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Review

Determination of hydroperoxides in foods and biological samples by the ferrous oxidation–xylenol orange method: A review of the factors that influence the method's performance

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The oxidation of lipids yields both primary and secondary oxidation compounds that produce undesirable biological effects [1–3] and includes loss of nutritional value and sensory problems in foods [4,5]. Primary oxidation products include lipid hydroperoxides (HP)[1] which can further decompose into secondary oxidation products and/or react with other compounds present in the food or biological material [6–8].

The measurement of HP, in connection with free radicals and other reactive oxygen species, has been used as indication of oxidative stress in biological samples [7,9–13] and associated with the pathogenesis of several diseases such as atherosclerosis [14,15], cancer [16,17], and neurodegenerative diseases [18–20]. Unfortunately, the majority of analyses to assess lipid oxidation in biological and food samples determine only secondary oxidation products, whereas the determination of HP could give an early and more accurate indication of the oxidative status. Hence, a proper assessment of the degree of oxidation in any kind of sample should be accomplished by the appropriate selection of the methods that include the determination of both primary oxidation products and their decomposition products [8,21,22].

However, the determination of lipid HP is quite challenging because many different kinds of HP are produced from lipid oxidation and HP are reactive compounds that rapidly react and decompose even at moderate temperatures [22]. Thus, a great variety of methods have been proposed to assess lipid HP in biological samples and foods [8,21–24]. Among them, chromatographic (GC and HPLC), spectroscopic (NMR and ESR), and enzymatic methods showed high sensitivity, selectivity, and reproducibility [21,24–30] but their application to routine analysis is compromised and not all laboratories have the necessary instrumentation. More simple methods that measure HP by titration and colorimetric methods based on iodide or iron oxidation are also available [31–33]. However, a simple routine method should be reproducible and sensitive also. In this frame, and based on the oxidizing properties of HP, the ferrous oxidation–xylenol orange (FOX) method is of interest.

Briefly, the FOX method is based on the ability of HP to convert ferrous ions into ferric ions which subsequently form a complex with xylenol orange (XO) that is determined through spectrophotometry. This method offers the possibilities to determine the total HP content rapidly and with low cost and to assess the susceptibility to oxidation [34]. Moreover, the FOX method has been reported to be simple, sensitive, and selective for the total amount of HP, with a good precision and potentially useful in a variety of matrices [34–36]. Despite that, many variables have been reported also to influence the performance of this method [37–39]. This paper reviews the major advantages and drawbacks of the FOX method, its applicability in different matrices, and the factors that influence its specificity, selectivity, and sensitivity.

The Fox Method: Principle, advantages, and disadvantages

Gupta [40] first described the FOX method for the analysis of hydrogen peroxide in irradiated solutions. Later, Jiang et al. [41]

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[1] Abbreviations used: FOX, ferrous oxidation-xylenol orange; HP, hydroperoxide; GC, gas chromatography; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; ESR, electron spin resonance; XO, xylenol orange; FTIR, Fourier transform infrared; TPP, Triphenylphosphine; BHT, butylated hydroxytoluene; LDL, low-density lipoprotein.

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adopted this method to demonstrate that mixtures of protein and glucose generate hydrogen peroxide under certain conditions. This latter research group realized that this method can also be used in the determination of lipid HP in liposomes, plasma, and lipoproteins [35,42–44]. The FOX method is based on the oxidation of ferrous to ferric ions by HP under certain acidic conditions at room temperature (Eq. (1)). The dye XO [3,3′-Bis(*N*,*N*-bis(carboxymethyl)aminomethyl)-*o*-cresolsulfonephthalein] tetrasodium salt shown in Fig. 1 then binds ferric ion to form a chromophore complex (Eq. (2)) which absorbs strongly at 540–600 nm.

$$ROOH + Fe^{2+} \rightarrow Fe^{3+} + RO^{\cdot} + OH^{-} \quad (1)$$

$$Fe^{3+} + XO \rightarrow Fe - XO. \quad (2)$$

Therefore, this method is simple to perform and the spectrophotometer, which is available in most laboratories, is the only required instrument. Moreover, it can be used routinely and it determines the total amount of HP. Nowadays, kits based on this method are available in the market and measure HP in oils and fats after 15 min [45,46].

Chromatographic techniques can be used for determining HP in different molecular classes [21] and then can be measured using a postcolumn FOX method [47] or through different detectors [21,48]. Separation techniques such as HPLC avoid most of the interference compounds that affect several methods, especially those based on chemilumiscence or fluorescence reactions [21,24,29,49,50]. Other separation techniques such as GC-MS can also be used, although they indirectly measure HP by their preceding reduction into hydroxy fatty acids [51,52].

In relation to iodometric methods, the classical titration methods, such as the AOAC Official Method [32] and the AOCS Official Method [53] in which HP reacts with iodide, have the advantages that they are simple to perform and the stoichiometry is known. This latter official method has been shown to be highly correlated ($r \geqslant 0.95$) with the FOX method, although it entails several drawbacks such as the high amount of sample needed and the uncertainty of the titration endpoint [46]. In addition, the iodometric reaction is affected by several conditions and is sensitive to interfering agents [33,54,55]. An important interfering agent in iodide-based methods is the presence of oxygen [33], whereas the FOX method is almost oxygen insensitive [34]. Therefore, several alternative iodometric methods, based on the colorimetric detection of the triiodide chromophore [33,56,57] and on the endpoint potentiometric detection [58], have been developed to overcome some of these problems and to increase the specificity and sensitivity up to 10-fold compared to titration methods.

Taking into account the simplicity, the costs, and the possibility to adapt the method to routine analysis, the spectrophotometric determination of conjugated dienes (absorption at 230–235 nm) is also an alternative with good sensitivity that has been applied to several matrices [59–63]. Nevertheless, as its name indicates, apart from HP, this method determines all kinds of conjugated dienes including those without HP and does not determine those HPs that do not have conjugated double bonds.

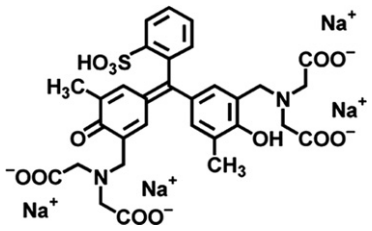

Fig. 1. Xylenol orange structure.

In neat fats and oils, Fourier transform infrared (FTIR) spectroscopy is a good alternative to routine analysis that has been applied successfully to measure HP [64,65]. In addition, increased selectivity and sensitivity can be achieved after the reaction of triphenylphosphine (TPP) with HP which forms TPP oxides that have characteristic FTIR bands [66,67].

Finally, a spectropothometic method based on the Standard Method of the International Dairy Federation [68] has been used in milk-based products [69–72] and has been adapted to fats and oils [31] and food lipids extracts [31,73–76]. The formation of the thiocyanate-ferric ion complex yields a red-violet color of strong absorption at 500–510 nm which allows the total amount of HP to be determined with the use of common laboratory instruments [31,77]. Like the FOX method, this method utilizes the oxidation of ferrous ions to ferric ions in acidic media by HP but then reacts with thiocyanate instead of XO, thus explaining the correlation between methods [31,55,78]. Both methods based on ferric ion complexes are simple and specific for HP, require low amounts of sample and solvents, and are sensitive [46,55]. In addition, these methods are pretty similar and the advantages such as the high specificity of the reaction for HP [43,74,79] or the disadvantages such as the spectral interference by some pigments [74,80] are common.

Nevertheless, the main advantage of the FOX method in relation to the latter is the broad applicability because it has been used in biological samples [81,82], in fats and oils [31,83], and in lipid extracts and homogenates from meat and vegetables [34,80,84]. Conversely, the main disadvantage of the FOX method seems to be the low linear range [55] and the low reproducibility [21] because it is affected by several factors that are reviewed below.

Influencing factors: Setting up the method

Although the FOX method is potentially useful in a variety of applications it requires a careful control of the conditions used. Moreover, the dependence of the apparent molar absorption coefficients (ε) on various variables makes it recommendable to set up this method and determine the absorption coefficients before analyzing each kind of sample. Among those variables, the kind of sample and the previous extraction and/or purification steps can influence HP determination. The majority of samples are extracts from protein precipitation with alcohols [34] or from lipid extraction [36] which are then added to the reaction media. Therefore, it is important to select the appropriate solvent to extract the HP in each case. In addition, it is important to determine the appropriate amount of extract to have sufficient sensitivity while being within the linearity range of the method.

Various compounds have been reported to interfere with FOX determination (Table 1). Some of these compounds are oxidizing/reducing agents that can be present in the samples endogenously [84–86], whereas others such as EDTA and other chelators can be added [43,86,87]. In relation to the reacting iron in the assay, it should be taken into account that Fe^{2+} rapidly converts to Fe^{3+} at pH above 7 so it has to be directly dissolved under acidic pH to make it more stable and further specific to HP reaction. Nevertheless, in some cases, even when the iron solution is acidic the iron has been reported to have a poor stability [31] so it is preferable to prepare the reactants extemporaneously.

