Evaluation of Assays for Epoxides in Oxidized Lipids

by

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ABSTRACT OF THE THESIS

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Epoxides have long been recognized as lipid oxidation products, and there is

recent evidence that epoxides may be as or more important than hydroperoxides under

many conditions. Nevertheless, epoxides are seldom analyzed when monitoring

oxidative degradation, at least in part because there are few established analytical

procedures. The goal of this thesis research, therefore, was to evaluate four available

epoxide assays for accuracy, sensitivity, stoichiometry, reproducibility, and handling

requirements, and from the results provide analytical protocols and practical guidelines

for selection and application of assays for lipid epoxides.

AOCS standard HBr titration of epoxides, nitrobenzylpyridine (NBP) reaction

with colorimetric endpoint, diethyl dithiocarbamate (DETC) complexation with high

pressure liquid chromatography (HPLC) separation and quantitation of adducts, and ^{1}H

nuclear magnetic resonance (NMR) analysis of epoxides were evaluated using

epoxybutane, epoxyhexene, and epoxydecene as standards.

The HBr assay is too insensitive (detects 0.0075-0.1M) for following lipid

epoxides in foods and biological materials. In addition, the reaction must be run under

inert atmosphere to prevent non-specific oxidation of the Br, and HBr degrades so

rapidly that frequent restandardization is necessary.

Nitrobenzylpyridine assay is more sensitive, detecting 0.5 mM epoxides. However,

the NBP reaction response varied considerably with time and temperature of reaction

and with epoxide structure (increased with epoxide chain length). Hence, a different

standard must be used for each epoxide analyzed, and selection of an appropriate

standard for epoxides of unknown structure is problematic.

For the DETC assay, reaction response was linear from 1μM to 1 mM for all three

epoxide standards ($R^2 > 0.99$) and oxidized methyl linoleate ($R^2 > 0.94$), and increased

with epoxide chain length. HPLC analysis of adducts allows differentiation and

quantitation of individual epoxides, so can provide important information about

oxidation chemistry as well as quantitation.

NMR offers the advantage of direct analysis of oils and extracts, detects

micromolar epoxides, and clearly distinguishes epoxides from other oxidation products

in lipids. Response curves were linear with concentration of epoxide standards,

oxidized corn oil, and oxidized methyl linoleate ($R^2 > 0.98$).

The DETC-HPLC and NMR assays hold the greatest promise for routine analysis

of epoxides in oxidized lipids.

ACKNOWLEDGEMENTS

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1. INTRODUCTION

Lipid oxidation is the major chemical reaction degrading food quality during food storage. During food processing and storage, lipids oxidize in the presence of oxygen, light, micro-organisms, and enzymes (Kan 2006), generating characteristic rancid off-flavors and odors, browning, and toxic products directly (Schaich 2005). In addition, all intermediates and products of lipid oxidation attack other molecules, leading to texture and functional changes, loss of natural colors, and loss of nutritional value (Schaich 1980; Kan 2006; Schaich 2008). Thus, the ability to accurately analyze the extent of lipid oxidation in foods is very important for both industrial quality control and research.

Tracking lipid oxidation is not an easy task because the reactions are complex and form many different products. In addition, these products are not stable, but decompose and transform over time. Traditionally, hydroperoxides have been the major product analyzed. Volatile breakdown products, particularly hexanal, are also commonly measured. However, these two products alone account for only some oxidation pathways and do not accurately portray the extent of lipid oxidation in any material. Current challenges of stabilizing foods reformulated with highly unsaturated oils for health as well as increased attention to presence of potentially toxic compounds now demand more complete accounting of lipid oxidation products.

Epoxides have long been recognized as lipid oxidation products yet they are seldom systematically analyzed when monitoring oxidation in any materials. One reason may be that epoxides are highly unstable due to high ring strain, and they are highly reactive with proteins and other compounds (Schaich 2008). Therefore, they they may not accumulate to detectable levels. A second reason is lack of sensitive methods for detecting epoxides. Whatever the reason, several studies have shown that epoxides are the dominant, if not only, product in aprotic systems (Gardner et al, 1978; Gardner and Kleiman, 1981; Haynes and Vonwiller, 1990) and recent research in our laboratory has verified epoxides as major products that parallel or exceed hydroperoxides in concentration. Consequently, it is mandatory that epoxides be accounted for systematically, in addition to hydroperoxides and other products, when analyzing lipid oxidation in any material.

To support development of standard protocols for analysis of lipid epoxides and encourage inclusion of epoxides in standard lipid oxidation analyses, this thesis evaluated four epoxide assays for accuracy, sensitivity and detection ranges, stoichiometry, effects of structure, and linearity of response, as well as required reaction conditions and handling procedures. The four assays were: NBP (4-p-nitrobenzyl-pyridine) assay, HBr (Hydrobromic acid) Titration assay, HPLC-DETC (N,N-diethyldithiocarbamate) assay and NMR assay. Each assay has

different limitations for detecting lipid epoxides, so a secondary goal was to determine

appropriate conditions for use of each assay. It is hoped that the information provided in

this thesis will arouse interest in epoxide assays and provide a starting point for more

extensive accounting of the role of epoxides in lipid oxidation.

2. BACKGROUND

2.1 General Overview of Lipid Oxidation

Lipid oxidation has huge negative impacts on food systems, including reduced

shelf life; production of off-flavored aldehydes, ketones and all other secondary

compounds; browning; and co-oxidation of other food molecules (Claxson et al. 1994;

Kamal-Eldin et al. 1997; Frankel 1998; Schaich 2008). Lipid oxidation is a constantly

changing process that is generally considered to occur in three stages -- initiation,

propagation and termination (Schaich 2005).

2.1.1. Initiation of lipid oxidation

Lipid oxidation is not a thermodynamically spontaneous reaction. Ground state

triplet oxygen has two unpaired electrons ($^{\bullet}$O-O$^{\bullet}$) with parallel spins that cannot

directly add to double bonds of unsaturated fatty acids (Frankel 1998), which are in

singlet state. Thus, initiators are required to start the reaction. During initiation,

initiators such as light, heat, metals, or other radicals provide the high energy needed to

remove hydrogen atoms from lipid molecules and form *ab* initio lipid radicals (L•)

(Schaich 2005):

$$LH \quad \xrightarrow{\text{initiator}} \quad L^{\bullet} + H^{+} \quad\quad\quad\quad\quad (1)$$

Metals: Transition metals are perhaps the most active initiator of lipid oxidation in

foods since metals are ubiquitous components and contaminants. Even at

concentrations as low as 0.1 ppm, transition metals possessing two or more valency

states can act as catalysts to initiate lipid oxidation, (Kan 2006). Higher valence state

metals act primarily by electron transfer to cause formation of radicals in lipids, while

reduced metals act primarily by reduction of the O-O bond in hydroperoxides (Schaich,

1992). These reactions will be discussed in more detail in Section 2.2.4.

Heat: Thermal energy at high temperatures (e.g. frying or baking) in the presence

of oxygen induces bond scissions to form radicals ($L^{\bullet}$, $LO^{\bullet}$ and $LOO^{\bullet}$) which can then

abstract hydrogens to start the radical chains of oxidation (Nawar, 1969, 1986). The

main effect of moderate heat, i.e. under most conditions except frying, is to break O-O

bonds of ROOH or LOOH already formed by enzymes, metals, photosensitizers, or

oxidation, since this is the only propagation step that has any significant activation

energy (Table 1) (Labuza 1971). The $RO^{\bullet}$, $LO^{\bullet}$, and $HO^{\bullet}$ •free radicals thus generated

then abstract nearby lipids for hydrogens to form L• and new radical chain reactions.

This greatly accelerates the rate of oxidation.

Table 1. Activation energy of different propagation reactions. Data from (Labuza 1971).

Reaction	Activation energy (E_a)
k_o ($L^\bullet + O_2$)	0 kcal/mole
k_p ($LOO^\bullet + LH$)	~5-15
k_t (2 $ROO^\bullet$)	~4
k_t (2 $R^\bullet$)	5
k_t ($R^\bullet + ROO^\bullet$)	1
k_d (monomolecular) (LOOH →)	31
k_d (bimolecular) (2 LOOH →)	50 uncatalyzed system

k_o, oxygenation; k_p, propagation; k_t, termination; k_d, decomposition

Light: Light initiates lipid oxidation less efficiently than heat and metals. Shorter wavelengths of ultraviolet light theoretically have enough energy to break C-C or C-H bonds in acyl chains (Table 2), but in practice ionization is more common. Thus, it is difficult to produce *ab initio* $L^\bullet$ radicals directly by light. The main effects of light are in two other directions (Schaich 2005):

a) Decomposition of low energy (157 kJ/mol) O-O bonds in hydroperoxides by ultraviolet light. This generates $LO^\bullet$ and $^\bullet OH$ radicals that abstract hydrogens more rapidly than $LOO^\bullet$ and accelerate lipid oxidation greatly.

b) Generation of free radicals or singlet oxygen, $^1O_2$, by visible light and photosensitizers. When exposed to light, photosensitizers absorb energy and

jump to an excited state. This energy is released in direct electron transfer to

form free radicals, or in electron transfer to oxygen. The energy transforms

ground state oxygen $^3O_2$ (unpaired electrons in parallel spin) to excited state

oxygen $^1O_2$ (unpaired electrons in opposite spin). Now having the same spin

state as double bonds, $^1O_2$ can add across lipid double bonds to form

hydroperoxides without generating radicals (Schaich 2005). Due to the high

energy of excited state oxygen $^1O_2$, the reaction rate of singlet oxygen is 1500

times greater than autoxidation (Kan 2006).

Photosensitizers in foods and biological materials are usually pigments such as

chlorophyll and hemoglobin. Type 1 photosensitization (free radical) is oxygen

dependent and is distinguished from normal autoxidation only by accelerated kinetics.

Type 2 photosensitization (singlet oxygen) is oxygen independent and related mostly to

the concentration of sensitizer present (Schaich 2005)

Table 2. Energies at various light wavelength compared to bond dissociation energies for typical chemical bonds (Schaich 2005).

	eV[a] (Physicists)	kJ[b] (Chemists)	kCal[b] (Biologists)	Bond Dissociation Energy ΔE		
				Bond	kJ/mol[c]	kCal/mol[d]
200	6.2	596	143	C=C	612	146
230	5.4	518	124	O–H	463	111
260	4.8	458	110	C–H	412	99
290	4.3	411	98	C–O	360	86
320	3.9	372	89	C–C	348	83
350	3.5	341	82	C–N	305	73
380	3.3	314	75	O–O	157	35
410	3.0	291	70			
440	2.8	271	65			
470	2.6	254	61			
510	2.4	234	56			
540	2.3	221	53			
570	2.2	209	50			
600	2.1	199	48			
630	2.0	189	45			
660	1.9	181	43			
700	1.8	170	41			

2.1.2 Propagation

During propagation, *ab initio* lipid radicals react with oxygen to form peroxyl radicals (LOO$^\bullet$). L$^\bullet$ radicals are relatively unreactive, but peroxyl radicals abstract hydrogens from nearby lipid molecules to form a stable intermediate hydroperoxide (LOOH) and generate a new lipid radical (L'$^\bullet$) at the same time (Schaich 2005). Hydroperoxides are decomposed by light, heats and metals to alkyl radicals (LO$^\bullet$) which are several orders of magnitude greater in reaction rate than peroxyl radicals (LOO$^\bullet$). In the propagation stage, hydroperoxide decomposition is the key rate-limiting step (Kamal-Eldin et al. 1997).

Table 3. Lifetimes and hydrogen abstraction rates of various radicals (Schaich 2005).

Radical	Half-life with Typical Substrate, 10^{-3} M, 37°C	Ave. rx Rate, k (L mol^{-1}sec^{-1})	Reference
HO$^\bullet$	10^{-9} sec	10^9–10^{10}	191
RO$^\bullet$	10^{-6} sec	10^6–10^8	191
ROO$^\bullet$	10 sec	10^1–10^3	191
L$^\bullet$	10^{-8} sec	10^4–10^8	191
AnOO$^\bullet$	10^{-5} sec		192[a]
O$_2^{-\bullet}$		~1	193[b]
HOO$^\bullet$		10^0–10^3	194[b]

The followings are some major competing propagation mechanisms of lipid peroxyl radicals (Schaich, 2005):

1. Hydrogen abstraction to form hydroperoxides:

$$LOO^\bullet + LH \longrightarrow LOOH + L^\bullet$$

$$LOO^\bullet + L'OOH \longrightarrow LOOH + L'OO^\bullet$$
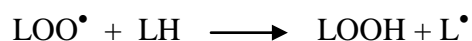
Peroxyl radicals formed from initiation reactions abstract hydrogen from nearby lipids or hydroperoxides to form hydroperoxides and new radical molecules (L$^\bullet$ and L'OO$^\bullet$, respectively); the new radicals add oxygen and start new chains. This cycle of reactions can continue indefinitely until the chain is intercepted.

2. Rearrangement/cyclization reaction to form epoxides, hydroxyepoxides and epidioxides. When abstractable hydrogens are not immediately available, peroxyl radicals add to nearby double bonds to form cyclic products. The most important internal rearrangement of peroxyl radical proceeds by 1,3-addition of the peroxyl radical to the nearby *cis*-double bonds to form a 5-exo ring (Rx.2). Initial

cyclization of peroxyl radical via 1,4-addition to form a 6-exo exocyclic peroxides

(Rx.3) forms only in fatty acids with four or more double bonds.

$$
\tag{2}
$$

$$
\tag{3}
$$
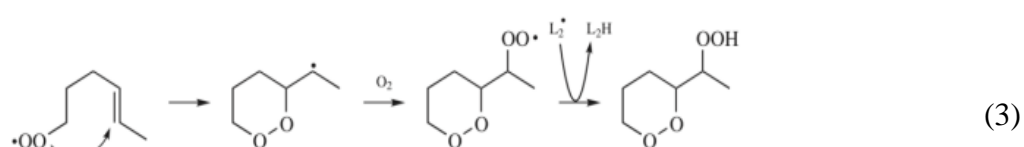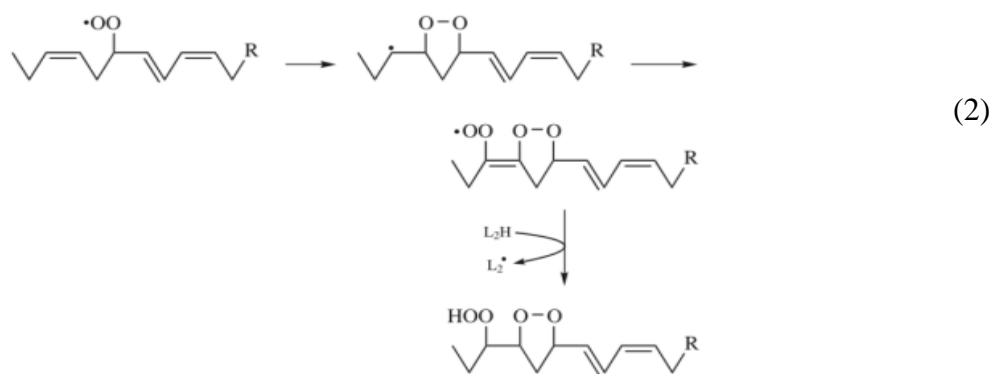
3.   Additions to double bonds to form dimer, polymers and hydropeoxy epoxides

(Kochi 1962,1973). Addition reactions compete with hydrogen abstraction. Although

perhaps less favored under general conditions, addition reactions become competitive

when the abstractable hydrogens are limited (aprotic solvent, low temperature). In early

stages of oxidation, LOO$^{\bullet}$ add to double bonds to form initial dimer complexes (Rx. 4)

which can further react to form new radicals. LOO$^{\bullet}$ add to isolated or nonconjugated

double bonds, then undergo 1,3-cyclization to generate an epoxide and new radical that

adds oxygen to produce a new peroxyl radical (Rx. 4).

$$LOO^\bullet + R_1-CH_2-CH{=}CH-R_2 \longrightarrow R_1-CH_2-\overset{\bullet}{C}H-\overset{\overset{\textstyle OOL}{|}}{C}H-R_2 \qquad (4)$$

$$R_1-CH_2-\underset{\underset{\textstyle OOL}{|}}{\overset{}{C}}H-CH-R_2 \longrightarrow LO^\bullet + R_1-\overset{\bullet}{H}C-CH-CH-R_2 \overset{O_2}{\longrightarrow} L_2(epoxy)OO^\bullet \quad (5)$$
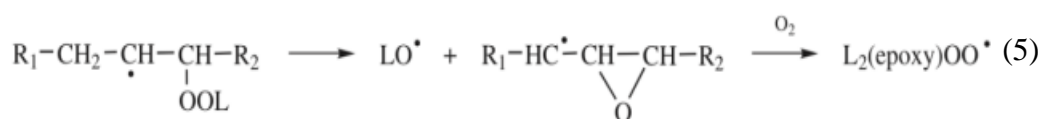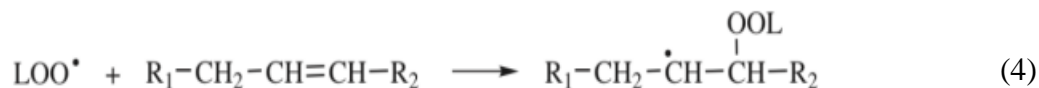
Alkoxyl radicals also have competing reactions that can change the course of lipid

oxidation (Schaich 2005). Of particular importance to propagation are:

a)   internal rearrangements to form epoxides and allylic radicals (Rx. 6) (Kochi

1962, Wu et al. 1977, Haynes and Vonwiller 1990). The radicals add oxygen to

become peroxy(epoxy) radicals, undergo additional reactions, and eventually

decompose to as yet unidentified products.

$$-HCH{=}CH-CH{=}\overset{\overset{\textstyle \bullet O}{|}}{C}H-CH\text{-}R_2 \longrightarrow -HCH{=}CH-\overset{\bullet}{C}H-\overset{O}{\underset{}{C}}H-CH\text{-}R_2 \qquad (6)$$
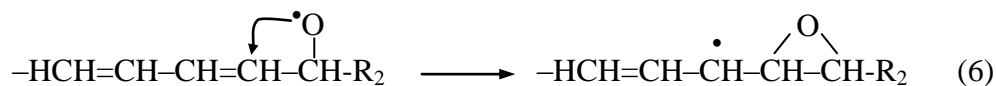
b)   addition to double bonds (Rx. 7) (Kochi 1962,1973). This occurs most readily

with conjugated double bonds, so increases in importance as oxidation

progresses. Unlike peroxyl radicals that add almost exclusively to trans double

bonds, alkoxyl radicals preferentially add to cis double bonds (Kochi 1962).

$$LO^\bullet + \quad \text{[structure]} \longrightarrow \text{[structure with LO]} \qquad (7)$$
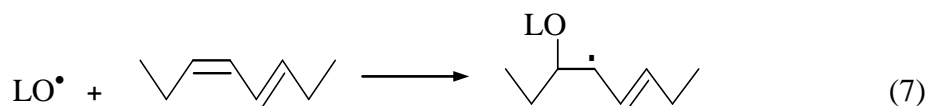
c)   scission of the acyl chains at positions α and β to the alkoxyl radical to form two

radicals (Rx. 8) (Chan et al. 1976). Some of the radicals convert immediately to

carbonyl compounds (termination products), but the remaining radicals can add

oxygen to form secondary peroxyl radicals, or can add to double bonds to generate more complex radicals. This source of propagating radicals creates complex product distributions yet is commonly overlooked.

$$R_1\text{-}CH\text{-}R_2 \xrightarrow{\quad} R^\bullet + CH\text{-}R_2 \quad \textbf{OR} \quad R_1CH + {}^\bullet R_2 \tag{8}$$
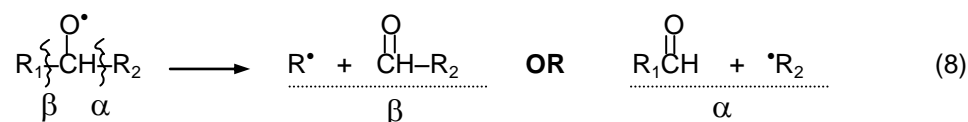
2.1.3 Termination

Propagation continues until there are no available hydrogens for abstraction or until non-radical products are formed by one of the following processes:

1. **Radical recombinations of two individual free radicals to form stable non-radical products.** These non-radical materials -- ketones, aldehydes, alcohols, alkanes and dimers -- are called secondary oxidation products and often result in flavor change, color change and texture degradation. The types of recombinations that occur are dependent on reaction conditions, that in turn determine the types of radicals formed. Oxygen and temperature play particularly important roles in radical recombination. When oxygen pressure pO_2 is low and temperature is high (rapid thermal scissions active), $L^\bullet$ recombination dominates. In contrast, when the oxygen pressure pO_2 is high and temperature is low, $LOO^\bullet$ reactions dominate as shown in Figure 1.
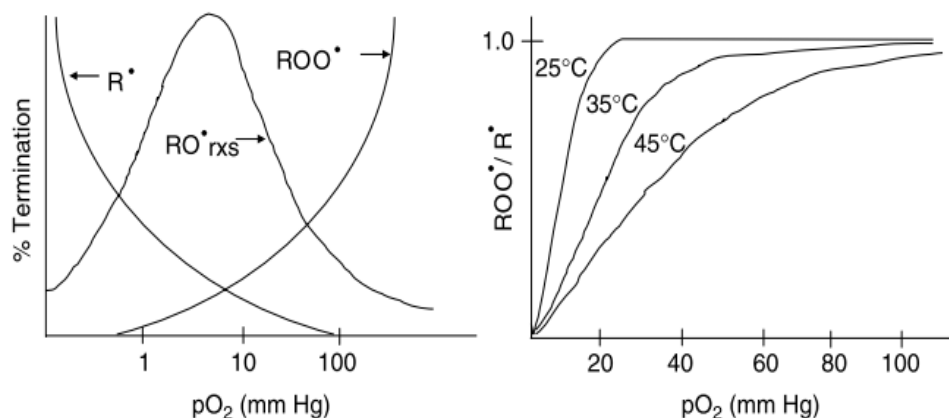
Figure 1. Effect of oxygen and temperature on termination processes in lipid oxidation

(Schaich 2005), redrawn from Labuza (1971).

2. **Cleavage of alkoxyl radicals when proton sources are present to stabilize

    products.** Alkoxyl radicals undergo scission on either side of the $-C(O^\bullet)-$ to form

    aldehydes, alkanes, and other products during lipid oxidation. Any fragments that

    contain double bonds can continue to oxidize to form carbonyl, alkane and other

    short chain products which contribute to the characteristic odors in lipid oxidation.

    Some examples of scission reactions are shown in Figure 2.

3. **Co-oxidation of other molecules (radical transfer).** Lipid radicals abstract

    available hydrogens even from non-lipid molecules such amino acid and protein

    (Schaich, 1980; Schaich, 2008; Guillén 2009). This quenches lipid radicals and

    ceases the propagation reactions, but transfers the oxidation potential to other types

    of molecules, thus broadcasting oxidation damage.
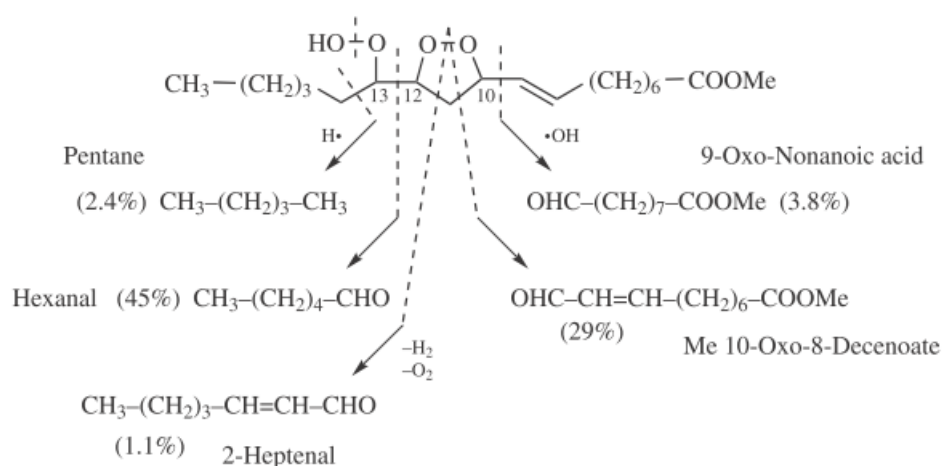
Figure 2. Example of an epioxide intermediate derived from photosensitization

undergoing secondary scissions to form short chain odor compounds and

aldehydes (Schaich, 2005; redrawn from Frankel et al., 1982).

4. **Eliminations.** OH⁻ and OOH⁻ can be cleaved from hydroperoxides and form an

internal ketones and paired desaturated products with an additional double bond,

respectively (Figure 3).
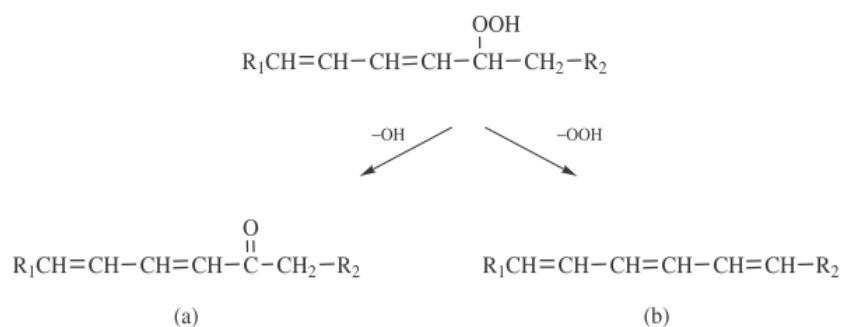
Figure 3. Example of a lipid hydroperoxide undergoing OH⁻ and OOH⁻

elimination (Schaich 2005).

2.1.4 Reaction schemes for lipid oxidation

2.1.4.1 Classical radical chain reaction

A reaction scheme showing how the free radical chains of lipid oxidation have been traditionally written is shown in Figure 4. In this series of reactions, hydrogen abstraction is the only chain propagating process, products do not accumulate until the end of oxidation, and no mechanisms are specified for the formation of different products. This scheme is oversimplified, does not account for the multitude of different products that arise from oxidizing lipids, and is inconsistent with observed kinetics and appearance of products. Thus, this simplistic reaction sequence must be incomplete.

