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**DEVELOPMENT OF NEW METHODS FOR ANALYZING LIPID OXIDATION:
ACCELERATED SOLVENT EXTRACTION AND HPLC-DNPH ANALYSES OF
CARBONYL OXIDATION PRODUCTS**

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ABSTRACT OF THE DISSERTATION

DEVELOPMENT OF NEW METHODS FOR ANALYZING LIPID OXIDATION: ACCELERATED SOLVENT EXTRACTION AND HPLC-DNPH ANALYSES OF CARBONYL OXIDATION PRODUCTS

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The basic reactions of lipid oxidation were first reported more than 50 years ago, yet accurate and reproducible measurement of lipid oxidation remains a serious problem and challenge for both industry and academia. This research addresses two critical issues in lipid oxidation measurements: 1) lack of extraction methods that do not induce adventitious oxidation or change existing oxidation products; 2) lack of sensitive reproducible methods for quantitating and identifying secondary non-volatile oxidation products such as carbonyls, core aldehydes in particular. This research aims to develop two reliable, sensitive and accurate methods for oxidation analyses: 1) a pressurized solvent extraction method that is efficient in removing lipid yet does not add to or destroy lipid oxidation products, and 2) an improved HPLC-DNPH assay to quantitate and identify soluble carbonyl oxidation products in individual lipid fractions.

Baked pet food biscuits and dry extruded pet food kibbles were chosen as samples to conduct lipid extraction in pressurized accelerated solvent extractor (ASE) by adjusting a variety of factors such as temperature, sample particle size, polarity of

extraction solvent, extraction static time and numbers of extraction cycles to investigate the effect of extraction conditions on lipid oxidation. Results showed extraction with two 15-minute cycles of chloroform:methanol at 40 °C gave lipid yields of 75-100%, depending on the kibble. Lipid oxidation was minimal when temperature was limited to 40 °C. Factors such as particle size, solvents and extraction static time and cycles could be combined and tailored to optimize extraction efficiency. Compared to traditional extraction methods, pressurized solvent extraction of lipids was significantly more efficient and induced less oxidation.

A reverse phase HPLC-DNPH (2,4-dinitrophenylhydrazine) assay capable of quantitating and identifying carbonyls in all lipid classes was developed using an acetonitrile–isopropanol-water gradient and diode array detection at 360 nm. Optical response decreased with fatty acid chain length; quantitation was achieved using average slopes for three size ranges. The assay was specific for carbonyls and detected 6-50 µg/L and accurately quantitated 20 µg/L standard aldehydes. LC-MS/MS Q-TOF verified hydrazone structures. Monomer carbonyls and core aldehydes were distinguished and quantitated in oxidized Trilinolein, commercial oils, and lipid extracts.

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LIST OF ABBREVIATIONS

ASE	Accelerated Solvent Extraction
HPLC	High Pressure Liquid Chromatography
DNPH	2,4-Dinitrophenylhydrazine
SFE	Supercritical Fluid Extraction
TBA	2-Thiobarbituric Acid
MS	Mass Spectrometry
MDA	Malondialdehyde
ECN	Equivalent Carbon Number
CN	Carbon Number
NDB	Number of Double Bond
ESI	Electrospray Ionization
APCI	Atomospheric Pressure Chemical Ionization
Q-TOF	Quadrupole Time-of-Flight
DAD	Diode Array Detector
LOD	Limit of Detection
LOQ	Limit of Quantification
TLC	Thin-layer Chromatogrpahy
TAG	Triacylglycerol
PL	Phospholipids
DAG	1,2-Diacylglycerols or 1,3-Diacylglycerols
FFA	Free Fatty Acid

EST	Ester
STE	Sterol
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
C	Chloroform
CM	Chloroform: Methanol (2:1,v/v)
HM	Hexane: Methanol (2:1,v/v)
H	Hexane
PTE	Petroleum ether
OCT	Octane
IPA	Isopropanol
ACN	Acetonitrile
CID	Collision Induced Dissociation
ELSD	Evaporative Light-scattering Detector
SSI	Stripped Soybean Oil (Incubated)
VGO	Vegetable Oil
ML	Methyl Linoleate

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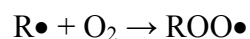
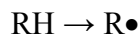
1. INTRODUCTION

The food industry and academia have been investigating basic reactions of lipid oxidation for decades to obtain detailed information in mechanism for quality control. Research in lipid oxidation stagnated during the low/no fat period because it was thought that everything was known about lipid oxidation and, in addition, without lipids, their oxidation to "rancidity" was not expected to be a problem. However, with the recent reawakening of interest in restoring healthful levels of essential polyunsaturated fatty acids to foods, lipid oxidation has again presented itself as a critical problem.

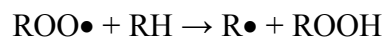
Dealing with lipid oxidation in foods presents a number of challenges, two of which are extracting lipids from complex matrices quantitatively and without modification or oxidation, and tracking multiple types of products with both sensitivity and specificity. Extraction of lipids without inducing adventitious oxidation, decomposition, or transformation of products already formed is no easy task. Previous methods used high temperatures or other extreme conditions to remove lipids trapped inside starch helices or complexed with proteins, and this virtually guarantees both new oxidation and alteration of existing products. Thus, new methods are needed to assure accurate evaluation of oxidation in all kinds of materials.

Some of the reasons for the analytical problems can be found in the complexity of the lipid oxidation reactions themselves. Traditional theory holds that lipid oxidation proceeds by a free radical chain reactions in three stages[1, 2] -- initiation, propagation and termination, shown below in oversimplified version:

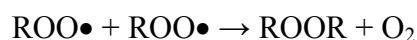
Initiation:



Propagation:



Termination:



Given these reaction expectations, current lipid oxidation analyses most commonly measure conjugated dienes and hydroperoxides as major oxidation products to determine the oxidation degree of food samples[3]. However, these initial oxidation products are not stable over the course of lipid oxidation but transform to a broad range of secondary products, so hydroperoxides alone cannot accurately reflect the extent of oxidation. Hydroperoxides decompose to secondary oxidation products or form alkoxyl radicals easily due to high temperature [4] and even light in laboratory, and subsequent scission reactions generate alkanes and aldehydes. These secondary reactions are typically monitored by measuring hexanal, the volatile product formed from scission of the C13-O• of linoleic acid. Little analysis of non-volatile carbonyls has been performed, particularly for identification of individual products.

Lipid oxidation can be grossly underestimated and misinterpreted, if only these the three traditional products -- conjugated dienes, hydroperoxides, and hexanal -- are measured. Many other products have been reported and there are inconsistencies in the literature about timing of formation of different classes of products. Schaich [5] has integrated a number of alternate pathways of lipid peroxy and alkoxy radicals that compete with hydrogen abstraction to make lipid oxidation much more complex than the simple radical chain reaction previously described. This integrated reaction scheme is shown in Figure 1. A major consequence of these alternate reactions is that many types of products are formed throughout the lipid oxidation process, not just at the end after hydroperoxides have been decomposed. Thus, accurately determining the true extent of lipid oxidation requires monitoring multiple pathways and classes of products, all at the same time. If only one or the wrong reaction pathway is measured, lipid oxidation can be missed. Unfortunately, dependable, sensitive, and accurate methods for analyzing all classes of lipid oxidation products are not available.

To provide improved tools for analyzing lipid oxidation, this research thus focuses on developing procedures for a) accelerated solvent extraction (ASE) as an improved lipid extraction method that is efficient and does not add to or destroy lipid oxidation products, and b) an HPLC-DNPH assay to identify and quantitate non-volatile carbonyl oxidation products.

For the first part of this study, ASE extractions were tested in baked and extruded dry pet foods. The pet food industry has been growing very rapidly in recent decades-- 12% from 2009 to 2014 in U.S. [6] -- and prices are predicted to increase in the future. In

addition, pet owners increasingly judge pet food quality on the same basis as their own food.

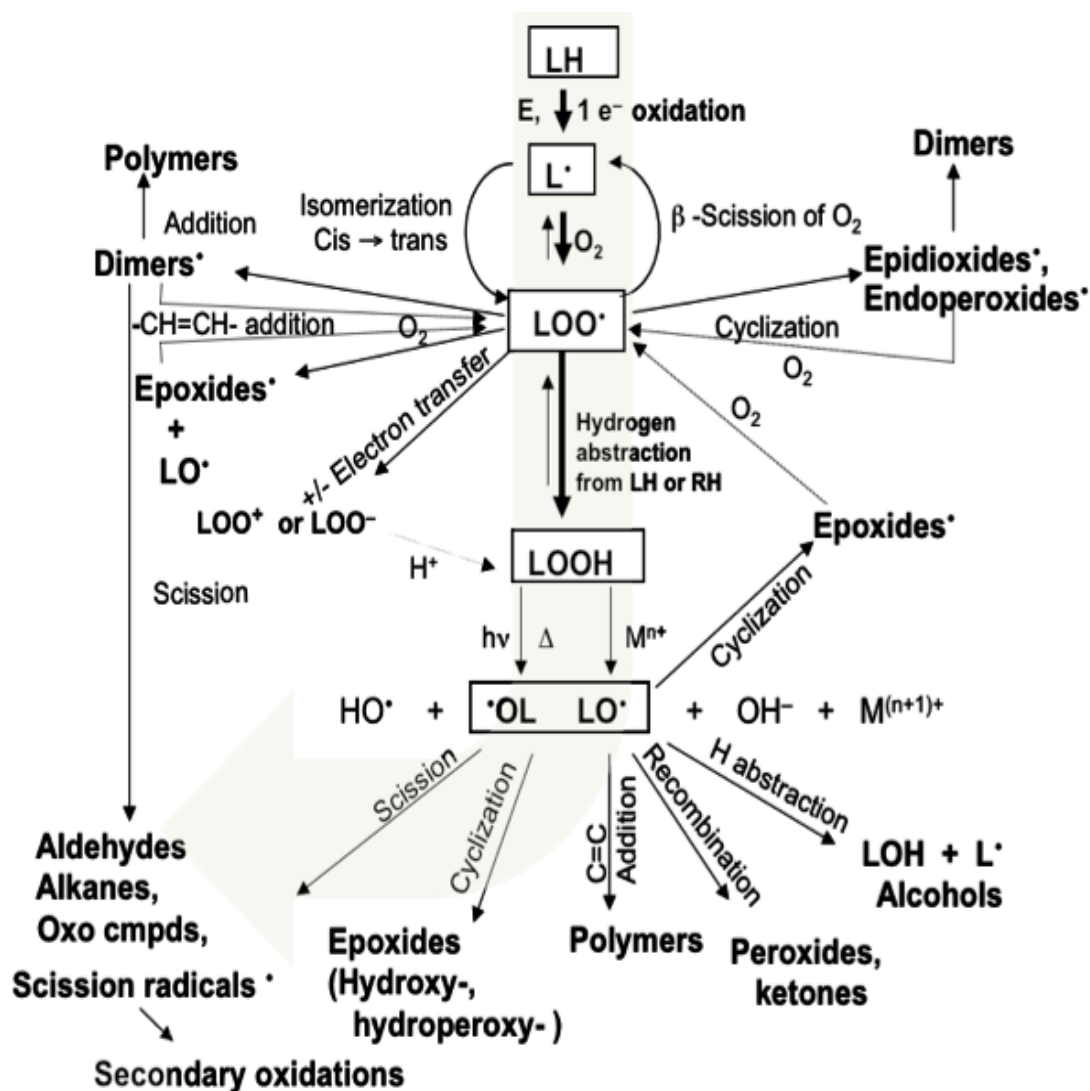


Figure 1. Typical lipid oxidation products analyzed in context of multiple alternate pathways, from Schaich [5].

They look for high nutrition and reject products with strong off-odors. In a survey conducted by Mintel, about 62% of subjects stated that they considered their pets as

family members and were willing to purchase quality food for them [7].

Edible fat and oils are important ingredients in pet food formulation to provide essential fatty acids, supplement energy and enhance palatability. As a result, commercial dry pet foods such as extruded kibbles or baked biscuits are subject to oxidation (development of lipid rancidity) during storage, resulting in production of off-odors that pet owners smell when they open the package, off-odors and flavors that cause pets' refusal to eat, and reactions reducing nutritional value in the food. Increasing consumer demands for higher quality pet foods and new legal standards for maintaining nutritional value are pressuring the pet food industry to pay more attention to oxidative stability in pet foods and to change formulations and processing to improve product stability.

Most pet food products are produced by high temperature extrusion cooking, which causes both oxidative rancidity and thermal degradation [8,9]. Both rate [10] and degree of lipid oxidation increases with temperature of extrusion[11,12]. In addition, during the production of pet food products, complex ingredients interact with each other and entrap or bind lipids, which severely limits their extractability for analysis. Traditional extraction methods such as Soxhlet, acid hydrolysis, ultrasonications, and microwave release lipids but also cause extensive damage to lipids. Thus, new extraction approaches are needed for accurate quality control and analyses of these important products.

The second part of this study focused on development of an improved assay for both quantitating carbonyls, secondary products from lipid oxidation, while at the same time identifying which carbonyls are produced and in which class of lipids (monomers, phospholipids, acylglycerols). To accomplish this, we adapted procedures for the

traditional dinitrophenylhydrazine (DNPH) assay to HPLC and extended previous methods to include separation and analysis of all lipid classes with a single gradient elution. Integration of total peak area gives quantitation of the carbonyls, while interfacing of LC to mass spectrometry detectors can identify specific carbonyl products. The method, therefore, should be very useful for both quality control and basic research.

2. LITERATURE REVIEW

2.1 Extrusion processing of dry pet food

Pet foods contain a variety of ingredients to satisfy daily nutritional requirements (carbohydrates, protein, fat, vitamins, minerals, etc.) of each animal species. Ingredients typically included are cereal grains (e.g., corn, rice, and wheat), meat products (raw meat, rendered and dried by-products or meat meals), fat sources (chicken fat and beef tallow), and vitamins and minerals [13]. Processing of dry pet foods involves combining ingredients to form a very complex food matrix that is then baked or extruded.

Extrusion processing is widely applied in the pet food industry, producing about 95% of dry pet food kibbles and other pet food products [14]. Particularly, in extrusion process, ingredients undergo shear stress and compression as a screw with kneading blocks forces the material through a heated barrel then through a shaping orifice in a continuous process [15], during which extensive physical interactions and chemical reactions occur.

In manufacturing of dry pet foods, slurries of proteinaceous materials are first adjusted to fat contents, elevated to temperature from 65°C to 80°C [16], then homogenized to reduce particle size and distribute the fat content uniformly in the mixture. At this point dried ground grain, vitamins, minerals, and preservatives are added. This dough is then extruded into many shapes and sizes of pet foods.

Individual steps in extrusion include mixing ingredients to form a moist dough, heating the dough in the preconditioner, introduction of dough to the extruder, cooking of dough under intense heat and pressure while it moves toward the open end of the extruder, shaping dough to kibbles or crackers by forcing through a [shaped] die, drying products

in oven until moisture content is low enough to ensure shelf stability, cooling, spray coating with flavors or nutrient mixes, and finally packaging (Figure 2) [17]. In the process, moisture drops from 7% to 15% [15]

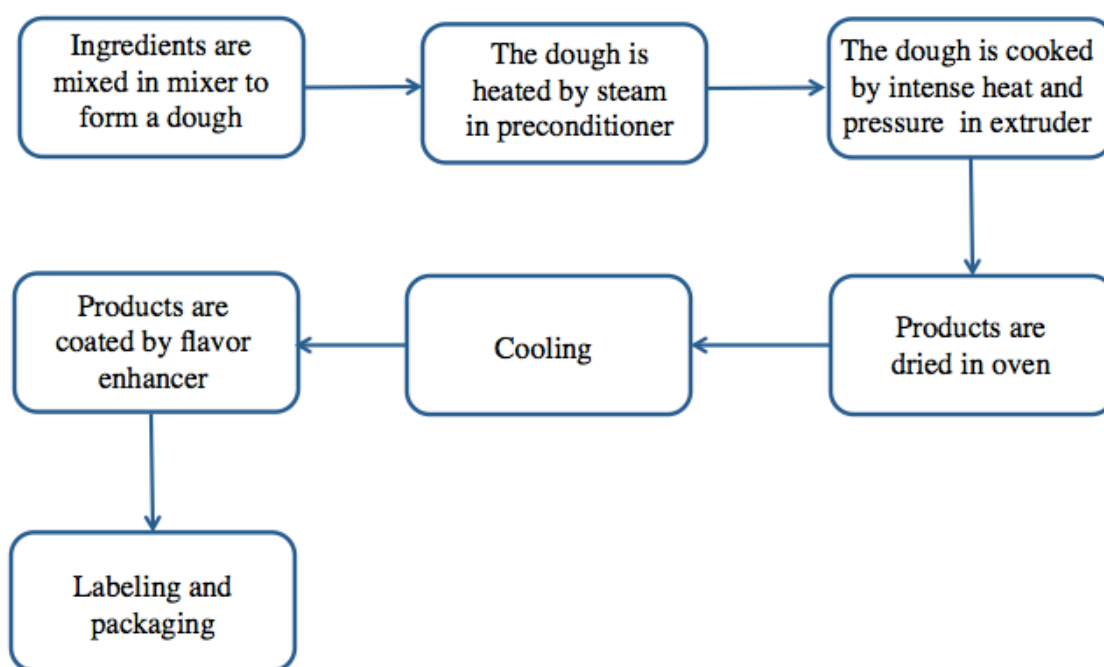


Figure 2. Flow diagram of dry pet food extrusion process [17].

2.2 Lipids in extrusion of dry pet foods

Lipids are added to dry pet foods not only for nutrition and palatability purpose but also as plasticizer and emulsifier that facilitate movement of the dough through the extruder. As plasticizers, lipids increase the mobility of food polymers and consequently decrease viscosity and glass transition temperatures of the polymer [15].

Lipids also form amylose-lipid complexes in which lipids become trapped inside amylose helices (Figure 3). The degree of complexation depends on heat, moisture

content, type of starch, type of lipids and degree of gelatinization [18]. During extrusion, lipids partially leach out of the complexes to protect the surface of starch granules [13]. It is very challenging for food analysts to extract these starch-entrapped lipids from extruded pet food samples.

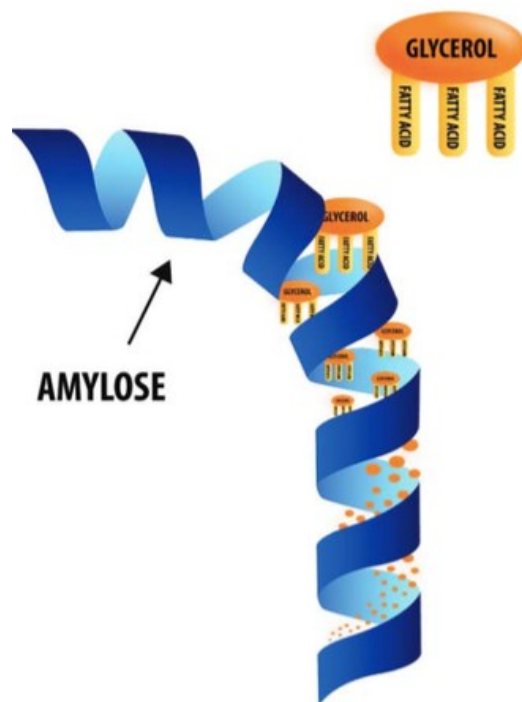


Figure 3. Schematic depiction of amylose-lipid complex formation during pet food processing [13].

High temperatures and moisture during extrusion catalyze protein denaturation, and the increased exposure of surface amino acid residues provides new sites for interactions with lipids; free fatty acids and polar lipids are especially reactive [19].

Chemical changes such as lipid oxidation, lipid binding, destruction of antioxidants, cis-trans isomerization of unsaturated fatty acids, deactivation of lipase and lipoxygenase

also take place in the extrusion.

Lipid oxidation in extrusion processing, in particular, has been studied by some research groups. In general, extrusion resulted in increased lipid oxidation and thermal degradation due to high extrusion temperatures. Alvarez [20] and Rao [11] both reported that peroxides were decomposed by the high temperature during twin-screw extrusion cooking of meat based products (71 °C to 115 °C) and products with soybean oil added (115°C to 175 °C), but then accumulated rapidly in extruded samples stored at 37°C [11]. Studies on other extrusion cooked products showed similar degradation of oxidative stability after extrusion [21, 22].

Lipid oxidation during and after extrusion is catalyzed by several other factors in addition to elevated temperature. Contact with the metal screw and barrel increases contamination by transition metals which greatly accelerates lipid oxidation [11]. The porosity and thinness of bubble cell walls facilitates oxygen penetration and increases contact area, further enhancing lipid oxidation [21]. Natural antioxidants degrade during extrusion [23], while some antioxidants can be formed due to Maillard reactions between proteins and sugars [15], so the net effect on lipid oxidation is not always predictable.

The binding between lipids and carbohydrates or proteins strongly inhibits the extractability of lipids. Free lipids can be extracted with petroleum ether or hexane at room temperature, but bound lipids usually need water-saturated butanol or chloroform-methanol mixture to be extracted at high temperature [24, 25]. Guzman et al. [26] reported that about two-thirds of the free lipids were bound when cornmeal was subjected to the shear conditions in extruder. Lipid binding is believed to affect lipid oxidation during extrusion. Zadernowski [21] investigated lipid changes in oat lipids during

extrusion and found that peroxide values increased in both free lipids and bound lipids, and that *cis-trans* isomerization of double bonds occurred as well.

Enzymes such as lipoxygenase, lipase and oxidase in cereal bran of wheat, rye, corn and oat lost their activities during extrusion process (temperature less than 120°C, 20% moisture and low mechanical stress) [27]. Therefore hydrolytic rancidity or lipid oxidation caused by enzyme during extrusion might not be the major concern for lipid changes.

2.3 Lipid extraction

Accurate and precise analysis of food lipids is demanded to determine lipid components and their nutritional value in foods, as well as to understand their effects on food functionality. Such analyses, in turn, require quantitative and qualitative extraction of lipids from samples, free of non-lipid contaminants. Lipid extraction procedures have been studied extensively, most from the standpoint of maximizing lipid yields from difficult matrices but also in attempt to optimize extraction conditions such as solvent combinations, extraction temperature, sample pretreatments etc.

Effect of extraction conditions were studied in combination or individually, for example, lipid extraction efficiency was compared between soybean meal and microalgae by using isopropanol [28], effect of different solvent combinations was studied in lipid extraction in frozen fish [29], and effect of enzyme hydrolysis before lipid extraction in extruded corn-soy blends [30].

2.3.1 Sample pretreatments before extraction

Samples with relatively high moisture content, such as animal or plant tissues, are not easily penetrated by the hydrophobic organic solvents required to dissolve the lipids. Drying such samples before extraction often improves extraction efficiency [31] but also increases exposure to oxygen and degradation of lipids.

Sample particle size greatly affects extraction efficiency of lipids, so grinding is usually required before extraction to reduce particle size and increase surface area, thus allowing solvents contact with samples more efficiently. In some extractions, solvents are added to samples to be homogenized simultaneously rather than conducting grinding sample and adding solvent separately [31].

Emulsion-based foods, particular dairy products, need to be alkali digested before extraction to break emulsions, neutralize fatty acids and dissolve proteins[31].

In complex food matrices, lipid often become covalently or ironically bound to protein and carbonhydrates. To make these bound lipids available to solvent, the most common approach has been acid (3-6M HCl under reflux conditions) or alkali hydrolysis. Enzymes have also been widely used to release the lipids by hydrolyzing food carbohydrates or protein by alpha-amylase [30] and protease[32].

2.3.2 Lipid extraction methods

2.3.2.1 Solvent

Lipids in food systems are a broad range of compounds varying in polarity and molecular size so selecting an appropriate and efficient solvent is not always straightforward. An ideal lipid extraction solvent must have specific combination of polar

and non-polar solvents to dissolve component lipids in each sample. The most common lipid extraction solvents are alcohols (isopropanol, methanol, ethanol and n-butanol), acetone, acetonitrile, ethers (diethyl ether, isopropyl ether, dioxane, tetrahydrofuran), chlorinated carbons (chloroform, dichloromethane), and hydrocarbons (hexane, benzene, cyclohexane, isooctane). These are used in various combinations to suit different materials.

Neutral lipids dissolve in organic solvents such as chloroform, benzene, diethyl ether, hexane, and iso-octane. Of these, only chloroform is polar enough to dissolve polar lipids or overcome the association between lipids and other constituents in the sample, such as protein or carbohydrates [33]. Chloroform is the universal solvent for lipids. However, due to its toxicity, there are strong efforts to replace it with dichloromethane, iso-octane, and other solvents. Hexane and isopropanol with or without water have been proposed as low-toxicity alternative for extracting lipids from biological materials[34].

Polar complex lipids (phospholipids and glycolipids) normally require a polar solvent such as methanol, ethanol, or isopropanol, and do not dissolve in hydrophobic solvents (except chloroform which is hydrophobic and also polar). However, when neutral lipids are present as co-solvents, limited amounts of polar lipids can sometimes be dissolved in non-polar solvents.

Two major factors affecting lipid solubility in organic solvents have been reviewed by Zalher and Niggli [35]: non-polar hydrocarbon chains of the fatty acid or other hydrophobic moieties and any polar functional groups such as phosphate and sugar residues. Triglycerides or cholesterol esters are readily soluble in hexane or benzene and also in slightly more polar solvents like chloroform and diethyl ether, rarely in polar

solvent as methanol. In some cases, non-polar lipids like TAGs can be extracted by methanol with phospholipids performing as co-solvent. In general, the shorter chain length of acyl groups, the greater they can dissolve in polar solvents. Polar lipids barely dissolve in non-polar solvents, but readily dissolve in polar solvents like methanol, ethanol or chloroform. In most of cases, organic solvents are combined to extract lipids from samples to ensure all lipid classes can be recovered.

In addition to dissolving power, solvent attributes that affect lipid environment and stability are critically important for lipid oxidation. Lipid solvents that disrupt associations of lipids with proteins or carbohydrates in foods (cell membrane or lipoproteins in tissues) facilitate release and improve yields, i.e. methanol [33]. Solvents should be able to penetrate sample matrices readily. Solvents also need relatively low boiling points to facilitate evaporation when recovering lipids. Particularly, for lipid oxidation analyses, evaporation temperature of lipid extracts should be limited to 40 °C [5], above which hydroperoxides undergo degradation and extra oxidation is induced. Since lipids often sit in solvents for long periods during extraction, oxygen solubility and content of the solvent can make the difference between a stable and degraded extract. Extraction solvents carry enough catalytic oxygen to trigger oxidation (Table 1), so solvents must be freshly distilled or sparged with inert gas. The solubility of argon is slightly higher than oxygen but about 2.5 times higher than nitrogen in water at 0-50°C [36]; in lipids, the solubility of argon is twice that of nitrogen. Therefore, nitrogen should be replaced by argon in sparging or headspace blanketing to more effectively prevent lipids.

Solvent stability is another factor to consider in extraction of lipids for oxidation analysis. Solvents such as chloroform and methylene chloride degrade over time, so they usually have stabilizer added [37]. For example, chloroform, forms toxic phosphene (COCl_2) over time, so it must be handled and stored with care (usually under inert gas after opening) [37]. More than 1% ethanol is also added to stabilize chloroform, but ethanol is susceptible to be attacked by radicals from oxidizing lipids, forming reactive radicals that accelerate oxidation [38]. Amylene (2-methyl, 2-butane), as free radicals scavenger, limits adventitious oxidation during extraction[39], but high background was reported by Richard [40] in the ferric thiocyanate hydroperoxide assay with amylene.

Table 1. Solubility of oxygen, nitrogen, and argon in common lipid solvents at room temperature (293 K), adapted from Schaich. [41]

Mol fraction x 10 ⁴			
Solvent	Oxygen	Nitrogen	Argon
Octane	21.22		
Iso-octane		14.13	29.2
Heptane	19.88		25.0
Hexane	19.60	14.0	25.3
Cyclohexane	12.31	7.55	14.9
Toluene	9.17		10.95
Acetone	8.34	5.19	
Tertrahydrofuran	8.03		
Butanol	8.03	4.52	
Chloroform	7.23	7.89	3.82
Dichloromethane	5.61	3.27	5.02
Ethanol	5.87	3.55	
Methanol	4.15	2.75	
Olive oil	0.51		

2.3.2.2 Solvent Extraction Methods

Developing strategies for oxidative stabilization requires constant monitoring of lipid oxidation in food products from starting materials through processing and storage. Accurately tracking lipid oxidation, in turn, presents a significant challenge because it requires extraction of lipids as fully as possible without inducing new oxidation or altering existing products. Furthermore, huge variations in food structures and lipid compositions means that extraction methods must be matched to the food matrix and the

component lipids. Thus, a number of extraction methods have evolved to tailor extractions to different materials.

For natural materials with cell structures, high concentrations of polar lipids, and high water content, the most commonly used methods have been the Folch wash [42] or its simplified version by Bligh and Dyer [43]. These extractions use chloroform-methanol (1:1 or 2:1, v/v) as solvents, water or salt solutions are combined with extraction to improve yields of very polar lipids, or after extraction to remove non-lipid impurities [43].

Manirakiza et al. [44] compared soxhlet, Roese-Gottlieb, Bligh & Dyer, and Modified Bligh & Dyer extraction methods for extracting lipids from samples such as powdered chocolate, chicken feed, fish flour, powder/liquid milk, eggs, margarine and human serum then reported that liquid-liquid extractions had some serious limitations depending on the type of sample. For example, the solid or liquid form of the food affected extraction efficiency greatly because of the difficulties in phase separation and handling. Soxhlet extraction might be suitable for lipid extraction from solid samples but leaves high concentrations of extracting solvents in the extracts.

Extraction of lipids from processed foods presents different challenges because lipids are not protected in compartments as in cells but exist in separate micro- and macro- phases that are exposed to many stresses during processing and storage. Lipid interactions with food macromolecules and entrapment in food matrices are additional problems. It is particularly difficult to extract lipids from extruded products where lipids are entrapped and bound in complex protein-starch matrices. Lipid extraction from such matrices have used hot water-saturated butanol [45], soxhlet refluxing [46], and some pre-digestions treatments such as acid [47] or enzymatic hydrolysis [30] conducted

before organic solvent extraction. Unfortunately, these methods decompose existing hydroperoxides, induce new oxidation, and enhance lipid hydrolysis [41] while still producing relatively low yields. For example, a simple soxhlet method with petroleum ether recovered only half the lipids present in extruded products [48]. Acid hydrolysis provides the basis for standard quantitative extraction methods for lipids in macaroni [49], flour [50], and pet food [51]. However, acid hydrolysis also results in decomposition of lipids, especially for hydroperoxides [52] and the lengthy digestion and handling required to obtain comparatively high lipid yields increases oxidation. Enzymatic digestion with α -amylase was developed as a milder alternative that minimizes modification of lipids while recovering high yields of lipids from extruded corn-soy blends [30]. While useful, this technique is quite time-consuming and difficult to use with large numbers of samples at once.

2.3.2.3 Treatments to Improve Solvent Extraction

Several pretreatments have been adapted to disrupt difficult matrices, including cells and foods, and improve extraction of lipids.

Microwave digestion has been studied for extracting lipids from microalgae [53]. It is believed that microwave energy reduces the energy required to disrupt hydrophobic associations by increasing the rotational force on bonds connecting dipolar moieties to adjacent molecules, thereby facilitating release of lipids to solvents [54]. Microwave extraction yields are comparable qualitatively and quantitatively to conventional methods such as Folch washing [55]. However, microwave radiation typically raises temperatures to 60 °C-120 °C for many minutes [56], and the heat accelerates lipid hydrolysis and lipids oxidation, breaking down existing hydroperoxides and increasing both radical

chain propagation and transformation of the secondary oxidation products. Wong reported that exposure times as short as 10-15 seconds (65°C-85°C) produced up to 11 nmol/mg malonaldehyde and formaldehyde in polyunsaturated methyl esters [57]. Ten minutes at high microwave power (around 210°C) induced significant increases in all oxidation products while augmenting hydrolysis of triacylglycerols and loss of polyunsaturated fatty acids [58].

Ultrasound-assisted extractions use high frequency acoustic waves to disrupt cell structures and molecular matrices. During ultrasonication, cavitation occurs in the solvent. When cavitation bubbles suddenly burst, the extreme high pressure and temperature destroy the adjacent tissue, releasing lipids. Ultrasound has been combined with microwave technology to extract lipids from microalgae [53]. However, this method has several drawbacks when lipid oxidation analyses are the end point. The cavitation caused by collapsing bubbles forms free radicals in any solvent present. Hydroxyl radicals formed during ultrasonication of water in solution of tissues [59,60] readily react with molecules in samples and are responsible for cell damages and lipid oxidation. There is also some evidence that ultrasound can induce decomposition of organic solvents, especially chlorinated hydrocarbons [61]. Paradoxically, sparging with inert gas during ultrasound extraction does not provide protection against oxidation because of cavitation. Argon has higher solubility than air in solvents, so more bubbles are created during irradiation. Friction between the bubbles generates much higher local temperatures and enhances bubble collapse; the fragments then become nuclei for further cavitation and radical generation [62].

Supercritical fluid extraction (SFE) with carbon dioxide has been investigated for lipid extractions because this gas is highly hydrophobic and it penetrates dense matrices easily. Above the critical point temperature (31.1°C) and pressure (72.9 atm), carbon dioxide does not liquefy but attains a dense gaseous state that behaves like a fluid of very low viscosity. When placed in a closed pressurized chamber with a food, the CO₂ flows through the molecular matrix and dissolves lipids that are then washed out with a stream of fresh CO₂ when the pressure is released [31]. CO₂ is particularly useful in penetrating starch helices, forming clathrates in the interior and allowing entrapped lipids to flow out, so SFE has received much attention for extracting lipids from extruded foods.

Supercritical carbon dioxide generally yields good recovery of non-polar lipids including esterified fatty acids, acylglycerols and unsaponifiable matter, but it is too hydrophobic to extract phospholipids and other polar lipids. For example, yields of *Mortierella alpine* single cell oil were significantly lower from SFE than from traditional soxhlet solvent extraction because SFE did not extract polar lipids [63]. Leaving behind the phospholipids can be an advantage in applications such as food oil extraction from oil seeds [31] where it eliminates the degumming step. However, when polar lipids are important, a polar solvent modifier such methanol, ethanol, or even water must be mixed with the CO₂ at levels of 5 to 15% to dissolve the polar lipids [64, 65].

While water is a common solvent modifier for SFE, the presence of moisture in foods being extracted decreases lipid yields, probably by enhancing swelling and gelatinization of the matrix under heat and pressure. Observations that lipid recovery increased with decreased moisture content of samples, for example in meat [66], have led

to a common practice of drying materials before extraction. However, adding this step also enhances lipid oxidation in both the materials and the extracts.

The disadvantage of SFE for lipid oxidation analyses is that SFE subjects lipids to elevated temperatures ranging from about 40 to 100 °C. In general, lipid extraction efficiency increases with temperature, but the high temperatures also greatly accelerate lipid oxidation and thermal degradation. In SFE of olive husks, peroxide values decreased as extraction temperature was raised 40 °C to 60 °C, due to enhanced peroxide decomposition. Peroxide values under all conditions were higher than human acceptance (8 to 43 meq/kg oil) [67]. Another disadvantage for lipid oxidation analyses is loss of secondary oxidation products as volatiles during SFE extraction [68].

2.3.3 Pressurized solvent extraction (ASE)

Pressurized solvent extraction (also called accelerated solvent extraction, or ASE) is a relatively new method that evolved to apply advantages of SFE while eliminating technical difficulties. ASE applies pressure and elevated temperature to facilitate solvent penetration into sample matrices. At higher temperatures, solvent molecules move farther apart, which increases their capacity to dissolve molecules and fit them into the solvent structure. At high temperatures, also, solvents have lower viscosity so they can penetrate the pores of food matrices more easily, and solvent molecules have greater Brownian motion so they diffuse faster through sample matrices. Finally, at elevated temperatures, solute-sample interactions (dipole attractions, van der Waals forces and hydrogen bonding, etc.) are disrupted more easily.

Pressure and moisture adsorbents are two additional critical components in

accelerate solvent extractions. Pressure forces solvent into the sample matrix to contact analytes more directly, and sustained contact under pressure during static cycles improves the dynamics of lipid dissolving [69]. Extracted lipids are then more efficiently washed out by solvent under pressure during dynamic cycles. As noted above, moisture decreased yields in supercritical extractions. To circumvent this problem and at the same time disperse the sample for more efficient solvent contact, ASE uses purified diatomaceous earth adsorbents to bind water from samples and prevent intermixing with solvents. Hence, ASE extracts usually have lower contamination with water-soluble materials such as starch and proteins.

One of the unique aspects of pressurized solvent extraction is the flexibility to adapt extraction conditions (solvent, pressure, temperature, time, etc.) to different materials to tailor and maximize removal of lipids.

2.3.3.1 Sample preparation

For efficient extraction, the solvent must make full contact with target analytes, so the more surface area that can be exposed in a sample, the faster and more completely the extraction can occur. For this reason, solid and semisolid samples must be ground or chopped to small particles to achieve good yields. Figure 4 shows the effect of sample particle size for ASE on extraction of lipids from mozzarella cheese [70] as one example.

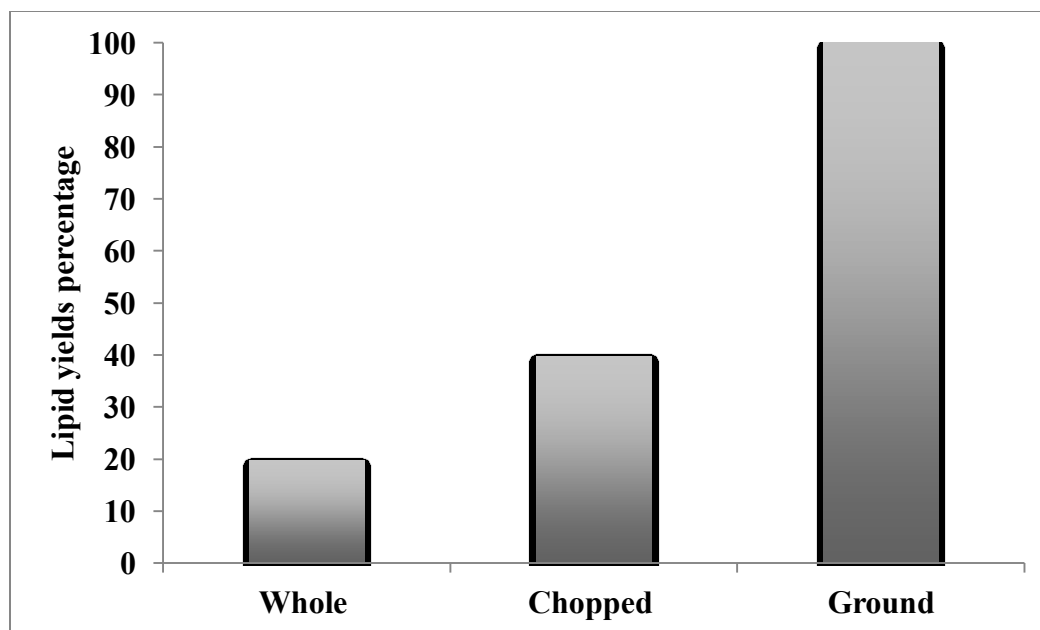


Figure 4. Effect of sample particle size for ASE extraction, whole cheese (0.5 to 1.5 cm size); chopped (2 to 5 mm); ground (mixed thoroughly with diatomaceous earth), obtained from Dionex [70].

Significantly more yields were recovered from ground cheese sample than chopped and whole samples. Result of Luo's [69] ASE studies on nylon-6 and PBT supported that smaller particle size of sample was able to improve extraction efficiency.

2.3.3.2 Solvent

In ASE, solvent polarity directs the specificity in solubilizing target analytes while leaving the sample matrix intact. The old adage that "like dissolve like" applies perfectly to ASE, so non-polar hydrophobic solvents must be present to extract neutral lipids while polar solvents must be present to extract phospholipids and glycolipids. Chloroform is unique in being both hydrophobic and polar, so it is the only solvent that alone is able to extract all lipid classes.

Because ASE has the capability of admitting different solvents in separate lines, even immiscible solvents can be combined in the cells to achieve specific solubilization properties and extract a broad range of compound lipid classes, collectively or separately. Hence, the solvent or mixture must be selected for the analytical goal. For example, Moreau et al. [71] tested hexane, methylene chloride, isopropanol and ethanol to extract polar and non-polar lipids from corn and oats by ASE. Results showed total lipid yields increased when polar solvents were present, and further, that solvents of different polarity favored different lipid fractions. This preference can be manipulated to selectively extract specific lipid fractions.

2.3.3.3 Temperature

In general, solvent viscosity decreases as temperature rises, enhancing the penetration of solvent molecules into food matrices to facilitate dissolution and removal of lipids. Temperatures in ASE can be varied to maximize lipid extraction from individual food matrices, which provides great advantage when total lipid extracts are required. For example, in Moreau's ASE study, lower lipid yields were obtained at 40 °C than at 100 °C from both corn and oats [71]. However, when extracts will be used for analysis of lipid oxidation, temperatures must be limited to 40°C to prevent inducing oxidation, degrading peroxides, and transforming other oxidation products [41].

2.3.3.4 Pressure

Unlike supercritical fluid extraction where pressure is a constant variable required to condense CO₂ and control its solubilization properties, in pressurized solvent extraction, pressures are usually fixed by the instrumentation at levels that exceed the critical pressure of most common solvents. Liquid solvents are relatively

noncompressible, so changing pressures above the critical point has little impact on analyte recovery [70]. Sufficient pressure must be present for the solvent used, but it is not an important variable condition for extractions.

2.3.3.5 Static Cycles

Static cycles introduce fresh solvent and hold it in contact with samples under pressure for varying times. The purpose of static cycles is to provide time for solvent to penetrate into food matrices, dissolve the lipid, and then move it out. A single cycle may be sufficient if lipid content is not too high and the matrix is relatively open. However, large amounts of lipid may exceed the solubility limit in the solvent, inhibiting further extraction, and dense compact matrices may impede solvent penetration and diffusion [70]. Under these conditions, use of multiple repeated cycles can greatly improve lipid yields and completeness of lipid extraction. Multiple extraction cycles can also compensate for lower yields obtained in low temperature extractions.

2.3.3.6 Time

Time is required for solvent molecules to diffuse into sample matrices and dissolve lipids. Thus, the static time must be coordinated with number of static cycles to achieve complete extraction in the most efficient way possible. A balance must be sought between providing sufficient time for solvent penetration and lipid dissolving, while not maintaining solvent contact so long that lipids degrade or migrate and bind to macromolecules in the matrices. This is an empirical process and must be determined and optimized for each food matrix and type of extraction.

2.4 Lipid oxidation

Lipid oxidation can largely affect consumers' acceptance by producing off-flavor, decreasing nutrition quality, altering texture of foods and even producing toxic compounds [31]. Temperature, presence of antioxidants or catalysts, and nature of the substrates all affect the rate of oxidation [72].