On the other hand, contamination with prooxidant metals such as iron is quite common because is a ubiquitous contaminant so it is recommended to use high-purity reagents and clean glassware [86]. To avoid metal leaching, glassware is reported to be cleaned with hot concentrated nitric acid and then rinsed with four-stage purification water [88]. However, glassware and cuvettes cleaned with a sulfuric acid-dichromate cleaning solution and rinsed with

Table 1
Summary of the reported matrix interferences in different ferrous–xylenol orange (FOX) methods

Reported matrix interferences	Brief description
Pigments	Unspecified pigments absorbing at 500-600 nm are found in centrifuged plant homogenates made with ethanol/water (80:20, v/v). The interference was not suppresed by catalase and TPP addition [80]. Carotenoid and chlorophyll interference found in plant extracts made with chloroform/methanol/0.15 M acetic acid (2.5:5:1, v/v/v). Their presence is confirmed by the typical absorbance spectra and by the nonsupression absorbance after TPP addition [89].
Ascorbic acid	100 μM in the assay caused 20% increase in color yield in the presence of 4 μM linoleic acid HP [43]. The addition of ascorbic acid (0.3–5 mM) to the reaction mixture after the complex formation caused a decrease in the absorbance but at 0.3 mM this effect was only transient [84]. Their presence at concentrations from 12–100 μM has no effect, whereas in plasma at concentrations higher than 200 μM the absorbance rises [86]. In the presence of air and using the FOX-1 method increasing concentrations of ascorbic acid to the media caused a decrease in the slope of the standard curve made with H_2O_2. Ascorbic acid at concentration of 20 μM yields almost no increase in absorbance with increasing standard H_2O_2 addition [90].
Free iron	Iron relase in blood samples as a result of hemolysis will overestimate the HP content [86].
Chelators	Addition of chelating agents used as anticoagulants during blood collection will underestimate the HP content [86,87].
Proteins	Using perchloric acid the addition of 10% human blood serum lowered the absorbance by 5.8% whereas using sulfuric acid the absorbance is much more lowered [39]. The addition of bovine serum albumin (1.5 μM) does not affect the color yield of added linoleic HP or H_2O_2 [43]. The addition of bovine serum albumin at 4.7 μM into the media reduced the response generated by the addition of peroxidized linoleic acid, whereas this is completely supressed with the addition at 44 μM [80]. Proteins are isolated by precipitation with 0.2 M perchloric acid and redissolved in 6 M guanidine HCl and then HP were measured through a FOX method [91].
Reducing compounds	At 100 μM uric acid increased color yield about 6%, whereas at the same concentration reduced glutathione had no effect on color yield [43]. The addition of cysteine ($\geqslant$0.2 mM) and glutathione ($\geqslant$0.2 mM) to the reaction mixture after the complex formation caused a decrease in the absorbance [84].
Lipid peroxides	The precentage reactivity relative to H_2O_2 of dicumyl, benzoyl, and lauroyl peroxides is 12, 9% and 12%, respectively in a FOX-2 method [43]. The precentage reactivity relative to H_2O_2 of dicumyl, and benzoyl,s is 1% and 5% in a FOX-1 method [43]. Serial cyclic peroxides, monocyclic peroxides, and bicyclic endoperoxides derived from cholesteryl arachidonate reacted with XO [93].
Chain–breaking antioxidants	The addition of BHT at 3.8 mM into the media containing linoleic acid HP and FOX reagents reduced color yield about 17%, whereas the addition of BHT before the FOX reagent reduced color yield about 44%. In addition, time course analysis showed that the response is lowered and reaching a steady point takes longer when samples contain increased levels of tocopherol or when BHT has been added [34,60,102]. The addition of tocopherol at 500 μM has no effect on color yield [43]. The addition of BHT at 4 mM has no effect on time course when H_2O_2 is added to the media, whereas the reaction induced by tissue extracts is inhibited [84].

BHT, butylated hydroxytoluene; HP, hydroperoxide; TPP, triphenylphosphine; XO, xylenol orange.

double-distilled water were reported to not have iron contamination problems in different food matrices [34,36].

Sample matrix

Two earlier versions of the same method depending on the type of matrix and HP to be determined have been developed [35]. The first version, also called FOX-1, consists of the addition of 950 μL of a solution reagent containing 100 μM XO, 250 μM ammonium ferrous sulfate, 100 mM sorbitol, and 25 mM sulfuric acid to 50 μL of aqueous test sample. Because the reagent is prepared in aqueous media together with sorbitol, which enhances the response, the measurement of low levels of water-soluble hydroperoxides such as hydrogen peroxide, butyl, and cumyl HP is possible [42,90,94,96,97]. However, in the presence of high amounts of nonperoxidized lipids, the aqueous media used in this method are not suitable for measuring small levels of lipid-soluble HP.

Therefore, a second version, called FOX-2, was developed to measure lipid-soluble HP in biological samples. Traditionally, this consists of the addition of 950 μL of a solution reagent containing 100 μM XO, 250 μM ammonium ferrous sulfate, 4 mM butylated hydroxytoluene (BHT), and 25 mM sulfuric acid to be dissolved in methanolic solution (90%, v/v). This is then mixed with 50 μL of aqueous or organic sample solutions which sometimes contain large concentrations of nonperoxidized material. This second method, thereafter referred to simply as the FOX method, omitted sorbitol to avoid extensive peroxidation and, thus, added a chain-breaking antioxidant to avoid artifact formation. This latter FOX method, with few modifications, has broader applicability and suitability and has been applied to biological samples such as plasma [9,81,98,99], serum [11], urine [100], and various animal tissues [84,101] and to meat [34,102], vegetables [80], and other food products [36,103].

There is a broad range of biological and food matrices (e.g., plasma, edible oils and fats) that can be added directly to reaction media which may contain different mixtures of solvents [31,35,46,78,81,83,99]. Other sample matrices such as animal tissues [84,101,104,105], meat [34,106,107], fish [103,108,109], shellfish [101,110], and plants, fruits, and nuts [80,111–114] are homogenated with a polar organic phase and then either added directly or are centrifuged to obtain a supernatant that is added into the reaction media. Organic-phase supernatant extracts are commonly made with alcohols, especially methanol [80,102,103].

Alternatively, lipid in the sample can be first extracted and then added to the reaction media as reported in human blood serum [91], meat [31], fried snack products [36], and fruits [89,115]. Lipid extraction is conducted with organic solvents of medium polarity such as chloroform/methanol (2:1 and 1:2, v/v) and hexane/isopropanol (3:2, v/v) to extract the lipid content including the lipid HP. Traditional extraction methods based on chloroform methanol mixtures [116,117] are commonly used. Unfortunately, little attention has been devoted to the selection of the appropriate solvent for HP recovery and to the effect of solvent type on the extraction of interfering compounds. Therefore, the selection of the appropriate solvent or mixture of solvents should take into the account whether they are added directly to the reaction media or used only to extract the lipids.

In relation to HP recovery in sample direct extracts, the ethanol/water (80:20, v/v) mixture seemed to be more effective in recovering HP than either 100% ethanol or ethyl acetate in plant extracts [80]. In addition, a relatively high-polarity solvent such as 100% methanol has been used in several food and biological samples

[34,84,103]. The use of methanol seems appropriate because it denatures proteins and allows for a good recovery of lipid HPs which are more polar than unoxidized lipids. Less polar solvents will extract the unoxidized lipid fraction together with the HP. For instance, ethyl acetate has been used to extract plasma HP present in a hydroalcoholic solution with relatively high recoveries [44]. Despite that, relatively more apolar solvents such as the mixture chloroform/methanol (2:1, v/v) are commonly used because they are efficient at extracting all lipids and thus give high lipid HP recoveries [36,70,91]. Once extracted, the extracting solvent is commonly evaporated before determining the HP content to increase the sensitivity [31,36,91].

In addition to HP, several pigments can be extracted by the solvents used in the FOX assay [89,118]. Therefore, special attention should be paid to those pigments that strongly absorb in the same range as the Fe–XO complex. For instance, some heme compounds have a strong absorbance around 560 nm and may cause interference although they are mainly soluble in aqueous media and/or easily denatured by alcohols. Anthocyanins at low pH (490–550 nm), chlorophyll derivatives (absorbance maximums at 400–455 and 645–665 nm), and carotenoids (350–550 nm) have spectra in the range from orange to blue regions [119]. Although these compounds do not absorb strongly in the absorbance range of the Fe–XO complex, at very high concentrations they would interfere with the assay.

Controversial results on the influence of ascorbate in the samples have been reported. Lowered responses are explained by the reduction of the formed ferric ions by ascorbate, whereas enhanced measures are attributed to the ferric ion possibly causing the oxidation of ascorbic acid in the presence of oxygen generating hydrogen peroxide. Those controversial effects seemed to be dose dependent [35]. For instance, at low concentrations (<20 μM) the *in vitro* activity of ascorbate in reducing ferric ions may lead to a lower formation of the ferric–XO complex [35]. Moreover, Hermes-Lima et al. [84] also suggested that ascorbate is able to reduce the ferric ion complex because of recorded decreases in the ferric ion–XO complex when ascorbic acid is added at doses $\geqslant$0.3 mM, although this effect is only transient at this latter concentration. Conversely, Nourooz-Zadeh [86] reported that at concentrations higher than 200 μM ascorbate would give rise to a high background signal, whereas at physiological concentrations (12–100 μM) ascorbate has little or no effect on HP determination [43]. In addition, ascorbic acid is often removed or significantly reduced by solvent extraction in FOX methods. However, this interference can be of importance in aqueous methods and/or in complex matrices that may contain high levels of ascorbic. Bleau et al. [90] reported that ascorbate at 20 μM increases the absorbance in the FOX-1 method. The authors suggested that this increase of absorbance can be explained because ascorbic acid, catalyzed by ferrous ion, can react with oxygen to generate a superoxide radical which then can form hydrogen peroxide. However, the addition of ascorbate oxidase has been suggested to overcome this interference [91].

