Grasas y Aceites 76 (1)
January-March 2025, 2201
ISSN-L: 0017-3495, eISSN: 1988-4214
https://doi.org/10.3989/gya.0759241.2201

# Electron spin resonance (microESR) to determine the peroxide value of virgin olive oil

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*Submitted: 10 July 2024; Accepted: 07 March 2025; Published: 23 February 2026*

**SUMMARY**: The present study aims to find correlation models between peroxide values (PVs), as determined according to the EU official method, and electron spin resonance (ESR) spectroscopy assay data, in order to use this method as an automated and solvent-efficient screening tool to assess the oxidative status of virgin olive oils (VOOs). The concentration of free radicals, measured at two times of a specific ESR procedure, and PVs were utilized to create linear regression models. To test them, the PVs of 7 VOO samples (3 VOOs for internal and 4 VOOs for external validation) were determined and, on the same oils, the ESR assay was performed. The results were interpolated in two models, and the PVs predicted by ESR were compared to those obtained using the official method. The two methods showed statistical consistency ($p \leq 0.05$). These preliminary findings suggest that the ESR assay is an effective and sustainable screening tool for monitoring the primary oxidative state of VOOs.

***KEYWORDS***: *Electron spin resonance; Free radicals; Oxidation; Peroxide value; Virgin olive oil.*

**RESUMEN:** ***Resonancia electrónica de espín (microESR) para comprobar el índice de peróxidos de aceites de oliva virgen.*** El presente estudio tiene como objetivo encontrar modelos de correlación entre los índices de peróxidos (PV), determinados siguiendo el método oficial de la UE, y los datos del ensayo de espectroscopia de resonancia electrónica de espín (ESR), para utilizar este método como una herramienta de detección automatizada y eficiente en disolventes para evaluar el estado oxidativo de los aceites de oliva vírgenes (AOV). La concentración de radicales libres, medida en dos momentos de un procedimiento de ESR específico, y los PV se utilizaron para crear modelos de regresión lineal. Para probarlos, se determinaron los PV de 7 muestras de AOV (3 AOV para validación interna y 4 AOV para validación externa) y, en los mismos aceites, se realizó el ensayo de ESR. Los resultados se interpolaron en dos modelos, y los PV predichos por ESR se compararon con los obtenidos utilizando el método oficial. Los dos métodos mostraron consistencia estadística ($p \leq 0,05$). Estos hallazgos preliminares sugieren que el ensayo ESR es una herramienta de detección eficaz y sostenible para controlar el estado oxidativo primario de los aceites de oliva virgen.

***PALABRAS CLAVE***: *Aceite de oliva virgen; Índice de peróxido; Oxidación; Radicales libres; Resonancia de espín electrónico.*

**Citation/Cómo citar este artículo**: Tura M, Mandrioli M, Valli E, Bendini A, Gallina Toschi T. 2025. Electron spin resonance (microESR) to determine the peroxide value of virgin olive oil. *Grasas Aceites* **76** (1), 2201. https://doi.org/10.3989/gya.0759241.2201