Lipids oxidize by two basic processes – normal autoxidation and photosensitized oxidation. Autoxidation proceeds by the standard free radical chain reaction in which initiators form free radicals in the lipid chains, and these then react with triplet oxygen to form peroxy radicals [73]. Note that the initiators are required here because ground state oxygen is in the triplet state (oxygen unpaired electrons oriented in the same direction) and thus cannot react directly with singlet state electrons in double bonds [74]. In contrast, photosensitization involves action of chemical photosensitizers, mostly pigments, which absorb low energy light and transfer that energy to induce radicals directly in lipids or to convert triplet ground state oxygen to singlet excited state oxygen [75]. With its spin state converted, singlet oxygen adds directly to double bonds to form hydroperoxides without formation of free radicals. Autoxidation radical chains are then set off when the hydroperoxides are decomposed by metals, ultraviolet light, or heat.

The key difference between autoxidation and photosensitization in terms of products is that radicals and hydroperoxides form preferentially at the outer carbons of double bonds in autoxidation (e.g. carbons 9 and 13 in linoleic acid, Figure 5), while singlet oxygen can react with either carbon in a double bond, thus generating internal hydroperoxides in lipids (carbons 10 and 12, Figure.5). Decomposition of these different hydroperoxides will generate different products in later stages of oxidation.

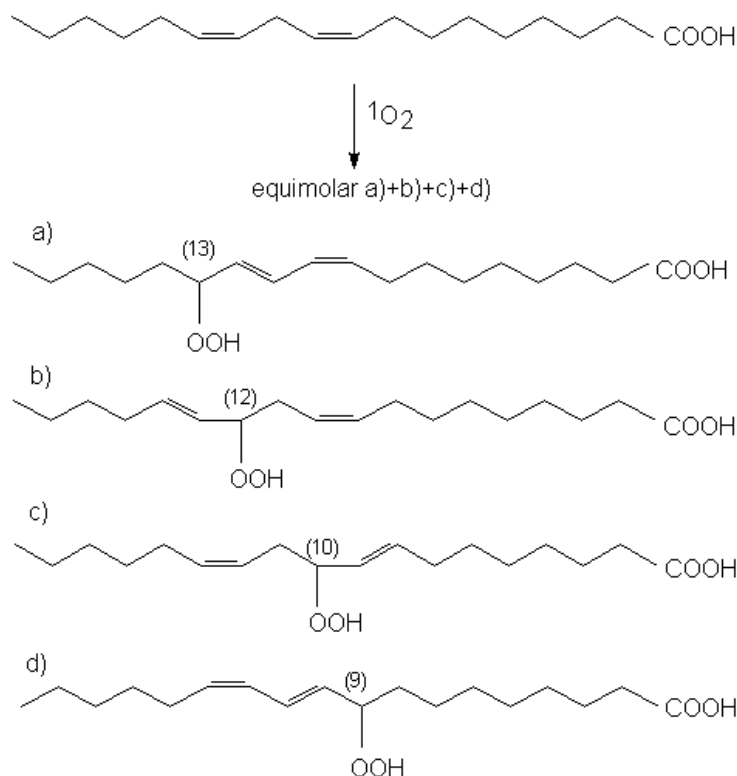


Figure 5. Singlet oxygen-promoted hydroperoxide formation in linoleic acid. Adapted from Min[76].

2.4.1 Additon reactions of lipid peroxyl radicals, LOO^\bullet

In addition to abstracting hydrogen atoms, peroxyl radicals add to double bonds to form peroxy dimers that decompose to epoxides and alkoxyl radicals, as shown in Figure 6 [5]. This reaction is very likely to occur undetected and provide a source of secondary products and strongly oxidizing radicals very early in oxidation. LOO^\bullet addition is favored under conditions where abstractable hydrogens are limited (aprotic solvents or low temperature) and there is a conjugated double bond, terminal or 1,1-disubstituted [5]. There are several factors controlling addition, such as strength of the bond formed, steric hindrance, polar effect and stability of the resulting radical adduct [79].

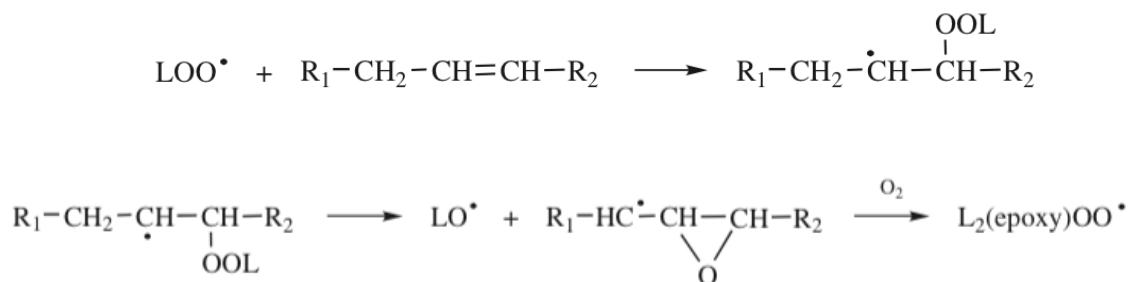


Figure 6. Peroxyl radical addition reaction and cyclization [5].

2.4.2 Hydroperoxide decomposition

Hydroperoxides themselves are relatively stable. However, in the presence of metals, ultraviolet light, or heat hydroperoxides decompose to alkoxyl radicals (LO^\bullet) that are orders of magnitude more reactive than alkyl radicals (L^\bullet) or peroxyl (LOO^\bullet) radicals [5] (Table 2). Alkoxyl radicals can undergo different reactions depending on the fatty acid type and the location of the original hydroperoxide on the fatty acids [76]. They are strong hydrogen abstractors so, once formed, become the dominant chain carrier in lipid oxidation. Products of these chain propagations should be lipid alcohols (LOH), which are almost never analyzed so there is little proof of their existence. In contrast, carbonyl decomposition products have been extensively studied and identified, and epoxides are being increasingly recognized. Reactions for generation of carbonyl products are described below.

Table 2. Lifetimes and hydrogen abstraction rates of various radicals that initiate lipid oxidation, adapted from [5].

Radicals	Half-life with typical substrate, 10^{-3} M, 37 °C	Ave. rx Rate, k ($\text{L mol}^{-1} \text{sec}^{-1}$)
LO^\bullet	10^{-6} sec	10^6 - 10^8
L^\bullet	10^{-8} sec	10^4 - 10^8
LOO^\bullet	10 sec	10^1 - 10^3

Figure 7 shows the decomposition of hydroperoxides to form alkoxyl radicals which then undergo scissions to yield short chain compounds including aldehydes, alcohols, alkanes and alkenes. Scissions will be discussed in detail in the Section 2.4.2.

In addition, as shown in Figure 2, LO^\bullet can also undergo rearrangement or internal cyclization by addition to the first carbon of an immediately adjacent double bond to form epoxides. In the process, the free electron migrates to the second carbon of the double bond and initiates oxidation and hydroperoxide formation there. Finally, LO^\bullet can add to lipid double bonds to form dimers with radicals on the next carbon of the double bond. These alternate reactions of LO^\bullet are shown in Figure 8.

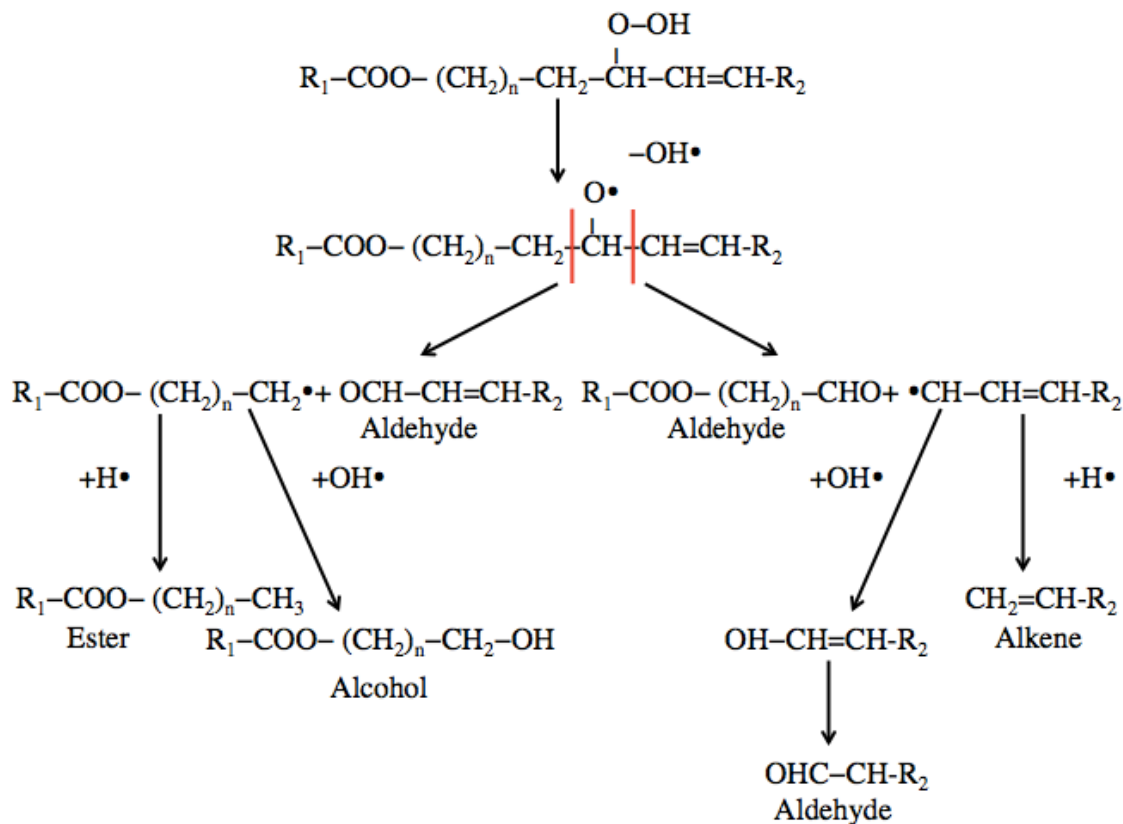


Figure 7. Decomposition of hydroperoxides to produce alkoxy radicals and short chain compounds, modified from AOCS lipid library [77].

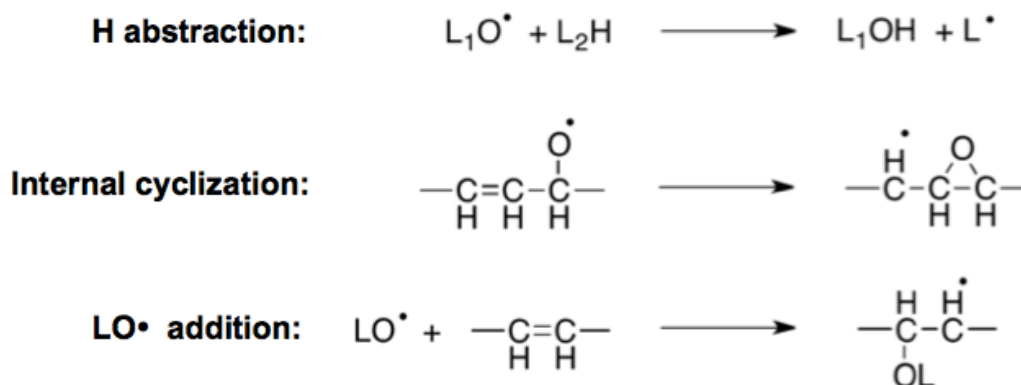


Figure 8. Alternate reactions of alkoxy radicals [5].

2.4.3 Scission of LO• and formation of carbonyl compounds

The main source of carbonyl products from lipid oxidation is scission of the C-C bond on either side of the LO• group -- α-scission occurs on the side closest to the acid group and β-scission occurs on the distal side. Products are a mixture of carbonyl products and free radicals, typically aldehydes, alkanes, and oxo-esters from initial alkoxy radicals (Figure 9.) [80]. Volatile scission products are characteristically associated with the off-odors of rancidity, while non-volatile scission products contribute to off-flavors and stability as well as texture changes during food processing and storage.

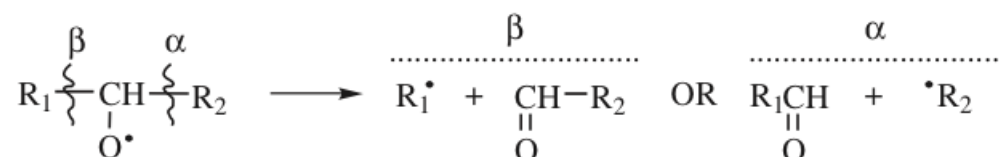


Figure 9. Simplified α or β-scission, adapted from Frankel [80].

Scission of alkoxy radicals is thought to be a solvent-dependent process in which protons stabilize charged transition states and also anneal resulting scission radicals [5]. Water and polar protic solvents stabilize the carbonyl products by providing solvation and hydrogen bonding to support the transition state and reduce the activation energy for bond rupture [81,82]. Alkoxy radical scission occurs 10-100 times faster in protic media than in non-polar organic solvents or neat lipids [1,83,84], but even in non-polar solvents proceeds at rates competitive with hydrogen abstraction (Table 4) [85]. In aqueous solution, scission and abstraction rates are about equal [86]. Scission accounted for 7-10% of oxidation products in neat trilinolein, but shifted to 66-75% in the presence of acid [87]. Another project in this laboratory is testing whether LO• scission follows this

predicted behavior [88].

Table 3. Solvent effects on rates of H abstraction (k_a) and β -scission (k_β) of cumyloxy radicals (CumO•), adapted from Schaich [5]

	H Abstraction $k_a \times 10^{-6} \text{M}^{-1} \text{S}^{-1}$	β -Scission $k_\beta \times 10^{-6} \text{M}^{-1} \text{S}^{-1}$	$k_a / k_\beta \text{ M}^{-1}$
CCl ₄	1.1	2.6	4.5
C ₆ H ₆	1.2	3.7	3.2
C ₆ H ₅ Cl	1.1	5.5	2.0
(CH ₃) ₃ COH	1.3	5.8	2.3
CH ₃ CN	1.2	6.3	1.9
CH ₃ COOH	1.3	19	0.7

Rate and tendency to undergo scission is strongly affected by temperature as well as solvent polarity. Heat provides thermal energy to accelerate scission of alkoxy radicals in all solvents even though distribution of scission products might shift at different temperatures [89].

Whether α or β scission dominates has been highly controversial. Theoretical consideration of bond dissociation energies suggests that scission should occur at the allylic bond ($-\text{CH}_2-\text{CH}=\text{CH}_2$, 60 kcal) rather than the vinyl bond ($-\text{CH}=\text{CH}_2$, 109 kcal) [10], which means α -scission should dominate at C-9 of linoleic acid but β -scission is preferred at C-13. This is indeed the pattern that has been observed in studies in this laboratory [88]. Nevertheless, some studies reported that scission occurring between the alkoxy radical and double bond generates the most stable products [90, 91] so should dominate. Another study proposed that the distance between the alkoxy radical and $-\text{COOH}$ controls the scission preference, with opposite results from bond dissociation energies [92]. Despite these mechanistic arguments, product analysis has detected

products from both types of scissions, so product mixes can become quite complex as oxidation progresses and secondary oxidation of scission radicals occurs, as shown for linoleic acid in figures 10 and 11. Clearly, more detailed research will be needed to resolve this issue.

In most food lipids, oleic and linoleic acid are the two main unsaturated fatty acid undergoing oxidation and producing volatile compounds from their hydroperoxides degradation. Frankel [72] reported volatile decomposition compounds from methyl oleate hydroperoxides generated by autoxidation and photosensitized oxidation (Table 3). Chan et al. [78] reported that the relative concentrations of hexanal, methyl octanoate, 2,4-decadienal, and methyl 9-oxononanoate from 9-LOOH of methyl linoleate were 1%, 37%, 51% and 12% respectively.

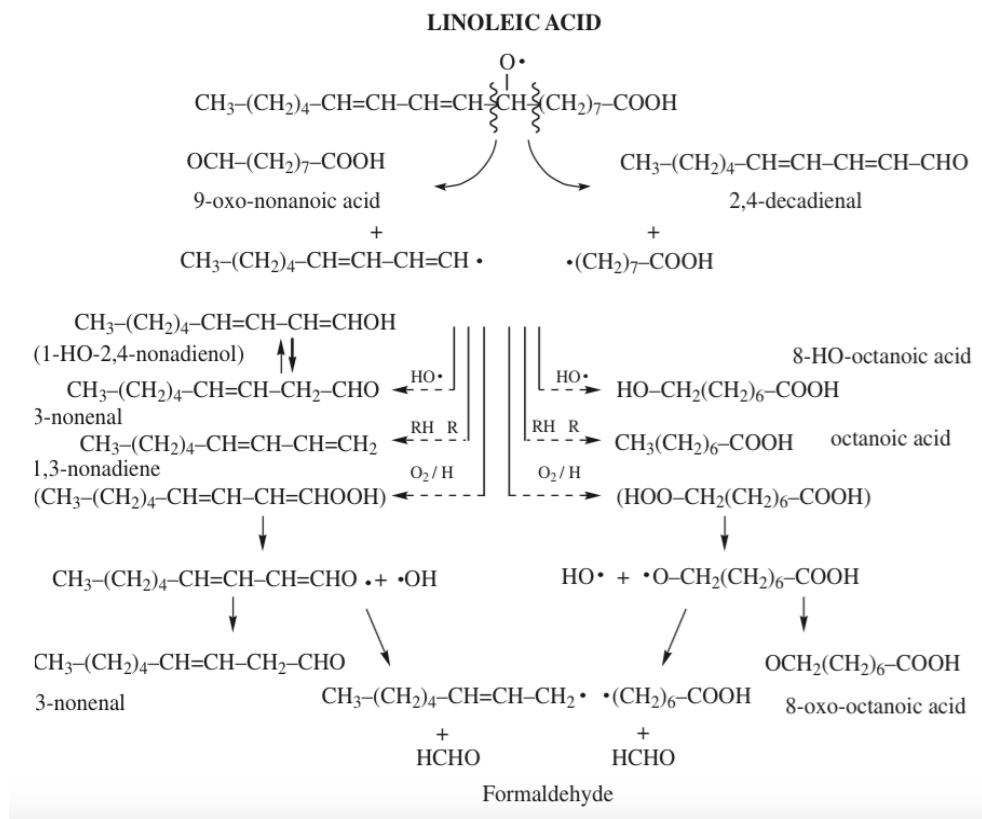


Figure 10. Typical scission reactions of oxidized linoleic acid (9-alkoxyl radical), adapted from Schaich [5].

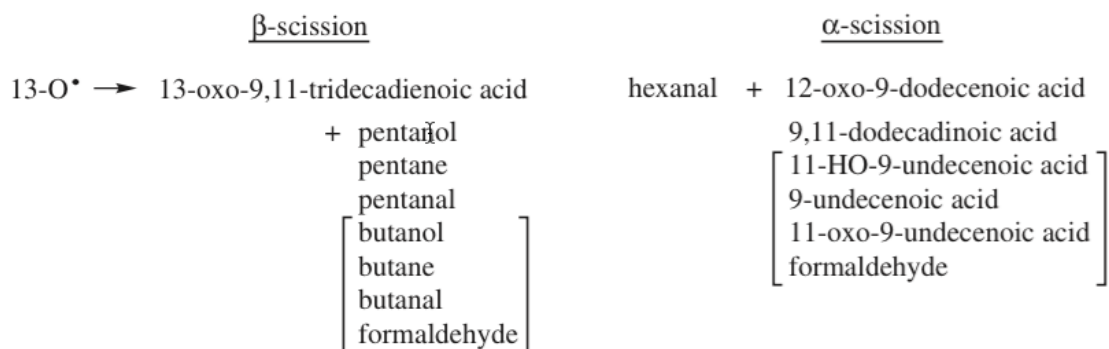


Figure 11. Scission products from oxidized linoleic acid (13) -alkoxyl radical [5].

Table 4. Volatile compounds formed from methyl oleate hydroperoxide [72].

Compound	Autoxidation (rel. %)	Photosensitized oxidation (rel.%)	Origin
Heptane	4.4	4.6	11-OOH
Octane	2.7	10	10-OOH
Heptanal	0.5	0.5	?
1-Heptanol	0.4	0.4	11-OOH
Octanol	11	3.8	11-OOH
Methyl heptanoate	1.5	4.9	8-OOH
1-Octanol	0.4	1.0	10-OOH
Nonanal	15	10	9/10-OOH
Methyl octanoate	5.0	9.7	9-OOH
2-Nonenal	0.5	0.7	?
Decenal	3.9	2.0	8-OOH
Methyl nonanoate	1.5	0.8	?
2-Decenal	5.4	12	9-OOH
2-Undecenal	1.7	7.1	8-OOH
Methyl 8-oxooctanoate	3.5	3.0	8-OOH
Methyl 9-oxononanoate	15	11	9/10-OOH
Methyl 10-oxodecanoate	12	1.7	11-OOH
Methyl 10-oxo-8-decanoate	3.4	5.0	10-OOH

2.5 Carbonyl monomers

When free fatty acids or their esters oxidize, scission products are all small molecules that can move away and become independent. When triacylglycerols or phospholipids oxidize, some small fragments are released and some remain bound to the glycerol backbone. The free migrating, independent cleavage products are referred to as monomers.

Carbonyl monomers may be either saturated and unsaturated. Saturated carbonyls are relatively stable but unsaturated carbonyls, such as 2-nonenal or 2,4-heptadienal, can

undergo further oxidation at their double bonds to generate alkanals, glyoxal and mixtures of α -keto aldehydes [80].

Carbonyl monomers are major contributors to off-odors and flavors in foods, and they are also the main target of carbonyl analyses.

2.6 Core aldehydes

During scission reactions, carbonyl fragments that remain associated with the glycerol backbone of acylglycerols and phospholipids (Figure 12) are referred to as core aldehydes. For every mole of volatile aldehydes, a mole of non-volatile oxidation residue remains bound to the parent acylglycerol molecules [94], as shown in Figure 13. Core aldehydes are non-volatile and their contribution to off-flavors is not clear. However, they serve as reservoirs of potentially toxic oxidation materials as well as agents for co-oxidation with proteins [93]. Little detailed research has focused on formation and reaction of core aldehydes, largely due to lack of appropriate analytical methods and requirements for sophisticated instrumentation, so relatively little is known about this class of carbonyls.

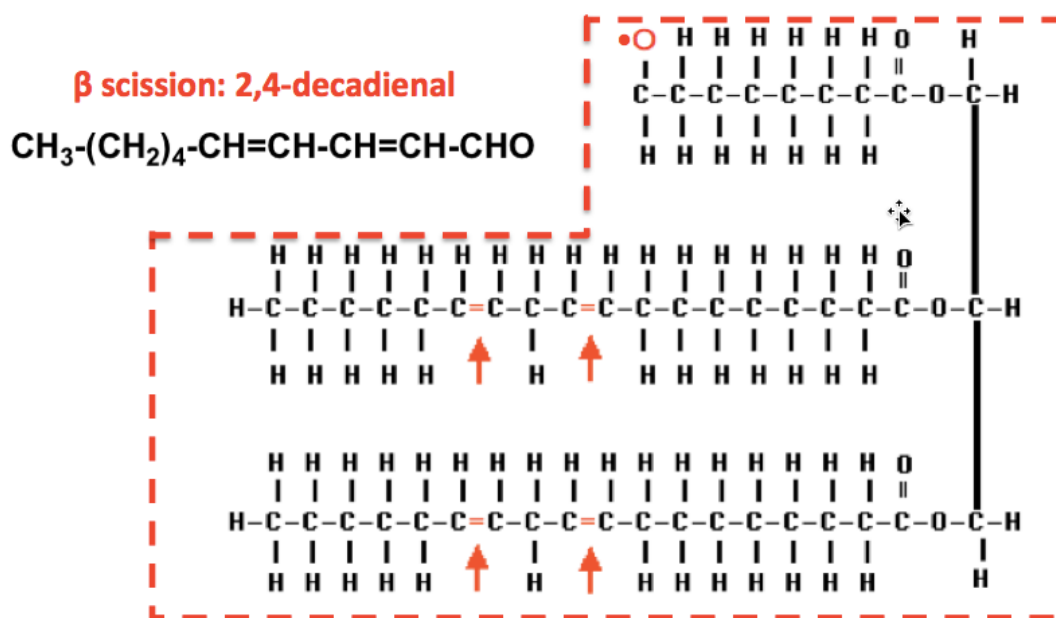


Figure 12. Structure of monomer carbonyl (2,4-decadienal) and core aldehyde cleaved from 9-hydroperoxide from oxidized trilinolein.

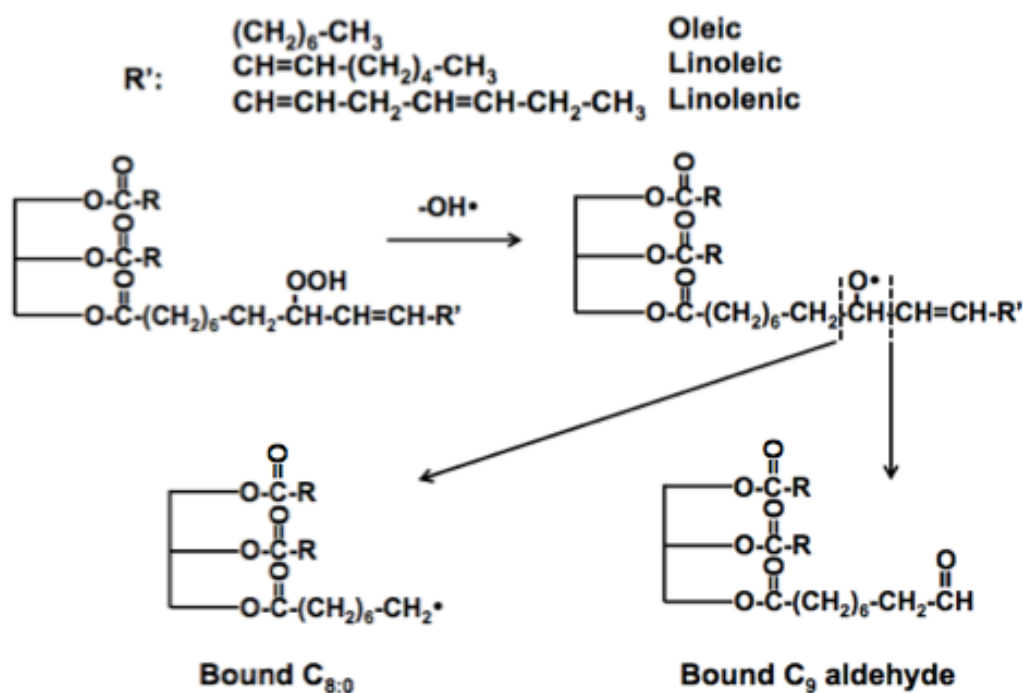


Figure 13. Formation of major short-chain oxidation compounds from 9-hydroperoxide of major fatty acyl groups, obtained from Velasco [95].

2.7 Measurements of lipid oxidation

To determine the lipid oxidation degree of a food sample, a variety of methods have been applied to measure the primary oxidation compounds (hydroperoxides and conjugated dienes) and a few secondary oxidation compounds (e.g. malonaldehyde and hexanal) [3]. However, as shown in Figure 1, lipid peroxy and alkoxy radicals can undergo multiple alternate reactions to generate a wide variety of primary and secondary oxidation compounds [41], certainly more than were previously recognized. The alternate pathways are very dynamic and competitive, so limiting assays to only hydroperoxides and/or volatile products such as hexanal may underestimate or miss the actual extent of oxidation.

A major problem in following lipid oxidation only by conjugated dienes and hydroperoxides is that they both decompose into other products. Thus, low values of either measure cannot distinguish whether oxidation has not begun or has progressed into secondary stages. This is a huge problem in the food industry where decisions about accepting batches may be based on peroxide values below allowable levels. Thus, sensitive, accurate measures of secondary lipid oxidation products are needed to supplement peroxide analyses and help interpret low peroxide values. Volatile carbonyls are easy to measure but are difficult to quantitate and detectability varies with the sampling method used [88]. Thus, being able to measure non-volatile carbonyls from lipid oxidation has become more important for providing valuable clues to supplement other analyses as well as to elucidate products from scission and other pathways more completely.

2.7.1 Carbonyls measurements

Analyses of volatile carbonyl products of lipids oxidation are being covered in detail in a parallel Ph.D. dissertation[88]. Only analyses of non-volatile carbonyls will be considered here.

2.7.1.1 Benzidine value

An old assay for lipid oxidation reacts carbonyls with benzidine to form a complex that can be detected optically at 350 nm (Figure 14) [96,97]. However, the sensitivity is low and quantitation is only relative since complexes of 2-alkenals and 2,4-alkadienals give higher absorbance due to their longer-chain conjugated double bond systems [98]. Additionally, benzidine is carcinogenic [99] and toxic, so this assay has been largely replaced.

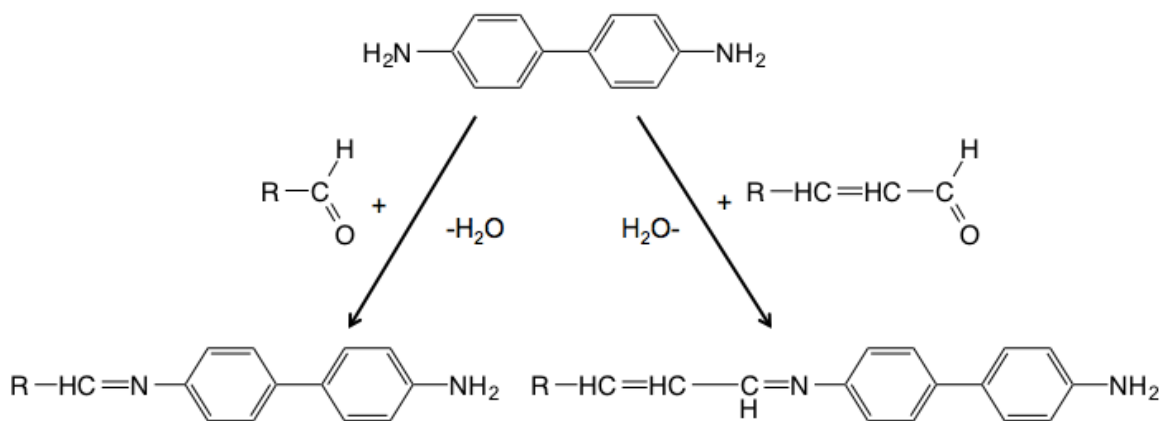


Figure 14. Reaction of benzidine and aldehydes [98].

2.7.1.2 2-Thiobarbituric Acid (TBA) Value

The method in perhaps longest use for measuring lipid carbonyls is the 2-thiobarbituric acid (TBA) value, expressed as milligrams of malondialdehyde (MDA) equivalents per kilogram sample or as micromoles MDA equivalents per gram sample.

The TBA assay is based on the reaction between TBA and carbonyls under acidic conditions to form red, fluorescent adducts with absorption maximum at 530-532 nm (Figure 15)[100, 101]. The assay can be conducted under a range of protocol temperatures (25-100 °C) and times (15 min to 20 h) [102] on whole samples, sample extracts, or distillates.

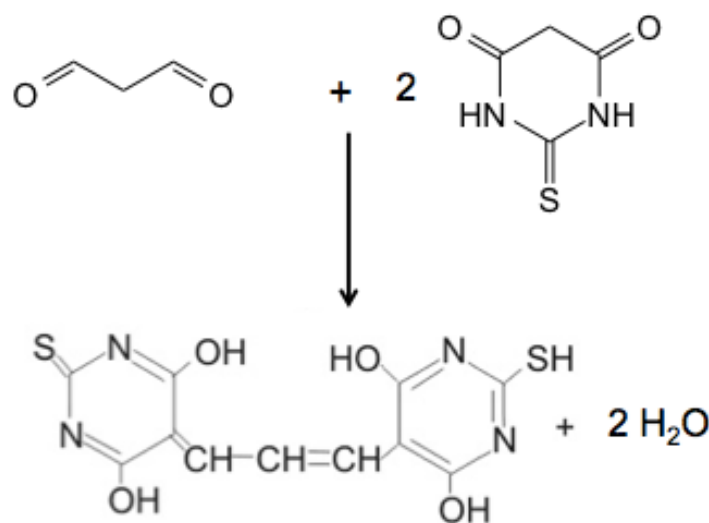


Figure 15. Reaction of 2-thiobarbituric acid (TBA) and malondialdehyde [31].

The TBA assay has many limitations that have earned severe criticisms for its use. The assay lacks sensitivity and specificity to determine aldehydes levels accurately. Authentic malonaldehyde is formed only by fatty acids with three or more double bonds, so it is not appropriate for use in most vegetable oils. Its main applications are with animal fats (high arachidonic acid content) and fish oils (high ω -3 fatty acid content). There is some evidence that TBA can also react with other aldehydes, especially unsaturated aldehydes, under some conditions, but the product is not the same as the TBA complex and must be distinguished by HPLC analyses. Most problematic, Dugan [103] and Baumgartner [104] showed that TBA was able to react with non-lipid carbonyls such

as ascorbic acid, sugars, and non-enzymatic browning products yielding color complexes with 450 nm-540 nm absorption range. Since the MDA assay is normally performed by heating intact foods in the reaction mixture, these interferences cannot be eliminated.

Modifications to improve specificity and sensitivity of the TBA assay have been proposed by Robbles-Martinez et al. [105], Pokorny et al. [106] and Shahidi et al, with particular focus on measuring lipid oxidation in marine lipids and poultry fat. However, food systems are quite complex and have many interfering ingredients exposed to each other. Thus, the TBA assay should be avoided if accurate and definitive oxidation analyses are required. Certainly, the TBA assay can provide no mechanistic information.

2.7.1.3 *p*-Anisidine Value

Levels of aldehydes (principally 2-alkenals, and 2,4-alkadienals) in animal fats and vegetable oils can be determined by reaction with *p*-anisidine under acidic conditions to form yellowish product with maximum absorption at 350 nm (Figure 16) [31]. The IUPAC standard method, defines the *p*-anisidine values as 100 times the optical density measured at 350 nm in a 1.0 cm cell of a solution containing 1.0 g of oil in 100 mL of mixture of solvent and reagent. List et al. [107] reported a highly significant correlation between anisidine values and flavor scores of salad oils processed from undamaged soybeans. However, anisidine requires relatively high carbonyl levels for reaction so it is most often used with degraded frying oils. The anisidine reaction is neither quantitative nor specific. Anisidine reacts slowly with hydroperoxides and more rapidly with all aldehydes, but unsaturated aldehydes give a higher color response. Thus, the results are only relative measures, not absolute concentrations. Furthermore, this assay cannot distinguish different types of carbonyls, so it is not useful in studies of lipid oxidation

mechanisms.

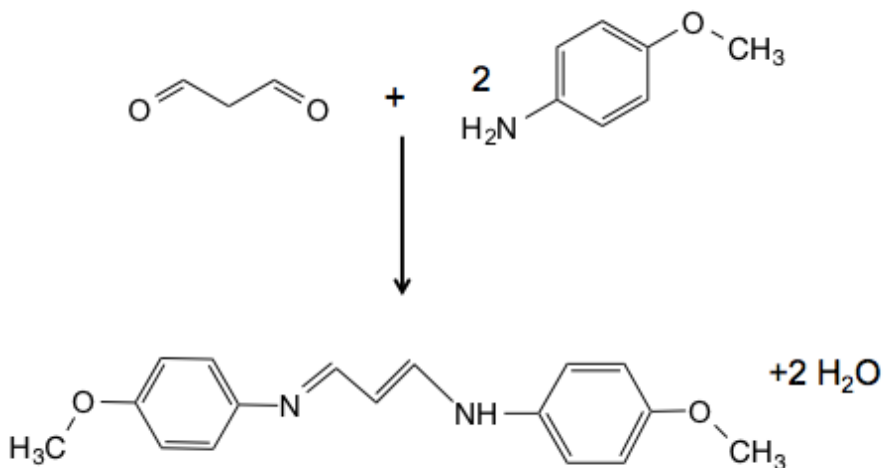


Figure 16. Reactions between *p*-anisidine and malonaldehyde [61].

2.7.1.4 DNPH (2,4-dinitrophenylhydrazine) assay

The reaction between carbonyl groups and 2,4-dinitrophenylhydrazine (DNPH) has been used to detect carbonyls for more than 100 years. Carbonyls (including ketones and aldehydes) react with DNPH to form dinitrophenylhydrazones (Figure 17) which have maximum absorption at 432 nm (saturated aldehydes), 458 nm (unsaturated aldehydes) in alkaline solution [108], and 360 nm in ethanolic solution [109].

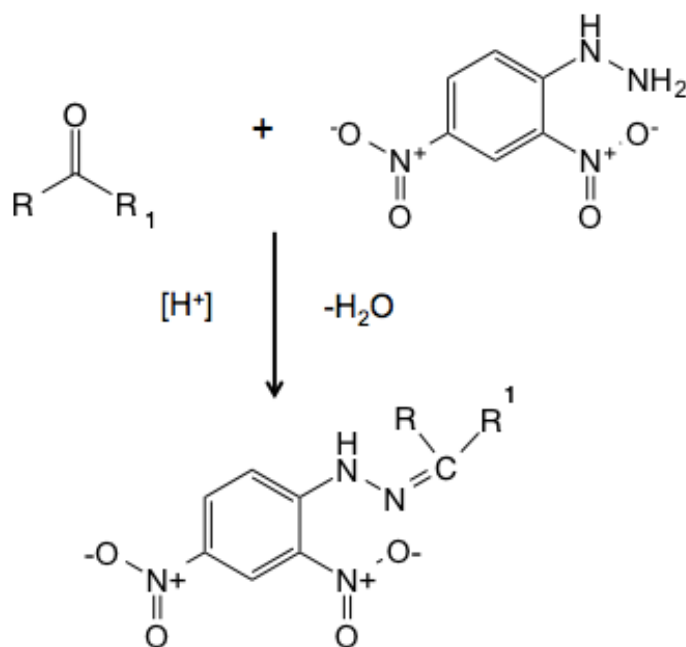


Figure 17. Reaction of carbonylic lipid oxidation products with DNPH [98].

The traditional DNPH solution assay monitored hydrazone formation by UV/Vis spectrometry. However, the excess DNPH required for the reaction but also causes instability and interference in optical measurements, and under some conditions the hydrazones fade rapidly. These difficulties may partially explain why the reaction is seldom used *per se*.

Stability and utility of the assay has been improved dramatically by adaptation to HPLC methods in which the unreacted DNPH can be separated from the hydrazone products, which are detected optically at 360 nm [110]. HPLC methods impart the additional advantage, especially for research on oxidation processes or analyses of specific aldehydes, of being able to separate polar and nonpolar reaction products from one another, and to determine individual DNPH derivatives.

DNPH-HPLC methods have been used to analyze non-volatile carbonyls in frying

oils and oxidized triacylglycerides [111, 112]. Solid-phase extraction cartridges impregnated with 2,4-dinitrophenylhydrazine (DNPH) are commercially available to collect and derivatize volatile carbonyls in air samples [113]. Other solid sorbents coated with DNPH, such as silica gel [114], XAD-2 [115], and octadecylsilane bonded silica gel [116], have also been used for sampling carbonyl DNPH derivatives. However, most cartridges have been designed to collect carbonyl compounds from air samples rather than aqueous samples. Research in our lab found that this method, though attractive, is difficult to apply to non-volatile aldehydes.

Currently, HPLC-DNPH methods appear to offer the greatest promise for quantitative and qualitative analyses of lipid carbonyls that are sensitive and specific. However, very few protocols have been developed and much is still to be learned about control factors in the reaction and the HPLC analysis.

2.7.1.5 High Pressure Liquid Chromatography-DNPH Analyses

High performance liquid chromatography (HPLC) and ultra-high performance liquid chromatography (UHPLC) have been used to investigate non-volatile lipid oxidation products. Typically, lipid class separations have been conducted by normal phase column [117], but reversed phase (C18) columns are more widely used to analyze lipid oxidation products [118] because they are generally easier to work with and are more compatible with solvents of different polarities [119]. Separation selectivity is provided by the column base materials and surface modifications, carbon loading, and solvent. Different manufacturers utilize different silica materials as supports, combined with different bonded phases to provide columns with a range of chromatographic retention and selectivity [119]. For reverse phase columns, relatively polar solvents such

methanol, acetonitrile, and isopropanol combined with water are very common solvents for elution of lipids or lipid oxidation compounds. In most of DNPH-HPLC carbonyl studies, acetonitrile combined with water or methanol in various proportions provided the mobile phase to elute and separate mixtures of short chain carbonyl DNPH derivatives [110,120-122]. Jeong et al. [123] used an isocratic mobile phase of acetonitrile and water (70:30,v/v) to analyze short chain carbonyls-DNPHs (propanal, pentanal, hexanal, *t*-2-heptenal, *t*-2-octenal and *t*-nonenal) from oxidized linoleic acid and lard model systems. The same solvents but in a gradient were also used to compare HPLC/UV and GC-MS for analyzing carbonyl compounds by DNPH derivatization [124]. Acetonitrile combined with water or methanol may be optimal solvents for most of short chain carbonyl DNPH derivatives, but they are too polar to elute large lipid molecules such as triglycerides. Therefore, adjustment of mobile phase polarity and solvent strength will be necessary when analyzing oxidized oil samples or extracts.

Simple UV/Vis or diode array (DAD) detectors connected to HPLC outlet lines detect analytes with chromophores, such as conjugated dienes that absorb at 234 nm [80]. The evaporative light scattering detector (ELSD) is a universal detector that can be used with any solvent or solvent mixture that is sufficiently volatile and pure [125]. ELSDs depend on analyte mass and are independent of optical absorption, so are often preferred for quantitative analyses.

Completely separating DNPH-carbonyl derivatives is deceptively difficult because the DNPH moiety dominates the migration through the column. Carbonyl adducts which need to be identified have less influence on elution. Substantial research has already focused on modifying DNPH derivatization and improving separation of

monomer carbonyls on HPLC [122, 126] but only a few studies of core carbonyls are available.

Co-elution of DNPH-carbonyl derivatives with different carbon number or different degree of saturation has been a major problem. For example, Brombacher et al. [110] found that 2-pentanone and cyclohexanone as well as n-pentanal and ethanedial were critical pairs eluting in common peaks. Zhu [126] reported that DNPH-carbonyls with smaller carbon number generally eluted before those with larger carbon number, and that derivatized carbonyls with higher degrees of unsaturation eluted earlier than the corresponding DNPH-carbonyls with lower degrees of saturations. This behavior is as predicted by the equivalent carbon number (ECN) concept proposed by Podlaha [127] in 1982 to illustrate the relationship between the peak resolution, carbon numbers, and saturation:

$$ECN = CN - 2 * NDB$$

where CN: carbon numbers, NDB: number of double bonds

This formula says that compounds having the same equivalent carbon numbers will elute at the same position, that compounds with lower ECN elute before those with higher ECN, and that the more double bonds a compound has, the smaller the ECN it will have and the earlier it will elute. Where this becomes important in DNPH-carbonyls analysis is that unsaturated aldehydes often co-elute with saturated aldehydes one or two carbons shorter. It is a problem of the assay that must be resolved before that assay can be applied routinely.

