Evaluation of Chemical Assays for Determining Hydroperoxides Levels in Oxidized Lipids

by

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and approved by

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ABSTRACT OF THESIS

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This thesis re-evaluated current analyses for hydroperoxides, the first stable product of lipid oxidation. The objective was to compare linearity of response, accuracy, limits of detection, active concentration range, reproducibility, and required conditions and handling for six commonly-used hydroperoxide assays, using cumene (lipid-soluble) and *tert*-butyl (water-soluble) hydroperoxides as test standards; optimized procedures were then applied to oxidized methyl linoleate.

Traditional iodometric titration method with thiosulfate is the most accurate assay chemically. It is stoichiometric, linear, and useful for high peroxide concentrations, but unclear endpoints limits sensitivity and many handling issues must be controlled to provide reproducible results. It is the only method providing absolute quantitation of hydroperoxides.

PeroxySafe^{TM} and PeroxoQuant^{TM} commercial kits based on the xylenol orange assay detected nanomoles of hydroperoxides, but samples with more than trace levels of hydroperoxides (the usual case with foods) must be diluted extensively before analysis. Variation of reaction response varied with hydroperoxide structure is a major

disadvantage for this assay, and the Fe^{3+}-xylenol orange complex was readily bleached by excess hydroperoxide, thus reducing apparent hydroperoxide levels. Reaction stoichiometry cannot be determined due to proprietary reagents of unspecified concentration.

The ferric thiocyanate method (chemical reaction or Cayman LPO^{TM} kit) is extremely sensitive, detecting as low as 5 nanomoles, but the reaction stoichiometry varies with solvent and hydroperoxide structure and concentration. Fe^{3+}-SCN complexes bleached at high hydroperoxide concentrations, causing underestimation of peroxide values. Extensive dilution of samples is thus required for analyses of lipid extracts from most foods.

Due to these complications, xylenol orange and Fe^{3+}-thiocyanate assays may be useful for monitoring changes of single materials over time or comparing extracts with comparable fatty acid composition, but they cannot determine absolute hydroperoxide concentrations. No optical assay tested matched peroxide values determined by iodometric assay.

Finally, hydroperoxides oxidize triphenylphosphine selectively and stoichiometrically to triphenylphosphine oxide that can be detected and quantitated by HPLC, detecting as low as 5 picomoles of hydroperoxide. The reaction has promise, but needs further investigation before adoption. Results for all methods highlight the importance of excluding oxygen during the assays and understanding the correct concentration range for each assay.

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1. INTRODUCTION

Lipid Oxidation has interested humans for centuries. In 1800 a Swiss Chemist by name of Nicolas-Théodore de Saussure observed a layer walnut oil exposed to air for a year using simple mercury nanometer (Leray, 2011). This observation led to the recognition that lipid oxidation is a major degradative reaction limiting shelf life and destroying quality of foods. Thus, over the years since, scientists have continued to study the causes, development, and products of lipid oxidation in foods and a wide range of model systems (Lea, 1946; Hills & Thiel, 1945: Gupta, 1973; Jiang et al., 1992; Eymard & Genot, 2003; Gay et al., 1999; Grau et al., 2000). While knowledge about lipid oxidation has grown as science has advanced, the process remains complicated. Even more importantly, with current trends in replacing stable saturated and monounsaturated fats with "healthy" polyunsaturated oils in foods, lipid oxidation has once again become a huge problem for the food industry.

The average consumer recognizes lipid oxidation as "rancidity". A consumer notices a food is rancid by odors or off flavors caused by volatile products released from oxidizing lipids. In general, these compounds are detrimental to food quality, although there are certain foods such as cheeses, fried foods, and dried cereal for which small amounts of lipid oxidation products are required to develop full characteristic flavors (McClements & Decker, 2008). Oxidation of the lipids are the main cause of chemical deterioration, reduced stability, and formation of off-flavors that reduce the quality, storage life, and consumer acceptance of most processed foods (Hornero-Méndez et al., 2001).

For food industry the oxidative deterioration of food has large economic

importance (Frankel, 1985) because lipid oxidation products can be found in all natural food materials (Kamal-Eldin & Pokorny, 2005). Even minute amounts present after processing can translate to greatly accelerate reactions during storage, with parallel changes in flavor, odor, texture, color, and loss of nutritional value (Schaich, 2009, 2012). There are even some claims that potentially toxic or carcinogenic products are formed (Shahidi, 1998; Schaich, 2012).Consequently, it is imperative that food scientists be able to track lipid oxidation at very low concentrations in order to control food quality all the way from ingredients through processing to products sitting for indefinite times in storage at consumers' homes.

Analysis of lipid oxidation is not simple. The complexity of lipid oxidation reactions and products formed means there is no straightforward way to monitor the entire process. One particular difficulty is that lipid oxidation reactions are at the same time both sequential and overlapping (Kamal-Eldin & Pokorny, 2005) with products constantly forming and decomposing to other products. Also, there is no set series of reactions – alternate pathways are active and the balance between them varies with conditions and reaction system (Schaich, 2005). This means that analyses of multiple products must be performed to obtain a complete picture of the oxidation process. To further complicate matters, not only do foods contain natural components other than lipids that can react with lipid free radicals, hydroperoxides, aldehydes, etc, but minor components of fats and oils also further react with radicals and hydroperoxides (Kamal-Eldin & Pokorny, 2005). These side reactions can make lipid oxidation appear to be low when in fact they have just been broadcast to other molecular targets. Thus, full tracking

of lipid oxidation requires analyses of co-oxidations, e.g. protein radicals and crosslinking, in addition to direct lipid oxidation products (Schaich, 2008).

Now add to these issues additional technical problems. First, all traditional analyses of lipid oxidation require extraction of lipids, and this process can itself induce oxidation or decompose some products into others. Second, analyses vary in their sensitivity, stoichiometry, detection ranges, and side reactions, yet these limitations are routinely ignored. Rather, the common method for selecting a lipid oxidation assay is to either

a) follow a method standardized by AOCS or AOAC, most of which were developed for oils decades ago using insensitive methods, or

b) Following a method cited in the literature, for which complete experimental details and appropriate concentration ranges are seldom available.

Both of these approaches can miss the target concentration range for lipid oxidation in foods, though in opposite directions. In particular, some of the more recent methods developed to detect ultralow levels of lipid oxidation products in living tissues have been adapted for use in foods, but these show response saturation at nano or micro molar levels. As a result, it is very easy to get results indicating no lipid oxidation in foods.

Returning to an earlier point, with current focus on reformulating foods with polyunsaturated oils to support improved health, the time has come to re-evaluate how lipid oxidation is measured in foods. As part of a larger program designed to establish a rational quantitative basis for selection of lipid oxidation assays for foods, this thesis focused on hydroperoxides assays. Five assays based on three fundamental reactions were tested for upper and lower detection limits, linear response ranges, stoichiometry of

reaction, accuracy and reproducibility, and required handling procedures and quirks. A sixth assay was given preliminary investigation. Comparisons of results provide some guidelines for selection and use of these assays for different types of samples.

It must be noted that this thesis focuses on optimizing assays to obtain the most accurate information possible, and comparing assays to determine appropriate concentration ranges and conditions for use of each assay. The expectation was some assays would be identified as useful for trace concentrations and others might be the method of choice for samples with high oxidation levels. This thesis is not addressing the issue of whether hydroperoxides should even be measured or whether hydroperoxides detected in extracts accurately reflect actual oxidation in original materials (foods or biological tissues). Kamal-Eldin has pointed out that analyzing whole lipid oxidation mixtures is generally easier than analyzing separate components, and separation of lipids can tremendously complicate interpretation of results (Kamal-Eldin & Pokorny, 2005). Isolating oxidized lipids from common foods is not easy, particularly when lipid oxidation products become bound to proteins and similar compounds with covalent bonds and thus are lost to normal analysis. The handling required in extraction and subsequent storage can induce extraneous oxidation or change existing products. Hydroperoxides are a particular case in point since they are decomposed by ultraviolet light and temperatures above about 40 °C. Finally, none of the various classes of lipid oxidation products are stable, and they rapidly undergo transformations with improper handling and storage (Kamal-Eldin & Pokorny, 2005). These are indeed important points to keep in mind when monitoring lipid oxidation, particularly hydroperoxides. However, the issues are beyond the scope of this research project.

2. BACKGROUND

2.1. General Overview of Lipid Oxidation

Lipid oxidation occurs in three stages. Lipid oxidation is not thermodynamically spontaneous. Although this is an oxidation reaction, oxygen cannot add directly to double bonds because the electrons in double bonds and in ground state oxygen are all parallel in the same direction. However, oxygen adds almost instantaneously to free radicals, so the initiation step in lipid oxidation requires high activation energy to produce alkyl free radicals on the lipid hydrocarbon chain (Schaich, 2005). This high energy comes most commonly from light, heat or metals, or radicals formed in other reactions such as decomposition of pre-formed trace hydroperoxides (Schaich, 2005). Alkyl radicals are formed at various positions depending on the initiator. Oxygen then adds to the alkyl radicals to generate peroxyl radicals, $LOO^{\bullet}$ (Kamal-Eldin & Pokorny, 2005; Schaich, 2005).

$LOO^{\bullet}$ are moderately reactive and abstract hydrogen atoms from a neighboring lipid chain to form stable hydroperoxides and new radicals. So each time a peroxyl radical is converted into lipid hydroperoxide, it produces another lipid radical which continues the chain. This basic process continues indefinitely, providing the basis for the second stage, Propagation, which establishes the characteristic free radical chain reaction of lipid oxidation (Schaich, 2006). Hydroperoxides are relatively stable. However, LOOH decomposes in the presence of metals to radicals plus ions,

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + {}^{-}OH,$$

$$LOOH + Fe^{2+} \rightarrow LOO^{\bullet} + {}^{+}H$$

or in the presence of light or heat to yield two even more reactive lipid alkoxyl and hydroxyl radicals,

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

All of these radicals abstract hydrogens to keep existing chains going and to generate new chains (called branching) (Schaich, 2005). As a result, oxidation rates begin to increase exponentially.

Free radical transfers continue and do not stop until there are no hydrogens available or the chain is interrupted (Schaich, 2009), i.e. the chain is terminated. In the termination step, two radicals can combine to form an infinite variety of nonradical products, or alkoxyl radicals. Then they can undergo scission on either side of the C-O$^{\bullet}$ bond to release mostly short chain alkanes and aldehydes, some of which are volatile. Figure 1 shows how these reactions are integrated into the three stages of lipid oxidation.

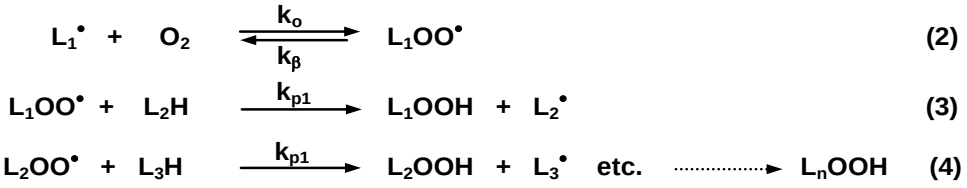
The rate of lipid oxidation depends on many factors, including system environment and solvent (temperature, light, oxygen pressures, etc), presence of other components (pro-oxidants, antioxidants, interceptors), nature or form of lipid (degree of unsaturation, trans vs cis isomers, phospholipids, etc), and molecular surfaces (Schaich, 2009). Rate information is important because a lipid analyst can slow oxidation during handling and analysis by decreasing temperature, or by storing samples under reduced oxygen pressure (partial to full vacuum) or inert gas. Addition of antioxidants can reduce oxidation rate, but not completely stop it, unless initiation is also blocked (Schaich, 2009). Most foods contain enough redox-active metal concentrations high enough to catalyze lipid oxidation; these are impossible to remove completely and very difficult to

CLASSICAL FREE RADICAL CHAIN REACTION MECHANISM OF LIPID OXIDATION

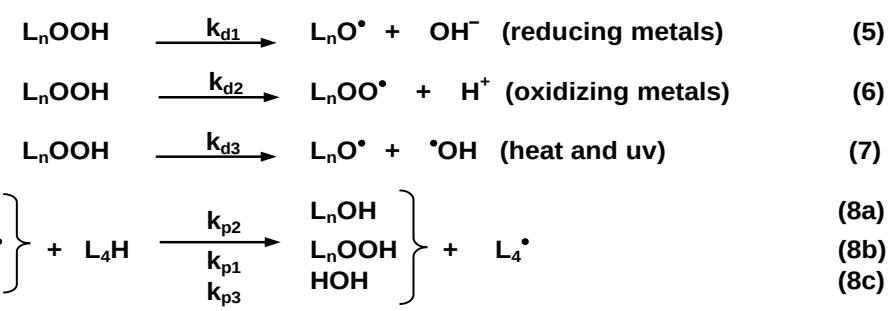
Initiation *(formation of ab initio lipid free radical)*

Propagation

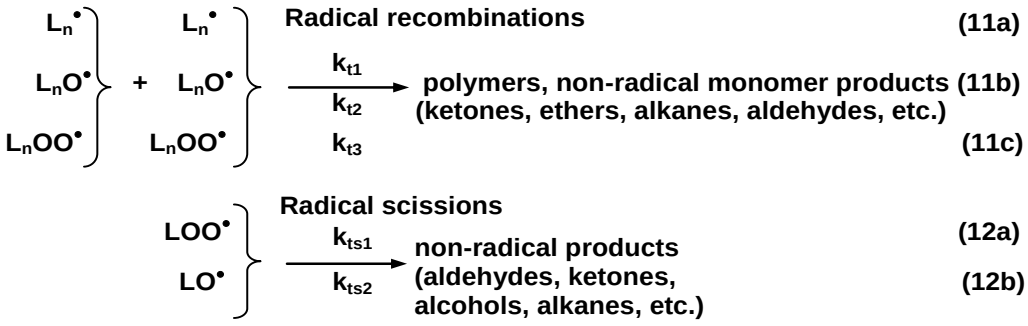
Free radical chain reaction established



Free radical chain branching (initiation of new chains)



Termination *(formation of non-radical products)*



i - initiation; o-oxygenation; β-O_2 scission; p-propagation; d-dissociation; t-termination; ts-termination/scission

Figure 1. Free radical reactions sequence in lipid oxidation described by classical theory (Schaich 2005)

bind and inactivate completely by chelators (Love, 1895; Schaich, 1992).

Polyunsaturated lipids oxidize more rapidly than monounsaturated (Uri,1961); saturated lipids are stable to autoxidation but can degrade at high temperatures (Nawar, 1986). Increasing the number of double bonds increases rates of lipid oxidation; ease of formation of fatty acid radicals increases with increasing unsaturation (Uri, 1961); (Frankel, 1985; Schaich, 2012) because the hydrogens on the methylene groups between two double bonds have much lower bond energies than at other positions on the acyl chain, and hence are more easily abstracted (Schaich, 2006).

All current analyses follow the three oxidation stages just described. Lipid radicals [$L^{\bullet}$ or $LO(O)^{\bullet}$] formed in any stage are short-lived to detect directly, even by electron paramagnetic resonance, although they can be trapped with spin traps (Schaich, 1980). The first detectable stable products are conjugated dienes formed when radical and hydroperoxide formation at external positions of 1,4 dienes structures, e.g. in linoleic and linolenic acids, forces a migration of the neighboring double bond:

$$\text{-CH=CH-}\dot{\text{C}}\text{H-CH=CH-} \rightarrow \text{-}\dot{\text{C}}\text{H-CH-CH-CH-CH-} \rightarrow \text{R-CH=CH-CH=CH-}\dot{\text{C}}\text{H-}$$

Conjugated dienes are the only lipid chromophore with significant absorption in the UV range, from 231-234 nm. Because of its ease and simplicity, this assay has been used extensively to follow lipid oxidation. However, the extinction coefficient varies with the solvent, conjugated dienes are not permanent but are generated even while the assay is underway and also are lost as lipids degrade to secondary products, and phenolic antioxidants in oils absorb in the same region and interfere with the analysis. Thus, conjugated dienes are best used when following the same system over time. They can only be considered general indicators of oxidation when spot-checking samples of

unknown history (e.g. pulling a sample off the shelf in a warehouse), and in most cases should be combined with other product analyses to accurate interpret the extent of lipid oxidation.

Termination products of lipid oxidation -- aldehydes, ketones, furans, alcohols, epoxides, and others -- are responsible for well-known flavors and odors of oxidized lipids. However, these products are difficult to detect because many of these products are not stable, they are present at very low concentrations, they are present in complex mixtures that are difficult to separate, and they are reactive so transform to other products and complex with food molecules, particularly proteins. Most commonly, secondary products are monitored as volatiles detected by gas chromatography (Frankel et al., 1977, 1979, 1984) or the Rancimat assay (Barrera-Arellano, 1992), but these approaches ignore non-volatile degradation products. A few chemical reactions are available for detecting aldehydes, e.g. the anisidine assay and formation of dinitrophenylhydrazones, but each has significant short-comings so are not used as routine measures (although they should be, in combination with conjugated dienes and other measures). It must also be noted that following or assessing lipid oxidation only by secondary products misses all the early reactions and can lead to incorrect interpretation of the extent of lipid oxidation. Secondary products are formed too late in the reaction sequence to be used for monitoring and control.

Last in this discussion but not in priority of oxidation products are the lipid hydroperoxides (LOOH), the first stable product that forms in all fatty acids regardless of degree of unsaturation. Hydroperoxides are the product most commonly monitored in the food industry and are also among the most controversial due to problems and with assays

and inconsistency of results (Schaich, 2012). Because of the serious issues with LOOH assays and the acute need for accurate improved LOOH assays in the food industry, this thesis research focused on hydroperoxides assays.

2.2. Assays for Lipid Hydroperoxides

2.2.1. General Considerations

Oxidation analysis is not simple ‘since food lipids can contain many different unsaturated fatty acids and can be exposed to several different prooxidants, hundreds of decomposition products can be formed (McClements & Decker, 2008). The complexity of pathways that consequently occur makes analysis very challenging.

Numerous analytical methods are available to measure lipid hydroperoxides, and choosing the best method for a particular application can be difficult (Dobarganes & Velasco, 2002). These methods can be separated into two groups based on endpoints: quantitative and qualitative. Chemical analytical methods are quantitative and determine the total amount of hydroperoxides, while chromatographic techniques are qualitative and give information about the structures and types as well as amounts of specific hydroperoxides present in test samples (Dobarganes & Velasco, 2002). Thus, the most appropriate method must be determined for each application, depending on what information is most needed. In most cases, qualitative assays that identify specific products are used mostly for basic research, particularly when reaction mechanisms are in question, whereas quantitative assays are used both in research and in practical applications such as storage stability studies and monitoring degradation of products on-line and after storage.

Each method has its positive and negative attributes, and these must be balanced when deciding upon an assay to use. For example one method may be ‘simple to apply but unreliable or expensive’, while another may be ‘highly sensitive, but difficult to control’ (Hara & Totani, 1988 p.1948). The nature of the sample matrix, number of samples to be analyzed, and quality parameters required also must be considered when determining the method which most consistently determines lipid hydroperoxides in food systems (Navas et al., 2004).

It is critical to keep in mind that every determination still only reflects oxidation status at the particular time of sampling, without history. Thus, due care must be exercised to avoid misinterpretation of results. For example, when primary lipid oxidation products are the only products measured, especially later stages of oxidation when formation rate becomes slower than decomposition rate, concentrations of these products will naturally decrease without a decrease in oxidation (McClements & Decker, 2008). Assays of a single product at a single time in food samples without prior testing or monitoring additional oxidation products to detect other pathways can lead to the erroneous conclusion of low lipid oxidation.

Another issue that can lead to misleading or erroneous results is that with certain conditions such as high temperature or high levels of reactive transition metals, primary product concentrations may show minimal net increase because decomposition rate is so high (McClements & Decker, 2008). Isolated biological samples often have peroxides naturally and if stored in presence of oxygen, these can contribute to overall oxidation products and can cause overestimation of sample composition (Fukuzawa et al., 2006). Endogenous peroxides in a sample can be detected in the assay in the same way the

hydroperoxides that are formed, leading to higher PV. Endogenous hydroperoxides are a particular problem in the presence of oxygen, where they serve as rapid initiators of new oxidation chains.

2.2.2. Iodometric Titration of Hydroperoxides with Thiosulfate

Volumetric methods of hydroperoxide determination, such as titrations, have been in use for more than 50 years (Pokorný, 2005). Titration methods are very simple, rapid, and require only unsophisticated equipment. Many titrations have 1:1 stoichiometry and are suitable for evaluation of large number samples (Dobarganes & Velasco, 2002).

Iodometric titration of hydroperoxides was developed and standardized, and official methods were published by AOCS in 1997(Method Cd 8-53). Since very little has actually changed in the methods, many references citing use of iodometric titration are very old, and the method is still used extensively in the food and oils industries. The discussions and points made in these articles are still very useful because many of the limitations and issues raised in these articles are not known and are not referenced in the official methods (Pokorný, 2005).

The reaction chemistry of the titration is very straightforward, developed from observations that all peroxidic compounds react with iodide ions, and are reduced to hydroxyl derivatives while at the same time, iodide ions are oxidized further to free iodine (Pokorný, 2005).The hydroperoxide reduction proceeds as:

$$LOOH + 2\,H^{+} + 2e^{-} \leftrightarrow LOH + H_2O$$

The electrons for this reduction are provided by the saturated KI which dissociates in solution:

$$2KI \leftrightarrow 2\,K^{+} + 2\,I^{-}$$

$$2\,I^{-} \leftrightarrow I_2 + 2\,e^{-}$$

This is an equilibrium reaction. When the electrons are removed, e.g. by reaction with oxygen or with LOOH, the reaction is driven to the right. Otherwise, I^{-} is favored.

In the net reaction, reduction of one LOOH releases one I_2 for reaction with thiosulfate.

$$LOOH + \underset{\text{(clear)}}{2\,H^{+}} + 2\,KI \rightarrow I_2 + \underset{\text{(yellow)}}{LOH} + H_2O + 2\,K^{+}$$

In presence of excess iodide, a complex ion that reacts in same way as free iodine is formed. The released iodine is then titrated, usually with standardized sodium thiosulfate, which becomes oxidized into a tetrathionate (reduction of free iodine with thiosulfate).

$$\underset{\text{(yellow)}}{I_2} + 2\,Na_2S_2O_3 \rightarrow \underset{\text{(colorless)}}{Na_2S_4O_6} + 2\,NaI$$

Starch is used as indicator; it forms a blue-purple complex with iodine molecules. When all the iodine has been reacted, the solution loses its color, and the transition from purple to clear marks the endpoint of the reaction (Skoog et al., 1998). The reaction should take place in the dark or in diffused daylight to prevent decomposition of hydroperoxides (Pokorný, 2005). If following the standard AOCS method, five grams of sample are recommended for peroxide values below 10, and about 1 gram of sample for peroxide values greater than 10 (Frankel, 1998) although certainly smaller quantities of lipids are routinely analyzed.

Despite the simplicity of the reaction chemistry and actual method, there are a large number of inherent disadvantages.

- The titration is highly empirical, so in order to replicate results special care must be taken to maintain exact conditions. Even small changes, such a new technician

or different grade of solvent, can cause variations in results. Consequently, it has been recommended that this method be used mainly for evaluation of related samples or determining evolution of hydroperoxides in the same samples over time (Dobarganes & Velasco, 2002).

- The chloroform used as solvent is organic and toxic, and replacement solvents introduce still more complications.
- This is one of the least sensitive methods (cannot detect PVs less than 0.5 meq/kg) because of difficulty in visually distinguishing the color change marking the actual end point determination. The color fades gradually in many shades rather than colored to clear instantaneously.
- The reaction is highly sensitive to oxygen, so accurate and reproducible results require scrupulous oxygen removal.
- Due to the empirical nature of the methodology, validity of results cannot be determined until the method used is also given (Gray, 1978).

Many papers have investigated various conditions that can affect the peroxide levels determined by the titration. Both ‘hot’ and ‘cold’ methods have been developed. Lea’s ‘hot’ method involved heating the solvents and oil/fat to boiling point (Lea, 1946). The ‘cold’ method usually referred to as ‘Wheelers’ is completed at room temperature, dissolving oil/fat in solvent without heat involved (Lea, 1946). In both the ‘hot’ and ‘cold’ methods (including modifications suggest by other authors) various factors such as method and length of deaeration, solvent composition, order of mixing reactants, effect of water, sample concentrations, and reaction time were all investigated (Lea, 1946, 1952; Stuffins & Weatherall, 1945).

