	500	0.989±0.030	0.004±0.003	0.8441
		25	600	1.021±0.059	0.014±0.006	0.9455
		60	400	0.997±0.038	0.011±0.004	0.9670
		60	500	0.986±0.078	0.023±0.008	0.9668
		60	600	1.059±0.206	0.040±0.021	0.9255
		80	400	0.961±0.148	0.039±0.015	0.9582
		80	500	0.995±0.306	0.069±0.031	0.9432
		80	600	0.876±0.451	0.066±0.046	0.8723
Granny Smith	First order	25	400	0.995±0.072	0.010±0.008	0.9544
		25	500	1.009±0.036	0.019±0.004	0.9597
		25	600	0.973±0.107	0.021±0.013	0.8881
		60	400	0.981±0.174	0.054±0.026	0.9455
		60	500	1.014±0.099	0.112±0.022	0.9929
		60	600	1.034±0.205	0.148±0.057	0.9785
		80	400	1.001±0.117	0.180±0.042	0.9930
		80	500	1.048±0.510	0.221±0.221	0.9096
		80	600	1.016±0.221	0.297±0.154	0.9812

Table 4 (continued)

	Kinetic model	T (°C)	Pres. (MPa)	RA_0 (−)	k_i (min^{-1})	R^2
Red Delicious	First order	25	400	0.982±0.051	0.010±0.006	0.9166
		25	500	0.978±0.053	0.010±0.006	0.9123
		25	600	0.996±0.027	0.017±0.003	0.9810
		60	400	0.982±0.046	0.013±0.005	0.9565
		60	500	0.992±0.136	0.032±0.017	0.9283
		60	600	1.033±0.173	0.055±0.024	0.9540
		80	400	1.086±0.373	0.072±0.056	0.8820
		80	500	1.091±0.737	0.181±0.181	0.8315
		80	600	0.994±0.264	0.278±0.171	0.9696

Significance level: α=0.05

Multivariate Study

In order to identify the general trends in the relations between variables and any possible group or patterns in the data, a Principal Component Analysis (PCA) was carried out. First of all, Fig. 3 corresponds to the scores of the samples in the two first principal components space, which represents 56 % of the overall variance of the original matrix (25 variables). This plot clearly shows six different groups of samples, which are distributed mainly along the maximum variance direction (PC1) and can be identified with the six different apple varieties. Therefore, the variety is the variable that contributes the most to the overall variance (i.e., the most important variable in the study). This clear separation of data groups suggests the need for building a separated PCA model for each one of these sample groups. Observing variable loadings (Fig. 4) in the PC1–PC2 space for the same global PCA model, some correlations between the variables may be established. In this way, juices density is highly correlated with total and reducing sugars, and acidity is negatively correlated with pH. Likewise, this plot also shows that Fuji is the variety with the highest PPO activity. As it was discussed before, PPO activity does not show any direct relationship with phenolics content. Maturity index and vitamin C content are the two physicochemical variables that show the highest (although weak) correlation with PPO activity.

When a particular PCA model is carried out for each one of the six apple varieties, the explained variance of the obtained models increases (80 % on average in the two first

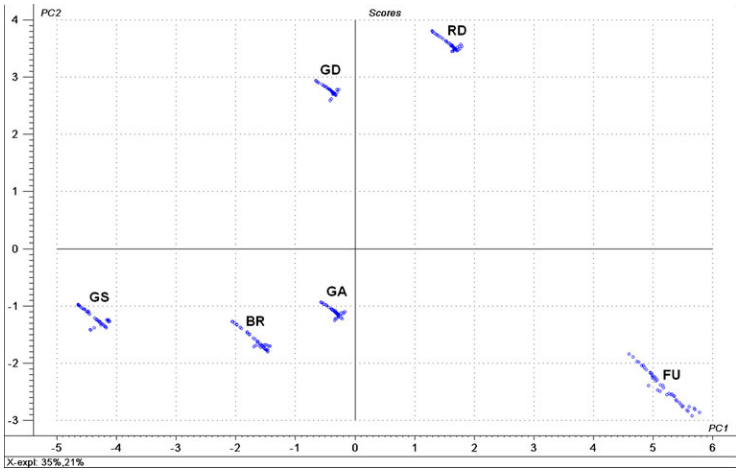

Fig. 3 Principal component analysis of the overall data. Sample scores in the two first principal components space (explained variance, 56 %). The samples are clearly distributed in six groups according to apple varieties: *BR* Braeburn, *FU* Fuji, *GA* Gala, *GD* Golden Delicious, *GS* Granny Smith, *RD* Red Delicious

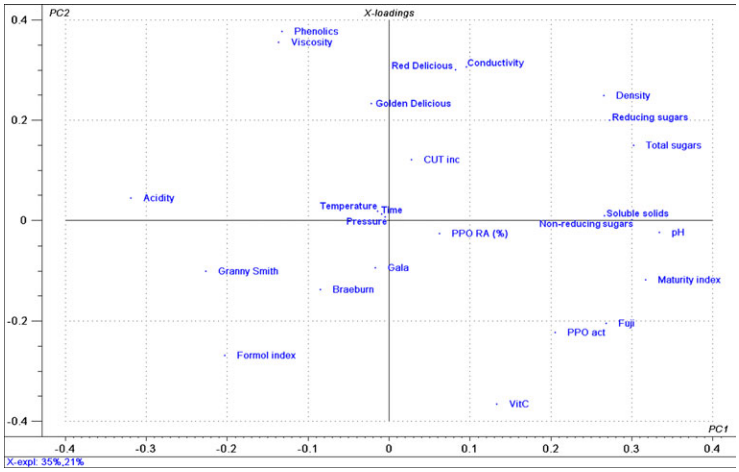

Fig. 4 Principal component analysis of the overall data. Variable loadings in the two first principal components space (explained variance, 56 %)

PCs), mainly due to the loss in variability caused by the matrix dimension reduction. Figure 5a shows sample scores from the PCA of Gala variety data, codified according to processing temperature. It can be easily seen that the samples are grouped following a clear trend along the two first PCs (especially the first one). It is also worth mentioning that, as processing temperature increases, the dispersion of the samples becomes greater. This fact reinforces the conclusion that at higher temperatures, the other variables (time, pressure, variety) have more effect on PPO inactivation rate (as it was stated analyzing kinetic constants in the previous section). As far as processing time is concerned (Fig. 5b), although a tendency appears especially in the vertical direction (PC2), no clearly defined groups appear, not being this effect as marked as in the case of temperature. In both diagrams, the unprocessed sample (20 °C raw; *20* in the upper graph, RW in the lower one) appears isolated. Codification for processing pressure is not shown since no pattern defining any phenomenon has been seen (the information regarding this variable appears randomly distributed in the samples, not only concerning the PC1–PC2 space, but also when more PCs are taken into account in calculating the model). The same trends were found in the PCA modeling of all the other varieties.

Regarding variable loadings for one single variety, their plot in the two first principal components space showed that the three variables related with PPO (absolute activity, relative activity and the activity increase produced in the come-up time—data from Table 2) were the ones that contributed the most to the overall variance. Among the parameters that define the experiment, the temperature is the most important one in the maximum variance directions, followed by the processing time and the pressure. This fact indicates that, in the variable range that has been studied, increasing the temperature has more effect on PPO inactivation than increasing processing time, and this than increasing the pressure. Analyzing kinetic constants from Table 4, it was also concluded that increasing the temperature has a greater effect on the inactivation rate than increasing the pressure. However, it must be remembered that the studied temperature range was 25–80 °C, while the pressure range was 400–600 MPa.