To sum up, even though the DNPH derivatization followed by HPLC analyses has been applied to detect carbonyl compounds from different types of samples, the methods

have been limited by a) the absence of elution system (mobile phase and gradient) to elute and separate short chain carbonyl monomers from core aldehydes in complex lipids such as phospholipids and acylglycerols in the same run; b) lack of an accurate and reproducible method to quantify the carbonyls with different chain lengths or saturation.

2.7.1.6 Mass spectrometry

Mass spectrometry (MS) is often coupled with HPLC to better identify and elucidate the structures of carbonyl-DNPH derivatives. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) interfaces are the major technique used to detect analytes and determine their fragmentation patterns in oxidized lipids [118]. ESI generates analyte ions in solution before the molecule reaches the mass spectrometer. As shown in Figure 18, the LC eluent is nebulized into a spray chamber at atmospheric pressure where it is charged by an electrostatic field. The mixture is then heated by drying gas to evaporate the solvent and yield bare ions before the mass is analyzed. Inert gas (e.g. nitrogen) is normally used to remove the solvent and decrease the droplet diameter. The Coulomb repulsion of ions will equal the surface tension when droplet diameter decreases, so the droplet explodes producing smaller droplets until bare ions are obtained [128]. The ESI is more well-suited for analysis of large biomolecules because it can provide multiple charging in ionization and analyze molecule as large 150,000 Da. Moreover, ESI is relatively mild and does not thermally decompose analytes [129].

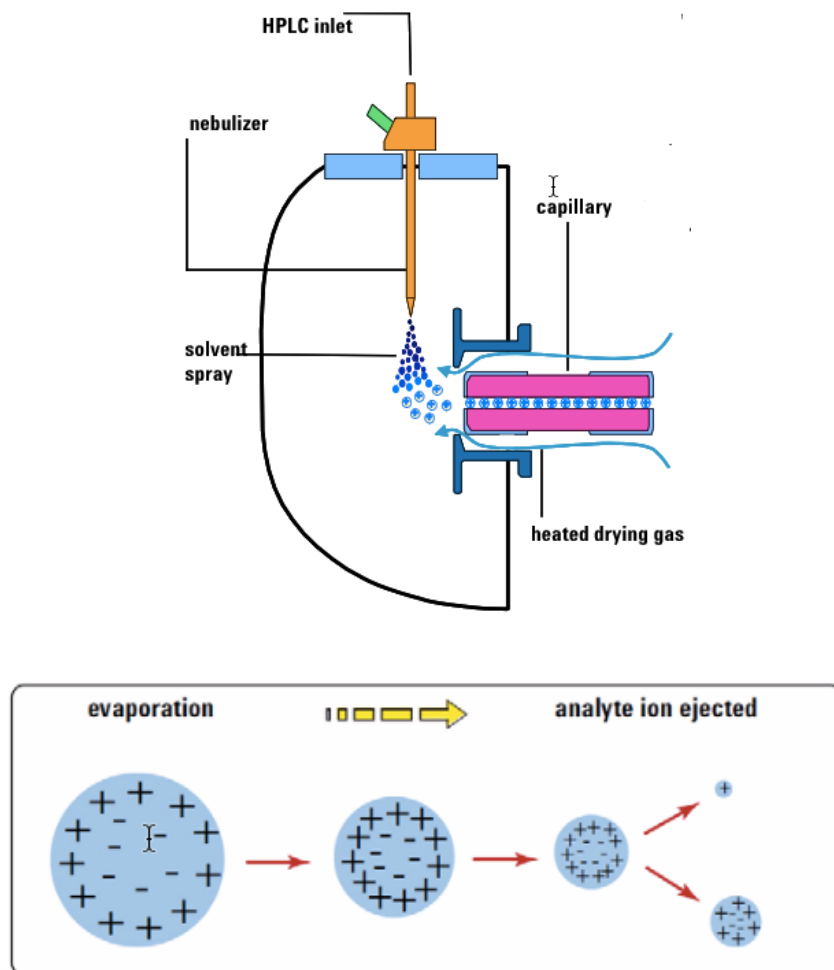


Figure 18. Atmospheric (ESI) and solvent evaporation process in mass spectrometry, obtained from Agilent Technologies Inc. [128]

APCI is gas-phase chemical ionization process. The eluent spray is created in a nebulizing needle and passed through a heated ceramic tube to be vaporized and then ionized by a corona needle (Figure 19). APCI requires that the analyte be in the gas phase to ensure that ionization occurs, so typically the vaporizer temperatures are 400 to 500 °C. Although APCI is applicable across a wide range of molecular polarities, the detectable molecular weight is limited to 1500 Da and molecules are not stable at high temperature.

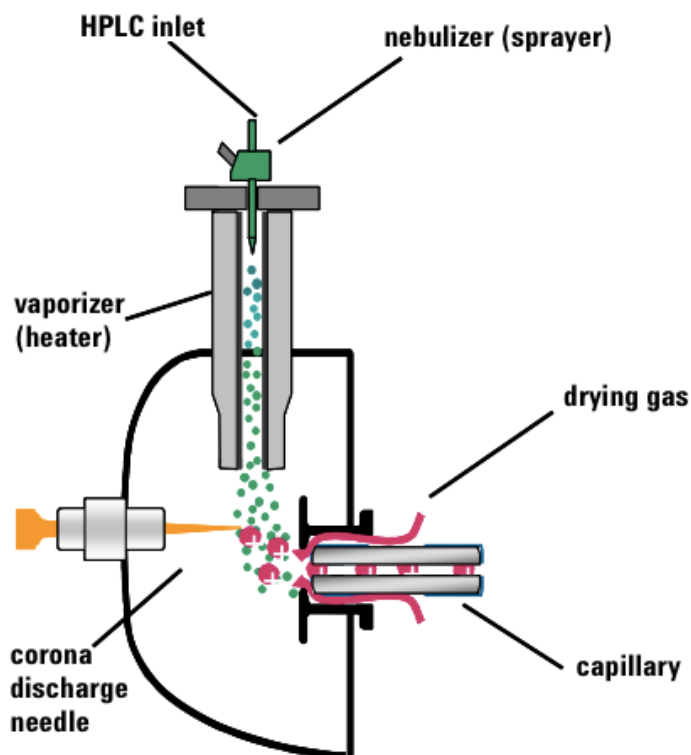


Figure 19. Atmospheric pressure chemical ionization (APCI), obtained from Agilent Technologies Inc. [128]

Zwiener [122] used LC-ESI-MS-MS to determine carbonyl compounds in disinfected water derivatized by DNPH and found that the excess of DNPH reagent, derivatization time, nature of buffer, dry gas temperature in ESI process as well as collision energy could affect MS analyses.

The negative ion mode is often chosen as the ion source in APCI-MS. Brombacher et al. [110] utilized negative $[M-H]^-$ mode for APCI to analyze carbonyls in ambient air. DNPH-carbonyl hydrazones structures were elucidated by APCI negative $[M-H]^-$ mode HPLC/MS [113], while positive ion mode of APCI was used in determining aldehydes in exhaled breath condensate [130]. Each ionization source and ion mode

should be tested and the final methods used should be based on the type of information needed as well as the nature of the analyte and the solvent chemistry[128].

Chemical structures of several DNPH-carbonyls have been elucidated by LC/MS/MS [113,121,131]. In Kölliker' studies on carbonyl compounds in air samples, carbonyl standards with different carbon number and different structure were analyzed, and DNPH hydrazones gave characteristic fragment ions at m/z 120, 122, 152 and 179 (Figure 20). Different substructures of DNPH-carbonyl hydrazones were investigated systematically. The scheme used for structure elucidation of DNPH-carbonyls under APCI (-) MS/MS is shown in Figure 21 [113].

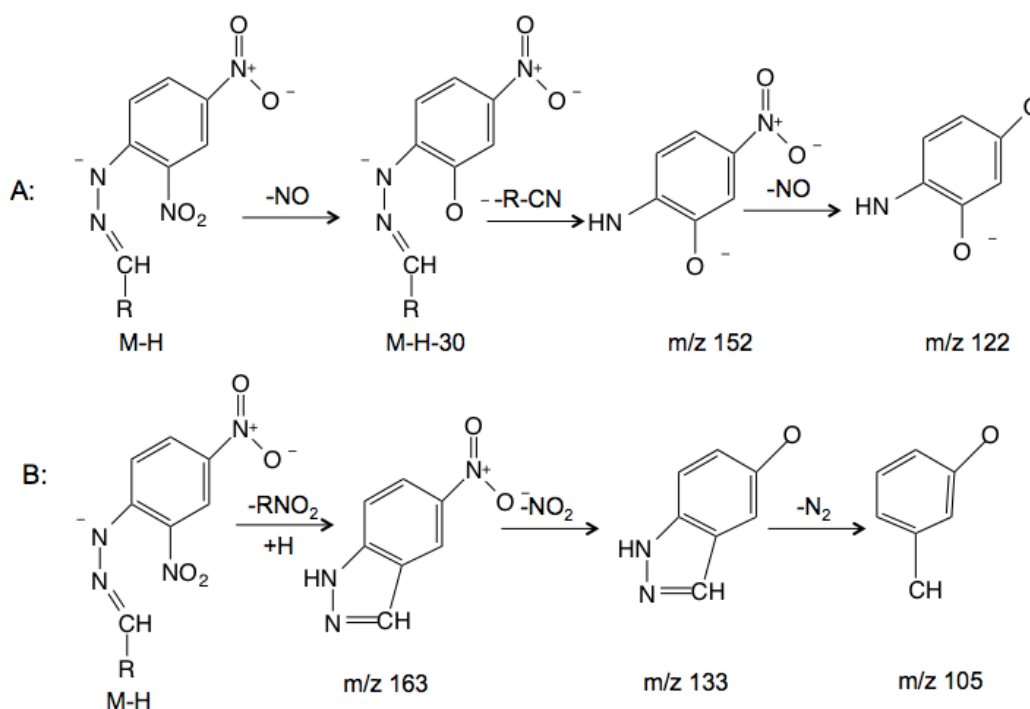


Figure 20. Characteristic fragments of DNPH-carbonyls derivatives under APCI (-) MSn,

A: formation of ions m/z 152 and 122, B: formation of ions m/z 163, product formed during derivatization. Obtained from Kölliker [113].

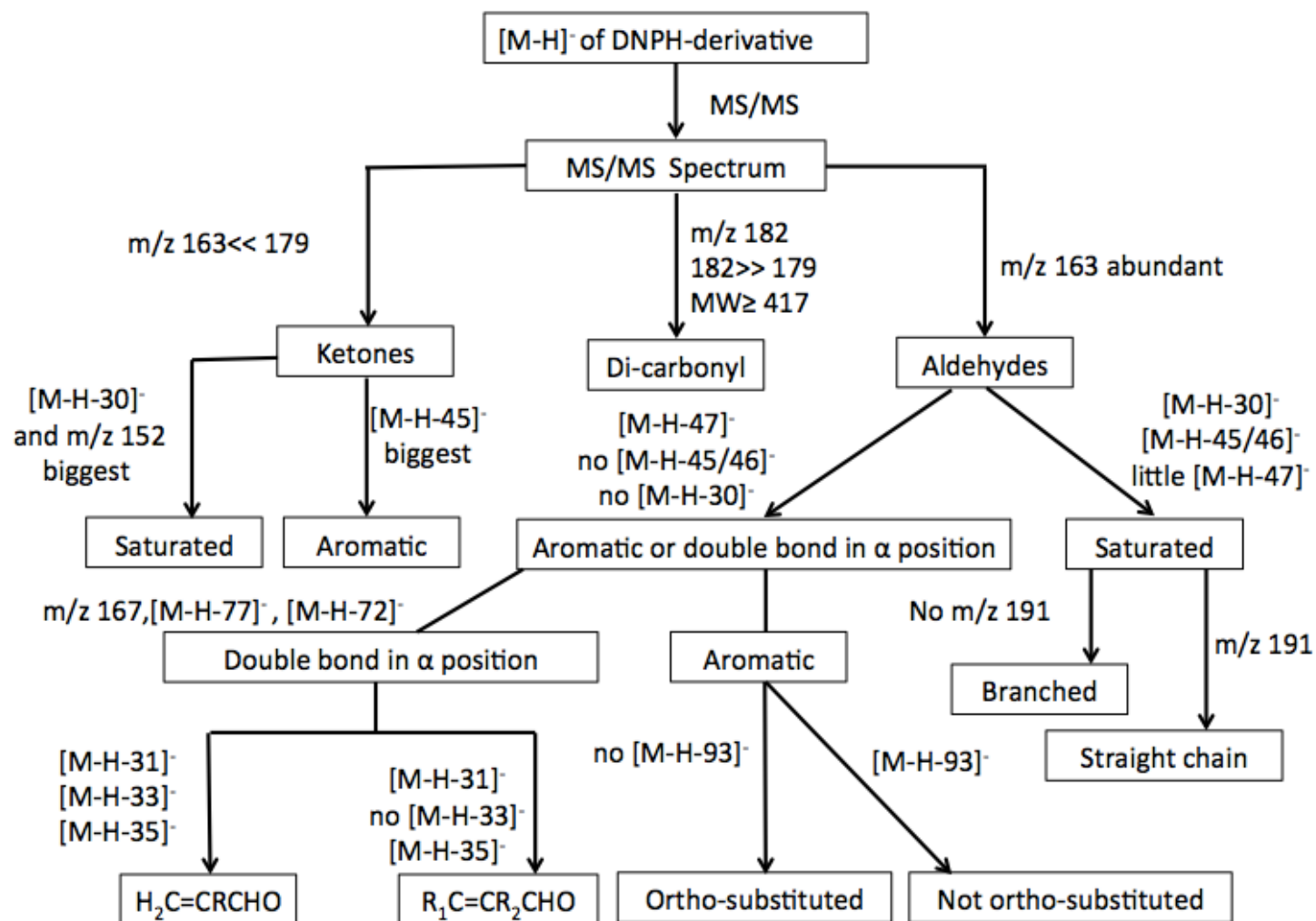


Figure 21. Structure elucidation scheme for the identification of different substructures in carbonyl-DNPH by APCI (-). Adapted from

Kölliker [113]

Non-volatile carbonyl products from lipid oxidation can also be analyzed directly by HPLC/MS. Byrdwell and Neff used RP HPLC-APCI-MS (positive mode) to analyze the autoxidation products of trilinolein and detected mono- and dihydroperoxide TAGs and epoxidised TAGs [132] by using methylene chloride-acetonitrile binary solvent and RP C18 column; they also detected non-volatile oxidation products of trilinolein under frying conditions [133]. According to their observations, APCI was more commonly used to ionize neutral lipids such as TAGs, while ESI was more suitable for more polar oxidized molecules [134].

Kuksis et al. [94] used negative HPLC-ESI/MS (negative mode) to detect gradual increases in core aldehydes in sunflower oil oxidized over 18 days at 60 °C. They also found that core aldehydes accumulated in sunflower oil after initial oxidation even at commercial storage temperature of 10 °C for 3 months. The content of hydroperoxide and hydroperoxide-core aldehydes combination were estimated to account for about 5% of total TAG. 9-oxononanoic (70%)-, 12-oxo-9,10-epoxydodecanoic (10%)-, and 13-oxo-9,11-tridecadienoic (5%)-containing acylglycerols were detected by ESI/MS as non-volatile oxidation products.

Overall, HPLC-MS appears to have great potential for identifying non-volatile lipid oxidation products, especially for triacylglycerol-backbone carbonyls. With this information from MS, products identities can be verified; compounds in co-elution of HPLC separation can be identified based on different ions fragments, and more details can be obtained to better understand the scission reactions of lipid alkoxyl radicals and track footprint of hydroperoxides decompositions.

3. RESEACH GAPS

Extensive studies of lipid oxidation conducted over decades have assumed that the driving force for the radical chain reaction was exclusively hydrogen abstraction. However, the literature also suggests that additional pathways are open to peroxy and alkoxy radicals, in parallel to and in competition with hydrogen abstraction. When these pathways are active, a wide variety of products can be generated even from the beginning of oxidation, rather than forming only after hydroperoxide decomposition. Tracking these alternate reactions requires much more sensitive and definitive analyses of multiple oxidation products simultaneously.

The first requirement for such analyses is extraction of lipids in a manner that does not induce adventitious oxidation or change existing oxidation products. Current extraction procedures have all focused on maximizing lipid yields rather than minimizing oxidation and degradation. This is perhaps understandable since oils that are 1% oxidized are considered inedible by taste panels, yet that lipid loss would barely be detectable in total lipid quantitation. However, there is a clear need to develop milder extraction procedures that give high yields (to ensure that the extract is representative) while protecting the lipid from degradation and new oxidation.

The second requirement is more complete analysis of oxidation products, certainly beyond the conjugated dienes, hydroperoxides, and hexanal commonly measured. Volatile carbonyls have been studied extensively as major contributors to rancidity development, due to ease of analysis and ready perception and recognition by consumers. However, volatiles alone give a distorted view of oxidation because they reflect mostly scission reactions. Transformations that generate non-volatile carbonyls

and other products remain analytically invisible in the oil phase. Here they contribute to off-flavors and interact with starches and proteins to mediate undesirable browning, texture change, and even the formation of toxic products. Chemical assays for non-volatile carbonyls currently available lack sensitivity and are difficult to handle and control, hence are not often applied in lipid oxidation analyses. Furthermore, they provide only total class analysis with no distinction of the types of carbonyls present or the molecules on which they have been formed. Hence, methods for determining exact carbonyl structures and distinguishing whether they are monomer degradation products or remain attached to phospholipids or acylglycerols are clearly needed, initially as a tool for more detailed research on lipid oxidation mechanisms and eventually as a quality control analysis.

4. RESEARCH OBJECTIVES

Our laboratory is engaged in broad-based research, the overall goal of which is to develop dependable, accurate and sensitive methods to analyze all products and stages of lipid oxidation. This dissertation research focuses on two critical deficiencies in lipid oxidation analyses: a) lack of a lipid extraction method that is efficient yet does not add to or destroy lipid oxidation products, and b) lack of a sensitive assay to identify and quantitate non-volatile carbonyl oxidation products.

The specific objectives of this research are as follows:

1) Develop an efficient accelerated solvent extraction method for extracting lipids from complex food matrices without inducing adventitious oxidation or destroying oxidation products.

- Use baked pet food biscuits and extruded pet food kibbles as model systems for complex, difficult to extract matrices.
- Determine the stability of hydroperoxides and other lipid oxidation products during ASE extraction.
- Investigate effect of extraction factors such as sample particle size, temperature, and solvent on ASE lipid extraction's efficiency and degree of oxidation.
- Optimize the combination of ASE extraction parameters such as solvent, static extraction time and numbers of extraction cycles to maximize the lipid extraction efficiency.
- Validate the ASE extraction method with known lipids.
- Compare extraction efficiency and lipid oxidation of ASE and manual extraction.

2) Convert the traditional DNPH carbonyl assay to an HPLC method that detects individual carbonyls and separates carbonyls in different lipid structural classes (monomers, phospholipids, acylglycerols).

- Test the specificity of DNPH derivatization to carbonyls.
- Develop gradients to elute a broad range of lipid sizes, from small monomers to large triglycerides on a reversed phase C18 column.
- Separate the monomers and core aldehydes carbonyl-DNPH derivatives on RP-C18 column.
- Improve the resolution of carbonyl-DNPH derivatives by adjusting the elution conditions such as solvents, or gradient and column.
- Determine the limit of detection, limit of quantification and RSD% of DNPH-HPLC on carbonyl-DNPH derivatives.
- Determine the stability of carbonyl-DNPH derivatives under different storage conditions.
- Quantitate the carbonyl-DNPH derivatives on DNPH-HPLC with diode array detection.
- Verify the carbonyl-DNPH derivatives on LC-MS.
- Investigate the formation of core aldehydes and scission carbonyls in oxidized trilinolein.
- Apply the DNPH-HPLC method to analysis of carbonyls from oxidized commercial oil and lipid extracts.

5. METHODS AND MATERIALS

5.1 Overall Experiment Design

The research was separated into two parts. The experimental designs are shown in Figure 22 for accelerated solvent extraction and Figure 23 for carbonyl analysis.

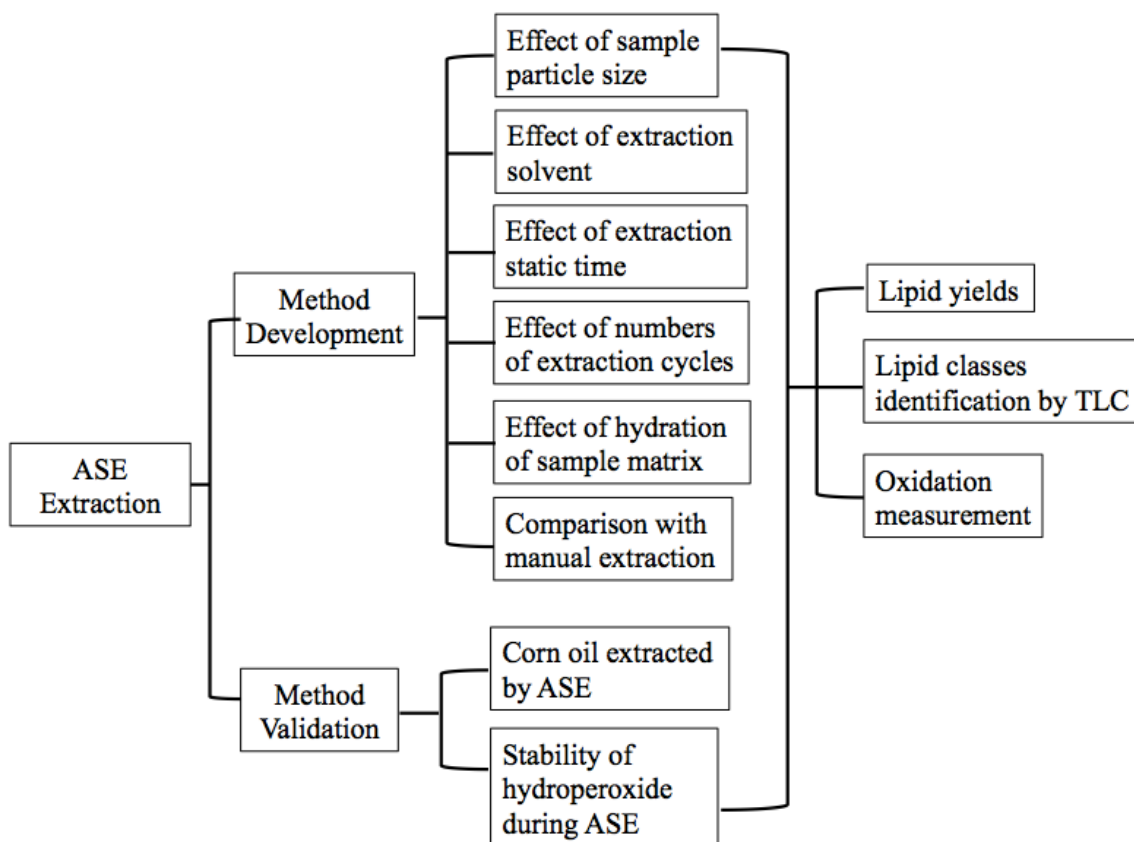


Figure 22. Overall experiment design for lipid extraction

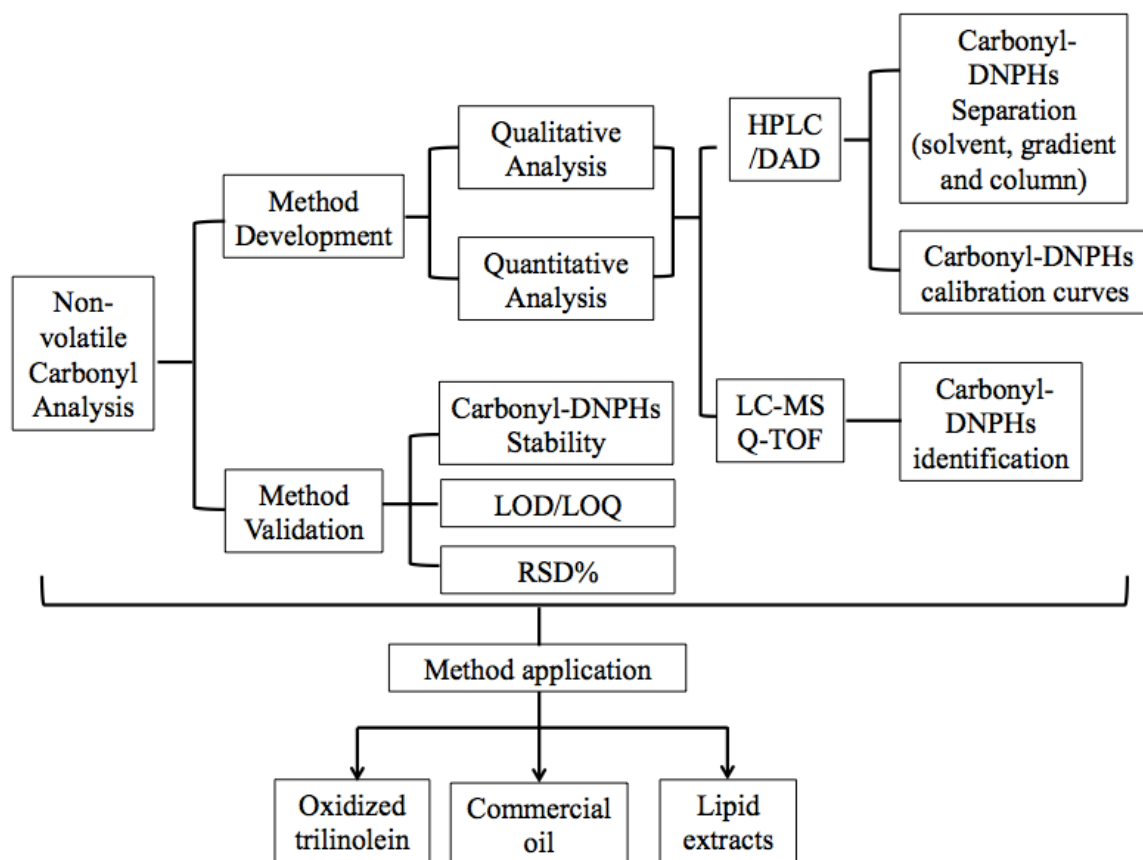


Figure 23. Overall experiment design for development of HPLC-DNPH analyses for lipid carbonyls

5.2 Materials

5.2.1 ASE extraction

Commercial pet food biscuits were purchased from a local supermarket, the crude fat content was 13.2% according to information provide by the manufacturer. Extruded pet food kibbles were provided by a pet food company; fat contents determined by acid hydrolysis are listed in Table 5.

Table 5. The fat content and peroxide value of the extruded pet food kibbles

Sample	Fat content (%)	Peroxide value (mEq/kg fat)
Lite	9.63	4.88
Lamb/rice	18.09	11.85
Chicken/rice	16.23	9.63
Chicken	16.82	3.67

All organic solvents used for lipid extraction were of HPLC grade or higher, >99% purity (Sigma-Aldrich, St. Louis, MO). All glassware used in the study was cleaned by washing with Citranox[®] (Alconox, White Plains, NY) acid cleaner and detergent, rinsing in water purified to 18 MΩ resistivity in a Milli-QTM water purification system (Millipore, Billerica, MA), soaking overnight in S-L-X denatured alcohol (W.M. Barr. Co., Memphis, TN) saturated with potassium hydroxide (Sigma-Aldrich, St. Louis, MO) to saponify any traces of lipids, rewashing with Citranox and rinsing with Milli-QTM water, soaking overnight in 0.1 N HCl (Ultrex, J.T. Baker, Phillipsburg, NJ) to remove metal impurities, rinsing in deionized water, and drying in an oven.

5.2.2 DNPH-HPLC analyses for lipid carbonyls

2,4-Dinitrophenylhydrazine was recrystallized three times from n-butanol. Sulfuric acid and the following carbonyl standards were purchased from Sigma-Aldrich (St. Louis, MO): butanal, pentanal, hexanal, heptanal, octanal, nonanal, decanal, undecanal, t-2-pentenal, t-2-hexen-1-al, cis-4-heptenal, t, t-2, 4-heptandienal, t-2-heptenal,

t-2-octenal, t, t-2, 4-nonadienal, t-2-nonenal, t, t-2, 4-decadienal, t-2-decenal, butanone, 2-heptanone, t-3-octen-2-one, dihexyl ketone, cummene peroxide, 1-octanol and 1,2-expoxy-9-decene. High purity monolinolein, dilinolein and trilinolein standards were purchased from Nu-check Prep (Elysian, MN). HPLC grade acetonitrile, isopropanol and chloroform were purchased from Fisher Scientific (Pittsburg, PA). Water was purified to 18 M Ω resistivity in a Milli-QTM water purification system (Millipore, Billerica, MA). The corn oil, olive oil and vegetable oil samples were purchased from local grocery store; stripped soybean oil was a gift of Bunge North America. (Waterloo, IN).

Lyophilized emulsion:

Methyl linoleate >99% was purchased from Nu-Check Prep Inc. (Elysian, MN). High purity alpha-lactalbumin was purchased from Davisco Foods International, Inc. (Eden Prairie, MN).

Crackers:

Natural mixed tocopherol were purchased from Jedwards International, Inc. (Brainree, MA). Flour and salt were purchase from local supermarket.

5.3 Methods

5.3.1 Accelerated solvent extraction of lipids

5.3.1.1 ASE effect on lipid oxidation

Lipid recovery and stability of lipid oxidation products during ASE were tested in two ways: 1) analyzing lipid oxidation in commercial corn oil purchased from a local grocery store before and after ASE extraction, and 2) spiking blank extractions with known amounts of cumene hydroperoxides. The experimental flow diagram for testing

oxidation in corn oil is shown in Figure 24. Details of spiking system are detailed in Table 6.

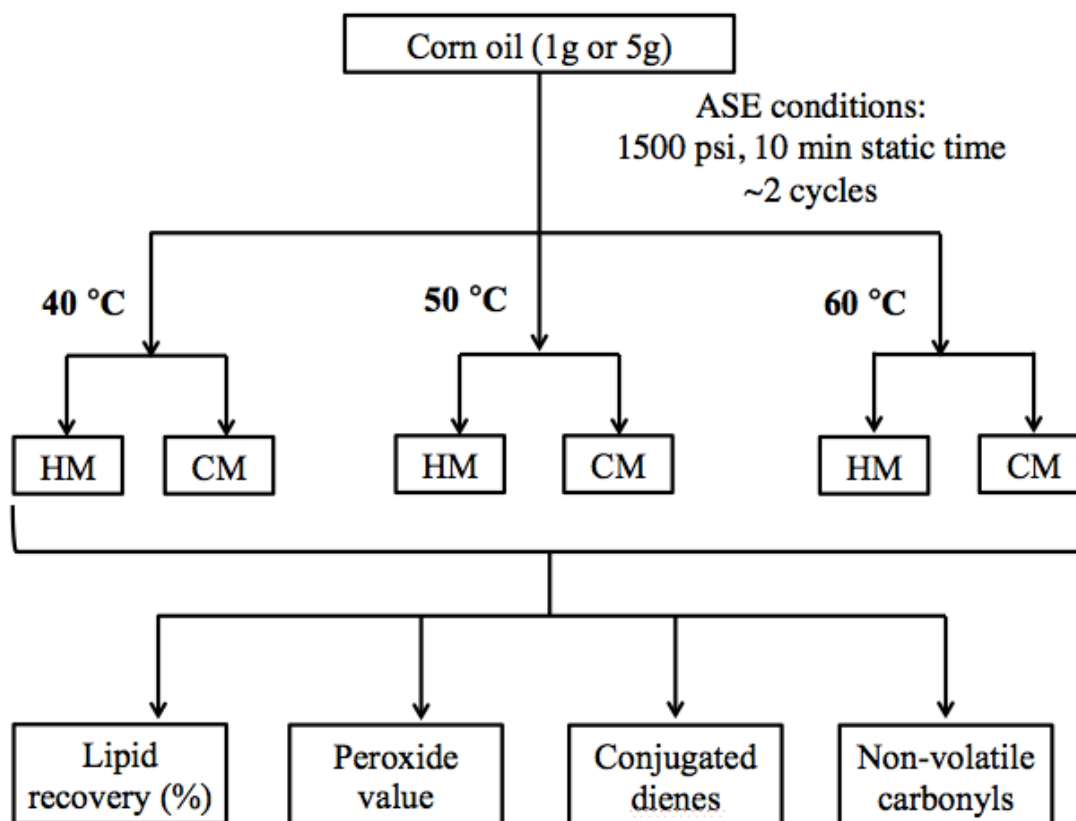


Figure 24. Experimental flow diagram for testing ASE effect on lipid oxidation, HM:

hexane: methanol (2:1, v/v) CM: chloroform: methanol (2:1, v/v).

Table 6. Cumene peroxide spike test to determine stability of hydroperoxides in ASE^a

Systems	Treatments
P+SD	0.2 and 0.02 mmol CuOOH dissolved in 51ml CM
(Peroxide + solvent, dried)	(2:1,v/v),dried at 40 °C by rotary evaporation
P+SI	0.2 and 0.02 mmol CuOOH dissolved in 51ml CM
(Peroxide + solvent, incubated, dried)	(2:1,v/v), incubated at 40 °C for 25 min, dried at 40° C by rotary evaporation
P+H ASE	0.2 and 0.02 mmol CuOOH mixed with hydromatrix, 10
(Peroxide + Hydromatrix, extracted)	min static extraction at 40 °C, two cycles, dried at 40 °C by rotary evaporation.
P+K+H ASE	0.2 and 0.02 mmol CuOOH mixed with hydromatrix and 1
(Peroxide + Kibble+ Hydromatrix, extracted)	g ground kibbles, 10 min static extraction at 40 °C, two cycles, dried at 40 °C by rotary evaporation.
K ASE	Mixed hydromatrix with 1 g ground kibbles, 10 min static
(Kibble+ Hydromatrix, extracted)	extraction at 40 °C, two cycles, dried at 40 °C by rotary evaporation.

^a Extractions were performed in a Dionex 350 Accelerated Solvent Extractor (Dionex Corp, Sunnyvale, CA). Up to 24 samples can be extracted in automated sequence in this system.

Stability of oil oxidation during ASE treatment of corn oil: Routine extractions used 1 g corn oil mixed thoroughly with 25 ml Hydromatrix™ (a pelletized diatomaceous earth material that absorbs twice its mass in water, Perkin Elmer/Agilent, Wilmington, DE) and transferred to 34 ml stainless extraction cells containing 30 mm diameter cellulose filters. For tests of scale-up, 5 g corn oil were mixed with 50 ml Hydromatrix™ and transferred to 100 ml extraction cells. Void volumes in the cells were filled with Hydromatrix™ and the cells were placed in the extractor sample tray. Extraction conditions were 1500 psi pressure, cell pre-heat period of 5 minutes, 10 minute static extraction for varying numbers of cycles, 150% dynamic volume (51 or 150 ml solvent percolated through cells, respectively, to wash out extracted lipid); 40°C, 50°C and 60°C cell temperature; chloroform:methanol (2:1,v/v) and hexane: methanol (2:1,v/v) solvents. Because ASE equipment has multiple solvent reservoirs, two immiscible solvents such as hexane and methanol can be delivered into cells and pressurized simultaneously. Repeat extraction cycles for the same sample were collected and pooled in the same sample bottle or collected separately, depending on the experiment. Extracts were transferred from ASE collection bottles to tared flat-bottom evaporator flasks, and solvent was evaporated under 110 mm Hg vacuum at 40 °C on a rotary evaporator (Model R-210, Buchi Corp., New Castle, DE). The extract residue was finish-dried in a vacuum oven for 30 minutes at room temperature to remove final traces of solvent. Flasks were weighed to determine presumptive lipid yield, flushed with argon, sealed, and stored frozen at –80 °C until oxidation analysis (peroxide value, conjugated dienes, carbonyls, epoxides) within 12 hours. Experiments were conducted in triplicate.

Spiking experiment: 0.2 mmol (29.8 μ l) and 0.02 mmol cumene hydroperoxide (1.03g/ml, 152.21 g/mol) were applied in five different systems designed to test effects of each extraction step on hydroperoxide stability, as detailed in Table 6.

5.3.1.2 Effect of sample particle size and solvents on lipid extraction by ASE

To determine optimum solvents or combinations and effects of sample particle size on lipid extraction efficacy, initial range-finding extraction experiments were conducted on baked pet food biscuits which had more open structure and less lipid binding in the sample matrix. The experimental flow diagram is presented in Figure 25.

Lipid recovery: Biscuits (fat content maximum ~13.2% according to manufacturer) were ground by high-speed food processor and separated by sieves into three particle sizes: >500 microns, 250-500 microns, and <250 microns. Ground samples were transferred to glass canning jars, flushed with argon, sealed, and stored at -80 °C during extraction set-up. Triplicate 1 g samples of each particle size were mixed thoroughly with 25 ml Hydromatrix™ and transferred to 34 ml stainless ASE cells as described above, then ASE-extracted by chloroform, chloroform: methanol (2:1,v/v), hexane, methanol, hexane: methanol (2:1,v/v, delivered simultaneously but separately), isooctane or petroleum ether. ASE conditions: 5 minute cell pre-heat to 40°C, 1500 psi pressure, 20 min static extraction time, 150% dynamic volume (51 ml), two extraction cycles. Lipid extracts sample were transferred to tared flat bottom flasks and solvents were removed by rotary vacuum evaporation at 40°C; then lipid residues were dried for 20 minutes at room temperature in vacuum oven to remove final solvent traces. Extract yields were determined as weight of the residue.

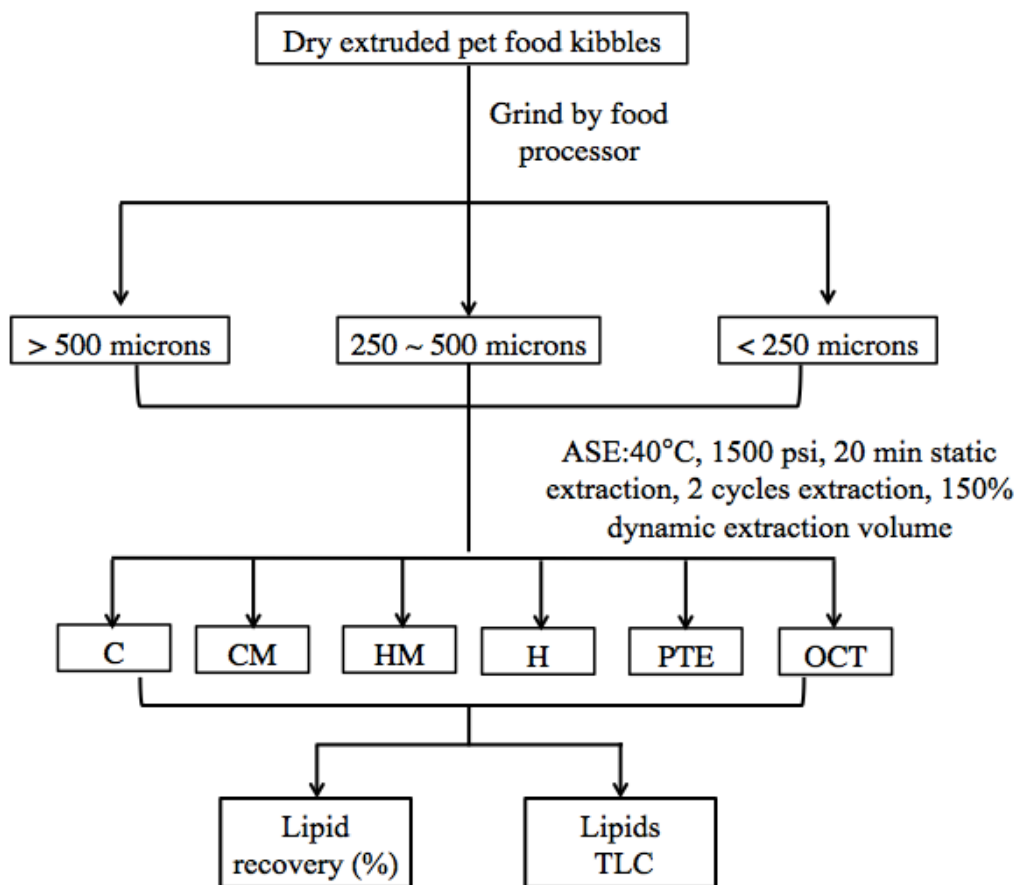


Figure 25. Experimental flow diagram for testing effects of particle size and solvent on lipid recovery from ASE extraction of baked pet food biscuits. C: chloroform, CM: chloroform: methanol (2:1,v/v), HM: hexane: methanol (2:1,v/v), H: hexane, PTE: petroleum ether and OCT: octane. TLC: thin-layer chromatography.

Thin-layer chromatography to identify lipid classes in extracts: Lipid classes in extracts were ascertained by HPTLC (high performance thin-layer chromatography) on glass TLC plates coated with Silica Gel G with Rhodamine 6G fluorescent indicator, 200- μm thickness (VWR, Radnor, PA). Plates were cleaned in methanol and activated by heating at 200 °C for 30 to 60 minutes to remove moisture. Two eluting systems were

used: Polar -- chloroform:methanol: acetone:acetic acid:water, 65:35:10:5:1; Non-polar -- hexane:diethyl ether:acetic acid 70:30:1, each with 100 ml total volume. To ensure complete equilibration of the solvent with the headspace in the tanks, eluting solvent components were mixed and poured into the bottom of standard glass TLC tanks (27W x 7D x 25H cm). Paper towels or large sections of filter paper were placed vertically along the sides of the tanks to facilitate migration of the solvent and provide a large surface area for evaporation. Tanks were closed and allowed to equilibrate for at least one hour before use. This procedure saturates the headspace with solvent vapor, decreases migration time, and improves homogeneous migration across the plate.

Lipid residues (~0.1 g) from ASE extractions were dissolved in 4 ml chloroform to eliminate non-lipid hydrophilic contaminants. 5 μ l of these diluted samples were spotted onto two plates under an argon stream using calibrated microcapillary tubes, and the spots were dried under argon. Smaller amounts of authentic standards were also spotted to establish R_f values and migration positions for specific products. Plates were placed into the polar and non-polar tanks and eluted until the solvent front reached ~ 2 cm from the top of the plate. Plates were removed from the TLC tanks and immediately placed under short and long wavelength UV light; patterns were recorded by camera and all visible spots were outlined with pencil. Plates were then dried and sprayed with 5% primuline in acetone and water (80:20 v/v) (TCI America, Portland, OR) to detect authentic lipids. After drying, primuline-stained lipids appear yellow under long wavelength UV light or purple under short wavelength UV.

