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Time Course Analysis of Fractionated Thermoxidized Virgin Olive Oil by FTIR Spectroscopy

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ABSTRACT: FTIR spectroscopy has been used to examine the spectral changes taking place in the polar fraction of thermoxidized virgin olive oil and compared to those changes occurring in the neat oil and the nonpolar fraction. It was demonstrated that examination of the polar fraction provides additional and substantially better information of the chemical changes taking place as oxidation proceeds because this fraction concentrates the oxygenated compounds formed. Of particular interest is the enhancement of the OH component of the spectrum (3600−3200 cm^{-1}) as well as tertiary alcohol formation (~1167 cm^{-1}), including the region associated with epoxides. Time course spectral changes for neat virgin olive oil and its polar and nonpolar fractions are illustrated, compared, and contrasted, demonstrating that the interpretation of neat oil spectra is greatly enhanced by fractionation and may in fact be a preferred means of studying thermoxidation processes.

KEYWORDS: *thermoxidation, virgin olive oil, fractionation, polar and nonpolar fractions, FTIR spectroscopy*

INTRODUCTION

The consumption of fried fast food products has increased substantially over the last 20 years and with it concerns about the thermal stability of frying oils in general as well as the toxicological implications thereof.[1,2] As a result, there has been increased interest in virgin olive oil (VOO), which is known for its stability to oxidation and hence its use as a frying oil.[3] Although not generally recognized, deep-frying and pan-frying represent the second most important application of olive oil in Mediterranean countries.[4] The excellent oxidative stability of VOO is mainly due to the low levels of polyunsaturated fatty acids and high content of phenols, which are excellent antioxidants. Thus, after 10 h of heating at 180 °C, the percentage total polar compounds (TPC) of VOO can be <25%,[5] the official limit used to discard frying oils in many countries,[6] while most other vegetable oils used for frying easily reach TPC levels of >29%.[7] Although the official IUPAC TPC method[8] correlates well with the thermal degradation of frying oils, its determination by column chromatography is both lengthy and laborious. Alternative methods based on the determination on free fatty acids (FFA), percentage of polymers, peroxide value (PV), and Lovibond Color, among others, have the general drawback of only providing partial information and are not necessarily correlated with the quality and safety of frying oils.[9]

Fourier transform infrared (FTIR) spectroscopy has been considered as a potential analytical technique for monitoring thermal degradation of frying oils because the mid-IR spectrum provides insight into the changes in the functional groups which are representative of the chemical processes that occur during frying, particularly hydrolysis and oxidation reactions. It has proven to be a very useful tool for the quantitative determination of key oil quality parameters such as FFA,[10] trans content,[11] PV,[12] and iodine value (IV).[13,14] Recently, we described an attenuated total reflectance (ATR-FTIR) method to predict the TPC of VOO as frying progressed,[5] the 978−960 cm^{-1} region, indicative of trans isomer formation, being directly proportionate to TPC formation. Although this relationship is relatively straightforward, many other degradation reactions of interest occur simultaneously, including hydrolysis and oxidation, but are difficult to elucidate because of the lack of sensitivity of ATR-FTIR due to its inherent short path length. In contrast, using transmission cells, such changes can be more readily tracked,[11,15] but the tracking of these changes is still problematic as the bulk of the oil masks these spectral changes. One means of improving this situation is to isolate the fraction of interest, such as unsaponifiable matter, which was shown to be helpful in detecting hazelnut oil adulteration of olive oil.[16] Fractionation of oil samples into polar and nonpolar fractions has been employed in other kinds of spectroscopies such as ultraviolet−visible (UV−vis) and by electron paramagnetic resonance (EPR) spectroscopies[17] and ^{13}C NMR spectroscopy.[18,19] Although FTIR has not been used to analyze polar fractions of thermoxidized oils, it has been used as a detector to analyze polar compounds separated by chromatography. Thus, Kuligowski et al.[20] determined total polymer triacylglycerols in olive oil by coupling a FTIR detector to online gel permeation chromatography.

In this paper, we separate thermoxidized virgin olive oil into its polar and nonpolar fractions and examine the two fractions spectroscopically and, more specifically, the polar fraction by high-performance size-exclusion chromatography (HPSEC), using this information to assist in interpreting the spectral changes taking place in olive oil during thermoxidation.

