This is the Submitted Manuscript version of an article accepted for publication in Food Control.
The Version of Record is available online at https://doi.org/10.1016/j.foodcont.2021.108295

Analysis of peroxide value in olive oils with an easy and green method

Francesco Longobardi[1]*, Francesco Contillo[1], Lucia Catucci[1,2], Luca Tommasi[3], Francesco Caponio[4], Vito Michele Paradiso[4,5]*

[1] Department of Chemistry, University of Bari, Via Orabona 4, I-70126, Bari (Italy); [2] CNR-IPCF Institute for Physical and Chemical Processes, Bari unit, Via Orabona 4, I-70126, Bari (Italy); [3] BonassisaLab, S.S.16 Km.684,300, I-71122 Foggia (Italy); [4] Department of Soil, Plant and Food Sciences, University of Bari, Via Amendola 165/a, I-70126, Bari (Italy); [5] Department of Biological and and Environmental Sciences and Technologies, Food Science Laboratory, University of Salento, I-73100 Lecce (Italy)

* corresponding authors: francesco.longobardi@uniba.it, (F Longobardi); vito.paradiso@unisalento.it, (V M Paradiso)

Abstract

A green, easy-to-use, and sensitive spectrophotometric iodide-dependant method for the quantitative determination of the lipidic hydroperoxides in virgin olive oils was developed. Virgin olive oils were added with 0.5 % HCl-ethanol, with a saturated KI solution and, after incubation, the resulting solution was filtered. Hydroperoxides were determined indirectly by reading the absorbance of the generated triiodide at 350 nm; the total time of analysis was about 7 min. A good linearity (with correlation coefficient (*r*) of 0.9997) of the calibration curve was obtained with purified olive oil spiked with tert-butylhydroperoxide at levels ranging from 1.0 to 10.0 meqO_2/kg, with variation coefficients less than 5% (n=3) and limit of detection and quantification of 0.3 and 0.9 meqO_2/kg, respectively. Results obtained with the spectrophotometric method showed good correlation with those obtained with the official method with a *r* of 0.9819 confirming the reliability of the developed method.

Keywords

Hydroperoxides, spectrophotometric method, virgin olive oil, green analytical chemistry

1. Introduction

Oxidation is of paramount importance for the quality and shelf life of vegetable oils, a fortiori for high quality products such as virgin olive oils (Paradiso, Pasqualone, Summo, & Caponio, 2018). National and international regulations (CodexAlimentarius, 2017; EEC Commission, 1991) set legal limits for the analytical parameters related to oil oxidation. Peroxide value, still the most representative parameter considered to measure oxidation in virgin olive oils, measures the level of primary oxidation products – hydroperoxides of fatty acids (ROOH) – deriving from H abstraction from the carbons (mainly bis-allylic and allylic) of the fatty acid chain (Schaich, 2005). The official method to determine peroxide value (PV) in olive oils is the iodometric titration assay, as reported in the Annex III of the Reg. (EEC) 2568/91 (EEC Commission, 1991), based on the oxidation of the iodide ion (I^-) by ROOH in acidic environment. Briefly, oil is dissolved in a mixture of chloroform/acetic acid and added with a saturated solution of potassium iodine. The reaction of I^- with ROOH leads to the formation of molecular iodine (I_2) which is titrated with a solution of sodium thiosulfate and starch as an endpoint indicator (Kiritsakis, Kanavouras, & Kiritsakis, 2002). The PV is calculated as milliequivalents of oxygen per kilogram of sample ($meqO_2/kg$). Nevertheless, this method presents several limitations: time-consuming and labour-intensive procedure, low sensitivity, need of large amounts of sample and to weight the sample according to the presumed number of peroxides, difficulty to determine the endpoint, large amounts of wastes, use of harmful/polluting solvents (Dobarganes & Velasco, 2002; Schaich, 2013; Shahidi & Zhong, 2005). Besides these drawbacks, the main limitation is the co-presence of the interfering reaction between oxygen present in solution and potassium iodide which again produce iodine causing an overestimation of the PV. In addition, absorption of iodine by unsaturated fatty acids (leading to PV underestimation), the oxidation of acetic acid and lipid oxidation (source of peroxides), and the sodium thiosulfate decomposition are possible sources of error.

