Novel methods and apparatus for analyzing the hydroperoxide content of a material comprising hydroperoxides. The novel methods comprise continuously flowing a carrier stream through a continuous flow reactor (2); mixing Fe(II) ions, thiocyanate ions, and a material comprising hydroperoxides in the carrier stream; reacting the hydroperoxides with the Fe(II) ions thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions; forming Fe(III)–thiocyanate complexes; and determining the amount of Fe(III)–thiocyanate complexes present in the carrier stream to obtain a measurement corresponding to the amount of hydroperoxides oxidized.
<table>
<thead>
<tr>
<th>Code</th>
<th>Country</th>
<th>Code</th>
<th>Country</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL</td>
<td>Albania</td>
<td>ES</td>
<td>Spain</td>
</tr>
<tr>
<td>AM</td>
<td>Armenia</td>
<td>FI</td>
<td>Finland</td>
</tr>
<tr>
<td>AT</td>
<td>Austria</td>
<td>FR</td>
<td>France</td>
</tr>
<tr>
<td>AU</td>
<td>Australia</td>
<td>GA</td>
<td>Gabon</td>
</tr>
<tr>
<td>AZ</td>
<td>Azerbaijan</td>
<td>GB</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>BA</td>
<td>Bosnia and Herzegovina</td>
<td>GE</td>
<td>Georgia</td>
</tr>
<tr>
<td>BB</td>
<td>Barbados</td>
<td>GH</td>
<td>Ghana</td>
</tr>
<tr>
<td>BE</td>
<td>Belgium</td>
<td>GN</td>
<td>Guinea</td>
</tr>
<tr>
<td>BF</td>
<td>Burkina Faso</td>
<td>GR</td>
<td>Greece</td>
</tr>
<tr>
<td>BG</td>
<td>Bulgaria</td>
<td>HU</td>
<td>Hungary</td>
</tr>
<tr>
<td>BJ</td>
<td>Benin</td>
<td>IE</td>
<td>Ireland</td>
</tr>
<tr>
<td>BR</td>
<td>Brazil</td>
<td>IL</td>
<td>Israel</td>
</tr>
<tr>
<td>BY</td>
<td>Belarus</td>
<td>IS</td>
<td>Iceland</td>
</tr>
<tr>
<td>CA</td>
<td>Canada</td>
<td>IT</td>
<td>Italy</td>
</tr>
<tr>
<td>CF</td>
<td>Central African Republic</td>
<td>JP</td>
<td>Japan</td>
</tr>
<tr>
<td>CG</td>
<td>Congo</td>
<td>KE</td>
<td>Kenya</td>
</tr>
<tr>
<td>CH</td>
<td>Switzerland</td>
<td>KG</td>
<td>Kyrgyzstan</td>
</tr>
<tr>
<td>CI</td>
<td>Côte d’Ivoire</td>
<td>KP</td>
<td>Democratic People’s Republic of Korea</td>
</tr>
<tr>
<td>CM</td>
<td>Cameroon</td>
<td>KR</td>
<td>Republic of Korea</td>
</tr>
<tr>
<td>CN</td>
<td>China</td>
<td>KZ</td>
<td>Kazakhstan</td>
</tr>
<tr>
<td>CU</td>
<td>Cuba</td>
<td>LC</td>
<td>Saint Lucia</td>
</tr>
<tr>
<td>CZ</td>
<td>Czech Republic</td>
<td>LI</td>
<td>Liechtenstein</td>
</tr>
<tr>
<td>DE</td>
<td>Germany</td>
<td>LK</td>
<td>Sri Lanka</td>
</tr>
<tr>
<td>DK</td>
<td>Denmark</td>
<td>LR</td>
<td>Liberia</td>
</tr>
<tr>
<td>EE</td>
<td>Estonia</td>
<td>LS</td>
<td>Lesotho</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LT</td>
<td>Lithuania</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LU</td>
<td>Luxembourg</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LV</td>
<td>Latvia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MC</td>
<td>Monaco</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MD</td>
<td>Republic of Moldova</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MG</td>
<td>Madagascar</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MK</td>
<td>The former Yugoslavia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ML</td>
<td>Mali</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MN</td>
<td>Mongolia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MR</td>
<td>Mauritania</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MW</td>
<td>Malawi</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MX</td>
<td>Mexico</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NE</td>
<td>Niger</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NL</td>
<td>Netherlands</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NO</td>
<td>Norway</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NZ</td>
<td>New Zealand</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PL</td>
<td>Poland</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PT</td>
<td>Portugal</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RO</td>
<td>Romania</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RU</td>
<td>Russian Federation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD</td>
<td>Sudan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SE</td>
<td>Sweden</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SG</td>
<td>Singapore</td>
</tr>
<tr>
<td>SI</td>
<td>Slovenia</td>
<td>SK</td>
<td>Slovakia</td>
</tr>
<tr>
<td>SN</td>
<td>Senegal</td>
<td>SZ</td>
<td>Swaziland</td>
</tr>
<tr>
<td>TD</td>
<td>Chad</td>
<td>TG</td>
<td>Togo</td>
</tr>
<tr>
<td>TJ</td>
<td>Tajikistan</td>
<td>TM</td>
<td>Turkmenistan</td>
</tr>
<tr>
<td>TR</td>
<td>Turkey</td>
<td>TT</td>
<td>Trinidad and Tobago</td>
</tr>
<tr>
<td>UA</td>
<td>Ukraine</td>
<td>UG</td>
<td>Uganda</td>
</tr>
<tr>
<td>US</td>
<td>United States of America</td>
<td>UZ</td>
<td>Uzbekistan</td>
</tr>
<tr>
<td>VN</td>
<td>Viet Nam</td>
<td>YU</td>
<td>Yugoslavia</td>
</tr>
<tr>
<td>ZW</td>
<td>Zimbabwe</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
METHODS AND APPARATUS FOR MEASURING HYDROPEROXIDES

BACKGROUND OF THE INVENTION

The present invention relates generally to methods and apparatus for measuring the hydroperoxide content of a material comprising hydroperoxides. More particularly, the present invention is directed to methods and apparatus for measuring lipid hydroperoxides by flow injection photometry.

Lipids such as oils and fats generally undergo oxidative decomposition over a period of time. The primary products of this decomposition are lipid hydroperoxides. These hydroperoxides can decompose further to form secondary products such as aldehydes, alcohols, carboxylic acids and epoxides. Lipid peroxidation in edible oils and fats is a primary cause of deterioration of the lipid. This deterioration can be manifested, for example, in the production of deleterious by-products, loss of palatability, and a rancid odor in the lipid.

Lipid peroxidation is a major concern in a variety of products for both human and/or animal use. For example, the formulation of antioxidants for incorporation in products ranging from potato chips to skin creams is currently a major area of commercial endeavor. Labuza, T. P., *Crit. Rev. Food Technol.*, 1971, 2, 355-405, generally discusses the kinetics of lipid oxidation in the context of foods.

The oxidation of the fat in animal feeds is a significant problem and can lead, for example, to a reduction in the energy value of the feed, thereby increasing the feed requirements of the animal to compensate for the reduction in energy value, as well as the generation of toxic byproducts. The hydroperoxides produced by oxidation can destroy other valuable nutrients such as the fat-soluble vitamins and can produce toxic by-products that negatively impact cell turnover in the liver and the immune response of the animal. In addition, the process of lipid peroxidation is believed to be the underlying cause of several diseases such as coronary heart disease caused by hypercholesterolemia, cerebral apoplexy and the general process of aging in living animals.

Accordingly, a number of methods have been developed for the determination of lipid hydroperoxide content. The status of oxidation of oil and fat samples is conventionally judged by their peroxide value ("PV"), an index that seeks to determine the hydroperoxide content by measuring how much iodine is liberated by the sample from iodide in an acidic medium in a specified time period. Conventional iodometric methods used for measuring lipid hydroperoxides in oils and fats include American Oil Chemist's Society Official Method Cd-8-53, 1996. In this iodometric method, a lipid sample is dissolved in acetic acid:chloroform (3:2 v/v), the dissolved sample is reacted with aqueous potassium iodide for
one minute, and the resulting solution is titrated with thiosulfate using a starch indicator. Due to concerns over use of a halogenated organic solvent, American Oil Chemist’s Society Official Method Cd-8b-90, 1996, also discloses an alternative method using isooctane in place of chloroform.