2.1.4.2 Integrated alternate pathways of lipid oxidation

There is ample evidence in the literature documenting reactivity of peroxyl and alkoxyl radicals beyond hydrogen abstraction and appearance of other products in competition with hydroperoxides. These alternate reactions, outlined above, have been reviewed in detail by Schaich (2005). Alternate reactions of peroxyl radicals, $LOO^\bullet$, that compete with hydrogen abstraction include β-scission of oxygen, internal rearrangement to epidioxides, addition to double bonds, and dismutation. Alternate competing reactions of alkoxyl radicals, $LO^\bullet$, include internal rearrangement to epoxides, addition to double bonds, and scission to secondary products. How these alternate reactions may be integrated into a more complete reaction scheme is shown in

Free Radical Chain Reactions of Lipid Oxidation

Stage 1: Initiation (starting the chain)

Energy, M, radicals

$$LH \longrightarrow L^{\bullet} + H^{+}$$
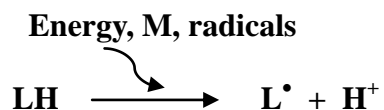
Stage 2 : Propagation (involving additional molecules in the chain)

$$L^{\bullet} + O_2 \longrightarrow LOO^{\bullet} \qquad k > 10^9 \ M^{-1}sec^{-1} \ \text{(very fast)}$$

$$LH + LOO^{\bullet} \longrightarrow LOOH + L^{\bullet}$$

$$LOOH \xrightarrow{\text{Energy, M}} LO^{\bullet} + {}^{\bullet}OH$$

$$LO^{\bullet} + LH \longrightarrow LOH + L^{\bullet}$$

Stage 3 : Termination

$$LOO^{\bullet} + LOO^{\bullet}$$

$$L^{\bullet} + LOO^{\bullet}$$

$$LO^{\bullet} + L^{\bullet}$$

$$L^{\bullet} + L^{\bullet}$$

Non-radical products

(aldehydes, alkanes, peroxides, epoxides,

ketones, polymers, …etc)
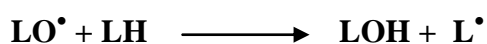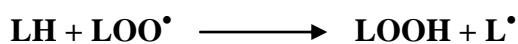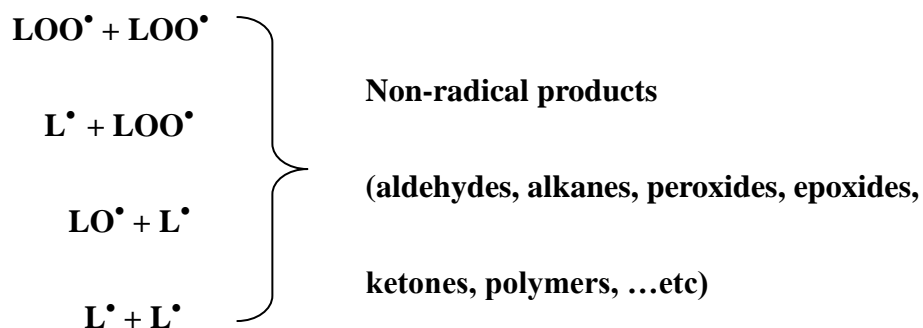
Figure 4. Free radical chain reactions of lipid oxidation proceed in three stages

Figure 5. The alternate reactions all compete with each other, and the balance between them changes with reaction conditions. Thus, the standard practice of following lipid oxidation by hydroperoxides alone, or perhaps in combination with hexanal as a secondary product, cannot accurately determine the extent of oxidation and may even be misleading (Claxson et al. 1994). Not only do hydroperoxides decompose, but when other pathways are active they form at much lower levels, later, or even not at all.

Similarly, when alternate pathways compete with scission of alkoxyl radicals, hexanal may not be formed. The problem is, favored pathways and types of products present in materials are seldom known before analysis, so it is impossible to predict ahead of time what products should be measured. Additional problems arise because different environmental factors or circumstances promote different lipid oxidation reaction pathways (Schaich 1992, Jie et al. 2003), and because food systems are complex matrices and lipid oxidation will be affected and altered by the presence of other ingredient within food (Marmesat 2008). Unless products from competing alternate pathways are measured, the wrong pathway can be followed and lipid oxidation can be missed. This means that accurate tracking of lipid oxidation products and pathways requires monitoring multiple products simultaneously on each sample (Schaich 2012).
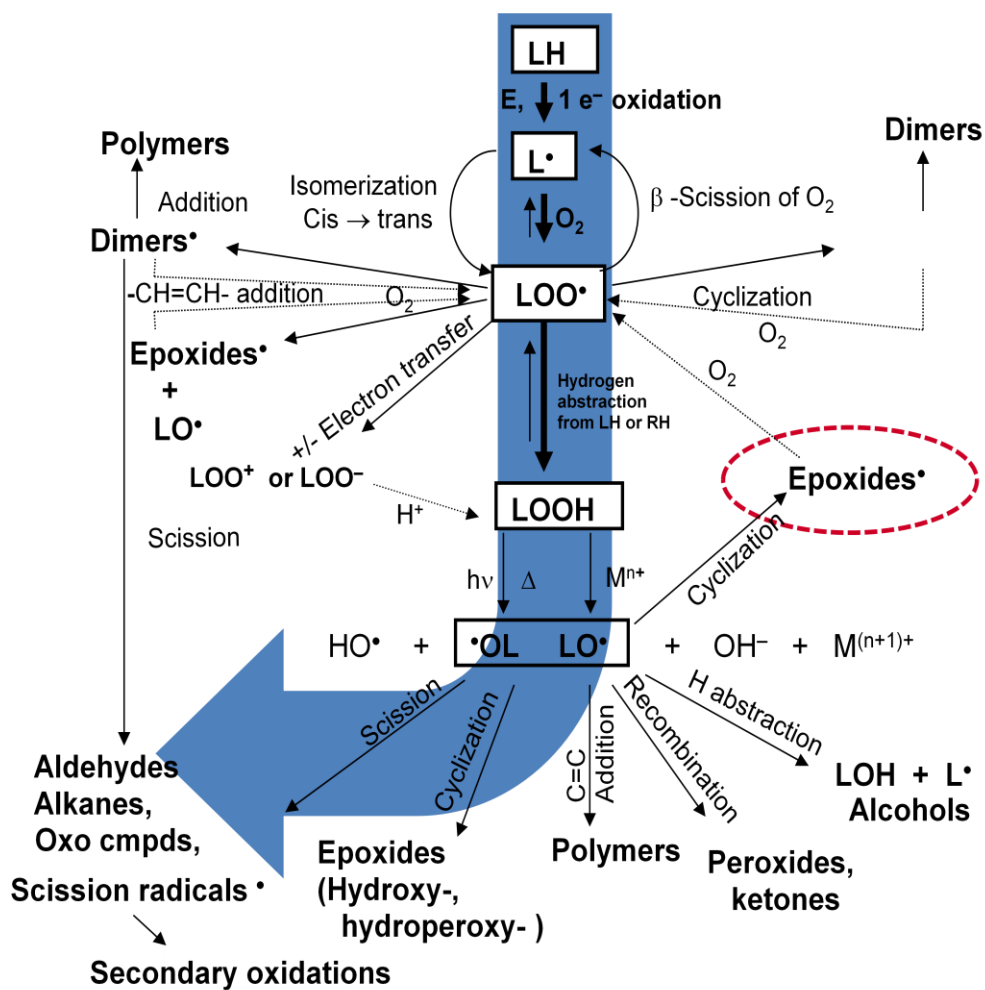
Figure 5. Integrated theory of lipid oxidation showing alternate reactions that compete with hydrogen abstraction (Schaich, 2005, 2012).

2.1.5 Factors that affect oxidation

Oxygen

Lipid oxidation is not a self-initiated process, but requires initiators to form the *ab initio* radical that starts the radical chains. This is the rate-limiting step. Heat, light, metals, lipoxygenase, and other radicals are the most common initiators (Schaich 1980). Oxygen adds to this radical almost instantaneously, that is, at rates controlled only by how fast oxygen can diffuse to the radical (Schaich 2005, Schaich 2012). Thus, addition of oxygen affects oxidation rates only at very low oxygen concentrations. When oxygen pressure is low (less than 1%), the rate of oxidation can be expressed as

$$\text{Rate of oxidation (low } pO_2) = k_2(k_1/k_8)^{1/2} [LOOH][O] \tag{9}$$

Under these conditions, the oxidation rate is dependent on oxygen and the balance between initiation and termination by alkyl radical recombinations (Schaich 2005). Above some level (few % O_2), more than enough oxygen is present to fill all radicals, so rates becomes independent of oxygen, and oxygen effects shift to product distributions. Under high oxygen pressure, the rate of oxidation can be expressed as following equation (Schaich 2005).

$$\text{Rate of oxidation (high } pO_2) = k_3(k_1/k_6)^{1/2} [LOOH][LH] \tag{10}$$

where K_3=H abstraction rate of $LO^{\bullet}$, K_1=initiation rate to form $L^{\bullet}$, and K_6=termination rate by LOOH recombination

Metals

Redox active metals are major factors catalyzing lipid oxidation in biological systems. All redox-active can catalyze lipid oxidation, although metals such Fe, Cu, and Co are the most common. The simplest mechanism for catalysis of initiation is direct electron transfer from higher valence state metals to double bonds in lipids to form lipid alkyl radicals, $L^\bullet$ (Schaich 1992, 2005):

$$RCH=CHR \ + \ M^{(n+1)} \ \longrightarrow \ RC^\bullet H\text{-}C^+HR \ + \ M^{n+} \tag{11}$$

$$RH \ + \ M^{(n+1)+} \ \longrightarrow \ R^\bullet + H^+ + M^{n+} \ [\ \xrightarrow{LH} \ L^\bullet + RH] \tag{12}$$

$$RCOOH \ + \ M^{(n+1)+} \ \longrightarrow \ RCOO^\bullet + H^+ + Mn^+ \ \longrightarrow \ R^\bullet \ + CO_2 \tag{13}$$

$$RCHO \ + \ M^{(n+1)+} \ \longrightarrow \ RCO^\bullet + H^+ + Mn^+ \ [\ \xrightarrow{LH} \ L\cdot + \text{scission products} \ ]$$
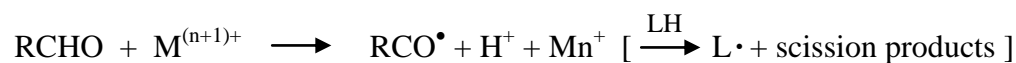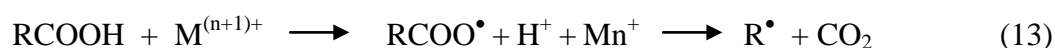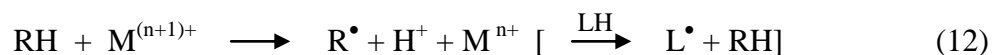
Lower valence state (reducing) metals require formation of complexes with oxygen that generate reactive oxygen radical species in order to initiate radical chains (Schaich, 1992, 2005):

$$M^{n+} \ + \ O_2 \ \longrightarrow \ [M^{(n+1)+}...O_2^{-\bullet}] \ \xrightarrow{LH} \tag{14}$$

(a) $\quad M^{(n+1)+} \ + \ O_2^{-\bullet} \ \longrightarrow \ HOO^\bullet \ \xrightarrow{LH} \ L^\bullet \ + \ H_2O_2$

(b) $\quad L^\bullet + \ [M^{(n+1)+}...\ ^-O_2H]$

(c) $\quad L^\bullet + \ M^{n+} \ + \ HOO^\bullet \ \xrightarrow{L'H} \ L'^\bullet \ + \ H_2O_2$

(d) $\quad LO^\bullet + \ [M^{(n+1)+}...\ ^-OH]$

(e) $\ [M^{(n+1)+} L^-] \ + \ M^{(n+1)+} + HOO^\bullet \ \longrightarrow \ L'^\bullet \ + \ H_2O_2$
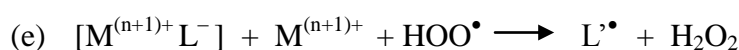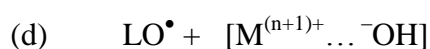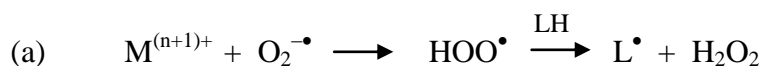
Redox-active metals are perhaps even more important in catalyzing propagation by decomposing relatively unreactive hydroperoxides to very reactive oxyl radicals (Schaich 1992, 2005):

$$LOOH \; + \; Fe^{3+} \; \longrightarrow \; LOO^{\bullet} \; + \; H^+ \; + \; Fe^{2+} \qquad\qquad (15)$$

$$LOOH \; + \; Fe^{2+} \; \longrightarrow \; LO^{\bullet} \; + \; OH^- \; + \; Fe^{3+} \qquad\qquad (16)$$
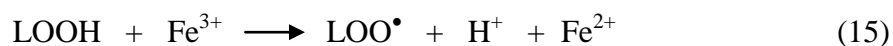
However, it must be noted that metals have some very different reaction mechanisms in aprotic solvents such as lipids, and as a consequence, oxygen insertion to form ketones is a common, if under-recognized reaction in oils, as is formation of metal-hydroperoxide complexes can generate hypervalent complexes that yield epoxides and alcohols in accelerated reactions (Schaich 1992, 2005). Overall, metal catalysis in multiphasic biological system can become quite complex and involve many different catalytic mechanisms depending on the phase of solubilization.

Enzymes

Lipoxygenase is an enzyme that specifically catalyzes formation of hydroperoxides in 1,4-pentadienes, preferably linoleic acid, without a lag period and without generating radicals. Catalysis rates are affected by several factors, most importantly pH, pO_2, and type and concentration of lipid (Kuo 2002). When lipoxygenases are active, hydroperoxides are generated at rapid rates to accumulate an oxidant reservoir. When metals, light or moderate heat are present, decomposition of

the hydroperoxides can lead to a cascade of damaging radicals that greatly accelerate and broaden propagation reactions.

Water

Water activity (Aw) strongly affects the rate and pathways of lipid oxidation. Oxidation occurs rapidly in very dry foods (Aw = <0.1~0.3), but as moisture is added and bound, when Aw reaches the monolayer value for the food, the rate of lipid oxidation drops to a minimum (Labuza 1971, Karel 1980). Reasons given for this behavior are that in dry systems, the matrix is open and air freely diffuses through it, metals are bare so are catalytically more active, and hydroperoxides have no stabilization from hydroigen bonding. As small amounts of water are added up to the monolayer value, reactive sites are hydrated and oxygen access is inhibited, metals are hydrated and electron transfer is impeded, and hydroperoxides are stabilized through hydrogen bonding. However, as water activity increases above the monolayer through the intermediate moisture region (Aw~ 0.33 to 0.73), the lipid oxidation rate increases again as catalysts become mobilized and more oxygen becomes dissolved in the water. Above Aw~0.73, concentrations of catalysts and reactants become diluted by large amounts of water so lipid oxidation rates decrease (Karel 1980).

2.2 Chemistry and Characteristics of Epoxides

2.2.1 Structure of epoxides

One class of lipid oxidation products that has been detected along with other products but seldom measured in focused analyses is epoxides, the topic of this thesis. Also known as oxirane and ethylene oxide, epoxides are ethers with the oxygen atom linked cyclically between the two carbons cyclic, thus forming the corner of a triangle ring (Clayden 2006).
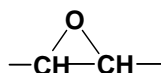
Epoxides are highly reactive and generally quite volatile. The average bond angle inside the ring is 60° (Figure 6), in comparison to the ideal angle of 109° for tetrahedral molecules. This places about 49° of strain on each corner, or approximately 150° of total strain within each epoxide ring. This strain gives rise to the instability and high reactivity characteristics of epoxides (Wade 2006).
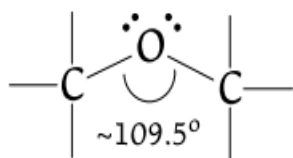
Tetrahedral sp^3 orbital
Bond angle = 109.5°
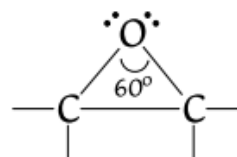
Planer sp3 Epoxide
Bond angle = 60°
Angle strain = 49° (109.5-60)
Total strain = ~150° (49*3)

Figure 6. Structure of epoxides relative to ethers.

2.2.2 Formation of epoxides

Epoxides are formed generally by addition of neighboring oxygenated compounds to double bonds. For example, epoxides are formed by addition of the peroxy oxygen of peroxy acids to double bonds (Clayden 2006):

$$-\overset{|}{C}=\overset{|}{C}- \quad + \quad R-\overset{\overset{O}{\|}}{C}-O-OH \quad \longrightarrow \quad -\overset{|}{\underset{|}{C}}\overset{O}{\triangle}\overset{|}{\underset{|}{C}}- \quad + \quad R-\overset{\overset{O}{\|}}{C}-OH \qquad (17)$$

alkene $\qquad$ peroxyacid $\qquad\qquad$ epoxide $\qquad$ acid
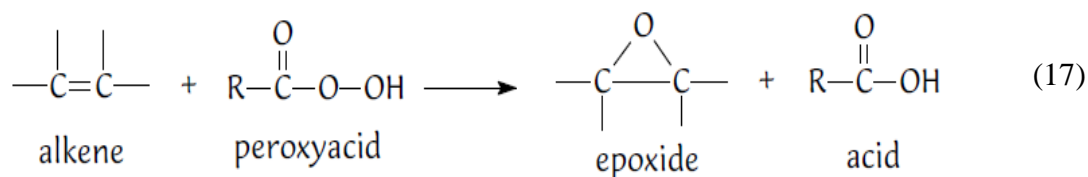
In lipids, epoxides form via two main mechanisms – addition of alkoxyl radicals to adjacent double bonds with formation of an allylic alkyl radical,

$$R_1CH=CH-CH=\overset{\overset{\overset{O\cdot}{|}}{}}{CH}-CHR_2 \quad \longrightarrow \quad R_1CH=CH-\overset{\cdot}{CH}-\overset{O}{\triangle}CH-CHR_2 \qquad (18)$$

and by elimination of an alkoxyl group from a hydroperoxyl adduct, followed by addition of the remaining oxygen to the adjacent radical:

$$R_1-CH_2-\overset{\cdot}{CH}-\overset{\overset{OOL}{|}}{CH}-R_2 \quad \longrightarrow \quad R_1-CH_2-\overset{O}{\triangle}CH-CH-R_2 \quad + \quad LO^\bullet \qquad (19)$$
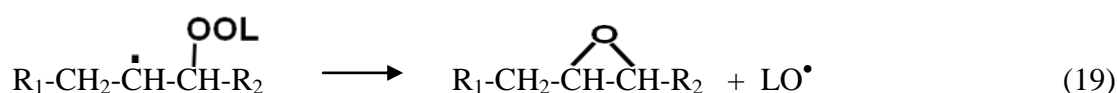
Epoxides can also be formed during heating of oils. Heating of olive and sunflower oils to 180℃ for 15 hours led to the formation of 1% monoepoxy fatty acids, largely epoxy oleic acid in olive oil and predominantly linoleic acid in sunflower oil (Fankhauser-Noti et al. 2006). Direct analysis of thermal oxidized methyl oleate and linoleate by GC-FID detected methyl trans-9,10-epoxystearate and cis-9,10-epoxy-stearate as products of methyl oleate while methyl trans-9,10-epoxystearate, cis-9,10-epoxystearate, methyl trans-12,13-epoxystearate and cis-12,13-epoxystearate

were the possible products of methyl linoleate (Giuffrida et al. 2004, Marmesat et al. 2008).

Epoxides are recognized as lipid oxidation products but are seldom measured. There are several reasons that account for this:

1. Concentrations may be low because epoxides are unstable and rapidly transform to other products –

    - Alkenals (Anderson, 1962)

    - metal-mediated cyclization and internal rearrangement to dihydroxy and hydroxyene compounds and their isomers (Acott and Beckwith, 1964)

    - Fe-catalyzed isomerization and conversion of OH-epoxides to ketols (Tung et al. 1962)

2. Lack of sensitive and accurate assays to detect and quantitate epoxides.

Because epoxides are seldom analyzed systematically but are rather detected adventitiously in other assays, the actual contribution of epoxides to lipid oxidation is unknown and most likely grossly underestimated (Schaich 2005).

2.2.3 Reactions of epoxides

The fast reactions of epoxides are important contributors to their instability, their toxicity, and difficulties in detection. Tracking epoxide formation and reaction is problematic for several reasons:

1. Epoxides are unstable -- after epoxides are formed, they are rapidly degraded to

other substances or products (e.g. alkenals) (Anderson 1962)

2. Metals especially Fe and Cu will catalyze cyclization and internal rearrangement

to form dihydroxy and hydroxyene compounds and their position isomers. (Acott 1964;

Schaich 2005)

3. Fe also catalyzes isomerization and conversion of OH-epoxides to ketols. (Tung

    1992)

2.2.4 Toxicity of epoxides.

Epoxides have been reported to be potent mutagens and carcinogens by way of

alkylating nucleic acids (Agarwal et al. 1979, Nelis and Sinsheimer 1981, Bush and

Kozumbo 1983) . Aliphatic epoxides are intermediates in bio-transformation of several

ethylenic compounds. Epoxy compound in vivo can degrade human transmission

molecules. The epoxy-compound 4,5-epoxy-2(E)-decenal is a precursor of

etheno-2-deoxyadenosine, a highly mutagenic substance that adds to human DNA (Lee

et al. 2001, Chen et al. 1998. Epoxides are also involved in a wide range of biological

effects including binding to cellular macro molecules (Agarwal 1979, Nelis and

Sinsheimer 1981, Bush and Kozumbo 1983). Lysine and histidine are the two most

susceptible amino acids in proteins that will be attacked by lipid epoxides (Lederer

1996).

2.3 Assays for Lipid Epoxides

2.3.1 General Considerations

Epoxides can be formed during lipid oxidation and they are also intermediate products during many chemical reactions and even in biochemistry pathways (Hammock 1974). Epoxide formation is one of the alternate lipid oxidation pathways that compete with hydrogen abstraction and each other for directing oxidative breakdown of lipids (Schaich 2005). Since the lipid oxidation reaction pathway is complicated with many products, both class and individual product analyses are needed to detect, quantitate, and identify epoxides in oxidizing lipids. Class analyses provide general mass balances of products from different pathways shown in Figure 5, and this is useful for tracking balance and direction of oxidation under different conditions. However, more detailed separations of products with mass spectrometry detection are needed to identify individual products and determine specific reaction mechanisms (Ehsan 2010, Guo et al. 2010).

One major reason why epoxides are seldom measured in lipid oxidation is the lack of sensitive, useful assays for epoxides. To fill this void, this thesis focuses on detecting epoxides as a whole, evaluating three chemical assays and one physical assay. Key issues will be sensitivity and accuracy of the chemistry, ease of handling, and conditions required for accuracy and reproducibility.

Even cursory evaluations of epoxide assay methods reported in the literature reveal diverse working conditions and detection ranges reported for each assay. Similarly, many precautions and handling issues must be addressed to ensure generating correct data. Each assay has both pros and cons. Some assays are easily performed but the detection range is too narrower or too high; some assays may be accurate but require expensive reagents or instrumentation not readily available in every lab. These issues should be considered carefully when selecting the assay most suitable for each application.

Because epoxides are highly reactive, the most common approach for class assays is to complex the epoxides to a target molecule that will give a detectable signal, or to convert the epoxides to a stable derivative that can be detected (Kim 1992). In this thesis, both chemical and physical assays were evaluated. Overall, chemical assays required more handling, and the resulting error or bias must be accounted for in calculations and interpretation. For example, when epoxides are titrated with hydrobromic acid as a reagent, judging the end point is a big challenge. Physical assays such as NMR or HPLC-DETC assays require less pre-treatment and handling and are probably more accurate, but how can we know that the experimental results reflect the actual epoxide composition? As a result, both chemical and physical assays need to be tested and compared.

2.3.2 HBr (Hydrobromic acid) Assay

Based on AOCS standard method Cd 9-57 (AOCS 1997), the HBr assay directly

determines the percentage of oxirane oxygen in samples by titration of the epoxide with

hydrobromic acid (Husain 1989). The acid catalyzes ring opening by an SN1

mechanism and the nucleophilic bromide ion with additional electrons (Br$^-$) adds to the

epoxy ring at the less substituted end, as shown in Rx. 20 (Claydon 2006).

(20)
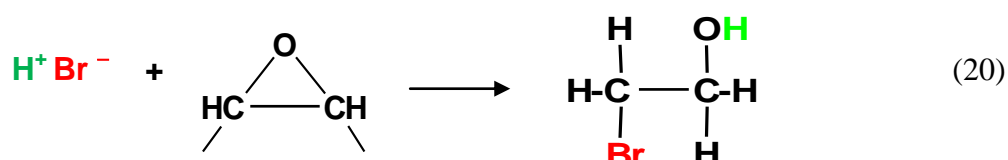
Since this assay is based on titration, stirring is required. Although little attention

has been given to how the speed of stirring influences the reactions, one study showed

that if an epoxy ring is to be formed from the mother material, the speed of stirring is

extremely important. Typically, the reaction rate increases with stirring rate --1000

rpm<1500 rpm< 2000 rpm<2500 rpm (Meshram et al. 2011). Another study showed

that when oxirane contents are less than 0.1%, hydrobromic acid titrations of epoxides

work best if oils are heated to decrease viscosity (Shahidi 1996). However, epoxy

groups degrade very rapidly at elevated temperatures (Earle 1970).

There is some evidence that the presence of cyclopropenoid acids and conjugated

dienols interferes with this reaction (Ansari 1986). Salts of epoxy acids can also react

with amine groups to form amine hydrohalide complexes (Durbetak 1958). Thus, back

titration has been introduced to minimize interference from cyclopropenoid, amine and

conjugated dienols or salts and other molecules that form bonds with hydrobromic acid

(Lee et al. 2009).

2.3.3 NBP (4-*p*-nitrobenzyl-pyridine) Assay

4-*p*-Nitrobenzyl-pyridine (NBP) alkylation of epoxides has been used as an assay

for epoxides for about 40 years. Briefly, the epoxide reacts with NBP to form a stable

complex that can be detected and quantitated by its violet color and optical absorbance

at 600 nm (Hammock 1974, Hemminki 1979, Chen et al. 1998). The detection

wavelength required may vary with the structure of the epoxide (Agarwal 1979). The

overall reaction between epoxides and 4-p-nitrobenzyl-pyridine is illustrated in Figure

7. The epoxy ring is attacked by the NBP reagent in an SN_2 reaction and the ring is

opened. Epoxides will react with amine group to give amine-alcohol products (Clayden

2006). Heat is generally required for this reaction to occur, but the temperature must be

maintained at less than 180 °C to avoid degrading the epoxides (Agarwal 1979).
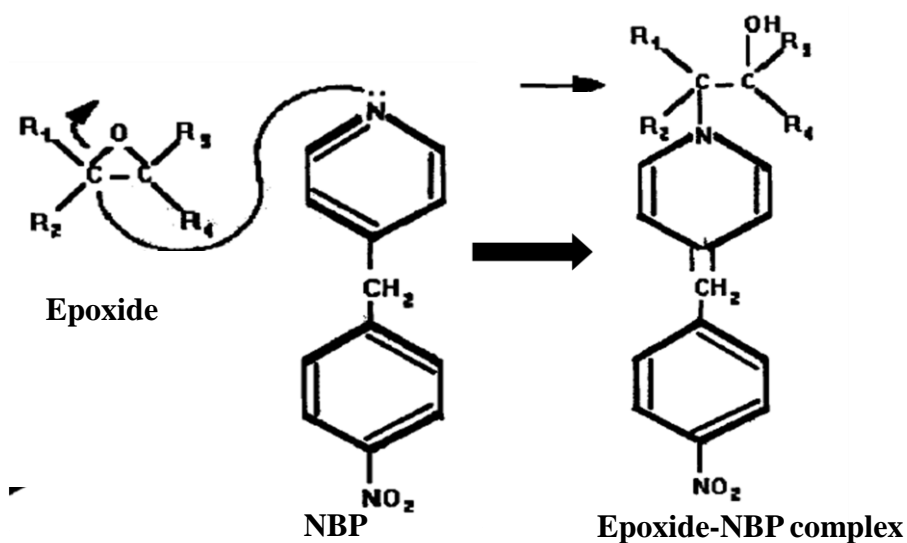
Figure 7. The mechanism of how NBP act as a cucleophile to react with epoxide to form

the NBP-epoxide complex (Kim and Thomas 1992).

