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Evaluation of the Influence of Thermal Oxidation on the Phenolic Composition and on the Antioxidant Activity of Extra-Virgin Olive Oils

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A comparison between the results obtained by using HPLC−UV, HPLC−MS, and CE−UV for characterizing the deterioration of extra-virgin olive oil during heating (180 °C) was investigated, taking into account phenolic compounds. The concentration of several compounds belonging to four families of phenols (simple phenols, lignans, complex phenols, and phenolic acids) was determined in the samples after the thermal treatment by all three techniques. Hydroxytyrosol, elenolic acid, decarboxymethyl oleuropein aglycon, and oleuropein aglycon reduced their concentration with the thermal treatment more quickly than other phenolic compounds present in olive oil. HYTY-Ac and Lig Agl were demonstrated to be quite resistant to this kind of treatment, and the behavior of lignans could be outstanding, as they belong to the family most resistant to thermal treatment. Several "unknown" compounds were determined in the phenolic profiles of the oils after the thermal treatment, and their presence was confirmed in refined olive oils. The oxidative stability index (OSI time) was reduced from 25 to 5 h after 3 h of heating, whereas the peroxide value showed a minimum after 1 h of heating.

KEYWORDS: Extra-virgin olive oil; HPLC; CE; phenols; heating degradation

INTRODUCTION

Virgin olive oil contains many minor compounds with a great importance, among which polyphenols stand out (*1*). These phenolic compounds have influence in the sensory quality (*2*, *3*), the beneficial biological activity (*1*, *4*, *5*), and the oxidative stability of olive oil (*6*, *7*). The major phenolic compounds identified and quantified in olive oil belong to five different classes: simple phenols (hydroxytyrosol, tyrosol), secoiridoids (oleuropein, ligstroside, and their hydrolysis derivatives), lignans ((+)-pinoresinol and (+)-acetoxypinoresinol), flavonoids (luteolin and apigenin), and phenolic acids (*p*-coumaric acid, vanillic acid, etc.) (*1*).

Virgin olive oil may be consumed raw as an ingredient in toast, salads, and other foodstuffs, but it is also consumed after domestic heating, such as frying, boiling, and microwave heating (*8*, *9*). Virgin olive oil is considered to be excellent for applications involving high temperatures, as it fulfills all the fatty acid criteria of the stable healthful frying oils, i.e., being rich in monounsaturated fatty acids, being low in saturated and polyunsaturated fatty acids, being very low in linolenic acid, and containing practically no *trans* fatty acids (*10*). Moreover, olive oil is considered to be a premium frying oil with added advantages linked to its relatively low melting point. That means that it drains from the dried food easily, leading to a low content of oil in the fried food (*11*).

During the thermal treatments, due to the high temperature and the absorption of oxygen and water, triacylglycerols in the oil suffer a series of reactions, namely hydrolysis, oxidation, isomerization, and polymerization (*12*). The thermal treatments are influenced by a large number of variables, among which the type of process, i.e., continuous or discontinuous, the surface-to-oil volume ratio, the food (in the case that there is some food in contact with the oil), the addition of fresh oil or not, the temperature, and the oil selected are of particular relevance (*13*). Due to the difficulties found in defining and/or controlling such variables and the additional strong interactions between them (*14*), it is not easy to replicate results from different laboratories.

The analytical methods used to evaluate fat degradation, i.e., carbonyl value (*15*) iodine value (*16*), viscosity (*16*), volatiles (*17*), room odor and flavor scores (*16*), triacylglycerol oxidation (*18*), polar compounds (*14*), peroxide value (*19*), and others, may also have contributed significantly to the variability found

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Table 1. Analytical Parameters of the HPLC Method

analyte	RSD[a] (%) (intermediate value)	detection limit (DL) (μg mL^{-1})	quantification limit (QL) (μg mL^{-1})	calibration range[b] (μg mL^{-1})	calibration equation	r^2
hydroxytyrosol	3.24	0.19	0.63	1500	$y = 25.76x - 200.67$	0.997
tyrosol	3.02	0.40	1.33	2000	$y = 12.11x - 43.92$	0.996
dopac	3.78	0.23	0.77	500	$y = 21.29x - 16.55$	0.997
oleuropein glucoside, $\lambda = 280$ nm	3.22	1.08	3.60	10000	$y = 4.45x + 15.93$	0.997
oleuropein glucoside, $\lambda = 240$ nm	4.21	0.29	0.97	10000	$y = 16.44x + 1587.60$	0.998

[a] RSD = relative standard deviation. [b] From QL to the value in the table.

in the results. These results would suggest interactions between the variables of the thermal process and dependence on the analytical method applied.