The conventional iodometric methods used to measure lipid hydroperoxides suffer from a number of problems. They are time-consuming and relatively insensitive. In addition, because a biphasic system is used, the precise degree of stirring affects results and careful shielding from light is also necessary. Direct UV absorbance measurement of the iodine liberated is substantially more sensitive, although there can be potential interference from other UV absorbing species. In addition, excessive agitation can result in oxygen being incorporated into the sample from air above the solvent. This agitation has the potential of producing an erroneous result. A fundamental problem with conventional iodometric methods, however, is that the kinetics of iodine liberation vary greatly among different hydroperoxides. While various catalysts such as silica gel, AlCl₃, and Fe(II) have been used, no systematic study has been conducted to establish that such approaches are effective for reliably measuring different hydroperoxides in real samples on an equivalent basis.

Other methods for determining hydroperoxide content have been reported in the literature. Heath, R. L., et al., Anal. Biochem., 1976, 76, 184-191; and Marshall, P. J., et al., Anal. Biochem., 1985, 145, 192-199, have reported several highly sensitive enzymatic methods for the determination of hydroperoxides in biological samples. On the basis of a comparative study of several methods, Frew, J. E., et al., Anal. Chim. Acta, 1983, 155, 139-150, disclose that the enzymatically mediated glutathione coupled oxidation of NADPH is a superior choice for abiologic applications as well. The applicability of the enzymatic methods for determining lipid hydroperoxides in nonaqueous media, as would be necessitated in measuring organic hydroperoxides in oils and fat samples, has never been established. In any case, the sophistication and the cost per assay for these methods generally precludes their routine use in the edible oil and fat industry. The same criticism also applies to processes using enzyme coupled chemiluminescence detection for hydroperoxides following high performance liquid chromatographic (“HPLC”) separation, such as those processes described in Yamamoto, Y., et al., Anal. Biochem., 1987, 160, 7-13; and Miyazawa, T., et al., Anal. Lett., 1988, 21, 1033-1044.

Meguro, H., et al., Methods in Enzymology, 1990, 186, 157-161, have reported an approach for measuring hydroperoxides that is based on the reaction of the hydroperoxides with diphenyl-1-pyrenylphosphine (“DPP”) to form the corresponding intensely fluorescent DPP oxide. Use of this approach for a variety of samples, directly or in the postcolumn reaction format after HPLC separation, has been reported in Akasaka, K., et al., Biosci.

The fluorometric DPP method is sensitive and selective. Akasaka, K., et al., Biosci. Biotech. Biochem., 1996, 60, 1772-1775, have disclosed the use of the fluorometric DPP method automated by flow injection to measure hydroperoxide content of edible oil and fat samples. Nevertheless, the fluorometric DPP is not well suited for flow injection automation because the DPP reaction is a slow reaction. The reaction time required, even at elevated temperatures, is generally around 5.6 to 7.6 minutes. Such residence times in turn require 30 m to 50 m reaction coils maintained at 80 °C. In addition, to keep dispersion minimized narrow bore tubes were used, thereby necessitating the use of high-pressure pumps. Overall, the relatively high cost of equipment and reagent for this approach is a significant drawback for this method.

Nakano, T., et al., Anal. Sci., 1993, 9, 459-465, have reported a colorimetric flow injection method that relies on the oxidation of a dye substrate by a hydroperoxide. Despite the use of an enzyme mimic as a catalyst, this oxidation reaction (like the fluorometric DPP oxidation reaction) proceeds slowly. For tert-butyl hydroperoxide the reaction generally requires about six minutes to reach 80% completion. Given the rate of reaction, it is unlikely that equivalent responses would be obtained from higher homologs within a reasonable period.

The determination of oxidants, including hydroperoxides, by oxidizing Fe(II) to Fe(III) in a batch reaction and then colorimetrically measuring the latter as the thiocyanato complex has been disclosed in, for example, Petruj, J., et al., Analyst, 1986, 111, 671-676; and Mihaljevic, B., et al., Free Radical Biol. Med., 1996, 21, 53-63. These references further disclose that the reaction is compatible with organic media such as benzene-methanol or chloroform-methanol, and that this method responds to a broad variety of hydroperoxides, including those of unsaturated fatty acids. These references additionally disclose that the reaction is generally more attractive than a competitive method involving the oxidation of Fe(II)-α-Phenanthroline. None of these references, however, disclose the use of such an approach in a continuous flow reactor.

**SUMMARY OF THE INVENTION**

Novel methods and apparatus for analyzing the hydroperoxide content of a material comprising hydroperoxides have been developed.
In one embodiment of the invention, the novel method comprises continuously flowing a carrier stream through a continuous flow reactor and mixing Fe(II) ions, thiocyanate ions, and a material comprising hydroperoxides in the carrier stream. The Fe(II) ions react with the hydroperoxides thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions. The Fe(II) ions can react with the hydroperoxides either as uncomplexed Fe(II) ions and/or in the form of Fe(II)-thiocyanate complexes. In the first situation, the Fe(II) ions are oxidized to form Fe(III) ions which then react with thiocyanate ions to form Fe(III)-thiocyanate complexes. In the second case the Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes. Additional Fe(III)-thiocyanate complexes are optionally formed by reacting uncomplexed Fe(III) ions formed in the hydroperoxide reduction step with thiocyanate ions. The amount of Fe(III)-thiocyanate complexes present in the carrier stream is then determined to obtain a measurement corresponding to the amount of hydroperoxides oxidized.

In another embodiment of the invention, the novel method comprises continuously flowing a carrier stream through a reactor under conditions wherein the carrier stream is maintained substantially isolated from external sources of oxygen. The reactor comprises a continuous flow reaction chamber having an inlet and an outlet. The inlet is in fluid communication with one or more flow means for controlling the introduction into the carrier stream of at least one member of the group consisting of lipids comprising hydroperoxides, reducing reagents comprising Fe(II) ions, and indicator reagents comprising thiocyanate ions. The outlet in fluid communication with a detecting means. Fe(II) ions, thiocyanate ions, and a lipid comprising hydroperoxides are mixed in the carrier stream thereby forming one or more reaction zones. Each reaction zone passes through the reaction chamber and represents a volume of the carrier stream containing the hydroperoxides, Fe(II) ions and thiocyanate ions introduced into that reaction zone. The Fe(II) ions react with the hydroperoxides thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions. The Fe(II) ions can react with the hydroperoxides either as uncomplexed Fe(II) ions and/or in the form of Fe(II)-thiocyanate complexes. In the first situation, the Fe(II) ions are oxidized to form Fe(III) ions which then react with thiocyanate ions to form Fe(III)-thiocyanate complexes. In the second case the Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes. Additional Fe(III)-thiocyanate complexes are optionally formed by reacting uncomplexed Fe(III) ions formed in the hydroperoxide reduction step with thiocyanate ions. The amount of Fe(III)-thiocyanate complexes present in the carrier stream is then determined to obtain a measurement corresponding to the amount of hydroperoxides oxidized.

In another embodiment of the invention, the novel apparatus comprises a continuous flow reactor comprising a reaction chamber having an inlet and an outlet and adapted to receive a
continuously flowing carrier stream. The apparatus further comprises a means for controlling the flow of the carrier stream and a means for mixing Fe(II) ions, thiocyanate ions, and a material comprising hydroperoxides in the carrier stream such that the reactions of the above-described method may be carried out thereby resulting in the formation of Fe(III)-thiocyanate complexes. The apparatus further comprises a means for detecting the Fe(III)-thiocyanate complex content of the carrier stream.

Other aspects of the invention will be in part apparent and in part pointed out hereinafter.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 schematically shows an analytical system for the determination of the hydroperoxide content of a lipid sample in accordance with one embodiment of the present invention.

Figure 2 schematically shows a colorimetric flow injection analysis system for the determination of the peroxide value of a material comprising a lipid in accordance with one embodiment of the present invention.

Figure 3 shows a profile of peroxide value against oxidation time for a conventional oxidation process employing cottonseed oil heated at 100 °C and sparged with air.

