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ARTICLE

Effects of Heating on Virgin Olive Oils and Their Blends: Focus on Modifications of Phenolic Fraction

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The phenolic profiles of two different virgin olive oils and their admixtures in different percentages have been analyzed after heating treatments by microwave or conventional oven. Changes in the phenolic profile upon heating were evaluated by chromatographic and spectroscopic methods, also monitoring the antioxidant activity by $ABTS^{\bullet+}$ test. 3,4-DHPEA-EA, *p*-HPEA-EA, and EA showed the highest decreases after thermal treatments. The only compounds that showed a clear increase with heating, in particular by conventional oven, were the dialdehydic form of elenolic acid (EDA) and *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid (*p*-HPEA-EDA). A comparison between the variations after heating of the sum of monoaldehydic and dialdehydic forms of phenolic compounds obtained by using different analytical approaches (HPLC-DAD/MSD and 1D and 2D NMR spectroscopy) was made. The results showed a good agreement of these two high-resolution techniques.

KEYWORDS: Virgin olive oil; phenols; heating; microwave oven; HPLC; NMR

INTRODUCTION

Extra virgin olive oil (EVOO) contains triglycerides, which represent >98% of the total oil weight and minor components, amounting to about 2% of the total oil weight. Minor components include more than 230 compounds, such as hydrocarbons, aliphatic and triterpenic alcohols, sterols, volatile compounds, and antioxidants (*1*). The main antioxidants of EVOO are carotenes, tocopherols, and polar phenols (*2*, *3*). Due to the presence of these compounds, EVOO is considered to be an excellent foodstuff compared to other vegetable oils, because of its excellent oxidative stability (*4*). Phenols are responsible for the organoleptic properties of EVOO (*5*,*6*); they also show beneficial biological activity, due to their anti-inflammatory (*7*), anticarcinogenic (*8*,*9*), and antioxidant (*10*−*12*) properties. The effects of thermal treatments on the phenolic pattern of EVOO have been well-studied in the literature. In a recent work (*13*) the authors found that the total phenol content, measured with Folin−Ciocalteu reagent, decreased by 55−60% after heat treatment at 100 °C for 142 h, with an air flow of 10 L/h. In particular, considering singular molecules, other researchers (*14*) showed that 3,4-dihydroxyphenylethanol (3,4-DHPEA), elenolic acid (EA), 3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid (3,4-DHPEA-EDA), and 3,4-dihydroxyphenylethanol linked to elenolic acid (3,4-DHPEA-EA) reduced their concentrations more quickly, among antioxidant compounds present in EVOO, with thermal treatments at 180 °C in a conventional oven for 30 min. Such a trend for 3,4-DHPEA was confirmed by Nissiotis and Tasioula-Margari (*15*): during heating, the amount of this molecule decreased more rapdily than the amounts of other phenolic compounds. This agrees with the positive correlation between the degradation rate of phenols in EVOO and their antioxidant capacity (*16*), which is very high for 3,4-DHPEA (*17*). However, lignans show a weaker antioxidant capacity in comparison with other phenolic compounds, so they are the molecules most stable to thermal treatment (*14*). In two papers (*13*, *18*), an increase in lignans and *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid (*p*-HPEA-EDA) was observed after heating of EVOOs, but was explained by the authors as a probable coelution with oxidized compounds. After thermal treatment in a microwave oven for 10 min with a power of 0.5 kW, the amounts of 3,4-DHPEA, 3,4-DHPEA-EDA, *p*-hydroxyphenylethanol (*p*-HPEA), *p*-hydroxyphenylethanol linked to elenolic acid (*p*-HPEA-EA), and 3,4-DHPEA-EA decreased (*18*).

The aim of this study was to determine how the heat treatments by microwave or conventional oven under routine home-cooking conditions may affect the phenolic patterns of two samples of virgin olive oils (VOO) and their blends at different percentages, characterized by a predictable quali-quantitative phenolic composition. This study has been carried out using HPLC-DAD/MSD and 1D and 2DNMR spectroscopy to permit comparison of the results with these different analytical techniques.

MATERIALS AND METHODS

Apparatus. HPLC-DAD/MSD analyses of phenolic compounds were performed with a HP 1100 series instrument (Agilent Technologies, Palo Alto, CA) provided with a binary pump delivery system, degasser,

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autosampler, diode array UV−vis detector (DAD), and quadrupole mass spectrometer detector (MSD). The HPLC column was a C18 Luna column, 5 μm particle size, 250 mm × 3 mm i.d. (Phenomenex, Torrance, CA), with a C18 precolumn (Phenomenex) filter. The separation and identification of the phenolic compounds by HPLC-ESI-TOF-MS was also performed using an Agilent 1200 series Rapid Resolution LC with a vacuum degasser, an autosampler, and a binary pump equipped with a RP C18 analytical column (4.6 × 150 mm, 1.8 μm particle size, Agilent ZORBAX Eclipse plus). The measurement of antioxidant activity of phenolic extract by $ABTS^{\bullet+}$ assay was carried out using an UV−vis 1610 instrument (Shimadzu Co., Kyoto, Japan), which had a six-slot shuttle and a system for temperature control of working conditions. NMR spectra were obtained using a Varian Mercury Plus 400 MHz instrument (Varian NMR systems, Palo Alto, CA) using library sequences.

Reagents and Standards. All solvents used were of analytical or high-performance liquid chromatography (HPLC) grade and filtered through a 0.45 μm nylon filter disk (Lida Manufacturing Corp., Kenosha, WI) prior to use. The standard used for evaluation of antioxidant capacity of phenolic extracts (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, Trolox), was purchased from Sigma-Aldrich (St. Louis, MO). 2,2′-Azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, diammonium salt (ABTS), luteolin (LUT), apigenin (API), 3,4-dihydroxyphenylacetic acid (3,4-DHPAA), sodium hydroxide, formic acid, and isopropanol were acquired from Fluka (Buchs, Switzerland).

Methanol, *n*-hexane, acetonitrile, and formic acid (of HPLC grade) were obtained from Merck (Darmstadt, Germany). Deuterated dimethyl sulfoxide (DMSO-d_6) was obtained from Cambridge Isotopes Laboratories, Inc. (Andover, MA).

