Phenol-enriched olive oil with its own phenolic compounds and complemented with phenols from thyme: a functional food development model

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PHENOL-ENRICHED OLIVE OIL WITH ITS OWN PHENOLIC COMPOUNDS AND COMPLEMENTED WITH PHENOLS FROM THYME: A FUNCTIONAL FOOD DEVELOPMENT MODEL

Doctoral Thesis

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“The doctor of the future will no longer treat the human frame with drugs, but rather will cure and prevent disease with nutrition”

Thomas A. Edison

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SUMMARY

Previous human studies have demonstrated an increase of the HDL cholesterol levels and a decrease in the *in vivo* lipid oxidative damage in a dose-dependent manner with the phenolic content of olive oil. Based on these studies, the EFSA has released a health claim concerning the protective effects of the ingestion of phenolic compounds (PC) from olive oil declaring that "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*". The EFSA Panel considers that in order to support this claim, 5 mg of hydroxytyrosol and its derivatives in olive oil should be consumed daily. However, the phenolic content in commercial virgin olive oil (VOO) is influenced by multiple agronomic and technological factors. As a result, phenolic concentration in most VOOs available on the market may be too low to allow the consumption of 5 mg of hydroxytyrosol and its derivatives within the frame of a balanced diet (20 g of VOO/day). In this context, the enrichment of VOO with its own phenolic compounds becomes an interesting strategy to increase and standardize the daily intake of hydroxytyrosol in the real food matrix and without increasing the caloric intake. Concerning VOOs with high phenolic content, however, on one hand they have a bitter and pungent taste, which could promote a refusal among consumers particularly those from non-Mediterranean areas. On the other hand, high phenolic compound-rich foods could have a dual action due to the fact that one type of antioxidants in high doses can also act as pro-oxidants. In this context, the strategy of the enrichment of VOO not only with its own phenolics but also with complementary phenols from aromatic herbs was outlined in this thesis with the hypothesis that it could not only provide improvements in oil stability and consumers' acceptation but also additional health benefits. So, the main aim of the present thesis was to develop a phenol-enriched olive oil using the aromatic herb of thyme as olive oil flavoring and phenol complementary source, and to assess the bioavailability of the phenolic compounds, evaluating possible interactions or synergies between both sources. An interventional human trial was also performed with the developed phenol-enriched olive oils, in which the compliance of the volunteers was assessed through the identification of the phenolic intake biomarkers, as well as the preliminary human efficacy regarding the oxidative damage protection and the promotion of the endogenous antioxidant system.

RESUMEN

Estudios realizados con humanos han demostrado un aumento de los niveles de colesterol HDL y una disminución en el daño oxidativo de los lípidos *in vivo* de forma dosis-dependiente con el contenido fenólico del aceite de oliva. En base a estos estudios, la EFSA ha publicado una declaración de propiedades saludables relativa a los efectos protectores de la ingesta de compuestos fenólicos del aceite de oliva que declara que "*los polifenoles del aceite de oliva contribuyen a la protección de los lípidos de la sangre del estrés oxidativo*". El Panel de la EFSA considera que con el fin de sustentar la declaración, se deben de consumir diariamiente 5 mg de hidroxitirosol y sus derivados. Sin embargo, el contenido de compuestos fenólicos en el aceite de oliva virgen comercial está influenciado por múltiples factores agronómicos y tecnológicos. A consecuencia de esto, la concentración fenólica en la mayoría de aceites de oliva disponibles en el mercado es demasiado baja para permitir el consumo de 5 mg de hidroxitirosol y sus derivados dentro del contexto de una dieta equilibrada (20 g de aceite/día). En este contexto, el enriquecimiento del aceite de oliva con sus propios compuestos fenólicos se convierte en una estrategia interesante para aumentar y estandarizar la ingesta diaria de hidroxitirosol en la matriz del alimento real y sin aumentar el consumo calórico. sin embargo, aceites con un alto contenido fenólico, por un lado tienen un sabor amargo y picante que podría provocar un rechazo entre los consumidores, en particular los de zonas no mediterráneas. Por otro lado, los alimentos ricos en compuestos fenólicos podrían tener una doble acción debido al hecho de que un tipo de antioxidante en altas dosis puede actuar como pro-oxidante. En este contexto, la estrategia de enriquecimiento del aceite de oliva no sólo con sus propios compuestos fenólicos, sino también con fenoles complementarios de las hierbas aromáticas se planteó en esta tesis con la hipótesis de que no sólo podrían proporcionar mejoras en la estabilidad del aceite de oliva y la aceptación de los consumidores, sino también beneficios de salud adicionales. Por lo tanto, el objetivo principal de la presente tesis fue desarrollar un aceite de oliva enriquecido con fenoles usando el tomillo como fuente complementaria de fenoles y aromatizante, con una posterior evaluación de la biodisponibilidad de los compuestos fenólicos mediante métodos *in vitro* e *in vivo*, evaluando posibles interacciones o sinergias entre ambas fuentes fenólicas. Con los aceites enriquecidos desarrollados en el marco de la tesis también se llevó a cabo un ensayo clínico sostenido en humanos, en el que el cumplimiento de los voluntarios se evaluó a través de la identificación de los marcadores fenólicos de cumplimiento, así como un estudio preliminar de su eficacia en relación con la protección contra el daño oxidativo y la promoción del sistema antioxidante endógeno.

RESUM

Estudis realitzats amb humans han demostrat un augment dels nivells de colesterol HDL i una disminució en el dany oxidatiu dels lípids *in vivo* de forma dosi-dependent amb el contingut fenòlic de l'oli d'oliva. En base a aquests estudis, l'EFSA ha publicat una declaració de propietats saludables relativa als efectes protectors de la ingesta de compostos fenòlics de l'oli d'oliva que declara que "els polifenols de l'oli d'oliva contribueixen a la protecció dels lípids de la sang contra l'estrès oxidatiu ". El Panell de l'EFSA considera que per tal de sustentar la declaració, s'han de consumir diàriament 5 mg d'hidroxitirosol i els seus derivats. No obstant, el contingut de compostos fenòlics en l'oli d'oliva verge està influenciat per múltiples factors agronòmics i tecnològics. A conseqüència d'això, la concentració fenòlica en la majoria d'olis d'oliva disponibles al mercat és massa baixa per permetre el consum de 5 mg d'hidroxitirosol i els seus derivats en el context d'una dieta equilibrada (20 g d'oli/dia). En aquest context, l'enriquiment de l'oli d'oliva amb els seus propis compostos fenòlics esdevé una estratègia interessant per augmentar i estandarditzar la ingesta diària d'hidroxitirosol a través de la matriu real de l'aliment i sense augmentar el consum calòric. Tot i així, olis amb un alt contingut fenòlic, d'una banda disposen d'un sabor amarg i picant que podria provocar un rebuig entre els consumidors, en particular els de zones no mediterrànies. D'altra banda, els aliments rics en compostos fenòlics s'ha demostrat que poden tenir una doble acció ja que altes dosis d'un sol tipus d'antioxidant pot actuar com a pro-oxidant. En aquest context, en la present tesi es va plantejar com estratègia l'enriquiment de l'oli d'oliva no només amb els seus propis compostos fenòlics, sinó també amb fenols complementaris d'herbes aromàtiques, amb la hipòtesi de que no només podrien proporcionar millores en l'estabilitat de l'oli d' oliva i l'acceptació dels consumidors, sinó que podria aportar a més a més beneficis de salut addicionals. Per tant, l'objectiu principal de la present tesi va ser desenvolupar un oli d'oliva enriquit amb compostos fenòlics utilitzant la farigola com a font complementària de fenols i aromatitzant, amb una posterior avaluació de la biodisponibilitat dels compostos fenòlics mitjançant mètodes *in vitro* i *in vivo*, estudiant possibles interaccions o sinergies entre ambdues fonts fenòliques. Amb els olis enriquits desenvolupats en el marc de la tesi, també es va dur a terme un assaig clínic sostingut en humans, en el qual el compliment dels voluntaris es va avaluar a través de la identificació dels marcadors fenòlics de compliment, així com un estudi preliminar de la eficàcia dels olis en relació amb la protecció contra el dany oxidatiu i la promoció del sistema antioxidant endogen.

CONTENT

INTRODUCTION

1. Functional Foods: Concept and interest

1.1 Defining the concept of Functional Food

The advent of functional foods and nutraceuticals on the market has blurred the distinction between pharma and nutrition (Eussen et al. 2011). Obviously, the concept of foods promoting health is not new. As early as 400 B.C., Hippocrates already stated, “Let food be thy medicine and medicine be thy food”, and in the countries of the Far East, influenced by Chinese culture, foods such as glutinous rice, wheat, sesame, jujube, gingiber, or leek were included in Chinese medicine books for their traditional use in the prevention of chronic diseases (Kwak & Jukes 2001). Nowadays, the development of functional foods is one of the most intensive areas of food product development worldwide, opening multiple challenges for countries with a vast biodiversity and long-established use of plant extracts.

Despite the ancestral knowledge of the link between diet and health, the appearance of specific terms related to foods promoting a health benefit is more recent. In 1984, the concept of functional food was first promoted in Japan by scientists, who were studying the relationships between nutrition, sensory satisfaction, fortification, and modulation of physiological systems (Hosoya 1998). The Japanese Ministry of Health and Welfare then introduced “Foods for Other Specific Health Use” (FOSHU) in 1991, promoting their use as a strategy to reduce healthcare costs. In the USA, evidence-based health or disease prevention claims have been allowed since 1990, when the Nutrition Labelling

and Education Act was adopted and the claims have to be approved by the Food and Drug Administration (FDA) (Arvanitoyannis, 2005).

In the latter half of the 1990s, the European Commission funded an activity to establish a science-based approach to exploring the concept of functional foods. This Concerted Action, "Functional Food Science in Europe" (FUFOSE), involved a large number of European experts in nutrition and related sciences and produced a consensus report that has become widely used as a basis for discussion and further evolution of thinking on the topic. (Diplock et al. 1999). Thus, a working definition was established whereby:

"A food can be regarded as functional if it is satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being or a reduction of disease risk".

They also established that functional foods must remain foods and demonstrate their effects when consumed in daily quantities that can be normally expected.

In the European Union, harmonization was achieved in 2006 with Regulation (EC) No. 1924/2006 *on nutrition and health claims made on foods*, which limited the use of nutrition and health claims by establishing the need for a strong substantiation of the evidence of the claimed effect.

In practice, there is a wide range of possibilities related with the concept of functional foods. A functional food can be:

(i) an unmodified natural food;

(ii) a food in which a component has been enhanced through special growing conditions, breeding or biotechnological means;

(iii) a food to which a component has been added to provide benefits;

(iv) a food from which a component has been removed by technological or biotechnological means so that the food produces benefits not otherwise

available;

(v) a food in which a component has been replaced by an alternative component with favorable properties;

(vi) a food in which a component has been modified by enzymatic, chemical or technological means to provide a benefit;

(vii) a food in which the bioavailability of a component has been modified;

(viii) or a combination of any of the above.

Regardless of the various definitions, the main purpose of functional food should be clear – to improve human health and well-being.

It is also important to highlight that functional foods are not medicines. Although they are intended to modify physiological functions within the body in a positive way, their mode of action is to restore, reinforce or maintain normal body processes in ways consistent with normal physiology. Some characteristics of functional foods and medicines are listed in **Table 1**.

Table 1. Main differences between functional foods and medicines.

	Functional food	Medicine
Mode of action	Modulation of a physiological process within the normal range	Intervention in a disturbed physiological process, or modulation of a physiological process outside the normal range
Purpose	To restore or enhance normal function in order to optimize health, well-being and performance To reduce risk factors for disease	To treat or prevent disease To enhance performance beyond normal range
Form	Food, consumed as part of the normal diet	Pill, tablet, capsule or syrup taken in controlled dose according to a timetable

The definition of functional food also establishes a clear separation from nutraceuticals, which can be considered as diet supplements that deliver a concentrated form of a presumed bioactive component from a food, presented in a nonfood matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods (Zeisel 1999). Nutraceuticals are sold in presentations similar to drugs: pills, extracts, tablets, etc. (Espín et al. 2007).

Other marketing concepts have arisen like "superfoods," which refer to natural products that have a low glycemic index and provide key nutrients that are lacking in the typical western diets. These include beans, dark green leafy vegetables, citrus, sweet potatoes, berries, tomatoes, fish high in omega-3 acids, whole grains, nuts, fat-free milk, and yogurt. A derivative concept, "superfruits," has emerged to designate natural fruits (including blueberries, blackberries, cranberries, pomegranate and exotic ones, such as açaí or goji) that have a high antioxidant capacity. However, neither of those terms has been recognized by the Food and Drug Administration (FDA) or the European Food Safety Authority (EFSA).

1.2 Lifestyle, diet & Functional Foods

Over recent decades, the socio-economic development has led to profound changes in consumer behavior, involving, in particular, the dynamics of food consumption. The elements that have most influenced and still influence consumer eating habits are the lengthening of life expectancy, the progressive aging of the population, rising economic and social costs of healthcare, the widespread desire for a better quality of life, and media and advertising (Gianetti et al. 2009).

Nowadays, current major problem areas for population health include obesity, cardiovascular health, age-related cognitive decline, metabolic syndrome, insulin resistance, and diabetes. All these concerns are expected to grow because of the change of the age pyramid towards aging societies. People are living longer, and so the incidence of specific diseases or conditions of the elderly, such as cardiovascular diseases, type-2 diabetes, osteoporosis, or neurodegenerative diseases, will increase. Cardiovascular diseases (CVDs) are one of the biggest concerns, representing 30–50% of mortality in developed countries. High blood pressure and serum cholesterol levels have reached pandemic dimensions, and it is predicted that the worldwide incidence of diabetes will exceed 450 million people by 2025 (Green 2003).

It is accepted that many of the major chronic diseases are substantially caused by poor diets. Mozaffarian et al. (2008) reported that the consumption of whole grains and better lifestyle choices could mean a reduction of over 100,000 deaths per year in the UK. Since a major issue for public research is targeting the prevention of diet-related diseases (McCarthy et al. 2011), there is among public health officials a growing interest in the links between food and health (Patel et al. 2008). In this context, functional foods have the potential to improve population health in line with the objectives identified by national public health strategies (Hellyer et al. 2012), promoting prevention and risk reduction of disease, and thus reducing mortality rates and medicinal costs associated with therapeutic treatments.

Green (2003) presented a model according to which major benefits for society will arise in the future from modest, individual improvements in health and well-being generating a cumulative, significantly improved pattern of health and well-being across entire populations. Indeed, population benefits bring improved health for all, while medicines, although irreplaceable and of immense value to individuals, only immediately benefit the current proportion of the population with treatable conditions (**Figure 1**).

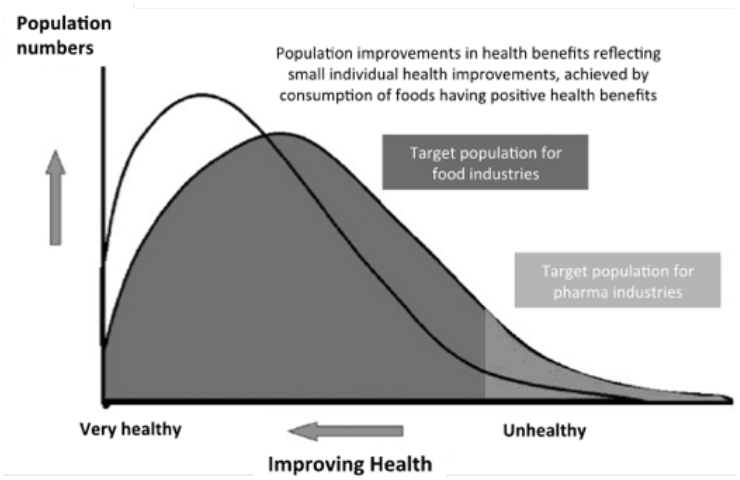


Figure 1. A vision for improving population health through consumption of foods (Green 2003).

1.3 How is food functionality assessed?

The effects of particular functional foods on human health must be based on scientific evidence of the highest possible standard. However, because a direct measurement of the effect a food has on health is not always possible, the key issue is to identify critical biomarkers that can be used to monitor how biological processes are being influenced (Howlett 2008). If appropriate biomarkers are chosen it is possible to monitor the effect of the food intake on the final endpoints by measuring the biomarkers modulation during or at the end of the food interventional period. The markers could be chosen to reflect either some key biological function or a key stage in the development of the disease that is clearly linked to the study endpoint (markers of an intermediate endpoint) (**Figure 2**) (Howlett 2008). In such a way, we can study the long-term effects in the shorter term. However, the selection of nutritional biomarkers, with beneficial

physiological effect, is a challenge. Many biomarkers have been proposed but relatively few have actually been established because of the complexity of disease mechanisms and the limited capability of a single biomarker to reflect the collective impact of multiple biochemical effects on clinical outcome.

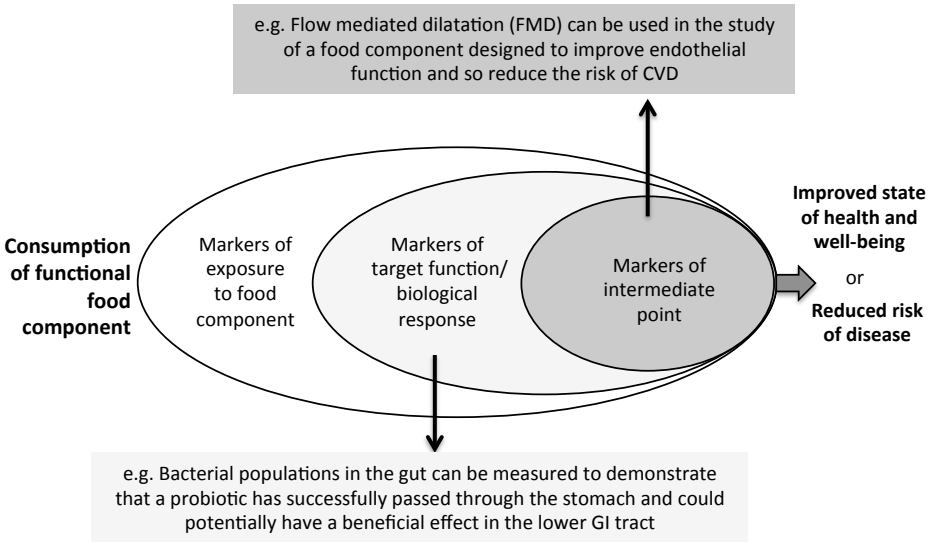


Figure 2. The use of markers to link food consumption to health outcomes (Adapted from Howlett 2008)

In Europe, a consensus on the criteria for the scientific support for claims on foods was reached in 2005 within PASSCLAIM – the Process for the Assessment of Scientific Support for Claims on Foods (Aggett et al. 2005). PASSCLAIM started by examining the available scientific data in seven different areas of physiological function, describing the scientific requirements for the quality of data to support claims that might be made and assessing the availability of markers to provide substantiation of these claims. These areas included diet-related cardiovascular disease, bone health, physical performance and fitness,

body-weight regulation, insulin sensitivity and diabetes risk, diet-related cancer, mental state and performance and gut health and immunity. From a broad-based analysis of the strengths and weaknesses of available methodologies for the assessment of health effects in these physiological areas, the criteria for the requirements for data provided as scientific evidence in support of claims was defined as presented in **Figure 3**.

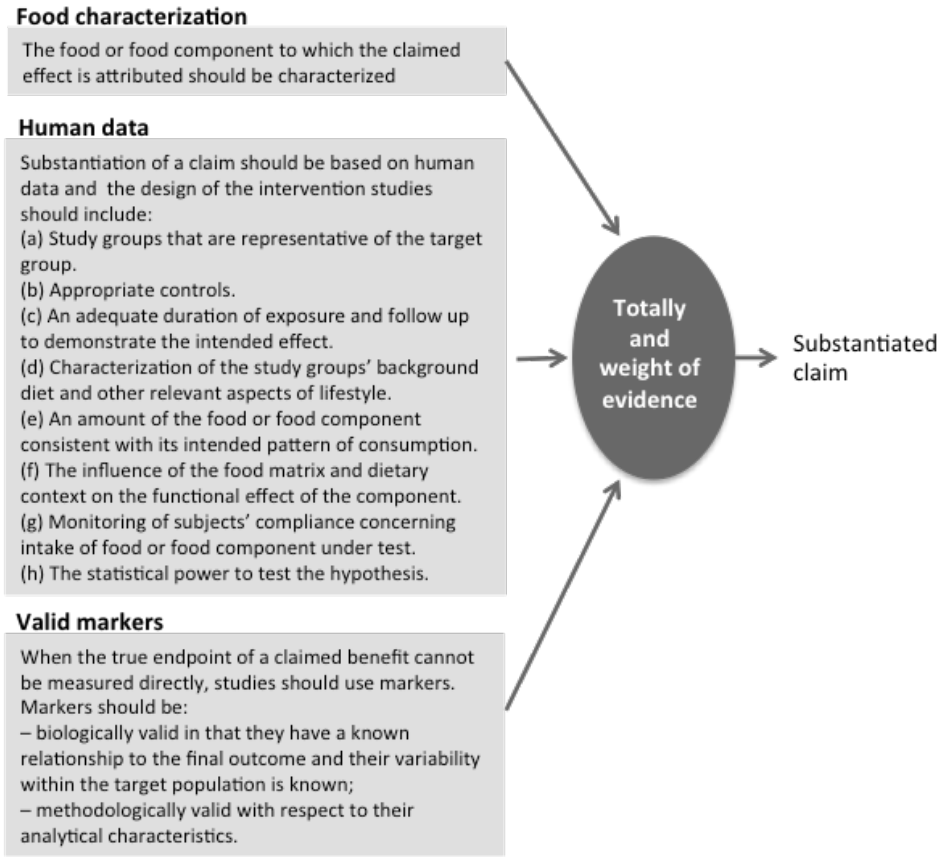


Figure 3. Criteria defined by PASSCLAIM for the scientific substantiation of health claims (Adapted from Aggett et al. 2005).

At this moment, in Europe, functional foods are most critically affected by the Regulation (EC) No 1924/2006 on nutrition and health claims made on foods (EC 2006). This regulation applies to all nutrition and health claims made in commercial communications, including trademarks and other brand names, which could be understood as nutrition or health claims. Regulation (EC) 1924/2006 defines a health claim as any claim that states, suggests or implies that a relationship exists between a food category, a food or one of its constituents and health. Basically, the regulation distinguishes two categories of health claims: (i) **General function claims** describe the role of a food in body functions, including the sense of hunger or satiety and without referring to children, and (ii) **disease risk reduction claims**, which state that the consumption of a food or food constituent significantly reduces a risk factor in the development of a human disease.

Before the legal authorization of the health claims, the Panel on Dietetic Products, Nutrition and Allergies (NDA) of the EFSA is the responsible for evaluating the evidence from all the studies presented, including human, animal and *in vitro* mechanistic studies and is asked to provide a scientific opinion. In synthesis, the main data required by EFSA to support the scientific evidence of a health claim related to foods are:

(i) The food or food ingredient to which the claimed effect is attributed will be sufficiently characterized.

(ii) The biomarker or biomarkers monitored should be biologically valid in that they have a known relationship to the final outcome and methodologically valid with respect to their analytical characteristics.

(iii) A cause and effect relationship needs to be established between the consumption of the food and the modulation of the biomarker selected.

(iv) When the health claim is scientifically supported, it is necessary to define the conditions of use of the claim in the context of a balanced diet.

Based on the scientific opinions delivered by the EFSA over the last decade, in 2012 the European Comission established the list of permitted heath claims for foods, other than those referring to the reduction of disease risk and to children's development and health (UE Regulation 342/2012), in which the specific wording of the claim is defined as along with the quantity of the food and pattern of consumption required to obtain the claimed beneficial effect.

As an example, a Scientific Opinion on the substantiation of health claims related to polyphenols in olive was recently released by the EFSA, in which it was stated that the only claim that had enough evidence to establish a cause and effect relationship with olive polyphenols consumption was the protection of LDL particles from oxidative damage (EFSA 2011). Other claimed effects, such as maintenance of normal blood pressure or anti-inflammatory properties, did not have enough scientific evidence, and therefore they do not appear on the list of permitted claims (UE Regulation 342/2012). **Table 2** shows the specific claim related to olive oil polyphenols, which is included in the list of permitted health claims.

Table 2 Health claim and conditions of use related to olive oil polyphenols (extracted from the EU 342/2012 Regulation).

Nutrient, substance, food or food category	Claim	Conditions of use of the claim
Olive oil polyphenols	*"Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress"*	The claim may be used only for olive oil which contains at least 5 mg of hydroxytyrosol and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of olive oil. In order to bear the claim information shall be given to the consumer that the beneficial effect is obtained with a daily intake of 20 g of olive oil.

2. Olive oil as a source of bioactive phenolic compounds

2.1 Mediterranean Diet and olive oil

The traditional dietary habits of the Mediterranean area have been consistently associated with lower incidence of CVDs and cancer (De Lorgeril 2007; Pauwels 2011) and perhaps other chronic conditions (De Lorgeril, 2008). In observational cohort studies (Sofi et al. 2010; Serra-Majem 2006) and a secondary prevention trial (De Lorgeril et al. 1999) increasing adherence to the Mediterranean diet has been consistently shown to be beneficial with respect to cardiovascular risk, the Mediterranean Diet being ranked as the most suitable dietary model to provide protection against coronary heart disease (Mente et al. 2009). Moreover, it was recently confirmed in the PREDIMED study that the Mediterranean Diet supplemented with extra-virgin olive oil or nuts reduced the incidence of major cardiovascular events among people with high cardiovascular risk (Estruch et al. 2013).

On one hand, the involvement of excessive free radical production in the development and progression of above mentioned diseases indicates that the abundant dietary antioxidants that characterise the Mediterranean Diet likely play a protective role (Seifried et al. 2007). On the other hand, a low content of saturated vs. a high content of monounsaturated fatty acids (mainly coming from olive oil as the main source of fat) in this diet has been associated with a lower risk of certain diseases, which this food element could be involved in (De Lorgeril & Salen 2007).

The Mediterranean Diet is characterised by: (i) the high consumption of vegetables, legumes, fruit and cereals; (ii) regular, but moderate, wine intake; (iii) moderate consumption of fish and white meat; (iv) a moderate intake of dairy products; (v) low consumption of red meat; and (vi) a relatively high fat consumption (up to 40% of total energy intake), mostly from monounsaturated fatty acids (up to 20% of energy) mainly provided by olive oil, the principal source of culinary (specially dressing) fat (Urpi-Sarda et al. 2012). However, olive oil consumption in Mediterranean countries varies. As an example, the three major producers of olive oil (Spain, Greece and Italy) consumed different amounts of olive oil per capita in 2011: 13.4 L, 10.7 L and 21.3 L, respectively (DG Agriculture & Rural Development, 2012).

The benefits of consuming olive oil have been known since antiquity and extensively reported (Quiles et al. 2006). Traditionally, the health effects of olive oil were attributed to its high content in oleic acid. Nowadays, the most recent scientific knowledge has demonstrated that these effects must also be attributed to the phenolic fraction of olive oil (Granados-Principal et al. 2010). This fraction has been shown to support antioxidant, anti-inflammatory and anti-microbial activities. The mechanisms by which olive oil phenolic compounds (OOPCs) can support these activities are varied and most probably, interconnected. On one hand, due to their antioxidant capacity, such as cell redox state modulating enzyme systems, OOPCs act as the first line of defence against free radicals in cellular compartments, and also extracellularly (Karihtala & Soini 2007). Furthermore, OOPCs are able to modulate gene expression, influencing gene and protein expression and, subsequently, metabolite production (Martín-Peláez et al. 2013). Studies supporting these statements are reviewed in detail in Section 3.3.

2.2 Olive oil phenolic fraction

The chemical composition of virgin olive oil (VOO) consists of major and minor components. The major components, which include glycerols, represent more than 98% of the total oil weight and the non-glycerol or unsaponifiable fraction consists of 0.4–2 % (Servili et al. 2004; Tripoli et al. 2005). Olive oil glycerol content is composed mainly of the mixed triglyceride esters of oleic acid and palmitic acid and other fatty acids (**Table 3)**. Oleic acid, a MUFA (18:1n-9), represents 70–80% of the fatty acids in olive oil (Abia 1999).

Table 3. Chemical composition of olive oil (Escrich et al. 2007).

Saponifiable fraction (98-99%)	Unsaponifiable fraction (about 2%)
Main fatty acids present in triacylglycerols:	Non-glyceride esters (alcoholic and sterol compounds, waxes)
Oleic acid (18:1n-9)	Aliphatic alcohols
Palmitic acid (16:0)	Triterpene alcohols
Linoleic acid (18:2n-6)	Sterols (b-sitosterol, campesterol,…)
Estearic acid (18:0)	Hydrocarbons (squalene, b-carotene, lycopene,…)
Palmitoleic acid (16:1n-9)	Pigments (chlorophylls,…)
Linolenic acid (18:3n-3)	Lipophilic phenolics (tocopherols and tocotrienols)
Miristic acid (14:0)	Volatile compounds
	Hydrophilic phenolics

Minor components, which are present in a very low amounts (about 2% of oil weight), include more than 230 chemical compounds, such as aliphatic and triterpenic alcohols, sterols, hydrocarbons, volatile compounds and antioxidants (Servili et al. 2004). The main antioxidants in VOO are the carotenes and the phenolic compounds that include lipophilic and hydrophilic phenols. While the lipophilic phenols can be found in other vegetable oils, the main hydrophilic phenols of VOO are not present in other oils and fats. Moreover, the hydrophilic

phenols of VOO constitute a group of secondary Olea europaea L. plant metabolites that show peculiar sensory and healthy properties (Servili et al. 2004). At least thirty-six structurally distinct olive oil hydrophilic phenolics that have been identified (Cicerale et al. 2009). They can be grouped as follows according to their chemical structure:

(i) **Phenolic acids.** These include caffeic, vanillic, *p*-coumaric, protocatechuic, sinapic, *p*-hydroxybenzoic and gallic acids; also ferulic and cinnamic acids have been quantified, but in lesser quantities (less than 1 mg analyte/kg VOO) (**Figure 4**). Phenolic acids are secondary aromatic plant metabolites spread over a wide range of plants and have been associated with the colour and sensory qualities of foods.

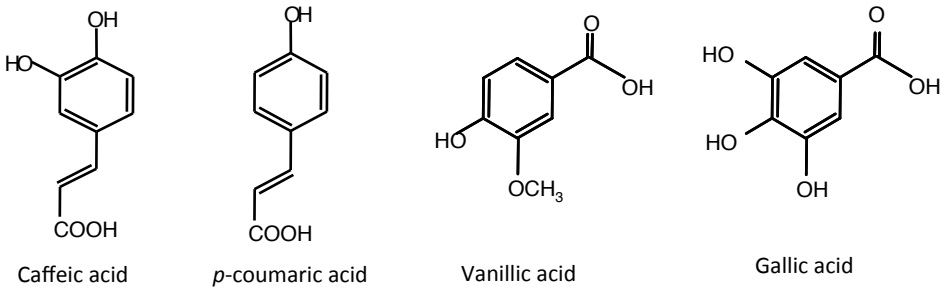
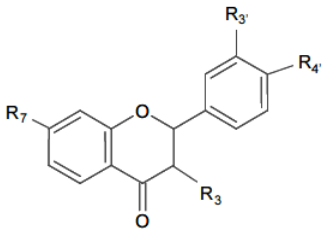


Figure 4. Chemical structure of main phenolic acids in VOO.

(ii) **Flavonoids**. These are compounds containing two benzene rings joined by a linear three carbon chain. Luteolin, apigenin and its glycosides forms are present in VOO (Rovellini et al. 1997).



Compounds	R_7	R_3	$R_{3'}$	$R_{4'}$
Luteolin	OH	H	OH	OH
Apigenin	OH	H	H	OH
Luteolin-7-G	Glu	H	OH	OH
Apigenin-7-G	Glu	H	H	OH

Figure 5. Chemical structure of main flavonoids in VOO.

(iii) **Lignans.** They were identified for the first time in VOO by Brenes et al. 2000. This group encompasses (+)-pinoresinol and (+)-1-acetoxypinoresinol (**Figure 6**), which are present in olive fruit and are trasferred to VOO during the mechanical extraction process.

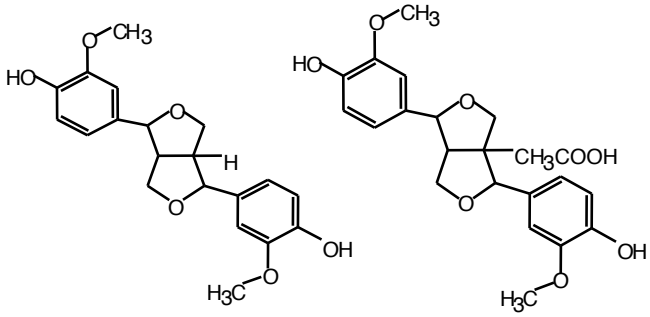


Figure 6. Chemical structure of lignans in VOO.

(iv) **Phenolic alcohols**. These are compounds with a hydroxyl group attached to an aromatic hydrocarbon group. The main phenolic alcohols in VOO are 3,4-dihydroxyphenyl ethanol, also known as 3,4-DHPEA or hydroxytyrosol (HOTYR), and p-hydroxyphenyl ethanol *p*-HPEA or tyrosol (TYR). These alcohols are found at low concentrations in fresh oils, but increase during storage as they derive from the hydrolysis of VOO secoiridoids containing HOTYR and TYR in their molecular structures (Brenes et al. 2001) (**Figure 7**). They are also the most important physiological compounds when olive oil is ingested, as they are released in large amounts after the hydrolysis of secoiridods in the stomach and small intestine.

(v) **Secoiridoids**. They are the main phenolics in VOO and are characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure. The most abundant secoiridoids in VOO are the dialdehydic form of decarboxymethyl elenolic acid linked to HOTYR or TYR termed 3,4-DHPEA-EDA and *p*-HPEA-EDA, oleuropein aglycon (3,4-DHPEA-EA) and ligstroside aglycone (*p*-HPEA-EA) (Montedoro et al. 1992). These compounds are intermediate structures of the biochemical transformation of secoiridoid glucosides of olive fruit oleuropein (OLE) and ligstroside (LGS), originated during the crushing process (**Figure 7**). They are also the main cause of the particular bitter organoleptic attribute and the oxidative stability of the VOO (Gutiérrez-Rosales et al. 2003; Andrewes et al. 2003; Busch et al. 2006).

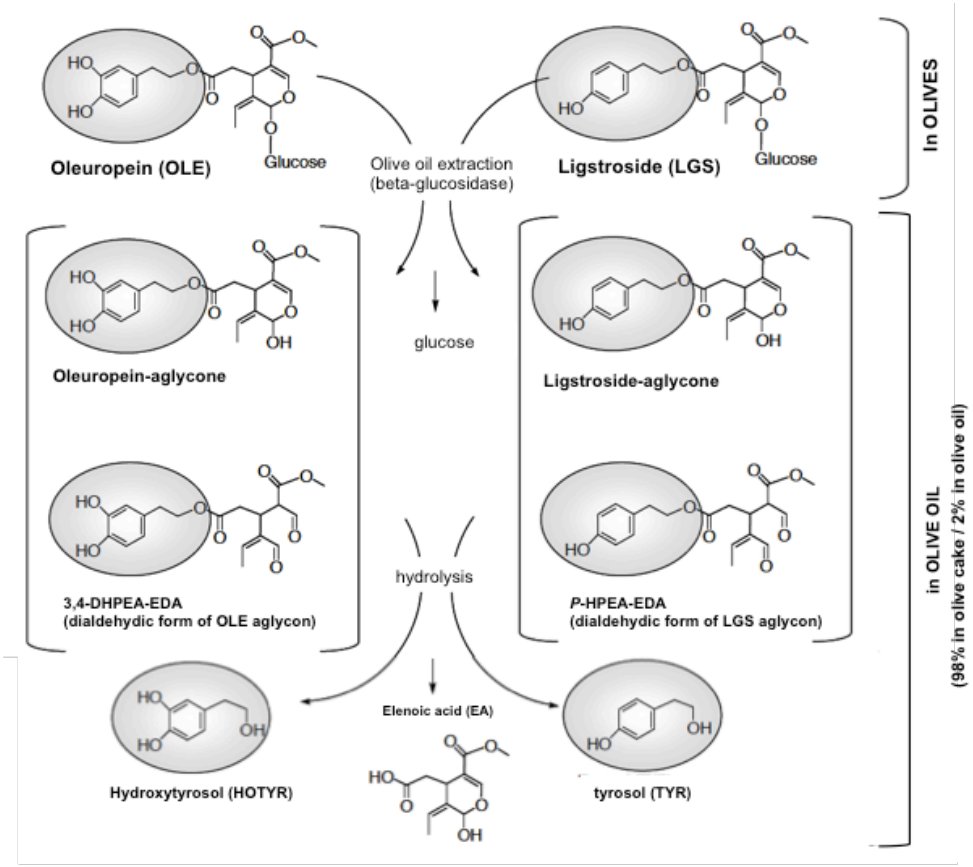


Figure 7. Chemical structures of the secoiridoid derivatives and phenolic alcohols in olives, olive oil and olive cake or alperujo.

The qualitative and quantitative composition of VOO hydrophilic phenols is the result of a very complex multivariate interaction between genotype and agronomic, environmental and technological factors, such as cultivar, fruit ripening, climatic conditions of production, and some agronomic techniques such as the irrigation (Servili et al. 2003; Kalua et al. 2006; Gómez-Rico et al. 2006; Morelló et al. 2006; Artajo et al. 2007; El Riachy et al. 2011a). Crushing and malaxation are the most important critical points of the mechanical oil

extraction process and where the most important changes in the VOO phenolic composition occur (Pasquale & Francesco 1996; Luquede Castro & Japón-Luján 2006). The average values of the common phenolic alcohols, phenolic acids and secoiridoids of VOO are shown in **Table 4**, and as seen, there is a huge variability in the phenolic concentrations among commercial VOOs.

Table 4. Mean values (mg/kg) of the common phenolic alcohols, phenolic acids and secoiridoids of VOO calculated using 116 olive oil samples (El Riachy et al. 2011b).

	Median	Lower quintile	Upper quintile
Vanillic acid	0.2	0	0.3
Caffeic acid	0.4	0.2	0.7
Hydroxytyrosol	1.9	1	3.9
Tyrosol	2.6	1.2	6.4
3,4-DHPEA-EDA	185.7	63.2	839.7
p-HPEA-EDA	22.4	15.4	33.3

There is an increasing interest in the phenolic compounds from VOO as functional ingredients due to their potential health benefits. However, it is difficult to ensure a fixed intake of phenols from VOO, which is subject to a high variability due to these aforementioned agronomic and technological factors. New research lines could be focused on olive cultivars with higher contents of phenolic compounds from classical breeding or cysgenic transformation. The initial steps in the breeding process for higher phenol contents are to study the genetic variability among the existing cultivars and determine the best ripening index for better comparison between genotypes. However, published information related to these topics is still scarce and very controversial (El Riachy et al. 2011b). Therefore, it appears to be interesting to forward research into the standardized development of phenol-enriched olive oils as a strategy for increasing and standardising the phenolic content of olive oils.

2.2.1 *Alperujo: olive oil by-product as a phenolic source*

Despite the great variety of phenolic compounds, it is important to highlight that only around 2% of the phenols from the olive fruit are transferred to the VOO during the extraction process. The other 98% are retained in the olive cake due to its hydrosolubility (EU Technical Report 2000). This byproduct (also called wet pomace or alperujo) is the most important waste generated in the VOO extraction process by the two-phase centrifugation system. The two-phase centrifugation system for oil extraction was developed during the early 1990s. Although this is called the ecological system because it greatly reduces wastewater generation and its contaminant load, it still produces the solid and very humid by-product alperujo. The production of olive oil in Spain with this new centrifugation system, which saves both water and energy compared with the three-phase system, is estimated to represent about 75% of the total and the system is used roughly by 90% of olive-mills. As can be seen in **Figure 8**, using the two-phase system approximately 800 kg of alperujo per ton of processed olives, meaning that the annual production of this by-product from the whole Spanish olive oil industry may approach four million tons (Alburquerque et al. 2004).

Agrochemically, alperujo is characterized by a high moisture content, slightly acidic pH values and a very high content of organic matter, mainly composed of lignin, hemicellulose and cellulose. It has also a considerable proportion of fats, proteins, water-soluble carbohydrates and the hydrosoluble phenolic substances represent a small (1.36% w/w) but active fraction (Morillo et al. 2009).

Alperujo has become a serious environmental problem due to its pollutant nature and high level of production. To avoid this problem, some alternatives have recently been studied, from using it as a fertilizer to its potential for energy recovery (Roig et al. 2006). Besides these applications, the use of olive cake as

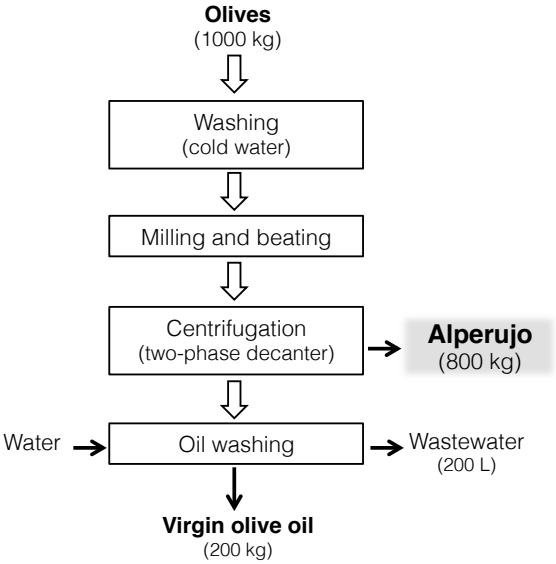


Figure 8. Flowchart of two-phase centrifugation system for olive oil extraction (Adapted from Alburqueque et al. 2004)

a natural source of phenolic compounds has recently been considered, and some studies have focused on the development of new extraction methods (Fernández-Bolaños 2002; Visioli et al. 1999; Suárez et al. 2010). The analysis of these phenolic extracts has demonstrated their high antioxidant activity and suggested their potential use as additives for the food industry (Obied et al. 2007). Thus, some experiments have been carried out to study the incorporation of phenolic extracts in real food matrices. Specifically, related to olive by-product, Japón-Luján & Luque de Castro (2008) studied the enrichment of edible oils with a phenolic extract obtained from olive leaves and concluded that its use considerably enhanced their concentration of phenolic compounds. Bouaziz et al. (2008) obtained similar results after adding olive leaf extract to husk olive oils, demonstrating the great potential of olive by-product extracts as antioxidants for the food industry. More recently, Suarez et al. 2010 evaluated

different combinations of phenolic extracts from olive cake at a range of concentrations to obtain the best prototype of a phenol-enriched olive oil.

The recent information supporting the health properties of VOO oil phenols and the interest in increasing the value of olive oil by-products, make the use of *alperujo* an interesting approach to obtain phenolic extracts and incorporate them into real food matrices. An example of an application is shown in the present thesis.

2.3 Flavoured olive oils: Herbs and spices phenolic fraction

Over recent years, some new products based on olive oil have appeared on the market. Some are olive oils flavoured with herbs or spices. Dry herbs or their extracts are used in oils or lipid-containing foods to retard oxidative deterioration (Gramza-Michalowska et al. 2011; Aladedunye 2014). Spices such as garlic, hot pepper and various aromatic herbs, including rosemary, bay leaf, oregano and thyme, are the most common flavor enhancers in the preparation of flavoured olive oils in the Mediterranean area.

Many essential oils and extracts obtained from these spices and aromatic herbs have recently drawn the interest of the scientific community as they contain a wide variety of active phytochemicals (**Table 5**) (including flavonoids, phenolic terpenes and phenolic acids). Apart from enhancing flavor, these provide a range of bioactive compounds with beneficial physiological effects. Among these are their beneficial influences on lipid metabolism (Naidu et al. 2002; Manjunatha & Srinivasan 2007), efficacy as antidiabetics (Tundis et al. 2010), antimicrobial action (Lai & Roy 2004), digestive stimulant action (Platel & Srinivasan 2004), anticarcinogenic potential (Lampe 2003), antioxidant property, and anti-inflammatory (Srinivasan 2005).

Table 5. Bioactive compounds isolated from the most common herbs and spices in Mediterranean

Herb/spice	Specie and family name	Bioactives compounds
Rosemary	Rosemarinus officialis (Labiatae)	Carnosic acid, carnosol, rosmanol, rosmarinic acid
Sage	Salvia officinalis (Labiatae)	Carnosol, carnosic acid,rosmarinic acid
Oregano	Origanum vulgare (Labiatae)	Phenolic acid derivatives, flavonoids
Thyme	Thymus vulgaris (Labiatae)	Thymol, carvacrol, rosmarinic acid, caffeic acid, flavonoids
Basil	Ocimum basilicum (Labiatae)	Eugenol, α-terpinene, carvacrol
Parsley	Petroselinum crispum (Apiaceae)	Caffeic acid, flavonoids
Marjoram	Majorana hortensis (Labiatae)	Flavonoids
Ginger	Zingiber officianale (Zingiberaceae)	Gingerol-related compounds
Black pepper	Piper nigrum (Piperaceae)	Phenolic amides, flavonoids
Red pepper	Capsicum frutescence (Solanaceae)	Capsaicin
Clove	Eugenia caryophyllata (Caryophyllaceae)	Eugenol, gallates

The active principles in spices and herbs with biological activities are none other than secondary metabolites produced by plants. The most abundant phytochemicals with a phenolic structure isolated from the most typical herbs and spices in the Mediterranean area include (i) phenolic terpenes (thymol, carvacrol, carnosic acid, carnosol and rosmanol), (ii) hidroxycinnamic acids and derivates (caffeic acid, ferulic acid, *p*-coumaric, rosmarinic acid and eugenol) and (iii) flavonoids (quercetin, luteolin, apigenin, eriodyctiol).

(i) **Phenolic terpenes.** Terpenes, also referred to as terpenoids or isoprenoids, are the largest class of natural products with > 55,000 known compounds structurally diversified (Chang et al. 2005). These chemicals are part of the secondary metabolism of plant and animal species and are derived from C_5 isoprene units joined in a head-to-tail fashion from two biosynthetic pathways, through the intermediates mevalonic acid or 1-deoxy-D-xylulose 5-phosphate. Among the compounds from this group, many are extensively used in the industrial sector as flavors, fragrances and spices, and also in perfumery and cosmetic products, as well as food additives. In the pharmaceutical industry, they are also used as active ingredients of drugs. Growing interest in the

clinical application of these compounds is due to the broad range of the biological properties that have been described for terpenoids, including cancer chemopreventive effects, antimicrobial, antifungal, antiviral, antihyperglycemic, analgesic, anti-inflammatory and antiparasitic activities (Paduch et al. 2007).

The monoterpenes (C_{10}) are formed from the coupling of two isoprene units. Thymol and carvacrol **(Figure 9**) are the most representative monoterpenes in herbs, and are mainly present in thyme and oregano. They contribute to their characteristic aromas and are described as potent antioxidants (Nguyen et al. 2000). Several studies have shown a variety of pharmacological properties for these compounds, among which they stand out as analgesics, as has been shown recently (Guimaraes et al. 2013).

Carnosic acid and carnosol (**Figure 9**), the main diterpenes (C_{20}) in aromatic herbs, together with rosmarinic acid, a hydroxycinnamic acid ester, are the main antioxidant compounds in rosemary (Wellwood et al. 2004). Among the herbal extracts reported to have antioxidative activity, rosemary is one of the most widely used and commercialized plant extracts, not only as a culinary

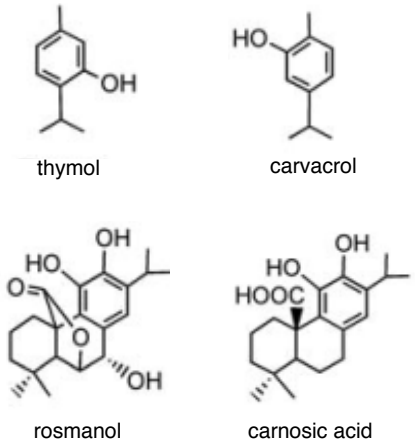


Figure 9. Most representative mono- and diterpenes present in aromatic herbs.

herb for flavoring but also as an antioxidant in processed foods and cosmetics (Zheng & Wang 2001). Carnosic acid is derived from isopentenyldiphosphate (IPP) via methylerythritol phosphate (MEP). It is located in chloroplasts and intracellular membranes, as is carnosol, formed from the oxidative degradation of carnosic acid (Almela et al. 2006).

(ii) **Hydroxycinnamates or phenylpropanoids**. This group of compounds (C_6–C_3) are secondary plant metabolites of phenylalanine and, to a lesser extent, of tyrosine via the action of phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL), respectively. Hydroxycinamic acids are important simple phenols as they are precursors for the synthesis of many other complex phenols. They are found in almost all food groups, although they are abundant in cereals, legumes, oilseeds, fruits, vegetables and beverages, and in large amounts in herbs and spices (Robbins 2003). Recently, the *in vitro* and *in vivo* antioxidant activity of hydroxycinnamates was reviewed and it was concluded that these phenolic compounds may exert a myriad of health benefits thus ameliorating chronic diseases associated with oxidative damage such as cancers, cardiovascular diseases, hypertension and neurodegenerative disorders (Shahidi & Chandrasekara 2010). *p*-coumaric acid has an important presence in oregano whereas eugenol (4-allyl-2 methoxyphenol) is the major component of cloves (Shan et al. 2005), and it is known for its aroma and medicinal values. Rosmarinic acid, a hidroxycinamic acid derivate, is an ester of caffeic acid and 3,4-dihydroxy-phenyllactic acid (**Figure 10**) typically found in Lamiaceae plants, such as basil, rosemary, thyme, mint and oregano (Petersen & Simmonds 2003). The characterization of 26 spice extracts and their phenolic constituents showed that all the spices in the Lamiaceae family tested contained very high concentrations of rosmarinic acid, mostly ranging from 1086 to 2563 mg/100 g of dry-weight, this being the major phenol in the Lamiaceae spices (Shan et al. 2005). Rosmarinic acid has two ortho-dihydroxy groups (catechol structures), which

is the most important structural feature for strong antioxidant activity in phenolic compounds.

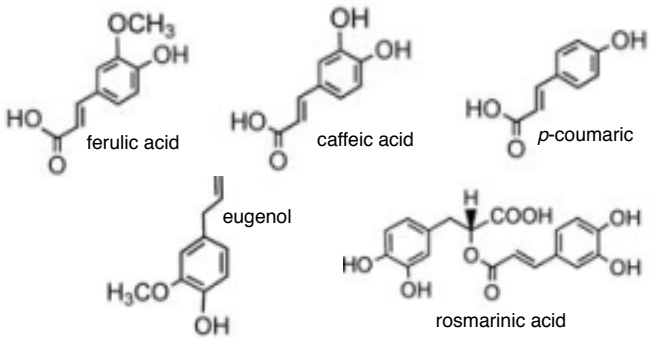
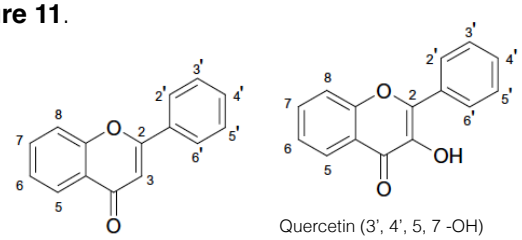


Figure 10. Most representative hydroxycinamic acids and derivates in aromatic herbs.

(iii) **Flavonoids.** Most classes of flavonoids are found in herbs and spices. Frequently these include relatively uncommon aglycones and/or common aglycones with comparatively uncommon substitution patterns. Flavonoid concentration in Lamiaceae herbs is around ~ 0.2–0.4 g/kg (Shan et al. 2005). There is a large body of evidence from epidemiological studies that long-term administration of flavonoids can decrease, or at least, tend to decrease, the incidence of CVDs and their consequences (Aherne et al., 2002; Peterson et al. 2012). The most common flavonoids found in herbs are shown in **Figure 11**.



Apigenin (4', 5, 7 -OH)
Luteolin (3', 4', 5, 7 -OH)

Quercetin (3', 4', 5, 7 -OH)
Myricetin (3', 4', 5', 5, 7 -OH)
Kaempferol (4', 5, 7 -OH)
Isorhamnetin (4', 5, 7 -OH; 3' -OCH_3)

Figure 11. Structures of major flavonoids identified in herbs and spices.

3. Biological effects of phenolic compounds

3.1 Antioxidants or signaling pathway modulators?

Oxidation[1] reactions are crucial for life and the generation of reactive oxygen species (ROS). These by-products of oxidation reactions are essential for maintaining homeostasis of the human organism. However in excess, as for example in situations of stress, exposure to environmental pollution and in aging, ROS can start chain reactions leading to cell damage and death. ROS are free radicals and highly reactive oxidizers that can bind DNA, lipids and proteins to attain stability, thereby turning a physiological condition into a pathological state (Del Rio et al. 2013). Considerable evidence now indicates that the role of ROS is more complex: they appear to play a central role in the key intracellular signal transduction pathways for a variety of pathophysiological cellular responses, such as inflammation, proliferation, migration, differentiation, angiogenesis, aging and apoptosis (Pan et al., 2009; Suvorava & Kojda, 2009).

The biological oxidative effects of ROS within organism/cell are controlled by a wide spectrum of antioxidants that together make up the cell/organism antioxidant defense system and keep the redox system in balance (Cutler 2005). As seen in **Figure 12**, the physiological balance between the ROS and antioxidative defenses is slightly moved towards an increased ROS production (discontinuous line), and prevents the accumulation of damage by ROS and

[1] Oxidation is a chemical reaction that transfers electrons/protons from a substance to an oxidizing agent, where an oxidizing agent (oxidant, oxidizer) is a chemical compound that readily transfers oxygen atoms, or a substance that gains electrons in a redox chemical reaction, and a reducing agent (reductant, reducer, antioxidants) readily donates its electrons/protons to another substance, and is, thus, oxidized itself.

enables enough ROS for signaling. Antioxidative defenses are made up of endogenous antioxidants and exogenous antioxidants.

Endogenous antioxidant compounds in cells can be classified as (i) enzymatic and (ii) non-enzymatic antioxidants. The major antioxidant enzymes directly involved in the neutralization of reactive species are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GS-R). The non-enzymatic antioxidants, usually low molecular weight molecules, are also called metabolic antioxidants. These compounds are produced by metabolism in the body, and include glutathione, L-arginine, coenzyme Q10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, etc.

Exogenous antioxidants or nutrient antioxidants on the other hand, are compounds that cannot be produced in the body and must be provided through foods or supplements. These include tocopherols, retinol, carotenoids and polyphenols among others (**Figure 12**). Tocopherols, retinol and carotenoids play a crucial role in specific organism functions and they need to be maintained at normal levels in the body, whereas phenolic compounds provided with the diet, despite exerting important antioxidant activity in the biological systems, they are treated by the body as xenobiotics. Despite the biological function of nutrient antioxidants, some studies have shown that when large amounts of exogenous antioxidants are taken, they can also act as prooxidants by increasing oxidative stress (Podmore et al. 1998; Palozza 1998). In this case, the physiological balance could be disturbed leading to “antioxidative” stress (**Figure 12B**). Thus, overconsumption of antioxidant supplements could be harmful (Bjelakovic et al. 2004; Miller et al. 2005).

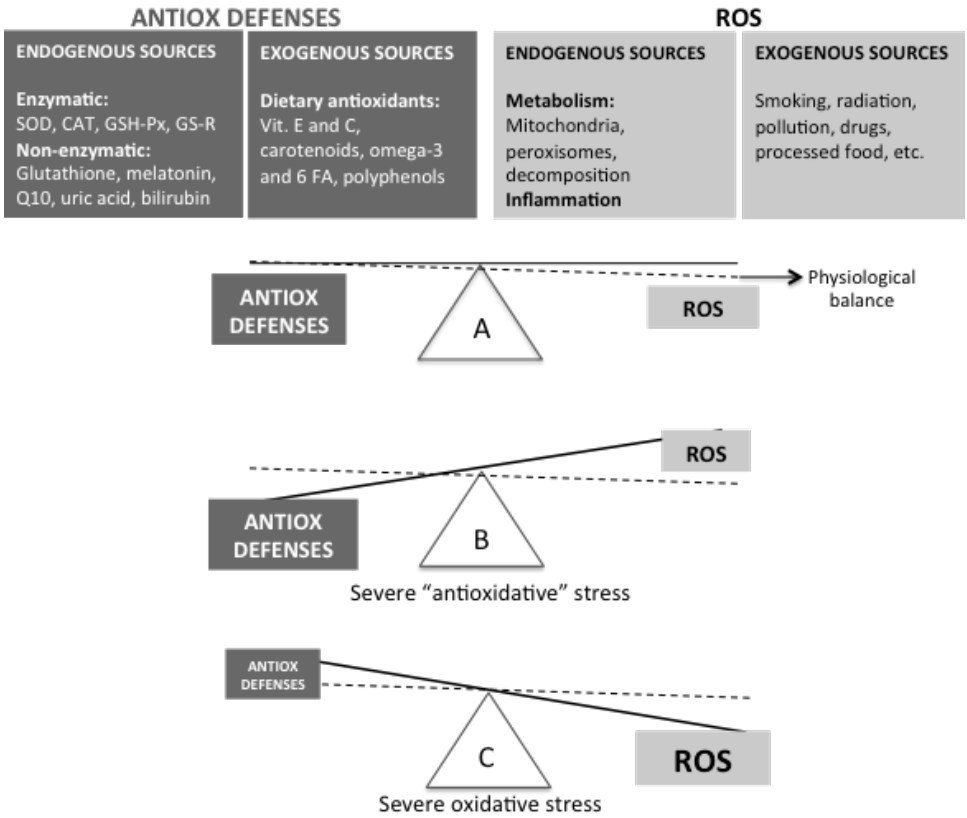


Figure 12. Physiological balance and disturbance between the ROS production and antioxidative defenses (Adapted from Poljsak et al. 2013).

Antioxidant therapies have been gaining recognition as a way to reduce ROS in the vasculature, thereby diminishing their detrimental effects (Hamilton et al. 2004). Inhibitors of angiotensin converting enzyme (ACE) that reduce circulating angiotensin II, have been shown to reduce oxidative stress in addition to their antihypertensive properties. Vitamins E and C have been used extensively as dietary aids in conjunction with other drugs to reduce oxidative stress (Hamilton et al. 2004). On the other hand, polyphenols are beginning to be recognised and accepted as potential therapeutic agents that could be beneficial in combating oxidative stress and thereby protecting individuals from cardiovascular diseases (Basu et al. 2010; Benetou et al. 2008). Historically, the

beneficial effects of polyphenols have been attributed primarily to their antioxidant capacity and their ability to modulate cellular antioxidant defense mechanisms by inducing the synthesis of detoxification enzymes like SOD, CAT, GPx and NAD(P)H quinone oxidoreductase1 (NQO1), amongst others (Rodrigo et al. 2011; Cao & Li 2004; Ungvari et al. 2010). However, recent research provides evidence of polyphenols as modulators of signaling pathways (Stangl et al. 2007; Vauzour et al. 2010).

Indeed, a direct antioxidant effect *in vivo* is less feasible because of the pharmacokinetic properties of polyphenols (presented in detail in the next section). Polyphenols have a limited bioavailability and only low concentrations are found in the systemic circulation and tissues compared with other endogenous and exogenous antioxidants. In addition, the extensive metabolism of polyphenols during absorption and distribution in the body greatly diminishes their antioxidant activity. Thus, it has recently been postulated that polyphenols do not prevent oxidative damage by direct antioxidant actions (Hollman et al. 2011).

A variety of studies encompassing clinical trials, epidemiological data as well as *in vitro* and *in vivo* studies with animals have been performed to establish first a cause and effect link between a diet rich in polyphenols and improvement in health, and secondly, to gain insight into the mechanisms of action and protection of these compounds. A significant mechanism to prevent the development of atherosclerosis is to protect the endothelium, reduce the oxidation of low density lipoprotein (LDL) and cholesterol levels and repress the synthesis of proinflammatory cytokines and adhesion molecules (Pandey & Rizvi 2009; Opie & Lecour 2007) .

In this scenario, polyphenols have been shown to modulate a variety of targets indirectly. These include endothelial nitric oxide synthase (eNOS) and nitric oxide (NO), inflammatory cytokines like tumor necrosis factor alpha (TNFα), interleukines (IL-6 and IL-8) in addition to vascular cell adhesion proteins

(VCAM-1 and ICAM-1) (**Figure 13**). They can also influence important signal cascades, such as mitogen-activated protein kinases (MAPKs), which control proliferation and apoptosis; protein tyrosine phosphatases (PTPs) and tyrosine kinases, which regulate the phosphorylation state of many important signaling molecules involved in the regulation of many cellular processes, kinase protein kinase C (PKC-a), involved in signal transduction to various effector pathways that regulate transcription and cell cycle control, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), a multiprotein complex known to activate genes involved in the early cellular defense reaction (Genestra 2007; Khurana et al. 2013).

Progress in the field of the health benefits of polyphenols can be made when the one-dimensional antioxidant view on polyphenols is replaced by another considering their multifaceted bioactivity. It should be realized that polyphenols are versatile bioactive compounds rather than mere antioxidants.

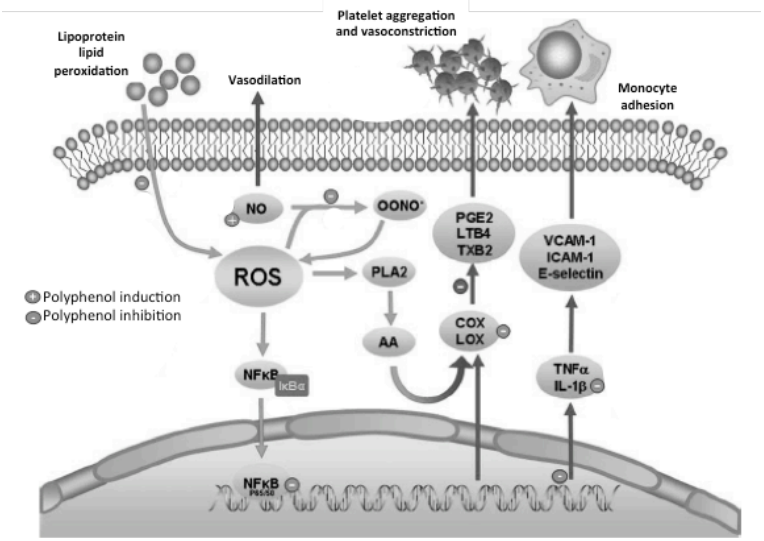


Figure 13. Some of the non-antioxidant action mechanisms of polyphenols related to atheriosclerotic target modulation, transcriptional gene regulation and signalling pathways modulation. NO, nitric oxide; ROS, reactive oxygen species; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; $OONO^-$, peroxynitrite; PLA2, phospholipase A2; AA, arachidonic acid; PGE2, prostaglandin E2; LTB4, leukotriene B4; TXB2, thromboxane B2; COX, cyclooxygenase; LOX, lypoxygenase; VCAM-1 and ICAM-1, vascular cell adhesion proteins; TNFα, tumor necrosis factor alpha; IL-1β, interleukin-1 beta (Adapted from Perona et al. 2006).

3.2 Bioavailability and metabolism of phenolic compounds

Although an ever-expanding amount of scientific evidence supports a protective effect by polyphenols against chronic degenerative diseases, it appears that there are insufficient studies to draw definitive conclusions for most diseases endpoints. The apparent shortcomings in the intervention literature stem primarily from a lack of pharmacokinetic studies. Establishing the biological activities of phytochemicals depends on a full understanding of their intake, absorption, metabolism and excretion. However, to date, this has only been carried out for a limited number of structures. These unknown metabolites are still a matter of much debate and most likely contribute to the bioactivity of bioactive compounds (Kay 2010). As suggested by Scalbert & Williamson (2000), researchers should focus less on the parent compounds as they are ingested and more on the biological activities of the metabolites present in our tissues. Kroon et al. 2004 highlighted that there have been significant advances in the understanding of polyphenols metabolism. However, there is still controversy about the nature and properties of its conjugates *in vivo*, and that uncertainty hinders progress towards understanding the real contribution of polyphenols as dietary protective agents against cancer, CVD or other diseases.

Kay (2010) recently stated that it is critical to use physiological conjugates at appropriate concentrations in *in vitro* studies to attempt to assess the effects of polyphenols in humans. Thus, the new tendency in the polyphenol research approach is to study the possible modifications that the bioactive compounds can undergo during food processing and establish the post-consumption pharmacokinetics and metabolism of the food that includes the bioactive compounds. This way, the relevant structures identified and the physiological concentration of metabolites can be established and assayed by *in vitro* or *in vivo* studies to evaluate their bioactivity at physiological levels (**Figure 14**).

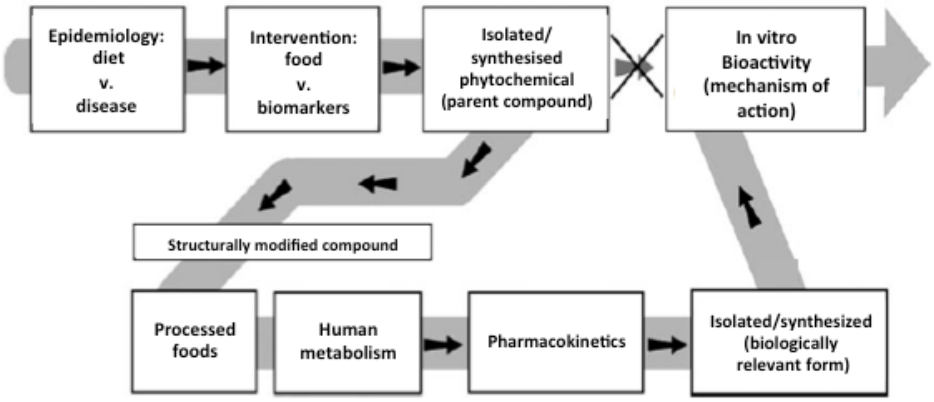


Figure 14. Multi-disciplinary approach of polyphenols research (Kay 2010).

The pharmacokinetics of polyphenols includes the same steps as those for orally ingested drugs (liberation, absorption, distribution, metabolism and excretion; LADME) and faces some of the same challenges, including transporters and enzymes. However, unraveling the bioavailability of polyphenols is even more challenging than with drugs, since many other factors, such variety in the chemical structure, food matrix and gut microbiota, can affect the bioavailability of polyphenols during digestion (Rubió et al. 2014. Appendix II).

The absorption of polyphenol glycosides, the most common phenolic structures in vegetables, is associated with the cleavage and release of the aglycone as a result of the action of lactase phloridzin hydrolase (LPH) in the brush border of the small intestine epithelial cells (**Figure 15**), so the released aglycone may then enter the epithelial cells by passive diffusion (Day et al. 2000). An

alternative hydrolytic step is mediated by a cytosolic β-glucosidase (CBG) within the epithelial cells. For CBG-catalyzed hydrolysis to occur, the polar glucosides must be transported into the epithelial cells, possibly with the involvement of the active sodium-dependent glucose transporter 1 (SGLT1) (Gee et al. 2000). Thus, there are two possible routes by which the glycoside conjugates are hydrolyzed, and the resulting aglycones appear in the epithelial cells, namely LPH/diffusion and transport/CBG (**Figure 15**).

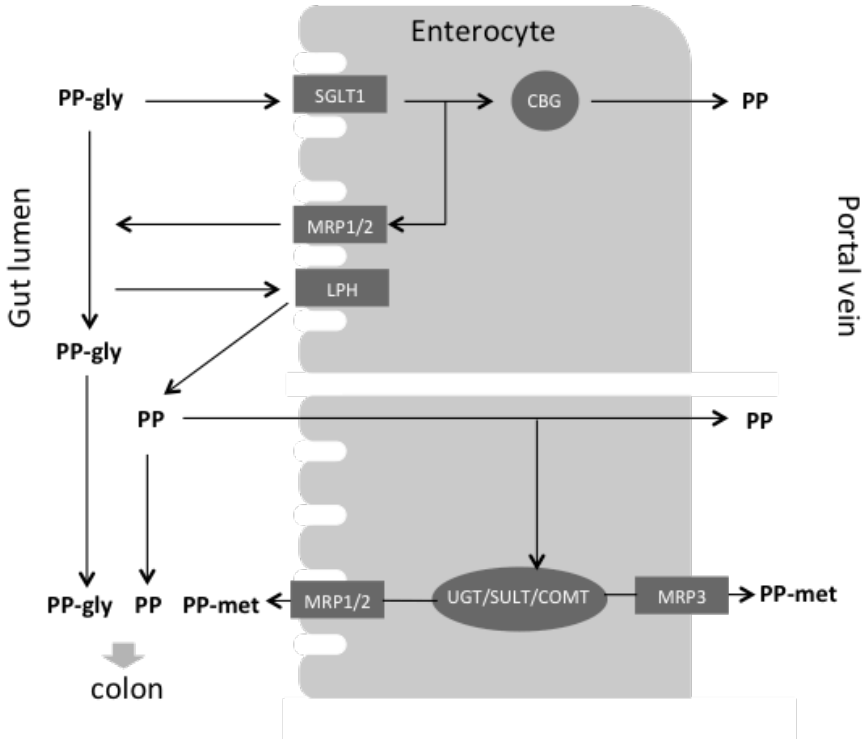


Figure 15. Proposed mechanisms for the absorption and metabolism of polyphenolic compounds in the small intestine. CBG, cytosolic β-glucosidase; COMT, catechol-O-methyl transferase; LPH, lactase phloridzin hydrolase; MRP1-2-3, multidrug-resistant proteins; PP, (poly)phenol aglycone; PP-gly, (poly)phenol glycoside, PP-met, polyphenol sulfate/glucuronide/methyl metabolites; SGLT1, sodiumdependent glucose transporter; SULT, sulfotransferase; UGT, uridine-5'-diphosphate glucuronosyltransferase (Adapted from Del Rio et al. 2013)

Before passage into the blood stream, the aglycones undergo some degree of phase II metabolism, forming sulfate, glucuronide, and/or methylated conjugates through the respective action of sulfotransferases (SULTs), uridine-5'-diphosphate glucuronosyltransferases (UGT), and catechol-O-methyl-transferases (COMTs). There is also efflux of some of the metabolites back into the lumen of the small intestine, and this is thought to involve members of the adenosine triphosphate-binding cassette (ABC) family of transporters, including multidrug resistance protein (MRP) and P-glycoprotein (**Figure 15**). Once in the portal bloodstream, metabolites rapidly reach the liver, where they can undergo further phase II metabolism, and enterohepatic recirculation may result in some recycling back to the small intestine through bile excretion (Donovan et al. 2007) (**Figure 16**).

Polyphenol conjugates with sugar moieties that are resistant to the action of LPH/CBG are not absorbed in the small intestine to any degree and pass to the colon (Day et al. 2000). Analysis of ileal fluid collected from ileostomists after the ingestion of various foodstuffs has shown that even when dietary polyphenols are absorbed in the proximal GIT, substantial quantities nonetheless pass from the small to the large intestine (Jaganath et al. 2006; Kahle 2006; Marka et al. 2009), where the colonic microbiota cleave conjugating moieties, and the resultant aglycones undergo ring fission leading to the production of smaller molecules, including phenolic acids and hydroxycinnamates. These can be absorbed and may be subjected to metabolism in the liver before being excreted in urine in amounts that, in most instances, are well in excess of the metabolites that enter the circulatory system via the small intestine (Jaganath 2006; Roowi et al. 2010; Stalmach et al. 2010) (**Figure 16**).

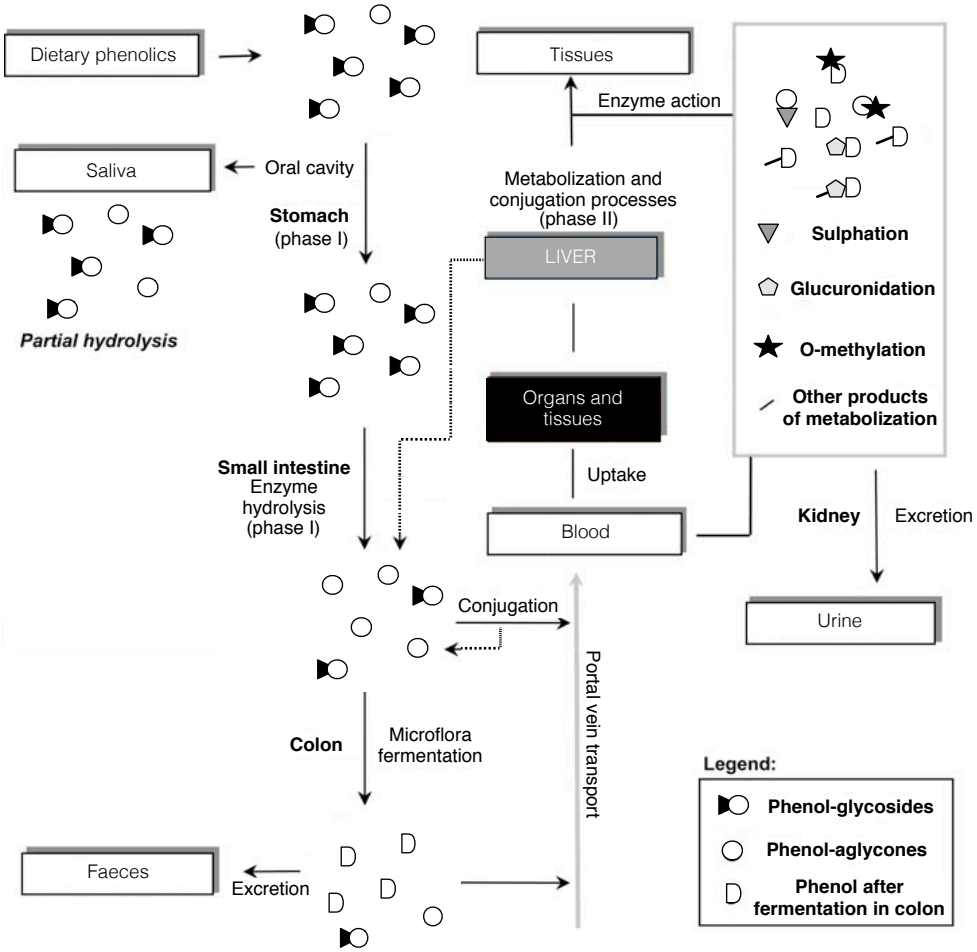


Figure 16. Schematic representacion of digestion, resorption, metabolization, and excretion of plant phenolics in mammals (Adapted from Vacek et al. 2010).

Phenolic conjugates are treated by the body as xenobiotics, and instead of accumulating in the circulatory system, they are rapidly turned over and removed by excretion via the kidneys. As a consequence, although the plasma pharmacokinetics of these metabolites provides useful information, estimates, such as area-under-the curve (AUC) values, do not necessarily yield accurate

quantitative data on absorption. In the circumstances, urinary excretion provides a more realistic assessment. However, as this does not include the possibility of sequestration in body tissues and organs, this too is theoretically an underestimate of absorption, but the degree to which this is so remains undetermined. The fact that tissue sequestration on any scale has yet to be convincingly demonstrated suggests that it can only be at low levels (Del Rio et al. 2013).

Most recent results also support the idea that the circulating conjugates in plasma behave as free active form carriers and that the aglycone released in the target organs after deconjugation is the final effector (Perez-Vizcaino et al. 2012). Hydrolysis of flavonoid glucuronides was found by and Shimoi et al. (2011) in human neutrophils and by Lee-Hilz et al. (2008) in carcinoma cell lines, who suggested that the activity of flavonoid metabolites depends on their deconjugation *in situ*. Terao et al. (2011) based on these and other indirect evidences, proposed that glucuronide conjugates of quercetin function not only as detoxified metabolites but also as precursors of the bioactive hydrophobic aglycone.

3.3 Biological effects of olive oil phenolic compounds

3.3.1 Antioxidant activities of olive oil phenolic compounds

The principal antioxidant properties of olive oil phenols described in chemical and physical models could explain to some extent the mechanisms underlying their antioxidant activities in more complex biological models and systems. A set of *in vitro* experiments have shown that olive oil phenols protect various macromolecules from oxidation, and could participate in the protection of cell

and the whole organism against oxidative processes primary involved in CVDs development and progression.

Among the various substrates that can be oxidized by free radical-mediated reactions, LDL is the most common. Several *in vitro* systems have been developed to mimic the reactions occurring *in vivo,* among which the susceptibility of isolated LDL and of lipid models to oxidation are the most common (Cheng et al. 2003; Paiva-Martins, 2006). HOTYR and OLE were reported to inhibit the radical induced lipid peroxidation of fatty acids in lipid model systems (micelles, vesicles and liposomes), but not TYR (Roche et al. 2005; Paiva-Martins et al. 2003; Saija et al. 1998). They act rather as retardants, reducing the initiating hydrophilic peroxyl radicals in the aqueous phase, than as chain breakers. In contrast to their monohydroxy counterparts (TYR and hydroxyphenylacetic acid), the o-diphenols (HOTYR, dihydroxyphenylacetic acid and OLE-aglycone) were reported to efficiently increase the *in vitro* resistance to oxidation of LDL isolated after being plasma pre-incubated with the tested compounds (Leenen et al. 2002). Both HOTYR and OLE potently and dose-dependently inhibit in vitro peroxyl radical–dependent and metal-induced oxidation of LDL isolated from plasma (Fitó et al. 2000; Visioli et al. 1995). The protective effect of olive oil phenols on oxidation of human LDL *in vivo* has been observed in several clinical and intervention studies, discussed later in this chapter.

3.3.2 Non-antioxidant activities of olive oil phenolic compounds

Olive oil dietary phenols exhibit several biological activities that are not directly related to their antioxidant properties. Most recent studies in relation to olive oil phenolics bioactivity show that the parent compounds and/or their metabolites:

(i) act as inhibitors/activators of regulatory enzymes;
(ii)have an impact on cellular signaling pathways;

(ii) influence the expression of certain genes;

In relation to enzymes modulation, HOTYR has shown several enzymatic activities linked to CVDs. They inhibit the proinflammatory 5-lipoxygenase activity in leukocytes (de la Puerta et al. 1999) and the expression of the inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in cells treated with lipopolysaccharide (Zhang et al. 2009). However in human endothelial cells under non-inflammatory conditions, HOTYR unable to modulate the eNOS enzyme either at the level of its expression or its activity (Schmitt et al. 2007). OLE was also suspected to cause some increase in the iNOS cellular expression (de la Puerta et al. 2001). An inhibition of cAMP-phosphodiesterase was proposed as one of the mechanisms through which olive oil phenols inhibit platelet aggregation (Dell'Agli et al. 2008).

The interaction of olive oil phenols with cell signaling systems and their influence on gene expression is in the preliminary stages of research. Many of these studies are dedicated to the analysis of their interaction with well-characterized signaling pathways mainly involved in carcinogenesis (Menendez et al. 2009; Corona et al. 2007). Nevertheless, some interesting data has been also obtained about their interaction with the cellular processes involved in the development and progression of CVDs. It was shown that the protective action of HOTYR and HVAlc against peroxide-induced injury in renal epithelium was linked to their potential to modulate the activation of extracellular signal-regulated kinases (ERK), protein kinase B (Akt) and c-Jun N-terminal kinases (JNK) and interaction with some of the apoptosis-related signalling pathways (Incani et al. 2009). Regarding the influence of olive oil phenols on the expression of CVDs inflammatory related proteins, it has been described that some phenolic compounds may inhibit cytokine and eicosanoid production by inhibiting IL-1β mRNA and protein expression and COX-2 activity and transcription (Carluccio et al. 2003; Petroni et al. 1997; de la Puerta et al. 1999). These interactions may

contribute to the anti-atherogenic properties ascribed to EVOO. Already at physiologically-relevant concentrations of phenolics, an extract from EVOO was reported to reduce the cell surface expression of ICAM-1 and VCAM-1 strongly, these being adhesion molecules involved in early steps of atherosclerosis, linked to a reduction in mRNA levels. OLE and HOTYR appeared to be the main components responsible for these effects (Dell´Agli et al. 2006).

