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EVIDENCE FOR MULTIPLE OXIDATION PATHWAYS FROM NON-VOLATILE
PRODUCTS OF METHYL LINOLEATE

By

JIA XIE

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ABSTRACT OF THE DISSERTATION

Evidence For Multiple Oxidation Pathways From Non-Volatile Products Of Methyl

Linoleate

by JIA XIE

Dissertation Director:
Professor Karen M. Schaich

Oxidation of unsaturated lipids is the most active chemical reaction leading to deterioration of food quality and shortening of shelf life. Lipid oxidation is known to be a free radical chain reaction driven by lipid peroxy and alkoxy radicals abstracting hydrogen atoms from neighboring molecules to form stable hydroperoxide intermediates and transfer a radical to a new molecule. In this process, products accumulate only after hydroperoxides decompose. However, this simplistic reaction fails to account for observed kinetics and compounds generated. Recently, a more complex reaction scheme for lipid oxidation was proposed which integrates traditional hydrogen abstraction with alternate reactions of peroxy and alkoxy radicals -- internal rearrangement, double bond addition, scission, dismutation. The alternate reactions run simultaneously and in competition with hydrogen abstraction, and alter the overall picture of lipid oxidation under different conditions. This dissertation research seeks to provide experimental proof of principle that these alternate pathways exist in lipid oxidation and have important consequences to timing and types of products developed.

Lipid oxidation was studied in pure methyl linoleate incubated for 20 days under a range of conditions to investigate early reactions. Four types of major oxidation non-

volatile products (conjugated dienes, hydroperoxides, epoxides, and carbonyls) were measured to determine product distributions and impact of reaction conditions on pathways.

Two observations supported activity of reaction pathways other than hydrogen abstraction. First, epoxides were the dominant product under all conditions, with levels higher than hydroperoxides and in some cases almost as high as conjugated dienes. Second, all products began accumulating immediately at start of oxidation, although at different rates. Carbonyls were produced at much lower levels than other products. Coordinated analyses of volatile products suggested that these unusual patterns could be explained by competitive activity of two alternate reactions: 1) peroxy radicals add to double bonds, forming a dimer that decomposes to an epoxide and an alkoxy radical which can either form more epoxides or undergo scission to carbonyl products; 2) alkoxy radicals add to an adjacent double bond to form epoxides and transfer the free radical down chain to a new site. Environmental conditions mostly affected initial oxidation rates.

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1. INTRODUCTION

The term lipid comes from the Greek for “fat” [1]. Later, the concept of lipid was broadened to include two kinds of material -- fat, which is solid at room temperature, and oil, which is usually liquid at room temperature, with exception of some tropical oils such as coconut oil. Both fats and oils are essential components in food. They provide calories, over 2.25 times greater than those provided by sugar and protein. They play essential biological roles in hormone production, cellular membrane synthesis, body insulation, signaling processes, and neural health. Fats and oils are also invaluable components of foods. They tenderize and moisturize breads; they provide structure and flavor in soups and sauces; they are also an excellent cooking medium with high heat transfer that allows cooking food faster with formation of unique crispy surfaces [2].

In early food industrialization, food manufacturers used animal fat, typically lard and tallow, as major food ingredients. As low production rate reduced supply of these by-products of the meat industry [3], oils from vegetable sources such as soybean and peanuts began to be used to augment the use of animal fats. However, people soon observed that vegetable oils became rancid very rapidly, leading to unpleasant odors and tastes. The culprits in vegetable oils were found to be unsaturated fatty acids, which in the presence of light, oxygen, and other catalysts, are more susceptible to oxidation than saturated fatty acids. To eliminate this problem, the food industry began converting vegetable oils into saturated solid fats via the well-known hydrogenation process, which adds hydrogen to the double bonds with the aid of catalysts. Full, directed, and brush hydrogenation were standard practices in industry for more than 30 years, but during the past decade claims of cardiovascular toxicity of studies *trans* fats, geometric isomers of

the naturally occurring *cis* double bonds, virtually halted this modification process. Whether *trans*-fat is bad for health is still highly controversial, but consumers have been shunning *trans*-fats, several cities have banned use of *trans* fats in food service establishment, and the USDA is very likely to ban *trans*-fats nationally in 2014 [4]. These changes have brought oxidation issues back to the table. Indeed, lipid oxidation has once again become one of the biggest challenges facing the food industry.

Lipid oxidation was extensively studied in the 1940's through the 1970's, starting with research of Farmer [5] and Bolland [6, 7]. More details were added to the reactions by extensive product analyses conducted by scientists such as Frankel [8-11], Grosch [12, 13], Barthel [14] and Schieberle [12, 15]. From these and other studies, lipid oxidation has been recognized as proceeding by a free radical chain reaction in three stages -- initiation, propagation and termination. During initiation, UV light, metals, heat or enzymes, and other initiators remove electrons from lipid molecules, generating *ab initio* carbon-centered radicals that are converted to peroxy radicals when exposed to oxygen. Peroxy radicals, in turn, abstract hydrogen atoms from neighboring molecules to form hydroperoxide products plus new lipid radicals that repeat the process described above to establish the chain reaction; this is the first part of the propagation stage. Hydroperoxides are reasonably stable, but their decomposition in UV light and heat generates alkoxy and hydroxyl radicals that react faster and less specifically than peroxy radicals. Like peroxy radicals, they abstract hydrogen atoms from neighboring molecules to initiate branching reactions that augment the main chain reaction and markedly accelerate oxidation; this is the second part of the propagation stage. During termination, radicals combine with each other or undergo scission, with both options resulting in non-radical products.

Over years of experimentation, a general timeline has evolved showing expected development of different classes of products in specific sequences. For example, the first change noted is appearance of conjugated dienes, and then hydroperoxides form and accumulate. Secondary products (epoxides, aldehydes, dimers, etc.) only appear after hydroperoxides begin to decompose [16]. These observations were based on old chemical assays and instrumental technologies with lower sensitivities and greater limitations. However, as analytical technologies have advanced, making it possible to obtain high resolution qualitatively and high sensitivity quantitatively, studies have observed additional products early in oxidation, unexpected mixtures of products, and kinetic patterns different than predicted from simple radical chain reactions. This new data raises questions about the accuracy and comprehensiveness of the classical autoxidation theory.

The core concept in traditional lipid oxidation theory has always been that the reaction is driven almost exclusively by hydrogen abstraction. However, inconsistent experimental data and detailed analysis of the free radical literature suggested that this concept was incomplete and oversimplified the complexity of the chemical reactions actually occurring [16]. A number of alternate reactions of peroxy and alkoxy radicals occur at rates comparable to hydrogen abstraction, so should compete with hydrogen abstraction in propagation and also alter observed products. Based on this analysis, in 2005 Schaich [16] proposed a revised mechanism of lipid oxidation that integrated alternate reactions of internal rearrangement, addition, scission, and disproportion of peroxy and alkoxy radicals with a core of traditional hydrogen abstraction.

The existence of alternate reactions has two immediate and obvious consequences. First, lipid oxidation cannot be adequately tracked and quantitated by merely measuring conjugated dienes, hydroperoxides, and perhaps also hexanal as is common practice. Measuring these products alone will underestimate the extent of lipid oxidation if other pathways are active. To avoid missing pathways, products from multiple pathways must be measured simultaneously, which is more demanding in time and analytical methodologies. Second, reaction conditions such as temperature, headspace, moisture, solvents, and lipid concentration should have a marked effect on the balance between the pathways and hence the kinetics and distribution of products observed.

Testing the integrated scheme of lipid oxidation proposed by Schaich [16] has been a major focus of our laboratory, to validate or refute it and to suggest modifications required to describe lipid oxidation accurately. This dissertation seeks to provide initial proof of principle that multiple pathways exist in competition with hydrogen abstraction by chemically analyzing conjugated dienes, hydroperoxides, epoxides, and carbonyls in oxidizing methyl linoleate. It also determines how reaction conditions of temperature, headspace oxygen, solvent, and lipid concentration affect product balance and dominant reaction pathways. Early reactions were emphasized in order to track products as they develop and transform.

The information gained can be used to design analysis strategies that provide a more accurate picture of the extent and direction of lipid oxidation. It will also be critical for developing more effective strategies for inhibition of lipid oxidation and limitation of co-oxidations that degrade food quality.

2. BACKGROUND

Oxidation of polyunsaturated fatty acids has long been recognized as the major chemical reaction limiting shelf life of foods via development of distinctive off-flavors and co-oxidation with proteins, pigments, vitamins, and other molecules [6, 7, 17, 18]. Lipid oxidation not only is responsible for quality deterioration of edible oils and food products formulated with them, but also produces toxic degradation products such as hydroperoxides [19], epoxides [20], and 4-hydroxynonenal [21] that pose threats to human health.

Early research on lipid oxidation focused on establishing oxidation pathway theories [22], measuring oxidation products [23], and learning how to control lipid oxidation [24]. By the 1990's when low fat foods came into vogue, the food industry considered that everything was known about lipid oxidation, and the process was considered controllable by a combination of low unsaturation, antioxidants, and low oxygen [25, 26] so research in lipid oxidation slowed markedly. However, during the past ten years as the importance of dietary lipids was again recognized and polyunsaturated fatty acids were added back to foods for health, the challenge for stabilizing these lipids against oxidation again loomed large. Difficulties in stabilizing a wide range of foods have brought lipid oxidation reactions to the forefront and are making reconsideration of lipid oxidation mechanisms timely.

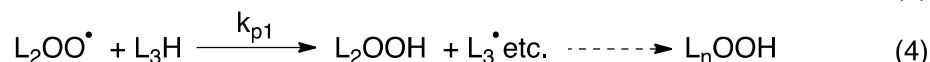
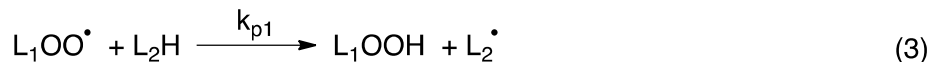
2.1 Traditional lipid autoxidation chain reaction theory

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

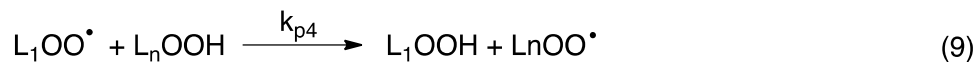
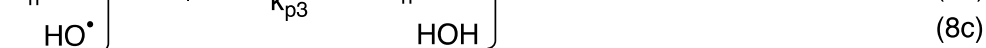
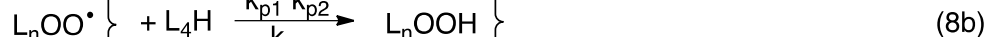
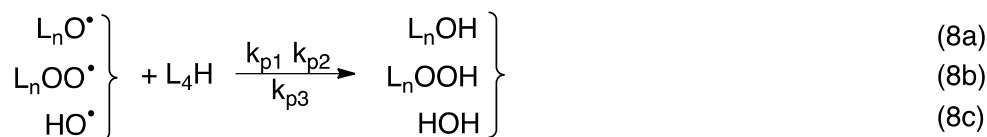
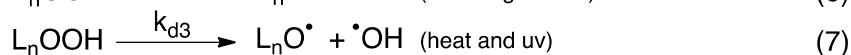
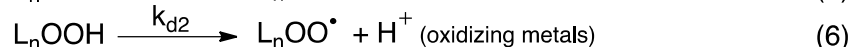
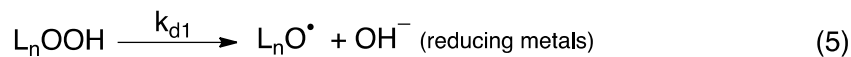


Propagation

Free radical chain reaction established



Free radical chain branching (initiation of new chains)



Termination (*formation of non-radical products*)

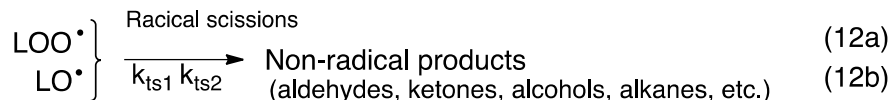
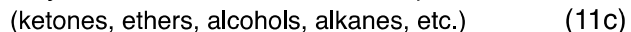
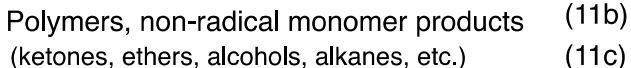
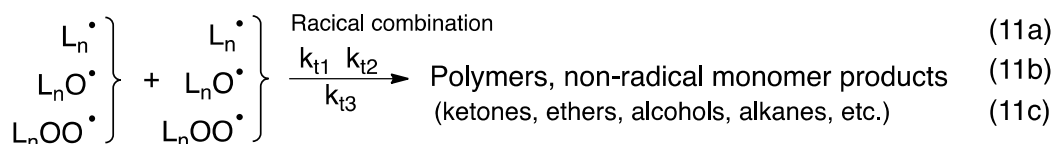


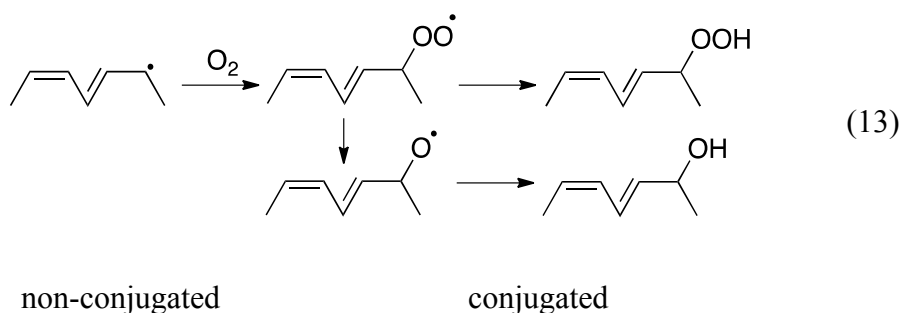
Figure 1. Classic free radical chain reaction mechanism of lipid oxidation. [16]

For years lipid autoxidation theory has been remained almost unaltered as the radical chain reaction mechanism shown in Figure 1 [27]. Three phases of this chain reactions are defined: initiation, propagation and termination [28]. Since the rate of

autoxidation increases with the degree of unsaturation [29], the duration of the initiation stage varies with the lipid content of food materials [30]. In initiation, a lipid molecule (LH) forms a lipid radical (L^\bullet) by attack of an initiator on a double bond or by abstraction of a hydrogen atom adjacent to a double bond by another radical (Reaction 1, Figure 1). An initiator is necessary because it is thermodynamically impossible for ground state oxygen (O_2) to react spontaneously with a double bond ($C=C$) due to differences in spin states: ground state oxygen is in triplet state (oxygen electrons in parallel spin), while electrons in the carbon double bond are in singlet state (electrons in opposite spin) [31]. The triplet state cannot invert to singlet state, and the energy barrier for singlet state double bond to be excited to triplet state is too high for a spontaneous reaction ($E_a=35-65$ kcal/mol) [16]. To overcome the barrier, either singlet state oxygen has to be available or a triplet state lipid molecule is needed[32]. This is accomplished by initiators that generate *ab initio* alkyl radicals, which are in triplet state and are readily attacked by oxygen. Important initiators include heat, metal catalyst and ultraviolet or visible light [33].

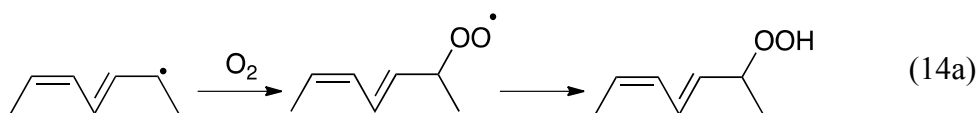
Initiation can be accomplished by attack on only a few molecules. The peroxy radicals thus formed begin the radical chain by abstracting hydrogen atoms from allylic carbons adjacent to a double bond in a neighboring molecule. The double bond remains intact and migrates one carbon position over. In polyunsaturated fatty acids with two or more double bonds, the doubly allylic carbon between two double bonds has the lowest C-H bond energy and this becomes the preferential abstraction site. The electron density concentrating on this carbon creates electron deficiency at the end carbons of the 1,4-diene system. Resonance and electron migration within the diene structure then leads to

formation of radicals at the two ends, with shifting of one double bond to form a conjugated structure (Reaction 13). Conjugated dienes are the first modified structure in lipid oxidation, and thus are often measured as an indicator of lipid oxidation.

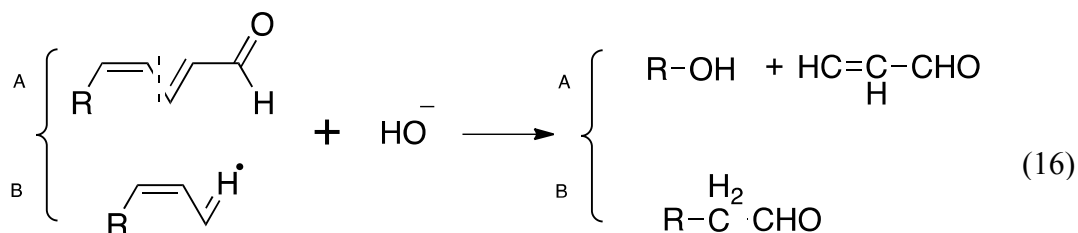
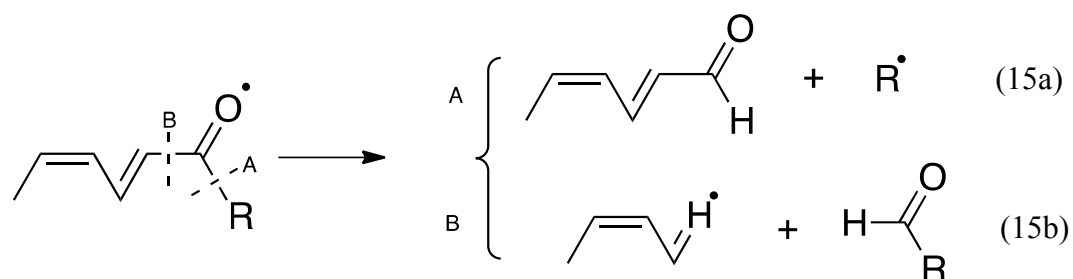


When oxygen is present, it adds almost instantaneously to lipid alkyl radicals to form peroxy radicals, LOO^\bullet (Reaction 2, Figure 1). The propagation stage starts when these peroxy radicals abstract hydrogen atoms from neighboring lipid molecules (Reactions 3 and 4, Figure 1) to form lipid hydroperoxides (LOOH) and a new lipid radical (L^\bullet) [34]. From then on, the chain reaction is autocatalytic: once it starts, it becomes self-accelerating and self-propagating.

Hydroperoxides are only relatively stable. They are readily decomposed by heat, light, and metals to form alkoxy, peroxy, and hydroxyl radicals (Reactions 5-7, Figure 1) [35], which in turn abstract hydrogens to start branching reaction (Reactions 8-10). In early stages of oxidation, the rate of LOOH formation exceeds that of decomposition. As oxidation progresses, LOOH decomposition becomes more prominent, and secondary branching reactions become quite complex. Conjugated diene structures may be retained if hydrogen abstraction reactions dominate (Reaction 14, below). However, conjugated diene structure will be lost if competing reactions such as internal rearrangement or addition to double bonds occurs.



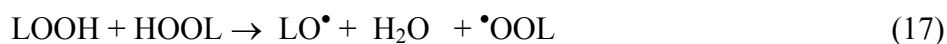
Reaction 11 and 12 in Figure 1 show termination reactions where formation of non-radical oxidation products such as polymers, aldehydes, ketones, ethers, alcohols, and alkanes stops the propagation of an individual chain [34], but also leads to a series of consequences like instability and rancidity odors [36]. Most commonly, termination is portrayed as occurring by radical recombination (Reaction 11) that generates a wide variety of products [28]. However, termination can also proceed via scissions of alkoxy radicals as shown in Reaction 15, below. Scission products may retain or lose the conjugated diene structure. For example, if an alkoxy radical undergoes scission reaction A, it will form an aldehyde with conjugated diene structure and an R radical group. If scission B occurs, a conjugated diene radical will form along with a free aldehyde [37]. Both products of scission A and B reactions can undergo secondary reactions that eventually lead to loss of conjugation (Reaction 16).



2.1.1 Sequence of product development under classic pathway

Some key studies that measured kinetics of lipid oxidation and followed multiple products have been largely responsible for the current general understanding of the progressive development of products in lipid oxidation, as illustrated in Figure 2. This graph has been reproduced many times in different forms, but surprisingly few studies measured enough different products to establish this sequence as unequivocal.

As oxidation starts, there is an induction period during which measurable oxidation remains very low, while lipid radicals are formed slowly behind the scene. As propagation becomes established and especially as faster-reacting alkoxyl radicals are generated, oxygen consumption increases dramatically and hydroperoxides (LOOH) begin to accumulate [38]. LOOH concentrations increase as long as formation rates exceed decomposition [39, 40]. In early oxidation, LOOH decomposition is thought to be monomolecular, i.e. $\text{LOOH} \rightarrow \text{LO}^\bullet + \bullet\text{OH}$ or OH^- [41]. However, at high concentrations hydroperoxides react with each other and bimolecular decomposition dominates (Reaction 17) [42]:



At this point, net LOOH concentrations decrease. Because of this, analysis of LOOH alone cannot determine extent of oxidation since low concentrations are present in both early and late stages of oxidation.

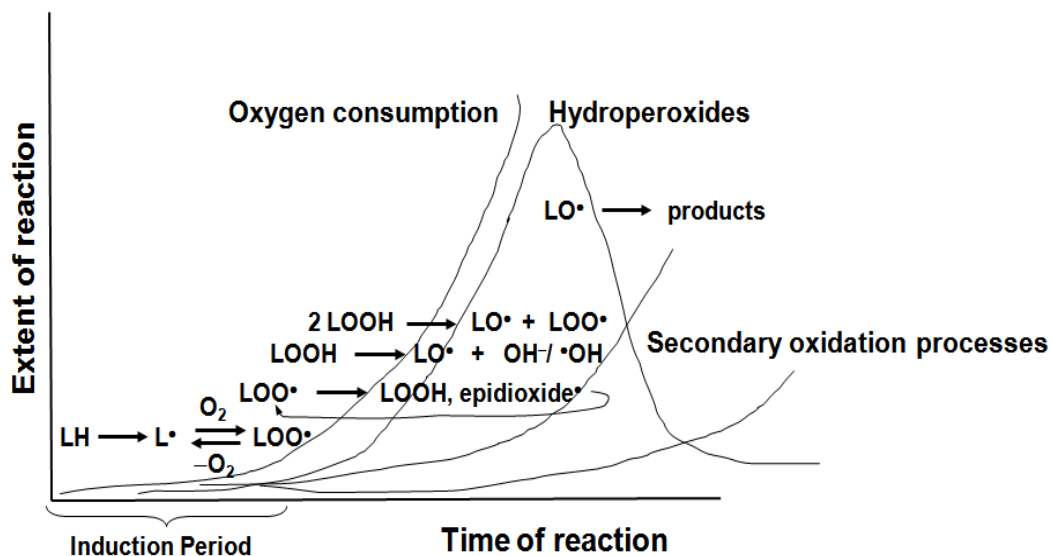


Figure 2. Progressive development of products during oxidation of lipids driven by H-abstraction. [16]

According to traditional thinking, secondary products such as aldehydes, epoxides, dimers, and polymers do not develop until after hydroperoxides decompose. Indeed, the appearance of secondary products at any point has been generally interpreted as an indication of advanced lipid oxidation [16].

2.2 Alternate pathways of lipid oxidation

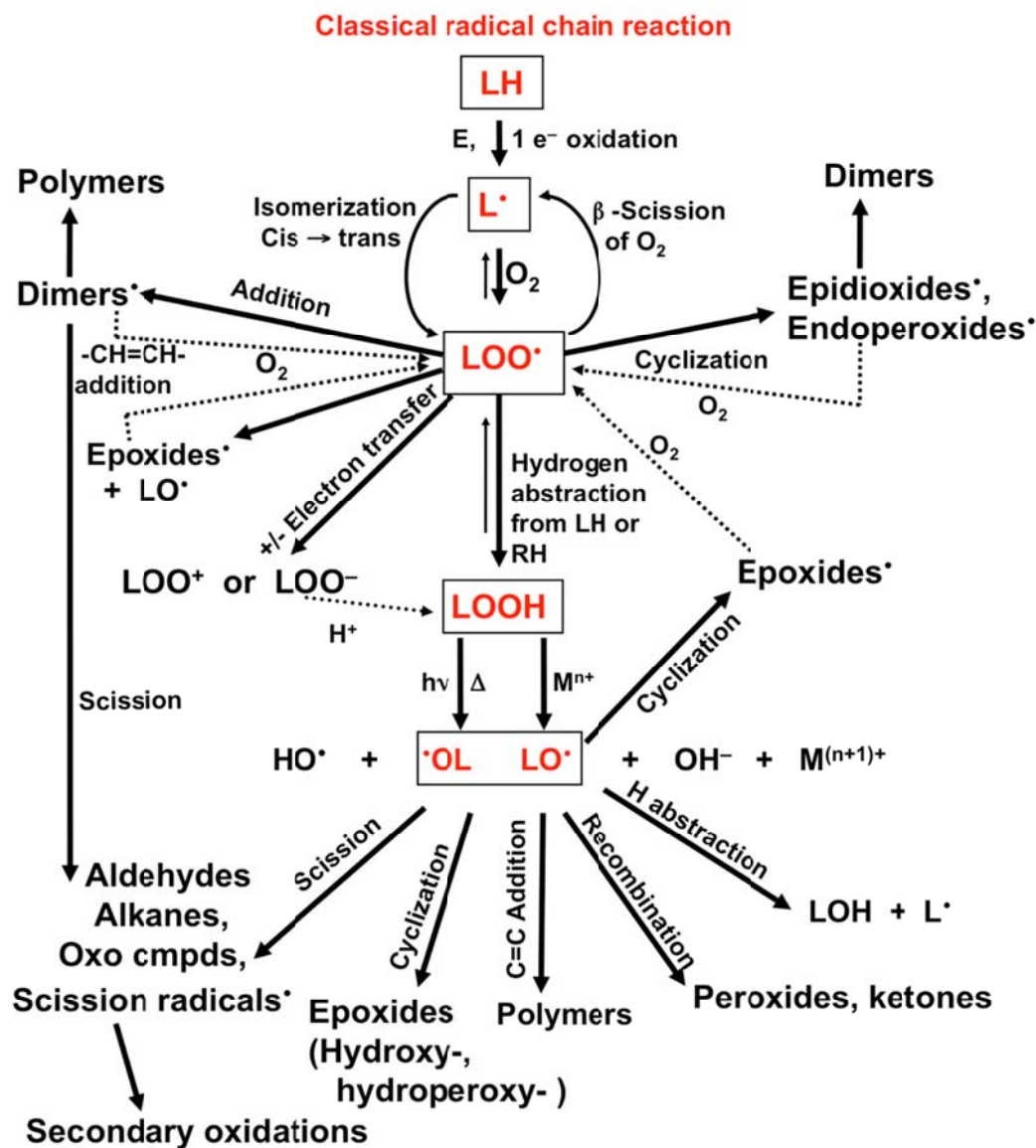


Figure 3. Reaction scheme for lipid oxidation, which integrates alternate reaction

Although the pattern of product development illustrated in Figure 2 is consistent with progressive changes expected from the three-stage oxidation reactions of the classical chain reaction theory, more and more experimental evidence has suggested that there may be no fixed sequence of lipid oxidation reactions and development of products. What's more, it has been observed that the dominating pathways change with reaction

conditions such as the type and concentration of lipids, the solvents, oxygen level, catalysts, and even antioxidants. This inspired the new thinking in the oxidation pathway theory. After extensively re-evaluating research in free radical reactions, Schaich identified a number of peroxy and alkoxy radical reactions that compete with hydrogen abstraction, and proposed a new oxidation pathway map that incorporated these.

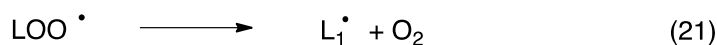
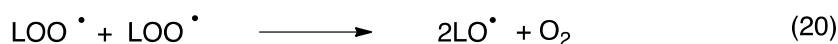
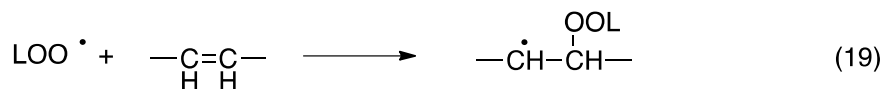
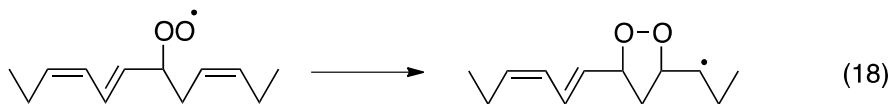
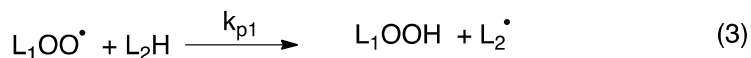
Figure 3 shows Schaich's proposed integration of alternate reactions of peroxy and alkoxy radicals (labeled) with hydrogen abstraction reactions in the classical free radical chain mechanism shown in red, and alkoxy radicals (internal rearrangement, addition, disproportionation, and scission) with hydrogen abstraction reactions of the classical free radical chain reaction [16]. The latter are shown in the vertical boxes with red labels running vertically in the center of the scheme. The branches of alternate pathways for LOO^\bullet show how products other than hydroperoxides can form early in the oxidation process (possibly even earlier and at higher levels than hydroperoxides), divert oxidation from products normally measured, and generate different products than expected. Indeed, products from these alternate reactions are not fully identified as yet, and on the scheme represent possible pathways and depict a comprehensive picture of lipid oxidation process. The branches of alternate pathways for LO^\bullet show how products can become quite complex, depending on which pathways are active. A critical consequence of these alternate reactions is generation of unexpected or unmeasured products, and hence common underestimation and misinterpretation of the extent of lipid oxidation when the wrong pathways are monitored.

Environmental conditions such as oxygen, solvents, presence of water, temperature, and lipid concentration can shift the dominance and balance between the

various reaction pathways [16]. For example, protic systems with a ready supply of abstractable hydrogens may push oxidation forward by traditional pathways and even stabilize LO^\bullet scissions, while aprotic systems reduce their energy faster in alternate reactions. All of the various reactions in lipid oxidation have different activation energies, some of which are surprisingly high. Thus, it should be expected that, as temperatures increase, the rates and pathways of product formation, transformation, and decomposition can change dramatically, completely altering the overall effect of oxidation. Available oxygen levels can also have a huge impact on oxidation since oxygen is the main fuel for this reaction. Secondary reactions also appear to be strongly affected by whether the system is open, allowing free volatilization of products, or closed where products accumulate and can undergo condensations.

The consequences of such shifts are far-reaching, once again leading to underestimation and misinterpretation of the extent of lipid oxidation when the wrong pathways are monitored. Thus, serious consideration of both the existence of alternate pathways and the effect of reaction conditions on the balance between them is critical for accurately understanding full lipid oxidation processes and eventually gaining control over them. The impact of these conditions will be elaborated in the following sections.

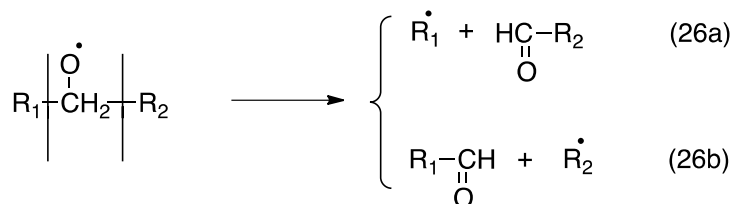
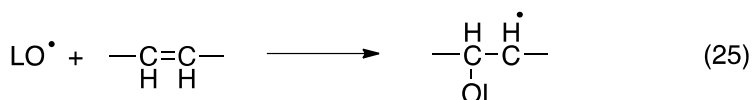
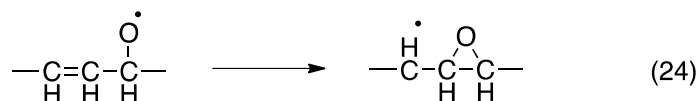
2.2.1 Alternate reactions of peroxy radicals



Reaction 3, Figure 1 was shown earlier as the reaction in traditional theory where an *ab initio* peroxy radical abstracts hydrogen from a neighboring lipid molecule to form new lipid radicals and start the chain reaction. However, hydrogen abstraction is not the only fate of peroxy radicals [43-45]. Peroxy radicals can undergo cyclization or rearrangement reactions to form epidioxides (Reaction 18 below) [46]. This reaction appears to be favored under low oxygen pressure and aprotic systems at low temperatures. Addition to a lipid double bond (Reaction 19) [47] can form peroxo dimers plus a new alkyl radical on the carbon adjacent to the link. This reaction is favored with conjugated double bond systems, and competes with H abstraction when proton sources are limited (aprotic system). In disproportionation (Reaction 20) [48] two LOO^\bullet radicals condense, releasing two LO^\bullet and one oxygen molecule. β -scission of peroxy radicals (Reaction 21)

involves reversible addition and release of oxygen to lipid radicals; it occurs during the induction period and mediates isomerization to form trans double bonds. Recombinations (Reaction 22) [49] (favored in viscous system) and electron transfers (Reaction 23) terminate radicals by forming dimers or lipid anions, respectively.

2.2.2 Alternate reactions of alkoxy radicals



Alkoxy radicals formed by decomposition of hydroperoxides are important propagators in established chain reactions [50]. As with peroxy radicals, hydrogen abstraction (Reaction 8a, Figure 1) may not be the only fate of alkoxy radicals in lipid oxidation [51]. LO^\bullet can undergo rearrangement or internal cyclization by addition to a double bond on the next carbon to form epoxides; at the same time, the free electron migrates to form a new alkyl radical on the second carbon of the double bond (Reaction 24) [20, 52]. Epoxidation is favored in neat lipid samples or low concentration lipids in aprotic solvents. Addition of LO^\bullet to lipid double bonds (Reaction 25) [53] results in a dimer with new radical on the second carbon of the double bond. Addition reactions are facilitated by conjugation and heat. Scissions are key reactions that probably generate

most of carbonyl compounds responsible for off-odors and flavors in rancidity. Both α (Reaction 26b) and β (Reaction 26a) scissions occur, generating non-radical carbonyl compounds (aldehyde or ketone) and an alkyl radical [54-56] which can oxidize further or be quenched by H atom donors. Hence, scission is reportedly favored in protic systems.

