ASSESSING PRESENCE OF ALTERNATE LIPID OXIDATION PATHWAYS FROM VOLATILE PRODUCTS DETECTED BY GAS CHROMATOGRAPHY

by

BRANDON A. BOGUSZ

A dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State of University of New Jersey

In partial fulfillment of the requirements

For the degree of

Doctor of Philosophy

Graduate Program in Food Science

Written under the direction of

Professor Karen M. Schaich

And approved by

_____

_____

_____

_____

New Brunswick, New Jersey

May 2015

ABSTRACT OF THE DISSERTATION

Assessing Presence of Alternate Lipid Oxidation Pathways from

Volatile Products Detected by Gas Chromatography

by: Brandon Bogusz

Dissertation Director:

Professor Karen M. Schaich, Sc.D.

Recently a new reaction scheme that integrates alternate lipid oxidation pathways including addition, cyclization, and scission with traditional hydrogen abstraction was proposed to more completely account for products and kinetics of lipid oxidation. These pathways compete with hydrogen abstraction during autoxidation, and the balance between them changes with reaction conditions, altering product levels and distributions. Alternate pathways, if present, must force a new paradigm in analysis of lipid oxidation since conjugated dienes, hydroperoxides, and perhaps hexanal alone can no longer adequately reflect lipid oxidation. Thus, it is critical to verify the presence of alternate pathways and learn how they can be used to more fully elucidate mechanisms of lipid oxidation.

Overall oxidation kinetics, individual kinetics, identifications, and distributions of volatile products formed during oxidation of methyl linoleate were tracked using three different gas chromatography (GC) methods -- static headspace (SHS), solid-phase

micro-extraction (SPME), and thermal desorption (TD). Methyl linoleate was incubated neat at 25, 40, and 60 °C under varying conditions of oxygen and solvent to investigate ability of reaction environment to force shifts in reaction pathways. Incubations were limited to 20 days to focus specifically on mechanisms involved in early oxidation.

Major early products pentane, hexanal, *t*-2-heptenal, *t*-2-octenal, and methyl octanoate were all detected by each GC method but in different amounts, distributions, and kinetics of formation. During early incubation, C-13 alkoxyl radical scission was the dominant pathway for generation of volatile products, with pentane as the overwhelming major product and hexanal at much lower levels even as the second largest peak. Levels and timing of appearance of remaining products were best explained by a process in which C-9 alkoxyl radicals internally rearranged to epoxides, moving radical sites down the acyl chain to C-11 in the process. Hydrolysis of the epoxides generates methyl octanoate. Oxidation at C-11, a secondary rather than initiating process, yields *t*-2-heptenal and *t*-2-octenal as products from β and α-scission, respectively.

Results verify existence of multiple pathways in oxidation of methyl linoleate and demonstrate that these must be considered to explain the distribution and kinetics of oxidation products.

ACKNOWLEDGEMENTS AND DEDICATION

I would first and foremost like to thank Dr. Karen Schaich, for believing in me while offering emotional and financial support and guidance through this entire dissertation.

I'd like to express my gratitude to my committee members, Dr. Chi-Tang Ho, Dr. Thomas G. Hartman, Dr. Chaim Frenkel, and Dr. Alberto Nunez for further assistance on editing, offering feedback and refining my dissertation.

I'd also like to thank the Department of Food Science faculty, staff, fellow lab mates, and officemates over the years. I'd specifically like to thank Bill Sumal and Dave Petrenka for their creative solutions to all of the tiny instrumental problems that have come up during my research.

To my creative inspirations, Anthony Irwin Kornheiser, Gonzalo Le Batard, and Zachary Harper, this research could not have been accomplished without your (at times) necessary life distractions.

I would like to recognize all the friends I've made here at Rutgers who have made these last 7 years the most impactful of my lifetime. Most importantly, SES, who has facilitated shaping me into the person I believe I am and want to be as my graduate experience comes to a close. You have shown me that personal success extends beyond the laboratory, classroom and even the basketball court.

Finally, I'd like to acknowledge my parents and brothers, Denise, Mark, Zachary and Shane, for their love and appreciation. Thank you for continuing to help and support

me in reaching my dream. I'm not sure what this experience will bring, but I do know my future plans include pride and happiness. Baba booey!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

Research of Farmer and Bolland[1–3] in the 1940s laid the groundwork for our current recognition that lipids oxidize via a free radical chain reaction with three stages -- initiation, propagation, and termination. During the 1960s through 1980s, studies discovering free radical mechanisms of lipid oxidation in foods were omnipresent in the food science community as research focused on identifying products, initiators, metal chelators, as well as effects of oxygen pressure on various oils and other food products[4–11]. However, little additional in-depth investigation on lipid oxidation mechanisms was published for the next 20 to 30 years as research focus shifted to approaches for inhibiting lipid oxidation in foods, most notably through the use of antioxidants, rather than elucidating more details of actual oxidation processes.

For the past several decades, the food industry has limited oxidation in foods by substituting more stable saturated and monounsaturated fats and by brush hydrogenating oils to eliminate the most reactive double bonds when unsaturated fats needed to be used in product formulations. However, with the resurgence of interest in formulating foods with more unsaturated fatty acids for potential health benefits, updating our understanding of lipid oxidation and elucidating incomplete lipid oxidation pathways has become critically important.

In the traditional free radical chain reaction sequence, propagation of reactions is driven entirely by peroxyl and alkoxyl radicals abstracting hydrogen atoms from neighboring molecules. However, this process alone cannot account for observed oxidation kinetics and products mixes. Integrating literature data for reactions of $LOO^{\bullet}$ and $LO^{\bullet}$ led to a proposal of alternate pathways of internal rearrangements, double bond

additions, scissions, etc. that occur in parallel and compete with hydrogen abstraction during lipid oxidation[12]. The balance between these pathways and hence the product distribution varies with reaction conditions. The presence of multiple pathways active simultaneously presents significant analytical challenges in that single products cannot accurately reflect the extent of oxidation – if a pathway other than the one analyzed is active, oxidation can be grossly underestimated. Thus, there is a great need for methods that detect multiple products in a single analysis.

Analysis of volatile oxidation products by gas chromatography is one method that can accomplish this. Volatile products are critical from a sensory aspect in that they are responsible for the off-odors that consumers smell -- one of the main reasons lipid oxidation is considered the most critical and limiting chemical reaction in foods. However, diagnostic analysis of mixed volatiles by GC can also be quite complex because the levels and types of volatiles detected vary with the sampling method used. Static headspace analysis has been most commonly applied and has contributed most of our current understanding of lipid oxidation reactions. It is a simple technique that requires no special equipment, but is also the least sensitive. Solid phase microextraction and thermal desorption are newer methods that improve volatile collection efficiency, but each is subject to some bias in sampling. Learning what information can be provided by each of these methods will be very important for applying these analyses to elucidate lipid oxidation reactions more fully and accurately.

The research reported here is part of a larger project that is investigating Schaich's[12] proposal that alternate $LOO^{\bullet}$ and $LO^{\bullet}$ reactions exist and compete with hydrogen abstraction in lipid oxidation. Lipid oxidation is actually a complex series of

reactions that integrates alternate pathways of internal rearrangements, radical addition to double bonds, scissions, and other reactions in addition to and in competition with hydrogen abstractions in free radical chain reactions. The balance between alternate pathways varies with reaction conditions and complicates both oxidation kinetics and product distributions. This work seeks to provide initial verification that alternate pathways are active in lipid oxidation, shed light on the reaction complexity, and demonstrate that more compounds than the few select products currently monitored must be measured to accurately assess extent of lipid oxidation in foods.

To generate evidence for multiple pathways, investigate whether and how reaction conditions cause shifts in dominant pathways, and to learn how best to use gas chromatography to elucidate lipid oxidation mechanisms, we studied model systems of methyl linoleate oxidized under a variety of conditions and monitored volatile products using static headspace, solid phase microextraction and purge and trap/thermal desorption analytical techniques. Levels and distributions of products were measured daily to assess active pathways. It is hoped that this research will contribute to clarifying lipid oxidation mechanisms and point the way to improved approaches to control. This more detailed and up-to-date understanding may also provide a starting point for investigating more complex omega-3 unsaturated fatty acids.

2. BACKGROUND

2.1 Traditional Lipid Oxidation Theory

Research that started in the 1940s[13,14] eventually led to compilation of the free radical mechanism that is commonly associated with modern lipid oxidation processes (Figure 1). This free radical chain reaction scheme shed light on the basic reaction pathways, and most importantly, introduced the separate processes of initiation, propagation, and termination. These three stages explained kinetics and primary products that formed during oxidation, but did not account for every type of product identified.

Research established that initiation is not spontaneous but requires initiators such as metals, light, or high temperatures. Once radicals are generated, the oxidation process becomes self-perpetuating and increasingly difficult to stop.

While initiation provides the starting point, it is radical propagation that presents the driving force for oxidation. Propagation is the most devastating step because repeated hydrogen abstraction expands oxidation, makes reactions self-propagating and self-sustaining, and allows lipid oxidation to continue indefinitely. The continuous hydrogen abstraction creates a body of radical intermediates that can change in number and intensity based on reaction conditions and incubation period.

The termination step generates a wide range of radical recombination products that include but are not limited to alkanes, aldehydes, ketones, epoxides, and dimers[15]. Termination is also responsible for creating off-flavors and other potentially harmful byproducts[16].

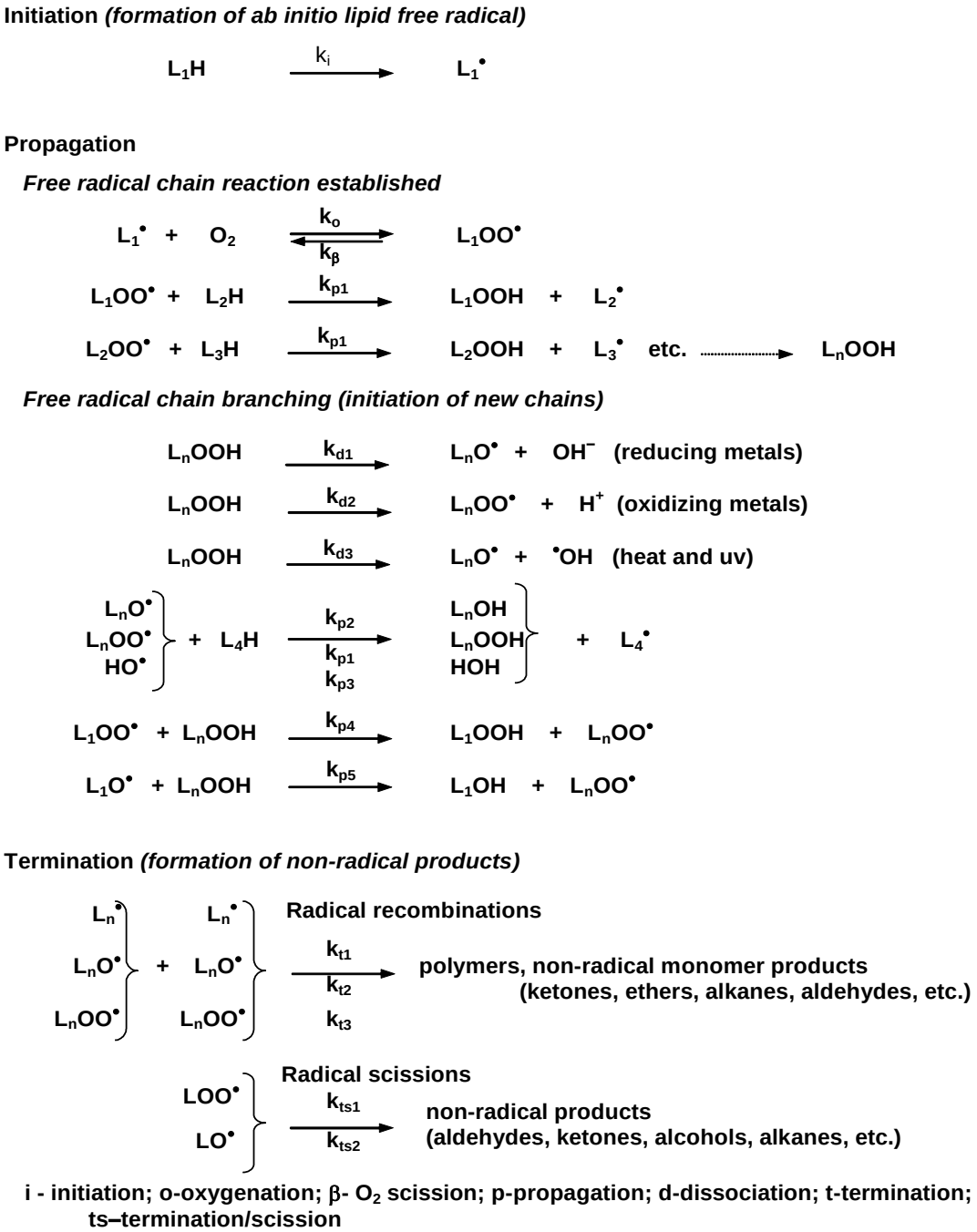


Figure 1. Classical free radical reaction sequence followed in lipid oxidation.[12]

2.1.1 Initiation

Oxygen found in the air lies in the triplet state (unpaired electrons on the oxygen atoms are parallel), as described in a core principle of quantum mechanics. This electron arrangement prevents oxygen from reacting directly with double bonds which are in the singlet state (unpaired electrons on the oxygen atoms are antiparallel). Thus, initiators or catalysts such as metals, light, heat, preformed radicals, or photosensitizers are required to change electron spin of either oxygen or the double bond before reaction can occur[17]. Absorption of visible light energy from sensitizers converts ground state oxygen to singlet oxygen (an excited state) which then adds directly to fatty acid double bonds at a rate at least 1450 times greater than that of triplet oxygen[18]. Alkyl radicals ($L^{\bullet}$) are formed by transfer of an electron to a metal from a double bond or by a free radical abstracting a hydrogen atom from a carbon adjacent to a double bond[12]. The newly formed radicals combine almost immediately with oxygen to form lipid peroxyl radicals, $LOO^{\bullet}$. This process is reflected in oxygen consumption and is difficult to stop once the process has started. Common practices to prevent radical formation utilize metal chelators, singlet oxygen scavengers, low oxygen pressure, low temperature, as well as blanching to inactivate lipoxygenases[12].

2.1.1.1 Types of initiators

Complete mechanisms of initiation are not well understood because the process is so fast and so isolated, but some information about activators or initiators is available. Metals are the chief initiators in foods and probably also biological tissues[19]. Higher valence state transition metals withdraw electrons directly from lipid double bonds,

generating the first radicals. Reduced metals initiate lipid oxidation primarily by autoxidizing to form superoxide anion radical, $O_2^{-\bullet}$. This radical reacts slowly with double bonds, bu converts to $HOO^{\bullet}$ and $H_2O_2/HO^{\bullet}$, both of which abstract hydrogen atoms very rapidly. High heat initiates oxidation by breaking C-C bonds in lipid acyl chains[15,20]. Moderate heat induces homolysis of preformed hydroperoxides, generating reactive alkoxyl and hydroxyl radicals that then abstract hydrogen atoms to generate new radical chains. Ultraviolet light acts by decomposing stable preformed hydroperoxides to reactive alkoxyl and hydroxyl radicals. The energy of visible light is too low to break bonds, but can be transformed to chemical energy by photosensitizers. Photosensitizers absorb the energy from visible light, become excited, and release the energy by forming radicals in lipids directly or by transferring energy to oxygen and exciting it to singlet oxygen. Singlet oxygen has no spin restrictions so adds across double bonds to form hydroperoxides (Figure 2) without generating radicals. Photosensitizers found in foods include chlorophyll, riboflavin, and many pigments[21]. Irradiated light energy also creates excited singlet photosensitizers which have the ability to turn into excited triple state photosensitizers through intersystem crossing mechanisms[22]. These excited triple state photosensitizers then are on the same energy level at triplet oxygen and can form singlet oxygen, which then can serve as the basis for oxidation.

Trace levels of initiators are sufficient to start lipid radical chain reactions. Except for metals, initiators usually act once and do not become involved in the chain reactions.

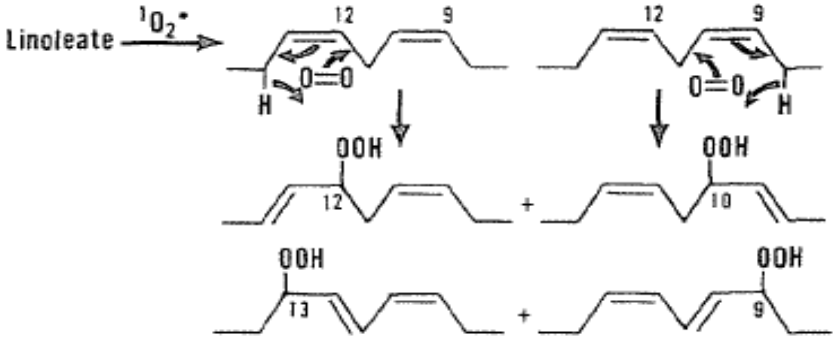


Figure 2. Mechanism for singlet oxygen reaction with methyl linoleate.[57]

2.1.2 Propagation

Traditional theory presents propagation as a process driven entirely by hydrogen abstraction. In early oxidation, initial peroxyl free radicals abstract hydrogens from neighboring lipid molecules to form hydroperoxides and new radicals (Reaction 1). As this action repeats itself, the radical chain reaction becomes established and self-perpetuating.

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet \quad (1)$$

As oxidation progresses, hydroperoxides are decomposed to alkoxyl (LO•) radicals[23] by light, heat, and metals (Reaction 2a) and, under heat and ultraviolet light, also to hydroxyl radicals (Reaction 2b).

$$LOOH \xrightarrow{\text{metals}} LO\bullet + {}^{-}OH \quad (2a)$$

$$LOOH \longrightarrow LO\bullet + \bullet OH \quad (2b)$$

Like peroxyl radicals, alkoxyl and hydroxyl radicals can abstract hydrogen atoms to propagate radical chains (Reactions 3a and b).

$$LO\bullet + LH \longrightarrow LOH + L^{\bullet} \quad (3a)$$

$$\bullet OH + LH \longrightarrow HOH + L^{\bullet} \quad (3b)$$

The importance here is that both of these radicals are orders of magnitude more reactive than the original peroxyl radicals. Thus, propagation by these radicals greatly amplifies lipid oxidation in a process referred to as branching.

Radicals preferentially abstract allylic hydrogen atoms on carbons adjacent to double bonds because these have lower C-H bond energies. Methylene groups ($-CH_2-$) between two double bonds of 1,4-diene systems such as those found in linoleic acid are doubly allylic, experiencing electron withdrawing effects of two double bonds. C-H bond energies here are the lowest in the acyl chain, so these carbons become the preferred sites for hydrogen abstraction by peroxyl radicals. The presence of many doubly allylic methylene groups in high polyunsaturated fatty acids explains their extraordinarily high susceptibility to oxidation. LO• and •OH radicals have higher energies so are able to abstract hydrogen atoms at single allylic sites as well[12,13].

Reactions of hydroperoxides distinguish two kinetic stages of propagation -- the monomolecular rate period and bimolecular rate period[24]. The monomolecular rate period in which hydroperoxides decompose independently is usually slow and is sometimes difficult to detect. In the bimolecular rate period, interactions of two hydroperoxides lead to rapid conversion of relatively unreactive LOOH to very active $LO^{\bullet}$ radicals. Although this usually results in a decrease of hydroperoxide levels, making oxidation appear to be decreasing, it actually leads to great expansion of oxidation and sets the stage for transformations to secondary products, as shown in Figure 3.

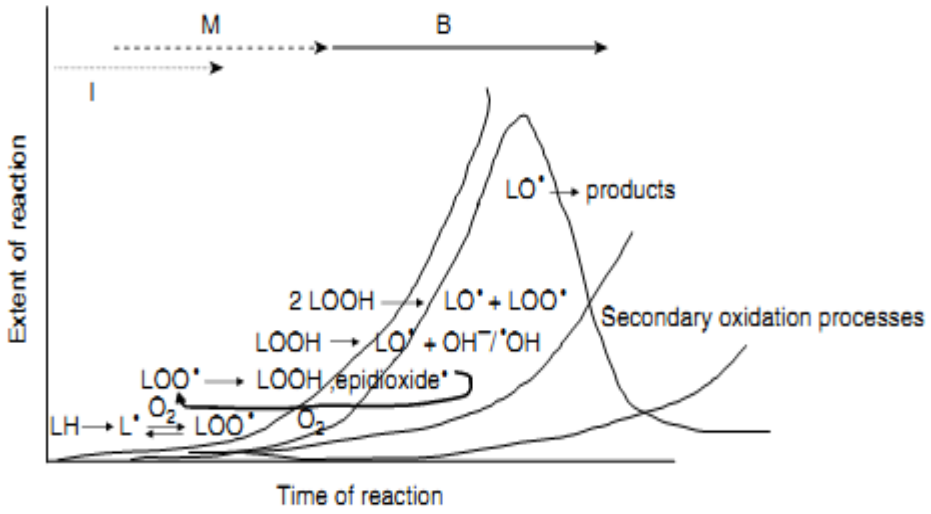


Figure 3. Representation of product formation during induction period (I), monomolecular rate period (M), and bimolecular rate period (B).[12]

2.1.3 Termination

The term "termination" is somewhat a misnomer since free radical reactions are self-sustaining and lipid oxidation never stops as a process. Chemically, termination refers to any action that quenches radicals and stops initiation of new chains. Termination stops individual radical chains and slows the overall process. Termination can occur by three pathways:

1) Radical recombinations (the reaction commonly drawn in traditional radical chain reaction schemes) to form non-radical products (Figure 4). Typical recombination products include acyl peroxides, ethers, alkanes, ketones, and alcohols. Ketones are unique to radical recombination. Types of products formed are strongly influenced by the amount of oxygen present.[12,25]

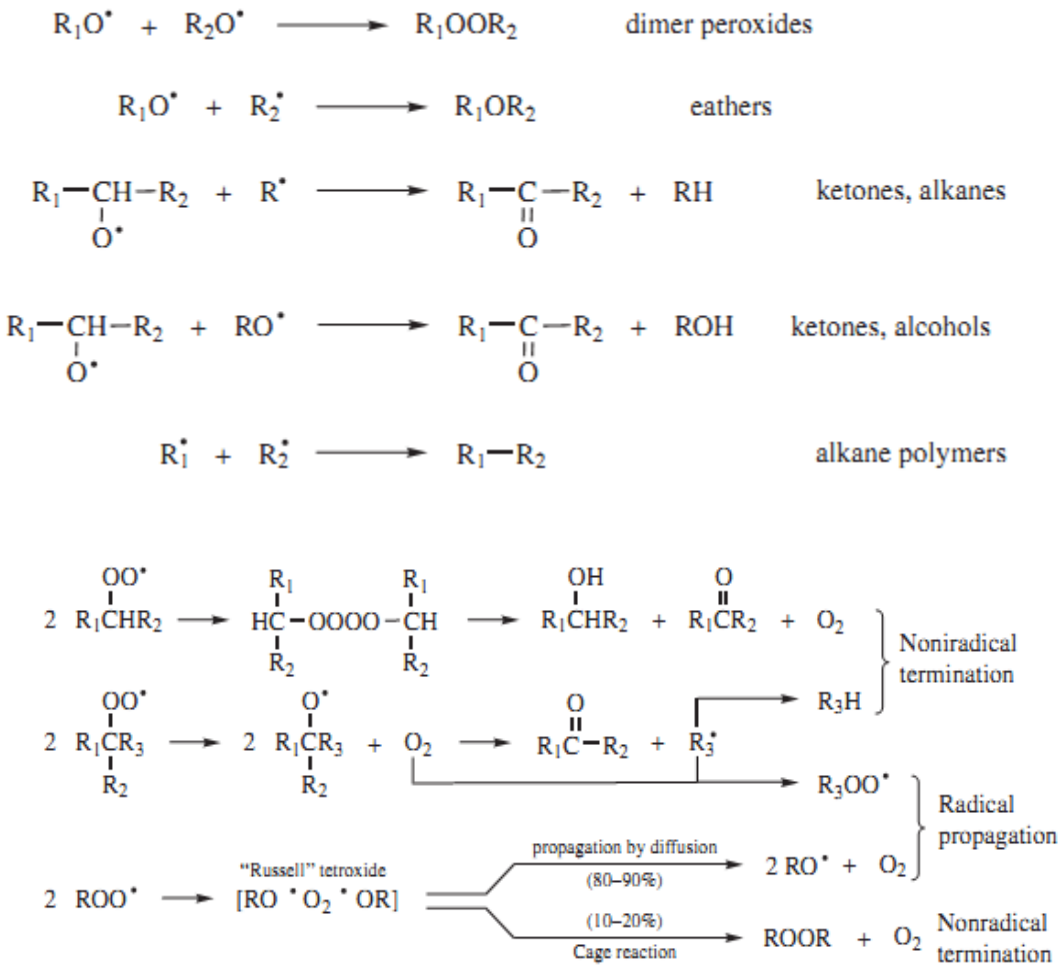


Figure 4. Examples of products formed from radical recombinations in lipid oxidation.[12]

2) Radical scissions to generate aldehydes, alkanes, and alkenes. It is important to note that scissions initially generate radicals that oxidize and continue to propagate radical chains. Scissions become an important termination reaction generating many products typically associated with "rancidity" in food

products when non-lipid proton sources (e.g. water, solvents, and antioxidants) are available to quench the radicals and form non-radical products. Dominant scissions in methyl linoleate will be discussed in detail in Section 2.4 – previous studies of methyl linoleate oxidation.

3) Quenching or interception of primary peroxyl and alkoxyl radicals by non-lipid sources such as water, solvents, antioxidants, and co-oxidation molecules (proteins and vitamins). Although the same products result as in lipid oxidation chains, the key difference is that a new lipid radical is not made in the process and hence the chain reaction stops.

2.2 Integrated Theory with Incorporation of Alternate Pathways

Observed lipid oxidation kinetics and products often cannot be explained by the straightforward free radical chain of traditional theory. The free radical literature cites a number of other reactions of peroxyl and alkoxyl radicals which have rate constants comparable to hydrogen abstraction (Table 1) and thus should be competitive in lipid oxidation. These alternative reactions contribute to shifts in kinetics and products under different reaction conditions, are important sources of products not accounted for in the traditional chain reaction scheme, and present distinct challenges in how to accurately determine extent of lipid oxidation. Ignoring the presence of alternate pathways can lead to gross underestimation of lipid oxidation and misinterpretation of effects of pro- and anti- oxidants.

Table 1. Comparison of rates for alternate pathways.[12]

		LOO•	LO•	
H abstraction, LH	nonpolar organic	<1-400 $M^{-1}s^{-1}$	$10^4 – 10^7$ M^{-1} s^{-1}	
	polar, aqueous		10^6 - 10^8 L $M^{-1}s^{-1}$	
H abstraction, LOOH	nonpolar organic	600 $M^{-1}s^{-1}$	2.5 x 10^8 M^{-1} s^{-1}	
	polar, aqueous	NA	NA	
Cyclization	nonpolar organic	10^1-10^3 s^{-1}	10^4 - 10^5 s^{-1}	
	polar, aqueous	NA	NA	
Addition	nonpolar organic	NA	$10^4 – 10^8$ M^{-1} s^{-1}	
	polar, aqueous	NA	NA	
β-scission	oleate	1-8 s-1	$10^3 – 10^5$ s^{-1}	org
	linoleate	27-430 s^{-1}	$10^4 – 10^5$ s^{-1}	polar org
			$10^6 – 10^7$ s^{-1}	aq
Dismutation	nonpolar organic	10^6-10^9 L $M^{-1}s^{-1}$	10^9 - 10^{10} $M^{-1}s^{-1}$	
	polar, aqueous	10^7-10^8 L $M^{-1}s^{-1}$	NA	
	oleate-OO•	10^6 $M^{-1}s^{-1}$		

2.2.1 Alternate Propagation Reactions of Peroxyl Radicals (LOO•)

Peroxyl radicals are formed first and are the major propagating species during early stages of lipid oxidation. Peroxyl radicals obviously abstract hydrogen atoms and they do this rather selectively, but they also undergo additional reactions that propagate the radical chain reaction, i.e. form new radicals on additional lipid molecules. These alternate reactions reactions include rearrangement/cyclization, addition to double bonds, disproportionation, β-scission, recombination or electron transfer, and occur in parallel and in competition with hydrogen abstraction[12].

Internal rearrangement (cyclization). In situations where allylic hydrogens are not readily available, peroxyl radicals have the ability to attack electron dense areas by adding to nearby carbon double bonds, thereby forming internal cyclization products. The most important cyclization by LOO• involves 1,3-addition to a nearby cis-double bond to create a 5-exo ring (epidioxides) while also generating a radical on the carbon γ to the

double bond (Figure 5). Reaction of this newly formed radical with oxygen forms an epidioxy hydroperoxide, which can subsequently undergo scission to generate secondary oxidation products. Because of the spatial requirement, LOO• cyclization occurs mostly from internal positions so is found mostly with higher polyunsaturated fatty acids and in singlet oxygen photosensitized linoleic acid.

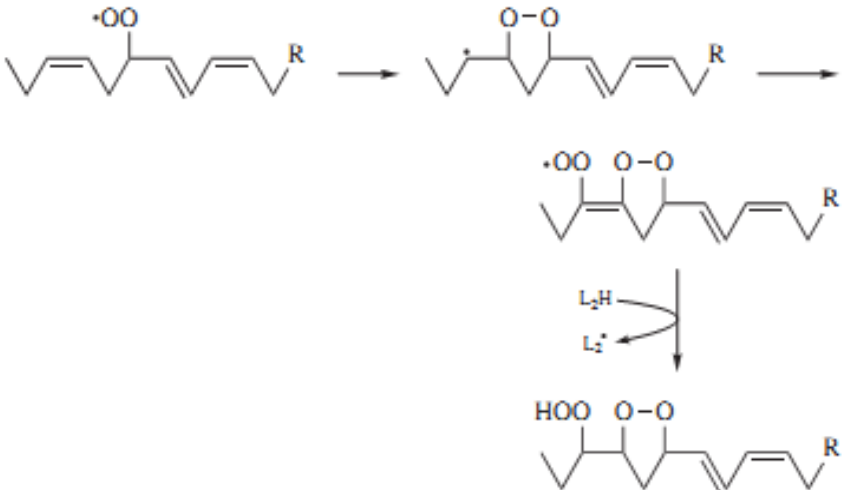


Figure 5. LOO• cyclization to the internal epidioxides (cyclic peroxides).

Addition to double bonds. Peroxyl radicals have the ability to add to double bonds, but competition with hydrogen abstraction makes it most active under conditions such as aprotic solvents or low temperature which limit available abstractable hydrogens. Addition theoretically should generate oxydimers and polymers (Reaction 4). However, the adducts are not stable and rapidly undergo elimination/fragmentation to yield epoxides and alkoxyl radicals (Reaction 5).

$$L_1OO^{\bullet} + R_1{-}CH_2{-}CH{=}CH{-}R_2 \longrightarrow R_1{-}CH_2{-}\overset{\bullet}{C}H{-}\underset{\displaystyle OOL_1}{\underset{|}{C}}H{-}R_2 \qquad (4)$$

$$R_1{-}CH_2{-}\underset{\displaystyle OOL_1}{\underset{|}{\overset{\bullet}{C}}}H{-}CH{-}R_2 \longrightarrow L_1O^{\bullet} + R_1{-}HC^{\bullet}{-}\underset{\displaystyle O}{CH{-}CH}{-}R_2 \xrightarrow{O_2} L_2(\text{epoxy})OO^{\bullet} \qquad (5)$$

Because of the peroxydimer instability, this reaction is not easy to track except through its products and the timing of their evolution. This reaction should be the main source of epoxides that form very early in oxidation, paralleling hydroperoxides, as opposed to epoxides generated from alkoxyl radicals that accumulate later in oxidation. Importantly, peroxyl radical additions simultaneously generate alkoxyl radicals with the epoxides. The $LO^{\bullet}$ can accelerate oxidations early by H abstraction, can undergo internal rearrangement to greatly amplify epoxide levels, and/or can undergo scissions to alkanes and aldehydes. Thus, $LOO^{\bullet}$ addition can easily be overlooked and products mistakenly attributed to other reactions.

Disproportionation. Disproportionation reactions occur when lipid peroxyl undergo self-recombination, sometimes generating oxygen while creating two new alkoxyl radicals (Reaction 6a) or an acyl peroxide (Reaction 6b).

$$R_1OO^{\bullet} + R_2OO^{\bullet} \longrightarrow [R_1OOOOR_2] \longrightarrow R_1O^{\bullet} + {}^{\bullet}OOOR_2 \longrightarrow R_1O^{\bullet} + O_2 + {}^{\bullet}OR_2 \quad (6a)$$

$$[R_1OOOOR_2] \longrightarrow R_1OOR_2 + O_2 \quad (6b)$$

Disproportionations are often discounted but occur when local radical concentrations accumulate in systems such as neat lipids or aprotic solvents where abstractable hydrogens are limited. Importantly, disproportionations return oxygen to the system and convert slowly reacting $LOO^{\bullet}$ to $LO^{\bullet}$ that react much faster and less selectively. The presence of disproportionation can be inferred from early acceleration of oxidation and early appearance of $LO^{\bullet}$ products such as alcohols and aldehydes without formation and decomposition of hydroperoxides. Epoxides may result from $LO^{\bullet}$ but early generation

may arise from $LOO^{\bullet}$ addition as well, so epoxide levels and kinetics are not conclusively diagnostic of disproprotionation.

β-scission. β-scission cleaves the C-O bond in peroxyl radicals, releasing oxygen and an alkyl radical (Reaction 7).

$$LOO^{\bullet} \longrightarrow L^{\bullet} + O_2 \qquad (7)$$

The rate of β-scission is competitive with hydrogen abstraction from allylic positions, so this reaction plays an important role in extending the induction period and preventing start of active oxidation. β-Scission also is the critical intermediate for isomerization of double bonds, e.g. 13-OOH to 9-OOH and cis to trans at high temperatures[26], which alters the distribution of end products.

2.2.2 Alternate Propagation Reactions of Alkoxyl Radicals ($LO^{\bullet}$)

Alkoxyl radicals are the main radical chain carriers during secondary and late stages of lipid oxidation. Alkoxyl radicals propagate free radical chains by hydrogen abstraction and also by rearrangement/cyclization, addition reactions to double bonds and α and β-scission.

Hydrogen abstraction. Alkoxyl radicals undergo hydrogen abstraction to create alcohols while also propagating the chain reaction. Alkoxyl radicals are more reactive and thus less selective in their type of hydrogen abstraction[27]. They can abstract both allylic and bis-allylic hydrogen atoms, most often in neat lipids, aprotic solvents, high lipid concentration, as well as at elevated temperature[12]. It is also common to have internal abstractions from distant positions on the acyl chain that mimic the reaction of

hydroxyl radical mechanisms (HO•). The results of these hydrogen abstractions are often hydroxy lipids.

Internal rearrangement (cyclization). Alkoxyl radicals undergo internal cyclization by addition to an immediately adjacent double bond to form epoxides. This occurs very fast and can be the dominant reaction in neat lipids, aprotic solvents, and at low lipid concentrations (Table 2). Epoxide formation is also common at room temperature and in environments with low oxygen pressure.

Table 2. Effects of solvent on internal cyclization in oxidized linoleic acid. [12]

	Product distribution (%)				
Solvent and system	LOH/LOOH[a]	Cyclic[b]	Scission	Other	Unknown
CH_2Cl_2, $FeCl_3$		100			
Anhydrous MeOH	3-8	75-80	13-15		
Cyclohexane, 7.5 mM	15	68		18[c]	
80% ethanol $FeCl_3$/cysteine	30	11	--	7[d]	7[e]

[a] total of all H abstraction products, all isomers
[b] total of all products that had any cyclic component
[c] oxo dienes
[d] hydroxyl ethoxylated products from rx with solvent radicals
[e] unidentified soluble products and volatile scission products

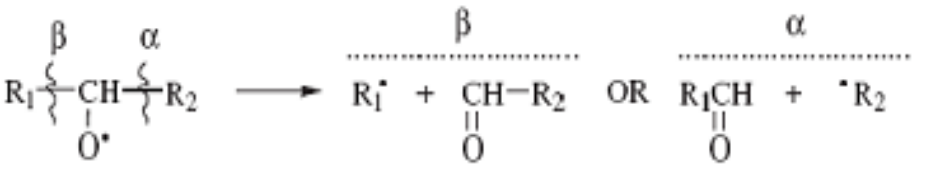
Addition of LO• to double bonds. While hydrogen abstraction and cyclization are most common, alkoxyl radical can also undergo addition to double bonds. LO• addition to double bonds transfers the radical to the acyl backbone of Lipid 2 and is facilitated by the lack of allylic hydrogens or by conjugation, creating dimers, polymers, and ketones. Alkoxyl radicals have a preference for allylic attack where hydrogen abstraction and internal cyclization also occur in completion. Since LO• addition is slower (Table 1), the other reactions are favored as along as allylic/bis-allyic hydrogens

are present. In the absence of allylic/bis-allyic hydrogens, alkoxyl addition is favored (Table 3).

