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RESEARCH ARTICLE

Improving the triphenylphosphine/triphenylphosphine oxide (TPP/TPPO)-based method for the absolute and accurate quantification by FTIR-ATR of hydroperoxides in oils or lipid extracts

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Abstract

Research on natural sources of polyunsaturated fatty acids (PUFAs) for both food and nutraceutical prospects has significantly grown in recent years. Some plant oils and lipid extracts contain carotenoids, xanthophylls, sterols, and/or phenolic compounds that can provoke a lipid hydroperoxides (LOOHs) overestimation when quantifying them using nonselective colorimetric assays. Herein, we have optimized a mid-infrared method using Fourier-transform infrared spectroscopy coupled with attenuated total reflectance (ATR) to quantify LOOHs in oils or lipid extracts. This method is based on the conversion of triphenylphosphine (TPP) to triphenylphosphine oxide (TPPO) in the presence of hydroperoxides, with a direct assessment of TPPO levels following the formation of an oil film on the ATR crystal's surface, allowing for a low detection limit of 0.5 mmol of LOOH kg^{-1}. The concentration of oil and TPP, as well as the reaction time, were optimized. It was demonstrated that the presence of pigments, unsaponifiable compounds, phenolics, and/or PUFAs on oils, do not disrupt the analysis. Furthermore, the stoichiometry of the TPP/LOOH reaction was examined, confirming the reliability of the method in detecting various forms of hydroperoxides. An accelerated oxidation study was carried out and the hydroperoxide contents measured using TPP/TPPO were found to be comparable to those obtained using the ferric thiocyanate method. Our method offers a fast, simple, robust, and sensitive approach to accurately quantify hydroperoxides, regardless of the chemical composition of oil or lipid extracts.

Practical Application: The hydroperoxide assay method outlined in this study allows for the rapid and straightforward detection of hydroperoxides in pure oil matrices. The

Abbreviations: ATR, attenuated total reflectance; CuOOH, cumene hydroperoxide; DLaTGS, deuterated L-alanine doped triglycine sulfate; EI-MS, electron-impact ionization mass spectroscopy; FAME, fatty acid methyl esters; FTIR, Fourier-transform infrared; GC–MS, gas chromatography–mass spectrometry; H_2O_2, hydrogen peroxide; HC, hydroperoxide content; HPLC-DAD, high-performance liquid chromatography diode-array detector; HPLC–UV, high-performance liquid chromatography-ultraviolet; LOOH, lipid hydroperoxide; MIR, mid-infrared; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TBuOOH, tert-butyl hydroperoxide; TPP, triphenylphosphine; TPPO, triphenylphosphine oxide.

method's elevated sensitivity facilitates the early identification of oxidation indicators in oils, especially those with significant carotenoid or xanthophyll contents since these compounds may affect the results when using methods based on colorimetric quantification of hydroperoxides. This accurate, precise, and reproducible approach requires only small quantities of test samples and chemical products, making it well suited to routine application in laboratories and industrial environments.

KEYWORDS
FTIR-ATR, hydroperoxide content, lipid oxidation, oil film, pigmented oil extract, TPP, TPPO

1 | INTRODUCTION

Lipid oxidation is a critical concern for the quality control of food products, especially when they contain polyunsaturated fatty acids (PUFAs), which are particularly sensitive to oxidative degradation. Various factors encountered during food processing, such as heat, oxygen, light, and metallic contaminants, contribute to PUFA degradation. Beyond the loss of the nutritional quality of foods, lipid oxidation initiates the formation of reactive molecules, including lipid hydroperoxides (LOOHs), aldehydes, or epoxides among others, which subsequently lead to the degradation of other compounds of interest (e.g., antioxidants, vitamins, etc.) and/or the production of toxic molecules, causing health problems to consumers. Moreover, the existence of such compounds may pose a risk to consumer health due to their potential toxicity.[1] Although the analysis of multiple products has to be performed to obtain a relevant picture of the lipid oxidation process, LOOHs appear as the first stable indicator of PUFA degradation. As a result, different methods have been developed to properly assess LOOH concentration and to track the lipid oxidation progress in foods. The Codex Alimentarius sets a permissible range for this value, typically between 5 and 10 mmol of LOOH kg^{-1}, with specific limits varying based on the type of oil.[2] This range is established to mitigate potential risks, including the occurrence of neurotoxic effects.[3,4]

Several well-established methods are widely employed for the determination of hydroperoxide content (HC), such as the iodometric[5] method or the spectrophotometric ferric thiocyanate method.[6,7] These methods, however, have certain limitations primarily due to their nonstoichiometric behavior and variable responses contingent on the structural diversity of LOOH. In addition, they can give rise to alternative reaction pathways and to the formation of reactive by-products. Furthermore, the outcomes of these methods can be influenced by various operating conditions, such as temperature, light exposure, oxygen pressure, reaction duration, and the characteristics of the sample (including the type and/or degree of lipid unsaturation and the presence of prooxidants, antioxidants, and/or other molecules as pigments). For instance, measurement discrepancies can be observed when analyzing samples rich in pigments because they may either absorb at the same wavelengths that are used for HC determination or impede the detection of color changes through visual examination, leading to a lack of analytical precision. Concomitantly, the interest in plant-derived PUFA sources naturally rich in pigmented carotenoids or xanthophylls has experienced significant growth, particularly for their use in food and cosmetic applications.[8,9] As a result, there is a growing need to develop HC determination methods for pigmented oils and oil extracts while avoiding discoloration of the sample by using organic solvents,[10] which leads to an underestimation of the HC content.

Accordingly, this work focused on improving the development of an alternative method based on the stoichiometric conversion of triphenylphosphine (TPP) into triphenylphosphine oxide (TPPO) by hydroperoxides.[11,12] This approach has been already investigated to estimate HC by quantifying TPPO concentration using HPLC–UV,[13,14] HPLC-DAD,[15] GC–MS, and EI-MS.[16] The main advantages of TPP/TPPO method lie in its capacity to provide an absolute and direct quantification of hydroperoxides in a very short reaction time and without introducing side reactions. Depending of the analytical tool to assess LOOH, the detection may be performed at the picomole level, meaning that the early stage of oxidation may be observed. Although HPLC-DAD analysis presents an interesting alternative for assessing TPPO quantities, it comes with certain limitations. This method requires the use of reverse-phase chromatography with a complex gradient to elute glycerides, a significant volume of organic solvent, and a relatively lengthy sample acquisition time of approximately half an hour.[15] The use of Fourier-transform infrared (FTIR) spectroscopy coupled with attenuated total reflectance (ATR) equipment has been already investigated for TPPO analysis by measuring its specific absorption band at 542 cm^{-1} with a single-element detector DLaTGS for convenience,[12] as it does not require cooling with liquid nitrogen.[17,18] The combination of this detector with ATR eliminates the requirement for labor-intensive sample preparation and additional processing steps typically associated with transmission or reflectance modes in this type of equipment. This approach streamlines the analytical process even when dealing with minimal sample quantities. Similar work has been conducted in the near-IR, involving the development of partial least square calibration for TPPO quantification.[19,20] This approach requires the collection of extensive data and the application of mathematical and statistical techniques, which may exhibit significant variations depending on the type of oil, especially pigmented oils. Unlike the other analytical tools, the FTIR-ATR spectroscopy allows for a faster measurement (<1 min), with minimal handling and limited

