HPLC SEPARATION OF TOTAL LIPID CLASSES AND SIMULTANEOUS

ANALYSIS OF LIPID OXIDATION PRODUCTS

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ABSTRACT OF THE DISSERTATION

HPLC Separation of Total Lipid Classes and Simultaneous Analysis of Lipid

Oxidation Products

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Reformulation of foods to include nutritionally adequate levels of polyunsaturated fatty acids is seriously challenging existing lipid analysis methods. Current label requirements with detailed accountability for food composition create increase demands for analysis of total lipid composition that are accurate, sensitive, and fast. In addition, presence of unsaturated lipids dramatically increases oxidation, which is perceived as "rancid" off-odors and flavors by consumers, and food companies must be able to track this degradation to adequately stabilize foods and know when to pull products from shelves. Traditional methods of lipid analysis do not adequately meet these challenges.

This dissertation research seeks to develop improved reversed phase high pressure liquid chromatography methods that simultaneously separate the lipid fractions most important in foods (free fatty acids, acylglycerols, and phospholipids) and quantitates each fraction while also analyzing lipid oxidation products. To achieve baseline separation of lipid lcasses, basic acetonitrile/water

gradients were modified with hexane and isopropanol to solubilize long chain saturated fatty acids and triacylglycerols, and the aqueous phase was buffered to pH 6.8 to dissociate fatty acids and facilitate their separation from monoacylglycerols. Under these conditions, fractions separated according to equivalent carbon number. A cyano column (to establish column triple bond interactions with lipid double bonds) in tandem with the reversed phase column did not increase separation of components within classes. Silver ion chromatography could not be applied because silver ions precipitate at neutral pH.

Evaporative light scattering successfully detected low concentrations of all lipid fractions, including saturated components, but slopes of concentration curves varied with each lipid component. This allows quantitation but necessitates separate calibration curves for each component.

A coulometric electrochemical cell with graphite electrode detected hydroperoxides but not aldehydes or epoxides. However, dinitrophenylhydrazones of aldehydes were detected by the coulometric cell; the response appears to be sensitive (micromolar) so may offer a superior alternative to optical detection of these products. Preliminary tests demonstrated superior detection of hydroperoxides with linear concentration response using an amperometric cell with glassy carbon electrode. Further studies with electrochemical detection of lipid oxidation products should focus on this method.

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1. INTRODUCTION

The first impression of lipids for most people is usually more negative than positive because of the common association with obesity, unhealthy food, cardiovascular disease, cancer, and more. While the "dark" side about lipids may have some truth, the positive flipside seldom receives attention. For food scientists, lipids are important from many aspects. First, lipids are a crucial energy source that, together with carbohydrates, provides the basic energy supply for humans. This makes them a target of controversy in food formulations where reduction of calories must be balanced against essential fatty acid requirements for brain, eye, and nerve health, prostaglandin production, skin integrity, and reproductive health. In the formulation of foods, lipids are also a key puzzle piece to sensory satisfaction because they provide a rich and creamy texture and mouth-feel. As the most important flavor solubilizer and carrier and a key source of natural and process flavors, lipids also bring pleasant taste and flavor to foods. Thus, tracking and documenting lipid composition of foods accurately is critical for both quality control and research.

A second important facet of lipids in foods is the ease of oxidation of unsaturated lipids, leading to production of off-odors and flavors referred to as "rancidity". Lipid oxidation has been long recognized as the major chemical reaction limiting the shelf life and the quality of the foods through several paths. Oxidation products generate off-flavors and co-oxidize proteins, vitamins and pigments. Emulsion structure can be compromised as polar products migrate to interfaces and alter interfacial tension; oxidation of lipid molecules used as the

emulsifiers also disrupts interfaces. Lipid oxidation mediates browning reactions through the generation of carbonyl compounds that condense with amino groups, and it produces toxic products[1]. Thus, detailed understanding of oxidation mechanisms, as well as accurate and detailed analyses of oxidation extent and product patterns, is crucial for stabilization of foods.

The food industry has evolved through several approaches to prevent lipid oxidation. Prior to the 1900s, animals were the only source of fats and oils for human consumption. Since these were highly saturated, oxidation was relatively easy to prevent. However, as the population boomed after 1900, availability of animal fats could not keep up with the increased demand for consumption, so fats and oils of vegetable origin were adopted as supplemental sources. Being highly unsaturated, these materials were much more prone to oxidation and development of rancidity, so to stabilize these products, hydrogenation was applied to remove sensitive double bonds, increase the saturation level, and create semisolid products.[2] After World War II, addition of antioxidants to slow oxidation also became commonplace[3–5].

More recently, consumption of saturated fats and trans fats was linked to development of atherosclerosis and cardiovascular disease[6–9]. These health concerns forced the industry to reformulate foods with polyunsaturated fatty acids (PUFAs), which in turn has presented significant new challenges in preventing oxidation and stabilizing foods. Potent synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been widely used for decades to prevent oxidation, but new concerns about the toxicity of

BHA and BHT are forcing restrictions on their use in foods[10,11]. Thus, the food industry is actively seeking safer alternatives. In response, natural antioxidants have gained attention and popularity from both food manufacturers and the general public, under the assumption that they are safe and also provide potential nutraceutical functions[12]. Unfortunately, attempts to use natural antioxidants in stabilizing foods have often been unsuccessful. It is clear that there is much to learn, both about mechanisms of action of natural antioxidants and about lipid oxidation reactions.

All these strategies for reducing lipid oxidation have significant shortfalls and limitations. In addition, results in our laboratory (data not shown) often contradicted classical lipid oxidation theory in a number of aspects. These two factors together led to re-evaluation of the standard theory, and in 2005 Schaich[13] proposed a new reaction scheme for lipid oxidation which integrates alternate reaction pathways such as internal rearrangements, addition, and scission reactions of lipid peroxyl and alkoxyl radicals with conventional hydrogen abstraction, as will be discussed in detail in Section 2.1.4. The integrated theory is solidly grounded in research observations but, for validation, the simultaneous presence of alternate competing pathways needs to be demonstrated in real systems. This presents two key challenges in lipid analysis.

The first challenge is how to identify and quantify lipids present in a given sample. Our laboratory has developed improved methods using pressurized solvents and mild heat to extract lipids quantitatively even from difficult matrices while minimizing oxidation[14]. This makes it possible to obtain clean extracts for

analysis. Paired with extraction, then, is identification of the lipid components in extracts (or oils). For this, thin layer chromatography (TLC) is a very handy and rapid technique, easy to set up and with low cost. Certainly, TLC has been used in our lab to produce valuable data regarding efficiency of extraction and lipid class composition of lipid extracts from various food sources. However, using only a general characterization of lipid species is inadequate for studying oxidation mechanisms because quantification is also critical for determining mass balance and stoichiometric yields.

High performance liquid chromatography (HPLC) is a more advanced alternative that is both more sensitive and capable of quantification. Lipid class separation by HPLC has been studied extensively, but mostly in the context of specific matrices. For example, some applications were developed for foods high in saturated fat, some targeted omega-3 rich foods, and others dealt with lipid molecule of medium saturation. It is truly difficult to find a universal program that will fit all lipid compositions and applications. Moreover, the gradient programs that are inevitably required vary tremendously from case to case and cannot be easily adapted to other applications without major change. Switching mobile phases even within systems can be disastrous for HPLC analyses, and especially problematic when switching between normal phase (NP) and reverse phase (RP) to fit a specific separation. Nevertheless, having a generic HPLC method to fit most, if not all, food matrices can be extremely beneficial for both academic research and industrial quality control labs. Such a methodology would eliminate the need for multiple systems and would tremendously reduce the

tedious and laborious work to make and change mobile phases from matrix to matrix.

The second significant challenge in validation of the alternate oxidation pathways proposed by Schaich[13] is lack of sensitive methods for detecting and quantifying multiple classes of lipid oxidation products beyond hydroperoxides, as well as lack of methods that detect multiple products simultaneously in the same sample. Lipid oxidation can be grossly underestimated when it is assumed that all oxidations follow the same pathway and only a select few products are measured. For example, epoxides are seldom analyzed yet are among the most reactive and toxic products of lipid oxidation, and recent results in our laboratory find epoxides at levels comparable to or higher than hydroperoxides[15,16]

In addition, analyzing multiple classes of products individually by separate chemical analyses is laborious and time-consuming, difficult for one technician to handle in timely fashion, and provides only quantitative information. It does not provide the individual component information that is required for mechanism studies.

Thus, there is practical urgency for both research and quality control in industry to develop new analytical methods that can do "double-duty" in separating, identifying, and quantitating lipid components of foods and oils and in detecting and quantifying multiple lipid oxidation products simultaneously. The methods must be sensitive and chemically accurate, and as much as possible, independent of the lipid structure beyond the active functional group.

This research thus evaluated a) applicability of reversed phase high

pressure liquid chromatography to separate total lipid classes and thus provide more detailed lipid component information, b) evaporative light scattering detection to quantitate lipid fractions, and c) capability of diode array and electrochemical detectors to identify lipid oxidation products in the different classes during separation.

2. BACKGROUND

2.1. Lipid Class Separation by High Performance Liquid Chromatography (HPLC)

2.1.1. Historical development

High performance liquid chromatography is an analytical technique that separates mixtures of chemicals into individual groups or molecules, which are then characterized and quantitated via a variety of detectors. The separation mechanism is based on competing polar and hydrophobic interactions among analytes, stationary phase and mobile phases. The strong separating power, variety of stationary phases available, and flexibility of detection methods of HPLC make it a very attractive candidate tool for our research.

Lipids are a group of naturally-occurring hydrophobic molecules that make up membranes, storage oils, surface films, adipose tissue, and more. A comprehensive classification initiated in 2005 categorized lipid molecules into eight major classes[17]: fatty acids (FA), glycerolipids (GL), glycerophospholipids (GP, or PL), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL) and polyketides (PK); each class was further divided into multiple sub-classes. Figure 1 shows structures of each class. This classification system was later updated and revised to accommodate more lipid molecules found in various sources[18]. Among the eight classes, FAs, GLs, GPs, STs and SLs have received greatest attention since they are common components of food sources as well as biological matrices.

While all these classes of lipids share some characteristics, they also vary significantly in basic properties (Table 1) that clearly support their physiological functions but also create some significant challenges in analysis.
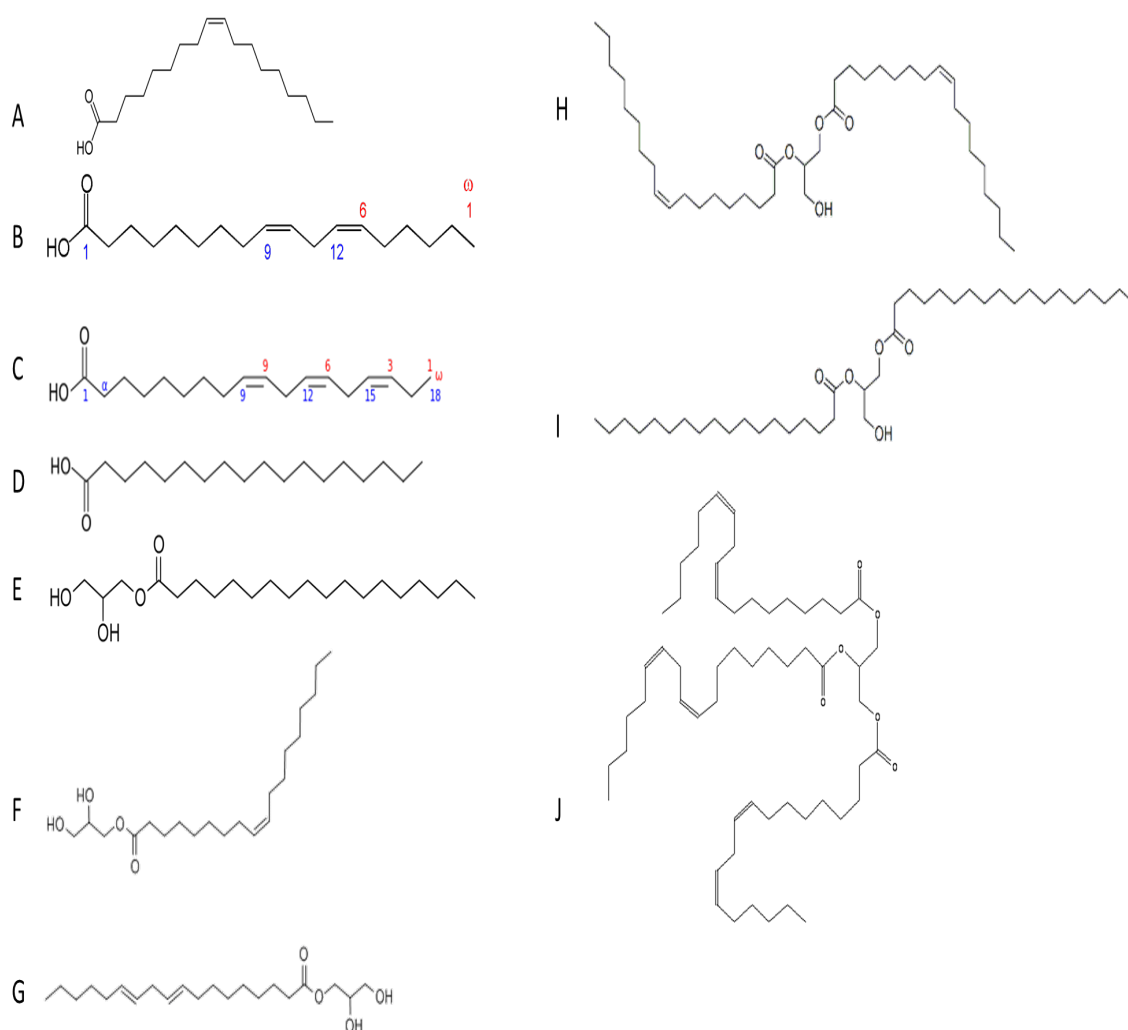
Figure 1. Structures of FFAs, MAGs, DAGs and TAGs. A: oleic acid, B: linoleic acid, C: linolenic acid, D: stearic acid, E: monostearin, F: monoolein, G: monolinolein, H: diolein, I: distearin and J: trilinolein[19].

Table 1. Physical properties of selected free fatty acids and triacylglycerols.[20]

Chemical Name	Vapor pressure (mm Hg at 25°C)	Melting Point (°C)	Boiling Point (°C)	pKa	Solubility[d]			
					Methanol	Ethanol	Acetone	Chloroform
Myristic acid	1.4×10^{-6}	53.9	326.2	4.8	S	S	S	S
Palmitic acid	3.8×10^{-7}	62.5	351.5	4.75	S	S	S	S
Stearic acid	4.28×10^{-8}	69.3	350	4.75	SS	SS	SS	S
Oleic acid	5.46×10^{-7}	16.3	286^a	5.02	S	S	S	S
Linoleic acid	8.68×10^{-7}	-8.5	230^b	4.77	S	S	S	S
Triolein	1.15×10^{-9}	-4	237^c	---	SS	SS	SS	S
Tristearin	5.4×10^{-17}	55	N/A	---	NS	NS	S	S

a: at 100 mm Hg
b: at 16 mm Hg
c: at 18 atm
d: S: soluble, SS: slightly soluble, NS: not soluble

2.1.2. Chromatography in lipid separations

Chromatography has been applied in the separation of lipid molecules for many decades. The first attempt to separate lipid classes was in the early 1940s, when Trappe[21] introduced silicic acid column chromatography to separate neutral lipid esters. Since then, chromatography has become a mainstay in lipid class analysis. Many methods used a step-wise strategy in which polar lipids and neutral lipids initially were separated into two groups, then further separation of each group was conducted separately. In his pioneering research, Borgstrom[22] reviewed three adsorbents that were popular in early normal phase

chromatography. The first adsorbent material, sucrose, was able to separate phospholipids (PLs), acylglycerols and FFAs, but its low loading capacity and high solubility in most eluents limited its general utility. Magnesium also was able to separate acylglycerols and PLs, but the resolution between PLs and FFAs was insufficient. The last adsorbent was silica acid, which proved to be the best material of the three: it separated PLs from both acylglycerols and FFAs and had high loading capacity as well.

Hamilton et al[23,24] used solid phase extraction on silica cartridges to separate PLs and neutral lipids, then each fraction was further separated on silicic acid with a simple isocratic program containing hexane, n-butyl chloride, acetonitrile and acetic acid. However, the normal phase column was not sufficiently selective to separate nonpolar acylglycerols. For example, triolein and trilinolein were incompletely resolved and even the separation of the FFAs was not very convincing. The author proposed that the polarity difference between one and two bonds was too small to be detected by the silica.

Hirsch[25] did achieve separation of complex lipid mixtures, including both neutral lipids [paraffin, squalene, cholesterol ester, cholesterols, triacylglycerols (TAGs), diacylglycerols (DAGs) and monoacylglycerols (MAGs)] and phospholipids in a single run, using silicic acid as the stationary phase. All long chain triacylglycerols ranging from C10 to C18 were separated from equivalent diacylglycerols as one group. However, the short chain TAG, triacylbutyrin, was found in the DAG fraction, and short chain MAGs from acetic acid co-eluted with PLs. The main cause of this co-elution phenomenon was mainly due to

equivalent carbon numbers on lipid molecules, which will be discussed in detail

later. Carroll[26] quantitatively separated standards of hydrocarbons, cholesterol

esters, triacylglycerols, free sterols, diglyacylglycerol, monoacylglycerols and free

fatty acids in one single run utilizing activated magnesium silicate.

The first successful application of reversed phase separation of lipid

molecules was not reported until 1950, ten years after this first successful normal

phase separation, when Howard et al[27] successfully separated long chain fatty

acids ranging from C11 to C18. In Howard's method, a mixture of diatomaceous

earth and paraffin was carefully hand-packed into a stainless steel column after a

complicated pretreatment process. A mixture of fatty acids was then loaded to

the column for separation. Despite the capability of this method to separate

individual FFAs that differed by two CH_2 groups, the handling required was

overwhelming and limited development of further applications. Limitations of

technology also hampered development of other early methods.

2.1.3. Forces that influence separation of lipid components

2.1.3.1. Equivalent carbon number

Historically, separation of lipid molecules by HPLC was divided into two

technical domains -- normal phase (NP-HPLC) and reverse phase (RP-HPLC) --

and it seemed that each technique acquired its own "success" in certain ways.

NP chromatography was the first applied to the separation of lipid

molecules. This type of chromatography utilizes polar stationary phases (silica

gel, polyvinyl alcohols, and amines) and non-polar mobile phases and separates

molecules by differential polar interactions between the stationary phase material

and analyte chemicals. In total lipid class separations, polarity is contributed by double bonds, hydroxyl groups, carboxylic groups and other functional group. Thus, NP has very great applications on separations that based on unsaturation level[28,29].

In contrast, RP chromatographic columns are usually made of silica with hydrophobic moieties such as C8, C18 and C30 acyl chains linked to the Si-OH groups. The mobile phases are polar organic solvents and water mixed in varying proportions. RP chromatography separates molecules according to hydrophobic interactions between the stationary phase and analytes. The higher the hydrophobicity of the analytes, the stronger the association with the stationary phase and the longer the compounds are retained on the column. In the separation of lipid molecules, the chain length of the molecules determines hydrophobicity. Nevertheless, the total carbon number is insufficient to explain the lipid retention properties comprehensively. This deficiency led to the development of the concept of equivalent carbon number[30–32].

In 1963, Hirsch[30] separated neutral lipids, including cholesterol, cholesterol ester, and mono, di and triacylglycerols by packing polymerized vegetable oil as the stationary phase. This was a landmark study because not only was overlapping elution of neighboring classes reported, but most importantly, in order to explain the co-elution and overlapping retention times, the concept of equivalent carbon numbers (ECNs) was proposed and defined as the total of number of carbons on the acyl chains minus two times of the number of

double bonds. This was later found to be the predominant factor affecting the elution order of lipid molecules, namely,

- the longer the acyl chain, the higher the ECN,

- every double bond decreased the ECN by two,

- molecules with higher ECNs are retained longer on reverse phase columns,

- for normal phase systems, retention is inversely proportional to the ECNs,

- lipid molecules with the same ECNs will co-elute or at least have very close retention times.

Under this concept, oleic acid (C18:1) and palmitic acid (C16:0) have identical ECNs so should have the same retention time and cannot be resolved on standard columns.

The concept of ECN was actually first addressed by Miwa et al in 1960[31]. In his characterization of FFAs by gas chromatography, the group proposed a linear relationship between the log scale of retention times of fatty acids and their Equivalent Chain Length (ECL). Retention times of other fatty acids could be predicted based on this linear relationship as long as separating conditions were the same. Coincidently, in the same year, the concept of ECNs was also proposed by another group of scientists in the Netherlands.[32] While solving the problem of peak shifts of fatty acids in GC analysis, Woodford and van Gent[32] claimed a similar semi-log plot of retention time against "designated carbon number" (CN). Furthermore, they also introduced "fractal" carbon numbers based

on the retention time. For example, palmitoleate was assigned with a CN of 15.75; linoleate is 17.5 and linolenate was 17.55. This assignment of CN varied with the operating conditions, especially the columns.

Despite different nomenclature systems, it is very clear that ECN/ECL influences elution behavior tremendously and lipid molecules with the same ECNs (also called critical pairs) are expected to co-elute. While the terminology and definition may vary, the physicochemical properties underlying the concept have been validated repeatedly, thus establishing a foundation for all separation research, particularly with lipids. For example, in a study determining adulteration of olive oil by canola oil using a tertiary gradient to separate lipid molecules from FFAs to TAGs[33], the separation was strictly according to the ECNs, all triacylglycerols from olive and canola oil were resolved based on their ECNs and critical pairs were not completely resolved. Despite author claims of complete separation, many compounds co-eluted or at least were not base-line separated. For example, lauric acid and 1-monomyristin co-eluted with partial resolution, and 2-monopalmitin, myristic acid and 1-monoolein co-eluted as a group.

Because of the dominant effects of ECNs, separation of critical pairs has emerged as a major obstacle in lipid class analysis. In a study by Lin et al[34], ECNs was claimed to be the most important factor controlling separation of 45 synthetic DAGs and TAGs (Table 2). However, careful examination of the retention times of individual compounds revealed very close time intervals (some of them were less than 1 second) between all critical pairs, indicating that base-line resolution was not achieved. Similar results were reported by Holcapek and

colleagues[35,36] who separated DAGs and TAGs on a C18 column. The overall eluting order followed the ECN predictions (molecules with lower ECN eluted first and higher ECNs eluted later) and lipid molecules with same ECNs were not resolved completely.

In summary, the ECN concept illustrates a challenge we must overcome in separating individual components within lipid classes where both saturated and unsaturated fatty acids are present.

Table 2. Relative retention time of DAGs and TAGs separated by C18 column. HPLC parameters: linear gradient from 100% methanol to 100% isopropanol in 40 minutes on C18 column and detection was with UV detector. Data selected from Lin et al.[34]

Diacylglycerols and Triacylglycerols	ECNs	Retention Time
1,3-Dipalmitin	32	16.17
1,3-Diolein	32	16.19
1,2-Dioleoyl-sn-glycerol	32	16.32
1,2-Dipalmitoyl-sn-glycerol	32	16.36
1-palmitoyl-3-stearoyl-rac-glycerol	34	18.45
1,3-Distearin	36	21.1
1,2-Distearoyl-rac-glycerol	36	21.35
Trilinolenin	36	23.28

Tripalmitolein	42	26.69
Trimyristin	42	26.8
Tripalmitelaiden	42	27.43
Trilinolein	42	27.92
Trilinolelaidin	42	28.41
1,2-Dimyristoyl-3-oleoyl-rac-glycerol	44	28.85
1,2-Dimyristoyl-3-palmitoyl-rac-glycerol	44	29.06
1,2-Dilinoleoyl-3-oeoyl-rac-glycerol	44	29.41
1,2-Dilinoleoyl-3-palmitoyl-rac-glycerol	44	29.5
1-Myristoyl-2-oleoyl-3-palmitoyl-rac-glycerol	46	31.02
1,2-Dioleoyl-3-linoleoyl-rac-glycerol	46	31.27
1,2-Dipalmitoyl-3-myristoyl-rac-glycerol	46	31.34
1-Palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol	46	31.35
1,3-Dipalmitoyl-2-linoleoyl-glycerol	46	31.43
Triolein	48	32.83
1,2-Dioleoyl-3-palmitoyl-rac-glycerol	48	32.93
1,3-Dioleoyl-2-palmitoyl-glycerol	48	32.96
1,3-Dipalmitoyl-2-oleoyl-glycerol	48	33.12
1,2-Dipalmitoyl-3-oleoyl-rac-glycerol	48	33.16
Tripalmitin	48	33.27
1 -Linoleoyl-2-oleoyl-3-stearoyl-rac-glycerol	48	33.32
Trielaidin	48	33.43
1,2-Dioleoyl-3-stearoyl-rac-glycerol	50	34.73

1,3-Dioleoyl-2-stearoyl-glycerol	50	34.76
1-Palmitoyl-2-oleoyl-3-stearoyl-rac-glycerol	50	34.91
1,2-Distearoyl-3-myristoyl-rac-glycerol	50	35.2
1,3-Distearoyl-2-oleoyl-glycerol	52	36.64
1,2-Distearoyl-3-oleoyl-rac-glycerol	52	36.72
1,2-Distearoyl-3-palmitoyl-rac-glycerol	52	37.07
Tristearin	54	38.86

2.1.3.2. Pi interactions between double and triple bonds

Effects of ECNs on elution of lipid molecules can be overturned only when other strong intermolecular force comes into play. Aveldano and coworkers[37] reported that one potential force for shifting the retention time of FFAs according the unsaturation was the triple bond (TB) in acetonitrile (ACN). Acetonitrile TB interactions with double bonds (DBs) in FFAs altered the retention properties in a binary mobile phase system consisting of acetonitrile and water. However, this observation was not very convincing since the alteration of retention time could purely be derived from the relative FFA solubility in the two phases. The separating power of the acetonitrile triple bond was further examined in resolution of conjugated linoleic acid (CLA) isomers by trace levels of ACN, propionitrile and butyronitrile[38]. It was very evident that the addition of nitriles to mobile phases exerted extra interaction between DBs and the nitrile triple bond

that made it possible to separate CLA positional isomers. Yet, it must be noted that this study was conducted with normal phase solid supports where lipid molecules had relative weak affinity to the polar silica gel (ECN regulation of overall eluting pattern still applies). Under such conditions, the DB-TB binding dominates and refines the isomer resolution. However, this type of interaction can be obscured in reverse phase systems where the hydrophobic interactions responsible for ECNs are much stronger. We have found no reports demonstrating that triple bond interactions are active in RP systems. Thus, in reversed phase systems, ACN may provide no resolving power other than polarity modification in the mobile phase[39].