Samples and Thermal Treatment. Two samples of virgin olive oils (designated VOO1 and VOO2) were analyzed as well as two blends of these oils, prepared at 70−30 percent (70−30, v/v) and 50−50 percent (50−50, v/v) of VOO1 and VOO2, respectively. These blends were prepared with the purpose of obtaining a predictable phenolic profile from a qualitative and quantitative point of view. Unheated oils (termed TQ, "tal quale", meaning "as such" in Italian) were sampled as a control. For analytical purposes, 300 g of each kind of sample was inserted in an open glass container and subjected to microwave (MW) or conventional oven (CO) heating; the heated samples were respectively named MW and CO. The surface/volume ratio was constant for the samples (256 cm^2/330 mL, total capacity of glass container = 1.5 L, oil thickness = 1.6 cm). The amount of olive oil subjected to thermal treatment was sufficient to carry out all of the analyses in triplicate, especially for the extraction of phenolic compounds. The time−temperature conditions for both heating treatments were similar to home-cooking or food catering: all of the samples were heated for either 1 h in a conventional oven (type M20-VN, Instruments s.r.l, Bernareggio (MI), Italy) at 180 °C or for 9 min at 750 W of power in a microwave oven (model AMW214/WH, Whirlpool, Benton Harbor, MI), with a frequency of radiation of 2450 MHz. These combinations of time and temperature for each type of heating system were necessary to reach similar final temperatures. During heating, the temperature was registered at fixed intervals of thermal treatment by a thermocouple HI 98804 (Hanna Instrument, Woonsocket, RI) inserted at approximately the center of the samples. Both unheated and heated samples were stored at 12 °C in a thermostat and some aliquots in freezer at −43 °C prior to analyses. All analyses were performed in triplicate.

Extraction of Polar Phenolic Extracts. The liquid−liquid extraction (LLE) procedure was carried out according to the method of Carrasco-Pancorbo et al. (*19*). Briefly, 60 g of oil was dissolved in 60 mL of *n*-hexane, and the solution was extracted successively with four 20 mL portions of methanol/water (60:40, v/v). The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator under reduced pressure at 40 °C. This extraction procedure was performed in triplicate, splitting each sample into two fractions of equal amount prior to the evaporation step (thus obtaining six dry extracts for each sample). Extracts were stored at −43 °C before analysis.

Measurement of Antioxidant Activity of Phenolic Extract by $ABTS^{\bullet+}$ Assay. The radical-scavenging capability of phenolic extracts was evaluated by $ABTS^{\bullet+}$ radical cation assay according to the procedure of Re et al. (*20*). ABTS was dissolved in H_2O at a concentration of 7 mM. The radical cation of ABTS was obtained by reaction with 2.45 mM potassium persulfate (final concentration) and allowing the stock solution to stand in the dark at room temperature for at least 12 h. Before use, the $ABTS^{\bullet+}$ solution was diluted with ethanol to reach an absorbance of 0.70 ± 0.02 at 734 nm at 30 °C. Next, 1 mL of this $ABTS^{\bullet+}$ solution was added to 0.01 mL of extract, and the decrease in absorbance was recorded for 10 min. Absorbance values were corrected for radical decay using a blank solution (0.01 mL of 50% aqueous methanol). Measurements were made in triplicate, and the antioxidant activity was calculated as the Trolox equivalent antioxidant capacity (TEAC, mmol of Trolox kg^{-1} of oil), using a calibration curve (equation: $y = 0.1304x - 0.0056$; $r^2 = 0.981$).

Determination of Phenolic Compounds by HPLC-DAD/MSD. The gradient elution was carried out using water/formic acid (99.5:0.5, v/v) as mobile phase A and acetonitrile as mobile phase B of the solvent system, according to the conditions described by Carrasco-Pancorbo et al. (*14*). The mobile phase flow rate was 0.5 mL min^{-1}, and the injection volume was 10 μL. UV−vis detection was set at 240, 280, and 330 nm. The detection was made using quadrupole MS as well, with an electrospray (ESI) interface operating in positive ion mode within the m/z 50−800 range and the following conditions: drying gas flow, 9 L min^{-1} at 350 °C; nebulizer gas pressure, 50 psi; capillary voltage, 3000 V. Nitrogen was used as nebulizer and drying gas. For HPLC analysis, the phenolic extracts were redissolved in 500 μL of methanol/water (1:1, v/v) and filtered through a 0.45 μm filter (VWR, West Chester, PA).

To carry out the quantification of phenolic compounds with HPLC-DAD, four standard calibration curves were made using three commercial reference compounds: 3,4-dihydroxyphenyl acetic acid (3,4-DHPAA), LUT, and API. EA and its dialdehydic form lacking a carboxymethyl group (EDA) were quantified using the calibration curve of 3,4-DHPAA at 240 nm (equation: $y = 11472x - 61846$; $r^2 = 0.999$); lignans, phenylethyl alcohols, and secoiridoids were quantified using the curve of 3,4-DHPAA at 280 nm (equation: $y = 14747x - 74555$; $r^2 = 0.999$); LUT and API were quantified with their respective calibration curves at 330 nm (for LUT, equation: $y = 57.22\,x$; $r^2 = 0.988$; for API, equation: $y = 108.18x$; $r^2 = 0.995$). All calibration curves show good linearity in the studied concentration range. The phenol content was expressed as milligrams of 3,4-DHPAA per kilogram of oil, except for LUT, which is expressed in milligrams of luteolin per kilogram of oil, and API, which is expressed in milligrams of apigenin per kilogram of oil.

Determination of Phenolic Compounds by HPLC-ESI-TOF-MS. Acidified water (0.5% acetic acid, v/v) and acetonitrile were used as mobile phases A and B, respectively. The mobile phase was programmed as follows: gradient from 5 to 30% B in 10 min, from 30 to 33% in 2 min, from 33 to 38% in 5 min, from 38 to 50% in 3 min, from 50 to 95% in 3 min, and from 95 to 5% in 2 min; an 8 min re-equilibration time was used after each analysis. The flow rate used was set at 0.80 mL min^{-1} throughout the gradient. The effluent from the HPLC column was split using a T-type phase separator before being introduced into the mass spectrometer (split ratio = 1:3). Thus, in this study the flow that arrived at the ESI-TOF-MS detector was 0.2 mL/min. The column temperature was maintained at 25 °C, and the injection volume was 10 μL.

The accurate mass data of the molecular ions were processed using the latest version of the Data Analysis 4.0 software (Bruker Daltonics, Bremen, Germany), which provided a list of possible elemental formulas by using the Smart Formula Editor. The Editor uses a CHNO algorithm, which provides standard functionalities such as minimum/maximum elemental range, electron configuration, and ring-plus double bonds equivalents, as well as a sophisticated comparison of the theoretical with the measured isotope pattern (sigma value) for increased confidence in the suggested molecular formula. The widely accepted accuracy threshold for confirmation of elemental compositions was established at 5 ppm.