Another important interference is the presence in the sample of free iron, for instance coming from hemolysis [86], and chelators that will affect the method by overestimating and underestimating the HP content, respectively [38,86,87,92]. DeLong et al. [80] assessed whether intrinsic Fe^{3+} would interfere with the FOX method by adding mercaptoethanol in samples to reduce ferric ions to the unreactive ferrous state. Using this technique, these latter authors found that iron in spinach samples did not increase lipid HP estimations.

Other compounds that can be present in complex matrices such as proteins, cysteine, glutathione, other thiols, uric acid, acidic phospholipids, and sugars have been also reported both to interfere and not interfere with either the FOX-1 or the FOX-2 methods [35,38,42,43,79,84,86,91]. The amount or absence of those compounds in the final solution would explain these results but, unfortunately, few data have been published. For instance, cysteine, glutathione, and ATP are reported to decrease the color yield of the HP determination [84,92] and a possible mechanism is the reduction of ferric ions or ferric complexes. However, other reductant compounds such as urate, glutathione, mercaptoethanol, cysteine, and other thiol groups were reported not to interfere at the acidic pH of the FOX-1 and FOX-2 methods [35,86,92] and uric acid has been reported to slightly increase the color yield [43] in the FOX-2 method. The chelation of transition metals and the promotion of sample oxidation are other possible mechanisms of interference. Interferences caused by thiol groups of proteins, peptides, and cysteine can be overcome by completely removing the proteins from the extract, whereas the aqueous soluble interferences can be avoided by selecting a more apolar solvent.

In relation to proteins, HP recoveries have been reported to decrease as protein content increases likely due to interactions between oxidized lipids and lipoproteins and/or other proteins [39,80,87,91]. DeLong et al. [80] found that a higher bovine serum albumin concentration yielded a lower amount of detected lipid HP even when a higher amount of HP was added. The authors suggested that peroxyl groups formed on the free fatty acids were either altered chemically or bound by the serum albumin, resulting in no ferrous reduction and no subsequent formation of the XO complex [80]. The ability to bind fatty acids explains why the majority of plasma HP was found in lipoproteins, especially in LDL which contained more than 65% of the total HP present in native plasma [99]. Therefore, because alcohols may lead to flocculated proteins and other materials, it seems more appropriate to remove them by centrifugation before [34,80,84,103] rather than after FOX reaction [35,98] to avoid interactions between HP and proteins. The method for protein removal should be taken into account because precipitation with cold methanol allowed a considerable fraction of proteins to remain in solution, whereas cold trichloroacetic (10%), metaphosphoric (1%), and perchloric acids (0.2 M) completely removed proteins from the original solutions [91]. Despite that, in methanol-based extracts, a linear relationship between volume extract and absorbance is recorded [34,84]. This latter and other above-discussed interferences have been summarized in Table 1, whereas interference caused by antioxidants is further discussed under Influence of time on complex formation and Improving specificity and selectivity subsections.

Some FOX method modifications add a fixed sample volume but to increase detection limits the majority of modifications involve variable sample amounts while keeping final concentrations of the reagents constant. Therefore, it should be taken into account that, whenever more concentrated samples or higher amounts of sample or extract are present, matrix effects may become more pronounced [39,87,103]. In relation to matrix effects, Hermes-Lima et al. [84] reported that the recovery of hydrogen peroxide was higher than that of cumene HP when they were added into mouse liver methanol extracts. These authors suggested that the interference caused by different reducing/oxidizing agents and proteins still remaining in methanol-based extracts may explain this effect.

Some authors reported that there is a linearity range up to certain absorbance units [36,103] or HP amounts [120,121] but when this range is exceeded the curve becomes asymptotic sometimes without a clear explanation of this phenomenon. Eymard and Genot [103] reported a linear region when absorbances were over 0.3 or under 0.6 when different volumes of horse mackerel methanol extracts were added but this linear region also occurs when the same extract was diluted twofold. Likewise, Navas et al. [36] reported linearity at 560 nm in lipid extacts from fried snacks, when the measurements of the absorbance were under 0.8.

Reaction media

As explained before, the FOX reagent consists of a mixture of the dye, i.e., XO, a ferrous source, an acid, and the solvent. The reaction between peroxides, iron, and XO is quite fast [34,43]. However, the preincubation of the HP with ferrous ion results in decreased FOX values [84]. Therefore, we recommend mixing the ferrous ion with the acid (to avoid iron oxidation) followed by XO and finally the sample containing the HP to standardize as much as possible the incubation times. This method has the advantage that it can be carried out under the presence of air because oxygen affects only slightly the measurements in comparison to those samples carried out under nitrogen-modified atmosphere [34]. However, several other factors have been reported to influence solubility, the sample molar absorbance coefficients (ε or extinction coefficients), color development, and stability [36–38]. The main factors that have to be considered in the reagent media are summarized in Table 2 and reviewed below:

Nature of the medium. As noted, the majority of the FOX methods utilize a methanol-based reaction medium [34,35,114]. However, a mixture of different solvents such as water, methanol, and chloroform may result after the addition of the sample into the reagent medium. In cases where sample amount has to be increased to increase sensitivity, some samples that contain apolar solvent extracts may present solubility problems in some reaction media [31,36,83]. For samples consisting of oil and fat extracts, replacement of methanol with a chloroform/methanol (7:3, v/v)-based reaction medium has been reported to overcome lipid sample solubility problems, whereas acetone, diethyl ether, and isopropanol did not [31]. Dichloromethane/ethanol (3:2, v/v) was also able to dissolve up to 25 mg/mL of fat extracts from fried snacks into a final reaction medium containing water/methanol/ethanol/dichloromethane (1:4:6:9, v/v/v/v) [36]. This amount of fat can be also dissolved in dichloromethane/methanol (7:3, v/v) and chloroform/methanol (7:3, v/v) but, because of its higher recorded response, the dichloromethane/ethanol (3:2, v/v) was preferred [36].

Apart from solubility issues, solvents must be chemically stable during analysis so the use of 2-propanol, ethyl acetate, and butanol is not recommended because they are prone to form HP [36]. To avoid this, some stabilizers such as BHT and ethanol are used in some solvents [122]. Actually, some FOX methods used solvents such as 2-propanol or ethyl acetate together with the addition of BHT [42,44,83,98]. However, as further discussed below, the use of this antioxidant in the reaction media is discouraged because it will interfere in the reaction and cause lower response [34,84]. Solvents must also allow formation of a stable color complex. In relation to this, Gay et al. [37] reported that the rate of complex formation was very rapid in 25 mM aqueous sulfuric acid, 25 mM methanolic sulfuric acid (methanol/water, 90:10, v/v) and aqueous acetic acid (water/glacial acetic, 1:1, v/v); however, the color development in acetic acid did not reach a stable plateau as shown in Fig. 2.

Dependence on pH. The FOX method is very dependent on the pH not only because ferrous ion rapidly converts to ferric ion under nonacidic conditions but also because HP oxidizes ferrous to ferric ion specifically in acid media [35]. That is because other ions can also form complexes with XO [123,124] and could potentially cause an overestimation of the Fe^{3+} derived from HP oxidation of Fe^{2+}. Fortunately, Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Se^{4+}, and Te^{4+} react weakly with XO at pH $\leqslant$ 2 and therefore do not seriously alter HP estimations [123–125]. Moreover, under this acidic medium the ferrous source is stable and ammonium ferrous sulfate is commonly used. However, some instability problems were recorded by some authors and were overcome either by adding the dye and the iron individually [31] or by using iron D-gluconate [87]. In addition, high pH may decrease the solubility of the XO in aqueous solutions, causing its precipitation.

Because some samples that contain high concentrations of natural buffer compounds (proteins) can modify the reaction pH, it is important to control the pH of the reaction. Proper pH conditions are commonly achieved by the addition of sulfuric acid at a concentration of 25 mM [34,35,42,44] although other concentrations and acids, such as 10 mM hydrochloric acid, have been also used in food analysis [31,78]. Color development was similar when comparing 25 mM both hydrochloric acid and sulfuric acid; however, the intensity of the absorption was higher in sulfuric acid [34,36] and showed a better precision [36]. Differences in oxidation potential might explain both why hydrochloric acid showed a much lower response than sulfuric acid [34] and why the reaction was slower and gave higher response with perchloric acid than with sulfuric acid [39]. However, these acids in the presence of iron record a very similar steady absorbance in aqueous medium (Fig. 3). Thus, the increased sensitivities can be attributed to several factors such as the optimum pH reached and that some of these acids may play a role in a set of reactions that propagate ferrous chain oxidation and, more probably, enhance the extinction coefficient because of a much better electron configuration of the resulting complex.

Nevertheless, for maximum color development each acid requires an optimum pH [38]. For instance, aqueous sulfuric acid has a narrow optimum pH of 1.7–1.8 [37,94], whereas, in an automated FOX method used to determine plasma lipid hydroperoxides, the optimum pH reaction for a mixture containing sulfuric acid (40 mM), formic acid (20 mM), and glycerol (1.37 M) was 1.3–1.4 [87]. In the presence of samples of biological origin the common final concentration of 25 mM sulfuric acid may not ensure the maintenance of the correct pH in aqueous-based media, whereas a concentration of 36 mM sulfuric acid seemed to be more appropriate to give optimum pH control [39]. The tolerance to pH changes in samples containing liposomes is greater than that in homogenous solution [95].