To overcome these drawbacks, other analytical methods have been proposed for PV measurements but neither of them is free from complications. Some proposed methods, either being green and almost solventless such as FTIR (Yu, van de Voort, & Sedman, 2007), or using organic solvents, such as RP-HPLC (Yang, 1992), require expensive equipment. Other methods

are based on the oxidation of iron with formation of ferric ions complexes (with thiocyanate or orange xylenol) that can be detected spectrophotometrically (Schaich, 2013). The ferric thiocyanate method is sensitive and reproducible, nevertheless it requires harmful solvents. The orange xylenol method, instead, is affected by many factors, such as the amount of sample, solvent used, and source of xylenol orange (Shahidi & Zhong, 2005). Moreover, these methods were conceived for peroxides in solvents or living tissues and may show lack of linearity in the concentration ranges expected in food samples, so that sample size and dilution should be adjusted, particularly in shelf-life studies (Schaich, 2013). Analogous considerations could be made for the methods developed using triphenylphosphine (Talpur, Sherazi, Mahesar, & Bhutto, 2010; Yu, Li, Sun, Dong, & Wang, 2015).

Almost three decades ago, Løvaas proposed a spectrophotometric iodide-dependant method to assess lipid hydroperoxides based on the absorbance of the triiodide (I_3) formed following the redox reaction between iodide and hydroperoxides (Løvaas, 1992). In fact, the liberated iodine (generated by the redox) and iodide (in excess) react to form I_3^-, which can be detected spectrophotometrically providing an indirect quantification of hydroperoxides. The method proposed by Løvaas was applied to capelin oil, a fish oil, which is quite a different matrix, compared to virgin olive oil, for fatty acid composition, as well as for the presence of potentially interfering substances. Moreover, though showing the feasibility of this analytical approach, the paper did not report method validation. Therefore, in view of the application of this method to virgin olive oils, possibly for in-situ analysis (e.g. in olive mills and bottling/storage plants) and oxidative status tracking, the following issues should be considered:

- Adaptation to the complexity of the virgin olive oil matrix
- Use of low volumes of reagents and solvents
- Reduction of the environmental and health impact of reagents and solvents
- Method validation.

In this framework, this research work was focused on the development of spectrophotometric iodide-dependant method for monitoring the oxidation of virgin olive oils during early stages of the oxidation process aiming to create an analytical protocol easy-to-use, as sustainable as possible

(green), with a reduced consumption of solvents and samples, with a high sensitivity and specificity towards primary oxidation products.

2. Material and Methods

2.1 Reagents and Apparatus

Potassium iodide (KI, purity ≥99.0%), tert-Butyl hydroperoxide solution 70 wt. % in H_2O (TBHP, Luperox® TBH70X), hydrochloric acid 37% v/v (HCl), glacial acetic acid (CH_3COOH, purity ≥99.0%), chloroform ($CHCl_3$ purity ≥99.5%), sodium thiosulfate ($Na_2S_2O_3$, purity=99%), and starch were purchased from Sigma Aldrich (Milan, Italy). Absorbance measurements were carried out with a Shimadzu (Shimadzu Corporation, Kyoto, Japan) UV-Vis spectrophotometer model UV-1700 PharmaSpec.

2.2 Samples and Calibration Curves

Oxidized olive oil (OOO) samples used for the development of the method were prepared by incubating a refined olive oil (ROO) at 60 °C for different times ranging between 3 and 14 days. The concentrations of hydroperoxide generated were determined by using the iodometric titration.

A purified olive oil (POO) having a negligible PV value was obtained as reported by Paradiso et al. (Paradiso, Gomes, Nasti, Caponio, & Summo, 2010).

Two matrix-assisted calibration curves were built with calibrants prepared as following: (a) by blending two OOOs having PV of 8.2 and 2.2 meqO_2/kg, respectively, obtaining calibrants with PV of 2.2, 3.7, 5.2, 6.7, and 8.2 meqO_2/kg; (b) by spiking the POO with increasing quantity of TBHP obtaining calibrants with PV of 1.0, 2.0, 4.0, 6.0, 8.0, and 10 meqO_2/kg. The limit of detection (LOD) was calculated as: $3\times s_{y/x}/b$ where $s_{y/x}$ is the residual standard deviation of the regression line and b is the slope of the calibration curve. The limit of quantification (LOQ) was calculated as: 3×LOD.