5.3.1.3 Effects of static time and numbers of extraction cycles on ASE extraction

In ASE, static time and numbers of extraction cycles can be combined and adjusted to enhance the efficiency of lipid extraction. The effects of static time and extraction cycles need to be tested and tailored to each sample to obtain maximum yields. The experimental flow diagram for testing effects of static time and number of extraction cycles on lipid yields and oxidation during extraction of extruded pet food kibbles is presented in Figure 26. Samples used in testing were dry extruded pet food kibbles (Test kibble A from pet food company) with known fat content (16.82%) determined from acid hydrolysis. Samples were ground and the 250-500 μm diameter fraction was separated and stored under argon at $-80\text{ }^{\circ}\text{C}$ in sealed glass jars during extraction set-up. Samples were extracted by chloroform: methanol (2:1, v/v).

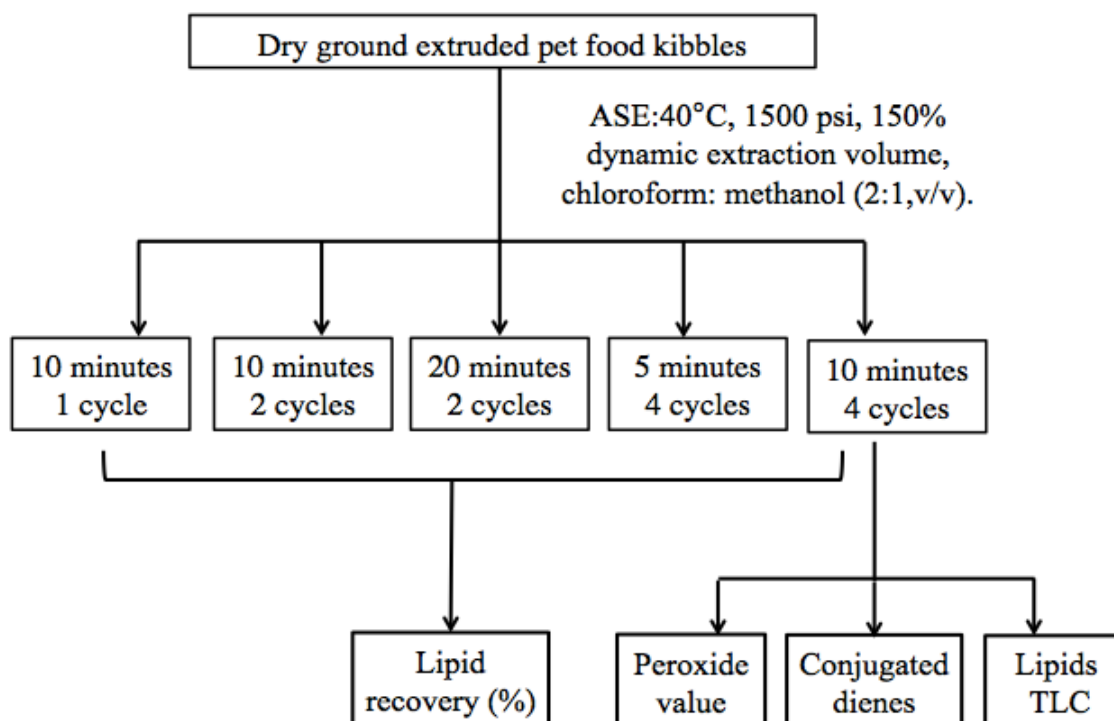


Figure 26. Experimental flow diagram for investigating effects of static time and extraction cycles on ASE.

1 g ground extruded samples were ASE-extracted at 40 °C, 1500 psi and 150% dynamic volume as described above. Different combinations of static time and cycle numbers were tested: 10 min/1 cycle, 10 min/2 cycles, 20 min/2 cycles, 10 min/4 cycles and 5 min/4 cycles. With multiple cycles, extracts from each cycle were collected and analyzed separately. Solvents were removed by rotary evaporator and vacuum oven as described above, then yields were determined by weight of residue and oxidation was evaluated by conjugated diene and hydroperoxide levels in extracts.

5.3.1.4 Effects of hydration to expand starch matrix in extruded kibbles

Lipids become entrapped in starch helices of extruded materials, and expansion of the starch helices by heat and moisture is often required for release. The traditional extraction method uses hot water-saturated butanol. To explore whether moisture added to the heated system during ASE extraction could similarly enhance lipid release, kibbles were extracted with and without hydration before ASE.

One gram ground dry samples were spread evenly in glass petri dishes, and allowed to hydrate for 12 hours at room temperature under vacuum in a desiccator containing 50 ml DI water. Weighed hydrated samples were then ASE-extracted at 40 °C, 1500 psi with 150% dynamic volume, 10 minutes static time, 2 cycles, and extract yields and oxidation levels were determined as described above. Results were compared to unhydrated samples.

5.3.1.5 Comparison of lipid yields oxidation in ASE vs. traditional manual extraction

Lipid extraction efficiency and oxidation extent of lipid extracts were compared for ASE and traditional manual extraction. The experimental flow diagram for the comparison is shown in Figure 27.

For manual extraction, one or ten grams ground samples were mixed with 50 ml chloroform: methanol (2:1,v/v) in 100 ml Erlenmeyer flasks covered with aluminum foil, flushed with argon, stoppered, and sealed with parafilm, then shaken at 150 rpm for 24 hour and 48 hours at room temperature. The solvent containing dissolved material was removed from sample solids in each flask by filtration, flushed with argon, clarified by 20 min centrifugation at 1000x g, then removed from sample residues by vacuum rotary evaporation and vacuum oven as described above. Extract yields and oxidation levels (conjugated dienes and hydroperoxides) were then determined.

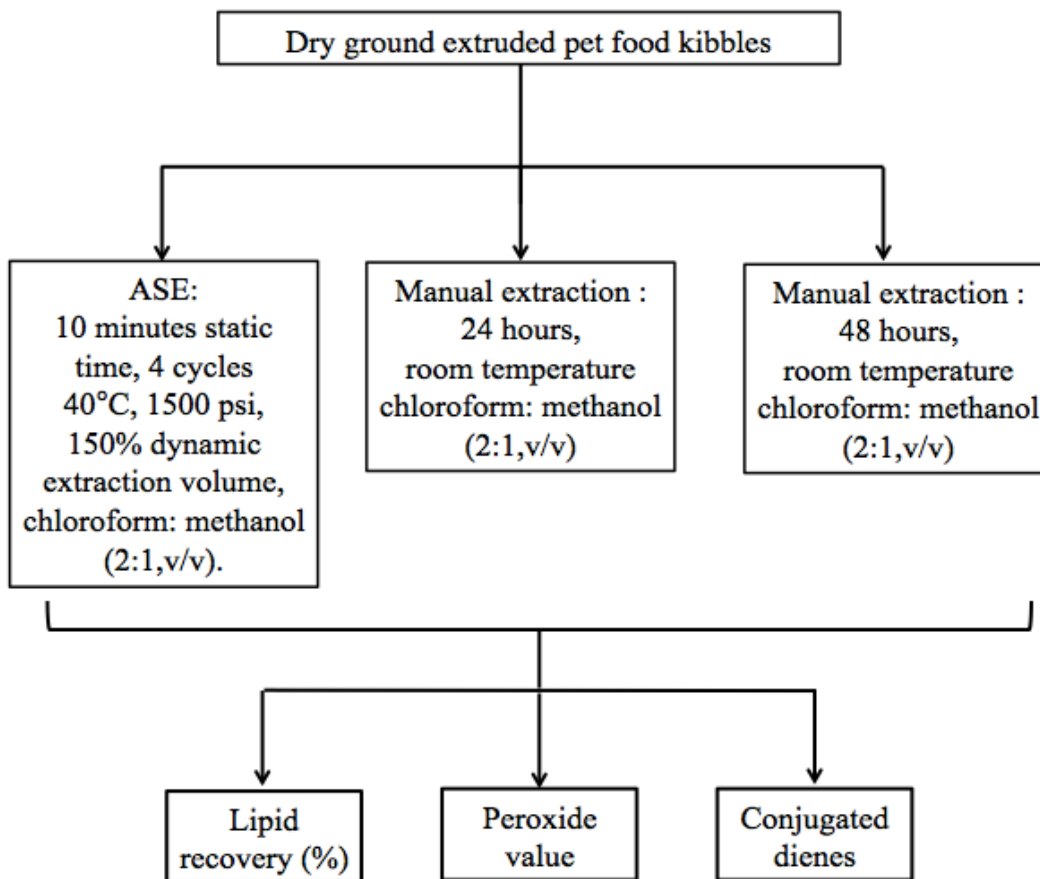


Figure 27. Experimental flow diagram for comparing extraction efficiency between ASE and manual extraction.

5.3.1.6 Analyses of lipid oxidation:

Conjugated dienes. Conjugated dienes were determined by a modification of the standard AOCS method, Ti 1a-64(09)[135]. 30 μ l of lipid extract was added to 10 ml argon-sparged iso-octane and briefly vortexed. An aliquot was transferred to a three ml quartz cuvette and the optical absorbance of the solution at 234 nm was measured in a Cary 50 spectrophotometer (Varian, Inc., Agilent Technologies, Santa Clara, CA). When absorbance was greater than 1, the sample was diluted and reanalyzed. The conjugated diene concentration (mmol/ mol lipid) was calculated as

$$\frac{\left(\frac{OD}{\epsilon} * Reaction\ vol(ml)\right)}{\left[\frac{Vol\ oil\ sample\ (ml) * oil\ density\ (g/ml)}{Oil\ or\ fatty\ acid\ mol\ wt}\right]} = \frac{mmol\ CD}{mol\ lipid}$$

using an average triacylglycerol weight of 885 in corn oil (12) and ϵ in iso-octane = 29500.

Hydroperoxides: Hydroperoxide concentrations in lipids and lipid extracts were determined using the PeroxySafe™ standard method (MP Biomedical, Solon, OH) modified to replace autopipettes with micropipettes for greater accuracy and reproducibility[136]. Oils, lipid extracts, or cumene hydroperoxide standards were diluted with Prep Reagent™ (10-100 times as necessary to keep the absorbance reading on scale) and vortexed several seconds to fully dissolve samples. The analysis was run by adding the following reagents in sequence to a clean 12x100 mm test tube: 1000 μ l reagent A, 25 μ l diluted sample, 100 μ l reagent B and 160 μ l reagent C. Samples were capped, vortexed for 30 seconds, and incubated for 15 minutes at 25 °C in a heating block. The test tubes were then inserted into the SafeTest MicroChem™ Analyzer equipped with a 570 nm filter and the optical absorbances of the reaction mixtures were read. Peroxide values of samples compared with cumene hydroperoxide standard curves and reported in meq/ kg fat by the analyzer were converted to mmol peroxide / mol triacylglycerol using an average corn oil triacylglycerol weight of 885[137].

Carbonyls: Carbonyl secondary oxidation products in corn oil during ASE validation and in pet food extracts were determined by reaction with dinitrophenylhydrazine using procedures described below.

5.3.2 Development and Testing of HPLC-DNPH Analyses of Carbonyls

The objective of this research is to develop a quantitative and qualitative method on HPLC or HPLC/MS/MS to analyze carbonyl-DNPH derivatives, including both monomers and core aldehydes. The separation of lipid carbonyl-DNPH derivatives on HPLC is controlled by several factors: eluent characteristics, elution solvents and gradient, and column. Lipid classes have a broad range of polarity. Hydrophobic triglycerides, large molecules with long acyl chains, are not easily eluted from reverse phase columns by the polar solvents such as acetonitrile or methanol used for carbonyl monomers. Therefore, factors such as solvent combination, elution gradient and column need to be manipulated to optimize resolution for both short chain carbonyl monomer and long chain core aldehydes. The experimental flow diagram for development and testing of the HPLC-DNPH analysis of lipid carbonyls is shown in Figure 28. The flow diagram for mass spectrometry analyses of DNPH-carbonyl structure identification is shown in Figure 29.

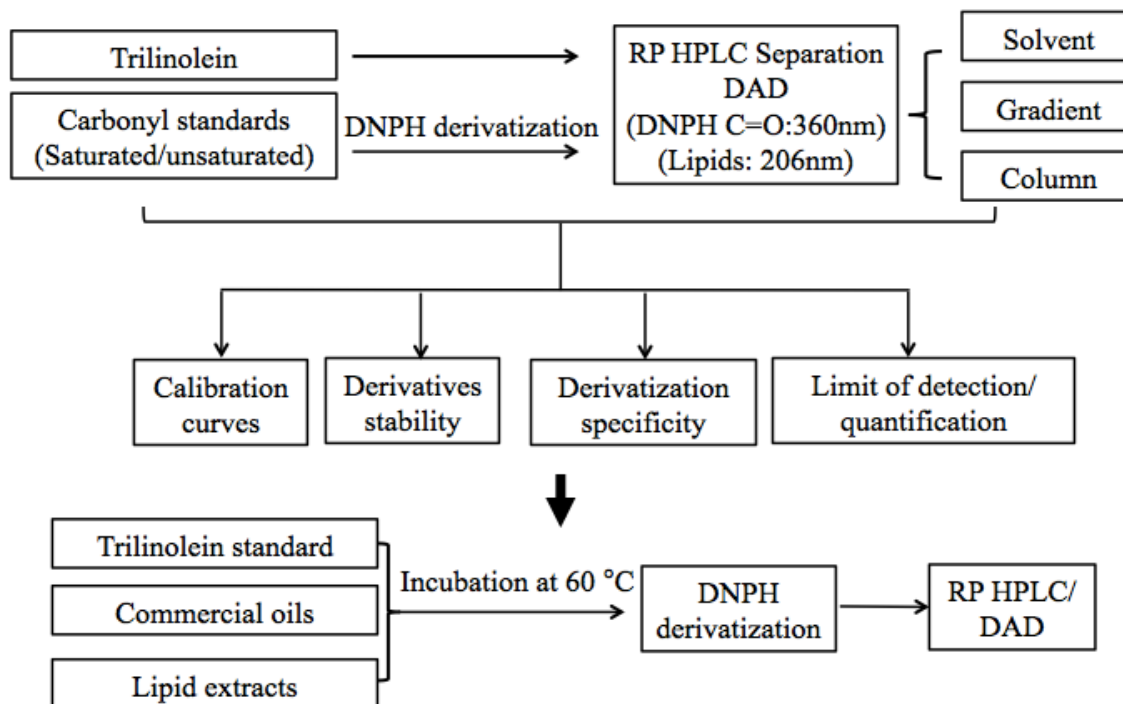


Figure 28. Experiment flow diagram for developing HPLC-DNPH analyses for identification of lipid carbonyls in total lipid classes.

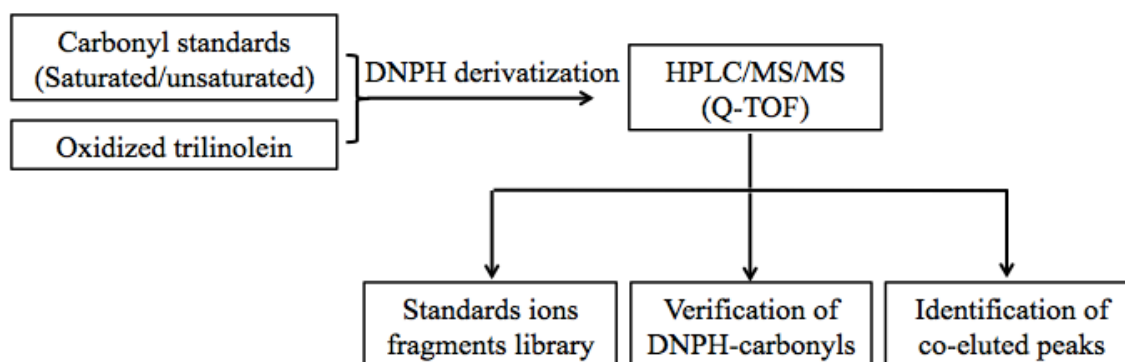


Figure 29. Experimental flow diagram for verification of DNPH-carbonyl structures by HPLC/MS/MS.

5.3.2.1 DNPH derivatization

DNPH reagent was prepared as follows: 40 mg recrystallized DNPH was dissolved in 990 μ l N, N-dimethyl formamide acidified by 10 μ l sulfuric acid (95%), vortexed and stored in refrigerator.

During experimentation, concern was raised about long-term degradation of HPLC columns by sulfuric acid. The possibility of using formic acid as an alternate acidifying reagent in DNPH preparation was thus tested. 30 μ l of formic acid was added to prepare DNPH reagent with the same acidity as 10 μ l sulfuric acid (1.84 g/ml), using the calculation below.

In 1 ml DNPH reagent:

Density of H_2SO_4 is 1.84 g/ml

Molecular weight of H_2SO_4 is 98 g/ mol.

Concentration of H^+ dissociated from $\text{H}_2\text{SO}_4 = \frac{0.01\text{ml} \times 1.84\text{g/ml}}{98\text{g/mol}} \times 2 = 3.75 \times 10^{-4} \text{ mol/ml}$

HCOOH : $K_a = 1.8 \times 10^{-4} = \frac{[\text{H}^+][\text{COO}^-]}{[\text{HCOOH}]}$

$[\text{HCOOH}] = (3.75 \times 10^{-4})^2 / (1.8 \times 10^{-4}) = 7.81 \times 10^{-4} \text{ mol/ml}$

Density of HCOOH is 1.22 g/ml

Molecular weight of HCOOH is 46 g/ mol.

So in 1 ml DNPH reagent solution:

Volume of $\text{HCOOH} = (7.81 \times 10^{-4} \text{ mol/ml} \times 46 \text{ g/ mol}) / 1.22 \text{ g/ml} = 0.02945 \text{ ml} = 29 \mu\text{l}$

5.3.2.2 Comparison of DNPH derivatizing ability of sulfuric acid and formic acid

Three μl of hexanal and *t,t*-2, 4-decadienal standards were added to 300 μl DNPH reagent (acidified by either sulfuric acid or formic acid), reacted for 20 minutes, then diluted with 697 μl acetonitrile. The hydrazones were separated by HPLC using the conditions described below. Peaks areas of DNPH derivatives compounds in HPLC chromatograms were compared to determine effects of the two acids on DNPH derivatization of carbonyls.

5.3.2.3 Carbonyl standard derivatization

150 μl DNPH reagent and 100 μl acetonitrile were mixed in a disposable tube, 2 mg of carbonyl standard were added, then each reaction tube was vortexed for 30 seconds and reacted at room temperature for 20 minutes. The derivative samples were diluted to 1 ml and stored at -20 °C until analysis, usually within 48 hours. For analysis of mixtures of saturated and unsaturated carbonyls, 100 μl of each DNPH derivatized standard were mixed and vortexed thoroughly. Samples were filtered by syringe filter (0.22 μm) before HPLC analyses.

5.3.2.4 Specificity of DNPH derivatization

To test the specificity of DNPH reactions with carbonyls, samples of other classes of lipid oxidation products were also reacted with DNPH and analyzed by HPLC. 3 μl cumene peroxide, 1-octanol and 1,2-epoxydecene standard were added to 100 μl DNPH reagent, reacted for 20 minutes, then diluted by 697 μl acetonitrile and analyzed by HPLC with diode array detection (DAD).

5.3.2.5 DNPH derivatives HPLC resolution studies

Chromatographic analyses of carbonyl-DNPH derivatives were performed on an Agilent (Santa Clara, CA) 1290 Infinity HPLC system equipped with 1200 Infinity Degasser, Quat Pump, HiP/ ALS Autosampler, column compartment and Diode Array Detector. Restek Ultra C18 (4.6mm* 150 mm) and C30 (4.6mm* 250 mm) columns were tested for sample elution and resolution studies. The injection volume was 5 µl and the solvent system was A: acetonitrile/ isopropanol (50:50); B: acetonitrile/ isopropanol/ DI water (25:25:50). The organic solvents were added to the aqueous phase to avoid bubbles from two phases mixing. Carbonyl-DNPHs were separated by C18 column with the following elution gradient: 16.7% - 100% A in 17 min (1.2 ml/ min), 100% A 1.2 ml/ min – 1.4 ml/ min in 2 min, remaining at 100% A 1.4 ml/ min for 16 min; returning to 16.7% A 1.2 ml/ min in 5 min and remaining for 5 min; the total run time was 45 min. The C30 column elution gradient was (only for standards separation): 16.7% - 100% A in 45 min (1.0 ml/ min), 100% A 1.0 ml/ min – 1.2 ml/ min in 2 min, remaining at 100% A 1.2 ml/ min for 3 min; returning to 16.7% A 1.0 ml/ min in 5 min and remaining for 5 min; the total time was 60 min. Absorbance of carbonyl-DNPHs was monitored at 360 nm; underivatized lipids were monitored at 206 nm.

5.3.2.6 Stability of DNPH derivatives

To test how long DNPH-carbonyl hydrazones were stable (i.e. whether they must be analyzed immediately or how long they can be held or stored until analysis), hexanal and *trans,trans*-2,4-decadienal DNPH derivatives were prepared at two concentration levels (2 µmol/ml and 2×10^{-2} µmol/ml) to test the stability of carbonyl-DNPH derivatives during holding and HPLC analyses. The injection program of the autosampler

was set up so that the needle pipetted samples from hexanal and *t,t*-2,4-decadienal sample vials separately and then injected them together onto the C18 column. Two groups of storage conditions were studied: 1) samples stored at room temperature in the autosampler without changing sample vial caps after injection; 2) samples stored frozen at -20 °C with sample vial caps changed immediately after injection. HPLC analyses were conducted at 0, 24, 48 and 72 hours after derivatization (designated in D0, D1, D2 and D3, respectively). Peak areas of samples were determined to follow changes in hydrazone concentration. All analyses were conducted in triplicate.

5.3.2.7 Calibration curves of carbonyl standards

Mixed carbonyl standards of butanal, 2-butanone, *t*-2-hexenal, hexanal, heptanal, 2-heptanone, *t*-2-octenal, *t*-3-octen-2-one, *t,t*-2,4-nonadienal, nonanal, *t,t*-2,4-decadienal, decanal and dihexyl ketone were derivatized by DNPH to investigate the linearity, relative response factors (RFFs), limit of detection (LOD) and limit of quantification (LOQ) of HPLC-UV method. A series of concentration points (0.5, 1, 2, 10, 20, 100, 200 µg/ml) were diluted to plot the calibration curves. All standards were prepared in three sets as well as all data points were analyzed in triplicate.

5.3.2.8 Method validation by analyses of carbonyls in oxidized pure lipids

Oxidized methyl linoleate: One ml methyl linoleate was incubated in each of two 10 ml vials (headspace was filled with air) at 40 °C and 60°C respectively for 3 days. Vials were vortexed thoroughly before sampling. 50 µl isopropanol:acetonitrile (1:1) and 200 µl DNPH reagent were added to a disposable tube in that order, then 50 µl oxidized sample was added to the tube and vortexed with the reagents thoroughly. After 20 min

reaction, 700 μ l mixed acetonitrile and isopropanol was added to dilute the sample to 1 ml.

Oxidized trilinolein: 0.5 gram pure trilinolein standard was dissolved in 2 ml isopropanol (100 μ l chloroform was added to facilitate solubilization of trilinolein) to prepare a 250 mg/ml solution in a 5 ml sealed vial (headspace was filled with air) and then incubated at 60 °C for 72 hours. The sample vial was vortexed thoroughly after incubation, 800 μ l oxidized standard solution was added to 120 μ l DNPH reagent and 80 μ l isopropanol:acetonitrile (1:1), then the mixture was vortexed thoroughly and reacted for 20 min.

5.3.2.9 HPLC-DNPH method application: determination of carbonyls in oxidized commercial oils and lipid extracts from baked crackers

Oxidized commercial oils: Five ml corn oil and olive oil were added to two separate sealed 20 ml tubes (headspace was filled with air). Samples were incubated at 60 °C for a week. Tubes were vortexed thoroughly before sampling. 100 μ l isopropanol:acetonitrile (1:1), 100 μ l chloroform, and 100 μ l DNPH reagent were added to a disposable tube in that order, then 50 μ l oxidized sample was added to the tube and the mixture was vortexed thoroughly. After 20 min reaction, 650 μ l mixed acetonitrile and isopropanol was added to dilute the sample to 1 ml.

Five ml stripped soybean oil and commercial vegetable oil were incubated at 60 °C for 6 days and 2 months, respectively. 25 μ l samples of oil were dissolved in 100 μ l isopropanol:acetonitrile (1:1) and 100 μ l chloroform, then 100 μ l DNPH reagent was added to the disposable tube and reacted for 20 min. 675 μ l mixed acetonitrile and isopropanol was then added to dilute the sample to 1 ml.

Lipid extracts from oxidized pet food kibbles: Lipids were extracted ASE-extracted (40 °C, 1500 psi, chloroform, 15 minutes, 2 cycles, 150% rinse volume) from one-year-old pet food kibbles (chicken and rice meal base) stored at room temperature in a sealed vial. Dried lipid extracts were redissolved in chloroform to remove non-lipid contaminants. 50 µl of this lipid extract were added to a mixture of 100 µl isopropanol: acetonitrile (1:1), 100 µl chloroform, and 100 µl DNPH reagent, then the mixture was vortexed well and reacted for 20 min at room temperature. The sample was diluted to 1 ml with 650 µl mixture of isopropanol: acetonitrile (1:1).

Lipid extracts from lyophilized emulsions (methyl linoleate:lactalbumin 1:4): Lyophilized emulsion samples that had been incubated for 11 days and 24 days at 40°C were ASE extracted (40 °C, 1500 psi, chloroform, 5 minutes, 1 cycle, 100% rinse volume) to recover the oxidized methyl linoleate using a Dionex 350 Accelerated Solvent Extractor (Dionex Corp, Sunnyvale, CA). 50 µl lipid extract were added to 50 µl isopropanol: acetonitrile (1:1) and 400 µl DNPH reagent, then the mixture was vortexed well and reacted for 20 min at room temperature. The sample was then diluted to 1 ml with 500 µl mixture of isopropanol:acetonitrile (1:1).

Lipid extracts from homemade baked crackers: Homemade baked crackers prepared with stripped soybean oil were incubated at 40 °C up to 32 days, and then ASE extracted (40 °C, 1500 psi, chloroform, 15 minutes, 1 cycle, 150% rinse volume) to recover the oxidized lipid. Lipid extracts were redissolved in chloroform to remove non-lipid contaminants. 50 µl lipid extract were added to mixture of 100 µl isopropanol: acetonitrile (1:1), 100 µl chloroform, 100 µl DNPH reagent, then vortexed well and

reacted for 20 min under room temperature. The sample was diluted to 1 ml with 650 μ l mixture of isopropanol: acetonitrile (1:1).

Preparation of lyophilized emulsions: 500 ppm tocopherol was added into 25 g of methyl linoleate. 125 g of high purity alpha-lactalbumin was dissolved in 1000 mL of deionized water and dispersed at 7000 rpm for 4-5 minutes then 10,000 rpm for another 10 seconds using an IKA T 25 Digital Ultra Turrax homogenizer (model# 3725001, Wilmington NC USA) with dispersing tool of IKA S 25 N-25 F stainless steel stator (model # 1713800) (Wilmington, NC). Once the protein was hydrated, 25 g methyl linoleate was gradually added into the protein suspension and homogenized, starting at 15,000 rpm for the first minute, then 20,000 rpm for the second minute, and the last 10 seconds at 25,000 rpm to ensure the oil was dispersed as tiny droplets. The prepared emulsion was immediately transferred into Erlenmeyer flasks, covered with a kimwipe, frozen rapidly in liquid nitrogen then transferred to a -80 °C freezer until lyophilization.

Preparation of homemade crackers: Crackers consisted of approximately 900 g all-purposed flour, 147.2 g stripped soybean oil, 470 g Milli-Q (18 M Ω resistivity) water, and 10 g salt, and 500 ppm tocopherol dispersed in stripped soybean oil. Dry ingredients (all-purpose flour, salt) were mixed with wet ingredients (water, oil with/without antioxidants) using a Hamilton Beach Commercial mixer (Model #: CPM700) with sigma blade. The dough was rolled out on a lightly floured surface to a thickness of less than 0.5 cm and cut into piece (2 * 3 cm). Crackers were baked in preheated convection oven at 350 °F for 15-20 minutes, then removed from the oven, cooled to room temperature, stored in double layer freezer bags, flushed with argon and stored in -80 °C freezer until oxidized at 40 °C.

5.3.2.10 HPLC-MS/MS Analyses

DNPH derivatives of carbonyl standard and oxidized trilinolein were separated by an Agilent liquid chromatography system containing G1367D Hip sampler, G1312B binary pump and G1316B column compartment, coupled with MS Q-TOF G6530A (Santa Clara, CA), Dual AJS/ ESI ion source. A 1.8u Exclips Plus C18 2.1* 100 mm column (Agilent, Santa Clara, CA) was employed to separate the carbonyls DNPH derivatives. The injection volume was 5 μ l and the mobile phase was A: acetonitrile/ isopropanol (50:50); B: acetonitrile/ isopropanol/ DI water (25:25:50) eluting at flow rate 0.3 ml/ min as gradient: 16.7% - 100% A in 17 min (0.3 ml/ min), 100% A 0.3 ml/ min – 0.5 ml/ min in 2 min, remaining at 100% A 0.5 ml/ min for 4 min; returning to 16.7% A 0.3 ml/ min and remaining for 7 min; the total time was 30 min. Carbonyl standards were analyzed under negative-ion mode; oxidized trilinolein were analyzed under positive-ion mode. The ESI temperature was at 200 °C, gas flow at 9 L/min and nebulizer at 35 psig; the voltage was 4500 v.

5.4 Data analysis

Experiment and analysis were both done in triplicate (n=9). Data were analyzed by ANOVA using DUNCAN multiple-range test. Unless otherwise indicated, difference are reported as statistically different with $p < 0.05$.

6. RESULT AND DISCUSSION

Results of the ASE extraction part of this dissertation research have already been published: Yao, L. and Schaich, K.M. 2014. Accelerated solvent extraction improves efficiency of lipid removal from dry pet food while limiting lipid oxidation. J. Amer. Oil Chemists' Soc. 92(1):141–151.

6.1 Accelerated solvent extraction of lipids

6.1.1 Effects of ASE conditions on lipids bonding and oxidation

Known amounts of corn oil were mixed with Hydromatrix and extracted by ASE to test whether ASE procedures can fully deliver extracted lipids, induce excessive oxidation, or break down existing oxidation products. At 40 °C, ASE was able to fully recover loaded corn oil using both chloroform: methanol and hexane: methanol solvent systems (Table 7), verifying that lipids were not retained in ASE vessels or on Hydromatrix. Similarly, as long as extraction temperatures were limited to 40 °C, conjugated dienes and hydroperoxides (mmol/mol triacylglycerol) increased only slightly during extraction and remained very close to unextracted control values in both solvents (Table 7). Surprisingly, oil oxidized more when extracted in hexane: methanol, even when oil was just dissolved in solvent and incubated for the same length of time as ASE extraction at all temperatures. Two explanations may be offered for this behavior (5): free radical stabilizers such as amylene (2-methyl-2-butene) or ethanol added to chloroform to prevent phosgene formation during long time storage also reduce lipid oxidation, and oxygen solubility is greater in hexane than in chloroform [41].

Table 7. Validation of ASE extraction: corn oil recovery and changes in oxidation products during ASE extraction with chloroform: methanol and hexane: methanol at 40°C, 50°C and 60°C.¹

	Chloroform: methanol				Hexane: methanol		
	Control	40 °C	50 °C	60 °C	40 °C	50 °C	60 °C
Recovery (%)	-	104.10±0.90 ^a	101.20±2.80 ^a	94.9±6.80 ^a	101.40±0.7 ^a	101.00±1.02 ^a	101.6±0.73 ^a
CD ²	1.85±0.00 ^h	2.22±0.00 ^g	2.75±0.01 ^e	2.93±0.02 ^d	2.58 ^c ±0.01 ^d	3.32±0.02 ^c	3.5±0.01 ^b
PV ²	0.79±0.01 ^m	0.77±0.10 ^m	1.11±0.2 ^k	0.85±0.06 ^m	1.21±0.01 ^k	1.56±0.02 ^j	1.3±0.17 ^k
C=O ²	1.95±0.02 ^t	2.49±0.08 ^s	2.35±0.03 ^s	2.38±0.12 ^s	2.98±0.09 ^r	3.07±0.06 ^r	3.42±0.10 ^p

¹ Values are means±SD, n=3. Letters that are different in each row indicate data sets that are significantly different, p<0.05.

² PV: peroxide value, CD: conjugated diene, C=O: carbonyls. All units are mmol/mol triacylglycerol.

Moreau et al. [139] compared ASE extraction of lipids from corn kernels over the temperature range 40°C to 100°C and concluded that higher extraction temperatures improved oil recovery by facilitating solvent mass transfer and interactions with samples. However, our results indicate that limiting extraction temperature to 40 °C is critical to avoid enhancing new lipid oxidation and hydroperoxide transformations (Table 9). At 50 and 60 °C, total extract yields decreased slightly (statistically not significant) with chloroform:methanol and remained constant with hexane:methanol, while conjugated dienes increased with temperature for both solvents, showing that even at moderately higher temperatures radical transfers and initiation of new oxidation chains was enhanced. Even though the differences were statistically significant, the oxidation levels remained remarkably low for extractions. Perhaps most importantly for extraction procedures, hydroperoxides increased at 50 °C then decreased again at 60 °C, presumably due to increased thermal decomposition into secondary products. This is a modification that should be avoided during extraction. Hydroperoxide decomposition did not generate characteristic secondary carbonyl products in chloroform:methanol but perhaps contributed to the slight increase in carbonyls with temperature in hexane:methanol. Clearly, there are alternate oxidation pathways active and generating products not measured in this study, but the key observation here is that levels of important oxidation products remain very low in ASE extraction. The temperature effects observed in ASE also raise questions about the appropriateness of any methods using heat to increase lipid yields from complex food and biological matrices when oxidation analysis is the analytical endpoint.

6.1.2 Hydroperoxide stability during ASE

Spike tests showed that preformed hydroperoxides were stable during exposure to ASE solvents and removal of same by evaporation (P+SD), time in solvent at 40 °C (P+SI), and contact with hydromatrix as a dispersant during ASE (P+H ASE). Extraction treatments had no effect on hydroperoxide levels at the lower spike level (0.02 mmol) (Figure 30, top). Although differences in recovered cumene hydroperoxides from solvent, incubation, and Hydromatrix were statistically significant at higher spike levels (0.2 mmol) (Figure 30, bottom), the actual quantitative differences were quite small. Recovery of hydroperoxides was slightly higher from Hydromatrix at both spike levels, probably because peroxide volatilization was impeded.

Known amounts of cumene hydroperoxide were also added to extruded kibbles to test recovery and stability from an active matrix. When spiked in the kibble, peroxides detected were higher than in the base kibble but substantially less than the cumene hydroperoxide added. The PeroxySafe assay cannot distinguish innate lipid hydroperoxides from added cumene hydroperoxide in the assay, but kibbles are a reactive system. It is highly likely that the added cumene hydroperoxides were decomposed by reaction with metals (components of the nutrient mix in the kibble), proteins, or other components of the kibbles. Thus, adding oxidation compounds to a reactive base system being extracted to test recovery efficiency of the extraction method is not appropriate.

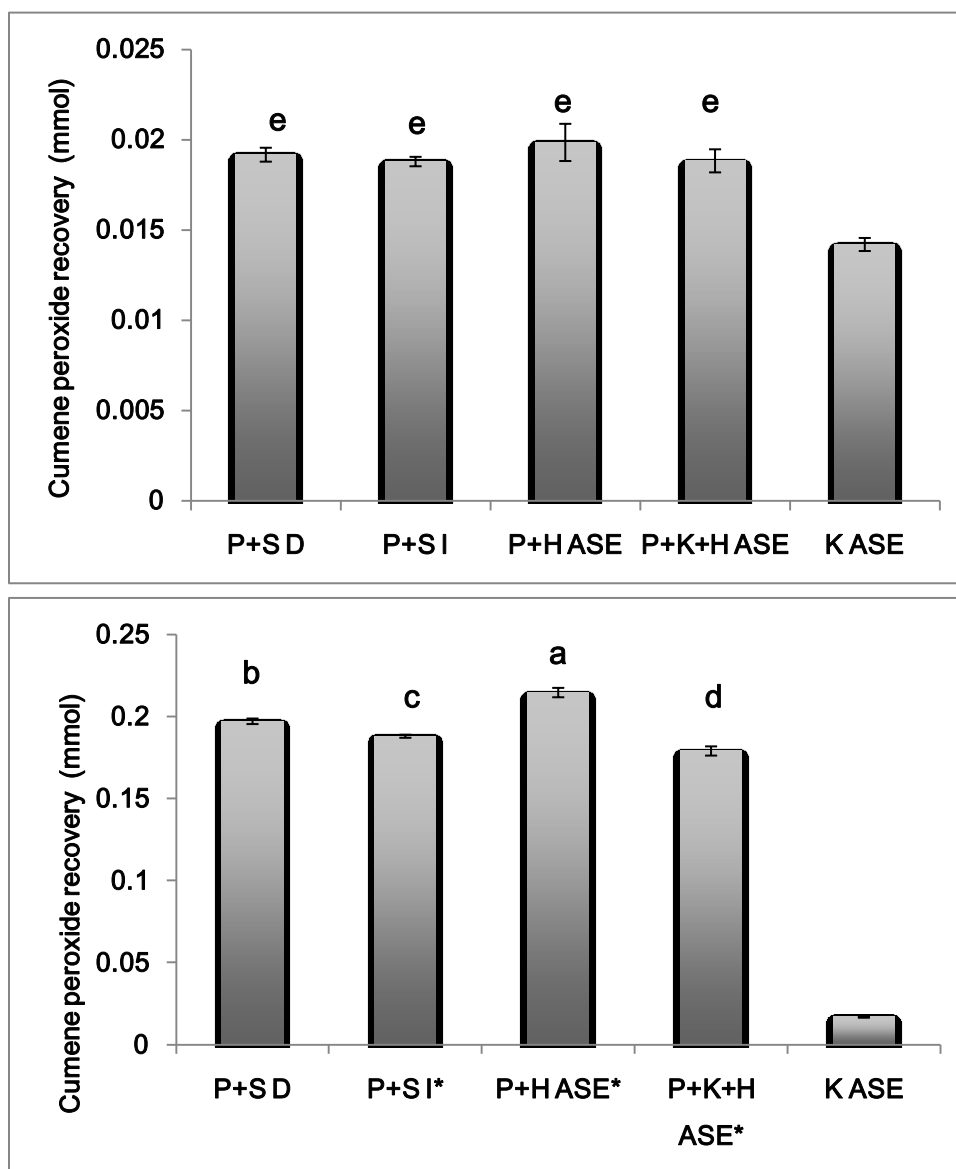


Figure 30. Recovery of 0.02 mmol (top) and 0.20 mmol (bottom) cumene hydroperoxide from spike test systems modeling the progressive exposure and handling in ASE extraction. Spike systems as described in text. Different letters indicate statistically significant differences.

6.1.3 Effect of particle size and solvent

Particle size: Commercial baked pet food biscuits (fat content claimed as 13.2% maximum by formulation according to manufacturer) were used in initial experiments because they had more open structure than extruded kibbles and hence solvent molecules could penetrate the matrix more easily. Extract yields increased significantly ($p < 0.001$) with each decrease in particle size (Figure 31), showing that grinding food samples to small particle sizes is critical for facilitating solvent penetration and access to all lipids in ASE. For dry pet foods, the optimum particle size was the 250-500 μm diameter range. Smaller particle sizes plugged the extraction cell frits, increased extraction of non-lipid components, and increased oxidation due to the extra handling required (data not shown). In larger particles, less material was extracted with the same volume of solvent. 250 μm particles can be easily generated in short times using a food processor and appropriate sieves. Stress in sensitive materials can be reduced further by use of a cryogenic grinder cooled by liquid nitrogen.

Solvents: Solvents can be tailored to the specific type of lipid to be extracted, but for total lipid extraction both polar and nonpolar solvents are generally required. The one exception to this is chloroform, which solubilizes all lipid classes because it is both hydrophobic and polar. In ASE extractions, chloroform alone gave the highest extract yields, nearly 17% of the sample weight, substantially above the manufacturer's claimed maximum lipid levels of 13.2% (Figure 31). Further investigation revealed that excess yield resulted at least in part from extraction of non-lipid components including food colorants (Figure 32). Extracts from chloroform/methanol and hexane/methanol were very clear (free from particulate and colored material in the dried residues) and for the

250-500 μm particles, the extract weights were close to the 13.2% fat content claimed by the manufacturer (14.10% for chloroform:methanol and 13.97% for hexane:methanol). Yields for these two solvents were statistically the same, demonstrating that ASE offers a means of replacing toxic chlorinated hydrocarbons while retaining yields in lipid extractions.

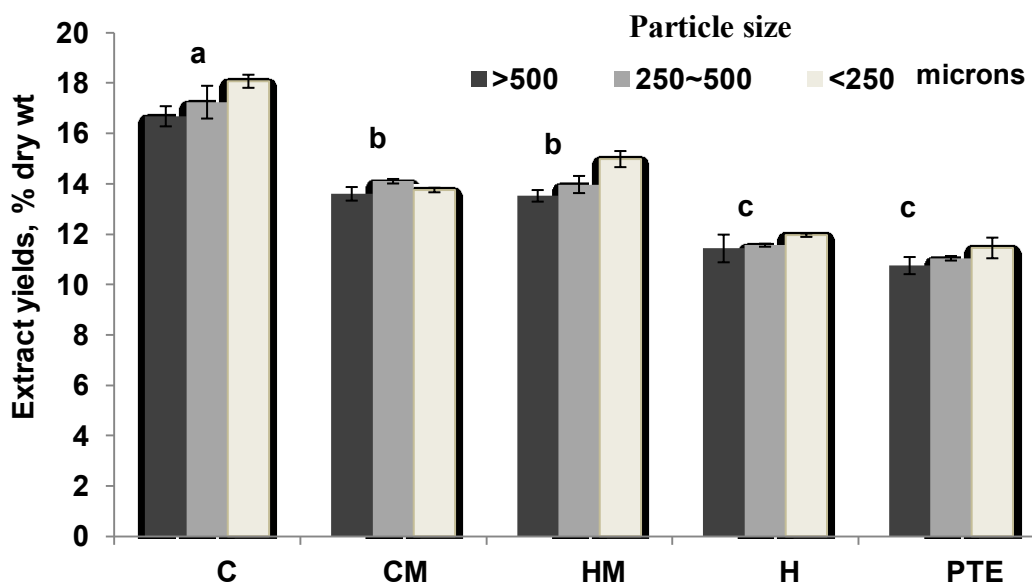


Figure 31. Effect of particle size (<500 microns, 250-500 microns, <250 microns) and solvent on ASE extract yields from baked pet food biscuits. ASE extraction conditions: 40°C, 1500 psi, 20 min static extraction, 2 cycles extraction, 50% dynamic extraction volume. Solvents: chloroform (C), chloroform: methanol (CM) (2:1,v/v), hexane: methanol (HM) (2:1,v/v), hexane (H), and petroleum ether (PTE). Different letters indicate significant differences between solvents. Yields increased significantly with particle size, all solvents.

