EVALUATION OF TOXICITY, MUTAGENICITY, METABOLISM AND FORMATION OF 2-DODECYLCYCLOBUTANONE IN IRRADIATED GROUND BEEF

by

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AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

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ABSTRACT

The effect of fat level and antioxidant Origanox on the formation of 2-dodecylcylobutanone (2-DCB) was investigated in fresh irradiated ground beef patties. Patties containing 15% and 25 % fat were irradiated by electron beam at 1, 2, 3, and 4.5 kGy. Ground beef patties with 0.08% Origanox were gamma irradiated at 3.0 kGy. Commercially available irradiated ground beef with different fat levels was analyzed in order to estimate dose absorbed by these samples.

The 2-DCB was extracted by Supercritical fluid extraction (SFE) and analyzed by gas chromatography-mass spectrometry (GC-MS). The concentration of 2-DCB increased linearly with dose with no significant difference in 2-DCB concentrations between the two fat levels. The estimated doses applied to the commercial samples ranged between 1.38 kGy and 1.55 kGy. Origanox did not affect the concentration of 2-DCB.

Mutagenicity of 2-DCB was evaluated by the Ames assay using five standard *Salmonella* tester strains with S9 enzyme activation. The Ames assay did not show a mutagenic effect of 2-DCB, including samples incubated with S9. Acute toxicity of 2-DCB was evaluated by the Microtox acute toxicity system and compared with cyclohexanone and 2-nonenal (both GRAS additives). The toxicity of 2-DCB was between that of cyclohexanone and 2-nonenal while the maximum toxic effect elicited by 2-DCB was the least of the three compounds.

Metabolism of 2-DCB was investigated in Female Sprague-Dawley rats. Hexane extracts of feces and fat were analyzed by GC-MS. Urine with and without added β-glucuronidase, was monitored for glucuronide complexes by hexane extraction GC-MS. The total amount of 2-DCB recovered in feces was 1.78 ± 0.63 mg over five days, about 3-11% of the total 2-DCB administered. The total amount recovered in fat was 0.08 ± 0.01 mg which was approximately 0.33% of the total 2-DCB administered. No metabolites were recovered in any of the urine extracts.

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PART I. REVIEW OF LITERATURE

INTRODUCTION

The presence of microbial pathogens in human foods is a serious global problem. Even in highly industrialized and developed countries like the United States, pathogen contaminated foods and the resulting health and economic impacts are significant. According to the Centers for Disease Control and Prevention, in the United States 76 million infections, 325,000 hospitalizations, and approximately 5,000 deaths due to pathogen-contaminated foods are reported annually (CDC 2004). These events carry an estimated annual healthcare cost totaling $7 billion (USDA/ERS 2000). About 70% of the 3.2 million deaths of children under 5 worldwide, are attributed to foodborne diarrheal diseases. In recent years, the U.S. food industry has made great advances toward improving the safety of our nation's food supply. A recent report from the CDC revealed that the incidence of *Escherichia coli* O157:H7 infections are down 36% from 2002 to 2003 (CDC 2004). Despite this, foodborne illnesses constitute an unacceptable health risk that make further improvements in food safety necessary.

Food irradiation is an important tool for increasing food safety and has the potential to dramatically decrease the incidence of foodborne infections. According to the CDC there would be 900,000 fewer cases of foodborne illnesses, and 352 fewer deaths annually if 50% of the meat and poultry in the United States were irradiated (Tauxe 2001).

There is ample data supporting the efficacy and safety of food irradiation. In 1994 the World Health Organization (WHO, 1994) declared that irradiation of food was safe from a toxicological and nutritional standpoint. Since then, many advances in the commercialization of

food irradiation have led to greater international trade of irradiated foods (Stewart, 2001a). As a result, laws and regulations dealing with the use of food irradiation have been implemented in various countries including United States.

Food irradiation is effective against most bacterial pathogens and parasites. In the United States food irradiation is approved for eliminating or sterilizing insects, extending shelf life, controlling pathogens and parasites, and inhibiting the sprouting of vegetables. Foods approved for irradiation in the United States include red meat, poultry, pork, fruits and vegetables, aromatic spices, seeds, herbs and seasonings, enzyme preparations, eggs, wheat, and shell fish. Ready to eat foods are under review for approval. Table 1 shows the various foods that have been approved for irradiation and the years the laws were passed.

In spite of the all the research and advances, consumer acceptance of irradiated food has been slow. Even though it is widely supported by medical, scientific and public health organizations, food irradiation is being underused. In the United States only 10% of spices and less than 0.002% of fruits, vegetables, meats and poultry are irradiated (Osterholm and Norgan 2004). Induced radioactivity, formation of toxic byproducts, and nutrient losses in food due to irradiation, are some of the concerns regarding food irradiation. These issues are discussed in detail later on in this review. It is important address these issues and educate consumers about the benefits of food irradiation so that it can be more widely utilized to ensure food safety.

Table 1: Applications of ionization radiation approved in the U.S. by the FDA (Smith and Pillai 2004, FDA 2005)

Product	Dose (kGy)	Purpose	Date
Wheat, Wheat flour	0.2 - 0.5	Insect disinfestation	1963
White Potatoes	0.05 - 0.15	Sprout inhibition	1964
Animal feed and Pet food	2 - 25	*Salmonella* control	1965
Pork	0.3 - 1.0	*Trichinella spiralis* control	1985
Enzymes	10 max	Microbial control	1986
Fruit	1 max	Disinfestation, Ripening delay	1986
Vegetables, fresh	1 max	Disinfestation	1986
Herbs	30 max	Microbial control	1986
Spices	30max	Microbial control	1986
Vegetable seasonings	30 max	Microbial control	1986
Poultry, fresh or frozen	3 max	Microbial control	1990
Meat, frozen, packaged *	44 max	Sterilization	1995
Meat, uncooked, chilled	4.5 max	Microbial control	1997
Meat, uncooked, frozen	7.0 max	Microbial control	1997
Molluscan shellfish, fresh and frozen	5.5 max	*Vibrio* spp, Microbial control	2005
Ready to eat products, chilled	4.5 max	Pathogen control	1991-Pending
Ready to eat products, frozen	10.0 max	Pathogen control	1991-Pending

* Used solely in the National Aeronautics and Space Administration space flight programs

FOOD IRRADIATION

History of Food Irradiation

The idea that irradiation could be used as a method for food preservation closely followed the discovery of radioactivity by Henri Bequerel in 1895. In 1905 a British patent described using ionizing radiation to destroy bacteria in food (Satin 1996) and in 1916 the USDA proposed using irradiation to eliminate the tobacco beetle (WHO 1994). Later, in 1921, X-rays were used to eliminate the *Trichinosis* parasite in pork. However, at the time a reliable and economic source for irradiation was not readily available and the technology was not commercially viable.

After the second world war spent fuel rods from nuclear reactors became available as experimental ionization sources. The US Atomic Energy Commission installed food irradiators at several universities to initiate research on food irradiation. The US Army commenced research on irradiated foods in 1953 and conducted some of the most extensive studies on the effect of irradiation on foods (WHO 1994). As a result of the research conducted by the Army and other studies by various researchers over the years, food irradiation has become the most studied of all the processes applied to foods. On the basis of decades of research, a FAO/IAEA/WHO joint committee declared that food irradiated up to 10 kGy was safe to consume, paving the way for utilizing food irradiation on a wider scale (WHO 1981).

Types of radiation and sources

Gamma

Gamma rays are photons with no mass, which originate within the nucleus of an atom. The two common radioisotopes approved by the FDA as gamma ray sources in food irradiation are cobalt-60 and cesium-137. Gamma irradiation facilities generally use the radionuclide cobalt-60 as the source of gamma rays because it is easier to obtain, has a lower environmental risk, and emits four times more energy per disintegration, as compared to cesium-137 (Urbain 1986).

Cobalt-60 is obtained by exposing pure, natural cobalt-59 pellets to a neutron source in a nuclear reactor to produce radioactive cobalt-60. The cobalt-60 pellets are encased in double stainless steel cylinders, called pencils. The gamma producing power of each pencil, measured in curies, is different and depends on the age of the pencil. The number of curies in a pencil continually declines as the radioactive cobalt-60 decays. Cobalt-60 has a short half life of 5.3 years which means that 12% of the source has to be replaced annually to maintain the original strength.

Gamma irradiation is the most penetrating of all the radiations. Even when a 1-m wide pallet of products is placed in front of the source rack, some of the gamma rays pass completely through the products (Olson, 1995). As the gamma ray photons penetrate a product, they lose energy. The absorbed dose from the photons is highest at the surface and diminishes exponentially as the rays travel through the product. Thus, the same percentage of photon energy is absorbed for each incremental thickness. The penetration depth of the photon is

determined by the density of the irradiated product. To compensate for the loss of energy of the photons, both sides of a product are irradiated to obtain better results.

Electron beam

Electrons need to have a minimum energy of 5 Mev to be useful for food irradiation, with higher energy electrons penetrating deeper into the product. The penetration power of electrons is much less than gamma radiation and as in the case of gamma irradiation, penetration depth depends on the density of the product. The electrons used for food irradiations are produced by an electron beam accelerator, a machine that generates and accelerates electrons under vacuum. Electron linear accelerators can be designed to produce electron energies sufficient to reach the maximum of 10 Mev allowed for food irradiation.

The major advantages of using electrons is that the source for the electrons is electricity. Thus, there is no radioactivity in the facility and the machine can be transported and installed without a biological shield. The machine can also be turned off when not in use unlike the cobalt-60 source which can never be turned off. With cobalt-60 it is necessary to have a biological shield to protect workers from exposure all the time. There is also a possibility that the cobalt-60 pencil may become defective or some could escape from the pencil. Thus electron beam processing is considered as a safer alternative. Additional advantages of using electrons are: 1) The source does not need to be replenished; 2) They are readily available; 3) They have an established history of use; and 4) they have a high throughput rate (WHO 1994).

The major limitation of electron beam is the lower penetrating power. Accelerated electrons lose energy as they enter the surface of the product (Olson, 1995). As they move

through the product, due their low mass, electrons are slowed down very quickly. This deceleration slightly increases the amount of energy absorbed just under the surface of the product. As the electrons move further into the product their speed and energy diminishes untill all the energy is lost and they can penetrate no further (especially if the product is thick). Therefore, more efficient irradiation can be achieved if the product is irradiated from both sides.

X-rays

Treatment with primary electron beams can provide the highest throughput rates and lowest unit costs. However electrons have limited penetration and are therefore unsuitable for thick products. To overcome the limitations of product thickness, the electrons can be directed at a metal target to produce Bremsstrahlung x-rays. In case of food irradiation tantalum or gold are permitted as X-ray sources (FDA 2004). These x-rays are as penetrating as gamma rays and have the same advantages as linear accelerators discussed previously.

The greatest limitations of using x-rays to irradiate foods are low efficiency and high cost of production. The overall efficiency of x-rays is about 4-8% compared with 10-25% for gamma rays and 40-80% (depending on product thickness) for electron beams. In December of 2004 the FDA increased the maximum permitted energy level of x-rays for treating food to 7.5 MeV from the previously permitted maximum level of 5 MeV (FDA 2004). Increasing the maximum permitted energy level of x-rays will result in higher efficiency and better penetration of these x-rays in food. This increased emission efficiency reduces treatment time for food resulting in higher production rates and lower treatment costs (FDA 2004). Currently the

efficiency is still not high enough for x-rays to be competitive as a viable alternative to gamma-ray or electron beam processing.

Radiation dose

The amount of irradiation energy that a food or biological system absorbs is measured in units called Grays (Gy). The unit refers to the amount of energy that 1 Kg of product receives from the ionizing radiation. One Gy is equivalent to one joule per Kg. The Gray replaced the earlier SI unit, the rad (1 Gray = 100 rad) in 1986.

The practical working range for food irradiation is generally from 0.05 kGy to 10 kGy (WHO 1994). There are three general applications and dose categories that are referred to when foods are irradiated as summarized in Table 2. Treatments aimed at inactivation of microorganisms fall under the following three categories depending on the desired function of the treatment.

Radurization - Irradiation of food with a dose sufficient to prevent spoilage by reducing a substantial number of spoilage organisms. The required dose range is 0.4-10 kGy.

Radicidation - Treatment of foods with a dose sufficient to reduce the level of non spore forming pathogens and parasites to an undetectable level. The required dose range is 2-8 kGy. Treatment of foods to eliminate pathogens is also referred to as cold pasteurization or electronic pasteurization.

Radapperization - Irradiation of food with ionizing radiation sufficient to sterilize it. Dose range for this process is 25-45 kGy.

Table 2: General applications and dose categories for irradiated foods. (Adapted from WHO 1994)

Function	Dose (kGy)	Products irradiated
Low-dose (up to 1 kGy)		
Inhibition of sprouting	0.05-0.15	Potatoes, onions, garlic, ginger
Insect and parasite control	0.15-0.5	Cereals, pulses, fresh and dry fruits, dried meat and fish, fresh pork
Delay of ripening	0.5-1.0	Fresh fruits and vegetables
Medium dose (1-10 kGy)		
Extension of shelf life	1.0-3.0	Fresh fish, strawberries
Pathogen and microbial control	1.0-7.0	Fresh and frozen seafood, raw and frozen poultry and meat
Improved food properties	2.0-7.0	Grapes (increased juice yield), dehydrated vegetables (reduced cooking times)
High dose (10-50 kGy)		
Sterilization	30-50	Meat, poultry, seafood, prepared foods, sterilized hospital diet
Decontamination of food additives and ingredients	10-50	Spices, enzyme preparations, natural gum, etc.

Science of food irradiation

Food irradiation is exposing food to ionizing energy, a process that results in the production of free radicals and ions. These ions and free radicals interact with the constituents of

the food and microorganisms. The interaction of ionizing radiation with food, or any matter is a complex process. The primary interaction that takes place is the transfer of energy from the incident radiation to the absorber, which in this case is food. Only the absorbed energy is effective in producing any changes. This primary process is followed by secondary effects that causes chemical and biochemical changes, leading to radiolytic products and microbial destruction.

Primary process

Atoms consist of a central nucleus with electrons orbiting around it. When a photon of high energy (gamma or X-ray) comes close to an atom, a part of the photon's energy can be transferred to an orbiting electron. The electron is thus excited and now has enough energy to leave its orbit around the nucleus, leaving behind a positively charged ion. A photon may transfer as much as half of its energy to the electron, and this ejected electron is known as a secondary electron. As a result of this energy transfer, the photon is deflected to a different path where it may again encounter and excite another electron. A number of such energy transfer events take place before the gamma ray exits the product, or its energy falls below what is needed for electron excitation. This is the most important mechanism for energy transfer in food irradiation and is called Compton Scattering or Compton effect (Stewart 2001b).

The ejected secondary electrons themselves interact with the molecules in the product by transferring part of their energy, creating more ions and excited electrons (Newton 1963, Sharpe, 1990). Consequently, each Compton event is accompanied by a large number of ionizations and excitations produced by energetic electrons. It has been estimated that one electron may produce

30,000-40,000 additional ionization processes and 45,000-80,000 excitations (Nawar 1986). Two other processes by which a photon can transfer energy are the photoelectric effect and pair production, but these are important only at energies lower and higher than those used for food irradiation

Secondary processes

At the beginning of the chemical changes, atoms have either had orbital electrons moved to higher energy states, or they have lost orbital electrons. Atoms which have gained energy without losing electrons are termed excited, and atoms that have lost electrons are ions. Excited molecules can lose energy by a number of pathways, including emission of energy as a photon, internal conversion to heat, transfer to a neighboring molecule or via a number of chemical reactions. Some of these reaction give rise to entities known as free radicals (an atom or molecule which has one or more unpaired electrons that are available to form a chemical bond). Both ions and free radicals are chemically very reactive and seek pathways to make them stable. They may capture an electron that has a normal energy or they may interact with an electron from a normal molecule causing a covalent bond to break. The chemical reactions of ions and free radicals with food components give rise to degradation products that are called radiolytic products (Stewart 2001b).

Radiolysis of water

Ionizing radiation can induce changes in foods by direct or indirect action. Direct action refers to the damage caused by the ionized particle itself to food components or microbial cells.

In indirect action the products of water radiolysis cause most of the changes of various food components. As water is a significant component in most foods, the radiolysis of water is of a particular importance. Figure 1 demonstrates ion and free radical formation when water is subjected to irradiation. These entities then attack the molecules of various food components leading to more ions and free radicals and eventually radiolytic breakdown products and microbial destruction.

Water can produce a solvated electron or can be excited and produce the hydroxyl ($^{\bullet}OH$) and the hydrogen ($H^{\bullet}$) free radical. The food components that may be attacked by the radiolytic products of water are represented by R and RH.

$H_2O \rightarrow H_2O^+ + e^-_{aq}$ (hydration)

or $H_2O^* \rightarrow {}^{\bullet}H + {}^{\bullet}OH$ (excitation* and dissociation)

$e^-_{aq} + R \rightarrow R^-$

$e^-_{aq} + RH \rightarrow {}^{\bullet}H + R^-$

$R^- + OH \rightarrow {}^{-}OH + R$

Figure 1: Radiolysis of water (modified from Stewart, 2001b)

Ions and free radicals can disperse rapidly in food products if water is present in a liquid form. In a dry or frozen product, the dispersion of the ions and free radicals is restricted, and

consequently the opportunity for reaction is limited. Some of the ejected electrons may be recaptured by the molecule as the electrons cannot disperse easily. Therefore, dry or frozen products require higher doses than moist products to obtain the same microbial destruction.

A quantitative basis for the chemical changes induced by radiation is provided by the G value. The G value is the number of absorber molecules changed or of new substances formed per 100 eV of energy deposited. Except for chain reactions, G values seldom exceed 10 (Urbain, 1986).

Effect on microbial cells

The purpose of irradiating foods, in most cases, is to destroy spoilage and pathogenic bacteria. Sometimes the objective of irradiation is control of normal deteriorative processes such as senescence of raw fruits or vegetables, but microbial destruction is by far the most important reason for food irradiation. When ionizing radiation acts on a biological system it does so at the cellular level, most often causing damage to the genetic material.

The genetic material deoxyribonucleic acid (DNA) is a very large molecule compared to proteins or enzymes that are present in the cell. The size and complexity of the DNA molecule make it especially vulnerable to change by radiation. Damage to the DNA occurs as a result of a direct collision between the radiation energy and DNA, or as a result of the ionization of an adjacent molecule, which in turn reacts with the DNA. The adjacent molecule is water in most cases (Grezc et al. 1983). A photon or electron strikes the DNA and causes a break in either a single strand, or if the orientation is correct, in both strands of the DNA.

Damage to the DNA is the principal mechanism that causes destruction of

microorganisms. About 90% of the damage is caused by ˙OH radicals from the hydration layer around the DNA molecule. Usually the purine and pyrimidine bases are chemically changed and the phosphodiester backbone is broken (Moseley 1989). Breaks in the DNA adversely affect various cellular processes like cell reproduction, protein production and cell metabolism. Even small changes in genetic materials can drastically affect cellular functions. Hence, a relatively small amount of ionizing radiation can have a tremendous effect on bacteria with only a small effect on food components.

Not all microorganisms are destroyed at the same dose, due to differences in their ability to repair their genetic material. In general, the sensitivity of a microorganism to irradiation is based on how efficiently it repairs DNA, and organisms that have a more efficient repair mechanism are more resistant to irradiation (Stewart 2001b). Other factors affecting sensitivity are size of the organism (the smaller the organism the more resistant), type of organism (cell wall characteristics, gram positive or gram negative), number and relative age of the cells and presence or absence of oxygen (Smith and Pillai 2005).

D_{10} values

The relative sensitivity of different microorganisms to ionizing radiation is based on their respective D_{10} values (which is the dose required to reduce the population by 90%). Lower D_{10} values indicate greater sensitivity of the organism in question. The physical and chemical composition of the food also affects microbial responses to irradiation. For example, as the temperature of ground turkey is decreased from 30 °C to –30 °C (Table 3), the D_{10} value for *Campylobacter jejuni* increases from 0.16 kGy to 0.29 kGy. The rate of migration of the

ionization products, including free radicals, decreases as the water in the product freezes, thereby requiring greater energy input to cause the collisions necessary to destroy the microbes (Thayer 2004).

Table 3: D_{10} Values of some common pathogens in meat and poultry products (Adapted from Dickson 2001, Smith and Pillai 2004)

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. meat	5	Turkey breast	0.71
Listeria monocytogenes	5	Beef	0.45
Escherichia Coli O157:H7	2-5	Ground beef	0.241
	-17		0.307

LIMITS OF FOOD IRRADIATION

Food irradiation cannot eliminate all foodborne contaminants. Bacterial spores and viruses are more resistant to irradiation than vegetative cells and require higher doses than those used in practice (Farkas 1994a). This is an important point for foods that will not be cooked or otherwise processed before consumption. Irradiation cannot prevent subsequent cross contamination after being processed by food service workers or consumers. Irradiation also does not destroy microbial toxins or prions, but neither does cooking (Osterholm and Nagan 2004).

Treatment with food irradiation is not meant to eliminate all bacteria in food, but rather to eliminate pathogenic organisms. The surviving non pathogenic bacteria can still spoil the irradiated foods, albeit more slowly. Irradiation can be looked upon as electronic pasteurization, which does not eliminate the need for safe handling and cooking, but reduces the dangers of primary contamination.

CONCERNS REGARDING FOOD IRRADIATION

Misuse to Avoid Plant Sanitation

A common concern stated by those opposed to food irradiation is that it would be used as an alternative to proper food-processing plant sanitation and cleanliness practices. A similar argument was used to dissuade implementation of milk pasteurization in the early 1900s. Today, milk pasteurization is a commonly used practice proven to have prevented countless cases of

milk-borne salmonellosis (Satin, 1996). Heavily contaminated food would require higher doses that decrease the acceptability of the product. Using food irradiation to overcome or conceal improper sanitation practices, would be counter productive and serve as a death knell to this food processing technology. Food irradiation is intended as the final step of a comprehensive HACCP program (Smith and Pillai 2005).

Background and induced radioactivity

The fear that irradiated foods will become radioactive is a major misconception amongst consumers. Most are unaware that people are exposed to background radiation from outer space (cosmic rays), the earth itself (terrestrial gamma rays) and natural radioactive elements in the environment. Foods contain low levels of background radioactivity due to natural radioactive compounds such as potassium-40. Potassium-40 produces high energy beta particles and gamma rays by an estimated 15 million disintegration/hour inside each of us (Stewart 2001b). Background radiation is estimated to cause between 0.3-1% of all cancers in humans (CAST 1986).

Foods contain elements with mostly low atomic numbers such as hydrogen (H), carbon (C), oxygen (O) and nitrogen (N). The principal reaction of radiation with atomic nuclei are those in which a neutron or proton is ejected. The energy of the incident radiation has to be of a certain minimum value in order to induce a radioactive change. The only nuclei that occur in foods with threshold values for nuclear reactions below 5 Mev are ^{2}H, ^{13}C and ^{17}O. As these nuclei are present in very minute quantities in foods and do not form unstable radioactive products, the chance of induced radioactivity is very remote (Urbain 1986). Electron beams can

produce Bremsstrahlung X-rays which can possibly induce radioactivity. However, the electrons have to possess a high enough energy before radioactivity is induced. The energy threshold for inducing radioactivity in food due to electrons through Bremsstrahlung is 10.5 Mev. The established limits of 5 Mev for gamma sources, 10 Mev for electron beams and 7.5 MeV for x-rays ensures that no radioactivity is formed in the food. In fact, it is estimated that not even one excitation of any nuclide took place in 1 Kg of meat irradiated sterilized with electron radiation (Urbain 1986). The FDA reported in its final rule of April 18, 1986 that there was no evidence to support the induction of radioactivity in foods (FDA 1986). Terry and McColl (1992) found that the induced activities in a wide range of foods less than 24 hours after irradiation were too low to elicit any concern. Therefore, it can be concluded that irradiation in the commercially useful range will not generate measurable radioactivity in foods.

Environmental concerns

There are lingering concerns among opponents to food irradiation regarding the environmental safety of irradiation facilities. Issues surrounding use, safety, and exposure to radioactive materials are often promoted as a concern relative to food irradiation, while similar concerns have not been major issues pertaining to the use of irradiation to sterilize medical equipment and other healthcare products (Derr 1993). Irradiation facilities are regulated depending on the source used. Gamma facilities have specific characteristics to protect workers (regulated by the Occupational Safety and Health Administration) and the surrounding environment from the radioactive isotopes. The isotope material is stored under water when not in use, as regulated by the U.S. Nuclear Regulatory Commission. Cobalt-60 requires 16–21 years

to decay to approximately 6–12% of its initial activity level, after which it is shipped back to the manufacturer in hardened steel shipping canisters to be regenerated and reused (Smith and Pillai 2005). Electron beam and X-ray do not employ radioactive sources, but contain a considerable amount of electrical circuitry, cooling systems, worker safety systems, and ozone attenuation capabilities (Olson, 1998). Production of ground-level ozone from E-beam facilities has been cited as a concern. Ozone is produced when the accelerated electrons come into contact with air and is routinely exhausted when interior levels reach maximum continuous exposure levels. The National Ambient Air Quality Standards have set the upper emission limit at 0.12 ppm/hr and irradiation facilities are not permitted to operate if ozone emissions exceed this limit (Smith and Pillai 2005).

Loss of Vitamins and Nutrients

Vitamins are present in relatively minor quantities in foods, but are nutritionally very important. Therefore, the fate of vitamins when foods are irradiated is of primary concern. Most of the information available on the effect of radiation on vitamins relates to how well they survive the process. There is not a great deal of information available on the identity of the resultant radiolytic products, because of the small amounts of vitamins present in foods. Studies of model systems can overcome this difficulty, but the effects observed in such systems may not convey what happens in a complex food system. In all but a few cases, vitamins survive irradiation quite well and therefore the lack of studies on radiolytic products is not considered significant (Urbain 1986).

Vitamin C is easily destroyed in sample aqueous solutions by irradiation. Dehydroascorbic acid, diketogluconic acid and other acids and compounds have been identified as radiolytic products. In contrast, only small losses of ascorbic acid have been observed in fruits and vegetables treated with doses up to 5 kGy (Urbain 1986).

Among the B vitamins, thiamine (B1) is the most radiation sensitive. This lability may be due to the presence of the C=N bonds in the molecule. Riboflavin also contains a number of C=N bonds and undergoes radiolysis in simple aqueous solutions. In food systems, however, the vitamin is reasonably resistant to ionizing radiation. This resistance is thought to be due its ability to bind proteins. Niacin (B5), pyridoxine (B6), and cobalamine (B12) undergo degradation when irradiated in aqueous solution, but are only moderately affected by irradiation (Urbain 1986).

Vitamin A and the provitamin carotenoids in the dry state are relatively radiation stable up to doses of 20 kGy (Lukton and MacKinney 1956). In foods, the losses of vitamin and carotenoids occur to varying degrees. In fats and milk the losses are high, while in fruits and vegetables the losses are negligible.

Vitamin D exists in two forms, D2 (calciferol) and D3 (cholecalciferol). Information on the radiation sensitivity of this compound is limited. Studies of D3 in isooctane indicate that it is not especially radiation sensitive (Knapp and Tapell 1961a). Irradiation of foods generally produces little loss of vitamin D. As with vitamin D, little information is available about the effects of radiation on vitamin K. Of the various forms, vitamin K3 is the most sensitive (Metta et al. 1959).

Vitamin E is the most radiation sensitive of the fat soluble vitamins (Knapp and Tapell 1961b). This effect is most pronounced in the presence of air and is not surprising as tocopherols are known as effective antioxidants.