Table 2
Summary of the reported media drawbacks assessed in different ferrous–xylenol orange (FOX) methods

Reported media drawbacks	Explanation
Nature of the solvent:	
solubility	Increased amounts of oils and food lipid extracts have been solubilized using more apolar solvents [31,36,83].
stability	Some organic solvents are prone to form hydroperoxides [36,86].
color stability	The stability of the formed Fe-XO complex depends on the solvent and acid used [37].
Stability of iron	The reagent absorbance containing iron and XO increases during storage [31,87].
Dependence of pH	Low pH prevents iron autoxidation and XO reacts specifically with ferrous ion [35]. Various acids and concentrations have been used providing different pH [34–39], however, each FOX method has an optimum pH range for the conditions and acid used [87,91,94,95].
Dye	A ratio XO:ferric ion in the system around 5 is recommended [37–39,78]. Different XO brands [36–38] and also batches [34] yield different spectra and responses.

XO, xylenol orange.

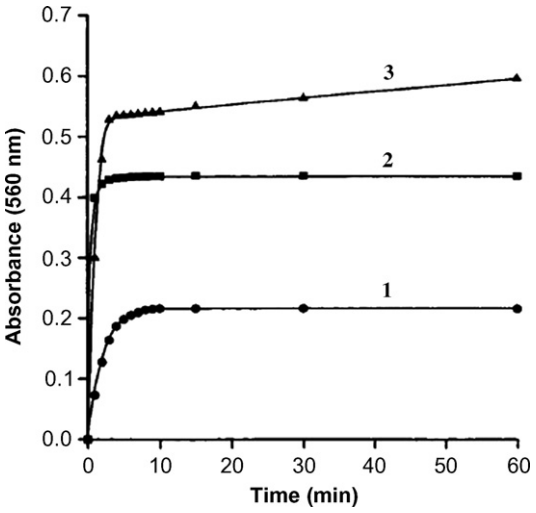

Fig. 2. Effect of solvent on the rate of formation and stability of the ferric–xylenol orange complex. 1, 90% methanol/25 mM H_2SO_4, 7.3 mM complex. 2, 25 mM H_2SO_4, 21.5 mM complex. 3, 50% aqueous acetic acid, 23 mM complex [37].

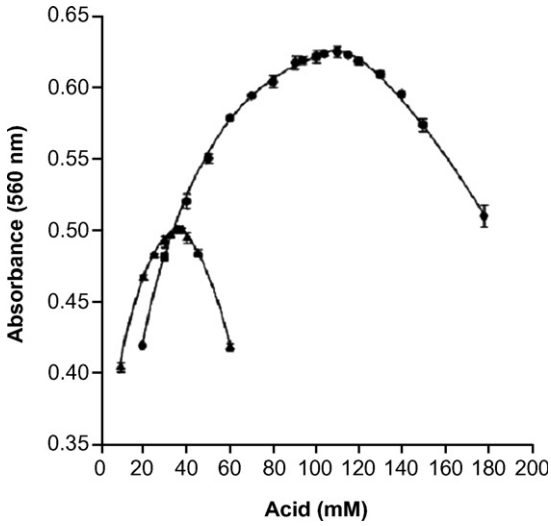

Fig. 4. Absorbance of the ferric–xylenol orange complex in different concentrations of perchloric (●) and sulfuric (▲) acids in aqueous medium [39].

Aqueous acetic acid (50%) gives a Fe–XO complex with a higher absorbance than sulfuric acid but the latter is preferred because the optimum pH of the former is in within an even narrower range (pH 1.6–1.7). In addition, as shown in Fig. 2, the color development was not as stable in acetic acid as in sulfuric acid [37]. However, absorption coefficients generated by various HPs made with 50% acetic acid were higher than those in 25 mM aqueous sulfuric acid [37,38] despite the linearity in both sulfuric acid and acetic acid at each optimum pH being good to at least 50 μM iron concentration [37,42]. The use of perchloric acid (110 mM) resulted in a lowering of the optimum pH of the assay to 1.1 in aqueous [39] and in methanol/chloroform (2:1, v/v)-based solutions [91]. In addition, as shown in Fig. 4, perchloric acid is much less sensitive than sulfuric acid to minor changes of pH in aqueous solutions [39]. Furthermore, the use of perchloric acid showed lower color decreases in the presence of buffering compounds than did 36 mM sulfuric acid [39].

Dye complex. Through the iminodiacetic group, XO is able to chelate metal ions; however, because it has multiple pK_a values, XO can form several different types of complexes with metals [37,123,124]. The complex Fe^{3+}–XO has a 1:1 stoichiometry [37,95] although 2:1 and 1:2 complexes can be formed also in the presence of excess Fe^{3+} and XO, respectively [125]. However, because the oxidation of ferrous ion by HP yields ferric ion and a radical (Eq (1)), this latter can cause further HP formation yielding to a stoichiometry higher than 1:1 ferric:HP and as a consequence more XO is required [44,78,86].

To obtain accurate measurements, the absorbance of the complex must be independent of XO concentrations to maintain a linear response [37,78]. These conditions are satisfied when the final ratio XO:Fe^{3+} is above 3 although it is recommended to maintain the ratio around 5 which gives a good safety margin for a ferric–XO 1:1 stoichiometry [37–39]. Assuming that the amount of Fe^{3+} formed by HP in the sample is unknown, these conditions can be verified by the color of the solution because a sample producing the Fe–XO complex while still containing free XO should be orange/brown instead of bluish/purple as reported in some earlier studies which could indicate the use of insufficient XO to complex all the Fe^{3+} present [39]. Low XO concentrations could explain the low linearity reported in the FOX method reported by Nielsen et al. [55]. Finally, a general recommendation is to carry out the analysis under attenuated light conditions, especially in complex matrices,

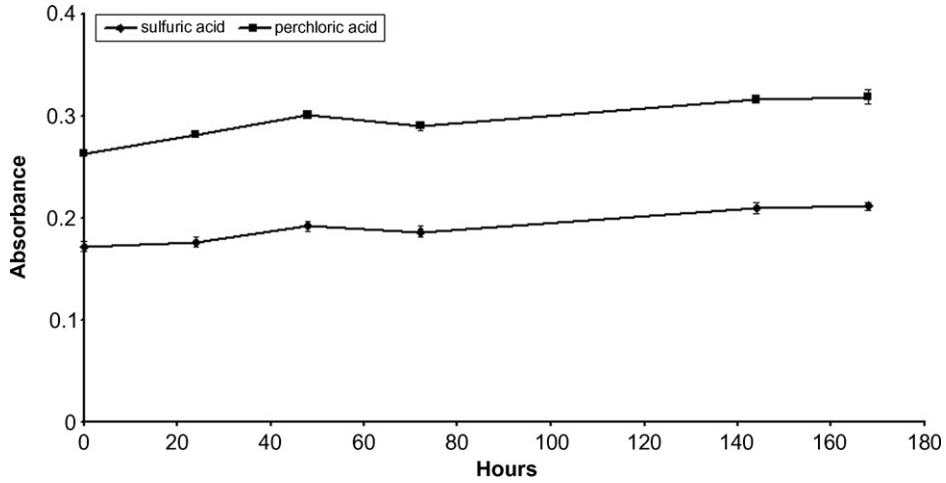

Fig. 3. Time course of absorbance of the ferric–xylenol orange (XO) complex at 560 nm performed using perchloric and sulfuric acids. Reaction medium consisted of 200 μL of 2.5 mM aqueous $Fe(NH_4)_2(SO_4)_2$, 200 μL of 2.5 mM aqueous XO, 200 μL of 1.1 M aqueous H_2SO_4 or $HClO_4$, and 1400 μL of water. Blank consisted of water.

although some authors reported that it is not necessary to keep the solutions in the dark unless the studied HP is light sensitive [38].

Absorbance spectra and wavelength selection

XO absorbance ranges 420–460 nm [43,84] whereas the ferric–XO complex absorbs strongly at 540–600 nm [34,37,40,84] (Fig. 5). The mixture of both, the complex and free XO, explains the orange/brown complementary color. Absorption characteristics of XO depend not only on the concentration of HP but also on the XO source. Several authors found that the slope of calibration curves and absorption spectra of the ferric–XO complex varies between XO suppliers [36,37] and between batches [34] with some sources of XO having greater absorption at 590 nm than that at 560 nm (Fig. 6).