2.2.3 Reaction dynamics

Table 1. Rate constant for alternate reaction pathways of peroxy radicals and alkoxy radicals in lipid oxidation^a. [16].

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

^a Data included authentic fatty acids whenever possible, plus primarily cumyl, tetralinyl, and *t*-butyl peroxy and alkoxy radicals. References for this table are available in [16].

(NA: data not available).

That rate constants for alternative reactions of LOO^\bullet and LO^\bullet or closely related radicals are comparable to hydrogen abstraction (Table 1) suggest that alternative pathways can certainly be competitive in lipid oxidation [16].

2.3 Analysis of Lipid Oxidation

As mentioned earlier, lipid oxidation has adverse effects on product shelf life and quality. Therefore, monitoring lipid oxidation products is very important in order to understand how far lipid oxidation has progressed to evaluate food quality as well as to track oxidation while designing new strategies for prolonging shelf life and improving product quality. Since the 1930s, a number of methods have evolved to measure lipid oxidation products; these include both physical and chemical methods, as well as quantitative and qualitative methods [23].

In modern lipid studies, numerous types of samples are measured, from edible oils to more complicated emulsions to extracted fat from food products. Most analyses focus on monitoring single types of products using one or more analytical methods. However, with recognition that LOOHs decompose during oxidation, analysis of lipid oxidation products expected before and after LOOH decomposition became necessary to distinguish early from late oxidation when LOOH was low. At the same time, analytical equipment and methods have evolved and improved considerably so that monitoring multiple oxidation products is now technically feasible. Fortuitously, this comes at a time when it is necessary to track the presence of multiple reaction pathways and distinguish which is dominant under different conditions. Monitoring oxidation products from multiple pathways will improve understanding of lipid oxidation by building a more complete picture of product distribution rather than focusing on a single product [57].

Moreover, it will reveal changes of product distribution in different conditions so that evaluation the extent of lipid oxidation will be more accurate.

2.3.1 Oxygen Consumption

One of the most direct methods for measuring lipid oxidation is oxygen consumption [58], either as total headspace pressure (e.g. in an oxygen bomb) or specific oxygen pressure (e.g. with a probe) [59]. Oxygen consumption offers the advantage that it is product and pathway independent, but at the same time has several disadvantages. It is a blind assay in not identifying where the oxygen adds. The drop of the headspace pressure from oxygen consumption can be compromised by the generation of new volatiles released into the headspace [60, 61]. In the same way, monitoring oxygen consumption by weight gain [58, 62] can also be inaccurate, because loss of volatiles counteracts weight gain from oxidation.

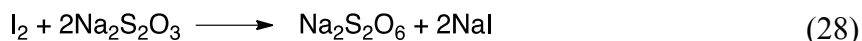
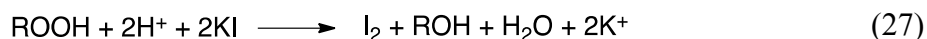
2.3.2 Conjugated dienes

Conjugated dienes, the first products generated in lipid oxidation, are very simple to measure by their optical absorption at 231-235 nm [62, 63], so this assay has been used quite extensively to follow lipid oxidation. The spectrometric detection of conjugated dienes is simple and fast. Sample size is relatively small, and the relationship between absorbance intensity and diene concentration is linear over a wide concentration range. Conjugated diene levels reportedly also have a good correlation with peroxide values [64, 65], at least in very early oxidation. Conjugated diene structure is retained in many peroxides and some scission products of alkoxyl radicals. Conjugated dienes are not a good absolute measure of the degree of oxidation because lipid oxidation is a dynamic

process and conjugated dienes transform to other products, but it can be used for relative determination of oxidation level [58].

2.3.3 Peroxide values

Peroxide values are a direct indicator of lipid oxidation and are probably the most widely used measure of lipid oxidation. A wide range of chemical methods have been used to measure peroxide values [66]. The classical and most common method to measure hydroperoxides is iodometric titration [58, 67], which is based on the oxidation reaction between I⁻ and hydroperoxides (Reaction 27-28). A saturated potassium iodide (KI) solution reacted with an oxidized oil sample reduces hydroperoxides and releases iodine.



Starch solutions added before titration chelate the free iodine (I₂) released in reaction and generate a blue-purple solution. The amount of free I₂ trapped by the starch is then measured by titrating with a sodium thiosulfate; the endpoint is marked by disappearance of blue-purple color [68].

This commonly used method is chemically totally accurate and stoichiometric in its detection of hydroperoxides, but it has also been notorious for technical difficulties. The reaction is a very complicated procedure that consumes a lot of time, samples and reagents. There are issues regarding the oxidation of iodine by dissolved oxygen in the solution and absorbance of iodine across unsaturated bonds [58, 62, 69]. Difficulties in visual determination of the endpoint, also can limit the accuracy of the assay [70].

Nevertheless, with careful attention to experimental details, this still remains totally accurate and is the gold standard for hydroperoxide assays [71].

Two other commonly used methods are based on oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron by reaction with peroxides in an acidic medium. Both are several orders of magnitude more sensitive (micromolar) than the thiosulfate titration [72] but have limitations associated with optical assays [71]. One method developed to detect trace levels of hydroperoxides in biological tissues complexes the Fe^{3+} with xylenol orange [73, 74]. Xylenol orange is a dye that forms a blue-purple complex (FOX) with Fe^{3+} and absorbs at 550-600 nm. In the second method, thiocyanate reacts with the released Fe^{3+} to form complexes with a red violet color and a characteristic absorbance at 500-510 nm [70]. Although the thiocyanate assay has been widely used, the chemistry is not stoichiometric, the extinction coefficient varies with the R group of the hydroperoxide so absolute quantitation of mixed hydroperoxides in oils and extracts is impossible, and the reaction quenches at very low concentrations of LOOH (i.e. excess LOOH oxidizes the SCN leading to loss of color [71]. There is also an FTIR assay base on IR spectroscopy of characteristic O-H stretching absorbance band that can quantitatively determine the peroxide value [75].

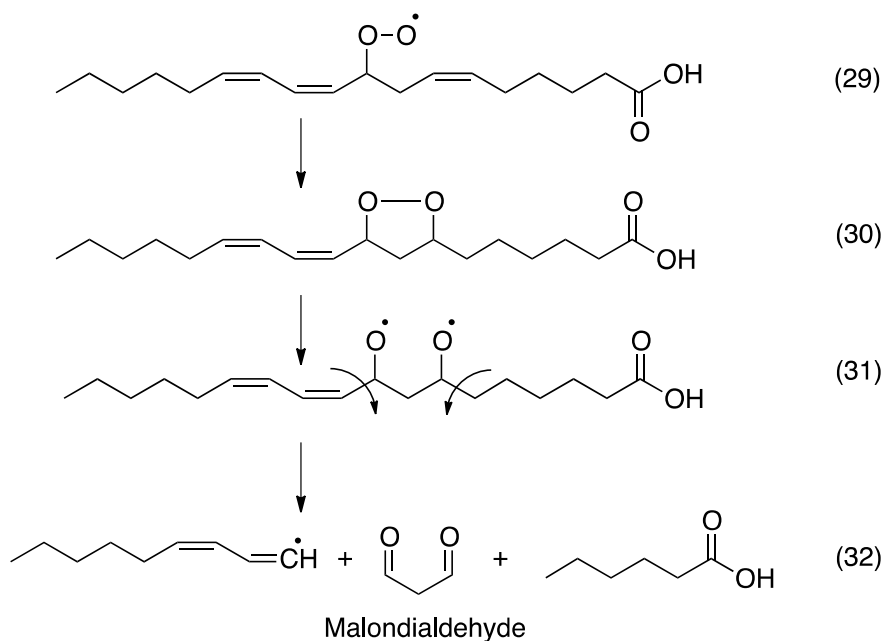
2.3.5 Carbonyl compounds

Secondary oxidation products including fatty acids, carbonyl groups (aldehydes, ketones), hydrocarbons, and epoxy compounds are also measured in oxidation studies using a variety of chemical and instrumental analyses.

2.3.5.1 Thiobarbituric acid test (TBA)

The TBA test is an old assay that has been used for more than 40 years to measure oxidation of oils as well as oil-containing food products [76], particularly meats.

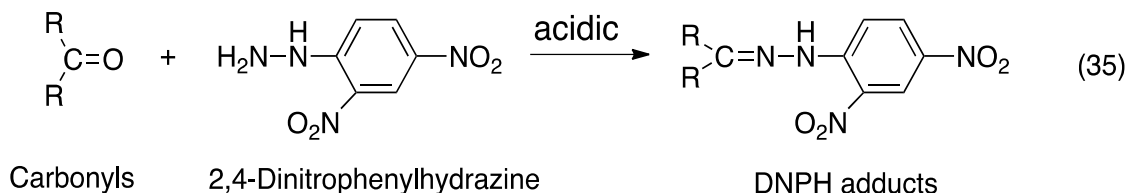
Malondialdehyde can be formed from multiple mid-chain scissions when polyunsaturated fatty acids with three or more double bonds oxidize (Reaction 29-32) [77].



In the TBA assay, thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) to form a pink-colored complex (Reaction 33) that absorbs 530-535 nm [78, 79]. A major limitation of this assay, however, is that MDA reacts with other alkanals and alkadienals, sugars, amino acids, and nucleic acids as well as TBA [80, 81]. For this reason, the assay is sometimes referred to as thiobarbituric acid reactive substances (TBARS). The TBA

2.3.5.3 DNPH assay

Unlike the TBA and *p*-AnV tests mentioned above, 2,4-dinitrophenylhydrazine (DNPH) assay measures not only aldehydes but also other compounds with carbonyl groups. Reactions between DNPH solution and compounds containing carbonyl groups in solution, generates hydrazone complexes that absorb at 360 nm (Reaction 35):



The DNPH assay has been adapted into chemical analyses of air pollution [91-94] by loading DNPH onto solid phase extraction cartridges. As air is drawn through the cartridges, any carbonyls present react with the DNPH and form hydrazones which can then be eluted with organic solvents and analyzed by a spectrometer or liquid chromatography. The advantage of the DNPH assay is that it not only measures the total amount of carbonyl groups in the sample, but can also identify specific carbonyl products when combined with liquid chromatographic methods to separate DNPH-carbonyl adducts by chain length [95].

The availability of detailed carbonyl group analyses enabled many studies to use DNPH to monitor hexanal, one of the most monitored products in lipid oxidation [96, 97]. The method shows strong linear correlation with the TBA test [98]. DNPH method has once been criticized for having the possibility of breaking down hydroperoxides thus increasing apparent aldehydes [90]; nevertheless, it is still an important method to assess oxidation in oil products.

2.3.6 Epoxide determination

Epoxy compounds (Figure 4) are a group of oxidation products that are not included in classic chain reaction theory. Epoxides are generated through internal cyclization of an alkoxyl radical (LO^\bullet), instead of hydrogen abstraction. Extensive studies in radical chemistry have shown that epoxide formation is competitive with other radical reactions [99, 100]. Epoxides are known to have toxicity and adverse effects for health [101], so it is very important to be able to monitor their levels in oils and foods. However, while epoxides are generally more stable to environmental conditions than are hydroperoxides, they are extremely reactive with other food components, particularly proteins, so it is often difficult to detect and quantitate epoxides in complex systems.

Some of the methods used to determine levels of epoxyl compounds in lipids and lipid extracts are described below.

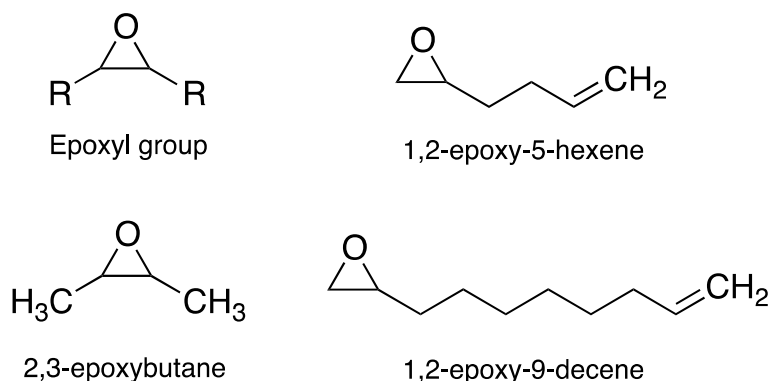


Figure 4. Compounds with terminal (right) and mid chain (left) epoxide structures.

2.3.6.1 4-(p-Nitrobenzyl)-pyridine assay

This method is based on the alkylation of 4-(p-nitrobenzyl)-pyridine (NBP) to form a blue derivative which can be detected optically at 600 nm [102]; it is also an AOCS standard method [103]. Solutions of potassium acid phthalate in H₂O and NBP in acetone are mixed in equal amounts, then the sample is added and the mixture is incubated at 100-110 °C for 45 min. The reaction is cooled rapidly, K₂CO₃ is added to neutralize the reaction, and the mixture is read at 600 nm immediately.

There are three challenges for adapting this method to measure lipid epoxides. First, reaction time is too long (>1 hour); second, reaction takes place at high temperature (105-110 °C), which might cause oxidation oil being studied; thirdly, there is a relatively high proportion of water in the reaction system, creating a solubility issue for fat samples [104].

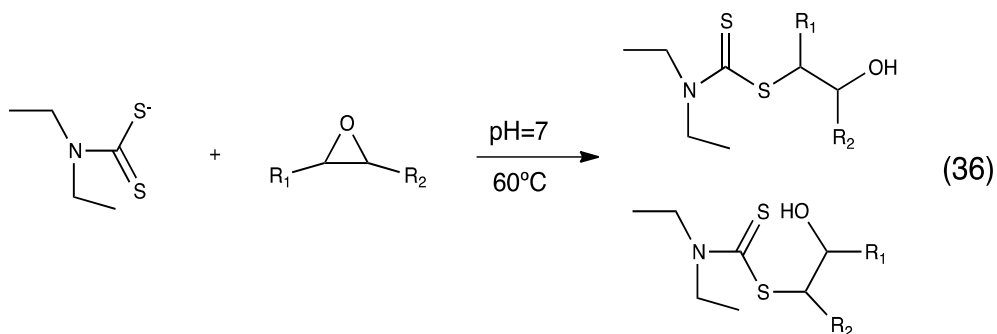
2.3.6.2 Picric acid assay

In this reaction, picric acid (2,4,6-trinitrophenyl) reacts with epoxy compounds, generating a chromophore with maximum absorption at 350-410 nm [105]. Epoxy compounds are added in 150 fold excess of picric acid in ethyl ether, then allowed to react for 24 hours. After a removal of excessive picric acid by NaHCO₃ and NaCl 5% aqueous solution, the organic phase of the mixture is drained and dried under nitrogen. The residue is then re-dissolved in a mixture of chloroform, methanol and water. The picrate adduct can be read by spectrometer at 350 and 410 nm for total epoxyl quantitation, or it can be injected into thin layer chromatography or liquid chromatography systems for detailed analysis of individual epoxides. Product concentrations can be calculated using Beer's law. The limitation of detection can be as

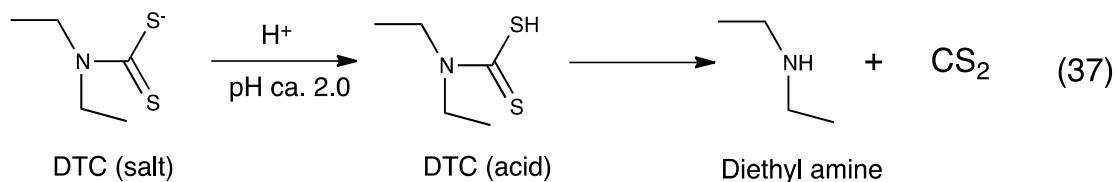
low as to 2 $\mu\text{g/ml}$ epoxide in cholesterol, suggesting high accuracy. Good quantification can be achieved as the reaction is linear from trace levels to 0.2 mg/ml of epoxides. However, this assay has a major disadvantage of long incubation time (24 hour) that limits its application to lipid oxidation. In most of lipid studies, short time and low temperature conditions are favored in order to minimize the oxidation during measurement.

2.3.6.3 N,N-diethyldithiocarbamate assay

N,N-diethyldithiocarbamate (DETC) forms adducts with epoxy compounds under relative mild temperatures (Reaction 36) [106]. The reaction produces two isomers, depending on which side of the epoxy group reacts with DETC. The total concentration of two isomers can be determined by chromatographic methods, although individual structure determination is challenging, even with mass spectrometry.



DETC solutions are first prepared in aqueous or organic solvent. The epoxide sample reacts with excessive amounts of DETC solution under neutral conditions at 60 °C for 20 minutes to ensure complete adduction of epoxy-DETC [106]. Excess DETC is removed by adding phosphoric acid to degrade DETC into diethyl amine and CS_2 (Reaction 37).



With the removal of excess carbamate, the adduct concentration is then determined from its absorbance at 278 nm. This method is accurate and has a short reaction time, so it has great potential in lipid oxidation measurement.

2.4 Factors that affect lipid oxidation

In food production and storage systems, products are often processed under a several conditions. For example, foods are cooked at different temperatures and for different lengths of time. Foods also come in contact with many catalytic chemical compounds during production, i.e. water (moisture), metals (cooking vessels) and solvents (industrial extraction). The quality of oil or food degrades during storage, with the extent of quality alteration influenced by several factors. First of all, the nature of lipids will inevitably affect lipid oxidation. For example, the degree of unsaturation, *cis* / *trans* structure, and pro-oxidant and antioxidant that naturally occurred or introduced into the lipid can accelerate or slow down the oxidation. These factors are often fixed by the formulation or composition of a specific lipid or food product. However, environmental factors such as temperature, light, oxygen level, solvent, moisture, pH can be manipulated to control lipid oxidation to enhance shelf life [107].

2.4.1 Temperature

As noted in Section 2.1, oxygen is in triplet state and double bonds on the lipid molecule are in singlet state. This means that oxidation requires energy overcome the spin restriction, e.g. by forming a singlet radical at or near the double bond. The

activation energy (E_a) for excitation is high ($16.2 \text{ kcal mol}^{-1}$) [38], so initiation occurs much more readily at elevated temperatures where thermal energy is supplied.

Traditionally, it has been assumed that elevated temperature accelerates oxidation according to the Arrhenius equation which holds that reactions do not change with temperature, but energy for activation becomes more available as temperature increases, so oxidation increases correspondingly. The Arrhenius relationship states that oxidation rate doubles for each 10 degree increase in temperature:

$$k = A \cdot e^{-\frac{E_a}{RT}}$$

Accelerated shelf-life tests in the food industry are based on this relationship and the assumption that reactions are accelerated but not changed at high storage temperatures (40°C or 60°C). However, this approach ignores a second well-known effect of temperature – that of accelerating decomposition of intermediates [41]. Indeed, perhaps the most important effect of heat at moderately elevated temperatures is decomposition of hydroperoxides to alkoxyl and hydroxyl radicals (Reaction 38), both of which then accelerate oxidation and open opportunities for alternate reaction pathways.



Thus, peroxide values do not accurately reflect extent of oxidation at elevated temperatures. Lipid oxidation is a dynamic reaction system. It is not one single reaction but involves a series of interrelated reactions that include formation, transformation, and decomposition of many types of products. Each of these component reactions has a different activation energy, so the elevation of temperature impacts each of them differently. Totally different oxidation patterns with altered product distributions and even formation of different products thus should be expected as temperature rises. The

chemical balance between the formation and breakdown of LOOH at different temperatures is the key factor that affects the determination of shelf-life of products. Hence, detailed information about how temperature mediates shifts in dominant reactions and lipid oxidation products is of great interest and importance to better product quality control and assurance for food industry and also to basic research on lipid oxidation mechanisms.

2.4.2 Oxygen Pressure

It has long been recognized that suppressing oxygen in the sample headspace can slow down oxidation because it limits formation of propagating peroxy radicals. This protection has often been used in the food industry where food packages are filled with inert gases, although 100% removal of oxygen is not guaranteed. However, it should be noticed that oxygen does not always accelerate the oxidation. Once a minimum oxygen level required to generate initiating LOO^\bullet is met (as low as it may be), further increases have no incremental effects, at least on oxidation rates. There is evidence that the formation of LOO^\bullet requires oxygen pressure only up to 10 mm Hg $p\text{O}_2$ (3%) in the headspace, which is much lower than regular air [108]. This raises a question of whether filling headspaces with inert gas is sufficient to reach this limiting value or more complete removal of oxygen is needed.

The impact of low oxygen pressure on alternate reactions of lipid oxidation is of great interest. Low $p\text{O}_2$ limits formation of LOO^\bullet and LOOH, so any products deriving from these two intermediates should also be blocked. This removes competition and allows alternate pathways that are independent of LOOH to become active, dominate the reaction scheme, and obviously cause a shift in products.

The surface to volume ratio of the sample controls contact with oxygen. Hence sample configuration as it relates to oxygen accessibility must also be considered.

2.4.3 Solvent effects on lipid oxidation

Solvent is not commonly considered as a main factor to affect lipid oxidation because organic solvents are rarely used in food production. However, in food oil refining where raw fats/oils are obtained from natural resources, different solvents such as hexane, and ethanol are applied in extractions, and chloroform is used extensively in research extractions of lipids. Extraction not only forces contacts between solvents and lipids but also often applies pressure and/or heat to the system, which can catalyze solvent interactions with oxidizing lipids.

Polarity and proticity are critical determinants of how a solvent affects lipid oxidation. Most lipid oxidation products (e.g. epoxides, carbonyls) are polar compounds that tend to accumulate in a polar environment. However, reactions generating these compounds have variable dependence (indeed, often opposite dependence) on solvent properties, where polarity can stabilize a transition state leading to one product or can inhibit interchain associations necessary for formation of another product (or vice versa). Solvents also play important roles as proton sources (or lack thereof) that stabilize the radicals or force alternate reactions. Most lipids oxidize *in situ* in a food or biological system where solvents are not an issue. Nevertheless, lipids are dissolved in solvents during industrial and research extractions, they are reacted in solvents during analyses, and they are stored in solvents in laboratories. Thus, it is critical to elucidate more fully how solvents may alter oxidation pathways and shift product distributions.

Table 2 [51] shows effects of solvent polarity on selectivity of lipid oxidation pathways. From CCl_4 to CH_3COOH , as the solvent polarity decreased, the rate of hydrogen abstraction remained almost constant. However, the rate constant for β -scission increased from $2.6 \times 10^{-5} \text{ s}^{-1}$ to $19 \times 10^{-5} \text{ s}^{-1}$, which is an increase of more than seven fold. The polar and protic CH_3COOH which dissociates in solution to provide free H^+ in solution, and these stabilize the scission reaction products, thereby driving the reaction forward. The change in ratio of these two reaction rates from 4.5 to 0.7 when increasing solvent polarity and proticity certainly suggests that the oxidation reactions occurring in the various solvents cannot be the same.

Table 2. Effect of solvent on comparative rates of H abstraction and β scission reaction of cumyl- O^\bullet radicals [16].

	H abstraction $k_a \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$	β -scission $k_b \times 10^{-5} \text{ s}^{-1}$	Ratio $k_a / k_b \text{ M}^{-1}$
CCl_4	1.1	2.6	4.2
C_6H_6	1.2	3.7	3.2
$\text{C}_6\text{H}_5\text{Cl}$	1.1	5.5	2.0
$(\text{CH}_3)_3\text{COH}$	1.3	5.8	2.2
CH_3CN	1.2	6.3	1.9
CH_3COOH	1.3	19	0.7

Another impact brought by solvents is that dilution increases the distance between lipid molecules so that the chance of them contacting each other to transfer radicals

correspondingly decreases. The most obvious effect is limitation of hydrogen abstraction and radical transfer, which in turn translates to increases in the alternate pathways. This includes increased self-rearrangements of LOO^\bullet and LO^\bullet . Increased formation of epidioxides and epoxides should be a major consequence.

3. SIGNIFICANCE AND PRACTICAL IMPORTANCE OF THE STUDY

This research breaks new ground in looking beyond hydrogen abstraction, tracking multiple products very early, and in investigating effects of conditions on product patterns. Demonstrating the existence of multiple pathways in competition with each other presents a number of technical challenges in managing sensitive, accurate assays for multiple products, but results will be of both theoretical and practical significance. This is very new thinking in lipid oxidation, and everything we learn from these studies should be greatly helpful in understanding how best to monitor foods for lipid oxidation and in designing more effective antioxidation methods for different products and processing. Major oxidation products can be accurately identified and then controlled by manipulating the reaction environment and by customizable antioxidants. The impact of environmental factors like oxygen and solvents can provide a lot more information on controlling or delaying oxidation process. For examples, specific oxygen levels can be controlled for packaged foods to maximize shelf life, and solvents can be selected for minimizing radical generation in oil extraction processes.

4. HYPOTHESIS

Multiple pathways for reaction of lipid oxyl radicals exist and compete with hydrogen abstraction to modify the progression of lipid oxidation. Alternate reactions include but are not limited to internal rearrangement, addition to double bonds, scission, and dismutation. Because each of these alternate reactions responds differently to the reaction environment, the balance between the pathways and associated products should change with reaction conditions. Measuring multiple pathways should provide a more complete and accurate picture of the progress and extent of lipid oxidation, both quantitatively and qualitatively.

4.1 Specific objectives

1. Provide proof of principle that alternate pathways of lipid oxidation exist and are active simultaneously by conducting class analyses of:

- conjugated dienes (measure of initiation) ,
- hydroperoxides (hydrogen abstraction by peroxy radicals),
- epoxides (internal rearrangement, presumably of alkoxy radicals), and
- carbonyls (scission of alkoxy radicals)

In model systems of methyl linoleate oxidized for up to 20 days. Methyl linoleate is the lipid of choice because it is a relatively simple and readily oxidizable molecule that has been extensively studied, so extensive product bases are available for comparison. Focus will be on short reaction times to detect reactions active in very early oxidation.

2. Investigate the effects of reaction conditions (temperature - 25, 40, and 60 °C, headspace oxygen in closed vs. open system, solvent - neat, chloroform and methanol, presence of water (2% in the system), lipid concentration (neat and 50%)) on the production rates and distributions of lipid oxidation products (and hence dominant reaction pathways) in oxidizing methyl linoleate.

3. Compare analyses of non-volatile products with those of volatile products generated in the same or comparable systems, in studies conducted by Brandon Bogusz, another student in our research group [109].

5. MATERIAL AND METHODS

5.1 Materials

Methyl linoleate (>99%, vacuum sealed in glass bulbs) was purchased from Nu-Chek-Prep (Elysian, MN). Dinitrophenylhydrazine (DNPH) and sodium diethyldithiocarbamate (DETC) were purchased from Sigma Aldrich (St. Louis, Mo). Carbonyl standards (butanal >99%, pentanal >99%, hexanal 98%, heptanal >96%, octanal 99%, nonanal >95%, decanal >98%, t-2-hexanal >95%, t-2-heptenal 97%, t,t-2,4-heptadienal >97%, t-2-octenal >94%, t-2-nonenal >97%, t,t-2,4-nonadienal >85%, t-2-decenal >95% and t,t-2,4-decadienal >85%) and epoxide standards (2,3-epoxybutane 97%, 1,2-epoxy-5-hexene 97% and 1,2-epoxy-9-decene 96%, cis-7,8-epoxy-2-methyloctadecane 96%) were also purchased from Sigma Aldrich (St. Louis, Mo). LC-MS grade iso-octane and LC-MS grade acetonitrile were purchased from Sigma Aldrich (St. Louis, Mo). 18 M Ω resistivity water was obtained by purification of doubly-distilled water through a four-cartridge Milli-QTM water purification system (EMD Millipore Corporation, Billerica, MA) with two ion exchange cartridges for removal of metals. PeroxySafeTM test kits for analysis of hydroperoxides were purchased from MP Biomedicals (Solon, OH).

5.2 Experimental design and sample set-up

5.2.1 Model system

Although many different systems have been investigated in lipid oxidation research, in this initial study a simple system is preferred to simplify kinetics and product identification in order to track alternate reaction pathways. Methyl linoleate was therefore chosen as the model system in this study (Figure 5).

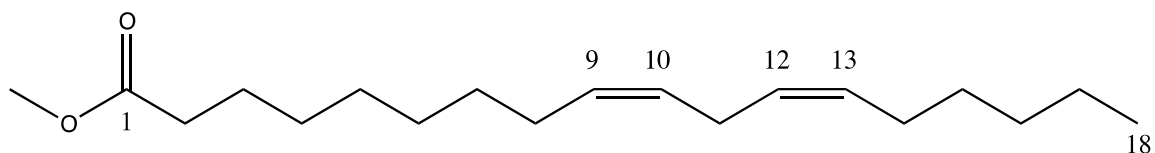


Figure 5. Structure of methyl linoleate

Methyl linoleate is the methyl ester of linoleic acid, which is the most prevalent fatty acid in food oils. In addition, methyl linoleate is more soluble in various organic solvents than are triacylglycerols, and it has been extensively studied, so there is an extensive data base of oxidation products for comparison.

5.2.2 Experimental Design

Figure 6 provides a flow chart for the experimental design of this research.

1. Methyl linoleate was oxidized in 10 ml vials, open and closed to the atmosphere, at 25, 40, and 60 °C in the dark for 20 days with shaking.
2. Oxidation products (conjugated dienes, hydroperoxides, epoxides, carbonyls) were measured each day during this period to track the progression of oxidation from initiation to established oxidation.
3. Oxidation conditions were varied to investigate effects of temperature, headspace oxygen, solvent, water, and lipid concentration on rates of oxidation, dominant products, and distribution of products. Each factor was studied independently; some factor combinations were also studied.
4. Data from daily tests were plotted versus incubation time (days) at the end of 20-day period. Development patterns and concentrations of each product, as well as relationship between them were determined from these plots and used to assess

the presence of multiple reaction pathways as well as conditions favoring one pathway over another.

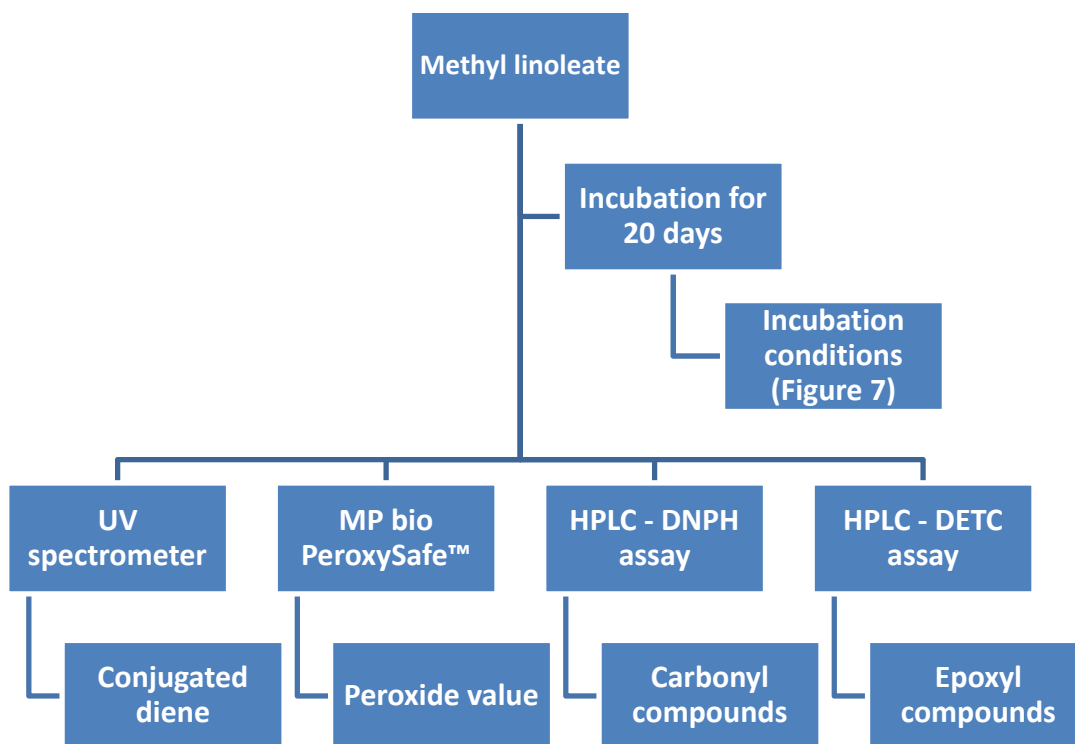


Figure 6. Experimental flow diagram for tracking degradation pathways in oxidizing methyl linoleate.

5.2.3 Methyl linoleate oxidation

500 μ L methyl linoleate (sufficient for 2 days of tests) were transferred to 10 mL glass crimp top GC vials (Thermo Scientific, Waltham, MA) wrapped with aluminum foil to exclude light and avoid photodegradation of hydroperoxides. A 10 mL vial volume ensures adequate oxygen and at the same time is economic in terms of glassware. Vials were then incubated in a Lab Companion IS-971 shaker (Ramsey, MN) at the temperature specified for the experiment. For closed samples, vials were sealed with

PTFE (teflon)/butyl caps (Perkin Elmer, Waltham, MA). For open-air samples, 5 mL methyl linoleate were incubated in a 100 mL beaker with top covered with 1 layer of kimwipes™ delicate task wipers (Kimberly-Klark, New Milford, CT) for dust protection. Samples were withdrawn daily for analysis of oxidation products. Triplicate samples were analyzed for each data point.

