A REVIEW OF ANALYTICAL METHODS MEASURING LIPID OXIDATION STATUS IN FOODS: A CHALLENGING TASK

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ABSTRACT

Lipid oxidation analysis in food samples is a relevant topic since compounds generated in the process are related to undesirable sensory and biological effects. As the process is complex and depends on the type of lipid substrate, oxidation agents and environmental factors, proper measurement of lipid oxidation remains a challenging task. A great variety of methodologies have been developed and implemented so far, both for determining primary oxidation products and secondary oxidation products. Most common methods and classical procedures are described, including peroxide value, TBARS analysis and chromatography. Some other methodologies such as chemiluminescence, fluorescence emission, Raman spectroscopy, infrared spectroscopy or magnetic resonance, provide interesting and promising results, so attention must be paid to these alternative techniques in the area of food lipid oxidation analysis.

KEYWORDS: Fat oxidation; Hydroperoxides; Secondary lipid oxidation products; TBA; Hexanal

ABBREVIATIONS

MDA	malondialdehyde
PV	peroxides value
AOAC	association of official analytical chemists
UV-Vis	ultraviolet-visible
TEP	1,1,3,3-tetraethoxypropane
TMP	1,1,3,3-tetramethoxypropane
TBA	thiobarbituric acid
TBARs	thiobarbituric acid reactive substances
PAV	para-anisidine value
HPLC	high perfomance liquid chromatography
ESI	electrospray ionization
MS	mass spectrometry
GC	gas chromatography
DNPH	2,4-dinitro-phenylhydrazine
FID	flame ionization detector
SOPs	sterol oxidation products
LDI-TOF	laser desorption/ionization-time of flight
HS	head space
SDE	simultaneous distillation extraction
RPDE	reduced pressure distillation extraction
SHS	static head space
DHS	dynamic head space
SPME	solid phase micro-extraction
CL	chemiluminescence
IR	infrared
FTIR	fourier transform infrared
SERS	surface enhanced Raman spectroscopy
NMR	nuclear magnetic resonance
EPR	electron paramagnetic resonance
HPSEC	high performance size exclusion chromatography

1. INTRODUCTION

Lipid oxidation in foods constitute a complex chain of reactions that firstly yields primary products (peroxides), that, when exposed to extended oxidation conditions, give rise to secondary oxidation products, including aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers. Most of them produce undesirable sensory and biological effects [1, 2]. Therefore, its control is of great importance.

Lipid oxidation occurs via different pathways: radical mechanism (known as autoxidation), singlet oxygen mediated mechanism (known as photooxidation) and also the enzymatic oxidation has been described, catalyzed by lipoxigenases. This review will be focused on the non-enzymatic routes. Both autoxidation and photoxidation give rise to identical or similar peroxides, differing just sometimes in position and stereoisomerism. The first mechanism requires an initial activation energy for the removal of a hydrogen atom, so it is enhanced by high temperatures and presence of double bonds. The latter is triggered by the highly reactive singlet oxygen specie, which is formed by excitation of triplet molecular oxygen, under light exposure and presence of photosensitizers [3, 4].

The first compounds formed during oxidation process are peroxides, especially hydroperoxides; hence they are called primary oxidation products. Despite being intermediate compounds of lipid oxidation process, they are relatively stable (depending on the lipid structure), and can be used to assess lipid oxidation status in food samples, providing not too advanced autoxidation is developed in the sample. Because of this intermediate characteristic, temperature conditions during analysis must be controlled to avoid hydroperoxide decomposition, and addition of antioxidant is often required.

Hydroperoxides usually suffer further oxidation to give secondary oxidation products. Silvagni et al. [5] proposed an alternative kinetic model where the aldehydes are generated not only via direct degradation of hydroperoxides but from peroxyl radicals through an independent pathway. This mechanism involves a bimolecular reaction to form intermediate tetraoxides, which are unstable at high temperatures and decompose to give alkoxyl radicals. The wide variety of secondary oxidation products to which oxidation gives rise includes aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers. Among them, both volatile and non-volatile compounds can be found, such as hexanal or malondialdehyde (MDA), respectively, as main representatives.

Evaluating lipid oxidation status is a challenging task due to a number of reasons. Firstly, different compounds are formed depending on the time, extent of oxidation and mechanism involved. Therefore, choosing just one parameter to analyse the oxidative status is rather difficult and it is frequently more convenient to combine different methods. Besides, as stated by Eymard et al. [6], not only nature and composition of lipid as the substrate of the reaction have an impact on lipid oxidation process, but also type and concentration of proteins, antioxidants and prooxidants present in the food matrix, as well as its physicochemical characteristics. In meat samples, Richards and Dettmann [7] suggested that rates of lipid oxidation may depend on the relative ability of haemoglobins from different animal species to promote it. Chen et al. [8] proposed that colloidal structures formed by phospholipids in vegetable oils could have an impact on the oxidative stability of food oils. Lipid oxidation was observed to be delayed in fish sausages after the addition of several antioxidants [9]. Milk samples oxidation has been recently studied in the presence of catechins and ascorbic acid [10]. On the other hand, each method allows a number of different experimental conditions, and this, together with the lack of uniformity among laboratories, leads to (at least for the moment unavoidable) dissimilar results. Finally, most of the oxidation compounds are prone

to be further degraded, which provides an added source of divergence. Therefore, a precise control of the experimental procedure must be kept.