For method validation, virgin olive oils (VOOs) were supplied by producers (year of production 2019) or purchased from supermarkets (year of production 2018) for a total of 23 samples.

2.3 Methods

Titrations were performed as described by the Reg. (EEC) 2568/91, Annex III (EEC Commission, 1991).

For spectrophotometric analysis an aliquot of 0.10 g of sample was added with 1 mL of 0.5% HCl-ethanol, 0.10 mL of saturated KI solution and incubated for 5 min. The resulting solution was added with 4 mL of water and transferred to a 1-cm UV cuvette with a plastic syringe equipped with a 0.45 µm pore size regenerated cellulose (RC) filter (Levanchimica s.r.l., Bari, Italy); the absorbance was measured at 350 nm against the blank (obtained by using the same procedure but without sample). The total time of analysis was about 7 min. Solutions showing absorbance values higher than 1.5 a.u. were suitably diluted and re-analyzed; the diluted factor was considered for the calculation of concentrations.

All experiments were done in triplicate.

2.4 *Statistical analysis*

Statistic analyses were performed using Statistica version 8.0 (StatSoft Italia srl, Padova, Italy).

3. Results and Discussion

Preliminarily, in order to verify the possibility of using the spectrophotometric detection rather than the manual titration for the determination of hydroperoxide contents, the absorbances of the aqueous solutions obtained by analyzing oxidized olive oil samples with the official method were measured between 250 and 500 nm. The use of the instrumental determination introduced immediate advantages, such as a higher inherent precision, a reduction of the total time of analysis (no titration was necessary), no thiosulphate decomposition risk, no need of trained operators, and a reduction of the used quantity of sample and reagents by a factor equal to 4 thanks to the reduce volumes required for the spectrophotometric measurement. As showed in Figure 1, two absorption peaks due to the formed chromophore at about 290 and 350 nm, respectively, were observed having intensities proportional to the PV of the analyzed OOOs (Løvaas, 1992). Nevertheless, also for the experiment carried out in the absence of sample, the I_3^- signals were still detectable, even if of low intensity. This confirms the presence of the interfering reaction between the dissolved

oxygen and iodide to form again triiodide, causing PV overestimation risks. Subsequently, aiming to improve the analytical performance, some factors such as nature of organic solvent and acid, acidic concentration, and intensity of the interfering reaction absorbance were investigated studying their effect on spectra and/or on the peak intensity at 350 nm.

In order to reduce the environmental impact of the method, the acetic acid/chloroform mixture used in the titrimetric method was replaced with an acidified alcohol. Firstly, 3% HCl-methanol solution was evaluated. However, the use of alcoholic solutions did not lead to a clear separation between the organic and the aqueous phase in the subsequent steps of the analysis, and required a filtration step before carrying out the spectrophotometric measurements. Therefore, to identify a filter with minimal retention of the chromophore of interest, experiments were initially carried out by using 3% HCl-methanol in the absence of olive oil and the absorption spectra due to the interfering reaction were recorded both without the filtering operation, and using two different filters, i.e. cellulose acetate (CA) and regenerated cellulose (RC) filter. As shown in Figure 2, similar absorbances were obtained from the unfiltered solutions and the solutions filtered with the RC with no statistically significant difference at 350 nm ($p \leq 0.05$; $n = 3$); on the other hand, the CA filter negatively affected the signal intensity reducing it significantly. Subsequently, the same experiments were repeated in the presence of an OOO having PV = 14.3 meq O_2/kg. Also, in this case, a signal intensity decrease was observed with the CA compared to the RC filter. Therefore, the latter was chosen as the optimal filter and used in subsequent experiments. It is important to highlight that even if the filtration step slightly complicates the analysis protocol, it simultaneously reduces the contact time between the iodine produced and the sample limiting the risk of undesirable absorption of iodine by unsaturated fatty acids.