Figure 4 shows a plot of the absorbance signal for seven lard samples having different peroxide values in the flow injection analysis system of Figure 2. Figure 4 reports triplicate measurements for each sample with the corresponding iodometrically measured peroxide value (in μeq/L) measured for each sample shown above each set of peaks.

Figure 5 shows a plot of normalized absorbance peak height as a function of flow rate for several samples in the flow injection analysis system of Figure 2 indicating that peak height generally does not increase with increasing reaction time.

Figure 6 shows a plot of the measured absorbance signal as a function of the corresponding and conventionally measured iodometric peroxide value for several different synthetic hydroperoxides in the flow injection analysis system of Figure 2.

Figure 7 shows a plot of the apparent peroxide value as function of the reaction time for iodine formation for several different sample types using the conventional iodometric method.

Figure 8 shows a plot of the absorbance signal for five polyol samples having different concentrations in the flow injection analysis system of Figure 2. Figure 8 reports triplicate measurements for each sample.

Figure 9 shows a plot of the measured absorbance signal as a function of the corresponding and conventionally measured iodometric peroxide value for the five polyol samples of Figure 8.
DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

In accordance with the present invention, novel methods and apparatus for analyzing the hydroperoxide content of a material comprising hydroperoxides have been devised. The novel methods comprise continuously flowing a carrier stream through a continuous flow reactor and mixing Fe(II) ions, thiocyanate ions, and a material comprising hydroperoxides in the carrier stream. The Fe(II) ions react with the hydroperoxides thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions. The Fe(II) ions can react with the hydroperoxides either as uncomplexed Fe(II) ions and/or in the form of Fe(II)-thiocyanate complexes. In the first situation, the Fe(II) ions are oxidized to form Fe(III) ions which then react with thiocyanate ions to form Fe(III)-thiocyanate complexes. In the second case the Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes. Additional Fe(III)-thiocyanate complexes are optionally formed by reacting uncomplexed Fe(III) ions formed in the hydroperoxide reduction step with thiocyanate ions. The amount of Fe(III)-thiocyanate complexes present in the carrier stream is then determined to obtain a measurement corresponding to the amount of hydroperoxides oxidized.

When the oxidizable material comprises an oil or a fat, for example, the status of oxidation of such samples is normally judged by the peroxide value of the sample. The peroxide value is an index for determining peroxide content by measuring the amount of iodine liberated by the sample from iodide in an acidic medium in a specified time period. At the peroxide levels of interest, this method requires considerable analytical skill. The method also is inherently flawed because of the considerable differences in the rates at which different peroxides liberate iodine from iodide and the potential for the consumption of nascent iodine by unsaturated sites.

The novel methods devised for the determination of hydroperoxides in a material comprising hydroperoxides, particularly oils and fats, overcome the problems associated with conventional iodometric peroxide value measurements. These novel methods are based on the oxidation of Fe(II) to Fe(III) by hydroperoxides followed by the colorimetric detection of Fe(III) as the Fe(III)-thiocyanate complex. The methods are simple, rapid, sensitive and do not depend on precise reaction time.

The present methods have several important advantages over previously reported ferric thiocyanate chemistry and previously reported automated systems. First, the peroxide value measurements can be carried out on a smaller scale than previously reported. This reduction in scale permits the use of shorter reaction times and, therefore, shorter reactor residence times. It also results in reduced sample size and a concomitant decrease in the quantity of solvent and reagents needed for the measurement.

Second, the methods avoid the inconsistent results and difficulty in reproducing results.
that are inherent in conventional methods. The peroxide values measured using conventional methods may be adversely affected by the differences in peroxide values from one sample to the next. Higher peroxide value samples generally will require more time for complete reaction than lower peroxide value samples. This time differential between samples before the reaction is completed can be significant. In the present methods, the reaction time is sufficiently short that reduction of the hydroperoxides contained in each sample is essentially complete before peroxide value is measured. The present methods avoid the reaction time differential associated with conventional methods and provide highly accurate and reproducible results.

Third, the present methods require no catalysts, heated reactors, high-pressure pumps or excessively long reaction coils. The costs associated with the methods are an improvement relative to the costs associated with presently available methods. Implementation with a peristaltic pump as shown in Example 1 below may necessitate periodic pump tube replacement. In principle, however, the methods can be implemented with other pumps or in the sequential injection format.

Fourth, the methods can easily be automated, preferably by flow injection photometry. Where peroxide value is determined manually in an open reactor, care is required in order to minimize the undesirable oxidation of Fe(II) by atmospheric oxygen. The variability of artifact Fe(III) production between the sample and the blank will quickly limit the minimum level of hydroperoxides that can be determined by the present method. Either all solvents are carefully deoxygenated or the Fe(II) and SCN⁻ reagents are mixed together immediately before use and then treated with a solid reducing agent that leaves no excess in solution, e.g., “active” silver. In contrast, errors due to the gradual oxidation of Fe(II) to Fe(III) are minimized or eliminated in the methods of the present invention. While a gradually increasing baseline might be possible, this result was not observed, for example, during the course of using the same batch of reagents over four days. If the reagent background does increase excessively, a fresh batch of reagent can be used. Any oxidized amount in the reagent at any point, however, is part of the baseline and causes no analytical error. For the same reason, batch to batch variation of the exact Fe(III) content of the reagent R1 (see Fig. 2) has no effect on the accuracy.

Fifth, in-line sample dilution (especially for liquid samples) can be incorporated into an automated system for many samples. This approach permits direct injection of the neat sample without any sample preparation.

One embodiment of the methods of the present invention is now described in detail with reference to analytical system 1 shown in Fig. 1. It will be understood by those skilled in the art that the present invention may be used in conjunction with continuous flow reactors of
various configurations and modes of operation, the following description is intended to be merely illustrative of the type of system in which the present invention may be applied. Analytical system 1 comprises a continuous flow reactor 2 in fluid communication with detecting means 3, which includes but is not limited to an optical detecting means such as a photometric detector. A carrier stream continuously flows through continuous flow reactor 2 to detecting means 3. During the operation of analytical system 1, Fe(II) ions, thiocyanate ions and a sample of a material comprising hydroperoxides are mixed in the carrier stream thereby forming a reaction zone, i.e., a volume of the carrier stream passing through reactor 2 and containing the lipid sample, Fe(II) ions and thiocyanate ions introduced into the reaction zone. The oxidizable material preferably is selected from the group consisting of lipids, hydrocarbons comprising an ester linkage and olefinic hydrocarbons. The hydroperoxides react with the Fe(II) ions thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions. As previously discussed, the Fe(II) ions can react with the hydroperoxides either as uncomplexed Fe(II) ions and/or in the form of Fe(II)-thiocyanate complexes. The uncomplexed Fe(II) ions are oxidized to form Fe(III) ions which then react with thiocyanate ions to form Fe(III)-thiocyanate complexes. The Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes. Detecting means 3 then analyzes the carrier stream to determine the amount of Fe(III)-thiocyanate complexes present and thereby obtain a measurement corresponding to the amount of hydroperoxides oxidized.

The definitions of the terms “lipid”, “fat” and “oil” are generally known to those of ordinary skill in the art and should be interpreted broadly. The term “lipid”, for example, generally refers to the group of biomolecules characterized by their insolubility in water and their solubility in fat solvents such as alcohol, ether and chloroform. Non-limiting examples of lipids includes fats, lipoids (such as phospholipids, cerebrosides and waxes), and sterols (such as cholesterol and ergosterol). The term “fat” generally refers, for example, to esters of fatty acids and glycerol. Non-limiting examples of fats include peanut oil, vegetable oil, corn oil, olive oil, cottonseed oil, safflower oil, coconut oil, palm oil, fish oil, tallow, poultry fat, bacon grease, butter and lard. The term “oil” generally refers to those fats that are liquids at 20°C.