This assay has a number of limitations in specificity. Epoxide structure affects

reaction of epoxides with 4-p-nitrobenzyl-pyridine in both pattern and extent of

reaction (Thomas et al. 1992). In addition, 4-p-nitrobenzyl-pyridine alkylates a wide

range of materials in addition to epoxides, including halogenated hydrocarbons,

hydrazine derivatives, aldehydes, thiuram and dithiocarba- mate derivatives

(Hemminki et al.1980, Nelis 1982). Assay results will be inaccurate if those molecules

are present in the sample.

The NBP assay has been used to detect epoxides in vivo as well as in vitro

(Hemminki 1979). As mentioned previously, epoxides are potential mutagens and

arcinogenic reagents, so being able to detect formation early is a great advantage.

2.3.4 DETC (N,N-Diethyldithiocarbamate) Assay with HPLC separation and

quantification of epoxy adducts

This assay reacts DETC (N,N-diethyldithiocarbamate) with epoxides to form

stable epoxide-DETC complexes (Benson et al. 1985) which are then separated by

HPLC (high performance liquid chromatography) and detected at 278 nm

(Dupard-Julien et al. 2007). Individual adducts can be identified by comparison with

authentic adducts or by interfacing HPLC with mass spectrometry detection.

This assay requires excess DETC to react with epoxide to ensure all the epoxide

binds to DETC. Two products are then formed -- a major form and a minor form (Figure

8). To eliminate interference with detection, phosphoric acid must be added to decrease

the pH and decompose the remaining DETC in the solution (Dupard-Julien et al. 2007).

Initial applications of this method used normal phase HPLC to analyze epoxide

complexes (Munger et al. 1977, Van Damme et al. 1995). However, normal phase

columns have some critical drawbacks. A normal phase column must be eluted by a

non-polar, non-aqueous mobile phase solvent such as chloroform. However,

chloroform is quite toxic and thus subject to severe restrictions on use. Normal phase

columns also exhibit poor reproducibility in retention time due to the presence of water

and protic organic layer on the column surface. Because of these shortcomings, normal

phase columns lost favor after reversed phase columns were invented (Hemström et al.

2006), and current HPLC-DETC assay applications have been modified for reversed

phase columns (Dupard-Julien et al. 2007).
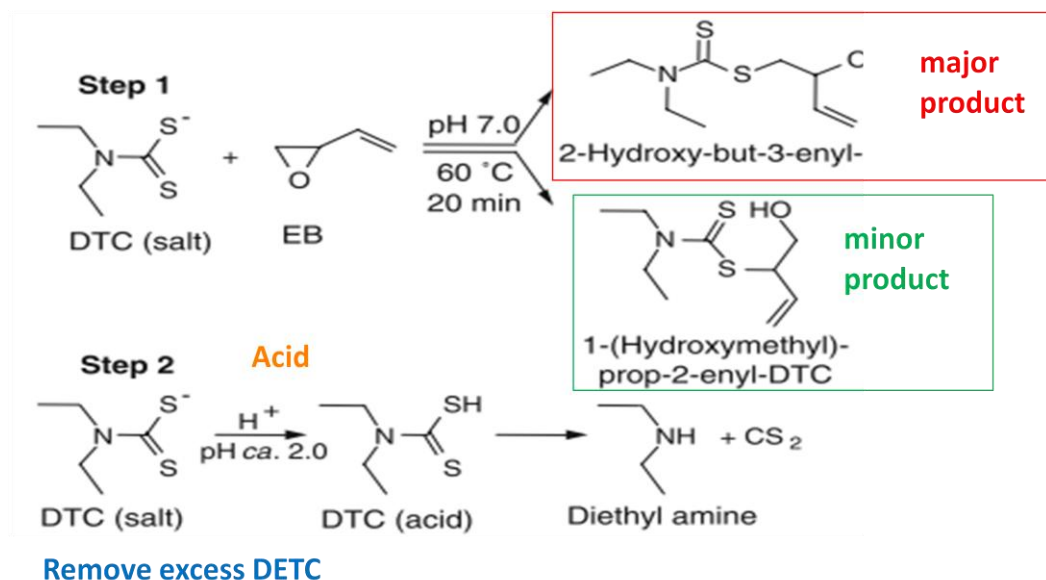
Figure 8. The mechanism of DETC to react with epoxide molecule and followed by an

acid decomposition reaction (Dupard-Julien et al. 2007).

The assay can be used to determine epoxide compounds in cells and tissues

(Rather et al. 2010). However, care is required in this application since DETC is a

reasonably strong metal chelator for metals such as lead (Pb), mercury (Hg), copper

(Cu) and cadmium (Cd) (Van Damme 1995). Thus, metals can be interferences with the

DETC assay, altering the efficiency and stoichiometry of the reaction.

2.3.5 NMR assay for epoxides

NMR itself as a tool is highly expensive and the background knowledge behind

NMR is very complicated. Nevertheless, NMR is unmatched as an analytical method

for analysis of molecular structure (Polozov et al. 1986, Baumann et al. 2002). NMR

can provide detailed information about the types and amounts of different functional

groups in a sample (e.g. edible oil) and these can be organized into an overall picture of

the structure and configuration of the (Guillén et al. 2004, Guillén 2006, Guillén 2009).

This includes the ability to accurately distinguish between (Z,E) and (E,E) isomers

(Guillén 2009).

Studies of edible oils by NMR have shown that there is a specific chemical shift

range for the epoxy ring. For ^{1}H NMR the chemical shift range for the epoxy hydrogen

is 2.5~3.5 ppm and for ^{13}C NMR the chemical shift range for epoxy carbon is 45~55

ppm (John et al. 2003, Ergozhin et al. 2004). Figure 9 shows the epoxy region near 2.9

ppm (–**CHOCH**–) in ^{1}H NMR of methyl oleate. This clearly increases as the peak at

2.01 ppm corresponding to (–**CH**2–CH=CH–**CH**2–) which gives rise to the epoxy

group disappears with incubation time (Aerts and Jacobs 2004). A similar phenomenon

occurs in a) palm oil where epoxide peaks at 2.9-3.1 ppm grow in with oxidation and

disappear when the epoxide group is cleaved (Guo et al. 2010), and b) oxidized epoxy

palm oil in biodegradable lubricants (Moreno et al. 1999, Piazza et al. 2003).
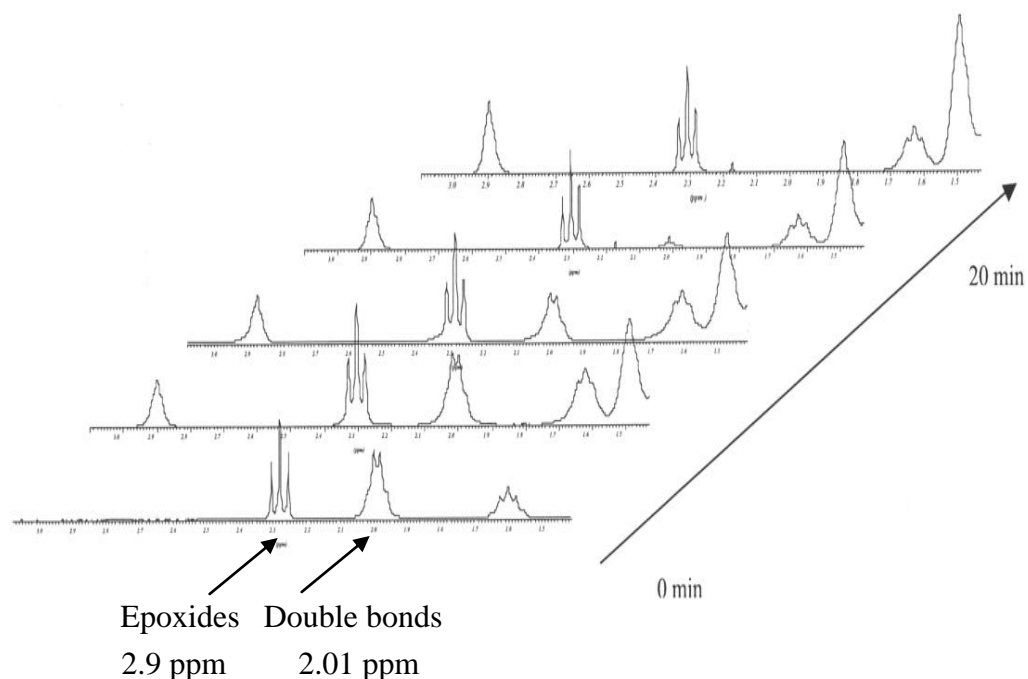
Figure 9. Changes in ^{1}H NMR spectra of oxidizing methyl oleate over time.

Double bond peaks at 2.01 ppm decrease as epoxide peaks at 2.9 ppm increase

(Aerts and Jacobs 2004).

NMR analyses of epoxides offer several advantages. First, NMR spectra provide

information about the total structure of the compound or material – not just epoxides.

In terms of epoxides, NMR can distinguish epoxy stereo isomers. This can be seen in

spectra of 6,7-epoxystearic acid where cis forms have shifts of 2.25 ppm while trans

forms of epoxy fatty acids have chemical shifts at 2.52 ppm (Kannan 1974).

$$H_3C - (H_2C)_6 - H_2C - \mathbf{CH} - \mathbf{CH} - CH_2 - (CH_2)_2 - CH_2 - COOH$$

O   6,7-epoxystearic acid
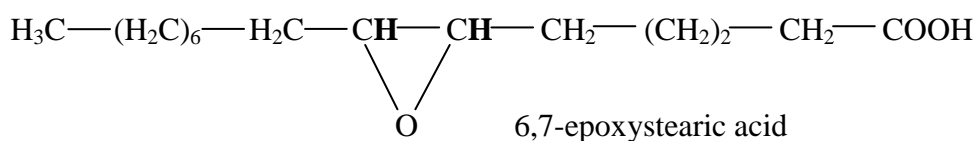
NMR also has the potential to detect other secondary oxidation products in the

same spectra as epoxides. This is possible because the chemical shifts for oxygenated

compounds are significantly separated from alkyl functional groups. As one example,

high proportions of cytotoxic and genotoxic 4-hydroperoxy-, 4-hydroxy- and

4,5-epoxy-trans-2-alkenals were formed and recognized during oxidation of sesame oil

at 70℃ (Guillen et al. 2005, Guillén et al. 2006). In addition, chemical shifts and

hyperfine structure of methylic, allylic and bis-allylic hydrogen atoms can provide

information about the degradation of different acyl groups during lipid oxidation

(Guillen et al. 2005).

From a practical standpoint, NMR is time-saving. An NMR spectrum can be

collected in as little as ten minutes -- one cycle (8 scans) (Claxson et al. 1994). Also,

samples require less pre-treatment and handling, so fewer artifacts and extraneous

oxidation are introduced. One issue of concern is eliminating water and other

non-sample related protons from the sample. To eliminate proton interference,

deuterated solvents such as $CDCl_3$ are used as solvents for lipids (Cravero et al. 2000).

3. EXPERIMENTAL PROCEDURES

3.1 Overall experimental design

Figure 10 shows the overall experimental design for testing four epoxide assays

with three standard epoxides, then applying the assays to analysis of epoxides in

authentic oxidized lipids methyl linoleate and corn oil. Each assay was tested with a

range of standard concentrations to determine linear detection range and detection

limits, R group effects, accuracy of assay, and handling issues.
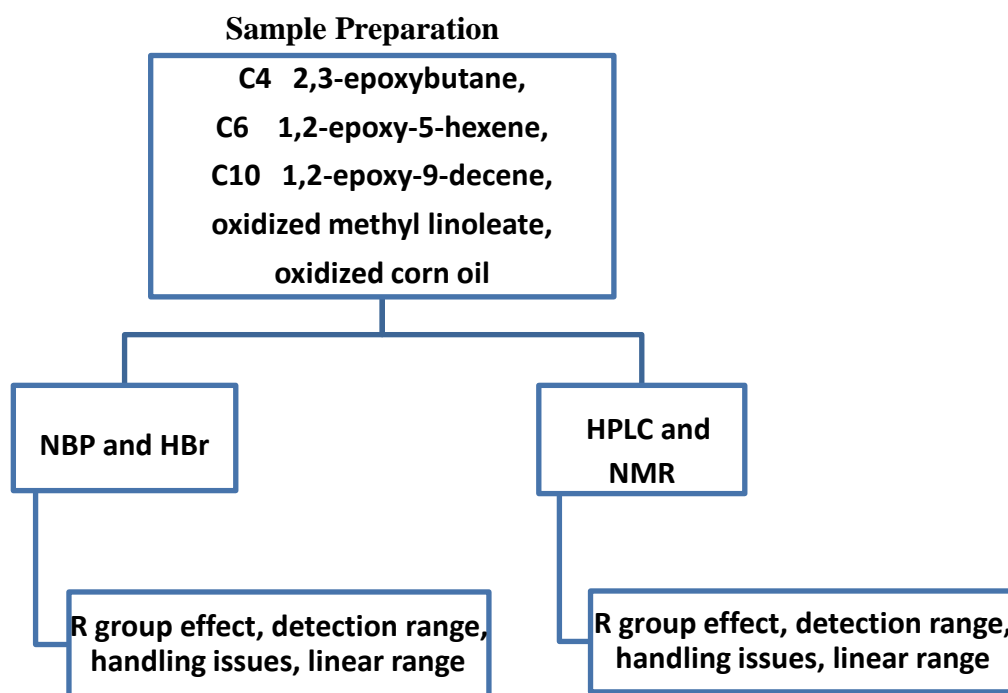
Figure 10. Schematic diagram of the experimental design used to evaluate epoxide

assays.

3.2 General handling issues

Keeping samples stable under experimental conditions was a challenge. Since

lipid oxidation can be initiated by the light and temperature and epoxides degrade

rapidly, all samples are wrapped in aluminum foil and stored frozen under argon until

analyses. Both test samples and epoxide standards were freshly prepared each day.

Only Milli-Q^{TM} purified water (18 MΩ resistivity) was used to prepare reagents.

3.3 HBr (Hydrobromic acid) Assay(AOCS Cd9-57) Methods and Materials

1. Apparatus/Instrumentation/Equipment

   A. Argon source - pre-purified compressed Ar, AirGas, Inc. (East Brunswick, NJ)

   B. Volumetric burettes

   C. Erlenmeyer flasks – 25 ml, 50 ml

   D. Analytical balance – M-310 (Denver Instruments, Bohemia, NY)

   E. Stir plate and mini stir bar

   F. Micro-pipettes – 1000 μl, 200 μl, 10 ul

2. Chemicals/Solvents

   A. Hydrogen bromide solution - 33 wt % in acetic acid (Sigma-Aldrich, St. Louis)

   B. Acetic acid - ≥ 99.7% ACS reagent, (Sigma-Aldrich, St. Louis, MO)

   C. Potassium hydrogen phthalate (KHP) – 99% (MP Biomedical, Solon, OH)

   D. 2,3-epoxybutane – >99% (MP Biomedical, Solon, OH)

E.   1,2-epoxy-5-hexene – 97% (Sigma-Aldrich, St. Louis, MO)

F.   1,2-epoxy-9-decene – 96% (Sigma-Aldrich, St. Louis, MO)

G. Methyl linoleate – >99% (NU-Chek Prep, Inc., Elysian, MN)

H. Corn oil – supplied by Libra Laboratories, Metuchen, NJ

I.   18 MΩ resistivity pure water –, Milli-Q™ Water Purification System (Millipore,

     Billerica, MA)

3.   Solutions/Reagents

A. 0.1 N HBr solution: Add 1 mL HBr (33% w/v) to 39 mL glacial acetic acid.

B. KHP primary standard solution (for standardizing the concentration of HBr

     solution):

   Dry KHP 1-2 hrs at 110 °C; cool. Accurately weigh 0.4 ± 0.0001 g KHP and mix it

     with 10 mL glacial acetic acid and warm gently to dissolve. Cool to room

     temperature.

D. Crystal violet indicator: Dissolve 0.1 g crystal violet in 100 mL glacial acetic acid.

E. Sparge all reagents with argon.

F. Working solutions for epoxides for preparing reaction curves: Add 183 μl

     1,2-epoxy-9-decene standard with 10mL acetic acid to make 0.1M solution in test

     tub and then dilute stock solutions 1:10 serially to prepare standard solutions ranging

     in concentration from 10E-1 to 10E-4 epoxydecene.

Concentration	C10 1,2-epoxy-9-decene gradient standard solutions
10^{-1}M	183 ul, C10 1,2-epoxy-9-decene in 10ml acetic acid
10^{-2}M	1ml 10^{-1}M + 9ml acetone
10^{-3}M	1ml 10^{-2}M + 9ml acetone
10^{-4}M	1ml 10^{-3}M + 9ml acetone

G. Add 113 μl 1,2-epoxide-5-hexene in 10mL acetic acid to make 0.1M solution.

and then dilute stock solutions 1:10 serially to prepare standard solutions ranging

in concentration from 10E-1 to 10E-4 epoxyhexene.

Concentration	C6 1,2-epoxy-6-dexene gradient standard solutions
10^{-1}M	113 ul, C6 1,2-epoxy-6-dexene in 10ml acetic acid
10^{-2}M	1ml 10^{-1}M + 9ml acetone
10^{-3}M	1ml 10^{-2}M + 9ml acetone
10^{-4}M	1ml 10^{-3}M + 9ml acetone

H. Add 87μl of 2,3-epoxybutane in 10mL acetic acid to make 0.1M solution and

then dilute stock solutions 1:10 serially to prepare standard solutions ranging

I. in concentration from 10E-1 to 10E-4 epoxybutane.

Concentration	C4 2,3-epoxybutane gradient standard solutions
10^{-1}M	87ul, C4 2,3-epoxybutane in 10ml acetic acid
10^{-2}M	1ml 10^{-1}M + 9ml acetone
10^{-3}M	1ml 10^{-2}M + 9ml acetone
10^{-4}M	1ml 10^{-3}M + 9ml acetone

J. Methyl linoleate and corn oil oxidation: 40°C for 3 days with shaking in an

incubator.

K. Methyl linoleate solutions:

Add 331 ul oxidized methyl linoleate to 10 mL acetic acid (Stock Solution).

Dilute stock solutions 1:10 serially to prepare standard solutions ranging in

concentration from 10E-1 to 10E-4 methyl linoleate.

Concentration	methyl linoleate gradient standard solutions
10^{-1}M	331ul, methyl linoletae in 10ml acetic acid
10^{-2}M	1ml 10^{-1}M + 9ml acetone
10^{-3}M	1ml 10^{-2}M + 9ml acetone
10^{-4}M	1ml 10^{-3}M + 9ml acetone

L. Corn oil solutions:

Add 315 ul oxidized corn oil to 10 mL acetic acid (Stock Solution). Dilute stock

solutions 1:10 serially to prepare standard solutions ranging in concentration

from 10E-1 to 10E-4 corn oil.

Concentration	corn oil gradient standard solutions
10^{-1}M	315ul, corn oil in 10ml acetic acid
10^{-2}M	1ml 10^{-1}M + 9ml acetone
10^{-3}M	1ml 10^{-2}M + 9ml acetone
10^{-4}M	1ml 10^{-3}M + 9ml acetone

M. Serial dilution of hydrobromic acid (HBr)

Concentration	HBr gradient standard solutions
$10^{-1}M$	1ml HBr in 39 ml acetic acid
$10^{-2}M$	5ml $10^{-1}M$ + 45 ml acetic acid
$10^{-3}M$	5ml $10^{-2}M$ + 45 ml acetic acid
$10^{-4}M$	5ml $10^{-3}M$ + 45 ml acetic acid

4. Standardize hydrobromic acid

   Titrate the HBr solution with KHP primary solution using no more than 2 drops

   crystal violet indicator /50 ml acid. The endpoint is determined as the point at which

   a faint purple color persists (all acid has been reacted and pH rises).

   Note: The HBr must be standardized daily, and even more frequently when results

   become erratic.

5. Determine Oxirane Oxygen %

   A. Weigh 0.39~0.41 g diluted epoxide solution into a 25 mL flask with 10 mL

      acetic acid (flush with Ar).

   B. Put the flask on the stir plate and mini stir bar in the flask.

   C. Load the standardized HBr solution in burette (flush with Ar).

   D. Use standardized HBr solution to titrate the epoxide solution with stirring

      (3drops of crystal violet are added as indicator).

E. Titrate until endpoint is reached (when the solution color changes from purple

to blue).

6. Calculations

A. Calculate concentration of HBr solution using the following equation:

$$\text{Normality of HBr} = \frac{\text{mass of potassium acid phthalate, g}}{0.2042 \times \text{mL HBr solution}}$$

B. Determine percentage Oxirane Oxygen:

$$\text{Oxirane oxygen, \%} = \frac{\text{mL HBr to titrate sample} \times N \times 1.60}{\text{mass of sample, g}}$$

Where—

N = normality of HBr solution

3.4 NBP (4-p-nitrobenzyl-pyridine) Assay Methods and Materials

1. Apparatus/Instrumentation/Equipment

A. Glass test tubes

B. Micropipets (1000 µl, 200 µl, 10 ul)

C. Spectrophotometer –Varian Cary 50 Bio UV-visible (Varian, Inc, Palo Alto, CA)

D. UV quartz cuvettes – 3.5mL (Sigma-Aldrich, St. Louis, MO)

E. Volumetric flask (10 ml, 25 ml, 100 ml)

F. Plastic squeeze bottle

G. Analytical balance: M-310 (Denver Instruments, Bohemia, NY)

2. Chemicals/Solvents

J. 4-p-nitrobenzyl-pyridine – $\geq$ 98% (Sigma-Aldrich, St. Louis, MO)

K. 2,3-epoxybutane – >99% (MP Biomedical, Solon, OH)

L. 1,2-epoxy-5-hexene – 97% (Sigma-Aldrich, St. Louis, MO)

M. 1,2-epoxy-9-decene – 96% (Sigma-Aldrich, St. Louis, MO)

N. Methyl Linoleate – >99% (NU-Chek Prep, Inc., Elysian, MN)

O. Corn oil – supplied by Libra Laboratories, Metuchen, NJ

P. Potassium hydrogen phthalate (KHP) – 99% (MP Biomedical, Solon, OH)

Q. Potassium carbonate – certified ACS $\geq$ 98.5% (Fisher Scientific, Pittsburgh, PA)

R. Acetone – Chromasolve$^{®}$, for HPLC, $\geq$99.9% (Sigma-Aldrich, St. Louis, MO)

S. 18 MΩ resistivity pure water –, Milli-Q™ Water Purification System (Millipore, Billerica, MA)

3. Reagents/solutions

A. 5% NBP reagent: 5 g NBP in 10 mL acetone

B. 0.1 N KHP solution: weigh 2.0422 g KHP in 100 mL Milli-Q water (use hot water to dissolve KHP)

C. 1 M K_2CO_3 solution: weigh 8.262 g K_2CO_3 in 50 mL Milli-Q water

D. 0.1 M 1,2-epoxy-9-decene: pipet 183 μl 1,2-epoxy-9-decene with 10 mL acetone in a test tube.

E. Working solutions for epoxide standard curves: Dilute stock solutions 1:10

   serially to prepare standard solutions ranging in concentration from 10E-1 to

   10E-6 epoxydecene.

Concentration	C10 1,2-epoxy-9-decene gradient standard solutions
10^{-1}M	183 ul, C10 standard + 10 ml acetone
10^{-2}M	1 ml 10^{-1}M + 9 ml acetone
10^{-3}M	1 ml 10^{-2}M + 9 ml acetone
10^{-4}M	1 ml 10^{-3}M + 9 ml acetone
10^{-5}M	1 ml 10^{-4}M + 9 ml acetone
10^{-6}M	1 ml 10^{-5}M + 9 ml acetone

F. 0.1 M 1,2-epoxy-5-hexene: pipet 113 μl 1,2-epoxy-5-hexene with 10 mL acetone

   in a test tube. Working solutions for epoxides for preparing reaction curves:

   Dilute stock solutions 1:10 serially to prepare standard solutions ranging in

   concentration from 10E-1 to 10E-6 epoxyhexene.

Concentration	C6 1,2-epoxy-5-hexene gradient standard solutions
10^{-1}M	113ul, C6 standard + 10 ml acetone
10^{-2}M	1ml 10^{-1}M + 9 ml acetone
10^{-3}M	1ml 10^{-2}M + 9 ml acetone
10^{-4}M	1ml 10^{-3}M + 9 ml acetone
10^{-5}M	1ml 10^{-4}M + 9 ml acetone
10^{-6}M	1ml 10^{-5}M + 9 ml acetone

G. 1 M 2,3-epoxybutane: pipet 87 µl 2,3-epoxybutane with 10 mL acetone in a test

tube. Working solutions for epoxides for preparing reaction curves: Dilute stock

solutions 1:10 serially to prepare standard solutions ranging in concentration

from 10E-1 to 10E-6 epoxybutane.

Concentration	C4 2,3-epoxybutane gradient standard solutions
10^{-1}M	87ul, C4 standard + 10 ml acetone
10^{-2}M	1ml 10^{-1}M + 9 ml acetone
10^{-3}M	1ml 10^{-2}M + 9 ml acetone
10^{-4}M	1ml 10^{-3}M + 9 ml acetone
10^{-5}M	1ml 10^{-4}M + 9 ml acetone
10^{-6}M	1ml 10^{-5}M + 9 ml acetone

H. Methyl linoleate and corn oil oxidation: Both ML and corn oil were oxidized at

40 ℃ in an incubator for 3 days oxidation.

I. ML serial dilutions: Dilute stock solutions 1:10 serially to prepare standard

solutions ranging in concentration from 10E-1 to 10E-6 ML as shown below.

Concentration	methyl linoleate gradient standard solutions
10^{-1} M	331 ul methyl linoleate in 10 ml acetone
10^{-2} M	1ml 10^{-1}M ML + 9 ml acetone
10^{-3} M	1ml 10^{-2}M ML+ 9 ml acetone
10^{-4} M	1ml 10^{-3}M ML + 9 ml acetone
10^{-5} M	1ml 10^{-4}M ML + 9 ml acetone
10^{-6} M	1ml 10^{-5}M ML + 9 ml acetone

J. Corn oil serial dilutions: Add 315 ul corn oil to 10 ml acetone. Dilute stock

solutions 1:10 serially to prepare standard solutions ranging in concentration

from 10E-1 to 10E-6 oil.

Concentration	corn oil gradient standard solutions
$10^{-1}M$	315ul corn oil in 10ml acetone
$10^{-2}M$	1ml $10^{-1}M$ + 9ml acetone
$10^{-3}M$	1ml $10^{-2}M$ + 9ml acetone
$10^{-4}M$	1ml $10^{-3}M$ + 9ml acetone
$10^{-5}M$	1ml $10^{-4}M$ + 9ml acetone
$10^{-6}M$	1ml $10^{-5}M$ + 9ml acetone

4. Epoxide assay

A. Add 1 ml epoxide soln to 1 ml acetone in 15 ml test tube (Blank will contain 2 ml

acetone). (Prepare analyses for each concentration dilution.)

B. Add 1 ml 0.1N KHP to 1 ml 5% NBP reagent in a small test tube or Eppendorf tube.

C. Transfer solution (B) quantitatively to solution (A) for each respective sample and

blank, shake or vortex to mix.

