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Critical Evaluation of Methods for the Measurement of Oxidative Rancidity in Vegetable Oils

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ABSTRACT

During storage and shipping, edible oils are susceptible to oxidative deterioration. To ensure high oil quality, it is essential to reliably analyze their oxidative status. The aim of this review was to critically evaluate published methods based on their value and applicability. This review focuses on major chemical, physical and chromatographic methods used for assessing the degree of lipid oxidation. Due to complexity of the mechanism of lipid oxidation, most of the methods determine either early or advanced oxidation products. Methods based on nuclear magnetic resonance spectroscopy, normal-phase high-performance liquid chromatography and high-performance size-exclusion chromatography are discussed as superior methods due to their capability of providing combined information about the extent of early and advanced oxidation stages.

Key words: lipid oxidation, vegetable oils, conjugated dienes, hydroperoxides, malondialdehyde

INTRODUCTION

Edible vegetable oils are often exposed to elevated temperatures during processing, shipping and/or storage. High temperatures and the presence of oxygen favor the process of lipid oxidation, which results in the formation of characteristic off-flavors and less palatable oils⁽¹⁾. The rate of lipid oxidation strongly depends on the degree of unsaturation of the fat^(2,3). The degree of unsaturation is determined by the number of double bonds in the fatty acid molecules – a neighboring double bond decreases the energy of the hydrogen binding to the carbon and a high number of adjacent double bonds facilitates the detachment of the hydrogen atom.

A high concentration of polyunsaturated fatty acids (PUFAs) renders a vegetable oil more prone to oxidation, resulting in a loss of PUFAs due to the formation of early and advanced volatile and non-volatile lipid oxidation products. As high levels of PUFAs are considered beneficial in terms of sustaining health⁽⁴⁾, controlling the oxidative stability of oils containing high amounts of PUFAs, such as soy bean oil⁽⁵⁾, becomes essential not only for the evaluation of the sensory quality of vegetable oils, but also for the determination of their nutritional value.

Oxidative stability is mainly affected by processing conditions that imply the application of high temperatures, oxidants, enzymes such as lipoxygenases, moisture, light

and oxygen^(6,7). Photosensitization facilitated by transparent packaging might induce the formation of singlet oxygen, which has been reported to react 1,500 times faster with linoleate than with oxygen in its triplet ground state⁽⁸⁾ and, thus, may promote lipid oxidation. In contrast to lipid peroxidation, which only occurs in the presence of enzymes, lipid autoxidation in foods is based on a free radical chain reaction, starting off with an endothermic reaction by the loss of a hydrogen atom in the presence of oxidation initiators, such as traces of metals, light or heat⁽⁹⁻¹¹⁾. After removal of the hydrogen atom at the initiation of lipid autoxidation, the carbon-centered radical undergoes molecular rearrangement to form stabilized conjugated dienes, followed by oxygen uptake to finally yield peroxy radicals. During the stage of propagation (Figure 1), the peroxy radical abstracts a hydrogen atom from another fatty acid to form the lipid hydroperoxide, a marker for early oxidation stages⁽¹¹⁾.

Due to the instability of lipid hydroperoxides, secondary decomposition products, such as aldehydes, ketones, alcohols, hydrocarbons, esters, furans and lactones, are generated. However, lipid hydroperoxides can also be oxidized to yield epoxyhydroperoxides, oxohydroperoxides, bihydroperoxides, cyclic peroxides and bicyclic endoperoxides⁽¹¹⁾. The secondary oxidation products can further decompose into monohydroperoxides, which may result in the formation of volatile products. Lipid hydroperoxides have also been reported to undergo condensation reactions that terminate the autoxidation process by forming dimers and polymers,

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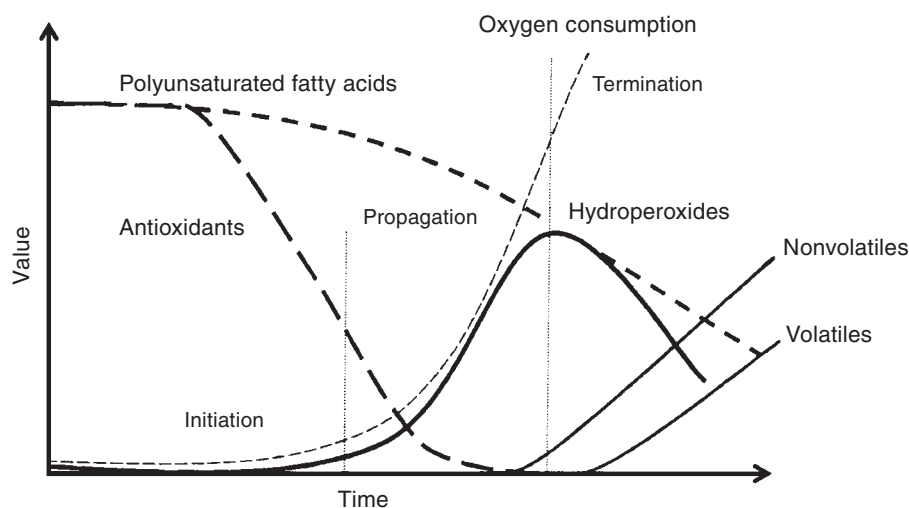


Figure 1. Time-dependent decrease of antioxidants, unsaturated fatty acids and formation of lipid autoxidation products in vegetable oils.