That XO needs to be present in excess with some remaining free after the formation of the Fe^{3+} complex means that at wavelengths lower than 550 nm absorbance by free XO can contribute to the total absorbance of the XO–Fe^{3+} complex [37] (Fig. 7). Because a significant amount of free XO remains after the formation of the complex, the use of a blank and a sample containing the same amounts of XO initially will underestimate the concentration of the Fe^{3+}. However, this concentration can be obtained by the measurement of the absorbance of test solution containing XO and the Fe^{3+} by means of the equation $C_{Fe\text{-}XO} = A_{obs}/(\varepsilon_{Fe\text{-}XO} - \varepsilon_{XO})$, where $C_{Fe\text{-}XO}$ is the Fe–XO complex concentration, A_{obs} is the absorbance recorded, and $\varepsilon_{Fe\text{-}XO}$ and ε_{XO} are the molar absorbance coefficients of the xylenol complex and free xylenol, respectively [37]. Therefore, by the measurement of the absorbance of test solution containing XO and the Fe^{3+}, against a blank made up with the same concentration of XO, and provided that the molar absorption coefficients of the complex and XO at the measured wavelengths are known, the amount of peroxides can be determined [37]. Nevertheless, because the extinction coefficients of the XO dye and the ferric–XO complex depend on several factors (e.g., wavelength, media composition, XO source, and pH), their calculation is very difficult because the extinction coefficients have to be determined for each particular assay. Therefore, the majority of authors proposed to measure the absorbance of the mixture of the complex and the uncomplexed XO at 560 nm against a blank containing the same initial concentration of XO. Then, the sample is read against this blank and by either applying a standard curve made with HP [34,37,80,112,126] or using their molar absorption coefficient [89,115] the equivalent HP concentration can be calculated. In this case, as shown in Fig. 7, by reading at 560 nm and not correcting the free XO effect, it has been estimated to cause an error of only 1.6% [37].

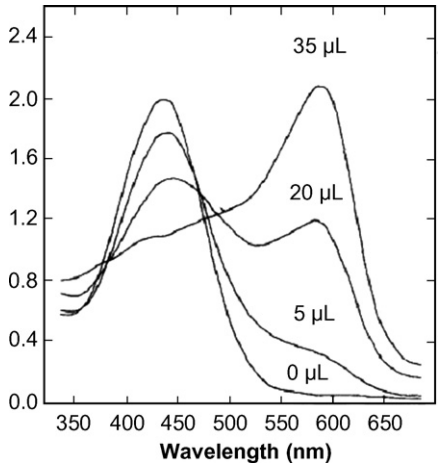

Fig. 5. Effect of different volumes of methanol extracts on the wavelength spectra of xylenol orange (XO). Extracts are added to 0.9 mL of the reaction medium containing 25 mM H_2SO_4, 0.25 mM Fe_2SO_4, 0.1 mM XO (Sigma) plus water up to a total final volume of 1 mL [84].

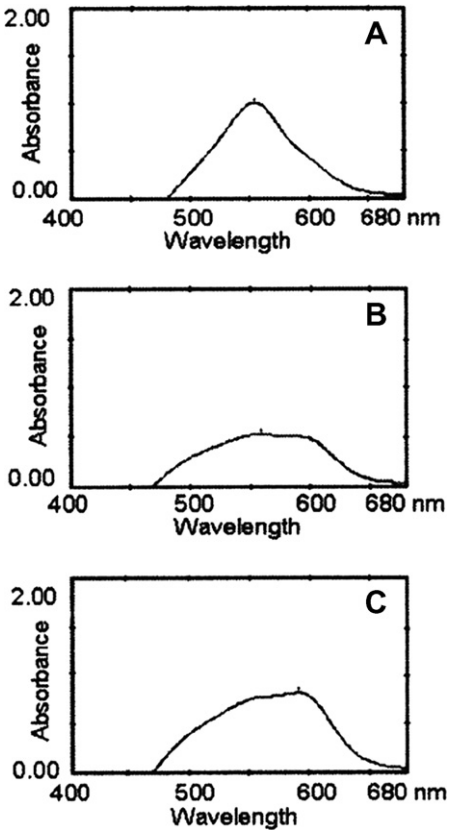

Fig. 6. Absorbance spectra of the ferrous–orange xylenol complex obtained after using xylenol orange (XO) from Sigma (A), Scharlau (B), and Aldrich (C). Reaction medium consisted of 100 µL 5 mM aqueous $Fe(NH_4)_2(SO_4)_2$, 200 µL 0.25 M methanolic H_2SO_4, 200 µL 1 mM methanolic XO, 1300 µL methanol, and 200 µL dichloromethane/ethanol (3:2, v/v) with different cumene HP standard additions up to concentrations of 10.6, 8.8, and 13.3 nmol/mL reaction medium in the three providers, respectively [36].

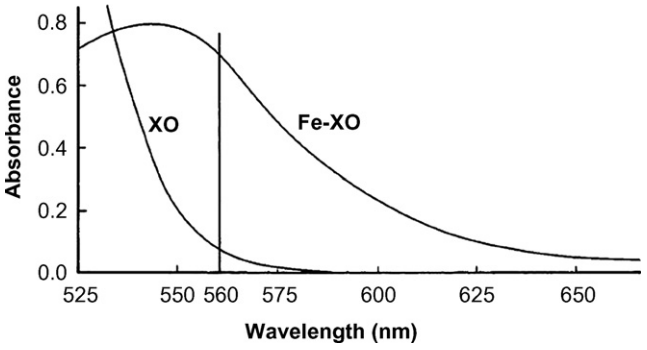

Fig. 7. Absorption spectra of xylenol orange (XO) (Sigma) and the ferric ion–XO complex in aqueous 25 mM H_2SO_4. The spectrum of the complex was obtained after substraction of a XO blank. Concentrations were 35 µM for the ferric–XO complex and 0.3 mM for free XO [37].

Wavelengths higher than 560 nm have been used also to determine lipid HP, for instance to assess the 5-lipoxygenase activity [92], whereas, in an automated XO method, Arab and Steghens [87] found that the absorption peak formed upon the addition of *tert*-butyl HP shifted from 550 to 570 nm in samples in which

0.4 g/L of bovine serum albumin or plasma was added (Fig. 8). Deiana et al. [79] found that the maximum absorbance of the ferric ion–XO complex in an aqueous medium was at 560 nm, whereas in 90% methanol it was at 580 nm. Likewise, Fukuzawa et al. [95] found that the presence of egg yolk phosphatidylcholine in 60% methanol shifted the peak from 560 nm to a sharp peak at 610 nm. The authors attributed this effect to the physical structure of the liposomes because this effect disappeared when surfactants or higher amounts of methanol were added to the sample. These shifts in absorbance maxima can be caused by either the solvents used or the type of sample matrix.

Because of shifts in absorbance maximum, measurements in food samples have been performed at 562 nm in fish tissues [108] and at 580 nm in other animal [84,104,127] and plant [128] extracts. Extinction coefficients determined from calibration curves at 592 nm in chicken meat [34], at 590 nm in fried snacks [36], or at 580 nm in fish meal [103] were lower than those compared at 560 nm in their respective samples. Therefore, the measurement of the absorbance at 560 nm seems to be appropriate in most cases [31,80,83,102]. In some cases, the measurements at the maximum absorbance wavelength in comparison to absorbance at 560 nm may improve the range of linearity [36]. In addition, slight wavelength changes may improve specificity and sensitivity compared to those at 560 nm because the interference with free XO can be avoided [95]. Likewise, other interferences such as those caused by pigments in vegetal extracts [80,89] could be minimized by selecting a wavelength with less interference. Interference by plant extracts is generally lowest over the range 520–580 nm. Thus, prior to analysis, we strongly recommend studing the absorption spectra of the dye being used. In addition, the same lot of XO should be used throughout the study.

Influence of time on complex formation

The development of color at room temperature using XO and ferric or HP standards is reported to reach a plateau after 30 min and to be stable overnight [34,37,43,89]. Despite that, several authors dealing with meat and plant extracts measured the absorbance after 10 min of incubation at room temperature [31,80,112],

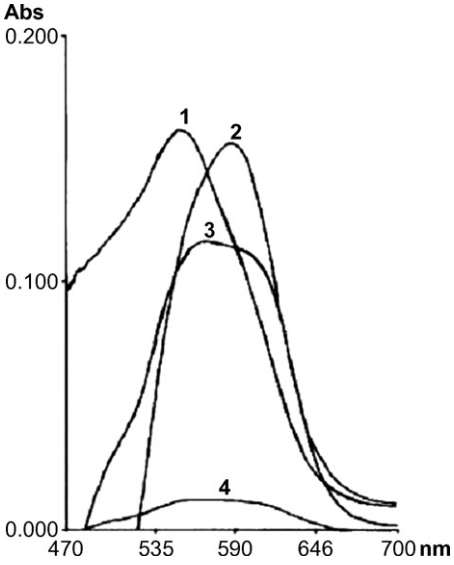

Fig. 8. Absorption spectra recorded by using an automated analyzer providing final concentrations of xylenol orange (Sigma) at 120 μM, ferrous D-gluconate at 150 μM, and *t*-butyl hydroperoxyde at 0.2 μM alone (1), the same conditions but with bovine serum albumin at 0.4 g/L (2), or in diluted human plasma (3). Its signal was suppressed by addition of KI (4) [87].

whereas others measured absorbances at much longer incubation times [34,84,102,103,106,128]. Actually, the stabilization of absorbance depends on various factors. The majority of studies using 25 mM sulfuric acid showed rapid and stable complex formation [37,39], whereas perchloric acid [39] seemed to require longer stabilization times and acetic acid did not reach a steady endpoint [37].

The kind of sample also influences the time for color stabilization which can range from 30 min to 2 h in most cases [80,84]. Hermes-Lima et al. [84] also suggested that unsaturated lipids present in the tissue extracts can undergo peroxidation, further influencing the assay. To check this possibility, these authors studied the effect of a standard addition of arachidonic acid on HP formation in the FOX assay. They found that for the first 12 h of incubation, HP concentrations were not changed by the presence of arachidonic acid. From these results, the authors suggested that the addition of BHT to the FOX assay is not necessary for short incubation periods. However, tissue extracts are complex matrices which can contain several compounds that may promote or inhibit oxidation of the nonperoxidized lipids and therefore could cause HP formation. In samples from chicken meat and other tissue extracts, a steady endpoint in dye complex formation is reached only after several hours, suggesting that oxidation is continuing during color development [34,84,102,106].