2.1.3.3. Hydrogen bonding

Another type of intermolecular interaction was also observed in normal phase HPLC applications when Silversand and Haux[40] used a single fatty acid component (oleic acid) in each class of neutral lipids. TAG (triolein) was the first compound eluted as expected, but the remaining compounds eluted in retention time order of FFA (oleic acid) < DAG (diolein) < MAG (monoolein). A LiChrosphere 100 Diol normal phase column was used in this study, and it is believed that the diols established stronger hydrogen bonding with glycerol OH groups in MAGs and DAGs than with the -OH on the carboxylic acid.

To further highlight conditions where ECN do not govern general eluting patterns, polyvinyl alcohol (PVA), another kind of widely-used normal phase material, demonstrated disruption of ECN patterns by H bonding. Deschamps et al. [41] systematically studied the influence of the mobile phase solvent powers on the

eluting order of lipid molecules on PVA. Each test solvent combined a weak and a strong eluent (chloroform/acetone, chloroform/methanol, chloroform/isopropanol, heptane/acetone and heptane/isopropanol). When chloroform was the weak solvent, the eluting order followed the ECN prediction (TAG < DAG <MAG < non-hydroxy FFA < hydroxy FFA) and the interruption from the H bonding on –OH group was obscured. One possible reason for this is that chloroform should not be considered a weak solvent -- it is the best universal solvent for all lipid classes, so it can move lipids powerfully through the column. In contrast, in the combinations where heptane was the weak solvent, the influence of H bonding became quite obvious (Table 3). First, when isopropanol was present in the binary solvent system, retention times of lipid molecules with hydroxyl functional groups were significantly earlier than the outcome with heptane/acetone system. Second, in the comparison of the eluting order using heptane/acetone vs. heptane/isopropanol, both hydroxy and non-hydroxy FFAs eluted earlier than MAG in the latter combination indicating competition between the hydroxyl group on the PVA support and isopropanol versus H bonding with hydroxyl-containing lipid molecules. What is not clear in these cases is that H bonding seems to act both ways in PVA columns, with dominant action determined by conditions. Similar results were also reported elsewhere[42].

Table 3. Retention times (minutes) of lipid molecule separated by PVA column. Data selected from Deschamps et al.[41]

Weak Solvent	Heptane	
Strong Solvent	Acetone	Isopropanol
TAG	1.73	1.75
Chlosterol	4.90	3.93
DAG	4.96-5.27	3.93-4.12
MAG	7.67	5.81
Non-Hydroxy FFA	10.03	5.49
Hydroxy FFA	8.29	5.01

To summarize the interruption of general elution patterns by H bonding, it is worth noting that like triple bond-double bond interactions, H bonding through solvent and stationary alcohol groups did not appear to be strong enough to force major alterations in elution, and changes of eluting pattern reported in NP systems have never been observed in RP-HPLC.

2.1.3.4. Ionic interactions

Explanations of elution order reported in the scientific literature raise questions about water involvement in elution of lipids. Water usually would be the last choice of solvent because of its poor solubilization of most lipid classes. Nevertheless, in Carroll's successful separation[26] of TAG (tripalmitin), DAG (dipalmitin), MAG (monopalmitin), cholesterol and FFA (palmitic acid) from biological samples on magnesium silicate as a support, it was observed that

when 7% water was added as a mobile phase, FFA eluted earlier than without

water due to the basicity of magnesium silicate. The possible explanation for this

is that basicity of magnesium silicate forced disassociation of FFA, so when

water was in the system ionic interactions between disassociated FFA and water

would move FFA out of the column faster. This observation could be

tremendously meaningful for lipid class separation. One of the most troublesome

overlapping of neighboring classes is always between FFAs and MAGs since

their ECNs are very close, if not the same. Potential utilization of strong ionic

interactions, which could be even stronger the ECN dominance, will be carefully

examined and discussed later.

Some HPLC methods that have been applied to separation of total lipids

in oils and extracts of natural materials on different columns are summarized in

Table 4.

Table 4. Selected applications of class separation using both RP-HPLC and NP-HPLC (argentation LC included) and various detection methods.

Column	Matrix	Mobile phases	Class Separated	Detector	Reference
NP: LiChrosorb Si 60	potato leaves, potato tubers, corn roots, tobacco leaves spinach, corn fiber and corn bran	Ternary Gradient: iso-octane, isopropanol and water	TAG, DAG, FFA, free sterols, beta-carotene, Chlorophyll A and B, monogalactosyldiacyglycerol (MGDG) and PLs	FID	[43, 44]
NP: LiChrosorb diol	Barley extract	Binary gradient: Hexane/acetic acid, Hexane/isopropanol	phytosterol fatty acyl esters, hydroxycinnamate phytosterol esters, triacylglycerols, free fatty acids TAG, PLs, Cholesterol, tocopherol	CAD	[45,46]

RP, C18	Rapeseed Oil	Binary: Methanol, Isopropanol/Hexane	Methyl ester, MAG, DAG AND TAGs	MS	[47]
Silver Ion Column	Animal Products, Pig	Isocratic: Hexane/diethyl ester/acetonitrile	Conjugated Linoleic acid Oleic acid isomers	UV	[48], [49]
On-line 2 dimensional: Silver Ion and C18	Corn oil	Argentation: isocratic MeOH/ACN C18, Isocratic MeOH/Methyltert-butyl ether	TAGs	ELSD, MS	[50]
Off-line 2 dimensional: Argentation and C18	Meadowfoam Oil	Argentation: isocratic dichloroethane/dichloromethane, Acetone, acetone/acetonitrile, RP-HPLC, isocratic,	TAGs	MS	[51]

		dichloromethane/dichloroethane/acetonitrile			
Off-line 2 dimensional: Argentation and C18	Beef tallow, Lard	RP: isocratic, acetone/ACN, Argentation, isocratic, Hexane/ACN	TAGs	UV, MS	[52]

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2.1.3.5. Silver ion chromatography for resolving critical pairs and isomers

Overall, none of the four forces discussed -- hydrophobic binding, pi interactions between double and triple bonds, hydrogen binding to -OH groups, and ionic interaction-- have the strength or delicacy to resolve three important structures in lipids:

- critical pairs -- compounds having the same ECNs but differing in structure by two methylene groups and one double bond, e.g. oleic acid and palmitic acid

- positional isomers -- compounds with the same structure but one or more groups in a different position, e.g. double bond shifted from C9 in oleic acid to C11 in vaccenic acid

- regioisomers -- same fatty acids but in different stereospecific positions on glycerol in MAGs, DAGs, TAGs, and phospholipids, e.g, 1-palmitoyl-2-oleoyl-3-linoleoyl-rac-glycerol, POL and 1-linoleoyl-2-palmitoyl-3-oleoyl-rac-glycerol, LOP.

Although the microstructure in each of these instances is distinctive, the physical properties are too similar for the four separation forces discussed above to effectively separate in reverse phase chromatography. In such cases, some specific interactions must be added to alter the molecular physical interactions efficiently and subtly.

One such supplementary interaction is silver ion chromatography, which separates critical pairs and isomers utilizing the interaction between silver ions and double bonds. As reviewed by Morris[53] in 1966 and Hartley in 1973[54], the

interaction involves formation of a sigma bond by overlapping of the olefin π orbital and the silver ion *s* orbital or silver ion's *d* orbital (Figure 2).
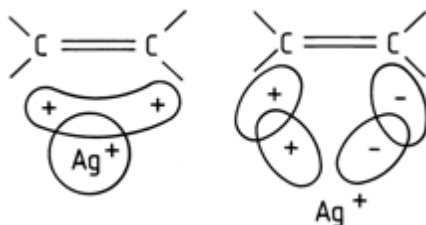
Figure 2. Diagrams of σ complex (left) and π complex (right) between olefin bond and silver ions[53].

The first successful application of silver ion chromatography was conducted by Nicholes[55] in separation of methyl oleate (cis) and methyl elaidate (trans) isomers. The *cis* isomer had much strong interaction with silver ions than *trans* isomer, and this became the basis for separating the isomers by both countercurrent distribution and paper chromatographies. This research did not compare interactions between silver ions and lipid molecules with different levels of unsaturation, nor did it consider competing ECN forces, but it did demonstrate the promise of argentation for separating critical pairs.

Gunstone et al.[56] used silver ion impregnated TLC plates to separate positional isomers of methyl oleate, claiming that when the double bond was on carbon 2, the interaction between the olefin and silver ion was the weakest while the strongest interaction occurred when the double bond was on carbon 6. On C2, the close proximity of the double bond to the ester group made it difficult for the silver ions in the stationary phase to gain access to the pi orbitals. In contrast,

on position 6, the molecule would have increased flexibility to bend and the resulting *cis* configuration positions the double bond close to the Ag ions on the silica gel. Wurster et al.[57] used an ion exchange resin impregnated with silver ions to separate oleate, linoleate, and linolenate methyl esters, reasoning that silver ions would bind more tightly to charges than to -OH groups.

Schofield[58] and coworkers employed countercurrent distribution with argentation to separate monoenoic, dienoic and trienoic methyl esters of partially hydrogenated linolenate with certain degrees of separation of cis/trans isomers. In this system, instead of having the stationary phase impregnated with silver ions, 0.2M silver nitrate was dissolved in the 90% aqueous methanol mobile phase. Schomberg and Zegarski[59] used the same approach in RP-HPLC to separate geometrical isomers of unsaturated lipid molecules. *Cis* isomers had stronger interactions with silver ions than did *trans* isomers. Applying silver ion in the mobile phase allowed Vonach and Schomberg[60] to also separate olefins. With silver ion concentrations as low as 1 mM, the retention time of unsaturated lipid molecules could be significantly shortened due to the enhanced interaction between olefins and mobile phases. Critical pairs such as C20:1/C22:2 and C18:1/C20:2 from C14 to C22 methyl esters of FFAs were similarly separated by Vereshchagin[61].

Dissolving silver ions in mobile phases gave comparable results in other HPLC studies[62,63] Ozcimder and Hammers[64] compared the resolvability of fatty acids by RP-HPLC and argentation HPLC, employing the relationship between capacity (the ability of molecule to interact with stationary phase, expressed as

k') and number of DBs as a parameter to determine the resolvability of each technique. Argentation chromatography successfully achieved separation of critical pairs of FFA methyl esters while RP-HPLC failed to do so. However, they also demonstrated that argentation HPLC is limited to low polarity solvents since high eluent polarity overpowers the interaction between olefins and silver ions in the silica gel stationary phase. This is important when considering potential applicability of argentation to RP-HPLC separation of lipid classes.

The separation of triacylglycerols (TAGs) can be even more complex than fatty acids since three fatty acids randomly esterified to the glycerol backbone generate a large number of regioisomers and positional isomers. Holcapek et.al.[65] calculated that using randomization procedures to transesterify six fatty acids (palmitic, stearic, oleic, linoleic, linolenic and arachidic acid) commonly found in natural materials, a total number of 6^3 =216 different TAG molecules could be generated. Separating all these would be very challenging indeed. When comparing silver ion chromatography versus non-aqueous RP-HPLC efficiency of separating TAGs, Holcapek found that separating power in non-aqueous RP-HPLC was mainly from acyl chain length via non-polar interactions, i.e. ECNs, and under such conditions, and separation isomers/critical pairs was fairly difficult to achieve. However, in silver ion chromatography, separation was dominantly from the π complex between double bonds and silver ions with very minor strength due to the ECNs, so separation of the isomers was much more efficient.

Using standard TAGS as model systems, Adlof and List[66] successfully

separated TAG regioisomers including POP/PPO, SLnS/SSLn and LOL/LLO (S=

Stearic acid, L=linoleic acid Ln=linolenic acid and O=oleic acid) using two normal

phase ChromSpher lipid columns coupled in series with an isocratic elution

program of 1.0% acetonitrile in hexane. For double bond positional isomers of

TAGs, silver ion chromatography also gave satisfactory performance that varied

with the mobile phase composition[39]. Hexane-based mobile phases seemed to

yield better and more predictable separation behavior, i.e., the closer the double

bonds to the carbonyl group, the more potent the interaction of the double bond

with the silver ions. Dichloromethane gave poorer resolution of double bond

positional isomers, and there was no apparent relationship between the relative

double bond positions to the carbonyl group. Furthermore, double bonds on

position sn-1 and sn-3 exerted stronger Ag binding than on sn-2, as exemplified

by ALnO/AOLn (A=Arachidic acid). However, this rule didn't always apply when

more highly unsaturated fatty acids such as O/L/Ln were present in TAG

systems.

Overall, RP-HPLC and argentation-HPLC techniques are being used

extensively in lipid analysis, but each technique has inherent drawbacks.

Although RP-HPLC provides a stable foundation for interactions between

stationary phase and lipid molecules across the spectrum, this technique falls

short when matrices contain isomers and critical pairs. Argentation HPLC, which

should be considered a kind of variant of normal phase system, makes up for this

insufficiency by establishing additional associations between lipid molecules and

stationary phase through π complexes, yet exerts very little selectivity in separating saturated lipid species.

.   In light of the many successful applications that have been developed for silver ion chromatography, it is very surprising that very few commercial vendors supply silver ion impregnated HPLC columns. Quite the opposite, most silver ion columns are constructed in-house. Christie[67] elaborated a procedure for impregnating silver ions on a normal phase bare silica column by continuously washing it with silver nitrate solvent for 20 minutes. Subsequent tests on the separation of fatty acids with zero to six double bonds showed found no silver salts eluting from the HPLC column.

An alternative procedure for in-house construction of silver ion solid phase extraction (SPE) columns was also reported[68]. Silver nitrate (80 mg/ml dissolved in acetonitrile-water 10:1 (v/v)) was percolated through a Bond Elut[TM] SCX solid phase extraction column, which was then flushed sequentially with acetonitrile, acetone and dichloromethane. Test results showed satisfactory separation for not only fatty acid methyl esters but also C16:1 *cis-trans* isomers. Other in-house procedures for producing silver TLC plates and HPLC columns were reviewed in detail by Nomchilova et al[69].

In developing new materials to enhance stability of silver ions and prevent their bleeding out during chromatography, Aponte et al.[70] synthesized two novel columns. The first one, called mercaptopropyl functionalized silica gel or AgTCM (Figure 3, left), covalently connected Ag to a thiol group by mixing SH and silver nitrate in either water-methanol (1:1) solution or acetonitrile under acidic pH for 2

hours, excess Ag was precipitated by 5% NaCl, and the reagent solution was then dried at 60 °C for 16 hours. The second material, called cyanopropyl functionalized silica gel (Figure 3, right), was synthesized by a similar process.

AgTCM (4)
($\equiv$Si-(CH$_2$)$_3$-S-Ag)

(5)
($\equiv$Si-(CH$_2$)$_3$-CN + AgNO$_3$)
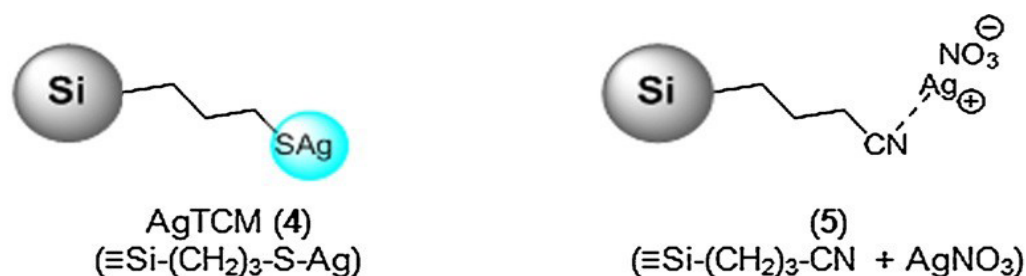
Figure 3. Molecular structure of mercaptopropyl functionalized silica gel (left) and cyanopropyl functionalized silica gel (right)[70].

In testing, these novel materials showed several advantages over traditional silver impregnated columns:

1) AgTCM exhibited weaker but more balanced interactions in a model system containing C14 hydrocarbons with 0, 1, 2, 4 double bonds and phenanthrene, a ring structure containing seven conjugated double bonds (Figure 4). In contrast, the traditional argentation column showed strong but nonselective interaction with the model compounds (overlap of C14 hydrocarbons containing two and four double bonds).

2) Silver ions and olefins in AgTCM were more stable and less sensitive to oxidation.

3) AgTCM significantly reduced the Ag leaching that is a serious problem with traditional argentation columns when polar solvents are present.

All these advantages resulted because covalent binding of silver ions to SH groups gave greater chemical and physical stability.

In summary, normal phase, reverse phase, and argentation chromatography each provide distinctive advantages for separation of lipid classes that normally occur in food matrices, but none of them alone can achieve complete resolution of all lipid components. Hence, combinations of one or more techniques are probably necessary for full resolution of individual lipid components in mixtures.
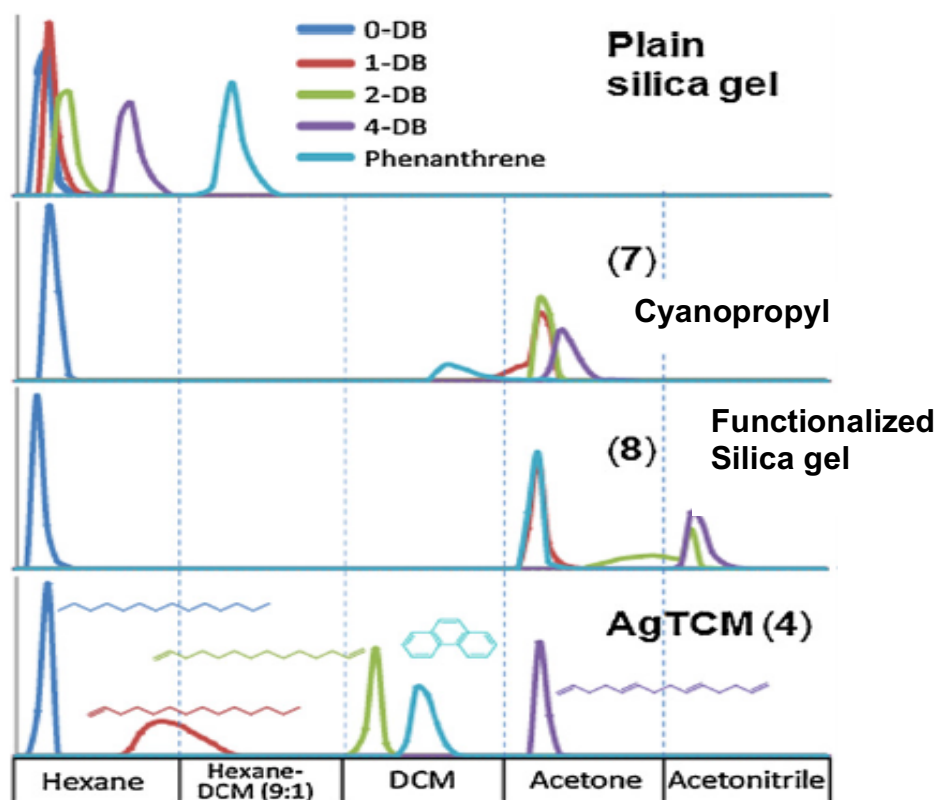
Figure 4. Comparison of double bond separations obtained by plain silica gel, cyanopropyl, functionalized silica gel, and AgTCM columns[70], 0-DB: 1 tetradecene, 1-DB: 1-tetradecene, 2-DB: 1,3-tetradecadiene, 4-DB: 1,5,9,13-tetradecatetraene.

2.2. Detection methods for lipid quantitation -- UV Detection VS. ELS Detection

2.2.1. UV detection

Along with advances in capability of chromatographic technology to resolve molecules with closely-related structure, a number of methods have now been developed and refined to increase the sensitivity and accuracy of peak detection. UV-Vis detection is the most widely used detection method. When placed in a beam of UV light (190 nm-350 nm) or visible light, functional groups (chromophores) on molecules absorb light at specific wavelengths where the light energy matches the group excitation energy.

UV-Vis detectors have limited applications for detecting lipid molecules because lipids contain few chromophores. 206 nm has been used as a general detection wavelength for lipids in many studies[38,71,72], as well as for fatty acid methyl esters and MAGs, DAGs and TAGs containing unsaturated fatty acids[34,50,66,73]. At this wavelength, double bonds and carbonyl groups in aldehydes, fatty acids, and esters are responsible for energy absorption, but the intensity is relatively weak. Absence of double bonds is normally not an issue for the detection of lipids at 206 nm since most naturally occurring lipids contain at least one unsaturated fatty acid moiety. However, UV detection fails with fully saturated lipids. Holcapek and colleagues[36] compared response factors of lipid molecules in UV-Vis, ELSD and mass spectrometer detection and showed that saturated lipid molecules had very weak absorption of UV light (Table 5). In this comparison, response factors of individual lipid molecules were expressed as the

ratio of triolein response (set as 1.00) to the response of the test molecule. Thus, higher signal responses give lower factor values. In this scheme, response factors for saturated fatty acids are 7-8 while those for polyunsaturated fatty acids are about 0.10 (Table 5) Similar results were reported by Heron et al[74]. Consequently, 206 nm is not particularly useful for detecting saturated lipid molecules, and detection at this wavelength is qualitative, not quantitative.

Table 5. Comparison of lipid response factors in MS/APCI, ELSD and UV detection. Data was excerpted from Holcapek[36]. CN=carbon number, DB=double bonds; HPLC gradient: 100% ACN at time 0, change to to 31% ACN and 69% 2-propanol in 106 minutes, then back to 100% ACN in 3 minutes on two C18 columns connected in series.

Lipid	Response Factor		
CN:DB	UV	ELSD	APCI
C7:0	7.57	0.32	97.2
C8:0	7.77	0.44	74.44
C9:0	8.00	0.48	38.91
C10:0	7.33	0.55	17.62
C11:0	7.85	0.36	10.85
C12:0	6.94	0.27	6.04
C13:0	8.11	0.15	4.31
C14:0	7.12	0.07	2.77
C15:0	7.43	0.07	1.75

C16:0	7.00	0.04	1.32
C16:1	0.79	1.16	1.33
C17:0	7.00	0.04	0.81
C18:0	6.46	0.03	0.61
C18:1	1	1	1
C18:2	0.16	0.49	0.57
α-C18:3	0.07	0.29	0.4
β-18:3	0.05	0.32	0.29
C19:0	5.88	0.02	0.49
C20:0	5.57	0.01	0.4
C20:1	0.86	0.02	0.36
C21:0	5.24	0.12	0.39
C22:0	6.07	0.07	0.46
C22:1	1.21	0.08	0.42
OPP	1.85	n/a	1.13
POP	1.45	n/a	1.1
OPO	1.33	n/a	1.05
OOP	1.03	n/a	1.02

UV detection is most commonly and appropriately used with lipids to detect conjugated dienes, the first products of lipid oxidation. Two double bonds in conjugation absorb UV light at maximum wavelengths between 231 and 235 nm[75]. Extinction coefficients are known for a wide range of solvents, so results can be made at least semi-quantitative. However, full quantitation requires knowledge of lipid weight in each peak as well.

2.2.2. Evaporative light scattering detection[76]

Recently, evaporative light scattering detection (ELSD) has been receiving more attention because it detects all molecules and quantitates by particle mass rather than by differential optical responses. Another great advantage of ELSD is that it is not susceptible to baseline fluctuation during complex gradients required to separate lipid mixtures with a wide range of polarity, e.g. from polar PL to very non-polar saturated TAGs. This greatly simplifies qualitative analyses of lipids.

Figure 5 is simple representation of ELSD. Analytes separated by the chromatographic system elute into the detector with the mobile phase. Here, they enter the nebulizer and become nebulized when heated to high temperatures under inert gas such as nitrogen. During this process, mobile phases (have lower boiling points than analytes) are evaporated, leaving the solute/analytes behind. Then a light (either laser or a visible light) shines on the molecular particles and is scattered. Light scattered at a certain angle impinges on the detector and is the energy is converted to an electronic signal for analysis. Most solvents used as mobile phases have boiling points (BPs) near ambient temperature; the BPs of even volatile lipid oxidation products are higher than this (Table 6), and BPs of fatty acids are well above 200 °C. This makes ELSD detection particularly appropriate for detection of lipid molecules, especially saturated lipid molecules that are not detectable under UV light.

Four major scattering mechanisms dictate the intensity of signal response in ELSD: Raleigh scattering, Mie scattering, reflection and refraction.[77,78] The scattering mechanism dominating in a given system is solely governed by the

particle size and shape. Raleigh scattering, which induces the oscillating dipole via light quanta, dominates with very small particles (about one twentieth of the light wavelength). Mie scattering plays a major role with irregular particles, where each point of the particle scatters light differently. As the particle size approaches that of the light wavelength (nm), reflection and refraction, which usually occur at the same time, dominate the scattering mechanism.
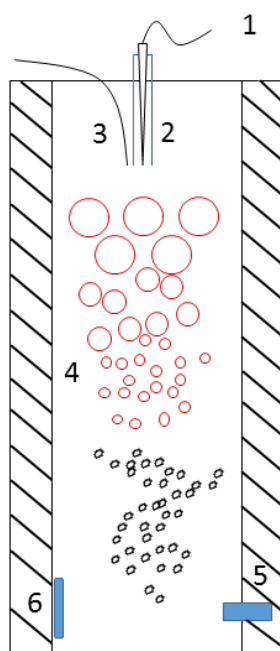
Figure 5. Schematic representation of ELSD, 1: Eluent from column, 2: nebulizer, 3: nebulizer gas, 4: drift tube, 5: laser source, 6: light detector.

Table 6. Chemical and physical properties of organic solvents and aldehydes.[20]

Chemicals	Vapor pressure (mm Hg@25ºC)	Melting point (ºC)	Boiling Point (ºC)
Methanol	127	-97.8	64.7
Ethanol	59.3	-114.1	78.3
Isopropanol	45.14	-87.9	82.3
Acetonitrile	88	-45	81.6
Hexane	153	-95.4	68.7
Dichloromethane	435	-95	39.8
Chloroform	197	-63.4	61.7
Butyraldehydes	111	-96.7	74.8
Pentanal	26	-91.5	103
Hexanal	11.3	-56	131
Heptanal	3.52	-43.4	152.8
Octanal	1.18	-23	171
Nonanal	0.37	---	191
Decanal	0.103	-3.9	212

While ELSD offers advantages in qualitatively detecting all lipid fractions, non-linear or semi-linear quantitative relationships between analytes concentration and signal response are typical in ELSD[42,79–82] due to variations in scattering mechanisms with particle size, molecular properties, and nebulizer

efficiency. A power regression model[80] was required to quantify phospholipids; R-squares were all above 0.990 indicating an excellent fit for all PLs tested over a 2 to 15 µg/ml range. Homan et al.[81] reported a quadratic fit for TAGs, PLs, cholesterol and cholesterol esters over the 0.05-10 µg range. A second order regression[42] fit neutral lipids, but the response factors for paraffin (mol wt 436.84 g/mol) and tripalmitate (mol wt 807.32 g/mol) were significantly different, indicating that each compound must have its own calibration curve for quantification.