Conclusions

Apple polyphenol oxidase from the six studied varieties (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith, and Red Delicious) is extremely pressure-resistant if the process is carried out at approximately room temperature (25 °C): after 16 min at 600 MPa, the maximum activity reduction (additional to the loss caused by compression and instant decompression) was found in Granny Smith juice (28.5 %), while in Braeburn and Golden Delicious juices only a reduction of 7 % was achieved under these conditions. As it may be easily observed, the apple variety does not only affect the initial PPO activity, but also the inactivation pattern and the achieved inactivation degree. In this way, the most appropriated kinetic model to describe PPO inactivation is the zero-order one for Golden Delicious and

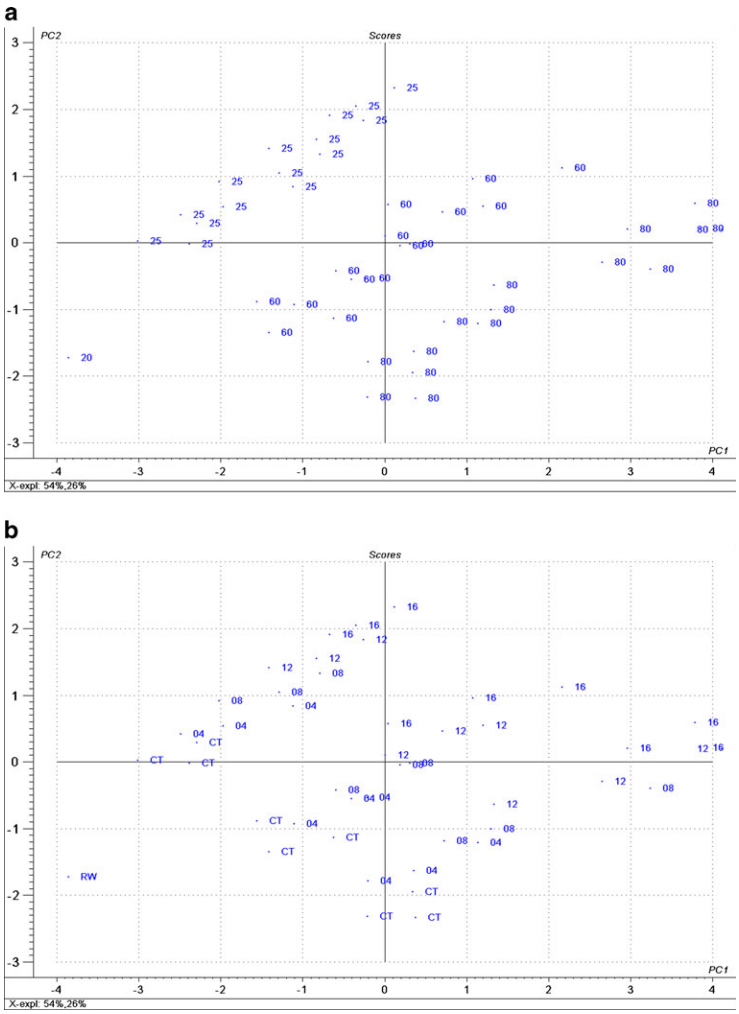

Fig. 5 Principal component analysis of the data corresponding to the Gala apple variety. Sample scores in the two first principal components space (explained variance, 80 %). **a** Codes correspond to nominal processing temperature. **b** Codes correspond to processing time

Gala juices and the first-order one for Braeburn, Granny Smith, and Red Delicious juices, while enzyme destruction in Fuji juice was found to follow a second-order kinetic expression.

Multivariate analysis (after eliminating unit scale effects) show that, in a process at temperatures between 25 and 80 °C, pressures from 400 to 600 MPa and processing times up to 16 min, among the variables that define the experiment the apple variety is the one with the strongest influence in PPO inactivation rate, followed by the temperature, the processing time and the pressure.

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APPENDIX I: SCIENTIFIC PUBLICATIONS IN ISI-INDEXED JOURNALS.

I.20.

Title: Changes on colour parameters caused by high pressure processing of apple juices made from six different varieties.

Authors: Víctor Falguera, Ferran Gatius, Albert Ibarz & Gustavo V. Barbosa-Cánovas.

Journal: *International Journal of Food Science & Technology* (in press).

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International Journal of Food Science and Technology 2012

Original article

Changes on colour parameters caused by high-pressure processing of apple juice made from six different varieties

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Summary The colour changes caused by high-pressure processing at different temperatures of apple juice were investigated. Six apple varieties were used (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith and Red Delicious), searching for the influence of the variety from two different approaches: a kinetic study, finding the best model for each one of them, and a multivariate study consisting of a principal component analysis of data. Although it had lower initial values, redness (a^*) was more influenced by high pressure than yellowness (b^*). The pattern followed by colour changes was found to be definitively dependent on the variety. High-pressure processing affects in a stronger way the absorbances located near 400 nm (i.e. 400.5 nm in Fuji and 434.0 nm in Braeburn).

Keywords Apple juice, colour, high pressure, multivariate analysis, processing effects.

Introduction

Nowadays, fresh-like quality attributes of food, such as flavour, texture, colour, vitamin content, aroma and overall appearance are at the forefront of consumers' demand. These attributes, as well as product shelf-life, are closely related to microbial proliferation and enzymatic reactions (Campus, 2010; Landl *et al*., 2010). Both micro-organisms and enzymes are being inactivated by means of thermal processes, which negatively affect these fresh-like properties. In this context, novel technologies such as high-pressure processing are being studied to obtain a better final product sensory quality, but without neglecting microbial safety (Falguera *et al*., 2011a). In this way, these processing techniques can be especially useful in keeping fresh-like features of fruit derivatives.

Colour plays an important role in the appearance and acceptability of foods, especially fruit juices (Hartyáni *et al*., 2011). High-pressure processing (HPP) has been widely reported to keep colour properties closer to the fresh-made juice than thermal processes, as well as other organoleptic and nutritional features (Cheftel, 1995; Oey *et al*., 2008; Bermúdez-Aguirre & Barbosa-Cánovas, 2011). However, its effects on these properties depend on each fruit and variety, so an individual study is required in each case to achieve quantitative kinetic data that may be useful in building predictive models (Buckow *et al*., 2009). From an industrial point of view, the applicability of a new technology will be subjected to assess its effects on each one of the varieties that each company uses for its final product, which always comes from a very specific variety combination that gives it an organoleptic and nutritional profile. Furthermore, not only the colour changes caused by the treatment itself are important for the industry, but also the evolution of the juice colour after the processing.

Thus, this study aims at the characterisation of the effect of high-pressure processing at different temperatures on colour parameters and absorbance spectra in juices from six different apple varieties. Then, the colour changes in the first 24 h after HPP have also been assessed for each variety–pressure–temperature–processing time combination. The dependence of these parameters on apple variety is discussed from two different approaches: a kinetic study and multivariate analysis.

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Material and methods

Apple juice preparation

Apples from six different varieties (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith and Red Delicious)

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were purchased from a local supermarket of Pullman (WA). The apples were washed, cut into four pieces and kept in water while waiting to be squeezed with a household juicer. The resulting juice was centrifuged in a Sorvall RC6 + centrifuge (Thermo Fisher Scientific Inc., Waltham, MA, USA) for 15 min at 7200 ***g***. This process was carried out at 4 °C to make separation of supernatant easier and to reduce enzymatic activities. After centrifugation was complete, the supernatant was recovered and the pellet was discarded. Fractions of 30 mL of juice were placed in 0.1016-mm-thickness polyethylene bags (Consolidated Plastics Company, Inc., Twinsburg, OH, USA), thermo-sealed and stored in a freezer at −20 °C.