5.2.4 Factors affecting lipid oxidation rates and reaction pathways

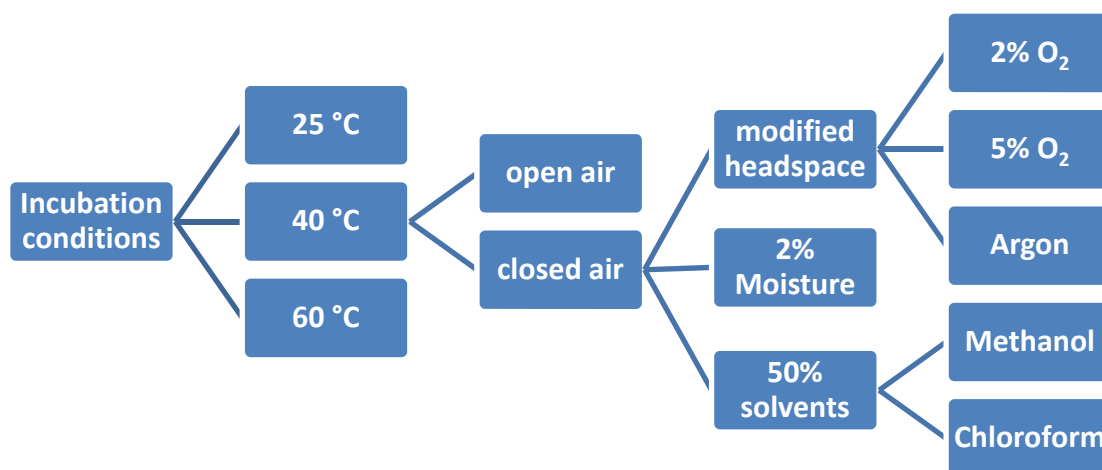


Figure 7. Incubation conditions. (this graph used 40 °C as an example to show how each factors are determined at such temp. Similar tests were also conducted under 25 °C and 60 °C).

Four catalytic factors were examined in this study: temperature, headspace oxygen, presence of water, solvents, and lipid concentration. Open-air and closed systems were tested for each temperature. Factors were studied individually and in some combinations only at 40 °C (Figure 7) because oxidation at 20 °C was too slow and oxidation at 60 °C altered pathways and is not representative of conditions foods normally encounter. Reduced oxygen concentrations were tested for each closed air condition.

5.2.4.1 Temperature

500 μ L methyl linoleate (sufficient for 2 days of tests) were transferred to 10 mL glass crimp top GC vials. 10 vials of methyl linoleate were incubated at each temperature (25 °C, 40 °C and 60 °C) in a shaker where temperature was strictly controlled. These three temperatures were chosen to mimic realistic environments encountered by foods. 25 °C is normal ambient room temperature; 40 °C simulates temperature abuse conditions (outdoor, shipping cargo, military supply storage); 60 °C is often used in accelerated shelf-life tests to predict long-term stability of foods within short periods of time.

5.2.4.2 Headspace oxygen

500 μ L methyl linoleate (sufficient for 2 days of tests) were transferred to 10 mL glass crimp top GC vials. 10 vials of methyl linoleate were used in each condition test. The effect of headspace oxygen was studied in both open and closed systems to test effects of volatile products escaping versus remaining in the system and reacting. To observe effects of unlimited oxygen, ML samples were incubated in uncapped vials with minimal but permeable covering for dust protection at the three test temperatures; vials were constantly agitated at 60 RPM to maintain mixing and prevent concentration gradients created by oxygen diffusion into the oil from a quiescent interface.

It is known that limitation of oxygen suppresses oxidation, yet the limiting oxygen concentration at which oxidation can be substantially inhibited remains equivocal. To test effects of low oxygen concentrations on oxidation rates and products, the headspaces of methyl linoleate samples were sparged with air ($O_2 \approx 21\%$), 5% O_2 , 2% O_2 , or Argon (Ar) for two minutes, and the vials were sealed for incubation. The headspace

in each vial was refreshed by the same method every three days to prevent oxygen limitation.

In preliminary studies, methyl linoleate stored in vacuum was carefully and quickly transferred to the sample vial pre-filled with modified gas to minimize contact with air. The methyl linoleate was then sparged with modified gas to saturate the oil phase. However, gas flow could not be reduced sufficiently to avoid splashing and dispersing the linoleate due to small sample and vial size, and these samples tended to oxidize more rapidly. Therefore, it was decided not to sparge the methyl linoleate with modified atmosphere gases directly.

5.2.4.3 Solvents

Solvents affect oxidation differently depending on their proticity and polarity. For example, Frankel claims that proton sources in the solvent are necessary to stabilize scission products [11], i.e. to increase carbonyl products, while internal rearrangements to epoxides are enhanced in neat oils or in aprotic solvents [110, 111]. Hence, three solvents with different proticity and polarity were tested: ethanol (protic/polar), ethanol/water mixtures, and chloroform (non-polar). 250 μL methyl linoleate and 250 μL solvent were added to each vial and mixed well using the same sample preparation protocol as in the neat ML tests described in Section 5.2.2 and 5.2.3. This gave a total sample volume of 500 μL , the same as for tests in neat ML, so the headspace volumes were also the same. Results from analyses were normalized according to the dilution factor.

5.2.4.4 Water

Water in lipid phases provides an environment in which polar oxidation intermediates or final products can partition. It also provides a proton source and

hydrogen bonding center that can stabilize transition states and some products, so it may shift the balance among alternate pathways, specifically enhancing alkoxyl radical scissions and accumulation of secondary products such as aldehydes and ketones. We specifically wanted to test Frankel's assertions [11], that the presence of water increases production of aldehydes and other scission products.

490 μ L ML and 10 μ L Milli-Q water were added to each 10 ml reaction vial, giving a final concentration of 98% methyl linoleate (2% water). 2% water was selected as a test concentration because it approximates the steady state concentration of water in early stages of deep fat frying, e.g. after potato chips have been added to the hot oil. To ensure full mixing between methyl linoleate and water, sample vials were sparged with air and crimp-top sealed, then vortexed for two minutes before incubating as described above for neat ML samples. Higher water content (10%) was originally planned to be tested, but dispersion of water at levels greater than 2% was not possible to maintain without emulsifiers, a complication we sought to avoid at this stage of testing.

5.2.4.5 Combinations of factors

In real food systems, more than one environmental factor usually affects oxidation. Therefore, it is important to also look at effects of combination of above factors. Multiple factors were tested together through a series of cross combinations. A total of 14 conditions tested in this research are listed in Table 3. These conditions are grouped into eight tests to facilitate comparison between samples.

Table 3. Experiment design – combinations of oxidation conditions.

Test 1	25 °C +open-air	40 °C + open-air	60 °C +open-air
Test 2	25 °C + closed air	40 °C + closed air	60 °C + closed air
Test 3	40 °C + closed, 2%O ₂	60 °C + closed, 2% O ₂	
Test 4	40 °C + closed, 5%O ₂	60 °C + closed, 5% O ₂	
Test 6	40 °C + closed, Argon	60 °C + closed, Argon	
Test 7	40 °C + closed air + 50% Ethanol	40 °C + closed air +50% Chloroform	
Test 8	25 °C + closed air +2%H ₂ O	40 °C + closed air +2%H ₂ O	60 °C + closed air +2%H ₂ O

5.3 Analyses of Lipid Oxidation Products

Conjugated dienes, hydroperoxides, carbonyls, and epoxides are most commonly measured in lipid oxidation studies. Determination of these four classes of products provides a starting point for critical comparison of active and dominant oxidation pathways in this study and for verification that multiple oxidation pathways exist.

5.3.1 Conjugated dienes

Conjugated dienes were determined using a modification of AOCS procedure Ti 1a-64 [112]. 30 µL of methyl linoleate were mixed with 10 mL iso-octane, and the solution was diluted as needed (usually 1:3) for accurate spectroscopic analysis. The

optical absorbance of the solution was measured at 234 nm against an iso-octane blank using a Varian Cary 50 UV/Vis Spectrophotometer (Varian Inc., USA). For extensively oxidized samples, the dilution factor was determined by preliminary testing so that final absorbance values were no higher than 1.0000. Concentration of conjugated dienes (mmols/mol ML) were calculated using Beer's Law using an extinction coefficient of 29500 L·mol cm⁻¹ in iso-octane [63].

5.3.2 Hydroperoxides

Considering the large quantity of samples to be analyzed, simple and fast procedures were clearly needed for hydroperoxide determination in this project. Hence, we used the standard SafTest™ (MP Biomedical, Solon, OH) for measurement of hydroperoxides. The PeroxySafe™ assay is based on LOOH oxidation of Fe²⁺ to Fe³⁺ that can be quantified by complexation with xylenol orange. There are four proprietary reagents in the kit: Reagent A, B, C and a Preparation Reagent. Standard curves for instrument calibration were prepared every other week using the calibrators provided. Methyl linoleate (ML) with different levels of hydroperoxides was diluted with PeroxySafe™ Preparation Reagent to a range of concentrations before each analysis. Dilution factors depended on degree of degradation and thus varied for each test. During testing, 25 µl of properly diluted ML sample, 1000 µl of PeroxySafe™ Reagent A, 100 µl Reagent B and 160 µl Reagent C were pipetted into 10 mm × 75 mm test tubes, followed by 30 seconds vortexing. The mixture was incubated at room temperature (25 °C controlled in a heating block) for 15 minutes, and absorbance at 570 nm was recorded using the SafTest MicroChem™ optical analyzer. Peroxide values reported by

PeroxySafe™ software in units of meq peroxide/kg fat were converted to mmols/mol ML to be able to compare all products on the same molar basis.

5.3.3 Analysis of Carbonyl Products

The 2,4-dinitrophenylhydrazine (DNPH) HPLC assay was adapted to determine carbonyl concentrations as well as to identify carbonyls formed during oxidation. The latter information will be very useful in deriving oxidation pathways. The method was developed and modified based on a number of DNPH methods in existing literature [91-94, 113-116] for measuring carbonyls in different systems.

Carbonyls reacts with DNPH and the resulting hydrazones can be separated by HPLC. For the analysis, a 100 mM DNPH solution was prepared by dissolving 0.0200 g 2,4-dinitrophenylhydrazine in 0.99 mL N,N-dimethyl formamide plus 0.01 mL (10 μ L) H₂SO₄. 50 μ L Methyl linoleate, 50 μ L acetonitrile, and 100 μ L DNPH solution were then mixed in a 10 mm capped test tube and incubated at room temperature for 15 minutes in the dark. 10 μ L sample was then injected onto an Agilent 1100 LC system (G1313A auto-sampler, G1310A isocratic binary pump, G1316A thermostatted column compartment and G1315A DAD detector) with Ultra C18 HPLC Column installed (150*4.6 mm, Restek) Individual hydrazones were separated by elution with a mobile phase composed of acetonitrile and water as described below. Hydrazones were detected at 360 nm by a diode array detector.

A series of experiments were conducted to determine best conditions for the HPLC separation of carbonyl compounds from methyl linoleate. DNPH hydrazones of a mixture of C4-C10 saturated aldehydes (butanal, pentanal, hexanal, decananal) were separated on a Restek® Ultra C18 column (4.6 mm ID), eluting with different acetonitrile and water

gradients. Figure 8 shows the first gradient elution we tested for DNPH methods; the corresponding chromatogram obtained using this gradient is shown in Figure 8. The gradient was then modified many times for optimization.

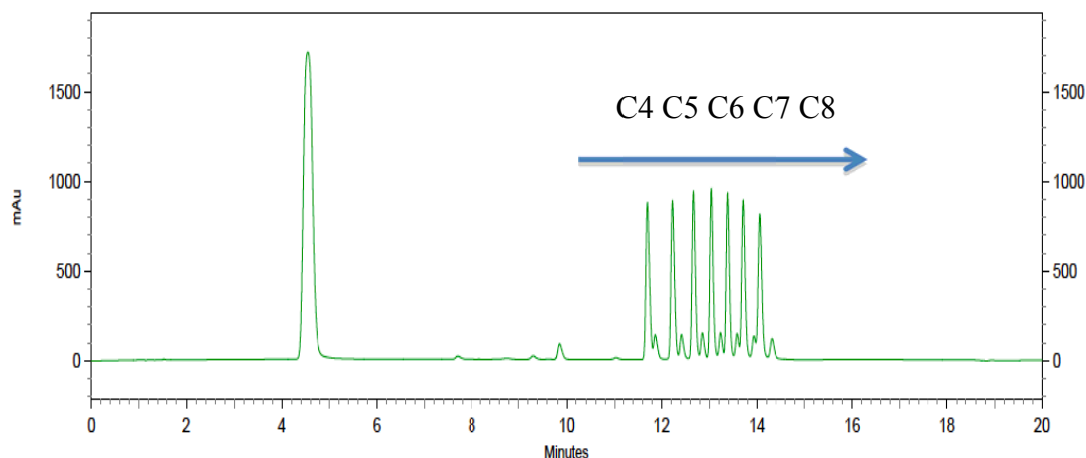


Figure 8. Chromatographic separation of C4-C10 saturated aldehydes by gradient elution method 1.

Figure 8 shows that separation of C4-C10 saturated aldehydes was achieved but was not optimal for quantitation. Seven major peaks from the C4-C10 aldehyde hydrazones were observed between 11 and 15 min, eluting in sequence by chain length. Butanal eluted first and decanal eluted last. Although each peak was separated, the time range was too narrow and the peaks were too close to each other. This was considered problematic because separation of mono- and di-unsaturated compounds with the same chain length will then become difficult.

To address this issue, a total of 44 mobile phase elution programs were tested to find optimal separation conditions. The final gradient (#44) giving best working separation of monomer aldehyde hydrazones in 20 minutes is shown in Table 4. The corresponding chromatogram of hydrazones from mixed saturated and unsaturated aldehydes is shown in Figure 9. The retention time of each standard peak is listed in

Table 6. This final version of gradient elution was applied to all carbonyl analyses of oxidized methyl linoleate in the study.

Table 4. Gradient #44 (the final version for carbonyl analyses in this study) optimized for separation of hydrazones of C4-C10 saturated and unsaturated aldehydes.

Time (min)	Acetonitrile (mL/min)	Water (mL/min)
0	0.8	0.4
15	1.2	0
17	1.4	0
18.5	0.8	0.4
20	0.8	0.4

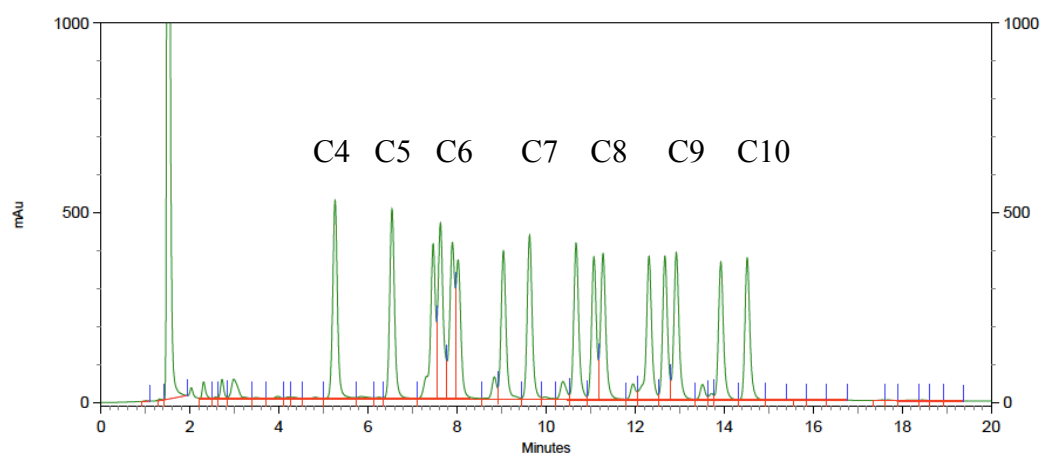


Figure 9. Chromatographic separation of DNPH hydrazones of saturated and unsaturated aldehydes C4-C10 by gradient elution method 44 (unsaturated aldehydes were not labeled).

Figure 9 showed the best separation of all aldehydes in 20 minutes. This final version of gradient elution will be applied to future carbonyl analysis. The retention time of each standard peak is listed in Table 6. According to the chromatogram, the area under the curve (AUC) of each peak is much closed to each other, suggesting it has no correlation with chain length; a standard curve for quantitating carbonyls was prepared from nonanal.

Table 5. Retention time for each aldehyde standard.

Carbon #	Aldehyde standards	Retention time (min)
C4	Butanal	5.26
C5	Pentanal	6.536
C6	t-2-hexenal	7.464
C7	c-4-heptenal	7.624
C6	Hexanal	7.85
C7	t,t-2,4-heptadienal	8.024
C7	t-2-heptenal	9.04
C7	Heptanal	9.628
C8	t-2-octenal	10.672
C8	Octanal	11.072
C9	t,t-2,4-nonadienal	11.28
C9	t-2-nonenal	12.312
C9	Nonanal	12.668
C10	t,t-2,4-decadienal	12.924
C10	t-2-decenal	13.924
C10	Decanal	14.516

Table 6. Area under the curve of different concentrations of nonanal.

	Area under the curve (AUC)				
[nonanal]	test 1	test 2	test 3	Average	Stdev
50 mM	21012.90	21047.60	21086.60	21049.03	36.87
10 mM	5065.30	5047.00	5056.40	5056.23	9.15
1 mM	514.20	512.70	513.80	513.57	0.78
0.5 mM	246.90	247.60	247.10	247.20	0.36
0.25 mM	122.10	122.70	121.00	121.93	0.86
0.1 mM	53.40	50.90	51.80	52.03	1.27

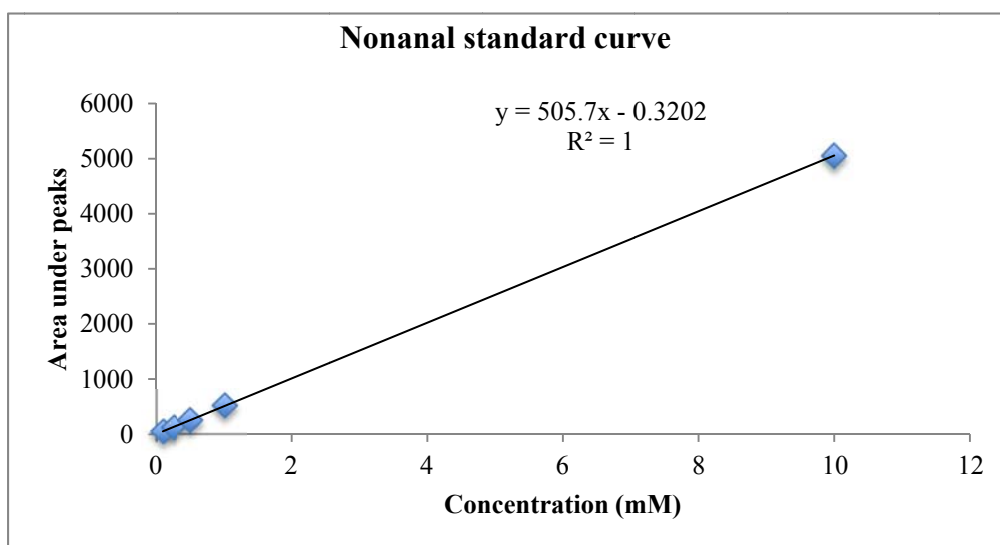


Figure 10. Standard curve for DNPH hydrazones of nonanal, used to quantitate carbonyls formed during oxidation of methyl linoleate.

Figure 10 is the plots of nonanal concentration vs. peak area yielded a straight line with regression equation $y=505.7x-0.3202$, with a $R^2=0.9999$, where Y is the area under curve and X is the concentration of nonanal peak. This regression equation was used to calculate concentrations of each individual carbonyl product from respective peak areas, as well as total carbonyls sum of from the sum of all peak areas.

5.3.4 Epoxide analysis

Epoxides were analyzed using the N,N-diethyldithiocarbamate (DETC) HPLC assay [106]. In this analysis, DETC reacts with epoxide groups to form a colored complex that absorbs at 278 nm. A DETC solution was prepared by dissolving 0.0685 g N,N-diethyldithiocarbamate in 20 mL methanol. 2 μ L methyl linoleate (ML) sample was added to 2 mL DETC solution in a 10 mm test tube and the mixture was incubated at 60 °C for 20 minutes in the water bath. 100 μ L 75% phosphoric acid was then added to decompose unreacted DETC. 20 μ L of the reaction mixture was injected onto an Agilent 1100 LC system (G1313A auto-sampler, G1310A isocratic binary pump, G1316A thermostatted column compartment and G1315A DAD detector) with Ultra C18 HPLC Column installed and component epoxide complexes were eluted and separated using a mobile phase composed of acetonitrile and water as described below. Epoxide peaks were detected by diode array at 278 nm.

A series of gradient elution sequences were tested to determine optimum separation of four epoxide standards with different chain lengths: 2,3-epoxybutane, 1,2-epoxy-5-hexene and 1,2-epoxy-9-decene, cis-7,8-epoxy-2-methyloctadecane. Table 8 shows the first gradient used for separation of DETC-epoxide adducts. Figure 11 shows the corresponding HPLC separation of the four epoxide standards. The tiny DETC

reagent peak near the solvent front confirms that excess DETC was eliminated by phosphoric acid. Epoxide peaks were well separated, but peak areas for the four epoxides analyzed were unequal due to volatility of 2,3-epoxybutane and lower purity of cis-7,8-epoxy-2-methyloctadecane.

Table 7. Test gradient elution method 1 for separation of DETC complexes with standard epoxides.

Time (min)	Acetonitrile (mL/min)	Water (mL/min)
0	0.8	1.2
5	0.8	1.2
10	1.2	0.8
13	1.4	0.6
16	2	0
20	2	0
25	0.8	1.2
30	0.8	1.2

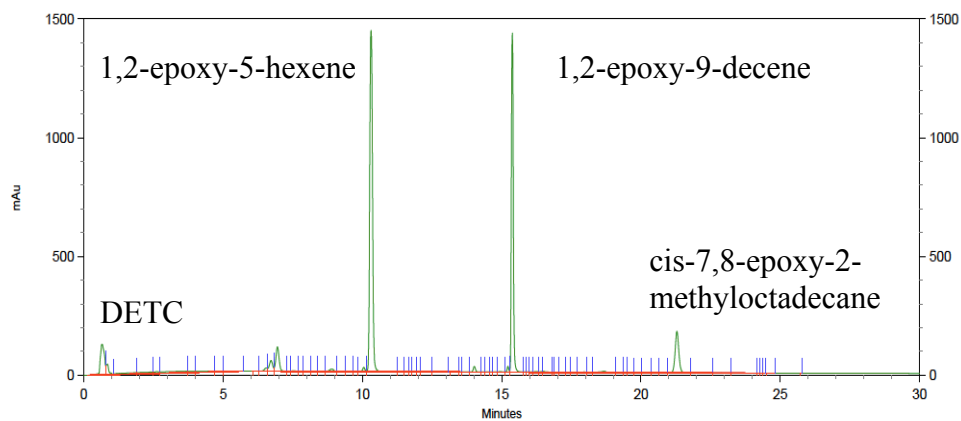


Figure 11. Chromatogram of DETC-epoxide standards separated by gradient elution method 1.

Table 8. Optimized solvent gradient for HPLC separation of DETC-epoxides. Method #4.

Time (min)	Acetonitrile (mL/min)	Water (mL/min)
0	0.8	1.2
2	0.8	1.2
10	1.6	0.4
12	2	0
17	2	0
19	0.8	1.2
20	0.8	1.2

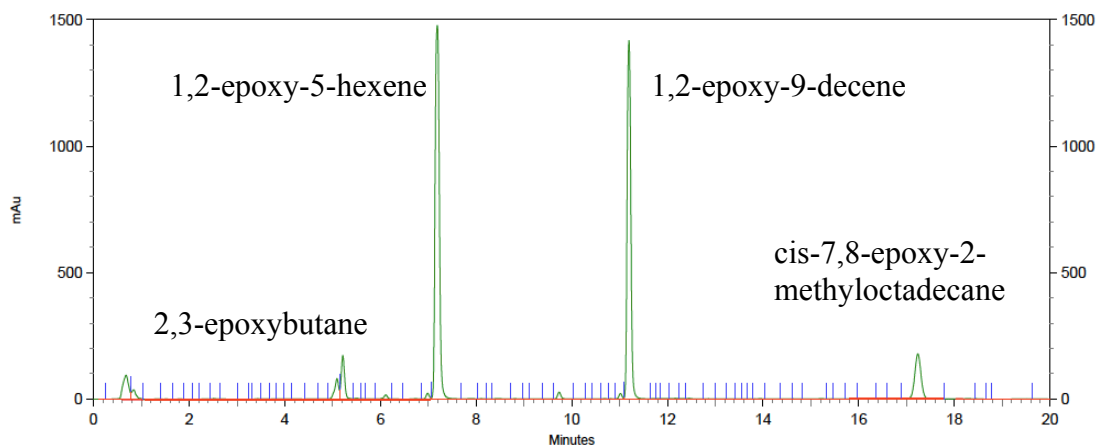


Figure 12. Chromatogram of DETC-epoxide standards separated by optimized gradient elution Method 4.

Table 9. Area under the peak for 1,2-epoxy-5-hexene and 1,2-epoxy-9-decene.

C6		C10	
Conc. (μM)	Peak Area	Conc. (μM)	Peak Area
1.97	13235	1.213	10050
3.94	25490	2.426	18307
7.88	45423	4.852	31217
15.76	99942	9.704	68116

Further testing of mobile phase elution programs yielded the optimized elution gradient (#4) with shorter elution times (Table 9). Epoxide separation achieved with this gradient is shown in Figure 11. Gradient #4 was used for all epoxide analyses in this study.

Epoxides were quantified by comparing analyte peak areas to a standard curve prepared from C6 and C10 epoxides (C4 and C18 epoxides could not be used accurately) analyzed under identical conditions. Peak areas over a range of concentrations for each

standard are shown in Table 10. This data was combined and plotted to derive a standard curve, for which the regression equation was determined to be $y=6281.6x+1482.9$, where Y is the peak area of both under curve and X is the concentration of epoxide standards. The combination of both standard curves showed excellent linearity ($R^2=0.9899$,) and was applied for quantification.

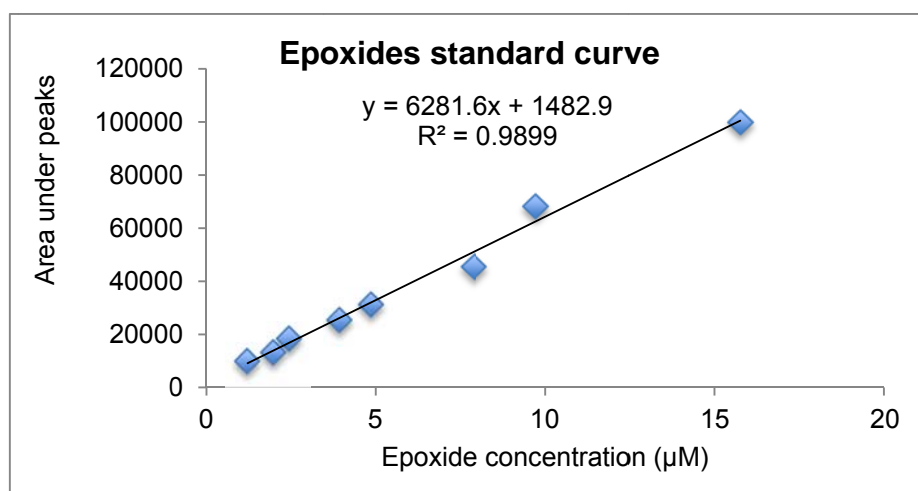


Figure 13. Standard curve from combination of 1,2-epoxy-5-hexene and 1,2-epoxy-9-decene.

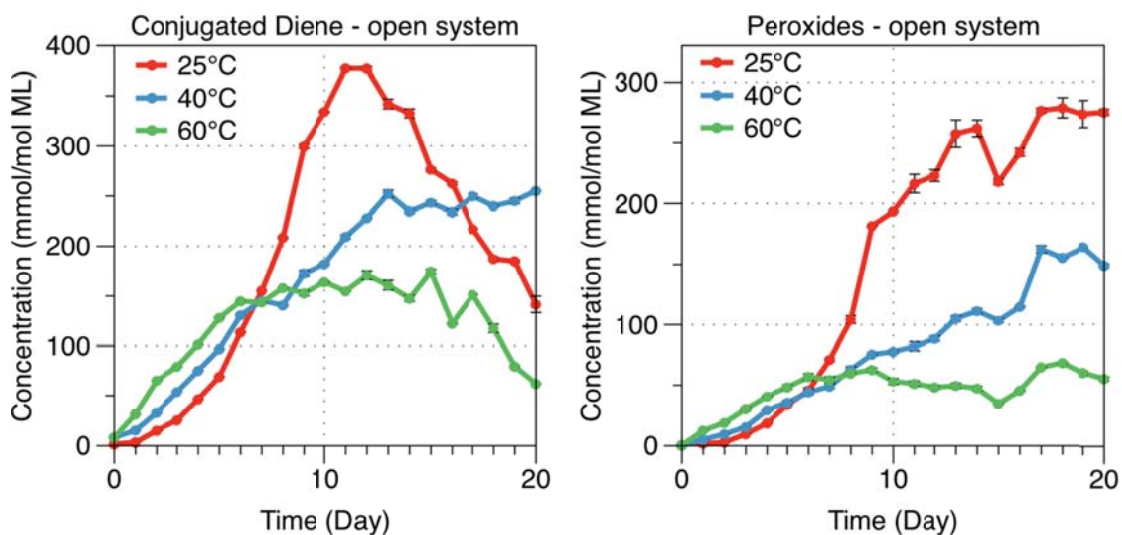
6. RESULTS

6.1 Effects of reaction conditions on kinetics and distribution of oxidation products.

All four groups of oxidation product (conjugated dienes, hydroperoxides, epoxides, carbonyls) were analyzed daily and curves describing the progression of oxidation over time for each product were plotted. Comparison of the distribution and balance between oxidation products under different environmental conditions provided evidence for other reaction pathways occurring in parallel to and in competition with hydrogen abstraction.

6.1.1 Effect of temperature on product dynamics -- Open-air

500 μ L samples of methyl linoleate (sufficient for 2 days of tests) were transferred to 10 mL glass crimp top GC vials and oxidized with shaking at 25 °C, 40 °C and 60 °C, covered with only a single layer of kimwipes™ delicate task wipers for dust protection. The time course for development of all products at the three temperatures is shown in Figure 14. Product distributions at each temperature are shown in Figure 15.



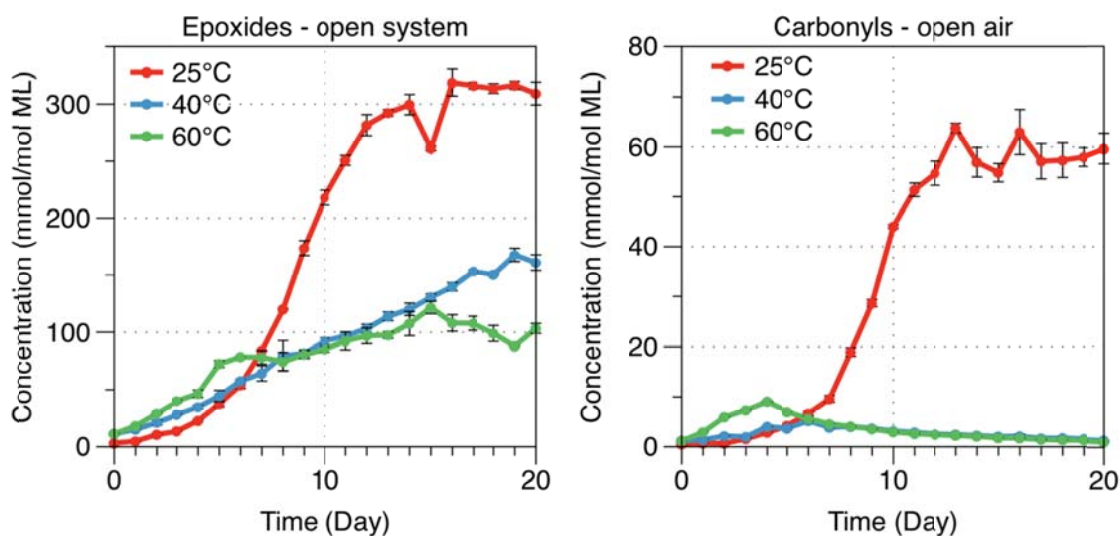
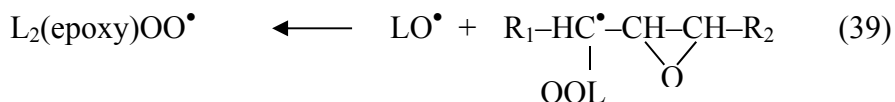
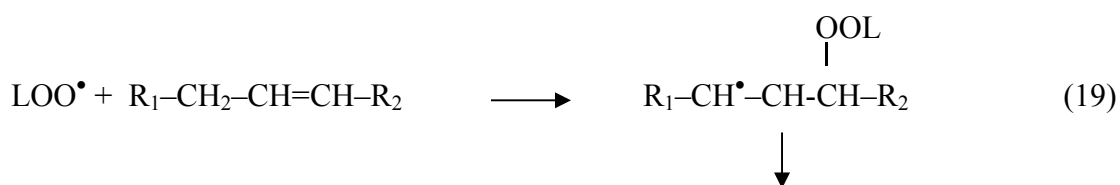


Figure 14. Effect of temperature on ML oxidation rates and levels of four major products over 20 days incubation in open system.