Changes to basic food components

Amino acids and proteins

Simple amino acids in the absence of oxygen yield ammonia, keto acids, fatty acids and small amounts of hydrogen, carbon dioxide and amines. Sulfur containing amino acids also yield hydrogen sulfide. The sulfur containing, and aromatic amino acids are most sensitive to irradiation as they act as scavengers and react more readily with free radicals (Liebster and Kopoldova 1964, Simic 1983). The aromatic acids primarily undergo hydroxylation of the aromatic ring. In general, amino acids are relatively stable to irradiation.

The radiation chemistry of proteins is complicated and cannot be predicted by the behavior of free amino acids. The complex structure of proteins influences the reactions that occur because of irradiation. Because of the secondary and tertiary structure, some groups which would have interacted with radiation as free amino acids may be protected from free radicals. As a result, reactions that occur with free amino acids may not take place when the same amino acids are the components of proteins. Absorption of ionizing radiation can lead to multiple ionizations and excitations within the protein molecule. The absorbed energy can be transferred from an initial site to another which may be more susceptible to bond breakage. Because of the complexity and size of the proteins a large number of sites are available for interaction with free

radicals. Therefore, the end products are diverse and the amounts formed, though dose dependent, are not likely to be large (Stewart 2001b).

Irradiation can denature native proteins mainly through breaking of hydrogen bonds and other linkages involved in secondary and tertiary structures. Breakage of peptide bonds can lead to the formation of smaller, lower molecular weight fragments. Sometimes cross-linking may result because of hydrogen bonding, leading to aggregation of the molecules. These kinds of changes result in altered normal properties of the protein. Enzymes are deactivated, chromoproteins undergo a change in color, and functional properties are altered. The type of reaction that predominates depends on the structure of the proteins. Globular proteins, which have a tighter structure, favor recombination reactions and are more resistant to change. Fibrous proteins like collagen are more susceptible to change (Delincee 1983). Generally due to the low doses used in food irradiation and the presence of other food components, denaturation, and not degradation of the protein is more likely.

The effect of radiation on proteins does not necessarily constitute a difficulty when considering food irradiation. It has been shown that protein quality is not significantly affected by doses up to 50 kGy (Eggum 1979). Amino acids, which are important from a nutritional standpoint, are protected within the protein complex and survive irradiation very well (Stewart 2001b). Enzymes and chromoproteins may need special considerations, and conventional uses of some proteins may be affected by structural changes that occur. But in general, at the doses used in food irradiation, radiation induced changes are minor and of no significance for the ordinary food uses.

Carbohydrates

Physical and chemical changes that occur on irradiation of low molecular weight carbohydrates, include a decrease in melting point, a decrease in optical rotation, changes in the absorption spectra and browning. Irradiation yields various gases such as hydrogen, carbon dioxide, methane and carbon monoxide, whose proportions are generally dependent on the type of sugar and absorbed dose. Pure carbohydrates in the crystalline states are especially radiation sensitive. Some reactions have a very high G value, which is attributed to chain reactions (Urbain 1986).

Low molecular weight sugars in aqueous solutions undergo oxidative degradation, partly due to direct action of radiation and partly due to interaction with hydroxy (˙OH) radicals (radiation products of water). The radiation chemistry of carbohydrates is complex, with many radiolytic products being possible. Thus, formation of large quantities of a few products is not commonplace.

The most important change in polysaccharides caused by irradiation is breaking of the glycosidic bonds. In starches, pectins and cellulose this results in the formation of smaller carbohydrate units. As a result, some foods like fruits may undergo softening and loss of texture when irradiated (Urbain 1986).

In the case of starches, irradiation leads to the formation of dextrins with varying lengths, resulting in reduction of viscosity. In addition to dextrins, lower molecular weight sugars such as glucose, maltose, erythrose, ribose and mannose are formed (Dauphin and Saint-Lebe 1983, Sokhey and Hannah 1993, Simic 1983). Further decomposition leads to products such as formic acid, acetaldehyde, methanol, acetone, ethanol and methyl formate. Furan is formed from some

sugars and has received attention recently due to its carcinogenic nature. It is discussed later in this review in greater detail. According to Anathswamy et al. (1970), the structure of irradiated starch undergoes change in sensitivity to action of the α- and β- amylases, leading to an increase in the maltose production compared to non irradiated starch. In general the end products obtained from irradiation of starch are the same regardless of the source (Raffi et al. 1981). Information for a particular starch may be used to predict the changes that occur in other starches.

The presence of amino acids and proteins have been shown to have protective effects on the radiolysis of carbohydrates. Proteins from wheat flour decreased the formation of radiolytic products from carbohydrates (Diehl et al. 1978) and the addition of cysteine or methionine to a glucose solution reduced the formation of carbonyl compounds. The protective action of amino acids and proteins is attributed to interference with the availability of the hydroxy (˙OH) radical for interaction with the sugar. The protective action is only quantitative and not qualitative as the same radiolytic products are formed in food products as in pure sugars, but in lesser amounts.

Lipids

Radiolysis of lipids is of interest in terms of nutritional, toxicological, functional and sensory characteristics. Ionizing radiation affects lipids in two ways; by catalyzing auto-oxidation, and by the direct or indirect action of high energy radiation on lipid molecules. Principal end-products of fatty acids are carbon dioxide, carbon monoxide, water, and various hydrocarbons (alkanes and aldehydes). Alkylcyclobutanones, the only cyclic compounds

isolated from irradiated foods are important by-products of lipid radiolysis and are discussed as a separate section.

In food systems, radiolysis of triglycerides is of main interest. In general, the amounts of end-products formed due to triglycerides are less than those formed from free fatty acids (Nawar 1977). In triglycerides, cleavage usually occurs in the vicinity of the carbonyl group but can sometimes occur at the other sites. The free radicals formed as a result of bond cleavage around the carbonyl group give rise to a number of stable end-products. The pathways that lead to these end-products are abstraction, dissociation, recombination, disproportionation and radical-molecule interaction.

Figure 2 shows the structure of a typical triglyceride molecule. Cleavage at various sites will give rise to different byproducts. The figure shows the sites that are cleaved leading to the formation of hydrocarbons and alkylcyclobutanones. Cleavage between carbons 1 and 2 (site c) of a fatty acid results in a free radical that can either accept or lose a hydrogen atom**.** The former gives rise to a Cn-1 alkane (alkane with 1 carbon less than the fatty acid) and the latter yields an 1-alkene. The alkane is produced in greater quantity and can be formed by hydrogen atom abstraction or by the fission of the C-H bond at various places on the hydrocarbon chain. If the fatty acid is cleaved at site d, the resulting free radical will decompose to yield a Cn-2 alkane and an 1-alkene. In this case the alkene is formed in greater amounts. The resulting unsaturated hydrocarbons can form dimers and polymers (Urbain 1986). The free radicals may form hydroperoxides in the presence of oxygen, finally yielding end products like aldehydes. Cleavage at site b gives rise to 2-alkylcyclobutanones.

The general pattern of radiolytic changes formed from saturated and unsaturated fatty acids is the same. Saturated acids give alkanes and 1-alkenes, monounsaturated acids give alkenes and alkadienes, while diunsaturated acids give rise to alkadienes and alkatrienes. Some hydrogenation of the unsaturated fatty acids occurs, giving rise to a small amount of saturated fatty acid (Stewart 2001b). Possible radiolytic products of triglycerides are listed in table 4.

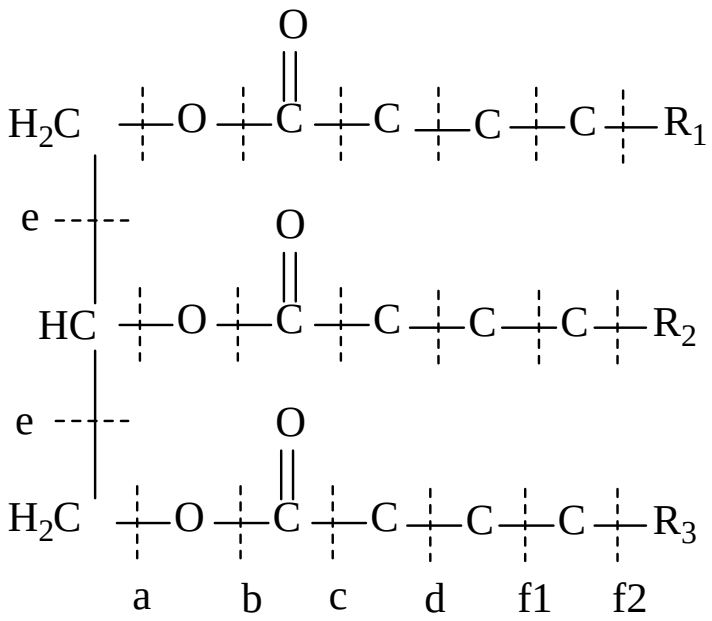


Figure 2. A triglyceride molecule showing the cleavage sites (broken lines) leading to the formation of hydrocarbons and alkylcyclobutanones (Stewart 2001b)

Table 4: Possible radiolytic products of triglycerides (Stewart 2001b).

x = any carbon number from 3 to –1

Site of Cleavage	**Primary products**	**Recombination Products**
a	Cn Fatty acid	Cn fatty acid esters
	Propanediol esters	alkanediol diesters, 2-alkyl-1,3-propanediol diesters, butanetriol triester
b	Propanediol diesters	
	Cn aldehyde	ketones, diketones, oxoalkyl esters
	Diglycerides	
	Oxopropanediol diesters	glyceryl ether diesters
	2-alkylcyclobutanones	
c	Cn-1 alkane	longer hydrocarbons
	Cn-1 alkene	
	Formyl diglycerides	triglycerides with shorter fatty acid
d	Cn-2 alkane	
	Cn-2 1 alkene	hydrocarbons
	Acetyl diglycerides	triglycerides with shorter or longer fatty acids
e	Cn fatty acid methyl ester	Cn fatty acid esters
	Ethanediol diesters	alkanediol diesters
f	C1-x hydrocarbons	hydrocarbons
	Triglycerides with shorter fatty acids	triglycerides with longer fatty acids

Irradiation promotes autoxidation if oxygen is present during, or after irradiation. Thus it is very important to minimize exposure to oxygen before, during or after irradiation. Exposure to ionizing radiation does not make a difference in the end products formed due to autoxidation (Vajdi et al. 1982). Protein or protein-carbohydrate interactions may protect lipids against oxidative changes, an effect that increases with irradiation dose (Green and Watts 1966).

Lipids contain other components like phospholipids, waxes, sterols, and pigments, besides triglycerides. There has been no significant research on the radiolysis of these components. Studies on phospholipids have shown that phosphatidic acid, lysophospholipids, fatty acids, phosphorylbases such as phosphocholine and volatile hydrocarbons are produced as by-products of irradiation (Stewart 2001b).

In general irradiation of fats does not pose significant problems. Physical properties like melting point or viscosity and chemical properties like peroxide value and iodine number, are not significantly altered (Urbain 1986).

Sensory changes

Foods such as milk, certain cheeses, eggs, and some fruits and vegetables are not likely candidates for irradiation because of the potential for undesirable off odors, flavors, and texture changes (WHO 1999). The bulk of sensory work has focused on muscle foods, because most of the emphasis for this technology has been on these foods (Molins 2001). Two studies have evaluated ground beef under various conditions of radiation dose (0–4.5 kGy), temperature (–25°C to room temperature), and packaging. The results have shown that irradiation causes no

significant differences in the flavor, texture, or color of beef irradiated at less than 3 kGy (Murano et al. 1998, Vickers and Wang 2002).

In a study using a trained panel and consumers, no significant differences were found in acceptance, meatiness, freshness, or juiciness in fresh or frozen irradiated boneless pork chops. The products were irradiated at 2.5 kGy or below (Luchsinger et al. 1996). However, it is known that poultry and pork are sensitive to flavor and color (pinking) changes (Houser et al. 2003, Nam and Ahn 2002). In order to address this issue, process techniques like packaging and antioxidants, that improve these meat characteristics have been evaluated (Bagorogoza and Bowers 2001, Nam et al. 2004). In one instance, consumers even preferred the pink color of irradiated turkey meat (Lee et al. 2003).

There are fewer studies with fruits and vegetables but the use of low-dose irradiation to offset quarantine (due to pest infestation) and/or for extension of shelf life is promising. Tropical fruits like papayas, rambutans, and Kau oranges were acceptable when treated with a quarantine level of 0.75 kGy. Chompoo and Biew Kiew fruit were more acceptable when treated with 0.40 kGy than with the currently used hot-water immersion (Follet and Sanxter 2002). Due to restrictions on chemical treatments like methyl bromide and the increasing demand of imported products, application of low dose irradiation has become an active area of research.

Radiolytic compounds

Ionizing radiation can cause changes to food components as noted earlier. Most of these changes are not unique to irradiation and are seen with other types of food processing techniques. Of the many radiolytic products, most attention has been paid to the production of

benzene and 2-alkylcyclobutanones (2-ACBs). Other compounds of interest are furans and trans fatty acids. The following paragraphs discussed each of these compounds. 2-ACBs which are the main focus of this dissertation are discussed as a separate section.

Benzene

The presence of several compounds, most notably benzene and toluene, have generated some concerns about the safety of irradiated foods. Originally it was thought that benzene, formed in trace quantities, was a unique radiolytic product. However, it was found that many foods contained trace amounts of benzene, some originating from decomposition of the preservative potassium benzoate (Zhu et al. 2004) and from cooking processes.

It is currently thought that oxidative/radiolytic cleavage of phenylalanine produces benzene and toluene. They have been reported in irradiated beef (Merrit et al. 1978) and poultry (Nam et al. 2003) in the 5-60 ppb range. Benzene and its derivatives are not typical components in raw food products, but thermal treatments appear to produce trace amounts in some cooked products. Benzene derivatives have been reported in scrambled eggs (Matiella and Hsieh 1991), and benzene was produced in butter, eggs, meat, and certain fruits with levels ranging from 0.5 ppb in butter to 500 -1900 ppb in eggs (McNeal et al. 1993). Angeliniey et al. (1975) evaluated volatile compounds in fresh and irradiated haddock and found benzene and toluene in all samples with larger quantities present in the irradiated ones.

Small amounts of benzene were noted in irradiated (56 kGy) and untreated beef (Chinn, 1979). Gamma and electron-irradiated beef contained about 18-19 ppm, which was reduced to 15 ppm upon cooking. Benzene was not detected in the thermally-sterilized, and frozen controls,

but on cooking the levels were approximately 2-3 ppm. Health Canada (Bureau of Chemical Safety, 2002) has estimated that approximately 3 ppb of benzene would be formed in irradiated beef at the typical dose ranges (1.5-4.5 kGy) and concluded that it is of insignificant health risk.

Furan

In a recent survey conducted by the FDA furan was detected in a large number of thermally processed foods, with levels up to 100 ppb in some foods (FDA 2004). Furan is a colorless volatile compound (Maga 1979), which is classified as "reasonably anticipated to be a human pathogen" by the U.S. National Toxicology Program (NTP 2004) and as "possibly carcinogenic to humans" by the International Agency for Research on Cancer (IARC 1995).

Furan has been found in irradiated orange and apple juices (Fan 2005a) at part per billion levels. Irradiation and thermal treatments induced formation of furan from ascorbic acid, fructose, sucrose, and glucose, all major components of fruit juices (Fan 2005b). The amounts of furan formed due to thermal treatments (sterilization) and irradiation were similar.

The FDA issued a call for information on the effects of furan and is currently evaluating the presence of furan in thermally processed and irradiated foods. The presence of furan in dozens of thermally processed foods, including baby foods, indicates that it is a common component of processed foods and irradiation may not pose an increased concern.

Trans fatty acids

Trans fat (also called trans fatty acids) is formed when liquid vegetable oils go through a chemical process called hydrogenation, in which hydrogen is added to make the oils more solid. Hydrogenated vegetable fats are used by food processors because they allow longer shelf-life and give food desirable taste, shape, and texture.

The majority of trans fat can be found in shortenings, stick (or hard) margarine, cookies, crackers, snack foods, fried foods (including fried fast food), doughnuts, pastries, baked goods, and other processed foods made with or fried in partially hydrogenated oils. Some trans fat is found naturally in small amounts in various meat and dairy products. The FDA estimates that the average daily intake of trans fat in the U.S. population is between 5.5 g/day -7.6g/day grams for individuals 20 years of age and older (FDA 1999).

Evidence suggests that consumption of trans fat raises the low density lipoprotein levels and lowers high density lipoprotein levels, enhancing the development of arterial plaques and increasing the risk of heart disease and stroke (FDA 1999). Thus the formation of trans fatty acids due to irradiation would be an undesirable result.

A review of available literature included one article that evaluated the formation of trans fatty acids in irradiated ground beef. The authors irradiated ground beef at various doses and compared the amounts of trans fatty acids formed with controls. They found that at 1 kGy there was a 3.4% increase in trans fatty acids while at 8 kGy there was a 6.4 % increase (Brito et al. 2002). The Center for Science in the Public Interest submitted this data to the FDA to oppose petitions seeking approval to irradiate shellfish and ready to eat foods (FDA 2003).

The natural background amount of trans fat in beef ranges from 3 to 10% and research performed by the FDA shows no change in the amount of trans fatty acids present when ground beef is irradiated at 25 °C. Furthermore, Consumer Reports (August 2003) found that no trans fats were produced when ground beef was irradiated (FDA 2005). The FDA reviewed the paper by Brito et al. (2002) and concluded that "the researchers did not demonstrate that there was an increase in the amount of trans fatty acid present in irradiated ground beef, or that irradiation showed a dose dependent response. In fact, the paper fails to demonstrate that the researchers were measuring the quantity of trans fatty acids." Thus at the current time it is not believed trans fatty acids are a concern with respect to food irradiation

Microbial virulence and resistance

There was some concern expressed that irradiation will induce mutations in microorganisms giving rise to more virulent or radiation resistant mutants. Such strains might pose problems of identification and diagnosis or they might survive irradiation. So far there has been no evidence to support this hypothesis. On the contrary, irradiation causes a loss of virulence and infectivity (Ingram and Farkas 1977, Farkas 1988), and the mutants are less competitive and adaptive (Farkas 1994b, WHO 1994). Other treatments such as thermal processing, food preservation, and drying can also increase mutations, but there has been no evidence to suggest that these processes increase the virulence of pathogenic bacteria (Moseley 1992).

Similarly, there is no evidence to suggest that ionizing radiation will give rise to radiation resistant mutants. It is very difficult to produce such mutations by a single treatment because of

the adequacy of the DNA repair mechanisms of wild type strains. It takes multiple cycles of irradiation exposure to produce radiation resistant populations of bacteria as in the case of *Salmonella enterica var typhimurium* LT2 (Ibe et al. 1982). This is the only known case of a radiation resistant population arising due to repeated irradiation and isolation. This phenomenon was also observed in thermal processing where many cycle of heat treatments gave rise to heat resistant salmonella mutants. This has not caused any problems in pasteurizing plants and is not expected to be of major concern with respect to food irradiation.

SAFETY OF IRRADIATED FOODS

Food irradiation is the most extensively studied type of food preservation technique. Studies evaluating the effect of irradiated food consumption were spearheaded by the US army in the 1950s. Since then hundreds of studies have examined toxicological safety and nutritional adequacy of irradiated foods.

The FDA reviewed the more than 400 studies on toxicology, after the publication of the 1981 report by the joint FAO/IAEA/WHO expert committee on the wholesomeness of irradiated foods (WHO 1994). These were subjected to a stringent guidelines and 150 studies that did not meet the acceptance criteria were rejected. The grounds for rejection included, no radiation dose reported, dose was less than 0.1 kGy or greater than 100 kGy, too few animals were used or number of animals was not reported, diet fed was nutritionally inadequate, no control animals included, and improper laboratory practices. Some of these reviewed studies are discussed in the following sections. It should be noted that the findings were similar in the accepted and rejected studies, and those rejected were only the basis of failure to meet the FDA guidelines.

Subchronic and chronic studies

A number of subchronic studies, 26 in rats, 2 in mice and 6 in dogs, covering a wide variety of irradiated foods, were examined. Radiation doses were between 0.1 - 55.8 kGy. The vast majority of study results were negative indicating a lack of toxicity of the irradiated foods. A few studies did show some effects, the most serious of these being anemia, a reduction of serum albumin transferase and body weight, and hemorrhagic diathesis in rats. A study showing anemia in rats was rejected by the FDA because of several experimental irregularities. The decrease in enzyme levels was questionable because of the lack of a dose response relationship. The study showed a statistical significance, which might have been due to higher enzyme activity in the controls. In the case of hemorrhagic diathesis, the high irradiation dose (55.8 kGy) destroyed vitamin K in the diet. The lack of vitamin K and not any toxic substances in the diet led to this effect. Only one of the 6 studies in dogs reported an adverse effect (anemia), but the study could not be evaluated because of the small number of animals used per group (WHO 1994).

Over 60 chronic studies were carried out using mice, rats, dogs, monkeys and pigs. Food irradiated at high doses was fed over the lifetime of the animal or for at least a year. Twenty six studies carried out in rats were over time periods sufficient to allow proper assessment of carcinogenicity. Many of them involved high irradiation doses (up to 93 kGy) where irradiated individual foods, mixtures of foods and whole diets were fed. According to the FDA no treatment-related increase in tumors was noted (FDA 1986). No consistent pattern or trend was observed in any of the chronic studies and they were overwhelmingly negative. The few adverse

effects that were noted were not deemed significant due to various problems with the experiments (WHO 1994).

A collection of toxicity tests was submitted to the FDA as part of a petition to permit irradiation of poultry in 1987. Studies included a multi-generational study in rats, a chronic study in rats and a one year toxicity study in dogs. No treatment related adverse effects were noted in these studies which were carried out at the Central Institute for Nutrition and Food Research in Netherlands (WHO 1994).

Reproduction and teratology studies

A total of 11 reproductive studies is rats, 5 in mice and 3 in dogs were reviewed. A variety of foods irradiated between 2 and 56 kGy were used. These studies were overwhelmingly negative and the adverse effects were either not statistically significant or could be explained by other factors such as inadequate diet (WHO 1994).

A study comparing growth, reproduction, hematology and histopathology between rats fed either an irradiated laboratory diet (50 kGy) or an autoclaved laboratory diet, found no adverse effect of irradiation (SCF 2003). A three generation study in pigs using the same laboratory diet found no irradiation related negative effects in growth and reproduction parameters (SCF 2003). Another multi-generation study in rats compared irradiated (3 or 6 kGy) and unirradiated chicken meat at 35% of their basal diet. Reproductive parameters (fertility, pup number per litter, post-implantation losses) were not adversely affected and no negative effects were reported on pup weight, pup mortality and pup growth rate (FDA 1987).

Mutations and polyploidy

The FDA reviewed about 20 studies that were designed to assess the potential to induce dominant lethal mutations in rats and mice. Only two studies conducted by Vijaylaxmi 1976, and Vijaylaxmi and Rao (1976) reported an increase in dominant lethal mutations due to consumption of wheat irradiated at 0.75 kGy (WHO 1994). As other studies using much higher doses gave negative results, the FDA concluded that it was unlikely that this positive result was due to irradiation (FDA 2005).

Irradiation of pure solutions of glucose or sucrose have been shown to produce mutagenic compounds as detected by the Ames *Salmonella* assay. Chromosomal breaks in human lymphocytes exposed to irradiated sucrose solution were observed by Shaw and Hays (1966) while mutations in *Drosophila melanogaster* exposed to irradiated glucose were noted by Rinehart and Ratty (1965). Such mutagenic effects, were not observed with irradiated foods like vegetables, fish, spices and chicken (van Kooij et al. 1978, Joner et al. 1978, Farkas et al. 1981, Thayer et al. 1987) or seen in any of the *in vivo* studies with irradiated foods. Further studies showed that chromosomal aberrations in bone marrow or spermatogonia of mice could not be induced by irradiated glucose.

The issue of chromosomal polyploidy was raised by a study conducted on malnourished children in India. The study showed the production of polyploid cells following the consuming of wheat irradiated at 0.75 kGy for 4-6 weeks. Five children each were place in three different groups, the first fed freshly irradiated wheat, the second, stored irradiated wheat and the third, non irradiated wheat. The percentage of polyploid cells was 0.8-1.8% for the groups fed

irradiated wheat, while no polyploid cells were reported for the control subjects (Bhaskaram and Sadasivan 1975).

There were several problems with this study. Only 100 cells were counted per child (500 cells per group) which is too low a number when checking for polyploidy per individual. The result that no polyploid cells were observed for the controls is suspect. Typically for a normal individual about 0.1-1% of the cells will exhibit polyploidy (WHO 1994). In addition, chromosomes of malnourished individuals sometimes appear fuzzy and are difficult to characterize (Satin 1996). None of this was taken into account in the published reports. An expert committee appointed by the Indian Ministry of Health, concluded that the experimental design and sampling technique was unsatisfactory and therefore the study could not establish a treatment related induction of polyploidy (WHO 1994).

A series of further studies in rodents failed to show that feeding wheat irradiated at 0.75 kGy produces chromosomal abnormalities. In addition, no chromosomal aberrations were reported in bone marrow cells of Chinese hamsters due to irradiated diet (10-100kGy) (Renner 1977). Tanaka (1992) found no increase in polyploidy in bone marrow cells and reticulocytes of Chinese hamsters fed wheat irradiated at 15 and 30 kGy.

Raltech study

In 1976, the U.S. government contracted with Raltech Scientific Services to carry out comprehensive nutritional, genetic, and toxicological studies of food irradiation. These studies are generally acknowledged as among the best and most statistically powerful of all the studies

on food irradiation. Mice, hamsters, rats, and rabbits were fed chicken as 35–70% of their total diet that had been irradiated at a minimum absorbed dose of 46 kGy. Dogs, rats, and mice were also fed the irradiated chicken at 35% of their diet during multi generational studies. They found no evidence of genetic toxicity or teratogenic effects in mice, hamsters, rats, or rabbits and no treatment-related abnormalities or changes in the multi generational studies (Thayer et al. 1987). Tests evaluating dominant lethal mutations and mutagenesis by the Ames assay were also negative.

Human feeding studies.

There are a limited amount of studies investigating the effects of irradiated food consumption in humans. Experiments carried out on volunteers consuming various irradiated foods (25-40 kGy, canned or frozen during irradiation and subsequently thawed and stored at room temperature), for 15 days showed no toxic effects and no clinical changes for up to one year after exposure. Fifty-four different irradiated foods, consisting of, meat, fish, vegetables, fruits, cereal products, and miscellaneous food items, were tested. The irradiated foods constituted up to 100% of the total calories (Plough et al. 1960).

In another series of studies, volunteers were fed a diet where 32% of the calories had been replaced by either unirradiated or irradiated canned pork (30 kGy, canned and stored 1 year at room temperature) for 15 days. The irradiated diet was not supplemented with vitamin K. No adverse clinical effects or prothrombin time abnormalities were noted (Plough et al. 1957).

In a third series of experiments volunteers had about 80% of the calories supplied by a variety of foods irradiated at 25-40 kGy and stored for three months at room temperature. No

clinical abnormalities were detected (Bierman et al. 1958). Overall there were no adverse findings with respect to cardiac performance, hematology, hepatic and renal function.

In a study conducted in China 36 male and 34 female healthy students were administered 35 different kinds of irradiated foods for 90 days. The same unirradiated diet was fed to a control group. The irradiation doses ranged from less than 1 kGy for fruits and vegetables to 8 kGy for meats. The storage periods for the irradiated diets were not reported. About 60% of the total diet consisted of irradiated foods. Physical examination was carried out before and after diet consumption. Numerical and structural chromosomal aberrations, sister chromatid exchanges and micronuclei in lymphocytes were determined and the urine was tested for induction of reverse mutations by the Ames assay (WHO 1994).