Interestingly, while conducting these early studies, Lea observed that that many scientists, when comparing effects of factors assumed that the highest reproducible value was the correct one, and this was an incorrect assumption (Lea, 1946). At the time, prevailing thought was that peroxide values were too low because of "incomplete reaction of more stable peroxides with the reagent, possible decomposition of very labile peroxides by side reactions, and loss of part of liberated iodine owing to re-absorption of fat" (Lea, 1946, p.290). While these are all valid problems to consider, Lea's assessment was still accurate. Even so, as Lea pointed out, studies assuming the highest value is correct could provide some valuable information, as long as absolute peroxide value was not needed. One recommended application was simple comparative studies that follow the oxidation of sample during different storage conditions (Lea, 1946).

In 1960, Mehlenbacher determined that there are two principle sources of error in iodometric titration; both have been widely observed and are considered to be true (Mehlenbacher, 1960). First, he identified of potassium iodide to molecular iodine by oxygen present in titrating and titrated solutions as the source of "oxygen error" that leads to high results in peroxide determinations. The second source of error is adsorption or addition of iodine to unsaturated bonds of fatty acids, which decreases PVs as proposed even by early scientists. Careful controls are necessary to avoid both kinds of error.

Issues with oxygen have been recognized since the earliest use of this method. Numerous articles have documented that when a titration is completed without deoxygenation, peroxide values are higher. However, there is no simple solution to the problem. To exclude oxygen from the reaction, Lea (1931) filled the sample tube with nitrogen at beginning of the test and assumed that evolution of chloroform would prevent

reentry of oxygen into the tube. Wheeler (1932) reformulated the reaction in homogenous solution to eliminate the need for shaking, thus minimizing oxygen effects. The 'hot' and 'cold' methods discussed above were both adapted to include inert gas bubbled through reaction mixtures throughout the reaction; flooding only the headspace with inert gas or not degassing at all gave much higher peroxide values (Stuffins & Weatherall, 1945). In 1954, rather than using inert gas, Sully mixed reactants in boiling acetic acid-chloroform solution to reduce dissolved oxygen (Sully, 1954). He observed that the iodine liberated was related, not to partial pressure of oxygen during reaction, but to the quantity of peroxide decomposed. He claimed that when iodide and peroxides react, radicals are formed, and even with short lifetimes these radicals react with gaseous oxygen and produce more peroxides (Sully, 1954). Until that time, it was believed that subtracting a blank could take care of the oxygen error. However, Sully asserted that the "effect of radical reactions cannot be estimated by blank determination" (Sully, 1954, p.86). Radical production in his system probably was due to thermal decomposition rather than iodide reaction, and these indeed cannot be covered by blanks which contain no lipid, but his observations are still quite interesting. Despite Sully's findings, standard official methods still include in their directions a blank subtraction step. This controversy illustrates why the method remains somewhat controversial and why results from different labs can be quite different. In fact, both blanks and argon sparging are required, but for different reasons. The blank accounts for reactive species in the solvents and reagents, while the argon eliminates oxygen that increases conversion of KI to I_2.

Oxygen error also brings up another important factor for iodometric titration, which is reaction time. Longer reaction time increases diffusion of oxygen into solutions

and emphasizes slow reactions at the expense of fast ones. Early methods of reaction at room temperature, e.g. Lea's cold method, had a reaction time of one hour in the dark (Lea, 1946; Stuffins & Weatherall, 1945). "Hot" iodometry always required less reaction time. Lea's 'hot' method had flask immersion in boiling water bath for only about 30 seconds and then transferred to 77 degree C bath for about 2 minutes, cooled, water added and then titrated (Lea, 1946). It was later determined that the LOOH-KI reaction is fast enough that when LOOH concentration is low, reaction time is not critical and PV does not change drastically with increasing reaction times (Dobarganes & Velasco, 2002).

Most methods today recommend only 1-2 minute reaction time. The AOCS (Method Cd 8 -53) standard procedure recommends 1 minute reaction time and specifies under diffuse daylight and room temperature (no inert gas) (Dobarganes & Velasco, 2002). However, three separate studies all concluded that one minute reaction time was not enough for complete reaction between lipid peroxides and iodide (or iodine) (Lezerovich,1985; Tian & Dasgupta, 1999). As cited by Pokorný (Pokorný, 2005), Yanishlieva and Popov in 1972 (Yanishlieva & Popov, 1972) found 5 minutes at room temperature was optimum for oxidized oils, while 1 minute reactions were sufficient for fresh fats and oils. IUPAC method agrees with this five minute reaction time at room temperature under diffuse daylight; like AOCS, IUPAC omits sparging with inert gas (IUPAC Method 2.501). The Japan Oil Chemists' Society official method (Std Method 2 4 12-71) also has a five minute reaction, but in contrast to AOCS and IUPAC, takes steps to avoid iodide autooxidation.

Perhaps because of radical complications from thermal decomposition of hydroperoxides, "hot" iodometry also appears to have fallen out of favor as a method. No current standard methods suggest heating solvents. Although an early study in 1945 found results between "hot" and "cold" method were similar, specifically when both bubbled with inert gas and saturated KI solution was used instead of solid KI, final recommendation were to use the "cold" or room temperature method (Stuffins, 1945). Several decades later, a study also showed that peroxide values of different oils were similar to the "cold" Wheeler and "hot" Sully method (though "hot" PV tended to be slightly lower), but further testing carried out by the group showed that 'hot' methods resulted in almost complete disruption of lipid hydroperoxides (Barthel & Grosch, 1974). At ambient temperatures, the LOOH-KI reaction is faster than LOOH breakdown to $LO^{\bullet}$ + $^{\bullet}OH$. Although LOOH-KI reactions accelerated at higher temperatures, the corresponding acceleration of hydroperoxide decomposition was even greater (Barthel & Grosch, 1974). Similarly, comparisons of a new hot method with Lea's hot and Stuffins 'cold' showed similar values, but "hot" values were slightly lower (Sully, 1954). In contrast, Amar et al showed the hot "Sully" Method PVs were significantly higher than "cold" Wheelers for Linseed Oil and Cotton seed oil (Amer et al., 1961).

Returning to the second error source cited by Mehlenbacher (1960), adsorption or addition of iodine to unsaturated bonds of fatty acids, in effort to perfect an assay based on oxidation of iron II to iron III (these type assays will be discussed later on), Tian and Dasgupta discovered another issue in iodometric methods that cannot be solved by increasing reaction time to allow from complete reaction or by subtraction of blanks. This issue was that all oils and fats of food origin contain some unsaturated sites that consume

iodine liberated by LOOH-KI reaction, leading to underestimation of actual peroxide content (Tian & Dasgupta, 1999).

Solvents have been another issue in iodometric titrations of lipid hydroperoxides, from several standpoints. One source of contention has been the ratio of chloroform to acetic acid. Stuffins and Weatherall study showed the when ratio of chloroform was greater than acetic acid then lower values are obtained (Stuffins & Weatherall, 1945). Acetic acid is needed to support ionization of KI, but Sully stated that present work showed that if insufficient chloroform is added to depress ionization of the acetic acid, potassium iodide precipitates and there is incomplete reaction (Sully, 1954). Another issue is that there must be sufficient H+ ions for complete reaction and acetic acid does not always provide this.

There have been attempts to replace toxic chloroform with acetone, ethyl alcohol, and isopropanol, but these solvents each react with iodine in the presence of water and acid so are unsuitable for this assay (Osawa et al., 2007). AOCS has approved an alternative official method using iso-octane as the lipid solvent (AOCS Cd 8b-90). However, poor miscibility with polar solvents creates new problems for the assay. For example, one study found that a 15-30 second delay in neutralizing the starch indicator at high PVs (>70 meq/kg) was caused by isooctane floating on the surface of the aqueous medium –extra time was required to adequately mix solvents when large volumes of titrant were added (Brooks & Berner,1990). Failure to note this condition can cause over-titration. As a result, the iso-octane official method recommends that it not be used for high peroxide analyses. Another problem is that while isoctane is less toxic, it can

create a fine emulsion that makes detecting the endpoint difficult and the assay overall less accurate (Kamal-Eldin & Pokorny, 2005).

The difficulty in visually determining the color endpoint is a perpetual problem that lowers the sensitivity of the assay and has spurred scientists to find alternative approaches. The most common is substitution of electrochemical or potentiometric determination of the endpoint while performing the titration in an electrochemical cell. The liberated iodine is reduced at a platinum electrode maintained at constant potential; the resulting current can be quantified by electronic integration, detecting with good precision peroxide values as low as 0.06 (Fiedler, 1974). It is essential that all solutions are dearated to prevent formation of peroxides when using this method (Shahidi & Wanasundara, 2002). Radicals can form from reaction of peroxide with iodide, which can react with oxygen and produce more peroxides (Sully, 1954).

In another approach, replacing the visual endpoint with potentiometric determination in the standard AOCS titration detected as low as 40 neq hydroperoxide (Hara & Totani, 1988). Two important changes helped achieve high accuracy (very small coefficient of variation). First, less than 1 mol of saturated potassium iodide was added when PVs were less than 100 meq/kg. At these peroxide levels, the free iodine formed by oxidation in the saturated potassium iodide solution increased COV, and decreasing the amount of iodide added limited the interference. Secondly, the reaction should be cooled (run on ice) and kept at a constant temperature because “solubility of potassium iodide in water is influenced by the atmospheric temperature”, and at higher temperatures, potassium iodide can more easily react with oxygen to produce free iodine and lipids also undergo oxidation more easily (Hara & Totani, 1988, p.1950; Schaich, 2005).

Another approach to increase sensitivity has been to abandon the thiosulfate titration altogether and convert the iodine detection to an optical assay. One really old method by Baldwin et al. (1944), cited by Hicks & Gebicki (Hicks & Gebicki, 1979), measured the iodine-starch complex at 560 nm. However, the method was not very adaptable because it was complicated and extinction varied with starch source.

A second optical method measured absorbance of the tri-iodide ion formed in the reaction:

$$LOOH + 2\,H^+ + 2\,KI \rightarrow I_2 + LOH + H_2O + 2\,K^+$$

$$I_2 + I^- \leftrightarrow I_3^-$$

In presence of excess I^-, the formation of I_3^- will by favored and it can be quantified by its absorbance at 360 nm (Gebicki & Guille, 1989). As with so many other assays, this one is also very sensitive to traces of oxygen due to oxidation of excesses iodide. Because of this sensitivity, reaction mixtures must be protected from light, solutions must be rigorously dearated before use, and during reaction there must be constant purging with inert gas to exclude atmospheric oxygen. Though some methods simply dearate by flushing with a gas (Takagi et al., 1977) , other procedures set up more elaborate gas flow systems with stoppered or sealed cuvettes (Gebicki & Guille, 1989; Hicks & Gebicki, 1979; Swoboda & Lea, 1958; Nouros, 1999). The procedures with the gas flow systems purge trace amounts of oxygen more fully so give better results. However, the added difficulty of this additional set-up may has prevented wide use of these colorimetric methods.

There are several methods with slight variations based on this chemistry (Gebicki & Guille, 1989; Hicks & Gebicki, 1979; Takagi et al., 1977). One used stoppered

spectrophotometric cuvettes with nitrogen flowing through to remove the oxygen (Hicks & Gebicki, 1979). The solvent and saturated KI are added to the cuvette (bubbled with nitrogen), absorbance recorded at 290 or 360 nm to ensure it did not increase by more than 0.005 units. Then the lipid sample was injected through a capillary, mixed with reagents, and absorbance was recorded until the change was linear with time (Hicks & Gebicki, 1979). Although this method was highly reproducible, had 1:1 stoichiometry, and was very sensitive (1 neq of lipid hydroperoxide), not all labs have the gas flow apparatus necessary.

A variation of this method is to add cadmium to complex with remaining I^- to prevent further oxidation by oxygen (Gebicki & Guille, 1989; Swoboda & Lea, 1958; Takagi et al., 1977). Addition of cadmium plus purging solutions with CO_2 and reacting in the dark has also been tested (Takagi et al., 1977). The I_3^- was measured at 358 or 410 nm, and peroxide values were calculated based on absorbance in a calibration curve. Another variation to improve sensitivity was to inject the reaction into an HPLC for detection instead of reading I_3^- on a spectrophotometer (Gebicki & Guille, 1989). Cadmium salts are extremely toxic (Kamal-Eldin & Pokorny, 2005), and perhaps because of this, these methods are not commonly utilized.

There is one last method based on measurement of triiodide ion. To eliminate oxygen interference, the assay was completed in solutions with low acid added, reduced iodide concentration, and Fe^{2+} as a catalyst (Løvaas, 1992). The acid was needed to supply protons for the reaction, but it also increased iodide oxidation and lipid peroxidation at the same time (Løvaas, 1992). Using less acid prevented autoxidation of lipids and decreased background oxidation of iodide. Fe^{2+} was added to accelerate I_3^-

formation from lipid hydroperoxide, assuming "that iron is kept at its reduced state by excess of iodine and that lipid peroxidation by Fe3+ thus was prevented" (Løvaas, 1992, p.780):

$$ROOH + Fe^{2+} \rightarrow RO^{\bullet} + OH^{\bullet} + Fe^{3+}$$

$$2RO^{\bullet} + 2H+ + 3I^{-} \rightarrow 2ROH + I_3^{-}$$

$$2Fe^{3+} + 3I^{-} \rightarrow 2Fe^{2+} + I_3^{-}$$

$$ROOH + 2H^{+} + 3I^{-} \rightarrow ROH + H_2O + I_3^{-}$$

This approach basically assumes that the iodide added incompletely reduces lipid hydroperoxides, so the reducing power of ferrous iron is needed to complete the reaction. Fe^{2+} did indeed induce a 30-80 fold increase in specific rate of I_3^{-} formation (Løvaas, 1992), but in preliminary evaluations, we found that the mix of aqueous and lipid phases caused serious solubility problems, the iron reactions did not run as proposed, and the reaction was very inefficient and poorly reproducible

2.2.3. Ferric Thiocyanate Assay for Lipid Hydroperoxides

The ferric thiocyanate (FeSCN) method also uses Fe^{2+} reduction of hydroperoxides in acidic media, but in this case the resulting Fe^{3+} ions are complexed by thiocyanate to form a red-violet complex that absorbs strongly between 500-510 nm. This method has greater sensitivity than iodometric titration, requires smaller samples (0.1g) (Frankel, 1998), and gives uncorrected peroxide values that are higher by a factor of 1.5 to 2 (Dobarganes & Velasco, 2002; Frankel, 1998). The latter occurs because the stoichiometry is actually 2:1 (LOOH and $LO^{\bullet}$ both oxidize Fe^{2+}) and the second oxidation by $LO^{\bullet}$ is seldom accounted for in calculations.

The FeSCN assay is an old method. While measuring hydrogen peroxide and succinyl peroxide, Young et al. were the first to see the potential of estimating peroxides based on the color that was produced by ferric thiocyanate complex (Young et. al, 1936). This finding sparked interest in the reaction as a way to quantify peroxide values and several methods were developed, differing in solvent choice, oxygen exclusion, reaction timing, and detection method (Kolthoff & Medalia, 1951; Lea, 1952).

Since then, the literature on the accuracy of this assay has remained very divided. An important aspect of the assay is the reaction stoichiometry, or the moles of Fe^{2+} oxidized by each mole of hydroperoxide. The reaction mechanism indicates that one lipid hydroperoxide should generate two ferric iron complexes:

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+}$$

$$LO^{\bullet} + Fe^{2+} + H^{+} \rightarrow LOH + Fe^{3+}$$

However, stoichiometry reported in the literature has been quite variable. Inclusion or exclusion of oxygen further complicates the assay's accuracy.

Very early it was observed that peroxide values obtained by FeSCN were considerably higher than with iodometric titration in three studies assessing rancidity in edible fats, each with slightly different variations (for variations see Table 1) of the FeSCN method (Chapman & McFarlane, 1943; Hills & Thiel, 1945; Lips et al., 1943). Initially, it was assumed that the FeSCN results were correct and differences were attributed to problems with the iodometric method (e.g., heating of solvent in the "hot" iodometric method destroyed peroxides, resulting in low values). However, at the time both the ferric thiocyanate and iodometric methods were still developing and many

variations were in use. The outcome of validation of the ferric thiocyanate method changed depending on which variant it was compared to.

A competing school of thought was that the iodometric method provided accurate values, and higher values in the FeSCN assay were due to the presence of oxygen that increased autoxidation of Fe^{2+} (Kolthoff & Medalia, 1951; Lea 1945,1946). Wagner also found lower peroxide values in the absence of oxygen but concluded that in presence of molecular oxygen, reduction of $LO^{\bullet}$ was faster than LOOH decomposition, leading to stoichiometric generation of ferric thiocyanate; the opposite was true in absence of molecular oxygen, resulting in lower formation of ferric thiocyianate) (Wagner et al., 1947). He concluded this after results showed comparable peroxide content detected when oxygen was carefully excluded using similar apparatus as Lea (Lea, 1945) and when air was passed for ten minutes through sample and reagent before mixing (Wagner et al., 1947). Additionally, if air was passed through a mixture of oxygen-free sample and reagent after reaction was allowed to take place, no additional color developed; which Wagner et al. stated was because peroxides were being decomposed rather than reduced (Wagner et al., 1947). Lea's results in 1952 agreed that there was no need to exclude oxygen since it complicated a simple method and results were always much lower than expected for oxidized fats (Lea, 1945) and oxidized pure esters (Lea, 1952).

Kolthoff et al. reported that, in the absence of oxygen, the ratio of Fe^{3+} formed to peroxides reacted was less than expected, and he attributed this to competitive peroxide decomposition by factors other than Fe^{2+} so that the iron did not get oxidized (Kolthoff & Medalia, 1951). In contrast, addition of oxygen (when present) to alkyl radicals generated by downstream reactions of $LO^{\bullet}$ (from decomposition of LOOH) forms peroxide free

radicals which further oxidize ferrous iron, causing "high analytical results" (Kolthoff & Medalia, 1951). Kolthoff went on to assert that the accuracy of all the procedures was questionable since there was very little difference in the protocols of the studies, whether results reported were correct, high, or low (Kolthoff & Medalia, 1951).

Table 1. Comparison of several methods by author.

Author	**Solvent**	**Applications**	**Comments**
Young et al. 1936	methanol	hydrogen peroxide succinyl peroxide	One of first to estimate peroxides based on reaction with ferrous thiocyanate
Bolland et al 1941	benzene: methanol 7:3	autoxidized rubber; succinyl peroxide, cyclohexane peroxide, dihydroxyheptyl peroxide	Application of Young et al 1936 method with change in solvent
Chapman & MacFarlane 1943	96% acetone	edible fats	
Wagner et al. 1947	methanol	organic peroxides hydrogen peroxide	Based directly on Young 1936 procedure

Solvents, as well as oxygen, can participate in radical reactions (Kolthoff & Medalia, 1951), leading to discrepancies in stoichiometry. Several solvents have been used in the FeSCN method. Older methods used 96% acetone and benzene-methanol (7:3) (Lips et al., 1943; Stine et al., 1954). Newer methods tend to use chloroform:methanol or (dichloromethane:methanol) (IDF 74A:1991; Mihaljevic et al., 1996; Richards & Feng, 2000). The problem is that the alkoxyl radicals formed in hydroperoxide decomposition can abstract hydrogens from solvents, forming solvent radicals. As with lipid radicals, radicals derived from organic solvents add oxygen to for peroxyl radicals, which in turn abstract hydrogens to form hydroperoxides. Solvent

radicals have different sensitivity to oxidative attack and different ability to propagate separate reactions (Kolthoff & Medalia, 1951), a behavior that depends at least in part on redox potential of the solvent radicals. This means solvent radicals can have significant effect on the overall reaction, most often increasing the stoichiometry to greater than expected stoichiometry, i.e. high results. Acetone was originally used because it was thought to suppress the induced decomposition of peroxides (Kolthoff & Medalia, 1951). However, acetone does not dissolve all oils. Removal of oxygen can be obtained by boiling aqueous phases for a short amount of time (Kolthoff & Medalia, 1951), or by sparging solvents with argon and maintaining inert gas in headspaces.

These problems with solvent radicals were known previously but ignored, and stoichiometry of the FeSCN assay was assumed to always be the predicted 2:1 ratio until a recent study raised the issue again. Using chloroform and methanol as solvents, Mihaljevic showed that solvent radicals cause additional reactions that change the yield of Fe^{3+} (Mihaljevic et al., 1996). When attacked by alkoxyl radicals, chloroform produces trichloromethyl radicals ($Cl_3C^{\bullet}$) which are strongly oxidizing and can continue to oxidize ferrous ions directly and after oxidation:

$$LO^{\bullet} + CHCl_3 \rightarrow LOH + {}^{\bullet}CCl_3$$

$$Fe^{2+} + {}^{\bullet}CCl_3 + H^{+} \rightarrow CHCl_3 + Fe^{3+}$$

$${}^{\bullet}CCl_3 + O_2 \rightarrow {}^{\bullet}OOCCl_3$$

$${}^{\bullet}OOCCl_3 + Fe^{2+} \rightarrow Fe^{3+} + {}^{-}OOCCl_3$$

In contrast, alkoxyl radical attack on methanol produces hydroxyl-methyl radicals which are strong reducing agents and recycle Fe^{3+} to Fe^{2+}.

$$LO^{\bullet} + CH_3OH \rightarrow LOH + CH_2OH$$

$$^{\bullet}CH_2OH + Fe^{3+} \rightarrow HCHO + Fe^{2+} + H^{+}$$

These are all competing reactions, so the resulting amount of Fe^{3+} generated in the reaction will be determined by nature of the solvent, levels and type of hydroperoxide, oxygen availability, and pH. In other words, the stoichiometry will depend on the analytical solution composition (Mihaljevic et al., 1996).

One additional comment about solvents needs to be made. Observations that at times bottles of chloroform caused high absorbance readings, and this prevented accurate quantitation of lipid hydroperoxides initiated an interesting on effects of chloroform stabilizers, amylene vs ethanol (Richards & Feng, 2000). Chloroform was originally chosen because it had been already verified as an appropriate solvent (Shantha & Decker, 1994). Chloroform stabilized with amylene caused not only very high blank readings, but also non-linear calibration curves with cumene hydroperoxide. Amylene did not prevent formation of trichlorometyl radicals in chloroform, and amylene did not prevent trichlromethyl radicals from reacting with ferrous ion. In contrast, ethanol as preservative either prevented trichloromethyl radicals from forming or stopped trichloromethyl radicals from reacting with the Fe^{3+} ions (Richards & Feng, 2000). Dichloromethane had less of sensitivity to amylene and gave lower blank readings (Richards & Feng, 2000).

Returning to issues of stoichiometry, peroxide values reported in the literature have confirmed inconsistencies in stoichiometry in this assay, and these create problems for absolute quantitation of hydroperoxides in this assay. Using the method of Stine et al with benzene-methanol as solvent (Stine et al., 1954), Barthel and Grosch saw a difference in stoichiometry of hydrogen peroxide and methyl linoleate hydroperoxide

(Barthel & Grosch, 1974). Hydrogen peroxide oxidized two equivalents of Fe^{2+}, while methyl linoleate hydroperoxide oxidized four equivalents of Fe^{2+}. The more extensive reaction with methyl linoleate was attributed to "secondary reactions of the methyl linoleate hydroperoxide acyl residue" (Barthel & Grosch, 1974, p. 540). Stoichiometric equivalents calculated for t-butyl hydroperoxide (2.5), cumene hydroperoxide (3), and MOPO/MLPO (~4) showed clearly that extent of reaction varied with the nature of the R group in the hydroperoxide. Barthel et al theorized that aromatic systems or double bonds increase Fe^{3+} production (Barthel & Grosch, 1974), although these explanations seem unlikely chemically.

In recent studies, Mihaljevic et al separated reactivity into three hydroperoxide classes based on the number of ferrous ions oxidized per molecule of ROOH: Hydrogen peroxide 2, small tertiary organic hydroperoxides (t-BuOOH and CuOOH) about 3, and fatty acid hydroperoxides about 4. Within the fatty acids, hydroperoxide structure -- primary vs secondary, positional of the -OOH, methyl esters or free acids – did not make a difference (Mihaljevic et al., 1996). Analyzing this hierarchy, redox potentials of the hydroperoxides and radicals increase in the same order. Thus, the most plausible explanation is that the stoichiometry of the FeSCN-LOOH assay increases with the redox potential of the hydroperoxide, i.e. the stronger the oxidant $RO^{\bullet}$, the greater the driving force for side reactions with that generate Fe^{3+} independently of LOOH.

These are critical observations for use of this assay with oxidized lipids where hydroperoxides are present on many different positions on different fatty acids. However, even more important for accurate quantitation of lipid oxidation was the observation that because of these stoichiometry differences, using tertiary hydroperoxides (t-butyl and

cumene) for calibration, as is customarily done, could lead to overestimation of LOOH (Mihaljevic et al., 1996).