Table 3. Effects of alkene structure t-BuO• preference for addition vs H abstraction.[28]

Alkene	Abstraction (%)	Addition (%)
R–CH=CH–R (trans)	95	3–4
R–CH=CH–R (cis)	83	17
R–CH=CH_2	97	3
R_2–C=CH_2	83	17

α- and β-scission reactions of LO•. Scission of C-C bonds on either side of alkoxyl radicals generates small volatile alkanes, aldehydes and other carbonyls that are often odor and taste components of what consumers refer to as "rancidity". β-Scission occurs on the distal side and α-scission occurs on same side of the alkoxyl carbon as the acid group. Scission produces free radicals that continue the radical chain as well as carbonyl products such as aldehydes, alkanes, and oxo-esters[15] (Reaction 8). Frankel has proposed that scissions are facilitated when proton sources are available to stabilize radical products.



(8)

System factors, most specifically solvent, play a role in determinin the preference for scission vs hydrogen abstraction of alkoxyl radicals. Table 4 shows that β-scission becomes more preferred as polarity of solvent in the system increases.

Table 4. Solvents effects on rates of hydrogen abstraction and β-scission of cumyloxyl radicals.[120]

	H Abstraction	β-Scission	
	$k_a \times 10^{-6}\ M^{-1}\varepsilon^{-1}$	$k\beta \times 10^{-5}\ \varepsilon^{-1}$	$k_a/k\beta\ M^{-1}$
CCl_4	1.1	2.6	4.5
C_6H_6	1.2	3.7	3.2
C_6H_5Cl	1.1	5.5	2.0
$(CH_3)_3COH$	1.3	5.8	2.3
CH_3CN	1.2	6.3	1.9
CH_3COOH	1.3	19	0.7

2.2.3 Integration of propagation reactions with hydrogen abstraction

The multiple reactions available to both alkoxyl and peroxyl radicals show the complexity of oxidation in even the most simple lipid systems. The sheer number of alternate reactions suggests there should be much more chemistry occurring than the traditional free radical mechanism portrays, and the similarity in reaction rates (Table 1) argues further that product mixtures should be much more diverse than the traditional reactions describe. Schaich[1] has proposed one way in which alternate pathways such as addition, cyclization, and scission can be integrated with hydrogen abstraction for each major type of reactive species (Figure 6), creating additional radicals and generating a much more complex mixture of products than predicted by the traditional scheme. This

integrated reaction scheme may better explain observed oxidation kinetics as well as varied mixtures of products formed during lipid oxidation while enabling scientists to accrue a more complete picture and more accurate assessment of the extent of oxidation in various systems.

Two key differences resulting from the integrated alternate pathways are that multiple products may be produced from the beginning of oxidation, and many more secondary products should be generated than expected from the traditional chain reaction. These two differences plus the multiple pathways active make it critical to monitor lipid oxidation frequently even in early oxidation, to track products from as many pathways as possible, and to develop analyses that detect and quantitate individual products as well as total classes. All three issues are being addressed in this research.

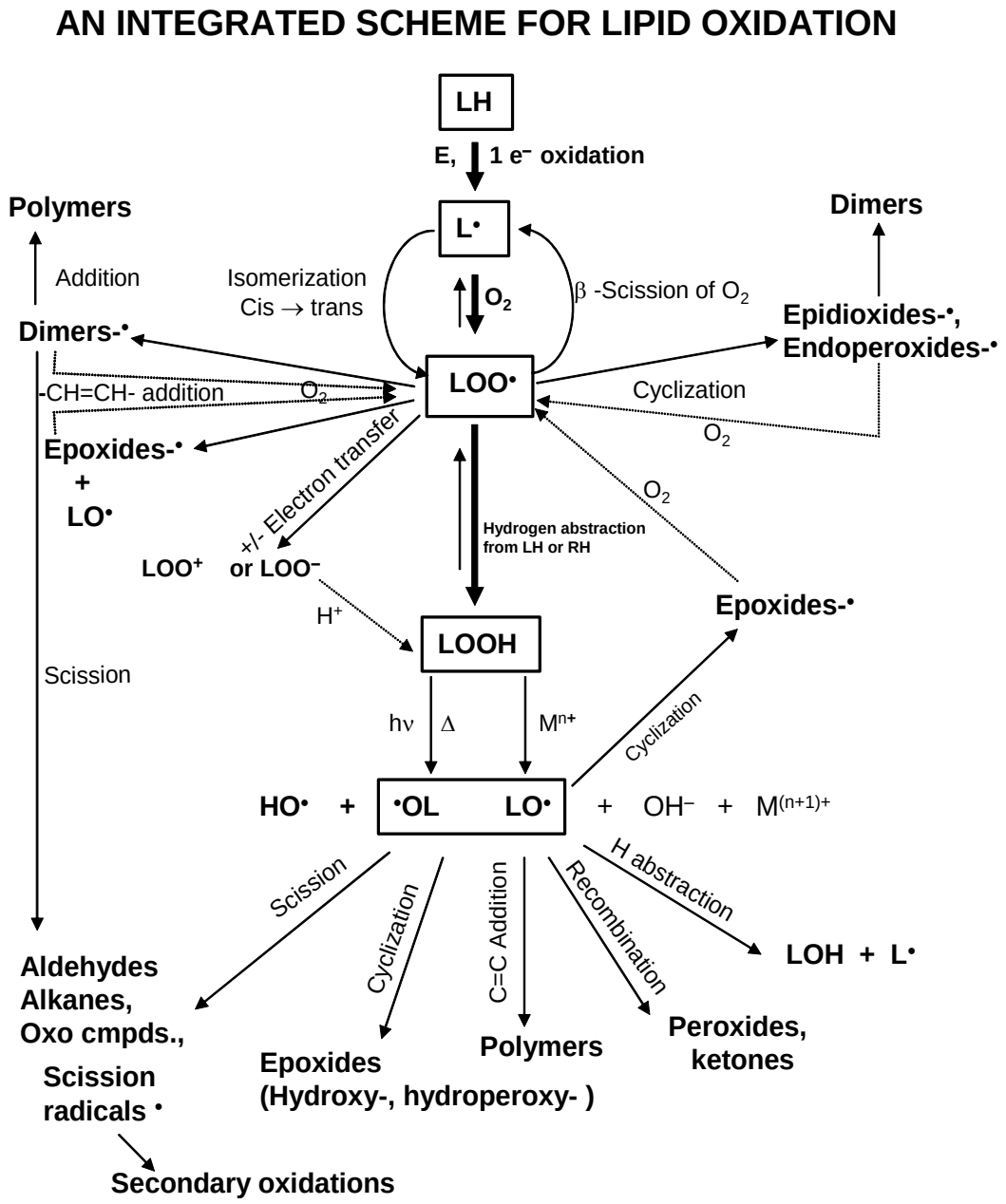


Figure 6. Proposed lipid oxidation reaction scheme integrating alternate pathways with traditional chain reaction driven by hydrogen abstractions.[12]

2.3 Analytical Techniques Used for Lipid Oxidation

As the previous discussion shows, lipid oxidation reaction mechanisms can be very complex with many reactions potentially active at the same time. The situation is complicated further by different activation energies for different pathways and constant changes in the competition between formation and transformation of various intermediates and products. Clearly, measuring oxidation by a single product or even a few standard products cannot give a full or accurate picture of oxidation.

No one method can provide information on all pathways. Traditionally, lipid oxidation has been followed by analyzing one or more soluble products by chemical class assays, e.g. conjugated dienes, hydroperoxides, carbonyls, or by instrumental analyses that can detect multiple products simultaneously without the need for lipid extractions. Examples here are infrared spectroscopy and gas chromatography. The specific method utilized depends on the food or oil system as well as analytical capabilities in individual laboratories[29]. The methods utilized most often due to their ease of handling, sensitivity, and accuracy have been high performance liquid chromatography (HPLC) and gas chromatography (GC) to analyze non-volatile and volatile products, respectively. Both offer advantages and disadvantages but most importantly require detection by mass spectrometry (MS) for identification of individual products.

2.3.1 High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) utilizes a mobile phase of a liquid as a solute/analyte undergoes interactions with liquids or mixtures of liquids. The polarity of the stationary phase (a packed column) needs to be selected based on the

interactions between the sample or solute to find an optimal interaction which enhances retention quality and time[30]. Few direct HPLC applications exist to measure lipid oxidation due to several critical limitations. First, lipids lack chromophores for optical detection; only lipids with conjugated diene structure are detectable in the ultraviolet range. Refractive index detectors (RID) can be utilized but are relatively insensitive[31]. Ultraviolet detectors require derivitization of lipids to find specific classes, such as aldehydes. In addition, despite the advances of mass spectrometry, the ability to have specific product identification in a sample as complex as lipids where standards are not available for every oxidation product is also somewhat difficult. Finally, lipid oxidation products are inherently unstable and reactive. They can only be analyzed in non-metallic chromatographic systems, e.g. all PEEK systems, which are not universally available, and in addition, lipid oxidation radicals and products rapidly degrade column materials. For these reasons, this dissertation will focus on the use of gas chromatography as the main investigative technique.

2.3.2 Gas Chromatography

2.3.2.1 Instrumentation and basic processes of gas chromatography

While the number of techniques utilized to measure lipid oxidation is varied[29], gas chromatography (GC) has received most interest due to its practicality and rapid sample run time. More importantly, GC is one of the best and most developed techniques used for analyzing and separating a wide variety of volatile products. GC has been of key importance to food applications since it was developed in the 1950s[32], and it continues to serve as a great resource for analyzing volatiles released from gases, liquids, and solids. It

is especially useful for identification purposes using either retention time or mass spectrometry.

Figure 7 shows a diagram of how gas chromatography typically works. The mobile phase is a gas, usually helium, nitrogen, or hydrogen. Hydrogen would provide the best resolution in theory, but cannot be used due to safety hazards. Instead, helium is used preferentially, with nitrogen sometimes being substituted for economic reasons.

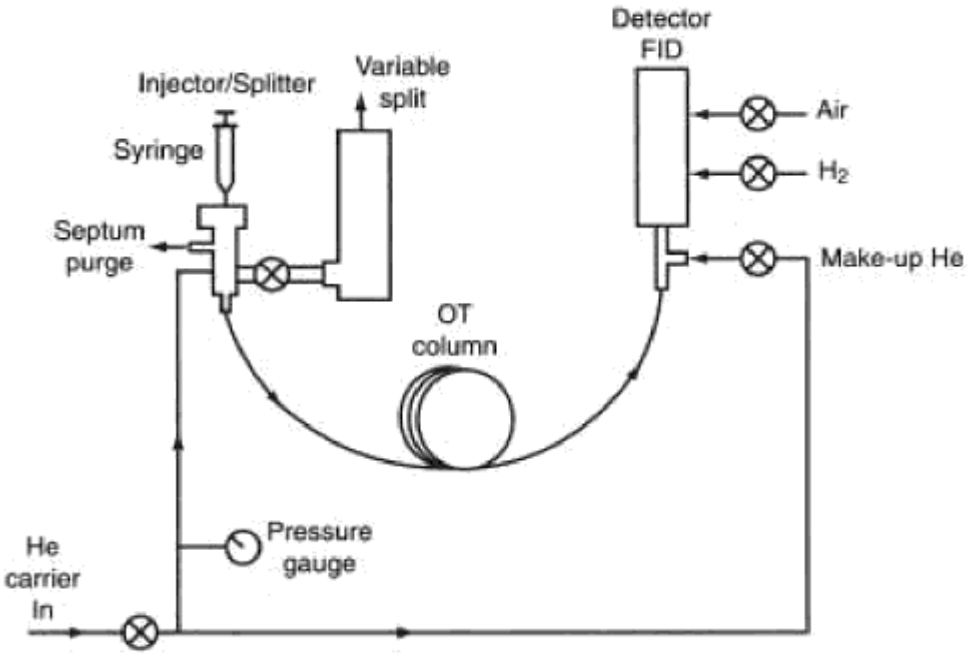


Figure 7. Typical gas chromatography setup.[33]

Four critical factors help determine which column should be used for an analysis: internal diameter, column length, film thickness, and stationary phase composition. The stationary phase is a microscopic layer of liquid or polymer placed inside a small column constructed of glass or metal tubing. Materials used for stationary phases provide a variety of polarities to separate compounds of interest. The main column used in GC is a

capillary column with inner diameter ranging from 0.1 to 0.53 mm. Most columns have internal diameters of 0.25 mm or 0.32 mm as they offer the best balance between resolution, speed, sample capacity, and ease of operation[33]. Column length varies from 5 m to 100 m and plays an important role in resolution of peaks. In general, longer columns provide higher resolution, but at the cost of longer run time. The stationary phase is coated as a film on the inside of the column, in thickness ranging from 0.1 μm to 5 μm with a 0.25 μm being the most common. Film thickness controls the column functionality, with lower thicknesses giving high resolution and higher thicknesses having a larger sample load capacity.

Stationary phase composition is perhaps the most critical factor to ensure efficient separation of compounds of interest. The two most common liquid phases for capillary columns are siloxane polymers or polyethylene glycol[33]. The most common column is a 5% (phenyl)-methylpolysiloxsane which has mostly non-polar interactions with the analytes. A laboratory with the following six stationary phases can conduct all GC analyses: 1) dimethylsiloxane, 2) 50% phenylpolysiloxane, 3) poly(ethylene glycol) of MW>4000, 4) DEGS, 5) 3-cyanopropylpolysiloane, and 6) trifluoropropylpolysiloxane[34]. The high methyl polymethylsiloxane columns are generally selected for oil analysis due to longer lifetime[35], although other columns may give better resolution. While most early research on oils and oxidation was completed on packed columns, the use of these capillary columns with specially selected polar phases eliminated problems associated with column bleed and showed a more reproducible peak retention time[36].

GC has the flexibility to investigate both gasses and liquid for analyses, as long as components of the liquid are volatile. Analytes are injected into the GC injection

port/inlet which is usually kept at high temperatures, around 250-275 °C. At this port, the sample meets the carrier gas, which pushes the sample through the column. Depending on the affinity of the analyte compounds to the stationary phase, each component will elute with unique time stamp known as retention time. The column is gradually heated to facilitate movement of analytes through the column, varying the rate and time pattern of heating to optimize resolution of compounds of similar molecular size, weight, and functional groups. At the end of the column, detectors monitor the different peaks and send the data output to the computer. Because GC is highly reproducible, compounds can be identified by their retention indices when the same flow rate, column, and temperature program are used.

Gas chromatography offers many advantages, but also has some limitations. The greatest difficulty arises with samples such as amino acids or carbohydrates that cannot be volatilized; these must be derivatized for GC analysis. GC cannot be used to analyze compounds that decompose at the high temperatures used in both the inlet and column. Quantitating volatile compounds, especially those originating from complex sample matrices, is complicated. In addition, when samples show only a low total volatile response, many large samples or special sample preparation are required to provide enough volatiles for detection.

2.3.2.2 Gas Chromatography Detectors

The most common general detectors in gas chromatography are the flame ionization detector (FID) and thermal conductivity detector (TCD). FID uses a combination of air and hydrogen to generate a flame that pyrolyzes analytes into small ion fragments. Each fragmentation then produces a small current that shows up as a peak.

FID is specific for hydrocarbons[33]. TCD measures the difference in thermal conductivity of an analyte compound relative to the thermal conductivity of the carrier gas; these detectors can track gases that are not hydrocarbons and were found on most early GCs[33]. Both of these detectors are very useful for quantitative (or semiquantitative) analyses but can only identify compounds indirectly by comparison of retention times.

The most critical detector for identification of compounds is a mass spectrometer interfaced to the gas chromatograph. Mass spectrometry provides structural, elemental, and, most importantly, molecular weight information about analytes in each peak by ionizing the compounds chemically (chemical ionization, CI) or electronically (electron ionization, EI) and breaking them into small fragments whose patterns generate a "fingerprint" specific for each molecule. Mass spec detectors have low sensitivity because they can handle only minute amounts of material, so they are usually combined with either FID or TCD detectors to provide both qualitative and quantitative information about compounds separated by gas chromatography.

2.3.2.3 GC Sampling Techniques

Extracting volatiles from a solid or liquid sample for gas chromatography is referred to as headspace analysis[33]. Headspace analysis is an umbrella term that encompasses both static and dynamic sampling. In static headspace techniques, volatiles are not forced out of their environment, but are allowed to accumulate within sealed containers until withdrawal for analysis. Obviously, compounds do not evaporate instantaneously, so time is required for volatiles to escape from each sample and reach equilibrate in the headspace before collection. The two static headspace techniques in common use are static headspace (SHS) and solid-phase micro-extraction (SPME).

Dynamic headspace analysis eliminates equilibration limitations by collecting volatiles constantly as they are released from a sample. Gas flow continually moves volatiles out of their container headspace and deposits them on an adsorbent “trap”, a process referred to as “purge and trap”[33]. Compounds on the trap are then transferred to gas chromatography columns by thermal desorption. This method is more exhaustive than static headspace or SPME so can often detect compounds not seen by static methods.

As an alternative to headspace sampling of volatiles, very small volumes of liquids (as little as 1 μL) can be injected directly into a GC. The liquid then volatilizes inside the GC inlet. This technique is useful for detecting less volatile compounds, but non-volatile components of samples can cause system contamination, polluting the injection liner, the column, or both.

This project will focus on headspace techniques to compare results with the huge data base in the literature, re-evaluate methods commonly used in academic and industrial laboratories, and provide a counterbalance to non-volatile products being studied in a parallel project.

2.3.2.3.1 Static Headspace (SHS)

Static headspace analysis relies on equilibrium being reached between a liquid/solid sample and the vapor phase. Obtaining a reproducible equilibrium between the two phases requires proper temperature control of sample vial[33]. SHS is fast, simple, and easy to complete, requiring only standard sample vials (typically 10 or 20 mL) and a gas-tight syringe, which can vary in size up to 5 mL. Most importantly, no sample preparation or solvent is necessary[37]. Samples of the vapor phase are then transferred

manually or by autosampler from the sample vial to the inlet of the gas chromatograph.

SHS offers advantages of low cost, simplicity of use, and extensive data base of volatile flavor and lipid oxidation compounds in food lipids. In addition, SHS allows volatile sampling without being destructive to the sample or the necessity of a solvent[38]. On the down side, headspace equilibria limit concentrations and types of compounds that accumulate in the headspace. The resulting low sensitivity in detection is the main reason for increasing interest in more versatile sampling techniques. In addition, for quantitative analysis, it may sometimes be necessary to understand more about specific compounds being tested as well how to optimize the matrix effects to ensure accuracy and precision[37]. Nevertheless, SHS is still used extensively to analyze lipid oxidation.

An additional consideration when using SHS to study lipid oxidation is that the elevated temperatures used to volatilize compounds (typically 60 to 120 °C) can also change or degrade them, and can also decompose hydroperoxides to products that were not there previously. Limiting research to 40 °C and 60 °C – temperatures at which foods and the oils contained in them are often processed, stored, or sometimes even cooked (smoked)[39] – can minimize artifacts but cannot guarantee that products observed are the same as those formed at ambient temperature.

2.3.2.3.2 Solid Phase Micro-extraction (SPME)

Solid phase micro-extraction (not to be confused with solid phase extraction) is a relatively new sampling technique developed in 1989 by Arthur and Pawliszn as a simple solvent-free sample extraction procedure[40]. This technique adsorbs volatile compounds onto a coated fused silica fiber attached to the end of a micro-syringe plunger that can be stored within the syringe barrel[33]. The glass fiber, usually 1-2 cm in length, is coated with

a variety of phases, applied singly or in combination, to provide selectivity in adsorption of analytes. Phases in common use include polydimethylsiloxane (PDMS), divinylbenzene (DVB), carboxen (CAR), and polyacrylate (PA), among others (Table 5). This wide variety of options allows for a diverse number of uses throughout the scientific community[41].

Table 5. SPME coatings available commercially.[42]

Fibre coating	Film thickness (μm)	Polarity	Coating method	Maximum operating temperature (°C)	Technique	Compounds to be analysed
Polydimethylsiloxane	100	Non-polar	Non-bonded	280	GC/HPLC	Volatiles
(PDMS)	30	Non-polar	Non-bonded	280	GC/HPLC	Non-polar semivolatiles
	7	Non-polar	Bonded	340	GC/HPLC	Medium- to non-polar semivolatiles
PDMS–divinylbenzene	65	Bipolar	Crosslinked	270	GC	Polar volatiles
(DVB)	60	Bipolar	Crosslinked	270	HPLC	General purpose
(a)	65	Bipolar	Crosslinked	270	GC	Polar volatiles
Polyacrylate (PA)	85	Polar	Crosslinked	320	GC/HPLC	Polar semivolatiles (phenols)
Carboxen–PDMS	75	Bipolar	Crosslinked	320	GC	Gases and volatiles
Carboxen–PDMS (a)	85	Bipolar	Crosslinked	320	GC	Gases and volatiles
Carbowax–DVB	65	Polar	Crosslinked	265	GC	Polar analytes (alcohols)
Carbowax–DVB (a)	70	Polar	Crosslinked	265	GC	Polar analytes (alcohols)
Carbowax-templated resin (TPR)	50	Polar	Crosslinked	240	HPLC	Surfactants
DVB–PDMS–Carboxen(a)	50/30	Bipolar	Crosslinked	270	GC	Odors and flavors

(a) Stableflex type on a 2 cm length fibre

The fiber is exposed to the sample, either in the headspace above the liquid or the liquid itself, and given time to adsorb analytes. The temperature and absorption time can be varied to fit the compound(s) of interest[43]; typical adsorption times are 10-30 minutes but can range as high as several hours[33]. The fiber is then pulled back into the protective syringe sheath, transferred to the gas chromatograph, inserted into the hot injection port, and analytes are desorbed thermally into the chromatograph. Desorption times vary with the analyte characteristics but average about 5 to 20 minutes (Figure 8).

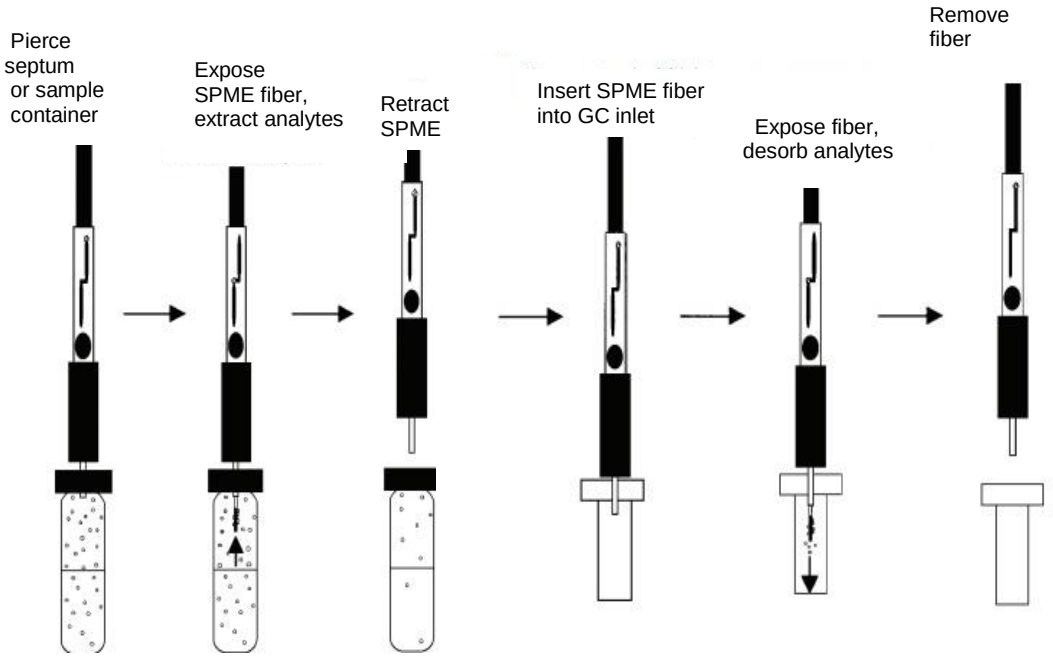


Figure 8. Simplified flow diagram of SPME extraction procedure[121].

Fiber selection is often a troublesome issue when using SPME as a sampling technique. Four characteristics that determine selection are molecular weight and size of desired analytes, analyte polarity, analyte concentration level and range, and complexity of the compounds of interest[40].

Studies on fiber selection showed that poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB) had the highest overall sensitivity, but carboxen/poly(dimethylsiloxane) (CAR/PDMS) was the most sensitive for small molecules and acids[44]. However, a three-phase fiber -- divinylbenzene/carboxen/poly(dimethylsiloxane) (DVB/CAR/PDMS) – has been developed by blending carboxen with PDMS in a 30 μm inner layer on a strengthened fiber core, then coating this with a 50 μm outer layer of DVB. This outer layer allows the fiber to extract the longer chain and semi-volatiles while still adsorbing the smaller more volatile molecules to the inside carboxen layer[45]. It is this mixture of qualities that makes the DVB/CAR/PDMS fiber the most preferred fiber for samples that have a broad range of analyte polarities. This fiber is specifically selective for compounds in C2-20 range[46].

SPME offers several advantages. It provides simple and fast sample preparation (as well as cleaning) with a solvent-free extraction that can be used to detect volatiles with a wide range of polarity and size while also increasing response and detection of semivolatile compounds coming from solid, liquid, or gaseous samples[47]. In addition, it has the flexibility to allow analytes to be transferred to either GC and HPLC[46]. These key advantages allow SPME to be used in several applications such as environmental[46], medical[48], as well as several food systems such as vegetable oils[45], milk[49], and cucumbers[50].

However, the wide variety fibers available also can work as a disadvantage because extraction and quantitative accuracy is dependent on experimental conditions (absorption/desorption times), sample matrix, analyte polarity, and calibration techniques[47]. Absorption time is critical since fibers come with linear ranges for

adsorbing compounds (usually under 1 ppm) for most compounds and when the range is exceeded, there is a decrease in the amount bound despite equal increases in concentration[44]. This effect can be neutralized by diluting the matrix or decreasing adsorption times. Alternatively, in rare cases when SPME is not sensitive enough or semivolatiles are present, salting out can be used to decrease solubility of the analytes, as is often done in studies of pesticides[51]. In addition, adsorption to the fiber may decompose some products or form new ones, e.g furan levels from 2-butenal increased with extraction time and temperature[52]. Additionally, depending on the analytes or fiber selected, cost can become an issue since fibers can be used only about 50 times before they begin to deteriorate[53].

2.3.2.3.3 Purge & Trap and Thermal Desorption

Dynamic headspace which allows volatiles to be continuously removed from a liquid or solid sample[54] was introduced in the early 1960s by Swinnerton et al.[55]. As noted in Section 2.3.2.3.1, static headspace analyses are limited by headspace equilibria in which high volatility of some products can impede volatilization of other products. Purge and trap with thermal desorption is a dynamic process developed to overcome these limitations and achieve more complete collection of products by continuously sweeping an inert carrier gas (usually nitrogen) across the sample to collect volatiles then deposit them onto a sorbent material to "trap" the analytes[37]. In this way, headspace pressure never develops and a strong driving force for volatilization is maintained. Constant removal of volatilized compounds facilitates a continuous flow of potentially volatile compounds in the sample to the surface where they can evaporate. Hence, product collection theoretically is much more complete than with static headspace or

SPME methods; it is also considered by some to be more accurate than the other methods.

In short path thermal desorption[56], a desorption unit with an injection needle afficed to the end is placed on top of the GC injection port (Figure 9). A sample tube filled with sample and absorbent material such as Tenax or Carboxen is inserted, and the injection is made by an electronic controlling unit that allows the user to control time of desorption as well as the desorption temperature[57].

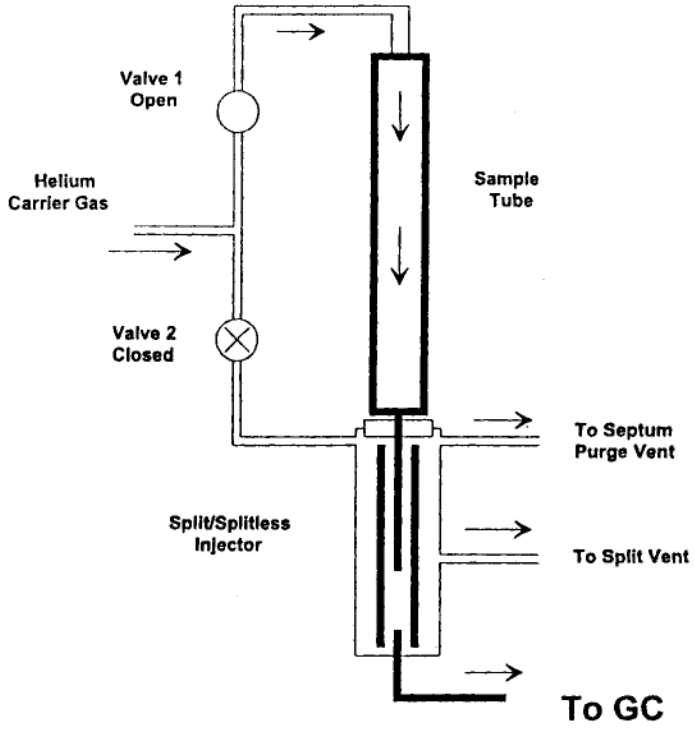


Figure 9. Model of short path thermal desorption.[57]

Alternatively, samples can be prepared with a purge and trap set-up where a flow of inert gas is bubbled through a liquid sample to purge volatiles (Figure 10). The purge gas is usually introduced through a needle or thin tube which is slightly below the surface of the analyzed liquid[38]. This bubbling causes a more exhaustive extraction, but depending on the liquid sample being analyzed, foaming could occur adding contamination or trapping volatiles. When this is a problem, the liquid can be deposited directly onto glass wool, followed by desorption directly into the GC[58].

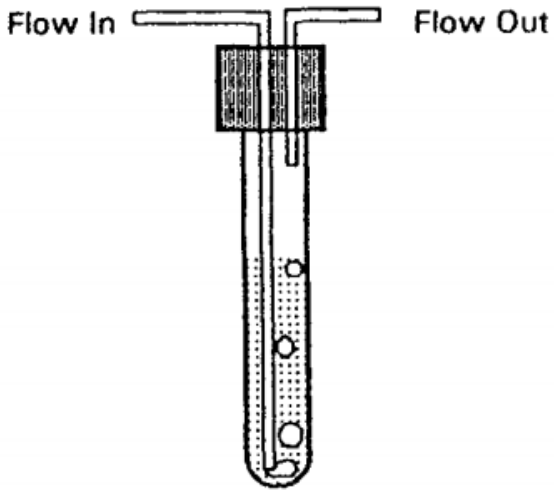


Figure 10. Purge and trap sample set-up.[38]

Thermal desorption offers particular advantages with solid samples because it provides a simple way to concentrate the headspace without the need for long extraction processes[59]. Volatiles present at trace levels – e.g. parts per billion (ppb) can routinely be analyzed[38]. Furthermore, ability to select different sorbents and extraction temperatures allows selectively in the range of volatiles collected, as unmatched volatiles would be

purged out of the trap[38]. In addition, can be accomplished easily by adding an internal standard to the trap between adsorption and desorption.

Disadvantages of thermal desorption include loss of volatiles due to leaks, purging inefficiencies, loss of purged volatiles through split/splitless injectors, carryover, and the way in which internal standards are added to samples provide some of the disadvantages of this technique[57]. Thermal desorption is more expensive than those of SHS and other extraction techniques. More importantly, with the electronic controller and the extraction process there are possibilities of heater damage, valve leaking, contamination, and cold spots during desorption[38]. Also, moisture content is important as excess water can result in shorten lifetime of both sorbent materials and the GC column[57]. Perhaps most importantly, high temperature needed for purging and desorption, it is possible the heat is creating artifacts or converted volatiles that are actually present in the sample[60,61].

In additional to traditional purge and trap, a specialized oxygen bomb can be used to collect volatiles from the headspace over liquid or solid samples. As one example, The Oxipres™ (Mikrolab, Aarhus, Denmark) was designed to monitor oxygen consumption in the headspace of lipid-containing materials, fats or oils is equipped with a quick-snap connector that allows the chamber to be vented through a thermal desorption trap after heating or incubation[62].

Purge and trap/thermal desorption techniques are applicable to both liquids and solids and have been used in agricultural[63,64], environmental[65,66] and pharmaceutical[67,68] analyses. The pairing of these extractions with a gas chromatography-olfactory (GC-O)

system has provided greater insight into the volatile compounds responsible for food odors and aromas, specifically classification of certain compounds into specific notes[69].

2.4 Previous Studies of Methyl Linoleate Oxidation

Fatty acid methyl esters have been the focus of most basic research with lipid oxidation. Methyl linoleate, in particular, has been extensively studied because linoleic acid is the main fatty acid of most food oils and biological materials, and the presence of two double bonds provides the simplest model for investigation of unsaturation and structural effects on oxidation and other properties. Most of our current understanding of lipid oxidation pathways and mechanisms has derived from studies of methyl linoleate oxidation[70–75].

Table 6 presents a summary timeline of important milestones for identifying reactions and products in lipid oxidation, with specific attention given to non-traditional products associated with methyl linoleate. The role of gas chromatography in elucidating lipid oxidation pathways is clear in this table. This is logical since most unoxidized lipids (e.g. fatty acids, acylglycerols, waxes) require derivatization to make the molecules volatile for detection by gas chromatography[76]. In contrast, most oxidation products are volatile up to relatively long chain lengths, and they can be collected without extraction or destruction of the samples. Hence, most of the discussion below will focus on what has been learned about the pathways that generated the volatile products typically associated with lipid oxidation.

Table 6. Timeline of products associated with methyl linoleate oxidation.

