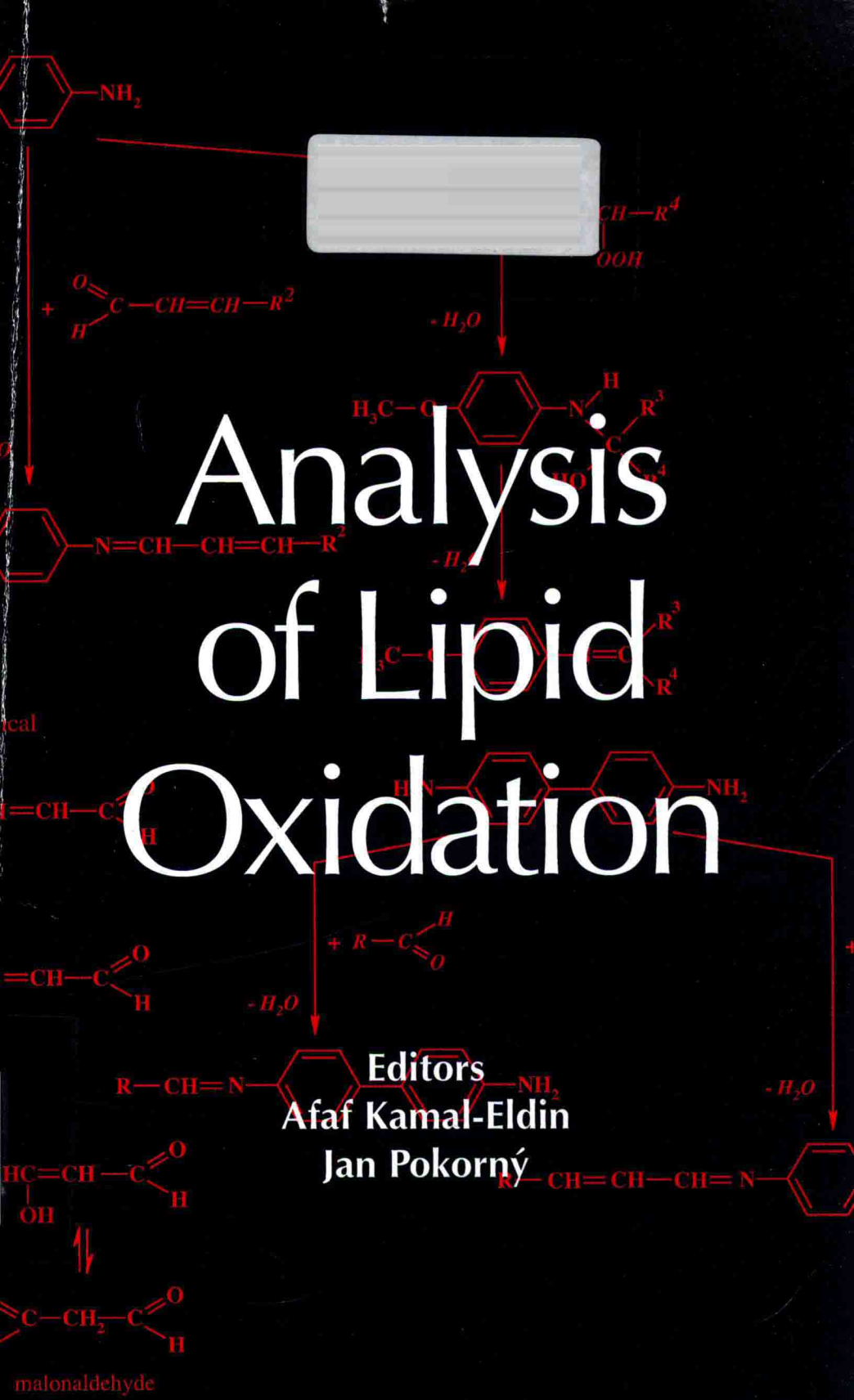

Analysis of Lipid Oxidation

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Preface

Lipid oxidation, though researched since the beginning of the 20th century, still gives no complete and satisfactory information on the composition of oxidized lipids. One important factor contributing to these gaps in our knowledge about lipid oxidation relates to the shortages in analytical methodology. Traditional analytical methods have been increasingly replaced by modern sophisticated instrumental methods, but lipid oxidation still presents a challenge in regard to its detailed mechanism, as well as its implications in the stability of biological tissues/compartments and *inter alias* human health. These shortages are very much connected to the complexity of parallel and consecutive, but overlapping, free radical-driven reactions and to the instability of a wide range of products.

