Detection of Radiolytes in Irradiated Foodstuffs by GC-MS Analysis

A thesis presented for the degree of Master of Science

by

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to

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This thesis is dedicated to Mum, for I now know what it would be like if she were not around

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Finally, I would not have made it to this stage if it were not for Rosemary I would like to express my gratitude for her patience, support and encouragement over the course of my research

Other parameters were investigated to establish if the method applied to detect the radiolytic products in question was commensurate with certain criteria considered important to an efficient detection method These were

(i) the effect of storage on the concentration of the radiolytes

(ii) relationship between concentration of radiolytes and dose

(iii) overall sensitivity and specificity of the method for each foodstuff

CONTENTS

CHAPTER ONE

INTRODUCTION

1.1. FOOD IRRADIATION

Food Irradiation is the process whereby food is treated with ionizing radiation in order to achieve a number of desirable effects including: the extension of shelf life: the destruction or inactivation of insects, parasites, pathogenic bacteria, moulds, and yeast; the delay of ripening of fruits and vegetables; and the inhibition of sprouting of tuber and bulb crops. Many of these effects can be achieved with relatively low radiation exposures. Some of the world's leading hygienists have advocated irradiation of feed materials and of certain foods as one of the most promising measures to fight the serious threat to public health posed by food poisoning and other food related diseases [1].

Food irradiation is not without controversy. The term initiates in many peoples minds the fear associated with nuclear radiation and the process has become linked with the general fear of the nuclear power industry. It is felt by some that food irradiation leads to radioactive food. Because of the fears and misconceptions associated with food irradiation, a clear and concise explanation of the process is required

1.1.i What is Irradiation?

In order to understand the term irradiation, it is first necessary to understand the word radiation.

The Van Nostrand's Scientific Encyclopaedia [2] defines radiation as follows;

1. The emission and propagation of energy through space or through a material medium in the form of waves: for instance the emission of electromagnetic waves or sound and elastic waves.

2. The term radiation or radiant energy, when unqualified, usually refers to electromagnetic radiation; such radiation is commonly classified according to the frequency, as radio frequency, microwave, infrared, visible (light), ultra-violet, x-rays, and γ (gamma)-rays.

It is the electromagnetic spectrum, Fig 1.1 that scientists and lay people alike most commonly relate to the term radiation. Radio waves, microwaves, infrared, ultra-violet, and x-rays are encountered regularly.

Fig. 1.1: The electromagnetic spectrum.

Therefore, irradiation refers to either exposure to or illumination by rays or waves of all types.

The type of radiation used in the food industry is called ionizing radiation because it has the ability to convert atoms and molecules to ions by the removal of electrons. Ionizing radiations can be energetically charged particles, such as electrons, or high-energy photons such as x-rays or gamma rays.

The Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food considered only the following types of ionizing radiation as

suitable for the irradiation of foods, and this has been supported by the Codex General Standard for Irradiated Foods [3]

(i) Gamma rays from the radionuclides ^{60}Co and ^{137}Cs,

(ii) X-rays generated from machine sources operated at or below a level of 5MeV,

(iii) Electrons generated from machine sources operated at or below a level of 10MeV

The eV (electron volt) is the unit of energy used to measure the energy of electrons and other forms of radiation The energy of one electron volt is equivalent to the kinetic energy acquired by an electron on being accelerated through a potential difference of 1V The eV is a very small unit of energy It is therefore more common to speak of keV (kiloelectronvolt = 1000eV) or MeV (megaelectronvolt = 1 million eV) To convert eV to units of energy one can use the conversion 1 MeV = 1 602 $\times 10^{-13}$ J (joule) [4]

When ionizing radiation penetrates a medium such as the food substrate, radiation is absorbed in some quantity This absorbed dose is quantified by the gray (Gy) The gray is defined as the absorption of one joule of energy per kilogram of matter The unit of absorbed dose most commonly used in food irradiation is the kilogray (1000 Gy) Formerly the dose unit was known as the rad and was defined as 100 erg/ g The relationship between the old and new units is 1000 rad = 10Gy

The dose rate describes the dose of irradiation per unit time and this is at a relatively low rate (e g 100-10,000 Gy/hr) for gamma ray sources In

comparison to this, electron accelerators operate at a higher level typically supplying 10^4-10^9 Gy/sec

Table 1 1 Units of Radiation Dose and radioactivity [4]

	Absorbed Dose	Radioactivity
Unit	gray (Gy)	becquerel (Bq)
Definition	1 Gy = 1 J/ kg	1 Bq= 1 disintegration/ sec
Old Unit	Rad	curie (Ci)
Conversion	1 rad = 0 01 Gy	1 Ci = 3 7 × 10^{10}Bq = 37 GBq
	1 krad = 10 Gy	1 kCi = 37 TBq
	1 Mrad = 10 kGy	1 Mci = 37 PBq

1 1 ii Gamma Sources

An element can exist as different isotopes with the same atomic number but different atomic mass i e the same number of protons but different numbers of neutrons Some of these isotopes, particularly among the heavy elements are unstable and decay by emission of particles and/or photons A number of such radioisotopes exist in nature e g $^{40}K_{19}$ or $^{238}U_{92}$ The disintegration of radioisotopes leads to the following forms of radiation

(i) α-Particles fast moving helium nuclei, consisting of two protons and two neutrons

(ii) β^+ and β^- particles high-speed positrons or electrons

(iii) γ-Photons high-energy electromagnetic radiation

(iv) Neutrons uncharged nuclear particles with the same mass as the proton

In addition to these naturally occurring radionuclides, atomic bomb test explosions and nuclear energy pollution have created anthropogenic (man-made) radioactive materials on earth When a uranium nucleus is split in nuclear fission reaction, many lighter elements (fission products) are formed Most of these have an excess of neutrons rendering them unstable Some have half-lives of seconds and some of years

Many experiments on food irradiation in the 1950's were carried out by the United States Atomic Energy Commission using spent fuel rods from nuclear reactors These fuel rods contained many fission products with varying half-lives and some neutrons were being emitted causing radioactivity This problem and that of dosimetry using the rods were the principal reasons for abandoning their use for irradiating food

^{60}Co has become the choice for gamma radiation sources It is not a fission product ^{60}Co is prepared by bombarding pellets of ^{59}Co in nuclear reactors for 1-1 5 years This is performed in selective facilities such as the CANDU reactors in Canada The ^{60}Co source is present in a water-insoluble form eliminating environmental contamination With a half-life of 5 27 years and with emission of gamma radiation (1 17 and 1 33 MeV) and beta-radiation (0 31 MeV) $^{60}Co_{27}$ disintegrates to stable $^{60}Ni_{28}$ (nickel) Opposition to food irradiation is often based on the belief that radioactive waste material is accumulated by the existence of these facilities This is a misconception as ^{60}Co is converted to non-radioactive nickel

1 1 iii Machine Sources

X-ray Machines

Some of the earliest systematic studies on food irradiation were carried out with x-ray machines X-rays are produced when matter is bombarded by electrons of sufficiently high kinetic energy However, x-ray machines are not used as commercial food irradiators due to their expensive running and maintenance costs

Electron Beam Accelerators

Electron accelerators capable of commercial food irradiation became available in the 1950's and have improved since Various designs are now available which are used for the sterilization of medical supplies and packaging materials, radiotherapy, removal of toxic components from exhaust gases and many other applications High-energy electron beam accelerators produce electrons with energies above one MeV For purposes of food irradiation 10 MeV is the upper limit As a rule of thumb, the depth of penetration of an electron beam in most foodstuffs is 5mm/ MeV A 10 MeV energy can thus be used for irradiation of thickness up to 5 cm if irradiated from one side or 10 cm if irradiated from two sides [4]

General conclusions about the relative economics of different types of food irradiation facilities are considerably affected by local conditions such as labour, transportation, and construction The economics also depend greatly on the throughput of a facility

1.1.iv Process Control

The dose of irradiation to be administered to a food depends on the effect that the irradiation is intended to bring about. The dose must be sufficient to bring about the desired effect in the food, but not enough to cause undesirable effects. Table 1.2 lists the current potential applications and their relevant dosage levels [5].

Table 1.2: Functions of food irradiation

Function	Dose (kGy)	Products irradiated
Low-dose (up to 1 kGy)		
(a) Inhibition of sprouting	0.05-0.15	Potatoes, onions, garlic, root ginger etc.
(b) Insect disinfestation and parasite disinfection	0.15-0.5	Cereals and pulses, fresh and dried fruits, dried fish and meat, fresh pork etc.
(c) Delay of physiological processes (e.g. ripening)	0.5-1.0	Fresh fruits and vegetables
Medium-dose (1-10 kGy)		
(a) Extension of shelf-life	1.0-3.0	Fresh fish, strawberries etc.
(b) Elimination of spoilage and pathogenic organisms	1.0-7.0	Fresh and frozen seafood, raw or frozen poultry etc.
(c) Improving technological properties of food	2.0-7.0	Grapes (increasing juice yield), dehydrated vegetables (reduced cooking time) etc.
High Dose (10-50 KGy)		
(a) Industrial sterilization (in combination with mild heat)	30-50	Meat, poultry, seafood, prepared foods, sterilized hospital diets.
(b) Decontamination of certain food additives and ingredients	10-50	Spices, enzyme preparations, natural gum etc.

Avoiding unnecessary high doses also makes good business sense from a cost perspective. It is important to ensure that a particular batch of food receives the

correct dose of irradiation Measurement of dose and dose distribution in the food helps to provide assurance that the radiation treatment is both effective and legally correct It is necessary, therefore, that every time a batch of food is to be irradiated the operator must establish the dose and dose distribution by strategically placing dosimeters into and between food packages and evaluating the dosimeter reading Once the process is running smoothly, it is not necessary to perform dosimetry on all the product Monitoring the process parameters and making occasional dosimetric checks is then sufficient [4]

1 1 v Interaction of Radiation with Matter

When high-energy electrons are absorbed by a medium, they lose their kinetic energy by interacting with the electrons of the medium The interaction of orbital electrons of the atoms of the medium causes ionization and excitation Ionization means that the electrons in the orbitals are ejected from atoms of the medium, excitation means that orbital electrons are transferred to an orbit of higher energy Ejected electrons also lose energy by interaction with other orbital electrons of the absorbing medium

When gamma or x-ray photons interact with matter, a number of interactions may occur, the most common being

(i) The photoelectric effect

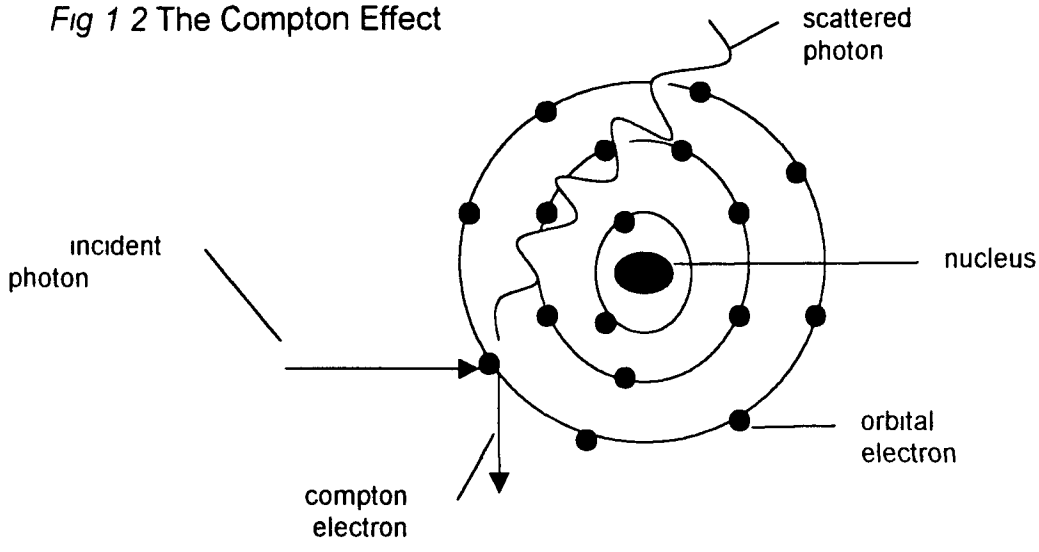
(ii) The Compton effect

(iii) Pair production (formation of pairs of electrons and positrons)

Photoelectric absorption usually occurs with photons of energies below 0 1 MeV and pair production usually occurs with energies above 10MeV Therefore for food irradiation purposes, the Compton effect predominates

As shown in Figure 1 2, an incident photon ejects an electron from the absorber In the Compton effect, the incident photon continues after the collision in a changed direction with less than its original energy The ejected electron (Compton electron) possesses sufficient energy to cause further excitation and ionization in the absorber atom [4]

Fig 1 2 The Compton Effect



1 1 vi Chemical Dose Meters

The purpose of dosimeters is to measure the amount of radiation energy absorbed by the irradiated product Reproducible changes caused by irradiation can be used to measure the absorbed dose as long as this change is for a stable time to allow measurement The chemical change is usually expressed as the G value, which is a measure of the number of atoms molecules, or ions produced (+G) or destroyed (-G) by 100eV of energy

Each dose range is measured by an effective meter for that range

1 2 HISTORY OF FOOD IRRADIATION

The first documented proposal to use ionizing radiation "to bring about an improvement in the condition of foodstuffs' and in 'their general keeping quality" was made ~90 years ago in the UK in a patent issued to J Appleby (Miller), and A J Banks (Analytical Chemist) [6] The inventors proposed the treatment of foods, especially cereals and their products with alpha- beta-, or gamma rays from radium or other radioactive substances A U S patent for an "Apparatus for Preserving Organic Materials by the Use of X-rays" was granted to D C Gillett of Tampa Florida in 1918 [7] In 1921 B Schwartz of the U S Department of Agriculture's Bureau of Animal Industry suggested the use of x-rays for inactivating trichinae in pork [8], the x-ray machines available at the time were not powerful enough to treat pork in commercially viable quantities In 1916, Mr G A Runner used x-rays to kill the insects, eggs and larvae in tobacco leaves in order to improve the quality of cigars [9]

Other studies and patents slowly followed The major limitations were the cost and availability of practical ionization sources Although x-rays proved to be effective in preserving ground beef, they were simply too expensive to be feasible

New interest was stimulated in 1947 by a publication [10] of two expatriate German scientists Arno Brasch and Wolfgang Huber, co-inventors of a pulsed electron accelerator They reported that meats and some other foodstuffs could be sterilized by high energy electron pulses, that some foodstuffs particularly milk and other dairy products, were susceptible to radiation and developed off-flavours and that these undesirable radiation effects could be avoided by irradiation in the absence of oxygen and at low temperatures At the same time,

J G Trump and R J van de Graaff of the Massachusetts Institute of Technology, who had developed another type of electron accelerator also studied effects of irradiation on foods and other biological materials [11] The foundations of food irradiation research had been laid down when B E Proctor and S A Goldblith reviewed these early studies in 1951 [12] Surveying the available radiation, these authors concluded that neutron radiation could not be used because it would produce radioactivity in the irradiated food, alpha particles and ultra-violet light were ruled out because of their low penetration, and x-rays were unsuitable because of insufficient power of available x-ray machines Gamma rays of radioactive isotopes were not even mentioned, presumably because suitable isotopes were not yet available on a sufficiently large scale

The U S Army supported research on both low and high dose food irradiations from 1953 to 1960 After 1960, the army concentrated its research efforts on developing radiation-sterilized meat products to substitute for canned or frozen military rations During 1961-1962, a large food irradiation laboratory was constructed at the U S Army Natick Laboratories in Natick Massachusetts It was equipped with a 1 3 million Ci ^{60}Co source and an 18kW electron linear accelerator The ready availability of gamma sources and electron accelerators in many parts of the United States then provided opportunities for food irradiation research and development work of which the earlier advocates of the use of x-ray machines could only have dreamt [4]

Reports from the United States about successful experiments of food irradiation stimulated similar efforts in other countries In the United Kingdom, investigation of the effects of radiation of food began in 1950 at the Low

Temperature Research Station at Cambridge and somewhat later at the Wantage Research Laboratories of the Atomic Energy Research Establishment By the mid to late 1950's, national research programs on food irradiation were also underway in Belgium, Canada, France, The Netherlands Poland, the Soviet Union and the Federal Republic of Germany Goldblith [13], Goresline [14] and Josephson [15] have reviewed this early history of food irradiation

In 1960, the first books on food irradiation appeared, written by Desrosiers and Rosenstock in the United States [16], Kuprianoff, and Lang in Germany [17] A first international meeting devoted to discussion of wholesomeness and legislative aspects of food irradiation was held in Brussels in 1961 [18] In the United Kingdom the report of a government working party on irradiation of food [19] summarized and evaluated the studies done until 1964

The first commercial use of food irradiation occurred in 1957 in the Federal Republic of Germany, when a spice manufacturer in Stuttgart began to improve the hygienic quality of his products by irradiating them with electrons using a van de Graaff generator [20] The machine had to be dismantled in 1959 when a new law was passed banning the use of ionizing radiation and the company turned to fumigation using ethylene oxide instead

In Canada, the irradiation of potatoes was permitted in 1960 to prevent sprouting, but financial difficulties caused the closure of the plant near Montreal in September 1965 [21]

In spite of these setbacks, there was considerable interest in food irradiation and at an International Symposium held by the International Atomic Energy Agency (IAEA), representatives of 28 countries discussed the progress made in

the research of the subject [22] At this time however, only three countries – Canada, the United States of America and the Soviet Union – had granted the permission for commercial irradiation of food at low dose levels Irradiated foods were still not marketed anywhere

The safety of the irradiation process was still strongly queried and this was recognized as the major obstacle to the irradiation of food worldwide Because of this recognition, the International Project in the Field of Food Irradiation (IFIP) was created in 1970, with the specific aim of sponsoring a worldwide research program on the wholesomeness and safety of irradiated food Under the sponsorship of the IAEA in Vienna, the Food and Agricultural Organization (FAO) in Rome and the Organization for Economic Co-operation and Development (OECD) in Paris, 19 countries joined their resources with this number later growing to 24 The World Health Organization in Geneva became associated with IFIP in an advisory capacity

The results obtained in the framework of IFIP and in numerous national testing programs were repeatedly evaluated by the Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food (JECFI), the internationally recognised arbiter in this field At its session in November 1980, this committee concluded "that the irradiation of any food commodity up to an overall average dose of 10kGy presents no toxicological hazard, hence, toxicological testing of foods so treated is no longer required" [23] Because of this landmark decision, many national governments have permitted the marketing of a number of irradiated foods

There have been a lot of objections subsequently to this decision but overall there has been growth in the use of food irradiation in countries like France, but

in other countries it has become somewhat stagnated. Nevertheless, many thousands of tons of food are irradiated each year. Both opponents and proponents of food irradiation have been sources of misinformation or valid information presented in a misleading manner [24]. As one leading British consumer representative put it: " The battle to get irradiation of food accepted as a beneficial food processing technique has been waged for some thirty years. It is an interesting case of warring factions glaring at each other across a gulf of incomprehension" [25].

There is a mass of scientific information on the subject of food irradiation but the difficulty lies in the collecting of it as there are so many sources. Useful documentation of developments in food irradiation research can be found in numerous analytical and agricultural journals as well as a computerised database called IRREFCO (Irradiation Reference Collection).

Finally, it is hoped that some information provided in this treatise may add to this long and ever growing list

1.3. CHEMICAL EFFECTS of IONISING RADIATION

Reaction Mechanisms

(i) Primary Effects

Irradiation of any material leads to the deposition of energy in that material. The deposited energy can cause chemical reactions, as demonstrated by chemical dose meters. If the irradiated material is a food, chemical changes can be expected, to an extent that increases with increasing dose of radiation. Consideration of the radiation-induced chemical changes is an important part of evaluating the safety of consumption of irradiated foods.

It may be of use to describe the effects of irradiation on a simple molecule such as methane (CH_4) When energetic electrons – either coming from an electron-generating machine or produced through Compton scattering – pass through a sample of methane, they cause *primary* effects [26]

$CH_4 \longrightarrow \cdot CH_4^+ + e^-$ ionisation

$CH_4 \longrightarrow CH_3\cdot + H\cdot$ dissociation

$CH_4 \longrightarrow CH_4^*$ excitation

The product of the ionization reaction is a cation, ~~characterized by a plus sign~~ It is also a free radical, ~~as indicated by a dot~~ Free radicals have an unpaired electron and are usually very reactive The dissociation reaction produces a methyl free radical and a hydrogen atom, which is also a free radical The primary effects are non-specific, they randomly hit any structure that is in the path of the incident or Compton electrons, without preference for any particular atoms or molecules The electrons removed in the primary process may possess enough energy to cause further ionization, dissociation, or excitation The excited molecules may undergo de-excitation, e g by giving off energy in the form of light (luminescence) They may also receive additional energy from a further interaction, so that dissociation or excitation can result

(ii) Secondary Effects

Because of the high reactivity of the free radicals produced as a result of the primary effect, secondary effects will occur The free radicals may undergo

reactions with each other. In the case of irradiation of methane this may result in *recombination*:

$$\cdot H + \cdot CH_3 \longrightarrow CH_4$$

or *dimerisation*:

$$\cdot CH_3 + \cdot CH_3 \longrightarrow C_2H_6$$
$$\cdot H + \cdot H \longrightarrow H_2$$

Another possibility is *electron capture*

$$\cdot CH_4^+ + e^- \longrightarrow CH_4$$

If other substances are present, the free radicals can also react with these. Newly formed compounds such as C_2H_6 (ethane) in the case of the irradiation of methane, will also interact with irradiation. A dissociation reaction could lead to $\cdot C_2H_5$. Two such ethyl radicals can react with each other by *dimerisation*:

$$\cdot C_2H_5 + \cdot C_2H_5 \longrightarrow C_4H_{10}$$

to yield butane or by *disproportionation*:

$$\cdot C_2H_5 + \cdot C_2H_5 \longrightarrow CH_2{=}CH_2 + C_2H_6$$

Disproprotionation reactions are those in which one reactant loses a hydrogen atom and the other gains one.

Which product or products predominate depends on various experimental conditions such as dose, dose rate, and temperature. While primary effects are largely non-specific, secondary effects depend on specific chemical structures. A substance, which reacts readily with a free radical, is known as a *scavenger*,

whereas a substance, which produces a more reactive free radical, is known as a *sensitiser*

When large molecules are irradiated, the absorbed radiation energy will be unevenly distributed in the excited molecule The energy is likely to be absorbed in those parts of the molecule having the greatest variation in electron density or where the bonds are weakest

The overall process that leads to more stable compounds is called *radiolysis* and the products of primary and secondary effects are known as radiolytic products This process occurs within fractions of a microsecond Because some end products are not completely stable, *post-irradiation* effects or radiation after-effects can occur in some systems for days or months after the treatment It should be noted that free radicals occur not only in irradiated materials Many biochemical reactions, both in plant cells and in mammalian organisms proceed through radical mechanisms [27] Grinding of dry powders [28] and heating of protein-rich foods [29] produces free radicals The question of whether consumption of foods containing a high concentration of free radicals could be damaging to health has been investigated in animal feeding studies (see section 1 4)

Influence of Dose and Dose Rate

It is generally expected that the level of products formed by primary and secondary effects of radiation will increase linearly with dose applied This may not hold for high dosage levels as products formed initially at the low level may themselves be destroyed by radiation at higher levels It is also possible that a

small amount of scavengers will be present in an irradiated system Therefore, this would suppress initial radiolytic development

Analytical Instruments in Radiation Chemistry

The most suitable technique for the investigation and detection of free radicals is electron spin resonance (ESR) spectroscopy

The ESR signals produced by irradiation have been extensively studied in Germany by Luck and associates [30-32] They investigated a series of fats in which free radicals could only be detected at higher doses and low temperatures The type of free radicals produced and their decay rates were markedly influenced by temperature In general they are much more stable at very low temperature e g -80°C and under vacuum These free radicals are capable of reacting with oxygen giving rise to the formation of new free radicals

Radiolytic Products of Water

In most foodstuffs, water is a significant, sometimes a major component Consequently, the radiation energy is absorbed largely by the water molecules and most of the radiolytic products are caused by the indirect effects of the resulting reactive species on organic molecules

The products formed in pure water and dilute aqueous solutions by irradiation can be summarized as follows

$$H_2O \xrightarrow{hv}$$

$$2\,7\ OH + 2\,7\ e^-_{aq} + 0\,55\ H + 0\,45\ H_2 + 0\,71\ H_2O + 2\,7\ H_3O^+$$

The hyroxyl radical is a powerful oxidizing species It can add to aromatic or olefinic compounds and abstract hydrogen atoms from carbon-hydrogen and sulphur-hydrogen bonds The hydrated electron e_{aq}, is also highly reactive, adding rapidly to most aromatics carboxylic acids, ketones, aldehydes and thiols Recombination can occur to form H_2O_2 but its concentration is low in the absence of oxygen

Direct Vs Indirect Effects

When an aqueous solution is irradiated, the molecules of the substrate may be directly affected by the incident or Compton electrons, or they may be affected by reactions with the reactive intermediary species of water radiolysis The former are direct effects, the latter are indirect effects The high reactivity of the intermediary radical species which are produced when water is irradiated is responsible for the often reported observation that a given irradiated dose will do more damage to a substance dissolved in water than to the pure dry substance, where only direct effects are possible [26]

Effects of Ionizing Radiation on Other Food Components

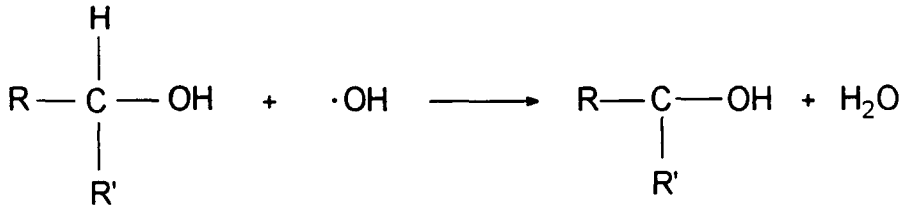
Minerals and trace elements are not affected by the process of food irradiation

Carbohydrates

Although the major products formed by irradiation in many pure sugars and saccharides have been studied [33,34], little research has been conducted on the radiolytic products derived from the carbohydrate portion in complex

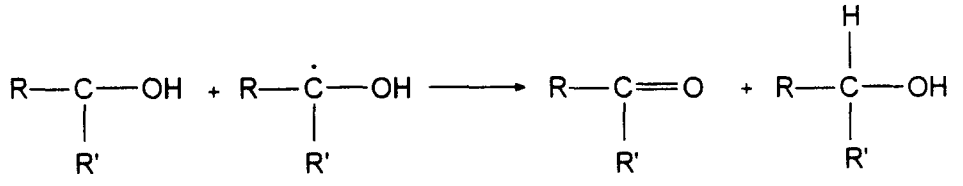
foodstuffs In aqueous systems, radiolysis of carbohydrates occurs mainly by indirect action of OH radicals that react primarily with C-H bonds

Hydrogen Abstraction

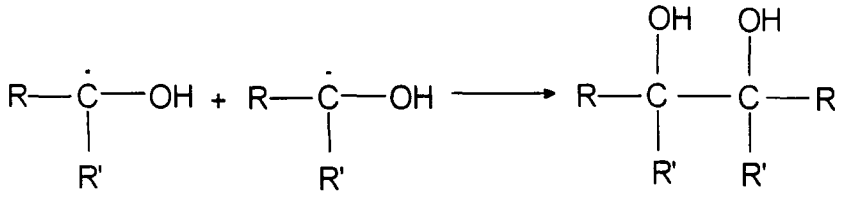


The resulting radicals react further by various mechanisms

Disproportionation



Dimerisation



Since OH radicals can abstract hydrogen from all six carbon atoms of a glucose molecule, a great number of compounds can be formed Von Sonntag's review listed 34 radiolytic products of glucose [35]

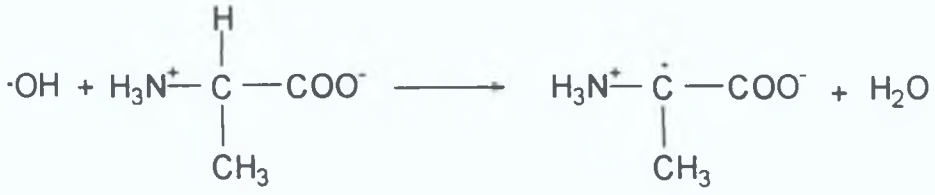
When disaccharides or polysaccharides are irradiated, the reactions observed with monosaccharides can also occur (i e formation of acids ketones, aldehydes etc) Additionally the glycosidic bonds that connect the monosaccharide units can be broken Dextrins, maltose and glucose are produced Winchester [36] reported that the radiation-induced formation of

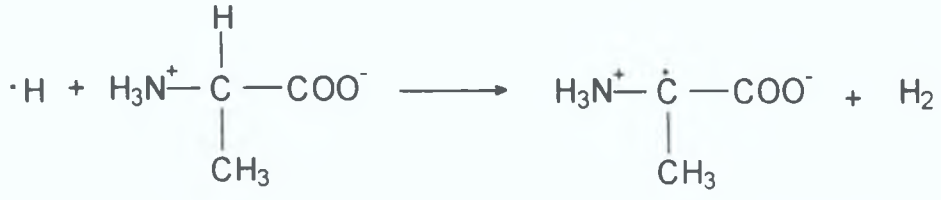
malondialdehyde permits identification of irradiated starch even at very low dose levels Very different results can be obtained when crystalline sugars are irradiated rather than aqueous sugar solutions This is not relevant in food irradiation However, the pharmaceutical industry uses crystalline sugars as carriers for medications in the manufacture of tablets When carbohydrates are irradiated as components of a food, they are much less radiation sensitive than in pure form This is because of the mutual protection exerted when different substances are irradiated together, and because most foodstuffs consist of a great number of compounds, food irradiation will generally not cause much chemical change in any one of these compounds The radiation damage will rather be distributed to all components, though not evenly Proteins offer a large amount of protection to carbohydrates during irradiation partly due to the scavenging effect of the amino acids

Proteins

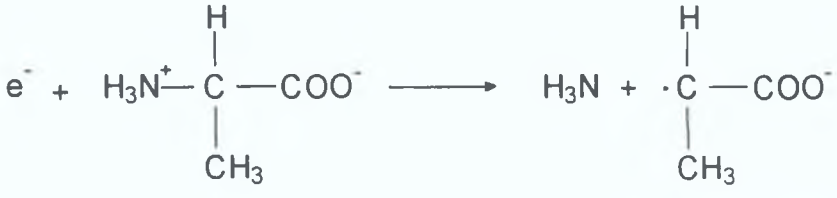
Proteins consist of chains of amino acids connected by peptide bonds The radiolysis of proteins can be largely ascribed to the reactions of their constituent amino acids and peptide bonds [37-42] Volatile decomposition products include ammonia, fatty acids and keto acids, aromatic compounds, amides and mercaptans Irradiation produced primary ionic and free radical intermediates leading ultimately to stable products Taking alanine as an example, the major radiolytic events in aqueous solution upon irradiation are

Abstraction of H



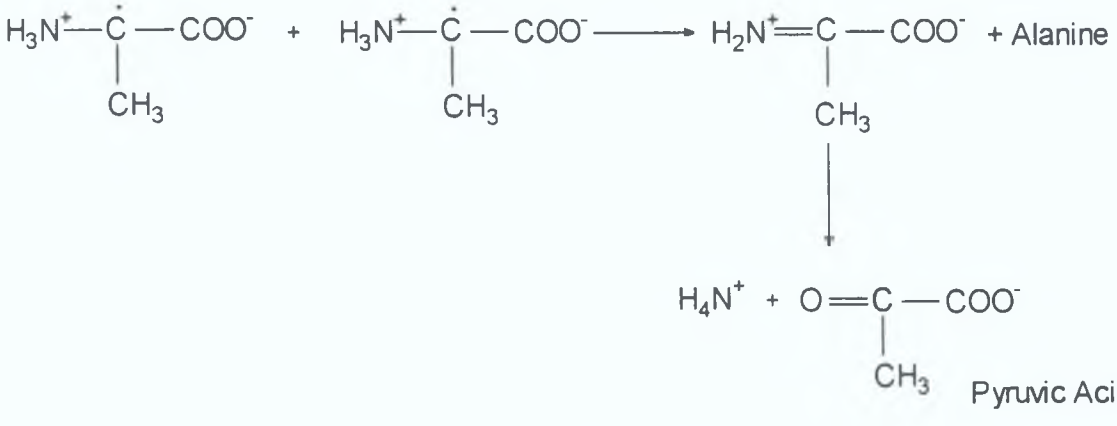


abstraction of H



reductive deamination

The produced radicals will react further



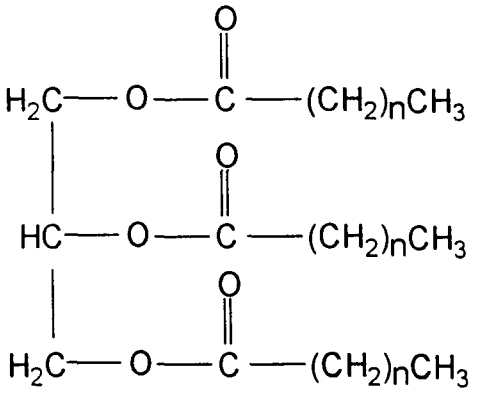
Pyruvic Acid

When proteins are irradiated in the presence of water, the reactions that are possible with amino acids are possible with a protein containing these amino acids. With some 20 amino acids present as constituents of proteins, complex interactions are possible.

Lipids

By far most of the volatile products formed in food by irradiation originate from the lipid fraction The main focus of this research lies in the chemistry of the radiolytes of lipids Irradiation can produce changes in lipids in two ways by catalysing their reaction with molecular oxygen, i e autoxidation, and by action of the high-energy radiation (direct or indirect) on the lipid molecules If oxygen is present, both effects will be superimposed

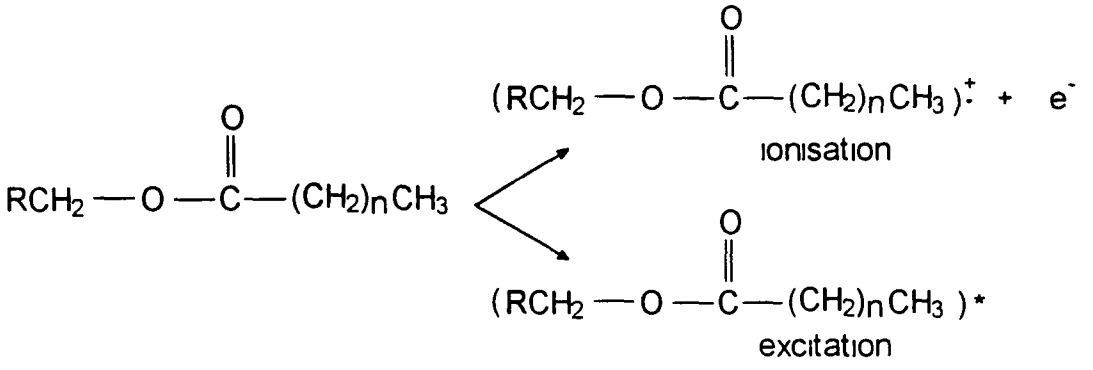
The lipid or fat portion of foods consists predominantly of triacylglycerols Milk fat, for instance, contains 94% triacylglycerols, soybean oil contains 88% triacylglycerols The following discussion of the effects of ionizing radiation will concentrate on the radiation chemistry of triacylglycerols



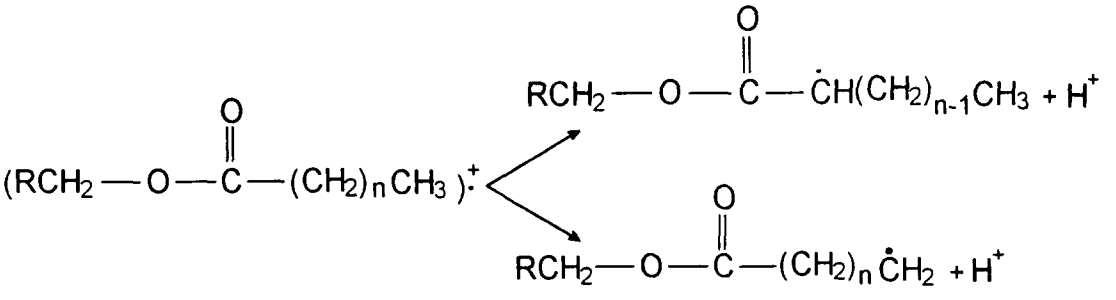
Triacylglycerol

Reactions of lipids with the reactive species of water radiolysis play a minor role in most situations, quantitatively at least

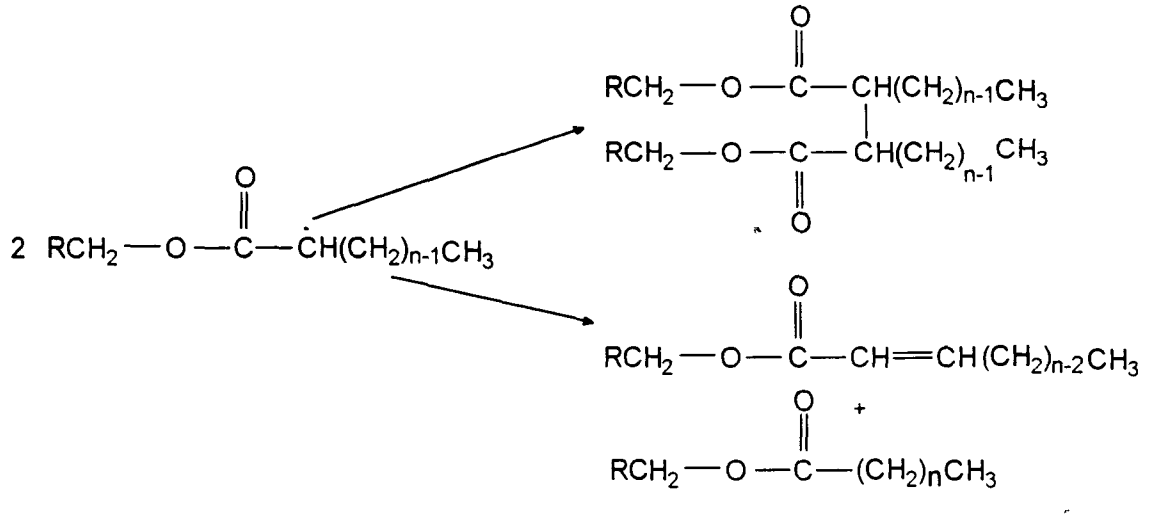
Upon irradiation of triacylglycerols, the primary effect of incident or Compton electrons leads to cation radicals and excited molecules [43]



The cation radical is shown generally with the localisation of the charge unspecified The main reaction of the cation radical is deprotonation

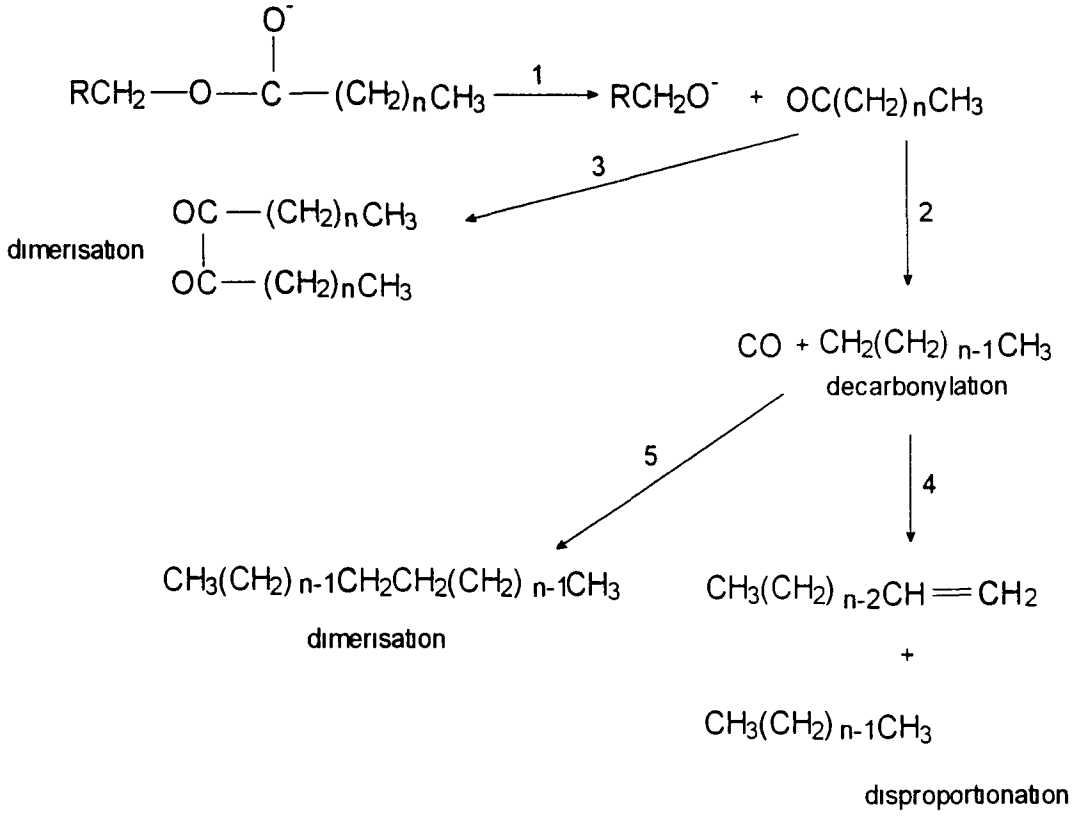


followed by dimerisation or disproportionation



Another primary effect is electron attachment

This may be followed by a series of reactions



The radiolysis of triacylglycerols of unsaturated fatty acids proceeds similarly, but the presence of double bonds, particularly if conjugated, will modify the range of products If the cleavage sites in the triacylglycerols are indicated by the letters a-f as shown, the products described in Table 1 3 are possible as a result of radiolysis

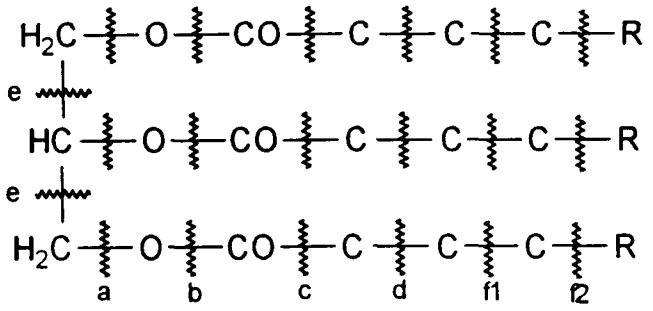


Table 1 3 Possible Radiolytic Products of Triacylglycerols

Site of cleavage	Primary Products	Recombination Products
A	C_n fatty acid	C_n fatty acid esters
	Propanediol diesters	Alkanediol diesters
		2-Alkyl-1,3-propanediol diesters
		Butanetriol triester
	Propenedioldiesters	
B	C_n Aldehyde	Ketones
		Diketones
		Oxoalkyl esters
	Diacylglycerols	
	Oxopropanediol diesters	Glyceryl ether diesters
		Glyceryl ether tetraesters
	2-Alkylcyclobutanones C_n	
C	C_{n-1} Alkane	Longer hydrocarbons
	C_{n-1} 1-Alkene	
	Formyl diacylglycerols	Triacylglycerols
D	C_{n-2} Alkane	
	C_{n-2} 1-Alkene	Hydrocarbons
	Acetyl diacylglycerols	Triacylglycerols with shorter or longer fatty acids
E	C_n fatty acid methyl ester	C_n fatty acid esters
	Ethanediol diester	Alkanediol diesters
		Erythritol tetraester
F_i	C_{n-x} Hydrocarbons	Hydrocarbons

Site of cleavage	Primary Products	Recombination Products
	Triacylglycerols with shorter fatty acids	Triacylglycerols with longer fatty acids

$_i$ = 1,2,.....n-3

x = any carbon number from 3 up to n-1 [43]

Detailed studies of the radiolysis of lipids have been previously reported, especially by Nawar and co-workers [44-46] at the University of Massachusetts, Amherst, and by Merritt and co-workers [47,48] at the U.S. Army Natick Research and Development Centre.

Most studies on the radiolysis of lipids are carried out under anoxic conditions. It is assumed that irradiation in the presence of oxygen leads to accelerated autoxidation, and that the pathways are the same as in light induced or metal-catalysed autoxidation.

Products from Radiation Induced Autoxidation

Irradiation and subsequent storage in oxygen accelerates lipid oxidation by

(i) enhancing the formation of free radicals which can combine with O_2

(ii) breakdown of the hydroperoxides

(iii) destruction of the antioxidants

Many of the products found in irradiated food are also, not surprisingly present in non-irradiated but oxidized fats.