In another embodiment, the instant method comprises continuously flowing a carrier stream through a reactor under conditions wherein the carrier stream is maintained substantially isolated from external sources of oxygen. The reactor comprises a continuous flow reaction chamber having an inlet and an outlet, the inlet in fluid communication with one or more flow means for controlling the introduction into the carrier stream of at least one member of the group consisting of lipids comprising hydroperoxides, reducing reagents comprising Fe(II) ions, and indicator reagents comprising thiocyanate ions. The outlet of the
reactor is in fluid communication with a detecting means. The Fe(II) ions, thiocyanate ions, and lipid comprising hydroperoxides are mixed in the carrier stream thereby forming one or more reaction zones, each reaction zone being a volume of the carrier stream containing the hydroperoxides, Fe(II) ions and thiocyanate ions introduced into that reaction zone and passing through the reaction chamber. The hydroperoxides then react with the Fe(II) ions thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions. The reacting Fe(II) ions can be either complexed Fe(II), uncomplexed Fe(II) ions or a combination of both. The complexed Fe(II) ions are present as Fe(II)-thiocyanate complexes formed by the reaction of uncomplexed Fe(II) ions with the thiocyanate ions. These Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes during the reduction of the hydroperoxides. Additional Fe(III)-thiocyanate complexes are optionally formed by reacting uncomplexed Fe(III) ions resulting from the hydroperoxide reduction step with thiocyanate ions. The carrier stream is then analyzed with the detecting means to determine the amount of Fe(III)-thiocyanate complexes present in the reaction zone of the carrier stream and thereby obtain a measurement corresponding to the amount of hydroperoxides oxidized.

In a preferred embodiment, the method is automated through flow injection photometry. A methanol-butanol mixed solvent (1:1 v/v) is used as the carrier stream. Lipid samples, generally pre-diluted in the same solvent, are injected into the carrier stream. Streams comprising Fe(II) and SCN⁻, respectively, are separately introduced, mixed in-line and merged with the carrier stream. After a reaction time of about 30 seconds, the optical absorbance of the resulting stream is detected by a light emitting diode ("LED") based photometric detector. The method exhibits a wide dynamic range, good linearity (linear $r^2$ 0.9943 for 0.1-120 meq/kg cottonseed oil hydroperoxides), and a good throughput rate (up to 60 samples/hour).

The amount of lipid sample mixed in the carrier stream with the Fe(II) ions and thiocyanate ions may vary depending upon such factors as the solubility of the lipid in the carrier stream and the size and configuration of the reactor used. Typically, the amount of lipid sample introduced into the carrier stream is between about 0.001 g/mL solvent to about 0.1 g/mL solvent, preferably between about 0.01 g/mL solvent to about 0.6 g/mL solvent, wherein solvent refers to the carrier stream solvent.

The source of Fe(II) ions can be any compound comprising Fe(II) that is sufficiently soluble in the carrier stream and ionizes to form Fe(II) ions under typical operating conditions. One preferred source of Fe(II) ions is (NH₄)₂Fe(SO₄)₂. Other sources of Fe(II) ions include but are not limited to, for example, ferrous sulfate, ferrous chloride, ferrous nitrate and ferrous perchlorate. When the source of Fe(II) ions is (NH₄)₂Fe(SO₄)₂, the concentration of (NH₄)₂Fe(SO₄)₂ in the carrier stream generally is less than about 8 mM,
preferably between about 4 mM to about 8 mM. Preferably, the hydroperoxides are reacted with Fe(II) ions in the presence of an organic or mineral acid. More preferably, the acid is a mineral acid such as sulfuric acid or hydrochloric acid.

The source of thiocyanate ions can be any compound comprising thiocyanate that is sufficiently soluble in the carrier stream and ionizes to form thiocyanate ions under typical operating conditions. An excess of thiocyanate ions is generally preferred. One preferred source of thiocyanate ions is NH₄SCN. Other sources of thiocyanate ions include but are not limited to, for example, alkali metal thiocyanates such as potassium thiocyanate and sodium thiocyanate. When the source of thiocyanate ions is NH₄SCN, the concentration of NH₄SCN in the carrier stream generally is at least about 100 mM, preferably between about 100 mM to about 2000 mM.

The lipid sample, Fe(II) ions and thiocyanate ions can be introduced into the carrier stream in any manner or sequence so long as they remain in relative contact with each other as the carrier stream containing them passes through the reactor. For example, the lipid sample, Fe(II) ions and thiocyanate ions may be simultaneously introduced into the carrier stream in the form of separate solutions of each. Alternatively, separate solutions of the lipid sample, Fe(II) ions and thiocyanate ions can be introduced into the carrier stream in temporal succession so long as they remain in relative contact with each other as the carrier stream containing them passes through the reactor. By way of further illustration, the lipid sample may be introduced first into the carrier stream followed by the subsequent introduction of a single solution comprising both the Fe(II) ions and the thiocyanate ions into the carrier stream.

Although the solvent employed by the novel method may comprise solvents or mixtures of solvents selected from a wide range of organic solvents (including aromatic solvents such as benzene and alkanes such as hexane and petroleum ether), solvents wherein each solvent molecule contains at least one functional group comprising oxygen are preferred. More preferably, the solvent comprises one or more members selected from the group consisting of alcohols, ketones, esters and ethers, or mixtures thereof. Still more preferably, the solvent comprises one or more members selected from the group consisting of methanol and butanol, or mixtures thereof. Still more preferably, the solvent comprises both methanol and butanol wherein the ratio of methanol:butanol is from about 2:1 to about 1:2 by volume, more preferably about 1:1 by volume.

The method of the present invention is effective over a range of carrier stream flow rates and for a range of continuous flow reactor designs. Flow rate and reactor design, however, will affect the residence time of the carrier stream in the reactor prior to measurement by the detecting means. The time period from when the hydroperoxides are first contacted with the
Fe(II) ions in the carrier stream until the amount of Fe(III)-thiocyanate complexes present in the carrier stream is determined by the detecting means generally is less than about two minutes. This time of reaction preferably is less than about 60 seconds, more preferably less than about 45 seconds and still more preferably is about 30 seconds. Carrier stream flow rates between about 0.1 mL/minute to about 1.0 mL/minute are typical although other flow rates may be used. An internal volume for the continuous flow reactor less than about 1000 μL, preferably between about 250 μL to about 1000 μL, is typical although other reactor volumes may be used.

Any detecting means capable of detecting Fe(II)-thiocyanate complexes may be employed. Optical detecting means such as photometric detects are preferred.

The method of the present invention may be carried out manually or may be automated. The method may be used to analyze a single lipid sample or to continuously analyze a plurality of lipid samples in succession. Preferably, the method is used to continuously analyze a plurality of lipid samples in succession. In a preferred embodiment, the novel method is implemented using automated flow injection photometry.

The present invention is further directed to an apparatus for analyzing the hydroperoxide content of a lipid sample in accordance with the above-described method. The apparatus generally comprises a continuous flow reactor comprising a reaction chamber having an inlet and an outlet and adapted to receive a continuously flowing carrier stream; means for controlling the flow of the carrier stream through the reaction chamber; means for mixing Fe(II) ions, thiocyanate ions and a material comprising hydroperoxides in the carrier stream; and a means for detecting the Fe(II)-thiocyanate complex content of the carrier stream.

It is to be understood that the term PV, to the extent used herein, is intended to refer to the peroxide value as determined by the standard iodometric method. The actual hydroperoxide content of the sample may differ for the reasons discussed in this application.

The present invention is further illustrated by the following examples which are merely for the purpose of illustration and are not to be regarded as limiting the scope of the invention or the manner in which it is practiced.

**Example 1**

The peroxide value of cottonseed oil, olive oil, lard, and poultry fat samples were evaluated as set forth below. The cottonseed oil, olive oil and lard used in this Example were obtained from local supermarkets. The poultry fat was obtained from a commercial rendering plant. All chemicals and reagents used were reagent grade or better and used without further purification.
To prepare samples having different peroxide values for each oil or fat tested, the sample was heated in a beaker on a hot plate to a temperature of about 100 °C and air was continuously bubbled through a 20 μm porous frit to form a fine stream of bubbles in the sample. About 5 g of sample was periodically withdrawn and its peroxide value was assayed according to the conventional iodometric method set forth in American Oil Chemist’s Society Official Method Cd-8-53, 1996, except that the assays for some of the samples used a potentiometric indication of end point instead of a visual indicator. Each assayed sample was frozen at -15°C for later use in the automated assay method.