Analytical methods suitable for oxidized lipids were often reviewed in the last decade, but mostly from the aspect of determination of individual oxidized lipid classes, such as peroxides, aldehydes, polar lipids or polymers. In this book, they are treated from the standpoint of types of analytical methods used. In modern lipid laboratories, analytical chemists are usually specialized to a single instrumental equipment so that the approach used in this book will be more useful than the traditional presentation. It will show, what could be achieved using the particular instrumental technique. On the contrary, for those, who are not familiar with the respective technique, and will thus be obliged to ask for help of a specialist, the book will give a basic information, what they can ask, and what they can expect from the technique.

The eleven chapters of *Analysis of Lipid Oxidation* aim to review the state-of-the-art of some of the methods currently used in studying lipid oxidation. Chapter 1 provides a short review of the primary and secondary products of lipid oxidation, as well as the problems associated with sample preparation and chemical and instrumental methods of analysis. Chapter 2 presents different volumetric methods used for the analysis of lipid hydroperoxides, free fatty acids, carbonyl oxidation products, epoxides, and residual double bonds following lipid oxidation. Chapter 3 reviews different UV-visible spectrometric methods used for the analysis of lipid radicals, hydroperoxides, and carbonyl compounds formed during the reaction. Analysis of non-volatile lipid oxidation products in different lipid matrices by high performance size-exclusion chromatography (HPSEC) is discussed in Chapter 4. Chapter 5 provides a review of the use of nuclear magnetic resonance spectroscopy (NMR) in the structural characterization of different compounds formed as a result of lipid oxidation. The analysis of intermediate radical species by electron spin resonance spectroscopy (ESR) is reviewed in Chapter 6. The use of differential scanning calorimetry (DSC) in the analysis of lipid oxidation is covered in Chapter 7, the use of chemiluminescence in Chapter 8, and the use of accelerated stability tests

in Chapter 9. Different approaches used for the evaluation of the kinetics of lipid oxidation are discussed in Chapter 10. The last chapter of the book reviews the analysis of interaction products of oxidized lipids with amino acids, proteins, and carbohydrates. This book is essential for further developments in analytical methodology and hyphenated techniques, with which more understanding of the reaction kinetics, mechanism, and implications will take place.

The editors are, indeed, grateful to the authors of the different chapters for making this publication possible. We also acknowledge, with great gratitude, the professional work of the AOCS staff that put this book into this shape.

Jan Pokorny
Afaf Kamal-Eldin
February 15, 2005

Contents

Chapter 1

Lipid Oxidation Products and Methods Used for Their Analysis

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Introduction

All natural food materials contain lipid oxidation products, at least in minute amounts. They are produced by the catalytic action of enzymes or by the action of singlet oxygen in living organisms, e.g., oilseeds and animal tissues used for fats and oils processing. During the isolation from the raw material, some oxidation can occur. Lipids are further oxidized either when stored or during heating in the course of food preparation. Lipid oxidation also proceeds *in vivo* after lipid ingestion or due to the leak of endogenous or exogenous free radicals and is implicated in a number of physiologic malfunctions that might lead to disease.

The analysis of lipid oxidation products is an important task, one often encountered by lipid analytical chemists. This task is difficult because the lipid oxidation reactions are consecutive but at the same type overlapping (see below). Therefore, the analytical methods used should be selected and/or adapted to the composition and amount of lipid oxidation products. The different chapters in this book provide different methods that can be used for the analysis of different oxidation products or stages. Knowledge of these alternative methods will enable the analyst to choose those appropriate for the question at hand.