Despite these differences, several studies claim that for very low sample concentrations (7.5×10^{-5} to 3×10^{-3} g/ml[78]) or weight per peak (50 to 200 µg[83,84]), ELSD responses were linear with mass, and response factors for molecules with similar size/molecular weight were not significantly different. Murphy et al.[83] claimed that the response factors were close enough for lipid molecules of same class that standard curves for each class could be prepared from only one representative lipid compound. This would tremendously reduce the workload required for calibration curves to be run for each different lipid molecule.

In summary, ELSD has been applied extensively in lipid class separation analysis due to its universal detection mechanism. Qualitatively, ELSD offers advantages over UV-vis in that it detects all lipid classes. However, use of ELSD for quantitation is controversial because light scattering involves particles rather than molecules, and scattering mechanisms that vary with particle size and shape lead to non-linear relationships between detector response to analyte size or mass. The problem is exacerbated because particle size depends on factors

not related to molecular structure – e.g. experimental conditions, nebulizer temperature, nebulizer gas flow rate and the efficiency and consistency of nebulizer, of each lab employed. Various algorisms, both linear and non-linear, have been utilized to fit calibration curves from various lipids, but most accurate quantitation appears to require separate standard curves for each type of molecule under conditions of analysis.

2.3. Lipid oxidation

Lipid oxidation has been long recognized as the major chemical reaction limiting the shelf life and the quality of the foods. The basis for the traditional understanding of lipid oxidation as a free radical chain reaction was established nearly 70 years ago by Farmer[85–87], Bolland[88,89], Holman[90], and Lundberg[91]. Additional details about breakdown pathways and major products generated under various conditions were added over the next 40 years, particularly by Frankel and colleagues[92,93].

The lipid oxidation chain reaction as classically understood is shown in Figure 6. Lipid oxidation is not spontaneous but requires an initiator to remove an electron or hydrogen atom to form the *ab initio* (first) radical. Mechanisms of initiation have been discussed in detail by Schaich[13,94]. Once the radicals are formed, oxygen reacts almost instantaneously – $k > 10^9$ L mol^{-1}sec^{-1}, i.e. diffusion controlled rates -- with the carbon-centered radical to form peroxyl radicals, which then abstract hydrogen atoms (H) from nearby lipid molecules to form a

**CLASSICAL FREE RADICAL CHAIN REACTION MECHANISM
OF LIPID OXIDATION**

Initiation *(formation of ab initio lipid free radical)*

$$L_1H \xrightarrow{k_i} L_1\cdot \qquad (1)$$

Propagation

Free radical chain reaction established

$$L_1\cdot + O_2 \underset{k_\beta}{\overset{k_o}{\rightleftharpoons}} L_1OO\cdot \qquad (2)$$

$$L_1OO\cdot + L_2H \xrightarrow{k_{p1}} L_1OOH + L_2\cdot \qquad (3)$$

$$L_2OO\cdot + L_3H \xrightarrow{k_{p1}} L_2OOH + L_3\cdot \quad etc. \quad \cdots\cdots\rightarrow L_nOOH \quad (4)$$
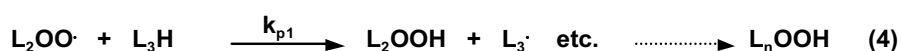
Free radical chain branching (initiation of new chains)

$$L_nOOH \xrightarrow{k_{d1}} L_nO\cdot + OH^- \ (reducing\ metals) \qquad (5)$$

$$L_nOOH \xrightarrow{k_{d2}} L_nOO\cdot + H^+ \ (oxidizing\ metals) \qquad (6)$$

$$L_nOOH \xrightarrow{k_{d3}} L_nO\cdot + \cdot OH \ (heat\ and\ uv) \qquad (7)$$

$$\left.\begin{array}{l} L_nO\cdot \\ L_nOO\cdot \\ HO\cdot \end{array}\right\} + L_4H \xrightarrow[\substack{k_{p1}\\k_{p3}}]{k_{p2}} \left.\begin{array}{l} L_nOH \\ L_nOOH \\ HOH \end{array}\right\} + L_4\cdot \qquad \begin{array}{l}(8a)\\(8b)\\(8c)\end{array}$$

$$L_1OO\cdot + L_nOOH \xrightarrow{k_{p4}} L_1OOH + L_nOO\cdot \qquad (9)$$

$$L_1O\cdot + L_nOOH \xrightarrow{k_{p5}} L_1OH + L_nOO\cdot \qquad (10)$$
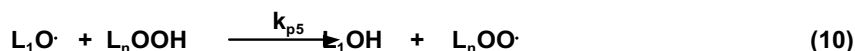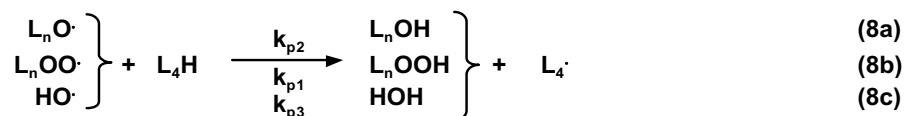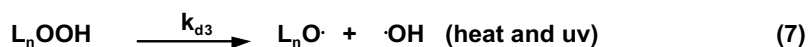
Termination *(formation of non-radical products)*

$$\left.\begin{array}{l}L_n\cdot\\L_nO\cdot\\L_nOO\cdot\end{array}\right\} + \left.\begin{array}{l}L_n\cdot\\L_nO\cdot\\L_nOO\cdot\end{array}\right\} \begin{array}{l}\text{Radical recombinations}\\ \xrightarrow[\substack{k_{t2}\\k_{t3}}]{k_{t1}} \text{polymers, non-radical monomer products}\\ \text{(ketones, ethers, alkanes, aldehydes, etc.)}\end{array} \begin{array}{l}(11a)\\(11b)\\(11c)\end{array}$$

$$\left.\begin{array}{l}LOO\cdot\\LO\cdot\end{array}\right\} \begin{array}{l}\text{Radical scissions}\\ \xrightarrow[k_{ts2}]{k_{ts1}} \text{non-radical products}\\ \text{(aldehydes, ketones,}\\ \text{alcohols, alkanes, etc.)}\end{array} \begin{array}{l}(12a)\\(12b)\end{array}$$
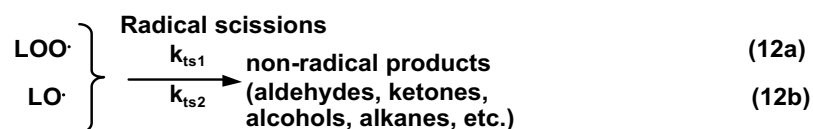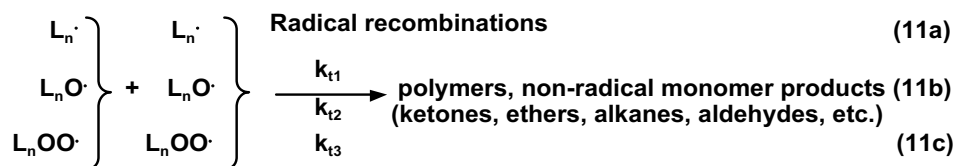
i - initiation; o-oxygenation; β-O_2 scission; p-propagation; d-dissociation;
t-termination; ts-termination/scission

Figure 6. Classical free radical sequence for lipid oxidation[13]; used with permission.

hydroperoxide and generate a new carbon-centered radical. This process, referred to as propagation, repeats itself indefinitely to broadcast and expand the oxidation to other lipid molecules. As a consequence, a single initiating event can end up oxidizing hundreds of lipid molecules in both direct main chains and side chains. Chain branching is established when hydroperoxides decompose to form alkoxyl radicals by reduction or peroxyl radicals by oxidation.

H abstraction rates by peroxyl radicals are relatively slow (10^1 - 10^3 L mol^{-1}sec^{-1}) but alkoxyl radicals abstract H atoms orders of magnitude faster (10^6 - 10^8 L mol^{-1}sec^{-1})[95]. Thus, oxidation processes greatly accelerate when hydroperoxides decompose and alkoxyl radicals take over as the main chain propagating species. The products always written for H abstraction by alkoxyl radicals are alcohols, but surprisingly, these products are almost never measured even though they should be stable products. Instead, attention is given to small volatile compounds released from foods or oils[92,93,96–100], including carbonyl and alkane breakdown products from α and β scission of alkoxyl radicals[92,93] and products formed from recombination of various radicals[101]. Indeed, most of the reaction sequences written for lipid oxidation were derived from studies of the small volatile products[99,102,103] formed during termination reactions (see Figure 6).

A key expectation of the traditional theory is that a relatively long period exists at the beginning of the oxidation process when chains are propagating and generating radicals, and that products other than hydroperoxides do not accumulate until late stages, after hydroperoxide decomposition. However, research on lipid oxidation processes and mechanisms in this laboratory

encountered many results that did not fit the classical theory just described. In particular, hydroperoxides did not always precede formation of secondary products and sometimes were not detectable at all while secondary reactions were obvious (unpublished observations). Also, alcohols that should be generated as major products via hydrogen abstraction by alkoxyl radicals were never observed as more than minor products, and secondary products detected did not fit decomposition pathways described in traditional reaction sequences[92,93,96,104,105].

The observed disparities led Schaich[13] to re-evaluate the free radical literature and propose of a modified theory that recognizes alternate reaction pathways for peroxyl and alkoxyl radicals and hydroperoxides. Internal rearrangement, addition, scission, and other reactions occur in parallel to and in competition with traditional hydrogen abstraction, and generate product mixtures that are much more complex than predicted by traditional understanding and include products seldom measured. These pathways were integrated with hydrogen abstraction by Schaich[13,106] in the comprehensive mechanism of lipid oxidation shown in Figure 7. This proposed comprehensive mechanism maintains the free radical chain reactions driven by hydrogen abstraction as the backbone (central vertical pathways in Figure 7), but also incorporates alternate reactions of each major lipid oxidation intermediate, shown in the competing reactions for each major oxidation intermediate -- LOO·, LOOH, and LO·.

INTEGRATED SCHEME FOR LIPID OXIDATION
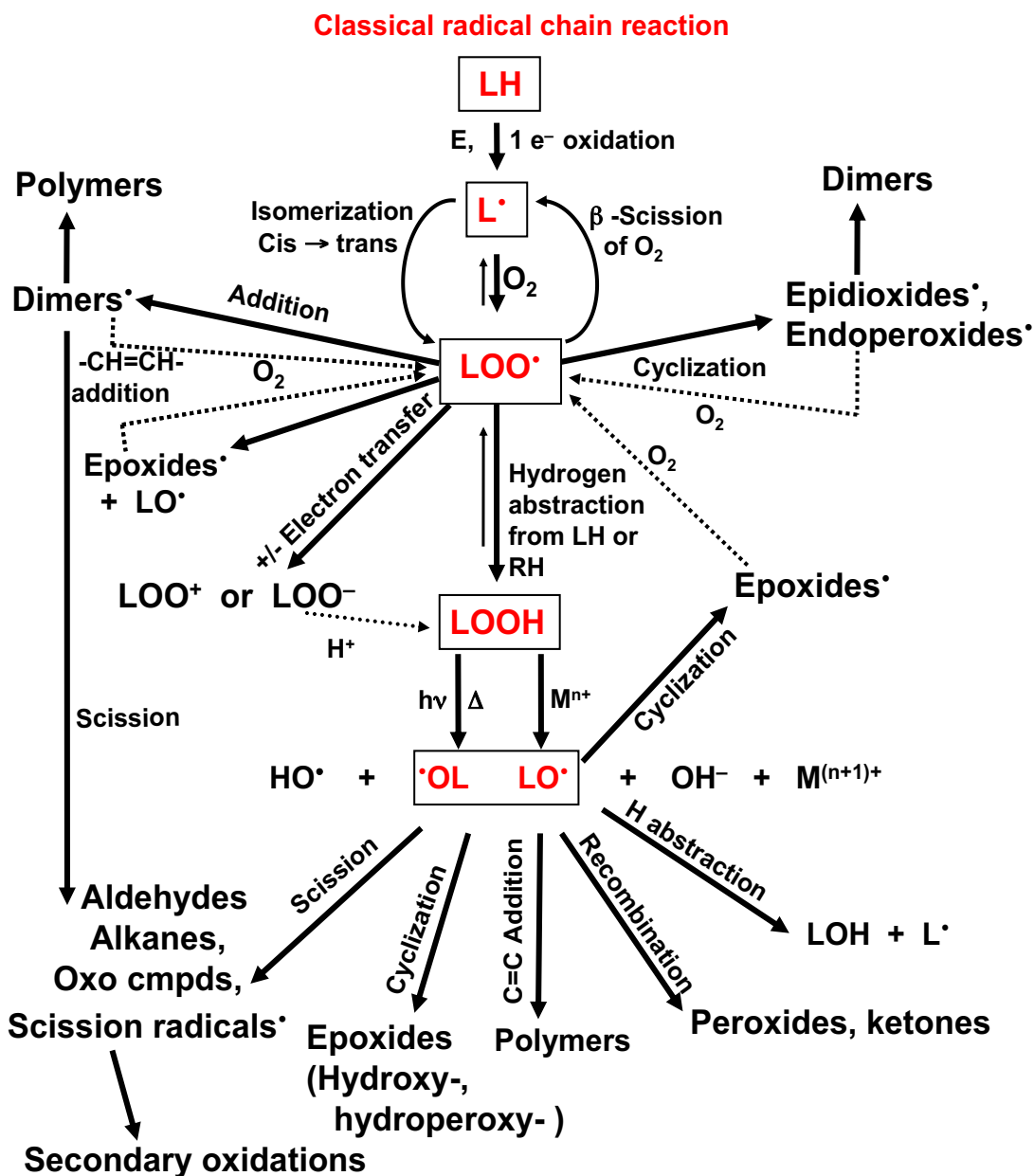
Figure 7. Comprehensive reaction scheme for lipid oxidation, integrating alternate reaction pathways with conventional hydrogen abstraction (noted in red).[9] Used with permission.

An important feature of this integrated reaction scheme is the attempt to account for as many products as possible to show what products must be measured to accurately determine extent of lipid oxidation. Current practice typically measures conjugated dienes and hydroperoxides by chemical assays, plus maybe some volatile secondary products (mainly hexanal) by gas chromatography. However, when alternate pathways are active, measuring only these products can tremendously underestimate the extent of lipid oxidation. For example, research in our laboratory has shown that non-volatile epoxides occur at levels equal to or higher than hydroperoxides, particularly at elevated temperatures[107]. Parallel research on volatile products has shown that C13 products dominate in linoleic acid oxidation, with the major product by far being pentane from β-scission; hexanal from α-scission is minor or even absent in many systems[108]. Thus, products from multiple pathways, at a minimum conjugated dienes, hydroperoxides, carbonyls, epoxides, and dimers need to be measured simultaneously to accurately track the progress of oxidation and determine the extent of oxidation.

Such a requirement presents several analytical challenges[109]:

1) When multiple pathways are active and change dominance with reaction conditions, measuring only one product may miss oxidation altogether and even the common practice of measuring conjugated dienes, hydroperoxides, and hexanal underestimates the full extent of oxidation.

2) Accurate assessment of the extent of oxidation requires measuring and identifying individual oxidation products from multiple pathways simultaneously.

Running analyses for many products simultaneously or at least close in time requires significant manpower.

3) Lack of available quantitative methods for all products, and variable detection efficiency for each assay makes comparison of pathways even more difficult.

4) Many current methods for analyzing lipid oxidation products are chemically inaccurate, reaction response rate and stoichiometry are not linear with product concentration, and reproducibility is lower than desirable.

These challenges make it clear that new and comprehensive analytical methods are needed to detect products from multiple pathways simultaneously in the same sample, to preferably differentiate individual products rather than general product classes, and to meet standards of quantification. Since the products are oxidized (oxygenated), they should also be susceptible to reduction, e.g. on an electrode. Hence, electrochemical detection may offer some unique opportunities to accomplish this on-line and detect products by their redox potential as lipid classes are being separated by HPLC.

2.4. Electrochemical detection of oxidation products during HPLC

Although ELSD may provide an optimum quantitative detection solution for dissolved lipid molecules, it fails for lipid oxidation products that are mostly volatile and evaporate along with mobile phases. In the search of the ideal detector for oxidation products, Electrochemical Detection (ECD) immediately attracted our attention because it specifically detects redox-active products. In

ECD, electrons transferred between an electrode and a susceptible functional group generates an electrical current that is registered by the detector[110]. How this occurs is shown in Figure 8.

Fundamentally, an ECD consists of a working electrode and a reference electrode linked by an electrical potential. When electrochemically active compounds pass over the surface of the working electrode, an applied potential forces the compound to either gain or to lose electron(s). The electron transfer forms an electrical current and is registered as a peak signal for the detector to analyze. Compounds with functional groups that are oxidized or reduced to different extents are detected as peaks when the applied current matches their redox potential (mV). Some ECDs run multiple parallel channels so can simultaneously detect products with different redox potentials[111].

Not all chemicals are electrochemically active, i.e. easily reducible or oxidizable. Active functional groups are usually categorized into three classes[112] according to their electrochemical activity. Normally compounds that contain primary amine groups in conjugation with pi electrons of an aromatic nucleus are very active under electrical potential. Chemicals like phenols, catechols and phenylamines also have pi electrons and an aromatic nucleus. Amines and thiols on aliphatic structure (non-aromatic) are less active, but would still give electrochemical signals at elevated applied potential. Surprisingly, alcohols and even carbonyl compounds, even though oxidized, have low response with electrochemical detection[113].
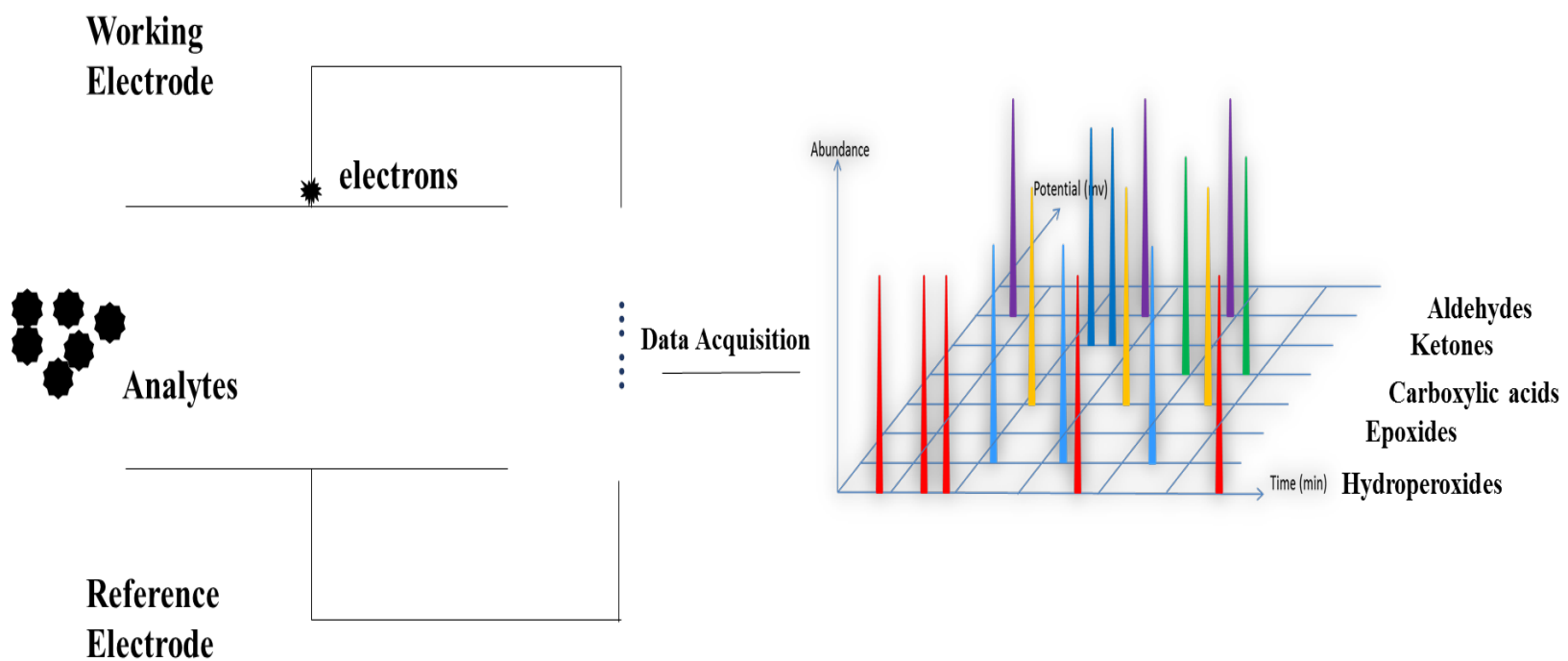
Figure 8. Schematic representation of ECD detection mechanism and data acquisition.

In addition to inherent chemical properties, other factors also affect electrochemical activity, including electrode material, the physical design of the electrode cell, the supporting electrolytes, and other experimental conditions.

Since redox reactions occur at the surface of the working electrode, electrode materials and properties are critical and determine the operating potential range.[114] Mercury formerly was used extensively in electrodes because of its wide range of negative potential. However, the positive range is limited as mercury itself is also prone to oxidation during the process[115]. Another drawback of mercury electrodes is the toxicity of its vapor (mercury has a relatively low vapor pressure), so these have increasingly been replaced by electrodes of solid materials. Noble metals like gold and platinum are good choices for working electrodes due to their inertness. Noble metal electrodes usually have an applicable electrical potential range sliding toward the oxidation end compared to the mercury electrode[116], which means that it possesses a higher oxidation potential yet limited reducing potential. Nevertheless, both platinum and gold electrodes have oxidation layers formed at the surface after extended use[117], so periodic polishing and re-surface is required to maintain the surface activity and reproducibility[115,118]. The carbon-based (graphite) electrode, developed to overcome the potential limitations of mercury and noble metals, has a larger working potential range on both reducing and oxidizing sides of neutral.

Because the electrochemical reaction occurs on the surface of the working electrode, the mass transfer of the active species to the surface is the key

process governing the efficacy of such reaction. Wang[115] summarized the

electrochemical process mathematically using Fick's second law (Equation 1),

$$\frac{\partial C}{\partial t} = D\left(\frac{\partial^2 C}{\partial r^2} + \frac{2\partial C}{r\partial r}\right) \qquad (1)$$

where C is the concentration of analyte on the surface of the electrode at the

time t, D is the diffusion efficiency, and r is the distance from the center of the

electrode. Therefore, according to this equation, a large enough surface area is

required so that active compound will have sufficient space and time to undergo

electrochemical reduction or oxidation. At the same time, a limited reaction

volume is also crucial to maintain a concentration gradient so that analyte could

be transferred to the electrode surface rapidly and efficiently.

2.4.1. EC detection of hydroperoxides

Hydroperoxides, a major class of lipids oxidation products that have an

easily reducible O-O bond, should be ideal analytes for ECD. Yamada et al[119]

reported successful detection of phosphatidylcholine (PC) and

phosphatidylethanolamine (PE) hydroperoxides at -300 mV vs Ag/AgCl vs AgCl

(-101 in NHE) while unoxidized PL showed no response at all. PL-OOH were

also detected at -50 mV using a mercury cathode[120] (194mv in NHE). In

comparisons of optimum detection potentials of cholesterol and unsaturated fatty

acid hydroperoxides using hydrodynamic voltammetry (HDV), 3-β-hydroxy-5α-

cholest-6-ene-5-hydroperoxide(5α -OOH), 3-β-hydroxycholest-4-ene-6-β-

hydroperoxide(6β-OOH), 3β-hydroxycholest-5- ene-7β-hydroperoxide(7β-OOH)

showed maximum redox response on a mercury electrode at around -250mv

against Ag/AgCl (-50 mV in NHE), a very low potential. In contrast, responses of oleic acid, linoleic acid, and cholesteryl linoleate hydroperoxides increased with applied potential to near 0 mv against Ag/ AgCl (200 mV vs NHE) and plateaued there, remaining constant at higher potentials[121,122].

It seems logical to expect that hydroperoxides should be detected under reducing mode since they are oxygenated products with a relatively weak O-O bond. However, Henderson et al.[123] detected linoleic acid hydroperoxides under oxidation mode instead. Reduction of linoleic acid hydroperoxides (LOOH) on glassy carbon electrode began at around +520 mV against Ag/AgCl (+719 mV vs NHE), but LOOH oxidation was observed over the potential range of +600 mv to +1300 mV against Ag/AgCl (+799 to +1499 mV vs NHE) with maximum response at the highest potential. This unexpected and interesting finding was also confirmed by our group, as will be discussed in more detail in the Results. Overall, hydroperoxides appear to possess very potent activity on electrochemical detector and offer promised for ECD analysis in HPLC.

2.4.2. EC detection of aldehydes

Early studies detected aldehydes with ECD by derivatization of aldehydes with 2,4-dinitrophenylhydrazine (DNPH) rather than directly. The aromatic ring on the DNPH makes hydrazones (DNPH complexes with carbonyls) detectable by optical methods[124,125], but also imparts electrochemical activity. When mixed with DNPH under acidic conditions, the amine group from the DNPH reacts with the carbonyl group of an aldehyde or ketone to form a Schiff base-like complex. Electrochemical activity arises not from the hydrazone structure but from the

remainder of the DNPH which has an amine group in conjugation with pi electrons of an aromatic nucleus (Group 1[112]). Goldring et al[126] detected 4-hydroxynonenal hydrazones over the range +300 to +800 mV (electrode not identified). Saczk et al[127] detected hydrazones of both aldehydes and ketones with ECD on a glassy carbon electrode, with maximum responses near +1197 mV against Ag/AgCl (1396 mv vs NHE). DNPH itself had an oxidation peak at +927 mv against Ag/AgCl (1126 vs NHE). Unlike hydroperoxides that responded under both oxidizing and reducing modes, DNPH hydrazones could only be oxidized; no reducing peaks were observed. Similar results were also reported elsewhere[128,129]. Thus, DNPH derivatization makes ECD detection of aldehydes and ketones completely feasible, provides selectivity advantages over other detection methods, and may increase sensitivity over optical methods (detection limit as low as ppb level[125]) .

Direct electrochemical detection of aldehydes has been mainly divided into two categories in terms of electrode action: coulometric and amperometric detection. Both techniques measure current generated from the transfer of electrons under a fixed potential. According to Kissinger[128,130] the difference is mainly due to the geometry: coulometry uses porous electrode with large surface area to electrolyze a compound completely when adequate voltage is applied, whereas amperometry uses a smaller smooth electrode surface and only partially electrolyzes the compounds (1-10%). Surprisingly, the higher conversion rate of coulometry does not always lead to higher sensitivity because the large electrode surface area causes significant increase in noise level that obscures actual

signals. Perhaps this explains why there are no reports of direct coulometric detection of aldehydes in the scientific literature, while numerous papers reported success with amperometric detection under specific conditions of electrode designs.