High-pressure processing

Polyethylene bags containing apple juice from the six different varieties were placed into a cylindrical liner made of white polypropylene (internal diameter 75 mm, external diameter 100 mm, height 21.5 mm; McMaster-Carr, Atlanta, GA, USA). The liner with samples was placed inside the cylindrical chamber vessel (internal height 25 cm, diameter 10 cm) of the high-pressure equipment (Engineered Pressure Systems, Inc., Andover, CA, USA). The unit was operated with an electrohydraulic intensifier pump (AP 10-0670-1116; Hochdruck-Systeme GmbH, Sigless, Austria) that pressurised the vessel to operating pressure in a few seconds (average 7 s). A 5% Mobil Hydrasol 78 water solution was used as pressure medium. To carry out the treatment, the equipment was preheated to target temperature (25, 60 and 80 °C). Pressure medium temperature was measured by means of three thermocouples placed inside the vessel. Processing pressures were 400, 500 and 600 MPa. Apple juice samples were processed for different times: come-up time (the time necessary to achieve the target pressure followed by immediate pressure release, average 7 s), 4, 8, 12 and 16 min.

CIELab colour parameters and absorption spectra measurement

Juices colour was monitored using a Minolta CM-2002 tristimulus colorimeter (Konica Minolta Sensing Inc., Sakai, Japan) in the CIELab colour space, with the D65 illuminant and the 10º observer. Lightness (L^*), greenness–redness (a^*) and blueness–yellowness (b^*) were determined. Absorption spectrum between 400 and 700 nm was measured in intervals of 0.5 nm with a Shimazdu UV-2550 spectrophotometer and recorded by means of its UV PROBE v.2.31 software (Shimazdu Scientific Instruments, Columbia, MD, USA), using a 1-cm-width quartz cell. This absorption spectrum was measured three times in each sample: on the fresh-made juice, immediately after high-pressure processing and after 24 h at room temperature (25 ± 1 °C).

Other physical and chemical analyses

Several physical and chemical variables were analysed to make the multivariate analysis more complete, taking into consideration other factors that describe the variability among the different apple cultivars. The results of these experiments were included into the matrix from which the principal component analysis (PCA) (described later) was accomplished.

pH and conductivity of the juices were measured with a Crison micropH 2000 pHmeter (Crison Instruments, S.A., Alella, Spain). Soluble solids content was assessed using an Atago RX-1000 Digital Refractometer (Atago Co. Ltd., Tokyo, Japan). Total phenolic content was determined as described by Shaghaghi *et al*. (2008), with a spectrophotometric method using the Folin–Ciocalteu reagent. Formol index was measured according to the official method of the International Federation of Fruit Juice Producers (IFFJP, 2001; method 30), by potentiometric titration up to a pH of 8.1. Total and reducing sugars were determined by the reduction of the Fehling reagent, according to the method of the AOAC (2000); (method 974.06). Titratable acidity was also assessed by the official method of the IFFJP (2001); (method 3), with a potentiometric titration with sodium hydroxide up to a pH value of 8.1. Results were expressed as malic acid mg L^{-1}. Vitamin C was quantified by the dichloroindophenol method (AOAC, 2000; method 967.21), expressing the results as ascorbic acid mg 100 mL^{-1}. The ratio of total soluble solids/titratable acidity has been calculated (Ladaniya, 2008).

Viscosity was assessed at 20 °C using a Haake RS-80 RheoStress Rheometer (Gebrüder Haake GmbH, Karlsruhe, Germany) with a Z40-DIN coaxial-cylinder sensor system (radii ratio 1.0847). For temperature control, a Thermo Haake C25P bath (Gebrüder Haake GmbH) was used, using a glycol–water solution (50% w/w) as coolant fluid, which allows an interval variation of 0.2 °C. The samples were sheared at a constant shear rate of 400 s^{-1} for 3 min, after which a downward ramp to 0 s^{-1} and another upward ramp until 400 s^{-1} were accomplished. The average shear stress values of these two ramps were used to calculate the juice viscosity by means of Newton's equation, because all the samples showed Newtonian behaviour (Ibarz *et al*., 2009).

Kinetic models and statistical analysis

To carry out the kinetic studies, colour difference (ΔE) was calculated as:

$$\Delta E = \sqrt{\left(L^* - L_0^*\right)^2 + \left(a^* - a_0^*\right)^2 + \left(b^* - b_0^*\right)^2} \qquad (1)$$

where L_0^*, a_0^* and b_0^* are defined as the reference colour parameters measured after the come-up time treatment (the time necessary to achieve the target pressure followed

by immediate pressure release) for each apple variety and processing conditions combination. During a reaction at constant pressure and temperature, changes in the colour difference (ΔE) with time (t) can be described by a differential balance (Ramirez *et al.*, 2009; eqn 1):

$$\frac{d(\Delta E)}{dt} = k \cdot (\Delta E)^n \quad (2)$$

where k is the rate constant for the reaction at a given pressure and temperature and n is the reaction order. Integration of this balance leads to expressions for zero ($n = 0$, eqn 3), first ($n = 1$, eqn 4)- and second ($n = 2$, eqn 5)-order kinetics:

$$\Delta E = (\Delta E)_0 + k_0 \cdot t \quad (3)$$

$$\Delta E = (\Delta E)_0 \cdot \exp(k_1 \cdot t) \quad (4)$$

$$\frac{1}{\Delta E} = \frac{1}{(\Delta E)_0} - k_2 \cdot t \quad (5)$$

The best kinetic expression describing the changes as a function of time was then used to determine k_i values as a function of temperature and pressure for each apple variety. The root mean square error (RMSE) index was used to decide which model provided the best fit for the experimental data. This index was defined as:

$$RMSE = \sqrt{\frac{1}{n}\sum \left[(\Delta E)_{\text{exp}} - (\Delta E)_{\text{pred}}\right]^2} \quad (6)$$

where n is the number of samples of each variety (i.e. considering all the experiments at different pressures and temperatures), $(\Delta E)_{\text{exp}}$ is the experimental colour difference and $(\Delta E)_{\text{pred}}$ is the predicted colour difference corresponding to each sample and fitting model.

Experimental data were fitted to the kinetic expressions by nonlinear regression procedures using STATGRAPHICS PLUS 5.1 statistical data processing software (STSC Inc., Rockville, MD, USA). The fittings and the estimates were calculated at a 95% significance level.

Multivariate analysis

Multivariate projection techniques (Martens & Naes, 1989) have been used in this study to determine the most important variables for data characterisation and to see whether there were any group patterns. These techniques have been widely applied for quality assessment, authentication or even classification of a great variety of food products, including vegetables, fruits, juices, wines, oils and others (Tzouros & Arvanitoyannis, 2001).

In this case, the data matrix consisted of 276 samples (six apple varieties × three pressures × three temperatures × five treatment times, plus six unprocessed samples) and 1228 variables. These variables included the ones defining the experiment (apple variety, pressure, temperature and processing time), the ones described in the previous sections (colour parameters and physicochemical measures) and two additional groups of variables from the absorbance of the samples in the visible region, at each wavelength from 400 to 700 nm in intervals of 0.5 nm (400.0 nm, 400.5 nm, 401.0 nm, and so on). The first group (601 variables) corresponded to the differences between the absorbance of the fresh juice and the absorbance measured after high-pressure processing, at each wavelength. The second group (601 variables) belonged to the differences between the absorbance immediately after HPP and 24 h later, so as to assess the evolution of juices' colour. Additional (PCA) were carried out with these spectrophotometrical data to infer the most important wavelengths to follow these colour changes. Such colour changes are expected to occur because of the resistance of apple polyphenol oxidase (PPO) to high-pressure processing (Buckow *et al.*, 2009). In a previous study (Falguera *et al.*, 2011b), this spectra/PCA method was used to indirectly characterise the effects of PPO activity in juices from other fruits.