In the nutrigenomic sense, olive oil bioactive constituents can be referred to as signals detected by cellular sensor systems and that affect the expression of the genome at several levels (mRNA and proteins) and subsequently, the production of metabolites. Thus, the nutrigenomic effects of olive oil on the development of atherosclerosis were analyzed in a series of experiments on genetically modified mice that spontaneously develop atherosclerosis (Acín et al. 2007; Alemany et al. 2010; Arbones-Mainar et al. 2007). Despite the limitations of this animal model given the morphological and physiological differences with humans, partially minimized by the similarity of the two genomes, several genes have been identified as responders to olive oil consumption (Guillén et al. 2009). Regarding olive oil-genome interaction in humans, to date, only one study reported by Farràs et al. 2013 has been performed, in which the up-regulation of the expression of cholesterol efflux related genes in human white blood cells occurred after a polyphenol-rich olive oil ingestion versus a moderate one. More studies related to the interactions between olive oil and the human genome are essential to gain mechanistic insights into its beneficial activity for health.

3.3.3 Human efficacy of olive oil phenolic compounds

Acute/postprandial studies

Several acute randomized and crossover clinical studies, summarized in **Table 6**, have examined the postprandial effect of olive oil phenolic compounds on the biomarkers of oxidative stress. Although they were well planed, the results of

these postprandial studies are difficult to evaluate and compare because the dosages of polyphenols chosen in some studies were quite dissimilar from representative dietetic levels of high and low phenolic content olive oil antioxidants (Visioli et al. 2000a; Ruano et al. 2005). Furthermore, the populations were usually small and mainly consisted of healthy male individuals. Only one study was performed on a mixed population (female and male) of hypercholesterolemic patients (Ruano et al. 2005).

In these postprandial studies with olive oil doses at which oxidative stress occurs, it was shown that VOO phenolic compounds increase serum antioxidant capacity and modulate the degree of lipid and LDL oxidation, in a dose dependent manner.

Sustained dose intervention studies

Single doses are not representative of the actual dietary situation with olive oil consumption. There are two main drawbacks:

(i) in most cases, consumption of olive oil, as a natural dietary component, is sustained;

(ii) the repeated administration of it could be necessary to make some of the effects of its actions visible.

In the majority of sustained dose interventions (summarized in **Table 6**), lipid oxidative damage was investigated. Two studies with a similar experimental design approach, a short-term intervention study (Weinbrenner et al. 2004a) with a strict very low antioxidant diet in both wash-out and intervention periods, and a 3-week intervention study (Marrugat et al. 2004) with a strictly controlled low antioxidant consumption diet, reported on the protective effects of olive oil phenols in vivo on the basis of two lipid oxidative damage biomarkers: plasma oxLDL and urinary MDA concentrations. Both the acute and short-term intervention studies were able to demonstrate that olive oil phenolic content

modulates the oxidative/antioxidative status of healthy subjects (Weinbrenner, 2004a).

These preliminary results were further supported by results obtained in a controlled, crossover international study (EUROLIVE) where participants (n=200) were randomly assigned to 3 sequences of daily administration of 25 mL of three olive oils for 3 weeks. The olive oils had a low (2.7 mg/kg of olive oil), medium (164 mg/kg), or high (366 mg/kg) phenolic content but were otherwise similar in composition. The phenolic content provided benefits in a direct dose-dependent manner for plasma lipids and lipid oxidative damage (Covas, 2006b). In a subset of subjects it was shown that all three olive oils caused an increase in the plasma and LDL oleic acid content ($P < 0.05$). In addition, olive oils rich in phenolic compounds led to an increase in their concentrations in LDL ($P < 0.005$) in a direct relationship with the phenolic content of the oils. This can account for the increased resistance of LDL to oxidation and the decrease of oxidized LDL observed within the framework of this clinical trial (Gimeno et al. 2007). The phenolic content of LDL was correlated with concentrations of HOTYR in plasma (Covas et al. 2006b) and its presence in LDL was demonstrated later (de la Torre-Carbot et al. 2007).

Previous studies were unable to demonstrate such findings due to several deficiencies in the design summarized as follows (Covas 2007):

(i) the experimental design of the studies (present/absent wash-out[2], period of intervention);

(ii) control and type of diet applied (diet compliance biomarkers, the amount of polyphenols consumed, type of olive oil pattern);

(iii) population sample (size and homogeneity);

(iv) the physiological characteristics of the participants (age, sex and oxidative status, etc.);

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[2] Wash-out periods is the minimum number of days between administrations of olive oil polyphenols needed to avoid influence of the previous administration on the plasma and urinary concentration levels of these polyphenols.

(v) the sensitivity and the specificity of the oxidative stress biomarkers evaluated.

The balance in pro-oxidant and antioxidant reactions is well regulated in the body and therefore the interventions with antioxidant-rich compounds at dietary doses exert only marginal effects on healthy volunteers. In addition, the detection of these effects is challenged due to the current state of the art of the oxidative biomarkers (Giustarini et al. 2009). In fact, the protective effect of olive oil phenolic compounds on oxidative damage in humans was better displayed in participants with a compromised oxidative status (males, males submitted to a low antioxidant diet, postmenopausal females) or in patients with high oxidative stress status (hyperlypemic, coronary heart disease, hypercholesteromic).

In summary, the overall results of sustained dose olive oil intervention studies in humans have provided evidence for (i) the *in vivo* protective role of olive oil phenolic compounds on lipid cardiovascular risk factors, including lipid oxidative damage, in humans and at real-life dosages, and (ii) the fact that olive oil phenolics contribute to the health benefits of olive oil and therefore this food can no longer be considered only as a source of MUFA fat.

Table 6. Randomized, crossover, controlled studies on the effect of phenolic compounds from olive oil on blood lipids, oxidation, inflamation, and oxidative dalage (Addapted from Covas, 2007 and Cicerale, 2009)

Treatment	Subjects (n, status)	Phenolic dose administered	Monitored markers	Findings	Ref.
Acute intervention studies					
EVOO vs OO vs CO (50 ml)	n=12 healthy	607 vs 16 vs 0 mg/ kg	Oxidative: Urinary hydrogen peroxide levels; serum antioxidant capacity; Inflammatory: plasma TXB(2) and LTB(4); Serum lipids: TC, HDL-C, LDL-C, TG, total plasma FA	↓ in TXB2 and LTB4 with ↑ in phenolic content; ↑plasma antiox. Capacity with ↑ in phenolic content	Bogani, 2007
High vs moderate vs low content of phenols in similar olive oils (40 mL)	n=12 healthy	366 vs 164 vs 2.7 mg/kg	Oxidative: 8-epi-$F_2\alpha$ and oxLDL in plasma; phenolic compounds, lipid composition and 3CT in LDL; Inflammatory: plasma TXB(2), LTB(4); Serum lipids: TC, hDL-C, TG, LDL-C	↑postprandial oxidative stress (by ↑ levels of F2-isoprostanes) for all oils tested; ↓the degree of LDL oxidation with ↑of phenolic content in oils	Covas, 2006
VOO vs OO (40 mL)	n=21 hyper-cholesterolemic	400 vs 80 mg/kg	Oxidative: plasma 8-epi-F2a, LPO, $NO_{(x)}$; Serum lipids: TC, HDL-C, TG, LDL-C, TRL; Thrombogenic state: PAI-1^m, FVIIa	↓in LPO, ↓in 8-epi-F2a, ↓ postpandrial thrombogenic state and ↑NO(x) production with ↑ phenolic compounds	Ruano 2005 and 2006
Phenol-rich vs phenol poor OO (100g)	n=14 healthy	303 vs 0.3 mg/kg	Oxidative: serum anrtioxidant capacity, antioxidant content of LDL and HDL	↑ plasma antioxidant capacity due to the olive oil phenol content at 2h postprandrial	Bonanome 2000
Different concentration of phenolics in olive oil (50 mL)	n=6 healthy	1950 vs 1462 vs 975 vs 487 mg/kg	Oxidative: urinary 8-epi-F_{2a}	↓in urinary 8-epi-F2a dose dependent on the phenolic concentration in olive oils	Visioli 2000
Short term intervention studies (up to 1 week)					
High vs moderate vs low content of phenols in olive oils (25 mL/day, 4days)	n=12 healthy	486 vs 133 vs 10 mg/kg	Oxidative: GSH-Px, GS-R and oxLDL in plasma; 8-oxo-dG in urine and mitDNA of MNC; MDA and 8-epi-F2a in urine; Serum lipids: TC, HDL-C, LDL-C, TG	↓plasma oxLDL, ↓urinary 8-oxo-dG and ↑plasma HDL-C and ↑GSH-Px in dose dependent manner with ↑of oils phenolic content	Weinbrenner 2004
Mid and long term intervention studies					
Phenol-rich vs phenol poor OO (50g/day, 4weeks)	n=14 healthy	303 vs 0.3 mg/kg	Oxidative: oxLDL in plasma; antioxidant contnet of LDL and HDL; resistance to oxidation	No changes in LDL oxidability due to the phenol content	Bonanome, 2000
VOO vs ROO (50ml/day, 3 weeks)	n=40 coronary heart disease	161 vs 15 mg/kg	Oxidative: LPO, GSH-Px, GS-R and oxLDL in plasma; LDL resistance to oxidation; Serum lipids: TC, HDL-C, LDL-C, TG	↓plasma oxLDL and ↓LPO with ↑ of oils phenolic content; ↑GSH-Px with ↑ of oils phenolic content	Fitó, 2005
VOO vs ROO (50ml/day, 3 weeks)	n=28 stable coronary heart disease	161 vs 15 mg/kg	Inflammatory; plasma IL-6, sICAM-1 and sVCAM-1 , CRP. Serum lipids: TC, HDL-C, LDL-C, TG	↓plasma IL-6 and CRP with ↑ of oils phenolic content; no changes in SVCAM-1 and s-ICAM-1	Fitó, 2008
High vs moderate vs low content of phenols in olive oils (25 mL/day, 3 weeks)	n=30 healthy	150 vs 68 vs 0 mg/kg	Oxidative: oxLDL in plasma; LDL resistance to oxidation; Serum lipids: TC, HDL-C, LDL-C, TG	↑HDL-C and ↑LDL resistance to oxidation with ↑of oils phenolic content	Marrugat, 2004

Mid and long term intervention studies (continuation)					
High vs low content of phenols in olive oils (70g/ day, 3 weeks)	n=25 healthy	308 vs 43 mg/kg	Oxidative: oxLDL and plasma antioxidant capacity	No significant changes	Moschandreas, 2002
EVOO high phenols vs EVOO low phenol content (50g/day, 8 weeks)	n=10 Healthy, post-menopausal	592 vs 147 mg/kg	Oxidative: plasma antioxidant capacity, DNA oxidation (by Comet assay); Serum lipids: TC, HDL-C, LDL-C, TG	↓DNA damage with ↑ of oils phenolic content; no changes in plasma antioxidant capacity	Salvini, 2006
EVOO vs ROO (40ml/ day, 7 weeks)	n=22 mildy dyslipemic	166 vs 2 mg/kg	Oxidative: plasma antioxidant capacity; urinary 8-epi-F_{2a} ; Inflammatory; plasma TXB (2). Serum lipids: TC, HDL-C, LDL-C, TG	↓plasma TXB(2) with ↑ of oils phenolic content; ↑plasma antioxidant capacity with ↑ of oils phenolic content; no changes in urinary 8-epi-F_{2a}	Visioli, 2005
VOO vs ROO (60g/day, 3 weeks)	n=46 healthy	308 vs 43 mg/kg	Oxidative: oxLDL, LDL and HDL resistance to oxidation; MDA, protein carbonyls, LPO and plasma antioxidant capacity; Serum lipids: TC, HDL-C, LDL-C, TG	No significant changes	Vissers, 2001
High vs moderate vs low content of phenols in olive oils (25g/day, 3 weeks) EUROLIVE study	n=200 healthy	366 vs 167 vs 2,7 mg/kg	Oxidative: DNA and RNA oxidation markers in urine, LDL fatty acid composition, oxLDL, urinary 8-epi-$F_{2a,}$, GSH and GSSG in plasma, LDL resisitance to oxidation; Serum lipids: TC, HDL-C, LDL-C, TG	↓of DNA and RNA oxidative markers non-related with olive oil phenols, ↑ liner for HDL-C and HDL-C/TC with ↑ of oils phenolic content; ↓ iner for plasma oxLDL and oxidative stress markers (GSH and GSSG) with ↑ of oils phenolic content; ↓TG levels for all oils; ↑ oliec acid and ↓ linoleic arachidonic acid in LDL	Machowetz, 2007; Covas 2006b; Cicero, 2008

EVOO - extra virgin olive oil; VOO - virgin olive oil; OO - olive oil; CO - corn oil; ROO - refined olive oil; SO - sunflower oil; TBX(2) - thromboxane B_2; LTB(4) - leukotriene B_4; TC - total cholesterol; HDL-C - high density lipoprotein cholesterol; LDL-C - low density lipoprotein cholesterol; TG - trglyceride; FA - fatty acid; 8-epi-F2a - 8-epi prostaglandin-F2a isoprostane; oxLDL - oxidated low density lipoprotein; PAI-1 - plasminogen activator inhibitor-1; FVIIa . activated factor VII; GSH-Px - gluctathione peroxidase; GS-R - gluctathione reductase; 8-oxo-dG - 8-hydroxy-7,8-dihydroxy-2'-deoxyguanosine; mitDNA - mitochondrial DNA; MNCs mononuclear cells; CRP - C-reactive protein

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HYPOTHESIS AND OBJECTIVES

Previous human studies have demonstrated an increase of the HDL cholesterol levels and a decrease in the *in vivo* lipid oxidative damage in a dose-dependent manner with the phenolic content of olive oil. Based on these studies, the EFSA has released a health claim concerning the protective effects of the ingestion of phenolic compounds (PC) from olive oil declaring that "*Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress*". The EFSA Panel considers that in order to support the claim, 5 mg of hydroxytyrosol and its derivatives in olive oil should be consumed daily. However, the phenolic content in commercial virgin olive oil (VOO) is influenced by multiple agronomic and technological factors. As a result, phenolic concentration in most VOOs available on the market may be too low to allow the consumption of 5 mg of hydroxytyrosol and its derivatives within the frame of a balanced diet (20 g of VOO/day). In this context, the enrichment of VOO with its own phenolic compounds becomes an interesting strategy to increase and standardize the daily intake of hydroxytyrosol in the real food matrix and without increasing the caloric intake. Concerning VOOs with high phenolic content, however, on one hand they have a bitter and pungent taste, which could promote a refusal among consumers particularly those from non-Mediterranean areas. On the other hand, high phenolic compound-rich foods could have a dual action due to the fact that one type of antioxidants in high dose can also act as pro-oxidants. In this sense, the strategy of VOO enrichment not only with its own phenolics but also with complementary phenols from aromatic herbs or spices could not only provide improvements in oil stability and consumers' acceptation but also additional health benefits based on the hypothesis that when multiple antioxidants are used in combination, they could synergistically potentiate their antioxidant properties.

The present Doctoral Thesis is framed on the context of the VOHF project: Virgin Olive Oil and HDL functionality: A model for Tailoring a Functional Food. The global aim of the project was to develop a functional olive oil for its best suitability for promoting one of the current goals for modern cardiovascular therapy: the increase in the HDL functionality. Prior to the efficacy studies related to HDL functionalilty, several studies were required, which have been carried out in the framework of this thesis.

The project is based on a close relationship between Food Science and Technology, Chemistry, Clinical, Human Pharmacology and Biochemical expertises. The added value of the project is its translational nature which allows the transfer from Food Science and Technology innovations to possible health benefits and health claims for the food developed products.

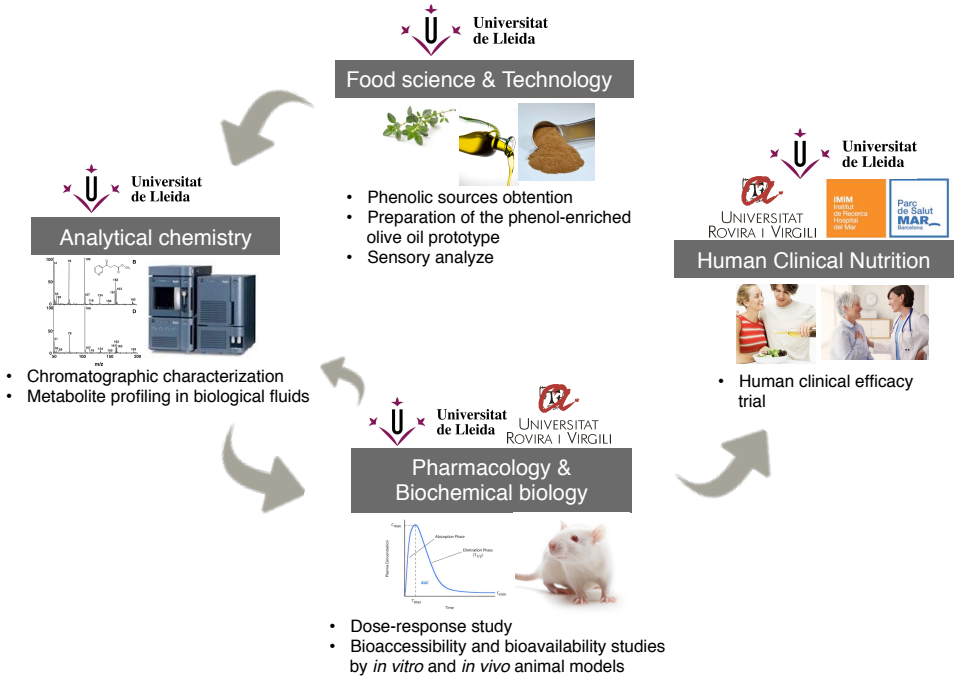

Figure 17. Multidisciplinary approach of the VOHF project. Participants: Cardiovascular Risk and Nutrition Research Group (IMIM-Institut Hospital del Mar d'Investigacions Mèdiques); Lipids and Atherosclerotic Research Unit (URV-Universitat Rovira i virgili); Antioxidants Research Group (UdL-Universitat de Lleida).

In the context of the multidisciplinary approach of the VOHF project, the **global objective** of the present Doctoral Thesis was to carry out the functional virgin olive oils preparation based on the phenol-enrichment with its own phenolic compounds and complemented with phenols from thyme, and to assess the bioavailability of the phenolic compounds as a step prior to human intervention studies, evaluating possible interactions or synergies on the bioavailability between both phenolic sources.

To achieve this goal, the **specific objectives** were defined:

I. To develop an olive oil enriched with both its own phenolic compounds and with additional complementary phenols from thyme and to establish the best ratio between the quantities of polyphenols from each of these sources based on the phenolic composition, oxidative stability, and bitter sensory attribute of the prepared oils.

II. To prepare similar phenol-enriched olive oils (enriched with their own phenolics), but with differences in their total phenolic content, and to carry out a dose-response study in humans in order to assess if there was a threshold in the bioavailability of phenolic compounds and establish the appropriate enrichment dose.

III. To develop and validate rapid, selective and sensitive analytical methods for the determination of plasma and urine metabolites derived from olive oil and thyme bioactive compounds.

IV. To assess the bioaccessibility, bioavailability and metabolism of olive oil and thyme phenolic compounds by combination of *in vitro* digestion and cell models (Caco-2 and HepG-2).

V. To assess possible interactions in the bioavailability and in the *in vivo* antioxidant capacity due to the co-ocurrence of olive and thyme phenolic compounds.

VI. To perform a sustained interventional human trial with the developed Functional Virgin Olive Oils (FVOO, enriched only with phenolics from olive, and FVOOT, enriched with both phenolics from olive and thyme), and to assess the compliance of the volunteers through the identification of the phenolic intake biomarkers in human plasma and urine.

VII. To assess the preliminary human biological efficacy of the functional olive oils in a sustained interventional trial regarding the protection against oxidative damage and the promotion of the endogenous antioxidant system.

RESULTS AND DISCUSSION

I. Preparation of phenol-enriched olive oils

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Publication I.

Development of a phenol-enriched olive oil with both Its own phenolic compounds and complementary phenols from thyme

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DEVELOPMENT OF A PHENOL-ENRICHED OLIVE OIL WITH BOTH ITS OWN PHENOLIC COMPOUNDS AND COMPLEMENTARY PHENOLS FROM THYME

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Abstract

A Besides affecting the oil' s sensorial characteristics, the presence of herbs and spices has an impact on the nutritional value of the flavored oils. The aim of the study was to develop a new product based on the phenol-enrichment of a virgin olive oil with both its own phenolic compounds (secoiridoid derivatives) plus additional complementary phenols from thyme (flavonoids). We studied the effect of the addition of phenolic extracts (olive cake and thyme) on phenolic composition, oxidative stability, antioxidant activity, and bitter sensory attribute of olive oils. Results showed that flavonoids from thyme appeared to have higher transference ratios (average 89.7%) from the phenolic extract to oil, whereas secoiridoids from olive presented lower transference ratios (average 35.3%). The bitter sensory attribute of the phenol-enriched oils diminished with an increase of the concentration of phenols from thyme, which might denote an improvement in the consumer acceptance.

KEYWORDS: bitter index / olive phenols / ORAC / phenol-enriched olive oil / Rancimat test / thyme phenols

INTRODUCTION

Over the past few years, the Mediterranean diet has gained in importance because of its relationship with a lower incidence of cardiovascular diseases and some types of carcinoma.[1] The irreplaceable elements in this dietary style include extra virgin olive oil and several spices and herbs.

Virgin olive oil (VOO) could be considered a functional food that, besides having a high level of monounsaturated fatty acids (MUFA), contains multiple minor components, such as phenolics, with relevant biological activity.[2,3] Recently, the European Food Safety Authority (EFSA) published a report substantiating the claims related to the health benefits attributed to polyphenols in olives and specifically to the protection of low-density lipoproteins (LDL) particles from oxidative damage, among other effects.[4] The scientific references backing this were basically human intervention studies. For instance, data from the EUROLIVE study showed that phenolic compounds from olive oil can modulate the postprandial oxidative stress after the ingestion of 40 mL of olive oil. [5] The degree of postprandial oxidative stress was inversely related to the phenolic content of olive oils from high (366 ppm) to low (2.7 ppm) phenolic content.

The daily intake of phenolics from VOO is low compared with that obtained from other phenolic sources. Therefore, the intake of VOO enriched with its own phenolic compounds could be of interest to increase the daily dose of these beneficial compounds. However, the enrichment of olive oil with its own phenolics has several drawbacks. In terms of sensory attributes, virgin olive oils with high phenolic contents have a bitter and pungent taste due to the presence of secoiridoids,[6] which could lead to rejection by consumers, particularly those from non-

Mediterranean countries. Moreover, phenolic-compound-rich foods could have a dual action due to the fact that antioxidants can also act as oxidants. The administration of high doses of a single type of antioxidant could even promote, rather than reduce, lipid peroxidation, and it has been shown to increase atherosclerotic areas in animal models.[7,8] In this sense, it has been reported that giving high-risk individuals supplements of vitamin E promoted lipid peroxidation,[9] while the combination of vitamin E and vitamin C was effective at reducing atherosclerosis in human trials.[10]

Besides, a novel therapeutic approach to suppress oxidative stress is based on the development of dual function antioxidants comprising not only chelating but also scavenging components.[11] Phenolic compounds present in virgin olive oil, specially secoiridoid derivatives of hydroxytyrosol, act similarly to phenolic acids, inhibiting the lipid oxidation by trapping free and peroxy radicals and also containing the ortho-diphenolic group necessary to chelate metals. Other antioxidants, such as flavonoids, also help to control the extent of lipid peroxidation by chelating metal (i.e., copper) ions.[11,12] However, this phenolic group makes a very small contribution to the total antioxidant capacity of VOO because of its low concentration. Due to all of the above, the enrichment of an olive oil complementing the olive phenols with other kinds of phenolic compounds was considered in order to improve the nutritional profile and sensorial characteristics of olive oil. Spices, such as garlic, hot pepper, and various herbs, including thyme and rosemary, are widely used as flavor enhancers of olive oils, and changes in the chemical and sensory profile have been studied.[13] Earlier studies on herbs and spices have considered their bioactive compounds from the perspective of antioxidants and anti-inflammatories, where the most important phytochemicals isolated include phenolic terpenes (thymol, carvacrol, carnosic acid), hydroxycinnamic acids and derivatives (rosmarinic acid, eugenol), and flavonoids (luteolin, thymusin, and xanthomicrol, among others).[14,15] Thyme is the herb selected in this study for the olive oil flavoring, as it is one of the richest sources of flavonoids.[16]

The aim of this study was to develop an olive oil enriched with both its own phenolic compounds and with additional complementary phenols from thyme and to establish the best ratio between the quantities of polyphenols from each of these sources.

MATERIALS AND METHODS

Samples

The virgin olive oils used as the matrix to carry out phenolic enrichments were from the olive-growing area of Les Garrigues (Lleida, Catalonia, Spain) and obtained by a two-phase continuous system from Arbequina cultivar. On the other hand, the samples of olive cake that were used to obtain the phenolic extract were from a commercial olive mill in the same area. These samples were taken at the decanter outlet, and liquid nitrogen was immediately added to avoid oxidative damage. The olive cake was then stored at –40 °C and freeze-dried in order to preserve it until the preparation of the phenolic extracts. Dried thyme (*Thymus zygis)* was supplied by Sabater Spices (Murcia, Spain).

Chemicals and reagents

Apigenin, apigenin-7-O-glucoside, luteolin, luteolin 7-O-glucoside, oleuropein, rutin, tyrosol, verbascoside, naringenin, kaemferol, eriodictyol, rosmarinic acid, taxifolin, quercetin-4′-O-glucoside, and vanillin were purchased from Extrasynthese (Genay, France). Hydroxytyrosol was purchased from Seprox Biotech, S.L. (Madrid, Spain). Caffeic, p-coumaric, and vanillic acids and fluorescein were purchased from Fluka Co. (Buchs, Switzerland), and (+)-pinoresinol was acquired from ArboNova (Turku, Finland). The dialdehydic form of elenolic acid linked to hydroxytyrosol (3,4- DHPEA-EDA), the dialdehydic form of elenolic acid linked to tyrosol (*p*-HPEA-EDA), and the lignan acetoxypinoresinol are not available commercially and were isolated from the VOO phenolic extracts by semipreparative HPLC[17] 2,2-Azobis(2-amidinopropano) dihydrochloride (AAPH) and Trolox were from Acros Organics (Geel, Belgium). Acetonitrile (HPLC grade), methanol (HLPC grade), ethanol, n-hexane, ethyl acetate, cyclohexane, and acetic acid were all provided by Scharlau Chemie (Barcelona, Spain). Water was of Milli- Q quality (Millipore Corp., Bedford, MA).

Preparation of the Phenolic Extracts

Eleven phenolic extracts (PE) were prepared from freeze-dried olive cake (OC) and dried thyme (T)

in different proportions, mixing previously to each extraction 10 g in total. The proportions varied from 100% OC to 100% thyme, and the different phenolic extracts were coded according to the percentage of thyme from 0% to 100% (PE0, 100% OC, 0% T; PE10, 90% OC, 10% T; PE20, 80% OC, 20% T; PE30, 70% OC, 30% T; PE40, 60% OC, 40% T; PE50, 50% OC, 50% T; PE60, 40% OC, 60% T; PE70, 30% OC, 70% T; PE80, 20% OC, 80% T; PE90, 10% OC, 90% T; PE100, 0% OC, 100% T). The phenolic extracts were obtained using an accelerated solvent extractor (ASE 100 Dionex, Sunnyvale, CA). This method was previously used and optimized by Suárez et al.[18] to prepare a phenol extract from olive cake. This equipment allows faster extractions by using solvents at high temperature and pressure. Ethanol/water (80:20, v/v) at 80 °C was used as the extraction solvent. To carry out the extractions, 5 g of diatomaceous earth was mixed with the sample (10 g) to increase the contact surface, avoid blockage of the cell, and improve the extraction of selected compounds. A 100-mL extraction cell was used, setting the flush volume at 60%. Two static cycles of 5 min were programmed in each extraction. After that, the sample was purged with nitrogen. The resulting extract was rotary evaporated until all the ethanol had been eliminated, freeze-dried, and stored at −80 °C until its use for oil enrichment. We obtained 1.96 ± 0.16 g of phenolic extract from 10 g of raw material.

Preparation of the Phenol-Enriched Olive Oils

Once these different PE extracts had been obtained, they were used to prepare olive oils with high phenolic content in the ratio of 2.5 g of extract/100 g of oil. Eleven phenol-enriched olive oils (EOO) were prepared and the oils were codified similarly by the percentage of thyme: EOO0, EOO10, EOO20, EOO30, EOO40, EOO50, EOO60, EOO70, EOO80, EOO90, and EOO100. To carry out the phenolic enrichment of the oils, 2.5 g of PE was dissolved in water (2% of the final oil volume) and mixed until complete dispersion with the vortex mixer. The water dispersion was then added to a small amount of oil (8 mL) to make a previous emulsion using an ultrasonic bath. This volume was then mixed with the rest of the oil (to 100 mL) using a Polytron (Kinematica, Littau, Switzerland) until completely homogenized. Finally, the EOO was centrifuged for 10 min at 3500 rpm to remove the extract solid waste. The oil was bottled in dark bottles until analysis.

Phenol Analysis of the Phenol-Enriched Olive Oils

The phenolic compounds of the olive oil samples were extracted following the method described in our previous paper.19 Briefly, 20 mL of methanol/water (80:20, v/v) was added to 5 g of oil and homogenized for 2 min with a Polytron. After that, the two phases were separated by centrifuging at 3000 rpm for 10 min, and the hydroalcoholic phase was evaporated to obtain a syrupy consistency at 31 °C and then purified by liquid−liquid extraction with acetonitrile. The acetonitrile solution was finally rotary evaporated to dryness and then dissolved in 5 mL of methanol and maintained at −40 °C before chromatographic analysis.

Chromatographic Analysis of the Phenols by HPLC–ESI-MS/ MS

The phenolic composition of the phenolic extracts (PE) and phenol-enriched olive oils (EOO) was analyzed by HPLC coupled with tandem mass spectrometry (MS/MS). The HPLC consisted of an AcQuity liquid chromatography system equipped with a Waters binary pump system (Milford, MA) using a SunFire C18 column (3.5 μm, 4.5 Å~ 150 mm), also from Waters. During the analysis, the column was kept at 30 °C and the flow rate was 0.8 mL/min. The mobile phase was Milli-Q water/acetic acid (99.8:0.2, v/v) as solvent A and acetonitrile as solvent B. The elution gradient was as follows. It started at 5% of eluent B for 5 min, was linearly increased to 40% of eluent B in 20 min, and further increased to 100% of eluent B in 0.1 min, and the reequilibration time was 1.9 min. The HPLC system was coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA). The software used was MassLynx 4.1. Ionization was done by using an electrospray (ESI) interface operating in the negative mode, [M−H]−, and the data were collected in the selected reaction monitoring (SRM) mode. The ionization source parameters were the same as those described in our previous report.[19] The SRM transitions and the individual cone voltage and collision energy were optimized for each phenolic compound. Two SRM transitions were studied in order to find the most abundant

product ions. The most sensitive transition was selected for quantification and a second one for confirmation purposes (**Supporting Information**). The phenolic compounds were quantified by the calibration curves for the respective commercial standards. The secoiridoid derivatives p-HPEA-EDA and the aldehydic form of elenolic acid linked to tyrosol (p-HPEA-EA) were quantified as p-HPEA-EDA; 3,4-DHPEA-EDA, 4-(acetoxyethyl)-1,2-dihydroxybenzene (3,4-DHPEA-AC), and oleuropein aglycon (3,4-DHPEA-EA) were quantified as 3,4-DHPEA-EDA; and elenolic acid, ligstroside derivative, and oleuropein derivative were quantified as oleuropein. The injection volume was 10 μL, and all the samples were filtered through a 0.22 μm Nylon filter (Tecknokroma, Barcelona, Spain) before analysis.

Oxidative Stability of the Olive Oils by the Rancimat Test

The oxidative stability of the olive oils (EOO) was evaluated by the Rancimat test (Metrohom, Herisau, Switzerland) using an air-flow of 20 L/h and a temperature of 120 °C to oxidize the samples (3 g of olive oil) (ISO 6886:1996). Changes in conductivity were measured continuously. The peroxidation curve was recorded, and the induction time, the time needed to reach the break point of this curve, was measured. All of the samples were analyzed in triplicate, and a control (VOO without addition of the phenolic extract) was incorporated into each experimental set. The results were expressed as the induction time in hours.

Antioxidant Capacity of the Olive Oils by the ORAC Assay

The hydrophilic ORAC was based on the methodology described by Huang et al.[20] with some modifications, and its value can be associated with the antioxidant activity of all the hydrophilic compounds of the oil. The assays were carried out on a FLUORstar optima spectrofluorometric analyzer (BMG Labtechnologies GmbH, Offenburg, Germany) in 96-well microplates, using an excitation filter at 485 nm and an emission filter at 520 nm. Trolox was used as the reference substance to express the results, whereas AAPH was used as an initiator. For the analysis, all the solutions were prepared using 0.075 M phosphate buffer at pH 7.4. The reaction mix consisted of 150 μL of 68 nM fluorescein solution, 25 μL of 74 mM AAPH solution (made immediately before use in phosphate buffer at 37 °C), and 25 μL of either olive oil phenolic extract used for chromatographic analysis (see Phenol Analysis of the Phenol-Enriched Olive Oils) or Trolox at different concentrations (from 0.415 to 4.15 ng/mL in the case of the oil phenolic extract and from 12.5 to 100 μM in the case of Trolox). The assay buffer was used as a blank. The experiments were carried out at 37 °C. The ORAC values were calculated on the basis of the area under the curve (AUC), and the data were expressed as micromoles of Trolox equivalents (TE) per 100 g of oil using Trolox and the sample calibration curves obtained in each analysis.

Phenol Transference Ratio

In order to know the effectiveness of the phenol transference from the extract (PE) to the oil (EOO), the transference ratio (TR) was calculated with the formula:

The oil phenolic content (PC) and the extract phenolic content were expressed as milligrams of phenol per kg of oil and extract, respectively. We took into account that the enrichment was carried out with 2.5 g of extract per 100 g of oil. The TR for each phenol was expressed as a percentage.

Bitter Sensory Attribute of the Enriched-Olive Oils

The aim of the sensorial analysis was to assess the bitter taste of the flavored oils in order to know if flavoring with thyme could improve the acceptance of the phenol-enriched olive oils in terms of bitterness. The analysis was carried out by a panel of six tasters, previously selected from 30 people according to the procedure of UNE 87–003–95,[21] where the tasters had to perceive differences in concentrations of various bitter solutions prepared with caffeine. This group selected had an intense sensitivity to the bitter taste. A specific profile sheet was set up for the flavored oils where the six judges were asked to assign a score for the bitter taste of six EOO (EOO0, EOO20, EOO40, EOO60, EOO80, and EOO100) on a 10-point scale going from “absent bitter taste” to “extremely perceptible bitter taste”. Before that, the members of the group were

trained together in order to reach a common agreement on the bitter scale points.

Statistical Analysis

All the data are expressed as the mean of three replicates. In order to simplify the results shown in the tables, we omitted the standard deviation, as all these values were lower than 10%. The data analyses were performed using the Statgraphics plus v. 5.1 software (Manugistics Inc., Rockville, MD). The data were analyzed by the one-way ANOVA test with a significance level of 0.05.

RESULTS AND DISCUSSION

Phenolic Composition of the Phenolic Extracts (PE)

Table 1 shows the phenol content of the 11 PEs determined by HPLC–ESI-MS/MS. Secoiridoid derivatives were the main compounds found in the PE0 extract obtained exclusively from olive cake, the 3,4-DHPEA-EDA being the most abundant (47.39 mg/g of PE0 extract). Curiously, the concentration of some secoiridoids (*p*-HPEA-EDA, *p*-HPEA-EA, and 3,4- DHPEA-EA) increased slightly in the PE10 compared with the PE0, indicating that the presence of phenols from thyme may have a protective effect on olive phenols by reducing their losses during the accelerated solvent extraction. In relation to flavonoids, their content in the PE extracts increased with the proportion of thyme in the sample used for phenol extraction. Thus, in the extract obtained exclusively from thyme (PE100) flavonoids was the main phenolic group. The main flavonoids provided by the thyme were thymusin (19.36 mg/g PE100), eriodictyol-rutinoside (13.76 mg/g PE100), xanthomicrol (7.98 mg/g PE100), and 7-methylsudachitin (6.12 mg/g PE100).

Apart from flavonoids, a dominant compound found in extracts containing thyme was rosmarinic acid (17.8 mg/g of extract in PE100). Rosmarinic acid is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid typically found in Lamiaceae plants, such as basil (*Ocimum spp.*), rosemary (*Rosmarinus spp.*), thyme (*Thymus*

Table 1. Phenolic Composition of the Control (olive oil used as enrichment matrix) and the 11 Phenol-Enriched Olive Oils (EOO) by HPLC– ESI-MS/MS. The concentration of each phenol in EOO is expressed as mg/kg oil after subtracting the amount of phenol in the control oil.

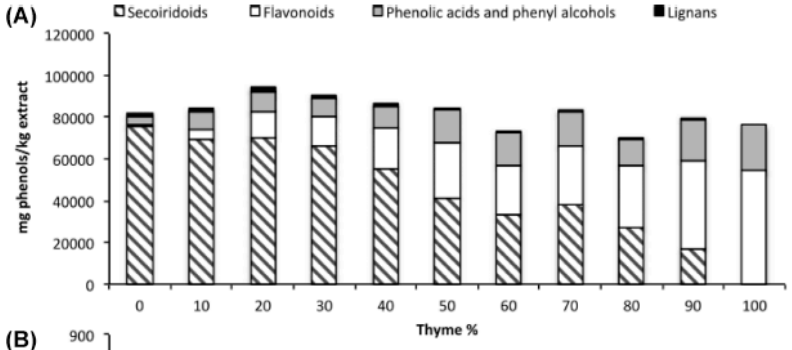
	phenolic composition (mg/g PE)										
compd	PE0	PE10	PE20	PE30	PE40	PE50	PE60	PE70	PE80	PE90	PE100
Phenyl Alcohols											
tyrosol	0.27	0.94	0.65	0.45	0.27	0.68	0.50	0.58	0.72	0.27	0.15
hydroxytyrosol	2.77	3.65	3.01	2.44	2.05	2.09	1.35	1.61	1.20	0.80	0.19
Phenolic Acids											
vanillic acid	0.67	1.52	1.51	1.16	1.63	1.32	1.36	1.27	1.02	1.63	1.28
caffeic acid	0.03	0.09	0.12	0.17	0.23	0.38	0.25	0.93	0.62	0.63	0.64
hydroxybenzoic acid	0.02	0.10	0.19	0.30	0.50	0.53	0.68	0.85	0.91	1.11	1.39
homovanillic acid	0.00	0.07	0.10	0.11	0.22	0.15	0.29	0.25	0.27	0.34	0.50
rosmarinic acid	0.73	2.24	3.81	4.43	5.07	10.6	10.8	10.7	8.33	14.9	17.8
Secoiridoid Derivatives											
3,4-DHPEA-AC	0.64	0.65	0.65	0.59	0.63	0.46	0.54	0.50	0.43	0.00	0.00
elenolic acid	13.2	9.93	9.58	9.55	9.46	6.22	4.00	4.31	2.51	1.48	0.00
p-HPEA-EDA	4.56	9.22	9.92	8.45	2.16	1.94	1.74	1.99	1.88	0.00	0.00
p-HPEA-EA	4.32	8.84	9.59	8.20	1.92	1.73	1.56	1.91	1.82	2.20	0.00
3,4-DHPEA-EA	5.36	9.61	10.6	9.06	11.1	8.97	9.00	13.4	9.41	6.42	0.00
3,4-DHPEA-EDA	47.4	31.1	30.1	30.6	29.6	21.7	16.2	18.5	11.1	7.17	0.00
Lignans											
pinoresinol	0.09	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
acetoxypinoresinol	1.17	1.66	2.34	1.24	1.67	0.41	1.11	0.76	0.24	0.12	0.00
Flavonoids											
apigenin	0.04	0.06	0.10	0.13	0.16	0.22	0.29	0.35	0.32	0.30	0.31
luteolin	0.49	0.59	1.01	0.98	1.09	1.27	1.10	1.24	1.73	1.22	1.45
naringenin	0.02	0.10	0.28	0.43	0.49	0.56	0.94	1.11	0.97	1.46	1.91
eriodictyol	0.00	0.10	0.34	0.51	0.70	1.05	1.17	1.21	2.13	2.19	2.79
taxifolin	0.02	0.05	0.08	0.15	0.19	0.16	0.26	0.24	0.72	0.57	0.47
eriodictyol-rutinoside	0.00	0.92	1.63	1.74	2.70	3.63	3.71	5.21	5.10	9.79	13.8
thymusin	0.00	2.17	7.24	7.19	10.8	12.8	8.94	10.5	11.1	16.6	19.4
xanthomicrol	0.00	0.26	0.90	1.20	2.05	3.29	3.28	3.32	4.24	4.93	7.98
7-methylsudachitin	0.00	0.24	0.72	1.42	2.13	3.83	4.46	5.04	3.70	4.97	6.12

spp.), mint(*Mentha spp.*), and oregano (*Origanum spp.*).[22] The evidence for the therapeutic potential of this compound as an antioxidant and anti-inflammatory compound has been recently reviewed.[15] The two o -dihydroxy groups (catechol structures) are the main active groups of the molecule and contribute to its strong potential as an antioxidant agent, which is mainly expressed by the H-abstraction reaction.[23]

Figure 1A shows the sum of total phenols by phenolic groups (phenyl alcohols and phenolic acids, secoiridoids, lignans, and flavonoids). The total phenolic content was similar in all the PEs. The main differences between the 11 PEs can be seen in the proportion of the major phenolic families. These results showed a direct relationship between the content of thyme in the sample and the flavonoid content in the phenolic extract and, similarly, between the content of olive cake in the sample used for obtaining the extract and the secoiridoid concentration. As seen, the strategy of mixing different plant sources rich in phenols may allow one to obtain custom-made extracts. In this sense, thyme clearly contributes to complementing the lack of flavonoids in the olive cake extract (PE0). Thus, extracts with different percentages of flavonoids or rosmarinic acid could be prepared by modifying the percentage of thyme in the sample used to prepare the phenolic extract.

Phenol-Enriched Olive Oil Composition and Transference Ratio

Eleven EOOs were prepared by the addition of different PEs to a base of VOO (ratio 2.5 g of PE per 100 g of VOO). The phenolic composition of the oils was analyzed by HPLC–ESI-MS/MS. As seen in **Table 2**, the main phenolics in the EOOs enriched with a high proportion of PE obtained from olive cake (EOO0) were the most



Lignans	38,6	30,9	23,7	31,3	32,2	28,3	25,3	45,0	12,0	0,0	0,0
Phenolic Acids and Phenyl alcohols	16.2	12.6	11.5	12.7	16.9	8.5	11.6	10.1	13.3	9.3	12.3
Flavonoids	82.3	89.7	56.2	68.8	56.4	41.3	46.3	41.0	39.0	31.0	33.5
Secoiridoids derivatives	35.3	28.7	25.7	23.2	21.9	26.2	24.9	9.1	8.6	0.0	0.0

Figure 1. Distribution of different phenolic groups of the phenolic extracts (A) and enriched olive oils (B). The table below shows the transference ratio from extract to oil (expressed as percentages) of each phenolic group for each experiment.

representative phenols of Virgin olive oils, the 3,4-DHPEA-EDA and *p*-HPEA-EDA (365 and 77.2 mg/kg of oil, respectively). These compounds are of special interest because they are precursors of hydroxytyrosol, the plasma concentration of which has been shown to increase in a dose-dependent manner with the phenolic content of the administered oil.[24] Therefore, higher quantities of these compounds could appear in the plasma, improving the health benefits of consuming olive oil. On the other hand, in EOOs with a high proportion of thyme, the most abundant phenolic compounds were again the same as in the extracts. For instance, EOO100 presented a high content of thymusin and xanthomicrol (268 and 60.9 mg/100 g of oil, respectively). As shown in **Figure 1B**, the concentration of major phenolic groups (secoiridoids, flavonoids, phenolic acids and phenyl alcohols, and lignans) quantified in different prepared oils is proportionally similar to the extract used for their preparation. However, the total amount of phenolic compounds was not the same for all the EOOs, although the enrichment was carried out with the same amount of extract (2.5 g per 100 g of oil). To understand these differences, a transference ratio (TR) (see eq 1) was calculated for each phenol. As shown in the table of **Figure 1**, flavonoids presented noticeably higher TRs than the other phenolic groups, ranging from 89.7% in EOO10 to 31.0% in EOO90, whereas the other major phenolic group, the secoiridoids, presented TRs between 35.3% in EOO10 and 0% in EOO90. Differences in the TR could be related with the molecular structure of

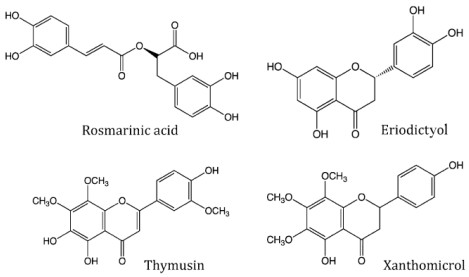


Figure 2. Chemical structure of the most abundant phenolic compounds found in thyme.

Table 2. Phenolic Composition of the Control (olive oil used as enrichment matrix) and the 11 Phenol-Enriched Olive Oils (EOO) by HPLC-ESI-MS/MS**.** The concentration of each phenol in EOO is expressed as mg/kg oil after subtracting the amount of phenol in the control oil.

	phenolic composition (mg/kg oil)											
compd	control	EOO0	EOO10	EOO20	EOO30	EOO40	EOO50	EOO60	EOO70	EOO80	EOO90	EOO100
Phenyl Alcohols												
tyrosol	1.50	6.68	7.75	4.90	7.33	3.61	2.66	1.65	1.34	2.55	0.00	0.00
hydroxytyrosol	0.27	5.47	4.98	5.92	4.76	7.37	3.05	2.72	3.96	1.45	0.93	0.00
Phenolic Acids												
vanillic acid	0.06	5.66	9.42	9.59	5.35	6.48	3.26	3.90	2.86	2.31	2.77	1.28
caffeic acid	0.00	0.17	0.22	0.38	0.65	1.12	1.21	1.19	1.94	2.10	2.07	2.68
hydroxybenzoic acid	0.00	0.26	1.28	2.24	3.17	6.27	5.88	8.39	10.6	12.2	13.6	19.5
homovanillic acid	0.00	0.00	2.10	2.68	1.32	1.41	1.70	1.62	4.48	3.03	3.03	3.32
rosmarinic acid	0.00	0.00	1.35	1.31	6.12	16.0	15.6	24.5	15.5	18.1	23.3	40.4
Secoiridoid Derivatives												
3,4-DHPEA-AC	0.00	3.93	3.43	3.52	3.25	3.00	2.83	2.53	2.33	2.11	0.00	0.00
elenolic acid	57.5	103	68.6	61.9	58.0	10.5	10.2	0.00	0.00	0.00	0.00	0.00
p-HPEA-EDA	52.5	77.2	73.2	71.1	68.8	15.7	17.1	13.14	11.9	10.7	0.00	0.00
p-HPEA-EA	15.4	67.9	68.1	69.9	65.4	14.6	13.1	10.7	8.77	0.00	0.00	0.00
3,4-DHPEA-EA	30.9	48.5	36.0	38.0	28.6	13.2	10.3	6.97	6.03	5.21	2.34	0.00
3,4-DHPEA-EDA	6.99	365	248	209	162	244	215	179	69.7	45.4	6.42	0.00
Lignans												
pinoresinol	1.36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
acetoxypinoresinol	60.09	23.1	12.9	8.03	9.71	13.5	11.6	6.99	7.57	0.72	0.00	0.00
Flavonoids												
apigenin	0.87	0.87	1.07	2.31	2.24	2.49	2.45	2.54	3.12	3.60	3.20	3.65
luteolin	1.37	10.2	8.79	6.42	3.64	9.28	6.08	7.46	5.73	7.46	4.90	5.78
naringenin	0.00	0.59	1.48	4.24	4.44	4.96	4.58	4.58	7.75	13.2	12.0	32.1
eriodictyol	0.00	0.00	1.32	2.53	3.38	9.59	5.90	6.97	12.4	10.9	10.7	15.1
taxifolin	0.00	0.02	0.18	0.27	0.34	0.61	0.55	0.83	0.86	1.02	1.04	1.33
eriodictyol-rutinoside	0.00	0.00	0.34	0.61	1.09	0.37	0.63	1.31	0.26	0.80	0.53	3.14
thymusin	0.00	0.00	102	135	189	207	201	197	175	184	189	268
xanthomycrol	0.00	0.00	6.66	10.0	16.6	26.2	29.1	30.6	42.5	48.6	52.5	60.9
7-methylsudachitin	0.00	0.00	6.62	11.3	15.4	25.5	26.9	28.8	41.9	23.1	51.1	62.4

phenols. Thus, the more apolar nature of flavonoids compared with the secoiridoids (monophenolic structures) could justify the higher transfer efficiency of flavonoids from the extract to a lipophilic matrix, such as olive oil.

According to the results, EOOs developed with a high proportion of thyme will provide an important amount of flavonoids, which have been shown to have multiple positive effects on human health. The direct scavenging potential of flavonoids has been traditionally related to their health benefits; however, current research has gradually changed this dogma by emphasizing their transition metal chelation properties and direct interaction with some enzymes and blood/vascular cells.[25,26] According to recent studies, the chelation activity of flavonoids depends on their molecular structure, and the most effective iron binding sites in flavonoids are those with two hydroxyl groups in an ortho position in the aromatic rings, the 4-keto with the 3-OH and the 4-keto with the 5-OH groups.[12,27] Most of the important flavonoids occurring in the phenolic extracts obtained with high proportions of thyme (**Figure 2**) appear to have these sites, and rosmarinic acid even has two potential chelating sites. This suggests that these phenolic molecules might act synergistically as potent iron chelators, thus, as aimed in this study, complementing the radical scavenging activity of the secoiridoid derivatives from the olive oil, their main antioxidant mechanism.

Oxidative Stability and Antioxidant Capacity

The oxidative stability of the oils was measured with the Rancimat test, and the results (**Figure 3A**) showed that in all the EOOs the phenol enrichment produced a significant increment in the oxidative stability compared with the control oil, indicating that both phenolic sources are able to improve the oxidative stability. Additionally, the oxygen radical absorbance capacity (ORAC assay) was selected to evaluate the antioxidant activity of the oils. This method has been widely used, as it is especially useful for food samples with complex reaction kinetics. The ORAC assay results again show that all the EOOs showed a substantial increase compared with the control oil, and antioxidant capacity increased gradually with the thyme phenolic enrichment (**Figure 3B**). This could be explained by the potent antioxidant capacity of the herbs reported before in different studies.[28,29] The last USDA database for ORAC of selected foods [30] provided very high values of hydrophilic ORAC for dried thyme, ranging from 131 000 to 139 400 μ mol TE/100 g. In this regard, the incorporation of active ingredients of the spices can be a good strategy to improve the oxidative capacity of the olive oil and for the development of new foods.

Bitter Sensory Attribute

In VOOs, bitterness and pungency sensations are related to the presence of phenolic compounds and can persist for rather a long time after

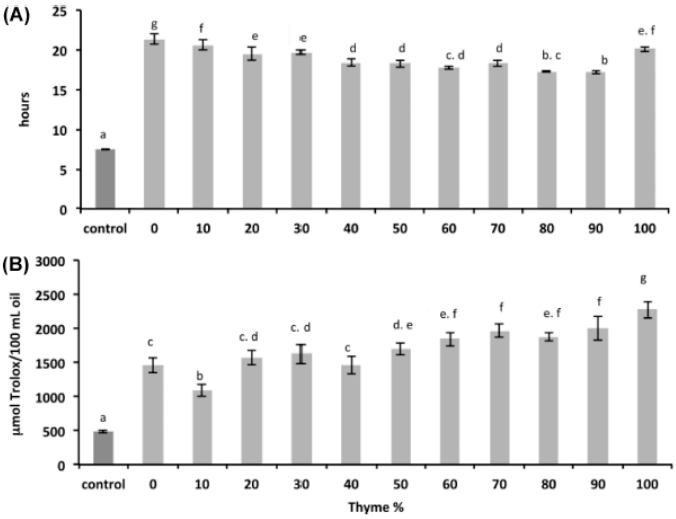


Figure 3. Effect of the phenolic enrichment on the oxidative stability by the Rancimat test (A) and antioxidant capacity by ORAC assay (B) of the control (virgin olive oil) and the 11 phenol-enriched olive oils. Different letters above indicate significant differences between the oils ($p < 0.05$).

deglutition, showing a clear after-effect that might affect consumer acceptance, especially if the olive oil contains a higher level of phenolic compounds.[31] Garcí a et al.[32] indicated the dialdehydic form of decarboxymethylelenolic acid linked to hydroxytyrosol (3,4-DHPEA-EDA) and oleuropein aglycon (3,4-DHPEA-EA) as the main compounds responsible for the bitter taste of VOO; Tovar et al. [33] attributed bitter and pungent notes to ligstroside derivatives, such as *p*-HPEA-EDA. As seen in **Figure 4**, the EOO0 rich in secoiridoid derivatives had the highest bitter score in the sensory test, compared with those with a lower proportion of olive phenols and higher proportion of thyme phenols. These results confirm the role of secoiridoids in the bitter attribute of VOO. Those EOOs combining olive extract and thyme had lower bitterness than EOOs enriched only with olive oil phenols, indicating that the use of herbs or species may improve consumer acceptance of high-phenol olive oils, besides the improvement in their oxidative stability. When combining both phenolic sources, aromatic components present in thyme could also reduce the perception of bitterness due to a bitterness-depressing effect caused by interference with the receptor sites as suggested in some studies. [34,35]

In summary, this study supports the original aim of developing a phenol-enriched olive oil tailored to provide the best relationship between different kinds of phenolic compounds. We conclude that the optimal enriched olive oil with the more balanced phenol composition appears to be EOO40 (prepared with phenolic extract obtained with 60% olive and 40% thyme), which provides the highest amount of secoiridoid derivatives and flavonoids. Moreover, this study revealed that working with extracts instead of infusions, as is traditional when preparing flavored olive oils, allows a phenol enriched and seasoned olive oil to be developed with exactly the amount of phenols required, which is an important factor when a functional food is produced.

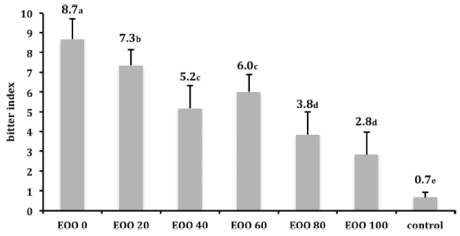


Figure 4. Sensory analysis comparing the bitter taste of the enriched olive oils with the control oil on a 10-point scale going from "absent bitter taste" (0) to "extremely perceptible bitter taste" (10). Different letters above indicate significant differences between the oils ($p < 0.05$).

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SUPPORTING INFORMATION

SRM conditions used for the quantification of the phenolic compounds by HPLC-MS/MS

Phenolic compound	SRM quantification	Cone voltage (V)	Collision energy (eV)
Olive cake			
p-hydroxybenzoic acid	137 > 93	30	15
Tyrosol	137 > 106	40	15
Cinamic acid	147 > 103	20	10
Vanillin	151 > 136	20	10
Hydroxytyrosol	153 > 123	35	10
p-coumaric acid	163 > 119	35	10
Vanillic acid	167 > 123	30	10
Cafeic acid	179 > 135	35	15
Homovanillic acid	181 > 137	25	10
Ferulic acid	193 > 134	30	15
3,4-DHPEA-AC	195 > 135	30	10
Elenolic acid	241 > 139	30	15
Apigenin	269 > 117	60	25
Luteolin	285 > 133	55	25
p-HPEA-EDA	303 > 285	30	5
3,4-DHPEA-EDA	319 > 195	40	5
Ligstroside derivate (1)	335 > 199	40	10
Chlorogenic acid	353 > 191	30	10
Pinoresinol	357 > 151	40	10
p-HPEA-EA	361 > 291	30	10
Oleuropein derivate	365 > 229	35	10
3,4-DHPEA-EA	377 > 275	35	10
Ligstroside derivate (2)	393 > 317	40	15
Acetoxypinoresinol	415 > 151	45	15
Luteolin-7-O-glucoside	447 > 285	50	25
Oleuropein	539 > 377	35	15
Rutin	609 > 300	55	25
Verbascoside	623 > 461	35	20
Thyme			
Thymol	149 > 134	40	15
Protocatechuic acid	153 > 109	45	15
Methoxyluteolin	229 > 119	35	15
Naringenin	271 > 151	40	15
Kaempferol	285 > 151	35	15
Eriodictyol	287 > 151	40	15
Dihydroxykaempferol	287 > 259	45	10
Epicatechin	289 > 245	45	15
Quercetin	301 > 151	40	15
Taxifolin (dihidroquercetin)	303 > 285	40	10
Thymusin	329 > 314	40	25
Xanthomicrol	343 > 328	40	20
Dihidroxanthomicol	345 > 301	40	20
Rosmarinic acid	359 > 161	40	20
7-Methylsudachitin	373 > 358	40	20
Methyl oleuropein aglycone	391 > 255	35	15
Kaempferol-rhamnoside	431 > 285	45	20
Naringenin-glucoside	433 > 271	45	10
Quercetin-arabinoside	433 > 301	45	20
Quercetin-rhamoside	447 > 301	40	15
Luteolin-glucuronide	461 > 285	40	25
Quercetin-4'-O-glucoside	463 > 301	45	25
Ligstroside	523 > 361	35	15
Naringenin-rutinoside	579 > 271	40	20
Luteolin-rutinoside	593 > 285	40	25
Eriodictyol-rutinoside	595 > 287	40	20

II. Studies of phenol digestibility, bioavailability and metabolism by *in vitro* and *in vivo* models

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Publication V *Serra et al. Analytical Bioanalytical Chemistry (2013) 405, 9179-9192*
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Publication II.

A new hydroxytyrosol metabolite identified in human plasma: Hydroxytyrosol acetate sulphate.

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A NEW HYDROXYTYROSOL METABOLITE IDENTIFIED IN HUMAN PLASMA: HYDROXYTYROSOL ACETATE SULPHATE

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Abstract

We report progress in the study of olive oil phenolic metabolites in humans and identify a new hydroxytyrosol metabolite called hydroxytyrosol acetate sulphate, which was determined using tandem MS, after ingestion of 30 ml of olive oil with a high phenolic content (500 mg/kg oil), reaching a maximum concentration of 1.63 μM. In order to understand and explain the generation of this metabolite, two different pathways are proposed.

Keywords: 3,4-DHPEA-EDA, bioavailability, hydroxytyrosol acetate sulphate, mass spectrometry, olive oil

1 INTRODUCTION

For the determination and identification of specific phytochemical metabolites as biomarkers after food consumption, it is necessary to use sensitive and selective analytical techniques to obtain reliable results. Normally, these metabolites appear at low concentration levels in biological samples, such as plasma, urine and tissues, and in general represent only a very small percentage of the ingested amount. Additionally, and especially in the case of polyphenols, the metabolites are difficult to identify and quantify, due to the lack of standards.

Mass spectrometry (MS) and tandem MS (MS/MS) with its different MS modes, such as full-scan, daughter-scan, neutral-loss scan and selected-reaction monitoring (SRM) are effective tools for analysing and identifying compounds that appear at low concentration levels in complex matrices, such as biological samples. Furthermore, apart from their high potential sensitivity, these detector systems have the capability to both determine the molecular weight and provide structural information about the molecule. As a consequence, this tool is very useful for analysing metabolites, especially when commercial standards are not available.

The metabolic fate of phenolic compounds after ingestion has been a subject of study by the scientific community, to find out the mechanisms

through which they exert their activity in the organism. In the case of virgin olive oil (VOO), studies have focused on some specific compounds, such as hydroxytyrosol and tyrosol, because they have been reported as the most biologically active. The importance of these compounds has been reinforced by the demonstration that they can help to prevent the oxidation of low density lipoprotein (LDL) (Marrugat et al., 2004). In consequence, chromatographic procedures to determine these compounds and evaluate their bioavailability have been proposed. A rapid method for detecting and quantifying the metabolites of specific olive oil phenolic compounds was first developed by De La Torre-Carbot et al. (2006) in LDL by SPE and HPLC/ESI-MS/MS. Later, Suárez et al. (2009) developed a modified method based on μSPE-UPLC–ESI-MS/MS, which was applied to determine phenolic metabolites in human plasma after the ingestion of a VOO.

In this work,we report progress in the study of the olive oil phenolic metabolites in humans, and identify a new hydroxytyrosol metabolite called hydroxytyrosol acetate sulphate. This was determined using tandem MS as the detector system with its different modes, after an acute ingestion of an olive oil with a high phenolic content.

2. MATERIAL AND METHODS

2.1 Chemicals and Reagents

Hydroxytyrosol and hydroxytyrosol acetate were purchased from Extrasynthese (Genay, France) and Seprox Biotech (Madrid,Spain), respectively. Methanol (HPLC grade), acetonitrile (HPLC grade), n-hexane and acetic acid were all provided by Scharlau Chemie (Barcelona, Spain). Water was of Milli-Q quality (Millipore Corp, Bedford, MA).

2.2. Olive oil with high phenolic content (HPOO)

HPOO was prepared by adding an extract rich in the main olive phenolic compounds to a virgin olive oil as described in Suárez, Romero, and Motilva (2010) to a total phenol content of 500 mg/kg oil. The concentrations of the hydroxytyrosol and oleuropein derivatives (secoiridoids) are shown in **Table 1.** Values are expressed as lmol of compound contained in 30 ml of HPOO, the dose ingested by the volunteers.

Table 1. Hydroxytyrosol derivatives composition of ingested olive oil with high phenolic content (HPOO). Values are expressed as lmol of compound/30 ml of HPOO.

Compound	μmol/30 ml oil
Hydroxytyrosol (3.4-DHPEA)	1.90 ± 0.07
Hydroxytyrosol acetate (3.4-DHPEA-AC)	3.08 ± 0.02
3.4-DHPEA-EDA	14.6 ± 0.13
3.4-DHPEA-EA	1.88 ± 0.05
Total hydroxytyrosol derivatives	21.5 ± 0.18

2.3. Plasma samples

Plasma samples were obtained by venipuncture from 12 healthy humans after the ingestion of 30 ml of HPOO. The blood samples were collected under basal conditions (after a 12-h overnight fasting period) and 15, 30, 45, 60, 120, 240 and 360 min after the consumption of the 30 ml of HPOO. To obtain the plasma samples, 50 ml of blood were collected in Vacutainer™ tubes, as reported In Suárez et al. (2011). The human study was approved by The Ethical Committee of Clinical Research in Sant Joan University Hospital, Reus, Spain (Ref 08-04-24/4proj5).

2.4. Analysis of phenolic metabolites in human plasma by off-line *μ*SPE-UPLC-MS/MS

Plasma samples were pre-treated by off-line microelution SPE plate (μSPE) and then the pre-concentrated phenolic metabolites were analysed by ultra-performance liquid chromatography coupled to tandem MS (UPLC–MS/MS), as reported in our previous study (Suárez et al., 2009).

3. RESULTS AND DISCUSSION

3.1. Identification of hydroxytyrosol acetate sulphate in human plasma

The characterisation of the metabolites in the plasma samples was based on their ion fragmentation in the MS and tandem MS modes. The full-scan mode by applying different cone voltages, the daughter-scan mode by applying different collision energies, neutral-loss scan and SRM transitions were used to identify and for confirmation purposes. **Table 2** shows the monitored metabolites from the hydroxytyrosol derivatives with the most sensitive SRM transition, which were used for quantification. The MS/MS fragments are also shown for the commercial

Table 2. MS/MS fragments, SRM transitions, cone voltage and collision energy and standard availability for the analysis of plasma samples.

Compound	MS/MS fragments of the standard	SRM transition	Cone voltage (V)	Collision energy (eV)	Standard availability
Hydroxytyrosol	153, 123, 95	153 > 123	35	10	Yes
Hydroxytyrosol sulphate	-	233 > 153	35	15	No
Hydroxytyrosol glucuronide	-	329 > 153	45	20	No
Homovanillic acid	181, 137, 122	181 > 137	25	10	Yes
Homovanillic acid sulphate	-	261 > 181	40	15	No
Homovanillic acid glucuronide	-	357 > 181	40	15	No
Homovanillic alcohol	-	167 > 137	40	15	No
Homovanillic alcohol sulphate	-	247 > 167	40	15	No
Homovanillic alcohol glucuronide	-	343 > 167	40	15	No
Hydroxytyrosol acetate	195, 135, 107	195 > 135	30	10	Yes
Hydroxytyrosol acetate sulphate	-	275 > 195	35	15	No
Hydroxytyrosol acetate glucuronide	-	371 > 195	35	15	No
3,4-DHPEA-EDA	319, 195, 183	319 > 195	40	5	Yes
3,4-DHPEA-EDA sulphate	-	399 > 319	40	15	No
3,4-DHPEA-EDA glucuronide	-	495 > 319	40	15	No

standards of the native molecules. In all the volunteers' human plasma, two main metabolites were quantified as a result of the HPOO ingesta, and neither of them appeared in plasma obtained under basal conditions (**Fig.1**). They corresponded to metabolites with SRM transitions of m/z 233 → m/z 153 and m/z 275 → m/z 195, respectively. The peak with the transition of m/z 233 → m/z 153 was identified as hydroxytyrosol sulphate, as reported in previous studies (De La Torre-Carbot et al., 2006; Suárez et al., 2009). It was the main metabolite identified with a maximum concentration (C_{max}) of 3.04 μM.

The other main metabolite quantified had an SRM transition of m/z 275?m/z 195 and a C_{max} of 1.63 μM. **Fig. 2A** shows the extracted ion chromatogram of the SRM transition of m/z 275 → m/z 195, by analysing the blank plasma, obtained under basal conditions, and the plasma obtained at 120 min. As can be seen, this transition did not appear under basal conditions. In our previous study (Suárez et al., 2011), we identified a metabolite with SMR transitions of m/z 275 → m/z 195 and m/z 275 → m/z 135, which also appeared after the ingestion of a phenol-enriched olive oil and was tentatively identified as dihydroferulic acid sulphate, due to the same molecular formula and weight (**Fig. 3**). However, we later observed that the pharmacokinetic profiles and the C_{max} of this compound and hydroxytyrosol sulphate were

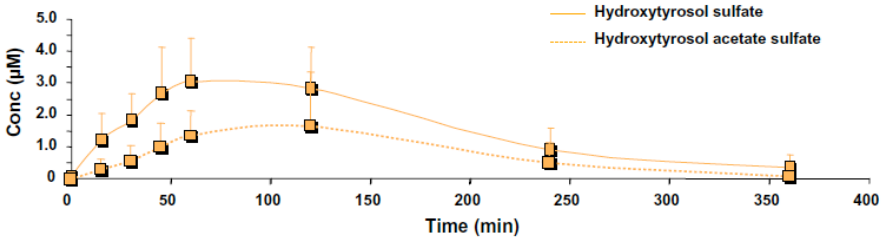

Figure 1. Pharmacokinetics of hydroxytyrosol sulphate and hydroxytyrosol acetate sulphate, the main metabolites quantified in the plasma of human subjects 0–360 min after the ingestion of the olive oil with a high phenolic content (HPOO). Data expressed in μM as mean values (n = 12) with the standard errors depicted by vertical bars. Hydroxytyrosol sulphate is tentatively quantified as hydroxytyrosol, and hydroxytyrosol acetate sulphate is tentatively quantified as ferulic acid.

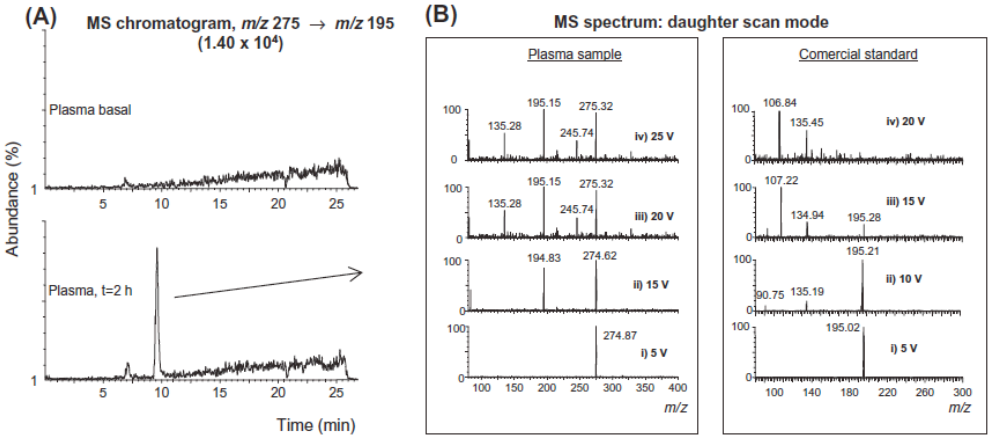

Figure 2. Pharmacokinetics (A) Extracted ion chromatogram of the SRM transition m/z 275 ?m/z 195 for the analysis of blank plasma and plasma at 2 h after the ingestion of 30 ml of HPOO; (B) MS spectrum obtained in daughter scan mode by applying different collision energies of the precursor ion m/z 275 when plasma sample at 2 h after ingestion of 30 ml HPOO was analysed (left), and of the precursor ion of m/z 195 when the commercial standard hydroxytyrosol acetate was analysed (right).

very similar. Therefore, we aimed to research this compound in greater depth, considering it more likely that this compound was a metabolism product of hydroxytyrosol or secoiridoids, as major oil phenols, rather than a ferulic acid metabolite described as a product of colonic fermentation.

The ESI-MS spectrum in full-scan mode of this metabolite that eluted at 9.54 min showed an intense ion at m/z 275 as the precursor ion. By applying different cone voltages in the full-scan mode only one ion fragment was formed, at m/z 195. This ion was related to the loss of the sulphate molecule (m/z 80). The fact that only one fragment (m/z 195) was observed could be due to the presence of the metabolite at low concentration and so the full-scan mode was not sensitive enough.

In order to identify other product ions from this metabolite, and therefore to know its chemical structure, the plasma extract was concentrated with nitrogen flow and additionally, the electrospray ionisation source (ESI) was brought closer to the cone. Under these special conditions, the concentrated plasma extract was analysed in the daughter scan mode, in the tandem MS mode, and by applying different collision energies. Under these conditions, apart from the precursor ion m/z 275, two major product ions were observed, one at m/z 246 and the second at m/z 135 (**Figure 2B**). These fragment ions were related to the loss of the methoxy and ester groups, respectively.

The presence of the product ion of m/z 135 allowed us to identify this metabolite as hydroxytyrosol acetate sulphate. This was confirmed by comparing its MS spectrum with the

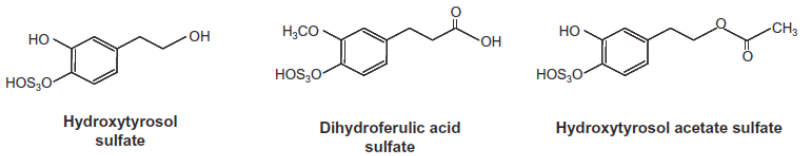

Figure 3. Molecular structure of hydroxytyrosol sulphate and hydroxytyrosol acetate sulphate, the main metabolites detected in plasma, and dihydroferulic acid sulphate, the metabolite tentatively identified in previous studies.

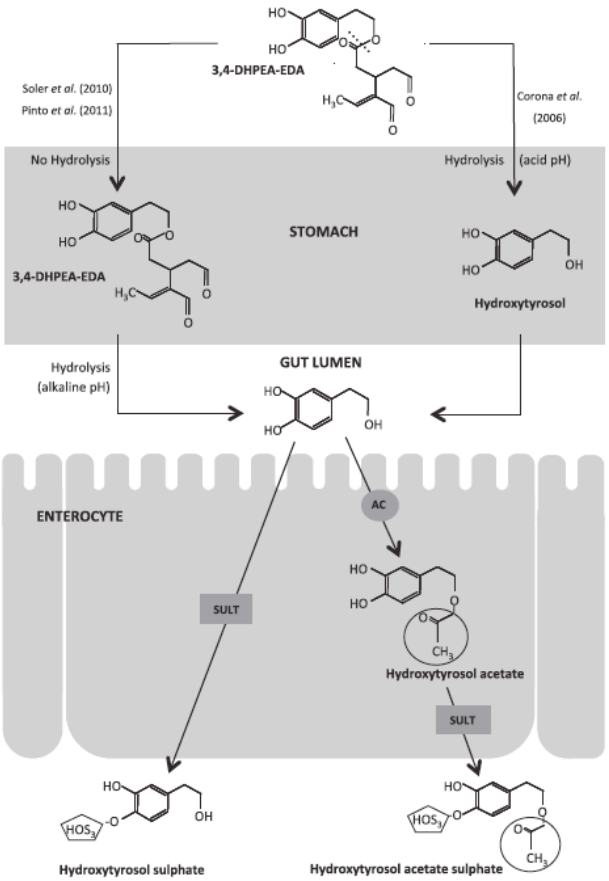

Figure 4. Proposed pathway for hydroxytyrosol acetate sulphate as a result of phase II conjugation metabolism in the enterocytes by acyltransferase enzymes (AC). Prior to the esterification of hydroxytyrosol, hydrolysis of 3,4-DHPEA-EDA occurs either in the stomach or in the gut lumen and a great amount of hydroxytyrosol is released. In parallel, hydroxytyrosol can also undergo phase II conjugation metabolism by sulfotransferase enzymes (SULT) producing hydroxytyrosol sulphate.

MS spectrum of a commercial hydroxytyrosol acetate standard. **Figure 2B** shows the MS spectrum obtained in the daughter scan mode by applying different collision energies to the peak m/z 275 (hydroxytyrosol acetate sulphate) and the commercial hydroxytyrosol acetate standard (m/z 195). By comparing their MS spectra, it can be seen that m/z 195 and m/z 135 ions are found in both spectrums.

3.2. Proposed metabolic pathways for hydroxytyrosol acetate sulphate

To the best of our knowledge, hydroxytyrosol acetate sulphate has been identified as a hydroxytyrosol metabolite for the first time in the present study. Once we confirmed the occurrence of this metabolite in the plasma after the ingestion of a single dose (30 ml) of HPOO, we considered possible pathways that could explain its presence in plasma.

3.2.1. Hydroxytyrosol acetate from olive oil

4-(Acetoxyethyl)-1,2-dihydroxybenzene or hydroxytyrosol acetate was described for the first time in olive oil by Brenes, García, García, Rios, and Garrido (1999) and is found in most Spanish virgin olive oils. In the present study, the HPOO contains hydroxytyrosol and hydroxytyrosol acetate at concentrations of 1.90 μmol/30 ml oil and 3.08 μmol/30 ml, respectively (**Table 1**). Recently, it was reported by Mateos et al. (2011) that hydroxytyrosol acetate is more soluble in the lipophilic phases than hydroxytyrosol, due to the presence of the ester group, which was demonstrated in a Caco-2 cell model. Thus, this increased lipophilicity means that hydroxytyrosol acetate is better absorbed across intestinal epithelial cell monolayers than free hydroxytyrosol. Although the concentration of hydroxytyrosol acetate in the HPOO ingested by volunteers in the present study was lower than other hydroxytyrosol derivatives as 3,4-DHPEA-EDA (**Table 1**), the presence of hydroxytyrosol acetate sulphate in plasma post-ingestion could be in part the result of direct sulfation by sulfotransferase enzyme (SULT) in the intestinal cells, or the result of the acetylation of free hydroxytyrosol as explained below (**Figure 4**).

3.2.2. Phase II metabolism acetylation

3,4-DHPEA-EDA is the phenolic compound most abundant in virgin olive oil (Suárez, Macià, Romero, & Motilva, 2008). Therefore, the main metabolites detected in the plasma could be related to the hydrolysis of this compound. Two recent studies by Soler et al. (2010) and Pinto et al. (2011) using an in vitro digestion model showed that 3,4-DHPEA-EDA remained stable under the acidic conditions of the gastric step. By contrast, Corona et al. (2006) reported previously that 3,4-DHPEA-EDA suffers a rapid hydrolysis producing free hydroxytyrosol in the same acidic environment

(**Figure 4**). According to Mateos et al. (2011), hydroxytyrosol derivates are more sensitive to the mild alkaline conditions in the small intestine enhancing the amount of free hydroxytyrosol released into the gut lumen. Once absorbed, hydroxytyrosol can undergo phase II conjugation metabolism by sulfotransferase enzymes (SULT) in the intestine and liver, producing hydroxytyrosol sulphate. Methylation, sulfation and glucuronidation represent the main phase II metabolic biotransformations for phenolic compounds. Acetylation has also been described in xenobiotics by a group of enzymes called acyltransferases, which catalyses the transfer of acetyl groups from acetyl-CoA (Levsen et al., 2005). These enzymes catalyse either N-acetylation or O-acetylation. Considering that a great amount of hydroxytyrosol is released after the ingestion of HPOO, another conjugation pathway is proposed, apart from the sulfation that could explain the presence of hydroxytyrosol acetate sulphate in the plasma. The acetylation of hydroxytyrosol has never been described, but due to the presence of acyltransferase enzymes, which have been previously related to drug metabolism, and the high amount of hydroxytyrosol, O-acetylation could be possible at the hydroxyl group of hydroxytyrosol as a metabolic transformation, in parallel to other well-known reactions (**Figure 4**).

4. CONCLUDING REMARKS

In the present study, progress was made in identifying the olive oil phenolic metabolites in humans, with a new hydroxytyrosol metabolite called hydroxytyrosol acetate sulphate being found. This was determined using tandem MS as the detection system. The pharmacokinetics and comparison of its MS spectrum with a standard of the native molecule (hydroxytyrosol acetate), led us to confirm the appearance of this new metabolite never previously described in plasma after the ingestion of VOO.

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Publication III.

Impact of olive oil phenols concentration on human plasmatic phenolic metabolites.

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IMPACT OF OLIVE OIL PHENOLIC CONCENTRATION ON HUMAN PLASMATIC PHENOL METABOLITES

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Abstract

Three different functional phenol-enriched virgin olive oils (FVOO) were prepared with a phenolic content of 250 (L-FVOO), 500 (M-FVOO), and 750 mg (H-FVOO) total phenols/kg. In a randomised, cross-over study with 12 healthy volunteers, the pharmacokinetics of phenolic biological metabolites was assessed. An increasing linear trend was observed for hydroxytyrosol sulfate, the main phenolic metabolite quantified In plasma, with C_{max} values of 1.35, 3.32, and 4.09 μmol/l, and AUC mean values of 263.7, 581.4, and 724.4 μmol/min for L-FVOO, M-FVOO, and H-FVOO, respectively. From our data an acute intake of phenol-enriched olive oils promotes a dose-dependent response of phenol conjugate metabolites in human plasma. Also, we point out for the first time hydroxytyrosol acetate sulfate as a main biological metabolite of hydroxytyrosol from olive oil ingestion.

Keywords: *Bioavailability / dose-response / Olive oil phenols / plasma phenolic metabolites*

1 INTRODUCTION

Recently the European Food Safety Authority (EFSA) (EFSA Panel On Dietetic Products, Nutrition and Allergies, 2011) has released a health claim concerning the protective effects of the ingestion of phenolic compounds from olive oil on low density lipoprotein (LDL) oxidation, one measure of atherosclerosis generation and progression. The EFSA Panel considers that in order to support the claim, 5 mg of hydroxytyrosol and its derivatives in olive oil should be consumed daily. However, the phenolic content in virgin olive

oil (VOO) is influenced by multiple agronomic and technological factors (Aparicio, Roda, Albi & Gutiérrez, 1999; Salvador, Aranda & Fregapane, 1999; Tous et al., 1997). As a result, it is difficult to ensure a fixed intake of phenols from a natural food, such as VOO, which is subject to a high variability. Furthermore, phenolic concentration in most VOOs available on the market may be too low to allow the consumption of 5 mg of hydroxytyrosol and its derivatives within the context of a balanced diet. For these reasons, the enrichment of VOO with its own phenols could be a possible strategy to increase and standardise the daily intake of hydroxytyrosol without increasing the caloric intake.

One requisite in order to develop functional VOOs is to assess whether very high doses of phenolic compounds could be absorbed from enriched VOOs in a dose-dependent manner in humans. High phenols doses could lead to the saturation of coupled metabolic pathways of conjugating enzymes and transporters. This could impair the efflux of the phenolic metabolites at the apical or basolateral sides of the enterocyte. As a result, a threshold could exist in olive oil phenolic absorption. In addition, high amounts of phenols increase the bitter and pungent sensory attributes of VOO. This fact can diminish consumer acceptance for the enriched VOO. Due all to the above-mentioned, a balance between the content of olive oil phenols, which can potentially increase health benefits, their absorption, and consumer acceptance must be achieved.