Related to lipid oxidation in food samples, other assessments can be also performed. On the one hand, determination of parameters highly indicative of lipid deterioration and subsequent enhanced susceptibility to oxidation (such as hydrolysis of triglycerides) is very common. On the other hand, measuring the time required by a sample to achieve a certain oxidative level through artificially promoting oxidation is another valid procedure to evaluate lipid susceptibility to oxidation (and/or oxidation stability). However, this review will only focus on methods determining the actual and current lipid oxidation of a sample, discarding procedures assessing hydrolytic status and those involving induction of oxidative degradation, since they are not properly indicators of oxidation status but of oxidative susceptibility and stability, respectively.

This review will describe traditional methods to determine both primary and secondary lipid oxidation products in foods, from spectroscopic to chromatographic techniques. Their characteristics, advantages and limitations will be pointed out. Then, alternative methodologies developed during last decades will also be revised in order to provide the complete oversight of possible options. Table 1 summarizes the main characteristics of the methods described in this review.

2. PRIMARY OXIDATION PRODUCTS

2.1 PEROXIDES

Hydroperoxides redox properties are the base of some of the key methods applied in their determination. A number of reagents can be oxidized by hydroperoxides, including simple inorganic ions, such as iodide or ferrous ion. These methods usually require subsequent complexation to improve the sensitivity.

2.1.1 Volumetric method

Among the different methods proposed for the analysis of peroxides, the iodometry has been the most conventional and widespread method mainly due to the simplicity of the experimental procedure. Although the procedure requires prior lipid extraction, rapid and easily understandable results are provided.

In acidic medium, hydroperoxides and other peroxides react with the iodide ion to generate iodine, which is tittered using a sodium thiosulfate solution, in the presence of starch solution. The AOAC offers an official method since 1965 [11]. According to this method, Peroxide Value (PV) is considered to represent the quantity of active oxygen (in meq) contained in 1 kg of lipid and which could oxidize potassium iodide.

It shows however some drawbacks, mainly derived from the iodide high susceptibility to oxidation in the presence of molecular oxygen and accelerated by light exposure. Also spontaneous hydroperoxide formation can occur (which would lead to overestimation) and absorption of iodine by unsaturated fatty acids (which would lead to underestimation) [12]. Moreover, it requires anhydrous systems to avoid interference problems, for what lipid extraction is required, and this procedure stage increases the contact with oxygen. In addition, the Peroxide Value determination does not give a real measure of the oxidative degradation, since peroxides are usually further degraded, so simultaneous measurement of secondary products would be appropriate.

2.1.2 VIS-UV spectroscopic methods

As well as the volumetric method, spectroscopic ones are rather simple and are moderately sensitive, reliable, and reproducible when carried out under standardized

conditions. However, they are highly empirical as they measure complex mixtures of oxidized molecules. In addition, they are generally work-intensive and use large amounts of solvents and reagents that might be hazardous [13].

2.1.2.1 Ferrous oxidation method

The ferrous oxidation method for determination of peroxide content is simpler to use than iodometry. The main reason is the lower sensitivity of ferrous ion to spontaneous oxidation by oxygen in air, as compared to high susceptibility to oxidation of iodide solutions. It consists of oxidation of Fe(II) to Fe(III), mediated by hydroperoxide reduction in acidic conditions and in the presence of thiocyanate or xylenol orange (in this later case, method is known as FOX). These two compounds provide the spectrophotometric properties, as they form complexes with the ferric ion, giving maximum absorbance peaks at 500 nm and 560 nm respectively, which can be measured with a UV-Vis Spectrophotometer [6, 14-18]. However, neither of the methods is free from complications [19]. The thiocyanate method requires large amounts of solvent, and as for the FOX, it detects in a small range of peroxides concentrations and molar absorptivity of the ferrilxylenol orange complex varies with different procedures of making the dye. Nuchi et al. [20] concluded that FOX results (from degradation of fat for feed uses) correlated better with other oxidation parameters than traditional iodometry.

2.1.2.1 Iodide oxidation method

A spectrophotometric iodide-dependant method has also been set to determine hydroperoxide content. In this methodology, not so commonly used [21], the lipid sample is placed in an acidic solution, which is then merged with iodide. The lipid hydroperoxide oxidises iodide to iodine. Then, generated iodine and iodide (in excess) react to give triiodide anion, which is detected spectrophotometrically at 350 nm. Bloomfield [22] used Fe (II) as a catalyst. The

closed conditions prevent interference from atmospheric oxygen and the short reaction time minimises interference from side reactions.