It is generally established that the autoxidation of fatty acids occurs via a free radical chain reaction mechanism. It has been proposed that the initiation step

may take place by decomposition of preformed hydroperoxides (via metal catalysis or heat), by exposure to light or by mechanisms where singlet oxygen is the reactive species involved The chain reaction is propagated by the abstraction of hydrogen atoms at a position alpha to the double bonds, followed by oxygen attack at those locations, and resulting in the production of peroxy radicals, ROO , which in turn abstract hydrogen from alpha methylenic groups of other molecules, RH, to form hydroperoxides, ROOH, and yield R groups which react with oxygen and so on

All of these studies on the radiolytic products formed in foods and food components have helped greatly in the evaluation of the health and safety of irradiated foods Such studies have made it clear, for instance, that radiation does not cause

(i) formation of aromatic rings

(ii) condensation of aromatic rings

(iii) formation of heterocyclic rings

all of which take place at higher temperatures of cooking

Irradiation of aqueous systems may produce hydrogen peroxide, particularly in the presence of oxygen During post-irradiation storage, hydrogen peroxide will gradually disappear, while some other constituents of the system are being oxidized Some oxidized compounds not present, or present in lower concentrations immediately after irradiation, will be present in higher concentrations after hours or days Many substances or foodstuffs undergo different chemical changes during storage depending on whether they have been cooked, frozen, dried or left untreated

Radiation effects on phospholipids in aqueous suspension [49], and on cholesterol in liposome preparation [50] and in meat [51] have been studied in Maerker's laboratory at USDA's Eastern Regional Research Centre, Philadelphia. Other investigations of radiolytic effects on cholesterol in foodstuffs are those carried out in Poland on meat [52] and in Hungary on egg powder [53].

Conclusions from Chemical Studies

The main effect of irradiation on food is fragmentation, resulting in the formation of smaller compounds. Quantitatively, the most important products of radiolysis are carbon dioxide, carbon monoxide, ammonia, hydrogen and short-chain alkanes, alkenes, aldehydes, cyclobutanones, and fatty acids. They are more or less volatile and tend to escape quickly or gradually from the food substrate. Experience has shown that many of these compounds are not detectable or are present at lower concentrations when analysed at periods after irradiation.

Nawar found mainly the same volatile compounds in irradiated foods that were also present in heated food [54]. Schubert has estimated that consumption of heat-processed food results in a daily intake of thermal decomposition products 50-500 times greater than those produced by an irradiated diet [55]. Merritt concluded in 1972 that no volatile compounds produced in foods by irradiation have been discovered, that were not found qualitatively and quantitatively in other products resulting naturally [56]. Since then however one such compound 2-dodecylcyclobutanone, around which the bulk of this research is centred, has been reported by Crone and her colleagues [57] after earlier reports by Le

Tellier and Nawar [58] This compound was not detectable in non-irradiated, raw, or cooked chicken meat Unless further studies reveal the presence of this compound in some non-irradiated food, it may indeed be a unique radiolytic product

Those responsible for deciding on the health, safety and legal acceptability of irradiated foods in the U S and other countries initially demanded long-term animal feeding studies for each food under consideration The uncertainty over the nature and extent of chemical changes in irradiated food was so great that evidence considered satisfactory for permitting irradiation of wheat was not considered sufficient for permitting irradiation of rye Acceptance of irradiated beef did not mean acceptance of irradiated pork The recognition of commonality and predictability of radiolytic changes in irradiated food [47,59] has greatly advanced the toxicological evaluation of irradiated foods and has contributed substantially to the conclusion that more animal feeding studies with irradiated foods are not needed, at least in the dose range up to 10 kGy

1 4 OTHER ASPECTS of IONISING RADIATION

Biological Effects of Ionizing Radiation

For a living biological system, the main target of ionizing radiation is chromosomal DNA, although other cellular components may be affected Ionization or excitation of nucleic acid molecules follows irradiation Indirect effects include excitation of water which may make contact with chromosomal material An exposure of 0 1 kGy results in 2 8% of the DNA being damaged,

whereas 0.14% of enzymes and 0.005% of amino acids are altered with the same dose [60].

It has been suggested that the use of irradiation may result in the production of a 'superbug' (bacterium or virus) that would be radiation resistant [61]. Added to this concern is that resistance to radiation would be associated with increased virulence. There is no record of the formation of a truly superior species through either selection or mutation. In its response to this concern, the FDA commented that:

> "Mutants produced during irradiation of food are essentially the same as those that occur naturally. The only real difference is in the rate at which mutations occur. Nor is there any reason to expect that the resulting mutants would be different to or more virulent than those created by nature"[62].

According to Nawar [45] it is hoped that knowledge regarding the structure of radiolytic products in foods, the mechanism of their formation, and the parameters which influence their quantitative production will make much of the questionable biological testing currently required unnecessary, thus accelerating the beneficial implementation of irradiation in food preservation.

Toxicological Aspects of Irradiated Food

With regard to irradiated foods, considerations of safety for consumption involve four aspects: radiological safety, toxicological safety, microbiological safety and nutritional adequacy.

The maximum energy supplied by the commonly used ^{60}Co source is too low to induce radioactivity in the constituent elements of foods.

The US Food and Drug Administration [62], and the Codex Alimentarius Commission [3] have all adopted the 10MeV limit for electron radiation of foods and the 5 MeV limit for x-radiation, as have the governments of most countries which have permitted irradiation of certain foodstuffs [63] This is to prevent any risk of radioactivity occurring in foods irradiated above these levels

Animal Feeding Studies

Animal feeding studies were performed in many countries because they were viewed as a prerequisite to the granting of permission for the marketing of irradiated food products

Numerous animal feeding studies have been performed to assess the effects of ionizing radiation on animals [64,65,66,67,19]

The Chemiclearance Approach

What is needed for proper evaluation of the safety and wholesomeness of irradiated food is a combination of animal feed studies and chemical testing Lehmann and Laug [68] of the FDA suggested in 1954

"Chemical and physical tests should always precede animal tests, for the advance clues may enable the investigator to plan the animal experiment with greater intelligence and insight In some instances even, animal experiments may be omitted when a radiation product has been adequately characterized "

Internationally the Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food (JECFI) recommended at it's meeting in Rome in 1964 the introduction of irradiated food to world markets [69] The estimation of radiolytic products as a basis for evaluating the wholesomeness

of irradiated foods and for extrapolating wholesomeness data from one irradiated food to another [70] was incorporated into the program of work of the International Food Irradiation Project (IFIP) in the second half of its existence [71]

The term chemiclearance was introduced by Basson of South Africa [72] and the concept was applied in evaluating the wholesomeness of irradiated fruits [73 59] It involves the detection of radiation induced compounds, monitoring their levels and granting clearance of the radiated product for consumption

A Long History of Safety Studies

The results of safety studies – animal-feeding studies, in vitro tests, chemical tests- carried out in various laboratories globally, have been periodically evaluated

The IFIP was created in 1970 to coordinate and implement proper laboratory practices Feeding studies contracted by the project involved a range of commodities irradiated at dose levels up to 10 kGy The work was limited to this dose range because most applications of food irradiation were not likely to exceed this level Two extensive monographs were published in 1977 and 1983 [74,75] Numerous documentation concerning the safety of irradiated food has been published by the various advisory committees on the safety of irradiated food [76-84]

The largest controversy in the history of food irradiation surrounded the report of Bhaskaram and Sadasivan in1975 [84] who claimed that malnourished children fed freshly irradiated wheat developed polyploidy This is a condition that renders a person seriously ill due to twice the number of chromosomes in

the body than normal Numerous subsequent reports have questioned the findings of Bhaskaram and Sadavisan criticizing, among other parameters, the numbers sampled and the finding of no polyploid cells in the control group, a highly improbable result [85,86]

Microbiological Aspects of Irradiated Food

Food is generally irradiated at doses less than 10 kGy, which are not sufficient to kill all microorganisms that may be present However, irradiation typically results in a massive reduction in the number and variety of micro-organisms, for example Table 1 4 lists the values of D_{10} for a group of foodborne pathogens in ground beef, fish, oysters, shrimps and liquid whole eggs (D_{10} is described as the dose of irradiation needed to produce a 10-fold reduction in the population of microorganisms)

Table 1 4 D_{10} values of selected nonsporogenic bacteria

Bacterium	Medium	D_{10} (kGy)	Reference
Vibrio parahaemolyticus	Fish[a]	0 03-0 06	Matches and Liston (87)
Pseudomonas fluorescens	Ground beef[a]	0 12	Maxcy and Tiwari (88)
Campylobacter jejuni	Ground beef[a]	0 14-0 16	Tarkowski et al (89)
Aeromonas hyrophila	Ground beef[b]	0 14-0 19	Palumbo et al (90)
Proteus vulgaris	Oysters[c]	0 20	Quinn et al (91)
Yersinia enterocolitica	Ground beef[a]	0 1-0 21	Tarkowski et al (89)
Shigella dysenteriae	Shrimp[d]	0 22	Mossel and Stegeman (92)
Shigella flexneri	Shrimp[d]	0 41	Mossel and Stegeman (92)
Brucella abortis	Ground beef[a]	0 34	Maxcy and Tiwari (88)
Escherichia coli	Ground beef[a]	0 43	Maxcy and Tiwari (88)
Salmonella anatum	Ground beef[a]	0 67	Tarkowski et al (89)
Salmonella enteritidis	Ground beef[a]	0 70	Maxcy and Tiwari (88)
Salmonella newport	Liquid whole egg[e]	0 32	Licciardello et al (93)

[a] Irradiated at ambient temperature
[b] Irradiated at 2°C
[c] Irradiated at 5°C
[d] Irradiated frozen
[e] Irradiated at 0°C
Adapted from Diehl (4)

As can be seen, doses below 10 kGy result in extensive destruction of common foodborne pathogens in typical foods. Consequently, doses between 1 and 10 kGy can be used in certain instances for the virtual elimination of foodborne pathogens.

Nutritional Quality of Irradiated Food

A large number of international reviews have supported the view that irradiated food is nutritionally sound and healthy to consume. The 1981 meeting of the Joint FAO/IAEA/WHO Expert Committee on the Wholesomeness of Irradiated Food [23] concluded that "irradiation of food up to an overall average dose of 10 kGy introduces no special nutritional or microbiological problems".

This view has been supported in numerous reports by governments throughout the world [69,94,95]

The publication "*Safety of Irradiated Foods*" by Diehl (1990) is concerned primarily with low and medium doses of irradiation. Chapter 7 of this book contains the following statement: "As with regard to potential microbiological problems, it can be stated that potential nutritional losses in irradiated foods are not different from losses in foods treated by other processes. On the contrary, heating, drying, and some other traditional processes may cause higher nutritional losses than irradiation" [4,60,96].

Macronutrients:

At the low and medium doses under consideration here, there are no significant effects on the nutritional value of proteins, carbohydrates, or saturated fats [4,60,96]

Vitamins

There is a voluminous amount of literature on the effects of irradiation on the retention and destruction of the water-soluble vitamins [95,97]

As with water-soluble vitamins, the sensitivity of fat-soluble vitamins varies greatly depending on the specific food involved, the radiation dose, and the environmental conditions during irradiation and storage Nevertheless, it can be stated that, in general, the order of sensitivity is as follows vitamin E >carotene >vitamin A >vitamin K >vitamin D

1 5 LIPID EXTRACTION

In this research, triacylglycerols are studied analytically by the composition of the total fatty acids, both qualitatively and quantitatively

Theory of Extraction

For the rapid and complete removal of lipids from tissues, three things must occur

(i) The tissue must be subdivided under conditions that do not favour breakdown of the lipids

(ii) The solvent used must be capable of penetrating the divided tissue and breaking the protein-lipid bond

(iii) The tissue must be washed completely free of the lipid by repeated treatment with lipid-free solvent

The effectiveness of the procedure will to a large extent depend on the chemical nature of the lipid components and the kind of cellular association they possess. There are three main types of association involved [98]:

(a) Van der Waals or hydrophobic association in which neutral or non polar lipids. such as sterol esters. glycerides, hydrocarbons, and carotenoids are bound by relatively weak non covalent forces through their hydrocarbon chains to other lipids and to hydrophobic regions of proteins; an example is adipose tissue.

(b) Hydrogen bonding or electrostatic interaction, in which polar lipids (phosphatides, glycolipids, and cholesterol) are bound to proteins by hydrogen bonding or electrostatic forces.

(c) Covalent association in which fatty acids, hydroxy acids, or complex branched acids are linked covalently as esters, amides or glycosides to polysaccharides of bacterial cell walls.

Lipids in hydrophobically associated form may be extracted with relative non-polar solvents such as hexane, diethyl ether, chloroform, and pet ether. Membrane-associated lipids, however, require polar solvents such as ethanol or methanol to disrupt the hydrogen bonding or electrostatic forces between the lipids and the protein. Covalently bound lipids, by contrast, cannot be extracted directly by any solvents but must first be cleaved from the complex by acid or alkaline hydrolysis.

Alcohol is an essential component of the extracting solvent, being required for disruption of lipid-protein complexes, dissolution of the lipids, and inactivation of degradative enzymes. However, there is one drawback introduced by the use of alcoholic solvents for lipid extraction, namely. the co-extraction of

cellular contaminants such as sugars, amino acids, salts, etc It is therefore essential that the crude lipid extract obtained be treated to remove these water-soluble contaminants The most commonly used procedure is to wash the extract with water, a procedure that may produce intractable emulsions This is the basis of the Folch extraction procedure, which has proven to be one of the most successful extraction procedures used in removal of lipids

The chemical nature of the lipids must also be taken into consideration in choosing an extraction procedure In general to avoid peroxidation of double bonds, all solvents used should be of the highest purity and peroxide free before use For highly unsaturated lipids, the solvents should be de-aerated by bubbling nitrogen through them

Storage of lipids

Correct storage of food samples is extremely important in order to minimise changes that may occur upon either hydrolysis or oxidation The oxidation reaction has such a low activation energy that it is impossible to avoid completely the reaction between oxygen and the unsaturated lipids This reaction may lead to contamination of the lipid with a series of oxidation products that may be falsely perceived as occurring originally from the natural lipid Precautions must be taken to minimize lipid oxidation The olive fruit possesses sufficient natural antioxidant to ensure minimal lipid oxidation Other methods of prevention are

(i) minimize the air content above the lipid extracts This is achieved by flushing storage containers with nitrogen and by evaporating solvents from lipid extraction in a rotary evaporator not exceeding 40°C, with the flasks being of as small a volume as possible

(ii) the lipid extract is not evaporated to dryness, leaving 3-5cm^3 in the flask to be removed under a gentle stream of nitrogen

(iii) the samples are kept in the dark as autoxidation is catalysed by light [99]

1.6. THIN LAYER CHROMATOGRAPHY (TLC) OF LIPIDS

Thin Layer Chromatography (TLC) is a well-established method for lipid separation and determination. Normal phase TLC is the term applied to the use of silica gel, a polar adsorbent, where polar lipids are adsorbed more strongly than non-polar lipids due to polar interactions. In the TLC separation of lipids, the non-polar lipids therefore migrate at the fastest rates (high R_f values) and the polar lipids at the slowest rates (low R_f values). By increasing the polarity of the developing solvent system, the R_f values of the components can be increased. The choice of the solvent system is critical in the separation of lipid classes.

Long chain hydrocarbons, alcohols, aldehydes, acids, mono-, di-, and triacylglycerols can be separated through adsorption TLC into compound classes of differing polarities, according to the nature and number of their functional groups.

The solvent systems used to separate simple neutral lipid classes most commonly contain hexane, diethyl ether, and acetic acid in various proportions, although other non-polar solvents are used. Table 1.5 shows the expected R_f values of different neutral lipid classes in various solvent systems using silica gel as adsorbent [100]. Authentic standards are always run simultaneously for more accurate identification of the individual lipid components. With all these solvent systems, complex polar lipids remain at the origin.

After development, the plates were stained by iodine to reveal the position of the lipids Iodine has been used for many years to visualise lipids on TLC plates

Table 1 5 Reference R_f values for TLC analysis using various solvent systems [100]

	1	2	3	4	5
Hydrocarbons	0 95	-	-	-	-
Trialkylglyceryl ethers	0 90	-	-	-	-
Steryl esters	0 90	0 94	0 94	0 95	0 59
Wax esters	0 90	0 92	0 94	0 91	-
Dialkyl monoacylglycerols	0 70	-	-	-	-
Alkenyl diacylglycerols	0 65	-	-	-	-
Fatty acid methyl esters	0 65	0 81	0 94	0 75	-
Alkyl diacylglycerols	0 55	-	-	-	-
Fatty aldehydes	0 55	-	-	-	-
Triacylglycerols	0 35	0 73	0 86	0 61	0 61
Fatty acids	0 18	0 33	0 39	0 35	0 35
Fatty alcohols	0 15	0 28	0 29	0 21	-
Sterols	0 10	0 24	0 26	0 19	0 45
1,2-O-dialkylglyceryl ethers	0 09	-	-	-	-
1,3-diacylglycerols	0 08	0 24	0 26	0 19	0 45
1,2-diacylglyceryls	0 08	0 21	0 24	0 09	0 54
Monoacylglyceryls	0 0	0 03	0 03	0 01	0 05
Chlorophyll/ Carotenoids	-	0-0 2	0-0 23	0-0 06	0 07-0 19 0 49- 0 69*
Complex polar lipids	0 0	0 0	0 0	0 0	0 0

Solvent Systems

1 Petroleum Ether (b p 60-70°C)/diethyl ether/glacial acetic acid (90 10 1, by vol) (163)

2 Hexane/diethyl ether/glacial acetic acid (80 20 2 by vol)

3 Hexane/diethyl ether/glacial acetic acid (70 30 3 by vol)

4 Hexane/Heptane/diethyl ether/glacial acetic acid (63 18 5 18 5 1, by vol) to 2cm from the top then full development in carbon tetrachloride (164)

5 Benzene/propan-2-ol/water (100 10 0 25, by vol) (165)

- = not determined,* pigments run in two separate regions in this solvent

1 7 DETECTION METHODS OF FOOD IRRADIATION

Criteria for a Detection Method

A Research Co-ordination Programme on Analytical Detection Methods for Irradiation Treatment of Foods (ADMIT) was initiated by the Joint Division of two United Nations organisations, the Food and Agriculture Organisation and the International Atomic Energy Agency At their meetings in Belfast in June 1994 [101], they re-confirmed the "General Principles for the Development of Detection Methods" which are ideally required or desirable It was concluded that not all the characteristics listed below are required for a method to be viable The list should serve as a guideline for the ideal method against which proposed methods should be measured

Two sets of criteria have been elaborated

Technical Criteria which are to be applied if a qualitative or quantitative test is to be administered, and

Practical Criteria, which will be utilised if a method is to be applied by a controlling body, concerned with the marketing of irradiated food

1 Technical Criteria

(a) **Discrimination**- the parameter measured in the irradiated substrate should not be present in the non-irradiated substrate of the same type, alternatively the parameter should be well characterised in the non-irradiated substrate, so that a distinctive difference can be recorded by the irradiation of the substrate

(b) **Specificity-** other food processing methods and storage should not induce comparable changes to irradiation

(c) **Applicability-** the test should apply throughout the dose range relevant to the irradiation of the food tested

(d) **Stability-** the parameter should be stable for at least the storage life of the irradiated food

(e) **Robustness-**the measurement should be insensitive to the following effects, or its response should be known with sufficient confidence, *e g*

- dose rate
- temperature at any stage of treatment or storage
- other variables (O_2, moisture *etc*)
- further processing
- admixture with other foods

(f) **Independence** –the method should not require samples from the non-irradiated food from the particular batch tested

(g) **Reproducibility** and **repeatability.**

(h) **Accuracy** and proper statistical **validation**

(i) **Sensitivity-** the method should be capable of detecting doses below the commercially applicable dose

(j) **Dose Dependence-** the method should be capable of generating a dose response curve This criterion concerns the measurement of dose applied to the food

2 ***Practical Criteria***

(a) **Simplicity-** the method should not demand high levels of technical skill, data interpretation or specialised equipment

(b) **Low cost**

(c) **Small sample size**

(d) **Speed of measurement**

(e) The method should apply to a **wide range** of food and food types

(f) **Non-destructive** measurement of the parameter

(g) The method should be capable of **easy standardisation** and **cross-calibration.**

(h) Confidence that the method is **resistant to fraud.** It would be desirable if for example, the parameters were inherent to food rather than to the associated packaging, mineral dusts *etc*

Standards of Health and Safety in Laboratory Practices should be adhered to

Detection Methods For Irradiated Foods

In spite of the many advantages of irradiation, consumers in many countries have remained sceptical of the technique However, when it has been possible to carry out properly conducted consumer trials the advantages of irradiated food have become evident Because improved safety does not readily lend itself to direct consumer evaluation, the point is often not appreciated that many irradiated products are considerably safer on account of the reduction in *Salmonella spp , Campylobacter spp , E coli* and other pathogenic organisms which can be present in non-irradiated foods One of the essential issues was the demand by consumers and their representative organisations for methods that would discriminate between irradiated and non-irradiated products In order to validate the correctness of labeling, analytical techniques were needed

which would allow irradiated and non-irradiated food to be differentiated. Thus, a practical basis was sought to allow consumers a free choice as to which food they wished to purchase.

A range of methods using a wide variety of chemical, physical, and biological techniques is now available. Some have the capability of acting as screening methods, while others can provide definitive discrimination. Some detection tests, such as luminescence, depend on changes in extrinsic components (*e.g.* adhering minerals) while other tests depend on changes to intrinsic components (*e.g.* production of 2-alkylcyclobutanones from food lipids). The choice of method will also depend on the food. Some methods, such as electron spin resonance spectroscopy, will not only permit detection of primary irradiated products but will also allow detection of these irradiated products in secondary and tertiary foods (*e.g.* the detection of irradiated mechanically recovered chicken meat in a cooked chicken-burger) such is the sensitivity and specificity of the method.

Physical Methods of Detection

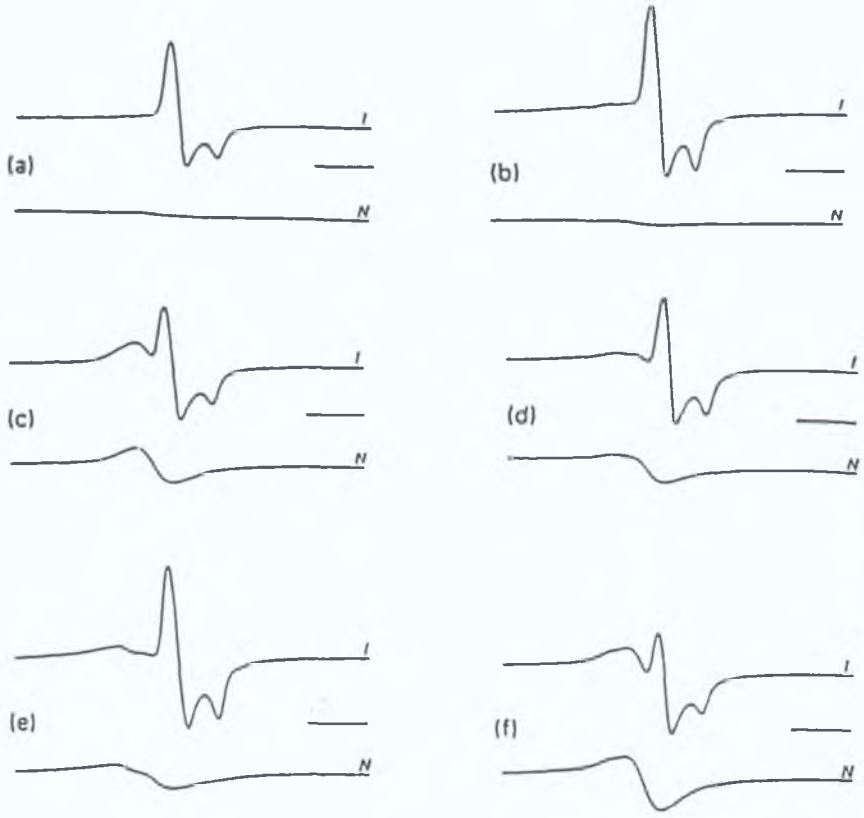
Electron Spin Resonance Spectroscopy

The advantage of ESR as a tool for the testing of irradiated food lies in the sensitivity and specificity of the method. Ionizing radiation produces free radicals, which disappear quickly due mainly to the presence of water. With increasing water content, the free radicals have increasing mobility and opportunity to react. In most foods the radiation-induced ESR signal can therefore be observed only if measurements are made immediately after irradiation. However as shown by Dutch authors in 1973, long-lived free

radicals can be observed even in wet food if it contains solid, crystalline, or semicrystalline regions where free radicals can be "trapped", e g in bone [102] Most studies have been carried out in chicken bone A linear dependence of ESR signal with irradiation dose was found in most cases The signal is stable during the expected shelf life of poultry products and survives cooking, although its intensity is decreased by cooking [103] A stronger signal is observed in older birds than in younger birds [104] Unirradiated bone gives a very weak ESR signal, which is intensified by heating or grinding, signal shape, however is quite different from that produced by irradiation as observed in Figure 1 3

Relatively stable radiation-induced free radicals are trapped in the achenes, pips, or stones of the fruits of certain species of plants [105,106 107], and in dried fruits [105], which are analysed by chemical methods in this research However, sunlight-induced pigments give ESR signals similar to those produced by ionizing radiation [108], and this can complicate the use of ESR for the detection of radiation-processed fruits and vegetables In figure 1 3, the magnetic field is characterised by the horizontal bar on the right of each pair of spectra, which represents 1 0 mT (millitesla) Fresh (a), freeze-dried (b), oven-dried (c), microwave-dried and ground (d), freeze-dried and ground (e), and oven dried and ground (f) are all represented

Fig 1.3: ESR spectra resulting from different sample preparation methods of irradiated (I) and non-irradiated (N) chicken bones.



The simple measurement of the ESR spectrum of an unknown sample will usually not permit an estimation of the dose because interpretation is complicated by many factors, such as sample history and degree of mineralisation of the sample. These potential obstacles can be overcome by using the *dose-additive* method [109,110]. A dose estimate can be obtained by re-irradiating the sample repeatedly and measuring the ESR signal each time, thus generating a dose-response curve for the sample being studied. Extrapolation of the curve to the negative dose axis yields an estimate of the initial absorbed dose of the sample.

Inter-laboratory tests between one laboratory in the USA, two in the UK, and one in France have yielded successful identification of irradiated chicken,

frogs, and pork. Good estimates of the absorbed dose were also achieved [111]. The ESR method for the detection of irradiated foods has been included in the compendium of official analytical methods in Germany and in the United Kingdom [112], and at the time of this research, is being reviewed for inclusion as a European standard method.

Luminescence Methods

As well as the production of free radicals as a result of irradiation, a series of excitations also occur. The presence of these excitations can be revealed by measuring the light emitted when the material is heated (*thermoluminescence*), stimulated by light (*photostimulated luminescence*), or dissolved in a liquid (*lyoluminescence*). The very weak light emission obtained by the latter method can be enhanced by addition of a chemical amplifier, such as luminol (chemiluminescence).

Thermoluminescence

The relatively cheap cost of this method makes it one of the most popular. Heat is used to stimulate the release of trapped energy in the form of light. The dry material (about 3-20 mg) is heated at a constant rate of 5-10 °C/ sec, up to a final temperature of 300-400 °C, and the light emission is recorded by a sensitive detector.

Studies performed in Scotland [113], Germany [114], and in Finland [115], demonstrated that the TL response of irradiated spices was coming not from the organic substance of the samples but from mineral debris adhering to the plant material examined. Wind-blown dust adheres to practically all foods of

plant origin and provides a basis for TL measurements Successful identification has been reported for strawberries [116] mushrooms and kiwi fruits [117], and table grapes [118] An inter-laboratory trial with coded samples in which 14 laboratories participated demonstrated high reliability of the detection of irradiated spices and herbs and less satisfactory results with fresh fruits and vegetables, where sometimes not enough mineral material could be isolated from the samples [119]

Other Luminescence Methods

Photostimulated Luminescence is proving to be a very successful screening method for the identification of irradiated food

Chemiluminescence (CL) measurements on dry spices and herbs received considerable attention in the 1980s [120 121,122] These studies showed large standard deviations of results, limited reproducibility, and therefore considerable uncertainties in the use of CL as a method of identifying irradiated food In view of the much greater success achieved with ESR TL and some other methods the use of CL has apparently been discontinued in most laboratories experimenting with it previously An interesting alternative to the usual CL technique of using luminol as a chemical amplifier is the application of liquid scintillation counting as described by Navraiz [123]

Other Methods Based on Physical Properties

Irradiation reduces the viscosity of carbohydrate polymers by causing chain breaks Mohr and Wichmann suggested that this effect be used for the identification of irradiated spices [124]

Electrical conductivity has also been used as it decreases with increasing dose and for a potato tuber disappears totally at a level of 1 kGy [125].

Differential scanning calorimetry was suggested as a method for detection of irradiated foods with a high water content [137].

Chemical Methods of Detection of Irradiated Food

Numerous chemical effects of ionizing radiation have been discussed in Section 1.3 of the Introduction. Because of most of the effects occurring from ionizing radiation also being caused by other processes such as heating, they are not suitable as a basis for a detection method. Other factors contributing to the unsuitability of a detection method are unstable reaction products, or too low a concentration for reliable analytical detection. This aside however, a number of analytical detection methods have been developed over the last 30 years which do enable the detection of irradiated food in a qualitative and quantitative manner.

Alkanes/Alkenes

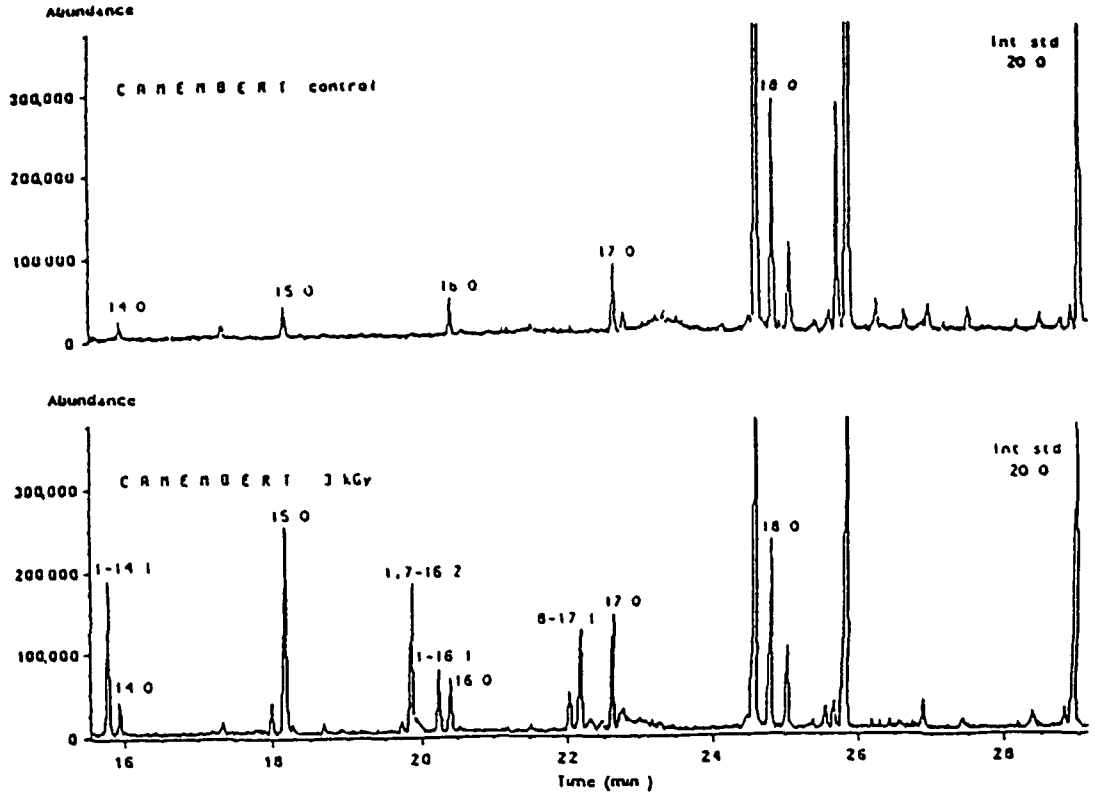
Gas Chromatographic (GC) determination of *hydrocarbons* as a method for the detection of irradiated fat-containing foods was proposed by Nawar and Balboni at the University of Massachusetts as early as 1970 [127]. The amounts formed were related to the fat content of pork analysed; the presence of air or moisture was found to have no significant effect on the quantitative pattern of the hydrocarbon formation. The radiolytic reactions leading from fatty acids to these hydrocarbons have been described in Section 1.3.

A later report from the same laboratory confirmed the reliability sensitivity and practicality of this method when applied to beef, pork and poultry [128] It was even possible to estimate the applied dose with a degree of accuracy

The results have been substantiated and the method optimised by Sjoberg and her group in Finland [129], Grob and her colleagues in Switzerland [130] Lesgard and his team in France [131], Morehouse and his team in the United States [132], Singh and collaborators in Canada [133], and Bogl's group in Germany [134] The hydrocarbons of interest are also present in some non-irradiated foods, as shown in Fig 1 4 for Camembert cheese but they are found in much higher concentration in irradiated samples [135 136]

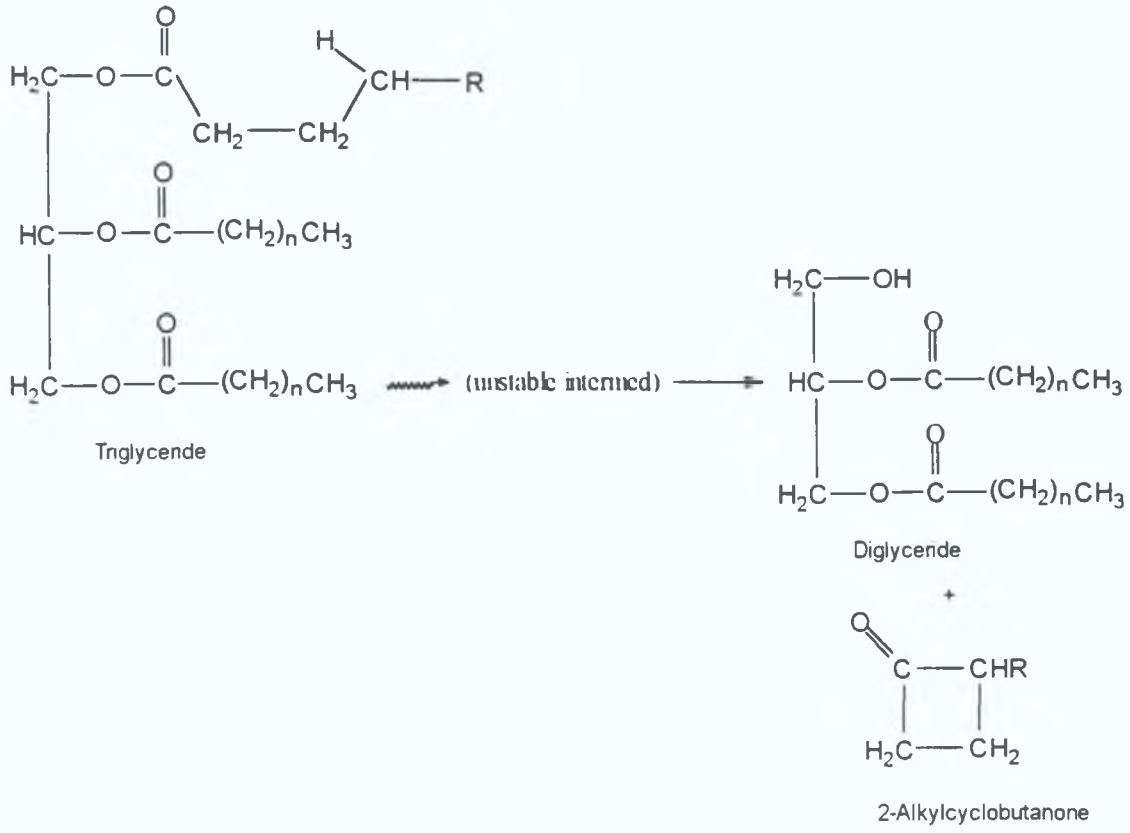
For quantitative determination of alkanes and alkenes these hydrocarbons must be separated from the bulk of the fat-soluble material by methods such as distillation, florisil column chromatography or HPLC before they can be separated from one another by GC and measured by mass spectrometry (MS) or flame ionisation detection (FID) The direct on-line coupling of HPLC and GC is probably the most advanced of the methods used The Swiss authors have pushed the degree of analytical sophistication one step further by developing a LC-LC-GC-FID technique [129] Regardless of the details of the method applied the determination of alkanes/alkenes can be considered as one of the most successful approaches to the identification of irradiated foods Its main drawback is the limitation to foods containing a substantial portion of fat

Figure 1.4 Gas Chromatograms of hydrocarbons from the fat fraction of unirradiated (above) and irradiated (3 kGy) Camembert cheese [From Ref 135]



2-Alkylcyclobutanones

Nawar's group had reported the GC identification of 2-alkylcyclobutanones among the radiolytic products of triglycerides [58] and phospholipids [137] irradiated at the high dose of 60 kGy The cyclobutanones have the same carbon number as the fatty acid they are derived from



With the much-improved GC/MS equipment now available, Stevenson and her colleagues found 2-dodecylcyclobutanone, a product of the radiolysis of palmitic acid, in chicken meat irradiated with a dose of 5 kGy [138]. This compound was not generated by cooking or spoilage [57] and persisted in chicken meat during long-term storage [139]. Irradiated liquid whole egg was found to contain 2-tetradecyl-, 2-tetradecenyl-, and 2-tetradecadienylcyclobutanone , radiolytic products of stearic, oleic, and linoleic acids respectively [140]. Results of interlaboratory trials on both irradiated chicken and liquid whole egg proved a great success [141].

Changes in Nonvolatile Lipid Fractions

Maerker et al. developed highly sensitive methods to detect and measure cholesterol A-ring and B-ring oxides in meat [51]. They consider 6-

ketocholestanol as a possible indicator of radiation treatment. This compound has not been detected previously in poultry but was found in some processed meats. Irradiation of chicken meat with a dose of 10 kGy increased the concentration of 6-ketocholestanol to four times the level found in non-irradiated chicken meat.

Katusin-Razem et al suggested the determination of hydroperoxides as a detection method for irradiated whole-egg powder. Although hydroperoxide levels declined during storage, irradiation at a dose level of 3-5 kGy could be detected up to 6 months after irradiation [142].

Whether these radiation-induced oxidative changes are sufficiently high above the background of autoxidation present in the non-irradiated food to permit unambiguous detection of coded irradiated samples remains to be demonstrated.

o-Tyrosine

o-Tyrosine is a radiolytic product of the amino acid phenylalanine. A detection method based on this compound would be very useful for foods low in fat content and high in protein. However, conflicting results have been obtained by different laboratories on the background levels of this compound. There are also conflicting views on the dose-effect curves produced by radiation induced o-tyrosine. Several observers have concluded that the levels of o-tyrosine in non-irradiated food, due to the presence of tyrosine hydroxylase, are too high to permit reliable identification of irradiated meats. Canadian authors who used HPLC with fluorescence detection for the determination of o-tyrosine in chicken meat have re-examined the issue and have expressed the opinion that with this

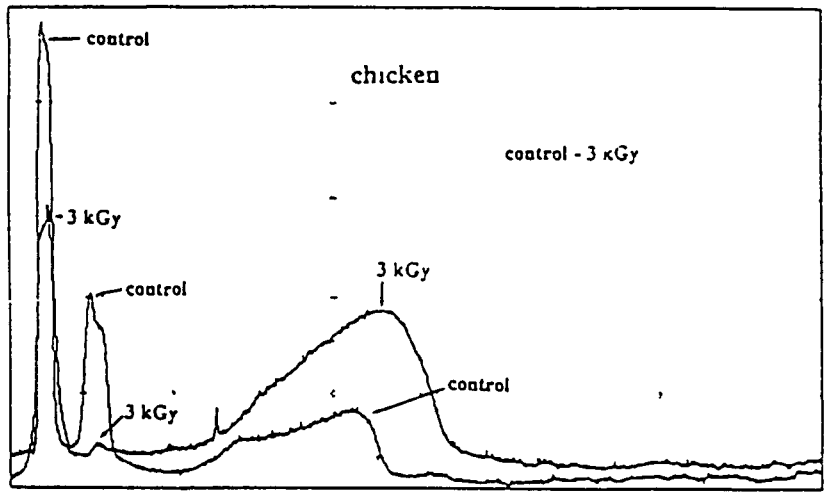
method o-tyrosine can be used as a marker for detection and dosimetry of irradiated chicken meat [143]

Methods based on radiolytic changes in DNA

The nucleic acids are minor constituents of food but they are extremely radiation sensitive

Radiation causes strand breaks in DNA, thus producing lower molecular weight fragments of DNA One of the methods used to detect these breaks is pulsed-field gel electrophoresis (PFGE), as reported by Mayer and colleagues [144] The method involves separation of the cell nuclei of a meat homogenate by centrifugation, digestion of proteins and nuclear membranes by incubation with proteinase/sodium dodecyl sulphate, and separation of DNA and DNA fragments by PFGE in a 1% agarose gel Staining with ethidium bromide reveals the position and concentration of unchanged and fragmented DNA DNA molecules too large to migrate in the gel stay at the point of application and are represented by the high peak on the left in the size distribution profile shown in Figure 1 5 The peak immediately to the right accounts for a group of DNA molecules with masses of approximately 2 2 MBP (megabase pairs) All DNA fragments below 0 9 MBP have moved further to the right and are indicated by the broad elevation in the profile A dose of 3 kGy has lowered the height of the peak that is due to large molecules (on the left), has almost eliminated the second peak, and has markedly increased the portion of fragmented DNA

Figure 1 5 Size distribution profile of DNA from non-irradiated and irradiated chicken meat, determined by pulsed-field gel electrophoresis



The main problem with most of the DNA methods described before is the fact that fragmentation of DNA also occurs in non-irradiated samples during storage due to the action of endogenous enzymes Working on the more stable mitochondrial DNA may be a solution to this problem It is too early to say whether this method is suitable for detection of samples of unknown history, but it is not unreasonable to expect a huge surge in DNA techniques as it is a rapidly growing area of analysis

Biological Methods of Detection of Food Irradiation

Radiation Effects on Microorganisms

The microbiological population in samples stored above the freezing point changes constantly, and this makes identification of irradiation very difficult At least one can say that an extremely low count on a food that normally contains a higher number of organisms can cause suspicion that this product has been irradiated (or fumigated), and this may give rise to further testing with one or several of the methods described previously

Radiation Effects on Plants

Irradiation can be used to inhibit the sprouting of potatoes, onions and garlic, and proposals have been made to use the ability or inability to sprout as a criterion of irradiation treatment

Conclusions

None of the methods described are suitable when compared to the standards set for an ideal detection method [145] However, several of the methods already fulfil most of these requirements The reliability of ESR spectroscopy of certain foods containing bones, shells, or other hard components, of thermoluminescence (TL) measurements of foods containing mineral dust particles, and of gas chromatographic (GC) measurements on fat-containing foods has been established in well-controlled collaborative tests These methods are being used in some countries for routine control of imported produce Several other techniques are ready for intercomparison tests and further promising approaches have been described

CHAPTER TWO

LIPID ANALYSIS

2 1 ANALYSIS OF LIPIDS

In this research, chicken, olives and figs were analysed for their lipid content The aim was to optimise the extraction of the lipids with respect to the following criteria

(i) Efficiency of extraction in terms of yield and reproducibility

(ii) Suitability of extraction procedure for incorporation into an overall methodology to detect radiolytes produced from lipids in irradiated foodstuffs

The identification and quantitative analysis of the fatty acid components of the extracted lipids were performed by GC-FID analysis of the methyl esters of the fatty acids, and the results are presented and discussed in this chapter TLC analysis was also performed for the qualitative analysis of the range of lipids extracted by the various methods employed

2.2 MATERIALS AND METHODS

2 2 1 Foods Extracted

Table 2 2 1 Extraction procedures used on each food substrate analysed

No	Food Substrate	Extraction Procedure	Extraction Solvent
1	Chicken (meat only, raw)	(i) Folch (ii) Soxhlet	(i)DCM[a]/ Meth[b] (2 1) (ii) TCE[c]/Methanol (2 1) Pet Ether (40-60) DCM/Methanol(2 1)
2	Chicken (meat only, raw)	(i) Folch (ii) Soxhlet (iii) Modified Fatty Foods	(i)DCM/Methanol (2 1) (ii)DCM/Methanol (2 1) (iii)Pet Ether (40-60)
3	Chicken (meat and skin, raw)	(i) Folch (ii) Modified Folch (iii) Bligh and Dyer (iv) Soxhlet (v) Foss-let (vi) Total Lipids (vii) Batch	(i)DCM/Methanol (2 1) (ii)DCM/Methanol (2 1) (iii)DCM/Methanol (2 1) (iv)Pet Ether (40-60) Hexane DCM/Methanol (2 1) (v) Perchloroethylene (vi)Methanol/Diethyl Ether (1 1) (vii)Hexane/IP[d] (3 2)

Table 2 2 1 continued

No	Food Substrate	Extraction Procedure	Extraction Solvent
4	Olives (black)	(i) Folch (ii) Modified Folch (iii) Bligh and Dyer (iv) Soxhlet (v) Foss-let (vi) Total Lipids (vii) Batch	(i) DCM/Methanol (2 1) (ii) DCM/Methanol (2 1) (iii) DCM/Methanol(2 1) (iv) Hexane Pet Ether (40-60) DCM/Methanol (2 1) (v) Perchloroethylene (vi) Methanol/ Diethyl Ether (1 1) (vii) Hexane/IP (3 2)

[a] Dichloromethane

[b] Methanol

[c] Trichloroethane

[d] Isopropanol

Each procedure described accounts for a series of approximately 10 extractions with any modifications made being mentioned where appropriate