A version of the ferric thiocyanate method was standardized by the International Dairy Federation. Now known as IDF (74A:1991), this variation was applied mainly to dairy products which normally have low peroxide values so need high sensitivity for detection (Frankel, 1998). Shantha and Decker then adapted the IDF method to non-dairy products such as chicken fat, cooked beef fat, fish oil, butter, and vegetable oil (Shantha & Decker, 1994). The IDF/ Shantha & Decker adaptation is the version most commonly used currently. Both methods retain the in situ generation of $FeCl_2$ from a displacement reaction with $BaCl_2$,

$$BaCl_2 + FeSO_4 \rightarrow BaSO_4(ppt) + FeCl_2$$

and construct standard curves using iron powder. Both these methods divide by results by a factor of two to express peroxide value in milliequivalents of peroxide instead of milliequivalents of atomic oxygen (O, not O_2) (Shantha & Decker, 1994).

There are a few other interferences that compromise the ferric thiocyanate method. In general, radical-quenching antioxidants have little effect on this assay, but strong metal chelators such as EDTA (0.02%) significantly decrease PV due to competition for the iron (Nielsen et al., 2003; Mihaljevic et al., 1996). Pigments, on the other hand, can be strong interferences if they absorb in the same wavelength region used to determine PV (Dobarganes & Velasco, 2002). To avoid this problem, Hornero et al modified the Shantha and Decker method by using diethyl ether to extract the pigments after oxidation reaction has completed, so that it could be applied to high carotenoid content (Hornero-Méndez et al., 2001).

2.2.4. Xylenol Orange Background

Originally developed to detect very low levels of lipid hydroperoxides in living tissues, the xylenol orange assay became widely used after initial testing showed it was simple with high sensitivity, and was not sensitive to dioxygen (Wolff, 1994). Ferrous ion oxidation occurs in presence of ferric ion indicator xylenol orange for measurement of hyroperoxides (Wolff, 1994). This assay is based on the oxidation of ferrous ions in an acidic medium containing xylenol orange dye. Binding of the resulting ferric ions by the dye produces a blue-purple complex with absorbance maximum between 550-600 nm (Dobarganes & Velasco, 2002; Grau et al., 2000). In simplest terms the mechanism can be shown as follows (Grau et al., 2000):

$$ROOH + Fe^{2+} \rightarrow Fe^{3+}$$

$$Fe^{3+} + XO \rightarrow (Fe^{3+}\ldots XO)\ \text{complex}$$

Gupta, in 1973, published one of the first papers that examined in detail the use of Fe-XO complex to determine concentrations of H_2O_2 (Gupta, 1973). Previous papers had documented binding of Fe^{3+} by xylenol orange; Gupta now investigated absorbance characteristics of the complex and ways to adapt the reaction as an assay for H_2O_2. Simon Wolff and collaborators extended applications of the xylenol orange assay to organic hydroperoxides, including cumene, *tert*-butyl, and lipid hydroperoxides (Jiang et al., 1990; Jiang et al., 1992), and probably contributed the most in formalizing this assay for trace levels of lipid hydroperoxides. Two procedures for the Fe^{3+}- xylenol orange (or FOX) assay were proposed. FOX1 is water-soluble version containing sorbitol as a reaction enhancer (Wolff, 1994). A second version was developed for samples in which high backgrounds of nonperoxidized lipids could interfere with measurement of low

levels of lipid hydroperoxides (Wolff, 1994). This version, FOX2, omitted sorbitol and added butylated hydroxytoluene (BHT) and methanol (Wolff, 1994).

As FOX1 and FOX2 procedures were applied to various systems, it became generally recognized that the stoichiometry was not constant and conditions of the original version of assay must be modified to fit individual sample types. If certain conditions of the assay are not adjusted, the hydroperoxide concentrations measured will not be accurate. One major problem discovered was that the recommended protocol for FOX (Wolff, 1994) was that it was only valid for the assay of H_2O_2, but not for other peroxides because not all hydroperoxides have the same molar absorption coefficients and reactivity of secondary alkoxyl and H abstraction radicals differ (Gay et al., 1999a, 1999b; Gay & Gebicki, 2000). A number of early papers recommended that a general extinction coefficient of 43,000 M^{-1} cm^{-1} could be used to quantitate hydroperoxides in this assay (Gay et al., 1999b). However, a survey of the literature and specific experimentation with different hydroperoxides showed clearly that this assumption is incorrect, that extinction coefficients vary with the reaction system and hydroperoxide, and that the FOX assay can be used if only relative hydroperoxide concentrations are needed, but not to obtain absolute concentrations of hydroperoxides (Gay et al., 1999a, 1999b; Gay & Gebicki, 2003).

Molar extinction coefficients and stoichiometry of the FOX reaction remain highly controversial. Molar extinction coefficients reflect the stoichiometry of the reaction, and variations in ε values indicate that the reaction is not running identically under all conditions or with all hydroperoxides (Fukuzawa et al., 2006). One paper claimed that 3 mole of Fe^{3+} were formed per mole of hydroperoxide, based on average

apparent extinction coefficient of 4.52 X 10 ^4 M^{-1} cm^{-1} (Jiang et al., 1992). This same paper found t-butyl and cumene hydroperoxides slightly less reactive than hydrogen peroxide, and linoleic hydroperoxide slightly more (Jiang et al., 1992). On the other hand, dicumyl peroxide, benzoyl peroxide, and lauroyl endoperoxides showed very little reactivity in this assay in comparison to hydrogen peroxide (Jiang et al., 1992). Yildiz (Yildiz et al., 2003) disagreed with these results. When comparing FOX assay with the AOCS PV method (Iodometric titration with thiosulfate), he found that if results were calculated in the manner suggested by Jiang et al. (Jiang et al., 1992) peroxide values were consistently overestimated (Yildiz et al., 2003). In order for results to compare with AOCS PV method where stoichiometry is unequivocal, Yildiz argued that two Fe^{3+} ions reacted with XO instead of one, and that 3 moles of ferric-XO complex were formed for every 2 moles of hydroperoxide (Yildiz et al., 2003). Seeking to clarify the issue, Gay and Gebicki examined the effect of solvent and hydroperoxide structure(Gay et al., 1999a, 1999b) as well as sorbitol (Gay, 2000) see below) on XO reaction stoichiometry. In their systems, Fe^{3+} ions generated per –OOH group were 2.5 for H_2O_2, 5 for cumene and tert-butyl hydroperoxides and, 2 for other hydroperoxides (Gay et al., 1999b; Yildiz et al., 2003). There was no obvious mechanism for production of five Fe^{3+} for t-butyl and cumene, but authors proposed that the cumene alkoxyl radical formed a methyl radical by β-elimination. Oxygen adds to this $CH_3^{\bullet}$ to form a peroxyl radical capable of oxidizing Fe^{2+} directly or via radicals initiated in other molecules by H abstractions (Gay et al., 1999b).

Another issue with this assay is its strong dependence on pH and source of acidity. The requirement of a low pH for this assay ensures the stability of iron ions in

reduced and oxidized forms. First suggested by Wolff (Wolff, 1994), 25 μM H_2SO_4 is commonly used in the FOX assay in order to maintain the pH consistently below pH 2. Reliable results can only be obtained with pH values below 2; maximum reaction occurs near pH 1.8. However, with H_2SO_4 the pH optimum of the assay is very narrow (pH 1.7-1.9) (Fukuzawa et al., 2006). This pH values is very difficult to maintain in the presence of compounds containing dissociable protons (Gay & Gebicki, 2002).

This problem has led investigators to evaluate other acids for replacing H_2SO_4. hydrochloric acid instead of sulfuric acid (Burat & Bozkurt, 1996; Shantha & Decker, 1994). Grau et al. found that HCl exerted the same stabilization as H_2SO_4 but found lower sensitivity so recommended no change in acids (Grau et al., 2000). A study determining LHP in snacks fried in sunflower oil agreed; sulfuric acid gave greater reaction sensitivity than hydrochloric acid, as well as better precision (Navas et al., 2004). In contrast, perchloric acid lowered the assay optimum pH to 1.1 (not so difficult to control), decreased dependence of ferric-xylenol orange complexes on acid concentration, decreased interference from added compounds, and increased molar absorption coefficients were higher for all hydroperoxides tested (hydrogen peroxide, cumene, t-butyl, lipid, protein) (Gay & Gebicki, 2002). A new protocol designated PCA-FOX was developed based on the observed 110 mM perchloric acid optimum (Gay & Gebicki, 2002).

The addition of sorbital to the assay has also been contested. Sorbitol, which acts as a radical chain carrier, was initially added to FOX-1 to increase assay response and sensitivity (Wolff, 1994; Gay et al., 1999b). Addition of 100 mM sorbitol increased the extinction coefficient for the H_2O_2 reaction from 4.46×10^4 to 2.24×10^5 M-1cm-1

(Wolff, 1994). Order of magnitude enhancement of H_2O_2 reactions by sorbitol was confirmed in later studies, sorbitol, but the amount of Fe^{3+} produced by organic hydroperoxides such as cumene, tert-butyl, and protein was only doubled (Gay & Gebicki, 2000). This difference was attributed to ability of the strongly oxidizing $HO^{\bullet}$ from H_2O_2 to abstract hydrogens from every carbon on sorbitol, thus creating at least six new radical sites for reaction with oxygen and/or Fe^{2+}. In contrast, alkoxyl radicals formed from organic hydroperoxides can abstract H only from terminal carbons of sorbitol:

$$RO^{\bullet} + RCH_2OH \rightarrow ROH + RC^{\bullet}HOH$$

In the end, the authors recommended against the use of sorbitol unless assaying H_2O_2 to avoid uncontrolled and unpredictable enhancement (Gay & Gebicki, 2000).

The FOX-2 assay contains BHT to prevent continued oxidation of lipids during handling and assay (Wolff, 1994). There is some disparity in the literature regarding effectiveness of BHT. Jiang et al. included it without notable problems in the procedures of at three studies (Jiang et al, 1990, 1991, 1992). However, in assays of phosphatidyl choline hydroperoxides (PC-OOH) in egg yolk, BHT decreased stoichiometry from 3.5 to 2.4 Fe^{3+} generated per PC-OOH (Fukuzawa et al., 2006; Grau et al., 2000). This decrease was considered tolerable and perhaps even desirable since the antioxidant was thought to inhibit generation of new radical chains that would, in turn, increase stoichiometry during the reaction. There are also authors who discourage use of BHT because BHT blocks reaction of $LO^{\bullet}$ with ferrous iron, thus diminishing Fe^{3+} generation and leading to pronounced underestimation of hydroperoxides (Hermes-Lima et al., 1995; Eymard & Genot, 2003; Grau et al., 2000).

While investigators disagree on details of the XO reaction, they all agree that the source of XO dye and even the batch number from the same source can significantly change the results (Gay et al., 1999; Grau et al., 2000; Navas et al., 2004). An example of this is XO dye from Sigma turned from red to purple (depending on hydroperoxide concentration) with one absorption maximum at 560 nm (Navas et al., 2004). In same study, XO bought from Aldrich or Scharlau resulted in colors ranging from brown to blue and the appearance of two absorption peaks at 560 nm and 590 nm (Navas et al., 2004).

If the same supplier and same batch is used in a study, presumably the color change should be due to hydroperoxide reaction. However, shifts in color with xylenol orange, iron, and hydroperoxide concentrations complicate interpretation. Both Navas (Navas et al., 2004) and Eymard (Eymard & Genot, 2003) agree that at low hydroperoxide concentrations (less than 10 uM), the absorption maximum is 550-560 and the color of the complex should be orange. At higher concentrations, however, absorption maximum shifts to 580-590 nm and the color turns purple. Supposedly, this shift in color from "reddish to purple" results mostly from iron concentration since the Fe3+ calibration curve was not linear over wide range; limitation of concentrations to 5-20 Fe^{3+} was thus recommended (Shantha & Decker, 1994). Burat and Bozkurt were able to extend the lower detection limit to 2-4 ug Fe^{3+} by increasing the XO concentration to 1.00 umol of (versus 0.5 umol in Shantha and Decker's assay) to prevent saturation of response at high LOOH and Fe^{3+} concentrations (Burat & Bozkurt, 1996). Gay and Bebicki also mention that a blueish/purple color indicates insufficient XO to complex of all Fe3+ present, resulting in underestimating of amount of hydroperoxide in sample (they claim brown/orange is correct color)(Gay & Gebicki, 2002).

Despite these many problems, the xylenol orange assay has its proponents and advantages. Its principal strong point is high sensitivity, detecting nanomoles of hydroperoxides in solution. The assay is felt to provide a reliable measure of concentrations of hydroperoxides in reasonably pure compounds under specific conditions (Gay et al., 1999a, 1999b), and can be used to compare relative concentrations of hydroperoxides in like samples or over time. The FOX assay was been applied to chicken, fish and lipid extracts from meat; edible oils and butterfat; foods; plant tissues and biological samples (Burat & Bozkurt, 1996; Eymard & Genot, 2003; Grau et al., 2000; Navas et al., 2004). Results from several papers conclude that FOX assay is comparable to the spectrophotometric iodometric assay (Jiang et al., 1991, 1992; Nourooz-Zadeh et al.,1995) and the official International Diary Federation (IDF 74A:1991) method (Burat & Bozkurt, 1996).

However, accurate quantitative measurements of absolute hydroperoxide concentrations are problematic, requiring at a minimum knowledge of hydroperoxide structure and apparent molar absorption coefficients as well as careful control of assay conditions (incubation time, solvent, source of XO etc, optimum wavelength, pH) (Fukuzawa et al., 2006; Gay et al., 1999b). This means the assay can be used to measure amounts of hydroperoxides at two levels of precision (Gay et al., 1999b). For high precision and absolute quantitation, the chemical identity of hydroperoxide must be known so that accurate extinction coefficient can be applied; if not yet determined, the hydroperoxide concentration must be measured by an independent primary method such as iodometric titration for calibration of XO results (Fukuzawa et al., 2006; Gay et al., 1999b; Gay & Gebicki, 2002). If the sample has unknown composition or contains

mixed hydroperoxides in unknown proportions, *approximate* ROOH concentrations can be calculated by using an appropriate general molar absorption coefficient (Gay et al., 1999b) or by expressing the concentrations as hydrogen peroxide equivalents (Fukuzawa et al., 2006; Gay et al., 1999b). Such values are useful for following oxidation of a single type of material over time or comparing like materials, but are not absolute and thus are not appropriate for mechanisms studies.

There is a second problem with consistent quantitation that is seldom recognized – bleaching of the Fe^{3+}-XO complex at high hydroperoxide concentrations. Thus, when samples are not sufficiently diluted, reaction can appear not to occur or to detect only low levels of hydroperoxides. Excess hydroperoxide probably explains results of Yildiz et al, who saw a good correlation of FOX assay with titration and PeroxySafe kit (also based on the xylenol orange reaction), but cited accuracy problems with samples of low PV (<2) and the uncorrected PV were consistently under-predicted (Yildiz et al., 2003).

2.2.5. PeroxySafe Background

Safety Associates, Inc (Temp, AZ) developed a colorimetric assay for peroxide value determination and marketed it as a kit under the brand name of SafTest™, more specifically PeroxySafe™. The assay is based on the xylenol orange assay described in the previous section, i.e. oxidation of ferrous ion in presence of xylenol-orange in an acidic medium (Osawa et al, 2008). PeroxySafe™ has been certified by as an official method by AOAC-RI #030501 (Gordon, 2005). The kits include optical reader, proprietary reagents, calibration standards, and autodispensers for reagent delivery.

SafTest™ offers three different kits; PeroxySafe™ MSA Kit (matrix special applications) which is designed for solid matrices such as pet foods, PeroxySafe™ STD

Kit (standard) recommended for pure oils or viscose substances with turbidity issues, and PeroxySafe™ HSY Kit (high sensitivity) designed for living tissues and samples with low oxidation levels. The company's website claims that all three kits "effectively measure peroxide levels up to 50 meq/kg with a high level of confidence", have an empirical lower detection limit of 0.02 meq/kg levels, and are "capable of analyzing a wide range of values with a high degree of accuracy and precision" (MP Biomedicals, 2012). The assay offers advantages of small sample size, rapid and straightforward performance, and reduced toxicity compared to traditional titration (Yildiz et al., 2003).

PeroxySafe is still a very new assay that has not yet been broadly accepted in the food industry, despite AOAC certification and internal studies by the manufacturer showing successful application of the assay to food systems (Foo et al., 2006). Research papers verifying accuracy and precision of PeroxySafe and correlating this assay with various other PV methods have shown mixed results. A study by Yildiz found a strong correlation between PeroxySafe assays and standard iodometric titration of hydroperoxides (Yildiz et al., 2003). Plots of PeroxySafe versus standard titration values were linear with slope close to one, indicating comparable response in the two assays (Yildiz et al., 2003). Foo et al found that calibration curves constructed from a single kit and multiple kits had good reproducibility with no significant lot to lot difference (Foo et al., 2006). However, diluted of samples to fit within the calibration curve markedly increases variability and decreased precision (Foo et al., 2006). After monitoring oil exposed to high heat for seven days, the study concluded that unless higher concentrated standards can be included in the kits to allow for an extension of the calibration curve, the PeroxySafe kit as currently sold should not be used to monitor the quality of heat abused

oil (Foo et al., 2006). [Note: the PeroxySafe assay has been used to track hydroperoxides in frying oil at Rutgers University with no problems. However, as noted for the xylenol orange assay above, constant argon atmospheres to avoid oxidation during handling and appropriate dilution of samples are critical for obtaining accurate and reproducible results.]

In analyses of refined vegetables oils, lard, and hydrogenated fats, reasonable correlations were observed between PeroxySafe kits (standard and HSY) kits and iodometric titration (AOCS method Cd 8b-90), although the SafTests were always higher. Differences in PVs between methods increased at PVs below 2 meq/kg and when lower concentrations of thiosulfate were used for titration (Osawa et al., 2007). The lower PVs obtained by iodometric titration were attributed to lack of sensitivity of iodometric method to low peroxides where endpoints are more difficult to see. However, the difference more likely stems from the stoichiometric variability of the xylenol orange reaction, which is at least 2:1, whereas Iodometric titration is 1:1.

In contrast, peroxide values in dried pet foods analyzed by the PeroxySafe MSA kits were less than half the peroxide values determined by iodometric titration (Osawa et al., 2008). Insufficient experimental detail was available to determine sources of differences.

2.2.6. Triphenylphosphine Oxidation using HPLC

High-performance liquid chromatography (HPLC) is considered a useful technique for hydroperoxide analysis (Dobarganes & Velasco, 2002). The biggest advantage HLPC has over Gas Chromatography is that it can be run at room temperature which decreases the risk of artifact formation, and lipid samples can be analyzed directly

without derivatization (Dobarganes & Velasco, 2002). However, lack of a universal detector causes difficulties in quantitative analysis (Dobarganes & Velasco, 2002).

One way to provide a detectable chromophore is to react the hydroperoxides with reagents that either bind or generate a modified species that can be followed independently. Triphenylphosphine (TPP) is one such reagent. TPP reduces hydroperoxides quantitatively to corresponding hydroxyl compounds, while being converted to triphenylphosphine oxide (TPPO) (Nakamura & Maeda, 1991). Either the TPPO produced or the TPP consumed in the reaction can be measured to quantify lipid peroxide content. (Nakamura & Maeda, 1991).

TPP → TPP=O (λmax = 560 nm)

It is particularly advantageous that even in high quantities (10 fold), TPP has no effect on hydrogen peroxide making it a useful tool to differentiate between H_2O_2 and non-H_2O_2 hydroperoxides (Nourooz-Zadeh et al., 1994).

Akasaka et al originally adapted the TPP reaction as a test tube assay, adding excess amounts of TPP, then measuring unreacted TPP after hydroperoxide reduction (Akasaka et al., 1987). However, at low lipid oxidation levels, accurate measurement of unreacted reagent was difficult since only trace amounts of TPP was consumed (Nakamura & Maeda, 1991). Thus, a new procedure was developed where even small amounts of TPO produced could be separated, detected, and quantified using reverse-phase or normal-phase HPLC with ultraviolet (UV)-detection at 220 nm (Nakamura & Maeda, 1991). The reaction is stoichiometric with cumene hydroperoxide or methyl 13-hydroperoxyoctadecadienoate and sensitivity is high (detection limit < 10 pmole) (Nakamura & Maeda, 1991).

Compared to the other hydroperoxide detection methods, TPP is a relatively new assay; and several papers have been published (Bauer-Plank & Steenhorst-Slikkerveer, 2000; Prior & Loliger, 1994; Yamada et a., 1987; Yamamoto et al., 1987).

3. OBJECTIVES

Inconsistencies in methodology and results in literature reports show that there are still many problems with lipid hydroperoxide analyses. Whether oxygen should be excluded, which solvent to use to solubilize both lipids and lipid oxidation products, which solvents are active radical substrates, how much response is generated per mole of hydroperoxide (i.e. stoichiometry), and which assay is best for different concentrations of hydroperoxides are all controversial issues that remain unresolved experimentally. Choosing an assay blindly from the literature without considering all these factors can lead to incorrect or invalid results.

The goals of this project, therefore, were:

- evaluate the three assays most commonly used to determine lipid hydroperoxides (iodometric titration, xylenol orange in two commercial kits, ferric thiocyanate chemical reaction and commercial kit).
- use two standard hydroperoxides (tert-butyl and cumene) which can be obtained commercially in high purity and known concentrations and provide distinctly different structure and solubility to determine for each assay

  linearity of response (and linear response concentration range)

  active concentration range (limits of detection and quantification)

  reaction stoichiometry

effects of hydroperoxide structure on quantitative response

reproducibility

required and optimum reaction conditions

handling quirks and limitations.

- apply optimized procedures for all assays to analysis of hydroperoxides in methyl linoleate oxidized to different extents; compare results (between assays and lipid vs standards) to determine most appropriate for use at different oxidation levels.
- use the information collected to develop guidelines and recommended procedures for hydroperoxide quantitation in research and industrial quality control.

4. EXPERIMENTAL PROCEDURES

4.1 Materials used for all assays:

Standard hydroperoxides: Tert-butyl hydroperoxide; 70% purity (MP Biomedicals,Solon, OH); cumene hydroperoxide; 88.1% purity (MP Biomedicals, Solon, OH)

Methyl Linoleate; > 99 %; NU-Chek Prep, Inc (Elysian, MN)

Other reagents as listed in the following protocols.

4.2. Experimental Protocols for Hydroperoxide Assays

4.2.1. Iodometric Titration with Thiosulfate

(Adapted from AOCS Cd 8-53 and AOAC 41.1.16)

I. Apparatus/Instrumentation/Equipment

A. Argon gas Source (AirGas, Inc, pre-purified Argon compressed)

B. Micro-pipettes; 1 ml, 200 µl, 100 µl

C. Erlenmeyer flasks (25 ml or 50 ml)

D. Stir plate and stir bar

E. Volumetric burettes

F. Analytical balance

II. Chemicals/Solvents

A. Hydroperoxide standard or lipid extract

B. Acetic acid, Glacial (CH3CO2H) – Sigma-Aldrich (St. Louis, MO), ACS reagent ≥ 99.7%

C. Chloroform (CHCl3) - Chromasolv® for HPLC ≥99.8%; (St. Louis, MO)

D. Potassium Iodide (KI) – Alfa Aesar: A Johnson Matthey Company;(Fair Lawn, NJ) ;99%

E. Distilled water – Milli-Q™ Water System

F. Soluble starch – Argo Corn Starch 100% (Englewood Cliffs, NJ)

G. Sodium Thiosulfate (Na2S2O3) – Canolco, Canal Industrial Corporation

H. Potassium dichromate (K2Cr2O7) – Mallinckrodt crystals, Analytical Reagent

I. Potassium oxalate ($K_2C_2O_4 \cdot H_2O$) – Fischer Scientific (St. Louis), certified A.C.S, crystals

J. Sulfuric Acid (H2SO4) – EMD, OmniTrace® (Gibbstown, NJ); 95.5-96.5%

III. Reagents/Solutions (make in appropriate quantities as needed)

For all reagents, use highest purity available. Use 18 MΩ water (Milli-Q or comparable) for all solutions.

A. 1% soluble starch solution in 18 MΩ water (bring water to a boil to solubilize starch and heat until solution is clear; mix continuously with stir bar until starch is in solution). Cool and store in stoppered bottles. Short term use – OK at room temperature. However, for long term storage and use, best stored in refrigerator to inhibit mold growth and yeast fermentation.

B. Acetic acid : Chloroform (3:2). HPLC grade or better.

C. Solution B: Dissolve 45 g of dry Potassium oxalate plus 20 g of KI in 500 ml 18 MΩ water.

D. Solution C: Dilute 59 ml of concentrated H_2SO_4 to1000 ml with 18 MΩ H_2O.

E. Saturated potassium iodide solution (make fresh daily). Bubble water on ice with Ar for 15 min. Immediately before use, dissolve 6 g KI in 5.0 ml water and keep covered with aluminum foil to prevent light access (or use red glass container). *Bubble with argon periodically throughout the day to prevent oxidation.*

F. 0.01 N $K_2Cr_2O_7$: Dry $K_2Cr_2O_7$ for 1-2 hrs at 150-200 °C. Accurately weigh 0.49036 g dichromate, dissolve in 100-200 mls 18 MΩ water, make up to 1000 ml with H_2O.