All the values included in the matrix were the average of six determinations, carried out on two different sample batches. Principal component analyses of the data obtained in the samples characterisation were carried out using the UNSCRAMBLER v.10.1 software (Camo Software AS, 2010; Trondheim, Norway).

Results and discussion

Initial parameters

Table 1 shows the CIELab parameters of the freshly squeezed apple juices from the six studied varieties. Granny Smith juice was the one with the highest initial brightness (L^*, 62.69) and the lowest a^* and b^* values (2.88 and 20.85, respectively). Meanwhile, Fuji juice had the lowest brightness (30.25) and the highest initial redness (a^*, 18.18), and the highest yellowness (b*) was found in Red Delicious juice (40.91). All the juices had a higher yellowness (b^*) than redness (a^*); in Granny Smith juice, b^* was more than seven times greater than a^*, while in

Table 1 CIELab parameters and maturity index of the six unprocessed apple juices

Variety	L^* (–)	a^* (–)	b^* (–)
Braeburn	48.37 ± 0.46	23.0 0.1	37.77 ± 0.04
Fuji	30.25 ± 0.23	58.3 ± 0.1	22.36 ± 0.25
Gala	47.11 ± 0.31	23.3 ± 0.1	37.07 ± 0.38
Golden Delicious	51.97 ± 0.19	17.4 ± 0.3	35.45 ± 0.10
Granny Smith	62.69 ± 0.06	13.1 ± 0.3	20.85 ± 0.04
Red Delicious	43.92 ± 0.54	32.6 ± 0.1	40.91 ± 0.31

Mean value ± Standard deviation.

Fuji, the b^*/a^* ratio was only 1.2. These initial parameters show the big colour differences that exist between the freshly squeezed juices form the different varieties.

Another type of measurement that may be useful to describe these differences is the absorption spectrum in the visible region (between 400 and 700 nm, Fig. S1). It can be clearly observed that Fuji and Granny Smith juices have the highest and the lowest absorbance in the entire visible region, respectively. The other four varieties (Braeburn, Gala, Golden Delicious and Red Delicious) offered closer spectra.

Colour difference (ΔE) caused by instant exposure to target conditions

Colour of fruit juices is extremely sensitive to processing. In this way, an instant exposure to high pressures and/or mild temperatures is enough to change these parameters. Table 2 shows colour difference (ΔE, eqn 1, using unprocessed samples of each variety as reference) caused by this instant exposure to processing conditions followed by immediate pressure release and cooling. First of all, it must be stated that in all cases, exposure for 1 s to processing conditions caused a decrease in L^* value and an increase in a^* and b^*, that is, the juice became darker and more coloured. The greater values for ΔE were found in Fuji juice, while Braeburn juice was the most stable one. In general, the produced colour change was greater as both temperature and pressure increased. Thus, colour of Fuji juice exposed to 400 MPa and 25 °C for 1 s changed 3.46 units, while at 600 MPa and 80 °C, a change of 19.33 units was found. Colour differences for Braeburn juice under the same conditions were 0.92 and 3.96, respectively.

CIELab parameters and absorbance spectra

As it has been described, apple juice samples were treated for different times: come-up time, 4, 8, 12 and 16 min. Experimental results showed that after the initial colour variations caused by the come-up time (described in the previous section), CIELab parameters behaved in the opposite way: as processing time increased, the juices became brighter (L^* increased) and less coloured (a^* and b^* decreased), even at 60 and 80°C. These results are consistent with those found by other researchers when processing fruit juices with high hydrostatic pressure (Sánchez-Moreno *et al.*, 2005; Hartyáni *et al.*, 2011), attributing colour changes to natural pigments impairment. On the contrary, Landl *et al.* (2010) found that a^* and b^* remained constant (although L^* increased) after high-pressure processing of apple purée at 400 and 600 MPa.

In addition to CIELab parameters, the changes in colour have also been assessed by means of the absorption spectrum in the visible region (400–700 nm). Figure 1 corresponds to the evolution of Gala juice spectrum after processing at 600 MPa and 80 °C. It can be seen that as processing time increases, the absorbance decreases in the whole region. Moreover, in the case of Gala juice, there is a greater shift between the samples corresponding to the come-up time and 4 min than between the other consecutive processing times, which was observed in almost all pressure–temperature combinations. However, this greater shift was found between different processing times depending on the variety (for example, in the case of Fuji juice, the greatest shift was found between the samples processed for 4 and 8 min, but was also repeated for almost all pressure–temperature combinations). This greater shift was observed easier as processing temperature was higher.

As a measure of the pigments formed by nonenzymatic browning during high-pressure processing, Table S1 shows the increase in absorbance at 420 nm after 16 min of treatment (these values have been taken from the absorbance spectra). In most of the samples (except in Granny Smith), this value increased, especially in those processed at 80 °C. This fact shows that dark

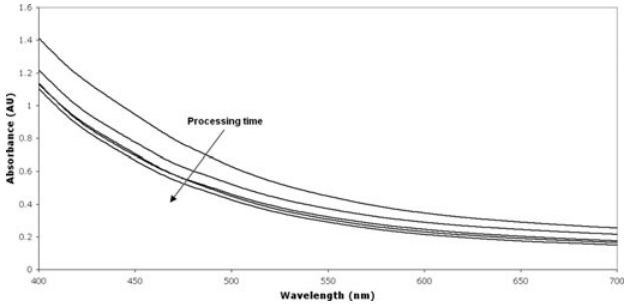

Figure 1 Absorption spectrum evolution of Gala juice processed at 600 MPa and 80 °C with processing time (come-up time, 4, 8, 12 and 16 min).

Table 2 Difference of colour (ΔE) relative to the freshly squeezed apple juice after come-up time (heating and pressure increase to target conditions followed by immediate pressure release and cooling)

		Colour difference (ΔE)		
	P (MPa)	25 °C	60 °C	80 °C
Braeburn	400	0.92	1.79	1.85
	500	2.04	1.81	1.89
	600	4.34	1.93	3.96
Fuji	400	3.46	3.60	13.91
	500	5.40	9.03	14.89
	600	7.70	15.27	19.33
Gala	400	2.22	6.01	6.57
	500	3.66	6.80	7.28
	600	5.14	7.67	7.69
Golden Delicious	400	1.59	2.36	1.76
	500	2.47	4.47	4.50
	600	3.74	5.03	7.30
Granny Smith	400	1.16	1.58	3.00
	500	1.25	3.07	4.71
	600	3.11	6.27	6.91
Red Delicious	400	3.84	3.31	3.64
	500	3.85	3.46	6.28
	600	4.05	5.88	7.09

(Shown results are the average of six determinations).

polymers appeared in apple juices. These compounds may appear as a result of Maillard reaction between sugars and amino acids (melanoidins) or as a product of PPO activity (melanins). However, CIELab parameters (previously discussed) showed that brightness (L^*) increased, while a^* and b^* decreased. Therefore, the formation of brown pigments leads to the conclusion that the evolution of CIELab parameters must be attributed to natural pigments destruction.

Colour of apple juices may also be affected after the treatment by the residual PPO activity. Table S2 shows the increase in absorbance at 420 nm produced in the samples that had undergone high-pressure processing for 16 min during the first 24 h after the treatment. Comparing the values of ΔA_{420} for each variety, browning was less severe (and therefore PPO was inactivated in a greater degree) as both temperature and pressure increased. In the case of Braeburn and Granny Smith processed at 600 MPa and 80 °C, browning because of remaining PPO activity hardly appeared.