MATERIALS AND METHODS

Sample Preparation. Generic, commercial Spanish VOO considered suitable for frying was purchased locally and used as a model system. Although the oxidative stability of virgin olive oil can

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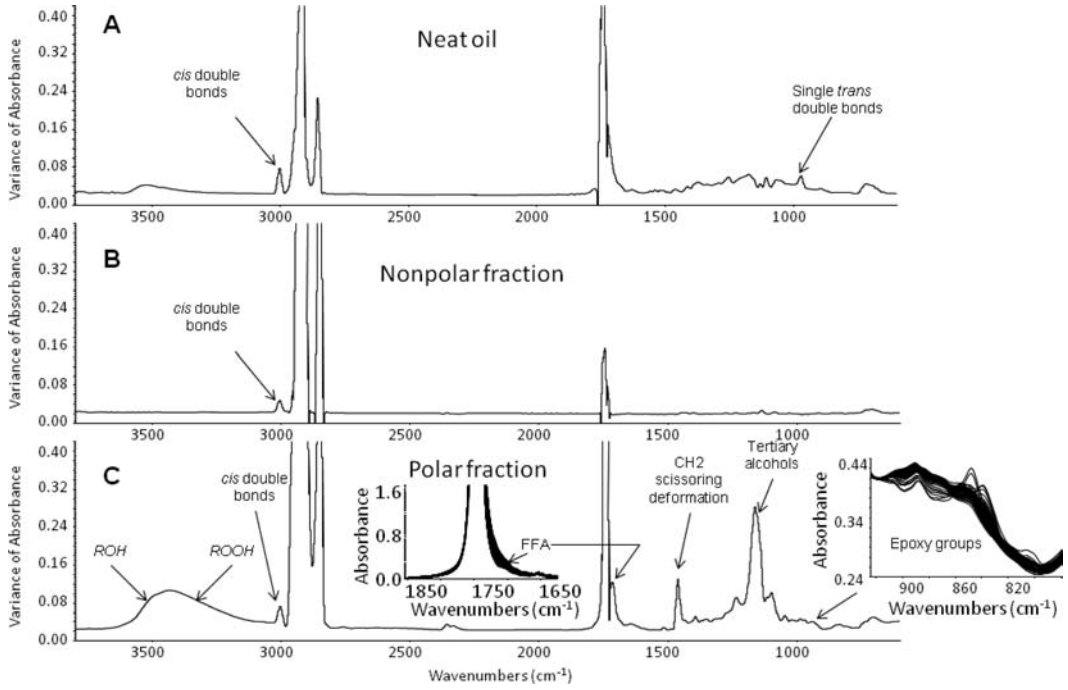

Figure 1. Variance spectra computed from the whole set of spectra of 47 thermoxidized samples for the neat oil (A), nonpolar (B), and polar (C) fractions.

vary substantially according to the cultivar, this oil was considered adequately representative for tracking and assessing oxidation for qualitative purposes. Thermoxidation was carried out in a 4 L domestic fryer with automatic temperature controller (Heidolph EKT 3001, Schwabach, Germany), with 3 L of oil heated at 190 °C for 94 h for 8 h cycles per day. Then 40 mL of sample were collected every 2 h until the end of the heating process, with a total of 47 samples collected, these kept at 4 °C in brown glass vials until analyses. This experiment was repeated with another commercially available VOO to validate the trends observed.

Analysis of Total Polar Compounds. The % TPC was determined gravimetrically according to the IUPAC Standard Method no. 2.507,[8] with the nonpolar and polar fractions separated from 1 g of oil by silica gel column chromatography using 20 g of silica adjusted to a water content of 5% w/w. Elution of the nonpolar fraction was carried out using 150 mL of *n*-hexane/diethyl ether (90:10, v/v), while the retained polar fraction was eluted with 150 mL of diethyl ether; the adequacy of separation confirmed by TLC using *n*-hexane/diethyl ether/acetic acid (80:20:1, v/v/v) and visualized with iodine vapor. The relative % polar and nonpolar fraction was determined gravimetrically after solvent evaporation, and these samples used for FTIR analysis.