No adverse effects were noted in the physical examination or in the frequency of chromosomal aberrations and polyploidy in the test and control groups. Although the polyploidy incidence was higher after completion of the study in both test and control groups, it was not treatment-related. There were no significant findings either for the micronuclei or the sister chromatid exchanges. The urine showed no evidence of any mutagenic activity (WHO 1994).

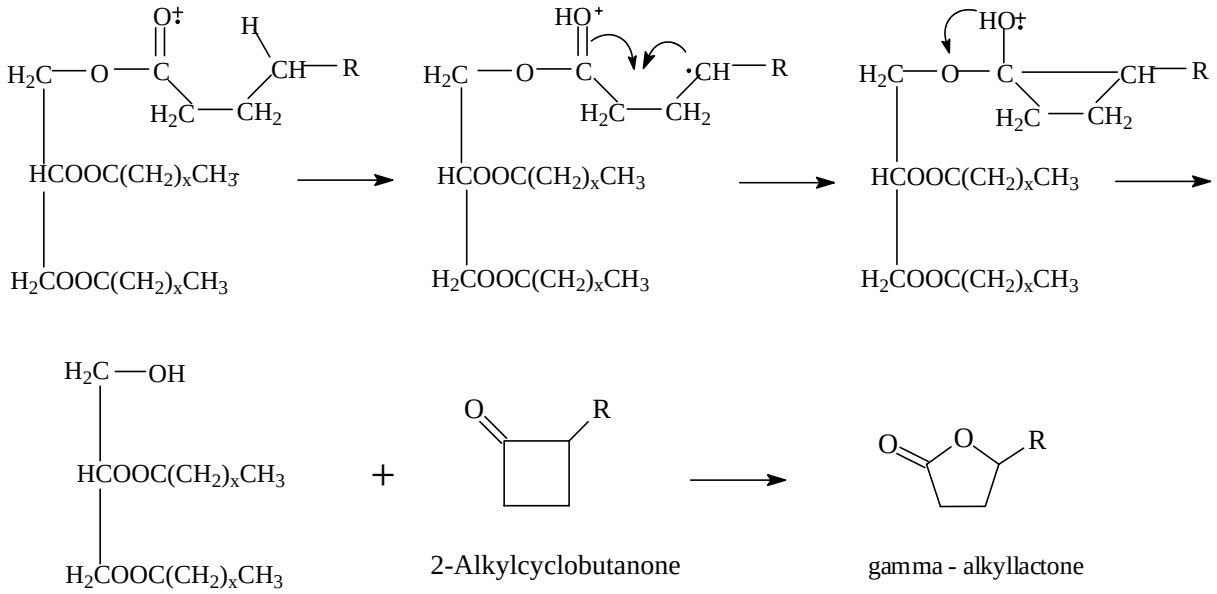
All the data from *in vitro* and *in vivo* tests indicate no adverse effects of irradiated food consumption. Thus it can be concluded that irradiation of foods in accordance with the established rules and good manufacturing practices does not pose a significant health risk.

ALKYLCYCLOBUTANONES

History of alkylcyclobutanones

Alkylcyclobutanones are four membered ring compounds that are generated from fatty acids present in irradiated foods. They were first discovered when LeTellier and Nawar (1972) isolated a cyclic compound from the radiolytic products of each of the simple triglycerides containing C6, C8, C10, C12, C14, C16, and C18 fatty acids. The simple triglycerides were irradiated in vacuum at 60 kGy. The first 2-ACB to be identified was 2-ethyl cyclobutanone, obtained from the radiolysis of tricaproin. Higher molecular weight alkylcyclobutanones were subsequently isolated from the radiolytic products of various triglycerides (Letellier and Nawar 1972).

The general reaction leading to the formation of these compounds is shown in figure 3. It is proposed that the compounds result from cleavage at the acyl-oxy bond via the formation of a six membered ring intermediate. The final product, gamma alkyllactone [5-alkyldihydro-2 (3H)-furanone], has not been observed in most irradiated products but is present as a minor breakdown product in pure 2-DCB solutions. The four most common fatty acids present in foods are palmitic, stearic, oleic and linoleic acid. The cyclobutanones formed from these fatty acids are shown in figure 3. The first compound of this class to be isolated and detected was 2-dodecylcyclobutanone (2-DCB). As this is the most common of the 2-ACBs and the commercial standard being readily available this is the most studied of the 2-ACBs.



Fatty acid	**2-alkylcyclobutanone (2-ACB)**	**R group**
Palmitic	2-dodecylcylobutanone (2-DCB)	$(CH_2)_{11}CH_3$
Stearic	2-tetradecylcyclobutanone (2-TCB)	$(CH_2)_{13}CH_3$
oleic	2-tetradec-5'-enylcylobutanone (2-TDCB)	$(CH_2)_4CH{=}CH(CH_2)_7CH_3$
linoleic	2-tetradaca-5',8'-dienylcyclobutanone	$(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$

Figure 3: Formation of 2-ACBs and the most common 2-ACBs found in foods.

To date, 2-DCB has never been detected in any non irradiated food products. Cooking, packaging in air, vacuum or CO_2, or storage did not give rise to 2-DCB (Crone et al. 1992a, Stevenson et al. 1993, Crone et al.1992b), indicating that the compound can be used as specific marker for irradiation.

Initial studies by Boyd et al. (1991), showed that 2-DCB could be detected not only in freshly irradiated chicken, but also in chicken stored at -4 °C for 20 days. Crone et al. (1992a), found a linear relationship between dose and the amount of 2-dodecylcyclobutanone formed over a dose range of 1-10 kGy, in irradiated fresh or frozen chicken meat. Cooking the chicken in a convection oven either before, or after irradiation did not affect the dose response relationship. When the chicken was re-evaluated after 18 days of storage, the amount of 2-DCB had decreased slightly but the relationship remained linear.

One study has demonstrated 2-DCB is stable and could be detected in irradiated chicken after 13 years of storage (Crone et al. 1993). The chicken had been sterilized by gamma and electron beam irradiation and stored at -40 °C. The compound was not present in thermally treated chicken stored for the same amount of time.

Initially all the studies focused on the detection on 2-DCB in chicken meat. With the synthesis of the 2-tetradecylcyclobutanone (2-TCB) standard it became possible to use this compound as a marker of irradiated chicken meat. As with 2-DCB, the amount of 2-TCB increased linearly with irradiation dose, although the amounts present were lower (Crone et al. 1993). This was thought to be because of the lower concentration of stearic acid in chicken meat. Further studies have detected 2-DCB and 2-TCB in irradiated liquid whole egg

(Stevenson et al. 1993), beef, lamb (Crone 1992, Stevenson 1994), mango, papaya, (Stewart et al. 1998, 2000), Camembert cheese, salmon (Stewart et al. 2000), prawns (McMurray et al. 1995), mechanically recovered meat (Crone 1992), peanuts (Lee et al. 1999), perilla seeds (Lee et al. 2000), and pork (Park et al. 2001).

The synthesis of 2-tetradecenyl-5'-enyl cyclobutanone (2-TDCB) allowed for its presence to be confirmed in irradiated chicken meat, papaya and mango (Hamilton et al. 1995, Stewart et al. 2000) but this 2-ACB was found to be more difficult to detect and quantify. Lee et al. (2000) and Park et al. (2001) did manage to quantify this compound in irradiated perilla seeds and pork and found that the concentration increased linearly with irradiation dose.

Research conducted in this lab showed that 2-DCB was produced in ground beef patties irradiated frozen by electron beam and gamma irradiated. The concentration of 2-DCB increased linearly with dose and it could be used to estimate irradiation dose (Gadgil et al. 2002).

Toxicity of 2-alkylcyclobutanones

Several studies have indicated potential toxic effects of 2-DCB. Delincee and Pool-Zobel (1998) investigated the genotoxic properties of 2-DCB using the Comet assay (Computerized Molecular Evaluation of Toxicity). Isolated rat and human colon cells were incubated with 2-DCB in the concentration range of 0.3-1.25 mg/mL (300-1250 ppm). Though a weak genotoxic effect was observed over the concentration range tested, the amounts used were very high compared to potential human intake. In addition, the comet assay has not been validated for the detection of weak genotoxins and can produce false positive results due to the chromosome degradation that occurs due to non-genotoxic cell death (Sommers and Schiestl

2004).

Burnouf et al. (2002) showed that 2-ACBs appear to induce DNA damage under unique experimental conditions. Cell cultures (HT 29 subcultures) were evaluated for toxicity when exposed to ACB concentrations of up to 100 ppm. Cytotoxicity was observed in some cultures at 12-13 ppm with most 2-ACBs exhibiting toxicity at approximately 25-26 ppm. Genotoxicity of the cultures was measured with the DNA Comet assay after a 30 minute exposure and no significant differences were found. HeLa and HT 29 DNA strand breaks were also measured with 2-ACB concentrations up to 40 ppm (90 ppm for 2-TCB). For some 2-ACBs, DNA breaks seem to appear at 10 ppm, though cell death that appears at 25 ppm may confound the data and call it into question. With HT 29 cells, there appeared to be less DNA breaks than controls at low levels of 2-ACBs (10 ppm). Although the breaks increased with concentration, there was extensive cell death at the higher levels.

Raul et al. (2002) examined colon cancer production in rats injected with azoxymethane (AOM), a known carcinogen, and then fed ACBs. They found that the total number of preneoplastic lesions for the treated and the control groups were the same indicating that the 2-ACBs did not increase colon lesions. The only significant difference was that treated animals developed larger and a greater number of larger tumors when fed ACBs. Control animals fed only 2-ACBs in the drinking water did not develop lesions unless they had been injected with AOM. This suggests that in this model experiment, although 2-ACBs do not induce carcinogenesis, they may promote the carcinogenic process.

In studies investigating mutagenicity, 2-DCB was not mutagenic in the *E. coli* TRP reverse mutation assay and the *Salmonella* reverse mutation assay. (Sommers 2003, Sommers

and Schiestl 2004). In forward mutagenesis assays, an entire gene is a target for mutagenesis, as opposed to single nucleotide changes that are detected in the bacterial reversion tests. Sommers and Mackay (2005) found that 2-DCB failed to induce mutagenesis in the uracil-phosphoribosyl transferase gene in *E. coli* following with or without exogenous metabolic activation.

Genotoxicity can also be determined by gene expression profiling which is capable of identifying many genotoxins that are not detectable using bacterial reverse mutation assays. An increase in expression in the UmuDC, RecA, DinD, and Nfo DNA genes of *E. coli* has been shown to increase following exposure to genotoxins (Orser et. al 1995a, b). 2-DCB was not able to induce gene expression from any of those gene promoters (Sommers and Mackay 2005).

2-DCB has also been tested for the ability to induce rearrangement of chromosomes in eukaryotic cells. The yeast (*Saccharomyces cerevisiae*) DEL assay measures a compound's ability to cause genomic rearrangements, induced by DNA strand breakage (Sommers and Schiestl 2004). The assay does not produce false positives due to cell death because only recombination events in live cells are selected. In contrast the Comet Assay only detects DNA strand break and not the actual genetic endpoint. The 2-DCB, failed to induce genomic rearrangements in the Yeast DEL Assay when concentrations up to 5 mg/ml were tested. (Sommers and Schiestl 2004). These concentrations reduced cell viability to 28%. On the other hand, benzene, which is commonly found in foods induces increases in intrachromosomal recombination in the yeast-based test (Sommers et al. 1995). Recent experiments have shown that 2-DCB had a genotoxic potential and caused chromosomal aberrations in human colon cells using Comet Assay to measure DNA strand breaks and 24-color-Fluorescence-In-Situ-Hybridization to estimate chromosomal abnormalities (Knoll et al. 2006).

That 2-DCB can cause DNA strand breaks in cell cultures is not surprising. Palmitic acid, from which 2-DCB is formed, is a strong inducer of oxidative DNA damage, DNA strand breaks, cell membrane damage, necrosis, and apoptosis in human and rodent cells *in vitro* at concentrations ranging from 13 to 51 ppm (Nogueira et al, 2005; Beeharry et al. 2003). Other fatty acids like linoleic acid (28), arachidonic acid (30 ppm), and thermally oxidized dietary oil, cause cell membrane damage and chromosome fragmentation in human and rodent cells *in vitro* (Nogueira et al. 2005, Udilova et al. 2003). Thus, the effects of 2-DCB on human primary cells and cells isolated from preoneoplastic lesions noted by Knoll et al. (2006), and Burnouf et al. (2002) appear to be similar to those caused by the palmitic acid parent molecule.

Metabolism of Alkylcyclobutanones

There has been only one published study on the fate of 2-ACBs after consumption. In a recent report, the metabolism of 2 alkylcyclobutanones was studied in animals (Horvatovich et al. 2002). Male Wistar rats were administered about 1 mg of 2-tetradecyl (2-TCB) or 2-tetradecenylcyclobutanone (2-TDCB) each day for four months after which the adipose tissue and feces for the last three days were analyzed. Although 2-ACBs are fat soluble substances, only a small amount of the ingested dose appeared in adipose tissue. The total quantity of 2-TCB in adipose tissue was 2 μg and that of 2-TDCB was 9 μg at the end of four months. In addition, less than 1% of the total amount of 2-ACBs consumed was detected in the fecal matter. This study provides evidence that these compounds do not accumulate in adipose tissue, but are rapidly metabolized and/or degraded in animals. They did not evaluate formation of metabolites or conversion of ACBs to carbon dioxide (CO_2).

In order to fully placate public concerns about consuming irradiated food, determining the fate of the ACBs in the body is of great importance. If the ACBs are metabolized rapidly, without adverse effects, and produce no harmful metabolites, public acceptance of irradiated foods should increase. As of now, it is not easy for the public to make an informed decision about the safety of ACBs.

REFERENCES

Anathswamy, H.N.; Vakil, U.K.; Sreenivasan, A. Effect of gamma radiation on wheat starch and its components. *J Food Sci*, **1970**, 35, 795-798.

Angelini P.; Merritt, C. Jr.; Mendelsohn, J. M.; King, F. J. Effect of irradiation on volatile constituents of stored haddock flesh. *J. Food Sci*. **1975**, 40, 197-199.

Bagorogoza, K. and Bowers, M. The effect of irradiation and modified atmosphere packaging on the quality of intact chill-stored turkey breast. *J. Food Sci*. **2001,** 66, 367-372.

Beeharry, N.; Lowe, J. E.; Hernandez, A. R.; Chambers, J. A.; Fucassi, F., Cragg, P. J.; Green, M. H. L.; and Green, I. C. Linoleic acid and antioxidants protect against DNA damage and apoptosis induced by palmitic acid. *Mutat. Res*. **2003,** 530, 27-33.

Bhaskaram, C.; Sadasivan, G. Effects of feeding irradiated wheat to malnourished children. *Am. J. Nutr*. **1975,** 28, 130-135.

Bierman, E. L.; Plough, I. C.; Sellars, J. H.; McGary, V. E.; Nevels, E. M.; Baker, E. M.; Harding, R.S. Short-term human feeding studies of foods sterilized by gamma irradiation and stored at room temperature. U.S. Army Medical Nutrition Laboratory, Fitzsimmons Army Hospital, Denver, CO. **1958,** Report No 224.

Boyd, D. R.; Crone, A. V. J.; Hamilton, J. T. G.; Hand, M. V.; Stevenson, M. H.; Stevenson, P. J. Synthesis, characterization and potential use of 2-dodecylcyclobutanone as a marker for irradiated chicken, *J. Agric. Food. Chem*. **1991**, 39, 789-792.

Britto, M. S.; A.L.C.H. Villavicencio, J.; Mancini-filho. Effects of Irradiation on Trans Fatty Acids in Ground Beef. *Radiat. Phys. Chem*. **2002**, 63, 337-340.

Burnouf, D.; Delincee, H.; Hartwig, A.; Marchioni, E.; Miesch, M.; Raul, F.; Werner, D. Toxicological study to assess the risks associated with the consumption of irradiated fat-containing foods. Federal Research Center for Nutrition Karlsruhe, Germany, **2002**. http://www.bfa-ernaehrung.de/Bfe-Deutsch/Information/e-docs/bfer0202.pdf). Accessed May 2006.

CAST. Ionizing energy in food processing and pest control: I Wholesomeness of food treated with ionizing energy. Council for Agricultural Science and Technology. Ames IA. **1986,** Report No. 109.

CDC. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food—Selected sites, United States 2003. *Morb. Mortal. Weekly Rep*. **2004,** 53(16): 338-343.

Chinn, H. I. Evaluation of the health aspects of certain compounds found in irradiated beef. I. Further toxicological considerations of volatile products. Federation of American Societies for Experimental Biology, Life Sciences Research Office, Bethesda, MD. Contract Rep. No. DAMD 17- 76-C-6055., **1979**, pp. 1-29.

Crone, A. V. J. The use of 2-alkylcyclobutanones as Markers for the Identification of Irradiated Lipid Containing Foods, Ph.D. Thesis, **1992**, The Queens University of Belfast, Northern Ireland, UK.

Crone, A. V. J; Hamilton, J. T. G.; Stevenson, M. H. Effect of storage and cooking on the dose response of 2- dodecylcyclobutanone, a potential marker for irradiated chicken. *J. Sci. Food. Agric.* **1992a**, 58, 249-252.

Crone, A. V. J; Hamilton, J. T. G.; Stevenson, M. H. Detection of 2- dodecylcyclobutanone in radiation sterilized chicken meat stored for several years. *Int. J. Food Sci. Tech.* **1992b,** 27, 691-696.

Crone, A. V. J.; Hand, M. V.; Hamilton, J. T. G.; Dharma, N. D.; Boyd, D. R.; Stevenson, M. H. Synthesis, characterization and the use of 2-tetradecylcyclobutanone together with other cyclobutanones as markers for irradiated liquid whole egg. *J. Sci. Food. Agric.* **1993,** 62, 361-367.

Dauphin, J. F.; Saint-Lebe, L. R. Radiation chemistry of carbohydrates in *Radiation Chemistry of Major Food Components,* Elias, P.S.; Cohen A. J. (eds), Elsiever biomedical Press, Amsterdam. **1983,** 131-185.

Delincée, H. Recent advances in radiation chemistry of lipids. in *Recent Advances in Food Irradiation*. Elias, P.S.; Cohen, A. J. (eds), Elsiever Biomedical press, Amsterdam, **1893,** pp 129-147.

Delincée, H.; Pool-Zobel, B. Genotoxic properties of 2-dodecylcyclobutanone, a compound formed on irradiation of food containing fat. *Radiat Phys. Chem*. **1998,** 52, 39-42.

Derr, D. D. International regulatory status and harmonization of food irradiation. *J. Food Prot.* **1993,** 56: 882-886.

Diehl, J. F.; Adams, S.; Delincee, H.; Jakubic, V. Radiolysis of carbohydrates and of carbohydrate containing foodstuffs, *J. Agric. Food Chem*. **1978,** 26, 15-20.

Dickson, J. S. Radiation inactivation of microorganisms, in *Food Irradiation Principals and Applications*, Molins, R. A (ed), Wiley-Interscience, New York. **2001,** pp 23-32.

Eggum, B.O. Effect of radiation treatment on protein quality and vitamin content of animal feeds, in Decontamination of Animal Feeds by Irradiation, Proc. Advisory Group

Meeting, Sofia, Oct Joint FAO/IAEA Division of nuclear techniques in Food and Agriculture, International Atomic energy Agency, Vienna, **1979,** pp 55-67.

Fan, X. Impact of ionizing radiation and thermal treatments on furan levels in fruit juice. *J. Food Sci*. **2005a**, 7, 409-414.

Fan, X. Formation of furan from carbohydrates and ascorbic acid following exposure to ionizing radiation and thermal processing. *J. Agric. Food Chem*. **2005b,** 53, 7826-7831.

Farkas, J.; Andrassy, E.; Incze, K. Evaluation of possible mutagenicity of irradiated spices. *Acta Aliment.* **1981,** 10, 129-135.

Farkas, J. Irradiation of dry food ingredients. CRC Press, Boca Raton, Florida. **1988,** 57.

Farkas, J. Tolerance of spores to ionizing radiation: mechanisms of inactivation, injury and repair. *J. Appl. Bacteriol*. Symp. Supp **1994a**, 76,815-905.

Farkas J. Special issue: predictive modelling. *Int J Food Microb* **1994b**, 23, 3-4.

FDA. Irradiation in the Production, Processing and Handling of Food. Final Rule. *Federal Register*, **1986,** 51, 13376-13399

FDA. Radiation Technology Inc. Filing of Food additive petition. *Federal Register*, **1987**, 52, 6391.

FDA. Food Labeling: Nutrition Labeling, Nutrient Content Claims, and Health Claims. Proposed rule. *Federal Register* **1999,** 64, 221, 62745-62825.

FDA. **2003,**
http://www.fda.gov/ohrms/dockets/dailys/03/Apr03/041403/01f-0047-c000073-01-vol7.pdf - public citizen oppose using trans fat data. Accessed May 2006

FDA. Irradiation in the Production, Processing and Handling of Food. *Federal Register*. **2004a,** 69, 76844-76847.

FDA. **2004b**. Exploratory Data on Furan in Food. May 7,; updated June 7, 2004 and June 15, 2005 http://www.cfsan.fda.gov/~dms/furandat.html. Accesses February 2006.

FDA. Irradiation in the Production, Processing and Handling of Food. *Federal Register*. **2005,** 70,157, 48057-48073.

Follett, P. A.; Sannxter, S. S. Longan quality after hot-water immersion and X-ray irradiation quarantine treatments. *Hort. Sci*. **2002,** 37: 571-574.

Gadgil, P.; Hachmeister, K. A.; Smith, J. S.; Kropf, D. H. 2-Alkyl-cyclobutanones as irradiation dose indicators in irradiated ground beef patties. *J. Agric. Food Chem.* **2002**, 50, 5746-5750.

Grecz, N.; Rowley, D.B.; Matsuyama, A. The action of radiation on bacteria and viruses, in *Preservation of Foods by Ionizing Radiation*. **1983,** Vol 2.

Green, B. E., Watts, B. M. Lipid oxidation in irradiated cooked beef, *Food Technol.* **1966,** 20, 111-114.

Hamilton, L.; Stevenson, M. H., Boyd, D. R.; Brannigan. I. N; Treacy, A. B.; Hamilton, J. T. G.; McRoberts, W. C.; Elliot C. T. Detection of 2-substituted cyclobutanones as irradiation products of lipid containing foods: synthesis and application of cis- and trans-2-(tetradec-5'-enyl)cyclobutanones and 11-(2'-oxocyclobutyl)-undecanoic acid, *J. Chem. Soc. Perkin. Trans*. **1995**, 1, 139-146.

Health Canada. Irradiation of ground beef: Summary of submission process. Food Directorate, Food Products and Health Branch, Ottawa. October 29 **2002**. http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/securit/gbeef_submission-soumission_viande_hachee_e.pdf. Accessed May 2006

Horvatovich. P., Raul, A. F., Miesch M., Burnouf, C. D., Delincee, D. H, Hartwig, E. A., Werner, F. D. Marchioni, E. Detection of 2-alkylcyclobutanones, markers for irradiated foods in adipose tissue of animals fed with these substances. *J. Food Prot.* **2002,** 65, 1610-1613.

Houser, T. A.; Sebranek, J. G.; Lonergan, S. M. Effects of irradiation on properties of cured ham. *J. Food Sci*. **2003,** 68, 2362-2365.

Ibe, S. N.; Sinskey, A. J.; Botstein, D. Genetic mapping of mutations in a highly radiation-resistant mutant of *Salmonella typhymurium* LT2. *J Bacteriol.* **1982,** 152, 260.

Ingram, M.; Farkas, J. Microbiology of foods pasteurised by ionizing radiation. *Acta. Aliment*. **1977**, 6, 123-185.

International Agency for Research on Cancer. Furan. In IARC Monographs on the evaluation of carcinogenic risks to humans, Vol. 63: Dry cleaning, some chlorinated solvents and other industrial chemicals; IARC: Lyon, France, **1995**, pp 393-407.

Joner, P. E.; Underwal, B.; Lunde, G. Mutagenicity testing of irradiated cod fillets. *Lebensmittel Wissenschaft und Technologie*. **1978,** 11, 224-226.

Knapp, F. W.; Tappel, A. L. Some effects of γ-radiation or linoleate peroxidation on α-tocopherol, *J. Am. Oil. Chem. Soc*. **1961a,** 38, 151-156.

Knapp, F. W.; Tappel, A. L. Comparison of the radiosensitivities of the fat soluble vitamins by gamma irradiation, *J. Agric. Food Chem*, **1961b,** 9, 430-433.

Knoll, N.; Weise, A.; Glei, M.; Claussen, U.; Marian, B.; Pool-Zobel, B. L. 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, causes DNA strand breaks and chromosomal aberrations in human colon adenoma cells. *Mutat. Res.* **2006**, 594, 10-19.

Lee H. J; Lee Myung-yul; Kim, K.S. Detection of radiation induced hydrocarbons and 2-alkylcyclobutanones from peanuts. *J. Food Sci. Nutr*. **1999**, 4 (4), 270-275.

Lee H. J; Byun, M.W; Kim, K.S. Detection of radiation induced hydrocarbons and 2-alkylcyclobutanones in irradiated perilla seeds. *J. Food. Prot*. **2000**, 63, 1563-1569.

Lee, E. J.; Love, J.; Ahn, D. U. Effect of antioxidants on consumer acceptance of irradiated turkey meat. *J. Food Sci.* **2003**, 68, 1659-1663.

LeTellier, M. H.; Nawar, W. W. 2-alkylcyclobutanones from the radiolysis of triglycerides. *Lipids*. **1972**, 7,73-76.

Liebster, J.; Kopoldova, J. The radiation chemistry of amino acids, *Adv. Radiat. Biol*. **1964,** 1, 157-226

Luchsinger, S. E.; Kropf, D. H.; Garcia Zepeda, G. M.; Chambers, E.; Hollingsworth, M. E.; Hunt,M. C.; Marsden, J. L.; Kastner, C. L.; Kuecker, W. G. Sensory analysis and consumer acceptance of irradiated boneless pork chops. *J. Food Sci*. **1996**, 61, 1261-1266.

Lukton, A.; MacKinney, G. Effect of ionizing radiation on carotenoid stability, *Food Technol*. **1956,** 10, (12), 630-632.

Maga, J. A. Furans in foods. CRC Crit. Rev. *Food Sci. Nutr*. **1979,** 1, 355-400.

Matiella, J. E.; Hsieh, T. C. Y. Volatile compounds in scrambled eggs. *J Food Sci*. **1991,** 56, 387-390.

McMurray, C.H.; McRoberts W.C.; Hamilton, J. T. G.; Elliot, C. T.; Stevenson. M. H. Detection of irradiated prawns using 2-alkylcyclobutanones, *Food Sci. Technol*. Today, **1995**, 9, 146-148.

McNeal, T. P.; Nyman, P. J.; Diachenko, G. W; Hollifield, H. C. Survey of benzene in foods by using headspace concentration techniques and capillary gas chromatography. *J. AOAC Int*. **1993,** 76, 1213-1219.

Merritt, C.; Angelini P; Graham. R. A. Effect of radiation parameters on the formation of radiolysis products in meat and meat substances. *J. Agric. Food Chem*. **1978,** 26, 29-35.

Metta, V.C.; Mameesh, M.S.; Johnson, B.C. Vitamin K deficiency in rats induced by feeding of irradiated beef, *J. Nutr*. **1959,** 69, 18-22.