G. 0.1 N sodium thiosulfate solution (weigh 12.4019 g of $Na_2S_2O_3$ and dissolve in 500 ml of recently boiled water).

H. Dilute 0.1 N sodium thiosulfate ten-fold to make a 0.01 N solution.

This solution can also be further diluted to 0.001 N if hydroperoxide concentration is very low.

IV. Standardization of Sodium Thiosulfate solution

A. Run at least three replications for all samples.

B. Mix together 5 ml of 0.01N $K_2Cr_2O_7$, 1.5 ml of Solution B and 1.5 ml of Solution C. Orange color will form.

C. Titrate with sodium thiosulfate (0.01 or 0.001N) until orange color turns lighter

D. Add about 1-3 drops of 1% starch solution; sample will turn blue. Add more starch if it does not.

E. Continue titration until blue color turns clear and colorless.

F. Calculate normality of Sodium Thiosulfate solution from

(ml of thiosulfate) X (Normality of thiosulfate) = (ml of chromate) X (normality of chromate)

Note: Sodium thiosulfate should be stored refrigerated and must be standardized each time a new solution is made. It is not necessary to re-standardize daily, but should be checked for degradation by repeating standardization steps about every 10 days.

V. Peroxide Value determination (*italic* text denotes critical handling required for accurate results)

A. Dilute standard hydroperoxide or extracted lipid if necessary. Sample size required is determined by extent of oxidation.

Note: The official method recommends 1-5 grams of extracted lipid per analysis, but these assays were designed for the oil industry where large volumes of oil are readily available. Another approach is to start the titration with whatever sample is available. We have run samples sizes as low as 30-40 mg to several hundred mg. If more than 10-15 ml of 0.01 thiosulfate is used, then the sample must be diluted for a more accurate reading. If less than 1 ml thiosulfate is used, 0.001 N thiosulfate must be used or the sample size must be increased.

B. Bubble $CHCl_3$:acetic acid with Ar for 15 minutes at 4 °C to avoid unequal volatilizing of solvents, seal bottle, bring to room temperature.

C. Dissolve aliquots of standard hydroperoxides, oil, or extracted lipid, accurately weighed to 0.1 mg, neat or diluted in solvent, in 10 ml of glacial acetic acid: chloroform (3:2).

D. *Bubble sample with argon for two minutes.* This can easily be done with a Pasteur pipette attached to a tube on a gas line.

E. Add 0.5 ml of saturated potassium iodide solution, *flush headspace with argon,* and stopper the flask.

F. After exactly one minute, add 15 ml of distilled water *(sparged with argon).*

G. *Set up an argon line so that argon is continuously bubbling in the solution during the titration.*

H. Add two drops of 1% starch solution, or enough drops to produce a noticeable bluish purple color.

I. Titrate above solution with 0.01 (or 0.001N*) sodium thiosulfate until the moment right when blue color disappears. *Titrate carefully and vigorously mix after each addition* either using hand stirring or a stir bar/stir plate with stir bar**.

*If less than 0.5 ml of 0.01 N sodium thiosulfate is used, repeat using 0.001N for better accuracy.

** Stir bar/stir plate is recommended for clearer endpoint and better reproducibility.

Additional Note: If solution is dark yellow in color (indicating high PV) before adding starch solution, start titrating with sodium thiosulfate until the yellow is lighter in color. Once the color is a 'light yellow', the starch can be added. This can help create a clearer endpoint.

VI. Calculations

A. Normality of standardized sodium thiosulfate

(ml of thiosulfate) X (Normality of thiosulfate) = (ml of chromate) X (normality of chromate)

Plug in known information and solve for Normality of thiosulfate.

B. Peroxide Value

PV = meq peroxide/ kg fat = ((1000)(ml of $Na_2S_2O_3$)(Normality of $Na_2S_2O_3$))/ (g fat or hydroperoxide)

4.2.2. Ferric Thiocyanate Assay for Lipid Hydroperoxides

Adapted from (Mihaljevic et al., 1996; Shantha & Decker, 1994).

I. Apparatus/Instrumentation/Equipment

C. Argon source (AirGas, Inc, pre-purified Argon compressed)

D. Centrifuge – Beckman Model TJ-6

E. Plastic centrifuge tubes

F. Micro-pipettes – 1ml, 200 µl, 100 µl

G. Spectrophotometer – Varian, Cary 50 Bio UV-visible

H. Crystal/glass 3 ml cuvettes

I. Glass test tubes (various sizes)

II. Chemicals/Solvents

A. Hydroperoxide standard or lipid extract

B. Methanol – Fluka Analytical, LC-MS Chromasolv® (St. Louis, MO); ≥99.9%

C. Chloroform (CHCl3) - Chromasolv® for HPLC (St. Louis, MO); ≥99.8%

D. Barium Chloride dehydrate – Fischer Scientific Company (Fair Lawn, NJ)

E. Ferrous Sulfate – Matheson Coleman & Bell Manufacturing Chemists (Phillipsburg, NJ), crystals

F. Ammonium Thiocyanate – Riedel-De Haen AG Seelze-Hannover; Reag. ACS, Reag ISO, Reag. Ph. Eur I.

G. Hydrochloric Acid (HCl; concentrated, 12N) – A.C.S reagent Sigma-Aldrich (St. Louis, MO); 37%

H. Ferric Chloride (FeCl3) – J.T. Baker Chemical, chloride hexhydrate (Phillipsburg, NJ)

I. Distilled water – Milli-Q™ Water System

III. Reagents/Solutions (make in appropriate quantities as needed)

A. Chloroform: Methanol (2:1) v/v

B. 10 N Hydrochloric acid in distilled water (remember to add acid to water!)

C. Ferrous Chloride Solution (make fresh daily):

1. 0.4 g barium chloride dehydrate dissolved in 50 ml distilled water
2. 0.5 g Ferrous sulfate dissolved in 50 ml distilled water
3. Barium chloride solution was slowly added with constant stirring to the ferrous sulfate solution
4. 2 ml of 10 N hydrochloric acid added to above solution

5. Solution was centrifuged for 3-5 minutes or until solution clear enough to siphon off
6. Clear iron (II) solution is pipetted out, leaving behind the barium sulfate precipitate). Store iron (II) solution in brown bottle or cover with foil to keep in the dark. Dearate solution with argon for 3-5 minutes.

D. 30 % Ammonium thiosulfate solution: 30 grams of Ammonium thiosulfate dissolved in water and volume brought up to 100 ml

E. Fe^{3+} standard curve solutions

1. Stock 1 mg/ml Fe^{3+}: dissolve 2.470 g of ferric chloride in a few mls of 10 N HCl, make up to 500 ml in a volumetric flask with 10 N HCl. Store in an amber-glass bottle at 4 °C.
2. Intermediate Standard Solution (0.1 mg Fe/ml): Prepare fresh daily. Add 1 ml of Stock 1 mg/ml

   Fe^{3+} to 9 ml $CHCl_3$:MeOH (2:1), v/v. Mix.
3. Working Standard Solution 1 (0.01 mg Fe/ml): Prepare fresh daily. Mix together 3 ml of intermediate standard solution and 27 ml $CHCl_3$:MeOH (2:1), v/v.
4. Working Standard Solution 2 (0.001 mg Fe/ml): Prepare fresh daily. Mix together 3 ml of Working Standard Solution 1 (0.01 mg Fe/ml) with 9 ml of $CHCl_3$:MeOH (2:1), v/v.

IV. Fe^{3+} Thiocyanate Standard Curve

A. Use Working Standard solution 1 (10 ug Fe/ ml) and Working Standard Solution 2 (1 ug Fe/ml) to construct Fe^{3+} standard curve.

B. Deaerate (on vacuum line or by argon sparging) enough Working Standard and solvent for analyses to be performed. See Table 2 for volumes.

C. Prepare reactions with a range of standard Fe concentrations in small test tubes, mixing reagents in volumes and order specified in Table 2. In particular, SCN must be added last.

D. Vortex solutions and react in dark for exactly 10 minutes.

E. Transfer reaction solutions to glass or quartz optical cuvettes (*disposable cuvettes cannot be used, plastic dissolves in chloroform:methanol*) and record absorbance at 500 nm.

F. Plot absorbance vs standard concentration to generate standard curve. Determine regression equation for curve using any standard math program (e.g. Excel or MatLab) or by determining the x-intercept and calculating slope of the linear region of the curve, then fitting these values to the equation for a straight line:

$$y = mx + b \quad \text{or} \quad y = \text{slope}\ (x) + \text{y-intercept}$$

Table 2. Reagents for preparation of Fe^{3+} Standard Curve

Standard Fe3+ Conc. (μM)	10 μg Fe/ml std (μl)	1 μg Fe/ml std (μl)	0.5 μg Fe/ml std (μl)	Chloroform: Methanol 2:1 (μl)	30% SCN (μl)*	Total (μl)
1.013	0	0	300	2337.5	12.5	2650
1.689	0	0	500	2137.5	12.5	2650
2.365	0	0	700	1937.5	12.5	2650
3.378	0	0	1000	1637.5	12.5	2650
5.067	0	0	1500	1137.5	12.5	2650
7.770	0	0	2300	337.5	12.5	2650
13.51	0	2000	0	637.5	12.5	2650
40.54	600	0	0	2037.5	12.5	2650
54.053	800	0	0	1837.5	12.5	2650
67.566	1000	0	0	1637.5	12.5	2650
135.133	2000	0	0	637.5	12.5	2650
168.916	2500	0	0	137.5	12.5	2650

*Note: Add 30% SCN last.

V. Peroxide Value Assay

A. Dissolve/dilute samples (hydroperoxide or extracted lipids) in appropriate solvent (usually water, chloroform, methanol, or chloroform:methanol for compatability) to give a stock concentration of about 5 mg lipid /ml or <20 uM hydroperoxides.

B. From this stock, prepare a series of dilutions in the same solvent to test the reaction response. This is particularly important with samples that have unknown hydroperoxides concentrations.

C. Dearate samples with argon for 3-5 minutes.

D. In a clean test tube take appropriate amount diluted sample from above step (hydroperoxide or extracted lipid) and mix with appropriate amount of deaerated solvent to give desired final concentrations. Total volume after all reagents add to 2.65 ml. See Table 3 for example of concentration curve for cumene hydroperoxide.

E. Add 12.5 µl of 30% SCN.

F. Add 12.5 µl of $FeCl_2$ solution and vortex and allowed to react in dark for 10 minutes.

G. Allow to react in dark for 10 minutes.

H. Transfer to glass or quartz optical cuvettes and record absorbance at 500 nm.

Table 3: Cumene hydroperoxide. Hydroperoxide Standard is first diluted to 1.64 ug/ml using Methanol.

Final Conc (µM)	1.64 ug cumene/ ml (µl)	30% SCN (µl)	Chloroform:Methanol (2.1) (µl)	Fe2+ solution (µl)	Total (µl)
6.1	1500	12.5	1125	12.5	2650
4.07	1000	12.5	1625	12.5	2650
2.033	500	12.5	2125	12.5	2650
1.22	300	12.5	2325	12.5	2650

VI. Calculations

A. Construct Fe^{3+} and hydroperoxide standard curves by plotting concentration versus absorbance.

B. Linear regression equation from Fe^{3+} standard curve can be used for following:

a. For hydroperoxide stoichiometry, plug concentrations into linear regression equation to find corresponding Fe^{3+} concentration. Divide iron concentration generated by hydroperoxides concentration reacted to determine corresponding reaction stoichiometry (mols iron consumed per mole hydroperoxides).

b. For lipid extract samples, use measured absorbance to calculate peroxide concentration from the linear regression equation of either Fe^{3+} standard curve or standard hydroperoxide standard curve.

c. Divide by lipid weight to normalize, and convert to meq LOOH / kg fat to express results as peroxide values.

4.2.3. Cayman Chemical Company: Lipid Hydroperoxide (LPO) Assay Kit

Adapted from booklet that came with kit (Catalog No. 70500) provided by Cayman Chemical Company; Ann Arbor, MI.

I. Apparatus/Instrumentation/Equipment

1. Plate Reader (500 nm filter) – Berthold Technologies Mirthras LB 940
2. Micro-Pipettes: 1ml, 200 µl, 100 µl
3. Glass 96-well plate – Cayman Chemical Company
4. Argon source (AirGas, Inc, pre-purified Argon compressed)
5. Test tubes: VWR Disposable Culture Tubes – Borosilicate Glass Size: 12 X 75 mm

II. Chemicals/Solvents

1. Standard Hydroperoxide or Extracted Lipid
2. Methanol – Fluka Analytical, LC-MS Chromasolv® (St. Louis, MO); ≥99.9% or comparable
3. Chloroform (CHCl3) - Chromasolv® for HPLC (St. Louis, MO); ≥99.8% or comparable
4. LPO Assay Kit:
    a. FTS Reagent 1: 4.5 mM ferrous sulfate in 0.2 M hydrochloric acid (ready to use as supplied; store in 4 degree C).
    b. LPO Assay FTS Reagent 2: 3% methanolic solution of ammonium thiocyanate (ready to use as supplied; store in 4 degree C).
    c. Lipid Hydroperoxide Standard: 50 uM ethanolic solution of 13-hydroperoxyoctadecadienoic acid (ready to use as supplied; store at -80 °C).

III. Reagents/Solutions <u>(make in appropriate quantities as needed):</u>

1. Chloroform: Methanol (2:1) v/v (deoxygenate by bubbling with argon)
2. Chromogen: in clean test mix equal volumes of FTS Reagent 1 with FTS Reagent 2 and vortex. Each test tube needs 50 ul of chromogen. (ex: replicates of five; 5 X 50 ul = 250 ul per sample).

   Must be freshly prepared.

IV. Calibration Curve: Lipid Hydroperoxide Standard supplied with kit

Note: Other standard hydroperoxides (cumene or t-butyl) can be used in addition to Lipid Hydroperoxide Standard supplied.

Dynamic range of kit; 0.25- 50 nmol per assay tube.

1. Mark 24 clean test tubes A-H (or with standard conc) in triplicates
2. Remove Lipid Hydroperoxide Standard from freezer and store on ice.
3. Aliquot Lipid Hydroperoxide Standard and solvent into each tube as shown in Table 4.

Table 4: Cayman Lipid Hydroperoxide Standard (13-HpODE) Curve *

Tube	**HP Standard (ul)**	**Chloroform: Methanol (2:1) (ul)**	**Final HP** (nmol)**
A	0	950	**0**
B	10	940	**0.5**
C	20	930	**1.0**
D	30	920	**1.5**
E	40	910	**2.0**
F	60	890	**3.0**
G	80	870	**4.0**
H	100	850	**5.0**

*Table copied from page 12 of booklet supplied in kit.
**This is the final amount of hydroperoxide in assay tube.

4. Start the reaction by adding 50 µl freshly prepared Chromogen added to each test tube. Vortex for 15 seconds or until well mixed. Use polypropylene caps to tightly seal test tubes.
5. Allow reaction to proceed at room temperature for five minutes.
6. Transfer contents of each tube each tube to a 1 ml glass/quartz cuvette and read absorbance at 500 nm against Tube 1 as the blank.

V. Standard Assay

1. Dissolve lipid extracts or oil samples in chloroform:MeOH. Record weight concentration (wt lipid/ml) for each sample.
2. In clean test tube aliquot 500 ul of lipid extract
3. Add 450 ul of chloroform: methanol solvent mixture.
4. Volume of sample can be more or less based on hydroperoxide concentration. Solvent volume must also be adjusted so that final volume is 950 µl before chromogen is added.
5. 50 µl of freshly prepared Chromogen added to each test tube. Vortex for 15 seconds or until well mixed. Use polypropylene caps to tightly close test tubes.
6. Leave assay tubes at room temperature for five minutes.
7. After 5 minute reaction time, color is stable for 2 hours. However, it is best to read absorbances immediately to avoid evaporation of solvent and corresponding change in absorbance.
8. Assay may be run in microplates by transferring 300 µl from each tube into a well of 96-well plate. Absorbance is read at 500 nm.

VI. Calculations

1. Before constructing lipid hydroperoxide standard curve, subtract average absorbance of standard A (the blank) from all other standards and samples.
2. Plot corrected absorbance of standards as function of final Lipid Hydroperoxide value from Table 4.
3. Determine hydroperoxide value by using linear regression equation obtained from standard curve. Corrected absorbance values for each sample are substituted in following equation:
4. Calculate concentration of hydroperoxide in original sample:

   HPST (nmol) = (sample absorbance – y-intercept)/ slope

   Hydroperoxide conc. in sample (μM) = (HPST/VE) x (1 ml/SV)

VE = volume of extract used for assay (ml)

SV = volume of original sample used for extraction (ml)

4.2.4. PeroXOquant™ Quantitative Peroxide Assay Kit, Lipophilic version

Based on xylenol orange assay (Jiang et al 1991; Jiang and Wolff, 1992); adapted from insert that came with kit available from Thermo Scientific (Pierce Biotechnology; Rockford, IL) Product number: 23285; Quantitative Peroxide Assay Kit (Lipid).

I. Apparatus/Instrumentation/Equipment

A. Spectrophotometer - Varian, Cary 50 Bio UV-visible (plate reader may be used in place of spectrophotometer)

B. Micro-pipettes

C. Glass test tubes -VWR Disposable Culture Tubes – Borosilicate Glass Size: 12 X 75 mm

D. Vortexer - Fischer Scientific Touch Mixer Model 232

E. Disposable Plastic cuvettes, 1 ml

II. Chemicals/Solvents

A. Standard hydroperoxide or lipid extract

B. Methanol - Fluka Analytical, LC-MS Chromasolv® (St. Louis, MO); ≥99.9%

C. Distilled water – Milli-Q™ Water System

D. PeroxoQuant^{TM} Kit – Reagents are ready to use/ store at 4 °C.

i. Reagent A, 1 ml, Composition: 25 mM ferrous (II) ammonium sulfate in 2.5 M H_2SO_4

ii. Reagent C, 2 X 50 ml, Composition: 4 mM BHT, 125 uM xylenol orange in methanol

III. Reagents/Solutions (make in appropriate quantities as needed)

Working Reagent (WR) : Mix 1 volume of Reagent A with 100 volumes of Reagent B

Example: Mix 10 ul of Reagent A with 1000 ul of Reagent C. *This solution is stable for 12 hours.*

Prepare at least 1 ml WR for each sample and standard replicate to be assayed in cuvettes.

(200 ul of WR for each sample and standard replicate if using a microplate).

A. Hydroperoxide Standard Curve: Serially dilute standard hydroperoxide (tert-butyl or cumene hydroperoxide for lipid analysis) so concentration range is between 1

uM to 1000 uM (working range of assay) using appropriate solvent. There should be 8-10 different concentrations.

C. Fe^{3+} Standard curve: Although not specified by Pierce, we prepared an Fe^{3+} standard curve to provide a basis for stoichiometry calculations and to determine completeness of reactions.

Fe^{3+} concentration range tested: 4.1 μM to 1000 μM.

D. Unless hydroperoxide levels are very low, sample should also be diluted so that absorbance is in the concentration range of hydroperoxide standard curve. Setting up a concentration curve for the sample can help ensure this, e.g. 1:10, 1:100, 1:1000.

Special note: High levels of peroxides bleach the dye and Fe-dye complexes, causing inaccurate low absorbance readings. It is recommended that all samples be analyzed over a range of dilutions, at least 1000x. If no reaction is obtained, continue diluting sample to 10^6 dilution. If diluted samples have higher or similar readings to undiluted samples, a bleaching effect has occurred and readings from the lowest concentrations giving a color should be used for calculations.

IV. Assay

A. Add 10 volumes of Working Reagent to 1 volume of sample (or standard) to a clean test tube. Example: 1000 μl of Working Reagent added to 100 μl of sample (or standard)

B. Vortex until mixed and leave reaction for 15-20 minutes at room temperature.

C. Transfer to plastic cuvette and measure absorbance at 560-600 nm (560 optimal) in a spectrophotometer (595 nm if using plate reader).

The complex formed is fairly stable, but samples should be measured the same day the reaction is performed and always after the same set reaction time, e.g. 20 minutes, 30 minutes, or four hours.

D. Using the measured absorbance, determine the hydroperoxide concentration from the regression equation for the standard curve as a reference.

Additional Notes:

1. In some cases depending on sample, it is necessary to prepare a blank that omits Reagent A. (for example: lipid sample is not extracted, sample contains endogenous iron interferences, sample contains other transition metals or a protein having chelating properties or strong absorbance characteristics at wavelengths used for measurement).

V. Calculations

Normally, the slope of the regression of A_{560} vs concentration of LOOH is equal to the molar absorption coefficient of the XO-Fe^{3+} complex, thus providing the basis for calculating unknown ROOH concentrations from Beer's Law. However, in this assay, none of the standard curves are linear (see below) except at very low concentrations, so a quadratic or best-fit curve to standard points must be used, or linear curves must be fit to individual100 uM increments. Also, the response curves are quite different, so serious questions must be raised regarding appropriate standard curves for use with lipids.

4.2.5. SafeTest PeroxySafe™ Assay for Lipid Hydroperoxides

Adapted from Protocol for PeroxySafe™ Standard Kit that is available from MP Biomedical, Solon, OH. This assay was developed specifically for use with oils.

Important Modifications:

1) The standard assay comes with reagents in bottles equipped with calibrated autopipettors. We found these units to be inaccurate, irreproducible, and extremely wasteful of expensive reagents due to the priming required, so we replaced them with direct pipetting of reagents using micropipettors.

2) Use a heating block set at 25 °C to maintain a constant "room temperature".

I. Apparatus/Instrumentation/Equipment

A. SafeTest Optical Analyzer provided with kit

B. Micro-pipettors (1 ml, 200 μl, 100 μl, 10 μl) and corresponding tips

C. Disposable Glass Test Tubes

a. Disposable Culture Tubes – VWR (West Chester, PA); Borosilicate Glass Size: 12 X 75 mm

b. Disposable Culture Tubes – VWR (West Chester, PA); Borosilicate Glass Size: 10 X 75 mm

D. Vortex

II. Chemicals/Solvents

A. Hydroperoxide standard or lipid extract

B. PeroxySafe Kit (store in 2-6 degree C): (All Reagents and calibrators are provided ready to use.)

a. Reagent A: Isopropyl Alcohol + Proprietary Compound

b. Reagent B: water, ultrapure + Chromogen (pH indicator)

c. Reagent C: Water, ultrapure + Sulfuric acid + Proprietary Compound (Iron salts)

d. Calibrators C1-C4: Fixed concentration of lipid peroxides; Isopropyl Alcohol + Proprietary Compound

e. Prep Reagent: Isopropyl Alcohol + Proprietary Compound (Stabilized alcohol)

II. Generation of Calibration Curve

A. Set-up Calibrators C1-C4 in the same way as regular samples. Follow steps in section III. In step III.B.in place of sample add 25 ul of the calibrator.

B. Press the calibration button and follow steps on machine

C. At 'Read Black" prompt insert test tube filled with distilled water in sample compartment. (Wipe outside of each tube with Kimwipe before inserting into analyzer)

D. At "Reagent Blank" prompt insert test tube containing reagent blank.

E. At "Cal # _, Insert tube" insert the Calibrators that were previously prepared.

F. Be sure to select "Yes" at "Store Cal Data" prompt.

G. Calibration is now set-up, stored in program, and will only need to be repeated when a new kit is opened. At the next prompt, regular sample tubes may now be inserted. More detailed instructions of sample preparation in III.A-F.

Note: If the "Corr" value under "Equation of Line" is less than 0.990, must redo calibration

III. PeroxySafe™ Assay

Note: 1. Assay is usually performed in triplicates.

2. Transfer reagents to be used into flasks; discard any unused portions when finished.

A. Dilute samples or standards in Prep Reagent to bring expected ROOH concentration into working range of the assay. Especially when working with unknowns (e.g. food oils or lipid extracts), we recommend diluting sample to at least three concentrations that are at least one order of magnitude different to make sure sample is detected. *If no reaction is observed, do not assume absence of hydroperoxides – excess hydroperoxides bleaches the detection complex*. When no reaction occurs, dilute the samples further sequentially until a response is observed. Record the dilution factor for each sample.