Title	Author	Year	Oxidation Procedures	Instrument	Products Found	Results
Displacement Analyis of Lipids IX. Products of the Oxidation of Methyl Linoleate	Khan, Lundberg, Holman	1953	In dark (-10° C), Visible light (18° C), UV light (35°C)	HPLC	No specific product details reported	hydroxyl, conjugated trans-trans diene, and onjugated cis-trans diene all differed depending on oxidation method
1-octen-3-ol and Its Relation to Other Oxidative Products from Esters of Linoleic Acid	Hoffmann	1962	Autoxidation to peroxide values 8 and 80	GLC	1-octen-3-ol , t-2-heptenal,	No t-2-octenal
Saturated Hydrocarbons from Autoxidizing methyl linoleate	Horvat, Lane, Ng, Shepherd	1964	Loboratory light at room temperature monitoring first 24 hours	GLC	butane, pentane, methane, ethane, propane	pentane major component (> 90% of fraction)
Identification of 2,4, 6-Trialkyl-1,3,5-trioxanes from Autoxidized Methyl Linoleate by MS	Horvat, McFadden, Ng, et al.	1965	oxidized on a film on clean glass wool by continous exposure to purified oxygen at room temperature. PV 1000 mmoles/kg	GLC	series of trioxanes generated by condensation of hexanal, pentanal, 1,1-dimethoxy hexane, 2-butyl-2-octenal	Trioxanes are secondary products (but oxidized conditions so harsh, difficult to confirm)
The flavour volatiles of fats and fat-containing foods. II*-A Gas chromatographic investigation of volatile autoxidation products from sunflower oil	Swoboda, Lea	1965	Autoxidation of refined sunflower oil at 37 C	GC	Attributes hexanal to β-scission at C-13 and 2,4-decadienal to α-scission at C-9	Preponderance of hexanal at low temperatures and products attributed to 2,4-decadienals
Edible Oil Quality as measured by Thermal Release of Pentane	Evans, List, Hoffman, Moser	1969	Aged edible oils were oxidized at 60 C in oven	GC	Thermal decombosition of hydroperoxy-9,11-octadecadienoic acid	pentane used as quality measure inverse related to sensory and linear relationship with peroxide values
Precursors of alk-2,4-dienals in autoxidized lard	Kimoto, Gassis	1969	Autoxidized lard on pyrex dishes at 20-40C	LC	Equal amounts of 9/13 isomers	hexanal shown as predominant product from C-13
Analysis of Autoxidized Fats by GC-MS: II Methyl Linoleate	Frankel, Neff, Rohwedder	1977	Autoxidized at 40 °C, 60 °C, 80 °C to various PVs from 93-1403	GC	equal amounts of 9/13 isomers; minor products: ketodienes, epoxyenols, di- and tri-hydroxyesters	Found 13:9 ratio to be ~1, formed in equal amounts at 40 °C, 60 °C, 80 °C. No evidence of C-11
Analysis of Autoxidized Fats by GC-MS: I Methyl Oleate	Frankel, Neff, Rohwedder	1977	Autoxidized at 40 °C, 60 °C, 80 °C to various PVs from 93-1403	GC	8/9/10/11-OH	Found 8/11 OH to be slightly preferred to 9/10 OH. Not all equivalent to equal attack. Greatest difference at 40 °C and smallest at 80 °C
Analysis of Autoxidized Fats by Gas Chromatography-Mass Sectrometry: V. Phhotosensitized Oxidation	Frankel, Neff, Bessler	1979	Photosensitized with methylene blue at 0 C	GC	No specific product details reported	Mixture of 66% (9 & 13) and 34% (10+12)
Lipid Oxidation	Frenkel	1980			No specific product details reported	conjugated 9/13 hydroperoxides

Analysis of Autoxidized Fats by GC-MS: VII Volatile Thermal Decomposition Products of Pure Hydroperoxides from Autoxidzed and Photosensitized Oxidized Methyl Oleate, Linoleate, and Linolenate	Frankel, Neff, Selke	1981	Autoxidation (27° C), PV 2970	GC	pentane (C13/9.9 rel %), hexanal (C12/13, 15), methyl octanoate (C9, 15), 2,4-decadienal (C9, 14), methyl 9-oxononanoate (C9/10, 19)	Same volatiles as photo, different values. Produced more pentane, 2-pentyl furan, 2,4-decadienal
Analysis of Autoxidized Fats by GC-MS: VII Volatile Thermal Decomposition Products of Pure Hydroperoxides from Autoxidzed and Photosensitized Oxidized Methyl Oleate, Linoleate, and Linolenate	Frankel, Neff, Selke	1981	Photoxidation (0° C), PV 1124	GC	pentane (C13/4.3 rel %), hexanal (C12/13, 17), methyl octanoate (C9, 7.6), 2,4-decadienal (C9, 4.3), methyl 9-oxononanoate (C9/10, 22)	Same volatiles as auto, produced more methyl 10-oxo-decenoate, 2-heptenal
Unified mechanism for polyunsaturated fatty acid autoxidation. Competition of peroxy radical hydrogen atom abstraction, β-scission, and cyclization	Porter, Lehman, Weber, and Smith	1981	Linoleic acid cooxidized at 30 C with 9, 10-dihydroanthracene (DHA) and 1,4-cyclohexadiene (CHD)	LC	No specific product details reported	Found hydrogen atom abstraction, β-scission, and cyclization were competitive
Compairons of Gas Chroatographic Methods for Volatile Lipid Oxidation Compounds in Soybean Oil	Synder, Frankel, Selke, Warner	1988	5 minutes at 180 C	GC	Found different major products from direct injection, SHS, and Dynamic HS	Relative percent volatile composition changed according to GC method. Found t-2-heptenal, 1-octene-3-ol attributed to
Photo-Initiated peroxidation of Lipids in Micelles by Azaaromatics	Barclay, Crowe, Edwards	1997	autoxidation carried out at 37° C, photo-initiated (Hg lamp)	GC	conjugated 9/13 hydroperoxides	Found 13:9 ratio to be > 1
Comparing Methylene Blue-Photosensitized Oxidation of Methyl-Conjugated Linoleate and Methyl Linoleate	Jiang, Eldin	1998	photosensitized oxidation at 25° C for 5 days	GC	No specific product details reported, but do suggest using more than PV to measure oxidation	ML yielded higher amounts of peroxides
Comparative Methyl linoleate (LMe) and Methyl Linolenate (LnMe) Oxidation in the Presence of Bovine Serum Albumin at Several Lipid/Protein Ratios	Zamora, Hidalgo	2003	incubated in dark for 6 days at 60° C	HPLC	No specific product details reported	Reaction rates much faster for LnMe than LMe
Analysis of Volatile Compounds from Chlorophyll Photosensitized Linoleic Acid by SPME	Lee, Min	2010	Photoxidation (4 °C), fluorescent lamps	GC	pentane, pentanal, hexanal	With chlorophyll: pentane ↑, hexanal ↓, t-2-heptenal ↑, 1-octen-3-ol ↑, 2-octenal ↑
Dihydroperoxidation facilitates the conversion of lipids to aldehydic prodcuts via alkoxyl radicals	Onyango, Kumura, Tominaga, Baba	2010	Fe mediated decomposition at 37 °C with or withot α-tocopherol	GC	No specific product details reported	Found products, potentially derived from C11 products; products not explained by current lipid aldehyde formation

2.4.1 Hydroperoxides

Studies have shown that hydrogen abstraction preferentially occurs (82% likelihood) from double-allylic sites, with much smaller abstraction from mono-allylic site (16%) and aliphatic sites (2%)[77]. Thus, in methyl linoleate, initial hydrogen abstraction occurs at carbon-11, generating a pentadienyl radical resonance system in which the electron density is localized on C-11, and C-9 and C-13 become electron-deficient[11]. Oxygen then adds preferentially to C-9 and C-13, and the first hydroperoxides are formed at these positions (Figure 11).

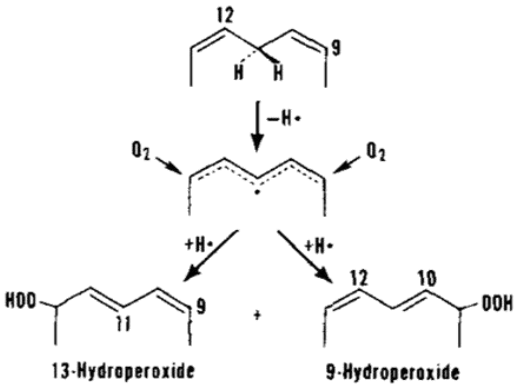


Figure 11. Positions of initial hydroperoxide formation during linoleate oxidation.[12]

Trans, cis and *trans, trans* hydroperoxide isomers are possible at each position, giving four potential structures as precursors for secondary products (Figure 12)[26].

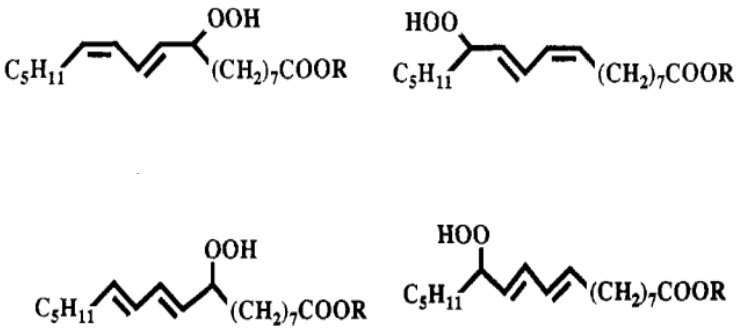


Figure 12. Four hydroperoxides formed from linoleate oxidation [28].

It has been reported, and indeed widely accepted, that equal amounts of 9- and 13-hydroperoxides are generated during autoxidation of methyl linoleate[78]. In contrast, photosensitized oxidation of linoleate, which involves singlet oxygen addition to double bonds, forms approximately 33% of the hydroperoxides at C-10 and C-12[79]. The presence of light and a photosensitizer (e.g. food pigments) thus adds considerable more complexity to the type and distribution of products[79].

Oxidation of other fatty acids has been studied, although less extensively than linoleic acid, most commonly oleic and linolenic. As the number of double bonds increase, oxidation mechanisms become more complex as reactions sites grow. Methyl linolenate (three double bonds) shows a clear preference for hydroperoxides at external positions (75-81% of the hydroperoxides at C-9- and C-16) hydroperoxides with only 18-25% at C-12 and C-13[80]. Arachidonic acid (four double bonds) has three different stabilized pentadienyl systems so the oxidation pattern becomes quite complex[11]. Higher systems such as of fish oils with 20:5 and 22:6 become are even more complicated as reactive sites increase. The increased complexity creates problems with derivation of products, which in turn difficult to understand what reactions are happening in these types of oils and systems.

2.4.2 Secondary Oxidation Products

During the propagation stage of oxidation, hydroperoxides decompose to alkoxyl radicals, which become the precursors for a wide range of scission products observed from methyl linoleate. Following just the traditional pathway, explanations have been proposed for products observed in many studies as arising from scission of carbon bonds on either side of the alkoxyl radical. Major oxidation products from C-13 alkoxyl radicals are pentane (β-scission) and hexanal (α-scission); pentanal and butanal are formed from secondary oxidations or alternate scissions, respectively (Figure 13)[81–83].

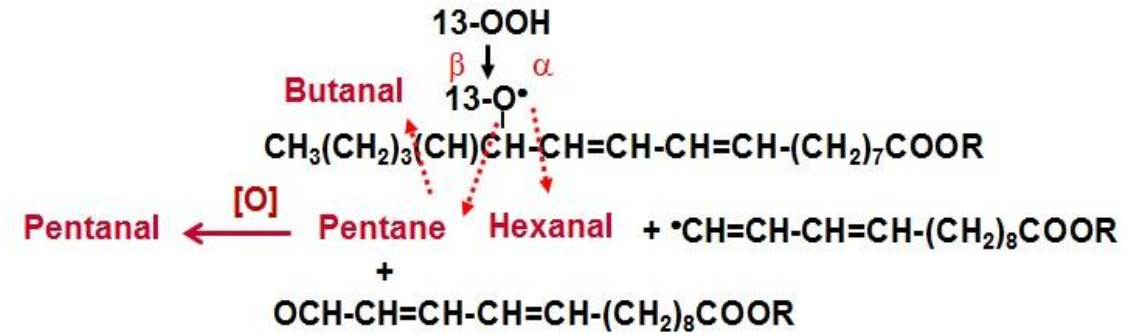


Figure 13. C-13 scission products that have been identified from oxidizing methyl linoleate.

Scission products from alkoxyl radicals at C-9 are *t,t*-2,4-decadienal and methyl octanoate from α-scission, and 9-oxo-methyl nonanoate and a 9-carbon conjugated diene radical from β-scission (Figure 14)[81,82,75].

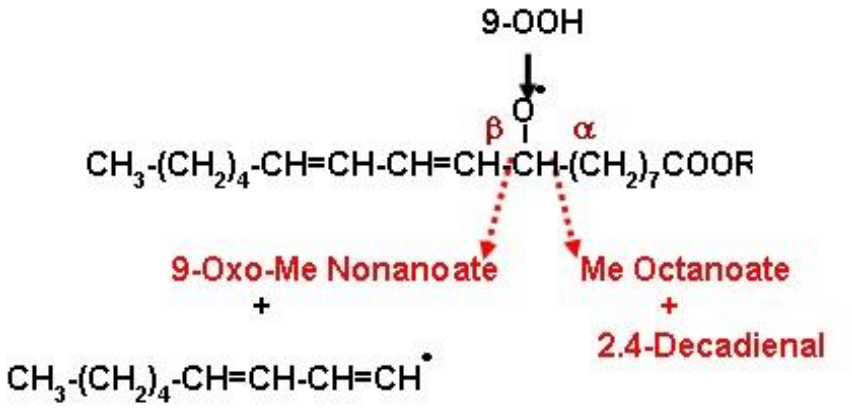


Figure 14. C-9 scission products that have been identified from oxidizing methyl linoleate.

These volatile products from C-9 were determined from GC monitoring of hydroperoxide isomer decomposition[75] as well as methyl linoleate oxidation[79]. Two isomers of 2,4-decadienal accounted for 45% of the total volatile response when soybean oil with a PV of 9.5 was loaded onto glass wool and placed in GC inlet at 180 °C (direct injection)[84]. Most often, analyses showing major peaks arising from C-9 scission are performed at higher temperatures, during extraction or oxidation, or at high excessively high PV when gross oxidation occurs.

As noted above, for many years oxidation was thought to occur almost exclusively (>99%) at the outer carbons of the 1,4-diene system, i.e. C-9 and C-13. However, recent research from Ned Porter's[85–88] laboratory and ours has observed products that arise from C-11 hydroperoxides as well. Scission products expected to arise from this central position in the methyl linoleate chain, *t*-2-heptenal and *t*-2-octenal, are shown in Figure 15[89].

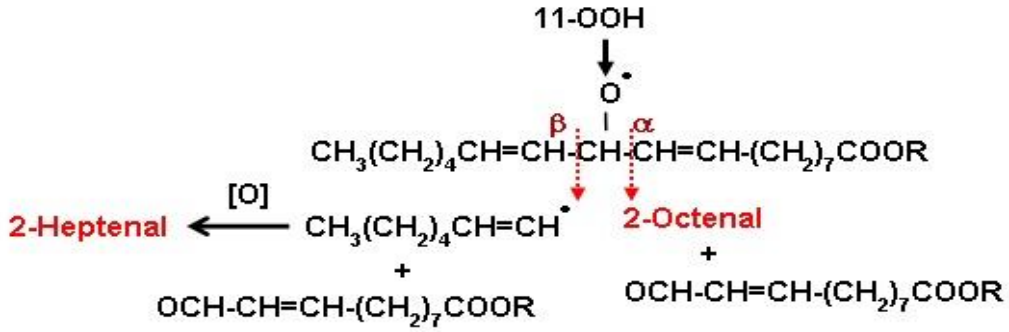


Figure 15. C-11 scission products that have been identified from oxidizing methyl linoleate.

The appearance of 2-heptenal is particularly interesting because it has normally been associated with photosensitization of methyl linoleate[79]. It has been reported in samples containing linoleate[90] or trilinolein[81] oxidized in the dark, but not in decomposition products but when analyzed looking at pure C-9 and 13 hydroperoxides, it does not appear[75]. Thus, they must arise from a secondary process that leads to formation of C-11 radicals but not necessarily involving a hydroperoxide precursor.

Pentane. The 13-OOH of linoleic acid attracted interest as an oxidation product early on when it was observed that its decomposition product pentane was directly related to PV in a variety of food oils, including soybean and cottonseed oils[91]. The plight of pentane as an oxidation product is very interesting. It has long been recognized as a major product, so much so that for a period its quantitation in expired breath was proposed as a measure of in vivo lipid oxidation[92]. Pentane has also been detected and reported in many oxidized oil and food systems[26,75,91,93,94]. That being said, pentane is routinely ignored in considerations of lipid oxidation mechanisms and analyses in oils and foods[95,96]. A conceptual problem is that pentane is odorless and escapes rapidly from packages with

any permeability, so the food industry does not consider it important. A technical problem is that cryotrapping is often needed to detect it by gas chromatography, specifically when using mass spectrometry as the detector. Thus, its full importance in lipid oxidation mechanisms remains undetermined.

Hexanal. Hexanal has been classified as one of the key indicators of lipid oxidation and is often the compound selected when monitoring in foods and other systems. Indeed, lipid oxidation products such as 2-pentyl furan and pentane[82] have been disregarded as not being important to lipid oxidation possibly due to low flavor significance, while hexanal has been given an almost god-like position. Some publications go so far as to measure lipid oxidation in nuts monitoring hexanal as the only volatile product to test for oxidation[43]. However, when analyzing lipid oxidation, considering only specific products drastically underestimates the true extend of oxidation. For examples, foods such as fish muscle showing that compounds such as propanal, 2-ethyl furan, and pentanal all appeared in larger proportions than hexanal[39].

Earlier oxidation assays that tracked production of only conjugated dienes, or for specific products (malonaldyde), or monitored only one regardless of food system, have become obsolete[11]. This dissertation research focused on tracking all volatiles in order to get rid of this "black box" way of thinking and provide concrete details to reveal the full complexity of lipid oxidation. As more sophisticated sample technique methods when monitoring lipid oxidation it has become clear that when investigating the same system, different volatile collection techniques will show different extents of oxidation. For example, in comparisons on solvent assisted flavor evaporation (SAFE), SPME, and thermal desorption, SAFE extraction appeared to be the most preferred method for

studying pea flour oxidation[97] because it collected the most volatiles. At the same time, SAFE results must be interpreted critically because high temperatures and long desorption times that potentially modify products are involved.

In general monitoring of lipid oxidation, most previous studies have focused on principal products such as pentanal, hexanal, amyl formate, methyl octanoate, and substituted dioxolanes while ignoring others, so there is relatively little information regarding the prevalence of products past the ones listed here.

Decadienal. Decadienal also has been used as an indicator for measuring oxidation as it favored during oxidation of methyl linoleate at high temperatures[81]. It is not normally a product associated with oleate or linolenate oxidation[89]. The largest issue with using decadienal as an indicator for oxidation is its volatility and instability (it decomposes to other products such as hexanal or *t*-2-octenal). Decadienal is a critical oxidation compound due to its low odor threshold[98], but does not appear to form greatly at low temperature[81].

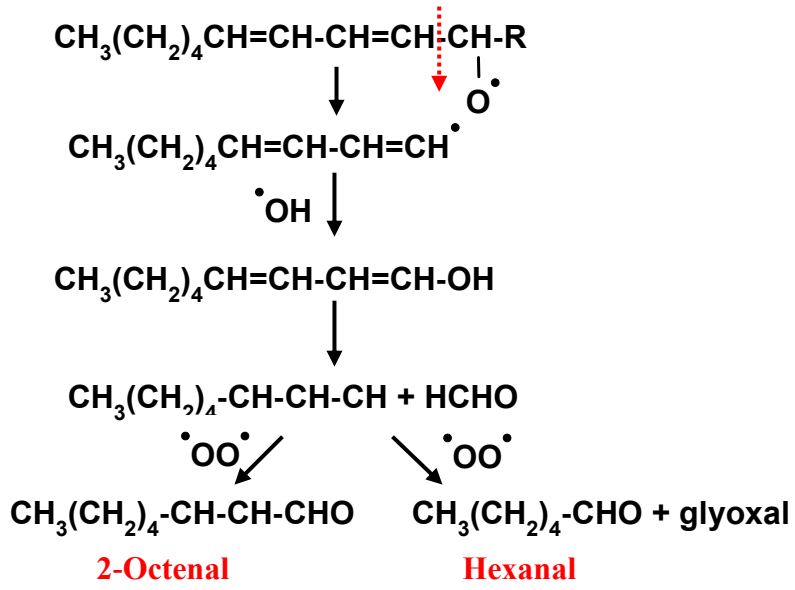


Figure 16. Formation of additional products through decadienal oxidation.[81]

Octanal. Octanal is a major product of oleic acid oxidation[12], specifically from the decomposition of the C-11 hydroperoxide[81,89]. It cannot be formed from linoleate or linolenate[81] due to the presence of additional double bonds, and less is produced during photosensitization than autoxidation of oleic acid because of the shift in dominant hydroperoxide as well as among the dominant in other studies[89]. Octanal is not an ideal measure of oxidation as it only can occur from systems containing oleic acid. Nevertheless, its presence is for smell, it is the most odor active compound in fresh oranges[47].

Other products (especially *t*-2-heptenal and *t*-2octenal). *t*-2-Heptenal was traditionally associated with photosensitization[22,79]. *t*-2-octenal was originally associated with formation of the C-11 hydroperoxide of methyl linoleate[89] but has more recently been linked to secondary oxidation of 2,4-decadienal[81,90].

Both products have been detected as major products from oxidation of methyl linoleate[12] and milk fat[99], and have been detected at levels higher than decadienal but still lower than hexanal in many other systems. Neither of these products have previously been considered important to monitor in lipid oxidation, for multiple reasons. Nevertheless, results of the present study will demonstrate that these two products are markers of a specific alternate reaction.

2.4.3 Headspace Analysis to Derive Oxidation Mechanisms

Most of the breakdown pathways described above were proposed based on products detected by gas chromatography, specifically headspace analysis. Frankel's research on fatty acid systems of different complexity[78,74,100] were particularly

instrumental in suggesting the origin of detected products. Photosensitization studies revealed differences in attack sites on the acyl chain (external for autoxidation versus internal for singlet oxygen reactions) and the importance of protecting samples from light[79]. As more system factors were investigated, the ability to monitor changes in product distribution under different conditions of environment and oil storage[101] as well as to identify volatile oxidation products that contributed to off-odors and flavors[102] became increasingly important.

Since equilibrium is being established between both the headspace and the sample, only low quantities of compounds are actually recovered. When soybean oil was oxidized to a PV of 1.7, major products appeared to be pentane and hexanal. However, as PV increased from 4.9 to 9.4, pentane peak area remained constant and despite the lack of change, the increased total peak area of longer chain products such as 2,4-decadienal was very minor (1.2% to 1.7%)[84]. This suggests that there is an upper maximum limit to how much pentane was being generated as despite doubling in PV value, and even at a PV of 9.4 hexanal and pentane dominated the overall volatile response (38.2% combined). So in general, it is the smaller compounds that are mainly seen in SHS, looking at generally compounds > 7 carbons or very volatile compounds that are sometimes lost during the extraction process[103].

2.4.4 Use of Solid Phase Micro Extraction (SPME) with Lipid Oxidation

The signature ability of SPME to increase sensitivity of volatile collection gives it some distinct advantages over SHS and has led to rapid increase in popularity and applications of SPME for lipid oxidation. At the same time, by detecting more products,

the increased sensitivity of SPME also shown supported proposals that lipid oxidation is much more complex than the simple free radical chain reaction suggests. For example, research with conventional and high-oleic sunflower oil detected 74 total volatile lipid oxidation compounds after only 14 days at 80 °C[93]. When monitoring the difference between the two types of sunflower oils, SPME provided a better assessment of overall lipid quality, because oils with different oxidative properties were not distinguishable by merely comparing one compound.

Another study monitored the oxidation of olive oil, oxidizing 20 grams in a 40 mL amber vial with a 60 minute equilibration time at 25 °C followed by extraction at 25 °C for 90 minutes, followed by a 4 minute desorption time[64]. This extraction time was chosen as volatile response continued until 90 minutes but plateaued thereafter. The study also looked at four different types of fibers: PDMS, CAR/PDMS, CW/DVB, and DVB/CAR/PDMS and results clearly showed the best results came from the DVB/CAR/PDMS fiber. Overall 34 compounds were extracted with the fiber, less than thermal desorption (60). Most of the products detected were aldehydes (52.9-63.1% across the four olive oils), including but-2-enal, 2-methyl butanal, hexanal, and 2-hexenal. Alcohols (10.8-22.2% of products) consisted of penten-3-ol, pent-2-enol, and hex-3-enal or hex-2-enal[64]. Alcohols, while mechanistically associated with lipid oxidation, are not traditionally analyzed chemically or detected in volatiles analyses. Thus, the ability of SPME to extract semi-volatile compounds such as alcohols, even if only median carbon length, could contribute significantly to elucidation of mixed pathways in lipid oxidation.

2.4.5 Use of Thermal Desorption (TD) with Lipid Oxidation

Purge and trap with thermal desorption studies have been conducted on lipid oxidation in a number of different meat and beef systems[98,104], soybean[36] and olive oils[64]; volatile release from vinegars[63], as well as packaging[105]; antioxidant effectiveness using a wide variety of sample preparation techniques ranging from steam distillation to simply purging heated solid samples at a wide range of temperatures[36,63,64]. Traditional products attributed to lipid oxidation such as the series of normal aldehydes (C5-9) have all been tracked using thermal desorption[106].

In the study cited in the SHS section 2.4.3 above, dynamic headspace was utilized to test the same soybean oil at PV of 1.7, 4.9, and 9.5 oxidized by heating to 180 C and purging onto Tenax traps. Thermal desorption detected a drastic increase in decadienal, in contrast to the low levels detected by SHS. At the PV of 9.5 both isomers of decadienal increased to 51.6% in thermal desorption analyses, while direct injection only showed 45.8%[36]. Compared to SHS at the same PV, hexanal and pentane decreased while compounds > C7 began to dominate the total volatile response. This strikingly demonstrates the marked differences in overall picture of oxidation that can be generated by different GC sampling procedures and points out the critical need to use multiple sampling methods when conducting analyses of volatiles to determine reaction mechanisms.

Thermal desorption analyses of beef and poultry at 110 °C reported a homologous series of aldehydes including pentanal, hexanal, heptanal, octanal, nonanal, and decanal. The concentration of hexanal appeared to increase more rapidly than the other aldehydes,

so hexanal became the recommended product to monitor for lipid oxidation[107] and this practice has been widely adopted[108].

In the olive oil study cited in the SPME section 2.4.4, four different olive oils were loaded onto glass wool in empty desorption tubes and desorption occurred from 20 to 80 °C at 60 °C/min with a 20 minute upper temperature hold. Results showed that while over 60 compounds were extracted (more than the 34 detected by SPME), semivolatiles were preferentially extracted and the main compounds identified were several sesquiterpenes such as farnescene and α-copane. These products would not be detected in SHS or SPME headspace extraction techniques[64].

Overall, these stark differences show that method and sample preparation drastically affect products and product distribution detected from SHS, SPME, and thermal desorption.

2.5 Pitfalls of Previous Research

The extensive literature that has led to the traditional understanding of lipid oxidation mechanisms has several critical limitations: 1) most analyses were conducted at very high levels of oxidation achieved under extreme conditions to enhance detection, 2) few products were accounted for, 3) reaction sequences were derived primarily from analysis of volatile decomposition products without corresponding analyses of non-volatile products, and 4) instrumentation at the time lacked the sensitivity and resolution of contemporary instrumentation.

As an example, in one study[109], methyl linoleate oil was held at room temperature while purified oxygen was passed through at a rate of 10 mL/min for eighteen days,

resulting in a peroxide value (PV) of 1000 mequiv/kg with volatiles being collected via a U-tube at -78 °C; approximately 0.5 g of condensate was isolated, most of which was water. This very high level of oxidation was studied despite the fact that industry professionals consider a PV of 4 already of questionable acceptability. Another study oxidized sunflower oil by oxygen bubbling for 3 days to generate a PV of 110 meq/kg. The high oxygen atmospheres and the extensive oxidation distort the distribution of volatile products that are generated and detected, provide no information on initiation or which non-LOOH products could possibly be early indicators of oxidation, and confound any judgments about oxidation processes.

Little prior research on methyl linoleate oxidation examined early stages of oxidation. Published research does not offer any insights regarding products formed in early oxidation stages, nor does it fully explain reaction pathways for additional products such as the minor compounds discussed above. Furthermore, most research focused on oxidation pathways was completed decades ago using instrumentation that lacked the sensitivity and resolution of today's instrumentation, particularly with advances in mass spectrometry.

Advanced instrumentation and methodology are now showing additional complexity in oxidation reactions. Methyl linoleate undergoing photosensitized oxidation at -60 °C gave the four expected monohydroperoxides at carbons 9,10,12, and 13 but also showed small percentages of three dihydroperoxides that add complexity to product pathways[110]. There is also some evidence for hydroperoxides at 8- and 14-, although in very low percentages[111]. Several saturated hydrocarbons -- methane, ethane, propane, butane, and pentane – have all been detected at substantial concentrations in headspace

analysis of autoxidized methyl linoleate[94]. Most importantly, these small hydrocarbons appeared early in the oxidation when ML had been exposed to air for less than 24 hours. Such observations are important in elucidating initiation processes and suggest that there may be a sequence of degradation along the acyl chain.

Finally, epoxides have frequently been reported as trace components in mixtures of products, but they have almost never been measured separately as a class of oxidation products along with hydroperoxides and aldehydes. Epoxides are difficult to analyze due to their reactivity, difficult to monitor and identify due to lack of sensitive detection methods, and even more difficult to get mass/molecular weight information, e.g. by mass spectrometry. However, there is new data supporting epoxides as major lipid oxidation products. After 6 hours of catalytic epoxidation, methyl linoleate yielded an approximately 50/50 split of a monoepoxide vs diepoxide; C-9 and C-12 monoepoxides were both present, but differentiating them structurally was difficult[112]. This observation suggests that epoxide formation during autoxidation is kinetically feasible. However, in model systems of methyl linoleate oxidized in aprotic solvents, epoxides were the ONLY product formed[113]. This raises questions about previous studies of oil oxidation where little oxidation was detected by peroxide values and aldehydes – was oxidation being completely missed? Secondary decompositions of epoxides have been largely unexplored, but presence of epoxides would definitely confound expected secondary reactions. The potential role of epoxides and their decomposition in generating volatile oxidation products needs to be investigated.

3. HYPOTHESIS AND SPECIFIC OBJECTIVES

3.1 Goals

This research is part of a larger project aimed at verifying or refuting the existence of multiple alternate pathways that compete with hydrogen abstraction to modify kinetics and product distributions during lipid oxidation. One component of the project is investigating non-volatile products (Jia Xie)[114]. This dissertation research focuses on what can be learned about reaction pathways by analyzing volatile products. Since volatiles detected are method specific, this research has two foci: 1) evaluation and comparison of sensitivity and accuracy of static headspace, solid phase microextraction, and thermal desorption methods for analyzing lipid oxidation products, and 2) verification or refutation of the presence of alternate oxidation pathways from the products detected. Where possible, information about volatile products will also be integrated with that from non-volatile products to derive breakdown sequences.

3.2 Basic Assumption

Lipid oxidation proceeds by a complex reaction system which includes not only traditional free radical chain reactions propagated by hydrogen abstraction but also internal rearrangements, double bond additions, scissions, and other reactions of LOO• and LO• that generate complex product mixtures even in parallel with hydroperoxides. Balance between the alternate pathways shifts with reaction conditions.

3.3 Experimental Hypothesis

Analyzing volatile compounds of lipid oxidation by gas chromatography can provide information to support or refute existence of multiple reaction pathways.

3.4 Specific Objectives

1. Incubate methyl linoleate (ML) under different conditions of temperature, oxygen, solvent, and concentration for 20 days to focus on early oxidation processes.
2. Monitor oxidation products daily using static headspace, SPME, and thermal desorption methods to compare sensitivities and product distributions detected by each method.
3. Identify oxidation products by comparison of chromatographic behavior with that of authentic standards and by mass spectrometric analysis.
4. Analyze data from each method to determine dominant reaction products, kinetics of development of different products, changes in product distribution with reaction and conditions and analysis method, and evidence supporting or refuting an integrated lipid oxidation scheme.
5. Integrate data with results for non-volatile products to compile most likely reaction pathways.

3.5 Significance of the Study

Previous studies investigating lipid oxidation have measured only limited products (e.g. conjugated dienes, hydroperoxides, and maybe also hexanal) and usually only non-volatile or volatile. To our knowledge, the larger project of which this

dissertation research is a component is the first study to attempt much more extensive product analyses and integration of information about non-volatile and volatile products from identical samples incubated in parallel. This approach is designed to track oxidation more completely in order to compile a more accurate picture of lipid degradation pathways and to document (or refute) the existence of multiple competing oxidation pathways.

Why is this important beyond theoretical or academic interest? The food industry and academic research have both selected oxidation analyses assuming the single pathway described in the traditional chain reaction. Results of this overall project initially will show how we need to rethink approaches to analyzing lipid oxidation. This includes re-evaluating methods that have been used blindly for decades and measuring more products in detail as well as in classes. Over the long term, what we learn from these integrated studies of alternate pathways may lead to a broader and more accurate paradigm of lipid oxidation that will facilitate development of more effective stabilization strategies and improved understanding of lipid oxidation chemistry in complex systems.

4. MATERIALS AND METHODS

4.1 Materials

High purity methyl linoleate (Nu-Chek-Prep Inc, Elysian, MN), the methyl ester of linoleic acid, was used as a model system.

Product standards were purchased from Sigma-Aldrich, Inc. (St. Louis, MO): 1-pentene, t-2-nonenal, pentane, butanal, 1-pentene-3-ol, 1-pentanol, 1-octene, hexanal, octane, t-2-heptenal, heptanal, 1-octene-3-ol, octanal, 2-nonanol, 2-nonanal, 1-nonanol, t,t-2,4 nonadienal, t-2-decenal, t,t-2,4 decadienal. Hexane, acetonitrile, ethanol, and chloroform solvents were also purchased from Sigma-Aldrich Co. (St. Louis, MO).

Two fibers were tested for SPME analyses: a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) Stableflex (2 cm) 23 gauge or 65um polydimethylsiloxane (PDMS)-divinylbenzene (DVB), both purchased from Supelco (Bellefonte, PA).

A TD-1 short path thermal desorption unit (Scientific Instrument Services, Ringoes, NJ) was utilized for thermal desorption analyses. Desorption traps were packed Tenax™ TA 60/80 mesh (50 mg):Carboxen™ 20/45 mesh (50 mg) = 1:1 (Scientific Instrument Services, Ringoes, NJ). Tenax was obtained from Scientific Instrument Services (Ringoes, NJ); carboxen™ was purchased from Supelco (Bellefonte, PA). Internal standards for TD were benzene-d6 and toluene-d8 (Sigma-Aldrich, St. Louis, MO) plus naphthalene-d8 (Isotec, Miamisburg, Ohio). Diafil 525 was obtained as a product sample from IMERYS Performance Minerals (Roswell, GA). During thermal desorption samples were incubated in either a Thermal Desorption Conditioning System

(Scientific Instrument Services, Ringoes, NJ) or Oxipres™ oxygen bombs (Mikrolab, Aarhus, Denmark).

4.2 Experimental Design

Methyl linoleate (ML) was selected as the model system to simplify product distributions. ML is the logical candidate because its fatty acid base, linoleic acid, is the most prevalent polyunsaturated fatty acid in foods and biological tissues, the ester form is volatile, and this is the lipid most commonly used for mechanism studies so there is an extensive data history available for comparison and for integration with new results.

Figure 17 shows the overall experimental flow diagram with incubation conditions and analyses for each GC method. The actual sequence in which the experiments were developed was 1) investigation of temperature effects on fundamental early oxidation processes using static headspace; 2) investigation of condition effects on oxidation product intensity and distribution using static headspace; 3) repetition of experiments using SPME and TD to determine sensitivity and patterns of information provided by each method.

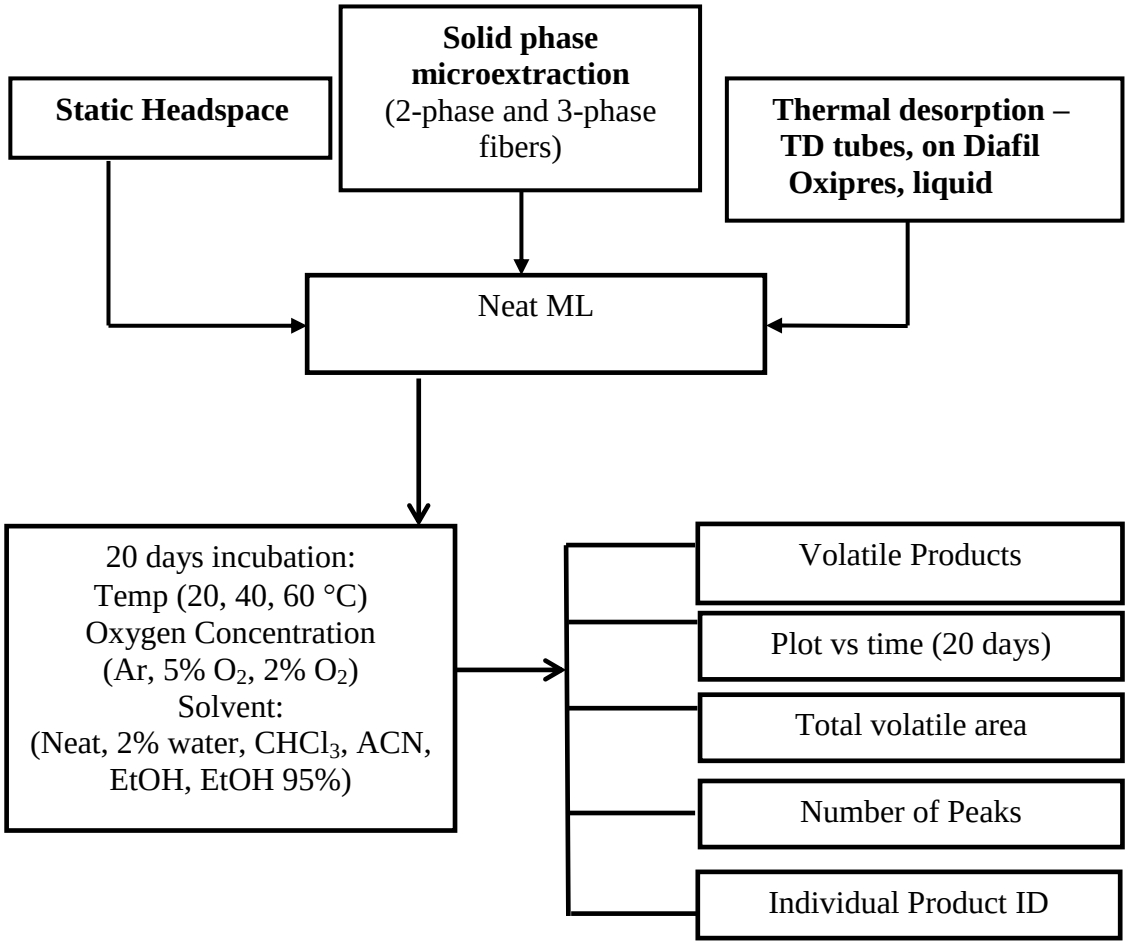


Figure 17. Experimental design: overall flow chart for conditions of incubations and types of analyses in this study.

4.3 Experimental Procedures

4.3.1 Lipid Handling

Methyl linoleate (ML) was provided in vacuum sealed vials. After the seal was broken to remove ML samples, the remaining oil in the vial was flushed with argon and the vial was resealed under vacuum, then stored frozen at -80 °C. Once ML was used for analyses, sampling vials were covered in aluminum foil to prevent exposure to light. Since this study was focused on the volatile portion, samples were left in incubators until prior to each run to prevent condensation of volatiles.

4.3.2 Glassware Cleaning

All glassware used in this study was cleaned using a three stage process to remove trace contaminants that might catalyze lipid oxidation or degrade products: 1) initial wash in hot tap water with Citranox® acid cleaner and detergent (Alconox, White Plains, NY), rinsed 3 times with tap water, rinsed 3 times with water purified to 18 MΩ resistivity in a Milli-Q™ water purification system equipped with Organex and double ion exchange columns (Model 13681, Millipore; Bedford MA), drain; 2) overnight soak in S-L-X denatured alcohol (W.M. Barr. Co., Memphis, TN) saturated with potassium hydroxide (Sigma-Aldrich, St. Louis, MO) to saponify any traces of lipids, rewash with Citranox as in Step 1); and 3), overnight soak in 1 N HCl (Ultrex, J.T. Baker, Phillipsburg, NJ) to remove metal impurities, rinse in deionized water, and dry in hot oven.