2.1.3 Chromatography

Methodologies explained up to here are in general quite simple regarding theory base, implementation of the procedure and ulterior interpretation of the data, presenting low to moderate selectivity and sensitivity, though. On the other hand, chromatographic techniques are far more accurate, sensible and specific for the compound in interest, allowing better identification of individual products. Indeed, their implementation for hydroperoxides determination instead of that of volumetric and spectroscopic measurements is growing up more and more over the last years. As an unavoidable consequence, chromatographic methods usually require long or meticulous experimental work, precise control of the experimental conditions and the data processing presents some complexity.

2.1.3.1 Liquid chromatography

High Performance Liquid Chromatography (HPLC) is being recently used to determine hydroperoxides. This method is highly sensitive and pretty versatile considering both column and detector properties, allowing to analyze compounds with different characteristics of volatility, molecular weight or polarity. On the other hand, sample preparation is frequently tedious and usually requires lipid extraction. Zeb and Murkovic [23] found the isocratic HPLC-ESI-MS a useful method for the identification and characterization of oxidized species of triacylglycerols (TAGs), i.e. mono- and bis-hydroperoxides. Gotoh et al. [24] developed a method for measuring the peroxide value in colored lipids on the basis of the reaction with triphenylphosphine, forming a compound which absorbs at 260nm. Sample then underwent HPLC separation and UV detection. Ferrous oxidation mediated methods have also been adapted to HPLC separation [25]. Specific hydroperoxides generated from sterols can also be

assessed by liquid chromatography. Saynajoki et al. [26] determined stigmasterol hydroperoxides by means of a normal-phase column and two types of detectors (UV and fluorescence).

2.1.3.1 Gas chromatography

Gas chromatography coupled to mass spectrometry (GC-MS) can also be used for the analysis of lipid hydroperoxides, but due to their thermo-lability, previous reduction is needed. This, along with the prior lipid extraction and subsequent derivatization step, makes it a cumbersome and time consuming method [27].

2.2 CONJUGATED DIENES/TRIENES

Hydroperoxide formation from polyunsaturated fatty acids is generally (over 90% of the cases) accompanied by stabilization of the radical state via double-bond rearrangement (electron delocalization), which gives rise to conjugated dienes and trienes. These relatively stable compounds absorb in the UV range (235 nm and 270 nm respectively) and this absorption can be measured by spectrophotometric techniques to assess oxidation level [28-29]. This technique is simple and rapid but not as widespread as determination of peroxides determinations, probably because it can lead to underestimation since oleic acid hydroperoxides, containing less than two double bonds, cannot be detected. On the other hand, overestimation is possible if conjugated double bonds are present in the original fatty acid. Furthermore, it is not suitable for oils that have been heated under conditions that decompose hydroperoxides because interference may occur with absorption of carbonyl compounds [30]. Even so, a number of studies have used them for the monitoring of lipid oxidation during heating treatments, especially in vegetable oils [31-33]. Correlation between 235nm absorption values and peroxide values has been reported [34].

3. SECONDARY OXIDATION PRODUCTS

Lipid primary oxidation products can generate, if submitted to further oxidation conditions, secondary oxidation products, including aldehydes, ketones, epoxides, hydroxy compounds, oligomers and polymers. These compounds show a wide variety of physico-chemical properties, differing mainly in volatility, polarity and molecular weight. Most relevant groups of compounds will be commented (aldehydes, volatiles and polymers), as well as a particular molecule very frequently used as oxidation marker (malondialdehyde).

3.1 MALONDIALDEHYDE

Malondialdehyde (MDA) is one of the most abundantly generated aldehydes during secondary lipid oxidation and it is probably the most commonly used as oxidation marker, too.

3.1.1 UV-Vis Spectroscopy

The most widely employed method for determination of MDA is the spectrophotometric determination of the red fluorescent MDA-thiobarbituric acid (MDA-TBA) complex.

Reaction occurs by attack of the monoenolic form of MDA on the active methylene groups of TBA, at low pH and high temperature, giving the mentioned chromophore which offers a maximum absorbance peak at 532nm. Reaction kinetics depends on the concentration of TBA solution, temperature and pH [35]. Several variations of MDA-TBA method exist, with different procedures currently performed in food analysis: direct heating of the sample, sample distillation, lipid extraction with organic solvents or aqueous acid extraction, followed by acid reaction with TBA. General procedure usually consists of homogenization and centrifugation at acidic medium (usually provided by trichloroacetic acid) and posterior reaction with TBA at high temperatures (around 90-100ºC). Nevertheless, there is quite a lot

of variability in reaction conditions, such as heat treatment exposure time; to illustrate it: Berasategi et al., Peiretti et al., Jung et al. and Jongberg et al. [36-39] left mixture react at boiling water bath for 15, 20, 30 and 40 minutes, respectively. On the other hand, trichloroacetic solution concentrations have also been reported to be different (from 3% to 15% w/v) among works [9, 40].

