HIGH-THROUGHPUT ANALYSIS OF LIPID HYDROPEROXIDES IN EDIBLE OILS AND FATS USING THE FLUORESCENT REAGENT DIPHENYL-1-PYRENYLPHOSPHINE

Short running title: DPPP method for high-throughput analysis of lipid hydroperoxides.

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ABSTRACT

A fluorometric method for the determination of hydroperoxides (HP) in edible oils and fats using the reagent diphenyl-1-pyrenylphosphine (DPPP) was developed and validated. Two solvent media containing 100% butanol or a mixture of chloroform/methanol (2:1, v/v) can be used to solubilize lipid samples. Regardless of the solvent used to solubilize the sample, the DPPP method was precise, accurate, sensitive and easy to perform. The HP content of 43 oil and fat samples was determined and the results were compared with those obtained by means of the AOCS Official Method for the determination of peroxide value (PV) and the ferrous oxidation-xylenol orange (FOX) method. The proposed method not only correlates well with the PV and FOX methods, but also presents some advantages such as requiring low sample and solvent amounts and being suitable for high-throughput sample analysis.

Keywords: diphenyl-1-pyrenylphosphine, hydroperoxides, oils, fats, xylenol orange, peroxide value, method comparison.

1. INTRODUCTION

Lipids undergo oxidation reactions in the presence of common catalytic systems such as light, heat, enzymes, metals and metalloproteins (Frankel, 1998; Shahidi & Zhong, 2005). The most common process of oxidation of edible fats and oils in bulk is autoxidation. In this, and other oxidation processes, there is a continuous formation of primary oxidation compounds, particularly lipid hydroperoxides (HP). These lipid HPs lack odour and flavour, but their instability leads to further free radical and oxidation reactions and thus a high variety of non–volatile and volatile compounds (Frankel, 1998). Overall, these latter secondary oxidation compounds are the major cause of lipid deterioration and are responsible for the development of off-flavours and rancidity in foods (Dobarganes & Velasco, 2002; Frankel, 1998; Shahidi & Zhong, 2010). Furthermore, lipid oxidation decreases the nutritional value and safety of food (Billek, 2000; Esterbauer, Schaur, & Zollner, 1991; Shahidi & Zhong, 2010). Different oxidation compounds can be absorbed and, although it is difficult to discern them from those produced *in vivo*, once in the organism they play a role in the development of different disorders and health conditions, including cardiovascular disease (Cohn, 2002; Staprans, I Hardman, D A Pan, X M Feingold,K R., 1999), Alzheimer's disease (Corsinovi, Biasi, Poli, Leonarduzzi, & Isaia, 2011), cancer (Kanazawa, Ayako Sawa, Tomohiro Akaike,Takaaki Maeda, Hiroshi, 2002) and ageing (Bokov, Chaudhuri, & Richardson, 2004; Pandey, 2010).

Therefore, the determination of HP is an important quality parameter in the food industry and is also very useful for assessing the progression of oxidation in *in vitro* and *in vivo* experiments. Consequently, the determination of HPs is of considerable interest

and explains why suitable analytical methods are being developed (Dobarganes & Velasco, 2002; Shahidi & Zhong, 2010). Because they are a highly reactive species, their determination is challenging. However, there is a wide range of methods (e.g. iodometry, spectrophotometry, spectroscopy, fluorometry, etc.), some of which use separation techniques (e.g. GC and HPLC), that can be used to determine the HP content of foods and biological samples (Barriuso, Astiasaran, & Ansorena, 2013; Bou, Codony, Tres, Decker, & Guardiola, 2008; Dobarganes & Velasco, 2002; Frankel, Neff, & Weisleder, 1990). With respect to fats and oils, the AOAC and AOCS provide official methods to determine peroxide value (PV), which is defined in terms of milliequivalents of peroxide per kg of lipid (AOAC, 2000; AOCS, 2006). These classical titration methods are based on the reduction of the HP group (ROOH) by iodide ion and have the advantage of being simple and inexpensive. However, these iodometric methods have some disadvantages such as being highly empirical, presenting interference and having a high detection limit (0.2 μmol H_2O_2) (Jessup, Dean, & Gebicki, 1994; Nielsen, Timm-Heinrich, & Jacobsen, 2003).

A simple alternative to these official methods that has a broad applicability to foodstuffs and biological samples is the ferrous oxidation-xylenol orange (FOX) method. This method consists of the spectrophotometric measurement of the chromophore complex formed after reaction of xylenol orange with ferric ions previously oxidized by the HPs present in the sample (Bou et al., 2008). Likewise, the spectrophotometric method, which is based on the standard method of the International Dairy Federation (International Dairy Federation, 1991; Shantha & Decker, 1994), utilizes the oxidation of ferrous ions to ferric ions in acidic media by HP; the ferric ions then react with thiocyanate to form a chromophore complex, instead of