Molins, R. Historical notes on food irradiation, in *Food Irradiation Principals and Applications*, Molins, R. A (ed), Wiley-Interscience, New York. **2001,** pp 1-21.

Moseley, B. Ionizing radiation action and repair. In: *Mechanisms of action of food preservation procedures*. Gould GW (Ed.). Elsevier Appl Sci, London, **1989,** pp. 43-70.

Moseley, B. Radiation, microorganisms and radiation resistance. In *Food irradiation and the Chemist.* Johnston, D. E and Stevenson, M. H. (eds.). Royal Society of Chemistry Special Publication 86, Cambridge. **1992,** pp 97-108.

Murano, P. S.; Murano, E. A.; Olson, D. G. Irradiated ground beef: Sensory and quality changes during storage under various packaging conditions. *J. Food Sci*. **1998,** 63, 548 –551.

Nam, K. C.; Min, B. R.; Park, K. S.; Lee, S. C.; Ahn, D. U. Effects of ascorbic acid and antioxidants on the lipid oxidation and volatiles of irradiated ground beef. *J Food Sci*. **2003,** 68, 1680-1685.

Nam, K.C.; Ahn, D. U. Mechanisms of pink color formation in irradiated precooked turkey breast meat. *J. Food Sci*. **2002,** 67, 600-607.

NTP. National Toxicology Program, Furan CAS No. 110-00-9. Report on carcinogens, 11th ed., U. S. Department of Health and Human Services, Public Health Service, **2004.** http://ehp.niehs.nih.gov/roc/eleventh/profiles/s090fura.pdf. Accessed May 2006.

Nawar, W. W. Radiation chemistry of lipids in *Radiation Chemistry of Major Food Components*, Elias, P.S.; Cohen A. J. (eds), Elsiever biomedical Press, Amsterdam. **1977,** pp 21-61.

Nawar, W. W. Volatiles from food irradiation, *Food Rev. Int*. **1986,** 2, 45-78.

Newton, A.S. Mechanisms of chemical effects of ionization radiation, in *Radiation Effects of Organic Materials*, Bolt, R. O. and Carroll, J. G (eds.), Academic Press, New York. **1963**, pp 35-63.

Nogueirra de Sousa Andrade, L.; Matins de Lima, T.; Curi, R.; Lauro Castrucci, A.. M. Toxicity of fatty acids on murine and human melanoma cell lines. *Toxicology In Vitro.* **2005**, 19, 553- 560.

Olson, D. G. Irradiation of food. *Food Technol.* **1998,** 52, 56-62.

Olson, D. G. Irradiation processing, in *Food Irradiation: A Sourcebook*, Murano, E. A. (Ed). Iowa State University Press, Ames. **1995,** pp 3-27.

Osterholm, M. T.; Norgan, A. P. The role of irradiation in food safety. *N. Engl. J. Med*. **2004**, 350, (18), 1898-1901.

Park, E. R; Kim, E. A; Kim, K.S. Detection of radiation induced hydrocarbons and 2-alkylcyclobutanones from irradiated pork. *Food Sci. Biotech*. **2001**, 10 (1), 84-89.

Plough, I.C.; Bierman, E.L.; Levy, L.M.; Witt, N.F. Human Feeding Studies with Irradiated Foods. *Federation Proceedings*, **1960**, 19, 4, pp. 1052-1054, http://www.nal.usda.gov/fnic/foodirad/000588.html, Accessed May2006.

Plough, I.C.; Sellars, J.H.; McGary, V.E.; Nuss, J.; Baker, E.M.; Harding, R.S.; Taylor R.L.; An Evaluation in Human Beings of the Acceptability, Digestibility, and Toxicity of Pork Sterilized by Gamma-Radiation and Stored at Room Temperature.U.S. Army Medical Nutrition Laboratory, Fitzsimmons Army Hospital, Denver, CO. **1957** Report 204. http://www.nal.usda.gov/fnic/foodirad/000585.html, Accessed May2006.

Raffi, J. J.; Agnel, J. P. L.; Thiery, C. J.; Frejaville, C. M.; Saint-Lebe, L. R. Study of γ irradiated starches derived from different foodstuffs: A way for extrapolating wholesomeness data, *J. Agric. Food. Chem*. **1981,** 29, 1227-1232.

Raul, F.; Gossé, F.; Delincée, H.; Hartwig A.; Marchioni, E;, Miesch M.; Werner D.; Burnouf D. Food-borne radiolytic compounds (2-Alkylcyclobutanones) may promote experimental colon carcinogenesis. *Nutr.Cancer* **2002,** 44, 188-191.

Renner, H. W.; Chromosome studies on bone marrow cells of Chinese hamsters fed a radiosterilized diet. *Toxicology*. **1977,** 213-222.

Rinehart, R. R.; Ratty, F. J. Mutation in Drosophila melanogaster cultured in irradiated whole foods or food components. *Int. J. Radiat. Biol*. **1967**, 12, 347-354.

Satin, M. Food irradiation in *Food Irradiation: A Guidebook*. 2nd ed. CRC Press, Boca Raton, Fla. **1996**, pp 1-26.

SCF (Scientific Committee on Food) Revision of the opinion of the Scientific Committee on Food on the irradiation of food. **2003.** http://europa.eu.int/comm/food/fs/sc/scf/out193_en.pdf. Accessed May 2006.

Sharpe, P. H. G., Dosimetry of food irradiation, in *Food Irradiation and the Chemist*, Johnston,

D. E and Stevenson, M. H. (eds.). Royal Society of Chemistry Special Publication 86, Cambridge. **1990,** 109-123.

Shaw, M. W.; Hays, E. Effects of irradiated sucrose on the chromosmes of human lymphocytes *in vitro. Nature.* **1966**, 211, 1254-1257.

Simic, M.G. Radiation chemistry of water soluble foods components in *Preservation of Foods by Ionizing Radiation*, Josephson E. S.; Peterson, M.S. (Eds), CRC Press, Boca Raton, FL, **1983,** Vol II, 1-73.

Smith, J. S.; Pillai, S. Irradiation and food safety: a scientific status summary. *Food Technol.* **2004,** 58,11, 48-55.

Sokhey, A.S.; Hanna, M.A. Properties of irradiated starches, *Food Struct.* **1993,** 12: 397-410.

Sommers, C. H.; Schiestl, R. H. 2-dodecylcyclobutanone does not induce mutations in the *Salmonella* mutgenicity test or intrachromosomal recombinationin *Saccharomyces cerevisiae*. *J. Food Prot.* **2004**, 67, 1293-1298.

Sommers C. H. 2-Dodecylcyclobutanone does not induce mutation in the *Escherichia coli* reverse mutation assay. *J Agric Food Chem*. **2003**, 51, 6367-6370.

Sommers, C. H. and Mackay, W. H. DNA Damage-Inducible Gene Expression and Formation of 5-Fluorouracil Resistant Mutants in *Escherichia coli* Exposed to 2-Dodecylcyclobutanone. *J. Food Sci.* **2005** 70, C254-257.

Stevenson, M. H.; Crone, A.V. J.; Hamilton, J. T. G; McMurray, C. H. The use of 2-Alkylcyclobutanones for the identification of irradiated chicken meat and eggs, *Radiat. Phys. Chem.* **1993**, 42, 363-366.

Stevenson, M. H. Progress in the identification of irradiated foods, Trends. *Food Sci. Technol.* **1994,** 3, 257-262.

Stewart, E. M.; Moore, S.; McRoberts, W. C.; Hamilton, J. T. G. 2-Alkylcyclobutanones as markers for exotic fruits, *Food. Sci. Technol. Today*. **1998**, 12, 103-106.

Stewart, E. M.; Moore, S.; Graham, W. D; McRoberts, W.C.; Hamilton, J.T.G. 2-Alkylcyclobutanones as markers for the detection of irradiated mango, papaya, Camembert cheese, and salmon meat. *J. Sci. Food Agric*. **2000**, 80, 121-130.

Stewart, E. M. Detection methods for irradiated foods in Food *Irradiation Principals and Applications*, Molins, R. A (ed), Wiley-Interscience, New York. **2001a,** pp 347-386.

Stewart, E. M. Food irradiation chemistry in *Food Irradiation Principals and Applications*, Molins,

R. A (ed), Wiley-Interscience, New York. **2001b**, pp 37-75.

Tanaka. N.; Induction of polyploidy in bone marrow cells and micronuclei in reticulocytes in Chinese hamsters. In: Final report of the Food Irradiation Research Committee for 1986-1991. Matsuyama A (Ed.), Japan Isotopes Association, **1992**, pp. 212-220.

Tauxe, R. V. Food safety and irradiation: protecting the public from foodborne infections. *Emerg Infect Dis*. **2001,** 7, Suppl, 516-521.

Terry, A. J.; McColl, N. P. Radiological consequences of food irradiation. **1992**, London, National Radiaological Protection Board.

Thayer, D. W.; Fox Jr, J. B.; Lakritz, L. Effects of ionizing radiation on vitamins, in *Food Irradiation*, Thorpe, S. (ed), Applied Science, London, **1991**, 285-325.

Thayer, D.W. Irradiation of food-Helping to ensure food safety. *N. Engl. J. Med*. **2004,** 350, (18), 1811-1812

Thayer, D. W.; Christopher, J. P.; Campbell, L. A.; Ronning, D. C.; Dahlgren, R. R.; Thomson, G. M.; Wierbicki, E. Toxicology studies of irradiation-sterilized chicken. *J. Food Prot.* **1987,** 50, 278-288.

Urbain W. U. Radiation chemistry of food components. In *Food Irradiation,* Academic Press Inc., Orlando Florida. **1986,** 37-82.

Udilova N.; Jurek D.; Marian B.; Gille L.; Schulte-Hermann R.; Nohl, H. Induction of lipid 4 peroxidation in biomembranes by dietary oil components. *Food Chem Toxicol.* **2003,** 41, 1481-1489.

USDA/ERS. Economics of foodborne disease: Feature. U.S. Dept. of Agriculture Economic Research Service, Washington, D.C. **2000.** www.ers.usda.gov/briefing/FoodborneDisease/features.htm. Acessed May 2006.

Vajdi, M., Nawar, W. W.; Meritt, C. Effects of various parameters on the formation of radiolysis products in model systems. *J. Am. Oil Chem. Soc*. **1982,** 55, 840-850.

Van Kooig, J. G.; Leveling, H. B.; Schubert, J. Application of the Ames mutagenicity test to food processed by physical preservation methods. In *Food Preservation by Irradiation*, Vol II. International atomic energy agency, Vienna. **1978**, pp63-71.

Vickers, Z. M.; Wang, J. Liking of ground beef patties is not affected by irradiation. *J. Food Sci*. **2002**, 67, 380-383.

WHO. *Wholesomeness of Irradiated Food*. Report of a joint FAO/IAEA/WHO Expert committee. Geneva, World health Organization, **1981,** WHO technical report series, No. 659.

WHO. Safety and nutritional adequacy of irradiated food. World Health Organization, Geneva, Switzerland. **1994**.

WHO. *Wholesomeness of Irradiated Food*. Report of a joint FAO/IAEA/WHO Expert committee. Geneva, World health Organization, **1999,** WHO technical report series, No. 890.

Zhu, M. J.; Mendonca, A; Min, B.; Lee, E. J.; Nam, K. C.; Park, K.; Du, M.; Ismail, H. A; Ahn, D.U. Effects of electron beam irradiation and antimicrobials on the volatiles, color, and texture of ready-to-eat turkey breast roll. *J. Food Sci*. **2004**, 69, C382-C387

[1]PART II: 2-DODECYLCYCLOBUTANONE AS AN IRRADIATION DOSE INDICATOR IN FRESH IRRADIATED GROUND BEEF AND EFFECT OF ORIGANOX ON 2-DCB FORMATION IN IRRADIATED GROUND BEEF.

ABSTRACT

Alkylcyclobutanones (2-ACBs) are radiolytic products formed when fatty acids are irradiated. These cyclobutanones are unique irradiation byproducts and therefore may serve as indicators of irradiation exposure. As only limited information exists about 2-ACB formation in retail meat products, reliable methods which can quantify 2-ACBs and thus estimate irradiation dose in commercial meat products are desired. The cyclobutanone studied in this experiment was 2-dodecylcyclobutanone (2-DCB) which is formed from palmitic acid. The formation of 2-DCB was evaluated in fresh irradiated ground beef patties at two fat levels. Patties containing 15% and 25 % fat were irradiated by electron beam at 1.0, 2.0, 3.0 and 4.5 kGy. Commercially available 1 lb irradiated ground beef chubs with different fat levels were analyzed in order to estimate dose absorbed by these samples. In addition, the effect of antioxidant Origanox on the formation of 2-DCB in irradiated ground beef was investigated. The 2-DCB was extracted using supercritical fluid extraction (SFE) and analyzed by gas chromatography-mass spectrometry (GC-MS) and was detected in all the irradiated samples. The concentration of 2-DCB increased linearly with dose with $R^2 = 0.9646$ for 25% fat samples and $R^2 = 0.9444$ for 15% fat samples. Further, there was no significant difference in 2-DCB concentrations between the two fat levels.

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The estimated doses applied to the commercial samples ranged between 1.38 kGy and 1.55 kGy, values consistent with doses normally used in the industry (1.0-2.0 kGy). Our results show that 2-DCB can be used to monitor fresh irradiated beef and approximate the adsorbed dose. There was no significant difference in the amounts of 2-DCB formed in the patties containing Origanox versus patties without the antioxidant. The results show that Origanox did not affect the formation of 2-DCB in ground beef under these experimental conditions.

INTRODUCTION

Treating food with ionizing radiation improves its safety and helps maintain its quality. The main selling point of irradiated foods is that it is safe from a microbial standpoint. Beginning October 2002 companies could petition the FDA for permission to use terms like "electronic pasteurization" on the labeling for irradiated foods (FDA 2002). Consumers are already familiar with pasteurization and associate the term with a safe product. Therefore, there must be a protocol in place to test for irradiation in order to check if the product meets with the regulations. Being able to differentiate between irradiated and non-irradiated foods will aid in proving the authenticity and safety of irradiated products, and in detecting mislabeled products. In November 2003, Excel Corporation (Dodge City, KS) voluntarily recalled 26,000 pounds of ground beef that was mislabeled as irradiated (USDA 2003). The incident appears to be the first case of its kind, and it emphasizes the need for a method which can reliably distinguish between irradiated and non irradiated foods.

Ionizing radiation causes changes in the food much like cooking (Stewart 2001). Many radiolytic products such as short chain hydrocarbons, aldehydes, ketones, and flavor volatiles are formed due to irradiation. Most of these changes are not unique to the process of irradiation, with these compounds being detected in non-irradiated foods as well. At the doses currently approved for food irradiation, the only unique radiolytic products that have been identified are alkylcyclobutanones (Stewart 2001). These are cyclic compounds formed by rearrangement of fatty acids when exposed to irradiation. The resulting compounds have the same number of carbon atoms as the precursor fatty acids, with an alkyl group attached at ring position two (LeTelleier and Nawar 1976). These compounds have been found in a wide variety of lipid

containing foods and have been universally accepted as indicators of irradiation exposure (Nawar and Zhu 1990, Lee et al. 1999, Lee at al. 2000).

Over the years various methods have been evaluated for the detection of alkylcyclobutanones in irradiated lipid-containing foods. These include solvent extraction, high performance liquid chromatography (HPLC), enzyme linked immunosorbent assay (ELISA) and SFE-GC-MS (Meier et al. 1996, Hamilton et al. 1996, Lembke et al. 1995). Of these, the solvent extraction method was adopted as a European Standard (EN 1785:2003) and Ministry of Agriculture, Fisheries and Food validated method (Ireland, MAFF V37) in 1996 (MAFF 1996). The method involves a long clean up and extraction procedure and uses large volumes of organic solvents. Recent studies using supercritical fluid extraction (SFE) have shown that this procedure offsets the limitations of the solvent extraction method and is better suited for the extraction of the 2-alkylcyclobutanones (Meier et al. 1996, Tewfik et al. 1998, Stewart et al. 2001). A SFE method previously optimized in this lab was used to extract and detect 2-DCB in gamma and electron beam irradiated frozen ground beef patties (Gadgil et al. 2002).

Recent concerns regarding toxicity of these compounds and lack of literature thereof have been misused by opponents of food irradiation to cast a negative light on food irradiation. Thus, all aspects of 2-ACB formation need to be studied in order to build up a body of literature that can be relied upon when discussing this issue. The 2-ACBs are thought to be formed by a free radical mechanism (Letellier and Nawar 1972). Therefore, antioxidants and free radicals scavengers, might be able to prevent or decrease the formation of 2-ACBs in irradiated products. Antioxidants have been shown to reduce levels of heterocyclic amines (HCAs), which are known carcinogens in grilled meats (Audi 2005). If 2-ACB levels can be similarly decreased by

antioxidants or other free radical scavengers, concerns and misgiving regarding irradiated foods should decrease.

As 2-DCB had been detected in frozen irradiated beef the next step was to evaluate 2-DCB formation in fresh irradiated ground beef. The effect of fat and added antioxidants are important aspects that had not been investigated in ground beef. Therefore, the formation of 2-DCB at two fat levels and the effect of antioxidant Origanox were studied in this experiment.

The antioxidant Origanox is a water soluble extract from *Oreganum vulgare* marketed by Barrington Nutritionals. The main active component of Origanox is rosmarinic acid, with thymol and carvacrol as minor components (Barrington Nutritionals 2006). The Origanox extract was investigated in this lab for its effectiveness against HCA formation concurrently with this research by Audi (2005).

The first objective of this experiment was to examine the dose response relationship of 2-DCB formation in fresh irradiated ground beef patties with two fat levels (15% and 20 % fat) irradiated at four doses. The second objective was to quantify 2-DCB in commercially available fresh irradiated ground beef using the data obtained from the first part, and estimate the absorbed dose. The third objective was to study the effect of Origanox on the formation of 2-DBC in irradiated ground beef patties at a level of 0.08%. The 2-DCB was selected as it was the most easily detected cyclobutanone according to previous research, and the standard was readily available (Gadgil et al. 2002).

MATERIALS AND METHODS

Reagents

Hexane, Florisil (60-100 mesh), anhydrous sodium sulfate, boron trifluoride-methanol (14%) were purchased from Fisher Scientific (Pittsburg, PA). Wetsupport (diatomaceous earth) and sand were obtained from Teledyne, Inc. (Lincoln, NE). The 2-DCB standard was obtained from Acros Organics (Fisher Scientific Co., Pittsburgh, PA).

Ground Beef Patty Preparation and Irradiation

Effect of fat level

Beef from round muscles and beef trimmings that were 48 hours postmortem was coarse ground and packaged in 4.54-kg chubs. Two coarse grind formulations (75/25 or 85/15) were formulated using a commercial processor (Tyson's IBP Fresh Meats, Emporia, KS), transported to Kansas State University (KSU), and stored for six days at 1 ± 1°C. After running the chubs through the grinder (Hobart model number 84145, Hobart Corp., Troy, OH), the fine grind was placed in a lug in a refrigerated unit set at -6.7 °C. Quarter pound ground beef patties (weight 115 ± 1 g) were made using a patty mold (1.2 cm height X 11 cm diameter) and Plexiglas press. The patties were placed in a single layer on metal trays lined with Butcher paper, crust frozen for 30 min in an open-top case (Model SM0781A11; Star Products Division, Hussmann Corporation, Dumas, AR), and individually vacuum-packaged in 3 mil standard barrier (nylon/Polyethylene, oxygen transmission rate of 9.3 cc/m^2/24 hrs at 0 °C) vacuum pouches (Koch Supplies Inc., Kansas City, MO) using a Multivac A300 Packager (Multivac Packaging Machines, Kansas City, MO). Vacuum was measured using a Kennedy Gauge (Kennedy Enterprises, Lincoln, NE), and the vacuum level was set at 90%. Patties were stored at 0 ± 1.5°C

until shipment to the irradiation facility. Patties were irradiated at the electron beam facility (SureBeam Corporation, Sioux City, IA). Temperatures were monitored during storage and throughout transportation using temperature loggers (Omega Engineering, Inc., Stamford, CT). Four patties were irradiated per dose. The dosimetry data is shown in table 5.

Table 5: Dosimetry data showing the maximum, minimum and average absorbed doses (kGy) for ground beef patties with two fat levels. Absorbed dose was measured by alanine dosimeters.

15% Fat			
Target Dose	Maximum dose	Minimum dose	Average dose
1	1.15	1.01	1.08
2	2.35	2.03	2.19
3	3.45	3.07	3.26
4.5	5.15	4.57	4.86
25% fat			
1	1.14	1	1.07
2	2.29	2	2.15
3	3.46	3.01	3.26
4.5	5.09	4.59	4.84

Effect of Origanox

Ground beef chuck (80/20) was purchased at the local supermarket (Dillons). Origanox (Barrington Nutritionals, Harrison NY) was dissolved in cold water (4°C) and added to the ground beef at 0.08% by weight of meat. The solution was sprinkled evenly over the beef and manually mixed for approximately 10 min. Uniform quarter pound ground beef patties (weight 115 ± 1 grams) were formulated using a burger press and irradiated to 3.0 kGy by gamma irradiation. Patties from the same meat formulation and same amount of Origanox were not irradiated and served as irradiated controls. Non irradiated patties served as simple controls.

Commercial samples

One lb chubs of commercially irradiated ground beef chubs were obtained from two sources. Two samples of Brand X containing 7% and 20% fat and two samples of Brand Y containing 10% and 20% fat were evaluated. These were stored at -80°C until ready for analysis.

Fatty acid profile

A fatty acid profile was obtained to determine the amounts of palmitic acid present in the beef fat. The fat from the ground beef patties was first extracted by blending 5 grams of the beef sample with 25 mL of hexane. The extract was passed through Whatman filter paper no. 4 and concentrated to dryness on a Rotavapor at 45 °C. The fatty acid were then converted to their corresponding fatty acid methyl esters (FAME) using Boron trifluoride-Methanol (14%), according to the procedure described by Ackman (1998). The extracts were analyzed by gas chromatography equipped with a flame ionization detector (FID). The percent of each fatty acid was determined from retention times based on comparison with fatty acid methyl ester standard

solutions (C8- C24, part # 18918, Sigma-aldrich St. Louis, MO). The GC-FID conditions were: Injector temperature 250 °C; initial temperature, 60 °C, hold 1 min; 20 °C/min, final temperature 195 °C, hold 15 min; detector temperature, 280°C. The instrument used was a Hewlett-Packard 5890 Gas Chromatograph (Hewlett- Packard, Palo Alto, CA), with a HP-23 cis/trans FAME column (Agilent Technologies Palo Alto, CA). The flow rate of carrier gas, helium, was 1 mL/min.

Preparation of patties for extraction

All patties and commercial samples were stored at -80 °C prior to analysis. Once ready for analysis, the patties were tempered at room temp for 20-30 min or until soft enough to cut. The patties were then cut into 1 cm^2 squares and immersed into liquid nitrogen. They were removed after the liquid nitrogen had stopped bubbling (about 30-45 seconds) and ground in a Waring blender fitted with a stainless steel blending container (Fisher Scientific Co. Pittsburgh, PA). The result was a fine homogenous powder which was used for the SFE procedure.

SFE procedure

An ISCO-Suprex prepmaster GA (Teledyne Inc. Lincoln, NE) fat analyzer was used for the SFE procedure. Ground beef was homogenized with Wetsupport in a Waring blender in the ratio of 1 part beef to 2 parts Wetsupport A 5 mL SFE cartridge (Teledyne Inc. Lincoln , NE) was loaded with sand, Florisil and about 1.5 g of the beef : Wetsupport mixture prior to being placed in the extractor. The sand protects the seals of the extraction cartridge while the Florisil serves to trap the fat. Three beef patties were extracted for each dose level and two cartridges were prepared per patty. The control and commercial samples were prepared and used in the

same way. The entire experiment was performed once. Patties containing Origanox were prepared and extracted similarly. Three replicates were performed for this part.

Extraction was carried out under the following conditions: pressure, 340 atm; temperature, 75 °C; 5 min static and 20 min dynamic with a flow rate of CO_2 of 1 mL/min. These parameters were modified from a previously described procedure (Tewfik et al. 1998). The 2-DCB was trapped on glass wool and eluted with about 25 mL of hexane. This extract was concentrated under nitrogen gas to 25 μL for control samples, samples irradiated at doses 1.0 and 2.0 kGy, 50 μL for 3.0 and 4.0 kGy and Origanox samples and 10 μL for the commercial samples and origanox samples. The extracts (1 μL) were then injected into the GC-MS.

GC-MS analysis

GC-MS was performed with a HP 5890 (Agilent Technologies, Palo Alto, CA) fitted with a HP-5 MS column (crosslinked 5% Ph Me siloxane, 30 m x 0.22 mm x 0.25 μm film thickness), and a HP MSD 5970 detector. The flow rate for the helium carrier gas was 1 mL/min. The GC temperature program was: injector temperature 250°C; initial temperature 55°C, hold 0.5 min; 20°C/min, final temp 200°C, hold 1 min; 15°C/min final temp 270°C , hold 1 min. The transfer line and ion source were held at 280°C throughout the runs. The MS was set to selected ion monitoring (SIM) mode and ions m/z 98 and 112 were monitored for the analysis of 2-DCB. Standard solutions of 1.0, 2.5, 5.0, 7.5 and 10.0 ppm were used to calibrate the standard curve for 2-DCB. The compounds were identified by comparing retention times and the ion ratios with the standards, and the concentration in the sample was determined from the standard curve.

Statistical analysis

The experimental design for investigating the effect of fat level was a completely randomized design with a 2 x 5 factorial structure. Analysis of variance was carried out on the data using the SAS software system release 8.1, (SAS Institute Inc. Cary, NC). For comparing the effect of Origanox comparison of means was performed using the Students t test.

RESULTS AND DISCUSSION

From fatty acid measurements the amount of palmitic acid in the beef fat was 25.4 ± 1.08 %. The fatty acid profile is included in the Appendix (Table A.1.). The 2-DCB was detected in all the irradiated samples and its concentration increased linearly with dose as illustrated in the response curve shown in figure 4. Figure 5 shows the chromatograms for the irradiated and control samples. There was no significant difference in the amount formed between the two fat levels. There might be an upper threshold beyond which the amount of fat does not effect 2-DCB formation. This indicates that the amount of fat may not be a factor affecting 2-DCB formation, at least at these fat levels. Thus, the absorbed dose can be estimated for commercial samples with a wide range of fat levels. In a commercial setting where there is considerable variation in product composition, this would be an advantage. The amount of 2-DCB for the two fat levels is shown in table 5.

The compound was detected in the all commercial samples and the absorbed doses as calculated from the dose response curves are shown in table 6. As the fat levels of all the commercial samples did not correspond exactly with the lab samples, line equations obtained from both 15% and 25% fat level samples were used to calculate the absorbed dose. Figures 6 and 7 show the

GC-MS chromatograms of the commercial samples. Our lab samples were irradiated at a SureBeam facility which also irradiated ground beef for retail sale. We expect our samples were processed in much the same way as commercial samples would be, and are suitable for estimating applied dose. It should be noted that the absorbed dose values are estimates. There were no true controls for the commercial samples and there was no information about when the samples were

irradiated. Therefore, the effect of storage conditions and/or time, if any, was unknown. However, these values are within the range of 1.0-2.0 kGy normally used in the industry (personal communication) indicating that this method was able to approximate the dose applied. The percent recovery for 2-DCB using the SFE-GC-MS was calculated as 99.48% (CV=18.81%).