B. Vortex hydroperoxide standard or lipid extract for several seconds with Prep Reagent to fully dissolve.

C. In a clean test tube:

1. Add Reagent A – 1000 µl
2. Add diluted sample – 25 µl
3. Add Reagent B – 100 µl
4. Add Reagent C – 160 µl

D. Start timer for 15 minutes at the moment Reagent C is added to the first sample.

E. Cap each tube and vortex each sample for about 30 seconds.

F. Let reaction incubate for 15 minutes at room temperature (25and then read immediately on SafTest analyzer. Wipe outside of each tube with Kimwipe before

inserting into analyzer. Follow the prompts on the machine (Sample #_, insert tube). The absorbance is read at 570 nm.

Additional Notes:

1. Values that are greater than highest calibrator will be flagged as HI. This means the sample must be further diluted and then the assay redone because the value is inaccurate. Values that are lower than value of lowest calibrator will be flagged as Lo. If possible the sample should be diluted less and retested. If sample has not been diluted then the sample value should just reported as 'less than (value of lowest calibrator)'. Coefficient of variation (%CV) will be flagged on screen if it is greater than 10% and assay should be repeated. If samples are being measured at low end of calibration curve, a high %CV is expected, so test may not have to be repeated.

IV. Calculations

A. Analyzer will calculate Peroxide Values as milliequivalents of peroxides per kilogram of sample using the calibration curve stored.

NOTE 1: These calculations are based on "per kg isopropanol". The assumption is that the sample is diluted to such an extent that the isopropanol weight accurately represents what is in the 25 µl of sample. Results for 25 µl are adjusted for IPA density (0.786) and multiplied by 40,000 (the number of sample wt units/kg, or 1000/0.025). The standard concentrations they specify are actually final meq/kg IPA, not the amount added in the sample.

NOTE 2: MP Bio uses cumene hydroperoxide as their standard. When standards are fresh and undegraded, the slope of the regression equation for the standard curve is very close

to 1. The slope decreases as the controls age. This needs to be monitored closely and controls replaced when the slope drops below a limit point (determination in progress) or PVs of samples are erroneously low.

B. Adjust results for any sample dilution:

Actual sample PV = SafTest PV * dilution factor

For example: SafTest result of 0.07 meq/kg with a dilution factor of 1:10:

Sample PV = 0.07 meq/kg * 10 dil = 0.70 meq/kg.

C. Adjust results for fat weight in sample to express PV as meq ROOH / kg fat.

Actual sample PV = SafTest PV (* dilution factor) (* % fat in sample)

For example, if sample has 11% fat and PV/kg sample was 0.70 meq/kg:

PV (meq/kg fat) = 0.70/(11/100) = 6.36

When testing standards, adjust for sample purity rather than lipid weight in this step.

4.2.6. LOOH Based on Triphenylphosphine Oxidation Using HPLC

Adapted from (Nakamura & Maeda, 1991)

A. Apparatus/Instrumentation/Equipment

A. Glass test tubes with cap

B. HPLC:

i. Autosampler**:** Shimadzu SIL-10ADvp

ii. Detector**:** Shimadzu SPD-M10Avp

iii. Pump: Shimadzu LC-10ADvp *2

iv. Column: Restek Ultra C18 (5um, 150*4.6mm, Serial# 10080457T, Lot#100617P); (reverse-phase)

C. Micro-pipettes

D. Aluminum foil

E. Water bath

F. Floating foam platform

II. Chemicals/Solvents

A. Hydroperoxide standard or lipid extract

B. Triphenylphosphine (TP); >99%; Alfa Aesar (A Johnson Matthey Company); Ward Hill; MA

C. Cyclohexane; Fischer Scientific (Fair Lawn, NJ); certified ACS

III. Reagents/Solutions

A. Triphenylphosphine solution (4 mM): Weigh 0.0052 grams of TP and dissolve in 5 ml of cyclohexane. TP reagent make fresh daily and prepare right before use.

IV. Peroxide Value Assay

A. Cover glass tubes with aluminum foil before adding reagents.

B. Add 750 ul of diluted hydroperoxide plus 750 ul of TP reagent to aluminum covered tube.

C. Cap, Mix, and place in 30 degree C water bath (used piece of foam with holes cut in it, so tubes could float in water bath).

D. Leave in water bath for 20 minutes.

E. Set-up HPLC: 1.35 ml/min ACN; 0.15 ml/min water (total flow rate 1.50 ml/min); 7 minute run; absorption at 220 nm; 20 ul of sample injected.

4.2.7. Modification of Iodometric Titration – Lovaas Method (Løvaas, 1992)

Chemicals: Potassium Iodide (KI) (Alfa Aesar: A Johnson Matthey Company (Ward Hill, MA); 99%); Methanol (Fluka Analytical, LC-MS Chromasolv® (St. Louis, MO); ≥99.9%); Butanol (HPLC Grade; Fisher Chemical/Fisher Scientific; Fair Lawn, NJ); Hydrochloric acid (concentrated, 12N; A.C.S reagent; Sigma-Aldrich (St. Louis, MO); 37%); Ammonium iron(II) sulfate $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (Fischer Scientific (Fair Lawn, NJ); certified ACS).

Assay: Mix 2.2 ml of solvent (MeOH:BuOH; 2:1 in 3% saturated KI solution), 100 µl HCl in MeOH (25 mM), 100 µl of hydroperoxide (diluted in BuOH), and 100 ul of Fe2+ solution (500 µM). Absorbance read for 15 minutes at 360 nm in spectrophotometer.

Please note: Protocol was not written for this assay because assay did not work

4.3. Autoxidation of Methyl Linoleate

About 15 ml of fresh methyl linoleate (stored in freezer) was poured into a round-bottomed flask. The flask was connected to a rotary evaporator, and rotated for thirteen days open to air with no protection light. Five milliliters of oxidized Methyl Linoleate was removed from flask on day 4, day 9, and day 13 for analysis. The sample was stored in three 2 ml vials covered with aluminum foil to block light. The sample was bubbled with argon for several minutes, capped tightly, and stored at -80 °C.

4.3.1. Assay of Methyl Linoleate

Samples were analyzed using the five assays described above over three days in different order (unable to analyze TPP Oxidation Assay because of technical problems with HPLC in lab). Each day the PeroxySafe assay was performed as a basis for comparison to ensure there were no changes in oxidation during analyses. Each assay day, a vial of sample was removed from the freezer and allowed to warm to room temperature (between 45-60 minutes). Each time the vial was opened it to remove a sample aliquot, it was again bubbled with argon, closed tightly and kept in the dark to prevent further oxidation.

5. RESULTS AND DISCUSSION

5.1. Iodometric Titration with Thiosulfate (TS)

The iodometric titration was assayed over a range of 0.118 umoles to 0.2 mmols of hydroperoxide added, requiring a thiosulfate concentration range of 0.001-0.01 N for titration. Lower concentrations were not tested because the endpoint color change became too difficult to determine visually. Higher ROOH concentrations can be detected but the reproducibility decreases due to the high concentration of 0.1 N thiosulfate in each drop.

As shown in Figure 2, reaction response for both hydroperoxides was linear (with average $R^{2 =}$ 0.997) and detectable from about 2 – 200 μmols added peroxide. While the regression correlations were quite high for the full range, reactions were surprisingly most linear (R^2=1) in the lowest concentration range (<50 μmol added ROOH). The assay was slightly more sensitive for cumene, with a lower limit of detection of 0.3 μmole ROOH compared to the lower limit of 0.88 μmoles t-butyl hydroperoxide using 0.001N thiosulfate. The lower limit was determined as the lowest concentration at which the endpoint could be comfortably and reproducibly detected by sight. The upper limit of the assay was arbitrarily set at 0.33 mmols t-butyl and 0.21 mmol for cumene using 0.01 N TS, the concentration of ROOH requiring 25 ml of 0.01 N TS to reach the endpoint. No true upper limit of detection was found. Samples requiring more than 25 ml 0.01 N TS must be diluted or titrated with 0.1 N TS. Each operation adds to variability in assay response.

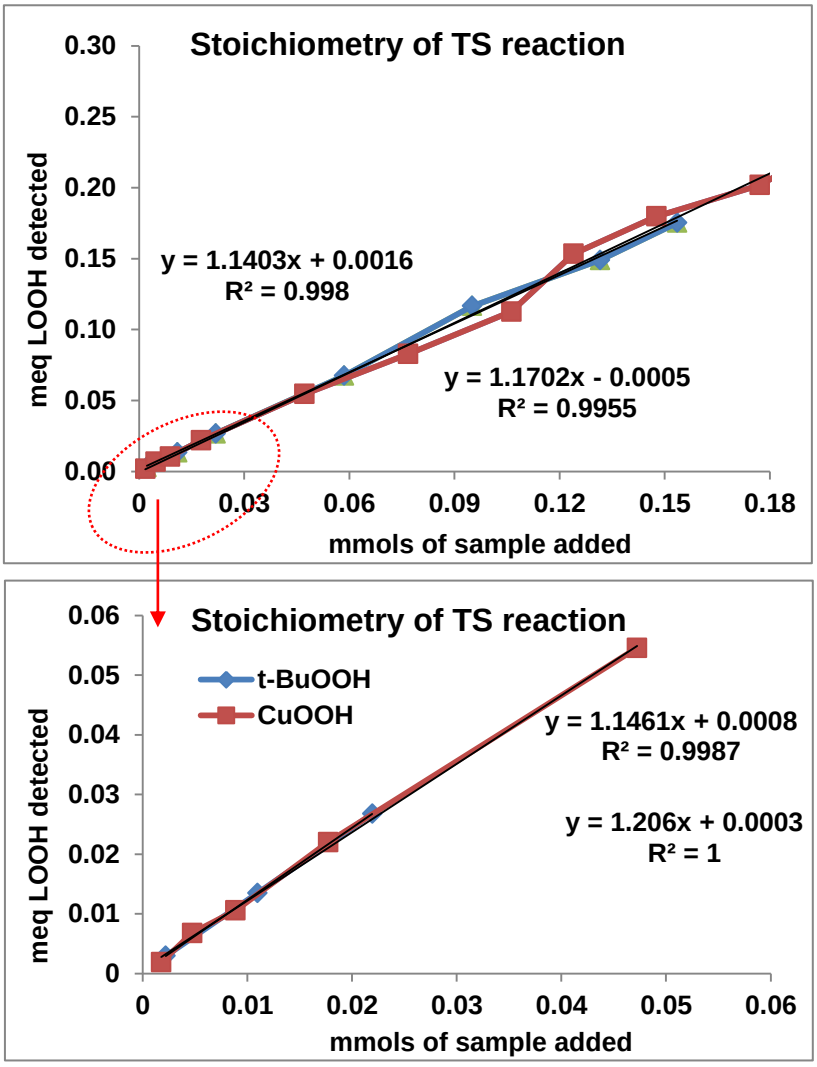


Figure 2. Concentration response curves for thiosulfate titration of cumene and t-butyl hydroperoxides. Plotted values are averages of five replicates. Standard error and replication data are presented in Table 1. Top: full concentration range. Bottom: expanded scale showing increased linearity at lowest hydroperoxide concentrations.

Slopes of 1.14 (t-butyl) and 1.17 (cumene) verify that the stoichiometry was very close 1:1 as expected theoretically from the reaction, i.e. the amount of iodine liberated was essentially equal to the ROOH concentration. It should be kept in mind that if thiosulfate solutions are prepared on a molar basis, the amount of thiosulfate consumed is twice the concentration of I_2 released and LOOH in the sample. However, if thiosulfate solutions are prepared as 1 N solutions (or dilutions thereof), the amount of thiosulfate consumed is equivalent to the I_2 released. Surprisingly, this was the most unambiguous stoichiometry and accurate reaction of all the assays tested. Comparable responses of the two hydroperoxides demonstrates that lipid extracts with different fatty acids and hydroperoxides structures can be analyzed accurately without concern that observed differences are due to different hydroperoxides rather than different hydroperoxides concentrations. The slight excess of reaction was probably due to traces of residual oxygen that are difficult to remove from hydroperoxide solutions.

Such excellent response was not obtained in first tests of this assay. This assay is known to be highly empirical and considered untrustworthy and inaccurate by some, and the problems described below verified these views.

1. Visual detection of the endpoint. The purple-blue color that formed after the starch was added turned various hues from oranges to pink (light purple/orange) and at times never fully cleared (staying a very light pink) when hand-swirling was used to add the thiosulfate. This was not observed when mechanical stirring (stir bar/stir plate) was utilized. Difficulty in distinguishing these changes were compounded by the swirling required to disperse the thiosulfate. Final color changes were not very distinct and often came suddenly in titration (Figure 3). At the endpoint the solution is colorless, though not

completely clear. The slight cloudiness is due to chloroform and water not mixing (Figure 3). Results ultimately depend on the titrator's perception of when color changed. Endpoint determination (light blue to colorless) was particularly problematic at low hydroperoxide concentrations.

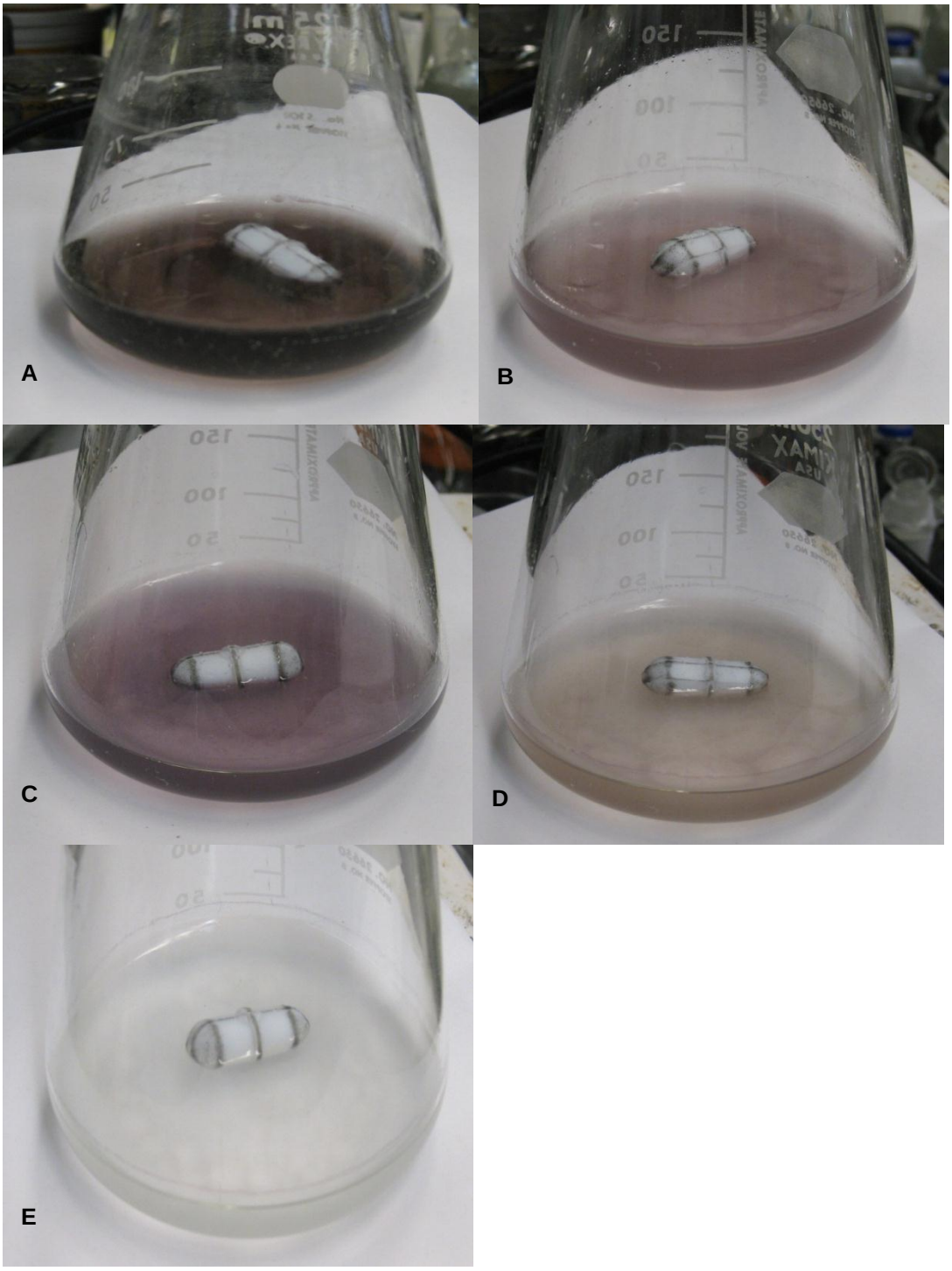


Figure 3. Progressive color changes during thiosulfate titration of iodine released after reduction of hydroperoxides. Change from D to E is often very fast. Note E is colorless.

2. Freshness of KI solutions. Protocols for Iodometric titrations always specify "prepare fresh daily". However, during handling the KI must be protected from light and bubbled with argon. When replicate samples show poor matches, making bubbling the KI with argon or preparing fresh KI restored reaction performance.

3. Transfer of hydroperoxide from organic phase to aqueous phase for reaction with I^-. Most organic hydroperoxides and certainly lipid hydroperoxides are soluble in the organic solvent but the iodine required to reduce the hydroperoxide is in the aqueous acid phase. Passive diffusion of hydroperoxides is inadequate so rigorous stirring/mixing must be provided to transfer the hydroperoxides from the organic to the aqueous phase of the reaction mixture. Normally, mixing is accomplished by hand swirling of the reaction flask during titration. However, reactions were incomplete (less ROOH detected than added) and the agitation this induced complicated endpoint detection. In addition, on numerous occasions reactions that appeared to be complete developed pink colors after standing as undetected ROOH diffused from the organic phases. To eliminate these problems, hand-stirring was replaced with mechanical stirring using a stir bar and stir plate. This modification clarified endpoints and greatly increased reproducibility. The purple-blue color no longer turned into the strange orange/pink colors, instead gradually lightening until colorless. An additional advantage of the stir bar/stir plate was that it freed the hands, making the entire titration easier to handle.

4. Oxygen. The most well-known problem with the iodometric titration is oxygen because in the chemical reaction of KI with ROOH, I^- can also react with any oxygen present, forming more iodine:

$$LOOH + 2\,H^+ + 2\,KI \rightarrow I_2 + LOH + H_2O + 2\,K^+$$

$$2KI \leftrightarrow 2\,K^+ + 2\,I^- \leftrightarrow I_2 + 2\,e^-$$

$$KI + O_2 \rightarrow I_2$$

The electrons for the ROOH reduction are provided by the saturated KI which dissociates in solution. This is an equilibrium reaction. Normally, I^- is favored. However, when the electrons are removed, e.g. by reaction with oxygen or with LOOH, the reaction is driven to the right. Consequently, unless oxygen is rigorously excluded, peroxide values can be significantly overestimated.

Initial titration data confirmed that the concentration of ROOH was several times higher than the theoretical amount when oxygen was present. Thus, several approaches were tested for eliminating oxygen.

1) To exclude oxygen, argon was bubbled through the solvents in every step. This included the saturated potassium iodide solution, acetic acid:chloroform solvent (3:2) before and after ROOH was added, and during the titration. Argon saturation combined with rigorous stirring reduced over-reaction and gave hydroperoxide yields very close to what was added.

2) A second solution to the problem was also attempted. Addition of sodium biocarbonate (sodium hydrogen carbonate) has been suggested as a way to provide a blanket of carbon dioxide over a solution and the layer of carbon dioxide would exclude oxygen from titration vessel (Skoog et al., 1998). However, sodium biocarbonate only made the solution more cloudy and the cloudiness obscured the endpoint. This approach was therefore abandoned.

Two other alterations to the procedure were investigated. The AOCS standard method has one variation in which the reaction time is extended to 2 minutes to ensure complete reaction after adding the KI and the distilled water; this option was tested. At lower hydroperoxide concentrations, one minute reaction time gave values that fit closer to the theoretical concentration, and at higher hydroperoxide concentrations, there was no significant difference between reaction times. Thus, one minute reaction time was retained since it gave the most accurate values and reduced opportunity for side reactions.

3:1 ratio of acetic acid: chloroform was tested as a replacement for the traditional 3:2 ratio of acetic acid: chloroform to investigate whether concentration of the hydroperoxides in the aqueous phase could increase sensitivity of the assay. No differences in response were observed so the original 3:2 ratio was retained.

After evaluating all these variations, it was clear that the assay accurately detected the standard hydroperoxides only when all solutions were sparged with argon, vigorous mechanical stirring was provided during titration, and argon sparging in and over the reaction solution was maintained throughout titration. These conditions were then used to test the reproducibility of the method. Five replicates of each hydroperoxide concentration were analyzed daily and repeated over three days. Results are presented in Table 5; the response curves shown in Figure 2 were also generated from this data. Within day reproducibility is just under 4% variance; variance between days is only slightly higher than this, just over 4%. Both values are surprisingly low for this assay that is notorious for its problems, and are well within acceptable limits for quantitative analyses. Replacing visual colorimetric detection of endpoint with electrochemical determinations may improve this reproducibility as well as method sensitivity.

Table 5a. Reproducibility of Iodometric titration for quantitating hydroperoxides. Values presented below are averages of 5 replicates. *0.01024 N TS **0.00104 N TS

Cumene Hydroperoxide		*mls TS required for titration*					
		With-in day			*Between Days*		
Mmols/rxn		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%COV**
0.00472**	**average**	4.03	4.19		4.11	0.11	2.71
	stdev	0.17	0.13				
	%cv	4.26	3.14				
0.00886**	**average**	9.83	10.49		10.16	0.47	4.59
	stdev	0.16	0.14				
	%cv	1.67	1.36				
0.018*	**average**	2.20	2.16	1.9	2.10	0.16	7.81
	stdev	0.07	0.09	0.08			
	%cv	3.21	4.14	4.40			
0.047*	**average**	5.23	5.51	5.26	5.33	0.15	2.88
	stdev	0.1823	0.07	0.17			
	%cv	3.4865	1.35	3.18			
0.0767*	**average**	8.38	7.94	8.10	8.14	0.22	2.74
	stdev	0.51	0.60	0.26			
	%cv	6.05	7.54	3.27			
Within day	**average % COV**	3.84		**Between Days**	**average % COV**	4.38	

Table 5b. Reproducibility of Iodometric titration for quantitating hydroperoxides. Values presented below are averages of 5 replicates. *0.01024 N TS **0.00104 N TS

t-Butyl Hydroperoxide		*mls TS required for titration*					
		Within day (ml)			*Between Days*		
mmols/rxn		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%COV**
0.0219*	**average**	2.52	2.56	2.76	2.61	0.13	4.92
	stdev	0.08	0.13	0.18			
	%cv	3.32	5.24	6.58			
0.0585*	**average**	6.63	6.00	7.14	6.59	0.57	8.67
	stdev	0.13	0.28	0.54			
	%cv	1.90	4.71	7.52			
0.0022**	**average**	2.92	2.78	2.81	2.84	0.07	2.60
	stdev	0.13	0.08	0.07			
	%cv	4.47	3.01	2.64			
0.0058**	**average**	8.73	8.83	8.72	8.76	0.06	0.69
	stdev	0.11	0.13	0.08			
	%cv	1.25	1.48	0.96			
Within day	**average COV%** 3.59			**Between days**	**average COV%** 4.22		

Overall, results showed why iodometric titration remains the gold standard to analyze hydroperoxides. However, oxygen and stirring must be controlled for accurate analysis. How the reaction is run strongly affects reaction stoichiometry, accuracy, and reproducibility. Results presented here prove that when there is strict control, the assay is linear and stoichiometric. Problems with weak endpoint can cause you to miss early oxidation products when levels are low. The assay may be time-consuming, and cumbersome; and does not lend itself to automation, but since it is a simple titration most labs would have the equipment necessary. This assay is least sensitive to low hydroperoxide concentrations, which may overestimate induction periods, but it is the

only assay useful for direct analysis of high hydroperoxide concentrations(no extensive dilution). High priority should be give to testing whether autotitrators can simplify the sample handling and amperometric endpoints can increase the sensitivity at low hydroperoxide concentrations.

5.2. Ferric Thiocyanate Assay for Hydroperoxides

There were various problems with the ferric thiocyanate procedure in the beginning. The absorbance was much lower than expected (less than 2:1 stoichiometry), so Fe^{3+} was not being formed. Results in lab were not matching the results in the literature. In order to find the source of the problem several modifications were tested, varying the. solvent, sources of Fe^{2+} (ferrous sulfate; ammonium ferrous sulfate) and NH_4SCN concentration (30% and 3 %; diluted in water and methanol). The issue of acidity, order of addition of reagents, and solubility issues were also investigated. Two papers in particular (Mihaljevic et al., 1996; Richards & Feng, 2000) were used as guidelines because they suggested that concentration of hydroperoxide should not exceed 20 uM. It is possible that the higher [ROOH] was causing problems. The final procedure presented in the Methodology section was based more on the Shantha & Decker Method (modified official IDF standard Method 74A:1991) than the Mihaljevic paper because results were closer to theoretical with 30% SCN than 3% SCN and adding Iron(II) and SCN separately, rather than together.