Aliphatic aldehydes could not be detected directly by a glassy carbon electrode, but when a Pt-Pb alloy was coated onto the electrode surface, aliphatic aldehydes responded electrochemically at potentials from 0.0V to 0.8V against a Ag/AgCl reference electrode[131]. Similarly, a rutheniun oxide-ruthenium cyanide film applied to a glassy carbon electrode allowed acetaldehyde, propionaldehyde, isobutyraldehyde and other aromatic aldehydes to be determined at around 1.1V against Ag/AgCl electrode with a reported detection limit of 0.1 ppb.[132] Other applications coated noble metals onto carbon-based electrodes for detection of formaldehydes.[133–137]

The explanation given for this behavior is that carbon electrodes cannot bind aldehydes, whereas the metal coatings provide a linking surface. The simple version of the explanation is the favored adsorption of aliphatic compounds on the surface of noble electrode surface provided by the partially unsaturated d-orbitals for compounds with less electrochemical activity.[117] Fedorowski and Lacourse[117] further elaborated that there are three detection modes at a noble metal electrode: mode I is through the direction detection of unfouled electrode surface that adsorbs analytes; Mode II is via adsorption of analytes to the surface oxide; Mode III is an indirect detection of electrochemical inactive analytes that suppresses the formation of surface oxide, which would otherwise produce a

normal positive signal, to generate a negative peak. Both of the first two major detection modes facilitate the oxidation of analyte by adsorption, but this is accompanied by enhanced formation of oxides on the electrode material that ultimately inactivate the working electrode.

Pulsed Amperometry Detection (PAD) was developed to avoid these problems of fouling by continually renewing the electrode surface, thereby maintaining performance. A PAD applies voltage in three minimum steps: a detection potential that detect analytes at optimum potential, a more oxidative potential to convert surface oxidation intermediates to oxides, then a large negative cathodic potential to remove surface oxides (Figure 9).

PAD has been used for direct detection of aliphatic aldehydes on noble electrodes. Casella and Coutursi[116] reported detection of formaldehyde, acetaldehyde, propionaldehyde, butyaldehyde and their isomers on a Pt electrode directly from 0.2-0.5V against Ag/AgCl reference electrode. The detection limit was from 10 to 200 ppb level. Hexanal, octanal, trans-2-hexenal and other flavor related aldehydes and even alcohols were detected and quantified directly from -0.5V to 0.2V on a gold electrode against calomel reference electrode under alkaline pH conditions.[138] These methods offer great sensitivity compared to optical detection, and display almost indiscriminative responses for both saturated and unsaturated aldehydes.

In summary, electrochemical detection, or more precisely, PAD offers a promising method for the detection of the less electrochemically active aldehydes and potentially other lipid oxidation products as well.
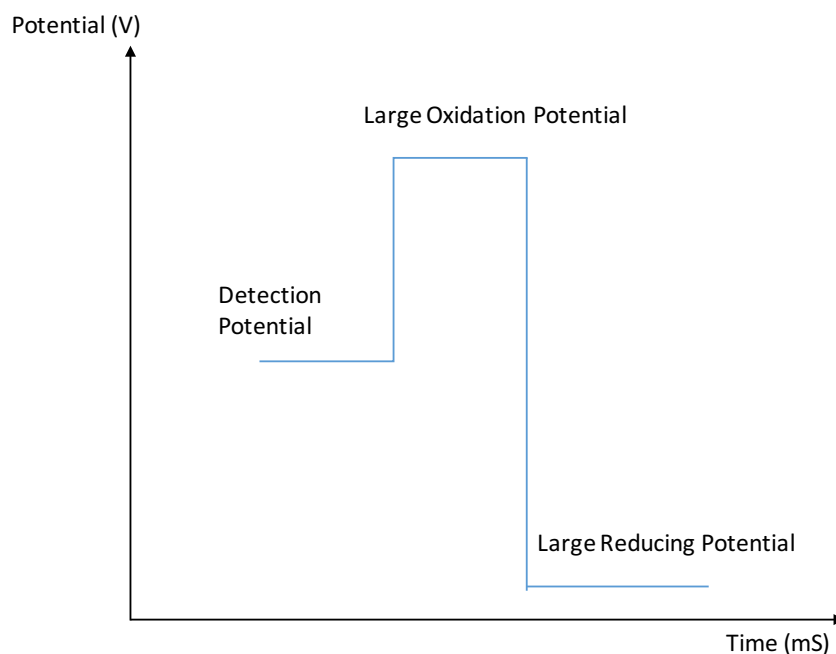
Figure 9. Three stages of Pulsed Amperometric Detection (PAD) for surface reactivation[117].

2.4.3. EC detection of epoxides

Epoxides are another group of lipid oxidation product attracting significant interest because of their potential toxicity. Epoxy products of lipids are formed through internal addition reactions of peroxyl radicals and cyclization reactions of alkoxyl radicals[13]. When abstractable hydrogen atom sources are not immediately available, peroxyl radicals add to double bonds either on β or γ position of the same molecule to form epidioxides[139] or on adjacent molecules to generate epoxides[140].

$$LOO\cdot \ + \ R_1-CH_2-CH=CH-R_2 \longrightarrow \ R_1-CH_2-\overset{\cdot}{C}H-\overset{OOL}{C}H-R_2$$

$$\downarrow O_2$$

$$LO\cdot \ + \ R_1-HC\cdot-CH-CH-R_2 \longrightarrow \ L_2(epoxy)OO\cdot$$
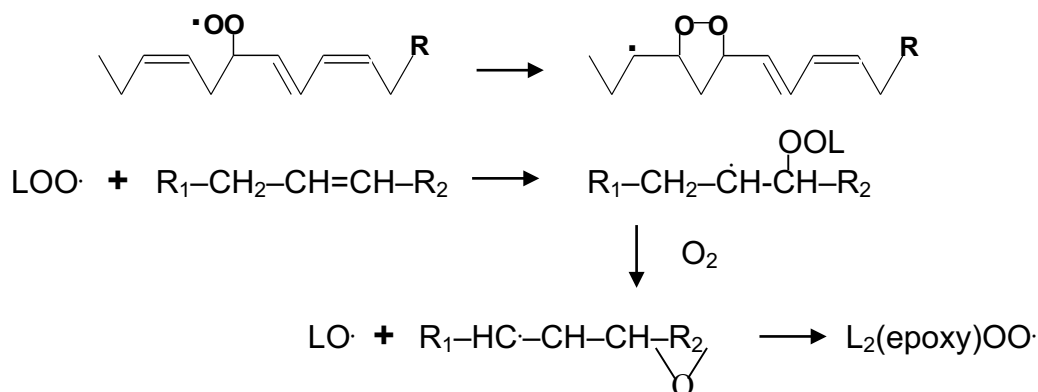
Epoxides can also be formed directly via addition of alkoxyl radicals to immediately adjacent double bonds:[141]

$$R_1-HCH=CH-CH=CH-\overset{\cdot O}{C}H-R_2 \longrightarrow \ R_1-HCH=CH-CH-CH-CH-R_2$$

Although epoxides have been largely ignored both analytically and mechanistically in previous studies, giving preference instead to hydroperoxides as the only major early oxidation product, the free radical literature suggests that epoxide formation should be expected[142–147], and studies testing the concept of multiple alternate reactions in competition with hydrogen abstraction have found epoxides as dominant oxidation products under many circumstances[16].

Like aldehydes, electrochemical detection of epoxides is not without challenges. Epoxy compounds have been detected in biological matrices with UV and MS detectors[148–151], but the epoxy compounds reported all have unique structures containing multiple rings with double bonds that contribute to the UV absorbance. In contrast, alkyl epoxides from fats and oils cannot be detected by UV absorbance due to the lack of chromophores. Thus, electrochemical detection, if achievable, could be very useful.

To improve electrochemical detection, some limited derivatization

methods that render products electrochemically-active are available. Most of the derivatization methods are based on tagging epoxides with compounds that bear one or multiple benzene rings with amine or nitro groups that have very high electrochemical activity. Colgan and Krull[152] reacted cyclohexene epoxide (electrochemically inert) with alkyl halides and silver picrate to form a picryl ether that was quite electrochemically active on a glassy carbon working electrode under both modes of EC detection; the approach was also quite sensitive, with a lower detection limit of ppb level. However, dry picric acid can be very explosive and extreme caution must be applied during handling.

8-amino-2-naphthoxide[153] was also reported as a derivatizing reagent of alkyl halides, acid chlorides and other electrophiles. The unsaturated double ring structure with amine adduct is very electrochemically active. However, it is not available commercially, and the derivatization process involves very complex synthesis of the starting material on a polymer immobilized resin, purification of the derivatizing reagent (recovery rate only ~35%), and derivatization reaction with electrophiles. The entire process requires days to complete, making the method not very practical for analyzing large numbers of samples.

Given reportedly low electrochemical activity of epoxides, lack of suitable derivatization methods to improve detection, and a paucity of research on epoxide interaction with different electrodes, it is perhaps not surprising that ECD determination of epoxides has received little attention and limited publications utilizing the technique are available. While potential is unknown, EC detection of epoxides still warrants further investigation.

3. RESEARCH GAPS

This research addresses two major challenges and critical lipid analytical needs that are distinct yet related. The first challenge is the separation, quantification, and identification of component lipid classes and component molecules in mixtures such as oils and extracts. Although lipid class separation has been accomplished extensively in column chromatography and in thin layer chromatography, these methodologies are laborious, slow, and they can expose lipids to substantial oxidation and degradation. Quantification is possible but certainly not easy. High pressure liquid chromatography has been applied much less, and mostly with normal phase. A few studies have reported reversed phase separations but factors controlling the separations have not been fully elucidated, incomplete resolution is common, and separation has not been combined with identification and quantitation of fractions and individual lipid compounds.

With interest in lipids and lipid composition of foods being reactivated, and food companies being held more accountable for the specific composition of their products, there is serious need for a general reversed-phase HPLC analytical method that can be easily and conveniently modified for different matrices to separate a wide range of food lipid compositions, from foods that contain high saturated fats to the ones composed of mainly high polyunsaturates or omega-3 oils, from oils with mixed acylglycerols to extracts with degradation products and phospholipids as well as acylglycerols.

The second challenge deals with detecting and quantitating a broad range of lipid oxidation products while separating total lipid classes. Previous HPLC

studies followed oxidation exclusively by absorbance of conjugated dienes at 234 nm. However, recent demonstration that multiple alternate degradation pathways exist and compete with hydrogen abstraction in lipid oxidation makes it imperative to monitor products from multiple pathways simultaneously for accurate assessment. Measuring many different products by separate chemical analyses is extremely time-consuming and subjects oils and extracts to substantial handling that can enhance oxidation and change products. In addition, detection sensitivity and linear response range varies with each assay, making it difficult to determine an accurate mass balance among all products. Thus, there is a critical need for methodologies that detect products from multiple oxidation pathways in a single analysis where detectability is comparable.

HPLC seems ideally suited to address both of these challenges since it can separate both classes of lipids as well as individual oxidation products. However, detection is limited since lipids lack optical reporter groups. ELSD is used frequently as a general detector for lipids, but its application for quantitation of lipids has not been systematically investigated. Some electrochemical detection (ECD) of hydroperoxides has been reported but the method has not been fully evaluated. Theoretically, other oxidized functional groups (aldehydes, ketones, carboxylic acids, epoxides, alcohols, etc.) should also be detectable electrochemically with high sensitivity and selectivity. Unfortunately, there is little information available demonstrating feasibility and practical application of ECD to these lipid oxidation products. Hence, detailed studies of ECD detection of various classes of lipid oxidation products are definitely warranted.

4. HYPOTHESIS AND OBJECTIVES

4.1 Hypothesis

Reversed phase HPLC can be successfully applied to separation and quantitation of total lipid classes using appropriate columns, solvent gradients, and evaporative light scattering detection. Interfacing of electrochemical detection, combined with UV detection of conjugated dienes at 234 nm may make it possible to simultaneously detect various oxidation products in the same process.

4.2. Specific Objectives

1) Develop a simple RP-HPLC separation of total lipid classes expected in foods (free fatty acids, phospholipids; mono, di, and triacylglycerols) without in-class discrimination

2) Modify this procedure to separate individual lipid components within classes by studying other intermolecular interaction.

3) Evaluate evaporative light scattering detection (ELSD) for quantitation of lipid fractions.

4) Evaluate the feasibility of using multichannel electrochemical sensors to detect, identify, and quantitate individual classes of lipid oxidation products (hydroperoxides, epoxides, alcohols, aldehydes, ketones, alcohols, etc) in oils and extracts, and if possible, develop methodology and guidelines for application.

5. MATERIALS AND METHODS

5.1. Experimental Design

An RP-HPLC method for separation of mixtures of lipid classes including FFAs, MAGs, DAGs, and TAGs was developed to provide improved information about content and concentrations of lipids in oils and lipid extracts of foods and biological tissues. Fractions were detected by diode array at 206/210 nm for general analyses. Detection with an evaporative light scattering detector (ELSD) in-line with the diode array detector was evaluated for qualitative sensitivity as well as for quantitation of lipid fractions. Electrochemical detection (ECD) was evaluated for detection and differentiation of oxidation products (hydroperoxides, epoxides, ketones, and aldehydes). It was expected that this approach would enable researchers to determine both lipid composition and extent of degradation in a single process, and might also contribute to tracking full extent of lipid oxidation more accurately.

Phase 1. For total class separation, "boundary lipids" were first used to define the elution time window of each class (Figure 10). According to the ECN theory, the elution order of acyl lipids should follow the carbon numbers from lower to higher ECN, so lipids with ECN in between should fit within the time window established by the boundary lipids of each class. For example, if the time window of FFAs is defined by myristic acid and stearic acid that have a ECN of 14 and 18 respectively, any other FFAs with an ECN in between (e.g. oleic acid and palmitic acid) should elute within the range. Elution boundaries were set similarly for acylglycerols. Gradients were developed using this model.
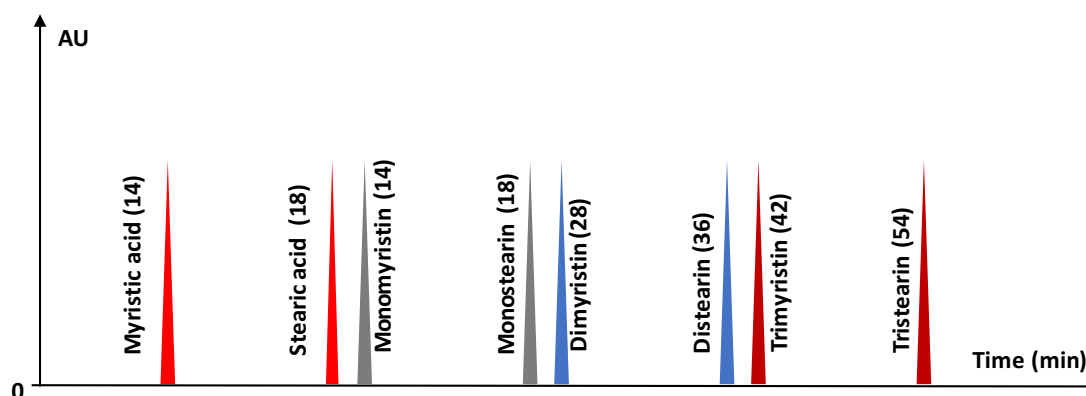
Figure 10. Boundary compound representation of each lipid class.

Phase 2 added phospholipids (PLs) to the mix, and gradient modification focused on the first 20 minutes where the PLs and FFAs overlap.

Phase 3 further modified elution to separate individual components within classes. Preliminary analyses showed that modification of solvents, gradients, and C18 column alone could not separate all the critical pairs, thus a second column -- silver ion column or cyano -- was connected in series with the C18 column and investigated.

Phase 4 used the best separation conditions identified in Phase 3 to evaluate evaporative light scattering detection for quantitating lipid components.

Phase 5 investigated the feasibility of using coulometric electrochemical (EC) detection for the determination of major oxidation products including hydroperoxides, aldehydes and epoxides. Various supporting electrolytes were tested and optimum detection potential was investigated. DNPH derivatization for aldehydes was also tested when direct coulometric detection was unsuccessful.

Linearity and concentration range of response were also measured for hydroperoxides and DNPH-aldehydes.

5.2. Materials

Reference standards for lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, linolenic acid, monomyristin, monopalmitin, monostearin, monoolein, monolinolein, monolinolenin, dimyristin, dipalmitin, distearin, diolein, dilinolein, dilinolenin, trimyristin, tripalmintin, tristearin, triolein, trilinolein, and trilinolenin were purchased in ultra-high purity from Nu-Check Prep (Elysian, MN). 1,2-dioleoyl phosphatidylcholine, 1,2-dioleoyl phosphatidic acid and 1,2-dioleoyl phosphatidyl-ethanolamine were generously donated by Dr. George Carman, Rutgers Food Science.

Acetonitrile, methanol, hexane, chloroform, lithium perchlorate, N,N-dimethylformamide and 2,4-dinitrophenylhydrazine were all HPLC grade (Sigma-Aldrich, St. Louis MO).

All water used in this study was purified to 18 MΩ resistivity in a Milli-Q water purification system (Model 13681, Millipore, Bedford, MA). It will hereafter be referred to in the text as MQ water.

5.3. Experimental Methods

5.3.1. RP-HPLC separation of lipid classes without in-class

discrimination

An Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) equipped with a G1329A autosampler, G1316A column oven, G1315A diode array detector, and G1311A quaternary pump was used for most experiments. The method for class separation without in-class discrimination used an Ultra Aqueous C18, 150×4.6 mm, 5 um column (Restek, College Station, PA) with flow rate 0.55 ml/minute, column temperature controlled at 40˚C, and injection volume of 20 μl. To optimize the gradient for separation of total lipid classes, the solubilities of the boundary compounds in Figure 10 were tested to identify appropriate mobile phase candidates. After solvents for mobile phases were selected, reference standards of boundary compounds were then tested with various gradient programs aiming to achieve the pattern of separation indicated in Figure 10, starting with polar solvent and gradually increasing the hydrophobicity of the system by introducing non-polar solvents. Once the pattern was achieved with boundary compounds, a master mixture of FFAs, MAGs, DAGs and TAGs reference standards was analyzed to confirm the separation pattern and dominance of the ECN mechanism in separation.

5.3.2. Quantitation of lipid components by ELSD

Routine detection of peaks (qualitative) used an Agilent diode array detector connected in series with an Alltech 500 ELSD. Data was recorded and

analyzed using Agilent ChemStation™ software. The Alltech 500 ELSD signal was converted to ChemStation™ signal by an Analog to Digital signal converter.

All quantification data was obtained on a Waters UHPLC e2695 system equipped with a 2424 ELS detector in the NJ Institute for Food Nutrition and Health. Data was collected and processed with Empower™ 2 software. ELSD condition was set as 2.5L/min nitrogen flow for the nebulizer that was kept at 50ºC.

5.3.3. Electrochemical detection, identification, and quantitation of lipid oxidation products.

An ESA coulometric electrochemical detector (ECD) connected in series with the Agilent diode array detector was tested for ability to detect lipid oxidation products as they eluted. Each detection cell contained a graphite working electrode with potential measured against a gold reference electrode. Eight of such micro flow cells connected in parallel allowed simultaneous monitoring of eight different electrical potentials from -1000 mV to +2000 mV. *Tetra*-n-butylammonium bromide and lithium perchlorate were tested as hydrophobic electrolytes. Based on background current levels, noise, and intensity of signal response, 1-10 mM lithium perchlorate, depending on the oxidation products, was selected as the supporting electrolyte.

ECD signals were recorded and analyzed using ESA Analyst software. Signal collection was initiated and synchronized to ChemStation™ by contact closure with the Agilent autosampler, which sent the starting signal simultaneously with injection of a new sample.

For regular maintenance of the microelectrode cell, the system was rigorously flushed with MQ water for 1-2 hours after the end the run to eliminate the deposition of electrolytes and other salts. Polishing to renew the electrode surface was not possible.

Electrochemical properties of pure cumene hydroperoxides, butyl hydroperoxides, aldehydes of C4 to C10, c-4-heptenal, t-2-octenal, t-2 nonenal, t-2-decenal, t,t-2,4-hetandienal, t,t-2,4-nonadienal, t,t-2-4,decadienal and 1,2-epoxy-5-hexene were studied first to determine the feasibility of using such detection technique for lipid oxidation products. Aldehydes were also tested as hydrazones of dinitrophenylhydrazine to determine whether sensitivity or specificity may be greater than in standard HPLC-DNPH assays with diode array detection.

5.3.3.1. ECD analysis of lipid hydroperoxides

HPLC conditions for elution of lipid hydroperoxides were tentatively adapted from Korytowski[121]. The eluting solvent was 250 ml MeOH, 112.5 ml acetonitrile, and 137.5 ml Milli-Q water mixed well together and made 5 mM $LiClO_4$ as electrolyte; flow rate was 0.8 ml/min isocratic. Electrical potential was scanned from –1000 mV to +2000 mV with 100 mV increments in 8 channels. Cumene hydroperoxide was used as a reference standard to test electrochemical activity, determine signal response factor, and develop a calibration curve for quantitation.

5.3.3.2. ECD analysis of lipid epoxides

For initial testing, HPLC conditions were 10 mM lithium perchlorate in acetonitrile:MQ water 9:1, with flow rate 1.2 ml/min. Reference standards of 1,2-epoxy-5-hexene and 1,2-epoxy-5-decene were tested for electrochemical activity.

5.3.3.3. ECD determination of aldehydes and DNPH derivatized aldehydes

EC detection of aldehydes was first tested without derivatization using the gradient listed in Table 7. Because of weak signal response and poor sensitivity, aldehyde derivatization by DNPH and EC detection of the resulting phenylhydrazones was also tested[154] using a procedure and gradient developed in our laboratory (Table 7). Mobile phase A was ACN:MQ Water 90:10 with 25 mM TBAB; mobile phase B was 20 mM citric acid, pH 6.5: ACN 99:1. The flow rate was set at 1.2 ml/minute and the electrical potential was applied from 200 mV to 900 mV with 100 mV increments. A stock solution of DNPH was prepared by dissolving 0.02 g 3x-recrystallized DNPH in 1 ml dimethylformamide with 10 μl 85% sulfuric acid. Hydrazones were prepared by mixing 100 DNPH stock solution with 100 μl of aldehyde reference standards, diluting the mixture to 2 ml with acetonitrile, and incubating at room temperature for 15 minutes.

Table 7. HPLC gradient for electrochemical analysis of aldehydes without derivatization. A: ACN:MQ Water 90:10 with 25 mM TBAB. B: 20 mM citric acid pH 6.5:ACN 99:1.

Time	A	B
0	66.7	33.3
15	100	0
17	100	0
18.5	66.7	33.3
23	66.7	33.3

For HPLC analysis, reaction mixtures were diluted an additional 20 times with acetonitrile to give the final sample concentrations as listed in Table 16 (Section 6.3). Hydrazones were separated by HPLC using the same gradient as in Table 7, with 10 mM lithium perchlorate. This method was extended to acylglycerols and mixtures of lipid classes by Lynn Yao using a modified gradient[155] to detect core carbonyls as well as monomer products.

6. RESULTS AND DISCUSSION

6.1. Development of Lipid Class Separation by RP-HPLC

The fairly wide polarity range of lipid fractions normally found in extracts presented some distinct challenges. Three particular problems that had to be resolved were low solubility of long chain saturated fatty acids and acylglycerols in HPLC solvents, and overlapping of monoacylglycerols with FFAs and FFAs with phospholipids in elution. Our approaches to overcoming these obstacles are described below.

6.1.1. Solubilizing and separating saturated fatty acids and acylglycerols

Saturated lipids have poor solubility in the organic solvents normally used in RP-HPLC. Acetonitrile, methanol, acetone, propanol and tetrahydrofuran were initially tested, but saturated lipids of FFAs and acylglycerols with ECN of 16 or more did not dissolve (e.g. stearic acid, monostearin, distearin and tristearin). These lipids did, however, dissolve easily in solvents such as hexane and chloroform. After considerable empirical testing, hexane:isopropanol 1:1 was found to provide a good combination of solvent strength and both polarities so was selected as the one of the mobile phases to elute long chain saturated lipid molecules. Isopropanol was the solvent of choice because, unlike acetonitrile, it mixed with hexane and provided an intermediate polarity that acted like a bridge to connect polar and non-polar mobile phases.

One observation worth noting is the minor negative effect of using hexane as a mobile phase in reversed phase HPLC, namely longer re-equilibration time is needed after each gradient run. Figures 11 shows two replicates of

monopalmitin elution using HPLC conditions as in Table 10. The first test of

monopalmitin was performed when the ultra AQ C18 column was freshly

equilibrated at the beginning of the day; a replicate test was conducted

immediately after the first run with about 10 minutes equilibration in between. Ten

minutes of re-equilibration, a time sufficient with other solvents, was obviously

incomplete when hexane was a gradient component since the peak eluted about

two minutes earlier than in the first run (Figure 11, top). After full equilibration, the

retention time of monopalmitin returned to the original value (shifted from 25.61

minutes to 27.88 minutes). We speculate that hexane may strongly associate

with the C18 acyl chain of the stationary phase and require a larger solvent

volume to clear it after runs. Under the conditions presented, residual hexane

from the previous run was still in the column and contributed to earlier elution of

the second run. This phenomenon actually resembles what frequently happens in

normal phase analyses when water is in mobile phases. A 30-minute

equilibration time was found to solve this problem, but the phenomenon is

important to keep in mind when using strong solvents for lipid class separations.
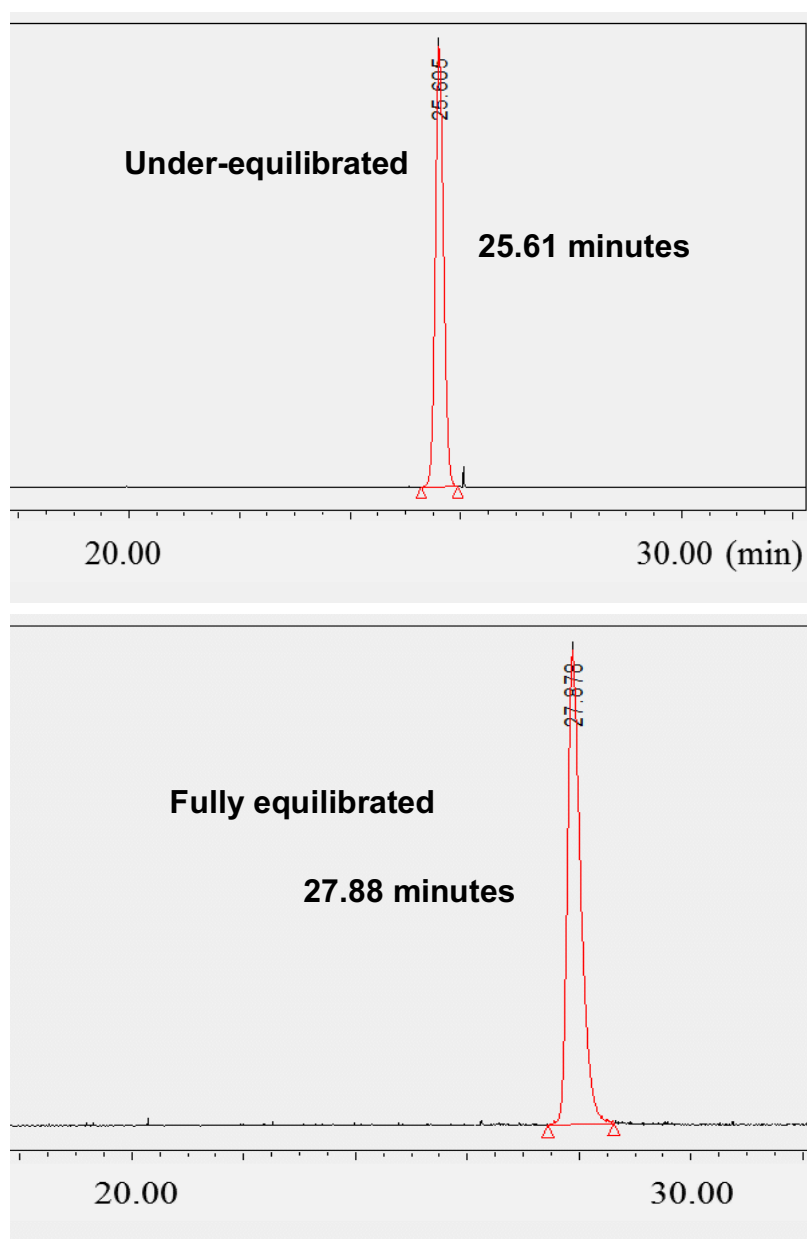
Figure 11. Monopalmitin peak shifting with incomplete re-equilibration when hexane is present in the elution gradient in RP- HPLC. Top, 10 minutes equilibration, hexane still bound. Bottom: fully equilibrated, 30 minutes. Gradient as in Table 10. ELSD detection was made on Waters E2695 UHPLC system, ELSD nebulizer was set at 2.5 L/minutes of nitrogen and temperature was set at 50°C.