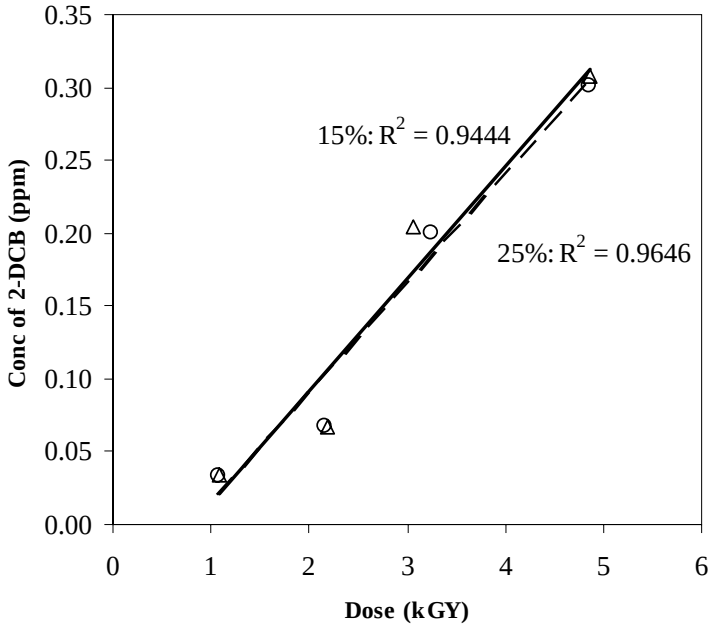

Figure 4. Response of 2-DCB (µg/g of beef) with increasing irradiation dose: **(**–△–) 15% fat samples; (--○--) 25 % fat samples.

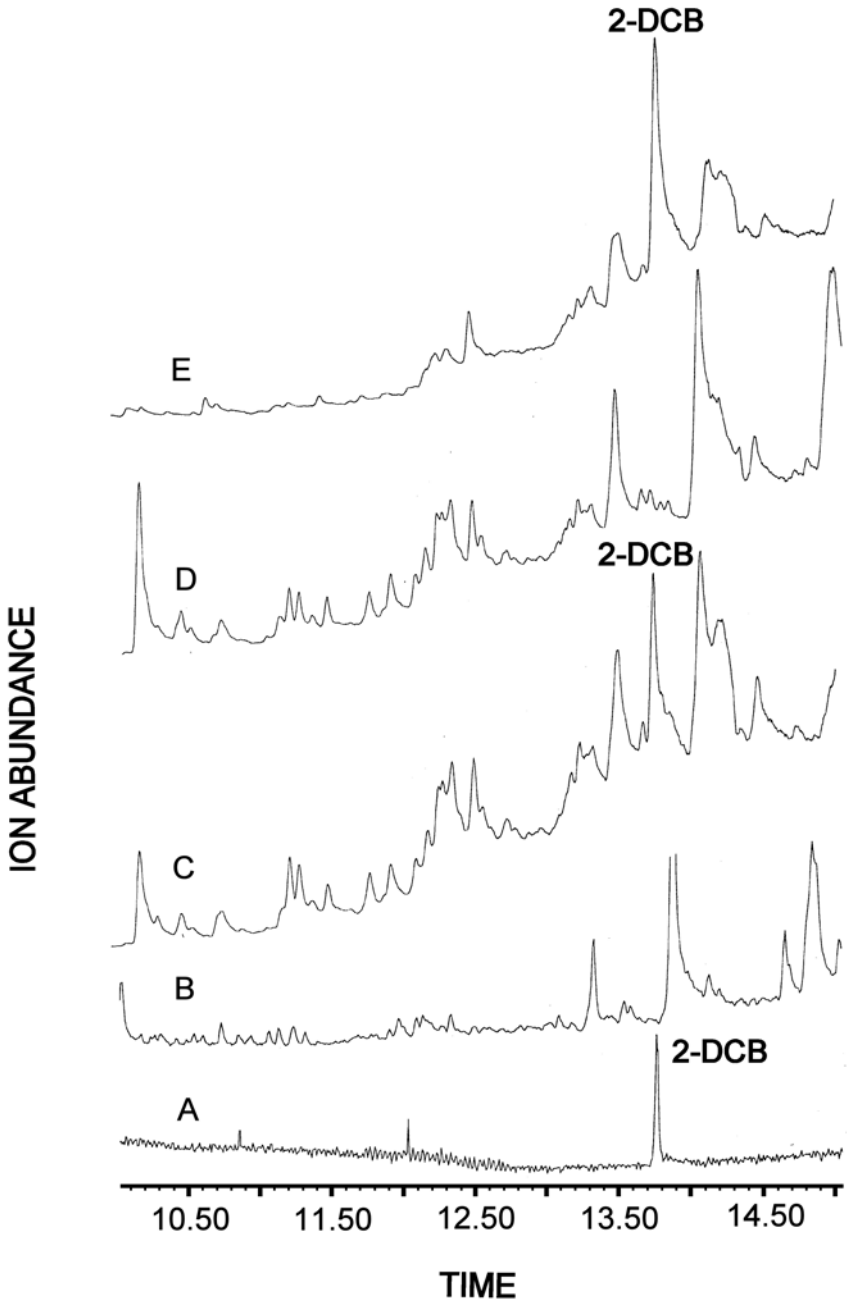

Figure 5: Chromatograms showing presence of 2-DCB in the irradiated samples. A) 2-DCB std, B) 25% fat control, C) 25% fat irradiated (4 kGy), D) 15% fat control E) 15% fat irradiated (4 kGy)

Table 6: Amount of 2-DCB in ground beef patties irradiated at 4 dose levels in µg/g of beef (ppm).

Targeted dose	Conc of 2-DCB (ppm)	
	15% fat	25% fat
1.0 kGy	0.03 ± 0.002	0.02 ± 0.004
2.0 kGy	0.06 ± 0.006	0.04 ± 0.003
3.0 kGy	0.20 ± 0.009	0.20 ± 0.008
4.5 kGy	0.31 ± 0.008	0.30 ± 0.009

Table 7: Estimated absorbed dose for all the commercial samples and average 2-DCB concentration using linear equations obtained from the 15 % and 25% samples

Using 15% line equation			
	Fat Level	Dose (kGy)	Ave conc of 2-DCB
Brand X	80%	1.54	0.06 ppm
	93%	1.45	0.05 ppm
Brand Y	80%	1.37	0.04 ppm
	90%	1.53	0.06 ppm
Using 25% line equation			
Brand X	80%	1.57	0.06 ppm
	93%	1.47	0.05 ppm
Brand Y	80%	1.39	0.04 ppm
	90%	1.56	0.06 ppm

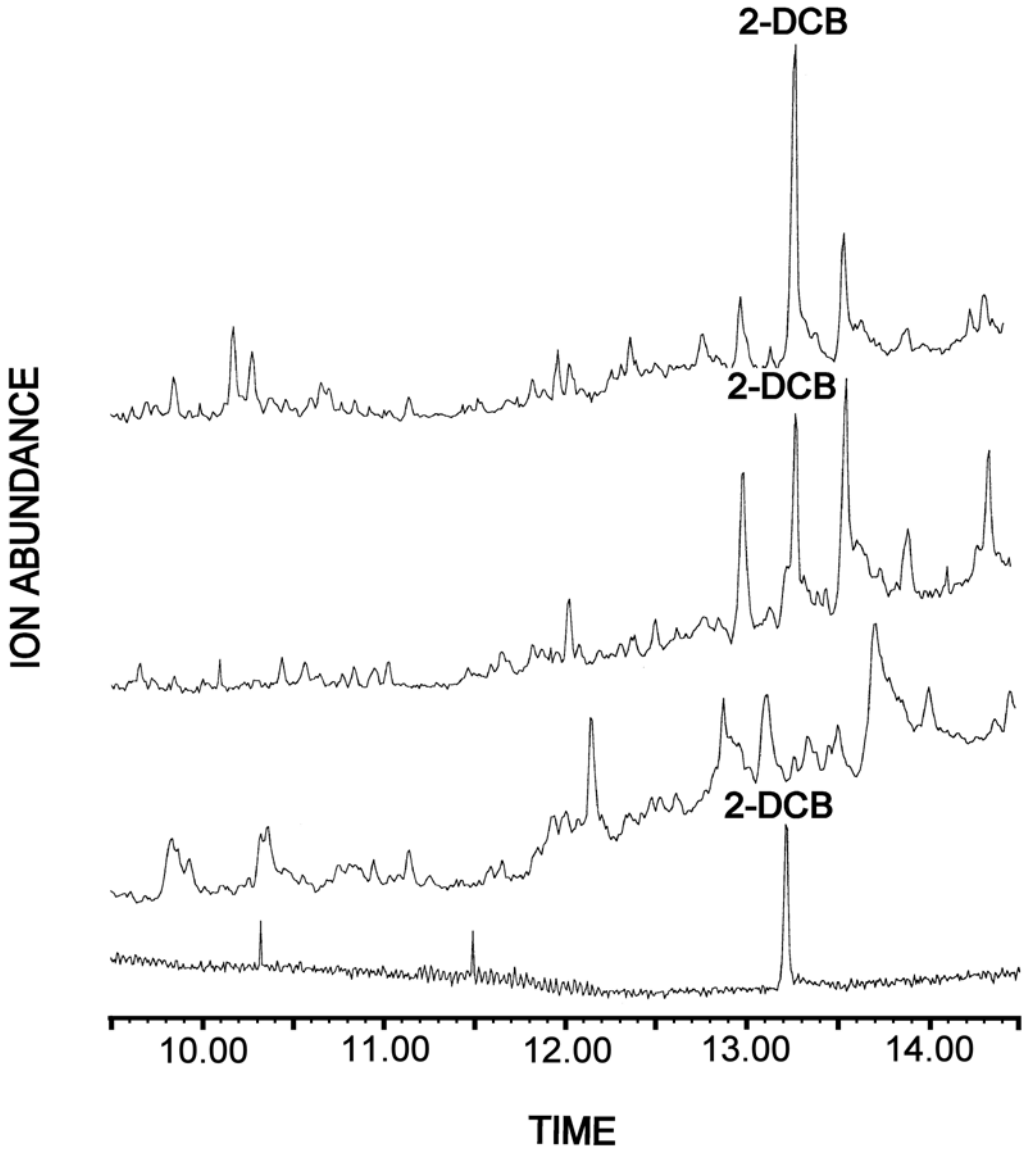

Figure 6: Chromatograms showing presence of 2-DCB in the commercially irradiated samples from Brand X. A) 2-DCB std, B) 25 % fat control, C) 20% fat irradiated, D) 7% fat irradiated

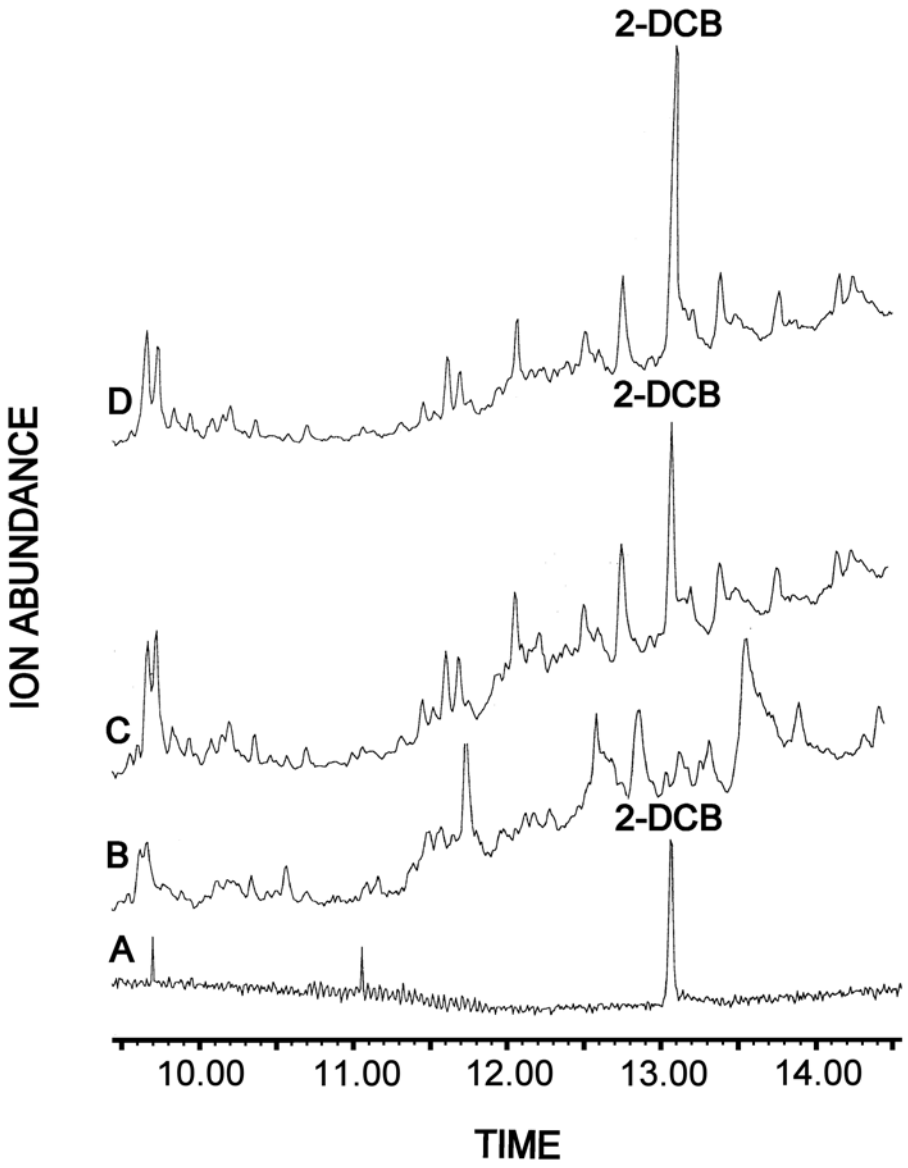

Figure 7: Chromatograms showing presence of 2-DCB in the commercially irradiated samples from Brand Y. A) 2-DCB std, B) 25% fat control, C) 20% fat irradiated, D) 10% fat irradiated.

The amount of 2-DCB was not significantly different in the patties formulated with or without origanox. The average amount of 2-DCB in the origanox patties (0.03 ± 0.006 ppm) was higher than in the irradiated controls (0.027 ± 0.003 ppm) but the difference was not significant (Figure 8).

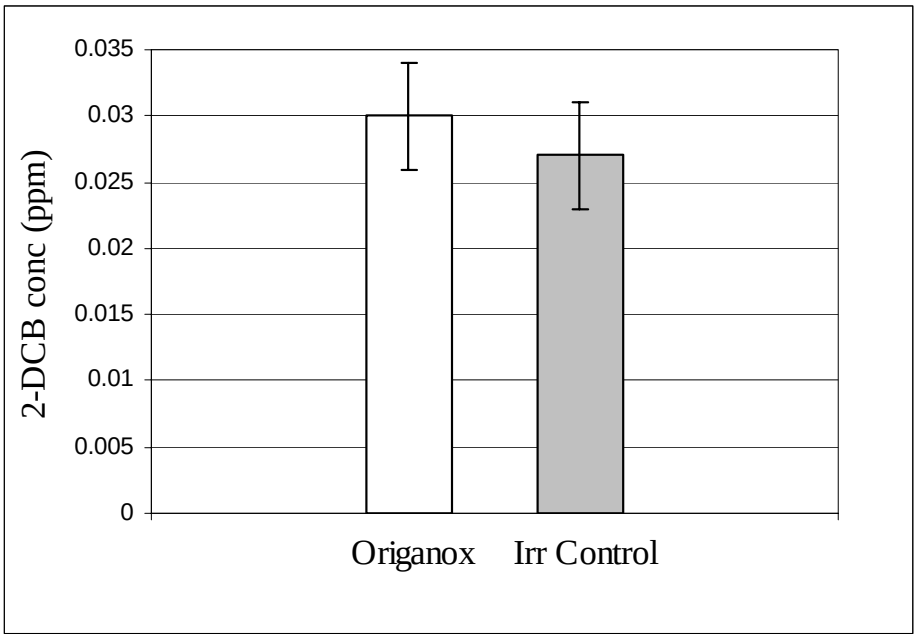

Figure 8: Amount of 2-DCB in patties irradiated at 3 kGy with and without Origanox

Figure 9 shows the GCMS chromatograms of patties with and without Origanox. It can be seen that there is no significant difference between the chromatograms. Audi (2005) found that Origanox was able to reduce some HCAs in beef patties but the results were not consistent. Therefore, Origanox may not be the most efficient of free radical scavengers for reducing 2-DCB. It is also possible that the level of Origanox was not high enough to be effective or that the irradiation temperature of 0 °C was too low. A higher irradiation temperature such 5 °C

might be more effective. Further studies using other antioxidants are required to see if 2-DCB formation will be affected. Some other antioxidants that can be tried include rosemary extracts, spice marinades, glutathione, etc.

Our results are in agreement with previous studies (Lee et al, 1999, Lee et al. 2000, Gadgil et al. 2002, Crone et al. 1992) and show that the applied dose can be estimated by monitoring 2-DCB formation. The results reiterated the efficacy of the SFE-GC-MS method in evaluating 2-DCB formation and consequently estimating the applied dose. The amount of 2-ACBs formed in irradiated ground beef is very low. Therefore, an extraction method that is efficient is very important. In addition to the high percent recovery, the method is rapid and does not involve excess use of organic solvents. The SFE method has the potential of being applied for routine monitoring of irradiated ground beef with respect to whether it has been irradiated and how much dose was absorbed. More studies are needed to investigate the influence of such variables as storage time and temperature on formation of 2-DCB and other 2-ACBs in ground beef. As further experiments are conducted, it will help establish the SFE-GC-MS method as the method of choice for evaluation 2-ACBs in irradiated ground beef and other lipid containing foods.

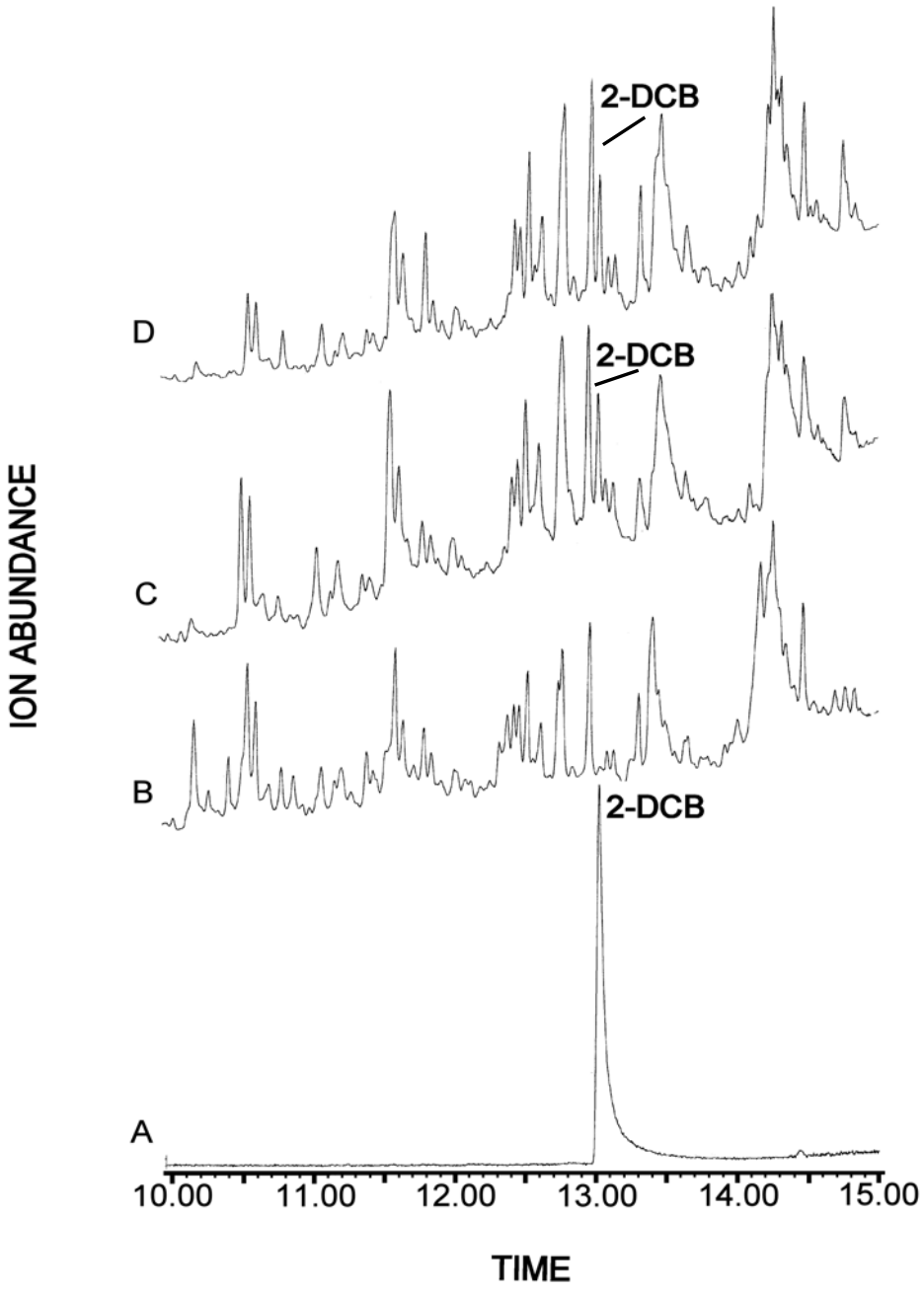

Figure 9: Chromatograms showing presence of 2-DCB in the irradiated samples with and without origanox. A) 2-DCB std, B) Control, C) 0.08% origanox, D) Irradiated control.

REFERENCES

Ackman, R. G. Remarks on official methods employing boron triflouride in the preparation of methyl esters of the fatty acids of fish oils. *J. Amer. Oil. Chem. Soc*. **1998,** 75, 541-545.

Audi, S. D. Evaluation of heterocyclic amine extraction and the analysis of antioxidants in spice extracts. MS Thesis. Kansas State University **2005.**

Barrington Nutritionals. http://www.barringtonchem.com/barNewart/therapeutic.htm. Accessed May 2006.

Crone, A. V. J; Hamilton, J. T. G.; Stevenson, M. H. Effect of storage and cooking on the dose response of 2- dodecylcyclobutanone, a potential marker for irradiated chicken. J. Sci. Food. Agric. **1992**, 58, 249-252.

European Union, Food and Feed Safety, European Committee for Standardisation (CEN). Detection of irradiated food containing fat - Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones. http://ec.europa.eu/food/food/biosafety/irradiation/1785-2003_en.pdf. (Accessed June 2006)

Food Safety and Information Service. Recall release FSIS-RC-061-2003. USDA, Washington, D.C **2003,** http://www.fsis.usda.gov/OA/recalls/prelease/pr061-2003.htm. Accessed May 2006.

Gadgil, P. G.; Hachmeister, K. A.; Smith, J. S.; Kropf, D. H.; 2-Alkylcyclobutanones as irradiation dose indicators in irradiated ground beef patties. *J. Agric. Food Chem*. **2002**, 50, 5746-5750.

Guidance on the petition process to request approval of labeling for foods that have been treated by irradiation; Availability. *Fed. Regist.* **2002**, 67 (194) 62487-62488.

Hamilton, L.; Elliot C. T; Boyd, D. R.; McCaughey, W. J.; Stevenson, M. H. The use of 2-substituted cyclobutanones in the development of an enzyme-linked immunosorbent assay (ELIZA) for the detection of irradiated foods. In *Detection Methods for Irradiated Foods-Current Status*, McMurray,, C. H.; Stewart, E. M.; Gray, R.; Pearce, J. (eds.), Royal Society of Chemistry, Special publication 171, Cambridge, UK, **1996,** pp 285-296.

Lee H. J; Lee Myung-yul; Kim, K. S. Detection of radiation induced hydrocarbons and 2-alkylcyclobutanones from peanuts. *J. Food Sci. Nutr*. **1999,** 4, 270-275.

Lee H. J; Byun, M. W; Kim, K. S. Detection of radiation induced hydrocarbons and 2-alkylcyclobutanones in irradiated perilla seeds. *J. Food. Prot.* **2000,** 63, 1563-1569.

LeTellier, M. H.; Nawar, W. W. 2-alkylcyclobutanones from the radiolysis of triglycerides. *Lipids.* **1972**, 7, 73-76.

MAFF validated Method V37. Method for the detection of Irradiated food containing fat. *J Assoc Publ. Analysts*, **1996,** 32, 39-52.

Meier, M.; Artho, A.; Nägeli, P. Detection of irradiation of fat containing foods by on-line LC-GC-MS of alkylcyclobutanones, *Mitt. Gebeite Lebensm. Hyg*, **1996a,** 87, 118-122.

Nawar, W. W.; Zhu, R.; Yoo, Y. J. Radiolytic products of lipids as markers for the detection of irradiated foods, in *Food Irradiation and the Chemist*, Johnston D. E.; Stevenson, M. H. (eds), Royal Society of Chemistry, Special Publication 86, Cambridge, UK, **1990,** pp 13-24

Lembke, P; Börnert, J; Engelhart, H. Characterization of irradiated food by SFE and GC-MSD. *J. Agric. Food Chem*. **1995,** 43, 38-45.

Stewert, E. M. Detection Methods for irradiated foods. In *Food irradiation: Principles and Applications*. Molins, R. Ed.; John Wiley & Sons Ltd: New York. **2001,** pp347-386.

Stewart E. M; McRoberts, W. C; Hamilton, J. T .G; Graham, W. D. Isolation of lipid and 2-alkylcyclobutanones from irradiated foods by supercritical fluid extraction. *J. AOAC. Int.* **2001,** 84, 973-986.

Tewfik, I. H.; Ismail, H. M.; Sumar, S. A rapid supercritical fluid extraction method for the detection of 2-alkylcyclobutanones in gamma irradiated beef and chicken. *Lebensm-Wiss. u.-Technol.* **1998**, 31, 366-370.

[2,3]PART III: ACUTE TOXICITY AND MUTAGENICITY OF 2-DODECYLCYCLOBUTANONE

ABSTRACT

Mutagenicity and acute toxicity of 2-dodecylcyclobutanone (2-DCB), a unique radiolytic product, were evaluated. Mutagenicity was evaluated by the Ames assay using 5 standard *Salmonella* tester strains with S9 enzyme activation and 5 concentrations of 2-DCB. Sodium azide (NaN_3), fenaminosulf, and 2-aminofluorene (2-AF) served as positive controls. The Ames assay showed no difference between the five concentrations of 2-DCB and the controls, including samples incubated with S9. The results indicate that 2-DCB does not produce point or frameshift mutations in *Salmonella* and is not activated by S9.

Acute toxicity of 2-DCB was evaluated by the Microtox acute toxicity system and compared with cyclohexanone and 2-nonenal (both GRAS additives). The effective concentrations that caused a 50% reduction in light emission by *Vibrio fischeri* cells (EC_{50}) were; 21.72 ± 14.57 ppm for 2-DCB, 37.40 ± 0.45 ppm for cyclohexanone and 1.65 ± 0.26 ppm for 2-nonenal. The maximum number of cells affected by 2-DCB was 65 ± 4 %, while it reached 90-100% for the other 2 compounds. Our results suggest that even though the EC_{50} for 2-DCB is lower than that for cyclohexanone, it was not toxic enough to decrease light emission of *V. fischeri* beyond 60-70%. These results indicate that the potential risk from 2-DCB, if any, is very low.

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2. Reproduced with permission from Gadgil, P., Smith , J. S. Mutagenicity and Acute Toxicity Evaluation of 2-Dodecylcyclobutanone. *J Food Sci.* **2004**. 69, (9), C713-6. doi:10.1111/j.1365-2621.2004.tb09921.x Copyright 2004 Institute of Food Technologists.