D. Heat test tubes in a boiling water bath for 40 minuts for the reaction to occur

E. Cap tubes with marbles to prevent evaporation.

F. Cool tubes to 40~50 °C on ice.

G. Add acetone to bring volume to 9 mL.

H. Shaking and return to ice bath to bring solutions to room temperature.

I. Add 1 ml K_2CO_3, mix well, transfer to optical cuvette, and read the absorbance at

   600 nm immediately. The violet color fades over time.

3.5 HPLC-DETC (N,N-Diethyldithiocarbamate) Assay Materials and Methods

1. Appraratus/Instrumentation/Equipment

   A. HPLC – LC-10AD (Shimadzu Scientific, Columbia, MD)

   B. Autosampler – SIL-10ADvp (Shimadzu Scientific, Columbia, MD)

   C. Detector – SPD-M10Avp (Shimadzu Scientific, Columbia, MD)

   D. Column – Ultra C18, 5um, 150*4mm (Restek Corp, Bellafonte, PA)

   E. HPLC sample bottle – 2 ml

   F. Test tubes – 5 ml

   G. Micro-pipettes – 1000 μl, 200 μl, 10 ul

   H. Analytical balance – M-310 (Denver Instruments, Bohemia, NY)

2. Chemicals/Solvents

   A. Methanol – ≥99.8%, Chromasolv® for LC-MS (Sigma-Aldrich, St. Louis, MO)

   B. Milli-Q™ Water System, 4 cartridge, 18 MΩ resistivity (Millipore, Billerica, MA)

   C. *O*-Phosphoric acid – 85% HPLC grade (Fisher Chemicals, Pittsburgh, PA)

   D. 2,3-epoxybutane – >99% (MP Biomedical, Solon, OH)

E. 1,2-epoxy-5-hexene – 97% (Sigma-Aldrich, St. Louis, MO)

F. 1,2-epoxy-9-decene – 96% (Sigma-Aldrich, St. Louis, MO)

G. Methyl Linoleate – >99% (NU-Chek Prep, Inc., Elysian, MN)

H. Corn oil – supplied by Libra Laboratories, Metuchen, NJ

I. Sodium diethyldithiocarbamate trihydrate – ≥99.0%, ACS reagent

   (Sigma-Aldrich, St. Louis, MO)

3. Reagents/solutions

   A. 0.01 M DETC: 0.0225 g DETC in 10 mL Methanol.

   B. 0.02 M 2,3-epoxybutane: add 17.5 μl 1,2-epoxybutane in 10 mL methanol.

   C. 0.02 M 1,2-epoxy-5-hexene: add 22.6 μl 1,2-epoxy-5-hexene in 10 mL

      methanol.

   D. 0.02 M 1,2-epoxy-9-decene: add 36.6 μl 1,2-epoxy-9-decene in 10 mL methanol.

   E. DETC and epoxide mixture(10^{-4}M): 4 mL, 0.01 M DETC + 20 μl, 0.02M

      epoxide solution in a test tube.

   F. Blank solution: add 4mL, 0.01M DETC in a test tube.

4. Reaction solutions for HPLC analysis

   A. Incubate the DETC and epoxide mixture solution: Incubate solution E in 60℃

      warm water bath for 20 minutes.

   B. Stop the reaction by adding 100 μl phosphoric acid to the mixture.

C. Dilute the DETC epoxide mixture for HPLC analysis:

2 mL DETC-epoxide mixture (10^{-4}M) + 8 mL methanol

D. Serial dilution solution of DETC-epoxide mixture with methanol

Concentration of DETC-epoxide mixture for HPLC	20uM DETC-epoxide mixture added (μl)	Methanol added (μl)
20 μM	1000	0
15 μM	750	250
10 μM	500	500
5 μM	250	750
2.5 μM	125	875

E. Blank solution: 1mL DETC (reagent F)

F. Put 1 mL each DETC-epoxide mixture in vial for HPLC

G. Inject 20 μL of each solution for HPLC analysis

5. Methyl linoleate and corn oil

A. ML and corn oil were oxidized in an incubator under 40℃ for 3 days.

B. Mix 0.112 g DETC and 33 μl methyl linoleate in 10 mL methanol in a test tube to make 0.01 M solution.

C. Mix 0.112 g DETC and 31 μl corn oil in 10 mL methanol in a test tube to make 0.01 M solution.

D. Incubate test tubes in a warm water incubation 60℃ for 20 minutes.

E. Add 200 μl phosphoric acid to the solution to stop the reaction and decompose

   excess DETC.

F. Vortex tubes well.

G. Dilute DETC-epoxide mixtures:

Concentration(M)	DETC-epoxide added	Methanol added
10^{-2} M	-	-
10^{-3} M	1000 μl 10^{-2} M	9000 μl
7.5×10^{-4} M	6000 μl 10^{-3} M	2000 μl
5×10^{-4} M	4000 μl 7.5×10^{-3} M	2000 μl
2.5×10^{-4} M	4000 μl 5×10^{-3} M	4000 μl
10^{-4} M	1000 μl 10^{-3} M	9000 μl

6. Inject 20 μl of each sample into the HPLC for separation and analysis.

7. HPLC instrumental conditions
   Instrument: Shimadzu
   Autosampler: Shimadzu SIL-10AD vp
   Injection volume: 20 μl
   Detector: Shimadzu SPD-M10Avp
   Pump: Shimadzu LC-10ADvp*2
   Column: Restek Ultra C18 (5 μm, 150*4.6 mm)
   Gradient:

M03 Mobile Phase: 2.0 mL/min
A: Acetonitrile
B: H_2O

Time (min)	0	2	10	12	17	19	20 (stop)
ACN (ml/min)	0.80	0.80	1.60	2.00	2.00	0.80	0.80
H_2O (ml/min)	1.20	1.20	0.40	0.00	0.00	1.20	1.20

3.6 NMR (Nuclear Magnetic Resonance) Assay Materials and Methods

1. Appraratus/Instrumentation/Equipment

   A. Test tubes – 5 ml

   B. NMR tubes – standard quartz, 5 mm OD

   C. NMR – Varian 400 MHz (Varian, Inc., Palo Alto, CA)

   D. Micro-pipettes – 1000 μl, 200 μl, 10 ul

2. Chemicals/Solvents

   A. 2,3-epoxybutane – >99% (MP Biomedical, Solon, OH)

   B. 1,2-epoxy-5-hexene – 97% (Sigma-Aldrich, St. Louis, MO)

   C. 1,2-epoxy-9-decene – 96% (Sigma-Aldrich, St. Louis, MO)

   D. Methyl Linoleate – >99% (NU-Chek Prep, Inc., Elysian, MN)

   E. Corn oil – supplied by Libra Laboratories, Metuchen, NJ

   F. Chloroform-d ($CDCl_3$) – Sigma 99.96%D

3. Reagents/solutions

   A. 1 M 1,2-epoxy-9-decene: Add 165 μl epoxide to 0.9 ml $CDCl_3$ in a test tube.

   B. Serial dilutions of C10 1,2-epoxy-9-decene from 1M to 10^{-6}M epoxide:

   Add C10 1,2-epoxy-9-decene 0.1 ml to 0.9 ml $CDCl_3$ and then dilute stock

solutions 1:10 serially to prepare standard solutions ranging in concentration

from 10E-1 to 10E-4.epoxide.

Concentration(M)	1,2-epoxy-9-decene gradient standard solutions
10^{-1}M	0.1 ml 1 M C10 + 0.9 mL $CDCl_3$
10^{-2}M	0.1 ml 10^{-1} M C10 + 0.9 mL $CDCl_3$
10^{-3}M	0.1 ml 10^{-2} M C10 + 0.9 mL $CDCl_3$
10^{-4}M	0.1 ml 10^{-3} M C10 + 0.9 mL $CDCl_3$
10^{-5}M	0.1 ml 10^{-4} M C10 + 0.9 mL $CDCl_3$
10^{-6} M	0.1 ml 10^{-5} M C10 + 0.9 mL $CDCl_3$

C. Methyl linoleate (ML) and Corn oil oxidation: ML and Corn oil were oxidized

at 40℃ with shaking in an incubator for 3 days.

D. Serial dilutions of methyl linoleate: Add 5.96 μl oxidized methyl

linoleate to 0.9 ml $CDCl_3$ then dilute stock solutions 1:10 serially to

prepare standard solutions ranging in concentration from 0.02M to

$2x10^{-6}$M.lipid.

Concentration	methyl linoleate gradient standard solutions
0.02M	5.96μl ML + 0.9ml $CDCl_3$
$2x10^{-3}$M	0.1ml 0.02M + 0.9ml $CDCl_3$
$2x10^{-4}$M	0.1ml $2x10^{-3}$M + 0.9ml $CDCl_3$
$2x10^{-5}$M	0.1ml $2x10^{-4}$M + 0.9ml $CDCl_3$
$2x10^{-6}$M	0.1ml $2x10^{-5}$M + 0.9ml $CDCl_3$

E. Serial dilutions of corn oil: Add 6.38 μl oxidized corn oil to 0.9 ml CDCl$_3$ then

dilute stock solutions 1:10 serially to prepare standard solutions ranging in

concentration from 0.02M to 2x10^{-6}M lipid.

Concentration	corn oil gradient standard solutions
0.02 M	6.38 μl corn oil + 1ml CDCl$_3$
2x10^{-3} M	0.1 ml 0.02 M + 0.9 ml CDCl$_3$
2x10^{-4} M	0.1 ml 2x10^{-3} M + 0.9 ml CDCl$_3$
2x10^{-5} M	0.1 ml 2x10^{-4} M + 0.9 ml CDCl$_3$
2x10^{-6} M	0.1ml 2x10^{-5} M + 0.9 ml CDCl$_3$

4. NMR analysis: Add 0.7 mL of each solution above in a NMR tube for ^{1}H NMR

analyses.

5. NMR running condiction

NMR	Varian VNMRS 400 MHz
Temperature	25 °C
Spin	20 Hz
Solvent	Chloroform-d (CDCl$_3$) – Sigma 99.96%D
Times of NMR scan	^{1}H NMR 8 scans

4 . RESULTS AND DISCUSSION

4.1 HBr (Hydrobromic acid) Assay

HBr titration provides a means of determining how much epoxy oxygen is present in a sample, expressed as the percent (%) oxirane oxygen. Although this assay has been accepted as a standard procedure for several decades, it was the most problematic of all assays we evaluated. This assay required more than average skill in titration and the end point (based on color change from purple-violet to blue-green) was difficult to determine accurately and reproducibly.

Detection range and accuracy. We found the assay was useful only when the epoxide concentrations were high. The detection range of the HBr assay was 0.0075 M to 0.1 M. However, the response was not linear below 10 mM epoxide and the response varied with epoxide structure, increasing with epoxide chain length (Figure 11, Table 4). At 0.1 M concentration, the detected % oxirane oxygen of 1,2-epoxy-9-decene was slightly above the theoretical value of 0.152, 1,2-epoxy-5-hexene was the most accurate, and 2,3-epoxybutane was slightly below the theoretical value. At 0.001 M standard, the experimental result of all 3 epoxide standard tested were about twice the theoretical value. At 0.0001 M epoxide standard, samples overtitrated by about ten times, probably due to the 0.1 M HBr prescribed by the standard assay which was designed for high epoxide concentrations in modified oils. However, at lower concentrations HBr was too volatile and autoxidized readily so titrations were erratic and inaccurate.
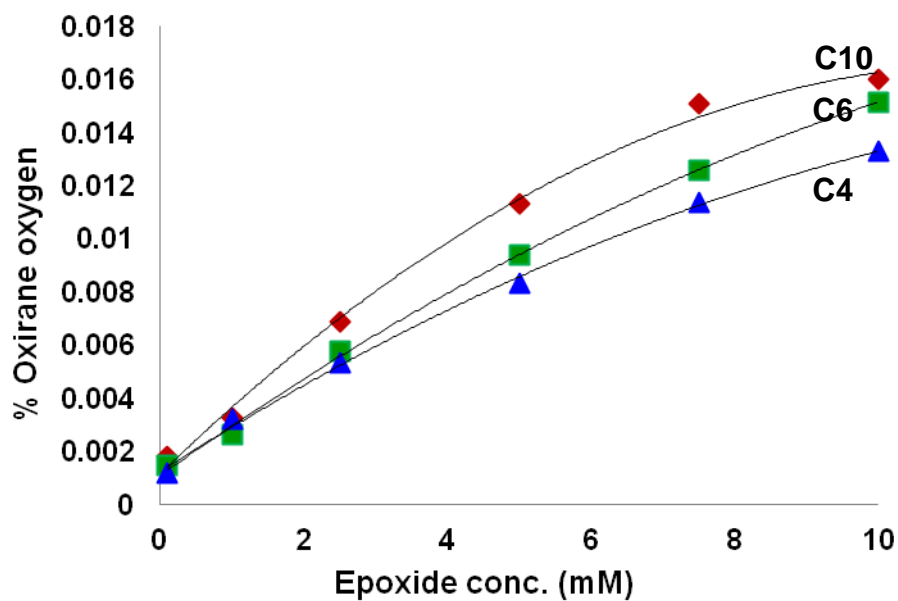
Figure 11. Titration curves for reaction of HBr with epoxybutane, epoxyhexene, and epoxydecene standards.

Table 4. Percent oxirane oxygen detected in different concentrations of epoxydecene, epoxyhexene and epoxybutane standards by HBr titration.

% of Oxirane Oxygen

Conc (M)	Epoxydecene	Epoxyhexene	Epoxybutane	Actual Value
0.1	0.16069	0.15336	0.11335	0.152
0.01	0.01601	0.01514	0.01329	0.0152
0.0075	0.01508	0.01257	0.01135	0.0114
0.005	0.01134	0.00938	0.00832	0.0076
0.0025	0.00687	0.00576	0.00533	0.0038
0.001	0.00326	0.00261	0.00321	0.00152
0.0001	0.00182	0.00146	0.00117	0.000152

Application to epoxides in oxidized lipids. To test suitability of the HBr assay with complex materials such as lipids, the assay was applied to analysis of epoxides in oxidized methyl linoleate and corn oil. Assessment of this application is hampered by not having a primary assay to determine actual spoxide concentrations. However, if this assay is correct, the oils either are barely oxidized or have very low levels (micromolar) of epoxide products present (Table 5).

Table 5. HBr titration detection of epoxides in oxidized corn oil and methyl linoleate.

% of Oxirane Oxygen

Oil Conc. (M)	Corn Oil	C10 equiv.(M)	Me Linoleate	C10 equiv. (M)
0.1	0.0023	0.00033	0.0021	0.0002
0.01	0.0017	0.00009	0.0016	0.00008
0.001	0.0016	0.00008	0.0011	0.00006
0.0001	0.0011	0.00006	0.0025	0.0004

Reproducibility. To test reproducibility of the assay, five replicate analyses of samples were performed on each of three days and within day and between day standard deviations (Tables 6-10) and coefficients of variance were calculated (Table 11). Within day reproducibility for the standards was excellent, 3% or lower variability. Between day variability was slightly higher, but still < 6% for the longer epoxides and <10% for epoxybutane. The high volatility of epoxybutane contributes to its higher

variability. Variability of methyl linoleate was comparable to the standards; corn oil

was slightly higher, reaching 12% between days. Corn oil by its nature is not totally

homogeneous, and it continues to oxidize and degrade even when frozen, so the higher

variability between days is perhaps to be expected.

Table 6. Reproducibility of HBr titration assay for C10 1,2-epoxy-9-decene. Five

replicates per day, three different days.

1,2-epoxy-9-decene		Day 1	Day 2	Day 3	Between days
		Within day % Oxirane O			
0.1M	Ave	0.1595	0.1642	0.1591	0.1610
	Stdev	0.0016	0.0061	0.0027	0.0028
0.01M	Ave	0.0149	0.0182	0.015	0.0160
	Stdev	0.0015	0.0018	4E-05	0.0019
0.0075M	Ave	0.0150	0.0154	0.0149	0.0151
	Stdev	0.0006	0.0002	0.0007	0.0003
0.005M	Ave	0.0114	0.0113	0.0113	0.0113
	Stdev	0.0004	9E-05	8E-05	3.1E-05
0.0025M	Ave	0.0069	0.0069	0.0069	0.0069
	Stdev	0.0001	0.0003	0.0002	2.6E-05
0.001M	Ave	0.0033	0.0032	0.0033	0.0033
	Stdev	0.0002	8E-05	0.0001	6.5E-05
0.0001M	Ave	0.0018	0.0018	0.0019	0.0018
	Stdev	0.0002	9E-05	0.0002	7.0E-05

Table 7. Reproducibility of HBr titration assay for 1,2-epoxy-5-hexene. Five replicates

per day, three different days.

1,2-epoxy-5-hexene		Within day % Oxirane O			
Conc. (M)		Day 1	Day 2	Day 3	Between days
0.1	Ave	0.1534	0.1536	0.1531	0.1534
	Stdev	0.0031	0.002	0.0035	0.0003
0.01	Ave	0.0128	0.0156	0.0171	0.0151
	Stdev	0.0013	0.0017	0.0002	0.0022
0.0075	Ave	0.0130	0.0123	0.0123	0.0126
	Stdev	0.0005	0.0007	0.0007	0.0004
0.005	Ave	0.0094	0.0093	0.0095	0.0094
	Stdev	0.0001	0.0001	0.0002	0.0001
0.0025	Ave	0.0058	0.0058	0.0057	0.0058
	Stdev	0.0001	0.0001	2.00E-05	0.0000
0.001	Ave	0.0023	0.0034	0.0022	0.0026
	Stdev	4.00E-05	3.00E-05	1.00E-05	0.0007
0.0001	Ave	0.0015	0.0015	0.0014	0.0015
	Stdev	1.00E-05	2.00E-05	2.00E-05	0.0000

Table 8. Reproducibility of HBr titration assay for C4 2,3-epoxbutane.

Five replicates per day, three different days.

2,3-Epoxybutane Conc. (M)		Within day % oxirane O			Between days
		Day 1	Day 2	Day 3	
0.1	Ave	0.1217	0.0943	0.1241	0.11335
	Stdev	0.0086	0.0481	0.0016	0.01654
0.01	Ave	0.0131	0.0134	0.0134	0.01329
	Stdev	0.0009	0.0002	6.00E-05	0.00016
0.0075	Ave	0.0112	0.0116	0.0113	0.0135
	Stdev	0.0002	2.00E-05	2.00E-05	0.00018
0.005	Ave	0.0083	0.0084	0.0083	0.00832
	Stdev	0.0001	0.0002	0.0002	4.03E-05
0.0025	Ave	0.0054	0.0053	0.0053	0.00533
	Stdev	0.0001	8.00E-05	3.00E-05	2.63E-05
0.001	Ave	0.0047	0.0027	0.0022	0.00321
	Stdev	0.0008	3.00E-05	2.00E-05	0.00135
0.0001	Ave	0.0011	0.0012	0.0012	0.00117
	Stdev	4.00E-05	0.0001	9.00E-06	4.23E-05

Table 9. Reproducibility of HBr titration assay for oxidized methyl linoleate.

Five replicates per day, three different days.

ML Conc. (M)		Within day % Oxirane O			
		Day 1	Day 2	Day 3	Between days
0.1	Ave	0.0018	0.0024	0.0021	0.0021
	Stdev	9.00E-06	9.00E-06	9.00E-05	0.0003
0.01	Ave	0.0018	0.0014	0.0016	0.0016
	Stdev	2.00E-05	0.0052	0.0001	0.0002
0.001	Ave	0.0011	0.0011	0.0011	0.0011
	Stdev	7.00E-06	4.00E-05	5.00E-05	0.0000
0.0001	Ave	0.0024	0.0026	0.0024	0.0025
	Stdev	1.00E-04	0.0002	0.0001	0.0001

Table 10. Reproducibility of HBr titration assay for oxidized corn oil. Five replicates per day, three different days.

Corn Oil Conc. (M)		Within day % Oxirane O			
		Day 1	Day 2	Day 3	Between days
0.1	Ave	0.0021	0.0023	0.0024	0.0023
	Stdev	5.00E-05	0.0002	4.00E-05	0.0001
0.01	Ave	0.0017	0.0017	0.0018	0.0017
	Stdev	0.0001	1.00E-05	3.00E-05	0.0001
0.001	Ave	0.0013	0.0013	0.0022	0.0016
	Stdev	4.00E-06	5.00E-05	0.0018	0.0005
0.0001	Ave	0.001	0.0011	0.0012	0.0011
	Stdev	7.00E-06	9.00E-05	5.00E-05	0.0001

Table 11. Coefficients of variation for HBr titration of standard epoxides.

	Coefficient of Variation (%)	
	Within day	Between Days
C4	3.38	9.17
C6	1.87	6.37
C10	2.13	3.10
Corn oil	5.64	12.93
Me Linoleate	3.89	7.93

Handling and Precautions. A number of factors contribute to increasing inaccuracy of the HBR assay in detecting lower concentrations of epoxides:

(1) **Innate instability and reactivity of hydrobromic acid (HBr).** Typically, acid with low pKa values dissociate more readily and thus donate H^+ to the surroundings. Acids with pKa values lower than -2 are considered strong acids. Hydrobromic acid has a pKa = -9 which means it is a very strong acid and is nearly totally dissociated under most conditions. The free bromine and H^+ ions then evaporate readily from the very beginning of the titration, contributing to variability and to apparent over-titrations because not all the hydrobromic acid leaving the burette reaches the epoxide. Volatility increases as the chain length decreases. Lower volatility thus may partially contribute to the apparent increased reactivity with HBr as chain length of the epoxide increases.

Also, HBr oxidizes readily, so less actual HBr is present in the titrant delivered to

the epoxide. As a consequence, oxirane values determined in air are consistently lower than when the solutions and headspace are sparged with argon (data not shown). To reduce variability and improve accuracy, we thus routinely use argon to flush the reagents, solutions, and flasks headspace before and during titration.

Finally, HBr reaction is not specific for epoxides; it also reacts with other substances. This is not a problem when pure compounds are being analyzed, but may be a significant limitation when titrating oxidized lipids that potentially contain a wide variety of oxidation products. HBr reacts with β –unsaturated ketones, cyclopropenes, conjugated dienols, soaps, and other oxygenated secondary products of lipid oxidation. Alternate targets compete with epoxides for HBr and may react in addition to epoxides, so whether the net effect is aynergistic or antagonistic is not clear. At any rate, results in complex systems (as opposed to pure materials) are questionable.

2) **Poor definition in endpoint.** The color does not change completely and instantly at the endpoint of HBR titration of epoxides. The indicator, crystal violet, has a purple violet color. As hydrobromic acid adds to the epoxide during titration, the color gradually fades and turns into blue. It is not easy to visually determine the exact point at which the color changes. In addition, the perception of color change is subjective and varies tremendously among individuals.

4.2 NBP (4-p-nitrobenzyl-pyridine) Assay

Procedures in original references for this method (Hammock et al. 1974, Agarwal et al. 1979) were mostly qualitative so required some adaptation to make them quantitative. The first problem encountered was determining the optimum heating time for the reaction. A range of heating times was tested, from 20 to 50 minutes (Figure 12). 40 minutes provided the most consistent and linear results so was selected as the standard time for all tests reported here.

Effect of epoxide structure on reaction response. The second problem was quantitation. Clearly, a standard curve constructed from the epoxide of interest can provide a basis for quantitation. However, as shown in Figures 12 and 13, the NBP reaction response varies markedly with epoxide structure, so an authentic epoxide standard is required for every epoxide analyzed. For lipids, such standards are very difficult to obtain commercially, and multiple epoxides are generated during lipid oxidation and they change with reaction conditions, so even determining which epoxides should be synthesized as standards is complicated. Thus, the assay can report relative concentrations and demonstrate changes over time, but it cannot provide the absolute quantitation of epoxides that is necessary for mass balance and comparison of alternate reactions in lipid oxidation. One option is to use epoxydecene or any longer epoxide that is commercially available as a standard and then report epoxide
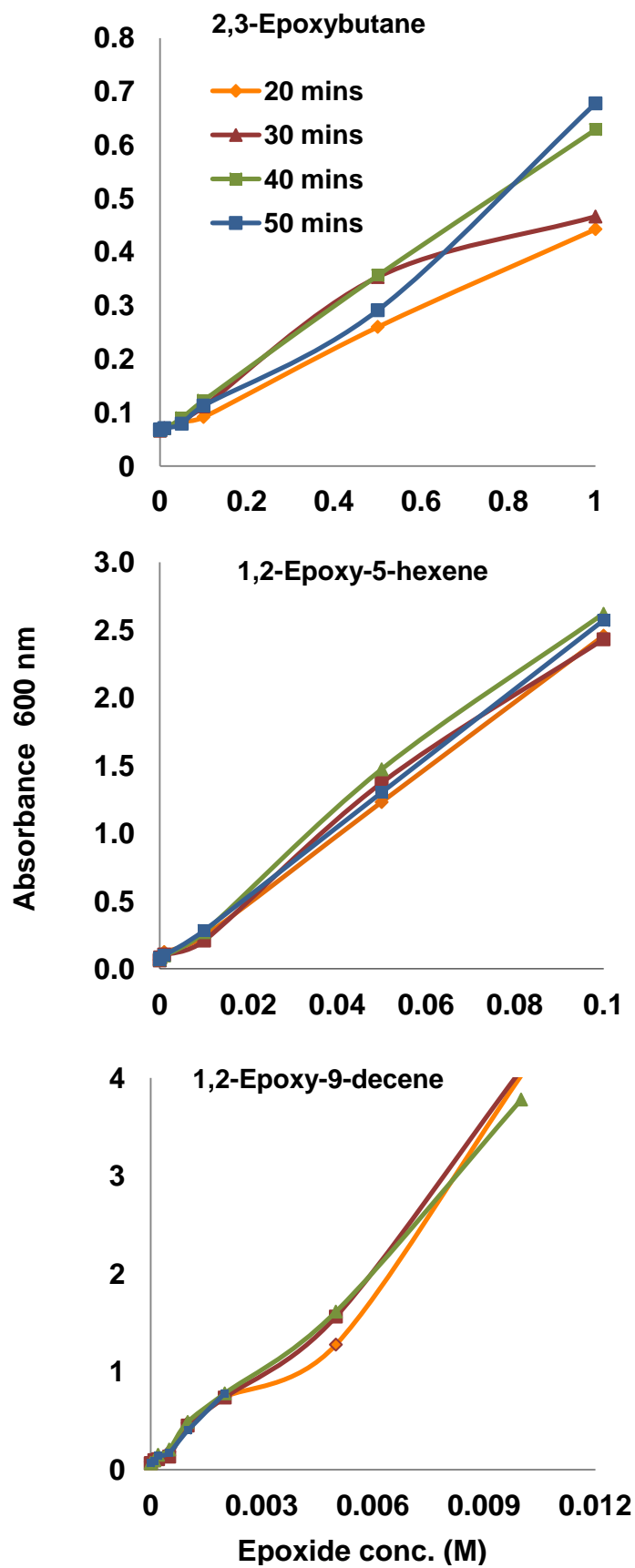
Figure 12. Effects of heating time on NBP reaction response of three standard epoxides.
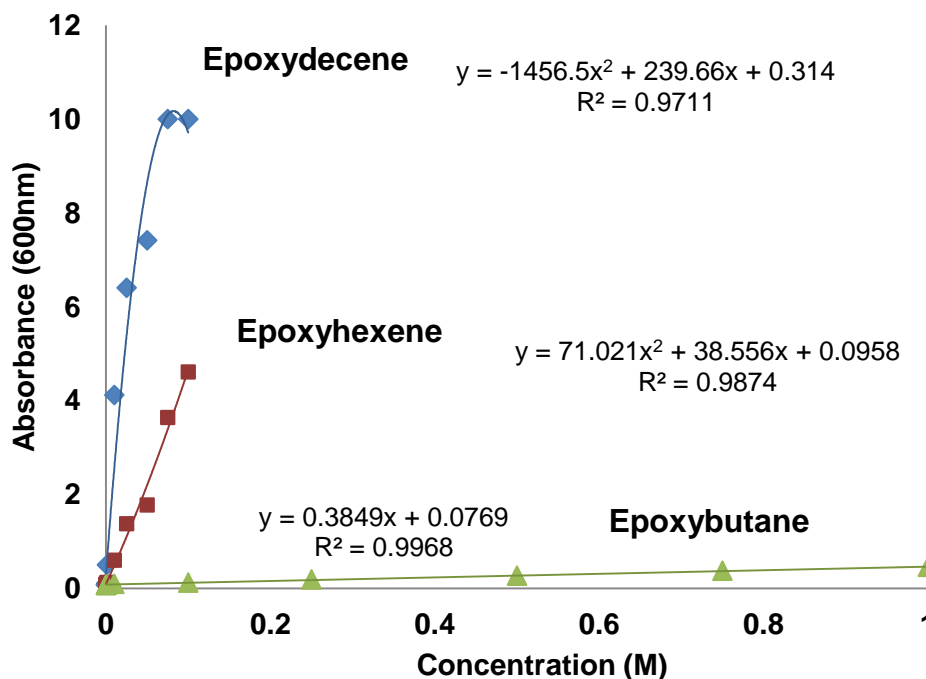
Figure 13. NBP reaction response curves for epoxybutane, epoxyhexene, and

epoxydecene standards. (40 minutes standard reaction time).

concentrations as equivalents of the standard used.