The aim of this exploratory study was to assess the bioavailability of the main phenolic biological metabolites from similar VOOs enriched with the full spectrum of their phenolic compounds, but differing in their total phenolic content. Pharmacokinetic parameters of phenolic metabolites in humans after the oral ingestion of 30 ml of three functional phenol-enriched virgin olive oils (FVOO) at 250 (L-FVOO), 500 (M-FVOO), and 750 mg (H-FVOO) total phenols/ kg of oil were calculated. We further evaluated the effects of phenol enrichment on the antioxidant capacity and sensorial acceptance of the three prepared FVOOs.

2. MATERIALS AND METHODS

2.1. Chemicals and reagents

Apigenin, luteolin, hydroxytyrosol, tyrosol, and p-coumaric acid, were purchased from Extrasynthèse (Genay, France). Hydroxytyrosol acetate was purchased from Seprox Biotech (Madrid, Spain). Caffeic and homovanillic acids were purchased from Fluka Co. (Buchs, Switzerland), (+)-pinoresinol was acquired from Arbo Nova (Turku, Finland), and catechol from Sigma–Aldrich (Germany). A stock solution of each standard compound was dissolved in methanol, and solutions were stored in a dark flask at 40 C. 2,20-Azobis(2-amidino-propano) dihydrochloride (AAPH) and Trolox were from Acros Organics (Geel, Belgium). Methanol (HPLC grade), acetonitrile (HPLC grade) and acetic acid were provided by Scharlau Chemie (Barcelona, Spain). Orthophosphoric acid (85%) was purchased from Panreac (Barcelona, Spain). Water was Milli- Q quality (Millipore Corp., Bedford, MA).

2.2. Preparation of the phenol-enriched olive oils

Three functional phenol-enriched virgin olive oils (FVOO) with phenolic content at 250 (L-FVOO), 500 (M-FVOO), and 750 mg (H-FVOO) total phenols/kg of oil were prepared. The FVOOs were prepared by the addition of an extract rich in the main VOO phenolic compounds to a VOO with low phenolic content (<80 mg/kg) used as enrichment matrix. This extract was obtained from olive cakes following the method described by Suárez, Romero, Ramo, Macià & Motilva, 2009a, and basically included secoiridoid derivatives (89.4%), phenyl alcohols (3.5%) and flavonoids (6.0%). To prepare the three phenol-enriched oils, 0.4 (L-FVOO), 2.5 (M-FVOO), and 5.3 g (H-FVOO) of olive cake phenolic extract/100 mL olive oil were dissolved in water (2 ml/100 mL oil) and mixed by vortex until well dispersed. A small aliquot of oil (VOO used as matrix enrichment) was added to the mixture and dispersed in the ultrasonic bath to make a preliminary emulsion. Finally, the whole oil (VOO used as matrix enrichment) volume was added using a Polytron (Kinematica, Littau, Switzerland) until complete homogenisation.

The three FVOOs were maintained in dark bottles before use. The total phenolic content of olive oils was determined spectrophotometrically at 725 nm following the Folin–Ciocalteu method (Vázquez-Roncero, Janer del Valle & Janer del Valle, 1973). Results are expressed as milligrammes of caffeic acid per kilogramme of olive oil. Subsequently, the phenolic composition of the three prepared FVOOs was analysed by HPLC coupled to

tandem MS (MS/ MS) (Suárez, Macià, Romero & Motilva, 2008).

2.3. Antioxidant capacity (ORAC value) and sensory analysis of the phenol-enriched olive oils

The antioxidant capacity of the FVOOs was measured by the oxygen radical absorbance capacity (ORAC) assay. This method has been widely used, as it is especially useful for food samples with complex reaction kinetics. The assay was carried out according to the method described by Suárez, Romero & Motilva, 2010. A sensory analysis was performed to assess the acceptance of the three EVOOs at the time of the human intervention. A specific profile sheet was set up where volunteers had to assign a score on a 6- point acceptance scale, going from "I dislike it very much" (0) to "I like it very much" (6), after the ingestion of 30 ml of each olive oil.

2.4. Human volunteers and experimental design

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the local IRB (Clinical Research Ethical Committee, University Hospital Sant Joan, Reus, Spain). The study is registered at ClinicalTrials.gov (Identifier: NCT01347515). The subjects gave their written informed consent before their participation.

A total of 12 healthy volunteers (six women and six men) were recruited to carry out the study. The volunteers, aged between 22 and 60 yrs, were considered healthy according to a physical examination and routine laboratory tests. Males had a mean weight of 85.1 ± 10.6 kg and the females had a mean weight of 63.6 ± 5.7 kg while the body mass index (BMI) was 28.3 ± 2.4 for men and 25.4 ± 1.8 for women.

A randomised, controlled, crossover trial with three treatment conditions FVOO (L-FVOO, M-FVOO and H-FVOO) was designed (**Fig. 1**). Subjects participated in three one-day experimental sessions separated by a 1 week washout period. Participants were instructed to follow a stabilisation diet containing 10% saturated fatty acids during the 2 weeks prior to the first postprandial test, for 1 week washout period between the first and second postprandial test, and another week before the third postprandial test. For 2 days before each postprandial test, the participants followed a polyphenol-free diet in which they avoided olive oil, olives, fresh fruit or juices, vegetables, legumes, soya, chocolate, coffee, tea, wine, and beer. Compliance with the polyphenol-free diet was assessed using a 24 h dietary recall of the day prior to the postprandial test day. Trained dieticians explained how to complete these questionnaires to the participants and talked them through the questionnaires on each postprandial test day. The participants were instructed to avoid intense physical exercise 3 days prior to the postprandial test. On the day of the experiment, the acute ingestion of 30 ml oil with 80 g white-bread was done at 8 a.m. after an overnight fast.

Plasma samples were obtained by venipuncture from volunteers and were collected under basal conditions (after a 12 h overnight fasting period) and at 15, 30, 45, 60, 120, 240, and 360 min after 30 ml of FVOO intake. Blood (10 ml) was collected in Vacutainer ™ tubes containing lithium-heparin

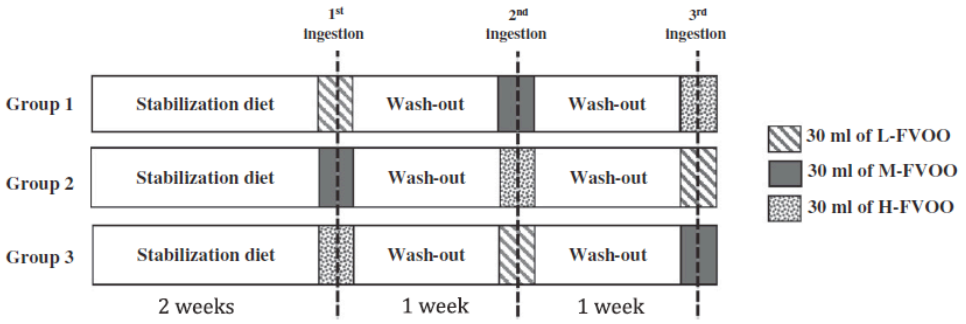

Figure 1. Experimental protocol for the intervention study

as anticoagulant. Collection tubes were protected from the light with aluminium foil and centrifuged for 15 min at 1,700g and 4 °C (Model H-103RS; Kokusan Corporation, Tokyo, Japan). Plasma was immediately separated from the cells and kept at 80 °C until analysis.

2.5. Analysis of phenolic metabolites in plasma samples

The extraction of the phenolic metabolites from the plasma samples was carried out using microelution plates (Waters, Milford, MA) packed with 2 mg of OASIS HLB sorbent (Waters) and the chromatographic analysis of the phenolic metabolites was carried out by UPLC coupled to tandem MS (MS/MS) with our previous validated method (Suárez et al., 2009b). The chromatographic system consisted of an AcQuityTM UPLC equipped with a Waters binary pump system using an AcQuity UPLCTM BEH C18 column (1.7 μm, 100 × 2.1 mm i.d.). During the analysis, the column was kept at 30 °C and the flow rate was 0.4 ml/min. The mobile phase was MilliQ water/acetic acid (100:0.2 v/v), as solvent A, and acetonitrile, as solvent B. Full-scan mode MS and MS/MS, based on neutral loss scan and product ion scan, were used to identify and quantify the phenolic metabolites from plasma samples. These techniques are very effective to verify the structural information of the compounds when standards are not commercially available. First, the analyses were carried out in full-scan mode (from m/z 80 to 800) by applying different cone voltages from 20 to 60 V. The MS spectrum obtained when low cone voltages were used provided information about the precursor ion or the [M–H]. Also, when high cone voltages were applied, specific fragment ions were generated and the MS spectrum gave information about their chemical structure. The structural information was also verified by using the product ion scan and neutral loss scan in the MS/MS mode. In the product ion scan experiments, the product ions are produced by collisionactivated dissociation of the selected precursor ion in the collision cell. Neutral loss scans of 80 and 176 units were used to characterise the sulfate and glucuronide forms, respectively. The detection and quantification of the phenolic metabolites were then performed based on their ion fragmentation in MS/MS by using SRM as the most sensitive mode.

Due to the lack of standards for olive oil phenolic metabolites, they were quantified with the calibration curves corresponding to their phenolic precursors. In this way, metabolites derived from hydroxytyrosol, hydroxytyrosol acetate, and tyrosol were quantified using the calibration curves of hydroxytyrosol, hydroxytyrosol acetate, and tyrosol respectively; homovanillic acid, vanillic acid, and vanillin metabolites were quantified using the calibration curve of homovanillic acid; p-coumaric, p-hydroxybenzoic, and ferulic acids metabolites were quantified by means of the p-coumaric acid calibration curve. Apigenin metabolites were quantified with the apigenin calibration curve.

2.6. Statistical analysis and pharmacokinetic parameters

Statistical analysis was performed using Statgraphics plus V.5.1 software (Manugistics Inc., Rockville, MA). Data was analysed via analysis of variance (ANOVA) followed by the post hoc Tukey's test (inter-treatment comparison). Values of p lower than 0.05 were regarded as statistically significant.

Kinetic parameters of the main metabolites of VOO phenolic compounds were calculated by means of pharmacokinetic (PK) functions (for Microsoft Excel). Area under the plasma concentration–time curve (AUC) was calculated using the trapezoidal rule method. Peak plasma concentration (C_{max}) and time to maximum plasma concentration (T_{max}) were the observed values. Data on pharmacokinetic parameters are presented as mean values ± standard error.

3. RESULTS

3.1. Characterisation of the phenol-enriched virgin olive oils

The phenolic composition of L-FVOO, M-FVOO, and H-FVOO was analysed by HPLC–ESI-MS/MS with the aim of establishing a relationship between the amount of ingested phenols and the pharmacokinetic response. Results of the phenol quantification of the VOO whose phenolic extract was used as enrichment matrix, and of each FVOO prepared are shown in **Table 1**. Oleuropein derivatives: 3,4-DHPEA–EDA (hydroxytyrosol linked to the dialdehydic form of elenolic acid), 3,4-DHPEA–EA (hydroxytyrosol linked to elenolic acid), and 3,4-DHPEA–AC (hydroxytyrosol acetate) were the major compounds in the three

Table 1. Phenolic composition of virgin olive oil (VOO) used as matrix enrichment, and the phenol-enriched olive oils L-FVOO (250 mg/kg), M-FVOO (500 mg/kg), H-FVOO (750 mg/kg) determined by HPLC-ESI-MS/MS. Values are expressed as mg compound or μmol (in brackets) by 30 mL of oil (ingested dose)

Compound	VOO	L-FVOO	M-FVOO	H-FVOO
Hydroxybenzoic acid	0.00	0(0.01)	0.02(0.11)	0.03(0.21)
Vanillic acid	0.00	0.02(0.12)	0.08(0.47)	0.10(0.59)
p-coumaric acid	0.00	0.02(0.13)	0.03(0.20)	0.08(0.48)
Total phenolic acids	0.00	0.04(0.27)	0.13(0.78)	0.21(1.28)
Hydroxytyrosol	0.05	0.21(1.35)	0.29(1.87)	0.37(2.45)
Hydroxytyrosol acetate	0.00	0.16(0.81)	0.60(3.06)	0.90(4.62)
3,4-DHPEA-EDA*	0.10	1.51(4.72)	4.67(14.64)	8.42(26.39)
3,4-DHPEA-EA*	0.32	0.36(0.95)	0.71(1.88)	1.18(3.12)
Total oleuropein derivatives	0.47	2.23(7.83)	6.27(21.46)	10.87(36.59)
Tyrosol	0.00	0.12(0.85)	0.17(1.27)	0.20(1.49)
p-DHPEA-EDA*	0.28	0.28(0.93)	0.31(1.03)	0.41(1.35)
p-DHPEA-EA*	0.46	0.52(1.45)	0.54(1.48)	0.58(1.61)
Total ligstroside derivatives	0.74	0.92(3.23)	1.02(3.78)	1.19(4.45)
Elenolic acid	0.70	1.22(5.05)	2.16(8.96)	3.76(15.61)
Pinoresinol	0.18	0.27(0.75)	0.19(0.11)	0.19(0.53)
Acetoxipinoresinol**	7.21	6.95(16.75)	5.98(5.80)	5.81(13.99)
Total lignans	7.38	7.22(17.50)	6.17(14.95)	6.00(14.52)
Luteolin	0.10	0.17(0.58)	0.28(0.97)	0.57(2.02)
Apigenin	0.06	0.06(0.21)	0.08(0.28)	0.10(0.36)
Total flavonoids	0.16	0.22(0.79)	0.35(1.25)	0.67(2.38)
Total phenols UPLC-MS/MS	9.45	11.85(34.67)	16.10(82.17)	22.70(74.83)

* quantified with calibration curve of hydroxytyrosol ** quantified with calibration curve of pinoresinol

FVOOs with a 20-fold or higher increase from the original VOO. Thus, the enrichment of VOO with its own phenols, giving a high content of phenolic compounds derived from hydroxytyrosol, is technically feasible.

Parallel to the increase of phenolic content, a similar increase of the antioxidant capacity, measured as ORAC values, of the FVOOs was observed (**Fig. 2A**). The effect of the phenol enrichment on the sensory acceptability of olive oils showed an inverse relationship between the phenol concentration and the acceptability level of the enriched olive oils (**Fig. 2B**). L-FVOO, (an extra virgin olive oil with a phenolic content that can be found in the market) with the lowest phenolic enrichment did not show significant differences with the VOO control (the matrix for enrichment), M-FVOO and H-FVOO with mean

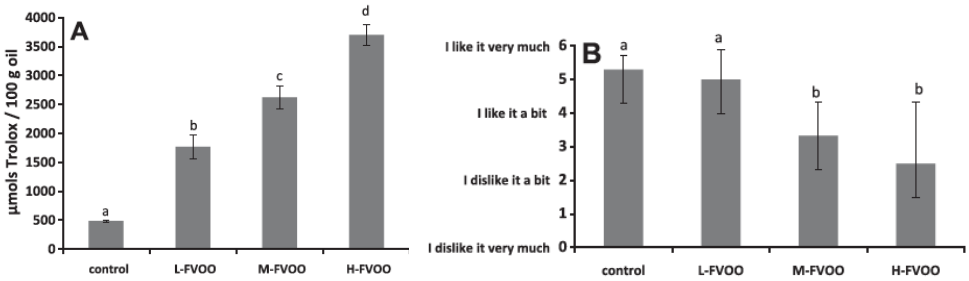

Figure 2. Phenolic (A): Effect of phenolic enrichment on the antioxidant capacity of the oils determined by ORAC assay of the control oil (matrix enrichment), L-FVOO, M-FVOO and H-FVOO, virgin olive oils enriched with 250, 500, and 750 mg/kg of phenolic compounds, respectively. (B): sensory evaluation of the enriched oils by a 7-point acceptance scale (n = 12). a,b,c Different superscript denotes significant differences ($p < 0.05$).

Table 2. Pharmacokinetic analysis of the main olive oil phenolic metabolites detected in human plasma following the ingestion of 30 mL of L-FVOO, M-FVOO and H-FVOO. Different letters show significant differences between oils ($p < 0.05$).

		AUC (μmol/min)		Cmax (μmol/l)		Tmax (min)	
		mean	SD	mean	SD	median	range
hydroxytyrosol-sulfate*	L-FVOO	264± 160 a		1.35± 0.72 a		60	75
	M-FVOO	581± 283 b		3.32± 1.56 b		60	75
	H-FVOO	724± 314 b		4.09± 1.99 b		90	75
hydroxytyrosol acetate-sulfate*	L-FVOO	85.5± 95.1 a		0.46± 0.26 a		60	90
	M-FVOO	292± 255 ab		1.89± 1.63 b		120	75
	H-FVOO	383± 297 b		2.24± 0.51 b		60	90
homovanillic acid**	L-FVOO	38.2± 50.6 a		0.17± 0.19 a		90	210
	M-FVOO	106± 64.0 a		0.63± 0.30 a		60	195
	H-FVOO	120± 116 a		0.65± 0.41 a		60	90
homovanillic acid sulfate**	L-FVOO	12.7± 13.1 a		0.12± 0.15 a		60	75
	M-FVOO	47.4± 43.7 a		0.27± 0.25 a		60	30
	H-FVOO	79.5± 103.7 a		0.53± 0.62 a		60	90

* Metabolites quantified in all the volunteers (n=12) ** Metabolites quantified in some volunteers (n=7)

scores of 3.3 and 2.5 out of 6, respectively, presented lower scores, probably due to the increased bitter taste of these oils.

3.2. Phenol metabolites plasma kinetics

The main phenolic metabolites detected in plasma samples 0–6 h after ingestion of the three oils were hydroxytyrosol sulfate, hydroxytyrosol acetate sulfate, homovanillic acid and homovanillic acid sulfate. Their pharmacokinetic parameters are presented in **Table 2**. Olive oils phenols showed a rapid absorption with Tmax around 1–2 h after ingestion (**Figs. 3 and 4; Table 2**). Plasma concentrations returned to baseline values 6 h after ingestion. In this study, sulfation was the main conjugation pathway for olive oil phenols, whereas the glucuronidated forms were not detected. Hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate, the main biological metabolites detected, showed a dose–response linear trend to the intake of the three phenol-enriched olive oils (**Fig. 3**). In accordance with a previous study (Suárez et al., 2011), hydroxytyrosol sulfate appears to be a good biomarker of olive oil phenols intake.

The dose–response effect was observed in AUC and Cmax values reached in human plasma as the phenolic metabolites increased in parallel with the amount of phenols ingested with FVOO (**Table 2**). However, no significant differences were observed between the higher doses (M-FVOO and H-FVOO) in the pharmacokinetic parameters AUC and Cmax. Homovanillic acid and homovanillic acid sulfate did not show significant differences between the three treatments in their pharmacokinetics (**Table 2; Fig. 4**). Tmax values observed for all metabolites ranged from 45 to 120 min.

The large interindividual variability could explain why no statistically significant differences were observed between M-FVOO and H-FVOO. **Fig. 5** shows the individual AUC values of hydroxytyrosol sulfate, the main metabolite of hydroxytyrosol quantified in plasma, from all the volunteers. Whereas hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate were detected and quantified in all plasma volunteers, homovanillic acid and homovanillic acid-sulfate, were only detected in seven subjects. Concentrations of these latter compounds were low at baseline (t =

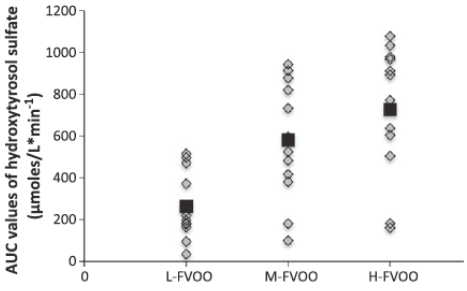

Figure 5. AUC values of hydroxytyrosol sulfate for all the volunteers after the ingestion of L-FVOO, M-FVOO and H-FVOO with phenolic contents at 250, 500 and 750 mg phenols/kg. Mean values are in black.

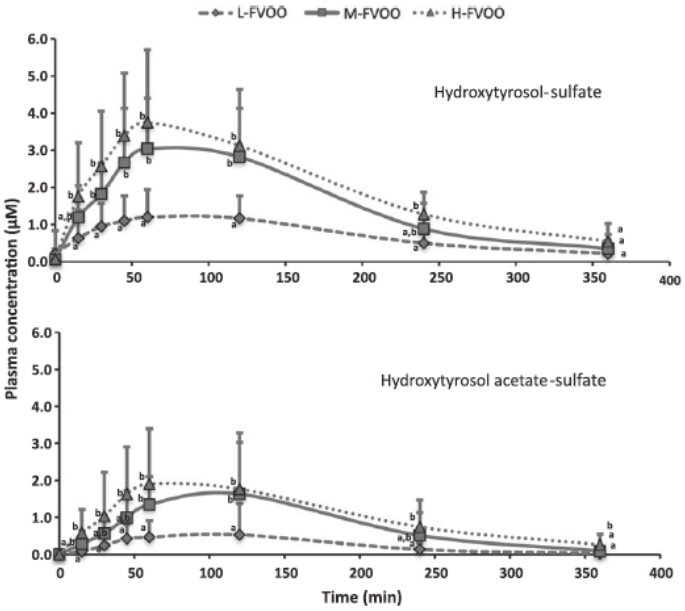

Figure 3. Human plasma pharmacokinetics of hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate, at 0–6 h after the ingestion of enriched olive oils with phenolic contents of 250 (L-FVOO), 500 (M-FVOO) and 750 mg (H-FVOO) phenols/kg. Data are expressed in μM as mean values, with standard errors (n = 12) depicted by vertical bars. [a,b,c] Different superscripts denotes differences between oils at each time ($p < 0.05$).

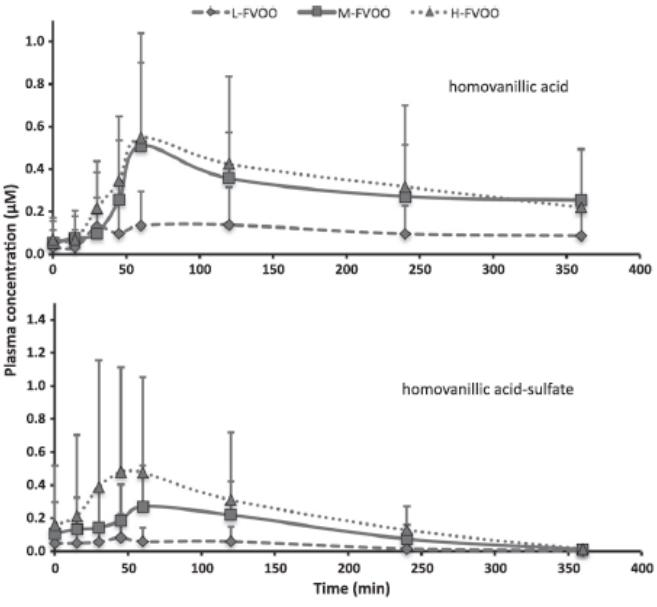

Figure 4. Human plasma pharma-cokinetics of homovanillic acid and homovanillic acid sulfate, at 0–6 h after the ingestion of enriched olive oils with phenolic contents of 250 (L-FVOO), 500 (M-FVOO) and 750 mg (H-FVOO) phenols/kg. Data are expressed in μM as mean values, with standard errors (n = 12) depicted by vertical bars. a,b,c Different superscripts denotes differences between oils at each time ($p < 0.05$).

0), their pharmacokinetic response being the lowest after the intake of LFVOO (**Fig. 4**). It is noticeable that the plasma concentration of homovanillic acid remained above the baseline concentration, even 6 h after of oil ingestion. This observation could be related to its endogenous origin from dopamine metabolism.

With regard to flavonoids, free luteolin and apigenin glucuronide were detected, but at very low concentrations, which could be due to the small amount of this phenolic group provided by the consumed oils. The chromatographic analysis of the plasma samples also revealed the presence of other compounds in similarly low concentrations (vanillin sulfate, hydroxybenzoic and hydroxyphenylacetic acids). However these minor metabolites were detected in basal plasma and did not show any dose response after the oil intake (**Table: Supporting Information**).

4. DISCUSSION

In the present study, we examined the dose–response pharmacokinetics in plasma of olive oil phenol biological metabolites. Analysis of these metabolites showed a dose–response relationship associated with the phenolic content of the olive oil. Cmax and AUC values after the ingestion of 30 ml of L-FVOO (250 mg total phenols/ kg) were significantly lower than those observed after the ingestion of M-FVOO or H-FVOO enriched with higher amounts of phenols. Hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate were identified as the main biological metabolites of olive oil phenolic compounds in plasma after olive oils ingestion.

It has been previously reported that an increase in the dose of olive oil phenols dose-dependently raised the concentration of glucuronide conjugates of hydroxytyrosol and tyrosol in urine (Miró-Casas et al., 2003; Salvini et al., 2006; Visioli et al., 2000). In our study the lack of differences between M-FVOO and H-FVOO in the AUC values of the main metabolites could be attributable to the high intra-individual biological variability (approximately 50% with respect to the CV% of AUC values). The fact that only 12 volunteers, and with gender and age differences, participated in the study could account for this. A high inter-individual variability related to metabolic processes has been previously described in human intervention pharmacokinetic studies (Borges et al., 2010). This inter-individual variability could be explained by modulations in the capacity of phase II enzymes toward phenolic compounds exerted by polymorphisms, epigenetic and genetic factors (Duthie, 2011).

Alternatively, it could be hypothesised that some sort of saturation of the intestinal transporters, or in the sulfation, after the intake of 30 ml H-FVOO could occur. In this sense, results of i*n vitro* and *in vivo* experimental studies showed that some saturation in the metabolism and absorption of phenols could occur (Hu, Chen & Lin, 2003; Silberberg et al., 2006). Phenol bioavailability is the result of coupled metabolic activities of conjugating enzymes and efflux transporters, which could represent a significant barrier to the oral bioavailability of phenols (Liu & Hu, 2007). Against this hypothesis, we observed that the fraction of hydroxytyrosol metabolites recovered in plasma when compared with the dose ingested is quite similar for all three EVOOs studied, ranging from 17% (H-FVOO) to 22% (M-FVOO). This suggests that there were no limiting processes in the bioavailability of phenols.

Hydroxytyrosol acetate sulfate has been recently identified in plasma as a key product of hydroxytyrosol metabolism, due to the high concentrations found in plasma after the ingestion of FVOOs. In a previous study we tentatively identified this compound as dihydroferulic acid sulfate (Suárez et al., 2011). The kinetics of this compound follows a similar shape to that of hydroxytyrosol sulfate. This suggested to us that it could be a metabolite from hydroxytyrosol or its secoiridoid derivatives, rather than a conjugate metabolite of ferulic acid, mainly described as a colonic fermentation product. Therefore, we decided to confirm the identification of this metabolite. The molecular weight and the comparison of its MS spectrum with the standard of hydroxytyrosol acetate, allowed us to identify it as hydroxytyrosol acetate sulfate (Rubió, Macià, Romero, & Motilva, 2012). This metabolite might come from the native compound of hydroxytyrosol acetate present in the oil. Hydroxytyrosol acetate could be effectively absorbed and then conjugated via sulfotransferase enzymes. A recent in vitro study performed by Mateos et al. (2011), with Caco-2 cells, showed that hydroxytyrosol acetate and its conjugates were much more bioavailable than hydroxytyrosol. This

could be due to the fact that the acetylation of hydroxytyrosol significantly increases its transport across the small intestinal epithelial cell barrier, due to its lipophilic structure. However, the relatively small amount of hydroxytyrosol acetate in the FVOOs also suggests other possible metabolic pathways. **Fig. 6** shows several proposed pathways for hydroxytyrosol acetate sulfate endogenous generation. One of the most plausible pathways could be the hydrolysis of the main oleuropein derivative (3,4-DHPEA–EDA), either during gastric digestion under acidic conditions according to Corona et al. (2006) or, by contrast, in the small intestine under mild alkaline conditions, having remained stable in the stomach (Pinto et al., 2011; Soler et al., 2010), both possibilities resulting in free hydroxytyrosol that reaches the gut lumen (Pathway 1, **Fig. 6**).

During the course of absorption, free hydroxytyrosol could undergo an acetylation mediated by acetyltransferase enzymes and a subsequent sulfo-conjugation in the intestine or liver. Alternatively, the hydrolysis of the 3,4-DHPEA–EDA molecule at the 4–5 carbon bond may result in a significant release of hydroxytyrosol acetate to be further sulfoconjugated (Pathway 2, **Fig. 6**). These proposed pathways may be responsible for the high concentrations detected in plasma of hydroxytyrosol acetate-sulfate and its kinetics following a dose–response trend after the intake of M- and H-FVOOs.

The detection of homovanillic acid and homovanillic acid sulfate in plasma reflects the methylation process of hydroxytyrosol in the organism, due to the activity of the enzyme cathecol-O-methyltransferase (COMT) (D'Angelo et al., 2001). The very low concentrations in postprandial human plasma of free hydroxytyrosol and its derivatives points out a high efficiency of the conjugation enzymatic systems in the generation of the sulfate or methyl sulfate conjugates which are more likely to be absorbed by the organism.

In our study, most of the identified metabolites appear in their sulfate conjugated forms, which suggests that sulfation is one of the main biological pathways in humans. Our results are in agreement with previous work (De la Torre-Carbot et al., 2006) in which hydroxytyrosol and tyrosol were identified in both their glucuron-idated and sulfated forms in human LDL after the intake of virgin olive oil. In our study apigenin glucuronide and ferulic acid glucuronide were also detected in plasma, but at very low concentrations.

Sensory analysis results are considered key factors for consumers. The sensory acceptance is the principal barrier for the acceptance of a functional food. The enriched olive oil with the middle dose (M-FVOO, 500 mg/kg) scored between "I like it a bit" (score 4 of the sensory analyses) and "I neither like nor dislike" (score 3) which implies a satisfactory level of acceptance, considering that it contained 5-fold the phenol content of a standard VOO. Considering both the dose-pharmacokinetic and sensory results, M-FVOO, with a content of 500 mg/kg of phenolics, appears to have an appropriate enrichment, as it had both a good pharmacokinetic response and a good sensory acceptability. Moreover, by consuming 30 ml of M-EVOO it is easy to reach the 5 mg daily dose of hydroxytyrosol and derivates recommended by the EFSA Panel.

5. CONCLUSIONS

In summary, we conclude that an acute intake of a dose of 30 ml of phenol-enriched olive oils promotes a dose-dependent response of phenol conjugates in the human systemic circulation. However, the pharmacokinetics of the hydroxytyrosol metabolites did not show a complete linear response after the intake of the H-FVOO (750 mg total phenols/kg oil) and the M-FVOO (500 mg total phenols/kg oil). This could be explained by the fact that only 12 volunteers were recruited and it was a highly heterogeneous population (males and females aged 25–60 yrs). A saturation of the transport systems involved in the intestinal efflux and conjugating enzymes could also account for this fact. From this exploratory approach we point out for first time hydroxytyrosol acetate sulfate as a main biological metabolite of hydroxytyrosol from olive oil ingestion.

Acknowledgements

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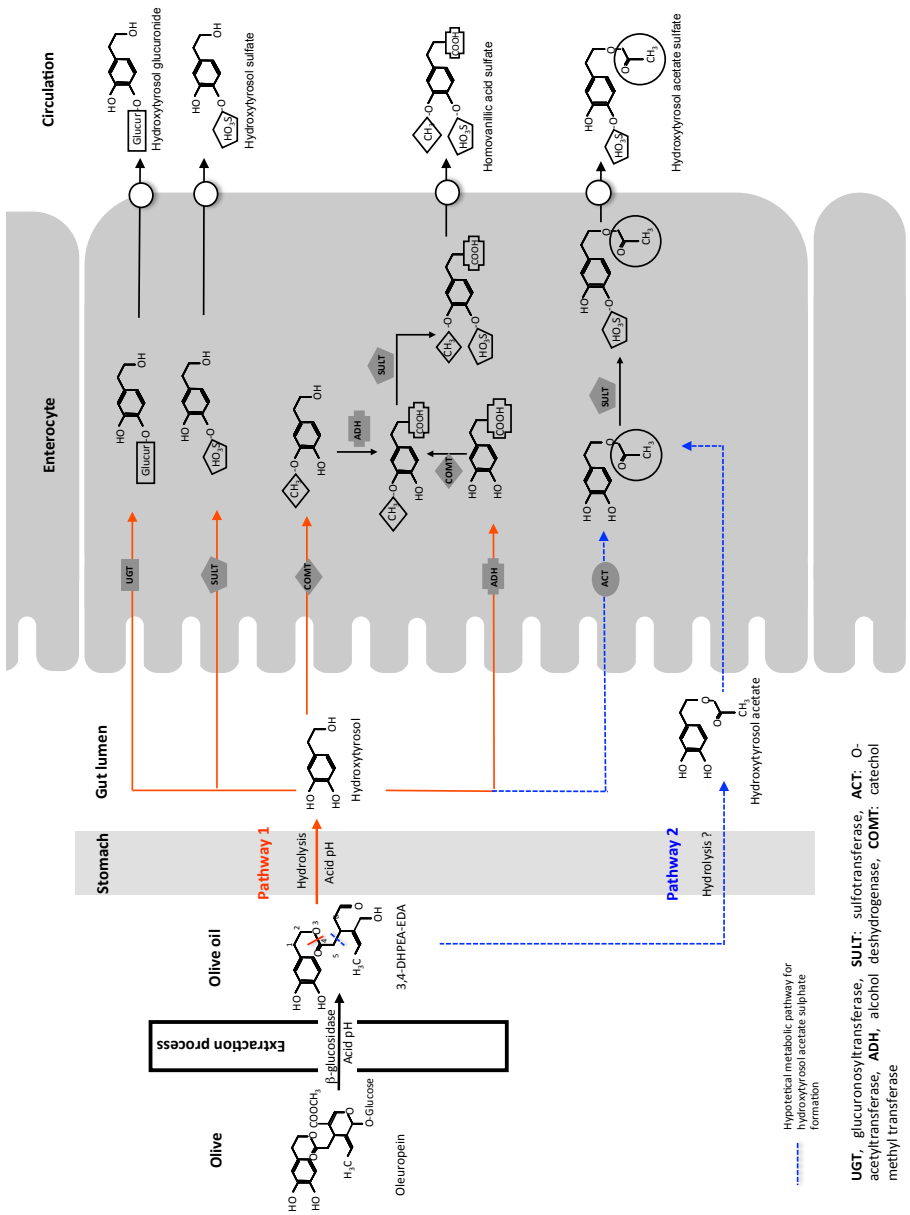

Figure 6. Metabolic pathway of 3,4-DHPEA–EDA, the main secoiridoid derivative present in enriched olive oils. Rapid hydrolysis occurs under gastric conditions resulting in significant increases of free hydroxytyrosol which undergoes extensive Phase II conjugation metabolism either in the enterocyte or hepatocyte. Discontinuous line indicates two possible pathways for hydroxytyrosol acetate. (UGT, glucuronosyltransferase; SULT, sulfotransferase; ACT, **O**-acetyltransferase; ADH, alcohol dehydrogenase; COMT, catechol methyl transferase).

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SUPPORTING INFORMATION

Concentration of phenolic metabolites quantified in the plasma of human subjects 0-6 h after the ingestion of 30 ml of enriched olive oils with phenolic contents at 250 (L-FVOO), 500 (M-FVOO) and 750 mg (H-FVOO) phenols/kg. Data are expressed in μM as mean values (n=12).

Phenolic metabolite (μM)		basal	15 min	30 min	45 min	60 min	120 min	240 min	360 min
p-hidroxybenzoic acid	L-FVOO	1.786	1.752	1.858	1.864	2.028	1.575	1.319	1.135
	M-FVOO	2.080	1.998	2.144	2.085	2.142	1.849	1.270	0.981
	H-FVOO	1.360	1.369	1.410	1.358	1.355	1.373	1.031	0.924
Hydroxyphenylacetic acid	L-FVOO	0.359	0.337	0.527	0.545	0.537	0.416	0.241	0.208
	M-FVOO	0.449	0.462	0.459	0.523	0.526	0.501	0.361	0.244
	H-FVOO	0.411	0.464	0.444	0.469	0.480	0.413	0.305	0.279
Vainillin	L-FVOO	0.000	0.007	0.007	0.01	0.007	0.014	0.016	0.010
	M-FVOO	0.000	0.000	0.005	0.014	0.015	0.019	0.009	0.005
	H-FVOO	0.000	0.004	0.021	0.020	0.016	0.015	0.005	0.000
Homovainillic acid	L-FVOO	0.018	0.018	0.075	0.057	0.079	0.080	0.056	0.050
	M-FVOO	0.031	0.044	0.057	0.150	0.297	0.208	0.158	0.149
	H-FVOO	0.040	0.041	0.126	0.201	0.319	0.248	0.185	0.129
Luteolin	L-FVOO	0.017	0.017	0.023	0.022	0.022	0.019	0.019	0.021
	M-FVOO	0.007	0.005	0.005	0.004	0.004	0.004	0.002	0.003
	H-FVOO	0.006	0.009	0.007	0.009	0.008	0.005	0.006	0.007
Tyrosol sulfate	L-FVOO	0.348	0.385	0.423	0.467	0.462	0.356	0.326	0.279
	M-FVOO	0.542	0.599	0.561	0.547	0.646	0.542	0.523	0.496
	H-FVOO	0.459	0.462	0.518	0.578	0.483	0.424	0.399	0.377
Vainillin sulfate	L-FVOO	2.640	3.119	3.512	4.634	5.358	6.044	4.720	4.554
	M-FVOO	3.170	2.342	2.672	2.827	2.715	2.791	1.655	0.612
	H-FVOO	2.497	2.604	2.844	2.299	2.713	2.835	2.099	1.764
Ferulic acid glucuronide	L-FVOO	0.018	0.019	0.018	0.019	0.018	0.016	0.009	0.010
	M-FVOO	0.021	0.022	0.025	0.023	0.02	0.018	0.016	0.015
	H-FVOO	0.019	0.028	0.026	0.029	0.028	0.025	0.015	0.012
Apigenin glucuronide	L-FVOO	0.017	0.017	0.016	0.018	0.017	0.016	0.015	0.015
	M-FVOO	0.024	0.022	0.022	0.024	0.024	0.021	0.021	0.019
	H-FVOO	0.018	0.018	0.017	0.018	0.019	0.017	0.017	0.017

Publication IV.

Validation of determination of plasma metabolites derived from thyme bioactives compounds by improved liquid chromatography coupled to tandem mass spectrometry.

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VALIDATION OF DETERMINATION OF PLASMA METABOLITES DERIVED FROM THYME BIOACTIVE COMPOUNDS BY IMPROVED LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

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Abstract

In the present study, a selective and sensitive method, based on microelution solid-phase extraction (SPE) plate and ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC–MS/MS) was validated and applied to determine the plasma metabolites of the bioactive compounds of thyme. For validation process, standards of the more representative components of the phenolic and monoterpene fractions of thyme were spiked in plasma samples and then the quality parameters of the method were studied. Extraction recoveries (%R) of the studied compounds were higher than 75%, and the matrix effect (%ME) was lower than 18%. The LODs ranged from 1 to 65 μg/L, except for the thymol sulfate metabolite, which was 240 μg/L. This method was then applied for the analysis of rat plasma obtained at different times, from 0 to 6 h, after an acute intake of thyme extract (5 g/kg body weight). Different thyme metabolites were identified and were mainly derived from rosmarinic acid (coumaric acid sulfate, caffeic acid sulfate, ferulic acid sulfate, hydroxyphenylpropionic acid sulfate, dihydroxyphenylpropionic acid sulfate and hydroxybenzoic acid) and thymol (thymol sulfate and thymol glucuronide). The most abundant thyme metabolites generated were hydroxyphenylpropionic acid sulfate and thymol sulfate, their respective concentrations in plasma being 446 and 8464 μM 1 h after the intake of the thyme extract.

KEYWORDS: UPLC-MS/MS / monoterpenes / phenolic compounds /plasma metabolites /thyme

1. INTRODUCTION

The interest in, and research on, the bioavailability and metabolism of bioactive compounds in leafy spices is well justified. Lamiaceae herbs are rich in various phenolic compounds and characterized mainly by the occurrence of phenolic acids, flavonoids and phenolic volatile oils [1]. The contribution of several culinary and medicinal herbs (oregano, sage, thyme, and peppermint) to the total intake of dietary antioxidants has been

assessed, and these foods were reported to be a significant source of dietary antioxidants, even superior to many other food groups [2].

Thyme comes originally from the regions around the Mediterranean and is used as cough medicine. It has also been commonly used as a culinary herb for adding flavor. The extensive phytochemical research into thyme has revealed the presence of several classes of bioactive compounds, such as phenolic acids, flavonoids, terpenoids and essential oils [3,4]. Among these compounds, rosmarinic acid (RA) is one of the most important anti-oxidative polyphenols in thyme and is also widely found in Lamiaceae herbs [5]. In addition to its potent antioxidant activity [6], many beneficial properties, including anti-inflammation [7], anti-mutagenicity [8] and prevention of Alzheimer's disease [9], have been attributed to rosmarinic acid. There are also large amounts of monoterpenes in thyme and their pharmacological properties have recently been reviewed, they are especially considered promising agents for the prevention or treatment of cardiovascular diseases [10].

Although thyme extracts (TE) and essential thyme oils have been shown to possess various pharmacodynamic activities [11–15], there have been no in vivo studies of the oral administration of thyme extract with all its bioactive compounds to clarify its bioavailability and metabolism.

Until now, the only in vivo studies reported in the literature that analyzed thyme derivates in plasma samples were based on the oral administration of rosmarinic acid [16–18] or thymol [19]. The analytical separation techniques used were highperformance liquid-chromatography (HPLC) coupled to DAD [18], mass spectrometry (MS) [17] and tandem MS (MS/MS) [16]; and gas chromatography (GC) coupled to a flame ion detector (FID) [19]. The plasma samples were centrifuged [16–18] for the sample pretreatment technique when HPLC was used. Headspace solid-phase microextraction (SPME) was the chosen method [19] when GC was used. Only the methodology reported by Kohlert et al. [19], based on off-line headspace – SPME-GC-FID, was validated for the analysis of thymol, after its sulfatase and b-glucuronidase enzymatic pretreatment, and its quantification limit (LOQ) was 7.1 µg/L in plasma samples.

In order to improve and enhance the reported methodologies for determining thyme metabolites in plasma samples, this paper presents and validates a rapid, selective and sensitive method to identify and quantify the metabolites generated after an acute intake of thyme extract. The analytical separation technique used is ultra-performance liquid chromatography (UPLC) coupled to tandem MS, and microelution solid-phase extraction (SPE) plates as the pretreatment technique. The method is validated in terms of linearity, calibration curves, precision, accuracy, recoveries and sensitivity with the most representative analytes from thyme. To observe and understand the future potential benefits of the minor components from thyme, taking their short life in plasma into account, the study was carried out during the postprandial state, which allowed their detection and quantification. This fact may be very useful in future repeated low-dose experiments, facilitating an understanding of long-term interventional studies. To our knowledge, for the first time, a liquid chromatographic (LC) method is validated for the analysis of thyme metabolites, and also for the first time, a thyme extract (TE) is administrated to determine the plasma metabolites generated.

2. EXPERIMENTAL

2.1. Chemicals and reagents

Catechol as internal standard (I.S.), p-hydroxybenzoic acid,thymol, hydroxyphenylacetic acid, dihydroxyphenylacetic acid, hydroxyphenylpropionic acid, caffeic acid, dihydroxyphenylpropionic acid, ferulic acid, naringenin, luteolin, eriodictyol, quercetin, rosmarinic acid were purchased from Extrasynthase (Genay, France). p-coumaric was purchased from Fluka Co. (Buchs, Switzerland). Acetonitrile (HPLC grade), methanol (HLPC grade), ethanol and acetic acid were all provided by Scharlau Chemie (Barcelona, Spain). The water was of Milli-Q quality (Millipore Corp, Bedford, MA).

2.2. Isolation of thymol sulfate as a standard

Thymol sulfate was isolated by using semi-preparative HPLC (Waters, Milford, MA) in the reversed phase from the SPE eluted solution of rat plasma after the ingestion of the thyme extract (TE). The HPLC system includes a Waters 1525EF binary HPLC pump, a Waters Flexinject, a Waters 2487 absorbance detector (280 nm) and a Waters Fraction Collector II. The HPLC system was operated using Brezze software. 50 L of the SPE eluted solution from

the rat plasma was injected manually into the injector module (1 mL sample loop). The method used was the same as reported in our previous study [20] except for the column. In this case, an XBridge BEH C18 (2.5 m, 4.6 mm x 100 mm) analytical column, also from Waters, was used. Prior to chromatographic isolation, the thymol sulfate was identified by MS/MS and its retention time was determined in order to collect the specific fraction. The fraction corresponding to thymol sulfate was collected manually by observing the detector output on the recorder according to its retention time. The organic solvent of the mobile phase was then removed by rotary evaporation (Buchi, Labortechnick AG, Switzerland) under a partial vacuum at 25 ○C. Finally, the aqueous extract was placed in a chromatographic vial, which had previously been weighed, and freeze-dried in a Lyobeta 15 lyophilizer (ImaTelstar, Spain). After freeze-drying, the vial was weighed and the difference in the weight was attributed to the isolated thymol sulfate. The thymol sulfate was stored in an N2 atmosphere at −40 ºC until its use as a standard for quantifying thymol metabolites in rat plasma. **Fig. 1** shows the precursor ion and the product ions of thymol sulfate obtained in the daughter scan mode and by applying different collision energies, from 5 to 40 eV, after its isolation from plasma samples.

In order to study the quality parameters of this method, the isolated thymol sulfate was dissolved with methanol and spiked in a rat plasma sample obtained under fasting conditions (control plasma).

2.3. Thyme extract (TE)

TE was prepared from dried thyme (***Thymus Zyguis***) with ethanol:water by accelerated solvent extraction (ASE) following the methodology optimized by Suárez et al. [21]. The bioactive compounds of the TE were analyzed according to the method in Rubió et al. by HPLC coupled to tandem MS [22]. Table 1 shows the results of the bioactive compound contents of TE and these are expressed as µmol in 1.5 g of extract, corresponding to the ingested dose.

Additionally, TE was subjected to gas chromatography (GC) analyses to identify and quantify the monoterpenes. The extract was analyzed using an Agilent 6890N GC interfaced to a 5973N mass spectrometry (MS) selective detector for identification and to a flame ionization detector (FID) for the quantification analysis. The column used was a ZB-50 (50% phenyl and 50% methylpolysiloxane), 30m x 250µm internal diameter (ID) and 0.25µm phase thickness (Pnenomenex, Micron Analítica S.A., Spain). The carrier gas was helium at a constant flow rate of 1 mL/min. One microliter of the extract was injected using splitless mode. The oven temperature was programmed at 100 ○C and ramped at 15 ○C/min up to a maximum of 280 ○C for 15 min. The GC–MS transfer line was held at 280 ○C, and the quadrupole analyzer and ion source heaters were maintained at 150 and 230 ○C, respectively. MS data were collected in the full-scan mode, with a scan range from 35 to 500 amu and at a rate of 3.5 scans/s. The monoterpene thymol was identified by comparing its mass spectrum with the commercial standard, and this spectrum was obtained under the same analytical conditions.

Carvacrol, borneol and a-terpineol were identified by comparing the mass spectra obtained with those found in the Nist/Wiley mass spectra database libraries [23]. Quantification of the

Table 1. Phenolic and monoterpene composition of the thyme extract (TE) administered to the rats.

compound	µmols in 1.5 g of TE
Phenolic acids [1)]	
p-hydroxybenzoic acid	4.27
Vainillin	0.59
p-coumaric acid	0.61
Vanillic acid	2.69
Caffeic acid	5.35
Homovanillic acid	4.09
Chlorogenic acid	2.09
Protocatechuic acid	2.75
Rosmarinic acid	236.80
Flavonoids [1)]	
Apigenin	1.72
Luteolin	7.60
Luteolin-7-O-glucoside	21.84
Naringenin	10.51
Eriodictyol	11.08
Dihidroxykaempferol	0.36
Quercetin-glucoside	4.54
Eriodictyol-rutinoside	2.96
Thymusin	23.84
Xanthomicrol	17.60
7-Methylsudachitin	13.23
Monoterpernes [2]	
Thymol	44.32
Carvacrol	16.59
α-terpineol	8.42
Borneol	5.58

[1)] compounds analyzed by HPLC-MS/MS
[2)] compounds analyzed by GC-MS and FID.

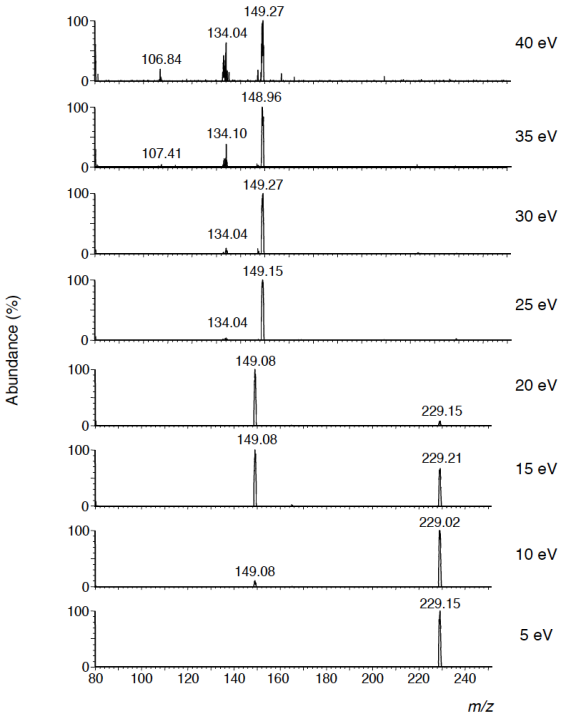

Fig. 1. Precursor ion and product ions of thymol sulfate obtained by daughter scan mode by applying different collision energy, from 5 to 40 eV, and 40 V as the cone voltage. Thymol sulfate was isolated by semi-preparative HPLC from rat plasma sample after TE ingesta. See Section 2.2 for the experimental conditions.

monoterpenes was performed by GC-FID, using the same analytical chromatographic parameters. The injector and detector temperatures were 250 and 300 °C, respectively. The calibration curve was prepared by using the external standard of thymol standard. **Table 1** also shows the results of the monoterpene contents of TE and these are expressed as µmol in 1.5g of extract, corresponding to the ingested dose.

2.4. Treatment of rats and plasma collection

Three-month-old male Wistar rats were obtained from Charles River Laboratories (Barcelona, Spain). The rats were housed in cages on a 12 h light–12 h dark schedule at controlled temperature (22 °C). They were subjected to a standard diet of a commercial chow, PanLab A04 (Panlab, Barcelona, Spain), and water ab libitum.

The animals were then kept in fasting conditions for between 16 and 17 h with only access to tap water. Subsequently, 12 rats were intra-gastrically gavaged with 1.5 g kg^{-1} BW dispersed in water. The rats were anesthetized with isoflurane (IsoFlo, VeterinariaEsteve, Bologna, Italy) and killed by exsanguinations 1 h (n = 3), 2 h (n = 3), 4 h (n = 3) and 6 h (n = 3) after the ingestion of the TE. Additionally, a control group of rats (n = 10) was maintained under fasting conditions
without extract ingestion and then similarly euthanized. The study was approved by The Animal Ethics Committee of the University of Lleida (CEEA 04-01/11, 26th January 2011). All experiments with rats were performed in compliance with the relevant laws and University of Lleida guidelines. Blood samples were collected by intracardiac exsanguination. The plasma samples were obtained by centrifuging (2000 x g, 30 min at 4 °C) and stored immediately with liquid nitrogen and then at –80 ○C until the chromatographic analysis of phenolic and monoterpene metabolites.

Table 2. Optimized SRM conditions for the analyses of the studied compounds and their plasma metabolites by UPLC-MS/MS.

Compound	SRM 1 (quantification)	Cone voltage (V)	Collision energy (eV)	SRM 2 (confirmation)	Cone voltage (V)	Collision energy (eV)
Standards						
Catechol	108.9 > 90.9	40	15	-	-	-
p-hydroxybenzoic acid	137 > 93	30	15	-	-	-
Thymol	149 > 134	40	15	-	-	-
Hydroxyphenylacetic acid	151 > 107	20	10	-	-	-
p-coumaric acid	163 > 119	35	10	163 > 117	35	25
Dihydroxyphenylacetic acid	167 > 123	20	10	167 > 95	20	15
Hydroxyphenylpropionic acid	165 > 121	20	10	165 > 149	20	15
Caffeic acid	179 > 135	35	15	179 > 117	35	20
Dihydroxyphenylpropionic acid	181 > 137	20	10	181 > 93	20	15
Ferulic acid	193 > 134	30	15	193 > 178	30	10
Thymol sulfate [1)]	229 > 149	40	20	229 > 134	40	30
Naringenin	271 > 151	40	15	271 > 119	40	20
Luteolin	285 > 133	55	25	285 > 151	55	25
Eriodictyol	287 > 151	40	15	287 > 179	40	25
Quercetin	301 > 151	40	15	301 > 179	40	15
Rosmarinic acid	359 > 161	40	20	359 > 161	40	25
Metabolites						
Coumaric acid sulfate	243 > 163	35	15	163 > 119	35	20
Hydroxyphenylpropionic acid sulfate	245 > 165	35	15	165 > 121	35	20
Cafeic acid sulfate	259 > 179	35	15	179 > 135	35	20
Dihydroxyphenylpropionic acid sulfate	261 > 181	40	15	181 > 137	40	20
Ferulic acid sulfate	273 > 193	35	15	193 > 134	35	20
Thymol glucuronide	325 > 149	20	25	325 > 134	20	30
Luteolin sulfate	365 > 285	35	15	285 > 133	35	20
Luteolin glucuronide	461 > 285	40	15	285 > 133	40	20

[1)] Standard isolated by semi-preparative HPLC from rat plasma samples, after ingestion of TE

2.5. Treatment of plasma samples and UPLC-ESI-MS/MS analysis of thyme metabolites

In order to clean-up the biological matrix and preconcentrate the phenolic and monoterpene compounds and their metabolites, the plasma samples were pretreated by microelution solid phase extraction (μSPE) plates following the methodology described by Suárez et al. [20]. Briefly the cartridges were firstly conditioned sequentially by using 250 μL of methanol and acidified Milli-Q water at pH 2. Then, 350 μL of plasma mixed with 300 μL of phosphoric acid 4% and 50 μL catechol (IS) at 10 mg/L were loaded onto the plate. After that, the clean-up of the plates was sequentially done with 100 μL Milli-Q water and 100 μL methanol 5% to eliminate any interference that the sample might contain. Finally the elution of the retained phenolic compounds was done with 100 μL of methanol. 2.5 μL of the eluted solution from the μSPE was injected directly into the Acquity UPLC–MS/MS system (Waters, Milford, MA, USA). The UPLC system was equipped with a Waters binary pump system (Milford, MA, USA) using an AcQuity UPLCTM BEH C18 column (1.7 μm, 100 mm x 2.1 mm i.d.). The flow rate was 0.4 mL/min using acetic acid 0.2% as solvent A and acetonitrile as solvent B. The elution started at 5% of eluent B for 5 min, then was linearly increased 40% of eluent B in 20 min, further increased to 100% of eluent B in 0.1 min and kept isocratic for 1.9 min. **Table 2** shows the quantification and confirmation transitions, and the cone voltage and collision energy for the commercial standards of the compounds studied and the thymol sulfate metabolite, which was isolated from the plasma samples as explained above in Section 2.2. In order to identify and quantify the bioactive compounds and their metabolites, different MS analyses were performed. These were based on MS (full-scan mode) and MS/MS (based on neutral loss scan and product ion scan). These techniques are excellent tools for verifying structural information about the compounds when

standards are not available [24]. Firstly, analyses were carried out in the full-scan mode (from 80 to 800 m/z) by applying different cone voltages, from 20 to 60 V. When low cone voltages were applied, the MS spectrum gave information about the precursor ion or the [M–H]–. In contrast, when

high cone voltages were applied, specific fragment ions were generated and the MS spectrum gave information about their chemical structure. This structural information was also verified in the MS/MS mode by using product ion scan and neutral loss scan. In the product ion scan experiments, the product ions are produced by collision-activated dissociation of the selected precursor ion in the collision cell. Additionally, neutral loss scans of 80 and 176 units were used to characterize the sulfate and glucuronide metabolites, respectively. Then, the detection and quantification of the phenolic and monoterpene compounds and their metabolites were performed based on their ion fragmentation in the MS/MS mode using SRM as the most sensitive. Table 2 also shows the SRM transitions, as well as the cone voltage and collision energy, used to quantify and identify the metabolites generated.

Due to the lack of standards for the great majority of these metabolites, they were tentatively quantified by using the calibration curveves corresponding to their phenolic precursors. This way, coumaric acid sulfate, ferulic acid sulfate, and caffeic acid sulfate were quantified using the calibration curves of p-coumaric, ferulic and caffeic acids, respectively. Dihydroxyphenylpropionic acid sulfate and hydroxyphenyl-propionic acid sulfate were quantified using the calibration curves of dihydroxyphenylpropionic and hydroxyphenylpropionic acids, respectively. Luteolin metabolites were quantified with the luteolin calibration curve. Finally, thymol metabolites were quantified with the calibration curve of thymol sulfate.

2.6. Validation procedure

The instrumental quality parameters of linearity, extraction recovery, calibration curves, precision, accuracy, detection limit (LOD), quantification limit (LOQ), and the study of the matrix effect were evaluated. These parameters were determined by spiking pool basal plasma (obtained in fasting conditions) with the standard phenolic and monoterpene compounds studied at known concentrations. This solution was then analyzed by off-line μSPE-UPLC– MS/MS. The linearity of the method was evaluated using these basal plasma samples spiked with the standards (phenols and monoterpenes) at different concentration levels. Calibration curves (based on

Table 3. Retention time (RT), extraction recovery (%R), linearity, calibration curves, reproducibility (%RSD), accuracy (%), LODs and LOQs for the determination of the phenolic compounds and the isolated thymol sulfate spiked in plasma samples by UPLC-MS/MS (1) 6 μM 2) 1.5 μM 3) 0.15 μM 4) 3 μM)

Compound	RT (min)	%R	Linearity (μM)	Calibration curve	RSD% (n=3), inter-day			Accuracy (%) (n=3) (6 μM)	LOD (μM)	LOQ (μM)
					12 μM	3 μM	0.3 μM			
Dihydroxyphenylacetic	3.00	77	0.30-11.90	y = 2.1242x – 0.0219	3.8	5.5	0.1	100	0.09	0.30
p-hydroxibenzoic acid	3.62	75	0.43-14.50	y = 39.806x + 1.0159	6.5	6.5	2.9	103	0.13	0.43
Hydroxyphenylacetic	5.11	79	0.16-13.15	y = 3.7609x + 0.5972	0.9	3.0	3.3	103	0.05	0.16
Dihydroxyphenylpropionic	5.29	81	0.50-10.99	y = 8.7028x + 0.0061	2.6	1.7	3.2	95	0.14	0.50
Caffeic acid	5.73	80	0.5-11.11	y = 12.118x + 0.1791	3.0	1.6	2.5	105	0.36	50
Hydroxyphenylpropionic	7.58	85	0.70-12.05	y = 1.6782x + 0.0298	1.5	2.8	10.5	101	0.23	70
p-coumaric acid	7.84	90	0.12-12.19	y = 13.342x + 0.1651	2.1	3.7	9.8	97	0.03	12
Ferulic acid	9.22	92	0.08-10.31	y = 10.192x – 0.0462	6.3	3.9	10.3	95	0.02	0.08
Rosmarinic acid	12.77	75	0.008-5.55	y = 8.0821x + 1.246	7.9 1)	0.3 2)	2.5 3)	95 4)	0.003	0.008
Quercetin	15.16	77	0.03-6.62	y = 0.6258x + 0.038	6.5 1)	0.3 2)	5.5 3)	105 4)	0.009	0.03
Eriodictyol	15.19	98	0.02-6.94	y = 17.982x + 0.2327	4.8 1)	5.4 2)	10.2 3)	103 4)	0.007	0.02
Luteolin	15.64	75	0.03-6.99	y = 8.6544x + 0.0533	9.9 1)	10.4 2)	0.1 3)	99 4)	0.007	0.03
Thymol sulfate	15.96	75	1.00-8.70	y = 3.1164x - 2.9851	5.3 1)	4.9 2)	6.7 3)	101 4)	0.30	100
Naringenin	17.65	98	0.02-7.35	y = 50.64x + 0.9056	3.7 1)	6.1 2)	9.5 3)	104 4)	0.004	0.02

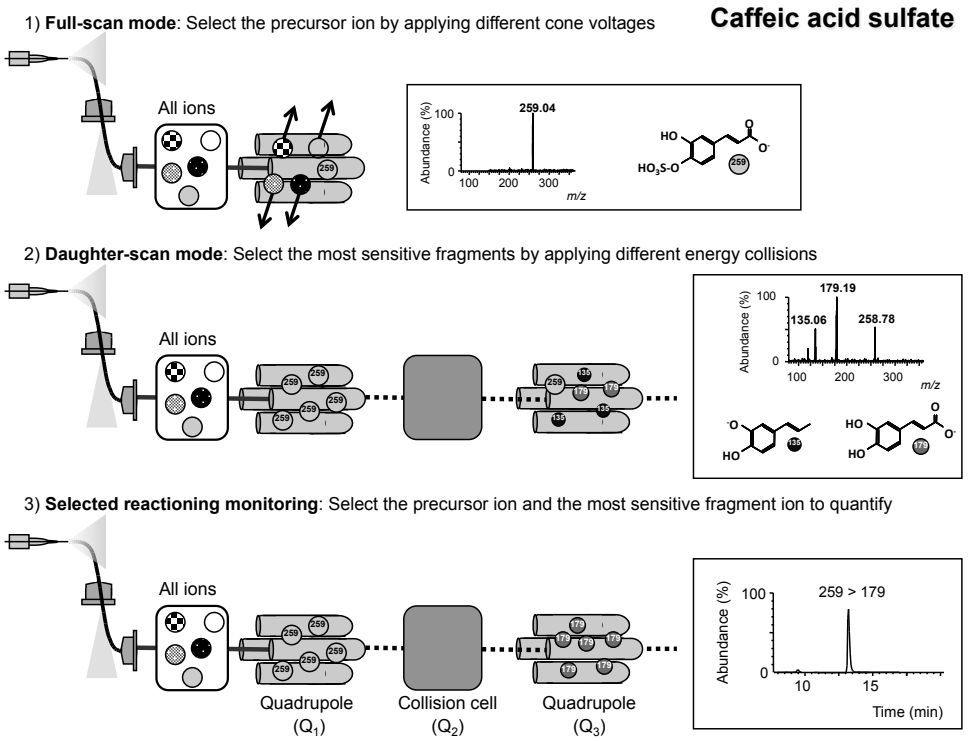

Fig. 2. MS analyses performed to identify and quantify the plasma metabolites, using caffeic acid sulfate as example.

peak area abundance) were plotted using y = a + bx, where y is the analyte/IS peak area abundance ratio and x is the analyte/IS concentration ratio. The concentrations of the metabolites were calculated by interpolating the analyte peak abundance onto their calibration curves. These curves were obtained by analyzing five points at different concentration levels and each standard solution was injected three times.

The precision of the method (inter-day and intra day precisions) were determined as the relative standard deviation (% RSD) of the concentration (n = 3). The accuracy (n = 3) was calculated as the ratio between the mean measured concentration and the nominal concentration multiplied by 100. The extraction recoveries (%R) of the sample pre-treatment method were calculated by comparing the responses of the analytes spiked in the plasma matrices before and after extraction. The matrix effect (%ME) was evaluated by comparing the peak abundances of the analytes spiked in the plasma matrices after the sample pretreatment, with the peak abundances of the analytes spiked in the elution solvent (methanol). The LODs and LOQs were calculated using the signal-to-noise criterion of 3 and 10, respectively.

3. RESULTS AND DISCUSSION

3.1. Quality parameters

The standards of the phenolic and monoterpene compounds studied were spiked in control plasma samples at known concentrations and analyzed by off-line μSPE-UPLC–MS/MS. Then, the quality parameters of the method, such as linearity, calibration curves, precision, accuracy, LOD, LOQ, extraction recovery and the study of the matrix, were evaluated. These results are shown in Table 3. The functions of the calibration curves were linear, with mean correlation coefficients >0.99. The inter-day and intra-day

Table 4. Metabolites from thyme identified in rat plasma by off-line μSPE and UPLC-MS/MS after ingesta of TE.

Metabolite	RT (min)	$[M-H]^-$ (m/z)	MS^2 ions (m/z)	Neutral loss scan
Coumaric acid sulfate	5.45	243	163, 119	80
Ferulic acid sulfate	5.85	273	193, 134	80
Hydroxybenzoic acid	9.12	137	93	-
Luteolin glucuronide	10.41	461	285, 133	176
Dihydroxyphenylpropionic acid sulfate	10.63	261	181, 137	80
Hydroxylphenylpropionic acid sulfate	11.93	245	165, 121	80
Caffeic acid sulfate	14.96	259	179, 135	80
Luteolin sulfate	14.97	365	285, 133	80
Thymol glucuronide	16.94	325	149, 134	176
Thymol sulfate	17.25	229	149, 134	80

precisions of the method, expressed as the relative standard deviation
(%RSD), were studied at three concentration levels, 0.3, 3 and 12 μM. These were lower than 10.5% and 12%, respectively, for all the compounds analyzed and the accuracy values (intra-day and inter-day) ranged from 95 to 105%. The precision and accuracy results appear to indicate that the methodology for extracting the compounds from the studied plasma matrix is highly reproducible and reliable. The extraction recoveries (%R) of the studied compounds were good, and these were above 75%. The matrix effect (%ME), which is due to the coeluting matrix components that compete for ionization, is observed by a decrease or increase of the analyte signal depending on whether the analyse is prepared in a sample matrix (plasma in our study) or in an organic solvent (methanol) [25]. In our study, the matrix effect (%ME) in absolute values was lower than 18% (values not shown). The LODs ranged from 0.003 to 0.36 μM. LOQ values ranged from 0.008 to 1.17 μM. These LOD and LOQ values were lower than those reported in the literature when phenolic compounds from olive oil and procyanidins and anthocyanins were studied in spiked plasma [20,26].

3.2. Identification of the plasma metabolites from the thyme extract

The validated method was applied to determine the bioactive compounds from thyme and their metabolites, in plasma samples, after an acute intake of TE. **Fig. 2** shows the different MS analyses performed to identify and quantify the plasma metabolites, using caffeic acid sulfate as example. Ten metabolites were generated and these are listed in **Table 4**. These compounds were not detected in The control plasma samples, showing that all metabolites identified in the plasma samples obtained after the ingesta of TE came from thyme. The native forms of the phenols and monoterpenes present in the TE (Table 1) were not detected in the plasma, except for hydroxybenzoic acid, indicating that the thyme compounds are absorbed and rapidly metabolized into sulfate and glucuronidate conjugates.

The metabolites generated, namely coumaric acid sulfate, ferulic acid sulfate, caffeic acid sulfate, hydroxyphenylpropionic acid sulfate and thymol sulfate, had already been identified in plasma samples after an oral administration of rosmarinic acid [16–18] or thymol [27]. The other metabolites generated (hydroxybenzoic acid, luteolin sulfate, luteolin glucuronide, dihydroxyphenylpropionic acid sulfate and thymol glucuronide) were first detected in our study after an acute intake of thyme extract. **Table 4** also shows the precursor ion, [M–H]–, the MS fragments or product ions (MS2 ions) and the neutral loss scan, m/z 80 or m/z 176, according to whether the loss is respectively a sulfate or glucuronide molecule, of the 10 identified and generated metabolites.

The metabolite coumaric acid sulfate was identified because its mass spectra showed a precursor ion of m/z 243, and two fragment ions at m/z 163 and m/z 119. These fragment ions are due to the loss of the sulfate molecule and carboxylic group (CO_2), respectively.

The ESI-MS spectra of the compound eluted at 14.96 min obtained in the full-scan mode showed an intense ion at m/z 259, which formed two major fragment ions, one at m/z 179 and the other at m/z 135. These ions could be related to the loss of the sulfate molecule and the caffeic acid rupture, respectively, and this suggests that the metabolite could be caffeic acid sulfate. Another metabolite was observed from the examination of the ESI-MS spectra at a retention time of 5.85 min and this was characterized by an ion at m/z 273.

This metabolite presents two fragment ions at m/z 193 and m/z 134, which can be explained by the loss of the sulfate molecule and the ferulic acid rupture, respectively, which suggests that the metabolite could be ferulic acid sulfate. This metabolite can also be generated by methylation of the caffeic acid molecule.

Two luteolin metabolites were identified by the fact that their fragment ions produce ions at m/z 285 and m/z 133, which demonstrates the existence of

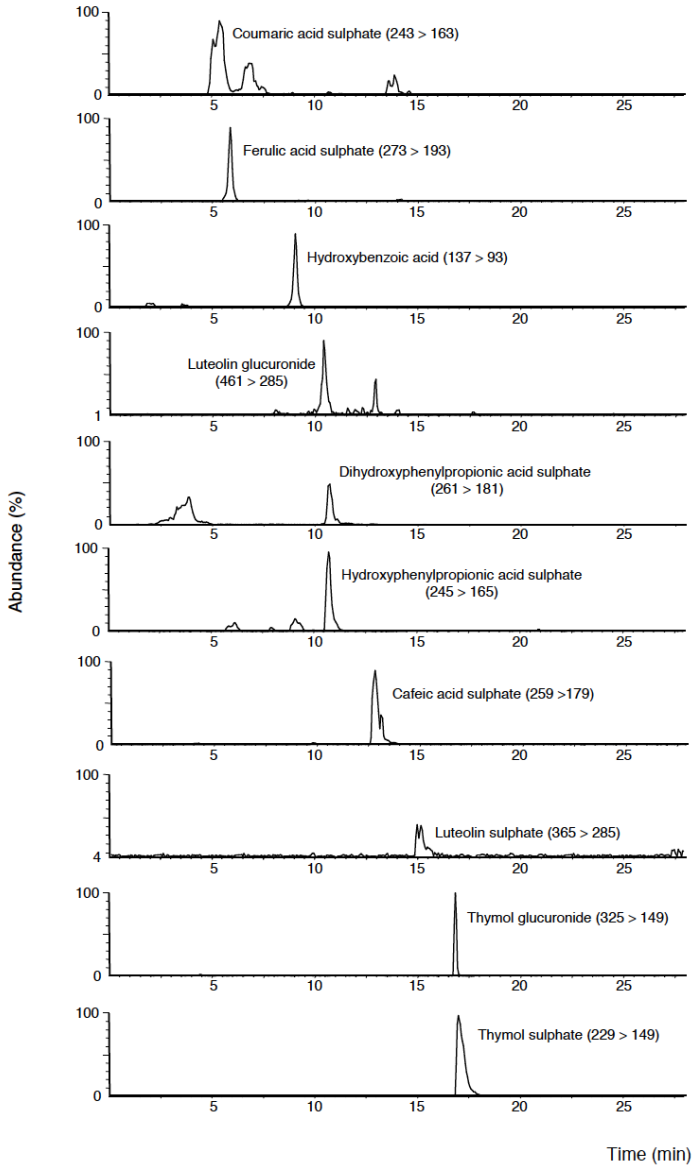

Fig 3. Extracted ion chromatograms of the 10 generated metabolites detected in rat plasma after the ingestion of TE.

the luteolin molecule. The first luteolin metabolite gave a precursor ion of m/z 365 and the second one of m/z 461. Therefore, these ions could be identified as luteolin sulfate and luteolin glucuronide due to the loss of the sulfate and glucuronide molecules, respectively.

The metabolite dihydroxyphenylpropionic acid sulfate was identified by a precursor ion at m/z 261 and two fragment ions, at m/z 181 and m/z 137 in its MS spectra. These ions can be described by the loss of the sulfate molecule and the carboxylic group (CO2), respectively. On the other hand, the metabolite with a precursor ion at m/z 245 was identified as hydroxyphenylpropionic acid sulfate. This metabolite presents a fragment ion at m/z 165 which can be described by the loss of the sulfate molecule, and a fragment ion at m/z 121 which can be explained by the loss of the carboxylic group (CO2).

The ESI-MS spectra of the thymol metabolites eluted at 16.94 and 17.25 min showed intense ions at m/z 325, and at m/z 229, respectively. These two precursor ions formed a major fragment ion at m/z 149, and a second, less-intense, one at m/z 134. These ions could be related to the loss of the glucuronide and sulfate molecules, and these could be identified as thymol glucuronide and thymol sulfate, respectively. Although carvacrol sulfate and glucuronide could give the same spectrum as thymol sulfate and glucuronide, the metabolites generated from thyme were thought to be thymol metabolites instead of carvacrol metabolites, because thymol was the most abundant in the TE extract, in comparison with carvacrol (see **Table 1**).

Fig. 3 shows the extracted ion chromatograms of the 10 metabolites generated, and **Fig. 4** shows the chemical structure, mass spectrum (obtained in daughter scan mode by applying different collision energy, from 5 to 35 eV) and the extracted ion chromatograms of the thymol conjugate metabolites.

Once the generated metabolites were identified, these were quantified. **Table 5** shows the plasma concentration of the 10 thyme metabolites, expressed as μM, at different times after the ingest of TE, the phenolic acids and monoterpenes being conjugated forms of the main metabolites. Firstly, the two thymol metabolites were tentatively quantified by thymol standard by UPLC–MS/MS. However, due to their volatility we scarcely detected them and were unable to obtain a reliable standard curve. Therefore, in order to quantify these metabolites with a reliable curve, it was proposed to isolate the thymol sulfate metabolite from the plasma samples, and quantify the thymol metabolites compared with thymol sulfate.

Hydroxyphenylpropionic acid sulfate, dihydroxy-phenylpropionic acid sulfate, and thymol sulfate

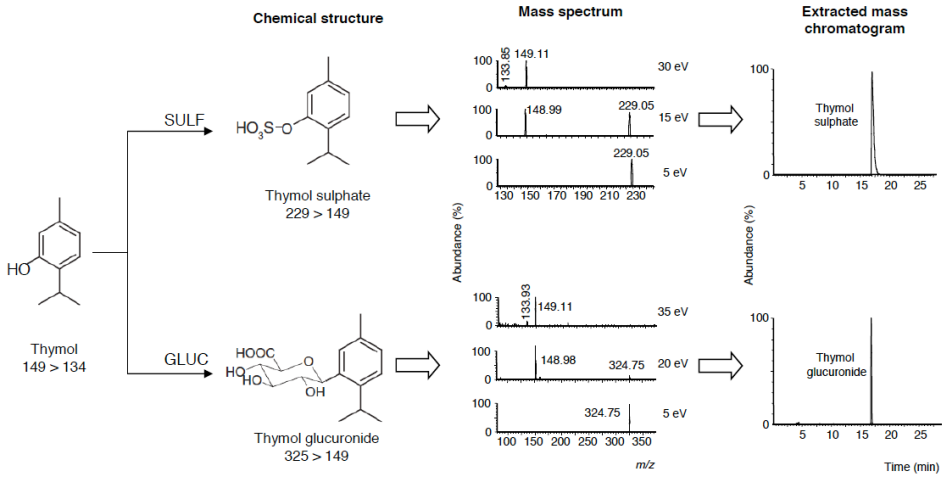

Fig 4. Chemical structure, mass spectrum (obtained in daughter scan mode) and extracted ion chromatograms of the thymol metabolites, thymol glucuronide and thymol sulfate.

Table 5. Concentration of thyme metabolites quantified in rat plasma at different times after ingesta of TE

Concentration (μM)	Control	1 h	2 h	4 h	6 h
Phenolic metabolites					
cafeic acid sulfate	n.d.	9.27± 2.58	3.32± 0.51	6.37± 1.53	10.85± 2.27
hydroxybenzoic acid	n.d.	1.16± 0.41	1.95± 0.46	3.91± 0.95	4.11± 0.61
coumaric acid sulfate	n.d.	0.75± 0.34	0.30± 0.08	0.76± 0.26	2.58± 0.51
ferulic acid sulfate	n.d.	4.42± 0.95	3.55± 0.64	3.67± 0.99	3.81± 0.66
ferulic acid glucuronide	n.d.	0.14± 0.01	0.13± 0.02	0.14± 0.02	0.14± 0.03
hydroxyphenilpropionic acid sulfate	n.d.	446.4± 109.6	159.3± 22.8	172.3± 11.3	369.3± 91.3
dihydroxyphenilpropionic acid sulfate	n.d.	24.81± 4.13	20.08± 2.89	13.51± 0.58	20.90± 3.44
luteolin sulfate	n.d.	0.03± 0.01	0.05± 0.02	0.05± 0.05	0.24± 0.09
luteolin glucuronide	n.d.	1.76± 0.52	2.16± 0.45	2.47± 0.86	4.02± 1.24
Monoterpene metabolites					
thymol sulfate	n.d.	8463.9± 827.9	3551.5± 116.4	5328.3± 239.5	6577.1± 932.5
thymol glucuronide	n.d.	2.43± 0.23	LOD- LOQ	30.67± 4.71	56.92± 4.38

n.d.: not detected
LOD-LOQ: its concentration is between its LOD value and its LOQ value LOD-LOQ

metabolites were the most abundant, and their concentrations 1 h after the ingesta of the thyme extract were 446, 25 and 8464 μM respectively.

The composition analysis of the TE revealed that the rosmarinic acid was the major phenolic compound in the extract (as shown in **Table 1**), and therefore most of the metabolites detected in the plasma could be linked to its metabolism. Similarly, thymol sulfate and thymol glucuronide were also quantified in the plasma as the main monoterpene metabolites from thyme. According to all this and the generated metabolites that have been identified in the plasma samples, pathways are proposed for both rosmarinic acid and thymol metabolites.

3.2.1. Proposed metabolic pathway for rosmarinic acid

Based on the major phenolic acids (hydroxy phenylpropionic acid, dihydroxyphenylpropionic acid and caffeic acid sulfate conjugates) quantified in the post-prandial plasma samples, we proposed a metabolic pathway for rosmarinic acid, illustrated in **Fig. 5.** Firstly, the microbial esterases in the digestive tract could hydrolyze the ester linkages into rosmarinic acid. The resulting caffeic and dihydroxyphenylpropionic acids could undergo p-dehydroxylation in the lower portion of the digestive tract. This type of transformation has been previously described by Goodwin et al. [28] and Wang et al. [29]. It has been demonstrated that the microflora in the digestive tract has the ability to catalyze the pdehydroxylation of polyphenols with a catechol moiety. The vicinal hydroxy group for this type of biotransformation is emphasized in these studies and that is why rosmarinic acid could be subjected to this reaction. The subsequent hydroxyphenylpropionic acid and coumaric acid derived from the p-dehydroxylation of rosmarinic acid could be absorbed, conjugated and methylated in the digestive tract and liver, resulting in a variety of sulfate conjugates of caffeic acid, ferulic acid, coumaric acid, dihydroxyphenylpropionic acid and hydroxyphenylpropionic acid detected in the plasma samples. Some of these, such as coumaric acid, coumaric acid sulfate and hydroxybenzoic acid appeared to have their maximum plasmatic concentration 6 h after the intake of the thyme extract indicating that rosmarinic acid could also be metabolized in the colon, these metabolites appearing as fermentation colonic products as described previously [30].