4.3.3 Measuring Volatiles Produced During Oxidation of Neat Methyl Linoleate at Different Temperatures by Static Headspace Analysis

The first experiment of the study diagrammed in Figure 17 was oxidation of neat methyl linoleate at three temperatures 20 (room temperature), 40 (body temperature and elevated storage temperature), and 60 °C (heat stress, accelerated shelf life). These experiments established the baseline of oxidation and provided an initial assessment of whether products matched those expected from previously-published lipid studies. In addition, product distributions were used to indicate whether alternate reactions could be competing with hydrogen abstraction to generate products not expected from proscribed breakdown pathways.

Lipid oxidation. For oxidation, 0.50 g samples of methyl linoleate were transferred to 10 mL glass headspace vials (Sun Sri (Rockwood, TN) and sealed with PTFE/butyl crimp top lids (Perkin Elmer, Melville, NY). All samples in vials were immediately wrapped with aluminum foil to protect them from light, then oxidized in an incubator (Jeio Tech, IS-971 and IS-971R, Seoul, Korea) at 25, 40, and 60 °C with 100 rpm shaking for up to 20 days. Triplicate samples were prepared for each experiment.

The optimum size of headspace vial was determined from preliminary experiments. Studies were completed with 0.25 g, 0.50 g, 0.75 g and 1 g ML in a series of 2 mL, 4 mL, and 10 mL vials with screw caps, screw caps with septa, and crimped cap lids with septa. In both the 2 mL and 4 mL vials, oxygen became limiting with all weights greater than 0.25 g ML. However, less than 0.50 g ML did not provide a large enough signal response in any vial size. Thus, to conserve oil and prevent oxygen

limitation while providing adequate volatile response, incubations were conducted with 0.50 g ML in 10 mL glass headspace crimp top vials.

Static headspace (SHS) analysis. Three sample vials were removed from incubators daily for volatiles analysis. One mL of headspace was extracted from each vial using a 1 mL gas-tight syringe (Pressure-Lok® 1 mL Series A-2 Syringe, Supelco, Bellefonte, PA), then injected into the GC. The removed headspace was replaced with 1 mL fresh air to maintain constant headspace volume and avoid oxygen depletion, and samples were then returned to incubation. The same three vials were analyzed each day during incubation to limit variation. Lids were replaced every three days to maintain sealing.

Headspace analyses were performed on a Hewlett Packard 6890 gas chromatograph equipped with an FID detector, split/splitless injector, and an Equity-5 fused capillary column (60 m x 32 mm x 1.0 µm film thickness, Supelco, Bellefonte, PA, USA). Helium carrier gas was passed through the column at 1.3 mL/minute under constant flow mode. The temperature program started with an initial oven temperature of 40 °C followed by 5 °C/min increase to 200 °C, then 10 °C/min increase to 315 °C, for a total run time of 43.50 minutes. The FID temperature was 300 °C and inlet temperature is 250 °C. Purge time is 0.30 minutes.

To analyze peaks, the Auto Integrate feature in Chem Station was used with the following integration events: slope sensitivity (221.93), peak width (0.118), area reject (1.7424), height reject (0.123), and shoulders (OFF). Peaks with an area of less than 1 were not counted or monitored.

Products were tentatively identified by matching retention times to those of authentic standards and verified by GC-MS analysis of parallel samples on a Hewlett Packard 6890 gas chromatograph equipped with an 5973 mass selective detector, split/splitless injector, and a DB-1701 fused capillary column (30 m x 25 mm x 0.25 µm film thickness, Supelco, Bellefonte, PA, USA). Helium carrier gas was passed through the column at 1.0 mL/minute under constant flow mode. The temperature program started with an initial oven temperature of 40 °C followed by 2 °C/min increase to 100 °C, then 15 °C/min increase to 315 °C, for a total run time of 44.33 minutes. The source temperature was 280 °C and inlet temperature was 250 °C. Purge time for static headspace samples was 0.30 minutes and 2.00 minutes for SPME samples. Solvent delay was 2.00 minutes and scans were made from 50 m/z to 350 m/z. Peaks were tentatively identified using the National Institute of Standards and Technology Mass Spectral Search Program Version 2.0f, 2008.

4.3.4 Influence of System Conditions on Competition Between Hydrogen Abstraction and Alternate Pathways (Product Distributions)

The next step of the experimental protocol was investigation of the effects of the environment or system factors on volatile oxidation product distributions in methyl linoleate. During this stage, oxygen concentration and varying solvents were varied to determine changes in types and levels of products or product distribution as a function of oxidation conditions. Volatile products were monitored daily by static headspace analysis as described in the previous section. Samples were analyzed in triplicate.

4.3.4.1 Modification of Methyl Linoleate with Water

Methyl linoleate (0.48 g) was weighed into 10 ml headspace vials, 200 µL water were added and samples were vortexed for 2 minutes (Day 0), wrapped in foil, then incubated at 25, 40, and 60 °C samples with 100 rpm shaking for up to 20 days. Headspace samples (1 ml) were removed daily for GC analysis as described in Section 4.3.3 Static Headspace Analysis. Triplicate samples were prepared for each experiment.

4.3.4.2 Methyl Linoleate Oxidation in Different Solvents

Methyl linoleate (0.50 g) was weighed into 10 ml headspace vials, 500 µL test solvents (100% ethanol (EtOH), 85% ethanol (85% EtOH), acetonitrile (ACN) and chloroform ($CHCl_3$)) were added, samples were vortexed for 2 minutes (Day 0), wrapped in foil, then incubated at 25, 40, and 60 °C samples with 100 rpm shaking for up to 20 days. Headspace samples (1 ml) were removed daily for GC analysis as described in Section 4.3.3. Static Headspace Analysis. Triplicate samples were prepared for each experiment.

With the focus of this work being on volatile products, the addition of solvents (all highly volatile small molecules) required some modification of the basic procedures used with neat methyl linoleate. Despite slightly changing the overall headspace volume by having both 0.50 g of methyl linoleate and 0.5 mL of solvent, it was deemed important to ensure changes in oxidation were due to effects from solvent, not from lower total amount of methyl linoleate. In addition, there was slight separation between methyl linoleate and solvent over over the course of 20 days, however solvents were not vortexed after day 0 as it would have drastically increased solvent volatility and altered headspace equilibrium. Since samples were shaking during incubation, the slight phase

separation was believed to not be an overpowering factor limiting oxidation, although it may have reduced solvent effects on pathways.

Initial experiments showed that solvent volatility caused problems with headspace analysis by both SHS and SPME. Levels of detected products were much lower than levels seen with neat ML so data analysis focused on product distribution patterns rather than total volatile production.

4.3.4.3 Methyl Linoleate Oxidation Under Different Oxygen Pressures

Methyl linoleate (0.50 g) was weighed into 10 mL glass headspace vials, and before vials were sealed, they were sparged for 2 minutes with modified gases 2% oxygen (O_2), 5% oxygen (O_2) and argon (Ar) for comparison with air in the base experiments. Samples were wrapped in foil to protect them from light, then incubated at 25, 40, and 60 °C samples with 100 rpm shaking for up to 20 days. Triplicate samples were prepared for each experiment.

Headspace samples (1 ml) were removed daily for GC analysis as described in Section 4.3.3 Static Headspace Analysis. This headspace was replaced with 1 mL of the respective gas for each sample, transferred from a separate sparged flask that was filled with gas for 5 minutes. To limit variation, the same three vials were analyzed each day during incubation. Every three days, lids were replaced to maintain sealing, and samples were sparged with the test gas again for two minutes to ensure that environment inside sample was not contaminated from normal air (~20%).

4.3.5 Effects of Sample Collection Analytical Technique on Product Intensity and Distribution Detected (Comparison of SHS, SPME, TD)

The final step of this work was to investigate the differences in the product picture generated by the three GC sampling methods – SHS, SPME, and TD. We were particularly interested in determining how different sample collection techniques can change the apparent oxidation intensity or product distribution. To accomplish this, all experiments performed with static headspace analysis (Sections 4.3.3 and 4.3.4) were repeated with SPME and thermal desorption detection.

4.3.5.1 Solid Phase Microextraction

Samples were monitored using both two-phase and three-phase fibers for comparison. The two-phase fiber was a 65 μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) 23 gauge fiber (Supelco, Bellefonte, PA) and the three-phase fiber was a 50/30 μm divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) Stableflex (2 cm) 23 gauge fiber (Supelco, Bellefonte, PA). Samples were prepared in triplicate 10 ml headspace vials and incubated as described in Section 4.3.3. Each day three sample vials were removed from incubators and placed in a water bath at 40 ºC. A SPME fiber is inserted into the headspace of each vial for 10 minutes then allowed to desorb into the GC inlet for 15 minutes at 250 ºC. After desorption, the fiber was transferred to a separate inlet at 250 ºC for an additional 15 minutes of desorbing to ensure complete cleaning before analyzing the next sample. The same three vials were analyzed each day during incubation to limit variation and maintain continuity in reactions. Lids were replaced every three days to maintain sealing. SPME analysis was performed on the

same HP 6890 gas chromatograph under conditions described above for SHS except the purge time was 2.00 minutes.

4.3.5.2 Thermal Desorption Analyses

For thermal desorption, samples were monitored using both the SIS thermal collection system and OxiPres™ oxygen bomb. Products were monitored every 5 days for the SIS system and daily for the OxiPres™.

4.3.5.2.1 Purge and Trap Collection of Volatiles (SIS System)

Three samples were prepared and incubated on a Thermal Desorption Conditioning System (Scientific Instrument Services, Ringoes, NJ) (Figure 18)[115]. Methyl linoleate (0.50 grams) was mixed with 0.50 grams of Diafil (IMERYS, San Jose, CA) and loaded into a 14 inch glass thermal desorption tube (0.5 inch O.D by 0.36 inch I.D) which was then plugged with 0.5 grams of glass wool at both ends. Tubes were incubated at 40 ºC in a sample collection oven, and volatiles were collected every 5 days. The sampling timing here was dictated by availability of dry ice for cryotrapping. The inlet fitting was connected to a gas supply (air) which maintained flow at constant rate of 40 to 50 ml/min; the exit fitting was connected to a thermal desorption trap packed with Tenax™ (50mg):Carboxen™ (50mg) = 1:1 (Scientific Instrument Services, Ringoes, NJ). To collect volatiles, air was purged over the sample for 10 minutes to move volatiles out of the incubation tube and into the trap.

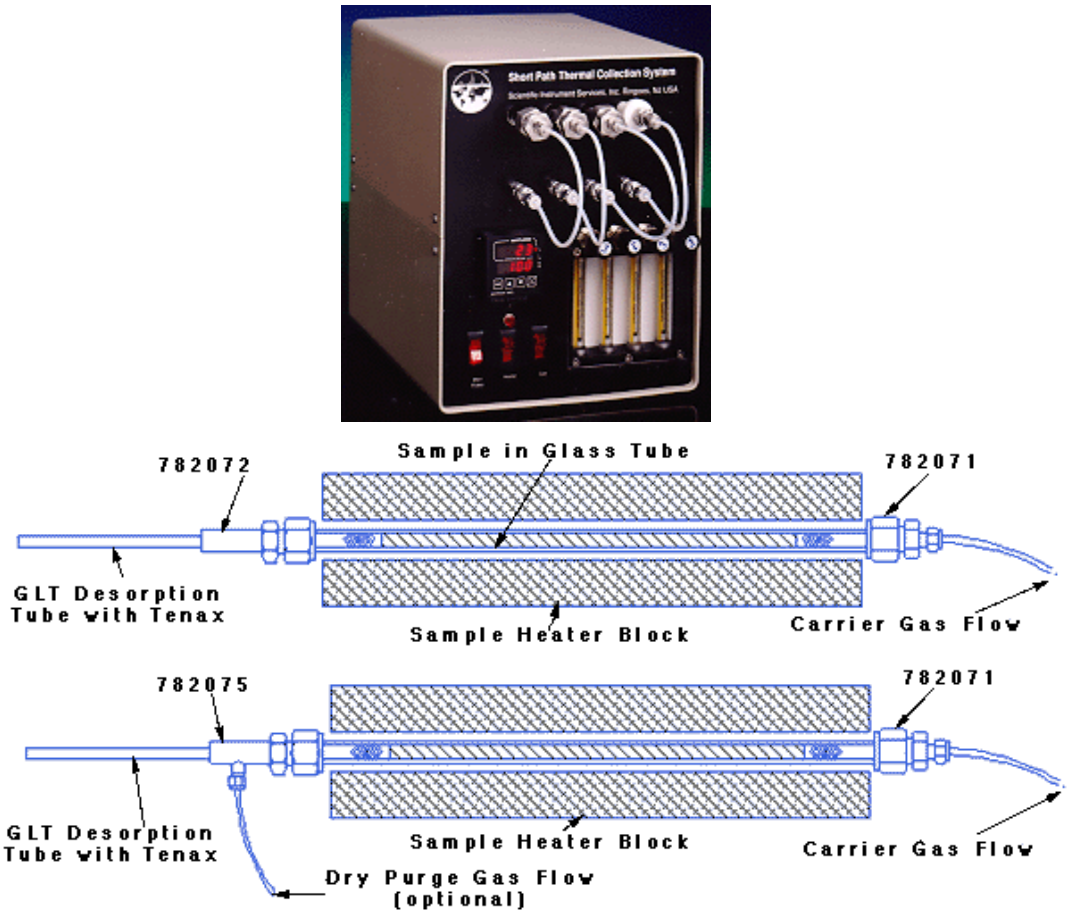


Figure 18. Thermal Collection System set-up and interior view including sample.[115]

Before desorption, an internal standard cocktail consisting of 1 µL each of 10 mg/mL benzene-d6, toluene-d8, naphthalene-d8 in methanol (Sigma-Aldrich, St. Louis, MO) was added to the trap using air as the carrier gas with a flow rate of 10 mL/minute for 2 minutes. This made it possible to report products more accurately by weight (ug/trap) rather than by peak area. Traps were connected to the desorption tube injection head to add the internal standard, before being added to the TD-1 unit.

4.3.5.2.2 Oxygen Bomb Generation of Volatiles

To eliminate possible effects of the solid support on lipid oxidation pathways and products, 0.50 grams of methyl linoleate were placed and weighed into an Oxipres™ flask and transferred to the Oxipres™ pressure cylinder, and covered with a loosely-fitting glass lid to prevent potential contaminants from falling into the oil. A lip under the cap provided open access to the headspace. The cylinder top was screwed onto the cylinder and hand tightened, then the cylinder was positioned in the heating block controlled electronically to within 0.1 °C of either 40 or 60 °C (Figure 19)[116]. The cylinder was pressurized to 1.00 Bar with air or gas mixture, and the inlet was sealed. Volatiles were vented daily onto Tenax™/Carboxen traps through a special quick-connect connection. Cells were then re-pressurized to 1.00 Bar and the incubation was continued. Oxipres™ units have capabilities to monitor oxygen consumption but that feature was not utilized during this study.

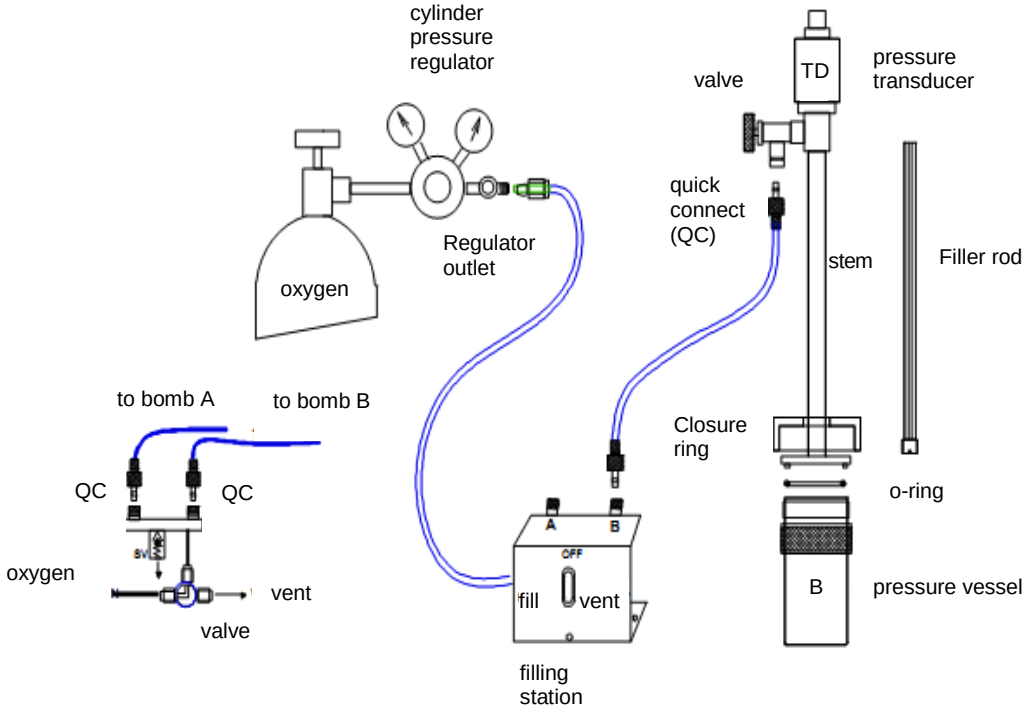


Figure 19. Scheme for pressurizing Oxipres™ pressure vessels.[116]

4.3.5.2.3 Gas Chromatography

Thermal desorption was performed on a Hewlett Packard 5890 series gas chromatograph which had the ability to manually cryofocus injections using dry ice without the necessity of an added cryovalve feature. Cryofocusing was included specifically to verify the presence of short chain products with elution close to pentane, as detected by SHS, and to prevent co-elution. The GC was equipped with an FID detector, split/splitless injector and an Equity-5 fused capillary column (60 m x 32 mm x 1.0 µm film thickness, Supelco, Bellefonte, PA, USA). Helium carrier gas was passed through the column at 1.0 mL/minute under constant pressure mode for both desorption and chromatography. For desorption, a TD-1 short path thermal desorption unit (Scientific Instrument Services, Ringoes, NJ) was connected to both air and helium, and temperature for the heating block was set at 250 ºC. Loaded thermal desorption traps were connected to the TD-1 via a 35 mm seal-autodesorb needle, and the side with the serial number was capped. The thermal desorption controller was set to 10 seconds to allow carrier gas (helium) to flush the needle before injection. The needle was injected through the injection port for 30 seconds to re-equilibrate the inlet pressure. The heating block was then closed and the trap was desorbed onto the column for 5 minutes.

For chromatography, the initial GC temperature was set to -20 ºC with dry ice for 5 minutes to ensure resolution of small volatiles; the temperature was then increased to 280 ºC at a rate of 10 °C/min. The FID temperature was 300 °C and inlet temperature was 250 °C. Purge time was five minutes.

4.3.5.3 Mass Spectrometry (MS) Identification of Products

Triplicate samples for SHS and SPME (different from FID samples) were also analyzed by GC-MS for peak identification on a Hewlett Packard 6890 gas chromatograph equipped with an 5973 mass selective detector, split/splitless injector, and a DB-1701 fused capillary column (30 m x 25 mm x 0.25 µm film thickness, Supelco, Bellefonte, PA, USA). Sampling conditions were the same as described for SHS and SPME methods run under splitless mode. Helium carrier gas was passed through the column at 1.0 mL/minute under constant flow mode. The temperature program started with an initial oven temperature of 40 °C followed by 2 °C/min increase to 100 °C, then 15 °C/min increase to 315 °C, for a total run time of 44.33 minutes. The source temperature was 280 °C and inlet temperature was 250 °C. Purge time for static headspace samples was 0.30 minutes and 2.00 minutes for SPME samples. Solvent delay was 2.00 minutes and scans were made from 50 m/z to 350 m/z. Peaks were tentatively identified using the National Institute of Standards and Technology Mass Spectral Search Program Version 2.0f, 2008.

5. RESULTS

5.1 Static Headspace Analysis

5.1.1 Effects of Temperature

Total volatiles were analyzed at 25 °C, 40 °C, and 60 °C. Measurements included total area, number of peaks and individual product identification.

5.1.1.1 Total Volatile Response

Total volatile response increased with incubation temperature (Figure 20), as would be expected. This phenomenon has been reported extensively in the literature. The data was recorded here not to compare temperatures per se but to provide a baseline for oxidation kinetics and product patterns during early incubation periods. Increase in volatiles at 25 °C and 40 °C was slow and gradual, with volatiles at 40 °C not increasing notably until about Day 15. In contrast, at 60 °C volatiles began accumulating immediately and increased continuously over the incubation period. At 60 °C, volatile response on Day 4 was already higher than the full incubation period of 20 days at 40 °C.

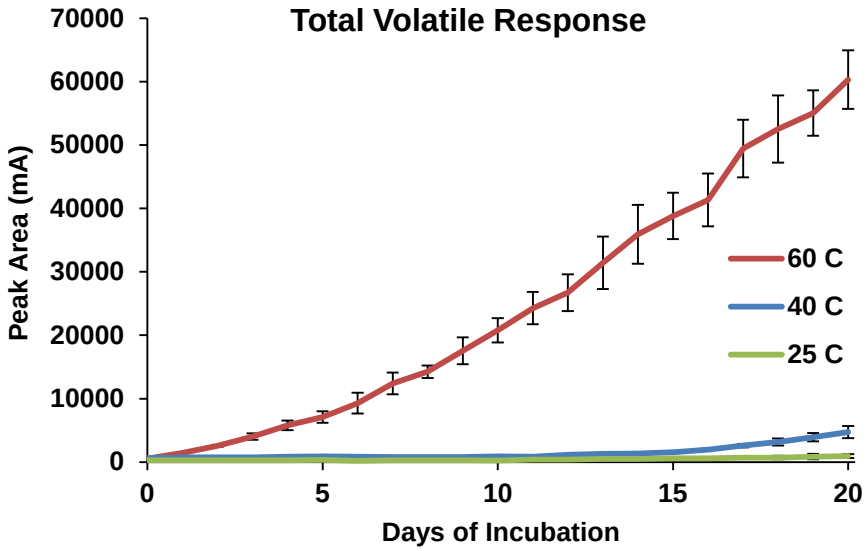


Figure 20. Effect of temperature on total volatile response measured by SHS.

5.1.1.2 Number of Peaks

Effects of temperature on the number of product peaks detected were not as clear as the total volatile response. As shown in Figure 21, the number of peaks at 60 °C increased steadily, nearly tripling by the end of the incubation. At 40 °C, the number of peaks increased over the first five days then decreased, while at 25 °C, the number of peaks increased only marginally during incubation. Differences in Day 0 number of peaks is due to experiments being completed at different times using different batches of methyl linoleate.

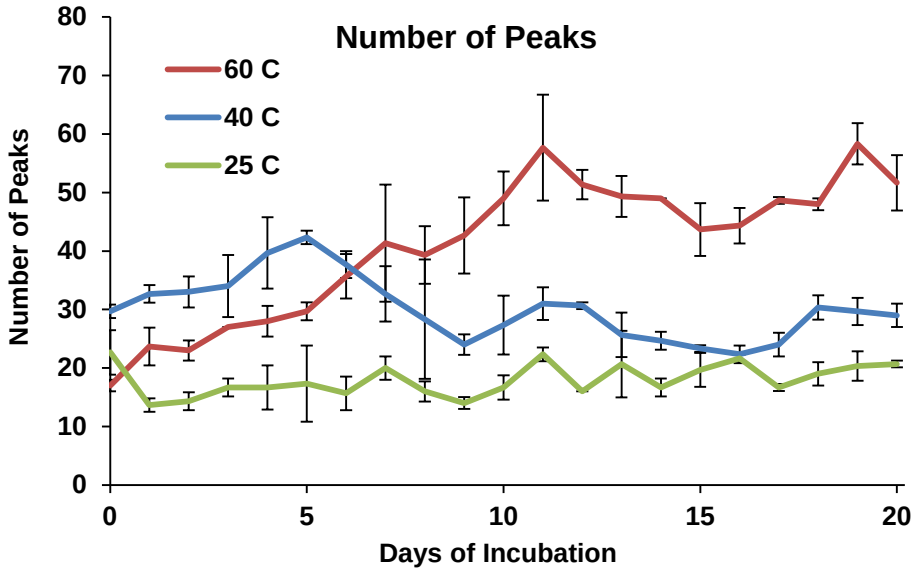


Figure 21. Effect of temperature on total number of ML oxidation product peaks detected by SHS.

Projecting these patterns to mechanism, with little change at 25 °C, the reactions appear to be set and products just accumulate over time. In contrast, the higher number of products at 40 °C indicates that more reactions are active, not just more products are produced. Furthermore, the rapid increase early in incubation while the total volatiles are relatively constant, followed by decreased numbers of volatiles indicates that the balance between reactions producing new products and reactions transforming initial products to secondary compounds must be rapidly changing during initial oxidation. The continual increase and the much higher numbers of products accumulating over time at 60 °C suggests that energy at this elevated temperature is sufficient to drive many more different reactions than at the lower temperatures. Production of mew products appears to dominate longer than at 40 °C – up to 11 days – before transformation or other degradation becomes competitive. These results suggest that accelerated shelf life studies at elevated temperatures are not studying the same reactions as at room temperatures, but are studying additional and different reaction pathways.

Interestingly, the number of peaks did not increase continuously at any temperature, but exhibited variable cycles in which number of peaks increased then decreased. These “variations” are not error or non-homogeneity of samples because our laboratory have observed the same pattern in both volatiles and non-volatile products in a number of food systems, including chocolate, pet food kibbles, processed cheese food, peanut butter, and a potato-cheese soup/spread.

5.1.1.3 Individual Product Identification

At all monitored temperatures, the major product by far was pentane (Figure 22) generated by β-scission of an alkoxyl radical at C-13 (Figure 13). In fact, pentane started

at about 30% of total products but accumulated to about 70% by the end of incubation (Figure 23) while other products remained at much lower levels. The overwhelming production of pentane detected by SHS suggests either that hydroperoxides (and their downstream products) do not occur equally at C-9 and C-13 as the literature reports, or that the hydroperoxides form equally but secondary reactions at the two sites are substantially different, e.g. with C13 preferentially undergoing scission reactions to generate small volatiles while other reactions occur at C9.

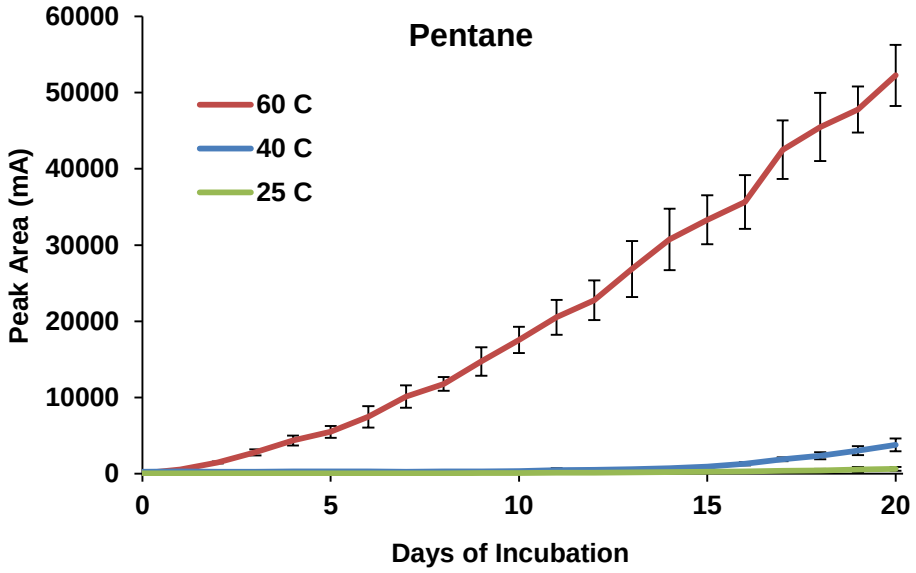


Figure 22. Generation of pentane in ML oxidized at different temperatures, as detected by SHS.

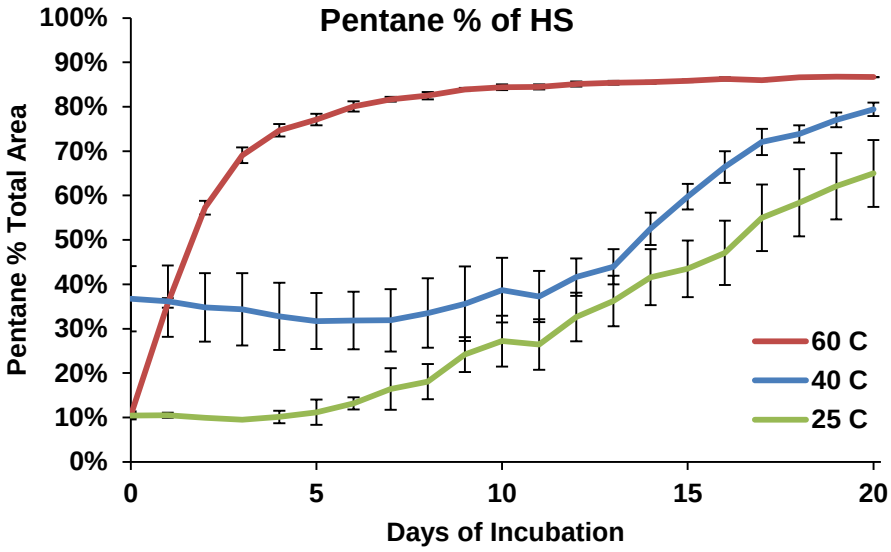


Figure 23. Pentane percentage of total volatile oxidation products in ML oxidized at different temperatures, as detected by SHS.

It could be argued that pentane is not mechanistically the most dominant product, but just the most volatile one due to its relatively low boiling point being (36 °C). This volatility issue is also related to a potential reason why pentane was not tracked or identified as a major product in the current understanding. Depending on how analyses are done and what type of product is being analyzed, pentane can escape before analysis.

The next most abundant product detected by static headspace was hexanal (Figure 24). Like pentane, this product results from scission of C-13 $LO^{\bullet}$, but from α-scission on the side closest to the carboxylic acid group. Hexanal is the volatile product most often monitored for extent of lipid oxidation.

Other products identified at all temperatures included t-2-heptenal, t-2-octenal, methyl octanoate, pentanal, and t-2-hexenal but are not all are shown at 25°C due to overall low oxidation levels (Figure 25A).

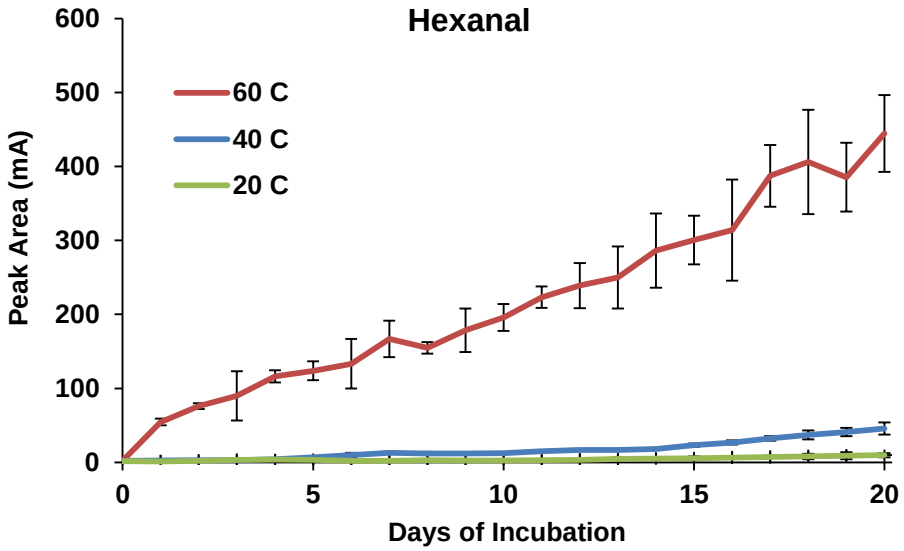


Figure 24. Generation of hexanal in ML oxidized at different temperatures, as detected by SHS.

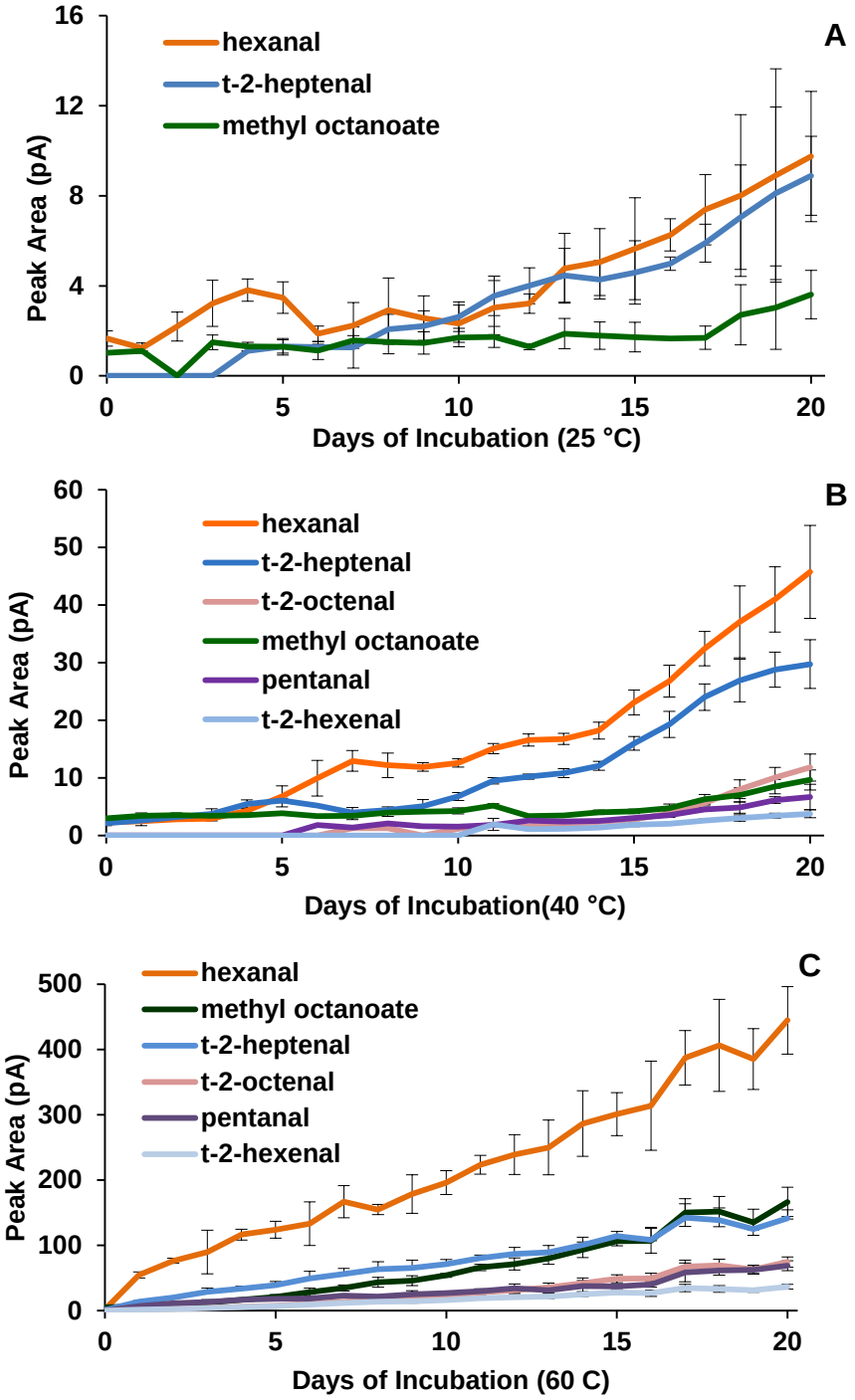


Figure 25. Generation of other identified products in ML oxidized at different temperatures, as detected by SHS.

At 40 °C products arising from both C-9 and C-11 positions were detected in addition to hexanal and pentane (Figure 25B). Two key compounds here are the appearance of t-2-octenal which could be coming from C-11 scission or potentially from the result of C-9 β-scission as a secondary oxidation product of 2,4-decadienal. The other compound here of interest is the appearance of pentanal, which most likely derives from oxidation of pentane.

At 60 °C, the same pattern of volatiles appeared, except in different proportions. Hexanal increased nearly 10-fold, but methyl octanoate (formed from α-scission of LO$^{\bullet}$ at C-9) also became more prominent (Figure 25C, Figure 26). Importantly, its scission partner, decadienal, was not detected. Enhanced production of these new compounds could result from increased volatility at the elevated temperatures, or could indicate that increased energy is necessary to drive scissions closer to the acid function on the acyl chain. That scission at C-13 were promoted most at higher temperatures (Figure 26) supports temperature-enhanced scissions but with preference still at sites distant from the acid group. Alternate reactions that may explain these results will be presented later.