use of chemicals and solvents. Our aim was to develop a more sensitive approach in the shortest analysis time, which may be used for accurate LOOH quantification. The application of FTIR spectroscopy can be optimized by employing materials such as polyethylene film placed on the ATR crystal surface, leading to an improved signal-to-background ratio.[21] To streamline the analytical process and concentrate on the sample's spectral fingerprint in the presence of TPP, we explored the concept of solvent evaporation, resulting in the formation of an oil film on the crystal. This approach simplifies the analytical procedure. In this context, it was necessary to optimize the ratio of lipid-to-TPP, the concentration of reactants, and the reaction duration. In addition, the effect of the oil composition and the presence of highly pigmented lipid extracts were evaluated. To confirm the absolute quantification of a large LOOH variety, the stoichiometry of the reaction was performed, and the method was finely tuned to enable rapid measurements with reliable HC determination as low as 0.5 mmol of LOOH kg^{-1} of oil. Finally, the newly optimized method was compared with iodometric and ferric thiocyanate assays for the HC of different oils stored under accelerated oxidative conditions.

2 | MATERIALS AND METHODS

2.1 | Oils, chemicals, and solvents

Rapeseed (*Brassica napus*), walnut (*Juglans regia* L.), and unfiltered and cold-pressed olive (*Olea europaea*) oils (one sample each) were purchased from a global consumer retailer. Raphia (*Raphia sese*) (Cuvette) pulp oil was extracted by cold pressing at room temperature (Komet CA 59 G, IBG Monforts Oekotec). Coconut medium-chain triglycerides (MCT) oil was sourced from Olisud. The microalgae extract from *Phaeodactylum tricornutum* was provided by Microphyt. Menhaden (*Brevoortia* spp.) oil and chemicals, such as TPP (>99%), TPPO (>99%), acetyl chloride, fucoxanthin, β-carotene, sodium methylate, sodium thiosulfate (99%), potassium iodide, thiodene, potassium permanganate (>99%), potassium dichromate, fucoxanthin, cumene hydroperoxide (CuOOH) (80%), undecanoic acid (C11:0), phenolphthalein, and tert-butyl hydroperoxide (TBuOOH) (5.6 M in decane), hydrogen peroxide solution (H_2O_2) (30%), along with solvents such as butan-2-one, chloroform, crystallizable glacial acetic acid, sulfuric acid (95%–97%), hexane, methanol, and butanol, were purchased from Sigma Aldrich.

Oils and reagents were weighed using a Precisa Semi-Micrometric EP 225SM-DR 0.01 mg precision balance from Precisa.

2.2 | FTIR-ATR signal acquisition

Measurements were conducted at 25°C using a FTIR instrument (Tensor 27, Bruker Optik) equipped with a GladiATR Single Reflection Diamond ATR unit, a DLaTGS detector, and a mid-infrared source. Sample (20 µL) was applied to the ATR crystal surface after a reaction time of 5 min between oil and TPP diluted in butan-2-one. After solvent evaporation (20 s), FTIR signal acquisition was initiated with 16 scans at a resolution of 4 cm^{-1}, covering a wavenumber range from 400 to 4000 cm^{-1}. Spectral analysis was carried out using the OPUS spectroscopy software provided by Bruker Optik. Background measurements (16 scans) were conducted prior to data acquisition without a sample on the crystal and every three depositions to ensure a stable baseline. The characteristic TPPO peak at 542 cm^{-1} was integrated using the software's integration method, taking the frequencies 535 and 560 cm^{-1} as bounds after correcting the spectra baseline.

2.3 | Fatty acid compositions of oils

Determination of oil fatty acid (FA) composition was made after methylation according to the procedure described in ISO 12966-1[22] with slight modifications. Oil (20 mg) and 1 mg of internal standard (C11:0) were added to 500 µL sodium methylate solution (0.5 M). Reaction medium was heated at 65°C for 10 min. Chlorhydric methanol (500 µL, 0.5 M) was added to phenolphthalein discoloration, and the mixture was again heated at 65°C for 10 min and then cooled to ambient temperature. Then, 2 mL of hexane and 2 mL of water were added. After centrifugation for 5 min at 1500 rpm (CR412 centrifuge, Jouan Thermo Electron), the organic phase was collected and analyzed by gas chromatography (8860 GC, Agilent Technologies) using a split injector ratio of 1/20, a CP-Cil 88 Varian capillary column (50 m × 0.25 mm with 0.2 µm film thickness; Agilent Chrompack), and helium (flow rate: 1 mL min^{-1}) as the carrier gas. Fatty acid methyl esters (FAMEs) were analyzed by flame ionization detector and OpenLab software data system (version B.01.18, 2019, Agilent Technologies). The column temperature started from 150°C, then reached 225°C with a rise of 5°C min^{-1} and was kept at 225°C for 10 min. The injector and detector temperatures were 250 and 270°C, respectively. FAMEs were identified using a mixture of methyl esters as external standards. For each FAME, the peak area was multiplied by the molecular weight, then the relative amount (%) for each FA was performed by considering the total of peak areas multiplied by the respective molar masses of each acid.

2.4 | Optimization of the oil film quantity on the crystal to improve the signal-to-background ratio

Different raphia oil contents (from 4 to 80 mg) were prepared in butan-2-one (200 µL), which means 20–400 mg mL^{-1}. The virtual HCs in the oil were set at 1 and 5 mmol TPPO kg^{-1}. For example, for 1 mmol of TPPO kg^{-1} with 4 mg of oil, 0.001 mg of TPPO is required, whereas 0.022 mg of TPPO is required for 80 mg of oil. For 5 mmol of TPPO kg^{-1} with 4 mg of oil, 0.005 mg of TPPO is required, whereas for 80 mg of oil, 0.11 mg of TPPO is required.

2.5 | Optimization of TPP concentration and reaction time

Solutions of TPP at 5, 15, 30, 40, and 60 mg mL^{-1} were prepared in butan-2-one. Oxidized rapeseed oil was prepared by incubating it at

40°C for 196 h in transparent flasks and stored at 40°C using an incubator (KS 4000 i control, IKA). Then, oxidized rapeseed oil (40 mg) was weighed into hemolysis tubes, and 200 µL of the five TPP solutions were added to the tubes and vortexed for 5 s. FTIR-ATR signal acquisition was performed after 1 min, 15 min, 1 h, and 17 h, as described in Section 2.2. Samples were stored in the dark between each measurement.