Traditional spectrophotometric TBA test has been criticised for some reasons. Firstly, TBA is not selective to MDA, since it also reacts with many other compounds, such as other aldehydes, carbohydrates, amino acids and nucleic acids [41], interfering in the TBA assay and resulting in considerable overestimation, as well as variability in the results. This is why it is also known as TBA reactive substances method (TBARS). There is also a risk of underestimating the response since malondialdehyde can, under *in vivo* conditions, form linear or cyclical Schiff bases, or even crosslinked bonds, with lysine and arginine from proteins. So poor quantification sensitivity and poor molecular specificity and selectivity can be attributed to this method. Furthermore, the high temperatures (95–100 °C), extended incubation times and strong acidic conditions commonly required for the reaction of MDA with TBA may cause an artifactual peroxidation of sample constituents even in the presence of added antioxidants. Note finally that malondialdehyde, which is mainly formed from linolenic acid oxidation, does not occur in other oxidized lipids (especially when only one double bond is present, i.e., oleic acid). So, it is often a minor secondary oxidation product, spoiling the role of lipid oxidation marker role usually assumed for this compound.
Despite the mentioned limitations, conventional spectrophotometric MDA-TBA methods are preferred because of their simplicity. In fact, it has been recently suggested as a more accurate and sensitive parameter in assessment of oxidative deterioration than p-anisidine test and hexanal determination [20, 42].

3.1.2 Chromatography

To overcome some of these limitations, more advanced chromatographic determinations have been developed. These techniques provide, as in the case of hydroperoxides measurement (section 2.1.3) more accuracy, sensitivity and specificity for MDA. Harder experimental work, and certain level of complexity in data processing are the drawbacks.

Some of them [43-48] involve the formation of MDA-TBA complex, purification by chromatography (GC or HPLC) and subsequent detection by MS, UV-Vis or fluorometric detector. And some others use derivatization of MDA instead of reaction with TBA, in order to obtain a detectable compound. Reaction with 2,4-dinitro-phenylhydrazine (DNPH) or pentafluorophenylhydrazine and conversion into pyrazole and hydrazone derivatives are the most commonly used procedures with HPLC separation and spectrophotometric/fluorometric detection [48-50]. On the other hand, conversion into tetramethylacetal or methylpyrazole is more common with GC separation, with Flame Ionization Detector (FID) or Nitrogen/Phosphorus specific detector [50]. Mendes et al. [48] and Marcincak [51] compared two HPLC separation methods for MDA determination (MDA-TBA and MDA-DNPH adduct) with the traditional spectrophotometric MDA-TBA test, in samples of chilled fish and pork. The methods were fast, simple, sensitive and stable and presented overall better performance (based on accuracy, specificity and recovery levels) than the traditional spectrophotometric MDA-TBA test, although MDA-DNPH showed a relatively high limit of detection and a lower reproducibility at lower MDA contents in standards and samples.

3.2 OTHER SECONDARY OXIDATION COMPOUNDS

3.2.1 UV-Vis Spectroscopy

A number of other aldehydes apart from MDA are generated during lipid secondary oxidation. A spectroscopic method to detect their presence is the p-anisidine value (PAV). It is one of the oldest methods for evaluating secondary lipid oxidation, especially in the analysis of animal fats and vegetable oils. It provides useful information on carbonyl compounds, especially non-volatile α-unsaturated aldehydes (such as 2-alkenals and 2,4-dienals) because it is based on the reactivity of the aldehyde carbonyl bond on the p-anisidine amine group, leading to the formation of a Schiff base that absorbs at 350 nm. The p-anisidine value is defined as 100 times the absorbance of a solution containing 1 g of fat in 100 mL of solvent. It is considered a very simple and rapid methodology. PV and PAV allow calculating total oxidation. This parameter (total oxidation) combines evidence about the past history and present state of an oil, so it allows to estimate the overall extent of oxidation in the food [12].

PAV has been recommended as a good control parameter for secondary oxidation control since it correlates well with peroxides content (FOX and PV), TBA and volatile aldehydes analysis [20, 52]. In the research field, it has remained a little backward, in favour of other techniques [53].

It is well known that the colorimetric response with p-anisidine varies according to the extent of aldehyde unsaturation. Hence, at identical concentrations, the response is more intense with di-unsaturated aldehydes than with mono-unsaturated aldehydes, which in turn are more sensitive than saturated aldehydes. Moreover, p-anisidine reacts with all aldehydes, irrespective of their origin. This is especially the case for some phenol compounds of virgin olive oil, such as decarboxymethyloleuropeine dialdehyde, which could interfere in the assessment. Finally, studies on correlations between PAV and the organoleptic quality highlighted the efficacy of this test for measuring oxidation in many different lipids.

However, these correlations may vary markedly between lipids and also according to the prevailing oxidation conditions. Caution is thus required when interpreting this index [28].

3.2.2 Chromatography

A number of other compounds apart from carbonyls are generated during lipid secondary oxidation.

Concerning fatty acids, they can suffer oxidation as free form, within triacylglycerols or bonded to phospholipids). Their secondary oxidation products can be assessed by HPLC [54]. However, while this technique may be useful to obtain a fingerprint of the oxidation status of the sample, only a minority of signals can be attributed unequivocally to a specific compound because separation is not good enough. Better quantitative analysis can be carried out by means of GC-FID and GC-MS after derivatization into methyl esters [55]. Development of LDI-TOF-MS and ESI-MS [56-58] has meant a great step forward in this field.

