Measurement of Lipid Oxidation: A Review

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ABSTRACT

Lipids become rancid as a result of oxidation, and this oxidative rancidity is a major cause of food deterioration. The acceptability of a food product depends on the extent to which this deterioration has occurred. Thus some criterion for assessing the extent of oxidation is required. This paper reviews the experimental techniques for the measurement of lipid oxidation. The spectrum of tests ranges from simple organoleptic evaluations to chemical and physical methods. There is no ideal chemical method which correlates well with changes in organoleptic properties of oxidized lipids throughout the entire course of autoxidation. The methods discussed each give information about particular stages of the autoxidative process, and some are more applicable to certain lipid systems than others. The method of choice depends on a number of factors including the nature and history of the oxidized sample, the type of information required, the time available, and the test conditions. Clearly, there is a need for a more thorough assessment of the available methods so that unreliable, cumbersome methods may be discarded and modifications made to the remaining methods to maximize the information obtained. The ultimate criterion for the suitability of any test is its agreement with sensory perception of rancid flavors and odors.

INTRODUCTION

Lipids can become rancid as a consequence of oxidation, and this oxidative rancidity is a major cause of food deterioration. Whereas other deteriorative reactions such as microbial or enzyme attack can be largely controlled by lowering the temperature, this is not particularly helpful in preventing oxidation since low energy thresholds are involved. Nor is exclusion of oxygen always practically possible. The oxidative deterioration of food lipids involves, primarily, autoxidative reactions which are accompanied by various secondary reactions having oxidative and nonoxidative character. From the standpoint of food oxidation, the important lipids are the unsaturated fatty acid moieties, particularly oleate, linoleate, and linolenate, the principal ones in foods (1). The susceptibility and rate of oxidation of these fatty acids increase in a somewhat geometric fashion in relation to their degree of unsaturation. Oxidation of unsaturated fatty acids has been well reported (1-5) and unless mediated by other oxidants or enzyme systems, proceeds through a free-radical chain mechanism involving initiation, propagation, and termination steps. These can be formulated as:

Initiation

$$RH + O_2 \rightarrow R. + .OH$$

Propagation

$$R. + O_2 \rightarrow ROO.$$

$$ROO. + RH \rightarrow ROOH + R.$$

Termination

$$R. + R. \rightarrow RR$$

$$R. + ROO. \rightarrow ROOR$$

$$ROO. + ROO. \rightarrow ROOR + O_2$$

RH refers to any unsaturated fatty acid in which the H is labile by reason of being on a carbon atom adjacent to a double bond. R. refers to a free radical formed by removal of a labile hydrogen.

Hydroperoxides (ROOH) are the major initial reaction products of fatty acids with oxygen. Subsequent reactions control both the rate of reaction and the nature of the products formed (Fig. 1). These compounds may be responsible for the development of off-flavors or for further reactions with other food constituents such as proteins.

The acceptability of a food product depends on the extent to which deterioration has occurred. Researchers are also interested in determining the effects of certain processes or antioxidants on the stability of a product. Thus some criterion for assessing the extent of oxidation is required. Although sensory analysis is one of the most sensitive methods available, it is not practical for routine analyses and generally lacks reproducibility. Consequently, many chemical and physical methods have been developed to quantify oxidative deterioration with the object of correlating the data with off-flavor development. When evaluating the usefulness of a particular test or procedure as a measure of the extent of oxidation certain questions must be answered:

1. Would the property being measured arise under any circumstances other than oxidation?
2. Is the property being measured found in all oxidizing systems?
3. Is the method specific for that particular property?
4. Does the property being measured adequately represent the extent to which oxidation has occurred?

The aim of this discussion is to review the experimental techniques for the measurement of lipid oxidation. The spectrum of tests ranges from simple organoleptic evaluations to chemical methods and electroanalyses. The advantages and limitations associated with each method are discussed.

Before one begins a detailed examination, a distinction should be noted between susceptibility tests and methods of measuring the extent of lipid oxidation. Susceptibility tests measure the stability of a lipid under conditions which favor oxidative rancidity and include tests such as the Schaal oven test, active oxygen method, and oxygen adsorption methods. They are, therefore, based on tests which measure the extent of oxidation to provide an indication of the point at which rancidity occurs. In this paper, it is solely this aspect, the method of evaluation, which is considered without reference to a particular stability test.

ORGANOLEPTIC EVALUATION

The consumer uses an organoleptic evaluation to judge the quality of fats and oils. Rancidity is considered to be

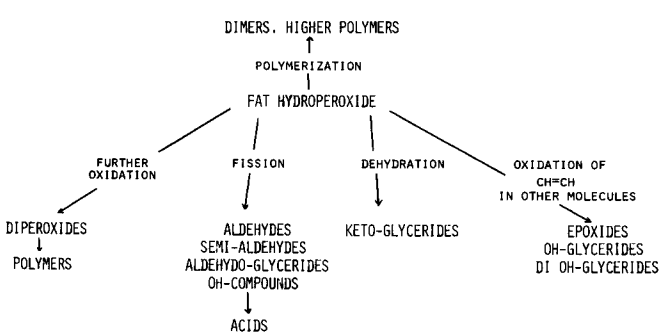


FIG. 1. Some routes of decomposition of fat hydroperoxide.