6.1.2. Separation of free fatty acids and monoacylglycerols

The second challenge encountered was separation of FFAs and MAGs whose ECNs were virtually the same. Early testing used acetonitrile-water-isopropanol gradients (Table 8).

Table 8. Method for initial separation of FFA, MAG, DAG and TAG using linoleic acid, monolinolein, dilinolein and trilinolein as model system

Column: Ultra AQ C18, 150*4.6 mm, 5 μm			
Flow rate: 0.55 ml/min			
Time	%ACN	%MQ water	%Isopropanol
0	70	30	0
20	100	0	0
36	100	0	0
132	40	0	60
140	70	30	0

Under all combinations we tested, the stearic acid used as a FFA class standard eluted much later than the MAG standard monolinolein, even though the latter was one class higher than FFAs. One fatty acid chain was present in each molecule but polarity added by the two –OH groups moved the MAG through the column faster than the fatty acids. Even though each peak resolved cleanly, this result failed to meet one of the goals of this project -- separating each class into its own time window. Manipulations of solvent polarity and

gradient to improve separation changed only the absolute retention times of these peaks without altering the relative eluting order. This behavior truly corresponds to the discussion of ECN dominance mentioned earlier. Based on ECNs of 18 for stearic acid and 14 for monolinolein, the elution pattern in Figure 12 should be expected and will not change unless another force greater than ECN dominance is imposed.
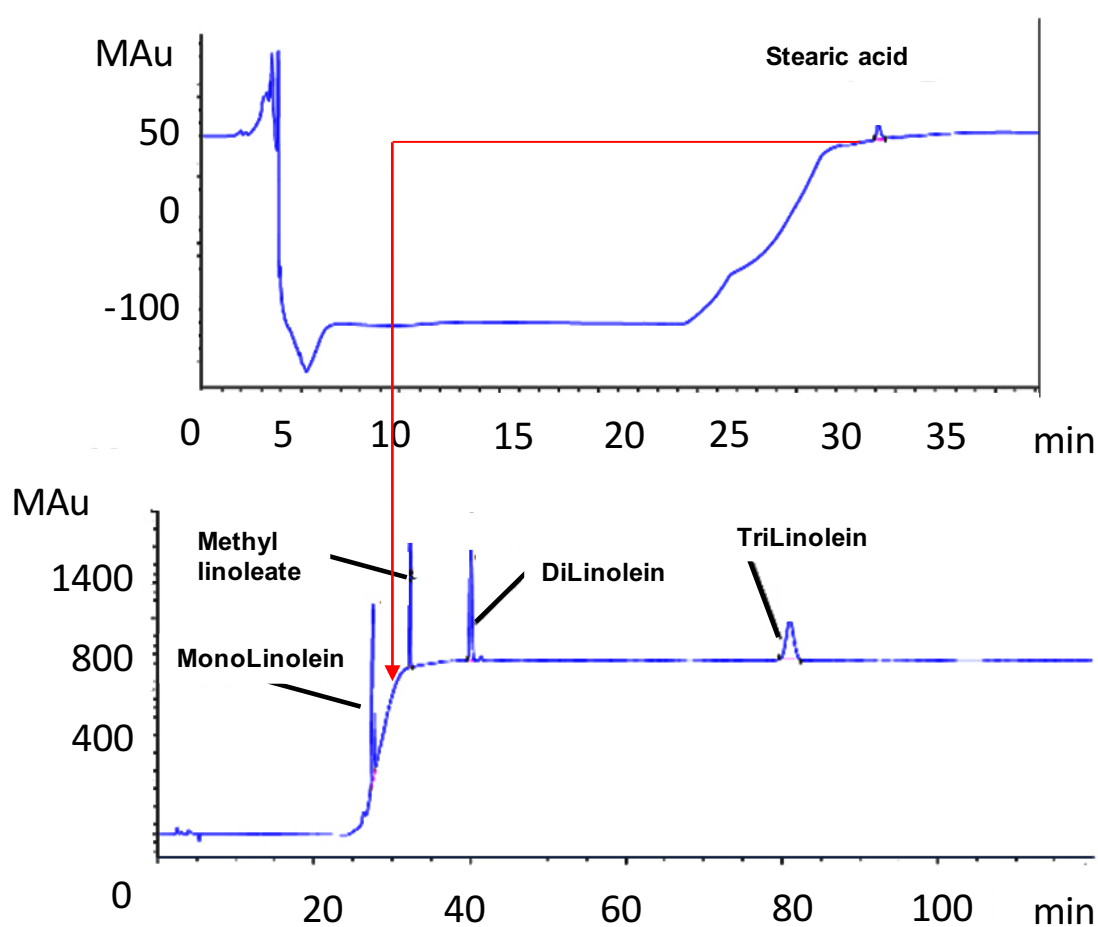
Figure 12. Separation of acyl lipids using stearic acid (top), monolinolein, dilinolein, trilinolein and methyl linoleate (bottom) to represent each class. Acetonitrile/water/isopropanol gradient as in Table 8; UV detection at 210 nm.

Mobile phase - analyte associations are weaker than ECN (acyl chain interactions with the column). However, ionic interactions between water and acid groups should be much stronger. To test whether the presence of charges in the eluting solvent could move fatty acids through the column faster, the pH of the aqueous phase was modified by addition of 0.1% of ammonium carbonate (w/w). The aqueous phase was first brought to 8.7 to assure that all FFAs were completely dissociated while maintaining a pH lower than 9, the limit specified for the column, and the run was started with 30% aqueous phase / 70% acetonitrile. The full gradient is shown in Table 9. Under these conditions, we observed poor reproducibility in a model mixture of monomyristin, dimyristin and trimyristin, with acylglycerol peaks missing (Figure 13). A huge peak that eluted very early in the run was determined to be myristic acid from alkaline hydrolysis of acylglycerols. Nevertheless, one positive observation was that the FFA peaks which normally appeared very close to the monoacylglycerol peaks (20-40 minutes) now eluted much earlier at 2-4 minutes (Figure 14). This shift suggested that optimum separation might be achieved with careful tuning of the pH.

Since the pKa's of the FFAs are lower than 5.0 (Table 1), greater than 99% of the FFAs should be dissociated at a pH of 7 where hydrolysis should not occur. Tests at pH 6.8 (Table 10) indeed showed that FFAs eluted much earlier than MAGs of equivalent or even longer chain length (Figure 15) as the ECN dominance was overpowered by the ionic strength, while the milder pH also stabilized the acylglycerols and prevented hydrolysis so that all fractions were separated (Figure 16).

Table 9. Gradient with high pH adjustments to aqueous phase.

Column: Ultra AQ C18, 150*4.6mm, 5um			
Flow rate: 0.55ml/min			
Time	%ACN	% 0.1% NH_4CO_3, pH=8.7	% Hexane:Isopropanol 1:1
0	70	30	0
10	70	30	0
20	100	0	0
36	100	0	0
120	50	0	50
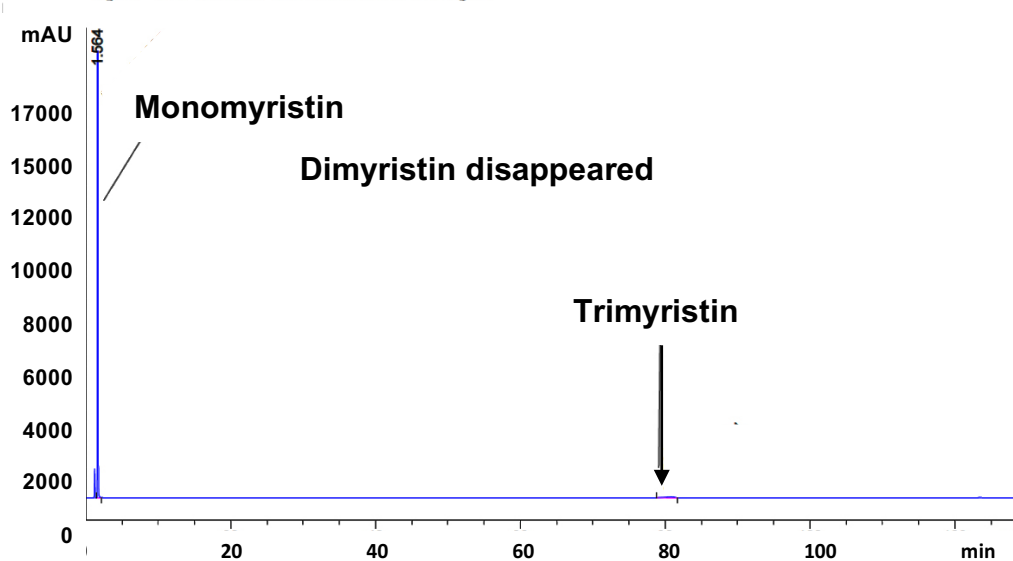
Figure 13. Elution of free fatty acids and acylglycerols at high pH (pH = 8.7) leads to hydrolysis of acylglycerols. Elution conditions as in Table 9. Detection with Alltech 500 ELSD, nebulizer flow was set at 2.5L/min of nitrogen and heated at 80°C.
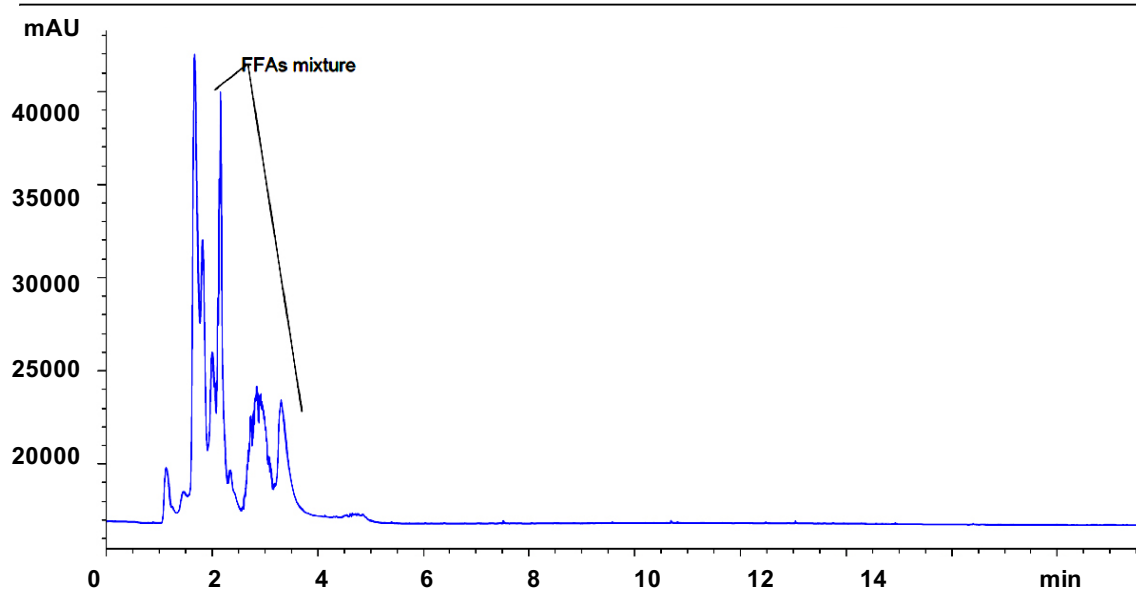
Figure 14. Early elution of FFAs with aqueous phase at pH 8.9. Elution conditions as in Table 9. Detection with Alltech 500 ELSD, nebulizer flow set at 2.5L/min nitrogen, heated at 80°C.

Table 10. Gradient with aqueous phase pH adjusted to 6.8.

Column: Ultra AQ C18, 150*4.6 mm, 5 μm

Flow rate: 0.55 ml/min

Time	ACN %	MQ Water with1 mM citric acid + NaOH, %,pH=6.8	Hexane:Isopropanol 1:1, %
0	60	40	0
10	60	40	0
20	100	0	0
36	100	0	0
120	50	0	50
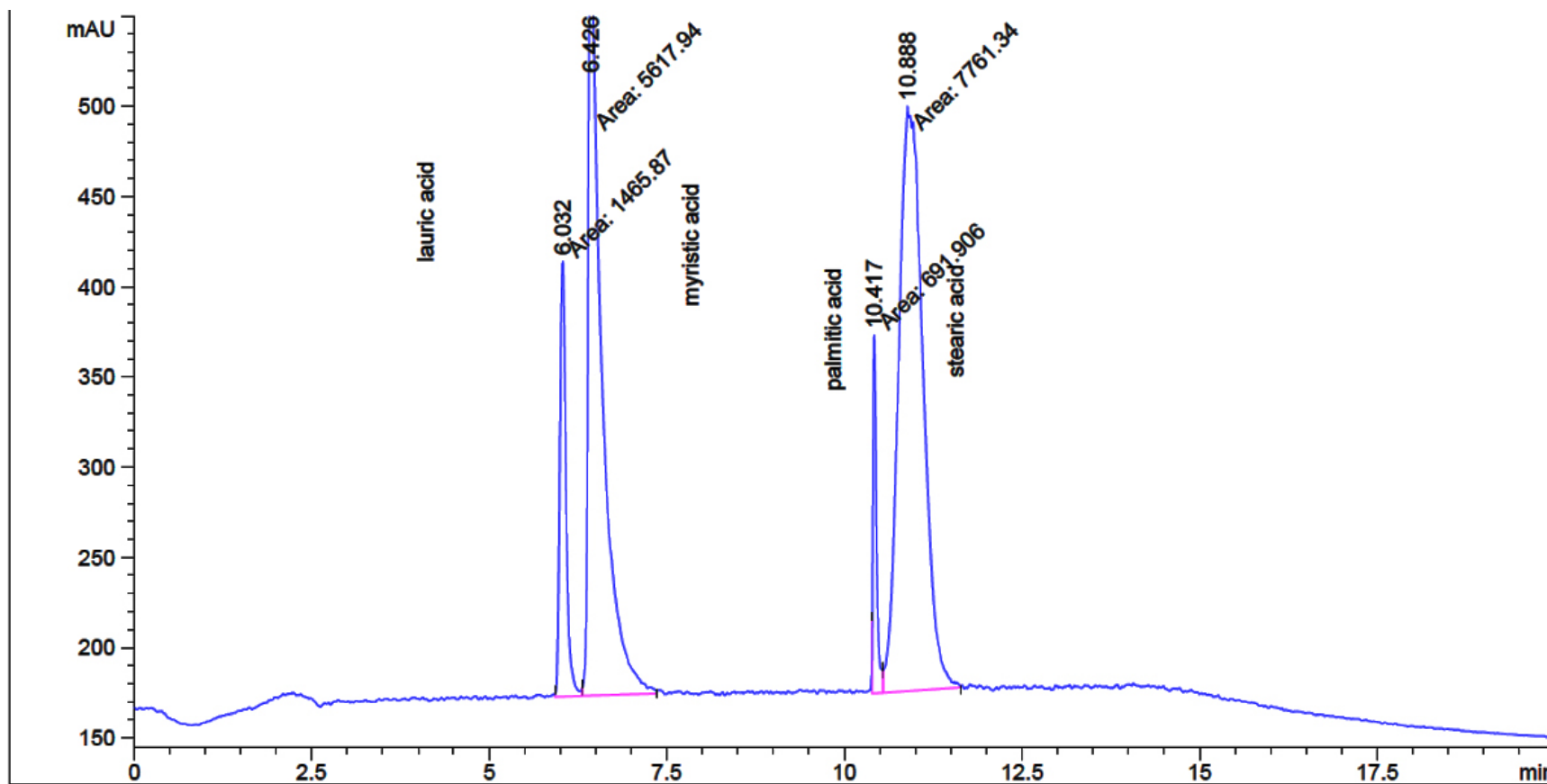
Figure 15. RP-HPLC separation of FFAs at pH of 6.8. Gradient as in Table 10. Detection was made using AllTec 500 ELSD, nebulizer flow was set at 2.5L/min of nitrogen and heated at 80°C.
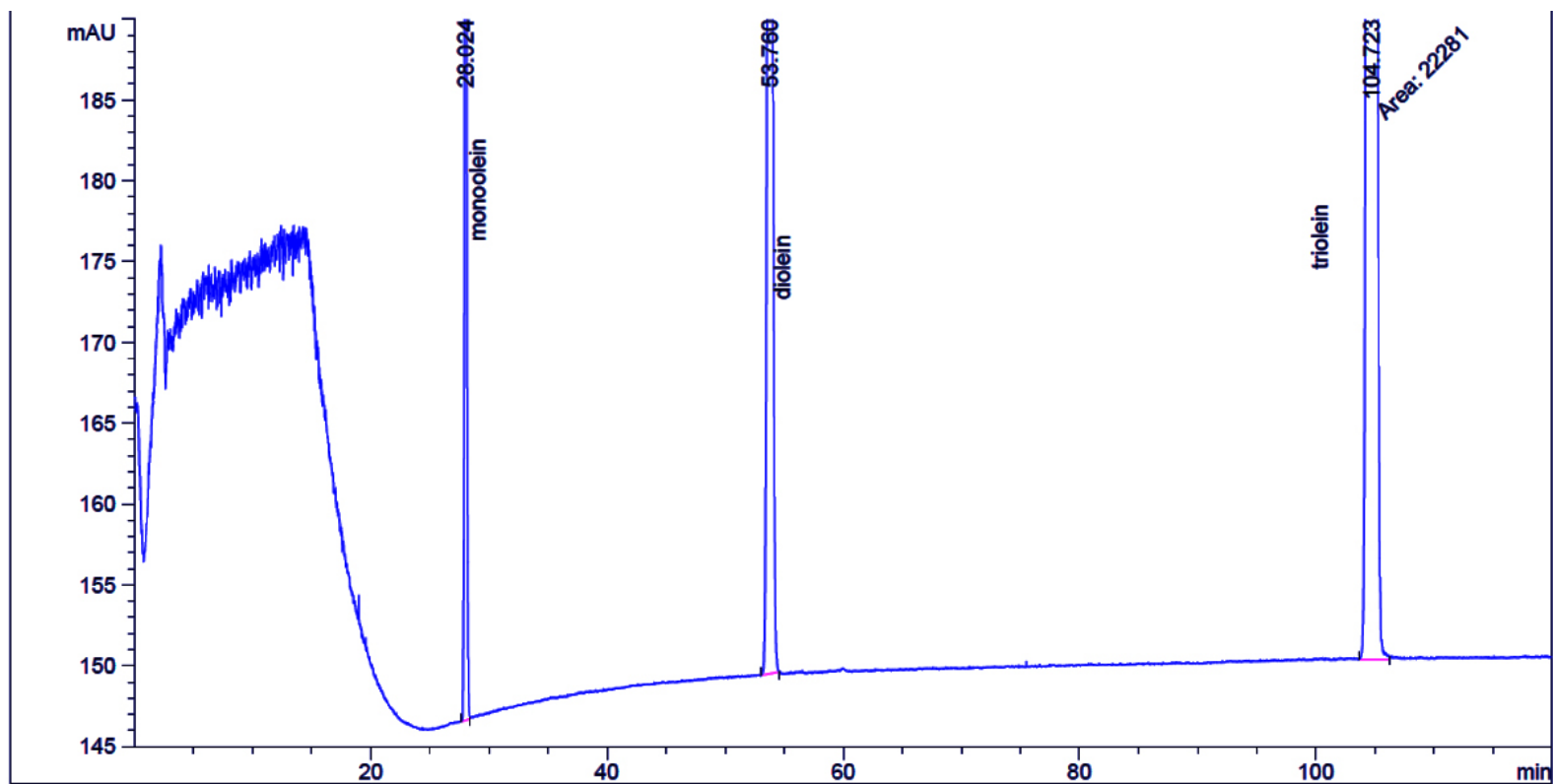
Figure 16. Normal eluting pattern for mono, di and triolein under neutral pH (pH 6.8). Gradient as in Table 10.

Detection was made using AllTec 500 ELSD, nebulizer flow was set at 2.5L/min of nitrogen and heated at

80°C.

6.1.3. Separation of FFAs, MAGs, DAGs and TAGs

The addition of hexane to maintain solubility of long chain fatty acids and acylglycerols was combined with pH control at pH 6.8 in order to obtain full separation of total lipid classes. Co-elution of FFAs was expected since the ionic strength of the citrate aqueous phase was strong enough to elute all the FFAs through the column in a non-discriminative manner. However, using what was learned with fatty acid separation, we combined early elution with a dominant pH 6.8 aqueous phase with gradual late introduction of hexane:isopropanol to elute saturated fractions and larger acylglycerols (Table 10) and found surprisingly good class and component separation (Figure 17). Thus, the gradient outlined in Table 10 provides a good starting point for general class separation of neutral lipids and fatty acids.
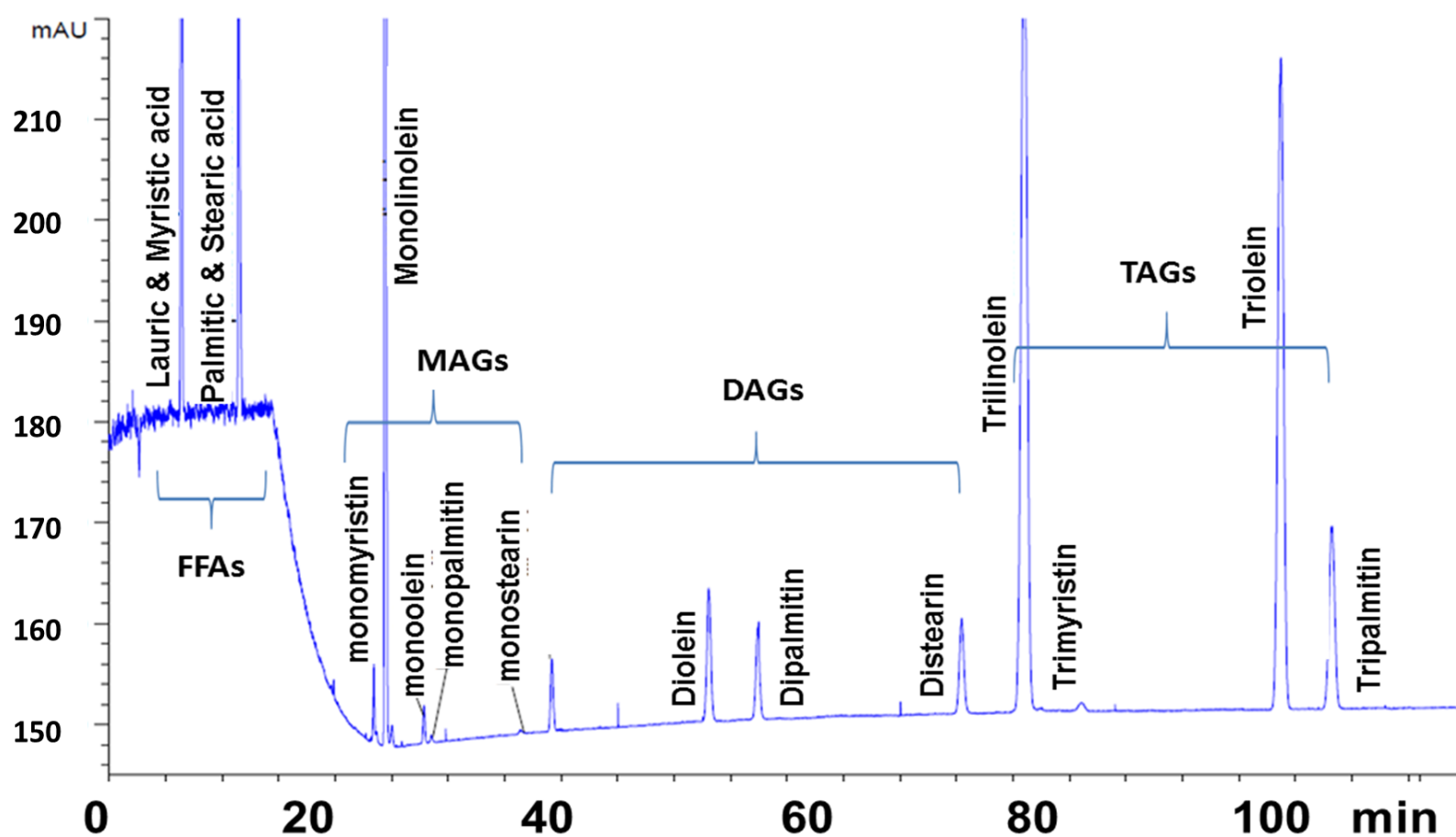
Figure 17. Lipid class separation without in-class discrimination. Elution with gradient listed in Table 10, detection was made using AllTec 500 ELSD, nebulizer flow was set at 2.5L/min of nitrogen and heated at 80°C.

6.1.4. Addition of phospholipid to the lipid mix – separation between PLs and FFAs

Including PLs in total class separations has always been a huge challenge due to the amphiphilicity of this lipid class. Because of the polar phosphate residue on the sn-3 position and two acyl hydrophobic chains on the sn-1 and sn-2 positions of phospholipids, neither ECN nor ionic interactions seem to predict retention of these molecules on the column. The effect of the phosphate group cannot practically be eliminated since it is charged over nearly the entire pH range[156] and the acyl chains on PLs are so characteristic of the source that they cannot be removed. Thus, the amphiphilic feature must be dealt with during the separation.