Mechanism of Lipid Oxidation

To be able to choose an appropriate analytical method, it is important to understand the complexity of the lipid oxidation reaction and the products thereof. The lipid oxidation reaction consists of the following steps: (i) formation of free lipid radicals, initiating the oxidation process; (ii) formation of hydroperoxides as primary reaction products; (iii) formation of secondary oxidation products; and (iv) formation of tertiary oxidation products. The reaction mechanism is very complex, and only a short review will be given here for information. More details can be found elsewhere (e.g., Chan 1987, Kamal-Eldin 2003).

Formation of Lipid Free Radicals and Primary Lipid Oxidation Products

Lipid oxidation is initiated by the formation of free radicals, even in the case of enzyme-catalyzed lipid oxidation. A relatively high activation energy, necessary for the formation of the first lipid free radicals, may be supplied by heat energy, natural radioactivity, singlet oxygen or other sources. Unsaturated fatty acids, especially di- and triunsaturated acids, are more easily converted into free radicals than saturated fatty acids because an atom of hydrogen is more easily abstracted from the molecule if a double bond is located on the adjacent carbon atom. Substantially lower energy is sufficient to produce free radicals from traces of lipid oxidation products, especially in the presence of transient valency metals, such as copper, iron, or manganese. Free radicals are also produced in the tissues of living organisms. The determination of the level of free radicals is important both for food scientists and in biological or medical research.

Oxygen is essentially a biradical so that it reacts with a free carbon-centered lipid radical with formation of a peroxy radical. Free radicals can be detected and investigated by electron spin resonance. Peroxyl radicals possess high energy so that they can abstract an atom of hydrogen from the lipid molecule. The peroxyl radical is thus converted into a molecule of lipid hydroperoxide, and another lipid radical is produced. The process can be repeated many times. When present in abundance, free radicals are very reactive and readily react with other free radicals forming dimers polymers. The general, often cited, scheme of lipid oxidation is presented below:

Initiation $X^{\bullet} + LH \rightarrow L^{\bullet} + H^{\bullet}$ [1]

Propagation $L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ [2]

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ [3]

Termination $2\ LOO^{\bullet} \rightarrow$ nonradical products [4]

$LOO^{\bullet} + L^{\bullet} \rightarrow$ nonradical products [5]

$2\ L^{\bullet} \rightarrow$ nonradical products [6]

The structure of lipid hydroperoxides depends on the structure of the original fatty acid, so that a mixture of isomeric hydroperoxides is produced. Fats and oils contain many different triacylglycerols, and each fatty acid bound in a triacylglycerol is oxidized with formation of a few or several isomeric hydroperoxides. To judge the progress of the overall oxidation reaction, it is sometimes sufficient to determine the total content of hydroperoxides (see Chapter 2), but for mechanistic understanding, it is important to determine the individual hydroperoxide species in

the mixture. To date, no satisfactory method is available to accomplish the later task.

Hydroperoxides present in food lipids are unsaturated, and can be oxidized by similar mechanisms into dihydroperoxides or hydroperoxides containing an additional cyclic peroxy group. Bicyclic diperoxides may also be produced from polyunsaturated fatty acids. Special methods exist for the determination of hydroperoxides in the presence of cyclic peroxides.

The rate of lipid oxidation depends on several factors. As mentioned above, polyunsaturated lipids are more rapidly oxidized than monounsaturated lipids, and saturated lipids are almost stable. The oxidation rate increases with increasing temperature, oxygen pressure, and irradiation. The oxidation is catalyzed by heavy metals, and inhibited by antioxidants. Water and various nonlipidic food components also can greatly affect the process. Lipid analysts should be aware that oxidation proceeds slowly in the case of refrigerated storage and under reduced oxygen pressure or in inert gas and that antioxidants can only reduce the rate of oxidation, not stop it completely.