2.6 | TPPO calibration curve

A stock solution of TPPO/TPP was prepared in a transparent glass bottle with, respectively, 10 mg mL^{-1} of TPPO (35.93 mM) and 30 mg mL^{-1} (114.38 mM) of TPP in butan-2-one. The solution was stirred until both compounds were dissolved. Subsequent dilutions of TPPO were made at 19.2, 9.58, 3.83, 1.92, 0.96, 0.38, 0.19, 0.1, and 0 mM with a solution of TPP at 30 mg mL^{-1} in butan-2-one. Then, 200 µL of each solution was mixed with 40 mg of MCT oil in hemolysis tubes, sealed, and vortexed for 30 s and FTIR-ATR signal acquisition was directly performed.

2.7 | Stoichiometry of TPP with different hydroperoxides

Solutions of CuOOH, H_2O_2, and TBuOOH were prepared at a concentration of, respectively, 3.81, 3, and 3 mM in butan-2-one. Subsequent dilutions of CuOOH, H_2O_2, and TBuOOH up to 7.63 µM were prepared in butan-2-one, and TPP was added to maintain its final concentration at 30 mg mL^{-1}. Then, 200 µL of each solution were introduced into hemolysis tubes and mixed with 40 mg of MCT oil. The tubes were sealed, vortexed, and analyzed by FTIR-ATR within the 5 min, as described in Section 2.2.

The stoichiometry between the introduced H_2O_2 molecules and the decrease in TPP content was also investigated. A TPP calibration curve was first generated by integration of peak at 742 cm^{-1} after preparing solutions with different concentrations of TPP (from 2.29×10^{-2} to 4.58×10^{-2} mM in 200 µL of butan-2-one) supplemented with TPPO (from 0 to 2.29×10^{-2} mM) to simulate the reacted hydroperoxides and added to 40 mg of MCT oil. Then, H_2O_2 at concentrations from 0 to 5.72×10^{-2} mM were added to TPP (4.58×10^{-2} mM) and MCT (40 mg) in 200 µL of butan-2-one. The peak areas at 742 cm^{-1} were integrated between 730 and 760 cm^{-1} using OPUS spectroscopy software (Bruker Optik), and the mmol of TPP remaining in the samples was calculated.

2.8 | Influence of the oil composition on the oil film formation and signal acquisition of TPPO

TPPO solutions with TPP were carried out in the same way as for the TPPO calibration curve in part 2.6. For each oil, 40 mg were placed in hemolysis tubes, mixed with 200 µL of each TPPO/TPP solution, sealed, and vortexed for 30 s. Then, FTIR-ATR signal acquisition was performed within the 5 min, as described in Section 2.2. The HCs ranged from ≈1 to 200 mmol of LOOH kg^{-1}. Rapeseed oil with 2500 mg kg^{-1} of fucoxanthin was prepared by mixing fucoxanthin (6 mg) with oil (2.4 g) for 20 min at 40°C with orbital shaking at 100 rpm using an incubator (KS 4000 i control, IKA). Carotenoid in raphia oil has been dosed by colorimetry using a UV spectrometer (Perkin Elmer Lambda) with UV WinLab software Lambda 2–40 version 2.8 (Perkin Elmer Lambda). One milliliter of oil was diluted in 1 mL of hexane, and the absorbance of each sample was measured at 453 nm.

The carotenoid content was expressed in β-carotene equivalent using a calibration curve ranging from 0 to 0.01 mg mL^{-1}.

2.9 | Comparison of TPP/TPPO, iodometric, and ferric thiocyanate methods

2.9.1 | Oil oxidation

Oils (25 mL) were placed in 50 mL transparent closed flasks and stored at 40°C under orbital shaking at 100 rpm using an incubator (KS 4000 i control, IKA). LOOHs were quantified using the TPP/TPPO, the iodometric, and the ferric thiocyanate methods immediately after preparation and then after 48, 96, and 144 h of storage.

2.9.2 | Iodometric titration

HC measurement in oils was assessed by iodometric titration according to the international norm ISO 3960.[23] Typically, a known quantity of oxidized oil (0.5–2 g) was dissolved in 10 mL of chloroform in a round-bottom flask, and 15 mL of acetic acid were added. To initiate the reaction, 1 mL of freshly prepared potassium iodide was added. The flask was then capped and stirred for 1 min. After 5 min in the dark, the reaction was stopped by adding 75 mL of distilled water. The manual titration was made with a sodium thiosulfate solution at 0.002 N with iodine as indicator, and the HC was determined as follows: $HC = ((V - V_0) \times N \times 1000/m)/2$. With V meaning volume (mL) of the sodium thiosulfate solution in presence of sample. V_0 meaning volume (mL) of the sodium thiosulfate solution in absence of sample, m meaning mass of the sample (g), and N meaning normality of the sodium thiosulfate solution. The results obtained using this method were expressed in mmol of LOOH kg^{-1} of oil.

2.9.3 | Ferric thiocyanate assay

HC was determined following the method of Shantha and Decker[6] (1994) and Mihalievic[7] (1995) protocols, adapted to microplate dimensions. Initially, 15 mg of oil were mixed in an Eppendorf tube with 1 mL of methanol/butanol (3/7, v/v) and vortexed (10 s). Then, 260 µL of this mixture were transferred into a 96-well microplate (Greiner), along with 2.5 µL of aqueous ammonium thiocyanate (30% w/v) and 2.5 µL of ferrous solution (0.066 M). The microplate was then incubated at

room temperature for 15 min and scanned at 500 nm using an Infinite 200 PRO microplate reader (Tecan) equipped with the Magellan Data analysis software (Tecan). The measurements were conducted in triplicate, and the results were expressed as mmol of LOOH kg^{-1} based on a calibration curve of CuOOH (from 0.78 to 65.62 μM) to compare with iodometric method results.

2.9.4 | TPP/TPPO method

Oils (≈40 mg) were weighed in hemolysis tubes, and the exact masses were recorded. A freshly prepared TPP solution at 30 mg mL^{-1} in butan-2-one was made in a 25 mL glass bottle. Once the TPP was completely dissolved through stirring, 200 μL of this solution was added to each oil sample, and the samples were vortexed for 30 s. FTIR-ATR signal acquisition was performed within the 5 min, as described in Section 2.2. The measurements were conducted in triplicate, and the results were expressed as mmol of LOOH kg^{-1} based on a calibration curve of TPPO (Section 2.6).

2.9.5 | Statistical analyses

Different tests were realized according to the number of samples, modalities, and the relationship between groups: one-way ANOVA using Tukey and REGWQ tests coupled with a two-tailed Dunnett test ($p < 0.01$), Friedman ($p < 0.05$), and Kruskal–Wallis test with Bonferroni-corrected ($p < 0.005$) tests. Statistical analyses were performed using XLSTAT.