Initial trials using the standard gradient (Table 10) and 1,2-dioleoyl phosphatidylcholine (PC), 1,2-dioleoyl phosphatidic acid (PA) and 1,2-dioleoyl phosphatidylethanolamine (PE) as model PLs indicated that FFAs and PLs eluted close together (Figure 17 and 18) with an elution time for all PL (about 9.6 minutes) that was in the same time window as FFAs in the standard gradient. Since PA, PC and PE nearly co-eluted, the alcohol groups are not contributing to their elution. However, it is not clear whether the charge on the phosphate group or the two acyl chains that are identical in all test PLs are driving the separation.

Retention of PLs and FFAs was manipulated without affecting the overall separation of MAGs, DAGs and TAGs by adjusting gradients in the early segments of the elution (Table 11). As the proportion of aqueous phase at the beginning of runs decreased, FFAs (e.g.oleic acid) tended to shift to later

retention times (Figure 19) while PLs (e.g. PA) moved out earlier (Figure 20).

However, there is a limit to which water can be removed since at 10% aqueous

phase, the peak shape of oleic acid became extensively broadened and distorted

(Figure 19, bottom). Under these conditions, there was insufficient buffered

aqueous phase to maintain a neutral pH required for dissociation of FFAs, and

the FFAs probably existed as both ionic and associated forms.

.
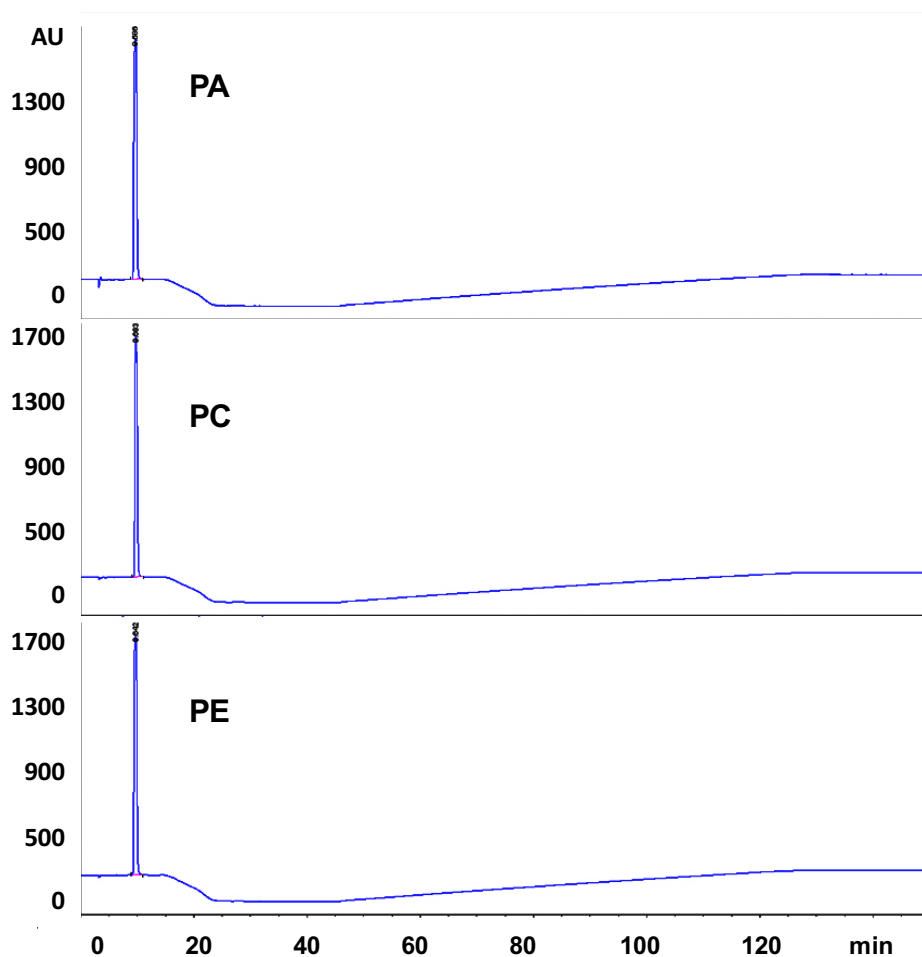
Figure 18. RP-HPLC elution of PA (top), PC (middle) and PE (bottom) using the

standard gradient listed in Table 10. Detection was made using Agilent DAD at

210 nm.

Table 11. Gradients tested to further separate PLs and FFAs on an RP-Aqueous

C18 column.

Time (minutes)	% ACN	% 0.1 M citric acid + NaOH, (pH=6.8)
Attempt 1		
0	70	30
10	70	30
20	100	0
Attempt 2		
0	80	20
10	80	20
20	100	0
Attempt 3		
0	90	10
10	90	10
20	100	10
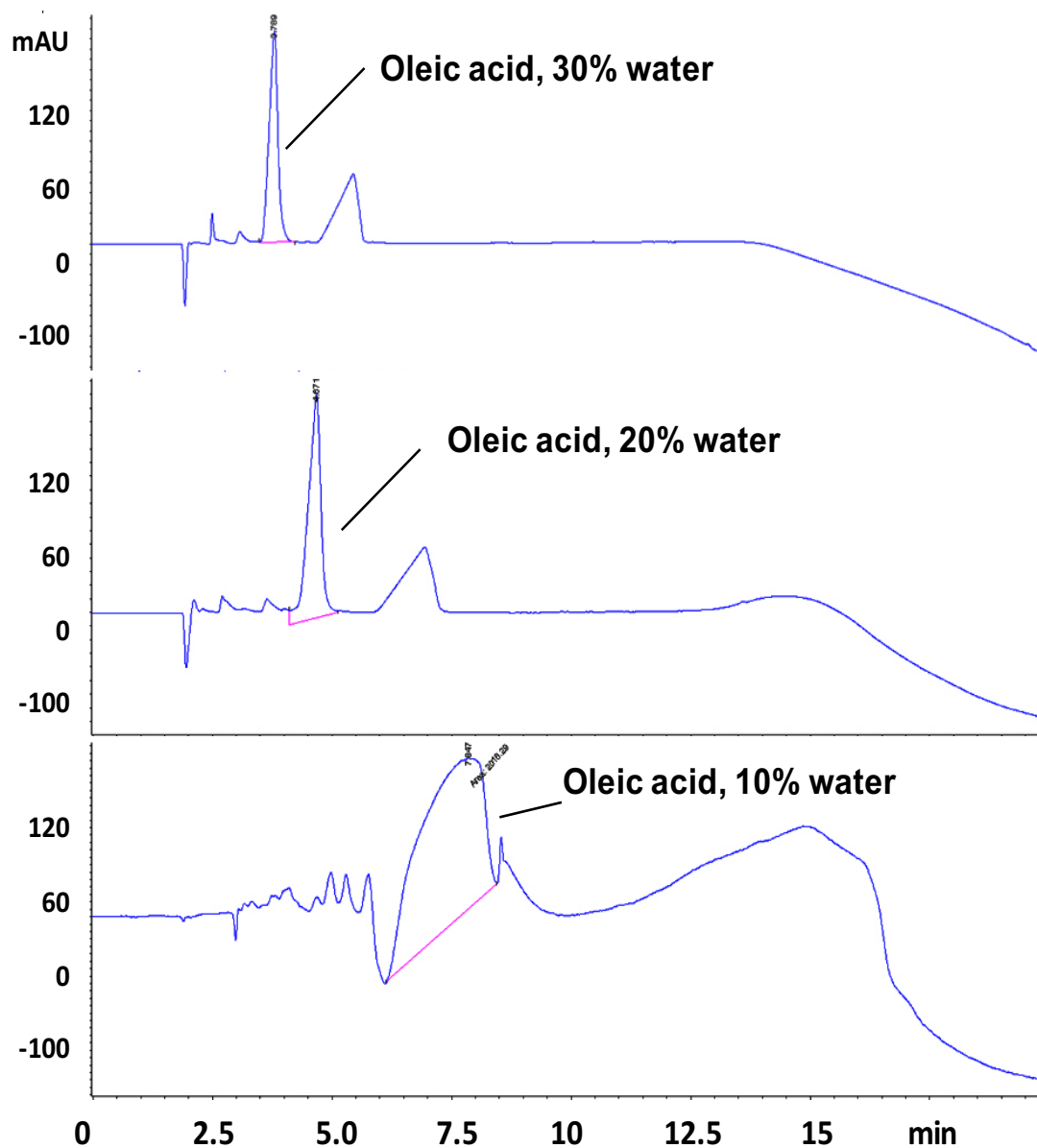
Figure 19. Peak shift of oleic acid caused by reducing water in the elution.
Gradient as in Table 11. Attempt 1 (top, 30% aqueous phase), attempt 2 (middle,
20% aqueous phase) and attempt 3 (bottom, 10% aqueous phase). Detection
with Agilent DAD at 210 nm. Extra peaks are impurities in oleic acid.
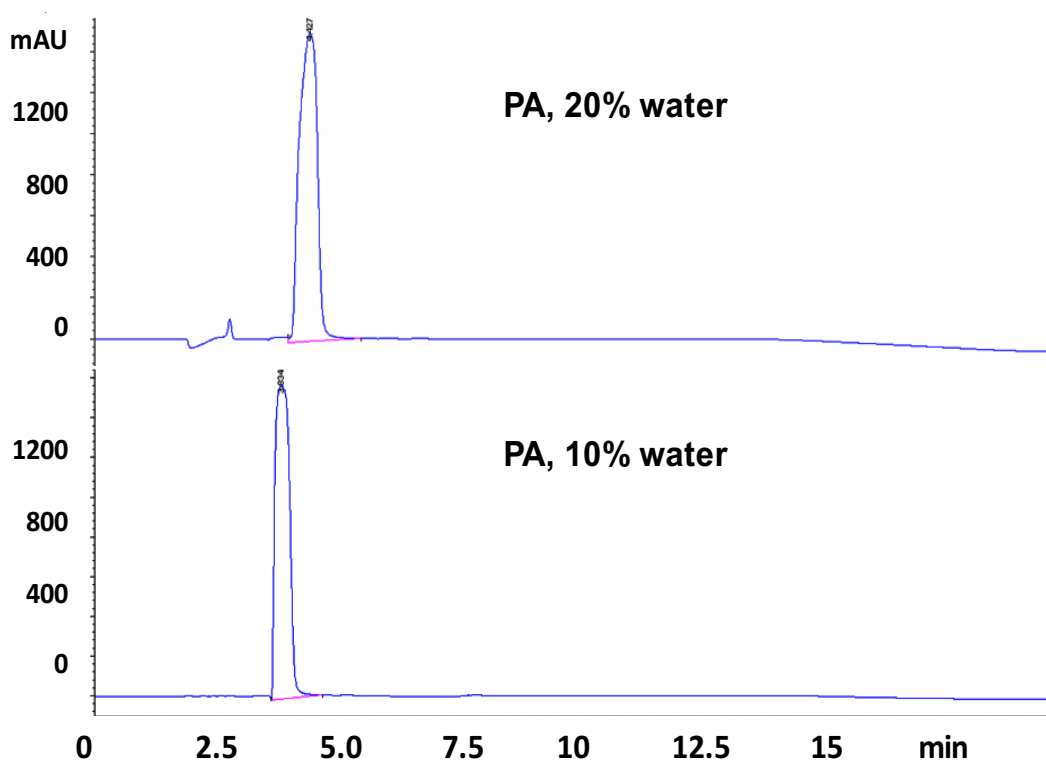
Figure 20. The peak shift of PA by increasing early % ACN. Gradient as in Table 11, attempt 2 (top), attempt 3 (bottom). Detection with Agilent DAD at 210 nm.

Based on these observations, a feasible solution for separating PLs and FFAs uses a high % ACN at the beginning of the gradient to elute the PLs while FFAs are retained on the column. However, maintaining high concentrations of ACN too long will push FFAs into the MAG region. In addition, a rapid transition from ACN to appropriate aqueous phase must be implemented to prevent deformation of FFA peaks. The gradient evolving from these requirements was 100% ACN for 3 minutes to elute PLs, then quickly (over three minutes) introduce 30% water buffered at pH of 6.8 to elute FFAs in ionic form, then hold

these conditions for15 minutes to allow full elution of all fatty acid chain lengths.

Separation of PA and oleic acid achieved by this gradient is shown in Figure 21.

Grafting this modified initial gradient onto the original gradient provides a master

gradient for separating all PLs, FFAs, MAGs, DAGs and TAGs. The final gradient

for total class separation without in-class discrimination is presented in Table 12.

Table 12. HPLC gradient separation of PL, FFA, MAG, DAG, and TAG lipid classes without in-class discrimination.

Time (minutes)	ACN (%)	% 0.1 M citric acid + NaOH (pH=6.8)	% IPA/Hexane (1:1)
0	100	0	0
3	100	0	0
6	70	30	0
15	70	30	0
20	100	0	0
36	100	0	0
120	50	0	50
140	50	0	50
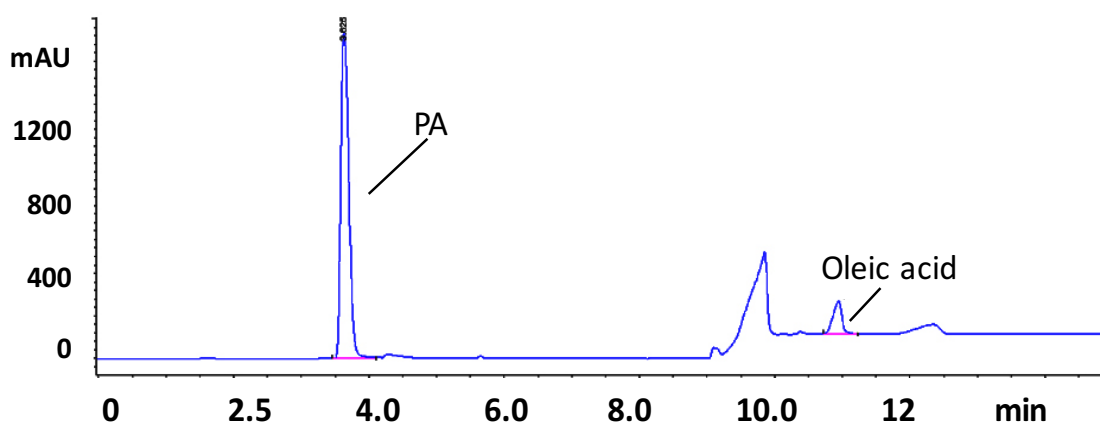
Figure 21. Separation of PLs and FFAs using the modified initial gradient (Table 12). Detection with Agilent DAD at 210 nm.

6.1.5. Testing of cyano columns to separate critical pairs

The initial separation described above separated total lipid extracts into individual lipid classes with partial resolution of components within classes. However, critical pairs of saturated and longer unsaturated fatty acids with the same ECNs could not be separated. We thus investigated two- dimensional LC techniques that could be added to the original separation to effect this separation.

A cyano column was selected to take advantage of double bond-triple bond interactions that may provide additional binding for unsaturated fatty acids. An Ultra AQ C18 column 150*4.6 mm, 5 μm, (Restek, College Station, PA) was connected in series with a Cyano 100*4.6 mm, 5 μm column (Restek, College Station, PA) in two sequences, and elution patterns of lipid reference standards were compared with the Ultra AQ C18 column alone. Retention times on the three systems are compared in Table 13.

Table 13. Comparison of retention using ultra AQ C18 and cyano columns in different combinations and sequences.

Lipid	ECNs	Retention Times (minutes)		
		C18+Cyano	C18	Cyano+C18
Monolinolein	14	25.75	24.01	25.80
Dilinolein	28	33.43	32.45	33.69
Trilinolein	42	81.06	85.89	82.86
OOL	46	101.86	107.15	100.44
Triolein	48	109.46	113.79	109.77
OOP	48	111.33	116.02	109.92
PC+PE+PA	32	7.04-7.05	7.03-7.05	9.68

These results were quite interesting and yet unexpected. We expected longer retention times for the unsaturated lipids. However, the cyano column added either before or after the C18 column neither prolonged retention times of unsaturates nor improved resolvability of the mixtures. It was first thought that the cyano column retained lipid too weakly, yet when the column was tested independently, trilinolein elution was 19.7 minutes (Figure 22). This extra retention was not reflected in the two-column setups, where retention times for all lipids tested were nearly the same as on C18 alone, or were even shorter.
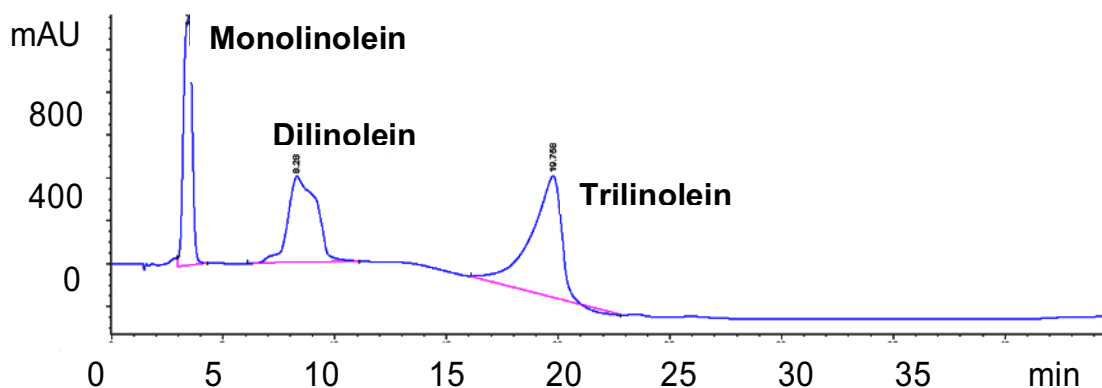
Figure 22. Elution of mono, di and tri-linoleins from a cyano column alone using the gradient in Table 12. Detection with Agilent DAD at 210 nm.

The unexpected loss of resolving power probably occurs because solvents move through the columns faster than the lipid molecules, so an advanced gradient stage with strong solvent is present in the second column when lipid fractions enter it. To see how this affects overall retention, consider how the lipid retention (R) of the RP column changes with time as specific solvents in the gradient flow through it. Eluting power (E) can be expressed as the reciprocal of polarity of solvents (Table 14). With only the Ultra AQ C18 column, the first 20 minutes of the gradient has relatively weak solvent strength (ACN and water combinations have low E) so acylglycerols elute slowly. As more iso-propanol and hexane are admitted into the system, the overall eluting power of the system gradually increases, and lipid molecules begin to elute much faster.

Table 14. Solvent polarity index and eluting power of HPLC solvents used with lipids.

Solvent	Polarity Index	Eluting Power (E)
Hexane	0.1	10
Iso-Propanol	3.9	0.26
Acetonitrile	5.8	0.17
Water	10.2	0.01

Applying this eluting power model to the Cyano + Ultra C18 column sequence, trilinolein elutes from the Cyano column at about 19 minutes, and when entering the C18 column, encounters much stronger solvent strength which moves it very rapidly through the column without the normal partitioning. Trilinolein thus elutes from the linked C18 column with overall retention time slightly shorter than with the C18 column alone. On the other hand, monoacylglycerols, e.g. monolinolein, which have a very short retention time (three minutes) in the leading cyano column, encounter only a mildly greater "initial speed" entering into the C18 column, and end up with retention in the two column system only slightly longer than on the C18 column alone.

When the Cyano column is second in series, the retention times comparable to C18 alone can be ascribed to the cyano column's inability to retain lipid molecules in later stages of the gradient where strong solvents dominate.

The Cyano column is technically a normal phase/hydrophilic column. Thus, as lipids exit the C18 column and enter the Cyano column, the high solvent strength (E) essentially washes lipids through the column with little or no interaction.

Furthermore, adding the Cyano column increased the overlap between critical pairs rather than improving their separation. The time interval between critical pairs of triolein and OOP was 1.87 minutes, 2.23 minutes and 0.15 minutes for C18+Cyano, C18 only and Cyano+C18, respectively. Thus, the Ultra aqueous C18 column alone presented the best, though not complete, separation of the critical pair of OOP and triolein.

In summary, even though successful separations of lipid molecules by Cyano columns have been reported, it appears that the Cyano column may be only useful for normal phase conditions. When coupled with an RP column and strong solvents, the lipid retention power of cyano column is largely obscured and even diminished for some compounds.

6.1.6. Issues with silver ion columns as the second dimension.

Adding triple bond-double bond interactions to facilitate separation of critical pairs using a Cyano column in sequence with a C18 column was unsuccessful. As an alternative approach, we looked to specific interactions of double bonds with silver ions (Ag) to refine the separation of unsaturated and saturated lipids with very close ECNs. The ability of Ag to distinguish lipids by unsaturation level and double bond positions on the acyl chain is well established in multiple chromatographies. However, our attempts to apply Ag to RP-HPLC separations encountered two major obstacles that ultimately ruled out this

approach. The first obstacle was commercial unavailability of Ag-impregnated columns. We contacted all major column manufacturers in U.S. and found that none of them make such columns. We next considered making the Ag column in-house since procedures were available[67], However, attempts to make the column revealed another obstacle that cause us to abandon this approach altogether. Separation of FFAs from MAGs that have virtually the same ECNs requires a pH of 6.8 at the beginning of the gradient to ionize the FFAs and elute them faster than their MAG counterparts. However, silver ions immediately precipitated under these conditions. An acidic pH system would make the incorporation of Ag ions possible, but this completely compromises the separation between FFAs and MAGs. Thus, alternate methods must be found for separating critical pairs within lipid classes.

6.2. Quantitation of lipid classes separated by HPLC

6.2.1. Preliminary exploration of calibration curves for lipid molecules using ELSD

ELSD is often a method of choice for detecting and quantifying molecules that lack an absorbing chromophore or that have large variations in their extinction coefficients so that standard curves are difficult to apply broadly. In this study, all lipid fractions were indeed detected by ELSD, but a critical "disadvantage" of ELSD (non-linear concentration response for different lipids as reported previously[42,79–82]) was also confirmed.

The ELSD detector was connected in series after the UV-Vis detector in

the HPLC system, then heated to 80°C with nebulizer gas set at 2.5 L/min to ensure total evaporation of the aqueous phase. When lipid mixtures were eluted with the gradient shown in Table 10, the qualitative advantage of ELSD is very obvious (Figure 23). Not only was the base line very smooth without any interference from the mobile phase gradient after 20 minutes (the initial drop was due to the use of water), but saturated lipids missing in UV detection were fully registered by ELSD. Response factors for the two detection methods have already been determined (Table 5)[36], so were not repeated here.

Quantitative results were more problematic. As noted above, response curves of monopalmitin, monostearin, dimyristin, tripalmitin and trilinolein analyzed over a range of concentrations were non-linear (Figure 24). Although R^2 for second order polynomial curves was 1, ELSD response intensity and regression equations were very different for each structure. Further testing of individual lipid structures should help determine whether the non-linearity derives from molecular size, acyl chain length, or other factors. Due to instrumentation problems, these extended studies could not be undertaken in this project.

Figure 23. Signal comparison between UV-DAD (upper) and ELSD (lower) detection of lipid mixtures. Gradient as in Table 10. DAD detection with Agilent DAD detection; ELSD detection with Alltech 500 ELSD, nebulizing gas flow set at 2.5 L/min, nebulizing temperature was 80ºC.
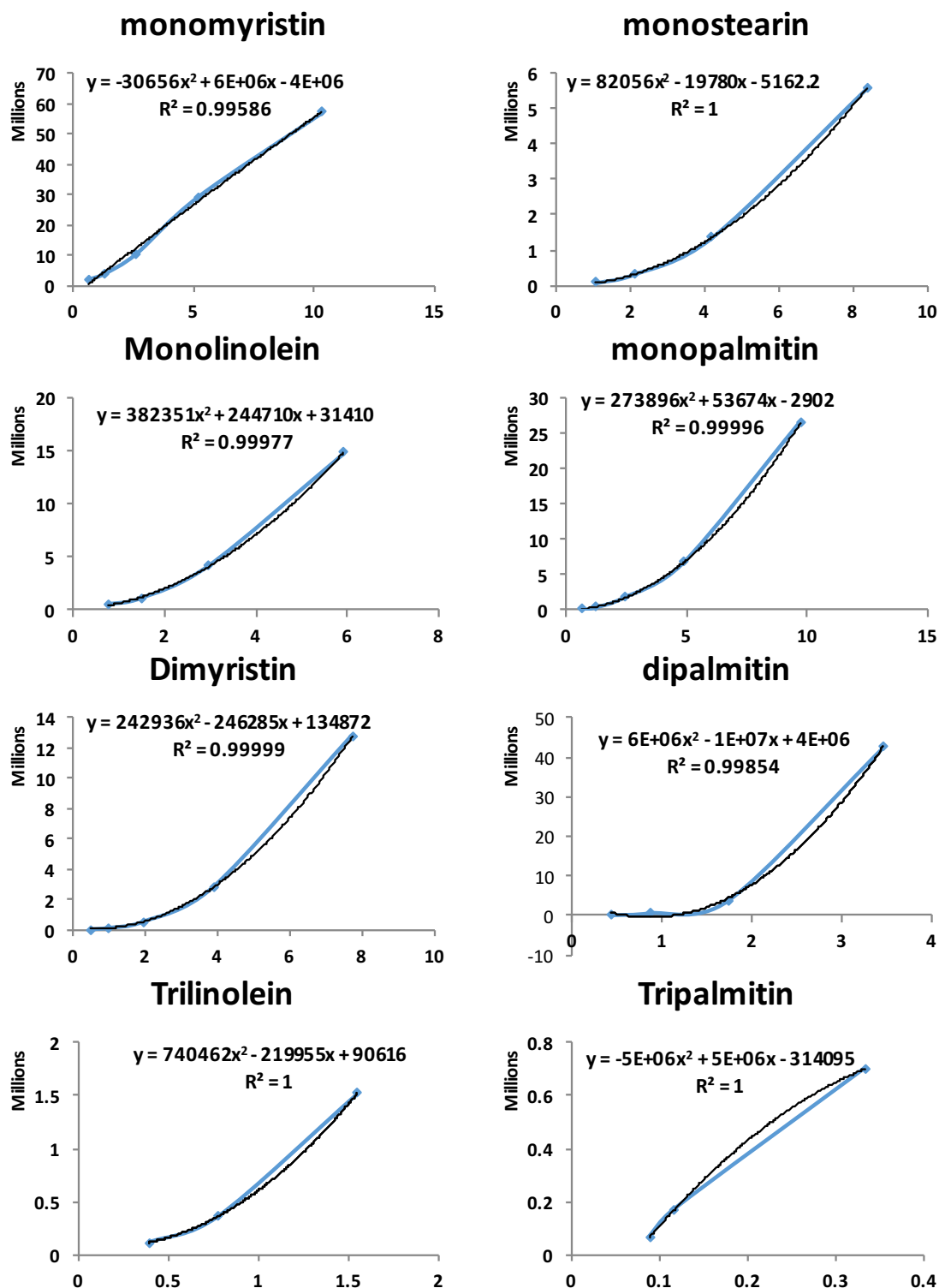
Figure 24. Calibration curves of acylglycerols detected by Waters e2695 UHPLC ELSD. Nebulizer flow at 2.5L/min nitrogen and temperature at 50°C.