3. Abstract presented in part at IFT Annual Meeting 2003. http://ift.confex.com/ift/2003/techprogram/paper_18601.htm

INTRODUCTION

Ionizing radiation can cause changes in irradiated foods in much the same way as cooking. Irradiation of lipid-containing foods leads to the formation of a certain class of compounds called alkylcyclobutanones, which so far have only been detected in irradiated foods (Stewart, 2001). These compounds are formed when fatty acids are cleaved at the acyl-oxygen bond and undergo cyclization resulting in a molecule with the same number of carbon atoms as the parent fatty acid (LeTellier and Nawar 1972).

Alkylcyclobutanones have been detected in numerous lipid-containing foods and are generally accepted as irradiation indicators (Stevenson 1994). Some recent studies have raised the possibility of these compounds being weak genotoxins or cancer promoters when tested at high concentrations (Delincee and Pool-Zobel 1998; Raul et al. 2002; Delincee et al. 2002,). The authors of these papers themselves caution about misreading these results as the concentrations tested were many times what humans beings might ingest from irradiated foods. In addition, there are numerous long-term and short-term toxicity tests that have demonstrated the safety of irradiated foods (WHO 1981, 1999, 1994; Thayer et al. 1987; Thayer 1994;). In spite of these reports, some claim that irradiated foods are unsafe and have used the previous studies as proof that alkylcyclobutanones are carcinogenic (Public Citizen 2003). Therefore more studies evaluating the toxicity of these chemicals at high and low concentrations are needed to conclusively prove their safety.

This work examines the mutagenicity and acute toxicity of 2-DCB which is formed from palmitic acid. This chemical was selected as it is one of the most commonly formed cyclobutanones, and previous research conducted in this lab has shown 2-DCB to be an

irradiation dose indicator in ground beef (Gadgil et al. 2002).

The Ames assay is a bacterial reverse mutation assay which can detect a wide range of chemical substances that lead to gene mutations (Maron and Ames 1983; Mortelmans and Zeiger 2000). The test uses several histidine-dependent *Salmonella* strains carrying different mutations in various genes in the histidine operon. When these strains are grown on media containing trace amounts of histidine only those cells that revert, either spontaneously or due to a mutagen, to wild type, can form colonies. The numbers of colonies usually increase in a dose dependent manner in the presence of a mutagen. As there is a high correlation between mutagenicity in *Salmonella* and carcinogenicity in animals (80-90%), this assay is widely used as an initial screen to determine the mutagenicity of various chemicals (Maron and Ames 1983).

The Microtox system is a screening tool used for a variety of toxicity testing applications. The assay utilizes *Vibrio fischeri*, a marine bioluminescent bacterium. The inhibition of light production by *V. fischeri* in the presence of toxins forms the basis of this assay. The advantages of this toxicity bioassay are its speed, simplicity, and relatively low cost (Giesy and Hoke 1989). The bioassay's strongest attribute is its usefulness as a primary screening test for a broad spectrum of toxins and its monitoring capability over time (Ribo and Kaiser 1987).

Accordingly the 2 objectives of this study were to determine the mutagenic potential of 2-DCB using the Ames assay, and to compare the acute toxicity of 2-DCB to cyclohexanone and 2- nonenal using the Microtox assay. Cyclohexanone and 2-nonenal are carbonyl compounds like 2-DCB, and GRAS substances (Newberne et al. 2000; FDA 2003).

MATERIALS AND METHODS

Ames assay

All chemicals were purchased from Sigma chemical Co. (St. Louis, Mo.) and Molecular Toxicology, Inc. (Boone, NC). The 2-DCB standard was purchased from Acros Organics (Fisher Scientific Co., Pittsburgh, Pa). The preparation of the various solutions and agars used in this assay was achieved as described by Mortelmans and Zieger (2000). Minimal glucose agar was used for the spot and plate incorporation assay. Several solutions were aseptically prepared and used in the in the assay as well. These included the Vogel-Bonner medium E ; 0.5 mM histidine biotin and biotin solutions; and 8 mg/mL ampicillin and tetracycline solutions; the microsomal fraction of rat liver (S9); as well as the NADP solution.

Diagnostic mutagens

Three known mutagens sodium azide (NaN_3), p-(dimethylamino)benzenediazo sodium sulfonate (fenaminosulf) and 2-aminofluorene (2-AF) I (Sigma) were used in the assay as positive controls. The sodium azide solution (100 mg/mL) was prepared in distilled water whereas fenaminosulf (50 μg/mL) and 2-AF (20 μg/mL) solutions were prepared in dimethyl sulfoxide (DMSO) (Sigma).

Salmonella enterica serovar Typhimurium tester strains

Five freeze-dried *Salmonella* tester strains, TA97, TA98, TA100, TA102, and TA 1535, were purchased from Molecular Toxicology, Inc. The vials containing these lyophilized strains on STDisc™ were warmed at room temperature for 5 min and aseptically uncapped. For each

strain, 2 discs were removed and introduced in a 125-mL flask containing 25 mL nutrient broth. Ampicillin was added to the TA 100 flask to achieve a final concentration of 25 μg/mL. The flasks were covered with aluminum foil and incubated at 37 °C in a Dubnoff metabolic incubator (Precision Scientific, Chicago, Il.) shaking at medium speed for 10 hours. Absorbance of the cultures was read at 650 nm with a Spectronic 1001 spectrophotometer (Bausch and Lomb, USA), using the non inoculated nutrient broth as reference. Absorbance values between 0.9-1.8 indicated there were enough viable bacterial cells for the Ames test. These fresh overnight nutrient broth cultures were used for the confirmation of the genotypes of the tester strains, for the spot test and the plate incorporation test.

Rat liver microsomes

Freeze-dried Moltox TM post-mitochondrial supernatant (S9) was purchased from Molecular Toxicology, Inc. This mitochondrial supernatant was prepared from the liver of male Sprague Dawley rats, which were induced with Aroclor 1254. Vials of Moltox^{TM} nicotinamide diphosphate (NADP) were also purchased from Molecular Toxicology, Inc. Forty six mg (1 vial) of NADP (Reagent B) was dissolved in 13.5 mL of a cofactor solution (Reagent A) to obtain a 10% S9 solution.

Confirmation of genotypes

After culturing the strains, their genotypes were confirmed prior to the bioassay. Five genotypic characteristics were verified as described by Maron and Ames (1983). These were *his* (histidine requirement), *rfa* mutation (crystal violet sensitivity), *uvrB* (UV sensitivity), *amp*^{r}

(ampicillin resistance, presence of R-factor), and *amp*^{r}, *tet*^{r} (ampicillin/tetracycline resistance, presence of pAQ1 plasmid).

Mutagenicity assay

The spot test and the plate incorporation test were performed as described by Mortelmans and Zeiger (2000). The spot test was carried out by spotting a single 2-DCB concentration of 0.1 mg/plate in the center of the petri dish containing minimal glucose agar. Four concentrations of 2-DCB in DMSO, 0.25, 0.5, 0.75, and 1.0 mg/plate, were used for the plate incorporation test. The spot test and plate incorporation tests were repeated with addition of S9 extract to check for microsomal activation. Three positive controls, 2-aminofluorene (20 μg/plate), sodium azide (1 μg/plate), and fenaminosulf (50 μg/plate) were included in the tests, in addition to negative controls.

For the plate incorporation test, 100μL of a fresh overnight culture of each strain, and the appropriate amount of 2-DCB were added to 2 mL of top agar (with traces of histidine and biotin), in a culture tube. For microsomal activation test, 500 μL of the 10% S9 extract was also added. The contents were mixed, poured onto minimal glucose agar, and allowed to harden on a flat level surface for a few minutes. For solvent controls 100 μL of DMSO was added to the plate instead of 2-DCB. To determine spontaneous mutants strains without added solvent were plated and served and negative controls. The petri dishes were then inverted and incubated at 37 °C for 48 h. The assay was repeated three times. Replicate one was plated in duplicate with replicates two and three were in triplicate. The colonies were counted at the end of 48 h and comparisons were made with positive, solvent and negative controls. In this study, the 2-fold

rule along with an examination of the dose-response relationship was applied to evaluate the data (Kim and Margolin 1999).

Microtox assay

Lyophilized (freeze-dried) Microtox reagent (*V. fischeri*), osmotic reconstitution solution, and diluent were purchased from Azur Environmental in Carlsbad, Ca. Dimethylsulfoxide (DMSO) was purchased from Sigma . Cyclohexanone and 2-nonenal were purchased from Fisher Scientific Co.

Apparatus

This investigation was carried out with a Microtox Model 500 Toxicity Analyzer System (Azur Environmental, Carlsbad, CA). The apparatus was equipped with a 30-well incubator block, a Reagent well and a Read well, all of which were temperature-controlled by an internal incubation unit. Operating temperatures were internally regulated, and set at 5.5 °C ± 1 °C for the reagent vials, and 15 °C ± 0.5 °C for both the Incubator block and the Read well. Light output was read from the digital display.

The organic solvent solubilization of sample test was carried out as described the basic test procedures manual (Azur Environmental 1995). The procedure measured the relative acute toxicity of each chemical by producing data for the calculation of chemical concentration effecting a 50% reduction of bioluminescence (EC_{50}). For each test run, 2 controls without any chemical and 8 sample two-fold dilutions with 2 replicates were used. The solvent was 1% DMSO in the assay diluent. The test samples and diluent controls were equilibrated to the

required temperature (15 °C ± 0.5 °C). One vial of lyophilized *V. fischeri* was hydrated in 1 mL of reconstitution solution, and the light outputs were measured before and after the desired incubation time (5 min). The diluent controls were run simultaneously, and the experiment was repeated 6 times for 2-DCB and 3 times each for cyclohexanone and 2-nonenal. Results were calculated in terms of the ratio of the light lost during test time to the light at initial time (time 0). A dose-response relationship curve was constructed by plotting the percentages of reduction in bioluminescence against test chemical concentrations. The EC_{50} values were determined as the concentrations corresponding to a 50% decrease in light output. Supporting computer software (Microtox Omni for Windows 95/98/NT) was used to calculate the light input, the ratio, EC_{50} , and to generate the dose-response curves.

RESULTS AND DISCUSSION

Mutagenicity assay

The strains acquired for the Ames test exhibited the expected phenotypes and with the the spot test similar revertant colonies were obtained with and without addition of the rat liver S9 mix (microsomal activation). Also, the number of revertant colonies observed with the tester strains were similar to those for the negative controls and solvent controls. The colonies were uniformly distributed indicating that 2-DCB was not mutagenic at a concentration of 0.1 mg/plate. A mutagenic chemical will rise to a ring of revertant colonies surrounding the area where the chemical was applied.

Figure 10 and Figure 11 show the results of the plate incorporation test on a range of 2-DCB concentrations (0.25,0.5,0.75, and 1.0 mg /plate) and with 10% S9. Over this

concentration range, the number of revertants were similar for each strain, and were comparable to the solvent controls and spontaneous mutation rates. Figures 1 and 2 indicate that there is no linear dose-response relationship between 2-DCB dose and the number of revertant colonies obtained for any of the 5 tester strains, with or without S9.

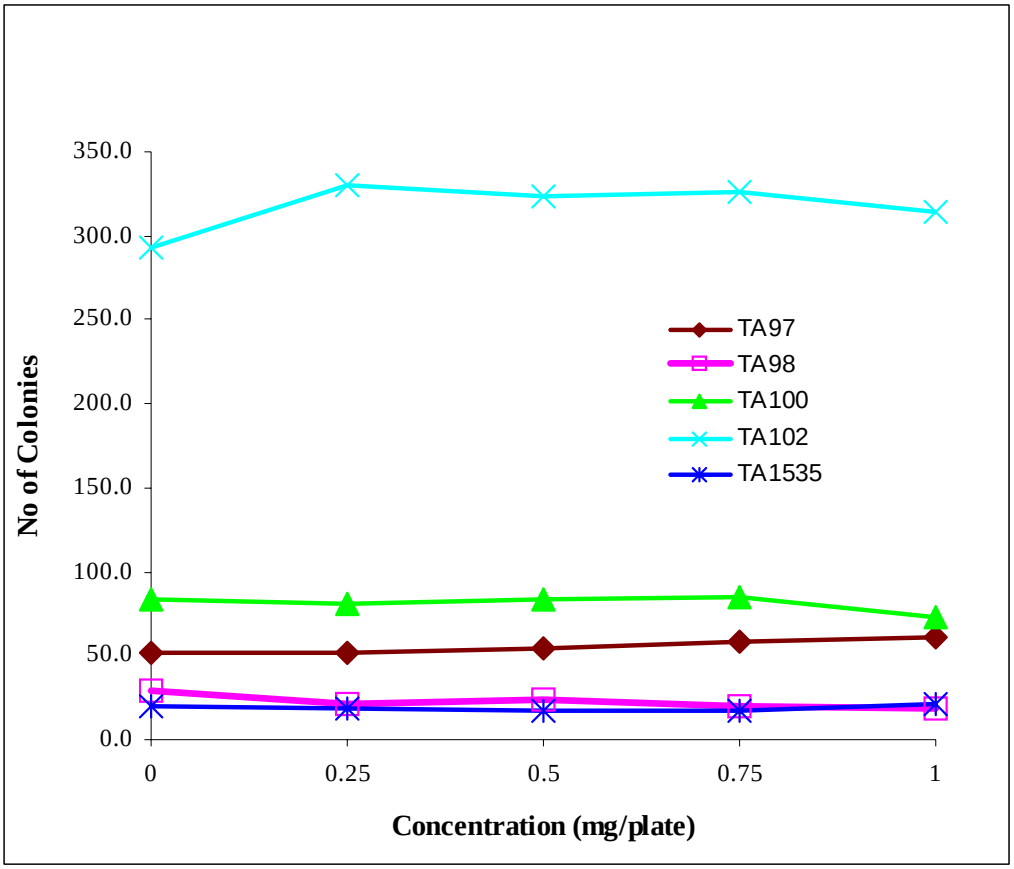

Figure 10: Effect of concentration of 2-DCB on the number of colonies in the plate incorporation test without S9. The concentrations tested were 0.25 0.5, 0.75, and 1.0 mg/plate without S9. Each point represents the average value from all three replicates (n=8).

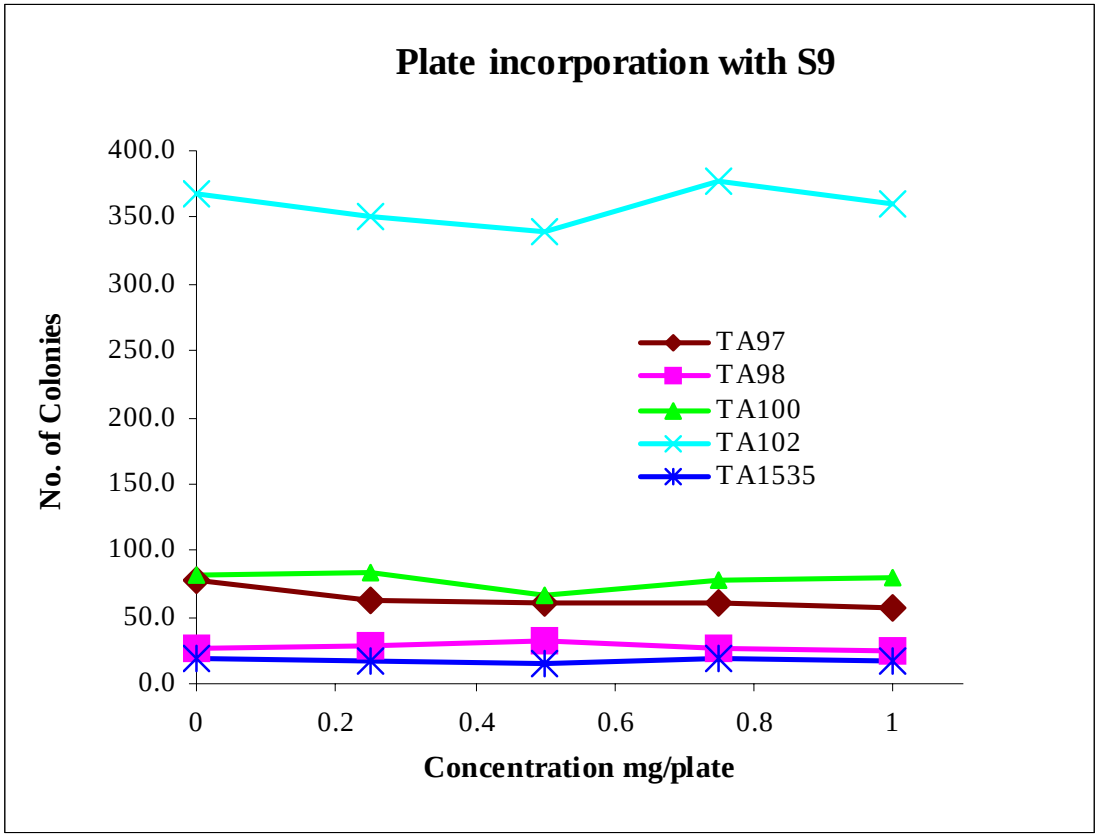

Figure 11: Effect of concentration of 2-DCB on the number of colonies in the plate incorporation test with S9. The concentrations tested were 0.25 0.5, 0.75, and 1.0 mg/plate with 500 µL of S9. Each point represents the average value from all three replicates (n=8).

The positive controls elicited the expected mutagenic response as described by Maron and Ames (1983) and Mortelmans and Zeiger (2000). The range of number of colonies obtained for the positive controls is shown in Table 1. Fenaminosulf and 2-AF caused mutations in all the strains tested (3 to 6 fold over controls). The positive result with 2-AF confirms the effectiveness of the S9 mix. This chemical is a pro-mutagen that must be bio-transformed to be mutagenic. In addition, NaN_3 was mutagenic to TA100 and TA1535.

Table 8: Average number of revertant colonies obtained per plate for each of the strains using positive controls (n=6).

Chemicals	**µg tested**	**Added S9 (µL)**	No. of colonies for each *Salmonella* tester strain				
			TA97	TA98	TA100	TA102	TA1535
Fenaminosulf	50 µg	0	497	188	NT	995	NT
2-AF	20 µg	500	509	1986	688	1098	NT
NaN_3	1 µg	0	NT	NT	710	NT	618
Negative control		0	42	29	100	266	21

NT = not tested on this strain.

The lack of dose-response relationship, in conjunction with the other results, like similar number of revertants for negative controls and test solutions, no activation by S9, suggest that 2-DCB is not mutagenic to the *Salmonella* tester strains used in this project. Therefore, 2-DCB did not cause any base-pair substitutions in TAI00 and TA1535 strains, nor did it cause any frameshift mutation in TA97 and TA98 strains, or an ochre mutation in TAI02.

Our results do not agree with studies which have shown 2-DCB to be cytotoxic and potentially genotoxic (Delincee and Pool-Zobel 1998; Delincee et al. 2002, Knoll et al. 2006). These studies used the comet assay to increases in DNA strand breakage which can produce false positives when cell viability is reduced (Sommers 2000). Furthermore, as the concentrations of 2-DCB tested in these tests were very high (1.25mg/mL), the authors of these papers themselves caution against using their data to infer that 2-DCB is a carcinogen.

Numerous long-term and short-term studies performed on the safety of irradiated foods have helped form a consensus about the safety of irradiated foods. Irradiated foods have not shown any mutagenic activity (Thayer et al. 1987) in bacterial reverse mutation assays, in contrast to some thermally processed foods where mutagenic activity is well known (Vizzani et al. 1983; Green and Fufua 1987). Our results support the findings of these studies and are in agreement with those of Sommers (2003) who found that 2-DCB is not mutagenic in the *E.coli* reverse mutation assay and did not cause chromosomal strand breaks in the Yeast DEL assay (Sommers and Schiestl 2004).

Microtox assay

Figure 12 shows the dose-response curves for the 3 compounds tested and it can be seen that the toxicity of 2-DCB is between than of cyclohexanone and 2-nonenal. The EC_{50} values for the three chemicals tested were 21.72 ± 14.57 ppm for 2-DCB, 37.40 ± 0.45 ppm for cyclohexanone, and 1.65 ± 0.26 ppm for 2-nonenal. Even if we consider the higher limit of the standard deviation for 2-DCB, we would get an EC_{50} value of 7.2 ppm which represents a lower toxicity than that of 2-nonenal. This indicates that according to the Microtox assay 2-DCB

would not represent a greater risk compared to 2- nonenal, a GRAS additive.

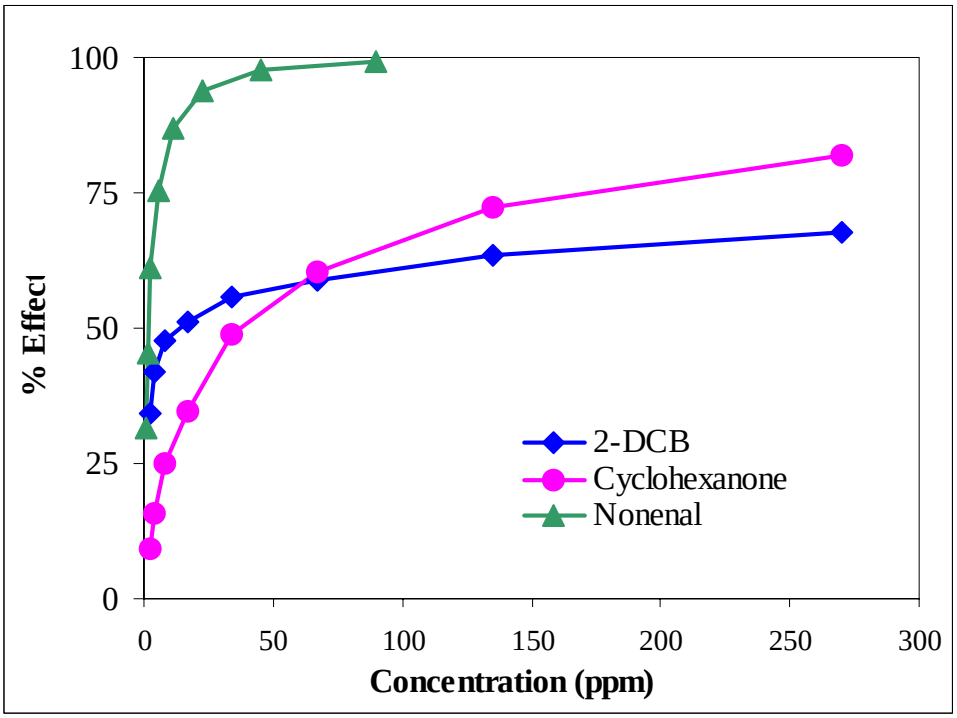

Figure 12: Effect of concentration of 2-DCB, cyclohexanone, and 2-nonenal on light emission by *V. fischeri*. Concentrations represent 8 serial 2 fold dilutions from a maximum concentration of 270 ppm for 2-DCB and cyclohexanone and 90 ppm for 2-nonenal.

The high variability for 2-DCB might be due to the poor solubility of 2-DCB in the test solution at higher concentrations. The maximum percent effect due to 2-DCB was 65 ± 4 %, while it reached 90-100% for the other 2 compounds. When comparing toxicity of chemicals, 2 parameters can be examined, namely, potency and efficacy. Potency is the range of doses over

which toxicity is observed and efficacy is the maximum toxic effect elicited by the chemical (Eaton and Klaasen 2001). The Microtox assay suggests that 2-DCB was not toxic enough to bring more than a 60-70% decrease in light production by *V. fischeri*. Therefore, compared to the other chemicals, 2-DCB had the lowest numerical value for maximum efficacy.

REFERENCES

Azur Environmental. Microtox - Acute Toxicity Basic Test Procedures. **1995**, Carlsbad, Calif. Delincee H, Pool-Zobel BL. Genotoxic properties of 2-dodecylcyclobutanone, a compound formed on irradiation of food containing fat. *Radiat Phys. Chem*. **1998**, 52, 39-42.

Delincee H.; Soika C.; Horvatovich P.; Rechkemmer G.; Marchioni E. Genotoxicity of 2-alkylcyclobutanones, markers for an irradiation treatment in fat-containing food-Part I: cyto- and genotoxic potential of 2-tetradecylcyclobutanone. *Radiat. Phys. Chem.* **2002,** 63, 431-435.

Eaton D. L.; Klaassen C. D. Principles of toxicology. In: Klaassen C. D. editor. Casarett and Doull's Toxicology: The basic science of problems. 6th ed. New York:McGraw Hill. **2001,** pp 11-34.

FDA. EAFUS: a food additive database. U.S. Food and Drug Administration. **2003.** http://vm.cfsan.fda.gov/~dms/eafus.html. Accessed May2006.

Gadgil P. G.; Hachmeister K. A.; Smith J. S.; Kropf D. H. 2-Alkylcyclobutanones as irradiation dose indicators in irradiated ground beef patties. *J Agric Food Chem.* **2002**, 50, 5746-5750.

Giesy J. P.; Hoke R. A. Freshwater sediment toxicity bioassessment: Rationale for species selection and test design. *J. Great Lakes Res*. **1989,** 15, 539-569.

Green N. R.; Fugua D. L.Mutagenic activity of heat processed foods as determined by the Ames *Salmonella*/ mutagenicity assay. *J Food Safety.* **1987**, 8, 261-266.

Kim S. B.; Margolin B. H. Statistical methods for the Ames *Salmonella* assay: a review. *Mutation Res. 1991,* 436, 113-122.

Knoll, N.; Weise, A.; Glei, M.; Claussen, U.; Marian, B.; Pool-Zobel, B. L. 2-Dodecylcyclobutanone, a radiolytic product of palmitic acid, causes DNA strand breaks and chromosomal aberrations in human colon adenoma cells. *Mutat. Res*. **2006**, 594, 10-19.

LeTellier M. H.; Nawar W. W. 2-alkylcyclobutanones from the radiolysis of triglycerides. *Lipids* **1972,** 7, 73-76.

Maron D. M.; Ames B. N.. Revised method for the Salmonella mutagenicity test. *Mutation Res* **1983,** 113, 173-215.

Mortelmans K., Zeiger E. The Ames *Salmonella*/microsome mutagenicity assay. *Mutation Res.* **2000**, 455:61-69.

Newberne P.; Smith R. L.; Doull J.; Feron V. J.; Goodman J. I.; Munro I. C.; Porthoghese P. S.; Waddel W. J.; Wagner B. M.; Weil C. S.; Adams T. B.; Hallagan J. B. GRAS flavoring substances 19. **2000,** *Food Tech*. 54, 6, 66-74.

Public Citizen. What's in the beef? A special report by Public Citizen and The Center for Food Safety, Washington, D.C. **2003.** http://www.citizen.org/documents/beeftesting.pdf Accessed May 2006.

Raul F.; Gosse F.; Delincee H.; Hartwig A.; Marchioni E.; Miesch M.; Werner D.; Burnouf D. Food-borne radiolytic compounds (2-alkylcyclobutanones) may promote experimental colon carcinogenesis. *Nutrition and Cancer* **2002,** 44, (2), 188-191.

Ribo J. M.; Kaiser K. L. E. *Photobacterium phosphoreum* toxicity bioassay. I. Test procedures and applications. *Toxicity Assess.* **1987,** 2, 305-323.

Sommers C. H. 2-Dodecylcyclobutanone does not induce mutation in the *Escherichia coli* reverse mutation assay. *J Agric Food Chem.* **2003,** 51, 6367-6370.