The peroxide values of the samples prepared above were subsequently determined using the flow injection analysis method described below. For each standardized oil or fat sample prepared above (and having a known peroxide value in meq/kg as determined by the conventional iodometric method), a dissolved sample for use in the flow injection analysis system was prepared by dissolving an amount up to 1.5 g of the original sample in an amount of 1:1 (v/v) 1-butanol:methanol sufficient to provide a dissolved sample volume of about 25 mL. The flow injection analysis system employed is shown in Fig. 2. Pump P was a Minipuls 2 multichannel peristaltic pump (Gilson). The carrier stream “CS” was 1:1 (v/v) 1-butanol:methanol. Reagents “R1” and “R2” comprised 7.7 mM (NH₄)₂Fe(SO₄)₂ + 45 mM H₂SO₄, and 0.13 M NH₄SCN, respectively, in methanol. The flow rates in each channel were the same. Although the system was successfully operated at all flow rates between 0.1 and 1.0 mL/minute (per channel), the data were obtained using a flow rate of 0.26 mL/minute unless otherwise stated. Solvent flexible pump tubes (Elkay Systems, Shrewsbury, MA) were used for pumping. Valve “V” was a standard electromechanically operated 6-port injector (5701P, Rheodyne) equipped with a 20 μL sample loop. Alternatively, other comparable valve arrangements could be used. The sample “S” was aspirated by the pump through valve “V”, any excess going to waste “W”. The reaction/mixing coil was a 0.7 mm diameter x 600 mm polytetrafluoroethylene (“PTFE”) tube. The reaction/mixing tube together with the connecting tube to the detector and the detector itself, provided a total residence volume of about 385 μL and resulted in a reaction time of about 30 seconds at the cited flow rates. All other tubing used in the flow system were 0.55-mm diameter PTFE tubes. Detector “D” was a laboratory-built flow-through photometric detector, equipped with a light emitting diode (LED) emitting at 555 nm (HBG5566x, Stanley Electric, Tokyo) with an output signal linearly related to the absorbance. Similar detectors are available commercially from, for example, Global FIA, Gig Harbor, Washington. The data were obtained using a personal computer “E” (Gateway 2000, P5-75) equipped with a DAS-1601 data acquisition board (Keithley-Metrabyte, Taunton, MA).

In general, the formation of peroxides, as a function of time, exhibits an induction period
followed by an sigmoidal increase due to the autocatalytic nature of peroxide formation. As time continues, the concentration of peroxides reaches a maximum when the rate of peroxide formation and decomposition are equal. This plateau is maintained until the source of fatty acid double bonds has been consumed, at which point the rate of peroxide formation declines. The peroxides already formed will continue to decompose and the net effect is an eventual decline in peroxide concentration. A typical pattern for the initiation of this sequence is shown in Figure 3.

1. Choice of Detection Wavelength

The colorimetric method used in the flow injection analysis system described in this Example is based on the detection of the red complex formed between SCN⁻ and Fe(III). With the large of excess of SCN⁻ present in this system, the absorbance maximum generally is at about 510 nm. This absorption band, however, is very broad (full width half maximum 130 nm). While ultrabright LEDs are available with an emission maximum at 495 nm that would increase the detection sensitivity by about 15% relative to the 555 nm LEDs used, detection sensitivity was not a problem for the samples analyzed in this Example. In addition, detection at longer wavelengths increases the immunity against interference from indigenous color (most typically yellow or brown) in the sample.

2. Choice of Reaction Medium

Solvents such as benzene, hexane, ethanol, and ethanol:1-butanol mixtures were studied as potential reaction media, as the carrier stream and as the sample solvent. The 1-butanol: methanol solvent performed better than any of these solvents. Benzene produced an unstable base line. Hexane and ethanol resulted in lower sensitivity of the signal, i.e., the response peak height was lower. Further, hydroperoxides did not show any unusual degradation in 1-butanol:methanol. Each oil or fat sample used in this Example, when diluted and stored at room temperature in this solvent, produced an identical analytical signal for at least 96 hours. Other oil samples that are very susceptible to oxidation, such as fish oil, may not be so stable. In addition, those samples that are solid at room temperature, especially fats, may not dissolve totally in the 1-butanol: methanol solvent. The solubility limit for lard, for example, is about 8 mg/mL. The hydroperoxides themselves, however, are polar and readily dissolve in the alcoholic solvent. If the sample is shaken well or heated with the solvent and then allowed to settle, the clear liquid contains the hydroperoxides that have been quantitatively extracted and which can be injected into the flow injection analysis system without further processing. The extraction is confirmed to be quantitative because a second extraction of the residue contains little or no hydroperoxide. A large amount of fat/oil sample is not needed
for analysis because the method is very sensitive.

3. Reagent concentrations

The two primary steps involved in the acid catalyzed oxidation of Fe(II) to Fe(III) by a hydroperoxide are hypothesized to be:

$$ROOH + Fe^{2+} + H^+ \rightarrow RO\cdot + Fe^{3+} + H_2O$$  \hspace{1cm} (1)

$$RO\cdot + Fe^{2+} + H^+ \rightarrow ROH + Fe^{3+}$$  \hspace{1cm} (2)

These reactions are facilitated by an increased concentration of Fe(II). Mohr’s salt, (NH$_4$)$_2$Fe(SO$_4$)$_2$, is the most stable among simple ionic Fe(II) salts and is therefore a preferred reagent. (NH$_4$)$_2$Fe(SO$_4$)$_2$, however, begins to precipitate in the solvent system of this Example around a concentration of about 8 mM, largely due to the common ion effect from H$_2$SO$_4$. This precipitate can cause spurious noise in the detector and blockage of system conduits when the system is shut down. A preferred concentration of 7.7 mM provides a margin of safety.

The Fe(III) that is produced by the reduction of the hydroperoxides forms a series of thiocyanato complexes, Fe(SCN)$_n^{(3-n)^+}$ up to $n = 4$. The absorption intensity increases and the absorption maxima shift to longer wavelengths as $n$ increases. To ensure maximum signal intensity and absorption at the longest possible wavelength, an NH$_4$SCN concentration of 0.13 M was used. This concentration was sufficient to ensure maximum absorption.

4. System Performance

The output of the flow injection analysis system used in this Example is shown in Fig. 4 for lard samples. A similar output was observed for all of the other samples. Cottonseed oil samples were most extensively studied. The precision of the measurements for replicate samples ($n=7$) of cottonseed oil was studied for peroxide values ranging from 0.03 to 2.5 meq/L. The measurements exhibited a range in relative standard deviation (“RSD”) from 0.67 to 1.4%. Based on the baseline noise, the limit of detection (“LOD”, S/N=3) was computed to be 2 μeq/L. This means, for example, that if 1 g of an unknown oil sample was dissolved in 25 mL of 1-butanol: methanol solvent, the LOD of hydroperoxides in the original sample would be 50 μeq/kg. To put this value in perspective, the maximum acceptable hydroperoxide concentration in an edible oil/fat sample is generally considered to be 20 meq/kg. In absolute amounts, the detection limit of hydroperoxide in a complex sample is 40 peq, a respectable limit for a colorimetric detection system. The response was
also linear with respect to the peroxide value ($r^2 = 0.9942$).

In comparison, the precision for poultry fat samples (original samples were dark brown in color) was 2.8% at 0.2 meq/L and 1.6% at 0.7 meq/L, where these concentrations refer to the diluted sample actually injected into the system. For highly colored samples, a blank measurement is recommended, with the NH$_4$SCN reagent being replaced with pure methanol. Even with this highly colored sample, however, the blank response was quite small (<4% of the total analytical signal).

5. Residence Time Independence

Relative to other reported automated methods that require residence times of many minutes even with heating, the present reaction is completed in seconds at room temperature. Although a minimum reaction time of 15 minutes for the ferric thiocyanate chemistry has been suggested in the literature in a benzene:methanol solvent, the data in Figure 5 indicate that reaction times as low as about eight seconds are sufficient for the assay reaction to go to completion in the present system. The data in Figure 5 were generated by adjusting the pump flow rate over a range between 0.12 and 1.0 mL/minute and illustrate that the signal output is not significantly affected by varying the reaction time in the system. To correct for changes in peak height due to changes in dispersion, H$_2$O$_2$ was used as a control and sample peak heights were calculated as the ratio of the measured peak height to the peak height of H$_2$O$_2$ at the same flow rate. The flow dispersion induced change in peak height for H$_2$O$_2$ across the entire flow rate range studied was less than 20%. Independent direct spectrometric experiments confirmed that H$_2$O$_2$ reacts essentially instantly with Fe(II). Finally, the ratio value was normalized to unity for a flow rate of 0.5 mL/minute. As the results in Figure 5 show, increasing reaction time (reciprocally related to flow rate and ranging from 8 to 64 seconds) did not result in a material increase in the signal. Similarly, there was no material change in the signal when the reaction coil was heated to 65 °C in a water bath. It appears that the reaction was completed by the time the measurement was made.