Table 1. Analytical methods to detect the formation of primary and secondary lipid autoxidation products

| Lipid peroxide product | Chemical structure | Analytical method |
|------------------------------|--------------------|-------------------|
| Polyunsaturated fatty acid | | GC/NMR |
| Carbon-centered radical | | ESR |
| Conjugated diene | | HPLC |
| Oxygen consumption | O_2 | Clark electrode |
| Peroxyl radical | | ESR |
| Hydroperoxide | | HPLC |
| Hydroxydiene | | GC |
| Aldehyde (hexanal, propanal) | | HPLC |
| Malondialdehyde | | HPLC |
| Epoxyhydroxy-compound | | HPLC |
| Oxodiene | | HPLC |

which may break down into volatile compounds⁽¹¹⁾. Due to this rather complex mechanisms of lipid oxidation, various analytical methods have been developed to investigate the different stages of lipid oxidation. However, a universal method suitable for the evaluation of early lipid autoxidation processes in edible oils is still lacking.

I. Evaluation of Analytical Methods

Upon oxidation of vegetable oils, unsaturated fatty acids and antioxidants decline over time, while an increase of oxygen consumption and the formation of hydroperoxides as primary oxidation products can be observed. However,

hydroperoxides readily decompose, aggravating their analytical monitoring. Advanced oxidation products such as volatile and non-volatile compounds have much longer half-life times, and are more commonly analyzed as markers of lipid autoxidation.

Table 1 depicts a simplified pathway of lipid autoxidation and provides an overview of analytical methods applied for the quantitative analysis of lipid oxidation products formed in vegetable oils. The loss of PUFAs and the formation of saturated fatty acids are commonly quantified by means of gas chromatography (GC) or nuclear magnetic resonance (NMR), whereas carbon-centered radicals can be analyzed by electron spin resonance (ESR). After immediate intramolecular rearrangement, the carbon-centered radicals form conjugated dienes, which are relatively stable, and commonly analyzed by high-performance liquid chromatography (HPLC)-UV techniques. Conjugated dienes may undergo oxygen uptake, analyzed by polarography such as Clark electrodes, to finally yield peroxy radicals which can be quantified by ESR. Peroxy radicals easily abstract hydrogen atoms from other fatty acids to form lipid hydroperoxides, also indicators of early oxidation stages⁽¹¹⁾, that are quantified by HPLC-UV. Lipid hydroperoxides may undergo either condensation or degradation reactions, resulting in the formation of dimers or polymers or aldehydes, hydroxydienes or epoxyhydroxydienes, respectively. All of these compounds may be analyzed by GC or HPLC techniques.

II. Chemical Evaluation

One of the most common methods for measuring the oxidative rancidity of vegetable oils is the peroxide value (PV)⁽¹²⁾. The PV determines the amount of peroxides formed during early oxidation stages, expressed as millimoles or milliequivalents of peroxide oxygen per one kilogram of oil. Numerous analytical protocols have been described for the measurement of PVs. The most widely used procedure utilizes the oxidative capacity of lipid (hydro) peroxides to generate iodine from potassium iodide⁽¹³⁻¹⁵⁾. Iodometric titration employs starch as the indicator and sodium thiosulfate as the titrant. One of the drawbacks of the PV method is the instability of hydroperoxides, which leads to an initial increase, followed by a decrease of hydroperoxides during the progress of lipid oxidation (Figure 1). Major concerns also relate to the accuracy of the measurement, pointing to the absorption capacity of unsaturated fatty acids for iodine and the additional release of iodine from potassium iodide by oxygen^(16,17). Other possible disadvantages comprise variation in sample weight, the type of solvent, variation in reaction time and temperature, and constitution of the peroxides⁽¹⁴⁾. Furthermore, it has been reported that this method failed to measure low PVs due to inaccurate determination of the titration end-point, leading to the introduction of electrochemical techniques⁽¹⁸⁾. Alternative empirical colorimetric assays were developed, such as the analysis of ferric thiocyanate formed by oxidation in the presence of peroxides⁽¹⁹⁾ or 1,5-diphenylcarbohydrazide, yielding a red color

after reaction with oxidized lipids⁽²⁰⁾. Although widely used, the different inherent drawbacks of the PV determination have prevented this method from being recommended as a standard method for evaluation of the oxidative rancidity of vegetable oils.