Besides 3% HCl-methanol, also 3% HCl-ethanol was evaluated. Results obtained analyzing olive oil samples (PV = 14.3 meqO_2/kg) revealed that the peak height was increased by 211±10% ($n = 3$) by using ethanol rather than methanol. This absorbance increment could be explained considering two aspects. On the one hand, I_3^- showed a greater extinction coefficient in ethanol than in methanol, on the other hand, even if the reaction of iodine and iodide is strongly in favor of I_3^- formation, the equilibrium constant (K) is inversely related to the dielectric constant of the

173 solvent determining a K value greater in ethanol than in methanol (Løvaas, 1992). Therefore, in the
174 following studies HCl-ethanol was used as organic solvent.
175 As known, the strongly acid conditions adopted in the iodometric methods represent a weakness of
176 these approaches because they also accelerate the iodide background oxidation. Taking into
177 account that acid cannot be omitted (being necessary for the reaction between ROOH and I^-),
178 different ethanol solutions containing decreasing amounts of HCl (3%, 2%, 1%, 0.5%), both in the
179 presence and absence of samples (PV = 5.0 meqO_2/kg), were used in order to evaluate these
180 background reactions.
181 As expected, as the amount of acid decreases, a significant decrease in the intensity of
182 absorbance was observed both in the presence and absence of sample (see Figure 3).
183 Specifically, it is important to note that the spectrum obtained by using the 0.5% HCl-ethanol
184 solution in the presence of the oxidized olive oil was still well observable allowing the ROOH
185 detection even at this low PV value. On the contrary, in the same acidic condition, the absorbance
186 obtained without sample was drastically reduced. Consequently, in the following experiments the
187 acid concentration was fixed at 0.5%.
188 To completely remove the influence of the absorbance due to the interfering reaction, the mixture
189 prepared following the analytical protocol but without olive oil could be used as spectrophotometric
190 blank and consequently its absorption spectrum as baseline. However, to be sure that this
191 approach did not introduce a source of error, it was considered necessary that the blank spectrum
192 should have never recorded an absorbance reading significantly varying during time. To test the
193 stability of the saturated solution of KI over time, so as to determine the same extent of the
194 interfering reaction and consequently a constant baseline, starting from the same saturated
195 solution of KI, different blank solutions were prepared at different times from KI solution
196 preparation, i.e. 10, 20, 30, 60, 120, 240, and 360 minutes, and their absorbance quickly read. The
197 experiments were carried out in triplicate: the mean values at 350 nm and their standard deviations
198 were calculated and showed in Figure 4. By applying an analysis of variance ANOVA, no
199 significant difference ($p \leq 0.05$) were found among the means, highlighting that the absorbance
200 could be considered constant at least during the investigated time range, i.e. up to 6 hours from the

preparation of the KI solution. Therefore, in the following experiments the contribution of the interfering reaction in the absorption of the analysis of oil samples was removed easily by using the spectrum obtained from the analysis of the saturated KI solution as baseline which was subtracted from subsequent sample readings.

Taking into account the results obtained from the experiments reported above, as well as the aims of this research work, it was decided to adopt the following analytical protocol. An aliquot of 0.10 g of sample was added with 1 mL of 0.5% HCl-ethanol, 0.10 mL of saturated KI solution and incubated for 5 min. The resulting solution was filtered on a RC filter and analysed spectrophotometrically. The absorbance was measured at 350 nm against the blank (obtained by using the same procedure but without sample). This protocol proved to be a good compromise between the desired characteristics in terms of sensitivity, quantity of sample and solvent, eco-compatibility of the solvent, and some practical aspects such as the maximum measurable absorbance fixed at 1.5 a.u.

Two calibration curves were built following the developed protocol. A first matrix calibration curve (Figure 5a), obtained by blending refined olive oils, showed a good linear response (r = 0.9991) obtained in the analysed concentration range (2.2-8.2 meqO_2/kg), with variation coefficients less than 5% (n=3) and limit of detection (LOD) and quantification (LOQ) of 0.4 and 1.2 meqO_2/kg, respectively. A second matrix calibration curve (Figure 5b), obtained by spiking a purified olive oil with TBHP (concentration range 1.0-10.0 meqO_2/kg), showed a good linear response (r = 0.9997), with variation coefficients less than 5% (n=3) and limit of detection (LOD) and quantification (LOQ) of 0.3 and 0.9 meqO_2/kg, respectively. By carrying out an Analysis of covariance (ANCOVA) between the two linear regressions, it was found that the regressions are not statistically different ($p \leq 0.05$); therefore the choice to use one method or the other for the preparation of the calibrants mainly depends on experimental considerations. In particular, the fortification approach of a purified oil has several advantages compared to the mixture approach. Fortification, in fact, allows to freely choose both the minimum level of fortification and, within the limits of linearity, the concentration range to be used for calibration. Otherwise, in the case of blending, the lowest point that can be analyzed depends on the availability of a poorly oxidized oil. In addition, the

