Report FD 11/01

FSA PROJECT FS231029 (A05021)

LITERATURE REVIEW AND PRACTICAL INVESTIGATION OF THE POTENTIAL FORMATION OF 2-ALKYLCYCLOBUTANONES IN NON-IRRADIATED FOOD

PROJECT INFORMATION

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Date:	August 2011
Project title:	Literature review and practical investigation of the potential formation of 2-alkylcyclobutanones in non-irradiated food
Sponsor:	Food Standards Agency Aviation House 125 Kingsway London WC2B 6NH
Sponsor's Project Number:	FS231029 (A05021)
Fera Project Number:	T7FY
Fera File Reference:	FLN 9000
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Distribution:	1. C. Thomas (FSA) 2. Fera Library

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Summary

Food irradiation is a processing technique that exposes food to high energy ionising radiation to improve shelf life and/or food quality. Although food irradiation is not common in the UK there is legislation in place to control its use. To support this there is a need for reliable analytical methods capable of detecting marker compounds that are specific to the irradiation process, such as the 2-alkylcyclobutanones.

A comprehensive literature review was carried out describing work in the area of identification of irradiated foods by the detection of 2-alkylcyclobutanones. The current state-of-the-art in the analytical methods was discussed and it was confirmed that to date these compounds have not been detected in food processed by non-irradiation based techniques and are still thought to be unique radiolytic products. However, one paper has been published claiming 2-alkylcyclobutanones have been found to be naturally occurring in cashew nuts and nutmeg.

Methods of analysis for 2-dodecylcyclobutanone (2-DCB), the only commercially available standard, were then developed and optimised for maximum sensitivity to allow very low irradiation doses to be detected. The gas chromatography with mass spectrometric detection (GC-MS) method used in the European standard method was re-established in our laboratory and two methods using liquid chromatography with mass spectrometric detection (LC-MS), one using time-of-flight (TOF)-MS and the second using tandem MS (MS/MS), were developed, neither of which has been previously reported for the detection of 2-DCB. The effect of various derivatisation reagents was also investigated. GC-MS without derivatisation and LC-MS/MS following derivatisation with 2,4-dinitrophenylhydrazine, were found to be the most suitable techniques as they had a similar limit of detection of approximately 1 ng/mL and both use equipment common to most food testing laboratories.

A range of fatty acids and triacylglycerols, both known to produce the 2-alkylcyclobutanones in food, were irradiated at a range of doses (0.3 – 5 kGy) by two different irradiation sources (gamma and electron beam). Of all the samples tested only those producing 2-DCB (palmitic acid, glyceryl tripalmitate and 1,3-dipalmitoyl-2-oleoylglycerol) were successfully measured, highlighting the need for authentic standards of the other ACBs to guide method development and validation.

Studies were carried out by treating samples of palmitic acid, glyceryl tripalmitate and 1,3-dipalmitoyl-2-oleoylglycerol with non-irradiation based food processing techniques. They were heated for various times at different temperatures in the presence and absence of oxygen, light, and redox active metal salts. They were also heated in a microwave oven, frozen and then defrosted in the microwave oven and heated to different temperatures in a pressure cooker. The processed fats were extracted, derivatised and analysed by LC-MS/MS. No 2-DCB was detected in any of these experiments, reaffirming the hypothesis that the 2-alkylcyclobutanones are unique radiolytic products and can be used as markers of irradiation in foodstuffs.

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Abbreviations

2-ACBs	2-Alkylcyclobutanones
ASE	Accelerated Solvent Extraction
CEN	European Committee for Standardization
DPOG	1,3-Dipalmitoyl-2-oleoylglycerol
DSE	Direct Solvent Extraction
GC-MS	Gas chromatography with mass spectrometric detection
GTO	Glyceryl trioleate
GTP	Glyceryl tripalmitate
GTS	Glyceryl tristearate
kGy	kilogray
LA	Linoleic acid
LLA	Linolenic acid
LC-MS	Liquid chromatography with mass spectrometric detection
LC-MS/MS	Liquid chromatography with tandem mass spectrometric detection
LC-TOF-MS	Liquid chromatography with time of flight mass spectrometric detection
MS	Mass spectrometry
OA	Oleic acid
PA	Palmitic acid
SA	Stearic acid
SFE	Supercritical Fluid Extraction
SPE	Solid Phase Extraction
SPME	Solid Phase Micro Extraction
TOF	Time of flight

1.0 INTRODUCTION

Food irradiation is a processing technique that exposes food to high-energy ionising radiation to improve shelf life and/or food quality. It is carried out to kill dangerous food-borne organisms such as *Salmonella*, *Campylobacter* and *Escherichia coli* (*E. coli*), to delay aging of fruit and vegetables by hindering ripening, germination and sprouting, and/or to decontaminate foods by killing invading insects. Some reports state that irradiation of foods does not change the sensory properties [1], however, other reports describe undesirable effects such as offensive odour, alterations in colour and changes in the oxidation and reduction environment of meat [2]. Most certainly this just reflects the different foods studied and the different doses given.

Although irradiation of foods is not common practice in the UK there has been an increase in using irradiation as a food processing technique elsewhere in the world. Irradiation of foodstuffs is heavily regulated around the world and in the EU is covered by European Directives 1999/2/EC and 1999/3/EC. These are implemented in England by the Food Irradiation (England) Regulation 2009, as amended by the Food Irradiation (England) (Amendment) Regulations 2010, with parallel legislation in Scotland, Wales and Northern Ireland. To support these Regulations there is a need for reliable analytical methods capable of identifying irradiated foodstuffs. One such method is the detection of marker compounds that are specifically produced by the irradiation process, such as the 2-alkylcyclobutanones (2-ACBs).

The detection of selected 2-ACBs in irradiated food is described in European Standard EN1785 [3]. This document describes a method for the determination of 2-dodecylcyclobutanone (2-DCB) and 2-tetradecylcyclobutanone (2-TCB) as marker compounds for irradiation, however other 2-ACBs may also be present depending on the fatty acid composition of the foodstuff. The method involves Soxhlet extraction, sample clean-up by adsorption chromatography and detection by gas chromatography with mass spectrometric detection (GC-MS). The method has been validated for the detection of irradiated raw chicken for doses of approximately 0.5 kGy and above. The detection of irradiated liquid whole egg, raw pork, salmon and Camembert cheese has been validated for doses of approximately 1 kGy and above. Using this method the occurrence of radiation induced 2-ACBs has been confirmed in other meat products (beef and lamb), other cheese products (Brie and cheese made from sheep's milk), seafood, fish, fruit, seed, nuts and cereals [see references in 3].

The use of 2-ACBs as markers for irradiation is only valid if these compounds do not form naturally in foodstuffs or as a consequence of any other processing techniques the foods may have undergone. Almost all foods undergo some form of processing before they are ready to eat. The simplest techniques include peeling, chopping, slicing and mincing. Foods may be preserved by processes of drying, smoking or curing. Cooking is also a food processing technique and pre-cooked ready meals that only require reheating are becoming more prevalent on the market. Other commonly used processing techniques include pasteurisation and sterilisation. To date 2-ACBs have not been detected in food processed by methods other than irradiation [4-5] and therefore these substances are postulated to be 'unique radiolytic products'. However it is also possible that the methods of analysis that are currently available are not sufficiently sensitive and that 2-ACBs may be present in non-irradiation processed foods but not at detectable concentrations. Therefore more sensitive methodology is needed to demonstrate that the 2-ACBs are not present in foods processed by techniques other than irradiation. To our knowledge there is no published evidence of food processing techniques causing the formation of 2-ACBs, however, recently a paper has been published claiming that 2-ACBs have been found to occur naturally in cashew nuts and nutmeg [6].

The aims of this work were to

i) carry out a review of the literature published in the area of measuring 2-ACBs as unique markers for irradiation,

ii) compare modern analytical approaches for detection of 2-ACBs to determine the technique(s) most likely to be able to detect the lowest irradiation doses,

iii) carry out model studies to check the 2-ACBs formed by irradiation of triacylglycerols and free fatty acids and the detection limits,

iv) determine the effect of other (non-irradiation) food processing techniques on 2-ACB formation.

The overall aim was to determine the continued suitability of 2-ACBs as unique marker compounds for food irradiation.

2.0 IDENTIFICATION OF IRRADIATED FOODS BY THE DETECTION OF 2-ALKYLCYCLOBUTANONES: A LITERATURE REVIEW (WORK OBJECTIVE 01)

There is an extensive list of publications describing food irradiation and identification and analysis of radiolytic marker compounds. The following pages give the literature review carried out under Work Objective 01. The review has also been reformatted and submitted for publication in the Journal of Food Comparison and Analysis.

The aim of this review is to investigate the current literature in respect of the analytical methods available to detect the irradiation of food by detection of 2-ACBs, describe the limitations of the methods, suggest possible modifications towards improvement, and offer alternative approaches that are within the capabilities of most testing laboratories. It also considers the evidence that the irradiation products might also be present in non-irradiated food.

Information was derived from in-house knowledge, peer-reviewed scientific literature, and 'grey' literature including trade journals. Several on-line databases were searched, principally ISI Web of Science, Food Science and Technology Abstracts (FSTA IFIS Publishing), Ovid databases, Royal Society of Chemistry FOODLINE: Science (LFRA), Chemical Abstracts (CAB International), CA SEARCH(R) (American Chemical Society), SciSearch(R) (The Thomson Corp), and Analytical Abstracts (Royal Society of Chemistry).

2.1 Glossary

2-ACBs 2-alkylcyclobutanones

ASE Accelerated Solvent Extraction - A technique for the extraction of analytes from samples using solvents at elevated temperature and pressure

CEN European Committee for Standardization

DSE Direct Solvent Extraction – An ambiguous term indicating a simple solvent extraction of analytes from samples using intimate mixing

Florisil A selective magnesium-silica gel adsorbent used in preparative scale chromatography

GC-MS Gas chromatography with mass spectrometric detection

kGy Kilogray - A unit of energy absorbed from ionizing radiation where 1 Gray is equivalent to 1 joule of energy absorbed per kilogram of sample

Soxhlet A technique for the extraction of analytes from samples by repeated leaching with cold solvent replenished by a distillation/siphoning apparatus

Soxtec A commercial apparatus for extraction using Soxhlet technology but with hot solvent

SFE Supercritical Fluid Extraction - A technique for the extraction of analytes from samples using a supercritical fluid (normally carbon dioxide) as solvent

SPE Solid Phase Extraction – A clean-up technique where the sample is supported on a solid medium and separated from impurities by use of solvents of different polarities

SPME Solid Phase Micro Extraction – A sampling technique where the sample is absorbed onto a coated needle which is then transferred to GC-MS by desorption by heating

2.2 Introduction

2.2.1 Background

There is a need to detect the use of food irradiation to ensure that food is labelled correctly and allow informed consumer choice. Several methods to detect irradiation are available and for fatty foods the standardised European Committee for Standardization (CEN) method EN1785 is based on measurement of 2-alkylcyclobutanones (2-ACBs) that are produced by the irradiation of lipids. Since this detection test was first developed in the 1990s there have been advances in analytical techniques and new methods of isolating and detecting 2-ACBs are available. Only recently have there been reports of the presence of 2-ACBs in food that has not been irradiated, and this work has yet to be confirmed.

Improvements to method EN1785 are required to lower detection limits of 2-ACBs. This will aid in an investigation into whether 2-ACBs can be detected in non-irradiated food or in food processed by means other than irradiation. Improvements could also widen the scope of the method and permit detection of the use of irradiated ingredients in composite foods, where lower detection limits will be required. Conversely, lowering of the limit of detection would increase the need to confirm the absence of 2-ACBs in foods that have not been irradiated and possibly the need to derive a threshold value to distinguish irradiated from non-irradiated foods.

2.2.2 Foods of relevance

Irradiation has been used for extending the shelf life and/or to help meet microbiological quality standards of food products such as chicken, pork, beef, rabbit, seafood such as prawns, frogs legs, and herbs and spices. It is a legal requirement in many countries that foods containing an irradiated ingredient are labeled as such. The use of irradiation as a preservation procedure requires the existence of tests capable of distinguishing between irradiated foodstuff and non-irradiated foods in order to regulate international trade and to meet labeling requirements. The legal aspects of food irradiation were briefly described in 1999 (Masotti and Zonta 1999). In the UK there are seven permitted categories of foods which may be irradiated: fruit; vegetables; cereals; bulbs and tubers; dried aromatic herbs, spices and vegetable seasonings; fish and shellfish; and poultry.

2.2.3 Detection of the irradiation of food

Several approaches have been made to detecting irradiation of foods. The methods include photostimulated luminescence and thermoluminescence, based upon changes in the luminescence of non-degradable components under different conditions, Electron Spin Resonance Spectroscopy, tests of microbiological quality, detection of damaged DNA by the comet assay, and detection of 2-ACBs. Several reviews have been published of the analytical methods for detection of irradiation, varying in their depth and scope (Bogl 1989; Rosenthal 1993; Schreiber and others 1993; Glidewell and others 1993; Stevenson 1994; Uchiyama 1995; Delincee 1993; Delincee 1998; Delincee 2002; Helle and others 1996; Haire and others 1997; Raffi 1998; Chauhan and others 2009; Arvanitoyannis and others 2009a; Arvanitoyannis and others 2009b). Many general reviews of the methods of irradiation and its detection (Fielding 2007; Arvanitoyannis and others 2009b) make little mention of 2-ACBs.

2.3 Formation of 2-ACBs

Food irradiation is carried out by the use of accelerated electron beams, X-rays, or γ radiation (^{60}Co or ^{137}Cs source). The irradiation dose varies with application with relatively low doses (0.15-0.5 kGy) used as phytosanitary measures to remove insect infestations or to prevent bulbs and tubers sprouting, medium level doses (0.5 to 1.0 kGy) to slow the ripening of fruits and vegetables and higher doses (1.0 – 10 kGy) used to reduce the level of bacteria causing food borne illness, primarily in meat products. Ionising radiation induces the formation of a variety of hydrocarbons and 2-ACBs from lipids. Their formation was first reported by Le Tellier and Nawar (1972) who irradiated triacylglycerols (triglycerides) at a very high dose (60 kGy). The formation was dose related, with hydrocarbons and 2-ACBs increasing in concentration as radiation dose increased.

2-ACBs comprise a four membered ring with a ketone group at position 1 and a side chain at position 2 (see Figure 1). The 2-ACBs are formed from fatty acids within the lipid molecule. During irradiation, the acyl-oxygen bond in the triacylglycerols is cleaved and this reaction results in the formation of alkanes, n-alkenes, lactones, ketones, esters, aldehydes and 2-ACBs (Stewart 2001a). The 2-ACBs contain the same number of carbon atoms as the parent fatty acid, four of which comprise the cyclobutane ring and thus the side chain has four fewer carbon atoms than the parent acid. If the fatty

acid composition of the lipid is known, the 2-ACBs formed can be predicted: palmitic acid (C16) forms 2-dodecylcyclobutanone (2-DCB), and stearic acid (C18) forms 2-tetradecylcyclobutanone (2-TCB). Irradiation of the monounsaturated oleic acid produces 2-tetradecenylcyclobutanone (Delincee and others 2002) where the ACB side chain carries the same C=C unsaturation as the starting fatty acid. This is of importance because oleic acid is the most abundant fatty acid found in foods. It is up to two times more abundant than palmitic acid in sheep's cheese and poultry meat and up to 4 times more abundant in liquid whole eggs, avocados, and papaya pips.

Synthesised standards of monounsaturated 2-ACBs have comprised mixtures of 75% *cis* and 25% *trans* isomers, but the *trans* isomers have not been detected in irradiated foods. The monounsaturated side chain 2-ACBs are fragmented during mass spectrometry and have thus been used much less as indicators of irradiation, despite their greater abundance (Kumar A 2008).

2.3.1 Mechanistic aspects

The mechanism of formation from triacylglycerols is shown in Figure 2.1 (Stewart 2001a) and it is proposed that radiolytic cleavage occurs preferentially at five positions of the triacylglycerol molecule. Of these, cleavage at the acyl-oxy bond produced the corresponding aldehyde, 2-ACBs, diacylglycerols, and oxo-propanediol diesters (LeTellier and Nawar 1972; Meidan and others 1977; Kumar 2008). The first step in the formation of 2-ACBs is loss of an electron from the outer shell of the oxygen atom of the fatty acid carbonyl group. This radical cation then abstracts a hydrogen atom from carbon atom 4 (C4) via a conformationally-favoured 6-membered transition state. Formal shift of an electron pair between C1 and C4 allows formation of the cyclobutane ring and then acyl bond cleavage and proton transfer plus gain of an electron (or loss of a proton and then hydrogen atom transfer) leads to the 2-ACB. Formation of 2-ACBs from free fatty acids essentially follows the same mechanism (Figure 2.2).

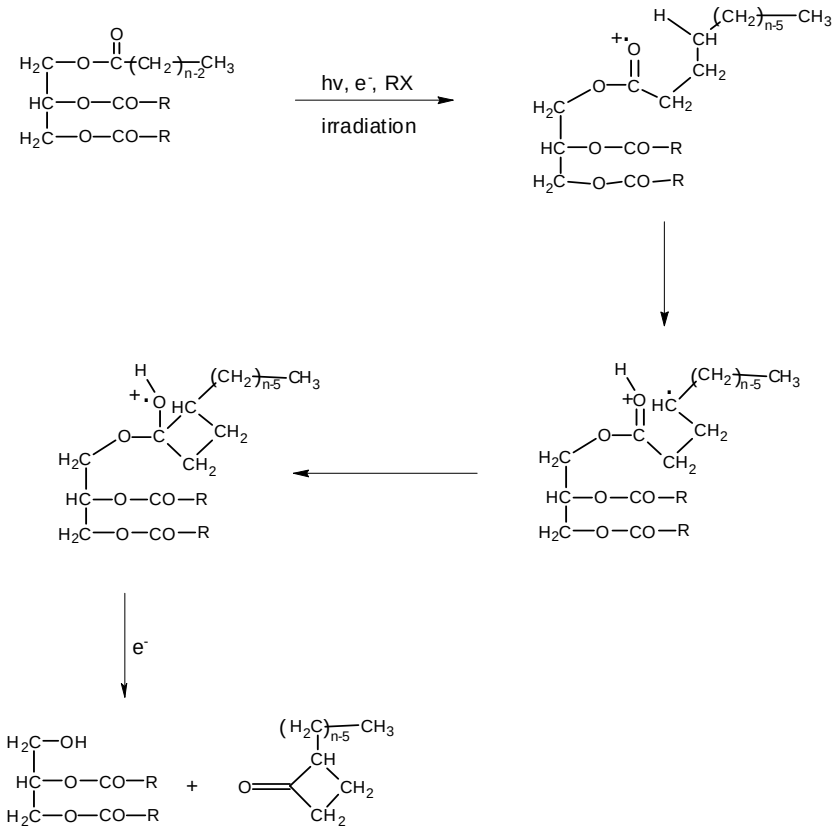

Figure 2.1. Formation of ACBs from triacylglycerols

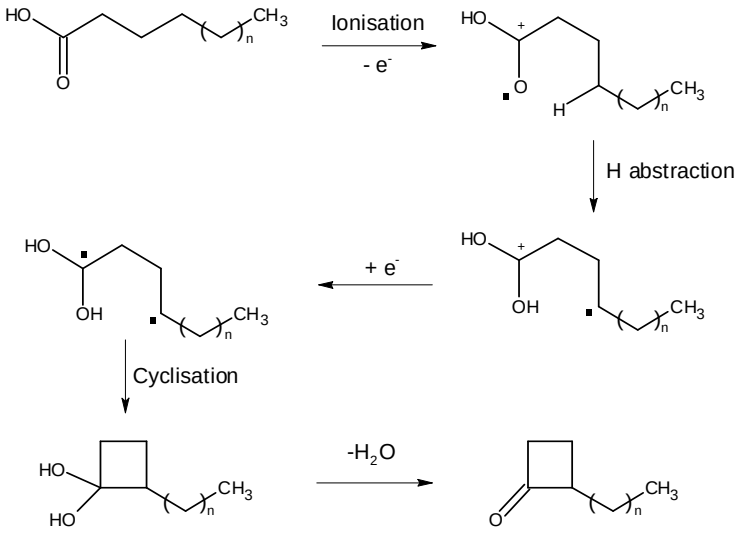

Figure 2.2. Formation of 2-ACBs from fatty acids

2.4 Occurrence of 2-ACBs

A list of the major 2-ACBs found in irradiated foods and their usual and reported acronyms is shown in Table 2.1. The use of acronyms has become more confusing as the range of 2-ACBs studied has increased. In earlier studies and in the bulk of reports published to date the major 2-ACBs, 2-dodecylcyclobutanone (C12 chain) and 2-tetradecylcyclobutanone (C14 chain), were referred to as 2-DCB and 2-TCB respectively. However, 2-decylcyclobutanone (C10 chain) has also been called 2-DCB, and 2-dodecylcyclobutanone has been called 2-dDCB. 2-Tetradecylcyclobutanone has also been called 2-tDCB, and the reporting of unsaturated side chain C14 cyclobutanones has introduced 2-tDeCB. As this report is concerned primarily with the determination of the major 2-ACBs formed, and those targeted in standardised methods, the more familiar terms will be used. 2-DCB will be used for 2-dodecylcyclobutanone from palmitic acid, 2-TCB will be used for 2-tetracylcyclobutanone from stearic acid and all other compounds will be named in full.

Table 2.1. Irradiation products, their precursors and acronyms

Fatty acid precursor	C number	Irradiation product	Acronym*
capric	C10:0	2-hexylcyclobutanone	2-HCB
lauric	C12:0	2-octylcyclobutanone	2-OCB
myristic	C14:0	2-decylcyclobutanone	(2-DCB)
palmitic	C16:0	2-dodecylcyclobutanone	2-DCB (2-dDCB)
palmitoleic	C16:1	cis-2-dodec-5'-enylcyclobutanone	(cis-2-dDeCB)
stearic	C18:0	2-tetradecylcyclobutanone	2-TCB
oleic	C18:1	cis-2-tetradec-5'-enylcyclobutanone	(cis-2-tDeCB)
linoleic	C18:2	2-tetradecyl5',8'-dienyl cyclobutanone	-
linolenic	C18:3	2-tetradecyl-5',8',11'-trienylcyclobutanone	-

* Acronyms in parenthesis are not used in this report.

The rate of 2-ACB production has often been described as increasing with the radiation dose and the temperature in a linear fashion. However Blanch and others (2009) found that the often quoted proportional relationship between irradiation dose and level of 2-ACBs formed may not hold true for lower irradiation doses, although it is possible that this may have been measurement error if in the SPME method used the fibre became saturated by the 2-ACBs present at the higher doses.

The quantity formed appears to be directly related to concentration of the precursor fatty acid, regardless of the food type. However the relative quantities formed do not always reflect the proportions of the fatty acids present. For example, mangoes contain approximately 47% oleic acid, 37% stearic acid and 7% palmitic acid. However irradiated mangoes contained approximately twice as much 2-TCB (from stearic acid) as 2-tetradec-5'-enylcyclobutanone (from oleic acid). This has also been shown to be true for frozen food. Irradiation of frozen meat, fish and chicken samples with γ-rays produced more 2-TCB (from stearic acid) than 2-DCB (from palmitic acid), and the ratios of 2-DCB to 2-TCB were less than those of palmitic acid to stearic acid (Obana and others 2007a). It was reported that the ratio of 2-DCB to 2-TCB in irradiated frozen

pork was 60% less than that in irradiated fresh pork (Stevenson 1994). 2-DCB production has been shown to be lower in irradiated frozen salmon than in irradiated chilled salmon but there was no difference in 2-TCB levels (Stewart and others 2000).

In a comparison of the effects of irradiation of beef at different temperature (room temperature, chilled on ice or frozen by dry ice) Obana and others (2007b) showed that the production of both 2-DCB and 2-TCB decreased considerably with lowering of the temperature, but that 2-DCB decreased more rapidly (Table 2). When expressed as a molar proportion of the precursor fatty acid the rates of production of 2-DCB and 2-TCB were similar at room temperature, but less 2-DCB was produced under colder conditions. The difference has been ascribed to the closer packing of triacylglycerol molecules when frozen (Stevenson 1994; Gunstone and others 1994).

Table 2.2 shows that on a fat basis the levels of 2-DCB and 2-TCB formed both decrease with temperature during irradiation and that the ratio of 2-DCB and 2-TCB also decreases. The quantities formed were related to the palmitic acid concentration but without a clear correlation with the precursor levels. The proportion of 2-DCB in irradiated pork is usually much lower than expected from the palmitic acid content. Stevenson (1994) has speculated that the position of palmitic acid and stearic acids on the glycerol backbone in pork can affect yield. Palmitic acid is usually found in the 2-position and stearic acids in the 1- and 3- positions (Gunstone *et al.* 1994) and the fatty acid in the 1- or 3- position may be more easily cleaved and cyclised (Stevenson 1994; Rahman *et al.* 1996a).

Table 2.2. Effect of temperature on 2-ACB formation in irradiated beef (5.3 kGy by gamma rays)

Temperature	mg/kg fat		ratio	% mole/mole ACB/precursor		ratio
	2-DCB	2-TCB		2-DCB	2-TCB	
Room	2.0	1.2	1.6	1.5	1.8	0.89
Chilled	0.70	0.80	0.88	0.60	1.2	0.48
Frozen	0.50	0.75	0.68	0.40	1.0	0.38

2.5 Analysis of 2-ACBS

The detection of 2-ACBs was based on research that showed that lipid degradation products (2-ACBs and hydrocarbons) could be used to detect irradiated foods that contain fat, including meat, fish, shrimp, cheese, and liquid egg products (Morehouse and Ku 1990). The major analytical methods are based on extraction of the lipid fraction of food using a lipophilic solvent such as hexane, fractionation of the lipid phase on a column of deactivated Florisil or silica, followed by separation of the 2-ACBs by gas chromatography and detection using mass spectrometry (GC-MS). Several variations of this approach have been proposed and used, and the procedure has been adopted by the European Committee for Standardization (CEN) as a standard method, initially in 1996 with an update in 2003.

2.5.1 Method EN1785

The Community Bureau of Reference (Brussels) studied analytical methods for the detection of irradiation of foods between 1989 and 1993 and proposed five methods to CEN (Raffi and others 1993) which were validated by collaborative trials and were adopted in 1996 (Delincee 1998). The procedures were electron spin resonance (ESR) spectroscopy methods for food containing bone or cellulose; thermoluminescence for spices and herbs, and chemical methods based on isolation and detection of hydrocarbons and cyclobutanones in foods containing fat. The national standards organizations of several countries including the UK are bound to implement this European Standard, and they have been adopted as CODEX methods. For fatty foods methods EN1784:2003 based on the detection of hydrocarbons and method EN1785:2003 based on the detection of 2-ACBs were adopted.

Prior to adoption the 2-ACB procedure was validated in a series of interlaboratory blind trials where the sample matrices were raw chicken, pork, liquid whole egg, salmon, and Camembert cheese. (Hasselmann and others 1996). The results were described in a series of reports (Meier and Stevenson 1993; Stevenson and others 1994a; Stevenson and others 1994b; Stevenson 1996; Stewart 2001). Many foods contain fat, and therefore, when linked with a suitable fat extraction procedure the method should be applicable to a wide range of foods (Stewart and others 2000). Several other studies, some using variations of the CEN method, have to various degrees confirmed the scope of the method.

In the CEN procedure the homogenised sample is mixed with an equal weight of anhydrous sodium sulphate and the fat extracted using a Soxhlet apparatus. The sodium sulphate is used to disperse the food sample within the extraction thimble thus providing a large surface area and enhancing solvent penetration. The solvent is removed by placing the samples in an oven and the lipid concentration measured gravimetrically using duplicate subsamples. A portion of the fat extract (approximately 200 mg) is applied to 30 g deactivated Florisil in a chromatography column. The column is cleaned with hexane and the 2-ACBs eluted using 1% diethyl ether in hexane. This is concentrated to dryness and re-dissolved in a solution containing the internal standard, 2-cyclohexylcyclohexanone. This solution is analysed by GC-MS with selected ion monitoring of the response for 2-ACBs.

2.5.1.1 Performance of the CEN EN1785 method

The performance of the method EN1785 has been summarised (Anon 2003). During the inter-laboratory trial five laboratories quantified 2-DCB in 15 samples of chicken which were either not irradiated or irradiated with doses of approximately 0.5 kGy, 3.0 kGy or 5.0 kGy (Stevenson and others 1994a; Stevenson 1996). They were analysed one and six months after irradiation. All of 74 results reported after one month identified the samples correctly; after six months only 2 of 60 reported results incorrectly identified the samples as false negatives (irradiated samples identified as non-irradiated). The method was also tested in an inter-laboratory trial carried out by the Food and Agricultural Organization of the United Nations and the International Atomic Energy Agency (Stevenson and others 1994b; Stevenson 1996). Eleven laboratories used determination of 2-DCB and 2-TCB to analyse 9 samples of chicken and liquid whole egg while 8 laboratories analysed pork. The samples were either not irradiated or given doses of 1.0 kGy or 3.0 kGy. Of the 99 chicken samples analysed only 1 irradiated sample was judged as non-irradiated and it appears that this sample was mislabelled. All 99 liquid whole egg samples and 72 pork samples were correctly identified. The method was further validated by means of an inter-laboratory trial carried out by the Ministry of Agriculture, Fisheries and Food (MAFF) in the UK (Stewart 2001). Seven laboratories measured 2-DCB and 2-TCB in 9 samples each of salmon and Camembert cheese. The samples were either not irradiated or given doses of 1.0 or 3.0 kGy. Of 63

samples only one Camembert cheese sample irradiated at low dose was declared not irradiated.

2.5.1.2 Limitations of the CEN EN1785 method

Systematic studies of the performance limits of method EN1785 have been reported (Stevenson 1996; Ndiaye and others 1999b). The method has been validated for 2-DCB and 2-TCB but can also detect 2-HCB, 2-OCB and 2 decylcyclobutanone.

Detection of irradiated raw chicken has been validated for doses of approximately 0.5 kGy and above. The detection of irradiated liquid whole egg, raw pork, salmon and Camembert has been validated for doses of approximately 1 kGy and above. Validation at these doses covers the majority of commercial applications, however there is a need for validated methods that perform with a lower level of detection in order to detect irradiation of ingredients or aged samples, and to improve confidence regarding the absence of irradiation of non-irradiated foods. EN1785 can only be used for the detection of foods irradiated at doses above 0.5 kGy when they contain at least 1 g of fat per 100 g of food. It cannot reliably detect irradiation of foods irradiated at lower doses or the irradiation of ingredients after mixing with low level into non-irradiated foods (Horvatovich and others 2006).

A major criticism of the EN1785 method has been that the Soxhlet extraction takes too long (6 hours) and uses too much solvent (McMurray and others 1994; Rahman and others 1996a; Tewfik and others 1999). Long Soxhlet extraction times using the flammable solvent hexane also present a fire hazard especially if run unattended. Thus several other approaches have been used or proposed to reduce sample processing time, as well as reducing solvent costs, and the amount of waste (Virgolici and others 2009). A further criticism is that the Florisil column clean-up has a poor capacity to retain fat compared to silica with the 30 g Florisil charge takes only 200 mg of fat.

Most procedures have been aimed at speeding up the extraction process, improving the clean-up step, and/or increasing the quantity of fat carried to the clean-up stage. These improvements have often been used in concert. Improving the clean-up provides an extract that can be more easily concentrated and shows fewer peaks in the chromatogram that might interfere with the 2-ACB response or suppress ionisation in the mass spectrometer. This increases the signal to noise ratio and improves the detection limit.

2.5.2 Extraction Procedures

2.5.2.1 Soxhlet extraction - Increasing the size of the fat sample

Increasing the size of the fat sample provides a more concentrated extract for GC-MS. Horvatovich pointed out that the retention of fat during the extraction of 2-ACBs is improved by using silica in place of Florisil (Horvatovich and others 2000). The capacity of silica gel to retain fat is significantly greater (3 g of silica can retain 100 mg of fat, five times more than the Florisil column used in the EN1785 method). By use of a column containing 60 g of silica the authors separated 2-ACBs from 2 g of fat in place of the usual 0.2 g (Horvatovich and others 2006). When using the larger silica column with 2 g fat the elution was carried out first with 300 mL hexane which was discarded. A second elution was carried out with 950 mL of 1% t-butylmethylether (TBME) in hexane, with the last 450 mL containing the 2-ACBs. The extract was free of triacylglycerols and contained mainly components of lipids having oxy groups. However the recovery of various 2-ACBs ranged between 57 and 68%, lower than the EN1785 method (91-98%).

Up to four extracts obtained by the EN1785 Soxhlet method have been combined and cleaned up using a cation exchange SPE column impregnated with silver ions (Ndiaye and others 1999a). This argentation method is useful for the separation of compounds by their degree of unsaturation. With GC-MS detection the method detected irradiated ingredients in chicken quenelles containing 2% mechanically recovered meat that had been irradiated at 5 kGy, and in cookies containing 3% liquid whole eggs (3%, w/w) irradiated at 4 kGy. It could also detect irradiation of rice at 0.1 kGy. Saturated 2-ACBs were eluted from the silver SPE column with 5% TBME in hexane, and unsaturated 2-ACBs were eluted with 5% TBME in methanol.

2.5.2.2 Direct solvent extraction

Direct solvent extraction (DSE) has been used as a rapid alternative to the Soxhlet method (Tewfik 2008a). A quantity of food sample (dependent on fat content) was mixed with sodium sulphate and shaken with a mixture (9:1) of hexane and heptane, and passed through Florisil prior to concentration and GC-MS analysis. The method produced clean chromatograms in a short time but the recovery of 2-ACBs was more variable between laboratories than was found with the EN1785 method. The method was tested in an inter-laboratory trial using irradiated chicken and liquid egg (Tewfik

2008b). All four participating laboratories could distinguish between 12 irradiated and non-irradiated samples. Tewfik concluded that this method is promising, rapid, simple, and robust for the analysis of irradiated lipid-rich foods (Tewfik 2008a).

Obana and others (2005) precipitated the fat from an accelerated solvent extraction (ASE) with ethyl acetate extract by mixing the solution with an equal volume of acetonitrile and precipitating fat by freezing. This extract was cleaned further using silica column SPE. Boyd and others (1991) extracted chicken with diethyl ether following an earlier procedure for pesticides, improved by the incorporation of a Florisil column clean up. No details of the method performance were provided but 2-DCB levels of 0.2 mg/kg were reported for a 10 g sample. The method was later applied by Crone and others (1992) to a study of storage and cooking but no method performance details were provided. Lee and others (2000) extracted 2-ACBs from the oilseed of the Perilla plant using *n*-pentane:iso-propanol (3:2). The ground seeds (30g) were homogenised with 30 mL solvent and centrifuged. The residue was re-extracted with 10 mL *n*-pentane:iso-propanol and the combined extracts evaporated to give about 15 g fat. A small portion (0.2 g) of the fat was taken for Florisil column removal of the hydrocarbon fraction. Elution was with 150 mL hexane (discarded) followed by 120 mL 2% ether in hexane, which fraction contained the 2-ACB.

A number of extraction procedures for the determination of the oil content of oilseeds have been compared (Matthaus and Bruhl 2001). These methods would be relevant to the extraction of fat for isolation of 2-ACBs. The methods included SFE, ASE, microwave-assisted extraction, solid fluid vortex extraction, Soxhlet, and Soxtherm. No significant differences in the quantity of fat extracted was seen and only extraction time and reagent volume varied, therefore a choice of these methods would probably also be available for ACB analysis.

2.5.2.3 Supercritical fluid extraction

Another procedure for extraction and purification of the 2-ACBs to be reported is supercritical fluid extraction (SFE) (Horvatovich and others 2000; Stewart and others 2001). SFE was applied to extract 2-ACBs from 2 g fat (10 times more than in the EN1785 method) extracted from food using the Soxhlet method (Horvatovich and others 2002). The recovery of the SFE method was 60-87% for minor 2-ACBs but 87-93% for 2-DCB. SFE was used by Tewfik and others (1998) on irradiated beef and chicken in a

comparison with the EN1785 method. Both methods could identify irradiation but 2-TCB in the beef sample was detected only in the SFE extracts in spite of the high content of the precursor stearic acid. Similar results were reported for egg (Victoria and others 1992), pork (Rahman and others 1996a), chicken (Rahman and others 1996b), and cheese (Lembke and others 1995).

Changing the SFE pressure and temperature has a major effect on the quantity of lipid extracted because it changes the solvating properties of the supercritical fluid. Therefore setting the optimum conditions to extract 2-ACBs, but retain other lipids, is complex (Horvatovich and others 2000). Little attention has been paid to modifications of the SFE solvent, which is invariably carbon dioxide. However it has been shown that for lean meat SFE with carbon dioxide alone provided less fat than petroleum ether extraction (Reichardt and others 1999). The addition of 2-propanol to the supercritical carbon dioxide co-extracted phospholipids and other compounds, giving a higher level than Soxhlet extraction with hexane and a chloroform-methanol extraction. The ratio of meat to 2-propanol affected the results considerably. It is apparent that SFE can be tailored for selective isolation of 2-ACBs, thus obviating the need for further purification on columns such as Florisil or silica gel (Variyar and others 2008).

In a comparison of SFE and the EN1785 Soxhlet method with Florisil clean-up applied to irradiated fish Tewfik and others (1999) showed that both methods could detect 2-DCB with the SFE extraction giving a more certain result. For 2-TCB which was present at low levels owing to the low level of stearic acid (0.02%) both methods could detect irradiation at 0.5 kGy only the SFE method could detect irradiation at 0.1 or 0.2 kGy.

Rahman and others (1996b) used SFE extraction of irradiated chicken followed by silica column clean-up but separated 2-ACBs by thin layer chromatography (TLC). The 2-ACBs were detected by spraying the plate with dinitrophenylhydrazine which produced a bright yellow spot. No limit of detection was provided in terms of irradiation dose (the samples received 5 kGy) but 2-DCB standards could be detected easily at 0.5 µg. Problems have been encountered with overloading of the SFE extraction cell, where a fat sample size in excess of 2 g of lipid resulted in a high content of triacylglycerol in the extract (Horvatovich and others 2000). This problem was overcome by the use of a TLC clean-up (Variyar and others 2008), and this combined method had a sensitivity about 20 times higher than using SFE alone.

2.5.2.4 Other extraction techniques

Total fat isolation by Soxhlet extraction can be accelerated considerably by the use of microwave heating focused at the cartridge zone using a technique called microwave assisted extraction (MAE). The time is reduced from 6 hours to less than 1 hour. Such a procedure has been validated (Priego-Capote and others 2004), and has been applied to bakery products (Priego-Capote and De Castro 2005), olives (Virot and others 2007), fish (Pena and others 2006), and cheese (Garcia-Ayuso and others 1999). Soxtec is a commercial alternative to the Soxhlet apparatus. The sample is placed in an extraction thimble as in the Soxhlet method but the thimble is placed directly into the flask containing boiling solvent. Extractable material is obtained by raising the thimble above the boiling solvent and rinsing it with freshly condensed solvent. ASE with hot ethyl acetate under pressure has also been used to extract 2-ACBs from irradiated meat and fish (beef, pork, chicken and salmon), followed by GC-MS (Obana and others 2005). Although shown to be excellent for increasing extraction efficiency these techniques (MAE, Soxtec and ASE) are not widely used as they each require specialised equipment not found in most laboratories

2.5.3 2-ACB analysis by GC-MS

In most cases, including method EN1785, GC-MS analysis is the final determination step for the 2-ACBs and work has been reported optimising both the chromatographic and mass spectrometric conditions used. The EN1785 method suggests use of a non-polar (100% dimethyl polysiloxane) column of short length (e.g. 12 m). In practice, longer (25-30 m) columns of slightly higher polarity (e.g. 5% phenyl) have more often been used. Even longer (60 m) GC columns improved the signal to noise ratio and hence the sensitivity in the detection of 2-DCB in cowpeas and rice, allowing an irradiation dose of 0.05 kGy to be detected. (Crone and others 1993). A longer or a more polar (OV-20-MS) column was used by Horvatovich and others (2005) with both the EN1785 method and supercritical fluid extraction to detect monounsaturated alkyl side chain 2-ACBs. Using chicken, salmon, crab and shrimp irradiated at 2-10 kGy, Virgolici and others (2009) optimised the EN1785 method and the EN1784 hydrocarbon method. This was achieved by evaluating injection parameters using the specified column, column flow, temperature programme and mass spectrometric parameters. The modified chromatographic method for 2-ACB enabled detection of much lower

(approximately 10 times) concentrations of ACBs, achieving detection as low as 0.025 mg/kg. The use of a PTV injector enabled the injection of 5 µL sample extract at low temperature (10ºC), resulting in a further ten-fold increase in sensitivity toward the 2-DCB standard.

2.5.3.1 Mass spectra of 2-ACBs

Electron impact (EI) ionisation is most commonly used in GC-MS analysis and the EI mass spectra of 2-ACBs have a weak molecular ion with predominant ions at m/z 98 and m/z 112. The ratio between these two ions (98/112) is 4:1 for the longer chain 2-ACBs including 2-DCB but increases to 6:1 for 2-OCB and to 56:1 for the 2-HCB. Monounsaturated 2-ACBs produce a mixture of the 2-ACB ions and those derived from the monounsaturated aliphatic hydrocarbons (Horvatovich and others 2005), the latter being fragments with a difference of m/z 14 (CH_2), e.g. 67, 81, 95, 109, 123, 137, 151, 165, 179, 193, 207, and 221. As described earlier, oleic acid is the most abundant fatty acid found in many foods, producing on irradiation the monounsaturated alkyl side chain 2-ACB, cis-2-tetradec-5'-enylcyclobutanone. In the mass spectrometer this fragments to a higher degree than saturated 2-ACBs, thus the base peak is less intense and the detection limit about 3 times higher. Because of this, cis-2-tetradec-5'-enylcyclobutanone is generally only monitored when the level of oleic acid is over three times that of palmitic acid. Several difficulties have been reported in the detection of cis-2-tetradec-5'-enylcyclobutanone, mostly related to the co-elution of impurities such as n-octadecanal and n-hexadecanal. These have been resolved by a combination of SFE and TLC (Variyar and others 2008).

Chemical ionisation (CI) GC-MS can increase the sensitivity and specificity of the determination by increasing the response of the 2-ACB molecular ion (or more likely pseudo-molecular ion) due to the lack of fragmentation. Usually only a single ion of the protonated compound is formed. A disadvantage is that the identity confirmatory information provided by the fragmentation is lost. When using isobutane as the chemical ionization reagent the predominant ions are the pseudomolecular ion $[M+H]^+$ and the ion produced by loss of water from the pseudomolecular ion $[M+H - 18]^+$, which for monounsaturated 2-ACBs is the predominant ion.

2.5.3.2 Internal standards

It is worth noting that since in the EN1785 method the internal standard is added after the Florisil column chromatography step, the quantitative data may contain an error unless a correction is made for the recovery. The compound normally used as an internal standard is 2-cyclohexylcyclohexanone. Isodrin has been used as an alternative (Tewfik 2008) on account of its favourable retention behaviour on GC-MS. Several procedures for the synthesis of standards have been published. All are relatively complex and expensive to carry out. With the commercial availability of standards these syntheses are perhaps of most interest in provision of isotopically labelled standards, to act as internal standards, which are not yet available.

2.5.3.3 Solid phase micro-extraction GC-MS

More recently solid phase micro-extraction (SPME) GC-MS has been applied to the determination of 2-DCB and hydrocarbons as markers of irradiation of cured ham (Blanch and others 2009). 2-DCB could be detected in cured ham irradiated with a low irradiation dose (0.5 kGy) and not in non-irradiated samples. The repeatability of the method using ham irradiated at 8 kGy was 18%, poorer than for a hydrocarbon determination by the same procedure. Caja and others (2008) used SPME to detect 2-DCB and 1,3-bis(1,1-dimethylethyl)benzene in irradiated ground beef. The SPME conditions were optimised, and different fibres (PDMS, DVB/CAR/PDMS, polyacrylate and PDMS/DVB), and different extraction times (10, 25 and 40 minutes) and temperatures (40^{o}C and 60^{o}C) were evaluated.

2.5.3.4 Derivatisation

Ketones, such as 2-ACBs, can be derivatised to enhance the GC-MS signal, most frequently by formation of hydrazones. Sin and others (2006) used the reagent pentafluorophenyl hydrazine to derivatise 2-ACBs in extracts of irradiated (1-5 kGy), chicken, pork and mangoes. Endogenous substances produced false positive results for one chicken sample out of five and one pork sample out of five irradiated at the lowest dose, but the method achieved better signal-to-noise ratios than the EN1785 method.

2.6 Method performance comparison

A brief summary of the performance for some of the more recent analytical methods is provided in Table 2.3. However, reliable comparison of method performance is made difficult by the lack of data provided in the literature and statistical studies of method performance are so uncommon that detailed comparison is impossible. The performance of the methods depends mainly on the limit of detection and the recovery of 2-ACBs. As most methods assume that 2-ACBs are absent from non-irradiated foods there has been little inclination to report the absolute level of 2-ACBs measured, or the recovery where the markers have been found. To confound this situation limits of detection have been quoted as µg/g of food or as µg/g of the extracted fats and often with no clarity which is quoted. Very little work has indicated the minimum irradiation dose detectable and the associated uncertainty of confirmation of ACB identity at low concentrations.

Table 2.3. Method performance summary for 2-DCB

Food	Extraction	Solvent	Clean up	LoD by weight	Minimum dose detected (kGy)	Reference
Chicken	Reflux	ether	Florisil		1.0	Boyd and others 1991
Chicken	Reflux	ether	Florisil	0.25 mg/kg fat	0.5	Crone and others 1992
Chicken	Soxhlet	hexane	Florisil (a)	0.064 mg/kgfat	Not reported	Elliott and others 1995
Cheese	Soxtec	hexane	Florisil	0.5 mg/kg fat	Not reported	Rahman and others 1996
Beef	SFE	CO2	none	0.1 µg (f)	Not reported	Tewfik and others 1998
Chicken	SFE	CO2	none	0.1 µg (f)	Not reported	Tewfik and others 1998
Beef	Soxhlet	hexane	Florisil	0.5 µg (g)	Not reported	Tewfik and others 1998
Chicken	Soxhlet	hexane	Florisil	0.5 µg (g)	Not reported	Tewfik and others 1998
Fish	SFE	CO2	none		0.1	Tewfik and others 1999
Fish	Soxhlet	hexane	Florisil	0.1 µg	0.5	Tewfik and others 1999
Various (b)	SFE/diatomaceous earth	hexane	Silica(c)	0.21 pmol	Not reported	Horvatovich and others 2000
Perilla seed	Homogeniser	pentane:iso-propanol	Florisil	0.022 mg/kg fat (e)	0.5	Lee and others 2000
Beef	SFE/Florisil	hexane	None(c)	0.02 µg	Not reported	Gadgil and others 2002
Dried squid	Soxtec	hexane	Florisil	0.07 mg/kg fat (e)	0.5	Kim and others 2004a
Dried shrimp	Soxtec	hexane	Florisil	0.014 mg/kg fat (e)	0.5	Kim and others 2004b
Meat & fish	ASE	ethyl acetate	silica	3 ng/g sample 0.1 mg/kg fat	0.5 (fatty) to 2.0 (fish)	Obana and others 2005
Various (b)	SFE/diatomaceous earth	hexane	Silica (d)	0.2 pmol	Not reported	Horvatovich and others 2006
Sesame seed	Homogeniser	pentane:propanol	Florisil	0.2 mg/kg fat (e)	0.5	Lee and others 2008
Chicken	Direct extraction	hexane:heptane	Florisil		Not reported	Tewfik 2008
Beef	SPME	none	none	0.35 µg/L (j)	2-4	Caja and others 2008
Various (h)	Soxhlet	hexane	Florisil (i)	0.025 µg/L (j)	Not reported	Virgolici and others 2009
Ham	SPME	none	none	0.3 µg/L	Not reported	Blanch et al 2009

a) ELISA, b) cheese, chocolate, egg, avocado, chicken, c) silica trap eluted with hexane TBME, d) CI GC-MS, e) lowest level detected, f) units apparently µg per 2 g food sample, g) units apparently µg/food sample, weight not given, h) rice, egg, chicken, i) large volume cold injection, j) 2-DCB standard

2.6.1 Detection Limit

The detection limit (or limit of detection, LOD) in food analysis by chromatographic methods is normally expressed as the signal that is three times the background noise. However in the determination of irradiation the detection limit is very often expressed as the minimum irradiation dose that can be demonstrated. In a study of the detection limit of 2-DCB using the EN1785 method Ndiaye and others (1999) quoted the value as being 0.2 pmol, which for a molecular weight of 210 is 42 pg on-column. Blanch and others (2009) using SPME-GC-MS found a limit of detection of 0.3 mg/kg 2-DCB. The detection limit for 2-ACBs from beef extracted by SFE (Gadgil and others 2005) was not provided but levels of 0.02 mg/kg were reported for a 0.5 g beef sample. Kim and others (2004b), using a method very similar to EN1785, did not provide detection limits but reported low levels (0.014 mg/kg) of 2-DCB in irradiated dried shrimp and lower levels (0.006 mg/kg) of 2-TCB although these would be higher when presented on a wet weight basis. Horvatovich and others (2006) compared the detection limit for synthesised standards of 2-ACBs and monounsaturated 2-ACBs when analyzed using both CI and EI ionization. With EI ionization and selected ion monitoring of the response at m/z 98 the detection limit of 2-ACBs was 0.21 pmol of 2-DCB, and in CI the limit was 0.07 pmol of 2-DCB on-column. For monounsaturated 2-ACBs, the limit of detection was about 5 times greater than for the saturated compounds. Horvatovich and others (2005) have pointed out that many researchers encountered difficulties in detection of monounsaturated 2-ACBs in irradiated food samples on account of misidentification and coeluting impurities.

2.6.2 Recovery

For an in-house trial on irradiated mango samples the average recoveries of the 2-ACB standards from the spiked control samples were 83% for 2-DCB, 92% and for 2-TCB (Stewart and others 1998). Similar results were obtained for papaya. The recoveries for 2-DCB were 89% and 91% for fresh and stored samples respectively, while recoveries of 2-TCB were 92% and 96%. The recoveries for 2-DCB from salmon were 73% and 83% for chilled and frozen samples respectively, while recoveries of 2-TCB were 76% and 83% (Stewart 2001). The recovery of 2-ACBs from meat and fish extracted by ASE with a comprehensive clean up (Obana and

others 2005) were 70-105%. The recovery of ACBs from cheese, mango, and chicken reported by Ndiaye and others (1999) were 97-98% for 2-TCB, and 95-97% for 2-DCB. With shorter alkyl chains and increased polarity the recoveries were lower, 90-92% for 2-DCB, 77-79% for 2-OCB, and 69-73% for 2-HCB.The recovery of 2-ACBs from chicken extracted by SFE (Gadgil and others 2002) was 100% for 2-DCB and 90% for 2-TBC. Slightly lower recoveries of 60% for 2-decylcyclobutanone, 75% for 2-DCB and 87% for 2-TCB were reported by Horvatovich and others (2000), who used a post-extraction cleanup.

2.7 Effects of storage and cooking on 2-ACB concentration

Decreases in 2-ACB concentration following storage of the foodstuff have been reported and have been attributed to the oxidation of 2-ACBs to lactones, but are more probably due mainly to volatilisation as the shorter chain 2-ACBs are lost more rapidly (Ndiaye and others 1999). Degradation does not significantly depend on the saturation state of the alkyl side chain (Horvatovich and others 2005).

Frozen storage reduced the level of 2-DCB in chickens irradiated at 1 kGy (Crone and others 1992). Storage did not have a significant effect on the 2-TCB content of irradiated mangoes, as the concentration remained constant over a 14 day storage period at 10℃, by which time it was observed that the quality of the mangoes had started to deteriorate (Stewart and others 2000). In an in-house blind trial carried out on 30 mango samples that had been irradiated at between 0.1 and 1 kGy, fifteen samples were analysed within 48 hours of irradiation, and 15 were analysed 14 days post-irradiation after storage at 10℃. All 30 samp les were identified correctly as either irradiated or non-irradiated using 2-TCB as the irradiation marker. Similar results were obtained for papaya (Stewart and others 1998) with 2-DCB used to detect irradiation at doses as low as 0.1 kGy, but only soon after irradiation. 2-TCB was detected for up to 3 weeks. A dose of 2.5 kGy is recommended for the irradiation treatment of Camembert cheese. After irradiation at 0.5-5 kGy detection of 2-DCB and 2-TCB in Camembert cheese was still possible after 26 days (Stewart 2001). Horvatovich et al. (2005) assessed the effect of post-irradiation storage on a number of different irradiated food commodities (ewe's cheese, 100 kGy), liquid egg (0.5, 1, 3, 5 kGy), avocado (0.1, 0.5, 1 kGy) and poultry meat (0.5, 1, 3, 10 kGy). Levels of the 2-ACBs decreased over time periods of up to 28 days. Losses of

2-ACBs on storage of lyophilized poultry meat of up to 28 days ranged from 40- 60% (Horvatovich and others 2000). Losses of 21-78% have been reported for irradiated poultry meat, cheese, and sardines irradiated at 3 kGy and stored at 4 ℃ (Ndiaye and others 1999).

Crone and others (1992) showed that 2-DCB persisted for at least 20 days in irradiated meat stored at 4ºC. It was very stable and detectable in frozen chicken meat that had been irradiated with γ-rays and electron beams 12 to 13 years earlier. Ndiaye analysed cheese, sardine and chicken stored for 1 month at 4℃, and three samples of mango stored at 20℃ for 1 month (Ndiaye and others 1999). Levels of 2-ACBs in irradiated meats, eggs and pancakes decreased only a little on frozen storage of one year, losses from dried animal feeds were greater and 2-ACBs could not be detected after a year (Obana and others 2007).

Cooking in a convection oven reduced the level of 2-DCB in chickens irradiated at 1 kGy (Crone and others 1992) but it could still be detected. Heating irradiated liquid whole egg at 64℃ for 2.5 minutes has been reported to cause loss of 2-DCB (Crone and others 1993). Cooking of irradiated meat, poultry and eggs frying, boiling or baking did not have a significant effect on the detection of 2-ACBs (Obana and others 2006).

2.8 2-ACBs in non-irradiated foods

There has been relatively limited analysis of non-irradiated foods for 2-ACBs and until very recently that work has not provided evidence of 2-ACB formation. For example Ndiaye and others (1999) found no 2-ACBs in non-irradiated ewe's cheese, sardine, trout, mango, rice, chicken or beef. However, evidence has been presented for the natural existence of 2-DCB, 2-tetradecenylcyclobutanone, and 2-tetradecylcyclobutanone in cashew nuts, and of 2-decylcyclobutanone and 2-DCB in nutmeg (Variyar et al 2008). The 2-ACBs were not detected using Soxhlet extraction of 30 g nuts with silica column clean-up of 2 g extracted fat, but they were detected by SFE following extraction of 30 g nuts with a TLC clean-up of the extract prior to GC-MS. The presence of 2-DCB in the non-irradiated cashew nut samples was confirmed from the retention time, the ratio of the ions at m/z 98 and 112 (4:1), comparable with that of the standard, and the full scan mass spectrum. The levels reported were low (2 µg/g DCB, 1 µg/g TCB) but the extraction and clean-up

methods were shown to give a lower limit of detection and confirmation than had been achieved elsewhere by other methods. The level of interferences (background signal) was also reduced. Possible reasons for doubting the findings include the fact that the cashew nut and the nutmeg samples were purchased from a local market and were therefore of unknown provenance and history. The 'fresh' cashew nuts had been shelled and dried and it is not stated that this processing took place in the laboratory. Six replicate analyses were carried out but there is no indication of how many nut samples were taken or whether or not they were from the same source. It is possible that 6 analyses were made of a single batch of market-bought nuts. The nutmeg samples were checked for prior irradiation using hydrocarbon analysis. However this method might not be sensitive enough to detect the low level irradiation that might form ACBs, and was probably not applied to the cashew nut samples.

Other studies have investigated the effect of different processing techniques on foodstuffs and in particular the formation of 2-ACBs. In model system studies aqueous suspensions of the triacylglycerols glyceryl tricaproate (tricaprin), glyceryl trilaurate (trilaurin), glyceryl trimyristate (trimyristin), glyceryl tripalmitate (tripalmitin) and glyceryl tristearate (tristearin) subjected to various physical treatments listed below did not form 2-ACBs (Ndiaye and others 1999).

Microwave treatment for 20 minutes, 750 W output, frequency 2450 MHz

Heating in an oven at 150°C for 30 minutes

UV irradiation (240-280 nm) for 1 hour

Pressure of 6000 bar for 1 hour

Sonication for 5 minutes (power 455 W, frequency 20 kHz)

All of the 2-ACBs likely to be formed from these triacylglycerols were detected after irradiation with 0.9 kGy. The formation of the cyclobutane ring seems in fact to require a high energy input over a very short time and at a specific site in the molecule, conditions which are expected to be fulfilled only by irradiation treatment. Similarly, reports suggest that 2-ACBs cannot be detected in non-irradiated foods that have been subjected to various forms of processing such as freezing, heating, microwave heating, UV irradiation, high-pressure processing, or simple preservation treatments (Ndiaye and others 1999; Crone and others 1992; Crone and others 1993).

2.9 Discussion

Many alternative procedures and variations to EN1785 have been proposed or adopted. However, few of these alternative procedures have been validated by inter-laboratory trials. Ndiaye and others (1999) have stated that in order to determine 2-ACBs in foods with low (<1%) fat content irradiated at low doses (0.5 kGy) or in foods containing irradiated ingredients in low proportions, the EN1785 method must be made more sensitive by a factor of 10 or 20. The authors suggested approaches to this are pre-column (presumably pre-GC-MS) concentration of the 2-ACB extract and/or using a more sensitive detector than the GC-MS. Ways of implementing these improvements are elaborated below and recommendations are made as to the best course of action.

2.9.1 Options for method improvement

Increasing the quantity of fat extracted from the food can be achieved only by increasing the size of the initial sample. This might cause problems in cases where the fat content is low. The use of alternative extraction procedure to the Soxhlet method will probably not have a significant effect on the method performance. The Soxhlet extraction procedure is often criticised for being lengthy, but by this means several samples, each of suitable weight, can be extracted unattended or overnight. It is also possible that a change to the nature of the extraction solvent will bring advantages. It is probably not feasible to use solvent mixtures in the Soxhlet method because of differences in boiling point (unless they form an azeotrope) but the use of TBME for example might be beneficial.