Sensitivity and linearity of response:

Standard curves prepared from authentic Fe(III) appeared to be linear up to optical limits of spectrophotometers (Figure 4). However, the FeSCN complexes are

intensely colored and have high extinction coefficients. Consequently, only very low concentrations are needed to give absorbances greater than 1, the dependable detection limit on most spectrometers. The upper limit with our Cary 50 spectrophotometer is about 70 μM or 180 nmoles Fe^{3+} added. Assuming that each LOOH reacts with 2 Fe, the upper limit for LOOH with our Cary 50 spectrophotometer is about 90 nmoles ROOH per reaction (half of the upper limit of Fe^{3+} in Figure 4). For other spectrophotometers, the upper limit is even lower. Using the full standard curve, the lower limit for most accurate detection must be set at about 14 μM Fe^{3+} or 36 nmols of Fe^{3+} added. Again assuming each LOOH reacts with 2 Fe, lower limit is about 18 nmoles of ROOH added (Figure 4). The lower limit was set there because the slope of the response curve increases at concentrations below this value (Figure 4). If using only the lower end of the standard curve (<14 μM), the lower limit is much lower; about 3 μM Fe^{3+} or 10 nmoles Fe^{3+} added (or 5 nmoles of ROOH added).

The actual reaction response for hydroperoxides was linear between 1 and 32 μM for CuOOH, and between 0.28 and 42 μM for t-BuOOH (Figure 5).

Accuracy and stoichiometry of response:

Two factors can lead to errors in calculation when using this assay with mixed or different hydroperoxides. As shown in Figure 5, the reaction response varied with the hydroperoxide, with cumene-OOH showing stronger reaction than t-Butyl-OOH. As will be shown in a later section, methyl linoleate hydroperoxide reaction is dramatically stronger than either of these standard hydroperoxides.

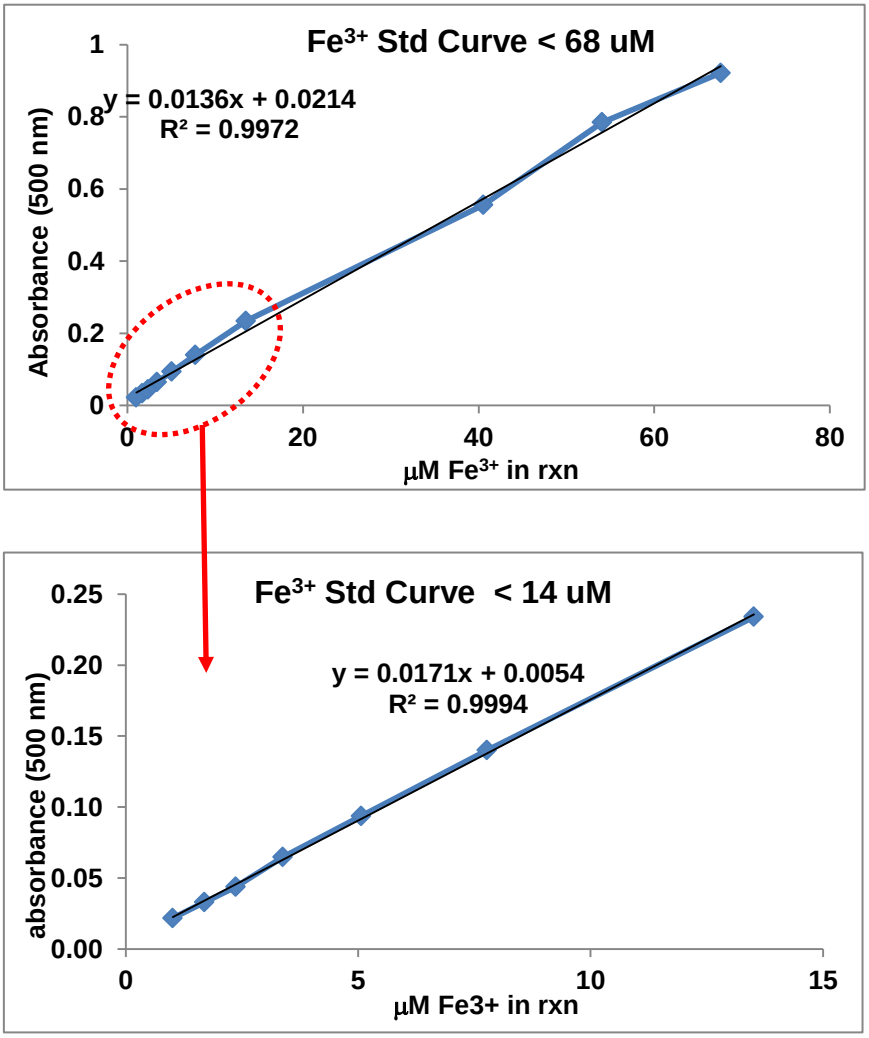


Figure 4. Iron standard curve for Ferric Thiocyanate assay for hydroperoxides. Top: full concentration range tested. Bottom: Expanded scale for lowest concentration ranges.

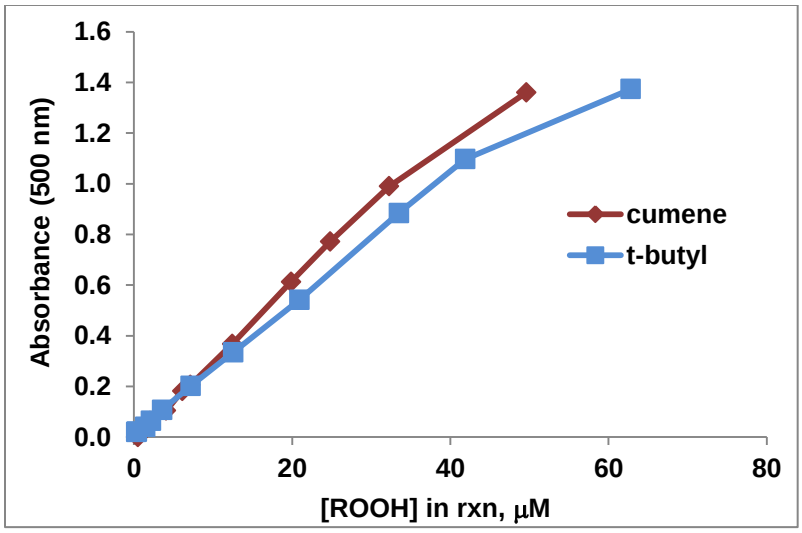


Figure 5. Reaction response of cumene and t-Butyl hydroperoxides in the FeSCN assay.

In addition, the calculated ROOH concentrations varied with the section of the regression curve applied (Figure 6). Thus, applying the slope of the higher concentration region (0.013) is probably accurate for that region but underestimates lower concentrations (some values were negative). On the other hand, using the slope for the lower concentration region underestimates the higher concentrations, and this discrepancy increases with concentration.

Both phenomena can lead to significant interpretation errors when comparing oxidation of oils/fats with different fatty acid compositions.

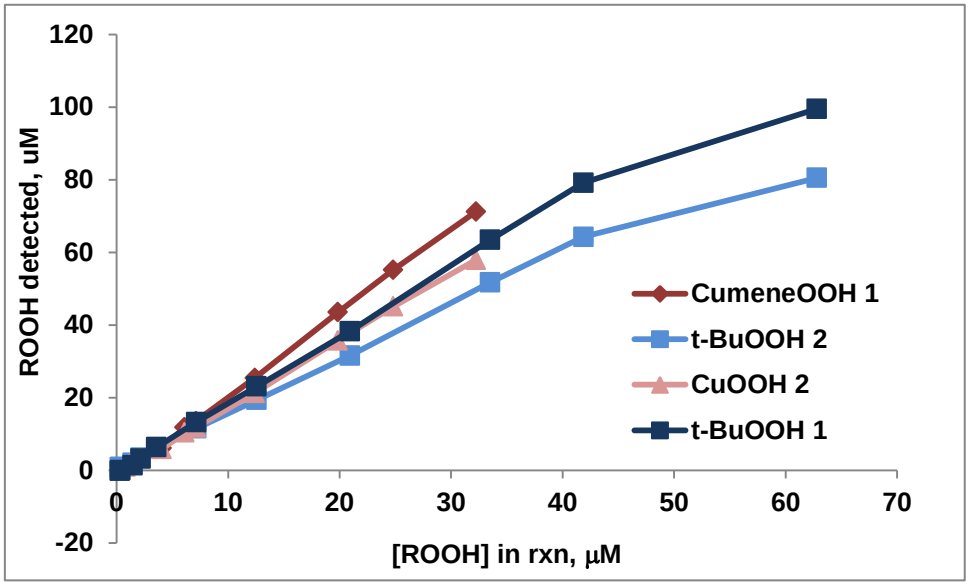


Figure 6. Response stoichiometry of cumene and t-Butyl hydroperoxides in the FeSCN assay. Curves labeled 1 were calculated using the slope of the upper region of the Fe^{3+} standard curve (0.013); curves labeled 2 were calculated using the slope from the lower region of the standard curve (0.017).

In terms of stoichiometry, Table 6 show that the reaction stoichiometry differed between hydroperoxides, was not constant with either ROOH concentration or direction of change, and was less than the 2:1 expected theoretically in all cases except the highest concentrations of CuOOH (also seen in Figure 6). CuOOH stoichiometry was at or near 2 and the highest concentrations but decreased dramatically as [CuOOH] decreased. t-BuOOH stoichiometry never reached 2 at any concentration and, opposite to CuOOH, was inversely related to [t-BuOOH] when the regression coefficient of the lower curve was used for calculations. Since the second electron is consumed in $RO^{\bullet}$ reactions, we

may speculate that the reaction is controlled by the redox potential of individual hydroperoxides in susceptibility to being reduced (Reaction 1) and by the redox potential of the resulting alkoxyl radicals in mediating secondary oxidations (iron or solvent) in reaction 2. This possibility will be discussed in more detail later.

Table 6. Stoichiometry of ferric thiocyanate reaction with cumene and t-Butyl hydroperoxides with comparison of regression curves.

CuOOH:	**y=0.0136X+0.0214**		**y=0.017x + 0.0054**	
[ROOH] reacted (µM)	**[ROOH] detected (µM)**	**Stoichiometry**	**[ROOH] detected (µM)**	**Stoichiometry**
32.25	71.20	2.21	57.90	1.80
24.81	55.19	2.22	45.10	1.82
19.85	43.49	2.19	35.73	1.80
12.40	25.40	2.05	21.26	1.71
7.12	13.53	1.90	11.76	1.65
6.10	11.78	1.93	10.37	1.70
4.07	6.11	1.50	5.83	1.43
2.03	3.08	1.51	3.40	1.67
1.22	1.08	0.89	1.81	1.48
0.81	0.00	0.00	0.94	1.16
t-BuOOH:	**y=0.0136X+0.0214**		**y=0.017x + 0.0054**	
[ROOH] reacted (uM)	**[ROOH] detected (uM)**	**Stoichiometry**	**[ROOH] detected (uM)**	**Stoichiometry**
62.81	99.48	1.58	80.52	1.28
41.87	79.13	1.89	64.24	1.53
33.50	63.44	1.89	51.69	1.54
20.94	38.28	1.83	31.56	1.51
12.56	23.06	1.84	19.39	1.54
7.15	13.24	1.85	11.54	1.61
3.58	6.32	1.77	6.00	1.68
2.15	3.15	1.47	3.46	1.61
1.43	1.37	0.96	2.04	1.42
0.36	-0.01	-0.04	0.93	2.60
0.29	-0.14	-0.49	0.83	2.89

Reproducibility of response:

The complexation of ferric iron by thiocyanate showed excellent precision and reproducibility, with an average coefficient of variance of 3.01% over all concentrations (Table 7). In contrast, reactions of cumene and t-Butyl hydroperoxides were less reproducible than the assays discussed above, with within-day and between-day COVs of 7.37 and 11.72%, and 5.75% and 19.78% respectively for CuOOH and for t-BuOOH. Reasons for the inconsistency have not been identified yet, but probably are related to complexity of the reaction system itself.

Table 7a. Reproducibility of ferric thiocyanate assay for hydroperoxides: ferric iron standard. Three replicates per day. Fe3+ Std curve

	Fe^{3+} Std Curve					
	µmoles added	**absorbance**	**µmoles added**	**absorbance**	**µmoles added**	**absorbance**
average	0.45	1.3594	0.11	0.5556	0.009	0.0648
stdev		0.0078		0.0174		0.0022
cov%		0.57		3.13		3.39
average	0.36	1.1740	0.04	0.2340	0.006	0.0438
stdev		0.0123		0.0002		0.0020
cov%		1.05		0.09		4.52
average	0.18	0.9215	0.02	0.1400	0.005	0.0330
stdev		0.0202		0.002		0.0011
cov%		2.19		1.37		3.34
average	0.14	0.7847	0.01	0.0937	0.003	0.0218
stdev		0.0082		0.004		0.0024
cov%		1.05		4.41		11.03
Within-day average COV	3.01%					

Table 7b. Reproducibility of ferric thiocyanate assay for hydroperoxides: cumene hydroperoxides. 5 replicates per day.

Cumene Hydroperoxide							
Final conc.		*With-in a day (absorbance)*			*Between Days*		
(µM)		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%cv**
6.10	**average**	0.1858	0.1747	0.1844	0.1816	0.0060	3.31
	stdev	0.0037	0.0052	0.0026			
	cov%	1.99	2.98	1.40			
4.10	**average**	0.0805	0.1111	0.1218	0.1045	0.02	20.50
	stdev	0.0091	0.0105	0.0035			
	cov%	11.29	9.4861	2.87			
2.03	**average**	0.0671	0.0564	0.0662	0.0632	0.0060	9.45
	stdev	0.0077	0.0064	0.0025			
	cov%	11.49	11.3990	3.79			
1.22	**average**	0.0276	0.0355	0.0367	0.0361	0.0049	13.62
	stdev	0.0037	0.0040	0.0027			
	cov%	13.29	11.19	7.31			
Within day average COV		7.37%		**Between-days average COV**		11.72%	

Table 7c**.** Reproducibility of ferric thiocyanate assay for hydroperoxides: t-Butyl hydroperoxides. 5 replicates per day.

t-Butyl Hydroperoxide							
Final conc.		*With-in a day (absorbance)*			*Between Days*		
(µM)		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%cv**
7.15	**average**	0.1699	0.2436	0.1986	0.2015	0.0331	16.45
	stdev	0.0067	0.0054	0.0047			
	cov%	3.95	2.22	2.38			
3.58	**average**	0.0827	0.1199	0.1193	0.1073	0.0213	19.86
	stdev	0.0045	0.0041	0.0022			
	cov%	5.46	3.46	1.81			
2.14	**average**	0.0518	0.0844	0.0565	0.0642	0.01764	27.46
	stdev	0.0023	0.0053	0.0008			
	cov%	4.52	6.22	1.43			
1.43	**average**	0.0335	0.0457	0.0410	0.0400	0.0062	15.37
	stdev	0.0055	0.0049	0.0043			
	cov%	16.39	10.64	10.56			
Within day average COV		5.75%		**Between-days average COV**		19.78%	

Handling issues, quirks and precautions:

The ferric thiocyanate reaction gives results only at very low hydroperoxide concentrations; hydroperoxide levels above the detection limit oxidize the complex and the reaction appears not to run. Consequently, when samples give very low optical absorbance with this reaction, they should be diluted sequentially and retested until a reactive concentration is found. Only when no reactions are found even at lipid concentrations below micromoles per reaction tube should a sample be considered to be without hydroperoxides. In fact, we recommend that a range of concentrations routinely be analyzed for each sample to make sure calculations are made in the linear response

range for the reaction. All solvents need to be sparged with argon and the reaction should be completed in the dark.

5.3. Cayman Hydroperoxide Kit (Thiocyanate Assay)

The Cayman Assay kit may be run using a microplate reader or spectrophotometer. The plate reader was chosen for this assay so that an assay for high throughput could be evaluated. The first task was plate reader optimization. The Cayman Assay states to run the assay at 500 nm. The plate reader came with a UV filter of 492 nm. In order to test whether this UV filter could be used, wavelengths were scanned between 200 nm – 650 nm and between 200 nm – 800 nm. The highest point of the peak for both was 504-507 nm, and it was only slight lower than absorbance at 492 nm measured on another spectrophotometer. It was decided that 492 nm UV filter could be used, though it must be noted that it is not the optimum wavelength. The plate reader lamp energy used for this assay was 10,000; at energies higher than 20,000 absorbance values began to decrease.

Sensitivity and linearity of response:

The Cayman Kit supplied a lipid hydroperoxide standard, 13-hydroperoxyoctadecadienoic acid (13-HpODE), to construct a calibration curve. A calibration curve using 13-HpODE was constructed from two different kits to compare reproducibility. The calibration curves were run several months apart, and show similar response (Figure 7). The dynamic range was between 0.5 – 5 nmols per assay which matched the claim on the Cayman assay kit. Above 5 nmols per assay was not tested, so

it cannot be verified if this is the true upper limit. Samples with < 0.5 nmoles could not be distinguished from the blank absorbance.

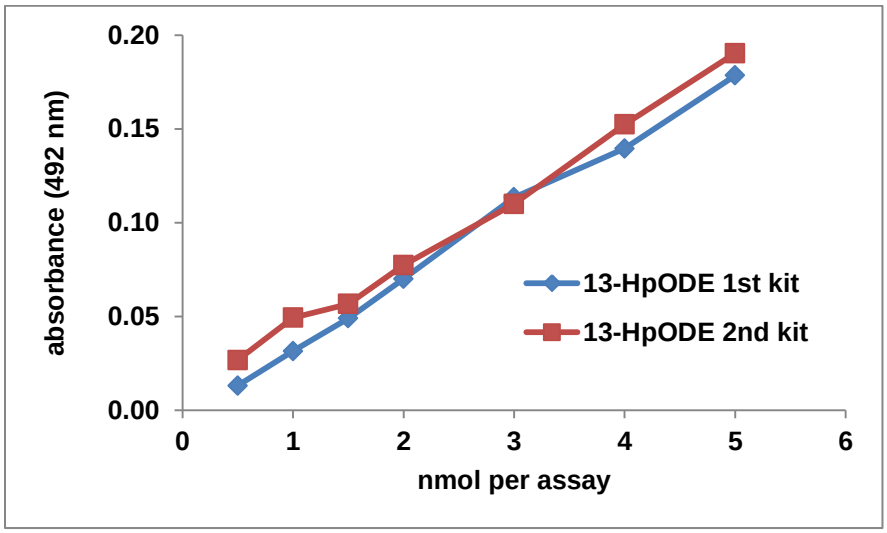


Figure 7. Reproducibility of standards and kits supplied for Cayman ferric thiocyanate assay of hydroperoxides. Average of three replicates; two different kits run several months apart.

When tested over the same concentration range (0 nmoles – 5nmoles per assay) to compare responses, CuOOH showed slightly higher reactivity than t-BuOOH (Figure 8). Both hydroperoxides had markedly lower reaction than the 13-HpODE standards (Figure 9), demonstrating that once again, the reaction response varied with the structure of the hydroperoxides. This means that in samples with unknown or mixed hydroperoxides, it cannot be determined whether differences in peroxide values result from differences in peroxide structures or peroxide levels. There was a solubility problem with t-butyl hydroperoxide which is water-soluble, but water interferes with this assay. t-BuOOH appeared to dissolve in chloroform:methanol, but the possibility that the lower reaction response of t-BuOOH resulted from incomplete solubilization cannot be excluded.

CuOOH had the same lower limit as 13-HpODE (0.5 nmols), but t-BuOOH was slightly less sensitive, with 1 nmol per assay as the lower limit.

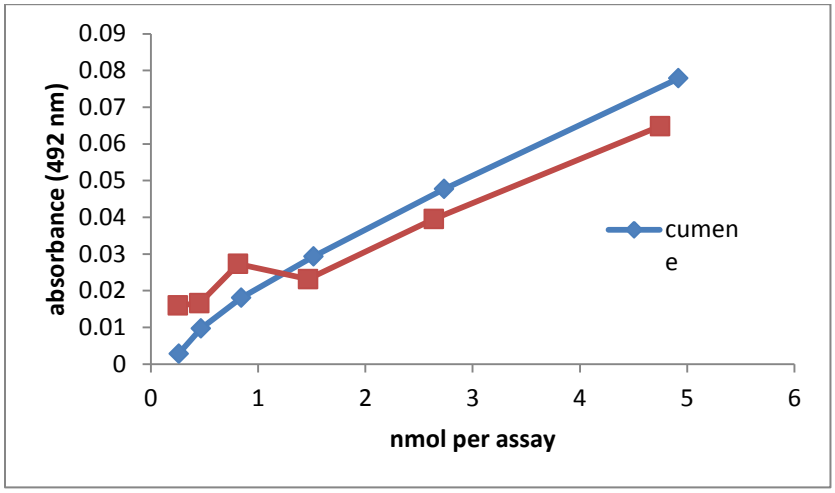


Figure 8. Reaction responses as a function of of cumene and t-butyl hydroperoxide concentrations in the Cayman Fe^{3+}-SCN assay.

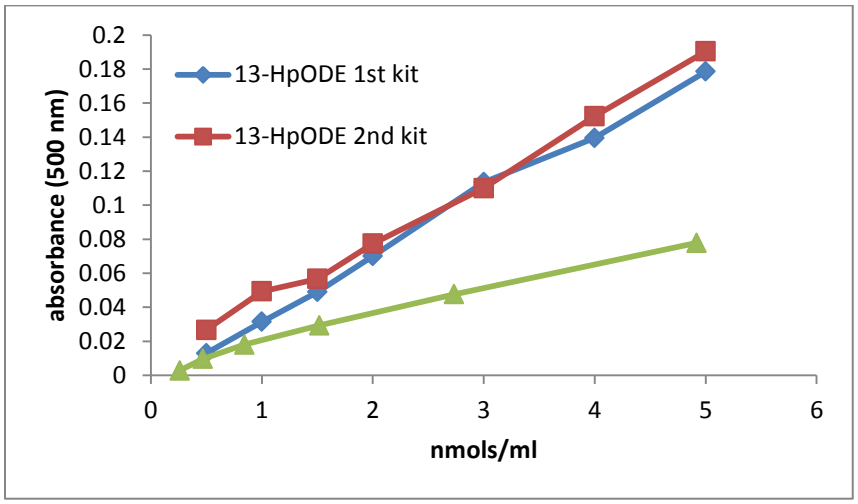


Figure 9. Comparison of reaction responses for cumene hydroperoxide and 13-HpODE standards in the Cayman Fe^{3+}-SCN assay.

Accuracy and stoichiometry of response:

Stoichiometry of the Cayman kit reaction could not be determined because an authentic Fe^{3+} standard curve matching the kit reagents could not be prepared.

Reproducibility of response:

Within-day variability for lipid standards (13-HpODE) was low -- 3.5% for the first kit with and 4.6% for the second kit (data not shown). Cumene and t-butyl hydroperoxides showed similar reproducibility with within-day cov of ~3.6% (Table 8). Between-day reproducibility was expectably somewhat lower: 5.3% for CuOOH and 6% for t-BuOOH. This reproducibility is still well within acceptable limits for assays (usually considered to be 10%), and was better than the original thiocyanate method.

Handling issues, quirks and precautions:

The solvent (chloroform) must be deoxygenated. The kit recommends use of nitrogen, but argon is heavier and does not carry oxygen contamination. High background absorbance (blank) indicates that solvent should be sparged longer with argon. The standard lipid hydroperoxide (13-HpODE) should be stored at -80 °C and held on ice during preparation of standard curves to prevent degradation. Running a standard curve with each set of samples should not be necessary.

It is very important to keep the tubes with reagents capped tightly and to cover the plates with aluminum foil after samples are added to prevent evaporation. A plastic cover cannot be used because the solvent will dissolve it. The reaction should be run in the dark. Tap the side of plates to eliminate any bubbles that form during dispensing. Inspect plate bottoms to be sure there are no scratches that can interfere with readings.

Table 8a. Reproducibility of Cayman ferric thiocyanate assay for cumene hydroperoxide.

5 replicates per day.