Kinetic study of colour difference (ΔE)

To find the kinetic pattern of colour changes for each variety–pressure–temperature combination, colour difference (ΔE) was calculated for each one of the ffity-four series (as described in *Materials and methods* section) and fitted to zero-order, first-order and second-order models (eqns 3, 4 and 5, respectively) by means of nonlinear regression procedures. These procedures allowed obtaining $(\Delta E)_0$ and k_i parameters for each model, from which predicted colour difference values were calculated. Table 3 shows RMSE values obtained when comparing the fourty-five (three pressures × three temperatures × five processing times) experimental vs. predicted values for each variety and kinetic model. Moreover, an additional RMSE value has been calculated for each kinetic model considering the samples from all apple varieties (270).

Zero-order kinetic model was the most appropriated one for describing colour difference evolution in Braeburn and Fuji juices, because the lowest RMSE values for these varieties were obtained with this expression (0.771 and 0.543, respectively). In the same way, the first-order model was the best one for Gala (1.148) and Granny Smith (0.805) juices, while the second-order equation was more appropriated for Golden Delicious (0.743) and Red Delicious (0.914) juices. These results show that colour changes kinetic pattern of high-pressure processed apple juice depends on the variety. If all the varieties are considered together, the RMSE value showed that the zero-order model (1.057) was the best one.

Table S3 shows the kinetic parameters ($(\Delta E)_0$ and k_i) obtained with the best model for each variety. As a general rule, kinetic constants (k_i) become higher as both pressure and temperature grow. As an example, in the case of Braeburn, k_0 at 25 °C is 0.156 min^{-1} for 400 MPa and 0.630 min^{-1} for 600 MPa, while at 80 °C, k_0 is 0.573 min^{-1} for 400 MPa and 0.676 min^{-1} for 600 MPa. Observing directly the kinetic constants, it is not possible to determine whether the pressure or the temperature has the greatest effect on colour variation, because the growth of these parameters with both variables follows different trends depending on the variety.

Multivariate study

A first (PCA) was carried out on the nonspectral data (twenty-six variables) to identify any possible sample

Table 3 RMSE values for the three kinetic models applied on colour difference (ΔE) data from the six different apple varieties

Variety	Zero order	First order	Second order
Braeburn	0.771	1.087	1.976
Fuji	0.543	1.001	1.174
Gala	1.711	1.148	1.581
Golden Delicious	0.948	1.288	0.743
Granny Smith	0.577	0.805	0.808
Red Delicious	1.033	1.344	0.914
All samples	1.057	1.143	1.328

groups and variable correlation trends. Observing samples scores in the PC1 vs. PC2 space (not shown), which represented 59% of the overall variance, six different groups of samples could be clearly identified, being distributed mainly along the maximum variance direction (PC1). All the samples of each group had in common the apple variety from which the juice was made. Therefore, the variety seems to be the variable that has the strongest contribution to the overall variance (i.e. the most important variable in the study). Observing this separation between groups, a particular PCA model for each one of these sample groups seems the most appropriate next step for the study of the relationship among the variables. Regarding loadings plot (Fig. S2), the ratio soluble solids/acidity was positively correlated with a^* and negatively correlated with L^*. b^* was not found to be correlated with the other CIELab parameters.

If additional PCA models are carried out for the samples of each variety (graphs not shown), the variance explained by the two-first PCs in each one of them increases (78% on average). In these new plots, samples scores codified according to processing time show a pattern distributed along the first PC, although no separate groups appear. Thus, this maximum variance direction can be identified with the processing time. In the same way, if the samples are codified according to processing temperature, they appear distributed vertically along the second PC, and therefore, this second maximum variance direction can be clearly associated with this variable. Regarding processing pressure, no pattern defining any trend was observed, because the information in this variable appeared randomly distributed.

As far as variable loadings for one single variety are concerned (graphs not shown), colorimetric variables are the ones with the strongest influence on the overall variance, because they have the highest values for PC1. In addition, L^* appears again strongly negatively correlated with a^*. As regards to processing conditions, time seems the most important variable and is more identified with the direction that PC1 represents, while temperature appears as the second most important variable and is clearly identified with PC2, as it was stated observing the codes for sample scores.

As it has been already introduced, the colorimetric study was completed with two additional PCA models on two spectral variable sets. In the first one, 601 variables were calculated as the difference between the absorbance in the visible range of the fresh juice and the absorbance measured after processing for each wavelength. By means of this analysis (Fig. S3), it can be stated that high-pressure processing affects in a stronger way the absorbance of colours located near 400 nm, because the loadings for the first PC (that explains 97% of the overall variance) are the highest ones in this region, with a peak in 401.5 nm. These results are consistent with Fig. S2, where a^* (redness) was the CIELab parameter with the highest loadings for the first PC. Equivalent results were obtained in the PCAs carried out only on the samples from each variety (not shown), obtaining maximum peaks between 400.5 (Fuji) and 434.0 nm (Braeburn).

For the second spectral variable set, 601 variables were calculated as the difference between the absorbance at each wavelength in the visible region immediately after high-pressure processing and after 24 h at room temperature (25 ± 1 °C). As it can be observed in Fig. S4, there is a maximum peak in 419.5 nm. If the PCA is carried out individually on the samples from each variety (graphs not shown), this peak is found between 416.5 (Gala) and 426.0 nm (Braeburn). These colour changes, affecting mostly the wavelengths around 420 nm, can be attributed to the enzymatic browning mainly caused by the residual PPO activity after the treatment (Falguera *et al.*, 2011b). Polyphenol oxidase has been reported to be highly resistant to high-pressure processing (Buckow *et al.*, 2009), and therefore, it could remain partially active after the treatment. Because PPO inactivation is a critical point for juice manufacturing, this assessment of colour changes by means of PCA may be a good indicator to prove the effectiveness of the inactivation of this enzyme by means of high-pressure processing.

Conclusions

To sum up, it can be stated that the colour of apple juice has very different properties depending on the variety (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith or Red Delicious) from which it is made. Although all the juices had a greater initial yellowness (b^*) than redness (a^*), the latter was more influenced by high-pressure processing. Compression to target pressure followed by instant decompression caused a decrease in L^* value and an increase in a^* and b^*, that is, the juice became darker. For longer processing times, the three parameters behaved in the opposite way, even at 80 °C. This effect can also be assessed by means of the decrease in absorbance in the entire visible region. Study of the increase in absorbance at 420 nm in the first 24 h after the treatment showed that residual PPO activity caused samples browning, which was only avoided in Braeburn and Granny Smith processed at 600 MPa and 80 °C.

In addition, the variety also determines the way in which colour is affected after the treatment. Colour difference (ΔE) was found to follow a zero-order increase in Braeburn and Fuji juices, while the first-order kinetic model was more appropriated for Gala and Granny Smith juices and the second-order one for Golden Delicious and Red Delicious juices.

Multivariate analysis showed that, in a process at temperatures between 25 and 80 °C, pressures from 400

to 600 MPa and processing times up to 16 min, among the variables that define the experiment, the apple variety is the one with the strongest influence in colour changes, followed by the processing time, the temperature and the pressure. Moreover, wavelengths between 400.5 and 434.0 nm were the most affected by high-pressure processing, while the wavelengths between 416.5 and 426.0 nm were the ones that changed the most in the first 24 h after the treatment because of residual PPO activity. On the other hand, the ratio soluble solids/titratable acidity was found to have a strong positive correlation with a^* and a strong negative correlation with L^*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Absorbance spectra of the unprocessed (raw) apple juices.