High-Performance Size-Exclusion Chromatography (HPSEC). HPSEC was used to facilitate the determination of polymerized triacylglycerols (PTG), oxidized triacylglycerols (OTG), and diacylglycerols (DTG) in the thermoxidized oils as the oxidation progressed. The polar fraction sample, dissolved in hexane (15 mg/mL), was analyzed by HPLC (Agilent Technologies 1200) by using two columns of Hewlett-Packard PL gel (30 cm ×0.75 cm i.d.) that were packed with high-performance styrene−divinylbenzene copolymer, with a particle size of 5 μm (Agilent Technologies, Madrid, Spain). The pore sizes were of 100 and 500 Å for the first and second column, respectively. These columns were connected in series, and tetrahydrofuran was used as eluent at a flow rate of 1 mL/min. The injection volume was 10 μL, and a refractive index detector (Agilent Technologies 1200) was used for monitoring the eluent. Quantification was done according to Dobarganes, Velasco, and Dieffenbacher.[21] The calculations assume that all compounds of the sample are eluted. The relative composition of PTG, OTG, and DTG were expressed as a percentage of the oil.

Fatty Acid Analysis. The fatty acid profile of VOO was monitored as thermoxidation progressed. The fatty acids in the oil were transmethylated according to ISO 5509,[22] with ∼0.2 g of oil dissolved in 4 mL of hexane and reacted with 0.4 mL of 2 N of KOH in methanol. The mixture was vigorously shaken for 10−15 s and the 0.2 μL of sample injected into a Varian 3900 gas chromatograph equipped with a split−splitless injector and a flame ionization detector; separation carried out using a Supelco SP-2380, 60 mm × 0.25 mm ID and 0.25 μm of thickness, column. Hydrogen was used as carrier gas at a flow rate of 1.0 mL/min, and the injector and detector temperatures were set at 225 and 250 °C, respectively; the oven temperature maintained at 170 °C for 10 min and then programmed from 170 to 210 °C at 1.5 °C/min and maintained there for the rest of the run. The mean of duplicate results were expressed as g fatty acid/100 g total fatty acids (%) normalized according to the procedure of Dobarganes and Pérez-Camino.[23]

Analysis for Phenols. The oils were analyzed for their phenol content by HPLC to monitor their loss over time. A standard solution (0.5 mL) of *p*-hydroxyphenylacetic (0.12 mg/mL) and *o*-coumaric acids (0.01 mg/mL) in methanol was added to a 2.5 g sample of filtered VOO. A rotary evaporator maintained at 40 °C under vacuum was used to evaporate the solvent and the resulting oily residue dissolved in 6 mL of hexane. The diol-bonded phase cartridge was conditioned according to Mateos et al.,[24] and after the sample loading, the final residue was extracted with 10 mL of methanol and evaporated at 40 °C under vacuum and the extract was diluted with 500 μL of methanol/water (1:1, v/v). A filtered aliquot (20 μL) of the final colorless solution was injected onto the HPLC system (LaChrom Elite Tokio, Japan), equipped with a diode array detector. The column was a Lichrospher 100RP-18 column (4.0 mm ID × 250 mm; 5 μm, particle size) (Darmstadt, Germany) maintained at 30 °C. The gradient elution, at a flow rate of 1.0 mL/min, was achieved by using the following mobile phases: a mixture of water/phosphoric acid (95.5:0.5 v/v) (solvent A) and methanol−acetonitrile (50:50 v/v) (solvent B). The change of solvent gradient was programmed as follows: from 95% (A)−5% (B) to 70% (A)−30% (B) in 25 min; 62% (A)−38% (B) in 10 min; 62% (A)−38% (B) in 5 min; 55% (A)−45% (B) in 5 min; 47.5% (A)−52.5%(B) in 5 min and 100% (B) in 5 min, followed by 5 min of maintenance. The chromatographic signals were

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obtained at 235, 280, and 335 nm, and the response factors and recoveries were based on the procedure used by Mateos et al.[24]

