METABOLISM AND FORMATION OF 2-DODECYLCYCLOBUTANONE IN IRRADIATED GROUND BEEF

by

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B.S., Birzeit University, Ramalla, Palestine, 1997
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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfilment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Food Science

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ABSTRACT

A rapid direct solvent extraction method for the extraction of 2-dodecylcyclobutanone (2-DCB) in irradiated beef using acetonirile was developed and evaluated. The 2-DCB in commercially irradiated ground beef patties was extracted with n-hexane by using a Soxhlet apparatus or with acetonitrile via direct solvent extraction. The hexane and the acetonitrile extracts were evaporated to dryness. Then, the fat in the hexane extract was removed with filtration by standing at -20 °C after the addition of a mixture of ethylacetate and acetonitrile. The defatted extract as well as the acetonitrile extract were purified with a 1 g silica cartridge and was injected into a gas chromatography-mass spectrometry (GC-MS). The 2-DCB concentration in irradiated patties was 0.031 ± 0.0026 ppm (n = 5) for the Soxhlet method and 0.031 ± 0.0025 ppm (n = 10) for direct solvent extraction.

The effect of low-energy X-rays on the formation of 2-DCB was investigated in frozen ground beef. Beef patties (85/15) were irradiated by low-energy X-rays and gamma rays at 3 targeted doses of 1.5, 3.0, and 5.0 kGy. The 2-DCB was extracted with n-hexane by using a Soxhlet apparatus and analyzed by GC-MS. There was no significant difference in 2-DCB concentration between gamma-ray- and low-energy X-ray-irradiated patties at all targeted doses.

The metabolism of 2-DCB was investigated *in vitro* and *in vivo*. The incubation mixture from the *in vitro* study was extracted with n-hexane by using a Soxhlet apparatus and injected into a GC-MS). The hexane extract from the *in vitro* study and of rat feces was also derivatized with a silylation reagent and injected into a GC-MS. The average percentage of 2-DCB recovered from the test incubations (2-DCB with S9 and NADPH) was 23%, compared with 50% from the controls (2-DCB in buffer). The GC-MS chromatograms of the derivatized samples showed that 2-DCB was metabolized to 2-dodecylcyclobutanol.

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PART I. REVIEW OF LITERATURE

INTRODUCTION

The Centers for Disease Control and Prevention reported that 76 million infections, 325,000 hospitalization and approximately 5,000 deaths occur annually due to consumption of contaminated food in the United States (CDC 2004). Tauxe (2001) estimated that if 50% of poultry, ground beef, pork, and processed meats in the United States were irradiated, the rates of morbidity and mortality caused by these infections could be reduced by 25%.

Although the implementation of strategies by the meat and poultry industry has reduced the microbial contamination of meat and poultry carcasses, these intervention efforts have not eliminated all microbial contamination (Eustice and Bruhn 2006). Between 1993 and 2003, the 10 largest beef-processing companies spent more than $ 650 million to fight *Escherichia Coli* O157:H7 (Kay 2003).

Increased number of foodborne outbreaks have been associated with fresh produce in the past 30 years. The recent increase in outbreaks associated with fresh produce has been attributed to its increased consumption and new production and processing technologies (Doyle and Erickson 2008). In 2006, spinach, lettuce and tomatoes were associated with 4 outbreaks. The first outbreak occurred in California and resulted from the consumption of spinach contaminated with *E. Coli* O157:H7. This outbreak resulted in 205 confirmed cases and three deaths, and 32 of the 103 hospitalized cases patients developed Hemolytic Uremic Syndrome (HUS). The second outbreak was reported in 21 states east of the Mississippi River after the consumption of tomatoes contaminated with *Salmonella*. The second outbreak resulted in 183 confirmed cases, and 22 of the 183 cases were hospitalized with most of these suffering from fever and diarrhea.

The third outbreak was reported in five states due to the consumption of lettuce contaminated with *E. Coli* O157:H7. This outbreak resulted in 71 confirmed cases, with 8 of the 53 hospitalized patients developing HUS. The last outbreak was reported in three states and was also due to spinach that was contaminated with *E. Coli* O157:H7. This outbreak resulted in 81 cases, with 2 of the 26 hospitalized cases patients developing HUS.

Because iceberg lettuce and spinach are often consumed raw, they lack the final microbial elimination step provided by cooking other foods (FDA 2008). However, irradiation of romaine lettuce and baby spinach at a dose of 1.5 kGy produced 3 to 4 $\log_{10}$ reduction in colony-forming units of *E. Coli* (Niemira 2007). The FDA (2008) has concluded that the irradiation of iceberg lettuce and spinach up to 4 kGy will reduce or eliminate bacterial population with no increased microbial risk from the pathogens that may survive the irradiation process.

Food irradiation improves food safety through destruction of microorganisms, increasing its shelf-life, controling parasites and insects, improving the quality of fruit and vegetables, inhibiting sprouting and delaying ripening (Urbain 1986a, Smith and Pillai 2004). Several types of foods have been approved for irradiation in the United States. They include wheat, potatoes, pork, herbs, spices, red meat and poultry, and iceberg lettuce and spinach. The complete list of foods approved for irradiation is shown in Table 1.

In spite of all the benefits of food irradiation, consumer acceptance of irradiated food has been slow; only 10% of spices and less than 0.002% of fruit, vegetables, meats and poultry are irradiated (Osterholm and Norgan 2004). Acceptance of irradiated food in the past has been slowed by several factors (Osterholm and Norgan 2004); the use of the term "irradiation" which, is associated with radioactivity, the poor understanding of the causes and prevention of foodborne

disease, the ignorance of health professionals and the media of the benefits of irradiating food, and the presence of anti-irradiation groups.

Table 1- Irradiation applications approved or under consideration by FDA and USDA (Smith and Pillai 2004, FDA 2008).

Product	Dose (kGy)	Purpose	Date
Wheat, wheat flour	0.2-0.5	Insect and mold control	1963
White potatoes	0.05-0.15	Sprout inhibition	1963
Pork	0.3-1.0	*Trichinella spiralis*	1985
Enzymes (dehydrated)	10.0 max.	Microbial control	1986
Fresh fruit and vegetables	1.0 max.	Disinfestation, ripening delay	1986
Herbs, spices, seasonings	30.0 max.	Microbial control	1986
Poultry, fresh or frozen	3.0 max.	Microbial control	1990
Poultry, fresh or frozen (USDA)	1.5-4.5	Microbial control	1992
Frozen meat for NASA programs	44.0 min	Sterilization	1995
Animal feed and pet food	2.0-25.0	*Salmonella* control	1995
Meat, uncooked, chilled	4.5 max.	Microbial control	1997
Meat, uncooked, frozen	7.0 max.	Microbial control	1997
Meat, uncooked, chilled (USDA)	4.5 max.	Microbial control	2000
Meat, uncooked, frozen (USDA)	7.0 max.	Microbial control	2000
Fresh shell eggs	3.0 max.	*Salmonella* control	2000
Molluscan shellfish, fresh or frozen	5.5 max	Microbial control	2005
Iceberg lettuce and spinach	4.0 max	Microbial control	2008
Ready to eat products, chilled	4.5 max.	Microbial control	pending
Ready to eat products, frozen	10.0 max.	Microbial control	pending

FOOD IRRADIATION

History of Food Irradiation

The notion that ionizing radiation could be used to kill microorganisms in foods was suggested in the year following the discovery of radioactivity by Henri Bequerel (1895). However, the first US patent on the use of ionizing radiation to kill parasites (*Trichinella spiralis*) in meat was not granted until 1921 (Hackwood 1991). In 1930, another patent was obtained in France for the preservation of foods using ionizing radiation.

During the 1950s and early 1960s considerable work on food irradiation was completed in the United Kingdom. From 1953 through 1970, the US Army sponsored many research projects on the wholesomeness of irradiated foods (EPA 2010). In 1970 the International Food Irradiation Project (IFIP) was launched by 19 countries to study the combined effect of irradiation and preservatives on food. In 1984, the Food and Agriculture Organization (FAO), the International Atomic Energy Authority (IAEA), and the World Health Organization (WHO) replaced the IFIP with the International Consultative Group for Food Irradiation (ICGFI). The ICGFI's responsibility was to sponsor and perform research related to food irradiation. In 1980, the FAO/IAEA/WHO Expert Committees on the Wholesomeness of Irradiated Foods (JECFI) concluded that the irradiation of food up to 10 kGy does not present a toxicological hazard (Hackwood 1991). In 1983, the Codex Alimentarius Commission adopted a revised "General Standard for Irradiated Foods" and a revised "Recommended International Code of Practice for the Operation of Radiation Facilities Used for the Treatment of Foods," which included the main conclusions of the 1980 JECFI regarding the safety of irradiated food.

Types of Irradiation

Gamma rays, energetic electrons, and X-rays are all ionizing irradiation. However, the choice of a radiation source depends on the thickness and density of the package, dose uniformity ratio, minimum dose, dose rate, and economics (Cleland 2006).

Gamma Rays

Gamma rays are electromagnetic radiation produced during the decay of certain radio-isotopes (Hackwood 1991). Gamma rays from cesium-137 (0.662 MeV) and cobalt-60 (1.17 and 1.33 MeV) are allowed by the U S FDA and by international standards for food irradiation (Cleland 2006).

The first source of gamma rays is made from cesium-137, which is produced as a result of uranium fission and may be reclaimed as a byproduct of nuclear fuel processing (Stewart 2001a). The use of cesium-137 for food irradiation has received much criticism from antinuclear opponents, who claim that food irradiation was created to dispose nuclear waste (Stain 1993). Since the practical capacity to extract cesium-137 from nuclear waste is limited and the act of processing nuclear waste is so controversial, the availability and common use of cesium as a food irradiation source is unlikely (Stain 1993).

The most common type of irradiator uses cobalt-60 as a source of radiation. Cobalt-60 is made from the natural nonradioactive cobalt-59 (Stain 1993). First, cobalt-59 is compressed into small cylindrical pellets, which are fit into stainless steel pencils. Then these pencils are bombarded by neutrons inside a nuclear reactor. This process produces purified cobalt-60, which is transformed into Nickle-60 upon the emission of gamma rays. Cobalt-60 is the radioisotope of

choice for gamma irradiation of food because it is readily available, the technology of its production is highly developed, and it has greater penetration power than cesium-137 (Stewart 2001a). However, cesium-137 has a longer half-life (30 year) than cobalt-60 (5.2 years). Because the supply of cobalt-60 is limited and consumers are concerned about the use of radioactive materials, electron-beam and X-ray machines are becoming the preferred means for food irradiation.

Electron Beams

Accelerated electrons with energies up to 10 MeV are allowed by the FDA (Cleland 2006). This energy limit was recommended to prevent the formation of radioactive nuclides in the food. The penetration of an electron beam depends on the electron's energy and most electron beam irradiators have a minimum energy of 5 MeV. For example, for treatment from one side, an electron beam with 10 MeV energy level can be successfully used to irradiate food products of 3.7 cm thickness. For treatment from opposite sides with 10 MeV, the thickness of the product can be increased to 8.6 cm.

More than 1,000 industrial electron beam accelerators have been built worldwide. Most of these accelerators are used for treating plastic and rubber and for sterilizing of medical devices. Only a few of these accelerators are used for food irradiation. Low-energy electron beam accelerators (0.1 to 0.3 MeV) can be used for treating grains and powders and for sterilizing the surfaces of packaging material (Cleland 2006). While high-energy electron beam accelerators are used for treating retail packages of foods. An obvious advantage of electron beam accelerators is that they can be switched on and off and are not related to the nuclear industry (Stain 1993).

However, electron beams have a limited penetrating power compared to gamma-rays.

X-rays

X-rays (bremsstrahlung) with energies up to 7.5 MeV are allowed by the FDA for food irradiation (Cleland 2006). X-rays are produced by the bombardment of a metal target with high power-electron beams (Stewart 2001a). The efficiency for converting electron beams to X-rays increases with the atomic number of the target metal and the electron energy (Cleland 2006). Nevertheless, 7.5 MeV X-ray cannot be used to irradiate full pallets of foods from opposite sides. Although the penetration of X-rays is higher than that of accelerated electrons, the efficiency of energy use, as accelerated electrons (5 MeV) are converted to X-rays is only 4-6% efficient (Stewart 2001a). Table 2 on the next page lists the differences and similarities among the three different types of irradiation.

Table 2- Comparison of the three different irradiation technologies. Adapted from Food Science Australia (2003).

	Electron beam	X-ray	Gamma ray
Technology	focused beam of electron	focused beam converted to X-ray	photons created from decay of radioactive material
Processing time	seconds	seconds	minutes-hours
Safety	safe; on and off	safe; on and off	always emits radiation
Penetration	3-6 cm	1-2 meters	1-2 meters
In-line-processing	yes	yes	yes
Chamber temperature	Ambient or controlled	Ambient or controlled	Ambient or controlled
Power source	electricity	electricity	radioactive isotopes

FOOD IRRADIATION CHEMISTRY

Because understanding radiation chemistry is necessary to understanding the safety of irradiated food, key aspects of radiation chemistry related to the safety evaluation of irradiated food are discussed here first. Radiation chemistry is defined as the chemical reactions that occur as a result of the absorption of ionizing radiation (FDA 2008). Radiation chemistry has been extensively studied and the basic principles are well understood (WHO 1999, Stewart 2001a). The radiation chemistry of foods like fresh vegetable that have a high water content is dominated by the radiation chemistry of water (FDA 2008). The primary radiolysis products of water are the hydroxyl and the hydrogen radicals. These radicals may recombine to form water, hydrogen gas, or hydrogen peroxide, or they may react with other food components. Although most of the chemical effects of radiation on foods results from the hydrogen and hydroxyl radicals, other food

components may absorb radiation directly and generate other radiolysis products (FDA 2008). In the following sections, the radiation chemistry of the major components of food will be discussed.

Carbohydrate

In the presence of water, carbohydrates react with the hydroxyl radicals generated by the radiolysis of water to form carbohydrate radicals and water. Direct radiolysis of carbohydrates is possible, but the effect is minor (FDA 2008). Irradiation of low-molecular-sugars and their derivatives cause a change in their physical and chemical properties (Stewart 2001a); irradiation reduces the melting point and the optical rotation of sugars. Browning has been observed after the irradiation of some sugars such as fructose, galactose, glucose, and xylose. The degradation of sugars during irradiation can result in the formation of a mixture of gases, mainly hydrogen and carbon dioxide, with traces of methane, carbon monoxide, and water. The irradiation of glucose, the main building unit of most carbohydrates, in aqueous solution resulted in the formation of 2- and 5-deoxygluconic acid and 2-deoxyribose (Dizdaroglu 1975). Irradiation also cleaved the glycosidic bonds in polysaccharides, resulting in short chain polysaccharides such as glucose, maltose, erythrose, and mannose (Stewart 2001a). Further decomposition of polysaccharides may result in the formation of formic acid, acetaldehyde, methanol, acetone, ethanol, and methyl formate. The radiolytic products of starch are similar and do not depend on the source of the starch. Although about 40 compounds have been identified in irradiated starch, all of these compounds have been found to be produced by heat treatment or the natural oxidation of starch. The average chain length of starch is reduced after irradiation, which may reduce starch viscosity and gelling capacity and increase starch sensitivity to α- and β-amylase. The FDA (2008)

concluded that the overall effect of ionizing radiation on carbohydrates is basically the same as the overall effect produced by cooking or by using other food processing treatments, and the irradiation of carbohydrate at 10 kGy or less has minimal effect on carbohydrate functionality or its nutrient value.

Proteins

Upon being irradiated simple amino acids such as glycine react with hydroxyl, hydrogen radicals, and aqueous electrons to form amino acid radicals, water, hydrogen, and ammonia (Stewart 2001a). The amino acid radicals may react further to form diamino acids, imino acids, and fatty acids. The imino acid can be hydrolyzed to give ammonium ions and a keto acids. Decarboxylation of the amino acid produces carbon dioxide and an amino acid with one carbon fewer than the original amino acid. The nature of the radiolytic products and the sensitivity to irradiation depend on the type of amino acid. Sulfur-containing and aromatic amino acids are the most sensitive to irradiation (Liebster and Kopoldava 1964). Upon being irradiated, cystine forms hydrogen sulfide, hydrogen, alanine, and water. The irradiation of methionine results in the formation of homocystine and methyl mercaptan. The production of hydrogen sulfide and methyl mercaptan can lead to the off-odors and off-flavors in irradiated foods (Stewart 2001a). As depicted in Figure 1 the irradiation of phenylalanine in aqueous solution causes its aromatic ring to be hydroxylated at different position to give *ortho*-, *meta*-, and *para*-tyrosine (Stewart 2001a). Simic and others (1983) proposed the use of *ortho*-tyrosine as a radiolytic marker for the detection of irradiated food. However, *ortho*-tyrosine was later shown to be present in non-irradiated food (Hart and others 1988).

The radiation chemistry of peptides is similar to that of amino acids. The reactivity of peptides increases as the number of the peptide bonds increases (Rustgi and Riesz 1978). The main radiolytic products of irradiation on peptides are ammonia, fatty acid, keto acids, amide, as well as diamino acids (Stewart 2001a). Most of the radiolysis products derived from proteins have the same amino acid composition, and they only differ in their secondary and tertiary structures (FDA 2008). Changes in irradiated proteins are similar to those changes that result from heating proteins, but in the case of irradiation, these changes are less significant.

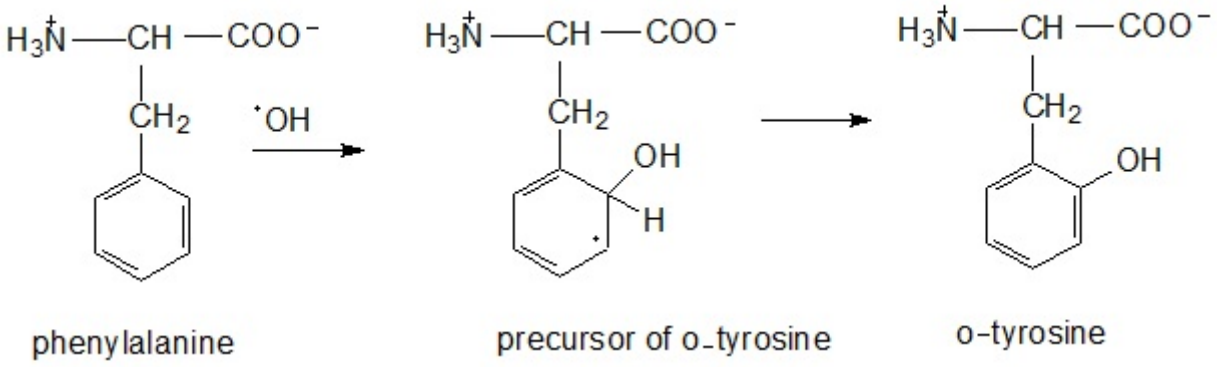


Figure 1- Mechanism of formation of o-tyrosine from phenylalanine (Stewart 2001a).

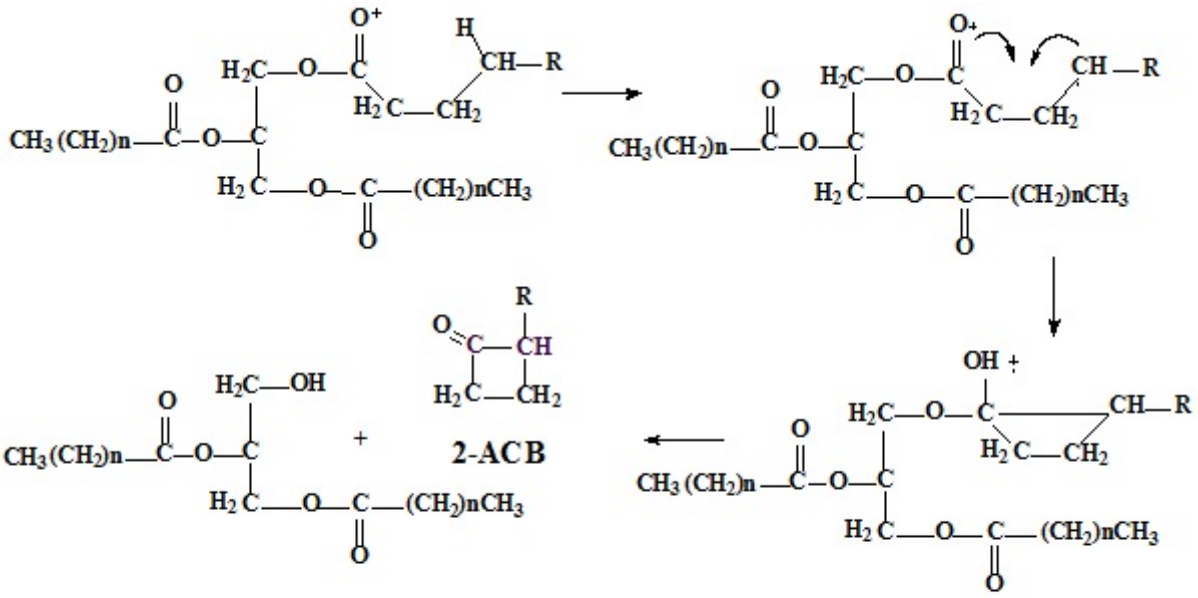
Lipids

The first studies used model systems (pure triglycerides) to study the basic mechanisms involved in the chemical changes occurring in lipids during irradiation (Dubravic and Nawar 1968, Nawar 1977, Nawar 1978). Ionizing radiation can change lipids by either catalyzing their reactions with oxygen (autoxidation) or by the action of high energy radiation (direct or indirect effect). If oxygen is present during irradiation, both oxidative and radiolytic effects will occur and, no

difference between them will be evident.

A number of radiolysis products have been identified in irradiated lipids. These products include fatty acids, esters, aldehydes, ketones, alkenes, alkanes, and other hydrocarbons (Stewart 2001a). Identical or similar products at higher concentrations were also identified in heat-treated food (FDA 2008). However, a class of radiolysis products derived from lipids, 2-alkylcyclobutanones (2-ACB), exists that does not form when fat is exposed to heat or other forms of processing. Cleavage at the acyl-oxygen bond in the triglyceride (Figure 2) result in the formation of the 2-ACBs in irradiated lipids (Nawar 1978). This class of compounds has the same number of carbon atoms as the parent fatty acid and an alkyl group with n-4 carbon atoms located at position 2. The 2-dodecylcyclobutanone (2-DCB) is formed in small amounts (> μg/g lipid/ kGy) from irradiated chicken and ground beef (FDA 2008). Some researchers also believe that 2-ACBs are present in non-irradiated food, but in amounts too low to be detected when conventional methods are used (Ndiaye and others 1999).

Recently, Variyar and others (2008) reported for the first time the natural occurrence of 2-ACBs in non-irradiated food using a combination of super critical fluid extraction (SFE) with thin layer chromatography purification (TLC). The concentration of 2-DCB was 2.7 μg/g in fresh cashew and 6.1 μg/g in irradiated cashews. Because this is the only study that demonstrated the natural existence of 2-ACBs in non-irradiated food, more studies are needed to confirm these results. If these studies show that 2-ACBs are present in non-irradiated lipids, then 2-ACBs will not be considered a unique radiolytic marker any longer.



Fatty acid	2-alkylcyclobutanone (2-ACB)	R group
Palmatic	2-dodecylcyclobutanone (2-DCB)	$(CH_2)_{11}CH_3$
Stearic	2-tetradecylcyclobutanone (2-TCB)	$(CH_2)_{13}CH_3$
Oleic	2-tetradec-5'-enylcyclobutanone (2-TDCB)	$(CH_2)_4CH{=}CH(CH_2)_7CH_3$
Linoleic	2-tetradeca-5',8'-dienylcyclobutanone	$(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Figure 2- Mechanism for the formation of 2-ACBs (Stewart 2001a).

Vitamins

Vitamins are present in food in relatively small quantities, but they are essential nutrients. Therefore, many studies have examined their survival after irradiation (Urbain 1986b). Vitamins in solution (model system) are more sensitive to irradiation than in food (Stewart 2001a). Thus, the effect of radiation on vitamins observed in the model system may not represent what occurs in the food system (Urbain 1986). Vitamin losses are affected by the applied dose, irradiation temperature, and the presence and absence of oxygen. Other food preservation methods, such as heat treatment, also destroy vitamins, so the effect of irradiation on vitamins is not unique to the irradiation process (Stevenson 1994).

Vitamins are subdivided into water-soluble and fat-soluble vitamins. The water-soluble vitamins include C, B, and folic acid, while fat-soluble vitamins include A, D, E, and K. The sensitivity of vitamins to irradiation varies depending on the type of the vitamin (Table 3).

Table 3- Sensitivity of fat- and water-soluble vitamins to irradiation.

Group	Most sensitive		⇒		Least sensitive
Fat-soluble	E	Carotene	A	D	K
Water-soluble	B_1	C	B_6	Folate, niacin	B_{12}

(Stewart 2001a).