Increasing the quantity of 2-ACB injected requires its separation from the extracted fat. It has been demonstrated that silica columns give better performance than Florisil in this task, and that TBME has potential to offer improved performance over hexane as a clean-up solvent. The removal of fat by freezing could also be investigated, although yields of 2-ACBs are likely to be reduced.

A novel and untested approach to the issue would be to remove the fat by saponification. This method is used to reduce the sample volume applied to a silica clean-up column in the official methods of analysis of steroidal hydrocarbons in olive oil where the non-saponifiable matter from a 20 g fat sample is applied to a 15 g

silica column. There is no obvious cause to expect that 2-ACBs will be affected by saponification procedures.

An improvement of the chromatographic resolution can be achieved by replacing the GC capillary column usually used for the analysis of 2-ACBs (low polarity) by a slightly more polar (OV-20-MS, 20% phenyl, 80% dimethylpolysiloxane) and longer (60 m) column (Horvatovich and others 2005). Improving detector sensitivity in the electron impact mass spectrometers in common use is largely a matter for improvement in instrument design. The use of cold, large volume injection has been shown to improve sensitivity but the use of such techniques is not widespread. The use of chemical ionisation MS both increases the sensitivity towards 2-DCB but also enables determination of the more abundant 2-TCB, but again the instrumentation is not widely used.

Derivatisation of 2-ACBs requires reaction of the ketone group. Derivatisation of ketones for GC-MS is generally achieved by preparing a hydrazone. Reaction with hydroxylamine or substituted hydroxylamines such as pentafluorobenzylhydroxylamine hydrochloride (PFBOA) is normally used. PFBOA produces a volatile derivative that can be detected by GC-MS or with an electron capture detector. Alternatively, reaction with cysteamine to form a thiazolidine has been used to determine volatile and non-volatile carbonyls in food (Yasuhara and others 1998).

A different derivatisation has been based on fluorescent labelling with 7-diethylamino-3-carbonylazide (Morsel and Schmiedl 1994), with separation and detection by liquid chromatography. The reaction with 7-diethylamino-3-carbonylazide was preceded by reduction of 2-DCB to 2-dodecylcyclobutanol with sodium borohydride. The chromatographic properties of the derivatives were somewhat poor. However the relatively easy reduction of 2-DCB to the alcohol would permit a wide number of derivatisation reaction to be targeted at the –OH group. This approach could for example improve the mass spectrometric properties of cis-2-tetradec-5'-enylcyclobutanone from the more abundant oleic acid.

A novel untested approach would be to brominate the unsaturated bond of cis-2-tetradec-5'-enylcyclobutanone, the 2-ACB produced by the most abundant fatty acid. This would be very likely to reduce the fragmentation, and introduce an intense and characteristic bromine isotope pattern into the mass spectrum. This reaction is

widely used and a number of simple reagents are available (Eissen and Lenoir 2008; Tang and Gong 2009).

2.9.2 Relative costs and effects on the testing laboratory

Increasing the quantity of fat extracted from the food will not usually increase the cost of the analysis but it might do so for foods with a low fat content where repeated extractions of each sample are required. There is likely to be no real advantage in using alternatives to the Soxhlet method, as this has a relatively low capital cost and does not require skilled operators. The use of MAE, Soxtec, SFE, and ASE all require considerable capital outlay. Procedures to increase the quantity of 2-ACB injected may be slightly more labour intensive but still good value. Removal of the excess fat by saponification would increase the time and labour costs a little but might offer very significant improvements in sensitivity. Changing the GC capillary column would have no effect on the cost of the analysis. Introduction of a derivatisation stage or a bromination reaction would have little effect on the cost of the analysis.

In summary most of the options available would result in little increase in the cost of the analysis. The use of alternative extraction procedures involving ASE or SFE would be faster but require a considerable capital outlay. The time saved might not be an improvement on using an unattended Soxhlet system.

2.10 Summary

A significant amount of work has been reported describing the detection of 2-ACBs in a variety of irradiated foods and a number of inter-laboratory trials have been carried out successfully. Until very recently 2-ACBs had not been reported as present in non-irradiated food but work has shown their presence in nuts and nutmeg, although this should be independently confirmed. A standard method (EN1785) exists but is based on analytical techniques and instruments from over ten years ago and with modern developments in techniques and apparatus there is much scope for improving sensitivity of the methods so that lower levels of 2-ACBS can be detected and thus foods treated with smaller doses of irradiation or foods containing smaller amounts of irradiated ingredients.

Knowledge is lacking regarding the true performance of the methods currently used, the optimum procedures for separating 2-ACBs from fat, the limits of detection of irradiated ingredients in foods, the relative formation rates of 2-ACBs from fatty acids in different parts of triacylglycerols, the longevity of 2-ACBs on storage, their fate on food processing, and most importantly the possible presence of 2-ACBs in non-irradiated foods.

2.11 References for literature review

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3.0 COMPARISON OF ANALYTICAL TECHNIQUES FOR THE DETECTION OF 2-ACBS (WORK OBJECTIVE 02)

3.1 Introduction

It is apparent from the literature review described in Chapter 2 that the majority of the 2-ACB detection is by GC-MS analysis based on method development carried out in most cases over ten years ago. Since then there have been many technological improvements and this chapter describes a comparison between modern state-of-the-art GC-MS and LC-MS techniques for the detection of 2-ACBs.

3.2 Availability of analytical standards

For the development of any analytical method authentic standards of the compounds of interest are needed to enable optimisation of instrument parameters and measurement of sensitivity. Difficulties were encountered when attempting to purchase standards of 2-ACBs for this project with companies having run out. Only 2-DCB was found to be commercially available and alternative sources were investigated. Synthetic pathways to the saturated and unsaturated 2-ACBs have been published and companies were approached to carry out customised synthesis. Quotes for this work were received and costs were £5000 for 1 g of the saturated 2-ACBs and £12000 for 1 g of the unsaturated 2-ACBs. An alternative approach would be to irradiate samples of triacylglycerols or free fatty acids to form the corresponding 2-ACBs. However, this would require clean-up and purification to produce the authentic standards, again taking extra resources and costs. The important point is that other testing laboratories would have the same difficulties obtaining these standards, and it would be these other testing laboratories that would eventually carry out the method, so it seems most appropriate to carry out this work with the commercially available 2-DCB (available from Sigma). This proposal was discussed and agreed with the FSA. Initially problems were also encountered sourcing cyclohexylcyclohexanone (CHCH), the internal standard used in the standard method. This was eventually found available from Tokyo Chemical Industry (Japan). The lack of availability of the authentic analytical standards indicates that testing for 2-ACBs is no longer commonly taking place as chemical companies seem not to have the demand to continue synthesising the compounds.

3.3 Gas chromatography with mass spectrometric detection

GC-MS analysis is the method used in the EN1785 standard method and in the majority of the published methods for 2-ACBs. For quantification it is usually used in selected ion monitoring (SIM) mode where ions derived from the 2-ACBs are measured making the method more sensitive. Confirmation is carried out comparing retention times and peak area ratios of sample to solvent standard and by re-running in full scan mode and comparing mass spectra. The following paragraphs describe re-establishing the GC-MS method at Fera and the optimisation carried out.

3.3.1 GC-MS experimental details

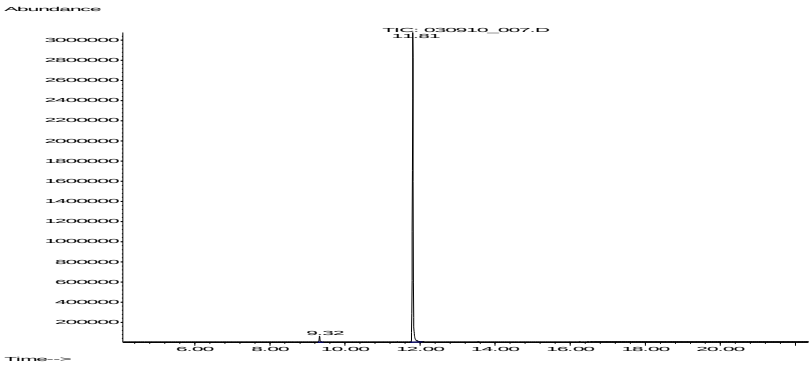
The samples were analysed by GC-MS using an Agilent 6980N gas chromatograph (Agilent, Palo Alto, CA, USA) coupled with an Agilent 5973inert mass selective detector. Splitless injection of 1 µL of solution was carried out into a DB-5MS capillary column (30 m x 250 µm i.d., 0.25 µm film thickness; J & W Scientific, Folson, Ca, USA). Following injection the oven was held at 55℃ for 1 minute and then raised at 15℃/minute to 300℃. The injector was held at 280℃. Helium (1 mL/min constant flow) was employed as the carrier gas. The MS was operated in electron impact mode with scanned monitoring between 40 - 600 amu or SIM mode measuring m/z 98 (quantification), 55 and 112 (confirmation) for 2-DCB and m/z 98 for CHCH.

3.3.2 GC-MS method development

The method parameters were taken from the standard method and solvent standards of 2-DCB were tested. Figures 3.1 and 3.2 show the total ion chromatograms (TICs) in full scan MS mode and the mass spectrum of the peaks corresponding to 2-DCB and CHCH respectively. It can be seen that the retention time for 2-DCB is 11.8 minutes and the mass spectrum shows that the most intense ions to be measured in the quantitative SIM mode are 55, 98 and 112. For CHCH the retention time is earlier at 9.3 minutes and the most intense ion is 98. These findings correspond with those found in the standard method.

Figure 3.1. a) Total ion chromatogram and b) mass spectrum of 2-DCB

a)



b)

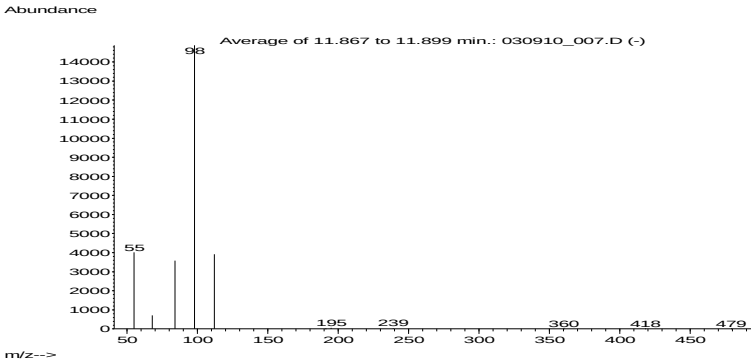
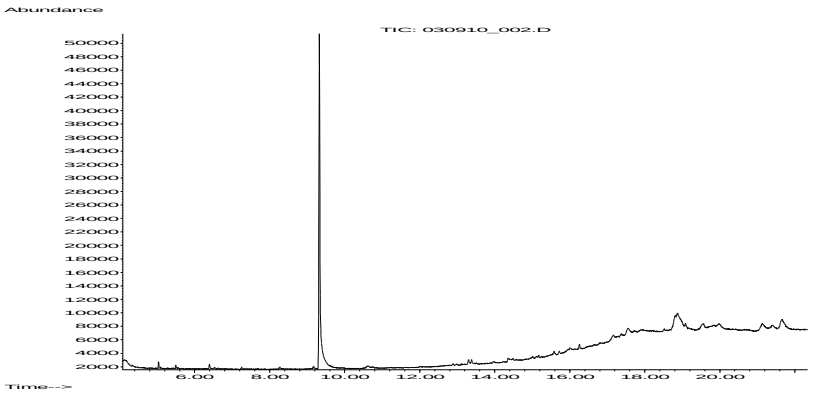
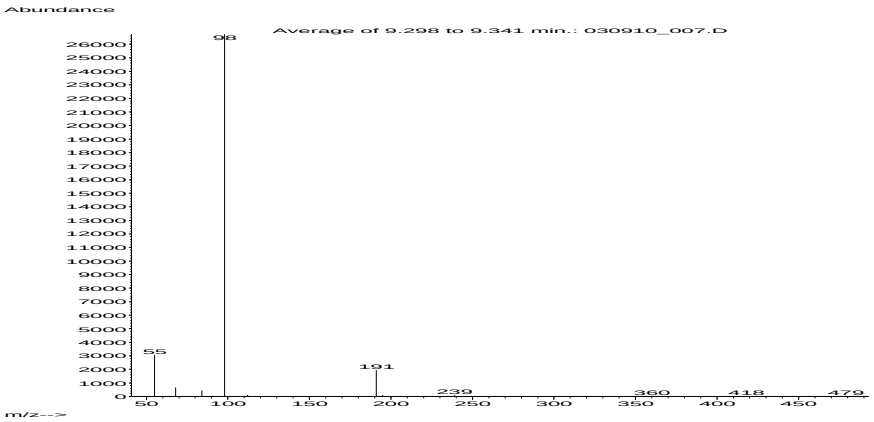


Figure 3.2. a) Total ion chromatogram and b) mass spectrum of CHCH

a)



b)

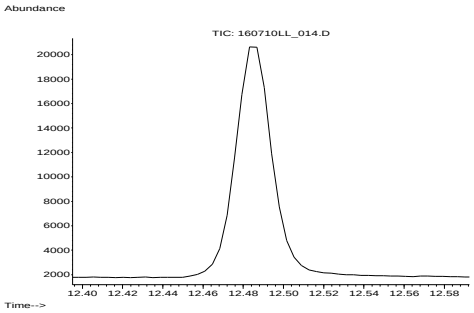


In the literature two main solvents are used for the final solution, hexane and isooctane. Tests were carried out to determine whether any differences in sensitivity or linearity occurred when using these solvents. A series of standards (nominal concentration at 0.001, 0.01, 0.1, 1, 10, 20, 50 and 100 µg/mL) were prepared in each solvent and injected onto the GC-MS. Figure 3.3 shows the extracted ion chromatogram (EIC) the ion at m/z 98 corresponding to 2-DCB. It can be seen that the peak shape was good for hexane and fronts slightly for isooctane, probably because of different interactions with the stationary phase of the column. A similar phenomenon is seen for the analyte and internal standard themselves, where the peak shape for 2-DCB (Figure 3.1), having a linear alkyl side chain, is far better than the peak shape for CHCH (Figure 3.2) having a cyclic alkyl side chain.

Figure 3.4 shows the two calibration curves for the series of standards in each solvent and it can be seen that good linearity was achieved in both cases with correlation coefficients greater than 0.99 over a large dynamic range. Finally the limit of detection (LOD) was determined in each solvent using three times the signal to noise ratio of the 0.01 µg/mL solvent standard and the extracted ion chromatograms are shown in Figure 3.5. The LOD was calculated as 0.003 µg/mL in isooctane and 0.001 µg/mL in hexane. Taking into account all of these facts it was determined that hexane was the better solvent to use in all further GC-MS analyses.

Figure 3.3. Exacted ion chromatogram (m/z 98) of the 2-DCB (0.1 µg/mL) peak in a) hexane and b) isooctane

a)



b)

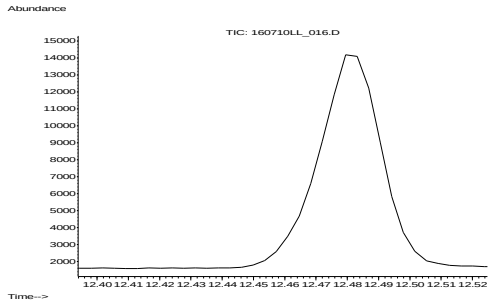
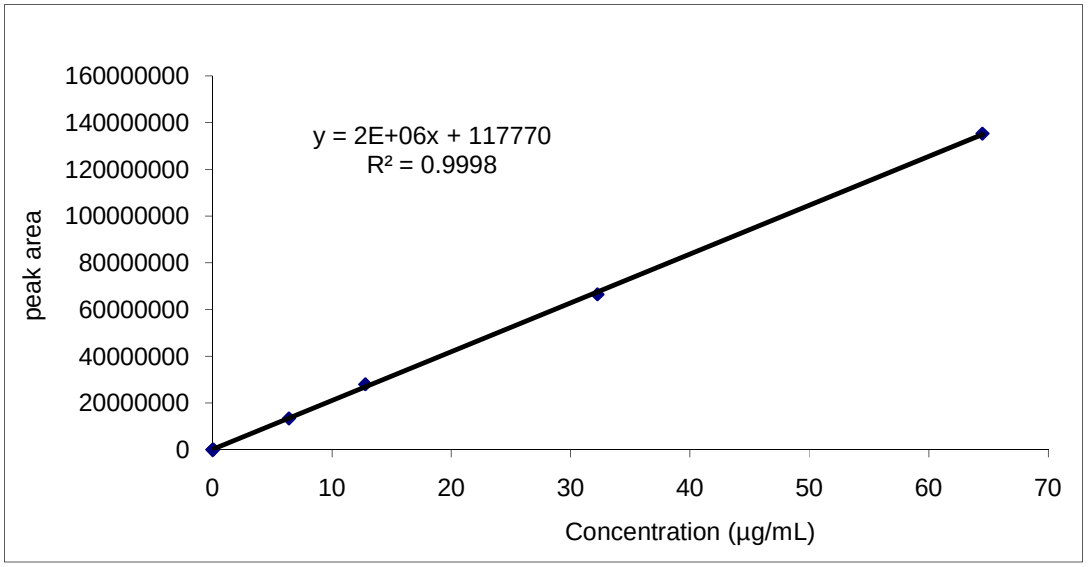


Figure 3.4. Calibration curves of 2-DCB in a) hexane and b) isooctane

a)



b)

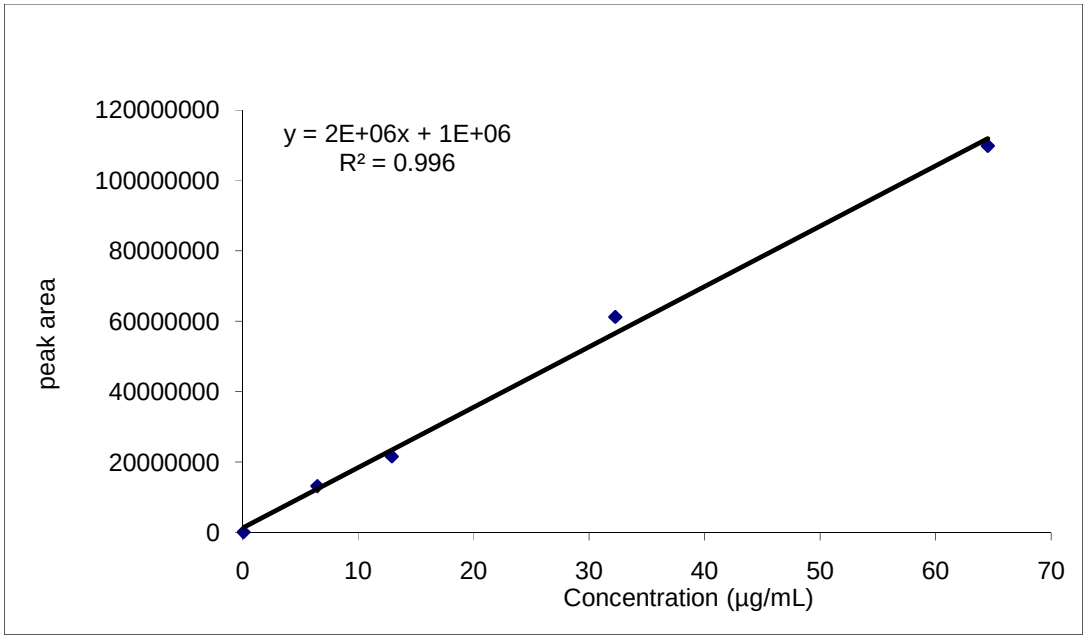
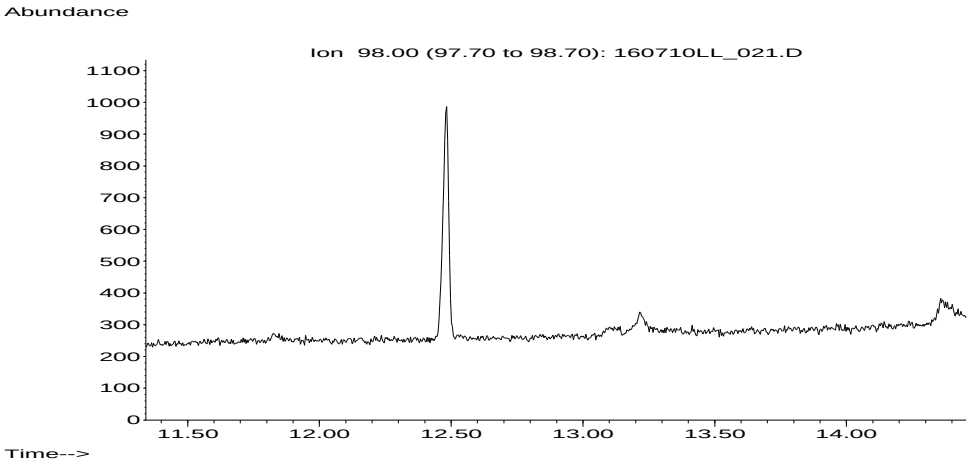
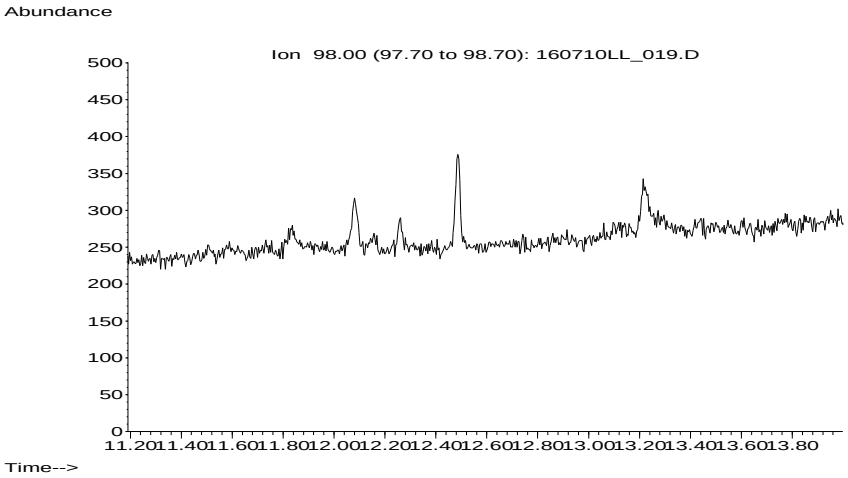


Figure 3.5. Extracted ion chromatograms (m/z = 98) of the 0.01 µg/mL 2-DCB solvent standards in a) hexane and b) isooctane

a)



b)



3.3.3 The standard method (EN1785)

The extraction and clean-up sections of the standard method were then re-established in the laboratories at Fera. This was to prove that the method could be successfully implemented and to determine whether interferences were carried through to the GC-MS analysis that might cause issues through either matrix ionisation suppression or co-eluting peaks. Samples of chicken and Camembert cheese (20 g) were homogenised using a food blender and spiked with 2-DCB (0.1 mg/kg). Anhydrous sodium sulphate (20 g) was added and the sample

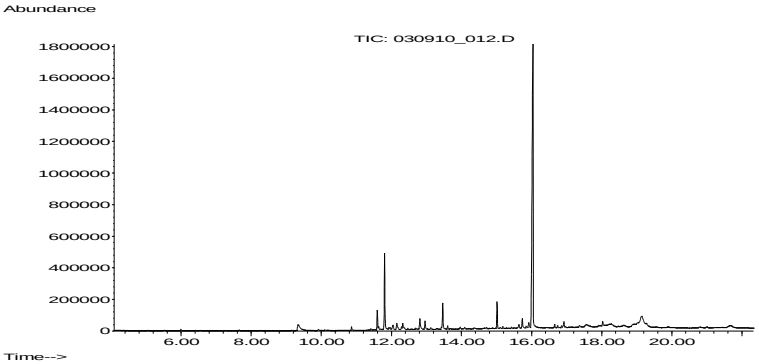
homogenised. This was then extracted using hexane (140 mL) in a Soxhlet extractor at reflux for 6 hours. The hexane was allowed to cool, made up to volume (100 mL) with further hexane, anhydrous sodium sulphate (10 g) was added and the flask left overnight. The lipid content was determined by taking a portion of the hexane extract (50 mL) to constant mass using a rotary evaporator. A Florisil column (20 cm) was prepared using deactivated Florisil and hexane, and a volume of extract providing approximately 200 mg of lipid was added to the top of the column. This was allowed to pass onto the column and further hexane (150 mL) was added. The eluent was collected following elution at a flow rate of 2 – 5 mL/minute. 1% Diethyl ether in hexane (150 mL) was then used to elute the 2-DCB and the eluent collected. This was rotary evaporated to dryness under a gentle stream of nitrogen at 40^{o}C. The residue was re-dissolved in hexane (200 µL) prior to GC-MS analysis.

Figures 3.6 and 3.7 show the TICs of the final extract from the spiked chicken and cheese samples respectively. As expected many more peaks were seen due to co-extractants from the food matrices along with the 2-DCB. The figures also show the extracted ion chromatograms for m/z 98, the ion used for quantification and 55 and 112, the ions used for confirmation. It is seen that the 2-DCB peak was fully resolved from all other peaks. The mass spectra of the 2-DCB peaks are also shown and can be seen to match those of solvent standards.

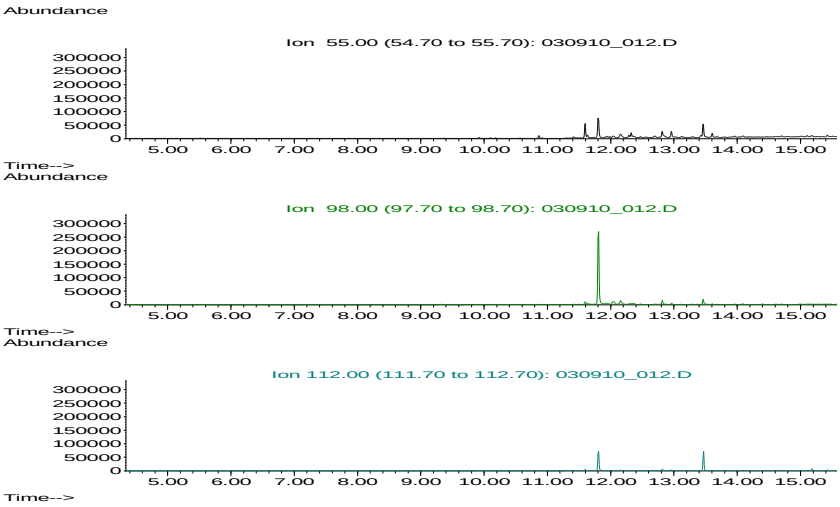
The recoveries were calculated from the 0.1 mg/kg over-spiked samples and were both acceptable at 82%. The confirmation calculated from the peak area ratios compared to those of the solvent standards were also acceptable (69-111%).

Figure 3.6. a) Total ion chromatogram, b) extracted ion chromatograms (m/z 55, 98 and 112) and c) mass spectrum for 2-DCB spiked onto chicken

a)



b)



c)

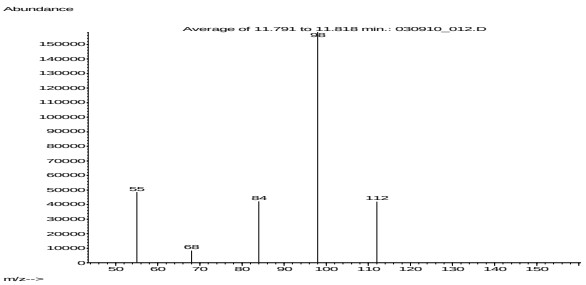
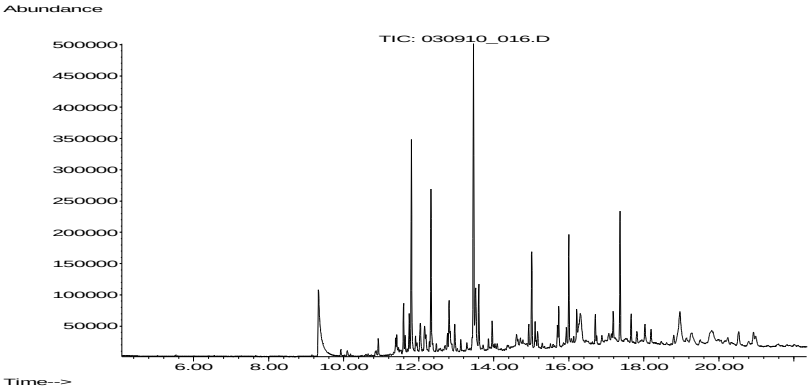
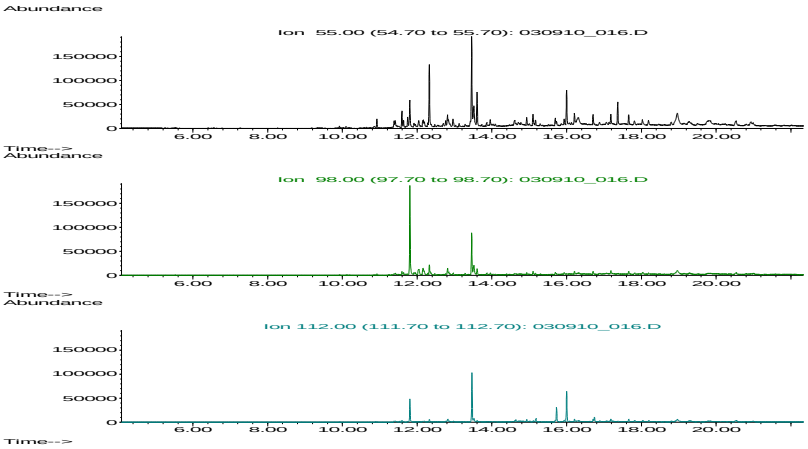


Figure 3.7. a) Total ion chromatogram, b) extracted ion chromatograms (m/z 55, 98 and 112) and c) mass spectrum for 2-DCB spiked onto Camembert cheese

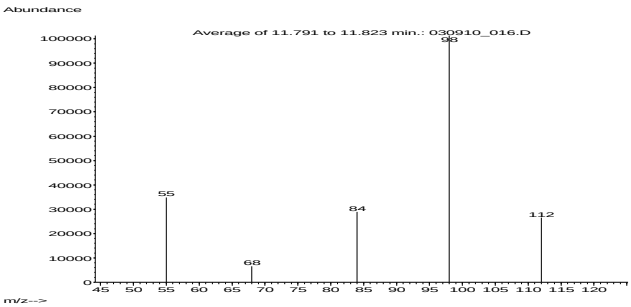
a)



b)



c)



3.4 Liquid chromatography with mass spectrometric detection

Figure 3.8 shows the chemical structure of 2-DCB. When considering the structure of this class of compounds LC-MS may not be considered to be the most appropriate method of analysis based on the lack of polar functionality and the long hydrocarbon chain present. These attributes make the compounds theoretically more amenable to GC-MS analysis. However, LC-MS and LC-tandem MS (LC-MS/MS) have become used more frequently over the last few years in the areas of food contaminant analysis and improvements and new developments have been made instrumentally. It was believed that this technique may prove useful for the detection of 2-ACBs.

When considering ionisation techniques for LC-MS atmospheric pressure chemical ionisation (APCI) theoretically seems the most likely to work well for the 2-ACBs. This is because the technique is more 'GC-MS-like' than electrospray ionisation (ESI) as the ionization occurs in the gas phase, unlike ESI, where the ionization occurs in the liquid phase. In APCI the mobile phase from the LC contains the analyte of interest and is heated to high temperatures (approximately 400^{o}C), sprayed with heated nitrogen gas and the entire aerosol cloud is subjected to a corona discharge where ions are created through analyte collisions with excited gas molecules, see Figure 3.9.

Figure 3.8.Chemical structure of 2-dodecylcyclobutanone

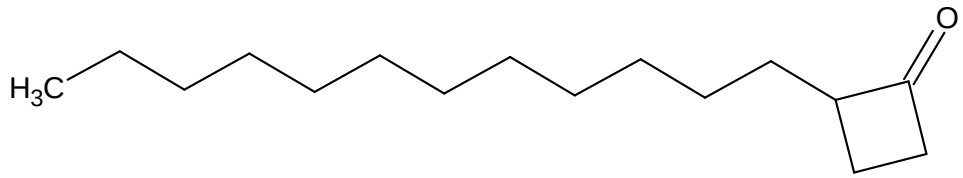
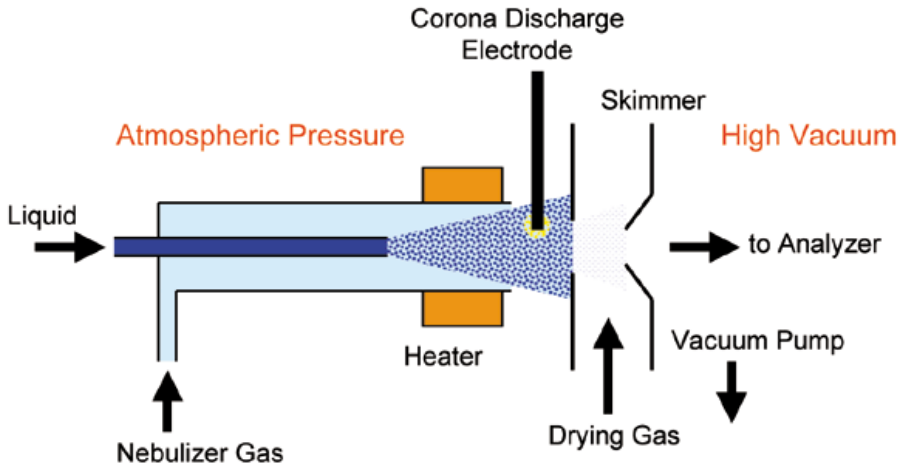
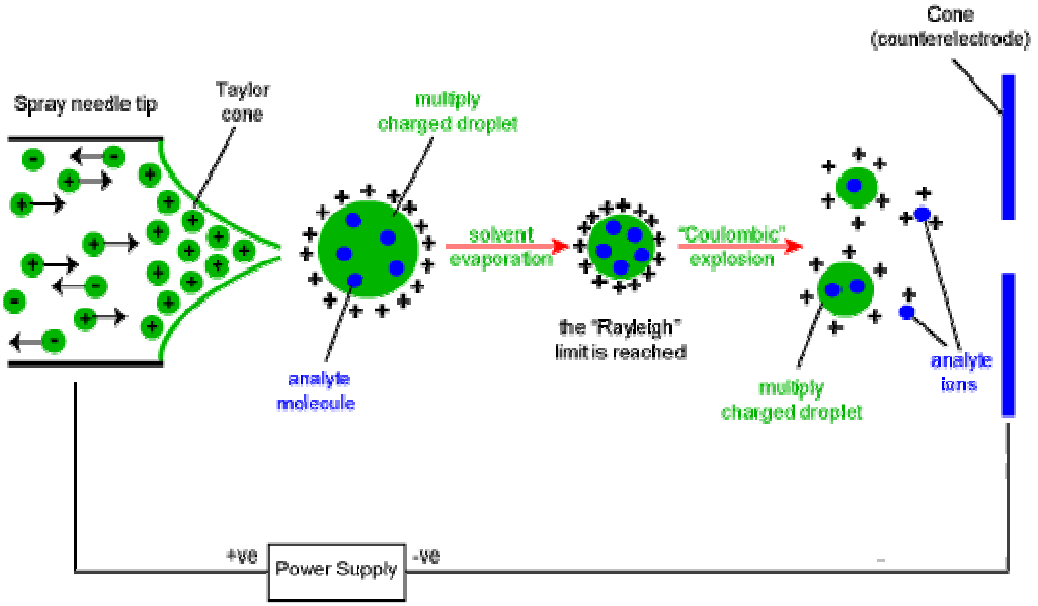


Figure 3.9. The principles of APCI in LC-MS



During ESI the mobile phase is dispersed into a fine aerosol called an electrospray by a voltage applied to the end of the capillary delivering the mobile phase to the source area, see Figure 3.10. Ion formation occurs following evaporation of the solvent and the charge transfers onto the analyte of interest.

Figure 3.10. The principles of ESI in LC-MS



Initial experiments were carried out on a LC-time-of-flight-MS (LC-TOF-MS) instrument as this system has a changeable source so that both APCI and ESI modes could be investigated.

3.4.1 LC-TOF-MS experimental detail

Solutions of 2-DCB were prepared in acetone at 1 mg/mL and 100 µg/mL. These were injected into an Agilent LC/MSD TOF (Agilent, Santa Clara, California, USA) consisting of a 1200 Series LC and a TOF-MS using flow injection analysis where the LC column was replaced by a plastic connector so no chromatography was carried out. The analyte was directly introduced into the TOF source in a mobile phase of 1+1 methanol+5mM ammonium formate (v/v). The gas was at 350^{o}C with a flow rate of 5 L/minutes and nebuliser pressure of 15 psi. The skimmer was at 65 V and the octopole RF was at 250 V. The fragmentor was tested at 120 – 200 V and mass range was m/z 100 - 1100. In ESI the capillary voltage was 3500 V and for APCI the capillary voltage was 3500 V and the corona current was 6 µA. Real time mass correction was performed using a solution including purine (C5H4N4 at m/z 121.05087) and hexakis (1H,1H,3H-tetrafluoro-pentoxy)-phosphazene (C18H18O6N3P3F24 at 922.00980).

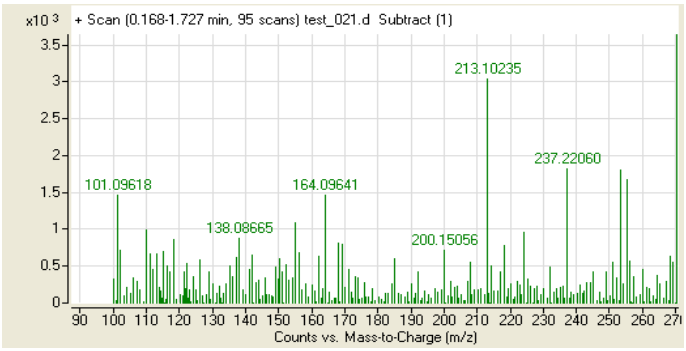
3.4.2 LC-TOF-MS results

Figure 3.11a shows the mass spectrum obtained from the 1 mg/mL solution of 2-DCB with the APCI source in positive ionisation mode. Surprisingly there were no significant peaks attributable to the expected 2-DCB molecular species. However, when analysed by ESI, see Figure 3.11b, the expected $[M+NH_4]^+$ adduct at m/z 256.26304 was present. Both sources were also tested in negative ionisation mode but no peaks attributable to the 2-DCB were detected. The 1 mg/mL solution is a high concentration and thus LC-TOF-MS is substantially less sensitive than published GC-MS methods. LC-TOF-MS is inherently less sensitive than LC-MS/MS methods as it is a full scan method compared to the more selective and hence sensitive LC-MS/MS. The method was transferred to a LC-MS/MS instrumentation where ESI sources were also available. LC-MS/MS works using a triple quadrupole MS system, see Figure 3.12, where one mass analyzer isolates the parent ion of

interest. A second mass analyzer then stabilizes the ion while it collides with a gas, causing fragmentation by collision induced dissociation. A third mass analyzer then detects the fragments produced from the parent ion enabling the sensitive selective reaction monitoring (SRM) measurements.

Figure 3.11. TOF-MS mass spectrum of 1 mg/mL solution of 2-DCB with a) APCI and b) ESI

a)



b)

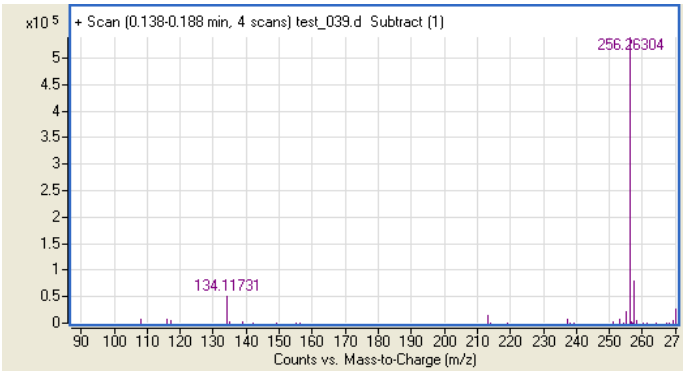
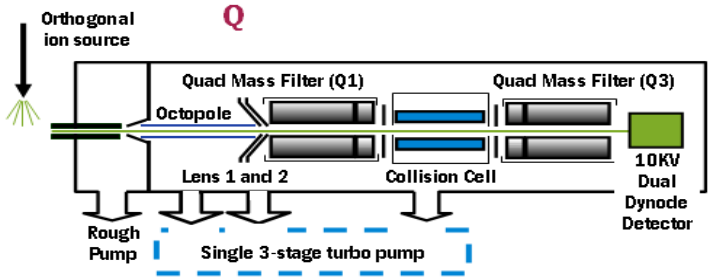


Figure 3.12. LC-MS/MS schematic



3.4.3 LC-MS/MS experimental detail

A Waters (Hertsford, UK) Alliance 2695 LC system was used with a Waters LC Sunfire column (C18), 150 mm long, 2.1 mm internal diameter, 3.5 µm particle size, maintained at 30^{o}C in a thermostated column oven. The mobile phase was aqueous based (A) and methanol (B) with a flow rate of 0.2 mL/min. For the ammonium adduct mobile phase A was 5 mM ammonium formate and for the protonated adduct was 0.1% acetic acid in water. A mobile phase gradient was used starting at 10% B changing linearly to 100% B over 10 minutes before the column was re-equilibrated at 10% B for 2 minutes. An injection volume of 20 µL was used.

The MS system was a Micromass (Manchester, UK) Quattro Ultima triple quadrupole mass spectrometer used in positive ion ESI. The capillary voltage was 3 kV with a source temperature of 120^{o}C and cone voltage of 35V. Desolvation gas was nitrogen at 700 L/hour and the desolvation temperature was 300^{o}C. Collision induced dissociation was performed using argon. RF lens 1 was set at 20 and RF lens 2 at 0.2. High and low resolution mass 1 were both set at 13.5 with an ion energy of 0.5 and high and low resolution mass 2 were both set at 15.0 with an ion energy of 0.5. MS/MS parameters were optimised for the protonated and ammoniated adducts separately.

3.4.4 LC-MS/MS results

Initially the system was set-up in LC-MS full scan mode to determine that the formation of the ammonium adduct seen by TOF-MS could be reproduced on this system. The full scan MS of 100 µg/mL 2-DCB in 1+1 methanol+5 mM ammonium formate is shown in Figure 3.13. The $[M+H]^+$ at m/z 239 and the $[M+NH_4]^+$ at m/z 256 peaks can both be seen and the general MS parameters (cone voltage, capillary current, desolvation gas temperature, desolvation gas flow and source temperature) were optimised to achieve the largest signal possible. The system was then changed into MS/MS mode and the collision energy was optimised. Here the collision energy was increased in increments and the fragments formed were measured. This process is carried out to achieve a balance between obtaining the largest fragment ion response whilst not fragmenting the ions so much that nothing can be detected.

The results of this process are shown in Figure 3.14. The results show that the optimum collision energy in this case is 5 eV.

Figure 3.13. LC-MS full scan mass spectrum of 100 µg/mL 2-DCB in 1+1 methanol+5 mM ammonium formate

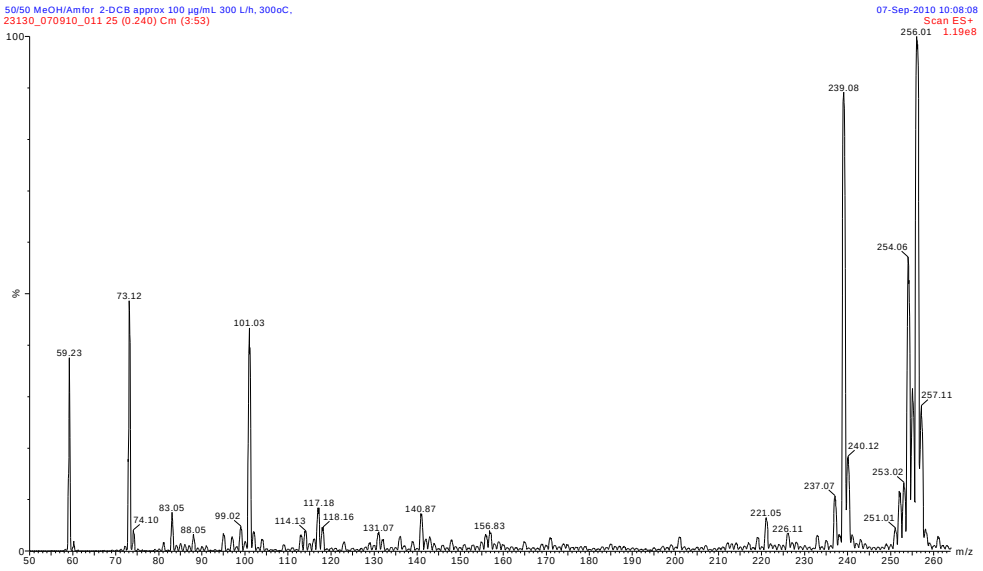
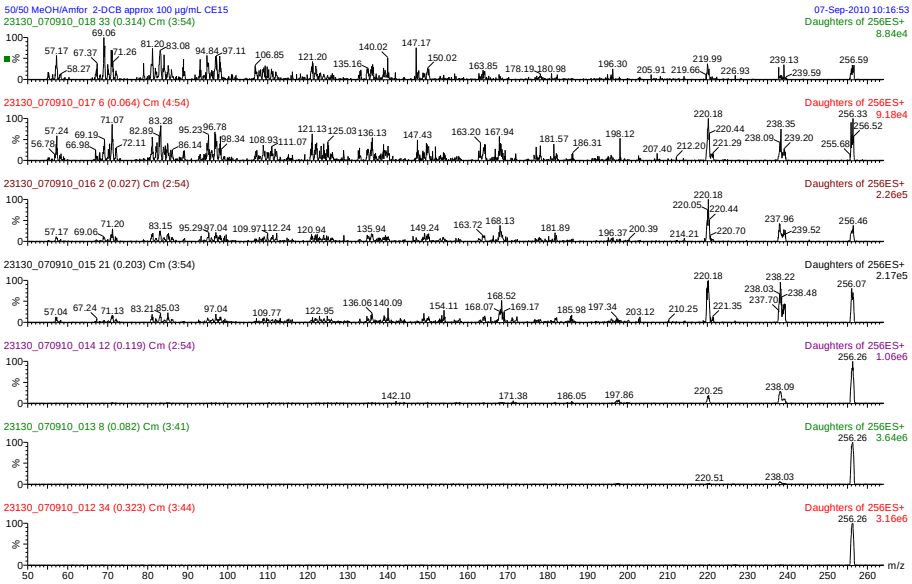
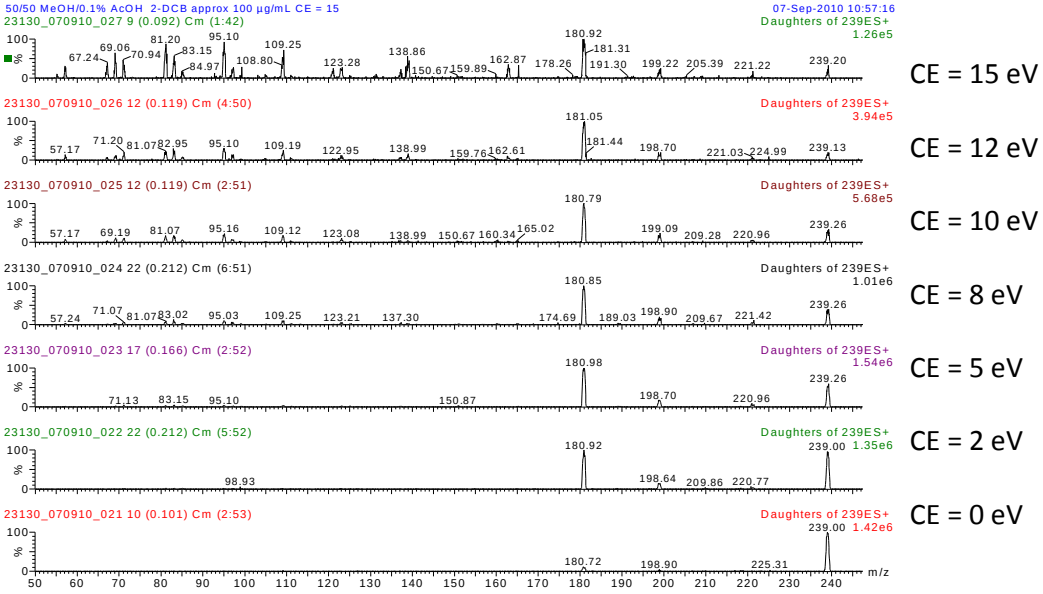


Figure 3.14. LC-MS/MS collision energy (CE) optimisation for the fragmentation of the $[M+NH_4]^+$ adduct of 2-DCB.



As the protonated molecular adduct was also observed in the full scan MS the MS/MS parameters were also optimised for this ion. The optimisation was carried out in the presence of methanol and 0.1% acetic acid in water to enhance ionisation by addition of protons. The collision energy optimisation is given in Figure 3.15 and was optimised at 5 eV for the SRM channel 239>181. The signal for the protonated adduct was stronger than for the ammoniated adduct so this was taken forward to the LC-MS/MS method.

Figure 3.15. LC-MS/MS collision energy (CE) optimisation for the fragmentation of the $[M+H]^+$ adduct of 2-DCB



The LC conditions were optimised and are given above in the experimental section. A series of 2-DCB solvent standards (10, 1.0, 0.1 and 0.01 µg/mL) were run to determine the limit of detection and this was then calculated as 0.03 µg/mL (30 ng/mL) based on three times the signal to noise for the 0.1 µg/mL standard. This standard was injected in triplicate to determine repeatability at this low level and the relative standard deviation of the peak area was acceptable at 4%.

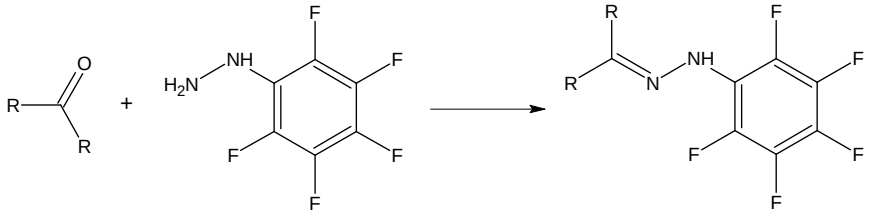
3.5 Derivatisation of 2-ACBs followed by analysis by GC-MS

Derivatisation can be used to make compounds more amenable to analysis by GC-MS by increasing the volatility. Although 2-ACBs can be analysed directly, reaction of the polar ketone group with a suitable derivatisation agent has been reported to result in improved measurement sensitivity and its use has been reported in the literature [8-9]. Derivatisation using pentafluorobenzylhydroxylamine (PFBOA) has been reported for the analysis of carbonyl compounds in foodstuffs, achieving detection limits at the parts-per-billion (µg/kg) level with good reproducibility [8]. Another derivatisation reagent, pentafluorophenylhydrazine (PFPH), has been applied for the detection of 2-ACBs in irradiated foods yielding a five-fold increase in sensitivity [9].

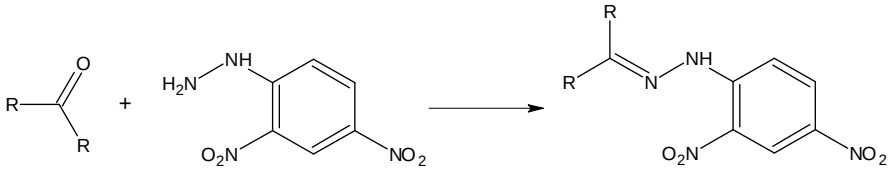
In an initial study the PFPH derivatisation method was tested. The EN1785 standard method was followed but instead of re-dissolving in CHCH solution the final residue was re-dissolved in PFPH (200 µL of 1500 µg/mL in 1 M potassium dihydrogen phosphate) and sulphuric acid (50 µL of 6 M). This was left for 1 hour at room temperature and then extracted with hexane (200 µL) prior to GC-MS analysis. Following this procedure no peaks attributed to the derivatised 2-DCB were detected. The reaction time and temperature were increased (60^{o}C and 6 hours) but still no peaks were detected. It was possible that the derivatisation reagent had decomposed so a fresh sample was purchased. Three other derivatisation reagents used commonly for carbonyl compounds were also tested: 2,4-dinitrophenylhydrazine (DNPH), methoxyamine (MOX) and pentafluorobenzylhydroxylamine (PFBHA). Figure 3.16 shows the structures of the derivatising reagents and the derivatives formed.

Figure 3.16. The reactions for the derivatisation used in this work. a) PFPH, b) DNPH, c) MOX, d) PFBHA

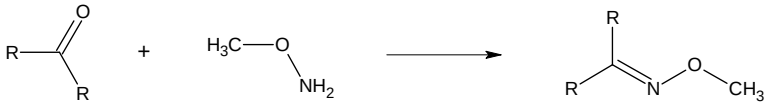
a)



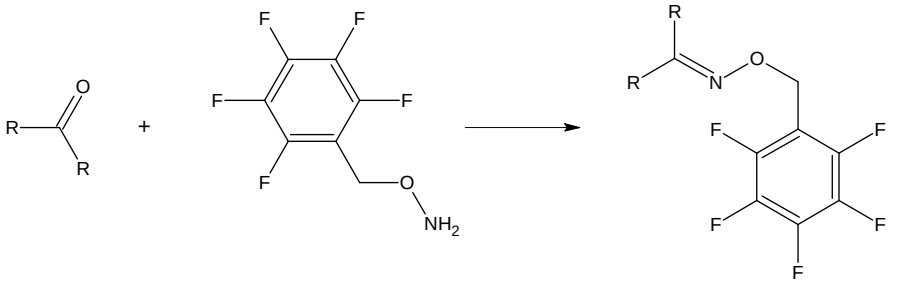
b)



c)



d)



3.5.1 Experimental details - Derivatisation reactions

3.5.1.1 PFPH derivatisation [9]

A solution of PFPH (1.5 mg/mL) was prepared in potassium hydrogen phosphate (1M). A portion of 2-DCB solution and CHCH solution (both equivalent to 50 µg in vial) were transferred to separate glass vials and the solvent evaporated to dryness under a gentle stream of nitrogen gas. A portion of the PFPH solution (200 µL) and sulphuric acid (50 µL, 6M) were added to each vial, the vials were sealed and left at room temperature for 1 hour. Hexane (500 µL) was added to each vial and the vials

carefully shaken. A portion of the hexane layer (200 µL) was transferred to a glass vial and analysed by GC-MS.

3.5.1.2 DNPH derivatisation [7]

A solution of DNPH (1.8 mM, 356 mg/mL)) was prepared in water. A portion of 2-DCB solution and CHCH solution (both equivalent to 50 µg in vial) were transferred to separate glass vials and the solvent evaporated to dryness under a gentle stream of nitrogen gas. A portion of the DNPH solution (1 mL) and water (700 µL) were added to each vial, the vials were sealed and left at room temperature for 30 minutes. Dichloromethane (2 mL) was added and the vial shaken. The dichloromethane was transferred to a clean vial and the extraction was repeated. After combining the dichloromethane portions the solution was evaporated to dryness under a gentle stream of nitrogen at 30^{o}C. The residue was re-dissolved in dichloromethane (1 mL) and analysed by GC-MS.

3.5.1.3 MOX derivatisation [10]

MOX hydrochloride (20 mg) was dissolved in pyridine (1 mL). A portion of 2-DCB solution and CHCH solution (both equivalent to 50 µg in vial) were transferred to separate glass vials and the solvent evaporated to dryness under a gentle stream of nitrogen gas. A portion of the MOX solution (50 µL) was added to each vial, the vials were sealed and left at 40^{o}C for 1 hour. This was transferred to a clean vial and hexane (200 µL) was added. This was capped, shaken and analysed by GC-MS.

3.5.1.4 PFBHA derivatisation [8]

PFBHA (125 mg) was dissolved in water (10 mL). A portion of 2-DCB solution and CHCH solution (both equivalent to 50 µg in vial) were transferred to separate glass vials and the solvent evaporated to dryness under a gentle stream of nitrogen gas. A portion of the PFBHA solution (500 µL) was added to each vial, the vials were sealed and left at 40^{o}C for 1 hour. Hexane (1000 µL) was added the vials shaken, a portion transferred to a clean vial and analysed by GC-MS.

3.5.2 GC-MS experimental details

The derivatised standards were analysed by GC-MS using an Agilent 6980N gas chromatograph (Agilent, Palo Alto, CA, USA) coupled with an Agilent 5973inert mass selective detector. Splitless injection of 1 µL was carried out into a DB-5MS capillary column (30 m x 250 µm i.d., 0.25 µm film thickness; J & W Scientific, Folson, Ca, USA). Following injection the oven was held at 55°C for 1 minute and then raised at 15℃/minute to 300℃. The injector was held at 280 ℃. Helium (1 mL/min constant flow) was employed as the carrier gas. The MS was operated in electron impact mode with scanned monitoring between 40 - 600 amu.

3.5.3 Results

The predicted molecular ions for the four derivatised 2-DCBs are shown in Table 3.1. These were looked for in each GC-MS chromatogram using the EIC mode.