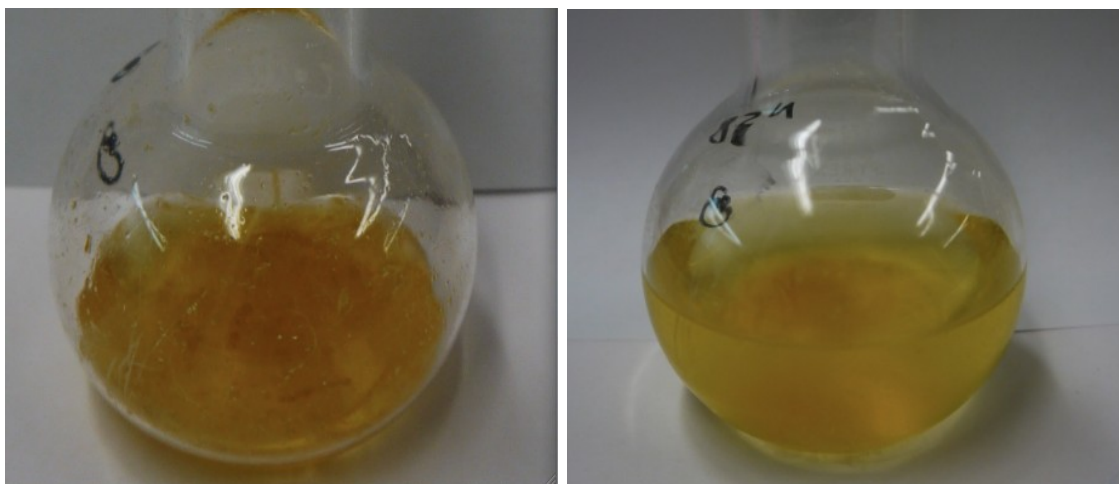


Figure 32. Photos of lipid extracts from different solvents: left: extracts dissolved in chloroform showing extraction of color particles from the pet food; right: extracts dissolved in chloroform : methanol(2:1, v/v)

Extract yields for very hydrophobic solvents hexane and petroleum ether (polarity index 0.1 for each) [140] were significantly lower than the solvents already discussed because polar lipids such as phospholipids were not extracted (see TLC discussion below). Hence, full extraction of lipids requires a reasonably polar hydrophobic solvent or inclusion of a polar solvent component (e.g. polarity index for chloroform= 4.1, methanol = 5.1) [140].

Isopropanol and hexane are miscible and their mixture has been proposed for replacing chlorinated solvents in lipid extractions [141]. Iso-octane is also a common lipid solvent [33]. In tests of iso-octane and isopropanol for ASE, the extracts were visibly clean but the temperatures required for evaporation (99 °C and 86 °C for iso-octane and isopropanol, respectively) led to substantial degradation of existing lipid oxidation products and initiation of new oxidation. In addition, solvent evaporation times

were very long. Furthermore, for lipid oxidation analyses, isopropanol is not the first solvent of choice since it so readily forms peroxides that catalyze lipid oxidation and interfere with many oxidation analyses. Hence, these two solvents were not considered useful for ASE extractions when lipid oxidation was a critical analytical endpoint.

6.1.4 Effect of ASE static time and numbers of extraction cycles

Since diffusion and mass transfer processes are major factors governing lipid extraction from dry materials, the length of time to which samples were exposed to solvent under pressure (static extraction time) and the number of extraction cycles including wash out should have critical effects on the total amount of lipid extracted. Multiple extraction cycles are especially important for lipid removal from difficult matrices. The optimum combination of static and dynamic times and number of extraction cycles must be tailored to the matrix complexity of individual materials.

Since two cycles of chloroform:methanol were sufficient to quantitatively extract lipids from the open-structured baked pet food biscuits, effects of static times and cycles were investigated in extruded kibbles that have a much denser structure and hence potentially greater need for multiple cycles. We also used materials for which lipid contents had been determined by acid hydrolysis in order to more accurately assess extraction efficiency. In Test Kibble A, a single 10-minute cycle with chloroform:methanol removed 77% of the lipid (assuming negligible non-lipid material in the extract) (Figure 33, left). Increasing the number of cycles with shorter static times enhanced extraction efficiency most. For example, for 20-minute extractions, 4 cycles of 5 min > 2 cycles of 10 min (13.52 vs 13.32, respectively); for 40 minute extractions, 4

cycles of 10 min > 2 cycles of 20 min (13.57 vs 13.44) (Figure 30). Increasing the static extraction time too long can be counterproductive, perhaps due to increased binding of lipids to starch and protein, or by compression of the matrix under extended pressure. Twenty minutes appeared to be the practical limit with the pet foods we tested.

With each additional cycle, less lipids were extracted but lipid oxidation increased (Figure 33, right), possibly due to: 1) minute amounts of lipid in the last two extract cycles were spread in thinner films in the evaporator flasks and hence had greater chance for oxygen exposure; 2) the last two cycles extracts were held longer at the elevated temperature of extraction and also (at refrigerated temperature under argon) before solvent evaporation; 3) the lipids buried more deeply in the kibble were oxidized more than at the surface. We feel that the thin films and longer heating times during extraction are more likely than greater oxidation in the protected interior of a kibble.

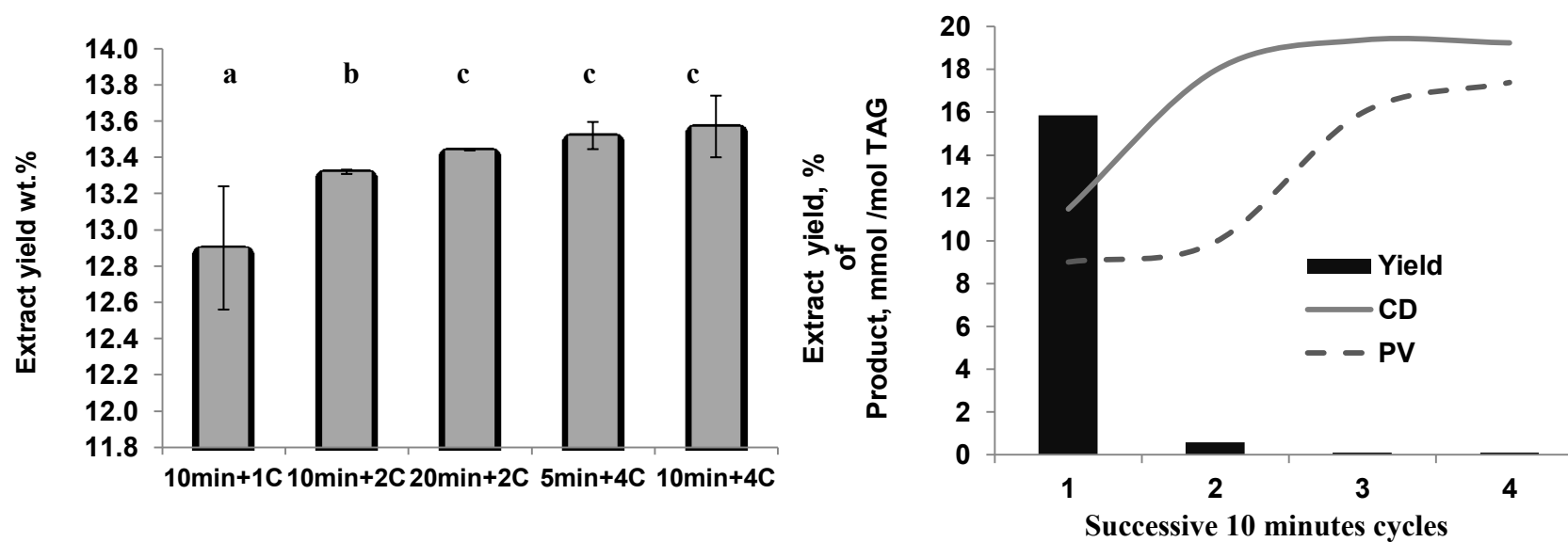


Figure 33. Effects of static extraction time and numbers of extraction cycles extraction yields from extruded Test Kibble A with 16.82% lipid. Left: Cumulative yields; Right: yields in successive cycles. Extraction solvent: chloroform:methanol 2:1. Extraction pressure 1500 psi, temperature 40 °C. Conditions with different letters are significantly different.

6.1.5 Effect of sample hydration

In flours and grains, lipids entrapped inside starch helices are difficult to extract unless the starch molecules are expanded with a combination of heat and moisture. Dry kibbles lack the requisite moisture so we investigated whether hydration of kibbles before ASE could improve lipid yields. Overnight hydration under vacuum increased moisture content in the kibbles by 10% but lipid yields by only 2% (Table 8). Furthermore, even though samples were held in the dark and under vacuum during hydration, the presence of additional moisture shifted lipid oxidation patterns, decreasing conjugated dienes while increasing hydroperoxides. These effects may result from expansion of the starch matrices, allowing increased oxygen diffusion; from enhancement of molecular conversions to secondary products; or hydrogen bonding of water to hydroperoxides, thereby stabilizing them. Thus, given the small increase in yield, the extra time and handling required, and the potential modifications of oxidation processes, hydration of kibbles before extraction does not seem to be warranted for routine analyses.

Table 8. Effect of sample hydration on presumptive lipid yields and lipid oxidation products in ASE-extracted Test Kibble A.¹

	Yield, wt.%	PV	CD
ASE	13.20 ^b ±0.12	11.28 ^d ±0.16	16.05 ^e ±0.01
Hydrated ASE	15.44 ^a ±0.43	23.52 ^c ±0.47	8.49 ^f ±0.01

¹ Values are means±SD, n=3. Letters that are different in each column indicate data sets that are significantly different, p<0.05

² PV = hydroperoxides, CD = conjugated dienes, units in mmol/mol triacylglycerol

6.1.6 Effect of composition and form

Extraction yields from extruded kibbles (~80%) were notably lower than for the baked biscuits (~100%) and longer extraction times were required. To investigate further the relationships between product structure and lipid extractability by ASE, we extracted three additional kibbles with different compositions, plus one lite (low fat) kibble.

Efficiency of lipid extraction varied from 75 to 90% with these kibbles, even though all were fresh and all had been ground to 250 microns (Figure 34). Hence, the molecular matrix is as important as particle size in extractions of lipids. In addition to differences in endogenous lipid content of the protein meals, kibbles also varied in size and shape as well as proportions of lipid mixed with the solids before extrusion versus sprayed on the surface after extrusion. Although these factors should be normalized by grinding to constant particle size, they nevertheless all appear to contribute to variability in kibble extraction efficiency. Lipids incorporated into the dough are more likely to become bound to starches and proteins during extrusion, while lipids sprayed on the surface should be readily accessible to solvents and hence easily removed. The Lite Adult kibbles (9.63% lipid) showed the lowest lipid extractability (~75%); this product did not have an obvious oily surface, so lipids were probably strongly associated with macromolecules in the dough. The other three kibbles appeared to have at least some fat/oil on the surface, hence their higher extraction yields, but the extraction efficiency did not parallel the total lipid contents of the kibbles. The lamb/rice kibble had 18.09% lipid but only 84% was extracted in two cycles, while lower fat chicken/rice kibbles (16.93 % lipid) had comparable yields (84%) and chicken kibbles (16.82% lipid) released more lipid (89.55% total yield). Starches are known to be strong lipid binders, so at least

some of the lower lipid yields may be attributed to binding by the rice components.

More detailed information about the proportions of lipids incorporated in the dough vs sprayed on the surface for each kibble, the lipid contents of the protein meals, and other factors will be necessary before extraction differences can be fully explained. Nevertheless, these results identify a number of endogenous product factors that affect extraction and demonstrate that “one size fits all” extraction protocols may not be appropriate for all kibbles.

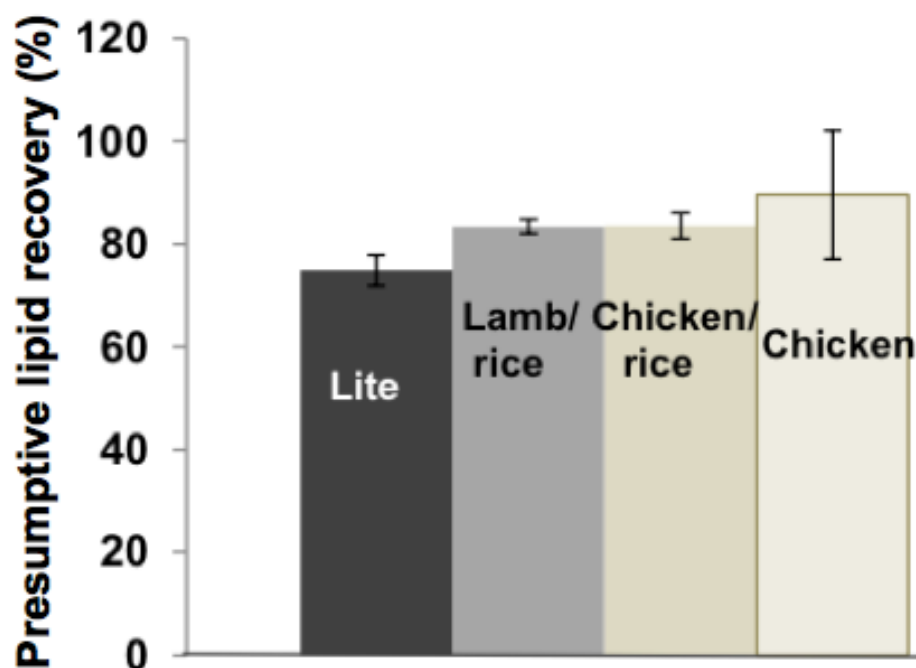


Figure 34. ASE extraction efficiency from four standardized kibbles. ASE conditions: 10 min/2 cycles, chloroform: methanol 2:1, 1500 psi pressure, 40 °C temperature, particle size 250 μ m.

6.1.7 Lipid classes extracted by ASE

To track which lipid classes were being extracted under the various ASE conditions tested, both non-polar and polar lipid components in extracts were analyzed by

high performance thin layer chromatography. All major lipid classes were extracted in ASE (Figure 35 A,B, Figure 36), and there was no obvious difference in lipids extracted by various solvents or under different extraction condition, except that very non-polar solvents such as hexane and petroleum ether did not dissolve polar lipids (Figure 35B, Figure 36, 37). Inclusion of polar solvent components is necessary to remove phospholipids and other polar lipids. Absence of polar solvents contributes to the lower extract yields reported for hexane and petroleum ether in Figure 31.

Of particular interest was the observation that the same pattern of lipids were removed with each successive extraction cycle (Figure 37), suggesting that lipids were distributed with reasonable uniformity throughout the kibble and that the extractions dissolved and washed away lipids in layers, like peeling back layers of an onion. A set penetration depth for each extraction cycle, determined by the matrix structure and solvent, would be consistent with increased extraction efficiency with smaller particle sizes.

Since additional extraction cycles increased lipid oxidation without isolating new or different lipid components, one to two ASE extraction cycles with static times less than twenty minutes appear to provide adequate lipid yields that accurately reflect the lipid composition while inducing minimal modification when lipid oxidation products are the analytical endpoint.

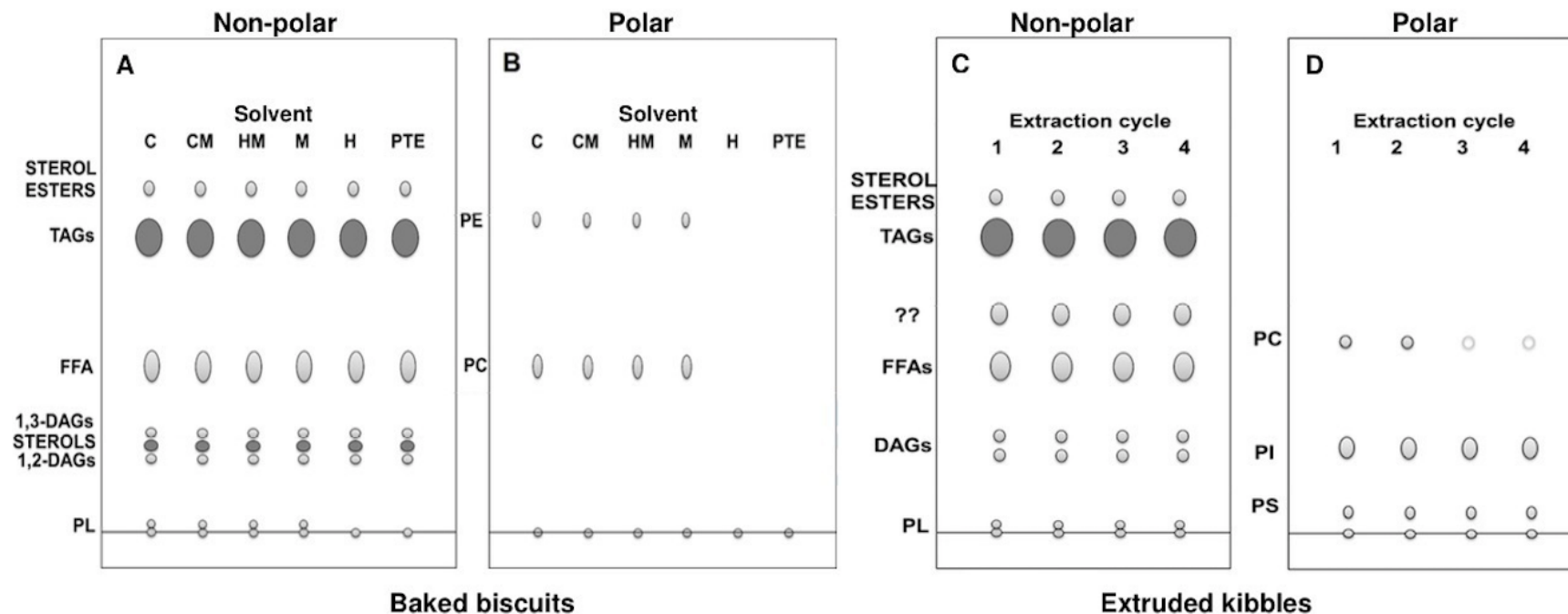


Figure 35. Tracings of TLC spot patterns for non-polar and polar lipids extracted from baked pet biscuits by different solvents (A, B) and from extruded kibbles with sequential extractions (C, D). PL: phospholipids; DAG: 1,2-diacylglycerols, 1,3-diacylglycerols: FFAs: free fatty acids, TAGs: triacylglycerols; PC: phosphatidylcholine; PE: phosphatidylethanolamine. C: chloroform, CM: chloroform: methanol 2:1, HM: hexane: methanol 2:1, H: hexane, PTE: petroleum ether. Equal lipid weights were spotted on each lane. (The original TLC photos for the samples are provided in Figure 36 and 37).

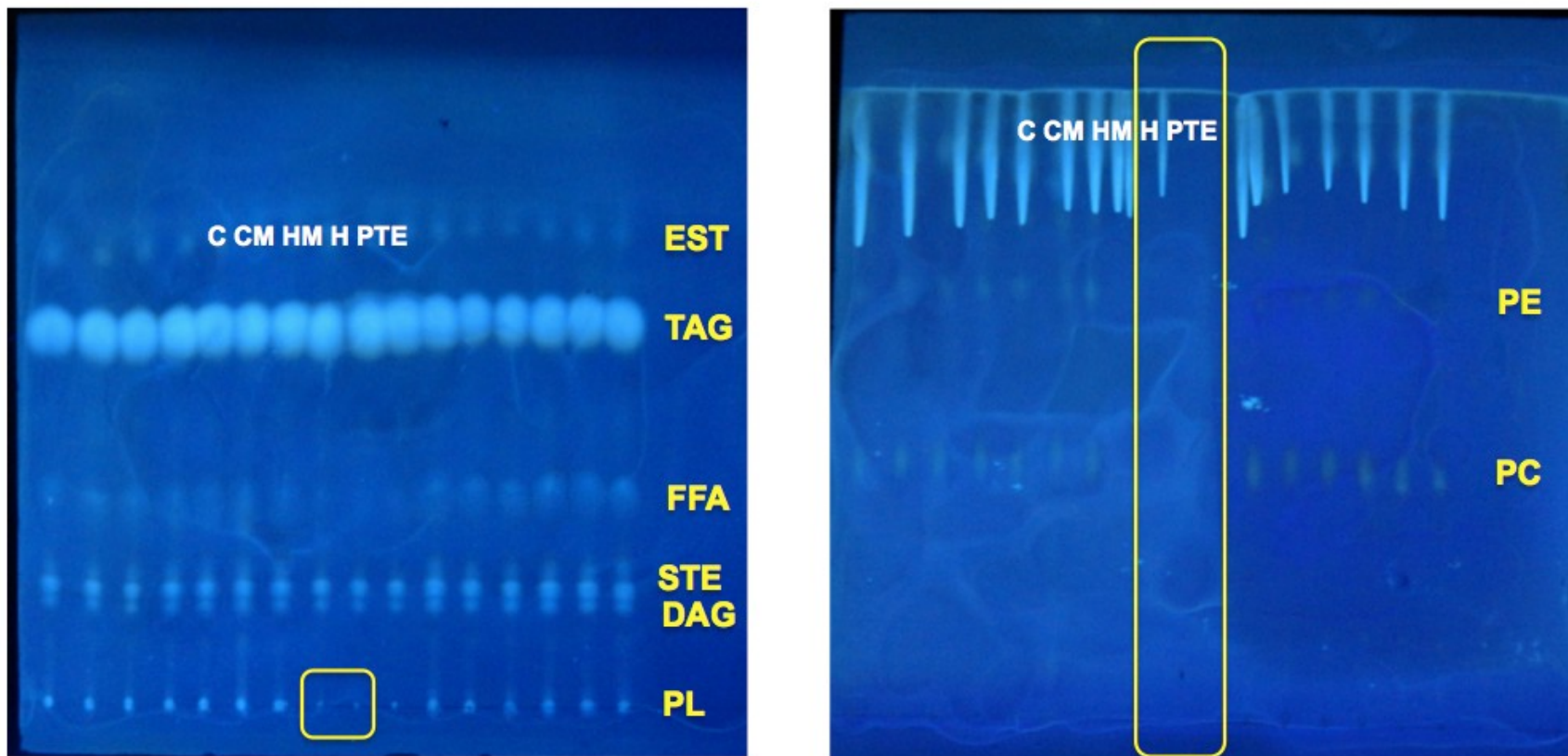


Figure 36. Original photos of TLC plates for non-polar (left) and polar (right) lipids extracted from baked pet biscuits by different solvents and PL: phospholipids; DAG: 1,2-diacylglycerols, 1,3-diacylglycerols; FFAs: free fatty acids, TAGs: triacylglycerols; PC: phosphatidylcholine; PE: phosphatidylethanolamine. C: chloroform, CM: chloroform: methanol 2:1, HM: hexane: methanol 2:1, H: hexane, PTE: petroleum ether. Equal lipid weights were spotted on each lane.

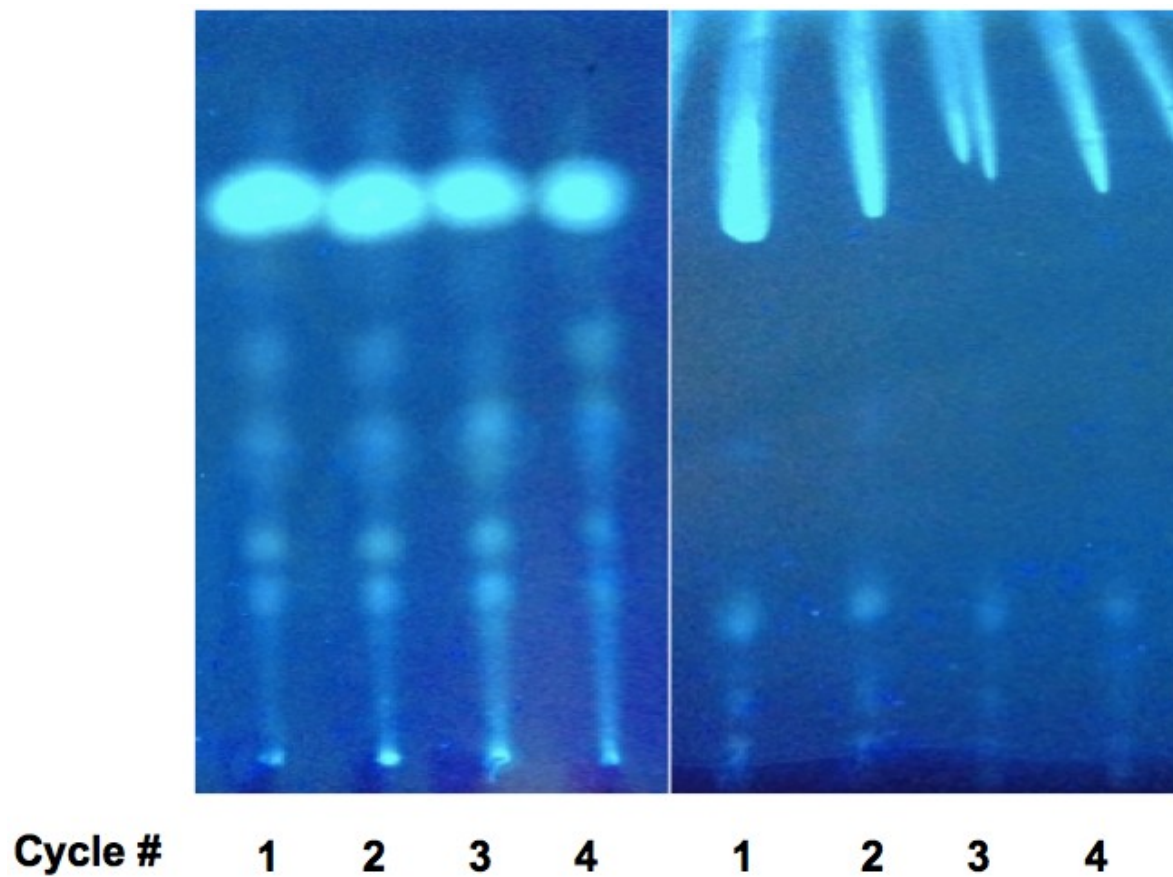


Figure 37. Original photos of TLC plates for non-polar (left) and polar (right) lipids extracted from extruded kibbles with sequential extractions. Equal lipid weights were spotted on each lane.

6.1.8 ASE comparison with traditional manual extraction

Traditional manual extractions which shake samples in solvent for hours to days to solubilize lipids are simple to run and provide a standard for comparison, but they are time-consuming, usually inefficient with complex matrices such as extruded products, and they pose great challenges in preventing new or altered lipid oxidation.

In contrast to previous results with extruded materials in this laboratory, manual extraction for 24 to 48 hours removed close to total lipid from the test kibble. This increased extractability may occur because a large part of the lipid in kibbles is sprayed on the surface, with lesser amounts mixed with the solid ingredient matrix rather than coming from endogenous components of flour and starch in other foods studied. Nevertheless, long contact times (days) are still needed in manual extractions of kibbles (Table 9), suggesting that lipid is trapped in matrices and time is needed for solubilization and mass transfer of lipid out of interior matrix spaces.

In manual extraction, mass transfer is a passive process and occurs slowly, requiring days for total release in the kibbles tested. The high pressure provided in ASE and application of multiple cycles enhances solvent migration into and out of kibble particles. Four 10-minute ASE cycles removed as much lipid as 24 hours of manual extraction, while at the same time maintaining lower oxidation levels of both conjugated dienes and hydroperoxides (Table 9) and other secondary products (data not shown). ASE extracts were also cleaner, with little or no particulate material in dried extract residues, while presence of non-lipid contaminants increased with incubation time in manual extractions. The much reduced extraction time, oxidation, and levels of

contaminants all demonstrate the superiority of ASE for extracting lipids from dry kibbles.

Table 9. Comparison of total extract yields and lipid oxidation in ASE (10 minutes static time, 4 cycles) and traditional manual (room temperature for 24h and 48h) extraction of Test Kibble A (lipid content by acid hydrolysis=16.82%).

	Yields wt.%			
	1g	10g	Peroxides	Conjugated dienes
ASE (10min, 4 C)	16.98 ^b ±0.16	17.20 ^c ±0.10	2.56 ^f ±0.08	10.01 ^j ±0.03
Manual 24h	17.33 ^b ±0.25	13.71 ^e ±0.66	4.08 ^g ±0.22	9.41 ^k ±0.04
Manual 48h	19.34 ^a ±0.45	15.50 ^d ±0.30	4.47 ^h ±0.18	10.42 ⁱ ±0.04

[†] mmol / mol triacylglycerol (p<0.05).

6.2 DNPH-HPLC assay for analysis of carbonyl from lipid

6.2.1 Development of solvent gradient for separation and detection of DNPH-carbonyls

HPLC-DNPH assays for carbonyls have been reported previously, usually for analyses of small monomer carbonyls. However, a) the handling that would be required to separate monomer products from the food matrix or lipid extract would alter the oxidation state of the lipids and potentially even decompose carbonyl precursors, leading to overestimation of oxidation, and b) core aldehydes and ketones remaining on acyl-glycerol or phosphoglycerol backbones are need to be analyzed along with the monomers.

The challenge in this project, therefore, is to minimize handling and lipid exposure while providing separation of lipid classes with a wide range of polarities and molecular size.

In general, the order of carbonyl-DNPHs elution is governed by the length of the carbon chain of the carbonyl and the number of double bonds in it [119]. The retention time increases with increasing chain length and decreases with number of double bonds. After considerable testing of solvents and instrumental conditions, a multistage gradient was identified that accommodated all lipid classes with their very different sizes and polarities. The first stage, designed to separate monomers fully, goes from 16.7% - 100% Solvent A (50:50 ACN:IPA) over 17 minutes (Figure 38). It was developed and tested using saturated and unsaturated carbonyl standards of varying chain lengths. As shown in Figure 41A, hydrazones of saturated carbonyls (from C4 to C11) were separated completely. Hydrazones of unsaturated carbonyl standards mostly separated, except t-2-hexenal and cis-4-heptanal hydrazones co-eluted in peak 2 (Figure 39 B).

In mixtures of saturated and unsaturated carbonyl standards, several critical pairs co-eluted with the same retention times (Figure 40A): t-2-hexenal and cis-4-heptanal (7.08 min); hexanal and t,t-2,4-heptadienal (7.49 min); octanal and t t-2,4-nonadienal (10.18 min), and nonanal and t,t-2,4-decadienal (11.45 min). Several modifications were tested to improve the resolution by manipulating the interaction between analytes and column coating material as well as mobile phase while retaining the same C18 column: extension of the gradient elution time, addition of formic acid to the mobile phase to regulate the polarity, decreasing the column temperature. None of these were effective in improving resolution.

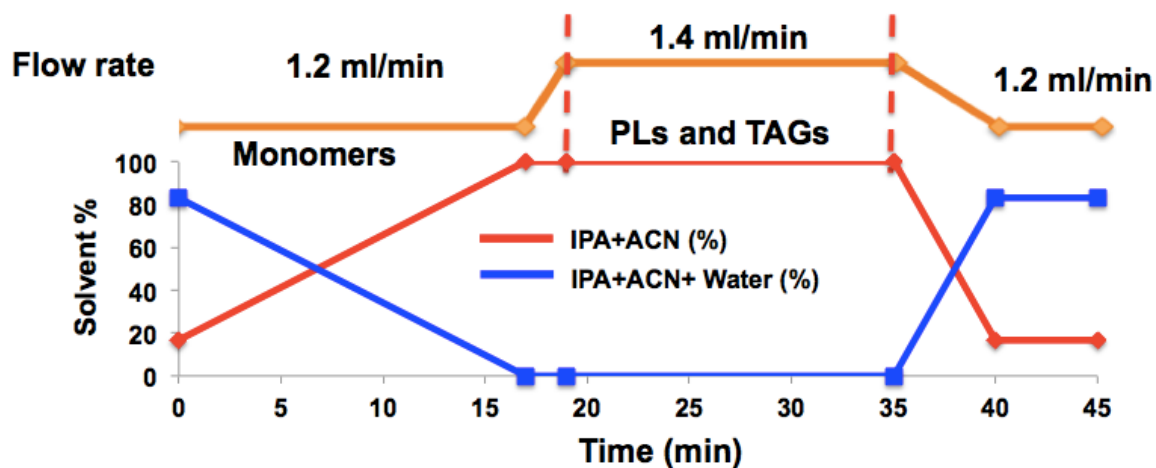


Figure 38. Two stage elution gradient for separation of carbonyls monomers and core aldehydes.

It was clear that increased resolving power was needed, so an Aqueous C30 column that had previously been useful for resolving isomers of tocopherol was tested. The C30 column improved resolution somewhat (Figure 40B), but now two new unsaturated critical pairs of *t,t*-2, 4-heptadienal and *t*-2-heptenal plus *t*-2-nonenal and *t,t*-2,4-nonadienal co-eluted and the total run time was doubled. Interestingly, saturated carbonyls eluted earlier than unsaturated carbonyls with the same carbon number, which was opposite to behavior observed on the C18 column. With its increased hydrophobicity and van der Waals interactions, the C30 column could discriminate subtle differences in structures of long chain compounds [142], but polar (more unsaturated) short chain carbonyls (C7, C9) had no column interactions so co-eluted. Identities of all co-eluting peaks were verified by mass spectra and comparisons of retention times to those of individual standards. Co-eluting peaks in HPLC/DAD analyses could be identified by LC/MS because different masses form different ions. DNPH hydrazones of 18 carbonyl

standards could be differentiated completely in total ion chromatograms (Figure 41).

LC/MS thus can be a useful adjunct method for to identifying carbonyl compounds.

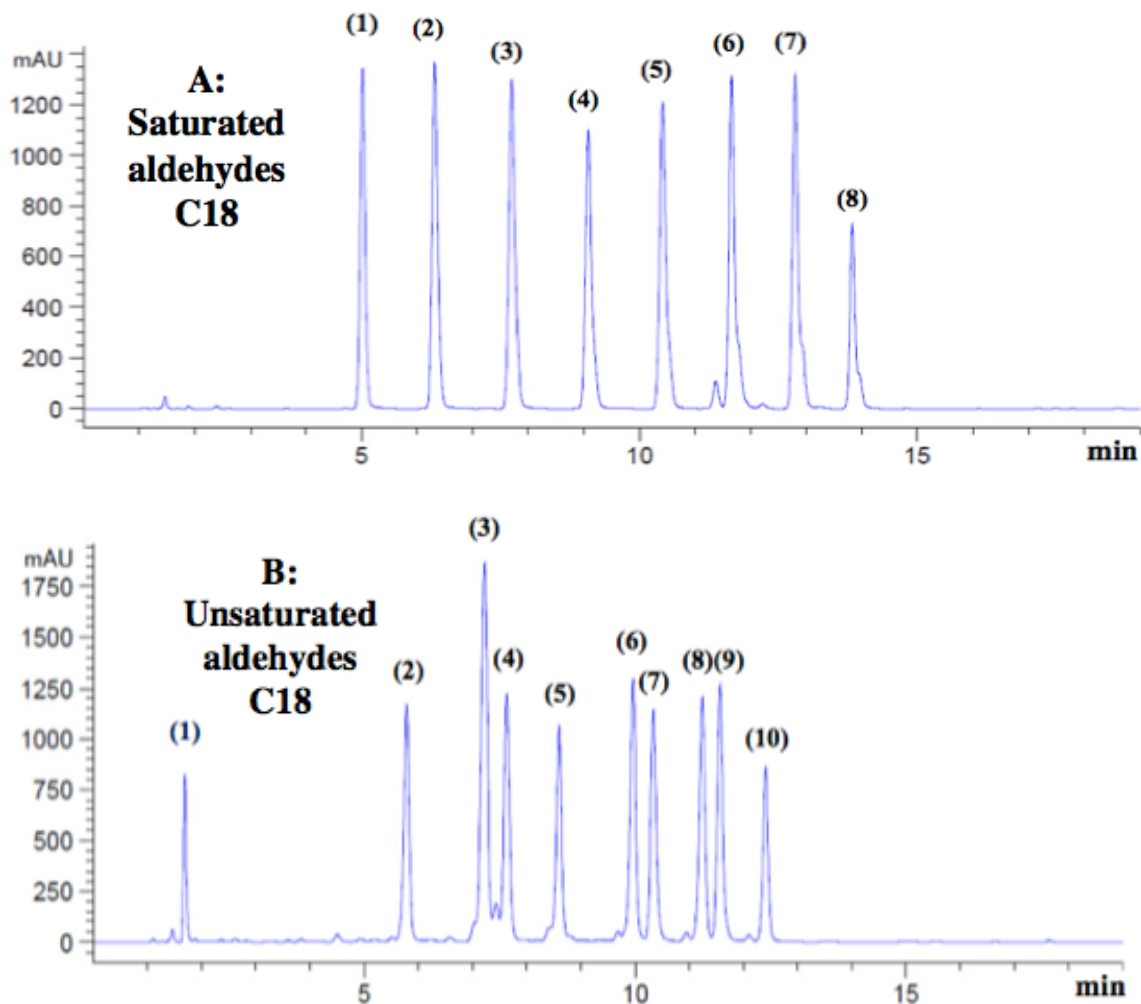


Figure 39. HPLC chromatograms of carbonyl-DNPH standards separated on a C18 column and detected at 360 nm. A: saturated aldehydes (1) butanal; (2) pentanal; (3) hexanal; (4) heptanal; (5) octanal; (6) nonanal; (7) decanal; (8) undecanal. B: unsaturated aldehydes (2) t-pentenal, (3) t-2-hexenal, cis-4-heptanal; (4) t, t-2, 4-heptadienal; (5) t-2-heptenal; (6) t-2-octenal; (7) t, t-2, 4-nonadienal; (8) t-2-nonenal; (9) t, t-2, 4-decadienal; (10) t-2-decenal.

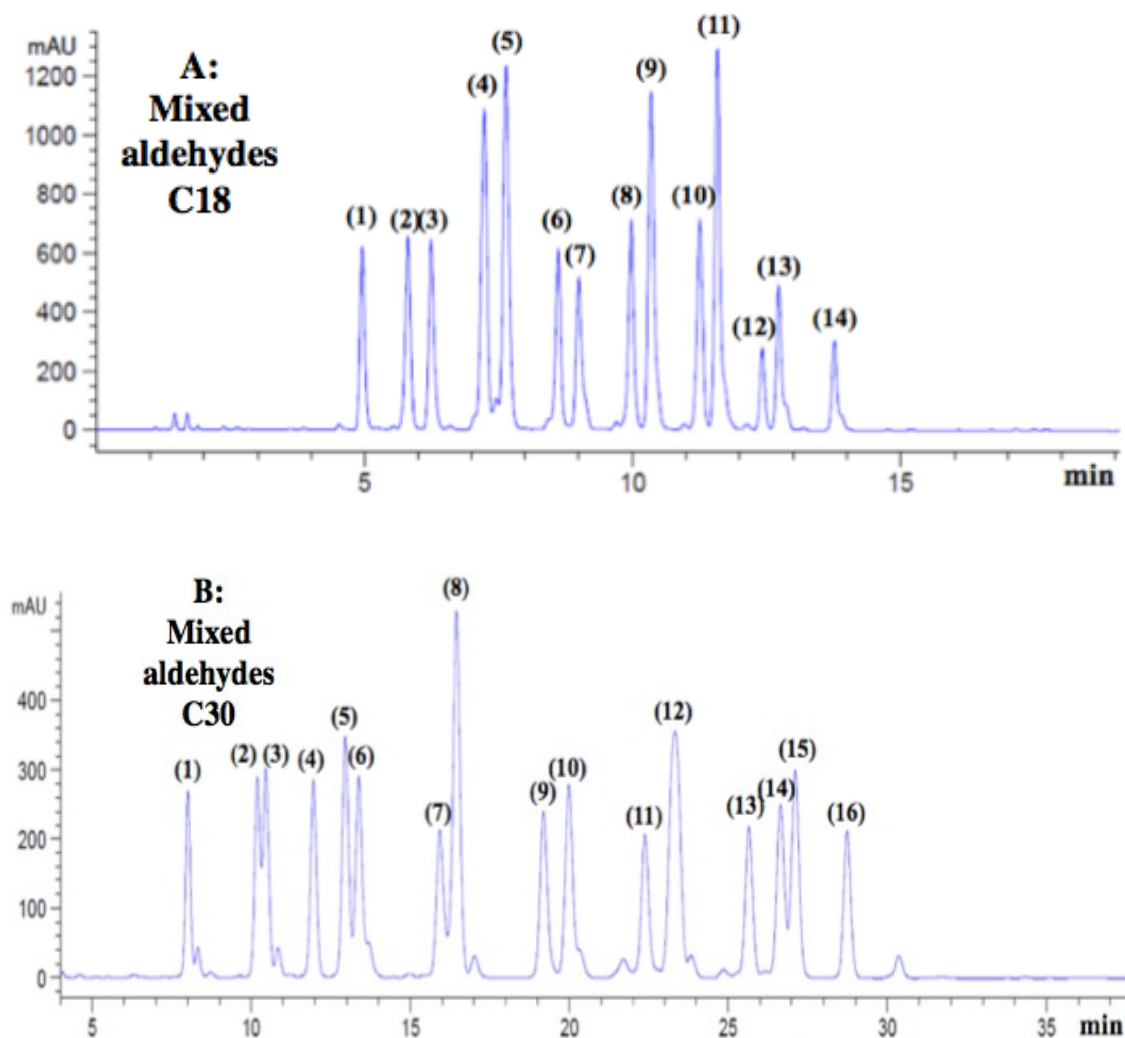


Figure 40. HPLC chromatograms of mixed carbonyl-DNPH standards separated on a C18 (A) and C30 (B) columns and detected at 360 nm. A: (1) butanal; (2) t-2-pentenal; (3) pentanal; (4) t-2-hexenal, cis-4-heptanal; (5) hexanal, t, t-2, 4-heptadienal; (6) t-2-heptenal; (7) heptanal; (8) t-2-octenal; (9) octanal and t, t-2, 4-nonadienal; (10) t-2-nonenal; (11) nonanal and t, t-2, 4-decadienal; (12) t-2-decenal; (13) decanal; (14) undecanal. B: (1) butanal; (2) pentanal; (3) t-pentenal; (4) cis-4-heptanal; (5) hexanal; (6) t-2-hexenal, (7) heptanal; (8) t, t-2, 4-heptadienal, t-2-heptenal; (9) octanal; (10) t-2-octenal; (11) nonanal; (12) t-2-nonenal, t, t-2, 4-nonadienal; (13) t-2-decenal; (14) t, t-2, 4-decadienal; (15) decanal; (16) undecanal.