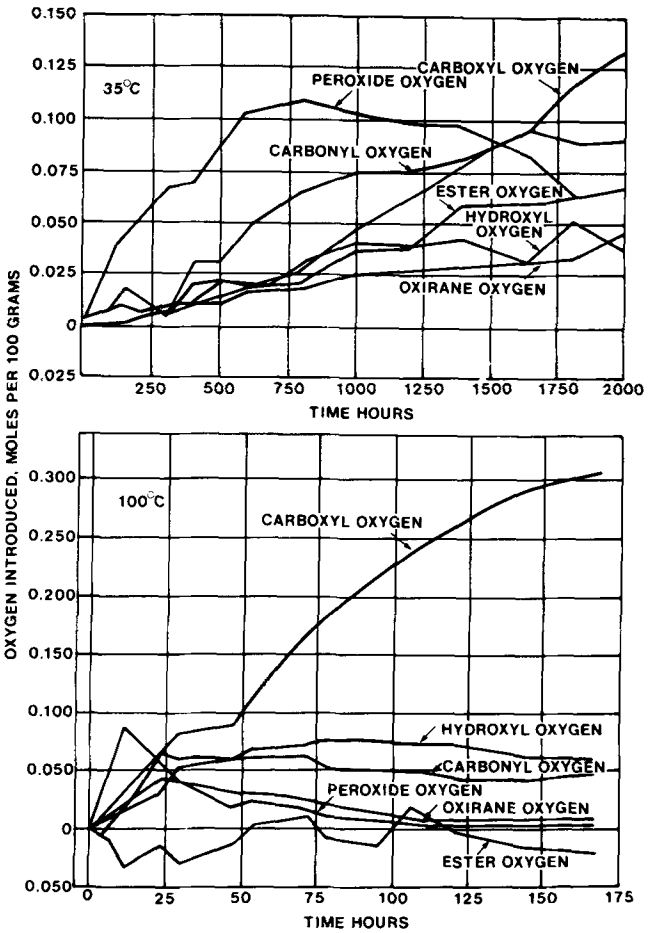


FIG. 2. Relationship of oxygen-containing functional groups in methyl oleate autoxidized at 35 and 100 C (6).

the objectionable flavors that result from the accumulation of decomposition products of the oxidation reaction. The testing method can be as simple as an individual tasting or smelling the oil. To minimize bias and human error, a multi-numbered panel is evaluated for odor and taste sensitivity. A statistically designed sampling experiment is then conducted. The disadvantages of this type of analysis are the length of time required and poor reproducibility. The chemical methods developed attempt to improve the reproducibility, sensitivity, and quantitativeness. Ultimately, these results must support the sensory measurements.

CHEMICAL METHODS

Peroxide Value

The primary products of lipid oxidation are hydroperoxides which are generally referred to as peroxides. Therefore, it seems reasonable to determine the concentration of peroxides as a measure of the extent of oxidation. However, this theory is limited due to the transitory nature of the peroxides. As shown in Figure 1, the peroxides are intermediate products in the formation of carbonyl and hydroxy compounds. The relationship between oxygen-containing functional groups and oxidation time is shown in Figure 2 (6). It clearly shows that the peroxide value passes through a maximum and is very sensitive to temperature changes.

Numerous analytical procedures for the measurement of the peroxide value are described in the literature. The results and suitability of the test depend on the experimental conditions and the reducing agent employed. As a result, whenever the soundness of an oil is expressed in terms of the peroxide value, the method used in the determination should also be given.

The iodometric methods of Lea (7) and Wheeler (8) are widely used, and these are based on the measurement of the iodine produced from potassium iodide by the peroxides present in the oil. According to Mehlenbacher (9), the two principal sources of error in these methods are (a) the absorption of iodine at unsaturated bonds of the fatty material, and (b) the liberation of iodine from potassium iodide by oxygen present in the solution to be titrated. The latter is often referred to as the oxygen error and leads to high results in the peroxide determination (10-13). Lea (7) attempted to eliminate this error by filling the sample tube with nitrogen at the beginning of the test and assuming that the evolution of chloroform thereafter would prevent the reentry of oxygen into the tube. Wheeler (8) used a homogeneous solution in an attempt to eliminate the need for shaking, thereby minimizing the effect of oxygen. It has also been established that other possible sources of error in the iodometric methods include variation in weight of sample, the type and grade of solvent used, variation in the reaction conditions such as time and temperature, and the constitution and reactivity of the peroxides being titrated.

Lips et al. (14) suggested a colorimetric method based on the oxidation of ferrous to ferric iron and the determination of the latter as ferric thiocyanate. This method has been found suitable for the estimation of fat peroxides in milk powder, and peroxide values determined by this method are considerably higher than by iodometric procedures. Stine et al. (15) used a nonionic surface-active agent to break the emulsion for the estimation of peroxide value by a modified ferric thiocyanate method. It appears to be a very satisfactory method for routine control purposes such as in milk, cream, condensed and dried milk products. Its reproducibility and sensitivity are excellent, but it is not suitable for estimating an oil sample from homogenized fluid samples.