6. Correlation With Iodometric Peroxide Value

The literature contains confusing and conflicting information as to the number of equivalents of Fe(II) oxidized per mole of hydroperoxide. At low H$_2$O$_2$:Fe(II) ratios relevant to analytical measurements, this number generally is accepted to be exactly two for H$_2$O$_2$.

Mihaljevic et al., *Free Radical Biol. Med.* 1996, 21, 53-63, however, point out that in addition to reactions (1) and (2) discussed above, the following reactions (3) and (4) can occur:
16

\[ \text{RO}^\cdot + \text{R'H} \rightarrow \text{ROH} + \text{R}^\cdot \]  

(3)

where R'H is the solvent present. The alkoxy radical RO\cdot also can react with a second molecule of hydroperoxide, generating an alkyl peroxy radical:

\[ \text{RO}^\cdot + \text{ROOH} \rightarrow \text{ROH} + \text{ROO}^\cdot \]  

(4)

At the low concentrations of ROOH that are characteristic of the analytical scale, reaction (4) generally does not materially contribute to the oxidation of the lipid.

A number of other reactions, specific to individual solvents, are possible. These additional reactions generally do not result in radical amplification or chain propagation involving oxidation of Fe(II) that can account for more than two equivalents of Fe(II) being produced per mole of ROOH. The only exception to this involves the production of alkyl peroxy radicals in the presence of oxygen:

\[ \text{R}^\cdot + \text{O}_2 \rightarrow \text{R'OO}^\cdot \]  

(5)

Reaction (5) provides a means for superstoichiometric oxidation:

\[ \text{R'OO}^\cdot + 2\text{Fe}^{2+} + 2\text{H}^+ \rightarrow \text{R'O}^\cdot + 2\text{Fe}^{3+} + \text{H}_2\text{O} \]  

(6)

Regardless of whether the reaction mechanistically proceeds in this manner, there can be no superstoichiometric oxidation unless another oxidant, notably molecular oxygen, can be involved.

Decomposition of a hydroperoxide into an alkoxy and a hydroxyl radical also is theoretically possible:

\[ \text{ROOH} \rightarrow \text{RO}^\cdot + \text{HO}^\cdot \]  

(7)

This reaction does not, by itself, provide a means for oxidizing more than two equivalents of Fe(II) per mole of ROOH. Mihaljevic, B., *Free Radical Biol. Med.* 1996, 21, 53-63, report that the number of equivalents of Fe(II) produced per mole of hydroperoxide is 3.1 for tert-butoxy hydroperoxide, 3.2 for cumene hydroperoxide ("CHP"), and ranges as high as 4.85 for higher hydroperoxides such as that of linoleic acid. In contrast, Petruj, J.; Zehnacker, S.; Sedlar, J.; Marchal, J. *Analyst* 1986, 111, 671-676, report that this stoichiometric coefficient is indistinguishable from 2.0 for tert-butoxy hydroperoxide and is 2.2 for CHP. They further
report that the stoichiometric factor for CHP increases with increasing cumene content of the CHP and suggest that at least part of the reason is that cumene autooxidizes to form CHP during the analysis process.

Figure 6 reports the absorbance signal for several different sample types of hydroperoxides in the flow injection system of this Example as a function of the peroxide value measured for the same samples using the conventional iodometric approach. In addition, results from careful manual experiments using the Fe(III) approach verified that, in agreement with the literature, two equivalents of Fe(II) are oxidized by one mole of H₂O₂. The slopes reported in Figure 6 are therefore normalized with the slope for H₂O₂ set to be 2.0.

The response for H₂O₂ and tert-butoxy hydroperoxide was experimentally determined and, within experimental error, was in agreement with the report of Petruj et al. The data for tert-butoxy hydroperoxide therefore are not separately shown. The results for CHP were significantly higher and might be due to autooxidation during the measurement. The results for the other samples were close to this value as well, approximately 2.5 times larger than for H₂O₂ except for one of the poultry fat samples (Poultry Fat 1) which was more than 4.5 times larger than for H₂O₂. These results appear to suggest that the findings of Mihaljevic et al. are correct and some types of hydroperoxides oxidize a greater number of equivalents of Fe(II) than others. In the present Example, as well as in those of Petruj et al. and Mihaljevic et al., however, the comparison standard was the iodometrically determined peroxide value. If the peroxide value thus determined underestimates the actual peroxide content, the Fe(II) oxidation will appear to be superstoichiometric. Each of the Fe(II) experiments of the Mihaljevic et al. were performed in carefully deoxygenated solvents. Experiments were conducted in which various hydroperoxide samples were measured in the flow injection analysis system where the samples and the reagents were carefully deoxygenated. The response was exactly the same with and without the deoxygenation step indicating that oxygen plays no significant role in oxidizing the Fe(II) in these experiments. It is unlikely therefore that large superstoichiometric oxidation of Fe(II) can occur under these conditions.

It is believed that the actual peroxide content of a sample is underestimated by the standard iodometric method for at least two reasons. First, the iodine liberation with some hydroperoxides is slow and is incomplete under the conditions of the measurement. Figure 7 presents data that show the impact on the peroxide value determined by the standard method when the reaction time for iodine formation is increased above one minute. The hydroperoxides in the poultry fat 1 sample are slow to liberate iodine and the reaction is far from complete when it is terminated at one minute. This sample in fact shows the greatest apparent superstoichiometric oxidation of Fe(II). A time dependence, albeit to a much smaller degree, is also apparent for the olive oil sample. In contrast to the response from the
poultry fat 1 sample, the response from the cottonseed oil or the other poultry fat sample is largely complete by the time it is measured at one minute. At three minutes, the reaction is complete.

While a much longer reaction time is prescribed in many recommended procedures for the measurement of hydroperoxide content, a longer reaction time for the standard method will not solve the problem. The liberation of iodine from iodide in an acidic medium by adventitious oxygen during an extended period creates a greater problem than the oxidation of Fe(II) to Fe(III) by oxygen. In addition, an altogether different issue is not addressed by extending the iodine liberation time or by performing a blank correction to correct for the liberation of iodine by oxygen. Almost all real oil and fat samples contain some unsaturated bonds. While molecular iodine does not readily add to such unsaturated linkages, it is conceivable that nascent iodine liberated by the iodometric procedure may be consumed by these unsaturated sites, thus leading to underestimation of the actual peroxide content. The determination of the extent of unsaturation in oil and fat samples by iodination, referred to as the iodine absorption number, is a separate analysis that is the basis of a standard method.

The method of the instant Example is suitable as an alternative to the conventional iodometric peroxide value method, a method that is empirical and manifestly cannot and does not produce the correct peroxide content for all samples. Where it is desirable to determine peroxide value iodimetrically and in accordance with the present method, however, it is possible to calibrate the method presented here by the particular sample type (with its peroxide value conventionally measured). The results of an analysis of a set of actual oil and fat samples using this approach (the samples analyzed were not a part of the calibration set) are presented in Table I.