Hydroperoxide Decomposition into Secondary Oxidation Products

Hydroperoxides, especially those of polyunsaturated fatty acids, are very unstable. The rate of their degradation is catalyzed by heavy metal traces, metal ions, as well as some complexes and undissociated salts. Three types of degradation products are formed:

1. Monomeric degradation products of hydroperoxides are formed by various reactions of hydroperoxides. Epoxides (oxirane derivatives) are produced by the interaction of hydroperoxides with a double bond; hydroperoxides are transformed by the reduction of the hydroperoxyl group into hydroxyl derivatives, or by dehydration into ketones. Cyclic monomeric derivatives may also occur.
2. Low-molecular-weight compounds result from the cleavage of the hydroperoxide chain, most often at a carbon atom adjacent to the hydroperoxyl group. Aldehydes, ketones, alcohols, and hydrocarbons are formed in these reactions. The rancid off-flavor of oxidized lipids is due to these volatile compounds.
3. High-molecular-weight combination products are formed in the course of polymerization of free radical degradation products of hydroperoxides, or of copolymerization of free radical decomposition products with other food components. Dimers or trimers may be aliphatic, monocyclic, or bicyclic.

Oxidized lipids contain a mixture of all of the above-mentioned secondary products. For the analysis of such complicated samples, prefractionation into simpler mixtures may be necessary before analysis by a technique such as high performance size exclusion chromatography (HPSEC). Monomeric and polymeric secondary oxidation products, which are still unsaturated, are further oxidized similarly to the original fatty acids. The oxidation also proceeds at carbon atoms adjacent to the oxygen-containing functional groups in the chain.

Further Reactions of Secondary Lipid Oxidation Products (Formation of Tertiary Oxidation Products)

The secondary lipid oxidation products are also unstable; aldehydes in particular are very reactive. They are easily oxidized into peroxoacids, which are unstable, and decompose into a mixture of other products. Unsaturated aldehydes, alcohols, or ketones are oxidized into hydroperoxides, and compounds with a shorter chain length are formed by their cleavage. Formic acid and other low-molecular-weight fatty acids are the end products that are measured by techniques such as the Rancimat or the Oxidative Stability Index. The formation of volatile fatty acids is used as an indicator in some accelerated methods for the determination of lipid stability. Aldehydes change even in the absence of oxygen by various aldolization and retroaldolization reactions.

Interactions of Lipid Oxidation Products with Other Food Components

Foods and biological tissues contain not only lipids, but also many other components that can react with lipid free radicals, hydroperoxides, aldehydes, epoxides, and other reactive oxidation products. Natural minor components of fats and oils, such as sterols, tocopherols, or other phenolic derivatives, readily react with lipid free radicals or with hydroperoxides so that oxysterols or tocopherol oxidation products are always found in oxidized lipids. Polyphenols, such as flavonoids and anthocyanins, are frequently found in foods of plant origin. They react with lipid free radicals or with hydroperoxides similarly to antioxidants. Phospholipids are present only in traces in refined oils, but their content is much higher in many foods of plant or animal origin. Because they are rich in polyunsaturated fatty acids, they are oxidized with the formation of products analogous to those of triacylglycerols. Free radicals, hydroperoxides, and aldehydes react with the nitrogen functional groups of the phospholipid molecules, forming brown-colored products. Amino acids, peptides, and proteins present in foods react with lipid hydroperoxides, epoxides, hydroxyketones, and aldehydes. Their reaction products cannot be determined by common analytical methods and require special procedures.

Problems with the Analysis of Lipid Oxidation Products

Before the analysis of oxidized lipids, it is necessary to decide whether the determination of individual species is required or whether the determination of classes of compounds is sufficient. Often, the individual species cannot be determined without previous fractionation and risk of artifact formation, whereas the determination of classes such as total peroxides or total aldehydes is much simpler. In isolated oxidized lipids, various oxidized minor components such as oxidized sterols are also present. They increase the fraction of oxidized lipids and may complicate the analysis. Usually, it is less difficult to analyze the whole mixture than to separate oxidized lipids from the oxidized minor substances and analyze each fraction separately. To achieve this goal, selective methods must be used.