FTIR Spectroscopy. Spectral data were collected on a Bruker 55 Equinox S FTIR spectrometer equipped with a DGTS detector (Bruker Optics, Ettlingen, Germany) and using a Specac (Orpington IK) KBr transmission cell with a path length of 150 μm. Each spectrum was recorded at room temperature over the region of 4000−400 cm^{-1} using 22 scans at a resolution of 4 cm^{-1}. The oils and their polar and nonpolar fractions were loaded into the IR cell by using a micropipet (~20 μL) and analyzed in duplicate. Between samples, the cell was thoroughly cleaned and dried by aspirating hexane through the cell using vacuum and its cleanliness verified spectrally. Spectra were examined using OPUS version 4.0 (Bruker Optics, Ettlingen, Germany) with peak heights and areas computed from the raw spectra and the results exported as ASCII data for further study using Statistica version 6.0 (Statsoft, Tulsa OK).

RESULTS AND DISCUSSION

Parts A−C of Figure 1 illustrate the variance spectra of the neat oil, its nonpolar fraction, and its polar fraction, these being indicative of the gross spectral changes having taken place over the 94 h, the sample was maintained at 190 °C. It is clear that on this basis, substantive changes are taking place throughout the spectrum but most obviously in the OH region (3584−3508 cm^{-1}), the single cis double bonds (~3005 cm^{-1}) region, the CH region (3970−2850 cm^{-1}), the C═O region (~3472 cm^{-1}), and the fingerprint region (1500−900 cm^{-1}), including the isolated trans portion (967 cm^{-1}). Changes in the OH region reflect the formation of alcohols and hydroperoxides, while variations in the CH region shows loss of cis double bonds and changes in the ratio of CH_2 moieties relative to CH_3 terminal groups. Variations observed in the C═O region point out hydrolysis of the ester linkage and formation of FFA, and the fingerprint region indicate structural changes in the fatty acids and in the trans region, such as the formation of trans double bonds. Variance spectra only indicate that changes have taken place but not their sequence, direction, or relationship to oil quality, but our previous work indicated that changes in the isolated trans region (976−963 cm^{-1}) correlated well with the % TPC.[5] On the other hand, Muik et al.[25] carried out a work with bidimensional correlation of FTIR and FT-Raman spectra in which the band at ~967 cm^{-1} also showed a linear relationship with the band assigned to carbonyl compounds at 1730 cm^{-1}, although in the present work that correlation was not observed due to the interfering effect of the strong ester linkage absorption at ~1746 cm^{-1}.

Figure 1B presents the variance spectrum of the isolated nonpolar fraction, which is quite different in its changes as most of the oxygenated constituents have been removed, effectively leaving mostly triacylglycerols and hydrocarbon based constituents. The predominant change observed is in the cis band and the split ester band, indicative of changes in the ester linkage as a result of hydrolysis and the concurrent formation of FFA. In this spectrum, no corresponding changes in the trans region is observed, implying they are associated with polar constituents. Thus, the nonpolar fraction is not static, however, it does not appear to undergo changes as dramatic as the polar fraction shown in Figure 1C. As can be seen, the changes taking place are substantially more detailed as all the oxygenated constituents formed as a result of thermoxidation are coalesced into one spectrum. Here changes in the OH region are dramatic, but complex, as alcohols (~3544 cm^{-1}), hydroperoxides (~3425 cm^{-1}), as well as C═O overtone changes (~3472 cm^{-1}), all overlapping each other. The changes in cis band (3005 cm^{-1}) are again evident, as are CH_2 and CH_3 (2925 cm^{-1}, 2854 cm^{-1}) changes which are off-scale, albeit CH_2 (1459 cm^{-1}) scissoring is on scale. We now clearly see OH absorptions of tertiary alcohols (~1167 cm^{-1}) as well as more detailed changes in the trans region (976−963 cm^{-1}), some of the contributing spectra being shown in the inset. Although FTIR spectroscopy has been used to monitor the formation and loss of hydroperoxides and the concurrent formation of alcohols produced during oxidation[26−28] in neat oils, the problem being that the polar constituents formed tend to be present at low concentrations (e.g., ~12 meq of O_2/kg hydroperoxides) and are also masked by the predominant presence of the nonpolar constituents[15] and are thus difficult to quantify accurately. These variance spectra illustrate that by fractionating the oil and concentrating the polar constituents, much more detailed and relevant spectral information is made available.