Table 3.1. Derivatised 2-DCB molecules

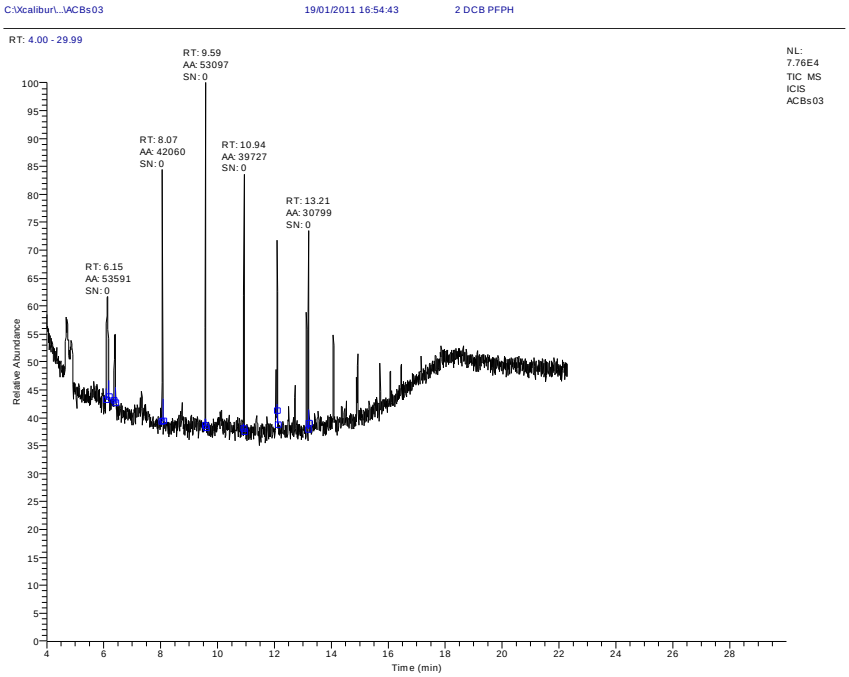
Derivative	Formula	Mass
PFPH-2-DCB	C22H31N2F5	418
DNPH-2-DCB	C22H34O4N4	418
MOX-2-DCB	C17H33NO	267
PFBHA-2-DCB	C23H32NOF5	433

The predicted ions for PFPH derivatisation were not seen (Figure 3.17); however large peaks of non derivatised 2-DCB and CHCH were observed indicating that the derivatisation had not taken place. Although it was later shown to have been successful by LC-MS, no peaks for DNPH-derivatised-2-DCB could be detected by GC-MS (Figure 3.18). This may be because the derivatives formed were not volatile enough to pass down the GC column, or the derivatised molecule may be thermally unstable and have decomposed during the GC-MS analysis. Derivatisation using MOX reagent was successful (Figure 3.19), however, the derivative was in the form of two peaks, presumably from two isomers forming upon derivatisation, this being

cis and trans about the C=N double bond (Figure 3.17) . Derivatisation using PFBHA reagent was also successful with the derivative again being in the form of two peaks. Comparing the abundances of the ions from both techniques it was decided to follow the PFBHA method as this produced the greater response. Figure 3.20 shows the full scan GC-MS TIC for the 1 µg/mL PFBHA-derivatised-2-DCB solvent standard. The peaks at 15.7 and 15.9 minutes were attributed to the derivatised 2-DCB and the mass spectra for these peaks were identical as can be seen in Figures 3.21 and 3.22.

Figure 3.17. Full scan total ion chromatogram for a) the 100 µg/mL 2-DCB solvent standard with PFPH derivatisation reagent and b) corresponding reagent blank

a)



b)

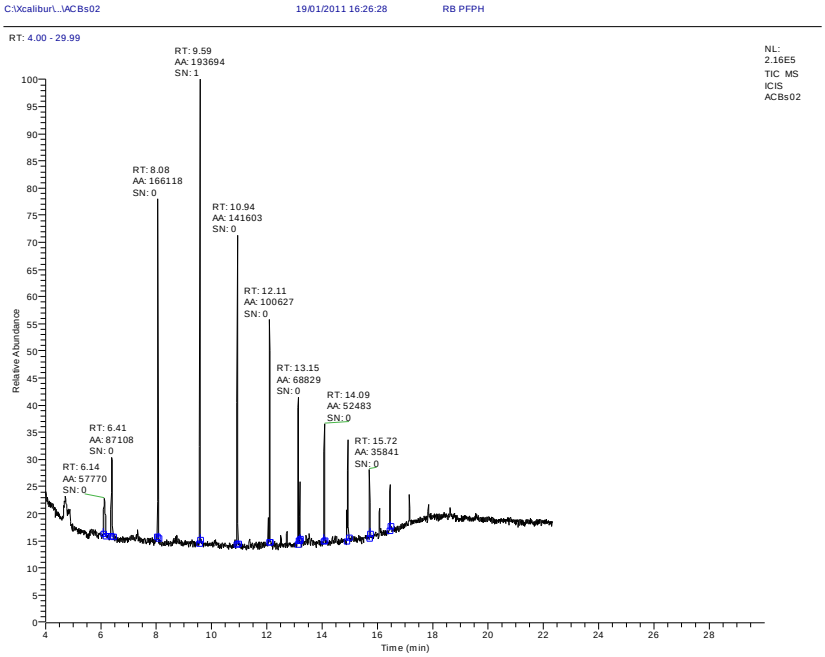
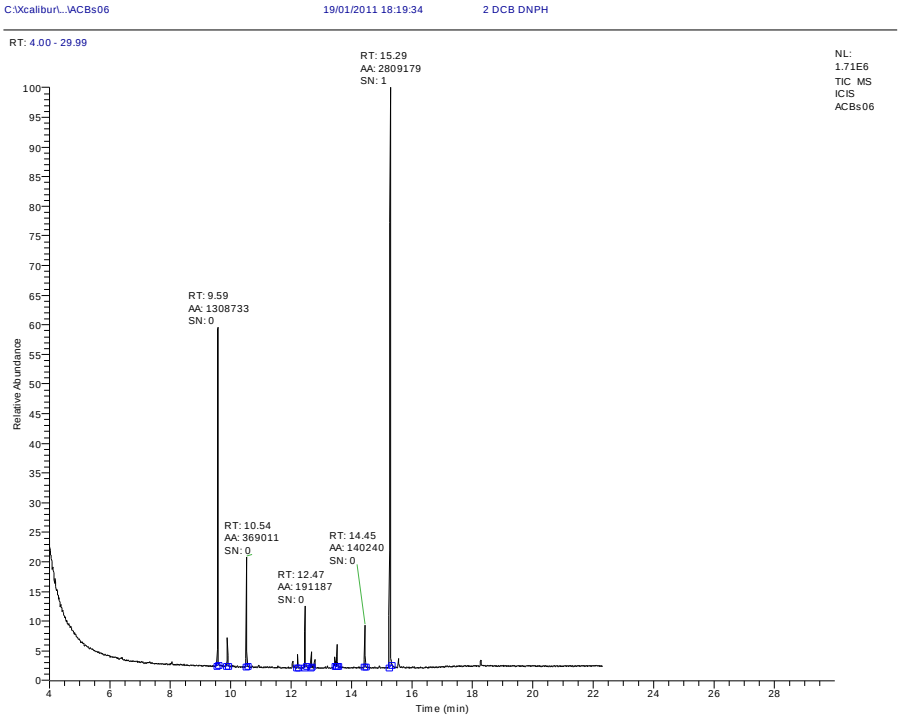


Figure 3.18. Full scan total ion chromatogram for a) the 50 µg/mL 2-DCB solvent standard with DNPH derivatisation reagent and b) corresponding reagent blank

a)



b)

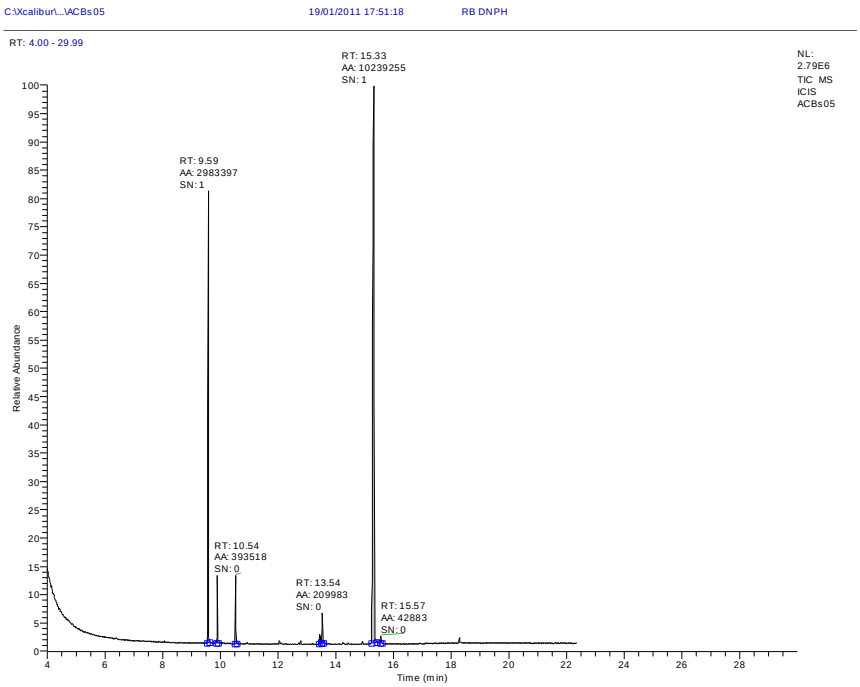
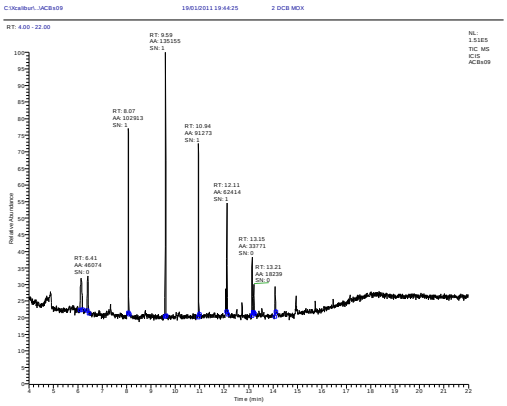


Figure 3.19. Full scan total ion chromatogram for a) the 200 µg/mL MOX-derivatised-2-DCB solvent standard, b) corresponding reagent blank, c) mass spectrum of the peak at 13.7 minutes

a)



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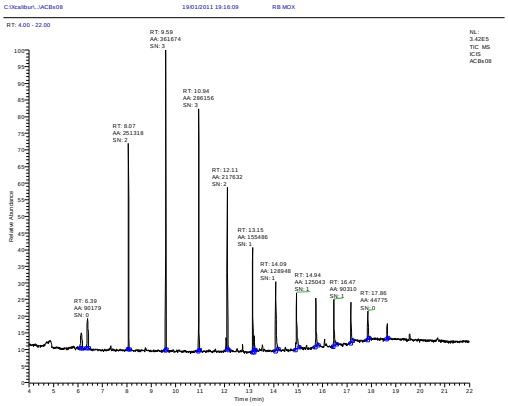


c)

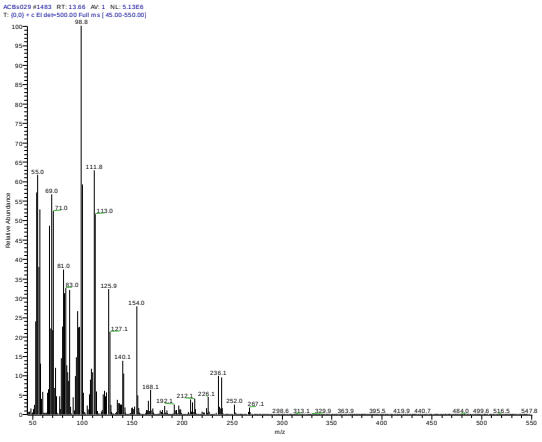
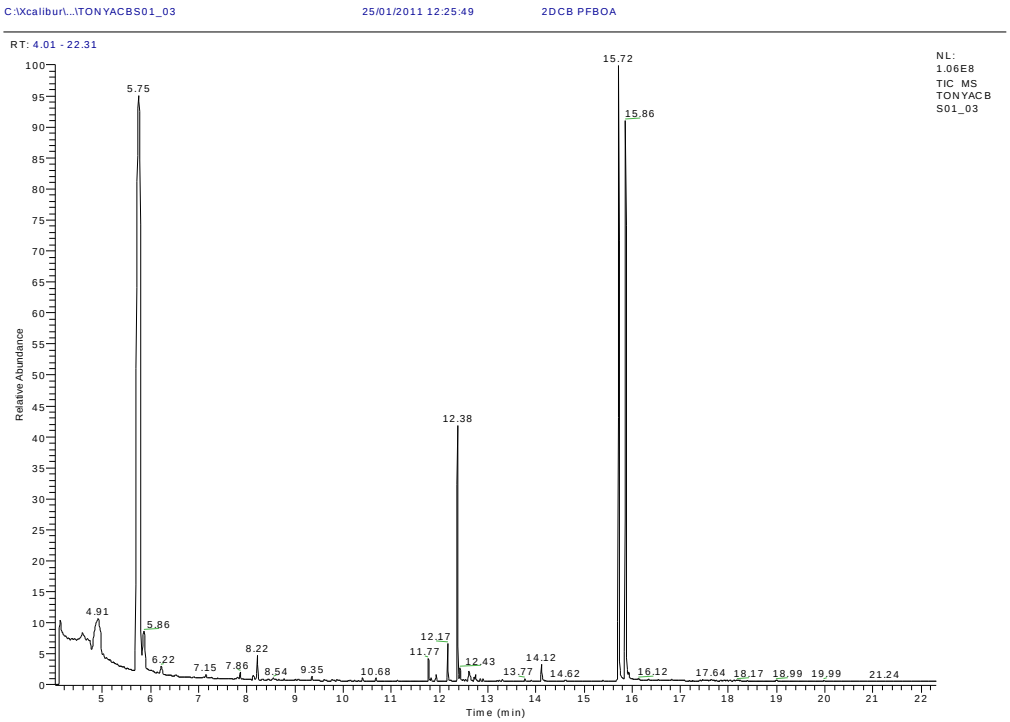


Figure 3.20. Full scan total ion chromatogram for the a) 50 µg/mL PFBHA-derivatised-2-DCB solvent standard and b) corresponding reagent blank

a)



b)

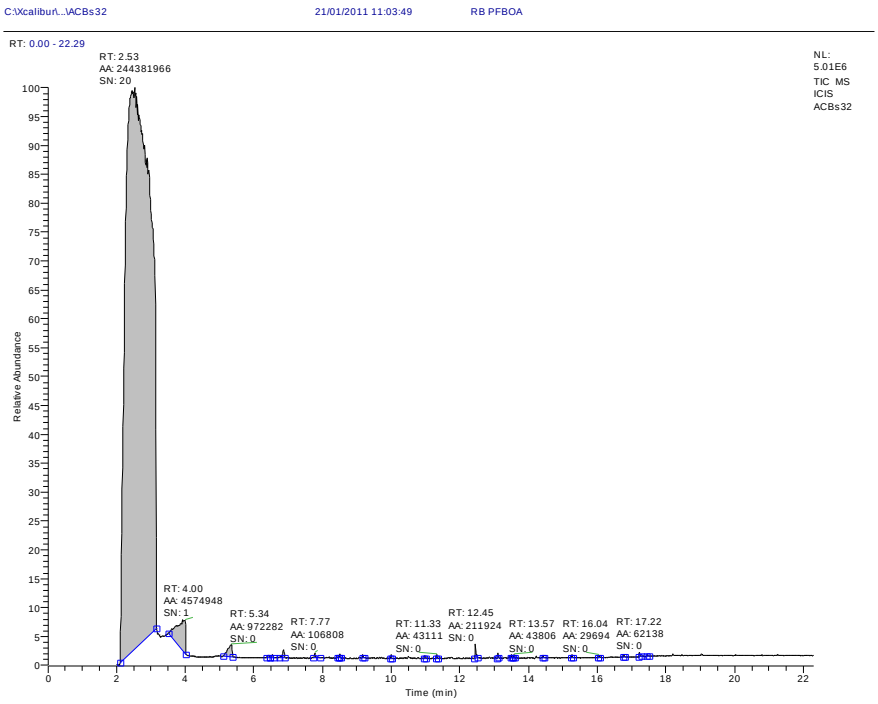


Figure 3.21. Mass spectrum of the peak at 15.7 minutes from PFBHA-derivatised-2-DCB

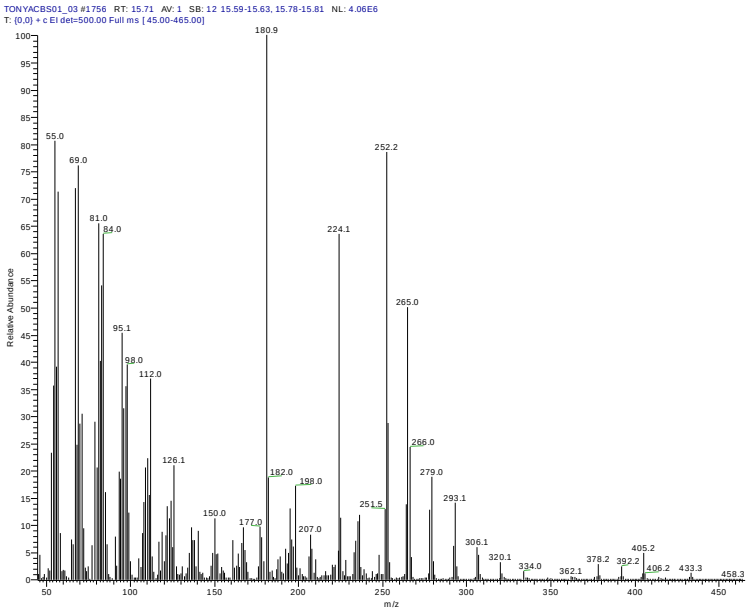
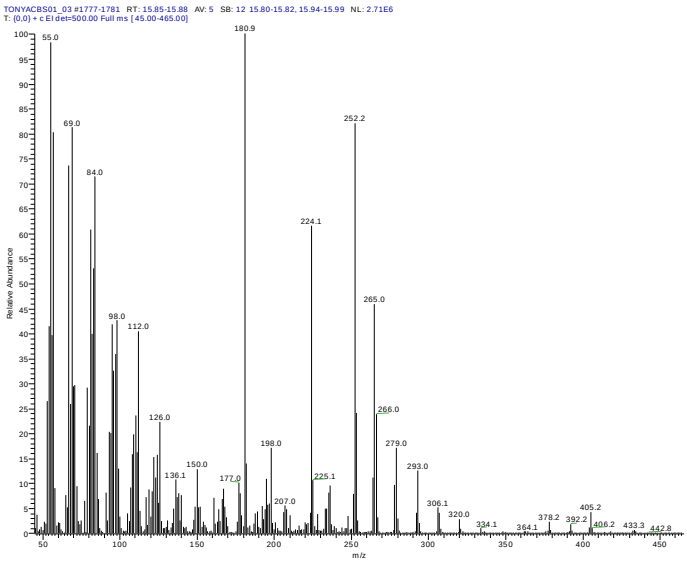


Figure 3.22. Mass spectrum of the peak at 15.9 minutes from PFBHA-derivatised-2-DCB



Figures 3.23 and 3.24 show the TIC and associated mass spectrum for the 50 µg/mL PFBHA-derivatised-CHCH solvent standard showing that this derivatisation reaction was also suitable for the internal standard.

Figure 3.23. Full scan total ion chromatogram for the 1 µg/mL PFBHA-derivatised-CHCH solvent standard

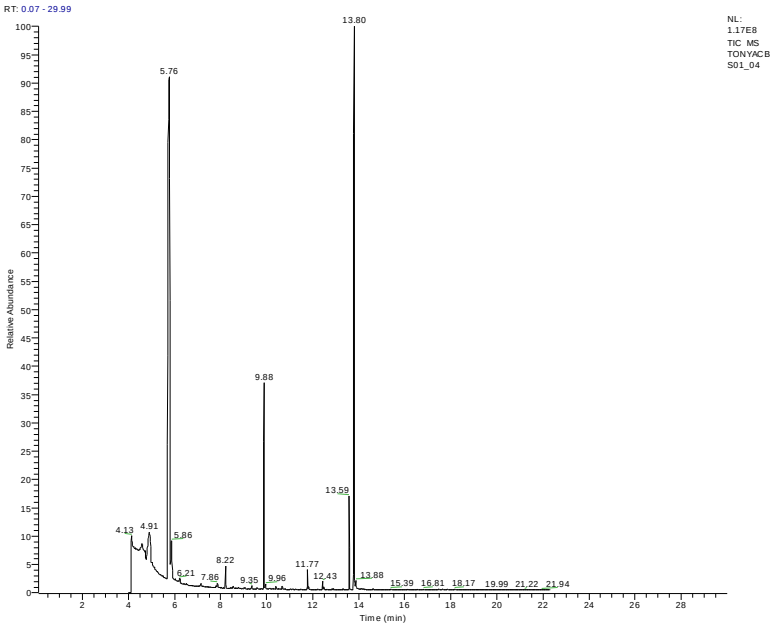
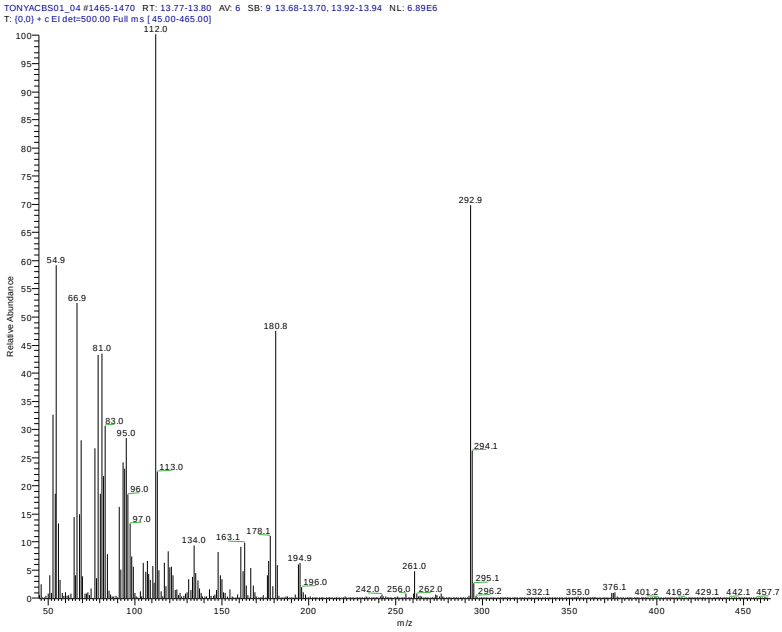


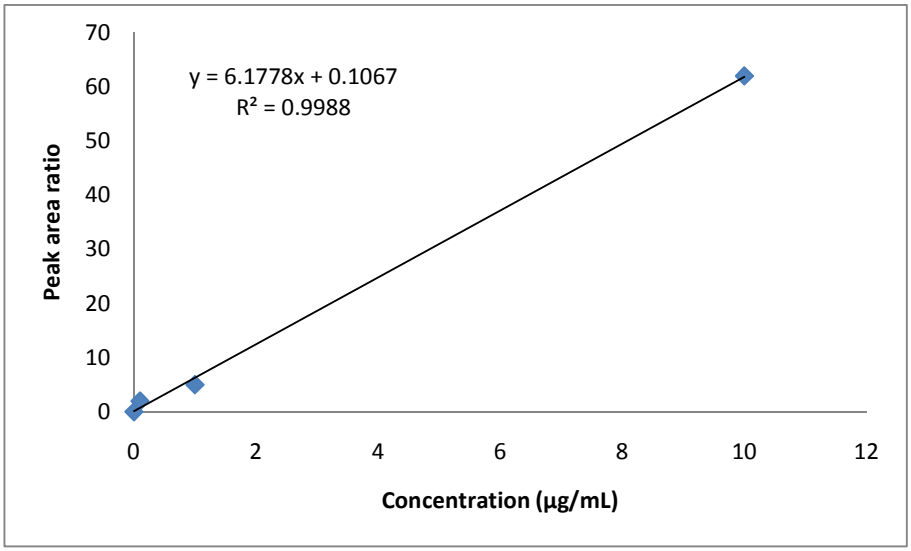
Figure 3.24. Mass spectrum of the peak at 13.8 minutes



A series of solvent standards of PFBHA-derivatised-2-DCB (10, 1.0 and 0.1 µg/mL) was then prepared to determine linearity. The calibration line for the base peak m/z 181 (Figures 3.21 and 3.22) is given in Figure 3.25 and an acceptable correlation

coefficient of 0.9988 was achieved. The 0.1 µg/mL standard was used to determine the limit of detection. This was calculated to be 0.02 µg/mL (20 ng/mL), based on 3 times the signal to noise.

Figure 3.25. Calibration line for a series of PFBHA-2-DCB solvent standards (m/z 181)



3.6 Derivatisation of 2-ACBs followed by analysis by LC-MS

Derivatisation of carbonyl compounds prior to analysis by LC-MS is standard practice to enhance ionisation and increase sensitivity. Derivatisation of aldehydes and ketones using hydrazine reagents and detection by LC-MS has been reported [7] and the reaction schematic is shown in Figure 3.17b for a common derivatising reagent used in LC-MS, 2,4-dinitrophenylhydrazine (DNPH).

3.6.1 Experimental details – DNPH derivatisation reaction for LC-MS

An aqueous solution of DNPH (356 mg/mL) was prepared. Portions of 2-DCB solution (equivalent to 10, 1, 0.1 and 0.01 µg in vial) and cyclohexylcyclohexanone (CHCH) internal standard solution (equivalent to 0.1 µg in vial) were transferred to separate glass vials and the solvent evaporated to dryness under a gentle stream of nitrogen gas. A portion of the DNPH solution (1 mL) and water (700 µL) were added

to each vial, the vials were sealed and left at room temperature for 30 minutes. Dichloromethane (2 mL) was added and the vial shaken. The dichloromethane was transferred to a clean vial and the extraction was repeated. After combining the dichloromethane portions the solution was evaporated to dryness under a gentle stream of nitrogen at 30^{o}C. The residue was re-dissolved in acetonitrile (1 mL) and analysed by LC-TOF-MS and LC-MS/MS.

3.6.2 LC-TOF-MS experimental details

An Agilent LC/MSD TOF (Agilent, Santa Clara, California, USA) consisting of a 1200 Series LC and a Jetstream TOF-MS was used with an Agilent LC Zorbax Eclipse Plus C18 column, 50 mm long, 2.1 mm internal diameter, 1.8 µm particle size, maintained at 50^{o}C in a thermostated column oven. The mobile phase was 0.1% ammonium acetate (A) and methanol (B) with a flow rate of 0.2 mL/min. A mobile phase gradient was used starting at 32% A changing linearly to 75% A by 7 minutes, 80% A by 7.1 minutes, 100% A by 9.5 minutes and held at 100% A until 20 minutes, before the column was re-equilibrated at 32% A for 5 minutes. An injection volume of 3 µL was used. The gas was at 250^{o}C with a flow rate of 5 L/minute, sheath gas flow at 12 L/minutes and nebuliser pressure of 40 psi. The skimmer was at 65 V and the octopole RF was at 250 V. The fragmentor was set at 150 V and mass range was m/z 50 - 1100. The system was used in electrospray ionisation mode and the nozzle voltage was 1000 V. Real time mass correction was performed using a solution including purine (m/z 119.0363) and hexakis (1H,1H,3H-tetrafluoro-pentoxy)-phosphazene (m/z 980.0164).

3.6.3 LC-MS/MS experimental details

A Waters (Hertsford, UK) Alliance 2695 LC system was used with an Agilent LC Zorbax Eclipse Plus C18 column, 50 mm long, 2.1 mm internal diameter, 1.8 µm particle size, maintained at 50^{o}C in a thermostated column oven. The mobile phase was 0.1% ammonium acetate (A) and methanol (B) with a flow rate of 0.2 mL/min. A mobile phase gradient was used starting at 32% A changing linearly to 75% A by 7 minutes, 80% A by 7.1 minutes, 100% A by 9.5 minutes and holding at 100% A until

20 minutes, before the column was re-equilibrated at 32% A for 5 minutes. An injection volume of 3 µL was used.

The MS system was a Micromass (Manchester, UK) Quattro Ultima triple quadrupole mass spectrometer used in negative electrospray ionisation mode. The capillary voltage was 3 kV with a source temperature of 120^{o}C and cone voltage of 35V. Desolvation gas was nitrogen at 360 L/hour and the desolvation temperature was 300^{o}C. Collision induced dissociation was performed using argon. RF lens 1 was set at 20 and RF lens 2 at 0.2. High and low resolution mass 1 were both set at 13.5 with an ion energy of 0.5 and high and low resolution mass 2 were both set at 15.0 with an ion energy of 0.5. MS/MS parameters were optimised for the $[M-H]^-$ adduct.

3.6.4 Results

LC-TOF-MS was chosen as the initial test method as the accurate mass information could be used to determine the molecular adducts produced. In the positive electrospray ionisation mode the expected $[M+H]^+$, $[M+NH4]^+$, $[M+Na]^+$ or $[M+K]^+$ adducts were not detected, as shown in Figure 3.26. None of the peaks present in the extracted ion chromatograms (EICs) were attributed to the correct molecular formula for the DNPH-derivatised-2-DCB (C22H34N4O4) even with the wide 100 ppm mass window. For example Figure 3.27 shows the mass spectrum of the peak at 9.8 minutes in the EIC for m/z 441.2472. It can be seen that the accurate mass of the peak is actually 441.2844, which is 84 ppm difference from the theoretical m/z of 441.2472 for the $[M+Na]^+$ adduct. An acceptable mass error by LC-TOF-MS is 5 ppm or less. The presence of the higher mass peaks, for example m/z 881.5629, in the mass spectrum also support this finding.

Figure 3.26. Extracted ion chromatograms (m/z 419.2653, $[M+H]^+$, m/z 436.2918, $[M+NH4]^+$, m/z 441.2472, $[M+Na]^+$, m/z 457.2212, $[M+K]^+$) in positive mode electrospray LC-TOF-MS for the 10 µg/mL DNPH-derivatised-2-DCB with a 100 ppm mass window

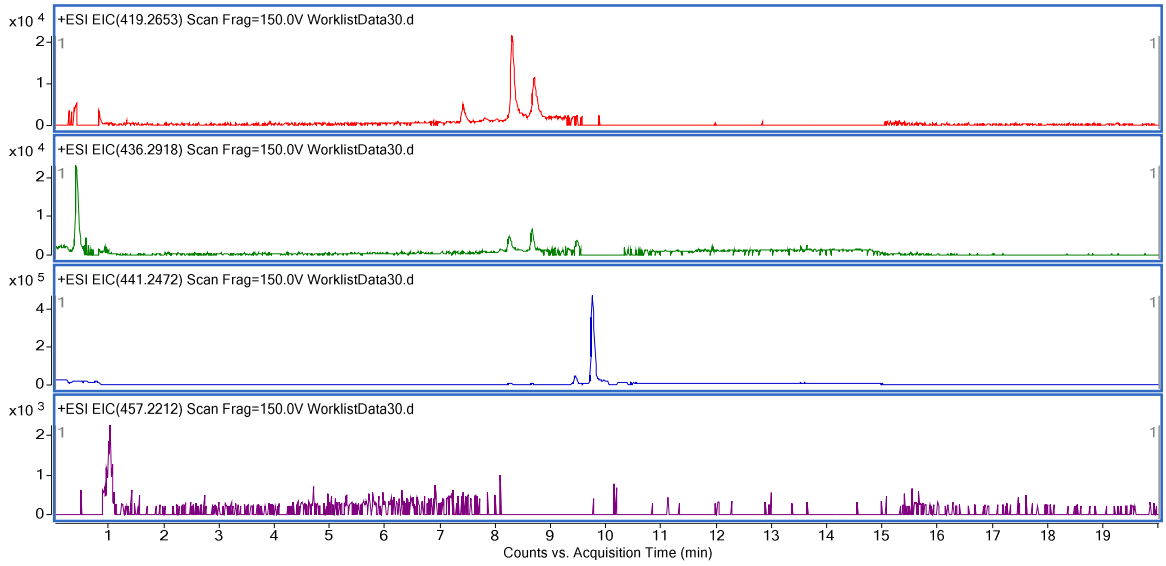
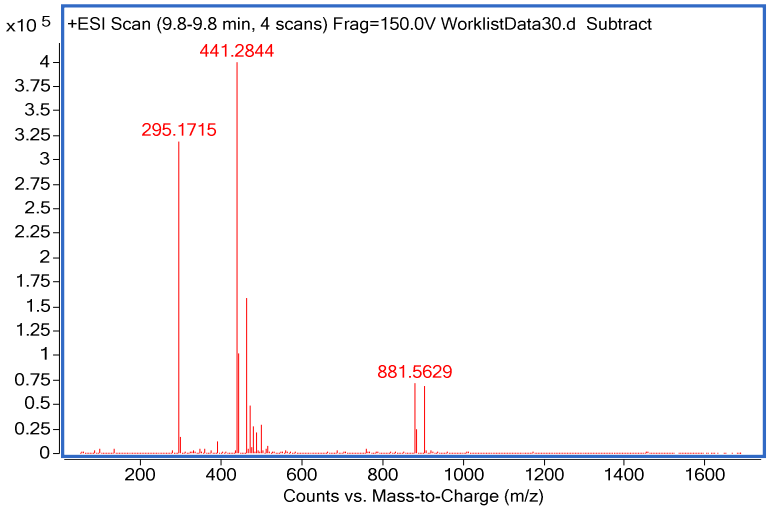


Figure 3.27. TOF mass spectrum of the peak at 9.8 minutes in the positive mode electrospray LC-TOF-MS for the 10 µg/mL DNPH-derivatised-2-DCB solvent standard



In contrast, for LC-TOF-MS in the negative electrospray ionisation mode it was shown that the 2-DCB was successfully derivatised with the $[M-H]^-$ adduct prominent.

Successful detection in the negative ion mode can be explained because of the electron-withdrawing properties of the two nitro groups (by both induction and resonance) will stabilise the negative ion but destabilise the positive ion. Figure 3.28 shows the EIC for a DNPH-derivatised-2-DCB standard (10 µg/mL) (m/z 417.2507, C22H33N4O4). Figure 3.29 shows the associated mass spectrum of the peak.

Figure 3.28. Extracted ion chromatogram (m/z 417.2507, $[M-H]^-$) in negative mode electrospray LC-TOF-MS for the 0.1 µg/mL DNPH-derivatised-2-DCB

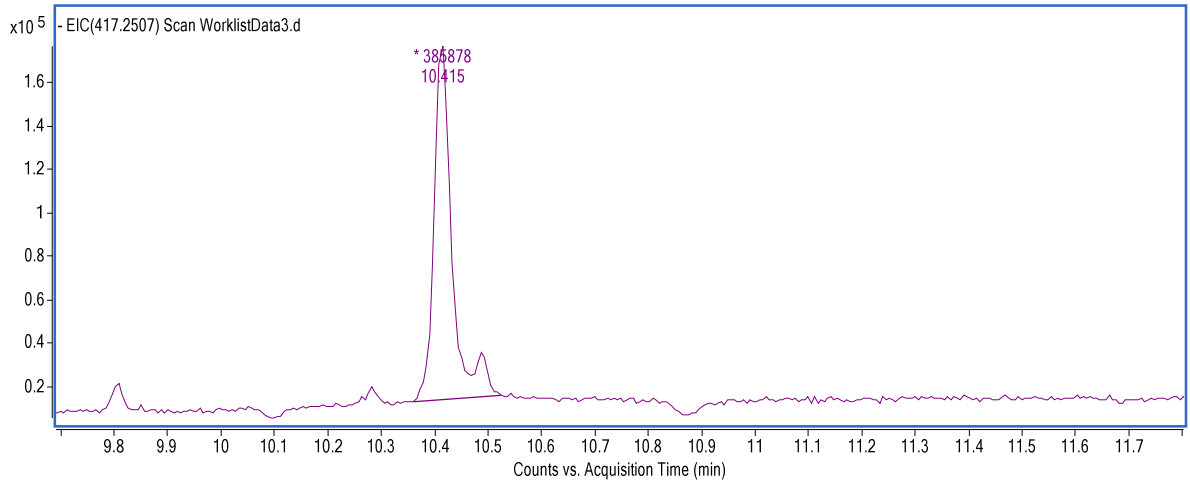
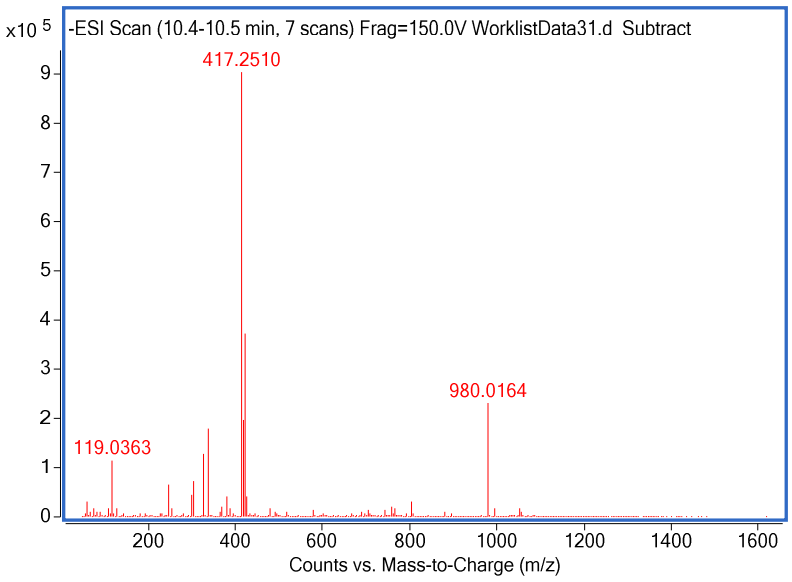


Figure 3.29. Mass spectrum of the peak at 10.4 minutes in the negative mode electrospray LC-TOF-MS for the 10 µg/mL DNPH-derivatised-2-DCB solvent standard



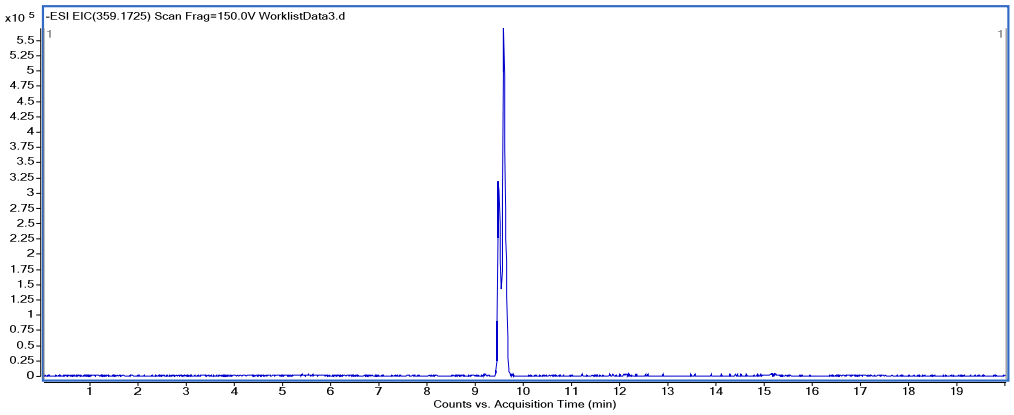
It can be seen that the accurate mass of the peak at 10.4 minutes is 417.2510, which is a mass difference of 0.72 ppm from the theoretical mass (417.2507), showing a very good match and adding confidence to the peak assignment. The masses at m/z 119.0363 and m/z 980.0164 are from residual reference mass solution. The limit of detection for the LC-TOF-MS was then calculated as 0.002 µg/mL, using three times the signal to noise for the 0.1 µg/mL DNPH-derivatised-2-DCB solvent standard.

Figure 3.30 shows the EIC and associated mass spectrum for the DNPH-derivatised-CHCH (internal standard), again successfully derivatised and detected in negative mode electrospray LC-TOF-MS. The mass difference between measured (m/z 359.1721) and theoretical (m/z 359.1725) mass was acceptable for the molecular formula at 1.1 ppm.

The DNPH-derivatised-2-DCB solvent standards were then analysed using LC-MS/MS. The selected reaction monitoring (SRM) transitions were determined and optimised following the same approach as described in section 3.4.4. The most appropriate transitions were 417.2>151.0 for DNPH-derivatised-2-DCB and 359.2>152.0 for the DNPH-derivatised-CHCH. Figure 3.31 shows the SRM chromatograms of the 0.01 µg/mL solvent standard. The limit of detection was calculated to be 0.0005 µg/mL (0.5 ng/mL), three times the signal to noise for the 0.01 µg/mL solvent standard.

Figure 3.30. a) Extracted ion chromatograms (m/z 359.1725, $[M-H]^-$) in negative mode electrospray LC-TOF-MS for the 0.1 µg/mL DNPH-derivatised-2-CHCHand b) associated mass spectrum

a)



b)

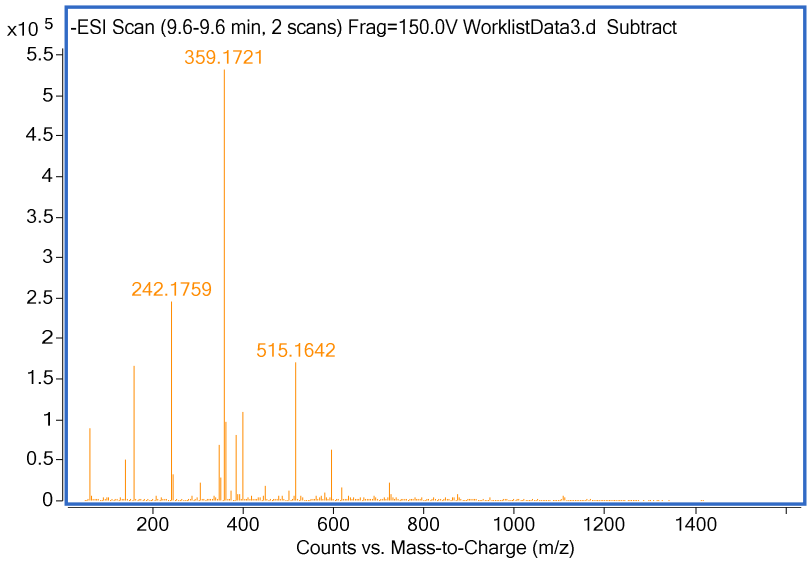
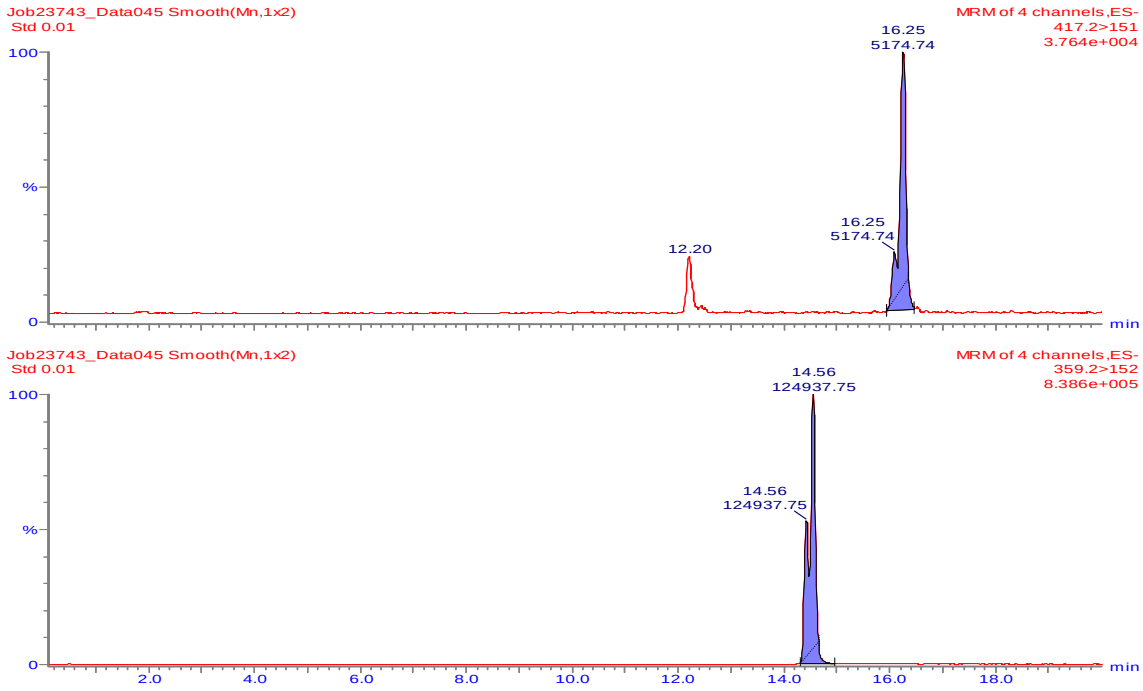


Figure 3.31. LC-MS/MS SRM chromatogram for the 0.01 µg/mL DNPH-derivatised-2-DCB (417.2>151.0) and DNPH-derivatised-CHCH (359.2>152.0)



3.7 Summary

Methods of analysis for 2-DCB, the only commercially available 2-ACB, have been developed by direct measurement of 2-DCB, and following derivatisation, by GC-MS and LC-MS techniques. Table 3.2 shows a comparison of the limits of detection determined for each technique tested.

For underivatised 2-DCB analysis the limit of detection of the GC-MS technique (SIM mode) is fifty times lower (better) than the LC-MS/MS (ESI positive) technique. Taking into account the structure of 2-DCB this is not surprising as the carbonyl chemical functionality and long hydrocarbon chain are both more amenable to GC analysis.

Table 3.2. Comparison of limits of detection for the analytical techniques used for the detection of 2-DCB

Technique	Limit of detection (ng/mL)	
	Without derivatisation	With derivatisation
LC-TOF-MS	Not measured (ESI pos)#	2.0 (DNPH - ESI neg)
LC-MS/MS	30 (ESI pos)	0.5 (DNPH - ESI neg)
GC-MS	1.0 (SIM)	20 (PFBHA - SIM)

Not measured but inherently less sensitive than MS/MS techniques

Derivatisation by PFBHA does not increase the limit of detection for 2-DCB by GC-MS and in fact reduces it approximately thirty-fold. Derivatisation by DNPH increases the limit of detection by the LC-MS techniques, again unsurprisingly taking into account that the aim of the derivatisation reaction is to make the 2-DCB molecule more amenable to LC-MS (or MS/MS) analysis. The limit of detection for the LC-MS/MS is lower than the LC-TOF-MS method, as expected due to the selectivity of the MS/MS technique, and the limit of detection for LC-MS/MS is of the same magnitude as the GC-MS analysis of the underivatised 2-DCB.

It should be noted that the derivatisation process does introduce extra steps into the procedure, increasing the time taken to get results, and there is the potential to introduce impurities and form side reaction products. However these are not a problem due to the selectivity of the MS/MS technique. LC-TOF-MS may also provide added information using the accurate mass of the LC-TOF-MS when authentic standards of the 2-ACBs are not available. In a similar way data from GC-MS when used in full scan mode can be compared to a spectral library to aid the identification of peaks.

When investigating the application of these different methods compared to GC-MS, the method used in the standard method EN1785 and the consequences for testing laboratories, then other practical issues such as cost and availability of instrumentation needs to be considered. Most Official Control Laboratories and other food testing laboratories will have access to GC-MS and LC-MS/MS instruments.

However the more expensive LC-TOF-MS equipment is not readily used for regular food testing.

In summary both GC-MS (without derivatisation) and LC-MS/MS (with derivatisation) would be available in most food testing laboratories, both have a similar limit of detection (approximately 1 ng/mL) and both are suitable for the low level detection of 2-DCB.

4.0 MODEL STUDIES (OBJECTIVE 03)

4.1 Introduction

Palmitic acid (PA), stearic acid (SA), oleic acid (OA) and linoleic acid (LA) are the major fatty acids that comprise the triacylglycerols found in foods. As described in the literature review in Chapter 2 it is from these compounds that the 2-ACBs are formed upon irradiation and the proposed mechanisms of formation are given in Figures 4.1 and 4.2.

Figure 4.1. Formation of ACBs from triacylglycerols

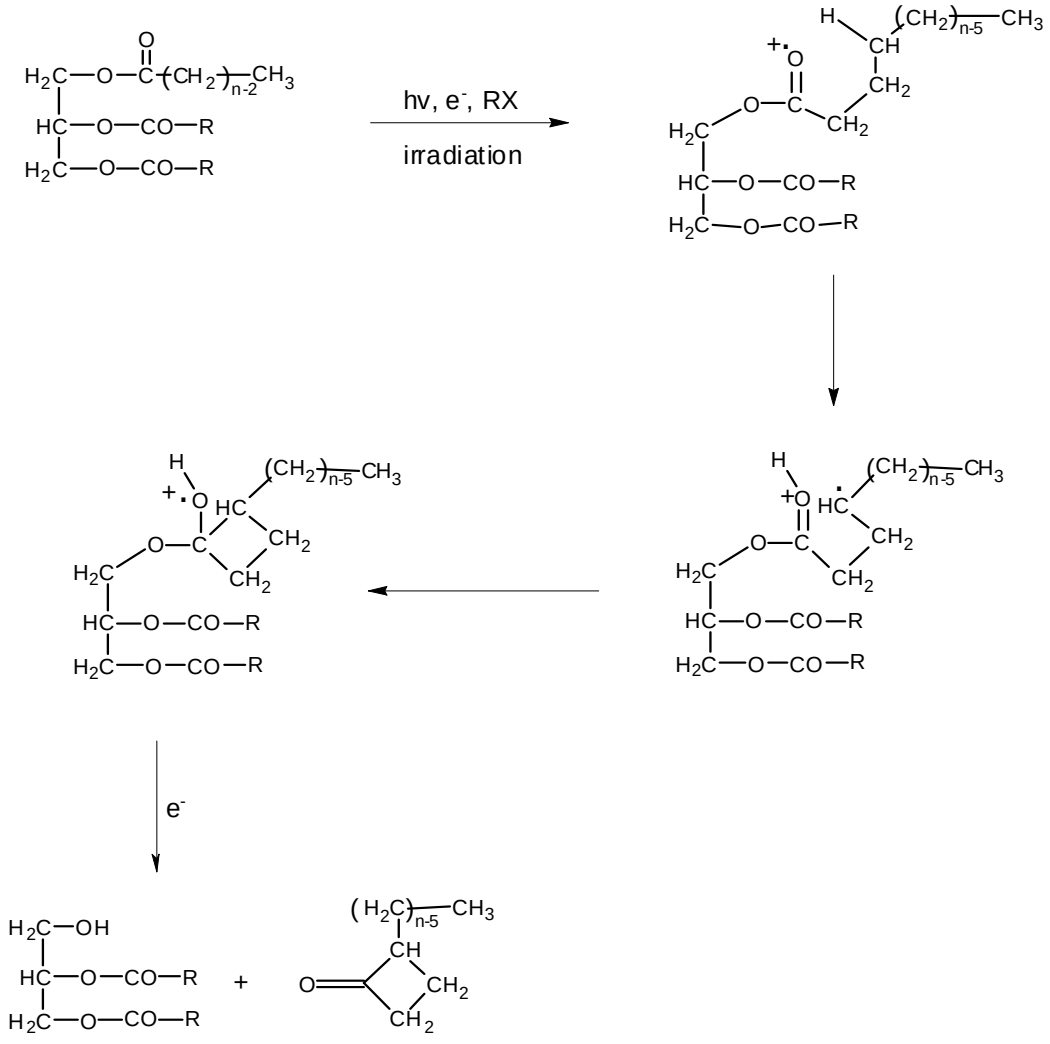
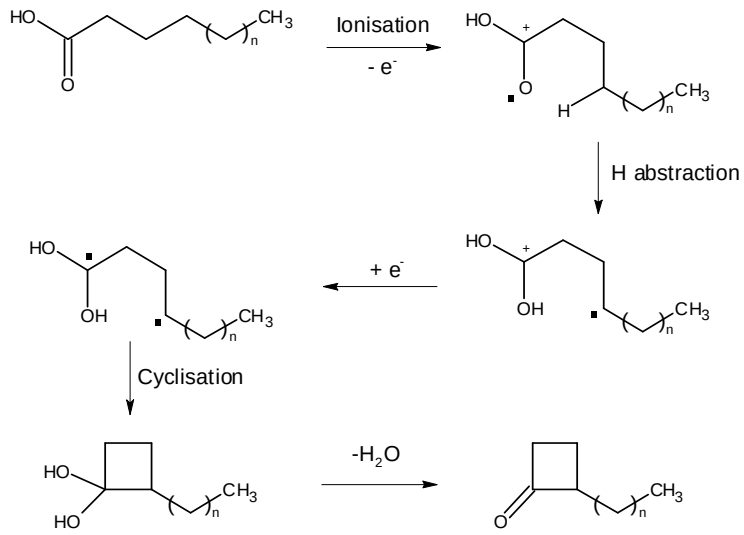


Figure 4.2. Formation of 2-ACBs from fatty acids



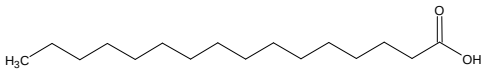
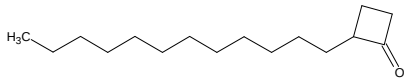
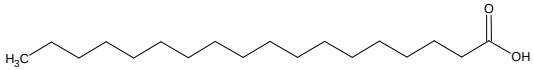
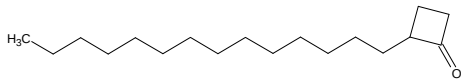
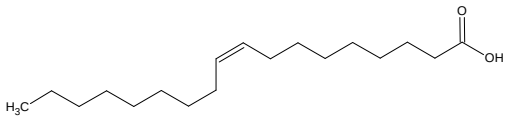
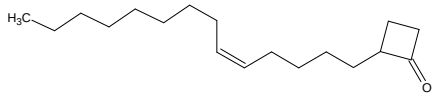
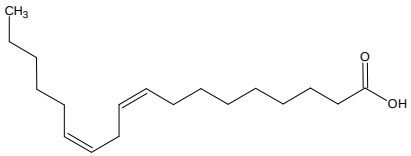
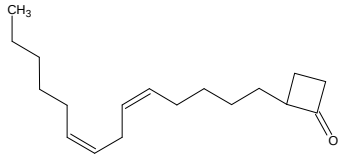
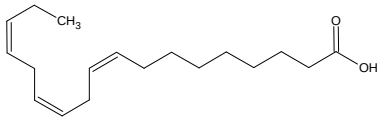
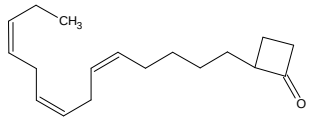
This chapter describes model studies carried out using triacylglycerols and fatty acids irradiated at different doses by different irradiation sources, and also treated by non-irradiation based food processing techniques.

4.2 Irradiation of triacylglycerols and free fatty acids (Task 01)

4.2.1 Samples used in the experiments

The fatty acids tested were palmitic acid (99%, PA), stearic acid (98%, SA), oleic acid (99%, OA), linoleic acid (97%, LA) and linolenic acid (99%, LLA). All of these were purchased from Sigma (Gillingham, UK). These were chosen as they relate to the triacylglycerols found most commonly in foods and also because they cover a range of saturation. PA and SA are fully saturated (no carbon carbon double bonds), OA has one double bond, LA has two and LLA has three carbon carbon double bonds. Table 4.1 gives the molecular formula, chemical structure and abbreviation of the fatty acids irradiated as well as the corresponding 2-ACB formed and its molecular formula, chemical structure and abbreviation used.

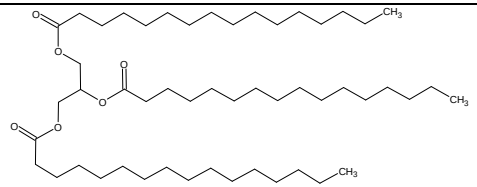
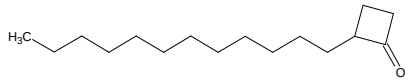
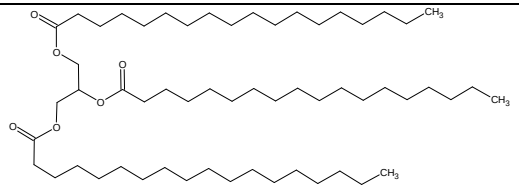
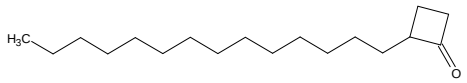
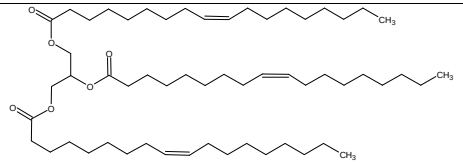
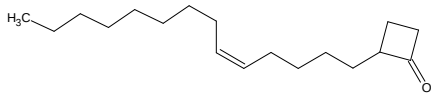
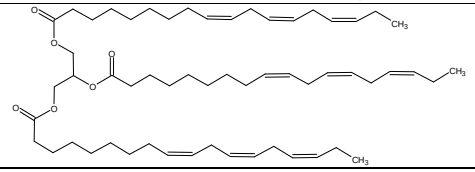
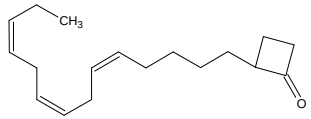
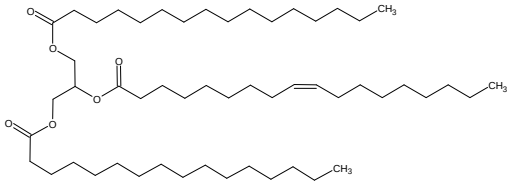
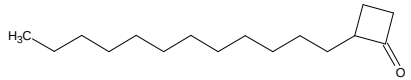
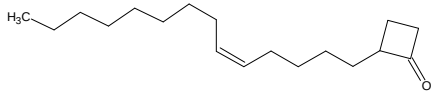
Table 4.1 Details of the fatty acids tested and irradiation products expected

Compound	Code	Formula	Structure	2-ACB expected	Code	Formula	Structure
palmitic acid	PA	C16H32O2		2-Dodecyl cyclobutanone	2-DCB	C16H30O	
stearic acid	SA	C18H36O2		2-Tetradecyl cyclobutanone	2-TCB	C18H34O	
oleic acid	OA	C18H34O2		2-(5'-tetradecenyl) cyclobutanone	TECB	C18H32O	
linoleic acid	LA	C18H32O2		2-(5',8'-tetradecadienyl) cyclobutanone	5',8'-TCB	C18H30O	
linolenic acid	LLA	C18H30O2		2-(5',8',11'-tetradecatrienyl) cyclobutanone	5',8',11'-TCB	C18H28O	

The triacylglycerols tested were glyceryl trioleate (>99%, GTO), glyceryl trilinolenate (99%, GTL), glyceryl tripalmitate (~99%, GTP), glyceryl tristearate (~99%, GTS) and 1,3-dipalmitoyl-2-oleoylglycerol (DPOG, 99%). All of these were purchased from Sigma (Gillingham, UK). Table 4.2 gives the molecular formula, chemical structure and abbreviation of the triacylglycerols irradiated as well as the corresponding 2-ACB formed and its molecular formula, chemical structure and abbreviation used.

The range of fatty acids and triacylglycerols were chosen because they break down into 2-DCB, the only 2-ACB where an authentic standard is available (PA, GTP); 2-TCB, the other common 2-ACB reported in the literature (SA, GTS); or they form unsaturated 2-ACBs from the unsaturated fatty acids and triacylglycerols (OA, LA, LLA, GTO, GTL). DPOG was also used as it is the main lipid found in chicken, a commodity likely to be processed by irradiation. However, this compound was expensive compared to the others and only available in small quantities and the experiments were reduced in scale accordingly.