Sommers, C. H.; Schiestl, R. H. 2-dodecylcyclobutanone does not induce mutations in the *Salmonella* mutgenicity test or intrachromosomal recombinationin *Saccharomyces cerevisiae*. *J. Food Prot.* **2004**, 67, 1293-1298

Stewart, E. M. Detection methods for irradiated foods. In: *Food Irradiation*. Molins R, editor New York: John Wiley and Sons. **2001**, pp 347-386.

Stevenson M. H. Identification of irradiated foods. *Food Technology*. **1994,** 48, 5, 141-144.

Thayer D. W.; Christopher J. P.; Campbell L. A.; Ronning D. C.; Dahlgreen R. R.; Thompson G. M.; Wierbicki E. Toxicology studies of irradiation-sterilized chicken. *J Food Prot.* **1987,** 50, 278-288.

Thayer D. W. Wholesomeness of irradiated foods. Food Technology **1994,** 48, (5), 132-135.

Vizzani A.; di Antonio E.; Domici S. Mutagenic activity in heat treated foods of animal origin. *Ind Aliment*. **1983,** 22(20):9-11 .

WHO. Wholesomeness of irradiated food report of a Joint FAO/IAEA/WHO Expert Committee, World Health Organization. **1981**, Technical Report Series No. 659.

WHO. Safety and nutritional adequacy of irradiated Food. Geneva: World Health Organization. **1994,** pp 81-107.

WHO. High-dose irradiation: Wholesomeness of food irradiated with doses above 10 kGy. Report of a Joint FAO/IAEA/WHO Study Group. **1999**,Technical Report Series No. 890.

[4,5]PART IV: METABOLISM OF 2-DODECYLCYCLOBUTANONE IN RATS

ABSTRACT

Alkylcyclobutanones (2-ACBs) are suspected cancer promoters and clastogens, which have raised concerns about the safety of irradiated foods. Currently there is little data on the metabolism of 2-ACBs which makes it very important to study this aspect of 2-ACBs in order to evaluate their safety. The objectives of this experiment were to quantify 2-DCB (formed from palmitic acid) in feces and adipose tissue of rats and to check for metabolites of 2-DCB in the urine. Six Female Sprague-Dawley rats were administered 2-DCB (5 mg/day) in corn oil for five days via gavage. Six control rats did not receive 2-DCB. Feces and urine were collected daily while adipose tissue was collected upon euthanasia. Hexane extracts of feces and adipose tissue were analyzed by gas chromatography-mass spectrometry (GC-MS). Urine with and without added β-glucuronidase, was monitored for glucuronide complexes by hexane extraction and GC-MS. The total amount of 2-DCB recovered in feces was 1.78 ± 0.63 mg at the end of 5 days which represents between 3-11% of the total 2-DCB administered. The total amount recovered in the adipose tissue was 0.08 ± 0.01 mg which was about 0.33% of the total 2-DCB administered. No metabolites were recovered in any of the urine extracts. The results show that at most 11% of the 2-DCB was recovered unchanged in the feces and adipose tissue. This indicates that either most of 2-DCB is metabolized, and rapidly eliminated from the body, or stored at sites other than adipose tissue.

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4. Publication: Metabolism of 2-dodecylcyclobutanone, a Radiolytic Compound Present in Irradiated Beef. *J. Agric. Food Chem*, 2006, 54, (13) 4896 - 4900; DOI: 10.1021/jf060840i. Copyright 2006 American Chemical Society.

5. Abstract presented in part at IFT Annual Meeting 2006.
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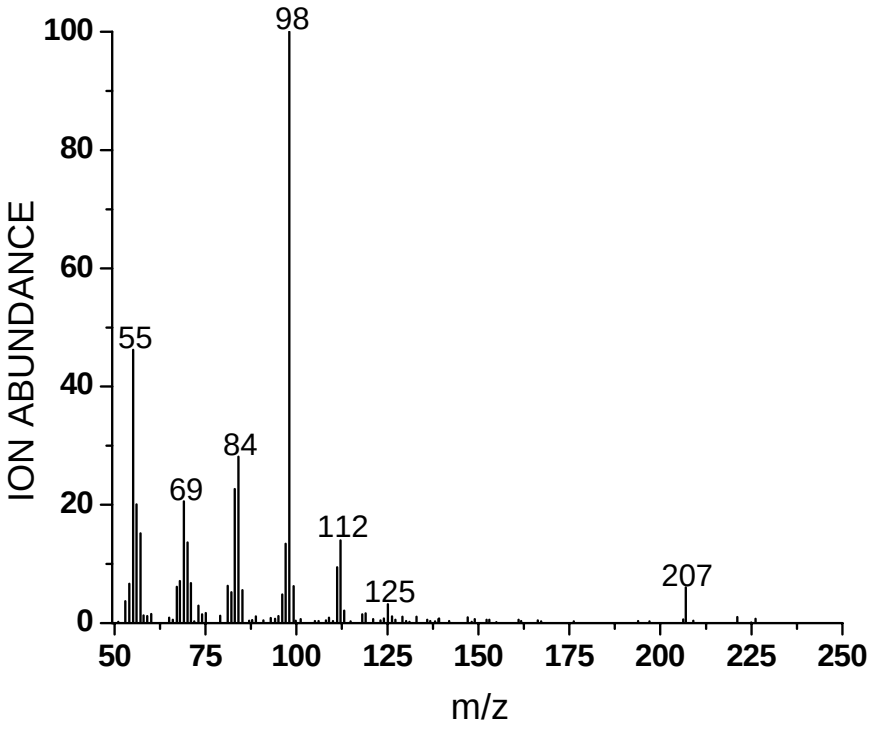

Figure 13 : Mass spectrum of 2-DCB

Table 9: Amount of 2-DCB recovered in the feces from days 3-5 combined and percent of the total amount of 2-DCB administered (25 mg). The total amount was averaged from duplicate extracts and assays

Rat number	Total amount of 2-DCB in feces	Percent of total 2-DCB
1	1.99 mg	7.97
2	0.96 mg	3.84
3	2.04 mg	8.17
4	1.75 mg	6.98
5	1.20 mg	4.81
6	2.71 mg	10.85
Average	**1.78 ± 0.63**	**7.10 ± 2.52**

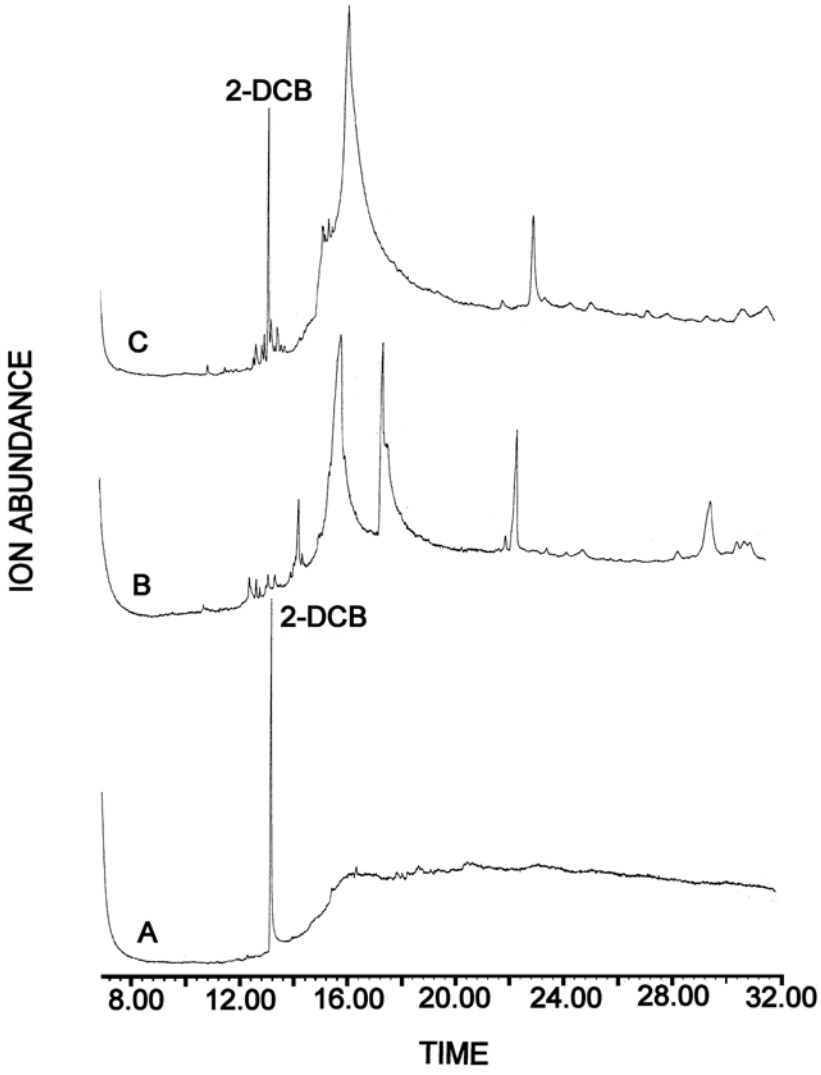

Figure 14: Chromatograms showing presence of 2-DCB in the feces of rats. A) 2-DCB std, B) Control feces, C) Treatment feces

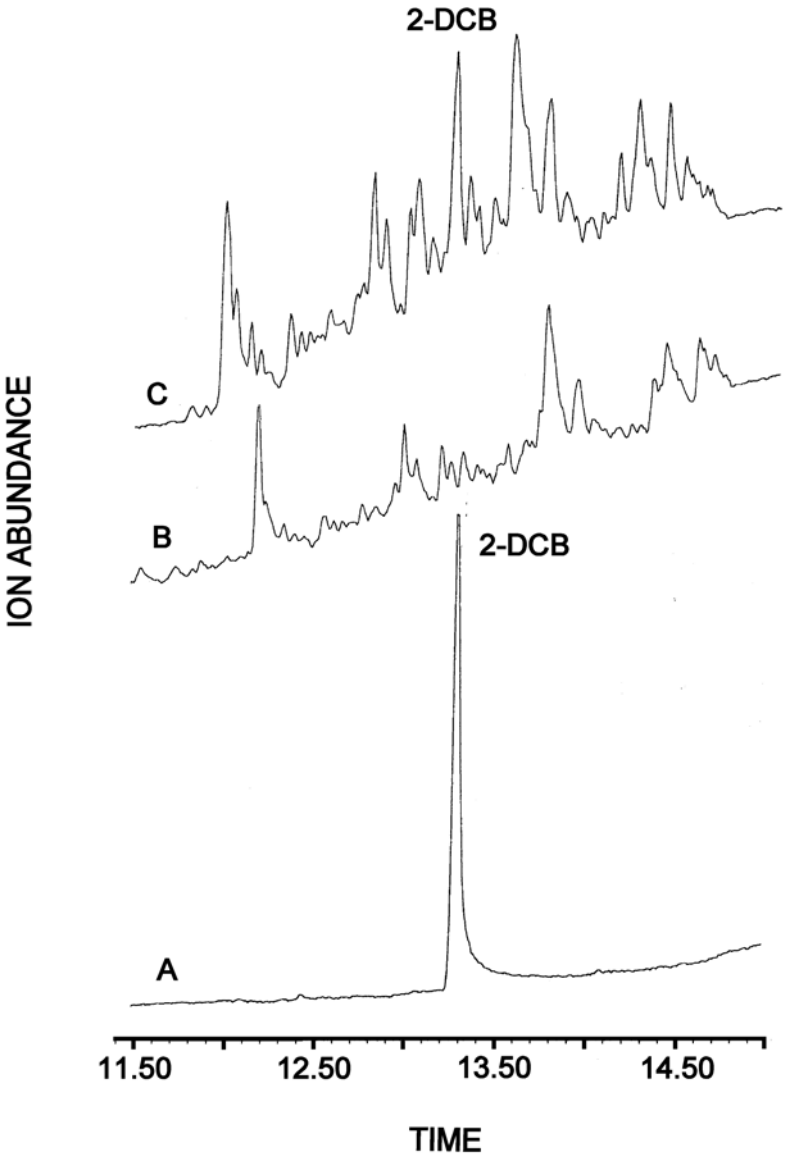

Figure 15: Chromatograms showing presence of 2-DCB in the adipose tissue of rats. A) 2-DCB std, B) Control fat, C) Treatment fat.

SUMMARY AND CONCLUSIONS

The SFE method was able to efficiently extract the 2-DCB from irradiated ground beef. The amount of fat in the beef did not significantly effect the amount of 2-DCB formed. This indicates that 2-DCB can be used to monitor the absorbed dose for samples containing different fat levels. The calculated absorbed dose for the commercial samples was within the limits used by the irradiation facility. Thus, 2-DCB can be used to estimate the absorbed dose for commercially available patties.

The 2-DCB was found to be non mutagenic in the Ames assay. It did not cause frameshift or point mutations in any of the *Salmonella* tester strains used in this experiment. Further, 2-DCB was not activated by S9 microsomal extract indicating that it was not biotransformed into mutagenic by-products. The acute toxicity of 2-DCB was between that of cyclohexanone and 2-nonenal as determined by the Microtox assay. The maximal efficacy of 2-DCB was the lowest of the three chemicals tested. Therefore it can be concluded the toxicity of 2-DCB was comparable to the two GRAS compounds.

When 2-DCB was administered to rats, an average of 7.2 ± 2.4% of the total amount was recovered from the feces. About 0.03% was recovered from the adipose tissue. Hypothesized metabolites of 2-DCB, the alcohol derivative or lactone derivative were not identified in the urine. The results indicate that most of the 2-DCB is metabolized by the rats. The metabolite/s may be excreted or stored in adipose or other tissues in rats. The best way to analyze for these metabolites would be to use a C-13 labelled 2-DCB standard. Analysis of various organs, urine and expired air can then be analyzed by an isotope ratio mass spectrometer. If 2-DCB metabolites are present in any samples, a larger than expected ratio of C-13 is detected. Thus, it

can be known where the metabolites may be concentrated and therefore isolation procedures can be worked out in order to isolate and identify these compounds.

The risk of 2-DCB consumption should be presented in the proper perspective. The toxicity 2-DCB is comparable to GRAS compounds and clastogeninc properties are comparable to palmitic acid. Furthermore, based on the research so far, 2-DCB has not been found to be more toxic or carcinogenic than furan or HCAs, both of which are known mutagens and are commonly found in cooked foods. Further research investigating other 2ACBs, and metabolic profiling of 2-DCB is necessary to augment the information about 2-ACB safety. At the present time, all the evidence indicates that the risks from 2-DCB consumption at expected levels in food are minimal.

APPENDIX

TABLES PAGE

FIGURES PAGE

Enzymatic Assay of β-glucuronidase
(EC 3.2.1.31) From Bovine Liver
http://www.sigmaaldrich.com/sigma/enzyme%20assay/bglucuronidase.pdf.
Accessed March 2006.

PRINCIPLE:

PheP-Gluc + H2O b-Glucuronidase> D-Glucuronate + Phenolphthalein

(Abbreviation: PheP-Gluc = Phenolphthalein Glucuronide)

CONDITIONS: T = 37˚C, pH = 5.0, A540 nm, Light path = 1 cm

METHOD: Spectrophotometric Stop Rate Determination

REAGENTS

A. 100 mM Sodium Acetate Buffer, pH 5.0 at 37˚C (Prepare 50 ml in deionized water using Sodium Acetate, Trihydrate, Sigma Prod. No. S-8625. Adjust to pH 5.0 at 37°C with 1 M Hcl.)

B. 1.2 mM Phenolphthalein Glucuronide Substrate Solution (PheP-Gluc) (Prepare 10 ml in deionized water using of Phenolphthalein Glucuronic Acid, Sodium Salt, Sigma Product No. P-0376.)

C. 200 mM Glycine Buffer Solution, pH 10.4 (Use Glycine Buffer Solution, Sigma Stock No. 105-2 or prepare 100 ml in deionized water using Glycine Free Base, Sigma Prod. No. G-7126. Adjust to pH 10.4 at 37 °C with 1 M NaOH.)

D. 0.2% (w/v) Sodium Chloride Solution (NaCl) (Prepare 20 ml in deionized water using Sodium Chloride, Sigma Prod. No. S-9625.)

E. E. b-Glucuronidase Enzyme Solution (Immediately before use, prepare a solution

containing 250 - 500 units/ml of b-Glucuronidase in cold Reagent D.)

F. 95% (v/v) Ethanol (Prepare 20 ml in deionized water using 200 Proof USP)

G. 0.05% (w/v) Phenolphthalein Standard Solution (Std Soln) (Prepare 2 ml by dissolving 1.0 mg of Phenolphthalein, Sigma Prod. No. P-9750 in Reagent F.)

PROCEDURE

Pipette (in milliliters) the following reagents into suitable containers:

	Test	Blank
Reagent A (Buffer)	0.70	0.70
Reagent B (PheP-Gluc)	0.70	0.70

Mix by inversion and equilibrate to 37˚C. Then add:

Reagent E (Enzyme Solution)	0.10	------

Mix by inversion and incubate at 37˚C for exactly 30 minutes. Then add:

Reagent C (Glycine Buffer)	5.00	5.00
Reagent E (Enzyme Solution)	------	0.10

Immediately mix by inversion. Transfer the solutions to suitable cuvettes and record the A540nm for both the Test and Blank using a suitable spectrophotometer.

COLORIMETRIC ASSAY

Standard Curve:

Prepare a standard curve by pipetting (in milliliters) the following reagents into suitable containers:

	Std 1	Std 2	Std 3	Std 4	Blank
Reagent A (Buffer)	0.70	0.70	0.70	0.70	0.70
Reagent B (PheP-Gluc)	0.70	0.70	0.70	0.70	0.70
Reagent G (Std Soln)	0.03	0.05	0.07	0.10	----
Reagent F (Ethanol)	0.07	0.05	0.03	----	0.10
Reagent C (Glycine Buffer)	5.00	5.00	5.00	5.00	5.00

Mix by inversion and transfer the standards to suitable cuvettes. Record the A540nm for each standard using a suitable spectrophotometer.

CALCULATIONS

Standard Curve:

ΔA_{540} Standard = A_{540} Standard - A_{540} Standard blank

Prepare a standard curve by plotting the ΔA_{540} for the Standard vs micrograms of Phenolphthalein.

Sample Determination:

ΔA540 Sample = A540 Sample - A540 Sample blank

Determine the total micrograms of phenolphthalein liberated using the Standard curve.

$$\text{Units/ml enzyme} = \frac{(\text{mg phenolphthalein released})(2)(df)}{0.1}$$

2 = Conversion factor from 30 minutes to 1 hour as per the Unit Definition
df = Dilution factor
0.1 = Volume (in milliliter) of enzyme used

$$\text{Units/g solid} = \frac{\text{units/ml enzyme}}{\text{g solid/ml enzyme}}$$

$$\text{Units/g protein} = \frac{\text{units/ml enzyme}}{\text{g protein/ml enzyme}}$$

UNIT DEFINITION:

One Sigma or modified "Fishman" unit will liberate 1.0 microgram of phenolphthalein from

phenolphthalein glucuronide per hour at pH 5.0 at 37˚C.

FINAL ASSAY CONCENTRATION:

In a 1.50 ml reaction mix, the final concentrations are 47 mM sodium acetate, 0.56 mM

phenolphthalein glucuronic acid, and 25 - 50 units b-glucuronidase.

REFERENCES:

Fishman, W. H.; Bernfeld, P. *Methods in Enzymology*. **1955**,Volume I, 262-269.
Combie, J., Blake, J. W., Nugent, T. E., and Tobin, T. (1982) *Clin. Chem* 28, 83-86.

Fishman, W.H. in *Methods of Enzymatic Analysis*. Bergmeyer, H. U. (Ed). 2nd ed., Academic Press, New York, NY. **1974,** Volume II, 929-932.

Dosimetry Data from SureBeam Corporation for Part II

Sample ID	Date	Time	Dose (kGy)	Alanine	Marker	Ratio	Frequency
1.0 kGy							
TOP 15%	5/21/2003	3:13:51 PM	1.01	15401.8	147341.3	0.1073	9.77402
BOTTOM 15%	5/21/2003	3:14:22 PM	1.15	17506.8	147175.3	0.1221	9.77404
TOP 25%	5/21/2003	3:14:54 PM	1.00	15254.8	148201.7	0.1057	9.77371
BOTTOM 25%	5/21/2003	3:15:19 PM	1.14	17311.2	147691.0	0.1203	9.77383
2.0 kGy							
TOP 15%	5/21/2003	3:15:58 PM	2.03	30830.0	147098.0	0.2150	9.77379
BOTTOM 15%	5/21/2003	3:16:22 PM	2.35	35193.0	145273.7	0.2485	9.77402
TOP 25%	5/21/2003	3:16:51 PM	2.00	30298.2	146480.3	0.2122	9.77388
BOTTOM 25%	5/21/2003	3:17:18 PM	2.29	34486.8	146092.7	0.2423	9.77391
3.0 kGy							
TOP 15%	5/21/2003	3:18:13 PM	3.07	45892.2	144741.3	0.3252	9.77399
BOTTOM 15%	5/21/2003	3:18:49 PM	3.45	51732.2	145402.7	0.3651	9.77379
TOP 25%	5/21/2003	3:19:23 PM	3.01	45263.8	146038.7	0.3181	9.77366
BOTTOM 25%	5/21/2003	3:19:57 PM	3.46	51786.0	145261.0	0.3658	9.77372
4.5 kGy							
TOP 15%	5/21/2003	3:26:44 PM	4.57	67949.6	144316.3	0.4833	9.77387
BOTTOM 15%	5/21/2003	3:27:05 PM	5.15	76192.6	143660.3	0.5444	9.77389
TOP 25%	5/21/2003	3:27:27 PM	4.59	68108.0	144187.7	0.4848	9.77391
BOTTOM 25%	5/21/2003	3:27:55 PM	5.09	75098.2	143226.0	0.5379	9.774

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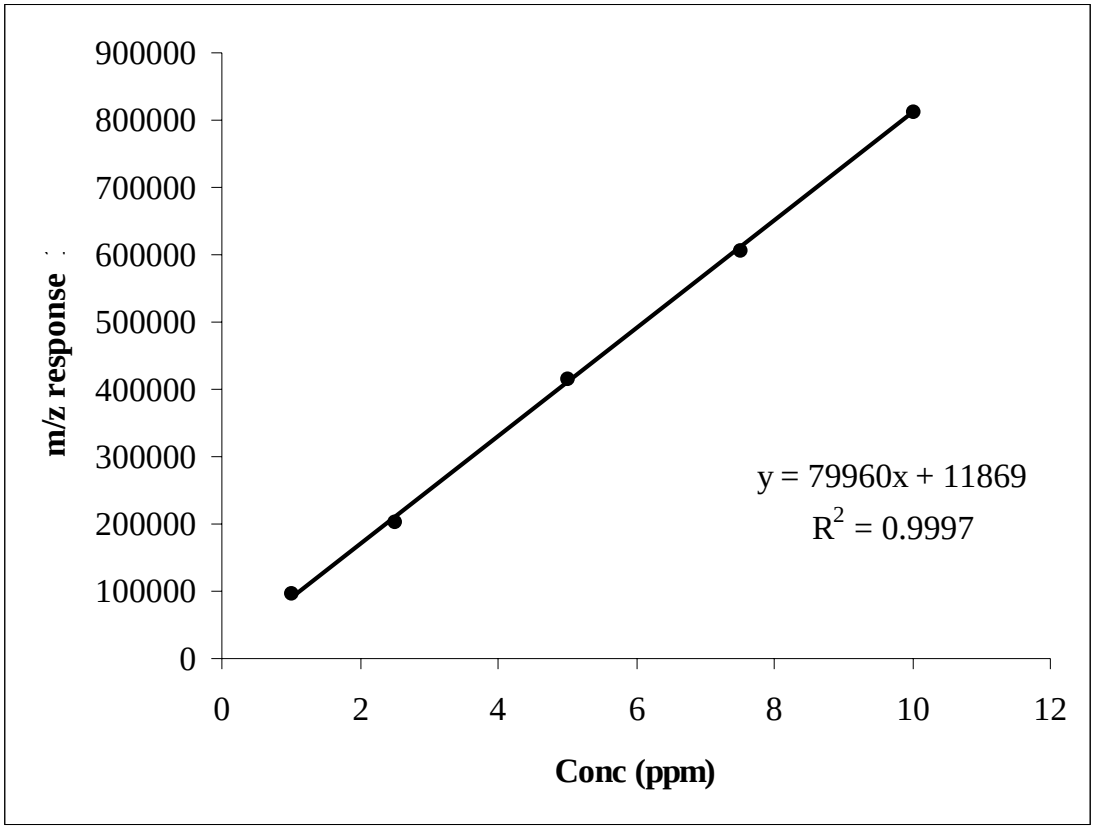

Figure A.1. Standard curve for 2-DCB used to calculate 2-DCB concentration in the irradiated samples in Part II

Table A.1: Fatty Acid Profile for irradiated ground beef patties containing 15% and 25% fat. Percent represents g/100g of beef fat

Fatty acid	**Average percent in sample**	**USDA value***
Lauric (C12:0)	0.3 ± 0.2	0.9
Myristic (C14:0)	4.6 ± 0.3	3.7
Palmitic (C16:0)	27.3 ± 0.4	24.9
Palmitoleic (C16:1)	5.0 ± 0.2	4.2
Stearic (C18:0)	15.1± 0.7	18.9
Oleic (C18:1)	43.4 ± 0.7	36
Linoleic (C18:2)	3.8 ± 0.7	3.1
Linolenic (C18:3)	0.3 ± 0.1	0.6

* USDA value obtained from the USDA National Nutrient Database for Standard Reference. Fat, beef tallow, NDB No: 04001. http://www.nal.usda.gov/fnic/foodcomp/search/

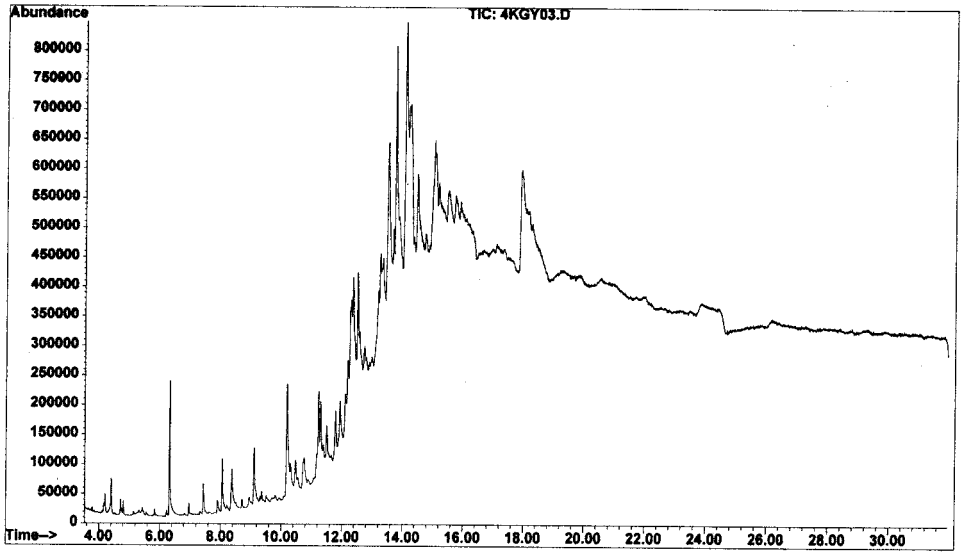

Figure A.2. Full (Total ion chromatogram) TIC for 25% fat sample irradiated at 4 kGy