Even though Sterol Oxidation Products (generally known as SOPs) present low levels in foods, they show a number of harmful effects in the organism [59], so a significant number of studies have focused their attention in their analysis. Experimental procedure involves lipid extraction, saponification, purification, derivatization and chromatographic analysis. That determination is challenging in many ways: artifact generation, very low concentrations, matrix effects, incomplete identification and reporting, to note a few [60,61]. GC-MS is the most accurate and commonly applied quantification method for this kind of compounds [62-66]. Clariana et al. [67] found this technique better than CG-FID in a study performed with pork meat. Due to the necessity of a derivatization process and the impossibility of analysing thermolabile molecules, some liquid chromatography methods have been recently developed [68-70]. However, liquid chromatography shows lower resolution than gas chromatography,

and the best way to overcome this problem is coupling it to a mass spectrometer detector, which in this case is quite complex and still has not been well solved. A new fast GC-MS method has been recently developed and applied to cholesterol oxidation products analysis, giving highly promising results [71]. Satisfactory resolution, good repeatability and sensitivity, together with the consequent reduction of the time of analysis and consumables make it a valid alternative to conventional CG-MS.

3.3 VOLATILES

Under this group of secondary oxidation products a great diversity of compounds has been included, presenting very different functional groups: aldehydes, ketones, alcohols, short carboxylic acids and hydrocarbons. They all share the property of giving from moderate to high smells and are related to rancidity in sensorial tests. Measurement of these secondary oxidation products is of great importance, since their formation closely relates to the deterioration of flavour. Some of these volatile compounds are highly specific to the oxidative degradation of a particular polyunsaturated fatty acid family: propanal is the main marker of oxidation of n-3 fatty acids, while hexanal and pentanal are markers of oxidation of n-6 fatty acids. Both propanal and hexanal are often used as indicators of lipid oxidation in foods because they can be measured in the sample headspace and their lack of double bonds makes them more stable towards oxidation than unsaturated aldehydes. Nevertheless, hexanal is more frequently measured as its formation is higher than that of most secondary oxidation products, apart from a few exceptions. However, measuring the extent of oxidation with just one or two markers is a rather coarse approach, so methods involving assessment of large set of compounds should be promoted [28].

Gas chromatography is the preferred method to quantify volatile molecules and mass spectrometry detection contributes to identify them. Different methods may be used to

recover volatile oxidation compounds before chromatographic analysis, including: (a) solvent extraction and (b) headspace (HS) techniques.

(a) Although liquid-liquid extractions are not very suitable to recover the volatile content (because they are long, laborious and require a solvent evaporation step, which leads to substantial volatile compound degradation), novel variants have been recently proposed to overcome some of these limitations. Note especially simultaneous distillation extraction (SDE) and reduced pressure steam distillation extraction (RPDE). Both allow to obtain compounds of relatively high boiling point, but with RPDE evaporation is reached with lower temperatures, avoiding possible artefact formation [72]. SDE and RPDE show the advantage of being able to extract high quantities of target compounds since the volatile fractions generally have high solubility in organic solvents [73,74]. Moreover, Ferhat et al. [75] developed a microwave energy-mediated extraction method. Liquid-liquid extractions are the preferred recovering methods whenever the samples require derivatization step previous to chromatographic analysis (HPLC and GC). DNPH, benzyloxime and thiazolidine derivatives are the most frequently used compounds to improve stability and/or detection by visible-ultraviolet spectrometry, flame-ionization, nitrogen-phosphorous and mass spectrometry detection [76].

(b) HS analysis can be performed by static headspace (SHS), dynamic purge-and-trap headspace (DHS) or headspace-solid phase microextraction (HS-SPME) techniques. All of them are prior to gas chromatography analysis.

In SHS method, the sample is placed in an airtight vial. Most compounds that are volatile at the analysis temperature evaporate from the liquid or solid fraction and pass into the overhead gas HS. At equilibrium, an aliquot is harvested and injected on the GC column. This method

is relatively inexpensive and easy to use, it does not require solvent extraction and can be automated. However, as equilibrium is established between the volatile compounds in the HS and those remaining in the sample, only low quantities of compounds are actually recovered, which limits the sensitivity. The increase in the extraction temperature could increase the volatilization of the target compounds and thus increase the quantities recovered, but the temperature must be kept as low as possible in order to minimize generation of new oxidation products and/or thermal degradation of oxidation markers. A number of authors [77,78] have applied this method in food samples analysis.

On the contrary, DHS technique does not require the establishment of equilibrium: the sample is continually purged by inert gas to extract volatile compounds. Then, the gas effluent passes through a porous polymer trap that collects volatile analytes. Among all available trap materials, tenax is the most commonly used. As volatiles contained in the sample are constantly released and trapped, a high concentration of compounds are injected on the GC column. Despite its high sensitivity, the instrumentation is complex and expensive, thus increasing the sources of error (trap drying, trap transfer, purging efficiency, etc.) and it is in general terms slower than SHS. Nevertheless, several studies have highlighted the efficacy of DHS-GC in assessing the oxidative status of different food matrix [79,80].