3 | RESULTS AND DISCUSSION

3.1 | Fatty acid composition of oils

The relative percentage compositions of FA in each oil are presented in Table 1. According to their FA composition, their amount of PUFAs, and in particular their omega-3 content, it is expected that the *P. tricornutum* extract and walnut oil are the more prone to lipid oxidation followed by raphia and rapeseed oil. However, this theoretical sensitivity to lipid oxidation depending on the FA composition only, can be also influenced by the content and nature of endogenous antioxidants that these oils may contain (e.g., tocopherols).

3.2 | Optimization of the signal-to-background ratio

Designing the FTIR-ATR spectroscopy method presented a significant challenge, primarily related to achieving a uniform oil film on the crystal's surface after the solvent evaporation (butan-2-one). This film, constituted by in situ lipids, plays a critical role in obtaining results that are not only representative but also highly reliable. In order to assess the method's sensitivity threshold, TPPO was added to raphia oil to simulate the formation of LOOH. Raphia oil, rich in carotenoids, was used as a model to prove that the TPP/TPPO method aims at being particularly suited for highly colored oils.

Each millimole of TPPO per kg of the sample corresponds to a HC of 1 mmol of LOOH formed. As the HC is expressed per kg of oil, it decreases when the quantity of oil utilized for the film increases while the amount of introduced hydroperoxides remains constant. Conversely, when measuring hydroperoxides in oil, the measurement's response (TPPO peak area at 542 cm^{-1}) increases with increased oil quantities. Thus, the optimization process involves the selection of an appropriate oil concentration to measure a substantial quantity of hydroperoxide while simultaneously limiting the sample size to enhance the sensitivity limit expressed as mmol of TPPO kg^{-1} of oil. Figure 1 illustrates the peak areas at 542 cm^{-1} after spreading various raphia oil concentrations on the ATR crystal and subsequently evaporating the solvent with fixed HC of (a) 1 and (b) 5 mmol of TPPO kg^{-1} of oil. For a HC of 5 mmol of TPPO kg^{-1}, the samples containing 80, 60, and 40 mg yielded the most substantial peaks. The addition of 10 and 4 mg of oil in 200 μL of butan-2-one did not result in a uniform oil film, leading to an uneven distribution on the crystal surface. This observation was consistent with experiments at 1 mmol of TPPO kg^{-1} of oil. Among these experiments, the addition of 40 mg of oil stood out, producing a significantly larger peak area at 542 cm^{-1}. This enhancement contributed to improved sensitivity and the limit of quantification, making it the chosen condition for the subsequent assays.

3.3 | Optimization of TPP concentration and reaction time

To ensure a complete reaction with the totality hydroperoxides present in the oil sample, a stoichiometric excess of TPP is required. Moreover, the reaction time and the stability of both reactants (TPP) and product (TPPO) must be considered to ensure measurement accuracy and prevent any side reactions (e.g., autooxidation of TPP). To validate these conditions and determine the optimal reaction time, a series of FTIR-ATR spectroscopy measurements were conducted at various time intervals (5 min, 15 min, 1 h, and 17 h) using different TPP concentrations (5, 15, 30, 40, and 60 mg mL^{-1}) added to an oxidized rapeseed oil sample (40 mg) (Figure 2). The HC of this oil was initially determined by iodometric and thiocyanate assays. HCs were, respectively, 22.95 ± 2.35 and 20.10 ± 1.65 mmol LOOH kg^{-1}, indicating that about 0.84 μmol of LOOHs were present in the 40 mg of oxidized oil sample. According to the stoichiometry of the reaction, the minimum concentration of TPP tested here (5 mg mL^{-1}) allowed for the quantification of 3.8 μmol of LOOH, equivalent to 95 mmol of LOOH kg^{-1} of oil. Thus, TPP was introduced in excess for all samples, with a TPP/LOOH mole ratios of ≈4.5, 13.5, 27, 36, and 54.

The peak areas of TPPO at 542 cm^{-1} from various TPP concentrations after 5 min, 15 min, and 1 h after contact between the oil and the TPP did not show substantial differences (Figure 2). This indicates that the reaction mostly occurs within the initial 5 min, confirming the rel-

TABLE 1 Fatty acid (FA) composition of coconut medium-chain triglycerides (MCTs), menhaden, olive, raphia, rapeseed, and walnut oils and of *Phaeodactylum tricornutum* extract.

FA composition (molar %)	Oils						
	MCT	Menhaden	Virgin olive	Raphia	Rapeseed	Walnut	*Phaeodactylum tricornutum* extract
C8:0	54.82 ± 0.12	nd	nd	nd	nd	nd	nd
C10:0	45.18 ± 0.12	nd	nd	nd	nd	nd	nd
C14:0	nd	10.21 ± 0.14	nd	0.20 ± 0.01	nd	nd	4.62 ± 0.56
C15:0	nd	nd	nd	nd	nd	nd	0.22 ± 0.02
C16:0	nd	19.87 ± 0.16	11.39 ± 0.02	41.16 ± 0.18	4.53 ± 0.01	6.72 ± 0.02	11.39 ± 0.02
C18:0	nd	3.34 ± 0.12	3.04 ± 0.01	10.12 ± 0.04	1.49 ± 0.01	2.73 ± 0.01	0.26 ± 0.01
C20:0	nd	1.65 ± 0.04	0.02 ± 0.01	0.19 ± 0.01	0.53 ± 0.01	0.1 ± 0.01	0.38 ± 0.01
Σ SFA	**100**	**35.07 ± 0.25**	**14.99 ± 0.03**	**51.67 ± 0.18**	**6.55 ± 0.02**	**9.45 ± 0.02**	**16.87 ± 0.56**
C15:1ω-9	nd	nd	nd	nd	nd	nd	5.39 ± 0.7
C16:1ω-9	nd	15.37 ± 0.11	1.16 ± 0.01	0.13 ± 0.01		0.07 ± 0.01	19.62 ± 0.14
C18:1ω-9	nd	9.72 ± 0.18	73.54 ± 0.05	11.86 ± 0.07	59.46 ± 0.03	14.57 ± 0.04	5.70 ± 0.03
C20:1ω-9	nd	3.09 ± 0.04	nd	nd	nd	nd	0.57 ± 0.01
C22:1 ω-9	nd	1.19 ± 0.15	nd	nd	nd	nd	2.09 ± 0.01
Σ MUFA	nd	**29.37 ± 0.26**	**74.7 ± 0.05**	**11.99 ± 0.07**	**59.46 ± 0.03**	**14.64 ± 0.04**	**33.37 ± 0.74**
C16:2ω-6	nd	nd	nd	0.09 ± 0.01	nd	nd	0.14 ± 0.01
C16:3ω-3	nd	nd	nd	nd	nd	nd	5.90 ± 0.03
C18:2ω-6 (LA)	nd	nd	7.29 ± 0.01	35.48 ± 0.05	19.94 ± 0.01	60.7 ± 0.10	2.28 ± 0.01
C18:3ω-3 (ALA)	nd	1.28 ± 0.05	nd	0.70 ± 0.04	8.38 ± 0.01	13.13 ± 0.01	0.56 ± 0.02
C20:3ω-3	nd	nd	nd	nd	nd	nd	nd
C20:4ω-6	nd	3.26 ± 0.03	nd	nd	nd	nd	1.85 ± 0.01
C20:5ω-3 (EPA)	nd	15.90 ± 0.10	nd	nd	0.13 ± 0.01	nd	19.27 ± 0.02
C22:5ω-3	nd	2.28 ± 0.60	nd	nd	nd	nd	nd
C22:6ω-3 (DHA)	nd	12.40 ± 0.02	nd	nd	nd	nd	nd
Σ PUFA	nd	**35.12 ± 0.61**	**7.29 ± 0.01**	**36.27 ± 0.60**	**28.45 ± 0.02**	**73.83 ± 0.10**	**32.84 ± 0.04**

