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Progress in Lipid Research 97 (2025) 101317

Contents lists available at ScienceDirect

Progress in Lipid Research

journal homepage: www.elsevier.com/locate/plipres

Navigating the complexity of lipid oxidation and antioxidation: A review of evaluation methods and emerging approaches

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ARTICLE INFO

Keywords
Lipid oxidation
Antioxidant
Mechanism
Method
Food

ABSTRACT

Lipid oxidative degradation contributes to the deterioration of food quality and poses potential health risks. A promising approach to counteract this is the use of plant-based antioxidants. However, accurately evaluating the antioxidant capacity and effectiveness of these compounds remains a challenge. While many rapid *in vitro* tests are available, they must be categorized according to their specific responses to avoid overinterpreting results. This review opens with an overview of current knowledge on lipid autoxidation and recent findings that highlight the challenges in measuring antioxidant capacity. We then examine various methods, addressing their limitations in accurately anticipating outcomes in complex compartmentalized lipid systems. The aim is to clarify the gap between predictions and real-world efficacy in final products. Additionally, the review compares the strengths and weaknesses of methods used to evaluate antioxidant capacity and assess oxidation degrees in complex environments, such as those found in food and cosmetics. Finally, new analytical techniques for multiproduct detection are introduced, paving the way for a more 'omic' and spatiotemporally defined approach.

Abbreviations: AAPH, 2,2′-Azobis(2-amidinopropane) dihydro-chloride; $ABTS^{+\bullet}$, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; AH, antioxidant; AIPH, 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride; ALA, α-linolenic acid; AMVN, 2′-azobis(2,4-dimethylvaleronitrile); AOCS, American oil chemists' society; ApoCAT, apolar conjugated autoxidizable triene; ATR, attenuated total reflection; ATR-FTIR, attenuated total reflectance with Fourier-transformed infrared; AV, p-anisidine value; BDE, bond dissociation energy; BHT, butylated hydroxytoluene; $BODIPY^{665/676}$, 4,4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora- 3a,4a-diaza-s-indacene; CAR, carboxen; CAT, conjugated autoxidizable triene; CD, conjugated dienes; CIS4–TDU, thermal desorption–cryofocalization; CLSM, confocal laser scanning microscopy; CMC, critical micellar concentration; cryo-CLEM, cryo-correlative light and electron microscopy; CUPRAC, cupric reducing antioxidant capacity; DEPC, 1,2-α-Eleostearoyl-sn-glycero-3-phosphocholine; DHA, docosahexaenoic acid; DHS, dynamic purge-and-trap headspace; DOSY, diffusion ordered spectroscopy; DPH-PA, diphenylhexatriene propionic acid; $DPPH^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl; DPPP, diphenyl-1-pyrenylphosphine; DPPPO, diphenyl-1-pyrenylphosphine oxide; DVB, divinylbenzene; EDTA, ethylenediaminetetracetic acid; EEM, excitation-emission matrix; EPA, eicosapentaenoic acid; ESI, electrospray Ionization; EPR, electron paramagnetic resonance; Fluor-DHPE, N-(Fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt; FOX, ferrous oxidation-xylenol orange; FRAP, Fe^{3+}-2,4,6-tripyridyl-*S*-triazine or ferric reducing antioxidant power; FT, Fourier-transformed; GC, gas chromatography; GC-FID, gas chromatography-flame ionization detector; HDAF, hexadecanoylaminofluorescein; H_2O_2, hydrogen peroxide; HLB, hydrophilic-lipophilic balance; HPLC, high performance liquid chromatography; $HO^{\bullet}$, hydroxyradicals; HS, headspace; HS-SPDE, dynamic headspace solid phase extraction; HS-SPME, headspace-solid phase microextraction; HSSE, HS-SBSE, high-capacity headspace sorbent extraction; INDEX, dynamic in-needle extraction; IR, Infrared; ITEX, in-tube extraction; IUPAC, international union of pure and applied chemistry; LA, linoleic acid; LH, Lipid; $LO^{\bullet}$, alkoxyradicals; $LOO^{\bullet}$, peroxyradicals; LOHs, hydroxylipids; LOOHs, lipid hydroperoxides; MALDI, matrix Assisted Laser Desorption Ionization; MCT, medium-chain triglycerides; MDA, malondialdehyde; MDMS-SL, multi-dimensional mass spectrometry-based shotgun lipidomics; MeO-AMVN, 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile); MS, mass spectroscopy; NaCl, sodium chloride; NIRS, near infrared spectroscopy; NMR, Nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; ORAC, oxygen radical absorbance; PCA, principal component analysis; PDMS, polydimethylsiloxane; PLSR, partial least squares regression; PLR, partial linear regression; PLS, partial least squares; PLS-DA, partial least squares discriminant analysis; PUFAs, polyunsaturated fatty acids; PV, peroxide value; ROS, reactive oxygen species; RSD, relative standard deviation; SHS, static headspace; SFS, synchronous fluorescence; SIFT-MS, Flow Tube Mass Spectrometry; SOD, superoxide dismutases; TAGs, triacylglycerols; TBA, 2-thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances; TocOH, tocopherol; TPP, triphenylphosphine; TOCSY, total correlation spectroscopy; TOTOX, total oxidation index; TPPO, triphenylphosphine oxide; TRAP, total radical-trapping antioxidant parameter; UV, ultraviolet; VesiCAT, vesicle Conjugated Autoxidizable Triene; VOC, volatile organic compounds.

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https://doi.org/10.1016/j.plipres.2024.101317
Received 23 September 2024; Received in revised form 10 December 2024; Accepted 11 December 2024
Available online 16 December 2024

1. Introduction

Lipid oxidation is a key factor in the deterioration of food quality, nutraceuticals and cosmetics, and can also pose significant health risks. As fats and oils undergo oxidation, they contribute to rancidity, loss of nutritional value, and the formation of potentially harmful compounds [1,2].

To address these issues, antioxidants are commonly added during product formulation. However, the range of authorized antioxidants available for industrial use is limited, and the approval process for new ones can be lengthy. Moreover, concerns about the potential health risks of synthetic antioxidants have led consumers to increasingly favor natural alternatives. Plant-based antioxidants have emerged as a promising solution, as these natural compounds can effectively scavenge free radicals, chelate pro-oxidant metals, and quench singlet oxygen, thereby slowing down oxidation. Despite their potential, accurately assessing the antioxidant capacity and effectiveness of these compounds in different systems remains a complex challenge.

Current methods for evaluating antioxidant capacity predominantly rely on rapid *in vitro* tests. While these tests offer valuable insights, they need to be carefully interpreted based on their specific responses to avoid misinterpretation. This review explores the complexities of lipid autoxidation and the challenges in evaluating antioxidant effectiveness. It begins by summarizing foundational knowledge on lipid oxidation and recent developments.

We then examine the limitations of current evaluation methods, particularly their difficulties in predicting outcomes in complex, nanostructured, and dynamic lipid systems. The goal is to bridge the gap between *in vitro* predictions and actual effectiveness in real-world products, including those found in food and cosmetics. By comparing the strengths and weaknesses of current evaluation techniques, this review provides a comprehensive overview of how antioxidant capacity and oxidation degree are assessed.

Finally, emerging analytical techniques offering multi-product detection capabilities are introduced. These advancements represent a shift towards more integrated, spatially defined, and dynamic analytical approaches, paving the way for more accurate and effective evaluation of antioxidant performance across diverse lipid-based systems.

2. A modern portrait of lipid autoxidation

Various mechanisms have been proposed to explain lipid oxidation in diverse systems, including consumer products, biological samples, and living organisms. The three main mechanisms are autoxidation, photosensitized oxidation and enzymatic oxidation. In the case of lipoxygenase-catalyzed oxidation of unsaturated lipids, which is the primary enzymatic oxidation route, the initial step clearly involves a radical mechanism with hydrogen abstraction, similar to typical initiation mechanisms. However, unlike autoxidation with standard free radical chain reactions, there is no propagation phase. This is because the peroxyradicals, $LOO^{\bullet}$, formed by lipoxygenases are reduced to LOO^{-} species. Likewise, in one type of photosensitized oxidation (mediated by type II photosensitizers), there is no radical mechanism at all. The distribution of hydroperoxides produced from oleate, linoleate and linolenate in the presence of singlet oxygen is thus very different from that obtained by autoxidation [3]. In this review, we focus solely on free radical chain autoxidation, which follows a different mechanism compared to the other two types of lipid oxidation. The structure of the present chapter follows our contemporary view of autoxidation which is largely inherited from its chemical description as a sequence of three reaction steps: *initiation*, *propagation*, and *termination*.

2.1. Initiation

In this section, two types of initiation reactions are described whether or not they depend on hydroperoxides.

2.1.1. Hydroperoxide-independent initiation

In the context of lipid oxidation, initiation refers to the production of lipid radicals from non-radical lipids. In simpler terms, this is the formation of lipid free radicals. Even though there is no large consensus, this reaction has generally been described as the production of a carbon-centered radical $L^{\bullet}$, hereinafter referred to as alkyl radical [4]. Unsaturated lipids which are prone to losing a hydrogen atom in the presence of heat, UV light, or catalysts such as inorganic free radicals can thus theoretically produce an alkyl radical (**Re. 1**).

$$LH \xrightarrow{catalyst} L^{\bullet} + H^{\bullet} \quad \text{(Re. 1)}$$

The loss of hydrogen atom by homolytic cleavage takes place most readily at the carbon site where the energetic cost associated to this hydrogen abstraction (dissociation energy) is the lowest. By decreasing order of dissociation energy for free radical formation, bis-allylic hydrogens (65 kcal/mol) covalently attached in α position of two double bonds come first, then followed by allylic hydrogens (77–85 kcal/mol) attached in α position of only one double bond, and finally alkylic hydrogens (100 kcal/mol). Bis-allylic hydrogens thus constitute the thermodynamically preferred targets for initiating oxidation, while alkylic hydrogens are not considered available for this type of reaction (Fig. 1a).

The reason why bis-allylic hydrogen atoms are more labile than mono-allylic hydrogens is because a pentadienyl radical is roughly 10–20 kcal/mol more stable than an allyl radical due to a much larger area of electron delocalization (Fig. 1b). The same thermodynamic reasoning applies to alkylic hydrogens: the resulting radical would not be able to delocalize the unpaired electron. Accordingly, the energetic cost associated to the abstraction of hydrogen decreases with unsaturation, all other things being equal.

As we will discuss further, **Re. 1** is likely not the predominant initiation mechanism in lipid autoxidation driven by natural processes, where the oxidation is not accelerated by artificial means. One common artificial method used for controlled initiation is the addition of an azo-initiator (R-N=N-R) to the lipid-based system. This compound generates $L^{\bullet}$ radicals through **Re. 2**, catalyzed by moderate heat (30–50 °C). Note that **Re. 2** is virtually similar to **Re. 1** as both yield carbon-centered radicals. The only difference is that azo-initiator derived radicals are not necessarily of lipid nature, hence they are denoted $R^{\bullet}$ instead of $L^{\bullet}$.

$$R-N=N-R \xrightarrow{heat} 2\,R^{\bullet} + N_2 \quad \text{(Re. 2)}$$

Azo-initiators can be classified according to their hydrophilic, lipophilic or amphiphilic nature. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride (AIPH) are the most hydrosoluble commercial azo-initiators, while 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) and its methoxyl derivative 2,2′-azobis(4-methoxy-2,4-dimethylvaleronitrile) (MeO-AMVN) are the most widely used oil-soluble azo-initiators.

2.1.2. Hydroperoxide-dependent initiation

Another set of reactions that can generate lipid radicals from non-radical species involves the decomposition of lipid hydroperoxides (LOOHs). Although LOOHs are the first primary stable products resulting from lipid oxidation, they are non-radical lipids, meaning that they can be considered a separate entity. Depending on the environment, they may initiate further oxidation mechanisms after decomposition leading to the formation of oxygen-centered radicals such as peroxyradicals ($LOO^{\bullet}$), alkoxyradicals ($LO^{\bullet}$) and/or hydroxyradicals ($HO^{\bullet}$) through four main different types of reactions: metal catalyzed-oxidation (**Re. 3**), metal-catalyzed reduction (**Re. 4**), heat or UV light-induced scission (**Re. 5**), and bimolecular decomposition (**Re. 6**). For metal-catalyzed reactions, iron cations are used as examples. Note that potential reactions with metallo-enzymes such as peroxidases, cytochromes and lipoxygenases largely proceed as described for iron.

$$LOOH + Fe^{3+} \rightarrow LOO^{\bullet} + H^{+} + Fe^{2+}\ \text{(Slow)} \quad \text{(Re. 3)}$$

$$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+} \ (Fast) \quad \text{(Re. 4)}$$

$$LOOH \xrightarrow{Heat, UV\ light} LO^{\bullet} + {}^{\bullet}OH \quad \text{(Re. 5)}$$

$$LOOH + LOOH \rightarrow LOO - H \ldots OOH \ (H-\text{bonded dimer}) \rightarrow LOO^{\bullet} + LO^{\bullet} + H_2O \quad \text{(Re. 6)}$$

Here, we assume that the decomposition of "pre-existing" LOOHs by any of the four above-mentioned reactions is the dominant initiation mechanism in autoxidation. Clearly, lipids, whether purchased from commercial sources or extracted from biological samples, are never entirely free of LOOHs. These compounds can be formed through photo- or enzymatic oxidation in vivo or can be generated by lipid autoxidation during handling or extraction. For instance, the purchase of high-purity (99.9 %) fatty acids is no safeguard, since purity refers to contamination with other fatty acids, not to hydroperoxide content [5]. In lipids containing hydroperoxides, the contribution of Re. 1 to initiation is quickly overshadowed by that of lipid hydroperoxide decomposition, especially in the presence of metal cations (Re. 3 and 4) or when heat and light are present (Re. 5). This statement is not new. One of the earliest mentions of this idea dates back over 50 years ago, when Ingold [4] observed that the heat-induced decomposition of LOOHs (Re. 5) becomes the predominant mode of initiation in the presence of even minute traces of hydroperoxides compared to heat-induced homolytic cleavage of LH (Re. 1). Even more significant is the fact that, historically, the first hypothesized initiation mechanism in lipid autoxidation was a hydroperoxide-dependent initiation [6]. Thus, the description of this type of initiation is as old as the proposition by Bolland that lipid autoxidation proceeds by a general scheme of initiation, propagation and termination. Additionally, measuring the oxygen consumption in a lipid dispersion of methyl linoleate oxidized by copper at 37 °C, it has been observed that appreciable oxidation does not take place if the lipid is completely free from contaminated hydroperoxides [7].

2.2. Propagation

Propagation is generally described as a fast addition of $^3O_2$ on the carbon-centered lipid radical, $L^{\bullet}$, that results from the hydroperoxide-independent initiation (Re. 1) to form a peroxyradical (Re. 7), followed by a slow hydrogen abstraction from a vicinal unsaturated lipid, L'H, by the formed peroxyradical (Re. 8).

$$L^{\bullet} + {}^3O_2 \rightarrow LOO^{\bullet} \ (\text{fast}, 10^9\ M^{-1}\ s^{-1}) \quad \text{(Re. 7)}$$

$$LOO^{\bullet} + L'H \rightarrow LOOH + L'^{\bullet} \ (\text{slow} < 10^3\ M^{-1}\ s^{-1}) \quad \text{(Re. 8)}$$

However, as discussed above, in the presence of trace amounts of LOOHs and/or in the absence of artificial azo-initiators, hydroperoxide-dependent initiation becomes the dominant mechanism. This process primarily produces peroxy- and alkoxyradicals, which are two types of oxygen-centered free radicals. Under these conditions, the oxygen addition reaction (i.e., the formation of the first H-abstracting radical) is no longer necessary, and the system progresses directly to the propagation step [8].

2.2.1. Peroxyradicals ($LOO^{\bullet}$) as chain carrier radicals

A pivotal event in autoxidation is the formation of a peroxyradical ($LOO^{\bullet}$). It can be formed from either (i) a hydroperoxide-independent initiation mechanism (Re. 1 followed by Re. 7) or (ii) a hydroperoxide-dependent initiation directly from Re. 3 or Re. 6. Whatever its source, $LOO^{\bullet}$ can then abstract a hydrogen atom from an unsaturated lipid molecule (L'H) to form LOOH and a carbon-centered lipid radical $L'^{\bullet}$ (Re. 8). $L'^{\bullet}$ has a very short life span (10^{-9} s) [9]. In presence of oxygen, it quickly reacts with triplet oxygen ($^3O_2$) to generate a new lipid peroxyradical (recapitulating Re. 7), thus replenishing the H atom transfer reaction with its reactive free radical. In this pathway, $LOO^{\bullet}$ plays the role of a chain carrier or a propagator: several $LOO^{\bullet}$ carry the propagation chain one H atom transfer at a time from the initiation point to the termination. This self-sustained radical chain reaction propagates indefinitely until no H donor (e.g. unsaturated lipid) is available or the chain is terminated in presence of a chain breaking antioxidant for example [10].