## 1. INTRODUCTION

The oxidation of oils and fats is known to affect their shelf-life (Frankel, 1983; Jiang *et al.*, 2020), and it is proceeded, initially, by the formation of free radicals (Frankel, 1980; Xie, 2019) which can form during the production process, prolonged storage or under stressful conditions (Ottaviani *et al.*, 2001). The oxidation of fats, oils, and in general, foods that contain lipids, results in sensory and nutritional degradations (Frega *et al*., 1999). Thus, the evaluation of the oxidative stability of vegetable oils is necessary to assess quality (Jiang *et al.*, 2020). In fact, lipid oxidation mechanisms are widely studied, as they involve a series of free radical reactions which generate primary and secondary oxidation products (Frankel, 1980; Cui *et al.*, 2017). According to the reaction products, various analytical methods have been developed for the evaluation of the oxidative state of the foodstuffs; for example, peroxides are considered primary oxidation products (Cui *et al.*, 2017). They are one of the indicators often used for assessing quality and estimated shelf-life. The peroxide value (PV) method measures the number of total peroxides (Lerma-García *et al.*, 2011). PV, has been extensively studied and cited (Frankel, 1980; Bajoub *et al.*, 2018; Conte *et al.*, 2020). It is one of the primary methods reported in the Commission Implementing Regulation (EU) 2022/2105 for the quality assessment of olive oil. Peroxides are not stable compounds and generally, their concentration in the VOOs increases until it reaches a maximum value and, then, decrease as they degrade into secondary oxidation products (Bajoub *et al.*, 2018). Moreover, the oxidative state of oils and fats can be evaluated by the detection of free radicals by electron spin resonance (ESR), a spectroscopy technique that allows for the determination of chemical species with unpaired electrons (Frankel, 2010; Xie *et al.*, 2019). The main radical species that are formed during the oxidation process of edible oils are LOO•, LO• and L• (Jiang *et al.*, 2020). ESR is an efficient method for the determination of free radicals. As reported in previous literature, it has been applied for the study of oxidative stability of different foods, such as mayonnaise (Altunkaya *et al.*, 2013, Raudsepp *et al.*, 2014), milk powder (Thomsen *et al.*, 2005), cheese (Kristensen and Skibsted, 1999), sunflower oil, rapeseed oil (Velasco *et al.*, 2004), extra virgin olive oils (Koprivnjak *et al.*, 2008), corn oil (Ramadan and Wahdan, 2012), hazelnut oil (Cui *et al.*, 2021), camelina oil (Yang *et al.*, 2022), hemp seed oil (Tura *et al.*, 2022), beef fat (Chen *et al.*, 2018), and fish oil (Yi *et al.*, 2011, Jiang *et al.*, 2020). Recently, Jiang *et al.* (2020) also correlated the results from an ESR analysis on different oils to the Rancimat analysis, thus allowing the evaluation of the oil resistance to oxidation by using the ESR spectroscopy approach. The detection of free radicals by ESR requires that the concentration of those compounds remains stable above $10^{-8}$ M. Unfortunately, the half-life of many radicals in solution is very short (< 1 ms) and steady-state concentration generally remains below $10^{-7}$ M (Shahidi and Wanasundara, 2002; Chen *et al.*, 2017a). The concentration of free radicals produced during oxidation is hard to be directly detected (Ricca *et al.*, 2012). In order to overcome this problem, the spin trapping technique is commonly used (Shahidi and Wanasundara, 2002). The detection of lipid free radicals can be effectively realized indirectly by the spin-trapping technique, which is based on the reaction of free radicals with diamagnetic compounds (spin traps) to form much more stable spin adducts (Chen *et al.*, 2017b). Among the spin-trapping agents, those that have a nitrone-type functional group are often used due to their ability to form a nitroxide (radical adduct) upon trapping of the free radical. Among these, N-t-butyl-α-phenylnitrone (PBN) is usually preferred for its lipophilicity and for the stability of the resulting spin adducts, even during high-temperature heating. PBN has already been used successfully for the entrapment of lipid-free radicals (Papadimitriou *et al.*, 2006; Chen *et al.*, 2017a; Chen *et al.*, 2017b). Although, PBN is not the optimal choice for identifying specific free radicals in the reaction (Chen *et al.*, 2017a) it does provide a good measure of the overall free radical flux due to its stability at various temperatures. On this basis, this work aimed to examine the correlations between the PV and the ESR assay data, in order to build a model that allows a rapid screening of oils based on the PV predicted by the ESR analysis. Thirty-five samples of virgin olive oils (VOOs) were analyzed by applying both the determination of peroxide values (according to the official Reg (EU) 2022/2104) and the ESR assay as described.

## 2. MATERIALS AND METHODS

### 2.1. Reagents and chemicals

Acetic acid (purity ≧ 99.8 %), chloroform (purity 99-99.4%) and absolute ethanol (purity ≧ 99.8%) were purchased from Sigma-Aldrich (St. Louis, USA). Sodium thiosulfate 0.1 N, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO, purity 98%) and mineral oil were supplied by Sigma-Aldrich (Milan, Italy). N-tert-butyl-α-phenylnitrone (PBN, purity ≥ 98%) was purchased from VWR-International (Milan, Italy).

### 2.2. Samples

The samples used for the construction of the model were 35 commercial VOOs (extra virgin, virgin and lampante olive oils). Of those samples, the titrimetric determination of PV and the ESR assay were performed simultaneously on the same day, in order to have no changes in the oxidative state of each sample (which could compromise the results). Three of the 35 VOO samples were interpolated in the models to make an internal validation. Moreover, four other commercial VOOs, not used for the construction of the model, were also analyzed for ESR and PV determination. These results were used for external validation of the model.