In terms of sensitivity. The quantitative limits for lipids tested ranged from 70 -700 ppm. The lower value is consistent with previous reports of Graeve and Janssen[79] who found quantitation limits of 50 ppm for both neutral and PLs (0.1 µg loaded on column). Rodriguez et al.[80] reported a lower quantitation limit of 30 ppm for PLs.. According to Holcapek et al.[36], UV detection of unsaturated lipids was orders of magnitude higher than by ELSD . At the same time, even though the peak heights are lower in ELSD, but this may be due to sensor differences, peaks are large enough for area calculations and at least all peaks are detected in ELSD, because the background is cleaner, while the baseline shifts with solvent gradient in UV detection obscure some peaks. Both methods require construction of standard curves for each lipid structure, so arguments can be made for preferential use of ELSD, or at least use of both UV and ELSD detection in series. In practice, probably each specific study will need to choose the most appropriate detection technique depending on lipid components present.

These results clearly show promise for use of ELSD for detecting and quantitating lipids in HPLC. However, more research is needed to optimize the ELSD and determine how best to use it for quantitation. Detection parameters need to be systematically studied, especially for nebulizing temperature and nebulizing gas pressure. These two factors directly influence the signal response since analytes can be partially evaporated during the nebulizing process. Moreau[157] reported that signal response of lipid molecules differed significantly

even between 40 and 60 °C. Analyses reported here were performed at 50 °C,

the temperature set on an ELSD on a Waters UHPLC-ELSD system, but the

ELSD condition was not optimized for nebulizer temperature and gas flow rate to

maximize signal to noise ratio. Effects of lipid structure on ELSD response also

need to be investigated in depth using contemporary instrumentation in order to

develop simpler methods for routine quantitative use of ELSD.

6.3. Feasibility study: using ECD to detect lipid oxidation products

6.3.1. Determination of lipid hydroperoxides using cumene

hydroperoxides as model system

Commercially available cumene hydroperoxides (CuOOH) and butyl

hydroperoxide were used as standard materials for feasibility tests. The mobile

phase prepared according to Korytowski[121] was 5 mM LiClO$_4$ in 250 ml methanol

plus 112.5 ml acetonitrile and 137.5 ml Milli-Q water, mixed well together and

flushed with argon for 30 minutes; flow rate was 0.8 ml/min isocratic. CuOOH

was first tested under reducing mode where greatest activity was expected. To

determine optimum potential, reducing potentials were set from -100 mV to -900

mV with 100 mV increments. A negative peak was detected at 4.3 minute

signaling the presence of cumene hydroperoxides (Figure 25). -800 mV gave the

strongest signal response without significant noise interference. A more negative

electrical potential did not lead to a stronger signal response without increasing

the noise more significantly.
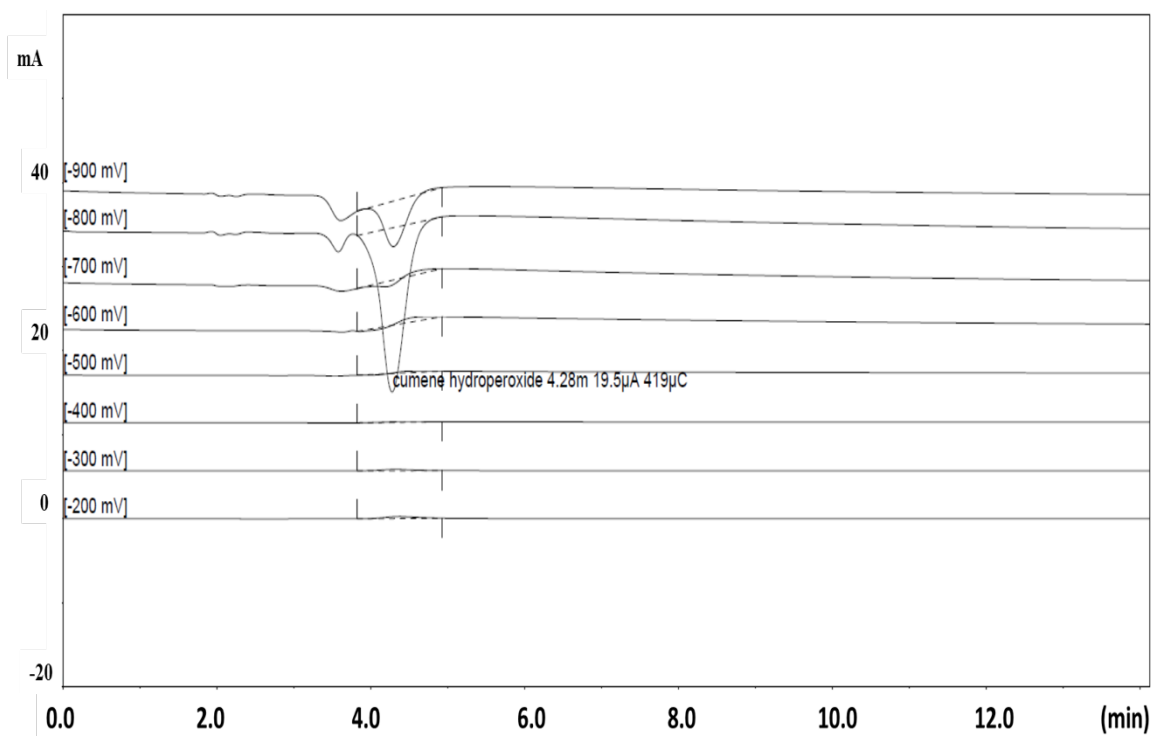
Figure 25. Electrochemical detection of cumene hydroperoxide under reducing mode. Detection by ESA coulometric detector.

Detection of a peak was encouraging. Unfortunately, attempts to determine the linear response range and develop a concentration response curve for quantitative calibration showed that response was not only non-linear, but it also decreased as hydroperoxide concentrations increased at low levels, then increased at higher hydroperoxide levels (Figure 26). This behavior was not due to ECD detection of contaminants since retention times with UV and ECD detection matched for both CuOOH and t-butyl hydroperoxide standards (Figures 27). One possible explanation of this behavior is that transition metal ions in the

HPLC system with stainless steel column and tubing decomposes low levels of

hydroperoxides, but at high hydroperoxide levels some peroxides remain.
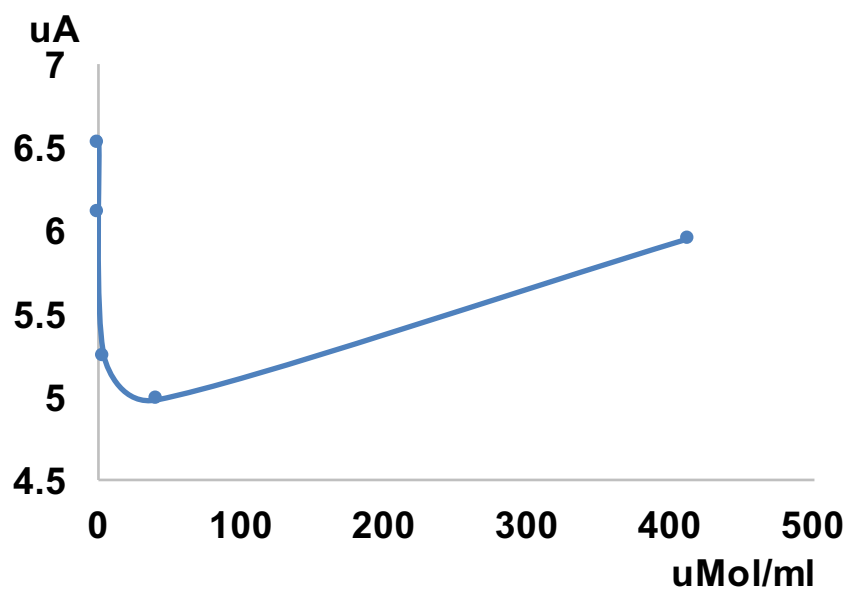
Figure 26. Concentration response curve for coulometric electrochemical

detection of cumene hydroperoxide at -800 mV with lithium perchlorate
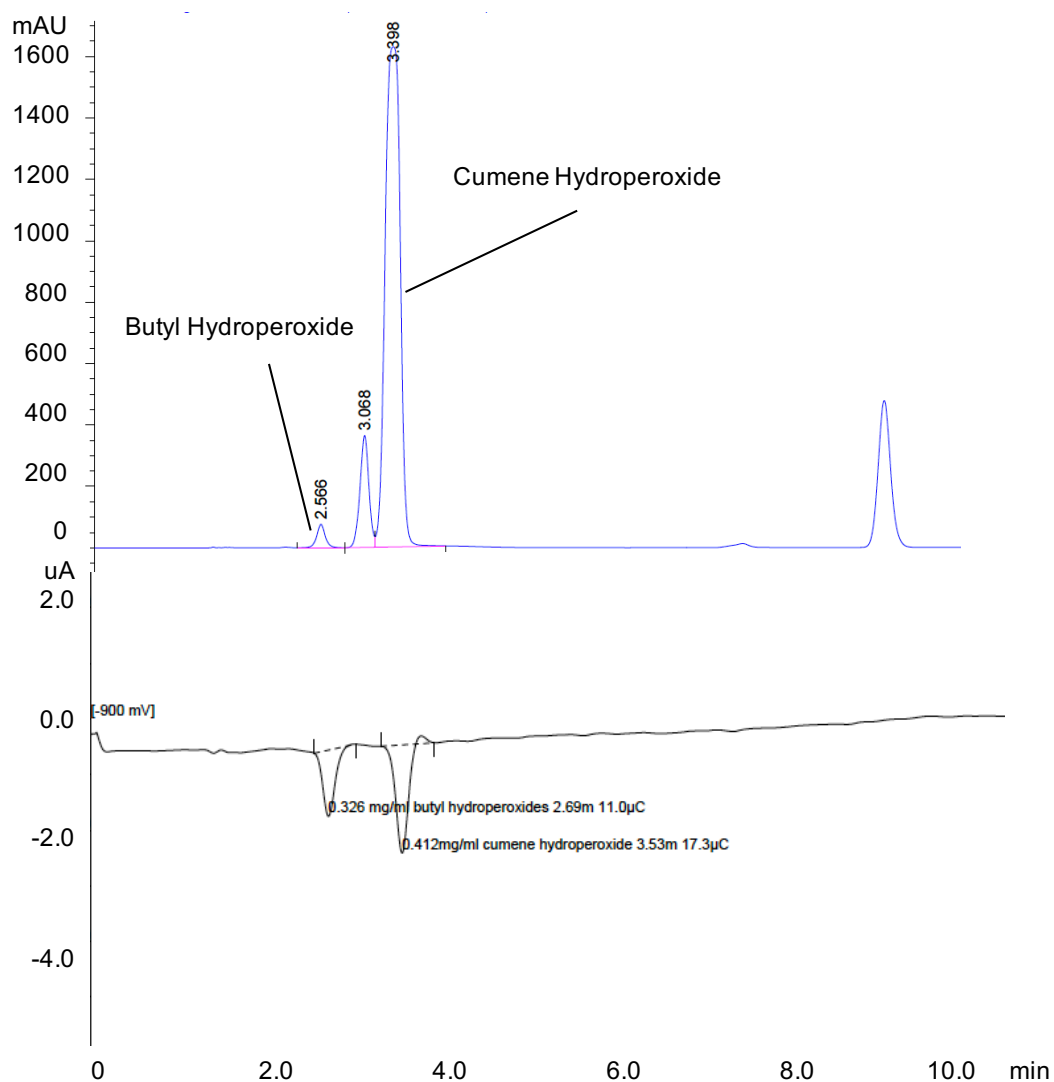
electrolyte.

Figure 27. UV (210 nm, top) and EC (-900 mV, bottom) detection of cumene.

Hydroperoxide with lithium perchlorate electrolyte. Detection with ESA

coulometric detector.

To test whether trace metals from the HPLC system were interfering with hydroperoxide detection, a collaboration was established with Dionex (Thermo Fisher Scientific) to utilize their all-PEEK Ion Chromatography system. The HPLC parameters used for the analyses are listed in Table 15. The metal-free system displayed a much superior detection of cumene and t-butyl hydroperoxides (Figure 28), giving linear response from 30 uM to 10 mM (4.6ug/ml to 1.5 mg/ml sample concentration) (Figure 29) and detection limit of 19.3 μM and 22.5 μM for butyl hydroperoxides and cumene hydroperoxides, respectively
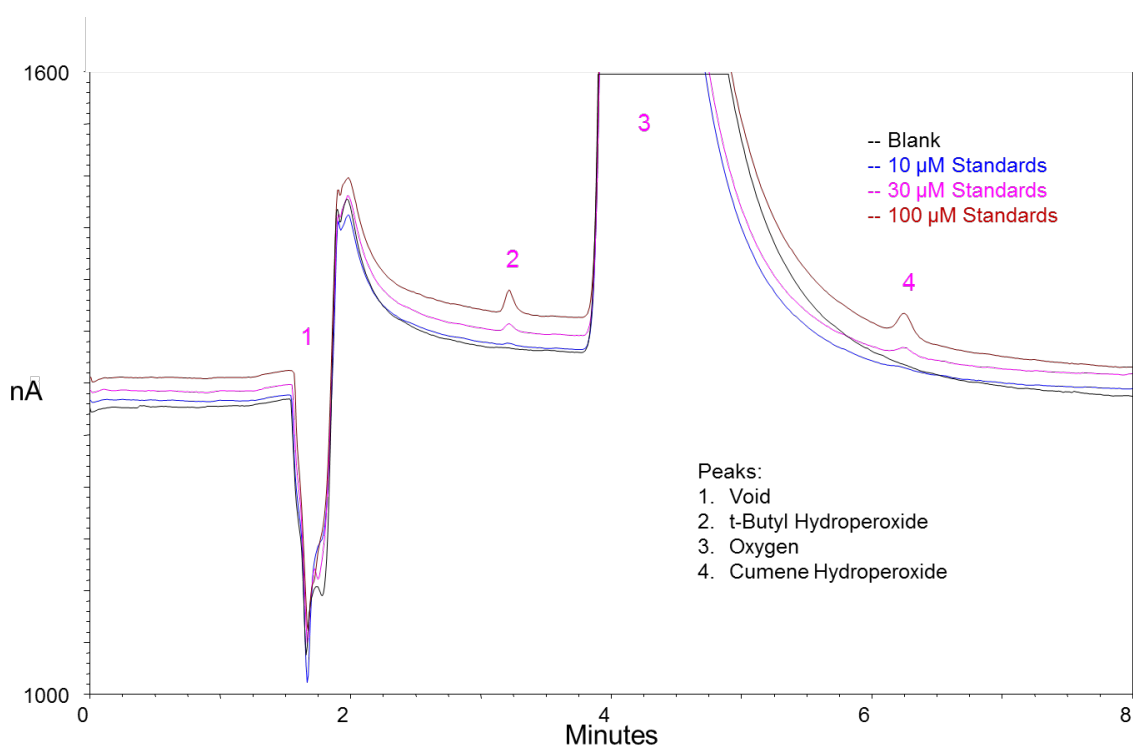
Figure 28. Amperometric electrochemical detection of t-butyl hydroperoxide and cumene hydroperoxide in a metal-free HPLC system. Top: 30 μM (4.6 μg/ml) to 10 mM (1.5 mg/ml). Bottom: 10 μM 1.5 ug/ml to 100 μM (15 μg/ml). Conditions as in Table 15.

Table 15. EC detection of cumene and butyl-hydroperoxides on Dionex ICS-5000 metal free system.

System:	Thermo Scientific Dionex ICS-5000
Mobile phase:	25 mM citric acid with 75 mM sodium citrate in water/methanol (45/55)
Column:	Acclaim® C18 120 (Anal.: 2.1 x 150 mm) ED cell equipped with an Ag/AgCl reference electrode and a glassy carbon working electrode (diameter: 1 mm) DC Amperometry: -1.25 V
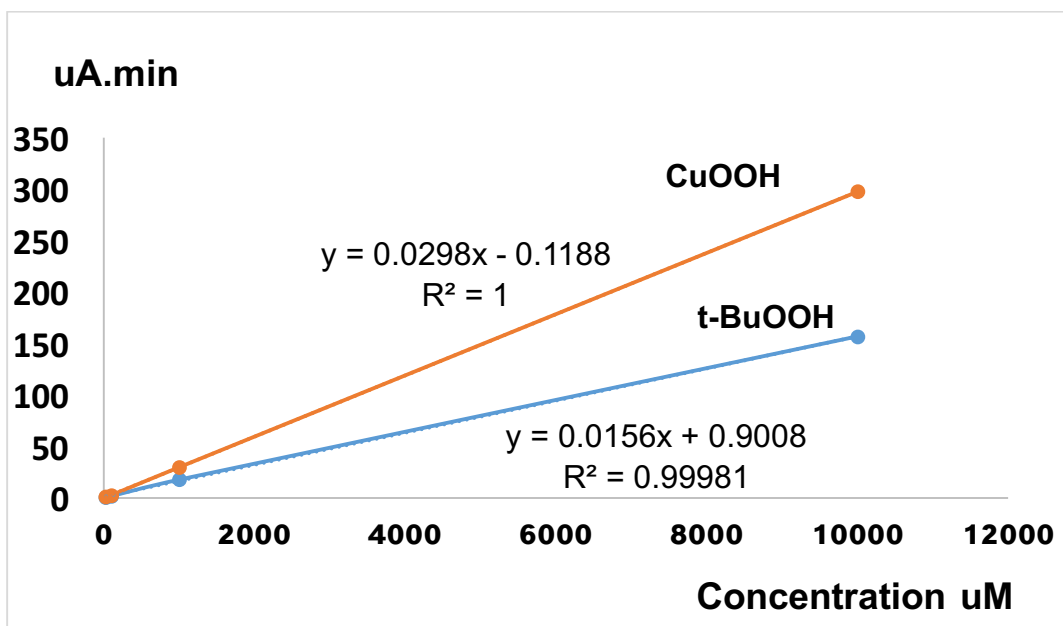
Figure 29. Concentration response curves for amperometric electrochemical detection of *t*-butyl hydroperoxide and cumene hydroperoxide using conditions listed in Table 15.

Several important points must be made about these results. First, the very

large oxygen peak in the chromatograms (Peak 3) demonstrates the critical

importance of fully degassing both the samples and solvents in electrochemical

detection. Second, multiple factors probably contribute to the increased detection

of hydroperoxides. The detector was amperometric rather than coulometric. As

explained in Background Section, although conversion rates are lower on

amperometric detectors, their background current is much lower so peaks are not

obscured and sensitivity increases. Also, the electrode in the Dionex system was

glassy carbon versus the graphite electrode in our coulometric system. Glassy

carbon electrodes have high density and low porosity, and the smooth surface

minimizes substrate double layer current, thus increasing polarization on the

surface. Graphite electrodes, in contrast, are formed from a crystalline form of

coal and are often contaminated with transition metals which would decompose

hydroperoxides and other lipid oxidation products. This would reduce, if not

prevent, their detection.

6.3.2. Electrochemical detection of aldehydes

6.3.2.1. Direct detection of aldehydes

Aldehydes and ketones were first tested for direct signal response under

ECD without derivatization. Solutions of 20 mM reference standards including,

butanal, pentanal, hexanal, decanal, trans 2-decenal, *t,t,*-2-4-decadienal,

nonanal, octanal, and 6-methyl-5-hepten-2-one were prepared in acetonitrile

The first attempt at ECD detection used the gradient shown in Table 7 with tetrabutylammonium bromide as the electrolyte running under oxidation mode. A second trial used lithium perchlorate as the electrolyte. No peaks of lipid carbonyl compounds were detected with either electrolyte at any voltage under both oxidation and reduicing modes.

6.3.2.2. Detection of aldehydes as dinitrophenylhydrazones

As an alternative approach, we also tested electrochemical activity of DNPH hydrazones of aldehydes using both TBAB and lithium perchlorate as electrolytes. To test simple hydrazones that were free and not attached to acylglycerols, separations use the gradient in Table 7. Thanks to the amine group conjugated with the DNPH aromatic ring, ECD detected weak but noticeable signals for hydrazones of butanal, hexanal and heptanal with TBAB as the electrolyte, although octanal, nonanal and decanal were not detected (Table 16, Figure 30). Neither increasing the electrolyte concentration nor monitoring at higher electrical potential improved the signal. However, when the electrolyte was changed to lithium perchlorate[127,158–161], response increased dramatically (Table 17, Figure 31) and hydrazones of all aldehydes tested were detected.
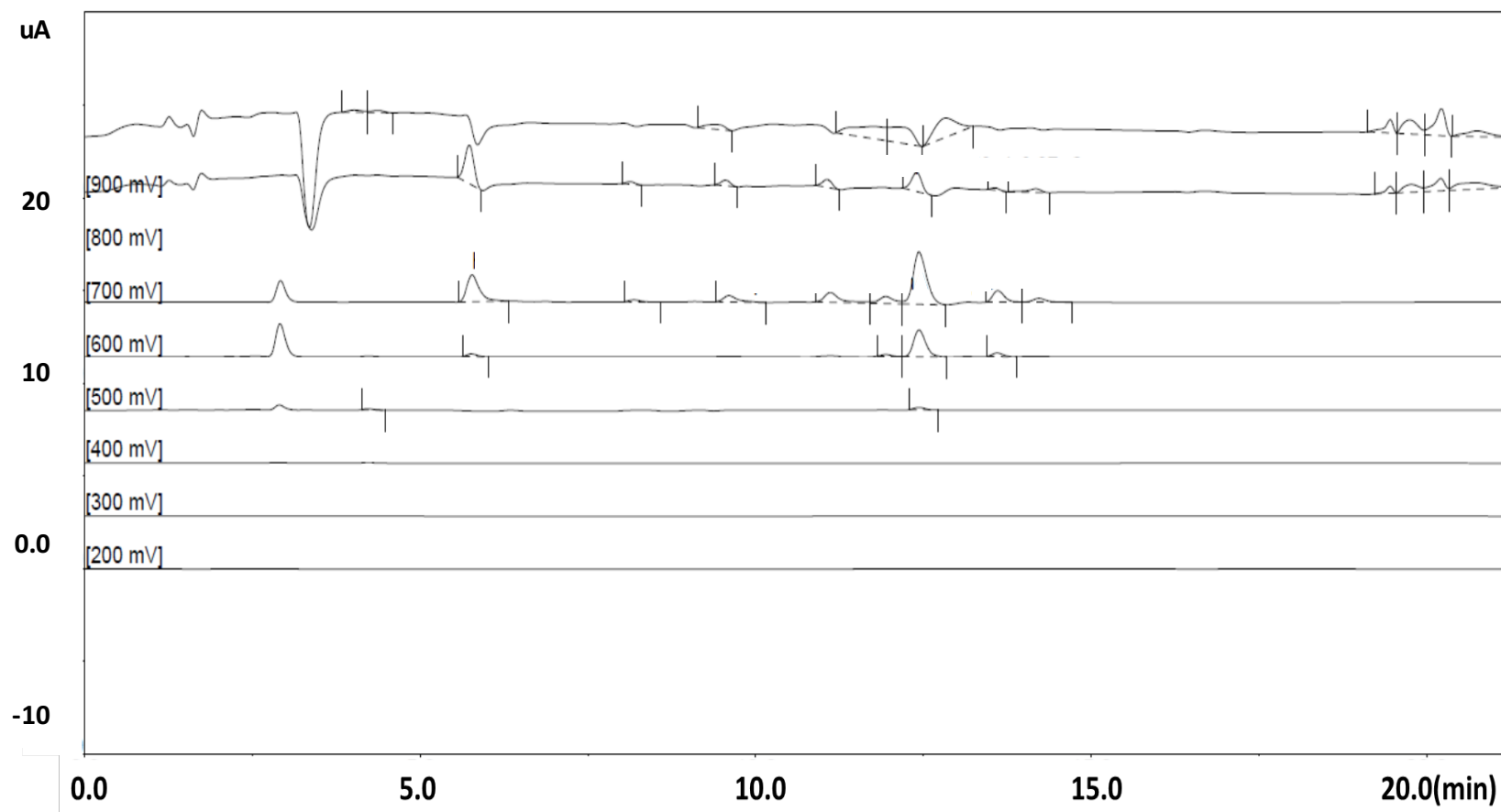
Figure 30. Direct coulometric electrochemical detection of aldehydes using TBAB as the electrolyte, gradient as in table 7. Detection was using ESA coulometric detector using oxidation mode.
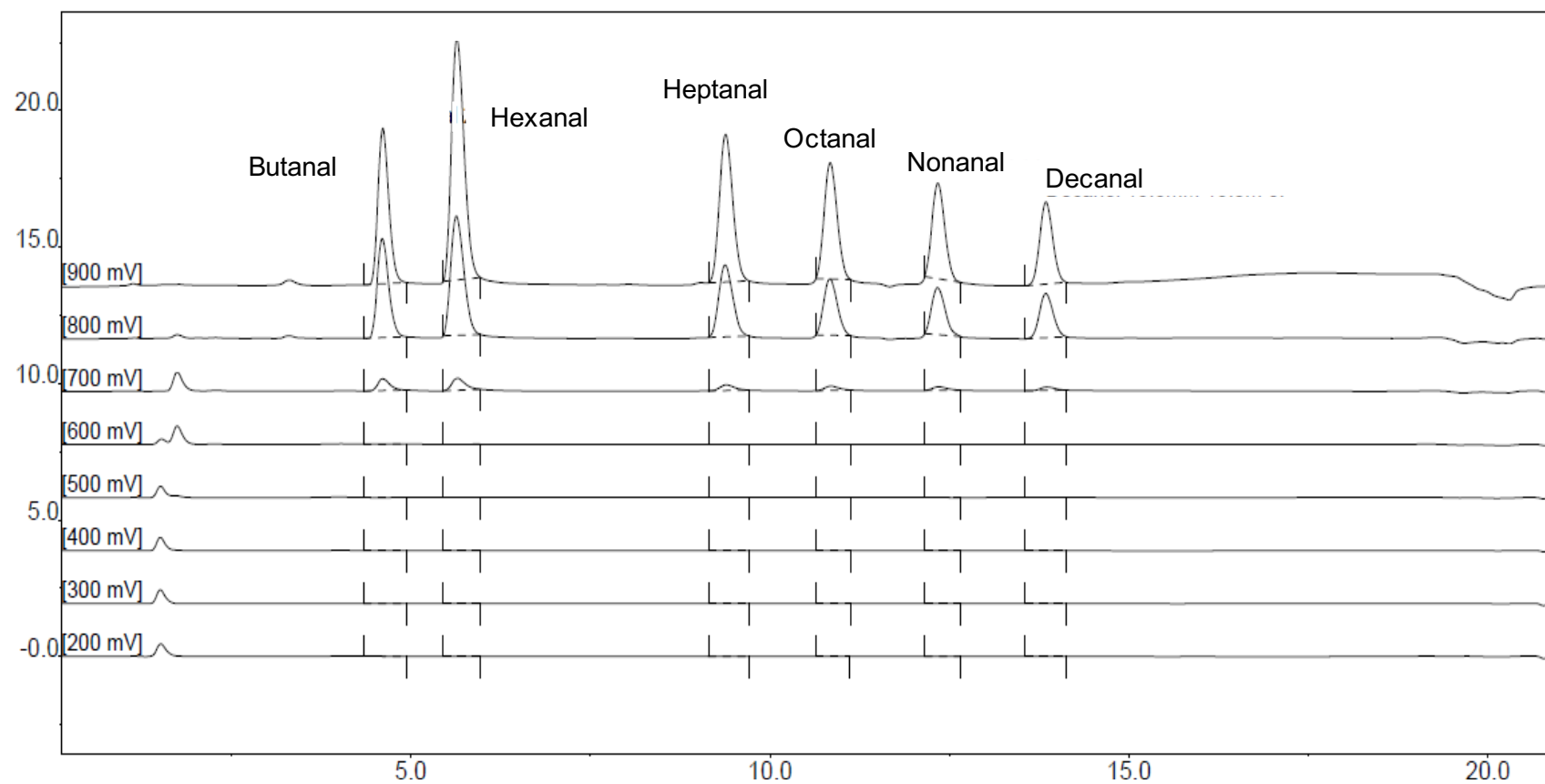
Figure 31. Direct electrochemical detection of aldehydes using 10 mM lithium perchlorate as the electrolyte, gradient as in table 7. Detection was using ESA coulometric detector using oxidation mode.