With regard to the bioavailability of rosmarinic acid, a previous study in rats showed that with an orally administered extract of Perilla frutescens, rosmarinic acid was present as intact and degraded and/or conjugated forms, such as hydroxyphenylpropionic acid, coumaric acid, and sulfate conjugates of caffeic acid, coumaric acid and ferulic acid that were subsequently excreted in the urine [18]. Later, Baba et al. [16] determined the absorption and metabolism of rosmarinic acid after a single intake of P. frutescens extract but only detected free rosmarinic acid, methylated rosmarinic acid and

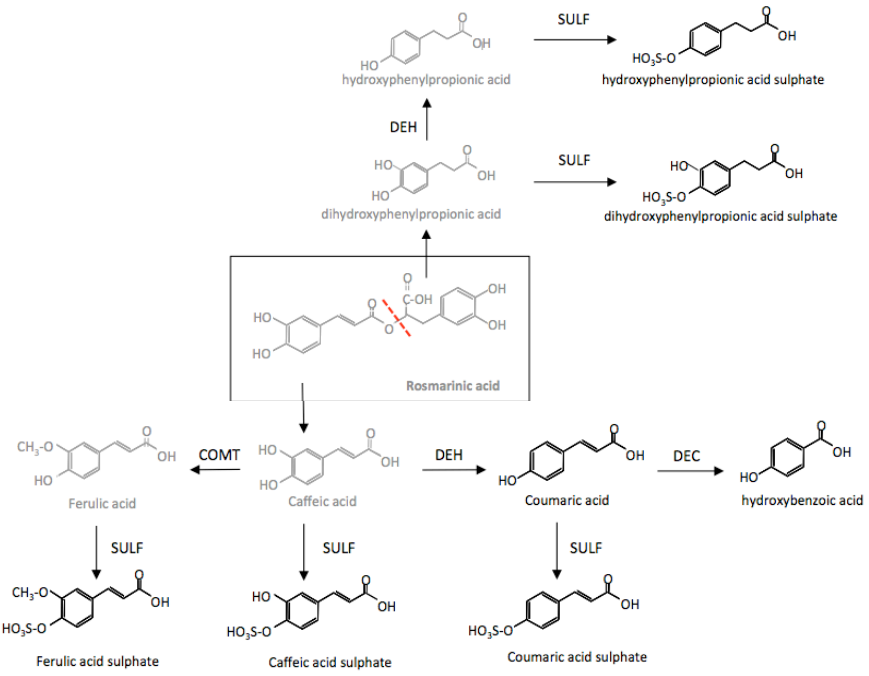

Figure 5. Proposed metabolic pathway of rosmarinic acid. DEC, decarboxylation; SULF, sulfate conjugation; GLUC, glucuronide conjugation; DEH, p-dehydroxylation; COMT, methylation.

ferulic acid in plasma, with the maximum levels obtained 0.5, 2 and 0.5 h after the intake of the extract, respectively. In contrast to these previous studies, our results suggest that rosmarinic acid is rapidly degraded into various components with a subsequent conjugation, as we detected no free rosmarinic acid in plasma. Going beyond previous results, we detected more metabolites related to rosmarinic acid metabolism and therefore we could elucidate a possible metabolic pathway, which improves the understanding of the bioavailability of rosmarinic acid.

3.2.2. Proposed metabolic pathway of thymol

The TE administered in the present study contained an importimportant amount of monoterpenes (see **Table 1**), thymol being the main monoterpene in the extract, followed by carvacrol, a-terpineol and borneol. However, with the exception of thymol, the metabolites of these monoterpenes were not detected in plasma samples after the ingesta of TE. Phase II conjugates, such as thymol sulfate and thymol glucuronide, were identified by means of UPLC–MS/MS analysis.

The maximum plasma concentrations were reached 2 and 6 h after the TE intake, respectively (**Table 5**). By contrast, thymol in its free form was not detected in the post-ingesta plasma samples. Considerable plasma concentrations of thymol sulfate were detected only 1 h after TE ingesta. This fast absorption indicates that thymol is mainly absorbed in the upper part of the gut. 6 h after the TE ingesta, thymol sulfate was still detectable in plasma.

Our results confirm previous observations made by Kohlert et al. [26], who determined the systemic availability and pharmacokinetics of thymol in a clinical trial with 12 healthy male volunteers, where both thymol glucuronide and thymol sulfate were identified in urine with sulfate being the main metabolite again, and no free thymol was detected. Hence, the data available so far (human and animal) indicate that thymol is

systemically available mainly as thymol sulfate. Thymol glucuronide was not detected by Kohlert et al., in plasma, and in our study, it appeared at very low concentrations, which could be the result of lower activity of hepatic UDP-glucuronyl-transferases compared with sulfotransferases.

4. Concluding remarks

In the present study, a rapid, selective and sensitive method to identify and quantify thyme metabolites in plasma samples is presented and validated. The use of off-line-μSPE as the sample preparation with UPLC–MS/MS allowed the rapid determination of the most important thyme bioactive compounds and their metabolites at low concentration levels in complex matrices, such as plasma samples. The analysis of the metabolites revealed that the potential bioactive compounds from thyme are absorbed and rapidly metabolized into simple phenolic acids and thymol as conjugated forms. In the present work, such thyme metabolites as hydroxybenzoic acid, luteolin sulfate, luteolin glucuronide, dihydroxy-phenylpropionic acid sulfate and thymol glucuronide were determined for the first time after an acute intake of thyme extract.

Additionally, it is the first in vivo study with oral administration of TE that proposes two metabolic pathways for rosmarinic acid and thymol, the main potential bioactive compounds from thyme. Given its speed, sensitivity, selectivity and low sample amount needed for the analysis, this method could be proposed as a routine analysis in human intervention studies with a large number of samples.

Acknowledgments

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Publication V.

Application of dried spot cards as a rapid sample treatment method for determining hydroxytyrosol metabolites in human urine samples. Comparison with microelution solid-phase extraction.

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RESEARCH PAPER

APPLICATION OF DRIED SPOT CARDS AS A RAPID SAMPLE TREATMENT METHOD FOR DETERMINING HYDROXYTYROSOL METABOLITES IN HUMAN URINE SAMPLES. COMPARISON WITH MICROELUTION SOLID-PHASE EXTRACTION

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Abstract

Two different rapid sample pretreatment strategies, dried spot cards, and microelution solid-phase extraction plates (μSPE), with ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) have been developed and validated for the determination of hydroxytyrosol and its metabolites in spiked human urine samples. Hydroxytyrosol, hydroxytyrosol-3′-*O*-glucuronide, hydroxytyrosol-4′-*O*-glucuronide, hydroxytyrosol-3- O-sulphate, and homovanillic alcohol-4′-*O*-glucuronide were used as the target compounds. Using the FTA DMPK-A dried urine spot card under optimum conditions, with 5 μL of preconcentrated urine volume and 100 μL of methanol/water (50/50, v/v) as the elution solvent, the extraction recovery (%R) of the compounds studied was higher than 80 %, and the matrix effect (%ME) was less than 8 %. The stability of these cards and punching at the centre or side of the card were also studied, obtaining an excellent stability after 7 days of storage and complete homogeneity across the surface of the dried drop. The different μSPE parameters that affect the efficiency were also studied, and under optimum conditions, the %R and the %ME were higher than 70 % and lower that 17 %, respectively. The linearity range in dried urine spot cards was 2.5-20 μM for all the metabolites, with the exception of hydroxytyrosol-3-O-sulphate and hydroxytyrosol, which were 0.3-70 μM and 2.5-50 μM respectively. With regards to μSPE, the linearity range was 0.5-5 μM for all the studied compounds, except for hydroxytyrosol-3-Osulphate, which was 0.08-5 μM. The quantification limits (LOQs) were 0.3-2.5 μM and 0.08-0.5 μM in dried spot cards and in μSPE, respectively. The two developed methods were then applied and compared for determining hydroxytyrosol and its metabolites in human 24 h-urine samples after a sustained consumption (21 days) of a phenol-enriched virgin olive oil. The metabolites identified were hydroxytyrosol in its glucuronide and sulphate forms, homovanillic alcohol in its glucuronide and sulphate forms, homovanillic acid sulphate and hydroxytyrosol acetate sulphate.

KEYWORDS: Dried spot cards / Hydroxytyrosol / Microelution SPE plate / Tandem MS / UPLC / Urine

1. INTRODUCTION

The beneficial effects of virgin olive oil consumption have been widely reported and have been attributed in part to the presence of phenolic compounds [1]. Owing to the properties of olive oil phenolics, research should be directed not only towards the characterization and study of its bioactivity, but also towards revealing its bioavailability in order to gain new insight into their in vivo physiological behavior. Therefore, the determination of virgin olive oil phenolic metabolites in biological samples is of relevance in order to understand how they are absorbed, metabolized, and cleared from the body. Hydroxytyrosol present in olive oil mainly as secoiridoid derivative, and to a lower extent as glycosylate and acetate derivative, is the most active polyphenol from olive oil [2].

Study of the recovery of polyphenols in plasma or urine after intake of olive oil is one way to access their absorption and metabolism in the human body [3]. Only five analytical methods for the determination of phenolic compounds from virgin olive oil in urine samples have been reported in the literature and the quality parameters have been studied by spiking the phenolic compounds in synthetic urine and real urine. These analytical methods are based on liquid chromatography (LC) [4–6] and gas chromatography (GC) [6, 7]. In these studies, mass spectrometry (MS) [5] and tandem MS (MS/MS) [4, 6, 7] were the detection systems of choice given their sensitivity, selectivity, and capacity to identify metabolites when commercial standards are not available.

Urine is one of the easiest biological samples to collect, but it contains high concentrations of different salts that can easily interfere with the ESI ionization and increase the matrix effect (ME). Therefore, their removal from the sample matrix before the chromatographic analysis is essential. The urine pretreatment techniques reported in the literature until now are offline liquid-liquid extraction (LLE) with ethyl acetate [4, 5, 8] and off-line solid-phase extraction (SPE) with hydrophilic lipophilic balanced (HLB) (60 mg) cartridges [6, 7].

In order to expand the analytical methods reported in the literature for determining phenolic compounds and their generated metabolites in complex biological samples, such as urine, plasma, and tissue samples, it is extremely important to develop novel approaches or strategies to improve the quality parameters of the analytical method, such as speed, robustness, sensitivity, and selectivity.

For this reason, we propose dried urine spot cards and microelution SPE plates (μSPE) as two different and rapid sample pretreatment strategies to determine hydroxytyrosol and its main metabolites in human urine after a sustained consumption (21 d) of a phenol-enriched virgin olive oil with 500 mg of total phenolic compounds kg–1 olive oil.

Dried spot cards have been reported as an easy, simple, speedy, and inexpensive sample strategy for collecting, shipping, and storing specimens, such as blood and urine [9–12]. Additionally, in preclinical studies, it allows the reduction of the number of animal tests, which is in accordance with the requirements to reduce, refine, and replace in animal studies.

Over recent years, the applications for dried spot card have increased significantly, and a range of applications has been reported for analyzing biomarkers and drugs in different biological samples, such as blood and urine [13–21]. μSPE also allows a small sample volume to be loaded onto the plate, eluting the retained analytes in a small volume and avoiding the evaporation step to preconcentrate the analytes, prior to the chromatographic analysis. Therefore, this technique also allows a rapid isolation of the target analytes, since the extraction time is considerably reduced [22, 23]. To our knowledge, no scientific research has yet been reported in relation to the application of the dried spot cards and μSPE for determining and quantifying phenolic compounds and their metabolites in urine samples.

After studying the optimum conditions for the two urine pretreatment strategies, the quality parameters of the two methods, based on off-line dried spot cards with UPLC-MS/ MS and off-line μSPE with UPLC-MS/MS, were evaluated. Then, in order to show the applicability of both methods, human urine samples were analyzed, and the results from the two sample pretreatment strategies were compared statistically.

EXPERIMENTAL

Chemicals and reagents

Hydroxytyrosol was purchased from Extrasynthese (Genay, France). Catechol as the internal standard (IS) was from Sigma-Aldrich (St. Louis, MO, USA). The hydroxytyrosol metabolites, hydroxytyrosol-3′-O-glucuronide (purity, 97.8 %), hydroxytyrosol-4′-O-glucuronide (purity, 96.4 %), and homovanillic alcohol-4-O-glucuronide (purity, 99.3 %) were synthesized according the method reported by Khymenets et al. [24].

Hydroxytyrosol-3-O-sulphate was custom synthesized by Toronto Research Chemicals Inc. (Toronto, ON, Canada), and its purity was 98.4 %.

Stock solutions of individual phenolic standard compounds were prepared by dissolving each compound in methanol at a concentration of 1000mg L–1, and storing these in a dark flask at 4 °C. The stability of the stock solutions was previously studied, and the stock solutions at the concentration of 1000 mg L–1 were stable at least for 3-mo time in which the experiments were carried out. After this period, storage stability changes were lower than 8 %.

Methanol (HPLC grade), acetonitrile (HPLC grade), and acetic acid were purchased from Scharlau Chemie (Sentmenat, Barcelona, Spain). Ortho-phosphoric acid (85 %) was purchased from Panreac (Barcelona, Spain). The water was Milli- Q quality (Millipore Corp., Bedford, MA, USA).

Human urine samples

Urine samples from five healthy volunteers were used for the application of the analytical methods developed to determine hydroxytyrosol and its metabolites. The subjects were subjected to a 2-week washout period in which they were only allowed to consume refined olive oil, prior to the administration of 25 mL day^{-1} of virgin olive oil with a high phenolic content (500 mg kg^{-1}) for a period of 3 weeks; 24-h urine was collected from each volunteer on the first day before (d 0) and after (d 21) the sustained consumption of the phenol-enriched olive oil. Subjects signed an informed consent prior to participation in the study. Details of the protocol have been previously provided [25].

Urine pretreatment strategies

In order to clean-up the biological matrix and isolate the phenolic compounds and their metabolites, the urine samples were pretreated using two different sample pretreatment

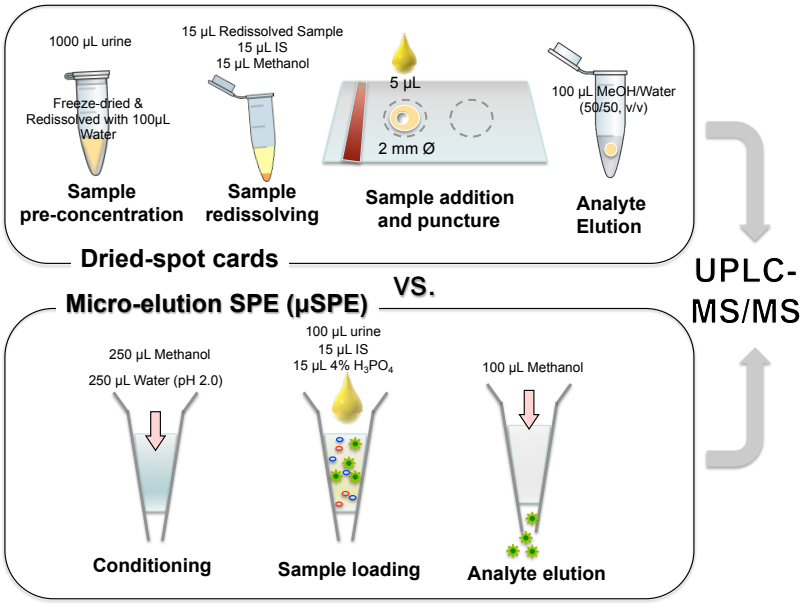


Fig. 1. Schematic procedure of the proposed methods: dried spot cards andmicroelution SPE plates

strategies, namely dried spot cards and μSPE. **Fig. 1** shows the schematic procedure of the proposed methods.

Dried spot cards

FTA DMPK–A, –B and –C cards (GE Healthcare, Buckinghamshire, UK) were tested as dried spots cards to pretreat urine samples. The different parameters that affect the extraction recovery (%R) and the matrix effects (%ME), such as the card type, urine volume, elution solvent nature, and elution volume, were optimized in order to obtain the maximum efficiency and potential of these cards.

One thousand μL of human urine sample was freeze-dried in a Lyobeta 15 equipment (ImaTelstar, Terrassa, Spain) and the dried urine samples were subsequently rehydrated with 100 μL of Milli-Q water (Millipore Corp., Bedford, MA, USA). In the validation study, the studied compounds were spiked in the blank urine samples before this preconcentration step. The blank urine was obtained from three volunteers who were non-consumers of virgin olive oil. A 15 μL volume of this pre-concentrated urine was mixed with 15 μL of IS (its concentration was 4000 mg L^{-1} and was prepared in methanol) and 15 μL of methanol. Then, 5 μL of this solution was spotted onto a premarked circle on the card (filter paper) and dried in the dark at room temperature for 2 h.

Afterward, a 2mm diameter disk was punched out from the card and placed into an Eppendorf with 100 μL of methanol/Milli-Q water (50/50, v/v). A Harris Uni-Core punch and a Cutting Mat were supplied by Whatman Inc. (Sanford, ME, USA). The Eppendorf was then vortexed for 5 min and centrifuged at 9000 rpm for 5 min at 4 °C. Afterwards, the supernatant was introduced in a chromatographic vial and 2.5 μL of this solution was directly injected into the UPLC-MS/MS system and the hydroxytyrosol metabolites were analyzed.

Microelution SPE plates (μSPE)

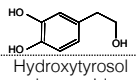
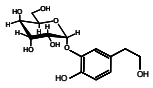
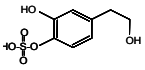
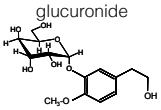
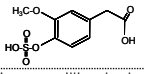
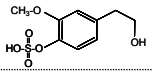
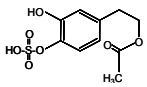
OASIS hydrophilic-lipophilic balance (HLB) μElution plates 30 μm (Waters, Milford, MA, USA) were used. These were conditioned sequentially with 250 μL ofmethanol and 250 μL of Milli-Q water at pH 2 with acetic acid. Aliquots of 50 μL of phosphoric acid 4 % and 50 μL of catechol, as the IS (its concentration was 2 mg L^{-1} and it was prepared in phosphoric acid 4 %), were added to 100 μL of human urine sample, and this solution was loaded into the plate. The retained hydroxytyrosol metabolites were then eluted with 2 x 50 μL of methanol. 2.5 μL of the eluted solution were directly injected into the UPLC-MS/MS system.

Liquid chromatography-tandem mass spectrometry

The UPLC analysis of hydroxytyrosol and its metabolites was performed using a Waters Acquity UPLC system (Waters), equipped with a Waters binary pump system (Waters). The UPLC system was coupled to a triple quadrupole detector (TQD) mass spectrometer (Waters). The analytical column was the Acquity UPLC BEH C18 (2.1 x 100 mm, 1.7 μm) also fromWaters. Themobile phase was 0.2%acetic acid as eluent A and acetonitrile as eluent B. The elution started at 3 % of eluent B and was kept isocratic for 2.1 min. Then it was increased linearly to 7 % of eluent B in 4.9 min, 12 % of eluent of B in 3 min, and further increased to 100 % of eluent of B in 0.1 min and kept isocratic for 4.9 min. Next, it was returned to the initial conditions in 0.1 min, and the re-equilibration time was 2.9 min. The flow-rate was 0.4 mL min^{-1}, and the temperature of the column during the analysis was kept at 30 °C. The injection volume was 2.5 μL, and the injection mode was "partial loop with needle overfill." Ionization was achieved with an electrospray (ESI) interface operating in the negative mode [M – H]– and the data were collected by selected reaction monitoring (SRM). The ionization source parameters were capillary voltage, 3 kV; source temperature, 150 °C; cone gas flow-rate, 80 L h^{-1} and desolvation gas flow rate, 800 L h^{-1}; desolvation temperature 400 °C. Nitrogen (99.99 % purity, N2LCMS nitrogen generator; Claind, Lenno, Italy) and argon (≥ 99.99 % purity; Aphagaz, Madrid, Spain) were used as the cone and collision gases, respectively. The SRMtransitions and the individual cone voltage and collision energy for IS, and hydroxytyrosol and hydroxytyrosol metabolites standards were evaluated by infusing 10 mg L^{-1} of each compound to obtain the best instrumental conditions. The most sensitive transition was selected for quantification and a second one for

confirmation purposes. **Table 1** shows the MS/MS transitions for quantification and confirmation, as well as the cone voltage and the collision energy values optimized for each of the studied standard compounds. In this table, the SRM parameters of the hydroxytyrosol metabolites, homovanillic acid sulphate, homovanillic alcohol sulphate, and hydroxytyrosol acetate sulphate are also reported. Their SRM parameters are derived from the

Table 1. Chemical structures and SRM conditions for the analysis of hydroxytyrosol and its metabolites by UPLC-MS/MS.

Phenolic compounds	Molecular weight (g mol^{-1})	SRM (quantification)	Cone voltage (V)	Collision energy (eV)	SRM (identification)	Cone voltage (V)	Collision energy (eV)
Standard compounds							
Catechol (IS) 	110	108.9 > 90.9	40	15	- - -		
Hydroxytyrosol 	154	153 > 123	35	10	153 > 95	35	25
Hydroxytyrosol glucuronide 	330	329 > 153	40	20	329 > 123	40	25
Hydroxytyrosol sulphate 	234	233 > 153	40	15	233 > 123	40	20
Homovanillic alcohol glucuronide 	344	343 > 167	40	20	343 > 152	40	35
No standard compounds[a]							
Homovanillic acid sulphate 	262	261 > 181	40	15	261 > 137	40	25
Homovanillic alcohol sulphate 	248	247 > 167	40	15	247 > 152	40	25
Hydroxytyrosol acetate sulphate 	275	275 > 195	35	15	195 > 135	35	10

[a] The SRM parameters were achieved by full-scan mode in MS and daughter scan mode in tandem MS from human urine sample obtained after 3 weeks of daily intake of 25 mL of virgin olive oil with high phenolic content (500 mg kg-1), and from the previous studies [20,24].

analysis of human urine samples (after 3 wk of a daily intake of 25 mL of high phenolic content virgin olive oil) by full-scan mode and daughter scan mode in MS and in tandem MS, respectively, and additionally from the previous reported studies [22, 26]. The dwell time established for each transition was 30 ms, and the software used was MassLynx 4.1.

The hydroxytyrosol metabolites, hydroxytyrosol-3′-O-glucuronide, hydroxytyrosol-4′-O-glucuronide, hydroxytyrosol- 3-O-sulphate, and homovanillic alcohol-4′-O-glucuronide were quantified respect to its own calibration curve (chemicals and reagents section). On the other hand, the metabolites homovanillic acid sulphate and homovanillic alcohol sulphate were quantified by using the calibration curve of alcohol homovanillic-4′-Oglucuronide, and hydroxytyrosol acetate sulphate was quantified by using the calibration curve of hydroxytyrosol-3-O-sulphate because of a lack of these standards.

Method validation

Validation parameters of the developed methods, such as the linearity, repeatability, accuracy, detection limits (LODs), and quantification limits (LOQs) were determined by spiking blank urine samples with known concentrations of standards of hydroxytyrosol and its metabolites. The calibration, precision, and accuracy were calculated by using the Microsoft Office Excel. In order to reduce inaccuracies attributable to the sample manipulation, the working solutions were spiked in urine samples at high volumes, from 100 to 500 µL, which were between 1 % and 5 % of the proportion of the total sample volume. In addition, the solutions used to prepare the standards and to study the precision/accuracy were the same ones.

A pool of human urine obtained under fasting conditions was used as blank urine. This urine pool was checked to verify that it was free of hydroxytyrosol metabolites. In the dried urine spot cards sample pretreatment, the working solutions were prepared in methanol, whereas for the µSPE sample pretreatment, phosphoric acid 4 % was used to prepare the working solutions.

The linearity range of the method was evaluated in different days by using blank urine spiked with the studied phenolic compounds. The calibration curves (based on peak area abundance) were plotted using the equation y=a+bx, where y is the (analyte/IS) peak area abundance ratio and x the (analyte/IS) concentration ratio.

The concentrations of hydroxytyrosol and its metabolites were calculated by interpolating their (analyte/IS) peak area abundance ratios on the calibration curve. The calibration curves were obtained by analyzing five points at different concentration levels and each standard solution was injected three times.

The precision of the method was determined by the relative standard deviations (%RSDs) of the concentration. The accuracy was calculated from the ratio between the concentrations of the phenolic compounds found compared with the spiked concentration. This quotient was then multiplied by 100. The LODs and LOQs were calculated using the signal-to-noise ratio criterion of 3 and 10, respectively.

The extraction recovery (%R) and the matrix effect (%ME) were also studied. To determine the %R of each phenolic compound in the two sample pretreatments, the peak areas of the analytes spiked in blank urine before and after sample pretreatment were compared. The %ME was evaluated by comparing the peak areas obtained from a blank urine sample spiked after sample pretreatment with those obtained from dissolving the standards with methanol/Milli-Q water (50/50, v/v) or methanol, according to whether the sample pretreatment was dried spot cards or µSPE, respectively.

The %R and the %ME were evaluated at three concentration levels with three replicates for each concentration. The concentration levels studied were 5, 10, and 20 µM for dried urine spot cards and 0.5, 1, and 5 µM for µSPE.

Statistical analysis

The quantification data of the different hydroxytyrosol metabolites studied were analyzed by Student's t-test in order to analyze the significant differences between the two urine sample pretreatments. In this sense, individual and average values of each urine specimen collected on d 0 were compared. Separately, individual and average values from the two pretreatments obtained from d 21 were also compared. Concentration values from d 0 and d 21 were not compared.

Significant differences were considered at a level of $P < 0.05$. All the statistical analyses were carried out using STATGRAPHICS Plus 5.1 (Manugistics Inc., Rockville, MD, USA).

RESULTS AND DISCUSSION

Dried spot cards as the urine pretreatment strategy

Dried urine spot cards were used as the urine pretreatment strategy in order to clean up the urine matrix and extract the target phenolic compounds. The absorption of dried urine spots onto the paper was reported as a type of pretreatment where several matrix components could be retained, while the target analytes could be released when the paper was treated with a suitable solvent.

For this sample pretreatment strategy, three FTA DMPK cards were tested, these being cardA (red), card B (black), and card C (blue). The FTA DMPK–A and –B cards are characterized by being chemically impregnated with proprietary mixtures that quickly lyse cells and denature proteins, including degradative enzymes. In contrast, the FTA DMPK–C cards contain pure cellulose, for applications that do not require those capabilities. Besides, the FTA DMPK–B cards are hygroscopic so they should be stored with effective desiccant In humid environments [27].

Owing to the low amount of the hydroxytyrosol standard metabolites available, the three cards were tested with hydroxytyrosol and catechol (IS) standards, and the extraction recovery (%R) and matrix effect (%ME) of these two phenolic compounds were evaluated. The matrix effect (%ME) is one of the main drawbacks of MS methods when ESI is used as the ionization technique. This effect is due to co-eluting matrix components that compete for ionization capacity and, therefore, it is essential to evaluate this effect when MS is used as the detector system [28]. When the different cards were compared, the conditions used were 10 µL of pre-concentrated urine sample, 2 h of incubation time, and 150 µL methanol/ water (50/50, v/v) of elution solvent.

The best results were obtained when card A (red) and card B (black) were used, and because of the hygroscopic characteristics of card B, card A was chosen. For card A, the %R and the %ME of hydroxytyrosol and IS were higher than 85 % and lower than 8 %, respectively. **Table 2** shows the results from the three cards tests. Once the A card was selected, the parameters that affect the efficiency of the urine sample pretreatment were studied. These parameters were the pre-concentrated urine sample volume, the nature of the elution solvent, and the elution volume. In a preliminary step, the urine sample (1000 µL) was freeze-dried in order to pre-concentrate the phenolic compounds, since with this sample pretreatment does not allow to pre-concentrate the analytes. The stability of the phenolic compounds during freeze drying was previously studied, and we proved that freeze-drying process did not alter the phenolic content of biological samples (data not published). Thus, we considered that this process and the later reconstitution was a very respectful treatment that could allow us to pre-concentrate the urine samples without altering the stability of hydroxytyrosol and its metabolites. After being freeze-dried, the urine was dissolved with 100 µL of Milli-Q water. Then, 15 µL of the reconstituted urine was mixed with 15 µL of IS and 15 µL of methanol. The mixture obtained was then pretreated with the dried spot cards.

Table 2. recovery (%R) and matrix effect (%ME) for the determination of hydroxytyrosol and the IS catechol in spiked urine samples by dried urine spot cards with UPLC-MS/MS.

	Dried urine spot cards: types					
	Extraction recovery (%R)			Matrix effect (%ME)		
	FTA® DMPK-A (red card)	FTA® DMPK-B (black card)	FTA® DMPK-C (blue card)	FTA® DMPK-A (red card)	FTA® DMPK-B (black card)	FTA® DMPK-C (blue card)
Catechol (IS)	102	86	132	-13	-15	27
Hydroxytyrosol	86	89	69	12	10	6

	Dried urine spot cards: urine volume							
	Extraction recovery (%R)				Matrix effect (%ME)			
	5 µL	10 µL	15 µL	20 µL	5 µL	10 µL	15 µL	20 µL
Catechol (IS)	110	85	66	60	-6	-9	-17	-21
Hydroxytyrosol	107	81	70	65	1	-7	-10	-14

The spiked concentration was 10 µM. The number of replicates was three (n =3).

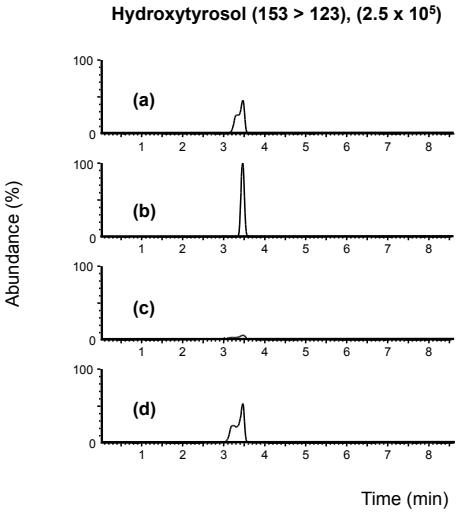


Fig. 2 Extracted ion chromatogram (EICs) of hydroxytyrosol obtained for the optimization of the elution solvent by dried spot cards in spiked blank urine sample. The experimental conditions were as follows: 5 µL pre-concentrated urine sample and 100 µL of elution solvent of (a) methanol, (b) methanol/ water (50/50, v/v), (c) acetonitrile, and (d) acetonitrile/water (50/50, v/v)

To optimize the volume of concentrated urine applied to the card, four volumes (5, 10, 15, and 20 µL) were tested. To test the urine sample volume, 2 h of incubation time and 150 µL methanol/water (50/50, v/v) as the elution solvent were used.

The best results in terms of %R and %ME were obtained with 5 µL (**Table 2**). Increasing volumes of concentrated urine resulted in a reduction of the %R and an increase of the %ME. These results could be due to an overloading in the card with the urine salts.

In order to elute the phenolic compounds and their metabolites from the paper with maximum efficiency, several assays were carried out. The suitable elution solvent composition and the volume were optimized. Pure organic solvents and aqueous/organic mixtures, such as methanol, methanol/water (50/50, v/v), acetonitrile, and acetonitrile/water (50/50, v/v), were tested. Elution volumes were tested in a range of 50 to 150 µL. The other conditions employed were 5 µL as the preconcentrated urine sample volume and 2 h as the incubation time. Figure 2 shows the extracted ion chromatogram (EICs) of hydroxytyrosol in the spiked blank urine sample using 100 µL of the four elution solvents tested, (a) methanol, (b) methanol/Milli-Q water (50/50, v/v), (c) acetonitrile, and (d) acetonitrile/Milli-Q water (50/50, v/v). As can be seen in **Fig. 2**, when the elution solvent methanol/Milli-Q water (50/50, v/v) was used, the peak shape was improved and the peak efficiency also increased in comparison with the other elution solvents. This could be due to the stronger elution capacity of methanol, acetonitrile, and acetonitrile/Milli-Q water (50/50, v/v) in comparison with the mobile phase when 50 µL was used, the hydroxytyrosol was not completely eluted from the card. The maximum extraction efficiency of hydroxytyrosol was reached with 100 and 150 µL of elution solvent with the same behavior for the extraction efficiency of the hydroxytyrosol metabolite standards studied. Therefore, 100 µL of methanol/ Milli-Q water (50/50, v/v) was chosen as the optimum volume and nature of the elution solvent in terms of peak shape, peak efficiency, %R and %ME of hydroxytyrosol in the spiked blank urine (Fig. 2b).

Under these optimum conditions, FTA DMPK-A as the card, 5 µL as the pre-concentrated urine sample volume, and 100 µL methanol/Milli-Q water (50/50, v/v) as the elution solvent volume and elution solvent nature, the %R and the %ME for standards of hydroxytyrosol and its metabolites were higher than 70 % and lower than 12 %, respectively. These results are shown in **Table 3.**

Once the parameters that affect the efficiency of the extraction were fully optimized, other parameters, such as the stability of the compounds in the dried spot cards and the puncture site, were studied.

Stability of the compounds on the dried spot cards

To evaluate the stability of hydroxytyrosol and its metabolites in the cards, blank urine spiked with all the studied phenolic compounds was applied to the cards and these were stored in the desiccator at room temperature for 2 d, 1 wk, and 1 mo. These were then analyzed following the described pretreatment method (see Experimental section).

The differences in peak area ratio between a stock solution after storage in the dark at room temperature in the desiccator for 2 d, 1 wk, and 1mo, and the freshly prepared solutions were 1.8 %, 2.5 %, and 10.6 %, respectively. This indicates

Table 3. Extracted Extraction recovery (%R) and matrix effect (%ME) obtained under the optimum dried urine spot cards and μSPE conditions for the determination of hydroxytyrosol and its metabolites and the IS catechol in spiked urine samples.

	Dried spot cards		*μ*SPE	
	Card: FTA® DMPK-A (red) Urine sample: 5 μL urine (previously freeze-dried) Elution solvent: 100 μL methanol/water (50/50, v/v)		Micro-cartridge: Oasis HLB 30 μm Conditioning and equilibration: 250 μL methanol and water at pH 2 Urine load: 100 μL urine Elution: 100 μL methanol	
	Extraction recovery (%R)	Matrix effect (%ME)	Extraction recovery (%R)	Matrix effect (%ME)
Catechol (IS)	89	-11	90	-9
Hydroxytyrosol	120	10	81	15
Hydroxytyrosol-4'-O-glucuronide	70	12	70	10
Hydroxytyrosol-3'-O-glucuronide	69	6	72	12
Hydroxytyrosol-3-O-sulphate	74	8	75	17
Homovanillic alcohol-4'-O-glucuronide	69	2	74	14

The spiked concentration was 10 μM for dried spot cards and 1 μM for μSPE, for all the compounds. The number of replicates was three (n =3)

that hydroxytyrosol and its metabolites are very stable for at least 7d in the card stored at room temperature, although after 1 mo storage, the stability could be considered also acceptable.

Puncture site

Punching out from the center or edge of the card after the urine was spotted onto the card was compared. This was studied at three concentration levels, 20, 10, and 5 μM, for all the studied compounds, an exception of hydroxytyrosol-3-O-sulphate, which were 50, 20, and 0.5 μM. The number of replicates of each concentration level was five (n =5). The average difference in the peak area ratio between the two puncture sites was 0.9 %. This result indicates that the circle of the card that contains hydroxytyrosol and its metabolites could be punched out anywhere in the dried drop in order to be eluted.

Figure 3 shows the total ion chromatogram (TIC) of (A) a blank urine sample, and (B) a blank urine sample spiked with hydroxytyrosol and its metabolites under the optimum conditions of the dried urine spot cards. The concentration of hydroxytyrosol-4′Oglucuronide, hydroxytyrosol-3′-O-glucuronide and homovanillic alcohol-4-O-glucuronide was 1 μM, and hydroxytyrosol-3-O-sulphate and hydroxytyrosol was 0.4 μM. As can be seen in this figure, no interfering compounds were eluted at the same retention time as the studied phenolic compounds.

μSPE as the urine pre-treatment strategy

Initial experiments to determine hydroxytyrosol and its metabolites in urine samples were based on our previous study where hydroxytyrosol metabolites were analyzed in plasma samples by OASIS HLB μSPE plate [22]. Briefly, in that report the conditioning and the equilibration were done by applying 250 μL of methanol and 250 μL of Milli-Q water at pH 2, respectively. Two hundred μL of plasma sample with 150 μL of phosphoric acid 4 % and 50 μL of IS (catechol) were loaded onto the plate. Then, 75 μL ofMilli-Q water and 75 μL of 5 % methanol were used to clean the plate. Finally, the retained phenolic compounds were eluted with 2×25 μL of 50 % acetonitrile.

By using these μSPE conditions to determine hydroxytyrosol and its standard metabolite compounds in spiked urine samples, the %R was lower than 50 % and the %ME higher than 35 %. This fact was probably due to the complexity of urine in comparison with plasma samples, because urine contains urea, creatinine and the chloride, sodium, and potassium lions that could interfere with the interaction between the sorbent and analyte of interest. It was observed that the phenolic compounds studied were almost lost in the load and clean-up steps. The target compounds (hydroxytyrosol and its metabolites)

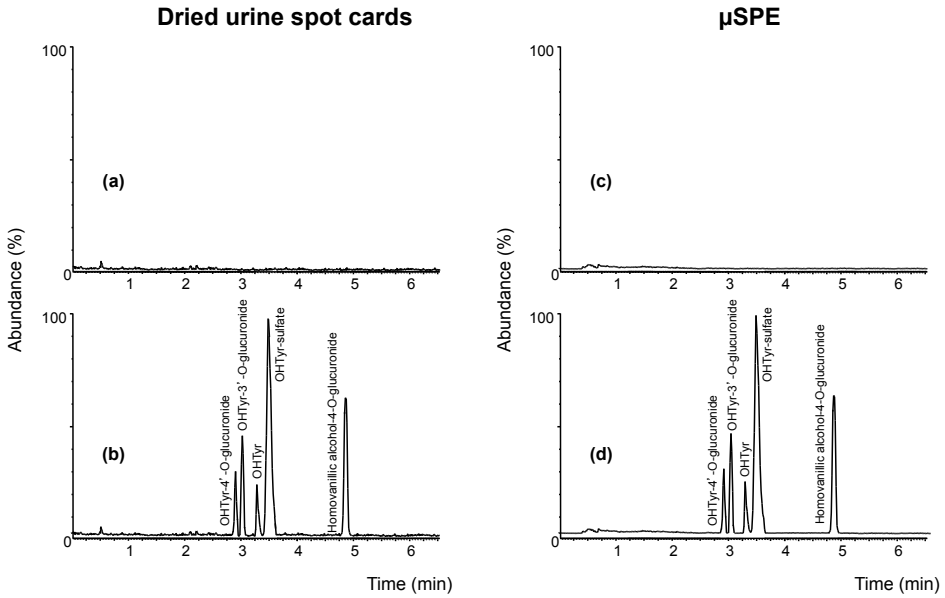


Fig. 3 Total ion chromatogram (TIC) for the determination of hydroxytyrosol and its metabolites in (a) a blank urine sample by dried spot cards, (b) a blank urine sample spiked with the studied phenolic compounds by dried urine spot cards, (c) a blank urine sample by μSPE, and (d) a blank urine sample spiked with the studied phenolic compounds by μSPE. The concentration of hydroxytyrosol-4′-O-glucuronide, hydroxytyrosol-3′-O-glucuronide and homovanillic alcohol-4-O-glucuronide was 1 μM, and hydroxytyrosol-3-O-sulphate and hydroxytyrosol was 0.4 μM

competed with other interference compounds in the urine matrix for the micro-sorbent and, additionally, because of the polarity of the hydroxytyrosol and its metabolites, these were lost in these steps.

In order to improve the efficiency of the extraction to determine hydroxytyrosol and its metabolites in urine samples, some parameters that affect the μSPE extraction were studied and optimized. These parameters were urine volume, clean-up solvent nature, clean-up solvent volume, elution solvent nature, and elution solvent volume.

First, various volumes of urine, from 50 to 350 μL, were loaded onto the plate. Samples of more than 350 μL of urine were not tested because this method involves loading the samples onto the plate mixed with the same proportion of phosphoric acid 4 % and the maximum sample volume that can be loaded is 750 μL [22]. Then, different solvents, such as Milli-Q water and 5 % methanol, were tested in the clean-up steps in volumes from 0 to 100 μL. The best conditions in terms of %R were achieved when 100 μL of urine with 50 μL of IS and 50 μL of phosphoric acid 4 % were loaded onto the plate and no clean-up step was used. No clean-up step was done because the target standard compounds were eluted when the micro-sorbent was cleaned up with water, and this was probably due to the polarity of these compounds. Furthermore, without this clean-up step, the %ME was good (lower than 18 %).

Then, different elution solvents, such as methanol, methanol/ Milli-Q water (50/50, v/v), acetonitrile, and acetonitrile/Milli-Q water (50/50, v/v), were evaluated in the volumes from 50 to 150 μL. Methanol was chosen as the elution solvent because it gave a better %R and a lower %ME than the other elution solvents tested when extracting the hydroxytyrosol metabolites. One hundred μL (2Å~50 μL) of methanol was the optimum elution solvent volume to elute the studied phenolic compounds. Lower methanol volumes were not enough to elute all these phenolic compounds, and higher methanol volumes decreased the sensitivity of the method. **Table 3** also shows the optimum μSPE conditions

for the extraction of the compounds studied in the spiked blank urine and their %Rs and %MEs. As can be seen, the %Rs were higher than 70 % and the %MEs were lower than 17 % for all the compounds although no clean-up step was used.

Figure 3 also shows the total ion chromatogram (TIC) of (C) a blank urine sample, and (D) a blank urine sample spiked with standards of hydroxytyrosol and its metabolites in the optimum conditions of μSPE. The concentration of these compounds was the same as that reported in the dried spot cards. No interfering compounds were eluted at the same retention time as the studied phenolic compounds (**Fig. 3a** and **b**). By comparing the two urine pretreatments (**Table 3**), good %Rs and %MEs were achieved for the extraction of the studied phenolic compounds, and these values were higher than 70 % and lower than 15 %, respectively. The studies reported in the literature based on the determination of phenolic compounds from olive oil in urine samples only studied the %R of hydroxytyrosol in synthetic urine samples, and these values were lower [4, 5, 8] or similar [6, 7] to those obtained in our study. Generally, in the reports in which the %R of hydroxytyrosol was similar to our results, the sample pretreatment used was SPE. As well as studying the %R of hydroxytyrosol, Khymenets et al. [6] studied the %R of other hydroxytyrosol metabolites, such as hydroxytyrosol glucuronides

and alcohol homovanillic alcohol glucuronide in synthetic urine and the obtained values were comparable with those in our study. On the other hand, no %ME values for the determination of these phenolic compounds in urine samples are reported in the literature.

Analytical quality parameters

The analytical quality parameters of the two developed methods, off-line dried urine spot cards with UPLC-MS/MS and off-line μSPE with UPLC-MS/MS, were studied by spiking the phenolic compounds studied in blank urine samples at different known concentrations. These parameters were linearity range, calibration curves, repeatability, accuracy, the LOQ, and the LOD. The results obtained for the two methods are shown in **Table 4**.

The respective linearity range for the analysis of hydroxytyrosol and its metabolites in dried urine spot cards was from 2.5 to 20 μM for all the

Table 4. Quality parameters of the two developed methods for the determination of hydroxytyrosol and its metabolites in spiked urine samples.

Compound	Dried urine spot cards and UPLC-MS/MS								
	RT (min)	Linearity (μM)	Calibration curve	%RSD (n=3), (intra-day)			Accuracy (%) (10 μM)	LOQ (μM)	LOD (μM)
				C1 (20 μM)	C2 (10 μM)	C3 (5 μM)			
Hydroxytyrosol-4'-*O*-Glucuronide	2.9	2.5-20	y=0.434x + 0.005	3.2	3.6	5.8	97	2.5	0.8
Hydroxytyrosol-3'-*O*-Glucuronide	3.4	2.5-20	y=0.855x – 0.013	2.7	3.4	6.3	95	2.5	0.8
Hydroxytyrosol-3-*O*-sulphate	3.5	0.3-70	y=3.978x – 0.096	2.0	2.6	5.0	99	0.3	0.1
Hydroxytyrosol	3.7	2.5-50	y=1.127x – 0.025	1.4	1.7	3.7	101	2.5	0.7
Homovanillic alcohol-3'-*O*-Glucuronide	4.8	2.5-20	y=0.337x + 0.011	0.1	1.5	3.1	103	2.5	0.6
	μSPE and UPLC-MS/MS								
Compound	RT (min)	Linearity (μM)	Calibration curve	%RSD (n=3), (intra-day)			Accuracy (%) (1 μM)	LOQ (μM)	LOD (μM)
				C1 (5 μM)	C2 (1 μM)	C3 (0.2 μM)			
Hydroxytyrosol-4'-*O*-Glucuronide	2.9	0.5-5	y=2.970x + 0.038	5.2	6.4	6.9	99	0.5	0.2
Hydroxytyrosol-3'-*O*-Glucuronide	3.4	0.5-5	y=2.936x + 0.081	5.5	6.0	7.1	101	0.5	0.2
Hydroxytyrosol-3-*O*-sulphate	3.5	0.08-5	y=8.978x + 0.036	6.0	6.6	7.8	102	0.08	0.02
Hydroxytyrosol	3.7	0.5-5	y=3.039x + 0.106	6.5	7.0	7.2	100	0.5	0.15
Homovanillic alcohol-3'-*O*-Glucuronide	4.8	0.5-5	y=2.101x + 0.003	7.0	7.5	7.9	98	0.5	0.15

Table 5. Concentration of the different generated hydroxytyrosol metabolites (μmols/24 h) in urine samples collected for 24 h, its average (n=5), its standard deviation (s) with applying the sample pretreatments dried spot cards and μSPE.

Compound (μmols/24 h urine)		Day 0		Day 21	
		Dried spot cards	μSPE plate	Dried spot cards	μSPE plate
Hydroxytyrosol sulphate	Subject 1	23.78 ± 0.95	25.75 ± 1.05	62.04 ± 13.46	53.93 ± 18.33
	Subject 2	90.03 ± 3.86	71.08 ± 10.23	261.67 ± 16.41	234.41 ± 15.77
	Subject 3	17.84 ± 1.13	16.94 ± 1.28	59.05 ± 10.11	43.13 ± 7.23
	Subject 4	24.27 ± 14.41	11.57 ± 3.72	111.91 ± 12.34	95.87 ± 4.42
	Subject 5	26.25 ± 7.74	18.55 ± 4.45	126.81 ± 12.51	90.54 ± 18.06
	Average	36.43 ± 30.13	28.78 ± 24.18	124.18 ± 82.16	103.58 ± 76.59
Homovanillic alcohol sulphate	Subject 1	9.58 ± 2.98	13.94 ± 2.28	30.16 ± 5.79	25.76 ± 5.76
	Subject 2	22.96 ± 5.82	13.43 ± 3.43	96.09 ± 10.78	124.86 ± 10.07
	Subject 3	6.22 ± 0.85	7.81 ± 0.98	25.24 ± 7.49	16.15 ± 2.58
	Subject 4	22.21 ± 1.20	18.55 ± 1.17	39.12 ± 4.75	24.32 ± 8.63
	Subject 5	33.79 ± 10.89	22.54 ± 6.54	68.82 ± 6.98	56.20 ± 6.03
	Average	18.95 ± 11.14	15.25 ± 5.58	51.89 ± 29.94	49.46 ± 44.82
Homovanillic Alcohol glucuronide	Subject 1	n.d.	n.q.	0.01 ± 0.00	0.01 ± 0.00
	Subject 2	n.d.	n.q.	13.17 ± 3.52	10.64 ± 2.12
	Subject 3	n.d.	n.q.	2.03 ± 0.25	2.32 ± 0.32
	Subject 4	n.d.	n.q.	2.99 ± 0.45	3.52 ± 0.50
	Subject 5	n.d.	n.q.	1.75 ± 0.23	1.48 ± 0.26
	Average	n.d.	n.q.	3.99 ± 5.24	3.59 ± 4.14
Homovanillic acid sulphate	Subject 1	43.38 ± 1.52	44.80 ± 1.37	95.59 ± 8.36	70.49 ± 15.21
	Subject 2	101.16 ± 20.57	64.50 ± 16.16	149.24 ± 10.54	197.75 ± 20.68
	Subject 3	26.22 ± 5.61	36.86 ± 5.33	44.79 ± 7.82	33.36 ± 5.10
	Subject 4	90.91 ± 2.20	87.67 ± 2.95	135.32 ± 31.59	174.54 ± 32.30
	Subject 5	103.57 ± 28.01	190.37 ± 47.60	241.37 ± 27.88	330.99 ± 64.95
	Average	73.05 ± 35.76	84.84 ± 62.18	133.26 ± 72.79	161.43 ± 117.17
Hydroxytyrosol acetate sulphate	Subject 1	31.69 ± 1.75	32.07 ± 1.88	62.84 ± 12.41	47.99 ± 9.89
	Subject 2	18.81 ± 2.75	21.91 ± 2.42	21.16 ± 5.55	15.26 ± 2.95
	Subject 3	14.79 ± 3.66	11.00 ± 3.95	24.58 ± 2.35	21.43 ± 2.99
	Subject 4	16.72 ± 4.85	13.24 ± 2.54	381.32 ± 64.21	254.70 ± 46.58
	Subject 5	22.17 ± 4.19	14.77 ± 4.67	96.95 ± 9.95	77.29 ± 16.17
	Average	20.84 ± 6.66	18.60 ± 8.57	117.17 ± 150.80	83.33 ± 98.89

n.d. = no detected.
n.q. = its concentration was between its LOD and its LOQ.
Values are not significantly different ($p < 0.05$). Comparisons were made separately on d 0 and on d 21.

metabolites, except for hydroxytyrosol-3-*O*-sulphate and hydroxytyrosol, which were from 0.3 to 70 μM and from 2.5 to 50 μM, respectively.

With regards to μSPE, the linearity range was 0.5 to 5 μM for all the studied compounds, except for hydroxytyrosol-3-Osulphate, which was from 0.08 to 5 μM. The calibration curves (based on peak area abundance) were calculated by using six points at different concentrations levels, and each concentration was injected three times. The determination coefficient (R^2) of the calibration curves was higher than 0.996.

The repeatability of the two analytical methods was determined by the relative standard deviation (% RSD) in terms of concentration, and this was calculated at three concentration levels of 20, 10, and 5 μM for dried spot cards for all the studied compounds, except for hydroxytyrosol-3-*O* sulphate, which was 50, 20, and 0.5 μM. Regarding for μSPE, the studied concentration levels were 5, 1, and 0.5 μM for all the compounds, except for hydroxytyrosol-3-O-sulphate, which was 5, 1, and 0.1 μM. For each concentration, three replicates were done. The %RSDs were lower than 6.3 and 7.9 %, respectively. As can be seen in Table 4, the %RSD increased as the concentration decreased.

The accuracy was calculated from the ratio between the concentration found for the standard phenolic compounds studied compared with the

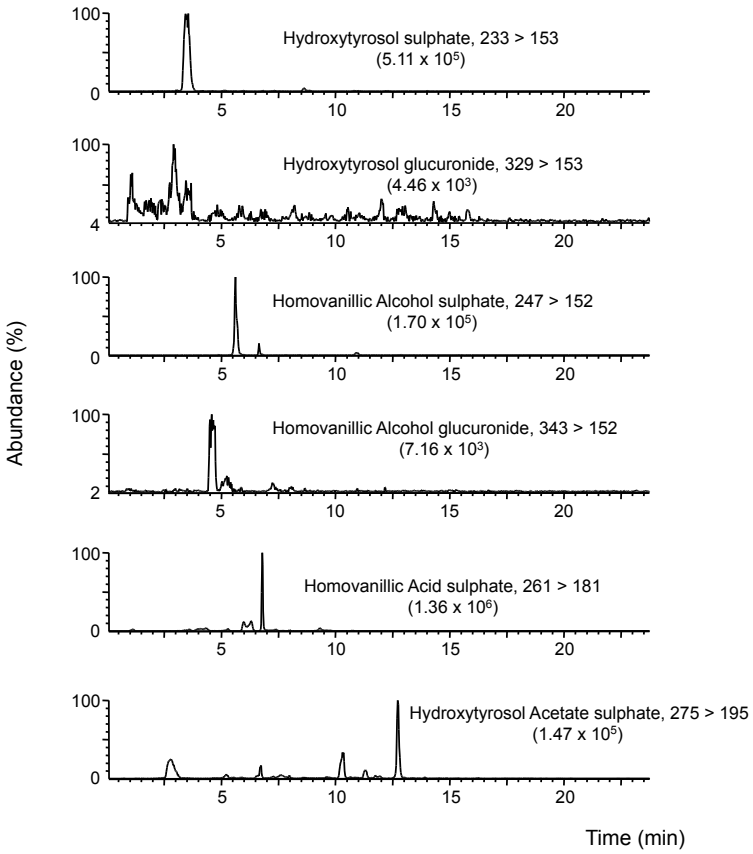


Fig. 4. Extracted ion chromatograms (EICs) of hydroxytyrosol metabolites obtained for the analysis of human urine sample obtained from a long-term intake of an enriched virgin olive oil (500 mg kg–1) for 21 d. The sample pretreatment was dried urine spots and the analytical technique, UPLC-MS/MS

spiked concentration. This quotient was then multiplied by 100. This quality parameter was also studied at three concentration levels, the same as the RSD%, and these ranged from 95 % to 103 % for the two sample pretreatment strategies.

The LOQs and LODs were calculated using the signalto-noise ratio criterion of 10 and 3, respectively. When the sample pretreatment dried spot cards were used, the LOQs and LODs for the analysis of hydroxytyrosol and its metabolites were in the range of 0.3–2.5 μM and 0.1– 0.8 μM, respectively, compared with a range of 0.08–0.5 μM and 0.02–0.2 μM when μSPE was used.

Comparing these values (LOQs and LODs) with the two sample pretreatment strategies, these values were around 5-fold lower when μSPE was applied. This could be explained by the large size of the pretreated urine sample (100 μL in μSPE compared with 5 μL in the dried spot cards). Although the urine sample in dried spot cards was first concentrated 10-fold by freeze-drying, the urine sample was then diluted 20-fold because 5 μL was spotted onto the card and the analytes were desorbed by contact during 5 min with 100 μL of elution solvent. In any further analysis, one approach to take into consideration could be to take more than one punch from the card.

The obtained results (LODs and LOQs) by the sample pretreatment μSPE were similar [6] and higher [4, 8] than those reported in the literature

for the extraction of hydroxytyrosol and its metabolites by off-line SPE, and hydroxytyrosol by off-line LLE, respectively, from synthetic urine sample. In these previous reports, a higher urine volume, from 500 and 1000 µL, was analyzed in comparison with the 100 µL used in µSPE. For hydroxytyrosol analysis, the reported LODs and LOQs were 10–160 nM and 31–487 nM, respectively [2, 5, 7]. For the analysis of hydroxytyrosol glucuronide metabolites, the eported LODs and LOQs were 10–16 nM and 32–50 nM, respectively.

In contrast, for the analysis of hydroxytyrosol sulphate no values could be compared with those reported in the literature because its LOD and LOQ have been determined for the first time. The stability of the studied phenolic compounds in urine samples was also studied. This was evaluated by using UPLC PDA system at 278 nm. The detector system tandem MS was not used in order to avoid inaccuracies derived from matrix effect. In this study, the analytes were spiked in urine samples before the sample pretreatments at different known concentrations and their peak abundances were compared with those obtained when the analytes were prepared in organic solvent (methanol/water, 50/50, v/v in dried spot cards or methanol in µSPE) at the same known concentrations. The concentration of the spiked urine was between 10 mg/l and the LOQ of each phenolic compound. The stability of these compounds in urine samples was higher than 92% in all the concentration ranges studied.

Application of the methods to human urine

In order to show the applicability of the two sample pretreatment strategies, dried spot cards and µSPE were used to determine hydroxytyrosol and its metabolites in human urine after a sustained consumption (21 d) of virgin olive oil with a high phenolic content (500 mg kg^{-1}). Urine samples collected on the first day of the intervention period (d 0) were also analyzed in order to apply the methods for the analysis of different concentration levels of hydroxytyrosol metabolites.

In this sense, 24 h urine from five healthy subjects was analyzed using the two sample pretreatment strategies and then the results were compared.

Apart from determining the hydroxytyrosol metabolites included in the validation study, full-scan mode, daughterscan mode, and neutral loss mode were also used in MS and tandem MS modes, in order to identify other hydroxytyrosol metabolites. The resulted SRM transitions are showed in **Table 1,** and these results were also reported in our previous studies [21, 25].

The same hydroxytyrosol metabolites were identified with the two sample pretreatment strategies. These were hydroxytyrosol in its sulphate and glucuronide conjugates, homovanillic alcohol in its sulphate and glucuronide conjugates, homovanillic acid sulphate, and hydroxytyrosol acetate sulphate. **Figure 4** shows the extracted ion chromatograms (EICs) of these hydroxytyrosol metabolites obtained using the sample pretreatment dried spot cards. Similar chromatograms were obtained when the µSPE was used (chromatograms not shown).

Table 5 shows the concentrations of these hydroxytyrosol metabolites on d 0 and 21, by applying the two different urine sample pretreatments. The concentrations are expressed in µmol in urine collected over 24 h. The individual and average concentrations from five subjects of each hydroxytyrosol metabolite generated in the two sample pretreatments with its standard deviation (s) are also shown. The concentration of the two hydroxytyrosol glucuronide metabolites in the two sample pretreatments is not shown in this table because their concentrations were below their LOQs.

For the determination of the studied compounds in urine sample by using the two sample pretreatment strategies, all the analytical ranges were suitable. So, it was not necessary to dilute the human urine samples, selected to application to the methods, in order to enable them fit in the analytical range.

No significant differences ($P > 0.05$) were detected when the individual and average concentration of each hydroxytyrosol metabolite determined by dried spot cards was compared with that determined in µSPE on either d 0 or d 21. One exception was homovanillic alcohol glucuronide on d 0. For the analysis of this hydroxytyrosol metabolite, all the subjects were not detected (n.d.) when the sample pretreatment dried spot cards were used because its concentration was below its LOD. In contrast, when µSPE was used for the analysis of this metabolite, its concentration was not quantified (n.q.) because this was found between its LOD and its LOQ for all the subjects. This fact could be

explained by the more sensitivity of the technique μSPE in comparison with dried spot cards. Although the sample pretreatment μSPE was more sensitive than the dried spot cards (the LODs and LOQs in μSPE were lower than in the dried spot cards), similar results were achieved in the quantification of the generated hydroxytrosol metabolites by the two sample pretreatments studied on d 0 and 21.

CONCLUSIONS

The present study reports two novel, rapid, selective, robust, and suitable sample pretreatment strategies for determining hydroxytyrosol and its metabolites in urine samples.

Technologically, the use of dried spot cards represents a step beyond in the actual sample pretreatment methodologies, since it has never been used for phenolic metabolites analysis. Compared with μSPE, it presents some advantages, as it is a simple, speedy, and inexpensive method. Another advantage of dried spot cards versus μSPE is that they can be analyzed up to 7 d since the studied compounds are very stable in the card stored at room temperature. On the other hand, μSPE shows some advantages above dried spot cards, such as its high sensitivity. For example, the urine samples can be analyzed directly without the need to be freeze-dried before its use in order to pre-concentrate the phenolic compounds.

Additionally, by using μSPE, analytes can be extracted in a shorter period of time compared with dried spot cards. The sample pretreatment μSPE, in addition to having higher sensitivity, smaller volumes of urine sample and extraction solvent are necessary compared with those required in the conventionally LLE and SPE with cartridges as the device format. It could be concluded that these two sample pretreatment techniques could be alternative strategies in human intervention studies for cleaning up biological samples when it is necessary to analyze a large number of urine samples with low concentration levels of phenolic metabolites. For the first time, these two sample pretreatment strategies were successfully applied to the analysis of urine phenolic metabolites as consumption biomarkers of virgin olive oil phenolics.

Acknowledgments

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Publication VI.

Effect of the co-ocurring olive oil and thyme extracts on the phenolic bioaccesibility and bioavailability assessed by in vitro digestion and cell models.

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EFFECT OF THE CO-OCCURRING OLIVE OIL AND THYME EXTRACTS ON THE PHENOLIC BIOACCESIBILITY AND BIOAVAILABILITY ASSESSED BY IN VITRO DIGESTION AND CELL MODELS

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Abstract

Olive oils flavoured with edible herbs have grown in popularity because of their added value and potential health benefits. However, the combined presence of different phytochemicals from olive oil and herbs requires study of their possible interactions during intestinal transport and metabolism. The aim of this study was firstly to evaluate the effect on bioaccessibility of the co-occurring bioactive compounds from olive oil and thyme through an *in vitro* digestion model of three extracts: olive extract (OE), thyme extract (TE) and a combination of both (OTE). The bioaccessible fractions were exposed to Caco-2 and HepG-2 cell models, as well as to a co-culture of both of these. Results indicated that the bioaccessibility of hydroxytyrosol was enhanced when OTE was digested. After Caco-2 cells exposure, no significant differences were observed in hydroxytyrosol transport, whereas the main flavonoids from thyme seemed to undergo an enhanced basolateral permeation when both phenolic sources where exposed.

KEYWORDS: Caco-2 / HepG-2 / Metabolism / Olive phenols / Thyme / Transport

1. INTRODUCTION

Phytochemicals from vegetal sources that are ingested daily, or natural extracts used to develop new functional foods are complex mixtures of molecules that have to be taken up and metabolised if they are to finally reach the cells in vivo (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005). Thus, one of the principal aspects that condition the beneficial effects of polyphenols is their bioavailability and metabolic fate. Bioaccessibility is defined as the amount of a food constituent, present in the gut as a result of its release from the solid food matrix, which might be able to pass through the intestinal barrier. Additionally, bioavailability is the proportion of the nutrient that is digested, absorbed, and metabolised through the normal pathways. The bioavailability of dietary compounds in general, and phytochemicals in particular, is dependent on their digestive stability, their release from the food matrix and the efficiency of their transepithelial passage. Therefore, bioaccessibility has to be

taken into account in studies concerned with bioavailability of polyphenols. Importantly, most studies on polyphenol bioavailability use pure single compounds (isolated from food or chemically synthesised), although their bioavailability when they derive from a mixture of different phenolic compounds might be substantially different (Saura-Calixto, Serrano, & Goñi, 2007). Interactions between phenolics from different sources may influence their bioaccessibility and bioavailability (Porrini & Riso, 2008; Scholz & Williamson, 2007) and therefore this should be analysed when studying the effects of a mixture of polyphenols from different origins.

In terms of biological effects, in previous in vitro (Herrmann & Wink, 2011) and in vivo studies (Kennedy, Scholey, & Wesnes, 2001), synergistic biological effects with stronger improvements have been reported when different phenolic compounds or extracts are combined, when compared with the single treatments. In this sense, it is important to study to what extent bioavailability is affected when a mixture of polyphenols is administered.

In a previous study (Rubió, Motilva, Macià, Ramo, & Romero, 2012a), an enriched olive oil with olive phenols and complemented with phenols from thyme was developed, which provided a balanced phenol composition from both sources with the aim of improving the nutritional profile and sensorial characteristics of the oil. The purpose of the present study was to verify to which extent the combination of the different kinds of phenolic compounds could influence their bioaccessibility, bioavailability and metabolism.

To do so, the phenolic extracts used for enriching the olive oil (olive extract, a thyme extract and a combination of both) were used. First, the effect on the bioaccessibility of the co-occurring bioactive compounds from olive oil and thyme through an *in vitro* gastrointestinal digestion model was evaluated. In the second part of the study, the bioaccessible fraction obtained from the in vitro digestion model was exposed to the epithelial and hepatocyte cell models to study the transport and metabolism of the phenolic compounds, as well as possible interactions in the absorption process.

2. MATERIAL AND METHODS

2.1. Chemicals and reagents

Apigenin, luteolin, oleuropein, naringenin, kaemferol, eriodictyol, taxifolin, quercetin, vanillin and thymol were purchased from Extrasynthese (Genay, France). Hydroxytyrosol and hydroxytyrosol acetate (3,4-DHPEA-AC) were purchased from Seprox Biotech, S.L. (Madrid, Spain). Hydroxytyrosol linked to the dialdehydic form of elenolic acid (3,4-DHPEA-EDA), hydroxytyrosol Linked to elenolic acid (3,4-DHPEA-EA) are not available commercially and were isolated from phenolic extracts of virgin olive oil using semi-preparative HPLC (Suárez, Macià, Romero, & Motilva, 2008).

Acetonitrile (HPLC-grade), methanol (HPLC-grade), glaciar acetic acid (P99.8%), formic acid, clorhydric acid and L(+)-ascorbic acid (reagent grade) were all provided by ScharlauChemie (Barcelona, Spain). Hydrochloric acid (37%) was from Prolabo (Badalona, Spain). Ortho-phosphoric acid 85% was purchased from Mont Plet&Esteban S.A. (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2. Olive oil and thyme extracts

Extracts were prepared from freeze–dried olive cake (OE) and dried thyme (TE) using an accelerated solvent extractor (ASE 100) (Dionex, Sunnyvale, CA), according to the methodology in Rubió, Motilva, et al. (2012a). Olive cake was used as source to obtain the main phenolic components of virgin olive oil, as it provides the same olive oil phenolic profile but in higher concentrations (Artajo, Romero, Suárez, & Motilva, 2007). In the manuscript we will refer to olive oil and olive cake phenolics indistinctly.

Briefly, the phenolics were extracted using ethanol/water (80:20, v/v) at 80 °C. The combined olive and thyme extract (OTE) was prepared by combining freeze–dried olive cake and dried thyme at a 1:1 proportion. The phenolic composition of the extracts is shown in **Table 1**. Values are expressed as mg compound/1.5 g of extract, since 1.5 g was the dose subjected to the in vitro gastrointestinal digestion.

2.3. Simulated gastrointestinal *in vitro* digestion

A gastrointestinal in vitro digestion model was performed based on the methodology described by Ortega, Reguant, Romero, Macià, and Motilva (2009). Each extract (OE, TE and OTE) was subjected to digestion in triplicate. The model describes a three-step procedure to mimic the digestive process in the mouth, stomach (gastric

digestion) and small intestine (duodenal digestion). The digestion Starts by adding amylase in phosphate buffer solution to 1.5 g of extract, which is incubated for 5 min. The gastric digestion includes the pH adjusted to 2 by adding HCl concentrate and porcine-pepsin solution. This mixture is shaken in an incubator for 2 h at 37 °C. Finally, for the duodenal digestion, 2.5 ml of bile salts and 2.5 ml of pancreatin (8 g/l) are added and the pH is adjusted to 6.5 by adding $NaHCO_3$. For this final stage a continuous-flow dialysed step was chosen. The proposed system was designed with an adapted Liebig-West condenser and some end-fitting fluid connectors.

The first chamber contained the dialysis tub (molecular mass cutoff at 12400 Da), through which the duodenal mixture flowed by using a peristaltic pump and a phosphate buffer solution, which covered the dialysis tub. A temperate water solution was pumped from a bath through the water jacket to keep the system's temperature constant, under 37 °C. (Ortega et al., 2009). At the end of the dialysed duodenal digestion step, two fractions were collected separately: the outside dialysis solution (OUT), which was considered the bioaccessible fraction that could be available for absorption; and the inside dialysis tub content (IN), referring to the digested fraction that would reach the colon fermentation intact. The bioaccessible percentage of each compound was calculated from the concentrations of the total amount recovered in the dialysable fraction (OUT) in relation to the concentration of the total amount (1.5 g) in the original extract subjected to the in vitro digestion. The OUT fraction was exposed to the cell lines in order to study the transport and metabolism of the phenolics.

2.4. Cell culture and treatments

Caco-2 and HepG2 cells lines were obtained from ATCC (American Tissue Culture Collection). The cells were cultured in DMEM supplemented as previously described (Castell-Auví et al., 2010).

Caco-2 cells were seeded onto a culture insert (12-well Millicell Hanging Cell Culture Inserts, Millipore) at a cell density of 5.3 x 10^4 cells/cm^2. The cells were used for the experiment after 21 days once the confluent monolayer had formed, and the cells expressed a constant trans-epithelium electrical resistance (TEER) measured with the Millicell-ERS system (Millipore). The volume of the culture medium was 0.4 ml on the apical side (AP) and 1 ml on the basolateral side

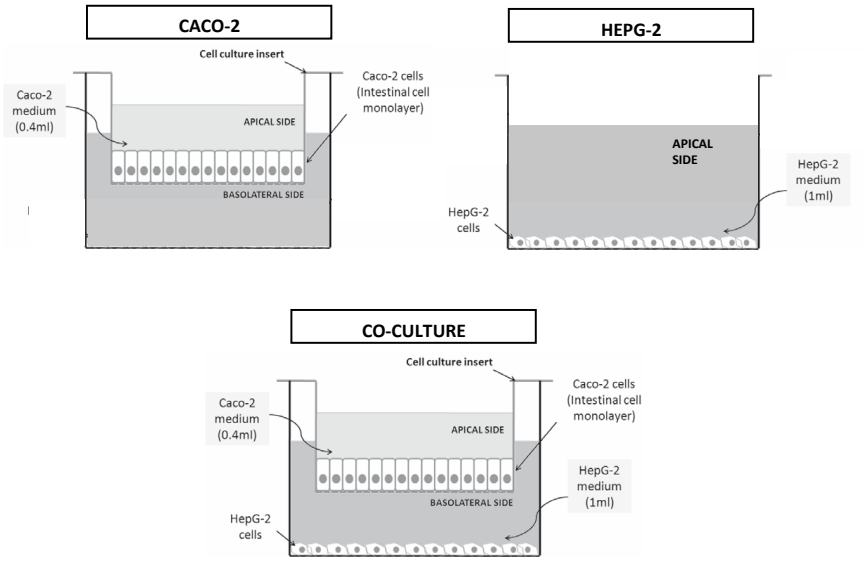

Figure 1. Scheme of the single and double-layered system of Caco-2 and HepG-2. Caco-2 cells were seeded alone at a cell density of 5.3 104 cells/cm2. HepG2 cells were seeded alone onto the 12-well plates at a cell density of 1 105 cells/cm2. At day 20 for Caco-2 and day 3 for HepG2, both cell types were co-cultured.

(BL). For the experiments, HepG2 cells were seeded onto the 12-well plates at a cell density of 1 x 10^5 cells/cm^2. The volume of the culture medium was 1 ml. Once the cells reached confluence, the following different culture systems were prepared: culture of Caco-2 cells, culture of HepG2 cells, and double-layered co-culture of Caco-2 cells and HepG2 cells (**Fig. 1**). The co-culture system was started on day 20 for Caco-2 cells and on day 3 for HepG2. The cells were treated after 1 day on co-culture. For the experiments, Caco-2 cells culture and double-layered co-culture were treated with 500 mg/l of the digested phenolic extracts, and the concentration was 50 mg/l for the HepG2 cell culture. The period of treatment was 4 h in all the culture systems.

2.5. Chromatographic analysis of phenolic compounds and their metabolites

The composition of phenolics in the extracts and the digested dialysable fraction (OUT) were characterised by the HPLC–MS/MS method described in Rubió, Motilva, et al. (2012a). Monoterpenes were quantified by GC–FID based on the method described in Rubió, Serra, et al. (2012b). Phenolic and monoterpene metabolites in the cell mediums were first extracted using microelution solidphase extraction (lSPE) and then quantified using a Waters Acquity UPLC–MS/MS system (Milford MA, USA), based on the method in Rubió, Serra, et al. (2012b). Briefly, the cartridges were firstly conditioned sequentially by using 250 ll of methanol and acidified Milli-Q water at pH 2. Then, 350 ll of cell medium mixed with 300 µl of phosphoric acid 4% and 50 µl catechol (IS) at 10 mg/l were loaded onto the plate. After that, the clean-up of the plates was sequentially done with 100 µl Milli-Q water and 100 ll methanol (5%) to eliminate any interference that the sample might contain.

Finally, the elution of the retained phenolic compounds was done with 100 ll of methanol. 2.5 µl of the eluted solution from the lSPE was injected directly into the UPLC–MS/MS.

3. RESULTS

3.1. Bioaccessibility of phenolic compounds using the gastrointestinal *in vitro* model

The main phenolics from the extracts used in the study were classified into three groups: hydroxytyrosol and secoiridoids (exclusively from olive), monoterpenes (exclusively from thyme), and flavonoids (mainly derived from thyme and, to a lesser degree, from olive). **Fig. 2** shows the bioaccessible fraction of the main phenolic groups from the OE, TE and OTE extracts after the in vitro digestion. In the case of hydroxytyrosol and secoiridoids, 13.3% and 23.0% was liberated through the in vitro gastrointestinal digestion from OE and OTE respectively, and was thus considered to be the bioaccessible fraction.

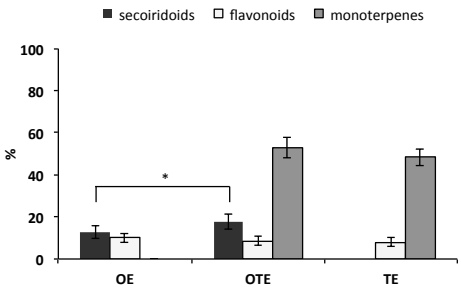

Figure 2. Bioaccessible percentages of the main phenolic groups from OE, TE and OTE extracts after the in vitro digestion. *indicates significant differences ($p < 0.05$).

Table 1 shows the detailed bioaccessible percentages of each phenol compound. Results show that the different % of bioaccessible secoiridoids is due to hydroxytyrosol.

Hydroxytyrosol presented a bioaccessible percentage of over 100% in both cases (132.3% with OE and 167.6% with OTE). This could be due to the release of hydroxytyrosol during the digestion of its precursors, the secoiridoids. Flavonoids were mainly found in the extracts containing thyme (OTE and TE), except for luteolin, which was also present in the olive extract (OE) but at a very low concentration. The bioaccessible percentages of this phenolic group varied from 1% to 33% with eriodictyol being the most bioaccessible. No significant differences between the single extract and the combined one were observed for any of the flavonoids. Finally, in the case of the monoterpenes from thyme, thymol showed the highest bioaccesibility with recoveries of 56.0% from the OTE and 53.7% from the TE compared with the initial concentration in the extract before the *in vitro* digestion. The bioaccessible percentages of the monoterpenes

Table 1. Phenol concentrations of the original extracts (in mg / 1.5 g of extract subjected to digestion) and phenol concentration of the dialysed fraction (OUT) considered the bioaccessible fraction (in mg / total grams of digested fraction). In a third column the bioaccessible percentage of each phenol. Data shown represent means ± SD (n = 3 independent experiments). Different letters in a row show significant differences in the bioaccessible percentages between EO, OTE and TE ($p<0,05$).

	OE			OTE			TE		
	Extract	OUT	%	Extract	OUT	%	Extract	OUT	%
secoiridoids derivatives									
hydroxytyrosol	1,43 ± 0,23	1,79 ± 0,13	132,3 [a]	1,34 ± 0,18	2,25 ± 0,29	167,6 [b]	n.d.	n.d.	-
3,4-AC*	1,73 ± 0,31	0,01 ± 0,00	0,6	1,31 ± 0,12	0,01 ± 0,00	0,7	n.d.	n.d.	-
3,4-EDA**	19,2 ± 1,79	1,08 ± 0,11	5,6	9,86 ± 0,84	0,68 ± 0,02	6,9	n.d.	n.d.	-
3,4-EA***	0,97 ± 0,11	0,13 ± 0,02	13,2	0,56 ± 0,05	0,07 ± 0,03	12,4	n.d.	n.d.	-
total	23,3 ± 2,26	2,98 ± 0,31	13,3 [a]	13,0 ± 1,72	2,31 ± 0,19	23,0 [b]			
flavonoids									
luteolin	1,58 ± 0,14	0,23 ± 0,02	14,6	3,05 ± 0,28	0,51 ± 0,04	16,7	5,79 ± 0,42	0,85 ± 0,08	14,6
naringenin	n.d.	n.d.	-	0,65 ± 0,07	0,11 ± 0,01	17,0	2,86 ± 0,26	0,50 ± 0,04	17,5
eriodictyol	n.d.	n.d.	-	1,45 ± 0,19	0,48 ± 0,04	33,1	3,19 ± 0,29	0,94 ± 0,08	29,4
thymusin	n.d.	n.d.	-	3,05 ± 0,24	0,04 ± 0,00	1,2	7,87 ± 0,61	0,08 ± 0,00	1,0
xanthomicol	n.d.	n.d.	-	3,07 ± 0,29	0,09 ± 0,01	2,9	6,05 ± 0,55	0,28 ± 0,02	4,6
me sudachitin	n.d.	n.d.	-	1,56 ± 0,14	0,08 ± 0,01	5,4	4,95 ± 0,51	0,26 ± 0,03	5,2
total	1,58 ± 0,14	0,23 ± 0,02	14,6	12,8 ± 0,15	1,31 ± 0,08	10,2	30,7 ± 3,15	2,90 ± 0,25	9,5
monoterperns									
thymol	n.d.	n.d.	-	3,90 ± 0,35	2,18 ± 0,18	56,0	6,65 ± 0,53	3,54 ± 0,27	53,7
carvacrol	n.d.	n.d.	-	1,05 ± 0,09	0,67 ± 0,08	63,8	2,49 ± 0,18	1,50 ± 0,12	60,3
α-terpineol	n.d.	n.d.	-	0,45 ± 0,05	0,10 ± 0,00	22,2	1,30 ± 0,11	0,23 ± 0,01	17,9
borneol	n.d.	n.d.	-	0,15 ± 0,01	n.d.	-	0,86 ± 0,07	0,10 ± 0,00	11,6
total				5,55 ± 0,40	2,95 ± 0,21	53,2	11,3 ± 0,12	5,37 ± 0,48	47,6

*hydroxytyrosol acetate; **Hydroxytyrosol linked to the dialdehydic form of elenolic acid; ***hydroxytyrosol linked to elenolic acid

from the single thyme extract and the mixed extract showed no significant differences.

3.2. Transport and metabolism through Caco-2 cells

After the incubation of the extracts with the Caco-2 cells grown in inserts, the transport and metabolism of the phenolic compounds was evaluated. This human small-intestinal epithelium cell model was used in order to study the metabolism yield of the parent compounds and to which extent the presence of both phenolic sources (olive and thyme) could influence the absorption of the phenolics through the epithelial cells. **Table 2** shows the proportion of the total recovered phenols and their metabolites in the extracellular culture medium (AP and BS) as well as in the cytoplasmatic contents of the cells. Hydroxytyrosol from the olive extracts presented a significant conjugate production with almost complete metabolic conversion. A high proportion of hydroxytyrosol sulphate (67.5% in the OTE and 62.3% in OE) was observed on the BL side, indicating a very efficient sulphation by Caco-2 cells and a preferential permeation across the BL membrane. Hydroxytyrosol was subjected to methylation and sulphation but no glucuronide metabolites were detected. The conjugates retained within the cells accounted for less than 0.6% of the total conjugates in both cases (OE and OTE). The percentages did not differ significantly between OE and OTE.

Luteolin, naringenin and eriodictyol and their metabolites were the only flavonoids detected after the incubation of Caco-2 cells with the three digested extracts. They were also the most bioaccessible in the simulated gastrointestinal digestion (**Table 1**). When cells were incubated with the TE, a higher rate of conjugation of luteolin into the sulphate and glucuronide forms was found compared with the luteolin metabolites when thyme was mixed with olive (OTE) or those from the OE alone, since the TE showed a significantly higher amount of metabolites and a lower amount of free luteolin on the AP side than the other extracts (**Table 2**). Both free luteolin and its conjugates were mostly detected on the AP side, indicating that the AP efflux was substantially higher than the BL permeation Eriodictyol and naringenin, both flavonoids exclusively from thyme, were detected mostly in their un-metabolised forms and both at a higher level on the BL side compared with luteolin. Eriodictyol had the highest bioavailability (15.2% of the free form was on the BL side in the TE) and was significantly higher in the OTE (28.9%). A higher percentage of eriodictyol was also retained within the cells. Similarly, naringenin was also more bioavailable when derived from the OTE than from the TE. Instead, it was less glucuronidated when derived from the OTE than from the TE.

The type of conjugation within flavonoids varied depending on the compound, with both the sulphated and glucuronidated forms of eriodictyol and only the glucuronidated form of naringenin. Thymol and its metabolites were the only monoterpenes detected in the cells, although the digested extracts also contained such other monoterpenes as carvacrol, but at lower concentrations. This could be explained by an isomerisation of carvacrol into thymol during the incubation. A significant first pass metabolism occurred in thymol presenting conjugation rates of 61.7% in the TE and 56.0% in the OTE, mainly into the sulphated form, which was efficiently permeated to the BL side. The un-metabolised thymol was distributed into the three compartments, most of it remaining on the AP side and unable to cross the epithelial barrier.

3.3. Intestinal and hepatic metabolism

The dyalised fractions of the extracts were also subjected to a HepG-2 cell culture and to a co-culture with a combination of Caco-2 and HepG2 in order to study the metabolism of the phenolic compounds in epithelial and liver cells in greater depth. **Table 3** shows the phenols and their metabolites detected in the BL media.

The metabolic profile of hydroxytyrosol was the same in the three cell cultures. The only difference was the detection of free hydroxytyrosol only on the AP side of the HepG2 and on the BL side of the co-culture. The metabolic proposal of hydroxytyrosol is shown in **Fig. 3.**

Flavonoids had a varied metabolic profile. Neither free nor sulphated luteolin was found after incubation of the HepG2 with any of the extracts. It was only found in the glucuronidated form in the TE and OTE. Instead, when Caco-2 is present (either alone or in co-culture), all three forms (free, sulphate and glucuronide) are found. Eriodictyol was subjected to sulphation only by Caco-2 cells and no glucuronide form was detected in any cell

Table 2. Metabolism yield of phenolic compounds after the incubation of the phenolic digested extracts in Caco-2 cell culture. Results are expressed as percentages of transformed phenol versus total initial concentration in the cellular medium. Hydroxytyrosol metabolites are expressed in relation to the total secoiridoids. Other metabolites are expressed in relation to each parent compound in the initial medium before incubation.