Figures 12 and 13 show clearly that epoxides with different chain lengths (or R-

groups), NBP reaction response can vary by orders of magnitude. 1,2-epoxy-9-decene

had the greatest response by far, reaching the detection limit of the spectrophotometer

at a concentration of 0.1M. Epoxyhexene also exceeded useful detection limits at 0.1 M

but the absorbance levels were lower. In contrast, epoxybutane did not reach optical

limts even at 1 M concentrations.

The strong effects of epoxide R group on reaction with NBP can create significant

problems in complex materials with unknown and potentially multiple epoxide

structures. Some of this difference may be attributed to high volatility in the shorter

chain epoxides. Epoxybutane is particularly volatile and thus difficult to handle and

prepare samples reproducibly. Another explanation for the more rapid and complete

reaction of long chain epoxides with NBP may arise from inductive effects of the side

chains on charge distribution in the epoxy ring. During the reactions, the acyl chains (R

groups) next to epoxy ring can act as electron donating groups that increase electron

density on the oxirane ring. This creates a polar gradient in the epoxy ring, with a partial

negative charge on the oxirane oxygen and a partial positive charge on one oxirane

carbon. Since larger molecule epoxide C10 1,2-epoxy-9-decene has a longer acyl chain

which will push orbital electrons much harder to the oxirane oxygen result in a more

partial negative charge on oxirane oxygen and a more partial positive charge on oxirane

carbon. At the same time, the NBP reagent possesses an extra lone pair of electrons

which are attracted by the positive charge of oxirane carbon, as shown below:

Because longer chains are more strongly electron withdrawing, the oxirane carbon is
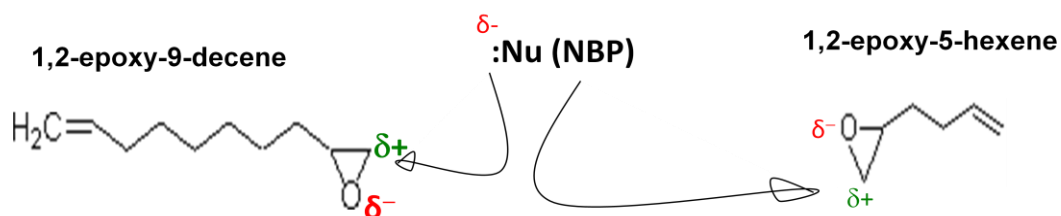
more strongly positive, and NBP thus reacts with these more readily. Hence, reactivity

increases in the order C4 < C6 < C10 epoxides.

Detection limits for assay. Useful detection ranges for the three standard

epoxides are presented in Table 12. Epoxybutane can be detected over the range 10 μM

to 1 M, but the response is thrd order polynomial, not linear, indicatint that

concentration is not the only factor affecting reactivity. C6 and C10 epoxides can both

be detected as low as 1 μM, but different upper limits – 100 mM for C6 and 50 mM for

C10 where the absorbance levels generated exceed the spectrophotometer limits.

The Cary 50 spectrophotometer used in this study uses a xenon lamp and has a quoted

optical limit of 10, although results above 3 are increasingly noisy. However, for most

spectrophotometers with monochomators and deuterium and hydrogen lamps, the

optical limit for accurate reading is an absorbance of 1. Thus, for most laboratories the

upper detection limits for the NBP assay are substantially lower than those reported

here.

Table 12. Active detection ranges for NBP assay.

	Low detection limt	High detection limit
C4	10 uM	1 M
C6	1 uM	0.1 M
C10	1 uM	0.05 M
ML	1 uM (oil)	1 M (oil)
Corn oil	1 uM (oil)	1 M (oil)

Linearity of reaction response. Advantages of increased reactivity at longer

chain lengths are counterbalanced by decreasing linearity of response. The reaction

response was low. Below 1 μM there was no detectable reaction. Response was linear over the range of 1 μM to 1 M for epoxybutane, but became increasingly non-linear (polynomial) as the R chain length increased. Responses for epoxyhexene were linear only at low concentrations, between 10^{-5}M and 10^{-6}M, but epoxydecene response remained polynomial even at the lowest concentrations (Figure 14). This suggests that there other factors such as steric accessibility or competing side reactions controlling the reactivity, in addition to inductive effects. In the same low concentration range, epoxybutane response was increasingly variable, reflecting the handling and volatility problems already noted.

When test concentrations span several orders of magnitude (0.1M to 10^{-6}M), semilog plots are sometimes more revealing about reaction patterns than standard concentration plots. Semilog plots of the low epoxide concentration range (10^{-5}M to 10^{-6}M) showed a linear relationship between log [epoxide] and absorbance (reaction response) for epoxydecene, but third order and second order quadratic relationships for epoxyhexene and epoxybutane, respectively (Figure 15). Hence, reactions of the three epoxides are controlled by different factors. Epoxydecene has a power (log) relationship with concentration, epoxydecene has a direct concentration relationship, and epoxybutane has other factors controlling reaction at low concentrations.
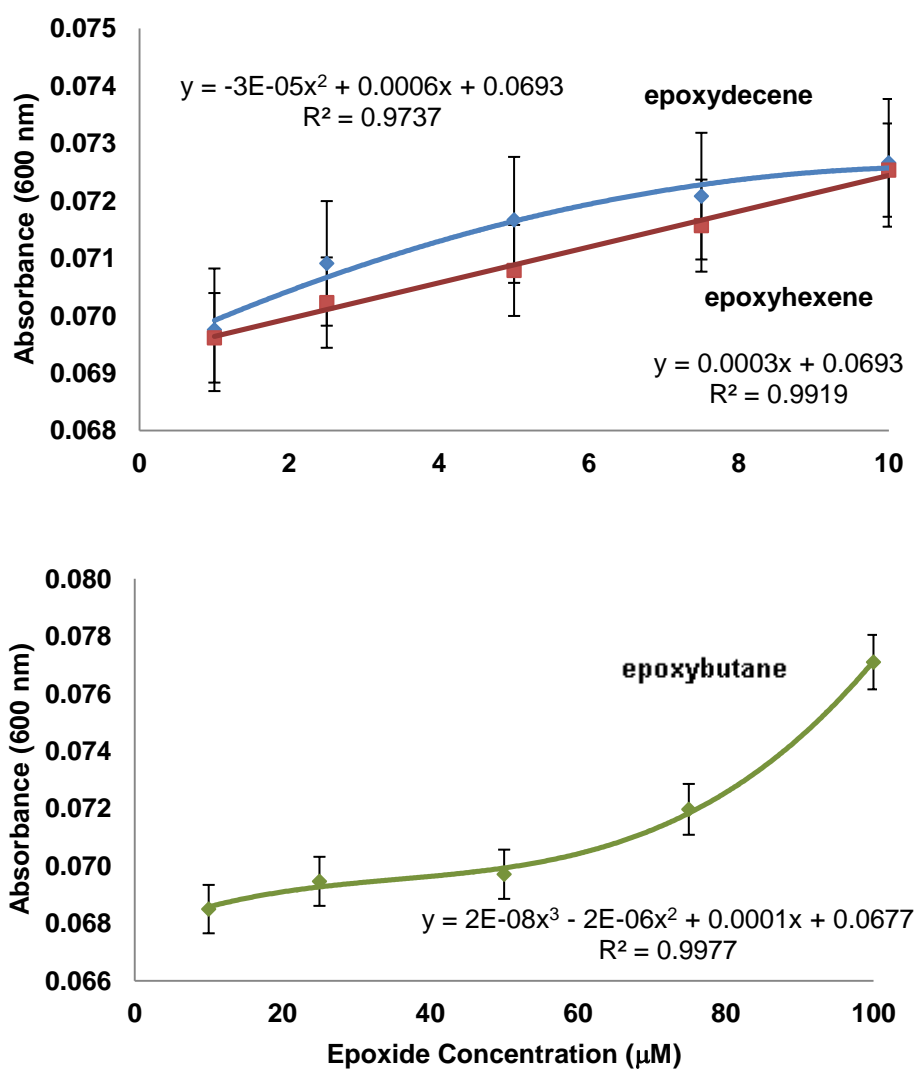
Figure 14. NBP reaction response at very low concentrations of epoxyhexene and

epoxydecene standards (top) and epoxybutane (bottom).
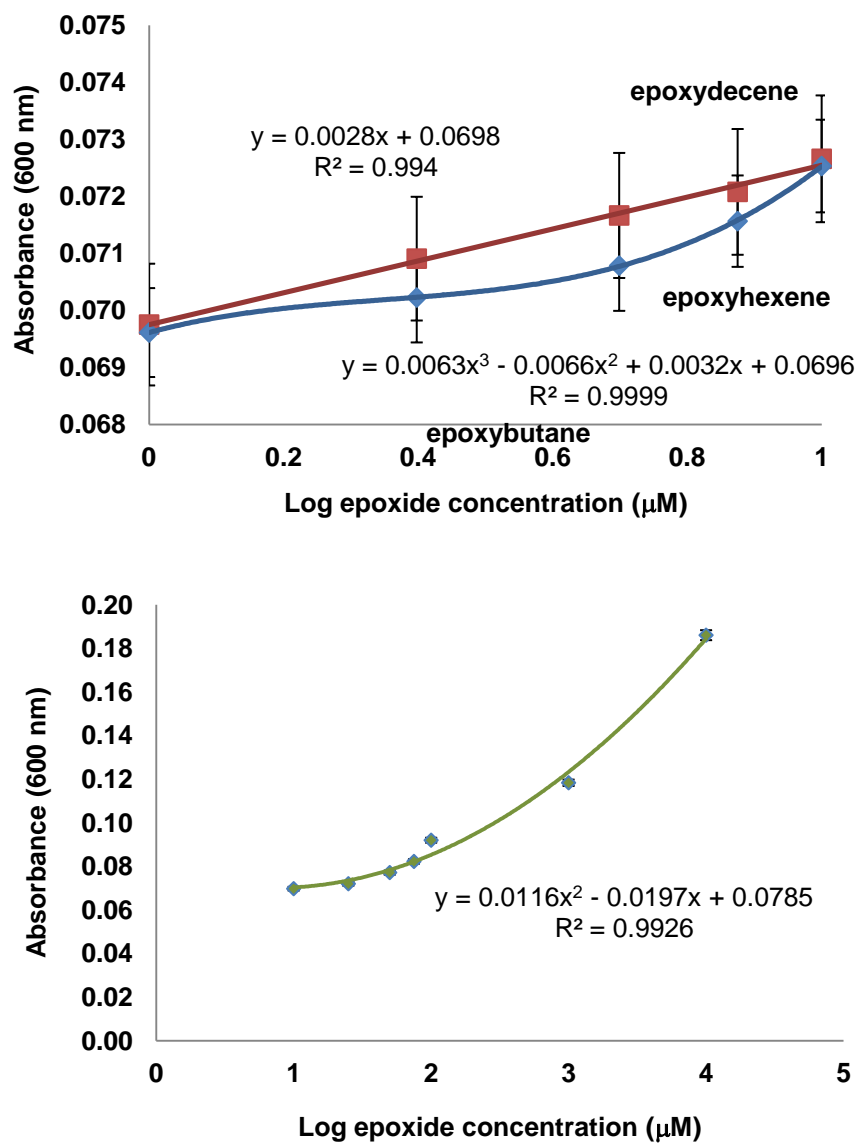
Figure 15. Log epoxide concentration vs absorbance (reaction response) for NBP assay.

Top: epoxydecene and epoxyhexene. Bottom: epoxybutane.

Application to epoxides in oxidized lipids. Similar results were found in methyl

linoleate and corn oil oxidized at 40 °C for 3 days (Figure 16). The concentration range

between 1M and 10^{-6}M oil was examined. Very little epoxide was detected in methyl

linoleate, but substantially more was detected in corn oil. The absorbance in corn oil

suggests a concentration of about 1 mM epoxydecene equivalents, while methyl

linoleate is less than 0.5 mM epoxydecene equivalents. These values are a bit higher

than detected by the HBR assay (0.33 and 0.20 mM for corn oil and ML, respectively)

consistent with the greater sensitivity of this assay. As with the HBr assay, reactions

were biphasic with epoxide concentration, possibly reflecting steric accessibility

limitations at higher epoxide/oil concentrations.

To examine the concentration relationships more closely, semilog plots of the

reaction responses were plotted (Figure 17). Rather than simplifying the relationships

to linear, these plots increased the complexity to third order. Confirming that factors

other than oil concentration are contributing to the reaction response. These factors may

be physical, such as increased viscosity slowing diffusion of the NBP to lipid sites,

steric hindrance by the long acy chains or triacylglycerols structure, or alterations in

NBP solubility. Or the factors may be chemical, related to the structure and functional

groups present in the lipids. More research will be needed to elucidate the reasons for

this variable reactivity..
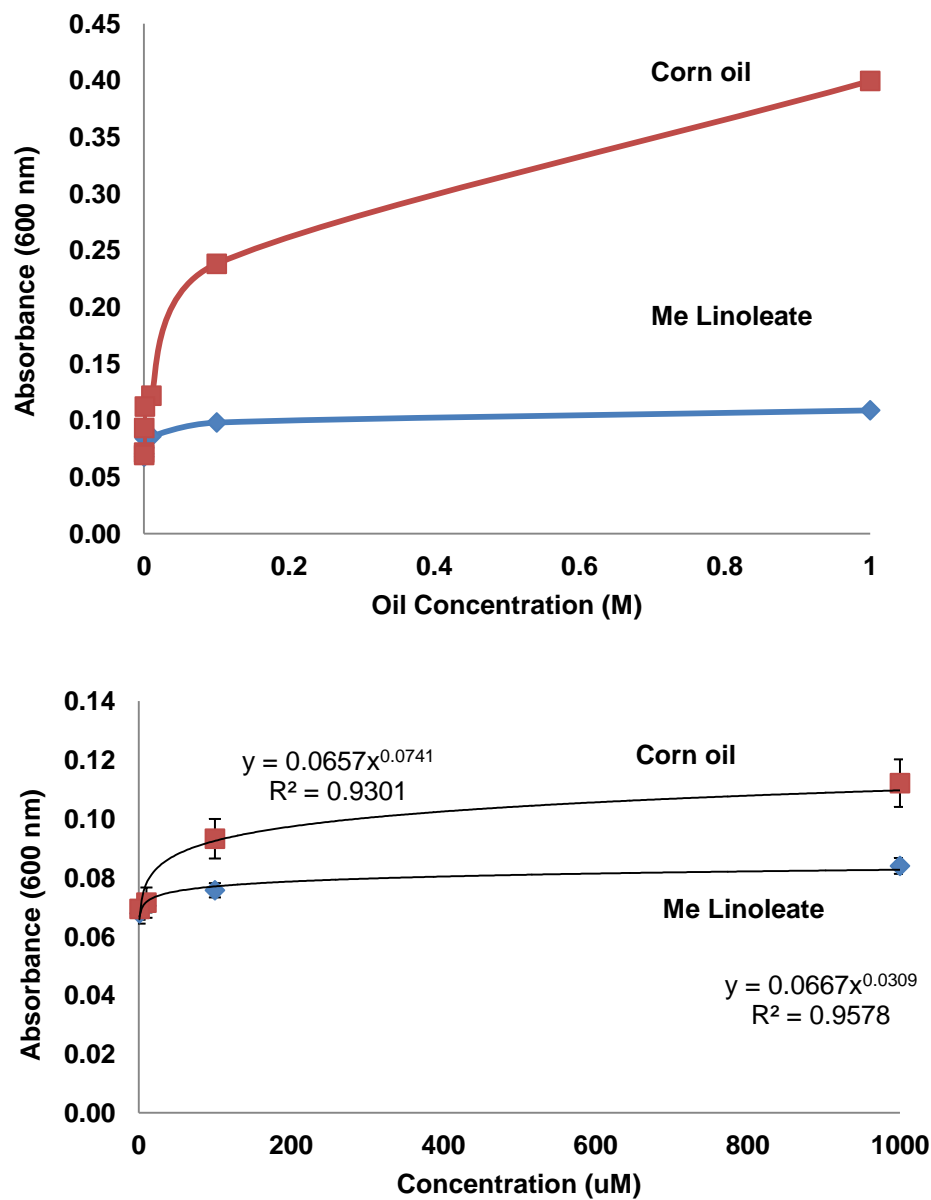
Figure 16. NBP response curves for oxidized methyl linoleate and corn oil. Top: Full

concentration range tested. Bottom: lower concentration range.
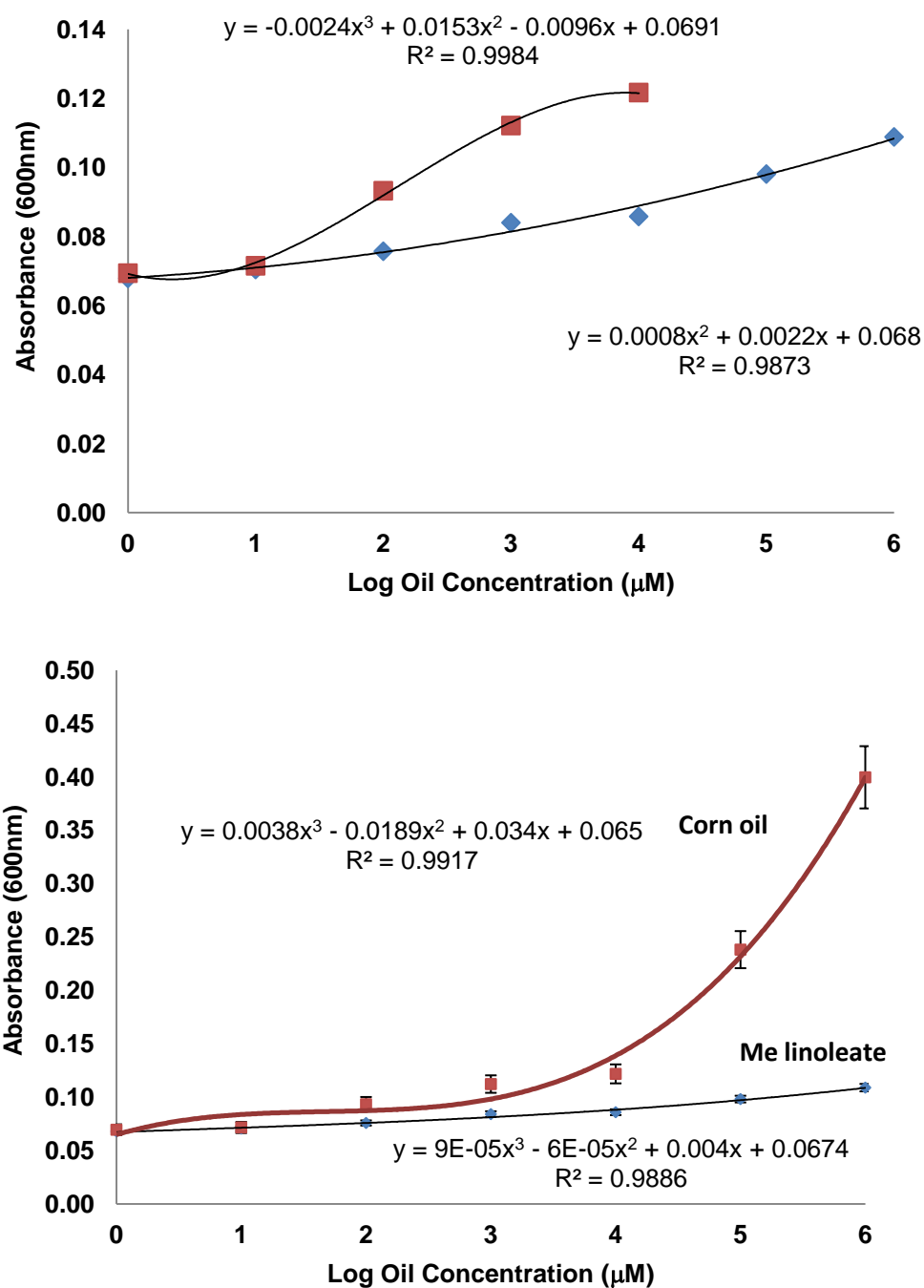
Figure 17. Log ML and corn oil concentration vs absorbance (reaction response) for

NBP assay. Top: full concentration range tested. Bottom: Lower concentration range

(<6 μM).

Reproducibility of assay. Five replicates of all samples were analyzed on each of

three days to test reproducibility of the assay. Coefficients of variation for the assay are

presented in Table 13. Results for epoxide standards are presented in Tables 14-16;

results for methyl linoleate and corn oil are in Tables 17 and 18. Reproducibility of the

assay with standard epoxides was exceptionally good -- 1-2% within days and 2-3%

between days. Variability with methyl linoleate was only slightly higher, 3-4% within

and between days, respectively. Variability of the assay with corn oil was significantly

higher – 7% within day and almost 12% between days. This probably reflected the

innate inhomogeneity of the material, or possibly sampling position, rather than

progressive oxidation since the epoxides di not increase each successive day. This is

still good reproducibility, but with some work on standardizing sampling methods the

variability could be reduced to less than 5% within days and 10% between days.

Table 13. Coefficients of variation for the NBP detection of epoxides in standards and
oxidized lipids. Averaged over all concentrations and days

	average COV(%)	
	Within day	Between days
C10	1.53	2.73
C6	1.12	1.87
C4	1.23	2.67
Corn oil	7.32	11.84
ML	3.21	4.17

Table 24. Reproducibility of NBP assay for 1,2-epoxy-9-decene standard. Five replicates per day, three different days.

1,2-epoxy-9-decene Conc. (M)		Within day (A 600 nm) Day1	Day2	Day3	Between Days
0.1	Ave	10	10	10	10
	Stdev	0	0	0	0
0.075	Ave	10	10	10	10
	Stdev	0	0	0	0
0.05	Ave	7.875	7.7145	6.657	7.4154
	Stdev	2.91	3.1365	3.055	0.6619
0.025	Ave	6.443	6.3835	6.384	6.4035
	Stdev	3.248	3.3014	3.301	0.0341
0.01	Ave	4.046	4.2131	4.095	4.1179
	Stdev	0.058	0.0829	0.054	0.0860
0.001	Ave	0.483	0.5103	0.497	0.4967
	Stdev	0.005	0.0114	0.002	0.0137
0.0001	Ave	0.102	0.1087	0.096	0.1021
	Stdev	0.008	0.0007	0.004	0.0066
0.00001	Ave	0.072	0.0734	0.073	0.0727
	Stdev	4.00E-04	0.0011	6.00E-04	0.0008
7.5E-06	Ave	0.072	0.0733	0.071	0.0721
	Stdev	7.00E-04	0.0007	5.00E-05	0.0011
5E-06	Ave	0.071	0.0722	0.072	0.0717
	Stdev	6.00E-04	0.0008	4.00E-04	0.0005
2.5E-06	Ave	0.071	0.0718	0.07	0.0709
	Stdev	7.00E-04	0.0003	0.001	0.0008
1E-06	Ave	0.068	0.0714	0.069	0.0698
	Stdev	0.003	0.0002	2.00E-04	0.0015

Table 15. Reproducibility of NBP assay for 1,2-epoxy-5-hexene standard. Five

replicates per day, three different days.

1,2-epoxy-5-hexene		Within day A 600 nm			
Conc. (M)		Day 1	Day 2	Day 3	Between Days
0.1	Ave	4.643	4.6834	4.502	4.6092
	Stdev	0.072	0.1792	0.003	0.0955
0.075	Ave	3.661	3.5916	3.663	3.6385
	Stdev	0.087	0.0352	0.008	0.0407
0.05	Ave	1.801	1.7546	1.763	1.7729
	Stdev	0.015	0.0002	0.004	0.0248
0.025	Ave	1.371	1.375	1.372	1.3729
	Stdev	0.006	0.0004	4.00E-04	0.0019
0.01	Ave	0.594	0.604	0.588	0.5953
	Stdev	0.003	0.013	3.00E-04	0.0081
0.001	Ave	0.13	0.1156	0.12	0.1219
	Stdev	6.00E-04	0.002	3.00E-04	0.0076
0.0001	Ave	0.096	0.0909	0.085	0.0907
	Stdev	5.00E-04	0.0019	6.00E-04	0.0055
0.00001	Ave	0.072	0.0729	0.072	0.0725
	Stdev	2.00E-04	0.0008	3.00E-04	0.0004
7.5E-06	Ave	0.071	0.072	0.072	0.0716
	Stdev	7.00E-04	0.0009	5.00E-04	0.0004
5E-06	Ave	0.069	0.0716	0.071	0.0708
	Stdev	8.00E-05	0.0003	4.00E-04	0.0013
2.5E-06	Ave	0.069	0.071	0.07	0.0702
	Stdev	2.00E-04	0.0002	7.00E-04	0.0008
1E-06	Ave	0.069	0.07	0.07	0.0696
	Stdev	1.00E-04	0.0002	4.00E-04	0.0005

Table 16. Reproducibility of NBP assay for 2,3-epoxybutane standard. Five replicates

per day, three different days.