Figure 4.2. Details of the triacylglycerols tested and irradiation products expected

Compound	Code	Formula	Structure	2-ACB expected	Code	Formula	Structure
glyceryl tripalmitate	GTP	C51H98O6		2-dodecyl cyclobutanone	2-DCB	C16H30O	
glyceryl tristearate	GTS	C57H110O6		2-tetradecyl cyclobutanone	2-TCB	C18H34O	
glyceryl trioleate	GTO	C57H104O6		2-(5'-tetradecenyl) cyclobutanone	TECB	C18H32O	
glyceryl trilinolenate	GTL	C57H92O6		2-(5',8',11'-tetradecatrienyl) cyclobutanone	5',8',11'-TCB	C18H28O	
1,3-dipalmitoyl-2-oleoylglycerol	DPOG	C53H100O6		2-dodecyl cyclobutanone	DCB	C16H30O	
				2-(5'-tetradecenyl) cyclobutanone	TECB	C18H32O	

4.2.2 Experimental

4.2.2.1 Irradiation experiments

The irradiation experiments were carried out by Isotron (Daventry) who have worked on another FSA sponsored irradiation project (FSA project A03068, Literature review, analytical screening and chemical migration studies on irradiated food packaging) and have previously carried out investigations to determine the relationship between the dose measured inside the empty vials and that measured outside by dosimeters, as the vials used for the experiments did not have dosimeters inside once the samples were present. A correction factor was determined and was applied to the doses measured for the samples.

Table 4.3 gives a summary of the doses used. The original plan was to irradiate each sample at five doses by both gamma ray and electron beam irradiation. However, during method development at Isotron (Daventy, UK) it became apparent that the two smallest doses, 0.3 and 0.5 kGy could not be achieved by gamma irradiation on the instrumentation available. The target dose of 2 kGy by EB was closer to 3 kGy (2.7-3.1) when measured.

Table 4.3. Target irradiation dose and actual dose measured

Irradiation type	Target dose (kGy)	Actual dose measured (kGy)
Gamma	1	1.1
	2	2.1
	5	5.1
Electron beam	0.3	0.27 – 0.34
	0.5	0.46 – 0.53
	1	0.93 – 1.1
	2	2.7 – 3.1
	5	4.5 – 5.1

For the irradiation experiments, the fatty acids and triacylglycerols (3 mg for DPOG, 50 mg for all others) were put into 10 mL glass vials and were capped and crimped shut. A number of empty vials were also capped and irradiated at each dose to act as procedural blank samples during analysis, allowing any interference produced from irradiation of the glass, caps or septa to be identified. All the samples were stored in the freezer at -20^{o}C prior to and following irradiation whilst awaiting analysis.

4.2.2.2 GC-MS

Hexane (5 mL) was added to the irradiated sample and was shaken overnight. A Florisil column (20 cm) was prepared using deactivated Florisil and hexane and the hexane from the irradiated sample was added to the top of the column. This was allowed to pass onto the column and further hexane (150 mL) was added. The eluent was collected following elution at a flow rate of 2 – 5 mL/minute. 1% Diethyl ether in hexane (150 mL) was then used to elute the 2-DCB and the eluent collected. This was rotary evaporated to dryness under a gentle stream of nitrogen at 40^{o}C. The residue was re-dissolved in a solution of CHCH (200 µL of 5 µg/mL) prior to GC-MS analysis.

The samples were analysed by GC-MS using an Agilent 6980N gas chromatograph (Agilent, Palo Alto, CA, USA) coupled with an Agilent 5973inert mass selective detector. Splitless injection of 1 µL of solution was carried out into a DB-5MS capillary column (30 m x 250 µm i.d., 0.25 µm film thickness; J & W Scientific, Folson, Ca, USA). Following injection the oven was held at 55℃ for 1 minute and then raised at 15℃/minute to 300℃. The injector was held at 280℃. Helium (1 mL/min constant flow) was employed as the carrier gas. The MS was operated in electron impact mode with scanned monitoring between 40 - 600 amu.

4.2.2.3 LC-TOF-MS

A portion of the DNPH solution (356 mg/L, 1 mL) and water (700 µL) were added to the irradiated sample, the vials were sealed and left at room temperature for 30 minutes. Dichloromethane (2 mL) was added and the vial shaken. The dichloromethane was transferred to a clean vial and the extraction was repeated.

After combining the dichloromethane portions the solution was evaporated to dryness under a gentle stream of nitrogen at 30^{o}C. The residue was re-dissolved in acetonitrile (1 mL) and analysed by LC-TOF-MS.

An Agilent LC/MSD TOF (Agilent, Santa Clara, California, USA) consisting of a 1200 Series LC and a Jetstream TOF-MS was used with an Agilent LC Zorbax Eclipse Plus C18 column, 50 mm long, 2.1 mm internal diameter, 1.8 µm particle size, maintained at 50^{o}C in a thermostated column oven. The mobile phase was 0.1% ammonium acetate (A) and methanol (B) with a flow rate of 0.2 mL/min. A mobile phase gradient was used starting at 32% A changing linearly to 75% A by 7 minutes, 80% A by 7.1 minutes, 100% A by 9.5 minutes and held at 100% A until 20 minutes, before the column was re-equilibrated at 32% A for 5 minutes. An injection volume of 3 µL was used. The gas was at 250^{o}C with a flow rate of 5 L/minute, sheath gas flow at 12 L/minutes and nebuliser pressure of 40 psi. The skimmer was at 65 V and the octopole RF was at 250 V. The fragmentor was set at 150 V and mass range was m/z 50 - 1100. The system was used in electrospray ionisation mode and the nozzle voltage was 1000 V. Real time mass correction was performed using a solution including purine (m/z 119.0363) and hexakis (1H,1H,3H-tetrafluoro-pentoxy)-phosphazene (m/z 980.0164).

4.2.2.4 LC-MS/MS

Samples were derivatised using DNPH as described above. An Agilent (Manchester, UK) Infinity LC system was used with a Zorbax column (C18), 150 mm long, 2.1 mm internal diameter, 3.5 µm particle size, maintained at 30^{o}C in a thermostated column oven. The mobile phase was 0.1% acetic acid in water (A) and methanol (B) with a flow rate of 0.2 mL/min. A mobile phase gradient was used starting at 10% B changing linearly to 100% B over 10 minutes before the column was re-equilibrated at 10% B for 2 minutes. An injection volume of 20 µL was used.

The MS system was an Agilent (Manchester, UK) 6490 triple quadrupole mass spectrometer used in negative ion ESI. The capillary voltage was 3 kV with a source temperature of 120^{o}C and cone voltage of 35V. Desolvation gas was nitrogen at 700 L/hour and the desolvation temperature was 300^{o}C. Collision induced dissociation was performed using argon. RF lens 1 was set at 20 and RF lens 2 at 0.2. High and

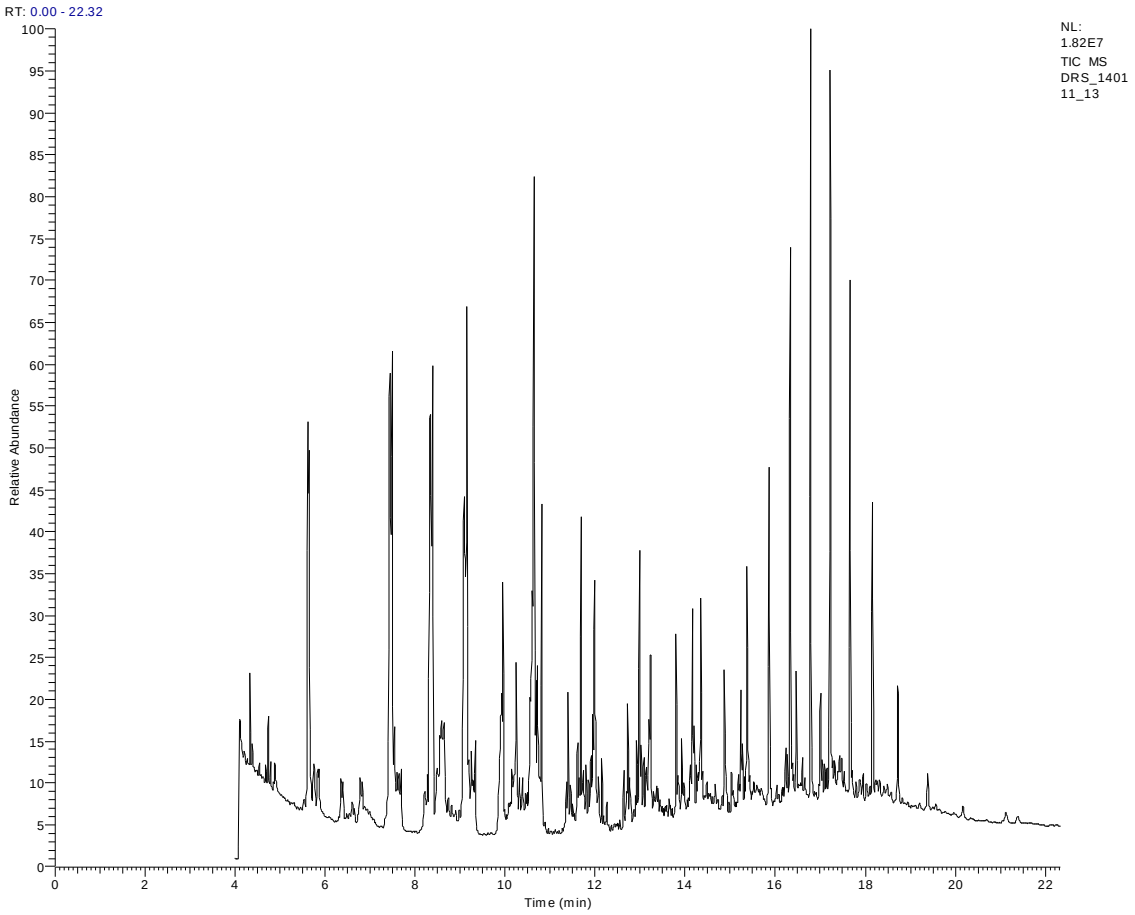
low resolution mass 1 were both set at 13.5 with an ion energy of 0.5 and high and low resolution mass 2 were both set at 15.0 with an ion energy of 0.5.

4.2.3 Results and discussion

4.2.3.1 GC-MS

The EN1785 standard method uses GC-MS analysis for both 2-DCB and 2-TCB and it was determined to have a low limit of detection in Chapter 3 so this approach was chosen to analyse the irradiated samples. In an attempt to speed up analysis extracts of irradiated GTP (gamma, 5 kGy) were tested directly without Florosil clean-up. The 5 kGy sample was chosen as this was the highest irradiation dose tested and the one most likely to produce the highest concentration of 2-DCB, for which there is an authentic standard available. The TIC is shown in Figure 4.3 and it can be seen to contain many peaks. When the data was interrogated the expected peak for 2-DCB could not be detected.

Figure 4.3. Total ion chromatogram of irradiated PA (gamma, 5 kGy), not cleaned-up by Florosil column chromatography

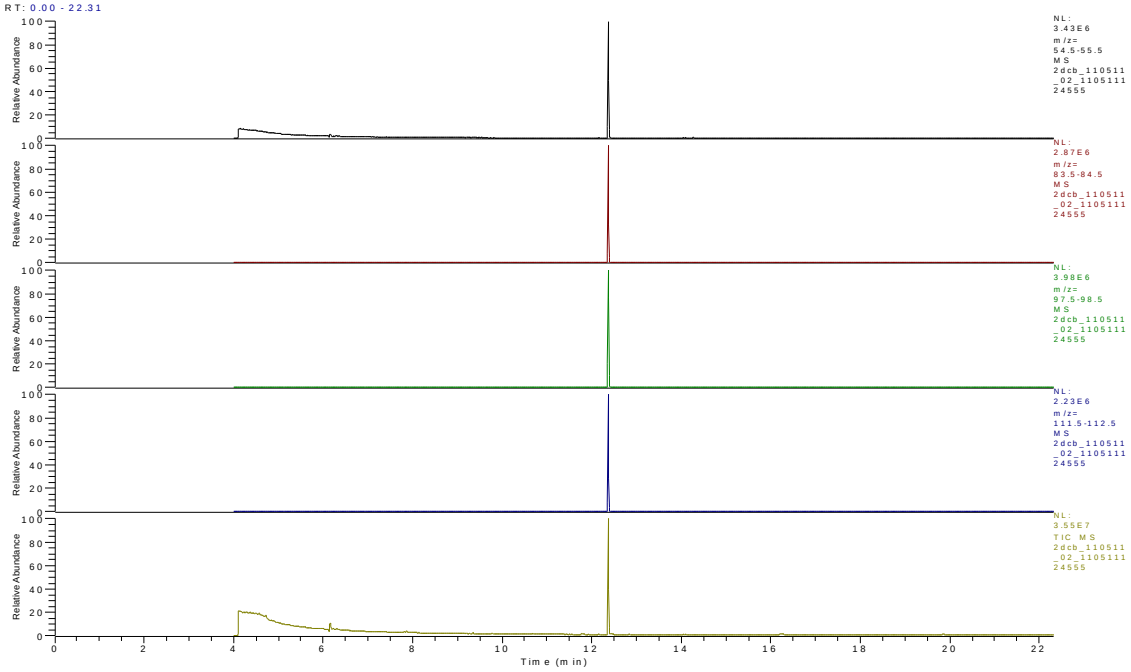


A 2-DCB solvent standard was passed through the clean-up procedure and was then analysed by GC-MS. Figure 4.4 shows the EICs at the expected m/z values for 2-DCB and the associated mass spectrum. A cleaned-up extract of the GTP (gamma, 5 kGy) was also tested, see Figure 4.5. For the solvent standard there is a clear 2-DCB peak at 12.35 minutes and the mass spectrum is as expected. For the irradiated GTP sample it can be seen that the 2-DCB peak is present at 12.35 minutes too and the mass spectrum is consistent indicating the method is working correctly. This suggests that components of the uncleaned-up sample were suppressing ionisation in the GC-MS and by passing the sample down the chromatographic clean-up column these have been removed. Following this the GTP sample irradiated at 5 kGy by electron beam irradiation was analysed and the results

are shown in Figure 4.6. This again shows successful irradiation of the GTP to form 2-DCB.

Figure 4.4. a) GC-MS chromatograms (EIC at m/z 55, 84, 98, 112 and TIC) of a cleaned-up 2-DCB standard (0.1 µg/mL) and b) mass spectrum of the 2-DCB peak at 12.35 minutes

a)



b)

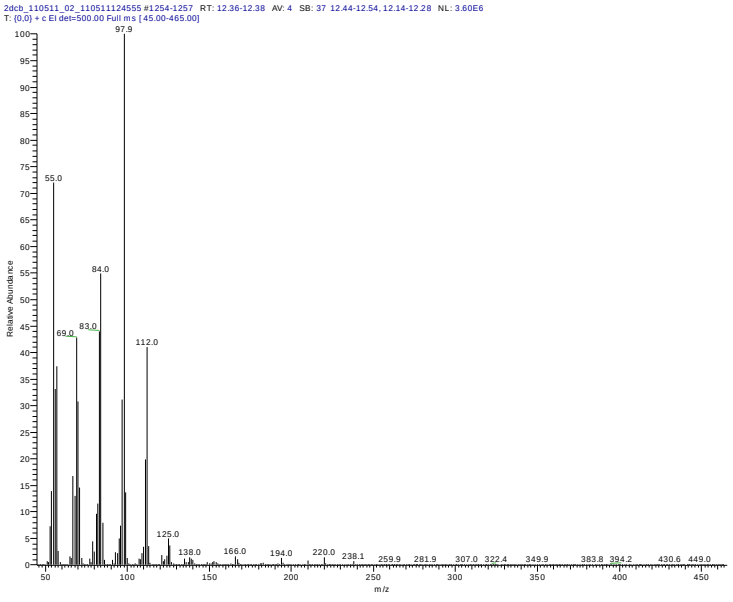
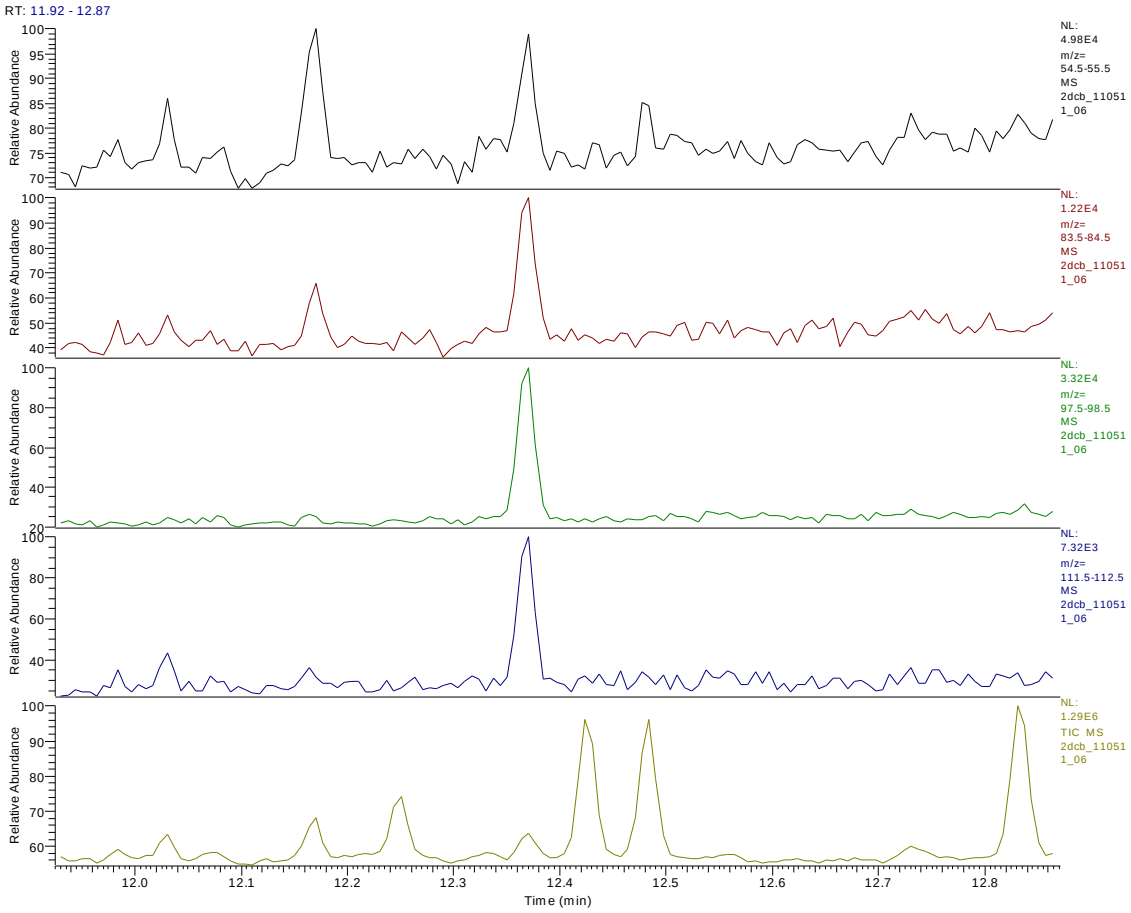


Figure 4.5. a) GC-MS chromatograms (EIC at m/z 55, 84, 98, 112 and TIC) of irradiated GTP (gamma, 5 kGy), b) mass spectrum of the peak at 12.35 minutes

a)



b)

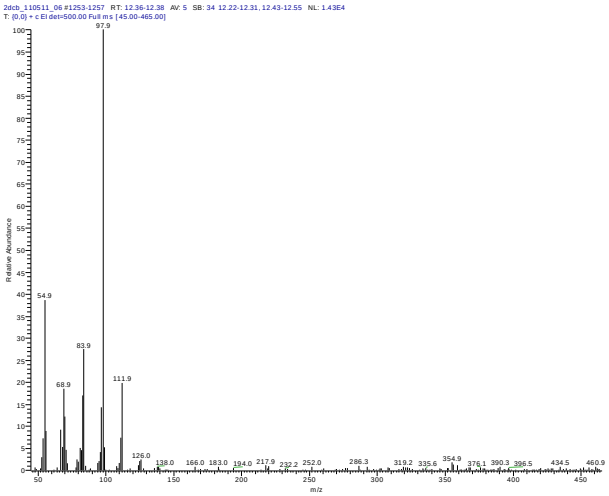
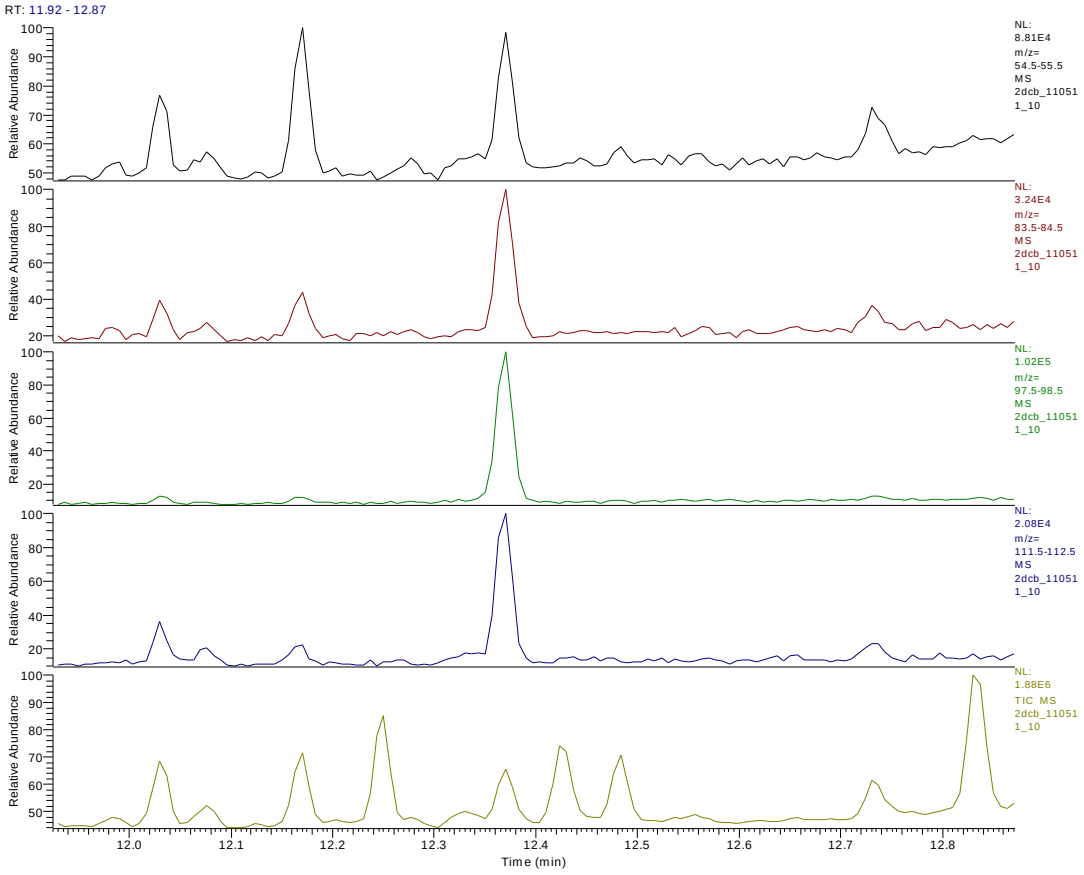
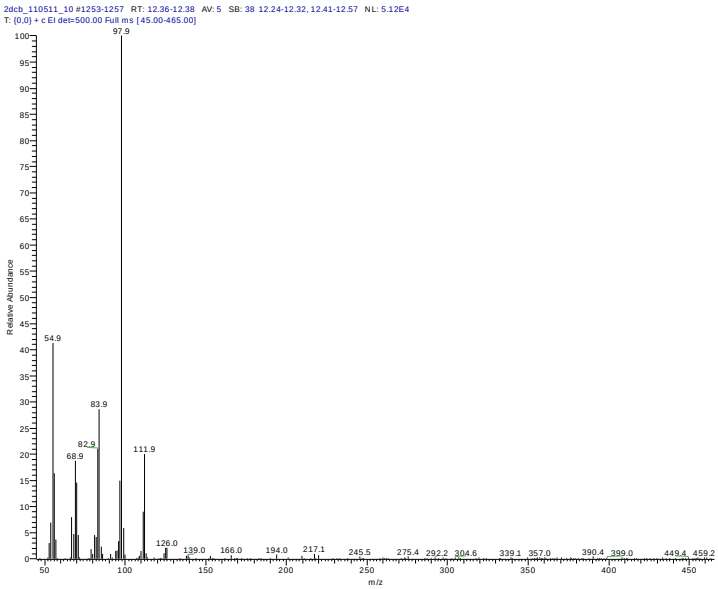


Figure 4.6. a) GC-MS chromatograms (EIC at m/z 55, 84, 98, 112 and TIC) of irradiated GTP (EB, 5 kGy), b) mass spectrum of the peak at 12.35 minutes

a)



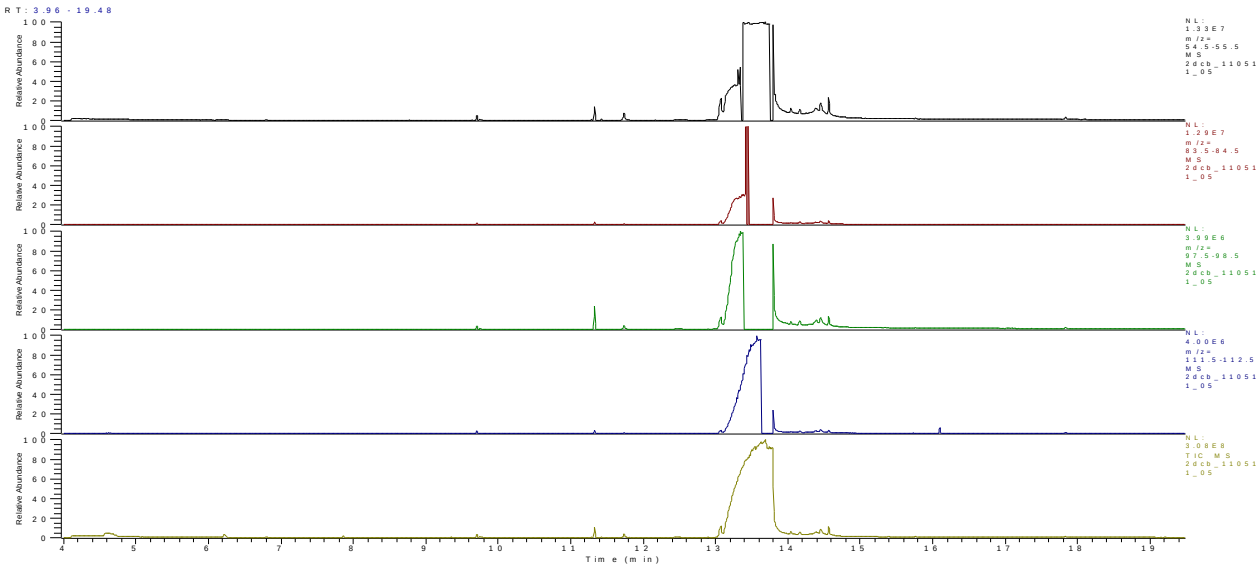
b)



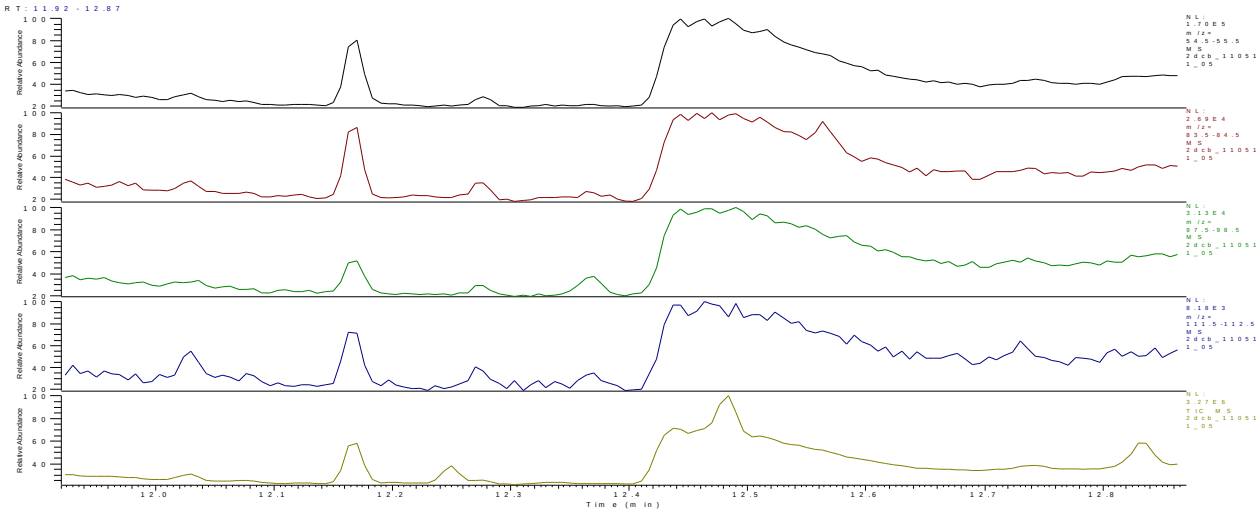
Figures 4.7 to 4.10 show the GC-MS chromatograms for the other samples tested which were expected to produce 2-DCB upon irradiation (PA and DPOG). It can be seen that for both PA samples (EB and gamma at 5 kGy) that a small peak is present at the expected retention time but that the mass spectral data (Figure 4.7c) does not confirm identity as 2-DCB, presumably because of the low amount of 2-DCB present. A large peak at 13-14 minutes is also present and the mass spectrum (Figure 4.7d) suggests that this is due to unreacted PA. The irradiated DPOG chromatograms again show a small peak in the expected EIC for 2-DCB and this time the mass spectral data confirms identity. Since only 3 mg of DPOG was irradiated compared with 50 mg of the other lipids, the effective detection limit is lowered about seventeen-fold (50/3) by comparison. Thus the detectable and confirmed 2-DCB peak shows it is formed in relatively good yield from DPOG.

Figure 4.7. a) GC-MS chromatograms (EIC at m/z 55, 84, 98, 112 and TIC) of irradiated palmitic acid (gamma ray, 5 kGy), full scale, b) zoomed in and mass spectrum of the peak at c) 13.5 minutes and d) 12.35 minutes

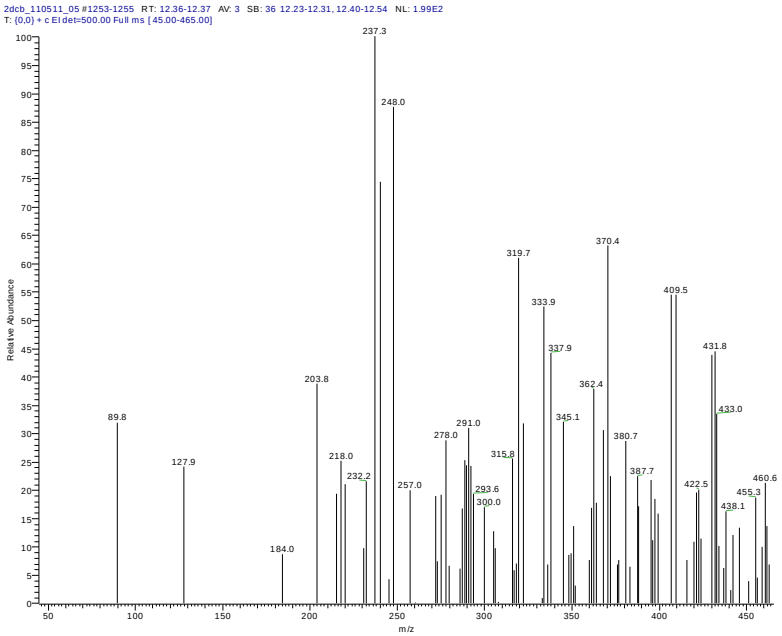
a)



b)



c)


2dcb_110511_05 #1253-1255 RT: 12.36-12.37 AV: 3 SB: 36 12.23-12.31, 12.40-12.54 NL: 1.99E2
T: {0,0} + c EI det=500.00 Full ms [45.00-465.00]
Relative Abundance
m/z
237.3
248.0
319.7
370.4
409.5
333.9
337.9
431.8
203.8
362.4
433.0
89.8
345.1
291.0
278.0
380.7
315.8
127.9
387.7
218.0
232.2
257.0
422.5
460.6
293.6
455.3
300.0
438.1
184.0

d)

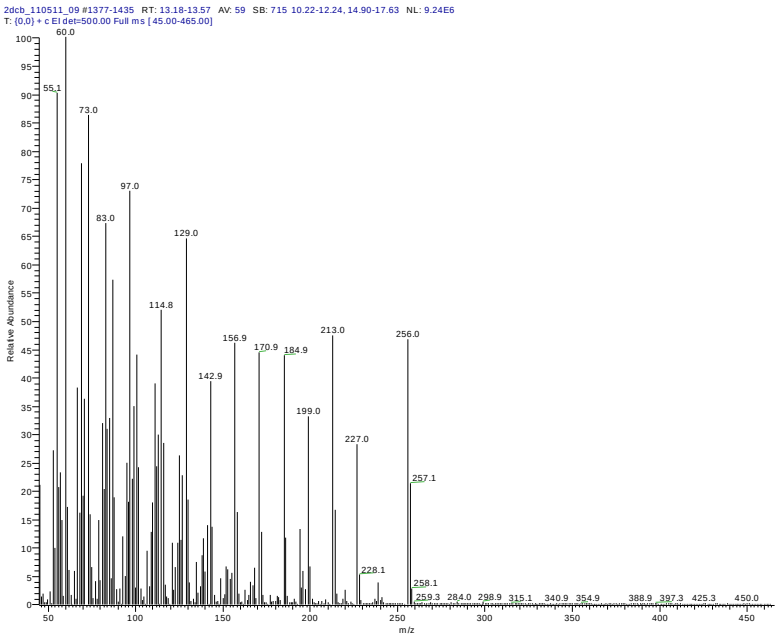
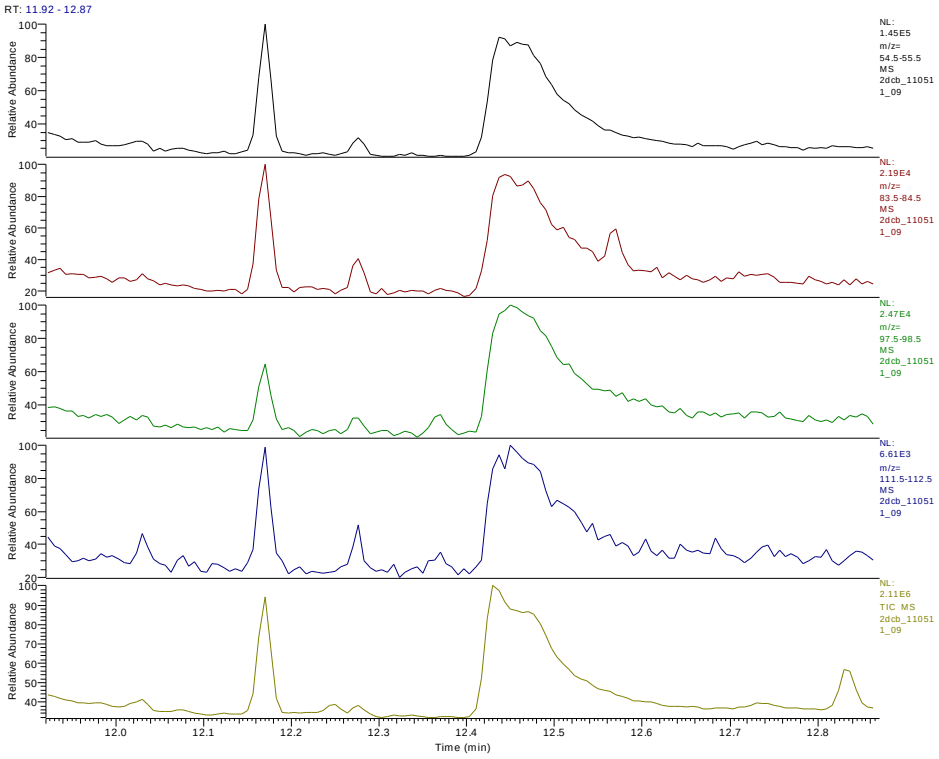

2dcb_110511_09 #1377-1435 RT: 13.18-13.57 AV: 59 SB: 715 10.22-12.24, 14.90-17.63 NL: 9.24E6
T: {0,0} + c EI det=500.00 Full ms [45.00-465.00]
Relative Abundance
m/z
60.0
55.1
73.0
97.0
83.0
129.0
114.8
213.0
256.0
156.9
170.9
184.9
142.9
199.0
227.0
257.1
228.1
258.1
259.3
284.0
298.9
315.1
340.9
354.9
388.9
397.3
425.3
450.0

Figure 4.8. a) GC-MS chromatograms (EIC at m/z 55, 84, 98, 112 and TIC) of irradiated palmitic acid (EB, 5 kGy), b) mass spectrum of the peak at 12.35 minutes

a)



b)

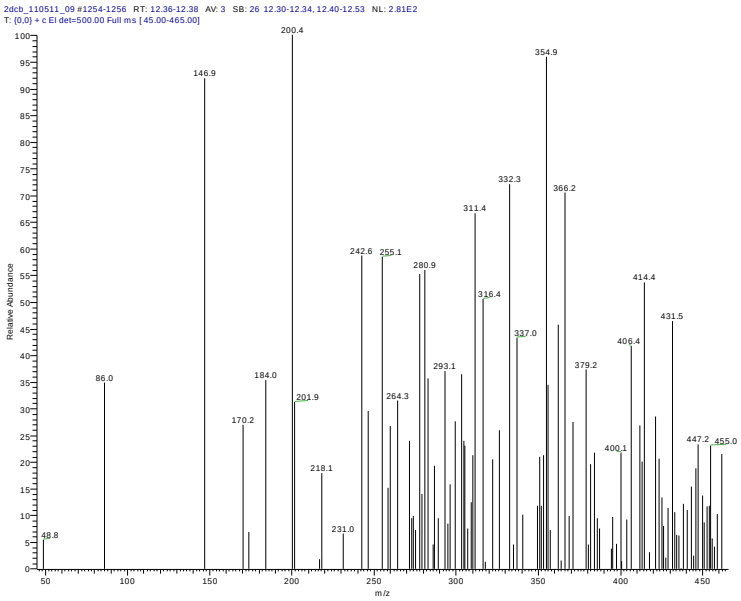
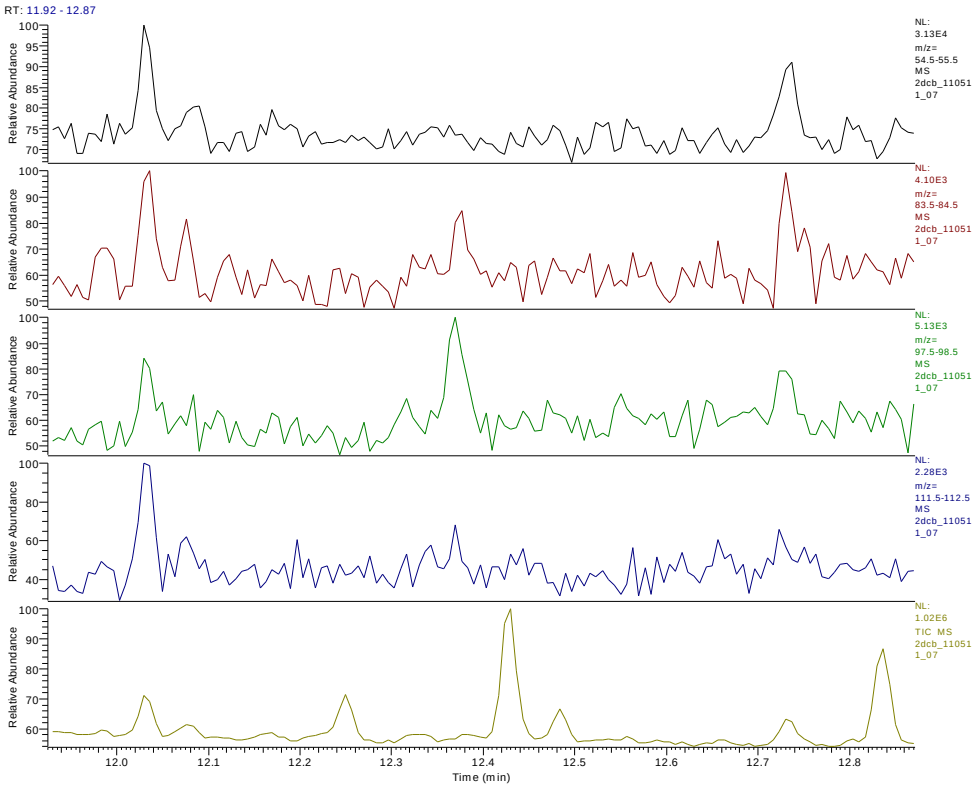


Figure 4.9. a) GC-MS chromatograms (EIC at m/z 55, 84, 98, 112 and TIC) of irradiated DPOG (gamma, 5 kGy), b) mass spectrum of the peak at 12.35 minutes

a)



b)

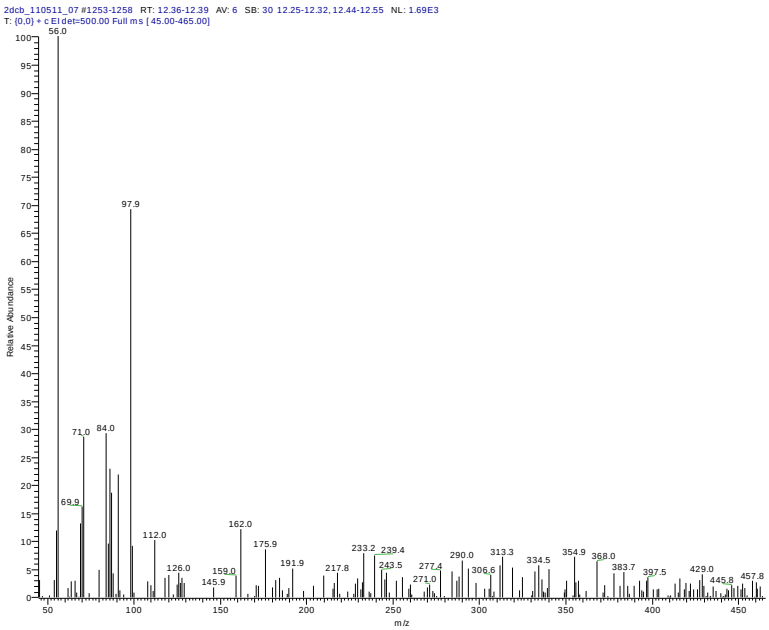
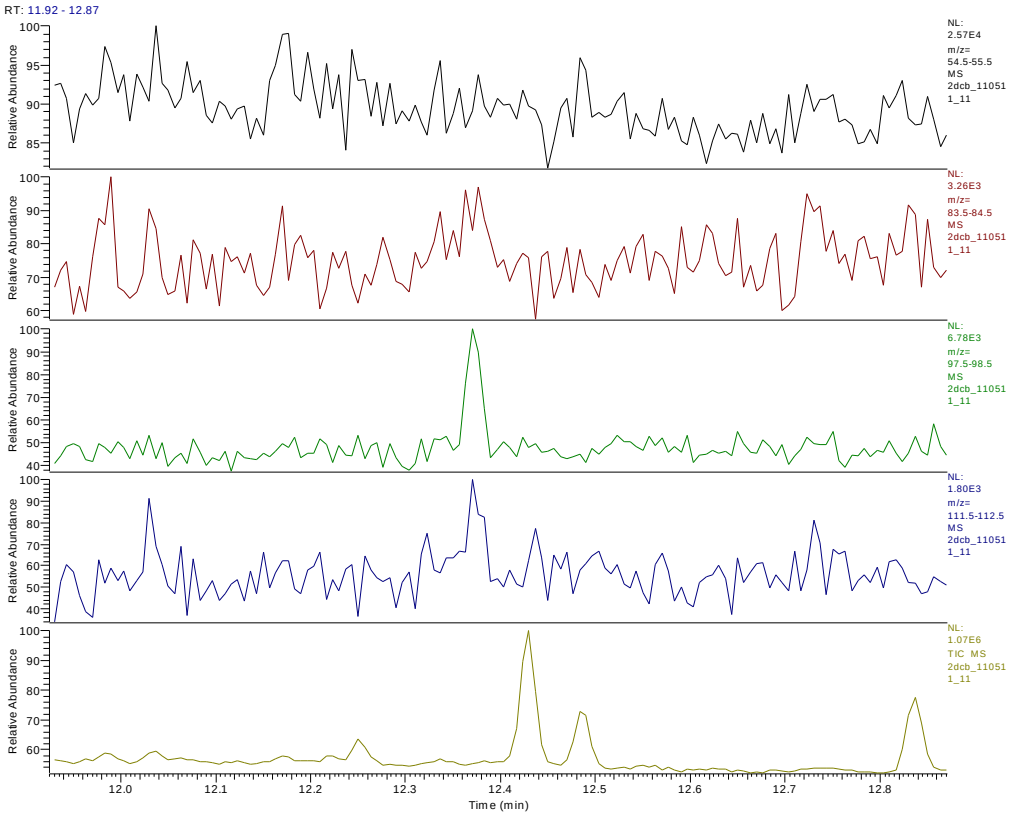
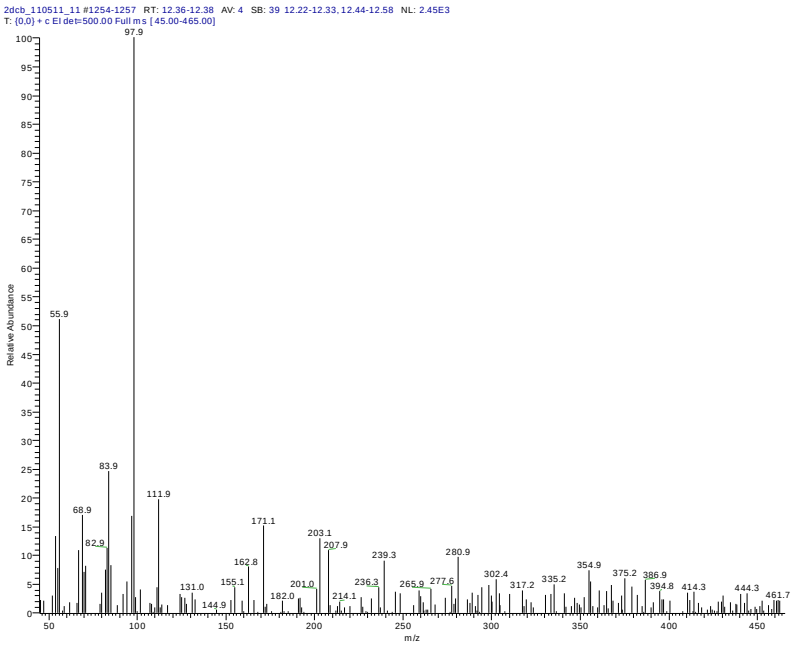


Figure 4.10. a) GC-MS chromatograms (EIC at m/z 55, 84, 98, 112 and TIC) of irradiated DPOG (EB, 5 kGy), b) mass spectrum of the peak at 12.35 minutes

a)



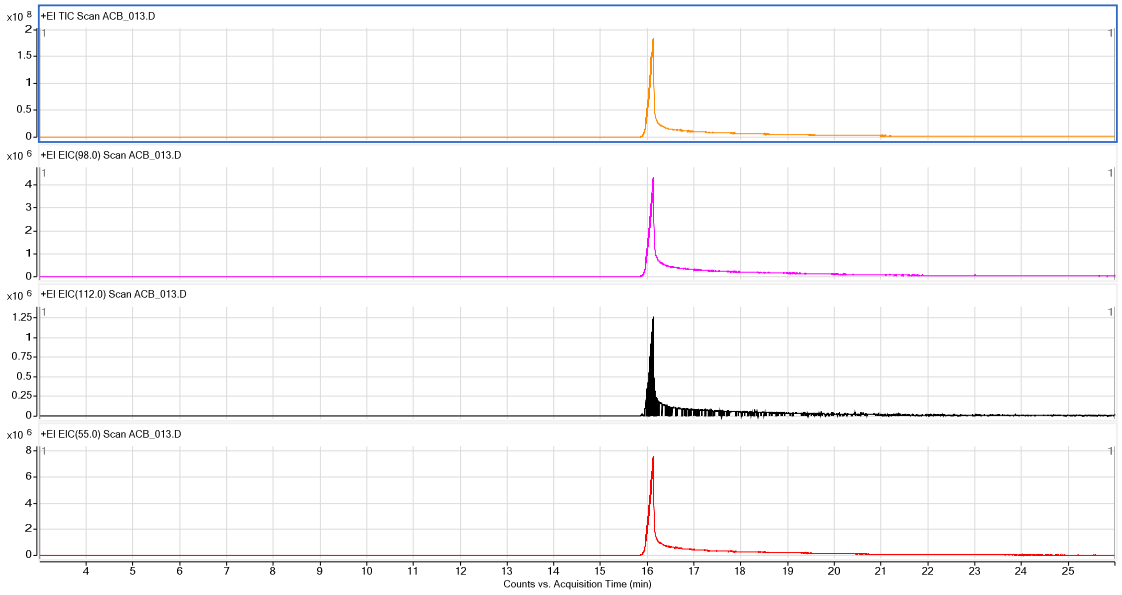
b)



Figures 4.11 to 4.16 show the GC-MS data for the SA and GTS non-irradiated and irradiated samples (5 kGy for gamma and EB). These are expected to produce 2-TCB upon irradiation, the other 2-ACB included in the standard method EN1785. The chromatograms were interrogated in an attempt to detect the 2-TCB peak but no 2-TCB peak could be found. The EICs for the expected ions (98 most intense, 112 and 55, the same masses as for 2-DCB due to the same fragments being formed) were plotted and are shown in the Figures but no 2-TCB attributed peak could be detected. There was a peak at 16 minutes in each chromatogram which could be attributed to SA, the parent compound and hydrolysis product for GTS.