86 being formed by xylenol orange. Consequently, there is a high correlation between
87 these two methods (Burat & Bozkurt, 1996; Nielsen et al., 2003; Shantha & Decker,
88 1994). They require common laboratory instruments and can be used routinely. In
89 addition, they are specific for HP, require low sample and solvent amounts, and are
90 fairly sensitive; all of these factors explain why they are so widely used. However, they
91 have the disadvantage of being affected by various factors such as the presence of
92 chelators and other chromophores, and have also shown low reproducibility (Bou et
93 al., 2008).
94 Akasaka, Sasaki, Ohrui, & Meguro (1992) were the first to describe a simple method to
95 determine lipid HP in oils and foods by means of the reagent diphenyl-1-
96 pyrenylphospine (DPPP). This is a non-fluorescent phosphine molecule that reacts with
97 HP to form DPPP oxide, which emits a strong fluorescence. The reaction is specific for
98 HP and the fluorescence intensity of DPPP oxide is directly proportional to the amount
99 of HP (Akasaka & Ohrui, 2000). Various publications have showed that DPPP is useful
100 for the determination of HP in biological samples using flow injection and HPLC post-
101 column methods (Akasaka, Ohrui, & Meguro, 1993; Akasaka, Takamura, Ohrui,
102 Meguro, & Hashimoto, 1996; Akasaka & Ohrui, 2000; Meguro, Akasaka, & Ohrui,
103 1990). The high sensitivity and specificity of these methods is considerable and useful
104 for some specific purposes in fields such as human health research. Nevertheless,
105 these more sophisticated methods have some drawbacks such as the need for
106 expensive apparatus and highly trained personnel, and this limits their implementation
107 in the food industry. The industry often demands cheap and robust routine methods
108 for the purposes of quality control and/or regulation. In terms of lipid HP
109 determination, the food industry is more concerned with being able to determine the

overall HP content easily and with having highly sensitive methods with a high-throughput of samples.

Therefore, the aim of this work was to develop and validate a routine method for the determination of total HP in edible oils and fats by means of the fluorescent reagent DPPP. In addition, the performance of this method was compared with the AOCS Official Method and the FOX method, which are probably the most frequently used titrimetric and spectrophotometric methods for HP determination.

2. MATERIAL AND METHODS

2.1. Materials

Cumene hydroperoxide (80% purity) (CHP) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dipheny-1-pyrenylphosphine (DPPP) was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA). HPLC-grade 1-butanol was obtained from Panreac (Barcelona, Spain), HPLC-grade methanol from Carlo Erba (Barcelona, Spain) and chloroform from Scharlau (Sentmenat, Barcelona, Spain). Bi-distilled water was obtained using a Milli-Q® Gradient System (Millipore Co., Billerica, MA, USA).

2.2. Samples

A total of 43 samples were used for the study. The samples were among the world's most commonly used vegetable oils or animal fats for edible purposes (USDA, 13/09/2013). The samples were: 4 coconut oils, 3 canola oils, 3 corn oils, 2 high-oleic sunflower oils, 3 palm kernel oils, 3 palm oils, 3 soybean oil and 3 sunflower oils, all of

which were donated by Lipidos Santiga. These oils were different because they belonged to different batches and were stored at room temperature thus explaining the different extents of oxidation. The remaining oils and fats were: 4 different brands of butter, 3 different brands of lard, 3 different brands of fish oil capsules, 1 grape seed oil, 1 hazelnut oil, 3 different brands of olive oils, 1 peanut oil, 1 safflower oil, 1 sesame oil, and 1 walnut oil, all of which were purchased from local shops.

The lipid fraction of the butters was extracted as follows: the butters were melted at 80°C, and the supernatant was separated and immediately centrifuged for 3 minutes at 1500 *g*. The upper phase was separated again and centrifuged for 3 minutes at 2700 *g*. The lard was melted at 55°C and filtered through a Whatman No. 54 filter paper. All samples were placed in 10 mL-vials with minimum head space and kept at -80°C until analysis.

2.3. Determination of the lipid hydroperoxide content by means of diphenyl-1-pyrenylphospine (DPPP)

A new method for the determination of HP in oils and fats using fluorescent probe DPPP was developed. The method was conducted under subdued light conditions, and positive displacement pipettes were used throughout the study. Two versions of the method were used depending on the solubility of the samples in the solvent media. The solubility of the different samples is shown in Table 1.

2.4. DPPP method version 1 (DPPP1)

An appropriate sample size, between 1 and 5 g of sample, was dissolved in butanol containing 4 mM BHT (between 10 and 50 mL depending on the expected peroxide