Figure 2 illustrates the hydroxyl region (3600−3200 cm^{-1}) collected from the polar and nonpolar fractions and the corresponding neat oil after 36 h of thermoxidation and having a TPC content of 31.03% .

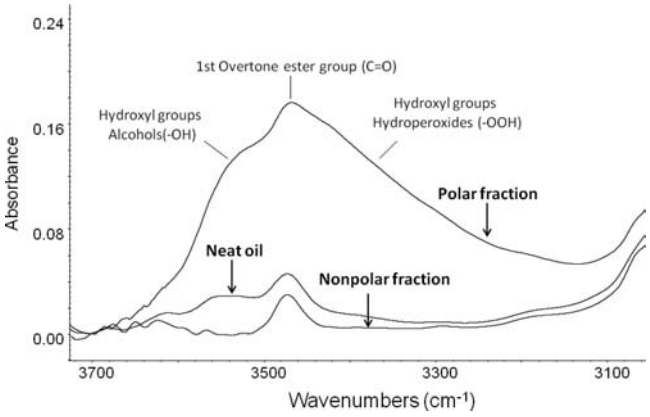

Figure 2. Spectra in the OH region (3600−3200 cm^{-1}) of a thermoxidized virgin olive oil (VOO) sample (thermoxidation time, 36 h; total polar compounds, 31.03%) and its corresponding polar and nonpolar fractions.

The spectrum of the nonpolar fraction only shows a single band centered within 3510−3430 cm^{-1}, which is due to the first overtone of the stretching vibration of the ester group in triacylglycerols.[26] By contrast, the polar fraction has a complex broad band that is mostly due to the hydroxyl groups of alcohols (~3544 cm^{-1}) and hydroperoxides (3425 cm^{-1})[29−31] but also includes the overtone of ester overtone band. These OH bands are also observed in the spectra of the neat oil, although at a lower intensity relative to the ester overtone (C═O ~3472 cm^{-1}), the latter clearly confounding the already complex overlapping OH bands and their hydrogen bond effects. Figure 3A tracks key spectral changes occurring in the polar fraction in more detail, while Figure 3B tracks and compares changes in the same regions to those occurring in the neat oil and the nonpolar fraction. The trends for validation samples are also shown.

The hydroperoxides, already present at the non thermoxidized sample, are decomposed into secondary oxidation products, reducing their concentration until 20 h of thermoxidation (14% of TPC). At this moment, the intensity of this band varies slightly with time and tends to reach a