In SPME analysis, volatile compounds make a first equilibrium between sample and HS, followed by a second one between the HS and the contact fibre (which is coated with a highly adsorbant polymeric film). Finally, the fibre is introduced in the GC injector. This method provides many advantages over other ones, including easy manipulation and experimental set up, short sampling times, easy automation and high sensitivity [81]. A number of authors have applied this method for food lipid oxidation determinations [80,82]. Its main drawback

is that fibre degradation and contamination occurs quite rapidly, thus replacement is required periodically.

Recent comparative studies performed with all these methods for capture of volatile content lead to the conclusion that each one presents its shortcomings and advantages [83,84], but HS-SPME is being used to an increasing extent on account of its most promising results.

3.4 OLIGOMERS/POLYMERS

During extended oxidation, a lipidic compound can be linked together with other one or several ones, giving rise to dimers, oligomers or polymers. Simultaneous analysis of oxidized forms of triacylglycerols and their oligo/polymers is very common to assess lipid oxidation progress. Monomers are very reactive and highly correlate with peroxide value, so they could give information about the primary oxidation level of a sample. On the contrary, triacylglycerols oligopolymers are rather stable compounds, being considered as good indicators of secondary oxidation status [85,86].

High Pressure Size Exclusion Chromatography (HPSEC) has demonstrated to provide satisfactory results in the analysis of this kind of oxidation products. It allows separation and subsequent identification and quantification of molecules according to their molecular weight. It is usually performed on polar compounds, so it requires a previous purification of the polar lipid fraction, which is usually done by silica gel column chromatography. Some studies [1, 87-88] have demonstrated the usefulness of HPSEC in the determination of the levels of the oxidative degradation of a variety of food samples, and particularly that of refined vegetable oils, whose technological process involves quality deterioration. Morales et al. [33] applied it for the determination of advanced oxidation in vegetable oils through the detection of fatty

acids polymers. Oligomers formation during thermo-oxidation of phytosterols has also been reported [89-92] by means of HPSEC analysis.

4. ALTERNATIVE METHODOLOGIES

The previous techniques are either too empirical or highly dependant on several experimental factors, such as technician skill, light exposure and atmospheric oxygen, apart from the fact of being time-consuming. To avoid these limitations, various methodologies have been proposed as good alternatives in analysis of both primary and secondary oxidation products. They are based on direct spectroscopic analyses of samples, such as magnetic resonance, fluorescence and vibrational spectroscopy, and on chemiluminescent properties. As general good points, preliminary treatment is minimal or unnecessary, low amount of sample is required and highly specific results are obtained.

4.1 Chemiluminescence

Certain chemical reactions generate electromagnetic radiation. This emission of energy is known as chemiluminescence (CL) and it can be applied to detect and quantify compounds of interest. However, light intensity is very low (ultraweak CL is accompanied during oxidation of hydrocarbons and lipids [93]), so light amplifiers should be introduced to increase it. One of the most commonly used one is the luminol. The luminol-enhanced chemiluminescence involves oxidation of luminol in basic solution generating a free radical intermediate which reacts with flux of oxidizing agents (active free radicals) present in the system, e.g. lipid hydroperoxides. This leads to formation of luminol derived product in excited state, which eventually returns to ground state emitting strong blue light at 430 nm [94]. Different versions of this method differ in the type of active free radical produced and

the way of free radical production as well as in details of the procedure. Robinson et al. [95] suggested the addition of *p*-iodophenol to provide more intensive, prolonged, and stable light emission as compared to the traditional luminol system. More recently, a new chemiluminescence method in non aqueous medium CL was developed to detect lipid peroxides in vegetable oils [96], presenting good correlation with spectrophotometric PV analysis.

Baj et al. [97] discovered that partial exclusion of oxygen from the reaction medium strongly influenced the light intensity of the luminol reaction, and the effect is dependent on the oxidant analyzed, so an alternative mechanism was suggested for some oxidant species. Besides, they stated that the oxygen concentration always affects the reproducibility of the results, so equilibrating the working solutions with oxygen or air should always lead to improved results.

The attractive features of CL methods are their higher quickness (taking only a few minutes), sensitivity (picomol levels have been assessed), low sample requirements, low cost and simplicity as compared with other methods [98]. As for shortcomings of this kind of methods, first of all, the kinetic theory and mechanism for chemical processes resulting in CL is not known in detail. This may mean problems with data interpretation. Furthermore, this method is not specific to the lipids (other oxidizing agents also give signal); but this opportunity can be seized to estimate the overall total oxidant status of the sample.

Bunting and Gray [99] developed an automated flow injection chemiluminescence system for measuring lipid hydroperoxide concentrations in oils and found good agreement with a traditional iodometric titration assay, what could denote the usefulness of CL methods to assess lipid primary oxidation; and also in vegetable oils, Yang et al. [100] found a similar trend for TBARs and CL measurements during oxidation.