Lea (16) determined peroxide levels using three methods: the iodometric, ferric thiocyanate, and 2,6-dichlorophenolindophenol procedures. Peroxide values were measured against oxygen uptake. For the iodometric method in the absence of oxygen, 91% of the oxygen absorbed by methyl linoleate was shown to be converted to peroxy compounds. It was suggested that the liberated iodine reacted with the unsaturated fatty acids or the oxidation products to account for the low value. The experimental procedure required that the entire reaction system be equilibrated to an oxidation temperature of 37 C, a fatty substrate free from pro- and antioxidants and rigid exclusion of oxygen during the peroxide measurement. The other two methods gave impossibly high peroxide values when correlated with the oxygen absorption. However, the ferric thiocyanate method was observed to give excellent reproducibility and to require much less sample. It was felt that this method might have more potential on a microscale and in the early stages of peroxidation than the iodometric method. The 2,6-dichlorophenolindophenol method had none of these advantages.

A variation in the iodometric method was reported by Swoboda and Lea (17). This was a colorimetric assay of liberated iodine which was converted into a blue starch-iodine complex. Since the quantity of material required for testing was 1 to 50 mg, this method has advantages for samples too small for convenient titration.

A recent comparison of the iodometric and ferric thiocyanate methods indicated that the results obtained vary with the experimental conditions and the reducing agent used (18). Two iodometric methods were compared: the Sully (19) and the Wheeler [modified by Hadorn et al. (20)] methods which differ primarily in the amounts of

TABLE I

Peroxide Values of Autoxidized Oils Determined by Various Methods (18)

Oil	Method		
	Wheeler (20)	Sully (19)	Stine et al. (15)
Sunflower	18.2	16.0	31.4
	57.0	51.6	108.3
Corn	6.6	6.5	11.5
	19.3	19.2	35.8
Walnut	17.7	24.5	58.6
Hazelnut	27.7	27.1	57.5
Olive	24.0	24.1	45.5

reducing agent employed and the fact that the former method involves boiling the substrate. The peroxide values of five oils were determined, and two different oxidation states were tested for sunflower oil and corn oil (Table I). The two iodometric methods gave similar results for hazelnut, olive, and corn oils, but the Sully method gave lower values for oils such as sunflower which have higher linoleic acid contents. A thin layer chromatographic analysis of the products of iodometry revealed that with the "cold" Wheeler method, the primary reaction taking place was the reduction of the hydroperoxides to the corresponding hydroxy acids. However, the more severe experimental conditions of the Sully method encouraged ca. 15% of hydroperoxide decomposition.

When ferrous sulfate [Stine et al. (15)] was used as the reducing agent, Barthel and Grosch (18) found that nearly double the reduction equivalents were required relative to the iodometric data. This was attributed to further reactions along the acyl chain and may explain the discrepancy in Lea's investigation (16).

The AOCS official iodometric method Cd 8-53 (21) for peroxide value determination is applicable to all normal fats and oils. This method is commonly used but is highly empirical and any variation in procedure may result in variation in results. Recently, the official method has been modified in an attempt to increase its sensitivity at low peroxide values (22). The official method fails to adequately measure low peroxide values because of difficulties with the determination of the titration end point. The modification involves the replacement of the titration step with an electrochemical technique in which the liberated iodine is reduced at a platinum electrode maintained at a constant potential. Peroxide values ranging from 0.06 to 20 have been determined with this method. It is essential, of course, during the determination to deaerate all the solutions since the presence of oxygen can lead to further formation of peroxides.

Stamm (23) reported that rancidity could be detected in oils by the red color which developed when such oils were heated with 1,5-diphenylcarbohydrazide. An investigation of Hamm et al. (24) revealed that oxygen in the solvent entered into the reaction, but this factor was constant. They derived a mathematical relationship to convert the absorbance of diphenylcarbazone to peroxide value.

$$PV = \frac{8{,}324\ A}{FW(15.71 - 0.1806\ t)}$$

where F is the empirical factor evaluated at 2.631 for soybean oil and W is the weight of sample in grams. The F factor is empirical and must be determined a priori for a particular oil rather than extrapolated to new conditions. This method is supposedly very sensitive to low concentrations of peroxides and hence could have some value for the study of early stages of autoxidation. However, it is a very empirical method, and changes in the order of the addition

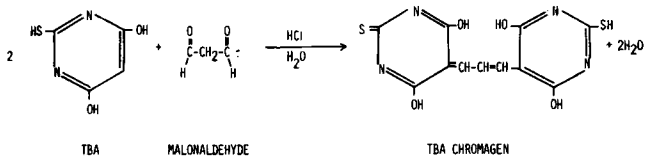


FIG. 3. Proposed TBA reaction (27).

of reagents, time and temperature of heating, acetic acid concentration, and polarity of the solvent used all have an effect on the results obtained. Any advantage of this method seems to be outweighed by the many disadvantages.

Hargrave and Morris (25) measured the hydroperoxide concentration using a method based on stannous chloride as a reductant. They noted that an oxygen balance for the reaction could not be based on only the hydroperoxide species. Other oxygen-containing functional groups were isolated during the reaction, and molecular scission gave rise to carbonyl compounds. An examination of the molecular weight of the oxidation products indicated that diperoxides were also formed. These workers showed that diperoxides were not reduced under the conditions of the chemical tests for hydroperoxides.