2.2.2. Alkoxyradicals ($LO^{\bullet}$): Second chain carrier radicals?

Alkoxyradicals can only be formed by a hydroperoxyde-dependent initiation reaction (Re. 4, 5 or 6). The implication of these radicals in the propagation step by hydrogen abstraction on an unsaturated lipid is still a matter of debate. Indeed, if alkoxyradicals (as well as peroxyradicals) were involved in hydrogen abstraction, it would logically lead to a concomitant accumulation of LOOHs (Re. 8) and LOHs hydroxylipids (Re. 9).

$$LO^{\bullet} + L'H \rightarrow LOH + L'^{\bullet} \quad \text{(Re. 9)}$$

But, as Schaich noted [3,10] and others have confirmed experimentally [11], LOHs are surprisingly present in very small proportions in oxidized lipids relative to LOOHs. At first sight, this suggests either that (i) LOHs are intermediates quickly converted into hitherto undetected molecules (very unlikely hypothesis), or that (ii) $LO^{\bullet}$ are

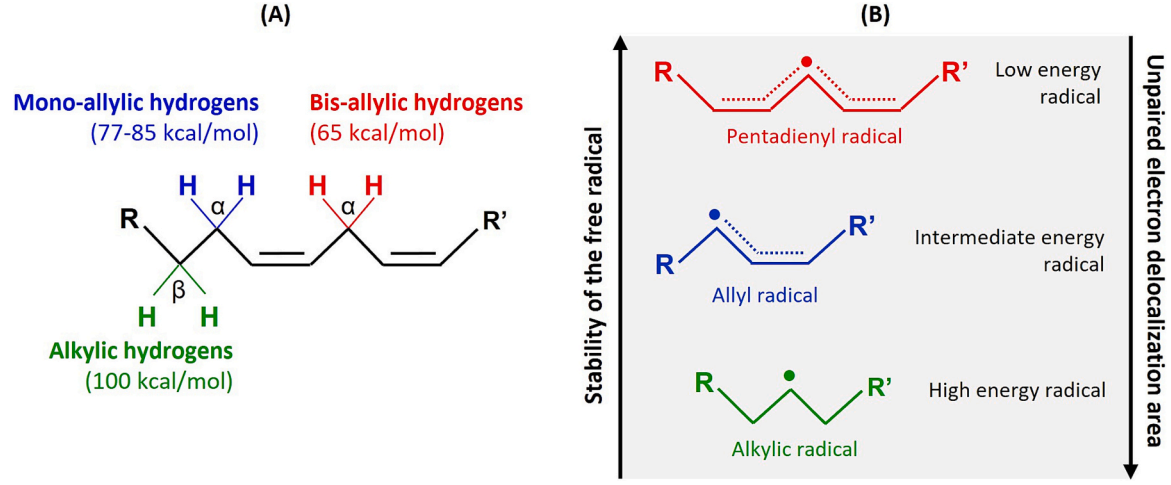

Fig. 1. Dissociation energy of the three main types of hydrogen atoms in unsaturated fatty acyl chain of methylene-interrupted acyclic lipids (a). Structures of the resulting free radicals (b).

somewhat converted into another radical before they abstract a hydrogen from a lipid. Regarding this matter, Gardner [12] suggested that « contrary to popular belief », LO• do not significantly abstract hydrogens, but rather are channeled into epoxide formation through intramolecular cyclization, a reaction proposed more than 60 years ago [13]. Epoxyallylic radical can thus be formed by cyclization of LO• to the alpha-unsaturation (Fig. 2). Epoxyallylic radicals are more stable than alkoxyradicals and can directly abstract a hydrogen atom from an unsaturated lipid to form an epoxide (first pathway, Fig. 2). Alternatively, the epoxyallylic radicals can bind to oxygen to form an epoxy-peroxyl radical (second pathway, Fig. 2). This second pathway has recently been shown to be dominant in epoxide formation from a linoleate model in bulk oils and emulsions [11]. The resulting peroxyradicals with a neighboring epoxide group (epoxy-peroxyl radicals) are among the most stable oxygen-centered radicals and are highly selective for hydrogen abstraction [14]. Thus, short-lived LO• that are the initial products of hydroperoxide decomposition through **Re. 4, 5 or 6** are converted into long-lived epoxy-peroxyl radicals that are efficient propagators of lipid autoxidation. In both pathways, the LO• is deviated from directly abstracting hydrogen atom by an internal cyclization, which could explain the low amounts of hydroxylipids (LOH) generally found during lipid autoxidation. In bulk oils and emulsions, in contrast, epoxides contribute up to 10–40 % of the total oxygenated products resulting from lipid autoxidation [3,11]. Finally, if the product of the first pathway (epoxide) is relatively stable, it is not the case of the epoxy-hydroperoxide formed at the end of the second pathway. This latter can be decomposed by any of the **Re. 3 to 6**, thus initiating a new propagation chain.

2.3. Termination

The termination step is generally less extensively described than the two preceding steps. According to IUPAC, termination may be defined as any chemical reaction in which a chain carrier is converted irreversibly into a *non-propagating species*, without the formation of a new chain carrier [15].

2.3.1. Radical recombinations

Termination is often viewed as a set of reactions called *radical recombinations* (or *self-recombination* by Ingold, 1961) in which radicals formed from oxidized lipids (L•, LO•, LOO•, etc.) react to eventually form nonradical compounds (**Re. 10–15**). Radicals can recombine in limitless combinations through homo- or hetero-couplings to generate a broad range of oxidation products such as alkanes, peroxides, alcohols, ketones, ethers and alkane polymers. As one radical propagates one chain, radical/radical recombination leads to the termination of two chains.

$$L^{\bullet} + L^{\bullet} \rightarrow LL\ (\text{homo} - \text{coupling}) \qquad \text{(Re. 10)}$$

$$LO^{\bullet} + LO^{\bullet} \rightarrow LOOL\ (\text{homo} - \text{coupling}) \qquad \text{(Re. 11)}$$

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOO - OOL\ (\text{tetraoxide})\ (\text{homo} - \text{coupling}) \qquad \text{(Re. 12)}$$

$$L^{\bullet} + HO^{\bullet} \rightarrow LOH\ (\text{alcohol})\ (\text{hetero} - \text{coupling}) \qquad \text{(Re. 13)}$$

$$LO^{\bullet} + L^{\bullet} \rightarrow LOL\ (\text{hetero} - \text{coupling}) \qquad \text{(Re. 14)}$$

$$LOO^{\bullet} + L^{\bullet} \rightarrow LOOL\ (\text{hetero} - \text{coupling}) \qquad \text{(Re. 15)}$$

Radical recombinations logically lead to the formation of homo/heterodimers of relatively high molecular weight. However, this termination mode can also give rise to small volatile molecules. The simplest example is the recombination of a small lipid radical (L•) with a hydroxyradical (HO•) to form a volatile alcohol (**Re. 13**, Table 1) or the corresponding aldehyde (Table 1). Additionally, dimers are not always stable products. For instance, while studying the kinetics of ethylbenzene autoxidation, Russell [16] observed peroxyradical homocoupling (LOO• + LOO•, **Re. 12**) followed by decomposition of the resulting tetraoxide intermediate (LOO-OOL) into a molecule each of alcohol, ketones and O_2 (Fig. 3). To conserve spin, cleavage of the tetraoxide intermediate must afford singlet oxygen ($^1O_2$) directly [17] or indirectly through exchange of ground state oxygen with excited triplet carbonyl [18] (Fig. 3).

2.3.2. Alkoxyradical scissions

The second termination type is called alkoxyradical scission. Through this mechanism, LO• undergo scission of the C—C bond on either side (α or β) of the alkoxyl group to yield a mixture of aldehydes final products which are termination products and alkyl free radicals (L•) which are not (yet) termination products [10] (Fig. 4). Indeed, these alkyl radicals can further abstract a hydrogen atom (H atom transfer) from a lipid to form the corresponding alkane and propagate the chain. A second route offered to these unstable alkyl radicals is the recombination with other radicals, especially the hydroxyradical (**Re. 13**), to give the corresponding alcohols or aldehydes (after keto-enolic tautomeric rearrangement in some cases, Table 1). In this example, alkoxyradical scissions can be seen as a multi-cascade process giving rise to many termination products on one side but leaking many unstable radicals (nontermination products) on the other side, these latest being possibly terminated by radical recombination; both types of termination working in sequence. Consequently, one might expect a very complex product mix of alkanes, alcohols, aldehydes, oxo-esters, and ketones in oxidized lipids.

Pioneering authors [19] have considered that major secondary oxidation products result from alkoxyradical scissions rather than from recombinations [10]. Therefore, hexanal is one of the most frequently monitored secondary oxidation products in lipid autoxidation studies. Yet, the structure and relative proportions of secondary oxidation products largely depend on the structure and proportions of the

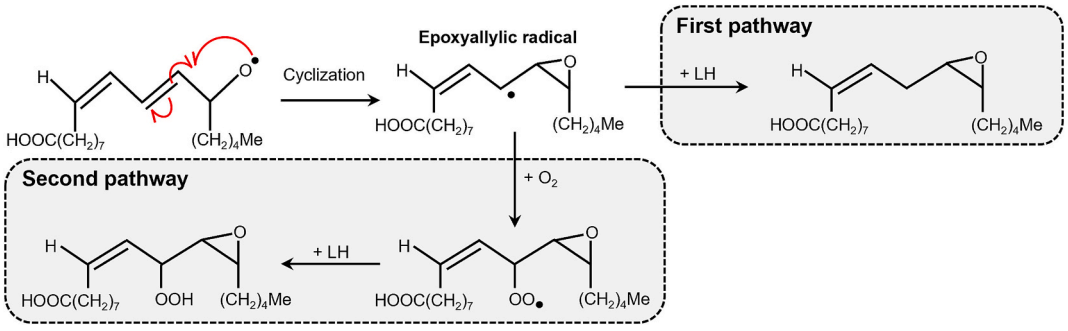

Fig. 2. Formation of an epoxide (first pathway) or an epoxy-hydroperoxide (second pathway) from a transient epoxyallylic radical derived from an initial linoleate alkoxyradical.

Table 1
114 compounds formed through homolytic scission of hydroperoxide-derived alkoxyradicals in oleate (Ol), linoleate (La), linolenate (α and γ; Lna), arachidonate (ARA), eicosapentaenoate (EPA) and docosahexaenoate (DHA) models. Structures are tentatively provided within this work. In bold, α-scission-derived aldehydes which are often measured as oxidation markers presents in the volatile fraction. *does not consider the possibility of double bond migration due to the presence of metals or radicals.

Fatty acyl group	LOOHs	α-Scission	β-Scission	
			Recombination with HO•	*H atom transfer*
Oleate	8-LOOH	Undec-2-enal	Decanal	Dec-1-ene
(18:1 n-9)	9-LOOH	Dec-2-enal	Nonanal	Non-1-ene
	10-LOOH	**Nonanal**	Octanol	Octane
	11-LOOH	Octanal	Heptanol	Heptane
Linoleate	9-LOOH	**Deca-2,4-dienal**	Non-3-enal*	Nona-1,3-diene
(18:2 n-6)	13-LOOH	**Hexanal**	Pentanol	Pentane
γ-Linolenate	6-LOOH	Trideca-2,4,7-trienal	Dodeca-3,6-dienal*	Dodeca-1,3,6-triene
(18:3 n-6)	9-LOOH	**Deca-2,4-dienal**	Non-3-enal*	Nona-1,3-diene
	10-LOOH	Non-3-enal	Oct-2-ene-1-ol	Oct-2-ene
	13-LOOH	**Hexanal**	Pentanol	Pentane
Arachidonate	5-LOOH	Hexadeca-2,4,7,10-tetraenal	Pentadeca-3,6,9-trienal*	Pentadeca-1,3,6,9-tetraene
(20:4 n-6)	8-LOOH	Trideca-2,4,7-trienal	Dodeca-3,6-dienal*	Dodeca-1,3,6-triene
	9-LOOH	Dodeca-3,6-dienal	Undeca-2,5-dienol	Undeca-2,5-diene
	11-LOOH	**Deca-2,4-dienal**	Non-3-enal*	Nona-1,3-diene
	12-LOOH	Non-3-enal	Oct-2-ene-1-ol	Oct-2-ene
	15-LOOH	**Hexanal**	Pentanol	Pentane
α-Linolenate	9-LOOH	Deca-2,4,7-trienal	Nona-3,6-dienal*	Nona-1,3,6-triene
(18:3 n-3)	12-LOOH	**Hepta-2,4-dienal**	Hex-3-enal*	Hexa-1-3-diene
	13-LOOH	Hex-3-enal	Pent-2-enol*	Pent-2-ene
	16-LOOH	**Propanal**	Ethanol	Ethane
Eicosapentaenoate	5-LOOH	Hexadeca-2,4,7,10,13-pentaenal	Pentadeca-3,6,9,12-tetraenal*	Pentadeca-1,3,6,9,12-pentaene
(20:5 n-3)	8-LOOH	Trideca-2,4,7,10-tetraenal	Dodeca-3,6,9-trienal*	Dodeca-1,3,6,9-tetraene
	9-LOOH	Dodeca-3,6,9-trienal	Undeca-2,5,8-triene-1-ol*	Undeca-2,5,8-triene
	11-LOOH	Deca-2,4,7-trienal	Nona-3,6-dienal*	Nona-1,3,6-triene
	12-LOOH	Nona-3,6-dienal	Octa-2,5-diene-1-ol*	Octa-2,5-diene
	14-LOOH	**Hepta-2,4-dienal**	Hex-3-enal*	Hexa-1-3-diene
	15-LOOH	Hex-3-enal	Pent-2-enol*	Pent-2-ene
	18-LOOH	**Propanal**	Ethanol	Ethane
Docosahexaenoate	4-LOOH	Nonadeca-2,4,7,10,13,16-pentaenal	Octadeca-3,6,9,12,15-pentaenal*	Octadeca-1,3,6,9,12,15-hexaene
(22:6 n-3)	7-LOOH	Hexadeca-2,4,7,10,13-pentaenal	Pentadeca-3,6,9,12-tetraenal*	Pentadeca-1,3,6,9,12-pentaene
	8-LOOH	Pentadeca-3,6,9,12-tetraenal	Tetradeca-2,5,8,11-tetraene-1-ol*	Tetradeca-2,5,8,11-tetraene
	10-LOOH	Trideca-2,4,7,10-tetraenal	Dodeca-3,6,9-trienal*	Dodeca-1,3,6,9-tetraene
	11-LOOH	Dodeca-3,6,9-trienal	Undeca-2,5,8-triene-1-ol*	Undeca-2,5,8-triene
	13-LOOH	Deca-2,4,7-trienal	Nona-3,6-dienal*	Nona-1,3,6-triene
	14-LOOH	Nona-3,6-dienal	Octa-2,5-diene-1-ol*	Octa-2,5-diene
	16-LOOH	**Hepta-2,4-dienal**	Hex-3-enal*	Hexa-1-3-diene
	17-LOOH	Hex-3-enal	Pent-2-enol*	Pent-2-ene
	20-LOOH	**Propanal**	Ethanol	Ethane

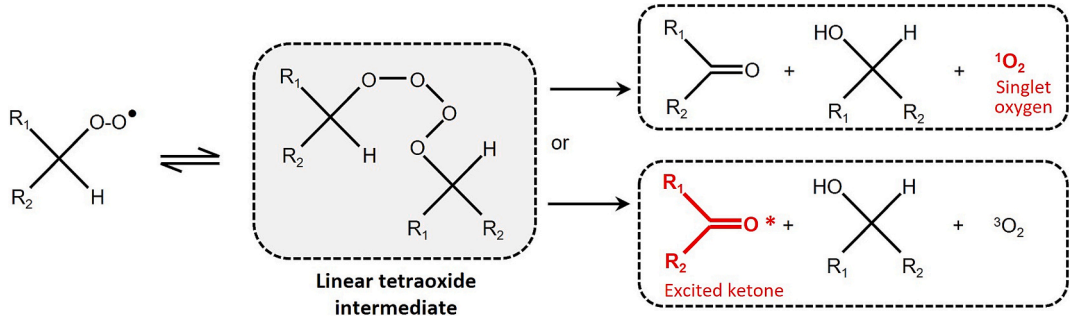

Fig. 3. Proposed Russell mechanism for the self-reaction of peroxyradicals involving a cyclic mechanism from a linear tetraoxide intermediate.

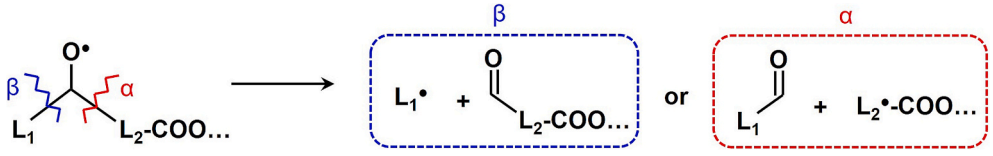

Fig. 4. The general scheme of α- or β-scissions (C—C cleavages) of alkoxyradicals. α-Scissions are involved on the bond closer to the carboxylate function, while β-scission referred to cleavage of the bond closer to the terminal methyl group.

dominant LOOHs (and their corresponding alkoxyradicals) formed from the various fatty acyl chains present in oils, fats, and biological samples. Obviously, the type of secondary oxidation products also depends on the type of scission and subsequent radical recombination. Table 1 reports the main α- and β-scission products obtained from most unsaturated fatty acyl chains. First, a redundancy can be seen in scission products between fatty acyl chains belonging to the same family (n-6 or n-3). Considering only α-scission products, hexanal and deca-2,4-dienal are virtually present in all n-6 fatty acyl chains (linoleate, γ-linolenate and arachidonate), while propanal, hex-3-enal, deca-2,4,7-trienal and hepta-2,4-dienal are virtually in all n-3 chains (α-linolenate, eicosapentaenoate and docosahexaenoate). Since the former aldehydes can be find in the n-6 family but not in the n-3 and vice versa for the latter aldehydes, they are used as secondary oxidation markers of each family. We can extend this to nonanal, dec-2-enal and undec-2-enal for the n-9 family, solely represented in this table by the oleate model.

To complicate matters further, most scission-products still contain abstractable hydrogens atoms of a bis- or monoallylic nature. They can thus be considered as oxidizable substrates (L'H) able to react with $LOO^{\bullet}$ through **Re. 8** to yield a radical, that can ultimately form a novel scission product. In case all bis- and monoallylic hydrogen atoms have been abstracted, scission product can still react through radical addition (not covered in this review) when they contain conjugated double bonds. In other word, most scission products presented in Table 1 are prone to subsequent and possibly multiple oxidation(s). Importantly, almost all scission products in a series can be obtained through hydrogen abstraction occurring on the longest α-scission product. The rare remaining scission products that cannot be "recapitulated" by H abstractions can be obtained by radical addition on conjugated double bonds. For example, in the DHA series, all the 27 α- and β-scission products mentioned in the table can be generated from a "parent" aldehyde, nonadeca-2,4,7,10,13,16-pentaenal, the highest α-scission homologue of the series. The same holds true for the oleate, linoleate, linolenate (α and γ), arachidonate and eicosapentaenoate series. Therefore, Table 1 shows that for each fatty acyl chain, there is a series of scission products which are structurally related. Exploring this chemical landscape and establishing formal intra- and inter-series relationship rules for scission products could help us gain a systemic understanding of the molecular diversity resulting from lipid autoxidation.