158 content) to lie within the linearity range of the method. Polypropylene microtubes
159 (8x44 mm; Deltalab, Rubí, Spain) held in 96 tube sample racks were used for the assay.
160 The rack containing the microtubes was placed in an ice bath and in each tube 100 µL
161 of dissolved sample was mixed with 100 µL of 130 µM DPPP dissolved in butanol
162 containing 4 mM BHT. Polypropylene caps were used to prevent solvent evaporation
163 during pipetting and incubation. Samples were incubated in a water bath at 60°C for
164 90 minutes under constant agitation. After incubation, the rack containing the tubes
165 was placed in an ice bath for 10 minutes to stop the reaction. Finally, samples were
166 allowed to stand at room temperature for 5 minutes and 100 µL was transferred to 96-
167 microwell plates. The fluorescence of the samples was measured using a Fluostar
168 Optima fluorimeter (BMG Labtech, Ortenberg, Germany). The apparatus was set at
169 30°C and the fluorescence measured with 360 ± 10 nm and 380 ± 10 nm filters for
170 excitation and emission, respectively. The signal was consecutively measured at 2-
171 minute intervals for 10 minutes. Since the signal was proven to be stable, the average
172 of the measurements was used for the calculations. The HP content of the samples
173 was determined using a calibration curve of R^2>0.99 and expressed as mmol of CHP
174 equivalents kg^{-1} of sample or transformed in mEq O_2 kg^{-1} of sample by multiplying the
175 former by a factor of 2 (Shantha & Decker, 1994).
176
177 **2.5. DPPP method version 2 (DPPP2)**
178 An appropriate samble size, between 1 and 5 g of sample, was dissolved in
179 chloroform/methanol (2:1, v/v) containing 4 mM BHT (between 10 and 50 mL
180 depending on the expected peroxide content) to lie within the linearity range of the
181 method. As with DPPP1, an aliquot of 100 µL of dissolved sample was pipetted in

polypropylene microtubes placed in an ice bath and mixed with 100 µL of 130 µM DPPP dissolved in butanol containing 4 mM BHT. Unlike DPPP1, and because of the higher degree of volatility of chloroform, the microtubes held in the rack had to be sealed using aluminium sealing foil (Deltalab, S.L., Rubí, Spain) instead of polypropylene caps. Samples were incubated in a water bath at 60°C for 150 minutes under constant agitation. After incubation, the rack was placed in an ice bath for 10 minutes in order to stop the reaction. Samples were allowed to stand at room temperature for 5 minutes and 100 µL was transferred to 96-microwell plates. The HP content was determined as described above for DPPP1.

2.6. Study of reaction kinetics

The reaction kinetics for DPPP1 were studied by mixing 100 µL of fish oil, sunflower oil or olive oil dissolved in 4 mM BHT butanol plus 100 µL of butanol containing DPPP and BHT at concentrations of 130 µM and 4 mM, respectively. The reaction kinetics for DPPP2 were studied by mixing 100 µL of fish oil, sunflower oil, palm oil and olive oil dissolved in 4mM BHT chloroform/methanol (2:1, v/v) plus 100 µL of butanol containing DPPP and BHT at concentrations of 130 µM and 4 mM, respectively. The fluorescence signal was assessed at 15, 30, 60, 90, 120, 150 and 180 minutes.

2.7. Determination of peroxide value (PV) and lipid hydroperoxide content by means of the ferrous oxidation-xylenol orange (FOX) method

The peroxide value (PV) of the samples was determined in accordance with the AOCS Official Method Cd 8-53 (AOCS, 2006). Results are expressed in mEq O_2 kg^{-1} of sample.

205 The lipid HP content of the samples was also assessed using the FOX method described
206 elsewhere (Navas et al., 2004). Briefly, samples were dissolved in
207 dichloromethane/ethanol (3:2, v/v), to fall within the linearity range of the method,
208 and 500 µL of dissolved sample was then mixed in 1-cm Teflon-capped glass cuvettes,
209 with the FOX reagent solution consisting of 100 µL 5 mM aqueous ferrous ammonium
210 sulphate, 200 µL 0.25 M methanolic H_2SO_4, 200 µL 1 mM methanolic xylenol orange
211 and 1000 µL dichloromethane/ethanol (3:2, v/v). Incubation was performed for 30 min
212 at room temperature under subdued light conditions. Absorbance was measured using
213 a Shimadzu UV-3600 UV-Vis-NIR spectrophotometer. Absorbance values were
214 measured at 560 nm. The results were expressed in mmol CHP equivalents kg^{-1} of
215 sample or transformed in mEq O_2 kg^{-1} of sample by multiplying the former by a factor
216 of 2.
217
218 **2.8. Statistical analysis**
219 Linear regression analysis and Pearson correlation coefficients were calculated using
220 SPSS v.17 for Windows (SPSS Inc. Chicago, IL, USA). A P-value of 0.05 or less for a one-
221 tailed test was considered significant.
222
223 **3. RESULTS AND DISCUSSION**
224
225 **3.1. Reaction kinetics of the proposed DPPP1 and DPPP2 versions of the method**
226 The development of fluorescence in the samples over incubation time at 60°C using
227 the DPPP1 and DPPP2 versions is shown in **Figure 1**. The signal was stable from 60 to
228 120 minutes for DPPP1 and from 120 to 180 minutes for DPPP2 for the oils assayed.