Condition effects: In contrast to Arrhenius predictions of steadily increasing oxidation at higher temperatures, very different patterns for formation of total oxidation products were observed at each temperature (Figure 15). At room temperature (25° C), there was a lower starting rate and induction period of about three days for all products. However, once started oxidation proceeded more rapidly and products accumulated to much higher concentrations than with either elevated temperature. At 40 °C, the induction period was shorter and all products accumulated steadily at a moderate rate, increasing over most of the incubation period but to lower total levels than at room temperature. At 60 °C, oxidation began rapidly with no induction period but product concentrations plateaued at lower overall levels after only about six days incubation. Although initial oxidation was fast, rates of transformations into secondary products also increased so that the net levels of the four products accumulating and detected over time actually decreased with temperature. This is quite different from the common

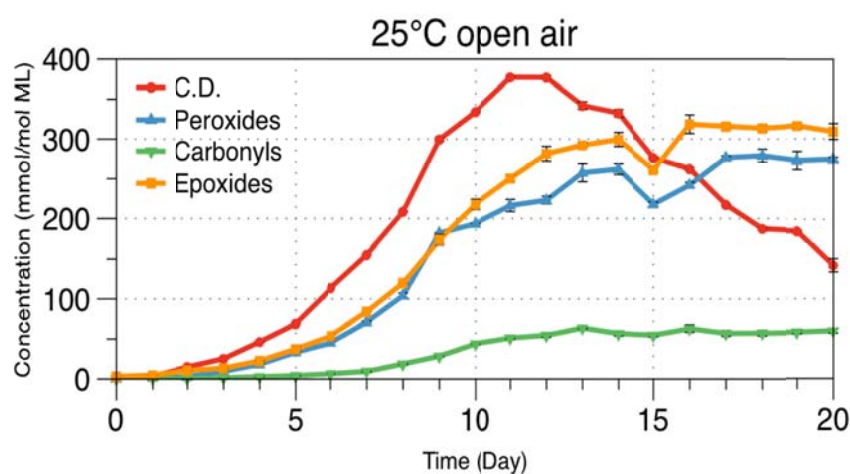
understanding that higher Temperature leads to faster oxidation and more concentrated products and raises serious questions about the validity of standard procedures used for accelerated shelf life studies.

Product distribution effects: Replotting the graphs to emphasize changes in product distributions at the three temperatures (Figure 16) showed three striking patterns: 1) conjugated dienes, hydroperoxides, and epoxides all began accumulating from the beginning of incubation, although at different rates, rather than appearing in sequence with a delay between, 2) epoxides were the dominant final product, forming in parallel to hydroperoxides and accumulating to comparable or higher levels (Figure 16); this was especially apparent at 60 °C where hydroperoxides decomposed but epoxides remained stable; and 3) conjugated dienes remained substantially higher than hydroperoxides under all conditions. Generally, you would expect that hydroperoxides would be very close to the conjugated diene levels since they retain that structure and very few other products have it. The large difference observed suggests either that the hydroperoxides are not being detected fully, the conjugated diene assay is not specific, or there are additional products with conjugated diene structure unaccounted for. This quandary also points out some of the analytical obstacles to sorting out multiple competing pathways.



Epoxides are most expected from internal rearrangement of alkoxy radicals. However, considering their immediate production during incubation, a likely pathway for their formation is via addition of lipid peroxyl radicals to ML double bonds (Reaction 39), followed by decomposition of the complex to yield epoxides (Reaction 40). It is important to note that the second product from this decomposition is alkoxy radicals (Reaction 40), which can rearrange to more epoxides or undergo scission to carbonyls, depending on conditions.

Individual products developed in different patterns over the 20 days period. At room temperature (Figure 15, top), conjugated dienes reached a peak of more than 400 mmol/mol ML at 11-12 days, then dropped to 150 mmol/mol ML by the end of incubation. The peroxide and epoxide accumulated in parallel, reaching a plateau after 16 days but not declining thereafter. Surprisingly, carbonyls also accumulated with only a moderate delay behind hydroperoxides and epoxides. The higher carbonyl levels attained may be attributed to early production from alkoxy radicals generated via Reaction 40 and from low volatility at room temperature.



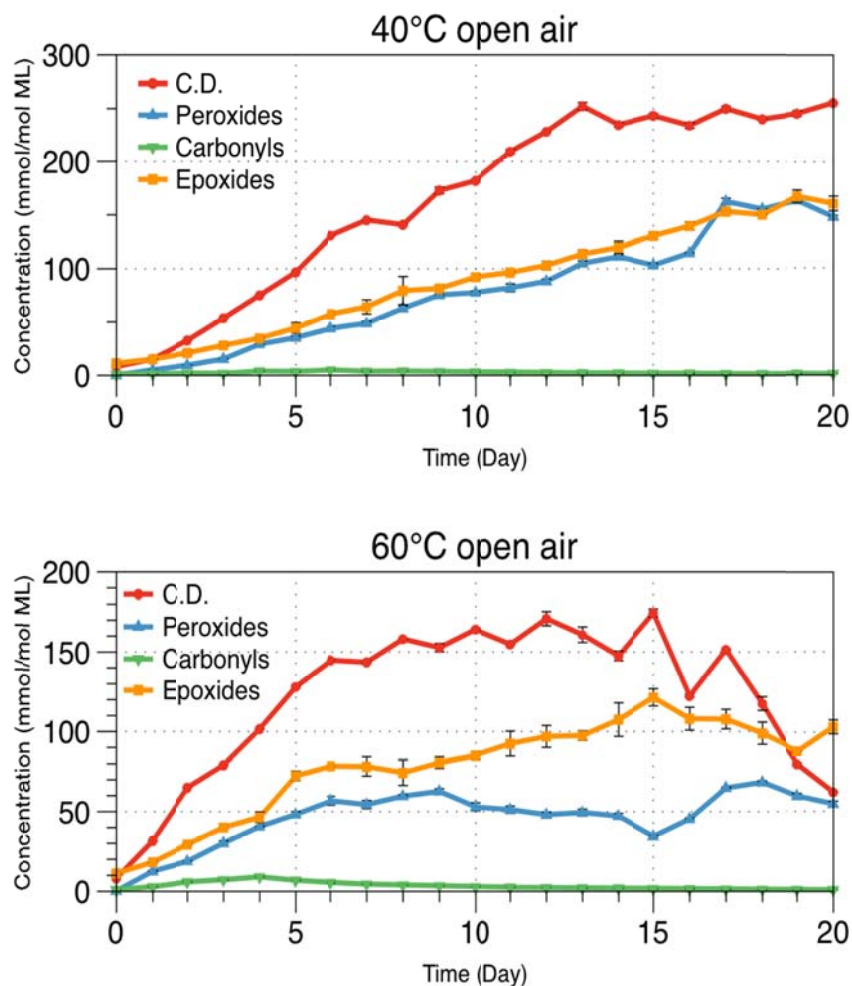


Figure 15. Effects of temperature on distributions of ML oxidation products during incubation in open systems.

At 40 °C (Figure 15 middle), conjugated dienes accumulated almost linearly up to about 14 days, then plateaued. Peroxides and epoxides also accumulated linearly but at a lower rate over the entire 20 days. This suggests that there was another unidentified reaction also occurring that transformed conjugated dienes into products other than epoxides and hydroperoxides. At this temperature, carbonyl compounds were detected only at very low levels for 6 days then decreased and remained under 10 mmol/mol ML for the rest of the incubation period. This loss of carbonyls may be explained by

increased volatilization at the elevated temperature, or there also may be increased rates of transformations to products to unidentified products, e.g. LOOH to non-carbonyl products, and carbonyls to other compounds, such as by carbonyl condensation reactions.

At 60 °C (Figure 15 bottom), oxidation began immediately and products accumulated at faster initial rates due to the higher thermal energy. However, the accumulation rate of conjugated dienes slowed down after only 7-8 days, which is much earlier than 40 °C. Also in contrast to 40 °C, the pattern of peroxides and epoxides showed opposite trends after nine days of accumulation: peroxides began to decompose while epoxides continued to increase until 15 days. This behavior is consistent with previous observations that at temperatures greater than 40°C, LOOH decomposition increases dramatically, while rates of epoxide formation from both LOO^\bullet addition and LO^\bullet rearrangement increase [117]. A shift in pathways is further supported by the negligible levels of carbonyls derived from LOOH decomposition and subsequent LO^\bullet scission. Of course, it could be argued that carbonyls were actually formed but were rapidly volatilized at the higher temperature. If so, longer chain carbonyls with lower volatility should still have been produced and accumulated in the ML, but these were not observed. Thus, although increased volatilization of breakdown products probably occurred, it seems clear that there was a strong major shift from H abstraction to internal rearrangement and radical additions. Future work needs to look for products from other pathways, e.g. alcohols from hydrogen abstraction, as well.

6.1.2 Effect of temperature on product dynamics --- Closed air

500 μL samples of methyl linoleate (sufficient for 2 days of tests) are transferred to 10 mL glass crimp top GC vials. 10 vials of methyl linoleate were oxidized in the

shaker at 25 °C, 40 °C and 60 °C. These were the same conditions as the open reaction systems except the crimp tops were sealed. Time course for development of all products at the three temperatures is shown in Figure 16. Product curves for each temperature are shown in Figure 17.

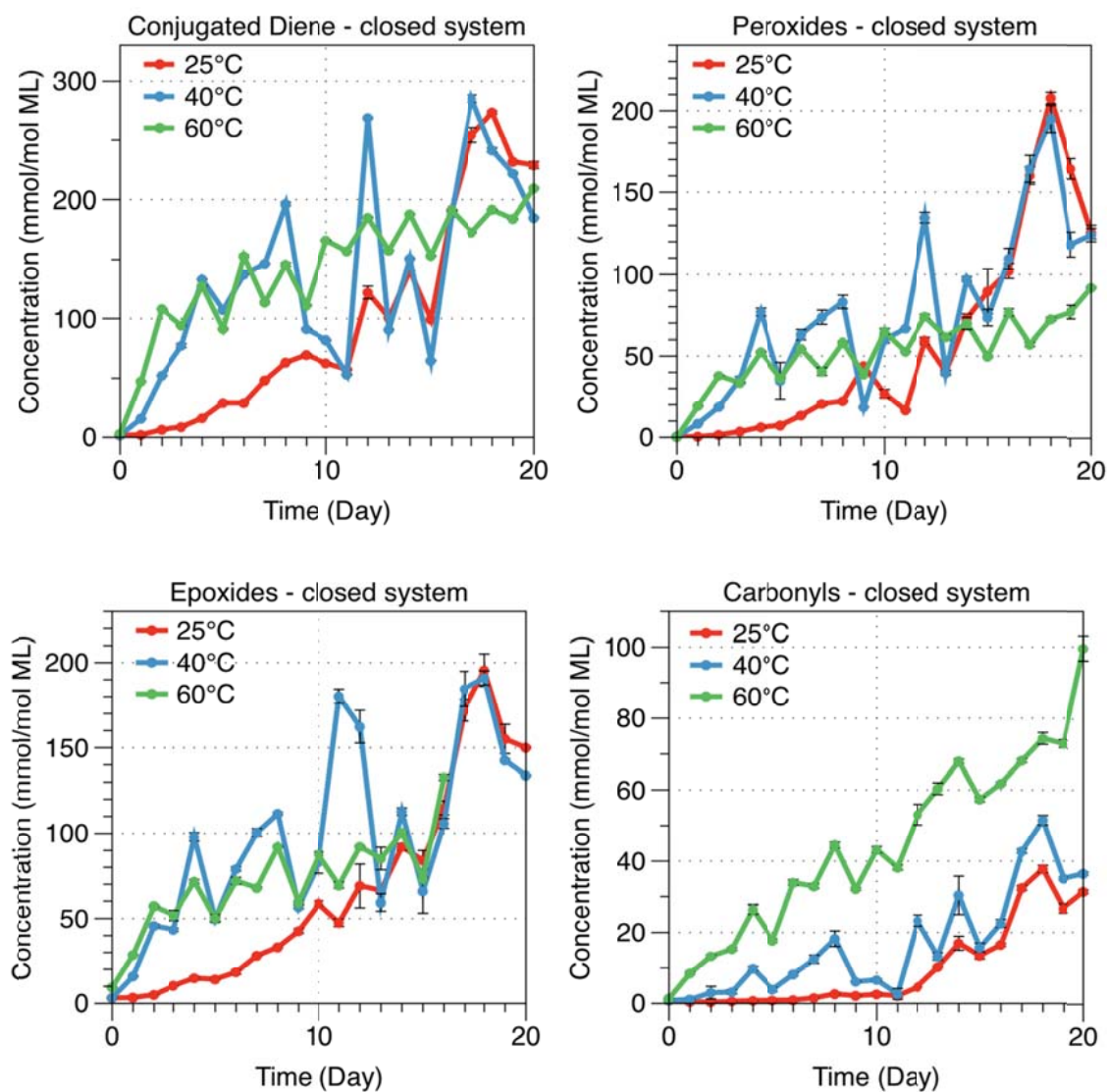


Figure 16. Effect of temperature on ML oxidation rates and levels of four major products over 20 days incubation in closed systems.

Condition effects: In contrast to the open reaction systems, oxidation levels in closed systems were lowest at room temperature, increased substantially at 40 °C, then dropped slightly at 60 °C (Figure 16), except for carbonyls which increased continuously with temperature, especially at 60 °C. Particularly interesting was the observation that temperature appeared to specifically affect initiation and the induction period -- the induction period decreased and initial oxidation rate increased with temperature -- but propagation rates afterward were reasonably comparable and oxidation curves were nearly parallel for the three temperatures

In closed systems, a surprising observation was that products cyclically increased and decreased throughout the incubation period, although the constant trend was upward. Cycling amplitude and frequency were least at room temperature, large and irregular at 40 °C, and moderate and constant at 60 °C.

The first and most obvious explanation was that the cycling was related to sampling in different vials or to replacement of air in the vials. However, the cycling periodicity changed with temperature, connecting it directly to chemical processes in the samples, it was not the same as sampling or aeration frequency, and we have seen similar cycling in many kinds of closed systems, including packaged foods. Thus, we speculate that this cycling arises from strong competition between several pathways that each of them dominated for a period, then reached to saturation and the production slowed down and degradation increased.

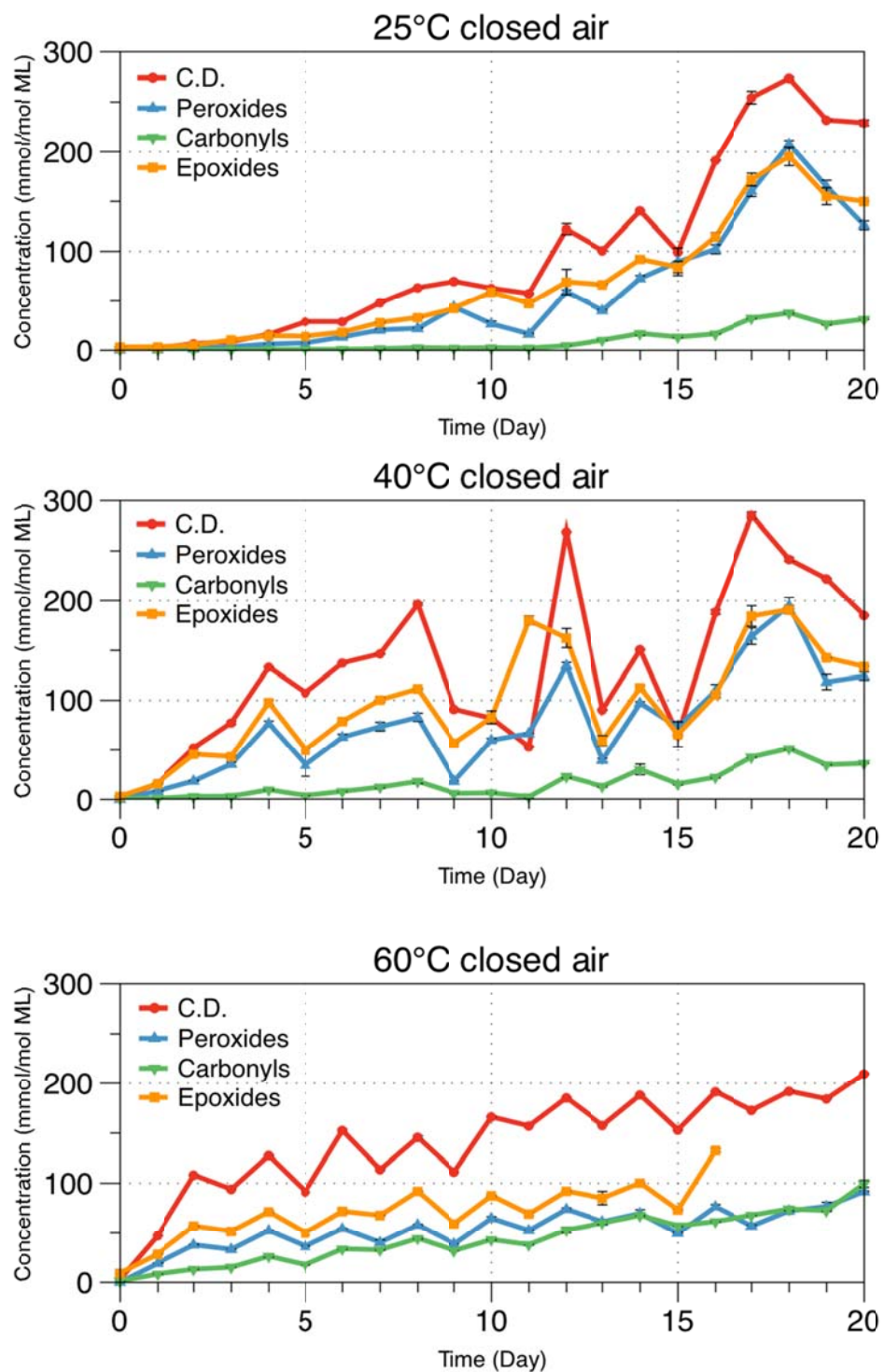


Figure 17. Effects of temperature on distributions of ML oxidation products during incubation in closed systems.

Looking at the oxidation and cycling more closely, in contrast to open systems, oxidation in 25 °C samples remained depressed through the first 15 days, then increased to the same level as samples at 40 °C and nearly as high as samples incubated in open air. Cycling peaks and valleys were minimal during early oxidation and increased with oxidation. These observations argue that oxygen limitation was minimal, if it existed. At 40 °C, products accumulated more rapidly, reaching the first peak after four days. Thereafter, cycling of all products was quite pronounced, suggesting strong interactions between oxidation intermediates. At 60 °C, a strong pattern of cycling remained but excursions were moderate and regular. Levels of the main three products were depressed relative to lower temperatures, but carbonyls began forming early and accumulated notably throughout the incubation.

As noted above, this cyclic pattern of oxidation has also been seen in many of our oxidation studies where closed systems exist, and so far, no explanation has been found. During lipid oxidation, products are mostly intermediates being generated and degraded. Therefore, this cycling pattern may very likely be reflecting the competition between a number of pathways including hydrogen abstraction and alternative pathways in the samples.

Product distribution effects: As in the open systems, epoxide levels were higher than hydroperoxides at all temperatures (Figure 17). Samples at room temperature showed an induction period, and this decreased with incubation temperature. Product levels were lowest at room temperature, increased at 40°C as initiation was enhanced, then decreased again at 60°C as secondary transformations were accelerated. Two key differences in closed systems were (a): oxidation took longer to develop and occurred at

lower levels overall, indicating that there was some oxygen limitation in the vials, and (b): all products developed cyclically, increasing and then decreasing repeatedly with some periodicity. In addition, carbonyls accumulated in parallel to epoxides and hydroperoxides, supporting the low levels of carbonyls in open systems as due to volatilization. Carbonyl levels increased with temperature, reaching levels comparable to hydroperoxides at 60 °C. This demonstrates increased scission of alkoxyl radicals at higher temperatures.

Conjugated dienes remained substantially higher than the sum of hydroperoxides and carbonyls throughout the oxidation period. Since nearly all of the hydroperoxides and up to half of the carbonyls should be conjugated (epoxides remove one double bond so cannot be conjugated), this pattern indicates the presence of products with structures and functional groups other than those measured.

6.1.3 Effect of moisture on product dynamics under closed air conditions

Moisture may exert both pro-oxidant and anti-oxidant effects depending on the levels and conditions. Tests of 2% water in this study were performed under closed air conditions at different temperatures.

490 μ L aliquots of methyl linoleate (sufficient for 2 days of tests) were transferred to 10 mL glass GC vials with 10 μ L of 18 M Ω water added. Vials were sealed with crimp tops and oxidized with shaking at 25 °C, 40 °C and 60 °C for 20 days. These were the same conditions as the closed reaction systems except the additional 2% of water. Time course for development of all products at the three temperatures is shown in Figure 18, Figure 20 and Figure 22. Product curves for each temperature are shown in Figure 19, Figure 21 and Figure 23.

6.1.3.1 Low temperature (25 °C)

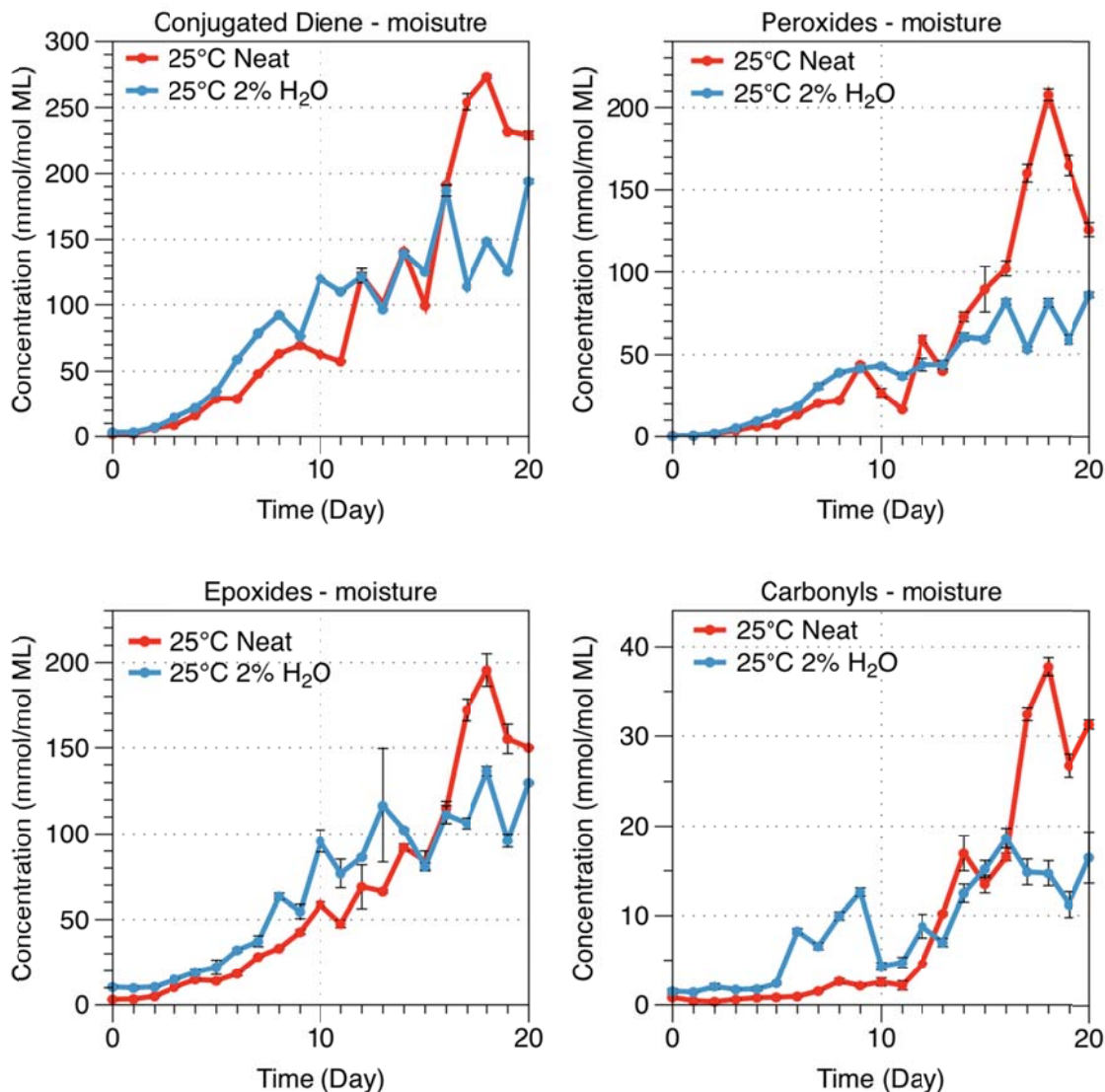


Figure 18. Effect of 2% added water on ML oxidation rates and levels of four major products over 20 days incubation at 25 °C.

Condition effects: Overall, at room temperature low moisture tended to decrease ML oxidation. However, effects on oxidation were variable across the 20 day incubation period (Figure 18). During the first five days, there were no differences in oxidation with or without water present. However, in the mid period between five and fifteen days,

water marginally increased all products except hydroperoxides, while during the last five days of incubation, water notably inhibited lipid oxidation. Since conjugated dienes also decreased, the effect was on initiation, so we speculate that water annealed radicals.

Product distribution effects: In neat ML, epoxide concentrations were slightly greater than hydroperoxides during the first fifteen days but thereafter concentrations of these two products were comparable (Figure 19, top left). The presence of water amplified epoxides and depressed hydroperoxides so that epoxides were clearly the dominant product throughout the incubation period (Figure 19, top right). This was somewhat unexpected since internal rearrangement has been shown previously to be facilitated by aprotic organic solvents and not water [118].

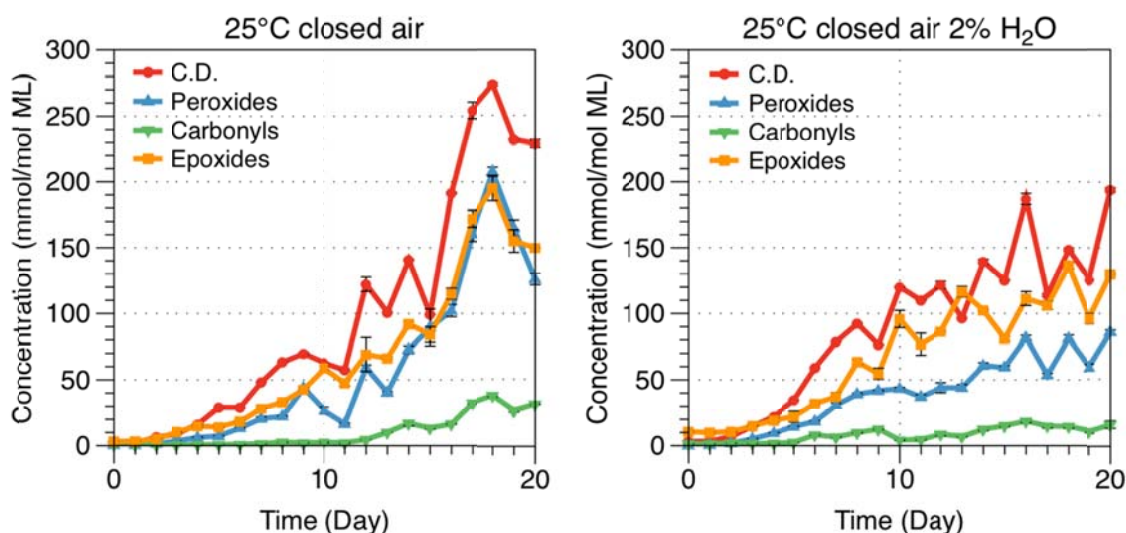


Figure 19. The effect of 2% water on distributions of ML oxidation products during incubation at 25 °C in closed systems.

Water is known to stabilize hydroperoxides via hydrogen bonding, but the reverse occurred in this system. Water also reportedly facilitates scissions of alkoxyl radicals to carbonyls by donating a proton to stabilize scission products such as carbonyls [119].

Accordingly, if LOOH were decomposing to LO[•] this system, such scissions would be expected to increase levels of carbonyls. Instead, there was a brief minimal increase in carbonyls between 5 and 10 days, but overall carbonyls levels decreased in the presence of water.

Discussion: The behaviors described above are inconsistent with water exerting its effects through polarity so other explanations must be found. One explanation for the shift to epoxides and decrease in other products in the presence of 2% water may be that the 18 MΩ water added to the ML contained enough trace metals (nM is sufficient), particularly Cu and Fe, to catalyze oxidation. Two reactions are possible. First, higher valence Fe and Cu both catalyze ML oxidation and formation of LOO[•] and they both also catalyze rapid conversion of LOO[•] to epoxides [118, 120, 121]. LOO[•] also generate epoxides via addition to double bonds [117]. Catalyzed oxidation would increase conjugated dienes but conversion to epoxides via either metals or radical addition would then remove conjugated dienes that had formed. As Cu²⁺ and Fe³⁺ react, reduced metals are generated, and these decompose LOOH to LO[•], which also rearranges to epoxides. The higher levels of epoxides thus may arise from any combination of (a): LOO[•] additions to double bonds, with subsequent decomposition of the complex, (b): metal catalyzed transformation of LOO[•], or (c): enhanced metal-catalyzed decomposition of LOOH to LO[•] followed by rearrangement. At the same time, more rapid transformations and decompositions in the presence of metals and water decrease net levels (not levels actually formed) of both conjugated dienes and hydroperoxides. Carbonyl levels increase slightly in the first few days of incubation due to higher levels of LOOH, but are halved

by the end of incubation, probably because LOO^\bullet and LO^\bullet are being rerouted to the epoxide pathway.

It is important to point out that if standard lipid oxidation assays for conjugated dienes, peroxide values, and hexanal were conducted on these samples, the conclusion would be that 2% water acts as an antioxidant. This conclusion would be in error because an alternate pathway not recognized or measured had been activated. As will be stressed many times in this dissertation, ignoring epoxides is dangerous because they are so much more reactive than other lipid oxidation products.

6.1.3.2 Moderate temperature (40 °C)

Condition effects: At moderately elevated temperature (40 °C) the main effect of moisture was to dampen the cycling excursion of conjugated dienes, hydroperoxides, and epoxides during ML oxidation (Figure 20). Total levels of conjugated dienes and hydroperoxides were also depressed. In contrast, carbonyls were notably enhanced during the middle period of oxidation (9-17 days) and inhibition at the end of incubation was less than the other three products. This would seem to support water facilitation of alkoxyl radical scissions, but also shows that elevated temperature is required as well.

Product distribution effects: Conjugated dienes and hydroperoxides were notably depressed in the presence of water while epoxides maintained levels until the end of incubation and carbonyls had periods of accumulation (Figure 21). This shows clearly that the balance between reaction pathways was shifting at the elevated temperatures.

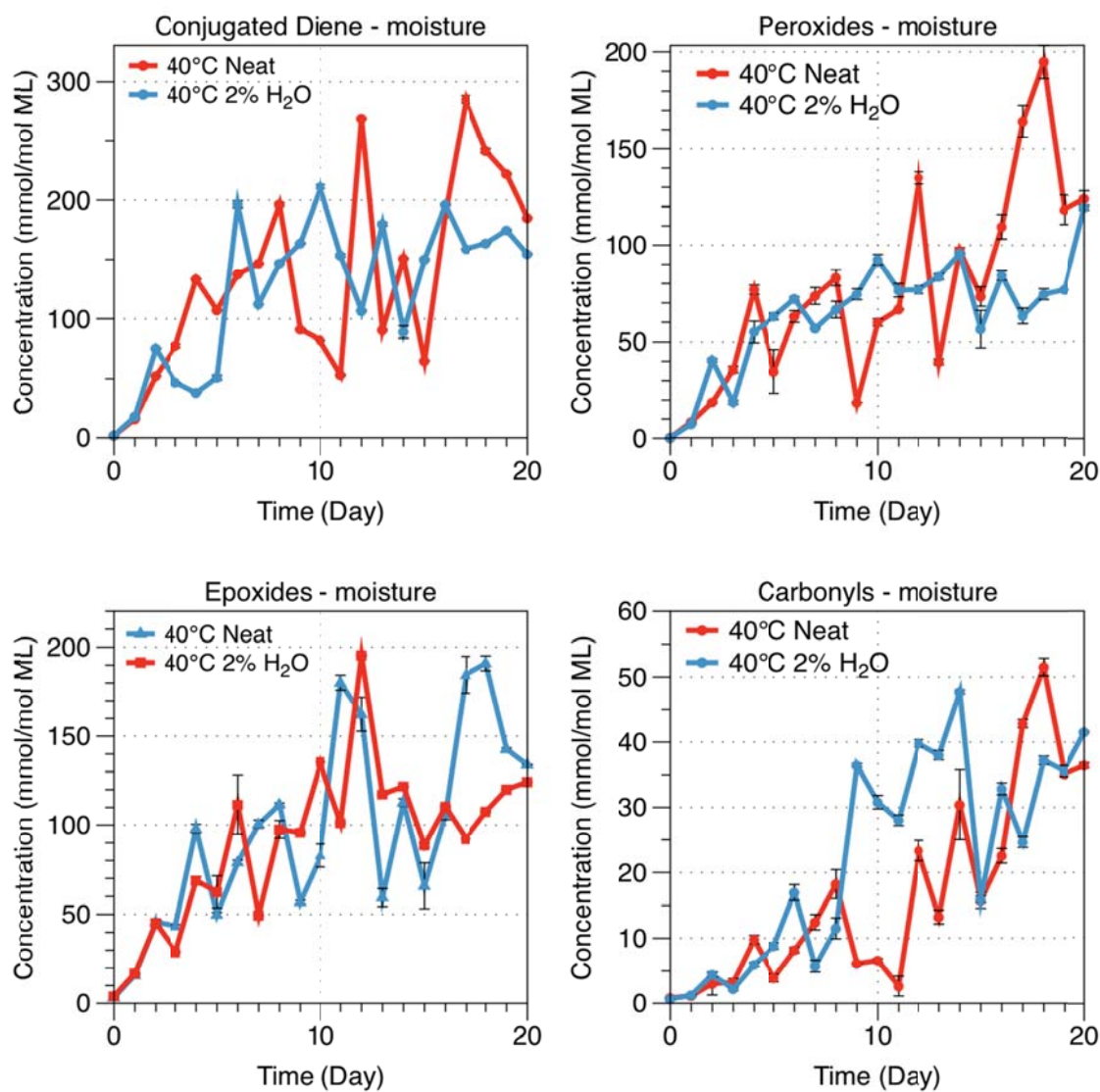


Figure 20. Effect of 2% added water on ML oxidation rates and levels of four major products over 20 days incubation at 25 °C.