Polar lipids such as monoacylglycerols, free fatty acids, or phospholipids have polarities similar to those of oxidized lipids and may interfere with their determination. Different classes of oxidation products give similar reactions, e.g., aldehydes and ketones, so that the use of specific analytical procedures is necessary. The polarities of oxidized monomers and dimers are often very similar, so that HPLC should be replaced by HPSEC.

The preliminary isolation of oxidized lipids often consists of several steps; because it is time consuming, further oxidation may take place. Use of inert gas or antioxidants will reduce the oxidation, but not eliminate it entirely. The isolation of oxidized lipids from common foods, which are mixtures of lipids with many nonlipidic components, is particularly difficult, especially in the case of those oxidized lipid fractions that are bound to proteins and similar compounds with covalent bonds. Different markers are often used instead of a complete analysis.

Instead of the determination of the oxidized products, it is possible to determine the loss of precursor substrates. The fatty acid composition is altered during the oxidation because of the different oxidation rates of individual fatty acids. The relative content of polyunsaturated acids decreases, whereas that of saturated fatty acids increases. This approach has the disadvantage that the fatty acid composition of the original, fresh sample should be known. The method is not very sensitive, because the relative increase of a saturated acid is much lower that the amount of oxidized polyunsaturated acid. The method is therefore suitable only for samples oxidized to a high degree.

No single analytical method gives reliable results. Therefore a few or several methods, based on different principles, are necessary. In the case of sophisticated instrumental methods, it is sometimes difficult to build an experienced team of operators. In the case of biological samples, such as blood plasma, the amount of sample is usually very small so that suitable microanalytical methods should be developed or modified. Some of these methods are available in the literature, but they should be adapted to the material undergoing analysis and the level of sensitivity required in most cases.

Preparation of Oxidized Lipids for the Analysis of Oxidation Products

The preparation of the sample for analysis is usually complicated, and it is better to test the procedure first using model samples, and then adapt it to the particular material. Extraction is frequently the first step. Nonpolar solvents, such as hexane or cyclohexane, used for the extraction of fresh lipids may not be suitable for oxidized lipids, and more polar solvents give better results. Polar groups of oxidized lipids are bound to proteins and other polar food components by hydrogen bonds, for example, so that mixtures containing methanol or ethanol may be necessary. In such a case, the extract is contaminated with water, and during evaporation of the solvent, some lipid components may distill off with water vapor. Most organic solvents are toxic; therefore, the selection of a suitable solvent or solvent mixture is

difficult because harmless solvents are often less efficient. Liquid carbon dioxide may be considered in future developments.

The solvent is removed from the lipid extract at high temperature, low pressure, or a combination of both principles. Lipids are not very volatile, but some oxidation products formed by the cleavage of hydroperoxides could be lost during the evaporation. If the evaporation is carried out in air, the extracted lipids could be oxidized. It may be preferable to use the extract without solvent removal for the analyses and to determine the weight of extracted lipids separately in an aliquot volume of the extract. If absolutely required, removal of solvents should be performed in an atmosphere of an inert gas, i.e., nitrogen or argon.

A mixture of original and oxidized lipids is obtained by extraction, so that the extract is purified mainly on prepacked silica gel or alumina. The unoxidized lipids are eluted with a nonpolar solvent, and the oxidized fraction is then eluted using a more polar solvent mixture. A risk always exists that some very polar or polymeric oxidation products will remain in the column. after the removal of nonpolar lipids. It is possible to fractionate the oxidized lipid fraction by column chromatography on various solid phases. It is also possible to use selective membranes or molecular sieves, but these methods are seldom used for the separation of oxidized lipids.

Chemical and Instrumental Methods of Analysis of Lipid Oxidation Products

After isolation of the fraction of oxidized lipids, they are analyzed by chemical, physical, or instrumental methods. The chemical methods are the oldest; they are very simple and relatively selective. Their disadvantage is the use of large amounts of organic solvents. Volumetric and colorimetric methods are commonly used, whereas gravimetric and electrometric methods are applied only rarely for the analysis of oxidized lipids.