External calibration was performed using sodium formate cluster by switching the sheath liquid to a solution containing 5 mM NaOH in the sheath liquid of 0.2% formic acid in water/isopropanol (1:1, v/v). Due to the compensation of temperature drift in the TOF, this external calibration provided accurate mass values (> 5 ppm) for a complete run without the need for a dual sprayer setup for internal mass calibration.

Determination of Phenolic Compounds by NMR Spectroscopy. For NMR spectroscopy, the phenolic extracts were redissolved in 750 μL of DMSO-d_6 and placed in 5 mm NMR tubes. After NMR spectroscopy, the same extracts were also analyzed by HPLC-DAD/MSD, for a comparative study. 1D and 2D NMR spectra of the phenolic fraction obtained as described above were recorded at 30 °C on a high-resolution spectrometer Varian Mercury Plus 400 MHz. The spectra were taken with

a 90° pulse angle of 6.3 μs at a power of 55 db, 10 s recovery delay, and 256−512 scans. All spectra were recorded without spinning the samples to avoid quantification problems due to the overlap of signals and spinning side bands. Heteronuclear multiple bond correlation (HMBC) spectra were recorded with the sequence provided by the Varian library of experiments, using standard parameters ($^1J_{CH}$ = 140 Hz and $^{2,3}J_{CH}$ = 8 Hz). NMR signals were assigned by comparison with Christophoridou et al. (*21*) and Montedoro et al. (*22*) and confirmed by following the connectivities provided by HMBC spectra. 2D NMR analysis was necessary as previous analytical work was carried out in a mixture of H_2O/CH_3CN/TFA (*21*) or in $CDCl_3$ (*22*) and the chemical shifts may differ from those recorded in DMSO. Next, the most representative signals for two classes of phenolic compounds (i.e., monoaldehydic and dialdehydic compounds) were integrated with the routine "fitspec" of the Varian "Vnmrj" software, in order to quantify them. ^{1}H NMR spectra were normalized with respect to the peak solvent area according to the literature (*23*).

Statistical Analysis. Means and standard deviations were calculated with Statistica 6.0 (2001, Starsoft, Tulsa, OK) statistical software. Statistica was used to perform one-way analysis of variance, and Tukey's honest significant difference test at a 95% confidence level ($p < 0.05$) to identify differences among groups.

RESULTS AND DISCUSSION

Measurement of Antioxidant Activity of Phenolic Extract by ABTS$^{\bullet+}$ Assay. To the best of our knowledge, there is only very limited information about the effects of heat treatments by microwave and conventional ovens on the phenolic profile of EVOO (*13*−*15*, *18*, *19*). As shown in **Table 1**, the antioxidant activity evaluated by ABTS$^{\bullet+}$ test, a parameter closely related to the total phenol content, was higher for VOO1 samples than VOO2 samples. Correspondingly, the total phenol amount was higher as well, according to Bendini et al. (*24*), in which EVOO samples (EVTQ, EVMW, EVCO) correspond to VOO1 samples (VOO1TQ, VOO1MW, VOO1CO, respectively), whereas DEO samples (DEOTQ, DEOMW, DEOCO) correspond to VOO2 samples (VOO2TQ, VOO2MW, VOO12CO, respectively). Their admixtures (70−30 and 50−50) showed a value of antioxidant activity near the mean value between the two whole samples (**Table 1**).

Table 1. Antioxidant Activity (ABTS) in Analyzed Samples before and after Thermal Treatment by Microwave (MW) or Conventional Oven (CO)[a]

	ABTS (mmol of Trolox kg^{-1} of oil)	
	mean	SD
VOO1TQ	0.935 a	0.032
VOO1MW	0.813 b	0.003
VOO1CO	0.793 bc	0.039
70−30TQ	0.749 c	0.023
70−30MW	0.684 d	0.015
70−30CO	0.659 d	0.017
50−50TQ	0.403 e	0.008
50−50MW	0.193 fg	0.014
50−50CO	0.162 g	0.019
VOO2TQ	0.222 f	0.006
VOO2MW	0.186 fg	0.006
VOO2CO	0.172 fg	0.006

[a] Data are expressed as mean of three determinations and standard deviation. The same letters within each column do not significantly differ ($p < 0.05$).

After thermal treatments, the total phenol content, determined with Folin−Ciocalteu reagent, significantly decreased ((*24*), the agreement of the codes of the samples is reported above), as reported in previous works (*13*−*15*, *25*). Upon heating, the same trend was seen for antioxidant activity, evaluated by ABTS$^{\bullet+}$ test. No significant variations were observed for the antioxidant activity between the two types of heating systems (**Table 1**).

Chromatographic Analysis of Phenolic Compounds by HPLC. On the basis of the study of UV and MSD spectra of phenolics carried out by HPLC-DAD/MSD, 12 different compounds were identified: 3,4-DHPEA, EDA, *p*-HPEA, EA, LUT, 3,4-DHPEA-EDA, PIN, AcPIN, *p*-HPEA-EDA, API, 3,4-DHPEA-EA, and *p*-HPEA-EA. The presence of these phenolic compounds was confirmed by micro-TOF-MS analysis, as reported in **Table 2**. A partial overlapping of (+)-1-acetoxypinoresinol (AcPin) and *p*-HPEA-EDA was noted. As reported in **Table 3**, 3,4-DHPEA, 3,4-DHPEA-EA, *p*-HPEA-EA, and EA showed the highest decrease with thermal treatment. Molecules with an *o*-dihydroxy structure (namely, *o*-diphenols), such as 3,4-DHPEA and 3,4-DHPEA-EA, are mainly responsible for the oxidative resistance of EVOO (*26*) and characterized by higher antioxidant activities; the ability to react rapidly with lipid radicals can partially explain their losses upon heating. On the other hand, EA, which is devoid of a phenolic ring and exhibits a weak antiradical capacity (*27*), decreased upon thermal treatment. This effect may be due to chemical conversion from its monoaldehydic to its dialdehydic form lacking a carboxymethyl group. This type of reaction was also described for oleuropein by Montedoro et al. (*28*, *29*) and Limiroli et al. (*30*) during crushing and malaxation of olives, and