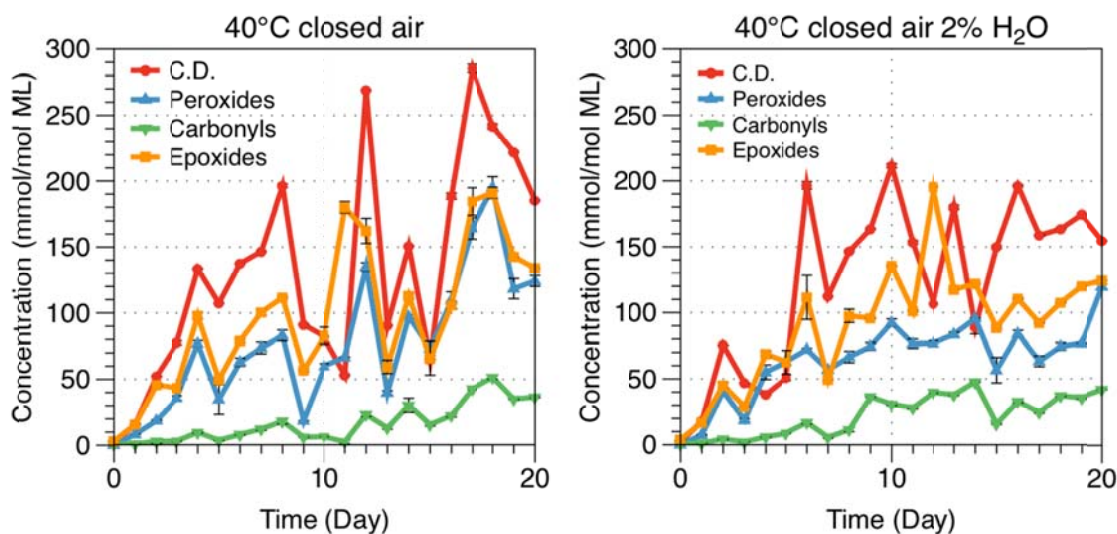


Figure 21. Effects of 2% water on distributions of ML oxidation products during incubation at 40 °C in closed systems.

Discussion: It seems counterintuitive that overall oxidation should decrease at elevated temperatures. As shown in the Temperature Effects section (6.1.2), overall oxidation increases at 40 °C but the increases are small, certainly not the quadrupling that would be predicted by the Arrhenius equation. In this context, it is important to keep in mind that lipid oxidation is a mixed series of reactions, each with its own activation energy and temperature effects. Reaction with low E_a will not be affected as much as temperature increases as reactions with high E_a . Complicating this further are counterbalancing decomposition reactions, particularly hydroperoxides. Epoxides, on the other hand, are more stable at high temperatures. Thus, it should be expected that the balance between alternate pathways will change with temperature. The current practice of limiting analyses to conjugated dienes, hydroperoxides, and perhaps hexanal will miss this shift and very likely give erroneous pictures of the true extent of lipid oxidation.

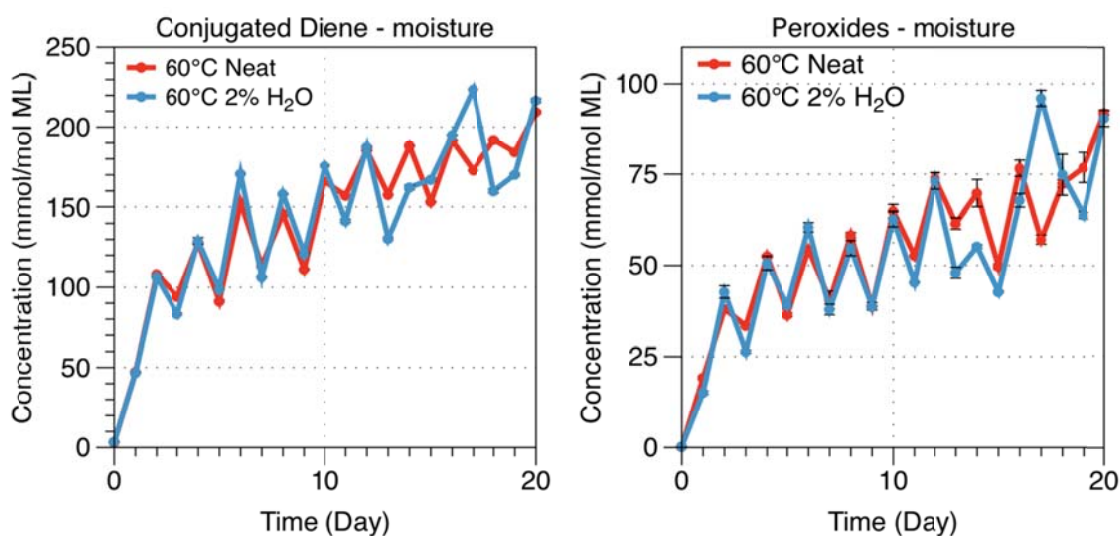
In future studies it will be critical to look for products other than those analyzed here to more fully account for alternate reactions competing at this temperature.

6.1.3.3 High temperature (60 °C)

Condition effects: At higher temperature all modifying effects of water on ML oxidation disappeared (Figure 22). The cycling for all products but carbonyls was very regular and unaffected by water, and oxidation levels in samples with water were virtually identical to those in neat ML. As in the neat ML systems, the main temperature effect was in increasing the initiation rate and decreasing the induction period. Products accumulated in parallel after initiation, and water did not alter this.

Product distribution effects: Product distributions and patterns remained the same as in neat mL -- conjugated dienes > epoxides > hydroperoxides > carbonyls (Figure 23). Carbonyls accumulated to higher levels than at lower temperatures, but water did not alter the levels.

Discussion: That 2% water had essentially no effect on oxidation at 60 °C may be attributed to evaporation of the small amount of water present, so the system behaved as if no water was present.



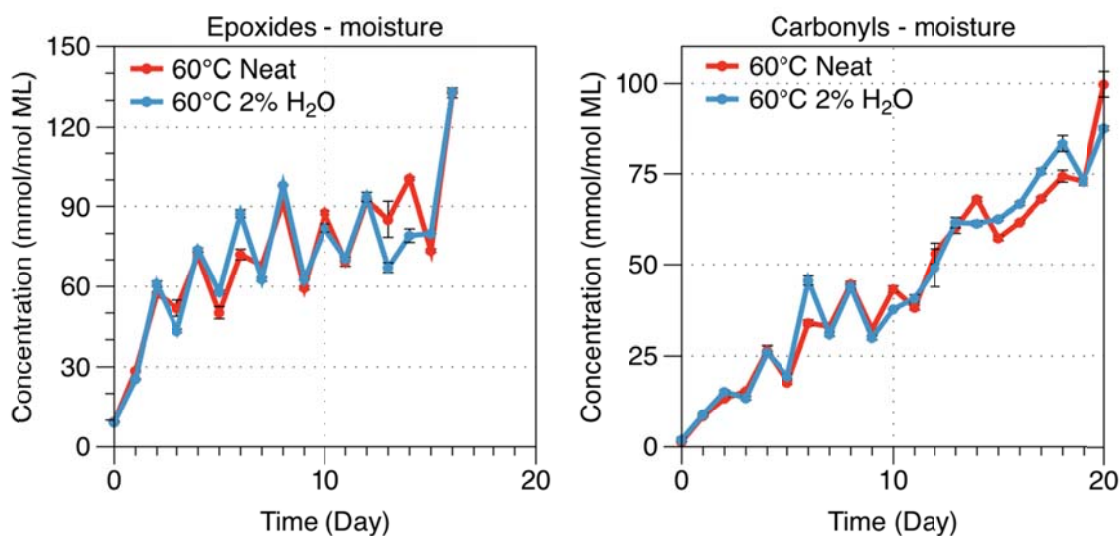


Figure 22. Effect of 2% added water on ML oxidation rates and levels of four major products over 20 days incubation at 25 °C.

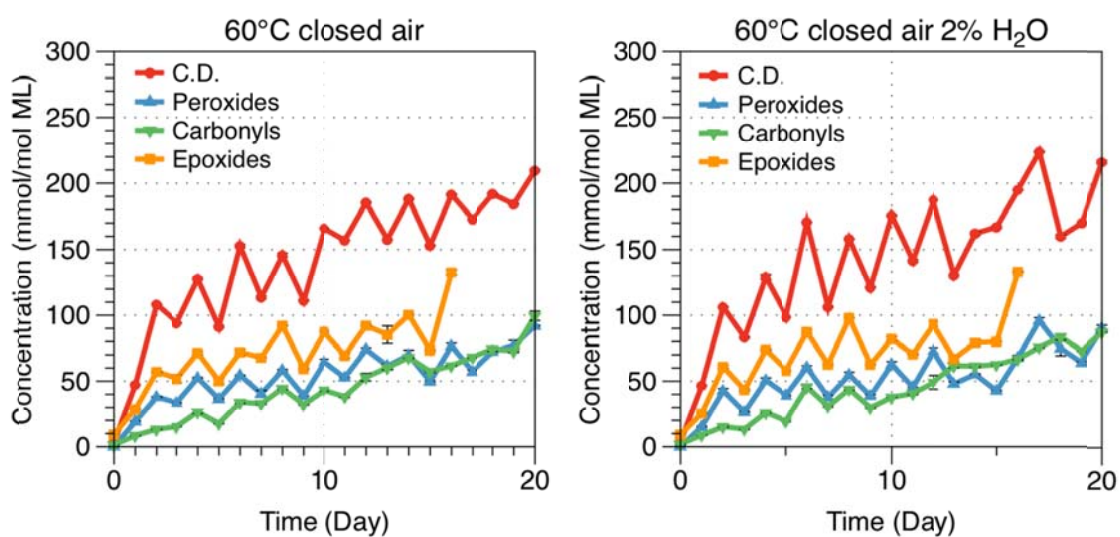


Figure 23. The effect of 2% water on distributions of ML oxidation products during incubation at 60 °C in closed systems.

6.1.4 Effect of solvent on product dynamics in closed systems

Literature reports have claimed that hydrophobic aprotic solvents favor internal rearrangement to epoxides while polar protic solvents provide hydrogen atoms to anneal radicals and stabilize alkoxyl radical scission products. If the latter actually occurs, carbonyls should increase in ethanol. To verify these patterns, methyl linoleate was oxidized in chloroform and ethanol (250 μ L methyl linoleate + 250 μ L solvents) at 40 °C in closed systems..

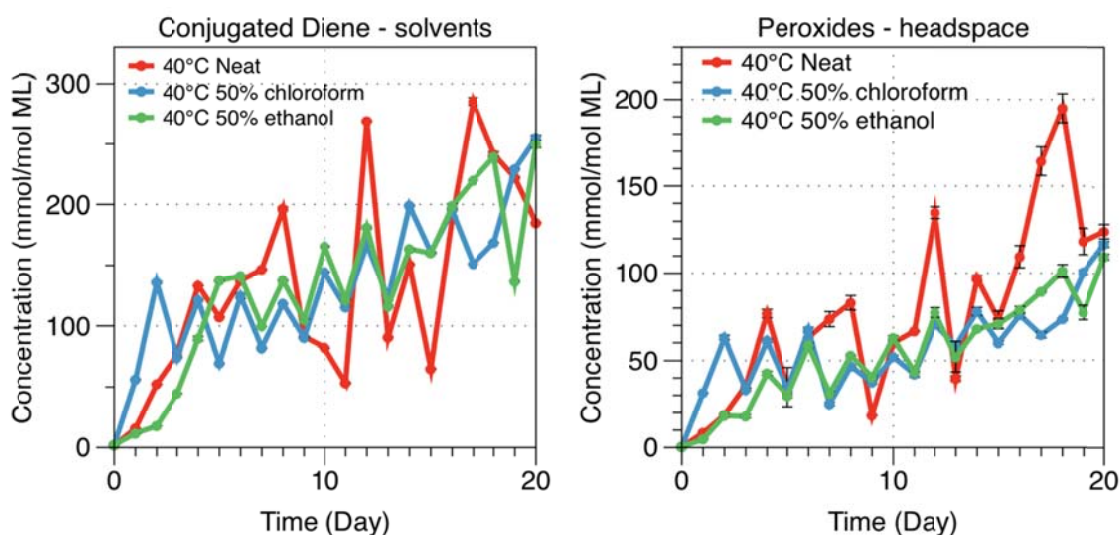
This experiment also tested dilution effects on oxidation. Dilution of methyl linoleate from both solvents was expected to impact the oxidation process by reducing molecular contact and availability of abstractable hydrogens. At the same time, the liquid solvent provided an environment for molecular movement and more random orientation which might either enhance or impede molecular contact, respectively.

Condition effects:

Figure 24 shows the progressive development of the four oxidation products neat versus diluted in the two solvents at 40°C under closed air conditions. During the first few days of oxidation (initiation), chloroform markedly enhanced oxidation (all products) while ethanol depressed products. As discussed in a previous section, chloroform may have carried additional catalysts or higher oxygen levels; ethanol is a radical scavenger. With extended oxidation, both solvents abated the extent of cycling that occurred and reduced overall oxidation levels slightly, but there was no favoring of any specific product – at least in terms of total production. The reduction of cycling may have resulted from the dilution of ML so that net product concentrations accumulating in microregions were lower.

Product distribution effects. Chloroform increased both hydroperoxides and epoxides as a proportion of conjugated dienes in early oxidation but increased epoxides at the expense of hydroperoxides during extended oxidation (Figure 25, Table 16 Section 6.2.5). In contrast, although ethanol depressed total oxidation, it increased all products relative to conjugated dienes much more than did chloroform. In particular, it stabilized hydroperoxides but at the same time nearly quadrupled epoxide levels and doubled carbonyls.

Discussion: All four products accumulated fastest in chloroform during initiation, possibly because chloroform facilitates iron catalysis and also has a higher oxygen solubility. Specifically, the surge in epoxide levels during the first two days and the increase of epoxides at the expense of hydroperoxides in long-term oxidation tends to support previous reports of preferential internal rearrangements in chloroform.



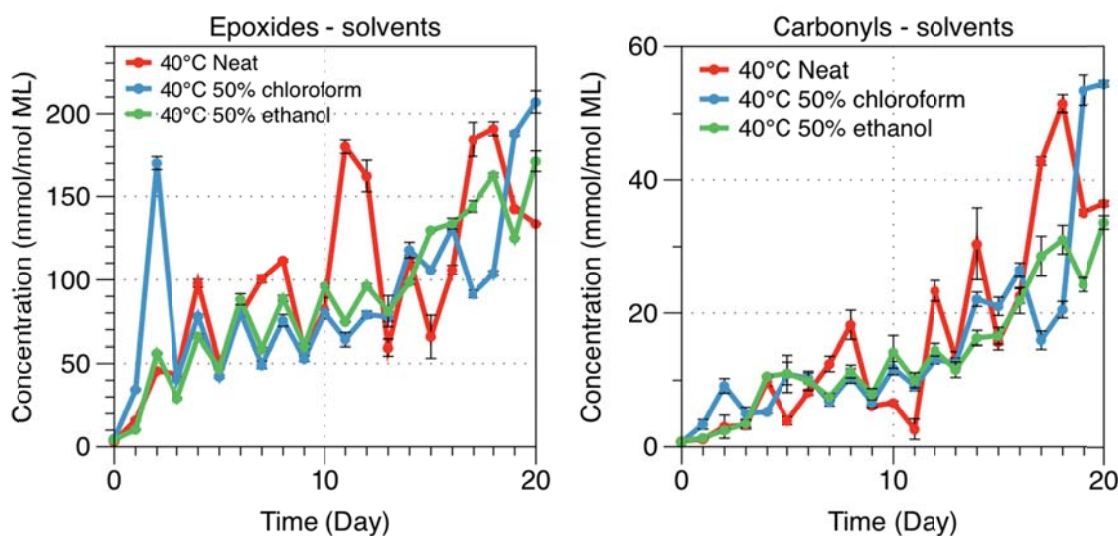


Figure 24. Effect of 50% added solvents on ML oxidation rates and levels of four major products over 20 days incubation at 25 °C.

Lower absolute levels of oxidation products in ethanol probably reflect effective radical quenching by hydrogen donation or by radical addition. Reaction curves show few obvious effects of ethanol but calculations of products as a proportion of conjugated dienes show that ethanol actually enhances or stabilizes *all* products relative to conjugated dienes. Epoxides and carbonyls are presumably produced via hydroperoxide decomposition, but then hydroperoxides should decrease while the other two increase. Instead, hydroperoxides greatly increased so they are not a likely source of epoxides and scission products. These results suggest that alternate pathways must be active in addition to (not in place of) hydroperoxide pathways in ethanol. Hydroperoxides probably become elevated due to stabilization by hydrogen bonding with ethanol. Greatly enhanced epoxide and carbonyl production is consistent with peroxy radical addition to double bonds, which generates epoxides and alkoxy radicals, which in turn decompose to more epoxides and carbonyls.

It was surprising to find that oxidation was only slightly lower when ML was diluted 50% in solvent than when neat. Since product concentrations are normalized to mol ML, this behavior suggests that oxidation was barely affected by either the solvent or the lipid concentration. Alternatively, it is possible that dilution decreased oxidation rate since it separates lipid molecules and disrupts radical transfer, but solvents counterbalanced that inhibition by accelerating oxidation. Both chloroform and ethanol form radicals that can participate in reactions for which they are carriers. Unfortunately it is not possible at this time to distinguish whether the acceleration of oxidation was due to solvents or to dilution. One possible approach to resolve the issue might be to dilute the ML in inert oil such as Migly oil or medium chain triglycerides that are saturated but liquid due to shorter chain fatty acids.

Finally, solvents blunted the extent of cycling. As was seen with addition of water, the cycling pattern became less extreme and more regular and predictable in the solvents. The factor reducing the cycling was unrelated to solvent proticity since cycling patterns were nearly the same with water, ethanol, and chloroform. One possibility is the dilution decreased the competition of different pathway so that the fluctuation was weakened in solvents. More research will be needed to answer this question.

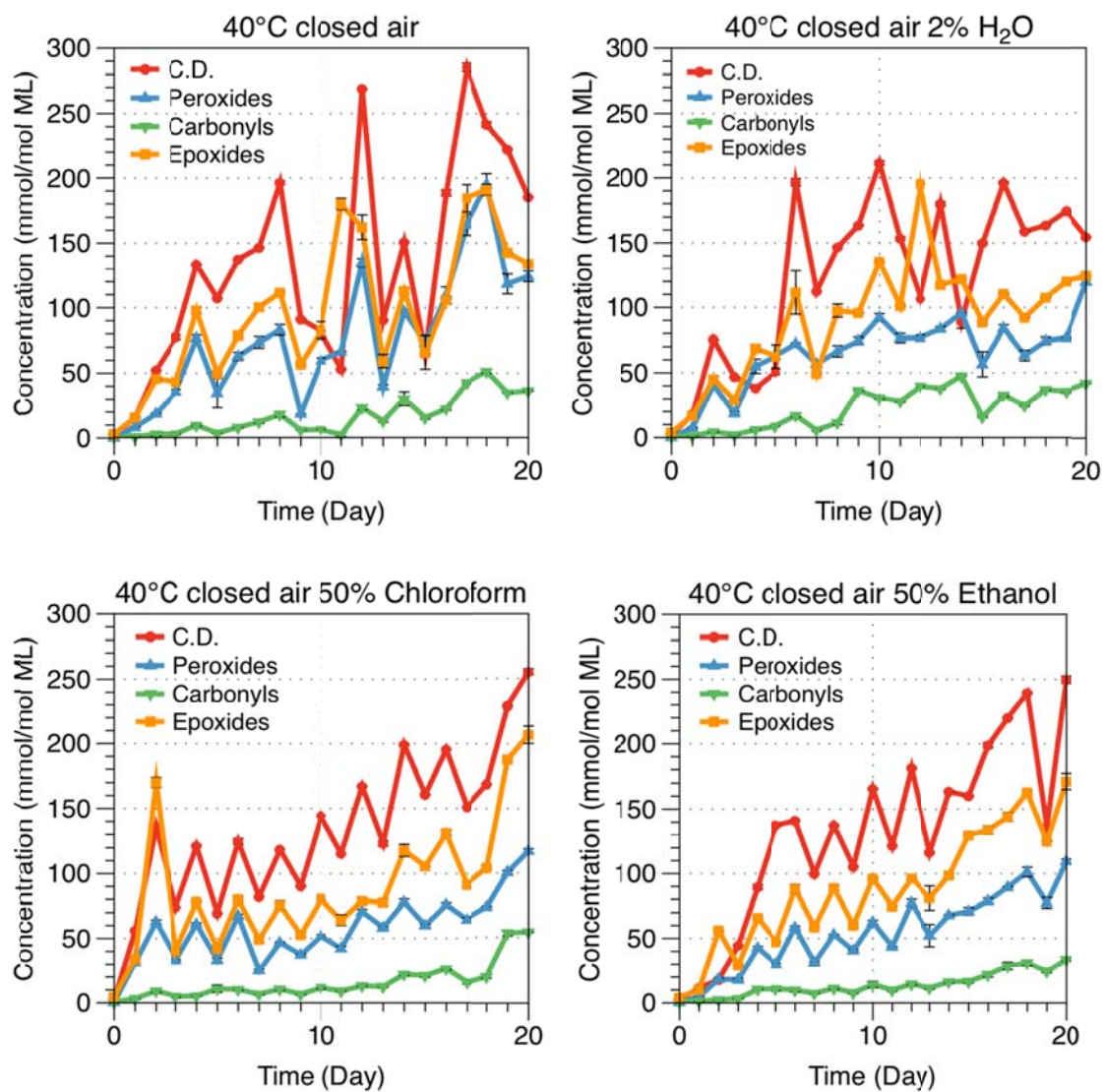


Figure 25. Solvents effect on methyl linoleate oxidation in 20 days at 40 °C closed air.

6.1.5 Effect of headspace on product dynamics under closed air conditions

The carbon-centered radicals that initiate lipid oxidation are relatively unreactive themselves as chain propagators. However, oxygen adds almost instantaneously to carbon-centered radicals, converting them to reactive peroxy radicals. Therefore, oxygen must play a critical role in lipid oxidation, and indeed, most strategies for controlling lipid oxidation involve some limitation of oxygen, e.g. in packaging. The perpetual question is, how much oxygen is actually needed to drive the reaction? Also, oxygen effects are linked to temperature due to changes in oxygen solubility and to thermal energy for activation of different reactions. This consideration is particularly critical when planning food stabilization strategies and accelerated shelf life testing.

Oxygen effects were tested by incubating neat methyl linoleate in closed vials purged with different gases. The headspace volume of each vial was 10 times larger than the methyl linoleate fluid. Oxygen levels were selected to model typical industrial oxygenation goals in food packages.

6.1.5.1 Headspace effect at medium temperature (40 °C)

Condition effects: Effects of oxygen limitation on lipid oxidation rates and levels were strongly dependent on temperature. At 40 °C, the overall effect of lower oxygen was to slow formation of all products except carbonyls, as shown by the regression lines in Figure 26. Oxygen effects were least in the first few days and increased over time as faster oxidation depleted existing oxygen further. It was surprising that 5% oxygen did not have a substantial impact on oxidation.

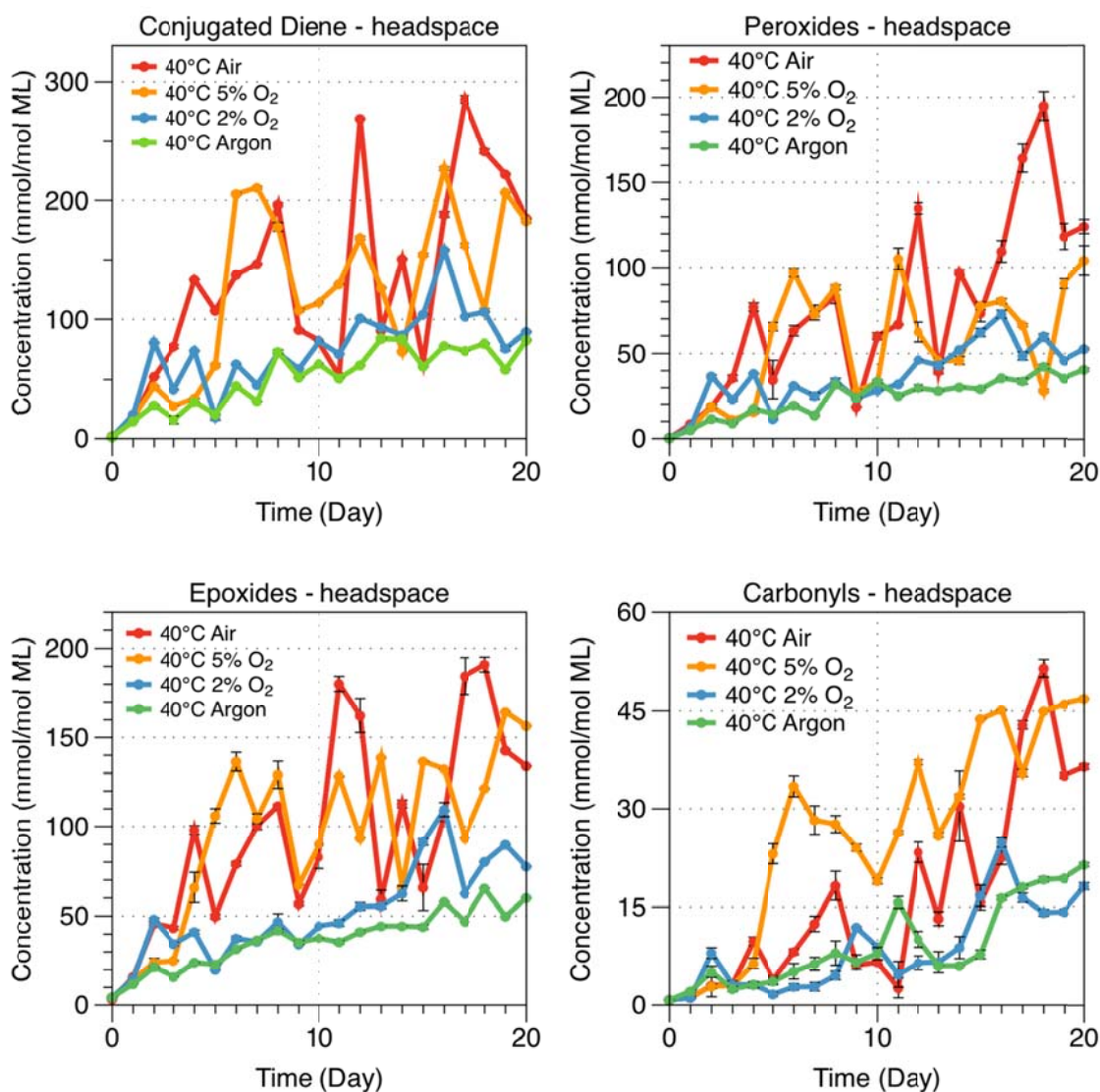


Figure 26. Effect of modified headspace on ML oxidation rates and levels of four major products over 20 days incubation at 40 °C.

Samples in 5% oxygen showed a surge in early incubation, comparable oxidation levels in mid-incubation, and but then oxidation rates began to plateau with longer incubation. Product cycling was comparable to that in neat ML. 2% Oxygen was still sufficient to fuel early oxidation during the first two days, but thereafter oxidation levels were substantially depressed, with most oxidation products restricted to < 100 mmol/mol

ML. Interestingly, oxidation in argon was not much different than 2% oxygen during the first ten days, but then oxidation plateaued and became increasingly more protective relative to oxygenated systems as incubation time increased. Cycling intensity decreased progressively as oxygen was lowered.

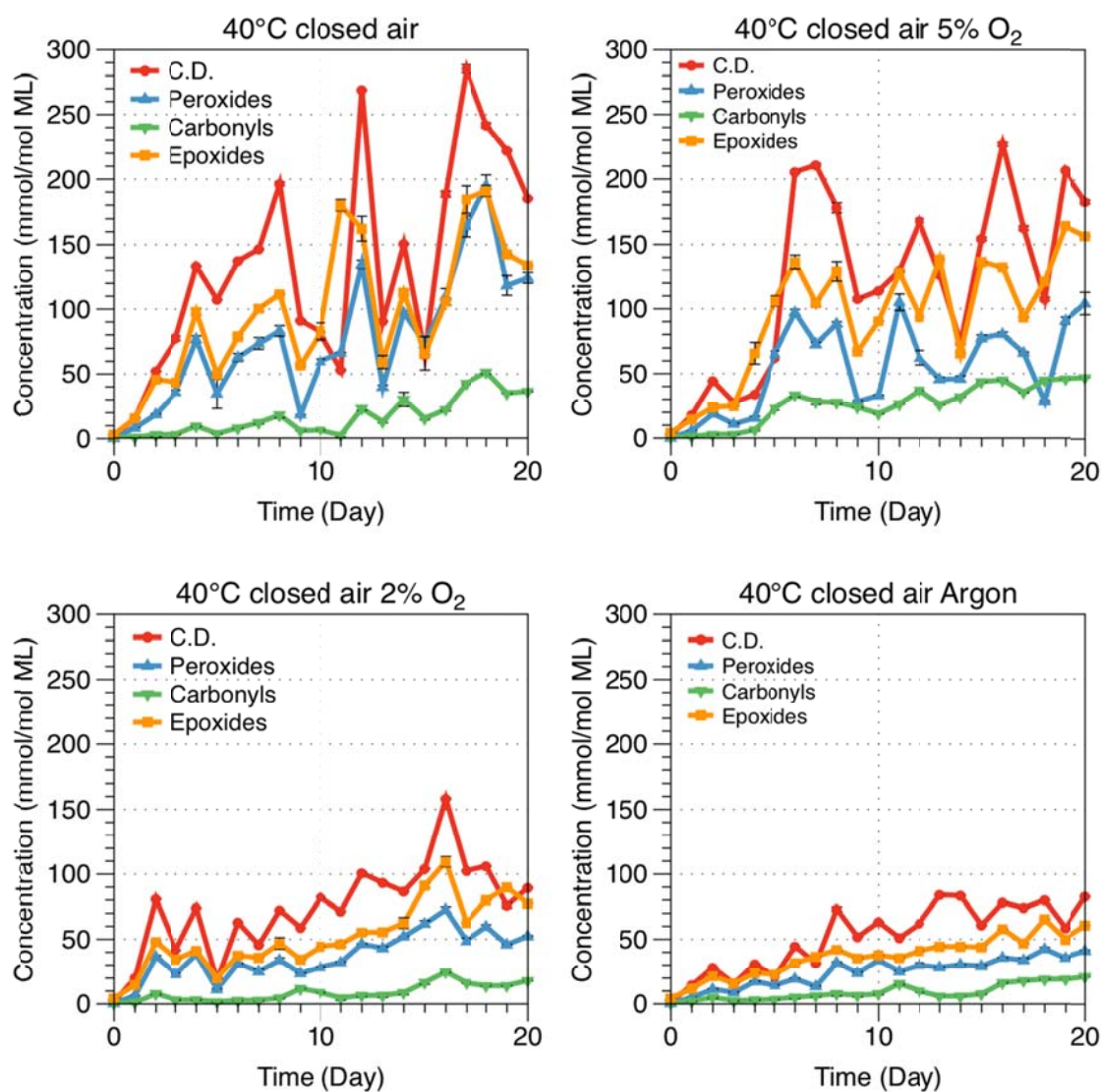


Figure 27. Effect of modified headspace on methyl linoleate oxidation under 40 °C closed air condition.

Product distribution effects: At 40 °C, 5% oxygen, epoxides and carbonyl levels as a proportion of conjugated dienes were comparable to those in air, but hydroperoxide levels dropped somewhat (Figure 28). This indicates that oxygen was not influencing initiation reactions (formation of conjugated dienes) or secondary pathways but was clearly required to form peroxy radicals, whether from initial radicals or those formed at any point in the radical chain.

6.1.5.2 Headspace effect at high temperature (60 °C)

At 60 °C, in contrast, oxygen limitation very strongly inhibited lipid oxidation, reducing levels of all products (Figure 28). Both 5% O₂ and 2% O₂ suppressed oxidation to the same level, and argon depressed oxidation still further. Initial surges in oxidation were still present, but were lessened. Argon atmospheres were not able to completely eliminate oxidation at either temperature. This is because minimum levels of oxygen already dissolved in the ML or stayed in the sample vial after purging, even with vacuum storage and careful handling, there were sufficient amount to drive low levels of oxidation.

It was interesting to observe that reduction of oxygen at all levels notably suppressed the cyclic production of all products. Argon in the headspace suppressed cycling further, providing additional support for products cycling arising from chemistry rather than physical handling.

It was also interesting to observe that only oxidation levels were depressed by low pO₂ and no one product was selectively affected. Although the carbonyl graph in Figure 29 suggests that carbonyls may be more strongly suppressed than other products, comparing all products under identical conditions shows that product proportional

distributions relative to each other were essentially constant (Figure 30). This seems to contradict observations in previous studies, but may just reflect the early stage in this study. Oxygen deficiency may affect secondary products farther downstream than the initial products analyzed here.