	Apical (AP) (% versus total initial)			Cells (% versus total initial)			Basolateral (BL) (% versus total initial)			Total metabolized (%)		
	TE	OTE	OE	TE	OTE	OE	TE	OTE	OE	TE	OTE	OE
HOTyr	-	0,3 ± 0,0	0,5 ± 0,0	-	n.d.	n.d.	-	n.d.	n.d.			
HOTyr sulf	-	13,7 ± 1,0	14,3 ± 1,1	-	0,3 ± 0,0	0,3 ± 0,0	-	67,5 ± 5,8	62,3 ± 5,6			
HOTyrAC sulf	-	1,5 ± 0,1	3,3 ± 0,2	-	n.d.	0,1 ± 0,0	-	6,2 ± 0,5	7,8 ± 0,6			
HVAlc sulf	-	2,4 ± 0,1	3,1 ± 0,1	-	0,1 ± 0,0	0,1 ± 0,0	-	8,6 ± 0,7	10,1 ± 0,8			
										-	99,7 ± 8,5	99,5 ± 8,1
luteolin	42,3 [a] ± 3,3	60,9 [b] ± 5,5	58,3 [b] ± 5,6	3,3 ± 0,2	3,4 ± 0,3	3,9 ± 0,2	4,2 ± 0,3	4,2 ± 0,4	4,3 ± 0,4			
luteolin sulf	22,8 [a] ± 1,8	10,2 [b] ± 0,9	8,1 [b] ± 0,6	n.d.	n.d.	n.d.	4,8 ± 0,4	1,7 ± 0,1	1,1 ± 0,1			
luteolin gluc	23,7 [a] ± 1,9	11,6 [b] ± 0,8	12,9 [b] ± 1,0	n.d.	n.d.	n.d.	2,4 ± 0,2	1,7 ± 0,1	1,4 ± 0,1			
										53,7 ± 4,8	25,2 ± 1,8	23,5 ± 2,1
eriodictyol	32,4 ± 2,8	34,8 ± 3,1	-	19,9 ± 1,3	20,8 ± 1,5	-	15,2 [a] ± 1,1	28,9 [b] ± 2,2	-			
eriodictyol sulf	8,8 ± 0,7	4,0 ± 0,2	-	n.d.	n.d.	-	8,8 ± 0,6	4,8 ± 0,3	-			
eriodictyol gluc	12,2 ± 1,1	9,4 ± 0,7	-	n.d.	n.d.	-	n.d.	n.d.	-			
										29,8 ± 2,2	18,2 ± 1,3	-
naringenin	63,4 ± 5,2	60,3 ± 5,4	-	1,5 [a] ± 0,1	6,3 [b] ± 0,4	-	6,3 [a] ± 0,4	16,7 [b] ± 1,3	-			
naringenin sulf	n.d.	n.d.	-	n.d.	n.d.	-	n.d.	n.d.	-			
naringenin gluc	11,5 [a] ± 1,0	6,3 [b] ± 0,4	-	n.d.	n.d.	-	15,4 [a] ± 0,9	10,2 [b] ± 0,9	-			
										26,9 ± 1,9	16,5 ± 1,1	-
thymol	23,3 ± 1,8	25,0 ± 2,1	-	5,5 ± 0,3	7,1 ± 0,5	-	14,2 ± 1,1	16,3 ± 1,2	-			
thymol sulf	7,3 ± 0,5	7,1 ± 0,6	-	1,3 ± 0,1	1,2 ± 0,1	-	51,5 ± 4,4	45,5 ± 3,9	-			
thymol gluc	0,5 ± 0,0	0,3 ± 0,0	-	0,1 ± 0,0	0,1 ± 0,0	-	1,0 ± 0,1	1,8 ± 0,1	-			

Sulf: sulphate; Gluc: glucuronide; HOTyr: hydroxytyrosol; HOTyrAC: hydroxytyrosol acetate; n.d.= not detected
Different letters in a row indicate significant differences between TE, OTE and OE in each cell culture compartment ($p<0.05$)

Table 3. Metabolic profile of individual phenol metabolites in CaCo-2, HepG2 and co-culture after the incubation of the extracts. **+** indicates the presence of the metabolite in the basolateral side, and **-** indicates not detected (apical medium in the case of HepG2).

	CACO-2			Co-culture			HepG-2		
	OE	TE	OTE	OE	TE	OTE	OE	TE	OTE
Hydroxytyrosol derivates									
hydroxytyrosol	-	-	-	+	-	+	+	-	+
hydroxytyrosol sulfate	+	-	+	+	-	+	+	-	+
hydroxytyrosol glucuronide	+	-	+	+	-	+	+	-	+
alcohol homovanillic sulphate	+	-	+	+	-	+	+	-	+
hydroxytyrosol acetate sulphate	+	-	+	+	-	+	+	-	+
Flavonoids									
eriodictyol	-	+	+	-	+	+	-	+	+
eriodictyol sulphate	-	+	+	-	+	+	-	-	-
eriodictyol glucuronide	-	-	-	-	-	-	-	-	-
luteolin	+	+	+	+	+	+	-	-	-
luteolin sulphate	+	+	+	+	+	+	-	-	-
luteolin glucuronide	+	+	+	+	+	+	-	+	+
naringenin	-	+	+	-	+	+	-	-	-
naringenin glucuronide	-	+	+	-	+	+	-	-	-
Naringenin sulphate	-	-	-	-	-	-	-	-	-
Monoterpenes									
thymol	-	+	+	-	+	+	-	+	+
thymol sulphate	-	+	+	-	+	+	-	+	+
thymol glucuronide	-	+	+	-	+	+	-	-	-

culture. The free form appeared on the BL side of the Caco-2 and co-culture, as well as on the AP side of the HepG2, indicating that the metabolic rate for eriodictyol was relatively low and it could be taken up efficiently from the AP side of the Caco-2 cell monolayers and transferred to the BL compartment without conjugation. Similarly, naringenin was not metabolised by the HepG2,

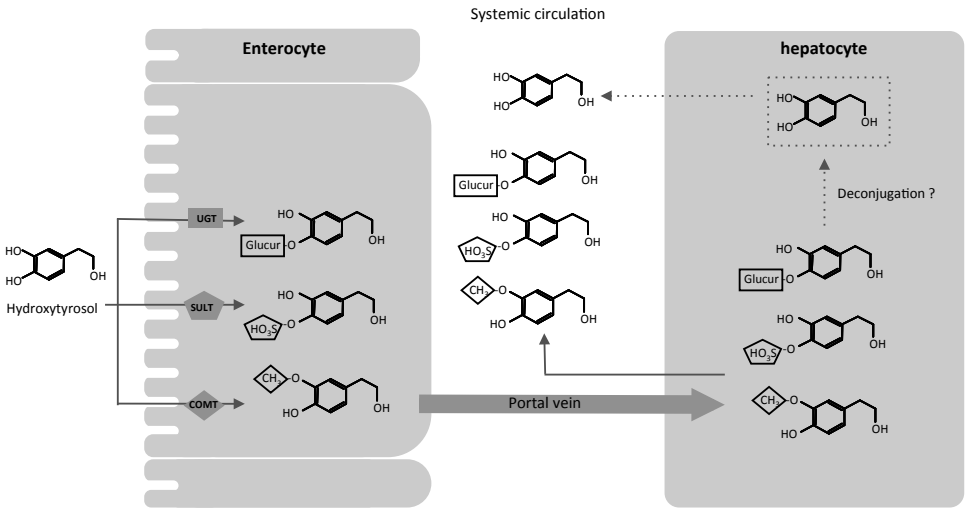

Fig. 3. Metabolic proposal for hydroxytyrosol. In the enterocyte hydroxytyrosol undergoes an intensive first pass metabolism of sulphation, methylation and glucuronidation and the resulting metabolites pass to the liver where a deconjugation process is hypothesised, resulting in the native form of hydroxytyrosol, which could enter the systemiccirculation together with the metabolites. UGT, glucuronosyltransferase; SULT, sulfotransferase; COMT, catechol methyl transferase.

and the Caco-2 led only to sulphated forms. Thymol was found in its free form in all three cultures, and sulphated forms were also observed in the three culture types. Glucuronidation was only observed when the Caco-2 cells (either alone or co-cultured) were present.

4. DISCUSSION

This research has documented the enhancing effect of thyme compounds on the bioaccessibility of secoiridoids from olive oil when the combined extract (OTE) was subjected to an *in vitro* digestion model and the influence of further metabolic changes through i*n vitro* cell models.

From the *in vitro* digestion, we observed that the presence of both phenolic sources together during digestion resulted in greater bioaccessibility of hydroxytyrosol in comparison with the single OE extract. In contrast, the combination of thyme and olive extracts showed no protective effects during the *in vitro* digestion of flavonoids and monoterpenes from thyme. A previous study (Gawlik-Dziki, 2012) demonstrated that, among raw vegetables, the best source of bioaccessible phenolics was a mixture of all of them. Similar effects were observed in another study, where antioxidant spices (turmeric and onion) prevented the loss of β-carotene during the *in vitro* digestion of raw vegetables (Veda, Platel, & Srinivasan, 2008). In keeping with these earlier findings, antioxidant spices, such as thyme or turmeric, probably exert a favourable influence on the bioaccessibility of antioxidant compounds by minimizing their loss during the digestion process. As seen in previous studies (Pinto et al., 2011) the stability of the secoiridoid derivatives is low during the intestinal digestion phase mainly due to the alkaline conditions. Therefore, mixing olive phenolic compounds with other bioactive substances, such as thyme, could be an interesting approach to minimise the low stability of secoiridoid derivatives. While other underlying mechanisms remain to be examined, we hypothesise that in this study, thyme bioactives might protect olive phenolics from degradation in the gastrointestinal tract before absorption, thus making them more bioaccessible.

After the in vitro digestion, the digested extracts considered the bioaccessible fraction, were subjected to a bioavailability study with Caco-2 cells. This cell exposure enabled us to study to which extent the transport across the cells and its first metabolism was affected by thyme co-occurring with olive bioactives. Similar proportions of metabolised hydroxytyrosol were detected after the extract exposure (OE or OTE), which indicated that hydroxytyrosol was not affected by the presence of thyme bioactives during the efflux across the epithelial cells. Hydroxytyrosol was almost completely converted into hydroxytyrosol sulphate, hydroxytyrosol acetate sulphate and homovanillic alcohol sulphate, with most appearing on the BL side, which indicates an extensive sulphation and methyl-sulphation during the first pass metabolism in the epithelial cells. In a previous study by Soler et al. (2010), isolated hydroxytyrosol was exposed to differentiated Caco-2 cell monolayers, and the main hydroxytyrosol metabolite corresponded with the formation of homovanillic alcohol, with a low conversion that ranged from 10.7% to 18.6%, with small quantities of sulphated and methyl-sulphated conjugates. In the present study, the digested extracts were used for the cell exposure instead of the isolated compounds, which could indicate possible interactions during the absorption and metabolism process when a mixture of phenolics is exposed to the cells.

Previous human studies after olive oil administration have also reported a high bioavailability of hydroxytyrosol and its extensive first pass metabolism, mainly into the glucuronidated and methylated forms (Miro-Casas et al., 2003). In contrast, our results suggest that hydroxytyrosol may undergo a significant conjugation into sulphated forms, which is in accordance with a most recent human study, where hydroxytyrosol sulphate and hydroxytyrosol acetate sulphate appeared to be the major metabolites in plasma after a phenol-enriched olive oil intake (Rubió, Serra, et al., 2012b). Also, the presence of both phenolic sources in the case of the OTE had no influence on the metabolism and absorption of hydroxytyrosol.

Flavonoids did not undergo such an extensive metabolism as hydroxytyrosol and the conjugation rates differed within the single and mixed extracts presenting lower metabolic change flavonoids from the mixed extract (OTE). Since flavonoids are also known to be metabolised by Caco-2 cells into glucuronidated and sulphonated metabolites (Williamson et al., 2007), a competitive inhibitory effect of hydroxytyrosol on the phase II metabolism might be the reason for the decrease

in the total amount of flavonoid metabolites formed by the Caco-2 cells upon co-exposure with olive phenols (OTE). The saturation of metabolic enzymes has been previously described with polyphenols (Liu & Hu, 2007) and could explain the reduced conjugation of flavonoids. Although flavonoids were not strongly conjugated, most of the non-modified flavonoids did not cross the epithelial barrier, and a significant percentage of the modified flavonoids permeated across the AP membrane, thus reducing their bioavailability. The results are in line with previous studies that also suggest a low bioavailability of flavonoids, 11–16% in the case of flavanones (Manach et al., 2005). Interestingly, although a significant amount of free flavonoids could not cross the epithelial cells, when the OTE was exposed to the cells, the percentage of native naringenin and eriodictyol on the BL side was significantly higher, indicating an enhanced bioavailability. This could also be due to the presence of hydroxytyrosol, which caused a reduction in the conjugation of naringenin and eriodictyol and consequently, an increased amount of the native forms were able to permeate to the BL side.

Thymol, the main monoterpene of thyme, underwent a significant metabolic conjugation into sulphated form in both cases (TE and OTE) and also a noteworthy excretion on the BL side of the Caco-2 cells, representing the bioavailable fraction on the blood/ plasma side of the intestinal barrier. These results are in accordance with a previous study where an acute intake of thyme extract was administered to rats, and thymol sulphate also appeared to be the most abundant metabolite in the plasma together with a small proportion of thymol glucuronide (Rubió, Serra, et al., 2012b).

Taking into account that bioavailability involves the interplay of the intestine (transport and metabolism) and the liver (secondary metabolism), the HepG-2 and co-cultured cell lines combining Caco-2 and HepG-2 appear to be a noteworthy approach for studying phenolic metabolism (Castell-Auví et al., 2010). Regarding hydroxytyrosol, the first pass metabolism of sulphation, methylation and glucuronidation occurred in both cell lines. The complete conversion of hydroxytyrosol in the single Caco-2 cell culture indicates an extensive metabolism occurring in the intestine. However, the remaining free hydroxytyrosol could undergo further metabolic changes in the liver. Earlier studies with a perfused rat intestinal model (Corona et al., 2006) also reported an important metabolic conjugation of hydroxytyrosol in the intestine. The second pass through the liver of hydroxytyrosol was also studied in HepG-2 cells. However, only methylated and glucuronidated forms of hydroxytyrosol were detected (Mateos, Goya, & Bravo, 2005). It is noteworthy that the free form of hydroxytyrosol only appeared in the co-culture and HepG-2 media but did not appear in the Caco-2, which could indicate that once the compound is conjugated, it can be deconjugated in the hepatocyte cells. Tissue deconjugation of phenolic compound is scarcely studied. However, it has been recently proposed that the delivery of the aglycone forms of polyphenols may be needed for them to exert biological effects. The hydrolysis of flavonoid glucuronides was found by Shimoi et al. (2001) in human neutrophils and by Lee-Hilz, Stolaki, van Berkel, Aarts, and Rietjens (2008) in carcinoma cell lines, who suggested that the activity of flavonoid metabolites depends on their deconjugation *in situ.* Based on this and other indirect evidences,

Terao, Murota, and Kawai (2011) proposed that glucuronide conjugates of quercetin function not only as detoxified metabolites but also as precursors of the bioactive aglycone. Besides, although hydroxytyrosol appears to be well absorbed in the gastrointestinal tract, its bioavailability is poor because of an important first pass metabolism in both the gut and liver. The concentrations of its free form in body fluids (plasma and urine) were almost undetectable in previous studies (Miro-Casas et al., 2003) so there is a drawback to our understanding of the antioxidant activity of this compound *in vivo* and the potential health benefits derived from its consumption. From our results, and considering these previous studies, we suggest that deconjugation of hydroxytyrosol could occur in the liver, and probably in other target tissues, which could generate the active form of hydroxytyrosol considered to be responsible for the biological effects *in vivo.*

Concerning flavonoids, on Caco-2 cultures a substantial portion of the compounds reaching the BL compartment was un-metabolised, indicating that hepatic metabolism may be important for these compounds. However, among the flavonoids, only luteolin appeared to be metabolised into glucuronide, which suggests a

lower conjugation activity of the HepG-2 cells. The same results were obtained in previous studies, where the *in vitro* glucuronidation of flavonoids by microsomes prepared from the human intestine was markedly higher than that by microsomes prepared from the human liver (Boersma et al., 2002). While Caco-2 showed a high sulphation activity on the flavonoids, HepG2 did not lead to sulphation of any of the flavonoids. Instead, the liver cells did induce sulphate conjugation of thymol. Thymol also appeared to undergo more conjugation under Caco-2 cells, and sulphation seemed to be the most probable metabolic change in this compound since a low percentage of free thymol was detected in Caco-2 cells cultured alone and no thymol glucuronide appeared with HepG-2 incubation. Our results are in accordance with a previous study, in which they reported that thymol was only systemically available as thymol sulphate after an oral administration to humans (Kohlert et al., 2002).

5. CONCLUSIONS

Our results demonstrate that when a mixed plant extract made from olive-cake and thyme with a higher diversity of polyphenols was studied in an in vitro digestion model, it resulted in greater stability and bioaccessibility of hydroxytyrosol compared with the single extract of olive. Furthermore, we showed that the co-exposure of olive and thyme bioactives led to fewer metabolic changes and higher bioavailability of eriodictyol and naringenin, the main flavonoids from thyme, while no variation was observed in the bioavailability and metabolism of hydroxytyrosol and thymol. Moreover, a deconjugation process in the liver has been hypothesised for hydroxytyrosol, which could be relevant to explain the biological effects in vivo.

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Publication VII.

Effect of the co-ocurring components from olive and thyme extracts on the antioxidant status and its bioavailability in an acute ingestion in rats

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PAPER

EFFECT OF THE CO-OCURRING COMPONENTS FROM OLIVE AND THYME EXTRACTS ON THE ANTIOXIDANT STATUS AND ITS BIOAVAILABILITY IN AN ACUTE INGESTION IN RATS

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Abstract

The aim of this work was to examine whether bioactives in thyme could enhance the antioxidant capacity of phenolics in virgin olive oil and their bioavailability in Wistar rats. After acute oral administration of extracts from olive cake (OE), thyme (TE) or their combination (OTE), blood samples were collected from 0 to 360 min. Plasma antioxidant status was analyzed by DPPH and FRAP in plasma and by SOD, CAT and GPx activities in erythrocytes. Plasma pharmacokinetics of the main metabolites of bioactives in olive oil and thyme were characterized. Plasma non-enzymatic antioxidant capacity was significantly modulated by OE, TE, and OTE in a time-, assay, and extract-dependent manner. OE, TE, and OTE all significantly decreased superoxide dismutase (SOD) and glutathione peroxidase (GPx) activity and catalase (CAT) activity was increased. Pharmacokinetic results showed that plasma concentration (C_{max}) of the main olive phenolic metabolites in rats fed with OTE were similar to those of OE. These results indicate that an enhanced bioavailability of olive phenolic compounds could occur in the presence of thyme, although any synergistic effect was observed in the antioxidant status when both phenolic extracts were administered. Antioxidant protection by phenolics from olive and thyme against oxidative stress occurs primarily through a direct antioxidant effect and may be related to the phenolic plasmatic metabolites.

1. INTRODUCTION

Numerous studies have shown that the Mediterranean diet is protective against all-cause mortality.[1] Since oxidative stress contributes to the initiation and progression of many diseases, antioxidants in which the Mediterranean diet is richmay account partially for these benefits. They exert effects via their large array of bioactions, such as free radical-scavenging, metal chelation, and enzyme modulation abilities, as well as their effects on signaling transduction pathways and gene expression.[2]

Oxidative stress is defined as a condition when the excessive formation of reactive oxidant species (ROS) overwhelms antioxidant defenses.[3] Given their main functionality as an oxygen carrier in the body, erythrocytes, or red blood cells (RBC), constantly cope with an elevated ROS concentration. Fortunately, RBC are equipped with an antioxidant defense system that efficiently fights against ROS attacks. The system includes SOD, CAT and GPx and other smallmolecule antioxidants, e.g., glutathione.

Moreover, up-regulating the blood antioxidant status has been proposed as a preventative means to decrease the risk of oxidative stress related diseases such as cardiovascular diseases, diabetes, and cancer.[4] Thus, exogenous dietary phenolics could ameliorate oxidative stress either by acting as a direct radical scavenger or modulating endogenous antioxidant defenses. Phenolics in virgin olive oil were found to modulate oxidative stress in vitro[5] and in vivo, [6] mainly by acting as a free radical scavenger via their hydrogen donation and electron transfer ability, as well as metal chelating activity.[7] Since olive oil is normally consumed with other foods, phytochemicals in olive oil could work with phytochemicals in other foods in the diet to modulate antioxidant defenses in an additive/ synergistic manner.

Flavored olive oils with herbs or species have become more common in the market in the last few years because of their added health benefits to consumers beyond that of olive oil. Thyme is a common aromatic herb rich in phenolics, e.g., favonoids (thymusin, eriodictyol, xanthomycrol, 7-methylsudachitin), phenolic acids (the conjugated form of caffeic acid, rosmarinic acid), and monoterpenes (thymol and carvacrol).[8,9]

Further, thyme has been frequently used in folk medicine in ancient times for treatment of a variety of diseases; and nowadays, scientific evidence reveals the mechanism of action for these medicinal benefits including antioxidant capacity [10].

On the basis of the complementary nature of structure/activity of phenolics existing in plant foods, we hypothesized that bioactives in thyme would enhance the antioxidant effect of phenolics in virgin olive oil. The primary objective of this study was to evaluate the acute effect of the oral administration of extracts of olive cake, thyme or their combination on biomarkers of antioxidant defense in plasma and erythrocytes in Wistar rats. The secondary objective was to characterize the plasma pharmacokinetics of metabolites of olive and thyme bioactive compounds and see the relation between the plasmatic concentration of the metabolites and the antioxidant effect.

2. MATERIALS AND METHODS

2.1 Chemical and reagents

Apigenin, apigenin-7-O-glucoside, luteolin, luteolin 7-O-glucoside, oleuropein, rutin, tyrosol, verbascoside, naringenin, kaemferol, eriodictyol, rosmarinic acid, taxifolin, quercetin-40-Oglucoside and vanillin were purchased from Extrasynthese (Genay, France). Hydroxytyrosol and hydroxytyrosol acetate (3,4- DHPEA-AC) were purchased from Seprox Biotech, S.L. (Madrid, Spain). Caffeic, p-coumaric, and vanillic acid, 2,4,6-tris(2-pyridy)-s-triazine (TPTZ) and fluorescein were purchased from Fluka Co. (Buchs, Switzerland). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, USA), and (+)-pinoresinol was acquired from ArboNova (Turku, Finland). Hydroxytyrosol linked to the dialdehydic form of elenolic acid (3,4-DHPEA-EDA), hydroxytyrosol linked to elenolic acid (3,4-DHPEAEA), tyrosol linked to the dialdehydic form of elenolic acid (p-HPEA-EDA) and tyrosol linked to elenolic acid (p-HPEA-EA) are not available commercially and were isolated from phenolic extracts of virgin olive oil using semipreparative HPLC.[11]

Acetonitrile (HPLC-grade), methanol (HPLC-grade), glacial acetic acid ($99.8%), formic acid, hydrochloric acid and L(+)-ascorbic acid (reagent grade) were all provided by Scharlau-Chemie (Barcelona, Spain). Hydrochloric acid (37%) was from Prolabo (Badalona, Spain). ortho-Phosphoric acid 85% was purchased from Mont Plet & Esteban S.A. (Barcelona, Spain). Ultrapure water

was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Olive oil and thyme extracts

Extracts were prepared from freeze-dried olive cake (OE) and dried thyme (TE) using an accelerated solvent extractor (ASE 100) (Dionex, Sunnyvale, CA), according to the methodology of Rubió et al.[8] Briefly, the phenolics were extracted using ethanol–water (80 : 20, v/v) at 80 °C using 10 g of freeze-dried material for each extraction. The compositions of phenolics and monoterpenes in the extracts were characterized by the LC-MS/MS method of Rubió et al.[8] Mixed olive and thyme extract (OTE) was prepared by combining freeze-dried olive cake and dried thyme in the proportion 1:1. The phenolic composition of the extracts administered to the rats is shown in **Table 1**. The total phenolic content administered to each rat was 35.2, 35.3 and 40.1 mg of phenolics with OE, TE and OTE respectively, which is 117.3, 117.6 and 133.6 mg kg^{-1} BW.

Olive cake was used to obtain the main phenolic components of olive oil, as it provides the same olive oil phenolic profile but in higher concentrations. In the manuscript we will refer to olive oil, olive or olive cake phenolics without distinction.

2.3 Treatment of rats and plasma and erythrocyte collection

Forty-six male Wistar rats of three months old were obtained from Charles River Laboratories (Barcelona, Spain). All rats were housed in cages in a 12 light–12 h dark cycle at controlled temperature (22 °C). They were fed ad libitum a standard commercial chow, PanLab A04 (Panlab, Barcelona, Spain) and had free access to water. After they were fasted for 16–17 hours but had access to water, rats were divided randomly into three treated groups and one control group. Twelve rats in each treated group were intragastrically gavaged with 1.5 g kg^{-1} BW dispersed in water of the extracts OE, TE or OTE, respectively.

After gavage, three rats in each group were sacrificed at 60, 120, 240, and 360 min post gavage. Rats were anesthetized with isoflurane (IsoFlo, VeterinariaEsteve, Bologna, Italy) and then euthanized by exsanguination. Ten rats in the control group did not receive any extract, were kept fasted up to 16–17 hours, and were euthanized by the same protocol. The study was approved by the Animal Ethics Committee of the University of Lleida (CEEA 04-01/11, 26th January 2011) and performed in compliance with the relevant laws and University of Lleida guidelines.

Blood was collected by intracardiac exsanguination with heparin-moistened syringes. Plasma was obtained after centrifugation at 2000 g for 30 min at 4 °C, snap frozen using liquid nitrogen, and stored at 80 °C until analyses. RBC were collected after removal of plasma and buffy coat, washed three times with 0.9% NaCl solution, snap frozen with liquid nitrogen, and stored at 80 °C until analysis. At the moment of analysis lysate RBC were washed with distilled water to ensure the lysis.

Table 1. Bioactive compounds composition of the extracts administered to the rats. Values are expressed as mg of compound in the administered dose. (1.5 g kg-1 BW)

Compound (mg)	Olive Extract (OE)	Olive and Thyme Extract (OTE)	Thyme Extract (TE)
Phenolic acids			
Rosmarinic acid	n.d.	6.8	17.1
Secoiridoid derivatives			
Tyrosol	0.1	0.2	n.d.
Hydroxytyrosol	1.4	1.3	n.d.
3,4-DHPEA-AC	1.7	1.1	n.d.
p-HPEA-EDA	1.2	0.7	n.d.
3,4-DHPEA-EDA	19.2	9.9	n.d.
Ole derivative	1.8	0.7	n.d.
3,4-DHPEA-EA	9.1	3.7	n.d.
Total secoiridoid derivatives	*34.5*	*17.6*	*n.d.*
Flavonoids			
Apigenin	0.1	0.3	0.5
Luteolin	0.6	1.7	2.2
Naringenin	n.d.	0.6	2.9
Eriodictiol	n.d.	1.2	3.2
Xanthomicol	n.d.	3.1	6.1
Total flavonoids	*0.7*	*6.9*	*14.9*
Monoterpenes			
Thymol	n.d.	2.9	5.6
Carvacrol	n.d.	1.1	2.5
Total monoterpenes	*n.d.*	*4.0*	*8.1*
Total phenols	35.2	35.3	40.1

3,4-DHPEA-AC: 3,4-Dihydroxyphenyl ethyl acetate (hydroxytyrosol acetate); 3,4-DHPEA-EA: 3,4-dihydroxy phenylethanol-elenolic acid (oleuropein aglycone); 3,4-DHPEA-EDA: 3,4-dihydroxy phenylethanol-elenolic acid dialdehyde (dialdehydic form of elenolic acid linked to hydroxytyrosol); ***p***-HPEA-EDA: ***p***-hydroxy phenylethanol-elenolic acid dialdehyde (dialdehydic form of elenolic acid linked to tyrosol).

2.4 Antioxidant capacity in plasma

Two non-enzymatic antioxidant capacity (NEAC) assays, Ferric Reduction Ability of Plasma (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical decomposition assay were performed in plasma. The DPPH assay was performed using the on-line HPLC method of Pellegrini et al.[12] with slight modifications. The HPLC system was equipped with a 600 Controller, 2996 Photodiode Array Detector, 717 Plus autosampler, on line Desgasser and Empower software (Waters, Milford, USA). The chromatographic column was substituted by a reaction coil (1.5 m). The methanol–0.1 mM DPPH solution (v/v : 50/50) was used as mobile phase in an isocratic elution mode with flow rate of 2 ml min^{-1} and total running time of 6 minutes. Prior to the DPPH analysis, deproteinized plasma was prepared by mixing 30 ml of plasmawith 90 ml of methanol, vortexing for 1 min, followed by centrifugation at 9000 rpm for 3 min at 5 ºC. 20 ml of supernatant was then diluted with 130 ml of methanol and an aliquot of 20 ml of diluted deproteinized plasma was injected into the injection valve connecting directly to the reaction coil. The quantitation of the antioxidant activity of the plasma sample injected was achieved by comparing the negative area of their decoloration peak to the dose–response curve constructed usingmultiple concentrations of caffeic acid. The total antioxidant capacity detected by the DPPH method was expressed as mg caffeic acid equivalents/l plasma.

A microplate spectrophotometer Multiskan GO (Fisher- Scientific, Madrid, Spain) was used to perform the FRAP assay of Benzie and Strain[13] with slight modifications. Briefly, 20 ml of plasma that was previously centrifuged at 1500 rpm for 7 min at 23 ºC (acetate buffer served as the blank) and 160 ml of acetate buffer (pH=3.6) were subsequently added to each well. After incubation for 2 min at 37 ºC, 40 ml of FeCl3 (10mM) and 40 ml of 33% TPTZ were added, followed by shaking for 30 s at 37 ºC in the platereader. The absorbance at 593 nm was monitored for 10 min in 15 s intervals. The first 4 min data were used for calculation of the FRAP value.13 The FRAP value was calculated based on the standard curve constructed using ferrous ion $FeSO_4$-H2O (0–1000 mM). The final value is expressed as mmol Fe equivalents/l plasma.

Limit of detection (LOD), limit of quantification (LOQ), accuracy, RSD% (n=3) intraday and interday for the analysis of the plasma non-enzymatic antioxidant capacity by FRAP and DPPH were determined (ESI†).

2.5 Activity of antioxidant enzymes in RBC

Determination of the protein content of lysate RBC was carried out using a BioRad DC™ protein assay kit (Hercules, CA). Protein was then adjusted to 5 mg protein per ml with phosphate buffer (pH . 7) and divided into aliquots. Lysate RBC aliquots were stored at 80 ºC. SOD activity was measured using a commercial colorimetric assay kit (Sigma-Aldrich, St. Louis, USA), which utilizes Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon reduction catalyzed by superoxide anions. SOD activity was quantified based on the percentage of inhibition on the formation of formazan dye. GPx activity was measured using a commercial colorimetric assay kit (Calbiochem, Merck, Darmstadt, Germany). The final value is expressed nM min^{-1} per mg protein. CAT activity was measured based on the method of Aebi, et al.14 with slight modifications. Briefly, 70 ml of phosphate buffer, 50 ml of RBC lysate (5 mg protein per ml) and 50 ml of 1%H_2O_2 were added in each well of a quartz microplate (Hellma, Müllheim, Germany). After shaking for 1–2 s in a platereader (FisherScientific, Madrid, Spain), the absorbance at 240 nm was monitored for 1 min in 15 s intervals. The final value is expressed as U per mg protein.

2.6 Determination of phenolic and monoterpene metabolites in plasma

Phenolic and monoterpene metabolites in plasma were first extracted using microelution solid-phase extraction (μSPE), based on the method of Suárez et al.[15] The metabolites in 2.5 ml elute were analyzed using a Waters Acquity UPLC-MS/MS system (Milford MA, USA). The same chromatographic method described by Rubió et al.[16] was employed to characterize the metabolites derived from olive oil and thyme. The identification and quantification of olive metabolites was based on the method of Suárez et al.[15] and the method described by Rubió et al.[16] was used to identify thyme metabolites.

2.7 Statistical analysis

Data are expressed as means SD. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Turkey-Kramer HSD multiple comparison test. A p value <0.05 was considered significant. All statistical analyses were performed using JMP software (Version 8.0.2, SAS Institute, Cary, NC, USA).

3. RESULTS

3.1 Plasma non-enzymatic antioxidant capacity (NEAC)

Plasma NEAC was signiFIcantly modulated by OE, TE, and OTE in a time-, assay-, and extract-dependent manner (Fig. 1). Plasma DPPH values at 60 and 120 min post-ingestion were significantly ($p < 0.05$) increased by at least 24% by TE, OTE, and OE as compared to the control rats sacrificed at 0 min (**Fig. 1**).

The plasma DPPH value in the rats fed with the OE stayed elevated up to 360 min while those of the OTE and TE at 360 min did not differ from that of the control. TE and OTE did not affect plasma FRAP values. OE increased significantly plasma FRAP values at 60 and 120 min post gavage by at least 72% as compared to the control ($p < 0.05$) (**Fig. 1**). The increases at 60 and 120 min noted in the OE rats were also significantly different from those of the TE and OTE ($p < 0.05$).

3.2 Antioxidant enzyme activities in RBC

The administration of the three extracts significantly decreased SOD activity as compared to the control ($p < 0.05$), OTE and OE at 60 min and TE at 120 min. CAT activity was significantly increased in all treated groups at 60 and 360 min post gavage as compared to the control group ($p < 0.05$) and the three extracts also showed a significant reduction in GPx activity at 240 min ($p < 0.05$) (**Fig. 2**).

3.3 Metabolites of phenolics and monoterpenes in plasma

Table 1 shows the content of phenols and monoterpenes administered with OE, OTE, and TE. Phenolics provided by olive cake extract were mainly secoiridoid derivatives. Flavonoids, phenolic acids (mostly rosmarinic acid) and monoterpenes were dominant in thyme.

Fig. 3 illustrates the pharmacokinetic profiles up to 360 min of the plasma metabolites of olive phenolics. Hydroxytyrosol sulfate was the main

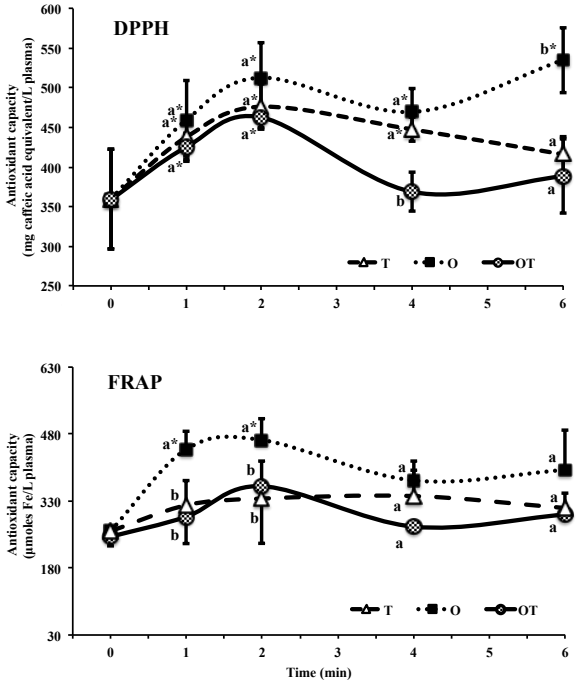

Fig. 1 Oxidative status determined in plasma at different times (0 min, 60 min, 120 min, 240 min and 360 min) after an acute intake of olive extract (OE), thyme extract (TE) and the combination of both extracts (OTE) analyzed on the basis of two different non-enzymatic antioxidant assays: DPPH (expressed as mg caffeic acid equivalent per l plasma ± standard deviation) and FRAP (µM Fe/l plasma ± standard deviation). [ab] Mean significant differences between treatments in the same tested time. * Mean differences between the treatment and the control (0 min).

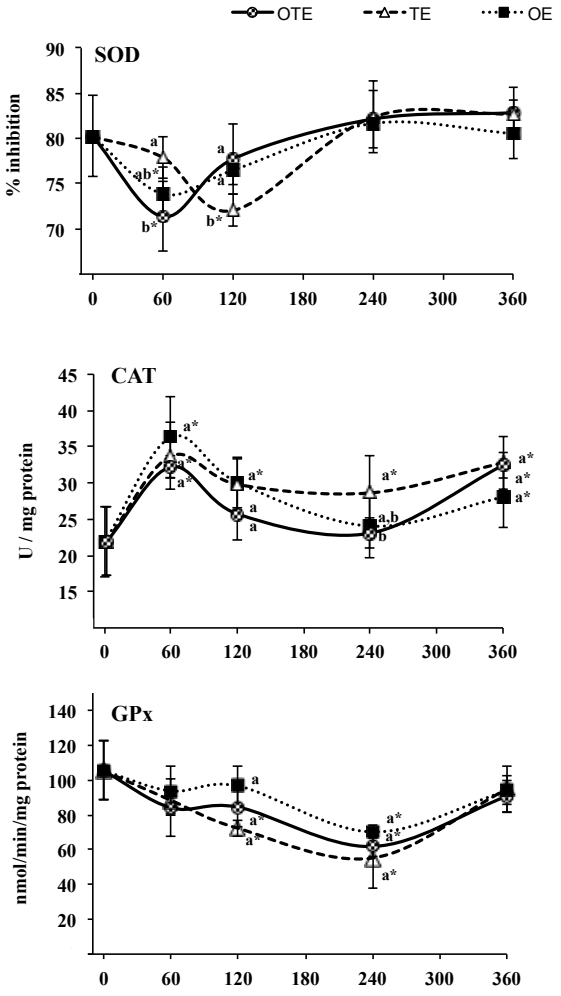

Fig. 2. Endogenous antioxidant enzyme activities determined in erythrocytes at different times (0 min, 60 min, 120 min, 240 min and 360 min) after an acute intake of olive extract (OE), thyme extract (TE) and the combination of both extracts (OTE). Results of super oxide dismutase (SOD) are expressed as % inhibition, results of catalase (CAT) are expressed as U per mg protein and results of glutathione peroxidase (GPx) are expressed as nmol min^{-1} ml^{-1}. ^{ab} Mean significant differences between treatments in the same tested time. *Mean differences between the treatment and the control (0 min).

metabolite with a maximum concentration (C_{max}) of 83 μM at 60 min after gavage. The time to reach Cmax (T_{max}) of all detected olive metabolites was 60 min after dosing. At 360 min, most metabolites remained detectable in the plasma. The C_{max} of hydroxytyrosol sulfate, homovanillic alcohol sulfate and hydroxytyrosol acetate glucuronide in plasma of rats fed OTE were similar to those of OE. Comparing the plasmatic olive phenolic metabolites with the olive phenolics administered, OE (100% olive phenolics) and OTE (50% OE and 50% TE), the plasmatic olive phenolic metabolites detected post gavage of OTE was proportionally higher than the expected concentrations which should be 50% of the concentrations of the respective metabolites in the OE rats.

Fig. 4 shows the major metabolites of thyme bioactives in the rats gavaged with TE or OTE. The main metabolites in plasma were phenolic acids and monoterpene thymol in a sulfate conjugated form. In contrast to one C_{max} noted in olive metabolites, there was a two-phase mode kinetic pattern for thyme metabolites with T_{max} at 60 and 360 min post gavage. Thymol sulfate was

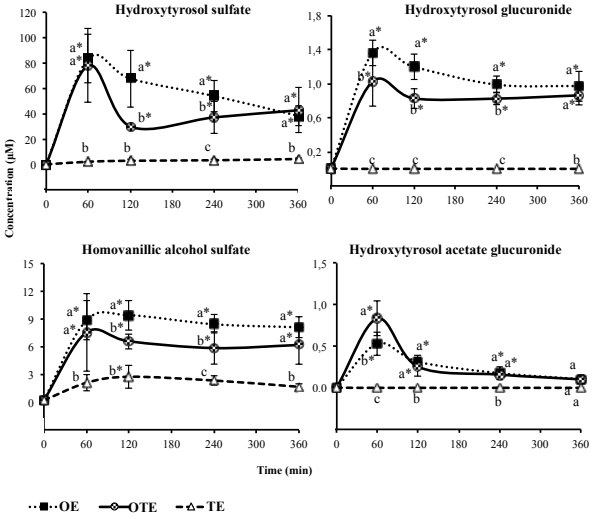

Fig. 3. Plasmatic concentration of olive metabolites determined in plasma at different times (0 min, 60 min, 120 min, 240 min and 360 min) after an acute intake of olive extract (OE), thyme extract (TE) and the combination of both extracts (OTE). Results are expressed as μmols/l plasma ± standard deviation. [ab] Mean significant differences between treatments in the same tested time. *Mean differences between the treatment and the control (0 min).

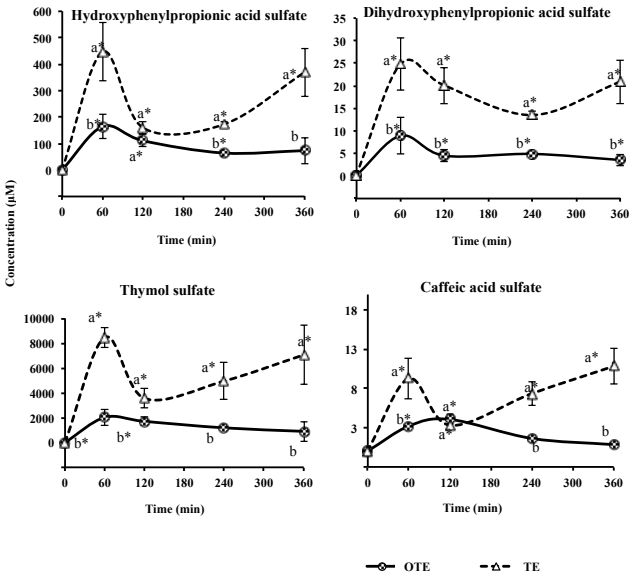

Fig. 4. Plasmatic concentration of thyme metabolites determined in plasma at different times (0 min, 60 min, 120 min, 240 min and 360 min) after an acute intake of olive extract (OE), thyme extract (TE) and the combination of both extracts (OTE). Results are expressed as μmols/l plasma ± standard deviation. [ab] Mean significant differences between treatments in the same tested time. *Mean differences between the treatment and the control (0 min).

the main metabolite in the TE and OTE rats, and its C_{max} was 8463 and 2021 μM, respectively.

4. DISCUSSION

In this paper we have established that phenolics derived from olive and thyme administered separately and together in rats have *in vivo* antioxidant protective effects in postprandial conditions, having an association with the metabolites accumulated in plasma which could participate in the antioxidant defense there.

The DPPH assay seemed to be more sensitive to modulation by the circulating metabolites than FRAP. FRAP showed an increasing trend but only OE gave a significant increase at 60 and 120 min, at the time that olive metabolites reached their Cmax. DPPH assay showed a significant increase of antioxidant capacity at 60 min after the intake of all the extracts, which was maintained in

significantly increased values until 240 min with TE and OTE and until 360 with OE. These results are in accordance with data obtained from the metabolite pharmacokinetics, observing that both olive and thyme metabolite concentrations follow the same profile, increasing at 60 min and remaining at high concentrations until 360 min. At 360 min an increasing trend is observed in antioxidant capacity levels associated also with the second plasma peak of the phenolic metabolites. Previous pharmacokinetic studies with polyphenols in rats have shown a second maximum plasma concentration after polyphenol administration, which is consistent with enterohepatic circulation.[16] As reported before, metabolites may contribute to increasing antioxidant capacity in plasma.[17] Indeed, previous bioavailability studies with phenolics from olive oil[18–20] and from other polyphenol-rich foods such as chocolate[21] have shown both increased polyphenol plasma levels and NEAC in plasma. The difficulty of comparing our results with previous reports is based on the fact that a single approach for quantifying NEAC in plasma or serum does not exist.[22] Based on our observations, the results in this study reveal a relationship between the intake of two phenol rich extracts and a significant increase in the antioxidant capacity of rat plasma measured by two different methods. Even though there is no consensus on the *in vivo* antioxidant actions of phenolics from olive oil, the present work could allow us to attribute these positive changes to the phenolic compounds, confirming that there is a strong relation between phenolic plasma metabolites and increasing values of plasma antioxidant capacity. In the present study phenolics were detected in plasma in their conjugated forms. Although there is controversy about the antioxidant activity of the conjugated forms of polyphenols, it has been established that polyphenols such as flavonoids, which mainly accumulate as conjugates in plasma, can participate in the antioxidant defense in blood serum.[23,24]

The modulating effect of thyme on antioxidant plasma status has not been thoroughly studied. El-Nekeety et al.[25] reported in a rat study that constituents in thyme exerted antioxidant activity especially in the liver and had a protective effect against aflatoxin-induced toxicity. Consistently, our study showed that TE enhanced plasma NEAC in healthy rats. Thymol sulfate appeared to be the major metabolite derived from thyme found in plasma, indicating that thymol, which was highly bioavailable, seem to be the compound most plausibly responsible for the observed increased antioxidant capacity in plasma after TE.

The antioxidant enzyme function was also improved by the decreased activity of SOD and GPx, and the increased CAT in RBC after the administration of the three extracts. In pathologies and oxidative stress situations CAT has generally been shown to be wasted[26–29] and SOD and GPx activities are usually increased,[27,30–32] so the three enzymes studied operate in a coordinated system and antioxidant protection does not involve the overexpression of all of them. Regarding the *in vivo* antioxidant enzyme modulation of olive phenols, our results are consistent with previous data obtained in humans after an intake of a phenol-enriched olive oil.[33] In addition, Paiva-Martins et al.[34] found that hydroxytyrosol and its metabolites were able to interact with RBC and protect them from oxidative hemolysis initiated by peroxyl radicals, indicating that hydroxytyrosol metabolites detected in plasma could be also responsible for the up-regulation of the antioxidant enzymes observed after the olive extract administration.

The *in vivo* antioxidant enzyme modulation of thyme bioactives is scarce in the literature. In a previous report, Rana et al.[35] noted in a rat study that a diet containing thyme diminished the impact of stress-induced reduction in the activities of SOD, GPx, and CAT as compared to the control normal diet. Youdim et al.[36] measured changes in the antioxidant enzyme activity of different organs during the lifetime of rats. They found out that dietary supplementation of thyme reduced the unfavourable age-related decline in activities of SOD in the liver and heart of old rats. Complementing these previous studies, we found that thyme bioactives could offer protection up-regulating antioxidant enzyme activities in healthy rats.

Currently, the precise mechanism by which antioxidant enzymes are modulated after the administration of polyphenols is unknown. The short time of the observed effects in our study support the hypothesis that the intake of polyphenols may act as a direct scavenger of reactive oxygen species reducing the body's need for certain antioxidant enzymes. Our suggestion is supported by recent reports[33,37] which also found a decrease of SOD and GPx

after antioxidant supplementation. Recent studies also postulate that this activation may occur at the post-translational level,[38,39] a situation that also deserves further attention.

When both phenolic sources (thyme and olive) were administered in a combined extract, we observed the comparable effect on the plasma NEAC and the antioxidant enzymes activity to the OE or TE, implicating additive interactions between bioactives of thyme and olive. Although any synergistic effect was observed in the antioxidant status when both phenolic extracts were administered, an enhanced bioavailability of olive phenolic compounds could occur in the presence of thyme phenolics when OTE was administered. It is noteworthy that the C_{max} of hydroxytyrosol sulfate and homovanillic alcohol sulfate in plasma of rats fed with OTE were similar to those of OE, or even higher, as in the case of hydroxytyrosol acetate glucuronide. These results suggest that thyme bioactives may facilitate the bioavailability of certain OE phenolics. In fact, in a previous study carried out in our lab with the same extracts, an *in vitro* digestion model was performed to evaluate the effect on bioaccessibility of the co-occurring bioactive compounds from olive and thyme. Results indicated that hydroxytyrosol bioaccessibility was enhanced when OTE was digested.[40] While the underlying mechanim(s) remains to be examined, we hypothesize that thyme bioactives might protect olive phenolics from degradation in the gastrointestinal tract before absorption. The mechanism of action of several phytochemicals is still unknown. Speculation as to the reason for this, whether it involves synergy, enhanced bioavailability, cumulative effects, or simply the additive properties of the constituents, requires further research.

In conclusion, results indicate that protection by phenolics from olive and thyme against oxidative stress occurs primarily through a direct antioxidant effect and may be related to the phenolic plasmatic metabolites. Although any synergistic effect was observed in the antioxidant status when both phenolic extracts were administered, an enhanced bioavailability of olive phenolic compounds could occur in the presence of thyme phenolics after an acute intake in rats due to protection in the gastrointestinal tract. Therefore, the combination of different phenolic sources might be a promising approach to improve the bioavailability.

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Publication VIII.

In vivo distribution and deconjugation of hydroxytyrosol phase II metabolites in red blood cells: a potential new target for hydroxytyrosol

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IN VIVO DISTRIBUTION AND DECONJUGATION OF HYDROXYTYROSOL PHASE II METABOLITES IN RED BLOOD CELLS: A POTENTIAL NEW TARGET FOR HYDROXYTYROSOL

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Abstract

Olive oil phenolic compounds, which have demonstrated several health benefits, are extensively metabolized into methylated and glucurono- and sufo-conjugates of hydroxytyrosol (HT), the detected circulating forms in plasma. However, these conjugates are considered to be pharmacologically inactive and most recent studies suggest that their in situ deconjugation in certain sites appears to be an absolute requirement to exert their beneficial effects. In the present study, red blood cells (RBCs), which are particularly exposed to oxidative damage, were analyzed after an oral administration in rats of an olive phenolic extract and their in vivo deconjugation capacity of hydroxytyrosol phase II metabolites was assessed. A decreasing trend of the conjugated forms of HT and a parallel free HT increasing in concentration up to 360 min was observed in RBCs. In the same study, free hydroxytyrosol was not detected in plasma, indicating that HT conjugates might be hydrolyzed intracellularly in RBC. Our results indicate for the first time, that RBC might be a potential cell target for HT, which could play a pivotal role in the distribution and bioavailability of circulating phenols and its metabolites and also in protection against cell oxidative damage.

KEYWORDS: deconjugation / hydroxytyrosol / phase II metabolites / red blood cells

1. INTRODUCTION

Recent intervention clinical trials have provided evidence that the phenolic content of an olive oil contributes to the protection against lipid oxidative damage in humans in a dose-dependent manner (Covas et al., 2006). One of the first steps in relating biological activities of dietary phenol compounds to health benefits in humans is to demonstrate their bioavailability from diet. It is well established that olive oil phenolic compounds are rapidly absorbed and extensively metabolized in the gut and liver (Visioli et al., 2000; Miró-Casas et al., 2001; Miro-Casas et al., 2003; Weinbrenner et al., 2004). As a result, olive oil phenols appear in biological fluids mainly as phase II metabolites (e.g., glucuronides, sulfates and methyl sulfates)

of hydroxytyrosol (HT) (Garcia-Villalba et al., 2010; Rubió et al., 2012a).

Phase II phenolic metabolites are generally considered to be pharmacologically inactive and targets for excretion. The information available about the in vitro effects of phenolic metabolites indicates that they are less active than the parent compounds and often totally inactive (Perez-Vizcaino, Duarte & Santos-Buelga, 2012). For instance, in contrast to the aglycones, glucuronidated and sulfated metabolites of quercetin lack a direct acute vasodilator effect in isolated arteries and they have only a partial effect in preventing acute endothelial dysfunction (Lodi et al., 2009). Based on these evidences, a recent study has suggested that in situ deconjugation of phenolic conjugated metabolites could play an important role in transiently providing bioactive/ unstable aglycones in the biological fluids, in particular within the sites of inflammation (Ishisaka et al., 2013).

Red blood cells (RBCs) are oxygen carriers with high polyunsaturated fatty acid content on their membranes and high cellular concentration of hemoglobin are particularly exposed to oxidative damage. The hemoglobin released from erythrocytes is potentially dangerous because in reacting with H_2O_2 it is converted into oxidized forms, which are powerful promoters of oxidative processes (van den Berg et al., 1992). For this reason, newer therapeutic agents, such as phenolic compounds from the diet that can target oxidative stress in RBC, may constitute a valuable means for preventing or delaying the development of organ complications (Silva, Belini Junior, De Almeida, & Bonini-Domingos, 2013).

In the present study free HT and its conjugates have been analyzed in RBCs for the first time after an oral administration of an olive phenolic extract, postulating that RBCs could be cell targets for HT and its metabolites within they could exert an antioxidant effect. The *in situ* deconjugation of HT conjugates into its free form was proposed, suggesting that it could be a mechanism of protection against cell oxidative damage.

2. MATERIAL AND METHODS

2.1 Chemical and reagents

The standard of hydroxytyrosol was purchased from Extrasynthese (Genay, France) and the secoiridoid derivatives 3,4-DHPEA-EDA and p-HPEA-EDA, which are not available commercially were isolated from virgin olive by semi-preparative HPLC as described previously (Suárez, Macià, Romero & Motilva, 2008). Hydroxytyrosol-3-O-sulfate, used for the quantification of HT conjugates, was custom synthesized by Toronto Research Chemicals Inc. (Toronto, ON Canada), and its purity was 98.4%. Catechol was acquired from Sigma-Aldrich (Germany) and was used as an internal standard (IS) prepared in 4% phosphoric acid.

Acetonitrile (HPLC-grade), methanol (HPLC-grade), glaciar acetic acid (≥99.8%), formic acid, clorhydric acid and L(+)-ascorbic acid (reagent grade) were all provided by ScharlauChemie (Barcelona, Spain). Hydrochloric acid (37%) was from Prolabo (Badalona, Spain). Orto-phosphoric acid 85% was purchased from Mont Plet & Esteban S.A. (Barcelona, Spain). Ultrapure water was obtained from a milliQ water purification system (Millipore Corp., Bedford, MA, USA).

2.2 Olive oil phenolic extract

Olive extract (OE) was prepared from freeze-dried olive cake using an accelerated solvent extractor (ASE 100) (Dionex, Sunnyvale, CA), according to the methodology described previously (Rubió, Motilva, Macià, Ramo & Romero, 2012b). Briefly, the phenolics were extracted using ethanol/water (80:20, v/v) at 80 °C using 10 g of freeze-dried material for each extraction. The phenolic composition is shown in **Table 1** and was characterized by LC- MS/MS as described in our previous report (Rubió, Motilva, Macià, Ramo, & Romero, 2012b).

2.3 Treatment of rats and erythrocytes collection

Seventeen male Wistar rats of 3 months old were obtained from Charles River Laboratories (Barcelona, Spain). All rats were housed in cages in a 12-light-12h dark cycle at controlled temperature (22°C). They were fed ad libitum a standard commercial chow, PanLab A04 (Panlab, Barcelona, Spain) and had free access to water. After they were fasted for 16-17 hours but had access to water, rats were divided into treated group and control group. Twelve rats were intragastrically gavaged with 1.5 g / kg BW dispersed in water of the extract OE. After gavage, 3 rats in each group were sacrificed at 60, 120, 240, and 360 min post gavage. Rats were anesthetized with isoflurane and then euthanized by exsanguination. Five rats in the

Table 1. Hydroxytyrosol and derivates content of the administered extract to the rats. Values are expressed as mg of compound in the administered dose (1.5 g/ kg BW).

Compound	(mg)
Hydroxytyrosol	1.4
3.4-DHPEA-AC	1.7
p-HPEA-EDA	1.2
3.4-DHPEA-EDA	19.2
Oleuropein derivative	1.8
3.4-DHPEA-EA	9.1
Total	34.4

3.4-DHPEA-AC: 3.4-Dihydroxyphenyl ethyl acetate (hydroxytyrosol acetate); 3.4-DHPEA-EA: 3.4-dihydroxy phenylethanol-elenolic acid (oleuropein aglycone); 3.4-DHPEA-EDA: 3.4-dihydroxy phenylethanol-elenolic acid dialdehyde (dialdehydic form of elenolic acid linked to hydroxytyrosol); p-HPEA-EDA: p-hydroxy phenylethanol-

control group did not receive any extract, were kept fasted up to 16-17 hours, and were euthanized by the same protocol. The study was approved by the Animal Ethics Committee of the University of Lleida (CEEA 04-01/11, 26th January 2011) and performed in compliance with the relevant laws and University of Lleida guidelines.

2.4 Pre-treatment of RBC and UPLC-ESI-MS/MS analysis of metabolites

The phenolic compounds were extracted from the RBCs samples with the SPE system using OASIS HLB 200mg cartridges (Waters Corp., Milford, MA). The conditioning of the cartridges was done by adding sequentially 2mL of methanol and 2 mL of milliQ water acidified at pH 2 with acetic acid. Extractions were done by loading 1mL of washed RBC, which had previously been mixed with 3 mL of distilled water and 20μl of phosphoric acid 85% to break the bonds between the proteins and phenolic compounds. The loaded cartridges were washed with 1mL of milliQ water and 1mL of methanol 5%. Finally, the retained phenolic compounds were eluted using 3 mL of methanol, which was evaporated to dryness and reconstituted with 100μL of methanol. HT and its metabolites were determined by UPLC-MS/MS using the method previously described (Serra et al., 2013), and the identification and quantification was based on the method already developed (Suárez et al., 2009).

3. RESULTS AND DISCUSSION

HT and its metabolites were detected in RBCs in the nanomolar range (**Figure 1**), which suggests that the quantities of phenolics detected in plasma do not reflect the true amounts of phenolics that gain access to the circulation, since these compounds might also be complexed with circulating RBCs.

The detected metabolites were mainly in its sulfated and methyl-sulfated forms (HT sulfate 52% and HValc sulfate 27% at 1h), being the glucuronide forms a minoritary proportion (HT glucuronide 2% and HValc glucuronide 1%) (**Table 2**). Free HT, which was not detected in plasma at any time in our previous report in which the same extract was administered (Rubió et al., 2014), followed an increasing trend in concentration up to 360 min, while its conjugates had the opposite behaviour as they reached its C_{max} at 1h and after that they decreased in concentration (**Figure 1**). The detection of free HT and its pharmacokinetic behavior together with its conjugates might indicate that both sulfates and glucuronidates could suffer a process of enzymatic hydrolysis intracellularly resulting in HT aglycone release. We hereby suggest that deconjugation of HT phase II conjugates can potentially occur in vivo to produce the active aglycone in RBCs **(Figure 2**). In accordance with our results, it was recently postulated in an *in vitro* study (Koren, Kohen & Ginsburg, 2010) that circulating RBCs and possibly also other blood cells might be constantly coated by polyphenols from diet, those acting as antioxidant depots which might act as protectors against the harmful consequences of oxidative stress.

Table 2. Content of hydroxytyrosol and its conjugates (nM) detected in RBC and percentage of the individual metabolites in relation to total at different time points (1 to 6 h) after the olive phenolic extract administration.

	0h	1h	(%)	2h	(%)	4h	(%)	6h	(%)
HT	n.d.	57,1 ± 16,6	26,7	172,5 ± 33,6	73,6	191,7 ± 22,1	92,8	176,0 ± 7,5	81,1
HT sulfate	n.d.	110,3 ± 25,7	51,5	30,3 ± 11,6	12,9	6,4 ± 1,3	3,1	23,4 ± 8,5	10,8
HValc sulfate	n.d.	41,3 ± 15,4	19,3	26,9 ± 3,0	11,5	5,5 ± 2,1	2,7	15,8 ± 3,6	7,3
HT glucuronide	n.d.	3,7 ± 1,2	1,7	3,2 ± 1,7	1,4	2,2 ± 0,8	1,1	1,1 ± 0,3	0,5
HValc glucuronide	n.d.	1,7 ± 1,0	0,8	1,3 ± 0,3	0,6	0,73 ± 0,9	0,4	0,7 ± 0,8	0,3
Total		214,0		234,2		206,5		216,9	

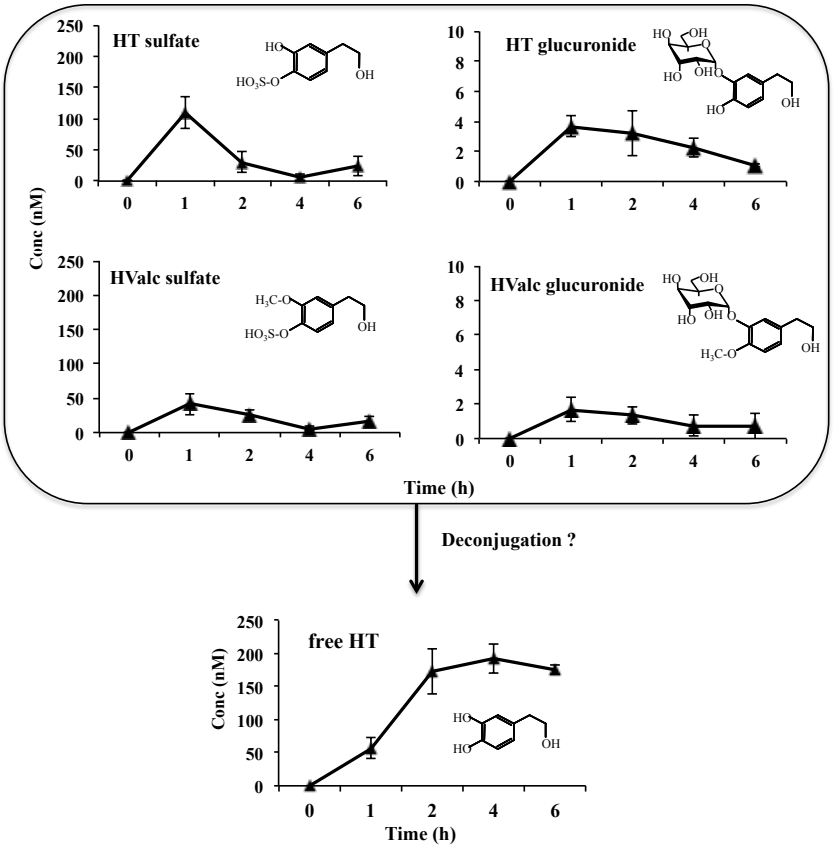

Figure 1. Pharmacokinetics of hydroxytyrosol and its conjugates in RBC at 0-360 min after the oral administration of olive extract (OE). Results are expressed as nM ± standard deviation.

In a preliminary in vivo study we also detected free HT in tissues like kidney and testicles (Serra et al., 2012) together with variable amounts of conjugated phase II metabolites. Similarly, other authors have detected quercetin aglycone in liver, kidney and intestine wall (Bieger et al., 2008), which suggest that an in situ deconjugation process could also occur at a tissular level. Some authors found hydrolysis of flavonoid glucuronides in human neutrophils (Shimoi et al., 2001) and in carcinoma cell lines (Lee-Hilz, Stolaki, van Berkel, Aarts & Rietjens, 2008), and they suggested that the activity of flavonoid metabolites depends on their deconjugation in situ. Based on these evidences, Terao, Murota and Kawai (2011) proposed that glucuronide conjugates of flavonoids such as quercetin, function not only as detoxified metabolites but also as precursors of the bioactive aglycone. Moreover, dietary polyphenols, such as quercetin, have long been recognized to protect blood vessels from atherogenic inflammation by yet unknown mechanisms. In a previous work (Kawai et al., 2008) the specific localization of quercetin-3-*O*-glucuronide (Q3GA) in macrophage cells in the human atherosclerotic lesions was discovered, and they further demonstrated the molecular basis of the interaction between quercetin glucuronides and macrophages, leading to deconjugation of the glucuronides into the active aglycone (Ishisaka et al., 2013). Based on this previous evidence, the selective deconjugation within the sites that tend to be more exposed to oxidative stress, such as RBCs, seems to be

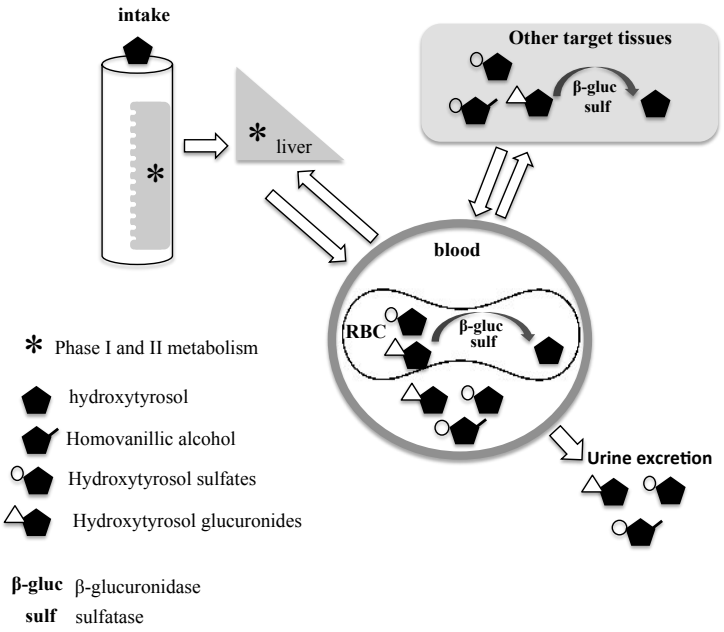

Figure 2. Schematic representation of hydroxytyrosol metabolism after absorption and RBC as potential target cells for its deconjugation.

crucial when it comes to bioactivity of polyphenols.

To sum up, RBCs, which are normally discarded from blood in pharmacokinetic studies, have been considered for the first time to be part of the bioavailable phenolic compounds after absorption. Results indicate that RBCs might be a first potential cell target for HT, which could play a pivotal role in the bioavailability and tissue distribution of circulating phenol metabolites. Moreover, the *in situ* deconjugation of HT conjugates into its free active form was proposed, suggesting that it could be a mechanism of protection against cell oxidative damage. Taken together, these findings open a hot topic to drive new antioxidant therapeutic opportunities with hydroxytyrosol.

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III. Sustained consumption of phenol-enriched olive oils: Human clinical trial

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Publication IX.

Metabolite profiling of olive oil and thyme phenols after a sustained intake of two phenol-enriched olive oils by humans: Identification of compliance biomarkers

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METABOLITE PROFILING OF OLIVE OIL AND THYME PHENOLS AFTER A SUSTAINED INTAKE OF TWO PHENOL-ENRICHED OLIVE OILS BY HUMANS: IDENTIFICATION OF COMPLIANCE BIOMARKERS

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Abstract

An understanding of causal relations between polyphenol intake and its beneficial effects on health is hindered by the lack of robust biological markers of its exposure. This is particularly relevant in mid/long-term nutritional intervention studies. An analytical methodology based on UPLC-MS/MS has been developed to determine the metabolites of the phenolic compounds from olive oil and thyme in biological fluids after a sustained intake of two phenol-enriched olive oils for their further use as compliance biomarkers. In a randomized, double-blind, controlled, cross-over trial, 33 hypercholesterolemic volunteers received during 3 weeks 25 ml/day of (1) raw virgin olive oil with a low phenolic content as a control (80 ppm; VOO), (2) Functional Virgin Olive Oil enriched with its own phenolics (500 ppm; FVOO), and (3) Functional Virgin Olive Oil enriched with its own phenolics plus complementary phenolics from Thyme (500 ppm, 50% from olive oil and 50% from thyme respectively; FVOOT). Plasma and 24 h-urine samples were collected. The results showed that some hydroxytyrosol (HT) metabolites presented low specificity as biomarkers of intake. However, hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate appeared to be suitable biomarkers for monitoring compliance with olive oil intake as their values in plasma or/and 24-h urine were significantly higher after FVOO compared to baseline pre-intervention concentrations. They were also significantly correlated with the monitored level of compliance. On the other hand, metabolites derived

from thyme were more specific, thymol sulfate and hydroxyphenylpropionic acid sulfate being the metabolites with the largest increase in both plasma and 24-h urine, whereas urinary *p*-cymene-diol glucuronide presented the greatest increase post-treatment. Their urinary excretion values also displayed significant correlations with the level of compliance and they were defined as FVOOT compliance biomarkers. This study enabled robust quantitative and qualitative compliance biomarkers after the ingestion of two phenol-enriched olive oils to be determined and provided a thorough analysis of the true phenolic exposure after a sustained consumption that could be further related to expected biological effects.

Keywords: compliance biomarkers / human intervention / olive oil / phenolic metabolites / thyme

1 INTRODUCTION

The European Food Safety Authority Scientific and Technical Guidance has recently provided the criteria for authorizing health claims for foods. This clearly establishes that relevant human interventional studies have to be presented to substantiate any claim. While studies in animal or *in vitro* models may provide supporting evidence (e.g. in support of a mechanism), human data are essential to substantiate the health claim (EFSA Panel on Dietetic Products, 2011). Over the past decade, a significant number of human nutrition intervention studies have been conducted with the goal of establishing the exact bioefficacy of various subclasses of polyphenols as protection against chronic degenerative diseases (Del Rio et al., 2013; Kay, Hooper, Kroon, Rimm, & Cassidy, 2012). Nevertheless, few validated biomarkers of polyphenols exposure are available, which hinders establishing any relationship between exposure and effects (Kay, 2010). This link is essential to decide whether the negative outcome of a controlled trial (i.e., a lack of functional change in response to supplementation) can be related to the basic hypothesis as a clinical effect or as a lack of compliance among the participants in the trial (Puiggròs, Solà, Bladé, Salvadó, & Arola, 2011).

The dietary intake of nutrients and non-nutrient components in human intervention studies is usually determined using dietary assessment methods, such as diet diaries. There are considerable difficulties in assessing polyphenol intakes using this traditional approach, thus highlighting the need for validated biomarkers of their intake. Biological markers have been used as an alternative over recent years (Kay, 2010). However, the relationship between dietary intake and the resulting concentrations of biomarkers in body fluids is highly complex, and, thus, very few validated biomarkers of dietary exposure are available. Various studies have measured total urinary polyphenols as biomarkers of fruit and vegetable intake in order to characterize and quantify habitual food intakes in a diet pattern (Krogholm et al., 2012; Krogholm, Haraldsdóttir, Knuthsen, & Rasmussen, 2004; Mennen et al., 2006; Nielsen, Freese, Kleemola, & Mutanen, 2002). Alternatively, more specific biomarkers can be monitored when we are interested in a given food or food ingredient.

Consistent clinical intervention trials have supplied evidence that the phenolic compounds (PC) of virgin olive oil contribute to the protecting humans against lipid oxidation in a dose-dependent way (Covas et al., 2006; Weinbrenner et al., 2004). The absorption and excretion of olive oil PC following an acute intake in humans have been studied previously (García-Villalba et al., 2010; Miro-Casas et al., 2003; Miró-Casas et al., 2001; Visioli et al., 2000; Vissers, Zock, & Katan, 2004) and attempts to monitor olive oil phenolic consumption as a biomarker for intervention compliance have focused in the analysis of total HT (Covas et al., 2006; Marrugat et al., 2004). However, no studies have been performed to determine individual polyphenol metabolites in biological fluids after a sustained intake of olive oil. Additionally, there is a modern trend towards flavoring olive oils with herbs and spices to improve their sensorial profile. This could be turned to advantage to look at the combined or synergic beneficial health effects of

polyphenols and a novel approach would consist of developing functional olive oils further enriched with their own PC combined with PC from other sources.

Our aim was to identify biomarkers for polyphenols from olive oil and thyme after a 3-week dietary intervention with phenol-enriched olive oils within the frame of a randomized, double-blind, crossover, and controlled nutrition intervention trial. Two functional phenol-enriched olive oils were evaluated, one with its own PC (FVOO) and a second one further enriched with thyme PC (FVOOT). A sensitive and reliable analytical method was developed to detect polyphenol metabolites in plasma and urine samples to identify the most appropriate compliance markers and eventually relate them to the expected biological effects.

2. MATERIAL AND METHODS

2.1 Olive oils preparation and characterization

VOO with a low phenolic content (80 ppm) was used as a control condition in the intervention and as an enrichment matrix for two FVOOs (500 ppm). FVOO was enriched with its own PC by adding a phenol extract obtained from freeze-dried olive cake. FVOOT was enriched with its own PC and complemented with thyme PC using a phenol extract made up of a mixture of olive cake and dried thyme. Hence, FVOOT contained 50% of olive PC (hydroxytyrosol derivates) and 50% of thyme PC (flavonoids, phenolic acids and monoterpenes) (**Table 1**). The procedure for obtaining the phenolic extracts and enriched oils was previously developed in our laboratory (Rubió et al., 2012a). For the wash-out period, a commercial commom olive oil (blend of refined and a small percentage of virgin olive oil) kindly provided by Borges Mediterranean Group was used. The total phenolic content of the olive oils was measured with the Folin-Ciocalteu method (Vázquez-Roncero, Janer del Valle & Janer del Valle, 1973). The phenolic profile of the olive oils was analyzed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) using the method previously described (Rubió et al., 2012a). Representative chromatograms of the three studied olive oils are shown in **Figure 1** of **Additional Information**.

A consumer acceptance test was performed to assess the overall opinion of the three olive oils at the time of the intervention. A specific profile sheet was set up where volunteers had to assign a score on a 7-point category scale (dislike very much, dislike moderately, dislike slightly, neither like nor dislike, like slightly, like moderately and like very much). The scores were converted to whole numbers between 0 and 6 respectively to calculate the means and the standard deviation.

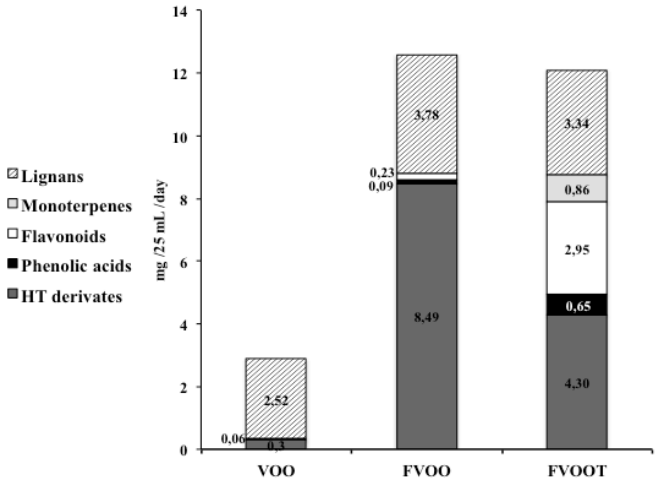
2.2 Study design

The study was a randomized, double-blind, crossover, controlled trial with 33 hypercholesterolemic volunteers (total cholesterol>200 mg/dL) (19 men and 14 women), aged 35 to 80. Exclusion criteria included the following: BMI>35 Kg/m^2, smokers, athletes with high physical activity (>3000 Kcal/day), diabetes, multiple allergies, intestinal diseases, or other disease or condition that could impede compliance with the measurements or treatment.

Subjects were randomized to one of three orders of administration of 25 mL/day of raw (1) virgin olive oil (80 ppm; VOO), (2) functional virgin olive oil enriched with its own PC (500 ppm; FVOO), and (3) functional virgin olive oil enriched with its own PC plus complementary PC from thyme (500 ppm; FVOOT) (**Figure 1**). In the crossover design, the intervention periods were 3 weeks long with a daily ingestion of 25 mL of raw olive oil free distributed along meals. Prior to the interventions, there was a 2-week wash-out period where common olive oil was the main fat consumed. To avoid an excessive intake of antioxidants, such as PC, during the clinical trial period, the participants were advised to limit their consumption of polyphenol-rich foods. A 3-day dietary record, which records the type, quantity, and preparation of food consumed, was administered to participants during the last three days at baseline and before and after each intervention period to control their diet throughout the study. A set of portable containers with the corresponding 25 mL of olive oil for each day of consumption were delivered to the participants at the beginning of each olive oil administration period. The participants were instructed to return the containers to the center after the corresponding olive oil consumption period in order to register the amount of olive oil consumed

Table 1. Phenolic daily intake through 25 mL of VOO (Virgin Olive Oil), FVOO (Functional Virgin Olive Oil enriched with its own phenolics) and FVOOT (Functional Virgin Olive Oil enriched with both its own phenolics and phenolics from Thyme). Values in the table provide the individual phenolic characterization of the olive oils expressed as means ± SD of mg phenols/25 mL oil/day. In the graph below the distribution by phenolic groups is represented with total values for each group.

Phenol (mg phenol /25 mL/ day)	VOO	FVOO	FVOOT
Hydroxytyrosol derivates			
hydroxytyrosol	0,01± 0,00	0,21± 0,02	0,12± 0,00
3,4-DHPEA-AC	n.d.	0,84± 0,06	0,39± 0,04
3,4-DHPEA-EDA	0,04± 0,00	6,73± 0,37	3,43± 0,29
3,4-DHPEA-EA	0,26± 0,04	0,71± 0,06	0,36± 0,03
Phenolic acids			
p-hydroxybenzoic acid	n.d.	0,02± 0,00	0,06± 0,00
vanillic acid	n.d.	0,07± 0,00	0,13± 0,01
caffeic acid	n.d.	0,00± 0,00	0,06± 0,00
rosmarinic acid	n.d.	n.d.	0,41± 0,03
Monoterpenes			
thymol	n.d.	n.d.	0,64± 0,05
carvacrol	n.d.	n.d.	0,23± 0,02
Flavonoids			
luteolin	0,04± 0,00	0,18± 0,02	0,21± 0,02
apigenin	0,02± 0,00	0,06± 0,00	0,10± 0,00
naringenin	n.d.	n.d.	0,20± 0,02
eriodictyol	n.d.	n.d.	0,17± 0,01
thymusin	n.d.	n.d.	1,22± 0,09
xanthomicrol	n.d.	n.d.	0,53± 0,06
7-methylsudachitin	n.d.	n.d.	0,53± 0,09
Lignans			
pinoresinol	0,05± 0,00	0,12± 0,00	0,10± 0,05
acetoxipinoresinol	2,47± 0,19	3,66± 0,31	3,24± 0,28



in the period. 24-h urine was collected in containers the 24 h before each visit. A phone call was done two days before each visit to remind them to collect the 24 h urine. The first morning urine on day 1 was discarded, and the first morning urine of day 2 was included. Blood samples were collected in litium heparin tubes, in fasting state at least of 10 hours. Plasma samples were obtained by centrifugation (1500 g, 15 minutes) of whole blood directly after being drawn. Urine and plasma samples were stored at -80°C until use. This protocol has been approved by the Local Ethics Committee (CEIC-IMAS 2009/3347/I) and is registered in the International Standard

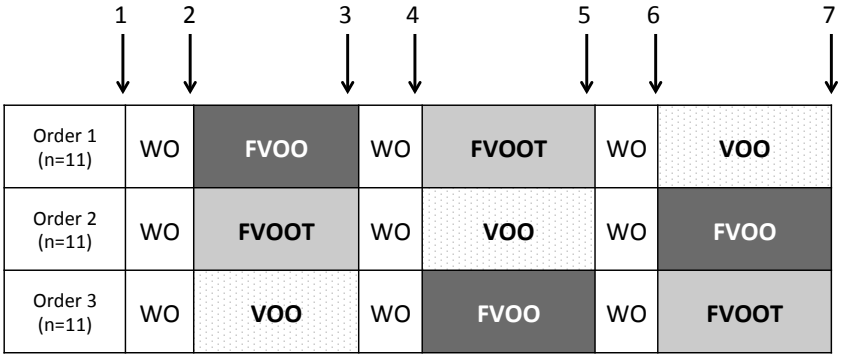


Figure 1. The study was a randomized, double-blinded, crossover, controlled trial with 33 volunteers (19 men and 14 women) who were randomized to one of 3 orders of administration of 25 mL/day of raw VOO (Virgin Olive Oil), FVOO (Functional Virgin Olive Oil enriched with its own phenols) and FVOOT (Functional Virgin Olive Oil enriched with both its own phenols and phenols from Thyme). The intervention periods were 3 weeks in length preceded by a 2-week washout period (WO). Examination number (blood and urine collection): 1, baseline; 2, pre-first intervention; 3, post-first intervention; 4, pre-second intervention; 5, post-second intervention; 6, pre-third

Randomized Controlled Trial register (www.controlled-trials.com; ISRCTN77500181).