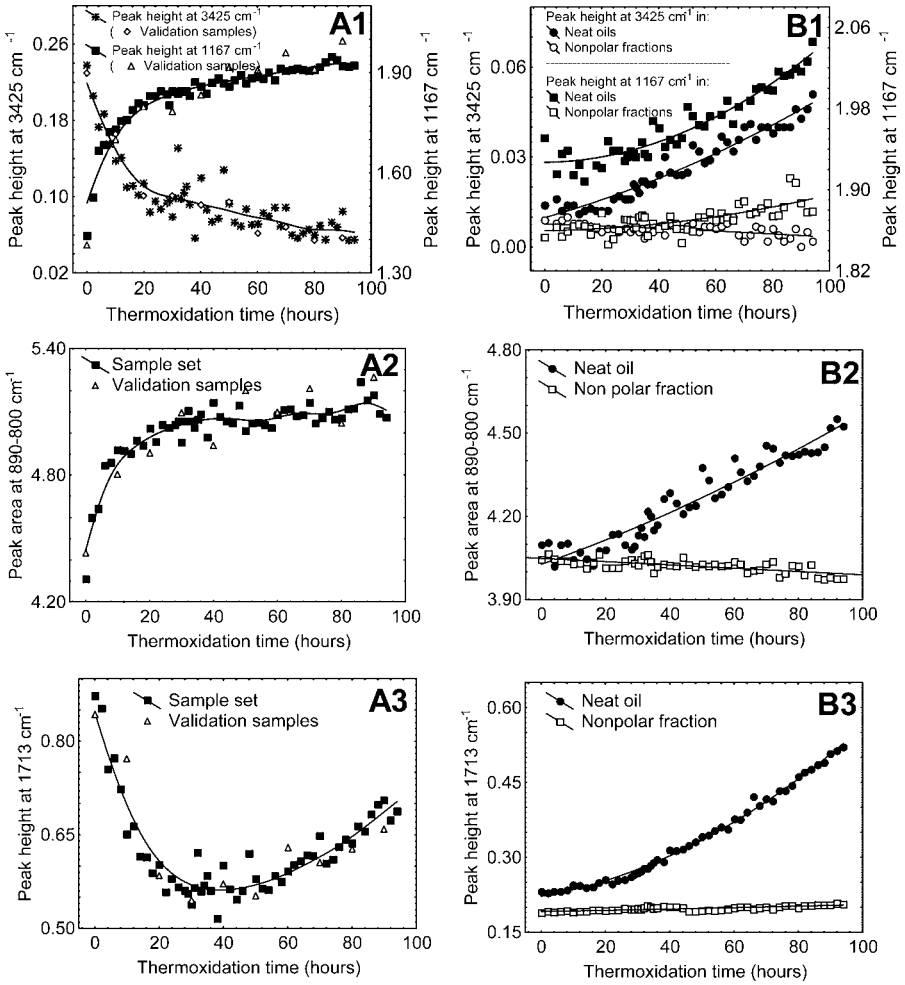

Figure 3. Time course plots of several FTIR bands measured in the polar fraction (A) and the nonpolar fraction and the neat oil (B) during the thermoxidation process of a virgin olive oil.

plateau. On the contrary, the tracking of this band in the spectra of the neat oils and the nonpolar fractions is unclear because of the low signal intensity and the resolving problems of this region (Figure 3B1).

The variance spectrum of polar fraction also allows the identification of other spectral changes. Thus, one of the most relevant bands is located around ∼1167 cm^{-1} (Figure 3A1). This band is assigned to tertiary alcohols (C−O stretching),[29] which can be formed as secondary oxidation products. This band rises during thermoxidation (Figure 3A1) following a trend that is opposite to the time course spectral change of the hydroperoxide band. Thus, this band reaches a maximum intensity at 20 h of thermoxidation (14% of TPC), probably due to the end of hydroperoxides breakdown. This band may also be assigned to phenols (∼1200 cm^{-1}, C−O stretching),[29] although their contribution to this band may be irrelevant because its intensity rises during the process while the concentration of phenols decreases, as it is shown in Table 1.[30] The evolution of this band is not significant in the case of spectra of nonpolar fractions and the neat oils (Figure 3B1).

The time at which the hydroperoxides and the tertiary alcohols reach a plateau matches the trend observed for the various polar groups analyzed by HPSEC. Table 1 shows the percentages of TPC, polymerized triacylglycerols, oxidized triacylglycerols, and diacylglycerols over the experiment. Polar polymers and oxidized triacylglycerols are among the compounds produced by oxidation at high temperatures,[31,32] and they account for 45.61% and 21.91% at the terminal stage of oil oxidation, with the concentration of these compounds rising more rapidly after 20 h of heating. This inflection at 20 h is not observed for the other polar compounds resulting from hydrolysis (e.g., diacylglycerols), whose formation is not directly related to hydroperoxide breakdown.

In addition to the O−H vibration observed for hydroperoxides at ∼3425 cm^{-1}, the C−O−O stretching of peroxides and epoxy groups produce a weak absorption in the range 890−800 in the polar fraction spectra (Figure 1C). Because it is a complex region characterized by weak bands, the spectral area was considered the best parameter for studying their evolution rather than peak height. Figure 3A2 illustrates the trend of the peak area in the region under study (890−800 cm^{-1}), which rapidly increases during the first hours of heating. However, unlike the trend observed for hydroperoxides and tertiary alcohols, the epoxy and peroxide band increase at a slower pace after 6 h of thermoxidation (9% of TPC). The epoxy groups responsible for this absorption are formed during thermoxidation, and they can be attached to oxidized monomeric triacylglycerols.[33] Bordeaux et al.[34] demonstrated the occurrence of *trans*-9,10- and *cis*-9,10-epoxystearate in an oxidation study carried out with methyl oleate and triolein, while the same experiment carried out with methyl linoleate and trilinolein produced a series of monounsaturated epoxides