Table 2. Phenolic Compounds Determined by HPLC-ESI-TOF-MS and Reported in Order of Elution

		m/*z*						
phenolic compound	time (min)	exptl	calcd	tolerance (ppm)	error (ppm)	σ value	formula	classification order
3,4-dihydroxyphenylethanol (3,4-DHPEA)	6.48	153.0565	153.0557	5	−4.8	4.2	$C_8H_{10}O_3$	1st (1)
dialdehydic form of elenolic acid lacking a carboxymethyl group (EDA)	7.69	183.0665	183.0663	5	−1.3	13.1	$C_9H_{12}O_4$	1st (1)
p-hydroxyphenylethanol (*p*-HPEA)	8.17	137.0613	137.0608	5	−3.5	11.4	$C_8H_{10}O_2$	1st (1)
elenolic acid (EA)	13.64	241.0700	241.0718	10	7.5	10.1	$C_{11}H_{14}O_6$	1st (3)
luteolin (LUT)	15.97	285.0387	285.0405	10	6.2	10.4	$C_{15}H_{10}O_6$	1st (4)
3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid (3,4-DHPEA-EDA)	16.20	319.1186	319.1187	5	0.5	7.8	$C_{17}H_{20}O_6$	1st (2)
(+)-pinoresinol (PIN)	16.74	357.1353	357.1344	5	−2.6	19.0	$C_{20}H_{22}O_6$	1st (2)
(+)-1-acetoxypinoresinol (AcPIN)	17.37	415.1415	415.1398	10	−1.1	12.9	$C_{22}H_{24}O_8$	2nd (5)
p-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid (*p*-HPEA-EDA)	17.81	303.1242	303.1238	5	−1.5	25.9	$C_{17}H_{20}O_5$	1st (2)
apigenin (API)	18.71	269.0445	269.0455	5	4.1	2.3	$C_{15}H_{10}O_5$	1st (1)
3,4-dihydroxyphenylethanol linked to elenolic acid (3,4-DHPEA-EA)	20.90	377.1262	377.1242	10	−5.4	5.9	$C_{19}H_{22}O_8$	1st (5)
p-hydroxyphenylethanol linked to elenolic acid (*p*-HPEA-EA)	23.28	361.1293	361.1293	5	−0.1	8.8	$C_{19}H_{22}O_7$	1st (3)

Table 3. Quantification of Phenolic Compounds (HPLC-DAD) before and after Thermal Treatments by Conventional Oven (CO) or Microwave Oven (MW)[a]

	EDA	EA	3,4-DHPEA	*p*-HPEA	3,4-DHPEA-EDA	PIN	*p*-HPEA-EDA + AcPIN	*p*-HPEA-EDA	3,4-DHPEA-EA	*p*-HPEA-EA	LUT	API
VOO1TQ	22.6 d	302.2 a	8.7 a	6.3 a	42.6 b	13.9 a	29.8 a	2.0 d	36.8 a	17.6 a	2.1 a	0.5 a
VOO1MW	23.9 c	276.4 a	9.3 ab	4.9 a	32.5 c	12.9 bc	27.5 b	3.8 c	19.4 cd	7.7 c	2.4 ab	0.5 ab
variation %	5.9	−8.5	7.1	−21.4	-23.6	−7.0	−7.9	90.8	−47.3	−56.3	17.8	7.8
VOO1CO	66.5 a	137.9 cd	5.2 abc	5.6 a	48.1 a	14.8 a	28.5 ab	7.9 a	12.5 e	5.5 cd	2.1 a	0.5 a
variation %	195.0	−54.4	−40.2	−10.9	12.9	6.8	−4.5	302.2	−66.0	−68.6	3.7	1.3
70−30TQ	19.8 cdef	198.3 b	8.7 ab	7.4 a	31.1 cd	10.6 de	21.4 c	2.0 d	25.9 b	10.9 b	1.3 ab	0.3 ab
70−30MW	17.7 cdef	176.1 bc	7.0 abc	7.0 a	22.3 e	10.3 de	18.5 de	2.2 d	19.3 cd	7.8 c	1.0 ab	0.3 abcd
variation %	−10.5	−11.2	−19.2	−4.7	−28.3	−2.3	−13.5	11.1	−25.6	−28.0	−19.6	−19.5
70−30CO	42.1 b	122.4 de	6.1 abcd	7.4 a	28.9 cd	11.1 cd	20.9 cd	4.5 cb	11.2 ef	6.8 cd	0.9 abc	0.3 abcd
variation %	113.0	−38.3	−30.3	−0.3	−7.3	4.7	−2.2	129.1	−56.9	−37.7	−29.8	−19.0
50−50TQ	21.1 cdef	171.4 bcd	8.3 ab	7.5 a	26.7 de	10.2 de	17.6 ef	2.3 d	24.3 b	10.4 b	1.2 abc	0.3 abc
50−50MW	14.5 ef	127.6 d	5.5 bcd	7.1 a	12.9 f	8.7 e	13.2 g	2.8 d	13.4 e	7.4 cd	0.8 bc	0.2 bcde
variation %	−31.1	−25.6	−34.5	−5.2	−51.5	−15.2	−24.9	21.6	−44.8	−28.2	−34.6	−28.9
50−50CO	44.8 b	65.4 f	3.8 cd	6.9 a	14.8 f	9.0 de	15.6 fg	4.8 bc	7.2 fg	5.8 cd	0.7 bc	0.3 bcde
variation %	112.2	−61.9	−54.5	−7.4	−44.5	−12.1	−11.6	110.6	−70.6	−43.9	−37.4	−22.8
VOO2TQ	16.4 def	74.1 ef	7.5 ab	7.9 a	6.3 g	5.3 f	4.6 h	2.1 d	14.1 de	7.8 c	0.3 c	0.1 de
VOO2MW	14.7 f	67.9 f	4.0 cd	8.4 a	4.5 g	4.7 f	4.1 h	2.2 d	8.0 g	7.3 cd	0.2 c	0.1 e
variation %	−10.4	−8.4	−47.3	5.6	−28.2	−11.1	−10.5	8.3	−43.7	−6.9	−28.2	−19.7
VOO2CO	22.6 cd	63.2 f	2.5 d	7.6 a	4.6 g	6.1 f	4.8 h	2.8 d	5.1 g	6.8 cd	0.2 c	0.1 cde
variation %	38.2	−14.7	−66.2	−4.5	−26.9	15.2	3.6	34.4	−63.6	−13.1	−35.3	0.0

[a] Expressed as mg kg^{-1} of oil. Data are related to phenolic extracts, redissolved in 500 μL of methanol/water (1:1, v/v). The abbreviations correspond to those in **Table 2**.