concentration values achieved through the use of the standard solution can be considered more accurate because, as mentioned above, they do not depend on the quantifications obtained by the official method; the latter, in fact, is affected by an higher uncertainty. For the reasons described above, the subsequent quantification analyses were carried out using the calibration curve obtained by fortification.

Finally, for the validation, 23 olive oil samples were analyzed by the proposed method and by the official iodometric titration. As showed in Figure 6, a very good correlation (r = 0.9819) with a slope very close to 1 and an intercept close to 0 was obtained confirming the reliability of the developed method.

4. Conclusions

In summary, a green and sensitive spectrophotometric iodide-dependant method for the quantitative determination of the lipidic hydroperoxides in virgin olive oils was developed in the present research. The method proposed shows different advantages in comparison with the official method in terms of analytical performances (time, accuracy, reproducibility, LOQ, and LOQ), sustainability of the solvent (substitution of chloroform/acetic acid with 0.5% HCl-ethanol), ease of use (no titration), reduction of sample amounts and solvent volumes, and reduction of interferences from side reactions. In particular, the use of the white absorbance as a baseline allows to remove or in any case drastically reduce interference from atmospheric oxygen without degassing. Moreover, the replacement of acetic acid with hydrochloric acid removes the risk of having a PV overestimation coming from the possible oxidation of acetic acid to peroxides. Finally, unlike the official method, for the method developed herein the amount of sample to be weighted does not depend on the expected peroxide value but is constant. This last feature makes the PV evaluation not influenced by the amount of sample, increasing both the performance and the ease to use of the method.

Declaration of competing interest

The authors have no competing interests to declare.

Acknowledgements

The work was carried out within the M3O3 project ("Multifunctional microsystems for the monitoring of olive oils oxidation" – project code XMPYXR1) founded by Regione Puglia (POR Puglia FESR-FSE 2014-2020 - Innonetwork call).

References

CodexAlimentarius. (2017). Codex Stan 33-1981. STANDARD FOR OLIVE OILS AND OLIVE POMACE OILS. *Codex*, *91*(5), 1689–1699. https://doi.org/10.1017/CBO9781107415324.004

Dobarganes, M. C., & Velasco, J. (2002). Analysis of lipid hydroperoxides. *European Journal of Lipid Science and Technology*, *104*(7), 420–428. https://doi.org/10.1002/1438-9312(200207)104:7<420::AID-EJLT420>3.0.CO;2-N

EEC Commission. EEC Regulation 2568/91, 37 Official Journal of the European Communities § (1991). https://doi.org/2004R0726 - v.7 of 05.06.2013

Kiritsakis, A., Kanavouras, A., & Kiritsakis, K. (2002). Chemical analysis, quality control and packaging issues of olive oil. *European Journal of Lipid Science and Technology*, *104*, 628–638. Retrieved from http://www.uclm.es/grupo/gao/aovc-upv-ehu/Tema3/Chemical analysis, quality control and packaging.pdf

Løvaas, E. (1992). A sensitive spectrophotometric method for lipid hydroperoxide determination. *Journal of the American Oil Chemists Society*, *69*(8), 777–783. https://doi.org/10.1007/BF02635914

Paradiso, V. M., Gomes, T., Nasti, R., Caponio, F., & Summo, C. (2010). Effects of free fatty acids on the oxidative processes in purified olive oil. *Food Research International*, *43*(5), 1389–1394. https://doi.org/10.1016/j.foodres.2010.04.015

Paradiso, V. M., Pasqualone, A., Summo, C., & Caponio, F. (2018). Everything Should Be as Simple as It Can Be. But Not Simpler. Does Food Lipid Oxidation Require an Omics

Approach? *European Journal of Lipid Science and Technology*, *120*(7), 1800103. https://doi.org/10.1002/ejlt.201800103

Schaich, K. M. (2005). Lipid Oxidation: Theoretical Aspects. In *Bailey's Industrial Oil and Fat Products* (Vol. 1, pp. 269–355). Hoboken, NJ, USA: Wiley. https://doi.org/10.1002/047167849X.bio067

Schaich, K. M. (2013). Challenges in Analyzing Lipid Oxidation: Are One Product and One Sample Concentration Enough?\rWhen, Where, and How Do Products Arise? In *Lipid Oxidation. Challenges in Food Systems* (pp. 53–128).