Accordingly, Grau et al. [34] suggested that the FOX method could also be used to measure susceptibility of a sample to oxidation when incubation is greater than 30 min. When measuring susceptibility to oxidation it is necessary to follow absorbance changes until they reach a stable plateau for each particular kind of sample to enable comparisons between samples. In the example shown in Fig. 9, the comparisons can be made after 25 h. Nevertheless, it should be taken into account that the number of readings (exposure to light) affects the color yield, so some differences can be found between those samples read several times and those read once [34].

When using the FOX assay to study susceptibility to oxidation, the addition of a radical scavenger such as BHT will have undesirable effects by inhibiting oxidative reactions [34,84]. Conversely, using this version of the FOX assay can allow differentiation of samples with different antioxidant activies and/or concentrations [34,102,106]. Moreover, when using the FOX assay to measure susceptibility to oxidation, it is important to check that reagents do not increase the absorbance over time which has been observed in cases when iron leaches from the glassware [86]. Thus, the FOX method has to be considered not only a method to measure lipid HP in foods and biological matrices but also a useful method to assess and compare susceptibility to oxidation when long periods of incubation are recorded [34].

Improving specificity and selectivity

As discussed under Sample matrix, the accuracy of the quantification of the method can be diminished by the presence of several components in the sample (see Table 1), thus affecting the FOX method specificity. Therefore, the use of separation techniques such as HPLC combined with the FOX method can be of interest because it allows one to determine and quantify different classes of lipid HP [47]. However, the use of these combined techniques is not very common because the analysis is more complex and tedious.

An alternate approach would be to use techniques to decompose different classes of HP so that they can be differentiated. A summary of these techniques is listed in Table 3. Jiang et al. [43] studied the effect of different enzymes known to metabolize peroxides on the quantitation of HP in LDL and liposomes. When samples were preincubated with Cu^{2+} to promote oxidation followed

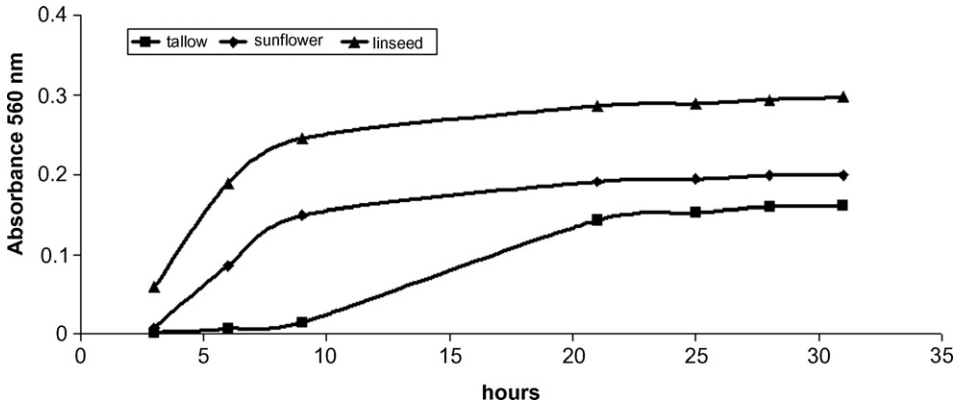

Fig. 9. Time course analysis of raw rabbit meat samples (50- μL extract aliquots) from animals fed different dietary fats (tallow, sunflower oil, and linseed oil). Unpublished data using the FOX method described elsewhere [34].

by addition of catalase, HPs in LDL and liposomes were reduced by 10–15% compared with the control, which indicated that hydrogen peroxide was in the samples. When peroxidized LDL and liposomes were incubated with glutathione, glutathione peroxidase, and phospholipase A_2, they recorded a 90–95% decrease in the color yield compared with the control presumably due to removal of lipid HP. From these results, Wolff [35] recommended treating samples with catalase or with glutathione–glutathione peroxidase to confirm HP authenticity. A less-selective method to confirm the presence of HP is to compare absorbance suppression in comparison to those samples preincubated with KI [87].

To detect only organic HP and, in consequence, increase the selectivity of the method, DeLong et al. [80] added catalase to plant tissue extracts prior to addition of FOX reagents to avoid hydrogen peroxide response. In samples with aqueous media, 1 mM sodium dithionite and 100 mM borohydride can be used to reduce peroxidized bovine serum albumin and other water-soluble HP [38,129]. Therefore, by subtracting the remaining absorbance found in the reduced samples, interferences in the sample can be calculated and the difference attributed to the current HP content.

The measurement of authentic lipid HP in FOX methods has been widely checked through the addition of TPP to the samples before iron–XO complexation [34,44,83,89,94,98]. TPP (30 min, 1–5 mM) reduces a variety of HPs, in hydroalcoholic and nonaqueous environments to their corresponding alcohols while it is being converted to triphenylphosphine oxide [44,94,130,131]. Linoleic acid, linolenic acid, endoperoxides, arachidonic acid, phosphatidylcholine, cholesterol, cholesterol ester, and protein HP are reduced to their alcohols by TPP [44,83,93,129]. TPP has no effect on hydrogen peroxide allowing it to be used to discriminate between hydrogen peroxide, and other HPs [44]. Using TPP, it has been found that lipid HP in liposomes, plasma, LDL, and edible oils are 80–85% of the total HP [43,44,83,98], whereas chicken meat contained over 98% lipid HP [34].

Table 3
Hydrophobicity and activity of the reducing agents commonly used to investigate the specificity of ferrous–xylenol orange (FOX) methods

Reducing agent	Phobicity	Reduce organic hydroperoxides	Reduce hydrogen peroxide
Glutathione/glutathione peroxidase	Hydrophilic	Yes	Yes
Catalase	Hydrophilic	No	Yes
Sodium dithionite	Hydrophilic	Yes	Yes
Sodium borohydride	Hydrophilic	Yes	Yes
Potassium iodide	Hydrophilic	Yes	Yes
Triphenylphosphine	Hydrophobic	Yes	No

Secondary oxidation products such as prostaglandin G_2 and H_2 and cyclic peroxides derived form cholesteryl arachidonate give positive responses to the FOX assay [93]. However, when TPP is added, cyclic peroxides survive to the reaction and, in consequence, its interference is not eliminated. On the contrary, prostaglandin G_2 and H_2 and other similar endoperoxides are reduced to F_2 isoprostane structures (metabolites produced by free radical damage of arachidonic acid) that then do not react with XO and, in consequence, will not interfere in the analysis by being considered lipid HP [93]. Fortunately, other endoperoxides such as dicumyl peroxide, benzoyl peroxide and lauroyl peroxide, have been reported to react with XO although to a much lower extent than HP [43,79].

In some studies the lipid-soluble chain-breaking antioxidant BHT was added (at doses about 4 mM) to the FOX-2 method during the analysis of liposomes, LDL, and plasma [35,42–44,86] and edible oils [83] to minimize oxidation that could occur during the assay. However, Hermes-Lima et al. [84] reported that BHT was not necessary during incubation times up to 12–24 h. Grau et al. [34] further investigated the effect of BHT on the FOX method by adding BHT immediately after the addition of the FOX reagent to a sample containing linolenic acid HP, which resulted in a decrease of absorbance at 560 nm of 17%, whereas when BHT was added just before the FOX reagent the absorbance decreased much more (44%). Because the reaction between lipid HP and FOX is instantaneous, the decrease in absorbance upon addition of BHT to samples after addition of the FOX reagents suggests that BHT is interfering with color formation and, therefore, its addition is not advisable [34]. Similar decreases in color yield when BHT is added were reported by several authors in other samples [79,84,95]. However, to avoid peroxidation during fat extraction the use of BHT has been recommended [31,89].

Some authors peroxidized different matrices or protein standards such as bovine serum albumin to check the performace of their methods [38,57,88,93,99]. Because protein peroxides have been detected in some protein samples, Gay and Gebicki [91] developed a methodology for the separation and measurement of lipid and protein HP which are extracted from biological materials with chloroform/methanol (2:1, v/v). Protein HPs are measured in an aliquot of the extract after precipitation of proteins with perchloric acid. The resulting pellet is then redissolved in guanidine hydrochloride and subsequently washed with chloroform to remove remaining lipid HP. Analysis of protein HP with this method showed that 100 μM dithiothreitol, mercaptoethanol, cysteine, or glutathione and 1 mM sodium dithionite did not interfere with analysis [91]. In addition, protein sulfhydryl groups did not interfere because there were no differences in samples treated with N-ethylmaleimidine to block free sulfhydryls.