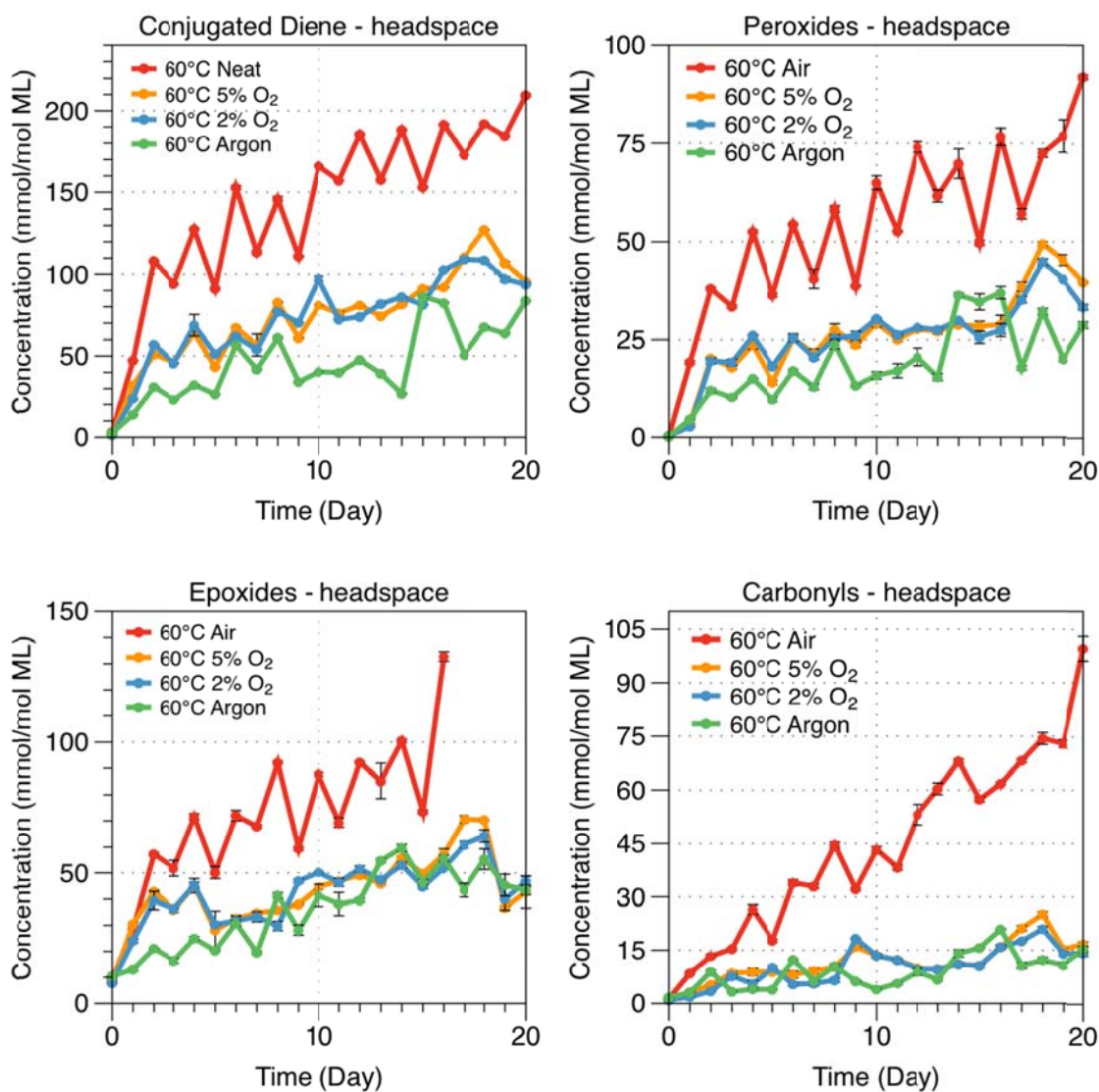


Figure 28. Effect of modified headspace on ML oxidation rates and levels of four major products over 20 days incubation at 60 °C.

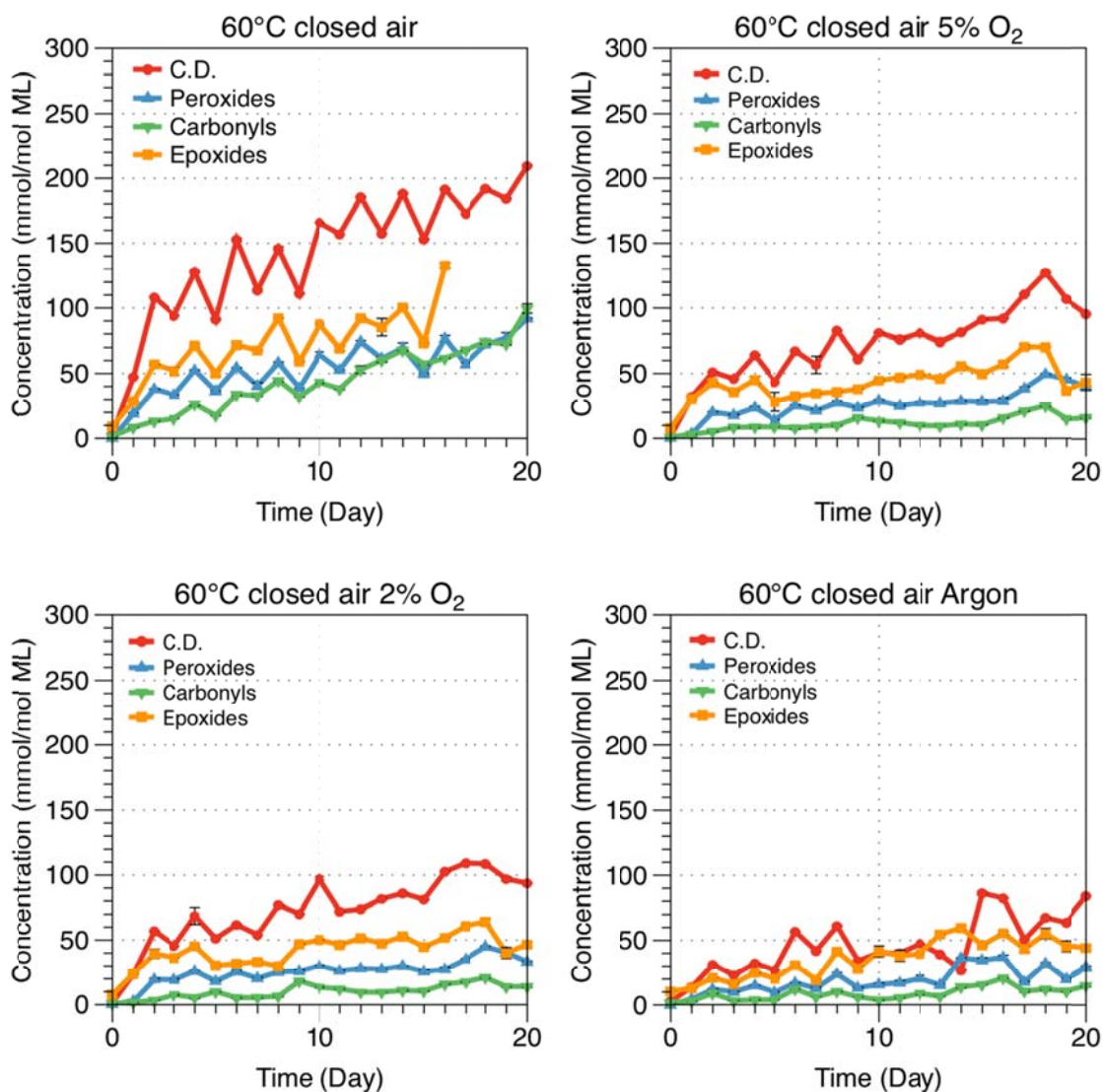


Figure 29. Effect of modified headspace on methyl linoleate oxidation under 60 °C closed air condition.

6.2 Development patterns of individual oxidation product groups from methyl linoleate incubated under different conditions

Previous sections discussed and compared how the four major oxidation products accumulate and distribute under different conditions at the same temperature. This section considers in detail how individual oxidation products change across all conditions.

Particular attention is given to the first few days where oxidation patterns were often different than in later oxidation. The impact of each environment factors on each oxidation product was also specifically evaluated to determine whether certain conditions favored specific products.

6.2.1 Conjugated diene

The formation of conjugated dienes in two days ranged from 6.56 to 136.01 mmol/mol ML (Table 11). The lowest level came from low temperature with limited oxygen supply (25 °C Closed air). Highest rate and levels of conjugated diene formation occurred with 50% dilution in chloroform. Possible reasons for this acceleration are dilution in low viscosity solvent which allows acyl chains to align more efficiently, aproticity of the solvent forcing lipids to interact with each other, and introduction of initiators in the solvent itself. The chloroform used in this study was stabilized with ethanol which can form radicals as chloroform oxidizes. No background levels of hydroperoxides, epoxides, or carbonyls were detected in the chloroform, but other catalysts were very likely present. These observations raise questions about which solvents best stabilize lipids without introducing artifacts during handling.

Table 10. Conjugated diene levels during the first two days of methyl linoleate oxidation under all conditions.

Conjugated diene (mmol/mol ML)			
	Day 0	Day 1	Day 2
25 °C open-air	1.73	3.41	15.17
25 °C Closed air	1.78	2.12	6.57
25 °C Closed air 2% H₂O	3.18	3.37	7.02
40 °C Open-air	8.32	15.35	32.95
40 °C Closed air	1.78	15.84	52.32
40 °C Closed air 2%H₂O	1.99	17.89	75.42
40 °C Closed air 50% Chloroform	2.30	56.09	136.01
40 °C Closed air 50% Ethanol	2.17	11.70	17.87
40 °C Closed air 5%O₂	2.03	17.89	44.32
40 °C Closed air 2%O₂	1.46	20.19	80.88
40 °C Closed air Argon	1.46	14.48	28.11
60 °C Open-air	8.32	31.55	65.16
60 °C Closed air	3.23	47.32	108.29
60 °C Closed air 2%H₂O	3.65	46.66	106.27
60 °C Closed air 5%O₂	1.44	32.15	51.03
60 °C Closed air 2%O₂	1.44	23.77	57.01
60 °C Closed air Argon	2.77	13.86	31.11

At the same time, conjugated dienes were tremendously suppressed in ethanol. It cannot be determined from present analyses whether these low levels reflected inhibition of oxidation due to ethanol's proton donating capacity or facilitated transformation to secondary products other than those analyzed (e.g alcohols). Interestingly, both solvents eliminated the apparent induction period for accumulation of conjugated dienes, although probably by different mechanisms. As will be amplified later, solvents have very strong effects on oxidation during the first few days, and this must be considered in extraction, handling, and storage of lipids.

2% Moisture did not affect induction periods but slightly depressed two-day accumulation of conjugated dienes at 25 °C, markedly enhanced conjugated dienes at 40 °C, and had no effect on either rate or levels of oxidation at 60 °C, probably due to volatilization. However, moisture effects changed to inhibitory at about 12 to 14 days for the two lower temperatures. Furthermore, the same pattern was observed for all products measured. This suggests that initially water may act by mobilizing or activating catalysts but as oxidation progresses, it interacts with lipid chains or lipid oxidation products to either block radical chain reactions and product transformations, or switch oxidation to pathways not measured in this study.

Rates and levels of conjugated diene formation are dependent on initiation processes. Electron and energy transfers are independent of oxygen, but secondary initiation by peroxy radicals requires oxygen. Thus, it is not surprising that oxygen effects on this product increased with temperature. The induction period of about 18 hours at 40 °C and lack of induction period at 60 °C totally reflect thermal energy effects on initiation. In contrast, decrease in conjugated diene production slopes with nominal

pO₂ in the headspace reflects reduced hydrogen abstraction by lower peroxy radical levels. Conjugated dienes were not completely shut down under argon because initiations (unknown processes) were not blocked and not all traces of oxygen had been removed from the oil.

6.2.2 Peroxides

Peroxide levels during the first two days of methyl linoleate oxidation under all conditions are compiled in Table 12. Unlike conjugated dienes whose formation depends on initiation processes, hydroperoxide formation depends more on chemistry and thus can be modified by many factors. In addition, the level of hydroperoxides detected is an instantaneous measure of peroxides formed minus those already decomposed. Thus, patterns of hydroperoxide accumulation may be complex and sometimes even apparently contradictory, and they certainly change over the course of oxidation.

Over the entire incubation period, contrary to expectations, hydroperoxides decreased with temperature in open systems but increased with temperature to 40 °C then plateaued in closed systems. In contrast, during the first two days, hydroperoxides increased with temperature and LOOH production curves were linear (no induction period) under both open and closed conditions, but hydroperoxide levels were higher in closed systems than in open. The latter results were particularly interesting since lipids and lipid extracts are normally held in closed systems during handling and analysis. These results suggest that over short times, thermal initiation is a stronger effect than hydroperoxide decomposition, but over longer times the effects reverse.

Table 11. Peroxide levels during the first two days of methyl linoleate oxidation under all conditions.

Peroxides (mmol/mol ML)			
	Day 0	Day 1	Day 2
25 °C open-air	0.50	1.78	2.92
25 °C Closed air	0.40	0.47	1.53
25 °C Closed air 2% H₂O	0.27	0.59	1.87
40 °C Open-air	0.34	5.21	9.57
40 °C Closed air	0.40	8.42	18.70
40 °C Closed air 2%H₂O	0.26	7.42	40.49
40 °C Closed air 50% Chloroform	0.37	31.43	63.02
40 °C Closed air 50% Ethanol	0.39	4.79	18.40
40 °C Closed air 5%O₂	0.23	5.76	18.85
40 °C Closed air 2%O₂	0.39	6.46	36.66
40 °C Closed air Argon	0.39	4.99	11.48
60 °C Open-air	0.34	12.59	18.85
60 °C Closed air	0.26	19.21	37.99
60 °C Closed air 2%H₂O	0.26	15.09	42.70
60 °C Closed air 5%O₂	0.36	3.47	20.23
60 °C Closed air 2%O₂	0.36	2.71	19.57
60 °C Closed air Argon	0.24	4.53	12.07

At 25 °C, water accelerated initiation during the first day but then oxidation proceeded in parallel to neat ML. The pattern reversed at higher temperatures. At 40 °C water inhibited initiation but the induction period had an increasing slope, and after 18 hours oxidation exploded. At 60 °C the induction period disappeared, and the 40 ° pattern remained but with lesser increases in hydroperoxides during Day 2. These results suggest that water participates actively in mobilization of reactants and overall chemistry of these systems, and that its dominant role changes with temperature and time. As was seen with conjugated dienes, at room temperature, water primarily mobilizes and activates catalysts, accelerating oxidation, but once propagation becomes established, these effects are minimized. At elevated temperatures where more bonds are being broken, water can initially act as H donor and anneal radicals, thus slowing oxidation. However, the point is eventually reached where water quenching ability is exceeded and its mobilization properties for both catalysts and products dramatically accelerate oxidation. At least at 40 °C, water may also stabilize hydroperoxides by hydrogen bonding.

As with conjugated dienes, hydroperoxides were formed immediately when methyl linoleate was incubated in chloroform, and their production was fast and linear (no induction period) while ethanol increased the inhibited LOOH production and made the induction period more pronounced. This once again indicates that the solvents are not innocuous but each influence lipid oxidation in different ways. Ethanol was probably acting as a radical scavenger, while chloroform apparently enhances electron transfer chemistry.

Hydroperoxides require oxygen for formation so it should be expected that their levels decrease as pO_2 is reduced. Interestingly, while reduced oxygen introduced a

twelve to eighteen hour induction period, oxidation did not stay depressed. When active oxidation began at 40 °C, hydroperoxides were produced faster with 5 and 2% O₂ than with neat methyl linoleate. At 60 °C, hydroperoxides formed at the same rate as neat ML. Since this occurred after one day and each vial held sample for two days, we suspect that this behavior resulted from air leaks into the vials through needle punctures. Argon maintained protection throughout the time period, probably because its higher density prevented ingress of air and oxygen.

6.2.3 Epoxides

Results showed that environmental conditions had different effects during initiation than over extended oxidation, which suggests but does not prove that different reactions are being influenced at different times.

In general, epoxides increased with temperature and in closed systems, though perhaps for different reasons. As noted above (Section 6.1.4), both chloroform and ethanol greatly enhanced epoxide formation relative to conjugated dienes over the entire oxidation period. Chloroform apparently redirected alkoxyl radicals because hydroperoxides barely increased, whereas ethanol allowed multiple reactions to occur simultaneously. Ethanol stabilized hydroperoxides by hydrogen bonding but at the same time facilitated peroxy radical addition to double bonds, generating epoxides and carbonyls.

Reduced pO₂ in the headspace reduced total levels of epoxides but increased epoxides as a proportion of conjugated dienes. More research will be needed to determine whether this results from increased transformation to epoxides under limited oxygen.

Table 12. The level of epoxides in the first 2 days of methyl linoleate incubation under all conditions.

Epoxides (mmol/mol ML)			
	Day 0	Day 1	Day 2
25 °C open-air	3.14	4.84	10.69
25 °C Closed air	3.26	3.47	5.13
25 °C Closed air 2% H₂O	10.58	10.01	10.62
40 °C Open-air	11.36	15.23	21.15
40 °C Closed air	3.26	15.95	45.79
40 °C Closed air 2%H₂O	4.10	16.99	45.26
40 °C Closed air 50% Chloroform	4.83	34.38	170.21
40 °C Closed air 50% Ethanol	4.26	10.39	56.12
40 °C Closed air 5%O₂	4.36	14.59	23.93
40 °C Closed air 2%O₂	4.45	14.85	48.04
40 °C Closed air Argon	4.45	11.95	21.53
60 °C Open-air	11.30	18.27	29.33
60 °C Closed air	9.41	28.61	57.23
60 °C Closed air 2%H₂O	9.24	25.44	60.95
60 °C Closed air 5%O₂	7.95	30.49	42.89
60 °C Closed air 2%O₂	7.95	24.06	39.48
60 °C Closed air Argon	10.54	13.04	21.12

6.2.4 Carbonyls

Unlike conjugated dienes and peroxides, carbonyl compounds are considered as the secondary products in traditional lipid oxidation chemistry. Thus, they may be expected to form in later oxidation after alkoxyl radicals have been formed and undergone scission reactions. However, in this project carbonyls began accumulating from the very beginning of oxidation.

Table 13 shows level of carbonyl compounds generated under all conditions during the initial stage. Since carbonyl compounds are formed much more slowly than other products, initial levels can sometimes be misleading. Thus, levels present on day 20 of incubation were also included for comparison.

Open air headspace had two contradictory effects on carbonyls. Unlimited oxygen enhanced oxidation and production of carbonyls, but also allowed virtually unlimited volatilization of shorter chain carbonyls as they formed. Carbonyls actually detected reflected competition between these two processes.

Scission is an energy-intensive process, so it is logical that the major environmental factor enhancing carbonyl production is increasing temperature (Table 14). However, levels generated are balanced by release and reaction. Thus, at room temperature, closed systems had lower levels of carbonyls due to low air, but enhanced levels of carbonyls during initiation and even more after extended oxidation due to increased production. Interestingly, carbonyls as a proportion of conjugated dienes (Table 16) decreased in closed systems, so oxidation pathways were being rerouted to other products.

Table 13. The level of carbonyls in the first 2 days of methyl linoleate incubation under all conditions.

Carbonyls (mmol/mol ML)				
	Day 0	Day 1	Day 2	Day 20
25 °C open-air	0.51	0.74	0.67	59.60
25 °C Closed air	0.84	0.46	0.37	31.41
25 °C Closed air 2% H₂O	1.53	1.45	2.06	16.50
40 °C Open-air	1.18	1.41	2.17	1.38
40 °C Closed air	0.84	1.13	3.02	36.50
40 °C Closed air 2%H₂O	0.66	1.24	4.42	41.58
40 °C Closed air 50% Chloroform	0.69	3.38	9.15	54.38
40 °C Closed air 50% Ethanol	0.81	1.35	2.48	33.65
40 °C Closed air 5%O₂	0.71	1.27	2.90	46.72
40 °C Closed air 2%O₂	0.79	1.11	8.01	18.15
40 °C Closed air Argon	0.79	2.13	5.13	21.43
60 °C Open-air	1.18	2.92	5.96	1.06
60 °C Closed air	1.39	8.48	13.31	99.54
60 °C Closed air 2%H₂O	1.95	8.88	15.21	87.45
60 °C Closed air 5%O₂	0.97	3.14	5.23	16.59
60 °C Closed air 2%O₂	0.97	1.81	3.45	14.08
60 °C Closed air Argon	1.71	3.08	8.95	15.19

Of particular interest was whether protic solvents water and ethanol would increase LO[•] scissions leading to carbonyls. Effects of water were interesting in that in terms of absolute carbonyl levels, effects appear small and occur only during initiation. However, absolute product levels underestimate water effects because carbonyls increased as a proportion of conjugated dienes during early oxidation. This substantiates reports that water facilitates LO[•] scissions and increases scission competition with other pathways. More studies are needed to determine how scission is related to water content.

Next to temperature, solvents were the factor most strongly facilitating production of carbonyls. For chloroform, both levels and proportions of carbonyls increased at all stages of oxidation. For ethanol, absolute levels of carbonyls were not changed much at any stage, but the proportion of carbonyls was tremendously amplified during initiation. We believe this was due to peroxy radical additions in early oxidation, generating alkoxy radicals that decomposed to early carbonyls. In later oxidation, scission reactions remained relatively minor contributors under the conditions of this study.

As would be expected, since carbonyls are secondary products deriving from oxygenated primary products, reduction of headspace pO₂ very effectively reduced absolute levels of carbonyls in parallel. At the same time, since primary products are affected more by oxygen, carbonyls remained high as a proportion of conjugated dienes. Under such conditions, foods may test low in oxidation by peroxide values and conjugated dienes but still have strong off-flavors.

6.2.5 Summary of environmental effects on levels and distributions of products during oxidation of methyl linoleate

Effects of specific reaction conditions were studied to verify literature reports regarding conditions that favored alternate reactions and to determine how product distributions (and the reactions generating them) change with conditions. Table 14 and Table 15 summarize general effects of reaction conditions on conjugated dienes, hydroperoxides, epoxides, and carbonyl products. Table 16 summarizes effects of reaction conditions on distributions of oxidation products expressed as a proportion of conjugated diene levels.

Some effects were expected, some were a surprise, and these differences may have a huge impact on oxidation in packaged foods and on lipid handling both on the production line and in the laboratory. One specifically interesting observation that deserves more detailed study is that reactions and system influences on them were not the same during initiation (first one to two days) as after oxidation became established. In most experiments of this study, there was an initial surge of oxidation during the first few days, followed by a decrease or stabilization of oxidation. This indicates that despite extensive glassware cleaning and very careful handling, initiators were present in the systems and became activated immediately. General thinking about lipid oxidation is that there is some induction period that buys time in handling during food production or lab analyses. Observations of this study suggest that every minute counts and that reaction are occurring in the background at all times. This is particularly important when lipid extracts are accumulated before analyzing all samples at once.

Table 14. Summary of environment effects on levels of major oxidation products oxidation of methyl linoleate, early period.

	Conjugated diene	Peroxides	Epoxides	Carbonyls
Temperature	Strong linear increase from 25 °C to 60 °C	Linear Increase from 25 °C to 60 °C	Increase from 25 °C to 40 °C, no further increase at 60 °C	No effect at 25 °C; Increase from 40 °C to 60 °C
Open-air system	Suppression at 25 °C	Suppression at 25 °C	Strong suppression at 25 °C	No effect at 25 °C and 40 °C
Closed air system	Increase at 40 °C and 60 °C	Increase 40 °C and 60 °C	Strong increase at 40 °C and 60 °C	Increase at 60 °C
2% Moisture	Increase at 40 °C; no effect at 60 °C	Increase at 40 °C; slight increase at 60 °C	No effect at 40 °C; very slight increase at 60 °C	No effect at 40 °C and 60 °C
50% Chloroform	Strong increase	Strong increase	Very strong increase	Strong increase
50% Ethanol	Strong suppression	No effect	Sight increase	Slight suppression
5% Oxygen headspace	slight suppression at 40 °C; strong suppression at 60 °C	No effect at 40 °C; Strong suppression at 60 °C	Strong suppression at 40 °C; suppression at 60 °C	No effect at 40 °C; Strong suppression at 60 °C
2% Oxygen headspace	Increase at 40 °C; Strong suppression at 60 °C	Strong increase at 40 °C; strong suppression at 60 °C	No effect at 40 °C, strong suppression at 60 °C	Increase at 40 °C; strong suppression at 60 °C
Argon headspace	Very strong suppression at both 40 °C and 60 °C	Very strong suppression at both 40 °C and 60 °C	Very strong suppression at both 40 °C and 60 °C	Increase at 40 °C; Slight suppression at 60 °C

Table 15. Summary of environment effects on levels of major oxidation products of methyl linoleate, extended period.

	Conjugated diene	Peroxides	Epoxides	Carbonyls
Temperature	Slowly decrease from 25°C to 60°C.	Slow decrease at 40°C; fast decrease at 60°C	No change in all from 25°C to 60°C	Slight fast increase at 40°C; very fast increase at 60°C
Open-air system	Decrease at 25°C and 60°C	Strong Increase at 25°C	Strong increase at 25°C	Huge increase at 25°C,
Closed air system	Increase at 40°C	No effect at 40°C, slight decrease at 60°C	No effect at 40°C, slight decrease at 60°C	Strong increase at 40°C and 60°C
2% Moisture	Decrease at 40°C; No effect at 60°C,	Strong decrease at 40°C, slight increase at 60°C	Decrease at 40°C, no effect at 60°C	Slight decrease at 40°C; No effect at 60°C
50% Chloroform	No effect at 40°C	Decrease at 40°C	No effect at 40°C	Slight decrease at 40°C
50% Ethanol	No effect at 40°C	Decrease at 40°C	No effect at 40°C	Decrease at 40°C
5% Oxygen headspace	Suppression at 40°C and 60°C	Strong suppression at 40°C and 60°C	Strong suppression at 40°C and 60°C	No effect at 40°C, strong suppression at 60°C
2% Oxygen headspace	Strong suppression at 40°C and 60°C	Strong suppression at 40°C and 60°C	Same as 5% oxygen	Strong suppression at 40°C and 60°C
Argon headspace	Slowly decrease from 25°C to 60°C.	Slow decrease at 40°C; fast decrease at 60°C	No change in all from 25°C to 60°C	Slight fast increase at 40°C; very fast increase at 60°C

Table 16. Effect of environmental conditions on distribution of products formed during oxidation of methyl linoleate, expressed as proportion of conjugated dienes. Bold font indicates conditions favoring specific product.

	CD	TWO DAYS			TWENTY DAYS		
		LOOH	Epoxides	Carbonyls	LOOH	Epoxides	Carbonyls
25°C open air	1.00	0.19	0.70	0.04	1.34	1.59	0.30
25°C Closed air	1.00	0.23	0.78	0.06	0.64	0.68	0.12
25°C Closed air 2% H ₂ O	1.00	0.27	1.51	0.29	0.46	0.81	0.11
40°C Open air	1.00	0.29	0.64	0.07	0.59	0.65	N/A
40°C Closed air	1.00	0.36	0.88	0.06	0.63	0.68	0.17
40°C Closed air 2% H ₂ O	1.00	0.54	0.60	0.06	0.49	0.67	0.20
40°C Closed air 50% in CHCl ₃	1.00	0.46	1.25	0.07	0.43	0.95	0.17
40°C Closed air, 50% in Ethanol	1.00	1.03	3.14	0.14	0.43	0.72	0.14
40°C Closed air 5% O ₂	1.00	0.43	0.54	0.07	0.41	0.77	0.25
40°C Closed air 2% O ₂	1.00	0.45	0.59	0.10	0.52	0.82	0.17
40°C Closed air Argon	1.00	0.41	0.77	0.18	0.50	0.79	0.26
60°C Open air	1.00	0.29	0.45	0.09	0.51	0.98	N/A
60°C Closed air	1.00	0.35	0.53	0.12	0.39	0.74	0.40
60°C Closed air 2% H ₂ O	1.00	0.40	0.57	0.14	0.40	0.72	0.40
60°C Closed air 5% O ₂	1.00	0.40	0.84	0.10	0.38	0.59	0.18
60°C Closed air 2% O ₂	1.00	0.34	0.69	0.06	0.35	0.59	0.17
60°C Closed air Argon	1.00	0.39	0.68	0.29	0.38	0.82	0.22

One observation that seems obvious is that in general oxidation increased with temperature. However, that pattern is very general and must be modified to account for a) enhanced transformation and degradation of individual products at elevated temperatures, b) ranges of activation energies for different oxidation pathways, c) volatility of some decomposition products.

These modifying factors have several consequences. One was the surprise that product accumulation decreased rather than increased with temperature in open systems, but the opposite occurred in closed systems. One possible explanation is that oxygen became somewhat limiting in the closed systems. However, another is that continual volatilization of secondary products provided a driving force for further oxidation in the open systems, while condensation of the products in closed systems modified secondary reactions in a way that slowed new oxidation but stabilized existing products. Since most food packaging of foods sensitive to oxidation focuses on limiting headspace volume and oxygen and foods are stored mostly at room temperature, this anomalous behavior deserves to be studied in more detail.

Another consequence was that individual products did not increase linearly with temperature but rather showed variable effects depending on their activation energies for formation and their secondary degradations. Hence, hydroperoxide levels increased with incubation time at temperatures that did not decompose them (limit is 40 °C), while at higher temperatures net hydroperoxide levels are a balance between increased production rates and increased decomposition rates. At 60 °C where many accelerated shelf life studies are conducted, hydroperoxide decomposition is dominant. Conversely, scission reactions to generate carbonyl products appear to require energy as well as protons for

stabilization, because in these short incubations their production remains at trace levels at room and moderately elevated temperatures and becomes significant only at 60 °C.

Limiting oxygen does not merely shut down oxidation across the board. Even though peroxy radical formation is inhibited, alternate reactions and secondary transformations are independent of pO_2 so these reactions continue and alter product distributions. This is particularly true for carbonyls, which may account for rancid odors even in foods that are vacuum packed and show low levels of other oxidation products.

Finally, next to temperature, solvents affected oxidation most strongly. Earlier studies have reported that chloroform and hydrophobic aprotic solvents enhance internal rearrangement to epoxides, while water and polar aprotic solvents facilitate scission reactions and stabilize resulting products. Results of this study only partially corroborate these behaviors. Chloroform notably increased early oxidation and formation of all products, especially epoxides, but after twenty days, only carbonyls were moderately augmented. In contrast, polar ethanol increased all products even more (especially epoxides) in early oxidation but decreased measured products with longer oxidation. Ethanol is known to form complexes with lipid radicals, so more detailed product analysis is needed to understand how this solvent acts in this system. Water enhanced carbonyl production at room temperature, as predicted but had no effects at elevated temperatures. This may result from water evaporation or water stabilization of hydroperoxides, probably from hydrogen bonding. These behaviors must be considered when choosing both solvents and analytical protocols for oxidation studies.

6.3 Effects of reaction conditions on formation of individual carbonyl compounds

Effects of environmental conditions on formation of four major classes of products during early stage oxidation of methyl linoleate were discussed in the previous section. However, while changes in classes of compounds can suggest shifts in pathways, more details of individual products are to verify directions. Data collected on individual carbonyl products during HPLC-DNPH analyses are thus presented here to facilitate consideration of alternate pathways, and in particular to coordinate with evolution of volatile products detected by gas chromatography. Although aldehydes remained at low levels in this study, they play a very important role in lipid oxidation because they can be perceived at trace levels, sub-ppb in some cases, by consumers. Odors deriving from the aldehydes contribute to both favorable flavor in fried foods and they signal rancidity in deteriorated foods.

6.3.1 Aldehydes formation under open air conditions

Aldehydes are highly volatile under open-air conditions which contributes to inaccuracies in carbonyl analyses in oils. As with hydroperoxides, carbonyl levels detected at elevated temperature reflect a balance between increased production rates and altered carbonyl products, increased transformation or decomposition, and increased volatilization. Taking these competing pathways into consideration, aldehydes analyses can still provide some important information that may elucidate active pathways.

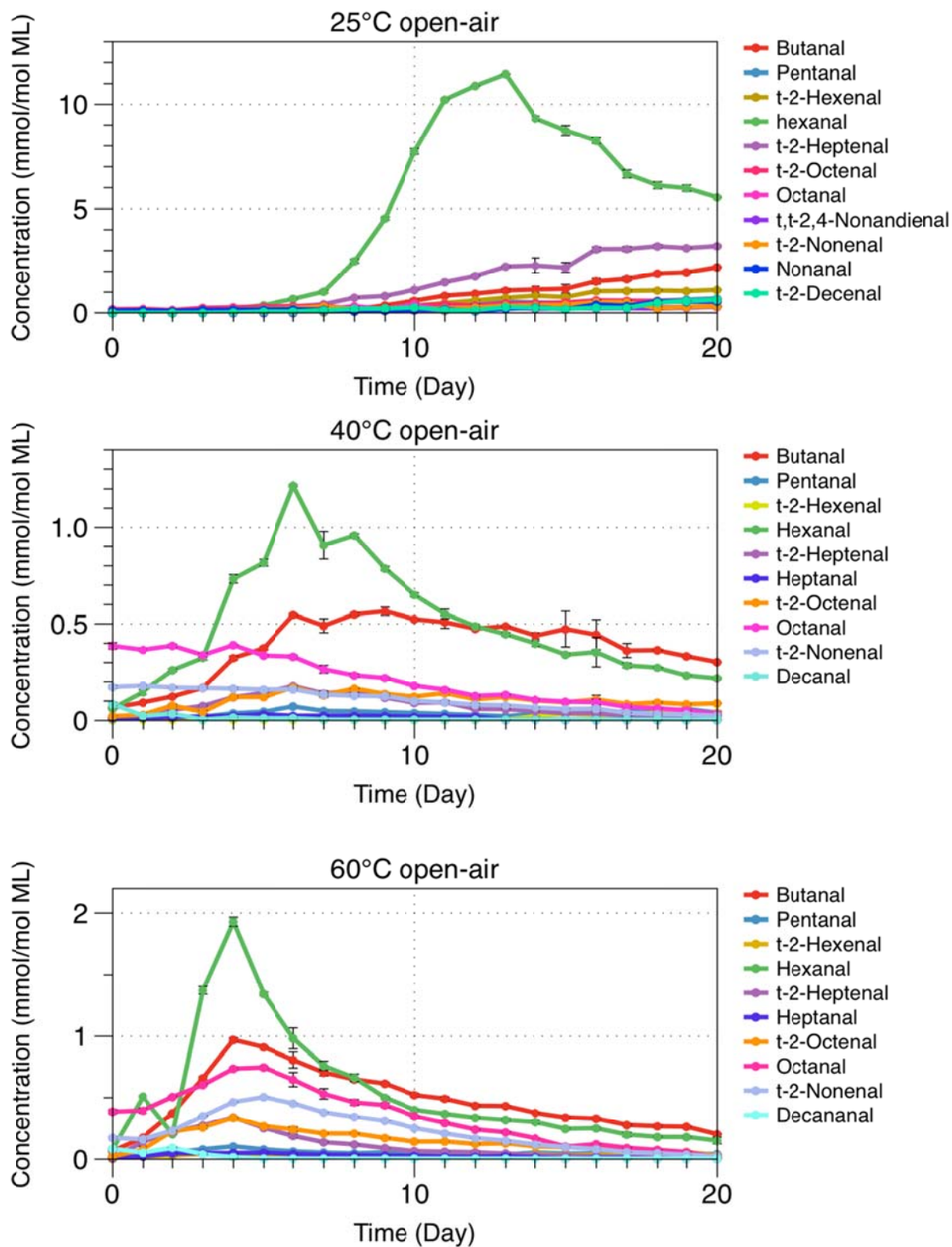


Figure 30. Patterns of aldehyde accumulation in methyl linoleate oxidized at 20, 40, and 60 °C in an open system for 20 days.