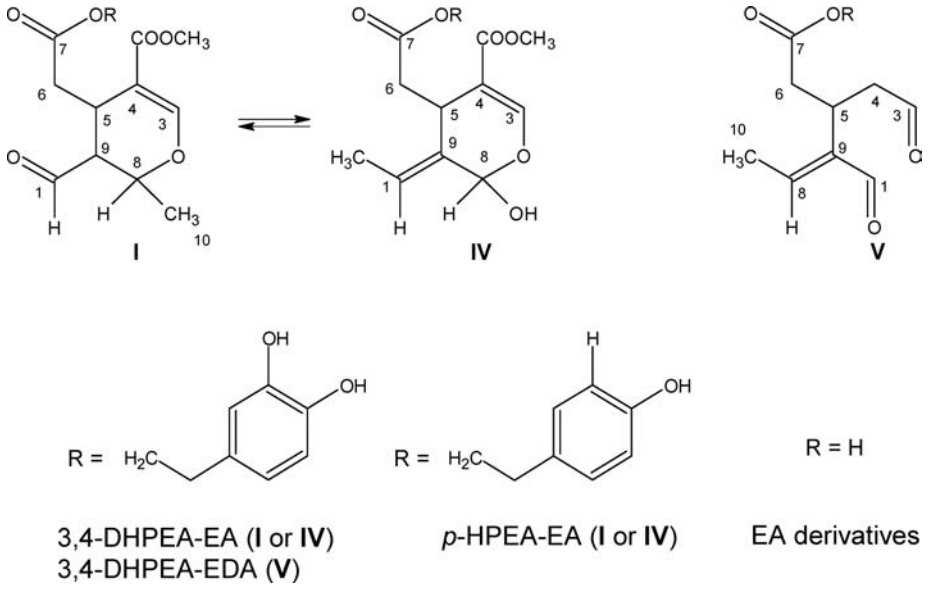

Figure 1. Type **I**, **IV**, and **V** structures. R = 2-(3,4-dihydroxyphenyl)ethyl, 2-(4-hydroxyphenyl)ethyl or H in 3,4-DHPEA-EA and 3,4-DHPEA-EDA, *p*-HPEA-EA, and EA derivatives, respectively.

it can be extended to EA, *p*-HPEA-EA, and 3,4-DHPEA-EA, which share the chemical moieties shown in **Figure 1**, distinguishing one from the other by a side group. EDA and *p*-HPEA-EDA were the only two compounds that clearly increased with heating, in particular by conventional oven (**Table 3**): thermal treatment by conventional oven led to a more intense oxidative and hydrolytic degradation of samples than microwave heating (*24*) and thus speeds the reaction pathway from monoaldehydic to dialdehydic forms of phenolic compounds. **Figure 2** shows the ratio between EA and EDA in all samples: the lowest values were reported after heating by conventional oven, suggesting a considerable decrease of the monoaldehydic structure and a concomitant increase of the dialdehydic form, both supported by strong thermal treatments. In fact, EDA and *p*-HPEA-EDA may originate from their respective monoaldehydic compounds (**Figure 3**). As seen in **Figure 3**, starting from a monoaldehydic structure (**I**), a retro-Michael equilibrium was proposed for structure **II**; the latter, via a keto−enol tautomerism, gives the dialdehydic form **III** that eventually yields **V** through loss of a carboxymethyl group, probably because of heating (*18*, *27*, *29*, *30*).

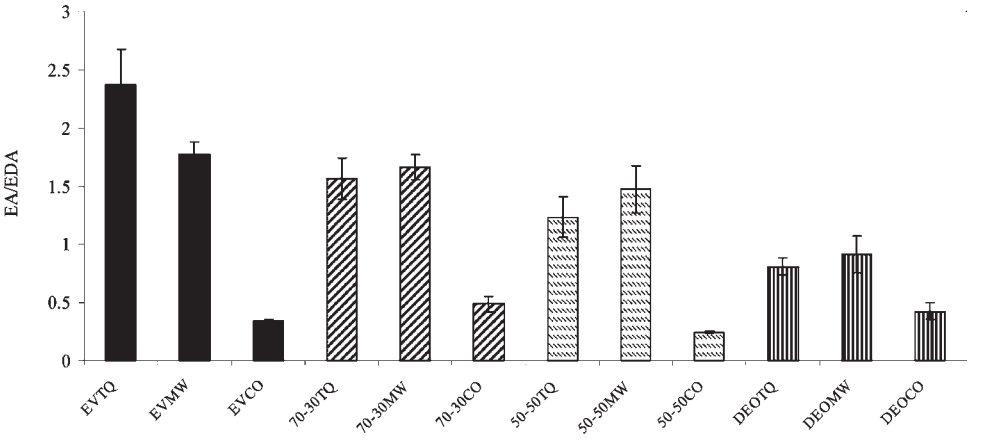

Figure 2. Ratio between EA and EDA in the samples. Data are referred to **Table 3**.