Vitamin E (α-tocopherol) is the most sensitive of the fat-soluble vitamins, and it is used as an indicator of the effects of irradiation on this class of vitamins (Knapp and Tappel 1961). Vitamin E is easily oxidized by the products of the oxidation of unsaturated fats. Destruction of vitamin E by irradiation is adversely affected by the presence of oxygen and the solvent degree of

saturation (Urbain 1986b).

Vitamin A (retinol) and pro-vitamin A (β-carotene) are relatively radiation stable, and they showed minimal destruction when irradiated at 20 kGy (Lukton and Mackinney 1956). Moreover, most foods such as milk and butter that are important sources of this vitamin are unlikely to be irradiated on a commercial basis because of off-flavor considerations (Stevenson 1994). β-carotene, which is found mostly in vegetables and fruits, showed little loss after being irradiated; tomatoes irradiated at dose as high as 200 kGy showed only 10-20% reduction in their β-carotene content, and carrots irradiated at 20 kGy showed only 5% reduction in β-carotene (Stevenson 1994).

Vitamin D exists in two forms: D_2 (calciferol) and D_3 (cholecalciferol). Little information is known on the radiation sensitivity of this vitamin, but when D_3 was irradiated in iso-octane, it was more stable than vitamin E and A (Urbain 1986b). Vitamin D was unaffected by gamma irradiation when it was irradiated in salmon oil because of the presence of α-tocopherol and other antioxidants.

All of the vitamin K-related compounds are derived from naphthoquinones (Urbain 1986b). The sensitivity of the vitamin K group to irradiation depends on the particular compound and the medium in which irradiation occurs. Vitamin K_3 is the most stable of the fat-soluble vitamins. However, it is the most radiation-sensitive of the vitamin K group (Knapp and Tapple 1961). The small amount of vitamin K_3 present in beef was totally destroyed by gamma irradiation at 30 kGy, while vitamin K_3 stability in plant foods was high (Urbain 1986b).

Vitamin B_1 (thiamin) is not only the most radiation sensitive of the water-soluble vitamins, but also the most heat labile (Stewart 2001a). The sensitivity of vitamin B_1 to heat and irradiation

is due to the presence of the hetro double bond between nitrogen and carbon in the molecule. Irradiation of thiamin in an aqueous system led to the formation of dihydrothiamin, which is biologically inactive. However, no radiolysis products have been isolated from foods. The destruction of thiamin in food increases when the applied dose and irradiation temperature is increased (Stewart 2001a). It has also been shown that greater destruction of thiamin occurs in the presence of oxygen during irradiation (Tappel 1956).

Vitamin C or ascorbic acid in aqueous solution is easily destroyed by radiation. Dehydroascorbic acid (Figure 3) and diketogluconic acids were among the radiolysis products identified in irradiated vitamin C solutions (Urbain 1986b). Since ascorbic acid and dehydroascorbic acid are both biologically active, each should be measured to ensure exaggerated losses are not reported (Stewart 2001a). Studies performed to determine the effect of irradiation on vitamin C content showed that doses up to 5 kGy did not markedly reduce the total ascorbic acid content of irradiated fruits and vegetables (Urbain 1986b). In conclusion, the effect of irradiation on vitamin C content of foods is comparable to the effect of heat.

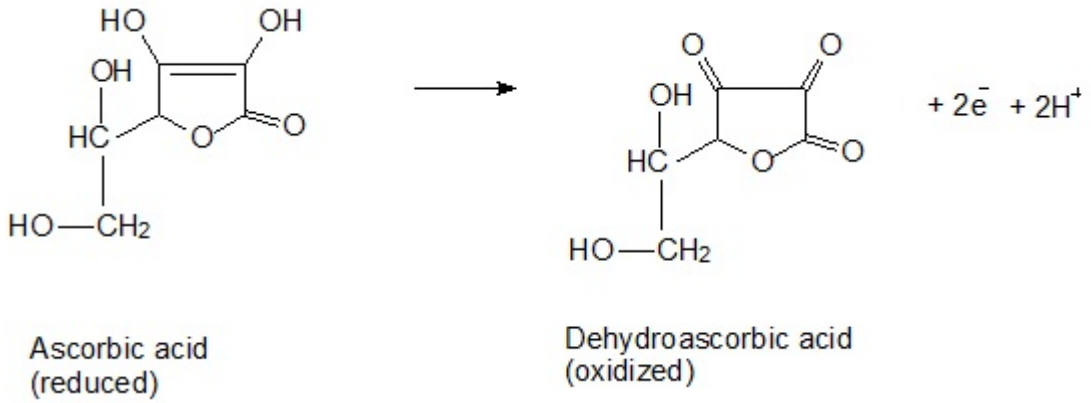


Figure 3- Reduced and oxidized form of ascorbic acid (Stewart 2001a).

Vitamin B_6 (pyridoxine or pyridoxol)) may be present in 2 other biologically active forms in which the alcohol group is replaced by an aldehyde (pyridoxal) or by a methylamine group (pyridoxamine). The pyridoxine content of food irradiated at commercial dose levels was not reduced significantly, and the changes that occurred were similar or less than those caused by heat (Richardson and others 1961).

Vitamin B_2 is present in two active forms in living tissue: as flavin mononucleotide (FMN) and flavin dinucleotide (FAD). Both are cofactors in the electron transport chain. Riboflavin is relatively resistant to irradiation in many foods. Irradiation of raw pork chops and chicken breast showed an initial increase in their riboflavin content (Fox and others 1995). Although folic acid (pteroylmonoglutamic acid), vitamin B5 (niacin), and vitamin B12 (cyanocoblamin) are sensitive to ionizing irradiation when they were treated in solution, they are stable when irradiated in foods (Stewart 2001a).

BIOLOGICAL EFFECTS OF IONIZING RADIATION

Ionizing radiation can damage the deoxyribonucleic acid (DNA) of living microorganism and ultimately kill the microorganism (Stewart 2001a). The DNA damage may result from direct or indirect action. In the direct action, the DNA is damaged directly by an ionizing particle or ray. In the case of indirect action, the DNA damage is caused by adjacent ionized molecules. Because water is the largest component of most food, indirect damage is often caused by the products of water radiolysis. Upon irradiation, water loses an electron and produces H_2O^+. The free electron and the H_2O^+ react with other water molecules to form hydrogen radicals, hydroxyl radicals, molecular hydrogen, oxygen, and hydrogen peroxide (Smith and Pillai 2004). Hydroxyl radicals

and hydrogen peroxide are very reactive and can cause single- and double-stranded breaks in the DNA. Biological systems can repair single- and double-stranded breaks of the DNA. However, because the damage caused by ionizing radiation is random and extensive, it is almost impossible for bacteria to repair the radiation damage.

As shown in Table 4 the lethal dose depends on the type of organism, and it decreases as the size of the organism increases.

Table 4- Approximate lethal dose for various organisms (Urbain 1986c).

Organism	Dose (Gy)
Mammals	5-10
Insects	10-1000
Vegetative bacteria	500-10,000
Sporulating bacteria	10,000-50,000
Viruses	10,000-200,000

Viruses

Although foods can carry viruses, foodborne illness are rarely caused by viruses (Urbain 1986c). Viruses can reproduce only by parasitizing living cells, and they do not multiply in foods. Because viruses are very small (16-200 nm diameter), they have greater resistance to radiation. The average D_{10} value for most viruses as they exist in foods is about 5 kGy when the food is irradiated at temperatures above freezing. The estimated dose required for viral inactivation in food is so high (20 to 100 kGy) that it makes irradiation an unlikely choice as a treatment procedure.

Bacteria

Bacteria cause most of the spoilage and foodborne illness, and most processing methods are designed to reduce or eliminate bacteria (Urbain 1986c). In general, spores are more resistant to irradiation than vegetative bacteria. However, some bacteria such as *Micrococcus radioudurans* and *Moraxella acinetobacter* isolated from meat, *Micrococcus roseus* No. 248 and *Micrococcus radiophilus* isolated from fish, and *Pseudomonas radiora* isolated from rice are highly resistant to radiation, their resistance being comparable to or exceeding that of spores (Table 5).

The resistance of microbial cells to irradiation depends on the size of the organism, type of organism, number and relative age of the cells in food, the absence or presence of oxygen, and the physical and chemical composition of the food (Smith and Pillia 2004). The rate of migration of free radicals decease as the water in the irradiated product freezes; therefore, more energy is needed to destroy the microbes and the D_{10} increases.

Table 5- D_{10} values for specific pathogens on meat and eggs products. Adapted from Smith and Pillia (2004) and Urbain (1986c).

Target organism	Temperature (°C)	Product	D_{10} value (kGy)
Staphylococcus aureus	5	Turkey breast meat	0.45
Campylobacter jejuni	30	Ground turkey	0.16
	5		0.19
	-30		0.29
Salmonella Heidelberg	0	Poultry (air packed)	0.24
	0	Poultry (vacuum packed)	0.39
Salmonella Enteriditis	5	Egg powder	0.6
	3	Ground beef	0.55-0.78
Salmonella spp.	5	Turkey breast meat	0.71
Listeria monocytogens	5	Beef	0.45
Escherichia coli O157:H7	5	Ground beef patties	0.27-0.38
Clostridium botulinum	0	Cooked beef	3.8
Moraxella osloensis	- 30	Raw beef	4.7-10.0

Yeast and Molds

Yeast and mold contamination is almost limited to fruit, vegetables, cereal grains, legumes, and baked products. Yeasts are single-celled organism, while molds are multicellular. Because determining the number of mold cells is difficult, the D_{10} value has not been applied to molds. Instead, the end point method (lethal dose) has been used for both molds and yeast. Both yeast

and mold are substantially resistant to radiation and that limits the use of irradiation to inactivate these organisms in some foods especially fruits. The lethal doses of molds is 4.6 -20.0 kGy and for yeast is 2.1-6.0 kGy (Urbain 1986c).

Parasites

Although a number of parasites can cause illness to humans, only a few are foodborne (Urbain 1986c). The two general groups of the foodborne parasites are protozoa and helminths. The most important foodborne parasitic protozoans are cysts of *Toxoplasma gondii* in meat and *Entamoeba histolytica* in raw fruit. Foodborne helminths include *Trichinella spiralis* and *Taenia solium* in pork, *Taenia saginata* in beef , *Fasciola hepatcia* in sheep, and *Anisakis* genera in fish. These organisms have a number of forms during their life cycle, but they are found in food only as larvae. The sensitivity of these parasites to radiation depends to a large degree upon their particular form at the time of irradiation. The lethal dose for most parasites is below 0.3 kGy. However, a relatively large dose is required to kill some parasites such as *Taenia solium*, *Taenia saginata*, *Trichinella spiralis*, and *Anisakis*.

Insects

The number of insect species is more than a million; however, only about 500 species are considered pests (Urbain 1986c). Insects may contaminate foods at any life stage and their radiation sensitivity varies with each life stage. However, regardless of the life stage of insect at the time of irradiation, radiation of appropriate amounts (0.01-1 kGy) produce either sublethal or lethal effects on all insects. In most cases, lethality is not always a necessary effect, but the

important requirement is to prevent insect infestation. Because some foods carry a low level of infestation, they can be treated at low doses. Irradiation is very effective in controlling insect infestations in foods. Doses of 0.13 to 0.25 kGy applied to eggs and larvae prevented their development into the adult stage, and pupae treated with 1 kGy did not develop into adults. Sexual sterility in most insects is produced with doses of 0.13 to 0.45 kGy.

WHOLESOMENESS OF IRRADIATED FOOD

Toxicological Consideration

The available information about food irradiation chemistry suggests that toxicological hazards are not associated with the consumption of irradiated foods. This is explained in the section on food irradiation chemistry on pages 8-17. The FDA (2008) noted that animal feeding studies provide an independent way to assess the toxicological safety of irradiated food.

Animal-testing procedures are often used to evaluate chemical food additives. However, the application of this method for the evaluation of irradiated food presents a serious problem because the test material is a food and not a food additive, and this does not allow testing at exaggerated levels (Urbain 1986d). Performing irradiation at high doses as a mean of paralleling the exaggeration of food additive concentration is invalid because high doses can change the food quality. Despite this difficulty, animal testing is the only approach that has been used to evaluate the toxicity of irradiated food.

The safety of irradiated food has been extensively studied since 1950s. The FDA reviewed more than 400 studies for their quality of experimental design, rigor, and statistical validity before

approving food irradiation (WHO 1994). Most of these studies showed that no adverse health effects were associated with the consumption of irradiated food, and only a small number of studies produced equivocal results. However, most studies that produced equivocal results were deficient in experimental design, used insufficient numbers of animals, or suffered from experimenter error (WHO 1994).

Animals fed radiation-sterilized foods for 40 generations did not exhibt any adverse health effect from the consumption of irradiated food (Swallow 1991). The Raltech study showed that rodents fed irradiated chicken (45-68 kGy) as 35% of their diet did not suffer an increased risk cancer or birth defects (Thayer and others 1987). The Raltech study confirmed the safety and wholesomeness of chicken sterilized by irradiation to a maximum dose of 68 kGy.

A multi-generation study and a two-year feeding study in rats also failed to find adverse effect associated with the consumption of irradiated chicken (Knecht-van Eekelen and others 1971, 1972). Rats fed radiation-sterilized ground beef as 45% of their final diet for two years showed no changes in survival rates, histopathology, or reproduction capabilities (Polling and others 1955).

The FDA evaluated a large number of studies in which irradiated (0.15-50 kGy) fruits or vegetables were fed to different animal species. Most of these studies showed no adverse effect. However, three studies reported some effects (FDA 2008). The FDA evaluated these studies and concluded that the effects reported were either not caused by irradiation or were not toxicologically significant. In the first study, dogs fed a diet containing 10% onions irradiated at 0.25 kGy were reported to developed anemia, increased spleen weights, myeloid metaplasia, and reticuloendothelial hyperplasia. However, because similar effects were reported in dogs fed nonirradiated onions, the FDA concluded that the effects were not caused by irradiation. In the

second study, rats fed a diet containing 35% oranges irradiated at 1.4 or 2.8 kGy showed a decrease in reproductive performance in the second breeding. Again, because these effects were observed in rats fed nonirradiated orange, the FDA concluded that these effects were not caused by irradiation. In the third study, a reduction in levels of alkaline phosphate in duodenal tissue of weanling rats fed a mixture of irradiated cabbage (6 kGy) and chicken stew (56 kGy) has been reported. The FDA reviewed this study in detail and concluded that the observed effect was not of toxicological significance.

Although most of animal studies showed that no adverse health effects were associated with the consumption of irradiated food, ataxia and paralysis were reported in cats fed a gamma-irradiated commercial pet food in Australia in 2008 (Child and others 2009). The pet food (Orijen) was irradiated at 50 kGy on entry to Australia for biosecurity purposes. The pet food manufacturer (Champion Petfoods Ltd, Morinville, Alberta, Canada) conducted extensive testing on Orijen pet food sold in Australia and concluded that the ataxia and paralysis were caused by two factors: depletion of vitamins and the formation and release of free radicals in irradiated foods (Champion Petfoods 2008). The manufacturer's testing showed that the irradiation of Orijen cat food at 50 kGy caused 77% depletion in vitamin A. Cassidy and others (2007) showed that depletion of vitamin A from irradiated cat food was associated with the same symptoms reported in cats in Australia. Orijen food is rich in omega-3 fatty acids, which are susceptible to oxidation following irradiation. Consumption of oxidized products with high levels of free radicals cause tissue damage. The manufacturer concluded that the loss of the antioxidants (vitamins) combined with the formation of free radicals were the major contributing factor to the toxicity of irradiated Orijen food. After this outbreak, Champion stopped exporting Orijen food to Australia. Because

Orijen food was not approved for irradiation and was irradiated by mistake, this case cannot be used to infer that food irradiated is not safe.

Furan and Benzene as Radiolytic Products

The presence of benzene, toluene, and furan following the irradiation of some foods has generated concern about the safety of irradiated foods. Originally, it was thought that these compounds were unique radiolytic products. Benzene and toluene are produced from the oxidative/radiolytic cleavage of phenylalanine (Sommers and other 2006) while furan is formed during irradiation of sugars or ascorbic acid (Fan 2005).

Trace amounts of benzene and toluene (5-60 ppb) were found in irradiated meat (Merritt and others 1978, Nam and others 2003). However, benzene derivatives have been reported in many cooked foods such as scrambled eggs (Matiella and Hsieh 1991), butter, meat, and certain fruits with levels ranging from 0.5 ppb in butter to 500-1900 ppb in eggs (McNeal and others 1993). Benzene and toluene were found in both irradiated and non-irradiated haddock. However, their levels were higher in irradiated samples (Angeliniey and others 1975). Health Canada has estimated that about 3 ppb of benzene would be formed in fresh irradiated beef at 4.5 kGy and concluded that no adverse health effects are associated with consumption of these small amounts (Health Canada 2002).

Irradiation of certain foods produce furan in low quantity (FDA 2008). However, some foods form furan when they are heated and other foods form furan during refrigerated storage. In 2004, the FDA issued a Federal Register notice requesting data and information from the public on furan in food and furan toxicology. The data showed that furan was present in a dozen thermally

processed foods with levels ranging from 3 ppb in apple juice to 125 ppb in beef and vegetable soup (FDA 2009). The levels of furan in irradiated iceberg lettuce and spinach were below the levels of furan formed during refrigerated storage (FDA 2008). Therefore, the FDA concluded that consumption of irradiated iceberg lettuce and spinach will not increase the amount of furan in the diet.

2-Alkylcyclobutanones

Although the safety of irradiated food has been studied extensively, little data are available about the genotoxic potential of 2-ACBs (Sommers and others 2006). Using the Comet Assay Delincée and Pool-Zoople (1998) showed that 2-DCB at concentrations of 0.3-1.25 mg/ml in vitro induced DNA strand breakage in human and rodent colon cells. Because the amounts of 2-DCB used in the experiment were very high compared to potential intake, and the Comet Assay has not been validated for the detection of weak genotoxins and may produce false-positve results (Sommers and others 2006), these results cannot be used to infer that irradiated foods are carcinogenic.

However, Sommers and others (2006) reported that a retest using the Comet assay of several of 2-ACBs at concentration up to 400 μM did not show significant levels of DNA strand breakage in human cell lines HT-29 and HT-29 cl 19 A. Sommers and Mackay (2005) concluded that 2-DCB did not increase expression of DNA damage-inducible genes in *E. coli* or the formation of 5-fluorouracil-resistant mutants in *E. coli*, with and without exogenous metabolic activation. Sommers (2006) tested the ability of 2-DCB to induce the formation of micronuclei (MN) in human TK6 lymphoplasts with and without exogenous metabolic activation (EMA). The

number of MN/1000 binucleated cells were induced 2.65-fold without EMA, and 2.85-fold in the presence of EMA when they were exposed to 53 μM 2-DCB. Because the amounts of 2-DCB that would be consumed is very small compared with that of its clastogenic parent fatty acid, Sommers (2006) concluded that 2-DCB is unlikely to have any adverse effects on human health.

All cyclobutanones tested produced negative results in the Ames test, a bacterial gene mutation assay. Sommers (2003) found that 2-DCB did not induce mutations in the *E. coli* tryptophan reverse mutation assay. Gadgil and Smith (2004) found that neither 2-DCB nor its metabolite was mutagenic in the Ames assay when 5 standard *salmonella* tester strains were used, indicating that 2-DCB was not biotransformed into a mutagenic metabolite. Hartwig and others (2007) reported that none of 2-ACBs tested reverted the histidine synthesis mutations in either test strain, neither in the presence nor in the absence of rat liver microsomal fractions.

Gadgil and Smith (2004) evaluated the acute toxicity of 2-DCB using the Microtox acute toxicity system and compared it with cyclohexanone and 2-nonenal (both GRAS additives). The acute toxicity of 2-DCB was between that of cyclohexanone and 2-nonenal. The authors concluded that the potential risk from 2-DCB, if any, is very low.

Because the adverse effects of 2-ACBs that have been noted refer almost exclusively to *in vitro* studies, it is not appropriate to evaluate the safety of irradiated fat-containing foods based on these studies, and determining the safety of irradiated fat-containing foods can be based on the results of the large number of feeding studies carried out with irradiated foods (Health Canada 2003).

Nutritional Consideration

Animal-feeding tests showed that the normal nutritional values of protein, fat, and carbohydrate are not significantly altered by irradiation (Urbain 1986d). Irradiation also does not alter the mineral content of food. However, levels of certain vitamins may be reduced by irradiation. The percentage reduction depends on the specific vitamin, the type of the food, and the conditions in which irradiation is performed (FDA 2008). A vitamin loss in specific food is considered significant if the food contributes highly to the total dietary intake of that vitamin and the vitamin levels are significantly reduced during irradiation. For example, if a specific food is considered an excellent source of vitamin A, but it only contributes no more than 1% to the total dietary intake of vitamin A. Then, a 50% reduction in vitamin A in that food will not be significant because it will reduce the total dietary intake by 0.5%. Urbain (1986d) concluded that in most cases, vitamin losses that result from irradiation are not large, and when they occur, they are not greater than losses caused by other types of food processing. The FDA (2008) also concluded that irradiation of iceberg lettuce and spinach up to maximum dose of 4.0 kGy will have no impact on the total dietary intake of vitamin A, vitamin K, or folate and will not have an adverse impact on the nutritional value of the overall diet.

Microbial Consideration

Two main concerns have been raised regarding the effects of irradiation on microorganisms: the effect of the reduction in the natural microflora on surviving pathogens and the possibility for the development of radiation resistant bacteria (Dickson 2001). Because ionizing radiation reduces the level of indigenous microflora in foods, some concern has been

expressed that irradiated foods will allow a rapid outgrowth of pathogenic bacteria. However, this is not true, and the growth rates of pathogenic microorganisms were the same in both irradiated and nonirradiated meats (Szczwiska and others 1991, Dickson and Olson 1999). During the evaluating of the irradiation on iceberg lettuce and spinach for the petition for its use, the FDA (2008) considered whether the irradiation process would increase the growth of *Clostridium botulinum* because this microorganism is relatively resistant to irradiation compared to non-spore-forming bacteria. The FDA concluded that the possibility of increased microbiological risk from *C. botulinum* is very small because the refrigerated storage necessary for these vegetables prevents the growth of *C. botulinum* and production of its toxin. Moreover, spoilage by other organisms will occur before the outgrowth and production of toxin by *C. botulinum*. The FDA also concluded that the irradiation of iceberg lettuce and spinach up to 4.0 kGy will reduce or eliminate bacterial populations without increasing the microbial risk of surviving pathogens.

Because ionizing radiation has been known to induce mutation, the concern with the development of resistant bacteria is significant (Dickson 2001). However, irradiation does not induce pathogenicity in nonpathogenic bacteria, and most bacteria that undergo radiation-induced mutation are vulnerable to stress (Ingram and Farkas 1997). A single irradiation does not produce resistant mutants because of the adequacy of the DNA repair mechanisms of wild strains. The only known case of a radiation-resistant population of bacteria as in the case of *Salmonella enterica var typhimurium* LT2 was due to repeated irradiation and isolation (Ibe and others 1982). Subjecting bacteria to several cycles of heat also gave rise to a heat-resistant Salmonella mutant. However, this is not considered a problem in pasteurization process and is not expected to be a problem in food irradiation.

EFFECT OF IRRADIATION ON MEAT QUALITY

Although irradiation is very effective in eliminating food pathogens, the adoption of irradiation technology by the meat industry has been limited because of quality and health concerns about irradiated meat (Ahn and Lee 2006). Irradiation produces off-odors and off-flavors, and alters meat color. Irradiation generates a pink color in cooked poultry, brown/gray color in raw beef, and an off-flavor and off-odor in irradiated meat and poultry. In general, consumers associate the pink color in cooked poultry and pork with contamination or with it being undercooked, the brown/gray color in raw beef with an aging or low quality product, and off-flavor with undesirable chemical reactions. Therefore, understanding the chemical changes in meat caused by irradiation is necessary to prevent those changes.

Lipid Oxidation

Irradiation accelerates lipid oxidation in meat due to the formation of free radicals (Ahn and Lee 2006). The rate of lipid oxidation in meat is affected by the applied dose and the presence of oxygen. However, oxygen plays a more important role in the development of lipid oxidation in meat than irradiation. Oxidation flavor and rancidity in cooked meat are mainly caused by aldehydes, with hexanal the most predominant aldehyde. Storage temperature and irradiation temperature also play an important role in quality changes of irradiated meat. Lipid oxidation was small in frozen-stored irradiated turkey due to the limited distribution of free radicals (Nam and others 2002a). At low temperatures, free radicals tend to recombine to form the original substances rather than diffuse through the food and react with other components (Nam and others 2002b).