CuOOH		*Within day (absorbance)*				*Between Days*	
nmol/ml		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%cv**
4.9	**average**	0.1684	0.1560	0.1552	0.1599	0.0074	4.63
	stdev	0.0024	0.0081	0.0029			
	%cv	1.43	5.17	1.90			
2.73	**average**	0.1318	0.1276	0.1296	0.1297	0.0021	1.62
	stdev	0.0036	0.0050	0.0051			
	%cv	2.70	3.94	3.96			
1.52	**average**	0.1046	0.1188	0.1106	0.1113	0.0071	6.40
	stdev	0.0035	0.0086	0.0034			
	%cv	3.35	7.23	3.04			
0.84	**average**	0.0954	0.1014	0.1034	0.1001	0.0042	4.16
	stdev	0.0027	0.0058	0.0011			
	%cv	2.83	5.70	1.10			
0.47	**average**	0.0830	0.0966	0.0955	0.0917	0.0076	8.24
	stdev	0.0023	0.0017	0.0031			
	%cv	2.83	1.73	3.25			
0.26	**average**	0.0784	0.0882	0.0878	0.0848	0.0055	6.54
	stdev	0.0063	0.0037	0.0019			
	%cv	7.99	4.20	2.19			

Overall average COV of the assay: within day 3.59**% between days** 5.27**%**

Table 8b. Reproducibility of Cayman ferric thiocyanate assay for hydroperoxides: t-butyl hydroperoxides. 5 replicates per day.

t-BuOOH		*Within day (absorbance)*				*Between Days*	
nmol/ml		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%cv**
4.75	**average**	0.1368	0.1498	0.1478	0.1448	0.007	4.83
	stdev	0.0016	0.0058	0.00239			
	%cv	1.20	3.88	1.61			
2.65	**average**	0.1166	0.1264	0.1156	0.1195	0.0060	4.99
	stdev	0.0033	0.0059	0.00289			
	%cv	2.82	4.63	2.50			
1.47	**average**	0.0998	0.1122	0.0974	0.1031	0.0079	7.70
	stdev	0.0063	0.00130	0.0034			
	%cv	6.31	1.16	3.45			
0.82	**average**	0.0994	0.1112	0.1112	0.1073	0.0068	6.35
	stdev	0.0023	0.0034	0.0025			
	%cv	2.32	3.08	2.24			
0.45	**average**	0.0912	0.0984	0.01	0.0965	0.0047	4.85
	stdev	0.0048	0.0048	0.0024			
	%cv	5.22	4.90	2.45			
0.25	**average**	0.0882	0.0998	0.01	0.096	0.0068	7.04
	stdev	0.0121	0.0015	0.0022			
	%cv	13.78	1.49	2.24			

Overall average COV of the assay: within day 3.63**% between days** 5.96**%**

5.4 Xylenol Orange – Pierce Assay Kit

Sensitivity of response:

The lower limit for cumene-OOH detection was 1-2 nmoles added, which agrees with what the kit claims (Figure 10). Detection of t-Butyl-OOH was less sensitive, with a lower limit of 7-8 nmoles added. The upper limit for the assay, based the instrument limitations (most spectrophotometers are not accurate much above absorbance of 1.0-1.5) was 45-50 nmoles for t-Butyl-OOH and 25-30 nmoles for cumene-OOH.

Linearity of response:

The Pierce kit warns that the hydrogen peroxide standard curve which they recommend for aqueous systems is not linear over the entire concentration range of the assay. Response curves for cumene and t-Butyl hydroperoxides and also the Fe^{3+} standard in the lipophilic system were not only non-linear, but were also difficult to fit equations of any form (Figure 10).

Accuracy and stoichiometry of response:

A standard curve was generated from authentic Fe^{3+} to provide a more accurate model of the reaction occurring in this solvent system. At low concentrations, the iron standard and t-Butyl-OOH response curves were quite close. However, above about 25 nmol test compound in the reaction mixture, the iron response plateaued while the t-Butyl-OOH response continued, albeit more slowly. The cumene-OOH response was far greater than t-BuOOH or Fe^{3+} and markedly non-linear, especially at higher concentrations. Thus, it is clear that much more complicated chemistry is occurring in the CuOOH system. It is likely that Cu-$O^{\bullet}$, which is a strong oxidizing agent, increases rate of conversion of Fe^{2+} to Fe^{3+} at lower concentrations. At higher concentrations this

action is counterbalanced by peroxide bleaching of the XO-Fe complex; this is the region where the curve slows and begins to level off.

The Pierce kit directions state that the Fe3+ and xylenol orange complex should yield a purple color. Lab testing showed a bluish color only formed at 100-50 nmoles of hydroperoxide added. At lower concentrations it turned into a muddy orange color. A wavelengths scan (Figure 11) between 350-700 nm was run for just xylenol orange, and then for cumene sample of 12.5 nmoles of added sample. At 560 nm there is a second peak with a high enough absorbance for the cumene, which is the wavelength were the Fe^{3+}-XO complex should be detected. This means that even though it is no longer a blue/purple color a reaction is still occurring.

The irregularity of response and the huge difference in R group effect on hydroperoxides reaction in this assay are of great concern. Both factors make it difficult to actually calculate ROOH concentrations unless there is an exactly matched hydroperoxide standard and concentrations are kept very low. Appropriate compounds to use as standards for lipid hydroperoxides are a particular problem. C18-OOH standards are commercially available at very high cost, but are not included in this kit (kit recommends hydrogen peroxide). Until issues of standards can be resolved, we consider this assay not acceptable for quantitative assays of lipid oxidation. It may, however, be useful for comparative assays of comparable materials and, despite being a lipophilic version, is probably more useful for hydrophilic systems than for lipid oxidation.

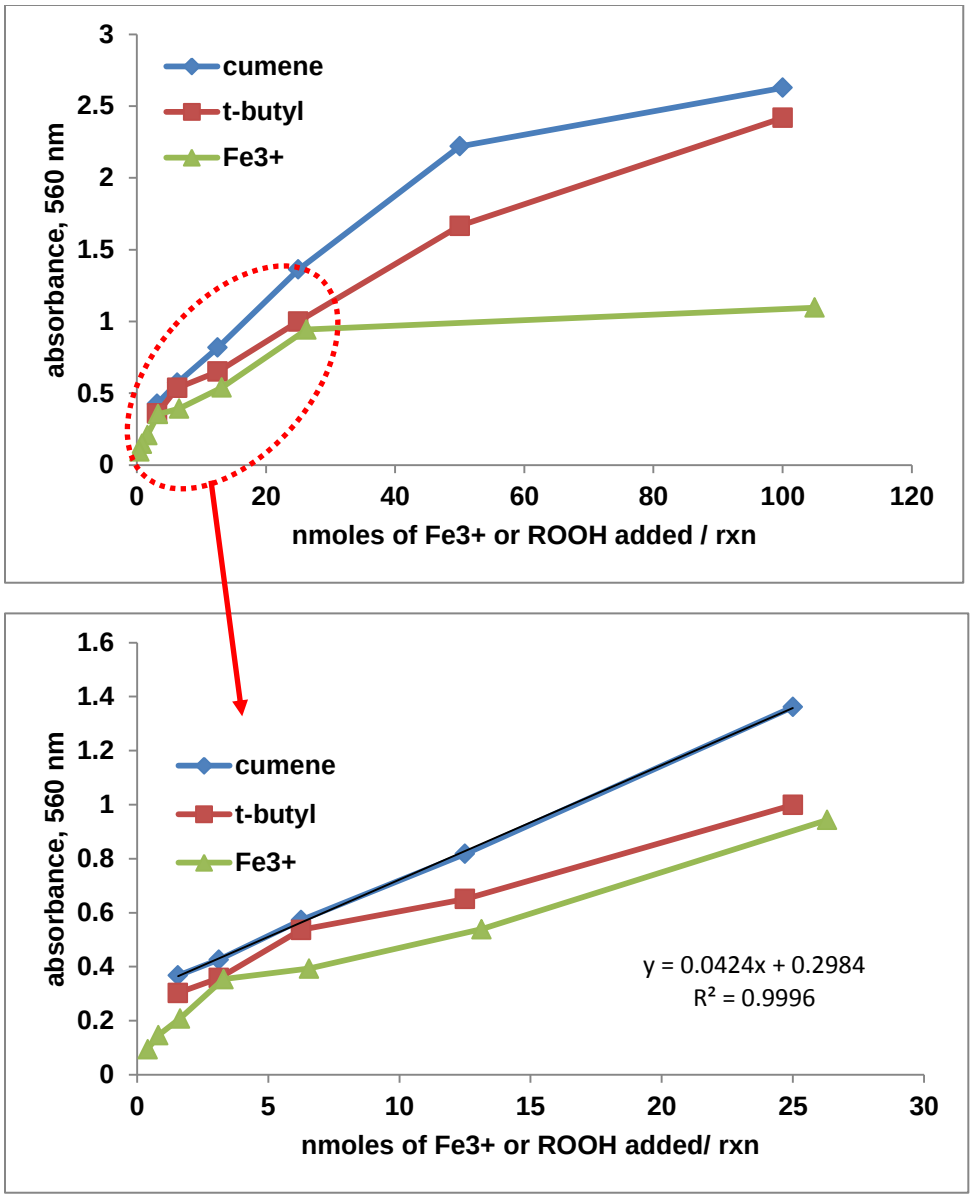


Figure 10. Response curves for reaction of Fe^{3+}, CuOOH, and t-BuOOH in the Pierce xylenol orange assay. Top curve: full concentration range. Bottom curve: lower concentration range on expanded scale.

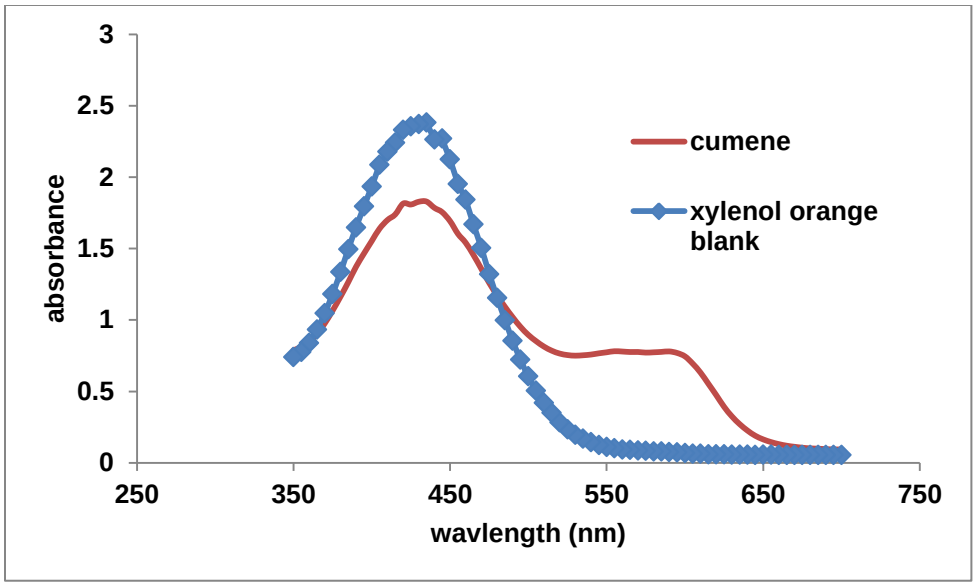


Figure 11. Optical spectra of xylenol orange blank and cumene hydroperoxide-XO.

Reproducibility of response:

Within day reproducibility of this assay is very good 1.4 and 2.5% for t-Butyl and cumene hydroperoxides, respectively (Table 9). However, between-day variance is notable larger than for the other assays. Possible explanation for variability are noted below.

Handling issues, quirks and precautions:

Special note: High levels of peroxides bleach the dye and Fe-dye complexes, causing inaccurate low absorbance readings. It is recommended that all samples be analyzed over a range of dilutions, at least 1000x. If no reaction is obtained, continue diluting sample to 10^6 dilution. If diluted samples have higher or similar readings to undiluted samples, a bleaching effect has occurred and readings from the lowest concentrations giving a color should be used for calculations.

Diluting cumene just in methanol creates substantial solubility problems that result in lower absorbances than expected. Since cumene is somewhat soluble in water (MSDS 1.5 g/ 100 ml), only the minimum methanol required to solubilize cumene-OOH was used. Further dilution was then done with water, which gave higher absorbances. Limiting methanol also minimized electron transfer differences between cumene and t-Butyl hydroperoxides.

Table 9. Reproducibility of Pierce xylenol orange assay for hydroperoxides, using standard cumene and t-Butyl hydroperoxides.

Cumene Hydroperoxide		*Within day (absorbance)*			*Between Days*		
uM cumene-OOH		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%cv**
250	**average**	1.4573	1.2746	1.3531	1.362	0.092	6.732
	stdev	0.0664	0.0420	0.0536			
	%cv	4.5573	3.2953	3.9637			
125	**average**		0.7959	0.8394	0.818	0.031	3.766
	stdev		0.0230	0.0146			
	%cv		2.8952	1.7384			
62.5	**average**	0.5902	0.5464	0.5815	0.573	0.023	4.052
	stdev	0.0171	0.0028	0.0070			
	%cv	2.9030	0.5120	1.1994			
31.25	**average**	0.4301	0.4089	0.4389	0.426	0.015	3.621
	stdev	0.0127	0.0055	0.0094			
	%cv	2.9498	1.3400	2.1365			
Within day ave COV		2.499	**Between-day ave COV**		4.543		

t-Butyl Hydroperoxide							
		Within day (absorbance)			*Between Days*		
uM t-Butyl-OOH		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%cv**
1000	**average**	2.34	2.49	2.43	2.42	0.08	**3.17**
	stdev	0.04	0.05	0.09			
	%cv	1.92	1.81	0.37			
500	**average**	1.55	1.82	1.62	1.67	0.14	8.50
	stdev	0.02	0.03	0.03			
	%cv	1.09	1.78	2.06			
125	**average**	0.62	0.71	0.62	0.65	0.05	8.16
	stdev	0.01	0.02	0.003			
	%cv	1.53	2.18	0.45			
31.25	**average**	0.35	0.37	0.36	0.36	0.01	2.56
	stdev	0.01	0.001	0.004			
	%cv	2.36	0.30	1.02			
Within day ave COV		1.40	**Between-day ave COV**		5.60		

Pierce Xylenol Orange Summary:

Sensitivity and reproducibility of this assay are both lower than the parallel xylenol orange assay in the Peroxy-Safe kit, and reaction responses are non-linear and vary with hydroperoxide structure. Issues of solubility in this assay may make cumene-OOH a poor choice for a standard (could not use methanol for dilution), while solubility mismatch may eliminate t-Butyl hydroperoxide as an acceptable standard for lipid hydroperoxides. In addition, high susceptibility of the XO-Fe^{3+} complex to bleaching by ROOH means that a series of sample dilutions must be run for each assay to make sure samples are in detection range. Until these issues can be resolved, we consider this assay not acceptable for quantitative assays of lipid oxidation. It may, however, be useful for comparative assays of comparable materials.

5.5. PeroxySafe Kit

Cumene and t-Butyl hydroperoxides were analyzed using the PeroxySafe™ Standard kit from MP Biomedical, Solon, OH. Since all of the hydroperoxides tested (including methyl linoleate) had to be diluted by several orders of magnitude for the STD assay, there was no reason to test the higher sensitivity HSY kit that was designed more for use with biological tissues. The kit assay was modified for delivery or reagents with a micropipette instead of the calibrated autopipettors supplied with the kit. We found these units to be inaccurate, irreproducible, and extremely wasteful of expensive reagents due to the priming required, so we replaced them with direct pipetting of reagents using micropipettors.

Sensitivity of response:

The technical sheets for all three SafeTest assays claim a working range of 0.01-50 meq/kg. The working concentration range observed in lab testing was 2-12 nmols ROOH in the sample added to the reaction, calculated against cumene hydroperoxide standards provided in the kit (Figure 12). This corresponds to about 0.01 to 0.5 meq ROOH/kg sample, so the assay is extremely sensitive but does not match the manufacturer's range specifications. Cumene and t-Butyl hydroperoxide had an upper limit of about 9 nmoles per reaction and lower limit of $< l$ nmoles/rxn.

Cumene-OOH response in the PeroxySafe assays was consistently about 12% greater than the t-Butyl-OOH response (Figure 12). Whether this difference is a solubility, steric, or chemical (e.g. inductive effects of chain length) issue is not yet known. Chain length effects of R in ROOH need to be investigated further to determine the potential variability among different lipid hydroperoxides. Variability of response according to ROOH structure is not a problem when the same samples are being followed over time or like samples are being compared. However, it is a distinct disadvantage and can lead to erroneous conclusions when multiple sizes of ROOH are present (e.g. in frying oils), when the nature of the hydroperoxides is unknown, or when samples with different fatty acid composition are being compared.

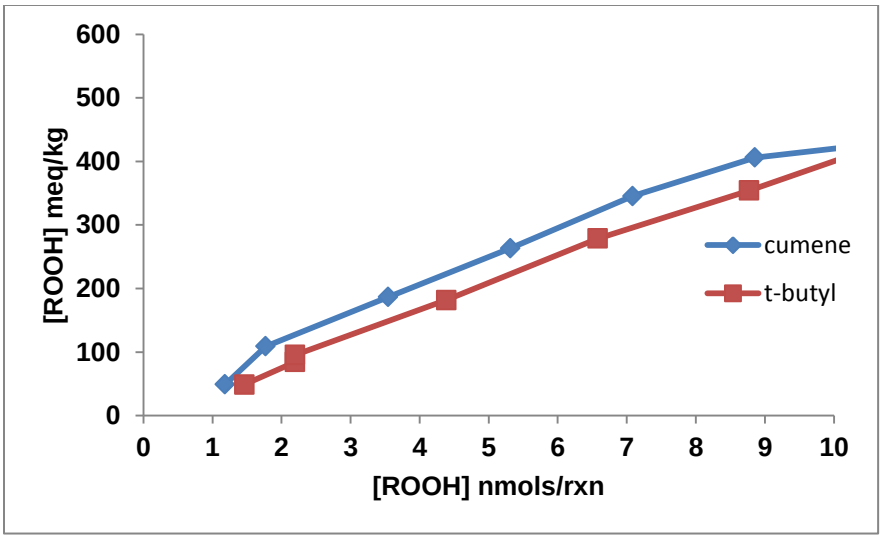


Figure 12. SafTest response curves for cumene and t-Butyl hydroperoxides. Each data point is an average of five replicates.

Linearity of response:

Lower concentrations of hydroperoxides gave linear responses with comparable slopes in the SafTest reaction with xylenol orange. However, close examination of the curves reveals that even in mid-concentration ranges, response drops off as hydroperoxide concentration increases (Figure 13).

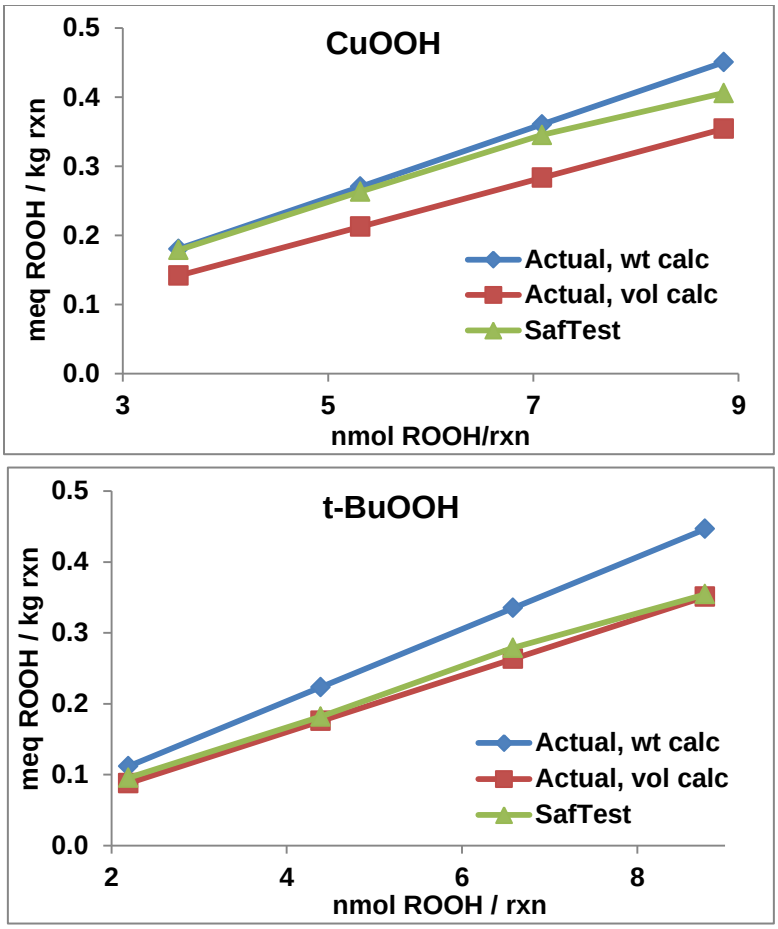


Figure 13. Comparison of SafTest peroxide values with actual peroxide concentrations added, calculated as / kg isopropanol (wt basis) or /L isopropanol (volume basis).

Accuracy and stoichiometry of response:

Since SafTest is a proprietary product, its components and concentrations are not made public. We tried to get detailed information about formulation, particularly xylenol orange concentrations and calculation procedures, but this information is unavailable. With some difficulty, we think we have tracked their calculations sufficiently to at least compare theoretical yields with results given by the SafTest "box". However, exact stoichiometry of reaction cannot be determined because reactant concentrations and exact methods for PV calculations are both unknown.

Particularly problematic is PV calculation method. PeroxySafe reports PVs as meq ROOH /kg sample (i.e. isopropanol). Thus, for comparing actual concentrations of ROOH added with those reported by these SafTests, methods for conversion must be known. Since isopropanol has a density notably lower than water (0.786), conversion of the initial sample to kg isopropanol should mean larger adjustment than volumetric (conversion to liter isopropanol) since 1 kg isopropanol = 1272 ml. The exact basis for the SafTest calculations is not clear (even from the company). Evaluating raw data printed out from the SafTest unit suggests that weights added to a sample are adjusted for isopropanol density (0.786) and multiplied by 40,000 dilution (the number of sample vol units/L, or 1000/0.025) to obtain the final meq/kg reported by the instrument. This gives a net multiplication factor of 50890 for conversion. However, some data were apparently only multiplied by a factor of 40000, which is simple volume conversion to ROOH /L isopropanol.

Obviously, the two conversions give different results, as shown in Table 10 and Figure 13. For weight conversions, hydroperoxide concentrations were underpredicted by SafTest, cumene-OOH only slightly but t-Butyl-OOH by up to 26%. In contrast, volume conversions overpredicted hydroperoxide concentrations, by up to 21% for t-Butyl hydroperoxide. It can be argued that these differences are very small on an absolute basis, but the inconsistencies and uncertainties are certainly sufficient to rule out SafTest as an accurate assay for absolute quantitation of hydroperoxides.

Table 10 . Detection efficiency of Peroxy-Safe assay for lipid hydroperoxides.

	actual ROOH (meq/kg ROOH)				measured ROOH
CuOOH nmol added	calc by wt *50890	% SafTest	calc by vol *40000	% SafTest	SafTest (meq/kg)
3.54	0.1803	100.91	0.1417	79.32	0.1787
5.31	0.2705	102.71	0.2126	80.73	0.2633
7.08	0.3605	104.40	0.2834	82.06	0.3453
8.86	0.4507	111.00	0.3542	87.25	0.4060
t-BuOOH					
2.19	0.1116	117.08	0.0877	92.03	0.0953
4.39	0.2232	122.88	0.1755	96.59	0.1817
6.58	0.3349	120.16	0.2632	94.45	0.2787
8.77	0.4465	126.01	0.3509	99.04	0.3543

Reproducibility of response:

Reproducibility and linearity drops off at less than 2 nmols/rxn and greater than 9 nmols/rxn (for cumene-OOH), so reproducibility tests (5 replicates each concentration * 3 days) were conducted within these concentration limits. Reproducibility data is reported Table 11. The SafTest software flags samples on the computer screen when the coefficient of variation (%CV) is greater than 10%. This indicates the assay should be repeated, perhaps with dilution. The average within-day reproducibility for t-Butyl hydroperoxide was 2.74% and between days was 7.1 %. The average reproducibility for cumene hydroperoxide was 2.66% within-day 4.65% between days. Within-day reproducibility was lower because a single batch of standards and reagents were used within a very short time (minutes), whereas even with great care in handling and argon sparging of reagents, reagents changed slightly between days.

Table 11a. Reproducibility of Peroxy-Safe assay for lipid hydroperoxides.