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Table 1. Percentages of Total Polar Compounds, Polar Polymers, Oxidized Triacylglycerols, Diacylglycerols, Oleic, Linoleic, and Linolenic Acids, and the Content of *o*-Diphenols (mg/kg) in the Virgin Olive Oil Samples During Thermoxidation Experiment[a]

thermoxidation time (h)	total polar compounds	polar polymers	oxidized TAG	DAG	C18:1	C18:2	C18:3	*o*-diphenols
0	3.45	0.15	1.44	1.32	75.93	4.55	0.67	134.12
6	5.10	1.54	1.68	1.39	72.92	4.03	0.56	70.00
10	7.11	2.71	2.46	1.51	72.35	3.90	0.54	29.78
16	9.40	3.93	3.45	1.61	72.20	3.41	0.53	11.61
20	13.02	5.89	4.93	1.76	71.38	3.40	0.49	8.71
26	18.56	8.86	7.23	2.03	70.83	3.12	0.47	7.43
30	20.76	10.94	7.90	1.64	69.52	3.04	0.47	7.19
36	31.03	16.34	12.73	2.19	69.31	2.65	0.41	tr
40	36.05	18.88	15.86	1.95	67.31	2.30	0.23	tr
46	37.90	21.56	14.48	2.55	65.21	2.04	0.20	tr
50	45.65	25.91	18.34	2.59	63.99	1.83	0.18	
56	50.62	30.45	18.79	2.97	62.98	1.64	0.16	
60	53.88	32.76	18.82	4.00	62.21	1.61	0.16	
66	60.51	37.22	21.66	3.96	60.83	1.39	0.16	
70	61.92	39.70	21.55	3.83	58.41	1.19	0.15	
76	63.75	40.30	21.90	4.91	57.99	1.20	0.14	tr
80	64.83	42.11	22.09	4.63	55.75	0.99	0.07	
86	66.02	42.76	22.25	5.13	53.75	0.77	0.06	
90	67.65	44.65	22.17	5.98	51.28	0.68	0.03	
94	68.11	45.61	21.91	5.51	50.53	0.65	0.01	

[a]Note: TAG, triacylglycerols; DAG, diacylglycerols; tr, trace levels.

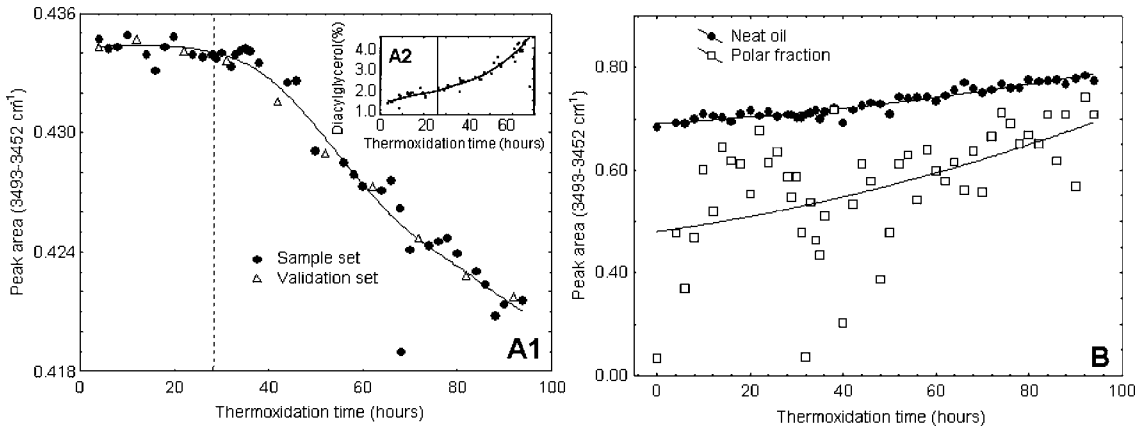

Figure 4. Time course plots of the band area of the first overtone of ester linkage absorption (3493−3452 cm^{-1}) measured in the nonpolar fractions (A1), in the polar fractions and in the neat oils (B) during the thermoxidation process. The time course plot of diacylglycerols content is shown as an inset (A2).