Off-Odor Production

All irradiated meat produced detectable irradiation odor regardless of the degree of lipid oxidation (Ahn and Lee 2006). Irradiated uncooked chicken breast and thighs produce a characteristic "bloody and sweet" aroma (Hashim and others 1995). Ahn and others (2000a) described the odor of irradiated pork as "barbecued corn-like." Although irradiation of meat increases the amount of volatile hydrocarbons and carbonyl compounds, the odor intensity of sulfur compounds was much stronger and more stringent. Volatiles from lipids account for only a small part of the off-odor in irradiated meat (Lee and Ahn 2003). This indicates that sulfur compounds are the major volatiles responsible for off-odor in irradiated meat, which are different from those of warmed-over flavor in oxidized meat. Ahn and others (2000a) showed that irradiated vacuum-packaged patties maintained irradiation odor for two weeks, but the intensity of the irradiation off-odor in aerobically packaged pork disappeared after one week of refrigerated storage.

The production of methyl mercaptan and hydrogen sulfide is important to irradiation odor (Ahn and Lee 2006). However, dimethyl trisulfide was the most potent off-odor compound in irradiated chicken meat, followed by bismethylthiomethane (sulfurous). Irradiation also greatly increased volatile sulfur compounds such as sulfur dioxide, methylthioacetate, dimethyl disulfide, and trimethyl sulfide from turkey and pork meat.

The side chains of amino acids are susceptible to radiolytic degradation (Ahn 2002). More than one site on the amino acid side chains is susceptible to free radical attack, and many irradiation products are produced by the secondary chemical reactions that follow the primary radiolytic degradation of the side chains (Ahn and Lee 2002). Most of the new compounds

generated by irradiation were sulfur compounds, indicating that sulfur-containing amino acids are the most susceptible to irradiation (Ahn and Lee 2006). The perception of odor from a sample containing sulfur volatiles depends on the composition and amounts present in the sample. Ahn (2002) showed that methionine was the major amino acid responsible for irradiation off-odor. Irradiated liposomes containing "sulfur amino acids" produced odor characteristics similar to those in irradiated meat, indicating that sulfur amino acids are the source of off-odor in irradiated meat. The volatile profiles and sensory characteristic of the amino acids show that irradiation odor is different from lipid oxidation odor, and lipid oxidation is responsible for only a small part of the off-odor in irradiated meat (Ahn and others. 2000b).

Ahn and Lee (2006) concluded that irradiation odor is different from lipid oxidation odor, and volatile hydrocarbons and carbonyls play a minor role in irradiation off-odor. The main source of irradiation off-odor is sulfur compounds derived from protein, not lipids.

Color Change in Meat by Irradiation

The color change in irradiated meat depends on various factors such as the irradiation dose, animal species, muscle type, and packaging type (Ahn and Lee 2006). Increased redness is a problem in irradiated light meat, especially poultry and pork, whereas brown discoloration is a problem in aerobically irradiated raw red meat.

Irradiation increases the redness value (a*) of both aerobically and vacuum-packaged raw chicken and turkey breast (Nam and Ahn 2003a). The color change is distributed over the whole meat sample, and the increased redness is dose dependent and stable for two weeks. The induced red color is more intense and stable in vacuum packaging during refrigerated storage (Grant and

Patterson 1991). Because the red color is associated with fresh meat, the induced red color in raw meat is not always detrimental (Lefebvre and others 1994). However, an objectional red color was observed in radiation-sterilized cooked chicken meat in the absence of oxygen (Hanson and others 1963). Irradiation also increases the redness of vacuum packaged cooked turkey, but the surface color of aerobically packaged cooked turkey turns grayish (Nam and Ahn 2003b).

Irradiation increases the lightness (L* value) of ground beef. The L* value increases as the storage time increased (Nam and Ahn 2003c). Irradiation reduces the redness (a* value) of ground beef and changes the color of ground beef from bright red to a greenish brown, which would be unattractive for consumers.

Mechanism of Color Changes in Irradiated Meat

In the beginning, it was believed that the pink color compound in irradiated meat was an oxmyoglobin pigment. However, this could not be true because the red color formed by irradiation was produced in anoxic conditions (Ahn and Lee 2006). Nam and Ahn (2002a) identified the pink pigment formed in irradiated raw and cooked turkey as carbon monoxide-myoglobin (CO-Mb). They found that production of CO, generation of reduced conditions, and CO-Mb formation were all essential for the pink color formation in irradiated light meat. Lee and Ahn (2004) showed that glycine, asparagine, glutamine, pyruvate, glyceraldehydes, alpha-ketoglutarate, and phospholipids were the major sources of CO production in meat.

Nam and Ahn (2002a) showed that irradiation lowered the oxidation reduction potential (ORP) of aerobically and vacuum-packaged raw and cooked turkey breast meat. However, the ORP was more stable in vacuum-packaging. Shahidi and others (1991) suggested that irradiation

might increase the reducing potential of sodium ascorbate because irradiated pork patties had higher a* values than nonirradiated patties in vacuum packaging. Irradiation generates hydrated electrons (aqueous e-) that act as a powerfull reducing agent and reduce the ferricytochrome. The decrease in the ORP in meat is necessary for the formation of CO-Mb because the CO-Mb complex can be formed only when the heme pigment is in its reduced form (Ahn and Lee 2006).

Houser and others (2005a) reported that irradiation-induced color fading in cured ham occured immediately after irradiation and the fading was dose-dependent. However, regeneration of cured color was confirmed after 7 days of refrigerated storage. Because reducing conditions were observed after irradiation and these conditions did not accelerate the reduction of residual nitrite to nitric oxide, Houser and others (2005a) assumed that free nitric oxide or other nitrite derivatives that are separated from the pigment during color fading can then become reattached to the pigment under highly reduced condition.

The mechanisms of color change in irradiated dark meat are different from those in light meat (Ahn and Lee 2006). The heme pigments in beef exist in much greater quantity than in light meat (10 to 1), and the amount of CO-Mb to total heme pigment in irradiated beef is small. Therefore, beef color is mainly controlled by the status of the heme pigment, which is controlled by the reducing potential of meat. The irradiation of meat under vacuum conditions or the addition of ascorbic acid to aerobically packaged meats creates a reducing environment and prevents the formation of brown color in ground beef (Wheeler and others 1996).

In conclusion, irradiation increases the redness of light meat but turns the red meat color to brown in the presence of oxygen. Irradiation generates CO and reducing conditions, which enhances the formation of CO-Mb complex in light meat. In dark meat, however, the contribution

of CO-Mb to the color is smaller than it is in light meat, and the most important factor is the status of the heme pigments.

Flavor Change in Irradiated Meat

Flavor results from the combined effects of basic tastes (sweet, sour, bitter, salt, and umami) derived from water soluble compounds and odor derived from volatile substances present in the food product (Brewer 2006). The odor of irradiated meat has been described as rotten egg, sweet, bloody, cooked meat, barbecued corn, burnt, sulfur, metalic, alcohol, acetic acid, liver-like, serumy, and bloody (Brewer 2009). Much of the work on irradiated meat odor and flavor has targeted specific components, especially lipids. At low doses, irradiation can initiate lipid oxidation, which results in undesirable off-odors and flavor (Lescano and others 1991). Degradation of sulfur-containing amino acids results in the production of volatile sulfur compounds, which produce cabbage-like, sulfury, putrid and/or rotten vegetable-like odors (Ahn 2002). Whether the odor and flavor changes that occur due to irradiation are due primarily to lipid oxidation or to generation of volatile sulfur compounds, or both, is unclear (Brewer 2009). The reaction of sulfur-containing amino acids with aqueous electrons appears to be the source of volatile sulfur compounds that contribute to off-flavors (Jo and Ahn 2000). Sulfur-containing compounds generally have a very low threshold. For example, the threshold value of 2-methyl-butanethiol is 0.00007 ppb (Brewer 2009).

The volatile compounds responsible for the off-odor in irradiated meat produced from impact of irradiation on proteins and lipids are different from those produced from lipid oxidation by itself (Jo and Ahn 2000). An increase in lipid peroxidation product (hexanal and trans-4,5-

epoxy-(E)-2-decenal) in combination with a loss of desirable meat odorants result in the development of the warmed over flavor (WOF) of cooked, refrigerated beef (Brewer 2009). In conclusion, because flavor results from the combination of the five basic tastes and from the volatile substances present in the food, it seems that volatile sulfur compounds are the main source of off-flavors because they have very low threshold.

Control of Quality Deterioration

Additives

The addition of antioxidants is very effective in inhibiting the formation of both hydrocarbon and volatile aldehydes in irradiated beef stored under aerobic conditions. The addition of antioxidants such as ascorbate, citrate, tocopherol, gallic esters, and polyphenols was effective in reducing the off-odor of irradiated meat (Huber and others 1953). The addition of acid to meat lowers the pH and increases the lightness of meat. The addition of citric or ascorbic acid did not increase the redness of irradiated meat but increased its lightness. Ascorbic acid added to ground beef at 0.1% (w/w) was very effective in maintaining the redness (a*) of irradiated ground beef, especially in "long-term-aged ground beef" (Nam and Ahn 2003c). Oxygen is an effective scavenger of aqueous electrons (e- aq). Therefore, in the absence of oxygen reducing environment is established in irradiated meat, which converts ferric myoglobin to ferrous myoglobin. Ascorbic acid with or without sesamol and tocopherol significantly lowered the oxidation reduction potential (ORP) of ground beef, that maintained the heme pigment in the reduced form and stabilized the color of irradiated ground beef.

In some studies, the addition of vitamin E (alpha-tocopherol) to animal diets prevented

lipid oxidation (Brewer 2009). Dietary vitamin E reduced lipid oxidation and off odor (sulfur compounds and aldehydes) in irradiated, aerobically stored turkey (Nam and Ahn 2003d). However, vitamin E-supplementation of swine diets had no effect on the off-flavor of irradiated pork, initially or during retail display (Ohene-adjei and others 2004). Dietary conjugated linoleic acid (CLA) reduced thiobarbituric acid reactive substances (TBARS), hexanal, and pentanal in irradiated raw chicken. CLA decreased TBARS, 18:3, and 18:1, and increased 18:0 and t, t-9,11-isomers in the fat of irradiated beef patties derived from steers fed CLA (Weigand 2001). However, CLA was ineffective in controlling volatiles in irradiated broiler breast fillets (Du and others 2001). Many studies have shown that natural antioxidants such as rosemary extract and synthetic antioxidants were effective in controlling oxidation in irradiated meat (Brewer 2009).

Packaging

Packaging is the major factor that influences the amounts and types of volatiles detected in irradiated meat (Ahn and others 2001). Nam and Ahn (2003a, 2003b) used a double-packaging concept to solve off-odor problems in irradiated meat. In the double packaging method, meat is first packaged in an oxygen-permeable bag, and then the meats are packaged again in a larger oxygen impermeable vacuum bag and irradiated. The meat irradiated in aerobic bags was vacuum-packaged one to three days, and the outer vacuum bags of the double packaged meat was removed after a few days. Double packaging was effective in controlling lipid oxidation (aldehydes) and off-odor (S-compounds). The redness (a*) of double-packaged meat was lower than that of the vacuum-packaged meats, but was not enough to reduce the pink color of irradiated raw turkey. The packaging condition is more important for dark meat than it is for light meat. Aerobic

packaging generate a greenish brown color in irradiated ground beef, but anaerobic packaging do not cause discoloration. Exposing vacuum-packed irradiated beef to aerobic condition in the middle of storage regenerates the fresh red color of the ground beef (Ahn and Lee 2006).

Packaging and Additive Combination

The addition of antioxidants to double-packaged irradiated meat has been shown to be effective in overcoming the problem of double-packaging (Nam and Ahn 2003b). Sesamol with alpha-tocopherol (S+E) and gallate with alpha-tocopherol (G+E) prevented lipid oxidation in aerobically or double-packaged irradiated turkey during storage. Double packaging in combination with G+E or S+E reduced the redness of irradiated turkey breast, but G+E was more effective than S+E. The combination of double-packaging (vacuum then aerobic) and ascorbic acid was effective in reducing off-odor volatiles and mainating the fresh red color of irradiated ground beef. Irradiation of meat under vacuum conditions and adding a reducing agent maintained low redox potential of irradiated beef and kept myoglobin in the reduced form (Ahn and Lee 2006).

Irradiation Dose, Oxygen, and Temperature

The quality deterioration of irradiated food also can be minimized by controlling the irradiation dose and temperature, and by excluding oxygen. Irradiation increases TBARS and carbonyl compounds in raw chicken breast in a dose-dependent fashion (Brewer 2009). More than 120 volatile flavors compounds were induced by the irradiation of chicken. These compounds include 62 hydrocarbons, 44 aromatic compounds, 9 aldehydes, 5 ketones, and 10 miscellaneous

compounds. Cyclotetradecene, 2-methylpentanal and 4-methylcyclohexane were generated specifically in response to irradiation, and cyclotetradecene concentration was dose dependent. Irradiation increases the concentration of dimethyl disulfide and off-odor scores as doses are increased to 2 kGy in irradiated ham (Houser and others 2005b). Therefore, the irradiation dose should be selected carefully to both kill microorganisms and to minimize quality deterioration.

Off-flavor, off-odor, and color change can be reduced by reducing the temperature during irradiation (Brewer 2009). Lower temperatures increase the matrix viscosity and decrease water mobility. Ion and free radical dispersion are lower when water is frozen. Free radical reactions with other food components are slowed down in frozen meat, and they tend to recombine because they are less likely to diffuse and react with other food components (Taub and others 1975). In conclusion, freezing limits free radical formation and oxidation initiation. Irradiation at - 20 °C reduces autoxidation and extends shelf life. Moreover, low storage temperature slows down lipid oxidation and microbial growth and extends the shelf life of irradiated meat.

Irradiated meat odor and flavor vary with the gas atmosphere (Brewer 2009). Because lipid oxidation requires the presence of oxygen, the off-odor and off-flavor can be reduced by oxygen exclusion. Irradiating meat in nitrogen environments under a vacuum improves the meat quality (color, flavor, vitamin retention, and lipid oxidation) (Groninger and others 1956). O'Bryan and others (2008) concluded that the combination of dosages, temperatures, dietary and direct additives, storage temperatures and packaging atmospheres can produce meats that will be indistinguishable from non-irradiated meats.

DETECTION OF IRRADIATED FOOD

The European Committee of Standardization (CEN) has published 10 official protocols for the detection of irradiated product. These official methods enable food quality control laboratories to identify irradiated foodstuffs, allow for a control of the international trade with respect to legal labeling, and give consumers a guarantee of choice (Horvatovich 2006). Six of these methods are reference methods and are based on the analysis of the primary radiolytic products using electron spin resonance (ESR) and thermoluminescence or on the analysis of secondary radiolytic products such as volatile hydrocarbons and 2-alkylcyclobutanones (Marchioni 2006). The four other methods are less specific than the reference methods, but they are of interest because they are easier, faster, and less expensive than the reference methods. The four screening methods are photo-stimulated luminescence, single gel micro-electrophoresis, and the bacteriological methods DEFT/APC (Direct Epifluorescence Filter Technique/Aerobic Plate Count) and L.A.L/G.N.B (Limulus Amoebocyte Lyaste/Gram Negative Bacteria) (Marchioni 2006). The methods of detecting irradiated foods can be divided into various classes including physical methods, chemical methods, DNA methods, and biological methods. The methods used for the detection of irradiated food will be discussed in the following sections.

Physical Methods

ESR Spectroscopy

Food with dry or rigid parts is able to trap free radicals or excited electrons for a period of time that can be longer than the shelf life of food (Marchioni 2006). Electron spin resonance can be successfully used to detect irradiated meat and fish with bones, eggs with shells, shellfish, fruit

with achenes, nuts with shells, dried fruit, and some seeds and spices. When the food sample is analyzed by ESR, the test sample is subjected to the simultaneous action of magnetic fields (intended to direct the magnetic moments) and electromagnetic microwaves of very high frequency (9 GHz). Unpaired electrons behave like tiny magnets and are lined up in an applied magnetic field. The energy of the applied alternating microwave radiation is absorbed when its frequency is identical to the energy required to flip the alined electrons. The derivative representation of the absorption of electromagnetic microwave as a function of the magnetic field gives the ESR spectrum (Marchioni 2006).

The application of the ESR is simple, consisting of drying the food sample and the recording ESR absorption spectrum (Marchioni 2006). The presence of water in the food sample prevents the ESR analysis because the O-H dipole absorbs the microwave energy. Therefore, food samples should be dried at reduced pressures and low temperatures to prevent the combination of the radicals and modification of food composition (sugar). The presence of free radicals in food is not radiation specific because they are also produced by heating or crushing (grinding), and low ESR signals are also present in non-irradiated bone. However, the shape of the spectra and gyromagnetic factors enable the analyst to determine whether the ESR signals are radiation specific or not (Figure 4). The presence of radiation specific ESR in food is a proof of irradiation history. However, the absence of such an ERS signal (except in case of mammal bone) does not mean that the food has not been irradiated.

ESR spectroscopy has many advantages: it is non destructive, simple, and rapid. However, it is costly, it cannot be used to analyze food with high moisture content, and it is not radiation specific.

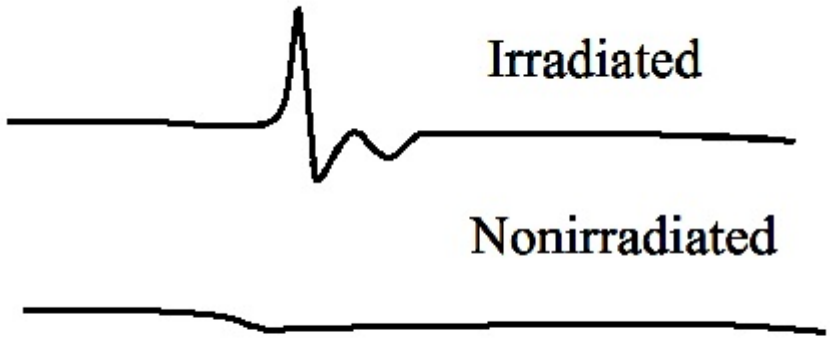


Figure 4- Electron spin resonance spectra from irradiated and nonirradiated bone (Stewart 2001b).

Luminescence

Luminescence is the emission of light when trapped energy is liberated by the addition of a chemical, heat, or light (Stewart 2001b). Ionizing radiation produces excited electrons in the irradiated foods, which can remain trapped in the crystalline lattice for several years. Heating irradiated foods (50 °C to 500 °C) releases part of the trapped energy in the form of light, which can be measured using by a thermoluminescence reader. Irradiated samples exhibit a sharp peak at 220 °C, whereas non-irradiated samples do not exhibit any signal at this temperature (Figure 5).

It was thought at first that thermoluminescence occurred as the result of organic components in the food. However, Sanderson and others (1989) showed that the origin of luminescence in spices was due to the presence of extraneous inorganic matter. In general, foods contain small quantities of silicate minerals, which come from the action of wind or from the contact with soil or sand (Soika and Delincee 2000). Because the standardized

thermoluminescence method requires separation of the minerals from the food matrix, the need for careful laboratory preparation and access to a calibrated source of ionizing radiation have limited the widespread use of thermoluminescence for routine commercial testing (Stewart 2001b). In addition, the thermoluminescence method is not suitable for food components such as bone or shell, where the intimate mixture of inorganic and organic components inhibits high temperature thermoluminescence analysis.

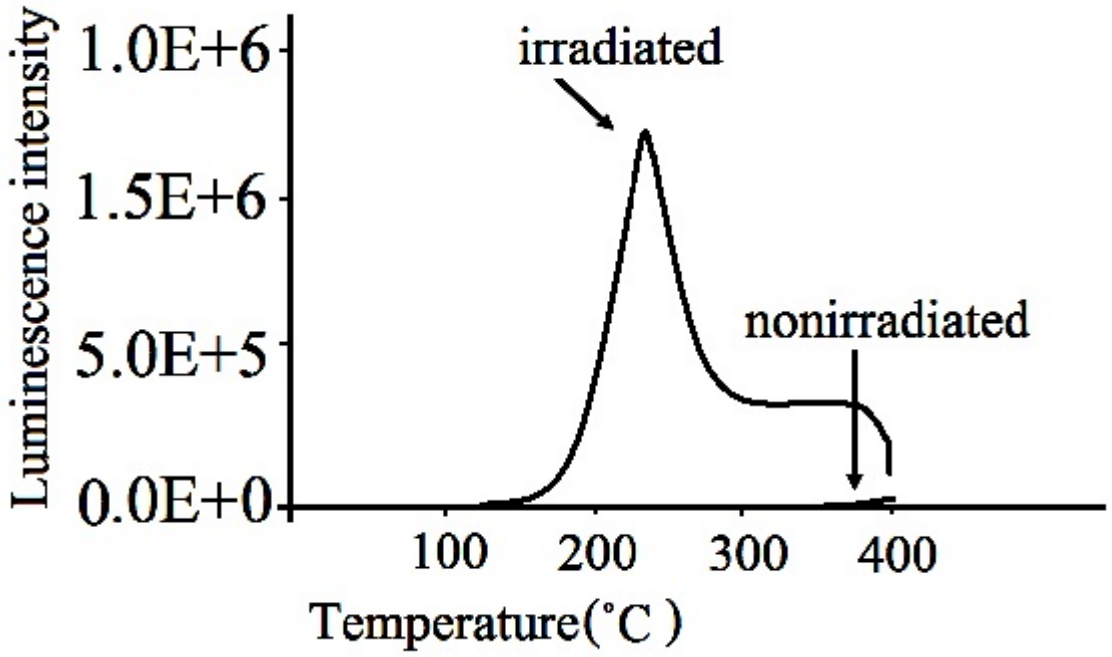


Figure 5- Thermoluminescence glow curves of silicate minerals extracted from irradiated (8 kGy) and nonirradiated sample of dehydrated asparagus powder (Marchioni 2006).

Other Physical Methods

A number of other physical methods such as viscosity measurement, electrical impedance measurement, and near infra red (IR) spectroscopy have been investigated for the detection of

irradiated foods (Stewart 2001b).

The decrease in viscosity of irradiated food is due to the molecular weight or methylation degree changes (Farkas and others 1990). However, the degree of viscosity changes depends on the type of material being irradiated. For example, changes in viscosity occurs at 0.2 kGy in case of pectin and at 10 kGy in cellulose (Stewart 2001b). Moreover, the variability of food viscosity depends on the food itself (the effect of origin, state of ripening, and conditions of culture and storage were much greater than the variability caused by irradiation) (Marchioni 2006).

Because radiolytic products may modify the electric impedance of irradiated food such as fish and potatoes, electric impedance may be used as a screening test for food irradiation detection (Ehlermann 1972, Hayashi and others 1993). However, because the variation of impedance between foods was higher than that induced by irradiation, the use of electric impedance was considered unreliable for the screening of irradiated foods.

A number of other physical properties have been proposed as screening tests for food irradiation, unfortunately without any real success. For example, degradation of irradiated lipids, oils, carotenoid, and starch can be detected using IR-spectroscopy. However, the changes observed in the IR-spectra depend on the radiation dose and the elapsed time after irradiation (Stewart 2001b).

Chemical Methods

Volatile Hydrocarbons from Lipid Containing Foods

Most of the volatile compounds formed in irradiated food originate from fat (Stewart 2001b). The composition of the products formed by the irradiation of lipids can be predicted if the fatty acid compositions of lipids are known (LeTellier and Nawar 1972). Hydrocarbons (Figure 6 position c, d, and f), aldehydes (Figure 6 position b), esters (Figure 6 position e), and free fatty acids (Figure 6 position a) are the major classes of volatile compounds produced by the irradiation of lipids (Stewart 2001b). Only two major hydrocarbons are produced in large quantities from each fatty acid by irradiation. The first hydrocarbon has one carbon atom fewer than the parent fatty acid, and results from the carbon-carbon bond alpha to the carbonyl group (Figure 6 position c). The other hydrocarbon has two carbon atoms fewer than the parent fatty acid and one double bond, and results from beta cleavage (Figure 6 position d) (Nawar and others 1990). Tetradecene, hexadecadiene, and heptadecene were found to be the most promising hydrocarbons for the identification of irradiated chicken. The concentration of these compounds increases linearly with the irradiation dose, and they showed only slight reduction during storage in frozen meat (Stewart 2001b).

Volatile hydrocarbons are extracted from the food sample by melting out or isolating using n-hexane. Then the hydrocarbons are separated from the fat by adsorption chromatography (Florisil) and analyzed with a gas chromatography (GC) coupled with a mass spectrometer (MS) or flame ionization detector (FID). Although the presence of hydrocarbons in irradiated food can be easily identified, these hydrocarbons are not unique radiolytic products and can be detected in

non-irradiated food. However, the presence of a hydrocarbon couple $C_{n-1:m}$ and $C_{n-2:m+1}$ (n= carbon atom, m = double bond) for each fatty acid confirms that the analyzed food has been irradiated (Marchioni 2006).