One of the most commonly used chemical assays to determine secondary oxidation products is the thiobarbituric acid (TBA) test⁽²¹⁾. At low pH and high temperature, two molecules of TBA condensate with one molecule of malondialdehyde, a decomposition product of oxidized PUFAs, to form a pink chromogen that can be quantified spectrophotometrically at a wavelength of 532 nm. The selectivity of the TBA assay can be further improved by utilizing the fluorescent property of the TBA chromogen⁽²²⁾. However, malondialdehyde is solely released from peroxides derived from fatty acids containing three or more double bonds⁽²³⁾. The fatty acid composition of the oil under investigation, therefore, has to be considered, rendering comparisons among different oils unreliable. It has also been noted that the TBA test is not specific to malondialdehyde, but also gives pink chromogens with sugars, lipids, urea, oxidized proteins and other aldehydes not derived from lipid oxidation⁽²⁴⁻²⁷⁾. Prior HPLC separation and the use of chromogens with different absorption maxima might reduce the limitations in specificity^(28,29). However, an inevitable drawback of the TBA assay might be the additional assay-borne generation of malondialdehyde as an artifact, due to heat and acid treatment of lipid hydroperoxides^(24,30). Despite some inherent disadvantages of the TBA assay, this method is widely used to rapidly screen large numbers of samples.

The Kreis test was one of the first methods to be applied to determine the oxidative deterioration of vegetable oils⁽³¹⁾. In this test, phloroglucinol reacts with epoxy aldehydes or their acetals under acidic conditions to form a red color. The Kreis test does not provide reliable information on the degree of lipid oxidation, since samples free from rancidity were shown to give positive results, probably due to the presence of aldehydes⁽¹⁷⁾. In addition, some food additives, such as vanillin, were identified to interfere with the Kreis test⁽³²⁾. The Kreis test is therefore not considered to be a reliable rancidity index.

III. Carbonyl Compounds

Several assays have been developed to analyze the carbonyl compounds derived from lipid autoxidation. One of the most commonly used assays for the detection of total carbonyl compounds is based on the formation of 2,4-dinitrophenyl hydrazones in the presence of carbonyl compounds and trichloroacetic acid⁽³³⁾. Due to the required experimental conditions of high temperature and low pH, the decomposition of lipid hydroperoxides is promoted, thereby interfering with the assay⁽³⁴⁾. However, prior reduction of hydroperoxides to non-carbonyl compounds could not prevent the generation of additional carbonyl substances^(35,36). Holm *et al.*⁽³⁷⁾ suggested quantifying aldehydes by using benzidine acetate without the application of high temperatures or strong acids.

Since benzidine acetate is carcinogenic, a similar method using *p*-anisidine has been proposed. *p*-Anisidine reacts with 2-alkenals, forming products that can be assessed by absorbance at 350 nm⁽³⁸⁾. However, this absorption maximum was demonstrated to shift towards longer wavelengths with increasing double bonds in the molecule, thereby aggravating comparisons among different vegetable oils⁽³⁹⁾.

To calculate the total oxidation (totox) value, the following equation including the *p*-anisidine value (AV) and the PV is used: totox value = 2 PV + AV⁽³⁹⁾. The totox value combines information about early and advanced stages of lipid deterioration, rendering it a valuable parameter for rancidity evaluation of vegetable oils.

Combination of the PV analysis with polarographic methods has also been demonstrated to provide valuable results up to PV of 250 mmol/kg⁽⁴⁰⁾. Polarographic methods are used to determine the oxygen consumption. The Clark-type electrode, a sensitive oxygen sensor, might be a promising tool to characterize early stage lipid oxidation.

IV. Physical Evaluation

The detection of conjugated dienes is a widely accepted physical method to assess the oxidative rancidity of vegetable oils⁽⁴¹⁾. Oxidation of PUFAs results in the generation of conjugated double bonds. Conjugated dienes were reported to absorb at 234 nm, whereas secondary oxidation products, such as diketones, were shown to have their absorption maximum at 268 nm⁽⁴²⁾. Due to their rapid and simple performance, and their independence of chemical agents and reactions or color development, analyses of conjugated dienes are widely accepted for the evaluation of lipid oxidation. However, this method shows limited sensitivity for more saturated oils due to the lower number of double bonds⁽⁴³⁾. Conjugation of PUFA dienes might also be assessed by qualitative methods such as refractometry⁽⁴⁴⁾ or infrared spectroscopy⁽⁴⁵⁾.