From an organoleptic standpoint, while lipid hydroperoxides are odorless and tasteless, their decomposition products through scission reactions are responsible for rancidity of lipid-containing foods. Generally, aliphatic carbonyl compounds such as alkanals, *trans*,*trans*-2,4-alkadienals, isolated alkadienals, isolated *cis*-alkenals, *trans*,*cis*-2,4-alkadienals, and vinyl ketones have the lowest threshold values [20].

A final consideration should be made on the fact that naturally occurring lipids are often constituted of triacylglycerols. While most mechanistic studies have been performed on free fatty acids or their methyl esters, these model lipids only represent a minor fraction of the lipids encountered in foods and biological media. As such, one might expect that radical scissions eventually create small volatile molecules, but also glycerol-bound compounds (often referred to as *core aldehydes* because most of these compounds are aldehydes) corresponding to the other part of the cleaved molecule. These glycerol-bound scission products consist in triacylglycerols containing short-chain acyl groups. While most of the scission products resulting from the terminal methyl fragment are relatively small and volatile, imparting odors and flavors to oxidized food lipids, glycerol-bound scission products are nonvolatile and consequently accumulate in the lipid phase.

2.3.3. Co-oxidation of non-propagating species

In addition to recombinations and scissions, the termination also includes a third type of reactions here called *co-oxidation of non-propagating species*, that allows the transfer of the radical state of the propagating lipids to non-propagating molecules, which are of lipidic (e.g. tocopherols and other chain-breaking antioxidants) or other (e.g. proteins, starch, etc.) nature. In this case, the termination merely transfers the radicals of one class of compounds (lipid-derived radicals) to another that is unable to propagate lipid oxidation, thereby irreversibly terminating a propagation chain in accordance with the IUPAC definition [15], without producing a non-radical product by itself.

Perhaps the most remarkable co-oxidations involve proteins nearby lipid molecules. This is the case for oil-in-water emulsions, in which the proteins can serve as natural emulsifiers to stabilize the oil droplets. It can also be seen in low-density lipoproteins, in which a lipoprotein is anchored in a lipid layer. Since side-chain amino and thiol groups contains available abstractable hydrogens, then histidine, lysine, arginine, and cysteine appear as prime targets for H transfer from proteins to lipid radicals [21,22].

Aside proteins, one could mention the *co*-oxidation of certain antioxidants called *chain-breakers* which are chain terminators such as α-tocopherol or many polyphenols bearing a catechol or a pyrogallol moiety [23]. Through this termination mode called *chain-breaking termination* by Ingold (1961), an antioxidant (AH) donates a H atom to the propagating $LOO^{\bullet}$ (**Re. 16**). In doing so, they are even more effective in counteracting lipid oxidation because the radical formed on the oxidized antioxidant ($A^{\bullet}$) is stable. Otherwise, the antioxidant-derived free radical could abstract a bis-allylic hydrogen on any unsaturated lipid and propagate the chain instead of terminating it.

$$LOO^{\bullet}\,(\text{propagating radical}) + AH \rightarrow LOOH + A^{\bullet}\,(\text{non-propagating radical}) \quad \text{(Re. 16)}$$

Finally, a common pitfall when trying to understand the termination step is the erroneous conclusion that this set of reactions stops the overall oxidation process. Therefore, [10] described it as an almost "misnomer". Another misunderstanding—which is somewhat related to the first one—is to consider that when the termination begins, the myriad of paralleled propagation chains is immediately blocked. Termination consists in converting a lipid radical to a nonradical product by scissions and/or recombinations or in transferring the radical state to non-propagating species. In fact, net oxidation slows down when the termination process exceeds the rate of new chain production, which does not mean that oxidation course starts to plateau or decline when the termination step begins. Termination reactions do not occur suddenly at the end of the oxidation process. It is not so much a sequence as a parallelized process. Termination occurs from the initiation point when the very first free radicals are generated. What changes significantly during lipid oxidation is the rate ratio. If the propagation rate decreases or the termination rate increases, or both simultaneously, the net oxidation slows down: more propagation chains are terminated than initiated and propagated.

Noteworthy, the three above-described reaction sets of termination do not occur in an all-or-nothing mode. They are in equilibrium with each other. The reaction set that dominates the termination step is influenced by the nature and concentration of the radicals, the temperature and oxygen pressure, as well as the viscosity, polarity and proticity of the medium [3,8,10].

2.4. Influential factors beyond thermodynamic considerations: The case of physical structures and interfaces

As discussed in section 2.1.1, the energetic cost of hydrogen abstraction decreases with increasing unsaturation, reaching its lowest for bis-allylic hydrogens. From this, we can infer the thermodynamic rule that lipids with lower degrees of unsaturation have higher oxidative stability, a relationship which is often quasi-exponential. In the specific example of triacylglycerols, trilinolein (TAG C18:2, LLL) is significantly more stable to oxidation in its bulk oil format or in solution in an organic solvent than trilinolenin (TAG C18:3, LnLnLn), as well as 1,3-dieicosapentaenoyl-2-palmitoyl glycerol (EEP or EPE) compared to trieicosapentaenoyl (EEE). However, this vulnerability to oxidation of fatty acids

can be strongly challenged when they are dispersed in micellar, liposomal, or emulsified systems; in other words, when they are assembled or structured in a dispersive phase (e.g., water). For instance, in these colloidal systems, free fatty acids may exhibit an opposite trend in oxidation with increasing oxidative stability with the degree of unsaturation [24,25]. Highly sensitive polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA, C22:6) or eicosapentaenoic acid (EPA, C20:5) even demonstrate surprising stability in emulsified systems. Here, a possible explanation lies in physical phenomena. The unsaturation system of PUFAs tends to be positioned near the water interface in lipid dispersions, such as emulsions or liposomal systems. Accordingly, the presence of these water molecules would inhibit or slow down hydrogen abstraction at their bis-allylic positions. The significant variation in the oxidation of fatty acids depending on whether they are in their free form or as TAGs is further evidence of factors beyond the thermodynamic ones discussed in previous sections that influence the oxidation of unsaturated lipids. This effect can probably be explained by the difference in surface activity, with free fatty acids (and even more so their hydroperoxide derivatives) being much more surface-active than their parent methylated fatty acids or triacylglycerols [26]. This characteristic renders free fatty acids highly active in oxidation pathways at multiple levels:

(i) By being more sensitive to oxidation as they can interact with transition metals in the surrounding aqueous phase, causing their decomposition into free radicals that propagate the oxidation reaction.
(ii) By acting as pro-oxidant agents due to their ability to influence the surface charge of lipid droplets (negatively charged when pH $>$ pKa) by attracting pro-oxidant metals.
(iii) By influencing the physical properties of lipid assemblies, or co-assemblies (lipids - surfactants).

It has been demonstrated that adding a small amount of oleic acid (~0.1 %) to a water-in-oil (W/O) emulsion increases the formation of hydroperoxides and volatile compounds, with the rate of oxidation correlated to the concentration of added free fatty acid [27]. This ability to promote oxidation decreases after lowering the pH and is nullified with the acylation of the fatty acid (methyl oleate). Furthermore, to confirm what was stated earlier, the geometry of the fatty acid (more than its chemical reactivity) is important since an increase in the degree of unsaturation of free fatty acids may decrease their ability to promote oxidation, as observed is emulsion systems [25]. The importance of transition metals in this reaction scheme is demonstrated by the ability of metal ion chelators to strongly inhibit oxidation in W/O emulsions [28]. Even though exceptions may be observed (e.g., inhibition of metal pro-oxidizing activity by binding with surface proteins [29]), generally, factors that promote the presence of metals at the interface increase oxidation rates (e.g., negatively charged emulsifiers). Conversely, factors that remove metals from the interface (e.g., positively charged emulsifiers or metal chelators in the aqueous phase) decrease oxidation rates.

Also, when lipids are dispersed as small particles in food products, there is a significant increase in the lipid interface (e.g., lipid-water interface). The specific surface area (As, surface per unit mass) of a spherical particle is related to its size by the following equation: $As = 6/\rho d$, where ρ is the particle density and d is its diameter. This expression shows that the specific surface area is inversely proportional to the particle size. The size of lipid particles in many food dispersions can be very small (less than one micron), greatly increasing their interfacial surface area. The dispersion of lipids in the aqueous phase makes them more vulnerable and sensitive to oxidation, most likely due to the increase in this interfacial surface area [30]. It can then be assumed that reducing the size of lipid particles in foods could accelerate lipid oxidation since smaller size results in larger surface area and shorter distance between the center and the interface, requiring less time for LOOH to move and accumulate at the interfaces. However, studies on the correlations between lipid droplet size and oxidative stability of the system have produced very mixed results. Some studies observe almost no effect of lipid particle size [31], while others conclude a negative effect of size reduction on oxidation [32–35] and yet others report a positive effect [36–38]. This disparity in interpreting the results once again highlights the impact of the nano- and microstructures of colloidal systems on oxidation, which can be affected by numerous parameters. For example, the composition of oils can lead to differences in the conformation of triacylglycerols (TAG) at the oil-water interface, which could explain why the oxidative stability of fish oil increases with decreasing droplet size while the opposite effect was demonstrated for soybean oil emulsions [39]. Furthermore, the lipid composition in the oil should have an influence on oxidation depending on whether we consider the early stage of progression (lag phase) or the exponential phase; a parameter often underestimated but which could explain, besides the physical conformation of the interface, the effect of controversial results related to droplet size.

The processing method can also strongly influence the interpretation of results. For instance, the energy level for preparing emulsions of different droplet sizes could modify the physicochemical properties of the system, as observed with milk protein exchanges between the continuous phase (water) and the interfacial layer [40]. Moreover, very often, preparing emulsions with the smallest size requires a higher energy input, which may be important for decomposing pre-existing LOOHs at "time 0" of the experimental kinetics, thus accumulating even more LOOHs and favoring the *"hydroperoxide-dependent initiation"*. Finally, most of these studies have used polydisperse emulsions, which contain both small and large lipid droplets, making it difficult to distinguish and classify the oxidation rates of existing droplet populations. It has been also recently shown that the lipid oxidation products were overrepresented in the smallest droplets of emulsion. These insights highlight the importance of the fraction of "tiny droplets" on the oxidative stability [41]. Additionally, small and large droplets do not have the same interfacial properties or surface curvature properties, which can alter the accumulation of emulsifiers (e.g. proteins).

Finally, and not least, when the lipid concentration is constant in a lipid dispersion, a decrease in droplet size will simultaneously increase the concentration of interfacial emulsifiers. The role of the emulsifier, and thus its nature, will have a greater impact on oxidation. This will also result in reducing the distance between droplets in a given formulation. With a reduced distance, one can expect exchanges of species (hydroperoxides, radicals, oxidizing species, surfactants) between neighboring droplets to be more pronounced. The notion of species diffusion and interactions among the many substances involved in lipid oxidation pathways is highly complex and warrants further research. Indeed, the transfer of molecules involved in oxidation pathways from one lipid droplet to another appears to be crucial, especially in liquid formulations. These mass transport phenomena have been recently detailed and discussed in recent reviews [8,42,43] suggesting that mechanisms of such mass transport could occur via three pathways: diffusion, inter-droplet collision, or transfer assisted by micelles. Water-soluble species are easily exchanged between droplets through diffusion in the aqueous phase of emulsions. More hydrophobic species are assumed to be transferred either by the collision of adjacent droplets or by micelle-assisted mechanisms [44]. Transfer would be faster via the micelle-assisted pathway and would depend on the size and concentration of micelles. Data support that transfer from one droplet to another is highly unlikely for $LO^{\bullet}$, whereas it may be feasible for $LOO^{\bullet}$. However, in the case of highly lipophilic $LOO^{\bullet}$ (especially when formed on a TAG), their potential diffusion distance may be shorter, and their transfer may only occur through collision, thus greatly limiting the inter-droplet pro-oxidant activity of TAG-OOH [45,46]. Conversely, studies have demonstrated the possibility for lipids, including LOOH (4-hydroperoxy-2-nonenal, or linoleic acid-derived LOOH), as well as secondary oxidation compounds (e.g., alkenals), to easily diffuse through

the emulsified medium and thus propagate oxidation onto neighboring lipid droplets [26,46–48]. As the majority of food products contain emulsifiers in quantities above their critical micellar concentration (CMC), the portion that is not adsorbed can readily self-assemble or co-assemble in the aqueous phase, forming (co-)micelles or aggregates. These structures could then impact the distribution and mobility of molecules (pro- vs. anti- oxidant), thereby influencing oxidative stability [43]. Finally, hydroperoxides on free fatty acids (LOOH) having higher surfactant properties than their radical counterparts ($LOO^{\bullet}$) with a longer half-life, suggest that they may be more involved in inter-droplet pro-oxidant phenomena [8]. In conclusion, in oil-in-water emulsion systems, it seems that the larger, more hydrophobic and reactive a molecular species is, the less its ability to propagate oxidation to surrounding droplets. Conversely, the smaller, more surface-active, and stable a molecule is, the greater its ability to migrate to a neighboring droplet.

Regarding bulk oil systems, primarily represented by vegetable oils, they have long been considered homogeneous continuous media where oxidation was assumed to occur at the lipid-air interface. However, edible oils are systems containing minor amphiphilic compounds such as mono- (HLB ~ 3.4–3.8) and diacylglycerols (HLB ~ 1.8), phospholipids (HLB ~ 8), sterols, and free fatty acids (HLB ~ 1) that are not completely eliminated during the refining process. Oils may also contain oxidation products (LOOHs, lipoperoxyl radicals) exerting surface activity. Additionally, edible oils contain traces of water (100–900 ppm) that can be trapped by the previously mentioned surfactant molecules within colloidal structures known as association colloids [49]. These aggregates are primarily represented, depending on the nature of the surfactant(s) involved, by lamellar structures and reverse micelles, uniformly dispersed in the oil. Several authors have highlighted the existence or formation of these colloidal structures, with the key structuring parameter being the surfactant/water molar ratio and the HLB value of the surfactant [50–52]. Bulk oil systems are therefore not necessarily homogeneous media; on the contrary, they are dynamic multiphase systems that evolve over time in terms of structure, composition, and reactivity during the oxidation process. Colloidal structures appear to be deeply associated with oxidation phenomena in bulk oil systems [49,50,53–56]. These studies suggest that lipid oxidation primarily occurs at the interface of colloidal associations, which act as reservoirs for LOOH and transition metals. Thus, colloidal associations promote the kinetics of lipid oxidation, and smaller-sized associations tend to concentrate more pro-oxidant metals near the lipid interface. Lastly, oxidation compounds (e.g., hydroperoxides, aldehydes, etc.) can influence the formation of these colloidal structures (alteration of CMC, size, or shape of aggregates, formation of reverse micelles), which could accelerate oxidation [57–61].

3. Inhibition of lipid oxidation by antioxidants

The term “antioxidant” is used in various contexts in the literature, leading to some drift in its understanding. For example, considering an antioxidant simply as a “scavenger” of free radicals is highly inaccurate. In 1989, Halliwell and Gutteridge defined an antioxidant as “a substance that, when present in low concentration compared to an oxidizable substrate, delays or prevents significantly the oxidation of this substrate”. A few years earlier, in 1962, Chipault admitted the same definition, also emphasizing this concentration ratio [antioxidants] $<<$ [substrates], but restricted the substrate(s) to easily oxidizable food material. Here, we define an antioxidant as any chemical species capable, at low doses, of reducing the overall oxidation degree of a system (e.g., a food) for a significant amount of time. The advantage of these different definitions is their observational nature. They do not stipulate a specific mechanism of antioxidant action. Then, the entire difficulty lies in assessing the oxidation level of the system, especially its “oxidant” chemical markers. The diversity of oxidation initiators and oxidants themselves means that we are faced with a multiplicity of oxidation pathways (**See section 2.**). As a result, the further oxidation progresses, the more difficult it becomes to differentiate between degrees of oxidation. Therefore, any comparison of the oxidation level of a system should be made at early stages of oxidation. Antioxidants are often classified as type I or type II, to differentiate between those (type I) that preferentially act during the propagation step of free radicals (“chain breakers”), and those (type II), more “preventive,” with little or no reactivity with free radicals (chelation of transition metals, deactivation of singlet oxygen, inhibition of pro-oxidant enzymes, etc.). Let us briefly review all the pathways on which a molecule could react and thus exert an antioxidant action.

3.1. Chelation of transition metals

The ability of a (macro)molecule to prevent the initiation or acceleration of oxidation by chelating transition metals such as iron or copper is of great importance. Metal chelators decrease (or suppress) lipid oxidation by preventing the various chemical mechanisms catalyzed by transition metals. The lipid oxidation catalysis is frequently attributed to the cyclic boost of LOOH decomposition by Fe^{2+} *(cf. 2.1.2)*. However, the chemical mechanisms of metal catalysis are more complex, especially in multiphase or compartmentalized reaction systems [62]. This could result from the direct oxidation of unsaturated lipids by metals in the higher valence state via electron transfer, or by metals in the lower valence state via the formation of metal-oxygen transition complexes or auto-oxidation [4]. As discussed in section 2.1.2., transition metals can also be involved in the oxidation or reduction of preformed lipid hydroperoxides (LOOH) or decompose hydrogen peroxide (H_2O_2), leading to the formation of free radicals that contribute to the overall increase in lipid oxidation because the rates of hydrogen abstraction by these substances (peroxides) are much faster than the rates of ab initio formation of the lipid radical L• [63,64]. Furthermore, metals can also alter the distributions of oxidation products, secondary reaction pathways, as well as the nature of termination reactions (e.g., rearrangement of LOOH into epoxides) [3,65]. Copper (Cu^{2+}) has received less attention than iron, yet it is known to be equally, if not more, effective in accelerating peroxide decomposition. Chelators such as proteins, polyphosphates, or polyacids (EDTA, citric acid), phenolic compounds (phenolic acids, flavonoids), or peptides can form insoluble metal complexes or provide steric hindrance between metals and oxidizable food components or their oxidation products [66]. The chelating agent can also physically separate the metal from lipids, as observed in studies conducted in oil-in-water emulsions with chelating agents present in the aqueous phase [30,67]. Note that pH can have a significant effect on the pro-oxidant activity of these transition metals, since it may affect their stability, solubility (hence concentration), and oxidation states, a criterion that is often overlooked.