Time curves for production of major aldehydes identified by DNPH analysis are presented in Figure 30. There were three patterns of note. The dominant aldehyde by far was hexanal. At elevated temperatures, butanal, octenal, heptenal, octanal, and nonenal were also notable products. Aldehyde levels were highest at 25 °C, decreased 10-fold at 40 °C, then increased again slightly at 60 °C. That lower levels at elevated temperatures were due to faster transformation was supported by the markedly reduced times to peak production – 13 days at 25 °C, 6 days at 40 °C, and 4 days at 60 °C (Table 17). Importantly, the distribution of secondary products changed at the higher temperatures. Heptenal, butanal, and hexenal were important secondary products at room temperature (Table 18), but butanal, octanal, and nonenal were present at higher concentrations than heptenal and octenal at elevated temperatures. These observations certainly suggest that oxidation pathways are not the same at the three temperatures. Such reaction inconsistency presents significant problems in accelerated shelf life studies if limited products are measured. For example, if hexanal was measured in these samples incubated at 60 °C for 15 days, the judgment would be that oxidation was low.

Note: these aldehydes levels may have been low due to loss by volatilization in the open systems.

Table 17. Hexanal accumulation in open-air system

Hexanal	Time to peak (days)	Peak level (mmol/mol ML)
25 °C open-air	13	11.46
40 °C open-air	6	1.22
60 °C open-air	4	1.93

Table 18. Levels of individual aldehydes produced from methyl linoleate oxidized at 25 °C in an open system for 20 days.

25 °C open-air	Cumulative levels^a
Butanal	1.80
Pentanal	0.61
t-2-Hexenal	1.06
Hexanal	*6.50
t-2-Heptenal	3.14
t-2-Octenal	0.39
Octanal	0.28
t-2-Nonenal	0.32
Nonanal	0.37
t,t-2,4-Decadienal	0.37
t-2-Decenal	0.43

^a Cumulative aldehyde levels = (Day 16-20 Avg value) – Day 0 value, mmol /mol ML

6.3.2 Aldehydes formation under closed air conditions

Closed conditions, although no oxygen limiting, effectively trapped aldehydes inside the vial (constant volume) where they established an equilibrium of evaporation and recondensation. Thus, detected aldehyde levels should be higher and more representative of the full products generated. Figure 31 shows accumulation curves for the main aldehydes detected in the closed systems incubated at 25 °C, 40 °C and 60 °C.

At room temperature, aldehyde levels were slightly lower in closed systems than open (Table 19), but at both elevated temperatures higher levels of products accumulated in the closed systems. Importantly, although gas law calculations for the sample vial volumes indicated that oxygen was not limiting and the headspace was replenished every three days, peak product levels were not reached until at or near the end of the full incubation period. Hexanal was again the dominant product at all temperatures, followed by butanal. Unlike the open systems where numbers of aldehydes were comparable at the three temperatures, in the closed systems, more products were formed and retained at the higher temperatures. Key secondary products again changed with temperature, supporting presence of different active pathways: 25 °C – octenal, nonanal, and heptenal; 40 °C – heptenal and pentanal; 60 °C – octanal, nonenal, heptenal, octenal (Table 20).

These results show once again that Arrhenius effects are not applicable to lipid oxidation and running accelerated shelf life assays at elevated temperatures are measuring different reactions than occur at room temperature.

Table 19. Comparison between 25 °C open-air and closed air

Hexanal	Initial period (days)	Time to peak (days)	Peak level (mmol/mol ML)
25 °C open-air	6	13	11.46
25 °C closed air	11	18	7.33

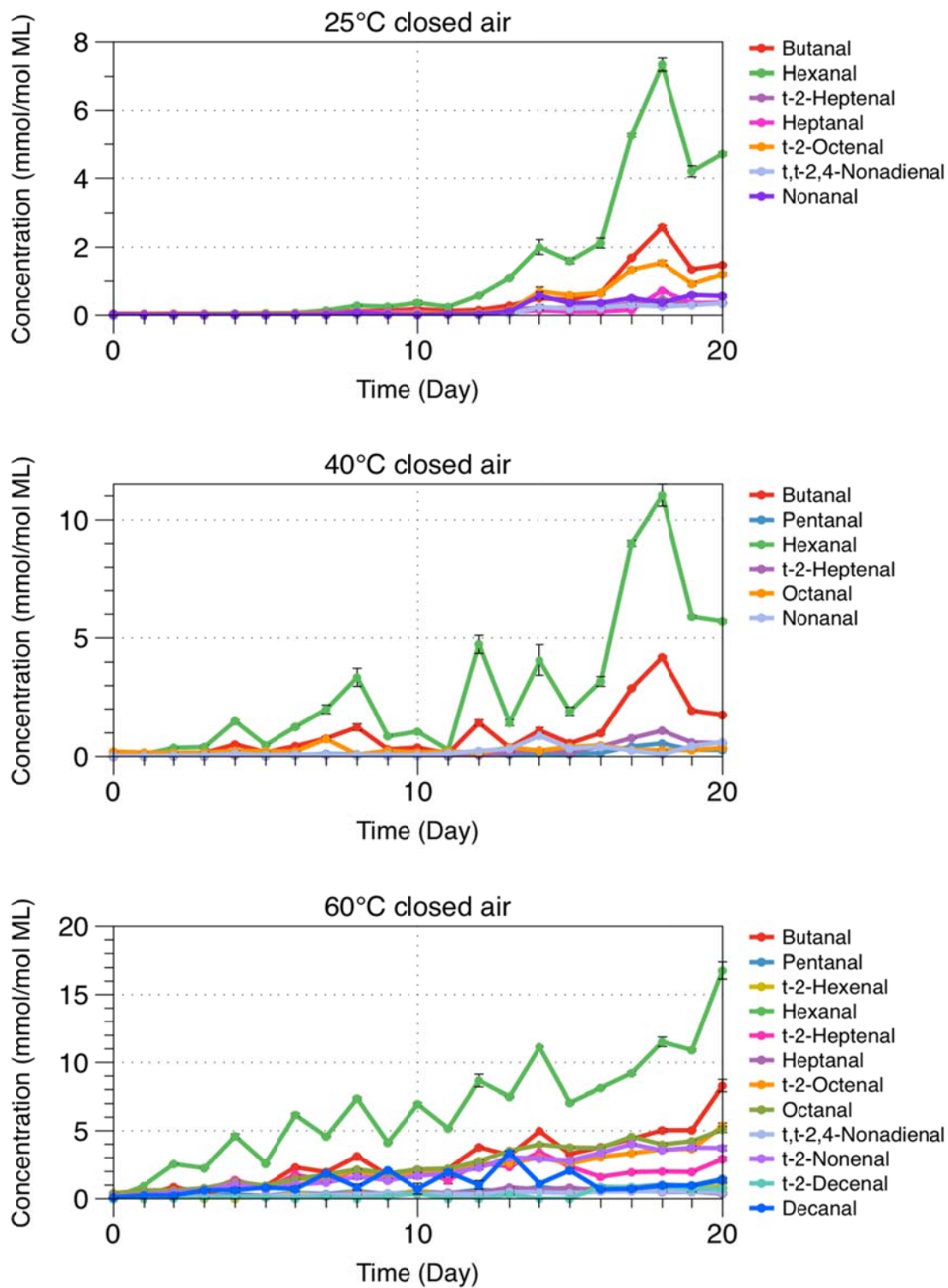


Figure 31. Aldehyde accumulation pattern of 20 °C, 40 °C and 60 °C closed air system in 20 days.

Table 20. Levels of individual aldehydes produced from methyl linoleate oxidized at 25, 40, and 60 °C in a closed system for 20 days.

	25 °C Closed air ^a	40 °C closed air ^a	60 °C closed air ^a
Butanal	1.50	2.31	5.24
Pentanal		0.34	1.02
t-2-Hexenal			0.78
Hexanal	4.70	6.93	11.23
t-2-Heptenal	0.35	0.69	2.07
Heptanal	0.35		0.54
t-2-Octenal	1.11	1.74	3.77
Octanal			3.91
t,t-2,4-Nonadienal	0.29		0.52
t-2-Nonenal			3.49
Nonanal	0.46	0.34	
t,t-2,4-Decadienal			
t-2-Decenal			0.81
Decanal			0.84

^a mMol aldehyde/mol ML calculated as [Day 16-20 Avg value – Day 0 value].

Some key “missing” products must also be noted. 2,4-Decadienal is a major α -scission product of C-9 alkoxyl radicals and is predicted to be generated in amounts approximately equal to hexanal. However, decadienal was detected only in trace amounts in open systems and not at all in closed systems. Products deriving from β -scission of C-9

alkoxyl radicals were similarly detected in only trace amounts, whereas dominant primary products came from C13 attack and secondary products mostly from C-11. These results are inconsistent with predictions from classical lipid oxidation theory.

6.3.3 Moisture effects

The main effect of 2% added moisture was a marked blocking of aldehyde production at room temperature, limiting both levels and types of aldehydes. No significant effects on aldehydes levels were noted at elevated temperatures. Moisture barely altered the distribution of aldehydes generated from oxidizing methyl linoleate at elevated temperatures (Figure 32). Hexanal, butanal, heptenal, octenal, and nonanal were still the major product at all temperatures. One difference was that in dry methyl linoleate, octanal was formed only at high temperature; with water added, low levels of octanal were also formed at 25 and 40 °C (Table 21).

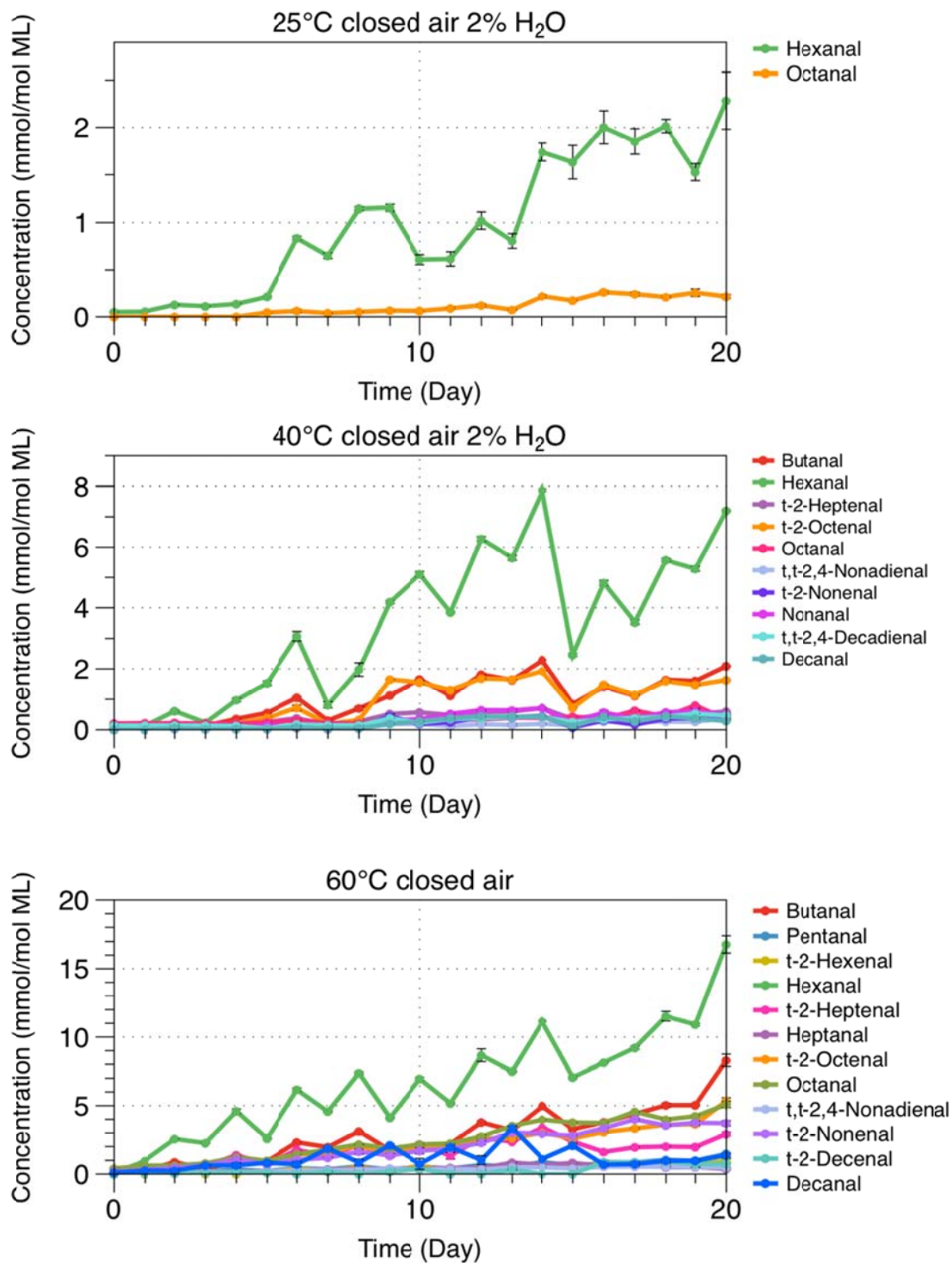


Figure 32. Patterns of aldehyde accumulation in methyl linoleate with 2% added H₂O oxidized for twenty days at 25 °C, 40 °C and 60 °C under closed conditions.

Table 21. Levels of individual aldehydes produced from methyl linoleate with 2% added moisture oxidized at 25, 40, and 60 °C in a closed system for 20 days.

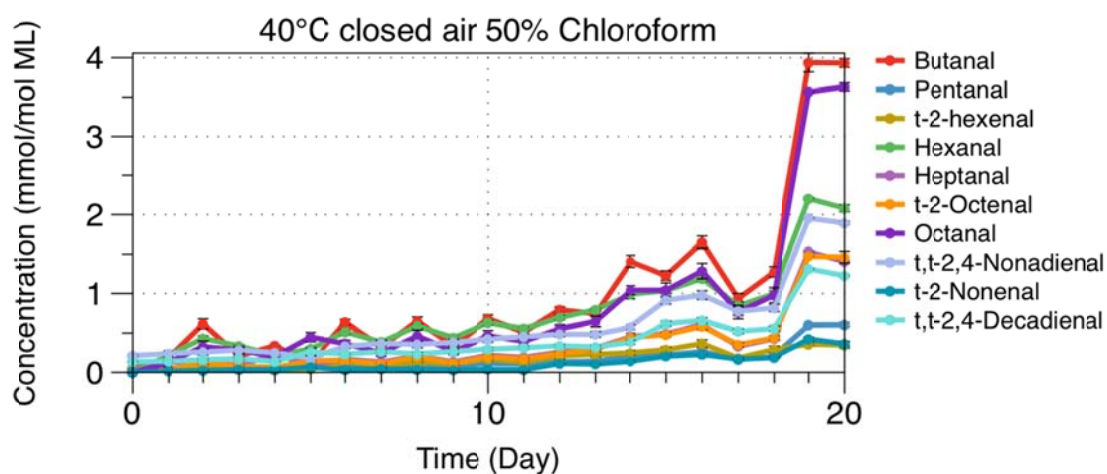
	Closed air condition ^a					
	25 °C	25 °C 2%H ₂ O	40 °C	40 °C 2%H ₂ O	60 °C	60 °C 2% H ₂ O
Butanal	1.50		2.31	1.53	5.24	6.21
Pentanal			0.34		1.02	0.97
t-2-Hexenal					0.78	0.65
Hexanal	4.70	1.88	6.93	5.26	11.23	11.94
t-2-Heptenal	0.35		0.69	0.51	2.07	2.11
Heptanal	0.35				0.54	0.45
t-2-Octenal	1.11		1.74	1.44	3.77	4.41
Octanal		0.24		0.30	3.91	3.35
t,t-2,4-Nonadienal	0.29			0.29	0.52	0.32
t-2-Nonenal				0.28	3.49	2.78
Nonanal	0.46		0.34	0.50		
t,t-2,4-Decadienal				0.29		
t-2-Decenal					0.81	0.65
Decanal				0.36	0.84	1.08

^a Values reported are mmol aldehydes/mol ML; calculated as [Day 16-20 Avg value – Day 0 value]. Aldehyde production < 0.25 mmol/mol ML were not reported here.

6.3.4 Solvent effects

In total class analyses (Section 6.1.4), we observed that chloroform markedly increased while ethanol inhibited initial oxidation (first two days), but overall the effects of dilution in solvents were to blunt the product cycling and slightly suppress all product levels thereafter (Table 22 and Figure 33). Formation of individual aldehydes similarly was suppressed by the solvents, but more important was potential shifts in product distribution that may reveal presence of pathways other than standard alkoxy radical scission. We also hoped to use specific aldehydes to identify active radical pathways.

Blunting effects of the solvents can be seen in the weaker but regular cycling of the aldehydes and prolonged induction periods of 15-18 days (Figure 34) as well as the reduced aldehydes levels relative to ML oxidized neat and with 2% added water (Table 27). Perhaps the most important observations were that hexanal (the dominant product under most other conditions) was greatly diminished while butanal and octanal became the major products over the entire incubation period for organic solvents. Water depressed overall carbonyl levels but enhanced scissions reactions at C9.



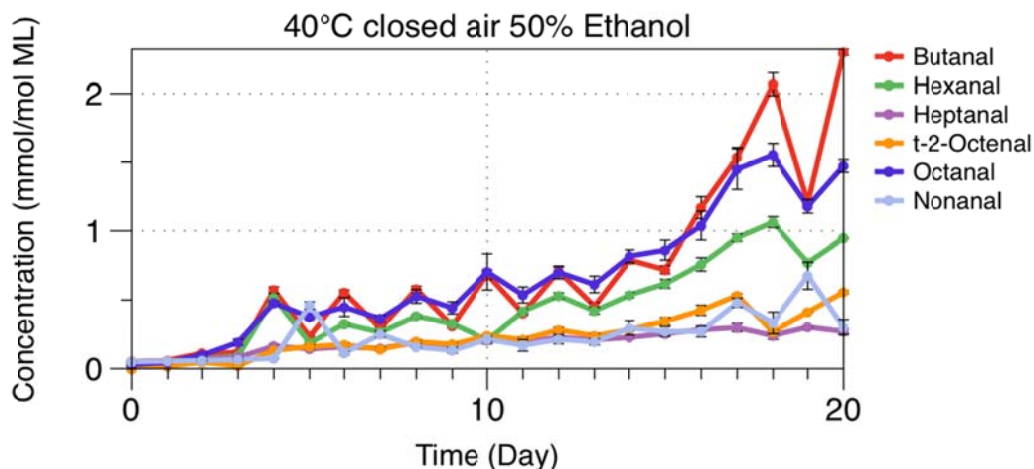


Figure 33. Aldehyde accumulation pattern of 40 °C closed air with 50% chloroform and ethanol in 20 days.

Chloroform appears to have directed a major shift in scission and precursor reactions. Four saturated aldehydes were dominant or major products – butanal > octanal > hexanal > heptanal. C-11 products that were important under other conditions were suppressed (octenal) or not present (heptenal). In contrast, C-9 scission products *t,t*-2,4-nonadienal and especially *t,t*-2,4-decadienal which were not present under other conditions, were increased. In ethanol, all aldehydes were generated at lower concentrations, contrary to predictions that this polar protic solvent would stabilize scission products. Major products again were saturated aldehydes – butanal > octanal > hexanal > nonanal > heptanal. Products expected from C9-O[•] scissions were missing. These are important observations because they indicate that both solvents alter normal pathways and direct oxidation into unidentified pathways. While these observations may not be relevant to foods directly, they certainly have great potential impact on how oils and lipid extracts are handled for analysis of lipid oxidation in the laboratory and they

raise an important question – do the solvents normally used to extract lipids from tissues and foods actually distort the product picture?

Table 22. Effects of solvent and 2% added water on aldehyde formation in methyl linoleate oxidized for twenty days at 40 °C.

	40 °C closed air		
	Neat ML	50% chloroform	50% ethanol
Butanal	2.31	2.30	1.60
Pentanal	0.34	0.37	
t-2-Hexenal		0.28	
Hexanal	6.93	1.45	0.90
t-2-Heptenal	0.69		
Heptanal		0.85	0.26
t-2-Octenal	1.74	0.86	0.44
Octanal		2.04	1.31
t,t-2,4-Nonadienal		1.07	
t-2-Nonenal		0.27	
Nonanal	0.34		0.36
t,t-2,4-Decadienal		0.72	

^a mmol aldehyde/mol ML, calculated as [Day 16-20 Avg value – Day 0 value].

Aldehydes with levels < 0.25 mmol/mol ML were not reported here.

6.3.5 Headspace effects

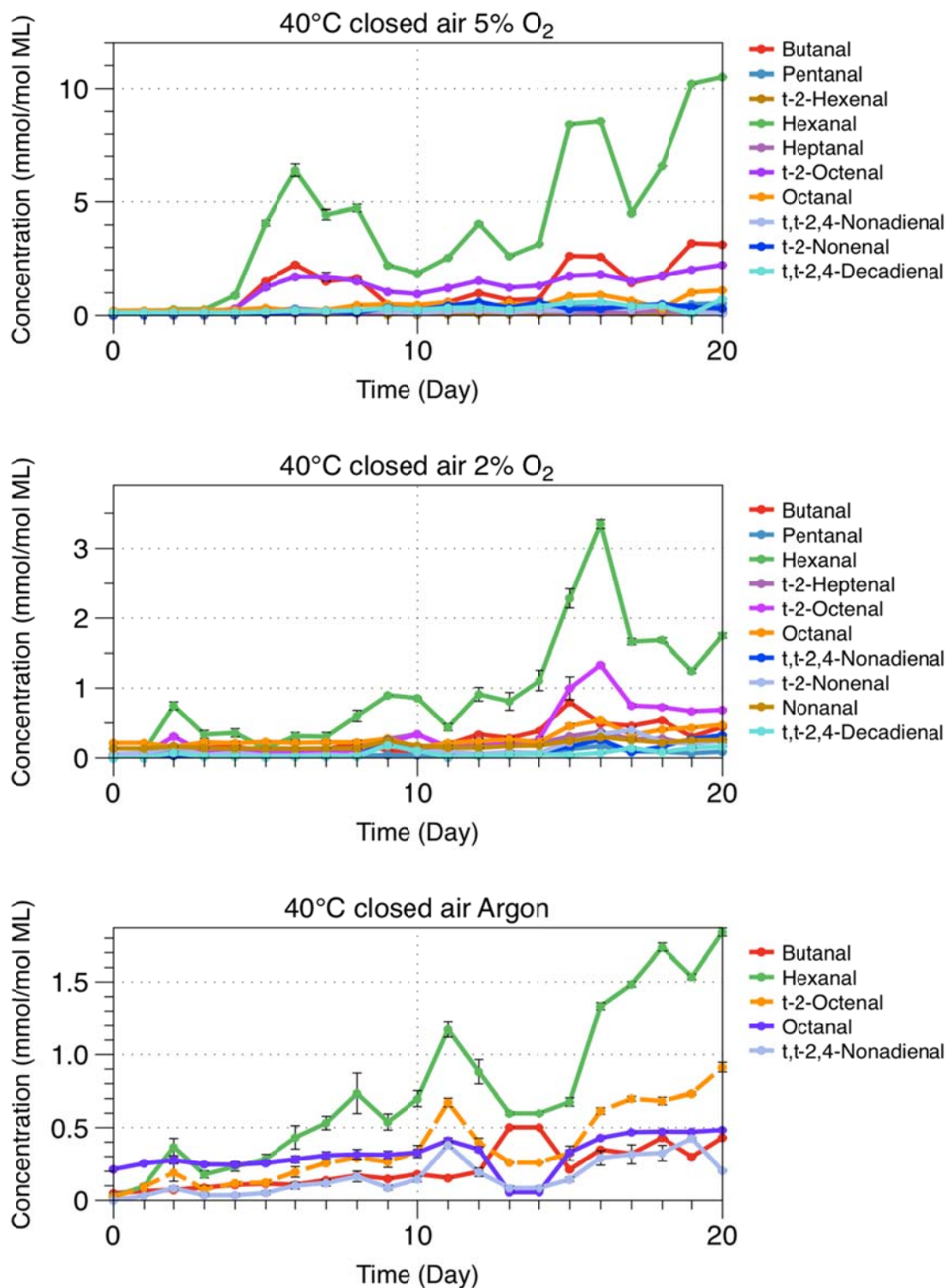


Figure 34. Aldehyde accumulation pattern of 40 °C closed air with 5% O₂, 2% O₂ and Argon in 20 days.

Low oxygen in the headspace was found to have tremendous suppression on major oxidation products total level. Here we are trying to find more information about the suppression on specific aldehydes.

From Figure 34 we can find that under 40 °C 5% O₂ suppressed the formation of aldehyde for 3-4 days, then hexanal level started to took off. This is almost the same delay period as the 40 °C closed air with regular air in the headspace. The final hexanal increase with 5% O₂ reached to a high level (>11 mmol/mol ML), suggesting that 5% O₂ was not low enough to suppress the formation of rancidity from hexanal, although some other primary oxidation product was suppressed.

Different from 5% O₂, 2% O₂ showed a strong suppression on aldehyde formation. It controlled the hexanal level under 1 mmol/mol ML for 14 days and eventually it reached to around 3.5 mmol/mol ML then dropped down to 2 mmol/mol ML. Most of the other aldehydes were controlled below 1 mmol/mol ML throughout the whole 20 days except for t-2-octenal, which also reached to a peak value 1.3 mmol/mol ML at 16th day. Argon showed a further suppression on all aldehydes, that hexanal was controlled to a lower peak level at around 1.9 mmol/mol ML. From Table 23 it is found that 5% O₂ in the system actually increase the level of aldehyde formation. Hexanal final increase rose from 6.93 mmol/mol ML to 8.06 mmol/mol ML. Butanal and t-2-octenal also had a slight increase. There were also more types of unsaturated aldehyde found with % O₂. t,t-2,4-Nonadienal, t-2-nonenal, t,t-2,4-decadienal, and decanal were all found with certain level of increase. The 2% O₂ was found to have substantial suppression on hexanal. This is suggesting a critical threshold between 2% and 5% of oxygen was found to have substantial suppression on hexanal. Argon had some further decrease on hexanal final

increase but it seemed changed a little bit on aldehyde distribution. t-2-Hexenal and t-2-heptenal was not found with Argon, but octanal and t,t-2,4-nonadienal occurred in low level.

Table 23. Comparison of aldehydes produced from neat methyl linoleate oxidized at 40 °C under different oxygen pressures.

	40 °C closed air^a			
	Air	5% O₂	2% O₂	Argon
Butanal	2.31	2.37	0.40	0.32
Pentanal	0.34	0.36		
t-2-Hexenal			0.25	
Hexanal	6.93	8.06	1.91	1.56
t-2-Heptenal	0.69	0.59	0.27	
Heptanal				
t-2-Octenal	1.74	1.84	0.81	0.71
Octanal		0.59		0.25
t,t-2,4-Nonadienal		0.26		0.31
t-2-Nonenal		0.34		
Nonanal	0.34	0.56		
t,t-2,4-Decadienal		0.31		
t-2-Decenal				
Decanal		0.41		

^a Values calculated as [mmol/mol ML Day 16-20 Avg value – Day 0 value]. Aldehyde production < 0.25 mmol/mol ML was considered as insubstantial so was omitted to show the signature pattern of aldehyde formation.

In previous chapters we observed that total oxidation product had similar suppression in both 40 °C and 60 °C with modified headspace. However, at higher temperature at 60 °C, oxidation process moved faster than 40 °C, the lack of oxygen may change the aldehyde formation and distribution pattern. Here we also compare how low oxygen environment may change the aldehydes at 60 °C.

Similar result of aldehyde formation as the total carbonyls level was observed with 60 °C modified headspace. As the temperature rose up, the threshold for aldehyde suppression actually rose up. Therefore, even with 5% oxygen, a strong suppression could still be found, because the oxidation pathway switched away from aldehyde formation to other chemicals. The lack of oxygen at such temperature had a strong decrease in aldehyde formation than 40 °C. Therefore, the high peak of hexanal in this situation dropped to 3 mmol/mol ML. With the same reason, 2% O₂ was found to give further suppression to a peak level lower than 2.5 mmol/mol ML. Not only did low oxygen environment had more substantial suppression on aldehydes at 60 °C, it also decreased the type of aldehyde that obtained accumulation. This observation continued with Argon at 60 °C. Hexanal peak level was furthered suppressed below 2.0 mmol/mol ML. Butanal was not found with a substantial increase at all with Argon; t-2-octenal also maintained below 0.5 mmol/mol mL. All the other aldehydes maintained a level below 0.6 mmol/mol ML in the whole 20 days. It was observed that most minor aldehyde

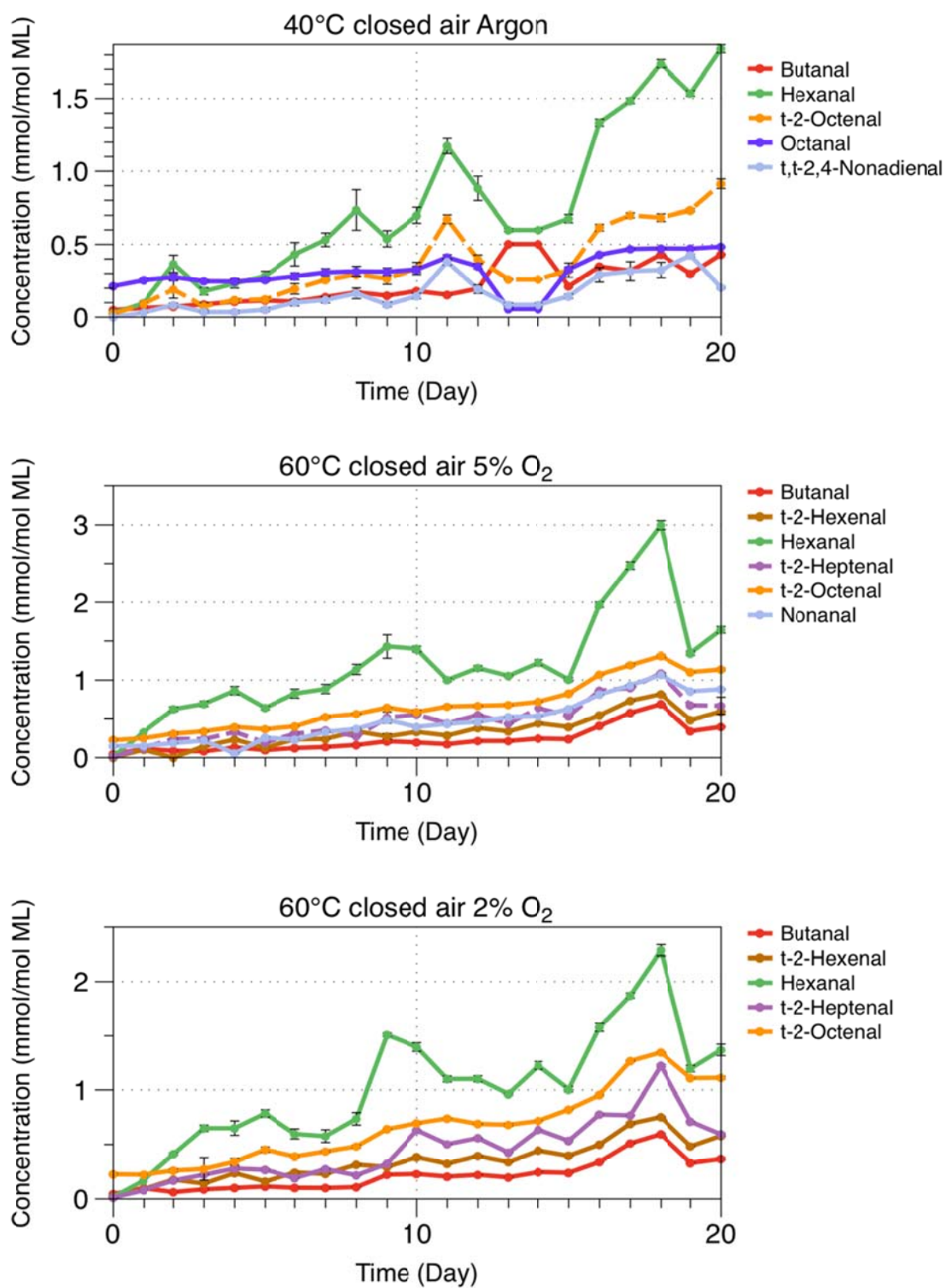


Figure 35. Aldehyde accumulation pattern of 60 °C closed air with 5% O₂, 2% O₂ and Argon in 20 days

accumulation occurred within the first two days, where very low levels of oxygen in liquid methyl linoleate or from processing methods was consumed.