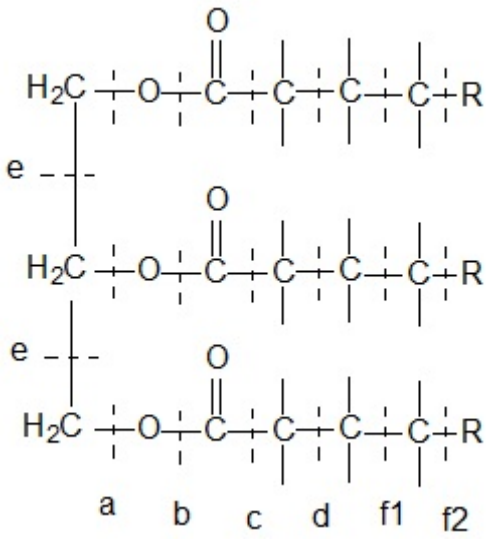


Figure 6- A triglyceride molecule showing the cleavage sites (broken lines) leading to the formation of hydrocarbons and alkylcyclobutanones (Stewart 2001a).

2-alkylcyclobutanone (2-ACBs)

Cleavage at the acyl-oxygen bond in triglyceride results in the formation of the 2-ACBs in irradiated lipid-containing foods (Nawar 1978). The 2-ACBs were first isolated from simple triglycerides irradiated in vacuum at 60 kGy (LeTellier and Nawar 1972). This class of compounds has the same number of carbon atoms as the parent fatty acid and an alkyl group with n-4 carbon atoms located at ring position 2. Stevenson and others (1990) for the first time reported the detection of 2-alkycyclobutanones in irradiated food. For more than 35 years, 2-ACBs were considered radiation specific because they were not detected in raw, cooked, frozen,

freeze-dried or spoiled chicken; thermally sterilized chicken; or chicken exposed to modified atomospheres (Sommers and others 2006). However, it is possible that the amount of 2-ACBs present in non-irradiated food is below the detection limit of the analytical method (Ndiaye and others 1999). Recently, Variyar and others (2008) demonstrated for the first time the natural occurrence of 2-DCB and 2-tetradecylcyclobutanone (2-TCB) in fresh cashews using a combination of super critical fluid extraction (SFE) with thin layer chromatography purification (TLC). The amount of 2-DCB was 2.7 μg/g fat and 6.1 μg/g fat in fresh and irradiated (1 kGy) cashews, respectively. Because the level of (2-ACBs) in non-irradiated cashew was very low and could not be detected using the traditional analytical methods (Soxhlet-silica or SFE-silica), 2-ACBs will continue to be used as radiolytic markers unless more research confirms their existence at higher concentrations in natural foods. The protocol used for the detection of 2-ACBs requires a Soxhlet extraction, a solid phase purification of the extract using Florisil, and separation using GC-MS (Marchioni 2006).

The official analytical method EN 1785 that has been adopted by the European Union as an official method for the detection of 2-ACBs from irradiated lipid-containing foods is time consuming and uses large volumes of organic solvent (Obana and others 2005). Many methods have been developed to reduce the extraction time of 2-ACBs. Tewfik and others (1999) developed a rapid SFE method that uses carbon dioxide for the isolation of 2-ACBs. The SFE method offset the limitations of the EN 1785 method and was more efficient in extracting low levels of radiolytic markers. Obana and others (2005) developed an analytical procedure that uses accelerated solvent extraction (ASE) to detect 2-ACBs in irradiated fat-containing foodstuffs. The method's time for sample preparation with 5 samples and 1 blank taking about 7- 8 h. Caja and

others (2008) developed a solid-phase microextraction (SPME) method to detect the occurrence of 2-DCB in irradiated ground beef. They showed that SPME can be used to detect 2-DCB in ground beef irradiated at 4 kGy or higher. However, the presence of 2-DCB was uncertain (its signal being approximately twice the background) when samples were irradiated at 2 kGy. Although the SFE and ASE methods have reduced the time required to extract 2-ACBs, both methods require costly equipment. Tewfik (2008) developed a rapid direct solvent extraction (DSE) method to detect chicken and whole liquid egg irradiated at various doses. In this method, a few grams of ground meat sample are mixed with sodium sulfate, and the 2-DCB is extracted manually with a mixture of hexane and heptane.

The 2-ACBs are relatively stable in irradiated food, and the moderate losses of these compounds during storage do not reduce the validity of this method (Marchioni 2006). Crone and others (1992) showed that 2-DCB was detectable in radiation-sterilized chicken meat stored for 13 years at room temperature although the amounts were reduced by 60%. Tewfik and Tewfik (2008) showed that it was still possible to identify irradiated lipid-containing foods towards the end of their shelf-life despite the significant drop in the levels of 2-ACBs with time. Moreover, 2-ACBs are not destroyed by conventional cooking. Obana and others (2006) were able to detect 2-DCB and 2-TCB in cooked food samples when the raw samples were irradiated with 3-6.5 kGy. However, these compounds were destroyed when heated to over 200 °C.

In an effort to obtain a rapid and inexpensive detection method for 2-ACBs, work has been conducted to develop an enzyme-linked immunosorbent assay (ELISA) (Elliott and others 1995, Hamilton and others 1996, Nolan and others 1998). Elliott and others (1995) showed that polyclonal antibodies raised to a cyclobutanone derivative with a side-chain length of 10 carbons

can be used to detect cyclobutanones in commercially irradiated chicken. Nolan and others (1998) were able to detect irradiated liquid whole egg using antibodies that were raised against a C12 cyclobutanones derivative. Though the ELISA method could be a good alternative to detect irradiated food, it is not widely used because it is not sensitive, requires a long cleanup procedure, and it not specific for 2-ACBs.

Ortho-tyrosine

The hydroxyl radicals generated from radiolysis of water react with phenylalanine to form *ortho*-, *meta*-, and *para*-tyrosine (Stewart 2001b). Simic and others (1983) proposed the detection of *ortho*-tyrosine assuming that it was a unique radiolytic product (URP). However, Hart and others (1988) showed that *ortho*-tyrosine was also present in non-irradiated meat prawns and strawberries. Nevertheless, they concluded that if the amount of *ortho*-tyrosine in irradiated food was much higher than that present in non-irradiated food, the compound could be used as an irradiation marker. *Ortho*-tyrosine can be measured using gas chromatography-mass spectroscopy, high performance liquid chromatography-UV detection, or by high performance liquid chromatography and fluorescent detection (Stewart 2001b). To date, this method has not been validated by blind tests or by interlaboratory studies.

Gas Evolution

The detection of irradiated foods by determination of evolved gases such as carbon monoxide, hydrogen, hydrogen sulfide, and ammonia has been extensively investigated (Stewart 2001b). This method requires the release of low-molecular weight gasses trapped in the food

matrix, which can be detected by multiple gas sensors even after several months of storage (Marchioni 2006).

Other Chemical Methods

Besides the chemical methods previously mentioned a number of other methods have been investigated. The lipid hydroperoxide test and the immunochemical method for detection of irradiated eggs are the most promising methods (Stewaert 2001b). The lipid hydroperoxide test is based on the determination of the organic peroxides formed during irradiation of fat in the presence of oxygen. Although the lipid peroxide test is rapid and the amount of peroxides can be measured using a simple iodometric titration, the peroxide test is not reliable because the peroxides are also produced by autoxidation and are unstable, the level of these peroxides in irradiated food is affected by the presence of antioxidants, oxygen levels, and the composition of the food. Irradiated eggs can be identified immunochemically by the detection of radiation-induced protein fragments of eggs using a specific antibody. This method can also be used to detect irradiated egg white protein that has been added to food; however, it cannot be used to detect irradiated chicken meat and shrimp.

DNA Methods

DNA "Comet Assay"

It is widely accepted that the DNA is the major cellular target for ionizing radiation (Stewart 2001b). Ionizing radiation induces three major classes of damage on DNA: double-strand breaks, single-strand breaks, and base damage (Von Sonntag 1987). Single- and double-strand breaks increase the mobility of the DNA in the electrophoretic gel. The DNA fragments of irradiated samples will leak from the nuclei during electrophoresis and a "tail" or "comet" is formed in the direction of the anode. When the gel is stained using fluorescent dye (acridine orange) and examined under fluorescent microscope, irradiated cells will have the appearance of a comet, whereas nonirradiated cells will appear as nuclei without or with a slight tail. The comet assay has been successfully applied to meats, fish, fruits, and vegetables (Stewart 2001b). The comet assay is simple, inexpensive, and rapid. However, it cannot be applied to food that has been cooked, repeatedly frozen-thawed, or subjected to high shear force because all of these treatments produce DNA damage similar to those produced by ionizing radiation.

Agarose Electrophoresis of Mitochondrial DNA

Another method is based on the analysis of the mitochondrial DNA (mtDNA), which is protected from enzymatic reactions by the mitochondrial wall, but is not protected from irradiation (Marchioni and others 1992). Because the mtDNA appears to be resistant to freeze thaw cycles, it is assumed that the DNA strand breaks in the mitochondria would be specific to irradiation (Marchioni and Hasselmann 1991). The mtDNA is smaller than the cellular DNA and is normally

in a supercoiled form. Irradiation transforms the supercoiled mtDNA to a relaxed circular and then linear DNA (Stewart 2001b). These three forms are then separated by agarose gel electrophoresis. Following irradiation (2-4 kGy) the percentage of supercoiled mtDNA in meat and fish is reduced while the percentage of circular and linear DNA increases. Although the supercoiled mtDNA remains stable during prolonged storage or during freeze thaw cycles, this test can not be carried out on frozen fish because it is not possible to extract the mtDNA from frozen fish by the technique that has been used for this purpose. The main disadvantage of the mtDNA test is that it is complicated and time consuming.

Immunologic Detection of Modified DNA bases

The irradiation of DNA results in the modification of purine and pyrimidine bases. Following, irradiation a proportion of the thymidine is converted to dihydrothymidine. Although DNA exposure to UV can result in the formation of dihydrothymidine, the yield of dihydrothymidine is much higher when DNA is exposed to ionizing radiation. Deeble and others (1994) developed antibodies specific to dihydrothymidine to distinguish between irradiated and non-irradiated DNA under anoxic conditions. The developed antibodies showed high sensitivity to irradiated DNA and were able to detect wheat irradiated at 2 kGY. Tyreman and others (1998) developed an indirect competitive ELISA for the detection of DNA base changes due to irradiation. The assay exhibited a dose response relationship for solutions of calf thymus DNA irradiated over a 0-25 kGy range. Their results showed that the ELISA can be used to detect irradiated prawns and it can be applied directly to the crude homogenates without any cleaning steps.

Biological Methods

Shift in Microbial Load

Ionizing irradiation like any other food processing (sterilization, pasteurization) destroys or changes the microbial flora of the food (Stewart 2001b). On this basis, the microbial content of irradiated and nonirradiated foods can be compared to determine whether the food has been irradiated. Tamminga and others (1975) showed that *Enterobacteriaceae* and species of genus *Pseudomonas* were found to be absent in strawberries irradiated at 2kGy, but were usually present in nonirradiated berries at levels of 10^5-10^6 colony-forming units/g (CFU/g). These results were valid for strawberries grown outdoors but not for green-house, or plastic-covered-grown strawberries. Because the microbial content of fruits is affected by climatological and environmental factors, data obtained for a foodstuff in a precise region can not be used to test the same kind of product in another region. The shift in the microbial profile of the food may indicate that the food has been irradiated. However, this is not considered proof of irradiation and the results should be confirmed using validated detection methods (Copin and Bourgëois 1991).

Direct Epifluorescent Filter Technique Combined with Aerobic Plate Count (DEFT/APC)

The DEFT combined with APC was devised as a method to identify irradiated food (Stewart 2001b). The APC gives the number of viable bacteria in the sample, while the DEFT count gives the total number of live and dead bacteria. In nonirradiated samples, the DEFT count is close to the APC because most of the cells are alive. However, the APC is significantly lower

than the DEFT count in irradiated samples (Figure 7). A difference between DEFT and APC indicates that irradiation has taken place. Jones and others (1994) showed that at 5 kGy and above the DEFT/APC method could be used for the identification of meat and fish that have been irradiated and stored chilled or frozen. However, this method could not be applied to samples irradiated at 5 kGy and stored in a chilled environment because the microbial growth during storage decreases the difference between DEFT and APC. Although the DEFT/APC method has been successfully applied to different types of foods, it is not irradiation specific (Stewart 2001b). The DEFT/APC difference in bacteria counts obtained after irradiation is similar to the difference obtained from chemical or heat treatment.

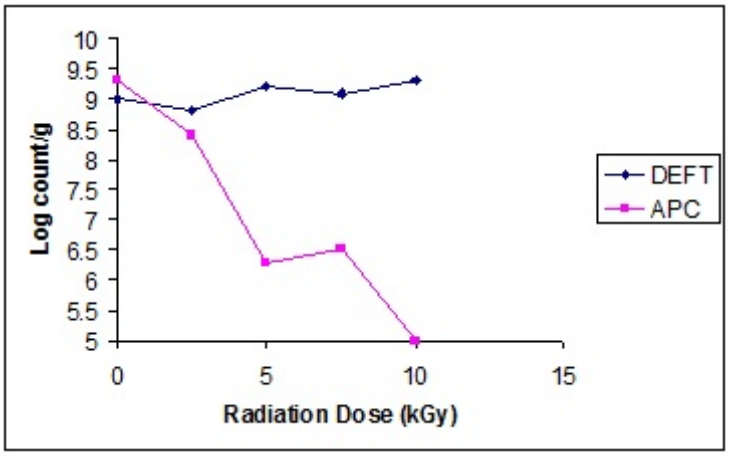


Figure 7- Effect of irradiation on the DEFT and APC bacterial count in ground beef (Stewart 2001b).

Limulus Amoebocyte Lysate Test Combined with Gram-Negative Bacterial Count (LAL/GNB)

The LAL/GNB test is based on the assumption that radiation kills most of the bacteria in food. The GNB enumerates the total gram negative bacteria and the LAL test measures the total

lipopolysaccaride (endotoxin) produced by the gram negative bacteria (Stewart 2001b). The level of the endotoxin is related to the total number of GNB. A high LAL titer in the absence of significant number of viable GNB indicates radiation treatment has taken place (Scotter and others 1990). Scotter and others (1994) conducted two inter-laboratory studies to evaluate the LAL/GNB method for the detection of irradiated chicken. The results from their trials showed that 86% of skin-on chicken portions were correctly identified as irradiated or non-irradiated, but the results for skinless samples were less satisfactory. Because the LAL/GNB ratio is also affected by other types of treatments, for example cooking, the LAL/GNB test is not radiation specific and any positive results should be reexamined using validated methods.

Half-Embryo Test to Measure Inhibition of Seed Germination

Ionizing radiation at doses of 1 kGy or lower inhibits or delays the germination of the embryo or germ of seeds. The inhibition of seed germination has been used to detect irradiated wheat, rye, barely grain and legumes, rice, and grape seed (Stewart 2001b). Although the inhibition of seed germination test is simple, it is unreliable because seed germination is affected by date and storage conditions. Because the seed germination test is too lengthy to be used for screening purposes, this test was improved and a "half-embryo test was developed for the detection of irradiated fruit seed" (Kawamura and others 1989). In the half-embryo test, the embryos are taken out of the seed shell to accelerate the germination and shorten the required time for the test. Kawamura and others (1996) showed that the test can be used for the detection of irradiated citrus fruit.

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PART II: A RAPID DIRECT SOLVENT EXTRACTION METHOD FOR THE EXTRACTION OF 2-DODECYLCYCLOBUTANONE FROM IRRADIATED GROUND BEEF PATTIES USING ACETONITRILE[1,2]

ABSTRACT

The amount of irradiated beef in the U.S. market is growing, and a reliable, rapid method is needed to detect irradiated beef. The official analytical method (BS EN 1785 2003) that has been adopted by the European Union is time consuming. The objective of this study was to develop a rapid method for the analysis of 2-dodecylcyclobutanone (2-DCB) in commercially irradiated ground beef. The samples were extracted with n-hexane by using a Soxhlet apparatus or with acetonitrile via direct solvent extraction. The fat in the hexane extract was precipitated with filtration by standing at -20 °C in a mixture of ethyl acetate and acetonitrile. Then, the defatted hexane extract and the acetonitrile extract were purified with a 1-g silica cartridge. The 2-DCB concentration was determined with gas chromatography-mass spectrometry. The 2-DCB concentration in the commercial samples was 0.031 ± 0.0026 ppm (n = 5) for the Soxhlet method and 0.031 ± 0.0025 ppm (n = 10) for direct solvent extraction. Recovery of 2-DCB from spiked beef samples in the direct solvent extraction method was 93.2 ± 9.0% (n = 7). This study showed that the direct solvent extraction method is simple and as efficient and reproducible as the Soxhlet method.

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INTRODUCTION

Food irradiation improves food safety and maintains food quality by controlling microorganisms and extending shelf-life (Deihl 2001, Molins 2001). To comply with legislation and enhance consumer confidence, a reliable, convenient method is needed to detect irradiated food and quantify the irradiation dose. Various chemical and physical methods have been developed to detect irradiated foods. These methods include electron paramagnetic resonance spectroscopy, thermoluminescence, and the detection of volatile hydrocarbon from irradiated fat (Elliott and others 1995). Recently, 2-alkylcyclobutanones (2-ACBs) have been used to detected irradiated lipid-containing foods (Boyd and others 1991, Ndiaye 1999, Gadgil and others 2002, 2005, Obana and others 2005, 2007). The 2-ACBs are radiolytic products formed from fatty acids (Le Tellier and Nawar 1972), and are considered radiation specific because they are not detected in raw, cooked, frozen, freeze-dried, or spoiled chicken; thermally sterilized chicken; or chicken exposed to modified atmosphere (Sommers and others 2006). However, it is possible that the amount of 2-ACBs present in non-irradiated food is below the detection limit of the analytical method (Ndiaye and others 1999). Recently, Variyar and others (2008) demonstrated for the first time the natural occurrence of 2-dodecylcyclobutanone (2-DCB) and 2-tetradecylcyclobutanone (2-TCB) in fresh cashew using a combination of super critical fluid extraction (SFE) with thin layer chromatography purification (TLC). The amount of 2-DCB was 2.7 μg/g and 6.1 μg/g in fresh and irradiated (1 kGy) cashew, respectively. Because the level of 2-ACBs in non-irradiated cashew was very low and could not be detected using the traditional analytical methods (Soxhlet-silica or SFE-silica), 2-ACBs will continue to be used as unique radiolytic products unless more research confirms their existence at higher concentrations in natural foods.

The official analytical method (BS EN 1785) that has been adopted by the European Union is the most common method used to isolate 2-ACBs from irradiated lipid-containing foods. However, this method involves a long cleanup and extraction procedure and uses large volumes of organic solvent (Tewfik and others 1999). Many methods have been developed to reduce the extraction time of 2-ACBs. Tewfik and others (1999) developed a rapid supercritical fluid extraction (SFE) method for the isolation of 2-ACBs that uses carbon dioxide. The SFE method offset the limitations of the BS EN 1785 method and was more efficient in extracting low levels of radiolytic markers. Horvatovich and others (2000) concluded the SFE method was faster than the BS EN 1785, and the minimal detection dose of the SFE method was lower than those of the reference methods. Gadgil and others (2002) also showed that the SFE procedure can be used to extract 2-ACBs from commercially irradiated ground beef. Although the SFE procedure has greatly reduced the extraction time to 30 min, the SFE instrument with a fully automated system is not available in most laboratories, and in some countries, legal permission is needed to possess and operate each instrument because of the extremely high gas pressure needed to operate the instrument (Obana and others 2005).

Obana and others (2005) developed an analytical procedure that uses accelerated solvent extraction (ASE) to detect 2-ACBs in irradiated fat-containing foodstuffs. In this method, the sample is extracted by ASE using hot, pressurized ethyl acetate, and the extracted fat is precipitated via filtration by standing at -20 °C after the addition of acetonitrile. The extract is further cleaned with a 1 g silica gel cartridge, and the 2-ACBs are determined by using gas chromatography-mass spectrometry (GC-MS) instrument. The operation time for sample preparation with 5 samples and 1 blank was about 7 to 8 h. The ASE method showed high

recoveries (70-105%) and was able to detect 2-DCB and 2-TCB in beef, pork, and chicken irradiated with gamma rays from 0.7 to 7.0 kGy at -19 °C. Obana and others (2005) concluded that total operation time and labor intensity for ASE were similar or better than those of SFE. Ethyl acetate was selected for extraction because it showed better extraction power than other solvents and was suitable for removing fat in an extract with the addition of acetonitrile. Because ethyl acetate is miscible with acetonitrile, which does not dissolve triglycerides, most of the fat (80-90%) in ethyl acetate solution could be removed by filtration. The defatting step was useful because it increased the volume of the extract that was charged to the 1 g silica cartridge, reduced the volume of the elution solvents, and reduced the operation time compared with the conventional florisil cleanup.

Caja and others (2008) developed a solid-phase microextraction (SPME) method to detect the occurrence of 2-DCB in irradiated ground beef. They showed that SPME can be used to detect 2-DCB in ground beef irradiated at 4 kGy or higher. However, the presence of 2-DCB was uncertain (signal approximately twice the background) when samples were irradiated at 2 kGy. Caja and others (2008) concluded that SPME could be used as an alternative to the Soxhlet method to detect 2-DCB in irradiated beef. The SPME is rapid (50 min), simple, and does not need large amounts of organic solvents. However, it cannot be used to detect 2-DCB at 2 kGy or lower to determine the irradiation dose.

Although the SFE and ASE methods have reduced the time required to extract 2-ACBs, both methods require costly equipment. Tewfik (2008a) developed a rapid direct solvent extraction (DSE) method to detect chicken and whole liquid egg irradiated at various doses. In this method, a few grams of ground meat sample are mixed with 10 to 20 g of sodium sulfate, and

the mixture is extracted by shaking it for 15 min with 35 mL of the extraction solvent (hexane:heptane, 9:1). Finally, the extract is drained through a 5 g bed of 20% of deactivated florisil and then concentrated and injected into the GC-MS. Tewfik (2008a) showed that the DSE method can be used to identify and quantify the presence of 2-DCB in irradiated chicken (1- 5 kGy) and liquid whole egg (1-3 kGy). Although levels of 2-DCB increased with the applied dose, extraction efficiency of the DSE method was 45% lower than that of the Soxhlet method. Tewfik (2008a) concluded that the DSE method was a promising, rapid, simple, and robust method for analyzing irradiated lipid-containing foods. Tewfik (2008b) also showed that DSE can be used to detect 2-DCB in irradiated cheese, and this method may be operated quantitatively or qualitatively. Tewfik (2008c) conducted an inter-laboratory trial to validate the DSE method for identification of 2-DCB in irradiated chicken (3-7 kGy) and whole liquid egg (3-6 kGy). All four laboratories that participitated in the study were able to correctly identify all 12 blind-coded samples as either irradiated (at medium and/or high dose) or unirradiated. Tewfik (2008c) concluded that the DSE method was fast and inexpensive and compared favorably to the existing standard Soxhlet method.

The amount of irradiated ground beef in the U.S. market is growing, and a reliable rapid method is needed to detect irradiated ground beef and quantify the irradiation dose. The objective of this study was to develop a new, rapid method for the analysis of 2-DCB in irradiated ground beef patties.

MATERIALS AND METHODS

Reagents

Hexane, acetonitrile, diethylether, 2-DCB, and anhydrous sodium sulfate were obtained from Fisher Scientific (Pittsburg, Pa.). Mega bond Elute 1 g silica cartridge were obtained from Varian Inc. (Palo Alto, Calif.). Commercially irradiated ground beef patty (90/10) were purchased from Schwan's Food (Marshall, Min), and unirradiated beef patty (90/10) were obtained from the Kansas State University Meat Laboratory (Manhattan, Kan.).

Experimental Design

The experimental design was completely randomized. Five commercially irradiated ground beef patties were randomly selected from 1 box (12 patties per box) and analyzed by either the Soxhlet method or DSE method. The objectives of the main study were to improve the method developed in the preliminary study and compare it with the Soxhlet extraction method.

Direct Solvent Extraction

A 5.0 g sample of commercially irradiated ground beef patty was mixed with 50 mL of acetonitrile in a 250 mL Erlenmeyer flask. The meat sample was mixed and crushed with a glass rod for 10 min, and then it was shaken for another 10 min in a wrist action shaker (Burrell Co., Pittsburg, Pa.). The extract was filtered through Whatman filter paper (No. 4) and transferred to a 500 mL round-bottom flask. The extraction procedure was repeated 3 times, and the acetonitrile was collected and evaporated to dryness in a rotavaporator. Ten milliliters of hexane:acetone mixture

(1:1) was added to the round-bottom flask and evaporated to remove any water. The round-bottom flask was washed with hexane, and the hexane was collected into a 50 mL volumetric flask. A 15 mL aliquot of the hexane was evaporated under a stream of nitrogen, and the sample was reconstituted in 1 mL hexane. The 1 mL sample was cleaned by using a 1 g silica cartridge as described by Obana and others (2005). The elution was concentrated to 40 µL with nitrogen stream, and 1 µl was injected into the GC-MS. A 5.0 g sample of unirradiated ground beef sample was used as a control and analyzed following the same procedure. To evaluate the new extraction method, a 5.0 g sample of unirradiated ground beef patty (90/10) was spiked with 200 µL of 10 ppm 2-DCB in hexane and analyzed as described previously.