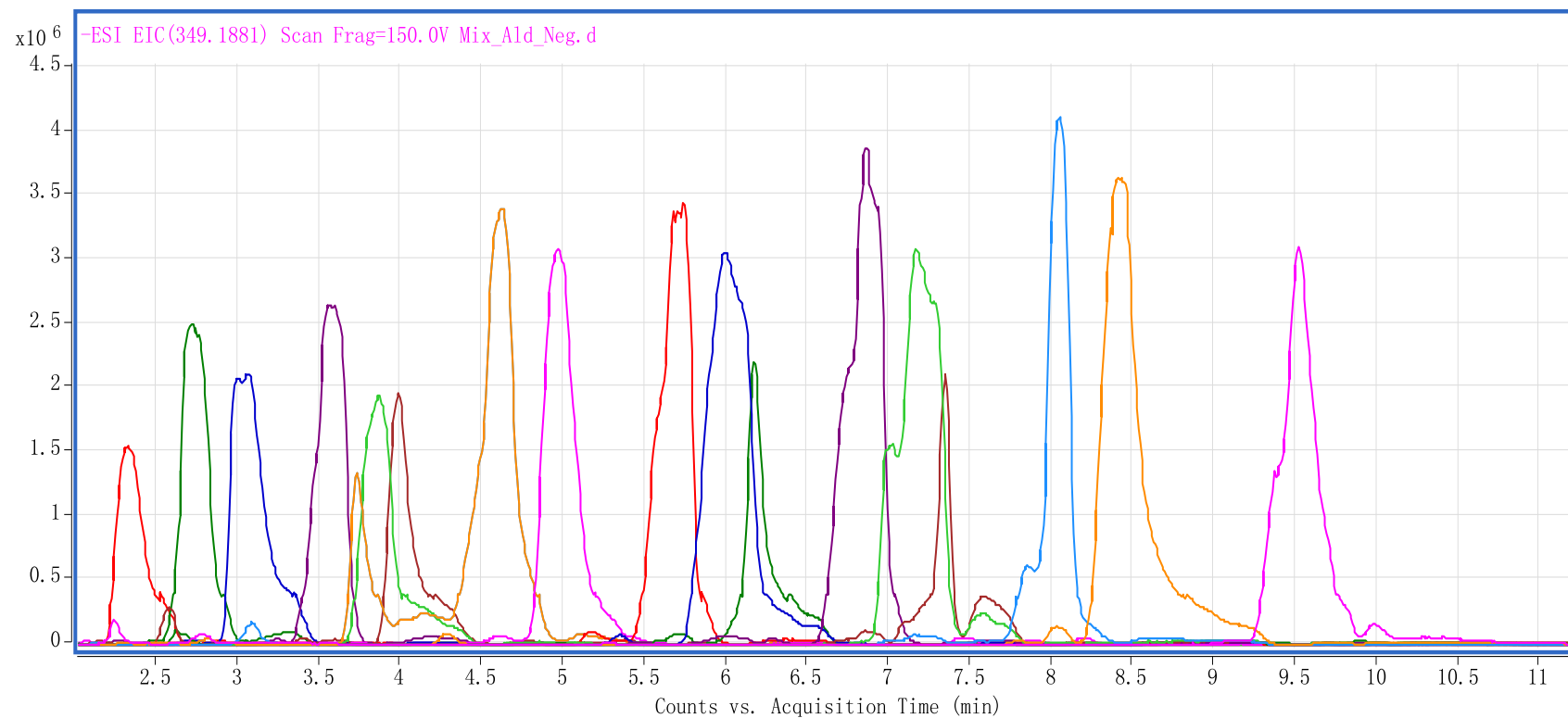


Figure 41. Total ion chromatogram of the mixture of carbonyl standard DNPH derivatives on LC/MS. Peaks in sequence left to right: butanal; t-2-pentenal; pentanal; t-2-hexenal, cis-4-heptanal; hexanal, t, t-2, 4-heptadienal; t-2-heptenal; heptanal; t-2-octenal; octanal and t, t-2, 4-nonadienal; t-2-nonenal; nonanal and t, t-2, 4-decadienal; t-2-decenal; decanal; undecanal.

The second stage of the elution gradient was designed to separate core aldehydes on larger lipid molecules such as phospholipids and triacylglycerides which have longer acyl chains and more complex molecular structure. Here, rapid flow of 100% organic solvent A was held for 18 minutes to elute the glycerol-based lipids. Isopropanol was included in Solvent A to reduce the polarity sufficiently to move the TAGs and PLs through the column. Acetonitrile alone was inadequate.

Monolinolein, dilinolein, and trilinolein standards were separated completely by this elution gradient (Figure 42). Retention times were 7.9 minutes, 16.1 minutes, and 19.5 minutes for monolinolein, dilinolein, and trilinolein, respectively. Note that these are parent acylglycerols, not hydrazones, so the peaks were detected non-specifically at 206 nm which detects all molecules. The climbing based line in chromatogram reflected the change in solvents.

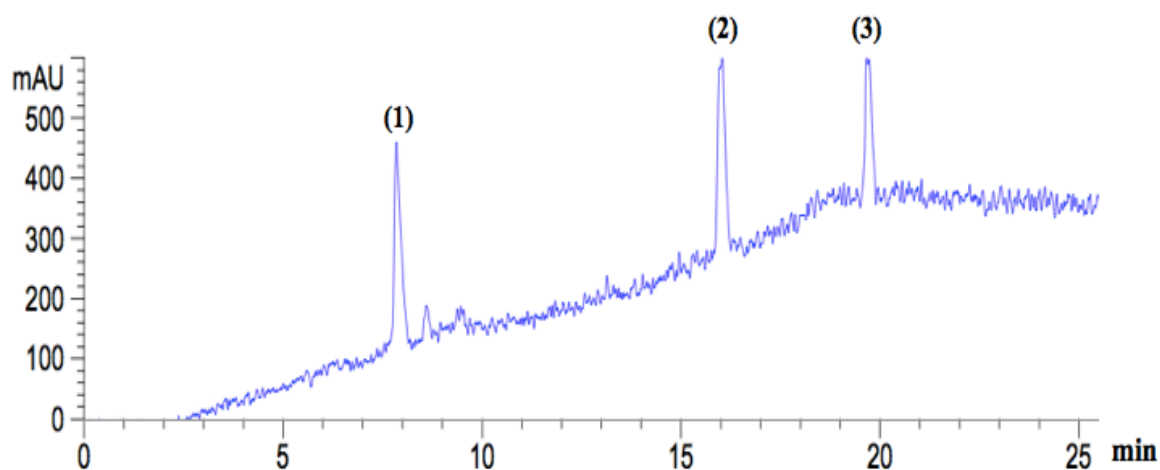
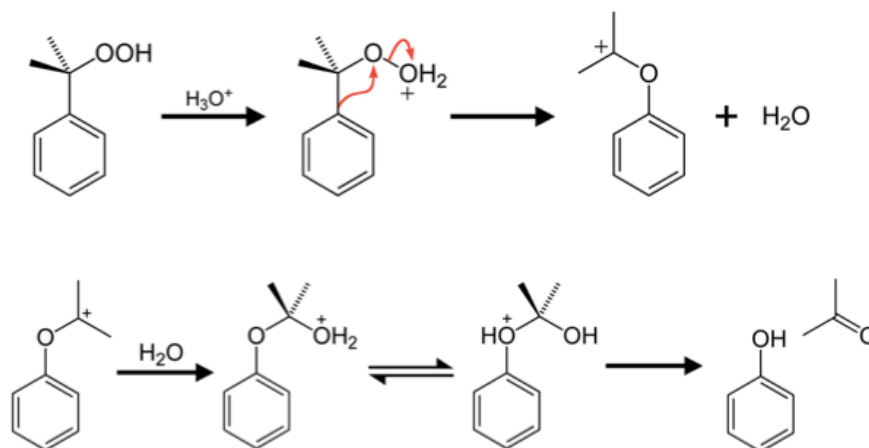


Figure 42. HPLC chromatogram of glyceride standards mixture on the C18 column: (1) monolinolein, (2) dilinolein, (3) trilinolein, detected at 206 nm, diode array detector

6.2.2 Specificity of DNPH reaction with carbonyls

To verify that the DNPH reaction was specific for carbonyls and inert to other lipid oxidation products, DNPH was reacted with cumene hydroperoxide, epoxydecene, and octanol and the reaction mixtures were subjected to HPLC analysis using the gradient conditions developed for the assay.

As shown in Figure 43, unreacted DNPH was detected at 1.68 minute. No hydrazone peaks were observed for any of the oxidation products, verifying the selectivity of DNPH reactions. Tiny peaks seen in the chromatograms of cumene hydroperoxide (3.6 and 6.249 min) and epoxydecene (2.8 minute) eluted much too early to be authentic hydrazones so probably result from reaction of contaminants or decomposition products. Short retention times are consistent with very small molecules, such as acetone which can be produced by Hock rearrangement of cumene hydroperoxide [143]:



Somma et al. [144] found that cumene hydroperoxide could undergo thermal decomposition to form acetophenone, methyl styrene or cumyl alcohol by autocatalytic kinetics. Epoxide degradation can also generate trace amounts of carbonyls as products.

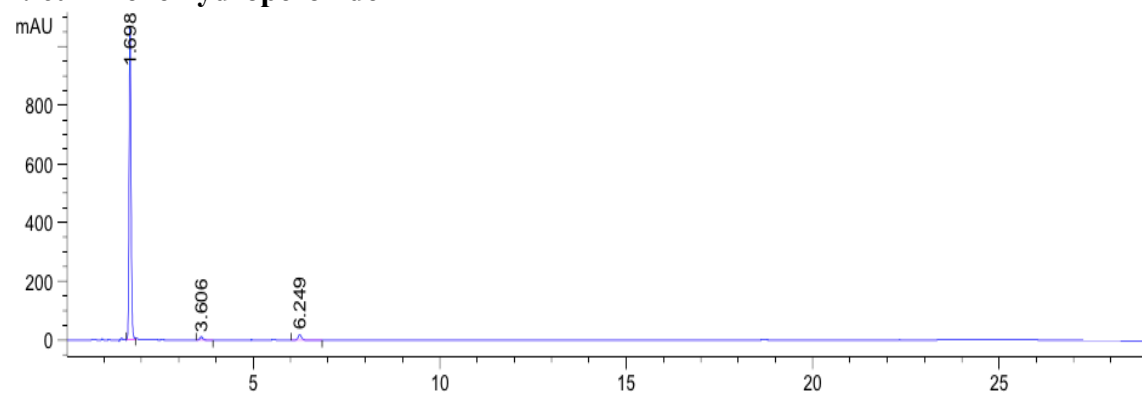
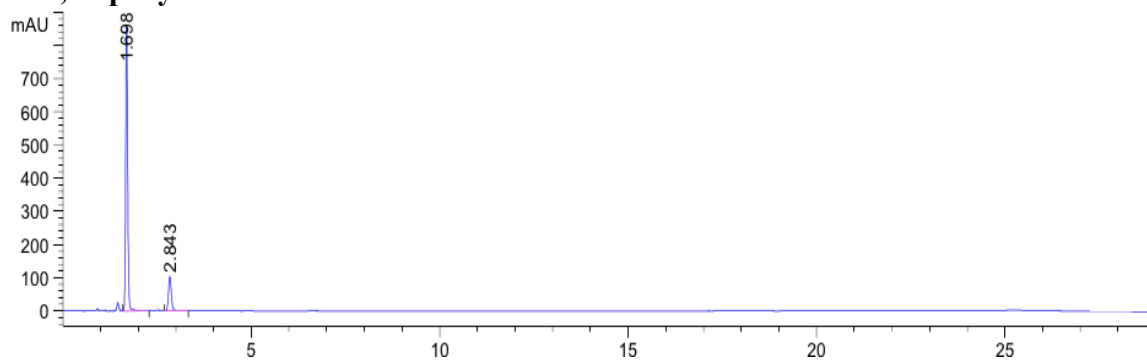
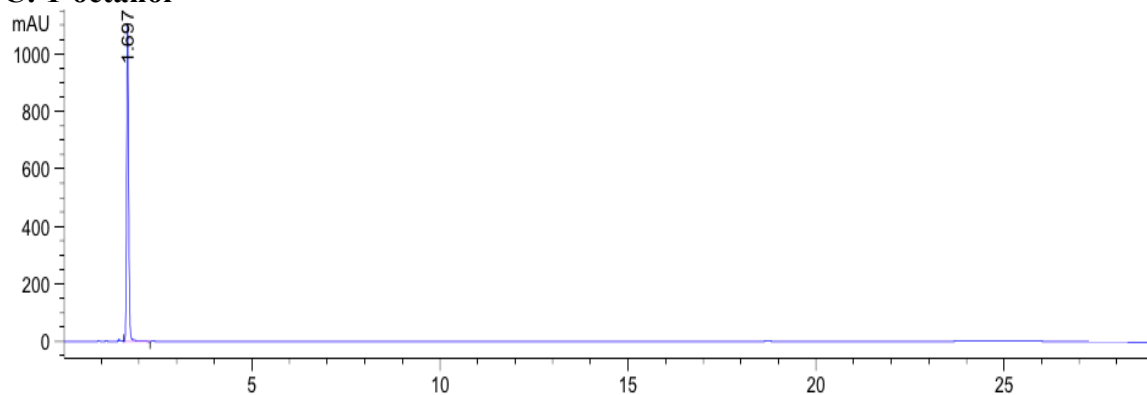
A: cumene hydroperoxide**B: 1,2-epoxy-9-decene****C: 1-octanol**

Figure 43. HPLC chromatograms of cumene hydroperoxide, 1,2-epoxy-9-decene and 1-octanol (A, B and C) reacted with DNPH reagent and analyzed by standard HPLC conditions. Peaks around 1.68 minute were unreacted DNPH.

6.2.3 Quantification of carbonyls

To determine linearity of response for the DNPH reaction, explore potential differences in reactivity, and establish a base for quantification, a range of concentrations for carbonyl standards of different chain lengths and saturation was reacted and response curves were plotted. Since this assay involves optical responses, it was not surprising to that each carbonyl standard gave a different calibration curve (Figure 44), with slopes of concentration response curves decreasing with carbonyl chain length (Figure 45, Table 10). Limits of detection and quantification for the different carbonyls varied from 6.2 to 50 $\mu\text{g/L}$ and from 18.4 to 166.7 $\mu\text{g/L}$, respectively, with an inverse relationship to carbon number (Table 11). Both limits increased with the carbonyl chain length. These behaviors make accurate absolute quantitation of carbonyls with this assay impossible under current conditions.

However, several alternative approaches may be used to quantify the carbonyls: 1) Total peak area except unreacted DNPH and solvent front could be used to compare carbonyl content for different samples. This provides relative values only but could be used for quality control. 2) Slopes of calibration curves can be separated into three categories based on relationship between chain length and slopes (Figure 45) and a separate calibration based on average slope of the range applied to each. Slopes of C4-C6 carbonyls: $\sim 4500 \text{ mAU}\cdot\text{L}/(\text{mmol}\cdot\text{s})$; C7-C10: $\sim 3000 \text{ mAU}\cdot\text{L}/(\text{mmol}\cdot\text{s})$; C11-C13: $\sim 2200 \text{ mAU}\cdot\text{L}/(\text{mmol}\cdot\text{s})$ (Table 12). Since carbonyl retention times vary with chain length, average slopes could be determined for different retention time/chain length categories. 3) Non-optical detection such as evaporative light-scattering (ELSD) might provide more equivalent responses for different chain length samples.

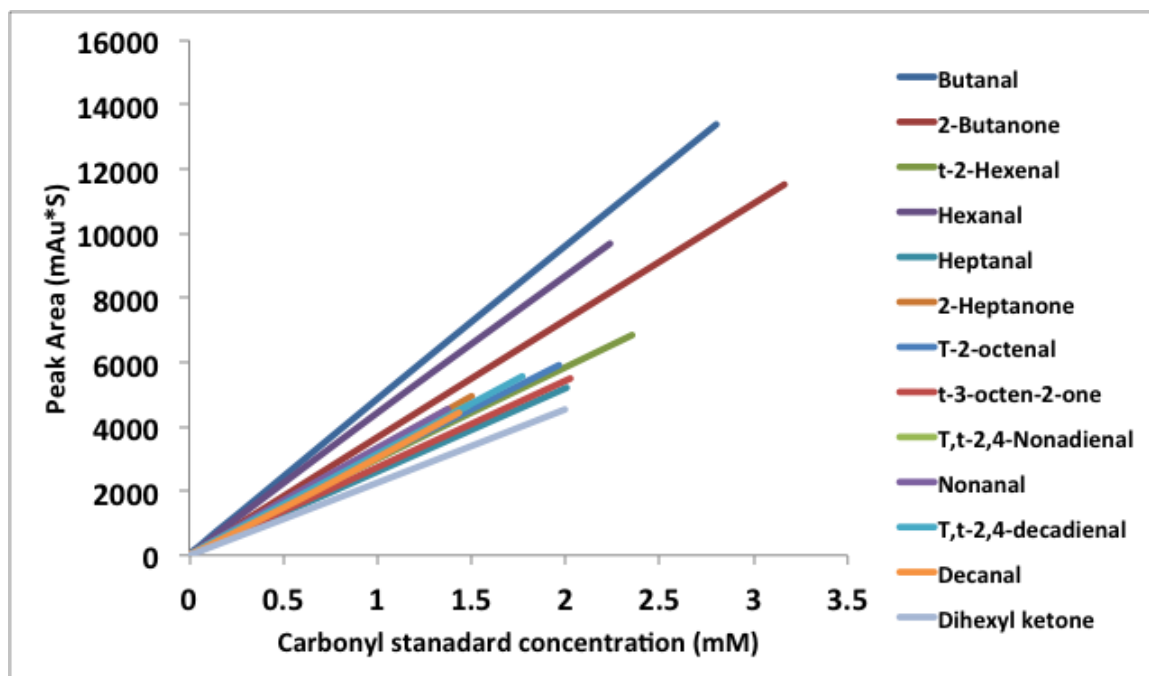


Figure 44. Normalized calibration curves of carbonyl-DNPH standards

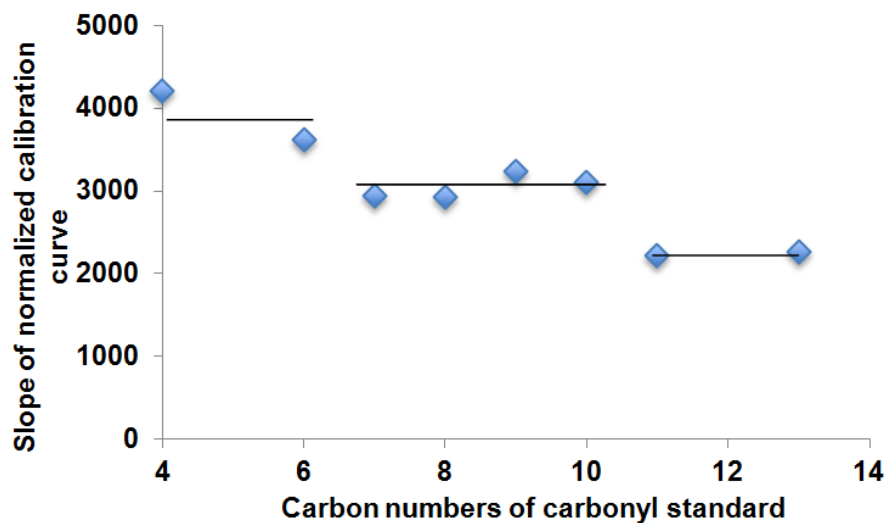


Figure 45. Relationship between carbon numbers (chain lengths) of carbonyl standard and the slopes of calibration curves.

Table 10. Calibration data (mM) for concentration response curves from reaction of carbonyl standards with DNPH; separation by HPLC with diode array detection.

Carbonyl	Molecular mass (g/mol)	Equations (Based on molarity)	R²
Butanal	72.11	$y = 4783.3x + 21.343$	0.9998
2-Butanone	72.11	$y = 3644.9x + 1.8328$	0.9999
t-2-Hexenal	98.14	$y = 2912.6x + 17.959$	0.9998
Hexanal	100.16	$y = 4339.7x + 14.555$	0.9999
Heptanal	114.19	$y = 2594.1x + 4.2909$	0.9999
2-Heptanone	114.16	$y = 3296.4x + 1.0884$	0.9999
t-2-Octenal	126.2	$y = 3008.1x + 13.143$	0.9999
t-3-octen-2-one	126.1	$y = 2714.5x + 3.4011$	0.9999
Octanal	128.21	$y = 3054.4x + 6.1784$	0.9999
t,t-2,4-Nonadienal	138.21	$y = 3158.4x + 7.1715$	0.9998
Nonanal	142.24	$y = 3314.9x + 5.2397$	0.9998
t,t-2,4-decadienal	152.23	$y = 3136.8x + 8.9496$	0.9999
Decanal	156.27	$y = 3076.3x - 12.905$	0.9999
Undecanal	170.29	$y = 2223.3x + 5.0198$	0.9999
Dihexyl ketone	198.34	$y = 2266.9x - 2.4865$	0.9999

Table 11. Limits of detection and quantification for reaction of carbonyls with DNPH detected; HPLC separation and diode array detection.

Carbonyl	Equations	R ²	LOD ¹	LOQ ²	RSD ³
			(µg/L)	(µg/L)	(%)
Butanal	$y = 66.333x + 21.132$	0.9999	6.2	18.7	0.16
2-Butanone	$y = 42.079x + 1.3378$	0.9999	6.1	18.4	0.46
t-2-Hexenal	$y = 31.781x + 11.169$	0.9998	12.6	42.0	0.54
Hexanal	$y = 43.328x + 12.996$	0.9999	13.1	43.6	2.67
Heptanal	$y = 22.717x + 3.7475$	0.9999	20.5	68.3	0.26
2-Heptanone	$y = 23.017x + 1.0125$	0.9999	19.5	64.9	0.33
t-2-Octenal	$y = 23.836x + 10.599$	0.9999	13.5	45.0	0.64
t-3-octen-2-one	$y = 19.567x + 2.4207$	0.9999	12.8	42.7	0.34
Octanal	$y = 23.829x + 4.3328$	0.9999	25.0	83.3	0.23
t,t-2,4-Nonadienal	$y = 22.205x + 6.7977$	0.9997	40.5	134.87	2.89
Nonanal	$y = 19.466x + 4.4784$	0.9999	45.3	150.9	2.73
t,t-2,4-decadienal	$y = 20.613x + 5.4971$	0.9999	41.2	137.2	0.25
Decanal	$y = 19.672x - 9.5351$	0.9999	48.1	160.2	0.30
Undecanal	$y = 18.925x + 4.8807$	0.9999	50.0	166.7	2.73
Dihexyl ketone	$y = 18.176x - 1.9972$	0.9999	46.8	155.9	0.53

1: limit of detection; 2: limit of quantification; 3: relative standard deviation

Table 12. Averaged calibration curve based on standard chain length ranges (mM)

Carbon numbers	Equations	R ²
	(Based on molarity)	
C4-C6	$y=3920x+13.92$	0.9999
C7-C10	$y=3039x+4.06$	0.9998
C11-C13	$y=2245x+1.27$	0.9999

6.2.4 Stability of DNPH derivatives

Stability of DNPH derivatives was investigated using hexanal (2 $\mu\text{mol/ml}$ and 0.02 $\mu\text{mol/ml}$) and 2,4-decadienal (3 $\mu\text{mol/ml}$ and 3 $\mu\text{mol/ml}$) DNPH derivatives at two concentrations stored under different conditions. At room temperature and without changing vial caps, hexanal hydrazones at both concentrations remained stable until Day 3, when apparent concentrations increased sharply due to solvent evaporation through needle perforations in the vial cap. 2,4-Decadienal hydrazone in high concentrations (2 $\mu\text{mol/ml}$) also remained at the same level for 4 days (Figure 46A). However, low concentrations of the 2,4-decadienal hydrazone decreased with the storage time as oxidation of its double bonds continued and degradation products increased in HPLC chromatograms. Solvent evaporation and 2,4-decadienal degradation counterbalanced each other in the high concentration sample, so the net change of 2,4-decadienal concentration was not remarkable. In contrast, all hydrazones were stable over four days when stored frozen with new vial caps (no needle punctures) (Figure 46 C and D). Thus, since composition of carbonyls in oils or extracts is seldom known, a general rule of

thumb for the DNPH analyses should be analyze as soon as possible after reaction, or freeze if there must be a delay in HPLC analysis.

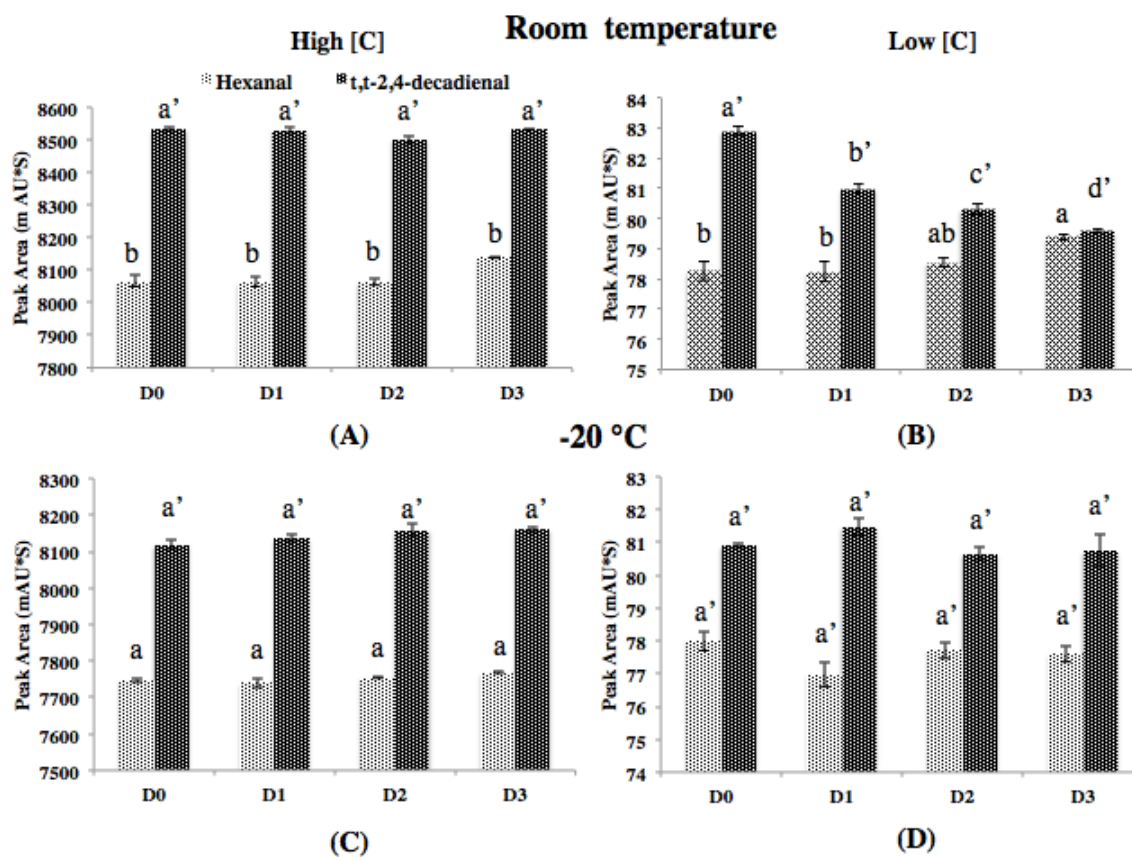


Figure 46. HPLC/DAD peak areas of hexanal (2 $\mu\text{mol/ml}$ and $2 \times 10^{-2} \mu\text{mol/ml}$) and 2,4-decadienal (3 $\mu\text{mol/ml}$ and $3 \times 10^{-2} \mu\text{mol/ml}$) DNPH derivatives stored at room temperature without changing vial caps for 4 days (A, B) and stored at -20 °C freezer and changed sample vial caps immediately after injection. Different letters mean statistically significant difference.

6.2.5 Effect of acidifying reagent on DNPH derivatizing ability

Phosphoric acid [145] and hydrochloric acid [122] are the traditional acids used in the DNPH test tube reactions but they are both harsh for HPLC applications. Formic acid offers a milder alternative [130] but has not been tested with HPLC versions of the DNPH assay. Comparisons of hexanal and decadienal reactions with DNPH in the presence of equal concentrations of sulfuric and formic acids showed identical peak virtually identical peak areas for the two acids (Figure 47). Therefore, formic acid can replace corrosive acids such as sulfuric acid in HPLC versions of the DNPH assay without changing reaction properties.

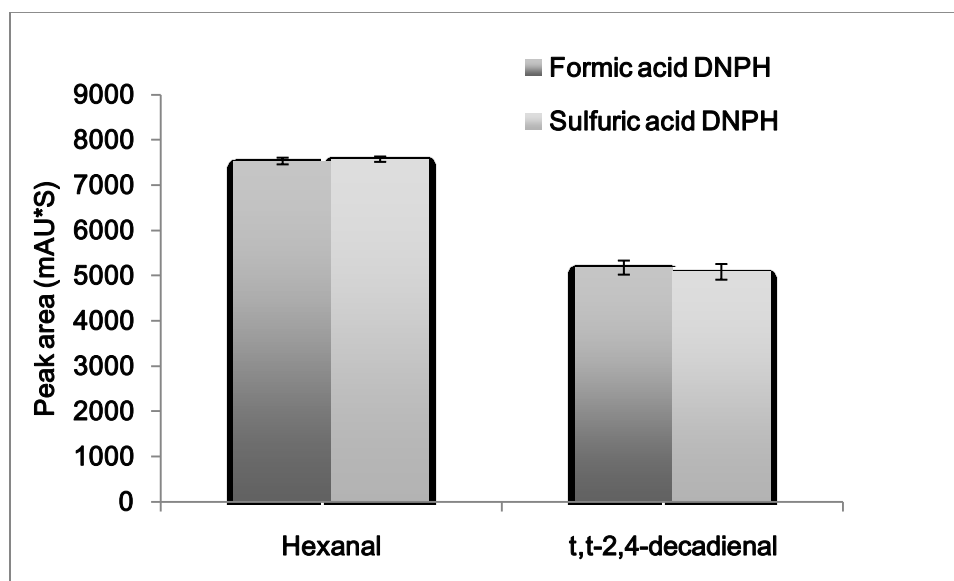


Figure 47. Comparisons of peak areas of hexanal and 2,4-decadienal derivatized by DNPH acidified by formic acid or sulfuric acid.

6.2.6 LC/MS/MS verification of hydrozone structure and identification of carbonyl adducts

All carbonyl standard DNPH derivatives were analyzed LC/MS/MS at different collision energy 10, 20 and 40 eV to yield characteristic fragment ions in negative mode. These were used to verify the hydrazone structure and begin building a library of signatures for known carbonyls. In structural studies of DNPH carbonyl derivatives by HPLC/MS and multiple MS/MS, ions such as m/z 152 and 122 163 and 179 were signature fragments for DNPH-carbonyl derivatives [110, 113, 122]. The fragmentation process is shown in Figure 48. (see also Figure 20). Observation of the same signature fragments in the present study verified the formation of authentic hydrazones.

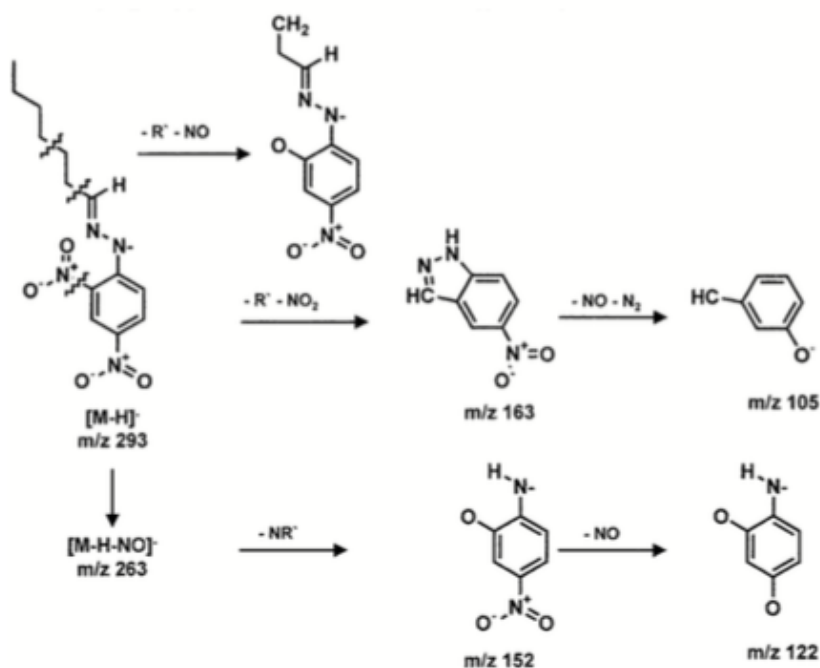


Figure 48. Structures tentatively assigned to CID (collision-induced dissociation) mass fragments of the DNPH derivatives of pentanal [113].

To obtain the typical fragments of DNPH derivatives, each carbonyl standard DNPH derivatives was analyzed and fragmented at different collision energy levels. Based on the spectra of carbonyl standards, a library was built as reference for further analyses. Figure 49 and 50 are an example of CID spectra of hexanal DNPH derivatives at different collision energy levels.

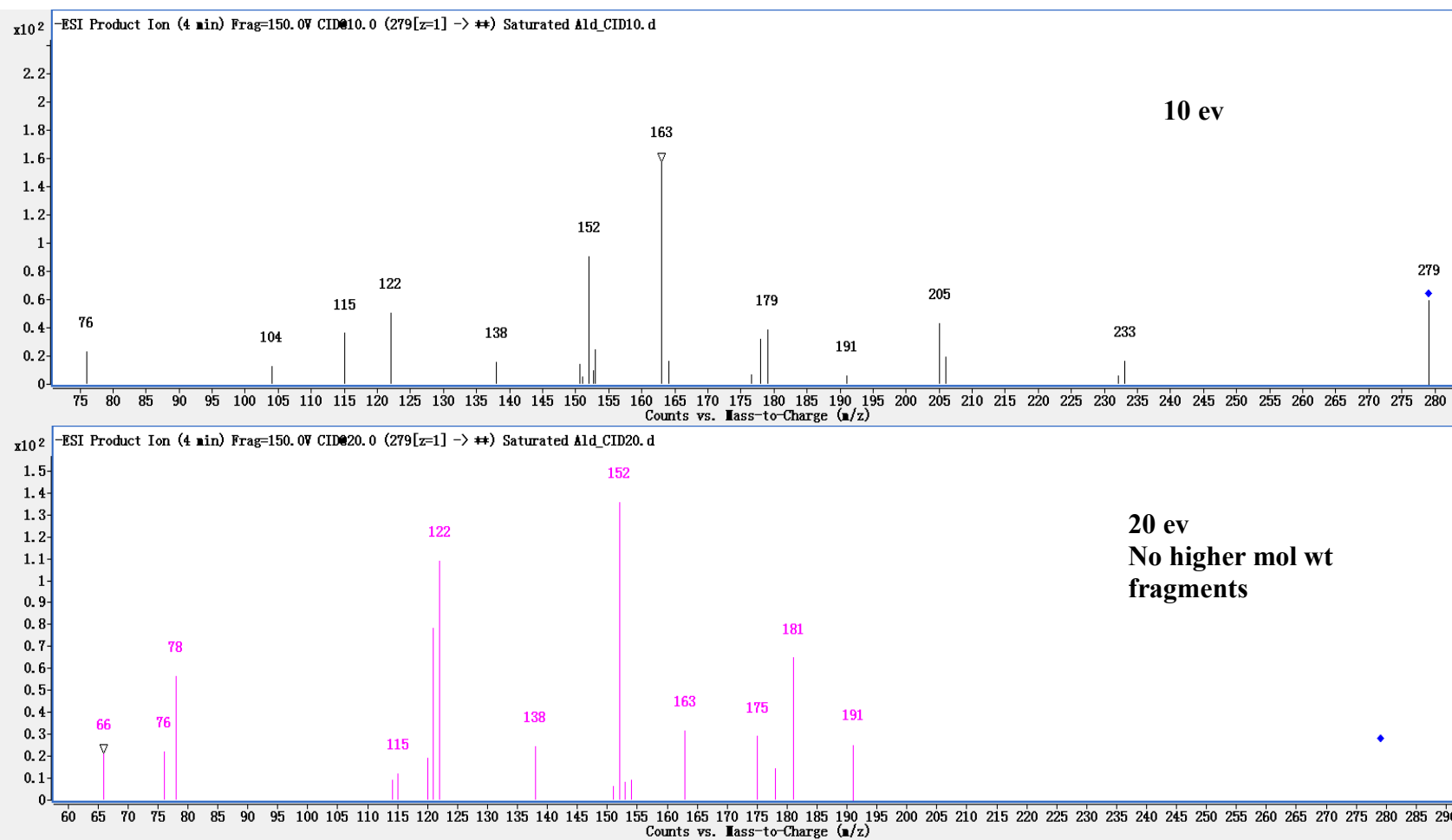


Figure 49. CID mass spectra of hexanal DNPH derivatives analyzed at 10 ev (top) 20 ev (bottom).

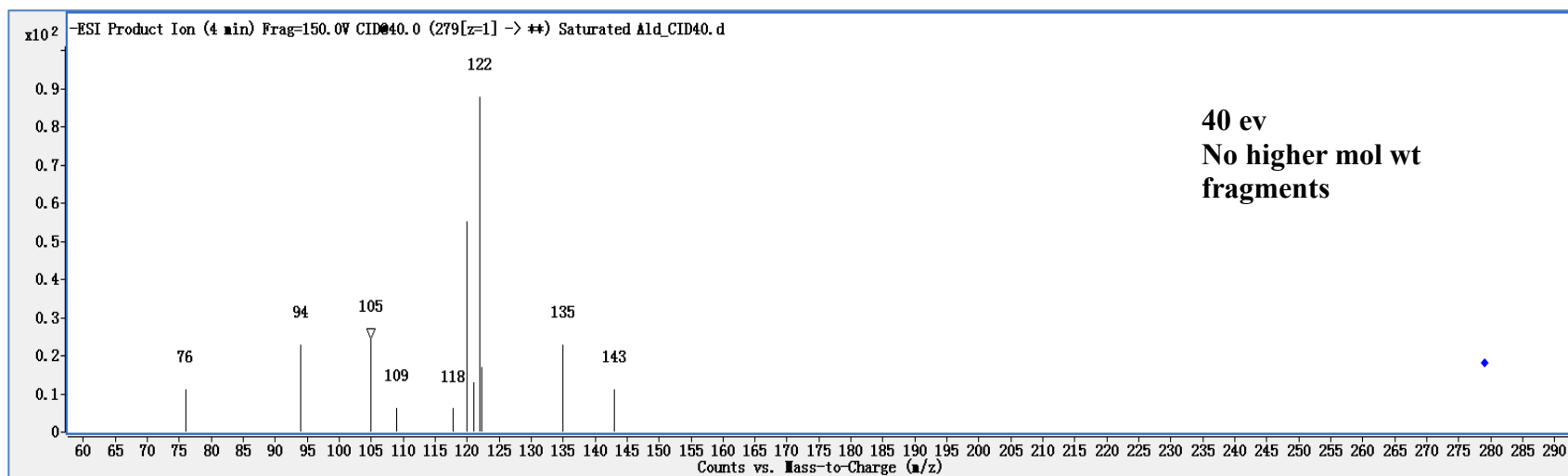


Figure 50. CID mass spectra of hexanal DNPH derivatives analyzed at 40 ev

As collision energy increases, more small fragments are presented. These are often useful for identifying DNPH adducts but sometimes can make the fragmentation pattern too complex for easy interpretation. Thus, several energy levels must usually be tested to determine optimum for different molecules.

CID mass spectra of butanal, butanone, t-2-octenal and octenone are shown in Figures 51 and 52 as examples how aldehydes and ketones can be differentiated by their fragmentation patterns. The m/z 152 and 163 fragments are both intense in aldehyde and ketone DNPH derivatives, but a strong m/z 163 is typical for aldehyde-DNPHs while no m/z 163 ions are present in ketone spectra. The ion of m/z 181 can also be an indicator to differentiate aldehydes DNPH derivatives from ketones.

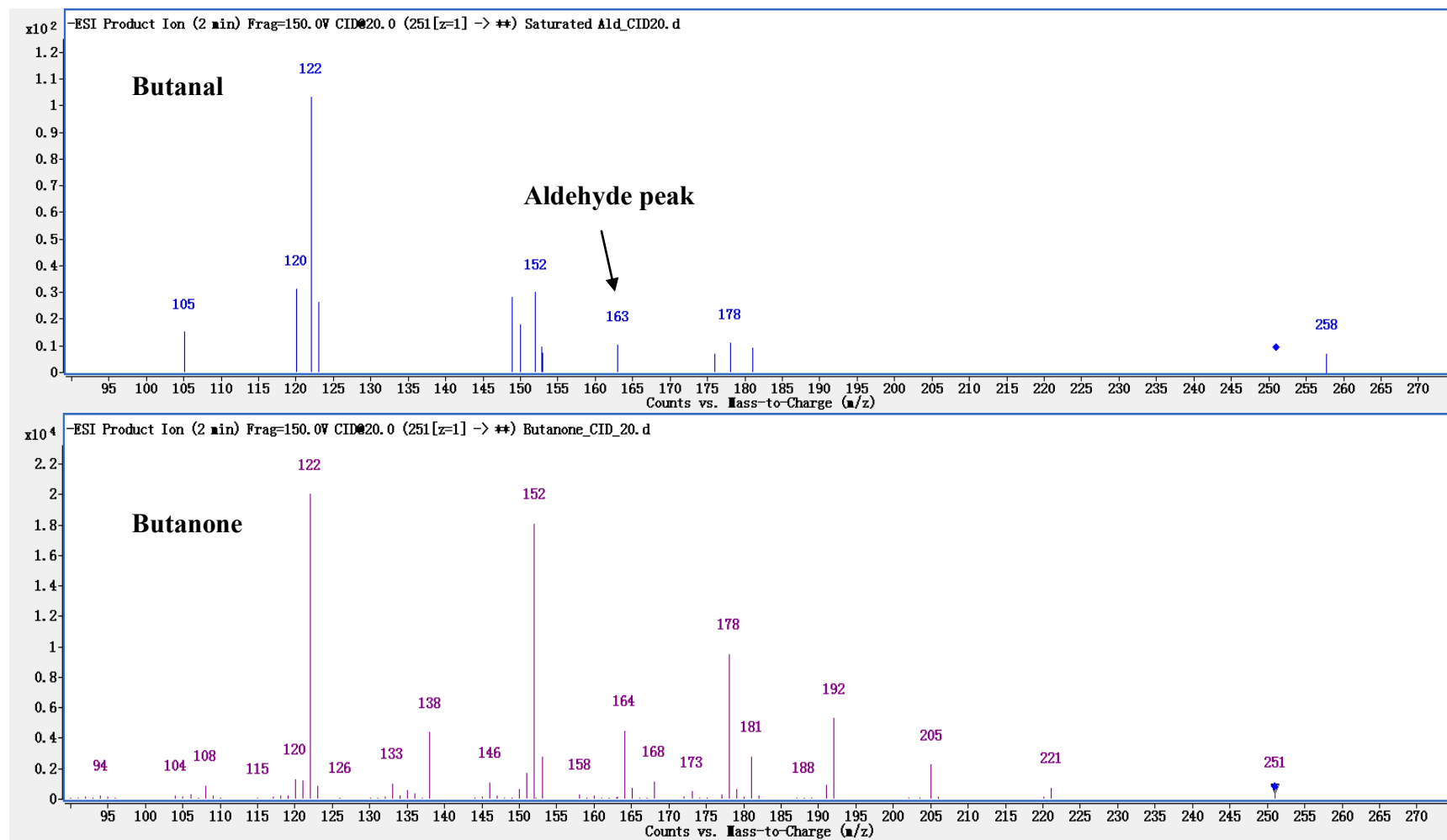


Figure 51. CID mass spectra of butanal (top) and butanone (bottom).