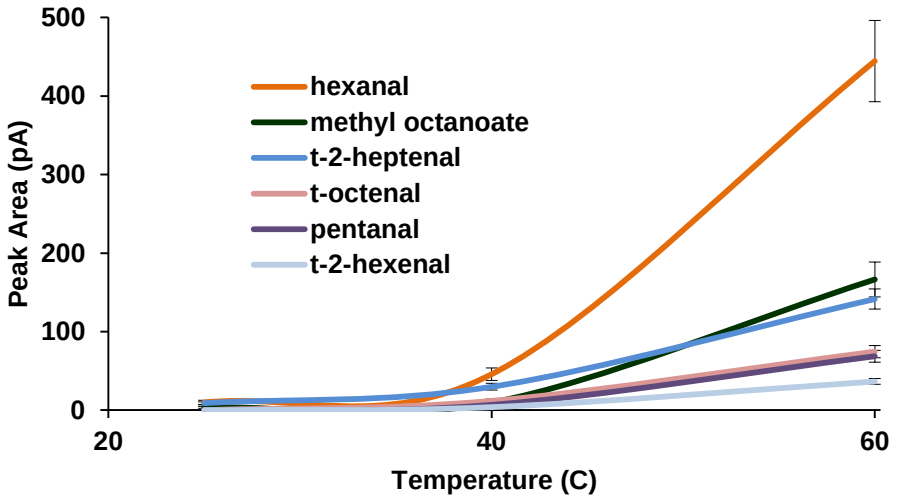


Figure 26. Peak areas of identified compounds across different temperatures by SHS.

5.1.2 Effects of System Factors

Obviously oxygen is required for oxidation but how much is required has been controversial. Also, oxygen has been shown to alter the balance between highly vs non oxygenated termination products, e.g. LOOL vs LOL vs LL, but whether oxygen availability also affects balance between propagation pathways has not been investigated. To learn more about oxygen direction of lipid oxidation, headspaces of 5% oxygen, 2% oxygen, and argon (0% oxygen) were investigated.

5.1.2.1 Effects of Oxygen Concentration at 40 °C

As total oxygen percentage decreased, total volatile response decreased (Figure 27). The slight drops in values do correspond to the days when the lid from each vial was removed and the headspace was replenished. Results showed that surprisingly little oxygen was needed to support oxidation since oxygen concentrations as low 2 and 5% gave produced more volatiles than under argon. These samples were not bubbled so it is clear that solubilized oxygen was enough to increase the extent of oxidation. Argon, due to its heavy mass, was able to remove more total oxygen from the headspace and the oil.

For all three concentrations, pentane again was the dominant peak despite the probability that some was lost during the purge days. As in air, hexanal, *t*-2-heptenal, *t*-2-octenal, and methyl octanoate were the next most prominent products identified (Figure 28). The final intensities of *t*-2-heptenal, octanoate, and *t*-2-octenal were the same at all reduced oxygen pressures; just hexanal increased with oxygen. This indicates that 5% or less oxygen was a limiting concentration for reactions generating the other products but did not affect the reaction generating hexanal. This phenomenon will be considered in

more detail in the Discussion section. Specific products detected were the same generated in air, just at different daily levels.

This helps show that at 40 °C, reaction pathways are constant and independent of oxygen concentration, and that these scission reactions are generating the same products based on available oxygen to drive reactions, just in different intensities.

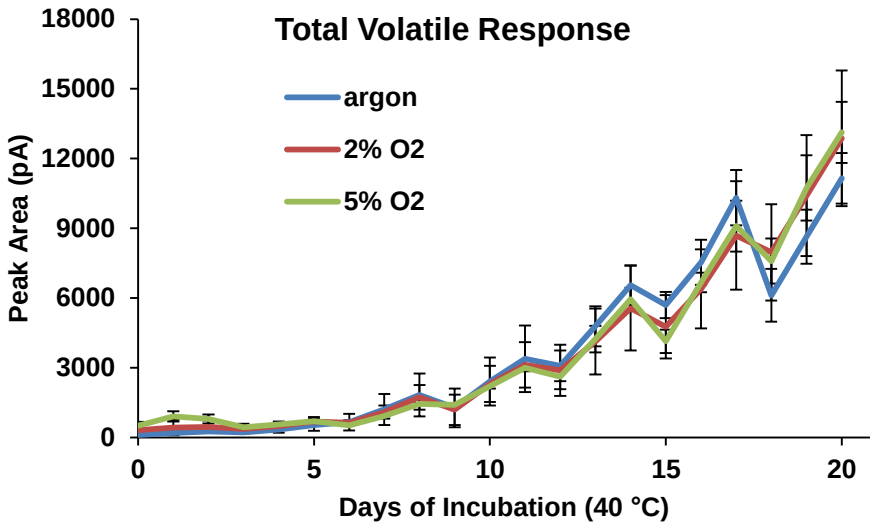


Figure 27. Effect of oxygen concentration on total volatile response from ML oxidized at 40 °C measured by SHS.

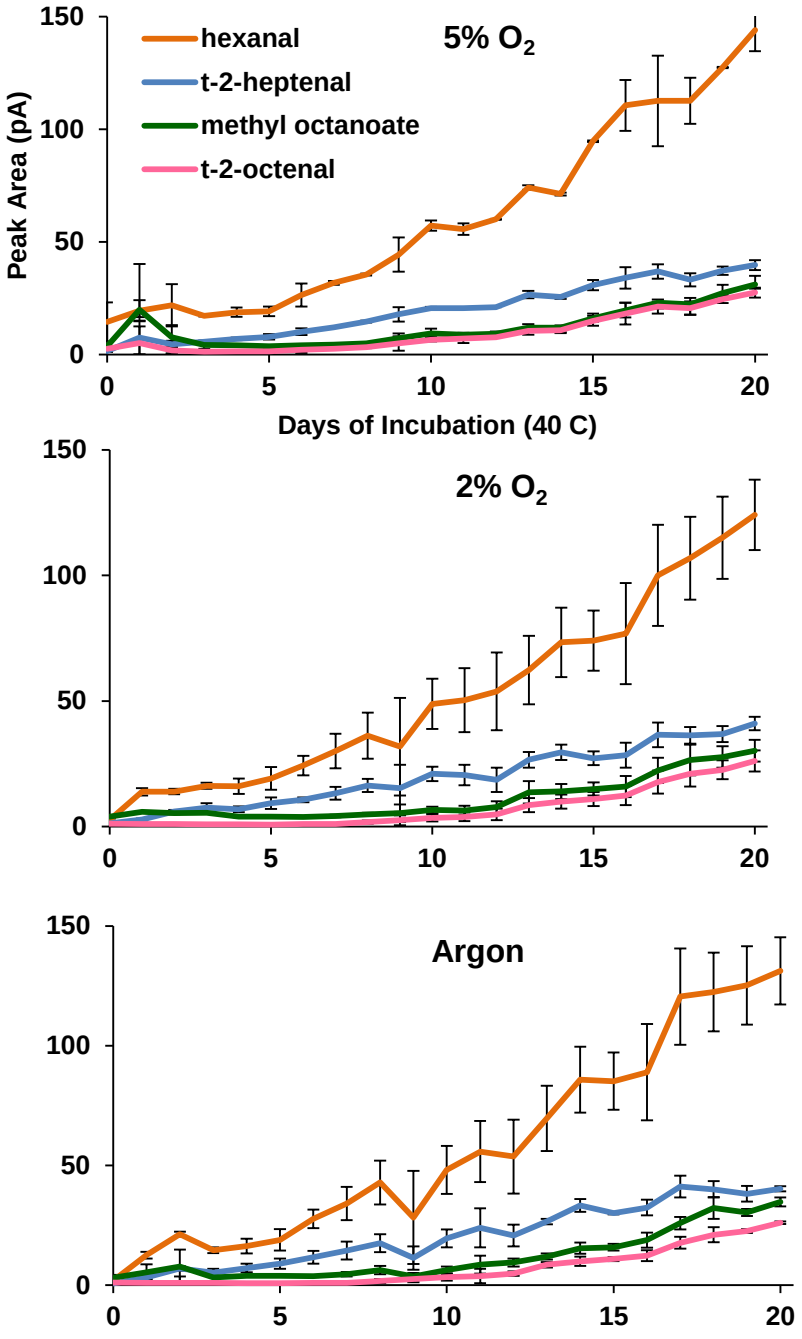


Figure 28. Generation of other identified products in ML oxidized in modified atmosphere at 40 °C, as detected by SHS.

Interestingly, as long as pentane is the dominant product, total volatile response presents a misleading picture. Contrary to expectations, total volatiles were higher under reduced oxygen than in air (Figure 20). This is largely due to pentane, which does not depend on secondary oxidation for generation. When pentane is subtracted from the total peak area, it becomes clear that all other volatiles together are diminished when oxygen is removed from the system, at least during the first two weeks of incubation (Figure 29). Thereafter, volatiles in the low pO_2 samples increased to greater than air. This could potentially be caused by metal contamination from transfer lines of gas tanks, but also the additional gas purges could have played a role in this effect. After each sample was purged to refresh the headspace, noticeable sharp decreases in total volatile response were seen the following day. It is possible that the purging forced the remaining volatiles out, regenerated the surface area of the oil which in turn allowed more pentane to be volatilized and released from the sample, filling up the headspace, thus having total volatile response appear as increased oxidation.

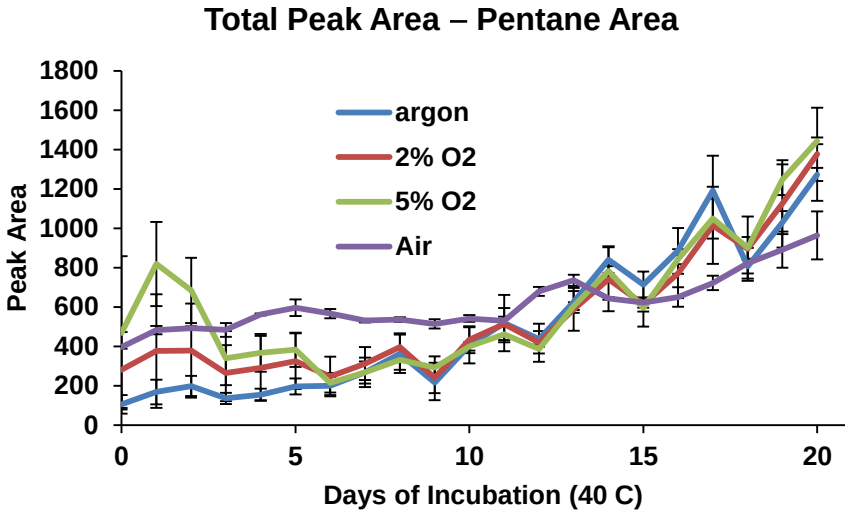


Figure 29. Total volatile response after subtraction of pentane peak area at 40 °C, SHS.

5.1.2.2 Effects of Oxygen Concentration at 60 °C

At 60 °C, the amount of oxygen plays an even larger role in driving oxidation, as shown in the notable reduction in total volatiles produced under argon compared to just 2 and 5% oxygen (Figure 30). The solubility of both oxygen and argon in oils increase at higher temperatures so small changes in headspace oxygen are magnified in the oil while at the same time argon becomes more efficient in displacing oxygen from oil. This demonstrates that to limit oxidation at higher temperatures, it is critical for the removal of oxygen from both oil and headspace.

Individual product showed a shift from distributions at 40 °C (Figure 31). As in air, more methyl octanoate was produced at higher temperatures, becoming the next major product after hexanal, whereas t-2-heptenal was dominant at 40 °C. However, the gap between methyl octanoate and t-2-heptenal lessened under the argon atmosphere.

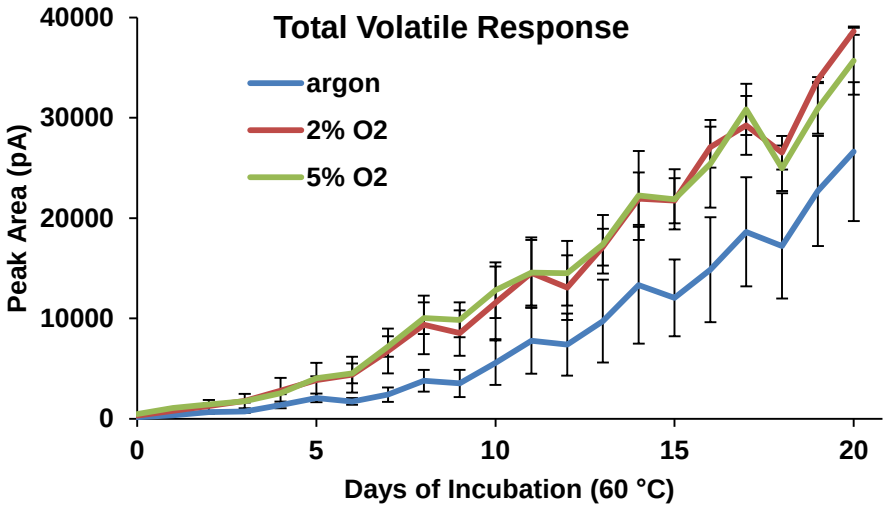


Figure 30. Effect of oxygen concentration at 60 °C on total volatile response measured by SHS.

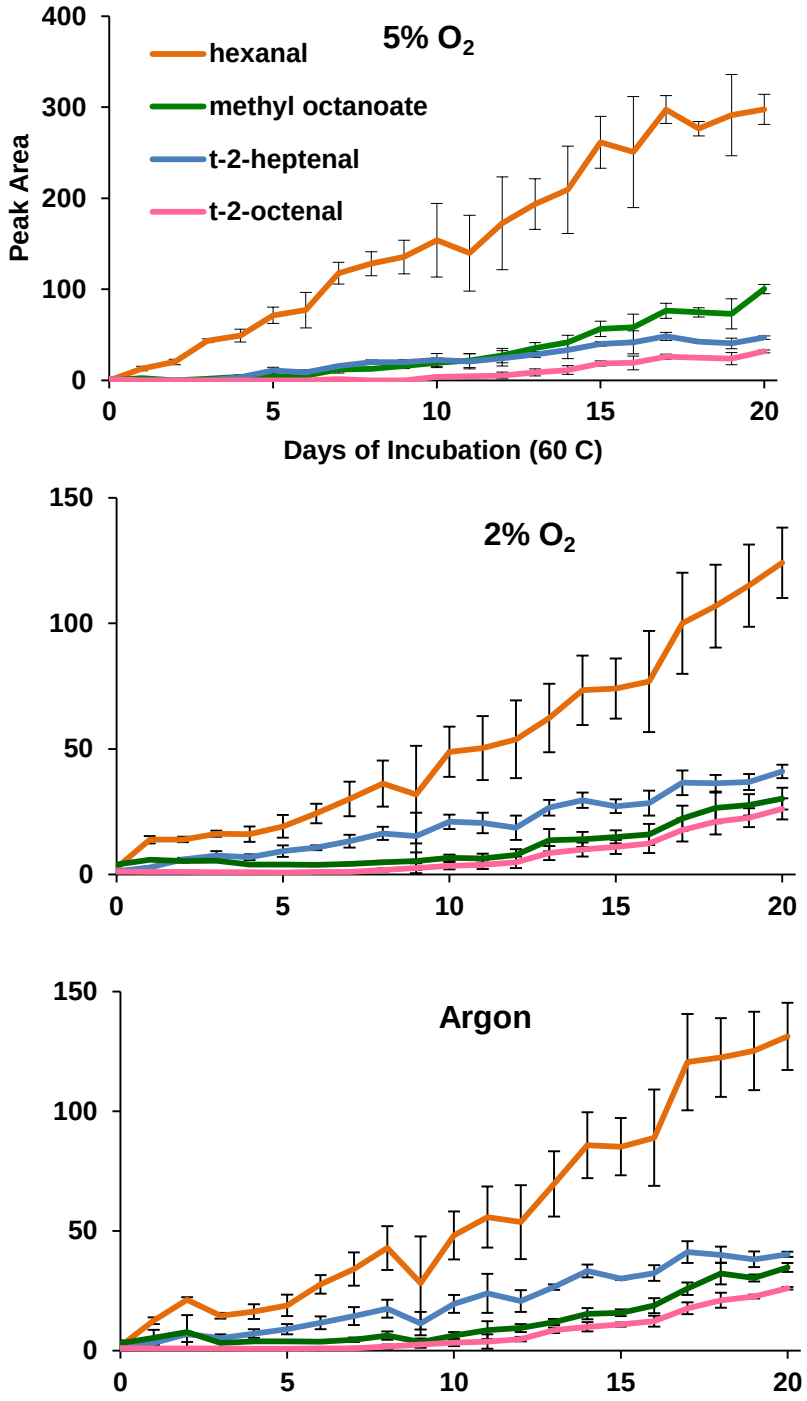


Figure 31. Other products identified in ML oxidized under modified atmosphere at 60 °C, as detected by SHS.

The same effect of pentane on total volatile response discussed for 40 °C was even more exaggerated at higher temperatures where pentane accounted for an even higher percentage of the products (Figure 32). When pentane area was removed, all other products were present at substantially lower levels under low pO_2 than in air. This observation was confirmed by significantly lower levels of non-volatile products in neat ML oxidized under reduced pO_2 versus in air[114].

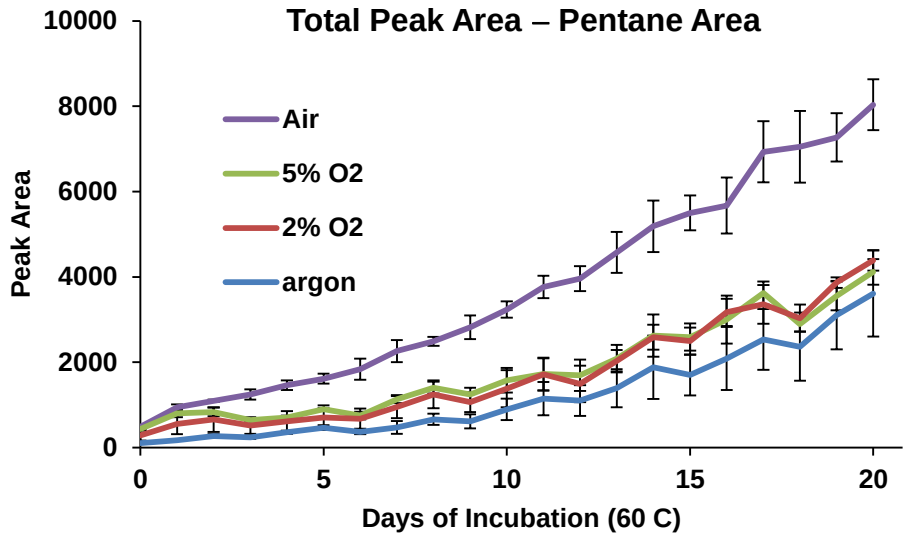


Figure 32. Total volatile response after subtraction of pentane peak area at 60 °C, SHS.

5.1.3 Lack of Solvent Data

Experiments investigating different concentrations of solvents such as chloroform, acetonitrile, 100% or 85% ethanol, and 2% water were attempted but could not be completed successfully because the strong volatility of each individual solvent overwhelmed lipid products peaks and introduced many interferences. Differences in volatility of the three solvents affected the volatiles that were carried into the headspace and the number of peaks detected. Since the headspace vials were closed systems, 500 µL

solvent was enough to saturate the headspace. In addition, pentane (the major product seen in SHS analyses) could not be monitored because it co-eluted with the solvents. Solvent volatility led to significant reproducibility problems. Despite ensuring syringe was clean of any solvent vapors that would drastically affect sample response if they were volatilized in the inlet, sharp increases and decreases were seen in overall total response and peak number. It is conceivable that the volatile solvents carried traces of liquid ML since products seen in the variable results were in fact lipid products such as hexanal, rather than being adducts formed between lipid and solvent.

Despite these limitations, some qualitative information can still be gleaned from the solvent data. Solvent drastically increased peak number (Figure 33), indicating an overall increase in active pathways in the presence of solvents, consistent with literature reports [12].

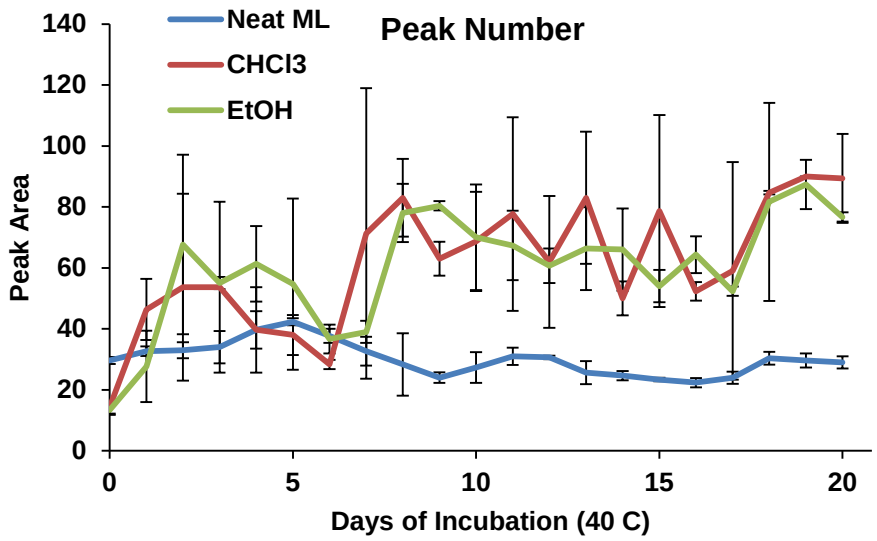


Figure 33. Number of product peaks detected in ML oxidized neat and diluted 50% in different solvents at 40 °C by SHS.

5.1.4 Summary of Static Headspace Results

Pentane appeared as the dominant product under all conditions, so SHS suggests that C-13 β-scission is the dominant pathway leading to formation of volatile oxidation products. The second most prevalent product, hexanal, also a scission product from C-13, further supported preferential scission activity at that site as the result of specific reaction rather than volatility. Longer chain aldehydes, e.g. deriving from C-11 and C-9, were detected over time, but at lower levels than pentane and hexanal. Scissions at C-9 and C-11 became more active at elevated temperatures and with longer incubation times. Headspace oxygen played critical roles in inhibiting production of most volatile oxidation products, although purging with modified oxygen concentrations appeared to drastically increase the amount of pentane produced at both 40 and 60 °C.

5.2 Effects of Sampling Techniques

Given the limitations of SHS for collecting volatiles (sensitivity and headspace equilibrium repression of volatilization), solid phase micro-extraction or thermal desorption sampling techniques were investigated to determine whether additional or different oxidation products could be detected with greater sensitivity. We were particularly interested in how the information gained might change interpretations of oxidation reaction chemistry.

5.2.1 Solid Phase Microextraction (SPME)

SPME analysis is a much more vigorous sampling technique since it collects products actively rather than passively. It is a very popular technique because

concentrating the products increases the sensitivity of detection and amplifies peaks that are very small in static headspace analysis. However, SPME also must be used with caution for lipid oxidation analyses because the fibers adsorb products selectively[47]. The three-phase SPME fiber adsorbs mixed compounds with chain lengths of three to twenty carbons, but it concentrates polar products with chain lengths of about six to eight carbons most strongly so the patterns of products detected shift to longer chain lengths than those detected by static headspace analysis. Results, therefore, may not include all oxidation products nor do they accurately portray actual product dominance or relative concentrations.

SPME results led to different interpretation of lipid oxidation extent than static headspace analysis. Quantitatively, SPME analyses collected more compounds and higher total concentrations of total volatiles than in static headspace analyses, although not all products have been identified. Results demonstrates the efficiency of volatile concentration by the SPME fiber, but the product levels detected also make lipid oxidation appear to develop more rapidly and more extensively than indicated by static headspace analysis, even though samples were incubated identically. In addition, product distributions detected by SPME suggested different reaction pathways than SHS.

Total Volatile Response. The total volatile response for SPME (Figure 34) increased with temperature, but at nearly double the level seen in SHS (e.g. 120,000 vs 60000 pA for 60 °C at day 20 per Figure 20).

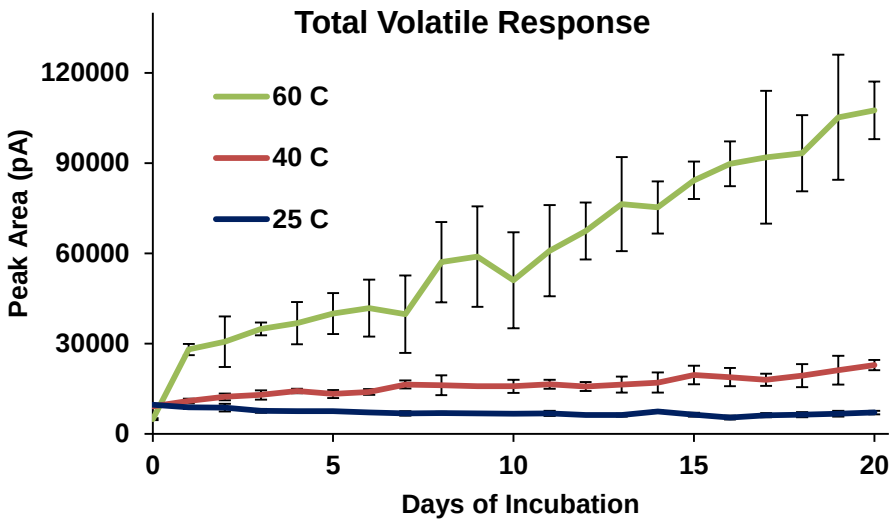


Figure 34. Effect of temperature on total volatiles from oxidized methyl linoleate detected by SPME.

40 °C. The major products detected by SPME were *t*-2-heptenal, hexanal, pentane, and methyl octanoate (Figure 35). Methyl octanoate (from C-9 attack) surprisingly was present in ML at time 0 and remained a key product throughout incubation. Hexanal increased rapidly during the first few days of incubation, and then slowed in production. *t*-2-Heptenal and pentane did not show an initial surge but increased steadily over time so that concentrations approached those of hexanal by the end of 20 days of incubation. Unlike SHS analyses, SPME was able to detect *t*,*t*-2,4 decadienal and 2-pentyl furan from scission reactions at C-9, although both were at trace levels.

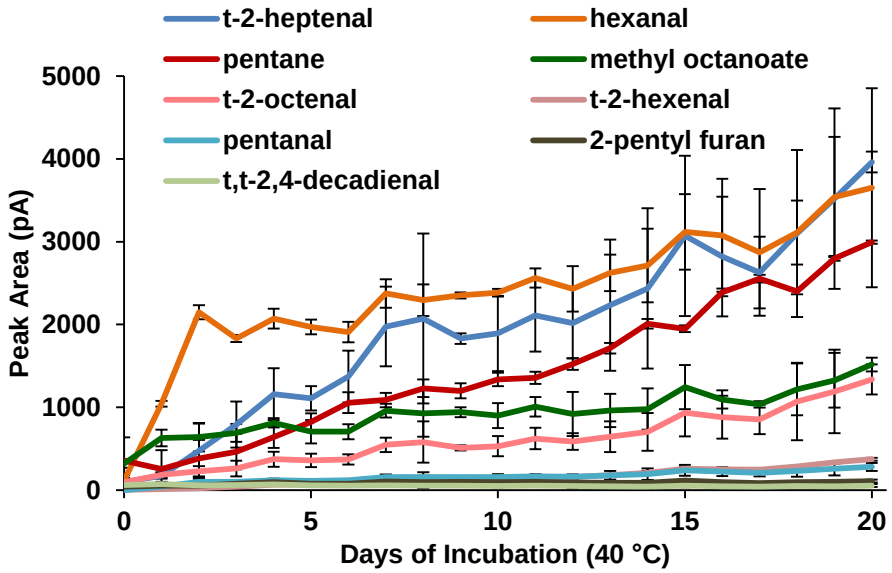


Figure 35. Volatile oxidation products from methyl linoleate incubated at 40 °C and detected by SPME.

SPME samples were also analyzed by GC-MS to help identify peaks for which we did not have standards for comparison of retention times. Figure 36 shows a typical chromatogram from ML incubated at 40 °C; products identified are listed in Table 7.

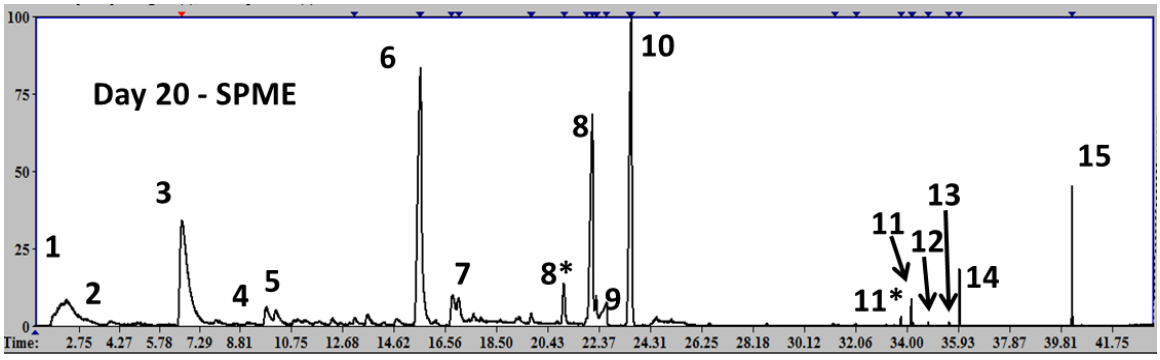


Figure 36. Total ion chromatogram of volatile products of methyl linoleate incubated at 40 °C at Day 20, detected by SPME.

Table 7. Volatile compounds identified in oxidized methyl linoleate after 20 days at 40 °C detected by SPME.

Day 20	Compound	RT	Probablity (%)
1	pentane	2.35	31.5
2	pentanal	3.96	32.5
3	hexanal	6.63	89.5
4	2-hexenal	9.80	49.2
5	pentyl-oxirane	10.18	55.7
6	2-heptenal	15.61	64.9
7	methyl heptanoate	17.06	77.5
8	2-octenal*	22.09	73.7
9	hexanoic acid	22.53	77.4
10	methyl octanoate	23.56	89.7
11	2,4-decadienal*	34.18	47
12	Heptanedioic acid, 3-methyl-, dimethyl ester	34.82	58.1
13	2(5H)-Furanone	35.61	67.6
14	Nonanoic acid, 9-oxo-, methyl ester	35.99	73.7
15	9,12-Octadecadienoic acid, methyl ester	40.25	26.2

The appearance of volatile pentyl oxirane could support internal rearrangements to epoxides, products expected in lipid oxidation but seldom tracked due to rapid reactivity. Oxirane identification remains to be verified. 9-Oxononanoic acid methyl ester (9-oxo-methyl octanoate) is generated by β-scission of the C-9 alkoxyl radical (Figure 14) and has also been found in oxidation studies with linoleic acid. Hexanoic acid is most likely generated from oxidation of hexanal, as has been observed in thermal degradation of linoleic acid. Compounds with asterisks have been identified in multiple peaks and are believed to be present as cis/trans isomers. SPME was able to help provide potential identification to *t*-2-heptenal, *t*-2-octenal, and methyl octanoate, compounds that were tracked by SHS-FID.

60 °C. At 60 °C, detected product patterns changed dramatically. Methyl octanoate became the dominant product, accumulating almost 30% of the total volatile response (Figure 37). This is the first time in this study that data indicated a focus of reaction at C-9, and it is different from results with SHS. The most obvious source of methyl octanoate would be α-scission of a C-9-O•. If so, the temperature effect suggests that this scission may require higher energy than the C-13-O•. The scission partner compound 2,4-decadienal, was detected, along with its rearrangement product pentyl furan, but both products were present at very low levels. While decadienal is known to oxidize and decompose to hexanal and *t*-2-octenal, the levels of these products are also very low, so there must be additional reactions generating octanoate. Alternate sources will be proposed in the Discussion section. The low levels of hexanal detected are interesting and important. The food industry uses SPME to measure lipid oxidation by hexanal production. This practice would give a false low oxidation value under accelerated shelf life conditions at 60 °C.

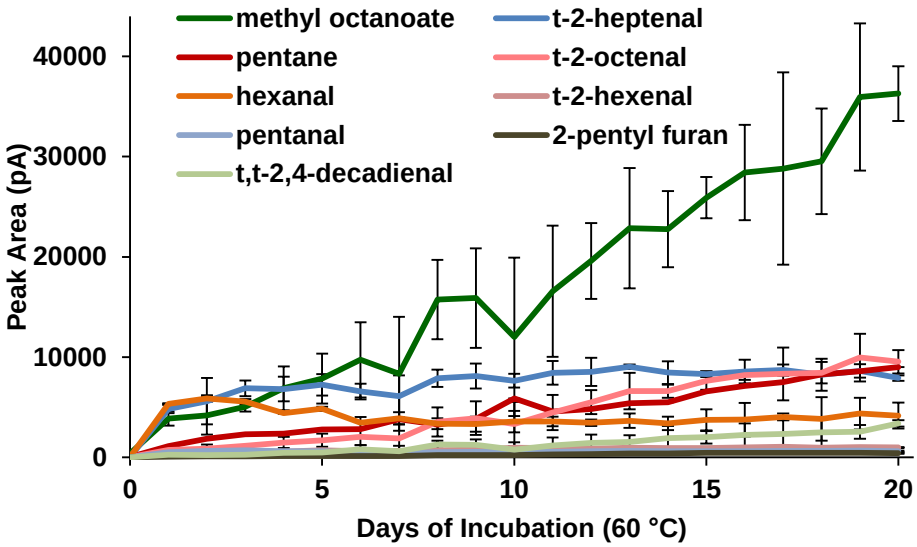


Figure 37. Volatile products from oxidation of methyl linoleate incubated at 60 °C and detected by SPME.

Products were also analyzed by GC-MS to identify additional peaks that did not match standards. The mass chromatogram is shown in Figure 38; products identified are listed in Table 8. A new compound verified by MS was 1-octene-3-ol, a compound with a flavor threshold similar to hexanal. This compound has been reported previously and attributed to reaction of a hydroxyl radical with the scission product of C-10 hydroperoxide (a singlet oxygen reaction point), followed by rearrangement[90]. This standard had been tested previously in FID analyses, but it co-eluted with other compounds under the temperature program used. Its presence is both puzzling and interesting. Formation of C-10 hydroperoxides does not usually occur in ML except via photosensitization, yet these samples were maintained protected from light. An alternative explanation might be double bond migration at elevated temperature since 1-octene-3-ol was only a trace compound at 40 °C but showed a huge increase in concentration at 60 °C (Figure 39). The appearance of this unusual product shows once again that reactions occurring under accelerated shelf life testing conditions at 60 °C are not the same as at room temperature or even 40 °C.

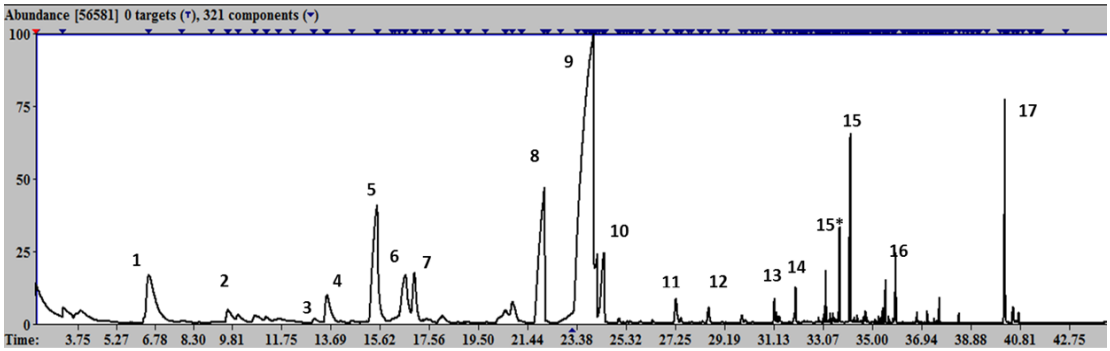


Figure 38. Total ion chromatogram of volatile products of methyl linoleate incubated at 60 °C at Day 20, detected by SPME.

Table 8. Volatile compounds identified in oxidized methyl linoleate after 20 days at 40 °C detected by SPME.

Day 20	Compound	RT	Probablity (%)
1	hexanal	6.54	85.2
2	t-2-hexenal	9.69	54.9
3	hexanal dimethyl acetal	13.07	92.4
4	2-pentyl furan	13.56	88.1
5	t-2-heptenal	15.54	68.7
6	1-octene-3-ol	16.63	85.1
7	heptanoic acid methyl ester	17.01	90.9
8	2-octenal	22.06	74.8
9	methyl octanoate	23.90	91.9
10	Hexanoic acid, vinyl ester	24.34	54.6
11	2-octenoic acid, methyl ester	27.27	32.1
12	2-nonenal	28.55	69.9
13	2,4 nonadienal	31.99	70.7
14	4-decenoic acid, methyl ester	33.18	57.4
15	2,4 decadienal	34.14	72.8
16	.Methyl 9-oxononanoate	35.93	97
17	methyl linoleate	40.23	57.8

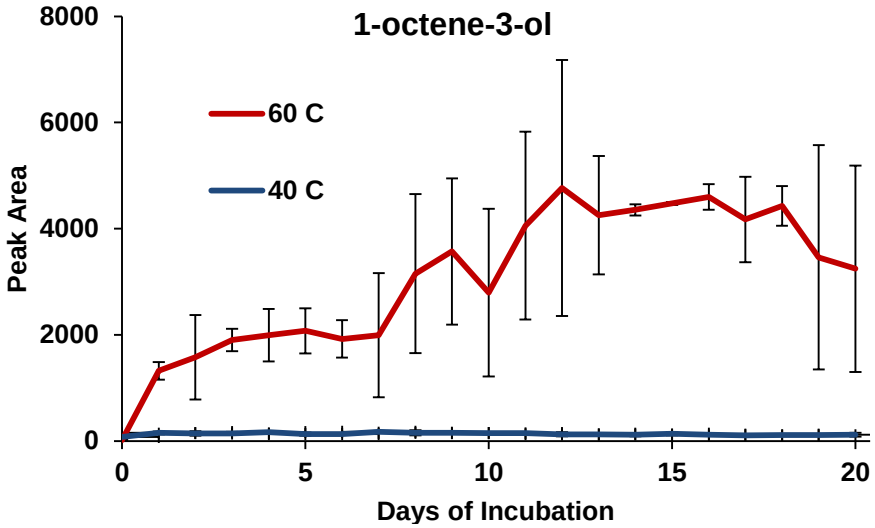


Figure 39. Generation of 1-octene-3-ol in ML oxidized at different temperatures, as detected by SPME.