229 The majority of DPPP methods that measure the HP content in cuvettes carried out the
230 reaction for 60 min at 60°C (Akasaka et al., 1992; Meguro et al., 1990). However, the
231 reaction time depends on various factors, such as the type of HP to be determined and
232 the reaction medium (Bou, Chen, Guardiola, Codony, & Decker, 2010; Okimoto,
233 Watanabe, Niki, Yamashita, & Noguchi, 2000; Santas, Guardiola, Rafecas, & Bou, 2013).
234 Therefore, it is advisable to measure the HP content after longer incubation times to
235 ensure a complete reaction and, so times of 90 min and 150 min were considered
236 optimal for the DPPP1 and DPPP2 versions, respectively.
237
238 **3.2. Sensitivity of the method**
239 The detection (LD) and quantification (LQ) limits were calculated for both versions of
240 the method in accordance with Long and Winefordner (1983). With respect to the
241 DPPP1 version, the LD and LQ were 0.02 and 0.18 nmol of CHP eq./well, respectively,
242 and for the DPPP2 version, the LD and LQ were 0.04 and 0.22 nmol CHP eq./well,
243 respectively. Overall, these limits are in close agreement with those reported for DPPP-
244 based methods consisting of batch and flow injection analysis (FIA) measurements of
245 edible oils and food samples (Akasaka et al., 1992; Akasaka et al., 1996; Bou et al.,
246 2010).
247
248 **3.3. Linear range**
249 The reaction was linear from 0.18 to 2 nmols of CHP in 100 µl of reaction media for the
250 DPPP1 method (R^2=0.9964) and 0.22 to 2 nmols in 100 µl of reaction media of CHP for
251 the DPPP2 method (R^2= 0.9928). This range is slightly narrower than that reported by
252 Akasaka et al. (1992). This shorter linear range is due to the fact that these authors

253 used 86 µM of DPPP in the reaction media, whereas our study used 65 µM in both
254 versions. Undoubtedly, the linear range of the method can be improved by increasing
255 the concentration of DPPP in the media, but this would also involve a higher cost, since
256 this reagent is relatively expensive.
257
258 **3.4. Precision and recovery**
259 The relative standard deviation (RSD) of the responses of the CHP standards that were
260 within the linearity range of the method ranged from 6.0% to 1.8% (n=3) for DPPP1
261 and from 7.0% to 1.3% (n=3) for DPPP2. In fish oil sample that was appropriately
262 diluted to fall in the middle of the calibration curve, the RSD for five HP determinations
263 was 2.6% (average concentration of 0.71 ± 0.018 nmol/well containing 100 µL of
264 reaction media) for DPPP1 and 3.8% (average concentration of 0.86 ± 0.032 nmol/well
265 containing 100 µL of reaction media) for DPPP2. The inter-assay reproducibility of
266 DPPP1 and DPPP2, defined as the RSD of the slope of the calibration curve on four
267 different days, was 2.1% and 3.8%, respectively.
268 Recoveries determined by adding an appropriate amount of CHP to the
269 abovementioned fish oils to increase the concentration in 0.5 nmol and 1 nmol of CHP
270 per well were 97-102% for DPPP1 and 101-108% for DPPP2. Taking into account all of
271 these parameters and based on AOAC recommendations, the precision and recovery of
272 the DPPP1 and DPPP2 versions of the method are satisfactory (AOAC, 1993).
273
274 **3.5. Method comparison**
275 The DPPP method was developed in two versions (DPPP1 and DPPP2) that differ in the
276 solvents used. Although DPPP1 uses butanol and DPPP2 uses a mixture of

277 chloroform/methanol (2:1, v/v), both displayed a similar response in HP measurement
278 (**Table 2**). Other than the time required for a complete reaction to take place, the
279 quality parameters of the two versions of the DPPP method showed only slight
280 differences. The DPPP1 presents some advantages, such as being faster and more
281 precise than DPPP2. However, the solubility of some lipid samples in butanol (DPPP1)
282 is limited, whereas all of the samples studied were found to be soluble in the
283 chloroform/methanol mixture (**Table 1**). Therefore, for practical reasons, DPPP2 was
284 used throughout to determine the HP content of all samples and compare them with
285 those obtained using other methods***.***
286 The relationships between the DPPP2 method and the two other common methods
287 used to determine HP in edible oils and fats are shown in **Figure 2.** The AOCS Official
288 Method for the determination of PV in fats and oils was chosen as the reference
289 method for the measurement of HP (AOCS, 2006). The FOX method was selected, as it
290 is used as an alternative method for the measurement of PV in many foodstuffs. In
291 fact, HP determination in foodstuffs and biological samples by means of the FOX
292 method offers several advantages compared to other methods, notably its sensitivity
293 and simplicity (Bou et al., 2008). This method has been subject to minor modifications
294 in order to adapt it to some specific needs and matrices (Gay & Gebicki, 2003; Long,
295 Evans, & Halliwell, 1999; Navas et al., 2004; Nourooz-Zadeh, Tajaddini-Sarmadi, Ling, &
296 Wolff, 1996). In this context, Navas et al. (2004) improved the FOX method to measure
297 low amounts of HP in oils and lipid extracts that at high amounts barely dissolve in the
298 traditional methanol-based medium. By changing the solvents and their relative
299 amounts, higher sample loads can be dissolved, thus improving the sensitivity of this