3.2. Singlet oxygen deactivation

To date, carotenoids are considered the best singlet oxygen ($^1O_2$) quenchers. With nearly 600 representatives, these liposoluble pigments are synthesized by numerous living organisms (bacteria, algae, fungi, chlorophyllous plants) and absorbed by animals and humans through diet. Regarding their mechanism of action, carotenoids act by deactivating $^1O_2$ into $^3O_2$ (**Re. 17**). In the example below, β-carotene in its excited state (β-carotene*), following the deactivation of a singlet oxygen molecule, dissipates its excess energy as heat through its long-conjugated polyene (**Re. 18**).

$$^1O_2 + \beta - \text{carotene} \rightarrow {}^3O_2 + \beta - \text{carotene}^* \quad \text{(Re. 17)}$$

$$\beta - \text{carotene}^* \rightarrow \beta - \text{carotene} + \text{heat} \quad \text{(Re. 18)}$$

Once regenerated, β-carotene can then initiate a new cycle of singlet oxygen deactivation. It is estimated that this type of non-stoichiometric quencher is capable of deactivating approximately 1000 molecules of

$^1O_2$ before chemically reacting and forming a product. Although less effective than carotenoids, there are other singlet oxygen quenchers such as tocopherols [68] and thiols [69].

3.3. Termination of propagating radicals: Chain-breaking antioxidants

This is arguably the most widely recognized and documented antioxidant mechanisms in the literature. Chain-breaking antioxidants (A-H), previously detailed in section 2.3.3, act as radical scavengers capable of reducing lipid radicals (e.g., LOO$^\bullet$) responsible for the radical propagation of oxidation through hydrogen or electron transfer (**Re. 16**).

This reduction, through homolytic cleavage of the A-H bond, is only possible if the molecule has a reducing power greater than that of LOO$^\bullet$.

3.4. Enzymatic trapping of reactive oxygen species (ROS)

This review focuses on oxidation in physical models such as food, cosmetics, and pharmaceuticals. However, in vivo, dysfunctions in oxygen metabolism can lead to increased production of reactive oxygen species (ROS), including free radicals (e.g., $^\bullet$OH, $O_2^{\bullet-}$, ROO$^\bullet$) and non-radical products (e.g., H_2O_2, ROOH). The body defends against these harmful species through various enzymes: superoxide dismutases (SOD) convert superoxide anions into hydrogen peroxide and oxygen; glutathione peroxidase detoxifies peroxynitrite, lipid hydroperoxides, and hydrogen peroxide; and catalase breaks down hydrogen peroxide into water and oxygen.

4. Methods for evaluating the antioxidant capacity

The research and study of molecules capable of counteracting oxidation phenomena in foods (or cosmetic products) have necessitated the implementation of rapid evaluation methods. This step is fundamental, not only to assess the potential and optimal conditions for a compound (or extract) to exert antioxidant activity, but also to assimilate fundamental knowledge about lipid oxidation to better understand the challenges and find suitable solutions for industrialized products. While there are numerous tests available today, it is often observed that they are conducted under very different conditions, resulting in the measurement of properties that are difficult to compare. The choice of test, based on the relevance of the information it will provide, is therefore of paramount importance (Fig. 5). All of these methods will inform us about the ability of a compound (or extract) to potentially act in limiting the various lipid oxidation pathways. The main difference between all of these tests lies in the presence (competitive methods) or absence (non-competitive) of oxidizable lipids, and thus the complexity of the study system.

4.1. Evaluation of the chelating capacity

One method for estimating chelating capacity involves measuring the concentration of free, uncomplexed metal ions (such as Fe^{2+} and Cu^{2+}), in the presence of an antioxidant molecule or extract. This is done by introducing the metal ions in the form of their sulfate (Metal-SO_4) or chloride (Metal-Cl_2) salts, and then performing an indirect titration with a chelating agent. Typically, Fe^{2+} is titrated by complexation with ferrozine or 2,2′-bipyridine, forming chromophore complexes that are quantified at maximum absorbances of 562 nm and 485 nm, respectively. For Cu^{2+}, titration can be performed by forming a blue-violet complex absorbing at 632 nm with pyrocatechol sulfone phthalein (pyrocatechol violet) [70], or at 485 nm with tetramethyl murexide [71]. The decrease in absorbance at these wavelengths, observed after adding an antioxidant chelating agent, indicates the formation of a metal-chelator complex. In many studies, the chelating capacities of metals by an antioxidant or extract are expressed in EDTA equivalents. The aqueous environment of these methods, along with measurement by visible spectrophotometry, may limit their utility. For instance, analyzing colored or poorly soluble samples in the aqueous phase may be challenging. Additionally, these methods require particular attention (such as choice of organic co-solvent, buffer choice and concentration, measurement time, etc.) to avoid shifting metal-chelator equilibria towards the metal-chromophore complex and thus underestimating the amount of metal ions initially complexed by the antioxidant. Lastly, the effect of the buffer (its nature and concentration) must be considered, as it can significantly change the observation made in an unbuffered aqueous system and thus, the transfer from prediction to real-world applications in lipid emulsion systems or actual foods. Infrared spectroscopy methods (ATR-FTIR) could be used to quantify metal-chelator complexes, but they are more complex to implement and less "universal" [72]. An approach using surface plasmon resonance can also determine an affinity constant between a chelating antioxidant and a transition metal ion immobilized on a molecular scale microchip [73–75]. Again, while these methods are valuable for assessing the ability of a given

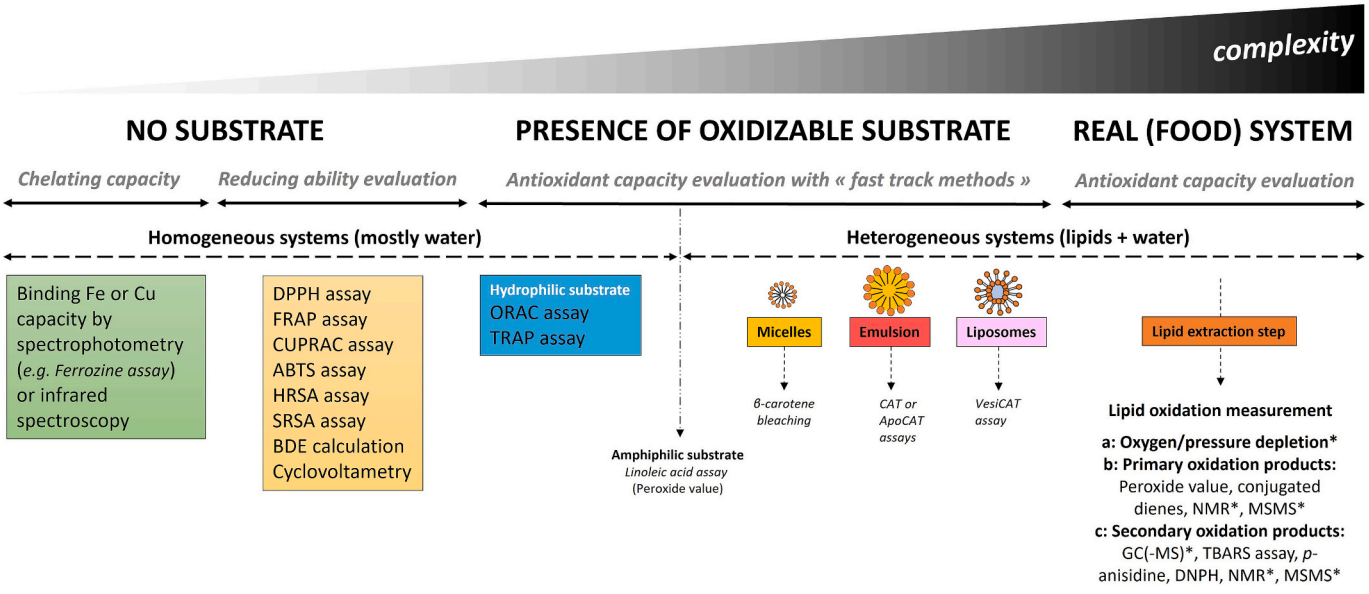

Fig. 5. Proposed classification of methods for evaluating antioxidant capacity based on the complexity of the study system. *May be implemented without the lipid extraction step.

compound to chelate transition metals, they should not lead to conclusions about its ability to prevent pro-oxidative pathways due to metal activity. This step is a necessary but not sufficient condition for expressing antioxidant activity. As mentioned earlier, the metal-chelator complex can have opposite effects on lipid oxidation since it results from a balance between its reactivity and distribution. This association can form an insoluble complex or steric hindrance to metals, which may be beneficial in reducing its reactivity. Conversely, this complex can alter the electron density and decrease the redox potential of the metal center, making it a better reducing agent for lipid hydroperoxides, for example [62,76]. Finally, complex formation can alter ion distribution in multi-compartmentalized lipid-based food systems. This observation is crucial for understanding the contradictory effects of metal chelating molecules, which may potentially act as antioxidants.

4.2. Evaluation of the reducing capacities

4.2.1. Evaluating the strength of chemical bonds or the redox properties/ electron transfer

The reducing capacity of a molecule can be estimated (either theoretically or experimentally) through its ability to donate a hydrogen atom (or an electron). This can be achieved through the calculation of the homolytic dissociation energy (bond dissociation energy, BDE) of the bond between the hydrogen and the rest of the molecule. A hydrogen will be more labile, meaning more easily donated or released as a radical, when the BDE value of its bond is lower. Experimental BDE values, mostly determined by techniques like EPR spectroscopy or photoacoustic calorimetry, can be adequately estimated through density functional theory calculations. In phenolic compounds, for example, it is primarily the aromatic hydroxyls that are involved, with significant differences depending on their number, position, and degree of substitution on the aromatic ring. For instance, the BDE of the O—H bond in catechol is lower than that of 2-methoxyphenol, which in turn is lower than that of 1,3-dihydroxybenzene [77,78]. Alongside these structural determinants, the number and position of phenolic hydroxyls, the presence of glycosylation(s), and the overall degree of conjugation significantly contribute to the antioxidant activity. In the case of phenolic compounds with the same number of hydroxyls, the presence of a methoxy group in ortho- or para-position to the hydroxyls can stabilize the aryloxy radicals resulting from electron donation and increase the reducing activity. This phenomenon is particularly true for hydroxycinnamic acids, which, moreover, have a higher activity than benzoic and hydroxyphenylpropionic acids due to the presence of the double bond -CH=CH-, which promotes resonance forms of the aryloxy radical.

Voltammetry (cyclic, square-wave, differential pulse, staircase, polarography, etc.) is an electrochemical method used to explore a compound's electron transfer properties, including the potential at which oxidation or reduction takes place, the reversibility of the redox reactions, and the kinetics of electron transfer processes. This knowledge can be used to thermodynamically predict the radical scavenging ability of a given compound. For example, in the series of tocopherols, the radical scavenging capacity is inversely proportional to the electrochemical potential: $\alpha > \beta \sim \gamma > \delta$ (+0.273, +0.343, +0.348, and + 0.405 V) $\alpha > \beta \sim \gamma > \delta$ (+0.273, +0.343, +0.348, and + 0.405 V) [68]. This pattern is attributed to the presence of CH_3 substituents in the ortho- and/or para-positions, which facilitate the homolytic cleavage of the O—H bond. These substituents stabilize the resulting radical (O•), prolong its lifetime, and enhance its reactivity with other radicals. This is why αTocOH (with two ortho-methyl substituents) is the most active in radical scavenging reactivity, followed by β- and γ-TocOH (with one ortho-methyl substituent), and δ-TocOH (with no ortho-methyl substituent).

Cyclic-voltammetry is the most extensively used electrochemical technique for the detection of antioxidant potential in extract-based samples [79–82]. However, a contradiction persists regarding the correlation between electrochemical techniques and other methods in determining antioxidant capacity. As previously noted, lipid oxidation - and thus the potential antioxidant action - is a complex process with numerous pathways, requiring a more nuanced understanding than merely the stabilization of peroxyl ($LOO^{\bullet}$) and alkoxyl ($LO^{\bullet}$) radicals. Supporting this point, it is important to consider how a molecule involved in these reduction pathways, which might be seen as an antioxidant, can paradoxically increase the oxidation level in a system, exhibiting a pro-oxidant effect. This phenomenon has been deeply documented for tocopherols [68], as well as for flavonoids [83], ascorbic acid [84], and carotenoids [85], which can display pro-oxidant behavior under certain conditions. Nevertheless, one of the key advantages of voltammetry is that it can be conducted without requiring reagents or sample pretreatment. Additionally, advancements in multiprobe approaches using simulations and data-driven techniques could further accelerate its development for exploring antioxidant potential. Thus, research on voltammetry for predicting antioxidant capacity continues, with new applications anticipated in the future [86].

4.2.2. Measuring the reduction of a radical (absence of oxidizable substrate)

One of the simplest and perhaps most common ways to anticipate the antioxidant capacity of a molecule (or extract) is by measuring its reducing capacity through the use of non-competitive methods (in absence of oxidizable substrate). This involves measuring the ability of a molecule to reduce a free radical (via hydrogen or electron transfer) or a transition metal (via electron transfer) in the absence of any oxidizable substrate. Methods based on this strategy are simple, quick to implement (using aqueous or organic homogeneous systems), and do not require advanced equipment, as most measurements are performed using a UV–Visible spectrophotometer. For example, tests are based on the reduction of the stable free radical 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$), the radical cation derived from 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid ($ABTS^{+\bullet}$), or the metal complexes such as Fe^{3+}-2,4,6-tripyridyl-*S*-triazine (FRAP) or Cu^{2+}-neocuproin (2,9-dimethyl-1,10-phenanthroline) (CUPRAC). However, these methods have several major drawbacks. They are not representative of the oxidation phenomenon in real lipid-based systems, which involves competition between oxidants, oxidizable substrates, and antioxidants (which can be seen as sacrificial oxidizable substrates). They do not take into account real conditions. For instance, the reduction of oxidizing species alone, especially a synthetic radical with relatively large steric hindrance such as $DPPH^{\bullet}$, may not be representative of lipid oxidation as described earlier. Reactivity takes place in a homogeneous environment, which may need adjustment based on the nature of the compounds/ extracts being tested, in order to prevent, for instance, bias in the reactivity of precipitated molecules. For methods using organic solvents as the reaction medium, the solvent itself can interfere with the measured chemical reactivity (e.g., Michael additions with methanol) [87]. Finally, measurements are often performed by UV–Visible spectrophotometry, which can introduce numerous biases, especially when samples (or their oxidized products) absorb at the wavelengths used. Despite these drawbacks, these methods, which are very popular, allow for the approximation of the intrinsic chemical reactivity of a pure compound or an extract.

4.2.3. Measuring the protection of a hydrophilic oxidizable substrate

In contrast to non-competitive methods, there are also competitive *in vitro* methods for evaluating antioxidant activity. These require an oxidizable substrate, favorable oxidation conditions (presence of initiator), and antioxidants (whose ability to protect the oxidizable substrate is evaluated). The antioxidant capacity of a given substance is then directly related to the degree of oxidation of the substrate, which is often measured by simple spectrophotometric methods (UV, visible, fluorescence). The most common methods rely on the use of probes such as fluorescein (ORAC method) or R-phycoerythrin (TRAP method), which

are artificially oxidized by the addition of hydrophilic radical initiators (AAPH, see section 2.1.1). The addition of antioxidants to the medium causes a difference in the degree of oxidation of the probe (speed and/or quantity), which can be exploited and used as an indicator of the antioxidant potential of the molecule (or extract). Although they allow us to go a little further by introducing a level of complexity beyond a simple bimolecular reaction between an oxidant (e.g., $DPPH^{\bullet}$, $ABTS^{+\bullet}$) and a reducer, these methods should not provide significantly different information compared to the DPPH and ABTS methods mentioned earlier. However, if we look at some studies, we may perceive some dissimilarity in the results, as observed with the series of esters of protocatechuic acid [88]. While the DPPH results seem to be identical regardless of the carbon chain length of the ester, the ORAC results decrease significantly as carbons are added, especially from six carbons onwards. This result, which could be interpreted as a loss of activity of the molecule, is actually due to a solubility problem in the aqueous phase for the most hydrophobic esters, highlighting a new limitation of these methods. In fact, these methods should be used to rank the reducing capacity of molecules, without venturing into predicting antioxidant efficacy in food, cosmetic, or pharmaceutical matrices formulated with lipids. It has been reported that homologous forms of tocopherols and tocotrienols have largely the same reactivity with free radicals in homogeneous systems [89] with the phytol tail playing no (or only very minor) role in this chemical reactivity compared to the chromane fraction. The same goes for the results obtained with protocatechuic esters [88]. However, the results of published studies on *in vitro* antioxidant activities in lipid dispersion systems or in crude oils do not necessarily follow these rules and rankings [68,88]. Similarly, it has been reported that ascorbic acid was twice as effective at trapping peroxyl radicals as propyl gallate with the ORAC test, while propyl gallate was a much better antioxidant in oil-in-water emulsions [90]. It goes without saying that for a reaction to occur, the reactant particles (e.g., oxidized lipid and antioxidant) must collide with each other (collision theory). However, except in exceptional cases, lipid formulations are heterogenous by nature, so that there are compartmentalized environments in which molecules are distributed and concentrated according to their affinities.