To reduce the extraction time, an 8.0 g sample of commercially irradiated ground beef patty was mixed with 75 mL of acetonitrile and blended for 1 min with a commercial blender. The extract was filtered through Whatman filter paper (No. 4) and transferred to a 500 mL round-bottom flask. The extraction procedure was repeated 3 times, and the acetonitrile was collected and evaporated to dryness with a rotavaporator. Ten milliliters of hexane:acetone mixture (1:1) was added to the round-bottom flask and evaporated to remove any water. The round-bottom flask was washed with hexane, and the hexane was collected into a 50 mL volumetric flask. A 10 mL aliquot of the hexane was transferred to a 10 mL vial and evaporated under a stream of nitrogen. The sample was reconstituted in 1 mL hexane and cleaned with a 1 g silica cartridge as described by Obana and others (2005). The elution was concentrated to 50 µl with nitrogen stream, and 1 µl was injected into the GC-MS.

Soxhlet Procedure

A 5.0 g sample of commercially irradiated ground beef patty was mixed with 10 g of anhydrous sodium sulfate and extracted with 150 mL hexane for 6 h by using a Soxhlet apparatus. At the end of the extraction time, the hexane was evaporated with a rotavaporator and the sample was dissolved in 50 mL hexane. A 10 mL aliquot of the hexane extract was transferred to a 10 mL vial, and the hexane was evaporated with a nitrogen stream. The fat inside the vial was dissolved in 5 mL ethyl acetate followed by 5 mL of acetontrile and was deffated and cleaned as described by Obana and others (2005). Finally, the elution was evaporated with a nitrogen stream, the extract was dissolved in 30 µl of hexane, and 1 µl was injected into a GC-MS.

GC-MS Analysis of 2-DCB.

Gas chromatography-mass spectrometry was performed with an HP 5890 GC (Agilent Technologies, Palo alto, CA) fitted with an HP-5MS column (cross-linked 5% Ph Me siloxane, 30 m × 0.22 mm × 0.025 µm film thickness) and a HP MS 5970 detector. The flow rate for the helium carrier gas was 1 mL/min. The injector temperature was set to 250 °C. The GC temperature program was as follows: initial temperature of 55 °C, hold for 0.5 min, ramp at 20 °C/min to 200 °C, hold for 1 min, ramp at 15 °C/min to a final temperature of 270 °C, and hold for 1 min. The MS was set to the selective ion mode, and ions *m/z* 98 and 112 were monitored for the analysis of 2-DCB (Gadgil and others 2005).

Statistical Analysis

Data were analyzed with SAS version 9.1 (SAS Institute, Inc., Cary, N.C.). Dunnett's test was used to compare all other methods with the control (Soxhlet method).

RESULTS AND DISCUSSION

Our study showed the DSE method developed in this study can be used to analyze 2-DCB in commercially irradiated ground beef patties (1.5-2.0 kGy). The concentration of 2-DCB in commercially irradiated ground beef patties was 0.031 ± 0.0032 (Table 6). This result is in agreement with Gadgil and others' (2002) results from SFE. Gadgil and others (2002) reported the concentration of 2-DCB in the same type of patties to be 0.033 ± 0.01. Because results of the DSE method were promising, we decided to compare it with the Soxhlet extraction method.

Our preliminary testing showed that solubility of 2-DCB in acetonitrile was more than 70,000 ppm. Acetonitrile was selected because it can dissolve 2-DCB but not fat. The polarity index of hexane is 0.01, whereas the polarity index of acetonitrile is 5.8 (Gupta and others 1997). Obana and others (2005) showed that fat can be removed from ethyl acetate extract via filtration by standing at - 20 °C after the addition of acetonitrile. This means that 2-DCB is still soluble at - 20 °C in a mixture of ethyl acetate and acetonitrile (polarity index ~ 5.1). Because 2-DCB is soluble in a mixture of ethyl acetate and acetonitrile at - 20 °C, we expected it to be soluble in acetonitrile at room temperature. Because acetonitrile was used to extract 2-DCB, the amount of extracted fat was low and the extract was charged to the 1 g silica cartridge without the deffating step.

The DSE method developed in this study is simple and rapid and may be operated quantitatively or qualitatively. The DSE method has been successfully used to identify commercially irradiated ground beef patties from nonirradiated ones by looking for the presence of 2-DCB (Figure 8). The level of 2-DCB in commercially irradiated samples was 0.031 (ppm) when extracted using the Soxhlet method, 0.029 (ppm) when extracted with acetonitrile using manual

mixing, and 0.033 when extracted with acetonitrile using a blender (Table 6). The precision of the new method was almost equal to that of the Soxhlet extraction method. The coefficient of variation for the Soxhlet method and the DSE method developed in this study were 8% and 10%, respectively.

Our study also showed that 2-DCB can be extracted from the food matrix by DSE. This result agrees with Tewfik's (2008a, 2008b, 2008c) results. The extraction efficiency of the DSE method developed in this study was equal to that of the Soxhlet method (Table 6), whereas the extraction efficiency of Tewfik's (2008a) method was 45% lower than that of the standard method (BS EN 1785 2003). The low extraction efficiency in Tewfik's (2008a) study could be due to the low polarity index of the hexane, short extraction time, and use of cold solvent.

Our results showed that 2-DCB can be extracted with a polar solvent such as acetonitrile, which is in agreement with Obana and others' (2005) results. They showed that 2-DCB can be extracted with ethyl acetate (polarity index 4.4) and separated from fat in a mixture of acetonitrile and ethyl acetate cooled at -20 °C.

Table 6- The 2-DCB concentration (ppm) in irradiated ground beef patties using manual and Soxhlet extraction.

Patty No	Soxhlet extraction	Manual (mixed with a glass rod)	Manual extraction (mixed with a blender)
1	0.030	0.028	0.034
2	0.030	0.030	0.032
3	0.030	0.027	0.037
4	0.028	0.033	0.033
5	0.035	0.025	0.028
Average	**0.031**	**0.029**	**0.033**
Standard deviation	0.0026	0.0031	0.0033
CV	8.5	10.7	10.0

The DSE method developed in this study was evaluated for recovery efficiency in which the 2-DCB was spiked at 0.4 mg/kg ground beef. The average percentage of 2-DCB recovered from spiked ground beef samples was 93.2 ± 9.0% (Table 7). Recovery of our proposed method was close to Obana and others'(2005) recovery using ASE. The average percentage of 2-DCB recovered from spiked minced beef using ASE was 83 ± 6% (n = 8). The cleanup procedure developed by Obana and others (2005) also was evaluated by eluting 1 mL of 10 ppm 2-DCB standard in the 1 g silica cartridge. The elution was collected after each 5 mL and injected into a GC-MS running in the selective ion mode. All the 2-DCB was eluted after the addition of the first 10 mL of 2% diethylether in hexane. This result showed that the cleanup procedure was efficient as and there was no loss of 2-DCB during this step.

Table 7- Recovery of 2-DCB from spiked ground beef patty extracted with acetonitrile and cleaned with 1 g silica cartridge.

Sample No.	Recovery (%)
1	80.5
2	102.9
3	82.8
4	91.6
5	95.5
6	95.2
7	103.70
Average	93.2
Standard deviation	9.00
CV	9.7%

The limit of detection (LOD) was calculated from the peak intensity at 4×10^{-5} ng. At this concentration, the signal-to-noise ratio was greater than 10. Obana and others (2005) reported a LOD of 2×10^{-5} ng. However, the-signal-to noise ratio was greater than 5. The LOD of our method in the tested samples (90/10) was about 1 ppb (calculated using 1.6 g sample and 50 µL final volume sample). This value is close to that of Obana and others (2005). Obana and others (2005) reported a LOD of 3 ppb in a high-fat sample and 1 to 2 ppb in a low-fat sample. Although the LOD in our test solution was higher than that reported by Obana and others (2005), the LOD in our test sample was lower because the final volume of the sample was 50 µL, compared with 2 mL in Obana and others' (2005) study. Moreover, the LOD can be lowered 3 times by using a 2 g silica cartridge, which can be used to clean 5.0 g of ground beef (90/10).

The operation time for sample preparation with 6 samples and 1 blank was about 5 h for manual mixing and 3 h when the sample was blended with a hand blender. This proposed method is rapid; it does not require defatting, lyophilization, or deactivation of silica. The total operation time and labor intensity of the new DSE method are better than those of the standard Soxhlet method and comparable to those of the SFE method. Our method has a greater extraction efficiency than the DSE developed by Tewfik and others (2008a), and the cleanup procedure is easier.

This study showed that the DSE method using acetonitrile is simple and rapid, does not require expensive instruments for the extraction, and is as efficient and reproducible as the standard Soxhlet method. Further studies are required to evaluate the use of this method for the detection of 2-DCB in other irradiated lipid-containing foods such as chicken, eggs, and cheese.

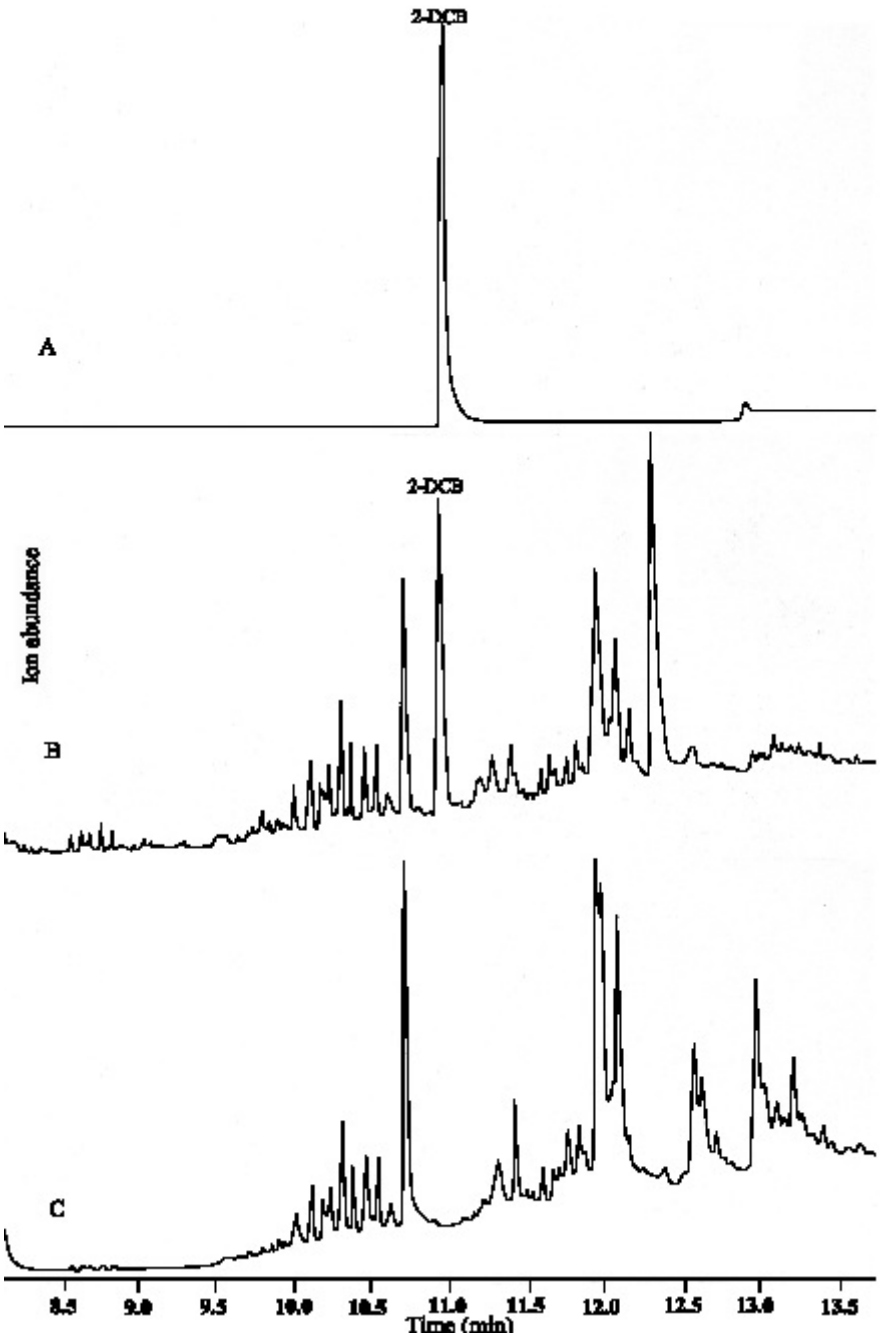

Figure 8- Gas chromatography-mass spectrometry chromatograms of 2-DCB standard (A), irradiated ground beef patty (B), nonirradiated patty (C). The MS was set to the SIM mode and ions 98 and 112 were monitored.

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PART III: LEVELS OF 2-DODECYLCYCLOBUTANONE IN GROUND BEEF PATTIES IRRADIATED BY LOW-ENERGY X-RAY AND GAMMA RAYS[1,2]

ABSTRACT

Food irradiation improves food safety and maintains food quality by controlling microorganisms and extending shelf life. However, acceptance and commercial adoption of food irradiation is still low. Consumers groups like Public Citizen and the Food and Water Watch have opposed irradiation because of the formation of 2-alkylcyclobutanones (2-ACBs) in irradiated, lipid-containing foods. The objectives of this study were to measure and to compare the level of 2-dodecylcyclobutanone (2-DCB) in ground beef irradiated by low-energy X-rays and gamma rays. Beef patties were irradiated by low-energy X-rays and gamma rays (Cs-137) at 3 targeted absorbed doses of 1.5, 3.0, and 5.0 kGy. The samples were extracted with n-hexane using a Soxhlet apparatus, and the 2-DCB concentration was determined with gas chromatography-mass spectrometry (GC-MS). The 2-DCB concentration increased linearly ($p < 0.05$) with irradiation dose for gamma-ray- and low-energy X-ray irradiated patties. There was no significant difference in 2-DCB concentration between gamma-ray- and low-energy X-ray-irradiated patties ($p > 0.05$) at the targeted doses.

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2. Abstract presented in part at IFT Annual Meeting 2010 http://www.abstractsonline.com/Plan/ViewAbstract.aspx?mID=2525&sKey=dde38d49-239a-489e-81e8-8a4d70dd08a6&cKey=1a69b79a-0edf-4ae8-a9d4-c1088325a9d0&mKey=%7b64C55C22-D314-40A2-98B8-3CE298279EC7%7d

INTRODUCTION

Electrons with energies up to 10 MeV, X-rays with energy up to 5.0 MeV, and gamma rays from cobalt-60 and cesium-137 are allowed by the U.S. Food and Drug Administration (FDA) for food irradiation (Cleland 2006). All of these energy sources produce similar effects in irradiated material because they transfer their energies to the materials by ejecting atomic electrons, which leads to ionization of other atoms in a cascade collision. Although all of these energy sources produce similar effects, the choice of radiation source for a particular product depends on density and thickness of the product, dose uniformity ratio, minimum dose, processing rate, and economics (Cleland 2006).

An important difference between these sources of irradiation is the penetration capacity. The penetration capacity of gamma rays and X-rays is 1 to 2 m, whereas penetration capacity of an electron beam is between 3 and 6 cm. Penetration capacity of electron beams depends on energy level. For example, penetration of an electron beam increases with its energy; electrons at 10 MeV can penetrate 4 cm, and electrons at 4 MeV can penetrate about 1.6 cm of material with a density of $1 g/cm^3$ (Hayashi 1991).

Another important difference between the three sources of irradiation is the dose rate. The dose rate of gamma rays is 1 to 100 Gy/min, whereas the dose rate of electron beams and X-rays is 10^3 to 10^6 Gy/min (Hayashi 1991). Ideally, effects of different types of ionizing radiation should be compared at the same dose rate, but this is not possible because irradiators differ in design. Although the dose rate is not critical in food irradiation, many studies have shown that dose rate can influence the effect of irradiation on food. Consequently, the difference between the effects of gamma rays and electron beams in food irradiation has been ascribed to differences in dose rate

rather than type of irradiation. The dose-rate effects are generally clear when electron irradiation at high dose rates is compared with gamma irradiation at relatively low dose rates. A higher dose rate decreases radiolytic products because it creates anoxic conditions in the reaction system and alters the end result of irradiation by reducing indirect reactions (formation of reactive oxygen species). It is also believed that the amount of combination of the primary radicals will be higher at a high dose rate than at a low dose rate. Hayashi (1991) concluded that most reports on chemical changes in irradiated food showed that chemical reactions caused by gamma rays and accelerated electrons are almost the same in irradiated food.

Although irradiation is very effective at eliminating food pathogens, acceptance and commercial adoption of food irradiation is still low because of the formation of 2-alkylcyclobutanones (2-ACBs) in irradiated, lipid-containing foods. The 2-ACBs are unique radiolytic products formed from fatty acids (Le Tellier and Nawar 1972), and were considered radiation specific because they were not detected in raw, cooked, frozen, freeze-dried, or spoiled chicken; thermally sterilized chicken; or chicken exposed to modified atmosphere (Sommers and others 2006). However, it is possible that the amount of 2-ACBs present in non-irradiated food is below the detection limit of the analytical method (Ndiaye and others 1999). Recently, Variyar and others (2008) demonstrated for the first time the natural occurrence of 2-dodecylcyclobutanone (2-DCB) and 2-tetradecylcyclobutanone (2-TCB) in fresh cashew using a combination of super critical fluid extraction (SFE) with thin layer chromatography purification. The amount of 2-DCB was 2.7 μg/g in fresh cashew and 6.1 μg/g in irradiated (1 kGy) cashew. Because the level of (2-ACBs) in non-irradiated cashew was very low and could not be detected using the traditional analytical methods (Soxhlet-silica or SFE-silica), 2-ACBs will continue to be

used as radiolytic markers unless more research confirms their existence at higher concentrations in natural foods. In animal studies, high concentrations of 2-ACBs, especially 2-DCB, which forms from palmitic acid, induce DNA strand breaks in cells (Delincée and Pool-Zobel 1998). Raul and others (2002) showed that although 2-ACBs did not affect the number of preneoplastic lesions in rats fed 2-ACBs along with azoxymethane (a carcinogen) injection compared with rats treated with azoxymethane alone, rats fed 2-ACBs developed larger tumors in their colons.

Beside the formation of 2-ACBs, irradiation produces off-odor and off-flavor and alters meat color (Ahn & Lee 2006). Antioxidants, vacuum packaging, and combinations of packaging and additive have been used to minimize quality change in irradiated meat. Although these treatments can reduce quality changes in irradiated meat, they cannot eliminate all drawbacks of irradiation, and the quality change in irradiated meat significantly alters consumer acceptance (Ahn & Lee 2006).

The Centers for Disease Control and Prevention estimated that if 50% of the meat in the U.S. is irradiated, there will be a 25% reduction in the morbidity and morality rate caused by foodborne illness (Eustice and Brubn 2006). Recently a low-energy X-ray instrument was developed to irradiate different types of food including meat and vegetables. Studies at Michigan State University showed that the low-energy X-ray can eradicate *Escherichia coli* O157:H7 in ground beef and other food products (5 log reduction). It is safe, needs only a few millimeters of shielding, can be placed into the production line, and is economical, and portable (Lindsay and others 2007). However, no information is available about the levels of 2-DCB in ground beef irradiated with this new technology. The objectives of this study were to measure and compare the level of 2-DCB in ground beef patties irradiated with low-energy X-rays and gamma-rays.

MATERIAL AND METHODS

Chemicals and Reagent

Hexane, acetonitrile, ethyl acetate, diethylether, 2-DCB, 10% boron trifluoride in methanol, and anhydrous sodium sulfate were obtained from Fisher Scientific (Pittsburg, Pa.). Mega bond Elute 1 g was obtained from Varian Inc., (Palo Alto, Calif.).

Meat Samples

About 10 Kg of ground beef (85/15) was obtained from the Kansas State University Meat Laboratory (Manhattan, Kan.) and stuffed into a 1-inch-dia sausage casing. Filled casings were frozen for 15 to 20 min in a blast freezer (-40 °C) and then stored overnight at -20 °C. The ground beef sausages were thawed overnight inside the refrigerator and then sliced into small patties (3 mm thick) with a commercial slicer. Sliced patties were frozen overnight at -20 °C and then packaged in Food Saver vacuum bags (Model No. FSFSBF0116-000, New York, NY) with a Food Saver V3840 vacuum sealer (New York, NY). Samples were stored at -20 °C until they were shipped for irradiation. The samples were shipped in dry ice to ensure they remained frozen.

Gamma Irradiation

Gamma irradiation of the ground beef samples was performed at the USDA Eastern Regional Research Center (Wyndmoor, Pa.). A Lockheed Georgia Company (Marietta, Ga.) self-contained 137Cs radiation source was used for all exposures. The radiation source consisted of 23 individually sealed source pencils placed in an annular array. The 22.9-cm × 63.5-cm cylindrical sample chamber was located central to the array when placed in the operating position. The dose rate was 0.079 kGy/min. The temperature during irradiation was maintained at -20 °C (±1.0) °C by the gas phase of a liquid nitrogen source that was introduced directly into the top of

the sample chamber. The temperature was monitored with two thermocouples placed on the side of the sample bags. The absorbed dose (0, 1.5, 3.0, and 5.0 kGy) was verified with film dosimeters (Far West, Inc., Seattle, WA), and the values were within 3% of the target dose (Table 8). Because of the annular array design of the irradiator, dose uniformity ratios were less than 1.1:1.0. Three bags, each bag as a replicate with 20 small patties were irradiated at each dose (1.5, 3.0, and 5.0 kGy).

Low energy X-ray

Frozen beef samples (25 mm dia and 3 mm thickness) were irradiated with a low-energy (70 keV) X-ray (Rainbow II™, Rayfresh Foods Inc., Ann Arbor, Mich.) food irradiator. A desired dose was achieved by simulating double-sided treatment (irradiating the sample by using two sources- one at the top and one at the bottom). To measure the dose rate at the top surface, a dosimeter (GAF3001DS, GEX Corporation, Centennial, Colo.) was inserted between the frozen beef sample and plastic bag and irradiated for 30 s. The dose rate at the bottom was measured for 120 s. Each measurement was repeated five times and averaged. The dose rate was 0.847 kGy/min. During irradiation, samples were placed on top of a block of dry ice (wrapped with aluminum foil) to maintain the frozen state of the sample. Twenty samples (one by one) were irradiated at each dose (1.5, 3.0, and 5.0 kGy). Actual absorbed (average) irradiation doses achieved with the low-energy X-ray instrument are shown in Table 8.

Table 8- Targeted and actual absorbed (average) irradiation dose at gamma-ray and low-energy X-ray facilities.

Targeted dose (kGy)	Delivered dose (kGy)	
	Gamma ray	Low-energy X-ray
1.5	1.5 ± 0.045	1.5 ± 0.049
3.0	3.0 ± 0.090	3.0 ± 0.098
5.0	5.0 ± 0.150	5.0 ± 0.163

Extraction of 2-DCB

All samples were stored at -80 °C until analysis and thawed at room temperature for a few minutes just before analysis. A 3-g sample of irradiated ground beef patties was mixed with 15 g of anhydrous sodium sulfate and extracted with 150 mL n-hexane for 10 h by using a Soxhlet apparatus. At the end of the extraction time, the hexane was evaporated with a rotavaporator and the sample was dissolved in 50 mL hexane. A-10 mL aliquot of the hexane extract was transferred to a 10-mL vial, and the hexane was evaporated with a nitrogen stream. The fat inside the vial was dissolved in 5 mL ethyl acetate followed by 5 mL of acetontrile and was deffated and cleaned as described by Obana and others (2005). Finally, the elution was evaporated with a nitrogen stream, the extract was dissolved in 50 µl of hexane, and 1 µL was injected into a gas chromatography-mass spectrometry (GC-MS) instrument.

GC-MS Analysis of 2-DCB

Gas chromatography-mass spectrometry was performed with an HP 5890 GC (Agilent Technologies, Palo alto, CA) fitted with an HP-5MS column (cross-linked 5% Ph Me siloxane, 30

m × 0.22 mm × 0.025 μm film thickness) and a HP MS 5970 detector. The flow rate for the helium carrier gas was 1 mL/min. The injector temperature was set to 250 °C. The GC temperature program was as follows: initial temperature of 55 °C, hold for 0.5 min, ramp at 20 °C/min to 200 °C, hold for 1 min, ramp at 15 °C/min to a final temperature of 270 °C, and hold for 1 min. The MS was set to the selective ion mode, and ions *m/z* 98 and 112 were monitored for the analysis of 2-DCB (Gadgil and others 2005).