Solvent Purification

When extracting minimal quantities of lipids it is important that the purity of the solvent is high to reduce contamination and to maximise yields Therefore, with respect to the extractions performed under this research the following solvents were purified

(i) Petroleum Ether

(ii) Diethyl Ether

Note: The remaining solvents used in the extraction procedures were purchased as high purity solvents requiring no further purification

(i) Petroleum Ether (40-60) [146]

Linde 5A sieve (200g) was placed into a Winchester of light petroleum (Bp 40-60°C) This was left to stand for 24 hours The drying agent was filtered off and the dried solvent (700cm^3) was placed in a 1 litre round bottomed flask The solvent was fractionally distilled, discarding the first 100cm^3 of distillate The distillation was allowed to proceed and the solvent boiling between 40-60°C was collected The distillation was halted when approximately 50-100cm^3 of the solvent remained in the round-bottomed flask The purified solvent was stored over sodium wire

(ii) Diethyl Ether [146]

Diethyl ether is often contaminated with peroxides, which were tested for as follows

Diethyl ether (2cm^3) was shaken with potassium iodide (10% w/v, 1cm^3) No colour change was observed and so the removal of peroxides was deemed unnecessary

Diethyl ether, which was previously dried over anhydrous calcium chloride, was fractionally distilled at approximately 1cm^3 per minute The distillation flask was covered with aluminium foil The first 50cm^3 of distillate were discarded and the liquid boiling at 35°C was collected The distillation was terminated before dryness

Sample Homogenisation

Food Samples were purchased from a local supermarket and were treated as follows

Chicken

Following removal of the skin of chicken samples 1 and 2 (the skin was left on chicken 3), whole chicken was deboned and homogenised in a blender [Dupont, Omni mixer 17106 (Chicken 1) and Moulinex Masterchef 650 (Chicken 2 and 3)] The homogenised meat was stored in glass or plastic containers and flushed for 10 minutes with dry nitrogen The stoppered containers were stored at –20°C until required for use Samples were then stored at 4°C until no longer required

Olives

Black olive samples were stored in brine for preservation purposes and so they were washed thoroughly with deionised water prior to use Each olive was then de-stoned manually and was homogenised in a blender (Moulinex Masterchef 650) The homogenised olives were placed in glass containers and were flushed with dry nitrogen for 10 minutes The stoppered samples were stored at -20°C until required for use Samples were then stored at 4°C until no longer required

2 2 2 Lipid Extraction Methods

The following extraction techniques were performed during the course of the research

1 Folch Extraction

2 Modified Folch

3 Bligh and Dyer Extraction

4 Soxhlet Extraction

5 Fosslet Extraction

6 "Modified Fatty Foods Extraction"

7 Total Lipids Extraction

8 Batch Extraction

1 Folch Extraction

The protocol followed was as cited by Folch, Lees, and Stanley [147] described in the lipid handbook of Hamilton [148]

The following procedure applies to both chicken and olive samples

Each sample (10g) of homogenised food was weighed accurately and homogenised (Dupont, Omnimixer 17106) with dichloromethane methanol (2 1 v/v) to a final dilution 20 times the volume of the tissue sample The homogenate was filtered and the crude extract was washed with 0 2% of its volume with water solution The filtrate was allowed to separate into two phases and the upper phase was removed The interface was rinsed three times with dichloromethane methanol water (3 48 47v/v, 30cm^3) The bottom layer was rotary evaporated to dryness leaving the lipid sample for gravimetric determination

2 Modified Folch [148]

Each sample (2g) was homogenised for one minute in methanol ($20cm^3$) Dichloromethane ($40cm^3$) was added The homogenisation was continued for a further two minutes The homogenate was filtered and the residue was suspended in dichloromethane methanol (2 1v/v, $60cm^3$) and homogenised for a further three minutes The homogenate was filtered and the residue was further washed with dichloromethane ($40cm^3$) and methanol ($20cm^3$) The combined filtrates were measured and an aqueous solution of potassium chloride (0 88% w/v) was added whose volume corresponded to one-quarter that of the combined filtrates The upper aqueous layer was removed by aspiration Water methanol (2 1 v/v, $30cm^3$) was added to the lower layer The upper layer was removed by aspiration The lipid was recovered from the lower layer by rotary evaporation and yield was recorded

3 Bligh and Dyer Extraction [149]

Each sample (20g) was homogenised in a blender with a mixture of dichloromethane ($20cm^3$) and of methanol ($40cm^3$) Dichloromethane ($20cm^3$) was added to this mixture and blended for 30 seconds Water ($20cm^3$, distilled) was added and the mixture was further blended for 30 seconds The homogenate was filtered The upper alcoholic layer was removed by aspiration The dichloromethane layer contains the lipid This fraction was transferred to a preweighed round bottomed flask ($250cm^3$) rotary evaporated to dryness and the yield of lipid was recorded

4 Soxhlet Extraction [150]

Table 2 2 2 List of Soxhlet extractions performed using various solvents

Solvent	Chicken 1	Chicken 2	Chicken 3	Olives
(i) TCE[1]/ Methanol (2 1)	3	-	-	-
(ii) DCM[2]/ Methanol (2 1)	5	12	5	5
(iii) Pet Ether	4	-	5	5
(iv) Hexane	-	-	5	5

[1] Trichloroethane

[2] Dichloromethane

(i) Trichloroethane/ Methanol (2 1 v/v)

Each food sample (5g) was weighed into an extraction thimble and was placed in the extraction chamber Trichloroethane/ methanol (2 1 by vol) was used as the extracting solvent The samples were gently refluxed to ensure that a continuous extraction occurred The solutions were cooled and rotary evaporated to dryness and the yield of lipid was recorded

The same procedure was also repeated using the following solvent combinations

(ii) Dichloromethane Methanol (2 1v/v)

(iii) Petroleum Ether

(iv) Hexane

5 Foss-let Extraction [151]

This is an automatic instrument for determination of the raw fat content of a series of foodstuffs The solvent involved in this particular method is tetrachloroethene, more commonly known as perchloroethylene

(i) Chicken

Chicken meat (22 5g) was weighed out accurately and placed in the extraction chamber The dispenser was filled with perchlorethylene (120 cm^3) which was added to the extraction chamber Calcium sulphate hemihydrate (50-60 g) was added and the extraction chamber was placed in the homogeniser The extraction chamber was placed in the measurement apparatus and after filtration the percentage fat was recorded

(ii) Olives

The same procedure was repeated for the olives samples except

1 Perchloroethylene = 60cm^3

2 Sodium sulphate (25-30g) instead of calcium sulphate hemihydrate

6 Modified Fatty Foods Extraction [152]

This extraction procedure is a modification of the organochlorine pesticide residue determination in fatty foods [152] as described by Morehouse, Kiesel and Ku in 1993 [132] The solvent used was petroleum ether Anhydrous sodium sulphate was used in an attempt to absorb the water that may hinder the extraction of lipids with an organic solvent

Each sample (10g) was weighed and homogenised with petroleum ether (40-60, 150cm^3) and anhydrous sodium sulphate (50g) for five minutes The residue was re-extracted with petroleum ether (2 × 100cm^3) for four minutes The combined extracts were dried over anhydrous sodium sulphate for 24 hours The petroleum ether was rotary evaporated and the yield of lipid was recorded

7 Total Lipids Extraction [153]

This procedure involved extracting food sample (10g) firstly with methanol and secondly with diethyl ether by using a pestle and mortar and heating at 55°C followed by centrifugation The combined extracts were then washed in a saline solution and after separation and rotary evaporation the yield was recorded

8 Batch Extraction

A food sample (10g) was homogenised with hexane isopropanol (3 2v/v, 80cm^3) and filtered The filtrate was transferred using a small volume of extracting solvent to a separating funnel The residue was re-extracted with extracting solvent and the combined extracts were evaporated to dryness until only pure lipids remained The yield was then recorded

Note:

A full description of all extraction procedures is contained in Appendix A.

2.2.3 Lipid Derivatization

The formation of methyl esters of fatty acids from the relevant triglycerides and free fatty acids of extracted food samples allows lipid profile analysis by GC-FID. The formation of these derivatives involves the saponification of the triglycerides into glycerol and sodium salts of the fatty acids followed by methylation to form the fatty acid methyl esters.

The methylating procedure of Metcalfe and Schmitz [154] was selected to derivatise the lipids to fatty acid methyl esters.

(i) Methylation using BF_3-Methanol [154]

Lipid (250mg) was placed in a round-bottomed flask ($50cm^3$). Sodium hydroxide (0.5M. $4cm^3$) was added. The reaction mixture was heated under reflux until the droplets of fat disappeared (10-20 minutes). Methanolic boron trifluoride (14%w/w. $5cm^3$) was added with a graduated pipette through the top of the condenser to the boiling liquid. This mixture was refluxed for two minutes. Hexane ($3cm^3$) was added through the top of the condenser and the reaction mixture was further boiled for a minute. The mixture was cooled to room

temperature A small portion of saturated sodium chloride was added and the flask was swirled gently Sodium chloride was added to the flask so that the top of the liquid was in the neck of the flask The upper hexane layer was transferred to a glass vial Anhydrous sodium sulphate was added to remove small traces of water The hexane was filtered through a microfilter (Sartorius 5 μm) and $1cm^3$ was transferred to a GC vial for analysis The hexane was evaporated under a gentle stream of Nitrogen and the FAMES were reconstituted in $1cm^3$ of hexane containing the internal standard Methyl Heptadecanoate (1000ppm)

Modifications

Series 1 All the lipid recovered was methylated Therefore relevant proportions of sodium hydroxide, BF_3-Methanol and hexane were used according to the yield recovered

2 2 4 Thin Layer Chromatography

Thin Layer Chromatography was considered a useful tool in the analysis of lipids for two reasons

(i) the component lipid classes of a lipid mixture may be identified

(ii) as a check to ensure that methylation was completed

(i) Identification of Component Lipids [100]

A TLC development chamber (10×21×21cm) was prepared by the addition of the relevant solvent system i e hexane/diethyl ether/glacial acetic acid (70 30 3 by vol) This was performed 30 minutes before analysis

A light pencil line was drawn horizontally 1 5cm from the bottom edge of a silica gel TLC plate (Polygram Sil G) Using a microcapillary tube, 2 spots for each sample were applied along with 4 standards Samples and standards were made up in hexane to a concentration of approximately 1mg/cm^3 Following solvent evaporation the plate was placed in the developing chamber and the separation process was begun ensuring the solvent depth was below that of the sample origin On removal of the TLC plate, the solvent front was marked The solvent was allowed to evaporate and the TLC plate was then placed in an iodine tank to allow identification of constituent lipids The component lipids turned a brown colour These stains were marked with a pencil The R_f value of each individual component for samples and standards were recorded and compared

The following solvent systems were employed in the TLC analysis

TLC of Lipid Samples

(i) Chicken 2 Sample

-*Folch Extraction* Hexane/ Diethyl Ether/ Glacial Acetic Acid (70 30 3 by vol)(100)

-*Modified Fatty Foods* Hexane/ Diethyl Ether/ Glacial Acetic Acid (70 30 1 by vol)

-*Soxhlet Extraction* Pet Ether (60-70°C)/ Diethyl Ether/ Glacial Acetic Acid (90 10 1 by vol)(100)

(ii) Chicken 3 Samples Hexane/ Diethyl Ether/ Glacial Acetic Acid (70 30 3 by vol)

(iii) Olives Hexane/ Diethyl Ether/ Glacial Acetic Acid (70 30 3 by vol)

TLC of Methylated Lipid Samples

(i) Chicken 2 Samples

-*Modified Fatty Foods Extraction* Hexane/ Diethyl Ether/ Glacial Acetic Acid (70 30 3 by vol)

-*Soxhlet Extraction* Pet Ether (60-70°C)/ Diethyl Ether/ Glacial Acetic Acid (90 10 1 by vol)

(ii) Chicken 3 Samples Pet Ether (60-70°C)/ Diethyl Ether/ Glacial Acetic Acid (90 10 1 by vol)

Note: Fatty acid methyl ester standards were not always spotted for TLC analysis Instead steryl esters were used which have an identical R_f value to the FAMES for solvent system hexane/ diethyl ether glacial acetic acid (70 30 3 by vol) This is a sufficient indication of whether the FAMES were present or not in the lipid extract by the non-detection of a spot at the appropriate R_f value Using the solvent system pet ether/ diethyl ether/ glacial acetic acid, the R_f values of steryl esters and FAMES differ, but for any analysis performed with this solvent system a fatty acid methyl ester standard was spotted

2 2 5 Detection of Fatty Acid Methyl Esters by GC-FID

A profile of the constituent fatty acids that make up the lipids of various foodstuffs was achieved by GC analysis of the methyl esters of these fatty acids The following procedures and conditions were involved in the analysis of the FAMES

GC Columns:

Two different columns were used in the analysis of the FAMES from the various samples extracted using different techniques

(i) Hewlett Packard Ultra 2 (5%-diphenyl 95%-dimethylpolysiloxane)

This column was 25m long with a 0 2mm internal diameter and a film thickness of 0 33μm (Used for the analysis of Chicken 1 Samples)

(ii) Macherey-Nagel Permabond FFAP-DF-0 25

This column was 25m long with an internal diameter 0 25mm and 0 25μm film thickness (Used for the analysis of Chicken 2, Chicken 3 and Olive samples)

All analysis was performed on a Shimadzu GC14A Gas Chromatograph

Conditions, calibration, and sample calculations are described in Appendix B

2 3 RESULTS and DISCUSSION.

2 3 1 Results and Discussion of Lipid Extraction Methods

For the purpose of comparison, reference values for the amount of fat present in chicken, olives, and figs have been included from the Composition of Foods Tables by McCance and Widdowson [155]

It must be noted that reference is made in this text that "the major source of variation in meat composition is the proportion of lean to fat and it is extremely difficult to define the level of average lipid content for a particular joint" [155]

Table 2 3 1 Total fat content of various foodstuffs used for analysis in this research [149]

Food Substrate	Total Fat (g/100g)	Fatty Acids (g/100g)		
		1	2	3
Chicken (meat only)	4 3	1 4	1 8	0 8
Chicken (meat and skin)	17 7	5 9	7 5	3 3
Olives	11 0	1 7	5 7	1 3
Figs (dried)	1 5	N	N	N

N = Nil 1=Saturated, 2=Monounsaturated, 3=Polyunsaturated

1 **Folch Extraction**

(i) **Chicken 1 Sample** (Meat only, raw)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	10 120	0 761	7 519
2	10 114	0 862	8 523
3	10 807	0 843	7 800
4	10 302	0 671	6 513
5	6 436	0 431	6 697
6	6 159	0 417	6 770
7	10 021	0 803	8 013
8	10 218	0 716	7 011
9	10 321	0 661	6 404
10	10 258	0 687	6 694

Average Total Fat = 7 194 g/100g

Standard Deviation = 0 723

Relative Standard Deviation = 10 05%

No Outliers (Outlier test applied was Dixon's Q test, see Statistics Appendix C)

(ii) **Chicken 2 Sample** (meat only, raw)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	9 929	0 287	2 890
2	10 178	0 404	3 969
3	10 068	0 363	3 605
4	10 061	0 361	3 588
5	10 116	0 232	2 293
6	10 126	0 238	2 350
7	10 119	0 340	3 360
8	10 093	0 356	3 527
9	10 153	0 263	2 590

Average Total Fat = 3 130 g/100g

Standard Deviation =0 613

RSD = 19 56%

No Outliers

(iii) **Chicken 3 Sample** (meat and skin, raw)

Extraction No	Sample Weight	Yield of Fat (g)	Total Fat (g/100g)
1	20 576	4 241	20 610
2	10 109	1 931	19 106
3	10 2353	2 246	21 945
4	10 173	1 954	19 209
5	10 122	1 942	19 178

Average Total Fat = 20 010 g/100g

Standard Deviation = 1 250

RSD = 6 25%

No Outlier

(iv) **Olives** (Black)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	10 162	2 523	24 825
2	10 545	2 417	22 917
3	10 212	2 349	23 011
4	10 853	2 444	23 936
5	10 158	2 381	23 445

Average Total Fat = 23 627 g/100g

Standard Deviation = 0 782

RSD = 3 31%

No Outliers

In the Folch procedure a 2 1 by volume ratio of dichloromethane methanol is used, the former to extract the neutral lipids and the latter polar solvent to extract the membrane-bound lipids and lipoproteins

From the analysis of the three different Chicken samples it is evident that the yields obtained varied greatly

Chicken 1 Average = 7 194 g/100g (no skin)

Chicken 2 Average = 3 098 g/100g (no skin)

Chicken 3 Average = 20 0096 g/100g (skin)

The large increase in yield for chicken 3 is mainly attributed to the addition of the skin to these samples, reflecting the large content of fat in the skin

There are further factors which may contribute to the variation in the yield as follows

(i) The homogeniser used in the extraction of lipids from chicken 1 was different to that used in chicken 2 and 3 (See section 2 2 1) This may help account for the variation between chicken 1 and 2

(ii) The final step of the Folch procedure involves the recovery of the lipid by evaporation of the solvent in a rotary film evaporator The solvents involved are dichloromethane, methanol and water (used as a washing solvent) The removal of traces of water proved extremely difficult as it did not azeotrope off with the dichloromethane, thus requiring further additions of the latter solvent in an attempt to solve the problem

For the removal of solvents in chicken 2, a freeze drier was used to try to remove these final traces of water A drop in the yields occurred as expected but it was also felt that some lipid content was lost This freeze drying procedure was not employed for chicken 3

A comparison of the yields obtained for the Folch procedure with the other methods used was very favourable but there are a lot of difficulties involved in using this method to achieve a smooth, simple and effective technique as part of a protocol for radiolyte detection The main difficulties encountered were removal of the aqueous non-lipid phase by siphoning, and the washing of the interface with dichloromethane methanol water 3 48 47 in such a manner as to ensure that the lower phase is not disturbed

As well as having the highest yields, the Folch procedure also provides the greatest RSD standing at 10 05, 19 56, and 6 25% respectively for chicken 1,2, and 3 This provides some confirmation as to the difficulty of some elements of this procedure resulting in a loss of precision

The ANOVA results (see Appendix C) show that there was no significant difference between the Folch method and the Soxhlet method using dichloromethane methanol, probably due to the solvent choice of both methods Also statistically there was no significant difference observed between the Folch method and the Foss-let method in the extraction of lipids from chicken 3

From the analysis of the olive sample the yield obtained was 23 627% This value is well in excess of the value quoted for the reference (11%) This reference value is quoted for green olives and the samples analysed in this research were black olives The reference value will also depend on the extraction procedure used to obtain it This is certainly portrayed in the results obtained for a range of extraction procedures used in this research The RSD value of 3 13% suggests that the extraction of lipids from olives is more precise than the extraction of lipids from chicken using the Folch procedure

Statistically, there is sufficient variation in the results obtained for the different extraction procedures with respect to the Folch method The Modified Folch method was nearest to being similar in yields

2 **Modified Folch Extraction**

(i) **Chicken 3 Sample** (meat and skin, raw)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	1 992	0 357	17 934
2	2 086	0 367	17 585
3	2 101	0 371	17 663

Extraction No.	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
4	1.998	0.356	17.811
5	2.019	0.359	17.792

Average Total Fat = 17.750 g/100g

Standard Deviation = 0.136

RSD = 0.76%

No Outliers

(ii) **Olives** (black)

Extraction No.	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	2.181	0.535	24.571
2	1.811	0.441	24.349
3	2.010	0.488	24.266
4	2.007	0.497	24.820
5	2.113	0.519	24.569

Average Total Fat = 24.51 g/100g

Standard Deviation = 0.217.

RSD = 0.88%

No Outliers

The Modified Folch method was applied to chicken 3 and an average yield of 17.757 g/100g was obtained This was the closest of all the methods of extraction used to the reference value of 17.7 g/100g.

The result was lower than the Folch method and statistically significantly different The slight variation in the extraction technique as well as random error contributes to this difference The precision is better than the Folch with an RSD value of 0 76%

The yield obtained for the extraction of the lipids from the olives was the highest of all the methods employed at 24 515% There is a difference statistically between this method and the Folch method Again the precision is good with an RSD of 0 88%

3 **Bligh and Dyer Extraction**

(i) **Chicken 3 Sample** (meat and skin)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	20 335	2 211	10 875
2	20 169	2 069	10 260
3	20 228	2 103	10 397
4	20 314	2 168	10 673
5	20 402	2 242	10 991

Average Total Fat = 10 639 g/100g

Standard Deviation =0 247

RSD = 2 32%

No Outliers

(ii) **Olives** (black)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	20 165	2 432	12 059
2	20 414	2 590	12 688
3	20 388	2 565	12 585
4	20 116	2 480	12 329
5	20 191	2 431	12 044

Average Total Fat = 12 34 g/100g

Standard Deviation = 0 295

RSD = 2 39%

No outliers

The Bligh and Dyer procedure is a simplified version of the classical Folch procedure and the average yield for chicken 3 was 10 639% This was considerably lower than the Folch result The different ratio of the solvents used may account for this difference

The yield of the fat content for olives is 12 341%, the lowest of all the methods used, suggesting possibly an incomplete extraction of the lipids

4 **Soxhlet Extraction**

(i) **Chicken 1 Sample (meat only, raw)**

(a) Trichloroethane Methanol (2 1 by vol) Extraction Solvent

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	5 024	0 313	6 249
2	5 011	0 321	6 413
3	5 164	0 328	6 366

Average Total Fat = 6 343 g/100g

Standard Deviation = 0 084

RSD = 1 33%

(b) Pet Ether (40-60) Extraction Solvent

Extraction No	Sample Weight	Yield of Fat (g)	Total Fat (g/100g)
1	10 081	0 470	4 663
2	7 592	0 183	2 410*
3	10 244	0 473	4 621
4	10 162	0 472	4 646

Average Total Fat = 4 643

Standard Deviation = 0 021

RSD = 0 45%

*Outlier not included in statistical result

(c) Dichloromethane Methanol (2 1 by vol) Extraction Solvent

Extraction Number	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	8 204	0 416	5 070*
2	9 938	0 741	7 456
3	10 040	0 701	6 988
4	10 011	0 542	5 411
5	10 108	0 687	6 796

Average Total Fat = 7 080 g/100g

Standard Deviation = 0 339

RSD = 4 79%

*Outlier

(ii) **Chicken 2 Sample (meat only, raw)**

(a) Dichloromethane Methanol (2 1 by vol) Extraction Solvent

Extraction Number	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	10 128	0 162	1 604
2	10 174	0 168	1 659
3	10 183	0 2096	2 058
4	10 008	0 136	1 355
5	10 014	0 133	1 331
6	10 224	0 171	1 677
7	10 201	0 180	1 770
8	10 195	0 164	1 616
9	10 003	0 168	1 697

Extraction Number	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
10	10 017	0 212	2 119
11	10 112	0 209	2 072
12	10 166	0 175	1 722

Average Total Fat = 1 723 g/100g

Standard Deviation = 0 255

RSD = 14 79%

No Outliers

(iii) **Chicken 3 Sample (meat and skin, raw)**

(a) Pet Ether (40-60) as Extraction Solvent

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	20 330	0 927	4 558
2	20 247	0 913	4 510
3	20 261	0 917	4 529
4	20 381	0 927	4 548
5	20 248	0 891	4 399

Average Total Fat = 4 509 g/100g

Standard Deviation = 0 064

RSD = 1 42%

No outliers

(b) Hexane as Extraction Solvent

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	20 690	0 609	2 947
2	20 049	0 624	3 113
3	20 183	0 628	3 058
4	20 241	0 605	2 991
5	20 169	0 607	3 010

Average Total Fat = 3 024 g/100g

RSD= 2 10%

Standard Deviation= 0 064

No Outliers

(c) Dichloromethane Methanol (2 1 by vol) as Extraction Solvent

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	21 009	2 590	12 327
2	20 686	2 689	13 001
3	20 549	2 596	12 636
4	20 331	2 585	12 714
5	20 494	2 595	12 661

Average Total Fat = 12 668 g/100g

Standard Deviation = 0 240

RSD = 1 89%

No Outliers

(iv) **Olives (black)**

(a) Pet Ether as Extraction Solvent

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	20 886	2 029	9 713
2	20 606	1 760	8 543
3	20 241	1 784	8 812
4	20 550	1 987	9 669
5	20 493	2 036	9 934

Average Total Fat = 9 334 g/100g

Standard Deviation = 0 615

RSD = 6 59%

No Outliers

(b) Hexane Extraction Solvent

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	20 450	1 772	8 667
2	20 031	1 745	8 714
3	20 011	1 713	8 559
4	20 310	1 771	8 720
5	20 309	1 768	8 707

Average Total Fat = 8 673 g/100g

Standard Deviation = 0 067

RSD = 0 77%

No Outliers

(c) Dichloromethane Methanol (2 1 by vol) as Extraction Solvent

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	20 837	3 547	17 025
2	20 845	3 588	17 213
3	20 666	3 430	16 599
4	20 414	3 475	17 023
5	20 381	3 266	16 026

Average Total Fat = 16 777 g/100g

Standard Deviation = 0 476

RSD = 2 84%

No Outliers

The Soxhlet procedure is an example of a continuous extraction procedure as opposed to the more conventional method of extraction This method more than any other used in this research demonstrates the difference in yields obtained by using different solvents for extraction of the lipids

Three different solvent systems were used in the analysis of chicken 1 The results obtained show that the average yield of lipid using petroleum ether was lower than that of tce/methanol (2 1 v/v) and dichloromethane/methanol (2 1 v/v), the latter two producing yields of somewhat similar magnitude

The RSD values were satisfactory at 1 33 0 45 and 4 79% respectively ANOVA tests were applied to all three systems i e non-polar/polar and non-polar systems and a significant difference was observed ANOVA test between

the non-polar/polar solvent systems showed no difference statistically between the methods

Due to a larger difference in the RSD value for dichloromethane/methanol system, a modification was introduced to the Soxhlet procedure for the extraction of lipids from chicken 2 using the same solvent system After rotary film evaporation, it was decided to remove any final remnants of solvent using a vacuum oven at 40°C in an attempt to achieve more consistent yields This had the adverse effect of increasing the RSD to 14 79% and so this modification was omitted in the analysis of chicken 3 The yields obtained for chicken 2 were also lower than those for chicken 1

The Soxhlet analysis of chicken 3 also employed 3 different solvent systems in order of increasing yield i e hexane (3 02%), pet ether (4 5%) and dichloromethane/ methanol 2 1 v/v (12 67%) This suggests that the more polar the solvent system the higher the yield, probably reflecting the extraction of the membrane-bound lipids by breakage of the hydrogen bonding by the polar solvent The drawback is the inconsistency of the precision A large difference was observed in the yields of the Soxhlet method when compared with the Folch procedure

A large difference was observed between the three sets of data using statistical analysis

Once more the average total fat of the olives was greatest using the non-polar/ polar solvent system The yields obtained were lower than those for the Folch method, with only the dichloromethane/ methanol yield (16 77%) being higher than the reference value of 11%

The RSD values for all the Soxhlet extractions were less than the Folch making it a more precise method of extraction. This is an important feature when considering a suitable method of extraction for the incorporation into a methodology of radiolyte detection.

Loss of accuracy in the Soxhlet method may be attributed to the loss of any volatiles during extraction, as this method involves the use of heat.

5 Modified Fatty Foods Extraction

(i) **Chicken 2 (meat only, raw)**

Extraction No.	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	10.152	0.258	2.541
2	10.295	0.276	2.681
3	10.159	0.262	2.579
4	10.170	0.273	2.684
5	10.250	0.222	2.166
6	10.176	0.220	2.162
7	10 172	0.244	2.395
8	10.166	0.246	2.421

Average Total Fat = 2.454 g/100g

Standard Deviation = 0.207

RSD = 8.44%

No Outliers

This extraction procedure was applied to chicken 2. The average yield obtained for the modified fatty foods extraction was 2.454% falling between the Folch

and Soxhlet extraction for the same chicken sample The RSD value for the extractions was also the lowest for all the extractions performed on chicken 2 at 8 44%, showing better reproducibility of this technique and this is reflected in the ease of the procedure An increase in the yield may have been achieved by reducing the volume of solvent for extraction but increasing the number of washes or re-extractions

6 Foss-let Extraction

(i) **Chicken 3 Sample (meat and skin, raw)**

Extraction No	Sample Weight (g)	Total Fat[a] (g/100g)
1	22 511	19 75
2	22 505	19 70
3	22 518	19 70
4	22 502	19 80
5	22 510	19 70

[a] Each value is an average of three readings

Note: Each measurement gave a value in g/100g and no yield was recorded

Average Total Fat = 19 730 g/100g

Standard Deviation = 0 045

RSD = 0 24%

No outliers

(ii) **Olives (black)**

Extraction No	Sample Weight (g)	Total Fat (g/100g)
1	22 501	13 75
2	22 509	13 85
3	22 510	13 85
4	22 518	13 90
5	22 522	13 75

Average Total Fat = 13 820 g/100g

Standard Deviation = 0 067

RSD = 0 48%

No Outlier

The Foss-let extraction method was the only automated fat content measurement system used in the course of the research

The average total fat yield was 19 73% for the extraction of lipids from the chicken 3 sample This is the closest value to the reference of 17 7% in Table 2 3 1 The RSD value is also the smallest, at just 0 24%

Calcium sulphate hemihydrate is used as a drying agent to prevent absorption of water by solvent and increase the efficiency of extraction of lipids

The yield of fat for the olives was 13 82% and was lower than the results obtained for the extraction methods employing polar/non-polar solvent mixtures but was higher than the other single solvent methods

The RSD is again very low for this procedure (0 485%) supporting the evidence of repeatability of automated systems

7 Total Lipids Extraction

(i) Chicken 3 Sample (meat and skin, raw)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	10 031	1 376	13 719
2	10 182	1 355	13 310
3	10 301	1 437	13 947
4	10 229	1 375	13 441
5	10 213	1 388	13 595

Average Total Fat = 13 602

Standard Deviation = 0 245

RSD = 1 81%

No Outliers

(ii) Olive Sample (black)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	10 051	1 681	16 722
2	10 012	1 669	16 668
3	10 063	1 642	16 316
4	10 058	1 651	16 412
5	10 009	1 620	16 189

Average Total Fat = 16 461

Standard Deviation = 0 228

RSD = 1 39%

No Outliers

The averge total fat extracted from Chichen 3 using this technique was 13 60% suggesting that the centrifugation step may be less efficient in the extraction process than the normal homogenisation step

Statistically, the method is significantly different than the others used, but the RSD value of 1 82% gives support to the reproducibility of the method

The average yield recorded for the extraction of the lipids from the olives using the Total Lipids extraction procedure was 16 46% This two component solvent system gave results higher than the Bligh and Dyer system but considerably less than the remaining two component solvent systems, the Folch, the Modified Folch, and the Batch extraction procedures The RSD value was low at 1 39%

8 Batch Extraction

(i) **Chicken 3 Sample** (meat and skin, raw)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Lipids (g/100g)
1	10 088	1 552	15 388
2	10 101	1 552	15 369
3	10 095	1 548	15 539
4	10 099	1 551	15 359
5	10 092	1 552	15 181

Average Total Fat = 15 367

Standard Deviation = 0 127

RSD = 0 83%

No Outliers

(ii) Olive Sample (black)

Extraction No	Sample Weight (g)	Yield of Fat (g)	Total Fat (g/100g)
1	10 126	1 889	18 661
2	10 122	1 863	18 404
3	10 118	1 876	18 539
4	10 102	1 863	18 444
5	10 124	1 868	18 448

Average Total Fat = 18 499 g/100g

Standard Deviation = 0 103

RSD = 0 56%

No Outliers

The average total fat yield recorded at 15 37% for this method was lower than the Folch procedure suggesting that the dichloromethane methanol solvent system is more effective than the hexane isopropanol one The yield was however higher than the Bligh and Dyer method which did use the former solvent system

The RSD value was 0 827% reflecting the ease of the extraction when compared to the Folch extraction

The method did vary from the others as proven by the statistical analysis, most probably due to the change in the solvent system An average yield of 18 49% and RSD value of just 0 557% makes this method an extremely effective method for the extraction of lipids from olives As with the chicken the yields

were greater than the Bligh and Dyer, Total Lipids, Soxhlet and Foss-let methods but less than the Folch procedures The RSD value is again low at 0 56% making this method of extraction more precise than some of the other more difficult techniques

Statistically this method is different from the other extraction methods used

Conclusion:

The following conclusions may be drawn from the results obtained, both in a general manner, and also with specific reference to a particular foodstuff analysed or an extraction procedure used

(i) It is apparent that the fat content may vary from one sample of a particular food to another This specifically refers to the results obtained for the extractions performed on chickens 1 and 2 There were certain modifications made to procedures between the analysis of one chicken and another but the fact that a lower value was observed for each procedure applied supports the lower fat content of chicken 2 This may be a result of dietary differences between samples or other factors such as age or sex

(ii) The amount of fat present in the skin of chicken is clearly demonstrated by the difference in the results obtained for chickens 1 and 2 (*which* were skinless) compared to chicken 3 (skin included)

(iii) The choice of solvent system for an extraction method is vital to the yield of lipids This is clearly demonstrated for the extraction of lipids from olives by the Soxhlet procedure using 3 different solvent systems i e

Dichloromethane Methanol> Pet Ether> Hexane

The use of a combination of non-polar polar solvents in a certain volume ratio (usually 2 1 v/v) produces the highest yields attainable

(iv) The variability between samples in a particular method of extraction was highest for procedures involving the removal of water as part of a solvent mixture to recover the final lipid i e Folch, Modified Folch and the Bligh and Dyer method The variability was also high for the Soxhlet extractions on chickens 1 and 2, attributable mainly to the inexperience of the analyst There was a major improvement in the precision of the Soxhlet method for the extraction of lipids from the chicken 3 sample

The Foss-let extraction method was found to be the most precise in terms of repeatability supporting the case for an automated system for precise analysis

(v) The experimental data gathered for this research is not attempting to prove that the methods of extraction provide similar results, but more to emphasise that yields obtained depend greatly on the method of extraction used

One of the main objectives of using a series of extraction methods in the research was to comment on the following

(a) efficiency of extraction in terms of yield and reproducibility and

(b) from performing the extraction procedures, to comment on the suitability of a procedure for incorporation into an overall method to detect radiolytes which are produced from food lipids, in irradiated foodstuffs

In relation to point (b) the Soxhlet extraction method offers certain advantages and this was chosen as the method of extraction of radiolytes from chicken, olives and figs It is a continuous process and can be carried out with intermittent observation Of the three solvent systems used in the Soxhlet experiments, the non-polar single solvent system hexane gave the lowest yield in terms of fat extracted from the foodstuffs However hexane is very volatile making recovery of the lipid extract from the solvent a rapid process This is also true for the pet ether solvent system which gave a slightly higher yield of fat than the hexane system

The Foss-let procedure is the method of highest precision due to its automated nature but has the disadvantage of the volatility of the solvent i e perchloroethylene with a boiling point of 110 °C as opposed to 69°C for hexane and 40-60°C for petroleum ether

In making a choice with respect to a particular solvent system for extraction of radiolytes from the foodstuffs analysed, consideration will have to be given to the type of lipids extracted by the various solvent systems

2 3 2 Results and Discussion of TLC Analysis of Lipids and Methylated Lipids

The main lipid classes identified by TLC were

Triacylglycerols, fatty acids, sterols, and fatty acid methyl esters (methylated samples only)

TLC of Lipid Samples

(i) Chicken 1 Sample

No TLC analyses were performed on the lipid extracts from the Folch and Soxhlet procedures of chicken 1 samples The main reason for this was that at the time only fatty acid profiles were being considered, and not until the next series of extractions was it deemed necessary to identify a wider range of lipid classes

(ii) Chicken 2 Sample

Folch extractions of chicken 2 sample The solvent system used was hexane/ diethyl ether/ glacial acetic acid 70 30 3 by volume R_f values obtained for the triacylglycerol standard (0 58) and sample (0 54)

Soxhlet extractions of chicken sample 2 The solvent system used was pet ether (60-70°C)/ diethyl ether/ glacial acetic acid (90 10 1 by vol) R_f values of all the lipid classes were reduced due to a decrease in the polarity of the solvent system A fatty acid methyl ester standard was spotted and as expected no corresponding R_f value was detected in the lipid extract samples

(iii) **Chicken 3 Sample**

A representative number of samples from each extraction method were analysed by TLC The solvent system used was hexane/ diethyl ether/ glacial acetic acid (70 30 3 by vol) The first three extraction methods i e the Folch, Modified Folch, and the Bligh and Dyer procedures, all produced very similar TLC results

No fatty acid methyl esters were detected in the lipid samples as expected The lipids of interest, free fatty acids and triacylglycerols were both present

The Soxhlet lipid extracts of chicken 3 sample, where 3 different extracting solvent systems were used provided an interesting result The non-polar extracting solvents used i e pet ether and hexane failed to extract the more complex polar lipids The most polar compound extracted by these solvents appears to be cholesterol The Soxhlet extracts using dichloromethane/ methanol as extracting solvent do yield complex polar lipids Therefore if interest lies in extracting the maximum amount of lipid from a sample the use of a polar solvent as part of the extracting system is very important

Finally the Foss-let, Batch, and Total Lipids extraction again provided very similar TLC results, with the common feature of the absence of fatty acid methyl esters in the lipid extract results

(iv) **Olive Samples**

The solvent system used in all TLC analyses of the olive samples was hexane diethyl ether glacial acetic acid (70 30 3 by vol) The main conclusion to be drawn from the TLC results of the olive samples is that qualitatively, there is

little difference between the lipid composition of the olives and that of the chicken.

The Folch, Modified Folch, and the Bligh and Dyer methods all produced very similar results, easily detecting the presence of free fatty acids and triacylglycerols. No fatty acid methyl esters were detected in any of these samples.

Further evidence is given of the absence of complex polar lipids in the Soxhlet non-polar extractions, and their subsequent detection using dichloromethane/ methanol as the extracting solvent.

The Foss-let, Batch and Total Lipids extractions also provided similar TLC results.

TLC of Methylated Lipid Samples

Modified Fatty Foods extraction of chicken 2 sample. The purpose of the analysis was to show the disappearance of the free fatty acids and the triacylglycerols upon their methylation to fatty acid methyl esters and this was dually observed. The only problem was the proximity of the newly formed fatty acid methyl esters (R_f = 0.96) to the standard triacylglycerol (R_f = 0.89). This was due to the solvent system used i.e. hexane/ diethyl ether/ glacial acetic acid (70:30:3 by vol.). The appearance of a polar constituent in the methylated sample may be due to some unsaponifiable material or glycerol being produced upon hydrolysis of the lipids and their subsequent methylation. As a result glycerol standard was spotted but no detection was achieved.

The closeness of the fatty acid methyl esters to the triacylglycerols prompted a change in the solvent system for the remaining extracts to be analysed. The new system was pet ether/ diethyl ether/ glacial acetic acid (90: 10: 1 by vol.).

A greater separation between the fatty acid methyl esters and the triacylglycerols was immediately observed in the analysis of the methylated extracts of the Soxhlet extractions of chicken 2 sample. The R_f value for the FAME was 0.69 and that of the triacylglycerol standard was 0.29. As expected the triacylglycerol and the free fatty acids were not detected in the methylated samples.

Chicken 3 and the olive samples all provided positive results for the detection of fatty acid methyl esters in the methylated samples of the various extraction methods, and no detection of free fatty acids or triacylglycerols supporting a 100% conversion during the methylation procedure. The non-polar Soxhlet extractions of chicken 3 and olives showed the presence of a polar constituent that was absent in the original lipid extracts. This may support the formation of glycerol upon hydrolysis of the lipids and their subsequent methylation.

Conclusion:

The following general conclusions can be made from the TLC analysis employed in the qualitative classification of the individual lipid components of both the chicken and olive foodstuffs, and the confirmation of their methylation upon hydrolysis.

(1) Thin layer chromatography of the lipid extracts provides a qualitative system of identification of the various lipid classes in the complex food matrices of chickens and olives.

(2) As the lipids of interest in this research were non-polar, and more importantly as the solid adsorbent silica is polar in nature, a polar solvent system was employed to effect the best separation of the various lipid components

Of the two solvent systems used, the pet ether (60-70°C)/ diethyl ether/ glacial acetic acid showed better resolution of fatty acid methyl esters and triacylglycerols

(3) There were no significant differences in the types of lipids present from variation of the methods of extraction and the solvents used for extracting them The major difference was observed in the Soxhlet results where the use of a non-polar solvent such as hexane yielded no polar lipids upon TLC analysis as opposed to the more polar dichloromethane methanol (2 1 by vol)

(4) There was also no observable difference in the lipid classification of chicken samples and those of olive samples, although there may be a difference quantitatively

2 3 3 Results and Discussion of Fatty Acid Content of Lipids from Chicken and Olives

This section is concerned with the qualitative and quantitative fatty acid composition of each lipid type extracted The purpose of the fatty acid profile analysis performed in this research is to ascertain the major fatty acids of the foodstuffs of interest, and from this to determine the main radiolytes that might form upon irradiation of these foods

The method of detection of the fatty acid content of these foodstuffs is by GC-FID analysis of the fatty acid methyl esters, which are prepared by hydrolysis of the parent triacylglycerols, followed by methylation

The results of the analysis are given in tabulated form under each foodstuff analysed Values are quoted in three ways Firstly the number of milligrams of fatty acid per gram of fat extracted is quoted This value considers the fatty acid content of the fat extracted which is dependent on the extraction method used The second table of each analysis quotes the concentration as the number of grams of fatty acid per 100 grams of meat (or fruit) extracted This is a direct reflection of the yield of fat obtained from each extraction method, as the quantity of fatty acid should increase with increased yield of fat from the extraction The third and final table of the fatty acid analysis shows the major fatty acid distribution in each food sample These table formats were chosen because it was felt they would give a clear indication of the results obtained and also would provide a means of direct comparison to results recorded in the literature

Note:

Calibration and Sample Calculations of Fatty Acid Methyl Esters may be found in Appendix D

The results are divided into the following sections

(i) Chicken 1 (meat only, no skin)

(ii) Chicken 2 (meat only, no skin)

(iii) Chicken 3 (meat and skin)

(iv) Olives

All results are an average of the extraction performed for each method

(i) **Chicken 1** (meat only)

n = number of samples analysed

Table 2 3 3 1 Concentration of palmitic acid per g of fat and per 100 grams of chicken meat

Extraction Method	mg palmitic acid/ g of fat	g palmitic acid/ 100 g of chicken
Folch n=10	**31.799** s=4 498	**0.227** s=0 034
Soxhlet[a] n=1	**45.257** (1 measurement)	**0.282** (1 measurement)
Soxhlet[b] n=3	**64.226** s=4 872	**0.299** s=0 212
Soxhlet[c] n=4	**45.716** s=6 63	**0 269** s=0 046

[a] Trichloroethane/ Methanol (2 1 by vol)

[b] Petroleum Ether (40-60)

[c] Dichloromethane/ Methanol (2 1 by vol)

s = Standard Deviation

(ii) **Chicken 2 (meat only)**

n = number of samples analysed

Table 2 3 3 2 Fatty acid profile of all extraction methods used

Extraction Method	mg fatty acid/ g of fat		
	16 0	18 0	18 1
Folch n=9	**199.646** s=25 258	**76.301** s=10 775	**344.469** s=37 026
Soxhlet (Dichloromethane/ Methanol) n=12	**220.750** s=10 398	**66.607** s=4 617	**354.116** s=5 737
Modified Fatty Foods n=8	**242.604** s=10 782	**67.275** s=10 782	**448.192** s=20 906

Table 2 3 3 3 Concentration of fatty acids per 100 grams of chicken meat

Extraction Method	g fatty acid/ 100g of chicken		
	16 0	18 0	18 1
Folch	**0.697** s=0 008	**0.269** s=0 017	**1.246** s=0 025
Soxhlet (Dichloromethane/ Methanol)	**0 390** s=0 051	**0.122** s=0 016	**0.656** s=0 062
Modified Fatty Foods	**0.609** s=0 028	**0.167** s=0 009	**1.134** s=0 012

(iii) **Chicken 3 (meat and skin)**

All values given are the average of five measurements

Table 2 3 3 4 Fatty acid profile of all extraction methods used

Extraction Method	mg fatty acid / g of fat					
	14 0	16 0	18 0	18 1	18 2	18 3
Folch	**2.719** s=0 350	**104.832** s=4 820	**21.781** s=1 589	**141.149** s=5 909	**77.733** s=3 148	**8.519** s=0 357
Modified Folch	**2.270** s=0 056	**90.778** s=0 634	**17.776** s=0 354	**122.291** s=0 557	**67.126** s=0 654	**7.689** s=0 741
Bligh and Dyer	**2.173** s=0 066	**89.450** s=1 924	**17.364** s=0 517	**120.01** s=3 429	**65.212** s=2 216	**7.035** s=0 178
Soxhlet[a]	**3.936** s=0 484	**153 929** s=18 11	**32.199** s=1 821	**241.202** s=4 826	**128 029** s=7 431	**18.299** s=2 321
Soxhlet[b]	**4.334** s=0 100	**166.496** s=3 056	**32.541** s=0 986	**260 956** s=1 678	**137 581** s=3 532	**14.020** s=1 970
Soxhlet[c]	**3.436** s=0 103	**130.826** s=3 564	**26.553** s=0 939	**205 556** s=5 065	**104.922** s=2 960	**11.324** s=0 372
Total Lipids	**2.226** s=0 019	**91.207** s=1 733	**17.558** s=0 367	**122.000** s=1 667	**66.554** s=0 724	**7.207** s=0 295
Batch Extraction	**2.195** s=0 027	**90.941** s=0 963	**17.348** s=0 414	**121.506** s=0 805	**66.190** s=0 592	**7.206** s=0 156

s = standard deviation

[a] Pet ether extracting solvent

[b] Hexane as extracting solvent

[c] Dichloromethane/ Methanol as extracting solvent

All values given are the average of five measurements.