4.3. Effect of physical structures on antioxidant activity

The intrinsic chemical reactivity of an antioxidant candidate can be anticipated from a limited number of structural traits, such as the number, type and position of electron or hydrogen donor groups, aromaticity, electronic effects of substituents, geometry, and planarity, and evaluated using the methods previously described. However, predicting its physical behavior and how that might influence its reactivity is more complex. And yet, this is important for anticipating and/or understanding its antioxidant capacity. One must go back to the 1980s to find traces of the first significant conclusions on how the physical structuring of lipid systems can influence antioxidant response [91]. Indeed, paradoxically, it was observed that polar and hydrophilic antioxidants are more active than their apolar analogs in hydrophobic bulk oil systems, while apolar antioxidants are more active in oil-in-water emulsions. Although purely empirical in nature, this simple rule is already a major advance that undermines the idea that an antioxidant can behave identically and have the same efficacy regardless of the lipid system considered. Furthermore, this observation renders obsolete the extrapolation of "antioxidant results" obtained in homogeneous systems to heterogeneous multiphase systems. The mechanistic hypothesis, supported a few years later by other works [92,93], would come from the fact that, in such emulsions, apolar antioxidants would concentrate at the oil-water interface and inhibit oxidation more effectively than polar antioxidants whose localization would be essentially in the aqueous phase, thus pointing to the interfacial region as a critical site of lipid oxidation. In bulk oil systems, the superior activity of hydrophilic antioxidants was initially attributed to their better ability to concentrate at the air-oil interface, where oxidation would be inferred, with hydrophobic antioxidants being diluted in the oil phase and therefore less effective. The growing interest in the role of colloidal associations (see section 2.4) and their influence on oxidation pathways in the early 2000s has profoundly reshuffled the cards on result interpretation. An antioxidant in the aqueous core of a colloidal structure, such as a reverse micelle, will generally interact more with pro-oxidant radicals and metals located in the interfacial zone, especially when the surface is negatively charged. In contrast, its lipophilic counterpart will have fewer such interactions. This is why α-tocopherol would be much less effective than its hydrophilic counterpart, trolox, in the presence of reverse micelles in bulk oil [94,95]. Furthermore, the co-distribution of antioxidants of different types in the interfacial zone of reverse micelles could promote their interactions, which could result in synergistic action [96].

Colloidal structures are undoubtedly closely linked to oxidation phenomena in bulk oil systems and contribute to the seemingly inconsistent effects of antioxidants, which may align or conflict with the polar paradox. Nevertheless, predicting their impact on the antioxidant activities of exogenous molecules is challenging due to the numerous physicochemical factors involved, which vary depending on the system. These factors include temperature, morphology, size and surface charge of colloidal objects, distribution of oxidation products and antioxidants, interactions between antioxidants, type and concentration of surfactant compounds, water content, presence of metal ions, etc. This explains the diversity of results obtained [94,97,98].

Regardless of whether it is a water-in-oil or oil-in-water system, numerous studies since the early 2000s have confirmed the importance of the interfacial region on lipid oxidation and the predominant role of the surface activity of a molecule in fulfilling its antioxidant capacity. If there is no theory capable of predicting the behavior of an antioxidant (in a given lipid system) based on its chemical structure, strengthening analytical methods that consider the effect of the physical structures and interfaces inherent to heterogenous media could help narrow the gap between prediction and actual efficacy. Therefore, it is important to make antioxidant evaluation methods more reflective of real-life conditions (i.e. with compartmentalization, interfaces, and colloid associations) to ensure their results are more relevant.

4.4. Fast track methods with compartmentalized and interfacial model systems to measure antioxidant capacity

Undoubtedly, the previous methods can provide important information, particularly on the intrinsic chemical reactivity of an antioxidant candidate. However, the introduction of an oxidizable lipid-based substrate, representative of those encountered in real-life conditions will make the system more complex but will render the response more informative. To this purpose, measuring the inhibition of linoleic acid solubilized in a hydroalcoholic solution (water-ethanol) represents an initial step towards increasing the complexity of these tests (Fig. 5). The introduction of colloidal systems, in the form of lipid assemblies dispersed in an aqueous phase, will considerably further improve matters.

Through these fast methods, the idea is to quantify, via spectroscopic approach and in a non-destructive manner (no extraction required), the oxidation degree of a lipid taken as an oxidizable substrate. This can be achieved by monitoring either the formation of oxidation products or the disappearance of the substrate. One of the most used methods involves measuring the appearance of oxidation products of linoleic acid, previously dispersed as micelles, by measuring conjugated dienes at 234 nm. This method is based on the fact that most hydroperoxides formed during lipid peroxidation have a conjugated diene system that absorbs in the UV range, typically between 230 and 235 nm. It is applicable only to systems involving unsaturated fatty acids with more than two double bonds in a methylene-interrupted configuration.

The other strategy monitors the fate of an oxidizable substrate rather than the formation of numerous oxidation products. This oxidation can

be tracked by (i) the decrease in absorbance, as in the case of the autoxidation of β-carotene (λ_{max} = 460 nm), or (ii) the decrease in fluorescence of different probes: cis-parinaric acid (conjugated tetraene with 18 carbons), BODIPY or C11-BODIPY, or other lipophilic derivatives of aminofluorescein (C11-fluor, C16-fluor, C18-fluor, Fluor-DHPE) [99].

In our research team, we have developed methods using a lipid substrate, eleostearic acid, possessing a conjugated triene system with 18 carbons (primarily in form Δ9c, Δ11t, Δ13t). This characteristic gives it both absorption and/or fluorescence properties in the UV-VIS spectrum (λ_{max} = 273 nm) and high sensitivity to oxidation. Eleostearic acid is found in nature, particularly in the oils of *Garcinia nutans* seeds (~89 %) and *Aleurites fordii* seeds (~80 %). The oil from *Aleurites fordii*, better known as tung oil or China wood oil, is readily available as it is widely marketed, especially due to its siccative properties. The oxidation of eleostearic acid present in this oil is characterized by a decrease in its absorption spectrum at 273 nm, which can be slowed down by the addition of an antioxidant. We utilized the characteristics of this fatty acid to develop rapid antioxidant methods in compartmentalized and interfacial model systems, called CAT (conjugated autoxidizable triene) method, allowing the measurement of activities in emulsified systems where oxidation was accelerated by the decomposition of AAPH at 37 °C [100]. More recently, a variant of the CAT method by initiating the generation of free radicals in the lipid phase (ApoCAT test) was implemented. For this purpose, the hydrophilic azo-initiator (AAPH) used in the CAT test was replaced by dimethyl 2,2′-azobis(2-methylpropionate) (or V-601), added in the form of an emulsion in MCT (medium-chain triglycerides) [101,102]. This strategy was further extended with the implementation of a new high-throughput method, called "Vesicle Conjugated Autoxidizable Triene" (VesiCAT test), allowing for the evaluation of lipid membrane oxidation and hence the antioxidant activity of molecules in the presence of membranous lipid assemblies [103]. This method is based on the UV absorbance spectral properties of a new phospholipid probe, synthesized from eleostearic acid extracted from tung oil (1,2-α-eleostearoyl-sn-glycero-3-phosphocholine (DEPC)). The VesiCAT test was developed with two different radical generators (2,2′-Azobis(2-amidinopropane) dihydrochloride; AAPH and 2,2′-azobis (2,4-dimethylvaleronitrile); AMVN), producing a constant flux of oxidant species, either at the membrane level or in the aqueous phase. The measurement of antioxidant capacity is performed after mathematical quantification of the slowing down of DEPC probe oxidation, by observing its absorption intensity at 273 nm. It is a completely unique system, the main advantage compared to other artificial probes used in this type of method (BODIPY and its derivatives, HDAF (hexadecanoylaminofluorescein), DPH-PA (diphenylhexatriene propionic acid)) being that the probe corresponds to real phospholipids. This method appears to be very effective for evaluating the ability of antioxidant molecules to preserve lipid oxidative degradation, structured in the form of membrane assemblies. Furthermore, oxidations induced by AAPH and AMVN offer the possibility to extract different but complementary information regarding the multifaceted effectiveness of antioxidants.

5. Methods for evaluating the oxidation degree in model formulations or real foods

5.1. Measuring the oxygen consumption

Since oxidation results from a reaction with oxygen, one method to assess the progress of oxidation in a system is to directly measure oxygen consumption. Because the rate at which oxygen adds to lipid radicals is diffusion-controlled and nearly instantaneous, monitoring oxygen consumption specifically highlights the duration of the lag phase and any potential extension of this phase when exogenous antioxidants are introduced into the system. Typically, oxygen consumption during lipid oxidation can be monitored using a variety of techniques, including optical sensors based on oxygen-sensitive fluorescent probes, which do not require gas extraction from the sample [104]. Other methods include gravimetric methods, Clark electrodes, paramagnetic oxygen analyzers, chemical titration methods (such as the Winkler method), polarographic techniques, gas chromatography [105–108]. Other indirect measurements of changes in conductivity or pressure using controlled atmosphere apparatus could also be used. Other methods (such as RapidOxy) indirectly quantify oxygen consumption by measuring changes in conductivity or pressure. However, these methods are performed under conditions of pressure and/or temperature that are quite different from real-life scenarios. This can introduce significant biases, particularly from a chemical perspective, by altering oxidation pathways and the products formed. For example, low pressure and temperature can lead to interference from volatile compounds, affecting pressure measurements, while high-moisture products can produce water vapor that may also create measurement artifacts [109]. Gas chromatography coupled with a thermal conductivity detector is well-documented for measuring headspace oxygen and evaluating lipid oxidation and antioxidant capacity. For instance, Tao et al. evaluated the effectiveness of γ-oryzanol and BHT in protecting canola oil from oxidation [110]. Similarly, Lai and Paterson measured headspace oxygen as an indicator of lipid oxidation in infant formula powders, noting a strong correlation between oxygen depletion and hexanal formation [111]. Berton et al. assessed oxidative stability in emulsions stabilized by various surfactants or proteins using headspace oxygen uptake measurement [112]. They concluded that this method, compared to other techniques such as measuring conjugated dienes or volatile compounds by GC, was the most rapid and reproducible for distinguishing between different emulsions based on oxidative stability.

However, methods measuring headspace oxygen consumption have some drawbacks, primarily related to the need for hermetically sealed assay equipment to prevent measurement artifacts. For example, Villiere et al. used such techniques to monitor linoleic acid oxidation with an oxygen-to-substrate molar ratio of about 1:5 [113]. In this setup, the total amount of oxygen limits oxidation in the vials, and the consumption of oxygen due to lipid oxidation can create a pressure drop. Consequently, if the syringe used to measure O_2 uptake is removed from the vials, air may enter through the needle, leading to measurement artifacts. Some other authors found that the measurement of the consumption of headspace oxygen is not accurate for the evaluation of the shelf life of 1 % oil-in-water emulsion [114]. Indeed, by monitoring both dissolved and headspace oxygen simultaneously using fluorescent optical sensors in the emulsion and headspace, the authors found that headspace oxygen does not decrease until the lag phase of lipid oxidation is complete. This delay in headspace oxygen consumption can lead to an overestimation of food stability. In contrast, measuring dissolved oxygen content provides a more accurate prediction of oxidative stability. The authors therefore recommend monitoring dissolved oxygen in the oil phase for a better assessment of oxidative stability.

Other important factors to consider when measuring the oxygen consumption include controlling the agitation conditions and headspace-to-emulsion ratio since both agitation and increased headspace volume significantly increased the oxidation [115]. In a near future, it can be expected that oxygen fluorescent probes with higher specificity and sensitivity will be further used to better evaluate lipid oxidation kinetics [116–118].

5.2. Measuring the early markers of oxidation (hydroperoxides and conjugated dienes)

5.2.1. The often overlooked yet crucial step of lipid extraction

Measuring early markers of oxidation generally require a preliminary step corresponding to the lipid extraction from the studied material (raw material, emulsion food or cosmetic products). This step must be carried out with full awareness of the potential effects it could have on the evaluation of the degree of oxidation [119]. Indeed,

extraction methods involving prolonged heating should be avoided. For example, Soxhlet extraction can modify peroxides content in oil samples, either by degrading them or increasing their content with the acceleration of lipid oxidation through heating [120,121]. Moreover, it is important to point out that depending on the solvent used, the different lipid classes (including various lipid oxidation products) may be extracted at different levels. The Folch method, a widely used lipid extraction technique, involves using a monophasic chloroform/methanol (2:1 v/v) solvent, known as the Folch solvent. This solvent is then separated by adding a saline solution (typically 0.58 % *w/v* NaCl) to achieve a final concentration of 8:4:3 v/v/v chloroform/methanol/water, and the lower chloroform phase is collected. Another common method, the Bligh & Dyer method, saves solvent and works directly on hydrated samples by extracting lipids with chloroform/methanol (2:1 v/v) to reach a final concentration of 2:2:1.8 v/v/v methanol/chloroform/water. Due to the toxicity of chloroform and methanol, alternative solvents like hexane or heptane/isopropanol and isooctane/isopropanol are increasingly used. The choice of solvent and extraction method is crucial to fully extract the target oxidation compounds and ensure valid quantification of the oxidative status of the sample. Typically, unoxidized lipids such as triacylglycerols are soluble in non-polar solvents such as hexane, heptane or isooctane. On the contrary, oxidation compounds are less soluble in these solvents and some aldehyde secondary oxidation compounds have even a good solubility in water [122,123]. In addition to being discriminant for specific lipid classes, recent studies have demonstrated that higher lipid extraction yields can be achieved using chloroform–methanol (2:1 v/v) (>95 wt%) compared to hexane (or isooctane)–isopropanol (3:1 or 3:2 v/v) (75–86 wt%) in oil-in-water emulsions. The extraction yield can vary depending on the emulsifier used and the duration, dropping as low as 26 wt% for WPI-stabilized emulsions [124]. The presence of non-lipid chemical species may influence the lipid extraction. For instance, in emulsified systems, lipid extraction can be hampered by the presence of other chemical species such as proteins. Last but not least, it is also worth noting that the extraction procedure itself can impact lipid oxidation [109]. For example, the presence of water in Folch or Bligh-Dyer procedures can favor scission of alkoxyradicals and may bring traces of pro-oxidant metals that would catalyze lipid oxidation. Solvents themselves may also contain catalytic level of oxygen that may contribute to lipid oxidation. Therefore, solvents should be freshly distilled or sparged with an inert gas before use. Additionally, the quality and potential degradation of the solvents used must also be taken into account. For instance, chloroform may be stabilized either by ethanol or cyclohexene. Both compounds are not inert in regards to lipid oxidation and are prone to lipid radical attack forming other radical species that can accelerate lipid oxidation kinetics. Chloroform may be also stabilized with 2-methyl-2 butene (amylene) which has been demonstrated to be an inappropriate preservative to measure lipid hydroperoxides by the ferric thiocyanate assay [125]. Similarly, degraded solvents may contain traces of peroxide compounds, which can contribute to lipid oxidation. For instance, peroxides have been observed in 2-propanol, ethyl acetate, and butanol [126].

5.2.2. Methodological considerations on lipid hydroperoxide measurement

LOOHs are assuredly the most studied primary products of lipid oxidation providing essential information on the early stages of the oxidation process. In media with high hydrogen-donating capacity, these compounds are formed in significant amounts. Unlike free radicals, they are more easily detected using various physicochemical methods. To be relevant, however, this approach must be considered with care since it may lead to misinterpretation. Indeed, at the very beginning of lipid peroxidation, hydroperoxides are faintly concentrated but, as their formation is faster than their decomposition, they gradually accumulate. Thus, during the exponential phase, hydroperoxides concentration increases until a maximum is reached. The subsequent decrease in LOOH content indicates that the decomposition rate has surpassed the formation rate, suggesting that the reservoir of double bonds is nearly depleted or no longer available. Consequently, since low levels of hydroperoxides can occur in both early and advanced stages of oxidation, their measurement should be considered primarily for samples that are only mildly to moderately oxidized. Otherwise, the extent of oxidation may be significantly underestimated. Additionally, because hydroperoxides are sensitive to temperature and light (**Re. 5**), it is crucial to keep the temperature as low as possible during sample preparation and analysis, also ideally protected from light. Another key factor to consider when quantifying peroxides is their tendency to undergo various rearrangements and reactions, particularly in acidic environments commonly found in analytical methods (see below). These reactions include well-known processes such as Baeyer–Villiger, Criegee, and Hock mechanisms, among others [127].

5.2.3. Measurement of hydroperoxides with iodometric titration

Iodometric titration, is one of the most commonly used method for quantifying total peroxides which include peroxides ROORs, hydroperoxides ROOHs (denoted LOOHs when derived from lipids), and hydrogen peroxide H_2O_2. It is expressed as the peroxide value (PV) in milliequivalents of active oxygen per kilogram of sample (meq O_2/kg). Standardized assays (ISO 3960:2017; AOCS, cd 8b–90) are based on the following principle.

First, in acidic medium (prevents hypoiodite formation that might interfere with the reaction), potassium iodide is stoichiometrically oxidized by peroxides into iodine (**Re. 19**):

$$R_1OOR_2 + 2I^- + 2H^+ \rightarrow I_2 + R_1OH + R_2OH \quad \text{(Re. 19)}$$

Where $R_1 = R_2 = H$, hydrogen peroxide; $R_2 = H$, Hydroperoxides.