Fatty Acid Profile

The fat from the ground beef patties was extracted with n-hexane using a Soxhlet apparatus and concentrated to dryness with a rotary evaporator (temperature = 60 °C). The triglycerides were converted to their corresponding fatty acids methyl esters (FAME) and analyzed with a Hewlett-Packard 5890 gas chromatography (Agilent Technologies, Palo Alto, CA) fitted with a flame ionization detector (FID) and an HP-23 cis/trans FAME column (Agilent Technologies) as described by Gadgil and others (2002).

Statistical Analysis

The experimental design was completely randomized with a 2 × 3 factorial treatment structure. Analysis of variance and regression analysis were carried out on the data by using SAS version 9.1 (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

Analysis of the fatty acid profile of the ground beef fat showed a typical composition. The fatty acid composition (g fatty acid per 100 g total fatty acids) was as follows: 2.73 ± 0.53 C14, 24.74 ± 1.51 C16, 5.0 ± 0.27 C16:1, 16.36 ± 1.68 C18, 46.80 ± 2.29 C18:1, 4.12 ± 0.41 C18:2, 0.25 ± 0.04 C18:3. Although the amount of oleic acid which is the precursor of 2-tetradec-5'-enylcyclobutanone (2-TDCB) is the highest in beef fat, Gadgil and others (2002) were not able to detect it in ground beef patties irradiated at 2.5 kGy or below. Obana and others (2005) showed that irradiation of frozen ground beef was likely to produce more 2-tetradecylcyclobutanone (2-TCB) from stearic acid than 2-DCB from palmitic acid and the ratio of 2-DCB to 2-TCB was less than those of palmitic acid to stearic acid. In this report, 2-DCB was analyzed as a chemical marker of irradiated ground beef because it was the most easily detected cyclobutanone (Gadgil and others 2002).

Table 9 shows the 2-dodecylcyclobutanone (2-DCB) concentrations in irradiated beef patties. These results are in agreement with recent studies on the levels of 2-DCB in irradiated ground beef. Gadgil and others (2002) reported 2-DCB concentrations in gamma-ray-irradiated ground beef patties (80/20) were 0.02 ppm ± 0.00 at 0.9 kGy, 0.03 ppm ± 0.00 at 2.3 kGy and 0.06 ppm ± 0.04 at 5.1 kGy. Gadgil and others (2002) also reported 2-DCB concentrations in the same patties irradiated with an electron beam were 0.03 ppm ± 0.00 at 1.7 kGy, 0.04 ± 0.01 at 2.6 kGy, and 0.12 ppm ± 0.02 at 4.6 kGy. Obana and others (2005) reported that 2-DCB concentration in gamma-irradiated minced beef (16.7% fat) was 0.077 ppm when irradiated at 3.9 kGy. Obana and others (2006) reported that 2-DCB concentration in electron-beam-irradiated beef patties (15% fat) was 0.107 ppm when irradiated at 5.3 kGy, and in gamma-ray-irradiated

patties (17% fat) was 0.081 ppm weight when irradiated at 4.7 kGy. In the present study, the 2-DCB concentration in gamma-ray-irradiated patties was 0.068 ppm when irradiated at 5 kGy (Table 9). Obana and others (2007) reported that 2-DCB concentration in gamma-irradiated beef (15% fat) was 0.068 ppm when irradiated at 5.1 kGy.

Table 9- Concentration of 2-DCB (ppm) in irradiated ground beef patties (n = 3).

Dose	Low-energy X-ray	Gamma ray
1.5 kGy	0.024 ± 0.004	0.028 ± 0.001
3 kGy	0.040 ± 0.008	0.042 ± 0.005
5 kGy	0.054 ± 0.011	0.068 ± 0.008

Figure 9 shows chromatograms of 2-DCB of non-irradiated and 5 kGy irradiated patties. The 2-DCB concentration increased linearly ($p < 0.05$) with irradiation dose for gamma-ray- and low-energy X-ray-irradiated patties (Figure 10). This result is in agreement with recent studies on the effect of irradiation dose on 2-DCB concentration (D'oca and others 2009; Tewfik 2008; Zanardi and others 2007; Obana and others 2005; Gadgil and others 2002, 2005).

There was no significant difference in 2-DCB concentration between gamma-ray- and low-energy X-ray-irradiated patties at all targeted doses ($p > 0.05$). Gadgil and others (2002) found no significant difference in 2-DCB concentration between gamma-ray- and electron-beam-irradiated patties at 0.5 and 1.0 kGy targeted dose levels. Obana and others (2006) also showed no significant difference in 2-DCB concentration between electron-beam- or gamma- ray-irradiated patties at 5.0 kGy targeted dose.

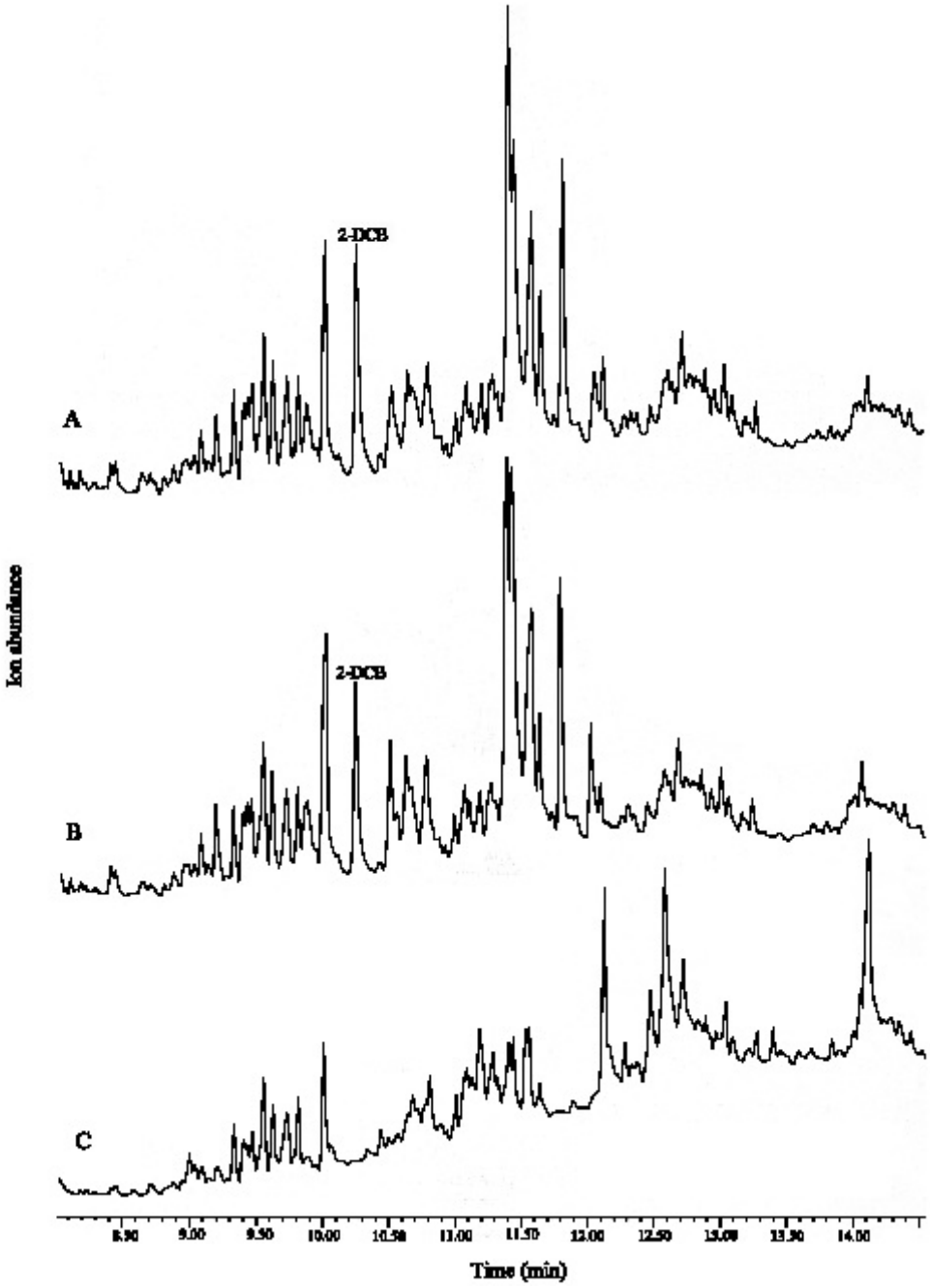

Figure 9- GC-MS chromatograms showing the detection of 2-DCB in irradiated beef patties: (A) 5.0 kGy (targeted) gamma-ray-irradiated patties, (B) 5.0 kGy (targeted) low-energy X-ray-irradiated patties, (C) control patties. The MS was set to the SIM mode and ions 98 and 112 were monitored.

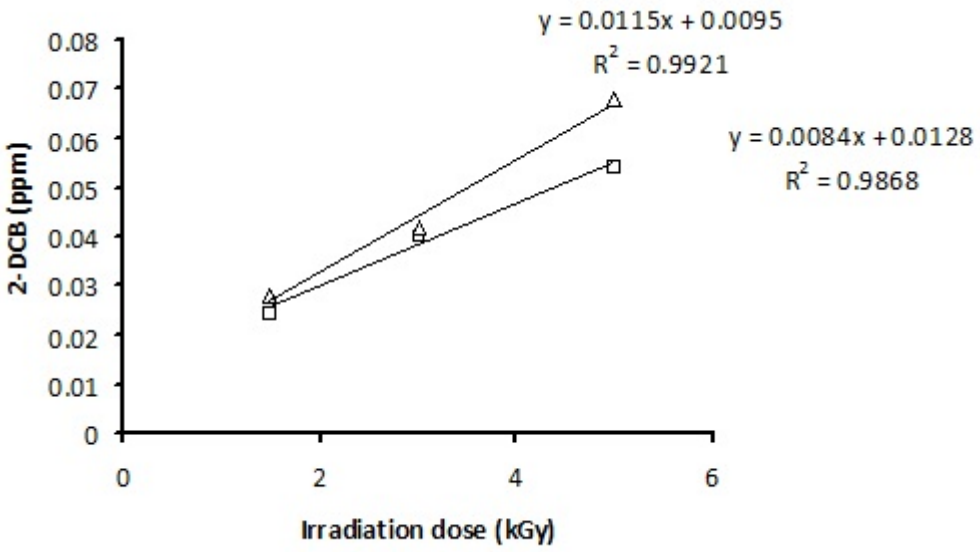

Figure 10- Response of 2-DCB (mg/kg of ground beef) with increasing irradiation dose: (▵) gamma-ray-irradiated patties; (▫) low-energy X-ray-irradiated patties.

Several factors may affect the actual absorbed dose during irradiation of beef patties: dose uniformity ratio (DUR), how the patties were irradiated (separate or in groups), placement of the dosimeter (middle, top, or bottom), and design of the radiator (annular, double, or single beam). For example, if 4 patties are stacked side by side and irradiated at the same time with double-electron-beam irradiation and the absorbed dose was measured in the middle, the 2 outside patties (top and bottom) will be exposed to a higher dose because the dose-depth distribution will be higher at the surface than in the middle. Although our results showed no significant difference in 2-DCB concentration between gamma-ray-irradiated and low-energy X-ray-irradiated patties ($p > 0.05$), 2-DCB concentration in low-energy X-ray-irradiated patties was lower than that in gamma-ray-irradiated patties at all targeted doses because the actual absorbed dose at the middle of the

patty was lower than that measured at the top or the bottom. To keep the dose uniformity ratio close to 1, it was necessary to use a small sample size (3 mm thickness). Ideally, effects of different types of ionizing radiation on the levels of radiolytic products in food should be compared at the same absorbed dose; however, it is hard to control the actual absorbed dose because it depends on irradiator design.

The literature provides limited information on the concentration of 2-ACBs in X-ray-irradiated food because only a few X-ray units have been built worldwide. However, gamma rays and X-rays are both electromagnetic radiation and are converted into fast electrons in irradiated media (Hayashi 1991). Because our results showed that 2-DCB concentration was consistently the same in gamma-ray- and low-energy X-ray- irradiated patties, there likely will be no significant differences in the effect on chemical reactions in food between low-energy X-ray and others types of food irradiation. Because 2-DCB concentration was the same in gamma-ray- and low energy X-ray- irradiated patties, there will be no difference in their lethal effects on microorganisms in food. In fact, Studies conducted at Michigan State University showed that low-energy X-ray can be used successfully to eradicate *E. coli* O157:H7 (5 log reduction) in ground beef and other food products (Lindsay and others 2007).

This study showed that 2-DCB produced in ground beef patties by low-energy X-ray irradiation are similar to those produced by gamma-ray irradiation. These result indicate that 2-DCB formation in irradiated ground beef is independent on the energy level or the type of the irradiation source.

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PART IV: *IN VITRO* AND *IN VIVO* METABOLISM OF THE RADIOLYTIC COMPOUND 2-DODECYLCYCLOBUTANONE[1]

ABSTRACT

Our knowledge about the metabolism of alkylcyclobutanones (2-ACBs) is limited, and the lack of literature on the metabolism of 2-ACBs causes consumers to doubt the safety of irradiated foods. The objectives of this study were to evaluate the metabolism of 2-dodecylcyclobutanone (2-DCB) and identify any possible metabolite. The 2-DCB was mixed with rat S9 (post-mitochondrial supernatant fraction) and β-nicotinamide adenine dinucleotide phosphate (NADPH) in phosphate buffer (pH 7.4) and incubated for 2 h at 37 °C. Then, the incubation mixture was mixed with sodium sulfate and extracted with n-hexane by using a Soxhlet apparatus. The hexane extract was concentrated under nitrogen and injected into the gas chromatography-mass spectrometry (GC-MS) machine running in selective ion monitoring mode (SIM) to measure 2-DCB concentration. The hexane extract from the *in vitro* and *in vivo* studies was also derivatized with a silylation reagent and injected into a GC-MS running in full scan mode. The average percentage of 2-DCB recovered from the test incubations was 23%, compared with 50% from the controls. The GC-MS chromatograms of the derivatized samples showed a unique peak in the *in vitro* test incubations and in the hexane extract of the rat feces that were given 2-DCB. This peak was later identified as 2-doecylcyclobutanol.

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1. Reproduced with permission from Hijaz, F, Shrestha TB, Bossman SH, Hussain F, Smith JS. 2010. *In vitro* and *in vivo* metabolism of the radiolytic compound 2-dodecylcyclobutanone. J Food Sci 75(4):T72-T80.

INTRODUCTION

Food irradiation improves food safety and maintains food quality by controlling microorganisms and extending shelf life. The Food and Drug Administration and the United States Department of Agriculture have approved the use of irradiation in food processing for many types of food. However, the use of irradiation as a way to enhance food safety is controversial. Consumer groups such as Public Citizen (Worth and Jenkins 2001) and the Center for Food Safety (Jenkins and Worth 2006) have opposed irradiation because of potentially toxic radiolytic products called alkylcyclobutanones (2-ACBs), which are radiolytic products formed from fatty acids (Le Tellier and Nawar 1972). In animal studies, high concentrations of 2-ACBs, especially 2-dodecylcyclobutanone (2-DCB), which forms from palmitic acid, induce DNA strand breaks in cells (Delincée and Pool-Zobel 1998). Raul and others (2002) showed that although 2-ACBs did not affect the number of preneoplastic lesions in rats fed 2-ACBs along with azoxymethane (a carcinogen) injection compared with rats treated with azoxymethane alone, rats fed 2-ACBs developed larger tumors in their colons.

Although there are many published studies on the occurrence of 2-ACBs in irradiated foods, only 2 studies on the fate of 2-ACBs after consumption have been published (Horvatovich and others 2002, Gadgil and Smith 2006). The lack of literature on the toxicity and metabolism of 2-ACBs is the biggest barrier to countering the claims of consumer groups that are opposed to food irradiation (Gadgil and Smith 2006).

Horvatovich and others (2002) administered 1 mg of 2-tetradecyl- or 2-tetradecenylcyclobutanone in 1% ethanol as a drinking water daily for 4 mo to male Wistar rats. During the last 3 d of the study, adipose tissue and feces were analyzed for 2-tetradecyl- and 2-

tetradecenylcyclobutanone. The total quantity of 2-ACBs recovered from adipose tissue was very low (10^{-5} times the total consumed), and less than 1% was recovered from fecal material. Horvatovich and others (2002) did not check for any metabolite and concluded that 2-ACBs either were not stored in animal fats (but might be stored elsewhere) or underwent metabolic transformation. The authors suggested further studies to determine the products produced by the metabolic breakdown of 2-ACBs.

In Gadgil and Smith's (2006) study, female sprague-Dawley rats were given 2-DCB (5 mg/d) in corn oil for 5 d via gavage. Feces and urine were collected daily and analyzed for 2-DCB. Adipose tissue samples were collected upon euthanasia and analyzed for 2-DCB. Urine was treated with β-glucuronidase and bis(trimethylsilyl)trifluoroacetamide (BFTSA) to determine if the ketone group of 2-DCB was converted to alcohol. Between 3 and 11% of the total 2-DCB administered was recovered in the feces, and about 0.33% was recovered in the adipose tissue. No metabolites could be identified in the urine samples that were derivatized with BFTSA or incubated with β-glucuronidase. Like Horvatovich and others (2002), Gadgil and Smith (2006) concluded that most of the 2-DCB was either metabolized and rapidly eliminated from the body or was stored somewhere other than in the adipose tissue.

Our knowledge about the metabolism of 2-ACBs is limited, and many assumptions have been proposed. Elliott and others (1995) proposed that 2-DCB is converted to 5-dodecyl-oxolan-2-one (γ-palmitolactone), noting that when 11-(2`-oxocyclobutyl)undecanoic acid was inoculated into the body of a rabbit, there was significant cross-reactivity between the antiserum generated and 2-substituted lactones (C-12 lactones, 72%). Recently, Hartwig and others (2007) reported that γ-palmitolactone formed when 2-DCB standard was kept at room temperature for a few

weeks. Vajdi and others (1979) detected long-chain gamma- and delta-lactones in beef irradiated at a very high dose (50 Mrad = 500 kGy). Horvatovich and others (2002) suggested that 2-ACBs oxidize to form lactones in animal tissues.

The concentration 2-ACBs in irradiated foods decreases during storage. Boyd and others (1991) reported a slight reduction in 2-DCB in irradiated meat stored for 20 d. The concentration of 2-tetradecyl-cyclobutanone (2-tDCB) and 2-DCB in irradiated feed was reduced by 50% during storage for 3 mo at room temperature. However, it took 6 mo for a similar reduction in frozen feed. Both 2-DCB and 2-tDCB were reduced by about 50% in irradiated chicken stored at 4 °C for 1 mo (Ndiaye and others 1999). Obana and others (2007) reported a 30% decrease in 2-DCB in irradiated meat stored at -20 °C for 1 yr; however, there was no clear reduction in 2-tDCB concentration.

Because the metabolite may be more active, equally active, or less active or possess an entirely different activity than the parent molecule (Jia and Liu 2007), identifying the metabolites of 2-ACBs is of great importance. The objectives of this study were to evaluate the metabolism of 2-DCB and identify any possible metabolite.

MATERIAL AND METHODS

Chemicals and Reagents

Hexane, 2-DCB, potassium phosphate (monobasic), potassium phosphate (dibasic), sodium sulfate anhydrous, hydrochloric acid, 95% ethanol, pooled male (Sprague-Dawley) rat liver S9 prepared by CellzDirect (Durham, NC), β-nicotinamide adenine dinucleotide phosphate (reduced β-NADPH 4Na), sodium borohydride, and dimethylsulfoxide (silyation grade) were purchased

from Thermo Fisher Scientific (Pittsburg, Pa.). The sylon BTZ kit [BSA (N, O-bis(trimethylsilyl)acetamide) + TMCS (trimethylchlorosilane) + TMSI (N-trimethylsilyimidazole), 3:2:3] was purchased from Sigma-Aldrich (St. Louis, Mo.). Chloroform-D was purchased from Cambridge Isotopes Laboratories, Inc. (Andover, Ma.).

Preliminary Study

Part one

The experimental design for this part was adapted from Jia and Liu (2007). To test if 2-DCB is metabolized by rat liver S9, 2-DCB was incubated with S9 and NADPH in potassium phosphate buffer (PPB) for 2 h at 37 °C. The concentration of PPB was 50 mM, and its pH was 7.4. The control was made by excluding the S9 and NADPH (Table 10).

Table 10- Experimental design for part one of the preliminary study (n = 9).

Groups	2-DCB	Potassium phosphate buffer	S9 (5 mg/mL) in potassium phosphate buffer	NADPH (2 mg/mL) in potassium phosphate buffer
Test	0.1 ml of 50 ppm in hexane[a]	0.8 mL	0.1 mL	0.1 mL
Control 1	0.1 ml of 50 ppm in hexane[a]	1.0 mL		

[a] Hexane was evaporated before the addition of potassium phosphate buffer.

Part two

To test if the metabolism of 2-DCB is energy and protein dependent, the preliminary study was repeated and two more controls were added (Table 11). The experimental design for this test also was adapted from Jia and Liu (2007).

Table 11- Experimental design for the second part of the preliminary study (n = 5).

Groups	2-DCB	Potassium phosphate buffer	S9 (5 mg/mL) in potassium phosphate buffer	NADPH (2mg/mL) potassium phosphate buffer
Test	0.1 ml of 50 ppm in hexane[a]	0.8 mL	0.1 mL	0.1 mL
Control 1	0.1 ml of 50 ppm in hexane[a]	1.0 mL		
Control 2	0.1 ml of 50 ppm in hexane[a]	0.9 mL	0.1 mL	
Control 3	0.1 ml of 50 ppm in hexane[a]	0.9 mL		0.1 mL

[a] Hexane was evaporated before the addition of the potassium phosphate buffer.

At the end of the incubation time, the samples were removed from the incubation bath and stored at -80 °C until they were extracted. The incubation mixture was mixed with 15 g of sodium sulfate and extracted with 150 mL hexane by using a Soxhlet apparatus for 6 h. At the end of the extraction time, the hexane was evaporated with a rotary evaporator and the round-bottom flask was washed with 10 mL hexane. The 10 mL hexane was evaporated under nitrogen, and the extract was dissolved in 0.5 mL hexane. A 1 µl aliquot of the concentrated extract was injected into a GC-MS running in the (SIM) mode.

GC-MS analysis of 2-DCB and its metabolite

GC-MS was performed with a HP 5890 GC (Agilent Technologies, Palo alto, CA) fitted with an HP-5MS column (cross-linked 5% Ph Me siloxane, 30 m × 0.22 mm × 0.025 μm film thickness) and an HP MS 5970 detector. The flow rate for the helium carrier gas was 1 mL/min. The injector temperature was set to 250 °C. The GC temperature program was as follows: 55 °C initial temperature, hold for 0.5 min, ramp at 20 °C/min to 200 °C, hold for 7 min, ramp at 15 °C/min to a final temperature of 270 °C, hold for 7 min (Gadgil and others 2005). The ions with mass to charge ratio (*m/z*) of 98 and 112 were monitored for the analysis of 2-DCB.

Main Study

The experimental design for the main study (Table 12) was adapted from Jia and Liu (2007). Dimethyl sulfoxide (DMSO) was added to increase the solubility of 2-DCB in the aqueous solution. The concentration of NADPH was increased from 2 mg/mL to 10 mg/mL because we increased the 2-DCB concentration five times. The 2-DCB was extracted with hexane by using a Soxhlet apparatus as in the preliminary study, except the final volume was 4.0 mL.

Identification of the metabolite (in vitro)

The hexane extract from the main study was concentrated to 1 mL and injected in a GC-MS running in full scan mode. The remaining part of the hexane extract was mixed with 3 μL of silylation reagent, and 1 μLwas injected in a GC-MS running in full scan mode using two different column (HP-5MS and DB-1MS).

Table 12- Experimental design for the main study (n = 3).

Groups	2-DCB	Potassium phosphate buffer	S9 (5 mg/mL) in potassium phosphate buffer	NADPH (10 mg/mL) in potassium phosphate buffer	Dimethyl sulfoxide
Test	50 µL of 1000 ppm in hexane[a]	780 µL	100 µL	100 µL	20 µL
Control 1	50 µL of 1000 ppm in hexane[a]	880 µL	100 µL		20 µl
Control 2	50 µL of 1000 ppm in hexane[a]	880 µL		100 µL	20 µL
Control 3		780 µL	100 µL	100 µL	20 µL

[a] Hexane was evaporated before the addition of potassium phosphate buffer.

In Vivo Metabolism of 2-DCB

The hexane extract (1 mL) of the rat feces and urine from Gadgil and Smith's (2006) study, which had been stored at -20 °C, was mixed with 3 µl of the silylation reagent, and 1 µl was injected into a GC-MS running in full scan mode.