Table 2 3 3 5 Concentration of fatty acids per 100g of chicken meat

Extraction Method	g fatty acid / 100g of chicken					
	14 0	16 0	18 0	18 1	18 2	18 3
Folch	**0.051** s=0 003	**2.199** s=0 284	**0.419** s=0 022	**2.799** s=0 149	**1 541** s=0 076	**0.169** 0 007
Modified Folch	**0.040** s=0 001	**1.612** s=0 010	**0.316** s=0 006	**2.171** s=0 009	**1.176** s=0 031	**0.136** 0 013
Bligh and Dyer	**0.023** s=0 005	**0.951** s=0 012	**0.184** s=0 004	**1.275** s=0 006	**0 694** s=0 007	**0.075** s=0 002
Soxhlet[a]	**0.019** s=0 003	**0.723** s=0 113	**0.138** s=0 062	**0.108** s=1 323	**0.575** 0 228	**0.082** 0 225
Soxhlet[b]	**0.015** s=0 002	**0 505** s=0 007	**0.099** s=0 003	**0.776** s=0 009	**0 417** s=0 007	**0.042** s=0 005
Soxhlet[c]	**0.043** s=0 002	**1.646** s=0 067	**0.334** s=0 015	**2.587** s=0 097	**1.321** s=0 053	**0.142** s=0 007
Total Lipids	**0.030** s=0 001	**1.243** s=0 001	**0.238** s=0 005	**1.659** s=0 010	**0.905** s=0 009	**0 101** s=0 006
Batch Extraction	**0.033** s=0 001	**1.399** s=0 008	**0.264** s=0 006	**1.867** s=0 010	**1 017** s=0 001	**0.110** s=0 002

[a] Pet ether as extracting solvent

[b] Hexane as extracting solvent

[c] Dichloromethane/ Methanol as extracting solvent

All values given are the average of five measurements.

Table 2 3 3 6 Average fatty acid distribution of lipids in each extraction method

Extraction Method	% fatty acid					
	14 0	16 0	18 0	18 1	18 2	18 3
Folch	**0.722** s=0 006	**29.427** s=0 177	**5.932** s=0 034	**39.685** s=0 210	**21 814** s=0 087	**2.362** s=0 072
Modified Folch	**0.729** s=012	**29 418** s=0 065	**5.792** s=0 090	**39.638** s=0 136	**21.754** s=0 125	**2.492** 0 229
Bligh and Dyer	**0.725** s=0 009	**29.772** s=0 114	**5.78** s=0 124	**39.688** s=0 114	**21 598** s=0 074	**2.342** s=0 041
Soxhlet[a]	**0.7125** s=0 005	**27.13** s=0 113	**5.447** s=0 062	**41.487** s=1 323	**21.795** 0 228	**3.310** 0 225
Soxhlet[b]	**0.708** s=0 009	**27.120** s=0 212	**5.360** s=0 165	**42.080** s=0 563	**22 410** s=0 480	**2.284** s=0 306
Soxhlet[c]	**0.709** s=0 008	**27.012** s=0 326	**5.532** s=0 040	**42.445** s=0 301	**21.662** s=0 048	**2.337** s=0 043
Total Lipids	**0.723** s=0 008	**29.644** s=0 133	**5.596** s=0 291	**39 588** s=0 202	**21.586** s=0 208	**2 452** s=0 235
Batch Extraction	**0.717** s=0 007	**28.046** s=3 401	**5.628** s=0 117	**39.742** s=0 136	**21.608** s=0 208	**2.352** s=0 046

[a] Pet ether as extracting solvent

[b] Hexane as extracting solvent

[c] Dichloromethane/ Methanol as extracting solvent

(iv) **Olives**

All values given are the average of five measurements.

Table 2 3 3 7 Fatty Acid Profile of all extraction methods used

Extraction Method	mg fatty acid/ g fat extracted				
	16 0	18 0	18 1	18 2	18 3
Folch	**75.926** s=5 702	**14.014** s=1 918	**405.197** s=31 929	**117.841** s=8 754	**4.858** s=0 406
Modified Folch	**69.042** s=2 185	**13.384** s=0 674	**359.992** s=8 501	**104.685** s=3 377	**3.615** s=1 399
Bligh and Dyer	**81.727** s=2 619	**16.449** s=1 163	**415.507** s=14 398	**124.269** s=5 188	**4.860** s=0 231
Soxhlet[a]	**97.998** s=6 482	**19.325** s=1 089	**495.846** s=27 828	**151 222** s=13 969	**5 848** s=0 496
Soxhlet[b]	**90.914** s=2 698	**16.041** s=0 545	**452.525** s=7 839	**140.112** s=5 338	**5.549** s=0 172
Soxhlet[c]	**89.682** s=1 656	**15.470** s=0 830	**454 513** s=8 005	**136.150** s=2 036	**5.090** s=0 533
Total Lipids	**84.462** s=2 8612	**17.750** s=1 363	**435.251** s=9 191	**132.046** s=3 461	**5.153** s=0 285
Batch Extraction	**87.859** s=1 565	**17.325** s=1 321	**445.871** s=2 749	**133.878** s=1 943	**5.167** s=0 162

[a] = Pet ether as extracting solvent

[b] = Hexane as extracting solvent

[c] = Dichloromethane/ Methanol as extracting solvent

All values given are the average of five measurements.

Table 2 3 3 8 Concentration of fatty acids per 100 grams of olives

Extraction Method	g fatty acid/ 100g olives				
	16 0	18 0	18 1	18 2	18 3
Folch	**0.179** s=0 017	**0.331** s=0 045	**9.464** s=0 915	**2.753** s=0 257	**1.135** s=0 117
Modified Folch	**1.634** s=0 11	**0.328** s=0 016	**8.826** s=0 190	**2.566** s=0 071	**0.103** s=0 003
Bligh and Dyer	**1.008** s=0 025	**0.201** s=0 010	**5.124** s=0 080	**1.551** s=0 035	**0.060** s=0 002
Soxhlet[a]	**0.912** s=0 046	**0.180** s=0 009	**4.621** s=0 273	**1.410** s=0 143	**0.054** 0 001
Soxhlet[b]	**0.788** s=0 020	**0.139** s=0 004	**4 090** s=0 327	**1.215** s=0 047	**0.048** s=0 001
Soxhlet[c]	**1.508** s=0 044	**0.259** s=0 017	**7.625** s=0 254	**2.284** s=0 052	**0.030** s=0 002
Total Lipids	**1.390** s=0 033	**0 292** s=0 021	**7.174** s=0 051	**2 170** s=0 037	**0.0846** s=0 004
Batch Extraction	**1.625** s=0 028	**0.320** s=0 025	**8.246** s=0 037	**2.476** s=0 035	**0.095** s=0 003

[a] = Pet ether as extracting solvent

[b] = Hexane as extracting solvent

[c] = Dichloromethane/ Methanol as extracting solvent

All values given are the average of five measurements.

Table 2 3 3 9 Fatty acid distribution of all the extraction methods

Extraction Method	% fatty acid				
	16 0	18 0	18 1	18 2	18 3
Folch	**12.359** s=0 298	**2.272** s=0 130	**65.080** s=0 795	**18.933** s=0 404	**0.779** s=0 012
Modified Folch	**12.671** s=0 268	**2.457** s=0 124	**65.312** s=0 712	**19.212** s=0 366	**0.774** s=0 021
Bligh and Dyer	**12.540** s=0 269	**2.539** s=0 132	**64.580** s=0 264	**19.589** s=0 264	**0.756** s=0 019
Soxhlet[a]	**12.744** s=0 256	**2.815** s=0 071	**63.898** s=0 641	**19.651** s=0 537	**0.997** s=0 495
Soxhlet[b]	**12.081** s=0 104	**2.261** s=0 044	**64.510** s=0 097	**19 514** s=0 096	**0 786** s=0 008
Soxhlet[c]	**12.805** s=0 101	**2.203** s=0 090	**64.748** s=0 104	**19.514** s=0 096	**0 782** s=0 014
Total Lipids	**12.540** s=0 269	**2.635** s=0 183	**64.506** s=0 356	**19.710** s=0 188	**0.764** s=0 026
Batch Extraction	**12.703** s=0 224	**2.506** s=0 193	**64.393** s=0 286	**19.362** s=0 286	**0.747** s=0 025

[a] = Pet ether as extracting solvent

[b] = Hexane as extracting solvent

[c] = Dichloromethane/ Methanol as extracting solvent

Discussion of Fatty Acid Analysis of Chicken and Olives

Results will be compared with those obtained from the literature Table 2 3 3 10 lists the levels of fatty acids per 100 grams of food [156], and Table 2 3 3 11 lists the levels of fatty acids per 100g of chicken obtained using the Modified Fatty Foods extraction by Morehouse and Ku [132] Finally the levels of fatty acid in olives and chicken fat as a percentage distribution is given in Table 2 3 3 12

Table 2 3 3 10 Levels of fatty acids in various foodstuffs (g/ 100g food) [156]

Food	saturated			mono-unsaturated		polyunsaturated	
	14 0	16 0	18 0	16 1	18 1	18 2	18 3
chicken[a]	0 05	1 08	0 29	0 29	1 62	0 55	0 03
chicken[b]	0 22	4 47	1 19	0 22	6 66	2 26	0 12
olives	0	1 26	0 24	0 11	7 57	1 16	0 07

[a] meat only, raw

[b] meat and skin, raw

Table 2 3 3 11 Levels of fatty acids in chicken meat extracted using the Modified Fatty Foods procedure [132]

Food	g fatty acid/ 100g meat						
	14 0	16 0	18 0	16 1	18 1	18 2	18 3
chicken (ground)	0 083	2 939	0 666	1 068	4 731	2 265	0 094

Table 2 3 3 12 Typical composition of common edible fats and oils

Food	% fatty acid						
	14 0	16 0	18 0	16 1	18 1	18 2	18 3
chicken fat	1 3	23 2	6 4	6 5	41 6	18 9	1 3
Olive Oil	0	13 7	2 5	1 2	71 1	10 0	0 6

It is important to note that a direct comparison with reference tables is difficult due to the following factors affecting the experimental results,

(i) which portion of the food constitutes a fair reflection on the fatty acid content Analysts may vary in the portion of chicken or olives taken

(ii) the method of extraction used i e the reference tables 2 3 3 10 and 2 3 3 11 themselves illustrate these difficulties as they show considerable variation in the levels of fatty acid per 100g of chicken

(iii) the diet of the animals concerned in the analysis i e in this case chickens

(iv) the sex of the animal will also vary the fatty acid content of the chicken

(v) the olives analysed in this research were black olives which are different to the reference olives which were green olives Black olives are riper than their green counterparts, and consequently variations in the fatty acid content are possible

A full range of levels of fatty acids from myristic (14 0) right through to linolenic (18 3) are reported for chicken 3 and the olive foodstuffs Levels of palmitic acid (16 0), stearic acid (18 0), and oleic acid (18 1) are reported for chicken 2 and palmitic acid (16 0) is reported for chicken 1

The two main fatty acids of interest with respect to this research are palmitic acid (16 0) and stearic acid (18 0), as they are the parent fatty acids from which the radiolytes 2-dodecylcyclobutanone and 2-tetradecylcyclobutanone respectively are formed and are used to detect irradiation of food

Chicken 1 and 2 Samples

A review of the results obtained for the concentration of fatty acids in chicken 1 and chicken 2, which were both skinless, reveals that the levels of fatty acids per 100g of fat are lower than the values in Tables 2 3 3 10 (chicken, meat only) except for those of chicken 2 extracted by the Folch method The concentration of palmitic acid was considerably lower in these results but both stearic acid and oleic acid levels were somewhat similar to the tables The levels of palmitic acid per 100g in chicken 1 were lower than expected with the Soxhlet method giving a higher yield than the Folch method In chicken 2 the reverse was obtained whereby the levels of palmitic, stearic, and oleic acid are higher for the Folch extraction than for the Soxhlet extraction TLC analysis established that all fatty acids extracted were converted to fatty acid methyl esters

Chicken 3 and Olives

The results obtained for the fatty acid analysis of chicken 3 are provided in Tables 2 3 3 4-2 3 3 6 A direct comparison with Table 2 3 3 10 reveals a lower level for all fatty acids except linolenic acid (18 3) from the GC-FID analysis The only exception to the lower level of fatty acids when compared with Table 2 3 3 10 is the polyunsaturated fatty acid linolenic acid (18 3) which shows

levels in some extraction procedures slightly higher than quoted values A possible reason for this is the dietary intake of the animal as discussed in the introduction (Chapter 1) Non-ruminants readily incorporate the unsaturated fatty acids of the diet into depot fats As mentioned a number of factors besides diet have influenced the fatty acid composition of the fatty tissue in meat animals of a given species These factors have included genetics and sex effect In general diet has had a more marked effect on fat quality than breed or sex, especially in non-ruminant animals which are susceptible to alteration of tissue fatty acid by dietary modification

The levels of fatty acids obtained for chicken 3 may be more favourably compared with those results obtained by Morehouse and Ku (Table 2 3 3 11) in their investigation into irradiated meat [132] The values obtained are slightly lower than the quoted values This is perhaps a more preferable comparison than Table 2 3 3 10, due to similar conditions of analysis being used

The fatty acid concentrations found in olives were similar to those cited in Table 2 3 3 10 While some concentration levels are higher in some extraction methods an overall consideration of the results shows good compatibility All fatty acids were detectable and quantified Once more the solvent combination of non-polar polar systems for extraction yields the greatest concentration of fatty acids per 100g of food substrate

The tables in the results indicating the concentration of fatty acid per gram of fat extracted provide some insight into the component fractions of the fat itself It was expected that extractions using non-polar solvents (e g Soxhlet-hexane) would contain less unsaponifiable matter than extraction methods that use

polar solvents (e g Folch etc) which would extract more polar lipids that cannot be hydrolysed As more of the lipid from a non-polar extraction can be converted to fatty acid methyl esters, the subsequent concentration of fatty acid per gram of fat will be higher This effect is demonstrated in all three chicken samples tested and in the olive samples across the range of extraction methods used The single solvent Soxhlet extraction system using either hexane or pet ether yielded the highest levels of fatty acid per gram fat extracted from the food samples Within the Soxhlet system itself this is best demonstrated in the chicken 3 sample

Table 2 3 3 4 quoting the concentration of fatty acids in mg per gram of fat extracted using the non-polar solvent hexane is considerably higher than those for the non-polar polar system of dichloromethane methanol The most abundant fatty acid, oleic acid, is present at a level of ~60mg/ g more in the non-polar extraction system This observation is in stark contrast to the yields of fat obtained by each solvent system, where the non-polar polar system has a much higher yield Therefore the result is more a direct reflection on the composition of the lipid classes in each fat sample This observation was also made in the TLC analysis of the extracted lipid extracts of the Soxhlet procedure where there was an absence of the more polar lipids The olive samples confirm these observations to a lesser degree, possibly due to lower levels of polar lipids in its total composition when compared to chicken

The final tables 2 3 3 4-2 3 3 9 presented in this chapter show the percentage distribution of the fatty acids analysed in chicken 3 and the olives This distribution reflects the proportion of fatty acids with respect to each other and

is therefore different to Table 2 3 3 12, which includes a wider range of fatty acids than analysed in this research However, as the extra fatty acids present in Table 2 3 3 12 are negligible in comparison (except for palmitoleic 16 1), the table is a good comparison for the results obtained by this research

The main observable difference in the results for the chicken is that myristic acid is slightly lower than the reference value, while linolenic acid is present at a greater level than expected This may be reflective of the dietary intake of the animal

The results obtained for the olives compare favourably for all fatty acids except linoleic acid (18 2) where it is higher than expected Consequently the level of oleic acid is slightly lower too It is interesting to note that the percentage of oleic acid is much higher in the olives than in the chicken This results in a drop in the levels of palmitic acid and stearic acid in the olives

The only other main observable difference between the fatty acid composition of chickens and olives is that the saturated fatty acid myristic acid while present at low levels in the chicken samples does not appear to be present at all in the olives

The two main fatty acids of interest, palmitic acid and stearic acid, are present in reasonable proportion These are the saturated fatty acids from which 2-DCB and 2-TCB are formed upon irradiation The main fatty acid present in both chicken and olives is the mono-unsaturated fatty acid oleic acid (18 1) The radiolyte formed upon irradiation of this fatty acid is 2-tetradecenylcyclobutanone, an unsaturated cyclic ketone This would be without doubt the most useful marker compound for the identification of irradiated food because it would increase the sensitivity of the method to a

large extent However, the synthesis of an authentic standard for this compound is extremely difficult and until this problem is resolved it will not be considered as a marker compound, thus leaving 2-dodecylcyclobutanone the most suitable compound for this purpose

The Soxhlet extraction method provided more than the 200mgs needed for the protocol to detect the concentrations of the radiolytes DCB and TCB Due to its advantages over the other methods as cited in section 2 3 3, this method was chosen to extract the radiolytes for the detection of irradiated food

CHAPTER THREE

DETECTION OF RADIOLYTES IN IRRADIATED FOODSTUFFS

3 1 INTRODUCTION

In recent times, differentiation between irradiated and non-irradiated food has focused on changes induced by ionising radiation [41]

These changes have been monitored more closely by the development of methods for the detection of specific compounds present only in irradiated food

The focus points with respect to the development of chemical methods have been the detection of a range of hydrocarbons and, more recently, 2-alkylcyclobutanones Champagne & Nawar [157], Kavalam & Nawar [158], and Dubravcic & Nawar [159] performed pioneering work on the detection of specific hydrocarbons in irradiated lipids or various meats As well as these hydrocarbons and other classes of compounds, Le Tellier & Nawar [58] first identified 2-alkylcyclobutanones as products of irradiation by using pure triacylglycerols irradiated at high dosage levels of 60kGy In 1981, Handel & Nawar also isolated 2-dodecylcyclobutanone from a synthetic phospholipid irradiated at 500kGy [137]

It was considered important that, in order to further progress this research, it was necessary to modify methods of detection to coincide with dosage levels more suitable to those specified in legislative documents [160] Pioneering work in this area has been carried out by a group of scientists in the Queen's University of Belfast (Boyd et al [161], Crone et al [139] & [140], and Stevenson et al [162])

Emphasis in their work was centred on the detection of 2-alkylcyclobutanones in meat and liquid egg irradiated at levels ranging from 0 5-10 kGy using GC-MS analysis [140,161] The detection method used was accepted as an official

European method of analysis passed by MAFF during the course of this research [150]

There was a need to expand the range of foodstuff analysed In this research work, a strategy of developing a chemical method for the detection of foods important to the Irish food industry was adopted Chicken meat was investigated as a reference guide as previous research results in this area were available Olives and figs are of importance to the pizza and biscuit industries respectively, and these were selected as novel foodstuffs for investigation into the detection of 2-Dodecylcyclobutanone and 2-Tetradecylcyclobutanone in irradiated samples of these fruits Figs were also chosen due to their relatively low fat content as they could provide information on the applicability of the method to low fat containing food Previous investigations had focused on high fat containing foodstuffs

The initial stage of the research was concerned with the characterisation of authentic standards of the 2-alkylcyclobutanones

Secondly, preliminary investigations into developing the detection method in a new analytical laboratory were carried out For this reason, chicken meat irradiated at 2 5 kGy was selected as an appropriate foodstuff as it was already reported in the literature

The results achieved for chicken, olives, and figs at various irradiation dose levels are reported and discussed in detail

The methodology developed for the detection of 2-alkylcyclobutanones is discussed with respect to the criteria that were considered necessary for an ideal detection method

3.2. EXPERIMENTAL

3.2.1. Irradiation of Food Samples

Irradiation was carried out in a Gamma Beam 650 (Nordion International Incorporated, Kanata, Canada) at QUB using cobalt 60 (Co^{60}) as the source of ionising radiation. Samples were irradiated on rotating turntables surrounding the Co^{60} source. In order to ensure that the dosage levels applied to all irradiated samples were correct, dosimeters were strategically placed on each sample to be irradiated Table 3.2.1 shows the type of dosimeter used in the analysis. To measure the irradiation dose applied to the samples the absorbance of the appropriate dosimeters were measured spectrophotometrically at 530 nm for the gammachrome YR and 603 nm for the amber dosimeters. Their thickness was measured using an electronic micrometer (RS Components Ltd, Japan) and the corresponding dose received was obtained from calibration graphs of the National Physical Laboratory, Teddington. The results are shown in Table 3.2.2.

Table 3.2.1: Dosimeters used to detect levels of irradiation. (UK Atomic Energy Authority, Harwell UK)

Dose Range (KGy)	Dosimeter	Wavelength (nm)
0.1-3.0	Gammachrome YR	530
3.0-10.0	Amber, Type 3042B	603
10.0-60.0	Red, Type 4034 AJ	640

Table 3.2.2: Dosimetry results of all irradiated samples

Dose Intended (kGy)	Dose Received (kGy)
0.5	0.44
2.5	2.53
5.0	5.35

3.2.2. **Sample Preparation**

As samples were irradiated at the Queen's University Belfast, they were frozen overnight. The following day, food samples were thawed and were treated as follows:

Chicken

The chicken was deboned with the aid of a scalpel into individual pieces (leg, breast). Each piece was homogenised in a blender (Moulinex Masterchef 650) and stored in glass containers. The containers were flushed with dry nitrogen for five minutes. The samples were then stored at -20°C or if required immediately, at 5°C. A control chicken was treated in the same manner.

Olives

Olives were de-stoned and homogenised in a blender (Moulinex Masterchef, 650 Series) and stored under the same conditions as used for the chicken samples. This was repeated for the control olives.

Figs

Irradiated and Control figs were homogenised in a blender (Moulinex Masterchef, 650 Series) and stored under the same conditions as the chicken and olives

3 2 3 **Extraction of Lipids**

The method of extraction chosen was the Soxhlet extraction

Anhydrous sodium sulphate (20g) and homogenised sample (20g) were weighed into an extraction thimble This was mixed thoroughly and plugged with cotton wool Hexane ($100cm^3$) was poured into a ($250cm^3$) flask and the extractor was placed on top The extraction thimble was placed in the extractor and further hexane was added ($40cm^3$) The sample was refluxed and extracted gently for six hours The flask was removed from the heat and allowed to cool to room temperature The thimble and the hexane in the extractor were disposed of The solvent in the round bottomed flask was transferred to a $100cm^3$ volumetric flask and the volume was adjusted to $100cm^3$ with more solvent Anhydrous sodium sulphate (5-10g) was added and the flask was stoppered and left overnight in the dark

Quality Control

The system was tested using an unirradiated control sample of the same type as the unknown sample and a duplicate unirradiated control spiked with DCB (200μl of 10μg/cm^3) and TCB (100μl of 10μg/cm^3) respectively Spiking was done immediately after sample preparation and prior to further treatment These samples were treated in the same manner as the unknown samples and the percentage recovery was measured

Modifications

(i) The first of the chicken samples (Batch 1) were extracted using pentane (GC grade 99+%)

3 2 4 **Determination of Lipid Content**

A series of test tubes were pre-weighed, two for each sample An aliquot of each sample (5cm^3) was added The solvent was evaporated from the tubes under a gentle stream of dry nitrogen and the tubes were re-weighed The yield was recorded and the volume of each extract required to give 200mg of lipid was calculated

3.2.5. Fractionation of Lipids (Florisil Column Cleanup)

Separation of the 2-alkylcyclobutanones was achieved by column chromatography using florisil (Mesh 60-100, PR Grade)

(i) Preparation of Florisil

The adsorbent was activated before use by heating at 550°C for at least 5 hours or overnight. It was cooled in a dessicator and sealed. Deactivated Florisil was prepared by adding 20 parts of water to 100 parts of adsorbent (w/w). It was ensured that the deactivated Florisil contained no lumps and that the powder flowed freely. It was then left to equilibrate overnight and always used within one week.

Procedure

The chromatography columns used for the procedure were of quickfit type with CR20/30 connections fitted with a teflon stopcock and a glass frit. Each column was rinsed out with hexane. They were then filled with hexane to approximately 20cm in height. Deactivated florisil was added gently with constant stirring using a glass rod to remove air bubbles. The florisil was added to about 10cm and the solution was mixed thoroughly to remove any air bubbles. The remainder of the florisil was added in the same manner. When all the florisil was added the sides of the column were tapped lightly with a wooden stick so as to pack the florisil in a compact manner. The level of the hexane was reduced to 1cm above the florisil. The volume of extract containing 200mg of lipid (which was concentrated to $5cm^3$ if necessary) was applied carefully to the column and the vessel was rinsed out with hexane ($5cm^3$). Hexane ($145cm^3$)

was added to a 250cm^3-dropping funnel fitted to the top of the column The hexane was eluted at 3 5cm^3/min and collected in a 250cm^3 conical flask When the dropping funnel was empty (ensuring that the level of hexane did not fall below the florisil), diethyl ether in hexane (1%v/v, 150cm^3) was added and eluted accordingly at the rate of 3 5cm^3/min into a 250cm^3 round-bottomed flask This was stoppered and left overnight

3 2 6 Preparation of Cyclobutanones for GC-MS Analysis

The 1% diethyl ether fraction was evaporated on a rotary film evaporator at 40° C to 5-10cm^3 and transferred to a test tube This was concentrated to dryness under a stream of dry nitrogen ensuring that the sample was not left under nitrogen flow once it was dry The sample was resuspended in 200µl of hexane containing 2-cyclohexylcyclohexanone (0 5ppm)

3 2 7 GC-MS Detection of 2-DCB and 2-TCB

The 2-alkylcyclobutanones were separated using a Hewlett Packard 6890 system The capillary column used was a HP5MS and the cyclic ketones were detected using a mass spectrometer (HP5972A MSD) operating in the selective ion monitoring mode for ions of mass/charge (m/z) 98 and m/z 112

The GC-MS parameters are described in Appendix E

3 2 8 **Preparation of Standards**

The linear range was checked regularly throughout the analysis of the irradiated samples at suitable concentrations (0 2-2 0ppm) Table 3 2 3 displays the standards used in the analysis of alkylcyclobutanones

Table 3 2 3 Preparation of 2-dodecylcyclobutane and 2-tetradecylcyclobutanone standards for calibration of the GC-MS Analysis

Std Conc (ppm)	Vol DCB ($10\mu g/cm^3$) (μl)	Vol TCB ($10\mu g/cm^3$) (μl)	Vol Int Std ($5\mu g/cm^3$) (μl)	Vol Hexane (μl)
0 2	20	20	100	860
0 5	50	50	100	800
1 0	100	100	100	700
2 0	200	200	100	500

Appendix G shows how the response factors for calibration are calculated and also how the final concentration of 2-DCB and 2-TCB in $\mu g/g$ are determined

3 3 **Characterisation Of Alkylcyclobutanones**

Characterisation of the target radiolytes was considered an important exercise to ensure the validity of the standards used and also to confirm that they complied with the intended method of analysis i e GC-MS Therefore, a series of physical and instrumental tests were performed on 2-dodecylcyclobutanone, 2-tetradecylcyclobutanone (QUB) and 2-cyclohexylcyclohexanone (internal standard, Fluka Chemicals) to assess their purity

3 3 1 2-Dodecylcyclobutanone

(i) Physical Test

DCB at room temperature was a colourless viscous oil with a boiling point of 83-95°C at 0 075 mm Hg (lit value 88-96 °C at0 075 mm Hg), [159]

It was noted that on storage at 5°C or less it yielded a white crystalline solid with an observed melting point of 25-27°C (lit value 25-27°C), [159]

(ii) Elemental Analysis

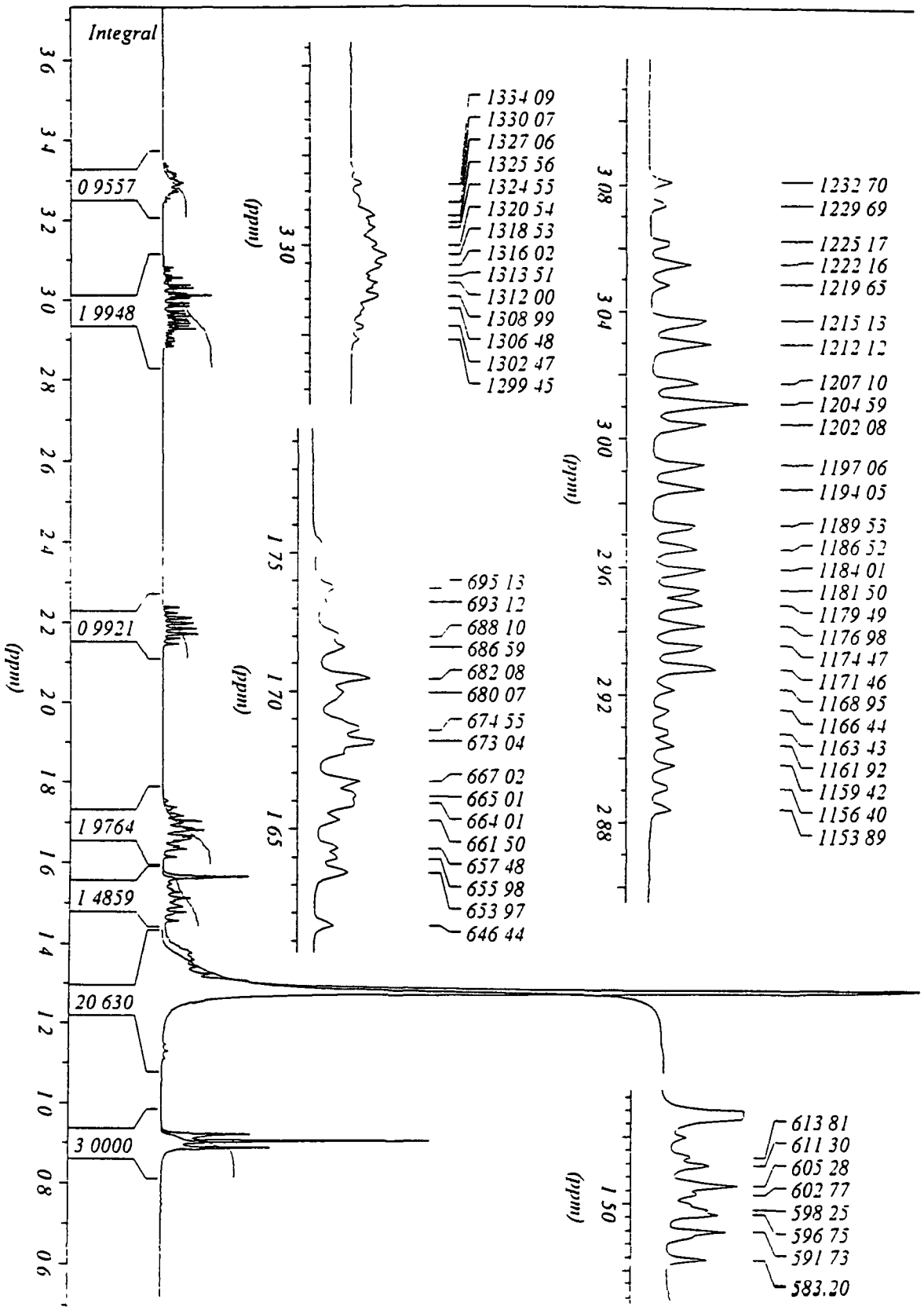
Elemental Analysis correlated very well with reference values

Found C 80 2%, H 12 5%, $C_{16}H_{30}O$ requires C 80 7%, H 12 6%

(iii) ^{1}H nmr Spectrum (400 MHz, $CDCl_3$)

0 88 (3H, triplet, Me), 1 22-1 37 (20 H, multiplet,CH_2 x 10), 1 49 (1 H, m, 1'-H), 1 66 (2H, m, 1'-H, 3-H), 2 17 (1H, m, 4-H), 3 0 (1H, m, 4-H), 3 28 (1H, m, 2-H)

Fig 3 3 1 ^{1}H nmr of 2-Dodecylcyclobutanone



(iv) EI Mass Spectrum

The electronic impact mass spectrum (Fig 3 3 2) obtained on the GC-MS 6890 system under the conditions specified in Section 3 2 7 shows a weak molecular ion peak at m/z 238 The most abundant peak is at m/z 98 and a major peak is observed at m/z 112 The method for monitoring the irradiated samples during the GC-MS analyses was by selective ion monitoring of these two ions The 98 ion was chosen as it was the base peak and the internal standard 2-cyclohexylcyclohexanone produced a similar peak result The ion at m/z 112 was chosen because it was a more selective ion for the cyclobutanones than m/z 98, which would be present in many other compounds Confidence in the results obtained for the irradiated samples and for the standards was achieved due to a high degree of selectivity in the method of detection and quantitation This spectrum compares favourably with the reference spectrum [163] of 2-DCB as shown in the appendix F

It had previously been noted that ketones of the cyclic form had a tendency to lose C_2H_4 groups from the hydrocarbon chain upon fragmentation in the mass spectrometer [163] It is suggested that on reaching m/z 98, a fragment is produced, hence forming the base peak

Fig 3 3 3 Proposed fragment m/z 98

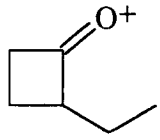
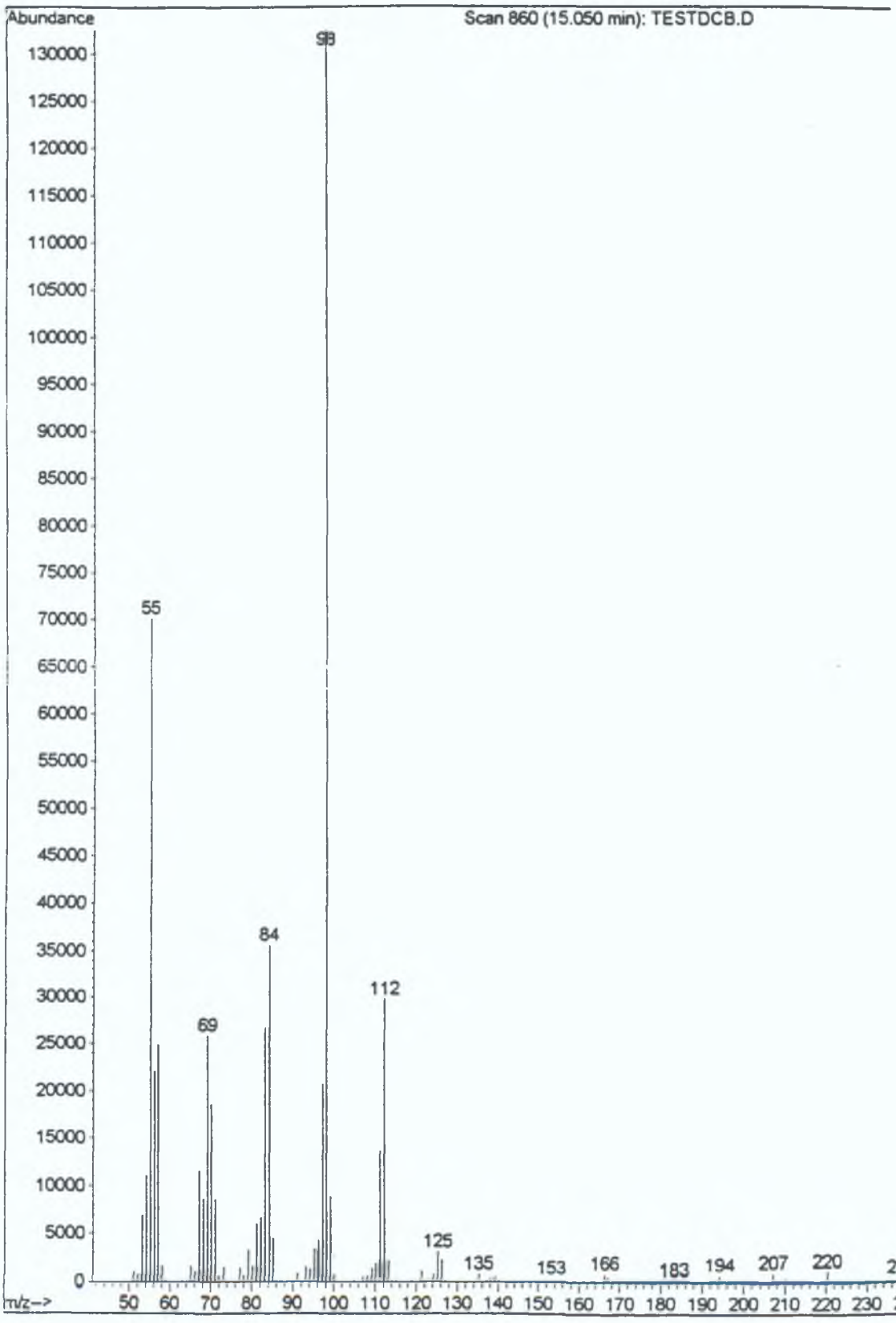


Fig 3.3.2 EI-Mass Spectrum of 2-Dodecylcyclobutanone



3 3 2 2-Tetradecylcyclobutanone

(i) Physical Test

TCB at room temperature was a white crystalline solid with a melting point of 34 5-36 0°C

(ii) Elemental Analysis

Found C81 13%, H12 88% $C_{18}H_{34}$ requires C81 2%, H12 8%

(iii) ^1H nmr Spectrum (400 MHz $CDCl_3$)

0 88 (3H, t, Me), 1 23 (24H, m, $(CH_2)_{12}$, 1 45 (1H, m, 1'-H), 159-1 70 (2H, m, 3-H, 1'-H), 2 18 (1H,m, 3-H), 2 97 (2H, m, 4-H), 3 28 (1H, m, 2-H)

(iv) EI-Mass Spectrum

As a result of the baseline noise in the spectrum, it was difficult to assign a molecular ion peak The unique fragmentation pattern associated with the 2-alkylcyclobutanones is observed with peaks at m/z 98 (base peak) and m/z 112 These peaks are both employed in the selective ion monitoring mode for the detection of DCB & TCB in irradiated food

Fig 3 3 4 ^{1}H nmr Spectrum of 2-Tetradecylcyclobutanone

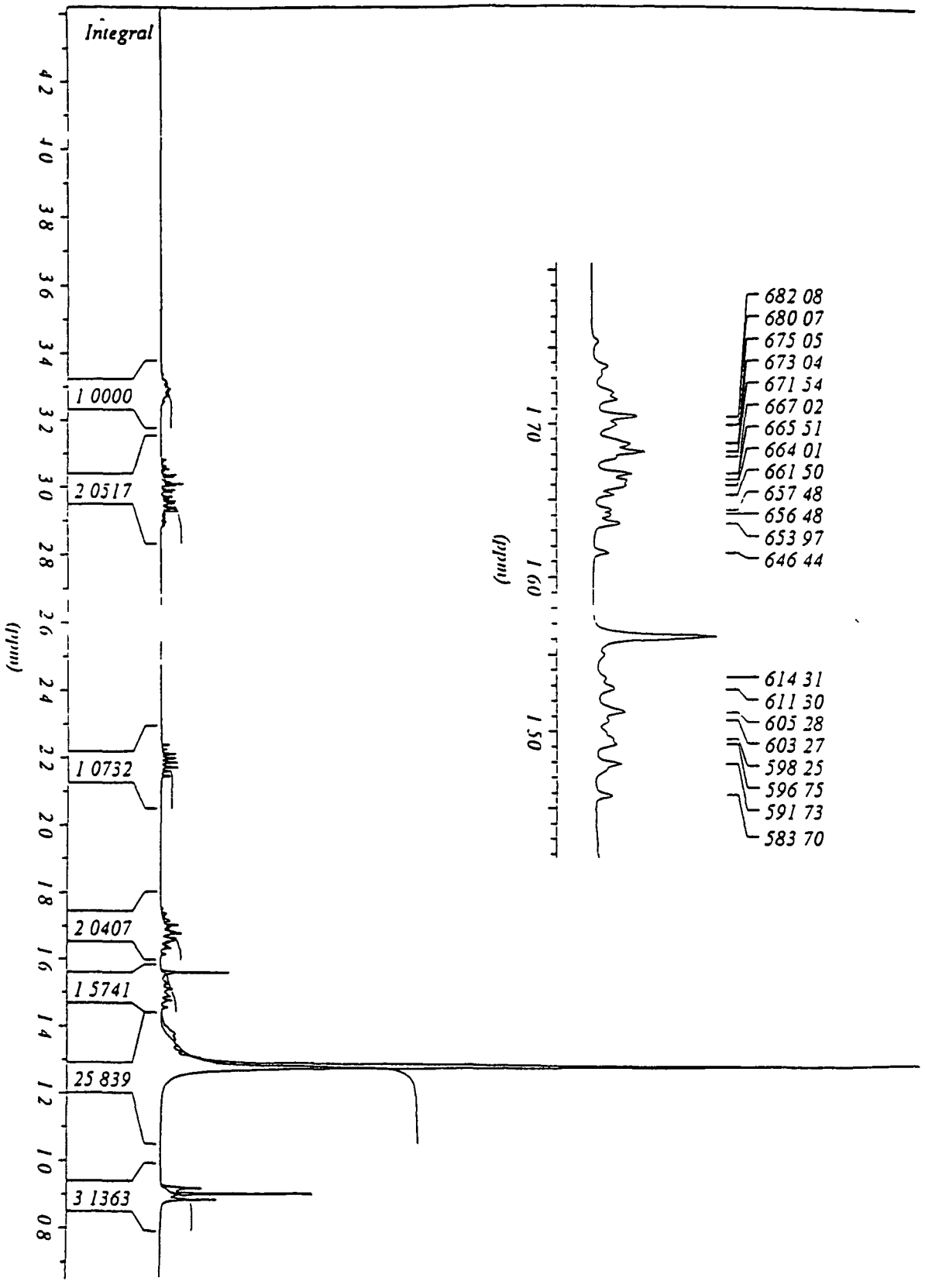
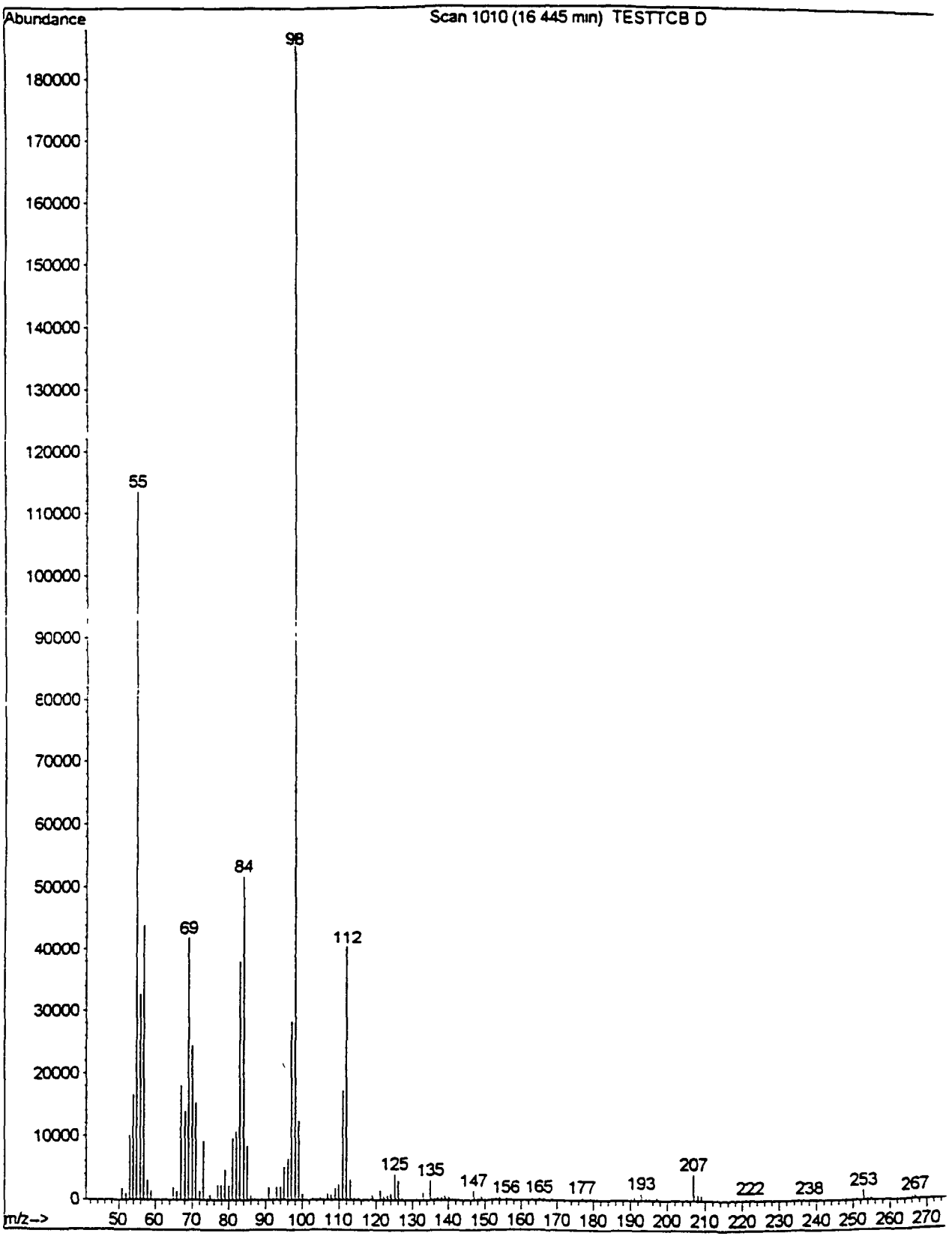


Fig 3 3 5 EI-Mass Spectrum of 2-Tetradecylcyclobutanone



3 3 3 2-Cyclohexylcyclohexanone

For effective and accurate calibration of the method employed, an internal standard was used 2-cyclohexylcyclohexanone was chosen because of its structural similarities with DCB and TCB, its retention time, and its stability during the GC-MS analysis stage

(i) Physical Test

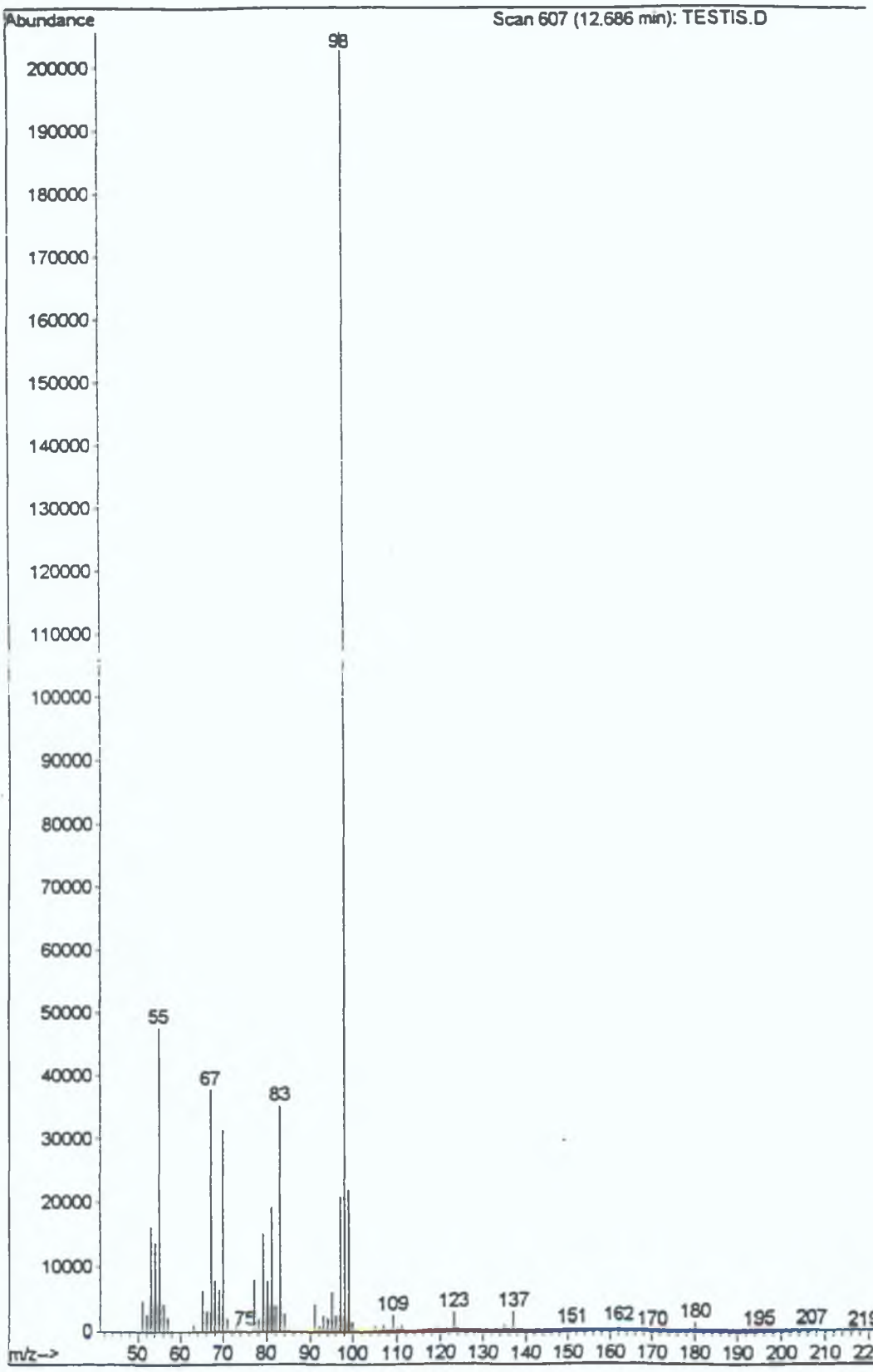
2-cyclohexylcyclohexanone was observed at room temperature as a colourless viscous liquid with a strong sweet odour Boiling point 129-131°C at 12mm Hg The reference value is 130-132°C at 12mm Hg

(iii) EI-Mass Spectrum

The electron impact mass spectrum was recorded Again, the base peak was observed at m/z 98 with no peak observable at m/z 112 No reference spectrum was available but as Fig 3 Appendix G reveals, a library search gave a very similar spectrum with a quality fit of 91%

This mass spectrum indicates the inability for quantitation of DCB or TCB using the ion m/z 112 because of its non-appearance in the internal standard mass spectrum The reason m/z 112 is used in the analysis protocol is because it forms a definite ratio with m/z 98 for all irradiated samples, and so helps to confirm the presence of the 2-alkylcyclobutanones after irradiation

Fig 3.3.6: EI-Mass Spectrum of 2-cyclohexylcyclohexanone



3.4. Detection of 2-Alkylcyclobutanones

The principal aim of this section of the research was to determine both qualitatively and quantitatively the presence of DCB and TCB in irradiated food.