Then, iodine (I_2) is titrated with a sodium thiosulfate standardized solution containing starch for endpoint determination (**Re. 20**). Note that, to avoid its decomposition, the starch solution must be added only near the endpoint where iodine concentration is drastically reduced.

$$I_2 + 2S_2O_3^{2-} \rightarrow S_4O_6^{2-} + 2I^- \quad \text{(Re. 20)}$$

The standard iodometric titration method is widely used for its simplicity, but it has several drawbacks including being time-consuming, labor-intensive, generating significant waste, requiring a large sample volume (5–10 g), and being limited to samples with a peroxide value (PV) lower than 30 meq O_2/kg. Accuracy depends on careful control of various factors, such as a correct visual endpoint detection (especially in low hydroperoxide or pigmented samples), temperature, light exposure, and minimizing oxygen and prooxidant contamination. Additionally, issues like iodine adsorption on unsaturated lipids and light-accelerated oxidation of iodide can lead to errors. Despite these limitations, iodometric titration remains the gold standard for hydroperoxide analysis due to its accuracy and reliability when properly managed. Future research should explore the potential of autotitrators to simplify sample handling and electrochemical methods to improve sensitivity and reproducibility. Another approach to increase sensitivity has been to abandon the thiosulfate titration and use an optical assay for I_2 detection. One really old method, measured the iodine-starch complex at 560 nm [128]. A second and more popular approach is to measure the absorbance of the tri-iodide ion (I_3^-) formed with excess iodide ions (I^-) (**Re. 21**), this latter being spectrophotometrically measured at 360 nm [129].

$$I_2 + I^- \rightarrow I_3^- \quad \text{(Re. 21)}$$

Due to its sensitivity, reaction mixtures must be shielded from light, solutions thoroughly de-aerated before use, and continuously purged with inert gas to exclude atmospheric oxygen. Various methods exist with slight differences in the chemistry. One method involves adding cadmium (though toxic) to complex with residual I^- and prevent further oxidation by oxygen [129,130]. UV detection sensitivity can be enhanced by separating triiodide using reverse phase HPLC [129], and

continuous-flow automated procedures have been successfully developed [131,132]. Another method measures triiodide ion and addresses oxygen interference by using solutions with low acid, reduced iodide concentration, and Fe^{2+} as a catalyst, though the acid also promotes iodide oxidation and lipid peroxidation [133].

5.2.4. Measurement of hydroperoxides by ferrous iron oxidation

The ability of hydroperoxides to oxidize ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions in acidic medium at room temperature (**Re. 4**), as well as the weak sensitivity of ferrous ions towards molecular oxygen or light exposure compared to iodide [134], has led to the development of alternative assays. One can cite the Ferric Thiocyanate Assay (standardized by the International Dairy Federation (IDF 74 A:1991) or the version adapted by Shantha & Decker [135] as well as the method known as FOX for ferrous Oxidation–Xylenol orange.

$$Fe^{3+} + SCN^{-} \rightarrow [Fe///SCN]^{2+} \text{ complex} \quad \text{(Re. 22)}$$

$$Fe^{3+} + XO \rightarrow \left[Fe^{3+}///XO\right] \text{ complex} \quad \text{(Re. 23)}$$

The principle of these methods is to complex the formed Fe^{3+} with a compound that produces a colored complex, which can be measured using simple spectrophotometry. For example, in **Re. 22**, the combination of Fe^{3+} and thiocyanate ions generates a red colored complex with maximum absorbance peak at ~500 nm. In **Re. 23**, Fe^{3+}reacts with xylenol orange to form a blue colored complex that strongly absorb at ~560 nm. Renowned for their speed, simplicity, and low cost, these methods have been reported as sensitive and suitable for assessing total hydroperoxides in a wide range of samples [135–138]. However, they have drawbacks and are particularly criticized for issues related to reproducibility and linearity range [139]. These problems arise from the intermediate formation of LO• (very reactive and capable of further oxidation reaction) and the oxidizing ability of Fe^{3+}, which may significantly affect Fe^{3+}/Fe^{2+} ratio and complicate the accurate quantitation of hydroperoxides (**Re. 24–25**).

$$LO\bullet + Fe^{2+} + H^{+} \rightarrow LOH + Fe^{3+} \quad \text{(Re. 24)}$$

$$\text{Reducing species} + Fe^{3+} \rightarrow Fe^{2+} + \text{oxidized species} \quad \text{(Re. 25)}$$

In their review, Bou et al. drew up an exhaustive inventory of the factors influencing performances of the FOX method, among which those related to the sample (preparation, homogenization, solubility, source of interferences such as pigments, reducing agents, proteins, chelators, etc.) and the reaction medium (pH, purity and concentration of the dye, neutralization of hydrogen peroxide, etc.) were of prime importance [140]. In addition, they pointed out that by selecting the most appropriate wavelength (the apparent extinction coefficients of ferric–xylenol orange complexes might vary from 15,000 to 150,000 M^{-1} cm^{-1} as a function of reaction conditions), and by adding sugars or polyols that promote ferrous oxidation, the specificity, sensitivity and linearity of the method can be greatly improved. Finally, to strengthen the FOX method and get reliable results, the same authors recommend to systematically determine the repeatability and reproducibility on several different samples through intra- and interlaboratory tests. In conclusion, the main advantage of the assay is its high sensitivity, allowing for the detection of nanomoles of hydroperoxides in solution. It is considered reliable for measuring hydroperoxide concentrations in reasonably pure compounds under specific conditions and is useful for comparing relative hydroperoxide levels in similar samples or over time. However, accurately quantifying absolute hydroperoxide concentrations is challenging. It requires detailed knowledge of the hydroperoxide structure, molar absorption coefficients, and careful control of assay conditions, including incubation time, solvent, optimal wavelength, and pH. FOX method has been also used for post column detection after HPLC separation of different lipid hydroperoxides classes [141,142]. Although the technique is rather tedious and complex, it allows nevertheless a more accurate quantification of individual hydroperoxides by avoiding most of the interference compounds present in the medium.

5.2.5. Measurement of hydroperoxides by phosphine derivatives oxidation

A simple way to determine the peroxide value (PV) is to analyze triphenylphosphine oxide (TPPO), which forms after the selective reaction between LOOHs and triphenylphosphine (TPP). In 1927, Challenger and Wilson discovered that benzoyl peroxide react with TPP to give benzoic anhydride and TPPO [143]. Later, Horner and Jurgeleit (1955) made a thorough study of the reaction between trisubstituted phosphine and various peroxides [144]. They were the first to describe hydroperoxide-mediated oxidation of phosphines into phosphine oxides (with the release of the corresponding hydroxy-derivatives) as an easy, fast, stoichiometric and quantitative reaction. TPP was firstly used to reduce LOOH into their corresponding hydroxy-derivatives for quantitation or structural elucidation purposes [145,146]. It was not until 1987, and the pioneer works of Akasaka, that fluorogen aryldiphenylphosphines were designed and successfully implemented in the quantitation of hydroperoxides, with or without a preliminary chromatographic separation [147]. The optimized method was found to be highly sensitive (up to 10,000 times more than standard iodometric assay) with linear response in a wide concentration range, so that phosphatidylcholine hydroperoxide contents of 20–40 pmol/mL were accurately determined in healthy adult human plasma [148]. The 1:1 stoichiometric reaction has already been used to estimate the concentration of hydroperoxides by measuring the concentration of TPPO using HPLC-UV [149], EI-MS [150], or infrared spectroscopy [151]. Although effective, the TPPO analysis procedure remains somewhat tedious (long and/or difficult sample preparation), time-consuming, and involves a significant volume of toxic organic solvent. With the recent development of Attenuated Total Reflection (ATR) infrared techniques, signal acquisition becomes faster, providing more precise results with a better signal-to-noise ratio, where only small volumes of solid or liquid samples (μL or mg) are required. Hence, a method for measuring PV in oils using FTIR-ATR spectroscopy based on the stoichiometric conversion of TPP to TPPO by hydroperoxides and measuring its specific adsorption band at 542 cm^{-1} has been developed [152,153]. Alternatively to TPP, diphenyl-1-pyrenylphosphine (DPPP) may be used as a fluorogen probe. It is weakly oxygen-sensitive, reactive towards all hydroperoxides but inactive towards secondary lipid oxidation products, and many antioxidants (tocopherols, BHT, BHA, β-carotene and propyl gallate) do not interfere with the fluorescence signal of diphenyl-1-pyrenylphosphine oxide (DPPPO). Thus, numerous procedures have been developed to assess total lipid hydroperoxides in various samples such as live cells or membranes [154,155], human plasma [156,157], edible oils and fats [158–160], or meat [161]. The implemented techniques are various, from the simple spectrofluorimetry including high throughput microplate reading [156,157], to the more complex flow injection analysis [159,160] or TLC blotting [162,163]. The post derivatization, after separation by HPLC, was also investigated by some authors [164]. The high fluorescence intensity of DPPPO at 378–380 nm (λex = 350–360 nm) allows its quantitation from few picomol, what is advantageous for LOOH evaluation in biological samples. Depending on sample matrix, hydrophobicity and solvent polarity, reaction with DPPP is usually performed at 60–80 °C for a duration from few minutes to three hours. A cooling step is however preconized to stop the reaction and to maintain the fluorescence intensity of DPPPO when the reading is not performed immediately. As demonstrated by Bou et al., the solvent plays a major role in the completion of the reaction, since it must solubilize all chemical species (DPPP, DPPPO and peroxides) while favoring their reactivity through optimal electron configuration [165]. Thus, for the quantitation of total lipid hydroperoxides, reacting samples solubilized in chloroform: methanol (2:1, v/v) with DPPP dissolved in butanol appeared as a good compromise, in terms of sensitivity and variability. To conclude, in addition to be accurate, sensitive and easy to perform,

methods based on DPPP oxidation are reported to correlate well with other methods (iodometric, thiocyanate and FOX assays), while requiring low sample and solvent amounts [157,165].

5.2.6. Measurement of conjugated dienes

At the early stage of the peroxidation process, alkyl radicals and hydroperoxides arising from polyunsaturated fatty acids, most often stabilize into derivatives bearing a conjugated diene (or conjugated triene in a less extend) system after rearrangement of the double bonds. Being quite stable and absorbing in the UV-domain with a large molar extinction coefficient, these compounds can be detected at 234 nm (268 nm for trienes) with common UV–Vis spectrophotometers. From a practical standpoint, assessing oxidation levels by measuring conjugated dienes is relatively simple and quick, requiring only a small sample amount and no preliminary chemical reactions. However, numerous limitations are associated with this technique, which explains why it has not become as widespread as other methods, such as the peroxide value. Firstly, this method can be applied only to samples with low oxidation level and that have been processed in mild conditions limiting hydroperoxide decomposition. Second, the method suffers from a lack of sensitivity and specificity. On one hand, the absorption peak of conjugated dienes is not well resolved due to a broad absorption band, between 200 nm and 255 nm, partly attributable to unoxidized unsaturated lipids. The resulting low resolution can nevertheless be improved using second-derivative UV-absorption spectroscopy [166–168]. On the other hand, depending on the nature of the sample, many compounds absorbing in the same UV region (e.g. original fatty acids containing conjugated double bonds, carotenoids, pigments, purines and pyrimidines, carbonyls from lipid oxidation) can interfere with the measurement at 234 nm [169] and lead to overestimation. Therefore, preliminary steps of extraction and purification of the samples are usually carried out to suppress undesirable compounds and associated interferences. Conversely, underestimation is possible for samples containing significant amount of oleic acid since the latter, with its unique double-bond, cannot generate hydroperoxides with a conjugated dienic system. This was well exemplified by Marmesat et al. who studied the correlation between the peroxide value and conjugated dienes during oxidation of stripped high-linoleic (conventional) and high-oleic sunflower oils [170]. Interestingly, they found in both cases a strict linear correlation but different slopes, even for high PV levels (400–1000 meq O_2/kg oil). They concluded that PV could be advantageously replaced with the measurement of conjugated dienes, providing that a preliminary calibration curve between the two parameters was established for each oil. Finally, despite the above-mentioned drawbacks, the measurement of conjugated dienes remains widely used to monitor lipid oxidation especially in simple or well-defined systems such as model emulsions [171,172] or bulk oils [173], but most of the time in addition to other methods.

5.3. Measuring the secondary/tertiary markers of oxidation

As mentioned above, primary oxidation products, namely hydroperoxides, are indicative of the oxidation state during the early stages of lipid peroxidation. In other words, as oxidation progresses, it becomes necessary to analyze additional oxidation markers to accurately assess the oxidation state and make valid comparisons when evaluating oxidative stability. Secondary (or tertiary) oxidation products are very diverse in nature, such as simple species including aldehydes, ketones, alkenes, or hydroxy-derivatives, to oligomers and polymers. As a result, these compounds exhibit very contrasting characteristics in terms of reactivity and physico-chemical properties (polarity, volatility, molecular weight), which determine the analytical methods used for their evaluation. Overall, these methods fall into two categories: the first is based on the chemical reactivity of certain secondary markers and their ability to form chromophores with specific reagents, and the second is based on the volatility of these markers. Thus, the three most important and commonly used methods, are the thiobarbituric acid-based assays (TBARS), the p-anisidine assay and the analysis of volatile compounds by gas chromatography techniques.

5.3.1. TBARS assay

Lipid oxidation leads to the formation of various end products, with aldehydes being particularly prominent. Among these, malondialdehyde (MDA) is the most extensively studied representative. It is associated with off-flavors development in presence of unsaturated fat-containing foods, and is considered as a major marker of lipid oxidation in biological systems [174]. Among all the method measuring MDA, the most widespread are based on the reaction of one mole of MDA with two moles of 2-thiobarbituric acid (TBA) and the spectrophotometric measurement of the resulting pink-colored adduct at 532–535 nm. Reaction kinetics depends on temperature and pH (usually medium-high and low, respectively), and TBA concentration [175]. Both the reaction and the measurements are quite easy to perform, which explains the widespread use of the method for assessing the extent of oxidation in foodstuffs, model lipid systems, and biological samples.

However, using MDA assessment with TBA can lead to an underestimation of lipid peroxidation extent. This is because MDA represents only a small fraction of possible secondary oxidation products and is formed only from fatty acids with at least three double bonds, excluding samples rich in oleic and linoleic acids, for instance. Conversely, the TBA reaction is not specific to MDA, and many other reactive compounds (such as aldehydes from lipid peroxidation or sugar autoxidation, Maillard reaction compounds, and amino acids) can also form colored complexes that absorb around 530 nm. This can lead to an overestimation of the results [176]. To overcome this lack of specificity and to improve accuracy, various methodologies have been developed and optimized including MDA distillation prior to reaction with TBA and subsequent analysis of the MDA-TBA complex by high performance liquid chromatography [177,178]. Note that direct and accurate MDA analysis by HPLC is however possible, as reported by Jung et al. who extracted free MDA with acetonitrile from meat products [179]. New developments regarding MDA analysis in biological matrices have been reviewed and pointed out several advanced techniques including liquid chromatography and gas chromatography coupled with tandem mass spectroscopy (MS/MS), as well as some alternative derivatization strategies to MDA-TBA adduct formation [180]. More recently, Bertolín et al. developed a procedure for accurate MDA determination in raw and processed meat using liquid chromatography coupled with a DAD or fluorometric detector [181]. Additionally, Grotta et al. examined sample preparation and treatment methods (such as distillation, acidic extraction, and the use of BHT antioxidant) as critical factors in assessing the oxidation of various meat samples [182]. Interestingly, Poyato et al. demonstrated that measuring TBARS at 390 nm offers greater sensitivity than at 532 nm for monitoring lipid oxidation in heated oils (180 °C – 4 h). This approach enables the prediction and semi-quantification of volatile aldehydes, regardless of the fatty acid profile of oils [183]. However, despite the aforementioned improvements, the spectrophotometric method remains the most popular due to its ease of use. Nonetheless, the term “TBARS” (thiobarbituric acid reactive substances) and the test of the same name were adopted to account for TBA’s lack of specificity towards malondialdehyde. As a consequence, the TBARS assay was adopted in many fields of study, especially those studying the action of antioxidants in food and complex lipid systems, especially in meat. On this particular point, we invite readers to refer to the recent works (experimental studies and exhaustive critical review) done by Ghani et al. [184,185].

5.3.2. Para-anisidine assay

The p-anisidine value (AV) is one of the oldest ways evaluating the secondary products of lipid peroxidation. The method is based on the nucleophilic addition of the p-anisidine amine group onto the carbonyl moiety of aldehydes followed, after dehydration, by the formation of

Schiff bases absorbing in the UV domain at ~350 nm. By convention (AOCS Official Method Cd 18–90), the p-anisidine value is defined as 100 times the increase in absorbance (1 cm cuvette, 350 nm) of a solution resulting from the reaction of 1 g lipids in 100 mL isooctane with 0,25 % p-anisidine solution in glacial acetic acid at a ratio of 5:1 (*v*/v). With the exception of oils and fats directly analyzable in this form, a lipid extraction step is therefore necessary. The p-anisidine can react with all aldehydes, but adducts arising from di-unsaturated aldehydes (2,4-alkadienals), especially nonvolatile species, are those absorbing the more strongly, followed by monounsaturated (2-alkenals) and lastly saturated (alkanals) [186]. Therefore, as a supplement to the AV standard assay, Zuo et al. developed a method for the specific determination of saturated aldehydes [187]. Additionally, an alternative NMR approach that enables the individual quantification of all aldehydes in edible oils was proposed by Skiera et al. [188]. Other aldehydes, unassociated with lipid peroxidation, such as those from the reducing sugar autoxidation or some naturally occurring phenolic antioxidants (vanillin, syringaldehyde, p-hydroxybenzaldehyde, decarboxymethyl oleuropeine dialdehyde) may bias the measurement when present in the sample.