300 version of the FOX method without substantially changing the method's other quality
301 parameters. We used this modified FOX method due to the increased sensitivity.
302 In accordance with the results shown in **Figure 2**, the Pearson's correlation coefficient
303 between the DPPP method and PV (r=0.998; P<0.001, n=37) indicates that the DPPP2
304 version has a high degree of accuracy. These results closely agree with those that
305 report a relationship between HP content measured using titration methods and that
306 which is different versions of the DPPP method involving batch measurements
307 (cuvettes) (Akasaka et al., 1992; Akasaka & Ohrui, 2000), FIA systems (Sohn, Taki,
308 Ushio, & Ohshima, 2005) and HPLC techniques (Nakamura & Maeda, 1991).
309 Furthermore, the Pearson's correlation coefficient between the DPPP2 and FOX
310 methods was also satisfactory (r=0.920, P<0.001, n=38). In fact, it is similar to the
311 correlation between the PV and the FOX method (r=0.933, P<0.001, n=38), thus
312 indicating that the DPPP2 method compares well with the other methods.
313 The DPPP2 method has several advantages compared with the PV determination and
314 FOX methods. Firstly, DPPP2 is not only precise, but also highly sensitive, and thus
315 detects HP at much lower levels than PV. Likewise, the FOX method is considered to be
316 very sensitive, but its level of detection (expressed per weight of sample) is highly
317 dependent on the solubility of the oil in the reaction media, which is usually composed
318 of 90% methanol or, in improved methods, H_20/methanol/CH_2Cl_2/ethanol (1:4:9:6,
319 v/v/v/v) in order to overcome lipid sample solubility problems (Bou et al., 2008). As
320 illustrated in **Table 3**, even an improved version of the FOX method was not sensitive
321 enough to determine the concentration of HP in samples such as butter or palm kernel
322 oil that have poor solubility in the reaction medium. However, using DPPP2 eliminates
323 this problem by dissolving lipid samples in chloroform/methanol (2:1, v/v).

Secondly, DPPP-based methods are not only sensitive, but also selective for HP determination (Akasaka & Ohrui, 2000), whereas the PV determination and FOX methods are known to present interference and specificity problems (Bou et al., 2008; Dobarganes & Velasco, 2002; Jessup et al., 1994; Nielsen et al., 2003). For instance, the PV method may be affected by the structure and reactivity of peroxides, the absorption of iodine at unsaturation sites in fatty acids and the liberation of iodine from potassium iodide by oxygen present in the solution to be titrated (Jessup et al., 1994). The FOX method is subject to interference from several factors, including oxidizing/reducing agents, chelators and chromophores (Bou et al., 2008). This latter method is based on the complexation reaction of ferric ions with xylenol orange and, since iron is highly ubiquitous, the use of high purity reagents and clean glassware is recommended (sometimes previously cleaned with a sulphuric acid/dichromate solution and double-distilled water), which makes this method tedious and time-consuming (Bou et al., 2008).

Thirdly, the low sample and solvent amounts required for the proposed versions of the DPPP method also represent a considerable advantage compared to the PV determination method, which requires large solvent and sample amounts (**Table 2**). It has been estimated that the cost of consumables in the determination of PV is about 5 times that of DPPP, in consequence, the environmental impact and cost of both DPPP versions is much lower.

Finally, the number of samples that can be analysed simultaneously using the DPPP1 and DPPP2 versions is much higher compared with previous DPPP-based methods consisting of cuvette measurements (Akasaka et al., 1992; Bou et al., 2010). The proposed method uses 96-well microplates and is therefore easier to perform than

348 other methods that use HPLC techniques (Akasaka & Ohrui, 2000) and, consequently,
349 very useful as a routine method for quality control measurements in the industry. It is
350 also worth noting that DPPP2 uses chloroform/methanol (2:1, v/v), which is the most
351 common solvent mixture used for the extraction of lipids in a broad variety of samples
352 (e.g. complex foods and biological samples), which would indicate that this method is
353 also promising for other applications.
354
355 **4. CONCLUSIONS**
356 Considering all the characteristics discussed above, both of the proposed versions of
357 the DPPP method for the determination of lipid HP content in edible oils and fats
358 proved to be precise, accurate and sensitive. In addition, this method requires low
359 sample, reagent and solvent amounts. It is also easy to perform and suitable for high-
360 throughput sample analysis. Therefore, DPPP2 (or alternatively DPPP1) can be used for
361 the routine analysis of HP in edible fats and oils in the food industry and in research
362 laboratories.
363
364 **Acknowledgments**
365 The authors thank Lipidos Santiga for kindly providing some of the oils analysed.
366

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Figure 1. Development of fluorescence of the reaction of DPPP with lipid hydroperoxides. A: DPPP1 (uses butanol) kinetics. B: DPPP2 (uses chloroform/methanol; 2:1 v/v) kinetics. Results represent means and SD error bars (n=3)

Figure 2. Fitted regression line and data points of DPPP2 *vs* PV (A), DPPP2 *vs* FOX (B), and FOX *vs* PV (C).