Abbreviations: MUFA, monounsaturated fatty acids; nd, not determined; PUFAs, polyunsaturated fatty acids; SFA, saturated fatty acids.
Note: Bold values is the sum of the fatty acids corresponding to the above lines

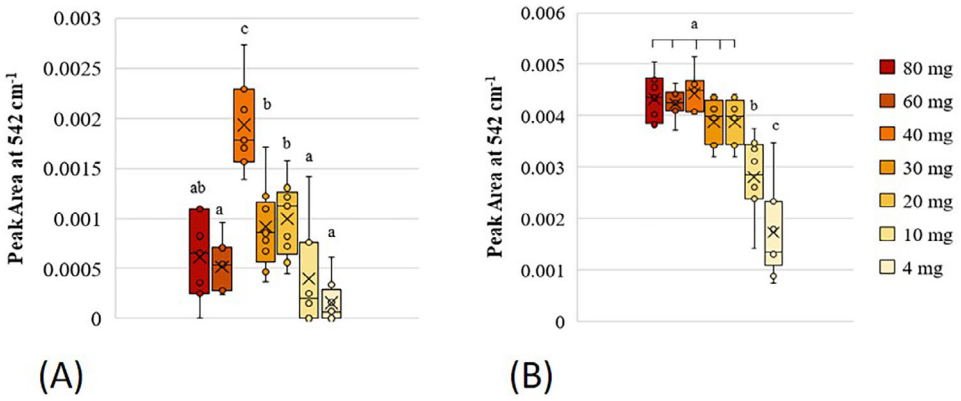

FIGURE 1 Peak area of triphenylphosphine oxide (TPPO) at 542 cm^{-1} for various raphia oil concentrations, with fixed hydroperoxide content at (A) 1 mmol of TPPO kg^{-1} of oil and (B) 5 mmol of TPPO kg^{-1} of oil, $p < 0.01$, ANOVA ($n = 9$).

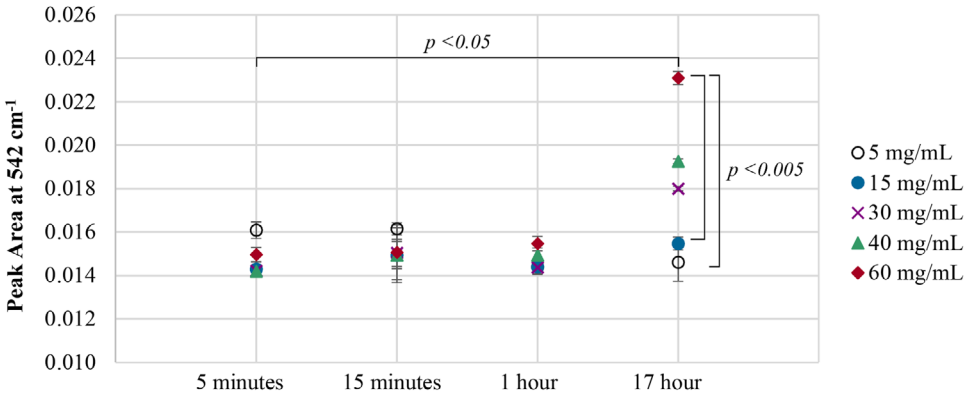

FIGURE 2 Evolution of triphenylphosphine oxide (TPPO) peak area at 542 cm^{-1} over time (5 min, 15 min, 1 h, and 17 h) after addition of oxidized rapeseed oil and with different triphenylphosphine (TPP) concentrations (5, 15, 30, 40, and 60 mg mL^{-1} in butan-2-one), $p < 0.05$ Friedman test, $p < 0.005$ Kruskal–Wallis test ($n = 3$).

atively rapid reaction rate between hydroperoxides and TPP.[12,13,24] This also confirms the good stability of TPPO over time.[15] This reaction time is notably faster than the recommended time for the ferric thiocyanate assay, which is 15 min after adding reactant.[25] As for the iodometric method, numerous adjustments and optimizations have been implemented, resulting in various reaction times spanning from 1 min to 1 h.[26] This fast reaction of TPP with LOOH significantly shortens the time required for a complete hydroperoxide assay, rendering it suitable for the routine analysis of a large number of samples. Furthermore, it allows for multiple measurements of the same sample within an hour, given that TPPO maintains its stability over this time. Nonetheless, it is worth noting that for the highest TPP concentration, exceeding 30 mg mL^{-1}, the hydroperoxide values determined after 17 h were notably higher. This observation raises the possibility of autooxidation of TPP, likely triggered by the diffusion of oxygen into the oil after extended exposure to air. Consequently, this test illustrates that there is no substantial difference in the estimation of oil hydroperoxides, regardless of the TPP concentration, as long as there is an excess of TPP available. To mitigate the potential autooxidation of TPP over an extended period, we opted for a 30 mg mL^{-1} solution, enabling the determination of hydroperoxides up to 572 mmol of LOOH kg^{-1} without the need for an oil dilution step.