4.2 Fluorescence spectroscopy

When a compound is irradiated with an electromagnetic energy source, some of their electrons promote from their fundamental state to an excited one, and subsequently they return to their original state, re-emitting the energy previously absorbed. Nevertheless, certain compounds can lose some of that energy as heat, what allows their electrons to return to a higher level than the original one, so emitted light is in this case lower than the absorbed one. This phenomenon is named as fluorescence, and compounds presenting this property, fluorescents. Beam of light is usually from the UV range and emitted energy is typically, but not necessarily, from the visible range. It can be used in analytical chemistry for both qualitative and quantitative determinations, as well as in isolated and coupled to chromatography equipments.

Regarding food field, its implementation is growing up more and more [101]. The free amino groups of proteins can react with aldehydes from lipid peroxidation or reducing sugars to give Schiff bases. These compounds present a high colour intensity (browning) and characteristic fluorescence spectra (excitation and emission wavelengths, and fluorescence intensity) according to the type of protein and adduct. Although its sensitivity is high, excitation and emission wavelength maxima vary depending on the food sample and the procedure followed. They range from 250nm to 500 nm for excitation, and from 280nm to 600nm for emission [53, 102-104]. Many authors have used the ability of these Schiff bases to emit fluorescence to monitor thermal oxidative processes, especially in dairy products [105,106], meat [107,108], fish [109,110] and oils [111], but fluorescence methodologies are still poorly documented in food lipid oxidation analysis. Both Gatellier et al. [104] and Nguyen et al. [110] found a high correlation between fluorescent pigments and TBARS of

meat and fish products, which demonstrated that the interaction between proteins and aldehyde products of lipid oxidation is mainly involved in the production of fluorescent pigments and these are good markers of lipid oxidation.

A different implementation of fluorescent properties was developed by Andersen et al. [112] with a cheese sample. They measured the fluorescence of the photosensitizers involved in the lipid oxidation mechanism of the cheese and used the spectra to successfully predict the content of volatile compounds.

4.3 Infrared spectroscopy

Infrared (IR) spectroscopy is also known as a very helpful way to study lipid degradation under oxidative conditions [113], particularly since it is an easy, rapid, economical and non-destructive technology. It is based on the determination of fundamental vibrational transitions of a particular compound and involves the absorption of discrete energy levels from the IR region. These discrete energy levels are characteristic of each of atom-atom linkage, so studying the IR spectrum can provide enough information to find out the nature of the analyzed compound. Mathematical tools, such as Fourier Transform (FT) or chemometric methods, permit data processing. Continuous ageing monitoring can be carried out with this methodology, although for the moment, most of the works have been assessed in discontinuous way. Some advances have recently been performed regarding technological devices [114].

IR has been applied to measure the peroxide value in oxidized lipids [115] and differences were found in the IR spectra of fresh and aged oils [116,117]; so IR spectra can be used to characterize the aging of various edible oils [118-122]. The investigation of the FTIR spectra of the treated oils revealed that the microwave heating of oils [123] caused significant changes in the intensities of their absorption bands and produced no shifts in the position of

the bands. These changes were attributed to the reduction in 18:2 and 18:3 fatty acids content due to the oxidation.

It has also been used for the analysis of edible oils [124], horse mackerel patties [125] and canned tomato juice [126], in combination with other analytical methods which lead to similar conclusions, and therefore providing marker bands to improve the understanding of chemical changes taking place during processing and storage.

4.4 Raman spectroscopy

Raman spectroscopy also detects fundamental vibrational transitions although (contrary to infrared spectroscopy) not by means of direct energy absorption, but through an energy (originated from a UV, visible or IR laser) scattering: promotion to a virtual vibrational state and subsequent relaxation to a fundamental vibrational state different from the original one. Therefore, Raman and IR spectroscopy are complementary techniques and provide complementary structural information about molecules. Actually, only some molecules show Raman scattering properties, and most of them at a very small intensity, so quite sophisticated and expensive optical detection equipments are required. This reduces its practical use to a few cases. Indeed, it is still very sparingly used in the food field, in spite of its interesting characteristics, which include being non-destructive, fast, relatively inexpensive, non-involving chemical products, requiring very little sample preparation, being highly sensitive to unsaturations and poorly sensitive to water [127,128]. Two instrumental methods can be employed with Raman spectroscopy: confocal Raman spectroscopy with a powerful laser in visible range and Fourier Transform Raman spectroscopy. Most of the applications on oils have been performed by the later [129]. However, a portable Raman spectrometer has been recently developed [130], which, on the other hand, shows lower resolution than classic ones. Zhang et al. [130] reported the first proof-of-concept study of surface-enhanced Raman detection of a TBA-MDA adduct using silver nanoparticles as the SERS substrate