Table I

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peroxide Value (meq/kg)</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Proposed Method</td>
<td>Std. Method</td>
</tr>
<tr>
<td>Cottonseed Oil</td>
<td>28.4±0.32</td>
<td>28.0±0.11</td>
</tr>
<tr>
<td>Olive Oil</td>
<td>54.8±0.19</td>
<td>52.4±0.25</td>
</tr>
<tr>
<td></td>
<td>4.44±0.61</td>
<td>4.36±0.19</td>
</tr>
<tr>
<td>Lard</td>
<td>26.9±0.65</td>
<td>27.2±0.08</td>
</tr>
<tr>
<td></td>
<td>2.75±0.48</td>
<td>2.68±0.51</td>
</tr>
<tr>
<td>Lard</td>
<td>71.1±0.04</td>
<td>71.6±0.37</td>
</tr>
<tr>
<td>Poultry Fat 1</td>
<td>1.53±0.07</td>
<td>1.48±0.35</td>
</tr>
<tr>
<td></td>
<td>1.53±0.09</td>
<td>1.49±0.22</td>
</tr>
</tbody>
</table>
Example 2

The peroxide value ("PV") of a sample of a polyol comprising hydroperoxides was measured by flow injection photometry in the same manner as previously described for the lipid samples in Example 1. The polyol used was voranol which was provided by Dow Chemical Company (Freeport, Texas). The polyol was heated to 100°C on a hot plate and air was passed through the polyol over about a four hour period. The oxidized polyol was standardized by the iodometric standard method. Test samples for later analysis were prepared by dissolving different amounts of the standardized polyol (ranging from 0-4.0 g) in a sufficient volume of 1:1 (v/v) 1-butanol:methanol to provide a total sample volume of 25 mL. The preparation and components of the carrier stream and reagents were the same as in Example 1 except as specified below. The flow rate in each channel was 0.30 mL/minute. The volume of polyol sample injected was 25 mL. The reaction/mixing coil was a 0.7 mm i.d. 800 mm PTFE tube. All other tubing used in the flow system was 0.55 mm i.d. PTFE tubes. The detector was the same as that previously used in Example 1 with a light emitting diode emitting at 555 nm.

Although the method and apparatus of the present invention when used to test vegetable oil samples exhibit good linearity between the signal from the detector and the peroxide value for reaction conduit lengths (i.e., coil lengths) as low as about 350 mm, greater reaction times generally are required to obtain a linear relationship for polyol samples. Linearity improves for polyol testing by increasing the coil length and/or increasing the acidity of the Fe(II) solution. In the present example, a longer reaction conduit (800 mm) was used.

Results are reported in Figs. 8 and 9. Fig. 8 shows a plot of the absorbance signal for five polyol samples of different concentrations. Fig. 8 reports triplicate measurements for each sample. Fig. 9 shows plot of the measured absorbance signal generated using this technique as a function of the corresponding and conventionally measured iodometric peroxide value for the same five samples (reflecting a concentration range of 1.9 to 16 ppm). Linear regression results based on the measured data reflect the following: \( Y = 939.6X \pm 0.18 - 8.827 \pm 0.031 \), and \( r^2 = 0.9985 \), where \( Y \) represents the signal (mV) from the detector and \( X \) represents the PV (meq/L) of sample solutions.

According to these experimental results, the peak shapes for polyol samples tested are generally better than that for vegetable oils tested, perhaps because the polyols have better solubility in the solvent. The solubility of the polyol in the solvent (1:1 butyl alcohol:methanol) can range up to at least 400 g/L. The signal-PV relationship is linear up to 400 meq/kg (linear \( r^2 = 0.9937 \)) and the limit of determination of the method is 0.1 ppm if the
amount of polyol added into the solvent is 400g/L.

In view of the above, it will be seen that the several objects of the invention are achieved. As various changes could be made in the above methods and apparatus without departing from the scope of the invention, it is intended that all matter contained in the above description be interpreted as illustrative and not in a limiting sense. All documents mentioned in this application are expressly incorporated by reference as if fully set forth at length.

All mentioned references are incorporated by reference as if here written. When introducing elements of the present invention or the preferred embodiment(s) thereof, the articles "a", "an", "the" and "said" are intended to mean that there are one or more of the elements. The terms "comprising", "including" and "having" are intended to be inclusive and mean that there may be additional elements other than the listed elements.

REFERENCES
32. Dong, J.; Ma K.; Voort, van de; Frederick, R.; Ismail, A. A. J. AOAC Int. 1997, 80, 345-352.


WHAT WE CLAIM IS:

1. A method for analyzing the hydroperoxide content of a material comprising hydroperoxides, the method comprising:
   continuously flowing a carrier stream through a continuous flow reactor;
   mixing Fe(II) ions, thiocyanate ions, and a material comprising hydroperoxides in the carrier stream;
   reacting the hydroperoxides with the Fe(II) ions thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions, wherein the Fe(II) ions that are oxidized can comprise complexed and uncomplexed Fe(II) ions, said complexed Fe(II) ions being present as Fe(II)-thiocyanate complexes formed by the reaction of uncomplexed Fe(II) ions with the thiocyanate ions, and wherein said Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes during the reduction of the hydroperoxides;
   optionally forming additional Fe(III)-thiocyanate complexes by reacting uncomplexed Fe(III) ions formed in the hydroperoxide reduction step with thiocyanate ions; and
determining the amount of Fe(III)-thiocyanate complexes present in the carrier stream to obtain a measurement corresponding to the amount of hydroperoxides oxidized.

2. A method of claim 1 comprising:
   continuously flowing a carrier stream through a continuous flow reactor;
   mixing Fe(II) ions, thiocyanate ions, and a material comprising hydroperoxides in the carrier stream thereby forming a reaction zone, the reaction zone being a volume of the carrier stream passing through the reactor and containing the hydroperoxides, Fe(II) ions and thiocyanate ions introduced into the reaction zone, wherein the material comprising hydroperoxides is selected from the group consisting of lipids, hydrocarbons comprising ester linkages and olefinic hydrocarbons;
   reacting the hydroperoxides with the Fe(II) ions thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions, wherein the Fe(II) ions that are oxidized can comprise complexed and uncomplexed Fe(II) ions, said complexed Fe(II) ions being present as Fe(II)-thiocyanate complexes formed by the reaction of uncomplexed Fe(II) ions with the thiocyanate ions, and wherein said Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes during the reduction of the hydroperoxides;
optionally forming additional Fe(III)-thiocyanate complexes by reacting uncomplexed Fe(III) ions formed in the hydroperoxide reduction step with thiocyanate ions; and
determining the amount of Fe(III)-thiocyanate complexes present in the reaction zone of the carrier stream to obtain a measurement corresponding to the amount of hydroperoxides oxidized.

3. A method for analyzing the hydroperoxide content of a lipid sample, the method comprising:
continuously flowing a carrier stream through a reactor under conditions wherein the carrier stream is maintained substantially isolated from external sources of oxygen, the reactor comprising a continuous flow reaction chamber having an inlet and an outlet, the inlet in fluid communication with one or more flow means for controlling the introduction into the carrier stream of at least one member of the group consisting of lipids comprising hydroperoxides, reducing reagents comprising Fe(II) ions, and indicator reagents comprising thiocyanate ions, and the outlet in fluid communication with a detecting means;
mixing Fe(II) ions, thiocyanate ions, and a lipid comprising hydroperoxides in the carrier stream thereby forming one or more reaction zones, each reaction zone passing through the reaction chamber and being a volume of the carrier steam containing the hydroperoxides, Fe(II) ions and thiocyanate ions introduced into that reaction zone;
reacting the hydroperoxides with the Fe(II) ions thereby reducing the hydroperoxides and oxidizing the Fe(II) ions to Fe(III) ions, wherein the Fe(II) ions that are oxidized can comprise complexed and uncomplexed Fe(II) ions, said complexed Fe(II) ions being present as Fe(II)-thiocyanate complexes formed by the reaction of uncomplexed Fe(II) ions with the thiocyanate ions, and wherein said Fe(II)-thiocyanate complexes are oxidized to Fe(III)-thiocyanate complexes during the reduction of the hydroperoxides;
optionally forming additional Fe(III)-thiocyanate complexes by reacting uncomplexed Fe(III) ions formed in the hydroperoxide reduction step with thiocyanate ions; and
analyzing the carrier stream with the detecting means to determine the amount of Fe(III)-thiocyanate complexes present in the reaction zone of the carrier stream and thereby obtain a measurement corresponding to the amount of hydroperoxides oxidized.
4. The method of claim 1 wherein the time period from when the hydroperoxides are first contacted with the Fe(II) ions in the carrier stream until the amount of Fe(III)-thiocyanate complexes present in the carrier stream is determined is less than about two minutes.

5. The method of claim 4 wherein said time period is less than about 60 seconds.

6. The method of claim 4 wherein said time period is less than about 45 seconds.

7. The method of claim 4 wherein said time period is about 30 seconds.

8. The method of claim 1 wherein the carrier stream comprises a solvent having at least one functional group comprising oxygen.