Although the peroxide value is a common measurement of lipid oxidation, its use is limited to the initial stages of oxidation. Since peroxides are vulnerable to further reaction, the complete oxidative history of the oil may not be revealed. Good correlations, however, have been reported between the official peroxide method and organoleptic flavor scores for various commercial fats such as lard, hydrogenated soybean oil, and corn oil (26).

Thiobarbituric Acid Test

The thiobarbituric acid (TBA) test is one of the more commonly used methods for the detection of lipid oxidation. However, the popularity of a method is not in itself ample proof that the method fulfills all the requirements of a reproducible technique. Certainly, much research in the past two decades has gone into determining some of the basic reactions involved with this test.

Early investigations by Sinnhuber et al. (27) helped to clarify the nature of the colorimetric reaction that occurs during the TBA test. They proposed that the chromagen was formed through the condensation of two molecules of TBA with one molecule of malonaldehyde (Fig. 3). However, no evidence was presented that malonaldehyde could be found in all oxidizing systems. Dahle et al. (28) postulated a mechanism for the formation of malonaldehyde, a secondary product in the oxidation of polyunsaturated fatty acids. This mechanism was based on investigations which showed that no color developed for linoleate even at peroxide values of 2000 or greater, but that for fatty acids with three or more double bonds the molar yield of the TBA color increased with the degree of unsaturation. These results indicated that only peroxides which possessed unsaturation, β, γ to the peroxide group were capable of undergoing cyclization with the ultimate formation of malonaldehyde. Such peroxides could only be produced from fatty acids containing three or more double bonds. It therefore becomes imperative to know the fatty acid profile of the sample to be tested. This work also indicated that meaningful results from the TBA test can only be obtained by comparison of samples of a single material at different stages of oxidation.

The TBA test has been criticized on several points. Tarladgis and his co-workers (29) considered the effect of acid, heat, and oxidizing agents on the TBA reagent. They suggested steam distillation of the product to remove the

volatile constituents which were assumed to be responsible for sensorial rancidity. These workers concluded that the structure of TBA was altered by acid and heat treatment as well as by the presence of peroxides and recommended that blank determinations be carried out in conjuction with the test. In another investigation of the effect of reaction conditions, Yu and Sinnhuber (30) suggested the following corrections to the conclusions of Tarladgis et al. (29): (a) the color interference was the result of impurities in the acid; (b) hydrogen peroxide reacted with TBA only if the acid concentration was high; and (c) since colored complexes formed when the TBA reagent was passed through a cellulose column, the column chromatography results were suspect. This investigation stressed the importance of using purified reagents.

The TBA test may be performed in two ways, either directly on a food product followed by extraction of the colored pigment, or on a portion of a steam distillate of the food. Both methods have in common the use of acid and heat. deKoning and Silk (31) reported that they were unable to successfully apply the TBA test in either of its forms to determine rancidity in fish oils. Poor results were attributed to the two-phase system of the direct method and to inefficient extraction of the malonaldehyde with the distillation method. The method was modified to a monophase system of ethanol, and good correlation between the TBA number and peroxide value was obtained. The observation that both values peaked at similar levels of oxidation led the authors to conclude that the TBA number was no more useful a criterion of rancidity than peroxide value after the initial stages of oxidation. Moreover, the monophase system was found to be light sensitive causing loss of intensity of the peak at 530 nm. Yu and Sinnhuber (32) attempted to improve the reliability of the TBA test by making further modifications. They observed that during the heating of the TBA reagent, a thin oil film was formed on the sides of the tube. They felt this could be a possible source of error since the reagents would not be in contact with the film, and further oxidation of the film might occur. This obstacle was overcome by coating the surface of the glass with silicone. Further oxidation of the sample under the conditions of the test was prevented by the addition of antioxidants. Under these modified conditions, the TBA number of fish oils was observed to vary linearly with peroxide values up to a peroxide value of 800.

Recently, a comparison of the direct TBA method with the distillation method for the determination of rancidity in mackerel was carried out by Vyncke (33). High correlation between the two methods was observed, although the TBA number as determined by the distillation method was twice as large as that from the direct extraction method. The author concluded that the simpler and quicker extraction method could be used instead of the distillation procedure for determining rancidity in mackerel.

Color development during the TBA test is usually assessed by measuring the absorbance of the red pigment at 532 nm. However, other pigments have been observed to form, notably a yellow pigment with maximum absorbance at 450 nm. Using a modified one-phase system, Jacobson et al. (34) observed that dienals showed an absorption peak at 432 nm while the TBA reaction products of the saturated aldehydes showed a peak at 452 nm. The location of the double bond of monoenes was found to influence the spectral behavior of the TBA reaction products. Marcuse and Johansson (35) also studied the reaction of TBA with various classes of aldehydes and found that alkanals, 2-alkenals, and 2,4-alkadienals produce a yellow 450 nm pigment with TBA, while only 2,4-alkadienals and to a lesser extent 2-alkenals produce the red 532 nm pigment. They suggested that the absorbance at 450 nm was indicative of rancidity as well as the value at 532 nm. The latter is generally taken as the characteristic absorbance of the TBA-malonaldehyde complex. Since the two pigments have different heat sensitivities, the color development conditions were modified to minimize the risk of interference. Patton (36), however, has questioned the value of the absorbance at 450 nm, since it can be produced by the reaction of TBA with aldehydes which are not oxidation products, e.g., glyceraldehyde, aromatic aldehydes, hydroxymethylfurfural. It may therefore not be a true monitor of oxidative rancidity.