### 2.3. Peroxide value

The determination of PV was performed according to the Reg (EU) 2022/2104 and Reg. (EU) 2022/2105. It is based on an iodometric titration where, following oxide reduction reaction in the presence of starch as an indicator, the quantity of peroxides present in the sample is measured and expressed as mEq of active oxygen per kg of oil (meq $O_2$/kg of oil). Analyses were performed at least in duplicate as indicated Reg (EU) 2022/2104 and Reg. (EU) 2022/2105.

### 2.4. Electron spin resonance (ESR) assay

The oxidation state was evaluated using the ESR spectroscopic analysis. In particular, 1 mL of oil was added to 40 µL of 2.5 M N-t-butyl-α-phenylnitrone (PBN,) in absolute ethanol and vortexed for one minute. Subsequently, the entire sample was transferred into a measuring tube (quartz ESR sample tubes, outer diameter 5 mm, length 103.5 mm caps included) and placed in a heating block at 80 °C. The measuring tube, and thus the sample, were in full contact with the heating surface of the block (sample chamber plus resonator cavity dimensions were 86 mm). ESR readings were taken with the Bruker microESR bench top spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) during a 240 minutes incubation period at 80 °C. ESR measurements were recorded every 20 minutes. The following parameters were used in the microESR instrument: Center field 3450 G; Microwave power 15 mW; Modulation amplitude 2.0 G; Number of scans 16; Number of points 1024; Conversion time 2.5 s. A calibration curve from signal intensity vs concentration of free radicals was used to express the measurements in µM units of the free radical nitroxide TEMPO. The TEMPO standard used for the construction of the calibration curve was prepared in mineral oil at concentrations of 1.0, 2.5, 5.0, 10.0, 20.0, 35.0, and 50.0 µM. The first analyses were carried out in triplicate. After observing an RSD% value lower than ±1.2, calculated on three independent replicates, and according to previous results obtained by the same instruments, it was decided to perform single replicate or two replicate measurements to save instrument time.

### 2.5. Statistical analyses

The data obtained from the PV analysis and ESR assay were statistically compared using the Pearson correlation test (α=0.05) with XLSTAT software, version 2018 (Addinsoft, New York, USA). The data related to peroxide value and the ESR assay were shown as box and whisker plots for the evaluation of their distribution using XLSTAT software. The data were compared applying a Student's test, α=0.05, using XLSTAT software, to study the accuracy of the predicted PV.

## 3. RESULTS

### 3.1. Construction of the correlation model between PV and microESR

The results obtained from the titrimetric determination of PV on 35 oils showed a balanced distribution of data in the range usually found for VOOs. The samples with the highest PV were also taken into consideration for the construction of the model allowing consideration for advanced oxidation states. Details of the ESR results can be seen in the file S1. The highest correlations (Pearson correlation matrix, α=0.05) between PV and the ESR assay data were seen after 40 and 60 minutes of heating at 80 °C (Table 1); the heating temperature was chosen after testing the kinetics at three different temperatures (70, 80, and 110 °C); at 80 °C, the highest correlation with PVs was found (data not shown).

**TABLE 1. Correlation values (Pearson correlation matrix, α=0.05) between the ESR assay times and PV.**

| | Concentration (μM) of free radicals at different times of ESR assay | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Minutes** | 40 | 60 | 80 | 100 | 120 | 140 | 160 | 180 | 200 | 220 | 240 |
| **PV** | 0.825 | 0.832 | 0.820 | 0.799 | 0.813 | 0.821 | 0.809 | 0.791 | 0.810 | 0.787 | 0.782 |

Values are different from 0 with a significance level alpha=0.05

The first measurement (0 minutes) and the second one (after 20 minutes of ESR assay) were not taken into consideration because the results were often below the noise of the instrument. Therefore, on the basis of the results shown in Table 1, the correlations between PV and ESR data after 40 and 60 minutes of the assay were both selected, as the highest correlation values for the construction of the models (Figure 1 and 2). This decision was also based on the fact that, despite the fact that the correlations observed between the PV and ESR data registered at longer assay times (e.g. 80 min, 120 min and 140 min) were also high, this would require an increase in the ESR analysis time. In fact, one major basis for this study was to find the conditions for a rapid screening procedure by the ESR method. The distribution of free radical concentration data after 40 and 60 minutes was similar to that obtained for the PV method (more detailed data can be seen in the file S1). The samples that presented higher PV were also characterized by higher concentrations of ESR-detected free radicals. For the 35 VOO samples, the results relating to the concentrations of free radicals at 40 and 60 minutes were plotted against the relative PV by linear regression ($y=4.7029x+6.5563$, $R^2=0.68$; $y=2.577x+7.0846$, $R^2=0.69$) (Figures 1 and 2).