Stoichiometry and sensitivity

As shown in Eq. (1), ferrous ion is oxidized by HP, yielding ferric ion and a radical. The radical species can then react with an additional ferrous ion to produce a second ferric ion (Eq. (3)).

$$RO^{\cdot} + Fe^{2+} + H^{+} \rightarrow ROH + Fe^{3+}. \quad (3)$$

On the other hand, the overall stoichiometry with H_2O_2 has been reported to be between 2 and 3 [38,42,98]. Gay et al. [38] reported a ratio Fe^{3+}:HOOH of approximately 2.5 and proposed the following mechanism:

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^{-} + HO^{\cdot}. \quad (4)$$

$$HO^{\cdot} + XO \rightarrow HOXO^{\cdot}. \quad (5)$$

$$HOXO^{\cdot} + Fe^{2+} \rightarrow HOXO^{-} + Fe^{3+}. \quad (6)$$

$$HO^{\cdot} + Fe^{2+} \rightarrow OH^{-} + Fe^{3+}. \quad (7)$$

In the presence of hydrogen peroxide, XO radicals are thought to be able to produce a third ferrous ion (Eqs. (5 and 6)). This is because the highly reactive hydroxyl radical is generated from the Fenton reaction (Eq. (4)) and this radical is scavenged mainly by XO (Eq. (5)), which competes with the direct oxidation of the ferrous ion. Because the reaction of hydroxyl radical with XO is faster than that with ferrous ion [40] this would explain the overestimation [38]. Therefore, through a series of reactions, 2 or 3 mol of Fe^{3+} was formed per mol of HP. This is supported by reports showing that the Fe–XO complex has an extinction coefficient of 15,000 $M^{-1}cm^{-1}$ at 560 nm when ferric iron is used to generate the standard curve, whereas the extinction coefficient is 45,000 $M^{-1}cm^{-1}$ when hydrogen peroxide is used as standard [35,42,43,83,86,93,98].

Similar extinction coefficients were reported for several acyl and alkyl HPs (*t*-butyl, cumene HP, linoleic acid HP, araquidonic acid HP, cholesteryl araquidonate HP) [35,42,43,86,93,98]. Nevertheless, for the Fe–XO complex generated by ferric ions, the extinction coefficients (ε) range 14,000–24,000 $M^{-1}cm^{-1}$ at 560 nm [37]. These differences in extinction coefficients depend on several factors such as the acid used, the pH, the dye, and the solvent as shown in Table 4. Despite that, the majority of lipid and protein HP in complex matrices seemed to yield about two ferric ions and this can be explained by Eqs. (1 and 3) whereas a third group of HP is formed by *t*-butyl and cumene HP which yielded five iron ions per HP [38]. There is no obvious mechanism for formation of these HPs to account for this yield although the formation of methyl free radical via β-elimination can produce a range of species capable of oxidizing ferrous ions. The nature and proportion of HP are mostly unknown in the majority of samples; thus, the majority of researchers express HP in terms of hydrogen peroxide equivalents [108,114] or cumene HP equivalents [34,103]. Cumene HP is generally preferred for these purposes because it is more stable than other HPs.

The use of chain amplifiers produces additional ferric ions and, in consequence, increases the recorded absorbance of the FOX method measurements [35,79,88]. Commonly in FOX-1 methods, these enhancers consist of sugars or polyols which react with the oxyl radicals generated from the reduction of HP by ferrous ions (Eq. (1)), propagating the ferrous oxidation step by the following proposed mechanisms

$$Polyol + RO^{\cdot} \rightarrow Polyol^{\cdot} + ROH. \quad (8)$$

$$Polyol^{\cdot} + O_2 \rightarrow PolyolO_2^{\cdot} \rightarrow ketose + HO_2^{\cdot}. \quad (9)$$

$$Fe^{2+} + HO_2^{\cdot} \rightarrow Fe^{3+} + H_2O_2. \quad (10)$$

$$2HO_2^{\cdot} \rightarrow H_2O_2 + O_2.$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe_{3+} + OH^{-} + HO^{\cdot}. \quad (11)$$

When the FOX-1 method was used to determine hydrogen peroxide, sucrose, sorbitol, glucose, and especially fructose were found to enhance color yields [41]. These authors found that color stability was higher in samples containing 100 mM sorbitol as the enhancer. The addition of sorbitol in the FOX-1 medium has been used to determine HP in some biological matrices [90,94]. The addition of 100 mM sorbitol produced a 15-fold increase in the extinction coefficient in comparison to a system without the enhancer [35]. Deiana et al. [79] compared 100 mM sucrose, mannitol, and sorbitol on the reaction of hydrogen peroxide with XO and found that sucrose increased the response the most (ε_{580} = 360,000 $M^{-1}cm^{-1}$). However, the enhancement factor in the FOX-1 method also depends on the HP used [41]. Furthermore, several authors reported that the inclusion or not of sorbitol (100 mM) in the aqueous assay solution yields different enhancement factors, ranging 1.9–9.4, depending on the hydrophilic HP [38,88]. However, the solubility of these polyols in the media limits their use to the FOX-1 method or variants of aqueous-based FOX methods [43].

Several alcohols (methanol and ethanol) and solvents (glycerol and dimethyl sulfoxide) at a dose of 100 mM have also been reported to increase color yield in an aqueous-based FOX-1 method [35,41]. These reports suggest that compounds that can propagate free radicals could be useful for increasing the sensitivity of the FOX method. The response of a certain amount of cumene HP in the FOX-2 is different depending on the solvents or mixture of solvents used in the media, obtaining higher responses with ethanol and methanol media than with some mixtures of these alcohols with dichlormethane or chloroform [36]. These latter authors compared the response of cumene HP in different reaction media based on ethanol, methanol, dichlorometane/methanol (at different proportions), dichloromethane/ethanol (at different proportions), and chloroform/methanol (7:3, v/v) and found that mixtures of dichloromethane/ethanol (7:3, v/v) and cholorofrom/methanol (7:3, v/v) recorded lower responses in comparison to methanol and ethanol media, whereas dichloromethane/ethanol (3:2, v/v) was similar to these latter. Furthermore, some proportions of dichloromethane/ethanol gave greater responses than methanol or ethanol but were not able to dissolve large amounts of lipid. Similarly, the addition of 1% ethanol in a FOX-1 medium containing 100 μM sorbitol enhanced the response of the assay by about 50% [97]. Conversely, the addition of ethanol into an aqueous-based FOX method containing sucrose as enhancer decreased the color yield of the latter although its effect can be overcome by using a high amount (400 mM) of sucrose [79]. Nevertheless, these authors also pointed out that even when using a high amount of sucrose the use of different volumes of several other solvents such as methanol, butanol, and dimethyl sulfoxide can cause a color yield decrease. Finally, as explained before, color yield can be also enhanced by the use of different acids such as perchloric acid [39] and formic and acetic acids [37,38,41,79], whereas the use of acids other than sulfuric acid such as hydrochloric acid led to lower responses [34].

Accuracy, precision, and quantification limits

FOX methods that measure the current level of HP have been reported to have a correlation coefficient with the AOCS iodometic official titration Cd 8b-90 and Cd 8-53 methods and with a NIR method higher than 0.93 in edible oils [45,46]. Spectrophotometic methods based on triiodide formation have also been reported to be correlated with the FOX values in oils [83], plant extracts [80], and liposomes [42,43].

However, as previously discussed, it should be taken into account that the response of the method depends on the type of HP present in the sample. In addition, the majority of FOX methods have been validated for a certain type of sample; thus, the applicability of those methods is quite limited to a defined type of matrix. In a recent study characterizing several categories of fatty by- and coproducts from the food chain (unpublished data), we observed that the results ob-

Table 4
Reported values of the apparent extinction coefficients (ε) of Fe–xylenol orange complexes using different FOX methods