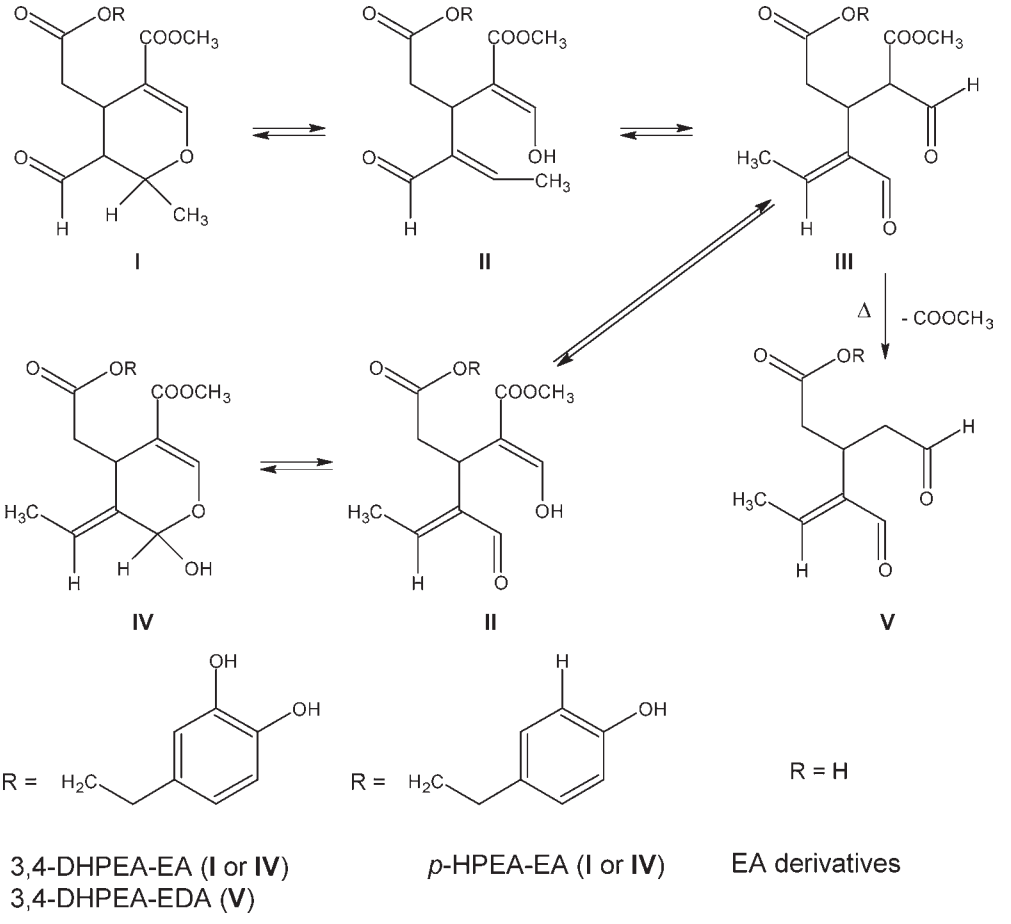

Figure 3. Chemical transformation induced by heating in type **I** and **IV** compounds to produce type **V** dialdehydic compounds.

This dialdehydic structure (**V**) may also originate from the corresponding aglycon **IV**, by a simple hemiacetal ring opening (**II**), followed by keto−enol tautomerism (**III**) and $-COOCH_3$ loss (**V**). It is worth noting that these chemical conversions do not modify the antiradical activity of 3,4-DHPEA-EDA because the catecholic ring of this molecule is unaffected by the reactions. No significant variations were seen for (+)-pinoresinol (PIN) or AcPIN upon heating (**Table 3**): in fact, lignans show a weaker antioxidant capacity in comparison with other phenolic compounds, and as suggested by several investigations (*14*, *18*), they are among the most stable compounds to thermal treatments. Finally, flavonoids (LUT, API) and *p*-HPEA also showed no significant variations after heating (**Table 3**).

Correlation between HPLC and NMR Analyses. To confirm the results for the variation of mono- and dialdehydic phenolic compounds with treatment (CO or MW), a comparison between the data obtained by HPLC and ^1H NMR spectroscopy was made for the VOO1 sample. In fact, provided that a spectrum has been recorded under proper conditions, the integrated areas of the NMR peaks are directly proportional to the relative concentrations of the compounds that yield the signals, and no calibration curve is necessary. A good correlation between NMR and HPLC results, therefore, helps strengthen the validity of the HPLC analysis if quantification of the analytes is obtained using a common calibration curve, as was done in our case, due to lack of commercial standards. Although it is true that the use of a

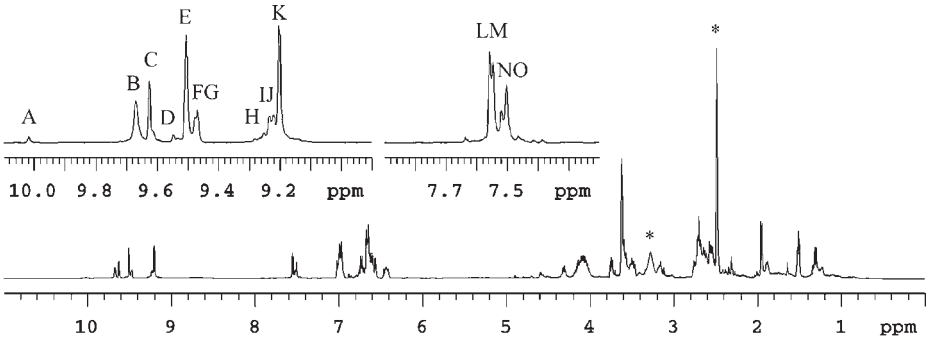

Figure 4. ^{1}H NMR spectrum of the phenolic fraction of a virgin olive oil (VOO1TQ). Close-up views of the aldehydic and enolethers regions are also shown.

Table 4. HPLC-DAD Quantification of Phenolic Compounds before (VOO1TQ) and after Thermal Treatments by Conventional Oven (VOO1CO) or Microwave Oven (VOO1MW)[A]

	VOO1TQ	VOO1MW	VOO1CO
EDA	24.4	31.5	43.4
EA	426.6	260	110
3,4-DHPEA	25.3	8.9	17.5
p-HPEA	16	8.6	12.9
3,4-DHPEA-EDA	51.5	51.9	34.1
PIN	17.1	19.6	15
p-HPEA-EDA + AcPIN	53.7	60.3	46.8
3,4-DHPEA-EA	45.3	28.1	10.5
p-HPEA-EA	19	5.2	2.3
LUT	3.8	3.8	2.1
API	0.8	0.8	0.5

[A] Expressed as mg g^{-1} of phenolic extract. These data are related to phenolic extracts redissolved in 750 μL of deuterated dimethyl sulfoxide (DMSO-d_6). The abbreviations correspond to those in **Table 2**.

common calibration curve does not impair the measure of the percentage variation of a *single* analyte along a series of experiments (it just multiplies the true concentration by an unknown coefficient that disappears in the calculation), it may give wrong results if percentage variations of classes of compounds (e.g., mono- vs dialdehydic) are to be measured (each true concentration, that is, summed to yield the total concentration of the class of compounds, is multiplied by a different unknown coefficient that does not disappear in the percentage calculation).

As the low sensitivity of NMR spectroscopy requires the use of concentrated solutions, the phenolic extracts were dissolved in DMSO-d_6, and the NMR results were compared to HPLC data obtained by specific experiments in the same solvent. **Table 4** shows the amount of phenolic compounds, determined by HPLC-DAD/MSD in DMSO-d_6 phenolic extracts of VOO1 samples, before and after the thermal treatments by microwave or conventional oven. EA was the most abundant compound, and in DMSO-d_6 extracts the increase in EDA with heating (especially by conventional oven) was quite evident.

The ^{1}H NMR spectrum of the phenolic fraction in DMSO-d_6 is quite complex (**Figure 4**), and the lines are broad, due to the superimpositions of signals from many different compounds. Due to high similarity of chemical shifts (*21*, *22*), it was almost impossible to discriminate among all of the signals of monoaldehydic (EA, 3,4-DHPEA-EA, and *p*-HPEA-EA) and dialdehydic compounds (EDA, 3,4-DHPEA-EDA, and *p*-HPEA-EDA) by NMR spectroscopy at 400 MHz. When assignment of a signal to a specific mono- or dialdehydic compound was not possible, the assignment was considered to a general "structure of type **I**" or "structure of type **V**" (**Figure 1**). Once identified, percentage variations of selected signals of these structures upon treatment will be compared to percentage variations of the pooled concentrations of the compounds in each class obtained by HPLC-DAD.