This section is divided into two parts

3.4.1. Preliminary studies of irradiated chicken meat to gain familiarity with the methodology and help incorporate it into a new analytical laboratory. Chicken meat irradiated at 2.5 kGy was chosen for this initial investigation as results for such irradiation were quoted in the literature [139,140].

3.4.2. Analysis of chicken, olives, and figs irradiated at various dosage levels for DCB and TCB with particular emphasis on linearity of concentration with dose and effects of storage on levels of DCB and TCB.

Various time periods between irradiation and analysis of samples for DCB and TCB were chosen to reflect various time delays that may arise between irradiation of the foodstuff, its passage to the market shelf and subsequent testing for the presence of radiolytes.

Table 3.4.1: List of relevant dates between irradiation and analysis

Date	Description
19 December 1996	Irradiation of 2.5kGy chickens (Preliminary studies)
26 January 1997	Analysis of 2.5kGy chickens (Preliminary analysis)
10 April 1997	Irradiation of foodstuffs
13 June 1997	Analysis of foodstuffs

It is also important to note that samples were analysed in batches rather than separately This was made possible by the convenience of the extraction method chosen, as it was deemed one of the most suitable for the extraction of the lipids from the food matrix i e the Soxhlet extraction (Section 2 3)

3 4 1 **Preliminary Investigation into Irradiated Chicken**

All samples analysed for DCB and TCB in this research were subjected to the same protocol (as described in section 3 2) The fat from each sample was extracted and DCB and TCB were separated from the lipid by adsorption chromatography GC-MS analysis was then used to confirm if DCB or TCB were present in each sample

In this batch, there were four irradiated samples, one control sample and one spiked sample The results obtained for each are given below

Note: This batch was re-analysed six months later

3 4 1 1 Control Sample

Table 3 4 1 1 Analysis of control chicken sample for DCB and TCB

Batch No	Sample Code	Sample Description	DCB		TCB	
			m/z 98	m/z 112	m/z 98	m/z 112
1	C5	Control Leg	ND	ND	ND	ND

D= Detected

ND= Not Detected

The lipids from all control samples were extracted simultaneously with irradiated samples and they were prepared for GC-MS analysis in exactly the

same manner as their irradiated counterparts The purpose of control samples is to confirm that the target radiolytes (namely DCB and TCB) are absent in non-irradiated chicken meat This is confirmed by observing the mass spectrum for the sample (figs 4 & 5, Appendix F) and noting the absence of peaks at the corresponding retention times of DCB and TCB (10 88 minutes and 12 31 minutes respectively)

3 4 1 2 Recovery Sample

Prior to extraction of the lipids, a non-irradiated chicken sample was spiked with 200 μl of DCB (10 μg/cm^3) and 100 μl of TCB (10 μg/cm^3) The two cyclobutanones were recovered and the yields are reported in Table 3 4 1 2

Table 3 4 1 2 Recovery sample for spiked control chicken for preliminary investigation

Batch No	Sample Code	Sample Description	Recovery (%)	
			DCB	TCB
1	C6	Spiked Leg	27 06	29 95

The method of calculating the percentage recovery is given in the next section The recovery rate for this particular batch is low As higher rates were recorded later in the research, the low yield may be accredited to inexperience on the part of the experimentalist and this is reflected in the improvement of recoveries with time as shown in the following section Other factors contributing to this low yield will also be discussed later

3 4 1 3 Irradiated Chicken Samples (Qualitative Detection)

The qualitative detection of the 2 5 kGy irradiated chicken samples is based on the ability to detect the presence of either DCB or TCB at the appropriate retention time in the relevant GC mass spectrum A positive identification of either compound is sufficient to confirm irradiation of the food sample Table 3 4 1 3 gives the results of this analysis

Table 3 4 1 3 Qualitative detection of 2 5 kGy irradiated chicken

Batch No	Dose kGy	Sample Code	Sample Description	Identification	
				DCB	TCB
1	2 5	C1	Irradiated Leg	D	D
		C2	Irradiated Leg	D	D
		C3	Irradiated Breast	D	D
		C4	Irradiated Breast	D	D

As observed, all chicken samples irradiated at 2 5kGy show the presence of 2-dodecylcyclobutanone and 2-tetradecylcyclobutanone

3 4 1 4 Irradiated Chicken Samples (Quantitative Detection)

Table 3 4 1 4 Concentration of DCB and TCB in 2 5 kGy Irradiated Chicken

Batch No	Dose kGy	Sample Code	Sample Description	Concentration (µg/g fat)	
				DCB	TCB
1	2 5	C1	Irradiated Leg	0 73	*
		C2	Irradiated Leg	0 76	*
		C3	Irradiated Breast	0 61	*
		C4	Irradiated Breast	0 65	*

* = Results not quantifiable due to error in detection of internal standard peak

The quantity of DCB was calculated as outlined in the experimental section

The values obtained compare well with those observed in the literature According to Stevenson et al [161], the concentration of DCB in 2 5 kGy irradiated chicken was calculated to be 0 75 µg/g lipid at day 0 and 0 83 µg/g lipid at day 21 following irradiation These results are discussed in section 3 5 Note that an increase in the concentration of DCB was observed after storage at 21 days but according to the reference this was within experimental error

As a result of the preliminary investigation, the following conclusions were drawn and a number of decisions were taken with a view to improving the consistency of detecting DCB and TCB in a variety of foodstuffs

(i) Organisation of each part of the protocol is extremely important as it is very much a continuous process and this aids with the accuracy and the precision of analysis

(ii) The washing and thorough drying of all glassware was most important in order to ensure that there was no contamination

(iii) As shown by the characterisation of the standards, it was very important to store them at -20°C to maintain their stability and integrity

3 4 2 Detection of 2-DCB and 2-TCB in Chicken, Olives and Figs Irradiated at Various Dose Levels.

On completion of the initial investigations into the identification of 2 5 kGy irradiated chickens, a range of experiments were carried out to establish whether 2-DCB and 2-TCB are suitable markers for the identification of irradiation in a variety of foodstuffs Tables 3 4 2 1-3 4 2 3 give details of these experiments Each batch contained a control sample and a spiked sample for quality control purposes

Table 3 4 2 1 List of chicken samples irradiated at various dosage levels

Batch No	Dose kGy	Sample Code	Sample Description
2	0 5	C8	Irradiated Leg
	0 5	C9	Irradiated Leg
	0 5	C10	Irradiated Breast
	0 5	C11	Irradiated Breast
	0	C12	Control Breast
	0	C13	Control Leg
	0	C14	Spiked Leg
	0	C15	Spiked Breast
	0 5	C16	Irradiated Leg
3	2 5	C17	Irradiated Leg
	2 5	C18	Irradiated Breast
	0	C19	Control Leg
	0	C20	Control Breast
	0	C21	Spiked Leg
	0	C22	Spiked Breast
4	5 0	C23	Irradiated Leg
	5 0	C24	Irradiated Breast
	0	C25	Control Leg
	0	C26	Spiked Breast
5	5 0	C27	Irradiated Leg
	5 0	C28	Irradiated Breast
	0	C29	Control Breast
	0	C30	Spiked Leg
6	0	C31	Control Leg
	0	C32	Control Breast
	0	C33	Spiked Leg
	0	C34	Spiked Breast
	2 5	C35	Irradiated Leg
	2 5	C36	Irradiated Breast

Table3.4.2.2: List of Olive Samples irradiated at various dosage levels

Batch No.	Dose (kGy)	Sample Code	Sample Description
7	0.5	O1	Irradiated Olives
	0.5	O2	Irradiated Olives
	0	O3	Control Olives
	0.5	O4	Irradiated Olives
	0	O5	Spiked Olives
	0.5	O6	Irradiated Olives
	0.5	O7	Irradiated Olives
8	0	O8	Control Olives
	0	O9	Spiked Olives
	2.5	O10	Irradiated Olives
	2.5	O11	Irradiated Olives
	2.5	O12	Irradiated Olives
	0	O13	Spiked Olives
	0	O14	Control Olives
9	0	O15	Spiked Olives
	0	O16	Spiked Olives
	5.0	O17	Irradiated Olives
	0	O18	Control Olives
	5.0	O19	Irradiated Olives
	0	O20	Control Olives
10	5.0	O21	Irradiated Olives
	5.0	O22	Irradiated Olives
	5.0	O23	Irradiated Olives
	0	O24	Control Olives
	0	O25	Spiked Olives
	0	O26	Spiked Olives

Table 3 4 2 3 List of Fig Samples Irradiated at 2 5 kGy

Batch No	Dose kGy	Sample Code	Sample Description
11	2 5	F1	Irradiated Figs
	0	F2	Control Figs
	0	F3	Spiked Figs
	2 5	F4	Irradiated Figs

3 4 2 1 **Control Samples**

The validity of any method of analysis is confirmed by the simultaneous analysis of control and recovery samples Each control sample used was a non-irradiated one and was treated and analysed in an identical manner to that of the irradiated counterparts

The function of control samples was to confirm that the target radiolytes were not present in the non-irradiated food samples Results of the analysis are given in the following tables

Table 3 4 2 4 Control Chicken Samples

Batch No	Sample Code	DCB		TCB	
		m/z 98	m/z 112	m/z 98	m/z 112
2	C12	ND	ND	ND	ND
	C13	ND	ND	ND	ND
3	C19	ND	ND	ND	ND
	C20	ND	ND	ND	ND
4	C25	ND	ND	ND	ND
5	C29	ND	ND	ND	ND
6	C31	ND	ND	ND	ND
	C32	ND	ND	ND	ND

Table 3.4.2.5 Control Olive Samples

Batch No.	Sample Code	DCB		TCB		Ratio 98/112*	
		m/z 98	m/z 112	m/z 98	m/z 112	DCB	TCB
7	O3	ND	ND	ND	ND	-	-
8	O8	D	D	ND	ND	4.28	-
	O14	ND	ND	ND	ND	-	-
9	O18	ND	ND	ND	ND	-	-
	O20	D	D	D	D	4.47	-
10	O24	D	D	D	D	4.40	-

*Ratio 98/112 = ratio of peak areas of m/z 98 relative to m/z 112.

Table 3.4.2.6 Control Fig Samples

Batch No	Sample Code	DCB		TCB	
		m/z 98	m/z 112	m/z 98	m/z 112
11	F2	ND	ND	ND	ND

Chicken Control

The retention times of DCB and TCB under the chromatographic conditions used were 10.88 and 12.30 minutes respectively. No peaks were observed at these times in any of the control chromatograms, indicating that DCB or TCB are not present in non-irradiated chicken.

Olive Control

A number of peaks with retention times similar to that of DCB and TCB were recorded in some of the control olive chromatograms

Sample O8 showed a peak with a retention time of 10 88 minutes and a m/z 98/112 ratio of 4 28 This result suggests that the sample contains DCB, but the concentration level is much lower than the corresponding irradiated sample

Samples O20 and O24 also showed peaks at the appropriate retention times of DCB and TCB However, m/z 98/112 ratio values could only be calculated for DCB Cross-contamination or more likely co-elution might explain the results observed for these control olive samples The latter reason may be valid because olives are a new foodstuff to be analysed in this manner and a co-eluent is a distinct possibility To overcome this problem, a modification in the chromatographic conditions is needed and this requires further investigation

These irregular control sample results have been quantified and they show low concentrations when compared to irradiated samples

Fig Control

The fig control sample investigated indicates that no detectable level of DCB or TCB is present in non-irradiated figs

3 4 2 2 **Recoveries**

Another feature used in the quality control of the method under investigation, is the percentage recovery of the compounds of interest With respect to the method of detection employed in this research for the detection of 2-DCB and 2-TCB in irradiated chicken, olives, and figs, the rate of recovery was

measured by spiking non-irradiated samples with a known concentration of DCB and TCB The concentration of both compounds was then calculated from the sample mass spectrum and a percentage yield value of each was recorded knowing the concentration of both at the initial stage of the experiment This provides information on the efficiency of the method used A recovery or spiked sample was run along with the samples of interest and the results obtained are provided in the following tables

Table 3 4 2 7 Recovery of DCB and TCB from Spiked Chicken

Batch No	Sample Code	Sample Description	% Recovery		Ratio 98/112	
			DCB	TCB	DCB	TCB
1	C6	Spiked Leg	27 06	29 95	4 17	3 77
2	C15	Spiked Breast	20 58	17 39	4 23	3 90
3	C21	Spiked Leg	46 12	38 75	4 24	3 89
4	C26	Spiked Breast	78 0	81 0	4 11	3 96
5	C30	Spiked Leg	73 12	80 0	4 22	3 92
6	C33	Spiked Leg	88 0	96 0	4 17	3 93

Table 3 4 2 8 Recovery of DCB and TCB from Spiked Olives

Batch No	Sample Code	Sample Description	% Recovery		Ratio m/z 98/112	
			DCB	TCB	DCB	TCB
7	O5	Spiked Olives	24 74	20 33	4 27	3 96
8	O9	Spiked Olives	67 0	69 0	4 11	3 86
	O13	Spiked Olives	77 0	77 8	4 22	3 52
9	O15	Spiked Olives	53 4	56 7	4 21	4 03
	O16	Spiked Olives	40 38	42 3	4 21	3 67
10	O25	Spiked Olives	57 8	64 74	4 24	4 17
	O26	Spiked Olives	70 8	72 14	4 25	4 15

Table 3 4 2 9 Recovery of DCB and TCB from Spiked Figs

Batch No	Sample Code	Sample Description	% Recovery		Ratio 98/112	
			DCB	TCB	DCB	TCB
11	F3	Spiked Figs	43 9	35 8	4 23	3 98

Sample Calculation

(a) Batch Number 4 *C26 Spiked Breast*

Refer to Sample Spectrum (Figs 6 & 7 Appendix F)

DCB

7134865[a]/2697916[b] -1 13 = 2 340 μg/cm^3 (1 13 = response factor from calibration Appendix E)

= 0 468 μg/200μl (Final concentration)

Spike 200μl of DCB (10μg/cm^3) in 100cm^3 = 0 02μg/cm^3

No of cm^3 of lipid extract required to give 200mgs of lipid = 30cm^3

Concentration of DCB in lipid extract = 30 × 0 02 = 0 6μg present at start

% Recovery

100 × 0 468 – 0 6 = **78 %**

[a] Peak Area due to DCB

[b] Peak Area due to internal standard 2-cyclohexylcyclohexanone

TCB

3472312/2697916 – 1 06 = 1 214 μg/cm^3 (1 06 = response factor from the calibration)

= 0 243/ 200μl

Spike 100μl of TCB (10μg/cm^3) in 100cm^3 = 0 01μg/cm^3

No of cm^3 of lipid extract required to give 200mgs of lipid = 30cm^3

Conc of TCB in lipid extract = 30 × 0 01 = 0 3 μg present at start

% Recovery

100 × 0 243 – 0 30 = **81 %**

(b) Batch No 10 *O26 Spiked Olives*

Refer to Sample Spectrum (Fig 8 & 9 Appendix F)

DCB

11663446/3211518 – 1 22 = 2 977 μg/cm^3 (1 22 = response factor)

= 0 595μg/ 200μl

Spike 200μl of DCB (10μg/cm3) in 100cm^3 = 0 02μg/cm^3

No of cm^3 of lipid extract required to give 200mgs of lipid = 42 1

Conc of DCB in lipid extract = 42 1 × 0 02 = 0 84μg present at start

% Recovery

100 × 0 595 – 0 84 = **70.8 %**

TCB

5783604/3211518 – 1 19 = 1 513 μg/cm^3 (1 19 = response factor)

= 0 303μg/ 200μl

Spike 100μl of TCB (10μg/ cm^3) in 100cm^3 = 0 01μg/cm^3

No of cm^3 of lipid extract required to give 200mgs of lipid = 42 1cm^3

Conc of TCB in lipid extract = 42 1 × 0 01 = 0 421 μg present at start

% Recovery

$$100 \times 0\,303 - 0\,42 = \underline{\mathbf{72.14\ \%}}$$

A wide range of recovery values was obtained and a number of factors are responsible for this Firstly, it is reflected in the improvement of results, that with time and practice, better yields are attainable As seen in table 3 4 2 7, batches 1, 2, and 3 produced recoveries all below 50%, whereas later batches 4, 5, and 6 produced recoveries over 70% Thus, practice has led to improved performance, as reflected in the results This emphasises the difficulties that arise when employing a technique for the first time

Secondly, the amount of florisil used in the separation procedure proved to be a very important factor In the first three batches, 46g of deactivated florisil were used in the chromatographic separation procedure This was altered to 30g in subsequent batches and considerable improvements were observed in the percentage recovery of 2-DCB and 2-TCB

An incorrect quantity of florisil, along with inexperience in the use of the method may have led to the poor recoveries observed in the early batches

The percentage recovery of batch 1 (the preliminary investigation), as mentioned earlier is low This result might imply that the recovery of DCB and TCB from the corresponding irradiated samples from batch 1 would be of a similarly low value, thus not reflecting the true concentration of DCB and TCB in the irradiated sample This same point was made by a group of researchers in an interlaboratory trial on the detection of DCB and TCB in irradiated chicken

meat [164] The authors of the report state "In some cases, the recovery of 2-DCB and 2-TCB from spiked control samples was low but nevertheless this did not appear to have a marked effect on the concentrations of the marker compounds measured in the irradiated samples"

A similar effect was observed in the present research Pecentage recovery for Batch 3 spiked control samples was 46% and 38% for DCB and TCB respectively Percentage recovery of spiked control samples for Batch 6 was 88% and 96% respectively However, the irradiated samples showed similar concentration levels of DCB and TCB, despite the difference in recovery

With respect to the olives, which were being analysed for the first time, there was a lack of consistency observed in the recovery between batches, reflecting possibly the difficulty of the technique, and its extension to a novel foodstuff

3 4 2 3 Qualitative Detection

The qualitative detection of 2-DCB and 2-TCB provides confirmation of whether or not a particular sample has been irradiated Irradiation of a sample is confirmed by the occurrence of a peak at the appropriate retention time of either of the two marker compounds and in the correct ratio of the ions m/z 98 to m/z 112

The following tables provide the results obtained for all the samples analysed and are discussed thereafter

Table 3 4 2 10 Qualitative Detection of Irradiated Chicken

Batch No	Dose (kGy)	Sample Code	Sample Description	Qualitative Identification		Ratio 98/112	
				DCB	TCB	DCB	TCB
1	2 5	C1	Irradiated Leg	D	D	4 23	3 95
		C2	Irradiated Leg	D	D	4 18	3 94
		C3	Irrad Breast	D	D	4 19	3 74
		C4	Irrad Breast	D	D	4 17	3 97
2	0 5	C8	Irradiated Leg	ND	ND	-	-
		C9	Irradiated Leg	D	D	3 40	1 71
		C10	Irrad Breast	D	ND	4 26	-
		C11	Irrad Breast	ND	ND	-	-
		C16	Irradiated Leg	D	ND	4 40	-
3	2 5	C17	Irradiated Leg	D	D	4 27	3 96
		C18	Irrad Breast	D	D	4 43	3 40
4	5 0	C23	Irradiated Leg	D	D	4 17	4 03
		C24	Irrad Breast	D	D	3 59	4 15
5	5 0	C27	Irradiated Leg	D	D	4 02	4 04
		C28	Irrad Breast	D	D	4 15	4 01
6	2 5	C35	Irradiated Leg	D	D	4 14	3 91
		C36	Irrad Breast	D	D	4 16	3 91

Table 3 4 2 11 Qualitative Detection of Irradiated Olives

Batch No	Dose (kGy)	Sample Code	Sample Description	Qualitative Identification		Ratio 98/112	
				DCB	TCB	DCB	TCB
7	0 5	O1	Irrad Olives	D	ND	4 17	-
		O2	Irrad Olives	D	ND	4 20	-
		O4	Irrad Olives	D	D	4 96	3 76
		O6	Irrad Olives	D	D	4 21	4 02
		O7	Irrad Olives	D	D	4 15	4 11
8	2 5	O10	Irrad Olives	D	D	4 22	3 51
		O11	Irrad Olives	D	ND	4 24	-
		O12	Irrad Olives	D	ND	4 19	-
9	5 0	O17	Irrad Olives	D	D	4 18	4 68
		O19	Irrad Olives	D	D	4 20	4 14
10	5 0	O21	Irrad Olives	D	D	4 22	4 02
		O22	Irrad Olives	D	D	4 24	4 17
		O23	Irrad Olives	D	ND	4 25	-

Table 3 4 2 12 Qualitative Detection of Irradiated Figs

Batch No	Dose (kGy)	Sample Code	Sample Description	Qualitative Detection		Ratio 98/112	
				DCB	TCB	DCB	TCB
11	2 5	F1	Irrad Figs	D	ND	3 89	-
		F4	Irrad Figs	D	D	4 23	4 50

(i) 0 5 kGy Irradiated Chicken

Due to the absence of DCB and TCB in two out of the five samples irradiated at this dose level it is difficult to draw definitive conclusions More samples need to be analysed at this level

(ii) 2 5 kGy Irradiated Chicken

2 5 kGy irradiated chicken samples were easily detected by a positive identification of both 2-DCB and 2-TCB by GC-MS analysis

The ion ratios calculated for each sample were correct except for Sample C18, where the TCB value was lower than expected

(iii) 5 0 kGy Irradiated Chickens

Excellent qualitative identification of 5 0 kGy irradiated chicken meat was achieved, as both retention times and ion ratios correlated well with DCB and TCB authentic standard ratios The only exception was sample C24 where the ratio of ions for DCB was lower than the expected value

(iv) 0 5 kGy Irradiated Olives

Using DCB and TCB as a marker, it proved possible to identify olives, which were irradiated at 0 5 kGy Only two olive samples were not detected for TCB and so the initial results for a novel foodstuff are very encouraging

(v) 2 5 kGy Irradiated Olives

Qualitative detection was again confirmed more strongly through DCB than TCB TCB was recorded for only one of the three samples analysed at this level

(vi) 5 0 kGy Irradiated Olives

Qualitative identification was extremely satisfactory TCB was not present in sample O23

(vii) 2 5 kGy Irradiated Figs

The presence of DCB was a good indicator for the identification of irradiated figs TCB was recorded in 1 sample with a slightly high ratio

The main conclusion to be drawn from these results is that 2-DCB proved to be a consistently more reliable marker compound than 2-TCB for the identification of irradiated food at all dosage levels This may well be due to the fact that palmitic acid is present at higher concentrations in all food samples analysed as shown in chapter 2

3 4 2 4 **Quantitative Detection of 2-DCB AND 2-TCB**

The calculation of the concentration of both 2-DCB and 2-TCB in all qualitatively detected samples was conducted

The relationship between concentration of the radiolytes and the dosage level applied was then investigated

The following tables provide all the results obtained for the quantitative detection of irradiated foodstuffs

Table 3 4 2 13 Quantitative detection of Irradiated Chicken

Batch No	Dose (kGy)	Sample Code	Sample Description	Concentration (µg/g lipid)		Ratio [dcb][a] [tcb][b]
				DCB	TCB	
1[c]	2 5	C1	Irradiated Leg	0 585	0 311	1 88
		C2	Irradiated Leg	0 568	0 470	1 21
		C3	Irradiated Breast	0 586	0 308	1 90
		C4	Irradiated Breast	0 549	0 515	1 07
2	0 5	C8	Irradiated Leg	ND	ND	ND
		C9	Irradiated Leg	0 0163	0 0126	1 29
		C10	Irradiated Breast	0 063	ND	ND
		C11	Irradiated Breast	ND	ND	ND
		C16	Irradiated Leg	0 077	ND	ND
3	2 5	C17	Irradiated Leg	0 665	0 296	2 24
		C18	Irradiated Breast	0 778	0 455	1 71
4	5 0	C23	Irradiated Leg	1 093	0 504	2 17
		C24	Irradiated Breast	1 052	0 500	2 10
5	5 0	C27	Irradiated Leg	1 269	0 576	2 20
		C28	Irradiated Breast	1 120	0 487	2 30
6	2 5	C35	Irradiated Leg	0 794	0 191	4 16
		C36	Irradiated Breast	0 662	0 171	3 87

[a] [dcb] = concentration of 2-DCB

[b] [tcb] = concentration of 2-TCB

[c] Batch 1 was analysed after six months

Table 3 4 2 14 Quantitative detection of Irradiated Olives

Batch No	Dose (kGy)	Sample Code	Sample Description	Concentration (µg/g lipid)		Ratio [dcb] [tcb]
				DCB	TCB	
7	0 5	O1	Irradiated Olives	0 055	ND	ND
		O2	Irradiated Olives	0 072	ND	ND
		O4	Irradiated Olives	0 046	0 042	1 09
		O6	Irradiated Olives	0 065	0 026	2 50
		O7	Irradiated Olives	0 085	0 318	0 27
8	2 5	O10	Irradiated Olives	0 416	0 131	3 175
		O11	Irradiated Olives	0 387	ND	ND
		O12	Irradiated Olives	0 340	ND	ND
9	5 0	O17	Irradiated Olives	0 664	0 262	2 534
		O19	Irradiated Olives	0 649	0 254	2 555
10	5 0	O21	Irradiated Olives	0 680	0 238	2 857
		O22	Irradiated Olives	0 539	0 189	2 852
		O23	Irradiated Olives	0 531	ND	ND

Table 3 4 2 15 Quantitative detection of Irradiated Figs

Batch No	Dose (kGy)	Sample Code	Sample Description	Quantity (µg/g lipid)		Ratio [dcb] [tcb]
				DCB	TCB	
11	2 5	F1	Irradiated Figs	0 032	ND	-
		F4	Irradiated Figs	0 045	0 045	1 000

Sample Calculation

(a) Sample C24 Irradiated Chicken Breast

Refer to sample spectrum Fig 10 & 11 Appendix F

Concentration of DCB

3193442/2684693 -1 13 = 1 052 μg/ cm^3

= 0 2104 μg/ 200μl

This is because the total volume of the final solution was only 200μl

However, according to the procedure, this volume only corresponds to 200 mg of lipid and the value is to be quoted per gram of lipid

0 2104μg/ 200mg × 5 = 1 052 μg/ g lipid

Concentration of TCB

=1463671/2684693 - 1 09 = 0 500 μg/ cm^3

= 0 100 μg/ 200 μl

This volume corresponds to 200mg lipid

Therefore 0 100 μg/ 200mg × 5 = 0 500 μg/ g lipid

(b) Sample O21 Irradiated Olives

Refer to sample spectrum Fig 12 & 13 Appendix F

Concentration of DCB

=2265759/2843860 - 1 19 = 0 680 μg/ cm^3

= 0 136 μg/ 200 μl

This volume is equal to 200mg lipid

0 136 μg/ 200mg × 5 = 0 68 g/ g lipid

Concentration of TCB

777395/2843860 – 1 15 = 0 238 μg/ cm^3

= 0 0476 μg/ 200 μl

This is equal to 200mg lipid

0 0476μg/ 200mg × 5 = 0 238 μg/ g lipid

3.5. **Relationship between concentration of 2-alkylcyclobutanones found in the current research with that observed in the literature.**

3.5.1. Irradiated Chicken

Listed below are values recorded in reference articles for concentration of DCB and TCB in chicken irradiated at various dosage levels.

Table 3.5.1: Concentration of DCB and TCB previously reported

Ref No.	Dose (kGy)	Concentration (μg/g lipid)		Storage (Days)
		DCB	TCB	
[57]	0.5	0.25		0
[139]	0.5	0.12 0.14 0.18 0.25 0.50		0 0 0 0 0
[164]	1.0	0.313 0.225 0.259 0.080 0.070 0.427	0.160 0.055 0.088 0.033 0.015 0.135	0 0 0 0 0 0
[139]	2.5	0.89 0.84	0.233	1 month 8months
[162]	2.5	0.750 0.830		0 21

Ref No	Dose (kGy)	Concentration (μg/g lipid)		Storage (Days)
		DCB	TCB	
[57]	2 5	1 72 1 43 1 75		0 18 0
[57]	2 5	1 64 1 27		0(precooked) 0(postcooked)
[164]	3 0	0 760 0 350 0 557 0 686	0 420 0 085 0 224 0 216	0 0 0 0
[139]	5 0	2 02 2 29	0 732	1 month 8 months
[141]	5 0	0 48 0 57 0 740 0 64		1 month
[57]	5 0	3 92 3 17		0 18
[57]	5 0	3 09 2 76		0(precooked) 0(postcooked)
[161]	4 7	0 244 0 180		0 20

Little consistency is found between the research groups in respect of the concentration of radiation induced cyclic ketones produced by the same irradiation dose There is some degree of consistency however, observed among samples within each batch and this is also reflected in the results obtained for the current research

(i) 0.5 KGy Chicken

It would appear that the quantitative validity of the 0.5 kGy irradiated chicken in this research would be extremely difficult to confirm because of the inconsistency of the results obtained. The closest concentration of DCB with those of references was sample C10 and C16, but even these were below the reference values. (The reason for these low results may be due to inexperience on the part of the experimentalist and the use of a greater quantity of florisil than required).

(ii) 2.5 kGy Chicken

The quantitation of 2-DCB and TCB in the 2.5 kGy irradiated chickens was achieved and a good degree of consistency was observed within each batch. The results obtained correlate well with some of the reference values, though these also vary somewhat.

(iii) 5.0 kGy Chicken

A good degree of consistency was achieved between samples in each batch analysed. The results obtained compare favourably with some of the quoted reference values especially those of the BCR interlaboratory trial [141].

It is important to note that DCB proved to be a more effective marker compound on a quantitative basis than TCB throughout the irradiation of the chicken sample.

3.5.2. Irradiated Olives

No reference values for irradiated olives were available.

(i) 0 5 kGy Olive

Table 3 4 2 14 shows some degree of reproducibility of samples irradiated at 0 5 kGy, for DCB and these initial results are encouraging for the development of this detection method to include olives TCB did not display this same reproducibility

(ii) 2 5 kGy Olives

Relationship between olive samples irradiated at 2 5 kGy shows a good level of detection with samples ranging from 0 340- 0 416 μg/ g lipid for DCB TCB range was wider and on two occasions was not detected

(iii) 5 0 kGy Olives

Results for DCB show good reproducibility and an improvement in TCB results were also observed, although in one sample neither was detected

The overall conclusion drawn from the results of the olive series is that DCB is a good marker for the identification of an irradiated sample TCB however, has shown some inconsistency and on the initial investigation, it would appear that it is not a suitable marker for the detection of an irradiated sample The parent fatty acid of TCB, stearic acid, was detected in the lipid profile of the olives, but possibly due to the lipid matrix, difficulties may arise in the detection of the corresponding cyclobutanone by GC methods

3 5 3 Irradiated Figs

Figs are also a novel foodstuff for the detection of DCB and TCB in irradiated samples, and so reference values are unavailable The quantitated results show that a lower concentration for DCB was obtained than 2 5 kGy irradiated chicken and olives The TCB concentration was almost equal to that of the DCB and because no fatty acid profiles were available due to the low fat content of the fruit the validity of this result must be questionable

3 6 **Linearity of Dose vs Concentration of DCB and TCB**

Another of the main criteria in considering the quantitative ability of the results is linearity of concentration with dosage level applied

The relationship between dosage level applied and concentration of relevant cyclobutanone may be best illustrated by graphical representation and Figs 3 6 1-3 6 4 provide the requisite data

Fig 3 6 1 Irradiation Dose vs Average Concentration of DCB in Chicken

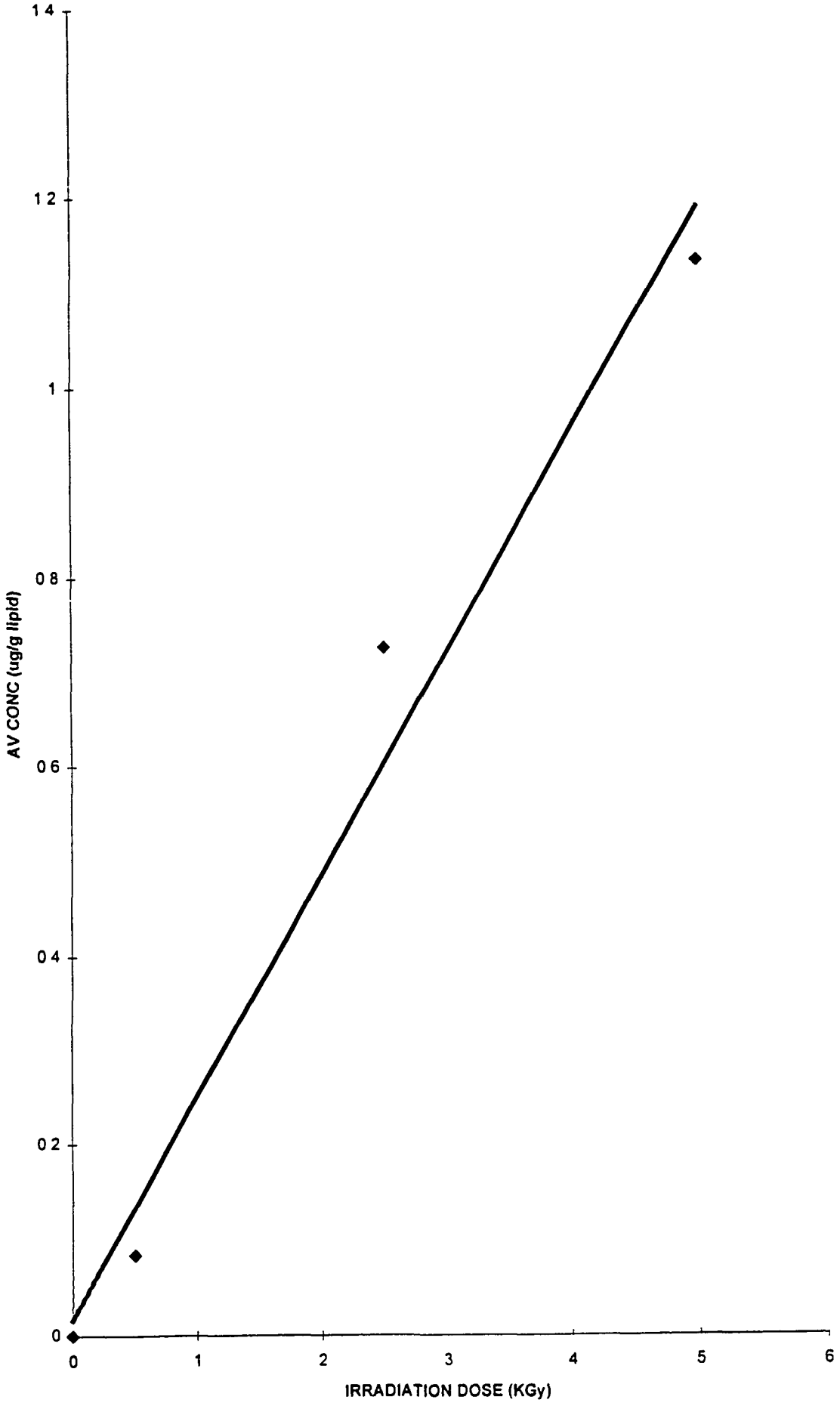


Fig 3 6 2 Irradiation Dose vs Concentration of TCB in Chicken

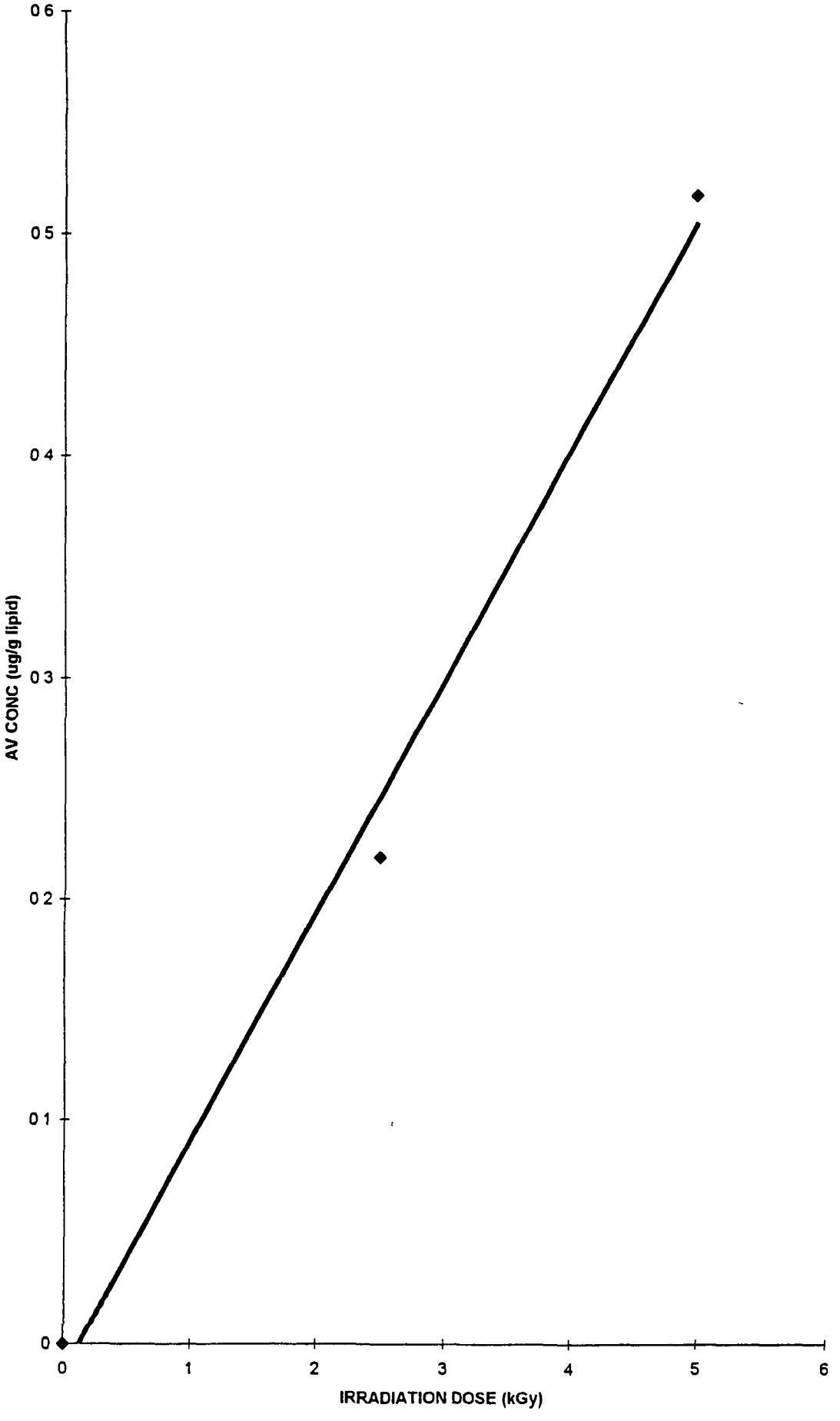


Fig 3 6 3 Irradiation Dose vs Concentration of DCB in Olives

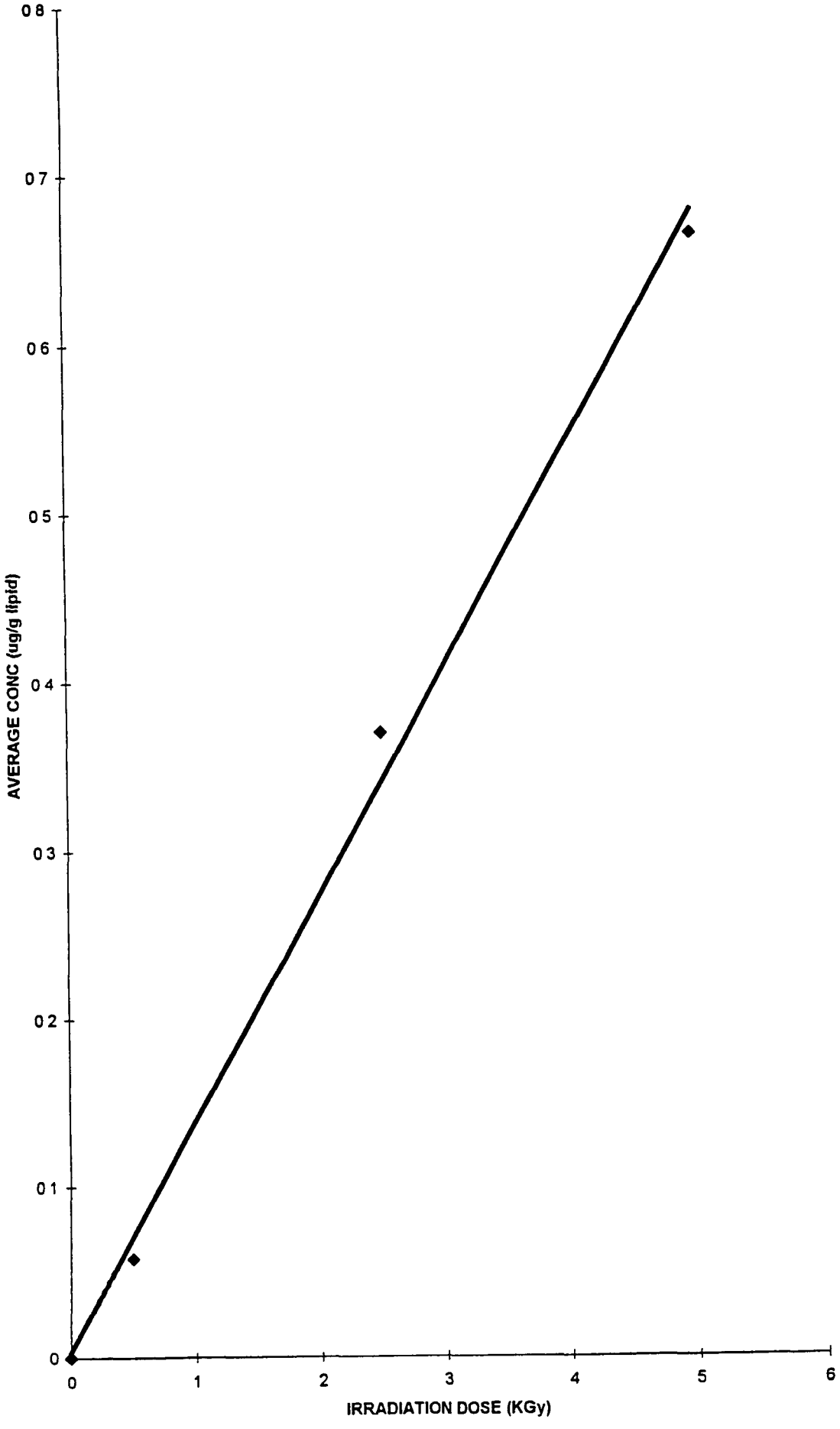
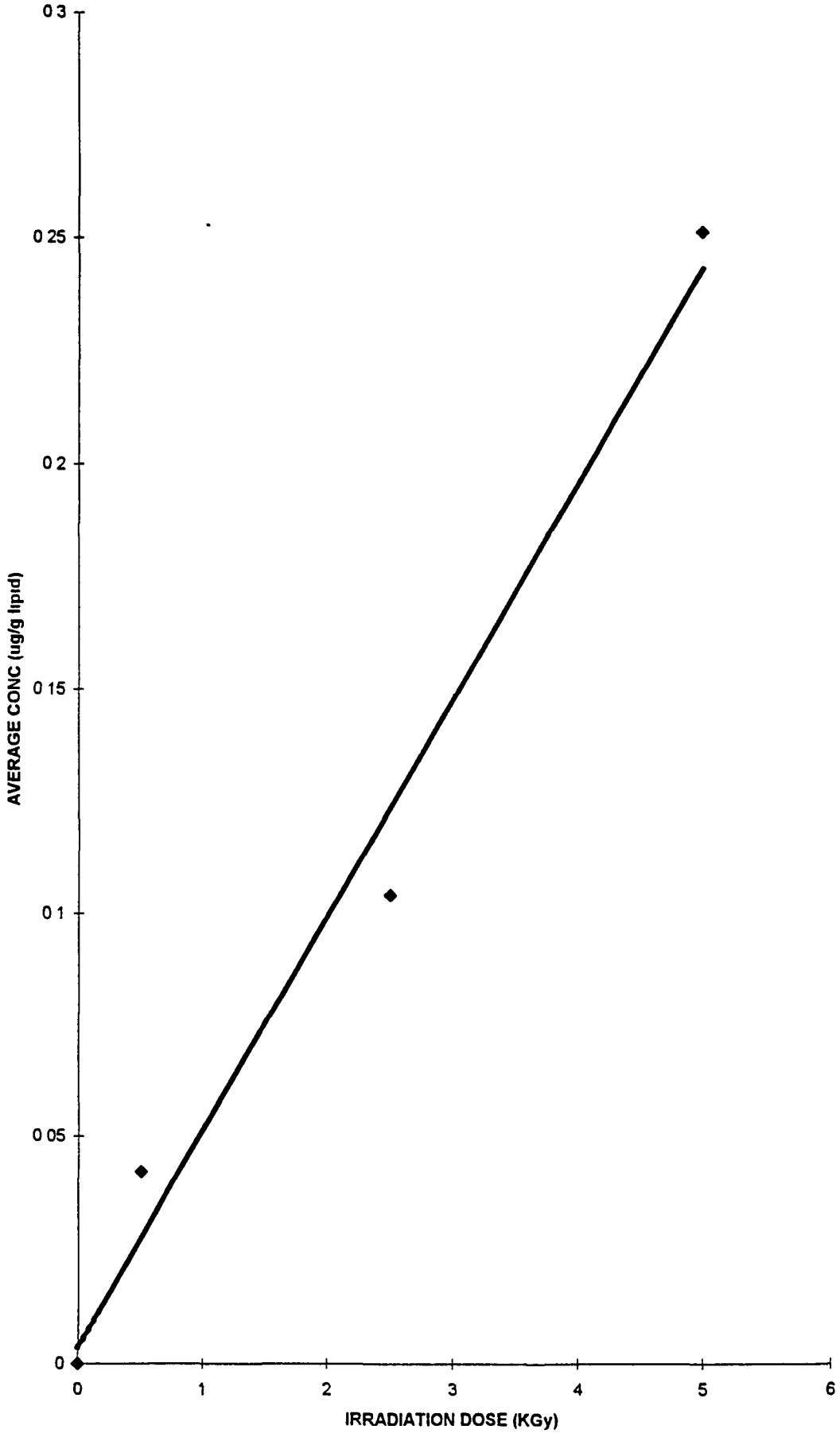