Raman spectroscopy results and oxidation levels were related in lipids extracted from several meat and fish products [128,132]. In line with peroxide values rises, Raman spectra data showed an increase in particular bands and regions of the spectra of oils extracted which could be attributed to alterations in lipids structure. Furthermore, Raman spectroscopy could be an alternative to gas chromatographic fatty acids analysis, since it successfully predicted total unsaturation and individual compounds several meat products [133]. Salmon Raman spectra [134] indicated differences in the fat fraction (as well as in protein fraction) in cold-smoked products. Regarding vegetable oils studies, Muik et al. [135] detected formation of aldehydes and conjugated double bond systems, as well as isomerization of *cis* to *trans* double bonds. The time dependent intensity changes in certain Raman bands were compared to conventional parameters used to determine the extent of oxidation in oils, such as anisidine value and K_{270}, and showed good correlation. El-Abassy et al. [136] assessed fatty acid content in olive oil. Zhang et al. [131] developed a method to determine MDA in a model system by means of this technique. They found that it was selective and specific for MDA-TBA adducts- in terms of differential spectra and high response- versus adducts formed by TBA and other TBARS different from MDA. Besides, they achieved better sensitivity than in works using UV-Vis or fluorescence detectors. Sometimes, reduction of carotenoids content measured by Raman spectroscopy has been used to monitor lipid oxidation process [137].

Simultaneous analysis of the oxidation of edible oils has been also performed by Infrared and Raman techniques [119]. These techniques led to improved information compared to isolated analysis concerning assignment of peaks, and therefore, compounds formed during oxidation.

4.5 Magnetic Resonance

The basis of Nuclear Magnetic Resonance (NMR) relies on the property of certain atoms of absorbing and re-emitting energy in the presence of a strong magnetic field due to the excitation of their atomic nuclei. This energy is at a specific resonance frequency which depends on the strength of the magnetic field and on the magnetic properties of the particular isotope of the atom in study. The energy absorptions of the atomic nuclei are affected by the nuclei of surrounding molecules, which cause small local modifications to the external magnetic field. Promising results are obtained by this alternative methodology considering reliability and specificity of the data since they provide an accurate fingerprint of the sample. It does not require extensive manipulation of the sample, thus preserving molecular integrity, and allowing detection of all the substances present in the sample at the same time. This, in addition to its high sensitivity even in complex matrices, highlights the necessity of improving and spreading its use. However, that is a very expensive methodology and requires special skills to interpret the spectra. The use of ^{1}H and ^{13}C NMR spectroscopy in food, applied by different research groups [138-146], has proved to be very useful in evaluating the oxidative status of the lipid fraction, as well as in providing information on the nature (main functional groups) and concentration of the compounds found (i.e. hydroperoxides, carbonyl compounds and dienes). It is considered a valuable tool for quantification of oxidation of food lipids [147], and good correlation with conventional analysis such as TBA has been reported [148]. Several multi-dimensional NMR techniques have been developed in last years (correlational spectroscopy, nuclear overhauser effect spectroscopy, diffusion-ordered spectroscopy…). They allow a better assignment than the one-dimensional spectra, improving the characterization of food lipid samples [143,149]. However, the main difficulty derived from the application of these tools is the high time required for the acquisition.

The basis of Electron Paramagnetic Resonance (EPR) is the same as that of NMR but in this case, energy excites spins of single electrons. So, only molecules presenting single electrons

(that is, radicals) have EPR spectra. It has been used to detect oxidant intermediate species in food matrices [150,151]. However, these radicals show quite short lives unless very low temperatures are guaranteed [13,151-152]. In an attempt to avoid this problem, some recently developed methodologies deal with the detection of unstable free radicals. Among them, spin trapping techniques allow the indirect detection of lipid-derived radicals by formation of stable spin adducts that can accumulate in detectable concentrations. This way, both identification and quantification of these intermediates is possible.Traps are not radical-specific, nevertheless particular traps are considered more or less useful for trapping particular radicals. Compounds such as PBN (α-phenyl-tert-butylnitrone) and DMPO (5,5-dimethyl-1-pyrroline-N-oxide) are frequently used for that purpose [150,153].

Combined application of both methodologies (NMR and EPR) is of great interest. In this sense, Silvagni et al. [5] used them in a study investigating the kinetics of thermally induced lipid peroxidation of peanut oil. The use of EPR allowed them to determine the primary alkyl radicals, and provided an estimation of the radical generation rate; whereas by means of NMR, simultaneously detection of primary and secondary oxidation products was performed, thus allowing a more detailed kinetic investigation.

5. CONCLUSION

Different kind of compounds can be used as lipid oxidation markers in food samples, among which hydroperoxides and a variety of aldehydes are the most common ones. Each one of them is indicative of a particular state of oxidation, so choosing just one parameter to analyse the oxidative status is rather difficult and it is frequently more convenient to combine different methods. Therefore, analyst must choose carefully the most adequate for his purpose, taking into consideration the most suitable molecules and experimental conditions

required in each case. First general decision is whether determining primary or secondary oxidation compounds, considering mainly the extent of oxidation. Afterwards, precision required and characteristics of the food matrix must be considered to follow one methodology or another. A variety of conventional and alternative methodologies have been developed and implemented. Considering the later, they have been proven to provide interesting and promising results, so attention must be paid to these alternative techniques in the area of food lipid oxidation.

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