Table 24. Comparison of aldehydes produced from neat methyl linoleate oxidized at 60 °C under different oxygen pressures.

	60 °C closed air			
	Regular air	5% O₂	2% O₂	Argon
Butanal	5.24	0.44	0.38	
Pentanal	1.02			
t-2-Hexenal	0.78	0.63	0.58	0.30
Hexanal	11.23	2.07	1.64	1.06
t-2-Heptenal	2.07	0.83	0.80	0.26
Heptanal	0.54			
t-2-Octenal	3.77	0.94	0.93	0.26
Octanal	3.91			
t,t-2,4-Nonadienal	0.52			
t-2-Nonenal	3.49			
Nonanal		0.76		0.33
t,t-2,4-Decadienal	0.12			
t-2-Decenal	0.81			
Decanal	0.84			

Table 24 showed the specific aldehyde formed at 60 °C, hexanal final increase of 5% O₂, 2% O₂ and Argon was 2.07mmol/mol ML, 1.64 mmol/mol ML and 1.06 mmol/mol ML respectively. Besides hexanal, butanal took a great decrease when Argon was applied. t-2-Heptenal and t-2-octenal also had a substantial decrease with Argon in the headspace, from 0.80-0.90 mmol/mol ML to 0.20-0.30 mmol/mol ML.

6.4 Epoxide structure validation by mass spectrometry.

A seminal observation of this study was that epoxides are major products that exceed hydroperoxides under most conditions. These observations depended on quantitative and qualitative accuracy of the DETC assay used to analyze epoxides. Thus, before moving forward to explain the high epoxides levels in terms of reactions, additional validation of the assay was sought.

The first step was to assure that the DETC assay was indeed measuring the epoxides other than peroxides, carbonyls, etc. A series of compounds with specific functional groups present in lipid oxidation were reacted with DETC following the method described in Section 5.3.4. No reaction was detected with hexanal, ethanol, stearic acid, cumene hydroperoxide or methyl ethyl ketone, while strong peaks were generated by 2,3-epoxybutane and 1,2-epoxy-9-decene (data not shown). Thus, the possibility of aldehydes, alcohols, carboxylic acids, peroxides and ketones reacting with DETC was ruled out.

A strong epoxide peak with retention time of 9.5 min accounting for >85% of the epoxides was found in oxidized methyl linoleate (Figure 36). To verify its identification as an epoxide, the peak was collected at the HPLC outlet for further analysis. This sample and two standard epoxides (2,3-epoxybutane and 1,2-epoxy-9-decene) were then

analyzed for structural verification by mass spectrometry using a Thermo Finnigan LCQ-DUO mass spectrometer with adjustable API-ESI (electrospray) Ion Source (Chemistry Department, Rutgers University). The electrospray source voltage was set as 4.5 kV and the heated capillary temperature was 150 °C. Positive ion mode was applied.

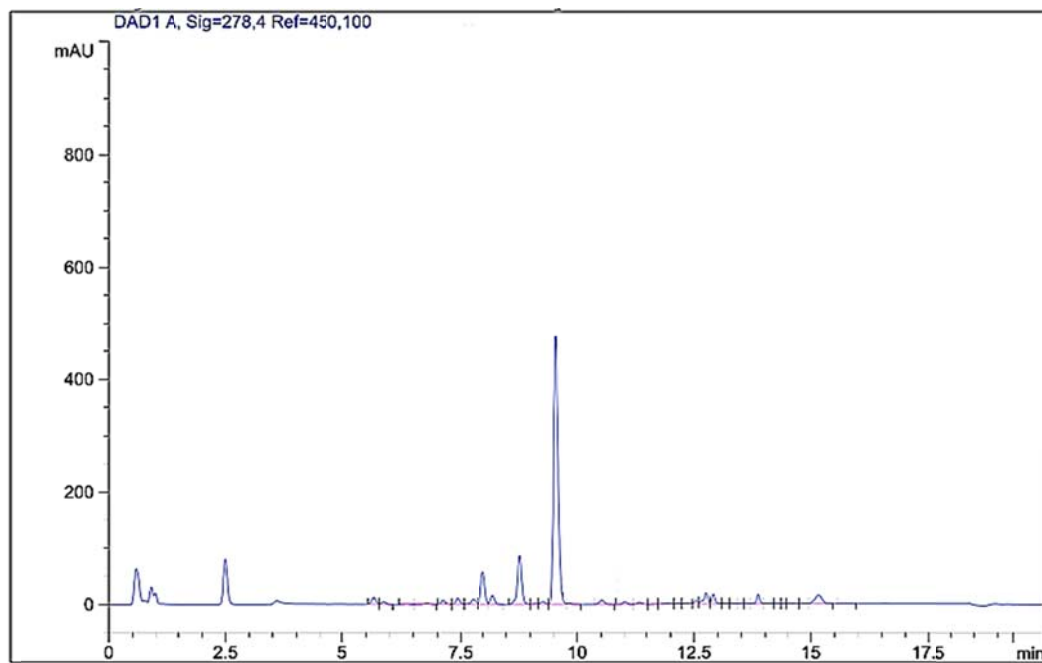


Figure 36. HPLC spectrometric graph of methyl linoleate reaction with DETC at 278 nM. Incubation condition: 60 °C, closed air, day 11.

Mass spectra of the two standards are shown in Figure 38. For both standards, the molecular ion of the predicted DETC-epoxide complex was present in the respective mass spectrum. The higher molecular weight fragments are contaminants in the mass spectrometer.

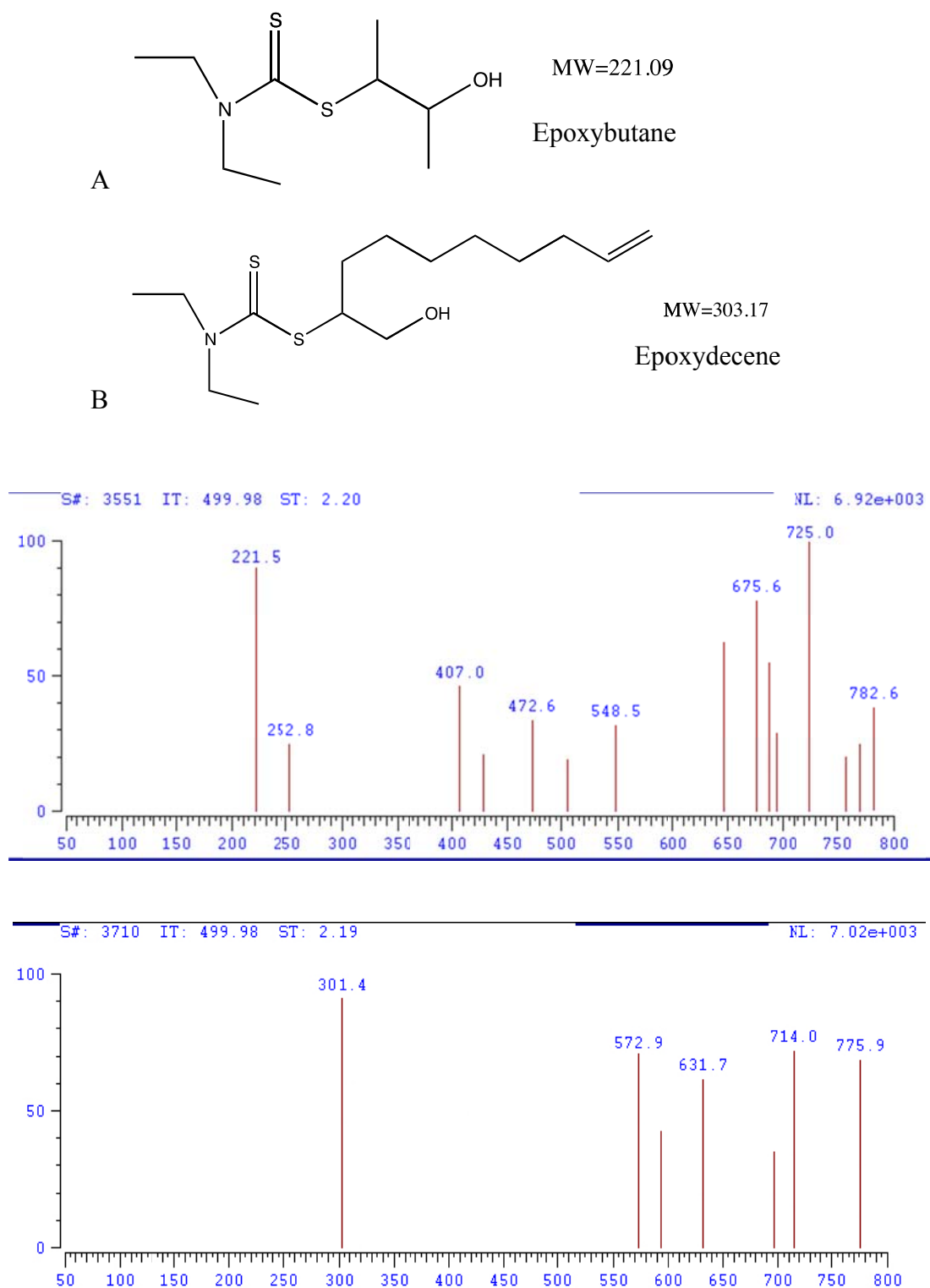
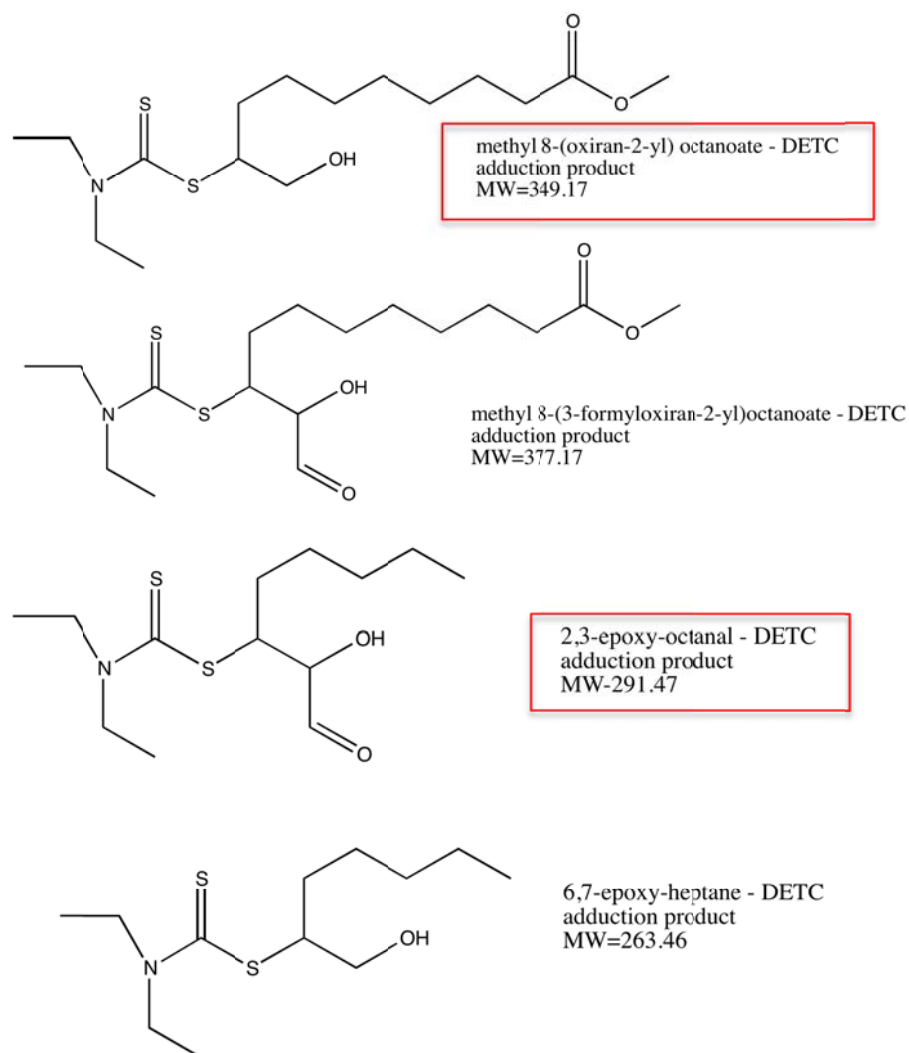


Figure 37. Mass spectra of 2,3-epoxybutane (top) and 1,2-epoxy-9-decene (bottom). Red arrow indicates DETC-epoxide molecular ion.

Figure 38 shows the mass spectrum from the DETC-epoxide peak collected from oxidized methyl linoleate. The sample gave two molecular ions of 286.9 and 351.2 from two co-eluting DETC-epoxides. The second molecular ion (351) is consistent with epoxydecene with a methyl ester group added. This epoxides could be generated from a C9 alkoxy radical. The smaller epoxides is consistent with formation of 2,3-epoxyoctanal formed from a C13 alkoxy radical. Structures of these epoxides and two other possible epoxides that are inconsistent with mass spectra are shown below. Reactions generating them will be discussed in the next section.



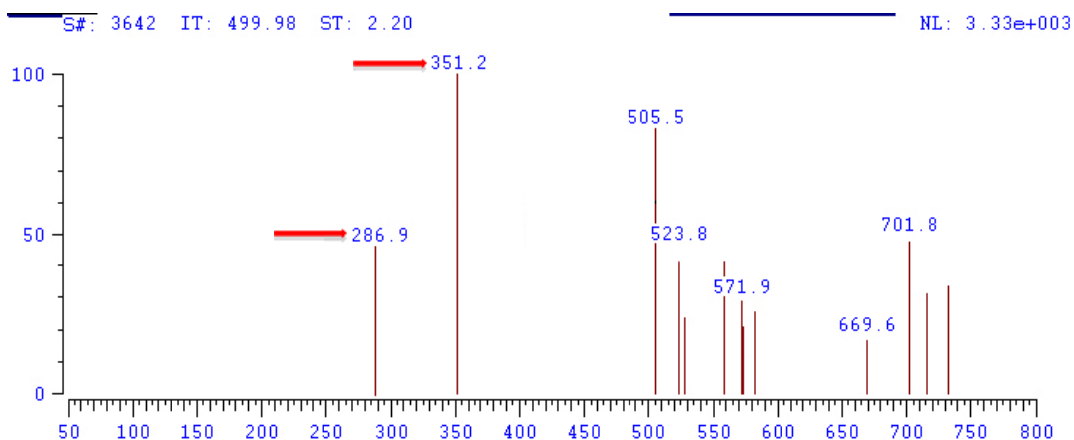


Figure 38. Mass spectrum of ML at peak at 9.5 min.

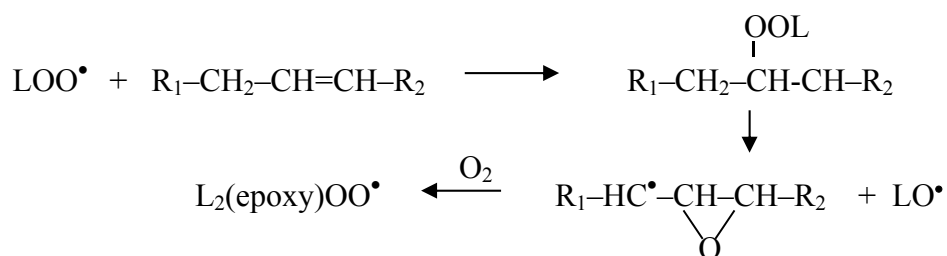
Red arrows indicate molecular ions of two co-eluting DETC-epoxides. Sample was tested with triplicate; only one representative graph is shown.

6.5 Reconciliation of observed chemistry

A key observation of this study was that epoxides are produced in parallel with and sometimes even before hydroperoxides, and their levels often exceed those of hydroperoxides in this model system of highly purified methyl linoleate. This pattern certainly does not fit with only hydrogen abstraction reactions as shown in the traditional scheme of lipid oxidation, so explanations of how such a dramatic change could occur are in order.

To propose underlying reactions, we considered the early appearance of epoxides, the presence of *t*-2-heptenal and *t*-2-octenal among the non-volatile aldehydes detected in this study and even more strongly among volatile products detected in a parallel study [109]. *t*-2-Heptenal and *t*-2-octenal are scission products from C11 alkoxyl radicals, but this carbon does not form stable hydroperoxides as initial products. Thus, radicals at this site must be formed in secondary reactions.

Reactions for formation of epoxides from standard internal rearrangement of C9 and C13 alkoxy radicals are shown in Figure 39 and Figure 40, respectively. Since epoxides begin forming immediately and alkoxy radicals require hydroperoxide formation and decomposition, addition of lipid peroxy radicals to double bonds, must also be considered. The peroxy dimer that results is unstable and decomposes very rapidly to an epoxy compound on the adduct and an alkoxy radical in place of the original peroxy radical:



Although two alternate reactions have not been written previously for lipid oxidation, together they account for both immediate and sustained high levels of epoxides observed in this study. Importantly, peroxy radical addition also provides early alkoxy radicals that can generate either epoxides or carbonyl scission products without forming hydroperoxides as intermediates.

The scission products are important distinguishers of reactions. The appearance of reasonable concentrations of heptenal and octenal among non-volatile aldehydes and as major components of volatile products (along with methyl octanoate) [109] strongly supports epoxidation at C-9 in methyl linoleate (Figure 39). They form when epoxidation at either C9 or C13 transfers the free electron to C11; subsequent oxidation generates the C11 alkoxy radical which undergoes α and β scission to generate these products.

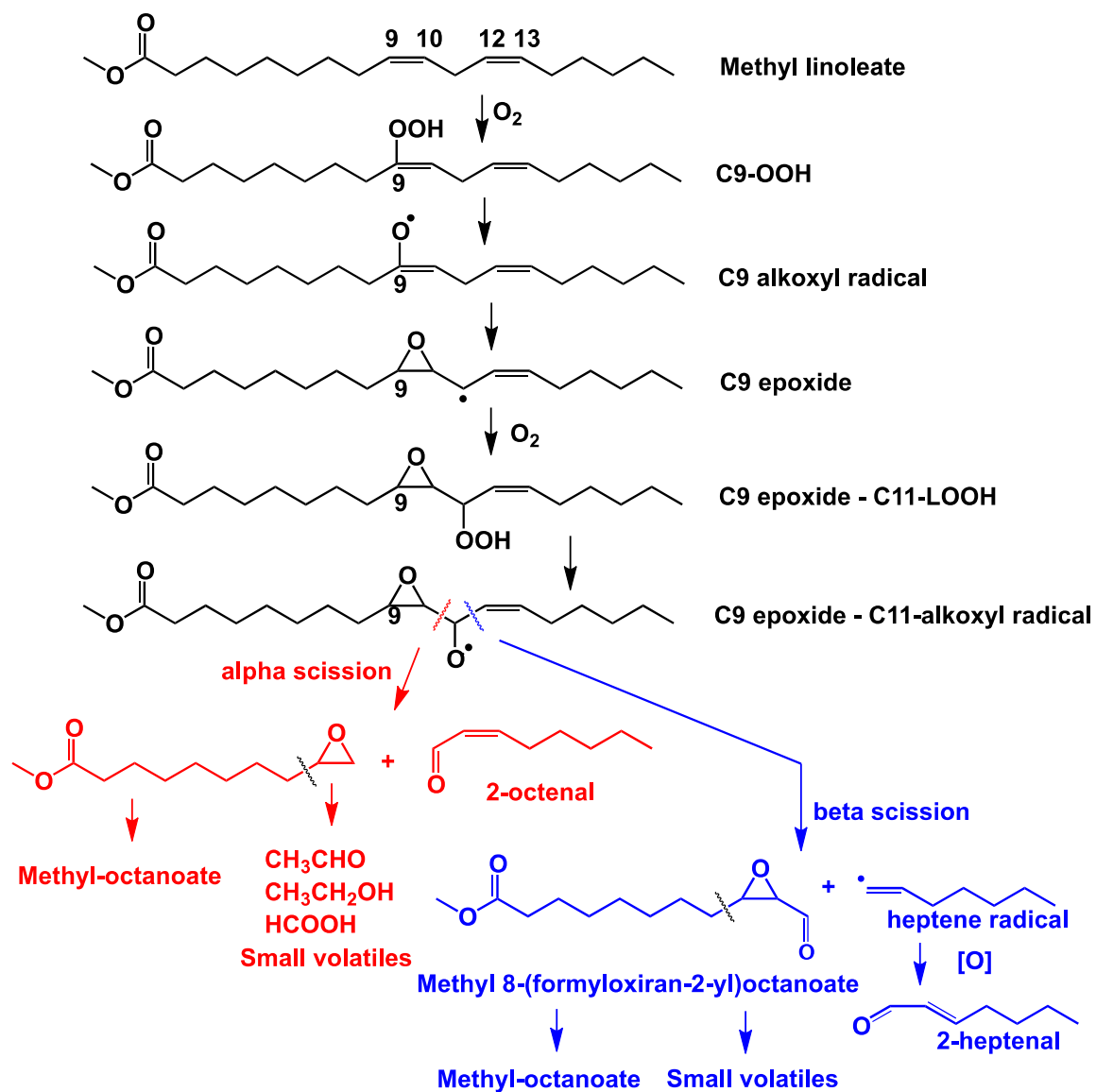


Figure 39. Possible epoxide formed from methyl linoleate (C9 epoxides scission product).

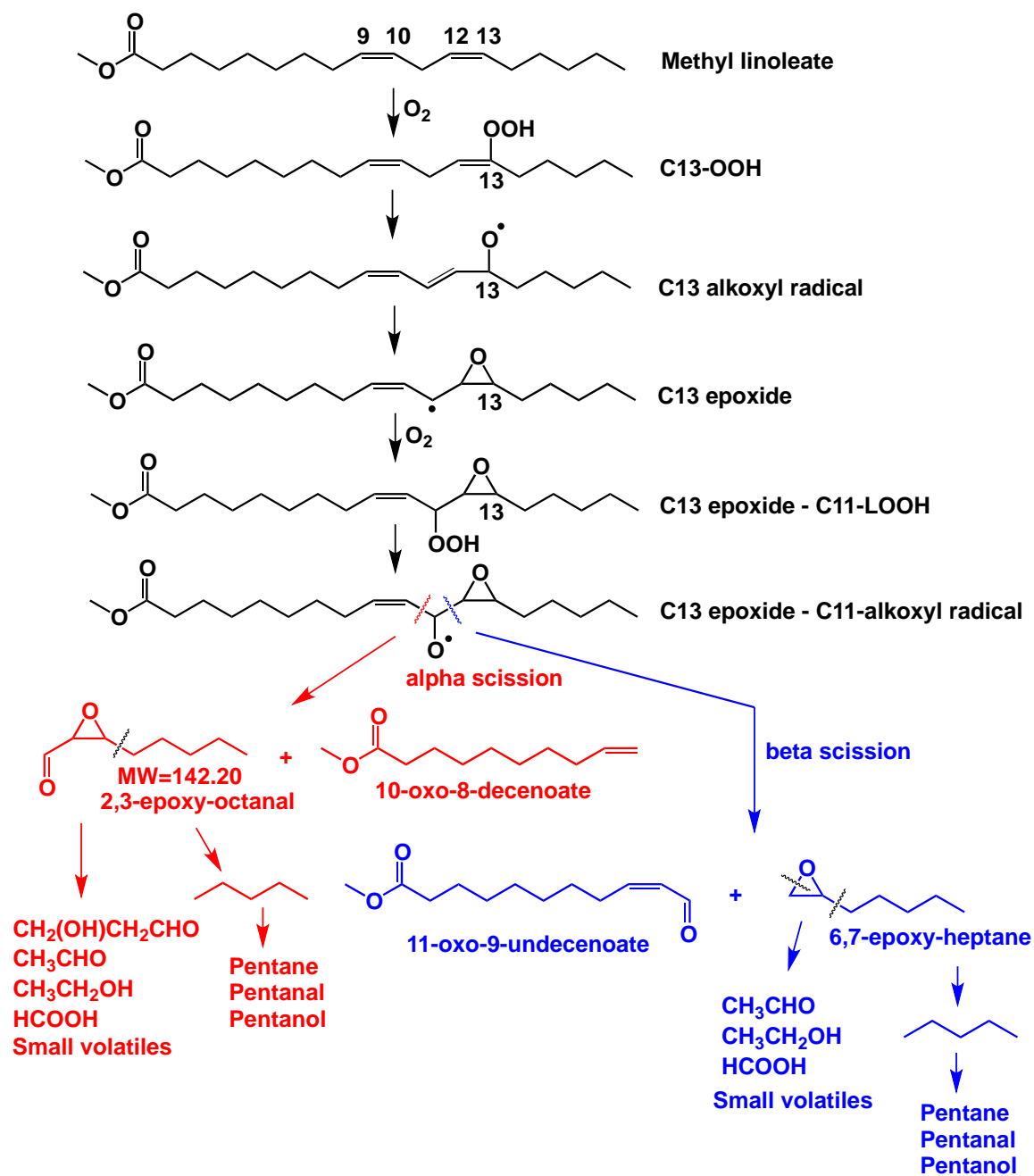


Figure 40. Possible epoxide formed from methyl linoleate (C13 epoxides scission product).

Lipid epoxides have previously been written as internal rather than terminal epoxides. However, MS analyses of DETC-epoxides from methyl linoleate tentatively identified methyl 8-(oxiran-2-yl) octanoate as a major epoxide component. This terminal epoxide results from α -scission of the C-11 alkoxyl radical, and its identification clearly establishes internal rearrangement as a competitive reaction in this system. Internal rearrangement at C13 also explains the epoxyactanal detected by MS, as well as the very high, sustained levels of pentane observed in volatile products [109].

Products from LOO \bullet addition reactions in other configurations are being studied as possible sources of unusual saturated aldehyde products (e.g. butanal and octanal) from methyl linoleate oxidation.

7. SUMMARY AND CONCLUSIONS

This project sought to provide initial direct evidence for multiple pathways in lipid oxidation and for possible shifts in pathways under different conditions by monitoring levels and distributions of conjugated dienes, hydroperoxide, epoxides, and carbonyl oxidation products in methyl linoleate. Concentrations of oxidation products were expressed on a common basis (mmol/mol methyl linoleate) to facilitate mass balance comparisons. Our analyses focused on the first 20 days of incubation to detect initial products rather than secondary reactions or transformed products.

Two observations provided evidence for multiple pathways. First and most dramatic was that under most conditions, epoxides rather than hydroperoxides were the dominant product. Considering the rapid rate of production, we propose that epoxides are most likely formed by addition of peroxy radicals to ML double bonds, followed by decomposition to epoxides and alkoxy radicals. This seems more reasonable kinetically than peroxy radicals \rightarrow hydroperoxides \rightarrow decomposition to alkoxy radicals \rightarrow rearrangement to epoxides. Internal rearrangement of alkoxy radicals to epoxides at both C9 and C11, verified by mass spectroscopy, accounts for appearance of *t*-2-heptenal and *t*-2-octenal in both non-volatile and volatile products and for high levels of pentane in volatile products. Preference for internal rearrangement in this system accounts for the dearth of C-9 scission products and the near absence of 2,4-decadienal. The second key observation was that all products began forming immediately, although at different concentrations. This is very different from the traditional understanding of oxidation in which peroxides accumulate first and secondary products follow hydroperoxide decomposition. For simultaneous generation of products to occur, either hydroperoxides

must decompose and move to secondary reactions extremely rapidly or not all products involve hydroperoxides as intermediates. The former seems inconsistent with accumulation of hydroperoxides as major products.

Results showed a number of interesting patterns in the early oxidations studied:

1. Open systems showed continual accumulation of oxidation products whereas cyclic increase and decrease occurs in closed systems. This cycling pattern of oxidation is of great interest, and to our knowledge has not been reported previously. At the present time, we speculate that the cycling pattern results from localized initiation in microregions, followed by accumulation of locally high concentrations of products which then undergo dismutation to secondary products. Initiation then begins again in another microregion, perhaps catalyzed by radicals remaining from the first cycle. It will be important to elucidate the cause of this cycling because most oxidation-susceptible food products are marketed in closed-system packaging.
2. In open systems, generation of the four products measured followed predictions of increase with temperature only for the first few days; thereafter product levels surprisingly decreased with incubation temperature. In closed systems, product levels increased at 40 °C then decreased again at 60 °C, reflecting more rapid rates of product transformation or degradation. These patterns point out a limitation in application of the Arrhenius equation when multiple reactions are active and products themselves are intermediates in a more complex process. They also raise serious questions about the rationale and practice of using elevated temperatures for accelerated shelf life studies, assuming generation of higher levels of the same products. Measuring only primary products at elevated temperatures is very likely to

underestimate oxidation (and perhaps overestimate antioxidant or packaging effectiveness) at higher temperatures where target oxidation products have been transformed into another compounds. This observation demonstrates the necessity of tracking multiple oxidation products in oxidation research.

3. Conditions for the most part affected rate and extent of oxidation rather than active pathways as reflected in product distributions and blunting of cycling excursions. Condition effects were usually most pronounced in very early oxidation (first few days) and near end of the short incubation period in this study. Additional studies will be needed to determine whether incubation conditions are more important in long term oxidations where they may alter secondary processes more than primary reactions.
4. Reduced headspace pO_2 was found to be generally very effective in delaying lipid oxidation, particularly at elevated temperatures (the higher the temperature, the stronger the inhibition by low pO_2). However, not all low oxygen levels provided the same effect, and effects were not linearly related to the oxygen level. Levels of products immediately dependent on oxygen for formation (conjugated dienes and hydroperoxides) dropped under reduced pO_2 . Secondary processes were less affected and continued despite reduced pO_2 . Hence, peroxides and carbonyls paradoxically increased.
5. Water is usually considered to be a pro-oxidant for lipid oxidation and has been proposed to enhance alkoxyl radical scission reactions to carbonyl products. In this study using dry ML, 2% moisture indeed showed moderate enhancement of all products during initial oxidation, especially at room temperature. However, by then

end of incubation water showed little or no impact on the total oxidation process, especially at elevated temperatures. On the other hand, water did alter product distributions, especially increasing epoxides and carbonyls relative to conjugated dienes. Water also appeared to stabilize hydroperoxides at 40 °. Hydrogen bonding of water to lipid hydroperoxides suppresses their decomposition, thus also preventing formation of carbonyls from scissions of subsequent alkoxyl radicals. More research will be necessary to verify whether water actively catalyzes LO• scissions to carbonyls and what moisture levels this occurs.

6. Effects of solvent proticity on product distributions were difficult to distinguish from effects of dilution and solvent radical interactions in this study. Both solvents impacted oxidation rates only in the initial 2-3 days of incubation where chloroform markedly increased oxidation rate, possibly due to activation of trace metal catalysts, while ethanol suppressed all oxidation products, most likely due to radical quenching. In longer incubations, levels of total products became comparable to those in neat ML. However, analyses of individual aldehydes revealed a very strong suppression of hexanal and formation of new aldehydes by both solvents, indicating a shift of oxidation sites on the ML chain or activation of different pathways for forming aldehydes. In addition, both solvents strongly directed primary conjugated dienes into secondary products, particularly elevating epoxides and carbonyls and activating additional pathways that have not yet been identified. Since chloroform is the solvent almost exclusively used for extracting lipids in oxidation studies, this observation raises concerns about use of product analyses in extracts to determine reaction

mechanisms, and perhaps more importantly, the measurement of hexanal levels to determine oxidation in food quality control.

7. Class analyses of products show general oxidation patterns but may be inadequate for detecting all shifts in pathways. Carbonyls, in particular, showed changes in individual aldehydes generated under different conditions, even when changes in total carbonyl levels were small. At 25°C under open-air conditions, carbonyls did not begin to accumulate until after 12 days when peroxides began to break down. However, at higher temperatures, aldehydes formed from the beginning of incubation and accumulated continuously over the entire 20 days. More importantly in terms of alternate pathways, elevation of temperature changed not only the rate of aldehyde formation but also the types of the aldehydes generated. In addition, some aldehydes accumulated cyclically while others did not. These observations certainly suggest that the same aldehydes can be formed by different pathways under different conditions and also that multiple pathways are active that generate different aldehydes, some of which are independent of hydroperoxide formation and decomposition.

In all, this study has provided experimental evidence for the existence of simultaneous multiple reaction pathways in oxidation of methyl linoleate, generating, in particular, high levels of epoxides that have not been noted previously. There was some shifting among pathways under different reaction conditions, but this was less evident from class analyses than from individual product analyses. Further studies of alternate pathways need to focus on detailed analysis of individual components in all product classes, include analyses of additional product classes (particularly alcohols, dimers, and

hydroperoxide isomers)), and consider pathways shifts caused by reaction conditions in order to elucidate lipid oxidation mechanisms more completely and accurately.

8. FUTURE WORK

This project provided a general proof of principle that multiple pathways of lipid oxidation do indeed exist. Areas now needing more detailed study include:

1. Continue to look for other oxidation products to support the multiple pathways theory. Sensitive quantification methods for determining hydroxy lipids, long chain aldehydes, long chain dimers and polymers, and radical recombination products are clearly needed to achieve this.

2. Simultaneous monitoring of more oxidation products need to be performed on ML and other lipids as well to begin building more complete understanding of oxidation patterns and to reveal interactions between each pathway.

3. Use HPLC-MS methods to separate and identify individual products in simple systems such as methyl linoleate. What is learned here can show what products we are currently missing and provide a basis for optimizing combinations of product analyses in more complex systems.

2. Further investigation on causes of the cycling patterns. Is it an authentic local phenomenon related to closed reaction conditions or some analytical artifact?

3. Test effects of more conditions (e.g. metals, higher water contents, multiphase systems, antioxidants) on patterns of methyl linoleate oxidation. Other studies in our lab suggest that metals may shift positional preference for radical attack and antioxidants preferentially block specific pathways.

4. Extend studies of multiple pathways to more complicated lipid systems such as oils (triacylglycerols) and phospholipids. Studies in complex lipids will be necessary for more accurate understanding of oxidation processes in foods.

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