Evidence that TBA can react with compounds other than those found in oxidizing systems to produce the characteristic red pigment has been presented in the literature. Dugan (37) has reported that sucrose and some compounds in woodsmoke react with TBA to give a red color so that cured and smoked meats require corrections for the sugar content and for the smoke in the outer layers. Baumgartner et al. (38) also found that a mixture of acetaldehyde and sucrose when subjected to the TBA test produced a 532 nm absorbing pigment identical to that produced by malonaldehyde and TBA. The authors suggested that this reaction may interfere with the assay of lipid peroxidation in organs such as the liver where ethanol can be converted to acetaldehyde.

Poor correlations between TBA values and other indicators of oxidation have sometimes been observed in complex biological materials such as animal tissues. Buttkus and Rose (39) explored this further by preparing various condensation products with malonaldehyde and amines and observing their effect on the color yield in the TBA test. They found that (a) open chain monoaddition products such as those formed between malonaldehyde and glycine or methionine and disubstituted products did not interfere in the test; (b) with cysteine-malonaldehyde complexes, only 70% of the malonaldehyde was recovered in the test; and (c) complexes in which a ring component is formed, as with citrulline and arginine, gave very low yields of malonaldehyde. These authors reason that the cyclic products in which malonaldehyde was incorporated were relatively stable under the conditions of the TBA test whereas straight chain products were readily hydrolyzed. The significance of these results to the TBA test is that in such oxidizing systems where proteins are also present, competition for the malonaldehyde may occur, resulting in lowered color yields. These competing reactions would also be expected to proceed more rapidly in dehydrated or concentrated systems since water also competes for the active sites.

In contrast to Buttkus and Rose (39), Shin et al. (40) found that there was little reactivity between malonaldehyde and sulfydryl groups. They suggested that cysteine was a poor compound to use to assess the effect of malonaldehyde on sulfydryl groups since cysteine also possesses an amino group which may alter the reactivity of the sulfydryl group. The pH and temperature of the reaction system and the concentration of malonaldehyde present all have an effect on the reactions which occur.

Several attempts have been made to establish a relationship between the TBA value and the development of undesirable flavor characteristics in fats. However, Pohle et al. (41) found that the flavor score could not be estimated for any given fat from the TBA value since the relative level varied from product to product. Thus the relationship between TBA value and change in flavors would have to be established for a given oil before the TBA value could be used as an index of flavor. Such reported incidents of poor correlation between TBA value and flavor scores were attributed by Wyatt and Day (42) to be the result of the type of flavor evaluation employed and not to any limitation in the TBA method. They used an evaluation based on the detection of flavor thresholds. This kind of flavor test does not require a judge to rank the samples or to assign a numerical

value to them but rather merely to indicate whether the samples taste rancid or not. The concentration of oxidized sample at which 50% of the judges detect rancidity is known as the average flavor threshold. When TBA number was plotted against the reciprocal of average flavor threshold, high correlations were obtained for corn, cottonseed, safflower, and soybean oils.

Kreis Test

The Kreis test (43), one of the first tests used to evaluate the oxidation of fats, involves the production of a red color when phloroglucinol reacts with oxidized fat in acid solution. Much of the literature on fats and oils prior to 1951, states without reservation that the compounds responsible for the Kreis color reaction of oxidized fats are epoxy aldehydes or their acetals. Most of these statements are based on the findings of Powick (44) who made a thorough study of the Kreis reaction. However, Patton et al. (45) presented evidence which suggested that epihydrin aldehyde (2,3-epoxypropanal) and its derivatives are not necessarily solely responsible for the Kreis color reaction. They showed that malonaldehyde also gave a positive reaction in the Kreis test and that the resulting color was spectrally similar to the Kreis colors obtained with 2,3-epoxypropanal, diethyl acetal, acrolein treated with H_2O_2, rancid lard, and oxidized milk fat. Lea (46) suggested that since 2,3-epoxypropanal and malonaldehyde are isomeric, 2,3-epoxypropanal may be formed by rearrangement of malonaldehyde in the oxidation of certain unsaturated fatty acids.

The most significant objection to the Kreis test is that the development of color does not necessarily parallel the development of rancidity (9). All too frequently samples which are entirely fresh and free from rancidity show some color when they are reacted with the Kreis reagent. In addition, it is sometimes difficult to obtain comparable results in different laboratories and in the hands of different operators. Although the Kreis test may serve a useful purpose in indicating slight changes in the condition of a fat under various circumstances, it does not provide a satisfactory index of rancidity.

Total and Volatile Carbonyl Compounds

An alternative approach to the determination of the extent of lipid oxidation is to measure the carbonyl compounds formed by the degradation of the hydroperoxides (Fig. 1).