(epoxyoleates). In this study, the epoxides arising from monounsaturated fatty acids show higher stability and thereby result in a higher concentration. Therefore, most of the absorption in the 890−900 cm^{-1} region can be assigned to the monounsaturated epoxides. The same band area quantified in the spectra of nonpolar fractions and in the neat oils resulted in a very different trend (Figure 3B2). Thus, the band area in the nonpolar fractions did not show any significant variation, while it rose in a linear manner, probably because of a baseline rise often observed in oxidation studies using FTIR spectroscopy.[35]

The increase of free fatty acids is also observed in the variance spectrum of the polar fraction in the range 1780−1670 cm^{-1} (Figure 1C). The band located at 1713 cm^{-1} is assigned to the carbonyl group of the carboxylic acid.[28] Although this band is more noticeable in the spectra of the polar fraction, it is not clear that this band is only due to free fatty acids because it decreases at the beginning of the process until it starts rising from 30 h onward (22% of TPC) (Figure 3A3). The dramatic shrink of this band at the beginning of the heating process is not observed in the case of the spectra of the neat oils (Figure 3B3), and it is probably due to the carbonyl group of ketones, which also absorb in this region (1725−1705 cm^{-1}),[29] in conjunction with the thermal deterioration of the existing free fatty acids of the unaltered oil.

Hydrolysis reactions also imply a decline of the concentration of triacylglycerols, and a concomitant reduction of the band at ∼1746 cm^{-1} assigned to the stretching vibration of ester linkage (C═O), this band being off-scale in all the spectra because of its strong intensity. Thus, the trend of this band was studied through its first overtone (∼3472 cm^{-1}). Although this overtone band interferes with the rest of absorptions of the hydroxyl region (3600−3410 cm^{-1}) (Figure 2), in the spectra of the nonpolar fractions this band is isolated and can be readily measured.

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Figure 4A1 shows the first overtone of ester linkage absorption (3493−3452 cm^{-1}) trend computed for the nonpolar fraction. The band area is almost constant, with a slight tendency to decrease until the oil reaches 26% of total polar compounds (32 h of thermoxidation). At this moment, the concentration of esters starts decreasing linearly. This trend matches with that of the band assigned to free fatty acids in the polar fractions and in the neat oils (1713 cm^{-1}) (Figure 3A3,B3), for which intensity increases at a faster pace from 30 h (TPC = 22%) onward. In contrast, the band areas computed for the polar fractions and in the neat oils do not show a clear trend (Figure 4B). The high dispersion of data in Figure 4B can be attributed to the interfering effect of the absorptions assigned to hydroperoxides and alcohols (Figure 2).

The decline of the ester linkage absorption associated with triacylglycerols is explained by the hydrolysis reactions that take place during thermoxidation, the principal byproducts being free fatty acids and diacylglycerols. Figure 4A2 shows the trend of the diacylglycerols content during the thermoxidation experiment. This trend is consistent with the decrease in intensity of the overtone band of the ester linkage band (Figure 4A1), which corroborates the relationship of the latter with hydrolysis.

In conclusion, the analysis of polar fractions of thermoxidized oils using a transmission cell permits tracking of weaker bands that evolve during thermoxidation, providing a sufficient signal-to-noise ratio compared to neat oils only. Because most of the chemical changes are associated with the polar fraction, the FTIR analysis of polar and nonpolar fractions separately allows one to better interpret neat oil spectra. The analysis of the hydroxyl region is simplified by isolating the hydroperoxide and alcohol bands in the polar fraction while gaining access to the ester linkage band via the nonpolar fraction. The spectra of the polar fraction also provides valuable information on the bands associated to free fatty acids, epoxy, and peroxide compounds because their absorptions are more intense compared to the spectra of the neat oil. This study indicates that more detailed insight into the concurrent chemical changes occurring during thermoxidation of virgin olive oil is possible and that this technique could be useful in the analysis of autoxidative procecess in general.

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Notes

The authors declare no competing financial interest.

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