AV is very often used together with PV in assessing the total extent of oxidation by the Totox value (AV + 2PV). Despite its simplicity, this empirical parameter lacks physical meaning because it results from combining two variables with different dimensions, necessitating caution in its use. Although it is considered a suitable indicator of oxidation extent, the correlation of acid value (AV) with other oxidation parameters significantly depends on the composition, physical state, and history (such as storage and heat treatment) of the samples. For example, AV was positively correlated with TBA in n-3 PUFA-rich fish oil samples ($r = 0.733$, $p = 0.025$; [189] and with odor intensity in fried soybean oil and shortening ($r = 0.82$, $p = 0.0001$; [190]). Conversely, no correlation was found between AV and the sensory quality of oxidized omega-3-enriched fat spreads [191] or oat-based biscuits [192]. These findings suggest that AV alone is not a sufficiently reliable indicator for evaluating the sensory quality of foods or the extent of lipid oxidation in complex food systems.

5.3.3. *Analysis of volatile compounds by gas chromatography*

Secondary oxidation products, mainly arising from hydroperoxide decomposition via alkoxyradical α- and β-scissions [193,194] (Fig. 4), correspond to a wide diversity of chemical species (carboxylic acids, aldehydes, ketones, alkenes, alcohols, hydrocarbons, etc.) of which, a significant part is volatile. The latter play a key role in the perception of rancid odors and off-flavors of foods, often at extremely low thresholds and can easily be chromatographically quantified. Aldehydes are the most abundant representatives of these volatiles, some of them, as propanal or hexanal, being specific markers of fatty acids oxidation of the n-3 and n-6 families, respectively (Table 1). In addition, due to its higher production over all other oxidized species, hexanal is considered as one of the best indicators of lipid oxidation, especially in meat and meat products. Although the separation of volatile products can be achieved by HPLC, customary gas chromatography (single column, 1D-GC) is by far the most employed technique in combination with mass spectrometry detection.

Anecdotally, comprehensive two-dimensional gas chromatography (GC x GC or 2D-GC) has been also used to exhaustively analyze volatiles from edible oils [195,196] or mayonnaise [197]. Despite its greater peak capacity, separation power, and sensitivity compared to 1D-GC, this technique is not yet widely used in research. It requires specialized equipment and advanced skills in cross-correlation data processing.

Apart from a few exceptions that will be exemplified later, a concentration step of the volatiles is most often carried out prior to chromatography separation and analysis. This can be achieved through traditional liquid extraction, simultaneous steam distillation solvent extraction, or reduced pressure steam distillation, where the use of appropriate organic solvents allows for high solubilization and quantitative recovery of VOCs. While these methods are well-suited for handling large sample volumes, they are time-consuming and labor-intensive. Additionally, depending on the temperature and duration of the process, degradation of compounds and accumulation of higher molecular weight molecules may occur. Conversely, headspace (HS) analysis, which involves examining volatiles in the vapor phase above a sample placed in an enclosed container, has become widely adopted as the most suitable method for assessing volatile oxidation markers. Developed since the 1950s, particularly for food quality assessment, HS analysis is typically conducted using one of three main techniques. The first, and simplest, is static headspace (SHS). The other two techniques are dynamic purge-and-trap headspace (DHS) and headspace-solid phase microextraction (HS-SPME). Regarding SHS, a liquid or solid sample is placed in a closed vessel where a volatile compound equilibrates between the sample matrix and the surrounding vapor phase. An aliquot of the headspace is then manually or automatically withdrawn and analyzed. This non-selective, inexpensive and easy-to-implement method requires no solvent, no sample preparation, and generates very few artifacts. It has been implemented in many fields, including the characterization of VOCs in edible oils for authentication, certification of origin, contaminant detection or oxidation assessment [198]. However, it is criticized for its lack of sensitivity because (i) only a small portion of the headspace is sampled and analyzed, and (ii) the diversity and amount of compounds extracted are significantly influenced by various factors. These factors include temperature, the amount and physical state of the sample (liquid or solid, specific surface area, particle size, viscosity, polarity), the ratio of sample volume to headspace volume, and the extent of sample homogenization during equilibration. In addition, the time and temperature applied to the sample can lead to degradation of labile molecules or the formation of additional oxidation products (via hydroperoxides decomposition), so that analytical results may not reflect the original VOCs composition and the true extent of oxidation. Nevertheless, a good and even high accuracy can be achieved if a careful optimization of the above parameters is performed. For instance, Azarbad and Jeleń [199] studied the formation of hexanal in different high-fat foods (oil, potato chips and mayonnaise) using automated SHS-GC-FID. By optimizing extraction parameters for each matrix (sample weight, water content, equilibrium temperature and time), the quantification limits were lowered to 0.2–0.3 mg/kg sample, with good linearity ($r = 0.999$), repeatability (<5 %) and intermediate precision (<6 %). To further improve the sensitivity, Jeleń et al. modified the method by trapping (Tenax TA adsorbent) headspace volatiles by successive extractions before desorption in GC–MS [200]. The study used a model oxidized oil with twenty volatile compounds (mainly aldehydes, plus some ketones and alcohols) in deodorized rapeseed oil. Comparing conventional SHS with optimized trapping-assisted SHS, the latter increased sensitivity by 10 to 30 times, provided a high linearity range (up to 0.1–50 mg/L), and demonstrated good reproducibility (RSD% <9 for 80 % of compounds).

With the dynamic headspace technique (DHS), equilibrium between sample and gas phase is no longer necessary since volatile compounds are continuously extracted and transported to a trap via an inert gas passing through (purge and trap) or above the sample (true dynamic headspace). VOCs are then released to the GC after a fast heating of the trap. This technique emerged in the early 1970s in its “purge and trap” version with the use, for the first time, of a trap covered by Tenax GC, a porous polymer material based on 2,6-diphenyl-*p*-phenylene oxide [201]. Through its high affinity for organic compounds, its moderate hydrophobicity and high thermal stability (375 °C), this adsorbent was particularly suited for DHS, overpassing other solid materials. More than a decade later, it is replaced with Tenax TA, an improved version for trapping medium to high boiling point compounds, giving less artifact background on thermal desorption [202]. Despite its propensity to degrade in the presence of oxygen, Tenax TA is likely the most widely used adsorbent. It is however not suited for all applications and other materials might be selected among polymer resins (Chromosorb,

Amberlite XAD), graphitized carbon (Anasorb, Carbotrap, Carbopack, Carbograph) or carbon molecular sieves (Anasorb, Carboxen). Numerous studies have successfully used DHS for the detection of lipid oxidation products in various matrices such as muscle foods, edible oils, emulsions, seafood products, milk, infant formula or topical skin care formulations [198,203–205].

The last technique, namely headspace solid-phase microextraction (HS-SPME), is based on the adsorption/absorption of the headspace volatile compounds onto a polymer material covering a fiber, followed by the thermal desorption of VOCs into the GC column. Developed more than 30 years ago for the extraction of analytes from aqueous solutions [206,207], SPME was quickly adapted to the analysis of flavors/volatile compounds in the agri-food sector where it has become one of the most popular technique [208]. The first step of trapping-assisted SHS is similar to standard SHS: VOCs equilibrate between the headspace and the sample in a tightly closed vial, heated for a fixed time. Temperature is crucial, as is relative humidity for solid samples. Damerau et al. found that water contents of 3.1 % and 5.2 % optimized the release of volatile lipid oxidation products from spray-dried protein-lipid emulsions, both qualitatively and quantitatively [209]. This highlights how hydration of the hydrophilic phase (sodium caseinate-maltodextrin) affects the partitioning and interaction of volatile oxidation products from oil droplet. After introducing the fiber (typically a coated silica or stainless-steel fiber inside the needle of a modified syringe) into the headspace, only a part of the VOCs adsorbs onto the fiber depending on (i) the applied temperature and exposure time, (ii) the nature and volume of the stationary phase, (iii) the competitive adsorption of VOCs onto the fiber and finally (iii) the partition of VOCs between the three phases of the system, i. e. sample, HS and fiber [198,210]. Note that the contact time between the fiber and the headspace can be drastically reduced by enhancing VOCs vaporization by laser irradiation-desorption (LID) of the sample. Applied to the study of tuna oil stability during storage, LID reduces adsorption time from 30 min to just 5 min while providing a similar VOC profile and performance compared to conventional heating [211]. Finally, the fiber is removed from the vial and inserted into the GC injection port where VOCs are thermally desorbed. A particular attention must then be paid to the temperature in order to ensure desorption of the less volatile compounds, while preserving the fiber coating from thermal degradations. HS-SPME is now a mature technology considered to be simple and fast, as well as highly reproducible (both in manual and automated configuration) and versatile due to the wide diversity of commercially available stationary phases and their associated adsorption/absorption properties. Some of them, including polydimethylsiloxane (PDMS), divinylbenzene (DVB) and carboxen (CAR), are now largely dominating the market, most often as composite material combining several of the aforementioned polymers. The main limitations of HS-SPME are (i) the relatively small amount of VOCs adsorbed on the fiber, (ii) the selectivity of the method due to adsorption competition of VOCs on fiber coating, (iii) the more or less rapid deterioration of the fiber coatings depending on the conditions of use and, (iv) for each matrix under study, the need for systematic method parameters optimization. Using HS-SPME, the monitoring of lipid oxidation in the presence or absence of antioxidants have been achieved on many food products such as meats [212,213], fishes and shellfishes [214,215], edible oils [198,211], milks and emulsions [209,216].

In addition to conventional HS-SPME, several other sorptive techniques have been developed to enhance the quantitative recovery of analytes in flavor and volatile compound analysis. These techniques include high-capacity headspace sorbent extraction (HSSE, HS-SBSE), dynamic headspace solid phase extraction (HS-SPDE), dynamic in-needle extraction (INDEX), and in-tube extraction (ITEX). Their principles, advantages, and drawbacks have been thoroughly reviewed [198,208]. To our knowledge, these techniques have been rarely applied to assessing lipid oxidation. Direct headspace analysis without extraction has been explored using techniques like thermal desorption–cryo-focalization (CIS4–TDU) coupled with GC–MS. This method, applied to infant powdered milk, involves thermal desorption of VOCs (primarily aldehydes) at 30 °C, followed by cryo-trapping at −40 °C in the programmable temperature vaporizer inlet. It allows for monitoring oxidation progress even at early stages under both normal storage and accelerated aging conditions [217]. Finally, Selected Ion Flow Tube Mass Spectrometry (SIFT-MS) allows direct analysis of headspace volatiles in air without preliminary sample preparation, pre-concentration, or chromatographic separation [218]. Utilizing ultra-soft chemical ionization with ions such as H_3O^+, NO^+, or O_2^+, SIFT-MS is well-suited for high humidity samples and enables real-time, quantitative analysis with detection limits in the ppt range (by volume).

6. Novel analytical methods for multiproduct detection: Towards a more 'Omic' and spatiotemporally defined approach

6.1. Mass (MS) spectroscopy

Advancements in ionization methods (MALDI and ESI) and mass analyzers/detectors have significantly enhanced the capabilities of liquid chromatography-mass spectrometry (LC-MS) for the detailed analysis of lipid molecules, including non-volatile and thermally unstable oxidized lipids. Furthermore, the identification of oxidized lipids (including eicosanoids and other oxylipins) as potential markers of oxidative stress, along with their role in physiological and pathological processes such as cardiovascular diseases, neurodegenerative disorders, and certain cancers, has driven the increased use of mass spectrometry for investigating oxidized lipids [219,220]. MALDI has been particularly helpful in practical imaging MS, which combines the powerful detection and identification capabilities of MS with microscopy to image biological tissues [221]. Multi-dimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) has advanced comprehensive analysis of cellular lipids, enabling the detailed study of nearly 50 lipid classes and thousands of individual lipid species with high accuracy and precision [222]. A selected reaction monitoring-assisted targeted analytical method using LC-MS/MS has been developed to evaluate fatty acid hydroperoxides [223]. Another study developed a non-targeted approach to analyze hydroxy fatty acids in vegetable oils such as flaxseed and rapeseed oils, providing new insights into the presence of oxidized fatty acids in plant oils and the effects of de-oiling seed processing [224]. In addition, Koch et al. [225] demonstrated that the comprehensive analysis of hydroxy-, epoxy-, and dihydroxy-LA/−ALA carried out by LC-MS after solid phase extraction using aminopropyl cartridges to remove the excess of triacylglycerols provided unique insights into plant oil composition and oxidation processes. They showed that oxylipin concentrations correlated well with the peroxide value (PV), whereas secondary volatile aldehydes did not reflect changes in oxylipins and PV. LC-MS was also used to quantify oxylipins derived from linoleic acid (LA) and α-linolenic acid (ALA) in various oils, including soybean, corn, olive, canola, and four high-oleic acid algae oils, at room temperature and after heating for 10 min at 100 °C [226]. Numerous papers published in the last decade have identified oxidized lipids in a variety of medicinal plants and natural or processed plant foods, such as cereals, nuts, oilseeds, macroalgae, some fruits, and legumes, using mass spectrometry techniques [227–229]. No doubt, the LC-MS approach is one of the most powerful tools for identifying oxidation progress. Moreover, with the growing and anticipated advancements in artificial intelligence and data processing capabilities of computers, LC-MS is expected to become incredibly effective for conducting "omic" analyses of lipids and proteins. However, it is important to recognize that MS generates vast amounts of data and requires extensive expertise in mass spectrometry, along with a significant budget for acquiring and maintaining such instruments. Hence, we believe that this approach will not be used for routine food oxidation analysis but rather reserved for more in-depth studies or integrated into food industry practices, to allow, for example, for more precise monitoring and optimization of processed food quality [230].

6.2. Fluorescence microscopy

Fluorescence spectroscopy involves studying the light emitted by a sample containing fluorophores, which become excited upon absorbing energy from photons and then return to their ground state, emitting light. The fluorophores can vary in polarity, and measurement geometry is important, especially for thick samples, where front-face spectroscopy (exciting the sample's surface) helps minimize reflection or scattering (Fig. 6). Classical modes include emission and excitation spectra, while combining both yields a fluorescence excitation-emission matrix (EEM), useful for capturing all fluorophores but time-consuming. A faster alternative is synchronous fluorescence (SFS) [231]. This technique is effective in monitoring food quality, particularly in fish and meat [232]. Since 1982 [233], many authors have suggested the use of fluorescence spectroscopy as a rapid technique for evaluating oxidation and other changes and determining quality. While the results obtained are interesting, the approach lacks detailed information, particularly regarding heterogeneous samples at the microscopic level. Additionally, there are challenges associated with understanding isolated mechanisms. In addition, not all materials can be excited to fluorescence due to the lack of intrinsic fluorophores. On the other hand, presence of several fluorophores in the examined samples may lead to overlapping peaks, which makes identification of specific fluorophores more complicated.

In this context, the development of confocal laser scanning microscopy (CLSM) enables observation without being constrained by sample structure, as long as the sample can receive fluorescence. To address the spatiotemporal aspects of lipid oxidation in food emulsions, CLSM is increasingly being utilized [234–237]. Fluorescent BODIPY dyes are widely used for this purpose due to their unique hydrophobic properties, making them excellent for staining lipids, membranes, and other lipophilic compounds [238]. They enable local, droplet-specific monitoring of lipid oxidation, as the spectral properties of these dyes change upon reacting with lipid radicals. For example, by using BODIPY$^{665/676}$ and flow cytometry, researchers emphasized how components such as micelles, free fatty acids, and secondary oxidation aldehydes may affect the transfer of oxidation spreading to the remaining emulsion droplets [235]. This work highlights the fact that, under certain conditions, lipid oxidation can spread from oxidizing droplets to non-oxidized ones. A different study using the same fluorophore confirmed that, under certain conditions, the oxidation does not spread rapidly to neighboring droplets [234]. Most current studies use a one-dimensional approach to measure markers of lipid oxidation from an extracted oil phase (see section 5). However, this method fails to capture the complexity of lipid oxidation kinetics occurring at the surface of lipid droplets or within colloidal structures (see section 2.4). The intricate nature of lipid oxidation in heterogeneous systems, considering both spatial and temporal aspects, has led to a shift in research towards spatiotemporally resolved methods. Recently, BODIPY$^{665/676}$ was used to monitor oxidation at the single-droplet level, in conjunction with local protein autofluorescence. The results revealed that the formulation in lipid-rich emulsions, such as mayonnaises, significantly influences the oxidative behavior of both lipids and proteins [237]. Later, by using the fluorescent spin trap CAMPO-AFDye^{647} in combination with BODIPY$^{665/676}$, researchers were able to visualize the different packing of proteins at the droplet interfaces in mono- and polydisperse WPI emulsions [239]. This approach confirmed that oxidation proceeds in a heterogeneous fashion, a detail that cannot be appreciated through one-dimensional measurements of lipid oxidation products. To go further in this research, a cryo-correlative light and electron microscopy (cryo-CLEM) platform for co-localizing the oxidation of lipids and proteins was implemented [240]. This approach revealed that more protein aggregates are present at the droplet interfaces in oxidized emulsions compared to fresh emulsions. Similarly, this approach revealed that in emulsions stabilized with legume protein isolates, oxidation kinetics in protein-lipid aggregates in the water phase proceed significantly faster than in the droplet phase and are influenced by tocopherols from the oil phase as well as by transition metals present as co-passengers in the protein isolates [241]. In conclusion, with anticipated advancements in imaging resolution and data processing [242], microscopy will significantly enhance our understanding of the relationship between physical distribution and oxidative stability in food emulsions, particularly in elucidating this spatiotemporal phenomenon.