The most reliable and widely used of the analytical methods is the one outlined by Henick et al. (47). The procedure is based on the formation of 2,4-dinitrophenylhydrazones of carbonyl compounds in the presence of trichloroacetic acid catalyst. However, the method has been criticized since hydroperoxides decomposed under the experimental conditions (48). Since this interfered with a quantitative determination of the oxidation products, several alternatives have been suggested.

In one method, the hydroperoxides were reduced to noncarbonyl compounds prior to the carbonyl determination. Mizuno and Chipault (49) established that under mild conditions with a stannous chloride reagent, there was no evidence of side reactions nor loss of carbonyl compounds. Hence the determination was free of interference from hydroperoxides. Fioriti (50) reported that reduction was not the complete answer since it is time consuming and also generates some additional carbonyl compounds. He suggested that interference from hydroperoxides can be reduced by carrying out the reaction at 5 C. However, under these conditions the reaction must be carried out for 20 hr.

Holm et al. (51) investigated a method based on the reaction of saturated and unsaturated aldehydes with benzidine acetate. The reaction occured in the fat sample without high temperature or addition of strong acid, both of which break down hydroperoxides. However, owing to the carcinogenic properties of benzidine, an alternative but similar method has been suggested (52) based upon anisidine (p-methoxyaniline). List et al. (53) reported a highly significant correlation between the anisidine values of salad oils processed from undamaged soybeans and their flavor scores. Multiple correlations between flavor scores, anisidine and peroxide values yielded a correlation of 0.81 and provided a method for predicting the initial flavor scores of salad oils processed from undamaged soybeans.

In an alternative procedure, Johnson and Hammond (54) used trichlorophenylhydrazine as the reagent. Although too time consuming for a routine assay of rancidity, the method allowed quantitative isolation, at the nanogram level, of carbonyl species from oxidized fat.

The reaction between carbonyl functional groups and hydroxylamine-hydrochloride has also been utilized as the basis of a test for measuring lipid oxidation (55). This test is based on the following reactions:

$$RCHO + H_2NOH.HCl \rightarrow RCH = NOH + HCl + H_2O$$
$$RCOR^1 + H_2NOH.HCl \rightarrow RR^1C = NOH + HCl + H_2O$$

The liberated acid is then titrated, and the end point may be detected visually or potentiometrically. However, the chief disadvantage of this method is the instability of the hydroxylamine. The hydroperoxides can also interfere in the reaction and give variable results or results that are too high. Hydroperoxides again must be reduced before the determination of the carbonyl compounds. However, it has been established that ester, carboxyl, primary and secondary amines, α-glycols, ether and allylic types of ethylenic groups do not interfere with this method. Trozzolo and Lieber (56) claimed that interfering factors such as steric effects, cyclization with the formation of isoazoxalones, conjugated unsaturation, and hydrogen bonding decreased the usefulness of such methods. Since fats contain some conjugated double bonds after oxidation, the hydroxylamine method is not really suitable for oxidation determinations.

The procedures discussed for carbonyl determination measure principally nonvolatile carbonyl compounds. Although these are probable precursors to more volatile, odorous compounds, they make no direct contribution to the flavor. Hence, it seems appropriate to attempt to recover and estimate the level of volatile degradation products.

In a 1966 review, Gaddis et al. (57) compared the isolation of carbonyl-2,4-dinitrophenylhydrazones by vacuum distillation and Girard T and Swartz procedures. The Girard T reagent which extracts carbonyls into an aqueous phase was recommended. It was also shown that precursors break down to carbonyl compounds under the other two experimental conditions. Lea and Swoboda (58) reported an improved vacuum distillation system for the recovery of low molecule weight carbonyls. Again the probelem of decomposition of precursors interfered with a quantitative determination of volatile products.

An advantage of these methods is that they attempt to measure products which contribute to the rancid flavor. These are not limited, as is the peroxide determination, to the early stages of oxidation (Fig. 2). Some are extremely sensitive and quantitative. However, it is necessary to establish that the experimental conditions do not decompose precursors to the carbonyl compounds being assayed.

Oxirane Determination

Oxirane compounds containing an α-epoxy group are formed during autoxidation of unsaturated lipid material (Fig. 2). The α-epoxy groups are usually determined by reacting the compound with an excess of halogen in a suit-

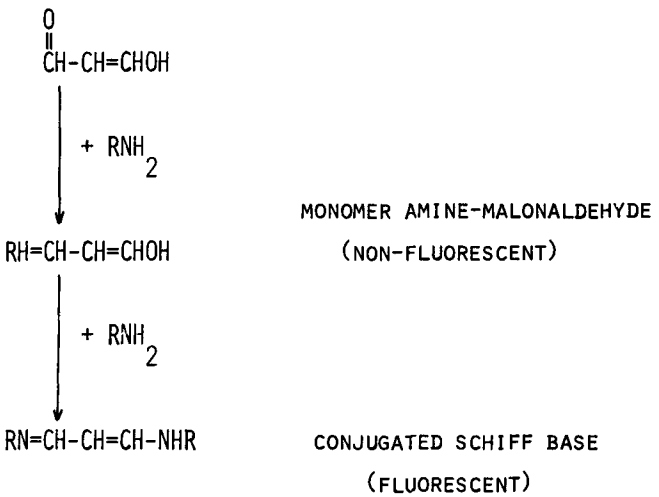
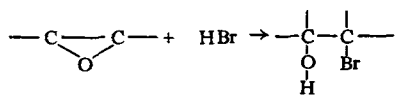


FIG. 4. Production of fluorescent chromophores by reaction of oxidation products and amines (71).

ble solvent, the halogen consumed being a measure of the α-epoxide. The reaction proceeds as follows:



The standard HBr titration method, AOCS Tentative Method Cd 9-57 (21) for oxirane determination was adopted as a result of collaborative analyses conducted in five different laboratories (59).