Figure 4.11. GC-MS chromatograms (TIC and EIC at 98, 112 and 55) of a) non-irradiated SA full scale, b) non-irradiated SA zoomed in

a)



b)

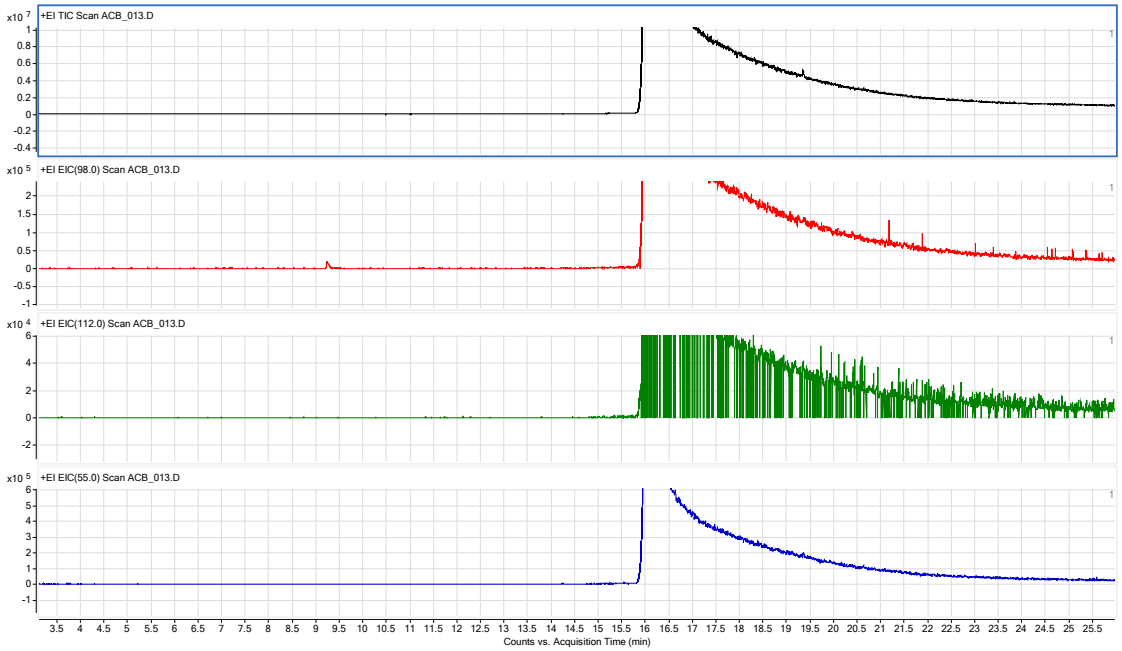
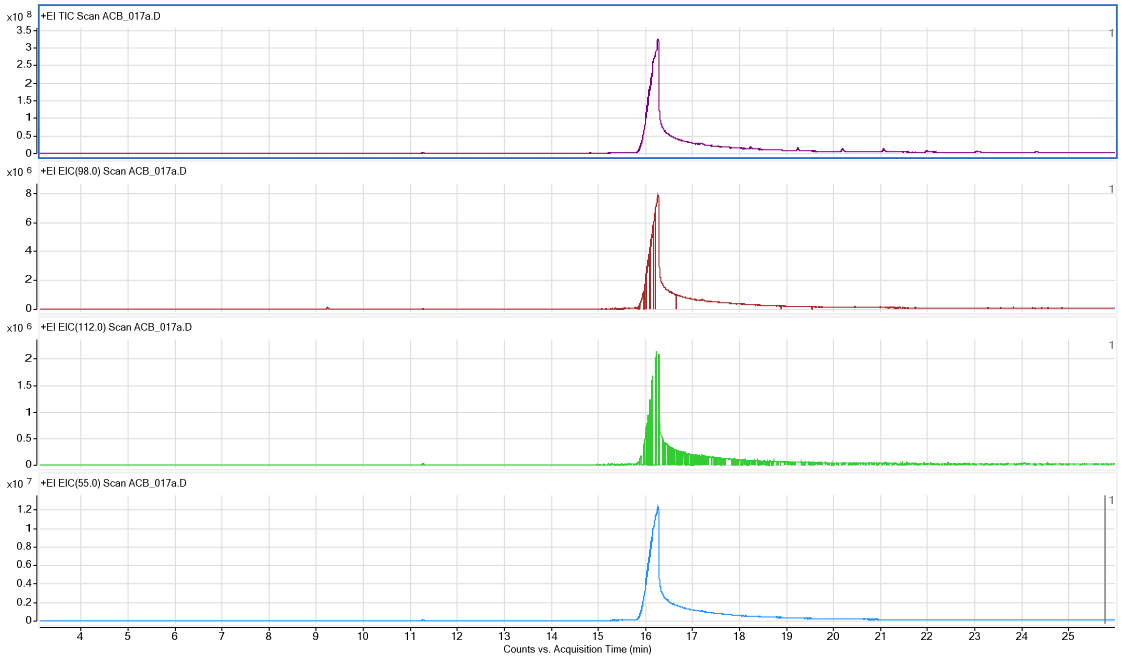


Figure 4.12. GC-MS chromatograms (TIC and EIC at 98, 112 and 55) of a) irradiated SA (gamma, 5 kGy) full scale, b) irradiated SA (gamma, 5 kGy) zoomed in

a)



b)

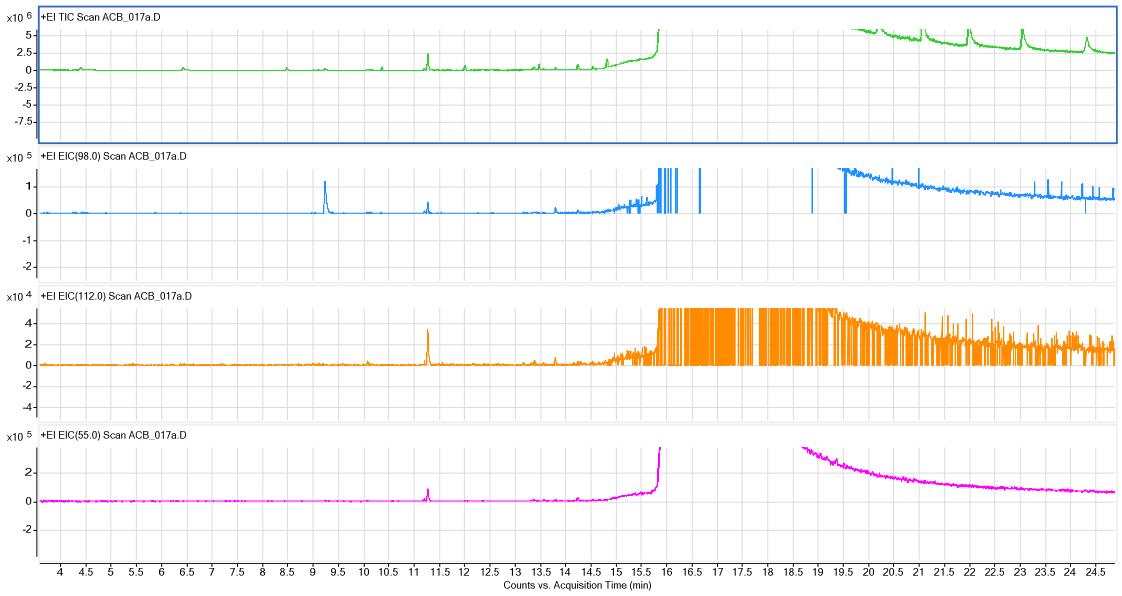
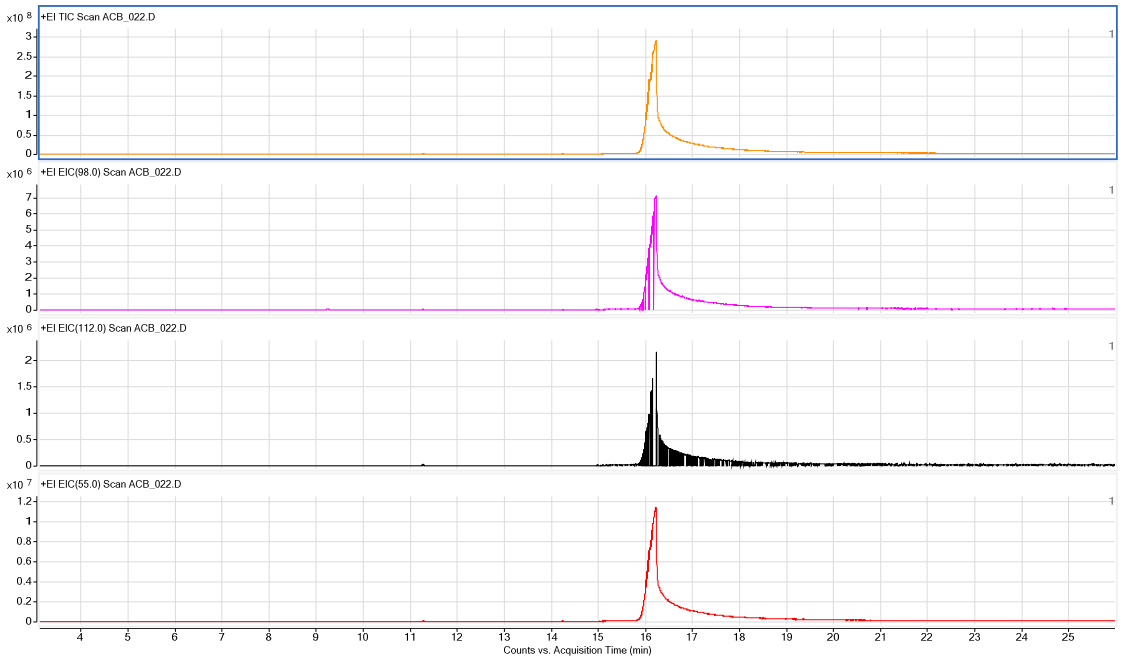
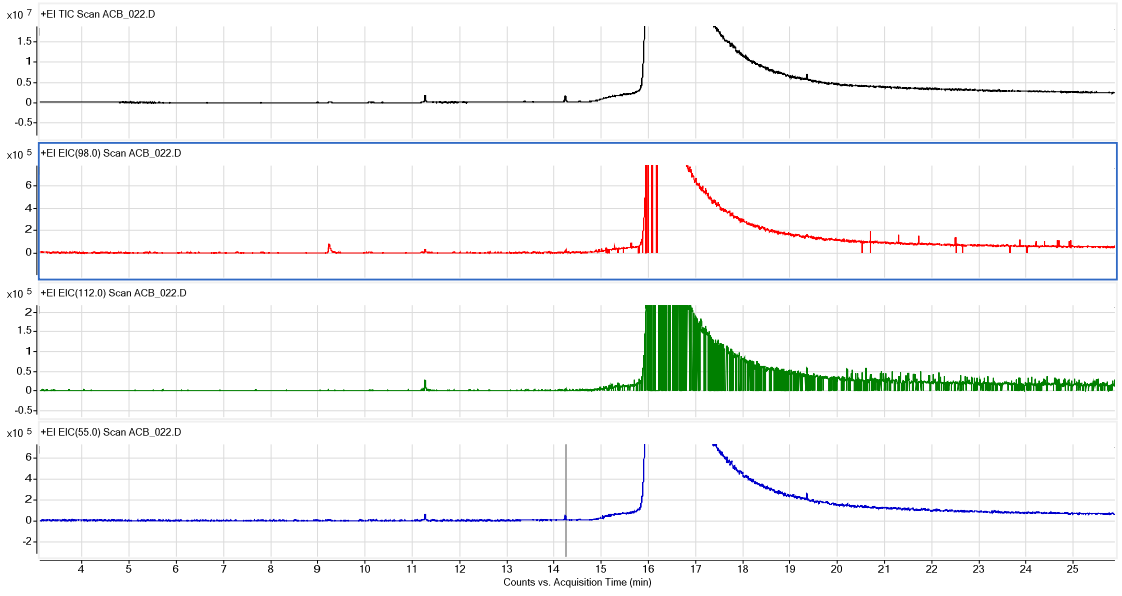


Figure 4.13. GC-MS chromatograms (TIC and EIC at 98, 112 and 55) of a) irradiated SA (gamma, 5 kGy) full scale, b) irradiated SA (gamma, 5 kGy) zoomed in and the mass spectra of the peaks at c) 11.27 minutes, d) 14.25 minutes, e) 16.20 minutes

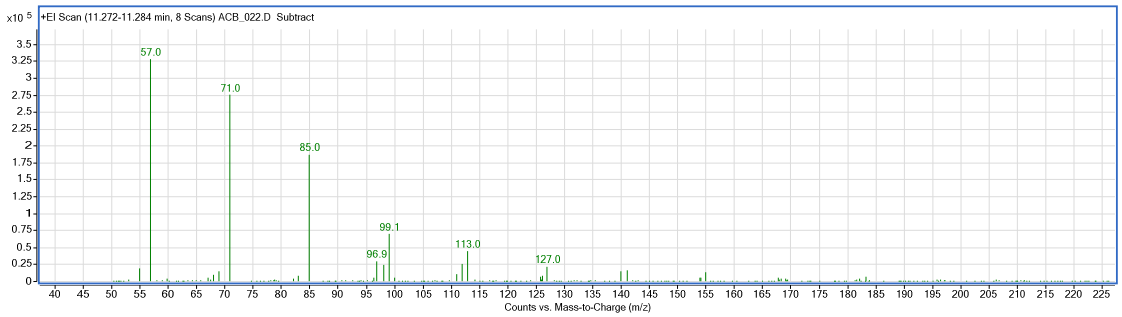
a)



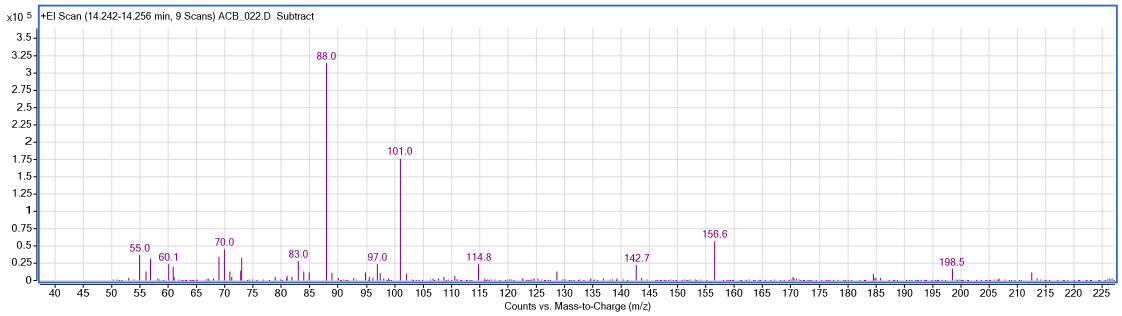
b)



c)


x10 5
+EI Scan (11.272-11.284 min, 8 Scans) ACB_022.D Subtract
57.0
71.0
85.0
99.1
96.9
113.0
127.0
Counts vs. Mass-to-Charge (m/z)

d)


x10 5
+EI Scan (14.242-14.256 min, 9 Scans) ACB_022.D Subtract
88.0
101.0
55.0
60.1
70.0
83.0
97.0
114.8
142.7
156.6
198.5
Counts vs. Mass-to-Charge (m/z)

e)

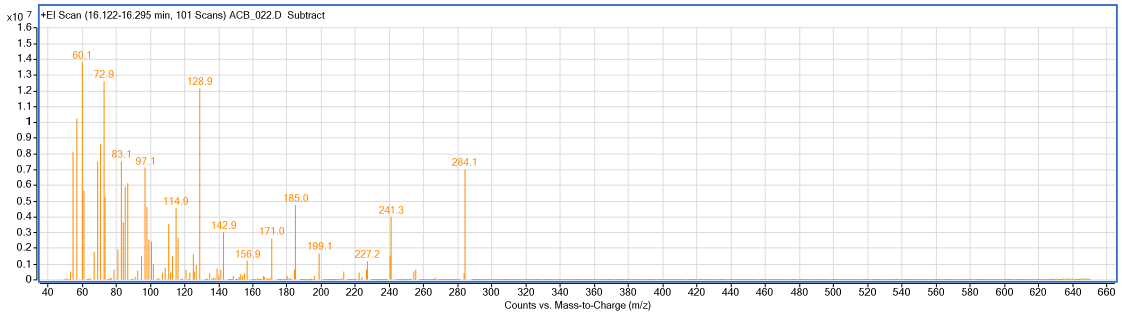

x10 7
+EI Scan (16.122-16.295 min, 101 Scans) ACB_022.D Subtract
60.1
72.9
128.9
83.1
97.1
284.1
114.9
185.0
241.3
142.9
171.0
156.9
199.1
227.2
Counts vs. Mass-to-Charge (m/z)

Figure 4.14. GC-MS chromatograms (TIC and EIC at 98, 112 and 55) of non-irradiated GTS

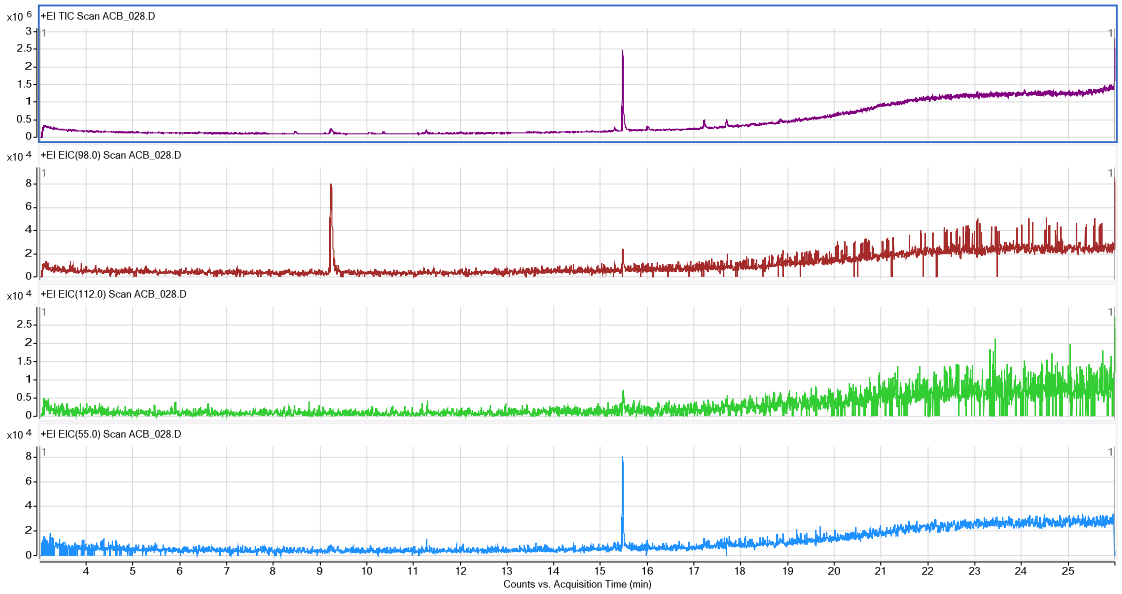


Figure 4.15. GC-MS chromatograms (TIC and EIC at 98, 112 and 55) of irradiated GTS (gamma, 5 kGy) full scale

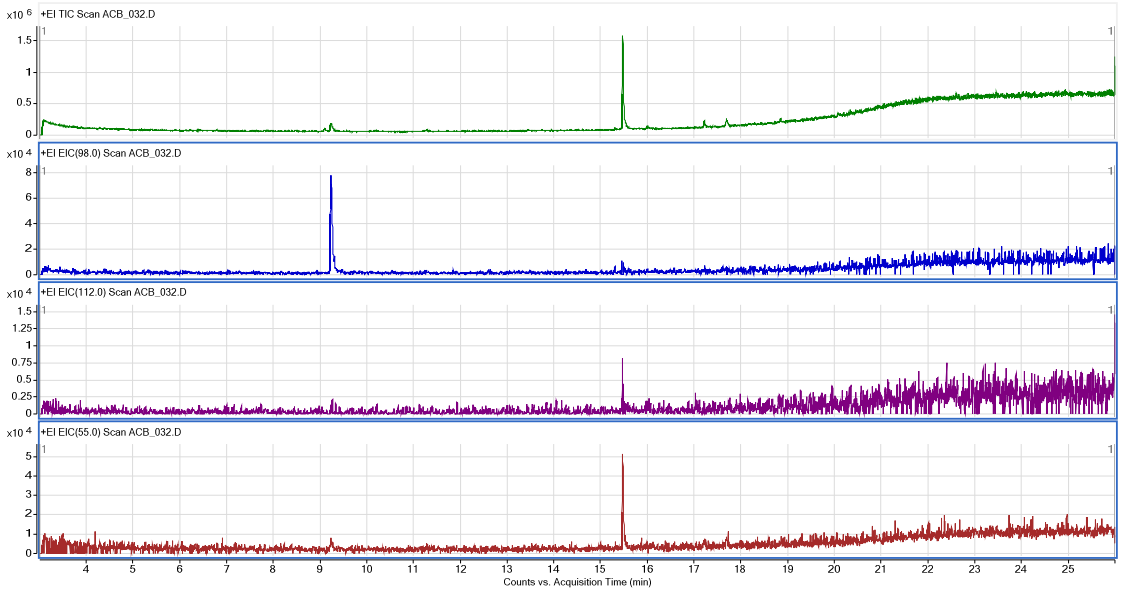
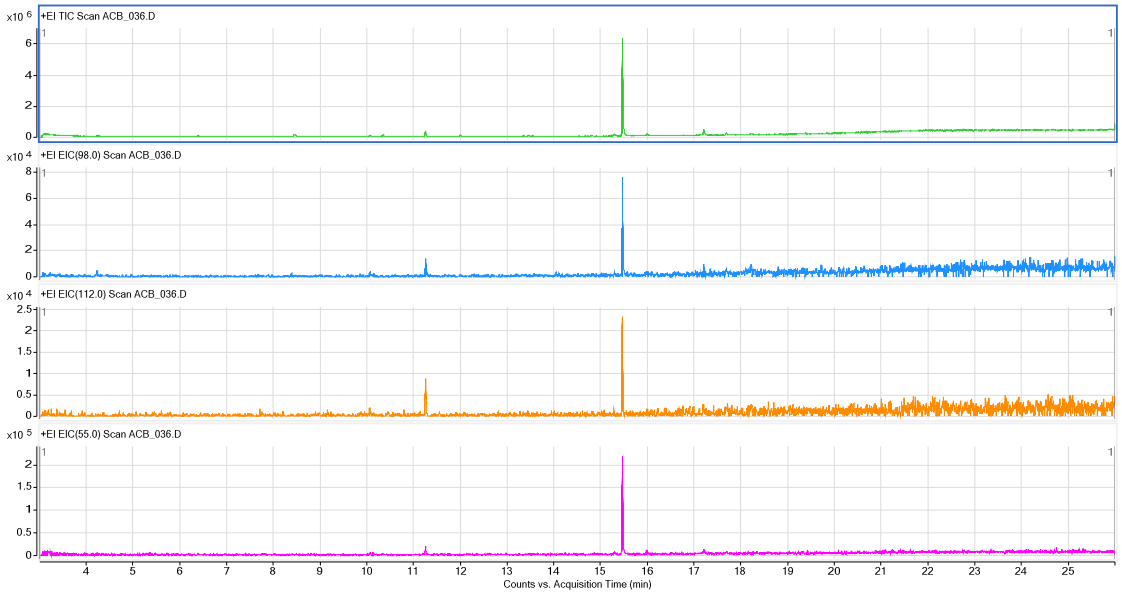


Figure 4.16. GC-MS chromatograms (TIC and EIC at 98, 112 and 55) of irradiated GTS (EB, 5 kGy) full scale



Figures 4.17 to 4.26 show the GC-MS results for the remaining samples, OA, GTO, LA, LLA and GTL and Figure 4.27 shows the TIC for the procedural hexane blank sample. For each of these compounds the expected irradiation products could not be detected. Reasons for this may include:

i) The clean-up procedure was not optimised for the 2-ACBs other than 2-DCB so the irradiation products may have been lost, either in the washing stage or by remaining retained by the column.

   This is unlikely as the procedure has been shown to be effective for 2-DCB in this work and the standard method EN 1785 is also validated for 2-TCB. The remaining 2-ACBs only differ by the number of carbon carbon double bonds and this is unlikely to effect the clean-up separation. This point is difficult to prove without authentic standards.

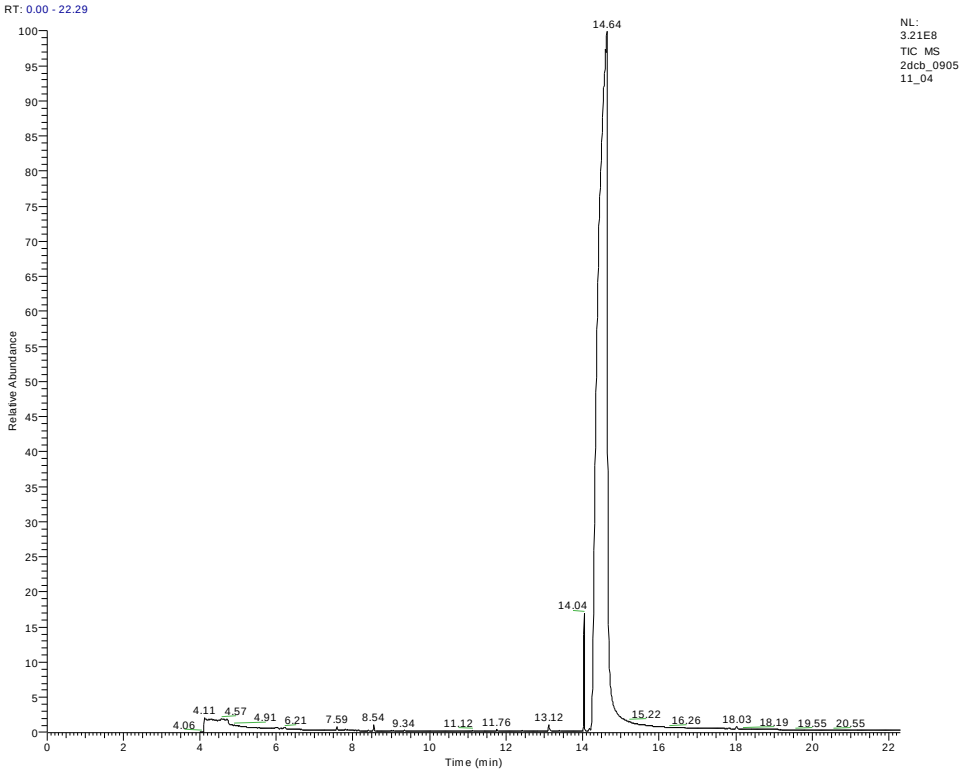
(ii) The irradiation yield may have been low so that the 2-ACBs may have been present but not in quantities sufficient for detection by GC-MS.

   This is possible as the 2-DCB peaks were small but the GC-MS was determined to be one of the most sensitive techniques currently available for detection of 2-DCB so no other technique would detect them. LC-MS/MS has a similar sensitivity but authentic standards would be needed to determine SRM channels to enable analysis.

A study of the radiolytic products of irradiated authentic fatty acids and triacylglycerides has been published [11]. This paper reports the formation of the expected 2-ACBs (as well as looking at the hydrocarbons formed) from PA, OA, SA, LA, LLA and GTP, GTO, and GTL by GC-MS in the concentration range 1-80 µg/g. The clean-up method and GC-MS analysis were similar to that used in this study. However, the study uses a higher irradiation dose (10 kGy) and does not state how much starting material was irradiated. It also does not state whether or not authentic standards of the 2-ACBs were used, but as no purchase details were reported this is unlikely. Also it is unclear how the 2-ACBS were quantified as no note is made to this and it appears unlikely that external calibration was possible without authentic 2-ACB standards.

Figure 4.17. a) GC-MS chromatograms (TIC) of irradiated oleic acid (gamma, 5 kGy), b) mass spectrum of the peak at 12.35 minutes

a)



b)

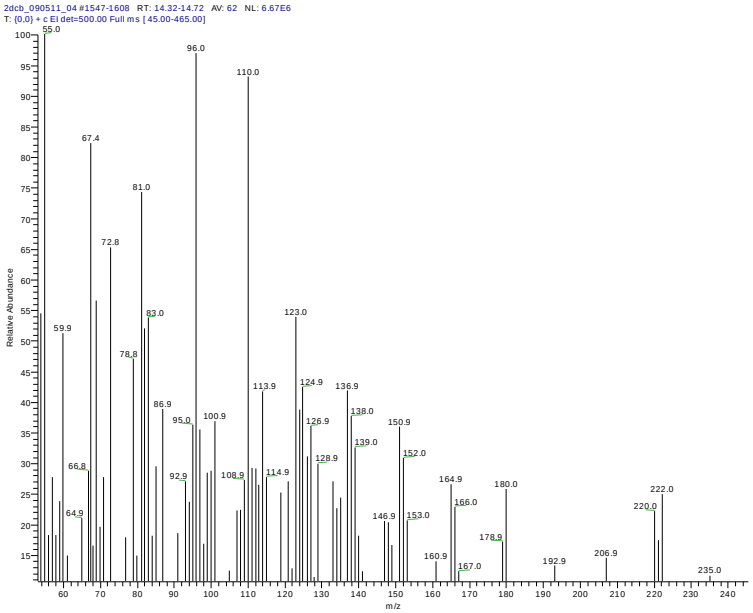
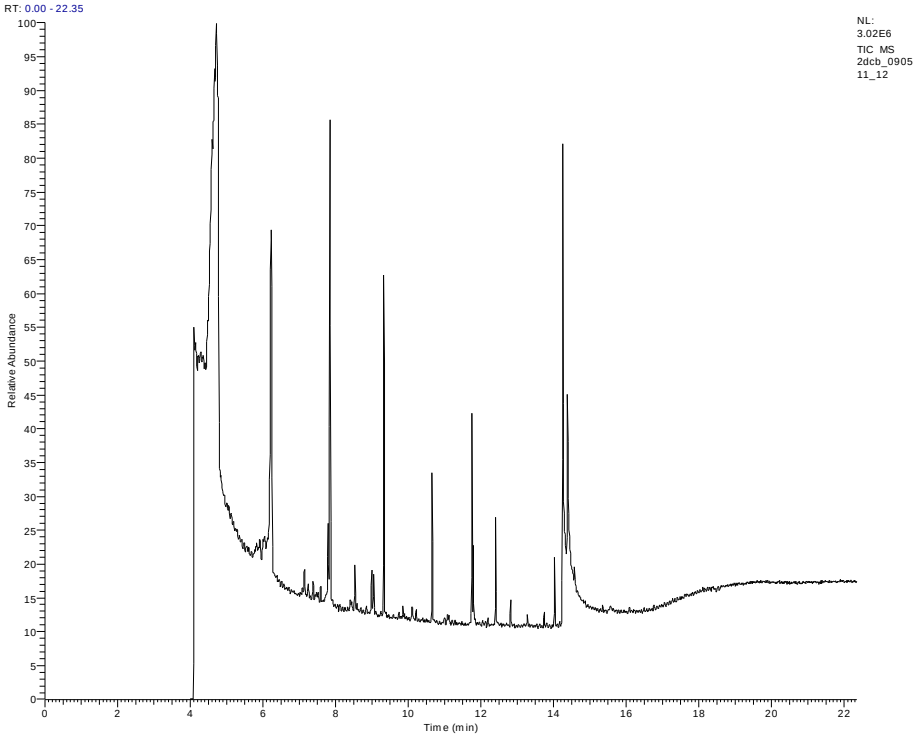
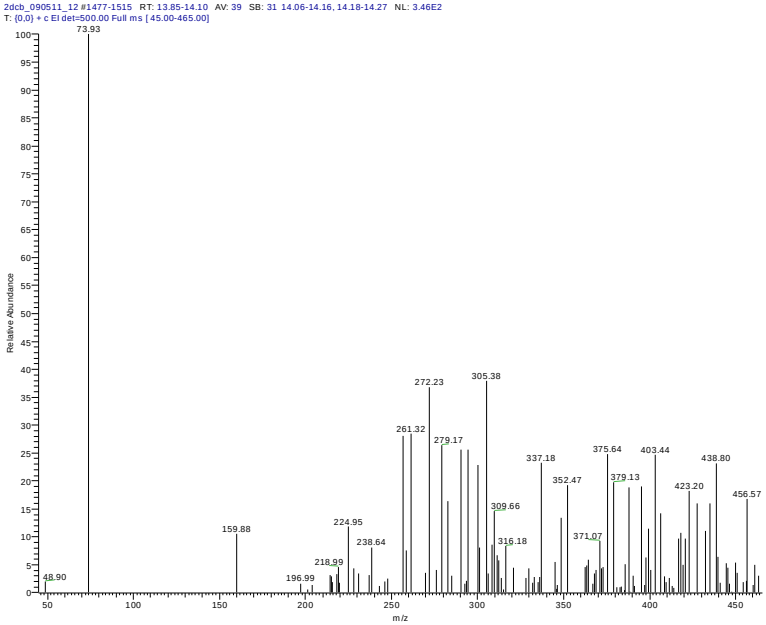


Figure 4.18. a) GC-MS chromatograms (TIC) of irradiated oleic acid (EB, 5 kGy), mass spectrum of the peak b) at 12.35 minutes and c) 15.00 minutes

a)



b)



c)

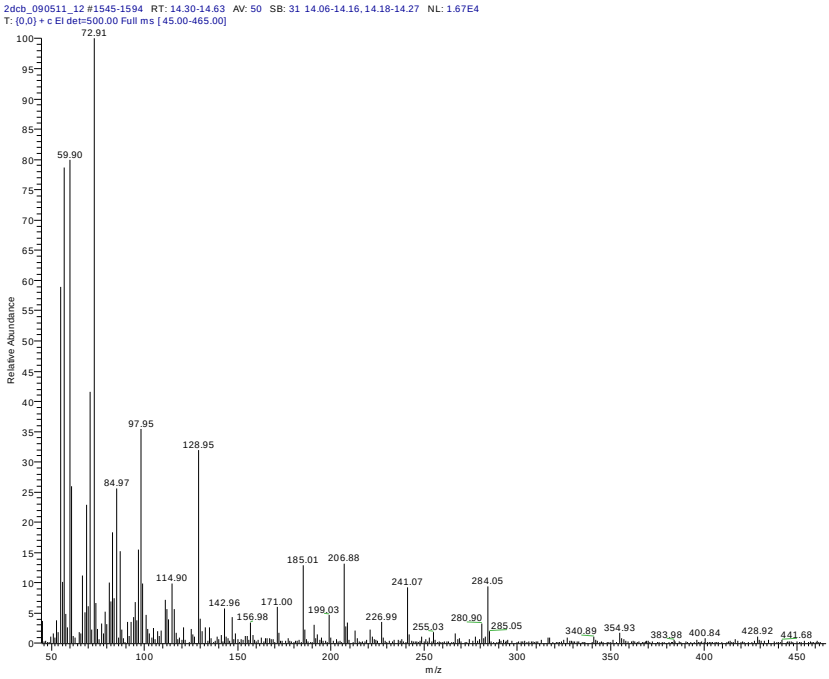
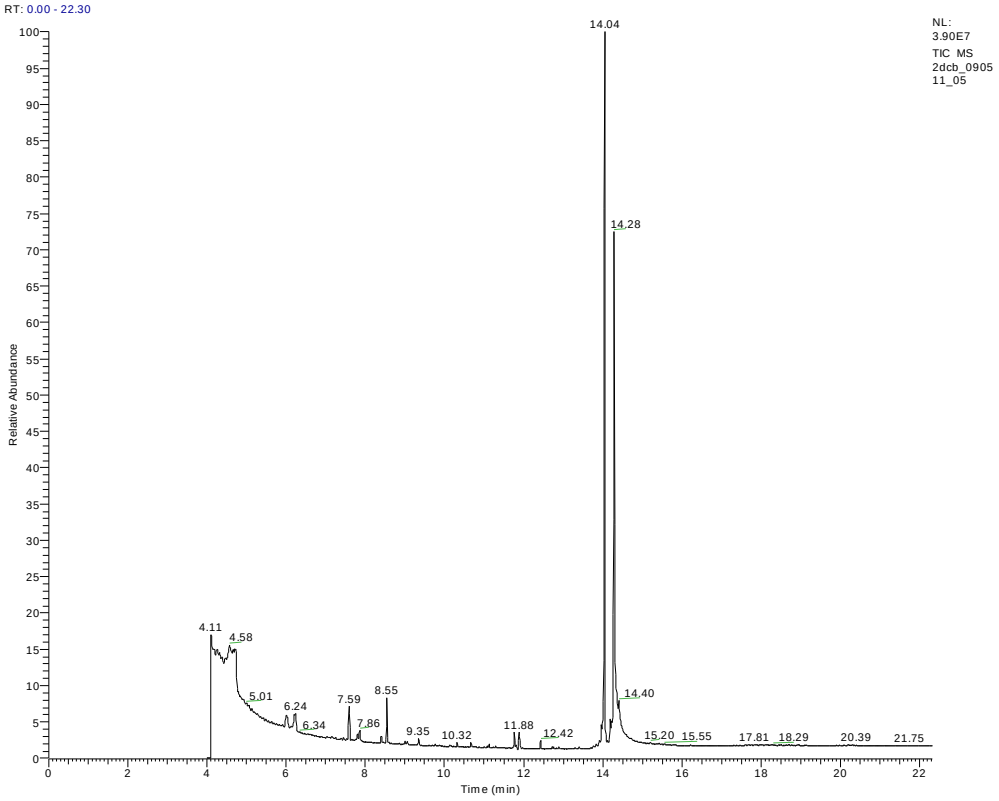
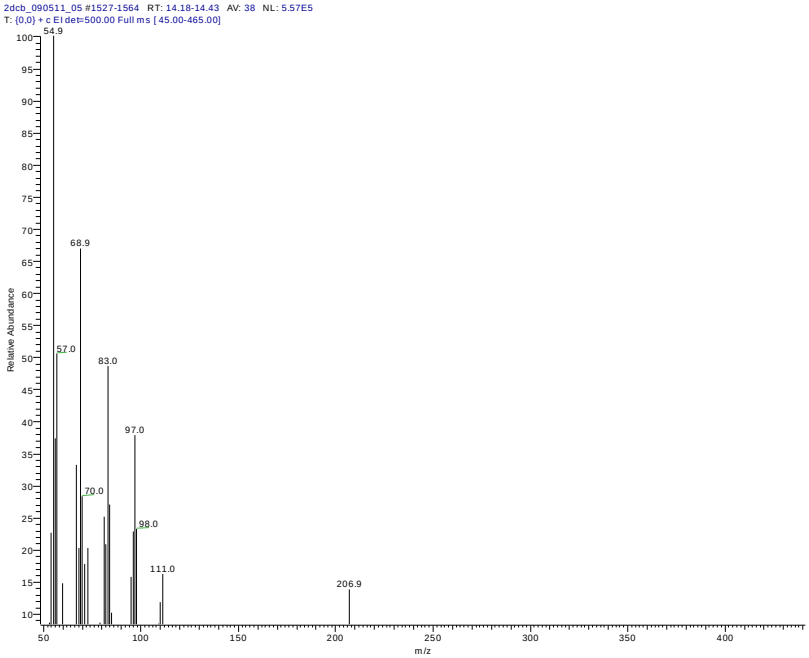

2dcb_090511_12 #1545-1594 RT: 14.30-14.63 AV: 50 SB: 31 14.06-14.16, 14.18-14.27 NL: 1.67E4
T: {0,0} + c EI det=500.00 Full ms [45.00-465.00]
Relative Abundance
m/z
72.91
59.90
97.95
128.95
84.97
114.90
185.01
206.88
241.07
284.05
142.96
171.00
156.98
199.03
226.99
255.03
280.90
285.05
340.89
354.93
383.98
400.84
428.92
441.68

Figure 4.19. a) GC-MS chromatograms (TIC) of irradiated GTO (gamma, 5 kGy) mass spectrum of the peak b) at 12.35 minutes and c) 14 minutes

a)



b)



c)

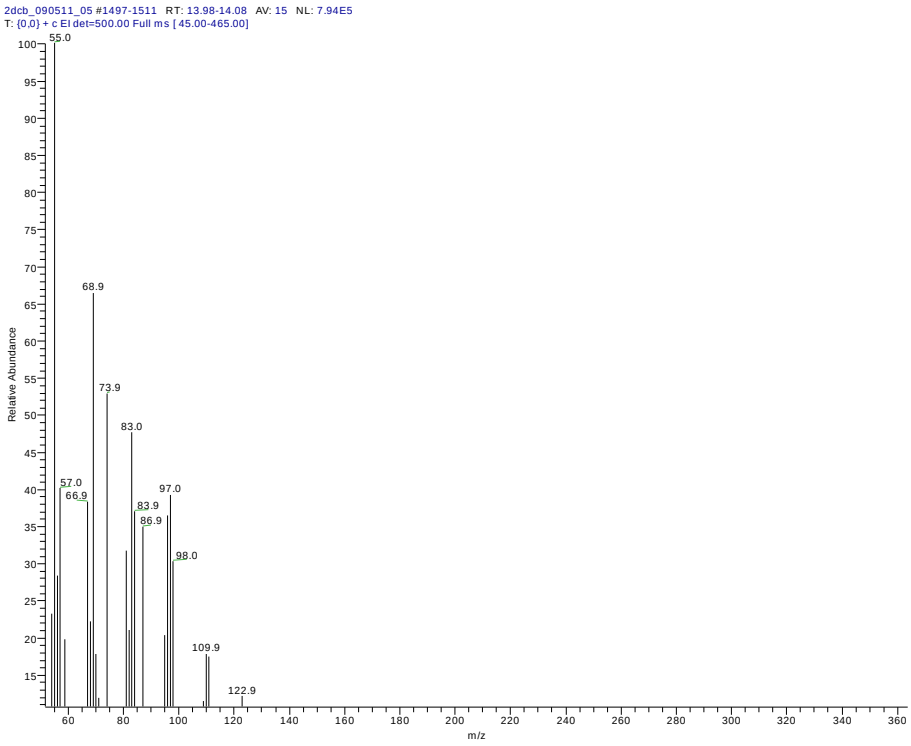

2dcb_090511_05 #1497-1511 RT: 13.98-14.08 AV: 15 NL: 7.94E5
T: {0,0} + c EI det=500.00 Full ms [45.00-465.00]
55.0
68.9
73.9
83.0
57.0
66.9
97.0
83.9
86.9
98.0
109.9
122.9
Relative Abundance
m/z

Figure 4.20. a) GC-MS chromatograms (TIC) of irradiated GTO (EB, 5 kGy)

a)

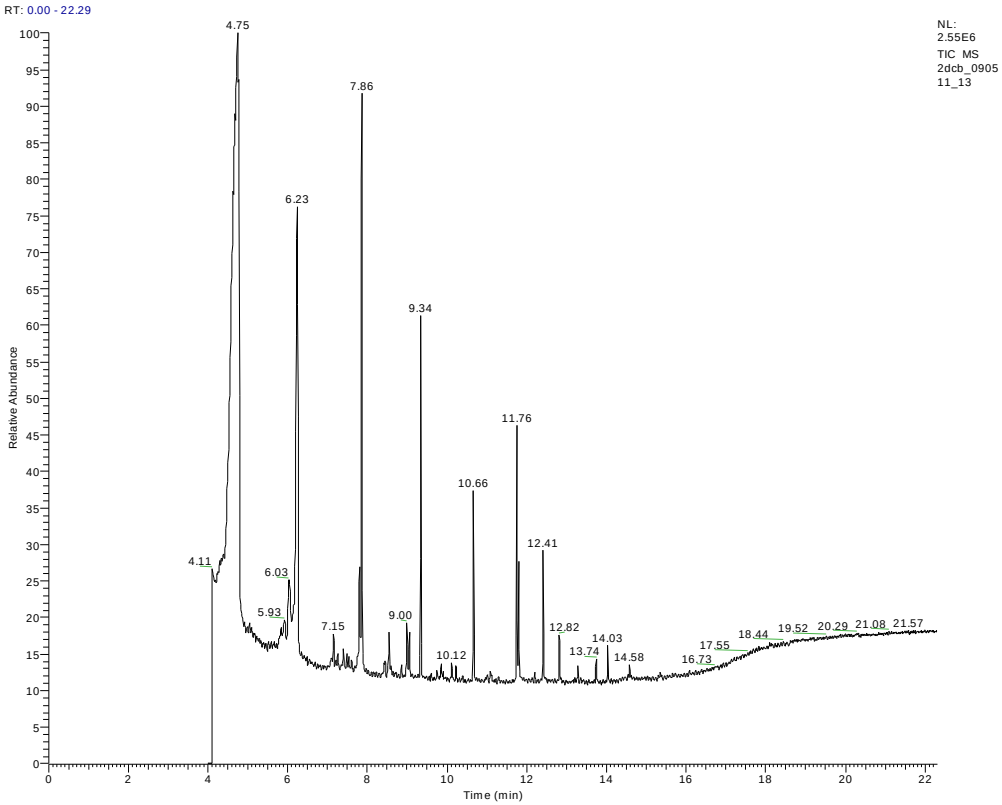
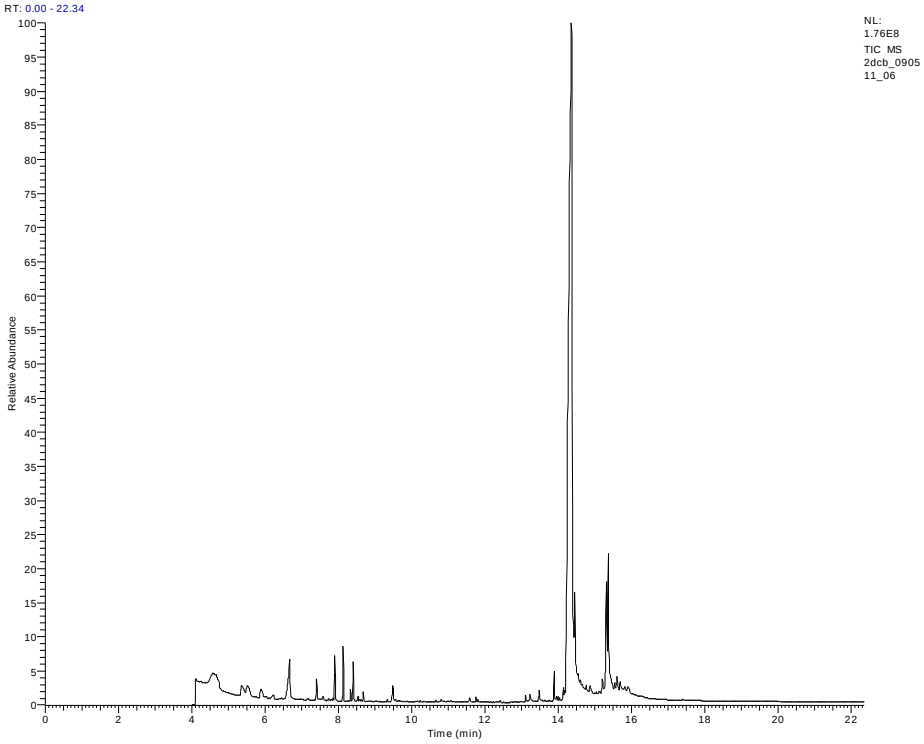
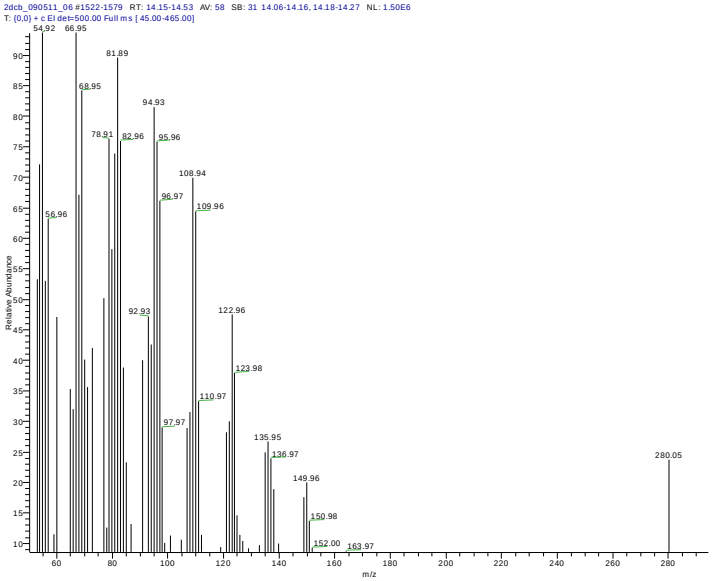


Figure 4.21. a) GC-MS chromatograms (TIC) of irradiated LA (gamma, 5 kGy), mass spectrum of the peak b) at 14 minutes and c) 15 minutes

a)



b)



c)

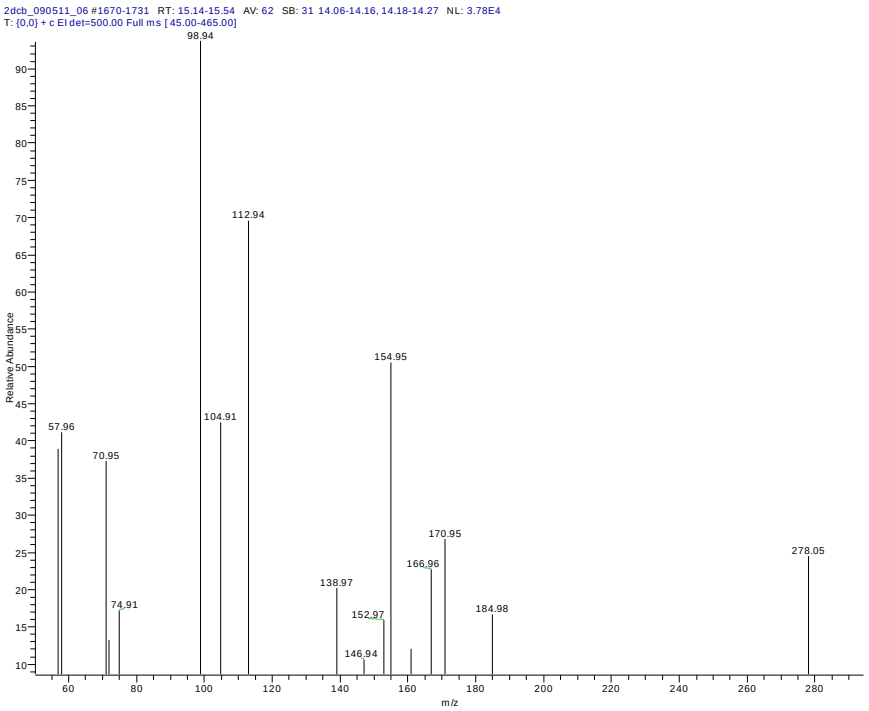

2dcb_090511_06 #1670-1731 RT: 15.14-15.54 AV: 62 SB: 31 14.06-14.16, 14.18-14.27 NL: 3.78E4
T: {0,0} + c EI det=500.00 Full ms [45.00-465.00]
Relative Abundance
m/z
98.94
112.94
154.95
104.91
57.96
70.95
170.95
166.96
278.05
138.97
74.91
152.97
184.98
146.94

Figure 4.22. GC-MS chromatograms (TIC) of irradiated LA (EB, 5 kGy)

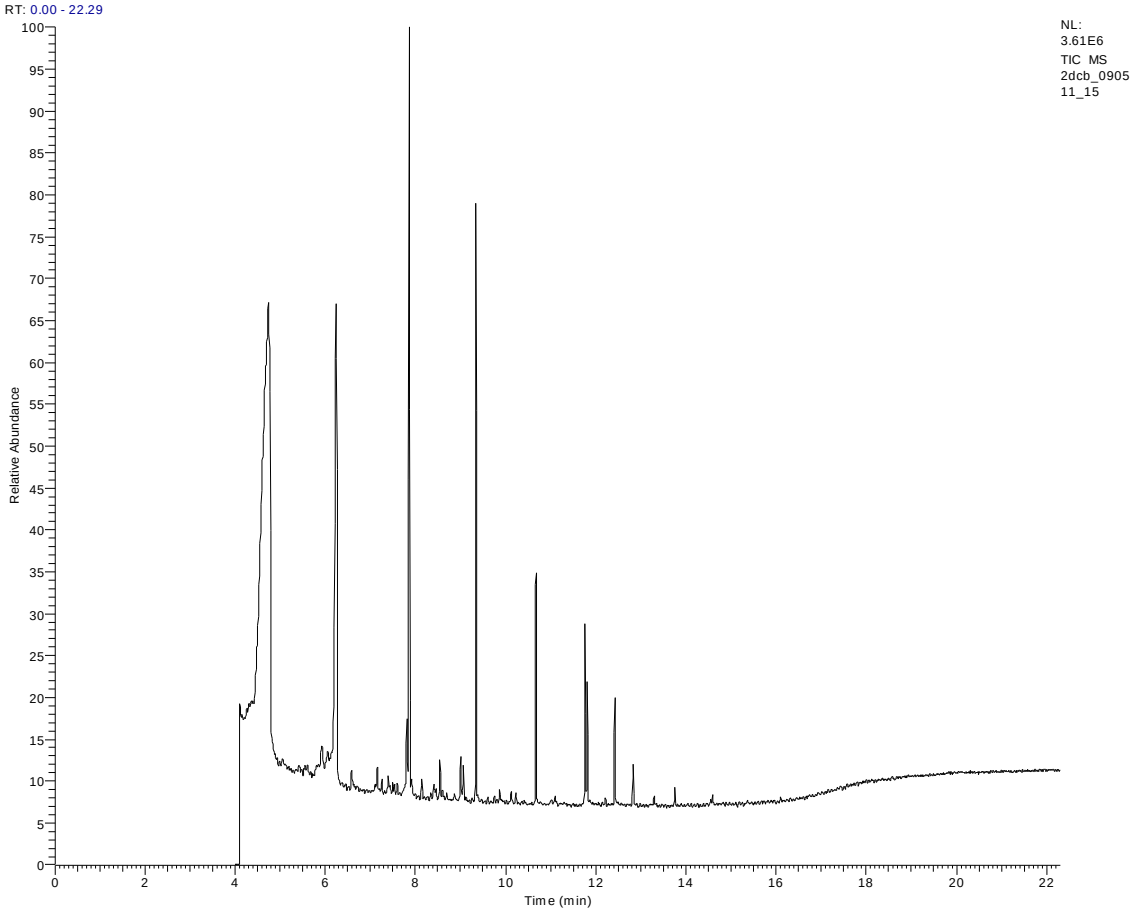


Figure 4.23. GC-MS chromatograms (TIC) of irradiated GTL (gamma, 5 kGy)

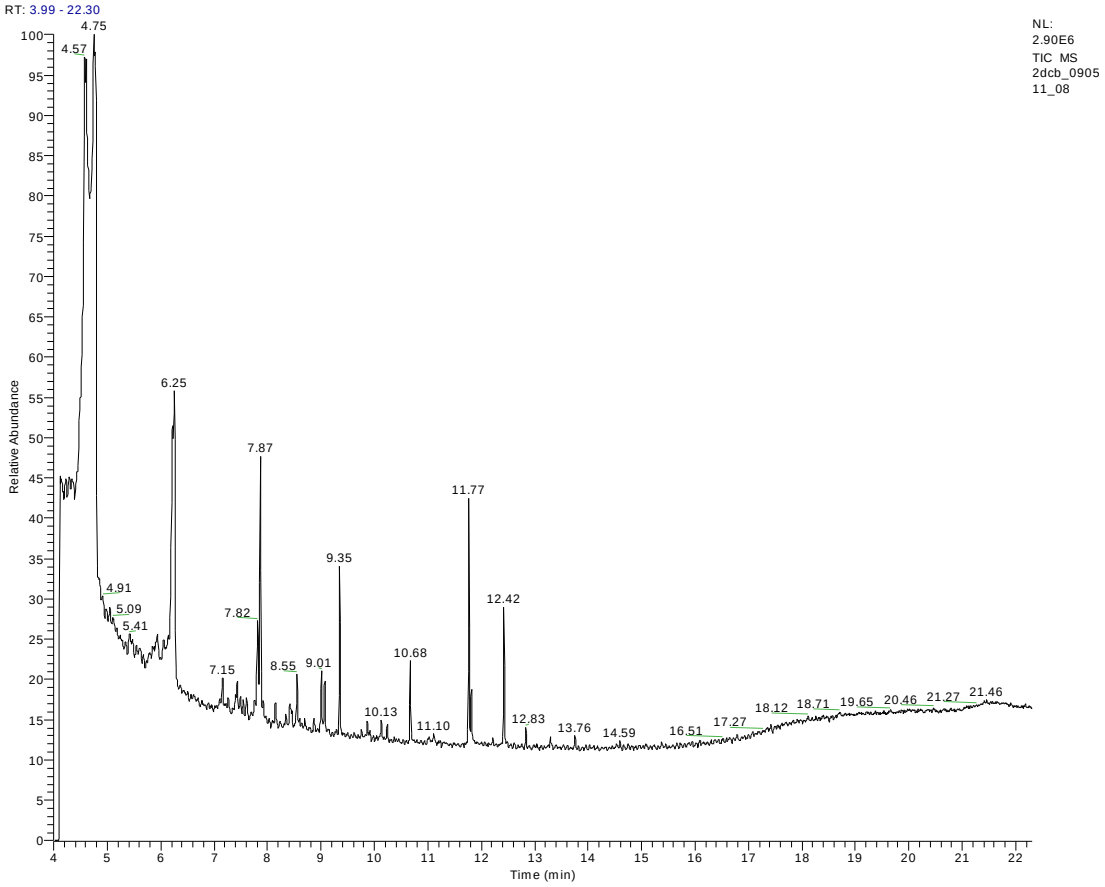


Figure 4.24. GC-MS chromatograms (TIC) of irradiated GTL (EB, 5 kGy)

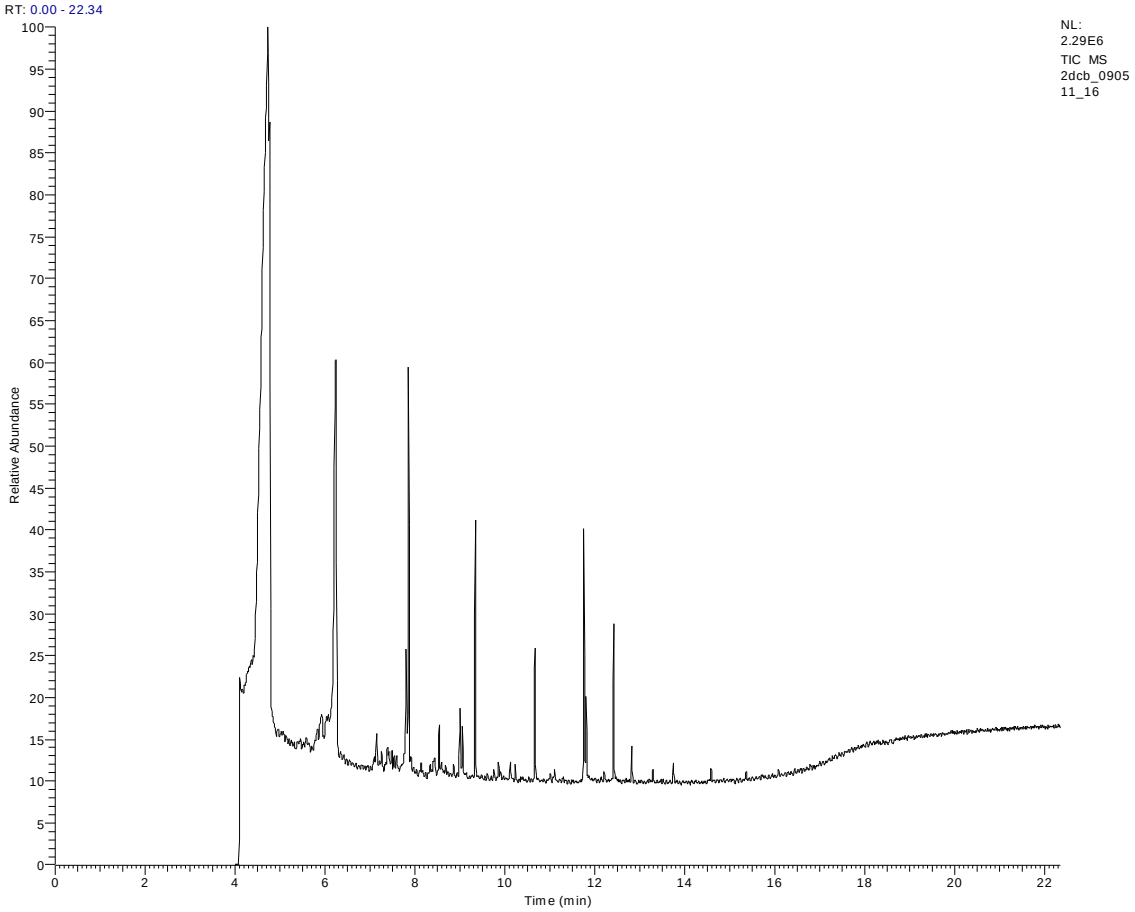
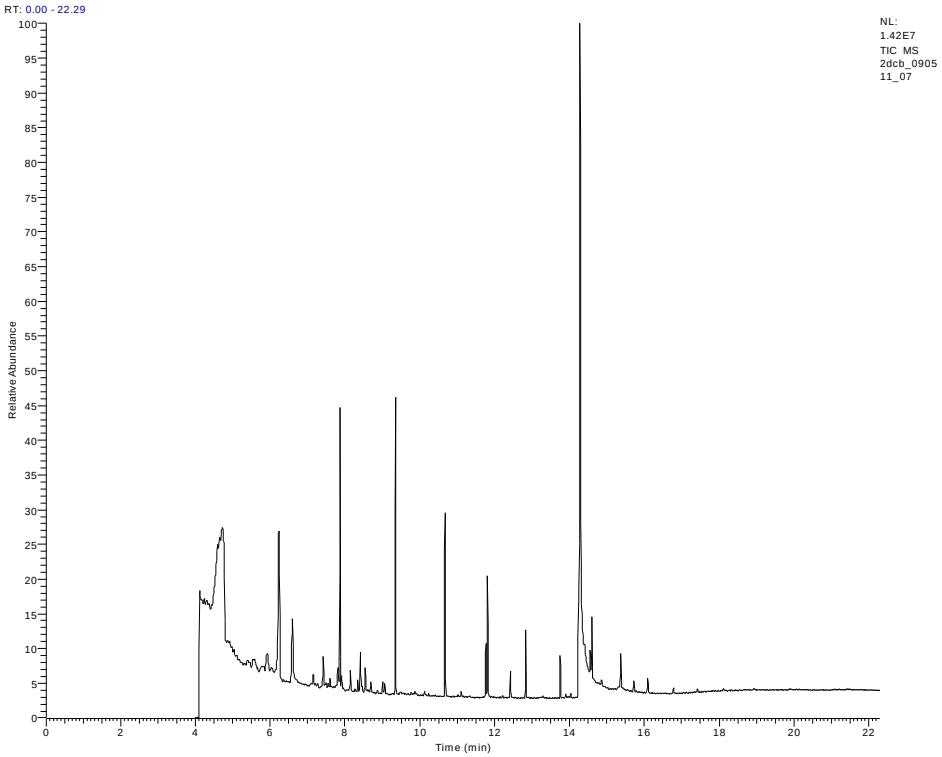


Figure 4.25. a) GC-MS chromatograms (TIC) of irradiated LLA (gamma, 5 kGy), b) mass spectrum of the peak at 12.35 minutes

a)



b)

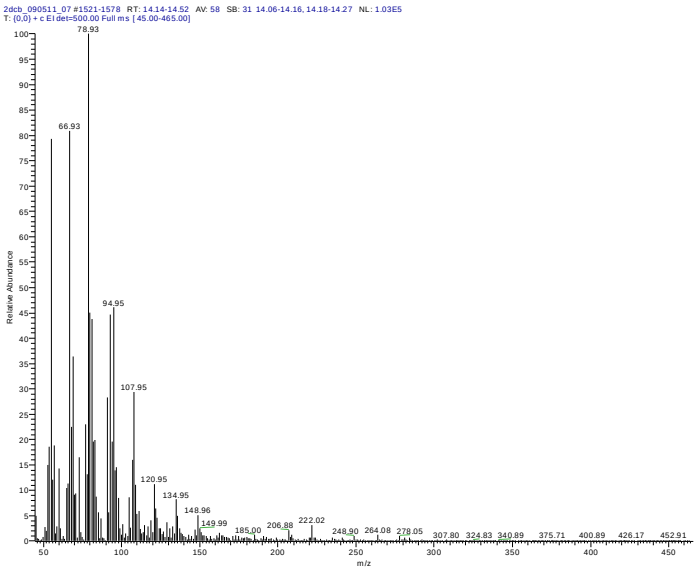


Figure 4.26. GC-MS chromatograms (TIC) of irradiated linolenic acid (EB, 5 kGy)

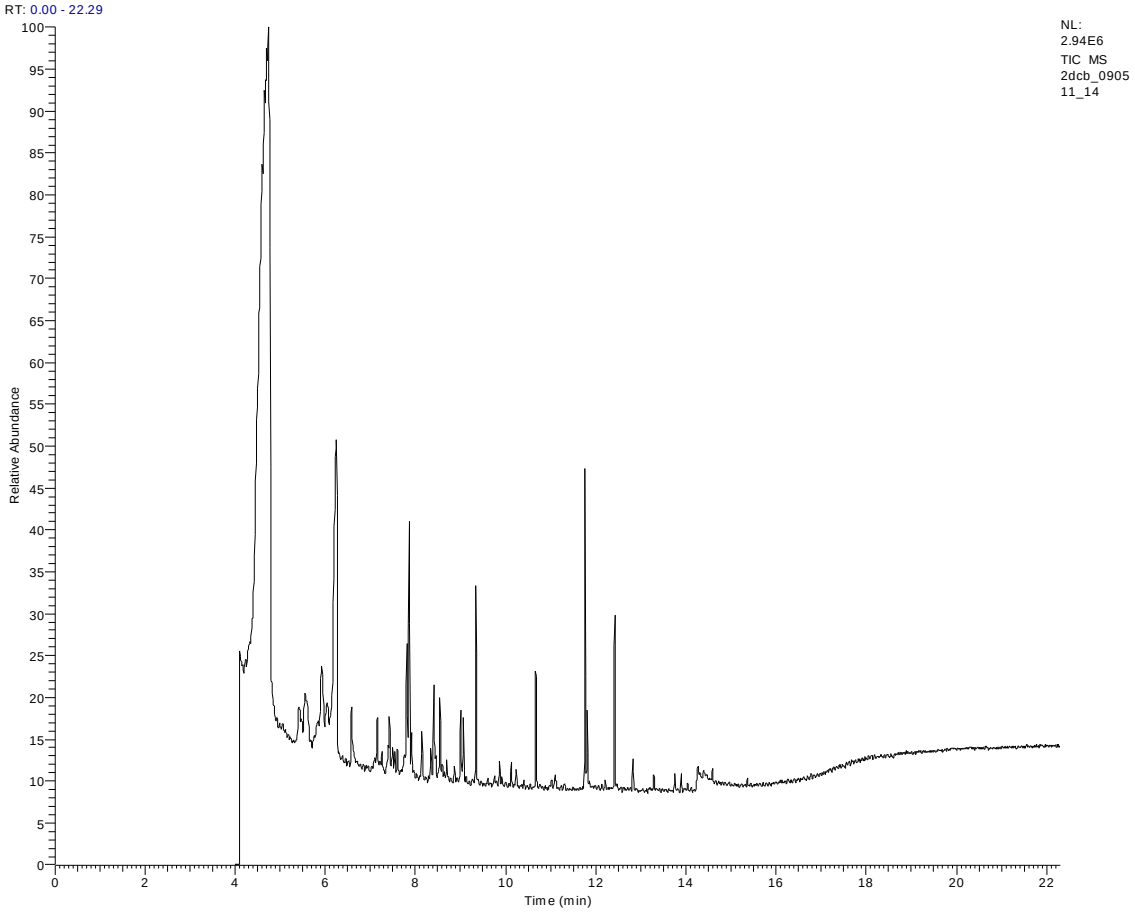
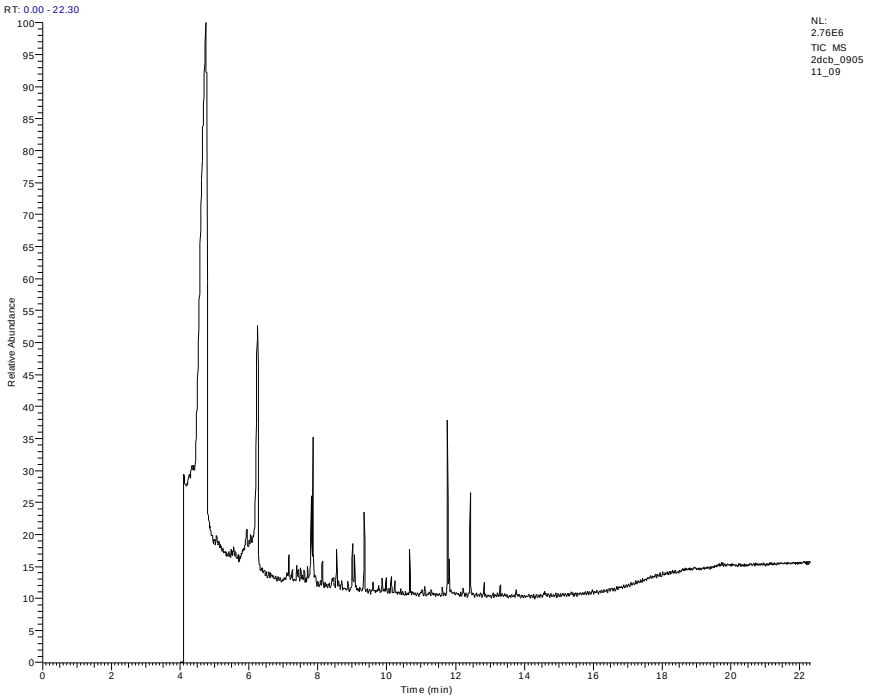


Figure 4.27. GC-MS chromatogram (TIC) of a hexane procedural blank



4.2.3.2 LC-TOF-MS

As limited information was determined from the GC-MS analysis of the irradiated fatty acid and triacylglyceride samples the same samples were analysed by LC-TOF-MS following DNPH derivatisation, with the aim of using the full scan accurate mass capability to investigate the samples further.