Fig 3 6 4 Irradiation Dose vs Concentration of TCB in Olives



The main statistical tool employed was regression analysis and the following results were observed

Table 3 6 1 Regression analysis of irradiated food

Foodstuff	Compound	R^2 Value
Chicken	DCB	0 976
	TCB	0 992
Olives	DCB	0 996
	TCB	0 982

(Outliers were omitted to increase accuracy)

A definite trend exists to suggest that unknown irradiated samples may be classified in a broader sense as low, medium, and high dosage foodstuffs

On a quantitative basis, the estimation of dose may be dependent on various factors such as temperature of irradiation, storage of sample before analysis and even possibly source of food sample The accuracy of dose estimation therefore, would be dependent on the history of the food sample In the case of blind samples, this information would more than likely be limited and it may only be possible to estimate the dose within bands such as low, medium, or high dose over the range of 0-10 kGy

It was very encouraging to see that this also applies to olives as they were being analysed for the first time using this method

3 7 **Effect of Storage on the Concentration of DCB and TCB.**

An investigation was carried out to determine the effect of storage on the concentration of DCB in the irradiated chicken

It is generally accepted that the concentration of DCB decreases with storage at room temperature and at a refrigerated temperature of 4°C [139] This is further supported by the current research in which the authentic standards of DCB were observed to decompose over a period while stored at -20°C

This investigation looked at the concentration of DCB in irradiated chicken meat analysed initially and six months later after storage at -20°C

Table 3 7 1 shows that the concentration of DCB decreases over time but the level is still significantly high to declare the sample as irradiated This would enable the analysis of samples over a long period provided they were stored sufficiently It also provides the possibility of detecting irradiated food that may have been stored in a commercial situation

Table 3 7 1 Concentration of DCB at initial irradiation time and after a storage period of six months at -20°C

Dose (kGy)	Sample Name	Conc DCB (µg/g lipid)	
		Month 0	Month 6
2 5	C1	0 73	0 585
	C2	0 76	0 568
	C3	0 61	0 586
	C4	0 65	0 549

The conclusion drawn from this investigation is that samples should be stored at a temperature of -20°C or less

3 8 Characteristics of a Detection Method

The purpose of this research was to optimise and develop the available methods of lipid extraction as well as optimise the detection of 2-alkylcyclobutanones in a complex irradiated food matrix and to progress this research to incorporate novel foodstuffs

Having shown that DCB and TCB are present in food samples following irradiation, it is necessary to consider how such a detection system would meet criteria considered essential for an efficient process

3 8 1 Selectivity and specificity to the irradiation process

The cyclic ketones 2-DCB and 2-TCB must be specific to the irradiation process and not present in non-irradiated products

During each batch experiment, non-irradiated control samples were prepared 2-DCB and 2-TCB were not detected in any of the chicken or fig samples, but a problem did present itself in the case of the olives, where false positives were recorded Therefore further research, most probably into the GC-MS method of detection is required, to try to address this problem as it is thought to centre around co-elution of a contaminant from extraneous substances

The following external events did not produce 2-dodecylcyclobutanone or 2-tetradecylcyclobutanone in non-irradiated samples

(a) mincing and packaging in plastic wrap or storage in glass or plastic containers

(b) freezing at temperatures below –20°C

(c) storage for periods in excess of 20 days at 4°C

Low dosage levels for DCB were detected for olive samples (quantitatively) and chicken samples (qualitatively) These levels are below that which is likely to be applied in commercial practice [5]

The results of all the experiments performed suggests that DCB and TCB are specific markers for the irradiation of food, and that DCB is a more reliable indicator in the detection of low doses of irradiation because of it's higher concentration

The specificity of this method over others has an advantage due to the low background levels present [41].

3.8.2. Accuracy and Reproducibility

Using 2-DCB and 2-TCB, it has been possible to distinguish between irradiated and non-irradiated chickens, olives and figs.

The reproducibility, which has been measured statistically, was demonstrated using the 2.5 kGy chicken samples measured six months apart and still producing very similar results. The results of all the experiments described suggest that DCB and TCB are suitable radiolytes for the detection of irradiated food on a qualitative basis, with DCB being a more reliable marker for quantitative estimation of irradiation dose.

Finally, the accuracy of dose estimation will be dependent on the history of the sample.

3.8.3. Detection Limit

The dosage level applied to a commercial foodstuff is the main factor when considering the limit of detection. The function of the irradiation of a particular food product is also a major consideration of the dose to be applied. In the case of meat products the function of irradiation would be to kill pathogenic material in order to prevent food poisoning. This would be achieved using doses between 1 and 7 kGy [5]. For fruit products, the main purpose of irradiation would be to delay ripening and prolong the shelf life. Therefore, a detection method must be capable of detecting doses of 0.5-1kGy [5]. Levels of

DCB and to a lesser extent TCB are detectable in chicken and olives at 0.5 kGy. The recorded levels are such that lower doses may well be detected. Improvement in technique would further enhance this assessment. Because of the lower level of TCB in chicken and olives. lower doses may prove more difficult for detection.

3.8.4. Dose Estimation

The linearity of increasing dose up to 5 kGy with concentration of 2-DCB and 2-TCB in chicken and olives (Fig 3.6.1-3.6.2) indicated that this method had potential for the estimation of irradiated dose.

On a quantitative basis, estimations of dose may be dependent on various factors such as temperature of irradiation, storage etc. The dose estimation would rely therefore on a history of the sample and classification may be better placed among low, medium, or high doses.

Finally, the concentration of DCB and TCB formed upon irradiation has been expressed as the weight per weight of extracted lipid. In the case of meat product, the fatty acid content of the animal is dependent on factors such as dietary intake, animal type, and location of fat in the carcass. For this purpose, all experiments were carried out using the same source of chicken.

3.8.5. Post Irradiation Processes.

All known irradiated samples of 2.5 and 5 kGy showed the presence of DCB and TCB upon analysis by GC-MS, although there was a significant time lapse in some instances. During this period, all samples were frozen until required

and this process was observed to have no effect on the concentration of DCB or TCB

3 8 6 Sample Size

The amount of the sample required for the analysis should be reasonable The weight involved for each sample was only 20g, which was deemed to contain enough lipids to provide 200 mg Indeed, both chicken and olives conformed to this supposition, but figs which contain a lower level of lipid, would require a substantially higher sample weight to achieve 200 mgs However, by modifying the method using a volume with a lower quantity of lipid, a valid result was attainable

3 8 7 Method applicable to wide variety of foodstuffs

Prior to the undertaking of the current research, meat products and liquid egg were the only foodstuffs reported in the literature as being analysed by the method prescribed As a result of this research, two new foodstuffs have been assessed and have shown promising results It is necessary for the food under analysis to contain a reasonable level of lipid, but as shown in this research irradiation of foods containing low levels of lipids, may be detected using this methodology

However, one feature to be considered is the dosage level applied to a particular food product In the case of figs which were irradiated at 2 5 kGy, this may be too high a dose level to be applied commercially (0 2-0 5 kGy is a more likely value) Therefore, further research is required to see if radiolytes in figs can be detected at these levels

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APPENDICES

APPENDIX A

Lipid Extraction Methods

The following extraction techniques were performed during the course of the research

1 Folch Extraction

2 Modified Folch

3 Bligh and Dyer Extraction

4 Soxhlet Extraction

5 Fosslet Extraction

6 "Modified Fatty Foods Extraction"

7 Total Lipids Extraction

8 Batch Extraction

1 Folch Extraction

Table 1 *Folch extractions performed on foodstuffs as batches*

Series Number	No of Extractions	Food Type
1	10	Chicken 1
2	9	Chicken 2
3	10	Chicken 3
4	10	Olives

The protocol followed was as cited by Folch, Lees, and Stanley [147] described in the lipid handbook of Hamilton [148]

The following procedure applies to both chicken and olive samples

Each sample (10g) of homogenised food was weighed accurately and homogenised (Dupont, Omnimixer 17106) with dichloromethane methanol (2 1 v/v) to a final dilution 20 times the volume of the tissue sample The time of homogenization was five minutes The homogenate was filtered through a Whatman No 1 filter paper using a buchner funnel The crude extract was washed with 0 2% of its volume with water solution The filtrate was allowed to separate into two phases and the upper phase was removed by siphoning off with a pipette The interface was rinsed three times with pure 'upper phase' i e dichloromethane methanol water (3 48 47v/v, 30cm^3) so that the lower phase was not disturbed This had the effect of removing any fluff at the interface The bottom layer was added to a 250 cm^3 round bottomed flask and rotary evaporated to 5-10cm^3 This was then transferred to a round-bottomed flask (50cm^3) and the remaining solvent was evaporated under a gentle stream of dry nitrogen leaving the lipid sample for gravimetric determination The temperature was kept below 40°C during the evaporation process

Modifications

Series 1

1 The crude extract was washed with sodium chloride (0 29%) solution

Series 2

1. The homogeniser used was changed to a mixer (Krups, 3 mix 1000plus) which was deemed more efficient than homogeniser in series 1, due to a mechanical fault in the latter. This was repeated in series 3.

2. There was difficulty in removing the water from the series 1 samples and so after the rotary evaporation stage of series 2, all samples were frozen and placed in a freeze drier for 6-12 hours to remove traces of water.

Series 3

1. The rotary film evaporation stage was carried out using a new pump (vaccubrand teflon diaphragm pump, type MX 2C).

2 Modified Folch [148]

Series 1: 5 Chicken 3 Samples.

Series 2: 5 Olive Samples.

Each sample (2g) was homogenised (Krups 3 mix, 1000 plus) for one minute in methanol ($20cm^3$). Dichloromethane ($40cm^3$) was added. The homogenisation was continued for a further two minutes. The homogenate was filtered through a buchner funnel and placed in a separating funnel ($250cm^3$) The residue was suspended in dichloromethane: methanol (2:1v/v. $60cm^3$) and was homogenised for a further three minutes. The

homogenate was filtered and the residue was further washed with dichloromethane (40cm^3) and methanol (20cm^3) The combined filtrates were measured and an aqueous solution of potassium chloride (0 88% w/v) was added whose volume corresponded to one-quarter that of the combined filtrates The mixture was shaken thoroughly and allowed to settle The upper aqueous layer was removed by aspiration Water methanol (2 1 v/v, 30cm^3) was added to the lower layer, the mixture was shaken thoroughly and allowed to settle The upper layer was removed by aspiration The lipid was recovered from the lower layer, which was transferred to a preweighed round bottomed flask (250cm^3) and the solvent was evaporated in a rotary film evaporator at a temperature below 40°C Any final remnants were removed under a gentle stream of nitrogen

3 Bligh and Dyer Extraction [149]

Series 1 5 Chicken 3 Samples

Series 2 5 Olive Samples

Sample (20g) was homogenised in a blender (Krups, 3 mix 1000plus) for 2 minutes with a mixture of dichloromethane (20cm^3) and of methanol (40cm^3) Dichloromethane (20cm^3) was added to this mixture and blended for 30 seconds Water (20cm^3, distilled) was added and the mixture was further blended for 30 seconds The homogenate was filtered through a Whatman No 1 filter paper on a Buchner funnel The residue was

compressed with a spatula to ensure maximum recovery of the filtrate The filtrate was transferred to a graduated measuring cylinder (250cm^3) After a brief settling period, to enable complete separation and clarification, the volume of the dichloromethane layer was recorded The upper alcoholic layer was removed by aspiration A small volume of the dichloromethane layer was removed to ensure complete removal of the top layer The dichloromethane layer contains the lipid This fraction was transferred to a preweighed round bottomed flask (250cm^3) rotary evaporated to dryness and the yield of lipid was recorded

4 Soxhlet Extraction [150]

Table 2 List of Soxhlet extractions performed using various solvents

Solvent	Chicken 1	Chicken 2	Chicken 3	Olives
(i) TCE[1]/ Methanol (2 1)	3	-	-	-
(ii) DCM[2]/ Methanol (2 1)	5	12	5	5
(iii) Pet Ether	4	-	5	5
(iv) Hexane	-	-	5	5

[1] Trichloroethane

[2] Dichloromethane

(i) Trichloroethane/ Methanol (2 1 v/v)

A round-bottomed flask (250cm^3) was preweighed to four decimal places Food sample (5g) was weighed into a cellulose extraction thimble and plugged with non-absorbent cotton wool The thimble was placed in the extraction chamber and trichloroethane/ methanol (2 1 by vol) (40cm^3) was added The remaining trichloroethane/ methanol (2 1 by vol) (110cm^3) was added to the round-bottomed flask and the apparatus was assembled The samples were gently refluxed to ensure that a continuous extraction occurred The extraction time was three hours The solutions were cooled and rotary evaporated to dryness and the yield of lipid was recorded

(ii) Dichloromethane Methanol (2 1v/v)

Samples (10g) were weighed into a cellulose extraction thimble and plugged with non-absorbent cotton wool The thimble was added to the extraction chamber Dichloromethane Methanol (2 1v/v, 40cm^3) was added to the extraction chamber and the remaining Dichloromethane Methanol (2 1v/v, 110cm^3) was added to a pre-weighed round-bottomed flask and the apparatus was assembled The samples were gently refluxed to ensure a continuous extraction occurred over a period of six hours The solvent was collected into the round-bottomed flask and rotary evaporated to remove the solvent The flask was re-weighed and the yield

of lipid was recorded

Modifications

1 Chicken 2 samples were freeze dried after rotary evaporation

2 20g of chicken were weighed out for chicken 3 and olive samples

3 Chicken 3 samples and olive samples contained 20g of anhydrous sodium sulphate

(iii) Petroleum Ether

A ($250cm^3$) round-bottomed flask was preweighed to four decimal places Food sample (10g) was weighed into a cellulose extraction thimble and plugged with non-absorbent cotton wool The thimble was placed in the extraction chamber and petroleum ether ($40cm^3$) was added The remaining petroleum ether ($110cm^3$) was added to the round-bottomed flask and the apparatus was assembled The samples were gently refluxed to ensure that a continuous extraction occurred The extraction time was six hours The solutions were cooled and rotary evaporated to remove solvent and the yield of lipid was recorded

Modifications

1 All samples were 10g except for chicken 3 and the olive samples where the weight was increased to 20g

(iv) Hexane

Sample (20g) was weighed into an extraction thimble, along with anhydrous sodium sulphate (20g), thoroughly mixed and placed in extraction chamber Hexane ($40cm^3$) was added Hexane ($110cm^3$) was added to a round-bottomed flask ($250cm^3$) and the Soxhlet apparatus was assembled The samples were gently refluxed to ensure a continuous extraction The extraction was left for six hours The solvent was collected in the round-bottomed flask, and this was rotary evaporated to remove solvent, and the yield of lipid was recorded

5 Foss-let Extraction [151]

Series 1 5 Chicken Samples

Series 2 5 Olive Samples

Calibration of Foss-let Instrument

The Foss-let calibration had to be carried out using a "0" and a "50%" calibration liquid

Preparation of liquids

"0" Pure perchloroethylene is used as "0" calibration liquid Since the perchloroethylene used is a technical grade, the "0" point may vary from one supply to another

"50%" A mixture of perchloroethylene and mineral oil with a known specific gravity is the 50% calibration liquid

Preparation of the 50% calibration liquid.

Standard oil (22 5g +/- 0 1g) was weighed into a conical flask with a lid Perchloroethylene (120 cm^3) was dispensed into the flask The flask was closed and shaken thoroughly

"0" point check.

The "0" point was checked before starting the actual measurements

The perchloroethylene was poured directly into the measuring chamber until the liquid appeared in the upper drain hose The outlet valve button was pressed This step was repeated Finally the perchloroethylene was poured into the chamber until the liquid appeared in the upper drain hose When the temperature control lamp was off the swimmer reset button was pressed The readout read 0-0 05

"50%" point check.

The 50% point was checked as follows before sample measurement

The zero point was checked and adjusted if necessary The 50% liquid was poured directly into the measuring chamber until the liquid appeared in the upper drain hose The outlet valve was pressed This step was

repeated The 50% liquid was poured into the measuring chamber until the liquid appeared in the upper drain hose When the temperature control lamp was off the swimmer reset button was pressed The readout was found to be 50 +/- 0 03

Adjustment of "0" point.

When the readout was outside the value permitted (i e 0 or 50 +/- 0 05) it was adjusted If the value was too low, the "0" adjustment knob was turned a little clockwise, and if the value was too high the "0" adjustment was turned a little anti-clockwise (The same procedure was repeated for the 50% point)

Analysis

(i) Chicken

Chicken meat (22 5g) was weighed out accurately and placed in the extraction chamber The extraction chamber was placed under the dispenser The dispenser was filled with perchlorethylene (120 cm^3) which was added to the extraction chamber Calcium sulphate hemihydrate (50-60 g) was added and the extraction lid was fitted The extraction chamber was placed in the homogeniser The extraction was performed for two minutes

Sample Measurement

The measurement chamber was previously emptied by pressing the outlet valve button

Preparation of the Filter Unit

The filter unit is a black cylindrical container with a flat piston and a removable bottom The bottom was removed and a piece of filter paper was inserted The filter unit was placed in the recess on the top of the measuring unit

Filtration

The lid was removed from the extraction chamber and the contents were poured into the filter unit The piston was fitted into the cylinder with the small hole facing upwards The recess cover was fitted on top and locked The large fastening screw was turned slowly clockwise to force the solvent containing the fat through the filter paper and retain the solid residue When the filtrate appeared in the upper draining tube the outlet valve was pressed to remove the excess solvent not required for the measurement of the fat When the filtrate appeared in the upper drain valve for a second time, the screw was loosened and the cover was removed The filter unit was removed and the recess cover was replaced

Measuring

The swimmer reset button was pressed when the temperature control lamp was off The display automatically counted up to the actual percentage fat in the sample

Emptying

After the digital read-out had been noted, the measuring chamber was emptied by pushing the outlet valve button The recess cover was cleaned out with a piece of fluffless paper in preparation for the next sample

(ii) Olives

The same procedure was repeated for the olives samples except

1 Perchloroethylene = 60cm^3

2 Sodium sulphate (25-30g) instead of calcium sulphate hemihydrate

6 Modified Fatty Foods Extraction [152]

Series 1 8 Chicken 2 Samples

Sample (10g) was weighed and homogenised with petroleum ether (40-60, 150cm^3) and anhydrous sodium sulphate (50g) for five minutes The supernatant was filtered through a Whatman No 1 filter paper The

residue was re-extracted with petroleum ether (2 × 100cm^3) for four minutes. The extracts were filtered through the same funnel and pressed to force out the remaining solvent. The combined extracts were dried over anhydrous sodium sulphate for 24 hours. The petroleum ether was rotary evaporated to approximately 10cm^3 and transferred to a pre-weighed 50cm^3 round-bottomed flask. It was then evaporated to dryness to remove solvent under a gentle stream of nitrogen and the yield of lipid was recorded.

Modifications

1. Samples 5 & 6 for chicken were dried for 3 hours over sodium sulphate

2 Sample 1 for chicken was dried for 24 hours over magnesium sulphate

7. Total Lipids Extraction [153]

Series 1: 5 Chicken 3 Samples

Series 2: 5 Olive Samples

Sample (10g) was weighed accurately and transferred to a mortar. Sodium sulphate (anhydrous, 10g) was added and the mixture was ground with a pestle. Methanol (5cm^3) was added and the mixture was ground further into a paste. The mixture was transferred to a 50cm^3 centrifuge tube using methanol (5cm^3) to rinse out the mortar. This tube was heated by

immersing in a water bath at 55°C for three minutes The tube was then allowed to cool to room temperature The contents of the tube were transferred back to the mortar with diethyl ether ($5cm^3$) and ground again The suspension was returned to the centrifuge tube with a further portion of diethyl ether ($5cm^3$) It was then centrifuged for five minutes in a clinical centrifuge (Gerber & Co) The supernatant fluid was decanted into $50cm^3$ Erlenmeyer flask The pellet was re-extracted with methanol diethyl ether (1 1v/v, 3 × $10cm^3$), heating cautiously in a water bath for three minutes during each extraction and the centrifugation was repeated The supernatant fluids were combined and were carefully poured through a funnel fitted with fluted filter paper into a $100cm^3$ graduated cylinder containing isotonic saline solution (0 9%w/v, $40cm^3$) The Erlenmeyer flask was rinsed with diethyl ether and transferred to the filtering apparatus The contents of the cylinder were mixed thoroughly with a glass rod and the mixture was allowed to settle into two distinct layers The clear diethyl ether layer was transferred to a pre-weighed $50cm^3$ round-bottomed flask where it was rotary evaporated below 40°C to approximately $5cm^3$ The remaining extract was dried under a gentle stream of nitrogen to yield a dry lipid extract for weighing

Modifications

1 Samples 3 & 4 of the chicken samples and samples 3 & 9 of the olives required the addition of more diethyl ether to allow separation into two distinct layers

8 Batch Extraction

Series 1 5 Chicken 3 Samples

Series 2 5 Olive Samples

A food sample (10g) was weighed accurately and transferred to a homogeniser (Krups, 3 mix, 1000plus) with hexane isopropanol (3 2v/v, 80cm^3) The mixture was homogenised for three minutes and was then filtered through a sintered glass funnel under pressure of nitrogen The filtrate was transferred using a small volume of extracting solvent to a separating funnel The residue was resuspended in extracting solvent (3cm^3) and allowed to stand in the sintered funnel for three minutes Pressure was applied to expel the wash This was repeated twice with 3cm^3 portions of extracting solvent The solvent containing the lipids was transferred to a round-bottomed flask and the solvent was evaporated to 5cm^3 on a rotary film evaporator The 5cm^3 were then placed in a pre-weighed glass vial The remaining solvent was evaporated under a gentle stream of dry nitrogen until only pure lipids remained The yield was then recorded

APPENDIX B

GC-FID Conditions used to Analyse Fatty Acid Methyl Esters

Instrument Shimadzu GC 14A

Gas Settings

H_2 ~0 6 kg/cm^2

Air ~ 0 5 kg/cm2

N_2(carrier) ~ 1 25 kg/cm^2

N_2(make-up) ~ 0 75 kg/cm^2

Temperature Settings

Injector 250°C

Detector 260°C

Temperature Programme

Initial Column Temperature 120°C

Initial Column Time 0 minutes

Column Programme Rate 1 15°C/min

Final Column Temperature 1 200°C

Final Column Time 1 5 minutes

Column Programme Rate 2 2°C/min

Final Column Temperature 2 210°C

Final Column Time 2 10 minutes

Injector

Mode = split 1/50

Flow rate = $1cm^3/min$

Injection volume = $1\mu l$

Detector

Flame Ionisation Detector

Parameter Settings

Instrument Integrator Shimadzu CR5A

Peak Width = 2

Slope = 200 or 1000

Minimum Area = 1000 or 5000

Attenuation = 2, 3, 4, 5

Stop Time = 20 minutes

APPENDIX C

Statistics

Statistical analysis was employed for the evaluation of all the results obtained in this research It is important from an analytical perspective to validate all quantitative results and provide an estimate of the error involved in their determination The statistical tests employed for this valuation are described below

(i) Average

$$x = \sum_i \xi_i/n$$

(ii) Standard Deviation

$$s = (\sum(x_i-\xi)^2/n-1)^{1/2}$$

This is the measure of the repeatibility or spread of a set of results

(iii) Relative Standard Deviation

$$100\ s/\xi$$

This is also referred to as the co-efficient of variation The CV or RSD, the units of which are obviously percent, is an example of relative error, i e an error estimate divided by an estimate of the absolute value of the measured quantity Relative errors are frequently used in the comparison of the precision of results, which have different units or magnitudes, and again are important in calculations of error propagation

(iv) Outlier Test

Q = | suspect value - nearest value | / | largest value - smallest value |

This is known as Dixon's Q test and if the value obtained is greater than the value for the 95% confidence level, then the suspect value is regarded as an outlier and is not included in any subsequent statistical testing

(v) Analysis of Variance

In these research results, there are two possible sources of variation The first which is always present, is due to random error in measurement It is the error that causes a different result to be obtained each time a measurement is repeated under the same conditions The second possible source of variation is due to what is known as a controlled or fixed-effect factor The controlled factor in this case is the method of analysis used Analysis of Variance (ANOVA) is an extremely powerful statistical technique which can be used to separate and estimate the different causes of variation It can be used to separate any variation, which is caused by changing the controlled factor, from the variation due to random error It can thus test whether altering the controlled factor leads to a significant difference between the mean values obtained

ANOVA tests are significance tests and of course, no clear-cut answers are derived directly from the data obtained Rather they aid the interpretation of experimental data by giving the probabilities that certain conclusions are valid

All the ANOVA tests applied to this research data are at the P=0 05 level, i e there is a 5% risk that a null hypothesis will be rejected even though it is true

This is known as Type 1 error It is also possible, however, to retain a null hypothesis even when it is false and this is referred to as Type 2 error

Comparison of all Extraction Methods using Anova Extraction Statistics

The following results were subjected to Single Factor Anova statistical analysis The purpose is to compare the yield of fat per 100g of sample using a variety of extraction methods as described in the following tables

1 Comparison of Folch extraction procedure with the Soxhlet procedure for Chicken 1 sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Folch	DCM/Meth[a]	7 1994	1 379	4 747
Soxhlet	DCM/Meth	6 662		

[a] Dichloromethane/Methanol (2 1v/v)

2 Comparison of Folch extraction procedure with the Foss-let extraction for Chicken 3 Sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Folch	DCM/Meth	20 0096	0 249	5 317
Foss-let	Perchloro-ethene	19 730		

3 Comparison of all extraction procedures performed on Chicken 3

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Folch	DCM/Meth	20 009		
Mod Folch	DCM/Meth	17 757		
Bligh & Dyer	DCM/Meth	10 639		
Soxhlet	Pet Ether	4 509		
Soxhlet	Hexane	3 024	926 686	2 208
Soxhlet	DCM/Meth	12 668		
Foss-let	Perchloro-ethylene	19 730		
Total Lipids	DE/Meth[b]	13 602		
Batch	Hexane/IP[c]	15 367		

[b] Diethyl Ether/Methanol (1 1v v)

[c] Hexane/Isopropanol (3 2v v)

4 Comparison of all the extraction procedures used performed on Chicken 3

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Folch	DCM/Meth	23 627		
Mod Folch	DCM/Meth	24 515		
Bligh & Dyer	DCM/Meth	12 341		
Soxhlet	Pet Ether	9 334		
Soxhlet	Hexane	8 673		
Soxhlet	DCM/Meth	16 777	1003 889	2 208
Foss-let	Perchloro-ethylene	13 820		
Total Lipids	DE/Meth	16 461		
Batch	Hexane/IP	18 499		

5 Comparison of Folch and Modified Folch extraction procedures for Chicken 3 sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(crit)
Folch	DCM/Meth	20 009	16 034	5 317
Mod Folch	DCM/Meth	17 757		

6 Comparison of Soxhlet extraction procedures for Chicken 1 sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Soxhlet	TCE/Meth[e]	6 343		
Soxhlet	Pet Ether	4 643	11 416	4 737
Soxhlet	DCM/Meth	6 663		

[e] Trichloroethane/Methanol (2 1v/v)

7 Comparison of Soxhlet extractions using a non-polar/polar solvent system

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Soxhlet	TCE/Meth	6 343	0 376	6 608
Soxhlet	DCM/Meth	6 663		

8 Comparison of Soxhlet extraction procedures used for Chicken 3 sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Soxhlet	Pet Ether	4 509		
Soxhlet	Hexane	3 024	6149 685	3 885
Soxhlet	DCM/Meth	12 668		

9 Comparison of Folch and Soxhlet extractions for Chicken 1 Sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Folch	DCM/Meth	7 194		
Soxhlet	TCE/Meth	6 342	11 528	3 239
Soxhlet	Pet Ether	4 643		
Soxhlet	DCM/Meth	6 663		

10 Comparison of Folch and Soxhlet extractions for Chicken 3 Sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Folch	DCM/Meth	20 0096		
Soxhlet	Pet Ether	4 509	761 465	3 238
Soxhlet	Hexane	3 024		
Soxhlet	DCM/Meth	12 668		

11 Comparison of extraction procedures performed on Chicken 2 Sample

Extraction	Solvent	Average Fat (g/100g)	F(calc)	F(tables)
Folch	DCM/Meth	3 130		
Soxhlet	DCM/Meth	1 723	33 197	3 369
Modified Fatty Foods	Pet Ether	2 453		

APPENDIX D

Calibration and Sample Calculations of Fatty Acid Methyl Esters

Before each foodstuff was analysed for the fatty acid methyl ester content by GC-FID analysis, the system was calibrated by running appropriate standards of the FAMES and constructing calibration graphs for the purpose of quantitation The internal standard method was used throughout the analysis (except for chicken 1) Methyl Heptadecanoate was chosen as the internal standard at a concentration of 1000 ppm It was chosen because it is not present in either foodstuff analysed and also because of its suitable retention time with respect to the other FAMES

Figure1 Calibration Graph for Palmitic Acid Methyl Ester for Chicken Sample 1

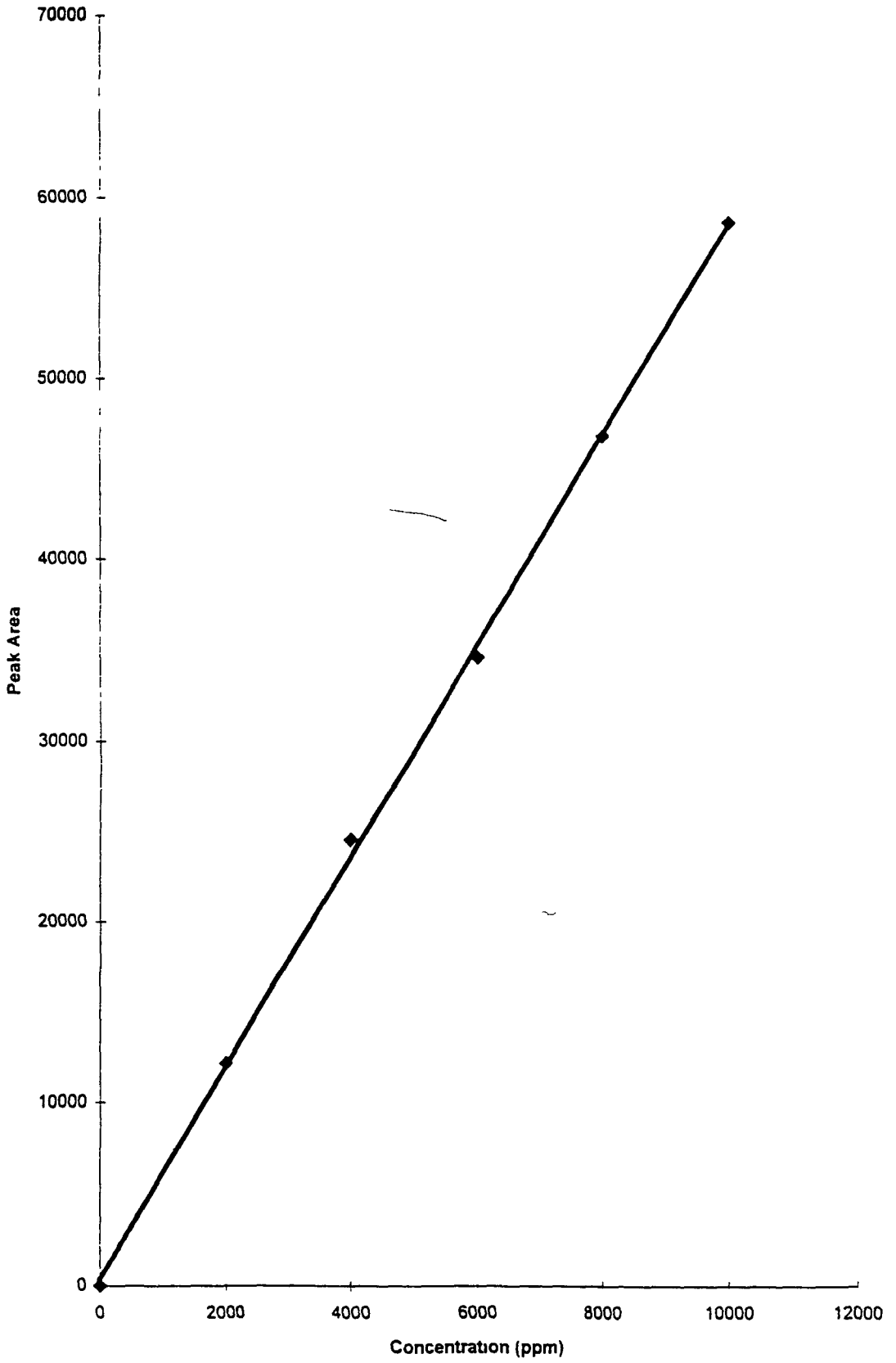


Figure 2 Calibration Graph for Palmitic Acid Methyl Ester using Heptadecanoic Acid Methyl Ester (HAME) as Internal Standard (Chicken 2)

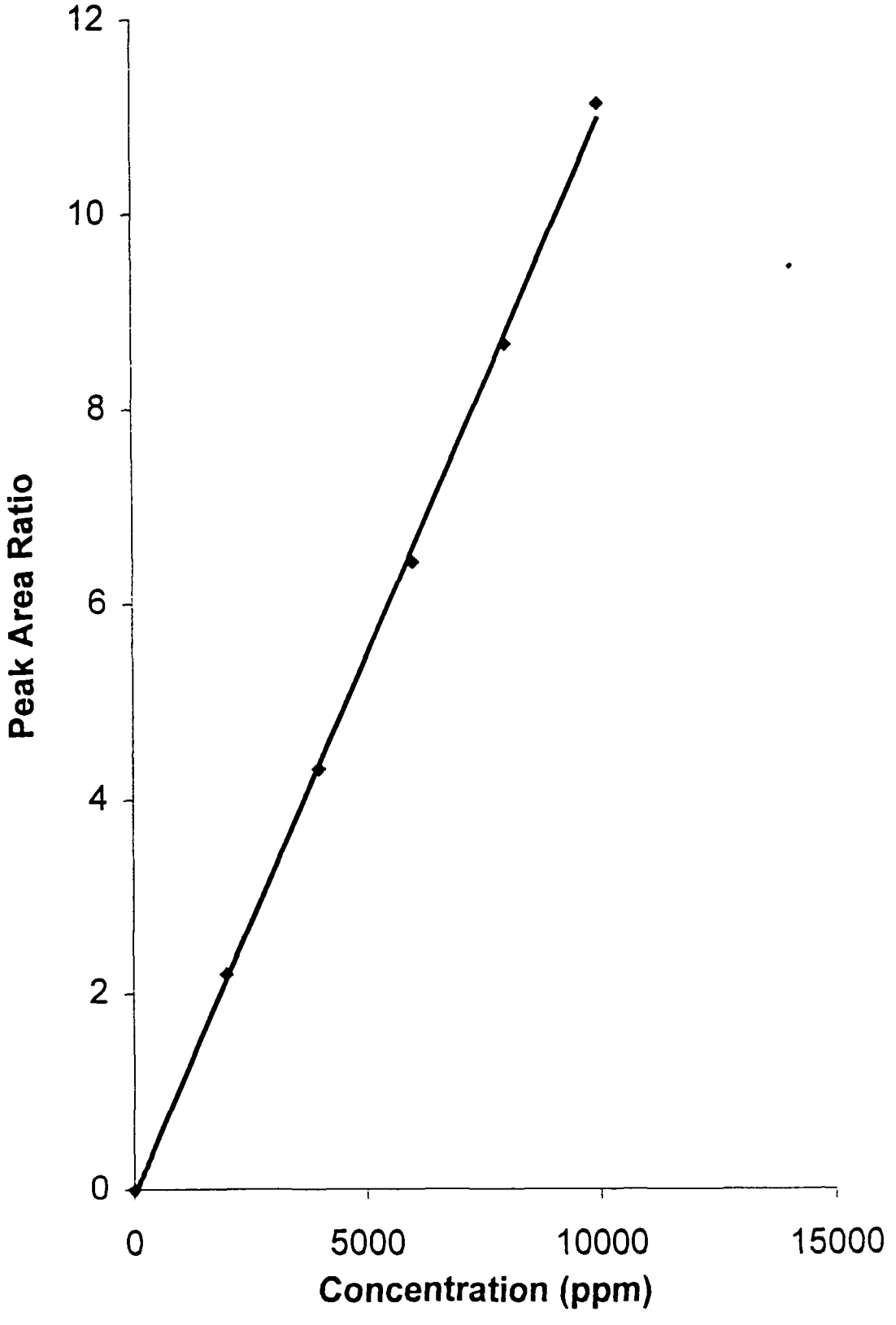


Figure 3 Calibration Graph of Stearic Acid Methyl Ester using HAME as Internal Standard (Chicken 2)

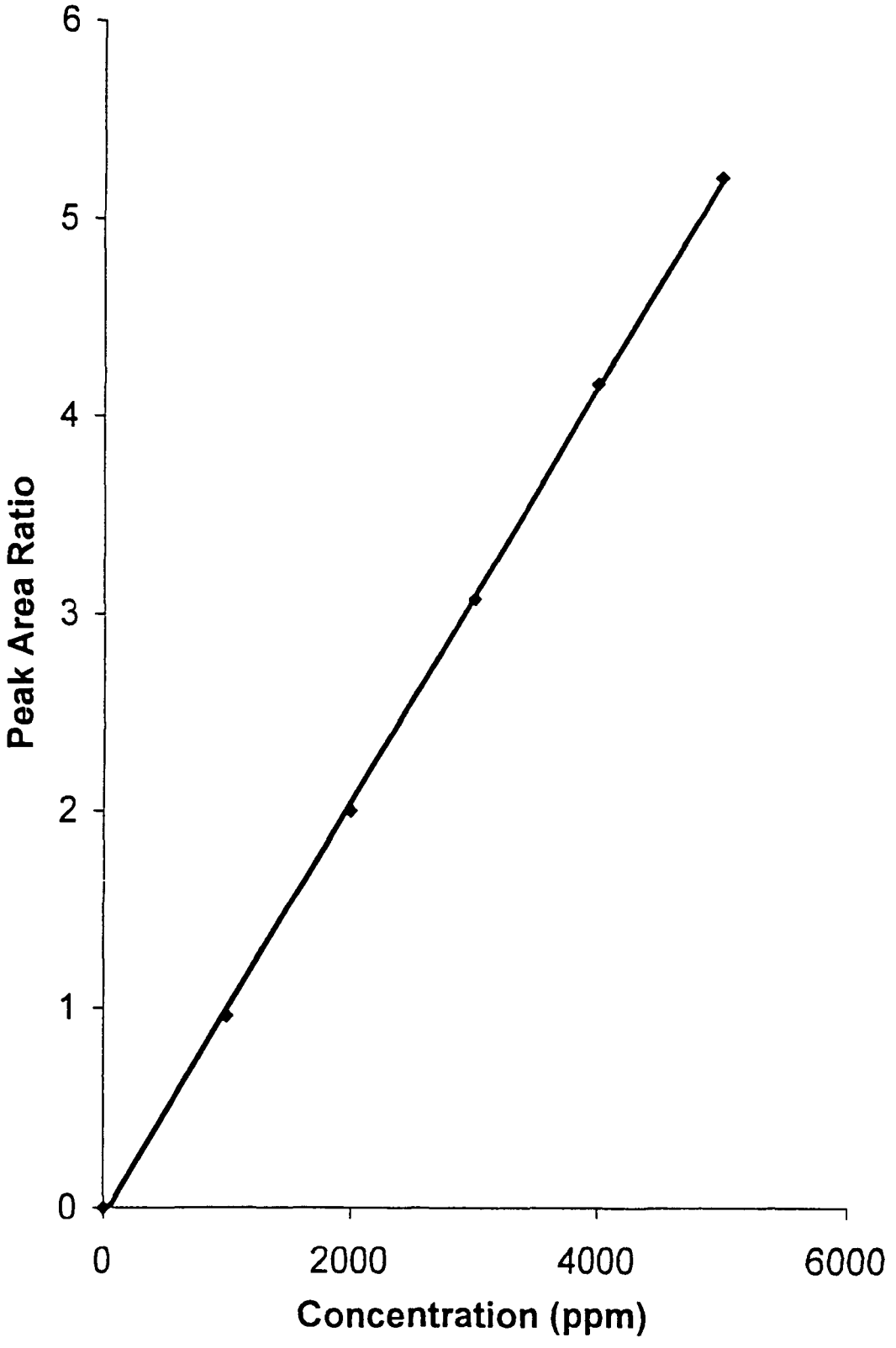


Figure 4 Calibration Graph of Myristic Acid Methyl Ester using HAME as Internal Standard (Chicken 3)