Even though many researches have studied polyphenols in raw and stored virgin olive oils (*1*, *6*, *7*, *20*), their influence on the stability and organoleptic properties of the oil (*2*, *3*), and nutritional benefits (*21*), there are not many data that report the availability of these compounds after domestic heating of the oil (*8*, *22*, *23*, *24*, *25*). In these cases, the phenolic compounds have been determined colorimetrically (*22*, *23*) and also by using HPLC (*8*, *24*, *25*, *26*). In these previously mentioned research works, different quantities of olive oil have been subjected to very different thermal treatments in various recipients inside distinct fryers or ovens (frying operation with potatoes (*25*); frying temperatures (160−190 °C) and heating times typical of domestic frying conditions (0.5−2 h) (*23*); thermal oxidation at 60 and 100 °C in an oven during a long time (*26*); twelve frying operations at 180 °C with potatos sliced into pieces (*24*); thermal oxidation of oils at 180 °C in a Rancimat apparatus, boiling a mixture of virgin olive oil and water in a pressure cooker for 30 min, and microwave heating of oils for 10 min (*25*)) and for this reason it is very difficult to compare all the data, although it is true that several general considerations can be taken into account.

Capillary electrophoresis (CE) is another separative technique which offers to the analyst a number of key advantages for the analysis of the compounds of foods. CE offers better resolution than HPLC and is more adapted to the simultaneous separation of a number of components of different chemistries within a single matrix (*27*). CE has been used for the analysis of phenolic compounds in olive oil and related samples, and its results represent a good compromise between analysis time and characterization of some classes of phenols in virgin olive oil (*28*, *29*).

The aim of this work was to study the changes in the phenolic profile of extra-virgin olive oil during thermal oxidation at frying temperatures and during different periods of time by using two separative techniques (HPLC and CE). The concentration of several compounds belonging to four families of phenols (phenolic alcohols or simple phenols, phenolic acids, secoiridoids, and lignans) was determined in the samples after the thermal treatment by both techniques, as well as the concentration of several "unknown" compounds which appeared after the treatment. The peroxide value trend during heating was evaluated, and changes in the antioxidant capacity of each virgin olive oil after different time intervals of treatment were indirectly measured by the OSI test.

MATERIALS AND METHODS

Instruments. HPLC analyses were performed with a HP 1100 series (Agilent Technologies, Palo Alto, CA), equipped with a binary pump delivery system, a degasser, an autosampler, a diode array UV−vis detector (DAD), and a mass spectrometer detector (MSD).

The HPLC column used was a C_{18} Luna column, 5 μm, 25 cm × 3.0 mm (Phenomenex, Torrance, CA), with a C_{18} precolumn (Phenomenex) filter. The mobile phase flow rate was 0.5 mL min^{-1}.

The CE instrument used was a Beckman 5500 capillary electrophoresis unit connected to a diode array detector. The system comprises a 0−30 kV high-voltage, built-in power supply.

All capillaries (fused silica) used were obtained from Beckman Instruments, Inc. (Fullerton, CA) and had an inner diameter (i.d.) of 50 μm, a total length of 47 cm, and an effective separation length of 40 cm. Data acquisition and processing were carried out with GOLD software installed in a personal computer.

The oxidative stability of the samples was evaluated by the oxidative stability index (OSI), using an eight-channel oxidative stability instrument (Omnion, Decatur, IL).

Reagents, Stock Solutions, and Reference Compounds. 2-(4-Hydroxyphenyl)ethanol (tyrosol) was acquired from Fluka, dopac (3,4-dihydroxyphenylacetic acid) was acquired from Sigma Aldrich (St. Louis, MO), and oleuropein (oleuropein glucoside) and hydroxytyrosol were obtained from Extrasynthèse (Genay, France). The stock solutions containing these analytes were prepared in methanol/water (50/50, v/v) at a concentration of 2000 μg mL^{-1} for tyrosol, 500 μg mL^{-1} for dopac, 10000 μg mL^{-1} for oleuropein, and 1500 μg mL^{-1} for hydroxytyrosol. These compounds were used to create the calibration curves.

Methanol, acetonitrile, and *n*-hexane, HPLC-grade, were from Merck (Darmstadt, Germany).

Distilled water with a resistance of 18.2 MΩ was deionized by using a Milli-Q system (Millipore, Bedford, MA).

Sodium hydroxide was purchased from Merck (Darmstadt, Germany); sodium tetraborate (borax) was obtained from Sigma and was used as running buffer at different concentrations and pHs.

Samples. A Bertolli commercial extra-virgin olive oil sample was used for this study (from Bertolli, Unilever Bestfoods Italia S.p.A., Inveruno-MI, Italy). We also used a commercial refined olive oil (Sagra, SALOV, Lucca, Italy) and a crude refined olive oil after and before the bleaching phase (these samples were kindly provided by an Italian mill for making olive oil). Therefore, four olive oils were used in this study.