2,3-epoxybutane Conc. (M)		Within day A 600 nm Day1	Day2	Day3	Between Days
0.1	Ave	0.448	0.4502	0.466	0.455
	Stdev	0.018	0.0261	0.005	0.0098
0.075	Ave	0.372	0.3767	0.368	0.3721
	Stdev	5.00E-04	0.0003	0.003	0.0045
0.05	Ave	0.263	0.2729	0.262	0.266
	Stdev	0.003	0.0014	4.00E-04	0.0060
0.025	Ave	0.193	0.1951	0.17	0.186
	Stdev	0.002	0.0015	0.046	0.0143
0.01	Ave	0.112	0.1223	0.121	0.1183
	Stdev	8.00E-04	0.0002	0.001	0.0056
0.001	Ave	0.089	0.0927	0.094	0.0919
	Stdev	0.002	0.0008	4.00E-04	0.0030
0.0001	Ave	0.077	0.0828	0.087	0.0822
	Stdev	0.001	0.0003	7.00E-04	0.0048
0.00001	Ave	0.075	0.0774	0.079	0.0771
	Stdev	3.00E-04	0.0006	4.00E-04	0.0028
7.5E-06	Ave	0.071	0.072	0.072	0.0718
	Stdev	6.00E-04	0.0003	6.00E-04	0.0004
5E-06	Ave	0.069	0.0698	0.07	0.0697
	Stdev	1.00E-04	0.0009	5.00E-04	0.0002
2.5E-06	Ave	0.069	0.0693	0.07	0.0695
	Stdev	1.00E-04	0.0004	6.00E-04	0.0003
1E-06	Ave	0.069	0.07	0.069	0.0693
	Stdev	2.00E-04	0.0011	4.00E-04	0.0007

Table 17. Reproducibility of NBP assay for oxidized methyl linoleate. Five replicates per day, three different days.

Methyl linoleate Conc. (M)		Within day A 600 nm Day1	Day2	Day3	Between Days
1	Ave	0.097	0.1103	0.119	0.1088
	Stdev	0.003	0.0008	0.0007	0.0112
0.1	Ave	0.094	0.0988	0.102	0.0981
	Stdev	0.0001	0.0005	0.0004	0.0038
0.01	Ave	0.087	0.0825	0.088	0.0858
	Stdev	0.002	0.00008	0.0004	0.0030
0.001	Ave	0.085	0.0811	0.086	0.084
	Stdev	0.00007	0.0011	0.0003	0.0025
0.0001	Ave	0.072	0.0757	0.079	0.0757
	Stdev	0.0003	0.00005	0.0004	0.0037
0.00001	Ave	0.07	0.0699	0.072	0.0704
	Stdev	0.0007	0.0004	0.0004	0.0012
1E-06	Ave	0.067	0.0674	0.069	0.0679
	Stdev	0.0006	0.0005	0.0001	0.0014

Table 18. Reproducibility of NBP assay for oxidized corn oil. Five replicates per day, three different days.

Corn Oil Conc. (M)		Within day Day1	Day2	Day3	Between Days
1	Ave	0.344	0.4765	0.378	0.3995
	Stdev	0.003	0.0146	1.00E-04	0.0689
0.1	Ave	0.238	0.2323	0.244	0.2381
	Stdev	0.032	0.0144	0.002	0.0061
0.01	Ave	0.133	0.0933	0.139	0.1217
	Stdev	0.008	0.0002	3.00E-04	0.0248
0.001	Ave	0.126	0.0898	0.121	0.1121
	Stdev	0.002	0.0008	8.00E-04	0.0195
0.0001	Ave	0.113	0.0745	0.092	0.0932
	Stdev	0.008	0.0005	2.00E-04	0.0192
0.00001	Ave	0.07	0.0714	0.073	0.0715
	Stdev	6.00E-04	0.0004	2.00E-04	0.0017
1E-06	Ave	0.069	0.0712	0.068	0.0694
	Stdev	0.001	0.0003	0.002	0.0017

Handling and Precautions. The following factors strongly affect experimental

results with the NBP assay:

1. Freshness of the reagent. The NBP reagent must be freshly made to provide

    accurate experimental results.

2. Handling issues. First is the degree of vortexing in the final step when potassium

    carbonate (K_2CO_3) is added to the epoxide standard dissolved in the acetone. The

    purple or violet color begins to form immediately after the potassium carbonate is

    added to the standard. However, the epoxide-NBP-carbonate mixture must be

    vortexed before the UV absorbance is read to avoid a color gradient within the tube

    (light violet color on top and darker violet color in the bottom of the tube). This

    arises because the potassium carbonate is dissolved in the water and epoxide is

    dissolved in the non-aqueous acetone. Thus, the samples must be vortexed

    vigorously to evenly distribute the violet color.

4.3 HPLC-DETC (N,N-diethyldithiocarbamate) Assay

Combination of HPLC to analyze DETC-epoxide complexes provided sensitive

detection of individual epoxides and also completely separated mixtures of epoxides

(Figure 18). C6 and C10 epoxides gave comparable responses, while epoxybutane

response was considerably lower. This method offers the additional advantage of

detecting trace epoxides not expected in the sample (note minor peaks int Figure 18).

Aswill be shown in subsequent discussion, the method is less subject to variation in

epoxide structure, has excellent linearity and acceptable reproducibility, so is suitable

for quantitation of mixed epoxides of unknown structures.
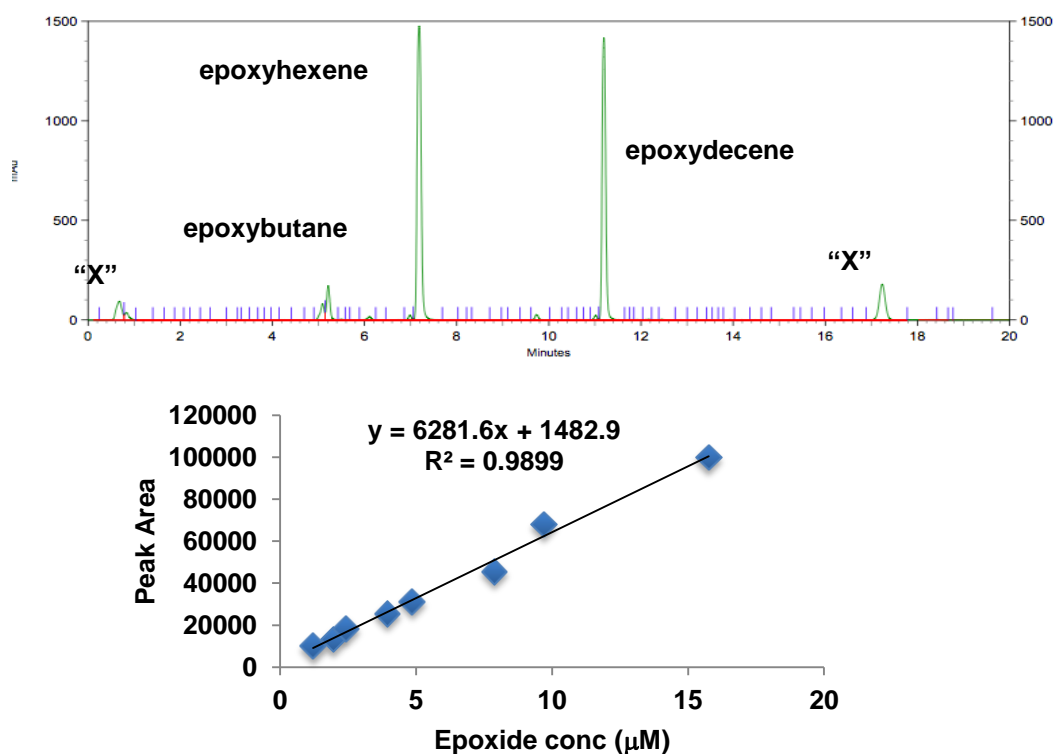

Figure 18. HPLC chromatogram of a 1:1:1 mixture of epoxy butane, hexane, and

decene (top). Bottom: Reaction response is linear for micromolar concentrations.

Detection limits. The HPLC-DETC assay was conducted over the concentration range from 2.5 μM to 0.01 M. This assay requires excess DETC to insure full interaction with the epoxides. Thus, analyzing higher concentrations of epoxide requires that additional DETC be added to the reaction mixture. However, high concentrations of DETC precipitate in the HPLC column and can crash the entire instrument. For this reason, the practical upper detection limit of the assay is around 0.01 M epoxide. For the short chain epoxide, epoxybutane, the lowest detection limit was 20 μM – about ten times higher than epoxyhexene and epoxydecene (2.5 μM). This lower sensitivity for epoxybutane was consistent with observations in the two assays previously discussed, so all three assays show some similar behaviors. As with the NBP and HBr assays the reasons for lower reaction of epoxybutane are probably loss of some of the reactants due to volatility, as well as lower inductive effect from fewer methylene groups on the carbon back bone. The resulting decreased polarity on the epoxy ring reduces reaction with DETC relative to mid-chain epoxyhexene and long chain epoxydecene.

Effects of epoxide structure. Interestingly, in this assay, although epoxybutane still showed much lower reaction, epoxy hexene and decene exhibited comparable reactivity in the higher concentration ranges, and epoxyhexene was only slightly less reactive in the lower concentration ranges (Figure 19). Thus, the DETC reaction is
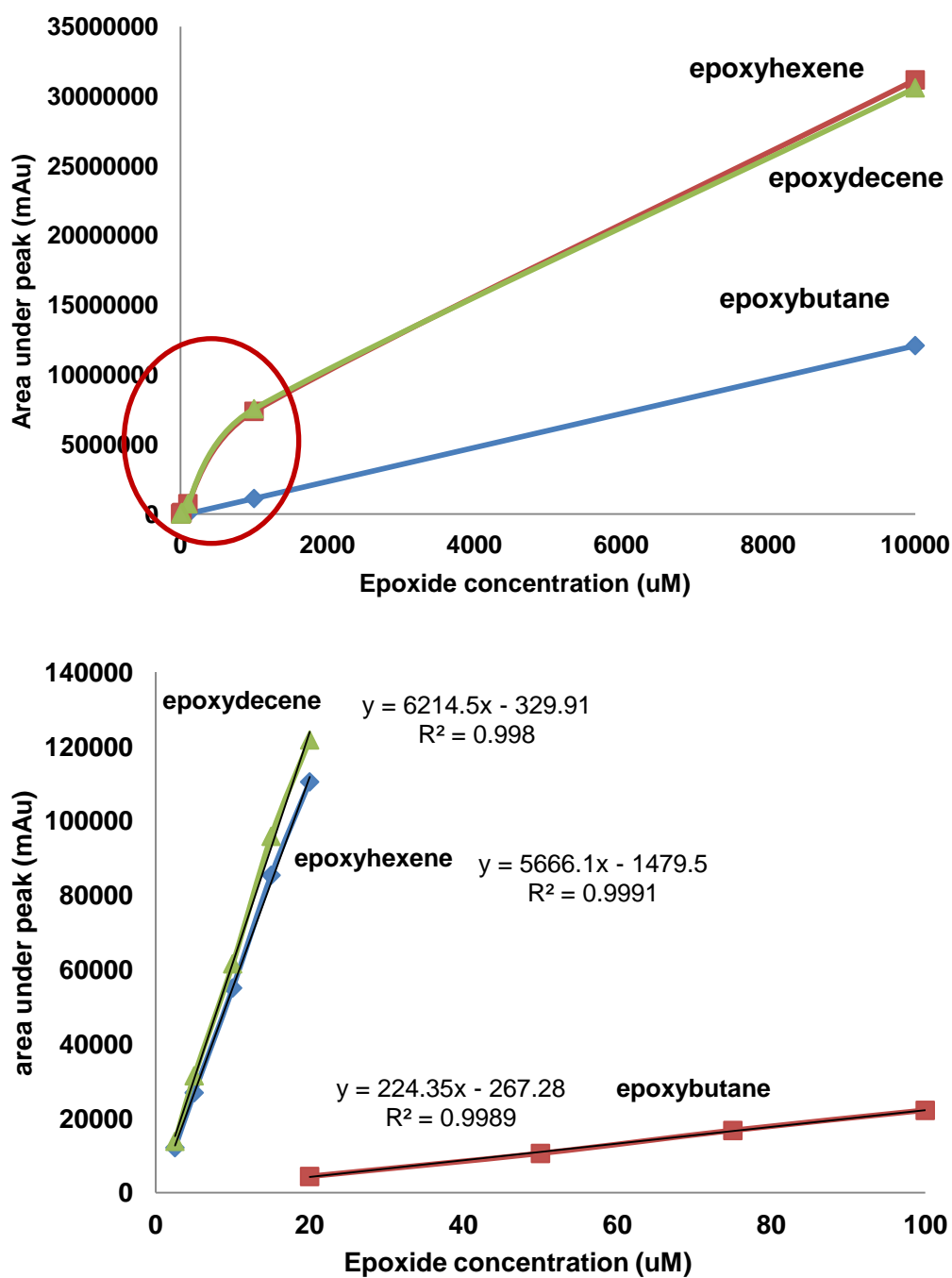
Figure 19. HPLC-DETC reaction response curves (reported as peak areas) for

epoxybutane, epoxyhexene, and epoxydecene standards. Top: full concentration range.

Circle denotes inflection region. Bottom: lower concentration range.

more independent of epoxide structure than the HBr and NBP assays. This is a significant advantage when analyzing complex systems.

Linearity of response. The overall reponse curve is biphasic, with an inflection point at or below 1 mM. Above and below this point the curves are remarkably linear (Figure 19), and in this regard the DETC assay is superior to the HBR and NBP assays for quantitation. As noted above, slopes are significantly steeper in the low concentration range, then decrease at higher concentrations.

Application to oxidizing lipids. Corn oil and methyl linoleate incubated for 3 days at 40 °C were reacted with DETC and applied to the HPLC column. Contrary to what was observed with the HBr and NBP assays, this assay found substantially higher epoxide concentrations in the methyl linoleate than the corn oil (Figure 20). The four epoxide assays were run back to back on the oils to keep the oils the same for each assay. While some small changes may be expected between assays, such a massive reversal is unlikely. Thus, the reversal must arise from differences in the way the methyl linoleate and triacylglycerols react with the DETC. One possibility is that epoxides on the triacylglycerols have hindered access to the DETC and hence show lower reaction than the free ester.

The oils showed reaction patterns comparable to the epoxide standards, with biphasic curves and inflections at or below 1 mM (Figure 20). Expansion of the lower
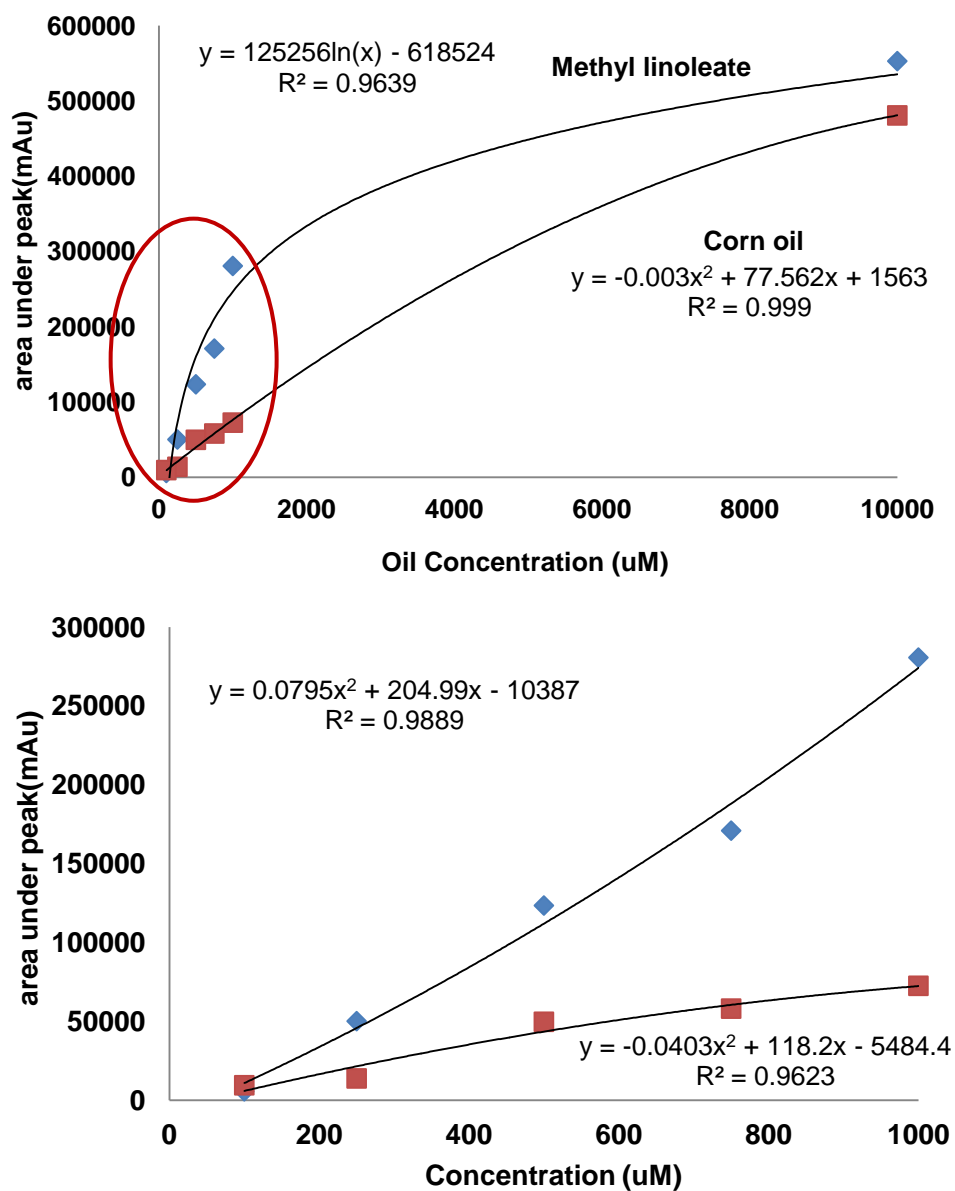
Figure 20. HPLC-DETC response curves for oxidized methyl linoleate and corn oil.

Top: full concentration range. The circle denotes the inflection region between the two

phases. Bottom: lower concentration range.

concentration region and calculation of the best fit curves shows that the relationship of

oil concentration is more quadratic (second order polynomial) than linear.   This

indicates that factors other than concentration affect the reaction. However, the R^2

values for linear regression curves are high enough (0.9842 and 0.9445 for oxidized

methyl linoleate and corn oil, respectively) that the response curves can be used for

quantitation with minimal error.

Reproducibility of epoxide detection by the assay. Coefficients of variation

are presented in Table 19; within and between day averages and standard deviations for

each standard and the oils are presented in Tables 20-24. This assay had good

reproducibility with epoxide standards, well within acceptable limits for quantitative

analyses, but the variability of results was a bit higher than the NBP and HBr assays –

3-5% within day and 5-7% between days. However, performance with methyl linoleate

and corn oil had some problems. Variation within day was 11-18% and between days

was 16-28%, with the higher values arising from the corn oil. This apparent variability

is most likely attributable to the greated sensitivity, detecting μM rather than mM

epoxides. Hence, tiny changes make a large difference in epoxide detected. A second

source of variability may be introduced by the HPLC separations. This would apply

also to the standards and account for the higher variability there.

Table 19. Coefficients of variation for HPLC-DETC assay of standard epoxides and

oxidized lipids, averaged over all concentrations and days.

| | average COV(%) | |
	Within day	Between days
C10	3.32	5.86
C6	5.52	7.29
C4	3.28	5.22
Corn oil	17.62	28.03
ML	10.66	16.07

Table 20. Reproducibility of HPLC-DETC assay for epoxybutane. Five replicates per day, three different days.

2,3-epoxybutane		Within Day (Peak area)			
Conc (uM)		Day1	Day2	Day3	Between Days
10000	Ave	10290486	10293769	10300000	10294752
	Stdev	31211	10235	10613	4833
1000	Ave	1118251	1116903	1116070	1117074
	Stdev	1162	1118	2264	1100
100	Ave	19818	21868	24833	22173
	Stdev	2660	3384	2394	2521
75	Ave	15872	16250	18133	16752
	Stdev	2224	1766	491	1211
50	Ave	10294	10347	11110	10584
	Stdev	1283	392	665	456
20	Ave	4217	4142	4807	4389
	Stdev	343	265	296	364

Table 21. Reproducibility of HPLC-DETC assay for C6 1,2-epoxy5-hexene.

Five replicates per day, three different days.

1,2-epoxy-5-hexene		Within Day (Peak area)			
Concentration (uM)		Day1	Day2	Day3	Average
10000	Ave	31161695	31141590	31197363	31166883
	Stdev	63879	62491	76735	28246
1000	Ave	7346637	7388211	7386759	7373869
	Stdev	56715	168439	82423	23595.01662
100	Ave	746736	725029	745802	739188.8
	Stdev	30953	2411	8181	12271
20	Ave	104278	108404	118875	110519
	Stdev	7330	6158	8932	7524
15	Ave	81539	81140	93554	85410.81
	Stdev	2804	2270	8251	7054
10	Ave	52371	52514	60360	55081.92
	Stdev	3738	3726	2050	4571
5	Ave	25125	23293	32344	26920.63
	Stdev	1934	1420	2182	4785
2.5	Ave	11455	10739	14228	12140.53
	Stdev	946	1118	873	1842

Table 22. Reproducibility of HPLC-DETC assay for C10 1,2-epoxy-9-decene.

Five replicates per day, three different days.

1,2-epoxy-9-decene Concentration (uM)		Day1	Within Day Day2	Day3	Between Day
10000	Ave	30541073	30595281	30656659	30597671
	Stdev	246178	192516	11585870	57830
1000	Ave	7519075	7534257	7627924	7560419
	Stdev	103969	160221	2883175	58952
100	Ave	686551	703687	822188	737475
	Stdev	24923	18055	310759	73862
20	Ave	132169	117481	115785	121812
	Stdev	29999	8893	44796	9010
15	Ave	95061	94678	97900	95879
	Stdev	11381	6053	37197	1760
10	Ave	68412	56938	59426	61592
	Stdev	17415	3413	22500	6036
5	Ave	35202	28922	30312	31479
	Stdev	15123	826	11532	3298
2.5	Ave	13455	14643	13444	13847
	Stdev	998	962	5243	689

Table 23. Reproducibility of HPLC-DETC assay for oxidized methyl linoleate.

Five replicates per day, three different days.

Methyl Linoleate Conc (uM)		Within Day (Peak Area)			Between Day
		Day1	Day2	Day3	
10000	Ave	538113	559270	560980	552788
	Stdev	5572	8799	12199	12737
1000	Ave	241431	335802	264894	280709
	Stdev	21736	134629	9098	49133
750	Ave	154429	178414	180044	170963
	Stdev	16009	7018	3399	14342
500	Ave	121257	122541	126650	123483
	Stdev	7887	6810	1316	2817
250	Ave	14285	68231	68018	50178
	Stdev	1533	3245	1615	31085
100	Ave	5424	5706	5877	5669
	Stdev	171	312	722	229

Table 24. Reproducibility of HPLC-DETC assay for oxidized corn oil. Five replicates per day, three different days.

Corn Oil Concentration (uM)		Within Day (Peak Area)			Between Days
		Day1	Day2	Day3	
10000	Ave	475227	498878	467920	480675
	Stdev	15989	10602	24328	16182
1000	Ave	66163	71271.2	80348	72594
	Stdev	5665	824	5557	7184
750	Ave	54951	59653	59775	58126
	Stdev	1511	1216	3471	2751
500	Ave	51291	43470	54547	49769
	Stdev	1548	1177	2996	5693
250	Ave	6418	17312	17831	13854
	Stdev	1629	1091	2069	6445
100	Ave	3899	19656	5032	9529
	Stdev	451	21071	738	8788

Handling and Precautions:

In general, the DETC reagent and reaction mixtures are much more stable than the HBr and NBP. However, use of HPLC introduces some additional complications.The mobile phase (combination of water and acetonenitrile) developed for this assay is sufficiently strong to elute epoxides up to C10 or possibly C12 but is inadequate to move long chain fatty acids through the column in reasonable time, and it does not dissolve triacylglycerols. Not eluting the epoxide complexes may be one reason for the lower levels of epoxides detected in oils by this assay. The gradient program has been modified to accommodate long chain epoxides and triacylglycerols, and this assay needs to be retested using the new solvent combination.

4.4 NMR (Nuclear Magnetic Resonance) Assay

Use of NMR to measure epoxide groups in compounds is fast and convenient. It can be preformed directly on extracts or oils and does not require any sample modification other than dissolving in deuterated solvents. Certainly NMR is an expensive instrument and is not available in every laboratory, and within institutions NMR analyses are uaually available only on a fee basis and with restrictions on access time. Because of these limitations, only three substances (C10 1,2-epoxy-9-decene, oxidized corn oil and oxidized methyl linoleate) were evaluated by NMR.

Both ^{1}H and ^{13}C NMR were initially evaluated. ^{13}C NMR more clearly

distinguished epoxide signals but sensitivity was substantially lower (Figure 21 A, B).

In ^{1}H NMR spectra, the three hydrogens on the terminal epoxy ring present a triplet

specifically in the specific chemical shift range between 2.5~3.5 ppm, while in 13C

spectra, the two carbons of the epoxide give two lines in the range 45-55 ppm. These

values are consistent with those reported for other terminal epoxides (Gunstone and

Knothe 2010), and the specified peaks are not present in decene (Figure 21 C,D). For

both forms of NMR, the peak areas were proportional to the standard epoxide

concentration analyzed.