Figure S2. Principal Component Analysis of the non-spectral data.

Figure S3. Principal Component Analysis of the spectral data for changes produced in the absorbance spectrum by high pressure processing in all samples.

Figure S4. Principal Component Analysis of the spectral data for changes produced in the absorbance spectrum by enzymatic browning, in all samples kept at 25 °C for 24 h.

Table S1. Change in absorbance at 420 nm caused by high-pressure processing of juices from the different apple varieties for 16 min.

Table S2. Change in absorbance at 420 nm of the samples that had undergone high-pressure processing for 16 min, immediately after the treatment and after 24 h at room temperature.

Table S3. Kinetic parameters obtained fitting the experimental ΔE values to the most appropriated kinetic model for each apple variety.

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Appendix II.
Language Appendix.

Resum.

En els darrers temps, les preocupacions dels consumidors per l'impacte dels aliments que consumeixen sobre la seva pròpia salut, així com les conseqüències socials i ambientals que això comporta, han portat a canvis importants en totes les etapes de la cadena alimentària, incloent-hi tots els agents des del productor fins al venedor. Aquestes modificacions han fet que la idea de *qualitat dels aliments* canviï substancialment, havent de tenir en compte nous factors i afegint nous paràmetres a la seva definició clàssica.

Entre aquestes noves tendències que la indústria alimentària es veu obligada a satisfer, apareixen dos corrents principals. D'una banda, els aspectes relacionats amb la salut: l'obtenció d'aliments que proporcionin beneficis per a la salut i evitar-ne la toxicitat potencial. De l'altra, les demandes referents a la percepció organolèptica dels aliments. I en el cas de fruites, verdures i bolets i també dels seus derivats, la polifenol oxidasa (PPO) afecta a ambdós tipus de qüestions que avui en dia són al capdavant del concepte *qualitat*.

De fet, la polifenol oxidasa, el principal enzim responsable de l'enfosquiment enzimàtic, és encara avui dia el principal problema en el maneig postcollita, l'emmagatzematge i el processament de les fruites. En aquesta Tesi, l'activitat de la PPO s'ha caracteritzat en diferents fruites i en diverses situacions, i s'han desenvolupat i validat models matemàtics per descriure la formació de melanines a partir de substrats monofenòlics i *o*-difenòlics. A més, en la línia de les qüestions relacionades amb la salut abans esmentades, la toxicitat potencial d'aquestes melanines sobre l'activitat de les proteases pancreàtiques carboxipeptidasa A, carboxipeptidasa B i tripsina ha estat estudiada.

La segona part de la Tesi comprèn estudis sobre mètodes de processament no convencionals per a la millora de la qualitat organolèptica i nutricional dels productes derivats de la fruita. Comprèn la inactivació de la PPO mitjançant tecnologies innovadores, així com els efectes secundaris que causen en diferents paràmetres. En aquest sentit, s'ha modelat la irradiació ultraviolada-visible en un fotoreactor pla amb una sola làmpada, i la inactivació de la PPO per aquest mètode s'ha avaluat en solucions model i en sucs de poma, pera i raïm. I, anant un pas més enllà, els efectes de la irradiació del most en la qualitat dels seus vins també han estat avaluats. A més, en un darrer enfocament diferent a les tecnologies innovadores, s'ha avaluat l'eficàcia de l'alta pressió hidrostàtica en la inactivació de la PPO de la poma, com també els seus efectes secundaris sobre el color dels sucs.

Cobrint tots els aspectes esmentats, aquesta Tesi intenta proporcionar una anàlisi exhaustiva d'un tema que actualment resulta clau en el disseny de nous productes i en la validació de nous processos. Diferents eines matemàtiques han estat aplicades, incloent el desenvolupament de models, anàlisis cinètiques, estadística clàssica i tècniques multivariants. Com a resultat, s'han realitzat diferents tipus d'experiments que han donat lloc a resultats molt diversos, tots ells amb un mateix objectiu: avançar en el coneixement de la polifenol oxidasa i les seves propietats.

Resumen.

En los últimos tiempos, las preocupaciones de los consumidores por el impacto de los alimentos que consumen sobre su propia salud, así como las consecuencias sociales y ambientales que ello conlleva, han llevado a cambios importantes en todas las etapas de la cadena alimentaria, incluyendo todos los agentes desde el productor hasta el vendedor. Estas modificaciones han hecho que la idea de *calidad de los alimentos* cambie sustancialmente, debiendo tener en cuenta nuevos factores y añadiendo nuevos parámetros a su definición clásica.

Entre estas nuevas tendencias que la industria alimentaria se ve obligada a satisfacer, aparecen dos corrientes principales. Por un lado, los aspectos relacionados con la salud: la obtención de alimentos que proporcionen beneficios para la salud y evitar su potencial toxicidad. Por otro, las demandas referentes a la percepción organoléptica. Y en el caso de frutas, verduras y setas, y también de sus derivados, la polifenol oxidasa (PPO) afecta a ambos tipos de cuestiones que hoy en día están a primera línea del concepto calidad.

De hecho, la polifenol oxidasa, el principal enzima responsable del pardeamiento enzimático, es aún hoy día el principal problema en el manejo, el almacenamiento y el procesado de las frutas. En esta Tesis, la actividad de la PPO se ha caracterizado en diferentes frutas y en situaciones diversas, y se han desarrollado modelos matemáticos para describir la melanogénesis a partir de sustratos monofenólicos y *o*-difenólicos. Además, la toxicidad potencial de estas melaninas sobre la actividad de las proteasas pancreáticas carboxipeptidasa A, carboxipeptidasa B y tripsina ha sido estudiada.

La segunda parte de la Tesis comprende estudios sobre métodos de procesamiento no convencionales para la mejora de la calidad organoléptica y nutricional de los productos derivados de la fruta. Comprende la inactivación de la PPO mediante tecnologías innovadoras, así como los efectos secundarios que causan en diferentes parámetros. En este sentido, se ha modelado la irradiación ultravioleta-visible en un fotoreactor plano con una sola lámpara, y la inactivación de la PPO por este método se ha evaluado en soluciones modelo y en zumos de manzana, pera y uva. Y, yendo un paso más allá, los efectos de la irradiación del mosto sobre la calidad de sus vinos también han sido evaluados. Además, en un diferente enfoque a las tecnologías innovadoras, se ha evaluado la eficacia de la alta presión hidrostática en la inactivación de la PPO de la manzana, así como sus efectos secundarios sobre el color.

Cubriendo todos los aspectos mencionados, esta Tesis intenta proporcionar un análisis exhaustivo de un tema que actualmente resulta clave en el diseño de nuevos productos y en la validación de nuevos procesos. Diferentes herramientas matemáticas han sido aplicadas, incluyendo el desarrollo de modelos, análisis cinéticos, estadística clásica y técnicas multivariantes. Como resultado, se han realizado diferentes tipos de experimentos que han dado lugar a resultados muy diversos, todos ellos con un mismo objetivo: avanzar en el conocimiento de la polifenol oxidasa y sus propiedades.

Conclusions.