2.3 Plasma and urine phenolic metabolites extraction

The extraction of the phenolic metabolites from the plasma samples was carried out using microelution plates (Waters, Milford, USA) packed with 2 mg of OASIS HLB sorbent (Waters, Milford, USA), using a method previously described (Suárez et al., 2009). Briefly, the conditioning and the equilibration were done by applying 250 µL of methanol and 250 µL of Milli-Q water at pH 2, respectively. Hydrochloric acid (HCl) was used to low the pH of water to equilibrate the plate. 350 µL of the plasma sample with 300 µL of phosphoric acid 4% and 50 µL of internal standard (catechol) were loaded onto the plate. Then, 100 µL of water and 100 µL of 5% methanol were used to clean up the plate. Finally, the retained phenolic metabolites were eluted with 2 x 50 µL of methanol. Two different methods with microelution plates were used to extract the phenolic metabolites from the urine. The metabolites derived from thyme were extracted with the same method used for plasma. However, for the extraction of hydroxytyrosol and their metabolites, an optimization of the method was required due to their low extraction recoveries obtained. The optimized extraction method used to extract hydroxytyrosol and their metabolites from urine is described in our previous report (Serra et al., 2013).

2.4 Identification and quantification of the metabolites by UPLC-ESI-MS/MS

The analysis of the phenolic metabolites was carried out by UPLC coupled to tandem MS (MS/MS) based on the method described in (Rubió et al., 2012b). Briefly, the chromatographic system consisted of an AcQuity™ UPLC equipped with a Waters binary pump system (Milford, MA, USA) using an AcQuity UPLC™ BEH C_{18} column (1.7 µm, 100 mm x 2.1 mm i.d.). During the analysis, the column was kept at 30° C and the flow rate was 0.4 ml/min. The mobile phase was water/acetic acid (99.8:0.2 v/v) as solvent A, and acetonitrile as solvent B. The elution started at 5 % of elution B for 5 min, then and it was linearly increased 40 % of eluent B in 20 min, further increased to 100% of eluent B in 0.1 min and kept isocratic for 1.9 min. Next, it was returned to the initial conditions in 0.1 min, and the reequilibration time was 2.9 min. The run time was 30 min, and the injection volume was 2.5 µl.

The phenolic and monoterpene metabolites were detected and quantified from their ion fragmentation in the MS/MS mode using selected ion monitoring (SRM) mode, being the most sensitive mode. In order to identify the metabolites, the full-scan mode in MS, and the

daughter-scan and neutral loss scan modes by tandem MS, were performed. These MS modes are excellent tools for verifying structural information about the compounds when standards are not available. Firstly, analyses were carried out in the full-scan mode, from 80 to 800 m/z, and cone voltages from 20 to 60 V were applied. In addition, the structural information was verified by using the daughter and neutral loss scans in the MS/MS mode. The neutral loss scans of 80 and 176 units were used to characterize the sulfate and glucuronide conjugates, respectively. The SRM transitions, cone voltage and collision energy values were optimized for each phenol metabolite to quantify the generated metabolites **(Table 1** of the **Additional Information**).

The metabolites hydroxytyrosol sulfate (sulfHT), hydroxytyrosol glucuronide (glucHT), homovanillic alcohol glucuronide (glucHVAlc) and thymol sulfate (sulfTHY) were quantified by using their own calibration curves. Due to the lack of reference standards, the rest of the metabolites were tentatively quantified with the calibration curves corresponding to their phenolic precursors or similar metabolite compounds. In this sense, the sulfate conjugates derived from hydroxytyrosol acetate sulfate (sulfHTAc), homovanillic alcohol sulfate (sulfHVAlc) and homovanillic acid sulfate (sulfHVac) were quantified with the calibration curve of sulfHT. p-cymene-diol glucuronide (glucCYM) and thymol glucuronide (glucTHY) were tentatively quantified using the calibration curve of sulfTHY. Caffeic acid sulfate (sulfCA) and hydroxyphenylpropionic acid sulfate (sulfHPPA) were tentatively quantified by using the calibration curve of caffeic acid and 3-(4-hydroxyphenyl) propionic acid, respectively. All calibration curves were performed in the corresponding biological matrix (plasma or urine).

2.5 Statistics

A paired Student t-test was employed to compare the post-treatment and baseline values. The changes in concentrations after each treatment of all the phenolic metabolites were compared between VOO, FVOO and FVOOT with an ANOVA analysis followed by a Tukey's multiple-difference test. Simple regression analysis was used to obtain correlation coefficients. The statistical analyses were performed with the Statgraphics plus v. 5.1 software (Manugistics Inc., Rockville, MD).

3. RESULTS

3.1 Phenolic profile and acceptance sensory test of different olive oil samples

Table 1 shows the amount of PC daily ingested with 25 mL of each olive oil. FVOO was mainly enriched with hydroxytyrosol related compounds, the major compound being 3,4-DHPEA-EDA (oleuropein-aglycone di-aldehyde). On the other hand, FVOOT phenolic enrichment provided 50% of the hydroxytyrosol related compounds and the other 50% consisted of different kinds of phenolic groups derived from thyme, with flavonoids being the most abundant, followed by monoterpenes and phenolic acids.

The results from the acceptance test showed that there were no significant overall differences in the opinions of the three olive oils VOO, FVOO and FVOOT, with mean scores of 4.4, 4.0 and 4.6 on a scale from 0 to 6, respectively (**Figure 2**). It is noteworthy that FVOOT was the best-rated oil and was not given the lowest score by any volunteer. These results confirmed that the phenol enrichment did not affect the acceptability of the oils. Therefore, this factor could not interfere in compliance with the intake of the olive oils.

3.2 Identification of the phenol metabolites in urine and plasma

After the sustained intake of the two phenol-enriched olive oils, eleven metabolites were detected and identified in urine and plasma samples, six derived from olive phenolics and five derived from thyme phenolics. **Table 2** shows the retention time, $[M-H]^-$ and MS^2 ions (m/z) of the metabolites. Metabolites M1, M2, M3, M4, M5, M6 were identified as hydroxytyrosol sulfate (sulfHT), homovanillic alcohol sulfate (sulfHVAlc), hydroxytyrosol acetate sulfate (sulfHTAc), homovanillic acid sulfate (sulfHVac), hydroxytyrosol glucuronide (glucHT) and homovanillic alcohol glucuronide (glucHValc) (see the extracted ion chromatograms in plasma and urine and the MS spectrum of each metabolite in **Figure 2** of **Additional Information**). These metabolites had already been identified in human urine samples after a sustained consumption of a phenol-enriched virgin olive oil in our previous study (Serra et al., 2013).

After FVOOT consumption, five main metabolites derived from thyme were identified (Table 2), which eluted at longer retention times,

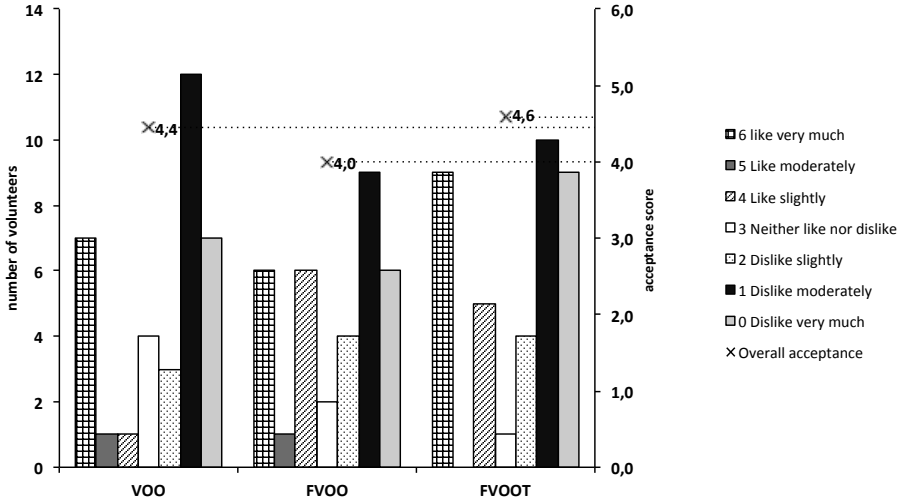


Figure 2. Results from the consumer acceptance test performed to assess the overall opinion of the three olive oils VOO, FVOO and FVOOT. Volunteers had to assign a score on a 7-point scale, ranging from "dislike very much" to "like very much". The scores were converted to whole numbers between 0 and 6 respectively to calculate the means and the standard deviation. The frequency of the acceptance scores and mean values for the

13.34, 15.41, 18.55, 18.66 and 19.55 respectively. M7, M8, M9 and M10 had precursor ions at m/z 245, m/z 259, m/z 229 and m/z 325, and were identified as hydroxyphenylpropionic acid sulfate (sulfHPPA), caffeic acid sulfate (sulfCA), thymol sulfate (sulfTHY) and thymol glucuronide (glucTHY), respectively (see the extracted ion Chromatograms and MS spectrums in **Figure 3** of the **Additional Information**). These metabolites had been previously identified in humans after the oral administration of rosmarinic acid (Baba et al., 2005; Baba, Osakabe, Natsume, & Terao, 2004; Nakazawa & Ohsawa, 1998), and thymol by the oral route (Kohlert et al., 2002), and in rats after the intake of a thyme extract (Rubió, et al., 2012b).

Table 2. Retention time (RT), $[M-H]^-$, and MS^2 ions (m/z) identified in urine and plasma samples after FVOO and FVOOT intake by off-line μSPE-UPLC-MS/MS.

Metabolite	RT (min)	$[M-H]^-$ (m/z)	MS^2 ions (m/z)	Identified metabolite (Abbreviation)	Biological fluids
Olive phenols					
M1	3.81	233	153, 123	Hydroxytyrosol sulfate (sulfHT)*	Plasma, urine
M2	5.49	247	167, 152	Homovanillic alcohol sulfate (sulfHVAlc)**	Plasma, urine
M3	9.94	275	195, 135	Hydroxytyrosol acetate sulfate (sulfHTAc)**	Plasma, urine
M4	5.12	261	181, 137	Homovanillic acid sulfate (sulfHVac)**	Plasma, urine
M5	2.05	329	153, 123	Hydroxytyrosol glucuronide (glucHT)*	Urine
M6	2.99	343	167, 152	Homovanillic alcohol glucuronide (glucHVAlc)*	Urine
Thyme phenols					
M7	13.34	245	165, 121	Hydroxyphenylpropionic acid sulfate (sulfHPPA)**	Plasma, urine
M8	15.41	259	179, 135	Caffeic acid sulfate (sulfCA)**	Plasma, urine
M9	18.55	229	149	Thymol sulfate (sulfTHY)**	Plasma, urine
M10	18.66	325	149	Thymol glucuronide (glucTHY)**	Urine
M11	19.55 and 20.14	341	165	p-cymene-diol glucuronide (glucCYM)**	Urine

* Metabolites identified by standards and fragmentation patterns
** Metabolites tentatively identified by fragmentation patterns

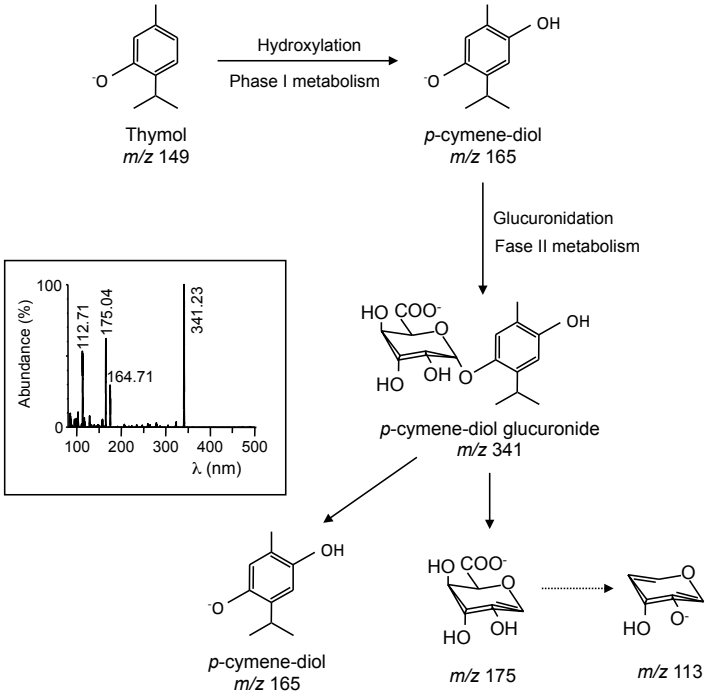


Figure 3. Proposed metabolic route of the metabolite p-cymene-diol glucuronide (341>165) detected in urine samples after FVOOT intake.

M11 appeared to be one of the most abundant and specific metabolites in urine after FVOOT intake. This metabolite, which eluted in two peaks at retention times of 19.55 and 20.14 min, showed an intense ion at m/z 341. This precursor ion formed a major fragment ion at m/z 165 and two more at m/z 113 and m/z 175. The product ion of m/z 165 is related to the loss of the glucuronide moiety (176 units). The other product ions, 113 and at m/z 175, have been reported as characteristic fragment ions derived from the glucuronide moiety (Huang, Niu, & Gu, 2008). The fragment ion of m/z 175 corresponds to the entire glucuronide molecule, and the fragment ion of m/z 113 is obtained after the loss of a water molecule (18 units) and a carboxylic group (CO_2) (44 units) from the m/z 175 ion. Thymol (m/z 149), the main monoterpene from thyme, could have been hydroxylated during phase I metabolism to generate the metabolite p-cymene-diol (m/z 165). The difference between thymol (m/z 149) and the aglycone of this metabolite (m/z 165) is 16 units, corresponding to a hydroxyl group. Thus, this metabolite was glucuroconjugated could have suffered phase II metabolism generating glucCYM (m/z 341). The proposed route for the generation of this metabolite is shown in **Figure 3**. Due to the low MS fragmentation and the long retention time, related to its hydrophobic character, M11 was tentatively identified for the first time as *p*-cymene-diol glucuronide. The two identified compounds were isomers, and the difference between both of them was the position of the glucuronide molecule. The metabolite *p*-cymene-diol generated during phase I metabolism, was previously identified by gas chromatography (GC)-MS in human urine samples after the oral administration of 50 mg of thymol. This shows that human metabolism leads to an hydroxylation of the aromatic ring of thymol (Thalhamer, Buchberger, & Waser, 2011). This metabolite has also been detected in rat urine samples, but only at trace levels (Austgulen, Solheim, & Scheline, 1987). In the present study, *p*-cymene-diol glucuronide was identified for the first time in

Table 3. Phenol metabolites derived from hydroxytyrosol in plasma (μM) and in urine (μmoles/24h urine) at baseline (day 0) and after each olive oil administration (day 21). Values are means ± SD and ranges in parentheses.

	VOO		FVOO		FVOOT	
Plasma	n	Day 0 Day 21	n	Day 0 Day 21	n	Day 0 Day 21
sulfHT	11	1,7 ± 1,21,7 ± 1,0 (0,0 to 3,3)(0,0 to 3,8)	18	1,2 ±1,33,0 ± 2,2 [1] (0,1 to 2,8)(1,3 to 9,8)	17	1,3 ± 1,32,1 ± 1,1 (0,1 to 2,8)(0,1 to 4,2)
sulfHTAc	13	1,6 ± 1,12,0 ± 0,9 (0,0 to 2,9)(0,1 to 3,0)	18	1,2 ± 1,32,8 ± 1,2 [1] (0,1 to 3,3)(2,1 to 6,7)	16	1,6 ± 1,52,3 ± 1,3 (0,0 to 3,8)(0,2 to 4,8)
sulfHVAlc	19	1,3 ± 1,11,4 ± 1,3 (0,2 to 4,0)(0,3 to 4,2)	23	1,4 ± 1,23,2 ± 2,9 (0,0 to 4,4)(0,8 to 11,4)	24	1,4 ± 1,21,9 ± 1,5 (0,0 to 4,2)(0,9 to 5,0)
sulfHVac	13	0,6 ± 0,50,6 ± 0,3 (0,0 to 1,7)(0,0 to 1,2)	15	0,6 ± 0,30,9 ± 0,7 (0,3 to 1,2)(0,2 to 3,0)	14	0,6 ± 0,60,8 ± 0,6 (0,0 to 2,1)(0,2 to 2,3)
24h-urine						
sulfHT	33	6,7 ± 4,86,7 ± 5,1 (0,4 to 18,4)(0,4 to 19,1)	33	5,8 ± 4,514,6 ± 10,8 [2] (0,5 to 19,7)(4,0 to 34,3)	33	5,8 ± 3,79,4 ± 6,0 [2] (0,2 to 15,8)(0,9 to 25,8)
glucHT	16	0,9 ± 1,31,5 ± 2,3 (0,0 to 5,5)(0,0 to 5,3)	15	0,6 ± 1,32,3 ± 2,2 (0,0 to 6,2)(0,1 to 8,7)	14	1,0 ± 1,52,5 ± 3,9 (0,0 to 2,5)(0,1 to 13,8)
sulfHTAc	33	5,2 ± 4,87,1 ± 7,7 (0,3 to 21,2)(0,4 to 29,8)	33	7,8 ± 6,317,0 ± 13,1 [1] (0,8 to 19,8)(6,5 to 55,0)	33	4,6 ± 4,77,8 ± 7,1 [1] (0,7 to 21,2)(1,2 to 22,2)
sulfHVAlc	33	7,0 ± 4,88,0 ± 6,2 (1,5 to 17,0)(0,5 to 26,0)	33	8,6 ± 5,112,8 ± 13,9 (1,8 to 17,5)(1,5 to 55,9)	33	5,8 ± 3,711,4 ± 14,0 (1,3 to 13,5)(0,9 to 39,6)
glucHVAlc	33	6,7 ± 5,78,6 ± 8,3 (1,3 to 25,4)(0,9 to 25,5)	33	7,8 ± 6,114,2 ± 16,8 (1,0 to 30,6)(1,4 to 71,2)	33	7,3 ± 7,911,2 ± 12,1 (1,4 to 27,18)(1,6 to 60,2)
sulfHVac	33	17,7 ± 13,817,6 ± 15,2 (4,0 to 45,3)(3,3 to 60,6)	33	17,5 ± 11,623,5 ± 17,9 (2,7 to 39,7)(5,5 to 85,2)	33	17,9 ± 15,023,0 ±17,4 (1,9 to 32,8)(2,6 to 64,8)

[1,2] Value post-treatment (day 21) is significantly different from baseline (day 0) (paired samples t test): [1] $p < 0,05$; [2] $p < 0,001$
SulfHT, hydroxytyrosol sulfate; sulfHVAlc, homovanillic alcohol sulfate; sulfHTAc, hydroxytyrosol acetate sulfate; sulfHVac, homovanillic acid sulfate; glucHT, hydroxytyrosol glucuronide: glucHVAlc, homovanillic alcohol glucuronide

human urine.

3.3 Quantification of olive phenolic metabolites after sustained intake of FVOO and FVOOT

Table 3 shows the plasma and urinary recoveries of the metabolites derived from the olive phenols detected at the baseline (day 0) and after each olive oil intervention (day 21). All the metabolites detected were derived from HT in its sulfated or methyl-sulfated form. The glucuronide conjugates of HT were only detected in urine, and free HT was undetectable. Olive phenolic metabolites were only detected in the plasma of 24 volunteers after FVOO or FVOOT, whereas all the studied metabolites except glucHT were detected in the urine of all the volunteers.

Despite the wash-out period, all the metabolites were present in the samples taken at the baseline and were also detected after the VOO treatment. Besides, an important inter-individual variability was observed in the urine and plasma values. Only two metabolites, sulfHT and sulfHTAc, presented significantly higher post-treatment values ($p < 0.05$) compared to the baseline in both the plasma and urine after FVOO. Urine recovery of sulfHT was also significantly different when compared to its baseline after FVOOT administration. When comparing changes in concentrations after VOO, FVOO and FVOOT, only sulfHT in urine and plasma and sulfHTAc in urine showed significant differences between the VOO control treatment and FVOO (**Figure 4**). It is worth noting that after FVOOT consumption, the plasmatic and urinary HT metabolites were proportionally higher than expected concentrations, which should be 50% of the concentrations of the respective metabolites after FVOO (**Table 4**).

3.4 Quantification of thyme phenolic metabolites after sustained intake of FVOOT

In contrast to the olive oil metabolites, phenol metabolites derived from thyme were detected in all the plasma and urine samples after FVOOT intake (**Table 4**). All the detected metabolites were markedly higher than its baseline ($p < 0.001$), sulfTHY and sulfHPPA being the metabolites that presented the largest increase in both the plasma and urine. In the plasma, these increased 42- and 37-fold and in the urine, 12- and 30-fold, respectively. Glucuronide conjugates were only detected in the urine, glucCYM being the metabolite with the largest increase post-treatment (172-fold). GlucCYM was also the only phenolic metabolite that reached zero at the baseline.

3.4 Correlations between phenolic metabolites and intake compliance

The volunteers were asked to return the containers after the corresponding olive oil consumption period, (either full, partially empty or empty). This enabled us to measure the volunteers' compliance in the study and further correlate these data with the concentrations of plasmatic and urinary phenolic metabolites. The number of bottles consumed varied from 21 to 15, with mean values for the number of bottles consumed of 20.2, 19.7 and 19.3 for VOO, FVOO and FVOOT, respectively (data not shown). The correlation analysis between the intake compliance, based on the number of bottles consumed per intervention period, and the phenol metabolite concentration in the plasma showed no significant positive correlations in either FVOO or FVOOT (**Table 5**). In contrast, there were significant positive correlation values for some of the urinary phenolic metabolites with both FVOO and FVOOT compliance. HT metabolites were all significantly correlated with FVOO or FVOOT intake, except glucHT and sulfHVac, sulfHT being the metabolite with the highest positive associations ($r = 0.575$; $p<0.01$ for FVOO; $r = 0.539$; $p<0.01$ for FVOOT). The levels of correlation for the urinary metabolites derived from thyme differed depending on the metabolite. sulfHPPA correlated best with FVOOT compliance ($r = 0.608$; $p<0.001$) followed by sulfTHY ($r=0.538$; $p<0.01$) and glucCYM ($r = 0.492$; $p< 0.01$).

4. DISCUSSION

In order to establish firm evidence for the health effects of certain foods or food components, it is critical to control the compliance level of the volunteers participating in the study. In this work, a sensitive and reliable analytical technique enabled us to detect the circulating and excretion metabolic forms of PC after a 3-week sustained intake of two phenol-enriched olive oils as a strategy to monitor the level of compliance during the study. An attempt was made to choose the most appropriate biomarkers from among all the detected metabolites that could be used to monitor the compliance of both

Table 4. Phenol metabolites derived from thyme in plasma (µM) and in urine (µmoles/24h urine) at baseline (day 0) and after each olive oil administration (day 21). Values are means ± SD and ranges in parentheses.

	VOO			FVOO			FVOOT		
Plasma	n	Day 0	Day 21	n	Day 0	Day 21	n	Day 0	Day 21
sulfTHY	23	1,2 ± 1,1 (0,1 to 4,1)	1,0 ± 0,7 (0,1 to 2,3)	25	1,0 ± 0,7 (0,1 to 2,4)	1,1 ± 0,9 (0,1 to 2,2)	33	0,7 ± 0,9 (0,0 to 3,2)	29,7 ± 20,3 [2] (5,9 to 80,4)
sulfCA	4	0,1 ± 0,1 (0,0 to 0,2)	0,5 ± 0,4 (0,0 to 0,5)	4	0,3 ± 0,3 (0,0 to 0,6)	0,9 ± 0,9 (0,0 to 1,7)	24	0,0 ± 0,1 (0,0 to 0,3)	4,2 ± 3,2 [2] (0,4 to 10,5)
sulfHPPA	9	1,5 ± 2,1 (0,0 to 5,5)	0,7 ± 1,1 (0,0 to 2,3)	11	1,1 ± 1,9 (0,0 to 6,6)	2,0 ± 2,9 (0,0 to 7,4)	33	0,8 ± 2,1 (0,0 to 9,1)	29,5 ± 24,4 [2] (4,7 to 93,9)
24h-urine									
sulfTHY	33	39,0 ± 20,4 (13,0 to 81,2)	38,1 ± 28,3 (5,5 to 85,2)	33	36,2 ± 28,5 (2,0 to 85,6)	34,4 ± 30,3 (4,2 to 98,3)	33	48,3 ± 40,3 (6,7 to 108,1)	596,3 ± 388,4 [2] (111,3 to 1469,6)
glucTHY	25	5,3 ± 5,9 (0,0 to 16,6)	7,8 ± 5,3 (0,1 to 21,0)	26	4,5 ± 4,3 (0,2 to 16,9)	4,8 ± 4,0 (0,1 to 14,4)	33	8,3 ± 6,9 (1,4 to 23,0)	41,7 ± 26,0 [2] (11,2 to 100,2)
glucCYM	3	0,1 ± 0,4 (0,0 to 1,7)	0,0 ± 0,1 (0,0 to 0,5)	4	0,2 ± 0,7 (0,0 to 2,7)	0,3 ± 1,4 (0,0 to 7,8)	33	0,4 ± 0,3 (0,0 to 8,8)	68,9 ± 38,9 [2] (6,0 to 206,5)
sulfCA	5	5,2 ± 5,9 (0,0 to 14,3)	3,8 ± 2,3 (0,0 to 8,8)	8	7,0 ± 5,7 (0,0 to 17,5)	5,3 ± 6,0 (0,0 to 15,7)	31	1,4 ± 3,2 (0,0 to 17,6)	15,9 ± 15,4 [2] (4,4 to 86,9)
sulfHPPA	14	19, 2 ± 30,6 (0,0 to 86,7)	19,6 ± 23,0 (0,0 to 68,1)	17	29, 2 ± 37,7 (0,0 to 86,1)	28,7 ± 29,4 (0,0 to 90,3)	33	11,5 ± 13,8 (0,0 to 52,7)	342,5 ± 200,5 [2] (58,9 to 692,2)

[1,2] Value post-treatment (day 21) is significantly different from baseline (day 0) (paired samples t test): [1] $p < 0,05$; [2] $p < 0,001$
sulfTHY, thymol sulfate; sulfCA, caffeic acid sulfate; sulfHPPA, hydroxyphenylpropionic acid sulfate; glucTHY, thymol glucuronide; glucCYM, p-cymene-diol glucuronide.

Table 5. Pearson correlation coeficients (r) between plasma and urine phenol concentration after 21 days of FVOO and FVOOT intake and olive oil compliance (numer of consumed bottles).

	plasma		24h-urine	
	FVOO	FVOOT	FVOO	FVOOT
Olive phenols	r	r	r	r
sulfHT	0,3271	0,0918	0,5752 **	0,5387 **
glucHT	-	-	0,3293	0,3301
sulfHTAc	0,3236	0,0073	0,3460 *	0,3773 *
sulfHVAlc	0,3387	0,0193	0,4033 *	0,3937 *
glucHVAlc	-	-	0,3517 *	0,3428 *
sulfHVac	0,3129	0,0436	0,3001	0,3148
Total	0,2414	0,1194	0,5374 **	0,5011**
Thyme phenols				
sulfTHY	-	0,1910	-	0,5383 **
glucTHY	-	-	-	0.3054
glucCYM	-	-	-	0.4922 **
sulfCA	-	0,2100	-	0,2531
sulfHPPA	-	0,2911	-	0,6075 ***
Total	-	0,2853	-	0,5531 **

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

phenol-enriched olive oils. Despite the blinding design of the study, the selected biomarkers also allowed the analysts to identify the order of intervention of the olive oils, denoting that the proposed analytical methodology is a reliable approach for phenolic biomarker profiling in nutritional intervention studies.

Although there is no consensus about the requirements for useful biomarkers for intake in nutritional studies, according to Spencer et al. (2008) there are some criteria that should be satisfied. The first requirement is the robustness of quantification and identification by sensitive methods. In the present study, the methods used to identify and quantify the metabolites were previously validated (Rubió et al., 2012b; Serra et al., 2013; Suárez et al., 2009) and the UPLC-MS/MS methodology was used. This is considered one of the most reliable approaches for profiling nutrient biomarkers (Puiggròs et al., 2011). Another prerequisite is that concentration of the biomarker of interest should be sensitive to changes in the intake of the dietary component. From this point of view, the results show that olive phenol metabolites appeared to be poor biomarkers after a sustained intake, which could be explained by several reasons. Firstly, it is worth noting that it was a mid-long sustained intake with a daily dose of PC that probably did not enable a clear change after consumption (8.49 mg HT derivates/day with FVOO; 4.30 mg HT derivates/mg with FVOOT). Besides, it is well established that PC from olive oil are rapidly absorbed and cleared (Visioli et al., 2000; Weinbrenner et al., 2004), and plasma concentrations are quite low. Olive oil PC are extensively metabolized in the gut and liver and, thus, they are mainly found in biological fluids as phase II metabolites (Miro-Casas et al., 2003; Miró-Casas et al., 2001). This extensive conjugation and further rapid excretion could also explain the low plasma concentrations and urinary excretion of HT metabolites. Although post-treatment changes in HT metabolites were not remarkable, fasting plasma values and 24 h-urine excretion of sulfHT and sulfHTAc after FVOO were significantly higher than the baseline prior intervention concentrations. Thus, sulfHT and sulfHTAc appeared to be the best candidates for monitoring the intake compliance of FVOO, which apart from showing a significant post-treatment increase both in plasma and urine they presented a significant change compared to VOO in urine (**Figure 4**). Previous studies have described the absorption and excretion of olive oil PC following an acute intake in humans (Miro-Casas et al., 2003; Miró-Casas et al., 2001; Visioli et al., 2000; Vissers et al., 2004; García-Villalba et al., 2010) and attempts to monitor olive oil consumption with biomarkers of intervention compliance have been focused on analyzing the total HT (Covas et al., 2006; Marrugat et al., 2004). However, no studies have been performed to determine the polyphenol metabolites in the biological fluids of individual after a regular intake of olive oil. In this sense, our

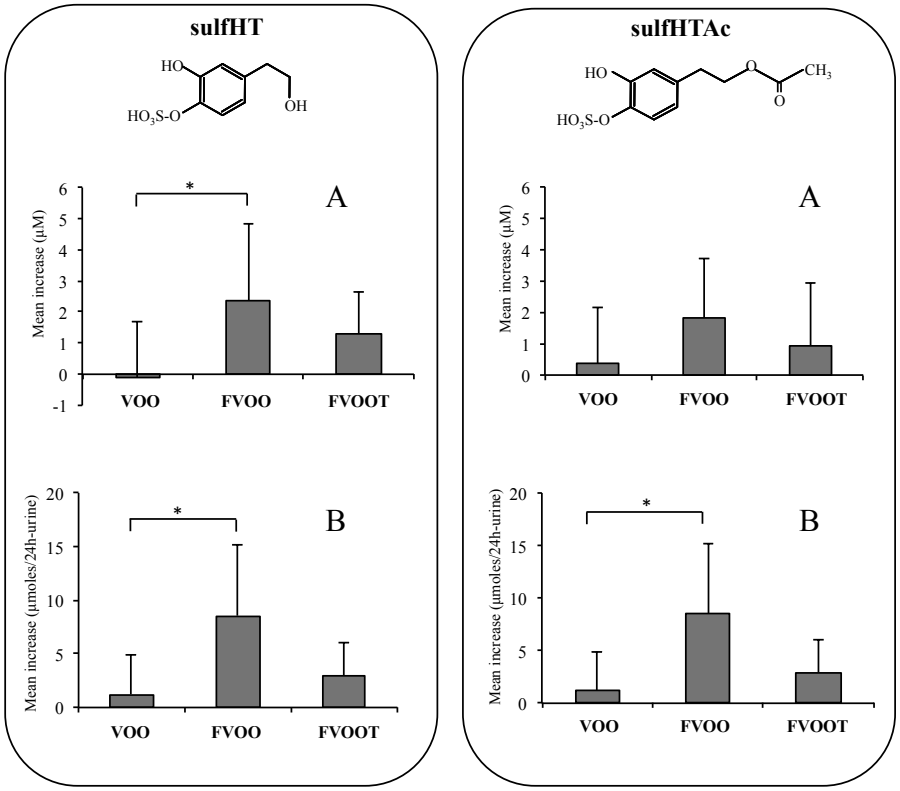


Figure 4. Selected compliance biomarkers of olive oil polyphenol intake. Plasmatic mean increase ± SD (A) and urinary excretion mean increase ± SD (B) of the biomarkers after each olive oil consumption. * indicates significant differences between VOO, FVOO and FVOOT increase values ($p < 0,05$).

study helps to extend the current knowledge about the intake biomarkers of olive phenols by providing more robust and specific biomarkers for compliance.

In contrast to olive phenol metabolites, those derived from thyme were more sensitive to changes after FVOOT intake and appeared to be better biomarkers. Among all the phenolic metabolites derived from thyme, sulfTHY and sulfHPPA appeared to be the best candidates as compliance biomarkers in plasma and urine in terms of increasing changes after treatment. GlucCYM was also a good biomarker in urine as it presented the greatest increase after FVOOT consumption. Urinary glucCYM, sulfTHY and sulfHPPA were also the only metabolites that showed a significant correlation with the monitored olive oil compliance. Based on these criteria, they were defined as the best compliance biomarkers for thyme phenolic intake (**Figure 5**). The extended half-life in plasma and the high urine excretion of thymol conjugates (sulfTHY and glucCYM) could be explained by the high liposolubility of their precursor thymol, which could probably enable a better absorption and accumulation of its conjugates in plasma or tissues. Our previous study in rats showed that thymol was heavily absorbed after an acute administration of a thyme extract, its metabolites also reaching high concentrations in plasma (Rubió et al., 2012b). There was also a significant increase in SulfHPPA after the administration of FVOOT. In rats, this appeared to be one of the most important metabolites derived from thyme and was defined as a product of the rosmarinic acid metabolism (Rubió, Serra, et al., 2012). It has been also reported that flavones and flavanones follow a scheme producing phenylpropionic acids

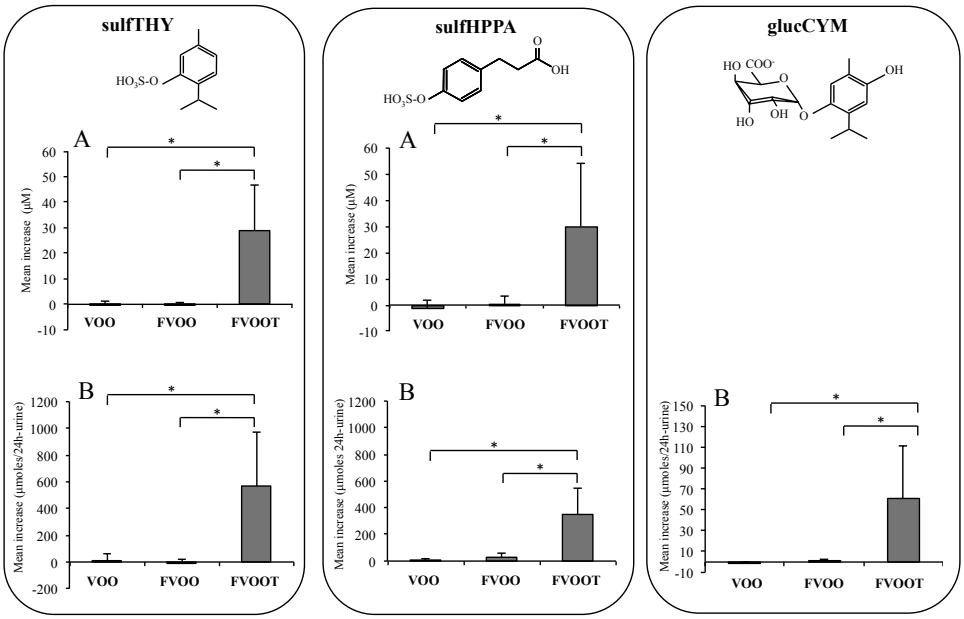


Figure 5. Selected compliance biomarkers of thyme polyphenol intake. Plasmatic mean increase ± SD (A) and urinary excretion mean increase ± SD (B) of the biomarkers after each olive oil consumption. * indicates significant differences between VOO, FVOO and FVOOT increase values ($p < 0,05$).

in the colon (Hollman, 2004). In this sense, the OHPPA sulfate metabolite detected could also be the result of the bacterial metabolism of flavonoids in FVOOT (2.95 mg flavonoids/day), which could be further reabsorbed and conjugated into a sulfated form. No flavonoids were detected in the plasma, either in their native or conjugated forms, suggesting that flavonoids are poorly absorbed and are more likely to suffer an intense metabolism in the colon, which is in line with the most recent studies (Del Rio et al., 2013).

Finally, another essential requirement for the characterization of a metabolite as a potential biomarker of intake is the specificity of the metabolite. According to Spencer et al. (2008) such a metabolite should only appear following the intake of a specific polyphenol without being formed as a product of the metabolism of any other compound either consumed or produced endogenously. In this sense, phenolic metabolites derived from thyme appeared to be more specific, with glucCYM being the most specific one presenting zero baseline levels both in plasma and urine. It is well known that, in addition to being a characteristic component of the phenolic fraction of virgin olive oil, HT is an endogenous compound derived from the oxidative dopamine metabolism and its metabolites are present in virtually all biological matrices of human and animal origin (Edwards & Rizk, 1981; Goldstein, Eisenhofer, & Kopin, 2003). Moreover, wine seems to be another source of HT in our diet (Minuti, Pellegrino, & Tesei, 2006), which seems to be well absorbed after red wine ingestion (De la Torre, Covas, Pujadas, Fitó, & Farré, 2006). Thus, HT metabolites are not specific biomarkers of the intake of olive oil phenols. In this study, the two-week wash-out period lowered their concentrations but it is hard to reach zero concentrations and consequently, changes after intake are hardly detected.

The correlation analysis between urinary phenolic metabolites and the monitored level of compliance, which also helped to choose the most appropriate biomarkers, corroborated that 24-h urine samples are more robust at monitoring daily intake than a spot measurement in plasma since urine provides a measure of the total output of polyphenol metabolites over a 24-h period, thus permitting the detection of the total concentrations

of small- and large-intestinal metabolites (Medina-Remón, Tresserra-Rimbau, Arranz, Estruch, & Lamuela-Raventos, 2012).

When comparing urinary levels of HT metabolites between FVOO and FVOOT consumption, it is noteworthy that although FVOOT contained half the amount of HT derivatives (Table 1), the excretion of its metabolites does not follow the ratio of doses administered, and the concentrations recovered were higher than expected (Table 3). This could indicate that when these PC were ingested together with thyme PC, absorption may be enhanced or bioavailability improved. This phenomenon was previously observed in an in-vitro digestion model where thyme and olive PC were digested together and separately. This showed an increase in the bioaccessibility of HT when it was digested together with thyme PC (Rubió et al., 2014). In keeping with these earlier findings, antioxidant spices such as thyme probably exert a favourable influence on the bioaccessibility of HT derivatives by minimizing their loss during the digestion process. Thus, mixing olive PC with other bioactive substances could be an interesting approach to minimize the low stability of secoiridoid derivatives in the development of functional olive oils (Pinto et al., 2011).

CONCLUSIONS

The analytical methodology used enabled the compliance to be monitored through the determination of biomarkers in human biological fluids after a sustained intake of phenol-enriched olive oils for the first time. The results show that the low specificity of HT metabolites implies certain limitations on the use of these metabolites as potential biomarkers of the intake of olive oil PC. However, sulfHT and sulfHTAc appeared to be suitable metabolites for monitoring the compliance of olive oil PC. On the other hand, sulfTHY, sulfHPPA and glucCYM were shown to be good compliance biomarkers for thyme PC, providing a good indication of thyme phenolic exposure. The fulfillment of the biomarkers of intake criteria denotes the usefulness of the provided intake biomarkers of polyphenols, which guarantees a successful dietary intervention. Despite the blinding design of the study, the selected biomarkers also allowed the analysts to identify the order of intervention of the olive oils, denoting that the proposed analytical methodology is a reliable approach for phenolic biomarker profiling in nutritional intervention studies.

Acknowledgments

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ADDITIONAL INFORMATION

Table 1. Optimized SRM conditions for the analyses of the studied metabolites by UPLC-MS/MS

Compound	SRM 1 (Quantification)	Cone voltage (V)	Collision energy (eV)	SRM 2 (Identification)	Cone voltage (V)	Collision energy (eV)	Standard Available
Hydroxytyrosol sulphate	233 > 153	40	15	233 > 123	40	20	Yes
Alcohol homovanillic sulphate	247 > 167	40	15	247 > 152	40	25	No
Hydroxytyrosol acetate sulphate	275 > 195	35	15	195 > 135	35	10	No
Homovanillic acid sulphate	261 > 181	40	15	261 > 137	40	25	No
Hydroxytyrosol glucuronide	329 > 153	40	20	329 > 123	40	25	Yes
Homovanillic alcohol glucuronide	343 > 167	40	20	343 > 152	40	30	Yes
Hydroxyphenylpropionic acid sulphate	245 > 165	35	15	165 > 121	35	20	No
Caffeic acid sulphate	259 > 179	35	15	179 > 135	35	20	No
Thymol sulphate	229 > 149	40	20	229 > 134	40	30	Yes
p-cymene diol glucuronide	341 > 165	40	25	- - -	- - -	- - -	No

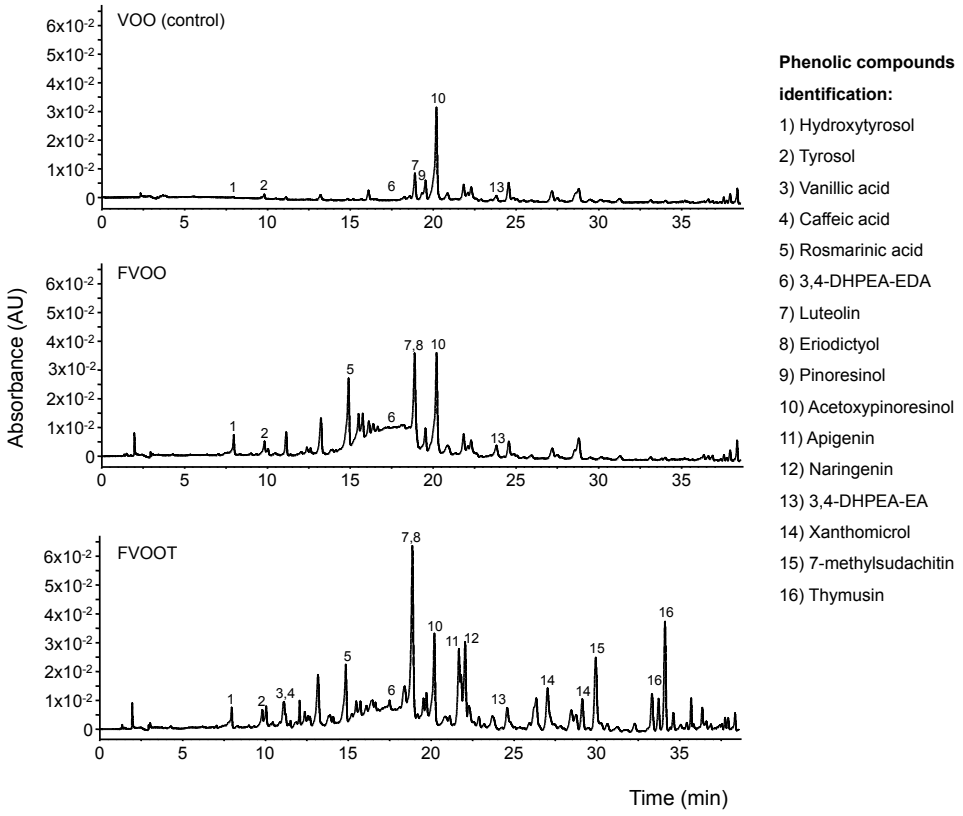

Figure 1.HPLC-DAD (at 278 nm) Representative chromatograms of the three olive oils

ADDITIONAL INFORMATION

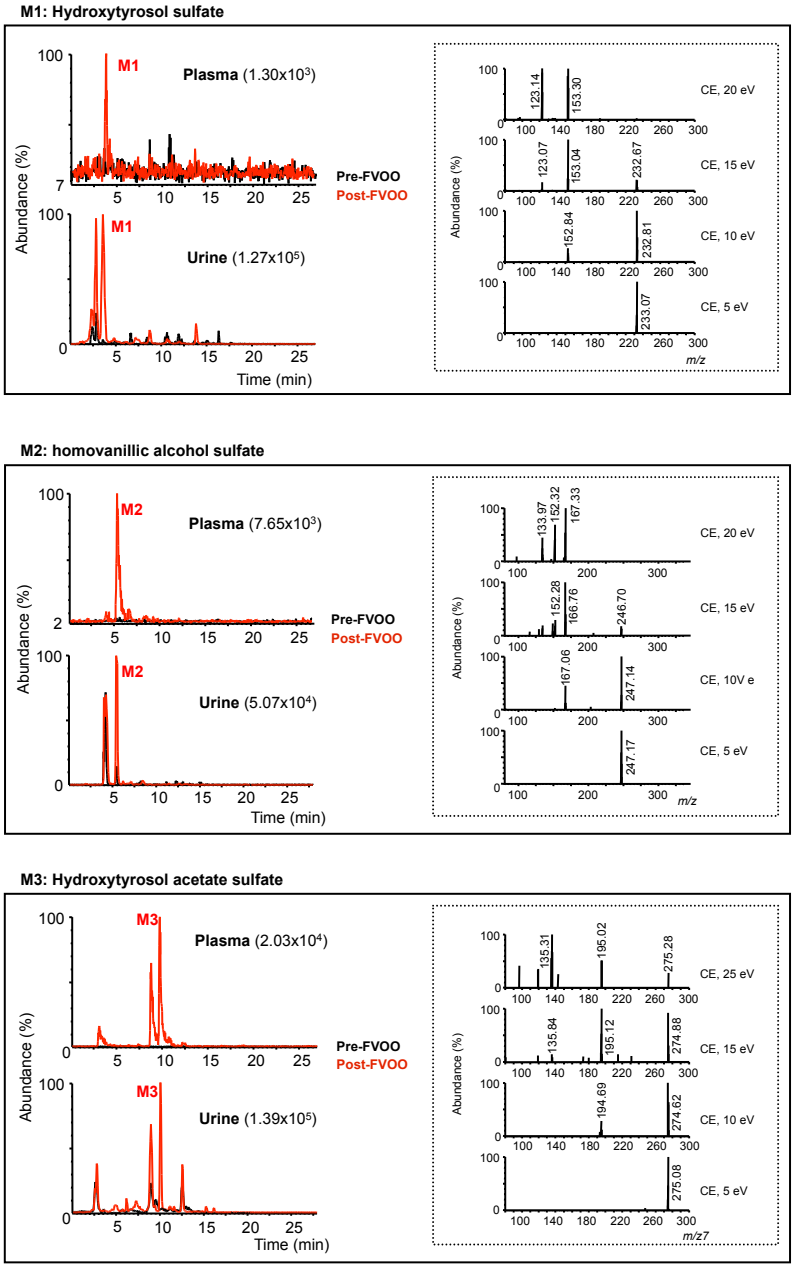

Figure 2. Extracted ion chromatograms in plasma and urine and the MS spectrum of phenolic metabolites derived from hydroxytyrosol before and after FVOO intervention.

ADDITIONAL INFORMATION

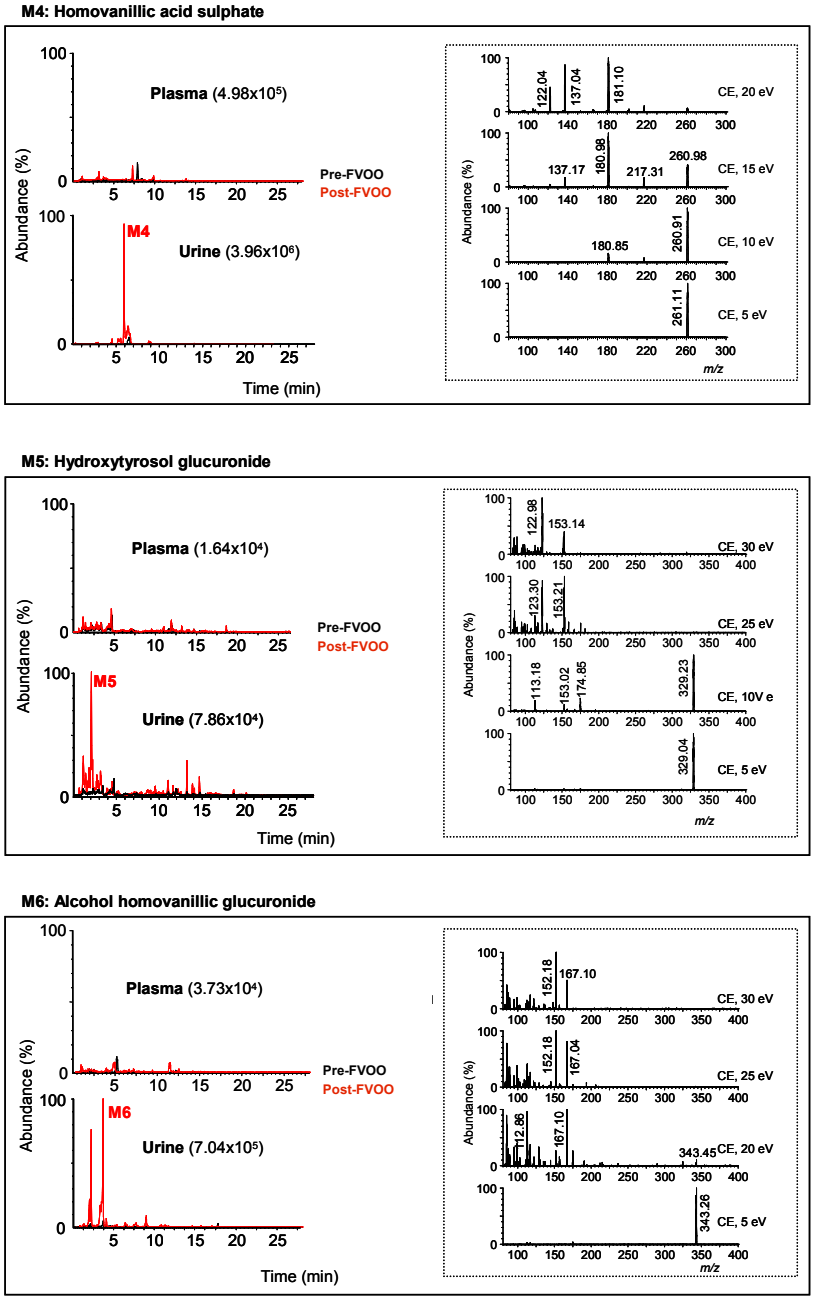

Figure 2 (continuation) Extracted ion chromatograms in plasma and urine and the MS spectrum of phenolic metabolites derived from hydroxytyrosol before and after FVOO intervention.

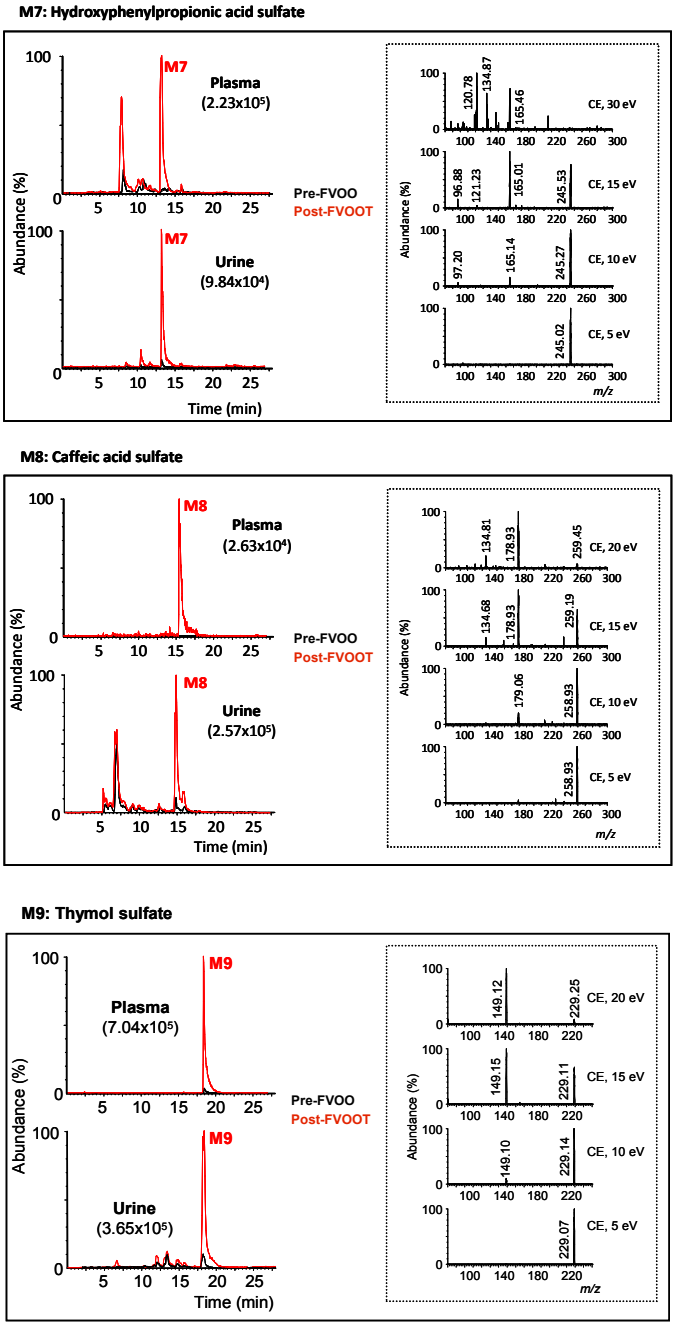

Figure 3. Extracted ion chromatograms in plasma and urine and the MS spectrum of phenolic metabolites derived from thyme before and after FVOOT intervention.

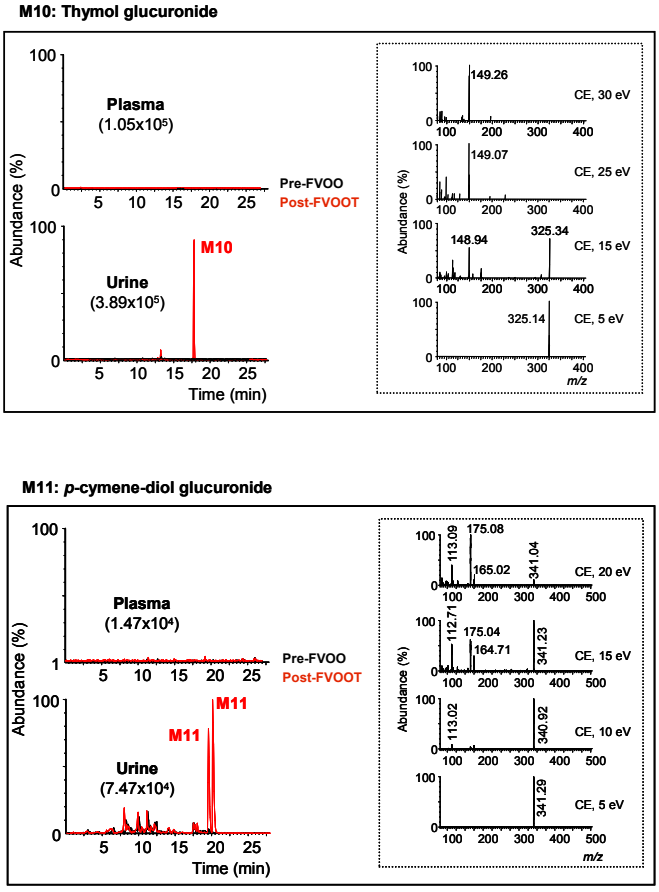

Figure 3 (continuation) Extracted ion chromatograms in plasma and urine and the MS spectrum of phenolic metabolites derived from thyme.

Publication X.

Endogenous antioxidant system and antioxidant stress biomarkers modulation after a sustained intake of phenol-enriched olive oils in hyperlipidemic patients: VOHF study

In preparation

ENDOGENOUS ANTIOXIDANT SYSTEM AND OXIDATIVE STRESS BIOMARKERS MODULATION AFTER A SUSTAINED INTAKE OF PHENOL-ENRICHED OLIVE OILS IN HYPERLIPIDEMIC PATIENTS: VOHF STUDY*

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Abstract

Phenolic compounds can modulate target functions related to oxidative stress. In this study, the effect of a well-established dose of phenolic compounds (PC) from virgin olive oil (mainly hydroxytyrosol derivatives) and PCs from thyme (flavonoids, monoterpenes and phenolic acids) on levels of endogenous antioxidants and oxidative stress biomarkers has been investigated in humans. For this proposal, 33 hyperlipidemic subjects consumed 25 mL/day of 3 types of olive oil: virgin olive oil control (VOO: 2.88 mg PC/day); VOO enriched with its own PC (FVOO: 12.59 mg PC/ day); and VOO enriched with its own PC and complemented with thyme PC (FVOOT: 12.10 mg PC/ day) during 3 weeks in a randomized, controlled crossover study (VOHF study). Samples of plasma and 24h-urine were obtained before and after each interventional period. To evaluate the antioxidant status, the concentration of endogenous plasma antioxidants retinol, tocopherols and carotenoids were determined. Additionally, several oxidation markers were measured: oxidized LDL, apolipoprotein B and methionine sulfoxide in plasma, and F2alpha-isoprostanes and 8-hydroxydeoxyguanosine in 24h-urine. Results of the study showed that the concentration of lutein and α-tocopherol in plasma were significantly increased in response to both FVOO and FVOOT intake, in comparison to the control olive oil (VOO) ($p = 0.010$), being significantly major the increase for FVOOT. Retinol and β-cryptoxanthin were also significantly increased compared to the control but only after FVOOT intake. In relation with oxidation markers in urine, 8-hydroxydeoxyguanosine significantly decreased after the intervention FVOO intervention when compared to VOO group indicating a protection effect against oxidative damage to DNA. From these results we concluded that the administered phenolic doses had no pro-oxidant effects, independently of its origin. In addition, the increase in plasmatic fat-soluble micronutrients suggests that PC provided with a sustained intake of phenol-enriched olive oils could optimize the endogenous antioxidant status and that the combination of olive and thyme phenolics might have a greater effect compared to the single olive phenols administration.

* *This work has been made in collaboration with Pharmacology Unit and Lipids & Atherosclerotic Research Unit of Universitat Rovira i Virgili (URV). In the present document the complete table of results is presented but we only include the discussion part made on plasma fat-soluble endogenous antioxidants results, which have been analyzed in our laboratory.*

INTRODUCTION

Olive oil is a food item typical of the Mediterranean diet and several studies have revealed that it has a unique phenolic profile with interesting biological properties (Martin-Peláez et al., 2013). Results from the European EUROLIVE study (Covas et al., 2006) showed that olive oil consumption promotes an increase in plasma HDL cholesterol and a decrease in oxidative damage in a direct relationship with the polyphenol content of the olive oil administered. Based on this and other studies, the EFSA has released a health claim concerning the protective effects of the ingestion of phenolic compounds (PC) from olive oil declaring that "Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress" (EFSA, 2011). The EFSA Panel considers that in order to support the claim, 5 mg of hydroxytyrosol (HT) and its derivatives in olive oil should be consumed daily. However, the phenolic content in commercial virgin olive oils (VOOs) is influenced by multiple agronomic and technological factors. As a result, phenolic concentration in most VOOs available on the market may be too low to allow the daily consumption of 5 mg of HT and its derivatives within the context of a balanced diet (20 g of VOO/day). In this context, the enrichment of VOO with its own phenolic compounds becomes an interesting strategy to increase and standardize the daily intake of HT in the real food matrix and without increasing the caloric intake. Moreover, the enrichment of VOOs with phenolic compounds of thyme was used for improving the nutritional profile and sensorial characteristics of the VOO and to assess possible synergies between different phenolic groups.

An imbalance of oxidants and antioxidants within the human body, in which either oxidants are high or antioxidant protection is low, will lead to a state of oxidative stress and it has been shown that oxidative stress is involved in over 100 diseases, as their cause or consequence (Halliwell et al. 1992; Gutteridge, 1993). Therefore, the measurement of the antioxidant status of biological fluids is used as an early warning sign of possible disease onset and also as an indicator of improvement of the endogenous antioxidant system. Currently there are two major ways to estimate antioxidant status of biological fluids: (i) direct measurement of levels of antioxidants in plasma (vitamins, phytochemicals, enzymes), and (ii) measurement of oxidative stress biomarkers in plasma or urine (lipid peroxides, malondialdehyde, isoprostanes, etc.). (Rabovsky et al., 2006).

In this context, we sought to investigate the effect of a well-established dose of phenol-enriched virgin olive oils on levels of plasma endogenous antioxidants (fat-soluble micronutrients) and oxidative stress biomarkers in plasma and urine, processes currently considered relevant for cardiovascular disease (CHD) in mildly dyslipidemic patients. In this sense, 33 hyperlipidemic subjects participated in the study and consumed 25 mL/day of 3 types of olive oil: virgin olive oil as a control (VOO: 2.88 mg PC/ day); VOO enriched with its own PC (FVOO: 12.59 mg PC/ day); and VOO enriched with its own PC and complemented with thyme PC (FVOOT: 12.10 mg PC/ day) during 3 weeks in a randomized, controlled crossover study.

MATERIAL AND METHODS

Olive oils preparation and characterization

VOO with a low phenolic content (80 mg total PC/ kg oil) was used as a control condition in the intervention and as an enrichment matrix for preparation of two phenol-enriched olive oils (FVOO and FVOOT) with a total phenolic content of 500 mg total PC/kg oil. FVOO1 was enriched with its own phenolic compounds, mainly hydroxytyrosol derivatives, by adding a phenol extract obtained from freeze-dried olive cake. FVOO2 was enriched with its own phenolic compounds and complemented with thyme phenols using a phenol extract made up of a mixture of olive cake and dried thyme leaves. Hence, FVOO2 contained 50% of olive PC (hydroxytyrosol derivates) and 50% of thyme phenolics (flavonoids, phenolic acids and monoterpenes) (**Table 1**). The procedure for obtaining the phenolic extracts and enriched oils was previously developed in our laboratory (Rubió et al., 2012). For the wash-out period, a commercial commom olive oil (blend of refined

and a small percentage of virgin olive oil) kindly provided by Borges Mediterranean Group was used. The total PC content of the olive oils was measured with the Folin-Ciocalteu method (Vázquez-Roncero, Janer del Valle & Janer del Valle, 1973). The phenolic profile of the olive oils was analyzed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) (Rubió et al., 2012). Tocopherols and fatty acids in olive oils were analyzed following the procedure described by Morelló et al. (2004) and the carotenoid content was analyzed as described before by Criado et al. 2008.

Human intervention study design

The study was a randomized, double-blind, crossover, controlled trial with 33 hypercholesterolemic volunteers (total cholesterol > 200 mg/dL) (19 men and 14 women), aged 35 to 80. Exclusion criteria included the following: BMI>35 Kg/m^2, smokers (>1 cigarette/day), athletes with high physical activity (>3000 Kcal/day), diabetes, multiple allergies, intestinal diseases, or other disease or condition that could impede compliance with the measurements or treatment. The subject flowchart is displayed in **Figure 1**.

The subjects were randomized to one of three orders of administration of 25 mL/day of (1) raw virgin olive oil (VOO), (2) virgin olive oil enriched with its own PC (FVOO1), and (3) virgin olive oil enriched with its own PC plus complementary phenols from thyme (FVOO2) (Figure 1). In the crossover design, the intervention periods were 3 weeks long with a daily ingestion of 25 mL (23 g) of raw olive oil distributed between meals. Prior to the interventions, there was a 2-week wash-out period where common olive oil was the main fat consumed. To avoid an excessive intake of antioxidants, such as PC, during the clinical trial period, the participants were advised to limit their consumption of polyphenol-rich foods. A 3-day dietary record was administered to participants before and after each intervention period to control their diet throughout the study. A set of portable containers with the corresponding 25 mL of olive oil for each day of consumption were delivered to the participants at the beginning of each olive oil administration period. The participants were instructed to return the containers to the center after the corresponding olive oil consumption period in order to register the amount of olive oil consumed in the period. 24-h urine was collected in containers. Blood samples were collected in fasting state and plasma samples were obtained by centrifugation

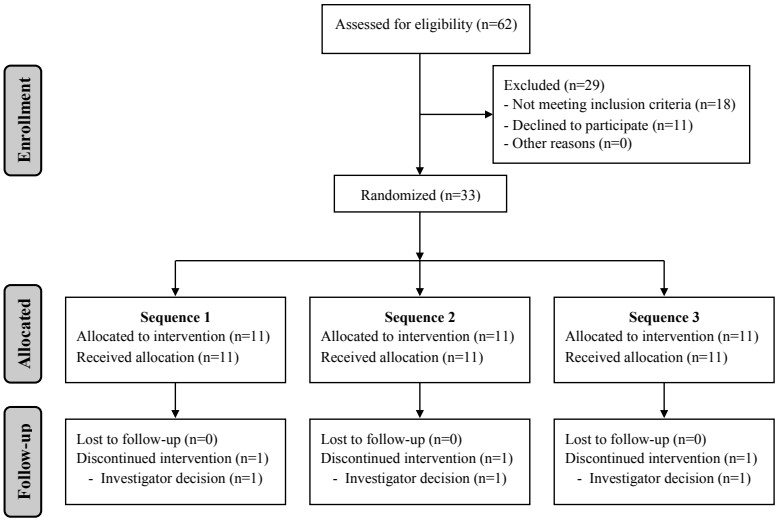

Figure 1. Subject flowchart. Data from all subjects for whom baseline and follow-up measurements were available were included in the analysis.

of whole blood directly after being drawn. Urine and plasma samples were stored at -80ºC until use. This protocol has been approved by the Local Ethics Committee (CEIC-PSMAR 2009/3347/I) and is registered in the International Standard Randomized Controlled Trial register (www.controlled-trials.com; ISRCTN77500181).

Plasma fat-soluble endogenous antioxidants extraction and chromatographic analysis

All sampling procedures were performed under low ambient light conditions. For extraction, 300uL of plasma was added to 300uL of ethanol containing internal standard (α-tocopherol acetate 100mg/L) and BHT (0.063%). After vortex mixing, the mixture was extracted twice with 900uL of n-hexane. Samples were centrifuged at 2000rpm for 5 min and the upper phases were carefully transferred to another tube. The obtained hexane phases were completely evaporated at room temperature under stream of nitrogen. The residue was re-dissolved in 75ul of methanol and analyzed in the HPLC the same day of extraction. The HPLC system was made up of a Waters 717 plus Autosampler, a Waters 600 pump, a Waters 996 Photodiode Array Detector and a Waters 2475 Fluorescence Detector managed by Empower software (Waters Inc., Milford, MA). A 150x4.6 mm i.d. YMC C30 analytical column (3µm) was used for the separation of all components and HPLC analysis was performed following the procedure of Gleize et al. 2007. Briefly, the mobile phase consisted of a gradient of methanol (A), methyl tert-butyl ether (B) and water (C). Flow rate was 1 mL/min. The gradient profile of the mobile phase (A:B:C) was set at 96:2:2 and changed linearly to 18:80:2 in 27 min, and then it changed back to 96:2:2 from 31 to 35 min. Carotenoids (lutein, β-crypthoxanthin and β-carotene) were detected at 455 nm, vitamin A (retinol) at 325nm and alfa-tocopherol acetat (IS) at 290 nm. γ- and α-tocopherol were detected using fluorescence detector with an excitation wavelenght of 285 nm and emission wavelenght of 325nm. All compounds were identified by their retention time compared with pure standards, or, when

Table 1. Characteristics of the participants at the begin of the study.

	Sequence 1 (n=11)	Sequence 2 (n=11)	Sequence 3 (n=11)	*p*
Gender, male/female	7/4	7/4	5/6	
Age, years	55.45 ± 7.84	55.18 ± 11.88	54.91 ± 12.57	
Body weight, kg	84.45 ± 17.74	74.60 ± 18.49	74.75 ± 16.80	
BMI, kg/m^2	27.85 ± 4.71	26.33 ±5.29	25.63 ± 3.68	
SBP, mm Hg	130 (106 – 166)	128 (96 – 151)	125 (104 – 153)	
DBP, mm Hg	72 (44 – 90)	72 (52 – 85)	68 (52 – 101)	
Glucose, mg/dL	90.91 ± 10.53	93.00 ± 13.33	88.55 ± 11.63	
Total colesterol, mg/dL	218.82 ± 82	231.91 ± 32.70	228.36 ± 42.70	
LDL colesterol, mg/dL	142.45 ± 25.64	152.00 ± 28.45	150.80 ± 34.08	
HDL colesterol, mg/dL	53.39 ± 9.55	52.96 ±12.82	52.78 ±11.75	
Tryglicerides, mg/dL	115.82 ± 32.49	134.36 ± 60.53	126 ± 86.68	
Plasma Oxidative biomarkers				
Oxidized LDL, U/L†	40.57 (35.6-56.7)	44.15 (42.4-56.2)	40.57 (36.6-49.3)	0.284
APO B, g/L	1.15 ± 0.2	1.17 ± 0.17	1.08 ± 0.16	0.529
Methionine, µM	19.48 ± 2.11	19.64 ± 1.68	20.36 ± 2.52	0.593
Methionine sulfoxide, µM	1 ± 0.24	1 ± 0.27	0.99 ± 0.14	0.986
Urine Oxidative biomarkers				
F2alpha-isoprostanes, µg/L	0.35 ± 0.24	0.50 ± 0.21	0.59 ± 0.23	0.060
8-Hydroxydeoxyguanosine, nM†	9.94 (8.1-13.7)	12.15 (9.7-18.1)	14.67 (10.1-18.3)	0.611
Plasma antioxidants				
Retinol, µM	18.06+ ± 4.69	19.64 ± 3.34	21.68 ± 3.05	0.103
beta-Carotene, µM†	4.81 (2-6.8)	4.21 (3.3-8.2)	5.49 (3.3-7)	0.920
beta-Cryptoxanthin, µM†	7.44 (6.8-8.8)	12,47 (9.6-14.9)	9.87 (7.7-11.9)	0.064
Lutein, µM	5.61 ± 4.31	5.76 ± 2.77	4.62 ± 1.71	0.654
alpha-Tocopherol, µM	16.74 ± 1.95	17.37 ± 1,17	16.83 ± 1.08	0.575
gamma-Tocopherol, µM	0.12 ± 0.05	0.19 ± 0.06	0.16 ± 0.07	0.063

Values are expressed as means ± SD; † Median (25^{th}-75^{th} percentile)
Sequence 1= control olive oil, FOO1 and FOO2; Sequence 2= FOO2, control olive oil and FOO1; Sequence 3= FOO1, FOO2 and control olive oil. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein; APO, apolipoprotein

Table 2. Changes in Outcome Measurements after Interventions

	Olive Oil Intervention							
	VOO		**FVOO**			**FVOOT**		
	Post-intervention	Change from Preintervention	Post-intervention	Change from Preintervention	p value	Post-intervention	Change from Preintervention	p value
Plasma Oxidative biomarkers								
Oxidized LDL, U/L†	1.60 ± 0.11	-5 (-8.67 to -1.34)	1.60 ±0.10	-2.49(-7.11 to 2.12)		1.61 ± 0.13	-1.68 (--7.14 to 3.77)	
APO B, g/L	1.08 ± 0.18	-0.005 (-0.058 to 0.048)	1.07 ±0.17	-0.038 (-0.1 to 0.024)		1.08 ± 0.21	0.02 (-0.029 to 0.069)	
Methionine, µM	20.57 ± 3.97	-0.45 (-1.96 to 1.87)	19.90 ±4.49	0.67 (-1.39 to 2.74)		19.56 ± 2.60	-0.16 (-1.22 to 0.89)	
Methionine sulfoxide, µM	1.21 ± 0.56	0.23 (-0.022 to 0.48)	1.20 ±0.34	0.18 (0.037 to 0.33)		1.08 ± 0.28	0.11 (-0.01 to 0.23)	
Methionine sulfoxide in Total Methionine, %								
Urine Oxidative biomarkers								
F2alpha-isoprostanes, µg/L	0.49 ± 0.30	0.048 (-0.08 to 0.18)	0.45 ±0.27	0.031 (-0.14 to 0.20)		0.46 ± 0.22	0.001 (-0.11 to 0.11)	
8-Hydroxydeoxyguanosine, nM†	1.11 ± 0.27	3.19 (-0.06 to 6.44)	1 ±0.25	-2.63 (-6.18 to 0.92)a	a=0.04	1.03 ± 0.23	1.01 (-2.39 to 4.42)	
Plasma fat-soluble antioxidants								
Retinol, µM	18.18 ± 3.52	-2.67 (-4.3 to -1.04)	20.23 ±4.85	-0.38 (-2.14 to 1.38)		22.32 ± 4.58	2.37 (0.94 to 3.81)a,b	a<0.01 b=0.046
beta-Carotene, µM†	0.63 ± 0.37	-0.25 (-1.49 to 0.99)	0.60 ±0.42	0.20 (-1.08 to 1.48)		0.74 ± 0.34	1.78 (-0.37 to 3.93)	
beta-Cryptoxanthin, µM†	0.99 ± 0.15	0.1 (-0.55 to 0.77)	1.05 ±0.20	0.85 (-0.62 to 2.31)		1.08 ± 0.18	2.28 (1.15 to 3.41)a	a=0.005
Lutein, µM	4.19 ± 1.90	-1.28 (-2.11 to -0.44)	4.83 ±2.23	0.20 (-0.11 to 0.51)a	a=0.005	5.87 ± 3.07	1.15 (0.33 to 1.97)a,b	a=0.01 b=0.014
alpha-Tocopherol, µM	16.56 ± 1.46	-0.42 (-0.72 to 0.12)	17.53 ±2.13	0.57 (0.03 to 1.11)a	a=0.01	18.59 ± 2.29	2.10 (1.08 to 3.12)a,b	a<0.01 b=0.014
gamma-Tocopherol, µM	0.17 ± 0.09	0.01 (-0.027 to 0.045)	0.18 ±0.09	0.033 (-0.01 to 0.07)		0.18 ± 0.11	0.008 (-0.04 to 0.06)	

Values are expressed as means ± SD and †Median (25th-75th percentile) for Postintervention and means (95% CI) for Changes from Preintervention.
a: p<0.05 compared to control; b: p<0.05 compared to FVOO

unavailable (lutein, β-cripthoxanthin), with compounds purified and gently provided by Dr. Gandul-Rojas research group (Minguez-Mosquera et al. 1992), which concentrations were determined by spectrophotometry using the molecular extinction coefficient (ε) of the molecule. For the plasma quantification of each analyte, five-point standard curves were constructed with stock solutions prepared individually with appropriate solvents (correlation coefficients <0.99).

RESULTS

Plasma fat-soluble endogenous antioxidants

To determine whether supplementation with phenol-enriched olive oils in hyperlipidemic patients modulates endogenous antioxidants, plasmatic concentration of tocopherols, vitamin A and carotenoids were determined. Results of the plasma analysis showed that the concentration of lutein and α-tocopherol were significantly increased in response to phenolic supplementation of both FVOO and FVOOT in comparison to the control olive oil (VOO) ($p = 0.010$), being significantly major the increase for FVOOT compared to FVOO for both lutein and α-tocopherol ($p = 0.014$). Retinol and β-cryptoxanthin were also significantly increased compared to the control but only after FVOOT intake. Other lipid-soluble antioxidants (β-carotene and γ-tocopherol) did not change significantly after olive oils consumption (**Table 2**).

DISCUSSION

The present study demonstrated that a sustained consumption of phenol-enriched olive oils with both olive and thyme phenolic compounds was associated with an increase in the concentration of several plasmatic fat-soluble vitamins. This increase could be attributed to the extra supplementation of phenolic compounds with the enriched olive oils (FVOO and FVOOT), as the three administered oils had the same fatty acid profile and similar content of α-tocopherol and carotenoids (lutein, β-cryptoxanthin, β-carotene) (**Table 1 Suplemmental information)**. The increase in the plasma concentration of α-tocopherol is especially interesting, because it is an antioxidant with well-characterized direct antiplatelet and antithrombotic effects (Abdala-Valencia et al. 2012; Da Costa et al. 2013; Freedman et al. 1996). The modest but significant increase observed in plasmatic α-tocopherol after the intervention period with both phenol-enriched olive oils is in accordance with previous studies reported by other authors in which they also found an increase in the plasma concentration of total α-tocopherol after consumption of grape-derived products rich in polyphenols (Freedman et al. 2001; Castilla et al. 2006). These authors suggested that phenolic compounds might protect LDL from oxidation either directly or indirectly by sparing vitamin E. Also in line with this hypothesis, flavonoids have been also shown to decrease the membrane lipid peroxidation with a consequent prevention of vitamin E loss in in vitro studies (Viana et al. 1996; Facino et al. 1998). In a previous mentioned study (Castilla et al. 2006) the increase in plasma of vitamin E was related to a parallel significant decrease in oxidized LDL in vivo. In our study, a modest but not significant decrease in oxLDL was observed after the consumption of the three olive oils. However, this decrease was not statistically significant when phenol-enriched olive oils (FVOO and FVOOT) were compared to the control oil (VOO). This could be related to the administration of a virgin olive oil as a control, which also contained a certain amount of PC (2,9 mg PC/25 mL oil/day) and probably did not allow detecting significant differences with the phenol-enriched olive oils FVOO and FVOOT (12,6 and 12,1 mg PC/25 mL/day, respectively). It could also be possible that a longer period of time would be necessary to observe an effective protection against lipid peroxidation.

Apart from α-tocopherol, other fat-soluble endogenous antioxidants in plasma (retinol, lutein and β-cryptoxanthin) increased after phenol supplementation with phenol-enriched olive oils in comparison to VOO, which could be also related to the prevention of plasmatic antioxidants loss in response to phenolic compounds absorption, as observed with vitamin E. It is remarkable that FVOOT showed a greater increase in fat-plasma endogenous antioxidants compared to FVOO, which might indicate that phenolic supplementation with thyme phenolics could exert a major benefit in promoting the antioxidant plasmatic status. In fact, as reported in our previous study in which the phenolic metabolites were determined in plasma and urine of the participants in the VOHF study (Rubió et al.

2014a), we observed that the concentration of the main phenolic metabolites reached in fasting plasma after the sustained intake of FVOOT was much higher compared to FVOO. In this sense, hydroxytyrosol sulfate, the main metabolite detected in plasma after FVOO intake, reached a concentration of 3,0 ± 2,2 μmols/L, and thymol sulfate, the main metabolite detected in plasma after FVOOT ingestion, reached a concentrations of 29,7 ± 20,3 μmols/L. This higher absorption of thyme PC compared to hydroxytyrosol and its derivates from olive could explain the differences between phenolic metabolites concentration in plasma, which was also confirmed in a previous study using a rat model (Rubió et al. 2014b). Therefore, the larger concentration of these phenolic metabolites in plasma could have lead to the greater increase in plasma endogenous antioxidants observed after FVOOT intervention.

Although an improvement in oxLDL and other plasma oxidative biomarkers was not observed after the phenol-enriched olive oils in comparison to control oil, the increased presence of endogenous antioxidants in plasma after the sustained intake of phenol-enriched olive oils might cause other beneficial effects modulating different biological pathways. Regarding α-tocopherol, in a previous large cohort study it was demonstrated that men in the higher quintiles of serum α-tocopherol had significantly lower risks of total and cause-specific mortality than did those in the lowest quintile (Wright et al. 2006). One of the primary biological functions of α-tocopherol is that of a chain-breaking antioxidant nutrient; as such, it protects lipids in membranes and lipoproteins from free radical damage, thereby limiting DNA mutation and oxidative modification of lipoproteins (Ricciarelli et al. 2001). α-Tocopherol may also affect human health through the inhibition of protein kinase C activity, which plays an important role in cell proliferation, adhesion, immune responses, and gene expression (Ricciarelli et al. 2001), or through the direct modulation of genes involved in growth, apoptosis, and inflammation (Azzi et al. 2004). Because these processes contribute to atherogenesis, the lower mortality from CVD in men with higher α-tocopherol concentrations was postulated to be biologically plausible.

In conclusion, the increase in plasmatic fat-soluble micronutrients suggests that PC provided with a sustained intake of phenol-enriched olive oils could optimize the endogenous antioxidant status and that the combination of olive and thyme phenolics might have a greater effect compared to the single olive phenols administration.

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SUPPLEMENTAL INFORMATION

Table 1. Chemical characterization of the olive oils used in the study. VOO (Virgin Olive Oil; 80 mg phenols/kg oil), FVOO (Functional Virgin Olive Oil enriched with its own phenols; 500 mg total phenols/kg oil) and FVOOT (Functional Virgin Olive Oil enriched with both its own phenolics (50%) and phenolics from thyme (50%); 500 mg phenols/kg oil). Phenolic compounds and fat-soluble micronutrients are expressed as means ± SD of mg /25 mL oil/day. The acidic composition is expressed as relative area percentage.