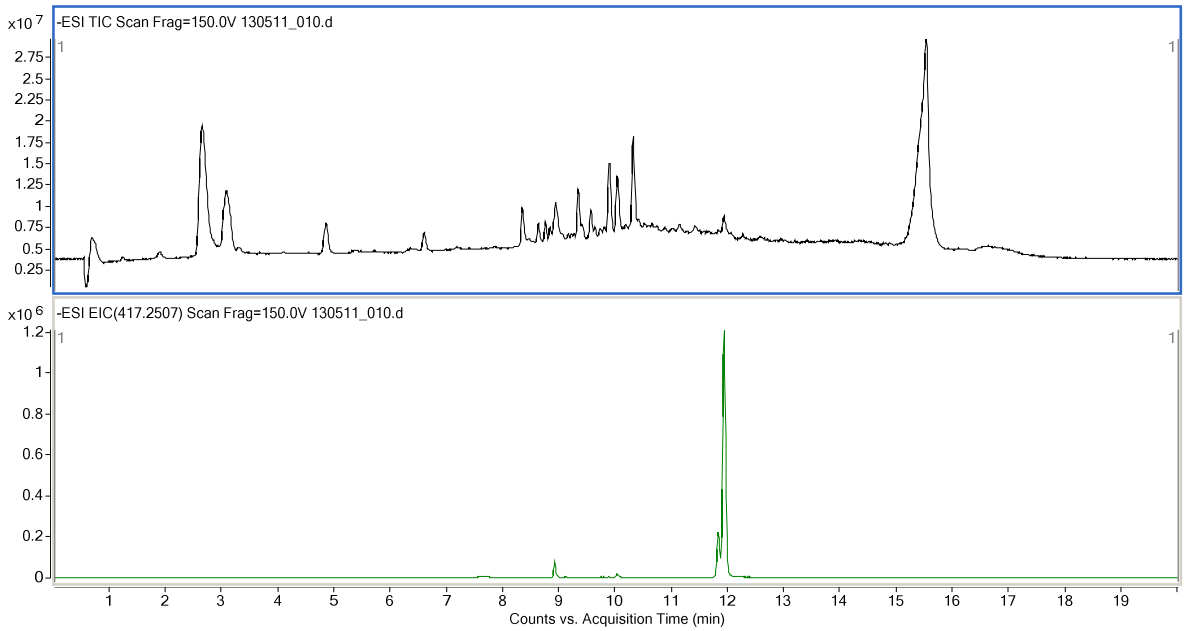
Initially the irradiated PA, GTP and DPOG samples were analysed as they were expected to produce 2-DCB upon irradiation, the one 2-ACB available commercially. Figures 4.28 shows the TIC, the EIC (417.2507 for the [M-H]- adduct) and associated mass spectrum of the DNPH-derivatised-2-DCB and Figure 4.29 shows the same information for the procedural blank. These were run to demonstrate that the instrument was performing as expected.

The EICs of the procedural blank sample, PA, GTP and DPOG are shown for the different gamma and EB doses in Figures 4.30 to 4.37. These figures show the presence of 2-DCB in most cases, indicating successful formation by irradiation and corroborating the results seen by GC-MS. As some of the peaks were very low in intensity, particularly for the lower irradiation doses and the DPOG samples, where a smaller amount of sample was irradiated, the samples were re-analysed and quantified by the more sensitive LC-MS/MS method and the results are described in section 4.2.3.3.

Figures 4.38 to 4.44 show the LC-TOF-MS data for the remaining fatty acids and triacylglycerols. Part a) of the figures shows the TIC and EICs of the expected DNPH-derivatised-irradiation products and the non-irradiated starting materials. Part b) of the figures shows the TIC of the irradiated samples overlaid with the TIC of the non-irradiated starting material extract in an attempt to identify new peaks formed upon irradiation and to determine whether the irradiation products had decomposed to other compounds.

Figure 4.28. LC-TOF-MS a) Total ion chromatogram and extracted ion chromatogram (417.2507, [M-H]-) for the DNPH-derivatised-2-DCB standard (1 µg/mL) and b) associated mass spectrum

a)



b)

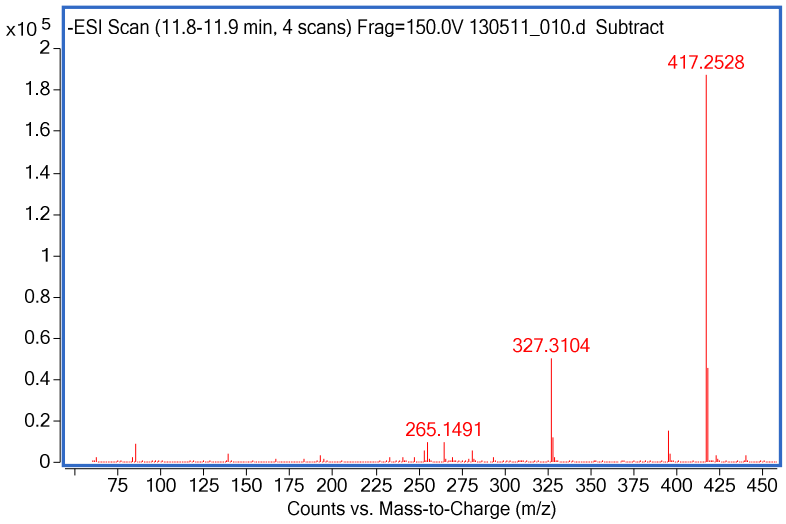


Figure 4.29. LC-TOF-MS total ion chromatogram and extracted ion chromatogram (417.2507, [M-H]-) for the DNPH-derivatised-procedural blank

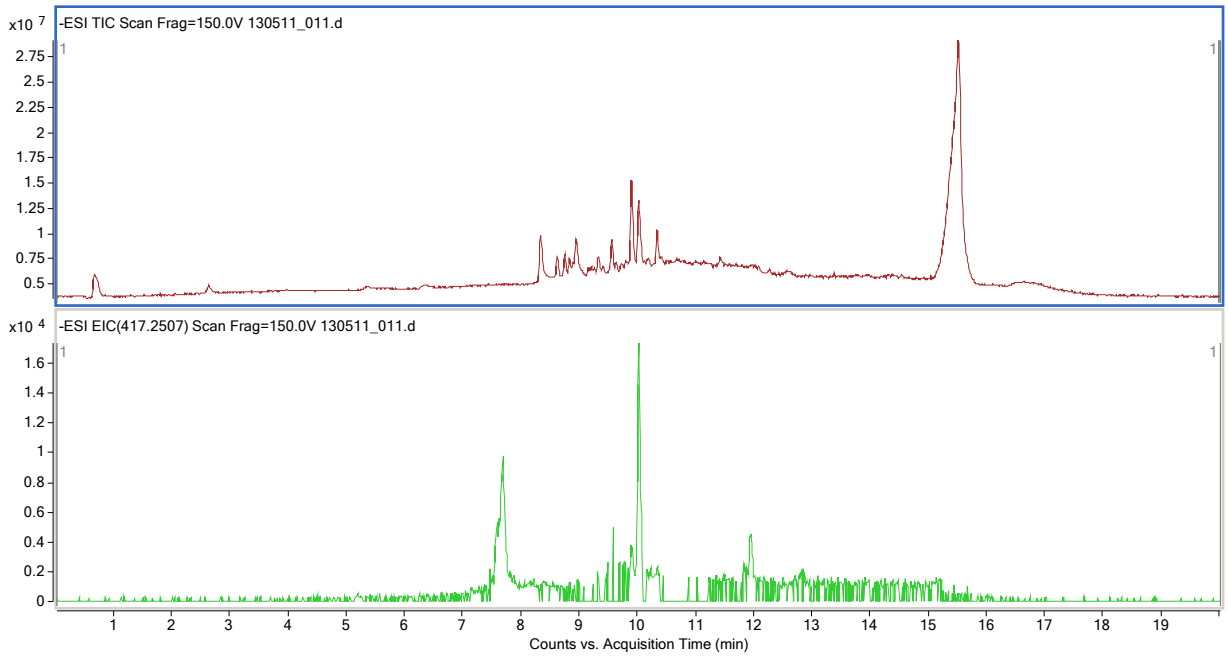


Figure 4.30. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-procedural blank samples gamma irradiated at 1, 3 and 5 kGy

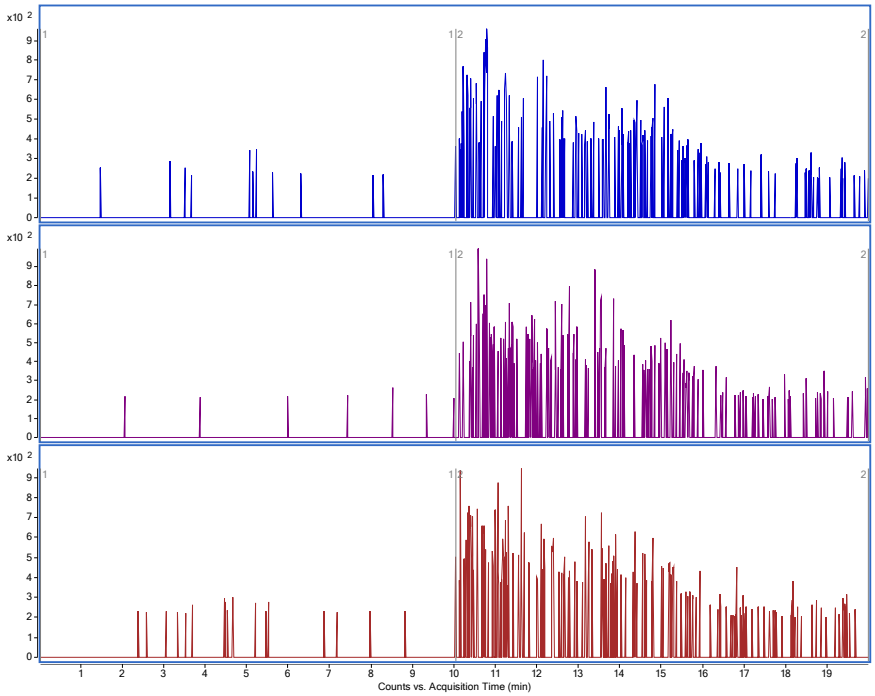


Figure 4.31. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-procedural blank samples EB irradiated at 0.3, 0.5, 1, 3 and 5 kGy

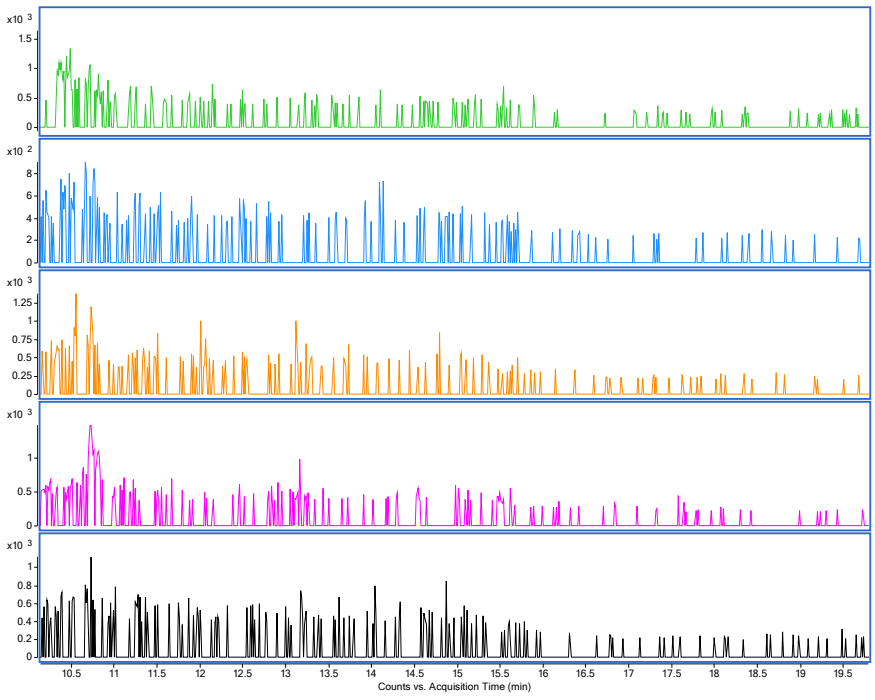


Figure 4.32. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-PA samples gamma irradiated at 1, 3 and 5 kGy

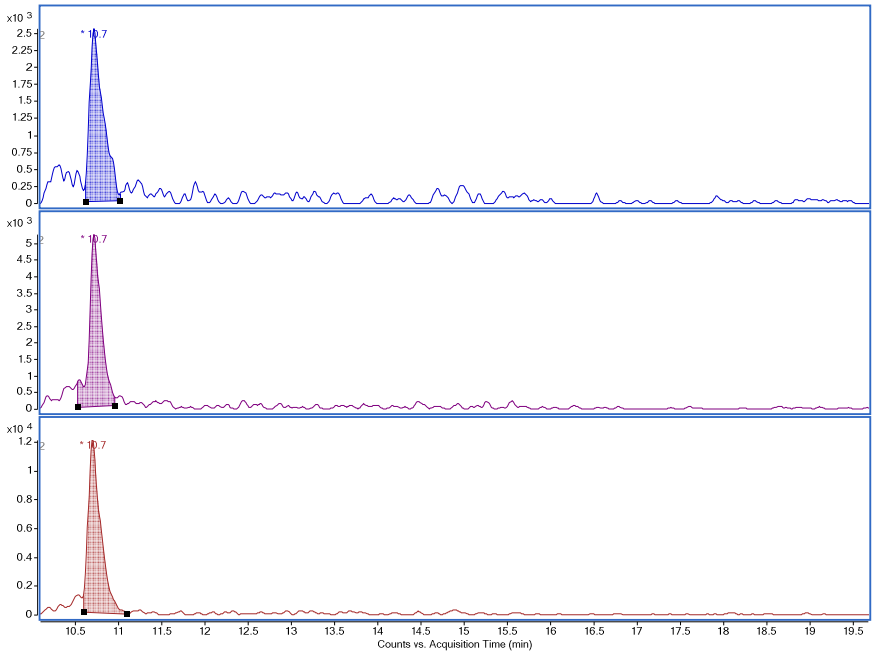


Figure 4.33. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-PA samples EB irradiated at 0.3, 0.5, 1, 3 and 5 kGy

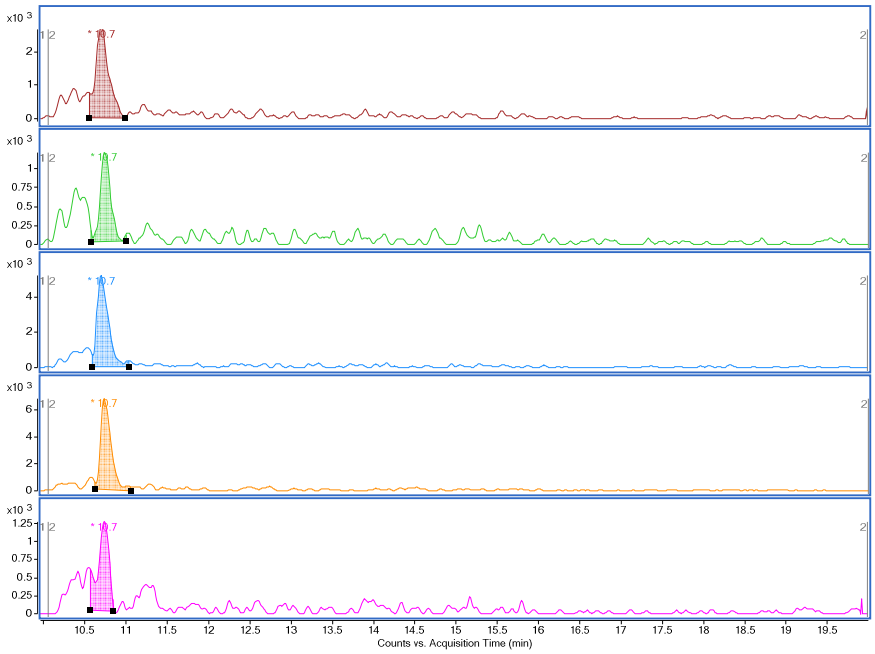


Figure 4.34. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-GTP samples gamma irradiated at 1, 3 and 5 kGy

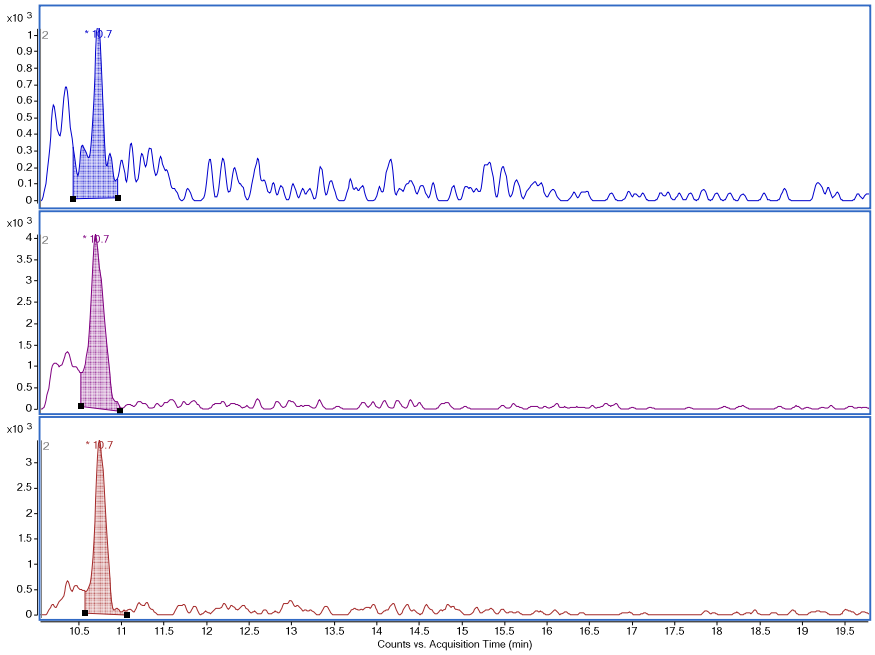


Figure 4.35. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-GTP samples EB irradiated at 0.3, 0.5, 1, 3 and 5 kGy

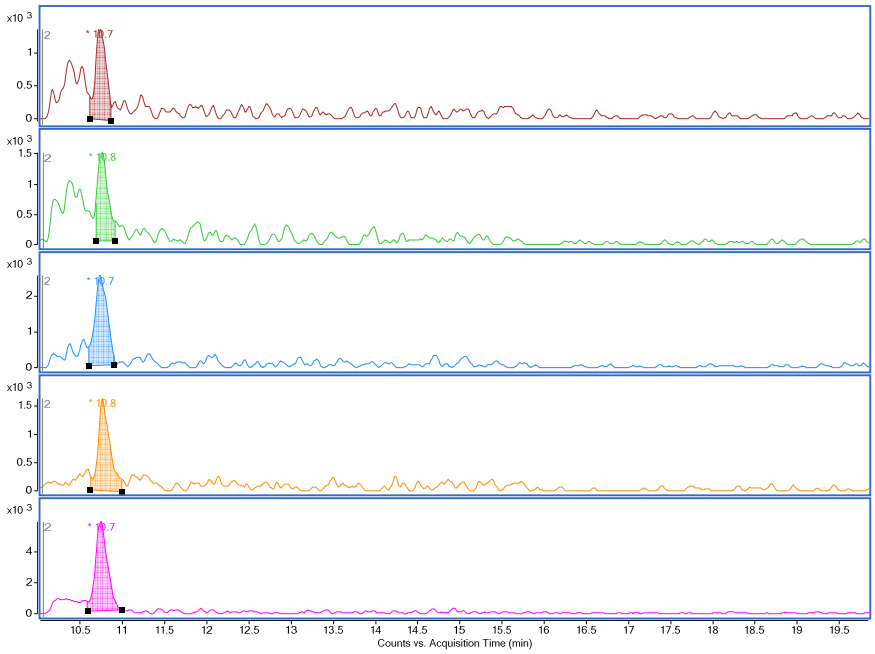


Figure 4.36. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-DPOG samples gamma irradiated at 1, 3 and 5 kGy

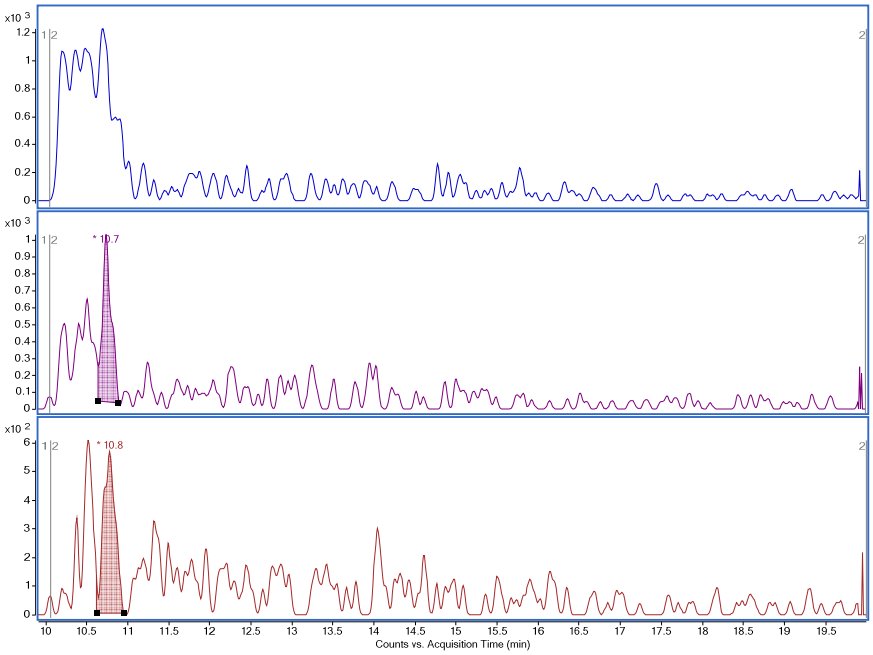


Figure 4.37. LC-TOF-MS extracted ion chromatograms (417.2507, [M-H]-) for the DNPH-derivatised-DPOG samples EB irradiated at 0.3, 0.5, 1, 3 and 5 kGy

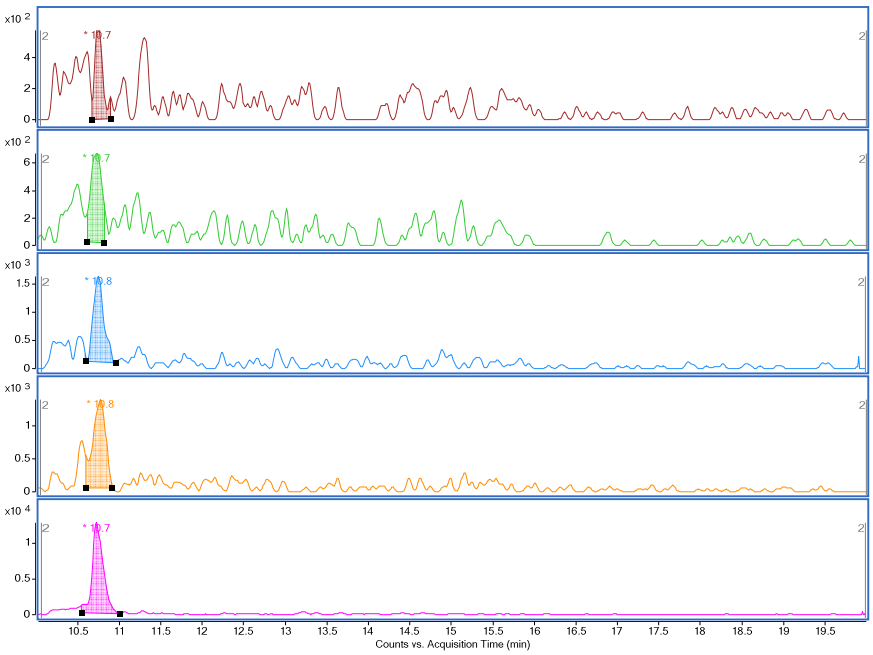
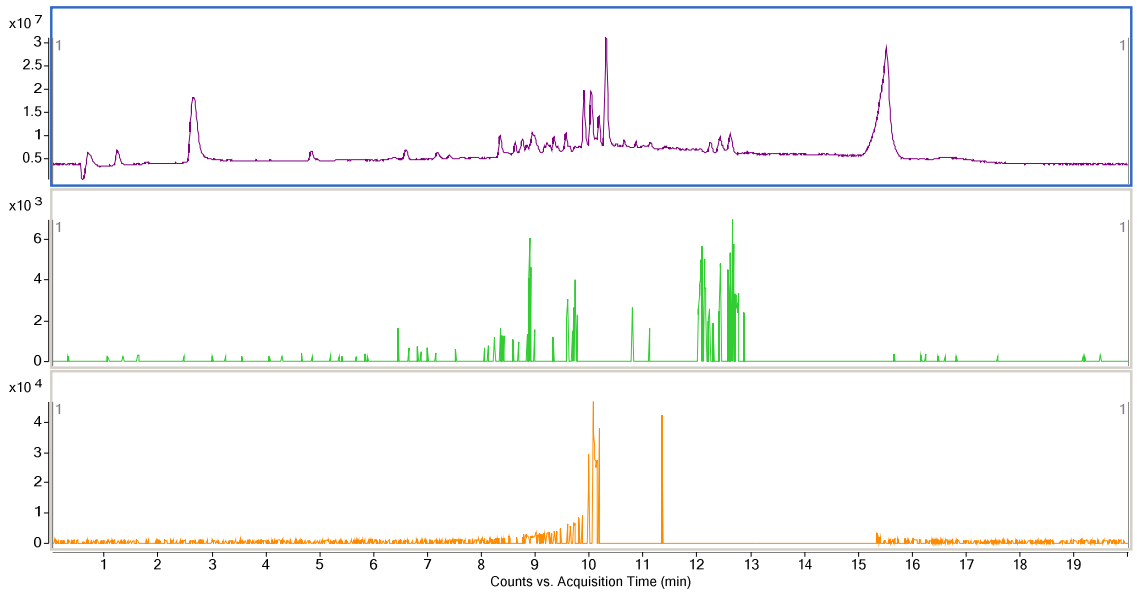


Figure 4.38. LC-TOF-MS a) total ion chromatogram, and extracted ion chromatograms of the expected $[M-H]^-$ adducts for the 2-ACB irradiation product and parent fatty acid for irradiated (gamma, 5 kGy) SA and b) total ion chromatogram of the irradiated SA overlaid with the non-irradiated SA

a)



b)

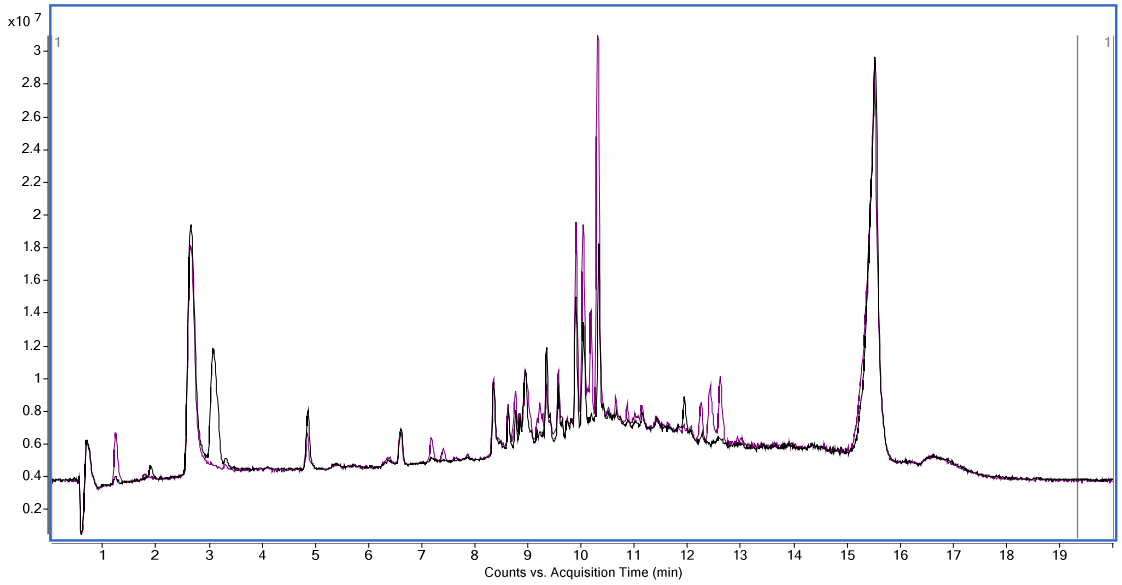
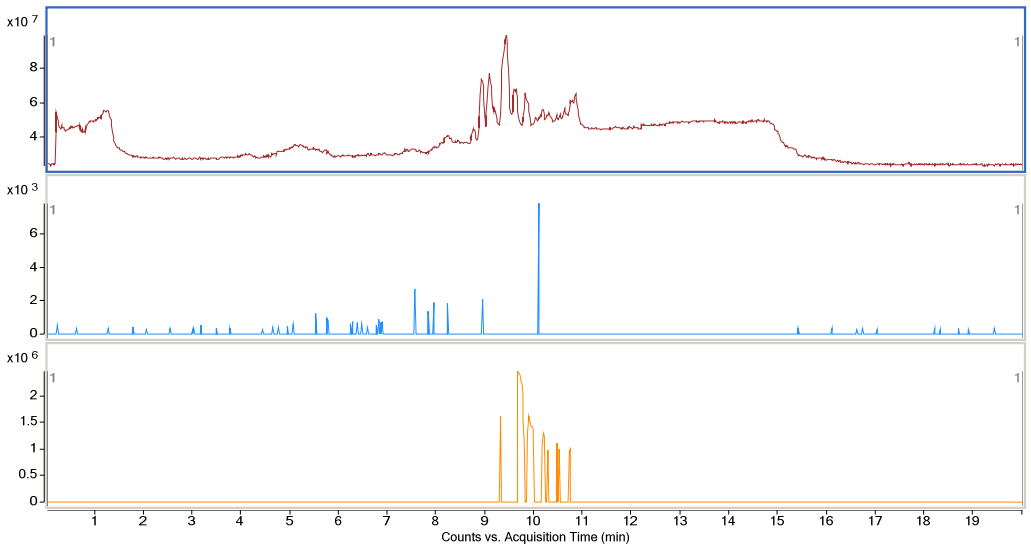


Figure 4.39. LC-TOF-MS a) total ion chromatogram, and extracted ion chromatograms of the expected $[M-H]^-$ adducts for the 2-ACB irradiation product and parent fatty acid for irradiated (gamma, 5 kGy) GTS and b) total ion chromatogram of the irradiated GTS overlaid with the non-irradiated GTS

a)



b)

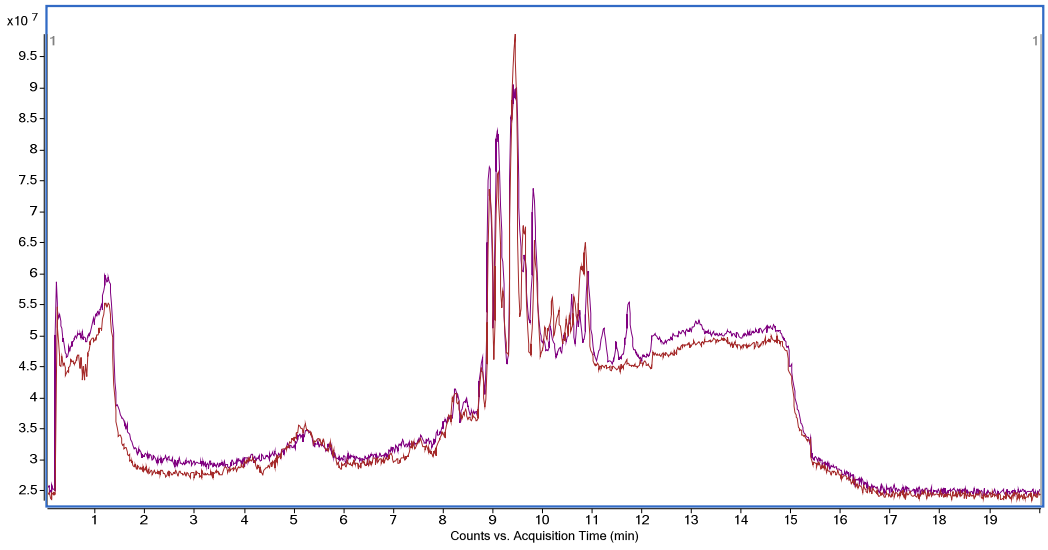
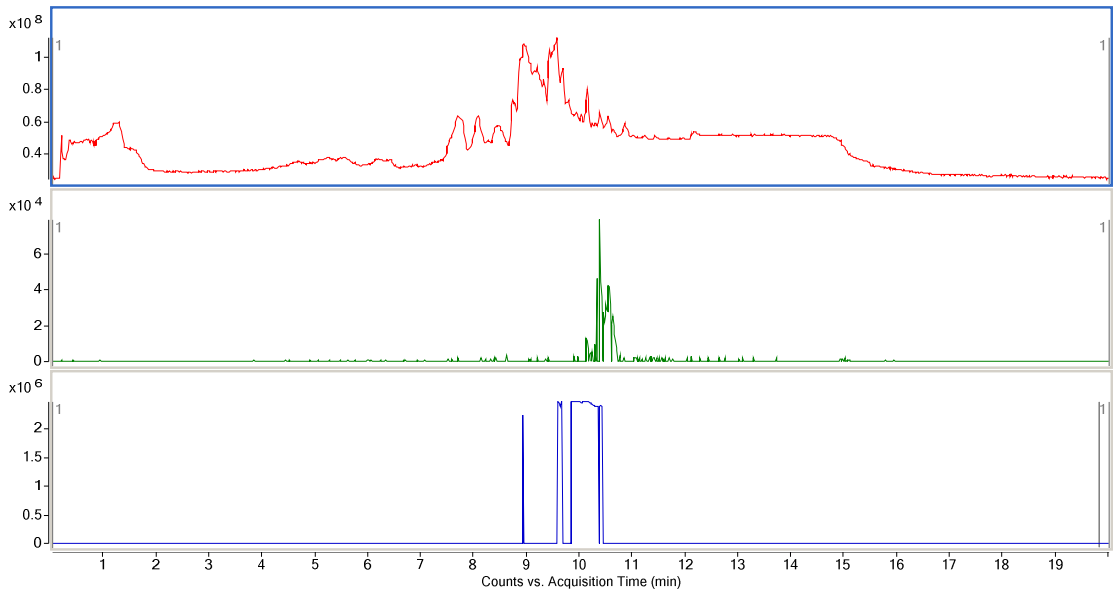


Figure 4.40. LC-TOF-MS a) total ion chromatogram, and extracted ion chromatograms of the expected $[M-H]^-$ adducts for the 2-ACB irradiation product and parent fatty acid for irradiated (gamma, 5 kGy) OA and b) total ion chromatogram of the irradiated OA overlaid with the non-irradiated OA

a)



b)

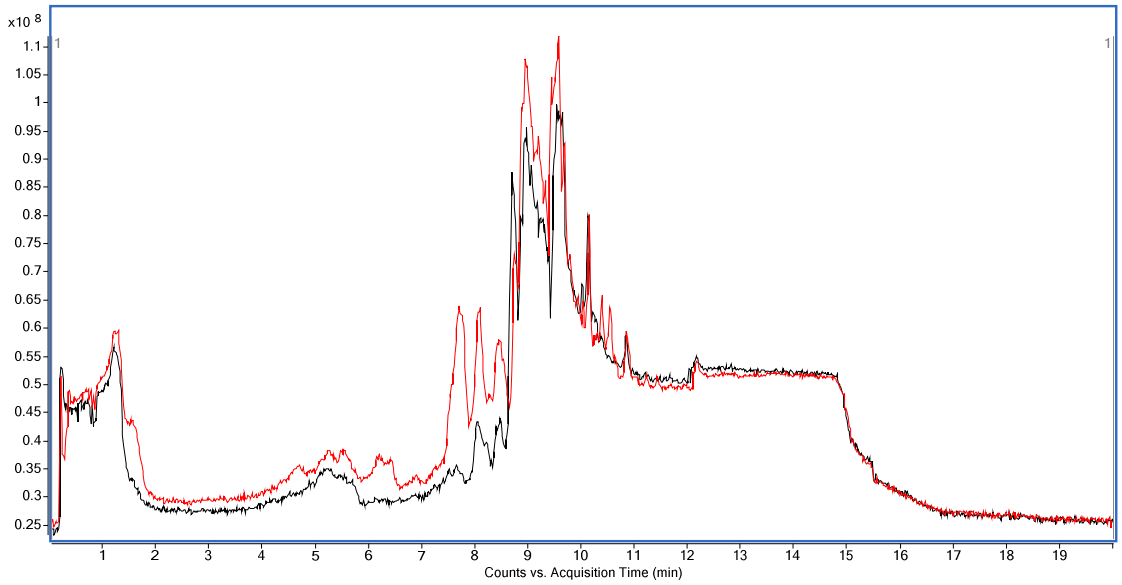
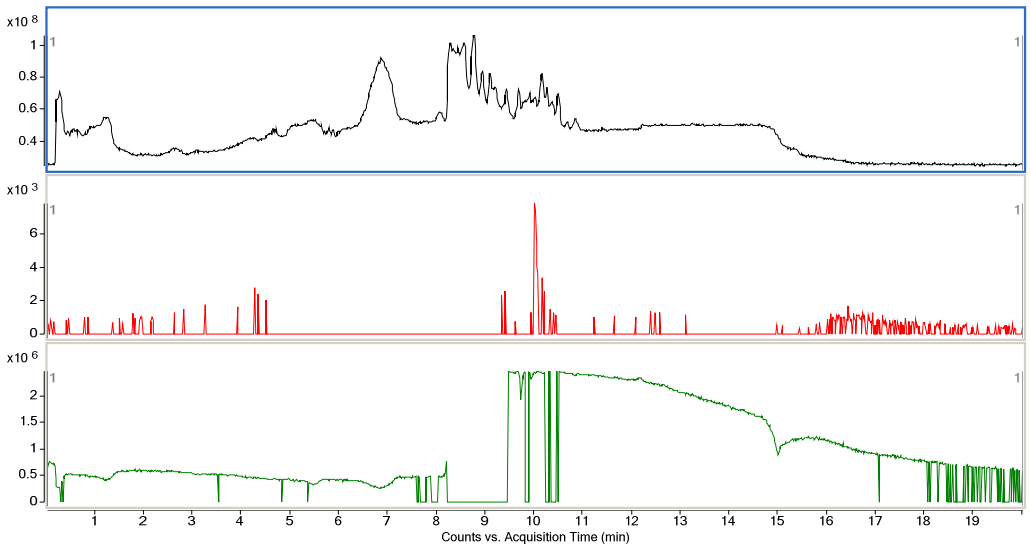


Figure 4.41. LC-TOF-MS a) total ion chromatogram, and extracted ion chromatograms of the expected $[M-H]^-$ adducts for the 2-ACB irradiation product and parent fatty acid for irradiated (gamma, 5 kGy) LLA and b) total ion chromatogram of the irradiated LLA overlaid with the non-irradiated LLA

a)



b)

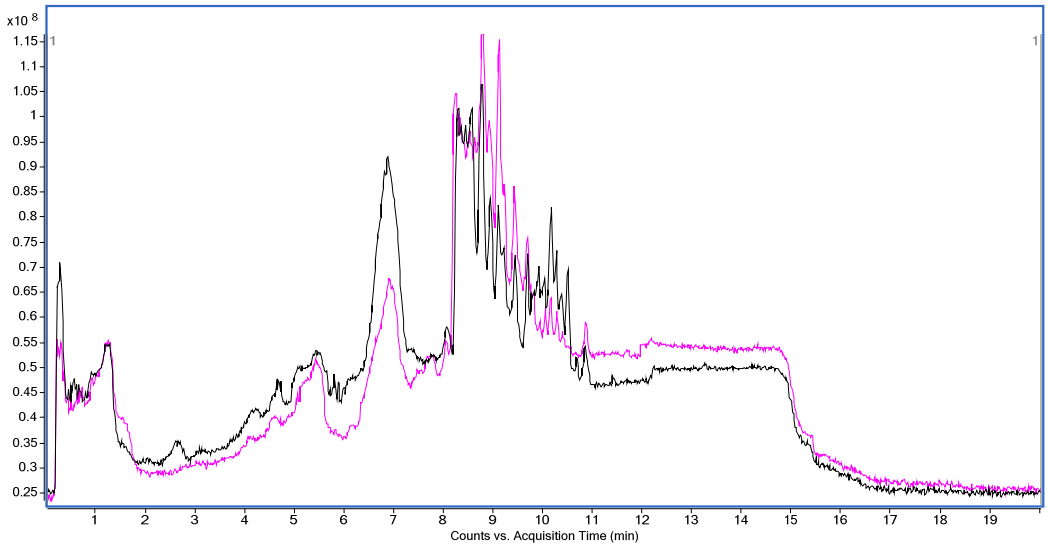
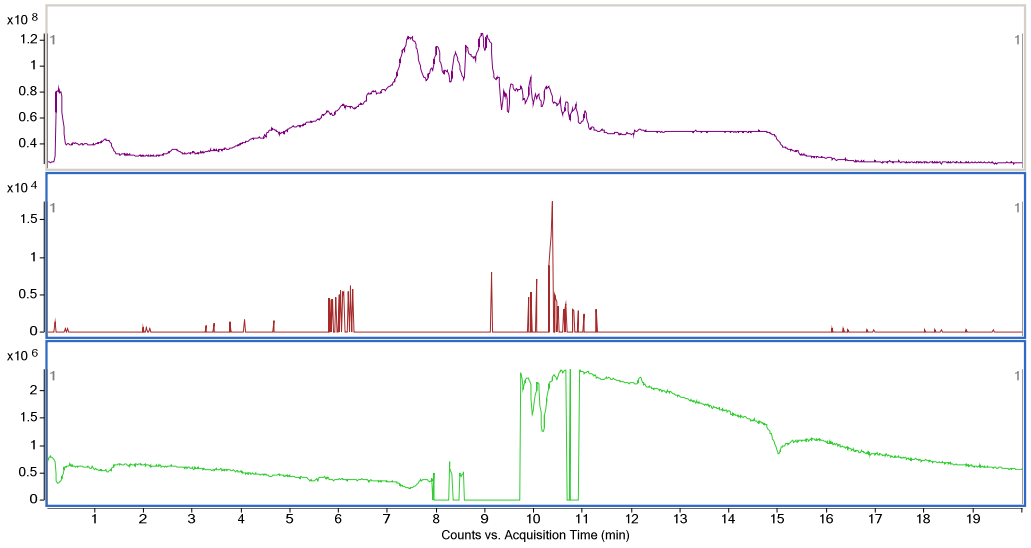


Figure 4.42. LC-TOF-MS a) total ion chromatogram, and extracted ion chromatograms of the expected $[M-H]^-$ adducts for the 2-ACB irradiation product and parent fatty acid for irradiated (gamma, 5 kGy) LA and b) total ion chromatogram of the irradiated LA overlaid with the non-irradiated LA

a)



b)

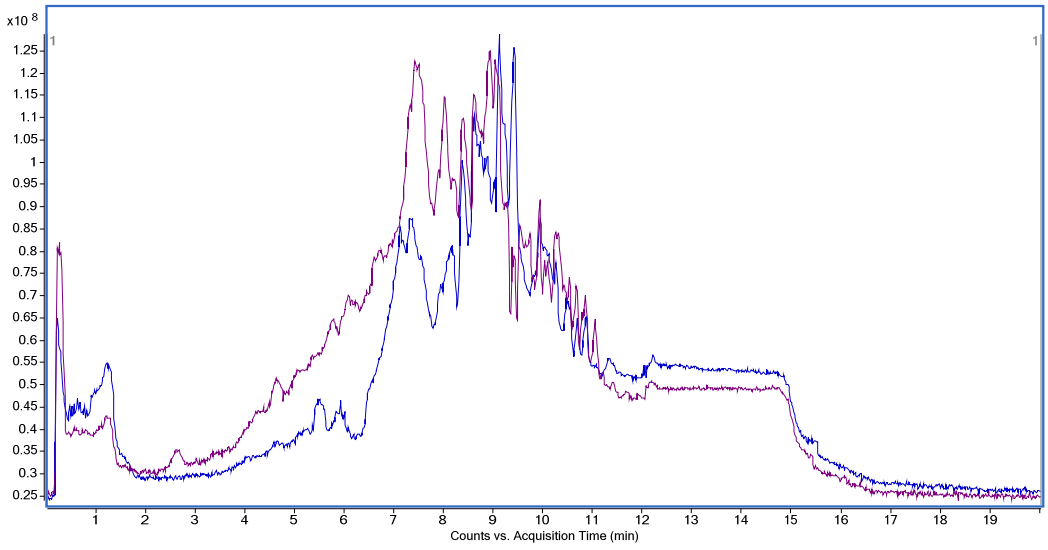
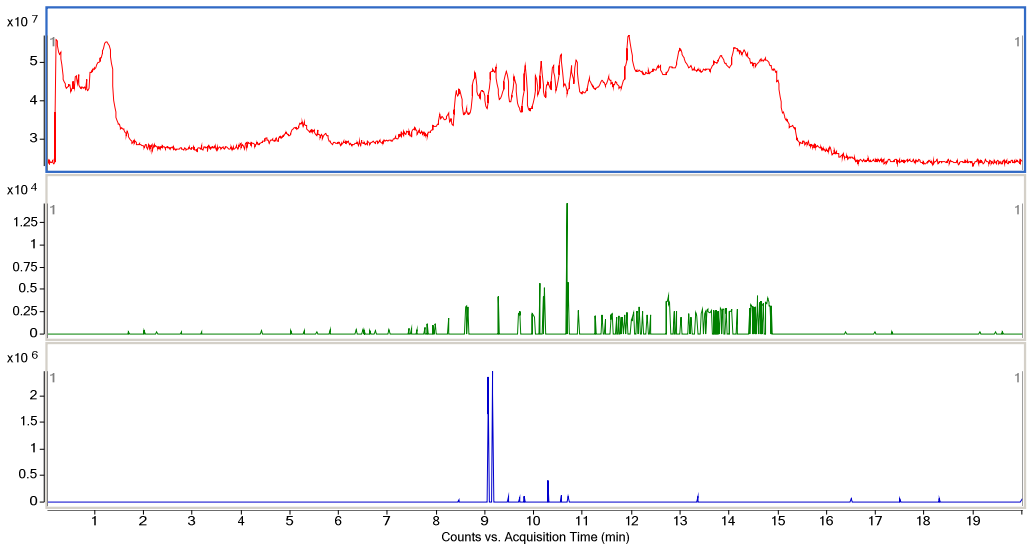


Figure 4.43. LC-TOF-MS a) total ion chromatogram, and extracted ion chromatograms of the expected $[M-H]^-$ adducts for the 2-ACB irradiation product and parent fatty acid for irradiated (gamma, 5 kGy) GTO and b) total ion chromatogram of the irradiated GTO overlaid with the non-irradiated GTO

a)



b)

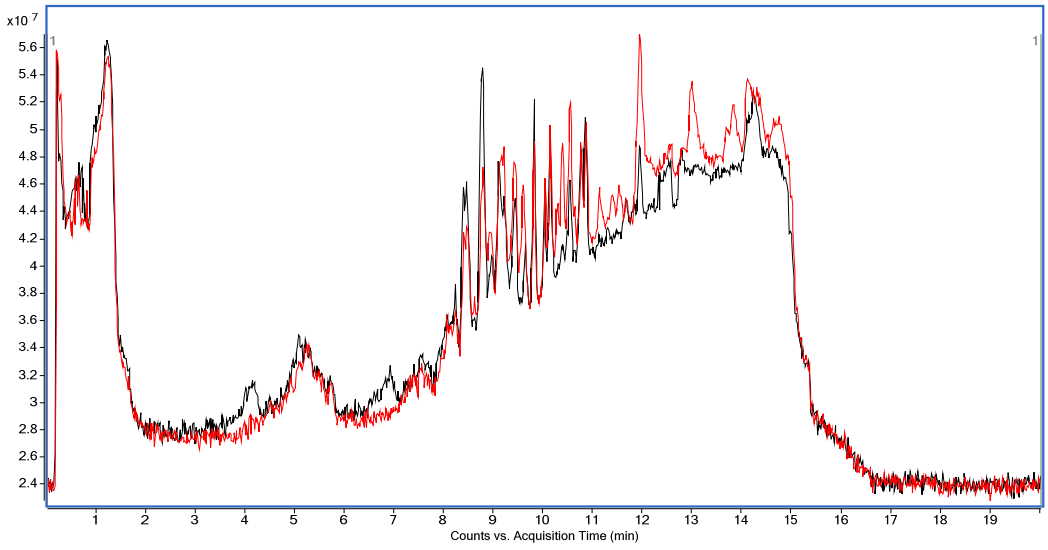
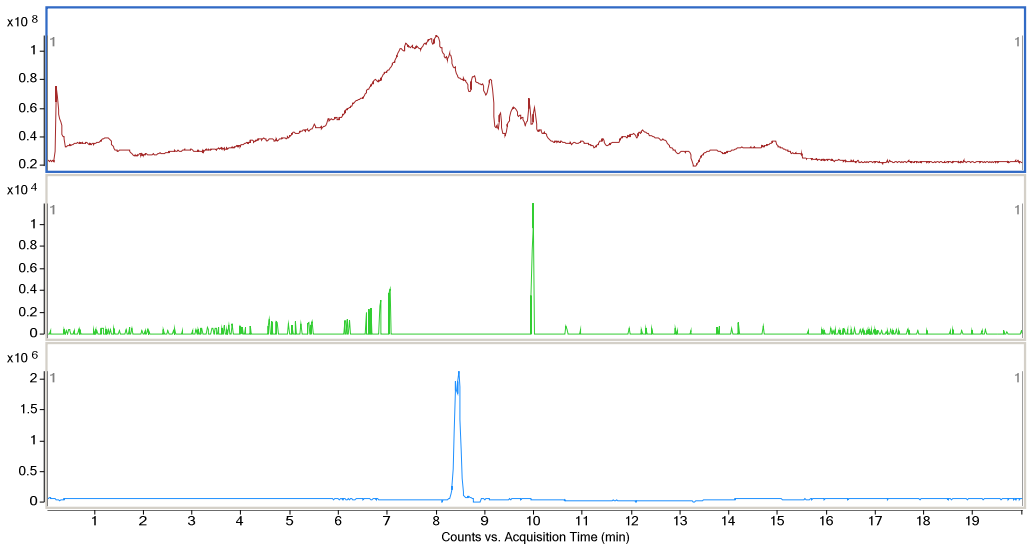
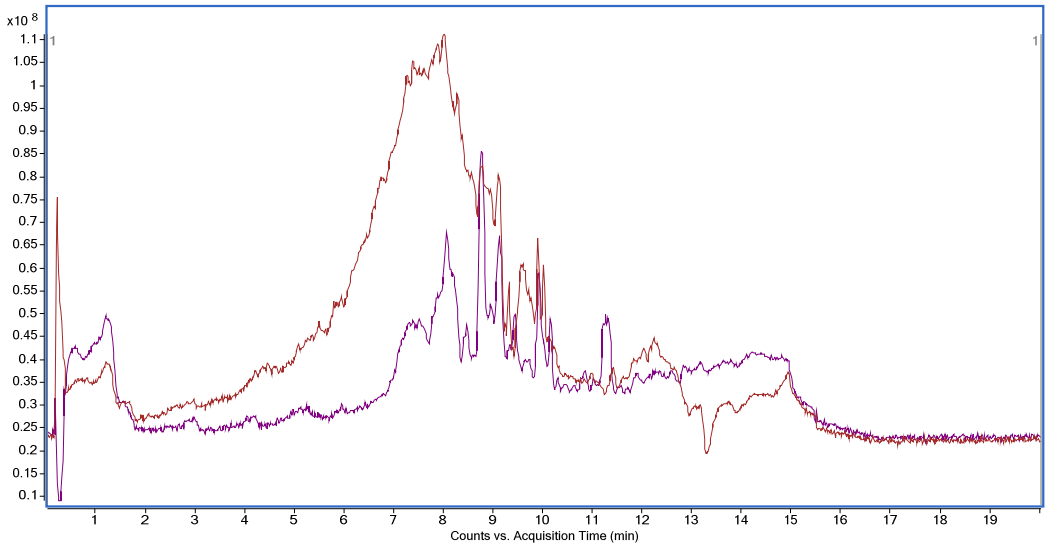


Figure 4.44. LC-TOF-MS a) total ion chromatogram, and extracted ion chromatograms of the expected $[M-H]^-$ adducts for the 2-ACB irradiation product and parent fatty acid for irradiated (gamma, 5 kGy) GTL and b) total ion chromatogram of the irradiated GTL overlaid with the non-irradiated GTL

a)



b)



It can be seen that no peaks are present that were attributable to the expected 2-ACB irradiation products. Also in most cases the irradiated sample overlays very closely with the non-irradiated starting material and where there appear to be new peaks formed, upon closer scrutiny of the mass spectral data indicates that the compounds are in fact in both samples, only at different concentrations. In some instances there is evidence for the starting materials, as was seen in GC-MS.

Some of the reasons for these observations may be:

(i) The derivatisation may not have worked effectively.

This is unlikely as the derivatisation was successful for PA, GTP and DPOG.

(ii) The irradiation yield may have low or the instrument sensitivity was not high enough to enable detection.

This is possible, however 2-DCB was detectable although the method was optimised as the authentic standard was available, and without this for the other 2-ACBs it is impossible to determine optimum conditions and sensitivity.

In summary without authentic standards it is extremely difficult to detect the expected 2-ACB irradiation products.

4.2.3.3 LC-MS/MS

As 2-DCB peaks were detected in PA, GTP and DPOG by GC-MS and LC-TOF-MS the samples were derivatised and analysed by the more sensitive technique of LC-MS/MS. Samples of non-irradiated PA (50 mg), GTP (50 mg) and DPOG (3 mg) were over-spiked with 2-DCB (0.01, 0.1 and 1 µg) and derivatised with DNPH to test the efficiency of the derivatisation process in the presence of the non-irradiated starting materials. Table 4.4 gives the recoveries. It can be seen that for the low level spike (0.01 µg 2-DCB) the recoveries were lower with an average of 51%, than for the two higher levels (0.1 and 1 µg 2-DCB) with average values of 93% and 75% respectively. This is more likely to be a consequence of the greater uncertainty when measuring at lower levels due to integrating smaller peaks than an issue with derivatisation efficiency, as the recovery in the presence of no sample (solvent only) also has a low recovery (50%) at the 0.01 µg spiking level. The presence of PA

(recoveries of 59%, 117% and 90%) does not significantly affect the recovery of the 2-DCB compared to the solvent derivatisation (50, 118 and 87%). The GTP recoveries were lower (52%, 71% and 72%) and the DPOG lower still (42%, 67% and 51%).