Shahidi, F., & Zhong, Y. (2005). Lipid oxidation: measurement methods. *Bailey's Industrial Oil and Fat Products*, (3). Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/047167849X.bio050/full

Talpur, M. Y., Sherazi, S. T. H., Mahesar, S. A., & Bhutto, A. A. (2010). A simplified UV spectrometric method for determination of peroxide value in thermally oxidized canola oil. *Talanta*, *80*(5), 1823–1826. https://doi.org/10.1016/j.talanta.2009.10.028

Yang, G. C. (1992). Detection of lipid hydroperoxides by high-performance liquid chromatography coupled with post-column reaction. *Trends in Food Science & Technology*, *3*, 15–18. https://doi.org/10.1016/0924-2244(92)90105-6

Yu, X., Li, Q., Sun, D., Dong, X., & Wang, T. (2015). Determination of the peroxide value of edible oils by FTIR spectroscopy using polyethylene films. *Anal. Methods*, *7*(5), 1727–1731. https://doi.org/10.1039/C4AY02718C

Yu, X., van de Voort, F. R., & Sedman, J. (2007). Determination of peroxide value of edible oils by FTIR spectroscopy with the use of the spectral reconstitution technique. *Talanta*, *74*(2), 241–246. https://doi.org/10.1016/j.talanta.2007.06.004

Figure captions

Figure 1. Triiodide absorption spectra of aqueous solutions obtained as follows: 0.25 g of oxidized olive oil was added with 6 mL of chloroform/acetic acid (3:2), 0.25 mL of saturated KI solution and incubated for 5 min. After 75 mL of water addition the resulting solution was transferred to a 1-cm UV cuvette and the absorbance was measured between 250 and 500nm. Sample peroxide value (meqO_2/kg): 43.1(•), 26.9(**–**), 8.2(•**—**); solid line was obtained without sample.

Figure 2. Triiodide median absorption spectra (n=3) of aqueous solutions obtained as follows: 3 mL of 3% HCl-methanol solution was added with 0.25 mL of saturated KI solution and incubated for 5 min. After 40 mL of water addition the resulting solution was transferred with a syringe equipped with a RC (**–**), CA(•), or no (solid line) filter to a 1-cm UV cuvette and the absorbance was measured between 250 and 500nm. The inset displays the analogous spectra obtained with 0.25 g of an oxidized olive oil with a peroxide value of 14.3 meqO_2/kg.

Figure 3. Triiodide median absorption spectra (n=3) of aqueous solutions obtained as follows: 0.10 g of oxidized olive oil (peroxide value = 5.0 meqO_2/kg) was added with 1.2 mL of 3%(•**—**), 2%(**–**), 1%(•) , 0.5% (solide line) HCl-ethanol solution, 0.10 mL of saturated KI solution and incubated for 5 min. After 16 mL of water addition the resulting solution was transferred with a syringe equipped with a RC filter to a 1-cm UV cuvette and the absorbance was measured between 250 and 500nm. The inset displays the analogous spectra obtained without sample.

Figure 4. Absorbance measurements at 350 nm of blank solutions prepared at different times after the preparation of the saturated KI solution. Each value represents mean ± standard deviation of triplicates.

335

336 **Figure 5.** Matrix-assisted calibration curve obtained by a) blending two oxidized olive oils having
337 PV of 8.2 and 2.2 meqO_2/kg (concentration range 2.2-8.2 meqO_2/kg); b) spiking a purified olive oil
338 with increasing quantity of TBHP (concentration range 1.0-10.0 meqO_2/kg).

339

340 **Figure 6.** Comparison of peroxides contents in virgin olive oil samples analyzed by
341 spectrophotometric and official method.