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Table 1. Solubility of oils and fats in pure butanol and chloroform/methanol (2:1, v/v)

Lipid	Butanol	Chloroform/Methanol
Butter	Insoluble	Soluble
Canola oil	Soluble	Soluble
Coconut oil	Partly soluble*	Soluble
Corn oil	Soluble	Soluble
Fish oil	Soluble	Soluble
Grape seed oil	Soluble	Soluble
Hazelnut oil	Soluble	Soluble
High-oleic sunflower oil	Soluble	Soluble
Olive oil	Soluble	Soluble
Palm kernel oil	Insoluble	Soluble
Palm oil	Insoluble	Soluble
Peanut oil	Soluble	Soluble
Pork lard	Insoluble	Soluble
Safflower oil	Soluble	Soluble
Sesame seed oil	Soluble	Soluble
Soybean oil	Soluble	Soluble
Sunflower oil	Soluble	Soluble
Walnut oil	Soluble	Soluble

* 7 ml of solvent is the minimum volume required to dissolve 1 g of sample

Table 2. Comparison of quality performance parameters of the proposed versions of the diphenyl-1-pyrenylphosphine method (DPPP1 and DPPP2), the ferrous oxidation-xylenol orange (FOX) method and peroxide value (PV) determination.

	DPPP1	**DPPP2**	**FOX**[1]	**PV**
Solvents in reaction medium	100% butanol	$CHCl_3$/methanol/butanol (2:1:3, v/v/v)	90% methanol or H_20/methanol/CH_2Cl_2/ethanol (1:4:9:6, v/v/v/v)	Acetic acid/isooctane (3:2, v/v)[2]
Volume of solvent	200 µL	200 µL	9 mL	50 mL
Reaction time	90 min	150 min	30 min	1 min
Incubation temperature	60°C	60°C	Room temp	Room temp
Sample amount	≤ 100 mg[3]	≤ 100 mg[3]	≤ 100 mg[3]	0.3 – 5 g
Limit of detection[4]	0.02 nmol/well	0.04 nmol/well	≈ 0.3 nmol/cuvette	≈ 0.2 µmol/flask
Sample throughput	high	high	medium-high	low
Linearity range	0.18 - 2 nmol/well	0.22 - 2 nmol/well	1 – 40 nmol/cuvette	0.2 – 1 µmol/flask[5]
Within day relative SD	1.8 – 6.0%[6]	1.3 – 7.0%[6]	0.3 - 10%	2.9 - 7.8%
Between day relative SD	2.1%[7]	3.8%[7]	--	--
Recovery	97-102%[8]	101-108%[8]	--	--

[1] The method described by Nourooz-Zadeh et al. (1995) uses 90% acidified methanol to measure hydroperoxides in edible oils and also in a broad variety of samples (Bou et al., 2008). The method described by Navas et al. (2004) uses a reaction medium consisting of methanol/CH_2Cl_2/H_2O/ethanol, which provides the same response level as the typical methanol-based method, but allows higher lipid amounts to be dissolved; this improves the limit of detection and quantification per weight of sample. Despite these differences, the performance of these methods is similar and therefore the parameters reported for the FOX method are in agreement with the literature (Bou et al., 2008; Navas et al., 2004; Nielsen et al., 2003; Nourooz-Zadeh et al., 1995; Shantha & Decker, 1994).

[2] The medium composition corresponds to that described in the AOCS Official Method (AOCS, 2006) and the performance parameters are in agreement with the literature (Nielsen et al., 2003; Shantha & Decker, 1994).

[3] Due to the high sensitivity of the method, it is often necessary to make a 10-fold or higher dilution when samples contain elevated hydroperoxide levels.

[4] In the DPPP1 and DPPP2 versions the limit of detection was calculated by multiplying by 3 the standard deviation of the blank and expressed as nmol of cumene hydroperoxide per well (100 µL). The limit of detection of the FOX method was in agreement with the literature (Dobarganes & Velasco, 2002) and also with that obtained by multiplying by 3 the standard deviation reported in oils and fats at low levels and considering 1 mg of sample weight in the cuvette after pipetting the previously diluted sample (Nourooz-Zadeh et al., 1995). The limit of detection of the AOCS method for determination of the peroxide value was in agreement with the data reported in the literature (Dobarganes & Velasco, 2002; Nielsen et al., 2003) and also with that obtained by multiplying by 3 the standard deviation reported in oils and fats at low levels and considering 1 g of sample weight in the Erlenmeyer flask (AOCS, 2006; Nourooz-Zadeh et al., 1995).

[5] As reported in the literature (Nielsen et al., 2003).

[6] This relative SD range was obtained from the standard solutions of the calibration curve that fell within the linear range of the method (n=3 for each concentration).

[7] This value is the relative SD of the slope of the calibration curve on four different days.

[8] This range was obtained from the standard addition of 0.5 nmol and 1 nmol of cumene hydroperoxide per well (n=3).

Table 3. Hydroperoxide content of samples determined by means of the PV determination, FOX and DPPP2 methods.