D'acord amb els diferents resultats exposats i discutits al llarg de tota la secció número 4, s'han obtingut les conclusions que es detallen a continuació.

a1. Entre les dotze fruites tropicals estudiades (lulo, mangostà, móra de Castella, mango, guaiaba-poma, tomàquet d'arbre, papaia, guanábana, curuba, badea, granadilla i maracuià), el mangostà i el lulo van mostrar la major activitat de polifenol oxidasa, mentre que el maracuià i la curuba en tenen les més baixes. L'activitat de la PPO a la papaia pot ser molt limitada a causa principalment del seu baix contingut en compostos fenòlics. Contràriament, el mango mostrà el major contingut de compostos fenòlics, però la presència d'inhibidors endògens de la PPO pot limitar-ne l'enfosquiment enzimàtic. La polifenol oxidasa provoca canvis de color en els sucs de fruites que poden ser fàcilment avaluats mesurant la disminució de brillantor per mitjà dels paràmetres CIELab. L'anàlisi de components principals revelà que el contingut en fenols totals i la variació de *L** estan fortament relacionats amb l'activitat de la PPO. Per tant, la PPO és més activa en les fruites amb més contingut de fenols, i aquest procés condueix a una major disminució de la lluminositat CIELab.

a2. La fertilització nitrogenada augmenta directament l'activitat de la polifenol oxidasa i també la consistència de la fruita (disminueix l'índex de Bostwick) en fruits de préssec, a banda de l'efecte que pot tenir el retard del procés de maduració. Per tant, si s'aplica la suplementació de nitrogen, encara que els fruits poden arribar a la maduresa comercial més tard, en aquest moment l'activitat de la PPO serà més gran, amb els conseqüents problemes en el maneig postcollita i processament. No obstant, no es van trobar diferències significatives entre l'aplicació de 60 i 120 kg N/ha.

a3. La melanogènesi a partir de substrats monofenòlics mitjançant polifenol oxidasa d'*Agaricus bisporus* és una reacció autocatalítica que pot ésser descrita pel model cinètic: $A_\lambda = \frac{C_A^0 \cdot \varepsilon_\lambda \cdot l \cdot k_1 \cdot \left[\exp\left[\left(k_1 + k_2 \cdot C_A^0\right) \cdot \left(t - t_0\right)\right] - 1\right]}{k_2 \cdot C_A^0 + k_1 \cdot \exp\left[\left(k_1 + k_2 \cdot C_A^0\right) \cdot \left(t - t_0\right)\right]}$. De la mateixa manera, la melanogènesi a partir de substrats *o*-difenòlics es pot dividir en dues etapes: a la primera, el procés enzimàtic pot ser descrit pel model cinètic: $\frac{A_\lambda}{\varepsilon_\lambda \cdot l \cdot t} = r_{\max} - \frac{K_M}{t} \ln\left(\frac{C_S^0}{C_S^0 - \frac{A_\lambda}{\varepsilon_\lambda \cdot l}}\right)$. La segona etapa (no enzimàtica) pot ser descrita per una expressió cinètica d'ordre zero. Entre les variables estudiades, el factor que té la major influència sobre la melanogènesi és la naturalesa del substrat. Quant a les altres dues variables que defineixen les condicions experimentals, el pH és més important que la concentració de substrat. El temps entre 240 i 306 minuts de reacció són els que proporcionen la major variació en les dades. Amb L-tirosina (monofenol), la PPO té la seva activitat òptima a pH 6,0. Amb 4-metilcatecol (*o*-difenol) el pH òptim és 8,0. En la formació de melanina a partir de L-tirosina, el coeficient d'extinció del producte resultant depèn de la concentració de substrat inicial i del pH. Inicialment, l'absorbància no augmenta a causa de la biosíntesi de substàncies intermèdies incolores, que condueix a un període de latència que creix en augmentar la concentració inicial de substrat. En aquesta reacció, la polifenol oxidasa té una activitat catalítica menor sobre la L-tirosina (activitat cresolasa) que sobre la L-DOPA (activitat catecolasa). D'altra banda, la transició entre les dues etapes definides en la melanogènesi a partir de 4-metilcatecol depèn del pH, donant-se més tard com menor sigui aquest, però no depèn de la concentració de substrat. La constant de Michaelis (K_M) i la velocitat de reacció màxima (r_{max}) augmenten a mesura que s'incrementen tant la concentració inicial de 4-metilcatecol com el pH. En la segona etapa, la constant cinètica K_0 manté un valor molt petit i gairebé constant per a totes les concentracions inicials de substrat a pH 5,0. En els altres casos, aquest paràmetre augmenta clarament amb la concentració de substrat i el pH de la solució.

b1. Les melanines sintetitzades a partir de L-tirosina i polifenol oxidasa d'*Agaricus bisporus* tenen un efecte inhibitori sobre les proteases pancreàtiques carboxipeptidasa A (CPA), carboxipeptidasa B (CPB) i tripsina. En els tres casos, la constant de Michaelis-Menten (K_M) augmenta amb la concentració de melanina en la solució, fet que indica que aquest polímer disminueix l'afinitat aparent entre els enzims i els seus respectius substrats. En contrast, la velocitat de reacció màxima (r_{max}) tendeix a disminuir amb l'augment del contingut de melanina seguint una tendència lineal. Aquests fets demostren que la melanina de L-tirosina inhibeix els tres enzims de forma mixta. Per al sistema CPA-melanina de L-tirosina, K_i epren un valor de 1,01 mg/mL, mentre que αK_i és 2,56 mg/mL i per tant α = 2,52. En el cas de la interacció entre la CPB i la mateixa melanina, K_i = 0,78 mg/mL, αK_i = 6,76 mg/mL i α = 8,64. I, per a la tripsina i la melanina de L-Tyr, el valor trobat de K_i és 0,148 mg/mL, αK_i 0,438 mg/mL i α = 2,95. Pel que fa a la melanina a partir de 4-metilcatecol, no es trobà que aquest polímer inhibeixi la CPA o la CPB.

c1. S'ha desenvolupat el modelat matemàtic dels perfils de radiació absorbida en un sistema format per un fotoreactor pla (amb les dimensions del disponible al Departament de Tecnologia d'Aliments de la Universitat de Lleida) i una sola làmpada. L'aplicació del model linial esfèric ha permès obtenir una equació senzilla una xarxa de valors de radiació absorbida en un conjunt discret de punts en tot el volum del reactor. La integració pas a pas per mètodes numèrics d'aquests valors en les tres dimensions de l'espai ofereix informació interessant sobre la quantitat de radiació que podria emprar-se en les reaccions fotoquímiques. Entre aquestes conclusions, per exemple, els perfils obtinguts han mostrat que, amb les dimensions i geometria considerades, si l'absorbància del medi de reacció és 0.1, apenes el 30% de la radiació emesa s'absorbeix, mentre que amb un valor de μ de 5 la meitat de la radiació emesa s'absorbeix en els primers 2 mm, i gairebé la totalitat és absorbida en el primer cm.