Fioriti et al. (60,61) found that picric acid was the best of several acidic chromophores in its reaction with epoxides. Despite a nonquantitative reaction, the product concentration followed Beer's Law. This method has been found to be particularly well suited to the determination of epoxides in heated fats where the oxirane level is often less than 0.1%.

PHYSICAL METHODS

Conjugated Diene Methods

Despite the claim by St. Angelo et al. (62) that the diene conjugation method is one of the three most widely used methods for determining the degree of rancidity in fat-containing food, little information dealing specifically with this method can be found in the literature.

Oxidation of polyunsaturated fatty acids is accompanied by increased ultraviolet absorption. Fatty acids with conjugated unsaturation absorb strongly in the region 230 to 375 nm, diene unsaturation at 234 nm, and triene unsaturation at 268 nm. The magnitude of change is not readily related to the degree of oxidation because the effects upon the various unsaturated fatty acids vary in quality and magnitude. However, the changes in the ultraviolet spectrum of a given substance can be used as a relative measurement of oxidation.

Oils containing linoleate or more highly unsaturated fatty acids are oxidized to conjugated diene systems that can be measured by ultraviolet absorption at 234 nm. Farmer and Sutton (63) indicated that the absorption increased proportionately to the uptake of oxygen and to the formation of peroxides in the early stages of oxidation. In the oxidation of ethyl linoleate the monohydroperoxide that forms was shown by ultraviolet absorption measurements to contain ca. 70% of conjugated isomers (64). Holman and Burr (65) suggested that development of ultraviolet chromophores cannot be taken as a measure of oxygen uptake (degree of oxidation) unless the composition of the fatty acid mixture is known. Privett and Blank (66) indicated that the plateau of the ultraviolet curve always occurred just prior to the end of the induction period.

St. Angelo et al. (62) studied the autoxidation of peanut butter by measuring the peroxide value and the increase in absorption at 234 nm due to diene conjugation. They concluded that the conjugated diene hydroperoxide (CDHP) method can be used as an index of progressive staling in place of, or in addition to, the peroxide value. The CDHP method is faster than the peroxide value method, is much simpler, requires no chemical reagents, does not depend upon chemical reaction or color development, and can be conducted on smaller samples. This method is applicable for the analysis of peroxides in vegetable oil products containing polyunsaturated fatty acids.

A recent paper by Parr and Swoboda (67) describes a new analytical procedure for determining lipid oxidation in stored foods. In this assay, hydroperoxides of polyenoic fatty acids as well as hydroxy and carbonyl compounds derived from them are converted by two chemical reaction steps, first reduction and then dehydration, into more conjugated chromophores. These yield "conjugable oxidation products" which are measured together and expressed as the "C.O.P. value." The first step of the analytical procedure, reduction by sodium borohydride, results in the disappearance of the characteristic ultraviolet absorbance of the carbonyl compounds of oxidized polyenoic fatty acids (oxodienes). The decrease in absorbance at 275 nm is known as the oxodiene value. Another feature of this assay is that it distinguishes between the oxidation products derived from dienoic fatty acids and those of more highly unsaturated polyenoic fatty acids and this is measured by the "C.O.P. ratio." These authors applied this assay to both fresh and stored foods and fats and compared their results with the traditional peroxide value. It was concluded that the peroxide value and the C.O.P. value had the same level of numerical significance.

Fluorescence

Recently, interest has been shown in the detection of oxidation products by fluorescent methods. These methods have been applied mainly as a measure of determining the extent of lipid oxidation damage in biological tissues. Tappel and co-workers have established that fluorescent compounds with the general structure of a N,N^1-disubstituted 1-amino-3-iminopropene (R - N = CH - CH = CH - NH - R) may develop through the interaction of peroxidizing lipids and cellular constituents such as certain phospholipids (68), deoxyribosenucleic acid (69), ribonuclease (70), and proteins which have free amino groups (71). Several advantages of the fluorescence method as a means of measuring lipid oxidation have been reported (70). The method which is very sensitive can detect fluorescent compounds at a level of one part per billion and was found to be 10 to 100 times more sensitive than the TBA assay. The development of fluorescence closely parellels oxygen absorption even in the later stages of peroxidation, and it can also be used to quantitatively assess accumulated damage in biological samples.

Dillard and Tappel (71) examined the production of fluorescent chromophores which they attribute to the reaction of an amino group with carbonyl compounds, mainly malonaldehyde. They observed that the mono-substituted product did not exhibit fluorescence since this required a conjugated Schiff base structure (Fig. 4). Buttkus and Rose (39) reported that the development of fluorescence depended not only on whether condensation products were formed between malonaldehyde and the free amino group of a compound but also on the nature of the substituents of

the amino groups. Different excitation and emission maxima were also observed with different condensation products.