Reduction of 2-DCB to 2-dodecylcyclobutanol and derivatization of 2-dodecylcyclobutanol

The procedure used to reduce 2-DCB to 2-dodecylcyclobutanol was adapted from Saeed and Ashraf (2006). Briefly, 26.0 mg of 2-DCB standard was dissolved in 5 mL of 95% ethanol in a 25 mL Erlenmeyer flask. Sodium borohydride (0.25 g) was added to the reaction flask and stirred for 20 min at room temperature. Then, 1 mL of water was added to the reaction mixture, followed by 1 mL of 3 M HCl. The reaction mixture was allowed to stand for 10 min until there was no more evolution of hydrogen gas, heated to boiling, and allowed to cool to room

temperature. The product (2-dodecylcyclobutanol) was manually extracted with 4 × 20 mL hexane. The hexane extract was collected in a round-bottom flask, and the hexane was evaporated with a rotary evaporator. The round-bottom flask was washed with 10 mL hexane, and the hexane was collected in a 10 mL vial. A 1 mL aliquot of the final solution was diluted to 2.0 mL with hexane, and 1 μL was injected into a GC-MS running in full scan mode to identify the product and ensure the 2-DCB had been completely reduced. A 1 mL aliquot of the diluted solution was mixed with 3 μL of the silylation reagent and injected into a GC-MS running in full scan mode. The derivatized test samples (*in vitro* and *in vivo*) also were injected into the GC-MS to compare the retention time and the mass spectra of the trimethylsilyl (TMS) derivative of the metabolite with that of the 2-dodecylcyclobutanol-trimethylsilane (2-dodecylcyclobutanol-TMS). The previous procedure was performed using an HP-5MS and PB35X (35% Phenyl Polysilphenylene-siloxane, 50 m × 0.32 mm × 0.25 μm film thickness) column to make sure that the retention time and the mass spectra of the TMS derivative of the metabolite match the retention time and the mass spectra of the TMS derivative of 2-dodecylcyclobutanol standard.

Infrared (IR) spectra and nuclear magnetic resonance (NMR) spectra of 2-DCB, 2-dodecylcyclobutanol, and the TMS derivative of 2-dodecylcyclobutanol.

The IR spectra of the neat 2-DCB, 2-dodecylcyclobutanol, and 2-dodecylcyclobutanol-TMS were recorded with a Thermo Scientific Nicolet 380 Fourier transform infrared spectrometer (Thermo Scientific, Waltham, Ma.). The ^{1}H and ^{13}C NMR of 2-DCB, 2-dodecylcyclobutanol, and 2-dodecylcyclobutanol-TMS were recorded in deuterated chloroform ($CDCL_3$) solutions at 200 MHz with a Varian Gemini 2000 spectrometer (Varian, Palo Alto, Ca). After the NMR and IR

spectra of 2-dodecylcyclobutanol were taken, the $CDCL_3$ was evaporated and 2-dodecylcyclobutanol was mixed with 20 μL silylation reagent in 1 mL hexane. The reaction mixture was left for 15 min at room temperature, and 1 mL of water was added to remove the excess silylation reagent. The trimethylsilyl of 2-dodecylcyclobutanol was extracted with 5 × 10 mL hexane. The hexane was evaporated, and the IR of the neat TMS derivative was taken. The ^{1}H and ^{13}C NMR of the 2-dodecylcyclobutanol-TMS were taken in $CDCL_3$.

Statistical Analysis

Data were analyzed with SAS version 9.1 (SAS Institute, Inc., Cary, N.C.). The experiment was created in a completely randomized design, and PROC GLM was used to detect statistical differences in 2-DCB concentration between the treatment and the control. The Dunnett test was used to compare all other treatments with control 1. The Tukey method, the least significant difference (LSD), the Student-Newman-Keuls (SNK) multiple range test, the Waller-Duncan test, and the Scheffe test were used for pairwise comparisons.

RESULTS AND DISCUSSION

Preliminary Study

Part one

The average percentage of 2-DCB recovered from the test treatments (2-DCB mixed with S9 and NADPH) was 28% less than that of the controls (Table 13). This indicates that 2-DCB was metabolized by the rat liver S9. Therefore, we designed another study to determine whether the reaction was protein and energy dependent.

Table 13- Recovery of 2-DCB after incubation with S9 and NADPH (n = 9).

Group	Average recovery (%)	Standard Deviation
Control (2-DCB in buffer)	88.36[a]	10.6
Test (2-DCB with S9 and NADPH)	60.07[b]	5.14

[ab] Means with different letters differ significantly ($P < 0.05$).

Part two

The average percentage of 2-DCB recovered from the test treatment (2-DCB incubated with S9 and NADPH) was 12.1%, compared with 67% recovered from control 1 (Table 14). This result also revealed that 2-DCB was metabolized by the rat liver S9. There was no significant difference between the average percentage of 2-DCB recovered from controls 1 and 3 (Table 15), which means that the metabolic process is protein dependent. The Dunnett test, Tukey test, LSD test, SNK multiple range test, and Waller-Duncan test showed that the average percentage of 2-DCB recovered from controls 1 and 2 were different ($P < 0.05$). However, the Scheffe test

showed no significant difference between controls 1 and 2. Because the average percentage of 2-DCB recovered from control 2 was much higher than that of the test treatment (53% compared to 12%) and the Scheffe test did not show any significant differences between controls 1 and 2, we concluded that the reaction was energy dependent. The low percentage of 2-DCB recovered from the test treatment revealed that 2-DCB is metabolized by rat S9 and the reaction is energy and protein dependent.

Table 14- Recovery of 2-DCB after incubation with S9 and NADPH (n = 5).

Group	Average recovery (%)	Standard deviation
Test	12.1[b]	1.5
Control 1 (2-DCB in buffer)	67.0[a]	5.8
Control 2 (no NADPH)	53.0[a]	11.5
Control 3 (no S9)	63.0[a]	3.6

[ab] Means with different letters differ significantly ($P < 0.05$).

Main Study

The average percentage of 2-DCB recovered from the test treatment group was about 27% lower than that recovered from controls 1 and 2 (Table 15), which means that 2-DCB was metabolized by S9 even though it was incubated at a higher concentration. There were no significant differences between the average percentage of 2-DCB recovered from controls 1 and 2 (Table 15). This result confirmed our findings from the preliminary study, proving that the metabolic process was protein and energy dependent.

Table 15- Recovery of 2-DCB after incubation with S9 and NADPH (n = 3).

Group	Average recovery (%)	Standard deviation
Test	23.00[a]	2.8
Control 1 (no NADPH)	49.80[b]	8.6
Control 2 (no S9)	53.87[b]	1.46
Control 3 (no 2-DCB)	NA	

[ab] Means with different letters differ significantly ($P < 0.05$).

Identification of the metabolite (in vitro)

The GC-MS chromatograms of underivatized test samples (incubated with S9 and NADPH) showed a unique peak (metabolite) that had a 74% match with 1-hexadecanol. However, this peak was not totally separated from the 2-DCB peak. The mass spectra of this metabolite is shown in Figure 11-A. Although this metabolite was observed in 3 test samples and was absent in the controls, the metabolite's peak disappeared (overlapped with 2-DCB) after a few injections. Because the mass spectrum of the potential metabolite showed a 74% match with 1-hexadecanol, we decided to derivatize the samples with the silylation reagent to test if 2-DCB was metabolized to alcohol.

The GC-MS chromatograms of the derivatized test samples showed a metabolite peak (Figure 12-B) that was absent in the controls (Figure 12-A, C, and D). These results were confirmed during the complementary GC-MS analyses using a DB-1MS column. The mass spectrum of the metabolite-TMS peak is shown in Figure 13-A. The mass spectrum of the metabolite peak was different from the mass spectra of 1-hexadecanol-TMS and 2-hexadecanol-

TMS derivatives. The 1-hexadecanol-TMS spectrum showed a major ion fragment at 299 *(m/z)*, which was absent in the metabolite's spectrum (Figure 13-A). The mass spectrum of 2-hexadecanol-TMS had a major ion fragment at 117 *(m/z)*; however, the metabolite-TMS spectrum showed a major ion fragment at 116 *(m/z)* (Figure 13-A). The metabolite-TMS derivative showed a 20% match with the spectrum of trimethylsilyloxycyclobutane (cyclobutanol-TMS). The major ion fragments of the metabolite spectrum (116, 73, 101, and 129) also were found in the cyclobutanol-TMS mass spectra.

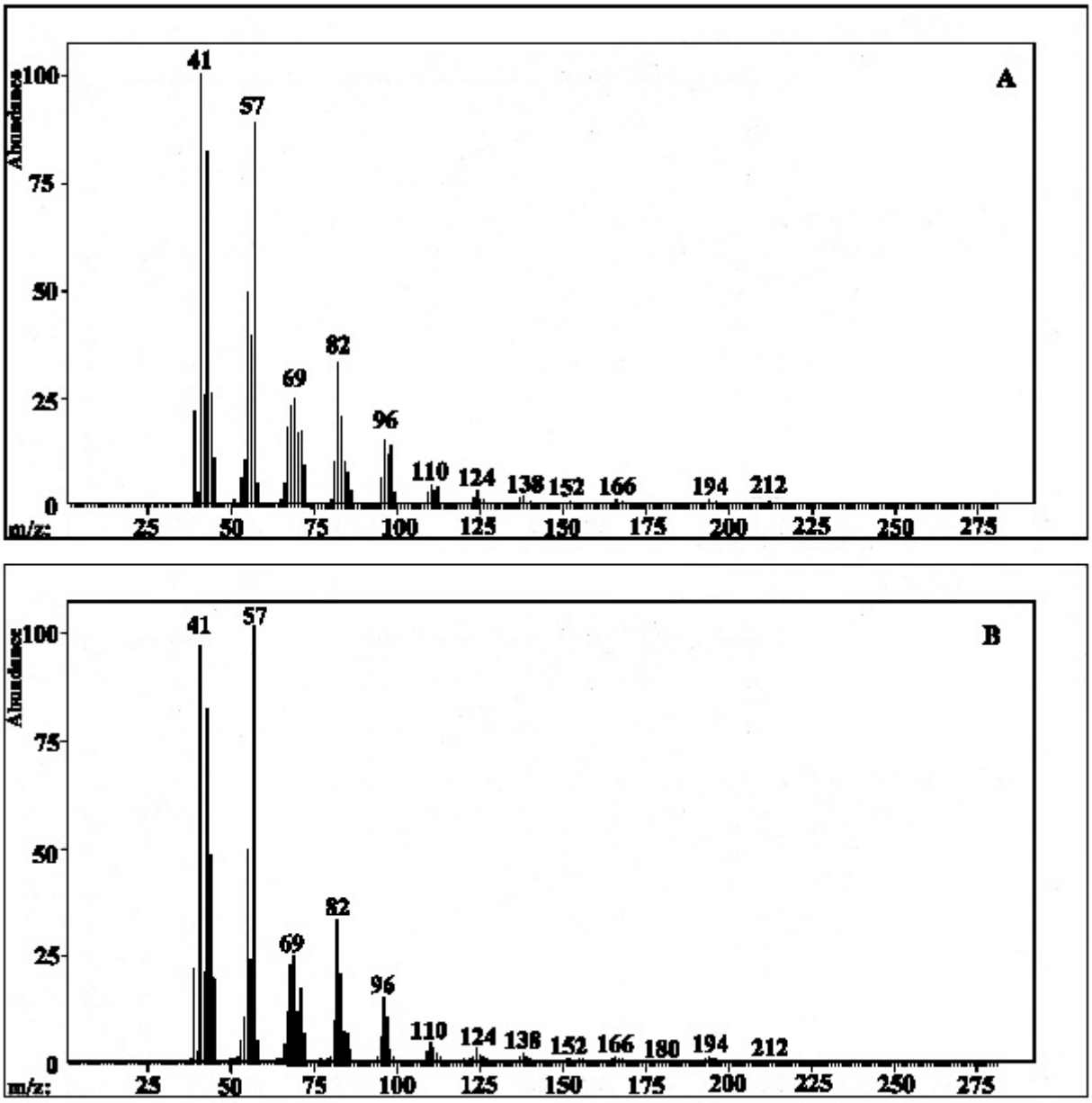

Figure 11- Mass spectra of *in vitro* metabolite of 2-DCB (A) and reduced 2-DCB standard (B).

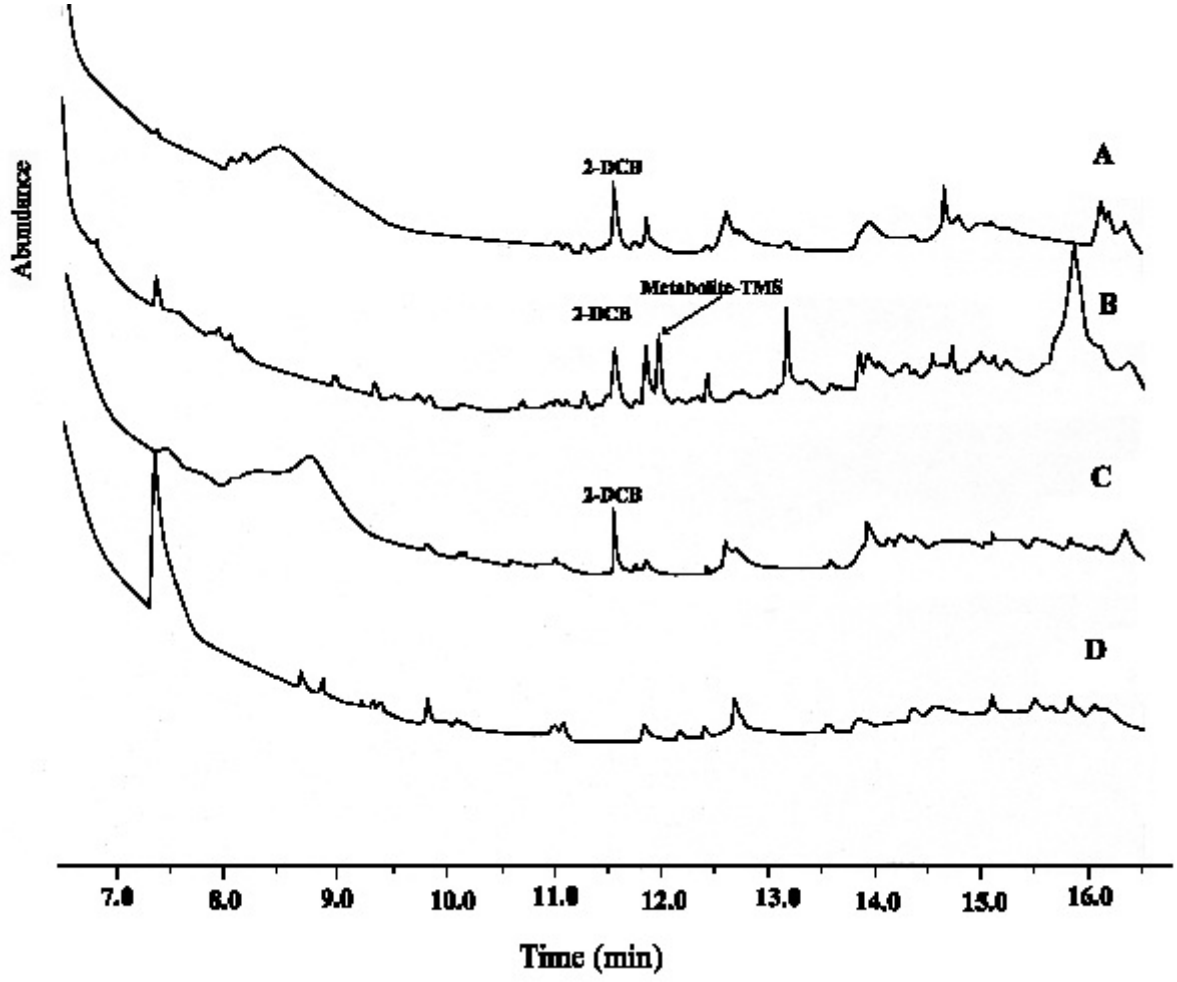

Figure 12- Total ion chromatogram (GC-MS) of the *in vitro* study (A) 2-DCB incubated with rat S9 only, (B) 2-DCB incubated with rat S9 and NADPH, (C) 2-DCB incubated with NADPH only, and (D) blank (no 2-DCB) .

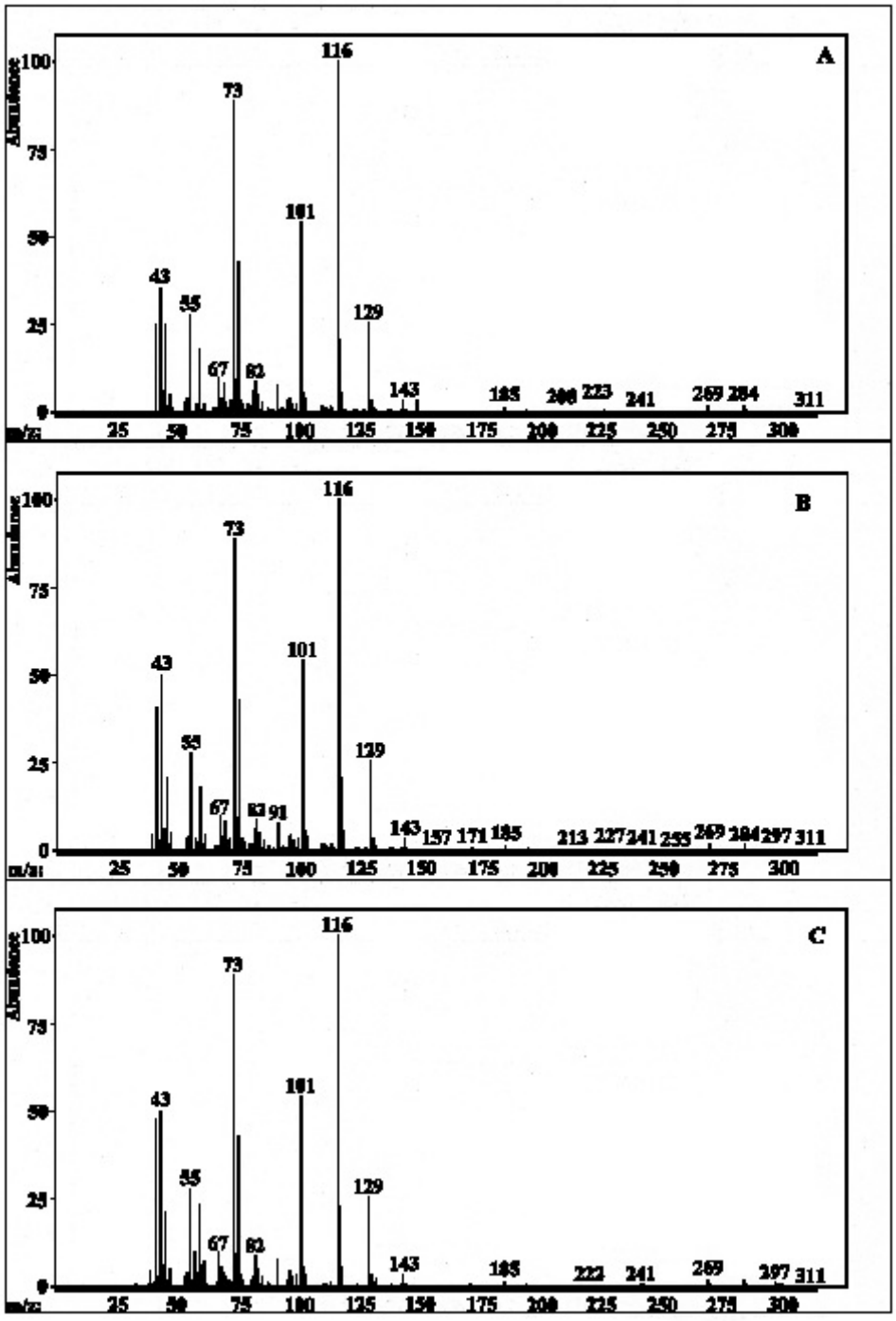

Figure 13- Mass spectra of TMS derivative of the 2-DCB metabolite *in vitro* (A), (B) mass spectra 2-dodecylcyclobutanol-TMS standard, and (C) mass spectra of TMS derivative of the 2-DCB metabolite in rat feces.

Because the mass spectrum of the metabolite-TMS was similar to that of cyclobutanol-TMS and the metabolic process reaction was energy dependent, we assumed that 2-DCB was metabolized by NADP(H)-dependent reductase to 2-dodecylcyclobutanol (Figure 14-B). To test this assumption, we reduced 2-DCB to 2-dodecylcyclobutanol and derivatized 2-dodecylcyclobutanol with the silylation reagent (Figure 14-A).

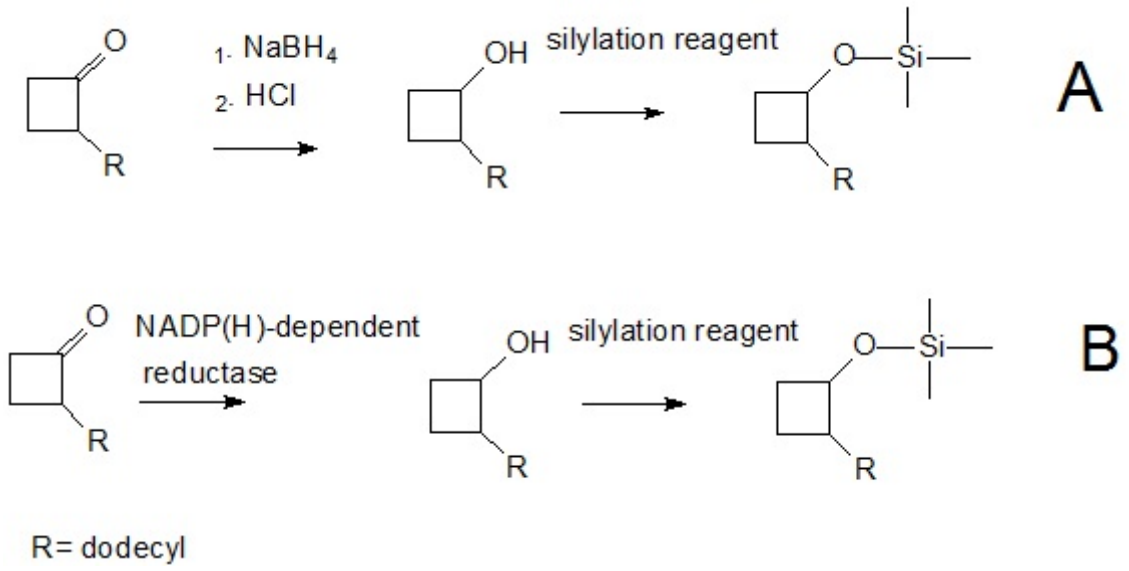

Figure 14- Reaction diagram (A) Reduction of 2-DCB by sodium borohydride followed by TMS derivatization and (B) reduction of 2-DCB by rat S9 followed by TMS derivatization.

The GC-MS chromatogram (full scan) of the reduced 2-DCB standard showed two major peaks (diastereoisomers). No 2-DCB was detected after treating 2-DCB with $NaBH_4$, which indicates that all of the 2-DCB was reduced to 2-dodecylcycolbutanol. The retention times of those two peaks were almost equal to the retention time of the 2-DCB standard, and matched the retention time of the 2-DCB's metabolite. The peak area of the first diastereoisomer was two

times larger than the area of the second peak; however, the mass spectra of those two peaks were exactly the same. (The mass spectrum of the first diastereoisomer is shown in Figure 11-B). The mass spectra of those two diastereoisomers were similar to the metabolite's mass spectra, which was identified in the underivatized test samples (Figure 11-A). These results showed that 2-DCB was metabolized by the S9, in the presence of NADPH, to 2-dodecylcyclobutanol.

The trimethylsilyl derivative of the reduced 2-DCB (2-dodecylcyclobutanol-TMS) showed only 1 peak (Figure 15-A), which means that the TMS derivatives of the two 2-dodecylcyclobutanol diastereoisomers are very similar and eluted as 1 peak. No 2-dodecylcyclobutanol was detected after the addition of the silylation reagent, which means that the 2-dodecylcyclobutanol was completely derivatized to its TMS derivatives. The retention time of the TMS derivative of the 2-dodecylcyclobutanol standard matched the retention time of the derivatized metabolite in the test treatments (Figure 15-A and 15-B). The mass spectra of the TMS derivative of the 2-dodecylcyclobutanol was exactly the same as the mass spectra of the TMS derivative of the metabolite in the test treatment (Figure 13-B and 13-A). The GC-MS analyses conducted using BPX35 column also showed that the retention time and the mass spectra of 2-dodecylcyclobutanol-TMS standard were exactly the same as the retention time and the mass spectra of the TMS derivative of the metabolite. These results confirmed the previous result, which showed that 2-DCB was metabolized by the S9, in the presence of NADPH, to 2-dodecylcyclobutanol.