Sample	Origin	PV[1] (mEq O_2/Kg)	FOX[2] (mEq O_2/Kg)	DPPP2 (mEq O_2/Kg)
Butter	Animal	ND*	ND	0.068
Butter	Animal	ND	ND	0.038
Butter	Animal	ND	ND	0.056
Butter	Animal	0.939	1.020	1.428
Canola oil	Vegetal	0.377	0.576	0.600
Canola oil	Vegetal	0.320	0.622	0.596
Canola oil	Vegetal	0.327	0.584	0.590
Coconut oil	Vegetal	ND	0.035	0.090
Coconut oil	Vegetal	ND	ND	0.082
Coconut oil	Vegetal	ND	0.048	0.086
Coconut oil	Vegetal	0.831	1.749	0.796
Corn oil	Vegetal	2.124	2.807	2.338
Corn oil	Vegetal	0.540	1.016	1.112
Corn oil	Vegetal	0.584	0.966	1.016
Fish oil	Animal	7.100	16.700	5.716
Fish oil	Animal	9.717	27.258	7.350
Fish oil	Animal	1.480	2.013	1.528
Grape seed oil	Vegetal	4.554	10.576	3.652
Hazelnut oil	Vegetal	5.137	6.242	4.132
Lard	Animal	1.427	2.139	1.418
Lard	Animal	3.655	4.602	3.094
Lard	Animal	4.472	5.982	3.732
Olive oil	Vegetal	17.836	26.974	15.454
Olive oil	Vegetal	6.879	17.002	6.474
Olive oil	Vegetal	24.802	27.362	21.764
Palm Kernel oil	Vegetal	0.186	ND	0.068
Palm Kernel oil	Vegetal	0.219	ND	0.082
Palm Kernel oil	Vegetal	0.726	0.471	0.340
Palm oil	Vegetal	0.426	0.441	0.436
Palm oil	Vegetal	15.490	20.963	13.362
Palm oil	Vegetal	5.906	8.901	5.182
Peanut oil	Vegetal	0.620	0.431	0.694
Safflower oil	Vegetal	0.487	0.696	0.628
Sesame oil	Vegetal	0.309	1.187	0.506
Soybean oil	Vegetal	0.464	0.816	0.920
Soybean oil	Vegetal	0.458	0.690	0.876
Soybean oil	Vegetal	0.806	1.374	0.910
Sunflower oil	Vegetal	13.335	28.131	11.474
Sunflower oil	Vegetal	0.018	0.204	0.300
Sunflower oil	Vegetal	1.004	1.849	1.118
Sunflower oil High-Oleic	Vegetal	0.317	0.447	0.464
Sunflower oil High-Oleic	Vegetal	0.244	0.505	0.616
Walnut oil	Vegetal	0.723	1.733	0.690

[1] In accordance with the AOCS Official Method (AOCS, 2006)

[2] In accordance with the method described elsewhere (Navas et al., 2004)