3.4 | Calibration curve for TPPO

The relationship between peak area and TPPO content (from 0 to 191.5 mmol kg^{-1} of oil; equivalent to 0 to 7663 nmol of TPPO in 40 mg of oil) has been represented in Figure 3. The correlation appears to be linear and shows a strong reproducibility ($R^2 > 0.99$). In addition, the smallest peak area values corresponding to 0.5, 1, and 2 mmol of LOOH kg^{-1} were significantly different from one another ($p < 0.01$). However, for HC lower than 2 mmol of LOOH kg^{-1}, it was necessary to establish a separate correlation specifically for TPPO contents below 80 nmol. This deviation from the origin in the linear relationship is attributed to the presence of TPPO in TPP or hydroperoxides in the solvent, even with freshly opened chemicals. In fact, we observed that

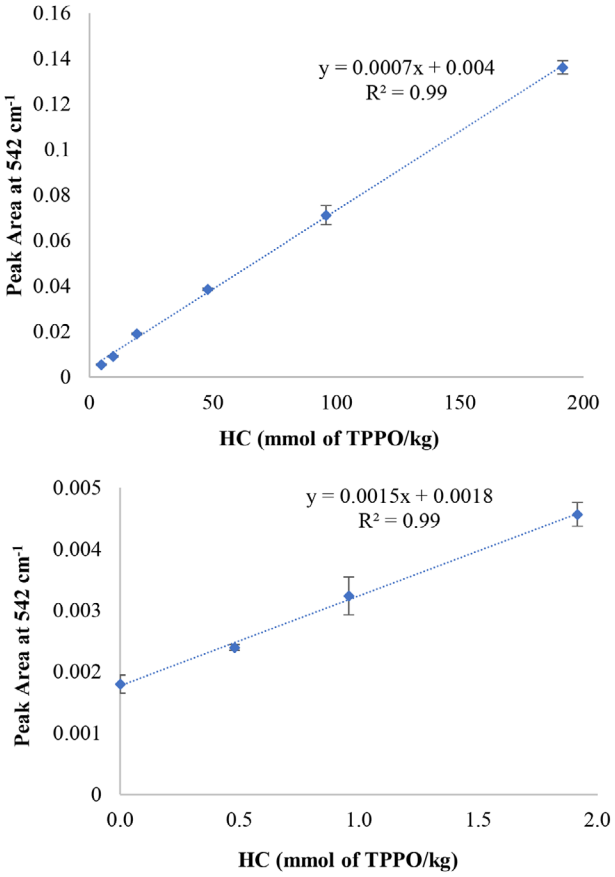

FIGURE 3 Calibration curve based on peak area at 542 cm^{-1} as a function of mmol of TPPO kg^{-1} of oil in triphenylphosphine (TPP)/medium-chain triglyceride (MCT)/butan-2-one (30/200/1 w/w/v). TPPO, triphenylphosphine oxide.

the addition of TPP to different concentrations of TPPO increased the sensitivity of the peak at 542 cm^{-1} for low HC. Due to this, the detection limit can be reached when the HC level is as low as 0.5 mmol of LOOH kg^{-1} (19.2 nmol of LOOH in 40 mg of oil), indicating the method's excellent sensitivity, suitability for rapid implementation, and potential for routine monitoring of oil oxidation.

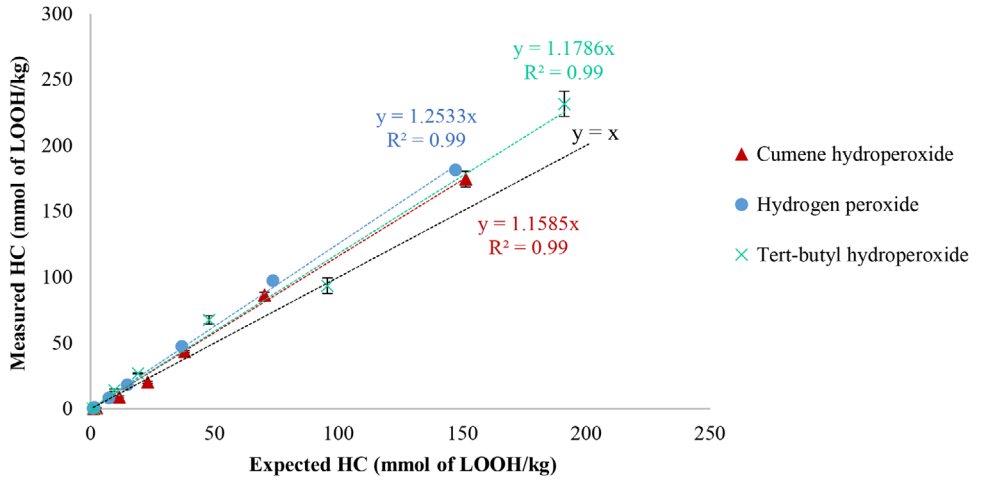

FIGURE 4 Measured versus expected hydroperoxide contents (in mmol of LOOH kg^{-1} of oil) from cumene hydroperoxide, tert-butyl hydroperoxide, and hydrogene peroxide in coconut medium-chain triglyceride (MCT) oil (40 mg in 200 μL of butan-2-one) using the implemented triphenylphosphine (TPP)/triphenylphosphine oxide (TPPO) assay. LOOH, lipid hydroperoxide.

3.5 | Stoichiometry of TPP and hydroperoxides in different molecular forms

A reliable method for measuring HC must be nonselective toward various forms of peroxides to provide results that are representative of the total composition of the sample. Therefore, all hydroperoxides, regardless of their form, must react with TPP. For this investigation, three standard peroxides were selected as reference compounds: CuOOH, TBuOOH, and H_2O_2. They were individually brought into contact to react with TPP. These experiments were carried out using hydroperoxide-free coconut MCT oil.

As illustrated in Figure 4, the measured LOOH contents in the coconut MCT oil spiked with CuOOH, H_2O_2, or TBuOOH closely matched the expected values. In addition, the responses were linear and demonstrated a stoichiometry close to 1. These measurements were conducted after a 5-min reaction, and our findings suggested that all three hydroperoxides underwent complete reaction, even though TBuOOH, with its tertiary structure, typically requires longer reaction times.[15] Interestingly, the stoichiometric reaction between TPP and H_2O_2 aligns with some previous works,[27] whereas it contradicts the results observed in other studies.[28] As both H_2O_2 and TPPO do not overlap with the aromatic C–H bending of TPP at 742 cm^{-1}, we assessed the consumption of TPP in the presence of H_2O_2 to definitively determine the stoichiometry of the reaction and its selectivity with this hydroperoxide (Figure 5). The TPP values were determined through a calibration curve, and upon increasing HCs, they were evaluated and compared with the theoretically loss of TPP contents. The TPP contents closely align with the expected 1:1 ratio.