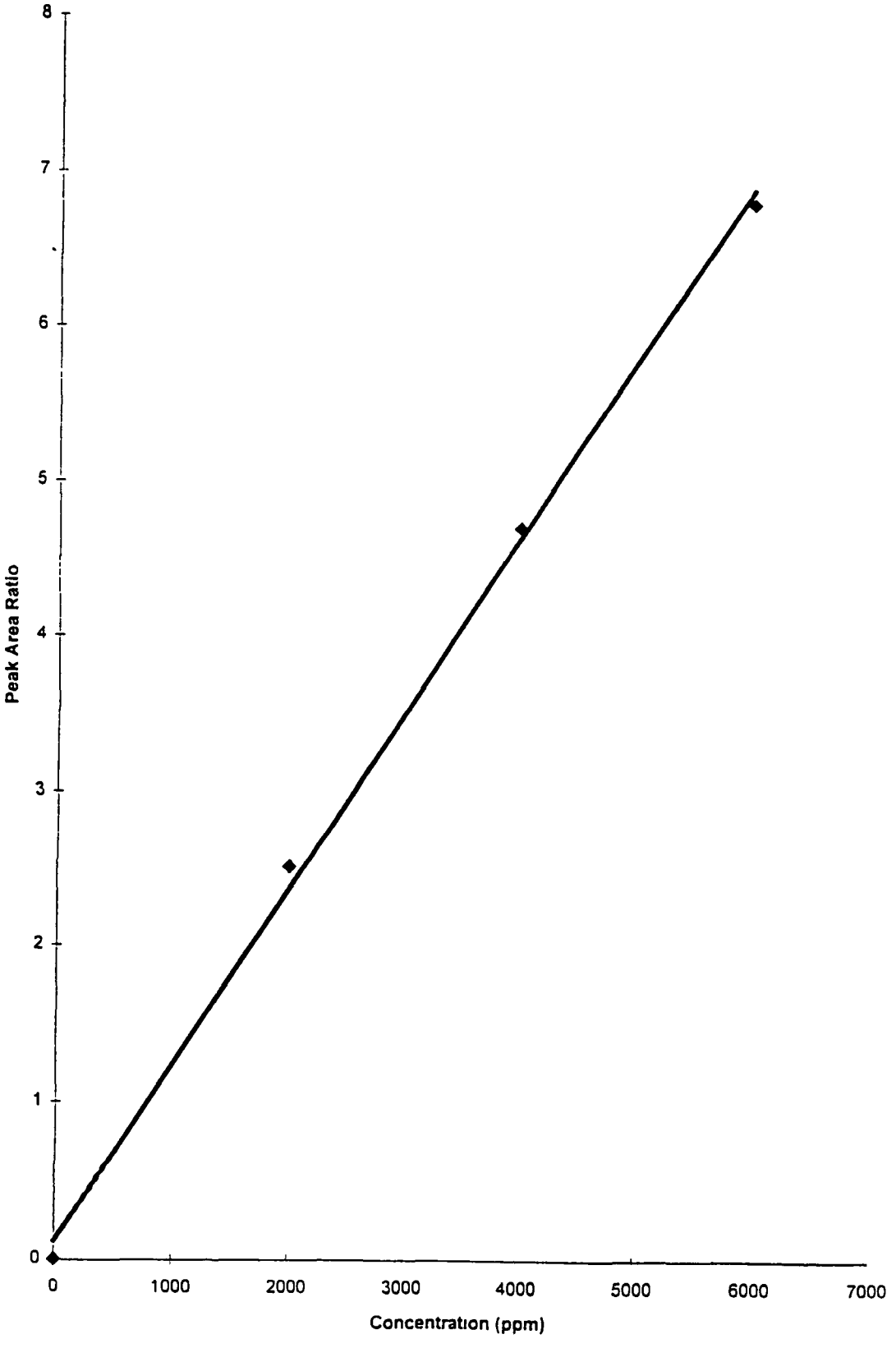


Figure.5: Calibration Graph of Palmitic Acid Methyl Ester using HAME as Internal Standard (Chicken 3)

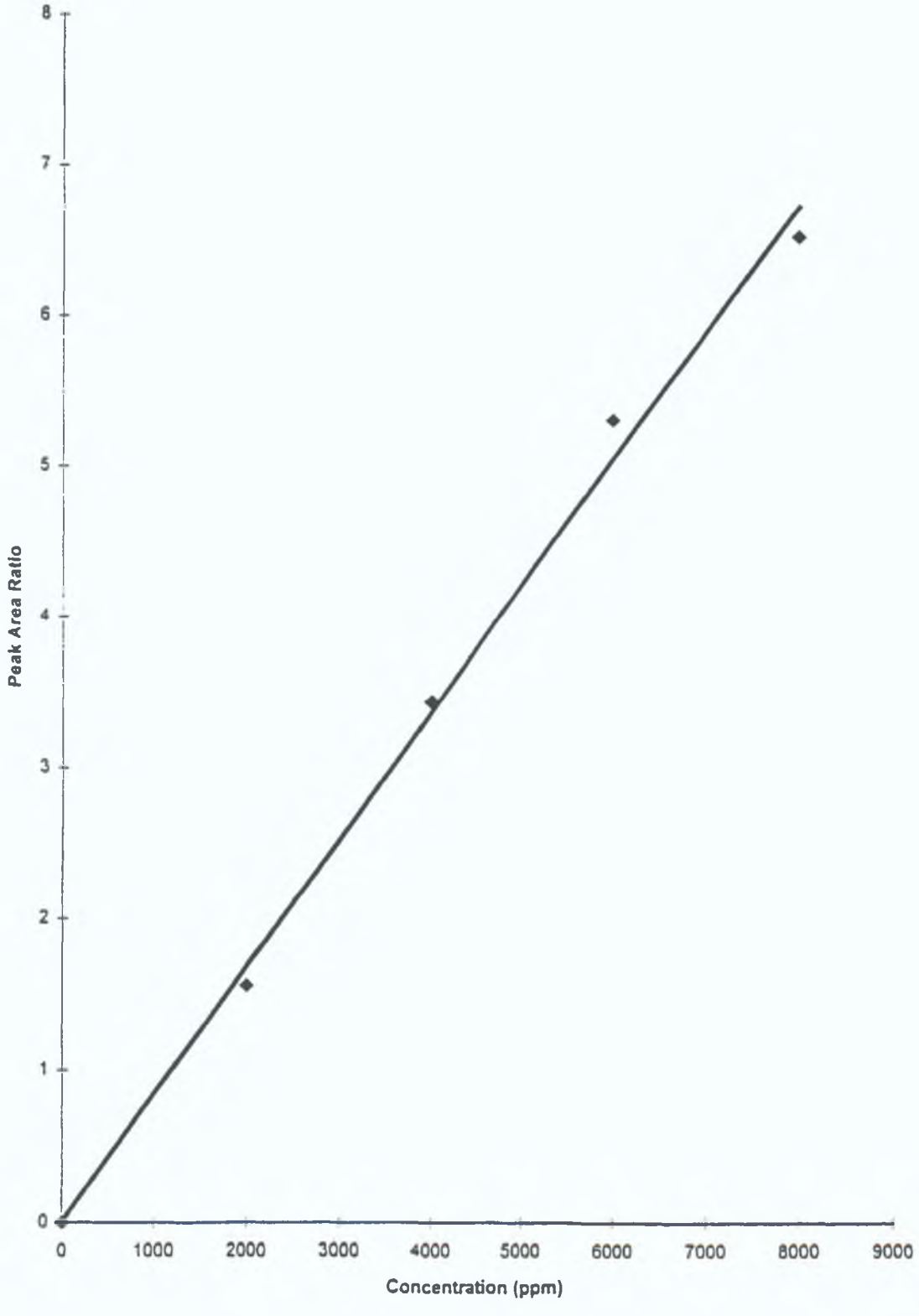


Figure6 Calibration Graph of Stearic Acid Methyl Ester using HAME as Internal Standard (Chicken 3)

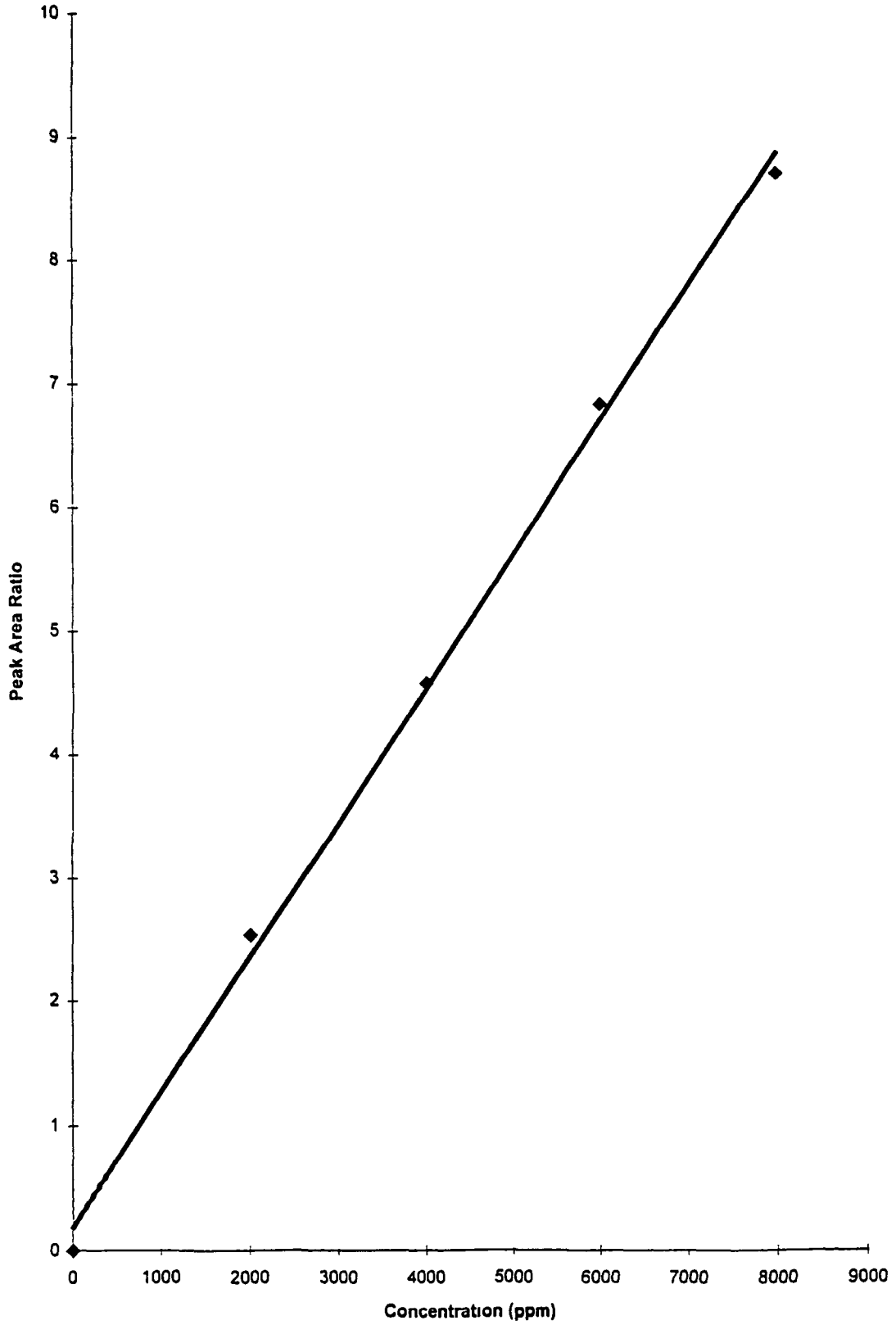


Figure 7 Calibration Graph of Oleic Acid Methyl Ester using HAME as Internal Standard (Chicken 3)

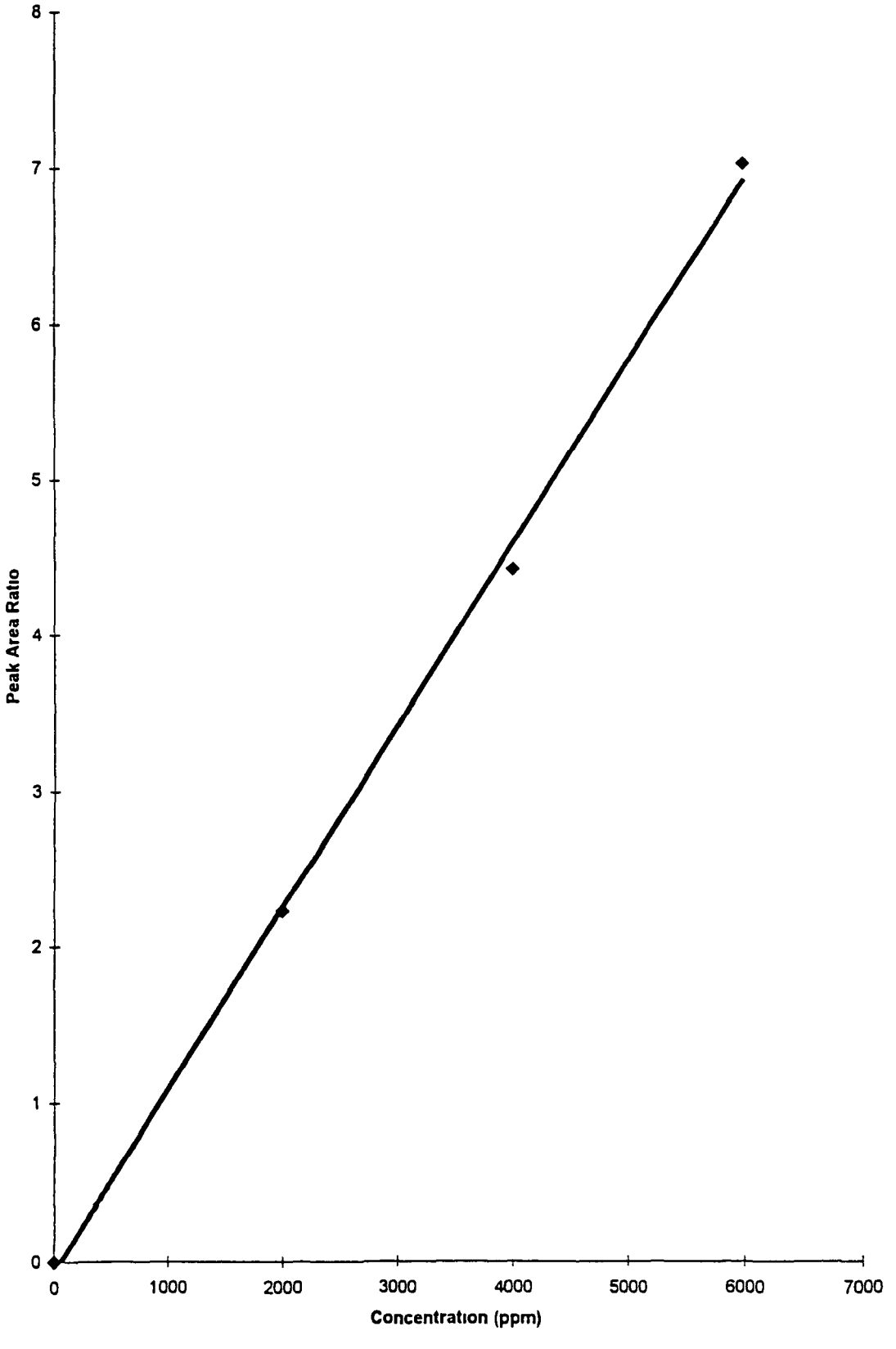


Figure 8 Calibration Graph of Linoleic Acid Methyl Ester using HAME as Internal Standard (Chicken 3)

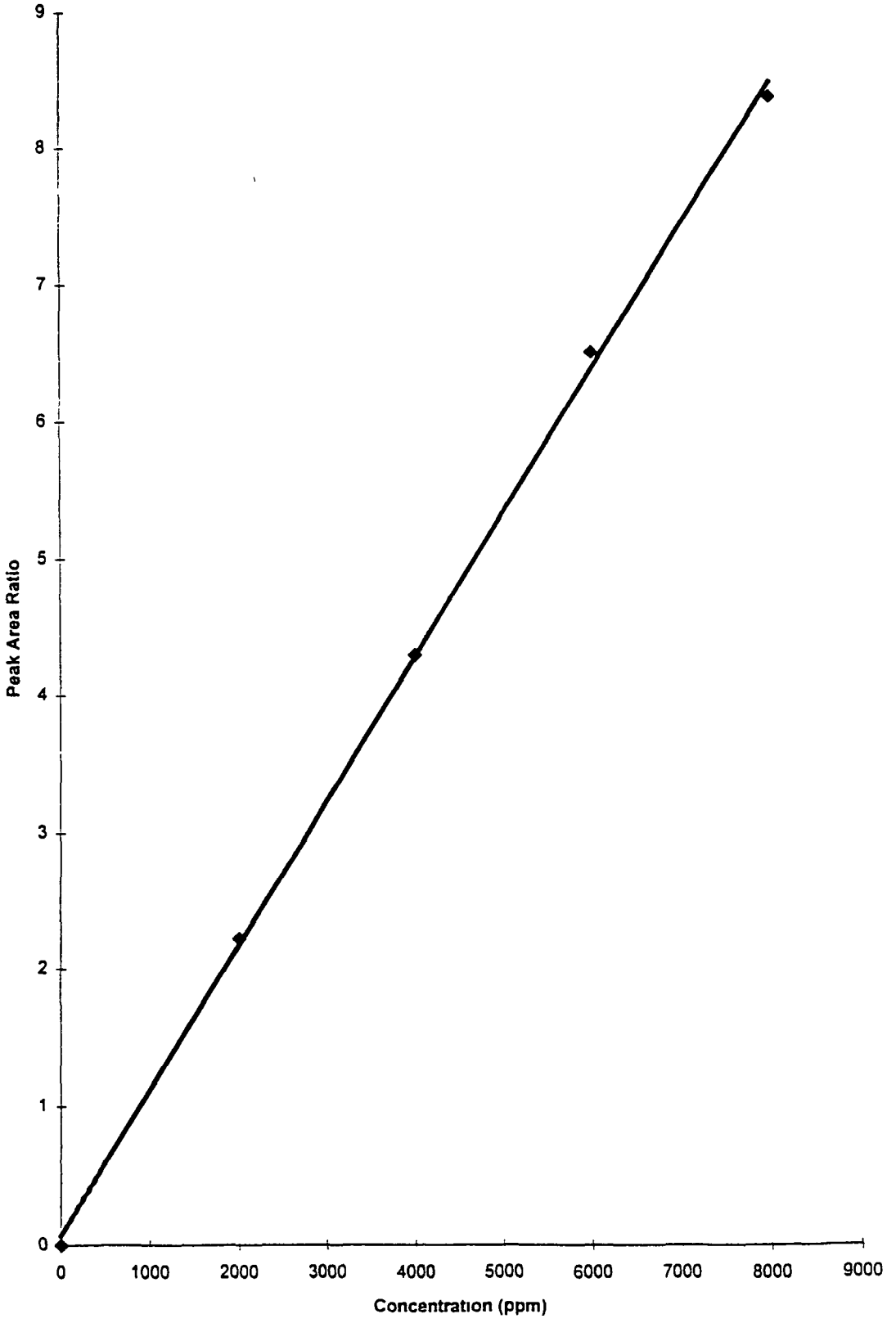
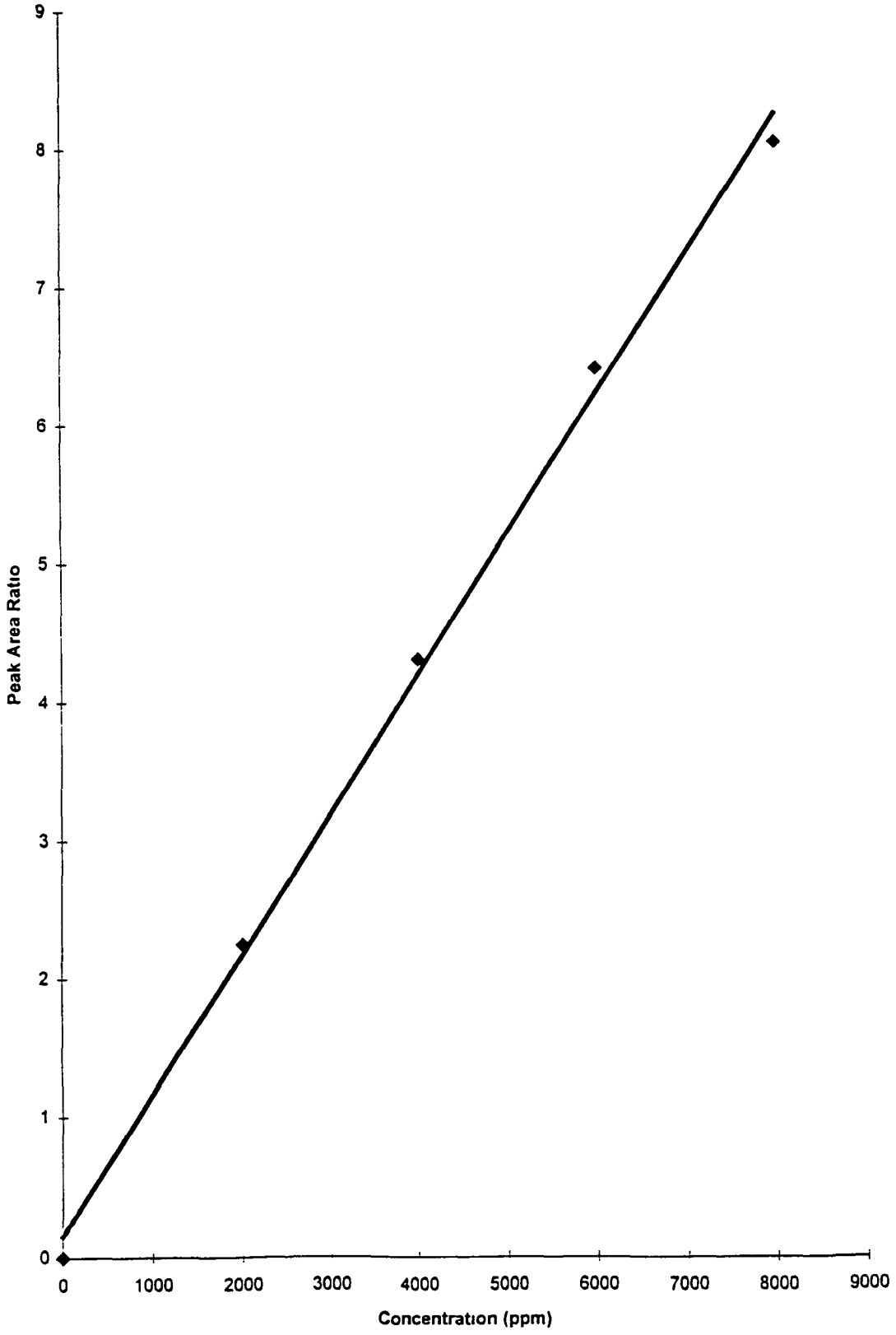


Figure 9 Calibration Graph of Linolenic Acid Methyl Ester using HAME as Internal Standard (Chicken 3)



Sample calculation of the concentration of the FAMES from the relevant calibration is given below

Examples (i) Chicken 3- Folch 1

(ii)Olives– Modified Folch 1

(i) **Chicken 3- Folch 1**

Myristic Acid

Equation of the line from calibration,

$$y = 0.001127x + 0.1175$$

Peak area ratio 0.429

0.429 = 0.001127x + 0.1175

x = 276.4 ppm

= 276.4 mg/ dm^3

= 0.829 mg/ 3 cm^3 (total sample volume)

= 0.829 mg/ 250 mg fat (weight for methylation to FAMES)

= **3.316 mg/ g fat**

= 14.06 mg/ 4.241 g fat (yield from extraction)

= 0.68 mg/ g chicken (sample weight = 20.576 g)

= **0.068 g/ 100 g chicken**

Palmitic Acid

Equation of the line from calibration,

$$y = 0.000839x + 0.0054$$

Peak area ratio = 0.913

0.913 = 0.000839x + 0.0054

x = 1081.76 mg/ dm^3

x = 10817 mg/ dm^3 (1 in 10 dilution)

= 32.453 mg/ 250 mg fat

= **129.811 mg/ g fat**

= 550 531 mg/ 4 241 g fat

= 26 76 mg/ g chicken

= **2.68 g/ 100 g chicken**

Stearic Acid

Equation of the line from calibration,

$y = 0\ 001084 + 0\ 1868$

Peak area ratio = 2 386

$2\ 386 = 0\ 001084x + 0\ 1868$

$x = 2028\ 78$ mg/ dm^3

= 6 086 mg/ 250 mg fat

= **24.345 mg/ g fat**

= 103 25 mg/ 4 241 g fat

= 5 018 mg/ g chicken

= **0.5018 g/ 100 g chicken**

Oleic Acid

Equation of the line from calibration,

$y = 0\ 001162x + (-0\ 0705)$

Peak area ratio = 2 36

$2\ 36 = 0\ 001162x + (-0\ 0705)$

= 2092 mg/ dm^3

$x = 20920$ mg/ dm^3 (1 in 10 dilution)

= 62 759 mg/ 250 mg fat

= **251.039 mg/ g fat**

= 51 743 mg/ g chicken

= **5.174 g/ 100 g chicken**

Linoleic Acid

Equation of the line from calibration,

$y = 0\ 001051x + 0\ 0692$

Peak area ratio = 1 0178

1 0178 = 0 001051x + 0 0692

= 902 568 mg/ dm^3

x = 9025 689 mg/ dm^3 (1 in 10 dilution)

= 27 077 mg/ 250 mg fat

= **108.308 mg/ g fat**

= 22 324 mg/ g chicken

= **2.232 g/ 100 g chicken**

Linolenic Acid

Peak area ratio = 1 152

1 152 = 0 001013x +0 1502

x = 988 94 mg/dm^3

= 2 967 mg/ 250 mg fat

= **11.867 mg/ g fat**

= 2 45 mg/ g chicken

= **0.245 g/ 100 g chicken**

(ii) **<u>Olives</u> -Modified Folch 1**

Palmitic Acid

Peak area ratio = 4 8517

4 8517 = 0 000839x + 0 0054

x = 5776 mg/ dm^3

= 17 239mg/ $3cm^3$ (total sample volume)

= 17 329 mg/ 250 mg fat (wt for methylation)

= **69.315 mg/ g fat**

= 37 083 mg/ 0 535g fat (yield from extraction)

= 17 003 mg/ g olives (sample weight =2 181g)

= **1.70 g/ 100 g olives**

Stearic Acid

Peak area ratio = 1 3365

1 3365 = 0 001084x + 0 1868

x = 1060 60 mg/ dm^3

= 3 182 mg/ 250 mg fat

= **12.727 mg/ g fat**

= 3 122 mg/ g olives

= **0.3122 g/ 100 g olives**

Oleic Acid

Peak area ratio = 3 4911

3 4911 = 0 001162x + (-0 0705)

x = 3065 06

= 30650 60 mg/ dm^3 (1 in 10 dilution)

= 91 952 mg/ 250 mg fat

= **367.807 mg/ g fat**

= 90 223 mg/ g olives

= **9.022 g/ 100 g olives**

Linoleic Acid

Peak area ratio = 9 305

9 305 = 0 001051x + 0 0692

x = 8787 53 mg/ dm^3

= 26 362 mg/ 250 mg fat

= **105.45 mg/ g fat**

= 25 867 mg/ g olives

= **2.587 g/ 100 g olives**

Linolenic Acid

Peak area ratio = 0 516

0 516 = 0 001031x + 0 1502

x = 355 346 mg/ dm^3

= 1 066 mg/ 250 mg fat

= **4.264 mg/ g fat**

= 1 046 mg/ g olives

= **0.105 g/ 100 g olives**

APPENDIX E :

GC-MS Parameters used in the Analysis of DCB and TCB

General Information

Acquisition Mode Selective Ion Monitoring

MS Information

Solvent Delay 7 minutes

EM Absolute False

EmV Offset 0 0

Resulting Voltage 1894 1

[SIM Parameters]

Group 1

Group ID Group 1

Dwell per ion 50 msec

Low Resolution Yes

Group Start Time 6 99

Plot 2 Ion 98 00

Ions in Group 98 00 112 00

[Real Time Plot Parameters]

Time Window 20 minutes

Iconize Real Time Display False

Plot 1 Type Total Ion

Scale Minimum 0

Scale Maximum 100000

Plot 2 Type single ion

Scale Minimum 0

Scale Maximum 75000

GC Temperature Information

[GC Zone Temperature]

Injector A 250°C

Injector B 250°C Off

Detector A 50°C Off

Detector B 280°C

Aux 50°C Off

[Oven Parameters]

Oven Equilibrium Time 0 50 minutes

Oven Maximum 325°C

Oven On

Cryo Off

Ambient 25°C

Cryo Blast Off

[Oven Programme]

Initial Temperature 55°C

Initial Time 1 00 minutes

Level	Rate (°C/ min)	Final Temp	Final Time(min)
1	15 00	300	5 00
2	0 00		

Next Run Time 22 33 minutes

Injector Information

Injection Source Auto

Injection Location Front

Sample Washes 0

Sample Pumps 5

Sample Volume 1 stop (s)

Viscosity Delay 0 sec

Solvent A washes (Acetonitrile) 3

Solvent B washes (Hexane) 3

On Column No

[Purge Information]

Purge A/B	Init Valve	On Time	Off Time
A	On	1 0	0 00
B	On	0 0	0 00

Timed MS Detector Entries

Time (min)	Stake (MS on/off)
14	Off

Percent Report Settings

Sort by Retention Time

Output Destination

Screen

Printer

Integration Events Events E

Generate Report Yes

Signal Correlation Window 0 020

Qualitative Report Settings

Peak Location of Unknown Apex

Library to Seach

NBS 75K

Integration Events Autointegrate

Report Type Summary

Output Printer

Generate Report during Run No

Quantitative Report Settings

Report Type Area Percent by Retention Time

Output Printer

Generate Report Yes

1 Internal Standard

Retention Time 8 14 min Extract and Integrate 7 80 to 8 30 min

Signal	Rel Resp	Pct Unc	Integration
Events			
Tgt 98 00			Events E
Lvl ID	Conc ( ) Response		
1	not used for this compound		

Qualifier Peak Analysis On

Curve Fit Linear

2 DCB

Retention Time 10 88 min Extract and Integrate from 10 58 to 11 18 min

Signal	Rel Resp	Pct Unc (rel)	Integration
Tgt 98 00			Events E
Q1 112 00	0 00	20 0	Events E
Lvl ID	Conc ( ) Resp		
1	not used for this compound		

Qualifier Peak Analysis On

Curve Fit Linear

3 TCB

Retention Time 12 30 min Extract and Integrate from 12 00 to 12 60 min

Signal	Rel Resp	Pct Unc (rel)	Integration
Tgt 98 00			Events E
Q1 112 00	0 00	20 0	Events E

Lvl ID	Conc ( ) Response
1	not used for this compound

Qualifier Peak Analysis On

Curve Fit Linear

APPENDIX F:

GC-MS RESULTS of IRRADIATED SAMPLES.

Retention time of internal standard (2-cyclohexylcyclohexanone) = 8 14 minutes

Retention time of 2-dodecylcyclobutanone = 10 88 minutes

Retention time of 2-Tetradecylcyclobutanone = 12 33 minutes

Figure 1 GC-MS of 2-Dodecylcyclobutanone [172]

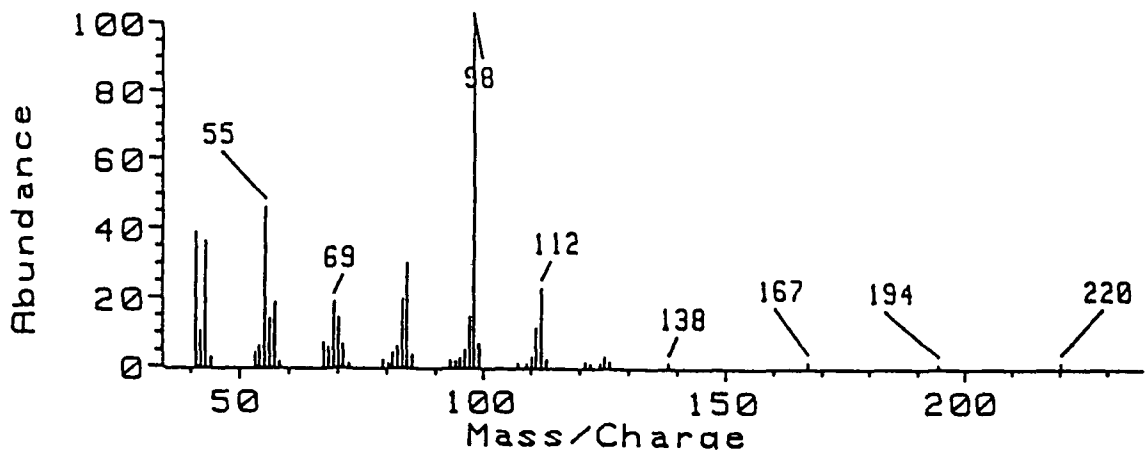


Figure 2 GC-MS of 2-Tetradecylcyclobutanone [140]

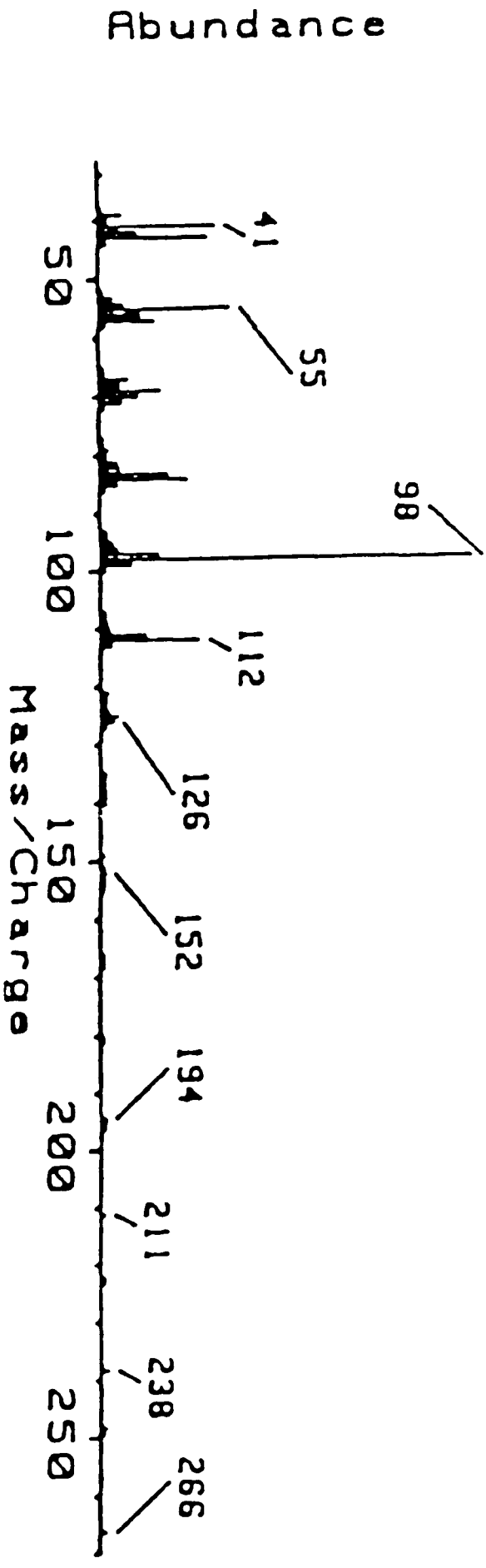


Figure 3: GC-MS of 2-cyclohexylcyclohexanone from the NBS 75K Library

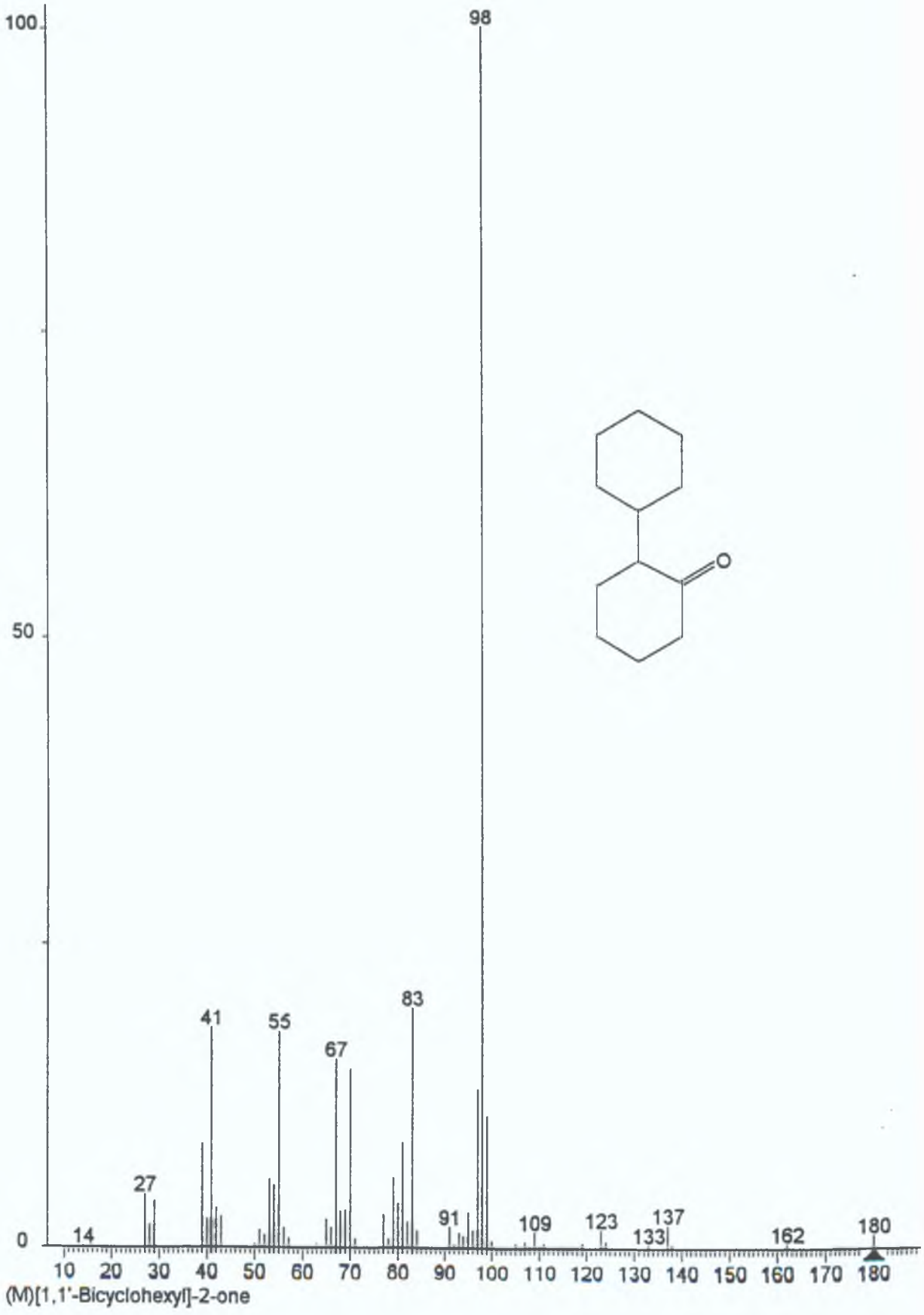
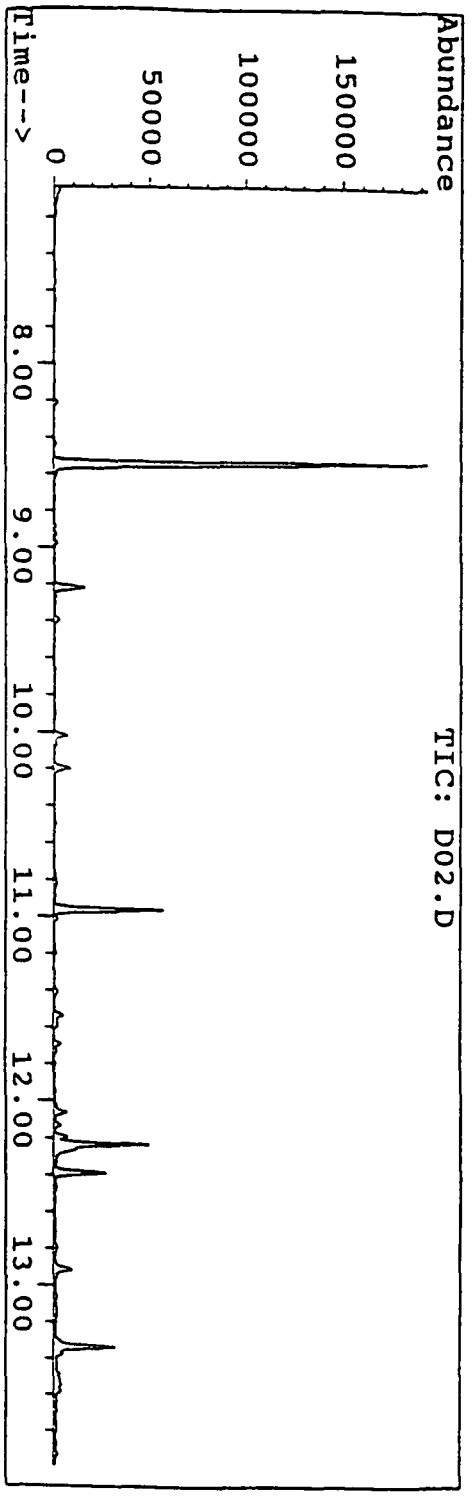
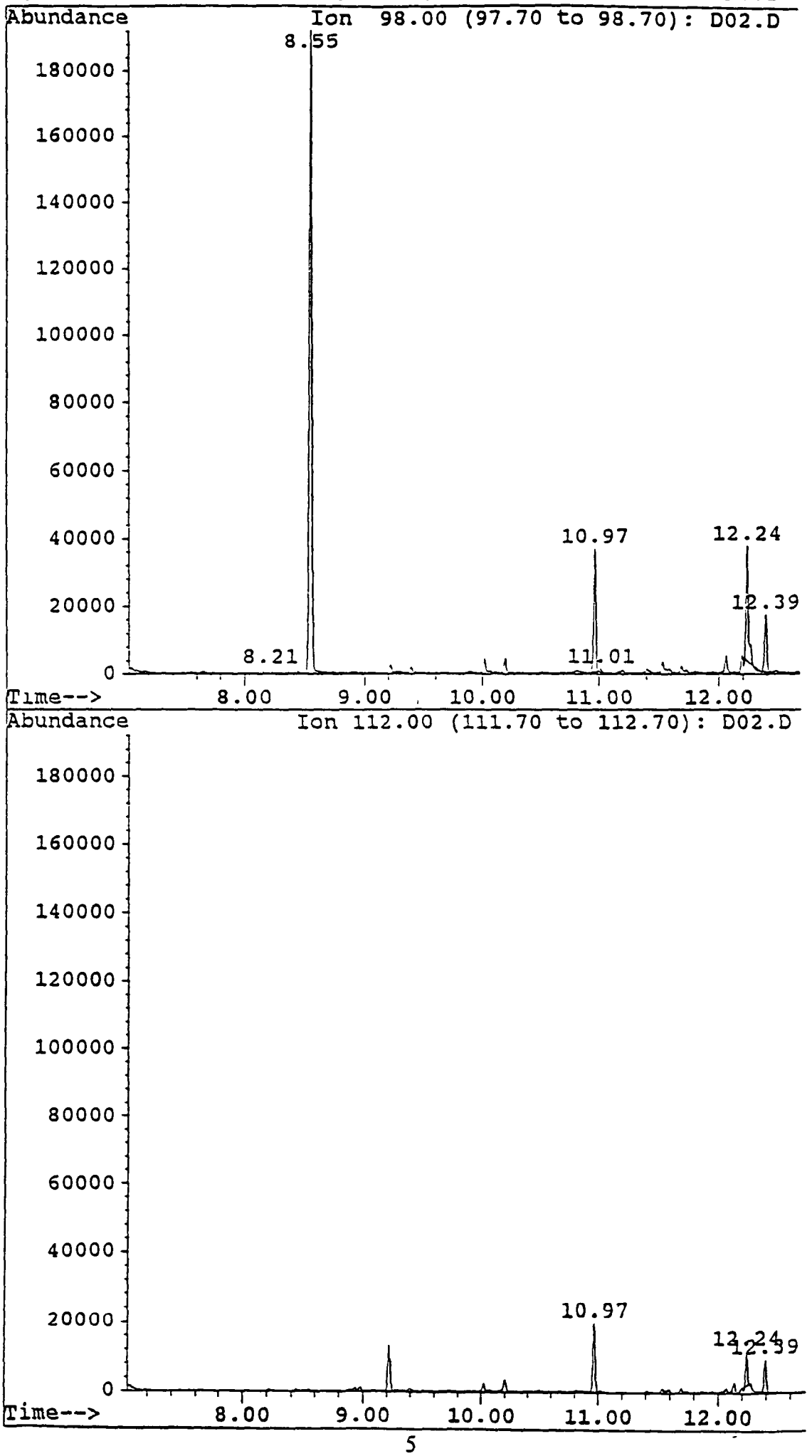