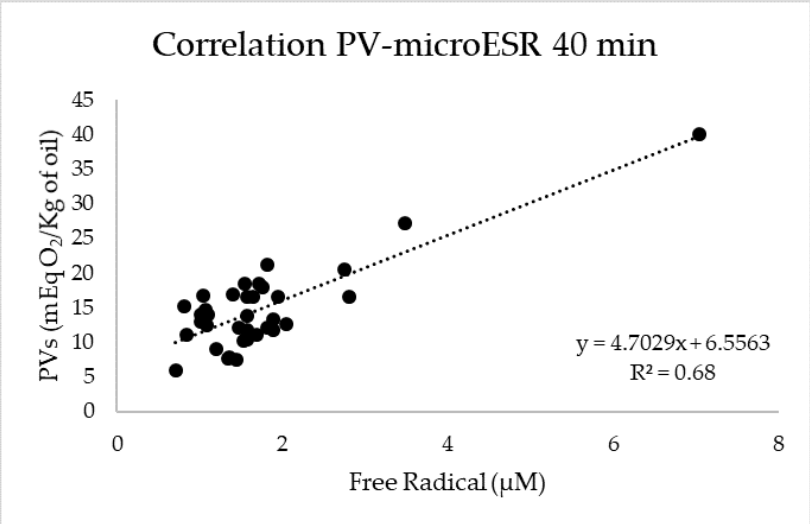


**FIGURE 1.** Linear regression between free radical concentration after 40 minutes of the ESR assay and peroxide value. Equation of the resulting straight line and R2 value were also reported.

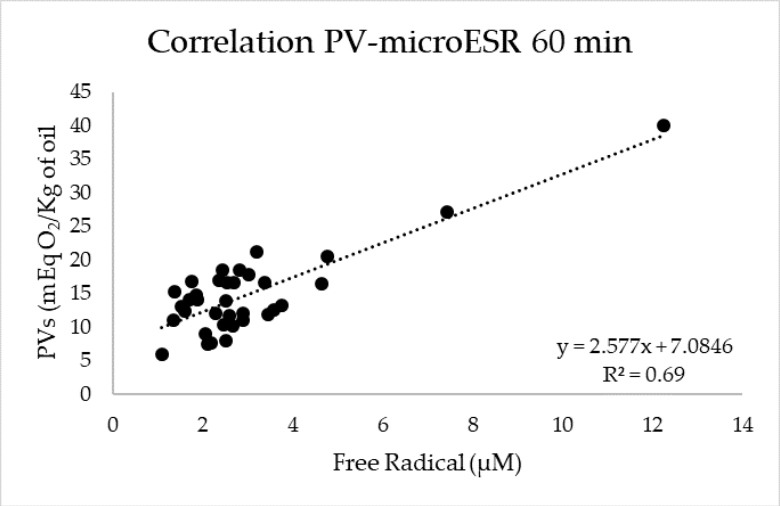


**FIGURE 2.** Linear regression between free radical concentration after 60 minutes of the ESR assay and peroxide value. Equation of the resulting straight line and R2 value were also reported.

A linear correlation was found between the measured ESR values and the peroxide values, as previously reported in the literature for different vegetable oils (Raitio *et al.*, 2011, Jiang *et al.*, 2020). Furthermore, as shown in figures 1 and 2, the correlations between the two measurements showed $R^2$ lower than 0.70. This is probably due to the different formation and degradation rates of peroxides and free radicals during the oxidative process, as found by Jiang *et al.* (2020). These correlation values were statistically significant according to the result obtained by the Pearson correlation matrix ($\alpha = 0.05$) (Table 1). Although these $R^2$ values are statistically significant, it should be noted that they are low: this result could be related to different levels of free fatty acids (FFA) and/or phenolic compound content. These two variables, as indicated in the literature (Fadda *et al.,* 2023), have been identified as impactful in ESR measurements. In particular, the concentration of free fatty acids can influence the oxidation kinetics of an oil. As reported by Paradiso *et al.* (2018), low doses of FFA can be responsible for an increase in hydroperoxide formation, in the early stages of oxidation, followed by a hydroperoxide decrease due to their decomposition. On the other hand, higher concentrations of free fatty acids promoted alternative oxidation pathways as the process progressed. These pathways involve the oxidation of FFAs, including those less susceptible to oxidation, which overlapped with the decomposition of hydroperoxides and the oxidation of esterified fatty acids. Furthermore, it has also been reported that the phenolic compounds in the oil may have interrupted the propagation of the radical chain reaction, reducing the number of radicals available for capture by PBN (Fadda *et al.,* 2023). These variables were not considered in the present study as the goal was to construct a correlation model between PVs and free radical values measured by ESR for a quick screening of different virgin olive oils of slightly different compositional characteristics. Certainly, oils that are very different in terms of composition (i.e., unsaturation degree, free acidity, and the presence of antioxidant compounds) cannot be compared solely in terms of peroxides using this approach.