Cumene Hydroperoxide							
		Within days (meq/kg)			*Between Days* (meq/kg)		
nmols/rxn	**day 1**	**day 2**	**day 3**		**average**	**stdev**	**%cov**
3.54	**average**	0.20	0.17	0.19	0.19	0.01	6.97
	stdev	0.01	0.003	0.01			
	%cv	5.10	1.70	3.20			
5.32	**average**	0.25	0.28	0.26	0.26	0.01	4.96
	stdev	0.01	0.01	0.01			
	%cv	3.50	2.700	3.100			
7.09	**average**	0.33	0.36	0.35	0.35	0.02	4.43
	stdev	0.003	0.01	0.01			
	%cv	0.90	2.80	2.400			
8.86	**average**	0.40	0.40	0.42	0.41	0.01	2.26
	stdev	0.01	0.01	0.01			
	%cv	2.50	2.50	1.50			
Within day	**Ave %COV** 2.66			**Between Days**	**Ave % COV** 4.65		

Table 11b. Reproducibility of Peroxy-Safe assay for lipid hydroperoxides.

t-Butyl Hydroperoxide							
		Within days (meq/kg)			*Between Days* (meq/kg)		
nmols/rxn		**day 1**	**day 2**	**day 3**	**average**	**stdev**	**%cov**
2.193	**average**	0.11	0.09	0.10	0.10	0.01	10.51
	stdev	0.003	0.003	0.003			
	%cv	2.90	3.50	3.20			
4.396	**average**	0.18	0.17	0.20	0.18	0.01	7.81
	stdev	0.01	0.01	0.01			
	%cv	4.00	2.70	2.80			
6.589	**average**	0.28	0.26	0.30	0.28	0.02	6.51
	stdev	0.01	0.01	0.004			
	%cv	2.30	2.70	1.30			
8.770	**average**	0.36	0.34	0.36	0.35	0.01	3.57
	stdev	0.01	0.02	0.01			
	%cv	1.30	4.60	1.40			
Within day	**Ave %COV** 2.73			**Between days**	**Ave %COV** 7.10		

Handling issues, quirks and precautions:

The PeroxySafe test is very sensitive, so dilutions in Prep Reagent are normally required to bring expected ROOH concentrations into working range of the assay when analyzing food oils or lipid extracts. In fact, however, the directions to dilute is a gross understatement of the requirement because excessively high levels of hydroperoxides bleach the Fe-XO complex and can eliminate detectable reaction almost altogether. In such cases, not even the Lo or Hi messages from SafeTests may catch the error. To avoid missing hydroperoxides, we thus recommend, especially when working with unknowns (e.g. food oils or lipid extracts), that samples be diluted to at least three concentrations that are each at least one order of magnitude different to make sure the sample is detected. Frequently, even further dilution is required.

MP Bio uses cumene hydroperoxide as their standard. When standards are fresh, the slope of the regression equation for the standard curve is very close to 1, but during even short-term storage there was a gradual and continual decrease in the slope of the SafTest standard curves. MPBio revealed that the standards were "stabilized" cumene hydroperoxide. There is no mention of this problem with the directions of the kit, but when the standard response drops, the SafTest overestimates very low peroxide concentrations and increasingly underestimates actual peroxide values as the LOOH concentration increases. An example of this is shown for a standard curve with m=0.79 in Figure 14. Thus, for accurate and reproducible results with this assay, it is critical to monitor the slope of the standard curve daily, and if necessary, substitute authentic CuOOH standards for the MP Bio reagents. The standard curve of the calibrators is

considered adequate as long as the slope remains close to 1; actual acceptable values must be determined by the deviation from absolute quantitation tolerable for each individual application.

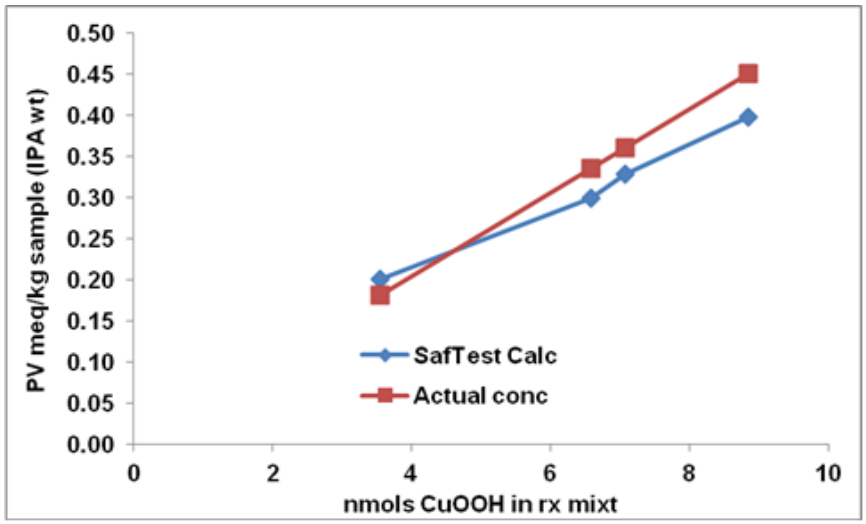


Figure 14. SafTest reported values for CuOOH reaction vs theoretical values (weights of standards) assuming that SafTest calculations are based on a sample weight of 25 µl isopropanol. Other calculation procedures attempted to account for sample weight do not give values anywhere close to reported PVs. Slope of standards: 0.79.

SafTest Summary:

There are numerous advantages to the PeroxySafe test. It is fast, easy, with minimal sample preparation since reagents are prepared and standardized. Unlike iodometric titration where only one sample can be run at a time, it is very easy to run multiple samples in this case. However, it has several important shortcomings. First, the autodispensers supplied with the kit are inaccurate so they need to be replaced with micropipettes. Also, it is a very sensitive assay (advantage), but this also means most

food extracts and oils must be diluted by 10-100 or more times to bring the response into range (excess peroxide leads to low or no response). Failure to dilute sufficiently can fail to detect hydroperoxides. Most importantly, however, the stoichiometry is unclear because reagent concentrations and exact calculation methods in the kit are unknown and the response level varies with the structure of ROOH. What this means is the assay may be used to determine or follow relative hydroperoxide concentrations in samples of the same or similar composition and structure, but it cannot provide absolute hydroperoxide concentrations and cannot be used to compare hydroperoxides of mixed, unknown, or very different structures. There is also the negative issue of high kit cost and short lifetime of reagents to consider.

5.6. Triphenylphosphine/ HPLC Assay of Cumene Hydroperoxide

Nakamura & Maeda published a method that claimed stoichiometric oxidation of triphenylphosphine (TPP) to triphenylphosphine oxide (TPPO) by hydroperoxides and detection of products by HPLC (Nakamura & Maeda, 1991). Their procedure was adapted and preliminary testing on cumene hydroperoxide was completed.

Several modifications were made to the procedure. The original assay agitated the mixture constantly at 30 °C. Testing showed that this mixing was not necessary – HPLC peak areas of tubes heated in a water bath with and without shaking or vortexing were the same (data not shown). The original method used a 30 minute reaction time even though the reaction reached a plateau at about 10 minutes. Reactions of cumene hydroperoxide after 10 and 30 minutes gave the same response, but to ensure a complete reaction all subsequent reactions were run for 20 minutes.

A reaction curve for cumene hydroperoxide constructed over the range of 0.005 nmoles (5 pmoles) to 10 nmoles showed that upper limit of reaction about 5 nmoles (curve departed from linear above this concentration) and lower limit of 5 pmoles (Figure 15). The response was linear even at the lowest concentrations and showed good reproducibility: within-day variation 0.5-1 % and between day variability 4-5% (Table 12). The 13% COV for one concentrations was likely caused by pipetting error since all other concentrations gave low variation.

TPP reactions with t-BuOOH and methyl linoleate hydroperoxides could not be analyzed. T-BuOOH was too hydrophilic to dissolve in cyclohexane (even as little as 50 ul in 1000 ul). MLOOH was soluble but the HPLC had technical problems that were not resolved before completion of this project. Nakamura found good correlation between this HPLC method and iodometric titration for detecting hydroperoxides in various oils and methyl linoleate (Nakamura & Maeda, 1991). At low oxidation levels, the two methods showed good agreement, but at higher hydroperoxide levels the TPP reaction leveled off and peroxide values were lower than those obtained by iodometry. The discrepancy was attributed to differences between the reducing abilities of KI in acidic medium (chloroform:acetic acid) vs TPP in cyclohexane. Further evaluation and development of this assay seems warranted.

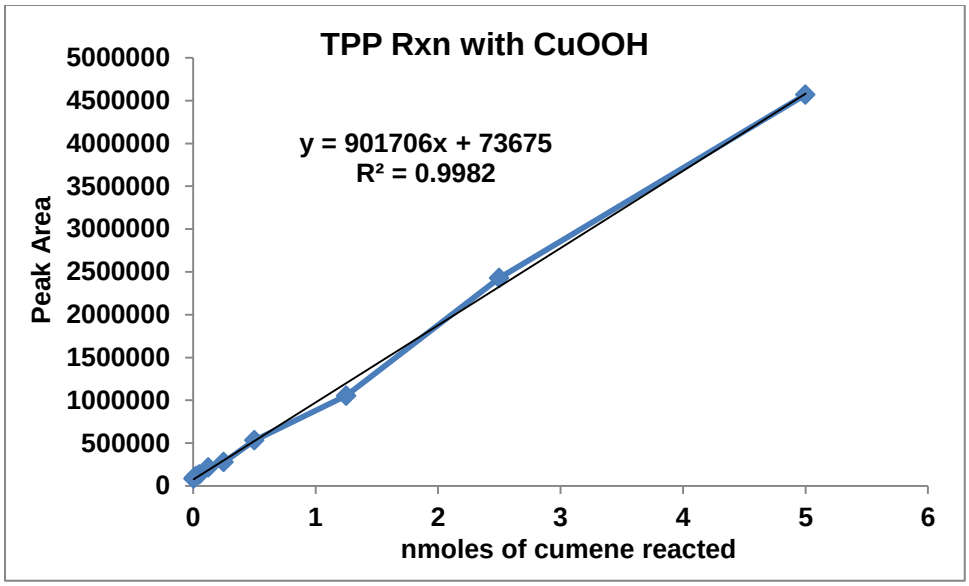


Figure 15. Response curve for cumene hydroperoxide with triphenylphosphine. Each point is the average of three analyses.

Table 12. HPLC peak area of TPPO formed in reaction of cumene hydroperoxide with TPP. Average of 3 analyses.

nmol CuOOH	Peak Area	Std Dev	COV (%)
2	2073147	37221	1.80
1	1096038	65464	5.97
0.5	605149	27424	4.53
0..2	345071	45955	13.32
0.1	199145	9577	4.81
0.05	144258	8254	5.7

5.7. Application of Assays to Determination of Hydroperoxides in Oxidized Methyl Linoleate

5.7.1. Iodometric Titration

The titration method successfully differentiated increasing levels of hydroperoxides in methyl linoleate oxidized for 4, 9, and 13 days with very low variability (Table 13). Results of this project show that when reaction conditions are carefully controlled, particularly deoxygenation, and adequate agitation, the iodometric titration method is the most accurate method for quantitating hydroperoxides, even if it has sensitivity limitations, so methyl linoleate PV's determined by this method were used as a standard against which all other assays were compared.

Table 13. Methyl Linoleate – Iodometric Titration. Three different concentrations; three replicates

Titration w/ TS	**Day 4**	**Day 9**	**Day 13**
Average meq/kg ML	304.012	1313.211	3153.201
Stdeva	6.182	27.894	39.389
cv%	2.033	2.124	1.250

5.7.2. PeroxySafe™ Xylenol Orange Assay

The PeroxySafe assay showed greater variability with methyl linoleate than with the two test hydroperoxides (CuOOH and t-BuOOH), and the variability increased with oxidation -- 6.29% cov on day 4 and >10% after day 9 (Table 14). Greater variability may be expected for ML since it is a natural material and probably does not oxidize homogeneously throughout the sample. However, there probably are also reaction issues also contributing to the variability since the peroxide values normalized to 1 kg varied

with sample size. Especially with higher oxidation (longer incubation time), peroxide values generally increased with sample size and the normalized peroxide values were far in excess of what would be theoretically possible for the weights of ML analyzed. The stoichiometry of the PeroxySafe assay is nominally two mols Fe^{3+} generated per mol hydroperoxide, and the calculated PVs must be adjusted for this. However, for the PeroxySafe PVs to be comparable to the Iodometric titration PVs requires assumption of a stoichiometry of about 4. If this is accurate, then the ML alkoxyl radicals react with the isopropanol in the SafTest Prep Reagent, augmenting the overall reaction. This is perhaps not surprising since the redox potential of ML alkoxyl radicals is higher than Cu-$O^{\bullet}$ and t-Bu-$O^{\bullet}$ and isopropanol is particularly prone to formation of hydroperoxides. Two additional Fe^{3+} could be generated by the following reactions:

$$\mathbf{LO^{\bullet} + IPA \rightarrow LOH + {}^{\bullet}IPA}$$

$$\mathbf{Fe^{2+} + {}^{\bullet}IPA + H^{+} \rightarrow IPA^{-} + Fe^{3+}}$$

$$\mathbf{{}^{\bullet}IPA + O_2 \rightarrow {}^{\bullet}OOIPA}$$

$$\mathbf{{}^{\bullet}OOIPA + Fe^{2+} \rightarrow Fe^{3+} + {}^{-}OOIPA}$$

Variable and uncertain stoichiometry is a serious problem with this and the other optical assays for hydroperoxides.

A second potential source of error leading to erroneously high lipid peroxide values is that this assay uses cumene hydroperoxide to prepare standard curves. Reaction response of CuOOH is much less than ML hydroperoxides, so using the CuOOH standards supplied with the kit will significantly overestimate peroxide levels in the lipid. Removing this excess calculation may drop the values to the 2:1 stoichiometry expected for this reaction.

Table 14. Detection of ML hydroperoxides by the PeroxySafe™ assay. Values are averages of three replicates.

Day 4		**Day 9**		**Day 13**	
µmole added	**meq/kg ML**	**µmole added**	**meq/kg ML**	**µmole added**	**meq/kg**
0.03	1473	0.008	5096	0.008	11370
0.05	1605	0.010	4545	0.009	12713
0.06	1716	0.013	4755	0.013	13064
0.08	1636	0.200	5852	0.015	14545
Avg	1608	**Avg**	5062	**Avg**	12923
Stdev	101.16	**Stdev**	573.45	**Stdev**	1304.93
COV (%)	6.29	**COV(%)**	11.32	**COV(%)**	10.10

5.7.3. Pierce Xylenol Orange Assay

The Iodometric titration gave peroxide values that varied little with ML samples size analyzed; PeroxySafe assay showed greater variability but peroxide values were sufficiently comparable to justify averaging values from different sample sizes. In contrast, PVs varies markedly with sample size in the Pierce xylenol orange assay, and detected values were more than an order of magnitude lower than the other two assays (Table 15). Sample sizes had to be reduced with incubation time to keep the PVs in detection range, and bleaching occurred at lipid concentrations ≥0.30 µmoles ML leading to lower apparent PVs on all three days. In hindsight, ML samples should have been diluted at least another few orders of magnitude to drop below the bleaching limit.

These results demonstrate quite pointedly that a sample concentration range must be analyzed for this assay to ensure accurate results; they also illustrate the inaccuracies that would result from using a single fixed sample size for analysis of all oxidation ranges. This would hold whether sampling products with unknown oxidation levels off the shelf or following oxidation of a sample over time.

Table 15. Detection of ML hydroperoxides by the Pierce Xylenol Orange assay. Values are averages of three replicates.

Day 4		Day 9		Day 13	
µmole added	**meq/kg ML**	**µmole added**	**meq/kg ML**	**µmole added**	**meq/kg ML**
0.06	41.16	0.03	86.30	0.03	192.05
0.15	47.93	0.06	43.25	0.06	274.67
0.30	41.99	0.15	102.96	0.30	222.18
3.01	20.16	0.3	105.47	0.50	148.30
6.04	10.91	1.0	61.36		
30.19	2.32				

5.7.4. Ferric Thiocyanate Assay

That the ferric thiocyanate method is very sensitive and works best at nanomolar hydroperoxide concentrations was shown clearly in reactions of this assay with oxidized ML (Table 16). Responses as a function of ML concentration were linear (R^2=0.9998) and most accurate when the hydroperoxide levels were lowest (Day 4). Although the raw peroxide values generated in the assay are higher than iodometric titration PV's, when adjusted for 2:1 stoichiometry, the net values were notably lower than for iodometric titration. Concentration curves departed from linear and the coefficient of variation increased as oxidation progressed and hydroperoxides increased. Thus, as was shown for xylenol orange assays, appropriate extensive dilutions are critical for obtaining accurate results from this assay.

Table 16. Detection of ML hydroperoxides by the ferric thiocyanate assay. Values are averages of three replicates.

Day 4		**Day 9**		**Day 13**	
µmole added	**meq/kg ML**	**µmole added**	**meq/kg ML**	**µmole added**	**meq/kg ML**
0.01	172.16	0.01	1763.15	0.01	3833.40
0.06	370.28	0.04	2079.51	0.03	5034.85
0.10	584.66	0.06	2066.46	0.04	3758.27
0.30	593.21	0.1	1776.68	0.06	4199.89
0.60	613.70				

	Day 4	**Day 9**	**Day 13**
average meq/kg ML	597.19	1970.88	4331.01
Stedeva	14.92	176.97	648.31
cov%	2.50	8.98	14.97

5.7.5. Cayman Kit - Thiocyanate Assay

Results show that reaction only works at the lowest hydroperoxide concentrations and becomes increasingly inaccurate as oxidation progresses and samples must be diluted further and further to keep the assay in range. On each analysis day, dilution curves increased to some maximum then decreased as samples were progressively diluted. Initial dilutions abate some of the color quenching by the excess hydroperoxides, while at concentrations below peak, hydroperoxide levels were too low for efficient reaction – hydroperoxides had to diffuse too far to reach an iron atom. Saturation and FeSCN quenching was seen even at the lowest hydroperoxide levels. As was seen before, the concentration needed to accurately analyze hydroperoxides change with the incubation day and level of oxidation. This is a distinct disadvantage because appropriate dilution for each sample must be determined independently by serial dilution. Our results suggest that samples should be diluted until the PVs reach a peak and then decrease. The peak value should then be used as the most accurate reflection of the peroxide contents. However,

this practice adds an additional complication: multiplying results by very large dilution factors gives peroxide values that are far in excess of what is possible for the materials. If these high values results because the [lipid] hydroperoxides are reacting with solvents to create new radicals and hydroperoxides, as has been discussed above, the assay is too inaccurate and too uninterpretable to be used with foods where PV's of at least 0.5-1 are characteristic of "fresh" materials and oxidized foods reach PV's of 10 or more.

Table 17. Detection of ML hydroperoxides by the Cayman ferric thiocyanate assay. Values are averages of three replicates.

Day 4		Day 9		Day 13	
nmole added	**meq/kg ML**	**nmole added**	**meq/kg ML**	**nmole added**	**meq/kg ML**
30.19	146.47	1.50	1051.55	0.01	753191.50
60.38	181.73	3.02	546.64	0.03	130817.47
167.72	192.73	6.04	972.26	0.15	30336.30
301.90	139.52	30.19	541.84	0.76	4898.87
754.75	125.03	60.38	465.48	1.50	4118.56
				3.02	2236.62

5.8. Comparison of Assays

The concentration ranges detected by each assay are listed in Table 18. The triphenylphosphine assay is the most sensitive, detecting picomoles of hydroperoxides, while Iodometric titration is the only assay that accurately quantitates high concentrations of hydroperoxides. What is more, it does so directly with minimal sample dilution.

Table 18. Comparison of detection ranges for various hydroperoxide assays.

Assay	ROOH Detection Range
Iodometric Titration	2-200 µmoles added
PeroxySafe	2-12 nmoles added
Xylenol Orange – Pierce	0.8- 50 nmoles added
Ferric Thiocyanate	5- 90 nmoles added
Cayman Assay(Ferric thiocyanate)	0.5- 5 nmols added

Comparison of hydroperoxides detected in common samples of oxidized methyl linoleate are presented in Table 19. Iodometric titration with thiosulfate (TS) is used as the base against which other assays are compared. Assay values are PVs determined directly from the assay without consideration of reaction stoichiometry. Comparing these values, all of the optical assays except the Pierce assay greatly overestimate peroxide levels. Samples probably require further dilution for accurate analysis in the Pierce assay. Adjusting these values for expected stoichiometry or using the maximum PV obtained in dilution series brings comparative levels of the assays into better agreement with iodometric titration, although now values are generally lower. Overall, these results suggest that carefully controlled iodometric titration is the best and most accurate assay, and also the only one that yields absolute concentrations of hydroperoxides. This assay

thus continues to be the gold standard, and efforts to refine endpoint detection should be given high research priority.

Table 19. Comparison of methyl linoleate hydroperoxide levels detected by the various assays.

	Assay values			**Adjusted for stoichiometry**		
	Day 4	Day 9	Day 13	Day 4	Day 9	Day 13
TS				**304**	**1313**	**3153**
Peroxy Saf	1608	5063	12923			
assume 2 Fe/LOOH				804	2532	6462
assume 4 Fe/LOOH				402	1266	3231
Pierce	42	105	222			
FeSCN						
ave PV, 2 Fe/LOOH	597	1971	4331	299	986	2166
use max PV	614	2030	5035	307	1015	2518
Cayman	193	1052	7532	97	526	3766

6. SUMMARY AND CONCLUSIONS

Hydroperoxide quantification assays have been used for decades. Nevertheless, as the results of this project have demonstrated, they remained plagued by handling, reaction, and stoichiometry problems. One of the biggest issues is when a scientist selects an assay blindly - whether standardized methods, methods cited in literature, and kits purchased – without thinking about the sensitivity, stoichiometry, detection ranges, and side reactions of the assay. In this study, protocols were evaluated with all these issues, as well as any handling quirks and limitations, in mind.

Detection range of assays is crucial, as shown in the comparison of detection ranges for the different assays (Table 18). The xylenol orange and ferric thiocyanate assays were first developed to detect trace hydroperoxide levels in solvents or biological tissues, and only later were adapted to food. Lipid extracts of food samples must be extensively diluted to reach these trace detection ranges; with inadequate dilution, samples can appear to have no or low lipid oxidation even in fact hydroperoxide levels are quite high.

Along with inappropriate detection range, assaying a single sample size can also give incorrect results, especially when degree of oxidation is unknown or oxidation is being followed in samples over time – the amount of sample required for the assay is constantly changing and must be determined empirically for each sample. To find the PV accurately, samples must be serially diluted until PVs reach a peak (bleaching effect eliminated) and then continually decreases, indicating that molecular migration rather than reactivity now controls the reaction. The peak PV in such a dilution series can be taken as the most accurate reflection of the actual peroxide value.

In terms of handling, critical factors include:

- All solutions, solvents, and reagents must be deaerated to exclude oxygen and limit side redox reactions.
- Light exposure must be limited to prevent hydroperoxide and reagent degradation.

The iodometric titration method has always been considered the gold standard of hydroperoxide assays but not very sensitive, empirical, and difficult to make reproducible. Results of this study agree with all these points but show that with strict control (ie: removal of oxygen, rigorous stirring, protection from light), accurate and highly reproducible results are attainable. Most importantly, the iodometric titration is only assay tested where the stoichiometry is clear (1:1) and independent of hydroperoxide structure, and potential side reactions can be eliminated as long as oxygen is excluded. It may be possible to overcome the issue of low sensitivity due to weak endpoint electrochemical titration, as will be further discussed in the future work section. The triphenylphosphine assay also shows promise because of its high sensitivity (0.5 nmol), selectivity and simple stoichiometry, but needs further evaluation.

Both assays using ferrous iron to decompose hydroperoxides, followed by complexation of the generated Fe^{3+} by some agent have complicated stoichiometry that is unclear, greater than 1:1, and changes with conditions, solvent, hydroperoxide level, and hydroperoxide structure. As hydroperoxide concentrations increase, both reactions become increasingly affected by side reactions with solvent that augment apparent hydroperoxide levels and also bleaching of the iron complexes that decrease apparent PVs; hence, there is always significant uncertainly in the hydroperoxide levels being detected. These factors present significant limitations when using these assays to obtain

absolute hydroperoxide concentrations (not possible) or comparing samples that differ in composition (different peroxide structures) or oxidation levels. Results of this study suggest that the xylenol orange and Fe^{3+} thicyanate assays can be used only for determination of relative concentrations in foods and only when samples are serially diluted to appropriate levels.

7. FUTURE WORK

A major recommendation is to find methods for improving the sensitivity of the iodide reaction (thiosulfate titration). One option is to use electrochemical instead of visual endpoints. A second is to adapt procedures for colorimetric assays. The two major drawbacks of the iodometric titration method are the need to eliminate oxygen and insensitive visual endpoints. Several papers have proposed methods adapting the iodide reaction to spectrophotometric methods. The method described by Lovaas (Løvaas, 1992) was investigated because the author claims to greatly reduce the interference of oxygen, and the assay appeared fast and simple to perform. Compared to other versions of the assay; it was completed in low acid and and Fe^{2+} was added to accelerate I_3^- chromophore formation. We found many problems with this assay, but it provides a model for redesigning reactions that can be quantitated by optical end points.

Add further investigation of the TPP assay to provide a more accurate alternative for detection of low hydroperoxide concentrations. It would be particularly important to determine stoichiometry and effects of hydroperoxide structure. Another issue to investigate is a replacement for the solvent cyclohexane, since it could not solubilize t-butyl hydroperoxide.

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