Two different parts of the ^{1}H NMR spectrum were considered for quantification (**Figure 4**): the aldehydic region (9−10 ppm), where 11 aldehydic signals appear, namely, A−K, which give information about the amount of compounds bearing one or two aldehyde groups and the enolethers region (around 7.5 ppm), which includes 4 main signals (L−O), typical of compounds containing the enolether moiety, such as the one present in compounds sharing structures such as **I** or **IV**.

A good starting point for the analysis of the HMBC spectrum is the group of signals around 7.55 ppm, typical of the enolether moiety (H-3 in **Figure 1**). This proton is connected to five carbons at 166.8, 106.8, 69.9, 27.2, and 156.0 ppm (peaks a, b, c, d, and e in **Figure 5B**) that can be assigned (*22*) to the carboxyl group of methyl ester (COOMe), C-4, C-8, C-5, and C-3, respectively (the latter via $^1J_{CH}$, yielding the doublet). It is worth noting that structures **I** and **IV** have different chemical shifts at C-8 (respectively, 70 and 95 ppm (*31*)). As no signals are present in the HMBC spectra around 95 ppm, it must be concluded that compounds containing structure **IV** were not present in the analyzed samples. Very similar connectivities were detected starting from a minor couple of enolether signals centered at about 7.50 ppm (peaks a′, b′, c′, d′, and e′ in **Figure 5B**), indicating presence of compounds containing the *R* epimer at C-8 of structure **I**, the chemical shift at H-3 of which is generally lower than in the *S* epimer (*21*).

To assign the aldehydic signals to aldehydic protons in compounds containing structure **I**, it is useful to start from the HMBC signal of C-5 of the major isomer (the one with *S* configuration at C-8) at 27.3 ppm, which is HMBC connected to two aldehydic protons at 9.68 and 9.62 ppm (peaks a and b in **Figure 5A**, corresponding to peaks B and C in **Figure 4**). These can be assigned (*21*) to the aldehydic protons of the *S* epimers of EA (peak B) and to the sum of the *S* epimers of 3,4-DHPEA-EA and *p*-HPEA-EA (peak C). Similarly, the C-5 of the *R* epimer at 26.1 ppm is connected to two aldehydic protons at 9.61 and 9.48 (peaks g and h in **Figure 5A**), which are assigned to the aldehydic protons of the *R* epimer of EA (peak C in **Figure 4**) and to the sum of the *R* epimers of 3,4-DHPEA-EA and *p*-HPEA-EA (peak F in **Figure 4**). The four assignments are corroborated by the presence of HMBC peaks between each of the aldehydic protons and their geminal carbons at C-9 (peaks c, d, i, and l in **Figure 5A**) with $^2J_{CH} \approx 23$ Hz (*32*) and by the presence of doublets due to residual $^1J_{CH}$ (peaks e, f, m, and n in **Figure 5A**). The nearly complete assignment of structure **I** in DMSO-d_6 is reported in **Table 5**.

The assignments of the signals of type **V** structure can be started by noting that H-5 is the only proton that can couple with two aldehydic carbons via HMBC; actually, the only proton

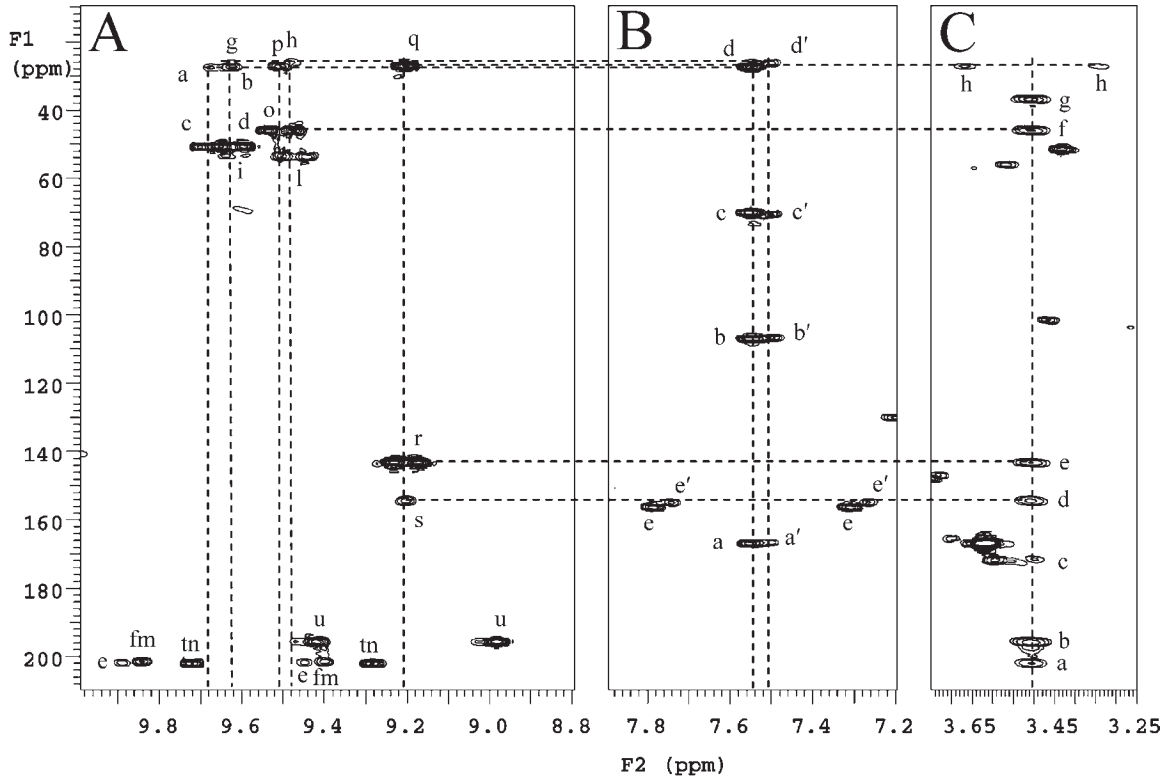

Figure 5. Portions of the HMBC 2D spectrum of the phenolic fraction of a VOO showing the relevant connections for the assignment of monoaldehydic and dialdehydic structures.