### 3.2. Internal validation of the models

Three oils, among the 35 VOO samples used for constructing the model, with different PV as determined according to the official EU method, were selected to internally validate the models (LP, PV = 11.0±0.3; MP, PV = 20.4±0.1 and HP, PV = 40.0±0.6). These three samples were selected to test the two models because they were very different in terms of their oxidative state; thus, to provide a proper internal validation of the models. A sample that presented a PV slightly higher than 20 was chosen because the related EU limit for the category of extra virgin and virgin olive oils, is 20 $mEqO_2$/kg in the oil. The free radical values obtained after 40 and 60 minutes of ESR analysis were interpolated for each sample in the respective equation. This provided the value for the dependent variable (y), namely the peroxide value predicted by each of the two models (Table 2).

The comparison between the ESR predicted PV and the PV determined by titration was performed studying test samples (Table 2). The differences between each pair of results were assessed using the two-tailed paired t-test, following the statistical approach reported by Reboredo-Rodríguez *et al.* (2016). The methods did not give statistically different values for the mean concentration [t < t (critical value)]. The ESR predicted PV compared to the official method for the three test olive oil samples (LP, MP and HP) did not show statistically significant differences (Student's t test, $p \leq 0.05$). As reported in Table 2, the results obtained by ESR prediction allow the discrimination of the samples on the basis of the PV measured using the official method. The ESR predicted values related to the sample MP should be carefully considered as the PV obtained by the official method for that sample is particularly close to the official legal limit for EVOOs and VOOs, equal to 20 meq$O_2$/Kg of oil. Therefore, this predictive method could be applied, but considering its approximation, especially on borderline samples between two commercial categories, it is advisable to perform the official titrimetric method. An increase in the number of samples used for the construction of the calibration data set should reduce this type of bias in the prediction.

**TABLE 2.** Prediction of the PV for the three selected samples considering the ESR analysis values after 40 and 60 minutes of the ESR assay (internal validation). Results were presented as mean of two replicates ± RSD%. The RSD% values related to the microESR measurements were calculated considering previous results obtained with the same instrument.

| Sample | PV official method (meq$O_2$/kg oil) | PV predicted with ESR model after 40 minutes | PV predicted with ESR model after 60 minutes |
|---|---|---|---|
| LP | 11.0±0.3 | 10.6±0.2 | 10.5±0.3 |
| MP | 20.4±0.1 | 19.5±0.2 | 19.4±0.3 |
| HP | 40.0±0.6 | 39.7±0.2 | 38.7±0.3 |
| Statistical analysis[1] | | | |
| $\bar{x}_d^2$ | | -0.557 | -0.960 |
| $S_d^2$ | | 0.343 | 0.452 |
| *t* observed value | | -0.046 | -0.081 |
| *t* critical value | | 2.776 | 2.776 |
| Conclusion | | *t<t critical value* | *t<t critical value* |

[1]Two-sample t-test. [2]Differences were calculated between the proposed method and the official method $\bar{x}_d$ and represented by the mean of differences ($S_d$ represents the standard deviation).

### 3.3. Application of the models on commercial olive oil samples

The predictive models were also tested on 4 commercial VOO samples which were not used for the construction of the model itself (external validation). ESR predicted PV for the four VOOs reported in Table 3 were compared to those obtained by the application of the official method. The ESR predicted PV were consistent with results from the official method. Some slight overestimation and underestimation were observed but were not statistically significant (Student's test, $p \leq 0.05$).