Table 4.4. Recoveries of solvent, PA, GTP and DPOG over-spiked with 2-DCB

	Recovery (%)			
	0.01 µg 2-DCB added	0.01 µg 2-DCB added	0.01 µg 2-DCB added	Average
Blank	50	118	87	85
PA	59	117	90	89
GTP	52	71	72	65
DPOG	42	67	51	53
Average	51	93	75	

These recoveries were taken into account and Figures 4.45 and 4.46 summarise the recovery corrected amounts of 2-DCB detected, in µg 2-DCB detected/g starting material (fatty acid or triacylglyceride). Figures 4.47 to 4.54 show the EICs (417.2>151.0) for the DNPH-derivatised-2-DCB formed for the procedural blanks, PA, GTP and DPOG following irradiation by gamma rays at 1, 3 and 5 kGy and EB at 0.3, 0.5, 1, 3 and 5 kGy.

2-DCB could be detected in each case, confirming irradiation, even at the lowest irradiation dose of 0.3 kGy. For PA and GTP the amount of 2-DCB formed increases as the irradiation dose increases. For DPOG the dose/concentration trend increases until 5 kGy when there is a small decrease in concentration when looking at EB irradiation. The amount of DPOG detected remains approximately the same for gamma irradiation. Another conclusion is that the amount of 2-DCB formed is approximately the same at a given dose when comparing gamma and EB irradiation types.

In the study mentioned earlier [11], also carried out investigating the formation of 2-ACBs from irradiated fatty acids and triacylglycerides, higher levels of 2-DCB were reported as being formed (approximately 20 µg/g at 10 kGy for GTP). This is a higher dose than those used here but it seems unlikely from our results that the 2-DCB concentration would increase by so much at the higher dose. Also the

quantification method is not clear as it is not apparent whether authentic standards of the 2-ACBs were used for comparison.

Figure 4.45. DNPH-derivatised-2-DCB results following electron beam irradiation at different doses (0.1, 0.3, 1, 3 and 5 kGy) for PA, GTP and DPOG

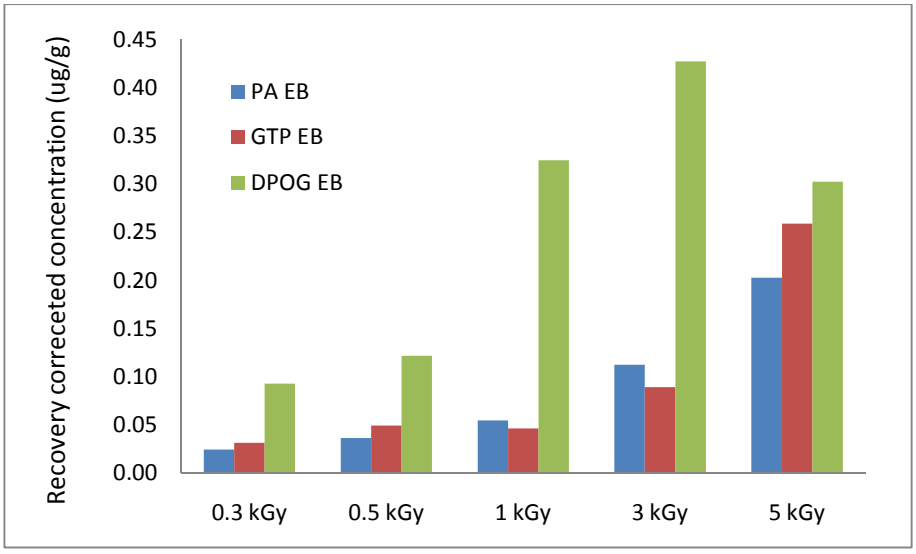


Figure 4.46. DNPH-derivatised-2-DCB results following gamma irradiation at different doses (1, 3 and 5 kGy) for PA, GTP and DPOG

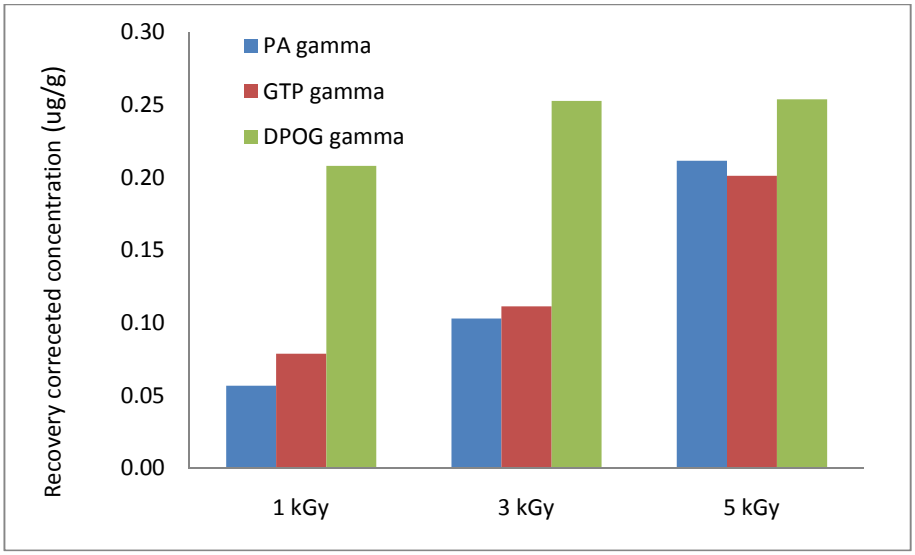


Figure 4.47. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-procedural blank samples gamma irradiated at 1, 3 and 5 kGy

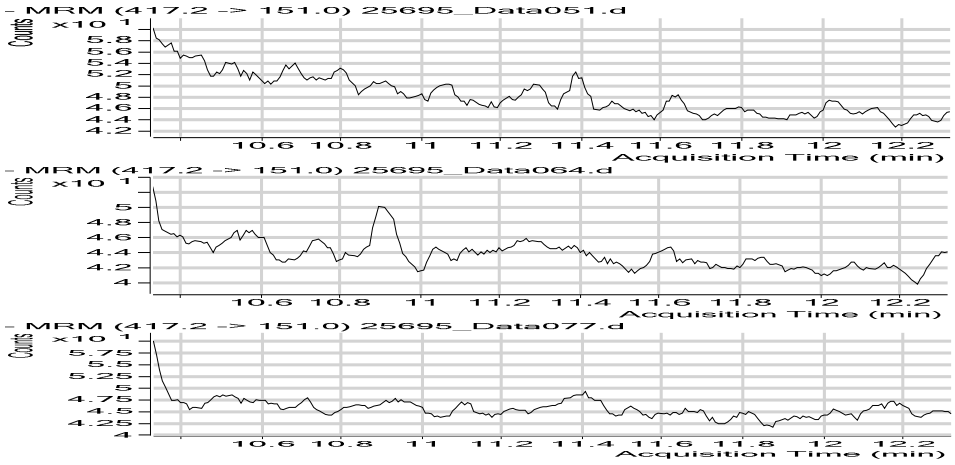


Figure 4.48. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-procedural blank samples EB irradiated at 0.1. 0.3, 1, 3 and 5 kGy

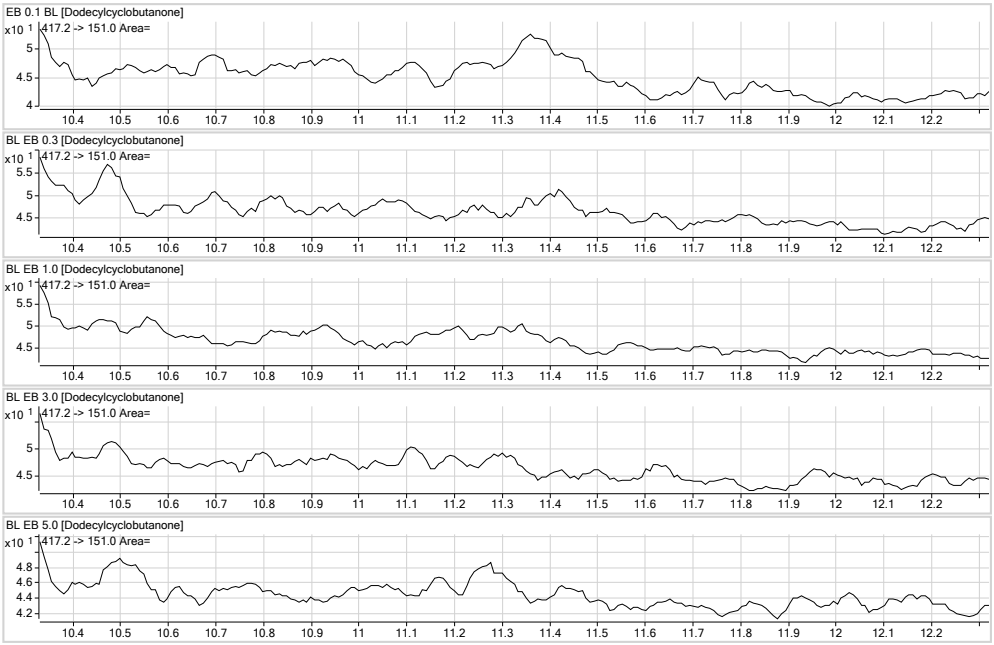


Figure 4.49. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-PA samples gamma irradiated at 1, 3 and 5 kGy

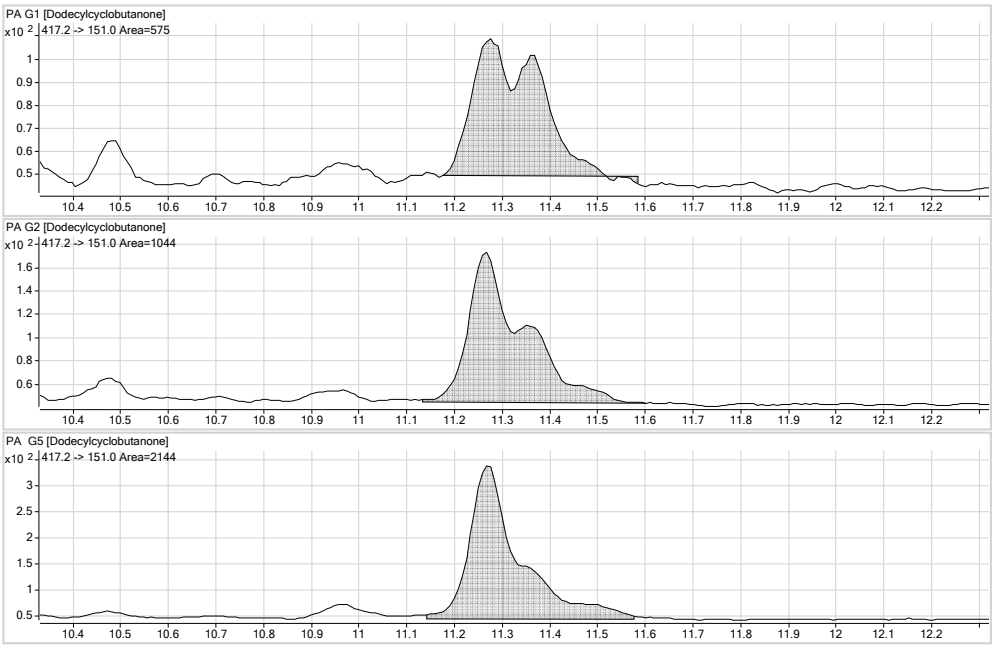


Figure 4.50. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-PA samples EB irradiated at 0.1. 0.3, 1, 3 and 5 kGy

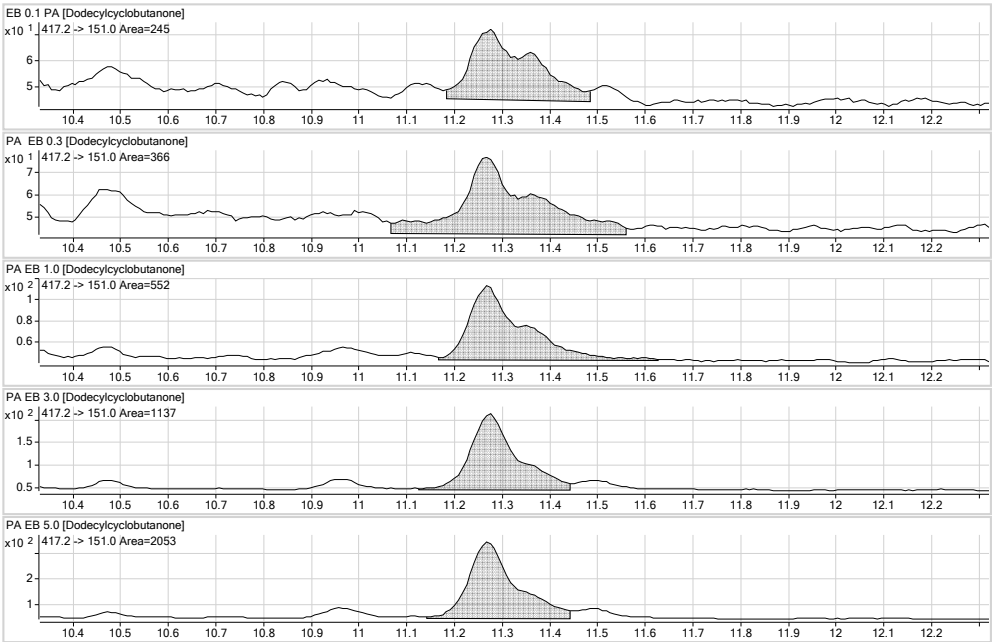


Figure 4.51. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-GTP samples gamma irradiated at 1, 3 and 5 kGy

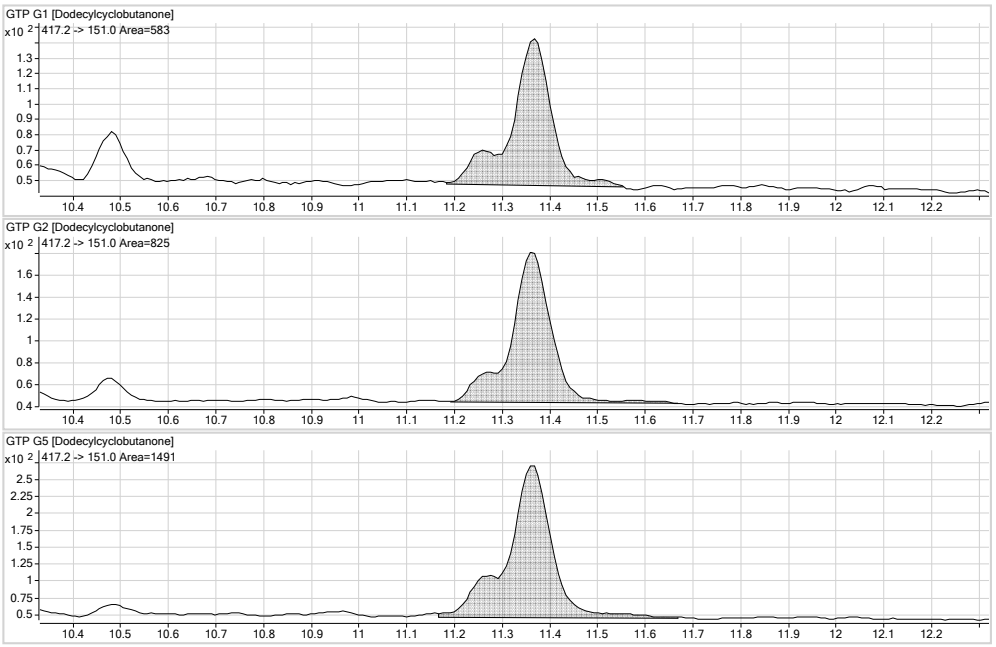


Figure 4.52. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-GTP samples EB irradiated at 0.1. 0.3, 1, 3 and 5 kGy

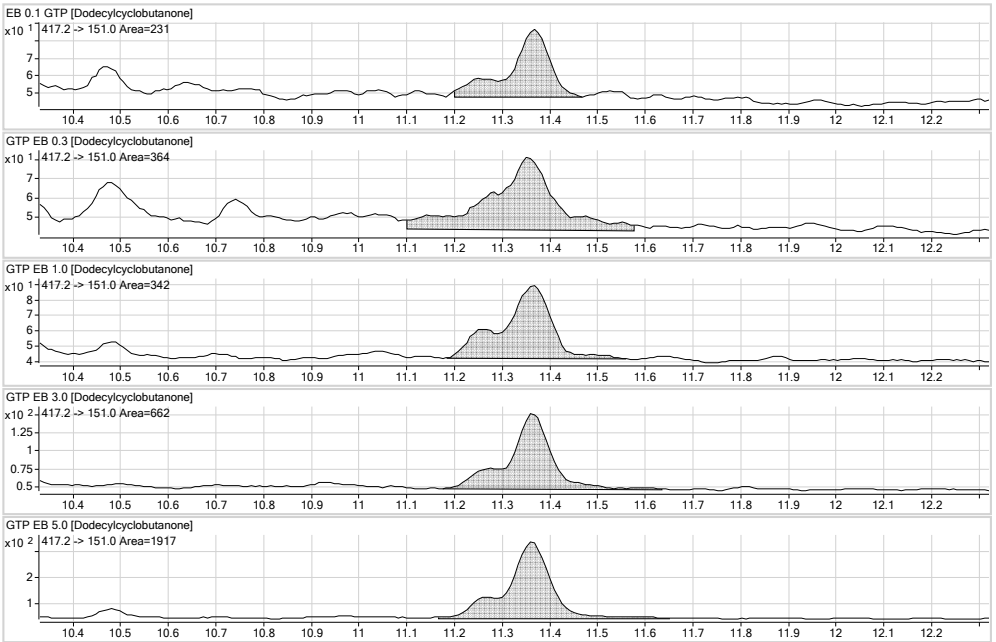


Figure 4.53. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-DPOG-procedural blank samples gamma irradiated at 1, 3 and 5 kGy

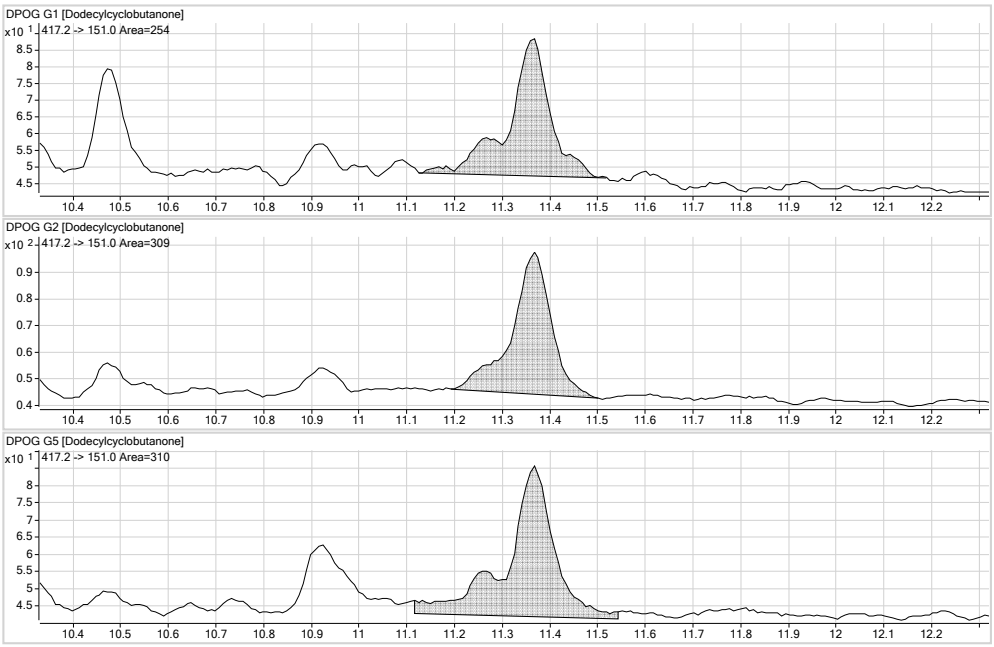
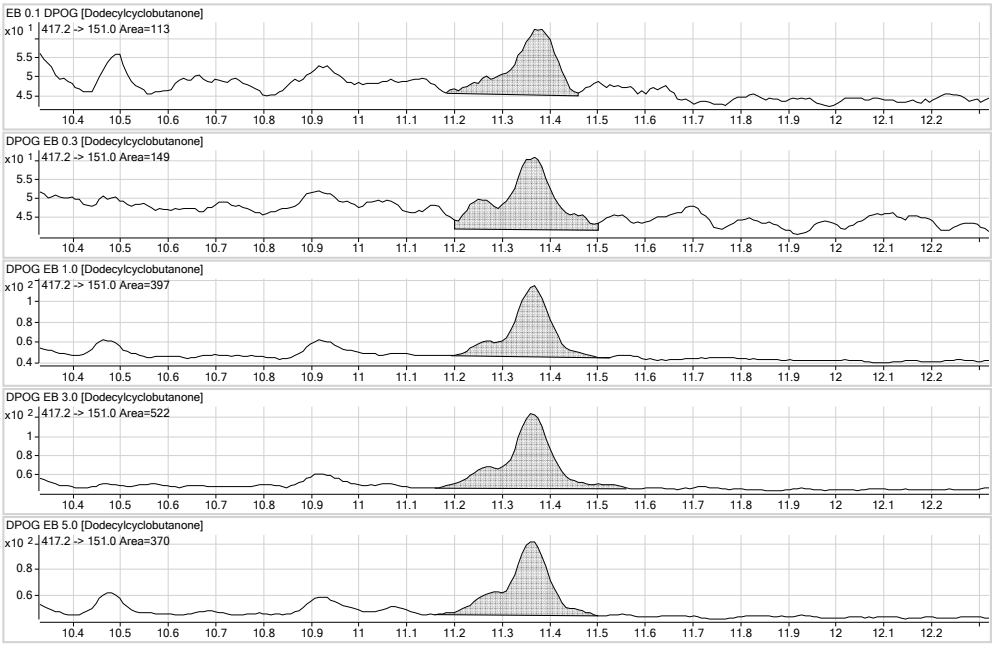


Figure 4.54. LC-MS/MS extracted ion chromatograms (417.2>151.0) for the DNPH-derivatised-DPOG-procedural blank samples gamma irradiated at 1, 3 and 5 kGy



4.3 The effect of non-irradiation food processing technologies on commercially available triacylglycerols and free fatty acids (Task 02)

4.3.1 Introduction

As mentioned previously, to date 2-ACBs have not been detected in food processed by methods other than irradiation [4-5] and therefore these substances are postulated to be 'unique radiolytic products'. However it is also possible that the methods of analysis that are currently available are not sufficiently sensitive and that 2-ACBs may be present in non-irradiation processed foods but not at detectable concentrations. Therefore more sensitive methodology is needed to demonstrate that the 2-ACBs are not present in foods processed by techniques other than irradiation. This section describes work carried out to determine whether 2-ACBs can be formed by food processing techniques other than irradiation.

As shown from Figures 4.1 and 4.2 the formation of 2-ACBs requires the excitation of the parent triacylglycerol or free fatty acid to form intermediate free radicals. Therefore their formation is most likely to involve processing parameters that can cause such excitation to occur. It was decided to investigate the effect of heating at different temperatures, in the presence and absence of light, air and redox-active metal salts (shown to catalyse lipid breakdown), at elevated pressure, and in the presence of microwaves, upon the three compounds tested previously that form 2-DCB upon irradiation (PA, GTP and DPOG). Following on from the results of the method development and comparison in Chapter 2 derivatisation with DNPH followed by LC-MS/MS was chosen as the analysis technique. This was because the detection limit of 2-DCB was the lowest (better) and that sample clean-up by Florisil chromatography was not required as with GC-MS. Although both clean-up and derivatisation add extra steps to the procedure more samples can be taken through the derivatisation more quickly.

4.3.2 Experimental details

4.3.2.1 Sample treatment – heating in a convection oven

Portions of PA (50 mg), GTP (50 mg) and DPOG (10 mg) were sealed in glass vials (the same sort as those used in the irradiation experiments) and were heated in a laboratory oven at 50℃, 100℃ and 250℃ for 10 min utes, 20 minutes, 30 minutes

and 60 minutes, in the presence of air and under a nitrogen atmosphere. The nitrogen was introduced by sealing the vials with a septa containing cap. Nitrogen was introduced with a needle through the septa and allowed to purge from another needle for 5 minutes. The needles were then removed sealing the vial again. The experiments at 250^{o}C were repeated but in amber vials to exclude light during treatment. Following treatment all samples were allowed to cool and stored in the freezer until analysis by LC-MS/MS.

4.3.2.2 Sample treatment – heating in a pressure cooker

Portions of PA (50 mg), GTP (50 mg) and DPOG (10 mg) were sealed in glass vials (the same sort as those used in the irradiation experiments) and were heated in a pressure cooker at 109℃ and 121 ^{o}C for 30 minutes. Following treatment all samples were allowed to cool and stored in the freezer until analysis by LC-MS/MS.

4.3.2.3 Sample treatment – heating in a microwave oven

Portions of PA (50 mg), GTP (50 mg) and DPOG (10 mg) were placed in glass vials (the same sort as those used in the irradiation experiments) but were not sealed as the caps were metallic and would cause problems in the microwave oven. The samples were then heated at 800 W for 2, 5, 10 and 20 minutes. Portions of sample were also frozen overnight and heated on defrost setting for 20 minutes to simulate frozen food being defrosted slowly. Following treatment all samples were allowed to cool and stored in the freezer until analysis by LC-MS/MS.

4.3.2.4 Sample treatment – redox active metal salts

Copper (I) chloride (10 mg) were added to portions of PA (50 mg), GTP (50 mg) and DPOG (10 mg) in glass vials. Ethanol (5 mL) was added and the vials were sealed and heated at 60^{o}C for 18 hours with occasional shaking. This was repeated with copper (II) chloride (10 mg) and iron (III) chloride (10 mg). All metal salts were Reagent Grade (97%) from Sigma (Gillingham, UK). Following treatment all samples were allowed to cool, the ethanol evaporated under a gentle nitrogen stream and stored in the freezer until analysis by LC-MS/MS.

4.3.2.5 Derivatisation

Samples were removed from the freezer and allowed to equilibrate to room temperature. CHCH internal standard solution was added (equivalent to 0.1 µg in vial). An aqueous solution of DNPH (356 mg/mL) was prepared and a portion (1 mL) added along with water (700 µL) to each vial. The vials were sealed and left at room temperature for 30 minutes. Dichloromethane (2 mL) was added and the vial shaken. The dichloromethane was transferred to a clean vial and the extraction was repeated. After combining the dichloromethane portions the solution was evaporated to dryness under a gentle stream of nitrogen at 30^{o}C. The residue was re-dissolved in acetonitrile (1 mL) and analysed LC-MS/MS.

4.3.2.6 LC-MS/MS experimental details

An Agilent (Manchester, UK) Infinity LC system was used with a Zorbax column (C18), 150 mm long, 2.1 mm internal diameter, 3.5 µm particle size, maintained at 30^{o}C in a thermostated column oven. The mobile phase was 0.1% acetic acid in water (A) and methanol (B) with a flow rate of 0.2 mL/min. A mobile phase gradient was used starting at 10% B changing linearly to 100% B over 10 minutes before the column was re-equilibrated at 10% B for 2 minutes. An injection volume of 20 µL was used.

The MS system was an Agilent (Manchester, UK) 6490 triple quadrupole mass spectrometer used in negative ion ESI. The capillary voltage was 3 kV with a source temperature of 120^{o}C and cone voltage of 35V. Desolvation gas was nitrogen at 700 L/hour and the desolvation temperature was 300^{o}C. Collision induced dissociation was performed using argon. RF lens 1 was set at 20 and RF lens 2 at 0.2. High and low resolution mass 1 were both set at 13.5 with an ion energy of 0.5 and high and low resolution mass 2 were both set at 15.0 with an ion energy of 0.5.

4.3.3 Results

The derivatised processed samples were analysed alongside derivatised solvent standards of 2-DCB so that quantification could be carried out. Figure 4.55 shows the calibration line and Figure 4.56 shows the EIC of the quantification channel

(417.2>151.0), the confirmation channel (417.2>122.0) and the internal standard channel (359.2>152.0) for the 0.005 µg/mL standard. The limit of detection was calculated as 1 ng/mL.

Figures 4.57 to 4.66 show the LC-MS/MS EICs for the PA samples treated under the conditions stated above, Figures 4.66 to 4.76 the same information for GTP and Figures 4.77 to 4.86 for the DPOG chromatograms. It can be seen that no peaks attributed to 2-DCB could be confirmed in any of the samples. The chromatograms either showed no peak at the correct retention time, or if a small peak was present then there was no peak in the confirmation channel, or the peak area ratios did not match that of the 2-DCB solvent standards.

As determined by the literature review this is not a surprising result as there were no reported occurrences of 2-ACBs forming from non-irradiation based processing.

Figure 4.55. Calibration line for the DNPH-derivatised-2-DCB solvent standards

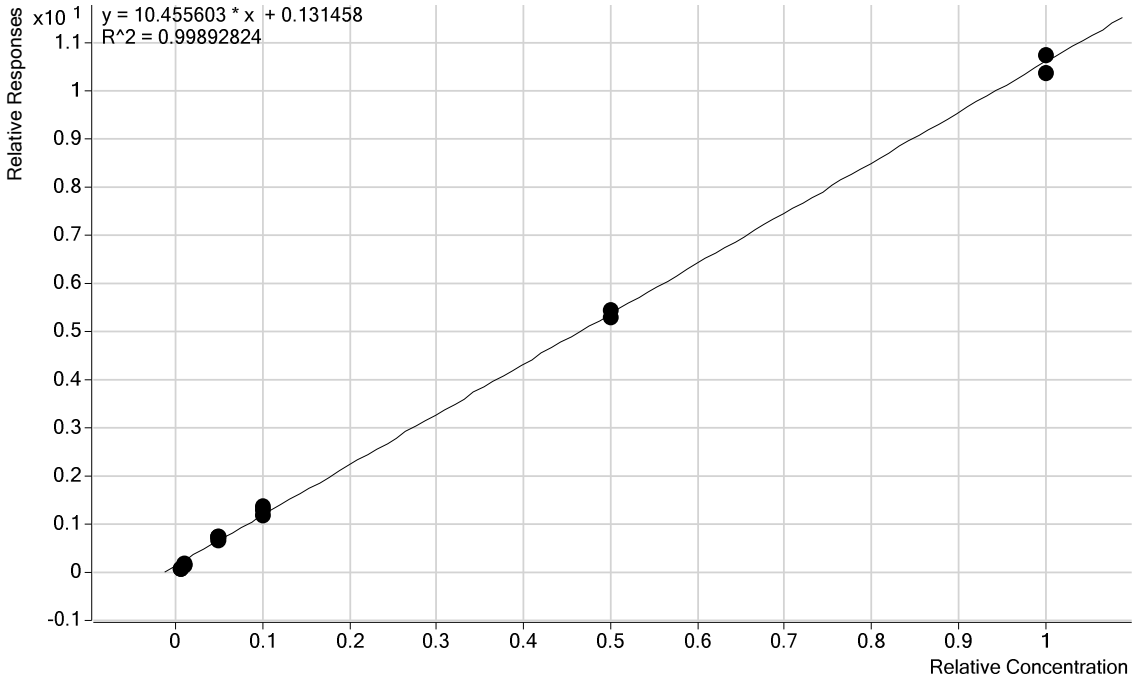
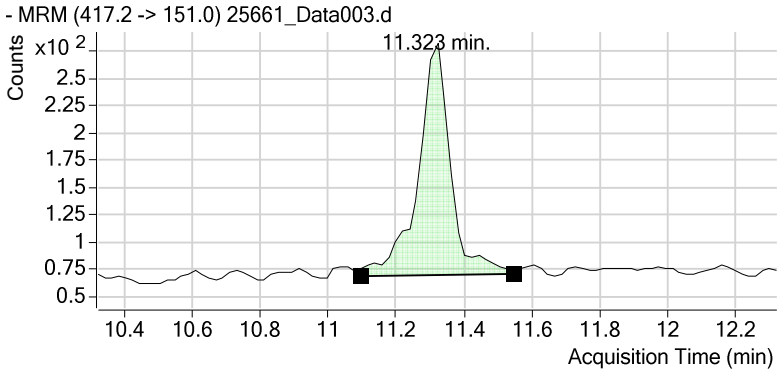
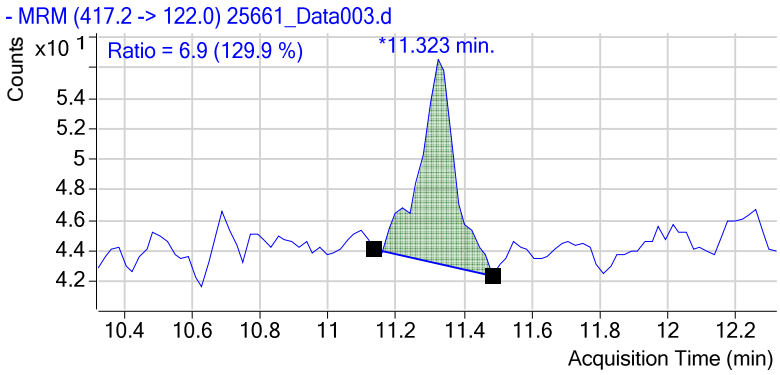


Figure 4.56. LC-MS/MS extracted ion chromatograms for the 0.005 µg/mL DNPH-derivatised-2-DCB a) quantification channel (417.2>151.0), b) confirmation channel (417.2>122.0), c) DNPH-derivatised-CHCH- internal standard (359.2>152.0)

a)



b)



c)

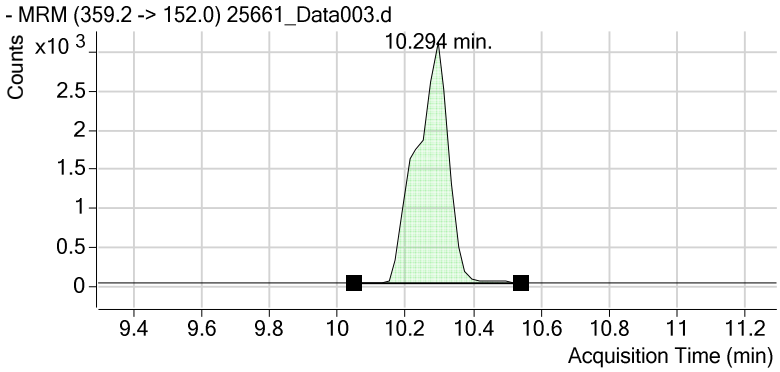


Figure 4.57. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated at 50^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

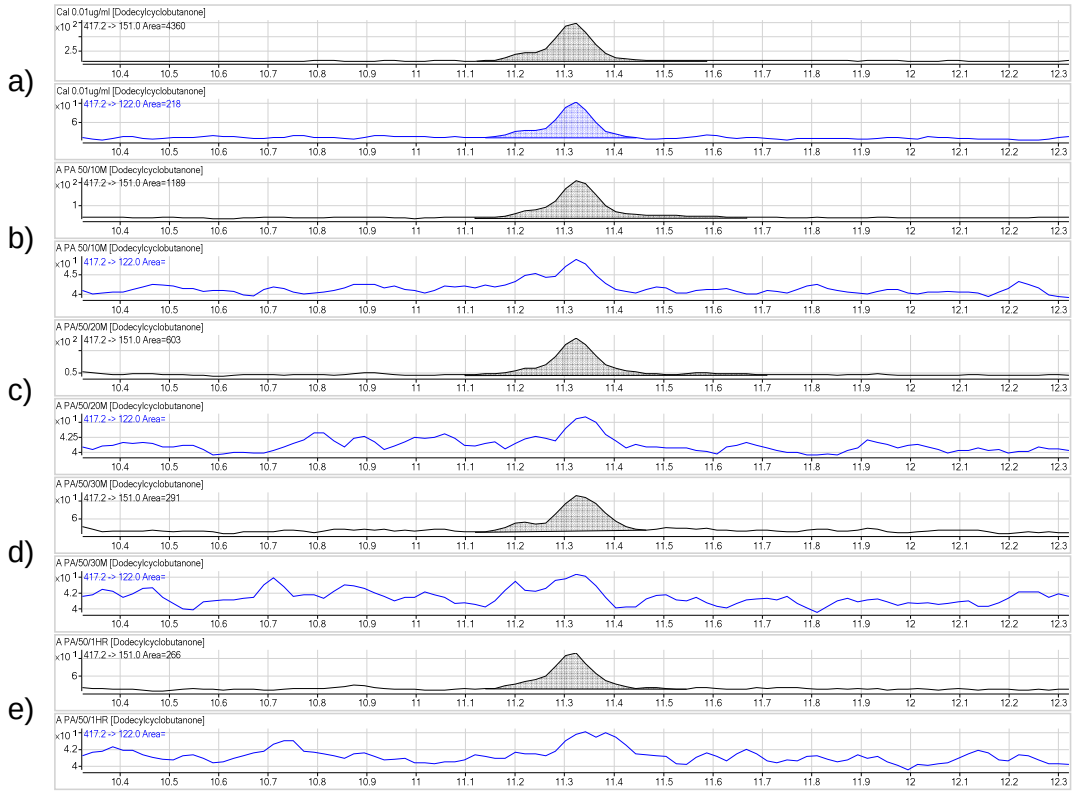


Figure 4.58. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated at 50^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

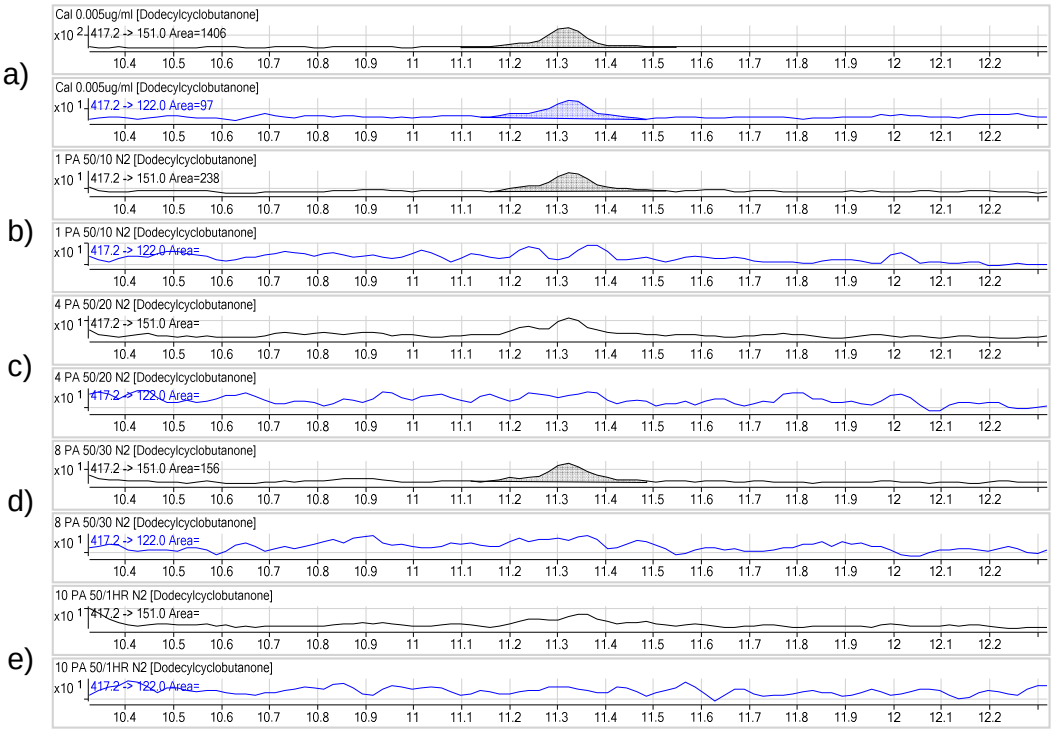


Figure 4.59. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated at 100^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

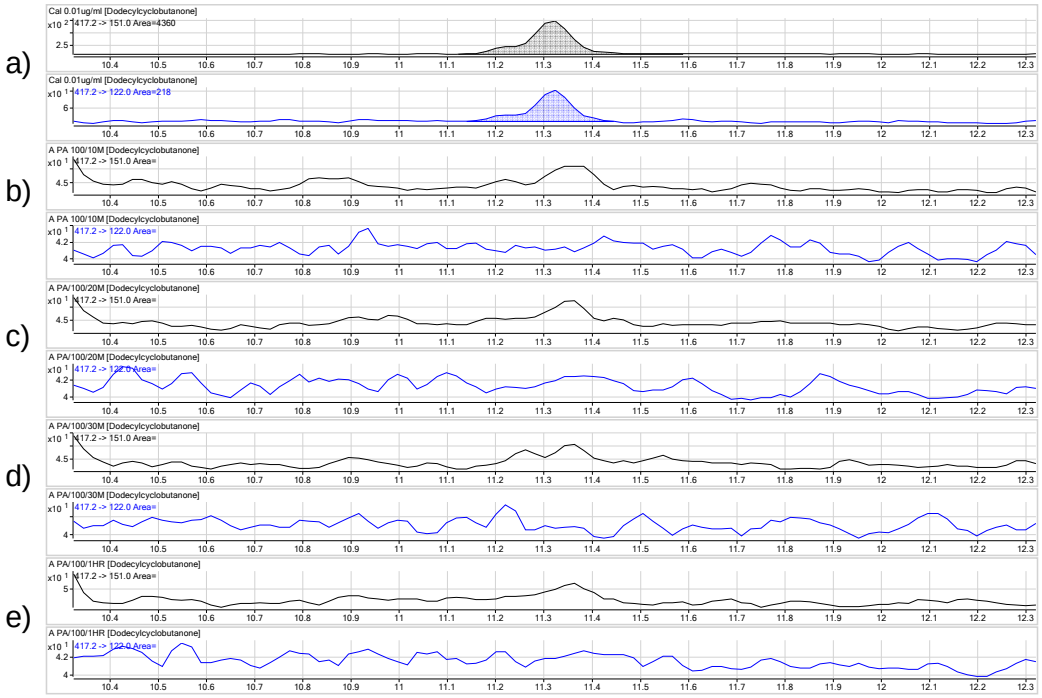


Figure 4.60. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated at 100^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

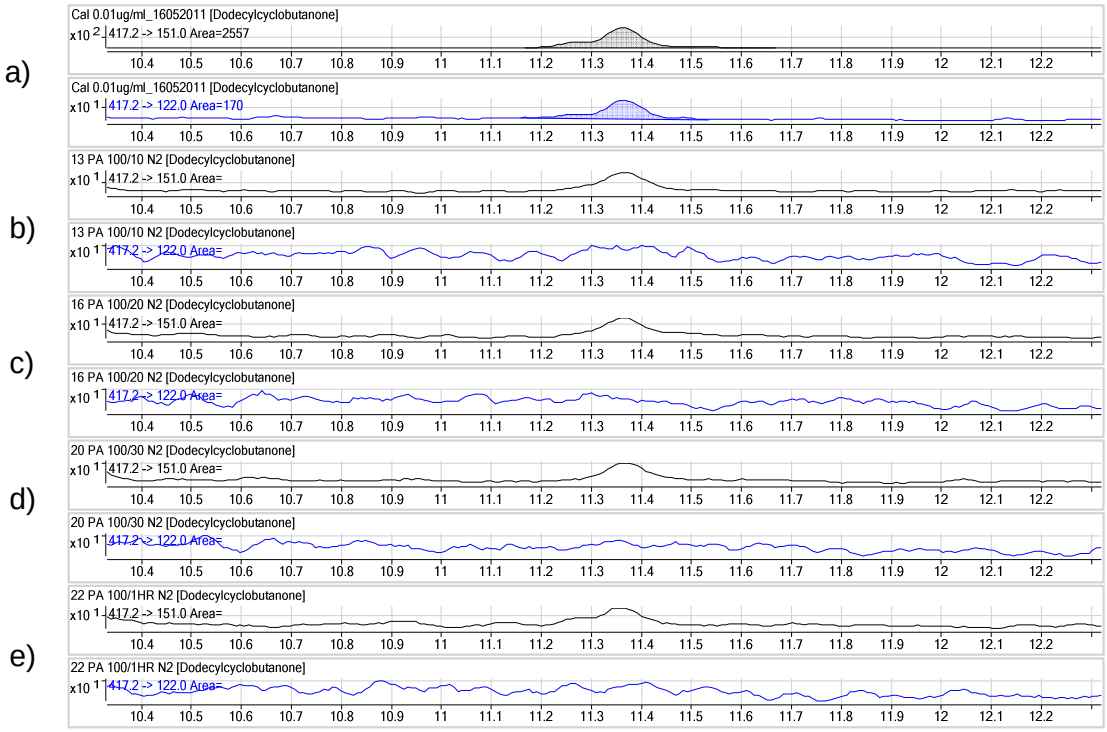


Figure 4.61. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated at 250^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

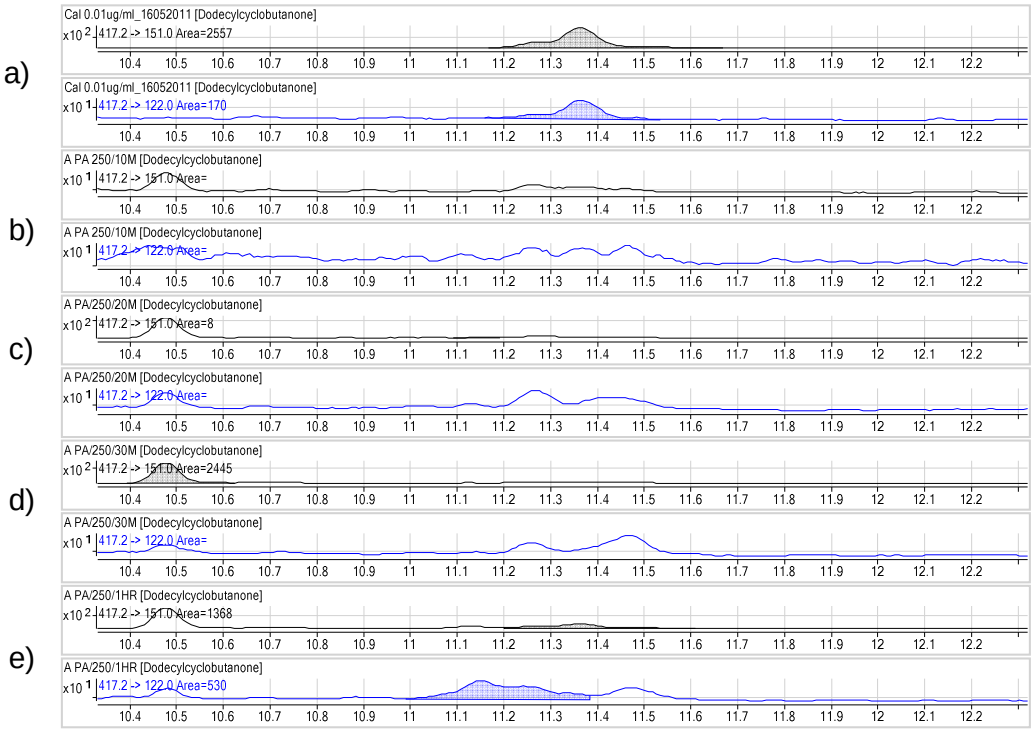


Figure 4.62. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated at 250^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

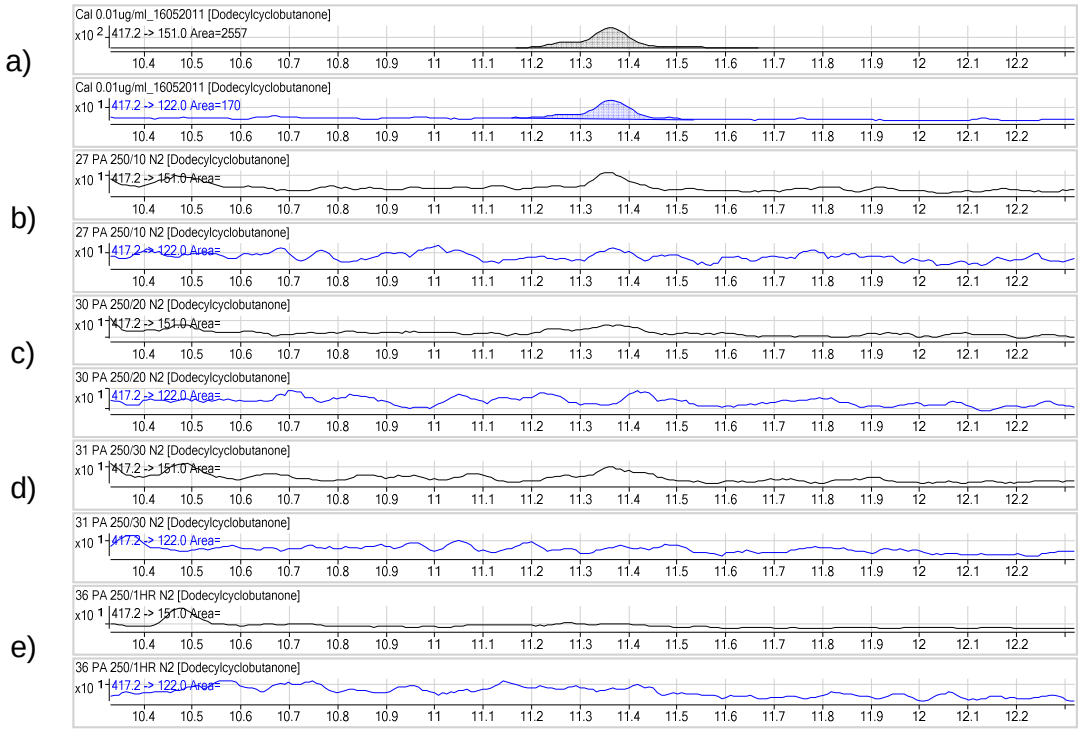


Figure 4.63. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated in the dark for 60 minutes at b) 50^{o}C, c) 100^{o}C, d) 250^{o}C. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

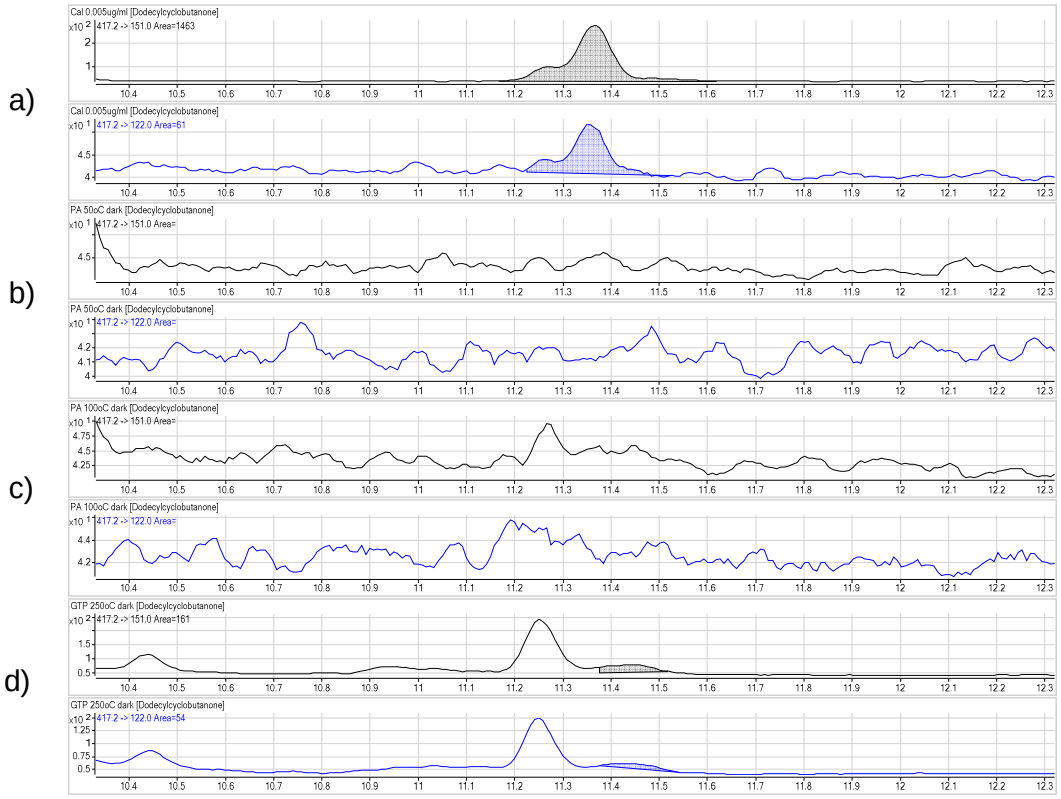


Figure 4.64. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated in a pressure cooker for 20 minutes at b) 109^{o}C, c) 121^{o}C. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

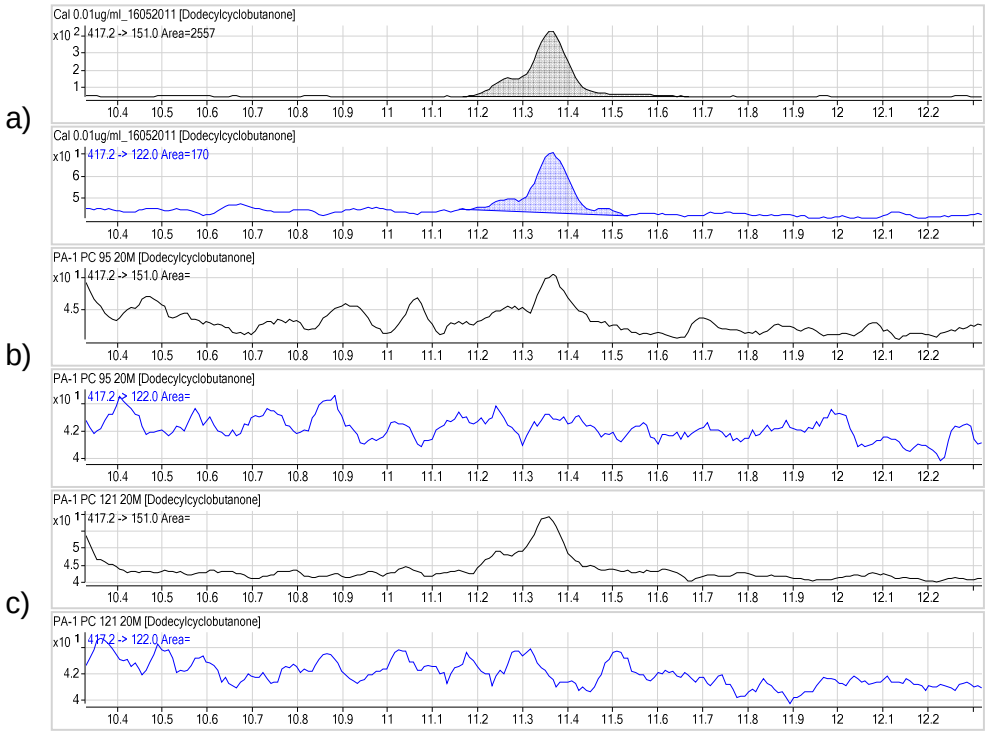


Figure 4.65. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated in a microwave at b) 800 W for 2 minutes, c) 800 W for 5 minutes, d) 800 W for 10 minutes, e) 800 W for 20 minutes, f) defrost setting for 20 minutes, following frozen overnight. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

a)

b)

c)

d)

e)

f)

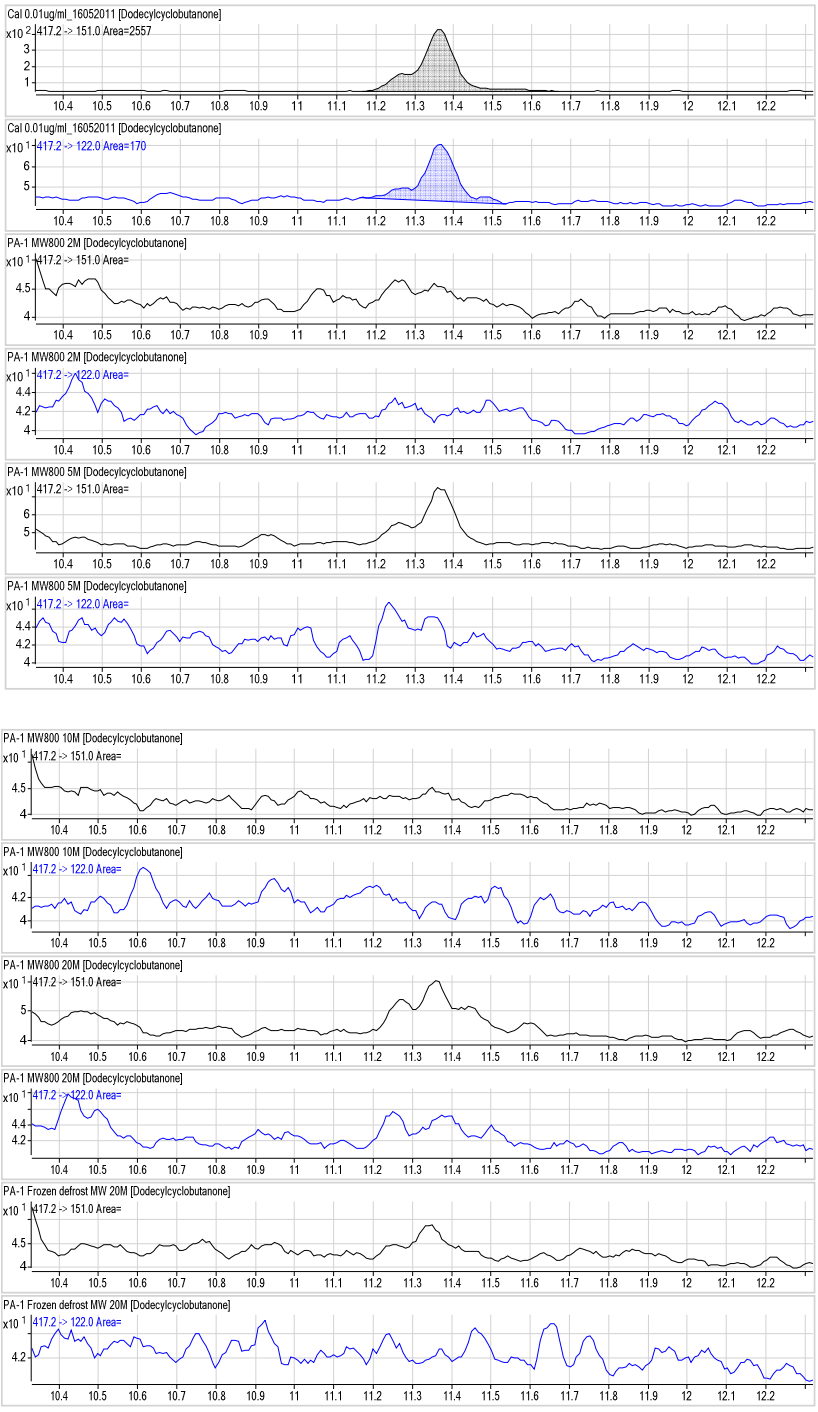


Figure 4.66. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the palmitic acid heated at 50^{o}C in the presence of b) Cu (I) salt, c) Cu (II) salt, d) Fe (III) salt. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