Table 5. NMR Assignments of Monoaldehydic Type **I** Structure (**Figure 1**) Contained in *p*-HPEA-EA, 3,4-DHPEA-EA, and EA

	chemical shift ^{1}H (^{13}C)	
atom no.	8*S* epimer	8*R* epimer
1	9.68[a] (201.7[a]) 9.62[b] (201.4[b])	9.61[a] (201.4[a]) 9.48[b] (201.5[b])
3	7.55 (156.0)	7.50 (154.5)
4	− (106.8)	− (106.6)
5	3.18 (27.2)	3.26 (26.1)
6	2.57, 2.64 (34.0[c])	2.50, nd[d] (36.7[c])
7	− (171.3)	− (nd)
8	4.31 (69.9)	4.57 (70.4)
9	2.64 (50.4)	2.61 (53.5)
10	1.51 (17.7)	1.30 (19.1)
$COOCH_3$	− (166.8)	− (166.4)
$COOCH_3$	3.62 (51.2)	3.57[c] (51.2[c])

[a] Values assigned to EA. [b] Values assigned to *p*-HPEA-EA and 3,4-DHPEA-EA. [c] Tentative assignment. [d] Not detected.

displaying the required connectivities is at 3.51 ppm, as it couples with two carbonyls at 201.8 and 195.6 ppm (peaks a and b in **Figure 5C**), corresponding via residual $^1J_{CH}$ doublets (peaks t and u, **Figure 5A**) to two aldehydic protons at 9.51 and 9.20 ppm (respectively, peaks E and K in **Figure 4**). Proton H-5 is also long-range connected to another five carbons at 171.7, 154.2, 143.2, 45.7, and 36.9 (peaks c−g in **Figure 4C**), assigned, respectively, to C-7, C-8, C-9, C-4, and C-6 both by comparison with the chemical shifts in $CDCl_3$ (*21*) and by following the key HMBC connectivities. In particular, the two olefinic carbons at 143.2 and 154.2 ppm are both coupled to the aldehydic protons at 9.20 ppm (respectively, peaks r and s in **Figure 5A**), only the first one showing a large $^2J_{CH} \approx 24$ Hz typical for a carbon geminal to a aldehydic proton (*32*). For the same reason, the carbon at 45.7 is assigned to C-4 (peak o in **Figure 5A**). Finally, C-5 (at 26.9 ppm) is HMBC

Table 6. NMR Assignments of Dialdehydic Type **V** Structures (**Figure 1**) Contained in *p*-HPEA-EDA, 3,4-DHPEA-EDA, and EDA

atom no.	chemical shift ^{1}H (^{13}C)
1	9.20 (195.6)
3	9.51 (201.8)
4	2.70, 2.70 (45.7)
5	3.51 (26.9)
6	2.53, 2.59 (36.6)
7	− (171.7)
8	6.74 (154.2)
9	− (143.2)
10	1.97 (14.9)

Table 7. Variations (Percent) of Monoaldehydic and Dialdehydic Forms of Phenolic Compounds after Thermal Treatment by Conventional (VOO1CO) or Microwave Oven (VOO1MW), Measured by NMR[a] and by HPLC-DAD/MSD [b]

	monoaldehydic forms			dialdehydic forms	
sample	B+C+F	L+M+N+O	HPLC	E+K	HPLC
VOO1CO	−69	−70	−75	−27	−4
VOO1MW	−57	−33	−40	14	11

[a] B+C+F corresponds to monoaldehydic signals; L+M+N+O corresponds to enolethers signals; E+K corresponds to dialdehydic signals. [b] Variations are calculated with respect to the untreated VOO1 (VOO1TQ).

connected with both aldehydic protons (peaks p and q in **Figure 5A**). The complete assignment is reported in **Table 6**.

From the assignments of the aldehydic (B+C+F) and enolether protons (L+M+N+O) to monoaldehydic compounds containing a structure of type **I** and aldehydic protons (E+K) to dialdehydic compounds containing a structure of type **V**, it is now possible to study the effect of microwave or conventional heating on aldehydic compounds by NMR spectroscopy and compare the results with the HPLC data (**Table 7**).

It is interesting to observe that the NMR results for the monoaldehydic compounds agree with HPLC data, although better agreement is found for CO than for MW treatment; however, even for the latter treatment, the HPLC value is close to the average variation measured by NMR spectroscopy (−40.3 vs −45%). Good agreement is also found for the variation of dialdehydic compound upon MW treatment, whereas the results for CO heating, while preserving the sign of the variation, are quite different. It is possible that this disagreement depends on the fact that *p*-HPEA-EDA and AcPIN coelute in a HPLC trace (see **Table 4**) so that the HPLC value may be biased.

In conclusion, an increase in dialdehydic forms of phenolic compounds (EDA, *p*-HPEA-EDA) has been observed after microwave and conventional heat treatments: NMR spectroscopy was able to confirm these results. It is likely that reasonable chemical conversions from EA, *p*-HPEA-EA, and 3,4-DHPEA-EA to their respective dialdehydic forms (EDA, *p*-HPEA-EDA, and 3,4-DHPEA-EDA) were induced by heating.

ABBREVIATIONS USED

1D NMR, monodimensional nuclear magnetic resonance; 2D NMR, bidimensional nuclear magnetic resonance; 3,4-DHPAA, 3,4-dihydroxyphenylacetic acid; 3,4-DHPEA, 3,4-dihydroxyphenylethanol; 3,4-DHPEA-EA, 3,4-dihydroxyphenylethanol linked to elenolic acid; 3,4-DHPEA-EDA, 3,4-dihydroxyphenylethanol linked to the dialdehydic form of elenolic acid; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline)-6-sulfonic acid, diammonium salt; API, apigenin; CO, conventional; COSY, correlation spectroscopy; DAD, diode array UV−vis detector; DMSO-d_6, deuterated dimethyl sulfoxide; EA, elenolic acid; EDA, dialdehydic form of elenolic acid lacking a carboxymethyl group; ESI, electrospray ionization; EVOO, extra virgin olive oil; HMBC, heteronuclear multiple bond correlation; HPLC, high-performance liquid chromatography; LLE, liquid−liquid extraction; LUT, luteolin; MSD, mass spectrometer detector; MW, microwave; *p*-HPEA, *p*-hydroxyphenylethanol; *p*-HPEA-EA, *p*-hydroxyphenylethanol linked to elenolic acid; *p*-HPEA-EDA, *p*-hydroxyphenylethanol linked to the dialdehydic form of elenolic acid; TOF, time of flight; TP, total phenol; VOO, virgin olive oil.

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