The results from the ESR assay and those from the official method (Table 3) did not yield statistically different values for the mean concentration [t < t (critical value)]. The ESR predicted PV with the two models provided the same product classification in quality grades, as the official peroxide value method. It should be noted that these four commercial VOO samples all showed a PV that was always below the limit established by the Commission Implementing Regulation (EU) 2022/2105 for EVOOs and VOOs. For this reason, it will be necessary to test the model with a higher number of samples, including those with a PV above this limit. The study reported here demonstrates that the ESR free radical assay can be useful to effectively evaluate the oxidative state of VOOs. Positive correlation with PV (a known primary oxidation product), suggests the ESR assay described here could provide one rapid method for the evaluation of PV.

**TABLE 3.** Prediction of the PV for the four commercial samples using ESR analysis after 40 and 60 minutes of the assay. Results were presented as mean of three replicates ± standard deviation.

| Sample | PV official method ($meqO_2$/kg of oil) | PV predicted with ESR model after 40 minutes | PV predicted with ESR model after 60 minutes |
|---|---|---|---|
| 1 | 12.2±0.0 | 14.9±1.3 | 14.3±0.6 |
| 2 | 11.2±0.4 | 9.3±0.3 | 9.5±0.3 |
| 3 | 8.8±0.0 | 9.3±0.1 | 9.4±0.1 |
| 4 | 10.9±0.0 | 11.4±0.1 | 11.4±0.1 |
| Statistical analysis[1] | | | |
| $\bar{x}_d^2$ | | 0.445 | 0.360 |
| $S_d^2$ | | 1.887 | 1.580 |
| *t* observed value | | 0.300 | 0.267 |
| *t* critical value | | 2.447 | 2.447 |
| Conclusion | | *t<t critical value* | *t<t critical value* |

[1]Two-sample t-test. [2]Differences were calculated between the proposed method and the official method $\bar{x}_d$ represents the mean of differences while $S_d$ represents the standard deviation.

## 4. CONCLUSIONS

The present study demonstrates a satisfactory prediction of the peroxide value in VOOs by electron spin resonance spectroscopy, in fact, the two models here were proposed to give results which are statistically consistent (Student's test, $p \leq 0.05$) with those obtained by the official method. Considering the wide potential of the ESR applicable studies published recently in the literature, this work provides further promise for the expansion of the ESR technique for the evaluation of the oxidative quality, stability and shelf-life of edible oils and fats. This technique would provide quality-control laboratories, that analyze high numbers of olive oil samples every day, with a single rapid tool for different assessments by using a benchtop automated ESR instrument equipped with an autosampler. The ESR method here reported requires minimal chemicals compared to the large quantities of acetic acid (15 mL for each replicate) and chloroform (10 mL for each replicate) foreseen by the official method reported in the Reg (EU) 2022/2105. In order to reach a high predictive ability of the ESR procedure for each kind of edible oil or fat, it will be necessary to analyze a broad number of selected samples. This will allow building even highly specific and robust calibration models, making it also possible to investigate whether or not it is necessary to consider correction factors in the prediction. With regards to VOOs, oils with significantly different compositions (such as varying degrees of unsaturation, free acidity, and antioxidant compounds), they cannot be compared purely based on peroxide levels using this method. In this case, the use of correction factors can be beneficial when the fatty acids profile differs from the average values typically occurring and used to build the calibration models, in particular, when linoleic or linolenic acids are high, oleic is low, or in relation to a particularly high or low polyphenol content. The final goal will be to extract multiple information on the oxidative state of an oil by performing a single dynamic microESR assay to find relations with both the official measure of the secondary oxidation products (i.e. $K_{270}$) and the non-official, but widely applied, indirect evaluation of the induction time by OSI/Rancimat.

## ACKNOWLEDGMENTS

The authors gratefully thank Bruker BioSpin Corp. for providing the microESR instrument.

## DECLARATION OF COMPETING INTEREST

The authors of this article declare that they have no financial, professional or personal conflicts of interest that could have inappropriately influenced this work.

## FUNDING SOURCES

The present study did not receive any funding.

## AUTHORSHIP CONTRIBUTION STATEMENT

M. Tura: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. M. Mandrioli: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. E. Valli: Conceptualization, Methodology, Writing – review & editing. A. Bendini: Conceptualization, Methodology, Writing – review & editing. T. Gallina Toschi: Conceptualization, Project administration, Writing – review & editing.

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