Research in the development of fluorescent compounds from oxidixed lipids is on-going and much more work is needed before the full potential of this method can be properly assessed. Trombly and Tappel (72) have indicated that definite analysis of purified fluorescent products of lipid oxidation cannot be accomplished by means of fluorescent characterization alone. Preparative thin layer chromatography of the lipid-soluble fluorescent components for infrared spectrophotometric or gas chromatography-mass spectrometry analyses could provide structural definition of the major fluorescent species resulting from lipid peroxidative damage. This method is very effective for analysis of the oxidation products.

Infrared Spectroscopy

Infrared spectroscopy is of particular value in the recognition of unusual functional groups and in the study of fatty acids with *trans* double bonds (73). As the compounds formed during the oxidation of fats change, it is possible using infrared spectroscopy to follow the course of oxidation. The significance of the infrared absorption spectroscopy was summarized by O'Connor (74). He reported that (a) the appearance of bands at about 2.93 μ indicated formation of hydroperoxides; (b) the disappearance of the band at 3.2 μ indicated the replacement of the hydrogen on a double bond with some other radical, probably indicating polymerization; (c) the appearance of additional bands at about 5.72 μ ester C = O stretching indicates the formation of aldehydes, ketones, or acids, and (d) changes in bands in the region 10 to 11 μ indicated *cis,trans*-isomerizations and probably formation of conjugated linkages.

Henick (75) applied infrared analysis to determine the oxidation products in milk fat. The spectral characteristics were detected before the off-flavors developed, and both loss of flavor and development of off-flavors were correlated with definite absorption bands. Ahlers and McTaggart (76) indicated that infrared methods were simple, rapid in operation, and required only small amounts of sample (20 mg). They claimed that the accuracy of each determination was similar to that of the corresponding conventional chemical methods. However, fats are complex materials, therefore the infrared method is only good as a qualitative index, especially in the induction and the early peroxide formation periods.

Polarography

Polarographic methods have been developed for quantitation of peroxides of fats, ethers, and hydrocarbons (77). In the early stages of oxidation of fats, a linear relationship exists between wave height and peroxide value. The accuracy of this technique is within 5% for fats with a peroxide value of 5 or higher. A linear relationship exists between the peroxide value determined by a modified Wheeler method and wave height until the peroxide value exceeds 250. Thereafter, wave height does not increase as rapidly as peroxide values. This method appears to be more beneficial for a study of the early stages of fat oxidation.

Ricciuti et al. (78) compared the polargraphic method with the Wheeler iodine and stannous chloride chemical methods. These three methods gave results which were not significantly different for high purity hydroperoxide, but with impure products, the polarographic method may give more reliable values because it is more specific than the chemical procedures.

The polarographic method can differentiate between the peroxide structures, -0-0 and -00H and give more precise results than other methods to evaluate peroxides. However, this technique is not widely used in the quality control industry.

Gas Chromatography

Gas chromatography has been more extensively used in the separation and identification of the products of lipid oxidation in model systems in order to elucidate the mechanisms of oxidation. However, some authors have developed gas chromatographic procedures for measuring rancidity in vegetable oils, ground beef, and cheese (79-83).

Oxidative studies with methyl linoleate (84) and soybean oil (85) revealed that saturated hydrocarbons arise early during autoxidation when aldehydes are either absent or not detectable. Evans et al. (86) reported that pentane is the predominant short chain hydrocarbon to arise through thermal decomposition. Correlations of flavor scores and pentane formation have been used to determine rancidity of oils after directly injecting the oils on a gas chromatographic column (82,87). A later study by Warner et al. (88) showed that significant correlations were obtained between the amount of pentane developed and the number of rancid descriptions of aged vegetable oils and potato chips. Samples needed only 0.08 ppm in the headspace to be described as rancid by 90% of the panel. Both oils and chips were more stable to the development of pentane as the linoleate content and the iodine value decreased.

Jarvi et al. (82) also designed a method to measure the development of rancidity in soybean oil by gas chromatography. They reported that very small amounts of hydroperoxides, too small to be detected by peroxide value analyses, can be visually detected by gas chromatography. An equation was developed to correlate flavor panel scores with the oxidation value measured gas chromatographically.

Gas chromatography can be very useful in determining the extent of rancidity in pure mixtures such as vegetable oils. However, in the case of more complex lipid systems such as foods, difficulties in identification and standardization will probably negate much of this usefulness.

Refractrometry

Ayra et al. (89) observed that a sharp increase in the refractive index coincided with the detection of rancid odors They also claimed that the refractive index changes according to the three known stages of fat autoxidation. In the induction period when peroxide formation is low, the refractive index remained constant. During the secondary stage of relatively more peroxide formation, the refractive index increased sharply until the peroxide value reached a maximum. This increase in refractive index was attributed to conjugation, known to precede hydroperoxide formation. In the tertiary stage of peroxide decomposition, the refractive index continued to increase at a steady rate, the increase being less sharp than in the second stage. Polymerization of partially oxidized fats was thought responsible for this change in refractive index.

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