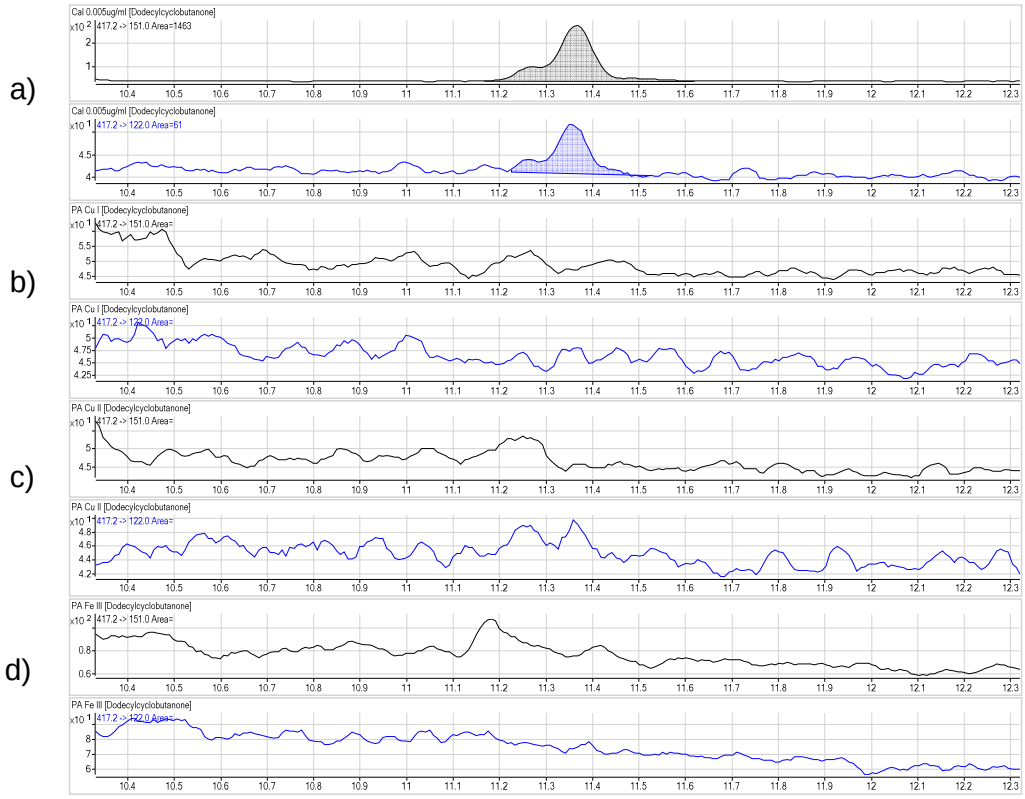


Figure 4.67. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and GTP heated at 50^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

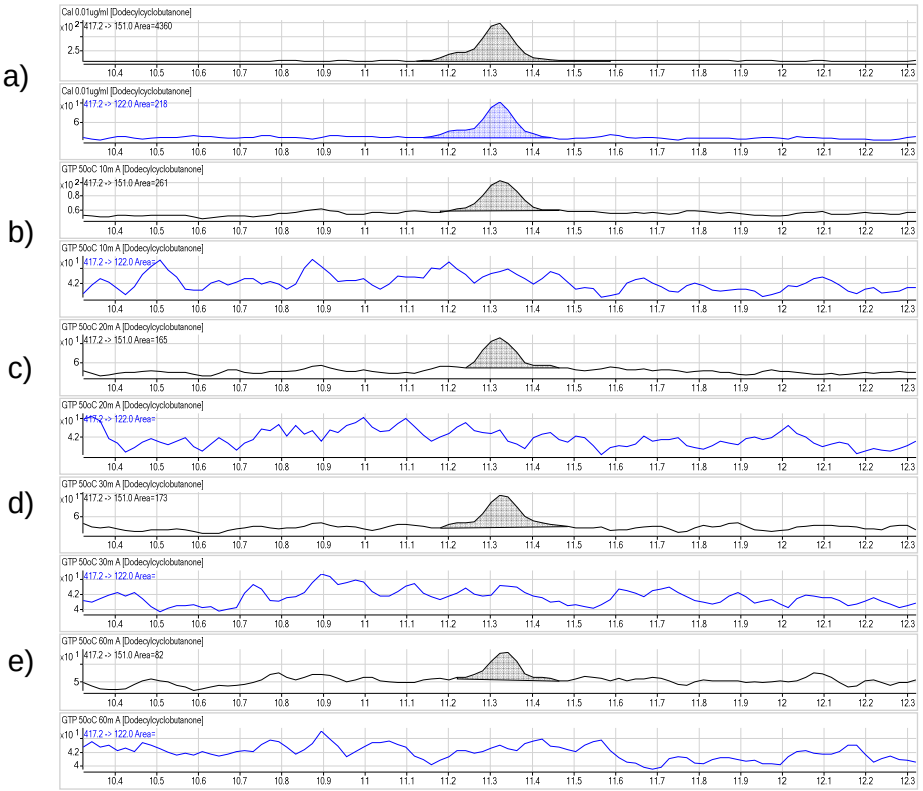


Figure 4.68. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and GTP heated at 50^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

a)

b)

c)

d)

e)

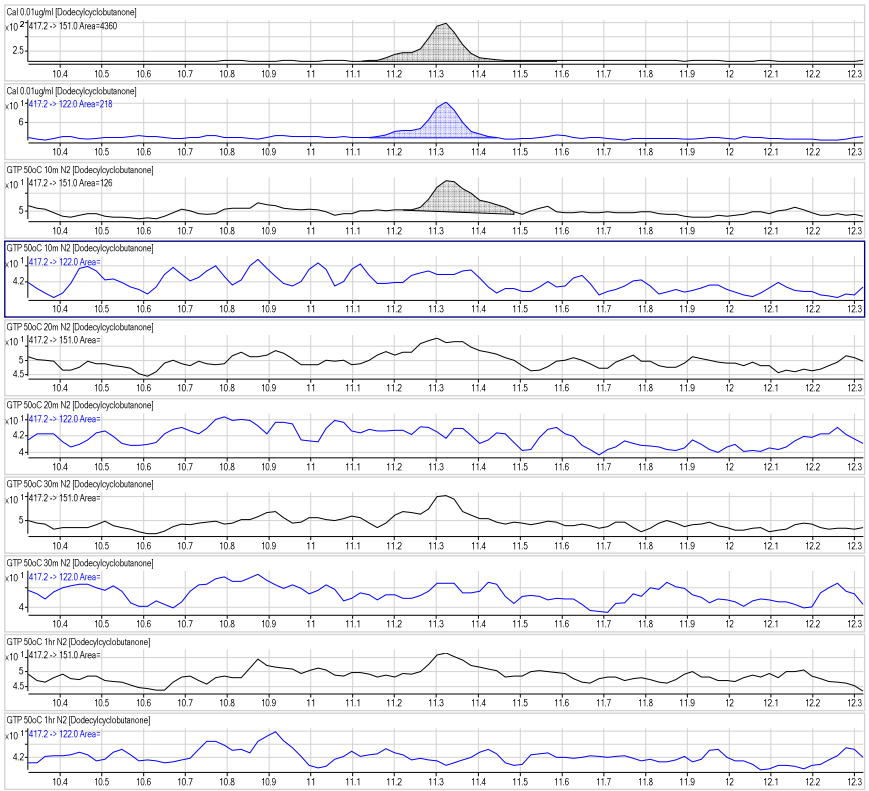


Figure 4.69. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated at 100^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

a)

b)

c)

d)

e)

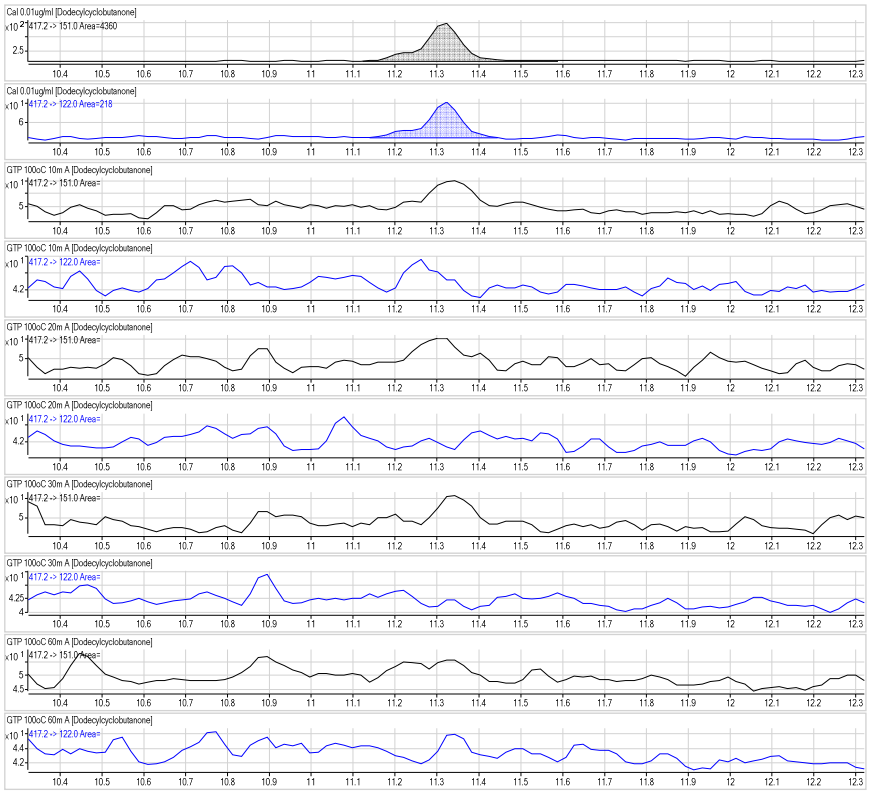


Figure 4.70. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated at 100^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

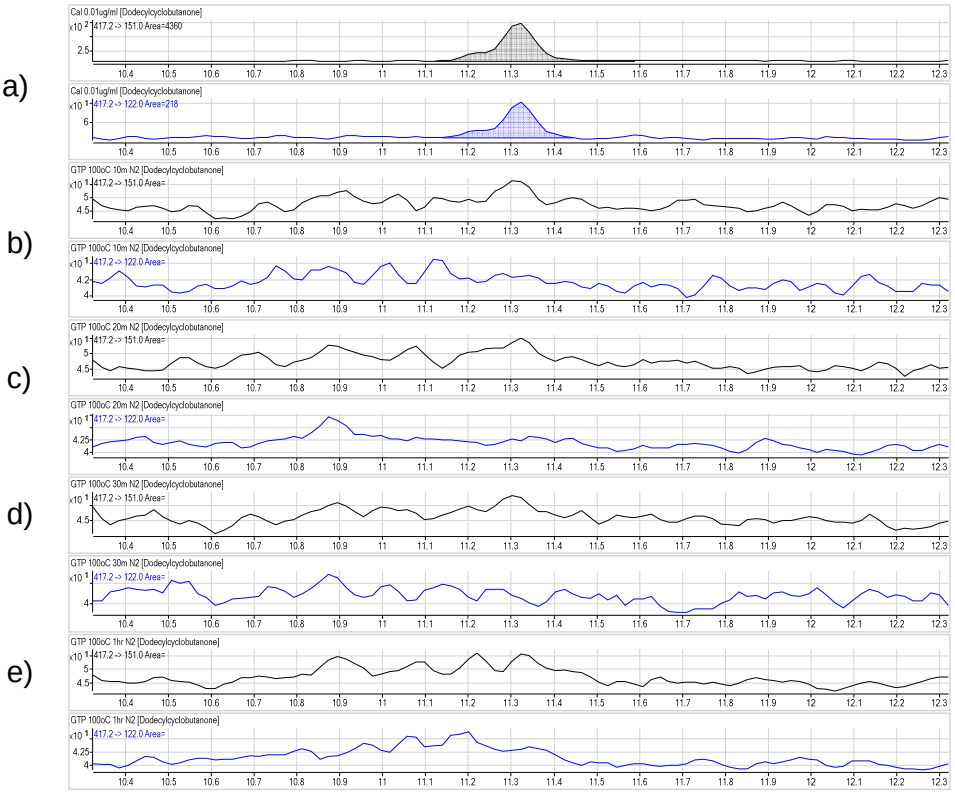


Figure 4.71. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated at 250^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

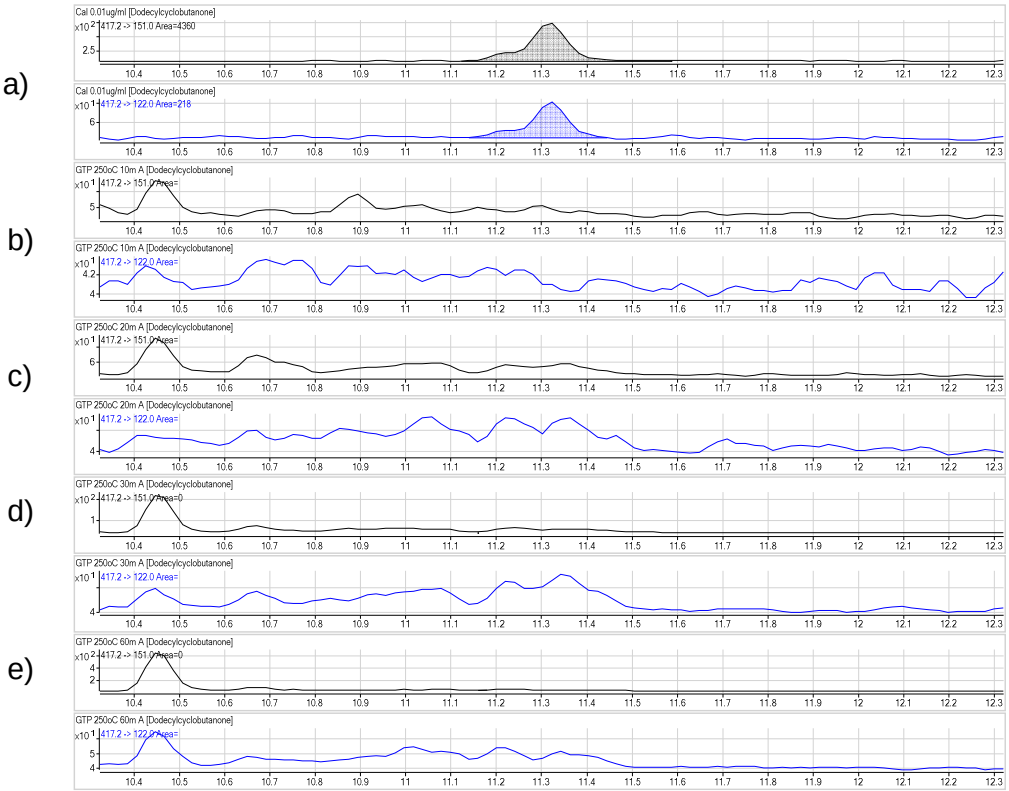


Figure 4.72. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated at 250^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

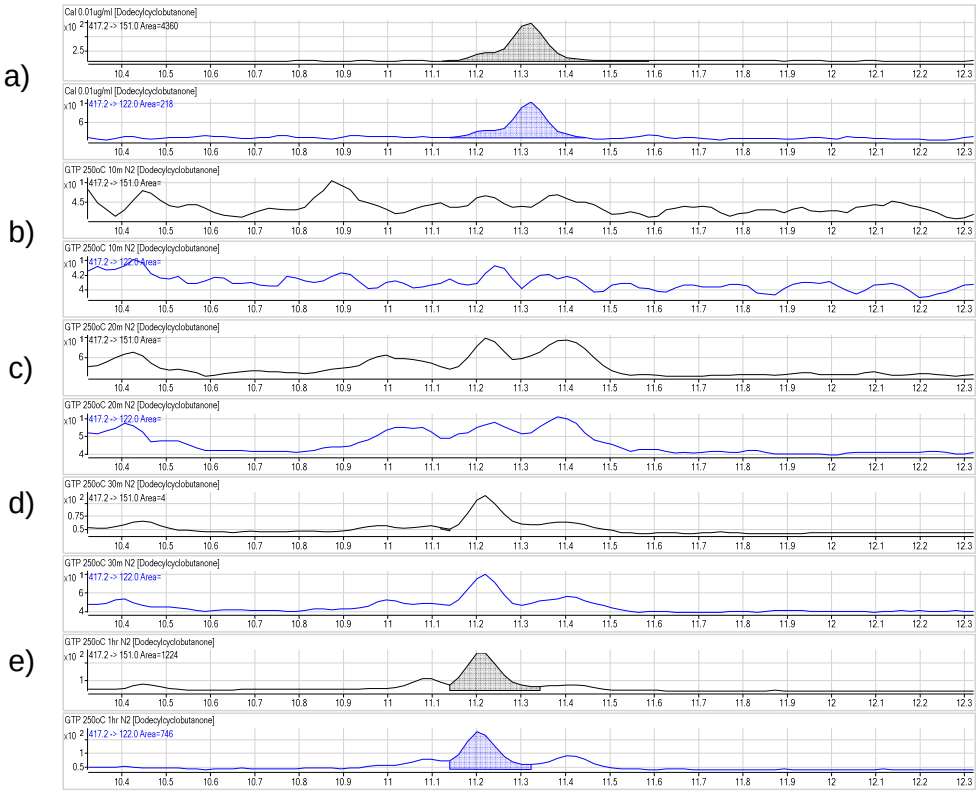


Figure 4.73. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated in the dark for 60 minutes at b) 50^{o}C, c) 100^{o}C, d) 250^{o}C. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

a)

b)

c)

d)

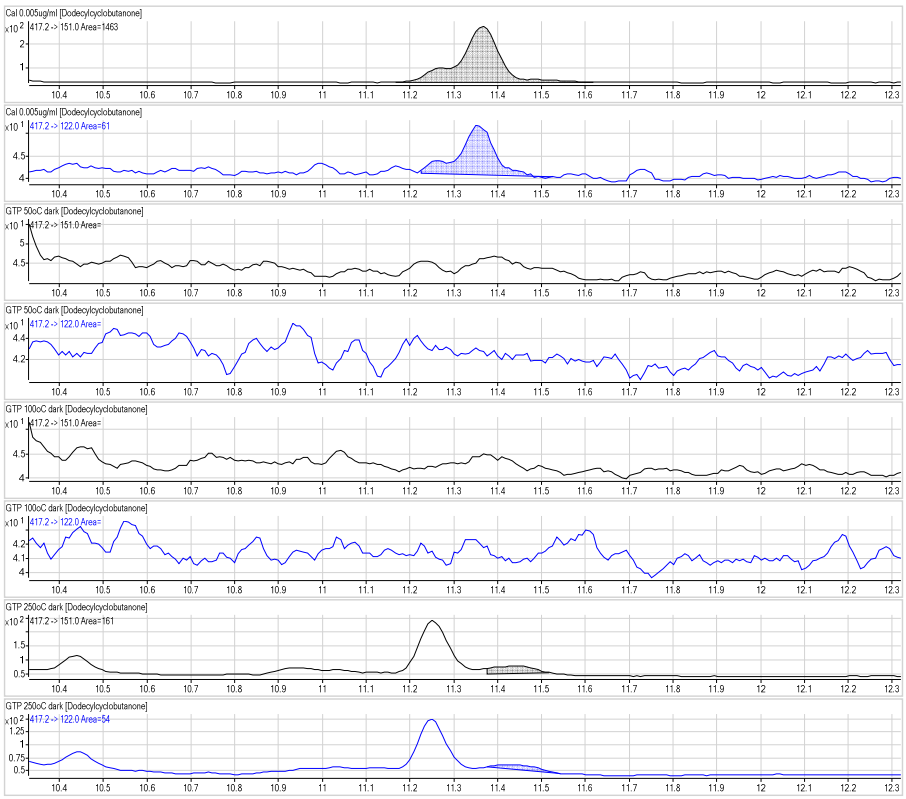


Figure 4.74. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated in a pressure cooker for 20 minutes at b) 109^{o}C, c) 121^{o}C. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

a)

b)

c)

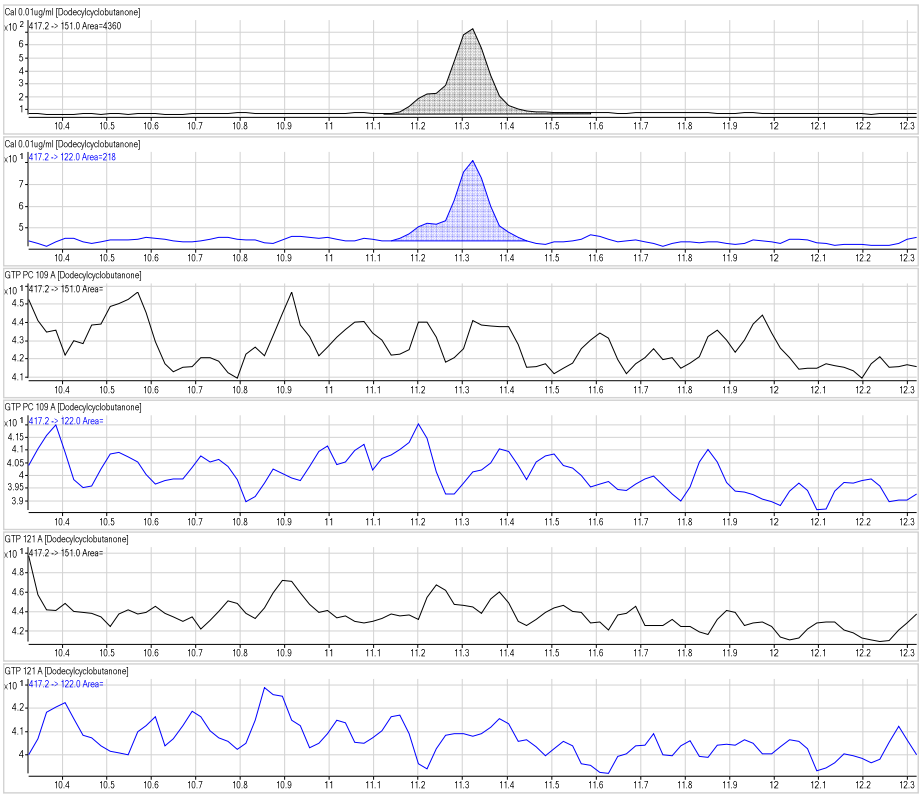


Figure 4.75. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated in a microwave at b) 800 W for 2 minutes, c) 800 W for 5 minutes, d) 800 W for 10 minutes, e) 800 W for 20 minutes, f) defrost setting for 20 minutes, following frozen overnight. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

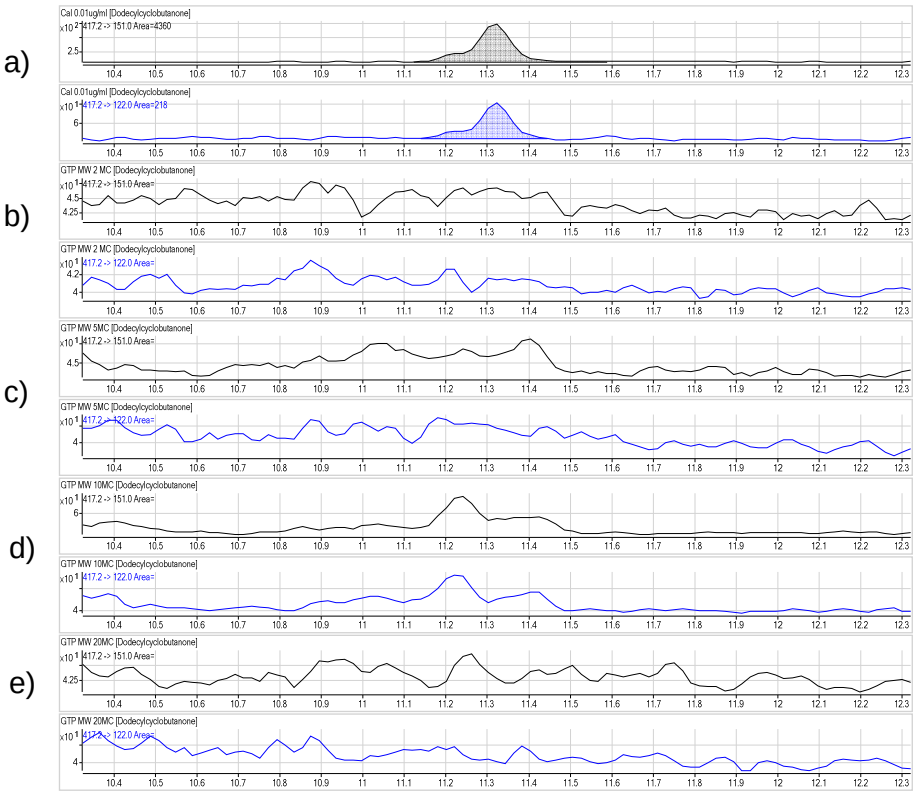


Figure 4.76. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the GTP heated at 50^{o}C in the presence of b) Cu (I) salt, c) Cu (II) salt, d) Fe (III) salt. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

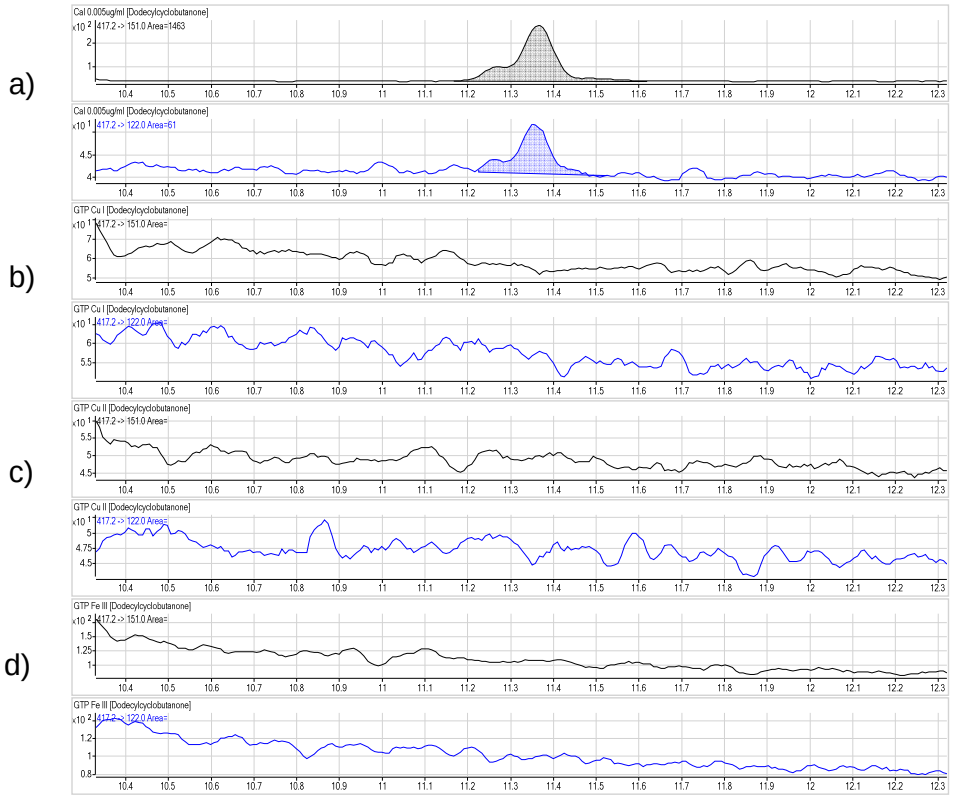


Figure 4.77. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated at 50^{o}C atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

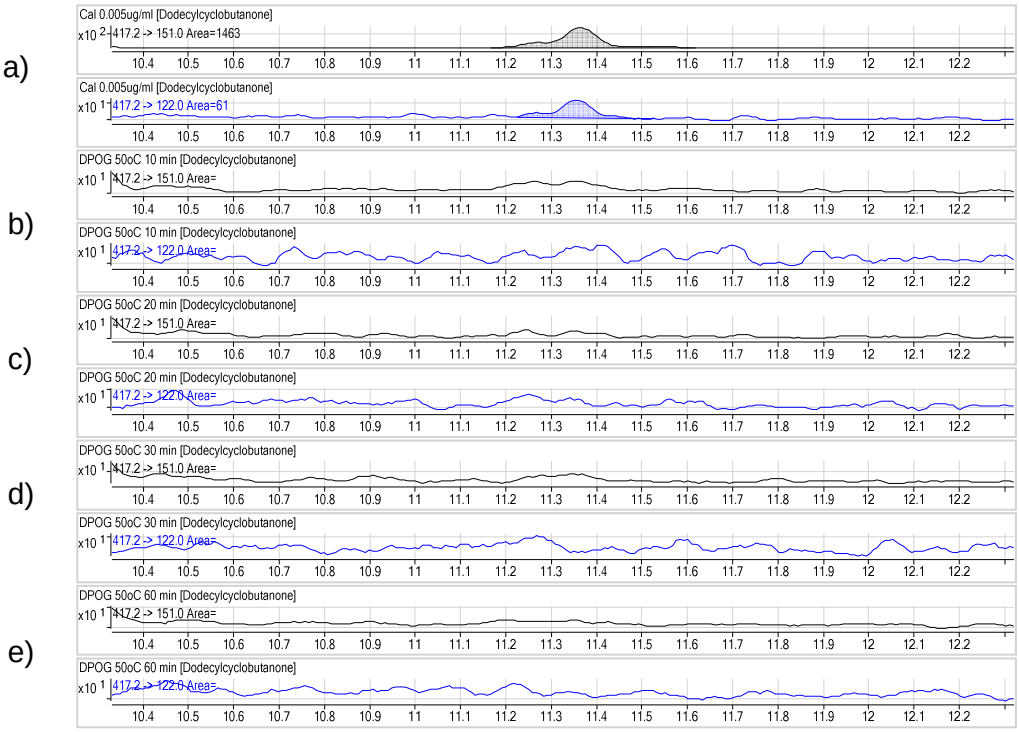
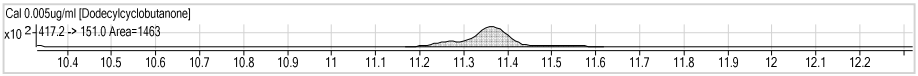
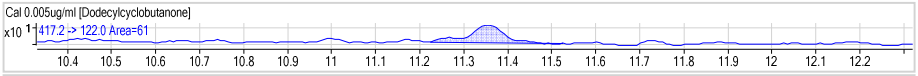


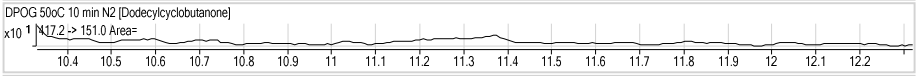
Figure 4.78. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated at 50^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

a)

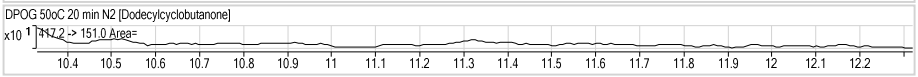


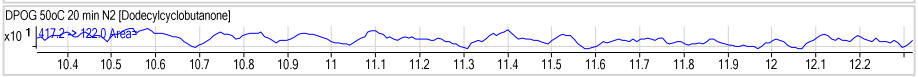


b)

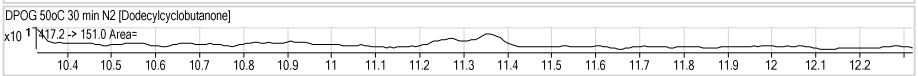


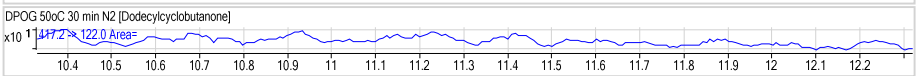
c)



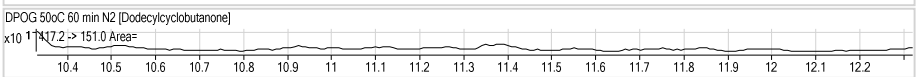


d)





e)



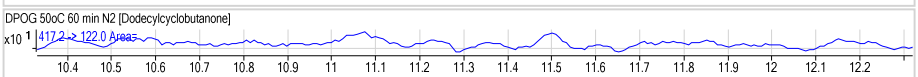


Figure 4.79. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated at 100^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

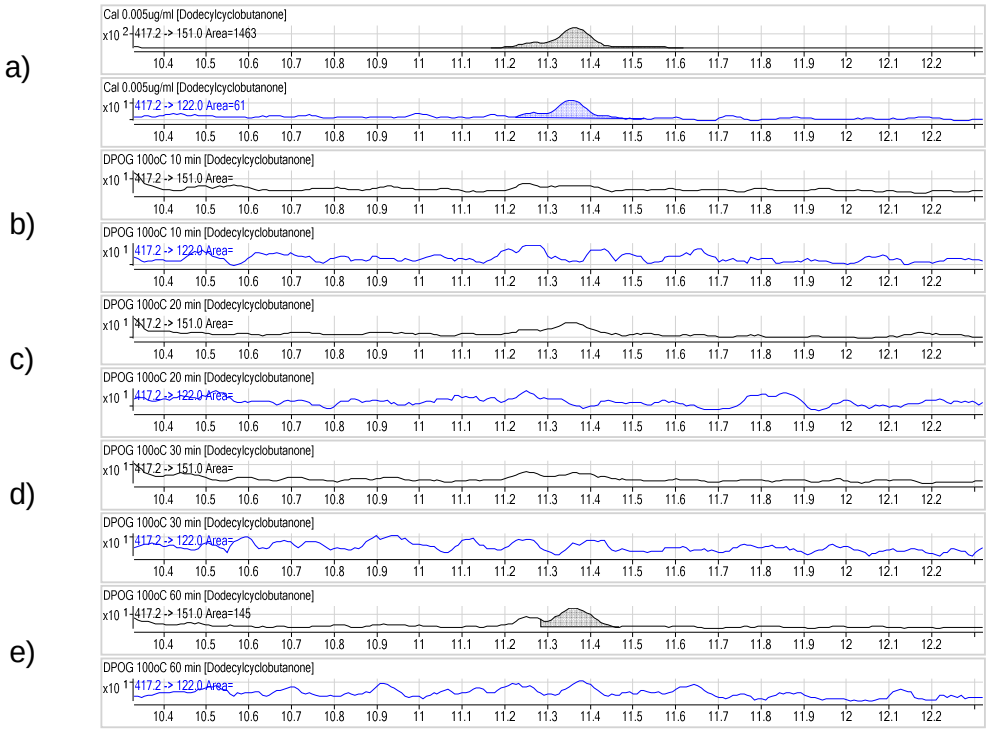


Figure 4.80. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated at 100^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

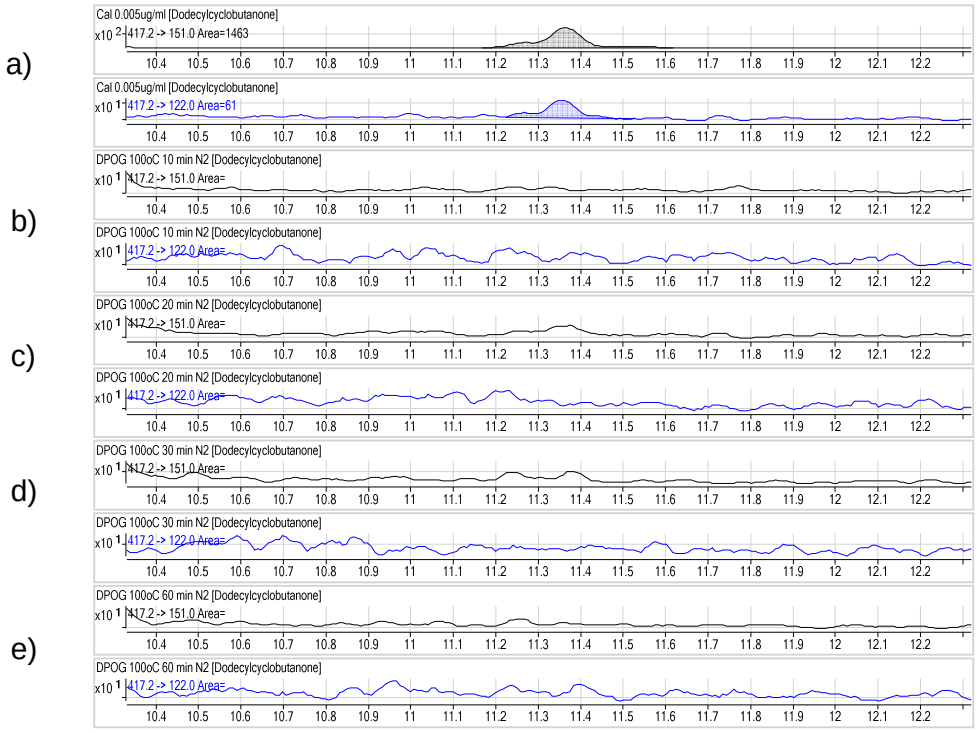


Figure 4.81. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated at 250^{o}C for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

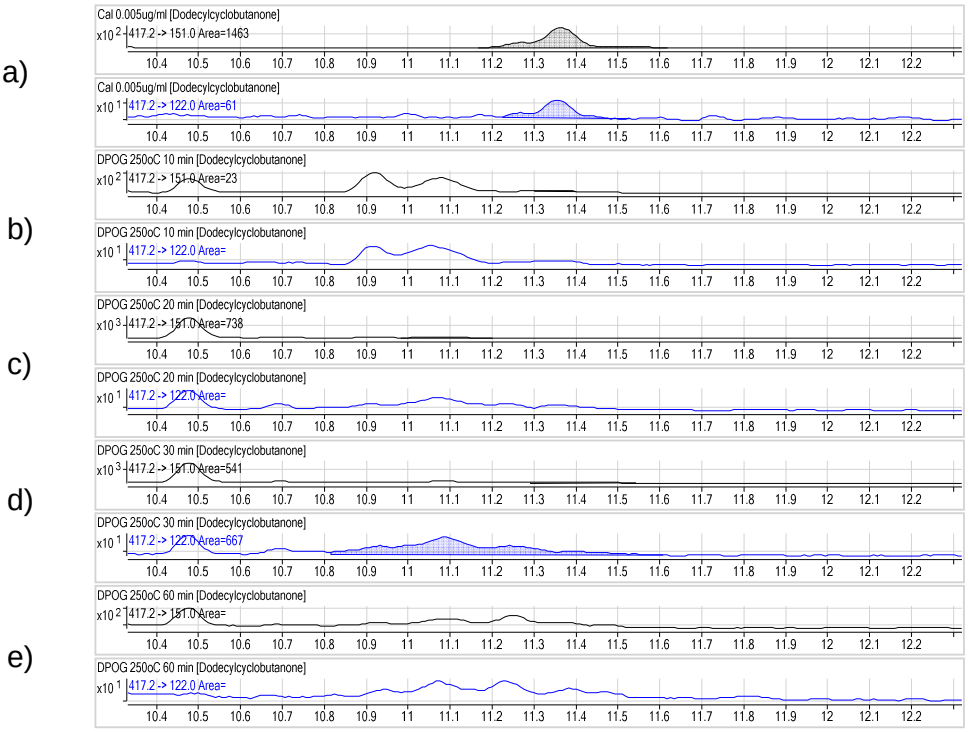


Figure 4.82. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated at 250^{o}C under a nitrogen atmosphere for b) 10 minutes, c) 20 minutes, d) 30 minutes, e) 60 minutes. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

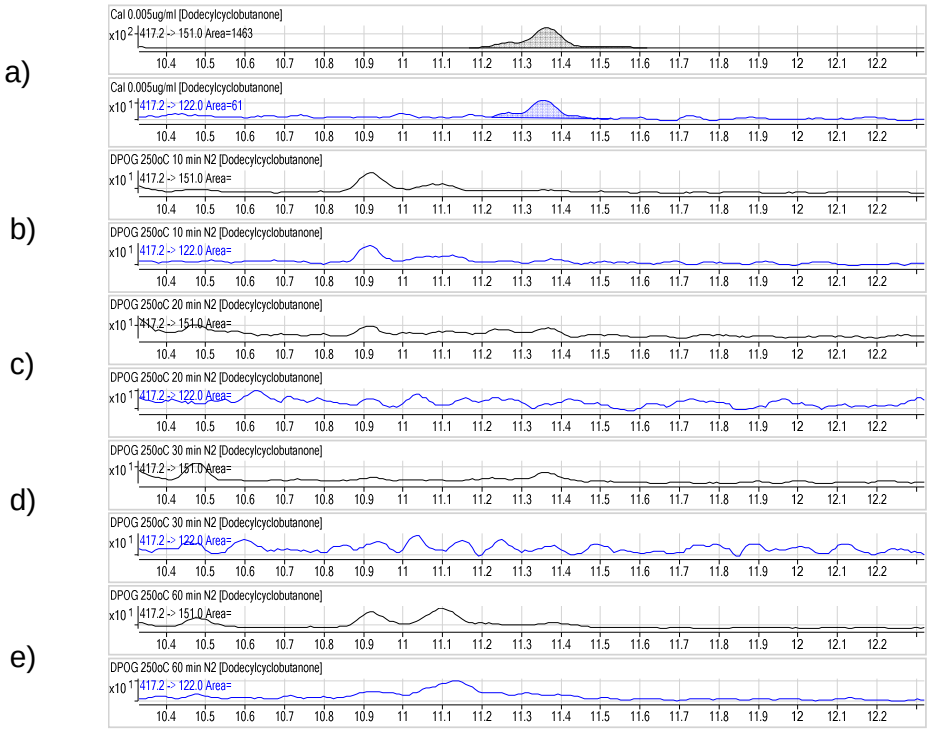


Figure 4.83. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated in the dark for 60 minutes at b) 50^{o}C, c) 100^{o}C, d) 250^{o}C. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

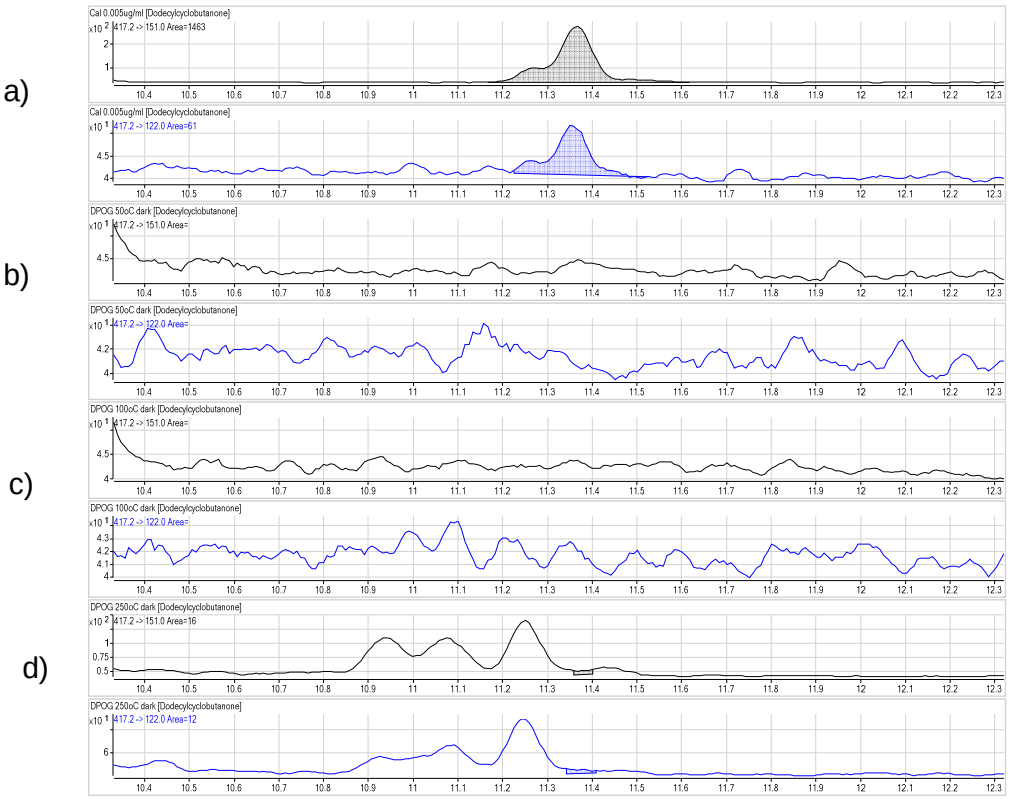


Figure 4.84. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated in a pressure cooker for 20 minutes at b) 109^{o}C, c) 121^{o}C. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

a)

b)

c)

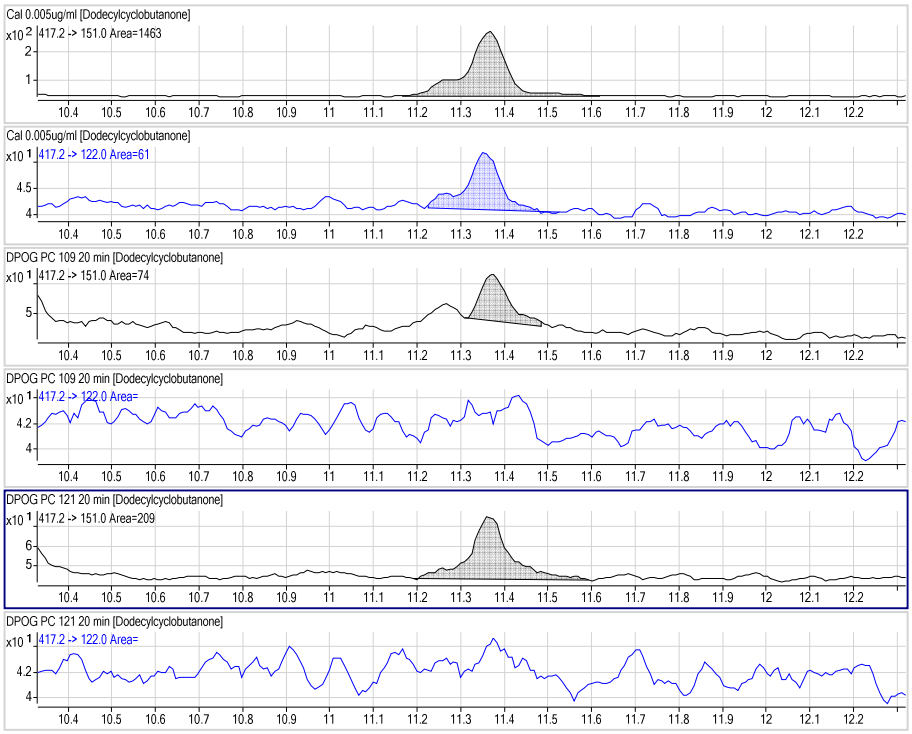


Figure 4.85. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated in a microwave at b) 800 W for 2 minutes, c) 800 W for 5 minutes, d) 800 W for 10 minutes, e) 800 W for 20 minutes, f) defrost setting for 20 minutes, following frozen overnight. 417.2>151.0 = quantification, 417.2>122.0 = confirmation

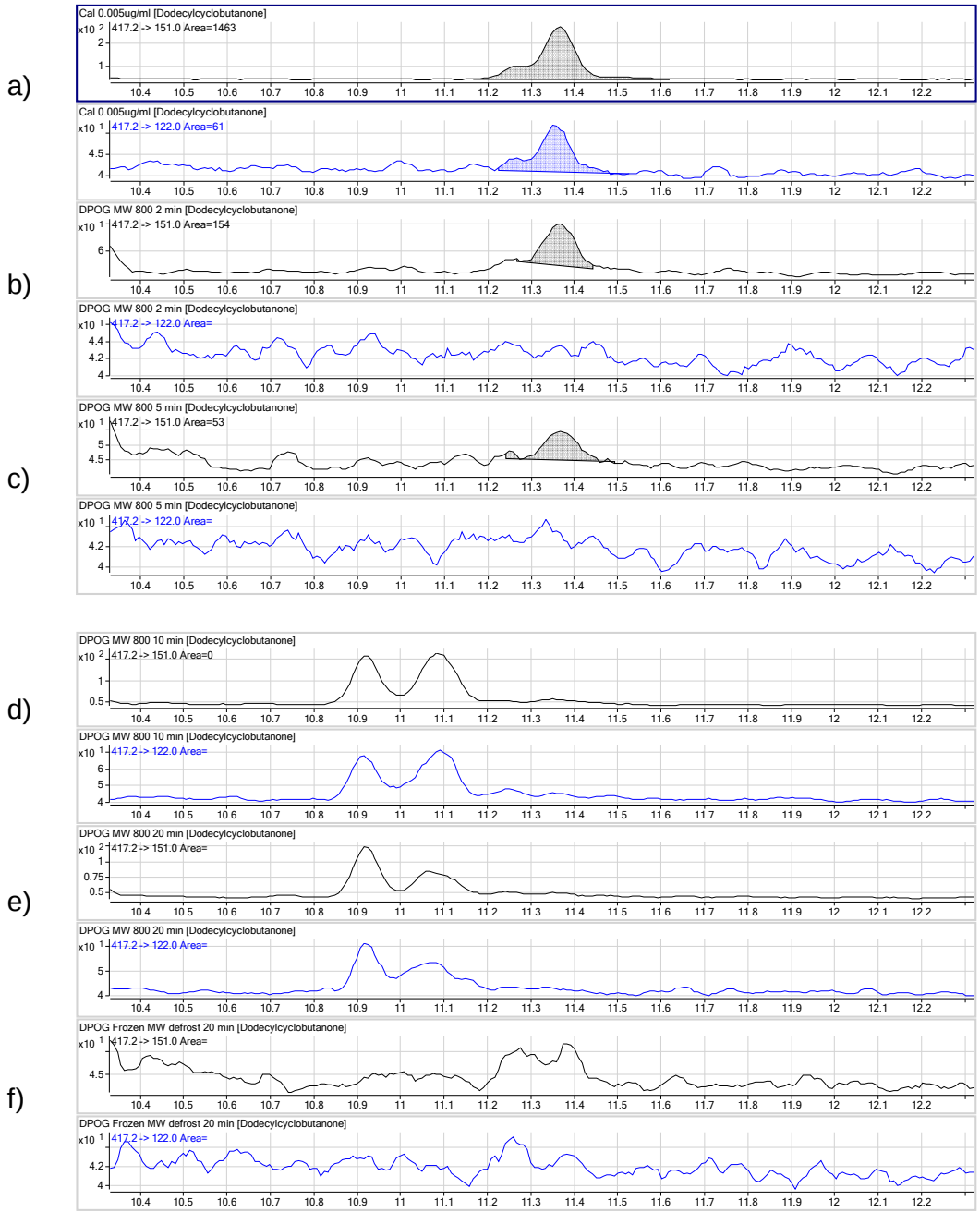
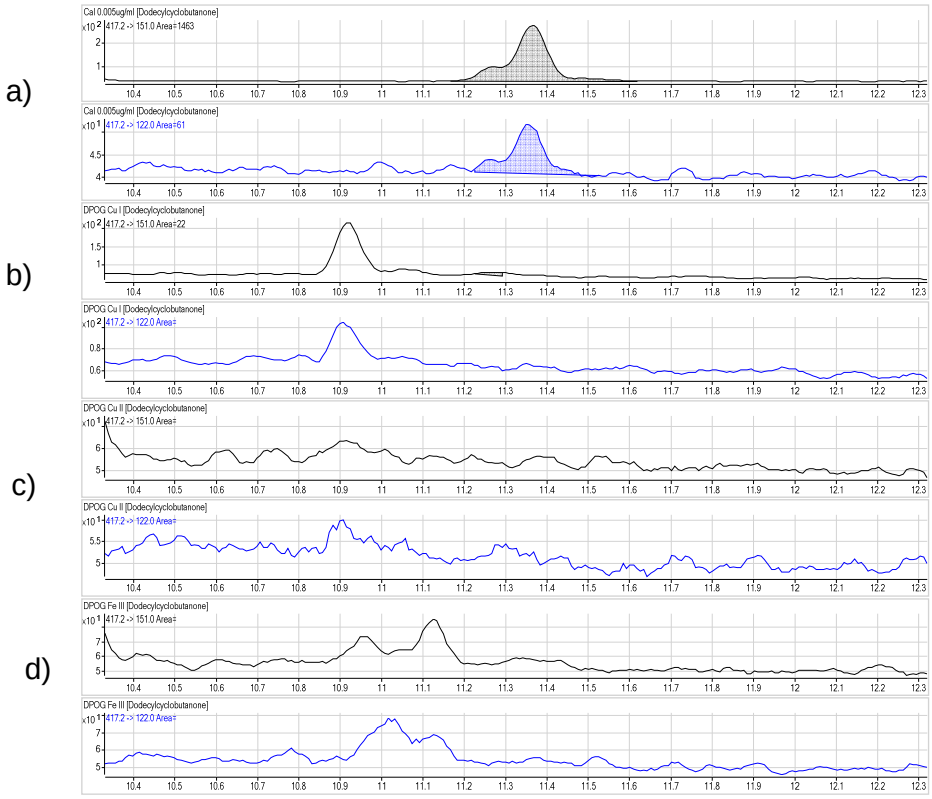


Figure 4.86. LC-MS/MS extracted ion chromatograms of a) DNPH-derivatised-2-DCB standard (0.005 µg/mL) and the DPOG heated at 50^{o}C in the presence of b) Cu (I) salt, c) Cu (II) salt, d) Fe (III) salt. 417.2>151.0 = quantification, 417.2>122.0 = confirmation



4.4 Summary

Model studies have been carried out looking at the effects of dose by different irradiation sources (1, 2 and 5 kGy by gamma ray and 0.3, 0.5, 1, 2 and 5 kGy by EB) on the fatty acids PA, SA, OA, LA and LLA and the triacylglycerols GTP, GTS, GTO, GTL and DPOG. Although analysis was carried out by GC-MS, LC-TOF-MS and LC-MS/MS the only 2-ACB detected was 2-DCB from PA, GTP and DPOG. This may have been because of a low irradiation yield or the instrument sensitivity not being high enough to enable detection. This highlights the need for authentic standards to allow method optimisation, as 2-DCB was the only 2-ACB currently available and used for method development. For PA and GTP the amount of 2-DCB formed increased as the irradiation dose increased. For DPOG the dose/concentration trend increased until 5 kGy when there was a small decrease in concentration when looking at EB irradiation. The amount of DPOG detected remained approximately the same for gamma irradiation. The amount of 2-DCB formed was approximately the same at a given dose when comparing gamma and EB irradiation types.

Studies were also carried out on PA, GTP and DPOG treated by a number of non-irradiation based processing techniques:

- Heating at 50, 100 and 250^{o}C for 10, 20, 30 and 60 minutes in the presence of air and nitrogen
- Heating at 250^{o}C for 60 minutes in the absence of light
- Pressure cooking at 109^{o}C and 121^{o}C for 30 minutes
- Microwaving at 800 W for 2, 5, 10 and 20 minutes
- Frozen samples microwaved on defrost setting for 20 minutes
- In the presence of redox active metal salts, Cu(I), Cu(II) and Fe(III)

No peaks attributed to 2-DCB were detected in any of the samples. This is not a surprising result as there have been no previous reports of 2-ACBs formed from non-irradiation based processing techniques, to the authors' knowledge.

5.0 CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK

One of the major conclusions from this work was the need for authentic standards for more 2-ACBs to be used in method development and optimisation. Quotations have been obtained from a custom synthesis company and were £5000 per gram for the saturated compounds and £12000 per gram for the unsaturated ones. With these compounds the methods developed here could be adapted and optimised for the other 2-ACBs enabling easier identification and detection and allowing greater investigation of the irradiation process and yields for the different food components.

All the work carried out in these studies used model compounds, namely the fatty acids and triacylglycerols known to be the precursors to the 2-ACBS. However, when present in food these compounds are in fact irradiated in the presence of all the other food components with many complex interactions taking place that may well effect the yield of the irradiation products. It is recommended that studies similar to those described here are carried out but from food samples, for example using chicken where DPOG is known to be present naturally. This will give a more realistic view of the amounts of 2-ACBs formed in irradiated food. It should be noted that even though the very low doses (0.3 and 0.5 kGy) were detected in the model compounds, these may not be detectable in foods if the irradiation yield is lower.

The standard method for the detection of 2-DCB and 2-TCB (EN1785) was developed over 10 years ago and with advances in technologies there are possibilities to improve the detection limits to allow lower doses to be detected. These suggestions were discussed in detail in the literature review (Chapter 2) and those most easily achievable with relatively low cost and effects on testing laboratories were highlighted. Some of these were investigated within the scope of this project, such as the studies on derivatisation and the development of LC-MS methods. Other options included:

- Increasing the sample size to provide more fat in the extract, and hence more 2-ACB.
- Investigating a variety of extraction procedures. Replacement of the Soxhlet method is unnecessary but the use of direct extraction with acetonitrile is worthy of further investigation as it would save time and hence reduce costs.

- Optimising the separation of 2-ACBs from the extracted fat. This should involve a study of the performance of various solvent mixtures on silica and Florisil columns, the use of two-stage clean ups using different columns, and the use of commercial SPE columns. This is probably the most promising and important approach. It should also include a brief study of the effects of saponification on the stability of 2-ACBs and their separation from fat on silica and Florisil columns.

Knowledge is also lacking regarding the true quantitative performance of the methods currently used, the limits of detection of irradiated ingredients in foods, the relative yield of 2-ACBs from fatty acids at different positions of triacylglycerols, the stability and fate of 2-ACBs on storage and food processing, and most importantly the possible presence of low levels of 2-ACBs in certain non-irradiated foods. It is recommended that research efforts are directed to address these knowledge gaps.

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