One key missing piece of the product puzzle at 60 °C is the low relatively low levels of pentane compared to SPME at 40 °C or SHS at any of the test temperatures. In general, volatile data has shown more peaks as temperature increases and more pathways become more active. However, because SPME has low binding preference for pentane compared to polar products, as more thermal energy is added to the system and more pathways become active, less pentane binds to the fiber due to unfavorable competition (Figure 40).

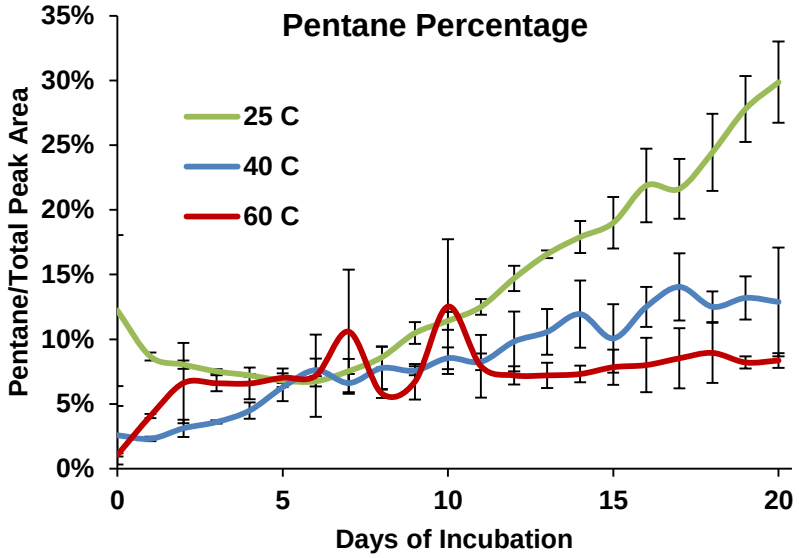


Figure 40. Pentane percentage of total volatiles detected by SPME.

Fiber Selection. Since the selection of the fiber in SPME is so critical to the product intensity and distribution detected, products detected at 60 °C by a 2-phase fiber consisting DVB/PDMS (no CAR) and the 3-phase fiber (DVB/PDMS/CAR) were compared to assess accuracy and usefulness for mechanism studies. As expected, presence of the CAR phase enhanced detection of small nonpolar compounds and increased total volatile response (Figure 41). CAR was particularly important for tracking

of pentane, showing nearly double the peak area compared to the DVB/PDMS fiber (Figure 42).

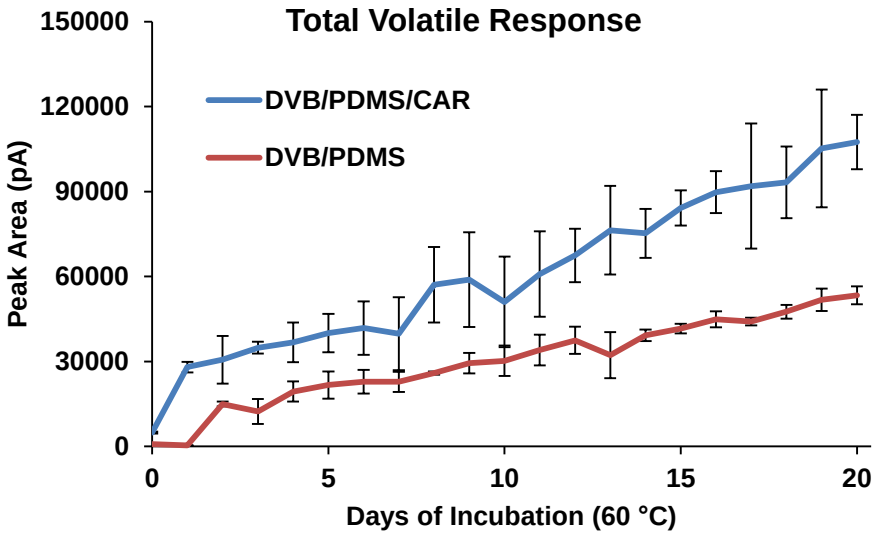


Figure 41. Comparison of total volatiles from methyl linoleate oxidized at 60 °C, detected by two different SPME fibers.

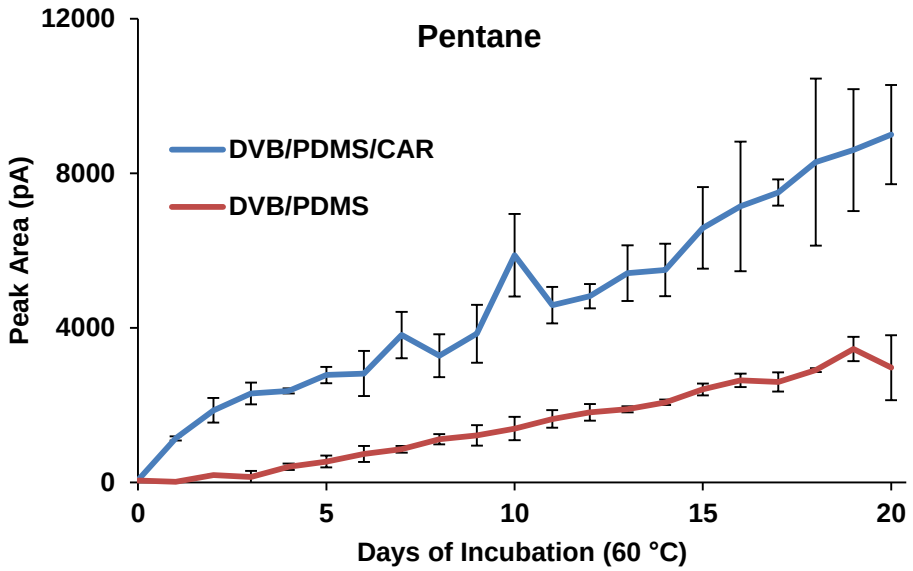


Figure 42. Detection of pentane by two different SPME fibers

However, pentane bound in competition with other compounds, so the price of its detection was lower binding of other compounds and fewer types of compounds bound. Lower binding of pentane allowed the DVB/PDMS fiber to adsorb additional compounds and the number of total peaks detected actually increased (Figure 43). This occurs because the DVB/PDMS fiber is designed for analysis of polar compounds and semivolatiles. This observation suggests that the DVB/PDMS/CAR is missing perhaps key product peaks while more accurately detecting pentane. The combination of Figure 42 and Figure 43 illustrates that the DVB/PDMS/CAR fiber adsorbs fewer compounds but at higher concentrations of each or of specific compounds.

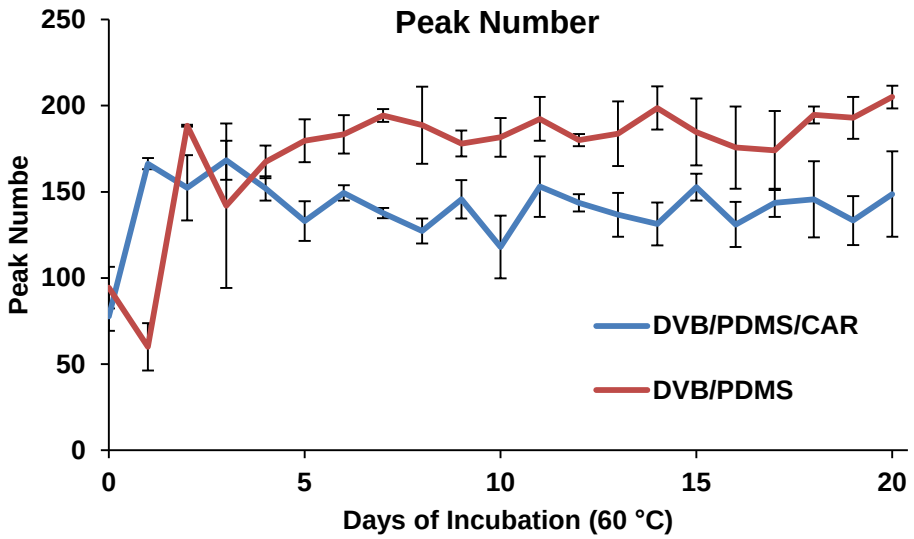


Figure 43. Total number of volatile peaks detected by SPME, using two different fibers.

Visual inspection of SPME chromatograms (Figure 44) reveal no obvious shifts in reaction pathways with either fiber. One noticeable difference, however, was a noticeable reduction in detected pentane value when the DVB/PDMS fiber was used. This behavior demonstrates the importance of fiber selection for analyses, especially when attempting

to drive mechanisms or monitor specific products. If the DVB/PDMS fiber was used to monitor oxidative stability in a different food or model system, without the ability to correctly monitor pentane, different conclusions about the extent of oxidation as well as mechanism or pathway would be drawn.

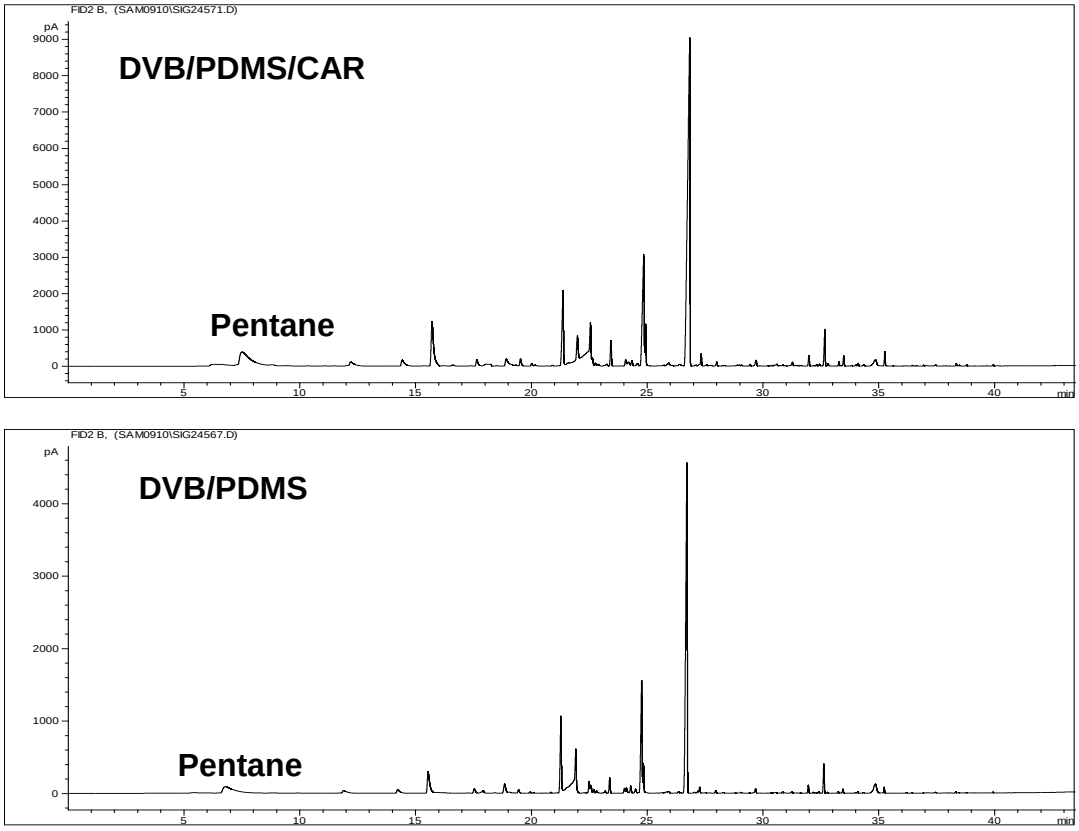


Figure 44. Comparison of ML incubated at 60 °C at day 20 monitored by SPME, with 2 different fibers.

Weekly Analysis. Since SPME is an extraction process it is possible that by monitoring samples daily rather than with a longer interval could change products detected and hence judgments made about reaction mechanisms. The dominance of methyl octanoate at 60 °C suggested a change in mechanism, so methyl linoleate was oxidized 40 °C monitoring samples weekly for 12 weeks. This time period was chosen

based on the industry standard that three months at 40 °C is equivalent to approximately one month at 60 °C. After taking into account differences in the quality of the starting ML in the two studies, monitoring weekly had no quantitative effect on total volatile response but it did miss short term cycling that was observed in daily analyses (Figure 45).

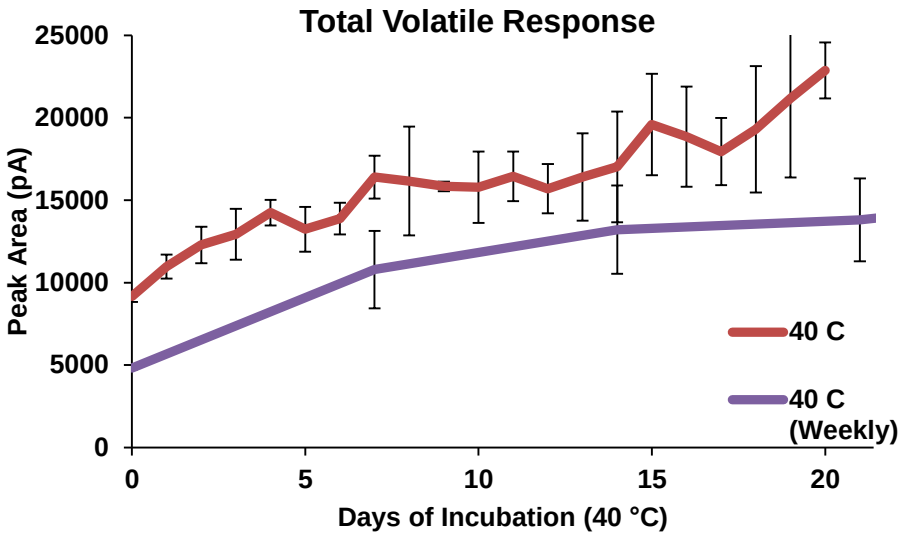


Figure 45. Total volatile response at 40 °C detected by daily versus weekly SPME analysis.

Long Term Storage. It could be argued that differences in oxidation patterns observed in this study from the literature because it monitored early oxidation rather than long-term secondary processes. To test this possibility, incubation times at 40 °C were extended to 80 days. Products detected were the same but their balance changed over time. Methyl octanoate, normally the dominant product at 60 °C, increased greatly after day 20 (Figure 46). However, pentane and hexanal were still high, so the ester was not

resulting from a shift in reaction to C-9. *t*-2-Heptenal and *t*-2-octenal, secondary products resulting from scissions, also increased notably. Thus, we speculate that some fundamental initial process was established during early oxidation, and extended incubations just enhance breakdown products deriving from it. Possible reactions will be considered in detail in the Discussion.

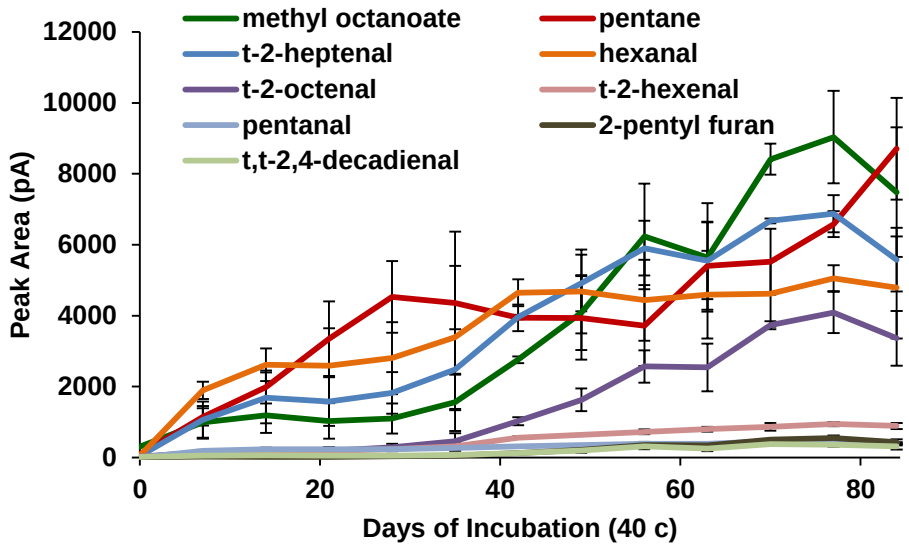


Figure 46. Volatile products from oxidation of methyl linoleate detected by SPME (DVB/PDMS/CAR) after long term incubation at 40 °C.

5.2.1.1 Summary of SPME Results

The picture of extent of ml oxidation and distribution of products (hence active reaction pathways) presented by SPME was very different from that gained from SHS. Total product levels (peaks areas) were 0.5 to 6 fold higher with SPME which collected compounds from the headspace to overcome limitations of volatilization equilibria. Although products at 25 °C were low with both methods, products began accumulating immediately at both elevated temperatures with SPME, in contrast to SHS which showed

an induction period of about 15 days at 40 °C with SHS. This is important from a practical standpoint since induction periods are used to judge stability of oils and foods. Which method generates more accurate estimates is arguable. SPME adsorbs compounds selectively and accumulates them while SHS is less sensitive and requires time to accumulate enough molecules in the headspace. It is likely, therefore, that SPME overestimates oxidation to some extent while SHS underestimates it

The major products detected by SPME were *t*-2-heptenal at 40 °C and methyl octanoate at 60 °C, and neither was in great excess over other products. In contrast, pentane was the overwhelmingly dominant product detected by SHS. These patterns present two very different pictures of oxidation pathways -- C-13 attack in SHS versus C-9 with also C-11 involvement in SPME.

Comparison of collection selectivity for DVB/PDMS fibers with and without CAR phases highlighted how apparent product distributions can be modified by fiber binding selectivity and competition. Limiting detection of certain compounds can allow additional compounds to be tracked. Despite limitations of adsorption specificity, results of the two fibers showed different products becoming prominent at different temperatures, suggesting that different pathways are active and can become more dominant based on the environment of the system.

While short incubation time periods suggested products from both C-11 and C-9 were dominant, longer incubation with less frequent sampling times detected appearance of pentane (C-13) as well as the overall increase of methyl octanoate at 40 °C. These observations raise cautions about interpreting SPME data because adsorption competition changes over time. Long term storage also showed that total volatile response as early as

day 8 at 60 °C already surpassed volatile levels from ML oxidized at 40 °C for 12 weeks (Figure 47); products formed at the two temperatures also did not match. These results demonstrate that different reactions occur and generate different products when foods are stored for short times storage at high temperatures, rather than merely accelerating the same reactions that occur at ambient temperatures.

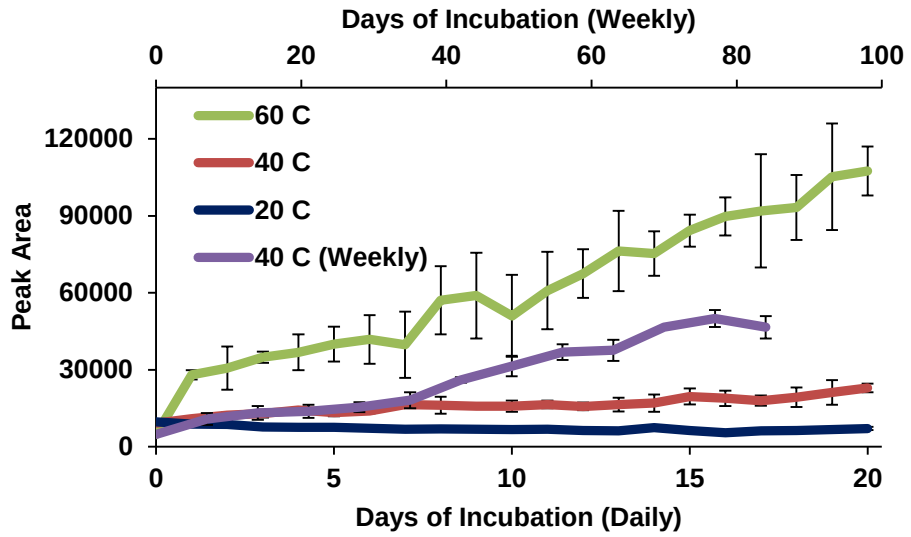


Figure 47. Total volatile response in methyl linoleate oxidized at different temperatures short-term and for extended time, detected by SPME.

5.2.2 Thermal Desorption

Thermal desorption (TD) offers a still different picture of lipid oxidation from collected volatile products. By continually sweeping volatilized products from the headspace, TD eliminates headspace equilibrium limitations while it collects products more exhaustively. Furthermore, the method should present the most accurate accounting

of lipid oxidation, although questions have been raised about potential modification of products on the traps.

Total peak area and retention times determined by TD in this study cannot be compared directly to SHS and SPME because sample configuration and gas chromatographs were different. Nevertheless, useful information can be gained for comparison and products can be quantitated more accurately (μg/trap rather than peak area) by comparison to standards loaded onto the traps. This section presents volatile products detected by thermal desorption using two different sample procedures -- the SIS Thermal Collection System and the OxiPres™ oxygen bomb. Effects of temperature on individual products will be shown first, followed by a comparison with between the two techniques including total volatile response and peak number.

5.2.2.1 Thermal Desorption Collection System

40 °C. Initial studies were conducted using the SIS Thermal Collection System where methyl linoleate was oxidized on Diafil inert solid support in sealed tubes at 40 °C and volatile products were collected every five days by purge and trap with 20 minute collection time. This procedure was used because preliminary experiments showed that short chain products were lost when volatiles were formed from ML in 10-mL glass vials (as in SHS and SPME), then removed and transferred to the thermal collection system. Also, it was judged that incubating neat ML in the glass tubes would greatly change the surface area of the oxidizing ML relative to SHS and SPME, especially at higher temperatures and long term storage. While we recognize that oxidizing methyl linoleate on the solid support may alter reaction mechanisms, the key goal of this study was to evaluate TD capabilities to track oxidation products, particularly short and long chain

volatiles that may be underestimated in other methods. In thermal desorption, removal of products as they volatilize prevents pentane repression of volatilization of longer chain products, and results thus may more accurately represent actual product distributions. Hence, we considered that the potential gains in information more than outweighed the differences in oxidation conditions for this comparison study.

Volatiles were initially collected for 20 minutes; total peak area and total number of peaks detected are shown in Figure 48 and Figure 49Figures 49 and 50, respectively.

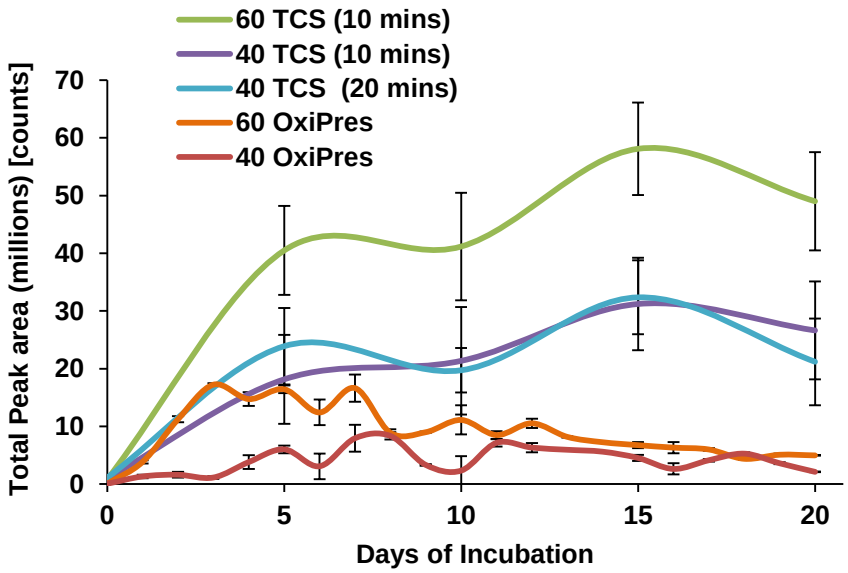


Figure 48. Total volatile response measured by two thermal desorption systems.

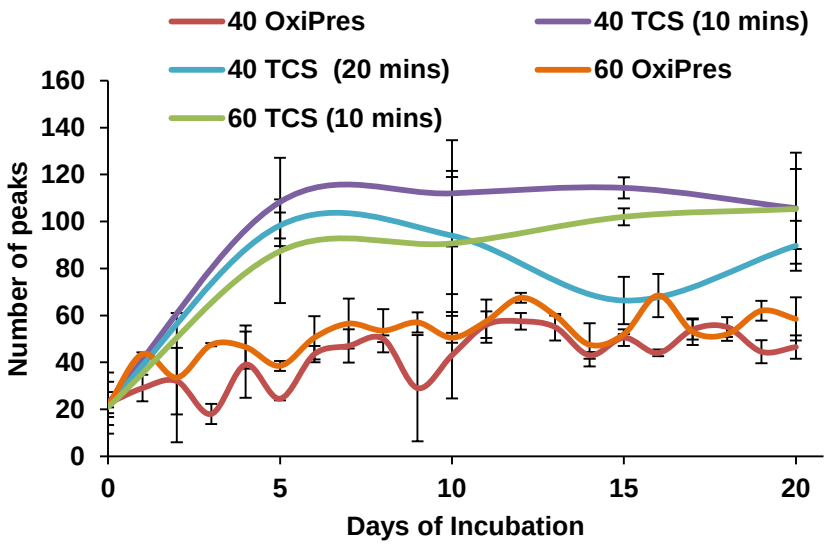


Figure 49. Total number of peaks measured by thermal desorption.

A typical chromatogram is shown in Figure 50. Peak areas cannot be compared directly to SHS and SPME because the detectors have different electronic bases and data is expressed in different units.

However, considering that thermal desorption is supposed to be a more exhaustive collection method, it was somewhat surprising to find that the average total number of peaks was substantially lower for thermal desorption (about 105) than for SPME (about 157). It is not known whether this difference is due to Diafil modifications of oxidation pathways and products, Diafil binding of products (preventing volatilization), loss of products during collection on trap, or conversely, enhanced breakdown of products on the SPME fiber.

In general, major volatiles observed were compounds also detected in SHS and SPME, including pentane, hexanal, pentanal, methyl octanoate, *t*-2-octenal, and *t*-2-heptenal. The majority of products had chain lengths between six and eight carbons; no

products with chain lengths longer than eight carbons were detected as no major peaks were seen after the standard methyl octanoate and *d*-naphthalene. However, some peaks were found (Unknowns A and B) that did not match previously run oxidation standards.

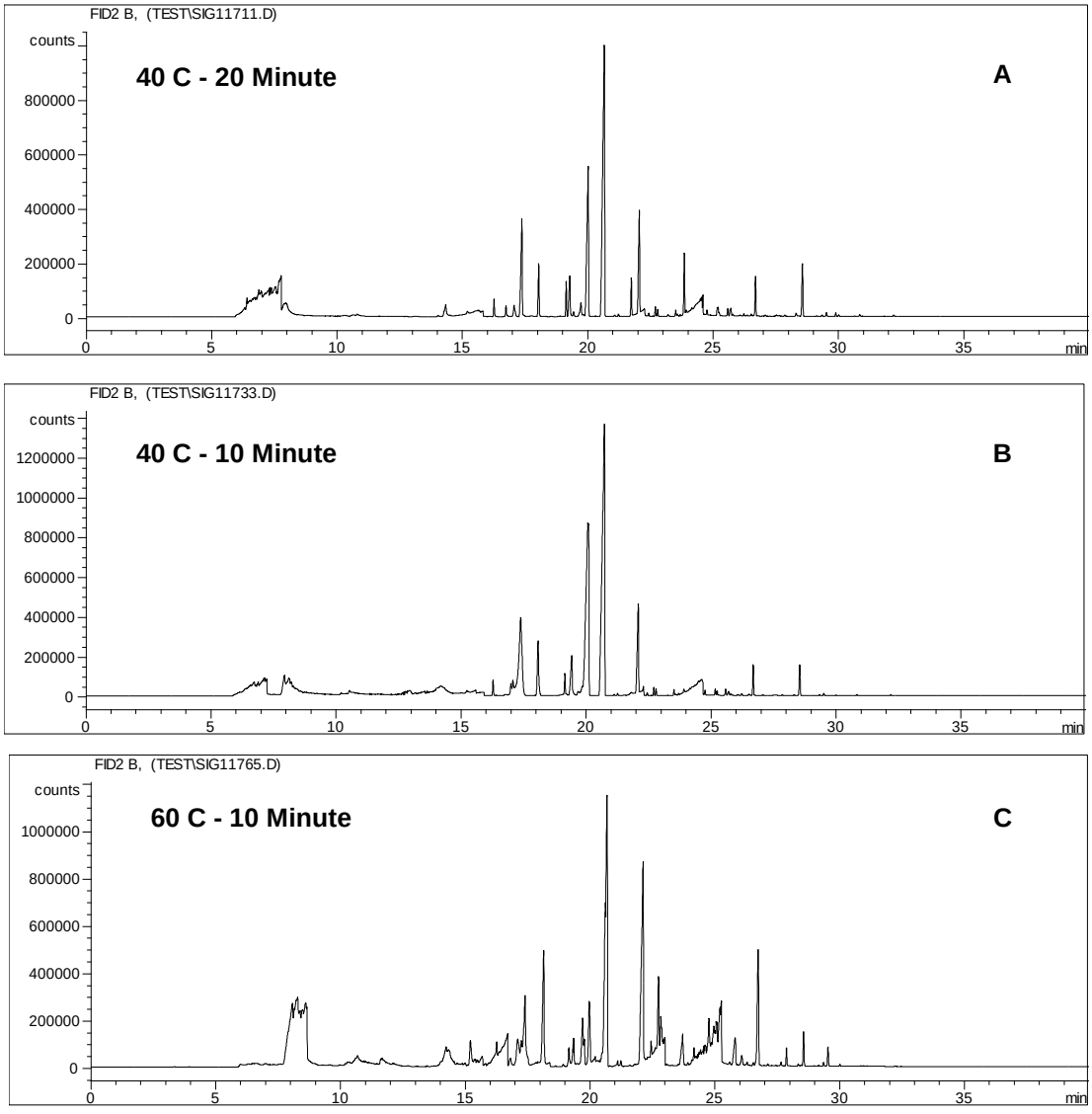


Figure 50. Day 20 chromatograms for both 40 and 60 °C sampled via the Thermal Collection System (TCS).

The distribution and timing of products differed in TD compared to SPME. Hexanal was the major product in early oxidation but Unknown A began to accumulate after Day 5 and became the dominant product by the end of incubation (Figure 51). Comparison with retention times for identified peaks suggested that unknowns A and B have chain lengths in the range of six to seven carbons, which would result from C-13 or C-11 preferential scission. This corroborates product patterns seen in SHS or SPME.

Surprisingly, pentane was a relatively minor product. It accumulated over the first five days, then decreased, and was not detected after ten days (Figure 51). This was the first analysis that showed pentane actually decreasing over time. Although pentane does not contribute to rancid odors and thus is often considered unimportant as a product, it is important mechanistically to fully understand how oil is oxidizing. Pentane is a small molecule so may not be retained efficiently if the long collection time led to overloading of the Tenax/carboxen trap. This seems reasonable since no products shorter than pentane were clearly detected, but possibly co-eluted with the solvent of the internal standard.

To test this possibility, the experiment was repeated with a 10 minute collection time. Now the picture of oxidation was very different (Figure 52). Pentane was barely detected at all, hexanal did not peak until Day 10, Unknown A accumulation was roughly comparable, and the other products had extended induction periods. Perhaps most importantly, hexanal and Unknown A levels were higher after ten than after twenty minute collection. This indicates either that overloading did occur at twenty minutes or that compounds began to degrade or be lost with the longer collection time. Whatever the explanation, it was judged advisable to use the shorter collection time in subsequent experiments to avoid problems.

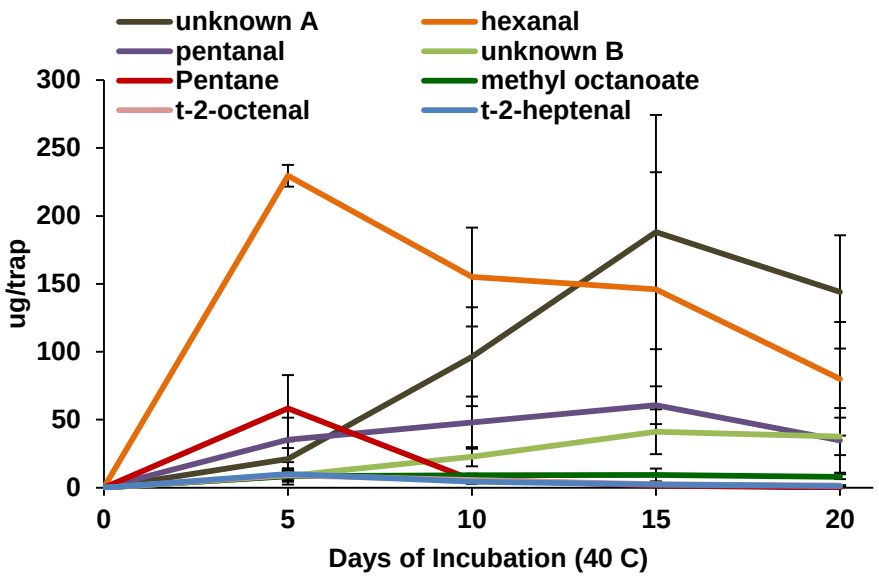


Figure 51. Volatile products from methyl linoleate oxidized at 40 °C and collected by purge and trap (ML on inert solid support)/thermal desorption. Twenty minute purge time.

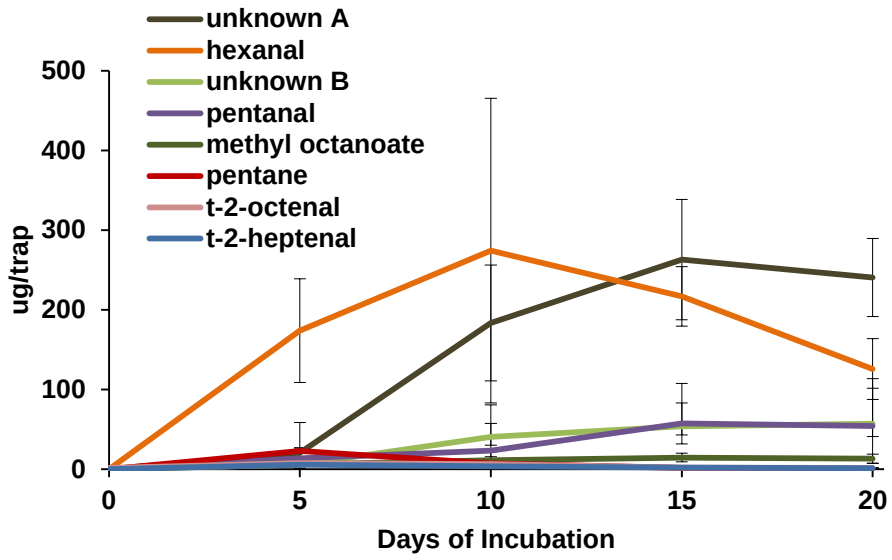


Figure 52. Volatile products from methyl linoleate oxidized at 40 °C and collected by purge and trap (ML on inert solid support)/thermal desorption. Ten minute purge time.

60 °C. Oxidation of ML at 60 °C on the solid support was also investigated (Figure 53). The higher temperature altered both oxidation kinetics and product distributions. Unknowns A, pentane, methyl octanoate, and Unknown B (in that order) showed an initial surge at five days, reaching levels approximately double those at 40 °C, then declined markedly to levels less than at 40 °C during the remaining incubation. At Day 20, the two Unknowns were the dominant products, but not by large margins. Other identified products stayed at low levels throughout the incubation period. Additionial peaks were detected but were too small for identification.

These are the only conditions under which such pronounced decomposition of products was observed over time. Whether contact with the Diafil catalyzed this decomposition or other factors were involved is not yet known.