Detection limits and linearity of assay. The concentration response curve for ^{1}H

NMR of 1,2-epoxy-9-decene is shown in Figure 22. As has been seen in all the assays

tested in this study, the concentration response was biphasic. There was a plateau range

from about 2 to 10 mM where the slope constantly varied. Below and above this range,

response was linear, although the slope was considerably steeper in the low

concentration range. This behavior is again consistent with all the other assays is fitting

a third order polynomial regression equation almost perfectly (R^2=0.994) (Figure 22,

bottom). For this epoxide, the lowest concentration detectable and quantifiable by

NMR was 0.5 mM. Below this concentration, epoxide peaks could be seen but were

barely distinguishable from background noise.
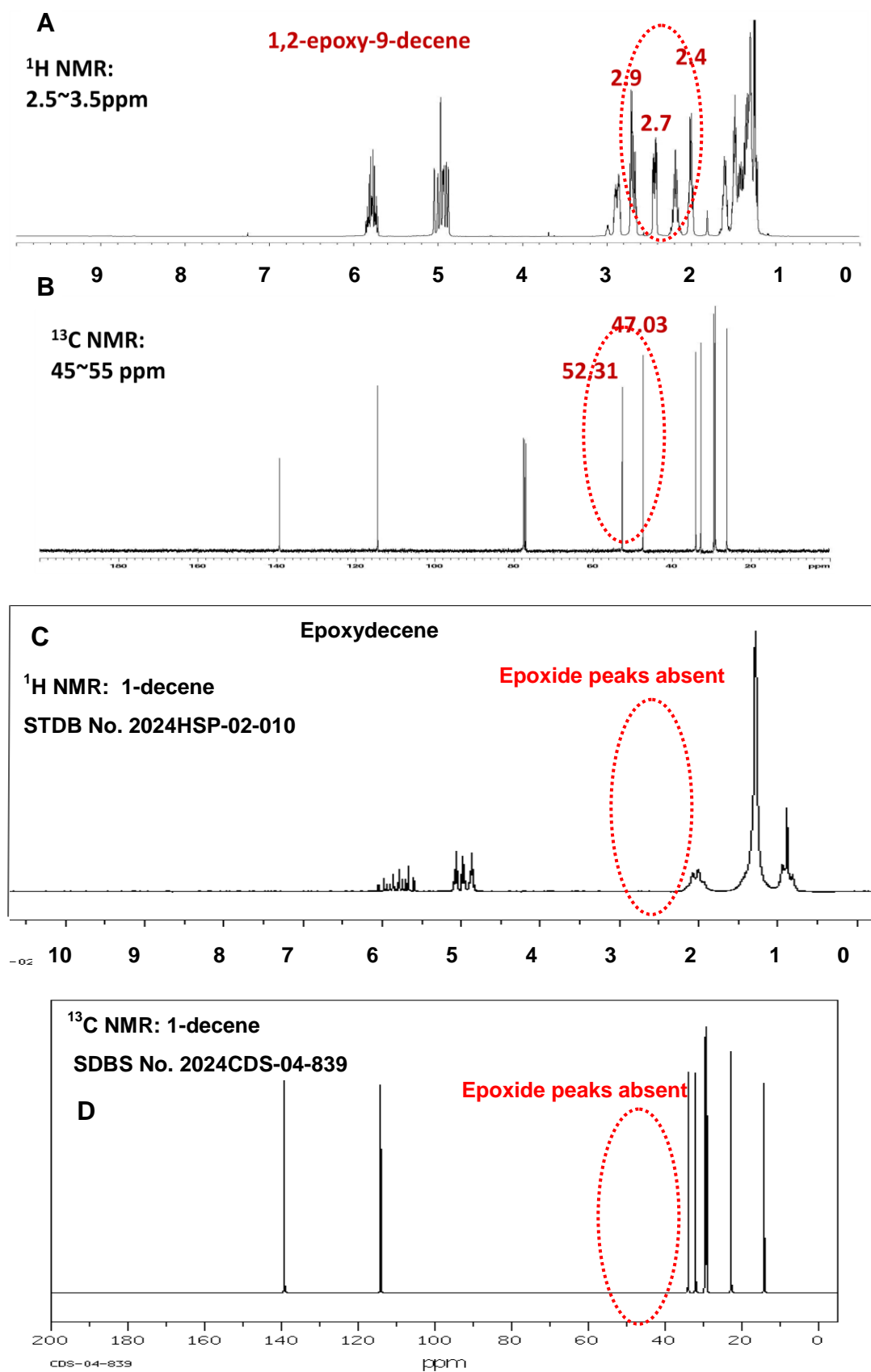
Figure 21. ^{1}H and ^{13}C NMR spectra of 1,2-epoxy-9-decene. A and B: this study. C and

D. Decene spectra fromSpectral Database for Organic Compounds (Yamaji et al. 2013).
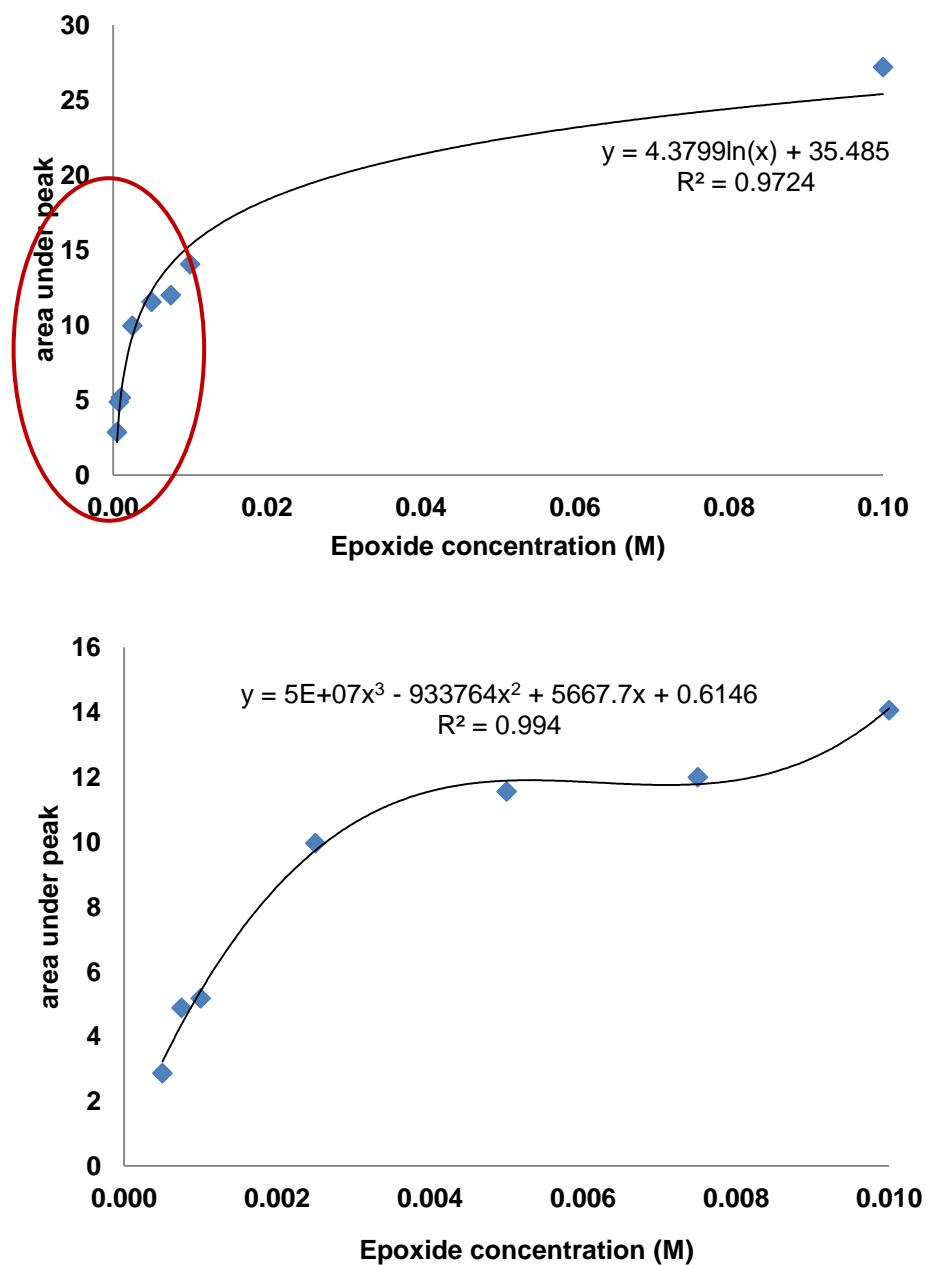
Figure 22. ^{1}H NMR peak area as a function of log epoxide concentration for

epoxydecene. Top: full concentration range. Bottom: lower concentration range.
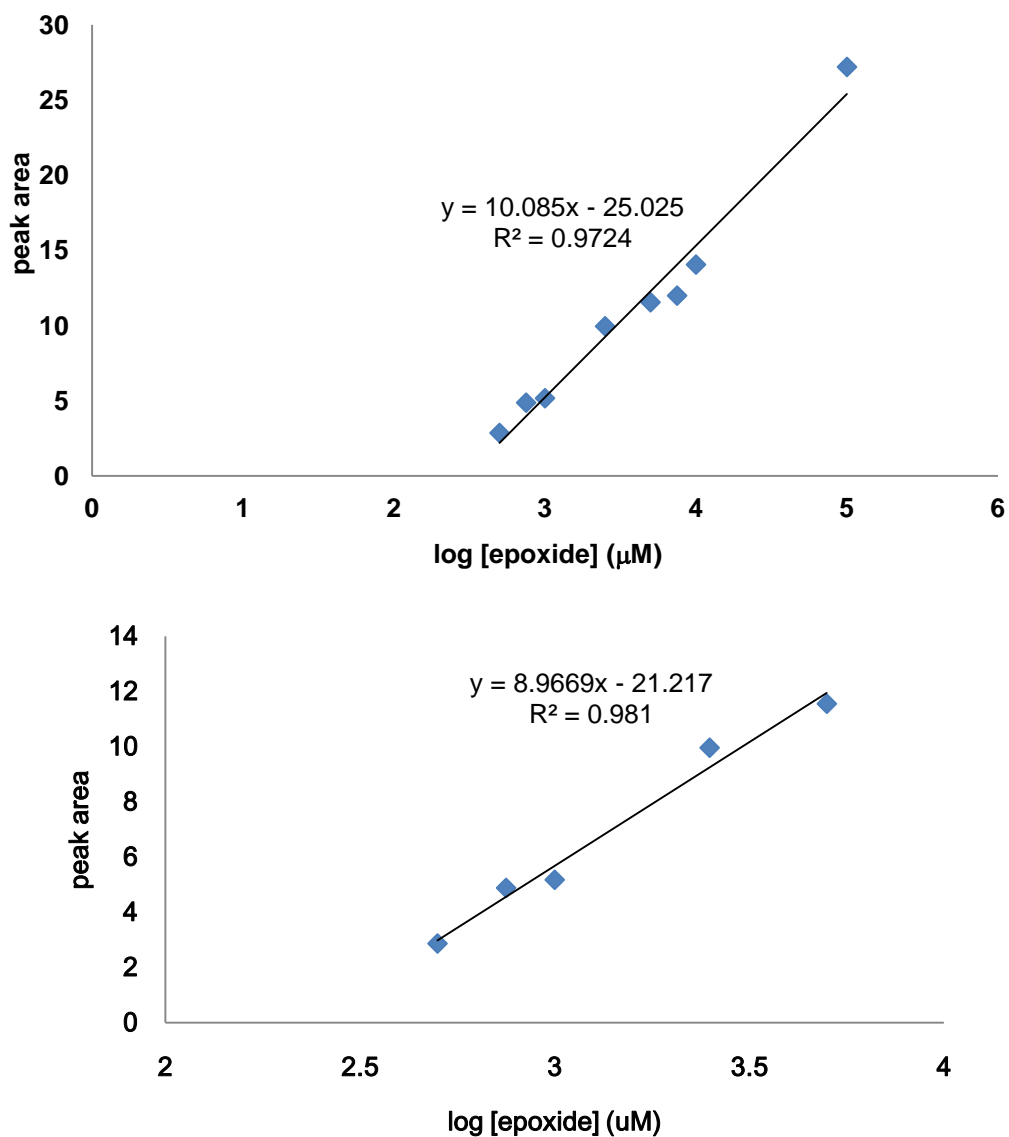
Figure 23. ^{1}H NMR peak area as a function of log epoxide concentration for

epoxydecene. Top: full concentration range. Bottom: lower concentration range.

Application to oxidized lipids. Methyl linoleate and corn oil oxidized three days at 40 °C were analyzed by NMR over a range of oil concentrations. 1H NMR spectra are shown in Figure 24. The three peaks within the epoxide chemical shift range 2.5~3.5 ppm were clearly present and reasonably strong. Plotting the integrated area under these three peaks against oil concentration (note oil, not epoxide) yielded the response curves shown in Figure 25.

The lowest detection limit for oxidized methyl linoleate we found is 0.25 mM oil (lower than epoxydecene); the lowest detection limit we found for oxidized corn oil is 0.5 mM (same as epoxydecene). As with epoxydecene in ^{1}H NMR and the epoxides in general in all the assays, the concentration response was biphasic, increasingly rapidly at low concentrations up to about 2 mM, then slowing at higher concentrations. However, responses were non-linear in all concentration regions (Figure 25).

Since the response curves over the full concentration fit log relationships reasonably well, the data was replotted based on log oil concentrations. As shown in Figure 26, the concentration relationships to response became even more complex rather than simplified to linear. Thus, in these oils, some complex factors other than oil concentration are controlling what is detected by NMR. One possibility is viscosity of the oil as the concentration increases. This phenomenon needs to be elucidated to provide a valid basis for quantitating epoxides in oils by NMR.

Methyl linoleate
¹H NMR
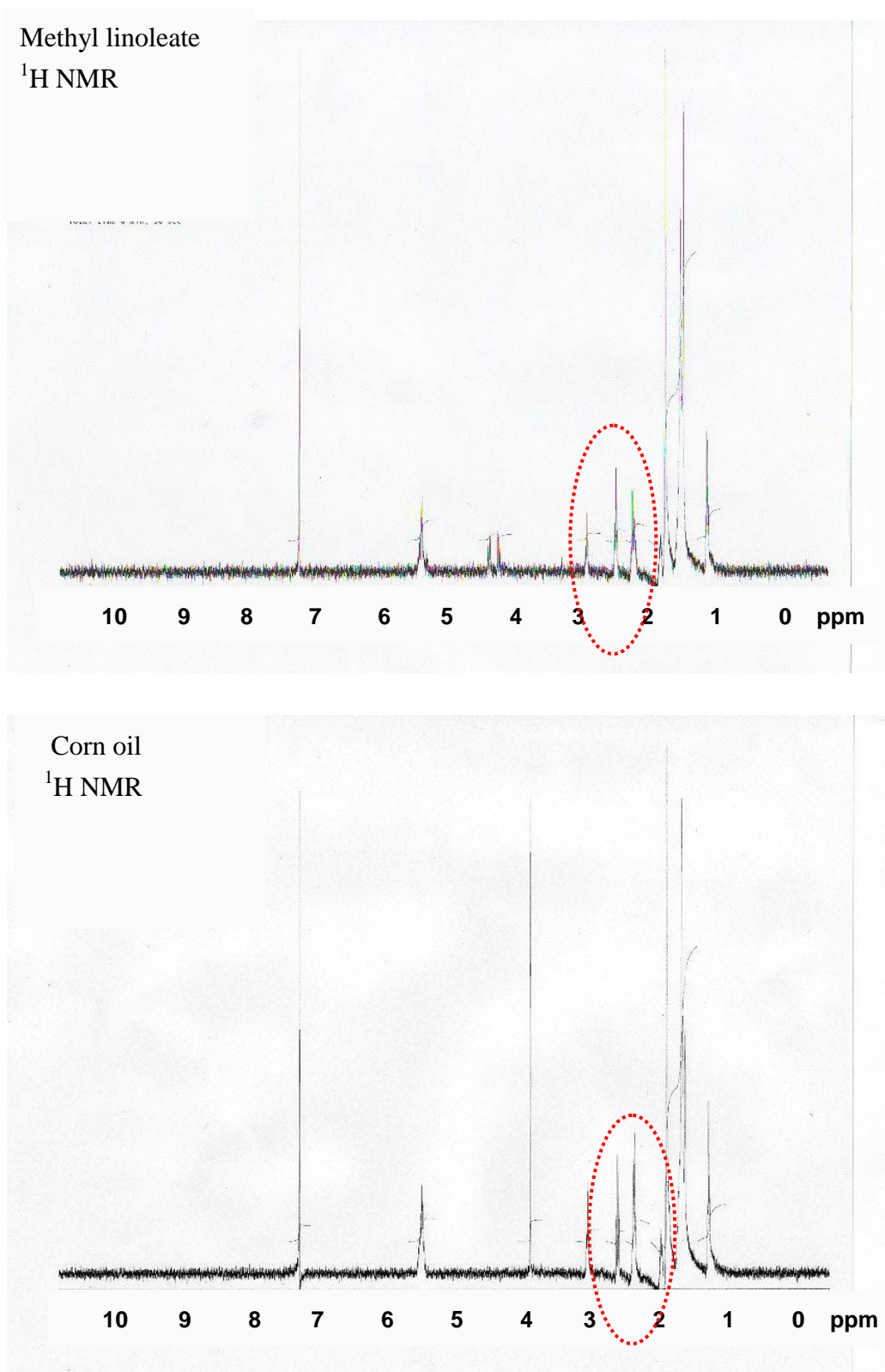
Corn oil
¹H NMR

Figure 24. ¹H NMR spectra of oxidized methyl linoleate (top) and corn oil (bottom).

Red circles denote the epoxide peaks that were integrated to determine epoxide

response.
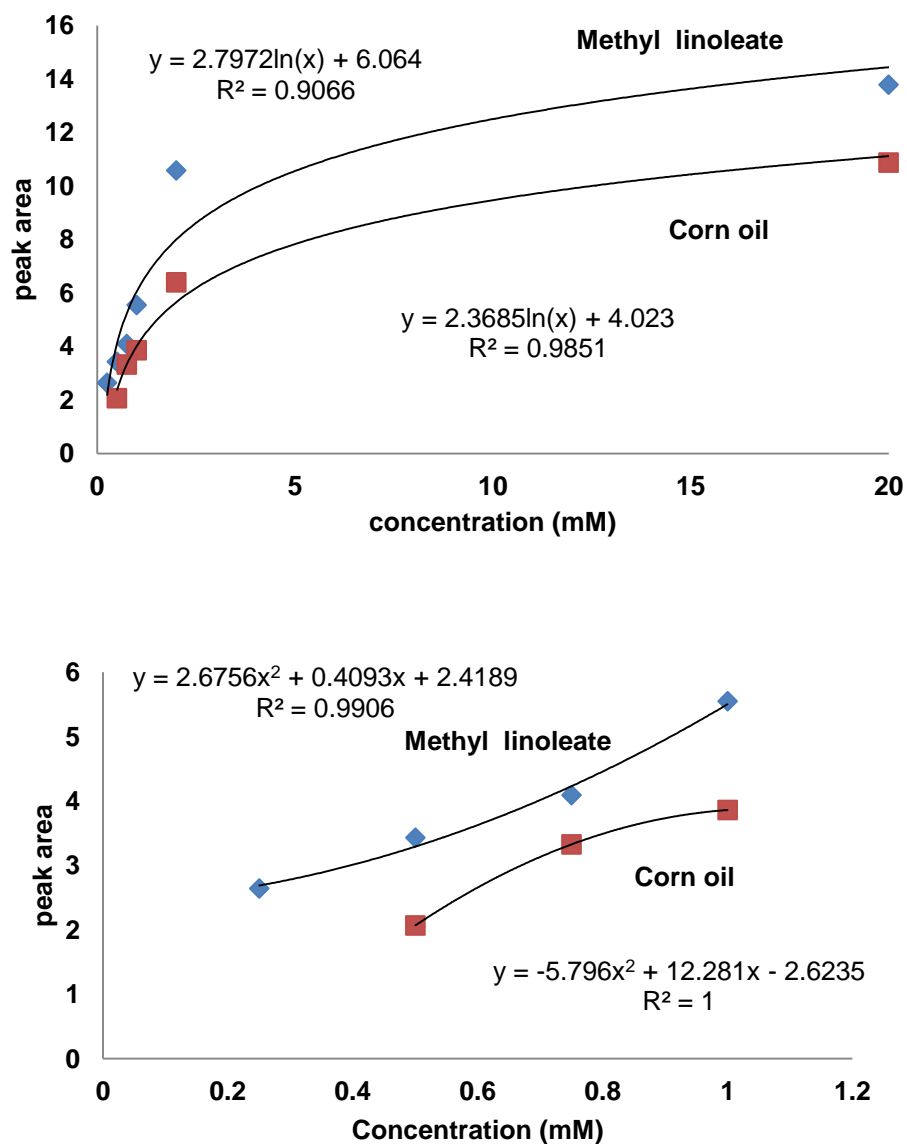
Figure 25. ^{1}H NMR peak area as a function of oil concentration for oxidized methyl linoleate and corn oil. Top: full concentration range. Bottom: lower concentration range.
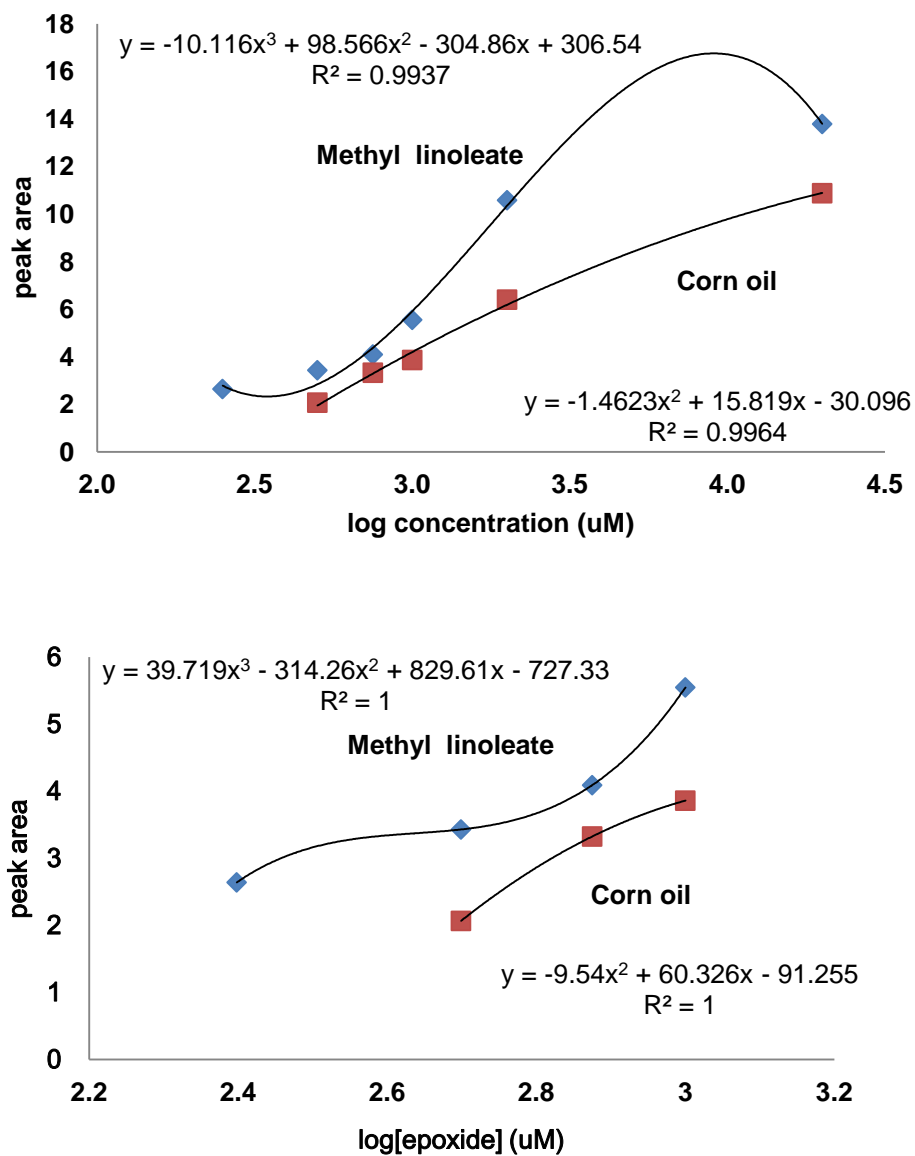
Figure 26. ^{1}H NMR peak area as a function of log[oil] for oxidized methyl linoleate

and corn oil. Top. Full concentration range. Bottom: lower concentration range.

Reproducbility of assay. Documentation of within day and between day variability in NMR analyses is presented in Table 25 for epoxydecene, Table 26 for methyl linoleate, and Table 27 for corn oil. Coefficients of variation for 1H NMR analysis of epoxydecene and oxidized methyl linoleate and corn oil are listed in Table 28. Reproducibility of epoxydecene analysis by NMR was the best of all the assays tested – about 1% within day and between days. Variability of the oils was slightly higher – about 5% within day and slightly higher between days, but oth values were well below the 10% limit usually applied to quantitative analyses. Examination of the day to day epoxide levels shows that the variation was not random, but systematic, decreasing on each successive day. Thus, the variation is due not to the NMR instrumentation itself but to non-homogeneity of oils and to slow degradation or continued transformation of the epoxides in the oil after the samples were oxidized and then stored refrigerated.

Considering the high precision attainable with this assay and the sub-millimolar sensitivity, NMR assays merit additional development.

Table 25. Reproducibility of NMR assay for C10 1,2-epoxy-9-decene. Five replicates

per day, three different days.

1,2-epoxy-9-decene		Within Day Integrated Peak Areas			
Conc. (M)		Day 1	Day 2	Day 3	Between Days
0.1	Ave	27.7733	27.3067	26.5133	27.1980
	Stdev	0.4771	1.7878	1.9634	0.6370
0.01	Ave	13.8633	14.4533	13.8633	14.0600
	Stdev	1.6187	0.9816	1.6187	0.3406
0.0075	Ave	12.1000	11.7867	12.1000	11.9960
	Stdev	0.3651	0.3885	0.3651	0.1809
0.005	Ave	11.5167	11.6267	11.5167	11.5530
	Stdev	0.2730	0.3646	0.2730	0.0635
0.0025	Ave	10.0000	9.8800	10.0000	9.9600
	Stdev	0.0600	0.0300	0.0600	0.0693
0.001	Ave	5.1133	5.2967	5.1133	5.1744
	Stdev	0.1557	0.1850	0.1557	0.1058
0.00075	Ave	4.8667	4.9067	4.8667	4.8800
	Stdev	0.1457	0.2001	0.1457	0.0231
0.0005	Ave	2.8033	2.9900	2.8033	2.8656
	Stdev	0.5437	0.1153	0.5437	0.1078

Table 26. Reproducibility of NMR assay for epoxides in oxidized methyl linoleate.

Five replicates per day, three different days.

Methyl linoleate Conc. (M)		Within Day Integated Peak Area			
		Day1	Day2	Day3	Between Days
0.02	Ave	14.1433	14.1067	13.1067	13.7860
	Stdev	0.5181	0.2003	0.2003	0.5882
0.002	Ave	10.4833	11.1000	10.1567	10.5800
	Stdev	0.8223	0.5292	0.0404	0.4790
0.001	Ave	5.9433	5.5067	5.2000	5.5500
	Stdev	0.1150	0.4727	0.4678	0.3736
0.0008	Ave	4.2367	4.0367	4.0033	4.0922
	Stdev	0.2237	0.8370	0.2409	0.1262
0.0005	Ave	3.2600	3.7100	3.3233	3.4311
	Stdev	0.2869	0.9305	0.1701	0.2436
0.0003	Ave	2.8400	2.5333	2.5533	2.6422
	Stdev	0.3208	0.3412	0.1914	0.1716

Table 27. Reproducibility of NMR assay for epoxides in oxidized corn oil. Five

replicates per day, three different days.

Corn Oil Conc. (M)		Within Day Integrated Peak Areas			
		Day1	Day2	Day3	Between Day
0.02	Ave	11.0033	10.8067	10.8133	10.8740
	Stdev	0.0473	0.1716	0.1888	0.1117
0.002	Ave	6.6767	6.2800	6.2400	6.3989
	Stdev	0.2098	0.2390	0.0954	0.2414
0.001	Ave	4.5100	3.6633	3.4100	3.8611
	Stdev	0.5507	0.5082	0.4597	0.5761
0.00075	Ave	3.7533	3.3200	2.9067	3.3267
	Stdev	0.3109	0.3961	0.0306	0.4234
0.0005	Ave	2.0333	2.2333	1.9367	2.0678
	Stdev	0.3570	0.2401	0.2139	0.1513

Table 28. Coefficients of variation for NMR assay of epoxydecene and epoxides in oxidized lipids. Averaged over all concentrations and days.

	average COV(%)	
	Within day	Between days
C10	1.01	1.72
Corn oil	5.32	7.95
ML	4.52	5.36

Handling and Precautions. ^{1}H NMR analyses specifically detected proton interactions in molecules, including any water present. Thus, it is crucial to exclude water from all test samples and solvents. In addition, also to avoid interference from extraneous protons, deuterated solvents must be used, e.g. deuterated chloroform ($CDCl_3$) for lipids. These solvents do degrade with time so it is prudent to purchase only small quantities of these solvents at a time and to not store these reagents for long periods.

5. SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

Since the importantce of lipid oxidation was recognized more than 50 years ago, epoxides have been overlooked as lipid oxidation products. Although epoxides are recognized as products, they are seldom analyzed or considered when examining reaction processes, perhaps because they form and react rapidly so are seldom detected among mixed products, because they do not produce known off-odors or flavors, or because there are few standard procedures or guides for analyses of epoxides. The main purpose of this thesis research was to evaluate four available epoxide assays to determine detection ranges, accuracy, reproducibility, and special handling issues in order to provide some guidelines and protocols for scientists for selecting and using appropriate methods when quantitating epoxides. Our main focus was on accurate quantitation rather than relative comparisons since we need to use the results in developing mass balances of alternate reaction pathways in lipid oxidation.

A major limitation to accurate quantitation that arose in the HBr and NBP assays was marked differences in reactivity that depended on epoxide structure. In these two assays, epoxide reactivity increased with epoxide chain length. One potential explanation for this enhancement is that the longer alkyl groups increase polarity of the oxirane ring more, and hence facilitate attack by nucleophiles such as HBr and NBP. Another possibility, at least for NBP, is that the NBP-epoxy adduct structure alters the

extinction coefficient and optical properties that are the basis for detection. Whatever

the explanation, non-constant reaction with epoxides is not an acceptable basis for

quantitative analyses of materials that have different or unknown epoxide structures, as

occurs with different oils and lipid extracts of materials.

Additional limitations that we noted in some assays was sensitivity to oxygen and

volatility/reactivity of epoxides. To maximize reagent stability and minimize side

reactions, we found it prudent to flush all samples, solutions, and sample headspaces

with argon and to minimize exposure to light as well. Samples, especially epoxide

standards, must be handled, transferred, and capped rapidly to avoid degradation and

loss. Strong odors in the laboratory are certain signs of epoxide loss.

Detection range is critically important when choosing a suitable assay. Detection

ranges and sensitivity vary with the assay. If you choose an assay with a detection range

that does not match your samples, the test results may well be erroneous, misleading,

and unreliable. The detection ranges for assays evalutated in this study are summarized

in Table 29. Perhaps because of limitations in useful stable HBr concentrations, the

HBr titration assay has a narrow detection range of only 0.1 M down to 7.5 mM epoxide.

This assay was originally developed to analyze epoxides in modified fuels and greases

where epoxide concentrations are high, and is probably borderline in being ablt to

detect epoxides in early stages of lipid oxidation. If this was applied to analyze lipid

Table 29. Summary of detection ranges and important characteristics of HBr, NBP, DETC, and NMR epoxides

HBr Assay

Limits of detection	7.5 mM to 0.1 M (1M for epoxybutane)
Linear detection range	non-linear response curves at all concentrations
Epoxide structure effects	marked, reaction increases with R chain length
Oxygen sensitive?	results lower in air than under Ar

Reproducibility (%)		Average within day	Ave between day
	Standards	2-3%	3-9%
	Oils	4-6%	8-13%

Handling issues	HBr volatile and degrades rapidly, frequent restandardization is necessary
	Endpoint difficult to determine visually
	Reaction less than stoichiometric, even under Ar
	Sparging with inert gas (Ar) increases yields -- inhibits Br⁻ oxidation and epoxide side reactions
Advantages	fast and relatively simple, standard equipment
Recommendations for use	use only for samples with high epoxides.
	can compare only samples with similar epoxide structures

_p_-Nitrobenzyl Pyridine (NBP) Assay

Limits of detection	C4: 10 μM - 1M; C6: 1μM to 100 mM; C10: 1μM – 50 mM
Linear detection range	1 μM to 1 M for epoxybutane
	1-10 μM for epoxyhexene
	polynomial at all concentrations for epoxydecene
Epoxide structure effects	**pronounced**, reaction increases with R chain length
Oxygen sensitive?	Yes, but with tube capped all the time it works

Reproducibility (%)		Average within day	Ave between day
	Standards	1-2%	2-3%
	Oils	3-7%	4-12%

Handling issues	reactions affected by unknown factors other than epoxide concentration
Advantages	high sensitivity and reproducility
Recommendations for use	gives relative rather than absolute qunatitation
	can compare only samples with similar epoxide structures

Diethyldithiocarbamate (DETC)-HPLC Assay

Limits of detection	2 μM, C6 and C10; 20 μM C4 epoxides
	upper limit ~10 mM (solubility limit of DETC)
Linear detection range	2 μM~100 μM epoxides
	appears to have a second linear range with lower slope at high epoxide concentrations
Epoxide structure effects	effects minimal mid chain length and above
Oxygen sensitive?	No effect noted

Reproducibility (%)

	Average within day	Ave between day
Standards	3%	5-7%
Oils	11-18%	16-28%

Handling issues	current procedures designed for monomer epoxides, HPLC gradient modifications required for epoxides on triacylglycerols and large molecules, in extracts
Advantages	fast, easy, sensitive
	provides information about individual epoxide species
	adaptable to microplate solution assays
Recommendations for use	Useful for research and quality control with gradient adjustment
	Merits detailed development and further testing

NMR Assay

Limits of detection	0.5 mM
Linear detection range	0.5 mM – 100 mM
Epoxide structure effects	Apparently minimal differences when samples are not volatile
Oxygen sensitive?	No, except for stability of the sample itself.

Reproducibility (%)

	Average within day	Ave between day
Standards	1%	2%
Oils	5%	5-8%

Handling issues	Requires dry, deuterated solvents
	Potential interferences in mixed extracts unknown
Advantages	Does not require derivatization or reaction
	Performed directly on oils, extracts, pure samples
	Provides both absolute quantitation and qualitative information about epoxide structure
Recommendations for use	**Merits detailed development and further testing**

oxidation in foods or biological tissues in the past, it most likely resulted in conclusions that epoxides were not present.

Based on accuracy of chemistry, linearity of response, sensitivity, lack of epoxide structure effects on reaction response or detection, and ability to obtain absolute quantitation, only two assays – DETC and NMR – are considered to be candidates for further development as routine analyses of lipid oxidation.

The DETC-HPLC assays seem to be the most promising method because of its sensitivity, linear response, and ability to separate epoxides of different structures. Combining this with mass spectrometry will create a very powerful tool for research on oxidizing lipids. The main limitation currently is the eluting solvent and gradient program, which was developed for monomer epoxides. When the solvent program recently developed in this laboratory is fully tested, the DETC-HPLC assay will be a very powerful tool indeed for analysis of oxidized lipids.

NMR is a very intriguing approach to analyzing lipid epoxides. Minimal handling is required – just dissolving sample or oil in deuterated solvents – and actual analysis is straightforward if NMR instrumentation is available. Samples can be analyzed in about 10 minutes on a 400 MHz NMR, with sufficient resolution and sensitivity. NMR also offers the potential to detect other oxidation products in the same same since oxygenated groups are shifted substantially downfield and are separated from each

other. values for each are substantially different. Responses were linear and sensitive

enough (about 0.5 mM as a lowe limit) to detect epoxides in many oil samples from the

laboratory. It may be possible to increase sensitivity and resolution by switching to

higher frequency NMRs, but this is probably more for research due to its high

associated user fees. Some attention will need to be given to effects of solvent and water

traces when extracts rather than refined oils are analyzed.

6. FUTURE WORK

To our knowledge, assays for lipid epoxides have not been evaluated previously, so this study begins the process. All of the assays still need work to make them rugged and dependable, and hopefully also more sensitive. As noted in the recommendations, the HBr and NBP assays are not appropriate for analyses of epoxides with mixed or unknown structures. However, the DETC and NMR assays offer potential for sensitive detection independent of epoxide structure, so merit considerable effort to optimize.

Modifications of the HPLC-DETC assay. Two major adaptations are needed here. First, there is the question of whether epoxides in oils exist as monomer scission products or are also retained on the triacylglycerols backbone, i.e. as core epoxides. In its current form, the elution gradient probably grossly underestimates epoxide levels of long chain fatty acids and triacylglycerols. This can be remedied by a) extracting the monomer epoxides with methanol and analyzing without interference from larger molecules, and b) adapting the solvent gradient to handle larger molecules such as triacylglycerols. A solvent gradient has bee modified for elution of triacylglycerols and this needs to be tested with epoxidized oils. In addition, stability of epoxides in lipid solvents must be tested. We have some evidence from extraction studies that epoxides degrade rapidly in organic solvents.

Questions have been raised about the specificity of the DETC assay. Our preliminary analyses found no reaction with other lipid oxidation products such as hydroperoxides and carbonyls, but this needs to be evaluated more systematically.

In addition, to make maximum use of the HPLC separation of individual epoxide adducts, LC needs to be interfaced with mass spectrometry detection to identify specific epoxide structures. This will contribute greatly to elucidating lipid oxidation mechanisms. MS will also verify that peaks detected are indeed from epoxides and not other degradation pathways or adventitious transformations. This information will allow exclusion of non-targeted peaks from integration, hence increasing sensitivity and improving accuracy of this assay.

Modifications of the NMR assay. Samples in this study were analyzed using a 400 MHz NMR spectrometer that provides middle of the road sensitivity. Since the end goal is to proved an assay that can be used at multiple levels, e.g. in quality control laboratories in industry as well as in academic research with materials ranging from biological tissues with barely trace levels of epoxides to foods in very early stages of oxidation to foods in later stages of degradation, it is imperative to determine what level of NMR is required to provide the sensitivity needed for each application. Thus, studies need to be conducted comparing sensitivity of table top NMRs to various frequency NMRs, e.g. 200, 400, 600, 800 MHz spectrometers. These range from rather crude

analyses to high sensitivity research instruments and will reveal which level is needed

for different applications. In addition, pure material were used in this study, but lipid

extracts are the samples analyzed in most routine quality control as well as basic

research. We noted problems with interference from water and solvent protons. Both

may be a significant problem when analyzing extracts. Detailed investigation of the

effects of solvent and water traces as well as multiple components on NMR analyses

needs to be undertaken.

7. REFERENCES

Acott, B.and Beckwith, A. L. J. (1964) "Reactions of alkoxy radicals. IV. Intramolecular hydrogen-atom transfer in the presence of cupric ion: A novel directive effect" *Austral J. Chem* 17(12), 1342-1353

Aerts, H. A. J. and Jacobs, P.A. (2004). "Epoxide yield determination of oils and fatty acid methyl esters using ^{1}H NMR." *Journal of the American Oil Chemists' Society* 81 (9), 841–846.

Agarwal, S. C., Van Duuren, B. L. and Kneip, T. J. (1979). "Detection of epoxides with 4-(p-nitrobenzyl) pyridine." *Bulletin of Environmental Contamination And Toxicology* 23 (6), 825–829.

American Oil Chemistry Society Official Methods (1997). Oxirane Oxygen. Method Cd 9-57.

Anderson, H. C. (1962), Thermal degradation of epoxide polymers. J. Appl. Polym. Sci., 6, 484-488.

Ansari, M.H. and Ahmad, M. (1986). "Acacia suma seed oil - characterization of a HBr-reactive acid." *Fette, Seifen, Anstrichmittel* 88 (10), 402–403.

Baumann, K., Hogenauer, K., Schulz, G. and Steck, A. (2002). "NMR study on 9-S and 9-R oxirane derivatives of ascomycin." *Magnetic Resonance in Chemistry* 40 (7), 443–448.

Benson, A. M., Barretto, P. B. (1985). "Effects of disulfiram , diethyldithiocarbamate, bisethylxanthogen , and benzyl isothiocyanate on glutathione transferase activities in mouse organs." *Cancer Research* 45, 4219–4223.

Bush, D. M. and Kozumbo, J.W. (1983). "Metabolism of polycyclic aromatic hydrocarbon derivatives to ultimate carcinogens during lipid peroxidation." *Science* 221 (4605), 77-79.

Chan, H. W. S., Prescott, F. A. A., and Swoboda, P. A. T. (1976) Thermal decomposition of individual positional isomers of methyl linoleate hydroperoxide: Evidence of carbon-oxygen bond scission. *J. Am. Oil Chem. Soc* 53, 572-576.

Chen, H.J., Gonzalez, F. J., Shou, M. and Chung, F. L. (1998). "2,3-Epoxy-4-hydroxynonanal, a potential lipid peroxidation product for etheno

adduct formation, is not a substrate of human epoxide hydrolase." *Carcinogenesis* 19 (5), 939–43.

Claxson, A.W.D., Hawkes, G. E., Richardson, D. P., Naughton, D. P., Haywood, R.M., Chander, C. L., Atherton, M., Lynch, E. J., Grootveld, M. C. (1994). "Generation of lipid peroxidation products in culinary oils and fats during episodes of thermal stressing: a high field ^{1}H NMR study." *FEBS letters* 355 (1), 81–90.

Clayden, J., Greeves, N. and Warren S. ed.(2006) Organic Chemistry, Oxford University Press, Oxford.

Cravero, R. M., Labadie G. R. and Sierra M. G. (2000). "Using empirical rules from ^{13}C NMR analysis to determine the stereochemistry of the epoxide located at the 5,6-position of decalinic systems." *Molecules* 5 (3), 323–324.

Derawi, D. and Salimon, J. (2010). "Optimization of epoxidation of palm olein by using performic acid." *E-Journal of Chemistry* 7 (4), 1440–1448.

Dupard-Julien, C. L., Kandlakunta, B., and Uppu, R. M. (2007). "Determination of epoxides by high-performance liquid chromatography following derivatization with N,N-diethyldithiocarbamate." *Analytical and Bioanalytical Chemistry* 387 (3), 1027–32.

Durbetak, A. J. (1958). "Determination of oxirane oxygen in salts of epoxy acids and in the presence of amines." A*naytical Chemistry* 30 (12), 2024-2025.

Earle, F. R. (1970). "Epoxy oils from plant seeds." *Journal of the American Oil Chemists' Society* 47 (12), 510–513.

Ehsan, S.D. (2010). "Optimization on Epoxidation of Palm Olein by Using Performic Acid." *E-Journal of Chemistry* 7(4):1440–1448.

Ergozhin, E. E., Bektenov, N. A. and Chopabaeva, N. N. (2004). "NMR study of the structure of polymers based on 2,3-epoxypropyl methacrylate." *Russian Journal of Applied Chemistry* 77 (5), 813–816.

Fankhauser-Noti, A., Fiselier, K., Biedermann-Brem, S. and Grob, K. (2006). "Assessment of epoxidized soy bean oil (ESBO) migrating into foods: comparison with ESBO-like epoxy fatty acids in our normal diet." *Food and Chemical Toxicology* 44 (8), 1279–86.

Fennema, O. R., ed. (1996). *Food Chemistry*, 3rd Edition, Macel Dekker Inc. New York.

Frankel, E. N., ed. (1998). *Lipid Oxidation ,* The Oily Press, Scotland.

Frankel, E. N., Neff, W. E., Selke, E., and Weisleder, D. (1982) Photosensitized oxidation of methyl linoleate: Secondary and volatile thermal decomposition products. *Lipids* 17, 11-18.

Gardner, H., .W., Weisleder, D., and Kleiman, R. (1978) Formation of *trans*-12,13-epoxy-9-hydroperoxy-*trans*-10-octadecadienoic acid catalyzed by either a soybean extract or cysteine-fecl$_3$. *Lipids* 13, 246-252.

Gardner, H. W. and Kleiman, R. (1981) Degradation of linoleic acid hydroperoxides by a cysteine-FeCl$_3$ catalyst as a model for similar biochemical reactions. II. Specificity in formation of fatty acid epoxides. *Biochim. Biophys. Acta* 665, 113-125.

Giuffrida, F., Destaillats, F., Robert, F., Skibsted, L. H. and Dionisi, F. (2004). "Formation and hydrolysis of triacylglycerol and sterols epoxides: role of unsaturated triacylglycerol peroxyl radicals." *Free Radical Biology & Medicine* 37 (1),104–14.

Guillen, M. D. and Goicoechea, E. (2009). "Oxidation of corn oil at room temperature: primary and secondary oxidation products and determination of their concentration in the oil liquid matrix from ^{1}H nuclear magnetic resonance data." *Food Chemistry* 116 (1), 183–192.

Guillén, M. D. and Ruiz, A. (2006). "Study by means of ^{1}H nuclear magnetic resonance of the oxidation process undergone by edible oils of different natures submitted to microwave action." *Food Chemistry* 96 (4), 665–674.

Guillén, M. D. and Ruiz, A. (2005). "Monitoring the oxidation of unsaturated oils and formation of oxygenated aldehydes by proton NMR." *European Journal of Lipid Science and Technology* 107 (1), 36–47.

Guillén, M. D. and Ruiz, A. (2004). "Study of the oxidative stability of salted and unsalted salmon fillets by ^{1}H nuclear magnetic resonance." *Food Chemistry* 86 (2), 297–304.

Gunstone, F. and Knothe, G. (2010) NMR spectroscopy of fatty acids and their derivatives., Lipid Library, http://lipidlibrary.aocs.org/nmr/nmr.html, accessed April 14, 2013.

Guo, R., Ma, C., Sun, S. and Ma, Y. (2010). "Kinetic study on oxirane cleavage of epoxidized palm oil." *Journal of the American Oil Chemists' Society* 88 (4), 517–521.

Hammock, L. G., Hammock, B. D., and Casida, J. E. (1974). "Detection and analysis of epoxides with 4-(*p*-nitrobenzyl)-pyridine." *Bulletin of Environmental Contamination and Toxicology* 12 (6), 759–764.

Haynes, S.C. and Vonwiller, R.K. (1990) Iron(III) and copper(II) catalyzed transformation of fatty acid hydroperoxides: efficient generation of peroxy radicals with Cu(II) trifluoromethane sulfonate. *J. Chem. Soc. Chem. Commun.*, 1102-1104.

Hemminki, K. (1979). "Correlation of mutagenicity and 4-(p-nitrobenzyl)-pyridine alkylation by epoxides" *Toxicology Letters* 4, 103–106.

Hemminki, K., Falck, K., and Vainio, H. (1980). "Comparison of alkylation rates and mutagenicity of directly acting industrial and laboratory chemicals." *Archives of Toxicology* 46 (3-4), 277–285.

Hemström, P. and Irgum, K. (2006). "Hydrophilic interaction chromatography." *Journal of Separation Science* 29 (12), 1787-1821.

Husain, S. R., Ahmad, M. S., Ahmad, Jr. F., Ahmad, M., and Osman, S. M. (1989). "Hibiscus mutabilis seed oil - characterization of HBr-Reactive Acids." *Fat Science and Technology* 91 (4), 167–168.

Kamal-Eldin, A., Marquez-Ruiz G., Dobarganes, C. and Appelqvist, L. A. (1997). "Characterisation of aldehydic acids in used and unused frying oils." *Journal of Chromatography* 776 (2), 245–254.

Kan, C. C. (2006). "Lipid" In *Food Chemistry*, 1st Edition, Lun, X. ed, New Wun Ching Developmental Publishing Co., Ltd., Taipei, pp.114-165.

Kannan, R., Subbaram, M. R., and Achaya, K. T. (1974). "NMR studies of some oxygenated. halogenated, and sulphur-containing fatty acids and their derivatives." *Fette, Seifen, Anstrichmeittel* 76, 344–350.

Karel, M. (1980) Lipid oxidation, secondary reactions, and water activity of foods. In *Autoxidation in food and biological systems*. Simic, M. G., & Karel, M., eds, New York: Plenum Press, pp. 191-206

Kim, J. H. and Thomas, J. J. (1992). "Use of 4-(nitrobenzyl)pyridine (4-NBP) to test mutagenic potential of slow-reacting epoxides, their corresponding olefins, and other alkylating agents." *Bulletin of Environmental Contamination and Toxicology* 49 (6), 879–85.

Kochi, J. K. (1962) Chemistry of alkoxyl radicals. *J. Am. Chem. Soc.* 84, 1193-1197.

Kochi, J. K. (1973) Oxygen radicals. In *Free Radicals*, Vol. 2 (Kochi, J. K., ed), John Wiley, New York, pp. 665-710.

Kuo, T. M. and Gardner, H. W. ed. (2002) Lipid Biotechnology, Marcel Dekker Inc, New York, NY

Labuza, T. P. (1972). "Kinetics of lipid oxidation in foods." *CRC Critical Reviews in Food Technology* 2 (3), 355–405.

Lederer, M. O. (1996). "Reactivity of lysine moieties toward γ-hydroxy-α,β -unsaturated epoxides: a model study on protein-lipid oxidation product interaction." *Journal of Agriultural and Food Chem.* 44 (9), 2531–2537.

Lee, C. S., Ooi, T. L., and Chuah, C. H. (2009). "The effect of reaction temperature on retaining oxirane oxygen contents in the synthesis of epoxidized diethanolamides." *American Journal of Applied Science* 6 (1), 72–77.

Lee, S. H., Oe, T., and Blai, I. A. (2002). "4,5-epoxy-2(E)-decenal-induced formation of 1,N-etheno-2'-deoxyadenosine and 1,N-etheno-2'-deoxyguanosine adducts." *Chemical Research in Toxicology* 15 (3), 300–304.

Lee, S. H., Oe, T., and Blair, I. A. (2001). "Vitamin C-induced decomposition of lipid hydroperoxides to endogenous genotoxins." *Science* 292 (5524), 2083–6.

Marcel, S. F., Jie, L. K., Lam, N. W., Ho, C. M. and Lau, M. L. (2003). "Epoxidation of a conjugated linoleic acid isomer." *European Journal of Lipid Science and Technology* 105 (8), 391–396.

Marmesat, S., Velasco J., and Dobarganes, M. C. (2008). "Quantitative determination of epoxy acids, keto acids and hydroxy acids formed in fats and oils at frying temperatures." *Journal of Chromatography A* 1211 (1-2), 129–34.

Meshram, P. D., Puri, R. G. and Patil, H. V. (2011). "Epoxidation of wild safflower (carthamus oxyacantha) oil with peroxy acid in presence of strongly acidic cation exchange resin IR-122 as catalyst." *International Journal of ChemTech Research* 3 (3), 1152–1163.

Moreno, M., Olivares, D. M., Amezquita Lopez, F. J., Martines, V. P., and Reig, F. B. (1999). "Study of the formation of carbonyl compounds in edible oils and fats by ^{1}H-NMR and FTIR." *Journal of Molecular Structure* 483, 557–561.

Munger, D., Sternson, L. A., Repta, A. J., and Higuchi, T. (1977). "High-performance liquid chromatographic analysis of dianhydrogalactitol in plasma by derivatization with sodium diethyldithiocarbamate." *Journal of Chromatography* 143 (4), 375–82.

Nawar, W. W. (1969) "Thermal degradation of lipids" *J. Agr. Food Chem* 17, 18-21.

Nawar, W. W. (1986) Lipids. In Fennema, *Food Chemistry* pp. 225-320. New York: Academic.

Nelis, H. J., Alry, S. C. and Sinsheimer, J. E. (1982). "Comparison of the alkylation of nicotinamide and 4-(*p*-nitrobenzyl)pyridine for the determination of aliphatic epoxide." *Analytical Chemistry* 54, 213–216.

Nelis, H. J., and Sinsheimer, J. E. (1981). "A sensitive fluorimetric procedure for the determination of aliphatic epoxides under physiological conditions." *Analytical Biochemistry* 115 (1), 151–7.

Nielsen, H. K., Löliger, J. and Hurrell, R. F. (1985). "Reactions of proteins with oxidizing lipids." *British Journal of Nutrition* 53 (1), 61-73.

Piazza, G. J., Nuñez, A., and Foglia, T. A. (2003). "Epoxidation of fatty acids , fatty methyl esters , and alkenes by immobilized oat seed peroxygenase." *Journal of Molecular Catalysis* 21, 143–151.

Polozov, G. I., Tishchenko, I. G., Suboch, V. P. (1986). "Mass spectra and ^{13}C NMR spectra of the adducts of epoxyenamines with sulfene." *Chemistry of Heterocyclic Compounds* 22 (2), 217–221.

Rather, L. J., Knapp, B., Haehnel, W. and Fuchs. G. (2010). "Coenzyme A-dependent aerobic metabolism of benzoate via epoxide formation." *Journal of Biological Chemistry* 285 (27), 20615–24.

Rebsdat, S., Mayer, D., ed (2001) "Ethylene Oxide" In *Ullmann's Encyclopedia of Industrial Chemistry*. Wiley-VCH, Weinheim, Germany.

Schaich, K. M. (1980). "Free radical initiation in proteins and amino acids by ionizing and ultraviolet radiations and lipid oxidation. Part II. Ultraviolet radiation and photolysis." *Critical Reviews in Food Science and Nutrition* 13 (2), 131–159.

Schaich, K. M. (1992). "Metals and lipid oxidation. Contemporary issues." *Lipids* 27 (3), 209–218.

Schaich, K. M. (2005). "Lipid oxidation: theoretical aspects." In Bailey's Industrial Fat and Oil Products, 6th Edition, Vol.1, Shahidi, F., John Wiley, ed, New York, pp. 269-355.

Schaich, K. M. (2008). "Co-oxidation of proteins by oxidizing lipids. In *Lipid Oxidation Pathways*, Vol. 2., Kamal-Eldin, A. and Min, D. ed, AOCS Press, Champaign, IL, pp. 181–272.

Schaich, K. M. (2012). "Lipid oxidation." In *Biochemistry of Foods*. Eskin, N.A.M. and Shahidi, F. ed, Academic Press, New York, pp. 419–478.

Schaich, K. M. (2012). "Thinking outside the classical chain reaction box of lipid oxidation." *Lipid Technology* 24 (3), 55–58.

Sevanian, A., Mead, J. F. and Stein, R. A. (1979). "Epoxides as products of lipid autoxidation in rat lungs." *Lipids* 14 (7), 634–43.

Shahidi, F. and Wanasundara, U. N. (1996). "Methods for evaluation of the oxidative stability of lipid-containing food." *Food Science and Technology* 2 (2), 73–81.

Solomons, T.W.G. (2004) "Epoxides" In Organic Chemistry, Brennan, D., ed, John Wiley and Sons. Inc, Danvers, CT, pp. 516-519.

Spiteller, G. (2003). "Are lipid peroxidation processes induced by changes in the cell wall structure and how are these processes connected with diseases" *Medical Hypotheses* 60 (1), 69–83.

Thomas, J. J., Kim, J. H. and Mauro, D. M. (1992). "4-(4-Nitrobenzyl)pyridine tests for alkylating agents following chemical oxidative activation." *Archives of Environmental Contamination and Toxicology* 22 (2), 219–227.

Tung, H.C., Kang, C. and Sawyer, D. T. (1992) "Nature of reactive intermediate from the iron-induced activation of hydrogen peroxide agents for the ketonization of methylenic carbons, the monooxygenation of hydrocarbons and the dioxygenation of arylolefins" J. Amer. Chem. Soc 114, 3445-3455

Van Damme, F., and Oomens, A.C. (1995). "Determination of residual free epoxide in polyether polyols by derivatization with diethylammonium N,N-diethyldithiocarbamate and liquid chromatography." *Journal of Chromatography A* 696 (1), 41–47.

Wu, G. S., Stein, R. A., Mead, J. F. (1977). "Autoxidation of fatty acid monolayers adsorbed on silica gel." *LIPIDS* 12 (11), 971-978.

Yamaji, T., Saito, T., Hayamizu, K., Yanagisawa, M. and Yamamoto, O. (2013) ^{13}C NMR of 9,10-epoxy-1-decene- Spectral Database for Organic Compounds, SDBS. National Institute of Advanced Industrial Science and Technology (AIST) Japan, http://sdbs.riodb.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi, accessed April 12, 2013.

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