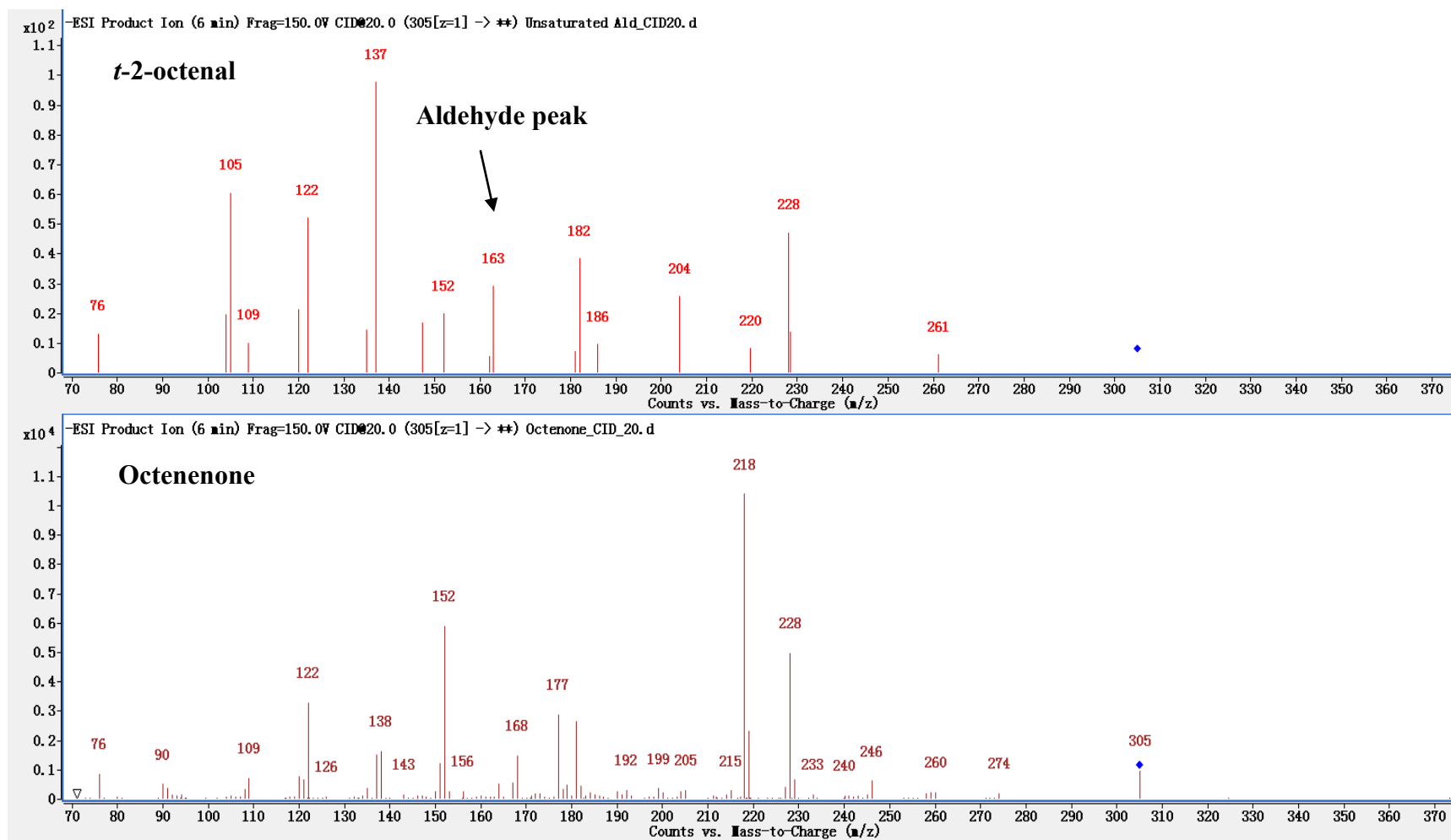


Figure 52. CID mass spectra of *t*-2-octenal (top) and octenenone (bottom).

6.2.7 Application of HPLC-DNPH assay to analysis of carbonyls in oxidized oils and lipid extracts

6.2.7.1 Methyl linoleate

To provide a simple model food system, methyl linoleate was incubated neat and also in lyophilized emulsions prepared with lactalbumin.

Neat. Neat methyl linoleate was incubated at 40°C for 3 days and 6 days, then reacted with DNPH to determine carbonyl oxidation products. From the HPLC chromatograms, we were able to identify <C4 carbonyls, (2) butanal, (3) pentanal, (4) hexanal after three days, and these plus t-2-decanal and t,t-2,4-nonadienal or octanal after six days (Figure 53). Individual and total carbonyls increased with incubation time (Figure 54), consistent with increased levels of hydroperoxides and conjugated dienes (data not shown).

Interestingly, this study detected <C4 carbonyls and decadienal as dominant initial products at three days, but by six days, pentanal, hexanal, and nonadienal (in that order) had accumulated to comparable levels. These product patterns are in contrast to other studies in our laboratory where decadienal, in particular, was detected at only trace levels, if at all. These results show that reaction pathways do shift with reaction conditions and once again raise questions about the common practice of using hexanal as the main secondary product marker of lipid oxidation.

ML oxidized with lactalbumin in a lyophilized emulsion. Methyl linoleate was incubated in lyophilized emulsions for 32 days, and samples were ASE- extracted at 11, 24, and 32 days for HPLC-DNPH analysis of carbonyl oxidation products. Total and individual carbonyls accumulated slowly from day 0 to day 11, then spiked at day 24 and

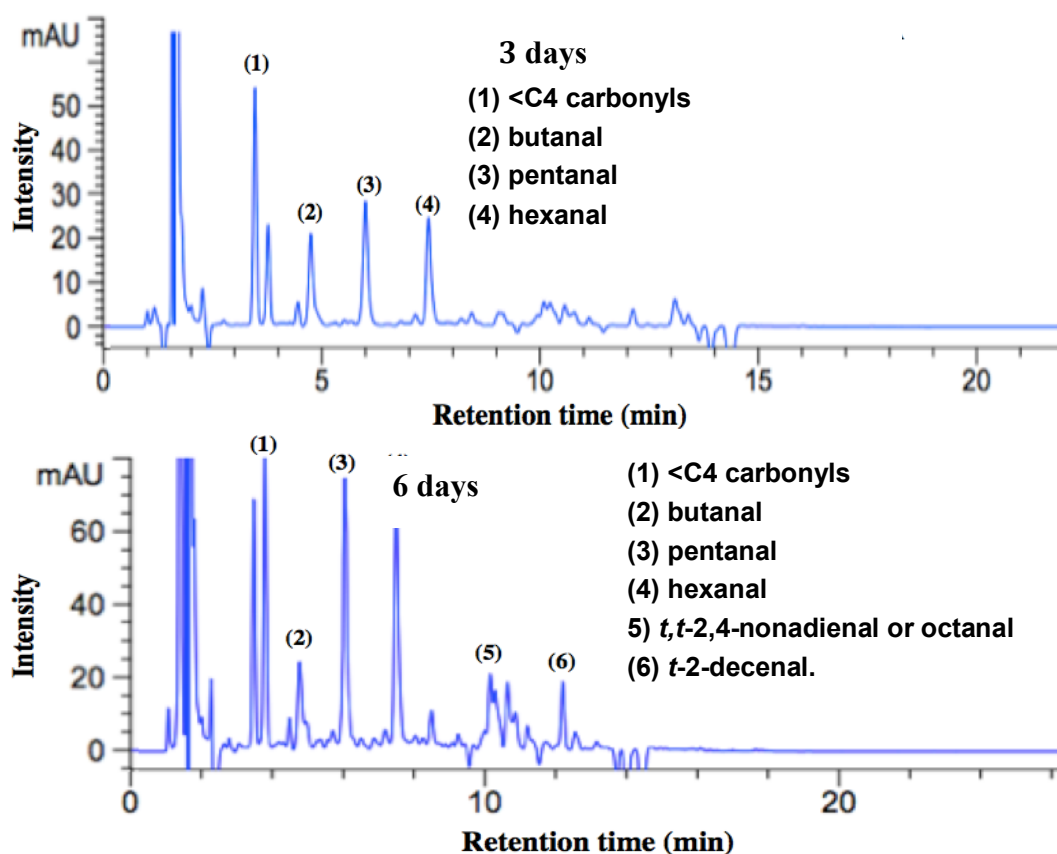


Figure 53. HPLC chromatograms of DNPH derivatives of incubated neat methyl linoleate 3 days (top) and 6 days (bottom) with 360 nm UV detection.

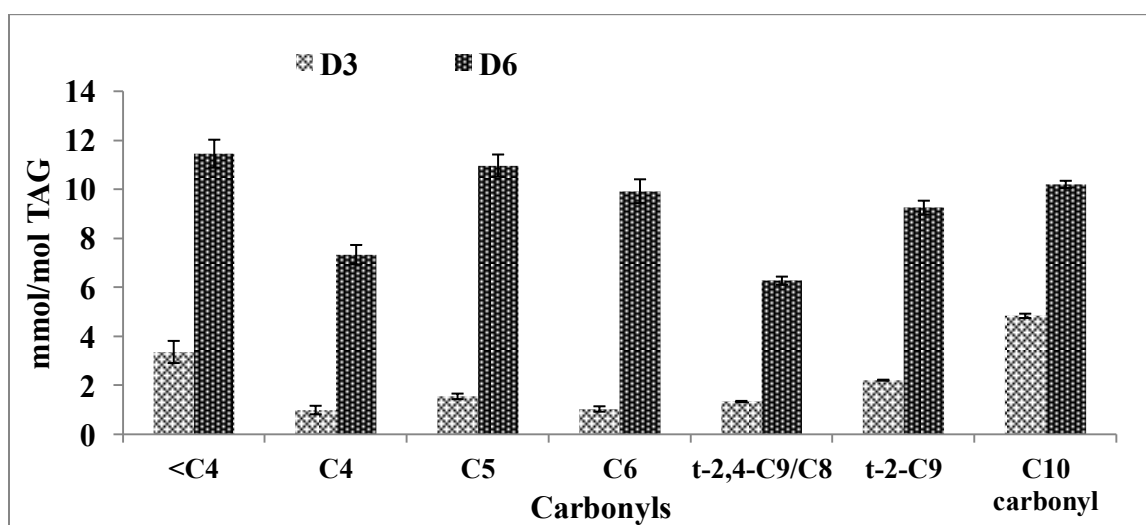


Figure 54. Carbonyls produced in neat methyl linoleate oxidized for 3 days and 6 days at 40 °C.

decreased at day 32 (Figures 55), in parallel with formation of conjugated dienes and hydroperoxides (unpublished data from another student). The decrease of carbonyls at day 32 (Figure 56) corresponded to a considerable increase in volatile carbonyls detected by GC analyses (data from K. Meyler, Geo. H. Cook Honors thesis [146]), so apparently scission reactions became active in this period. The color of the emulsion at day D32 was visibly more yellow than before oxidation, indicating occurrence of Maillard reactions of lipid carbonyls with protein amine groups. Pentanal and decenal were present in highest concentrations; both are generated in post-secondary decompositions of methyl linoleate. Lower levels of hexanal may be due to altered balance of oxidation pathways in the emulsions, or perhaps to preferential reaction of hexanal in Maillard reactions. These observations support the need for much more detailed analysis of lipid oxidation products to fully elucidate pathways active under different oxidation conditions.

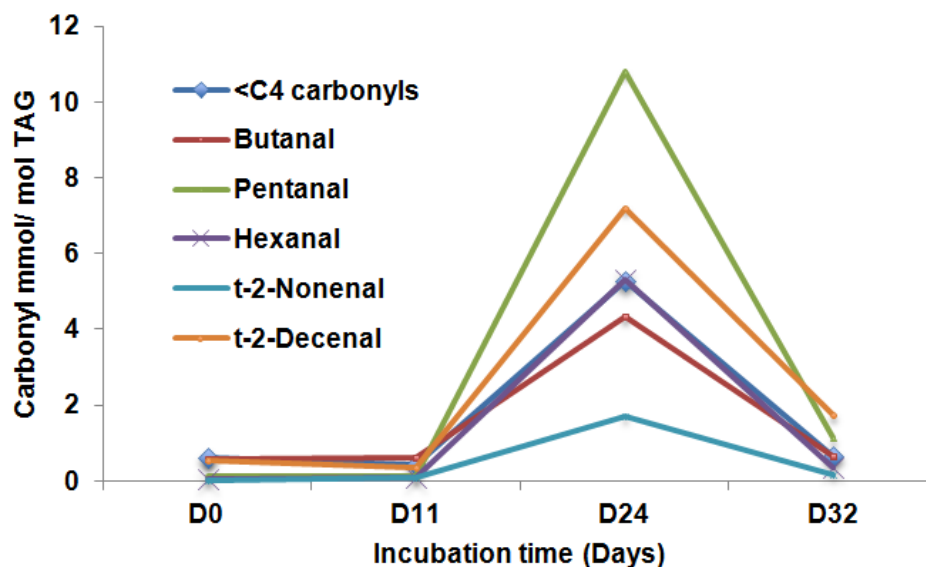


Figure 55. Carbonyl production of oxidized methyl linoleate extracted from lyophilized emulsions incubated at 40 °C for 0, 11, 24 and 32 days.

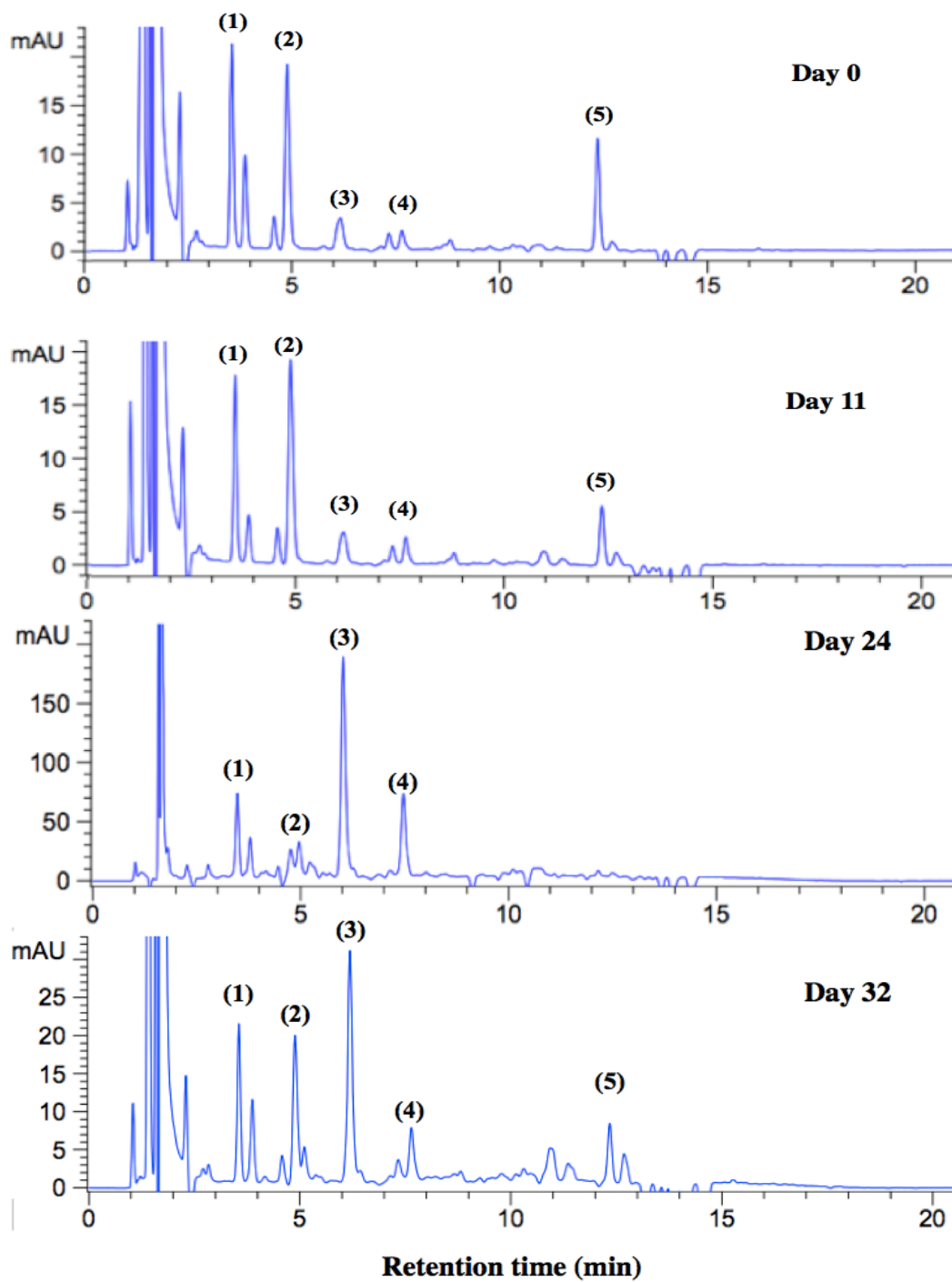


Figure 56. HPLC chromatograms of DNPH hydrazones of methyl linoleate extracted from lyophilized emulsions incubated at 40 °C for 0, 11, 24 and 32 days. (1) <C4 carbonyls, (2) butanal, (3) pentanal, (4) hexanal, (5) t-2-decenal.

6.2.7.2 Oxidized trilinolein

Ability of the developed HPLC-DNPH procedures to distinguish monomer scission carbonyls and core aldehydes in an actual oil was tested in a model system of trilinolein incubated at 60 °C for 72 hours, then reacted with DNPH. Resulting hydrazones showed short chain monomers eluting between 6 and 12 minutes while core aldehydes eluted at 18 to 22 minutes (Figure 57). This chromatogram provides strong evidence for the existence of core carbonyls remaining on the glycerol backbone, in addition to released monomer carbonyls.

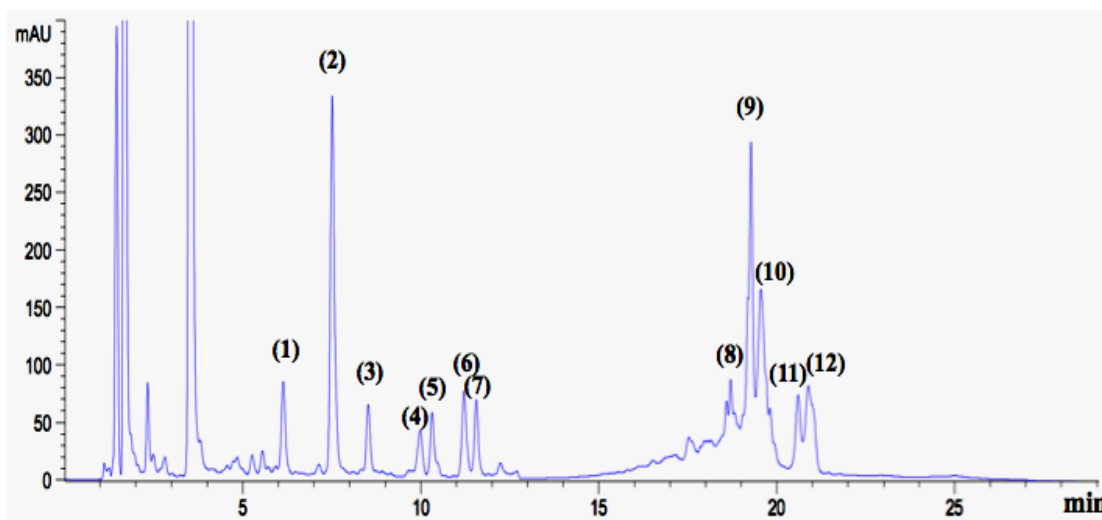


Figure 57. HPLC chromatogram of DNPH hydrazones from oxidized trilinolein separated on a C18 column: (1) pentanal, (2) hexanal, (3) 2-heptenal, (4) 2-octenal, (5) *t*, *t*-2,4-nonadienal, (6) 3-nonenal and (7) *t*,*t*-2,4-decadienal; peaks (8) to (12) were not identified except as core carbonyls. Peaks eluting before 5 minutes were unreacted DNPH and solvent front.

Comparison with retention times of carbonyl standards identified seven peaks in the HPLC chromatogram as (1) pentanal, (2) hexanal, (3) 2-heptenal, (4) 2-octenal, (5) *t*,

t-2,4-nonadienal, (6) 3-nonenal and (7) *t,t*-2,4-decadienal. LC/MS/MS, which is more sensitive, detected hexenal and heptanal in addition to the base monomers (Figures 58-63). Pathways for generation of these products from scissions of C9, 11, and 13 alkoxyl radicals are shown in Figure 64. It should be noted that C-11 is not considered to be a feasible initial attack site on linoleic acid[90-92], but our laboratory has recently shown that radicals can be transferred to this carbon by internal rearrangements of C9 and C13 alkoxyl radicals to epoxides[88, 147]. This pathway is shown in Figure 65.

Monomer carbonyls were not formed equally at the three positions (Table 13). Concentrations of hexanal (C13 product) were significantly higher than other scission products, consistent with what was observed in volatile [88] and non-volatile [147] products of oxidizing methyl linoleate. Heptenal was the next prevalent, followed by decadienal, then octenal and nonadienal. This pattern suggests that the preferential reaction for C-13 alkoxyl radicals is scission, while C-9 alkoxyl radicals undergo, in order, epoxidation followed by β -scission at C-11, α -scission to release decadienal, and epoxidation followed by α -scission at C-11 equal to C-9 β -scission to release octenal and nonadienal, respectively.

Multiple core carbonyls were detected in oxidized trilinolein (Figure 57). This should be expected since, theoretically, the oxidations shown in Figure 54 for the sn-1 position on the triacylglycerol should occur on fatty acids at all three sn positions. All core carbonyls contained parent ions m/z 687, 698 (Figures 66) and 1000 (Figures 67) and fragment ions m/z 122, 152, 163 and 181 in their CID mass spectra. Better resolution will be needed to fully identify specific aldehydes and position.

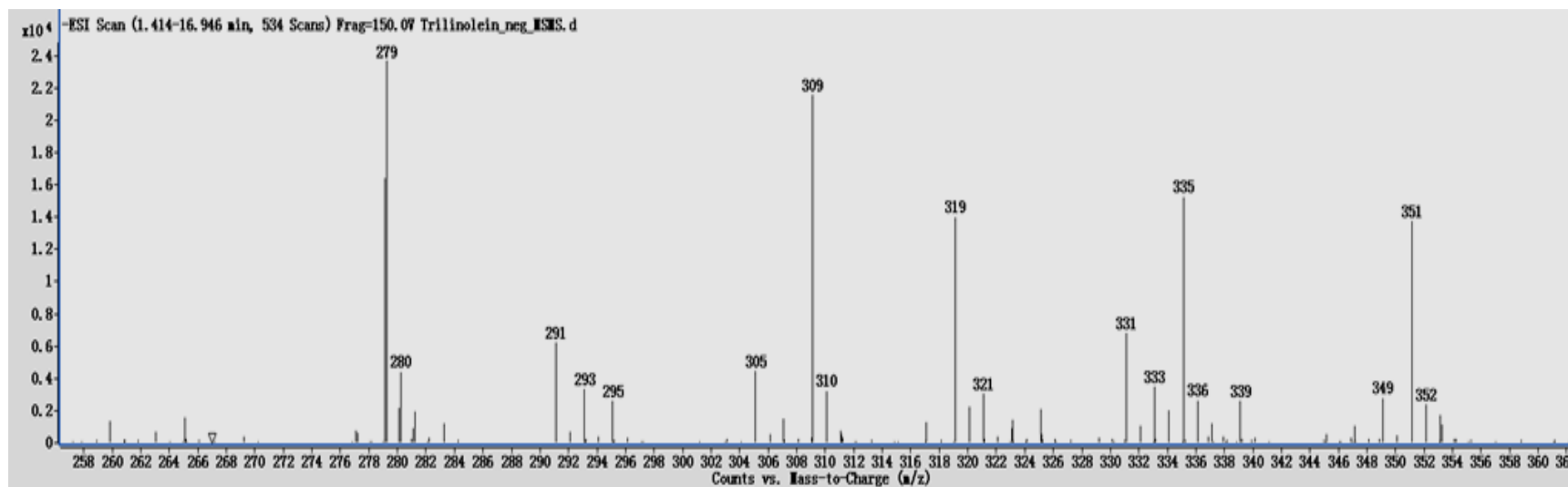


Figure 58. Mass spectrum of total ions of DNPH derivatized carbonyl monomers from oxidized trilinolein.

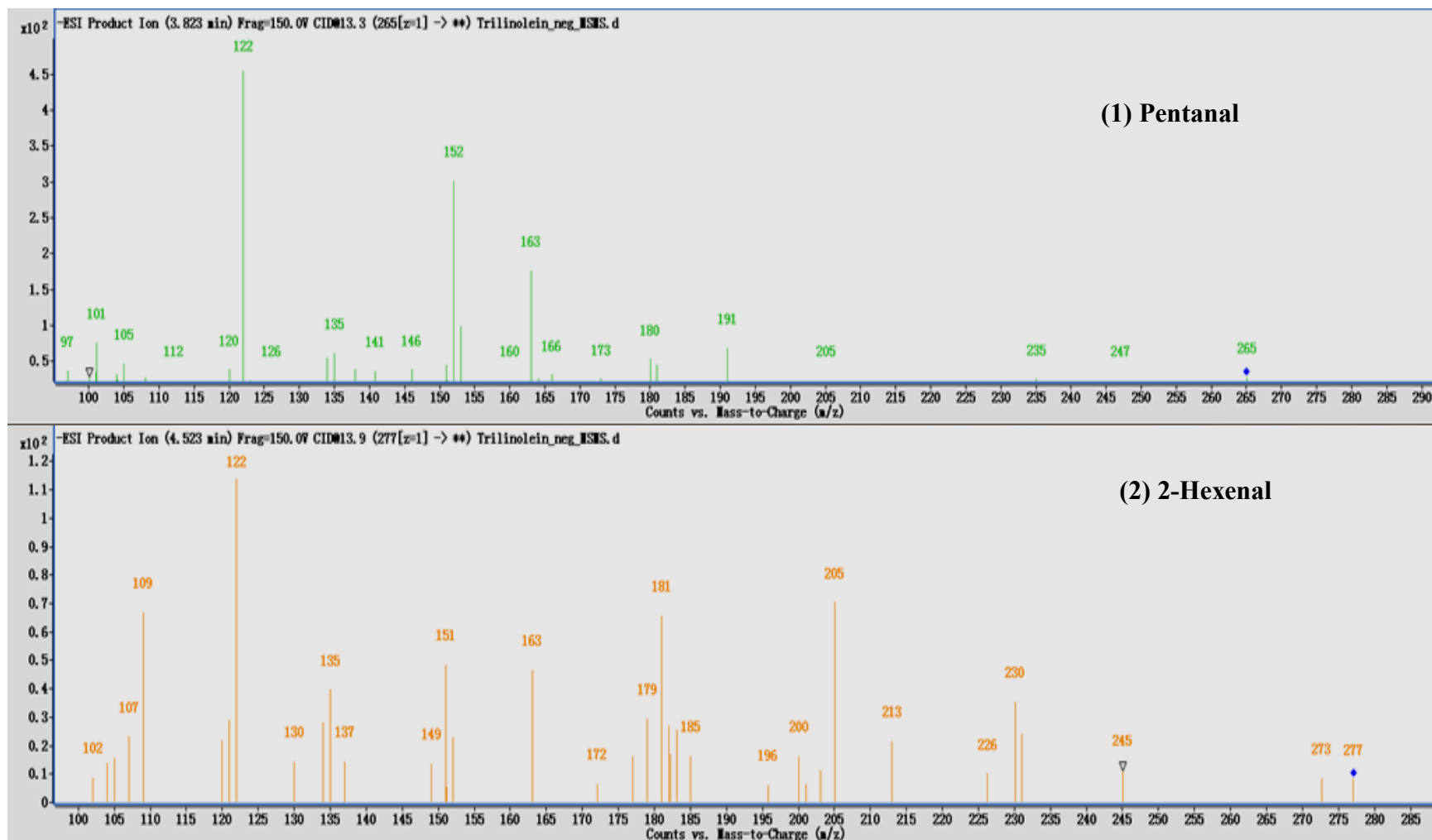


Figure 59 . CID MS spectra of pentanal (top) and 2-hexenal (bottom) DNPH hydrazones.

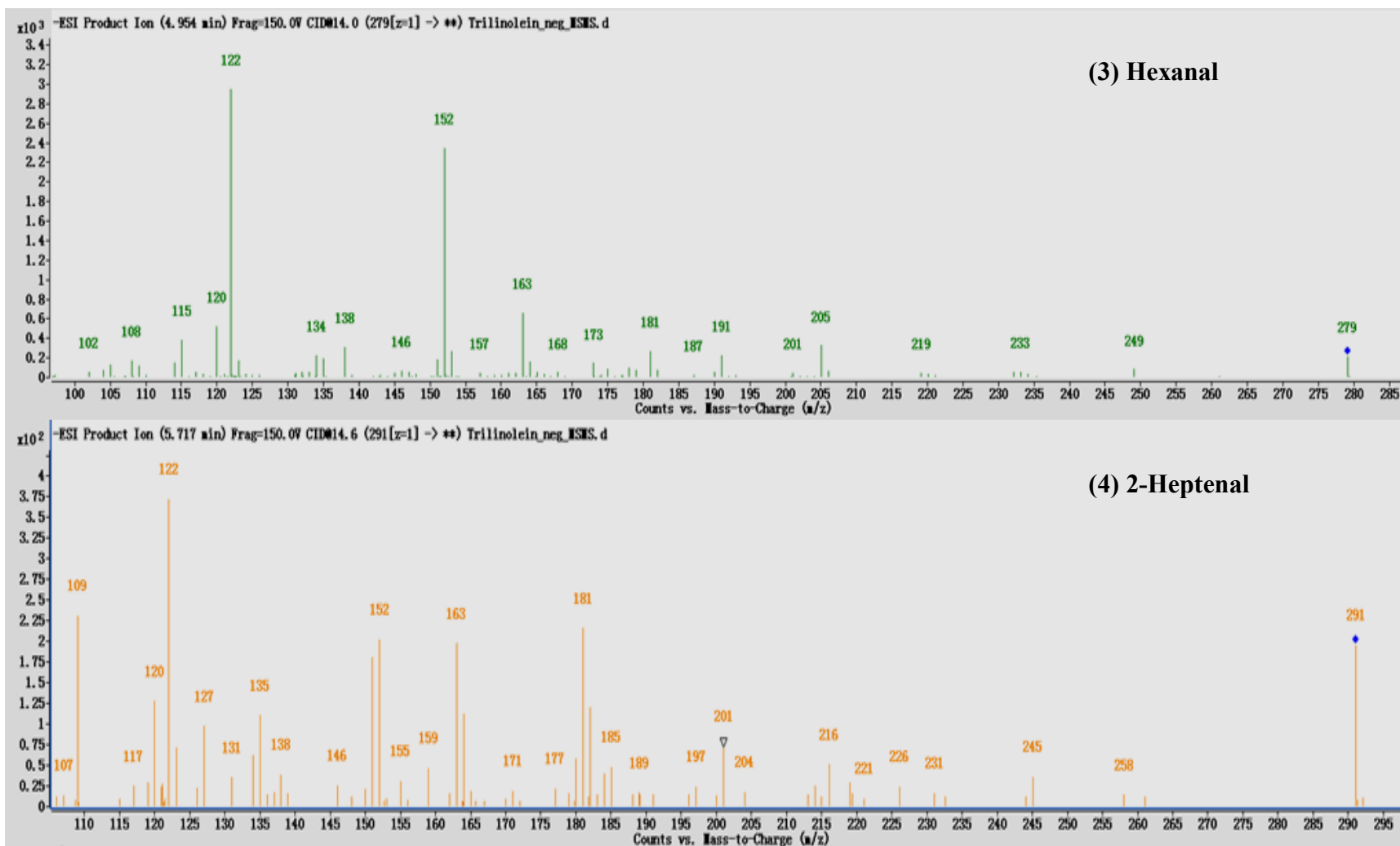


Figure 60. CID MS spectra of hexanal (top) and 2-heptenal (bottom) DNPH hydrazones.

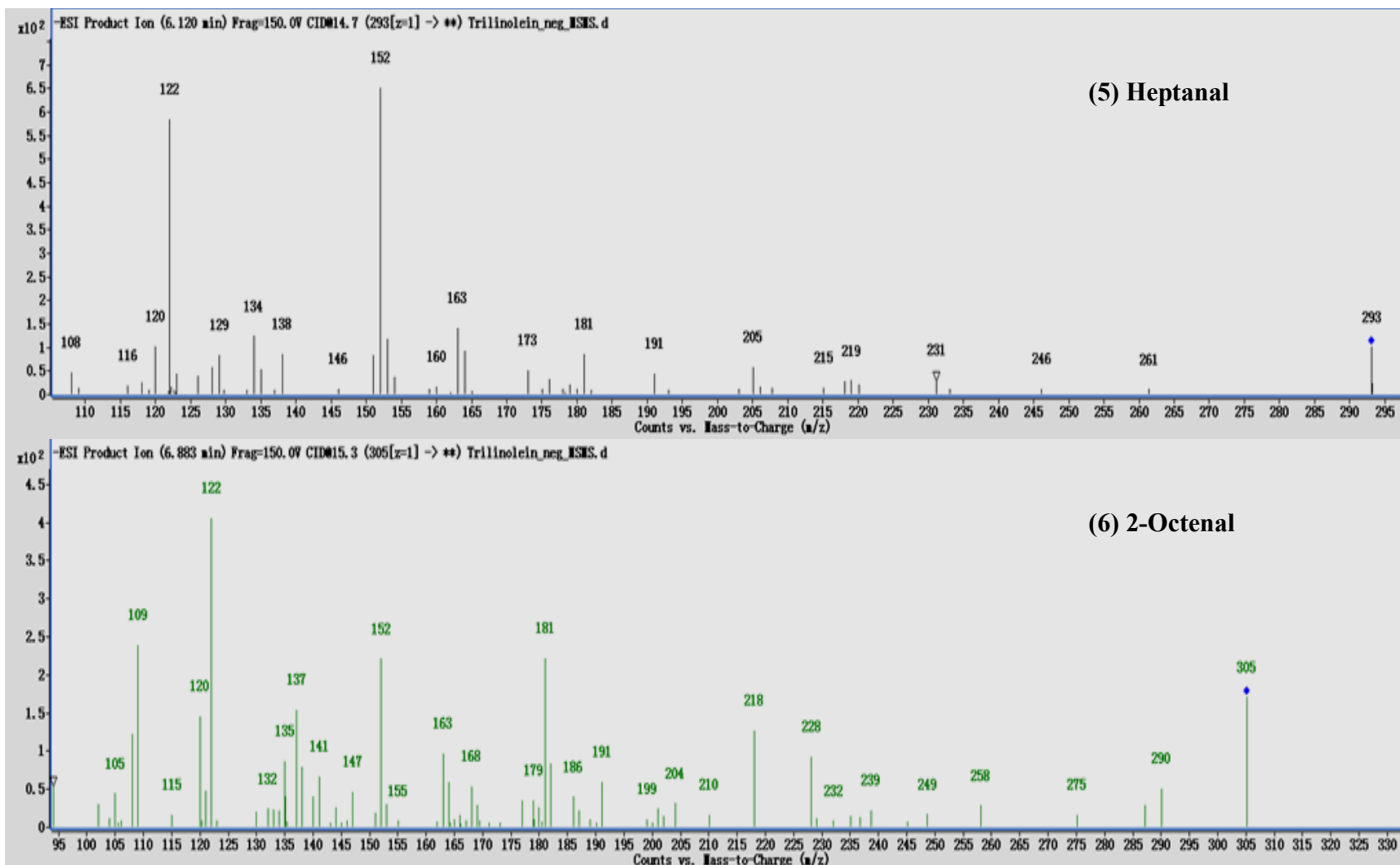


Figure 61. CID MS spectra of heptanal (top) and 2-octenal (bottom) DNPH hydrazones.

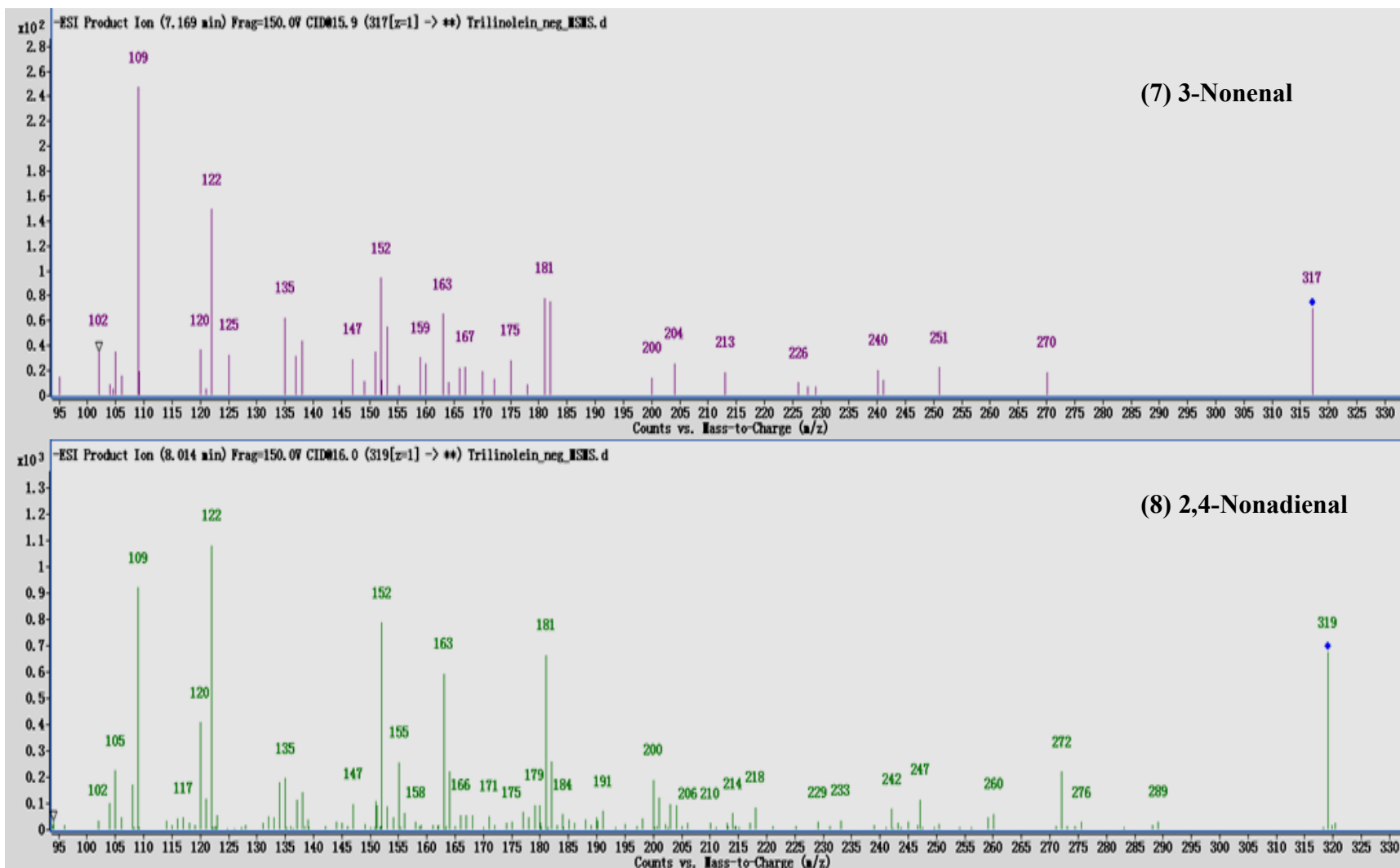


Figure 62. CID MS spectra of 3-nonenal (top) and 2,4-nonadienal (bottom) DNPH hydrazones.