	VOO	FVOO	FVOOT
PHENOLIC COMPOUNDS (mg/25 mL/day)			
hydroxytyrosol	0,01± 0,00	0,21± 0,02	0,12± 0,00
3,4-DHPEA-AC	n.d.	0,84± 0,06	0,39± 0,04
3,4-DHPEA-EDA	0,04± 0,00	6,73± 0,37	3,43± 0,29
3,4-DHPEA-EA	0,26± 0,04	0,71± 0,06	0,36± 0,03
Total HT derivates	0,30	8,49	4,30
p-hydroxybenzoic acid	n.d.	0,02± 0,00	0,06± 0,00
vanillic acid	n.d.	0,07± 0,00	0,13± 0,01
caffeic acid	n.d.	0,00± 0,00	0,06± 0,00
rosmarinic acid	n.d.	n.d.	0,41± 0,03
Total phenolic acids	-	0,09	0,65
thymol	n.d.	n.d.	0,64± 0,05
carvacrol	n.d.	n.d.	0,23± 0,02
Total monoterpenes	-	-	0,86
luteolin	0,04± 0,00	0,18± 0,02	0,21± 0,02
apigenin	0,02± 0,00	0,06± 0,00	0,10± 0,00
naringenin	n.d.	n.d.	0,20± 0,02
eriodictyol	n.d.	n.d.	0,17± 0,01
thymusin	n.d.	n.d.	1,22± 0,09
xanthomicrol	n.d.	n.d.	0,53± 0,06
7-methylsudachitin	n.d.	n.d.	0,53± 0,09
Total flavonoids	0,06	0,23	2,95
pinoresinol	0,05± 0,00	0,12± 0,00	0,10± 0,05
acetoxipinoresinol	2,47± 0,19	3,66± 0,31	3,24± 0,28
Total lignans	2,52	3,78	3,34
FAT SOLUBLE MICRONUTRIENTS (mg/25 mL/day)			
α-tocopherol	3,27± 0,01	3,40± 0,02	3,44± 0,01
lutein	0,05± 0,00	0,06± 0,00	0,06± 0,00
β-cryptoxanthin	0,02± 0,00	0,03± 0,00	0,02± 0,00
β-carotene	0,01± 0,00	0,02± 0,00	0,02± 0,00
FATTY ACIDS (relative area %)			
Palmitic acid	11,21	11,20	11,21
Stearic acid	1,92	1,92	1,92
Araquidic acid	0,36	0,36	0,36
Behenic acid	0,11	0,11	0,11
Total saturated	13,75	13,74	13,75
Palmitoleic acid	0,70	0,70	0,69
Oleic acid	76,74	76,83	76,75
Gadoleic acid	0,27	0,27	0,27
Total monounsaturated	77,71	77,80	77,72
Linoleic acid	7,43	7,36	7,43
Timnodonic acid	0,36	0,36	0,35
Linolenic acid	0,43	0,43	0,43
Total polyunsaturated	8,22	8,15	8,22

GENERAL DISCUSSION

As presented in the objectives, the overall aim of the VOHF project which this thesis is framed in was to assess whether phenol-rich olive oils, enriched both with their own phenolic compounds or with these plus complementary phenols from thyme, could act as nutraceuticals concerning the *in vivo* quantity and quality (functionality) of the human high density lipoproteins (HDL) *and* their *in vivo* protective effects on oxidative damage. To accomplish this aim two steps were necessary: 1) to develop functional olive oils enriched with their own and other natural phenolic compounds by tailoring the quality and quantity of total polyphenols for the best taste and bioavailability; and 2) to assess the human efficacy of the phenolic compounds from the phenol-enrich olive oils developed. The activities carried out during this thesis have mainly focused on the first step and, to a lesser extent, on the human interventional study, in which the phenolic compliance markers were defined and biomarkers related to the protective effect on oxidative damage were also determined. The assessment of the HDL functionaly modulation is still in progress. An overview of the results obtained is presented and discussed in this “General discussion”section.

1.1 Preparation of phenol-enriched olive oils

Following the lessons learned by the European Food Safety Authorization (EFSA) for the evaluation of health claims, three different aspects have to be assessed to justify a health claim:

(i) Characterization of the food or ingredients
(ii) Relevance of the sustained effect claimed
(iii) Scientific evidence provided by efficacy studies

The first criterion establishes that the food constituent for which the health effect is claimed needs to be sufficiently characterized in terms of identity and content. This step is one of the most crucial prior to the efficacy studies. Although many

of the functional ingredients (vitamins, minerals, omega-3 fatty acids, etc.) possess reliable methodologies of quantification, this aspect is sometimes incomplete for plant extracts or vegetal foods. In some cases, there is no reference to the contents of the bioactive compounds, the part of the plant used for the preparation, no indication of daily doses, or if the extracts are used as multi-botanical combinations without specifying the other components. One of the main reasons for this lack of concretion is the complexity of plant matrices, whose diversified secondary metabolism includes a vast number of different compounds with similar chemical structures that can be hard to identify. So, in order to obtain standardized extracts or food products and also to understand their mechanism of action, the application of validated methodologies of identification, analysis, and quantification of individual bioactive components is required by the EFSA.

One of the most important phytochemicals present in plant extracts with a well-documented bibliography related to health effects is the subject of our interest, namely the phenolic compounds. In the context of this thesis, the phenolic sources used in the development of the phenol-enriched olive oils were, on the one hand, olive cake to provide the oil with enrichment of its own phenolic compounds, and on the other, thyme was the candidate to provide the oils with flavour and complementary phenolic compounds. A wide range of aromatic herbs was considered, but thyme was finally chosen as it is one of the most common and well-accepted aromatic herbs in the Mediterranean area. Moreover, according to the literature (Yanishlieva et al. 2006; Viuda-Martos et al. 2007; Vallverdú et al. 2014), thyme provides a wide range of different kinds of phenolic compounds (flavonoids, phenolic acids and phenolic terpenes).

The first step for preparing the phenol-enriched olive oils was to select the best strategy for transferring the phenolic compounds from the thyme to the oil. Two strategies were testes for the process of flavouring the olive oil with thyme, one

based on the traditional infusion of the thyme leaves in the oil, and another based on the preparation and application of phenolic extracts prepared from thyme leaves. The results showed that good transference ratios for the phenolic compounds derived from thyme were obtained with both methods. However, when the infusion was applied, high temperature and long times (40-50ºC, 48 h) were required to obtain the same phenolic enrichment, which could jeopardise the quality of the prepared oil. From this first study (data not shown), we concluded that working with phenolic extracts instead of infusions, as is traditional when preparing flavoured olive oils, allows a phenol enriched and seasoned olive oil to be developed with exactly the amount of phenols required, which is an important factor when a functional food is produced, and which also does not endanger the quality of the product.

Once the strategy of phenol enrichment with extracts was adopted, the process for obtaining phenolic extracts was carried out using a method based on the solvent accelerated extraction developed previously in our laboratory (Suárez et al. 2009a), and which was effectively applied to olive cake phenol extraction. In this case, it was used for the first time to extract the phenolic compounds of an aromatic herb (thyme) in combination with olive cake, which also resulted to be successfully applied. The phenolic extracts obtained were then characterized using a chromatographic method based on HPLC-MS/MS previously validated in our lab. This allowed us to identify and quantify a broad range of phenolic compounds in the extracts, such us phenolic acids, phenyl alcohols, lignans, secoiridoid derivatives and flavonoids. Regarding the characterization of the thyme phenolics, HPLC-MS/MS again appeared to be a reliable tool for the structural elucidation of the phenolic compounds in such a complex sample, and thus our study provides a comprehensive identification of thyme phenolic profile, which was lacking in the literature mainly due to the wide variety of structures involved. The more volatile compounds in thyme (phenolic monoterpenes) were further characterized in the extracts and enriched olive oils

using GC-FID, which also appeared to be a good methodological approach for identifying and quantifying these compounds.

When mixing both sources (olive cake and thyme leaves) in the preparation of the phenolic extracts during the extraction process, a protective effect of the olive phenols in the presence of thyme was observed. Thus, our results suggest that a new strategy for preparing phenolic extracts based on a mixture of different phenolic groups could be an interesting approach for increasing and better preserving the bioactive compounds.

The flavonoids appeared to be one of the major phenolic groups supplied by thyme. In this sense, thyme clearly contributes to complementing the lack of flavonoids in the olive cake extract. Rosmarinic acid, an hydroxycinamic acid derivate, was also detected in large amounts in the thyme extract, which appears to be of high interest as a therapeutic agent due to the two catechol groups in its structure and its antioxidant potential (Kelsey et al. 2010; Rubió et al. 2013). Apart from the flavonoids and phenolic acids, it was further elucidated that thyme extract also provided bioactive compounds of the phenolic terpene group, specifically thymol and carvacrol. As it will be discussed in the next section, the *in vivo* experiments performed with thyme showed that monoterpenes have demonstrated a high bioavailability, their metabolic structures reaching high concentrations in plasma and urine.

Once the phenolic extracts had been characterised, the enrichment process was carried out using different proportions of both sources, olive cake and thyme, and evaluating the transference ratios of different phenolic groups from the extract to the oil. From this study, it was concluded that the optimal enriched olive oil with the most balanced phenol composition appeared to be the one prepared with the phenolic extract obtained from 60% of olive cake and 40% of thyme, providing the highest amount of olive phenolic compounds (mainly secoiridoid derivatives) and thyme phenolics (mainly flavonoids and phenolic

acids). One of the aims of flavoring with thyme was also to reduce the bitterness and pungency, leading to higher consumer acceptance. In this sense, our results confirmed that phenol-enriched olive oils combining olive and thyme extracts had lower bitterness than those enriched only with olive oil phenols, indicating that the use of herbs or spices may improve consumer acceptance of high-phenol olive oils (**Figure 18).**

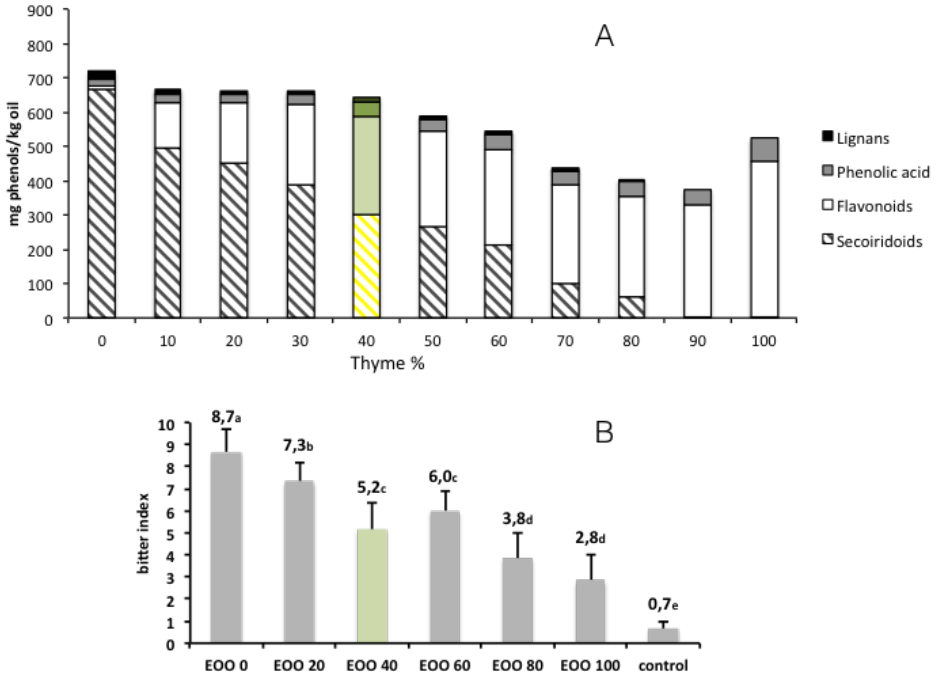

Figure 18. A: Distribution of phenolic groups after the enrichment of the olive oils prepared with phenolic extracts with different proportions of thyme and olive cake. **B**: Bitter sensory analysis of the enriched oils. Highlighted in color the phenol-enriched olive oil with the most balanced composition and with a light-moderate bitter score.

In summary, our study supports the original aim of developing a phenol-enriched olive oil tailored to provide the best relationship between different groups of phenolic compounds by combining different proportions and types of phenolic sources. Moreover, this study revealed that working with the direct addition of phenolic extracts instead of the traditional infusion process could be a feasible for implementing in the food industry and could allow phenol enriched and seasoned olive oils to be developed with exactly the amount and nature of phenols required. Moreover, an appropriate method for the phenolic characterization of the enriched olive oils enables to the exact nature of its active phenolic compounds to be measured, which is an essential step prior to determining of its biological efficacy.

1.2 Studies of phenol digestibility, bioavailability and metabolism by *in vitro* and *in vivo* models

As reported by Kay et al. (2010), establishing the biological activities of phenolic compounds depends on a full understanding of their intake, absorption and metabolism. After reviewing the most recent research related to the efficacy of phenolic compounds, it has been postulated that one of the most important shortcomings in the intervention studies with phenolic compounds stems from a lack of standardized biomarkers of intake and the study of the pharmacokinetics. In fact, the EFSA (2011) established that "*in order to have the food constituent well characterized it is necessary to show that this constituent is bioavailable and provide a rationale of how the constituent reaches the target site*". For these reasons, in order to evaluate the stability during digestion and potential bioaccessibility and bioavailability of the phenolic compounds of interest in this study and to establish valid biomarkers of intake, several *in vitro*

and *in vivo* experiments were carried out before the evaluation of the efficacy of the developed phenol-enriched olive oils.

Primarily, a dose-response study in humans was performed (Rubió et al. 2012 P-III). Understanding the dose-response relationship of a compound or group of compounds is a fundamental aspect of research into the early stages of the product development (Ruberg 2005), as one may only wish to know, "*Is there any evidence of effect?*" and, "W*hat is the optimal dose according to the effect and the pharmacokinetic response?*" This study followed a multidisciplinary approach, and the aim was to evaluate the bioavailability of the phenolic compounds from similar virgin olive oils (VOOs) enriched with the full spectrum of their phenolic compounds, but differing in their total phenolic content and to assess whether very high doses of phenolic compounds could be absorbed in a dose-dependent way in humans. The pharmacokinetic parameters of the phenolic metabolites in humans were assessed after the oral ingestion of 30 ml of three different phenol-enriched virgin olive oils (FVOO) with a phenolic content of 250 (L-FVOO), 500 (M-FVOO), and 750 mg (H-FVOO) total phenols/ kg, which provided 2.2, 6.3 and 10.9 mg of hydroxytyrosol and derivates, respectively.

Certainly, we observed a dose-dependent response of phenol conjugates in the human systemic circulation after the acute intake of the three olive oils. However, the pharmacokinetics of the hydroxytyrosol metabolites did not show a complete linear response, as observed in the C_{max} values of hydroxytyrosol sulfate as the main plasma metabolite, reaching concentrations of 1.35, 3.32, and 4.09 μmol/l for L-FVOO, M-FVOO, and H-FVOO respectively. The minor differences observed between the M-FVOO and H-FVOO C_{max} values could be explained by a saturation of the transport systems involved in the intestinal efflux or the conjugating enzymes, a possibility that has been postulated for polyphenols by other authors when high doses are administered (Hu, Chen & Lin, 2003; Liu et

al. 2007). Additionally, the sensory evaluation of the three oils indicated that the phenol-enriched olive oil with the medium dose (M-FVOO, 500 mg/kg) scored between ‘‘like it a bit’’ (score 4 out of 6) and ‘‘neither like nor dislike’’ (score 3 out of 6), a satisfactory level of consumer acceptance considering that it contained 5 times the phenol content of a standard VOO, whereas the olive oil with the highest dose (H-FVOO, 750 mg/kg) received much lower consumer acceptation (**Figure 19**). Considering both dose-pharmacokinetic and sensory acceptance results, M-FVOO, with a content of 500 mg total phenols/kg, appeared to have an appropriate phenol-enrichment, as it had both a good pharmacokinetic response and a good sensory acceptability.

In parallel to the pharmacokinetic evaluation of the phenolic compounds, the dose-effect of the three olive oils was assessed by measuring the *in vivo* endothelium-dependent vasodilation, which the EFSA considers a biologically valid biomarker related to the maintenance of an adequate blood flow to body cells and tissues (EFSA 2012). The endothelial-dependent vasomotor function was measured as ischemic reactive hyperemia (IRH) at the baseline and at 4h and 6h after each olive oil intake. The results showed that the IRH time-course increased in a linear trend after L-FVOO and M-FVOO ingestion from the baseline to 6 h postprandial (p=0.012 and p=0.005), indicating an improvement in the endothelium-dependent vasodilatation (**Figure 19**). It is noteworthy that compared to L-FVOO and H-FVOO, only M-FVOO presented a sustained increase in IRH, as it increased significantly first at 4h and continued until 6h postprandial. In a recent scientific opinion on the substantiation of a health claim related to cocoa flavanols, the EFSA considered that a sustained increase in endothelium-dependent vasodilation in fasting conditions in response to a regular or acute intervention represents a beneficial physiological effect (EFSA 2012). So, according to the IRH behaviour, the M-FVOO prepared in the context of this doctoral thesis also appeared to be the optimal phenolic dose (publication in preparation).

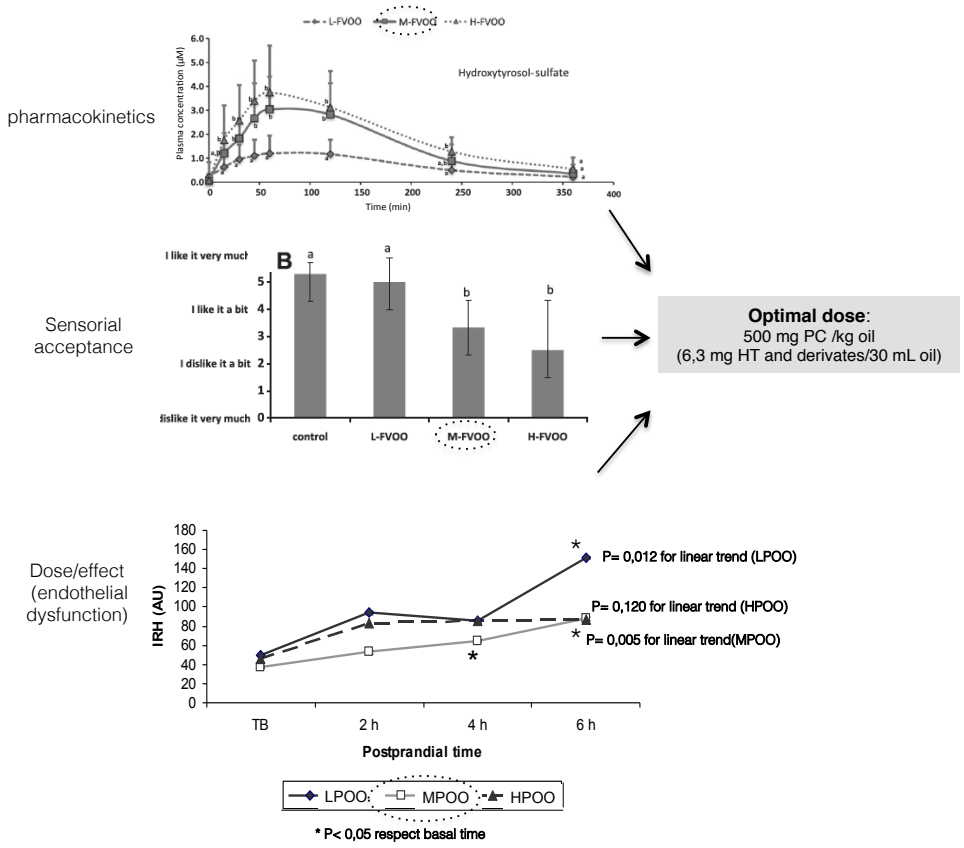

Figure 19. Criterion used to decide the optimal dose of phenolic compounds in the enriched olive oils for the human clinical efficacy trial.

To sum up, it was concluded from the dose-response study that the olive oil enriched with 500 mg total phenols/kg oil which provided 6.3 mg of hydroxytyrosol and derivates (30 ml), appeared to be the optimal phenolic dose considering the pharmacokinetic, sensory and dose-effect results **(Figure 19**). Accordingly, this phenol enrichment level was used later in the sustained human intervention study (see section 1.3).

It is important to highlight that in the framework of this thesis, we also reported progress in the study of the olive oil phenolic metabolites in humans, as a new hydroxytyrosol metabolite called *hydroxytyrosol acetate sulfate* was identified in plasma (Rubió et al. 2012 P-II). The metabolic fate of olive phenolic compounds has been a subject of study by the scientific community to find out the mechanisms through which they exert their activity in the organism. Thus, sensitive and selective chromatographic methods to determine these compounds in human plasma have been optimized by various authors (De La Torre-Carbot et al. 2007; Suárez et al. 2009b). The importance of these compounds has been reinforced by the demonstration that they can help to prevent the oxidation of low density lipoprotein (LDL) (Marrugat et al. 2004; EFSA 2011). In the present doctoral thesis, the determination of the phenolic metabolites in plasma samples was based on their ion fragmentation in the mass spectrometry (MS) and tandem MS modes. In the dose-response study, two main metabolites were quantified in all the volunteers' plasma as a result of the olive oil intake. The most abundant peak corresponded to hydroxytyrosol sulfate, which was identified and defined as one of the most important olive oil phenolic metabolites in a previous work performed by our group (Suárez et al. 2011). The other main plasmatic metabolite, which presented the same pharmacokinetic profile, had to be studied in greater depth to be identified. Its pharmacokinetics and the comparison of its MS spectrum with the standard of the native molecule (hydroxytyrosol acetate), finally led us to finally confirm the appearance of hydroxytyrosol acetate sulfate as a new metabolite never previously described in plasma after the ingestion of VOO. After this identification, we proposed the most feasible metabolic pathway based on the acetylation of hydroxytyrosol (**Figure 20**). Considering that a great amount of hydroxytyrosol is released after the ingestion of phenol-enriched olive oil as a consequence of the hydrolisis of secoiridoids during digestion, we suggested that acetylation of hydroxytyrosol pathway was more feasible than the direct sufation of hydroxytyrosol acetate, as

this compound appeared at a very low concentration in the oil compared to secoiridoids. The acetylation of hydroxytyrosol has never been described, but due to the presence of acyltransferase enzymes, which have previously been related to drug metabolism (Levsen et al. 2005), and the high amount of hydroxytyrosol released form the hydrolisis of the main olive oil secoiridoid (3,4-DHPEA-EDA), we postulated that *O*-acetylation could possible be a metabolic transformation, in parallel with other well-known phase II metabolism reactions.

To sum up, from this exploratory approach we showed for the first time that hydroxytyrosol acetate sulfate is one of the main biological metabolites of hydroxytyrosol formed after the olive oil phenol ingestion. This is a step forward in the metabolic fate of olive oil phenolics research.

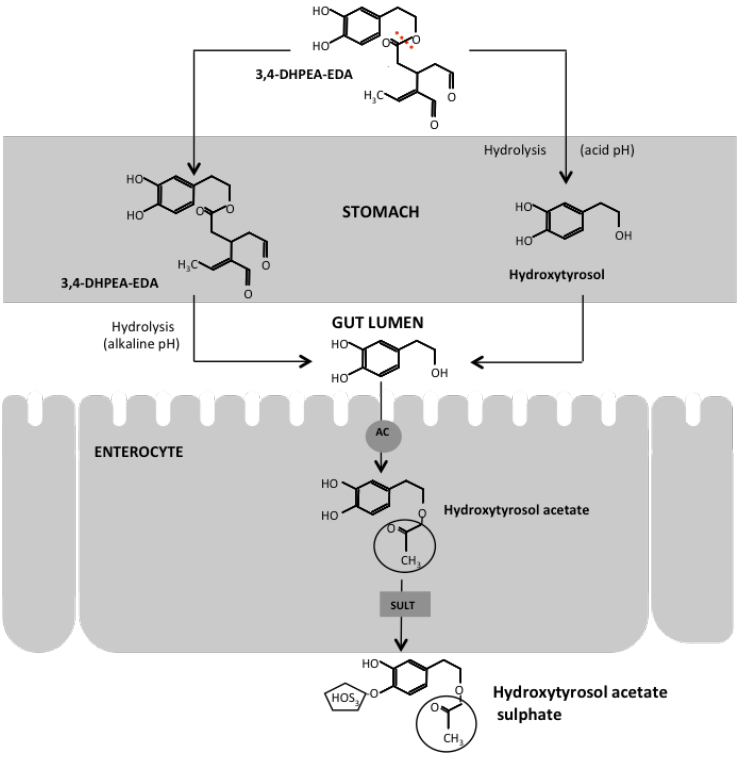

Figure 20. Proposed pathway for the new identified metabolite hydroxytyrosol acetate sulfate as a result of phase II conjugation metabolism in the enterocytes by acyltransferase enzymes (AC) and further sulfation by sulphotrasferase enzymes (SULT). Firstly, hydrolysis of 3,4-DHPEA-EDA occurs either in the stomach or in the gut lumen and a great amount of hydroxytyrosol is released.

Once the metabolites derived from olive oil phenolic compounds had been well characterized in plasma (Suárez et al. 2008; Rubió et al. P-II; Rubió et al. P-III), one of the aims of this thesis was to study the metabolic fate of thyme phenolic compounds, which was scarcely described in the literature. Our aim was to develop and validate a rapid, selective and sensitive analytical method to identify and quantify the metabolites generated after the intake of thyme phenolic compounds as a preliminary phase to understanding the future potential benefits of these components from thyme. Taking into account the short life of phenolic metabolites in plasma, the study was carried out during a postprandial state to enable their detection and quantification. For this purpose, twelve rats were intra-gastrically gavaged with a thyme phenolic extract at a dose of 1.5 g kg^{-1} body weight and sacrificed at different times from 1 h to 6 h. Additionally, a control group of rats (n=10) was maintained under fasting conditions (**Figure 23**).

In order to identify the thyme phenolic metabolites in plasma, the analytical separation technique used was ultra-performance liquid chromatography (UPLC) coupled to tandem MS, and microelution solid-phase extraction (μSPE) plates as the pretreatment technique. The analytical method was validated in terms of linearity, calibration curves, precision, accuracy, recoveries and sensitivity using the commercial standards of the most representative phenols from thyme. Besides the use of very sensitive and robust methodologies and taking into account the relatively low sensitive of the full-scan mode of the ESI-MS/MS detector, the targeted metabolomics require detailed knowledge of the potential analytes that could be detected after the ingestion of the phenolic compounds. Due to the lack of commercial standards of phenolic metabolites, and the complexity and high cost of their chemical synthesis, carrying out an acute intake study with experimental animals (rats in our case) allows valuable biological samples with high concentration of phenolic metabolites to be obtained. These biological samples (plasma, in this case) were very useful for

identifying and tentatively quantifying the metabolites generated after the intake of thyme phenolic compounds. In this study, ten metabolites were identified in the plasma samples, most of them derived from rosmarinic acid and the monoterpene thymol. The native forms of the thyme phenolic compounds were not detected in plasma, indicating that thyme compounds were absorbed and rapidly metabolized into sulfate and glucuronidate conjugates, similarly to what was observed with olive oil phenols. The most abundant thyme metabolites generated were hydroxyphenylpropionic acid sulfate and thymol sulfate, whose concentrations in plasma appeared in the mM range. The phenolic composition of the thyme extract administered revealed that rosmarinic acid was the major phenolic compound in the extract, and therefore most of the metabolites detected in plasma could be linked to its metabolism. Similarly, thymol sulfate and thymol glucuronide were also quantified in plasma as the main monoterpene metabolites from thyme. Given this, metabolic pathways were proposed for both thymol (**Figure 21**) and rosmarinic acid metabolites (**Figure 22**).

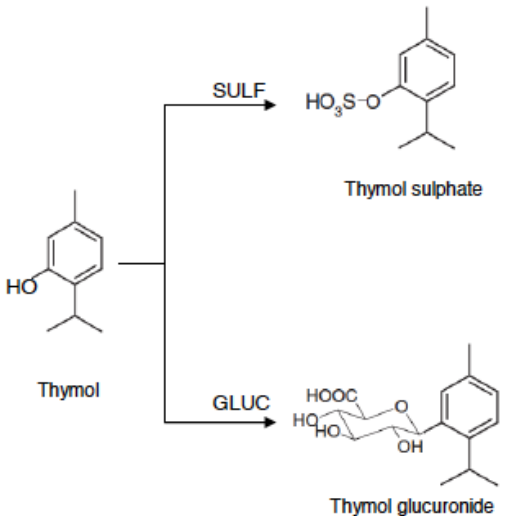

Figure 21. Proposed pathway for thymol metabolites detected in plasma.

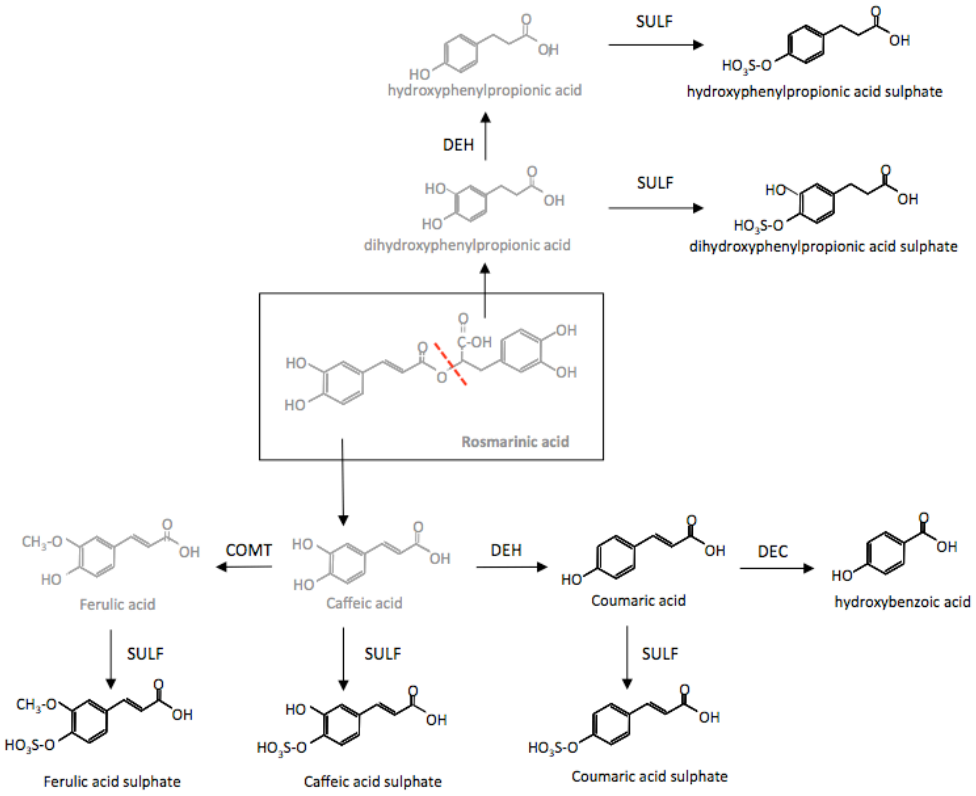

Figure 22. Proposed metabolic pathway of rosmarinic acid. DEC, decarboxylation; SULF, sulfate conjugation; GLUC, glucuronide conjugation; DEH, *p*-dehydroxylation; COMT, methylation.

Apart from enabling the identification of the metabolites and elucidating the metabolic pathways, another advantage of performing acute intake studies with phenolic compounds is that high concentration found of their conjugated forms can be isolated from biological fluids in sufficient quantities to be used as standards, representing, to some extent, an alternative to synthesizing conjugate metabolites. This novel approach, which was also recently reported by Radko et al. (2013) for the isolation of resveratrol metabolites, can also be

used to test their bioactivity instead of their aglycone forms, as they represent the physiological compounds found in tissues after ingestion. In our case, thymol sulfate, the main metabolite found after the intake of thyme extract, was isolated from the plasma. The acute intake of the extract in rats enabled this metabolite to be isolated in large enough quantitites for the circulating thymol sulfate to be quantified more accurately, and it was also well preserved at -80ºC for further use as a standard in our next studies. Thymol sulfate has not previously been isolated from any biological source or synthesized and it is therefore considered to be a novel compound.

To determine the bioavailability of polyphenols in intervention studies, their concentration is usually calculated via the area under the curve from multiple blood sampling over a period of time, or urinary excretion of phenol metabolites, which is generally consistent with plasma kinetic data. It is thought that urine samples (the most common is 24h-urine) offer advantages over plasma measurements, mostly because they allow an accurate evaluation of the total polyphenols absorbed. Urine samples are mainly appropriate for phenols with short plasma half-lives, where plasma measurements may fail to monitor the phenolic intake (Medina-Remón et al. 2012). In this sense it is also important to develop rapid and reliable methodologies to be able to analyze urinary phenolic intake biomarkers in human intervention studies. Despite being one of the easiest biological samples to collect, urine contains high concentrations of different salts that can easily interfere with the chromatographic analysis and increase the sample matrix effect. Therefore, it is essential to remove them from the sample matrix before the chromatographic analysis. The urine pretreatment techniques reported in the literature until now are off-line liquid-liquid extraction (LLE) with ethyl acetate (Del Boccio et al. 2003; Garcia-Villalba et al. 2010; Miro-casas et al. 2001) and off-line solid-phase extraction (SPE) with hydrophilic lipophilic balanced (HLB) (60 mg) cartridges (Khymenents et al. 2011; Bazoti et al. 2005). In order to expand the analytical methods reported in the literature for

determining phenolic compounds and their generated metabolites in urine, it is extremely important to develop novel approaches to improve the quality parameters of the analytical method, such as speed, robustness, sensitivity, and selectivity. For this reason, in this thesis, two sample pretreatment strategies were proposed, which were dried urine spot cards and microelution SPE plates (μSPE). The methods were developed and validated for the determination of hydroxytyrosol and its metabolites in spiked human urine samples and were then applied and compared in human 24h-urine after a sustained consumption of a phenol-enriched virgin olive oil. After the optimization of the conditions, both strategies presented good quality parameters, and compared with conventional LLE and SPE with cartridges, both can be considered as improved methodologies as smaller volumes of urine sample and extraction solvent are required.

Over recent years, the applications for dried spot cards have increased significantly, and a range of applications has been reported for analyzing biomarkers and drugs in different biological samples, such as blood and urine (Spooner et al. 2009; Mercolini et al. 2010; Barfield et al. 2011; Nirogi et al. 2012). Technologically, the validation of dried spot cards as a sample pre-treatment for phenolic metabolite analysis represents a step beyond in the actual sample pretreatment methodologies, since it has never been used for this purpose. Compared with μSPE, it presents some advantages, as it is a simple, inexpensive and highly stable (up to 7 days at room temperature). However, μSPE shows some advantages over dried spot cards, such as its high sensitivity and the rapid isolation of the target analytes, since the extraction time and pre-concentration is considerably reduced compared to dried spot cards. Due to these reasons, μSPE was finally chosen as the pre-treatment strategy for analysising intake biomarkers in urine in the sustained human intervention study (see Section 1.3). In future human intervention studies, dried spot cards could

be a very interesting approach for analysing metabolites in blood as it could avoid the blood sampling via venipuncture.

At this point, with the methodological approaches for analysing phenolic metabolites in biological fluids developed and validated, we evaluated through different models (*in vitro* digestion, *in vitro* cell models and *in vivo* rat model) (**Figure 23**) the effect of co-occurring olive oil and thyme phenolic compounds on their digestibility, bioavailability and metabolism.

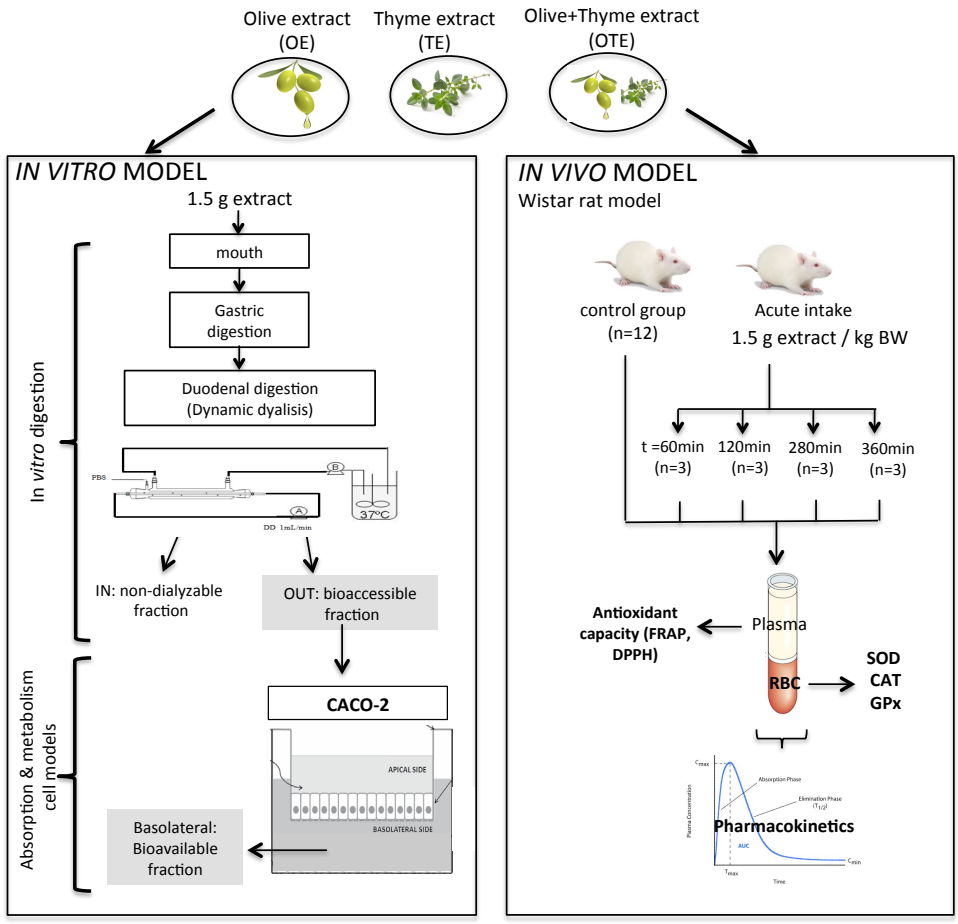

figure 23. Summary of the experimental design of the *in vitro* and *in vivo* experiments perfomed with the phenolic extracts.

In a first experiment, the stability during digestion and the potential bioaccessibility of the phenolic compounds was evaluated through an *in vitro* digestion model of three extracts: olive extract (OE), thyme extract (TE) and a combination of both (OTE). (Rubió et al. 2014 P-VI). Although the *in vitro* digestion method has some limitations for mimicking *in vivo* gastrointestinal conditions accurately, the stability achieved under the conditions generated by the gastrointestinal digestion method applied appears to be crucial for understanding the potential bioavailability of phenolic compounds (Mcdougall et al. 2005). The results from this study showed that hydroxytyrosol appeared to be the most bioaccessible phenolic compound, followed by monoterpenes (thymol), with flavonoids being the phenolic group with the lowest bioaccessible rates. When the combined extract (OTE) was digested, it is noteworthy that an enhancing effect of thyme compounds on the bioaccessibility of hydroxytyrosol and derivates was observed. It appeared that the codigestion of both phenolic sources resulted in greater stability and bioaccessibility of hydroxytyrosol compared with the single OE extract.

There is a wealth of articles in the literature dealing with the bioaccessibility of specific types of polyphenols in single foods, but the information only refers to specific types of phenolic compounds such as flavonols, flavones, catechins or phenolic acids (Manach et al. 2005). In this sense, Saura-Calixto et al. (2007) reported that studies addressing mixtures of polyphenols reflecting the real diet are necessary because when complex mixtures of different kinds of phenolic compounds are ingested, the gastrointestinal tract becomes a major site of their synergistic action. In our *in vitro* digestion model with olive and thyme extracts we report an example of this synergistic phenomenon. In keeping with earlier findings (Veda et al. 2008; Frontela et al. 2011; Kamiloglu et al. 2014), certain phenolic compounds could probably exert a favourable influence on the bioaccessibility of other antioxidant compounds by minimizing their loss during the digestion process. Accordingly, we hypothesize that thyme bioactives might

protect olive phenolics from degradation in the gastrointestinal tract before absorption, thus making them more bioaccessible. However, other underlying mechanisms remain to be examined.

What is also remarkable, is that in our further *in vivo* study with rats (Rubió et al. 2014 P-VII), an enhanced bioavailability of olive phenolic compounds occurred in the presence of thyme phenolics when the combined extract was administered to the rats. This confirmed the enhanced bioaccessibility observed in the *in vitro* digestion and could probably explain the enhanced bioavailability. In terms of biological effects, in previous *in vitro* (Herrmann & Wink 2011) and *in vivo* studies (Kennedy et al. 2001), synergistic biological effects with stronger improvements were reported when different phenolic compounds or extracts were combined, when compared with the single treatments. In this sense, it is important to study the extent to which bioaccessibility and bioavailability are affected when a mixture of polyphenols is administered.

In the second part of this experiment, the bioaccessible fraction obtained from the *in vitro* digestion model (OUT fraction after dynamic dyalisis) was exposed to the epithelial (Caco-2) and hepatocyte (HepG2) cell models to study the transport and metabolism of the phenolic compounds (**Figure 23**). When the digested phenolic extracts were exposed to Caco-2 cells, we observed that hydroxytyrosol was almost completely converted into hydroxytyrosol sulfate, hydroxytyrosol acetate sulfate and homovanillic alcohol sulfate, with most appearing on the basolateral side, which indicates high bioavailability of olive oil phenolics and an extensive sulphation and methyl-sulfation during the first pass metabolism in the epithelial cells. These results are in accordance with our *in vivo* studies in rat and human models (Rubió et al. 2012 P-III; Rubió et al. 2014 P-VII), in which a similar phenolic metabolic profile was observed after the acute intake. This indicates that the Caco-2 cell model appears to be a reliable model for simulating the *in vivo* absorption of olive phenolics.

However, depending on the model used (*in vitro*, rat or human models), as shown in **Table 7**, slight differences were observed in the metabolic profile. For instance, hydroxytyrosol acetate sulfate, the new hydroxytyrosol metabolite identified in humans in the present thesis frame, was only detected in the Caco-2 cell medium and human plasma, suggesting that *O*-acetylation might be a predominant metabolic pathway in humans and less expressed in rats. Another difference observed among the models applied to metabolism studies is that the glucuronide conjugates of hydroxytyrosol were only detected in rat plasma, indicating that this conjugation pathway might be more feasible in rats than in humans. In accordance, a recent study reported the relevance of the glucuronidation as a conjugation pathway after the administration of hydroxytyrosol as a standard in a rat model (Kotronoulas et al. 2013). It is also remarkable that all models have the complete conversion of hydroxytyrosol into their conjugated forms in common. These findings are in line with studies in humans showing that the fraction of hydroxytyrosol excreted in its free form in urine is very low or undetectable (Khymenets 2010; Visioli 2000; Miro-Casas

Table 7. Comparison of the metabolic profile observed after the administration of olive oil phenolic compounds in different models (*in vitro*, rat or human model).

Model	Administration form	Phenolic dose	Metabolite profile (basolateral side or plasma)	Ref.
Caco-2 cell model	Digested fraction of an olive extract	2,98 mg of HT and derivates	Main metabolites: hydroxytyrosol sulfate Minor: hydroxytyrosol acetate sulfate, homovanillic alcohol sulfate	Rubió et al. 2014 *Food Chem*
Rat model (acute intake)	Olive extract (1.5 g/ kg BW)	34,5 mg of HT and derivates	Main metabolites: hydroxytyrosol sulfate Minor: hydroxytyrosol glucuronide, homovanillic alcohol sulfate	Rubió et al. 2014 *Food & Function*
Human model (acute intake)	Enriched olive oil (30 ml olive oil)	6,3 mg of HT and derivates	Main metabolites: hydroxytyrosol sulfate, hydroxytyrosol acetate sulfate Minor: homovanillic acid, homovanillic acid sulfate	Rubió et al. 2012 *Food Chem*
Human model (3 week sustained intake)	Enriched olive oil (25 ml olive oil/ day)	8,49 mg of HT and derivates	Main metabolites: hydroxytyrosol sulfate, hydroxytyrosol acetate sulfate, homovanillic alcohol sulfate Minor: homovanillic acid sulfate	Rubió et al. 2014 *Food Res Int*

2003; Kotronoulas 2013). In synthesis, hydroxytyrosol is typically absorbed in a dose dependent manner and is rapidly metabolized. Therefore, the contribution of its metabolites to bioactive effects cannot be completely discarded. In this sense, it has been postulated that certain hydroxytyrosol conjugates may contribute to the health benefits derived from their consumption (Tuck et al. 2002), this topic being an interesting research line for future studies.

Regarding flavonoids, mainly characteristic from the thyme extracts, they did not undergo such an extensive metabolism and their conjugation rates differed within the single and mixed phenolic extracts presenting lower metabolic change when they were administered with the mixed extract (OTE). Since flavonoids are also known to be metabolized by Caco-2 cells into glucuronidated and sulfonated conjugates (Williamson et al. 2007), a competitive inhibitory effect of hydroxytyrosol on the phase II metabolism might be the reason for the decrease in the total amount of flavonoid metabolites formed by the Caco-2 cells upon co-exposure with olive phenols. However, most of the non-modified flavonoids did not cross the epithelial barrier, and the same happened with their conjugated forms, which most of which permeated across the apical membrane, thus reducing their bioavailability. These results were also consistent with our *in vivo* rat study with the same phenolic extracts, where the concentration of flavonoids in plasma was very low or undetectable. Our results are in line with previous studies that also suggest a low bioavailability of flavonoids (Manach et al. 2005), and in consequence, they are increasingly being studied as a fermentation source for colonic microbiota (Del Rio et al. 2013). Interestingly, although a significant amount of free flavonoids could not cross the epithelial cells, when the OTE was exposed to the cells, the percentage of native naringenin and eriodictyol on the basolateral side was significantly higher, indicating an enhanced bioavailability of flavonoids in presence of olive oil phenols. As mentioned above, this could also be due to the presence of hydroxytyrosol, which caused a reduction in the conjugation of

flavonoids and consequently, an increased amount of the native forms were able to permeate to the basolateral side.

Regarding the monoterpene fraction from thyme, it showed very high bioaccesibility rates with recoveries of around 50% from the initial extracts, compared to the lower percentages observed with flavonoids (10%). When they were exposed to the Caco-2 cells, monoterpenes also appeared to be one of the most efficiently permeated to the basolateral side, with a significant first pass metabolism, mainly into the sulfated form. These results were in accordance with the acute intake in rats performed with the same phenolic extracts, in which we observed that thymol was highly bioavailable, reaching concentrations in plasma at the mM range, in comparison with other metabolites which appeared at lower concentration levels (μM range). When we performed the human intervention trial, these results were confirmed again, as thymol sulfate appeared to be one of the most important intake biomarkers after a sustained intake of an olive oil enriched with thyme phenolics (Rubió et al. P-IX). Considering the whole set of bioavailability studies performed with thyme, we concluded that despite the initial interest on flavonoids as thyme bioactive compounds complementing olive oil phenolics, in the end, they became less interesting due to their low bioavailability. In contrast, monoterpenes, that appeared to have a lower occurrence in thyme, were remarkably more bioavailable, and thus, in future studies the attention should be focused on these compounds as they might have greater possibilities of reaching the target sites.

The combination of Caco-2, HepG2 and a co-culture of both also allowed us to hypothesize a possible deconjugation process of hydroxytyrosol phase II metabolites into free hydroxytyrosol in the liver, since the free form was only detected in the basolateral media of the co-culture model and the HepG2 media, but did not appear after Caco-2 exposure. Currently, tissue

deconjugation is a hot topic in the polyphenol research community as it is considered to be the next step in the understanding of the phenolic bioactivity (Perez-Vizcaino et al. 2012). In this sense, phenolic conjugates are thought to be pharmacologically inactive and some recent studies suggest that their *in situ* deconjugation with the delivery of the active aglycone forms in certain target sites appears to be an absolute requirement for them to exert beneficial effects (Terao et al. 2011; Perez-Vizcaino et al. 2012; Ishisaka et al. 2013). From our results, and considering these previous studies, we suggest that deconjugation of hydroxytyrosol could occur in the liver, and probably in other target tissues, which could generate the active form of free hydroxytyrosol considered to be responsible for the biological effects *in vivo.* In our later *in vivo* rat study, red blood cells (RBCs) were also collected and analyzed (**Figure 23**), and we observed that when the olive phenolic extract was administered to the rats the free form of hydroxytyrosol, which was not detected in plasma, appeared in RBCs with an increasing trend in concentration up to 360 min and a parallel decreasing trend of its conjugated forms. These results reinforce our hypothesis that RBCs, which are particularly vulnerable to oxidative damage, could hydrolyze hydroxytyrosol conjugates into their free active form, becoming a potential cell target for hydroxytyrosol.

Furthermore, from the *in vivo* study with rats, the antioxidant status was analyzed by DPPH and FRAP in plasma and by SOD, CAT and GPx activities in erythrocytes. The results showed that phenolics derived from both olive and thyme might have *in vivo* antioxidant protective effects in postprandial conditions, with an association being observed between the metabolites accumulated in the plasma which could participate in the antioxidant defense there, and the modulation of the antioxidant status in the blood. Although there is controversy about the antioxidant activity of the conjugated forms of polyphenols, it has been established that polyphenols, such as flavonoids, which mainly accumulate as conjugates in plasma, can participate in the

antioxidant defense in blood serum (Terao 1999; Beekmann et al. 2012). In this sense, our work allowed us to attribute the increase in the antioxidant capacity in plasma to the increase in the concentration of the phenolic metabolites. In fact, we observed that the plasmatic pharmacokinetic profile of the phenolic metabolites was in accordance with the modulation of the antioxidant capacity in plasma. On the other hand, the precise mechanism by which antioxidant enzymes are modulated after the administration of polyphenols is still unknown. The short time of the observed effects in our study (at 1 and 2 h after the extracts intake) supports the hypothesis that the intake of polyphenols may act as a direct scavenger of reactive oxygen species reducing the body's need for certain antioxidant enzymes. Recent studies also postulate that this activation may occur at the post-translational level (Carrera-Quintanar et al. 2012; Quirantes-Piné et al. 2013), a situation that also deserves future attention.

1.3 Sustained consumption of phenol-enriched olive oils: Human clinical trial

Once the development and characterization of the phenol enriched olive oils had been carried out in terms of phenolic composition and bioavailability studies, scientific evidence needed to be provided through an efficacy study with humans, as a primordial and final step required by the EFSA for the development of a functional food. This task is still in progress in coordination with all research groups participating in the VOHF project and the preliminary results related to the *in vivo* protective effect of the phenol-enriched olive oils against oxidative damage are presented in this thesis.

For the efficacy study, 33 hypercholesterolemic volunteers participated in a 3-week dietary intervention with phenol-enriched olive oils within the frame of a randomized, double-blind, crossover, and controlled nutrition intervention trial. The volunteers were administered 25 ml/day of (1) control olive oil with low

phenolic content (VOO), (2) Functional Virgin Olive Oil enriched with its own phenolics (FVOO), and (3) Functional Virgin Olive Oil enriched with its own phenolics plus complementary phenolics from Thyme (FVOOT). In this sense, both phenol-enriched olive oils had a total phenolic content of 500 mg total phenols/kg oil but with different phenolic composition. As seen in **Figure 24**, FVOO was enriched basically with hydroxytyrosol and its derivates, whereas FVOOT was enriched with 50% of hydroxytyrosol and derivates and 50% of thyme phenols, mainly flavonoids, phenolic acids and monoterpenes. The phenolic group of lignans also had an important presence in the oils, however no significant difference was observed among them, indicating that the effects observed after the phenol-enriched olive oils can not be related to this phenolic group.

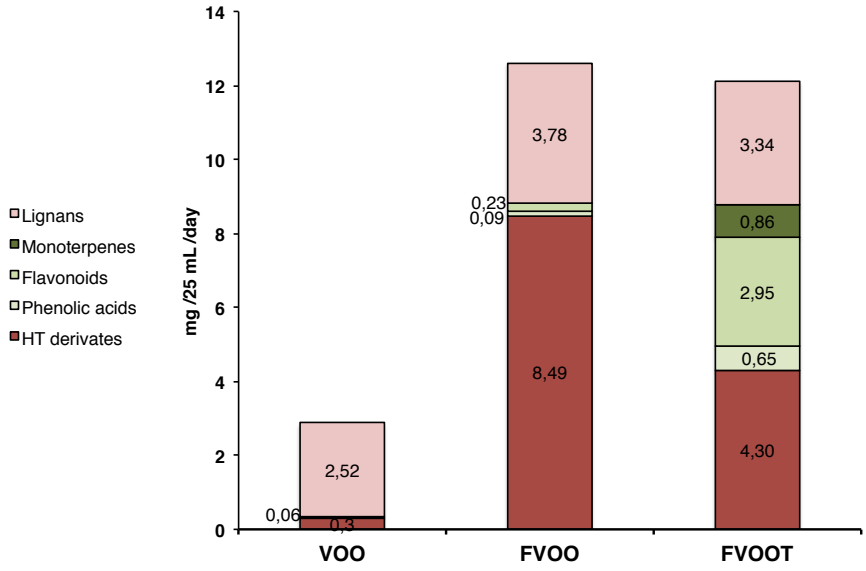

Figure 24. Phenolic composition of the olive oils used in the VOHF sustained study represented by phenolic groups. Compounds derived from olive are displayed in reddish colors and compounds derived from thyme are represented in greenish colors.

Concerning the study design, the requisites established by the EFSA are accomplished as it includes a group of participants that is representative of the target group and also presents an adequate duration of exposure and follow up to demonstrate the intended effect. Moreover, it provides the amount of food (in this case 25 mL/23g of olive oil) consistent with its intended pattern of consumption.

Although the human data is indispensable to substantiate a health claim, an understanding of causal relations between polyphenol intake and its beneficial effects on health can be hindered by the lack of robust biological markers of its exposure. Therefore, an essential requirement for establishing polyphenol bioactivity is to develop validated *in vivo* biomarkers of intake as a prior step in order to monitor the volunteer's compliance (Kay 2010). Indeed, the criteria defined by the PASSCLAIM (Aggett et al. 2005) for the scientific substantiation of health claims specifies that *the subjects' compliance concerning the intake of food or food component under test must be monitored*. In this sense, the first task in the human intervention study was to identify compliance biomarkers for phenolic compounds from olive oil and thyme after the sustained intake of olive oils.

It is noteworthy that a sustained intake of a phenol-rich food provides relatively low phenolic doses per day. In our case, phenol-enriched olive oils provided around 12 mg/day of total phenolics. This represents a challenge for quantifying the expected low concentrations of phenolic metabolites in plasma and urine. Thus, we required the use a sensible and reliable analytical technique. Sensitivity is an extremely important quality parameter of the analytical method because phenolic compounds are present at low concentration levels (low μM range). On the other hand, the quality and relevance of the results are related to the accuracy of the measurements (Panteghini 2006), and unreliable results could represent underestimations of effects, false interpretations, and unwarranted conclusions (Peters et al. 2007). Therefore, only a validated

methodology can be objectively judged as an adequate method (Peters et al. 2007). In our case, the methods used to identify and quantify the metabolites in plasma and urine are based on UPLC-MS/MS methodology and were previously validated (Rubió et al. 2012 P-IV; Serra et al. 2013; Suárez et al. 2009). This methodology is considered one of the most reliable approaches for profiling nutrient biomarkers (Puiggròs et al. 2011) and enabled us to detect a wide range of phenolic metabolites in plasma and urine derived form olive and thyme. Among these, the most robust quantitative and qualitative compliance markers in plasma and urine were selected (**Figure 25**).

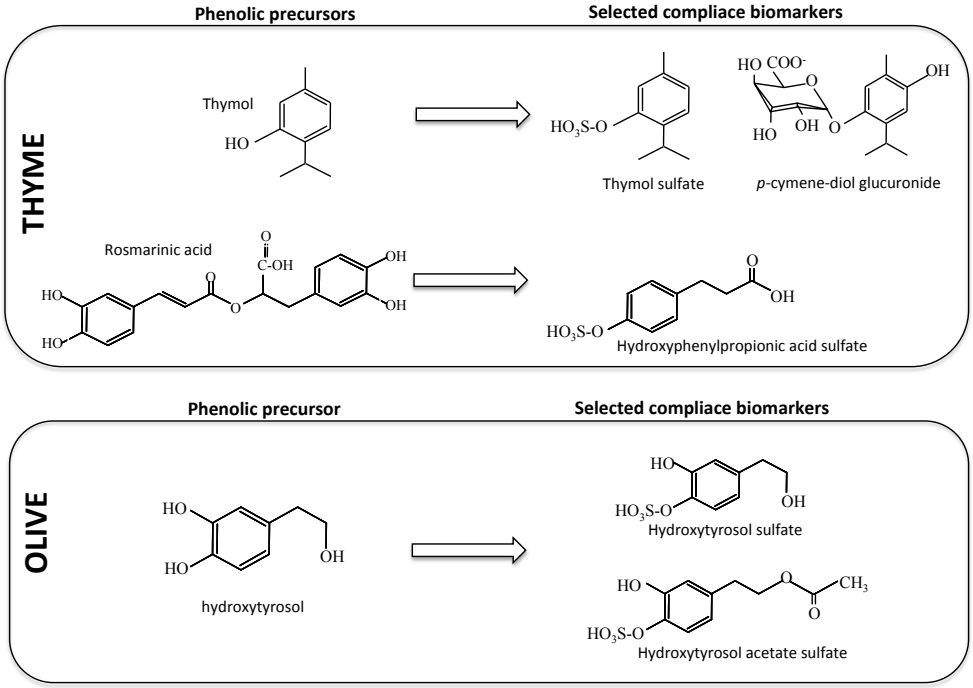

Figure 25. Selected compliance biomarkers of thyme and olive polyphenol intake in the VOHF study.

According to Spencer et al. (2008) specificity is an essential requirement for the characterization of a metabolite as a potential biomarker of the intake of the metabolite, and such a metabolite should only appear following the intake of a specific polyphenol without being formed as a product of the metabolism of any other compound. Our results showed that olive phenolic metabolites detected in plasma and urine (mainly from hydroxytyrosol metabolism) after 21 days of phenol-enriched olive oils intake presented low specificity, as they were also detected at baseline before the phenol-enrichment consumption. These results indicate certain limitations on the use of these metabolites as potential intake biomarkers of olive oil phenolic compounds. It is well known that, in addition to being a characteristic component of the phenolic fraction of virgin olive oil, hydroxytyrosol is an endogenous compound derived from the oxidative dopamine (Edwards & Rizk 1981; Goldstein et al. 2003). Moreover, wine seems to be another important source of hydroxytyrosol in our diet (Minuti et al. 2006), which could also have interfered in our study. Despite the low specificity, two hydroxytyrosol metabolites, hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate, appeared to be good candidates for monitoring the intake compliance of olive oil phenolic compounds, as their concentration in plasma and 24h-urine after the intervention period of 21 days with FVOO, were significantly higher than the baseline prior to the intervention concentrations. These results were in accordance with our previous studies performed with *in vitro* cell models and the *in vivo* rat model, in which hydroxytyrosol sulfate was always the metabolite with major occurrence after olive oil phenolic exposure or intake.

In contrast to olive phenol metabolites, those derived from thyme were more sensitive to changes after FVOOT intake and appeared to be better biomarkers. Despite the concentration of thyme phenolics administered with the FVOOT being the same as the olive oil phenolics (**Figure 24**), the amount of thyme phenolic metabolites in plasma and urine was much higher, indicating that they were more bioavailable than olive phenolics. These results confirmed our

previous studies in cell and animal models, in which thyme phenolics also presented higher bioavailability compared with olive phenolics. Among all the phenolic metabolites derived from thyme, thymol sulfate (sulfTHY) and hydroxyphenylpropionic (sulfHPPA) acid sulfate appeared to be the best candidates as compliance biomarkers in plasma and urine in terms of increasing changes after treatment. *p*-cymene-diol glucuronide (GlucCYM) was also a good biomarker in urine as it presented the greatest increase after FVOOT consumption. Urinary glucCYM, sulfTHY and sulfHPPA were also the only metabolites that showed a significant correlation with the monitored olive oil compliance. Based on these criteria, they were defined as the best compliance biomarkers for thyme phenolic intake.

The fulfilment of the intake biomarkers criteria defined by Spencer et al. (2008) denotes the usefulness of the intake biomarkers of polyphenols provided, which guarantees a successful dietary intervention. Moreover, despite the blinding design of the study, the selected biomarkers allowed the analysts to identify the order of intervention of the olive oils, denoting that the proposed analytical methodology is a reliable approach for phenolic biomarker profiling in nutritional intervention studies.

In the present thesis, preliminar human biological efficacy of the phenol-enriched olive oils in relation to the promotion of the endogenous antioxidant system is also presented. Results showed that FVOO and FVOOT lead to an increase in the plasmatic fat-soluble endogenous antioxidants lutein, α-tocopherol, retinol and β-cryptoxanthin. This increase was attributed to the extra supplementation of phenolic compounds with the enriched olive oils, as the three administered oils had the same fatty acid profile and similar content of α-tocopherol and carotenoids. The increase in the plasma concentration of α-tocopherol is especially interesting, because it is an antioxidant with well-characterized direct antiplatelet and antithrombotic effects (Abdala-Valencia et al. 2012; Da Costa et al. 2013; Freedman et al. 1996). Moreover, it was also

demonstrated in a large cohort study that men in the higher quintiles of serum α-tocopherol had significantly lower risks of total and cause-specific mortality than did those in the lowest quintile (Wright et al. 2006). As suggested by other authors, the increase of α-tocopherol in plasma after a phenolic intake could be due to the protection of LDL from oxidation exerted by phenolic compounds either directly or indirectly by sparing vitamin E (Freedman et al. 2001; Castilla et al. 2006).

It is remarkable that FVOOT showed a greater increase in fat-soluble endogenous antioxidants compared to FVOO. In fact, if we compare the phenolic metabolite concentrations in plasma after FVOO and FVOOT (Rubió et al 2014 P-IX), the concentration of the main phenolic metabolites reached in fasting plasma after the sustained intake of FVOOT was much higher compared to FVOO, which might indicate that the greater bioavailability of thyme phenolics could lead to a major benefit in promoting the antioxidant plasmatic status.

In conclusion, results suggest that phenolic compounds provided with a sustained intake of phenol-enriched olive oils could optimize the endogenous antioxidant status and that the combination of olive and thyme phenolics might have a greater effect compared to the single olive phenols administration.

Further results provided by the other reseach groups related to HDL functionality are needed to corroborate that the phenol-enriched olive oils have relevant physiological effects.

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CONCLUSIONS

Answering the specific targets proposed in this thesis, the main achievements of the research are summarized below:

Target I: Phenol-enriched olive oils development

- The direct application of thyme phenolic extracts instead of thyme leaves infusions, as is traditional when preparing flavoured olive oils, enables a phenol enriched and seasoned olive oil to be developed with exactly the amount of phenols required, thus enabling a functional virgin olive oil with standardized content and profile of phenolic compounds to be obtained.

- The optimal enriched olive oil with the more balanced phenol composition appeared to be the one prepared with phenolic extract obtained with 60% of olive cake and 40% of thyme, providing the highest amount of olive oil phenolics (mainly secoiridoid derivatives) and the highest quantity of thyme phenolics (mainly flavonoids, phenolic acids and monoterpenes).

- Phenol-enriched olive oils combining olive cake and thyme extracts had lower bitterness than those enriched only with olive oil phenols at the same concentration, indicating that the use of aromatic herbs or species may improve consumer acceptance of high-phenol olive oils.

Target II: Olive oil phenolic dose-response study

- Human pharmacokinetics of hydroxytyrosol metabolites do not have a complete linear response after the intake of phenol-enriched olive oils with different phenolic contents, indicating that a threshold could exist in olive oil phenolic absorption. Considering both the dose-pharmacokinetic and sensory acceptance, the olive oil with a content of

500 mg total phenols/kg oil appeared to have the optimal enrichment, as it had both a good plasma pharmacokinetic response and a good sensory acceptability.

- We pointed out that, for the first time, hydroxytyrosol acetate sulfate was identified as one of the main biological human metabolites of hydroxytyrosol after olive oil ingestion. This was a step forward in research into the metabolic fate of olive oil phenolics.

Target III: To develop and validate analytical methodologies for the analysis of phenolic compounds in plasma and urine

- The development and validation of the methodology based on ultra-performance liquid chromatography coupled to tandem (UPLC-MS/MS) after an acute intake of thyme extract in rat enabled the plasmatic metabolite identification and the metabolic pathways elucidation of thyme phenolic compounds, which was scarcely described in the literature.

- Dried urine spot cards (DSC) and microelution SPE plates (μSPE) were proposed as two new sample pre-treatment strategies for the analysis of olive phenolic metabolites in urine, representing a step forward in the development of novel pre-treatment approaches for the analysis of phenol metabolites in biological samples.

Targets IV and V: To assess the bioaccessibility and bioavailability of olive oil and thyme phenolic compounds by combining in vitro and in vivo models and assess their in vivo antioxidant effects

- The phenolic bioaccessibility study revealed that thyme phenolics might have an enhancing effect on the bioaccessibility of secoiridoids (hydroxytyrosol and derivates) from olive oil when the combined extract was subjected to an *in vitro* digestion model, suggesting that when a

mixture of phenolics is ingested, the gastrointestinal tract becomes a major site for their synergistic action.

- Regarding thyme phenolics, monoterpenes appeared to be remarkably more bioaccessible than flavonoids, with recoveries of around 50% compared with 10% for flavonoids after the *in vitro* digestion. After that, monoterpenes were the most efficiently permeated to the basolateral side in the Caco-2 cell model, with a significant first pass metabolism, mainly into the sulfated form.

- The bioavailability of olive phenolic compounds was enhanced in the presence of thyme phenolics when the combined extract was administered to the rats, which was in accordance with the enhanced bioaccessibility observed in the *in vitro* digestion model that could probably explain the improved bioavailability *in vivo*. These results suggest that the combination of different phenolic sources might be a promising approach for improving the bioavailability of certain phenolic compounds.

- Both phenolics derived from olive and thyme might have *in vivo* antioxidant protective effects in postprandial conditions, observing an association between the metabolites accumulated in plasma, which could participate in the antioxidant defense there, and the modulation of the antioxidant status in blood.

Target VI: To perform a sustained human interventional trial with the developed phenol-enriched olive oils, and assess the compliance of the volunteers through the identification of the phenolic intake biomarkers in human plasma and urine.

- The methodological approach based on UPLC coupled to MS/MS enabled to monitoring the volunteers' compliance in a sustained intake of phenol-enriched olive oils through the determination of specific phenol metabolites

in human plasma and 24h-urine for the first time. Despite the low specificity of hydroxytyrosol metabolites, hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate appeared to be valid compliance markers. Regarding the phenolic compounds from thyme, the metabolites derived from thymol (thymol sulfate and *p*-cymene-diol glucuronide) and from rosmarinic acid (hydroxyphenylpropionic acid sulfate) appeared to be the best compliance markers with a very high specificity.

Target VII: To assess the preliminary human biological efficacy of the functional olive oils in a sustained interventional trial regarding the protection against oxidative damage and the promotion of the endogenous antioxidant system.

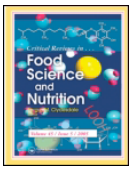
- The sustained daily intake (21 days) of 25 ml of the phenol-enriched olive oils developed (FVOO or FVOOT) resulted in an improvement in the antioxidant status based on the increase of the plasma concentration of fat-soluble endogenous antioxidants (α-tocopherol, carotenoids and vitamin A). Additionally, the olive oil enriched with its own polyphenols might protect against oxidative damage to DNA. The modulation of these biomarkers opens news perspectives for the use of phenol-enriched olive oils as a strategy to improve antioxidant defenses and oxidative damage protection.

APPENDICES

APPENDIX I

REVIEW: Recent Advances in Biologically Active Compounds in Herbs and Spices: A Review of the Most Effective Antioxidant and Anti-Inflammatory Active Principles

Critical Reviews in Food Science and Nutrition (2013) 53, 943-953



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RECENT ADVANCES IN BIOLOGICALLY ACTIVE COMPOUNDS IN HERBS AND SPICES: A REVIEW OF THE MOST EFFECTIVE ANTIOXIDANT AND ANTI-INFLAMMATORY ACTIVE PRINCIPLES

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Abstract

Spices, like vegetables, fruit, and medicinal herbs, are known to possess a variety of antioxidant effects and other biological activities. Phenolic compounds in these plant materials are closely associated with their antioxidant activity, which is mainly due to their redox properties and their capacity to block the production of reactive oxygen species. More recently, their ability to interfere with signal transduction pathways involving various transcription factors, protein kinases, phosphatases, and other metabolic enzymes has also been demonstrated. Many of the spice-derived compounds which are potent antioxidants are of great interest to biologists and clinicians because they may help protect the human body against oxidative stress and inflammatory processes. It is important to study the bioactive compounds that can modulate target functions related to defence against oxidative stress, and that might be used to achieve health benefits individually. In the present review, an attempt has been made to summarize the most current scientific evidence about the in vitro and in vivo effects of the bioactive compounds derived from herbs and spices, focused on anti-inflammatory and antioxidant effects, in order to provide science-based evidence for the traditional uses and develop either functional foods or nutraceuticals.

KEYWORDS: spices / herbs / antioxidant activity / terpenes / phneolic acids / flavonoids

INTRODUCTION

Spices and herbs are common food adjuncts, which have been used as flavouring, seasoning, and colouring agents and sometimes as preservative, throughout the world for thousands of years, especially in India, China, and many other south-eastern Asian countries. While bringing colour and taste to the food, some spices have long been considered to possess medicinal value and have been effectively used in the indigenous systems of medicine (Nadkarni and Nadkarni, 1976). Apart from the traditional use, a host of beneficial physiological effects have been brought to the fore by extensive animal studies during the past three decades (Srinivasan, 2005a). Among these are their beneficial influences on lipid metabolism (Naidu et al, 2002; Manjunatha et al., 2007), efficacy as anti-diabetics (Tundis et al, 2010), antimicrobial (Lai et al, 2004), digestive stimulant action (Platel et al, 2004), anticarcinogenic potential (Lampe, 2003), antioxidant property and anti-inflammatory (Srinivasan, 2005b).

Much of the earlier studies on herbs and spices have viewed their bioactive compounds

from the perspective of antioxidants. The antioxidant property of these molecules was later explained on the basis of the availability of –OH and the system of conjugated double bonds present in these molecules. However, many other effects such as anti-inflammatory, anti-tumor, anti-atherogenic abilities could not be explained solely on the basis of their antioxidant properties. Investigations into the mechanism of action of these molecules have thrown light on the fact that polyphenols may not merely exert their effects as free radical scavengers, but may also modulate cellular signaling processes during inflammation or may themselves serve as signaling agents (Aggarwal and Shisodia, 2004).

Moreover, the most recent and large Antioxidant Food Database (Carlsen et al., 2010) developed from the analysis of 3,100 foods, beverages, spices and herbs, shows that the most antioxidant rich products in the human diet are spices and herbs, some of them exceptionally high. The elevated concentration of antioxidants observed in several dried herbs compared to fresh samples, is a normal consequence of the drying process leaving most of the antioxidants present in the fresh vegetal tissue intact in the dried end product. They are phytochemically complex and variable geographically within a species or taxon but with major contributions from the *Apiaceae* and *Lamiaceae.*

The active principles in spices and herbs with biological activities are none other than secondary metabolites produced by plants. The most recent phytochemicals isolated from herbs and spices include: phenolic terpenes (thymol, carvacrol, carnosic acid, carnosol and rosmanol) (**Table 1**), hidroxycinnamic acids and derivates (caffeic acid, ferulic acid, p-coumaric, rosmarinic acid, eugenol and curcumin) (**Table 2**), flavonoids (quercetin, luteolin and apigenin) (**Table 3**) among others.

To-date, randomised controlled trials (RCT) considering the intake of single antioxidants have provided no evidence of health-benefit effects. So, if benefit is to be gained, it might be better to consume a wide range of antioxidants, such as occurs naturally in certain foods (Howlett, 2008). However, it is important to study individually bioactive compounds that can modulate target functions related to defence against oxidative stress and inflammation, and that might be used to achieve such benefits. Oxidative damage and inflammatory processes at the cellular and subcellular level is now considered to be an important event in disease processes like cardiovascular diseases (CVD), inflammatory disease, carcinogenesis, and ageing, so the use of herbs and spices as source of bioactive compounds to combat oxidation warrants further attention. In the present review, an attempt has been made to summarize the most current scientific evidences about the in vitro and in vivo effects of the bioactive compounds derived from herbs and spices, focused on anti-inflammatory and antioxidant effects.

Antioxidant activity

Reactive oxygen spices (ROS) are considered the major contributors to many of the diseases associated with ageing, including cardiovascular diseases (CVD), cancer, cataracts, age-related decline in the immune system, and degenerative diseases of the nervous system such as Parkinson's and Alzheimer's disease (Howlett, 2008). These free radicals generated in vivo damage many targets, including lipids, proteins, DNA, and small molecules. The body's innate defences need to be supported by a wide variety of small-molecular-weight antioxidants found in the diet, and many of them are of plant origin. Hence, natural antioxidant molecules can counter ROS directly or boost regenerative systems to restore antioxidant capacity (Naghavi et al., 2003).

Spices, like vegetables, fruit, and medicinal herbs, are known to possess a variety of antioxidant effects and properties (Zheng et al., 2001). The antioxidant effect of phenolic compounds is mainly due to their redox properties and their capacity to block the production of ROS formed in several in vitro and in vivo systems. This is the result of various possible mechanisms: free-radical scavenging activity, transition-metal-chelating activity, and/or singlet-oxygen-quenching capacity. It has been demonstrated in in vitro and in vivo models, that the antioxidant activity of these bioactive compounds found in herbs and spices could play an important role in suppressing cell growth, viral replication, inhibiting allergy and arthritis, preventing cancer and heart diseases, and abrogating several other pathological conditions responsible for the early stages of multiple pathologies (Aggarwal et al., 2002).

Recently, a growing bulk of experimental evidence indicates that the cellular effects of non-enzymatic antioxidants do not simply depend on their free radical chain-breaking activity. Practically all of them have repeatedly been shown to act also at several key sites in the complex network of functional signalling within tissues and cells, involving various transcription factors, protein kinases, phosphatases, and other metabolic enzymes (Leonarduzzi et al., 2010). For instance, over expression of antioxidant enzymes can block activation of the nuclear transcription factors NF-κB and AP-1, the stress activated protein kinases, and apoptosis. Thus, non-enzymatic antioxidants appear to contribute to maintaining cellular behaviour and function within the physiological range, and might protect against atypical cell proliferation; but especially they may effectively down-regulate the inflammatory process. Thus, antioxidant ingredients derived from the spices, by blocking transcription factors and kinases, have potential for blocking various diseases, including cancer and cardiovascular diseases, two major causes of death in developed countries.

From a prevention standpoint, the antioxidant properties of herbs and spices are of particular interest in view of the impact of the oxidative modification of low-density lipoprotein cholesterol in the development of atherosclerosis. It has also been reported that various spice-derived ingredients are potent inhibitors of lipid peroxidation in cell and human LDL (Akhilender, 2002).

These multiple potential mechanisms of antioxidant action make the diverse group of phenolic compounds in herbs and spices an interesting target in the search for health-beneficial phytochemicals. In this review, various biological activities that have been assigned to the antioxidant ingredients derived from herbs and spices are listed in Tables 1-3 and extended to every group of bioactive compounds.

Anti-inflammatory activity

Various spice and herb derived-compounds have also been shown to exhibit anti-inflammatory activity and a review of the most recent studies is presented here. Different studies have demonstrated an association between the typical Western diet rich in refined starches, sugars, saturated and transfatty acids and poor in fruit, vegetables, fibre, ω-omega-3 fatty acids and whole grains, and an increased tendency towards inflammatory disorders and related diseases, such as cardiovascular diseases, arthritis or diabetes (Giugliano et al., 2006). In order to reduce inflammation, a diet rich in fruits and vegetables has been negatively correlated with various diseases that are associated with inflammatory disorders. In this sense, several dietary polyphenols were shown to ameliorate inflammatory stages via diverse mechanisms (González-Gallego et al., 2010).

Chronic inflammation plays an important role in the development of atherosclerosis, a progressive disease characterized by the accumulation of lipids and fibrous elements in the large arteries. This inflammation is the mechanism with which the body responds to the interactions between modified lipoproteins, monocytes, macrophages, T-cells and arterial endothelial cells (Libby, 2008). Activated leukocytes, endothelial cells and macrophages produce pro-inflammatory cytokines including interleukin (IL)-1b, IL-6, as well as tumor necrosis factor-alpha (TNF-α) and anti-inflammatory cytokines, like the cytokine IL-10 (Li et al., 2005). These cells also produce pro-inflammatory enzymes, the inducible forms of nitric oxide sintase (iNOS) and cyclooxygenase (COX), which are responsible for increasing the levels of nitric oxide (NO) and prostaglandins (PEG2), and are known to be involved in various chronic diseases, including multiple sclerosis and colon cancer (Yan et al., 2007). The NF-κB transcription factor also plays an important role in the inflammatory response by regulating the expression of various genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes, such as COX-2 (Hanada et al., 2002). The use of plants with pharmaceutical properties has received increased interest nowadays from both the homeopathic and allopathic branches. Besides, these medicinal plants play an important role in public health, especially in developing countries.

Although inflammation is primarily a protective response (against micro-organisms, toxins or allergens, for example), chronic and uncontrolled inflammation becomes detrimental to tissues. Since ancient times, in various cultures worldwide, inflammatory disorders and related diseases have been treated with plants or plant-derived formulations (Krishnaswamy, 2008). Over

the last decade, the anti-inflammatory activity of several spice ingredients and isolated compounds has already been scientifically demonstrated and this review pretends to summarize the most recent advances obtained from the in vitro and in vivo studies.

BIOACTIVE COMPOUNDS IN HERBS AND SPICES

The antioxidant and anti-inflammatory activities of herbs and spices have been related with three main groups of compounds: terpenes, phenolic acids and flavonoids.

Terpenes

Terpenes form structurally and functionally different classes. They are made from combinations of several 5-carbon-base (C_5) units called isoprene. The biosynthesis of the terpenes consists of synthesis of the isopentenyl-diphosphate (IPP) as a precursor, repetitive addition of IPPs to form the prenyldiphosphate precursor of the various classes of terpenes, modification of the allylicprenyldiphosphate by terpene specific synthetases to form the terpene skeleton and finally, secondary enzymatic modification (redox reaction) of the skeleton to attribute functional properties to the different terpenes. We will focus here on the main terpenes in herbs and spices, these being the monoterpenes and diterpenes.

The monoterpenes are formed from the coupling of two isoprene units (C_{10}). They are the most representative molecules, constituting 90% of the essential oils, and in terms of health, the most important are thymol and carvacrol (Table 1). The antioxidant properties of thymol and carvacrol have been demonstrated in several studies, suggesting their use as nutraceutical ingredients in the development of novel functional foods. The derivatives of thymol and carvacrol have been described as antioxidant according to the DPPH radical scavenging method (Mastelic et al., 2008). Essential oils from oregano and their components (carvacrol and thymol) inhibited 3-nitrotyrosine formation, a biomarker of oxidative stress, supporting the nutraceutical value of oregano and the potential of thymol and carvacrol for preventing the formation of toxic products by the action of reactive nitrogen species (Prieto et al., 2007).

Also, thymol and carvacrol act in preventing the autoxidation of lipids (Yanishlieva et al., 2006). In another recent study the antioxidative effect of essential oils from oregano and thyme, and aqueous tea extracts, on the oxidation susceptibility of LDL has been studied (Kulišić et al., 2007). The results indicate a dose-dependent protective effect of both the essential oils and the aqueous tea extracts on the copper-induced LDL oxidation. The protective effect is assigned to the presence of phenolic monoterpenes, thymol and carvacrol, which are identified as the dominant compounds. The strong protective effect of aqueous tea infusions is proposed to be the consequence of the presence of both components and large amounts of other polyphenols, like rosmarinic acid and various flavonoids. These findings may have implications for the effect of these compounds on LDL in vivo.

A recent study showed the possible anti-inflammatory effects of these two components based on the decrease in pro-inflammatory TNF-α, IL-1b and IL-6 cytokines synthesis, as well as an increase in the production of the anti-inflammatory cytokine IL-10 (Ocaña-Fuentes et al., 2010). These results may suggest an anti-inflammatory effect of oregano extracts and their compounds in a cellular model of atherosclerosis.