Table 16. Electrochemical detection of DNPH-derivatized C4-C10 aldehydes using 25 mM TBAB as the supporting electrolyte.

Chemical	Concentration (mM)	Retention time (min)	Response (μC)
Butanal	28.0	5.83	10.6
Hexanal	20.4	4.75	14.2
Heptanal	17.7	9.73	14.2
Octanal	15.9	n/d	n/d
Nonanal	14.5	n/d	n/d
Decanal	13.3	n/d	n/d

Table 17. Electrochemical detection of DNPH-derivatized C4-C10 aldehydes using 10 mM LiClO$_4$ as the supporting electrolyte.

Chemicals	Retention time (min)	Response (μC)	Maxi. Response (mv)
Butanal	5.63	110	900
Hexanal	4.60	85.9	900
Heptanal	9.33	79.0	900
Octanal	10.83	69.7	900
Nonanal	12.34	63.3	900
Decanal	13.83	52.8	900

Using these same HPLC conditions, six replicates of C4-C10 aldehydes were then systematically tested to determine response factor and reproducibility of hydrazone detection at 900 mV. From 0.002 mg/ml to 0.1 mg/ml (0.001 to 1 μmol/ml) range, all saturated and poly-unsaturated aldehyde-DNPH showed reasonable linearity (>0.99). However, the monounsaturated aldehydes, *c*-4-heptenal, *t*-2-nonenal and *t*-2-decenal displayed a poor linearity for the concentration tested.
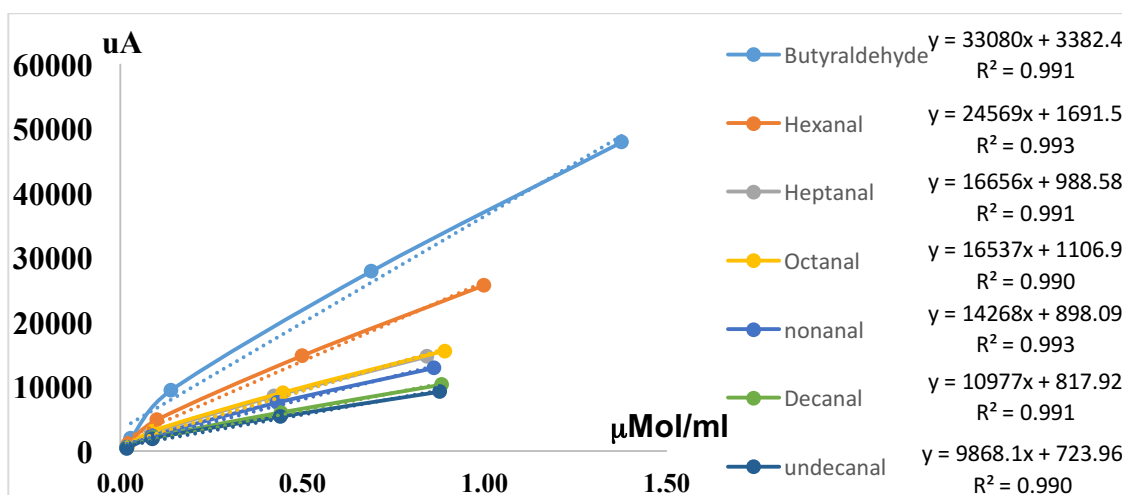
Figure 32. Calibration curves for hydrazones of saturated aldehydes expressed as μmol/ml, electrochemical detection at 900 mV.
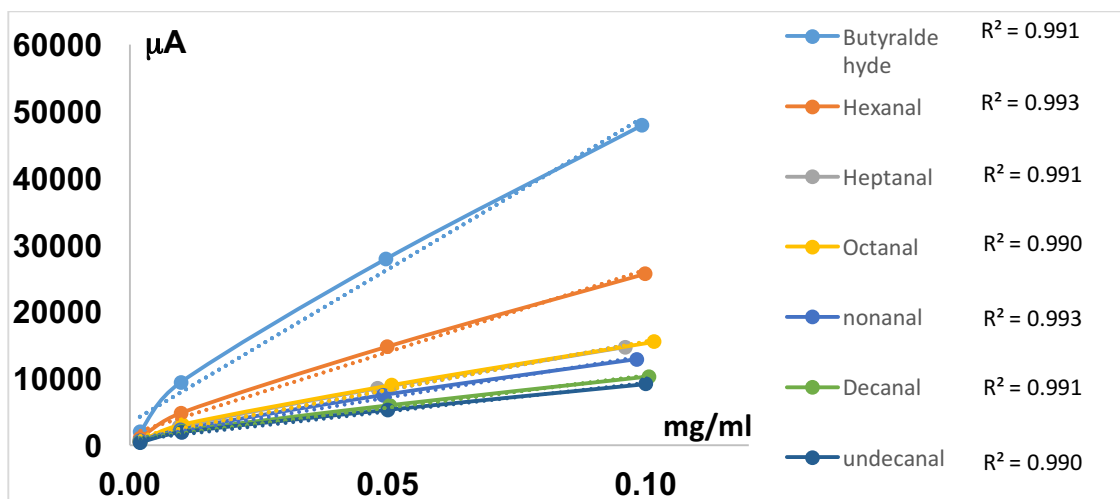
Figure 33. Calibration curves for hydrazones of saturated aldehydes expressed

mg/ml, electrochemical detection at 900 mV.
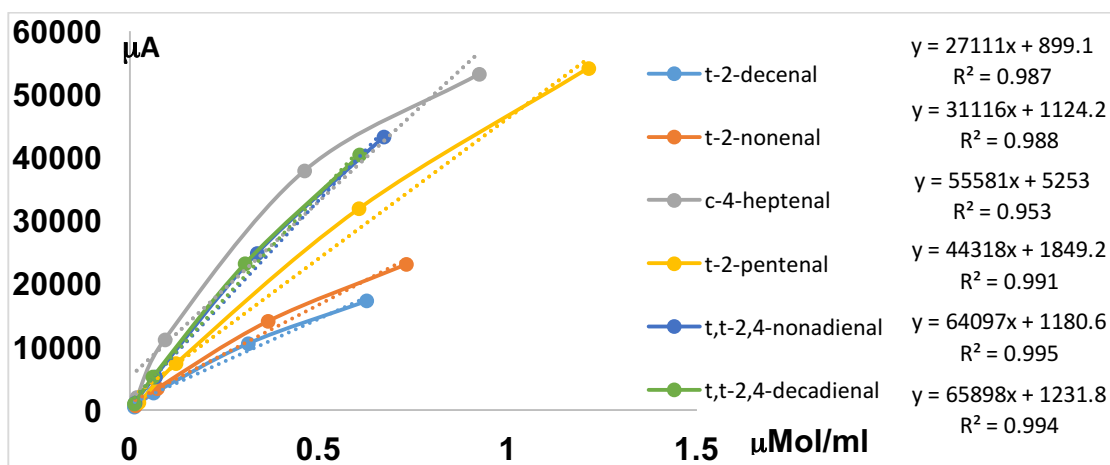
Figure 34. Calibration curves for hydrazones of unsaturated aldehydes

expressed as umol/ml, electrochemical detection at 900 mV.
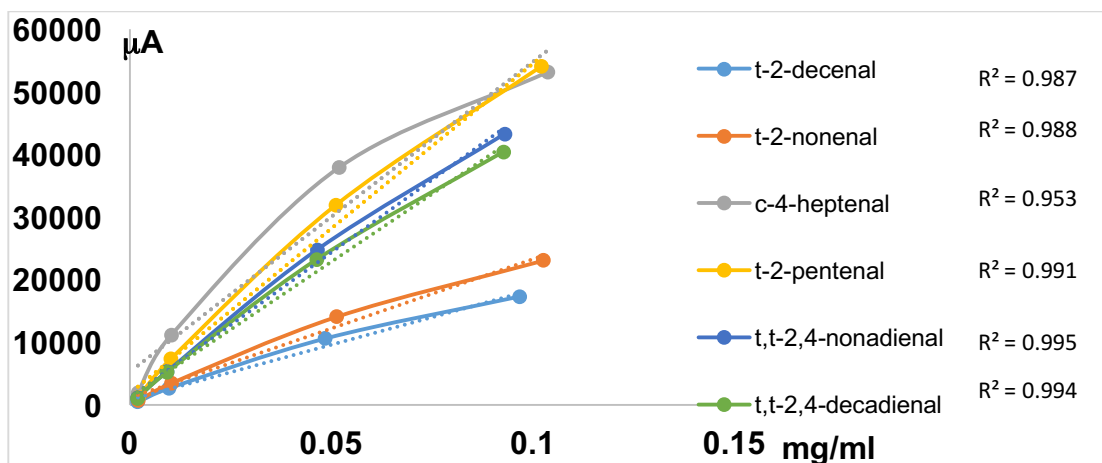
Figure 35. Calibration curves for hydrazones of saturated aldehydes expressed as umol/ml, electrochemical detection at 900 mV.

At first glance, it may appear that each hydrazone has different interaction with the electrode. However, the difference in the response curves results not from differences in actual reaction at the electrode surface but from different amounts of hydrazones formed in the fixed reaction time, as was also observed with optical detection of hydrazones in HPLC[162]. The time required for full reaction of aldehydes with DNPH varies with carbon chain length, increasing tremendously for longer chain aldehydes and decreasing with degree of unsaturation (Chris Izzo, unpublished data).

The contribution of reaction time (rather than specific interaction of aldehyde with the electrode) to quantity of aldehyde detected is shown in variation of signal response with order of addition of aldehydes to standard reaction mixtures. Test mixtures were prepared by dissolving excessive amount of DNPH in 1 ml dimethylformamide, and 10 μl of H_2SO_4 was added and mixed

well. For reaction, 700 μl DNPH solution was pipetted into a reagent vial, then

100 μl reference standard of undecanal, decanal, nonanal, octanal, heptanal,

hexanal and butyraldehyde were immediately added to the DNPH in order. This

aldehyde-DNPH mixture (with resulting hydrazones) was then diluted in series for

analysis. When aldehydes were added to DNPH from short chain to long chain,

peak area decreased markedly with chain length (Figure 36). However, when the

order of addition was reversed (longest chain to shortest chain), peaks of

aldehydes added early, especially undecanal, increased while the peak of

aldehydes added late (heptanal through butanal) decreased (Figure 37).
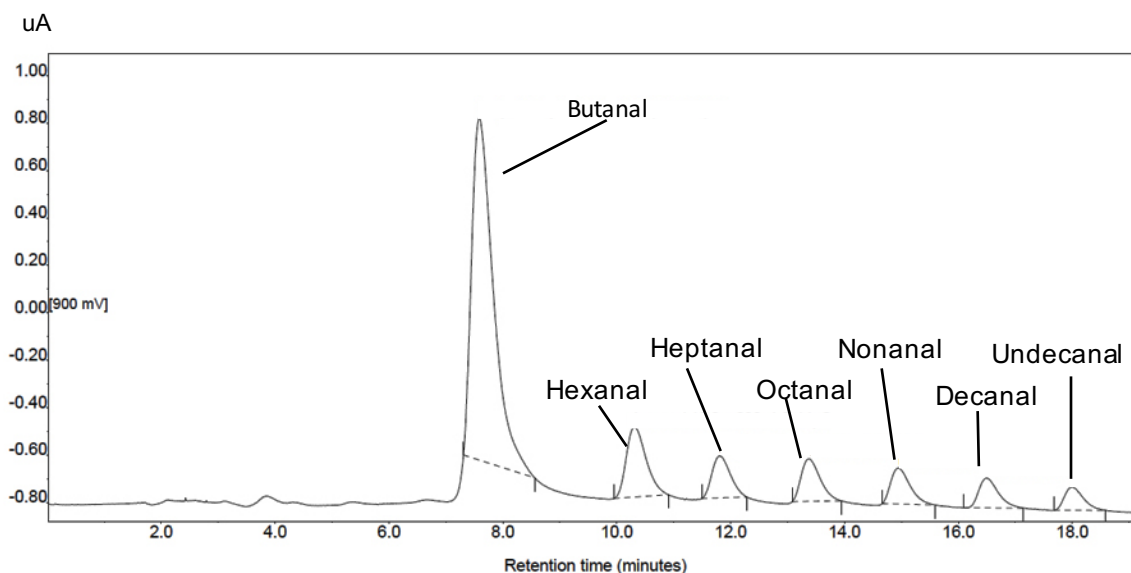
Figure 36. Relative pattern of signal response of 0.1mg/ml saturated Aldehyde-

DNPH when adding aldehyde standards from short chain to long chain

aldehydes. Detection was using ESA coulometric detector using oxidation mode.
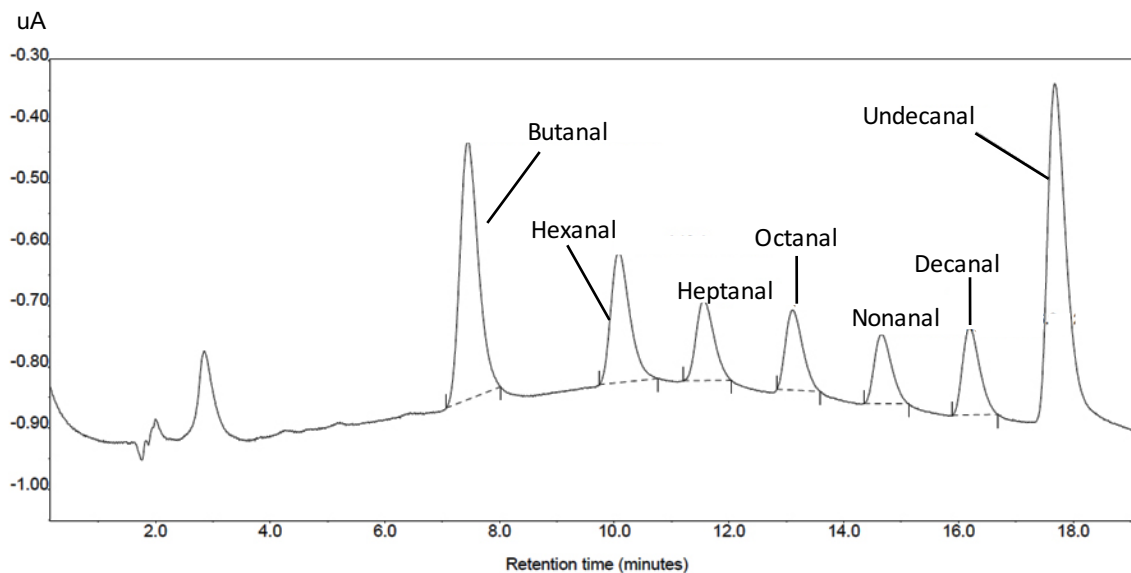
Figure 37. Relative pattern of signal response of 0.1mg/ml saturated aldehyde-DNPH when adding aldehyde standards from long chain to short chain aldehydes. Detection was using ESA coulometric detector under oxidation mode.

Differences in times required for reaction of each aldehyde with DNPH thus poses significant limitations when using this assay for quantitation of aldehydes, whether detection is optical or electrochemical. Thus, full evaluation of this method can only be completed after issues with the reaction are resolved (being studied by another student in this laboratory). At that point, electrochemical and optical detection of hydrazones needs to be compared in detail. Currently, ECD appears to be more sensitive, which would provide an advantage, but this must be verified.

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6.3.3. Electrochemical detection of lipid epoxides

Since reference standards for frequently-occurring lipid epoxides are hard to obtain, commercially available 1,2-epoxy-5-hexene and 1,2-epoxy-5-decene were used to test the feasibility of EC detection. Mobile phase was prepared by dissolving 10 mM lithium perchlorate in 90% acetonitrile in Milli-Q water; flow rate was set at 1.2 ml/min. Epoxide standards were tested under both oxidizing and reducing mode across all electrical potentials. Surprisingly, no detector response was observed despite the known reactivity of epoxides. The research in this area is truly limited so we do not yet have a definitive explanation for this lack of electrochemical response. However, we can speculate about likely issues.

1) Epoxides are quite reactive so may not survive passage through the column, particularly when column and liquid lines are stainless steel.

2) As shown with the hydroperoxides, high background current or resistance from the coulometric detector may obscure epoxide signals. Detection by an amperometric detector in a metal-free HPLC system must thus be tested.

3) Epoxides are not strongly electrochemical so may need increased contact or contact time with the electrode for response. This may require changing the design of the electrode cell to use a different electrode material or perhaps a coated electrode that will bind epoxides more effectively.

6.4. Assessment of HPLC-electrochemical detection of lipid oxidation products

We began this research expecting that coulometric detection would be much more sensitive than amperometric electrochemical detection. That assumption appeared to be in error since detection of lipid oxidation products by a coulometric detector with graphite electrode was absent (epoxides) or weak (hydroperoxides and aldehydes), while promising linear responses of hydroperoxides were obtained with an amperometric detector with glassy carbon electrode. Thus, the concept of electrochemical detection of lipid oxidation products still offers promise and merits full and systematic investigation using amperometric systems with different electrodes and electrolytes. These follow-up studies will be undertaken in collaboration with electrochemical system designers.

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7. **SUMMARY AND CONCLUSIONS**

RP-HPLC methods for improving analysis and quantitation of lipid composition in extracts and oils while simultaneously detecting lipid oxidation products were developed or evaluated.

RP-HPLC was applied to separation of total lipid classes to develop a method superior to thin layer chromatography for identifying and quantitating all lipid classes in lipid extracts, determining effectiveness of oil refining, and detecting degradation (e.g. hydrolysis) in oils and extracts. Although total lipid class separation has been achieved previously by normal phase HPLC, reversed phase HPLC was investigated here because it can be run with a greater variety of columns and solvents.

Free fatty acids and mono- di-, and tri-acylglycerols were successfully baseline separated in classes using an RP-Aqueous 150mm x 4.6mm, 3um with gradients based on acetonitrile and water modified to accommodate specific lipid fractions. Hexane-isopropanol was added to fully solubilize long-chain saturated fatty acids and all triacylglycerols. pH of the aqueous phase adjusted to 6.8 effectively separated free fatty acids from monoacylglycerols. Presumptive evidence was obtained for capability of simultaneous separation of phospholipids by beginning the gradient with 100% acetonitrile to clear phospholipids before fatty acids, then returning to the established aqueous to organic gradient separated remaining lipid classes.

Attempts were made to improve separation of critical pairs and individual components within classes. Cyano columns, which should have enhanced

interactions of lipid double bonds with column triple bonds, had no effect or worsened lipid class separations when placed in sequence before or after an RP aqueous column. Silver columns, which are well-known for their ability to separate based on unsaturation, could not be used in the reversed phase system because Ag ions precipitated at the pH required for fatty acid separation. Clearly, further investigation of different combinations of columns will be needed to achieve full detailed separation of lipids by individual component.

Detection of lipids in HPLC is a challenge due to lack of chromophores absorbing either ultraviolet or visible light. Evaporative light scattering detection (ELSD) was investigated as a potential replacement to UV 206/210 nm for detection of lipids while also quantitating lipid fractions. Initial study on the quantification of lipid molecules using ELSD produced very positive and promising evidence that this technique is feasible and practical for the determination of lipids regardless their unsaturation status. A particular advantage was full detection of saturated fatty acids that are invisible to UV 206 nm. At the same time, a major disadvantage is that response is based on particle size rather than molecular size, so there is no consistent, predictable relationship between ELSD response and molecular size or other properties. As a result, quantitation is possible, but it requires a separate standard curve applied to each individual molecule (e.g. every different fatty acid). The method merits further detailed investigation to determine factors that control response and to develop optimized methods for application using contemporary instrumentation.

Oxidation is the major mode of lipid deterioration and there is great need to be able to detect and quantitate multiple oxidation products simultaneously. Based on the assumption that oxidation products such as hydroperoxides, epoxides, and aldehydes should be electrochemically active at different potentials, electrochemical detection of these three lipid oxidation products was investigated monitoring eight different potentials in a coulometric system with a graphite electrode. Cumene and *t*-butyl hydroperoxides were detected with lithium perchlorate as electrolyte in oxidizing mode. A major problem was encountered by apparent decomposition of hydroperoxides in the HPLC system with stainless steel column and lines. However, sensitivity and linearity of response was greatly improved when detection was switched to metal-free HPLC system with a glassy carbon electrode in an amperometric detector. Epoxides and aldehydes showed no direct response in the coulometric detector, but dinitrophenyl-hydrazones of aldehydes were well-detected. However, concentration response curves were non-linear and complicated by differences in reaction rates of different aldehydes with DNPH.

While these studies of electrochemical detection of lipid oxidation products during HPLC separations were not fully successful, they were still worthwhile in that they did identify several critical factors that affect and control detection. First, since metals react with all lipid oxidation products, not just hydroperoxides, it is very likely that all electrochemical analyses of lipid oxidation products will require metal-free HPLC systems. Second, contrary to expectations, amperometric detectors appear to be more effective than coulometric systems for focusing

interactions of lipid oxidation products with electrodes, and therefore increasing sensitivity. Finally, not all electrodes and electrolytes are equally applicable to lipid oxidation products. Graphite electrodes did not work well while glassy carbon electrodes gave promising initial results, and lithium perchlorate and citric acid were effective electrolytes while *t*-butyl ammonium bromide was not. Hopefully additional research with different electrodes and electrolytes will verify the usefulness of electrochemical detection and determine optimum conditions for detecting lipid oxidation products.

8. FUTURE WORK

Goal 1: Develop a simple RP-HPLC separation of total lipid classes expected in foods (monomer oxidation products, free fatty acids, phospholipids; mono, di, and triacylglycerols) without in-class discrimination.

The procedures developed in this study with pure compounds provide excellent separation of all major lipids classes intended, including PLs, FFAs, MAGs, DAGs and TAGs, and with certain capability of also splitting critical pairs. Now this procedure must be tested with full mixtures of lipid standards and with actual oils where composition is much more complex, then refined as necessary to provide a robust procedure for routine analysis of lipid extracts.

Goal 2: Modify this procedure to separate individual lipid components within classes.

The method developed separates lipids from different classes and even some lipids with different ECNs within classes very well, even without second dimension intervention. Neither the Cyano column or Ag chromatography were found suitable for increasing separation of critical pairs when linked with the RP column. The next step will be testing other columns, column configurations, and elution conditions in reversed phase to improve total separation of individual components.

Goal 3: Determine the feasibility of using multichannel electrochemical sensors to detect, identify, and quantitate individual

classes of lipid oxidation products (hydroperoxides, epoxides, alcohols, aldehydes, ketones, alcohols, etc) in oils and extracts.

Results of this study demonstrated that theoretical considerations suggesting that coulometric electrochemical cells should provide superior detection of lipid oxidation products were incorrect. Preliminary investigations with a Dionex metal-free HPLC system and amperometric detector showed strong promise with detection of hydroperoxides. Studies with amperometric detectors and non-graphite electrodes now must be extended to epoxides and aldehydes, and systematic investigations developed to optimize conditions, identify critical control factors, and provide robust standard procedures for direct electrochemical detection of lipid oxidation products during elution from HPLC columns. In particular, since amperometric detectors run at a single potential, it must be determined whether specificity is lost while sensitivity is gained in these analyses.

8. SIGNIFICANCE OF PROJECT

Lipid oxidation processes and products were studied extensively from the 1950's through the 1980's, then interest was lost as fat was removed from foods. Understanding of oxidation at the time was developed through relatively low sensitivity class assays (mostly hydroperoxides) and gas chromatographic analyses of volatiles released after hydrolysis. Even with current renewed concern about adding essential unsaturated fatty acids to foods and challenges in stabilizing these new formulations, methods used to analyze oxidizing lipids have not changed.

This project contributed new lipid analysis procedures that are sensitive, robust, chemically accurate, and reasonable to handle and control in a typical quality control or research laboratory. Development of HPLC procedures that can separate and quantitate lipids by classes (and can be automated) will greatly facilitate tracking composition and degradation of foods during formulation, processing, and storage. For example, the total lipid class separations by HPLC could become a routine check of lipid ingredient composition and quality, rather than running peroxide values and perhaps some TLC plates. The analysis will be much more accurate and sensitive, and it will provide more detailed information than TLC alone or in combination with chemical analyses. HPLC class analysis will also be faster and more efficient overall since samples can be loaded onto autosamplers, sealed and stabilized under argon, and analyzed automatically. Such a procedure itself would be invaluable.

However, just as important is the need to track lipid oxidation, not just as hydroperoxides but through multiple alternate pathways. Running even four separate product analyses (conjugated dienes, hydroperoxides, epoxides, and carbonyls) on oils and extracts can be overwhelming when many samples must be handled. Developing a procedure that can track multiple specific oxidation products while separating and identifying total lipid components will certainly make life easier in basic research and quality control alike. More importantly, even the short time we have been running these four analyses in our laboratory have shown us that there are lipid oxidation products we have not accounted for, perhaps even conceptually. It is hoped that integration of the methods developed here with parallel product structure identification by mass spectrometry will also provide new information about lipid oxidation mechanisms and products

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