Physical methods, such as the determination of viscosity, specific density, refractometry, dielectrical constant, optical rotation, for example, are suitable only for orientation or monitoring of a process. The conductivity measurements are used in some accelerated tests of lipid stability against oxidation.

Instrumental methods are progressive methods, used increasingly in recent years. Instruments necessary for the analysis are expensive, but the analysis itself is short and relatively inexpensive. The selectivity can be improved by derivatization. Spectral, calorimetric, and chromatographic methods will be discussed in detail in different chapters of this book.

For the analysis of oxidized lipids, it is better to combine several methods, e.g., chemical, chromatographic, and spectrometric methods. Because most operators are experts in only a single instrumental method, it is better to form a team of analysts. The work should be coordinated so that the analyses are conducted at the same time or within short time intervals. It is important that a specialist in the analysis of oxidized lipids be present.

Concluding Remarks

The analysis of oxidized lipids is a difficult task because the material, which is a very complex mixture of different compounds, is unstable during storage and analytical operations. The best procedure is to use at least three analytical methods based on different principles. The interpretation of results requires a scientist with long experience in lipid analysis. Different techniques that can be used for the analysis of different products are discussed in the ensuing chapters of this book.

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Chapter 2

Volumetric Analysis of Oxidized Lipids

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Introduction

Chemical methods were the first analytical methods used for the estimation of lipid oxidation products. Volumetric methods, i.e., methods based on titration, were proposed more than 50 years ago because they are very simple, rapid methods that require no specific equipment. Their disadvantage is that they require the use of organic solvents and other toxic chemicals. Most volumetric methods have been replaced by instrumental methods, but some of them are widely used even now.

Volumetric methods were developed and standardized several decades ago, particularly for fresh fats and oils; they have changed little since that time. Therefore, most of the references cited in this chapter are very old. Nevertheless, their discussion is useful even now because most lipid scientists and technologists, who use them for the analysis of oxidized lipids, are not familiar with their limitations, the effect of various factors on the results, or the environmental aspects.

Iodometric Determination of Lipid Hydroperoxides

Principle of the Reaction

Hydroperoxides belong to the most important primary oxidation products. The hydroperoxide group is located on the carbon atom adjacent to a double bond or to a system of two conjugated double bonds. Hydroperoxides can be still further oxidized because they are unsaturated compounds. Dihydroperoxides or cyclic peroxides are formed in the later oxidation stages. All of these peroxidic compounds react with iodide ions. They are reduced to hydroxy derivatives, whereas iodide is oxidized into free iodine (Fig. 2.1A). In the presence of excess iodide, a complex ion that reacts in the same way as free iodine is formed (Fig. 2.1B). Iodine is then titrated, usually with a solution of sodium thiosulfate, which is oxidized into a tetrathionate (Fig. 2.1C). Reducing agents other than sodium thiosulfate may be used for the titration, e.g., sodium arsenite, which would be preferable but is unfortunately much more toxic than thiosulfate. A starch solution is added as an indicator because it forms a deep violet product with iodine. At the end of titration, the reaction mixture is decolorized.

(A) reaction of hydroperoxides with iodide ions

$$R\text{-}OOH + 2\ I^- + 2\ H^+ \rightarrow R\text{-}OH + I_2 + H_2O$$

(B) complex formation of iodine with iodide ions

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

(C) reduction of free iodine with thiosulfate

$$I_2 + 2\ S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2\ I^-$$

Fig. 2.1. Mechanism of iodometric determination of the peroxide value.

Analytical Procedures and the Effect of Air Oxygen

The result of the analysis is called the peroxide value (PV) for historical reasons, but the hydroperoxide value would be a more appropriate term because only lipid hydroperoxides react quantitatively, and the other peroxides only partially because of their low reactivity. Fortunately, hydroperoxides are present almost exclusively in fats and oils at a low degree of oxidation.