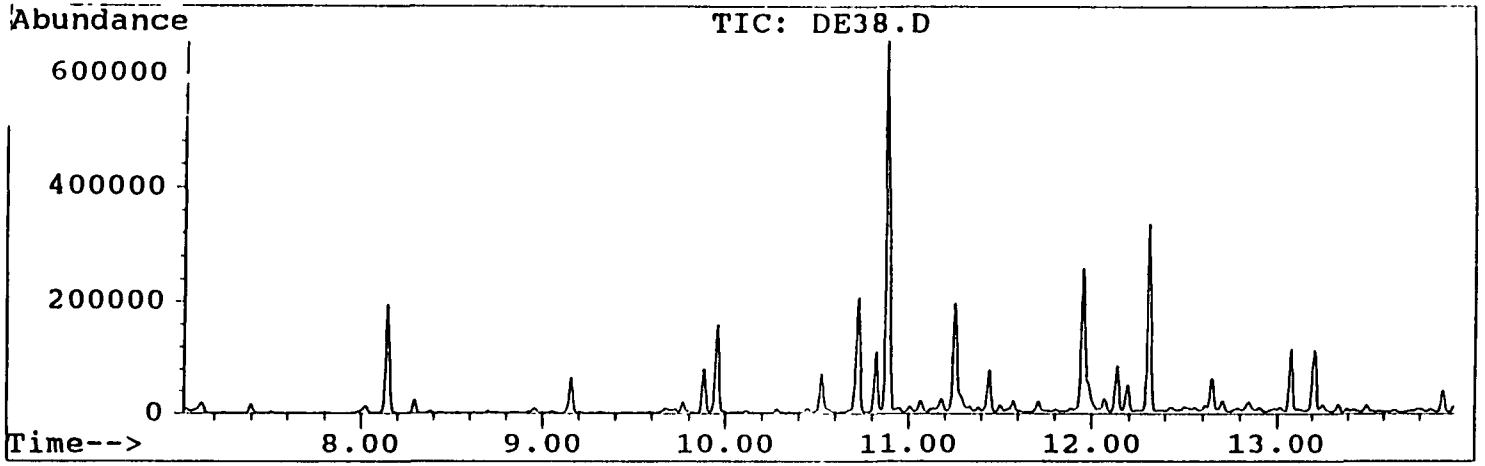
Figure 4 Total Ion Chromatogram of Sample C5 Control Chicken Leg



Ret Time	Signal Descr	Area	% Pk	%LPk
8.211	98.00 amu	15699	100.000	100.000
8.548	98.00 amu	2788165	100.000	100.000
10.965	98.00 amu	490114	64.369	100.000
10.966	112.00 amu	271297	35.631	55.354
12.236	98.00 amu	463161	82.767	100.000
12.237	112.00 amu	96435	17.233	20.821
12.390	98.00 amu	231696	65.451	100.000
12.391	112.00 amu	122305	34.549	52.787

Figure 5 Selective Ion Monitoring of Sample C5 at Ions m/z 98 and m/z 112

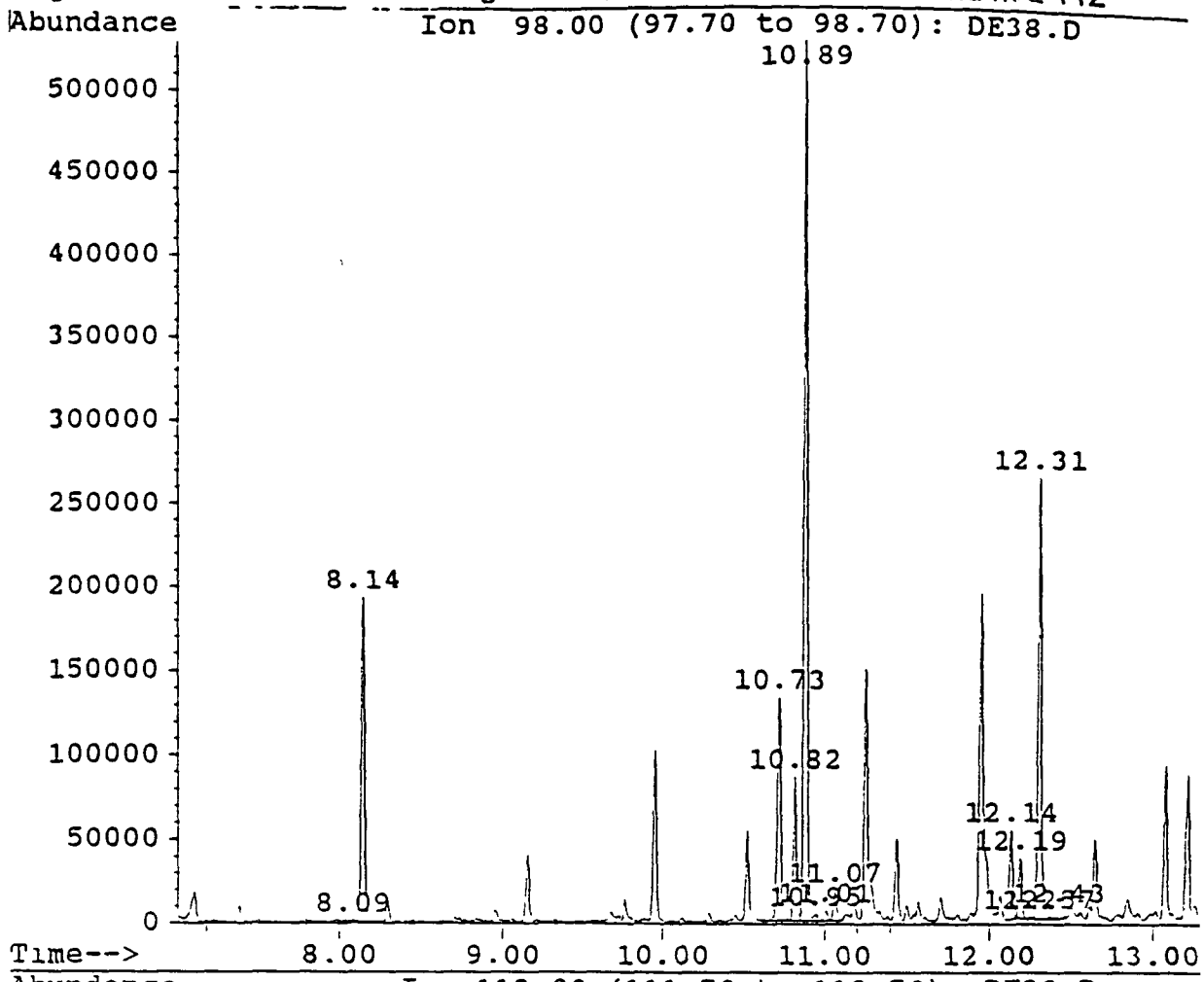


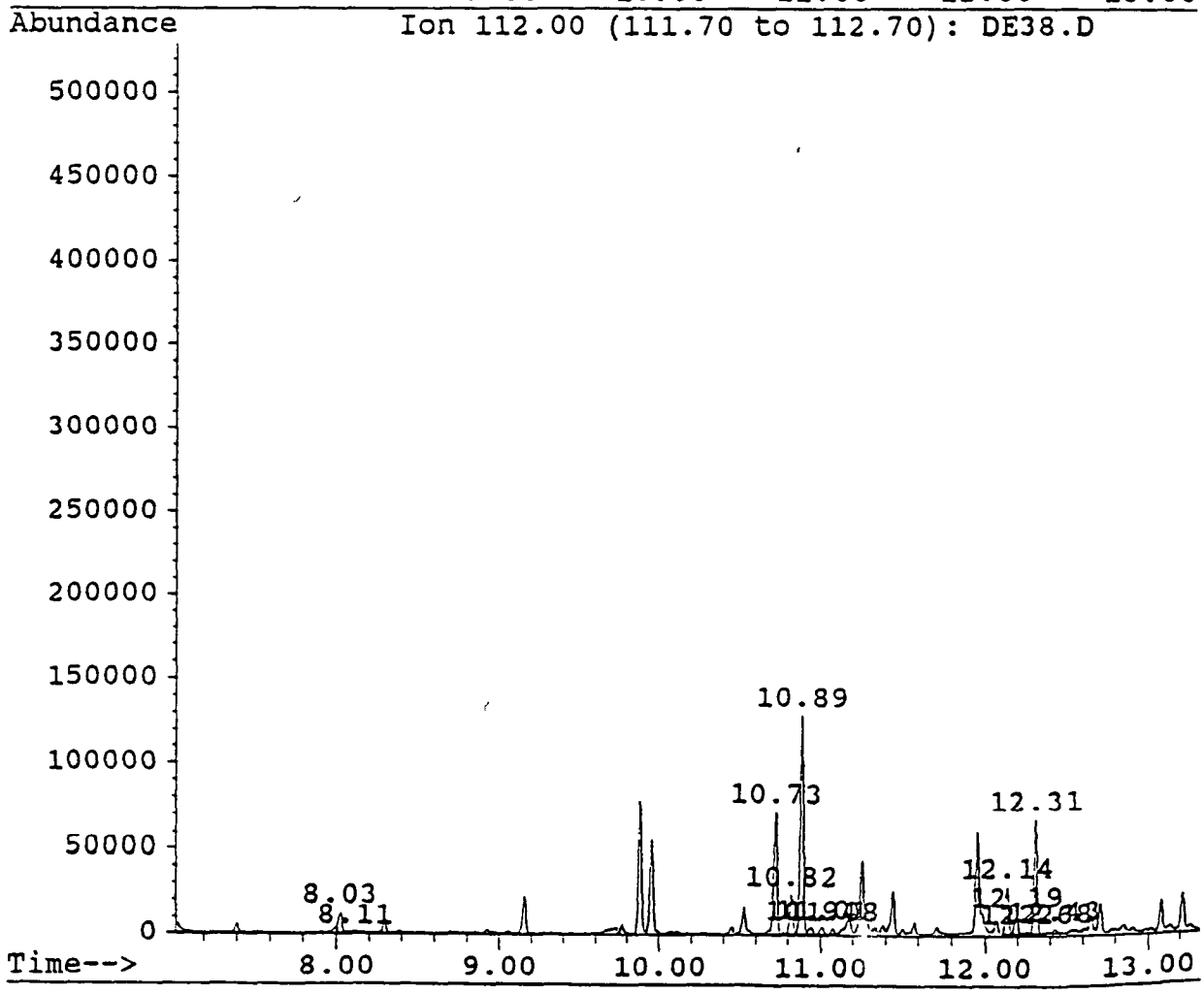


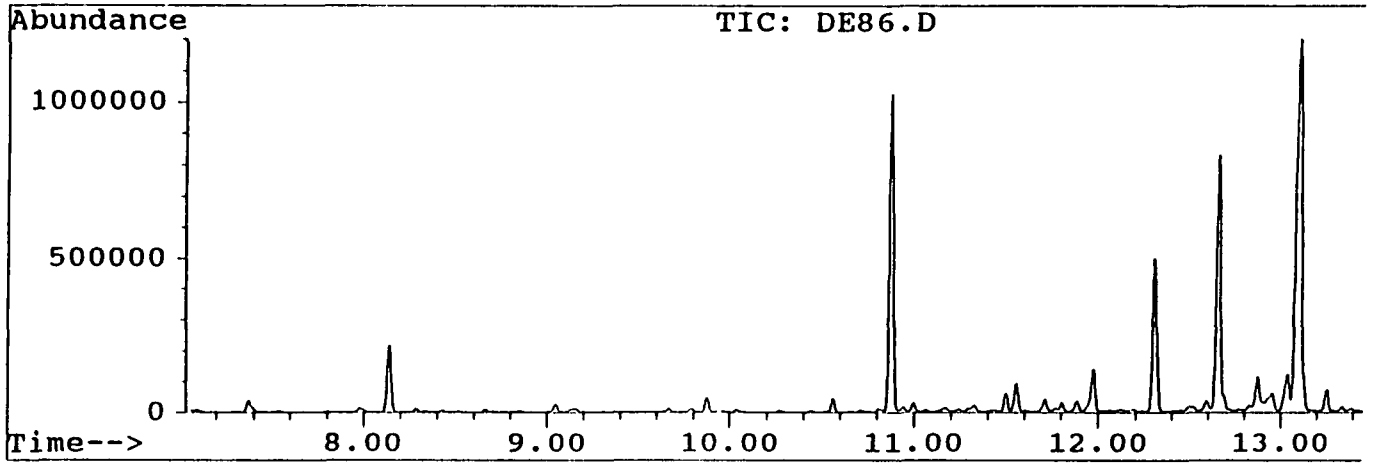
Ret Time	Signal Descr	Area	% Pk	%LPk
8.086	98.00 amu	16664	100.000	100.000
8.145	98.00 amu	2697916	100.000	100.000
10.886	98.00 amu	7134865	80.421	100.000
10.888	112.00 amu	1736979	19.579	24.345
10.944	112.00 amu	78948	54.389	100.000
10.947	98.00 amu	66205	45.611	83.860
12.310	98.00 amu	3472312	79.845	100.000
12.311	112.00 amu	876487	20.155	25.242

Figure 6 Total Ion Chromatogram of C26 Spiked Chicken Breast

Figure 7 Selective Ion Monitoring of Sample C26 at Ions m/z 98 and m/z 112



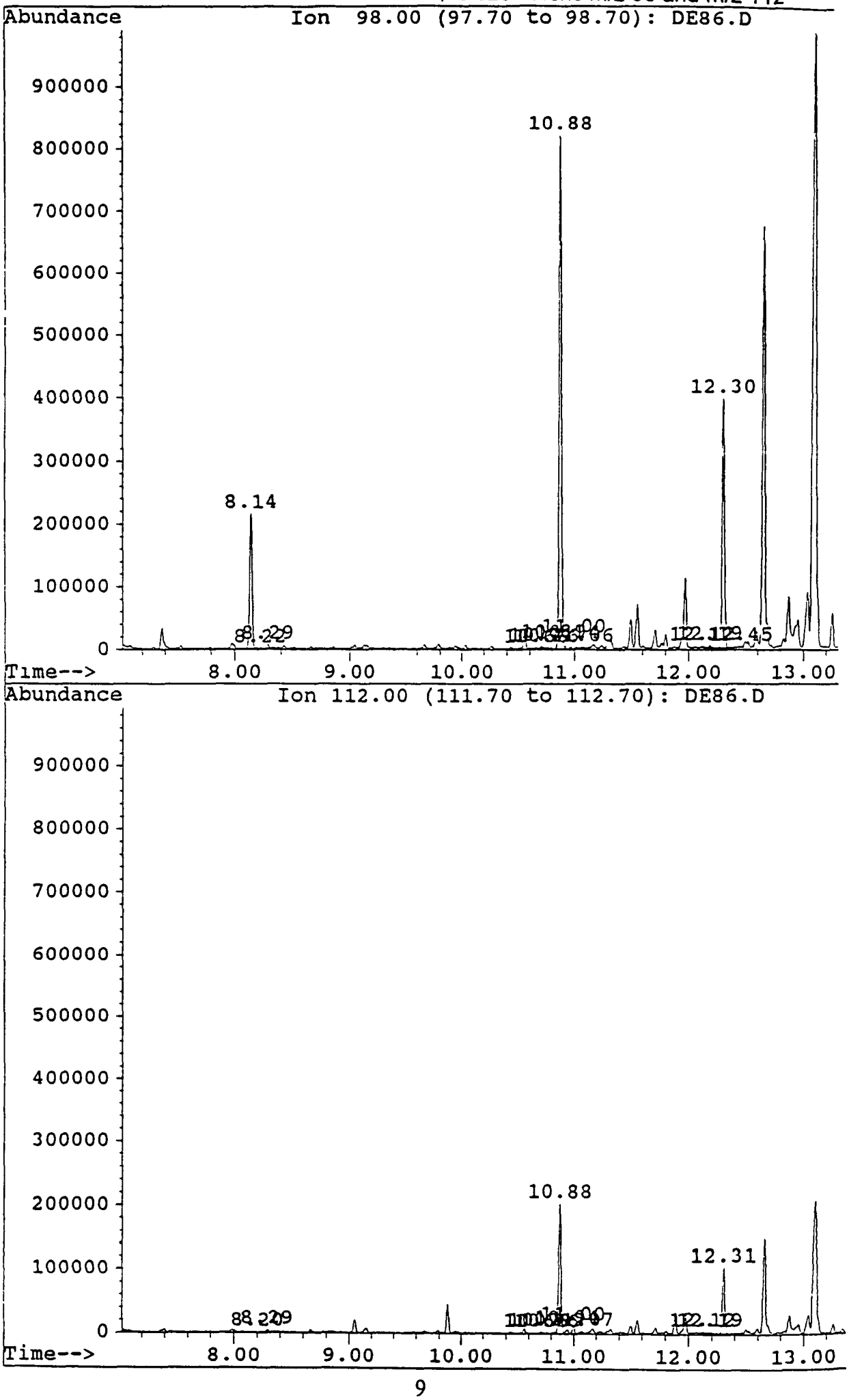


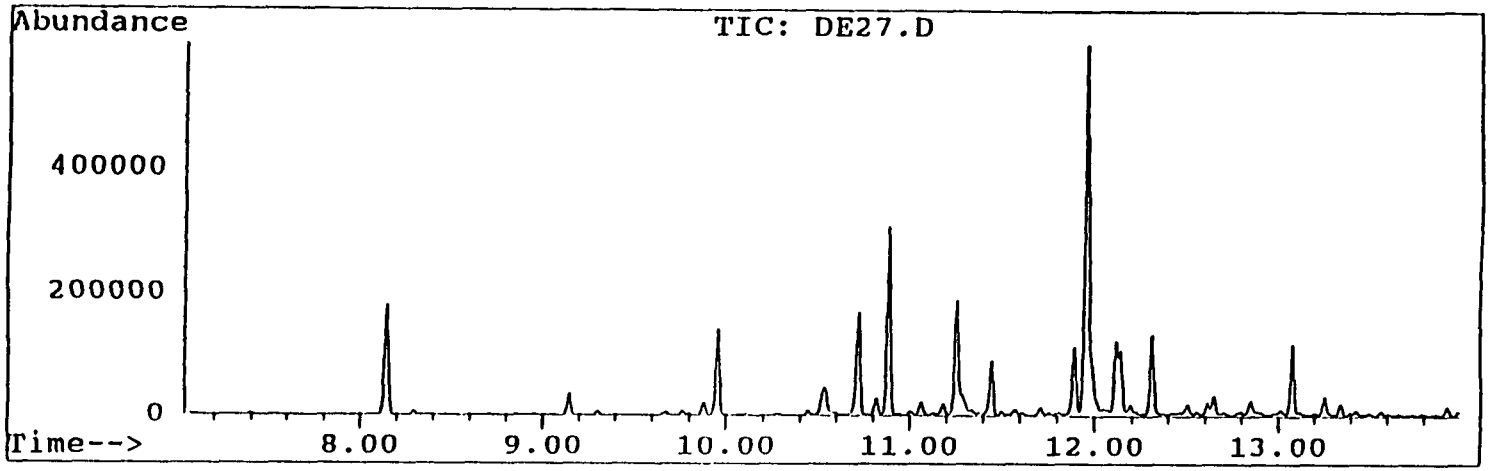


Ret Time	Signal Descr	Area	% Pk	%LPk
8.140	98.00 amu	3211518	100.000	100.000
10.877	98.00 amu	11663446	80.298	100.000
10.878	112.00 amu	2861665	19.702	24.535
12.304	98.00 amu	5783604	79.622	100.000
12.305	112.00 amu	1480259	20.378	25.594
12.451	98.00 amu	25225	100.000	100.000

Figure 8 Total Ion Chromatogram of Sample 026 Spiked Olives

Figure 9 Selective Ion Monitoring of Sample 026 at Ions m/z 98 and m/z 112

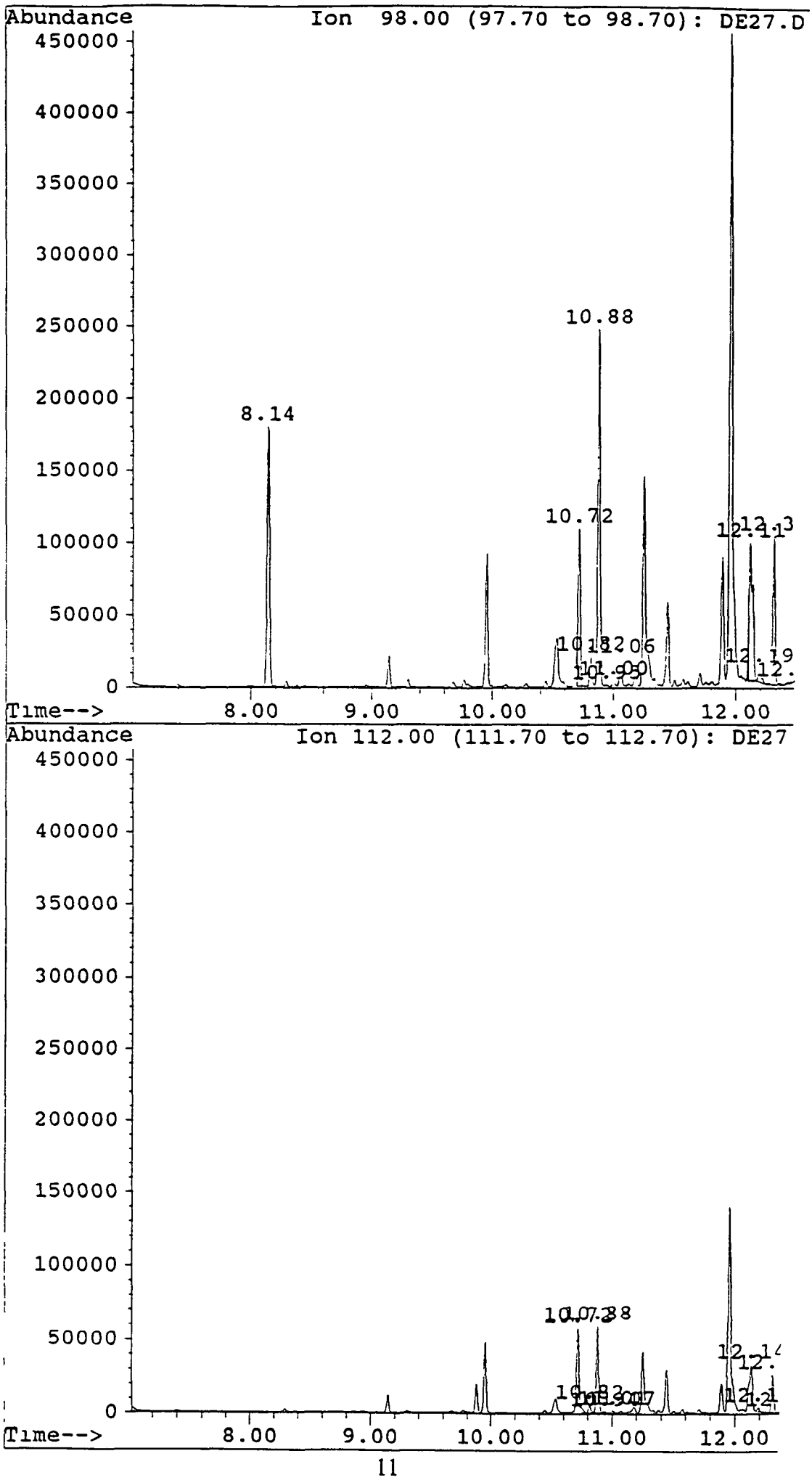




Ret Time	Signal Descr	Area	% Pk	%LPk
8.143	98.00 amu	2684693	100.000	100.000
10.882	98.00 amu	3193442	78.224	100.000
10.883	112.00 amu	888972	21.776	27.837
10.942	112.00 amu	188792	91.552	100.000
10.946	98.00 amu	17422	8.448	9.228
12.308	98.00 amu	1463671	80.581	100.000
12.309	112.00 amu	352736	19.419	24.099
12.371	112.00 amu	10350	100.000	100.000

Figure 10 Total Ion Chromatogram of Sample C24 Irradiated Chicken Breast

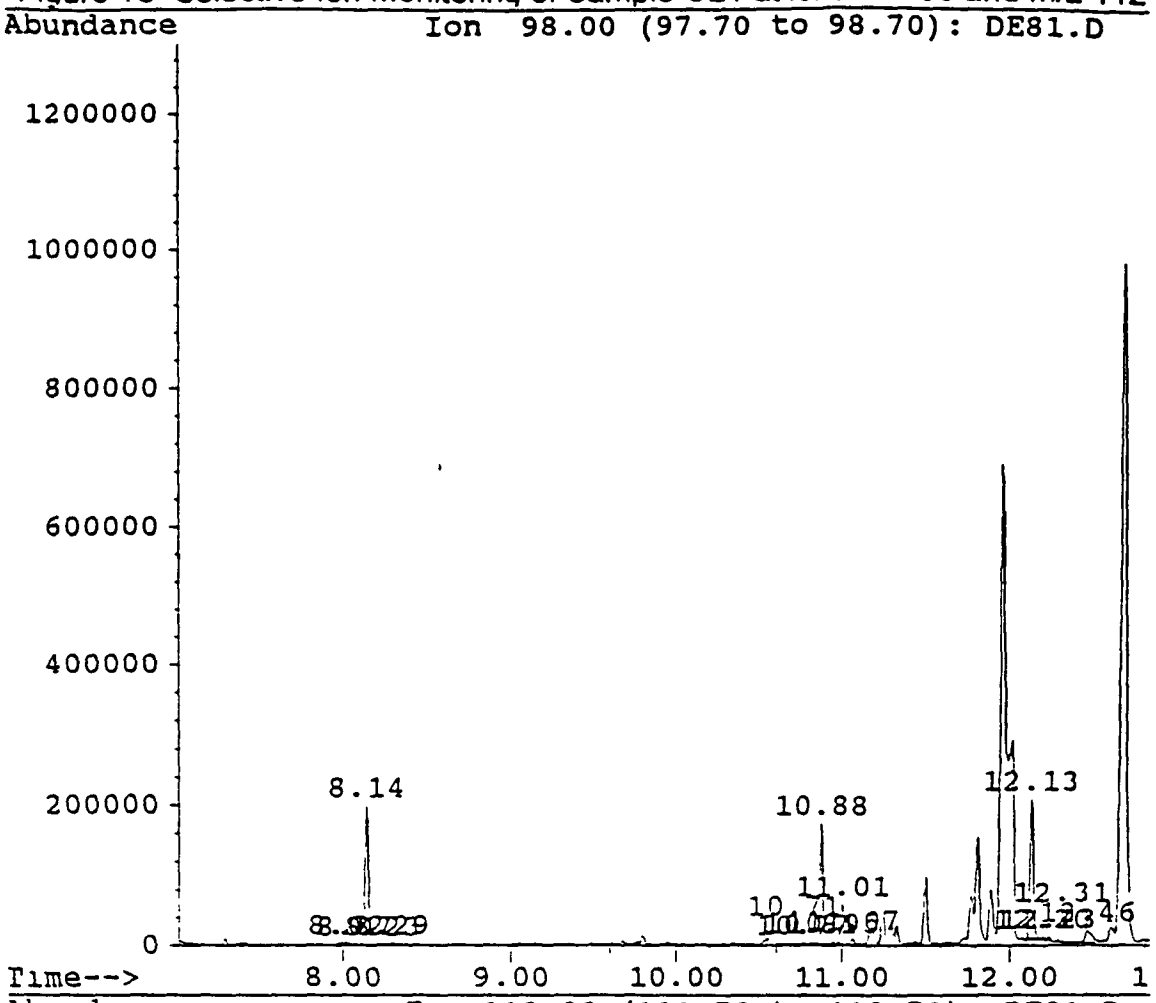
Figure 11 Selective Ion Monitoring of Sample C24 at ions m/z 98 and m/z 112

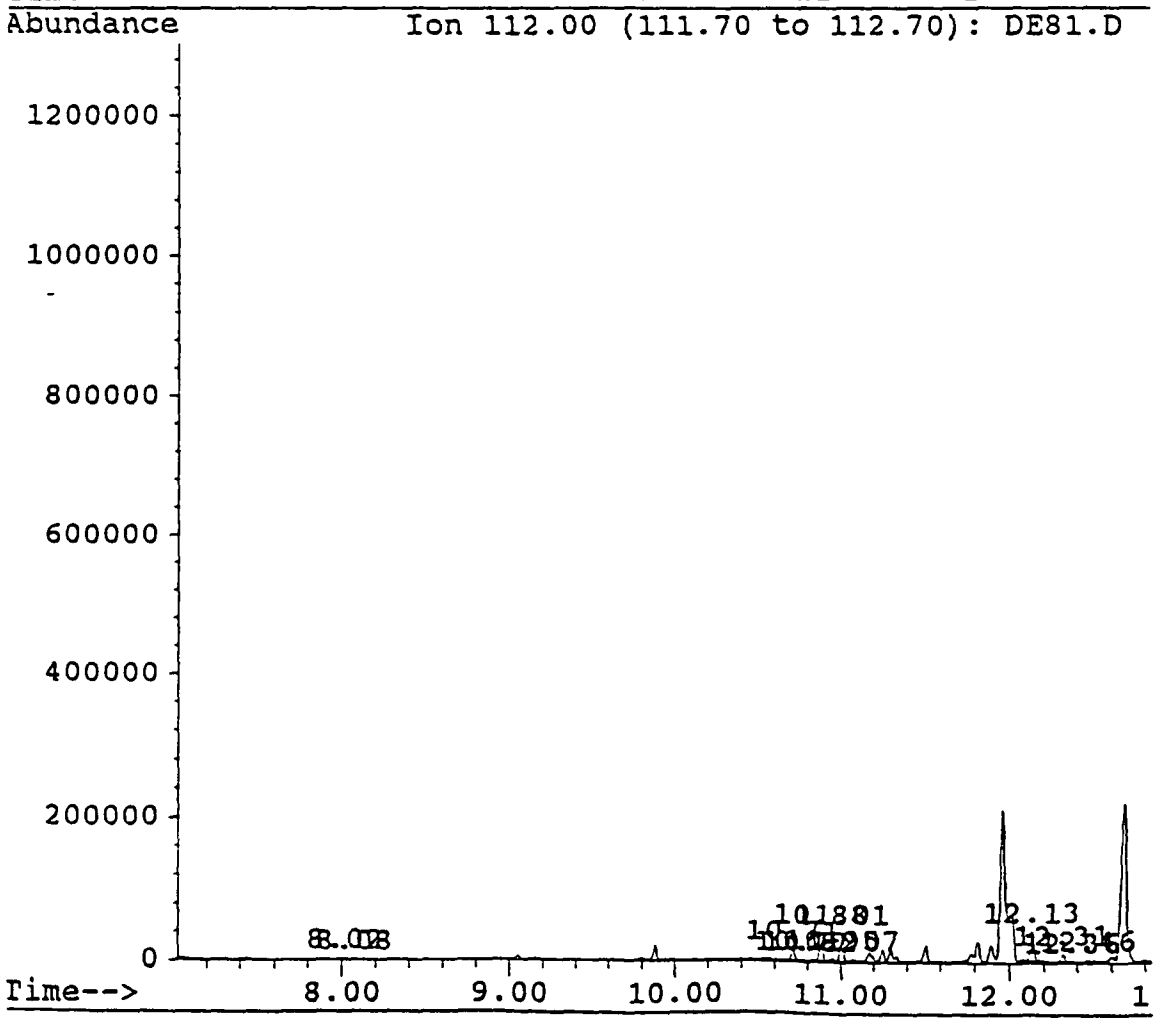


Ret Time	Signal Descr	Area	% Pk	%LPk
8.141	98.00 amu	2843860	100.000	100.000
10.713	98.00 amu	414181	62.713	100.000
10.714	112.00 amu	246259	37.287	59.457
10.880	98.00 amu	2265759	80.855	100.000
10.881	112.00 amu	536480	19.145	23.678
10.948	98.00 amu	68892	49.236	96.988
10.949	112.00 amu	71031	50.764	100.000
12.312	98.00 amu	777395	80.099	100.000
12.313	112.00 amu	193154	19.901	24.846

Figure 12 Total Ion Chromatogram of Sample O21 Irradiated Olives

Figure 13: Selective Ion Monitoring of Sample O21 at ions m/z 98 and m/z 112





APPENDIX G:

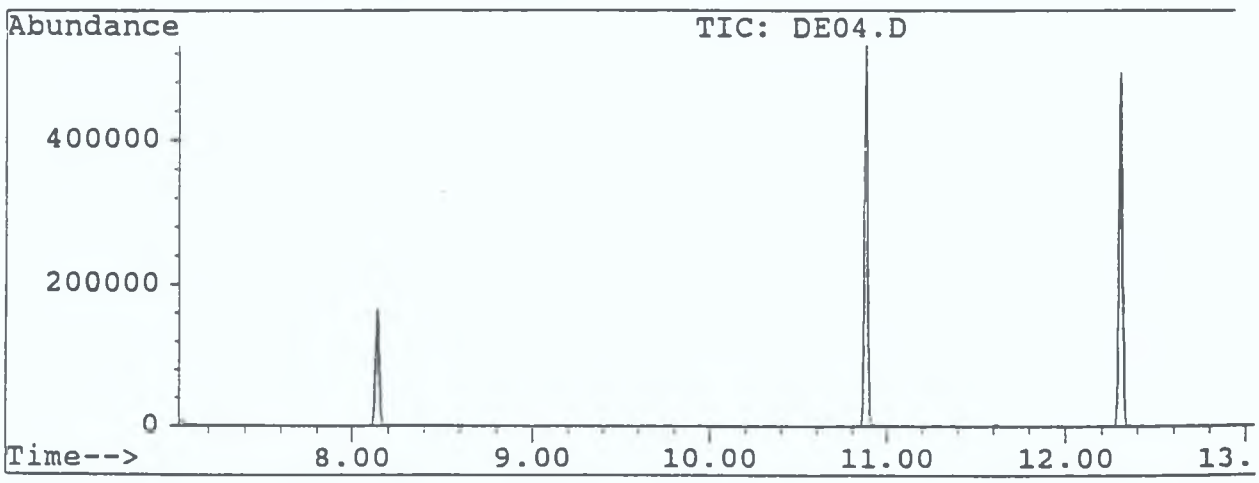
Calibration of 2-DCB and 2-TCB using the Internal Standard Method

During the analysis of 2-DCB and 2-TCB in chicken and olives a series of standards of each cyclic ketone were run after every ten samples for calibration purposes. From these the response factor of each ketone for chicken and olives

was determined.

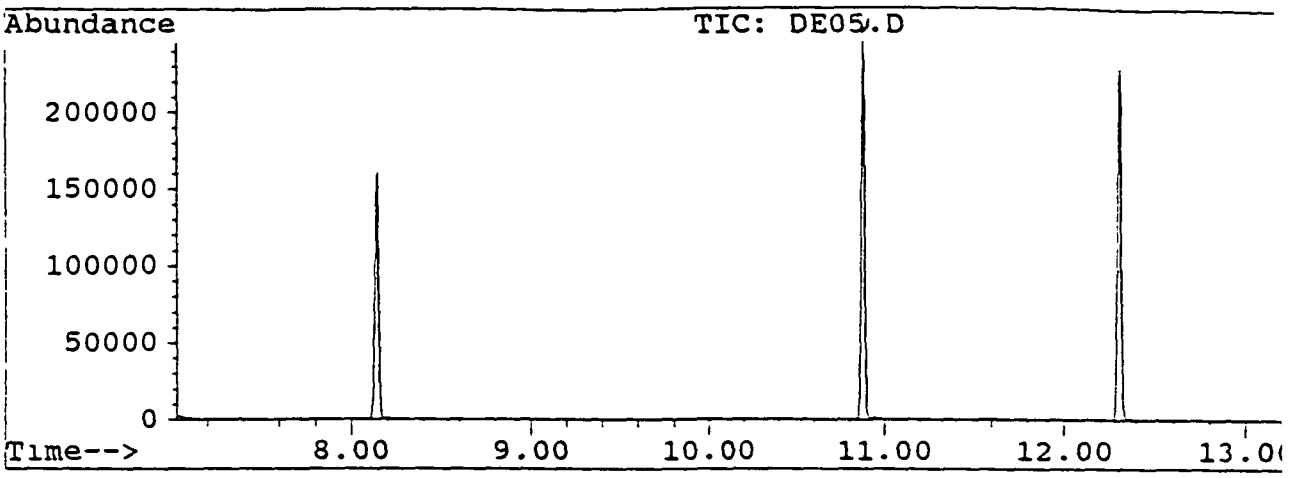
Figures 1-4 give an example of a series of standards (0.5-2.0ppm) that were run for calibration of 2-DCB and 2-TCB.

Figure 1: Total Ion Chromatogram of 2ppm DCB and 2ppm TCB standard.



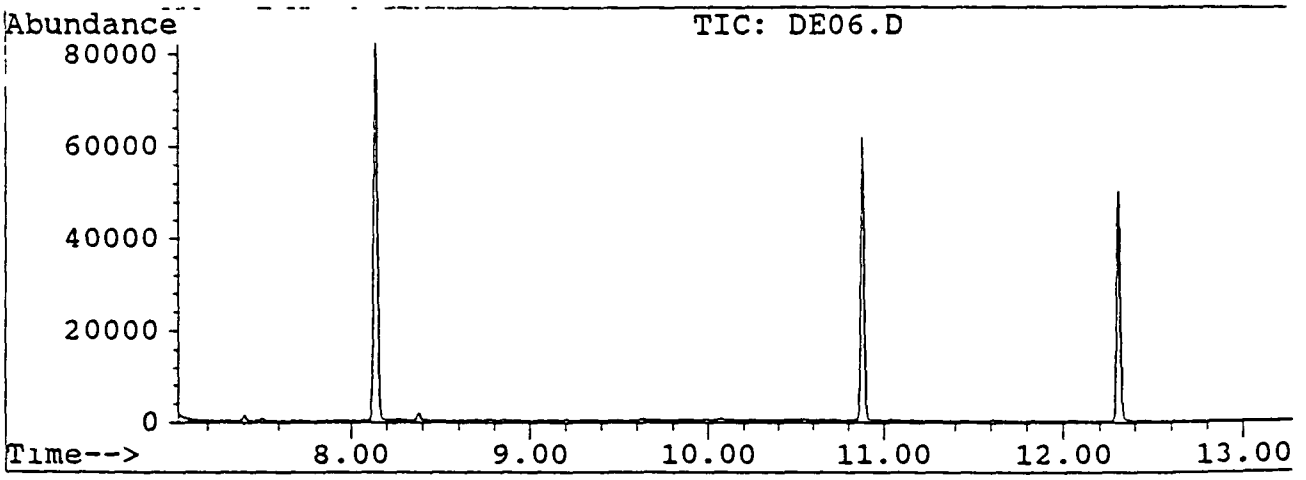
Ret Time	Signal Descr	Area	% Pk	%LPk
8.141	98.00 amu	2374303	100.000	100.000
10.879	98.00 amu	5776041	80.909	100.000
10.881	112.00 amu	1362852	19.091	23.595
12.308	98.00 amu	5524878	80.092	100.000
12.309	112.00 amu	1373278	19.908	24.856

Figure 2 Total Ion Chromatogram of 1ppm DCB and 1ppm TCB standard



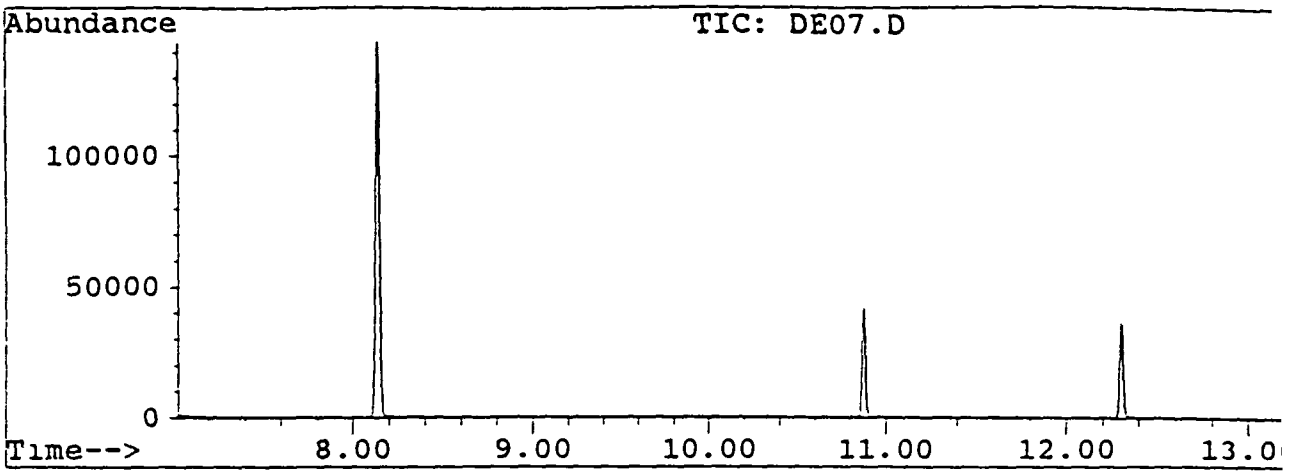
Ret Time	Signal Descr	Area	% Pk	%LPk
8.143	98.00 amu	2241693	100.000	100.000
10.878	98.00 amu	2645496	81.131	100.000
10.880	112.00 amu	615288	18.869	23.258
12.306	98.00 amu	2480036	80.257	100.000
12.307	112.00 amu	610068	19.743	24.599

Figure 3 Total Ion Chromatogram of 0 5ppm DCB and 0 5ppm TCB standard



Ret Time	Signal Descr	Area	% Pk	%LPk
8.141	98.00 amu	1202151	100.000	100.000
10.880	98.00 amu	633738	81.246	100.000
10.881	112.00 amu	146286	18.754	23.083
12.308	98.00 amu	547171	80.222	100.000
12.309	112.00 amu	134897	19.778	24.654

Figure 4 Total Ion Chromatogram of 0 2ppm DCB and 0 2ppm TCB standard



Ret Time	Signal Descr	Area	% Pk	%LPk
8.141	98.00 amu	2152460	100.000	100.000
10.877	98.00 amu	435626	81.093	100.000
10.878	112.00 amu	101567	18.907	23.315
12.305	98.00 amu	396050	80.182	100.000
12.306	112.00 amu	97890	19.818	24.716

Calculation

Standards either side of the samples being measured were treated as follows

Peak Area = area of ion m/z 98

$$\frac{\text{peak area of 2- Dodecylcyclobutanone}}{\text{peak area of internal standard}} - \text{concentration of 2-dodecyl-cyclobutanone (ppm)} = \text{ratio (r)}$$

$$\frac{\text{peak area of 2- Tetradecylcyclobutanone}}{\text{peak area of internal standard}} - \text{concentration of 2-tetradecyl-cyclobutanone (ppm)} = \text{ratio (r)}$$

These ratios so produced are averaged to give r_{av}

Sample r_{av}

Chicken r_{av} DCB = 1 13 Chicken r_{av} TCB = 1 09

Olives r_{av} TCB = 1 19 Olives r_{av} TCB = 1 15

Spike r_{av}

Chicken rav DCB = 1 13 Chicken rav TCB = 1 06

Olives rav DCB = 1 22 Olives rav TCB = 1 19

Samples are treated as follows

x sample = peak area of ion m/z 98 corresponding to 2-dcb (or 2-tcb) in sample

y sample = peak area of ion m/z 98 corresponding to internal standard in sample

[x sample] = concentration of 2-dcb (or 2-tcb) in sample

$$\frac{\text{x sample}}{\text{y sample}} - r_{av} = [\text{x sample}]\ (\mu g/\ 200\mu l)$$

$$\frac{[\text{x sample}]}{5} = [\text{x sample}]\ (\mu g/\ ml)$$