*ND: Not detected

Figure 1 revised

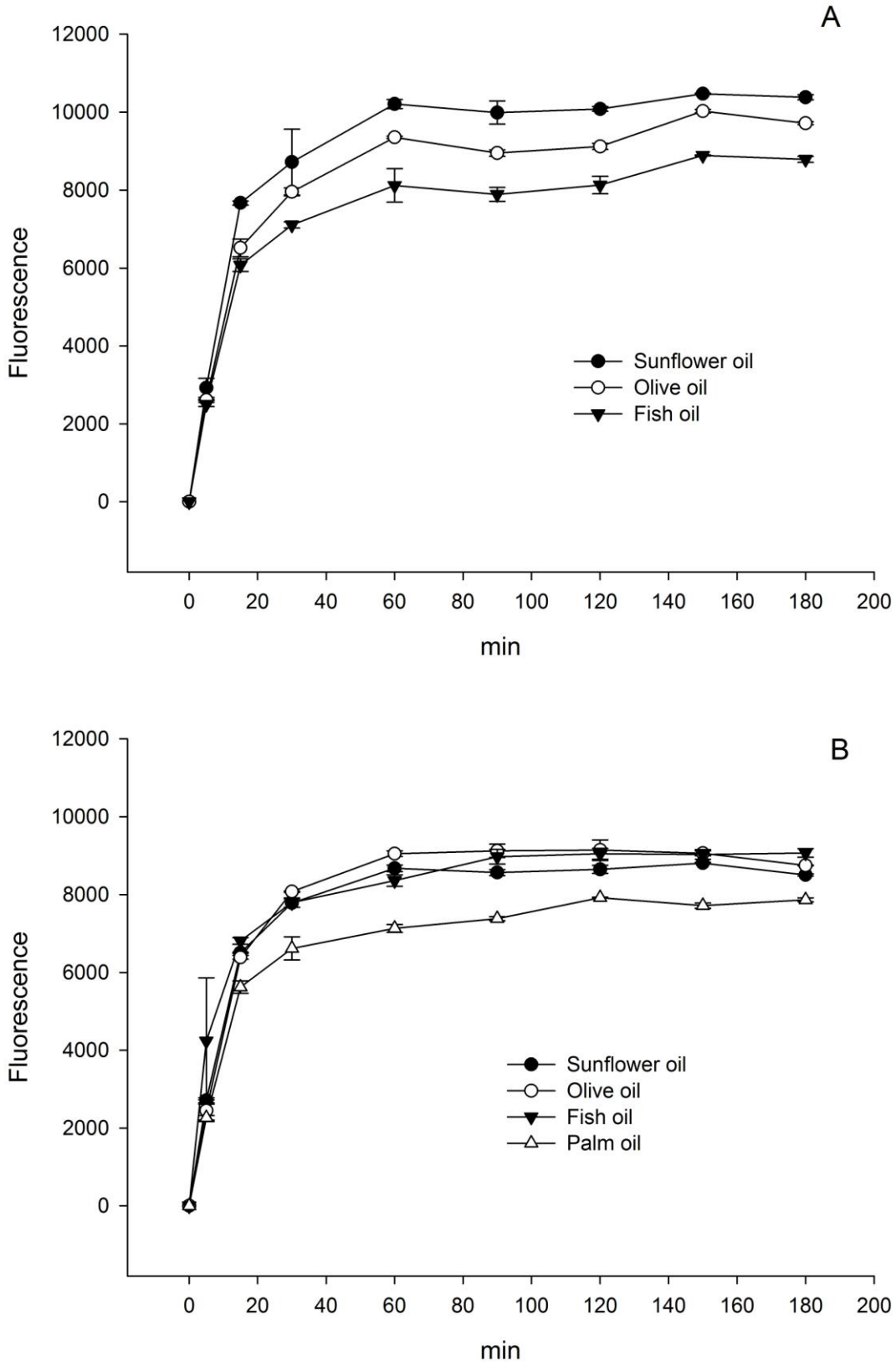

Figure 1.

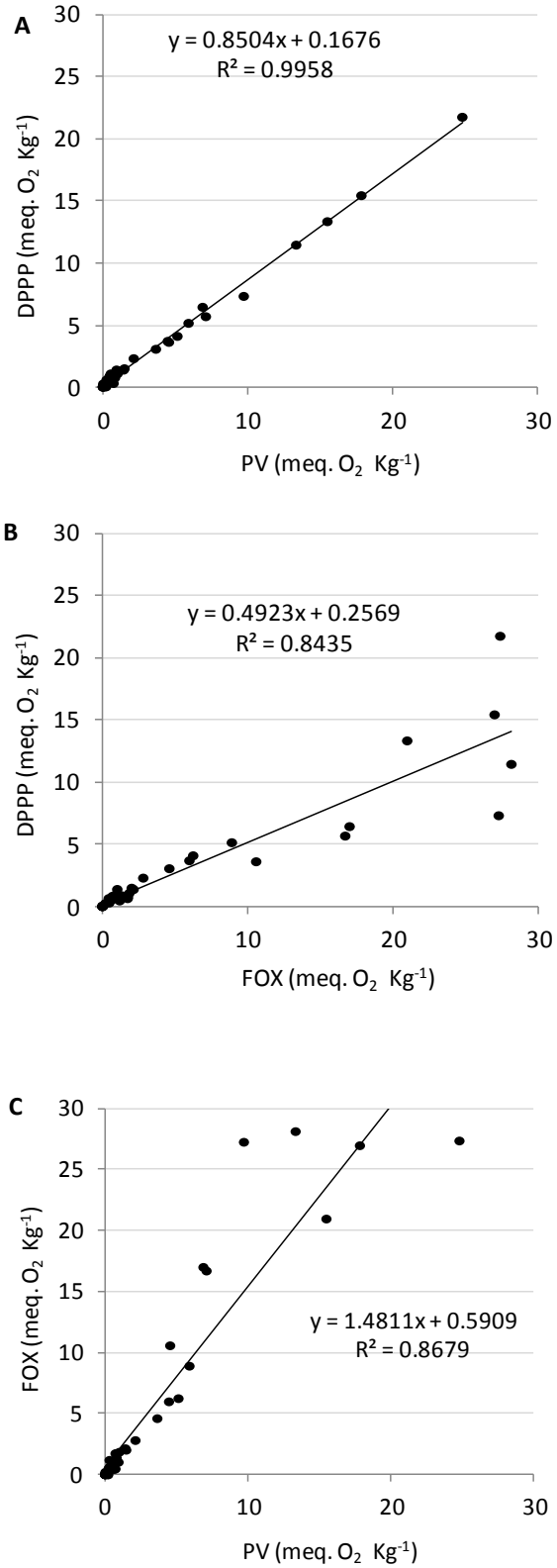
A
y = 0.8504x + 0.1676
R² = 0.9958
DPPP (meq. O2 Kg-1)
PV (meq. O2 Kg-1)
B
y = 0.4923x + 0.2569
R² = 0.8435
DPPP (meq. O2 Kg-1)
FOX (meq. O2 Kg-1)
C
y = 1.4811x + 0.5909
R² = 0.8679
FOX (meq. O2 Kg-1)
PV (meq. O2 Kg-1)

Figure 2.