9. The method of claim 8 wherein the carrier stream comprises a solvent selected from the group consisting of alcohols, ketones, esters and ethers.

10. The method of claim 8 wherein the carrier stream is substantially exclusive of solvents that do not have at least one functional group comprising oxygen.

11. The method of claim 8 wherein the carrier stream comprises an alcohol.

12. The method of claim 8 wherein the carrier stream comprises butanol.

13. The method of claim 8 wherein the carrier stream comprises methanol and butanol.

14. The method of claim 13 wherein the ratio of methanol to butanol is between about 1:2 to about 2:1 by volume.

15. The method of claim 1 wherein the carrier stream comprises a solvent selected from the group consisting of alkanes and aromatic hydrocarbons.

16. The method of claim 15 wherein the carrier stream comprises a solvent selected from the group consisting of hexane, petroleum ether, and benzene.

17. The method of claim 1 wherein the source of the Fe(II) ions is \((\text{NH}_4)_2\text{Fe(SO}_4)_2\).
18. The method of claim 1 wherein the hydroperoxides are reacted with the Fe(II) ions in the presence of an acid.

19. The method of claim 18 wherein the acid is a mineral acid.

20. The method of claim 1 wherein the source of the thiocyanate ions is NH₄SCN.

21. The method of claim 1 wherein the source of the Fe(II) ions is (NH₄)₂Fe(SO₄)₃ and the source of the thiocyanate ions is NH₄SCN.

22. The method of claim 1 wherein the source of the Fe(II) ions is (NH₄)₂Fe(SO₄)₃, the hydroperoxides are contacted with (NH₄)₂Fe(SO₄)₃ in the presence of a mineral acid, and the source of the thiocyanate ions is NH₄SCN.

23. The method of claim 17 wherein the concentration of (NH₄)₂Fe(SO₄)₃ in the carrier stream is less than about 8 mM.

24. The method of claim 20 wherein the concentration of NH₄SCN in the carrier stream is at least about 100 mM.

25. The method of claim 21 wherein the concentration of (NH₄)₂Fe(SO₄)₃ in the carrier stream is between about 4 mM to about 8 mM and the concentration of NH₄SCN in the carrier stream is between about 100 mM to about 2000 mM.

26. The method of claim 1 wherein the carrier stream has a flow rate between about 0.1 mL/minute to about 1.0 mL/minute.

27. The method of claim 1 wherein the reactor has an internal volume of less than about 1000 µL.

28. The method of claim 1 wherein the presence of Fe(III)-thiocyanate complexes is determined using an optical detection means.

29. The method of claim 1 wherein the optical detection means comprises a photometric detector.
30. The method of claim 1 wherein the method is automated.

31. The method of claim 1 wherein a plurality of lipids comprising hydroperoxides are continuously analyzed in succession.

32. An apparatus for analyzing the hydroperoxide content of a lipid sample, the apparatus comprising:
   a continuous flow reactor comprising a reaction chamber having an inlet and an outlet and adapted to receive a continuously flowing carrier stream;
   means for controlling the flow of the carrier stream;
   means for mixing a lipid comprising hydroperoxides, Fe(II) ions and thiocyanate ions in the carrier stream such that the hydroperoxides can react with the Fe(II) ions to reduce the hydroperoxides and oxidize the Fe(II) ions to Fe(III) ions, wherein the Fe(II) ions that are oxidized can comprise complexed and uncomplexed Fe(II) ions, said complexed Fe(II) ions being present as Fe(II)-thiocyanate complexes formed by the reaction of uncomplexed Fe(II) ions with the thiocyanate ions, and wherein said Fe(II)-thiocyanate complexes can be oxidized to Fe(III)-thiocyanate complexes during the reduction of the hydroperoxides, and wherein additional Fe(III)-thiocyanate complexes optionally can be prepared by reacting uncomplexed Fe(III) ions formed in the hydroperoxide reduction step with the thiocyanate ions; and
   means for detecting the Fe(III)-thiocyanate complex content of the carrier stream.
FIG. 6

Fe (II) OXIDATION ABSORBANCE SIGNAL, 555 nm

IODOMETRIC PEROXIDE VALUE OF SAMPLE, meq/L

+ POULTRY FAT 1, $r^2 = 0.9892$, SLOPE 9.4
+ POULTRY FAT 2, $r^2 = 0.9986$, SLOPE 5.4
● LARD, $r^2 = 0.9973$, SLOPE 5.0
□ OLIVE OIL, $r^2 = 0.9997$, SLOPE 4.7
○ COTTONSEED OIL, $r^2 = 0.9997$, SLOPE 4.7
■ CUMENE HYDROPEROXIDE, $r^2 = 0.9981$, SLOPE 4.4
□ HYDROGEN PEROXIDE, $r^2 = 0.9965$, SLOPE NORMALIZED TO 2.0
FIG. 7

- **Apparent Peroxide Value Relative to 1 min Reaction Time**
- **Iodine Development Reaction Time, min**

Legend:
- Diamond: Poultry Fat 2
- Solid Circle: Cottonseed Oil
- Open Square: Olive Oil
- Plus Sign: Poultry Fat 1
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N35/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, INSPEC

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>EP 0 047 130 A (VICKERS LTD) 10 March 1982 (1982-03-10) claims 1,11</td>
<td>1,17-21, 28-30,32</td>
</tr>
<tr>
<td>Y</td>
<td>PETRUV J ET AL: &quot;TRACE DETERMINATION OF HYDROPEROXIDES BY SPECTROPHOTOMETRY IN ORGANIC MEDIA&quot; ANALYST, vol. 111, June 1986 (1986-06), pages 671-676, XP000934144 LONDON, GB cited in the application page 672, right-hand column, paragraph 9 -page 673, left-hand column, paragraph 1 page 674, left-hand column, paragraph 7 -right-hand column, paragraph 2</td>
<td>1,17-21, 28-30,32</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents:
* "A" document defining the general state of the art which is not considered to be of particular relevance
* "E" earlier document but published on or after the international filing date
* "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
* "O" document referring to an oral disclosure, use, exhibition or other means
* "P" document published prior to the international filing date but later than the priority date claimed

Date of the actual completion of the international search: 16 August 2000

Date of mailing of the international search report: 22/08/2000

Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel: (+31-70) 340-2040, Tel: 31 651 epi rl, Fax: (+31-70) 340-3016

Authorized officer: Krametz, E
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>US 4 520 108 A (YOSHIDA KASUMI ET AL)</td>
<td>1,32</td>
</tr>
<tr>
<td></td>
<td>column 2, line 16 - line 22</td>
<td></td>
</tr>
<tr>
<td></td>
<td>claim 1</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>MIHALJEVIC B ET AL: &quot;THE REEVALUATION OF</td>
<td>1-3,17, 28,32</td>
</tr>
<tr>
<td></td>
<td>THE FERRIC THIOCYANATE ASSAY FOR LIPID</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HYDROPEROXIDES WITH SPECIAL CONSIDERATIONS</td>
<td></td>
</tr>
<tr>
<td></td>
<td>OF THE MECHANISTIC ASPECTS OF THE</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RESPONSE&quot;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FREE RADICAL BIOLOGY &amp; MEDICINE,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>vol. 21, no. 1, 1996, pages 53-63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>XP000934162</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cited in the application</td>
<td></td>
</tr>
<tr>
<td></td>
<td>page 55, left-hand column, paragraph 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>page 55, right-hand column, paragraph 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>-page 57, right-hand column, paragraph 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>page 61, right-hand column, paragraph 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## INTERNATIONAL SEARCH REPORT

**Information on patent family members**

<table>
<thead>
<tr>
<th>Patent document cited in search report</th>
<th>Publication date</th>
<th>Patent family member(s)</th>
<th>Publication date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DE 3168903 D</td>
<td>28-03-1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 4032345 B</td>
<td>29-05-1992</td>
</tr>
<tr>
<td></td>
<td></td>
<td>JP 58087464 A</td>
<td>25-05-1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DE 3275659 D</td>
<td>16-04-1987</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EP 0081116 A</td>
<td>15-06-1983</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 4645647 A</td>
<td>24-02-1987</td>
</tr>
</tbody>
</table>