Standard	Source	Wavelength (nm)	Media Final Concentration	ε ($M^{-1}cm^{-1}$)	Reference
Ferric chloride	Aldrich	560	Aqueous – 25 mM H_2SO_4	15000	[41]
H_2O_2	Aldrich	560	90% methanol – 22.5 mM H_2SO_4	43000	[42]
t-Butyl HP	Aldrich	560	90% methanol – 22.5 mM H_2SO_4	43000	[42]
Cumene HP	Aldrich	560	90% methanol – 22.5 mM H_2SO_4	43000	[42]
H_2O_2	Aldrich	560	90% methanol – 22.5 mM H_2SO_4	45600	[43]
Linoleate HP	Aldrich	560	90% methanol – 22.5 mM H_2SO_4	47000	[43]
H_2O_2	Sigma	580	10% methanol – 22.5 mM H_2SO_4	40000	[84]
H_2O_2	Sigma	560	90% methanol – 22.5 mM H_2SO_4	38600	[81]
Ferric sulfate	Sigma	560	Aqueous – 50% acetic acid	20100	[37]
Ferric sulfate	Sigma	560	90% methanol – 25 mM H_2SO_4	24167	[37]
Ferric sulfate	Sigma	560	Aqueous – 25 mM H_2SO_4	30375	[37]
Ferric sulfate	Aldrich	560	Aqueous – 50% acetic acid	14500	[37]
Ferric sulfate	Aldrich	560	Aqueous – 25 mM H_2SO_4	16750	[37]
H_2O_2	Sigma	560	Aqueous – 25 mM H_2SO_4	44000	[38]
H_2O_2	Sigma	560	Aqueous – 50% acetic acid	59800	[38]
H_2O_2	Aldrich	560	Aqueous – 25 mM H_2SO_4	34360	[38]
H_2O_2	Aldrich	560	Aqueous – 50% acetic acid	45360	[38]
BSA HP	Sigma	560	Aqueous – 25 mM H_2SO_4	35500	[38]
BSA HP	Sigma	560	Aqueous – 50% acetic acid	48000	[38]
BSA HP	Aldrich	560	Aqueous – 25 mM H_2SO_4	30250	[38]
BSA HP	Aldrich	560	Aqueous – 50% acetic acid	32500	[38]
Linoleate HP	Sigma	560	90% methanol – 25 mM H_2SO_4	60000	[38]
Linoleate HP	Aldrich	560	90% methanol – 25 mM H_2SO_4	44000	[38]
t-Butyl HP	Sigma	560	Aqueous – 25 mM H_2SO_4	98000	[38]
t-Butyl HP	Sigma	560	Aqueous – 50% acetic acid	115700	[38]
t-Butyl HP	Aldrich	560	Aqueous – 25 mM H_2SO_4	78900	[38]
t-Butyl HP	Aldrich	560	Aqueous – 50% acetic acid	84500	[38]
Cumene HP	Sigma	560	Aqueous – 25 mM H_2SO_4	99300	[38]
Cumene HP	Sigma	560	Aqueous – 50% acetic acid	116200	[38]
Cumene HP	Aldrich	560	Aqueous – 25 mM H_2SO_4	79000	[38]
Cumene HP	Aldrich	560	Aqueous – 50% acetic acid	88750	[38]
Ferric Sulfate	Sigma	560	Aqueous – 36 mM H_2SO_4	20550	[39]
Ferric sulfate	Sigma	560	Aqueous – 110 mM $HClO_4$	25400	[39]
H_2O_2	Sigma	560	Aqueous – 36 mM H_2SO_4	54660	[39]
H_2O_2	Sigma	560	Aqueous – 110 mM $HClO_4$	75000	[39]
Cumene HP	Sigma	560	Aqueous – 36 mM H_2SO_4	105720	[39]
Cumene HP	Sigma	560	Aqueous – 110 mM $HClO_4$	148430	[39]
t-Butyl HP	Sigma	560	Aqueous – 36 mM H_2SO_4	105590	[39]
t-Butyl HP	Sigma	560	Aqueous – 110 mM $HClO_4$	150260	[39]
BSA HP	Sigma	560	Aqueous – 36 mM H_2SO_4	20950	[39]
BSA HP	Sigma	560	Aqueous – 110 mM $HClO_4$	37000	[39]
Ferric sulfate	Sigma	560	Methanol/$CHCl_3$/H_2O (5:2.7:1, v/v/v) – 102 mM $HClO_4$	45710	[91]
Lipid HP	Sigma	560	Methanol/$CHCl_3$/H_2O (5:2.7:1, v/v/v) – 102 mM $HClO_4$	51200	[91]
Ferric sulfate	Sigma	560	Aqueous – 25 mM $HClO_4$	30390	[91]
BSA HP	Sigma	560	Aqueous – 25 mM $HClO_4$	35900	[91]
Cumene HP	Sigma	560	75% methanol – 25 mM H_2SO_4	44000	[34]
Cumene HP	Sigma	592	75% methanol – 25 mM H_2SO_4	47500	[34]
Cumene HP	Sigma	560	Methanol/CH_2Cl_2/H_2O/ethanol (8.5:0.6:0.5:0.4, v/v/v/v) – 25 mM H_2SO_4	92400	[36]
Cumene HP	Aldrich	560	Methanol/CH_2Cl_2/H_2O/ethanol (8.5:0.6:0.5:0.4, v/v/v/v) – 25 mM H_2SO_4	58800	[36]
Cumene HP	Aldrich	590	Methanol/CH_2Cl_2/H_2O/ethanol (8.5:0.6:0.5:0.4, v/v/v/v) – 25 mM H_2SO_4	62700	[36]
Cumene HP	Scharlau	560	Methanol/CH_2Cl_2/H_2O/ethanol (8.5:0.6:0.5:0.4, v/v/v/v) – 25 mM H_2SO_4	58500	[36]
Cumene HP	Scharlau	590	Methanol/CH_2Cl_2/H_2O/ethanol (8.5:0.6:0.5:0.4, v/v/v/v) – 25 mM H_2SO_4	63200	[36]
Cumene HP	Scharlau	560	Methanol/CH_2Cl_2/H_2O (5:4.5:0.5, v/v/v) – 25 mM H_2SO_4	58600	[36]
Cumene HP	Scharlau	590	Methanol/CH_2Cl_2/H_2O (5:4.5:0.5, v/v/v) – 25 mM H_2SO_4	54000	[36]

HP, hydroperoxide; BSA, bovine serum albumin.

tained using the FOX method for animal fats and fish oils correlate well (r = 0.83 and 0.77, respectively) with the results obtained using the EU iodometic official titration method [132], which is similar to AOCS Cd 8b-53 method [53]. However, the FOX method does not correlate well with the titration method for those acid oils from chemical refining and acid oils from physical refining (r = 0.33 and 0.07, respectively). Thus, the nature of the sample clearly affects the results of both methods. In fact, AOCS recommends different iodometric titration methods depending on the nature of the sample, i.e., normal fats and oils, margarines, lecithins [53]. Therefore, it is necessary not only to optimize some of these FOX methods but also to define their applicability (i.e., edible oils, plasma, or meat extracts).

The results obtained from a FOX method using appropriate sample preparation, homogenization, extraction, and conditions of analysis for each type of sample can be precise and much interference avoided. In fact, FOX methods can be even more accurate and precise than those, for instance, generated through iodometric techniques because they have been reported to be much more influenced by several known and reviewed factors such as the protein content in the serum [33,38]. HPLC methods not only can overcome interference but they also involve the separation of HPs of cholesteryl ester, phosphatidylcholine, and triacylglycerides in human plasma [133–136]. Despite HPLC methods being more selective and able to quantify at nanomolar levels, they showed discrepancy in reporting normal values among healthy subjects [133–135,137], whereas FOX methods showed more consistent results for the total amount of HP found in human plasma [9,81,86,87,98].

However, after optimization of some representative FOX methods, paying attention to the above reviewed critical points, it is necessary to perform interlaboratory studies using several selected samples (i.e., edible oils, plasma, or tissue extracts) to determine their accuracy, precision, and reproducibility. This applies to those FOX methods that measure the real extent of peroxidation in samples, whereas that would not be necessary for those methods that measure the susceptibility to oxidation. That is because in these latter methods the absolute value of HP present in the sample is not important because they are to be used only in making comparisons between treatments when run under the same conditions. The inclusion of other methods to measure hydroperoxides (i.e., official titration methods) in these interlaboratory studies will allow study of whether there is any equivalence between the results of these methods. That may be the case in very uniform samples such as edible oils.

Performing the analysis under appropriate conditions and within a range of concentrations, the precision (% RSD) of the FOX-2 method has been reported as 7–9% in meat homogenates [34,102], 4–20% in plant extracts [89], 7–12% in plasma [81,86], and 0.3–10% in edible oils and food lipid extracts [31,36,78,83]. Similarly, the FOX-1 method precision ranged 2–15% in biological tissues [35,90] and 4–13% in plant extracts [97].

Finally, by multiplying 10 times the standard deviation found at low levels in collaborative studies, the approximate limit of quantification of the official titration methods in oils can be set around 0.5 mEquiv HP/kg [53], whereas that in meat homogenates, edible oils, and lipid extracts using the FOX method can be set from 0.1 mEquiv HP/kg [21,31,83,102,138] to less than 7 μEquiv HP/L in plasma, lipid extracts, and plant extracts, when optimized methods are used [36,80,81]. Moreover, by taking into account the low amount of sample needed to perform the analysis (the meat extract required to run the analysis can be equivalent to weigh 0.01 g of sample), this method is at least 500 times more sensitive than the iodometric titration methods.

Conclusions

This review covers the major issues related to the determination of HP using the FOX method. As summarized, many factors influence this assay. Those factors related to the matrix and the precision, specificity, and linearity range of the method can be improved or minimized by using appropriate steps of homogenization, separation, and/or extraction and the correct range of sample amount added to the assay. Sample solubility into the medium can be increased through the appropriate selection of solvents which in turn can affect the sensitivity of the method. In relation to the medium, sensitivity and precision can be improved by using acids such as sulfuric and perchloric acid at their corresponding optimum pH. In addition, the source and concentration of the XO dye can affect the reproducibility, precision, and linearity of the method. The selectivity of the method can be increased through the use of enzymes such as catalase which remove hydrogen peroxide or reducing agents such as TPP which remove those organic HPs. Finally, in the FOX-1 methods, response can be improved through the addition of sugars and polyols propagating the ferrous oxidation step.

Once all the conditions of the analysis have been established, it is wise to study the absorbance spectra to select the most appropriate wavelength that will give the maximum range of linearity, specificity, and sensitivity. Then, we can choose to run the method to measure the current level of HP in the samples or use it as an induced method to measure the susceptibility to oxidation. As the presence of different HPs yield different reponses and the nature of the HP composition is unknown in the majority of samples, the results should be expressed as equivalents of a selected HP using a standard curve. Therefore, as the results obtained will depend on the samples and conditions used, is advisable to use the HP content as a relative value when making comparisons between treatments rather than as an absolute content in HP. Despite this, the FOX method is very useful to measure the HP content in very different types of matrices at low concentrations.

Following the recommendations given in this review and under well-defined analysis conditions it is necessary to study the reproducibility of the FOX methods which has been sometimes criticized. To do this, intra- and interlaboratory studies are required to determine the repeatability and reproducibility of the FOX methods which, also will allow comparisons with other methods, thus assessing their accuracy.

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