These results validate the hypothesis of a stoichiometric reaction between TPP and hydroperoxides, without selectivity based on their nature. This observation presents an advantage in the context of lipid oxidation, where various peroxide forms can arise due to the complex

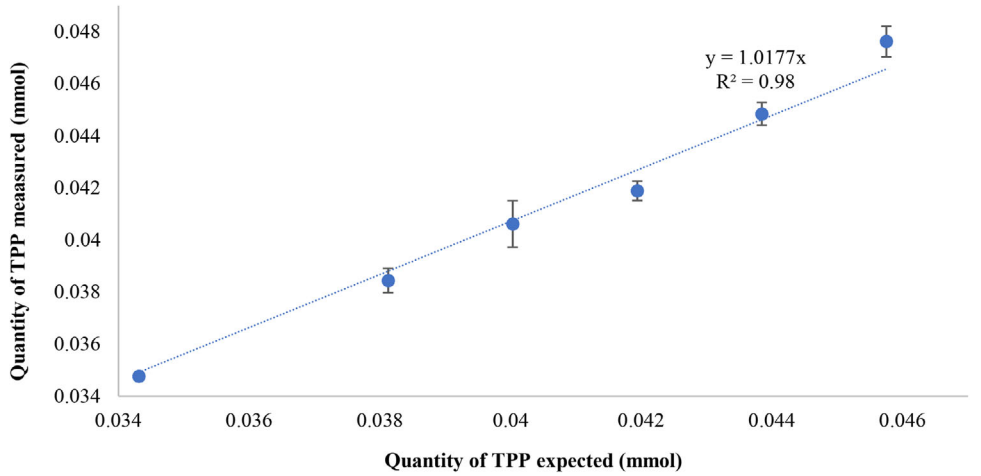

FIGURE 5 Measured versus expected quantities of triphenylphosphine (TPP) (in mmol) in samples with different hydrogen peroxide contents in coconut medium-chain triglyceride (MCT) oil (40 mg in 200 μL of butan-2-one) by monitoring the peak area at 742 cm^{-1}.

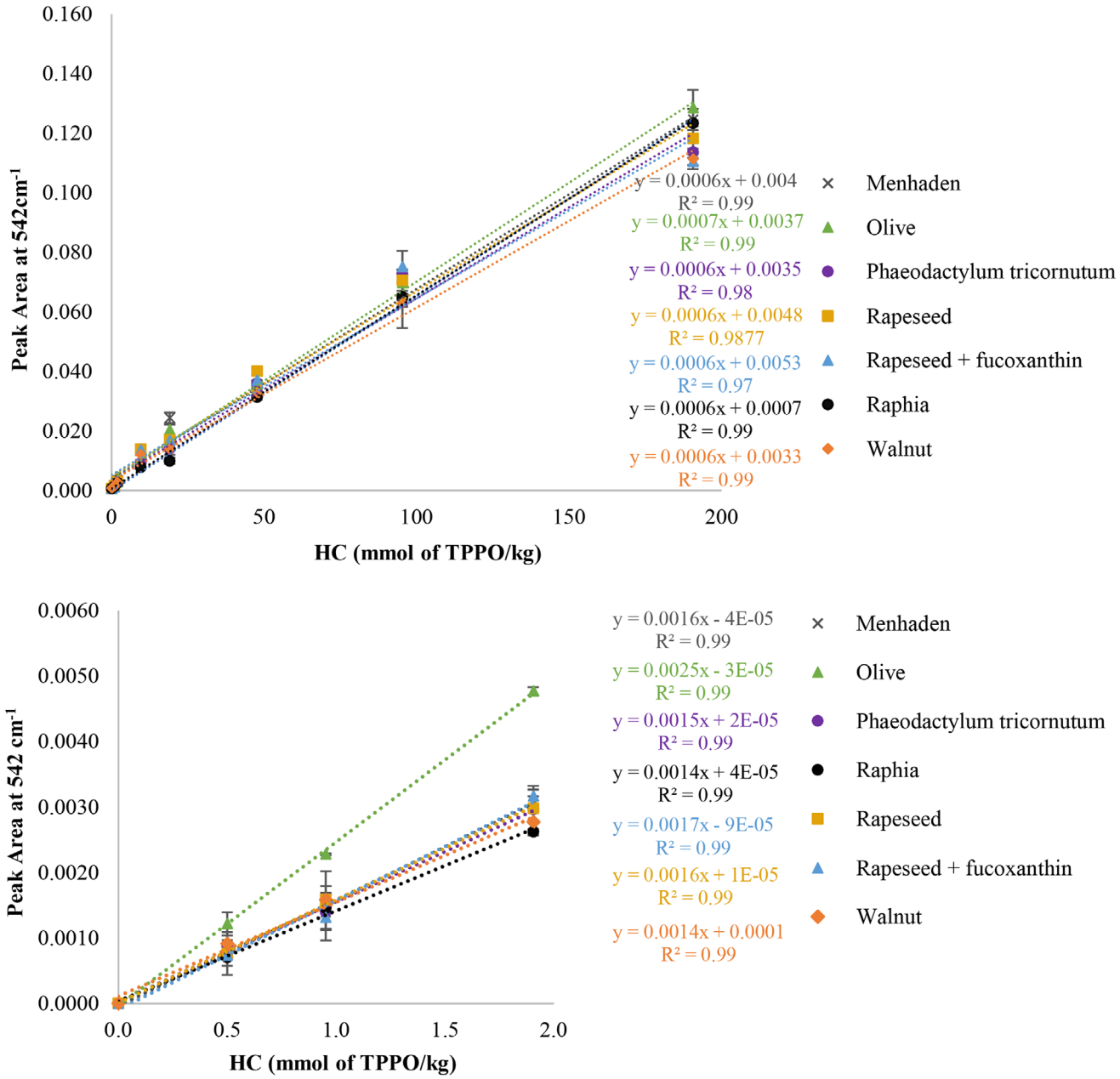

FIGURE 6 Peak areas at 542 cm^{-1} as function of the hydroperoxide content (mmol of TPPO kg^{-1} of oil or lipid extract) in menhaden oil, virgin olive oil, *Phaeodactylum tricornutum* extract, raphia oil, rapeseed oil, rapeseed oil + fucoxanthin (2500 mg kg^{-1}), and walnut oil (after normalization). TPPO, triphenylphosphine oxide.

FA compositions of vegetable oils. Further studies, involving the addition of hydroperoxides derived from PUFAs (LA, ALA, EPA, and DHA), could enhance and complement these findings.

3.6 | Influence of the oil composition on the oil film formation and signal acquisition of TPPO

A selection of five oils and one lipid extract were used to explore the potential impact of oil composition on various aspects, such as the formation of the oil film, the integration of the characteristic TPPO peak, and the subsequent quantification of LOOH in the respective oils. The lipid extract from *P. tricornutum* was specifically selected due to its composition, which contains both fucoxanthin and chlorophyll. Raphia oil was included in the study for its notable pigment content, comprising approximately of 118.57 ± 0.23 mg equivalent β-carotene/kg of oil. Unfiltered and cold-pressed olive oil was selected for its chlorophyll and its content in phenolic compounds.[29] Menhaden and walnut oils were introduced to assess the influence of PUFA, particularly omega-3 FAs. Finally, the rapeseed oil was spiked with fucoxanthin at 2500 mg kg^{-1} (a similar amount found in the *P. tricornutum* lipid extract) to assess the influence of a high concentration of pigments.