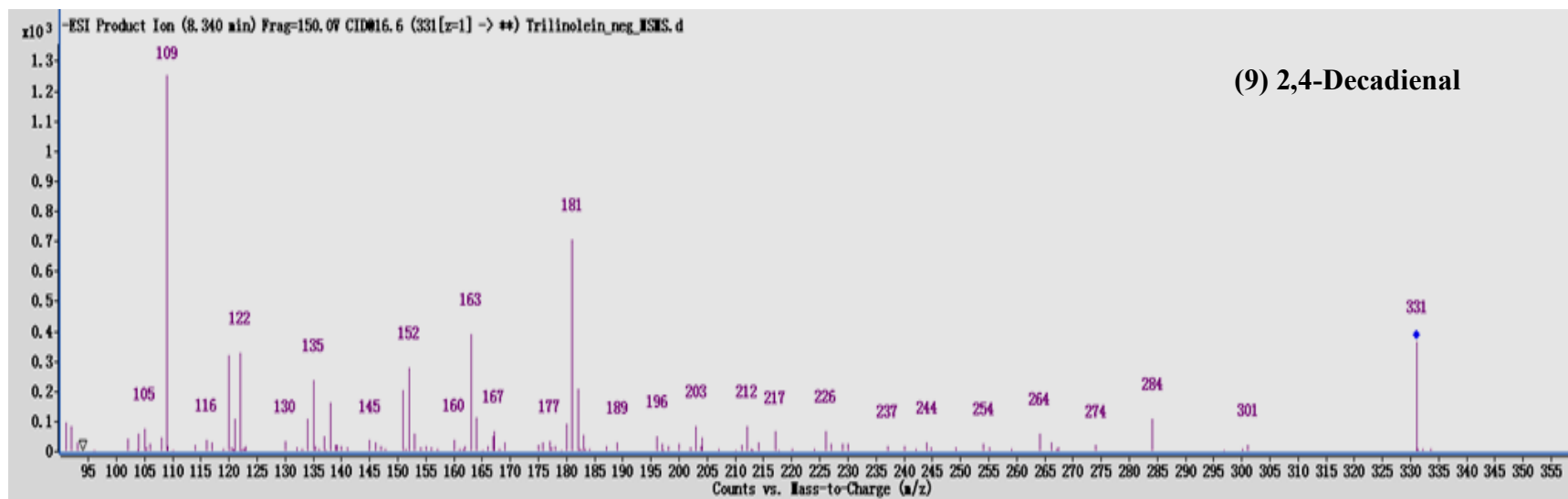
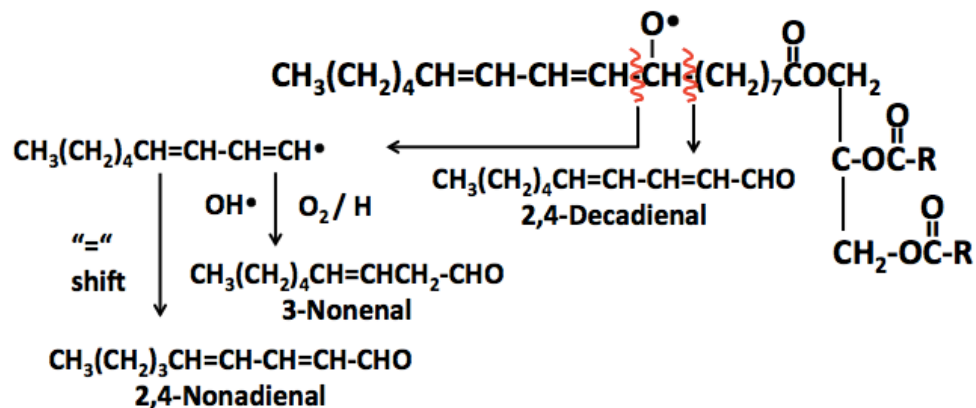
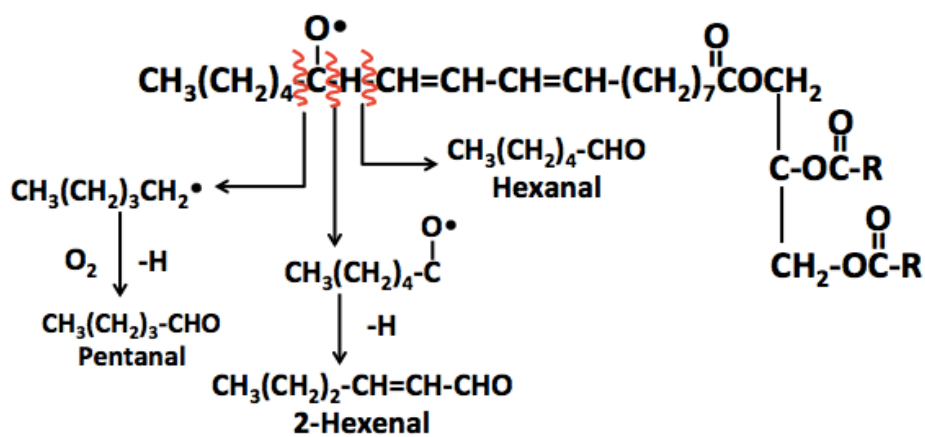


Figure 63. CID MS spectrum of t,t-2,4-nonadienal DNPH hydrazones.

9-LO•



13- LO•



11- LO•

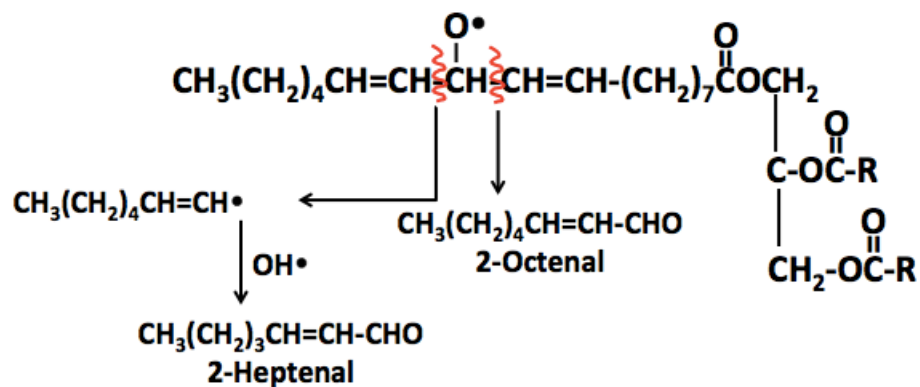


Figure 64. Pathways for generation of observed carbonyl products from scissions of C9, 11, and 13 alkoxy radicals.

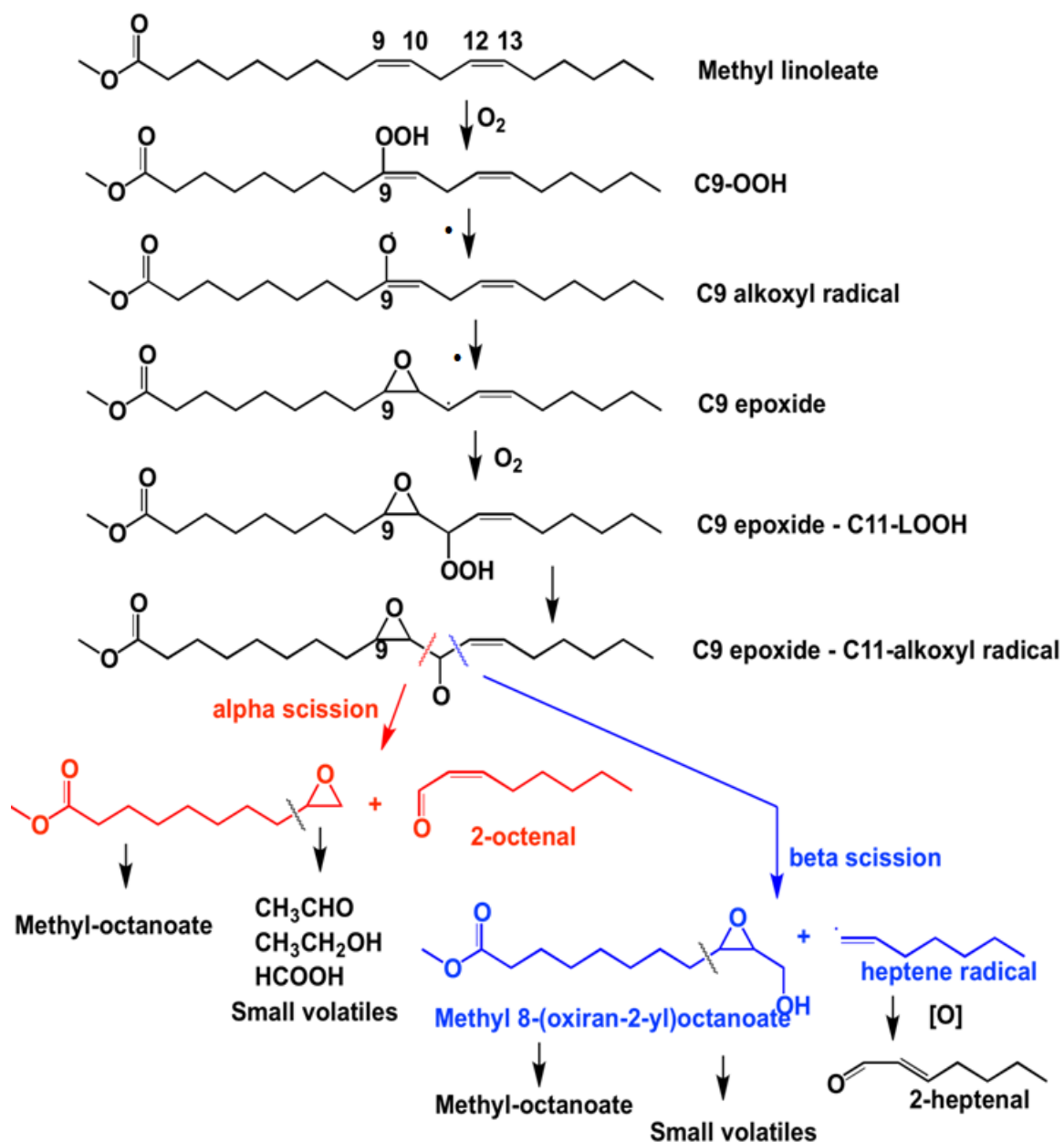


Figure 65. Pathways for formation of heptenal and octenal from internal rearrangement of C9 alkoxy radicals to epoxides, with transfer of a free radical to C-11, followed by oxidation and scissions at that site.

Table 13. Quantification of carbonyl monomers from oxidized trilinolein using standard calibration curves and averaged chain length calibration curves

Carbonyls	Individual standard (mM)	Average standard (mM)
Pentanal	N/A	0.14±0.00
Hexanal	0.52±0.01	0.58±0.02
2-Heptenal	0.19±0.00	0.16±0.00
2-Octenal	0.12±0.00	0.13±0.00
2, 4-Nonadienal	0.12±0.01	0.13±0.01
2-Nonenal	N/A	0.17±0.01
2, 4-Decadienal	0.15±0.00	0.16±0.00

Note: Concentrations of monomer carbonyls were determined from both individual standard curves for each carbonyl, identified by its retention time, and from the average standard curve for the chain range based on chain length classification ($\leq C6$, $C7-C10$, and $\geq C11$) (Table 12). Results from the two calculations are very close, validating the use of average regression equations for quantification of unknown carbonyls based on their retention times.

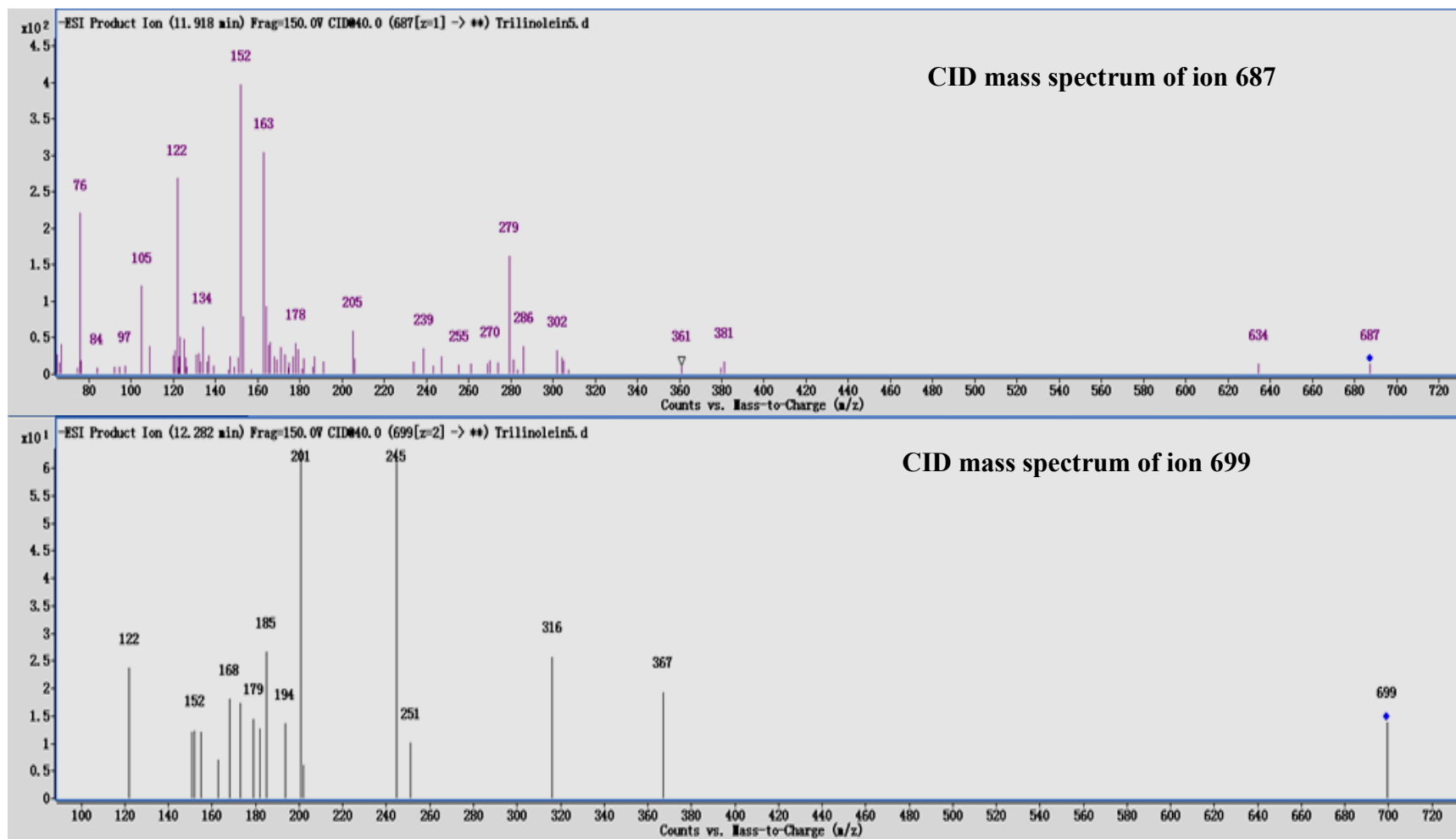


Figure 66. CID mass spectra of core aldehydes ions of 687 (top) and 698 (bottom).

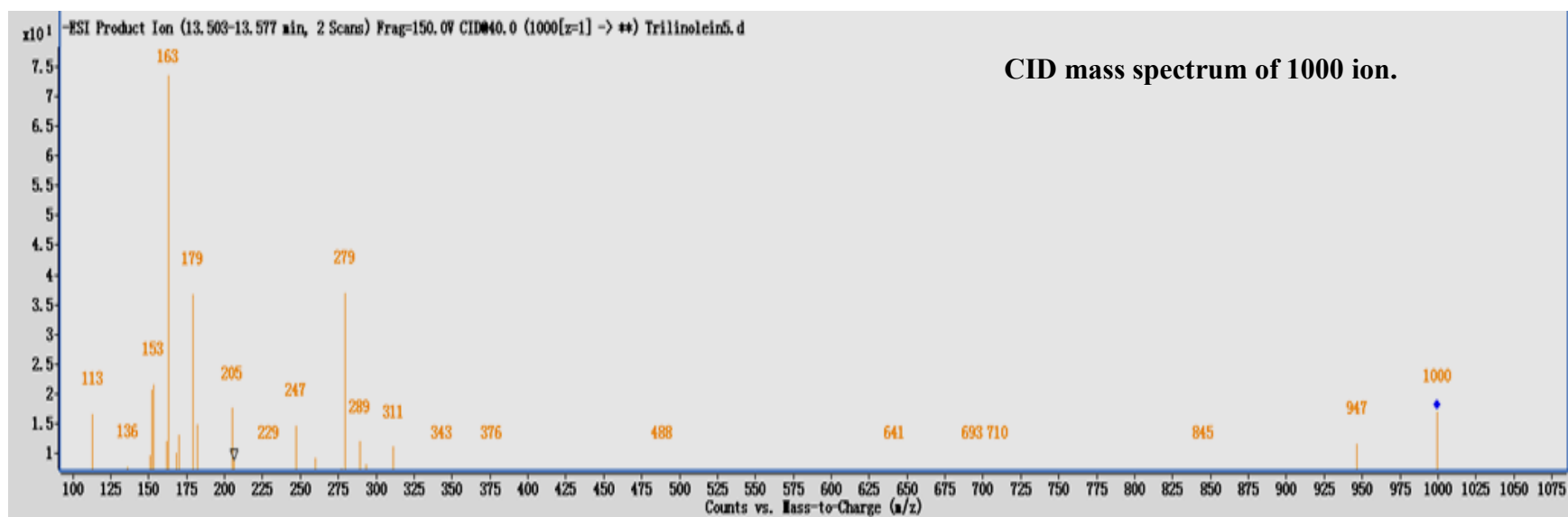


Figure 67. CID mass spectra of core aldehydes ions of 1000.

6.2.7.3 Commercial food oils

To further test general applicability, the HPLC-DNPH method was used to detect carbonyl products in commercial corn oil and olive oil oxidized for 6 days. HPLC chromatograms are shown in Figure 68. Since these oils contain substantial levels of natural antioxidants, the extent of oxidation in these samples low, but both monomer and core carbonyls were detected in the oils. Surprisingly, despite lower unsaturation (mostly oleic acid), oxidized olive oil appeared to have higher levels, more different core carbonyls, and better resolution than corn oil (mostly linoleic acid). In contrast, core carbonyls in oxidized corn oil showed poor resolution with substantial co-elution over a broad retention time range. One possible explanation is that corn oil has 60% of linoleic acid in its fatty acid profile [148] while olive oil has 77% of oleic acid. With dominant monounsaturations, oleic acid has fewer options for core carbonyls composition while corn oil has multiple fatty acids plus multiple positions for oxidation within linoleic acid, so can potentially form many types and isomers of core carbonyls.

Many carbonyl peaks in Figure 68 were too tiny to identify and quantify, indicating that six days incubation might not be long enough for the commercial oils to accumulate quantifiable carbonyls. To test the DNPH methodology further, another two oil samples were oxidized (commercial vegetable oil incubated at 60 °C for 2 months and stripped soybean oil incubated at 60 °C for 6 days) and analyzed by the DNPH-HPLC assay. Results showed notably higher levels of monomer and core carbonyls in both oxidized oils than observed in the first study (Figures 69,70). The vegetable oil (soybean oil) presented more carbonyls than the stripped soybean oil, probably because it had been oxidized longer. Major monomer carbonyl compounds were identified were butanals and

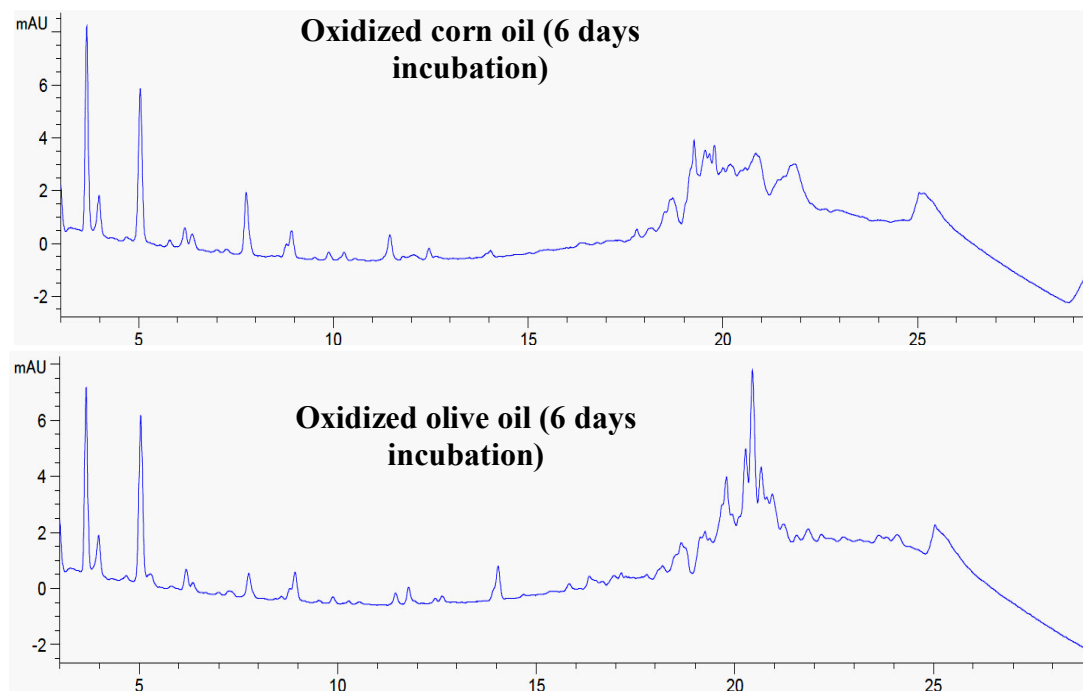


Figure 68. HPLC chromatogram of DNPH derivatives of oxidized commercial corn oil, olive oil, 360 nm detection.

<C4 aldehydes rather than the expected product hexanal. This suggests that hexanal cannot be used as a universal oxidation indicator in all materials. Even more striking was the high levels of core carbonyls in the vegetable oil, showing the importance of carbonyls that are not volatilized but remain behind attached to the glycerol backbone in oils. That the peaks around 20 minutes retention time were from derivatized triacylglycerols was verified by 206 nm detection of unoxidized triacylglycerols eluting at 21-29 minutes (Figure 69).

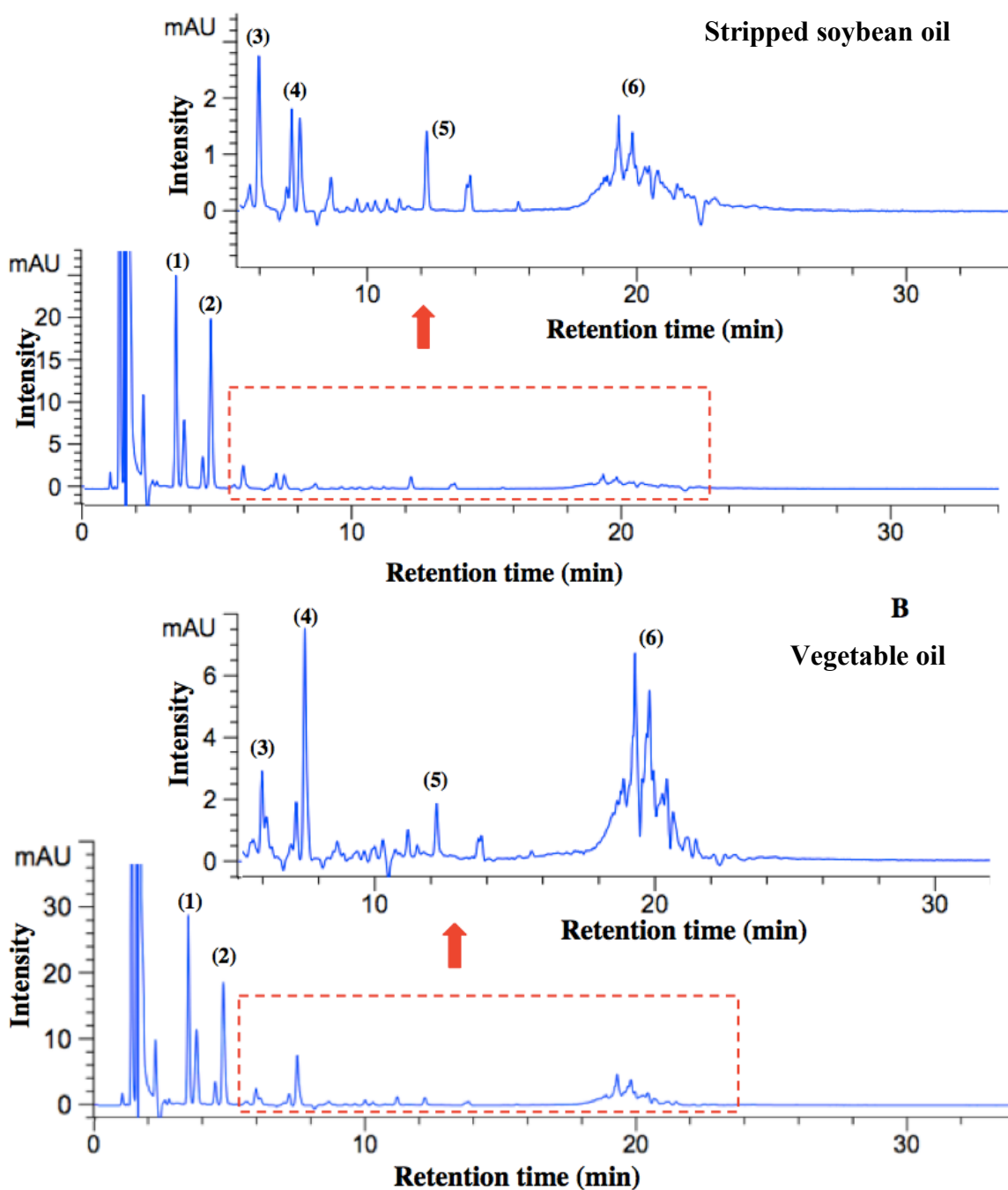


Figure 69. HPLC chromatogram of DNPH derivatives of oxidized stripped soybean oil (A, upper) and oxidized vegetable oil (B, lower) at 360 nm UV detection. A/B: (1) <C4 carbonyls, (2) butanal, (3) pentanal, (4) hexanal, (5) t-2-decenal and (6) core aldehydes.

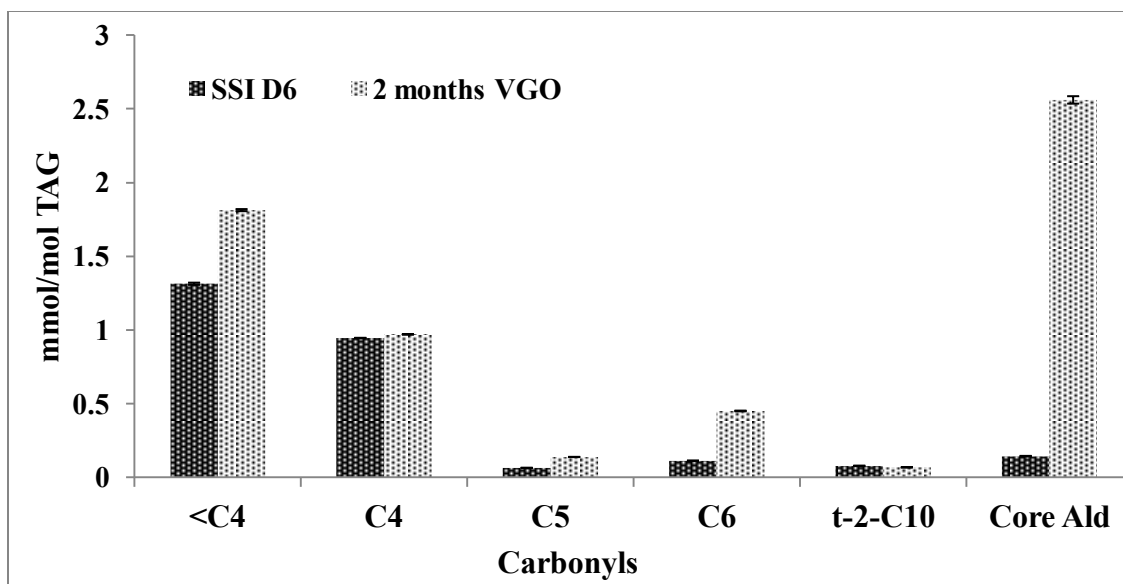


Figure 70. Comparison on carbonyl amount (mmol/ mol TAG) between six days incubated stripped soybean oil and 2 month incubated commercial vegetable oil.

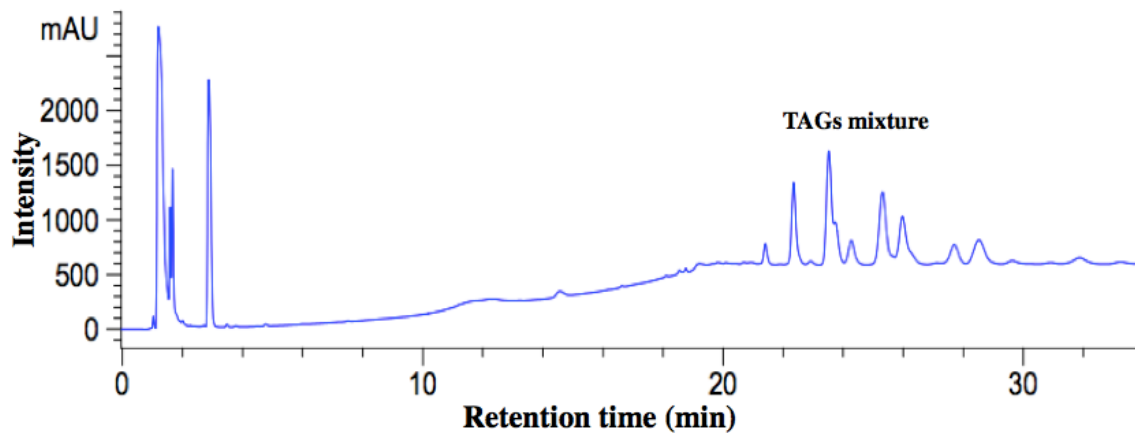


Figure 71. HPLC chromatogram of DNPH derivatives of oxidized vegetable oil at 206 nm UV detection

6.2.7.4 Lipid extracts from pet food kibbles and homemade crackers

ASE lipid extracts from oxidized pet food kibbles were reacted with DNPH and the resulting hydrazones were separated by HPLC. Both carbonyl monomers and core aldehydes were detected (Figure 72). Monomers identified by comparison with standards were <C4 carbonyls, butanal, pentanal, hexanal, *t*-2-decenal, and core aldehydes. Two distinctive core aldehyde peaks were detected at 17.5 and 18.5 minutes in chicken & rice (A) and fish & potato kibbles (B) respectively (Figure 72). Unlike the oils analyzed in experiments described above, lipids from pet food kibbles contained considerable amounts of phospholipids which are also likely to form core carbonyls. In Figure 72, peak (6) was not fully identified, but according to its retention time should be between monomers and triglycerides in size. Thus, it is not unreasonable to assign this peak to phospholipid core carbonyls. Interestingly, carbonyls were higher in kibbles with fish than with chicken, as might be expected from the high n-3 polyunsaturated fatty acid content.

That the profiles of the core aldehydes in the two kibbles were much simpler than the commercial oils probably resulted from interactions of the core aldehydes with proteins in the two kibbles. This observation stresses the importance of the role of core carbonyls in co-oxidations of proteins and other macromolecules in complex food systems.

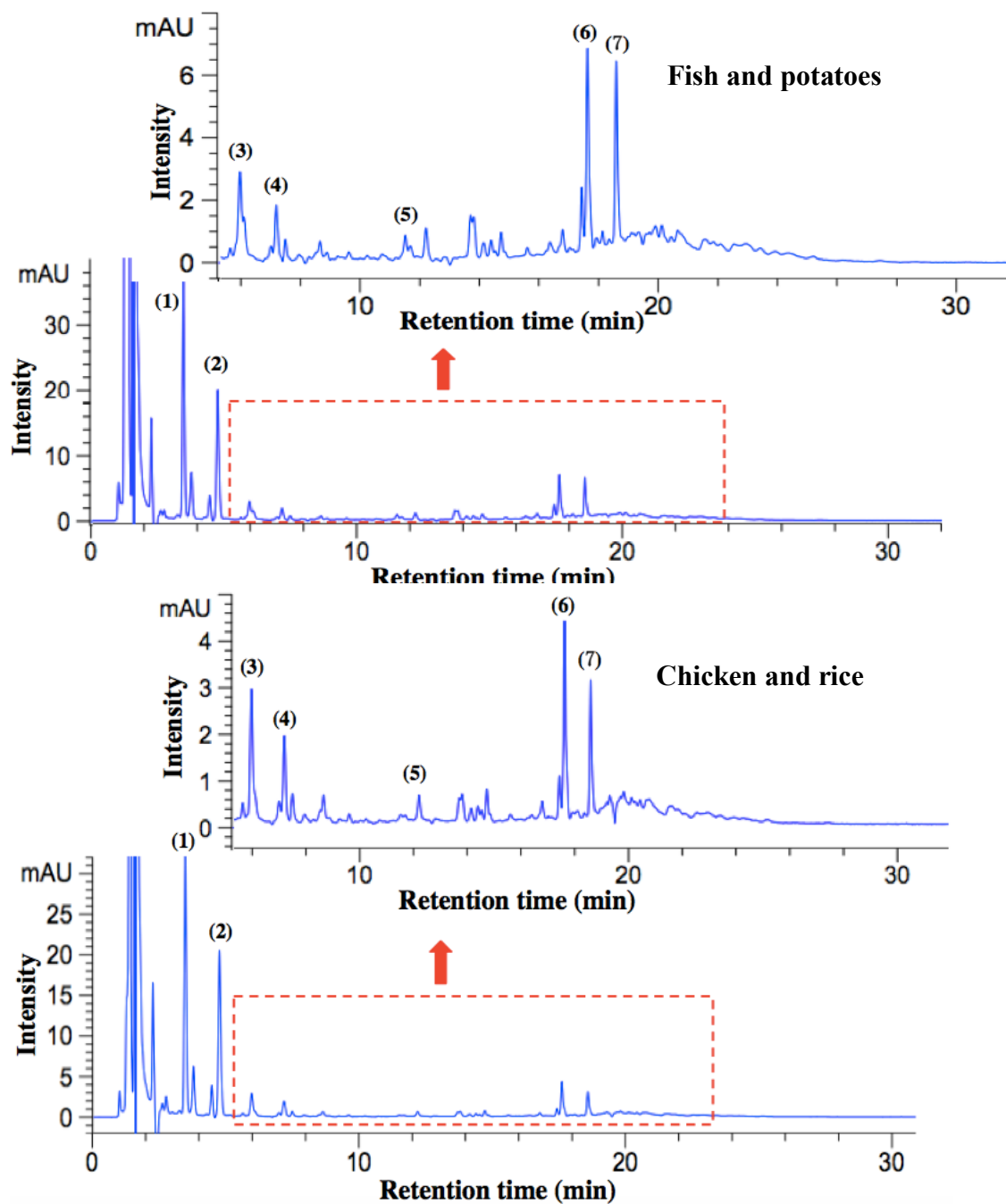


Figure 72. HPLC chromatogram of DNPH derivatives of lipid extracts from oxidized fish & potato kibbles and chicken & rice kibbles at 360 nm UV detection. In A/B: (1) <C4 carbonyls, (2) butanal, (3) pentanal, (4) hexanal, (5) t-2-decenal, (6) and (7) core aldehydes.

In a second food test, tocopherol was added to stripped soybean oils and combined with flours and other ingredients to make baked crackers. Crackers were incubated at 40 °C in the dark, ASE-extracted at 18 and 32 days, then the lipid extracts were analyzed for carbonyl products by the HPLC-DNPH assay. The carbonyl profiles observed were similar to those in stripped soybean oil incubated alone (Figure 73), and both total and individual carbonyls increased with incubation time. Pentanal, hexanal and *t*-2-decenal showed slight increases over time; <C4 carbonyls, butanal, and especially core aldehydes increased more dramatically.

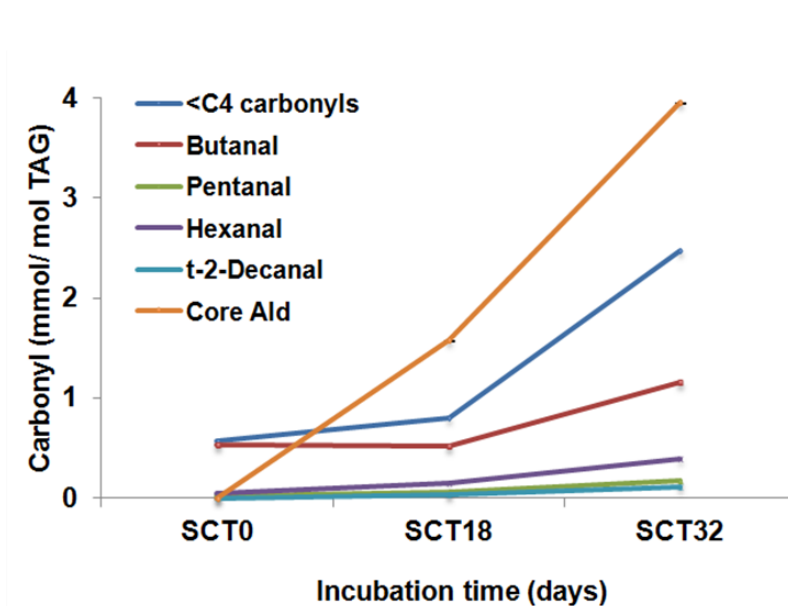


Figure 73. Carbonyl oxidation products in lipid extracts from stripped soybean oil crackers incubated for 0, 18 and 32 days.

7. CONCLUSIONS

This project addressed two important challenges in lipid oxidation analyses: the absence of lipid extraction methods that are efficient yet protect lipids from oxidation, and lack of sensitive, useful methods for analyzing non-volatile carbonyl products and distinguishing monomer from core aldehydes.

To address the first challenge, pressurized solvent extraction (or accelerated solvent extraction) was evaluated for effects on lipid oxidation and ability to extract lipids from a difficult food matrix where extreme extraction procedures are usually required. Exposure of corn oil to each step of ASE extraction conditions showed that little new oxidation was induced and existing oxidation products were not decomposed as long as extraction temperature was limited to 40 °C and samples were protected during handling and preparation.

Baked and extruded dry pet foods were used as a model system to test extraction efficiency and optimization requirements of accelerated solvent extraction. Effects of solvent, static extraction time and number of static cycles, extraction temperature, sample pre-treatments such as grinding to small particle size and hydration were investigated. Results provided a number of guidelines that can be applied to ASE extraction of any material. First, extraction conditions (especially solvent, static time, and numbers of extraction cycles) must be tailored and optimized for each different product. Lipid extraction efficiency increases as particle size decreases, so foods should be ground to particle sizes of about 250 μm for maximum extractability. Of course, samples must be protected from oxidation and light during this grinding. Extraction temperatures must be limited to 40° C to limit lipid oxidation. More short extraction cycles (10-15 min) remove

more lipid than fewer long extractions (20-30 min) -- long times under pressure in the extraction cells appear to compact the food matrix or enhance lipid binding to macromolecules, reducing extractability.

All extraction methods offer options for using different solvents to selectively extract specific lipid classes. However, ASE appears to be unique in making it possible to use two immiscible solvents because they are injected into the extraction cell separately. This capability allowed use of a combination of hexane and methanol, which are normally immiscible. Extractions of both baked and extruded dry pet foods showed that the 2:1 hexane:methanol combination gave lipid yields from pet foods comparable to traditional chloroform:methanol, thus providing an option for eliminating toxic chlorinated hydrocarbons in lipid extractions. However, oxygen is more soluble in hexane, so extra degassing is necessary when this solvent is used.

Chloroform, chloroform:methanol 2:1, and hexane:methanol 2:1 all gave good lipid yields – nearly 100% from baked biscuits which had a more open structure, and >90% after optimization from extruded kibbles which had very dense structure. Even extractions that were not optimized with several different kibbles gave yields of 75-85%. These yields were better than manual extractions and were obtained in 30 to 60 minutes, compared to 24-48 hours for manual extractions.

Overall, ASE extractions of lipids from pet foods were fast, efficient, and easy to handle while not inducing or modifying lipid oxidation. Altogether, our results suggest that accelerated solvent extraction should become the preferred method for extracting lipids when analysis of lipid oxidation is the end point.

To address the second challenge, procedures for a basic DNPH (2,4 - dinitrophenylhydrazine) analysis of carbonyls were upgraded to develop an HPLC method capable of detecting, quantifying, and identifying non-volatile carbonyl oxidation products in a broad range of lipid molecules, from simple to complex, in pure materials, oils, and extracts. A particular focus of the analysis was distinction between monomer (free) carbonyls and core carbonyls attached to phospholipids and acylglycerols.

DNPH was shown to be specific in derivatizing carbonyls without reacting with other oxidation products such as hydroperoxides, epoxides, and alcohols. The sulfuric acid traditionally used in DNPH reactions degrades HPLC columns over time, but can be effectively replaced by formic acid for HPLC applications. Hydrazones of saturated carbonyls were reasonably stable (days) at both room and refrigerated temperatures; hydrazones of unsaturated carbonyls in HPLC vials began degrading after one day at room temperature, but were stable at freezing condition in a sealed vial.

The HPLC-DNPH assay was very sensitive, detecting 6-50 $\mu\text{g/L}$ and quantitating 18-156 $\mu\text{g/L}$ over all the monomer standards tested aldehyde.

DNPH hydrazones of saturated carbonyls (C4 to C11) could be baseline separated on a reverse phase C18 column, but when mixed with hydrazones of unsaturated carbonyls, some co-eluting peaks presented. Elution of saturated/unsaturated carbonyl mixtures showed reasonable separation of peaks but some critical pairs (saturated aldehyde and monounsaturated aldehyde one carbon longer) still co-eluted. Normal adjustments in solvents, elution gradient, flow rate, and column temperature were unable to provide baseline resolution. A C30 column provided better resolution but still could not completely separate two critical pairs of diene aldehydes, and the elution time was

more than doubled. Thus, some additional optimization of column is still needed, and choice of column will be largely controlled by end-use of the analyses (quality control vs quantitative research vs research on oxidation mechanisms).

HPLC coupled with MS verified that detected peaks were indeed DNPH derivatives of carbonyls and identified components of overlapping peaks by the distinctive parent ions. Quantitatively, the extinction coefficients and slopes of concentration response curves of DNPH hydrazones decreased with the chain length of the carbonyl. Thus, a single carbonyl standard cannot be applied for all carbonyls in HPLC-DNPH analyses. Options are expressing results as equivalents of a given carbonyl standard or using the average regression equation for standards in different chain length ranges (short, medium, and long carbonyls). Applying individual vs average regressions for the standard aldehydes gave very close results, demonstrating that unidentified carbonyls can be quantitated with reasonable accuracy using average regressions determined by retention times (chain lengths).

A two-phase solvent gradient based on acetonitrile, isopropanol and water was able to separate carbonyl monomers from core aldehydes on acylglycerol backbones. In application tests of the assay, both carbonyl monomers and core aldehydes were detected in oxidized trilinolein, demonstrating that core aldehydes could be formed during scission reactions of lipid oxidation and left behind on glycerol backbones. Carbonyl monomers from oxidized trilinolein were identified and quantified. Hexanal was the major monomer carbonyl, although other short-chain products were present, supporting evidence from other studies that scissions occur predominantly at C13. Carbonyl monomers and core aldehydes were detected clearly in oxidized corn oil, olive oil and lipid extracts,.

These two analytical methods should make it possible to determine the degree of lipid oxidation more accurately and reproducibly in both quality control and research, and provide a better understanding of lipid oxidation mechanisms.

8. FUTURE WORK

Studies done in this project provided two analytical tools to analyze lipid oxidation degree quantitatively and qualitatively, but there are still some areas worth investing to obtain more details in understanding the mechanism of lipid oxidation.

1. ASE extraction method can be applied on known composition extruded pet foods to study extractability of lipids and lipid-starch or protein bonding in extruded foods.
2. Investigate stability of the other oxidation products such as carbonyl and epoxides by spiking these compounds in intact corn oil and run the ASE process, then optimize the ASE handling to protect these existing oxidation products from being decomposed.
3. Isolate and separate the core aldehydes and develop a more specific elution conditions and LC-MS/MS method to identify and quantitate core aldehydes in oxidized trilinolein.
4. Investigate detection limit of ELSD detector on carbonyls-DNPH compounds to avoid analytes' extinction coefficient issues and develop a more accurate quantitative method.
5. Study the core aldehydes formed on phospholipids glycerol backbones by HPLC and LC-MS/MS. More specific elution conditions might be required to obtain better resolution.
6. Shelf life studies can be done in real food system by applying the ASE method and DNPH-HPLC-ESI-MS/MS method on food oil and lipid extracts to determine the lipid oxidation degree quantitatively and qualitatively.

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