c2. És possible inactivar completament i irreversible la polifenol oxidasa d'*Agaricus bisporus* amb un procés d'irradiació UV-vis amb una làmpada de 400 W de potència nominal que emet en un rang entre 250 i 740 nm. La presència de melanines o melanoidines en el medi de reacció protegeix l'enzim, fent el procés d'inactivació més lent a mesura que augmenta la seva concentració. Si el medi de reacció conté melanines sintetitzades a partir de L-tirosina, quan el seu contingut és inferior a 0,05 mg/mL un model cinètic compost és necessari per explicar la inactivació més ràpida en les primeres etapes. Quan el contingut de pigment és més gran, aquestes molècules també protegeixen la PPO d'aquesta desnaturalització ràpida, fent que un model cinètic simple de primer ordre sigui vàlid per explicar tot el procés. Amb melanina de 4-metilcatecol en el medi de reacció, ambdós models resulten vàlids. El model cinètic de primer ordre és més adequat per descriure i quantificar fàcilment l'efecte protector per la seva simplicitat matemàtica. No obstant, l'ús del model cinètic compost aporta informació addicional sobre la transformació de l'enzim natiu en una forma intermèdia, i a la forma inactiva (desnaturalitzada) després. Amb la mateixa concentració, la melanina de 4-metilcatecol té un major efecte protector que la melanina formada a partir de L-tirosina. Pel que fa a l'efecte protector de les melanoidines sintetitzades a partir de fructosa i àcid glutàmic, els graus d'inactivació obtinguts després de 90 minuts de tractament i l'estudi cinètic porten a la conclusió que les melanoidines amb massa molecular menor de 150 kDa tenen un efecte protector major que les molècules majors de 150 kDa. Si les melanoidines obtingudes no es fraccionen, l'efecte protector que exerceixen és menor, a causa de fenòmens d'agregació afavorits per la presència de polímers de diferent longitud de cadena, fet que condueix a una menor absorbància en la regió ultraviolada de l'espectre. A la mateixa concentració, aquestes melanoidines (sigui quina sigui la fracció considerada) tenen un major efecte protector que les melanines. L'estudi de l'efecte protector de les diferents solucions de melanoidines i els seus espectres d'absorbància mostra que la radiació més eficaç per inactivar la PPO és la corresponent a longituds d'ona entre 260 i 310 nm.

c3. L'eficàcia de la irradiació UV-vis per inactivar la polifenol oxidasa en sucs de poma, pera i raïm depèn de cada fruita i varietat. En els sucs de poma, independentment de la seva activitat inicial, la polifenol oxidasa és inactivada per complet després de 100 minuts de tractament. En els sucs de pera, l'activitat residual després de 120 minuts de tractament és gairebé nul·la (a excepció de la varietat Conference, l'activitat inicial de la qual és la més alta). A més, després de 20 minuts, és possible per reduir l'activitat en més del 50%. En els mostos, la PPO no s'inactiva completament després de 140 minuts d'irradiació UV en qualsevol de les quatre varietats estudiades. En raïms blancs (Victoria i Dauphine), s'aconsegueix una inactivació al voltant del 80%, mentre que en les mostres a partir de raïm de color rosa (Red Globe i Emperor) només es redueix el 50% de l'activitat inicial. No es van observar variacions significatives en el pH, contingut de sòlids solubles, índex de formol i sucres en qualsevol dels sucs de poma, pera o raïm. En els sucs de poma, el contingut de vitamina C en els de Golden, Starking i Fuji es va reduir lleugerament durant l'experiment (4,0-5,7%), mentre que en el suc de King David la pèrdua va ser del 70,0% a causa de la manca de pigmentació d'aquest suc. El mateix fenomen, encara que a una escala diferent, s'observà en els sucs de pera: en els d'Abate Fetel i Ercolini, que tenien les pigmentacions més baixes, el contingut de vitamina C al final del tractament va tenir una disminució del 60,5% i el 50%, respectivament. En les altres varietats (Conference, Passa Crassana, Flor d'hivern i Blanquilla) aquesta disminució es trobà en l'interval del 20% al 30%. No obstant, en els mostos, el contingut de vitamina C disminuí (entre el 12 i el 30%), essent impossible trobar una relació directa entre la pigmentació del suc i la protecció contra la degradació de la vitamina C. Pel que fa al color, com a norma general, la irradiació UV-vis causà un augment de lluminositat (L^*) en els sucs, el que significa que la radiació afecta alguns dels pigments presents en el suc. Aquest deteriorament és especialment notable en els pigments vermells, ja que en totes les mostres (independentment de la fruita o varietat) s'observà una disminució del paràmetre CIELab a^*, juntament amb la desaparició dels pics en els espectres d'absorbància que pertanyen a aquests pigments. Contràriament, el comportament dels pigments grocs (b^*) depèn de cada fruita i varietat.

c4. La irradiació UV-vis dels mosts abans de la fermentació en la vinificació és una tecnologia que pot ajudar a reduir la quantitat de SO_2 que s'afegeix als vins. Un ús residual d'aquest producte és necessari per evitar un embruniment excessiu, ja que la polifenol oxidasa no és completament inactivada (sobretot en els mostos de raïm negre, on les antocianines absorbeixen part de la radiació reduint-ne la dosi disponible per inactivar l'enzim). A més a més, les anàlisis de qualitat química indiquen que la irradiació pot assegurar una major estabilitat del producte final, ja que destrueix els llevats i bacteris presents de forma natural. Com a resultat, els vins de mostos irradiats fermenten més lentament i tenen una acidesa volàtil menor. No es van trobar diferències significatives en el grau alcohòlic, pH i contingut d'àcid tartàric entre els vins de mostos irradiats i els de mostos tractats amb SO_2. Pel que fa als paràmetres colorimètrics, la irradiació de mostos blancs (Xarel·lo i Parellada) provoca un increment del color vermell, mentre que en els negres (Cabernet Franc) hi ha una important reducció del color blau. Aquesta reducció constitueix un problema en l'elaboració de vins negres, però resulta interessant per a la producció de rosats. Congelar i descongelar el most abans del processat causa algunes variacions en els paràmetres de qualitat dels vins de Xarel·lo, però no en els de Parellada.

d1. La polifenol oxidasa de poma de les sis varietats tractades per alta pressió (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith i Red Delicious) és extremadament resistent a la pressió si el tractament es fa a temperatura ambient (aprox. 25 ºC): després de 16 min a 600 MPa, la inactivació obtinguda (a banda de la variació causada per la compressió i descompressió instantània) és d'entre el 7% (Braeburn i Golden Delicious) i el 28,5% (Granny Smith). La varietat de poma no només afecta a l'activitat inicial de la PPO, sinó també al patró i el grau d'inactivació assolit. L'anàlisi multivariant (després d'eliminar els efectes d'escala) mostra que, en un procés a temperatures entre 25 i 80 ºC, pressions de 400 a 600 MPa i temps de processat de fins a 16 minuts, entre les variables que defineixen l'experiment la varietat de poma és el que té la major influència en la inactivació de la PPO assolida, seguida per la temperatura, el temps de processat i la pressió.

d2. El color del suc fresc de poma té propietats molt diferents depenent de la varietat (Braeburn, Fuji, Gala, Golden Delicious, Granny Smith i Red Delicious) de la qual prové. Malgrat tots els sucs tenen un color groc (*b**) inicialment major que vermell (*a**), aquest últim es veu més influenciat pel tractament a alta pressió. La compressió fins a la pressió objectiu seguida per una descompressió instantània causa una disminució en el valor de *L** i un augment de *a** i *b**, és a dir, el suc s'enfosqueix. Per a temps de processat més llargs, els tres paràmetres es comporten de manera oposada, fins i tot a 80 °C. A més, la varietat també determina la manera com el color evoluciona després del tractament. Així, l'anàlisi multivariant mostra que, en un procés a temperatures entre 25 i 80 ºC, pressions de 400 a 600 MPa i temps de tractament de fins a 16 minuts, entre les variables que defineixen l'experiment la varietat de poma és el que té una influència més forta sobre els canvis de color, seguit pel temps de processat, la temperatura i, en darrer lloc, la pressió. D'altra banda, les longituds d'ona entre 400,5 i 434,0 nm són les més afectades pel tractament d'alta pressió, mentre que les longituds d'ona entre 416,5 i 426,0 nm són els que més varien en les primeres 24 hores després del processat a causa de l'activitat residual de la PPO. D'altra banda, s'ha trobat que l'índex de maduresa té una forta correlació positiva amb la vermellor (*a**) i una forta correlació negativa amb la lluminositat (*L**).