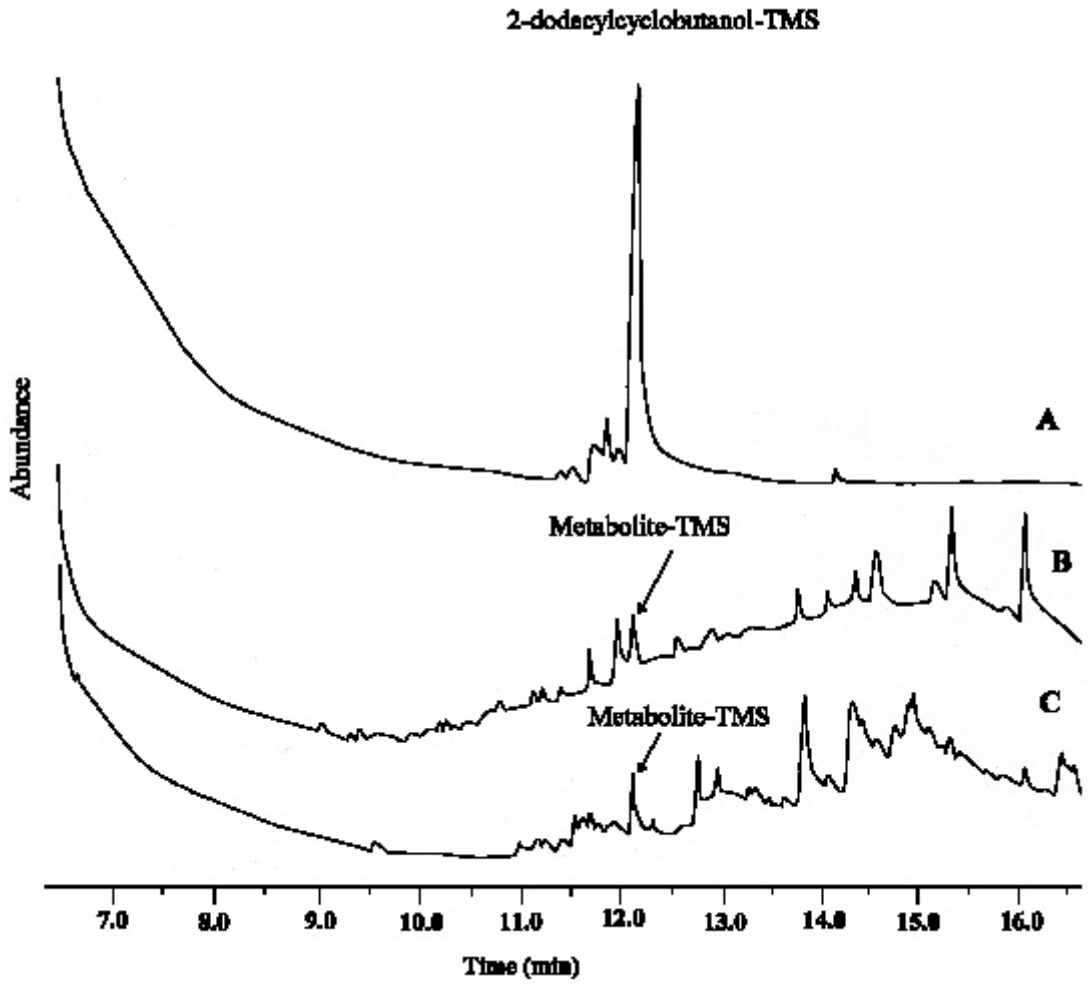

Figure 15- Total ion chromatograms (GC-MS) (A) 2-odecylcyclobutanol-TMS standard, (B) 2-DCB metabolite-TMS *in vitro*, and (C) metabolite-TMS in rat feces.

In vivo metabolism of 2-DCB

To confirm that 2-DCB was metabolized to 2-dodecylcyclobutanol *in vivo*, we derivatized the hexane extract of the rat feces and urine samples from Gadgil and Smith's (2006) study, which were kept frozen in our laboratory. The GC-MS chromatogram of the derivatized rat feces samples from the treatment group that was given 2-DCB showed a unique peak (Figure 15-C), which was absent in the controls. The retention time of this peak matched the retention time of the TMS derivative of the 2-dodecylcyclobutanol (Figure 15-C and 15-A). The mass spectrum of this peak (Figure 13-C) also matched the mass spectrum of the TMS derivative of the 2-dodecylcyclobutanol (Figure 13-B) and the mass spectrum of the TMS derivative of the *in vitro* metabolite (Figure 13-A). These previous results generated using an HP-5MS column were also confirmed using BPX35 column. No metabolite could be identified in the urine extract, which agrees with Gadgil and Smith's (2006) results. These results showed that 2-DCB was metabolized in the body to 2-dodecylcyclobutanol and excreted in the feces.

Infrared (IR) spectra and nuclear magnetic resonance (NMR) spectra of 2-DCB, 2-dodecylcyclobutanol, and the TMS derivative of 2-dodecylcyclobutanol.

Reduction of 2-DCB and the TMS derivative of the resulting alcohol were confirmed by ^{1}H NMR, ^{13}C NMR, and IR spectroscopy. The 2 peaks at 2.9 and 3.3 ppm in the ^{1}H NMR of 2-DCB (Figure 16-C) moved up field after reduction, and 2 new quartet peaks formed at 3.8 and 4.35 ppm (Figure 16-B). These 2 new peaks are due to the formation of 2 isomers (diastereisomers) of 2-dodecylcyclobutanol during the reduction of 2-DCB. Similarly, the TMS derivatives of 2-dodecylcyclobutanol were confirmed by the presence of the TMS proton signal at 0.1 ppm

(Figure 16-A). Additional proof for the reduction of 2-DCB to 2-dodecylcyclobutanol was provided by ^{13}C NMR (Figure 17). The carbonyl carbon peak of 2-DCB at 213 ppm (Figure 17-C) shifted to 67.82 ppm and 72.68 ppm (Figre 17-B). Furthermore, the TMS carbon peak at 0.8 ppm (Figure 17-A) strongly supports the formation of the TMS derivatives of 2-dodecylcyclobutanol. Finally, the functional group transformation was further confirmed by the disappearance of the carbonyl (C=O) stretching frequency of 2-DCB at 1779.62 cm^{-1} (Figure 18-A) and the appearance of the new broad peak around 3318 cm^{-1} (hydroxyl (-OH) stretching) in the infrared spectrum in the reduced 2-DCB (Figure 18-B). As anticipated, the broad peak around 3318 cm^{-1} (Figure 18-B) disappeared after protecting the -OH group with the TMS group (Figure 18-C). Because the amount of 2-DCB incubated with S9 and NADPH was very small (0.05 mg), it was impractical to isolate the metabolite and identify it by NMR, or IR spectroscopy.

Although our results showed that 2-DCB was metabolized by the NADP(H)-dependent reductases to 2-dodecylcyclobutanol, we were not able to test if 2-DCB was metabolized to 1 or 2 diastereisomers because the metabolites were not separated from the parent compound (2-DCB). However, ketone reduction generates alcohols of S-configuration more frequently than R-configuration because of the hidride addition to the *re* face of the prochiral carbonyl group (Rosemonod and Walsh 2004). Because the carbonyl reductase enzymes are present in many tissues, we assume that 2-DCB also is metabolized to 2-dodecylcyclobutanol in irradiated meat during storage. However, further research is needed to confirm this assumption.

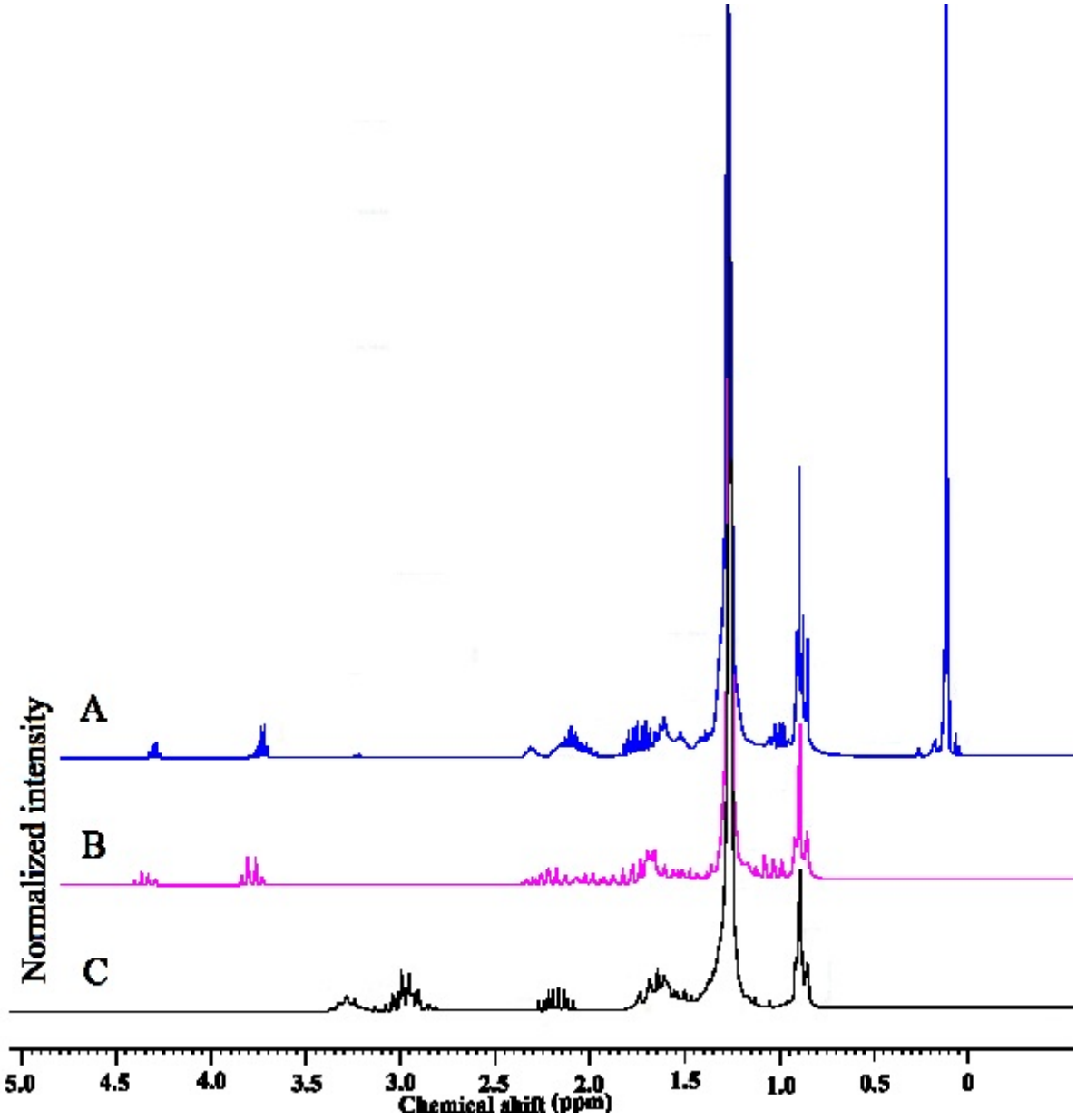

Figure 16- ^{1}H nuclear magnetic resonance spectra of the trimethylsilyl derivative of 2-dodecylcyclobutanol standard (A), 2-dodecylcyclobutanol (B), and 2-dodecylcyclobutanone (C).

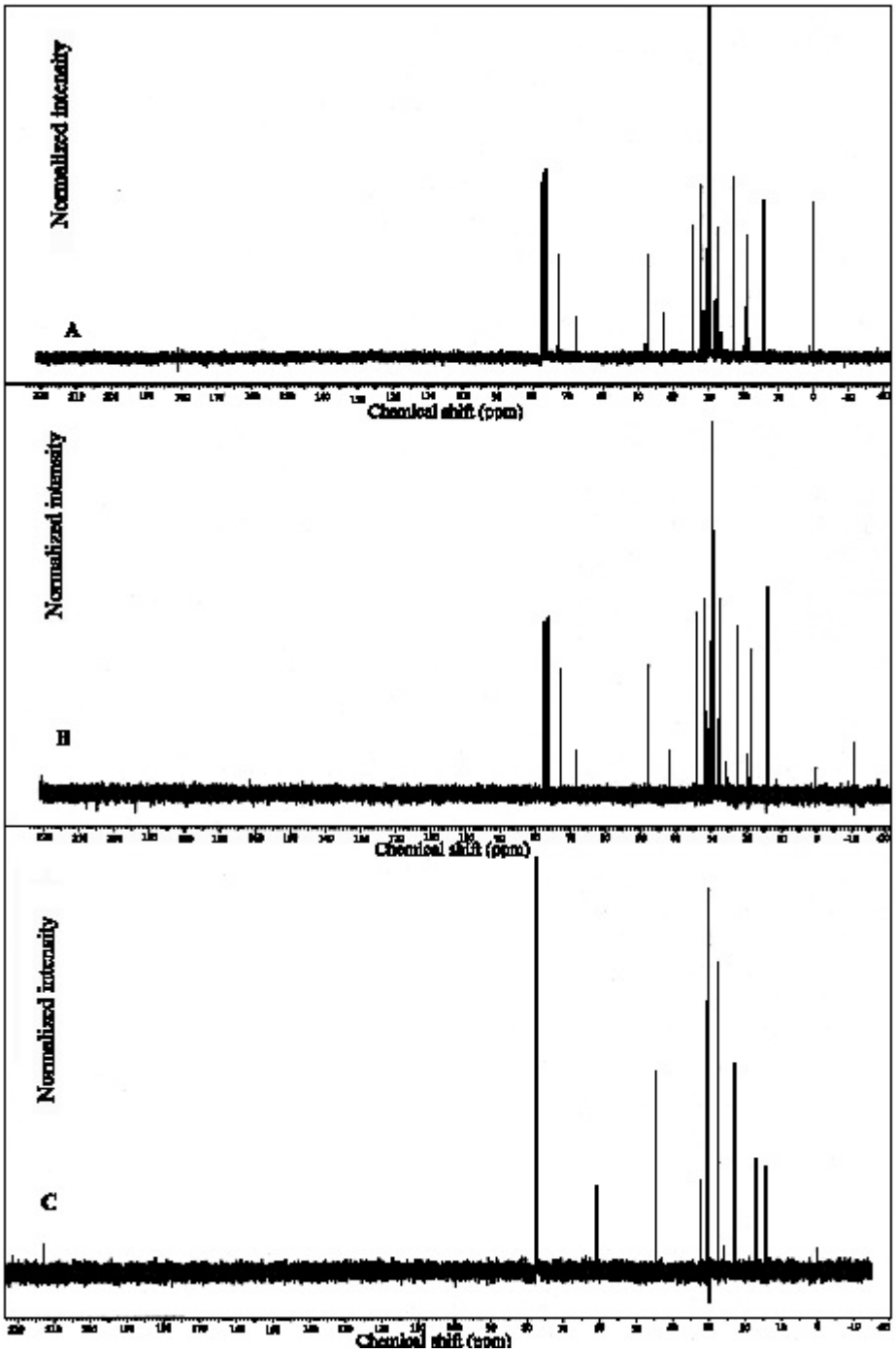

Figure 17- ^{13}C nuclear magnetic resonance spectra of the trimethylsilyl derivative of 2-dodecylcyclobutanol standard (A), 2-dodecylcyclobutanol (B), and 2-dodecylcyclobutanone (C).

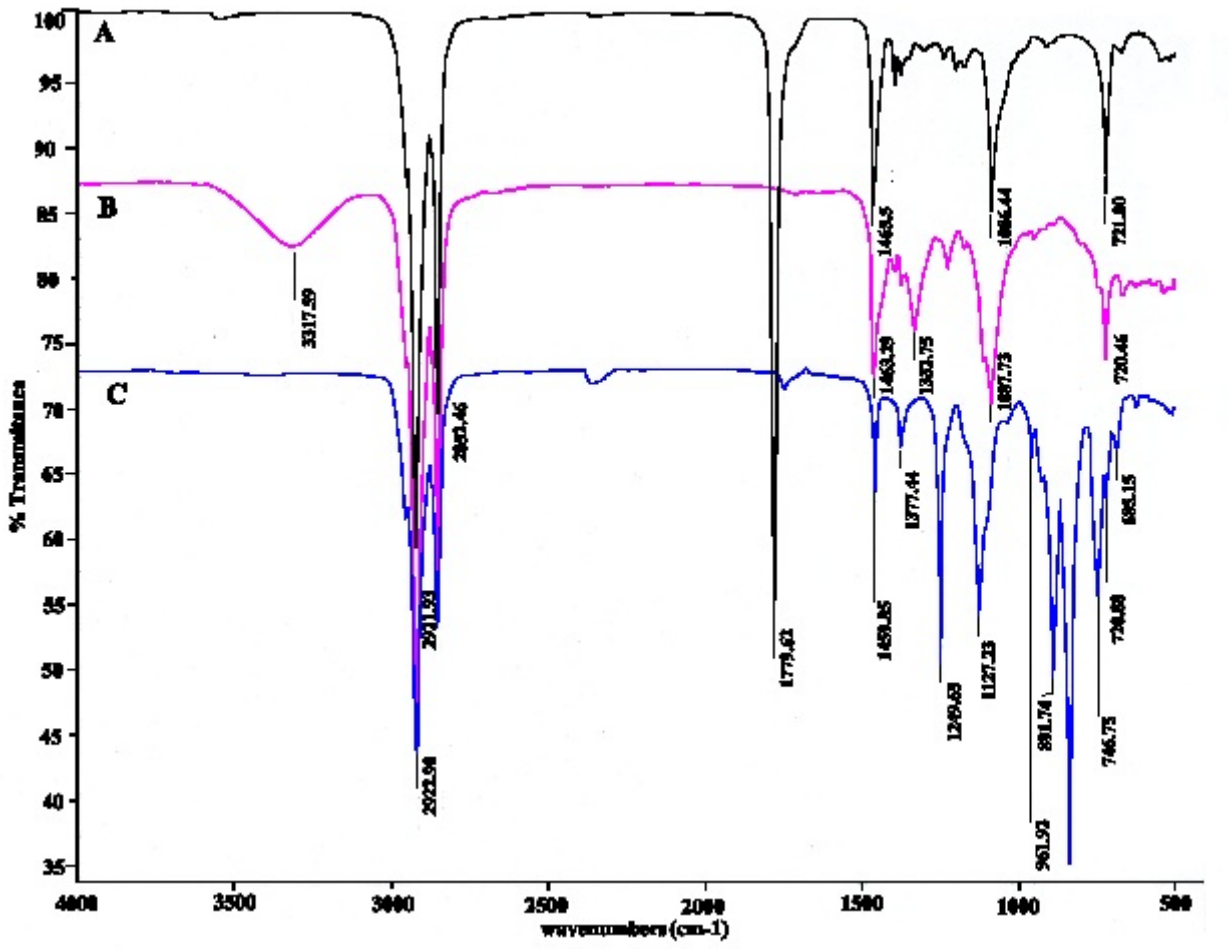

Figure 18- Infrared spectra of 2-dodecylcyclobutanone (A), 2-dodecylcyclobutanol (B), and the trimethylsilyl derivative of 2-dodecylcyclobutanol.

The results of this study showed that 2-DCB was metabolized by the NADP(H)-dependent reductase to 2-dodecylcyclobutanol and excreted in the feces. These results are in agreement with the 2 recent studies conducted on the metabolism of 2-DCB (Horvatovich and others 2002, Gadgil and Smith 2006). Horvatovich and others (2002) showed that amounts of 2-ACBs detected in the adipose tissue of the rats fed 2-DCB were very low (10^{-5} times the total consumed) and that less

than 1% was recovered from fecal material. Their result indicated that 2-ACBs were metabolized, but they could not confirm that because they were not able detect any metabolite. Gadgil and Smith's (2006) results were similar to those of Horvatovich and others (2002). However, the amount of 2-DCB recovered in the rat feces was higher (3 to 11% of the total 2-DCB ingested by these animals), which could be due to the high 2-DCB dose (5 mg/d per rat) that was given to these rats (Horvatovich and others used 1 mg/d per rat). In Gadgil and Smith's (2006) study, no metabolites were identified in the feces extract or in the urine samples derivatized with BFTSA or incubated with β-glucuronidase. However, the hexane extract of rat feces was not derivatized with BFTSA, which explains why the researchers were not able to identify any metabolite. As mentioned before, it is not easy to separate the metabolite (2-dodecycyclobutanol) from the parent compound (2-DCB) because they have a very similar structure and elute about the same retention time. In general, small-molecular-weight (>350 Da) and water-soluble compounds are excreted in the urine (Lehman-McKeeman 2008). Because 2-dodecylcyclobutanol is not water soluble (long-chain alcohol), it is expected to be excreted in feces, not in urine. In our study, no metabolites were detected in the urine extract that was treated with the silylation reagent. This result is in agreement with Gadgil and Smith's (2006) results, in which no metabolites were identified in the feces extract or the urine samples derivatized with BFTSA or incubated with β-glucuronidase.

Our study showed that 2-DCB was metabolized to cyclic alcohol (2-dodecylcyclobutanol), and cyclic alcohols are not known as genotoxins or carcinogens. These results are in agreement with several recent studies conducted on the genotoxicity and mutagenicity of 2-DCB (Sommers 2003, Gadgil and Smith 2004, Sommers and Mackay 2005, Sommers 2006, Hartwig and others 2007). Sommers (2003) found that 2-DCB did not induce mutations in the *Escherichia coli*

tryptophan reverse mutation assay. Gadgil and Smith (2004) found that neither 2-DCB nor its metabolite was mutagenic in the Ames assay using 5 standard *Salmonella* tester strains, indicating that 2-DCB was not biotransformed into a mutagenic metabolite. Sommers and Mackay (2005) concluded that 2-DCB did not increase the expression of DNA damage-inducible genes in *Escherichia coli* or the formation of 5-fluorouracil-resistant mutants in *E. coli*, with and without exogenous metabolic activation. Sommers (2006) tested the ability of 2-DCB to induce the formation of micronuclei in human TK6 lymphoplasts with and without exogenous metabolic activation (EMA). The number of micronuclei per 1000 binucleated cells were induced 2.65-fold without EMA and 2.85-fold in the presence of EMA when exposed to 53 μM 2-DCB. Sommers (2006) concluded that 2-DCB, like palmitic acid, may be weakly clastogenic. Hartwig and others 2007 reported that none of the 2-ACBs they tested reverted the histidine synthesis mutations in either test strain, both in the presence and absence of rat liver microsomal fractions.

Results of this study showed that 2-DCB is metabolized to 2-dodecylcyclobutanol and excreted in the feces. Considered with results of previous metabolism and mutagenicity studies of 2-ACBs as well as with long-term feeding studies in multiple animal species that show no adverse health effects associated with consumption of irradiated foods, these results support the position of the Food and Drug Administration and the World Health Organization on the safety of irradiated foods.

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SUMMARY AND CONCLUSIONS

The direct solvent extraction method was able to extract the 2-DCB from irradiated ground beef. There was no significant differences in 2-DCB concentration between ground beef patties extracted using the Soxhlet method and those extracted using the direct solvent extraction. The concentration of 2-DCB in commercially irradiated patties analyzed using the direct solvent extraction method was 0.031 ppm, which is very close to the concentration 2-DCB in ground beef patties irradiated at 1.5 kGy (Table 9, section III). Thus, the direct solvent extraction method can be used to identify irradiated ground beef and estimate the absorbed dose.

There was no significant difference in 2-DCB concentration between gamma-ray- and low-energy X-ray irradiated patties at all targeted doses. Thus, we expect that the chemical reactions produced in foods by low-energy X-ray irradiation are similar to those produced by gamma-ray irradiation. Although the low-energy X-ray irradiation has a lower penetration capacity than gamma-ray irradiation, this technology may be used to irradiate grains, dried vegetables, spices, and other foodstuffs.

Incubation of 2-DCB with rat S9 (post-mitochondrial supernatant fraction) and β-nicotinamide adenine dinucleotide phosphate (NADPH) showed that 2-DCB was metabolized. The average percentage of 2-DCB recovered from the test incubations was 23%, compared with 50% recovered from the controls. The GC-MS chromatograms of the derivatized samples (treated with silylation reagent) showed that 2-DCB was metabolized to 2-dodecylcyclobutanol. The 2-dodecylcyclobutanol was also identified in the rat feces that were given 2-DCB. The result of this study confirmed that 2-DCB was metabolized and excreted in the feces.

Because 2-DCB was found to be non mutagenic, nontoxic, relatively weak clastogenic, and

is metabolized to alcohol and excreted in feces, consumption of irradiated foods containing trace amounts of residual 2-ACBs is safe. This conclusion about the safety of irradiated fat-containing food has also been confirmed by a large number of feeding studies carried out with irradiated foods.