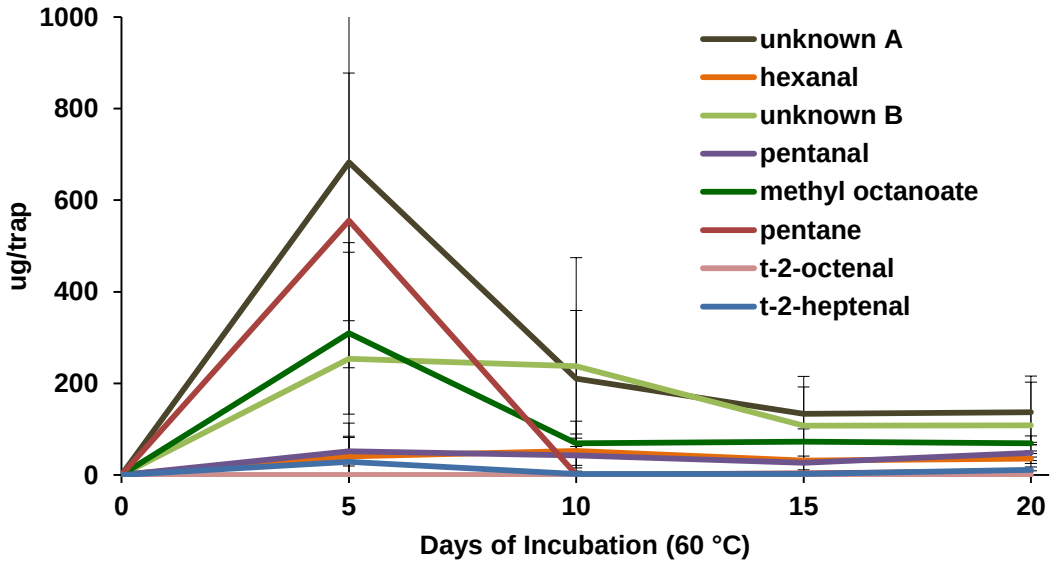


Figure 53. Volatile products from oxidation of methyl linoleate incubated at 60 °C and collected by purge and trap (ML on inert solid support)/thermal desorption. Ten minute purge time.

5.2.2.2 Volatile Products Collected From Oxygen Bomb by Thermal Desorption

We were concerned that loading the ML onto the Diafil might have changed oxidation pathways by interactions between the solid material (or contaminants), or by spreading the ML more as a film, hence increasing surface are and orientation of the acyl chains. To investigate this possibility, ML was oxidized in an Oxipres oxygen bomb and volatiles were vented through Tenax/carboxen traps for analysis. In this case, headspace also was sampled daily to track products more accurately. The oxidation system was different – 0.5 g in a headspace of about 150 ml – but oxidation products from the same amount of ML as in all other experiments were concentrated on the traps during the approximately 2 minute purging, so direct comparisons are relevant.

Contrary to expectations, much lower levels and numbers of oxidation products were collected from the oxygen bomb (Figure 48 and Figure 49, respectively). Whether this results from the configuration of the incubation system (small ML volume, large headspace), loss of volatiles during rapid venting through the trap, reaction of volatiles while in the headspace (e.g. radical recombination to ketones), or other factors is not yet known.

40 °C. There were three important observations in the Oxipres system (Figure 54, Table 9). Mass spec analyses were extended for longer times and to higher temperatures, but no peaks past 15 minutes (longer chain products) were detected. Most importantly, the presence of Unknown A indicates that this product does not result from ML interactions with Diafil, although the possibility of some transformation on the trap itself cannot be eliminated. Retention indices suggest that Unknown A is a C5 derivative, possibly 1-methyl butanol or 3-butene-1-ol[117]. Products detected here were quite different

than in the other systems, showing significant presence of ketones and small scission products (<five carbons).

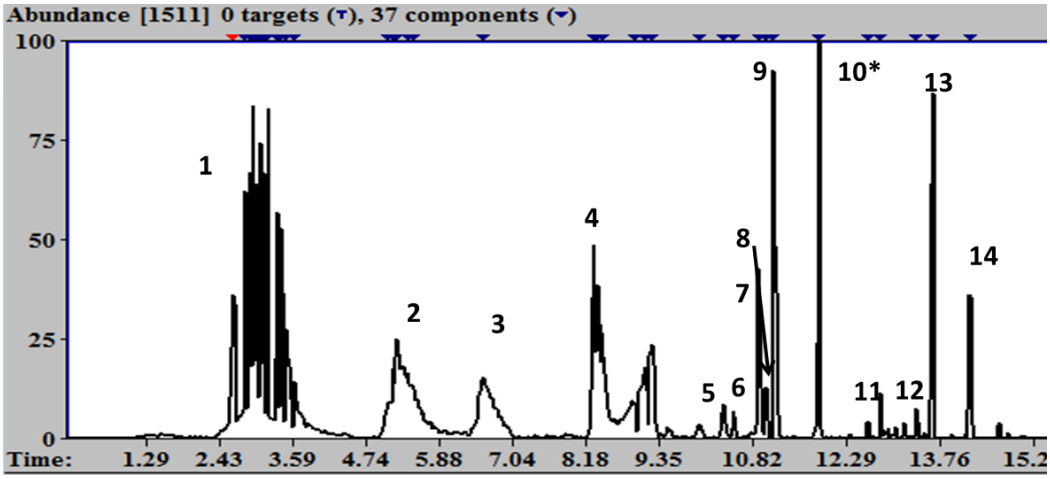


Figure 54. Total ion chromatogram of ML oxidation products after 42 days of incubation at 40 °C, collected on Tenax/carboxen traps after venting from OxiPres™ oxygen bomb.

Table 9. Volatile compounds identified in oxidized methyl linoleate after 42 days at 40 °C collected by thermal desorption via the OxiPres™.

Day 42	Compound	RT	Probablity (%)
1	MW 60 - 100 Abundance	3.00	N/A
2	mixture of pentane/small hydrocarbons	5.22	
3	Acetic Acid	6.58	81.2
4	2-butanone	8.31	78.8
5	1-butanol	10.35	24.5
6	Tetrahydro-2-methylfuran	10.51	93.1
7	2-pentanone	10.91	93.1
8	1-heptene	11.04	43.1
9	pentanal	11.15	52.5
10	Unknown A*	11.84	
11	pentanol	12.83	53.2
12	2-hexanone	13.39	79.5
13	hexanal	13.64	89.4
14	pentyl formate	14.23	63

Also, major products in this system all showed the repeated cycles of product accumulation and decomposition that we have observed in many closed incubation systems, including packaged foods.

Pentane was the dominant early product, accumulating to nearly 150 μg per collection at 7 days then declining to very low levels after ten days (Figure 56). Hexanal began to accumulate as pentane levels declined and became the dominant product after ten days. Unknown A was negligible until after ten days.

These results raise questions about conditions that apparently lead to shifts of β-scission (yielding pentane) to α-scission (yielding hexanal). It seems unlikely that pentane is lost due to poor absorption on the carboxen/tenax trap (traps are fresh each day), loss during venting (high levels were trapped on some days), or overloading of the trap (volatile levels are lower here than in TD experiments). Clearly, the reaction environment in the Oxipres^{TM} is different in unidentified ways that modify the progress of oxidation. This system needs to be investigated further in great detail to determine factors that are affecting lipid oxidation.

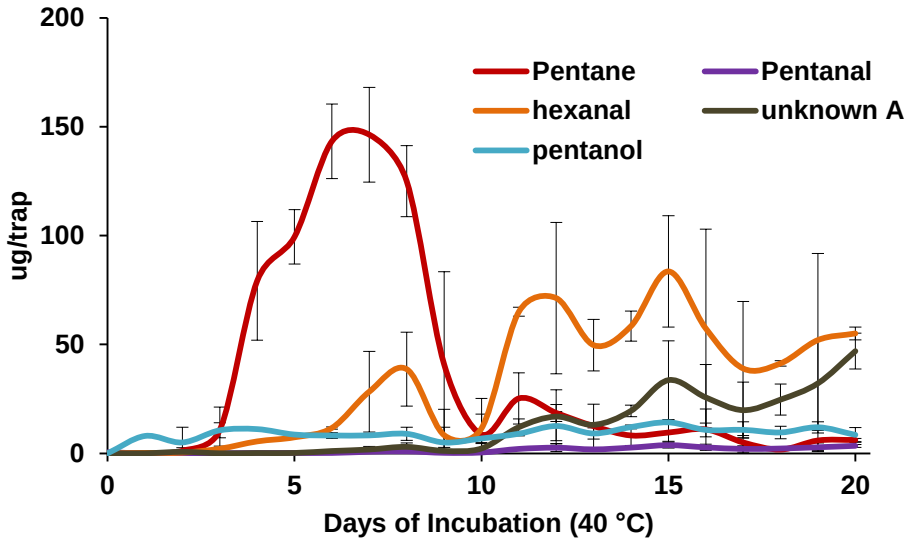


Figure 55. Volatile products from oxidation of methyl linoleate incubated at 40 °C and collected by OxiPres™ .

60 °C. Product formation at 60 °C was comparable to that at 40 ° in the Oxipres except that the timing of product development was moved up and peak product levels were higher, as would be expected from thermal influences (Figure 56). One compound that showed a notable difference in intensity from 40 °C was pentanal[118]. Pentanal has been proposed to form via secondary oxidation of pentane by hydroxyl radicals. This is a very slow process at lower temperatures, but at 60 °C, lipid hydroperoxides decompose homolytically to generate alkoxyl and hydroxyl radicals:

$$LOOH \rightarrow LO^{\bullet} + {}^{\bullet}OH$$

Reaction of $^{\bullet}OH$ with the pentyl scission radical yields pentanol and pentanal, both of which were observed in this experiment:

$$CH_3CH_2CH_2CH_2CH_2^{\bullet} + {}^{\bullet}OH \rightarrow CH_3(CH_2)_4OH + CH_3(CH_2)_3CHO$$

Unknown A was seen at 60 °C at even higher levels than at 40 °C. As noted previously, this observation demonstrates that Unknown A was not an artifact introduced by the Diafil solid support; however, the possibility that it is a derivative product induced on the trap still exists. Unknown B was not identified at either temperature.

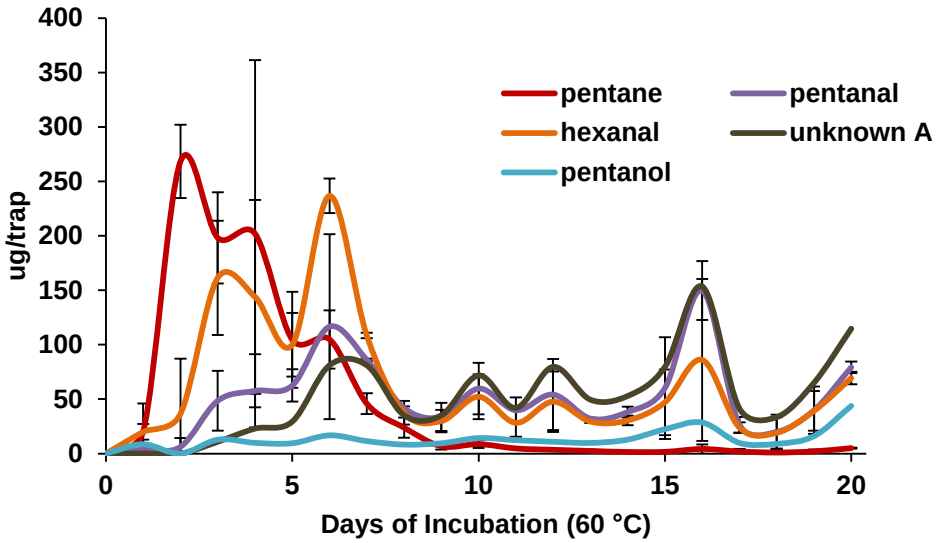


Figure 56. Volatile products from methyl linoleate oxidized at 60 °C in an OxiPres™ oxygen bomb and collected by venting onto a Tenax/carboxen trap.

5.2.2.3 Comparison of SIS Purge and Trap Tubes and Oxygen Bomb

Thermal desorption increases detection sensitivity and eliminates some shortcomings of static head space and SPME analyses. However, differences in results from the Thermal Collection System (TCS) and the Oxipres oxygen bomb highlight some serious issues with using thermal desorption as a collection system when results will be used to derive oxidation mechanisms. First, sampling collection procedure clearly affects total volatile response, total products detected, individual product distributions, and apparently can also intervene in oxidation and alter degradation pathways. Collection time that is too short limits sensitivity but too long risks saturating the trap and increases

both losses and transformation of products that would affect any proposed mechanisms. Second, problems with leaks are common with thermal desorption, during both sample collection and desorption onto the GC column. Thus, important products can be lost, changing the picture of oxidation, and sample reproducibility is usually much lower than SHS or SPME.

Quantitatively, samples oxidized using the TCS showed a much higher volatile response. It is possible that the solid support catalyzed oxidation or decomposition of primary products to secondary volatile compounds. However, it is also likely that the higher detected product levels resulted because the sample was being continually swept for 10-20 minutes and all volatiles were collected and concentrated on the trap. In contrast, only volatiles that had collected in the headspace of the Oxipres were trapped in a single venting.

Qualitatively, except for Unknowns A and B, TCS products were comparable to those detected by SHS and SPME. In contrast, products detected from Oxipres^{TM} venting were predominantly ketones and short chain decomposition products. As noted in the previous subsection, this difference appears to be related to the Oxipres set up, but further detailed investigation will be required to ascertain what is causing the product decomposition.

5.2.2.4 Summary of TD results

Thermal desorption results clearly agreed with SHS and SPME in the dominance of volatile products from C13. However, no longer chain products from C9 or C11 were detected by TD. This was surprising since TD was expected to collect volatile products more exhaustively than SHS or SPME. To test whether this was a volatility issue, ML

was spiked with nonadienal and decadienal in the Oxipres and purged for 10 minutes; large peaks of both products were detected (data not shown), demonstrating that limited volatility did not account for the lack of C9 products. Similarly, lack of C9 products in the Oxipres samples eliminates the possibility that the Diafil was binding longer chain products. Thus, the observation of volatile products only from C13 appears to be real and not experimental artifact. At the present time, we speculate that the difference in products arises because the ML in both thermal desorption systems was dispersed in films rather than being oxidized in bulk.

Another interesting difference observed with TCS was that dominant products changed in sequence over time. Pentane was the first dominant product, and as it declined, hexanal ascended, then pentanal and Unknown A in sequence. Distinguishing this sequence pattern may be uniquely possible with thermal desorption because volatiles are fully removed from the headspace and do not remain to react further with the lipid phase.

Certainly, these changes are inconsistent with a straightforward radical chain reaction driven entirely by hydrogen abstraction. In contrast, the constant shifts among detected products supports the presence and activity of other pathways.

5.3 Discussion

Gas chromatography has been used extensively to study lipid oxidation, both for routine quality control evaluations (especially in hexanal analyses) and for derivation of oxidation mechanisms, as in the extensive body of research from Edwin Frankel and Werner Grosch which has been cited throughout this dissertation.

The current project was initiated to not to test the differences between GC methods (those have been well-documented), but to determine how best to gain information about oxidation reactions, particularly pathways other than the hydrogen abstraction and scission reactions that form the basis for current understanding of lipid oxidation. Having a mechanistic end-goal imposes special requirements on GC analyses for both quantitative and qualitative accuracy. Hence, we expected to examine product distributions, product levels, and kinetics and timing of individual product generation to obtain evidence for active reactions.

Each sampling method for GC has its own limitations. Static headspace has low sensitivity so oxidation must progress more to detect products. SHS is also controlled by headspace equilibrium so high levels of very volatile products can repress volatilization of other products. As a result, product distributions viewed may miss key players that reveal oxidation mechanisms or alternate pathways.

In SPME, compounds and quantities detected are not what was produced but what was bound and concentrated. The fiber used governs selectivity of product adsorption so different fibers give different pictures of overall oxidation. Competition between products for binding further skews product distributions and apparent product dominance. Timing and conditions for adsorption also affect products bound. Potential for modification of products on the SPME fibers has been claimed. Thus, SPME may be useful for detecting compounds not seen in SHS, but apparent relative concentrations cannot be viewed as representative and SPME results, by themselves, should not be used to derive mechanisms.

Thermal desorption theoretically should overcome sensitivity and headspace equilibrium limitations of SHS as well as the specificity limitations of SPME by continual sweeping of headspace with collection and concentration of all products. However, how the sample is handled markedly affects product levels and distributions collected. Oxidation rates and pathways are modified by whether the oil is bubbled directly, loaded onto a solid adsorbent, or spread on a glass surface. Further product modifications from contact with trap sorbents are also possible, and there are losses from poor trapping, leaks, and transformation during desorption, as well. TD system configuration also appears to be critical since samples incubated in the Oxipres showed much more extensive secondary decomposition (source unidentified) than samples analyzed in the SIS thermal collection tubes.

Results of this study show that each analytical technique detects a different major oxidation product and suggests a different kinetics, total extent, and product distribution in ML oxidation (Figure 57Figure 51 and Figure 58). Taken altogether, these observations demonstrate that no one GC method alone – or indeed volatiles analysis alone -- can provide adequate information to derive mechanisms because key information is missing. Accurate assessment of mechanisms and detection of alternate pathways will require integration of multiple GC methods for volatiles as well as integration of information about multiple volatile and non-volatile products. At least some small steps have been achieved in this regard.

Most results from the current project, especially from SHS, suggest that accepted dogma and understanding of lipid oxidation chemistry is probably incomplete. As portrayed extensively in the scientific literature, the mandatory process in lipid oxidation

is establishment of a free radical chain, formation and subsequent decomposition of hydroperoxides, and recombination or scission of radicals to generate final products. If this is true, alkane and ketone recombination products as well as alkane and aldehyde scission products should be readily detectable and quantifiable. However, ketones were detected only in samples from the oxygen bomb; other recombination products, if present, may have been among the unidentified peaks, but they were not obvious or readily identifiable by mass spectrometry.

Similarly, there is a common expectation that equal hydroperoxides are formed at C-9 and C-13 of linoleic acid, and that equal α and β scissions occur for each derivative alkoxyl radical from the hydroperoxides. Hence, equal amounts of octanoate and decadienal (α-scission) plus 9-oxo-nonanoic acid and nonadienal (β-scission) should be produced from C-9 attack, while pentane and a 13-carbon dienal plus hexanal and a 12-carbon product should be produced from C-13 attack. These products were not all observed. Long chain products are not volatile and will not be detected by GC, so it is not surprising that pentane and hexanal were the main products from C-13. However, it was very puzzling why a) only methyl octanoate was detected from C-9 while decadienal (a volatile compound commonly analyzed by GC) was apparently not present -- detected only by SPME and then only in trace concentrations, and b) why *t*-2-heptenal and

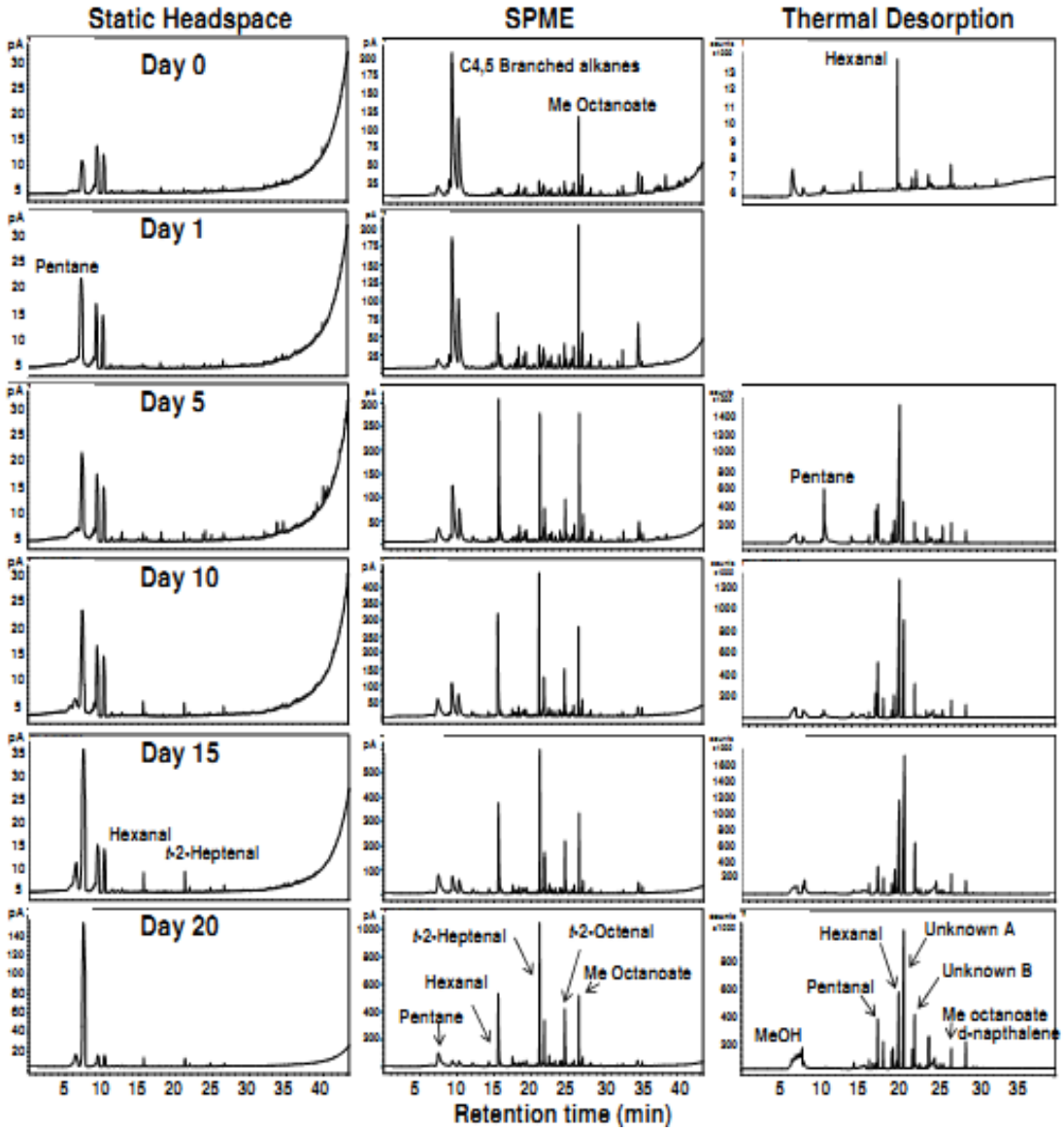


Figure 57. Comparison of chromatograms for SHS, SPME, and TD (TCS) at 40 °C.

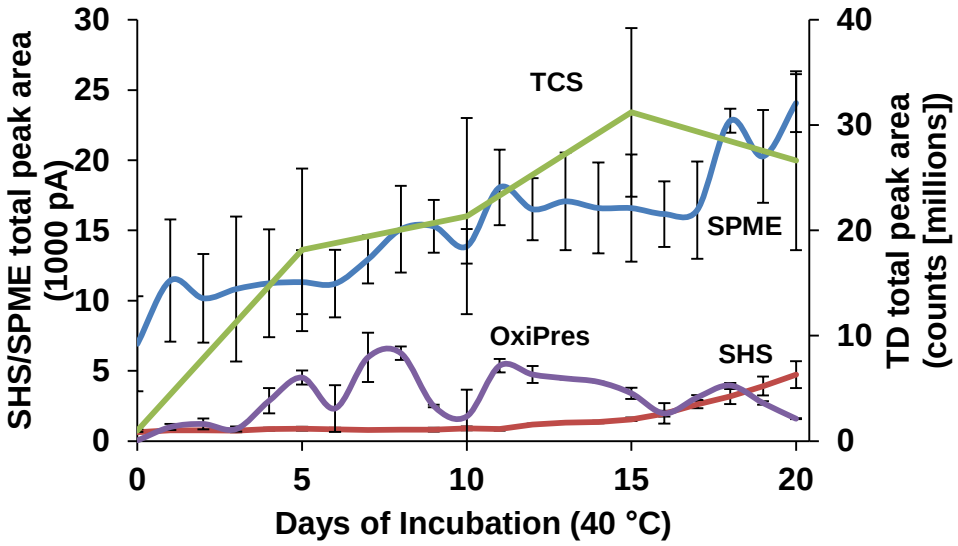


Figure 58. Comparison of total volatile response from methyl linoleate oxidized at 40 °C, detected by SHS, SPME, and TD.

t-2-octenal (scission products from C-11-O$^{\bullet}$) should be present at all if this doubly allylic position is involved in oxidation only as the initial hydrogen abstraction site.

Granted, many products remain unidentified in this study, so judgments about reactions must be limited to only products that have been fully identified by mass spectrometry and by comparison of retention times to authentic standards with FID detection. Nevertheless, even without full identification, the number of products detected and the timing of their development suggest that there must be additional active reaction pathways over and above the main ones predicted.

Total peak areas for all volatile products reveal differences in both oxidation kinetics and product levels detected by the three GC methods for both elevated temperatures used in this study. Results for 60 °C are presented in Figure 59 .

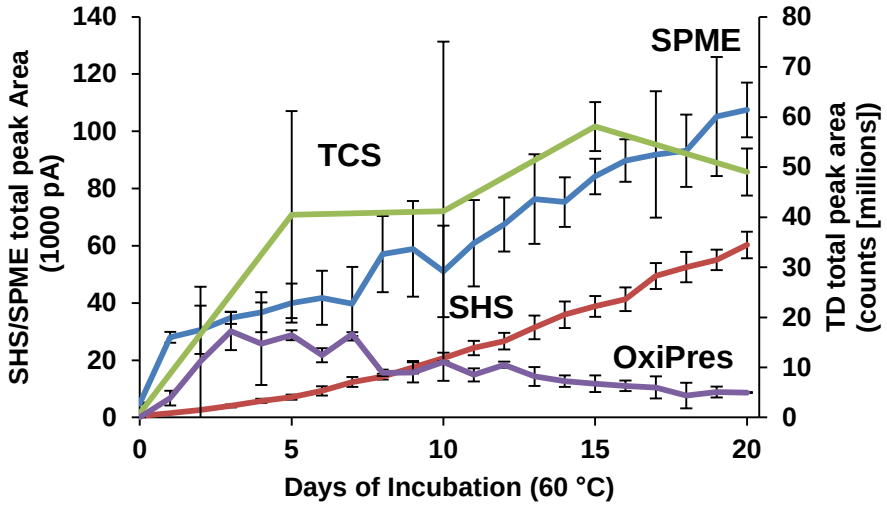


Figure 59. Comparison of total volatile response from methyl linoleate oxidized at 60 °C, detected by SHS, SPME, and TD.

SHS shows only slow and gradual accumulation of products. However, SPME and TD both detect an initial burst of products that could be important precursors to secondary products but are missed by SHS. After the first few days, the SPME product accumulation rate is about the same as for SHS, just at a higher level (parallel curves) so they are probably monitoring comparable processes. In contrast, products detected by TD reach a higher maximum later and begin to drop off before the end of the incubation period. While it could be argued that this pattern results from more exhaustive removal of volatiles from the samples, it could also reflect a very different reaction system in which the solid support and/or the Tenax/carboxen trap bind or modify some of the volatiles. Attempts to eliminate interference from the solid support by repeating the experiment in an oxygen bomb were not successful. The system configuration was too different and

something in the setup led to extensive changes in the ML oxidation, generating high levels of ketones and small scission products. Thus, further investigations are needed to determine an optimum configuration that will allow TD to provide accurate and sensitive data bout ML oxidation processes. For this study, most judgments about reaction pathways will be based on SHS and SPME results.

All of the products detected in this study, except perhaps the two Unknowns from TD, have been reported before and are well-known lipid oxidation products. So what can this study contribute that is new and different? By integrating multiple analytical methods and analysis of volatile and non-volatile products, we hope to provide new insights into reaction pathways, specifically looking for evidence of reaction mechanism other than hydrogen abstraction, as well as for other details that help explain oxidation.

Pentane was by far the most dominant product detected, followed by hexanal. Pentane has been reported extensively in the scientific literature but it is routinely ignored in industrial analyses of lipid oxidation and is undervalued in consideration of oxidation mechanisms. Hexanal was the next most dominant product in most cases (but not all). Both of these products derive from reaction at C-13 in ML and were the earliest seen as well as being quantitatively the most prevalent.

The next product appearing and accumulating was t-2-heptenal, then t-2-octenal and methyl octanoate appeared in order and relative levels that varied with conditions. No long-chain products were detected. Taken at face value, this pattern suggests that, contrary to current understanding of lipid oxidation, initiation starts at C13, progresses to C11, and finally affects C9. This is certainly inconsistent with theory.

To understand these heretical results, it must be remembered that GC detects only volatiles, so we are viewing only part of the oxidation picture. Detailed HPLC analyses of hydroperoxides in ML are currently being conducted to verify which positional and geometric isomers are present. For the present discussion, it will be assumed that both C9 and C13 hydroperoxides are formed in comparable if not equal concentrations. Thus, any imbalance in products from the two positions will occur because secondary and decomposition reactions at the two positions do not follow the same pathways.

A number of observations from this study are inconsistent with current lipid oxidation theory and need to be explained:

- Literature predicts equal C9 and C13 hydroperoxides and equal scission products from each, yet we saw overwhelming dominance of C13 volatiles
- The levels of *t*-2-heptenal and *t*-2-octenal detected in some cases approached (or exceeded) hexanal levels so were much too high to have derived directly from C-11 hydroperoxides, which are unstable and do not form, according to much of the literature.
- C9 α-scission that gives methyl octanoate should also yield equal amounts of decadienal, which was only detected by SPME and then only in trace amounts. Decadienal oxidizes to hexanal and t-2-octenal, but not so fast that the parent compound is immediately transformed and never observed.
- Dominant products and kinetic patterns from ML oxidations change as incubation temperature increases. In particular, hexanal which is used routinely in the food industry as a measure of lipid oxidation decreases relative to other products. Thus,

the assumption that elevated temperatures in accelerated shelf life studies merely mimic room temperature oxidations, only much faster, is erroneous.

- C-9 and C-13 products showed different sensitivity to reduced oxygen.

Finding evidence for actual involvement of the alternate reactions proposed by Schaich was a goal of this study. It is indeed interesting, then, that some alternate reactions may explain most of the inconsistencies outlined above. Epoxides were not detected among the volatile products. However, observations of epoxide levels comparable to hydroperoxides in the parallel study of Jia Xie[114] suggested strongly that reactions involving epoxides should be considered when explaining the chemistry observed.

Closer examination of the timing and levels of octanoate, *t*-2-heptenal and *t*-2-octenal, plus integration of volatile and non-volatile product observations from this dissertation and that of Jia Xie[114]suggest that C-9 and C-13 hydroperoxides undergo separate degradation processes, at least under the conditions of this study. C13 hydroperoxides, which are farthest away from the acid functional group and experience less inductive effect, preferentially go through decomposition and $LO^{\bullet}$ scissions to pentane, hexanal, and some additional short-chain products. In contrast, the two C-C bonds at C-9 are effectively stronger because they are closer to the acid group. Thus, when $LO^{\bullet}$ radicals form from LOOH decomposition, rather than undergo α and β scissions, it is energetically more feasible for them to internally rearrange to epoxides and transfer a radical to C-11.

A plausible reaction sequence for epoxidation at C-9 is shown in Figure 60. Reactions at C-13 are shown in Figure 61. At C-9, internal rearrangement provides an alternate route to methyl octanoate and accounts for lack of typical C-9 scission products from $LO^{\bullet}$ (e.g. 2,4-decadienal) as well as unidentified small products that elute early. It also provides a means of involving C-11 as a secondary process without oxidation chains beginning there. *t*-2-Heptenal and *t*-2-octenal arise from scission of C11-$O^{\bullet}$. That *t*-2-heptenal appears earlier and at higher levels than *t*-2-octenal suggests that β- is preferred over α-scission of C11-$O^{\bullet}$, a pattern that is consistent with increase in β-scission preference as the carbon position moves away from the acid group[119].

The balance between methyl octanoate, *t*-2-heptenal, and *t*-2-octenal is determined by reaction time and conditions. All three products increased with temperature, but at different rates (Table 10). At room temperature and 40 °C, *t*-2-heptenal appeared first and at highest concentrations. Methyl octanoate was present but at lower concentrations because under these conditions, hydrolysis of the epoxide group is slow. *t*-2-Octenal was not detectable at room temperature but grew in at higher temperatures and with longer oxidation times. Methyl octanoate was affected most by increased temperature because it was generated by several reactions. Higher thermal energy should increase α-scission to *t*-2-octenal and hydrolysis of its partner 9,10-epoxide, and it also increases hydrolysis of the oxo-epoxide partner of *t*-2-heptenal.

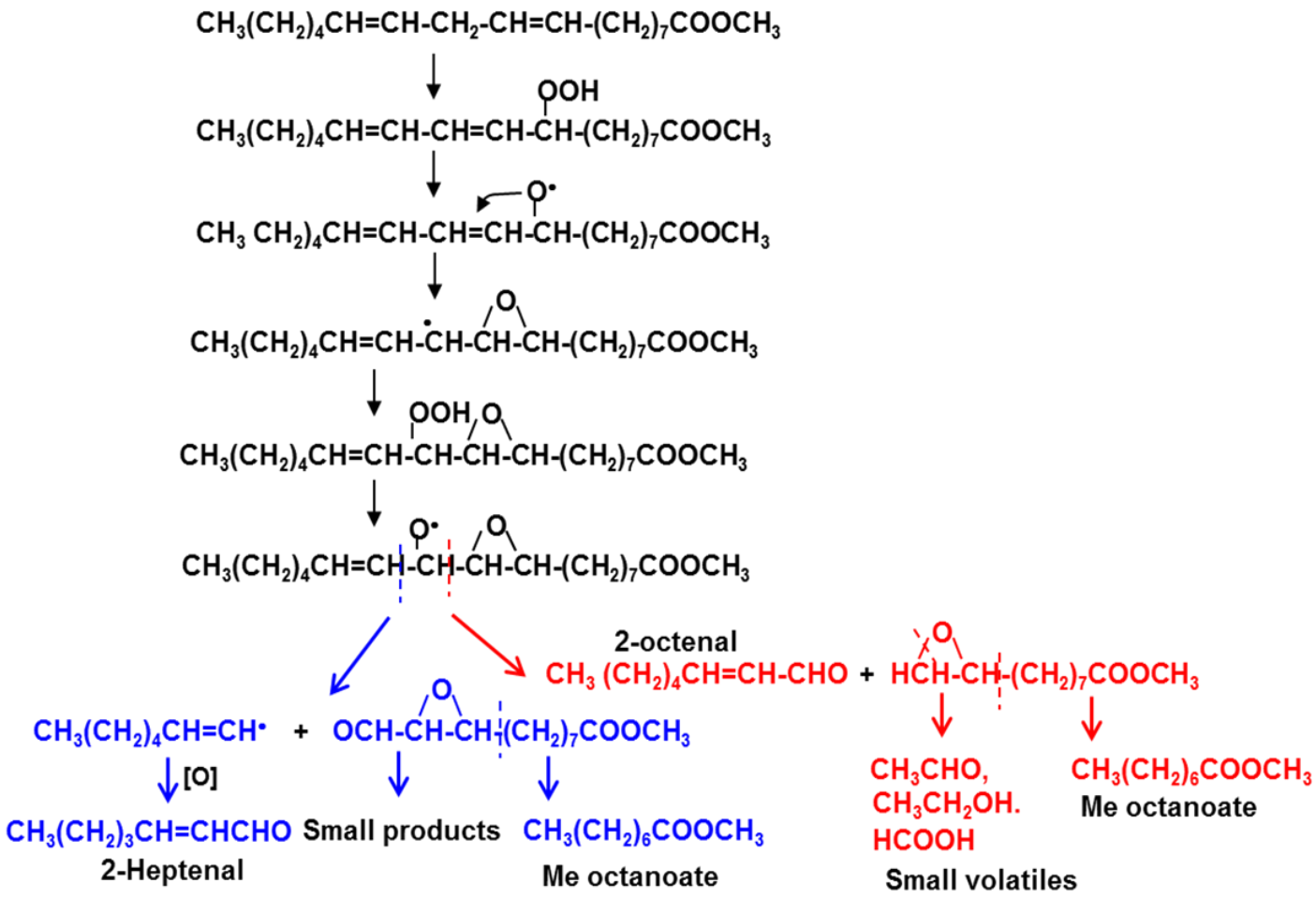


Figure 60. Proposed reaction sequence for internal rearrangement of alkoxyl radicals at C-9 to generate the C-9 and C-11 volatiles observed by gas chromatography.