Carnosic acid and carnosol, the main diterpenes in aromatic herbs, together with rosmarinic acid, a hydroxycinnamic acid ester, are the main antioxidant compounds present in rosemary (Wellwood et al., 2004; Peñuelas et al., 2005). Among the herbal extracts reported to have antioxidative activity, rosemary is one of the most widely used and commercialized plant extracts, not only as a culinary herb for flavoring but also as an antioxidant in processed foods and cosmetics (Zheng et al., 2001). Carnosic acid and carnosol, together with such other isoprenoids as sterols, tocopherols or carotenoids, play a photoprotective role and are considered as bioactive constituents. Carnosic acid is originated from isopentenyldiphosphate (IPP) via methyl-erythritol phosphate (MEP). It is located in chloroplasts and intracellular membranes, as is carnosol, formed from the oxidative degradation of carnosic acid (Almela et al., 2006).

The antioxidant activity of rosemary extracts and their respective major compounds (carnosic acid, carnosol and rosmarinic acid) was analyzed in a study by Laura et al. (2010) and compared by using different in vitro systems.

Whereas rosmarinic acid, carnosic acid, and carnosol exhibited similar antioxidant activity in a phospholipid membrane-free assay, carnosol behaved as an extremely potent antioxidant in a membrane-based assay (4-6 times stronger than the rest of the compounds). This differential antioxidant behavior suggests that factors other than the radical scavenging capability may be involved. All of the diterpenes induced changes on the lipid order and packing of phospholipid model membranes. These effects may contribute to membrane stabilization and the interruption of radical propagation, which may cooperate with the electron donor ability of rosemary diterpenes to protect the membranes against oxidative damage (Laura et al., 2010).

In a very recent study the anti-inflammatory activity of various fruits, herbs and spices has been demonstrated (Mueller et al., 2010). Sage, a herb rich in carnosol and carnosic acid, presented anti-inflammatory activity, improving the anti-inflammatory profile of the secreted cytokines and inhibiting notably the expression of the pro-inflammatory enzyme, iNOS. According to the literature, carnosic acid and carnosol can inhibit the formation of pro-inflammatory leukotrienes and LOX-5 (Poeckel et al., 2008). Based on the positive results of their effects on cytokine profiles and inhibition of iNOS and COX-2 expression, several plant extracts tested in this study could potentially be used as food supplements with the purpose of providing anti-inflammatory effects.

The pharmacokinetics and the absolute bioavailability of carnosic acid in rats have also been examined (Yan et al., 2009). The absorption of carnosic acid was slow (T_{max}=125.6 min) after i.g. administration (90 mg/kg). However, the maximum plasma concentration was high and retained for a long time. The absolute bioavailability of carnosic acid was also high [F (%)=65.09], which would be a useful feature in future clinical applications of the drug as an antioxidant.

Recent studies (Lai et al., 2009) demonstrated that rosmanol, another natural diterpene from rosemary, down regulates inflammatory iNOS and COX-2 gene expression by inhibiting the activation of NF-κB and STAT3 through interfering with the activation of phosphatidylinositol 3-kinases (PI3K/Akt) and MAPK signalling. Taken together, rosmanol with carnosic acid might contribute to the potent anti-inflammatory effect of rosemary and may have the potential to be developed into an effective anti-inflammatory agent.

Hidroxycinamic acids and derivates

Hydroxycinnamates or phenylpropanoids (C_6–C_3 compounds) are secondary plant metabolites of phenylalanine, and to a lesser extent, of tyrosine via the action of phenylalanine ammonia lyase (PAL) or tyrosine ammonia lyase (TAL), respectively. Hydroxycinamic acids are important simple phenols as they are precursors for the synthesis of many other complex phenols. They are found in almost all food groups, although they are abundant in cereals, legumes, oilseeds, fruits, vegetables, beverages and herbs and spices (Robbins, 2003).

The latest studies into the antioxidant and anti-inflammatory activities of hidroxycinnamates derived from herbs and spices are summarized in Table 2. Recently, the in vitro and in vivo antioxidant activity of hydroxycinnamates was reviewed and the revision concluded that these phenolic compounds may exert a myriad of health benefits thus ameliorating chronic diseases associated with oxidative damage such as cancers, cardiovascular diseases, hypertension and neurodegenerative disorders (Shahidi et al., 2010).

Among these, the inhibition of LDL peroxidation by supplementation of antioxidants has become one of the most attractive therapeutic strategies for preventing atherosclerosis. A study by Cheng et al. (2007) used the in vitro peroxidation of LDL as a model to evaluate the free radical-induced damage of biological membranes and the protective effect of hydroxycinnamic acid derivatives i.e., caffeic acid, chlorogenic acid, sinapic acid, ferulic acid and p-coumaric acid. The kinetic analysis of the antioxidation process demonstrates that these hydroxycinnamic acid derivatives are effective antioxidants against both AAPH- and Cu^{2+}-induced LDL peroxidation with the activity sequence of caffeic acid~chlorogenic acid > sinapic acid > ferulic acid > p-coumaric acid, and caffeic acid~chlorogenic acid > sinapic acid ~ ferulic acid ~ p-coumaric acid, respectively. Caffeic acid, one of the most effective antioxidants in this study, is found in great amounts in a number of Mediterranean culinary herbs,

including sage, parsley, thyme and oregano (Wojdyło et al., 2007).

p-coumaric acid has an important presence in oregano (Shan et al., 2005) and there is evidence that it has an antioxidant effect in vivo, protecting LDL oxidation and reducing LDL levels in serum (Zang et al., 2000). In this study, rats were administered with p-coumaric acid in drinking water at low and high doses for 10, 21 and 31 days, and the oral administration of 317mg/day for 30 days significantly inhibited LDL oxidation and reduced LDL levels. Blood levels of 8-epiprostaglandin F2α were monitored as a marker of LDL oxidation. If p-coumaric is an efficient antioxidant for LDL, it may play a key role in the purported effect of oxidized lipoprotein on platelet activity to slow the progression of atherosclerosis.

Eugenol (4-allyl-2 methoxyphenol), the major component of cloves (Shan et al., 2005), is known for its aroma and medicinal values. In a very recent study the antioxidant activity of eugenol was evaluated by the degree of protection offered against free radical-mediated lipid peroxidation using both in vitro and in vivo models (Nagababu et al., 2010). The in vitro lipid peroxidation was induced in mitochondria by (Fe (II)-ascorbate) or (Fe(II) + H_2O_2), and eugenol completely inhibited both iron and Fenton reagent-mediated lipid peroxidation. The inhibitory activity of eugenol was about five times higher than that observed for α-tocopherol. The in vivo lipid peroxidation-mediated liver damage was induced by administration of CCl_4 to rats. Eugenol significantly inhibited the rise in serum glutamic oxalacetic transaminase (SGOT) activity and cell necrosis. The protective action of eugenol has been found to be due to interception of secondary radicals derived from endoplasmic reticulum (ER) lipids rather than interfering with primary radicals of CCl_4($\bullet CCl_3$/$CCl_3OO\bullet$).

In several previous studies, there is also evidence of the in vitro inhibition of LDL oxidation by eugenol. The activities of 23 selected essential oils in inhibiting the copper-catalyzed oxidation of human LDL were determined in vitro (Teissedre et al., 2000) and antioxidant activity average levels from different plant varieties showed that when eugenol is the major component, the inhibition of LDL oxidation ranged between 68% (clove) and 100% (St. Thomas Bay). In a study by Ito et al. (2005), the antioxidant action of eugenol compounds was analyzed in relation to the role of transition metals. Iron-mediated lipid peroxidation and autoxidation of Fe^{2+} ion were markedly inhibited by isoeugenol, and less effectively by eugenol. Copper-dependent oxidation of LDL was potently inhibited by eugenol and isoeugenol to the same extent. This study evidenced that antioxidant properties of eugenol compounds can be explained by forming complexes with reduced metals. The potent inhibitory effect of isoeugenol on lipid peroxidation may be related to the decreased formation of perferryl ion or the iron-oxygen chelate complex as the initiating factor of lipid peroxidation by keeping iron at a reduced state. Inhibition of LDL oxidation by eugenol compounds could be due to the suppression of free radical cascade of lipid peroxidation in LDL by reducing copper ion. Another recent study (Shukri et al., 2010) attempts to evaluate the organ and tissue protective in vivo effects of dietary cloves (Eugenia aromaticum) in chronic hyperglycaemia. The cloves (equivalent to 100 mg total eugenol + eugenyl acetate per kg body weight/day) were administered orally to streptozotocin-induced diabetic rats. Dietary supplementation with cloves reduced tissue injuries, especially in the lens and cardiac muscles, and to a lesser extent in the liver, but not the kidneys. Additionally, the cloves treatment significantly reduced blood sugar increases and lipid peroxidation in rats by restoring the antioxidant enzyme levels. Cloves inhibited hyperglycaemia-induced oxidative tissue damage and cataract formation in the eye lens. This study also demonstrates the in vivo antioxidative organ protective effects of clove in diabetics.

Rosmarinic acid, a hidroxycinamic acid derivate, is an ester of caffeic acid and 3,4-dihydroxy-phenyllactic acid typically found in Lamiaceae plants, such as basil (Ocimum spp.), rosemary (Rosmarinus spp.), thyme (Thymus spp.), mint (Mentha spp.) and oregano (Origanum spp.) (Petersen et al., 2003). The characterization of 26 spice extracts and their phenolic constituents showed that all spices in the Lamiaceae family tested contained very high concentrations of rosmarinic acid, mostly ranging from 1086 to 2563 mg/100 g of dry-weight being the major phenol in the Lamiaceae spices (Shan et al., 2005). Rosmarinic acid has two ortho-dihydroxy groups (catechol structures), which is the most important structural feature for strong antioxidant activity in phenolic compounds. This

compound may function as an antioxidant, scavenging superoxide, hydroxyl radicals and inhibiting oxidation of low-density lipoproteins (Nakamura et al., 1998; Fuhrman et al., 2000). In addition there is recent scientific evidence about the anti-inflammatory activity of rosmarinic acid. It has been recently demonstratedthat in a lipopolysaccharide-stimulated macrophage model, rosmarinic acid may contribute to the reduction of the inflammatory response by increasing the secretion of IL-10 (Mueller et al., 2010). Many anti-inflammatory studies have been performed on oregano extracts where the water soluble extract was reported to inhibit COX-2 secretion in humane epithelial carcinoma cells (Lemay, 2006), and it also exhibited anti-inflammatory activities in mouse models of stress-induced gastritis and contact hypersensitivity (Yoshino et al., 2006). However, these anti-inflammatory studies were all performed on oregano crude extracts without any information as to which compounds may be responsible. Recently, all of these compounds were elucidated coupled with bioactivity-guided isolation, and one of the anti-inflammatory constituents identified was rosmarinic acid (Shen et al., 2010). It was then subjected to the LPS-induced nitrite production assay and Western blotting of LPS-induced iNOS and COX-2 protein levels in murine cells, and all showed stronger or comparable anti-inflammatory activities compared with indomethacin, a recognized anti-inflammatory agent used as a control.

Curcumin (diferuloylmethane), a polyphenolic compound derived from the dietary spice turmeric (also an ingredient of curry powder), has been used for centuries as a treatment for inflammatory diseases. Extensive research within the past two decades has shown that curcumin, a complex molecule with multiple biological targets, mediates its anti-inflammatory effects through the down regulation of inflammatory transcription factors (such as NF-kB), enzymes (such as COX-2 and LOX-5) and cytokines (such as TNF-α, IL1 and IL-6) (Aggarwal et al., 2009). Most recent studies about its biological activities are summarized in Table 2. Because of the crucial role of inflammation in most chronic diseases, the potential of curcumin has been examined in neoplastic, neurological, cardiovascular, pulmonary and metabolic diseases. It is known that bis-α,β-unsaturated β-diketone, two methoxy groups, two phenolic hydroxy groups and two double-conjugated bonds might play an essential role in the antiproliferative and anti-inflammatory activities assigned to curcumin (Sandur et al., 2007).

A recent publication by Epstein et al. (2010) reviewed the evidence for the therapeutic potential of curcumin from in vitro studies, animal models and human clinical trials, and conclude that in complex multifactorial illnesses, such as systemic inflammatory diseases and cancer, an agent like curcumin that acts at a number of different cellular levels, perhaps offers a better chance of effective prophylaxis or treatment. The wealth of in vitro and pre-clinical data has provided a strong basis from which to progress to the trialling of curcumin in humans. Many of the molecular efficacies of curcumin demonstrated in cell culture systems and animal models are comparable to those seen in human subjects. The anti-inflammatory targets of curcumin including reduction of NF-kB, COX-2 and pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, translate into clinical anti-inflammatory efficacy with improvement of rheumatoid arthritis, psoriasis, post-operative inflammation, chronic anterior uveitis and orbital inflammatory pseudo-tumours (Epstein et al., 2010). Concordant with the finding that high concentrations of curcumin are achievable in gastrointestinal tissue, curcumin shows clinical benefits in some diseases, such as irritable bowel syndrome (Bundy et al., 2004) and gastric ulceration (Prucksunand et al., 2001). The in vitro findings of enhanced PPAR-γ expression and modulation of NOS, glutathione and other antioxidant activities are supported by the clinical power of curcumin to lower serum cholesterol (Soni et al., 1992) and improve the endothelial function in type 2 diabetes mellitus (Usharani et al., 2008).

There is also evidence that curcumin significantly inhibits both the initiation and propagation phases of LDL oxidation measured by TBARS and by relative electrophoretic mobility (REM) of LDL on agarose gel (Akhilender Naidu et al., 2002). These data suggest that curcumin, which constitute about 1-4% of turmeric, could be an effective antioxidant and offer protection against oxidation of human LDL, although it requires further investigations on in vivo models.

Its non-toxicity (Aggarwal et al., 2003) and good tolerability in human subjects, in

combination with strong promising results from cell line, animal and early human clinical studies support the ongoing research and development of curcumin as a preventive and disease-modifying agent. Nevertheless, curcumin exhibits poor bioavailability in humans (Anand et al., 2007). Major reasons that could contribute to the low concentration of curcumin in plasma and tissue could be related with its poor absorption, its rapid metabolism through conjugation, and its rapid systemic elimination (Garcea et al., 2005). To improve the bioavailability of curcumin, numerous approaches have been tested (Anand et al., 2007). These involve, first, the use of pipeline that interferes with glucuronidation; second, the use of liposomal curcumin; third, curcumin nano-particles; fourth, the use of curcumin phospholipid complex; and fifth, the use of structural analogues of curcumin (e.g., EF-24). The latter has been reported to have a rapid absorption with a peak plasma half-life.

Despite the lower bioavailability, the therapeutic efficacy of curcumin against various human diseases, including cardiovascular and neurological diseases (Aggarwal et al., 2009), diabetes (Usharani et al., 2008), arthritis (Venkatesha et al., 2011), cancer (Garcea et al., 2005) and Crohn's disease (Claramunt et al., 2009) has been documented. Enhanced bioavailability of curcumin in the near future is likely to bring this promising natural product to the forefront of therapeutic agents for the treatment of human disease.

Flavonoids

Most classes of flavonoids are found in herbs and spices. Frequently these include relatively uncommon aglycones and/or common aglycones with comparatively uncommon substitution patterns. Flavonoids do not generally exceed ~ 0.2–0.4 g/kg in Lamiaceae herbs but reach ~ 1.5–3 g/kg in Apiaceae herbs, ~ 3.5 g/kg in cloves and ~ 7 g/kg in bay leaf (Shan et al. 2005). Although flavonoids make a rather small contribution to the total antioxidant capacity of the spice extracts because of their low concentrations, in this review they are not discriminated because it has been shown that they are very powerful antioxidants with interesting biological activities, which are reviewed in Table 3. There is a large body of evidence from epidemiological studies that long-term administration of flavonoids can decrease, or at least, tend to decrease, the incidence of cardiovascular diseases and their consequences (Aherne et al., 2002; Mennen et al., 2004).

Besides their antioxidant properties, many reports have focused on the possible role of flavonoids in the modulation of ROS-dependent cell signaling pathways, emphasizing that their beneficial effects may be ascribed to other than simple antioxidant activity. Direct interactions between polyphenols and signaling proteins may occur, likely because of flavonoids' hydrophobicity and the presence of multiple hydroxyls on the favonoid rings, which enable hydrogen bonding to occur with protein functional groups (Fraga, 2007).

Flavonols are the most ubiquitous flavonoids in foods, and the main representative is quercetin. Among herbs and spices, quercetin is found in a great amount in dill (Proestos et al., 2005). The flavonoid quercetin has been proven to be an excellent antioxidant that also possesses anti-inflammatory, anti-proliferative and gene expression changing capacities in vitro. Within the flavonoid family, quercetin is the most potent scavenger of ROS, including O^{2-}, and RNS like NO^{-} $ONOO^{-}$ (Heijnen et al., 2002). These antioxidative capacities of quercetin are attributed to the presence of two antioxidant pharmacophores within the molecule that have the optimal configuration for free radical scavenging, i.e. the catechol group in the B ring and the OH group at position 3 on the AC ring.

Quercetin is known also to possess strong anti-inflammatory capacities. Several in vitro studies, using different cell lines, have shown that this molecule is capable of inhibiting LPS-induced cytokine production. For instance, quercetin inhibits LPS-induced TNF-α production in macrophages (Manjeet et al., 1999) and LPS-induced IL8 production in lung cells (A549) (Geraets et al., 2007). Moreover, in glial cells, it was even shown that quercetin can inhibit LPS-induced mRNA levels of two cytokines, i.e. TNF-α and IL-1 (Bureau et al., 2008). A possible explanation for these anti-inflammatory effects of quercetin may be found in the interplay between oxidative stress and inflammation. ROS are not only involved in the occurrence of oxidative stress, but also in the promotion of inflammatory processes via activation of transcription factors, such as NF-κB and activator protein (AP)-1 which induce the production of cytokines like TNF- α.

Indeed, it has already been shown that quercetin can inhibit the production as well as the gene expression of TNF- α via modulation of NF-κB in human peripheral blood mononuclear cells (Nair et al., 2006). In another recent study, it is suggested that the modulation of iNOS, COX-2 and CRP by quercetin may contribute to the anti-inflammatory effects of these in Chang Liver cells, via mechanisms likely to involve blockade of NF-κB activation and the resultant up-regulation of the pro-inflammatory genes (García-Mediavilla et al., 2007).

Until now, only the antioxidative and anti-inflammatory effects of quercetin have been shown in vivo as well. Interestingly, these two effects of quercetin appear to be more pronounced when the respective basal levels of the occurring oxidative stress and inflammation are high. This indicates that the use of quercetin supplementation is especially fruitful in people suffering from a disease that is associated with both processes, such as hypertension (Boots et al., 2008).

Flavones are much less common than flavonols in fruit, vegetables and herbs. Flavones consist chiefly of glycosides of luteolin and apigenin. One of the most important edible sources of flavones identified to date in herbs and spices is parsley, which contains a great amount of apigenin (2 g/kg) (Justesen et al., 1998). Luteolin is also the main flavonoid in thyme and mint (Proestos et al., 2005). Both compounds have been linked to antioxidant and anti-inflammatory effects. In a recent study the anti-inflammatory activities of several flavonoids were evaluated (Lee et al., 2010). Luteolin and apigenin strongly inhibited LPS-induced nitrite production in a dose-dependent manner, mainly due to the suppression of inducible NO synthase. Luteolin showed the strongest inhibitory activities on 15-LOX and a potent inhibition on COX-2 reaction. Recently, different compounds from plant extracts were also tested in the LPS-stimulated macrophage model to see their anti-inflammatory response, and luteolin and apigenin reduced IL-6 and TNF-α secretions, and also reduced the expression of iNOS and COX-2 (Mueller et al., 2010). These findings further the idea that a diet rich in fruits, herbs and spices may contribute to the reduction of inflammation and help prevent related diseases.

The hypolipidemic effect of these compounds was also proven in hyperlipidemic rats induced by high fat diet, combined with the oral administration of Perilla Frutescens, a herb used in traditional Chinese medicine. The main flavonoids present in this herb are luteolin and apigenin. The results of this study showed that Perilla Frutescens was highly effective at decreasing the levels of serum total cholesterol, triacylglycerols, LDL-c and adipose tissue accumulation and in increasing the level of serum HDL-c (Feng et al, 2011). In conventional therapy, steroidal and non-steroidal anti-inflammatory drugs that inhibit COX are used to treat acute inflammation, but are unsuccessful at curing chronic inflammatory diseases, such as rheumatoid arthritis or osteoarthritis. Furthermore, these compounds exhibit several undesirable side effects. Therefore, alternative treatments with safer compounds are needed (Yoon et al., 2005).

CONCLUSIONS

Research on the structure activity relationships in spice components has become an exciting field as these compounds play a major role in the culinary, industrial and pharmacological fields. A range of bioactive compounds in herbs and spices has been studied for antioxidant and anti-inflammatory properties in vitro and in vivo animal models, but the challenge lies in integrating this knowledge to ascertain whether these effects can be observed in humans, and within defined cuisines.

In summary, as several metabolic diseases and ageing-related degenerative disorders are closely associated with oxidative and inflammatory processes in the body, the use of herbs and spices, or their bioactive principles, as a source of antioxidants to combat oxidation warrants further attention. Immediate studies should focus on validating the antioxidant capacity of herbs and spices, as well as testing their effects on markers of oxidation and inflammation. This should be done in parallel with clinical trials that are aimed at establishing these bioactive compounds reviewed here as mediators of disease prevention. Based on the positive results from the latest research in this field, several of the plant extracts tested could potentially be used as food supplements to provide beneficial effects.

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ABBREVIATIONS

ROS = Reactive oxygen species
CVD = Cardiovascular diseases
ROI = Reactive oxygen intermediates
NF- κB = Nuclear factorκB
TNF-α = Tumor necrosis factor
COX = Cyclooxygenase
LOX = Lipooxygenase
IL = Interleukin
LDL-c = Low-density lipoprotein cholesterol
LPS = Lipopolysaccharide
HDL-c = High-density lipoprotein cholesterol
NO = Nitric oxide
oxLDL = Oxidised low-density lipoprotein
PPARγ = Peroxisome proliferator activated receptor
iNOS = Inducible nitric oxide synthase
STAT3 = Signal transducer and activator of transcription 3
MAPK = Mitogenactivated-proteinkinases
CRP = C- reactive protein

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Zang, L. et al. 2000. Effect of antioxidant protection by p-coumaric acid on low-density lipoprotein cholesterol oxidation. American Journal of Physiology - Cell Physiology, 279:C954-C960.

Zheng, W. et al. 2001. Antioxidant activity and phenolic compounds in selected herbs. J.Agric.Food Chem., 49:5165-5170.

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Table 1 Recent advances in biological activities of phenolic terpenes in herbs and spices.

Active principle	Molecule	Origin studied	Biological activity	Reference
Monoterpenes				
Thymol		Oregano, thyme	Inhibits LDL oxidation in vitro	Kulišić et al., 2007
			Prevents peroxynitrite-induced formation in vitro	Prieto et al., 2007
			Anti-inflammatory: inhibits oxLDL induced pro-inflammatory cytokines secretion (TNF-α, IL-1b, IL-6), and increases the anti-inflammatory cytokine IL-10 in vitro	Ocaña-Fuentes et al., 2010
Carvacrol		Oregano, thyme	Inhibits LDL oxidation in vitro	Kulišić et al., 2007
			Prevents in vitro peroxynitrite-induced formation	Prieto et al., 2007
Diterpenes				
Carnosic acid		Rosemary, sage	Protects lipid membranes against oxidative damage in vitro	Laura et al., 2010 Pérez-Fons et al., 2006
			Anti-inflammatory: inhibits the formation of pro-inflammatory leukotrienes and 5-LOX	Mueller et al., 2010
Carnosol		Rosemary, sage	Protects lipid membranes against oxidative damage in vitro	Laura et al., 2010 Pérez-Fons et al., 2006
			Anti-inflammatory: inhibits the formation of pro-inflammatory leukotrienes and 5-LOX	Poeckel et al., 2008 Mueller et al., 2010
Rosmanol		Rosemary, sage	Anti-inflammatory: inhibits the activation of NF-kB and STAT3	Lai et al., 2009
Epirosmanol		Rosemary	Inhibits lipid peroxidation in the cell membrane and human LDL	Hui-Hui et al., 2001
			Inhibits the formation of apo B in LDL	

Table 2 Recent advances in biological activities of hydroxycinnamic acids and derivates in herbs and spices.

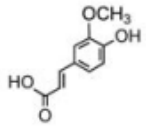
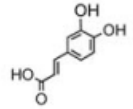
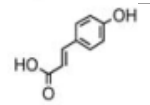
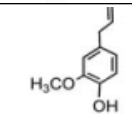
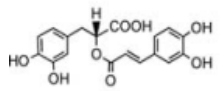
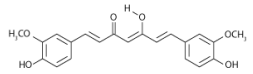
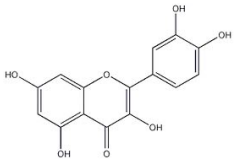
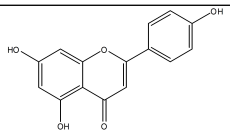
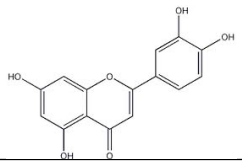
Active principle	Molecule	Origin studied	Biological activity	Reference
Ferulic acid		Mint	Inhibits LDL peroxidation in vitro	Cheng et al., 2007
Caffeic acid		Sage, parsley, lemon balm, thyme, oregano	Inhibits LDL peroxidation in vitro	Cheng et al., 2007
p-coumaric		Oregano	Inhibits LDL oxidation and reduces LDL cholesterol in vivo	Zang et al., 2000
Eugenol		Clove, basil	Inhibits LDL oxidation in vitro and in vivo Antioxidative organs protection effects	Teissedre et al., 2000 Ito et al., 2005 Nagababu et al., 2010 Shukri et al., 2010
Rosmarinic acid		Oregano, sage, basil, rosemary, thyme, mint	Anti-inflammatory: increases secretion of the anti-inflammatory cytonkine IL-10 Reduces expression of iNOS and COX-2 protein	Mueller et al., 2010 Shen et al., 2010
Curcumin		Turmeric	Anti-inflammatory: reduction of NF-kB, COX-2 and pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α in vitro and in vivo. Antioxidant: increase in PPARγ, glutathione, Haem oxygenase-1, superoxide dismutase and ROS; reduction of NO synthase Reduction in colonic NF-kB inducible NO synthase and various measures of oxidative stress such as myeloperoxidase and lipid peroxidation in vivo. Inhibition of LDL oxidation in vitro.	Epstein et al., 2010 Aggarwal et al., 2009 Akhilender Naidu et al., 2002

Table 3 Recent advances in biological activities of main flavonoids in herbs and spices.

Active principle	Molecule	Origin studied	Biological activity	Reference
Quercetin		Dill	Anti-inflammatory: inhibits iNOS, COX-2 and CRP, and down-regulates NF-kB and TNF-αsecretion. ROS scavenging in vitro and in vivo. Anti-inflammatory effects in vitro and in vivo. Modulation of gene expression in vitro.	García-Mediavilla et al., 2007 Boots et al., 2008
Apigenin		Parsley	Anti-inflammatory: Suppression of inducible NO synthase. Inhibition of LOX and COX-2 Reduccion IL-6 secretion and TNF-α and expression of iNOS.	Lee, et al., 2010 Mueller et al., 2010
Luteolin		Thyme, mint	Decreases the levels of serum total cholesterol, triacylglycerols, LDL-c and increases the level of serum HDL-c	Feng et al., 2010

APPENDIX II

REVIEW: Impact of Various Factors on Pharmacokinetics of Bioactive Polyphenols: An Overview

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IMPACT OF VARIOUS FACTORS ON PHARMACOKINETICS OF BIOACTIVE POLYPHENOLS: AN OVERVIEW

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Abstract

Several epidemiological studies throughout the years have suggested that polyphenols from fruits and vegetables promote health and reduce the risk of certain chronic and neurodegenerative diseases. Yet, it has been proved to be extremely difficult to quantitatively establish the benefit afforded by polyphenols, principally due to the limited understanding of the extent of its absorption and metabolic fate. Pharmacokinetics includes the study of the mechanisms of absorption and distribution of an ingested polyphenol, its chemical changes in the body (e.g. by metabolic enzymes), and the effects and routes of excretion of the metabolites. In recent years, there have been major advances in our knowledge of polyphenol absorption and metabolism, and it is apparent that most classes of polyphenols are sufficiently absorbed to have the potential to exert biological effects. The pharmacokinetics of polyphenols includes the same steps as those for orally ingested drugs (LADME) and faces some of the same challenges, including transporters and enzymes. However, unraveling the bioavailability of polyphenols is even more challenging than with drugs, since many other factors, such as the variety in the chemical structure, the food matrix and the gut microbiota, can affect bioavailability of polyphenols during digestion. This review focuses on the most relevant factors that influence polyphenol pharmacokinetics, and also on the most recent technological strategies developed to overcome the poor bioavailability of phenolic compounds and thus increase their potential for greater health benefits.

KEYWORDS: bioavailability / food matrix / metabolism / polyphenols / processing

1. INTRODUCTION

The role of polyphenols, as the components responsible, in part, for the protective effects of a fruit and vegetable-rich diet has become an increasingly important area of human nutrition research [1-3]. There is increasing evidence that modest long-term intakes of phenolic compounds can have favorable effects on the incidence of cancers and chronic diseases, including cardiovascular disease (CVD), type II diabetes, and impaired cognitive function, which are increasingly frequent in Western populations [4]. However, it has been proved to be extremely difficult to quantitatively establish the benefit afforded by polyphenols, principally due to the limited understanding regarding the extent of absorption and the metabolic fate of individual polyphenols from particular foods.

Pharmacokinetics describes how the body affects the compound of interest after administration through the mechanisms of absorption and distribution, as well as the chemical changes the substance undergoes in the body (e.g. by metabolic enzymes), and the

effects and routes of excretion of the metabolites. Pharmacokinetics of bioactive food compounds includes the same steps as those for orally ingested drugs (LADME) and faces some of the same challenges, including transporters, molecular structures, and enzymes. However, the pharmacokinetic profiles differ markedly among the different classes of polyphenols due to the different chemical structures. Unraveling the bioavailability of bioactive food compounds is even more challenging than with drugs, since many other factors, such as the variety in the chemical structure, the food matrix and the gut microbiota, can affect bioactive food compounds during digestion [5]. In this sense, bioaccessibility is a crucial factor in polyphenol bioavailability. This is influenced by the composition of the digested food matrix, the synergisms and antagonisms of the different components [6], but also by physicochemical properties, such as pH, temperature and the texture of the matrix [7].

Polyphenols are relatively poorly absorbed, the absorption ranging from 0.3% to 43%, and the circulating plasma concentrations of their metabolites can be low [8]. Yet, recently it has been established that due to the presence of significant amounts of polyphenols in the colon, one cannot infer biological responses from polyphenols dietary intake records without considering polyphenol–microbiota interactions [9]. Therefore, polyphenol-metabolizing phenotypes or 'metabotypes' have to be considered as an important aspect influencing polyphenol bioavailability.

The methodology generally used to study the bioavailability of polyphenols also has to be considered. The in vivo approach most widely used is the single-dose design. It involves the intake of one portion of food containing the tested polyphenol. In such a way, the increase in blood concentration is transitional and mainly reflects the ability of the organism to take up the polyphenol from the food matrix. Therefore, the observed increase can only have a minor implication for tissue uptake and bioactivity. On the contrary, under conditions of regular intake, even low amounts of polyphenols can be "repeatedly" absorbed and can significantly increase the concentrations at both the plasma and cellular levels [10].

All these factors are of increasing interest as food industries are continually involved in developing new products, defined as "functional" by virtue of the presence of specific antioxidants or phytochemicals. In this sense, bioavailability is a key step for functional foods and the health claims related to food components, followed by knowledge of the circulating metabolites, which leads to anv understanding of the mechanisms of action in relation to the benefit [11].

In the present contribution, the discussion of factors influencing the bioavailability of polyphenols will focus, on one hand, on food-related factors such as the polyphenols' chemical structure, the nature of the food matrix or food processing, and on the other hand, on human-related factors such as metabolic enzymatic activity, transporters and gut microbiota. In the second part of the review, the most recent technological strategies for overcoming the poor bioavailability of phenolic compounds have been highlighted, which are of particular interest in the formulation of functional foods.

2 FACTORS AFFECTING BIOAVAILABILITY OF PHENOLIC COMPOUNDS

2.1 Structure of phenolic compounds

Bioavailability is mainly influenced by the chemical structure of the phenolic compound, and the structure determines the extent to cross membranes and the rate of absorption in the gut to form the active metabolites [12]. Their chemical structure depends on different factors, such as the degree of glycosylation, acylation, conjugation with other phenolics, molecular size, the degree of polymerization, solubility, hydrophobicity and pk_a. **Figure 1** and **Figure 2** show the classification of most common dietary polyphenols, with characteristic examples of each phenolic family and its chemical structure.

In general terms, the highest bioavailability has been reported for isoflavones, followed by flavanols, flavanones and flavonol glycosides, while the proanthocyanidins, flavanol gallates and anthocyanidins are the most poorly absorbed [8].

The glycosylation of a phenolic compound influences its chemical, physical and biological properties [13, 14]. Flavonoid glycosides (**Figure 3**), with sugars such as glucose, galactose, or xylose, are first deglycosylated by hydrolysis prior to intestinal absorption as a result of the action of lactase

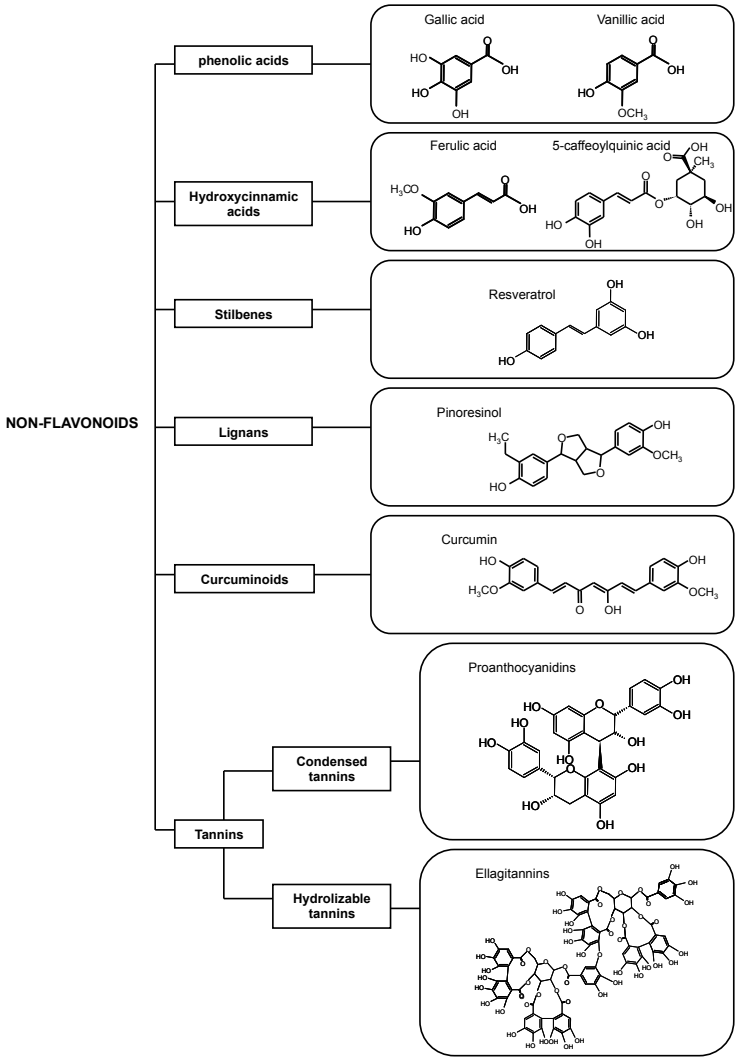

Figure 1. Classification of common dietary non-flavonoids polyphenols with characteristic examples of each phenolic gamily.

phloridzin hydrolase (LPH) in the brush-border of the small intestine epithelial cells. The resulting aglycones may then enter the epithelial cells by passive diffusion as a result of their increased lipophilicity and proximity to the cellular membrane [15, 16]. However, when flavonoids glycosides are attached to a rhamnoside moiety instead of glucose, galactose, or xylose, these flavonoids cannot be absorbed through the small intestine. They are degraded by the action of rhamnosidase enzymes produced by the colonic microflora [17].

The sugar moiety is a major determinant of the absorption site and bioavailability of the flavonoid since flavonoid monoglucoside bioavailability is several times higher than of flavonoid rutinosides (rhamnose-glucose) [16]. Flavonoid mono-glucosides are absorbed in the small intestine

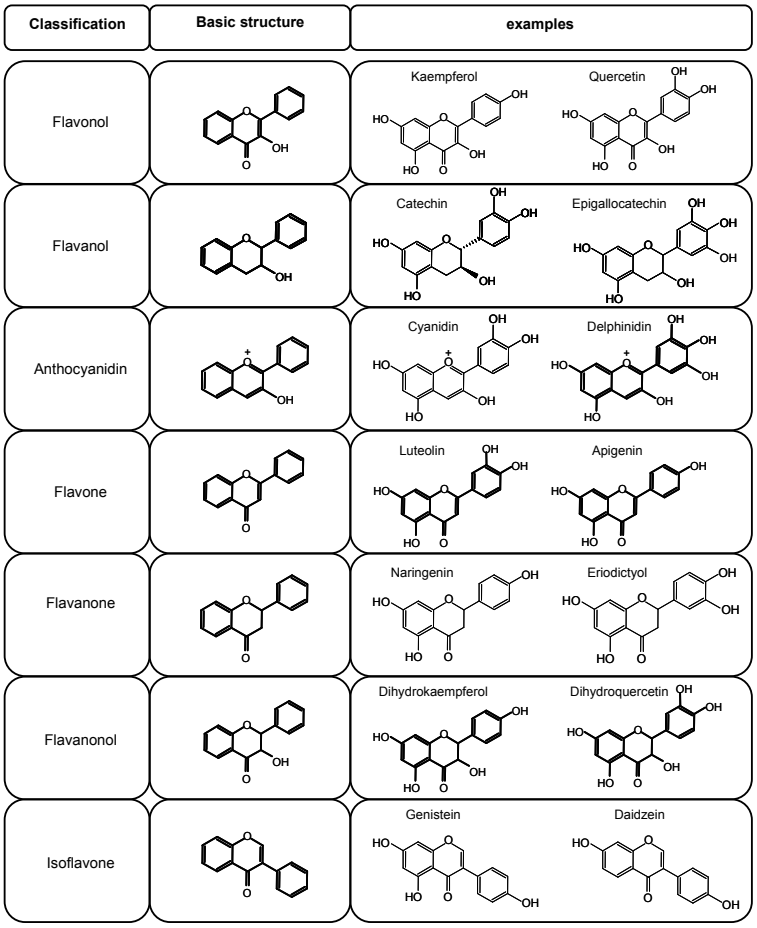

Figure 2. Basic structure skeleton of flavonoids and the chemical structure of the most common flavonoids in each group.

after hydrolysis, whereas flavonoid rutinosides must reach the microflora. By enzymatic treatment with α-rhamnosidase, the rutinoside moiety can be hydrolyzed to yield glucoside moiety [18]. A recent study showed that after an α-rhamnosidase-treatment in orange juice, bioavailability of naringenin-7-O-glucoside was increased about 4-fold based on AUC compared to naringenin-7-O-rutinoside (untreated orange juice) in healthy human subjects.

The absorption of oligomeric and polymeric proanthocyanidins across the intestinal epithelium requires a preliminary degradation from their high molecular weight into lower molecular-weight compounds [19, 20]. There have been numerous feeding studies with animals and humans, indicating that the oligomeric and polymeric proanthocyanidins are not absorbed to any degree. A study in which ileostomists consumed apple juice indicates that most pass unaltered to the large intestine [21], where they are catabolized by the colonic microbiota, yielding a diversity of phenolic acids and aromatic components, which are absorbed into the circulatory system and excreted in the urine. There is a report, based on data obtained from an in vitro model of gastrointestinal conditions that procyanidins degrade, yielding more readily-

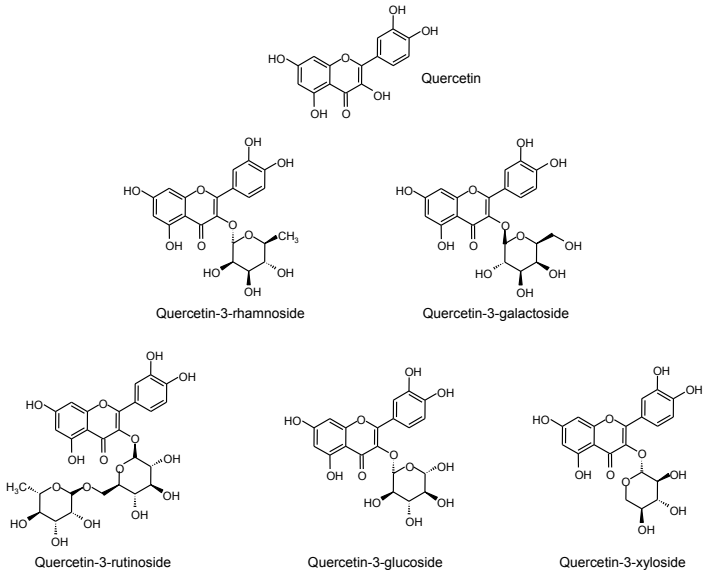

Figure 3. Chemical structure of most common flavonoid glycosides.

absorbable flavan-3-ol monomers [22]. However, in vivo studies [23-25], including feeding to ileostomists [21], have not supported this conclusion, suggesting that not more than 10% of procyanidin dimers are converted into monomers in this way [26, 27].

Apart from the chemical structure of phenolic compounds, their isomeric configuration has also been reported to affect their absorption. Flavonoids with different stereochemistry can exhibit different bioavailability and bioefficacy. An example is the case of the isomer of (-)-epicatechin and (+)-catechin, where, despite having the same molecular weight, (+)-catechin has been reported to have a higher bioavailability than (-)-epicatechin [12, 28, 29].

2.2 Food matrix

The absorption of polyphenols and other food constituents can be very complex depending upon the food matrix consumed, and research into this aspect is providing a better understanding for developing a tailored approach. Bioaccessibility has been defined as the fraction of a compound which is released from the food matrix in the gastrointestinal lumen and thereby made available for intestinal absorption. Mastication in the mouth initiates the process and several digestive fluids containing different enzymes continue to break down the food matrix in the stomach and throughout the remainder of the gastrointestinal lumen [29]. Bioaccessibility is influenced by the composition of the digested food matrix, the synergisms and antagonisms of the different components [30], but also by physicochemical properties, such as pH, temperature and texture of the matrix [31]. Table 1 summarizes most recent studies assessing the effect of different kinds of food matrix on the availability of phenolic compounds.

The simultaneous intake of different foods can affect polyphenol absorption. Administering blackcurrant anthocyanins extract (delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, cyanidin 3-O-glucoside, and cyaniding 3-O-rutinoside) dissolved in water, with or without the addition of oatmeal, rats showed a different pattern of anthocyanins in plasma over time in rats [32]. The time required to reach the maximal plasma concentration of anthocyanins was shorter after administering anthocyanin/water than after anthocyanin/oatmeal. In consequence, the total amount of anthocyanins absorbed between 0 and 7 h was significantly higher after the

Table 1. Studies related to the effect exerted by the food matrix on the bioavailability of polyphenols.

	Phenolic compounds	Food matrix studied	Administration	Effect on bioavailability	Reference
Anthocyanins	Delphinidin 3-O-gluc, delphinidin 3-O-rut, cyanidin 3-O-gluc, and cyaniding 3-O-rut	Blackcurrant extract with water vs. with oatmeal	Acute oral intake (rats)	Total amount of anthocyanins absorbed was significantly higher after anthocyanins/water administration than after anthocyanins/oatmeal administration	Walton et al., 2009
	Malvidin-3-gluc	Anthocyanin extract with ethanol vs. w/o ethanol	Caco-2 cell model	Ethanol increased anthocyanins bioavailability in vitro	Faria et al., 2009
	Malvidin, peonidin, cyanidin, p-coumaroyl-acylated	Red wine vs. dealcoholized red wine	Sustained oral intake (humans)	Alcohol had no influence in the formation of phenolic metabolites by the gut microbiota	Jiménez-Girón et al., 2013
Hydroxycinnamic acids	Caffeoylquinic acids (CAA)	Instant coffee vs. apple smoothie vs. cloudy apple juice	Ileostomy (humans)	Coffee and apple smoothie are better matrices for delivery of CAA to the colon, whereas after cloudy apple juice consumption, CAA are more likely to be distributed systemically	Erk et al., 2013
	Chlorogenic acid	Coffee vs. coffee with milk	Caco-2 cell model and sustained intake (rats)	The presence of milk proteins had no effect on CQA absorption in both in vitro and in vivo experiments.	Dupas et al., 2006
Flavanones	Naringenin	Tomato sauce with oil vs. w/o oil	Acute oral intake (humans)	Lipid food matrix potentially enhanced the plasma half-life of glucuronide metabolites of naringenin	Tulipani et al., 2012
Flavanols	Catechin and epicatechin	Sugar-free cocoa vs. cocoa with carbohidrate-rich meals	Sustained oral intake (humans)	The uptake of flavanols was significantly increased by concurrent carbohydrate consumption	Schramm et al., 2003
	Procyanidins	Grape seed procyanidin extract vs. GSPE with carbohydrate-rich food	Acute oral intake (rats)	An enhanced uptake of the monomeric forms was observed When GSPE was ingested with carbohydrate-rich food	Serra et al., 2010
	Epicatechin	Apple puree vs. epicatechin enriched drink	Acute oral intake (humans)	Plasma absorption and urinary excretion were all significantly higher after ingestion of epicatechin enriched drink compared with apple puree	Hollands et al., 2013
	Catechin and epicatechin	Cocoa vs. cocoa with milk	Acute oral intake (humans)	Plasma epicatechin levels were reduced by almost 2-fold when dark chocolate was accompanied by milk	Serafini et al., 2003
		Cocoa beverage vs. cocoa beverage with milk	Sustained oral intake (humans)	Drinking a cocoa beverage with milk does not influence the concentrations of flavan-3-ol metabolites in plasma or excreted in urine	Roura et al., 2007 Roura et al., 2008
Flavonols	Quercetin and kaempferol	Tea vs. tea with milk	Sustained oral intake (humans)	Flavonols are absorbed from tea and that their bioavailability is not affected by addition of milk	Hollmann et al., 2001
	Quercetin aglycone and quercetin 3-O-gluc	Quercetin with low fat meal vs. high fat meal	Acute oral intake (pigs)	Bioavailability from both quercetin aglycone and quercetin 3-O-glucoside is enhanced in a diet enriched with fat compared with a low-fat diet	Lesser et al., 2004
Stilbenoids	Resveratrol	Red wine and grape juice vs. grape extract	Acute oral intake (humans)	Bioavailability of resveratrol from wine and grape juice was 6-fold higher than that from grape extract	Ortuño et al., 2010

administration of anthocyanin/water, than after anthocyanin/oatmeal. The reason for the delayed absorption of anthocyanins from the oatmeal treatment might be the higher viscosity of the food matrix and the longer transit time of the chyme through the gastrointestinal tract, or the potential binding of anthocyanins to other food components.

Erk et al. [33] studied the possible effects of different food matrices on the bioavailability of caffeoylquinic acid (CQA) using ileostomy volunteers. They chose three different food sources: a cloudy apple juice (CAJ), an apple smoothie (AS) and an instant coffee beverage. Results showed that coffee and AS may be better matrices for delivering of polyphenols to the colon, whereas after CAJ consumption, CQA and its metabolites are more likely to be distributed systemically or degraded during passage through the gastrointestinal tract.

Evidence suggests that variations in polyphenol absorption also occur due to interactions between polyphenols and other food components. Tulipani et al. [34] carried out a randomized controlled cross-over study to evaluate the effect of adding an oil matrix during the processing of tomato sauce on the bioavailability of tomato phenolics. They suggested that the lipid matrix added to the sauce might stimulate the occurrence of re-absorption events by enterohepatic circulation, potentially enhancing the apparent plasma half-life of glucuronide metabolites of naringenin prior to excretion. In another study, the influence of dietary fat on the oral bioavailability of quercetin was assessed in pigs. They concluded that bioavailability of both quercetin aglycone and quercetin-3-O-glucoside was enhanced in a diet enriched with fat compared with a low-fat diet [35]. In agreement with this study, Azuma et al. [36] showed that in rats fed with 150umol/kg of quercetin, its absorption was enhanced significantly when it was complemented with lipids and emulsifiers, which seemed to be affected by quercetin's solubility in the vehicles used to administer it.

Other compounds, such as ethanol, seem to be important when present in the same food matrix, and appeared to increase the in vitro bioavailability of anthocyanins by using a Caco-2 cell model [37]. In a more recent study, Jiménez-Girón et al. [38] assessed the composition of microbial phenolic metabolites in human feces collected after regular consumption of either red wine or de-alcoholized red wine, to study the effect of alcohol on anthocyanin absorption and gut metabolism. Alcohol did not seem to influence the formation of phenolic metabolites by the gut microbiota.

Carbohydrates may also exert an effect on phenolic absorption. Schramm et al. [39] suggested that the uptake of flavanols from cocoa (catechin and epicatechin) can be increased significantly by concurrent carbohydrate consumption (bread, sucrose and grapefruit juice). They also observed a limited influence of protein (whole milk and steak) and lipid-rich meals (whole milk and butter) on the parameters of AUC and C_{max}, and suggested that protein-flavanol and lipid-flavanol interactions have little effect on flavanol bioavailability. In agreement with this study, when a grape seed procyanidin extract was ingested with carbohydrate-rich food, an enhanced uptake of the monomeric forms was observed [40]. This enhanced effect with carbohydrate-rich foods may have been mediated by a carbohydrate-specific effect on gastrointestinal physiology (e.g. motility and/or secretion) or a carbohydrate-specific enhancement of the activity of a yet-unidentified carbohydrate–flavanol transporter.

Several studies have also hypothesized that milk proteins could inhibit absorption of flavonoids. However, these reports appear to be contradictory. Serafini et al. [41] indicated that plasma epicatechin levels were reduced by almost 2-fold when dark chocolate was accompanied by milk. On the contrary, two more recent studies have shown that drinking a cocoa beverage with milk does not affect plasma flavan-3-ol monomer concentrations [42] and does not influence the concentrations of flavan-3-ol metabolites in plasma or excreted in urine [43, 44]. In agreement with these studies, some other reports indicate that milk does not affect the absorption of catechin, quercetin or kaempferol when added to green or black tea [45, 46]. Dupas et al. [47] assessed the effect on CQA bioavailability of adding milk to coffee. CQA absorption and bioavailability were studied in vitro using a Caco-2 cell model coupled with an in vitro digestion process, and in vivo in a chronic supplementation study in which rats were fed with coffee or coffee and milk daily for 3 weeks. Both experiments showed that CQA absorption is

Table 2. Studies related to the effect exerted by the food processing on the bioavailability of polyphenols.

Phenolic compounds	Food processing	Administration	Effect on bioavailability	Reference
Ferulic acid, vanillic acid, sinapic acid, and 3,4-dimethoxybenzoic acid	Whole wheat bread vs. bread with bioprocessed bran	Acute oral intake (humans)	Bioprocessing (fermentation with enzyme treatment) can remarkably increase the bioavailability of phenolic acids and their circulating metabolites	Anson et al., 2011
Ellagitannins and ellagic acid	Raw vs. thermal processed strawberries	Acute oral intake (humans)	Processing increased the amount of free ellagic acid 2.5-fold, but this had no effect on the transformation in urolithins by the gut microbiota or in the excretion of urolithin metabolites in urine	Truchado et al., 2012
Naringenin and chlorogenic acid	Fresh vs. cooked tomatoes	Acute oral intake (humans)	Plasma concentrations of naringenin and chlorogenic acid increased significantly after administration of cooked tomatoes, but not after administration of fresh tomatoes	Bugiansesi et al., 2004
Naringenin and hesperidin	Orange fruit vs. orange juice	Acute oral intake (humans)	No difference was observed in absorption or excretion of either flavanone between the fruit and juice matrices	Brett et al., 2009

unmodified by the addition of milk proteins.

2.3 Food processing

Processing of plant foods can influence the bioaccessibility of nutrients, mainly through changes in the plant cell wall structure and properties. Several studies have been carried out to assess how food processing can affect polyphenol bioavailability, and these are summarized in Table 2.

Since plant cell walls are largely resistant to degradation in the upper gut, they represent an important barrier for the release of bioactive compounds. One example is ferulic acid in whole grain wheat, where the bioavailability of ferulic acid is limited due to its high binding affinity to polysaccharides. Mateo Anson et al. [48] studied the bioaccessibility of ferulic acid from wheat fractions and breads consumed by human subjects. The authors observed that wheat ferulic acid had a low bioaccessibility (<1%). However, the bioaccessibility increased when free ferulic acid was added to flour (~ 60%). Cereal bioprocessing is receiving increasing attention as a technique for purportedly improving the bioavailability of bound phytochemicals in grains. This technique uses hydrolytic enzymes to release phytochemicals selectively from the bran layer. Recently, the same authors (49] developed a bioprocessing technique whereby wheat bran undergoes a yeast fermentation and enzyme treatment procedure. When this wheat bran was incorporated into wholemeal bread and consumed by volunteers the plasma concentrations of ferulic, vanillic and sinapic acids, and 3,4-dimethoxybenzoic acid were 2- to 3-fold higher than in the control bread [49].

Several studies also suggest that thermal processing of raw products may increase the amount of free phenolic compounds in the final product by releasing them from the cells. Takenaka et al. [50] found an increase in 3-caffeoylquinic, 4-caffeoylquinic, 3,4-dicaffeoylquinic and 4,5-di-caffeoylquinic acids in boiled sweet potatoes due to the isomerization of 5-caffeoylquinic and 3,5-di-caffeoylquinic acids, the major phenolic compounds of this vegetable. The authors hypothesized that heat is one of the factors causing isomerization. A significant increase (ranging from 66 to 94%, depending on the cooking method applied) in the total caffeoylquinic acids, was also observed in artichokes after boiling, steaming and frying [51]. This enhancement of polyphenolic content after processing could result in a consequent enhanced bioavailability, but few studies have reported this effect in vivo. Truchado et al. [52], studied the effect of the thermal processing of strawberries on their polyphenol bioaccessibility and metabolism, particularly of ellagitannins and ellagic acid. These compounds are transformed into urolithins by the gut microbiota, which exert several biological effects that could be responsible for the health benefits of strawberries. The study showed that processing caused a 2.5-fold increase in the amount of ellagic acid, but this had no effect on the transformation in urolithins by the gut microbiota or in the excretion of urolithin metabolites in urine, showing that the release of ellagic acid from ellagitannins is not a relevant factor affecting the microbial metabolism. Bugianesi et al. [53] carried out a human cross-

over study to evaluate the effect of domestic cooking on the bioavailability of antioxidants (i.e. carotenoids and polyphenols) after the administration of a test meal containing cherry tomatoes. The results demonstrated that plasma concentrations of naringenin and chlorogenic acid increased significantly over the baseline after the administration of cooked cherry tomatoes, but not after the administration of raw cherry tomatoes. In contrast to these studies, analysis of the plasma pharmacokinetic and urinary excretion data on a dose-adjusted basis indicated no difference in the absorption or excretion of flavanones (hesperetin, naringenin and eriodictyol) between the raw orange fruit and juice matrices [54].

2.4 Polyphenol metabolism and intestinal transport

Once polyphenols are released from the food matrix and are bioaccessible in the gastrointestinal tract, relative concentrations of free aglycones and conjugates in the plasma are determined by the metabolic activities of conjugating enzymes and efflux transporters. These coupling processes are present in disposition tissues and represent a key factor in the inter-individual variation of polyphenol bioavailability. Since significant numbers of bacteria also appear to participate in the metabolism of polyphenols, the gut microbiota has also been considered in this review as an important metabolic factor influencing polyphenol bioavailability.

2.4.1 Metabolic enzymes

Once a bioactive compound or drug has entered into an enterocyte, it is subjected to a battery of phase I and II enzymes. The phase I reactions include oxidation, reduction and hydrolysis, which primarily serve to increase the hydrophilicity of the molecule, and expose or add a functional group (such as a hydroxyl group) to facilitate phase II conjugation reactions. Oxidation is the most predominant reaction involved in the phase I metabolism of xenobiotics, and is carried out mainly by a family of closely related isozymes known as the cytochrome P450-dependent mixed-function oxidases (CYPs) [55].

The parent polyphenols (or their phase I metabolites) that contain suitable functional groups (e.g., a hydroxyl group) undergo conjugation reactions with endogenous compounds to yield more polar and water-soluble compounds, which are ideal substrates for active transport out of the cell and eventually excretion from the body. The principal conjugation reaction is the formation of glucuronides catalyzed by a family of enzymes known as the uridine diphosphoglucuronosyl transferases (UGTs), and conjugation with a sulpho moiety ($SO3^-$) catalyzed by various sulphotransferases (SULTs). Less-polar conjugates may also be formed by methylation, catalysed by catechol-O-methyl transferase (COMT).

The metabolizing enzymes in the gut and liver represent a major factor responsible for the wide variation in a population's response to drugs, toxins and polyphenols. For example, UGT activity has been shown to vary 6- to 15-fold in human liver microsomes and 10- to 100-fold in intestinal microsomes [56]. Both environmental and genetic factors contribute to this wide variation in the rate and extent of polyphenol metabolism and disposition between individuals. It is now well recognized that genetic polymorphisms of the xenobiotic-metabolizing enzymes and active transport proteins are responsible for a large part of the variable exposure at their site(s) of action, and consequently the response [55]. Most information is available for the CYPs, and genetic polymorphisms have been identified for most CYP isozymes involved in xenobiotic metabolism (57]. Combinations of different CYP gene variants determine different phenotypes: poor (PM); intermediate (IM); extensive (EM); and ultra-rapid metabolizers (UM). Important interethnic differences in the distribution of the different phenotypes have been observed, and these are particularly relevant to drug therapy, often resulting in adverse reactions and toxicity in PMs, or no response in UMs. Many variants of UGT 1A1 and 2B7 have also been identified, which could have also functional significance with regard to xenobiotic metabolism [58]. It is highly likely that these polymorphisms play an important part in the variable bioavailability and, thus, the efficacy of polyphenols.

The human body is continually exposed to innumerable foreign chemicals and phytochemicals through our diet, which, if absorbed, these can have a profound influence on our capacity to metabolize xenobiotics, either by inducing the synthesis of more enzymes, or by

inhibiting the activity of these enzymes. With regard to polyphenols interacting with these enzymes, probably the best-known example is the co-administration of certain drugs with grapefruit juice that could markedly increase drug bioavailability [59]. Further studies have indicated that the main mechanism for this interaction is the inhibition of intestinal CYP3A4 by components of grapefruit juice, with flavonoid naringin and its aglycon metabolite, naringenin being the most likely inhibitors [60, 61].

In terms of useful synergies between polyphenols whose bioavailability is limited by phases I and II enzymes in the creation of a functional food, a study by Fuhr et al. [62] showed that the maximum plasma concentration (C_{max}) and half-life of caffeine, which is metabolised by both CYP1A2 and 3A4, could be increased by 23 and 31%, respectively, through the co-consumption with grapefruit juice.

2.4.2 Efflux transporters

Once metabolized, the hydrophilic conjugates have to be transported out of the cells by transporter(s] because they lose their ability to diffuse across the cells via passive diffusion. The best-known efflux transporters in the intestine mostly belong to the ATP-binding cassette (ABC) transporter family [63].

Polymorphisms in the ABC transporters have also been identified, and most studies have focused on P-glycoprotein (Pgp) efflux pumps [64]. ABCs are widely distributed in many tissues, including the liver, kidney, small intestine, colon, placenta and the capillary endothelium of the brain. Like the CYPs and UGTs, their expression varies widely between individuals because of environmental and genetic factors. Genetic variations in the ABCB1 gene, and their codes for Pgp, have been correlated with drug exposure for a number of commonly used drugs [65, 66], and it is to be expected that these polymorphisms, and their differences in interethnic frequency, would have a similar impact on the exposure of polyphenols in human populations. This may require a nutrigenomic approach, where functional foods are tailored to a consumer's-specific genotype.

Polyphenols and their conjugates that are transported to the liver may be subjected to additional metabolism or excretion via the bile. The liver is the main and central organ for xenobiotic disposition, especially for metabolism, and possesses a variety of transporters and metabolic enzymes that are capable of clearing of xenobiotics from the body rapidly [67]. These transporters in the liver usually have a very high capacity and can excrete flavonoid metabolites and biliary concentration can reach several millimolars in an intestinal perfusion model in mice [68]. The efficient biliary excretion is enabled by apical efflux transporters located at the bile cannicular membrane. These transporters in the apical side of the liver include multi-drug resistance proteins, sodium taurocholate cotransporting protein and human prostagrandin transporter. On the contrary, transporters localised on the basolateral side of the hepatocytes are involved in the efflux from hepatocytes to the blood [69]. The coupling of apical transporters and metabolic enzymes in the liver enables the enterohepatic recycling of polyphenols and drugs because conjugated metabolites that are excreted via the bile may enter the large intestine, be hydrolyzed (by microflora) and return as absorbed aglycones from the large intestine. In conjunction with enterohepatic recycling, enteric recycling may increase the apparent half-lifes, which explains why flavonoids have poor systemic bioavailabilities, but remain in the plasma for an extended period of time (i.e., hours) [70]. Many studies have also reported that flavonoids are well known substrates for the ABC family of transporters involved in the efflux mechanism, and affinity with these transporters has also been suggested as one of the main reasons for the poor bioavailability of these bioactive compounds [71-73].

Recent advances, however, have generated hope of overcoming these obstacles in bioavailability. Apparently, kaempferol has a higher affinity for ABCG2 than quercetin. Administration of both flavonols simultaneously revealed that kaempferol blocked the efflux of quercetin, allowing quercetin to remain inside and exert its effects [74]. Therefore, kaempferol could possibly be combined with another substance with an even higher affinity for ABCG2, which would leave kaempferol inside to cause destruction of the malignant cells. In addition, kaempferol has been found to lower mRNA levels of ABCC6, another ATP-binding cassette transporter [75]. ABCC6 is involved in the transport of cisplatin, a chemotherapy drug, outside of the cell [76]. The introduction of

kaempferol was found to enhance cisplatin's cytotoxic effects on cancer cells significantly [75]. Ostensibly, even if kaempferol itself has poor bioavailability, it does at least seem to improve the bioavailability of other substances meant to fight cancer. These transport proteins are a good potential research focus for increasing the body's access to polyphenols and other drugs.

2.4.3 Gut microbiota

Although significant quantities of polyphenols are absorbed in the gut, a large proportion of dietary polyphenols remains unabsorbed in the gut lumen and becomes concentrated in the ileal and colorectal lumen [77]. For example, while most apple juice polyphenols are absorbed in the small intestine [78, 79], up to 85% of blueberry anthocyanins enter the colon [79].

Once in the colon, a significant number of bacteria appear to participate in the metabolism of polyphenols. Colonic microflora is exposed to two sources of polyphenols: dietary polyphenols (mostly in glycoside forms] that are not absorbed and hydrophilic polyphenol conjugates (sulfates and glucuronides) that are excreted by the intestine and liver. Microflora bacteria employ a large number of extracellular glycosidases (secreted by bacteria) and can break down all known glycoside bonds formed between polyphenol aglycones and sugars. These bacteria also express a high concentration of glucuronidases and sulfatases, which also release aglycones from their respective glucuronides and sulfates [80]. The released aglycones can be taken up by the colonic cells in humans or animals, or they can be further metabolized into other products, contributing to their bioavailability. Unlike humans and animals, bacterial metabolism can cause ring-fission and reduction reactions, producing a wide range of low-molecular-weight metabolites, which may be absorbed by the host [20, 81].

The composition of the gut microbiota varies substantially between individuals [82]. Consequently, each intestinal microbial community can be expected to display its own characteristic metabolic profile. For some polyphenolic compounds, bioconversion has been shown to vary between individuals due to differences in the metabolic potential of their endogenous microbiota [83]. Culture models of human colonic microbiota that simulate microbial processes in the large intestine have proven to be useful tools for investigating the extensive microbial metabolism of natural polyphenols [84]. In contrast to more sophisticated, but time consuming, in vitro gut models, batch fermentations provide a feasible possibility for assessing multiple experimental conditions using fecal samples from several subjects [85].

Recent in vitro studies suggest that the polyphenol food source influences the amount and profile of microbial metabolite that may be produced by the colonic microbiota [86-88]. These changes in the metabolome may have profound implications for health at both the gut level and systemically, because for some polyphenols microbial catabolism constitutes a way of their conversion into more bioactive forms [89]. Although the known bioactive properties of microbial metabolites are still limited, some metabolites have revealed antioxidant, antithrombotic, anti-inflammatory, and antiproliferative activities, as well as inhibiting pathogenic bacteria and modulating lipid metabolism [89]. Recent studies also indicate that flavan-3-ol-rich sources such as chocolate, green tea, blackcurrant or grape seed extracts, may modulate the intestinal microbiota in vivo, producing changes in beneficial bacteria such as Lactobacillus, but inhibiting other groups such as Clostridium spp. [90-93].

Despite these modulatory effects found in vivo, very few studies have attempted to study the metabolome of the corresponding fecal samples [38, 94, 95] limiting the analysis to urine samples. The integration of both microbiome and metabolome analyses in fecal samples from in vivo polyphenol feeding trial studies is crucial not only to link bacteria to the production of certain metabolites but also to understand how the modulatory effects of these compounds result in benefits to the bacteria–host mutualism, in favor of disease prevention or health improvement.

3. TECHNOLOGICAL STRATEGIES TO IMPROVE BIOAVAILABILITY

Improving the bioavailability of bioactive food compounds is fundamental to improving their bioefficacy. Several approaches to improve the bioaccessibility and bioavailability of bioactive ingredients and drugs have been evaluated. The most common strategy for

overcoming the poor bioavailability of polyphenols and therefore enhances its bioaccessibility is to entrap or encapsulate the phenolic compound with an encapsulating material [96-98]. Microencapsulation is described as a technique where a bioactive compound is encapsulated by a biopolymer to protect it from oxygen, light, water or other conditions to improve its stability, water solubility and bioavailability [96]. Thus, the bioactive compound can be transported through the cell membranes much more easily, resulting in an increase in the phenolic concentration in plasma and bioavailability. The bioactive compound to be encapsulated usually stays in the core of the capsule surrounded by the encapsulating agent or dispersed in one matrix containing the encapsulating agent. The sealed capsules can then release their contents (bioactive compounds) at controlled rates under specific conditions. Thus, the bioactivity of the compounds can be protected during food processing and the subsequent passage through the gastrointestinal tract.

There are many studies in the literature where the encapsulation techniques have been used to improve the stability of phenolic compounds. Recently, Fang et al. [96] reviewed these encapsulation techniques for these compounds. However, only few reports in the literature are based on using these encapsulating techniques to enhance the bioavailability of phenolic compounds, and these are discussed below. Strategies such as nanoencapsulation [99-104], liposomes [105-110], emulsions [111] and spray drying [112, 113] have been reported to increase the water solubility of phenolic compounds and permit their controlled delivery into the gastrointestinal tract. These encapsulation techniques have been applied to the phenolic compounds curcumin [100-109, 111, 112], ellagic acid [99], quercetin [110] and flavanols and phenolic acids [113]. Curcumin is the phenolic compound that the encapsulation techniques have been applied to most. Although clinical studies in humans have shown that curcumin is safe and well tolerated even at very high doses, its use as a therapeutic agent is limited by its low bioavailability, poor absorption, rapid metabolism, and systemic clearance [114, 115]. Table 3 summarizes the different encapsulation strategies reported in the literature to enhance the in vivo bioavailability of phenolic compounds.

3.1 Nanoencapsulation

The nanoencapsulation technique involves forming active-loaded particles at the nano-scale. The generated nanocapsules are vesicular systems in which the phenolic compound is confined to a cavity consisting of an inner liquid core surrounded by a polymeric membrane. This technique has been applied to encapsulate hydrophobic phenolic compounds, such as curcumin [100-104] and ellagic acid [99]. These phenolic compounds have been encapsulated with biodegradable nano-particulates based on poly(lactic-co-glycolic acid] (PLGA) and a co-solvent and stabilizer polyethylene glycol (PEG) [99, 101, 102], poly (vinyl alcohol) (PVA) [100, 103] or (didodecyldimethylammomium bromide (DMAB) [99].

Different methodologies have been used to prepare these nanoparticles. These include nanoprecipitation [102], emulsion-diffusion-evaporation [99, 100], high-pressure emulsification-solvent evaporation [103] and solid-in-oil-water solvent evaporation [101]. The entrapment efficiency has been reported to be between 77 and 97% for curcumin [100, 102, 116] and 50% for ellagic acid [99]. Apart from the copolymer PLGA, colloidal nanoparticles have also been used to encapsulate curcumin [104].

The in vivo bioavailability of curcumin encapsulated with nanoparticles has been compared with its bioavailability in the native form in several studies. After the oral administration, intragastric or intravenous injection of encapsulated curcumin and the native curcumin, biological samples such as plasma [100, 101, 103, 104], serum[102], urine [103] and faeces [103] were analyzed. The results obtained showed higher concentrations of encapsulated curcumin in these biological samples and longer half-lifes compared with free curcumin. Encapsulated curcumin showed between 5.6-fold and 40-fold increase in apparent bioavailability [101, 104] and around 640-fold in water solubility [102] than the native curcumin when the same concentration of this phenolic compound encapsulated and freely was administered or injected in rats. The 40-fold enhancement of bioavailability was obtained when colloidal nanoparticles were used to encapsulate curcumin. In humans, after the oral administration of 30 mg/kg of encapsulated curcumin and native curcumin, the AUC of the encapsulated form was

Table 3. Strategies to encapsulate phenolic compounds in order to enhance their bioavailability *in vivo*

Encapsulation technique	Phenolic compound	Entrapment	Entrapment efficiency (%)	Administration	Phenolic dose	Increased bioavailability	Reference
Nanoencapsulation	Curcumin	Nanoparticles: PLGA Co-solvent: PEG	91.96	Intragastric (rats)	CUR: 100 mg/kg CUR-encap: 100 mg/kg	5.6-fold	Xie et al., 2011
	Curcumin	Nanoparticles: PLGA Co-solvent: PEG	97.5	Intravenous (rats)	CUR: 2.5 mg/kg CUR-encap: 2.5 mg/kg	- - -	Anand et al., 2010
	Curcumin	Nanoparticles: PLGA Co-solvent: PVA	76.9	Oral (rats)	CUR: 250 mg/kg CUR-encap: 100 mg/kg	9-fold	Shaikh et al., 2009
	Curcumin	Nanoparticles: PLGA Co-solvent: PVA	- - -	Intravenous (rats)	CUR: 10 mg/kg CUR-encap: 2.5 mg/kg	22-fold	Tsai et al., 2011
	Curcumin	Colloidal nanoparticles	- - -	Oral (rats)	CUR: 30 mg/kg CUR-encap: 30 mg/kg	27-fold	Sasaki et al., 2011
				Oral (humans)	CUR: 50 and 300 mg/kg CUR-encap: 50 and 300 mg/kg	40-fold	
	Ellagic acid	Nanoparticles: PLGA Co-solvent: PEG	50	Intestinal perfusion (rats)		- - -	Bala et al., 2006
Liposomes	Curcumin	Phospholipids complex	- - -	Intravenous (rats)	CUR: 40 mg/kg CUR-encap: 40 mg/kg	- - -	Li et al., 2005
	Curcumin	Phospholipids complex	- - -	Oral (rats)	CUR: 300 mg/kg CUR-encap: 100 mg/kg	3-fold	Liu et al., 2006
	Curcumin	Phospholipids complex	- - -	Oral (rats)	CUR: 10 mg/kg CUR-encap: 30 mg/kg	- - -	Takahashi et al., 2008
	Curcumin	Phospholipids complex	68.0	Oral (rats)	CUR: 100 mg/kg CUR-encap: 100 mg/kg	5-fold	Takahashi et al., 2009
	Curcumin	Phospholipids complex	82	Oral (rats)	CUR: 50 mg/kg CUR-encap: 50 mg/kg CUR-encap: 25 mg/kg CUR-encap: 12.5 mg/kg CUR-encap: 1 mg/kg	39-fold 59-fold 32-fold 155-fold	Kakkar et al., 2011
	Quercetin	Phospholipids complex	60-80	Oral (rats) Oral and intranasal (rats)	QUER: 300 mg/kg / day QUER-encap: 20 µg / day	- - -	Priprem et al., 2008
Emulsions	Curcumin	Silica nanoparticle stabilized Pickering emulsion	- - -	Intestinal perfusion (rats)		- - -	Tikekar et al., 2013
Spray drying	Curcumin	Cellulose derivative	- - -	Oral (humans)	CUR: 800 mg / day CUR-encap: 800 mg / day	7-fold	Vitaglione et al., 2012
	Flavanols (FLA) and phenolic acids (PA)	High amylose maize starch	- - -	Oral (human)	FLA&PA: 385 µmol FLAV/ day 28 µmol PA /day FLA&PA-encap: 385 µmol FLAV/ day 28 µmol PA /day	~ 13.9-fold	Vitaglione et al., 2013

27 times higher than the native curcumin [104]. On the other hand, when the concentration of the encapsulated curcumin was 2.5 [100] and 4 times [103] lower than the curcumin in its native form, the bioavailability increased 9-fold and 22-fold, respectively. It was observed that after intravenous injection of encapsulated curcumin, the curcumin content in faeces decreased, but increased in urine, compared with the native curcumin [103]. Apart of the enhanced bioavailability, a long half-life of encapsulated curcumin was also observed, in comparison with the native curcumin. Encapsulated curcumin was constant released over 48 h, while the native curcumin was not detectable in plasma beyond 6 h [100]. The improved bioavailability of curcumin reported in all these studies could be associated with the improved water solubility, the higher release rate in the intestinal juice, the enhanced absorption by improved permeability and the increased residence time in the intestinal cavity [101]. These findings demonstrate that the use of nanoencapsulation is a good strategy for protecting curcumin through in vivo biotransformation, thus increasing its circulating concentration compared with the free ingredient.

Nanoencapculation has also been applied to ellagic acid to improve its bioavailability [99]. In this study, in situ perfusion of intestinal segments of rats was used to study the permeability and absorption kinetics of ellagic acid. Results showed higher uptake of encapsulated ellagic acid in nanoparticles than pure compound. The permeation of ellagic acid from jejunum was found to be 66%, while drugs encapsulated in PLGA nanoparticles using PVA and DMAB as stabilizers showed 75 and 87% permeation, respectively

3.2 Liposomes

Liposomes are colloidal particles consisting of a membranous system formed by lipid bilayers encapsulating an aqueous space (phospholipids). Owing to the possession of both lipid and aqueous phases, liposomes can be used to entrap, deliver, and release water soluble, lipid soluble, and amphiphilic materials. There have been several reports on the employment of liposomes to protect such pharmaceuticals products, such as insulin [117], peptides [118], and cyclosporin [119] from enzymatic degradation and to deliver them into the bloodstream efficiently.

Liposome encapsulation has successfully been applied for quercetin [110] and curcumin [105-109] to entrap the phenolic compounds to enhance their bioavailability. The entrapment efficiency was reported to be between 60 and 80 %. The trapping efficiency of curcumin could be increased with an increase in the lecithin concentration up to 10% [108]. A bioavailability enhancement between 5- [108] and 39-fold [109] was reported when using liposome encapsulation after an oral rat administration of encapsulated curcumin in comparison with the native form, at concentration of 100 and 50 mg/kg, respectively. Further enhancement of bioavailability by up to 155-fold was observed when the concentration of encapsulated curcumin was 50-times lower than the free curcumin [109].

A faster rate of absorption was also observed when quercetin was encapsulated with liposomes after intranasal administration (20 µg/day) for 7, 14, 21 and 28 days, in comparison with a higher dose (300 mg/kg) of oral quercetin without encapsulating [110]. Several advantages were reported for quercetin encapsulation. The advantages included effectiveness at a lower dose and a significant improvement in both anxiolytic and cognitive-enhancing effect, that appeared much sooner than with quercetin in its native form. Quercetin liposomes were described as a potential strategy for delivering quercetin into the central nervous system via the intranasal route.

3.3 Emulsion technology

Emulsion technology is generally applied to encapsulate bioactive compounds in aqueous solutions, which can either be used directly in the liquid state or can be dried to form powders after emulsification. Stabilizers, such as emulsifiers or texture modifiers, are commonly added in the emulsion systems to obtain a kinetically stable solution.

Curcumin has also been encapsulated by using emulsions, and the bioavailability of this phenolic compound in simulated gastric and intestinal digestion was evaluated [111]. Results showed a rapid and efficient release of this compound in simulated intestinal medium when it was encapsulated. In addition, the stability of the curcumin encapsulated in emulsion was 100-times higher than its native form in water.

3.4 Spray drying

Spray drying is a simple and low-cost microencapsulation technology by which a liquid product is atomized in a hot gas (generally air) to obtain a powder instantaneously. It can both protect bioactives from environmental pressures (e.g. oxygen or moisture) and mask the unpleasant taste of bioactives through the encapsulant physical barrier. Vitaglione et al. [112] [113] encapsulated the phenolic compounds, curcumin, flavanols and phenolic acids with the spray drying technique in order to increase the bioavailability of these compounds. In these studies, cellulose derivate was used to entrap curcumin [112], and flavanols and phenolic acids from cocoa-nut cream were entrapped with high-amylase maize starch [113].

In the first study, Vitaglione and colleagues assessed the bioavailability and biotransformation of curcumin by comparing the human oral administration of bread enriched with curcumin in its native form, and bread enriched with encapsulated curcumin. Serum concentrations of curcuminoids over 6 h after consumption of the bread with encapsulated curcumin were significantly higher than those after consumption of the bread with the free ingredient. In addition, fecal curcuminoids were 6-times more abundant after the administration of encapsulated curcumin rather than free curcumin. Thus, the use of encapsulated curcumin can protect the phenolic compound by in vivo biotransformation [degradation intestinal], thus increasing its circulating concentration compared with the curcumin in its native form [112].

In a further study, the same authors investigated the bioavailability of flavanols and phenolic acids from a cocoa-nut cream enriched with cocoa phenolic extract and a cocoa-nut cream enriched with encapsulated cocoa phenolic extract [113]. The subjects consumed the cream portion distributed throughout the day and then serum, urine and faecal biological samples were collected for that day (24 h). A reduced bioavailability (13.9-fold) of catechin and epicatechin was reported in the cream enriched with encapsulated phenolics than that was found with the cream enriched with cocoa phenolic extract in its native forms. In faeces, the concentration of flavanols after the encapsulated cocoa phenolics was higher than after the cream with free forms. From these studies, it was concluded that encapsulation allowed the delivery of bioactive compounds in the lower gut and their successive metabolism by local microflora. Although the encapsulation of cocoa polyphenols caused a reduced 24-h bioavailability of the phenolic compounds studied, the encapsulated cocoa polyphenols may be considered a functional prebiotic ingredient. Moreover, the use of the encapsulation technique effectively masked the bitter taste through the use of an encapsulated agent inaccessible by salivary enzymes, such as high amylose maize starch.

CONCLUSIONS

The study of the numerous factors influencing bioavailability is an important step towards understanding the role of polyphenols in human health and optimizing dietary advice to the population. The bioavailability of bioactive food compounds follows the same steps as orally ingested drugs (LADME) and faces some of the same challenges, including transporters, molecular structures, and enzymes. However, unraveling the bioavailability of bioactive food compounds is even more challenging than with drugs since many other factors, such as bioaccessibility, food matrix and the gut microbiota, can affect bioactive food compounds during digestion. Although there are many studies reporting the bioavailability and the bioefficacy of polyphenols, understanding their interactions, metabolism and mechanism of action warrants further work. In this sense, for future studies of the efficacy of specific phenolic compounds, it is crucial to assess their absorption and metabolism from the food/beverage. The next important step in understanding the bioavailability of bioactive food compounds is to properly identify the circulating metabolites in order to have a better understanding of the fate of the parent compounds. Only when the circulating forms of a bioactive food molecule or a drug are known can a more complete picture related to bioavailability and possible correlation to bioefficacy be obtained. For drugs, this is a requirement when performing bioavailability studies and this type of approach can also be applied to nutrition research. Moreover, metabolism by microflora needs to be well understood, because the gut microflora probably plays a major role in the biological activity of many polyphenols. Finally,

strategies to improve the bioavailability of the polyphenols need to be developed and it is necessary to determine whether these methods translate into increased biological activity.

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