The sample is dissolved in a mixture of chloroform and acetic acid (or another solvent mixture of a similar polarity), and a saturated aqueous potassium iodide solution is added. The reaction takes place in the dark or at least under diffuse daylight. After the reaction, water is added, and the liberated iodine is titrated with a solution of sodium thiosulfate. Near the end point, when the colour of dissolved iodine becomes faint, the starch solution is added, and the titration is finished. The solution should be shaken only gently; otherwise, the reaction mixture would be contaminated with air oxygen. The resulting PV would then be higher than the actual value.

Several slightly diferent procedures are available for the determination of peroxides; those proposed by Wheeler (1932), Lea (1952), and Sully (1954) are the basis of further development of procedures that are now standardized. Amer *et al.* (1961) compared three methods of iodometric PV determination, and observed great differences among the results. These methods differed in the reaction time, temperature, and the presence of inert gas. The factors affecting the results are reviewed in Table 2.1. If the peroxide content in a sample is low, the reaction time has no great effect, and determination at ambient temperature is satisfactory. A reaction time of 5 min at ambient temperature was selected as the optimum (Yanishlieva and Popov 1972), but only 1 min is generally sufficient in the analysis of fresh fats and oils.

The presence of oxygen leads to an overestimation of the PV and is an important factor, especially in the case of low PV. Thus, it is preferable to remove oxygen from the reaction vessel by a stream of nitrogen or carbon dioxide before the analysis. The gas exchange takes rather a long time, e.g., a level of 5% oxygen in the atmosphere is attained after >7 min in a 300-mL flask with a flow rate of nitrogen >100 mL/min (Kubota *et al.* 1974). The gases should be free of oxygen

TABLE 2.1
Factors Affecting the Peroxide Value (PV)

Factor	Effect on the PV
Time	Increasing
Temperature	Increasing
Shaking	Increasing
Oxygen	Increasing
Inert gas (nitrogen, carbon dioxide)	Decreasing
Increasing pH value	Decreasing
Water	Increasing
Light	Increasing
UV radiation	Increasing
Polyunsaturation	Decreasing
Cupric and ferric ions	Increasing

because traces of oxygen could affect the results. Iodide ions are stabilized against oxidation by air oxygen by the addition of cadmium salts (Takagi et al. 1978), but cadmium is a toxic metal. If the blank is carried out in the same way and the difference between the sample and the blank is recorded, the inert gas is often omitted. In the case of a 1-min reaction time, the introduction of an inert gas is unnecessary. Water added after the end of the reaction also must be free of oxygen and trace metal ions. In samples with a high PV, complete elimination of oxygen is not so crucial as in fresh samples. The PV rises by the action of other oxidants, such as ferric ions (Gutfinger *et al.* 1976). Wheeler's procedure was found suitable for the analysis of dry soap (Popov and Yanishlieva 1968), in which 0.1–1.0 g of sample was dissolved directly into the solvent mixture.

According to the IUPAC standard procedure (Paquot and Hautfenne 1987), the reaction time is 5 min in diffuse daylight at ambient temperature, and the inert gas is not required. According to the AOCS standard procedure (Firestone 1996), the reaction time is 1 min at ambient temperature and under diffuse daylight, and the inert gas is also omitted. Isooctane can be used instead of chloroform. The standard method as proposed by Wheeler was modified to a micromethod that requires <0.1 g sample and a 2-min reaction time (Yanishlieva *et al.* 1978).

Lipid hydroperoxides may partially polymerize during the reaction, leading to the formation of less reactive products. This side reaction becomes important at high sample PV. Hydroperoxides may be stabilized by the addition of boric acid so that the PV is higher and corresponds better to the real concentration of the hydroperoxides than if the standard method is used (Amer *et al.* 1961). The authors suspected that a part of the iodine, formed by the reaction of hydroperoxides, was reabsorbed on double bonds of the analyzed sample, thus reducing the PV (Amer *et al.* 1960).

Chloroform and acetic acid are usually used as solvents. Chloroform is a good lipid solvent, and the addition of acetic acid is important as a medium suitable for the interaction of the reactants. For the analysis of biological samples, chloroform