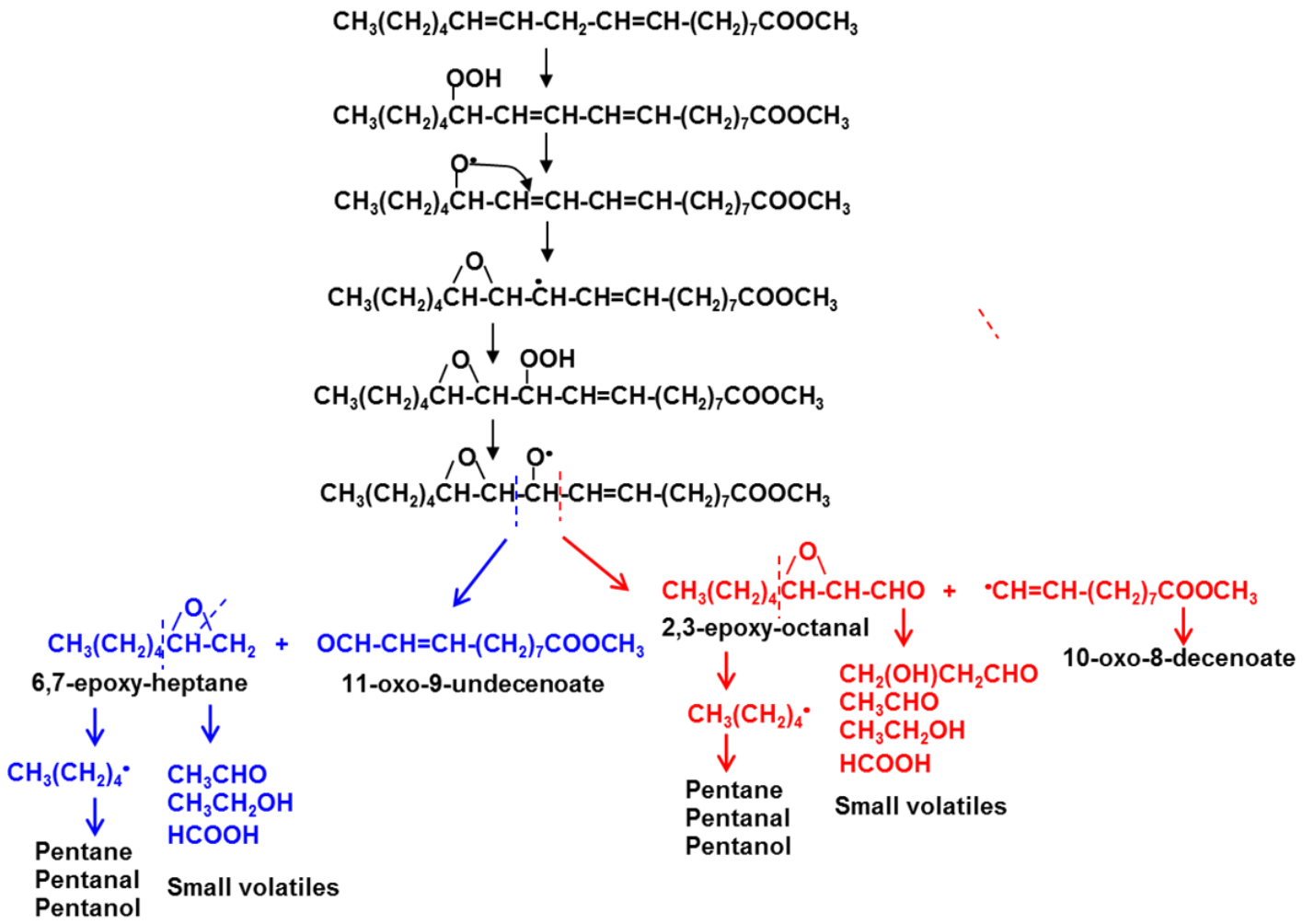


Figure 61. Proposed reaction sequence for internal rearrangement of alkoxyl radicals at C-13 to generate C-13 volatiles observed by gas chromatography.

Table 10. Peak areas (pA) of C-9 and C-11 products from oxidation of methyl linoleate at different temperatures and under different oxygen pressures.

	Hexanal	t-2-Heptenal	Octanoate	t-2-Octenal
T °C 25	10	9	4	0
40	46	30	10	12
60	445	141	166	74

Oxygen levels surprisingly decreased hexanal but had no effect on levels of octanoate, t-2-heptenal, and t-2-octenal (Table 11). This would seem to indicate that precursors for the epoxides are already saturated but enough oxygen remains in the oil to drive C13 oxidation. While the phenomenon needs further investigation, the difference in oxygen sensitivity of C9 vs C13 products supports different processes occurring at the two sites.

Table 11. Peak areas (pA) of C-9 and C-11 products from oxidation of methyl linoleate at different temperatures and under different oxygen pressures.

	Hexanal	t-2-Heptenal	Octanoate	t-2-Octenal
pO_2 5%	144	40	31	27
2%	124	41	30	26
Ar	131	40	35	26

It must be stressed that the alternate reactions proposed in Figure 6 are not mutually exclusive but are expected to be integrative, competing with each other and

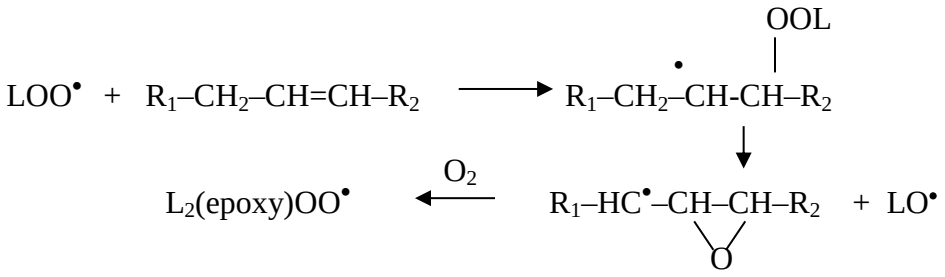
occurring at rates consistent with their individual activation energies. Hence, the internal rearrangements proposed to explain results of this study appear to be the dominant reaction under most conditions investigated, but they are not the only reaction that occurs. Decadienal was detected by SPME when ML was oxidized at elevated temperatures, especially 60 °C, although it was still a very minor product. That it appeared at higher temperatures here and is used in the food industry as a marker of frying oil degradation suggests that the scission generating decadienal requires higher energy than is available under ambient conditions. On the other hand, the scission partner of decadienal, methyl octanoate, occurs at reasonable to high concentrations under even mild oxidation conditions. These patterns certainly support the co-existence of multiple oxidation pathways that shift in balance and dominance as reaction conditions change. By extension, then, accurate analyses of lipid oxidation should require measuring a broad range of products to be able to detect these shifts.

Internal rearrangement to epoxides can also explain products observed from C13 (Figure 61), particularly the huge excess of pentane over other products. Epoxidation occurring in competition with scission of C13-O$^{\bullet}$ eliminates hexanal as a product while retaining pentane and its derivative products. Granted, pentane formation in this case requires hydrolysis of the epoxide so epoxidation is not the main route nor is it the initial reaction. However, since both α and β scission of the secondary epoxy-C11-O$^{\bullet}$ generates pentane, epoxidation occurring in addition to scissions of C13-O$^{\bullet}$ would greatly enhance pentane at the expense of hexanal and contribute to the pentane excess that increased over time in SHS analyses.

The partner compounds from the epoxy-C11-O• scissions are on the borderline of chain lengths expected to be volatile, so they may not be detectable by GC. However, these structures should be evaluated in mass spectrometry libraries, and they certainly should be sought among non-volatile products remaining in the oil phase.

Finally, the reactions proposed above assume that epoxides are formed via alkoxyl radicals, which puts them somewhat downstream in the oxidation process. Analyses of non-volatile products in neat methyl linoleate showed that epoxides began to form at the same time or even before hydroperoxides[114]. Such rapid epoxide generation cannot depend on alkoxyl radicals deriving from hydroperoxide decomposition. Therefore, to complete the evaluation of alternate reactions that may contribute to product patterns observed in this study, epoxide generation by peroxyl radical addition to double bonds must also be considered.

As another alternate reaction, lipid peroxyl radicals can add to double bonds, preferably isolated double bonds, as would occur in the methylene-interrupted configuration of unoxidized ML. The peroxy dimer that results is unstable and decomposes very rapidly to an epoxy compound on the adduct side and an alkoxyl radical in place of the original peroxyl radical:



This process is reportedly very rapid while the rate of initiation is usually slow on a molecular scale. Therefore, if it occurs, it has the effect of transforming less reactive

peroxyl radicals to very reactive alkoxyl radicals in parallel with initiation, and it is a process that bypasses formation of hydroperoxides. For non-radical products, addition provides a source of early epoxides in competition with and independent of hydroperoxides. For this study, peroxyl radical additions provide an early source of alkoxyl radicals as precursors for epoxides, plus epoxides that move radicals to new sites on the acyl chains, thereby generating products not predicted by the traditional hydroperoxide formation-decomposition-scission sequence.

It is interesting to note that addition of a C13-OO$^{\bullet}$ to a C13 double bond could result in a 13,14-epoxide on ML, the decomposition of which might be a source for four carbon products that were observed in this study.

Finally, when comparing results of this study to reports of lipid oxidation processes in the literature, a reminder must be put forward that this study was different in focusing on very early reactions and in monitoring oxidation products daily. Most older studies monitored oxidation products in much more extensively oxidized lipids and allowed relatively long times (weeks to months) to elapse between analyses. Under such conditions, many products that were rapidly changing would have been missed and the secondary products detected would probably have gone through multiple transformations. In contrast, following development of total and individual products daily, as reported here, revealed the differential timing and levels of products as well as inconsistencies with existing dogma. This information was key in indicating the likely presence of alternate reactions.

Results of this study are seminal in suggesting new reactions – peroxyl radical additions and alkoxyl radical rearrangements to epoxides -- that are active and

competitive with hydrogen abstraction and alkoxyl radical scissions in early oxidation and can both change direction of oxidation and account for products that are not predicted by conventional understanding of lipid oxidation.

6. SUMMARY AND INTEGRATION OF DATA

Results of this study suggest current understanding of lipid oxidation is at least incomplete. The dictum that C-9 and C-13 are attacked equally in methyl linoleate oxidation was particularly brought into question based on the strong dominance of C13 products that were detected and expected C9 products that were missing. Three different sampling methods for gas chromatography gave consistent overall pictures of oxidation, although they differed in details, so it is unlikely that the observations are artifacts of analysis. Hydroperoxide positions in ML are being analyzed to verify attack at both C9 and C13 as previously reported. For the purpose of evaluating alternate explanations of the products detected, we assumed approximately equal levels of C9 and C13 hydroperoxides as a starting point. Results of this study provided strong evidence for alkoxyl radical internal rearrangement to epoxides and probably also peroxyl radical addition pathways occurring in competition with or in addition to standard hydrogen abstraction and alkoxyl radical scission, and these reactions must be considered to account for oxidation kinetics and products generated.

Important observations of this study:

1) Even though hydroperoxides may form equally at both C9 and C13 in methyl linoleate, subsequent reactions of the hydroperoxides are not identical. Results of this study suggest that in oxidation of neat ML, internal rearrangement to epoxides dominates at C9, moving radical sites down the acyl chain to C11 in the process, whereas scission preferentially occurs at C13, generating the volatiles that dominated all analyses.

During early incubation, C-13 scission is the dominant pathway for generation of volatile

products, possibly because the C-C bond energy is effectively reduced by distance from the acid group.

2) Static headspace analysis showed pentane as the overwhelming major product, with hexanal at much lower levels even as the second largest peak. Taken at face value, this suggests that at C-13, β-scission (resulting in pentane) is strongly preferred to α-scission (resulting in hexanal). However, this huge discrepancy in product levels are difficult to reconcile based on preference for α vs β scission alone. Theoretical consideration of alternate pathways of C13-O$^{\bullet}$ reveal that internal rearrangement to epoxides provides a second source of pentane and its derivatives. Coexistence of scission and internal rearrangement pathways will contribute more pentane to the product mix and is a more defensible explanation of both the high levels of pentane and its increased accumulation over time.

3) The appearance of *t*-2-heptenal and *t*-2-octenal over time and at higher temperatures suggested direct oxidative attack at C-11, which has been discounted in the literature. Similarly, appearance of methyl octanoate as an important product along, under some conditions in excess of hexanal, suggested scission reactions of C9-O$^{\bullet}$, but the scission pair, 2.4-decadienal was not detected. These two apparent disparities can be explained by internal rearrangement of C9-O$^{\bullet}$ to epoxides with concurrent transfer of the free radical down-chain to C-11. Normal oxidation sequences at this site result in C11-O$^{\bullet}$ which undergo β-scission to generate 2-heptenal and α-scission to generate 2-octenal plus epoxide scission pairs. Hydrolysis of both epoxide functions yields methyl octanoate. All of these reactions bypass formation of C9 hydroperoxides and scission of

C9 alkoxyl radicals. Hence, analysis protocols that look for decadienal as the "traditional" expected product will miss the oxidation altogether.

4) Numbers of products appeared to be relatively constant over time, determined more by detection method than reaction conditions, while product concentrations increased over time. This suggests that reaction pathways may be set from the beginning of oxidation and that products just accumulate to fill in existing pathways. Clearly products change over time, so the total number of products remains constant by decline of some products while others are generated.

5) Accelerated shelf-life oxidations at 60 °C change reaction pathways as well as kinetics so should not be used to predict reactions under ambient conditions.

6) Many more products were detected than could be identified, and some products (e.g. *t*-2-hexenal) were detected but could not be accounted for by standard reactions. In order to fully elucidate lipid oxidation reactions, considerable effort needs to be focused on full identification of products and use of computer modeling to predict products that would be generated from various alternate reactions. This will be especially important for studies of more unsaturated fatty acids where product mixes will become very complex.

7) Focus of this study on analysis of volatile products to derive information about lipid oxidation processes shows clearly that using volatile products as the only assay of lipid oxidation can be inaccurate for both quality control and determination of mechanisms. Indeed, using only one of any assays causes problems because each assay provides information about specific products but none represents the total oxidation. For example, data from the parallel study monitoring non-volatile products showed a decrease in hydroperoxides as incubation temperature increased[114], but volatile response

from the same systems was opposite this. Furthermore, not all breakdown products are volatile and many are not detected in current assays (e.g. hydroxylated products). Thus, analyses of lipid oxidation should move to integration of assays for multiple products, both volatile and non-volatile, as standard protocol to gain the accurate pictures of lipid oxidation.

7. FUTURE WORK

While this work establishes the groundwork for investigating alternate pathways of lipid oxidation, more work is needed to fully elucidate oxidation pathways and products.

Further studies of SHS, SPME and TD are needed to determine how best to obtain results that are sensitive and both quantitatively and qualitatively accurate.

Now that alternate pathways have been demonstrated, experiments need to move to systems that can provide complete product identification so that additional pathways can be tracked and all pathways can be balanced.

Further clarification is needed to understand the apparent preference for scissions at C-13 and internal rearrangement to epoxides at C-9, plus conditions that shift the balance between α and β scissions of alkoxyl radicals.

More detailed studies of water, solvent, and other environmental conditions need to be completed to determine what physical conditions, if any, force shifts in reaction pathways.

Eventually, these mechanistic studies must be extended to more complex systems such as triacylglycerols and n-3 fatty acids.

8. REFERENCES

1. Farmer EH, Koch HP, Sutton DA. The course of autoxidation reactions in polyisoprenes and allied compounds. VII. Rearrangement of double bonds during autoxidation. *J Chem Soc*. 1943:541-547.
2. Farmer EH. Peroxidation in relation to olefinic structure. *Trans Faraday Soc*. 1946;42:228-236.
3. Bolland JL. Kinetics of olefin oxidation. *Q Rev Chem Soc*. 1949;3(1):1-21.
4. Swern D. Primary products of olefinic autoxidation. In: Lundberg WO, ed. *Autoxidation and Antioxidants*. New York: Interscience; 1961:1-54.
5. Uri N. Physico-chemical aspects of autoxidation. In: Lundberg W, ed. *Autoxidation and Antioxidants*. New York: Interscience; 1961:55-106.
6. Kochi J. Oxygen Radicals. In: Kochi J., ed. *Free Radicals, Vol.2*. New York: John Wiley; 1973:665-710.
7. Scott G. *Atmospheric Oxidation and Antioxidants*. London: Elsevier; 1965.
8. Ingold KU. Peroxy Radicals. *Acc Chem Res*. 1969;2:1-9.
9. Frankel E. Hydroperoxides. In: Schultz H, Day ED, Sinnhuber W., eds. *Symposium on Foods: Lipids and Their Oxidation*. Westport, CT: AVI Publishing; 1962:51-76.
10. Porter NA, Lehman LS, Wujek D. Oxidation mechanisms of poly-unsaturated fatty acids. Bors W, Saran M, Tait D, eds. *Oxyg Radicals Chem Biol*. 1984:235-237.
11. Porter N. Mechanisms for the autoxidation of polyunsaturated lipids. *Acc Chem Res*. 1986;105(12):262-268.
12. Schaich KM. Lipid Oxidation: Theoretical Aspects. In: Shahidi F, ed. *Bailey's Industrial Oil and Fat Products*. NY: John Wiley & Sons; 2005:269-310.
13. Farmer E., Bloomfield GF, Sundralingham A, Sutton DA. The course and mechanism of autoxidation reactions in olefinic and polyolefinic substances, including rubber. *Trans Faraday Soc*. 1942;38:348-356.
14. Bolland JL. Kinetic studies in the chemistry of rubber and related materials. I. The thermal oxidation of ethyl linoleate. *Proc R Soc A Math Phys Eng Sci*. 1946;186(1005):218-236.
15. Nawar W. Thermal degradation of lipids. *J Agric Food Chem*. 1969;17(1):18-21.
16. Morales J.J.; Aparicio, R. MTR. Changes in the volatile composition of virgin olive oil during oxidation: flavors and off-Flavors. *J Agric Food Chem*. 1997;45(7):2666-2673.
17. Kaplan ML. "Singlet" oxygen. *Chem Tech*. 1971:621-626.
18. Yang W, Min DB. Chemistry of Singlet Oxygen Oxidation of Foods. In: Ho C-T, Hartman T, eds. *Lipids in Food Flavors*.; 1994:15-29.
19. Schaich KM. Metals and lipid oxidation. Contemporary issues. *Lipids*. 1992;27(3):209-218.
20. Tian X. Factors affecting stability of frying oils. 2013.
21. Choe E, Min D. Mechanisms and factors for edible oil oxidation. *Compr Rev Food Sci Food Saf*. 2006;5:169-186.
22. Lee J, Min DB. Analysis of volatile compounds from chlorophyll photosensitized linoleic acid by headspace solid-phase microextraction (HS-SPME). *Food Sci Biotechnol*. 2010;19(3):611-6116.

23. Erben-Russ M, Michel C, Bors W, Saran M. Absolute rate constants of alkoxyl radical reactions in aqueous solution. *J Phys Chem*. 1987;91(9):2362-2365.
24. Labuza TP, Dugan LR. Kinetics of lipid oxidation in foods. *C R C Crit Rev Food Technol*. 1971;2(3):355-405.
25. Lindsay D, Howard J. The bimolecular self-reactions of secondary peroxy radicals. Product studies. *Can J Chem*. 1973;(10):870-880.
26. Porter NA, Lehman LS, Weber BA, Smith KJ. Unified mechanism for polyunsaturated fatty acid autoxidation. Competition of peroxy radical hydrogen atom abstraction, .beta.-scission, and cyclization. *J Am Chem Soc*. 1981;103(21):6447-6455.
27. Schaich K. Lipid Oxidation in Specialty Oils. In: Shahidi F, ed. *Nutraceutical and Specialty Lipids and Their Co-Products*. Boca Raton: CRC Press; 2006.
28. Kochi J. *Free Radicals*. (Kochi J, ed.). New York: John Wiley & Sons; 1973:665-710.
29. Barriuso B, Astiasarán I, Ansorena D. A review of analytical methods measuring lipid oxidation status in foods: a challenging task. *Eur Food Res Technol*. 2012;236(1):1-15.
30. Siouffi AM. HPLC. In: Nollet LM., ed. *Food Analysis by HPLC*.Vol 16. 2nd ed. NY: CRC Press; 2000:1-55.
31. Marini D. HPLC of Lipids. In: Nollet LM., ed. *Food Analysis by HPLC*. 2nd ed. New York; 2000:169-251.
32. Grob RL. Introduction. In: Grob, Robert L; Barry EF, ed. *Modern Practice of Gas Chromatography*. 4th ed. Hoboken, New Jersey: John Wiley & Sons; 2004:1-24.
33. McNair Miller, M. James HM. *Basic Gas Chromatography*. 2nd ed. Hoboken, New Jersey: John Wiley & Sons; 2009.
34. McCloskey DH, Hawkes SJ. Choosing standard stationary phases for gas chromatography. *J Chromatogr Sci*. 1975;13(1):1-5.
35. Jennings W, Mittlefehldt E, Stremple P. Selected Applications. In: *Analytical Gas Chromatography*. 2nd ed. London: Academic Press; 1997:286-307.
36. Snyder JM, Frankel EN, Selke E. Capillary gas chromatographic analyses of headspace volatiles from vegetable oils. *J Am Oil Chem Soc*. 1985;62(12):1675-1679.
37. Snow, Nicholas H.; Slack GC. Sample Preparation Techniques for Gas Chromatography. In: Grob, Robert L; Barry EF, ed. *Modern Practice of Gas Chromatography*. 4th ed. Hoboken, New Jersey: John Wiley & Sons; 2004:547-604.
38. Wampler TP. Analysis of Food Volatiles Using Headspace-Gas Chromatographic Techniques. In: Marsili R, ed. *Flavor, Fragrance and Odor Analysis*. New York: Marcel Dekker; 2002:25-55.
39. Medina I, Satue-Gracia M, Frankel E. Static headspace gas chromatographic analyses to determine oxidation of fish muscle lipids during thermal processing. *J Am Oil Chem Soc*. 1999;76(2):231-236.
40. Pawliszyn J. SPME Commercial Devices and Fibre Coatings. In: *Handbook of Solid Phase Microextraction*. Beijing: Chemical Industry Press of China; 2009:99-133.

41. Zhang Z, Pawliszyn J. Headspace solid-phase microextraction. *Anal Chem*. 1993;65(14):1843-1852.
42. Vas G, Vékey K. Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis. *J Mass Spectrom*. 2004;39(3):233-254.
43. Pastorelli S, Valzacchi S, Rodriguez A, Simoneau C. Solid-phase microextraction method for the determination of hexanal in hazelnuts as an indicator of the interaction of active packaging materials with food aroma compounds. *Food Addit Contam*. 2006;23(11):1236-1241.
44. Roberts DD, Pollien P, Milo C. Solid-phase microextraction method development for headspace analysis of volatile flavor compounds. *J Agric Food Chem*. 2000;48(6):2430-2437.
45. Page BD, Lacroix G. Analysis of volatile contaminants in vegetable oils by headspace solid-phase microextraction with Carboxen-based fibres. *J Chromatogr A*. 2000;873(1):79-94.
46. Wardencki W, Curyło J, Namieśnik J. Trends in solventless sample preparation techniques for environmental analysis. *J Biochem Biophys Methods*. 2007;70(2):275-288.
47. Marsili R. SPME Comparison Studies and What They Reveal. In: Marsilli R, ed. *Flavor, Fragrance and Odor Analysis*. 1st ed. New York: Marcel Dekker; 2002:205-229.
48. Grote C, Pawliszyn J. Solid-phase microextraction for the analysis of human breath. *Anal Chem*. 1997;69(4):587-596.
49. Marsili RT. SPME-MS-MVA as an electronic nose for the study of off-flavors in milk. *J Agric Food Chem*. 1999;47(2):648-654.
50. Zhou A, McFeeters RF. Volatile compounds in cucumbers fermented in low-salt conditions. *J Agric Food Chem*. 1998;46(6):2117-2122.
51. Jiménez J., Bernal J., del Nozal M., Martín M., Mayorga A. Solid-phase microextraction applied to the analysis of pesticide residues in honey using gas chromatography with electron-capture detection. *J Chromatogr A*. 1998;829(1-2):269-277.
52. Adams A, Van Lancker F, De Meulenaer B, Owczarek-Fendor A, De Kimpe N. On-fiber furan formation from volatile precursors: a critical example of artefact formation during solid-phase microextraction. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2012;897:37-41.
53. Supelco. *Solid Phase Microextraction Troubleshoting Guide*. Bellefonte, PA; 2004:1-12. https://www.sigmaaldrich.com/content/dam/sigma-aldrich/docs/Supelco/General_Information/1/t101928.pdf.
54. Curvers J, Noy T, Cramers C, Rijks J. Possibilities and limitations of dynamic headspace sampling as a pre-concentration technique for trace analysis of organics by capillary gas chromatography. *J Chromatogr A*. 1984;289:171-182.
55. Swinnerton JW, Linnenbom VJ, Cheek CH. Determination of dissolved gases in aqueous solutions by gas chromatography. *Anal Chem*. 1962;34(4):483-485.
56. Manura JJ., Hartman TG. Applications of a short-path: thermal desorption GC accessory. *Am Lab*. 24(8):46-52.

57. Grimm C, Lloyd S, Miller J, Spanier AM. The Analysis of Food Volatiles Using Direct Thermal Desorption. In: Marsili R, ed. *Flavor, Fragrance and Odor Analysis*. New York: Marcel Dekker; 2002:55-75.
58. Dupuy HP, Rayner ET, Wadsworth JI, Legendre MG. Analysis of vegetable oils for flavor quality by direct gas chromatography. *J Am Oil Chem Soc*. 1977;54(10):445-449.
59. Eri S, Khoo BK, Lech J, Hartman TG. Direct thermal desorption−gas chromatography and gas chromatography−mass spectrometry profiling of hop (Humulus lupulus L.) essential oils in support of varietal characterization. *J Agric Food Chem*. 2000;48(4):1140-1149.
60. Spanier AM., Grimm CC., Miller JA. Sulfur-containing flavor compounds in beef: Are they really present or are they artifacts? *ACS Symp Ser 558*. 1994:78-97.
61. Block E. Flavor artifacts. *J Agric Food Chem*. 1993;41(4):692-692.
62. Trojakova L, Reblova Z, Pokorny J. Determination of oxidative stability in mixtures of edible oil with nonlipidic substances. *Czech J Food Sci*. 1999;19(1):19-23.
63. Manzini S, Durante C, Baschieri C, et al. Optimization of a dynamic headspace-thermal desorption-gas chromatography/mass spectrometry procedure for the determination of furfurals in vinegars. *Talanta*. 2011;85(2):863-869.
64. Cavalli J-F, Fernandez X, Lizzani-Cuvelier L, Loiseau A-M. Comparison of static headspace, headspace solid phase microextraction, headspace sorptive extraction, and direct thermal desorption techniques on chemical composition of French olive oils. *J Agric Food Chem*. 2003;51(26):7709-7716.
65. Montero L, Popp P, Paschke A, Pawliszyn J. Polydimethylsiloxane rod extraction, a novel technique for the determination of organic micropollutants in water samples by thermal desorption–capillary gas chromatography–mass spectrometry. *J Chromatogr A*. 2004;1025(1):17-26.
66. Rodríguez-Navas C, Forteza R, Cerdà V. Use of thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) on identification of odorant emission focus by volatile organic compounds characterisation. *Chemosphere*. 2012;89(11):1426-1436.
67. Tienpont B, David F, Benijts T, Sandra P. Stir bar sorptive extraction-thermal desorption-capillary GC–MS for profiling and target component analysis of pharmaceutical drugs in urine. *J Pharm Biomed Anal*. 2003;32(4-5):569-579.
68. Akoto L, Vreuls RJJ, Irth H, Pel R, Stellaard F. Fatty acid profiling of raw human plasma and whole blood using direct thermal desorption combined with gas chromatography-mass spectrometry. *J Chromatogr A*. 2008;1186(1-2):365-371.
69. Hartvigsen K, Lund P, Hansen LF, Holmer G. Dynamic headspace gas chromatography/mass spectrometry characterization of volatiles produced in fish oil enriched mayonnaise during storage. *J Agric Food Chem*. 2000;48(10):4858-4867.
70. Khan NA, Lundberg W., Holman R. Displacement analysis of lipids. IX. Products of the oxidation of methyl linoleate. 1954;17(9).

71. Hoffmann G. 1-Octen-3-ol and its relation to other oxidative cleavage products from esters of linoleic acid. *J Am Oil Chem Soc*. 1962;39:439-444. doi:10.1007/BF02637660.
72. Horvat RJ, McFadden WH., Ng. H;, Lane WG, Shepherd A. Identification of 2, 4, 6-trialkyl-1, 3, 5-trioxanes from autoxidized methyl linoleate by mass spetrometry. 1965;43:350-351.
73. Karel M, Schaich K, Roy RB. Interaction of peroxidizing methyl linoleate with some proteins and amino acids. *J Agric Food Chem*. 1975;23(2):159-163. http://www.ncbi.nlm.nih.gov/pubmed/1133287.
74. Frankel E, Neff W., Rohwedder W. Analysis of autoxidized fats by gas chromatography-mass spectrometry: II. Methyl linoleate. *Lipids*. 1977;12(11):908-913.
75. Chan HW, Prescott F, Swoboda P. Thermal decomposition of individual positional isomers of methyl linoleate hydroperoxide: Evidence of carbon-oxygen bond scission. *J Am Oil Chem Soc*. 1976;53(9):572-576.
76. Gordon MH. Lipids. In: Gordon MH, ed. *Principles and Applications of Gas Chromatography in Food Analysis*. West Sussex; 1990:145-176.
77. Bors W, Erben-Russ M, Saran M. Fatty acid peroxyl radicals: Their generation and reactivities. *Bioelectrochemistry Bioenerg*. 1987;18:37-49.
78. Frankel EN, Neff WE, Rohwedder WK, Khambay BP, Garwood RF, Weedon BC. Analysis of autoxidized fats by gas chromatography-mass spectrometry: II. Methyl linoleate. *Lipids*. 1977;12(11):908-913.
79. Frankel E, Neff W, Bessler T. Analysis of autoxidized fats by gas chromatography-mass spectrometry: V. Photosensitized oxidation. *Lipids*. 1979;14(12):961-967.
80. Frankel, EN; Neff, WE; Rohwedder, WK; Khambay, BPS, Garwood RWB. Analysis of autoxidized fats by gas chromatography-mass spectrometry: III. methyl linolenate. *Lipids*. 1977;12(12):1055-1061.
81. Frankel EN. Volatile lipid oxidation products. *Prog Lipid Res*. 1982;22:1-33.
82. Frankel EN. Review. Recent advances in lipid oxidation. *J Sci Food Agric*. 1991;54(4):495-511.
83. Jung D-M, Ebeler SE. Headspace solid-phase microextraction method for the study of the volatility of selected flavor compounds. *J Agric Food Chem*. 2003;51(1):200-205.
84. Snyder JM., Frankel EN., Selke E., Warner K. Comparison of gas chromatographic methods for volatile lipid oxidation compounds in soybean oil. *JAOCS*. 1988;65(10):1617-1620.
85. Tallman KA, Pratt DA, Porter NA. Kinetic products of linoleate peroxidation: rapid β-fragmentation of nonconjugated peroxyls. *J Am Chem Soc*. 2001;123(47):11827-11828.
86. Pratt DA, Mills JH, Porter NA. Theoretical calculations of carbon-oxygen bond dissociation enthalpies of peroxyl radicals formed in the autoxidation of lipids. *J Am Chem Soc*. 2003;125(19):5801-5810.
87. Yin H, Porter NA. New insights regarding the autoxidation of polyunsaturated fatty acids. *Antioxid Redox Signal*. 2004;7(1-2):170-184.

88. Pratt DA, Tallman KA, Porter NA. Free radical oxidation of polyunsaturated lipids: New mechanistic insights and the development of peroxyl radical clocks. *Acc Chem Res*. 2011;44(6):458-467.
89. Frankel E, Neff W, Selke E. Analysis of autoxidized fats by gas chromatography-mass spectrometry: VII. Volatile thermal decomposition products of pure hydroperoxides from autoxidized and. *Lipids*. 1981;16(5):279-285.
90. Frankel E. Chemistry of Autoxidation: Mechanism, Products and Flavor Significance. In: *Flavor Chemistry of Fats and Oils*. Champaign, Illinois: American Oil Chemists' Society; 1985:1-34.
91. Evans C, List G, Hoffmann R, Moser H. Edible oil quality as measured by thermal release of pentane. *J Am Oil Chem Soc*. 1969;46:501-504.
92. Dillard CJ, Tappel AL. Volatile hydrocarbon and carbonyl products of lipid peroxidation: A comparison of pentane, ethane, hexanal, and acetone as in vivo indices. *Lipids*. 1979;14(12):989-995.
93. Petersen KD, Kleeberg KK, Jahreis G, Fritsche J. Assessment of the oxidative stability of conventional and high-oleic sunflower oil by means of solid-phase microextraction-gas chromatography. *Int J Food Sci Nutr*. 2012;63(2):160-169.
94. Horvat R, Lane W, Ng H, Shepherd A. Saturated hydrocarbons from autoxidizing methyl linoleate. *Nature*. 1964;203:523-524.
95. Shahidi, Fereidoon; Pegg RB. Hexanal as an indicator of meat flavor deterioration. *J Food Lipids*. 1994;1(3):177-186.
96. Pastorelli S, Torri L, Rodriguez A, Valzacchi S, Limbo S, Simoneau C. Solid-phase micro-extraction (SPME-GC) and sensors as rapid methods for monitoring lipid oxidation in nuts. *Food Addit Contam*. 2007;24(11):1219-1225.
97. Murat C, Gourrat K, Jerosch H, Cayot N. Analytical comparison and sensory representativity of SAFE, SPME, and purge andtrap extracts of volatile compounds from pea flour. *Food Chem*. 2012;135(3):913-920.
98. Ross CF, Smith DM. Use of volatiles as indicators of lipid oxidation in muscle foods. *Compr Rev Food Sci Food Saf*. 2006;5:18-25.
99. Nielsen NS, Yang T, Xu X, Jacobsen C. Production and oxidative stability of a human milk fat substitute produced from lard by enzyme technology in a pilot packed-bed reactor. *Food Chem*. 2006;94(1):53-60.
100. Frankel EN, Neff WE, Rohwedder WK, Khambay BP, Garwood RF, Weedon BC. Analysis of autoxidized fats by gas chromatography-mass spectrometry: I. Methyl oleate. *Lipids*. 1977;12(11):901-907.
101. Frankel EN, Neff WE, Selke E, Brooks DD. Thermal and metal-catalyzed decomposition of methyl linolenate hydroperoxides. *Lipids*. 1987;22(5):322-327.
102. Ullrich F, Grosch W. Identification of the most intense odor compounds formed during autoxidation of methyl linolenate at room temperature. *J Am Oil Chem Soc*. 1988;65(8):1313-1317.
103. Blank I. Gas Chromatography-Olfactometry in Food Aroma Analysis. In: Marsili R, ed. *Flavor, Fragrance and Odor Analysis*. Marcel Dekker; 2002:297-333.
104. Lee YS, Shin HS, Han JK, Lee M, Giacin JR. Effectiveness of antioxidant-impregnated film in retarding lipid oxidation. *J Sci Food Agric*. 2004;84(May):993-1000.

105. Wessling C, Nielsen T, Giacin J. Antioxidant ability of BHT- and alpha-tocopherol-impregnated LDPE film in packaging of oatmeal. *J Sci Food Agric*. 2001;81(2):194-201.
106. Sunesen L., Dorigoni V, Zanardi E, Stahnke L. Volatile compounds released during ripening in Italian dried sausage. *Meat Sci*. 2001;58(1):93-97.
107. Dupay H, Bailey M, St. Angelo A, Vercellotti J, Legendre M. Instrumental analsysis of volatiles related to warmed-over flavor of cooked meats. In: St. Angelo A, Bailey M, eds. *Flavor Quality of Fresh Meats: Warmed over Flavor*. Orlando, Fla: Academic Press; 1987:165-190.
108. Shahidi F, Pegg RB. Hexanal as an Indicator of the Flavor Deterioration of Meat and Meat Products. In: Ho C-T, Hartman TG, eds. *Lipids in Food Flavors*. 1994th ed. USA: American Chemical Society; 1994:256-279.
109. Horvat, R.J; McFadden, W.H.; Ng, Hawkins; Black, D.R., Lane, W.G, Teeter RM. Volatile products from mild oxidation of methyl linoleate. analysis by combined mass spectrometry-gas chromatography. *J Am Oil Chem Soc*. 1965;42(12):1112-1115.
110. Zhang W, Sun M, Salomon RG. Preparative singlet oxygenation of linoleate provides doubly allylic dihydroperoxides: Putative intermediates in the generation of biologically active aldehydes in vivo. *J Org Chem*. 2006;71(15):5607-5615.
111. Haslbeck F, Grosch W, Firl J. Formation of hydroperoxides with unconjugated diene systems during autoxidation and enzymic oxygenation of linoleic acid. *Biochim Biophys Acta*. 1983;750(1):185-193.
112. Du G, Tekin A, Hammond E, Wood L. Catalytic epoxidation of methyl linoleate. *J Am Oil Chem Soc*. 2004;81(4):3-6.
113. Haynes RK, Vonwiller SC. Iron(III) and copper(II) catalysed transformations of fatty acid hydroperoxides: efficient generation of peroxy radicals with copper(II) trifluoromethanesulphonate. *J Chem Soc Chem Commun*. 1990;(16):1102.
114. Xie J. Evidence for Multiple Oxidation Pathways from Non-Volatile Products of Methyl Linoleate. 2015.
115. Thermal Desorption Sample Collection System. *Serv Sci Instrum*. http://www.sisweb.com/sptd/sampoven.htm. Accessed February 27, 2015.
116. A/S MA. ML Oxipres Instruction Manual. 2012:1-15. http://www.mikrolab.dk/Admin/Public/Download.aspx?file=Files/Filer/PDF/Manualer/Oxipres.pdf.
117. Adams R. *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*. 4th ed. Carol Stream, IL: Allured Publishing Corporation; 2007.
118. Frankel EN. Hydroperoxide Decomposition. In: *Lipid Oxidation*. Elsevier Science; 2005:67-99.
119. Selke E, Frankel EN, Neff WE. Thermal decomposition of methyl oleate hydroperoxides and identification of volatile components by gas chromatography-mass spectrometry. *Lipids*. 1978;13(7):511-513.
120. Avila D V, Brown CE, Ingold KU, Lusztyk J, Ingold U. Solvent effects on the competitive. beta.-scission and hydrogen atom abstraction reactions of the

cumyloxyl radical. Resolution of a long-standing problem. *J Am Chem Soc*. 1993;115(1):466-470.

121. Supelco. *Capillary GC Column Choices for Residual Solvent Analyses Using Direct Injection or Solid Phase Microextraction* (SPME)*. Bellefonte, PA; 2004:1-12.