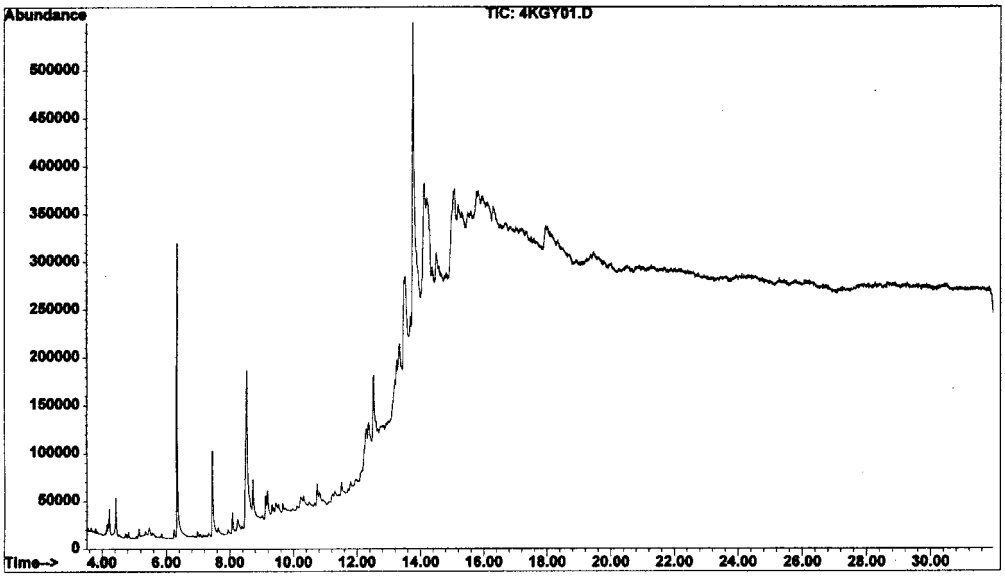

Figure A.3 . Full TIC for 15% fat sample irradiated at 4 kGy

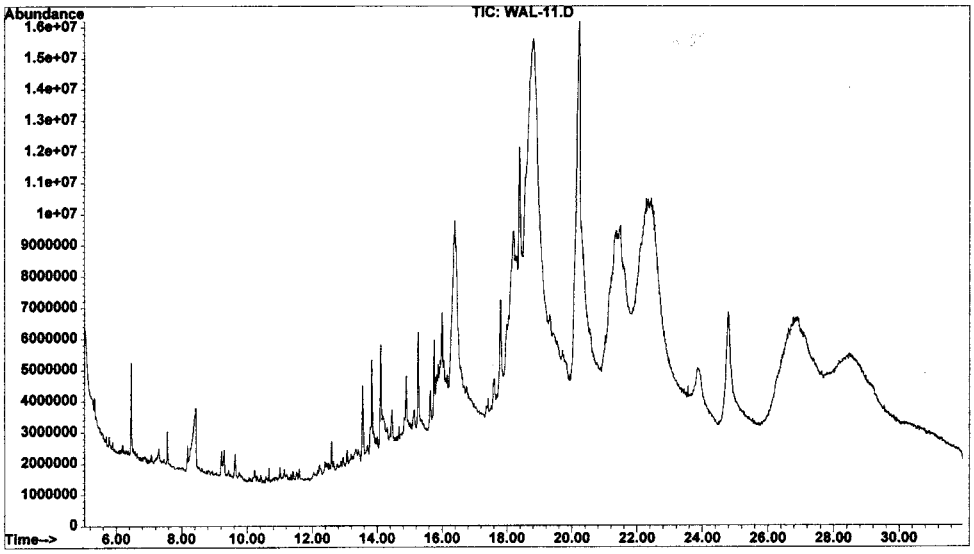

Figure A.4. Full TIC for brand X 80 % fat sample

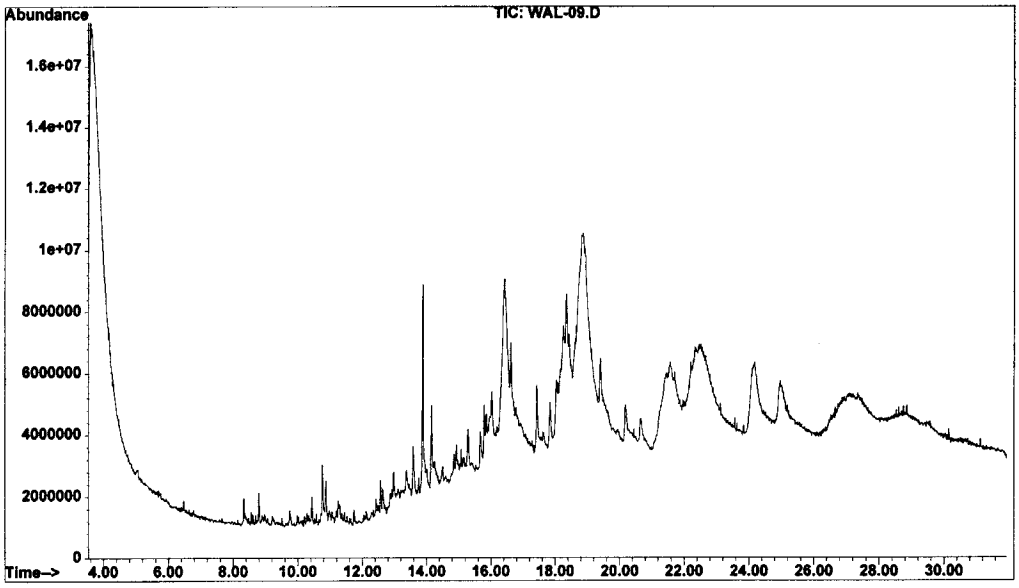

Figure A. 5. Full TIC for brand X 93 % fat sample

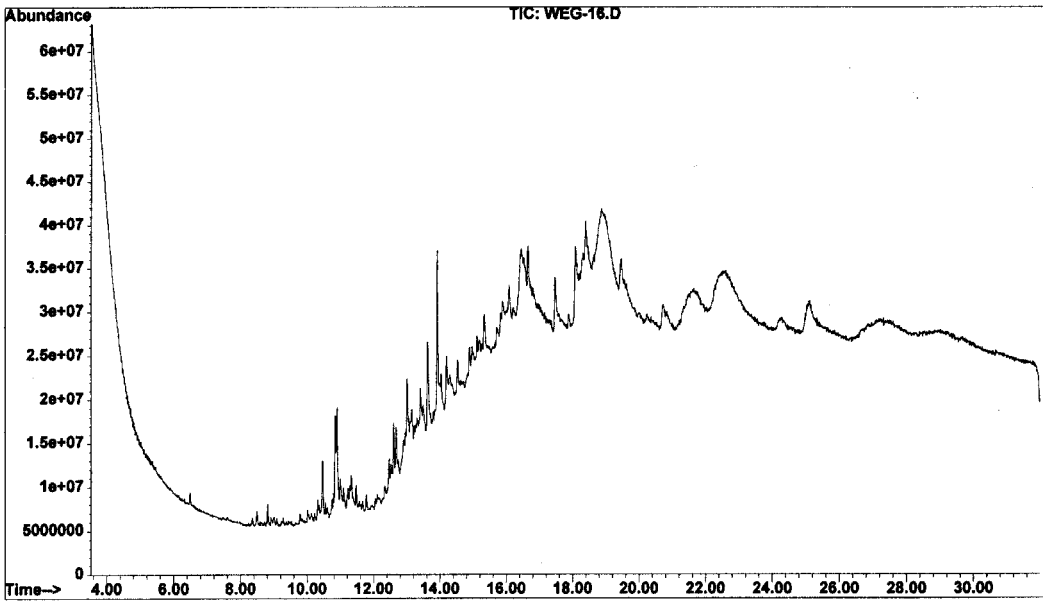

Figure A.6. Full TIC for brand Y 80 % fat sample

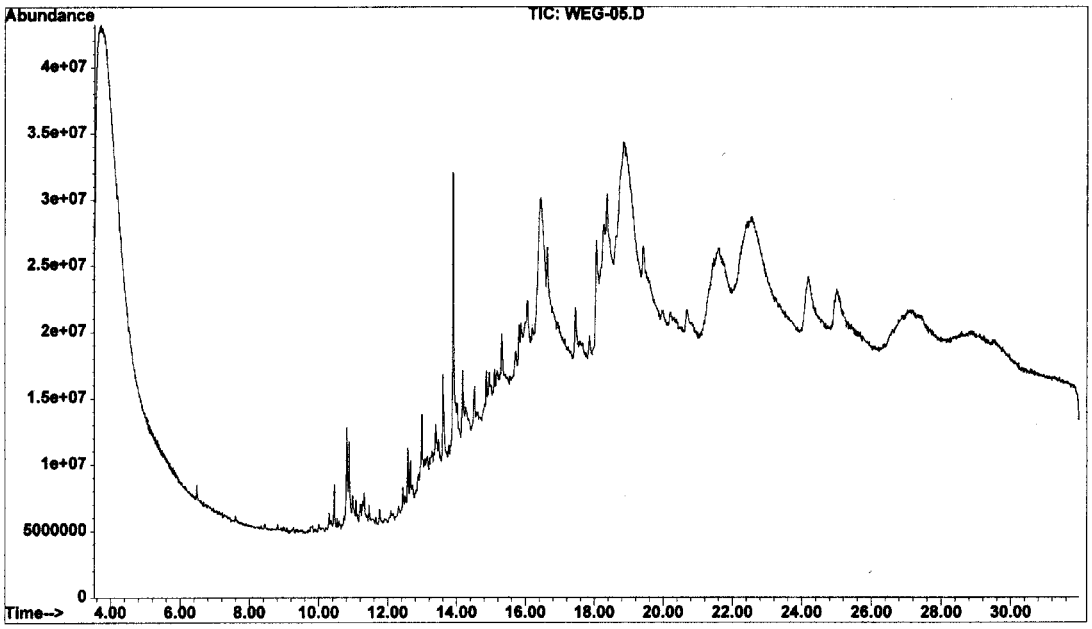

Figure A.7. Full TIC for brand Y 90 % fat sample

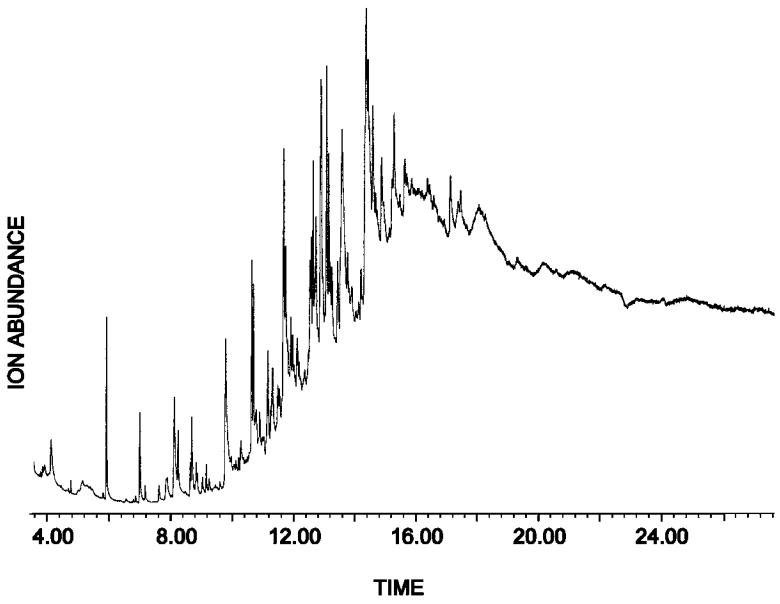

Figure A.8. Full TIC for origanox sample irradiated at 3 kGy

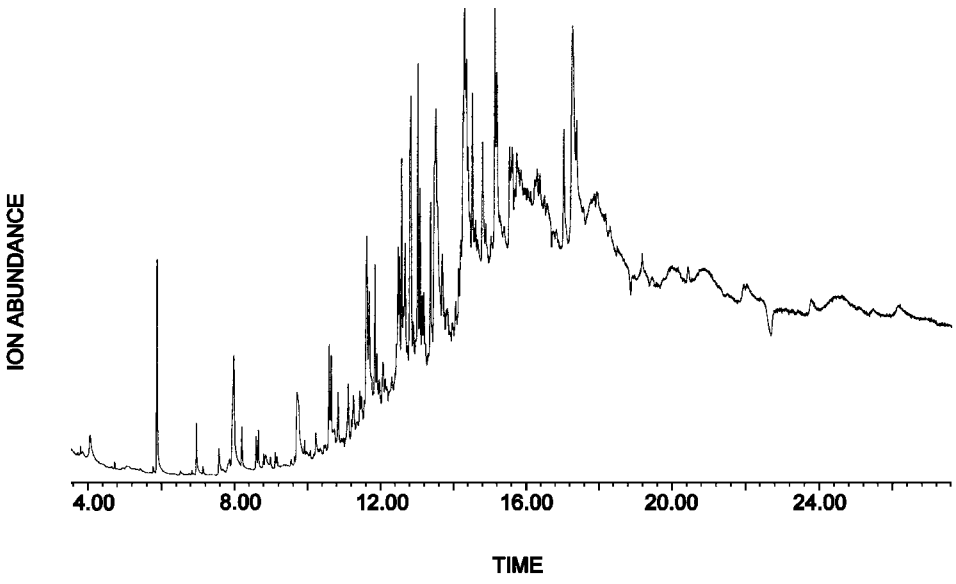

Figure A.9. Full TIC for sample without origanox irradiated at 3 kGy (irradiated control)

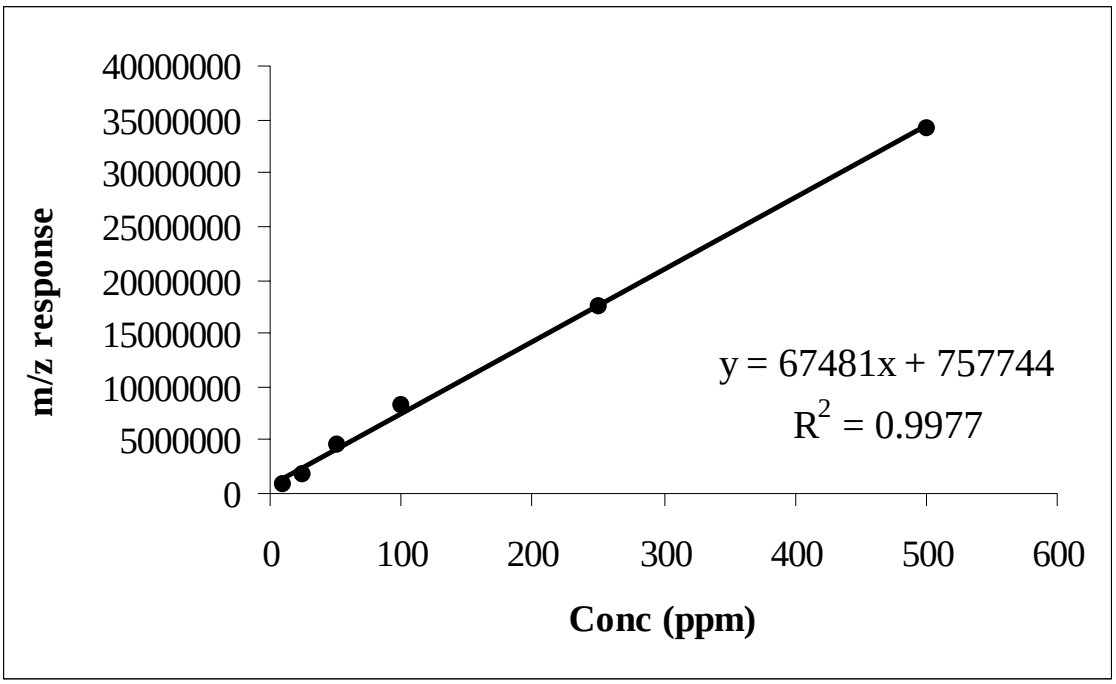

Figure A.10. Standard curve for 2-DCB used to calculate 2-DCB concentration in the feces and adipose tissue of rats.

Laboratory Rodent Diet 5001*

DESCRIPTION

Laboratory Rodent Diet is a Constant Nutrition™ formulation recommended for rats, mice and hamsters. The constant formula feature is designed to minimize nutritional variables in long-term studies. It is formulated for life-cycle nutrition; however, it is not designed for maximizing production in breeding colonies. This product has been the standard of biomedical research for approximately fifty years. Refer to the Shelf Life section at the end of this book for product longevity information and storage suggestions.

Features and Benefits

- Constant Nutrition™ formula helps minimize nutritional variables
- Formulated for multiple species for single product inventory
- The rodent diet standard for biomedical research

Product Forms Available

- Oval pellet, 10 mm x 16 mm x 25 mm length (3/8"x5/8"x1")
- Meal (ground pellets)

GUARANTEED ANALYSIS

Crude protein not less than	23.0%
Crude fat not less than	4.5%
Crude fiber not more than	6.0%
Ash not more than	8.0%
Added minerals not more than	2.5%

INGREDIENTS

Ground corn, dehulled soybean meal, dried beet pulp, fish meal, ground oats, brewers dried yeast, cane molasses, dehydrated alfalfa meal, dried whey, wheat germ, porcine meat meal, wheat middlings, animal fat preserved with BHA, salt, calcium carbonate, choline chloride, cholecalciferol, vitamin A acetate, folic acid, pyridoxine hydrochloride, DL-methionine, thiamin mononitrate, calcium pantothenate, nicotinic acid, dl-alpha tocopheryl acetate, cyanocobalamin, riboflavin, ferrous sulfate, manganous oxide, zinc oxide, ferrous carbonate, copper sulfate, zinc sulfate, calcium iodate, cobalt carbonate.

FEEDING DIRECTIONS

Feed ad libitum to rodents. Plenty of fresh, clean water should be available to the animals at all times. Refer to the "Animal Care and Biological Values" section of this manual for detailed feeding directions.

Rats- All rats will eat varying amounts of feed depending on their genetic origin. Larger strains will eat between 15-30 grams per day. Smaller strains will eat between 12-15 grams per day. Feeders in rat cages should be designed to hold two to three days supply of feed at one time.

Mice-Adult mice will eat 4 to 5 grams of pelleted ration daily. Some of the larger strains may eat as much as 8 grams per day per animal. Feed should be available on a free choice basis in wire feeders above the floor of the cage.

Hamsters-Adults will eat 10 to 14 grams per day.

CHEMICAL COMPOSITION[1]

Nutrients[2]	
Protein, %	**23.4**
Arginine, %	1.38
Cystine, %	0.32
Glycine, %	1.20
Histidine, %	0.55
Isoleucine, %	1.18
Leucine, %	1.70
Lysine, %	1.42
Methionine, %	0.43
Phenylalanine, %	1.03
Tyrosine, %	0.68
Threonine, %	0.91
Tryptophan, %	0.29
Valine, %	1.21
Serine, %	1.21
Aspartic Acid, %	2.83
Glutamic Acid, %	4.54
Alanine, %	1.44
Proline, %	1.55
Taurine, %	0.02
Fat (ether extract), %	**4.5**
Fat (acid hydrolysis), %	**5.5**
Cholesterol, ppm	200
Linoleic Acid, %	1.16
Linolenic Acid, %	0.07
Arachidonic Acid, %	<0.01
Omega-3 Fatty Acids, %	0.26
Total Saturated Fatty Acids, %	1.50
Total Monounsaturated Fatty Acids, %	1.58
Fiber (Crude), %	**5.3**
Neutral Detergent Fiber[3], %	14.3
Acid Detergent Fiber[4], %	6.8
Nitrogen-Free Extract (by difference), %	**49.9**
Starch, %	31.9
Glucose, %	0.23
Fructose, %	0.30
Sucrose, %	3.68
Lactose, %	1.67
Total Digestible Nutrients,%	**76.0**
Gross Energy, kcal/gm	**4.00**
Physiological Fuel Value[5], kcal/gm	**3.34**
Metabolizable Energy, kcal/gm	**3.04**

Minerals	
Ash, %	**6.9**
Calcium, %	0.95
Phosphorus, %	0.67
Phosphorus (non-phytate), %	0.40
Potassium, %	1.10
Magnesium, %	0.21
Sulfur, %	0.28
Sodium, %	0.40
Chlorine, %	0.65
Fluorine, ppm	18
Iron, ppm	270
Zinc, ppm	70
Manganese, ppm	64
Copper, ppm	13
Cobalt, ppm	0.6
Iodine, ppm	0.8
Chromium, ppm	2.0
Selenium, ppm	0.27

Vitamins	
Carotene, ppm	4.5
Vitamin K (as menadione),ppm	0.5
Thiamin Hydrochloride, ppm	17
Riboflavin, ppm	8.0
Niacin, ppm	124
Pantothenic Acid, ppm	24
Choline Chloride, ppm	2250
Folic Acid, ppm	5.9
Pyridoxine, ppm	6.0
Biotin, ppm	0.2
B_{12}, mcg/kg	22
Vitamin A, IU/gm	22
Vitamin D_3 (added), IU/gm	4.5
Vitamin E, IU/kg	49
Ascorbic Acid, mg/gm	—

Calories provided by:	
Protein, %	28.049
Fat (ether extract), %	12.137
Carbohydrates, %	59.814

*Product Code

1. Based on the latest ingredient analysis information. Since nutrient composition of natural ingredients varies, analysis will differ accordingly.
2. Nutrients expressed as percent of ration except where otherwise indicated. Moisture content is assumed to be 10.0% for the purpose of calculations.
3. NDF = approximately cellulose, hemi-cellulose and lignin.
4. ADF = approximately cellulose and lignin.
5. Physiological Fuel Value (kcal/gm) = Sum of decimal fractions of protein, fat and carbohydrate (use Nitrogen Free Extract) x 4,9,4 kcal/gm respectively.

LabDiet
www.labdiet.com

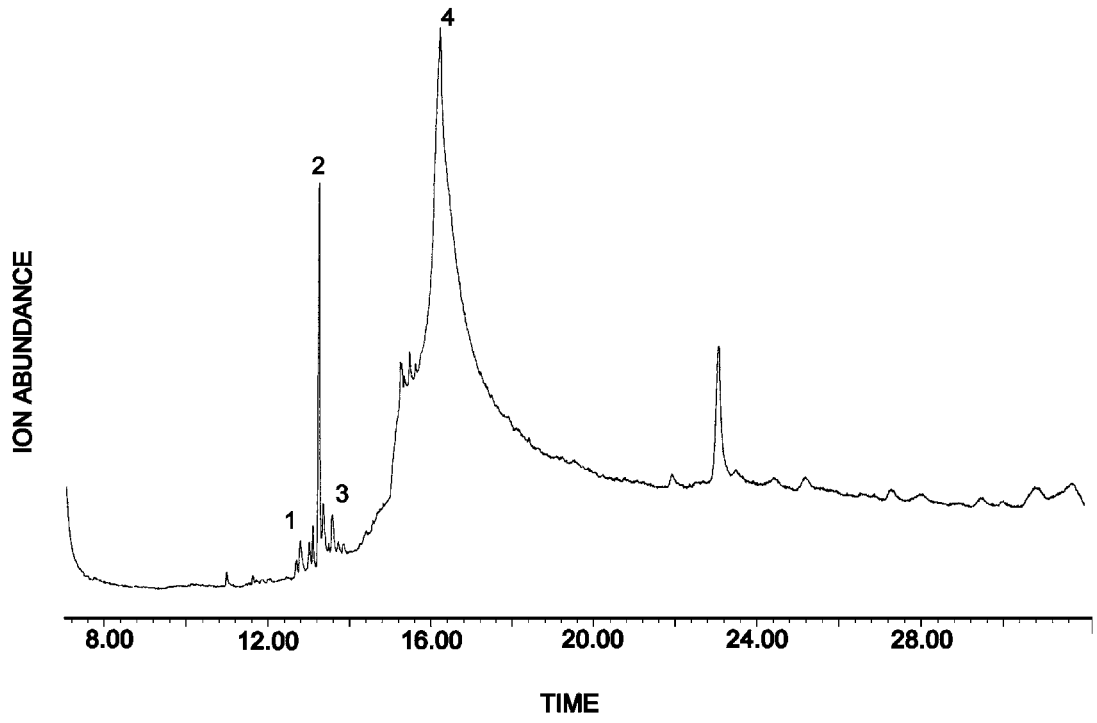

Figure A.11. Full TIC for hexane extracts of rat feces sample

List of compounds identified in the above samples. All samples had match values of (90% or higher

1. 1-Pentadecene
2. 2-DCB
3. Tetradecanal
4. Linoleic acid

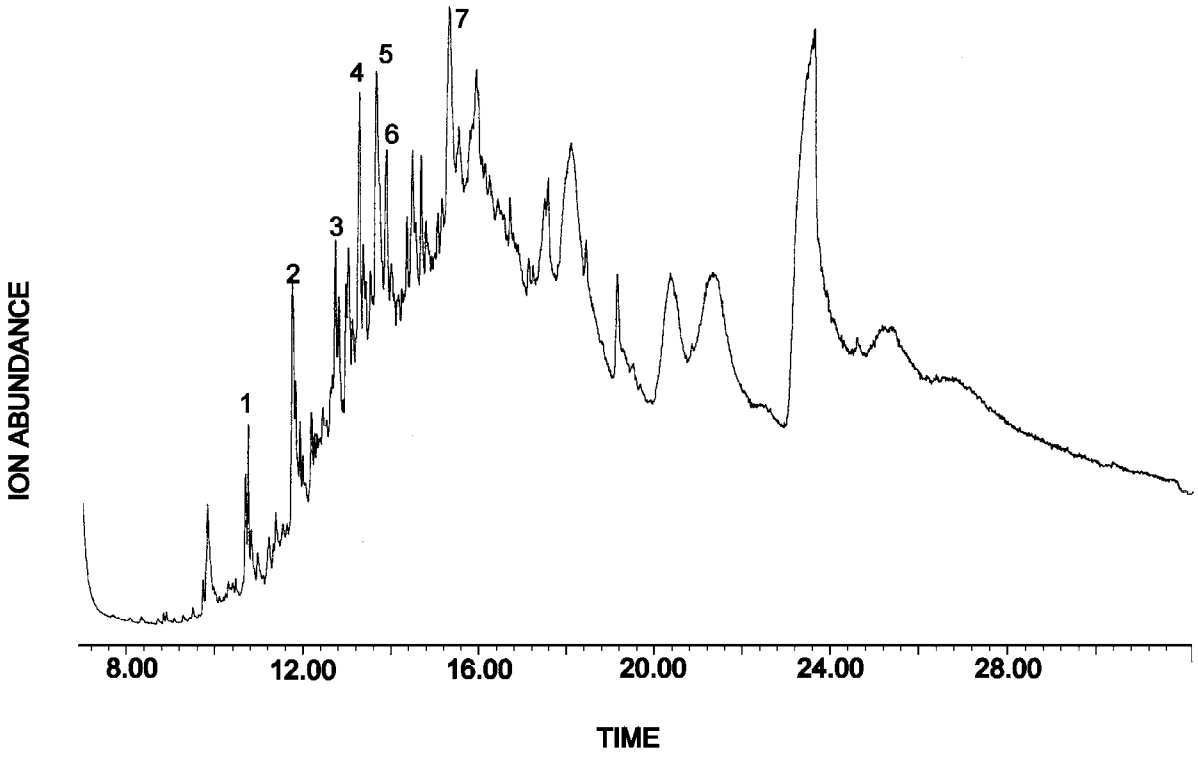

Figure A.12. Full TIC for the SFE -hexane extract of rat adipose tissue sample

List of compounds identified in the above samples. All samples had match values of (90% or higher

1. Hexadecane
2. Heptadecane
3. Octadecane
4. 2-DCB
5. 1-Octadecene
6. Pentadecanoic acid methyl ester
7. Oleic acid

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