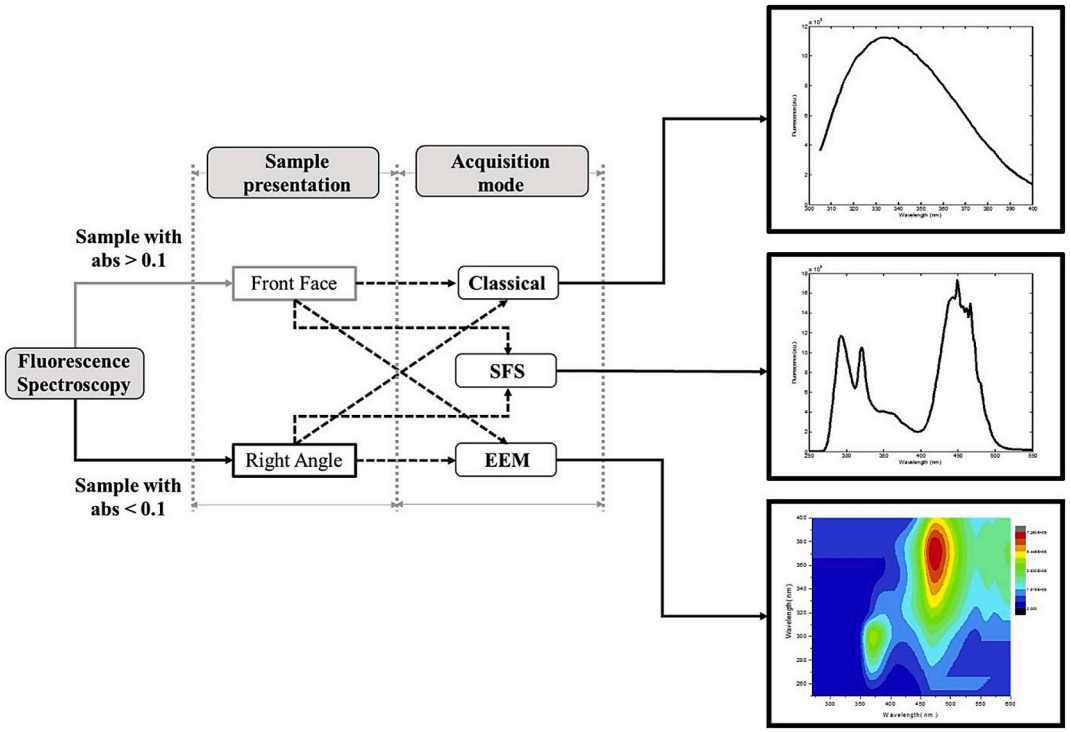

Fig. 6. Principal acquisition modes of fluorescence spectroscopy reproduced with permission from [232].

6.3. Infrared (IR) spectroscopy

Modifications of spectral properties of a sample across the wavelength range of 14,000 to 4000 cm^{-1} (Near-IRS) or 4000 to 400 cm^{-1} (Mid-IRS) have been widely employed to monitor qualitative or quantitative changes in food matrix quality. Since the 90's the development of Fourrier-transformed (FT) but also attenuated total reflectance (ATR) have helped the development of this method to follow oxidation. FT-IR is fast and non-destructive as the sample can be scanned without any specific preparation. (ATR)-FTIR or NIRS have been used by several authors to measure lipid oxidation in various matrices: in bulk oils first [243–246] and more recently in more complex emulsions [247–250]. Infrared spectroscopy emerges as a rapid and straightforward method for measuring lipid oxidation in complex foods by analyzing marker bands that indicate chemical changes during processing and storage.

The primary advantage of this approach is that it eliminates the need for the tedious lipid extraction step. To this purpose, specific calibration methods using multivariate analyses and chemometric tools such as Partial Least Squares Regression (PLSR) are generally necessary to develop models that will correlate with one or several oxidation indicators, such as peroxide value (PV), TBARS, conjugated dienes (CD), p-anisidine values, or volatiles, etc. and thus be able to determine the system's oxidation state. For instance, strong correlation coefficients ($R^2 > 0.9$) were achieved between volatile content and infrared spectroscopy, enabling the detection of spectral changes specific to lipid oxidation in infant formula containing proteins and sugars. In this context, Near-Infrared Spectroscopy (NIRS) outperformed ATR-FTIR, with prediction errors of 9 % and 18 %, respectively [249]. Similarly, in oil-in-water emulsions, ATR-FTIR and NIRS were also employed to monitor oil oxidation, predicting conjugated dienes (CD) levels without sample preparation using PLSR models based on different spectral regions [247,248]. The prediction accuracy ranged from 7.8 % (NIRS) to 18 % (ATR-FTIR). Recently, a model using NIRS spectra and partial least squares discriminant analysis (PLS-DA) was developed to predict the oxidation state of a water-in-oil-in-water emulsion by utilizing peroxide and anisidine values. Results showed that a predictive and appropriated model can be successfully applied to determine the lipid oxidation state of an emulsion stored in a glass-sealed container, instead of using a fiber optic probe [250]. The presence of overlapping peaks in complex mixtures complicates the identification of individual functional groups, making it challenging to evaluate oxidative degradation of lipids in food systems. However, the resolution and accuracy of IR spectroscopy for a specific system can be improved through multiple correction techniques, spectral preprocessing, and extensive data collection. Developing a versatile model with high predictive accuracy across different lipid-based systems could significantly boost the adoption of this approach.

6.4. Nuclear magnetic resonance (NMR) spectroscopy

The use of NMR (^1H or ^{13}C) to assess the lipid oxidation status of samples is highly promising, as it allows for the elucidation and quantification of newly formed chemical structures over time. This makes NMR a valuable tool for evaluating chemical pathways and identifying new oxidation products. However, the interpretation of the collected NMR data requires high skills and experimented operator. Although the elucidation of hydroperoxide structures dates back to the 1970s [251,252], one of the earliest studies demonstrating the potential of ^1H NMR to evaluate lipid oxidation and its multicomponent analytical capabilities was conducted more recently [253]. In this work, the authors used ^1H NMR (270 MHz) to determine the ratios of aliphatic to olefinic or/and to diallylmethylene protons in fish oil. These ratios were then compared with peroxide values and acid value. The authors concluded that, despite the limitations of their 270 MHz equipment at that time, this spectroscopic technique showed promise for assessing lipid oxidation kinetics. The same approach (ratio of aliphatic to olefinic or/and to diallylmethylene protons) determined by ^1H NMR were also used by others to assess the oxidative status of lipids in mackerel muscle [254] and in sesame oil [255]. Going beyond the determination of these ratio by NMR, [256] focused on identifying PUFA oxidation products generated in culinary oils subjected to standard frying procedures. The authors observed the appearance of primary oxidation compounds, including conjugated diene olefinic protons (multiplets at 5.4–6.7 ppm), broad hydroperoxide group -OOH protons (singlets at 8.5–8.9 ppm), and CH(OOH) protons (multiplet at 4.35 ppm). They also identified various secondary oxidation compounds of the aldehyde series, such as trans-2-alkenals, alka-2,4-dienals, and 4-hydroxy-trans-2-alkenals. Further on, their use of two-dimensional COSY ^1H NMR favoured the distinction between the four classes of aldehydes and allowed the identification of trans-2-heptenal and trans-2-octenal, and hexanal that are generated upon oxidation of linoleic acid. In a similar manner, sunflower oil oxidation has been studied by ^1H NMR, to follow the appearance of many oxidation products, namely hydroperoxides, conjugated dienes, aldehydes including oxygenated alpha, beta-unsaturated aldehydes, and mono and diepoxides [257]. Depending on the oxidation conditions (70 or 100 °C), different kinetics and distributions of the primary oxidation compounds were observed, although the same types of aldehydes were formed. Interestingly, the authors used characteristic NMR spectra to demonstrate the simultaneous formation of mono and diepoxy derivatives along with aldehydes, showing that both aldehydes and epoxy acyl groups are generated as soon as hydroperoxides begin to degrade. Later, the quantification of these epoxides by ^1H NMR was further developed by [258]. Soybean oils with various concentrations of synthesized epoxides were analyzed using the signal areas at 2.90 and 3.24 ppm, with sn-1,3 glycerol protons (4.18 and 4.33 ppm) used as internal references. The NMR results were compared to epoxide content determined by titration with hydrogen bromide (HBr)–acetic acid solution, showing a very good correlation. When comparing the evolution of epoxide content with peroxide values, contrasting patterns were observed, suggesting that epoxide content offers a different perspective on the extent of lipid oxidation compared to peroxide values. The correlation between ^1H NMR spectroscopy and other "classical" methods to evaluate lipid oxidation was performed on different lipid matrices. For example, the oxidation of ethyl docosahexaenoate was evaluated by ^1H NMR (600 MHz) and compared with traditional measurements using PLSR regression [259]. The NMR detection limit for selected oxidation products was found to be <0.01 mM. Regression analysis of each classical method, along with the increase in peak intensity at specific areas of the NMR spectra, allowed for a good correlation with peroxide values (PV), conjugated dienes, or TBARS. In contrast to previous results, the authors concluded that the ratio between olefinic and aliphatic hydrogen atoms was not a reliable indicator for tracking oxidation. Instead, they recommended a detailed multivariate data analysis of the 9–10.5 ppm region of the spectra, as this region is expected to contain only oxidation products (peroxides and aldehydes) and no other components. Moreover, as hydrogen signal of undegraded fatty acids are much higher than the ones of oxidation products, an enlargement (x10) of this specific area must be carried out.

Other authors evaluated ^1H NMR as a tool to quantify aldehydes (n-alkanals, trans-2-alkenals, 4-hydroxy-trans-2-alkenals, alka-2,4-dienals) in olive, soybean and sunflower oils subjected to frying conditions (180 °C, 3 h) [260]. They found a good linear relationship between aldehyde levels and the determination of total polar compounds. Similarly, the formation of aldehydes and hydroperoxide was followed and quantified in thermally oxidized peanut oil (180 °C) [261] or in sea bass oil [262]. [263] evaluated the oxidation of fish oils using ^1H NMR and compared the results with those obtained from traditional methods such as peroxide value (PV), anisidine value, TOTOX value, and acid value. Again, PLSR models were employed, and specific spectral regions were identified. Results showed excellent correlation with the traditional oxidation metrics, with $R^2 = 0.949$, 0.962, 0.991, and 0.977 for PV, anisidine value, TOTOX value, and AV, respectively.

In addition to providing information on oxidative status and kinetics,

^{1}H NMR can also assess the oxidative stability of oils based on their fatty acid compositions and regiodistribution. [264] investigated the impact of linoleic acid's positional distribution in triacylglycerols on oil stability. They compared the oxidation and stability of soybean oils with similar total fatty acid compositions but different positional distributions (non-modified versus interesterified oils). Using ^{1}H NMR, they quantified remaining linoleic-bound triacylglycerol and its oxidation products, including cis-trans- and cis-cis-conjugated dienes, hydroperoxides, and aldehydes. The results indicated that oil with linoleic acid predominantly at the sn2 (central) position exhibited greater resistance to oxidation.

In another study, ^{13}C NMR spectroscopy was used to investigate the lipid oxidation mechanisms during the heating of salmon oil [265]. The study demonstrated that allylic sites closest to the carbonyl group were more susceptible to oxidation, followed by those near the methyl terminal group. In contrast, unsaturated bonds in the middle of the carbon chain showed minimal damage. Still focusing on fish oil, ^{13}C NMR spectroscopy analysis has been conducted to monitor the formation of stable hydroxy derivatives from the oxidation of linoleate esters or fish oil [266]. The study concluded that, compared to HPLC analysis, this method lacks sensitivity for detecting hydroxy products at the early stages of oxidation.

Recently, [267] evaluated the potential of quantifying oxidation compounds by ^{1}H NMR (600 MHz, equipped with a 5 mm cryoprobe) in complex matrices such as food emulsions (mayonnaises). To achieve this, the authors first used a freeze-thaw process to break the emulsion, allowing clear separation of the aqueous and lipid phases, with the lipid phase subsequently withdrawn for NMR analysis. Since their method aimed to detect lipid oxidation at early stages, they applied specific signal treatment to reduce digital noise in the hydroperoxide and aldehyde regions and suppress signals from abundant unoxidized lipids. A 1D band-selective gradient pulse was applied to specifically excite the signals of oxidation compounds, allowing distinct acquisitions in the hydroperoxide (δ 11.5–10.5 ppm) and the aldehyde (δ 10.0–9.0 ppm) regions. Through band selective NOESY and TOCSY experiments and principal component analysis (PCA) modeling, twenty hydroperoxides and aldehydes were simultaneously and accurately quantified, with quantification limits for individual hydroperoxides and aldehydes around 0.01 and 0.03 mmol/kg, respectively, which is comparable to traditional PV determination. This method has also been used to investigate and uncover the distinct mechanistic effects of α-tocopherol and γ-tocopherol, as well as their concentration-dependent influences, on soybean oil peroxidation. After dissolving the oil samples in deuterated chloroform, they simultaneously monitored the formation of hydroperoxides, associated conjugated dienes, conjugated hydroxy-dienes, keto-dienes, aldehydes, epoxides, and alcohols [268,269].

To expand the quantification of underexplored oxidation products such as epoxides, a 2D ^{1}H—^{13}C HSQC NMR spectroscopic method has been developed [11]. Through this study, the authors demonstrated that epoxides, formed following hydroperoxide accumulation via alkoxyl radical intermediates, accounted for 10–40 % of the oxidation products. Furthermore, epoxides, formed via the cyclization of alkoxyradicals (Fig. 2)—competing with their cleavage into aldehydes (Fig. 4)—have emerged as critical markers for modeling lipid oxidation mechanisms, as previously suggested by Schaich et al. [3,10].

In addition to these chemical and reactive aspects, NMR has recently shown its capability to report on the microstructural characteristics of emulsions and the diffusional behavior of their main components. For instance, 2D Diffusion Ordered Spectroscopy (DOSY) NMR was developed to characterize the distribution and diffusion of medium-chain triglycerides (MCT) and the emulsifier Tween 80 (5 wt%) in model oil-in-water emulsions, by monitoring their translational self-diffusion coefficients [270]. They demonstrated that when MCT concentrations were below 1 wt%, Tween 80 micelles coexisted with thermodynamically stable microemulsion droplets of similar size (~12 nm in diameter), composed mainly of Tween 80 with a small amount of dissolved MCT. At higher MCT concentrations (up to 5 %), these small microemulsion droplets (~12–22 nm in diameter) persisted alongside kinetically stable nanoemulsion droplets (less than ~200 nm in diameter) and larger emulsion droplets (greater than ~200 nm in diameter) that contained minimal emulsifier. The authors suggested that the small microemulsion droplets, present irrespective of the MCT concentration, could act as active carriers, facilitating the dissolution and transfer of molecules between the emulsion droplets and thereby promoting the spread of potential chemical reactions throughout the emulsion.

In summary, NMR spectroscopy is a promising tool for the rapid evaluation of lipid oxidation. Within a short period of analysis and using a small amount of sample without derivatization, it allows to obtain a more global overview of lipid oxidation pathways through the determination of the structure of various oxidation compounds and their concomitant quantification. The primary drawback is that, like many other techniques, it necessitates the extraction of the lipid fraction when working with complex media, such as emulsified systems.

7. Conclusion and perspectives

As we have seen, lipid oxidation is a complex dynamic phenomenon that results in the formation of numerous products families, some of which are particularly suitable as markers for studying the various stages of the peroxidation process. This has resulted in the development of a wide range of analytical methods focusing specifically on one type of oxidation product (primary or secondary), none of which, taken separately, allowing an exhaustive account of all the oxidation mechanisms involved. Although numerous tests are available to evaluate the antioxidant efficacy of a compound or extract, it is clear that these tests are often conducted under varying conditions, making the resulting measurements difficult to compare. Caution is needed to avoid predicting antioxidant activity based on overly simplistic tests with limited interpretative value, and conclusions drawn from the data should not be overstated. Therefore, selecting the appropriate test is crucial, based on the relevance of the information it offers.

In this context, many authors have embarked on the path of multivariate analysis, but the number of data to be processed with the available computer resources (in particular raw data from spectroscopy techniques) has proven to be the main limiting factor. However, advances in computing technologies over the past 15 years now make it possible to routinely process huge datasets with minimal loss of information. Thus, chemometrics, which consists in the computational analysis of multivariate results, has been seen as a powerful and particularly appropriate tool for unravelling the complexity of oxidation phenomena and the action of antioxidants. Among multivariate analysis techniques, PCA, an unsupervised method, is the most frequently used. It is often utilized alongside other multivariate methods which are unsupervised such as cluster analysis (CA) or are supervised such as PLSR and PLS-DA, to name a few [271].

Today, the abundant literature on chemometrics and metabolomics applied to the elucidation of oxidation phenomena in foodstuffs, testifies to the strong interest and expectations aroused by these approaches. In a general way, multivariate analyses are applied on datasets often associating global oxidation measurements such as PV, TBARS or AV (p-anisidine value), with chromatographic, spectroscopic, or sensory analyses. For instance, Ritter and Budge [272] used oxidation descriptors to predict fish oil sensory quality, identifying eight key volatile aldehydes and ketones responsible for sensory degradation. However, PV and AV showed weak correlations with sensory properties, making them poor quality indicators. In another study, Mancebo-Campos et al. [273,274] developed a shelf-life predictive model for virgin olive oil using PCA and multivariate regressions, allowing the calculation of oxidation progress based on various parameters. Tan et al. studied milk photooxidation, identifying four biomarkers affected by oxygen and light, with a strong correlation between sensory scores and oxygen content [275]. These examples illustrate the general trend of applying multivariate analyses

to data that (i) come from various measurement methods, each with its own biases and limitations as discussed earlier, and/or (ii) derive from the analysis of a specific component of the system being studied.

From our point of view, this only partially meets the requirements of an omic approach, i.e. holistic and non-reductionist, as proposed by several authors [276–278]. Ideally, this would require a multi-scale approach that enables both the simultaneous and precise monitoring of key oxidation products and antioxidants (both spatially and quantitatively). Lastly, and just as importantly, these data should be time-resolved to provide a comprehensive and dynamic understanding of the entire system. In this context, recent advancements in NMR spectroscopy, fluorescence imaging, and mass spectrometry hold significant promise.

CRediT authorship contribution statement

Erwann Durand: Writing – review & editing, Writing – original draft, Conceptualization. **Mickael Laguerre:** Writing – review & editing, Writing – original draft, Conceptualization. **Claire Bourlieu-Lacanal:** Writing – review & editing, Writing – original draft, Conceptualization. **Jérôme Lecomte:** Writing – review & editing, Writing – original draft, Conceptualization. **Pierre Villeneuve:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

None.

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