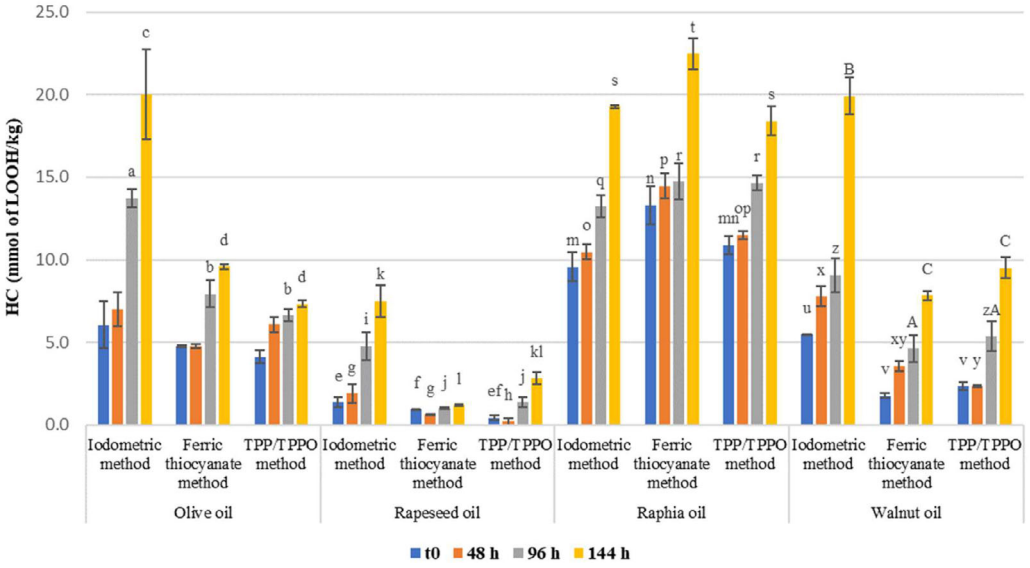

FIGURE 7 Evolution of hydroperoxide contents (HC in mmol of LOOH kg^{-1} of oil) in virgin olive, rapeseed, raphia, and walnut oils by iodometric, ferric thiocyanate, and triphenylphosphine (TPP)/triphenylphosphine oxide (TPPO) methods at 0, 48, 96, and 144 h (corrected with TPP/TPPO), p values < 0.05, Kruskal–Wallis test, the HC for the same time and the same oil without letter (virgin olive oil at 0 and 48 h) did not show p-values lower than the significance level alpha of 0.05 and therefore belong to similar groups ($n = 3$). LOOH, lipid hydroperoxide.

The peak area values for samples of rapeseed oil + fucoxanthin, *Phaeodactylum tricornutum* extract, rapeseed, and walnut oils with only TPP and no addition of TPPO were very low, resulting in HC levels close to 0 (data not shown). This suggests that the minor components present in these oils (pigments and unsaponifiable) did not react with the TPP. The peak area values of samples of raphia oil and menhaden oil under the same conditions exhibited higher peak area values, most likely due to the presence of hydroperoxides related to the raphia extraction process or the inherent sensitivity of fish oil (data not shown). Therefore, the intrinsic HC on the native oils was normalized to zero in order to enhance the comparability of the different calibration curves following the addition of TPPO. As shown in Figure 6, the chemical characteristics of the studied oils, such as chlorophyll pigments, carotenoids, or polyphenols, did not prevent the film formation nor the signal acquisition at 542 cm^{-1}. However, it is worth noting that variations in peak intensity may exist among different oils, as observed with virgin olive oil. To enhance the accuracy of quantifying hydroperoxides, a practical recommendation is to establish a calibration curve using TPPO/TPP with the same oil or lipid extract.

3.7 | Comparison of TPP/TPPO, iodometric, and ferric thiocyanate methods

Raphia, rapeseed, virgin olive, and walnut oils, which differ in term of composition (Table 1), were subjected to accelerated oxidation conditions at 40°C for 144 h (Figure 7). Significant disparities were observed between the standardized methods, particularly at 96 and 144 h. The iodometric method yields overestimated results, especially with virgin olive, rapeseed, and walnut oils, potentially arising from various biases such as increased exposure to oxygen leading to the oxidation of iodine or presence of reducing molecules.[15] Overall, when considering these three oils, the results obtained using the TPP/TPPO method are generally closer to those obtained with the ferric thiocyanate method than those obtained with the iodometric method. Unexpectedly, the results obtained for raphia oil exhibit minimal deviation between the iodometric, TPP/TPPO, and ferric thiocyanate methods, justifying further clarification.

Assays, including iodometric, ferric thiocyanate, and TPP/TPPO methods, were conducted to assess HC in samples with a high chlorophyll content, such as the *P. tricornutum* lipid extract. Measurements with the iodometric method were unsuccessful, even after removing the chloroformic phase, as the sample remained too dark to observe the color change during titration. In contrast, both the TPP/TPPO and ferric thiocyanate methods provided reproducible results, confirming their adaptability. However, there may be variations HC values and kinetics oxidation behavior among different methods. Therefore, additional tests and comparisons involving oils with varying chlorophyll contents and types, particularly chlorophyll a and b (the most common in the plant kingdom), must be performed to accurately correlate HC with the oxidative stability of chlorophyll-enriched lipid samples. Indeed, careful consideration of multiple factors[30–33] is crucial when monitoring the oxidative stability of lipids enriched in chlorophyll.

Finally, in comparison with our previous work,[12] the sensitivity limit has significantly improved. Previously, quantification below 2 mmol of LOOH kg^{-1} was not feasible, but now we can accurately measure down to 0.5 mmol. Reproducibility has also been enhanced substantially, with significantly lower standard deviation. Moreover, there is a notable reduction in the use of chemicals and solvents. Additionally, the amount of oil required has decreased considerably, from 500 µL to ≈50 µL.

4 | CONCLUSION

This study deals with a new methodology for the quantification of hydroperoxides in oil matrices using the TPP/TPPO method coupled with FTIR-ATR spectroscopy. It offers numerous advantages over the standardized approaches and holds significant potential for application in both industrial and laboratory settings for routine analysis. The method allows for a fast, simple, reproducible, accurate, and absolute quantification of hydroperoxides, which remains unaffected by the presence of pigmented compounds such as carotenoids or xanthophylle. The ability to detect hydroperoxides at the nanomolar level enables the early detection of oil oxidation. The setup time for the assay is significantly reduced compared to alternative methods, as it does not necessitate the pre-preparation of glassware or solutions, therefore drastically reducing chemical and solvent usage. In continuation of this work, assessments on oils rich in chlorophyll or more complex systems such as emulsions can be undertaken.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author.

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