Application of nuclear magnetic resonance (NMR) spectroscopy has also been shown to be a promising technique^(46,47). The NMR method analyzes the change of the fatty acid profile in oils by calculating the ratio of aliphatic to olefinic protons. Wanasundara *et al.*⁽⁴⁷⁾ observed a constant increase in the ratio of aliphatic to olefinic protons during the storage of canola and soybean oil, concluding that storage of these oils was associated with the progressive oxidation of unsaturated fatty acids.

Electron spin resonance (ESR) spin trapping techniques have also been applied to determine early oxidative events in vegetable oil⁽⁴⁸⁾. A widely applied spin trap that studies free radical formation during the storage of oil is *N*-tert-butyl- α -phenyl-nitron (PBN), which gives a linear correlation with the PV and ESR results⁽⁴⁹⁾, although higher sensitivity for early oxidative changes is achieved by ESR techniques. Disadvantages of the ESR techniques include the fact that spin adducts are only stable for a short period of time, requiring the addition of the spin trap after sampling and immediate recording of the ESR signals. In addition, the ESR spin trapping technique has not been recommended for

application in complex food systems containing antioxidants due to possible interactions with PBN⁽⁴⁹⁾.

V. Chromatographic Evaluation

Gas chromatography (GC) is widely used to determine volatile compounds, such as hexanal and pentane, that are generated by hydroperoxide scission⁽⁵⁰⁻⁵²⁾. GC provides information about the underlying mechanism of lipid oxidation. Convenient GC methods have been developed for direct on-column injection of the oil onto the column, however, resulting in an unstable chromatographic baseline and the shortening of column life⁽⁵³⁾. Thus, a U-tube, which is connected to the GC, was implemented for the isolation of volatile compounds⁽⁵⁴⁾. Another possibility with regard to on-column injection is to vacuum distill the volatiles into a cold trap prior to GC analysis⁽⁵⁵⁾. Alternatively, GC is often used for headspace analyses⁽⁵⁶⁾, whereby the volatile is accumulated by heating in the headspace of a tube and subsequently injected onto the GC capillary. Since high temperatures are required for this method, further reactions of peroxides and secondary oxidation products might be initiated. Furthermore, partitioning of the volatiles between the vapor and the liquid oil might pose some uncertainties. Lipid oxidation was monitored by combining solid phase microextraction (SPME) with GC⁽⁵⁷⁾. Prior extraction of volatiles with SPME has been assessed to be an environmentally-friendly method due to omission of toxic solvents. Furthermore, the results obtained are in good correlation with the AV.

Nonvolatile lipid oxidation products are favorably analyzed by means of HPLC coupled with UV or MS detectors⁽⁵⁸⁾. Steenhorst-Slikkerveer *et al.*⁽⁵⁸⁾ developed a normal-phase HPLC/MS method for the detection of nonvolatile triacylglyceride oxidation products, such as epoxy-triacylglyceride, oxo-triacylglyceride, hydroperoxy-triacylglyceride, hydroxyl-triacylglyceride and glycerides with two intact fatty acid chains and one short chain with an aldehyde or hydroxyl group. This method can be used to characterize and quantify nonvolatile triacylglyceride oxidation products, although reference standards have to be synthesized for this method. Besides normal phase chromatographic methods, reversed-phase HPLC techniques have also been described to separate and analyze mixtures of conjugated diene hydroperoxide isomers, but only based on their geometrical isomerism, while normal phase-HPLC separates based on the position of the hydroxyl or hydroperoxide group⁽⁵⁹⁾. Normal phase-HPLC techniques, therefore, allow differentiation between different isomers, such as 13-hydroperoxy dienes and 9-hydroperoxy dienes. Normal phase-HPLC coupled with MS or UV detection might have the potential to emerge as a method that combines the determination of early and advanced lipid oxidation products. Another promising HPLC method, concomitantly evaluating primary and secondary oxidation products, was developed by Márquez-Ruiz *et al.*⁽⁶⁰⁾. Trilinolein samples were first fractionated using solid phase extraction to separate polar from non-polar fractions. The polar fraction was then analyzed by high-performance

size-exclusion chromatography using a refractive index detector. With this method, oxidized monomers, dimers and polymers of methyl linoleate were detected. It could be shown that the amount of oxidized monomers represents the early oxidation stage, while dimers and polymers were indicative of advanced oxidation. This method has been successfully applied to study oxidative alterations in sunflower oils⁽⁶¹⁾.

VI. Concluding Remarks

Most of the assays developed to evaluate lipid oxidation detect either primary or secondary oxidation products, thereby limiting conclusions on the progress of lipid oxidation. Methods that combine the concomitant detection of primary and secondary oxidation products are scarce. NMR, HPLC and high-performance size-exclusion chromatography methods are discussed as superior methods applied for the detection of the changes of the fatty acid profile, conjugated diene hydroperoxides/hydroxides and condensation products of hydroperoxides.

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