Refined olive oil is the olive oil obtained from virgin olive oils by refining methods which do not lead to alterations in the initial glyceridic structure. It has a free acidity, expressed as oleic acid, of not more than 0.3 g per 100 g of oil (0.3%), and its other characteristics correspond to those fixed for this category. On the other hand, the purpose of bleaching is not only to provide a lighter colored oil but also to purify it in preparation for further processing, since refined oil could contain traces of a number of undesirable impurities either in solution or as colloidal suspensions.

Liquid−Liquid Extraction (LLE) of Phenolic Compounds from Olive Oils. A LLE system was used to extract the phenolic compounds present in VOO. The LLE was carried out with the same extraction conditions and amounts of oil that are reported by Carrasco-Pancorbo et al. (*29*). Briefly, 60 g of oil (±0.001 g) were 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) solution. The combined extracts of the hydrophilic layer were brought to dryness in a rotary evaporator

Table 2. Analytical Parameters of the Electrophoretic Method[a,b]

analyte	RSD[d] (%) (intermediate value)	detection limit (DL) (μg mL^{-1})	quantification limit (QL) (μg mL^{-1})	calibration range[e] (μg mL^{-1})	calibration equation	r^2
tyrosol	2.03	1.14	3.80	1500	$y = (7 \times 10^{-6})x + 5 \times 10^{-6}$	0.994
oleuropein glucoside, $\lambda = 200$ nm[c]	2.51	0.10	0.33	10000	$y = (8 \times 10^{-5})x + 3 \times 10^{-4}$	0.997
oleuropein glucoside, $\lambda = 240$ nm[c]	2.04	0.40	1.33	10000	$y = (2 \times 10^{-5})x - 8 \times 10^{-5}$	0.995
hydroxytyrosol	2.34	0.40	1.33	500	$y = (2 \times 10^{-5})x + 8 \times 10^{-6}$	0.992
dopac[c]	1.67	0.04	0.13	2000	$y = (2 \times 10^{-4})x + 4.8 \times 10^{-3}$	0.997

[a] Data obtained using the software Microcal Origin, Microcal Software, Inc., USA. [b] $y = bx \pm a$, where y is the peak area (au), x is the concentration (μg/mL), a is the y intercept, and r^2 is the correlation coefficient. [c] $y = bx \pm a$, where y is the time corrected area counts [= peak area (au) × migration velocity (cm/min)]. [d] RSD = relative standard deviation. LD = $3\sigma_b/b$ ($\sigma_b = 2.66 \times 10^{-6}$, calculated using 100 data points). LQ = $10\sigma_b/b$. [e] From QL to the value in the table.

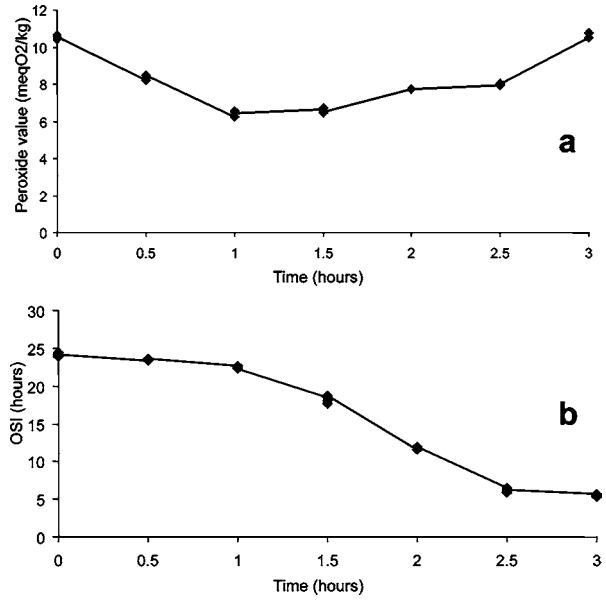

Figure 1. **(a)** Changes in the peroxide value after thermal treatment (180 °C) from 0 (without thermal treatment) to 3 h ($n = 5$). **(b)** Trend of the oxidation process evaluated by the determination of volatile secondary oxidation products by the OSI test.

under reduced pressure and a temperature of 40 °C. Finally, the residue was redissolved in 0.5 mL of methanol/water (50:50, v/v) and filtered through a 0.20 μm filter.

Thermal Treatment. We compared changes in the antioxidant capacity of olive oil and its phenolic composition, due to degradation of antioxidant compounds, after thermal treatment, simulating frying temperatures (180 °C) during different time intervals (30, 60, 90, 120, 150, and 180 min). The samples (100 mL) were poured into appropriate glass beakers (15 cm diameter), thus providing an excellent control of the surface-to-oil volume ratio. The samples were then removed from the oven, cooled to room temperature, and analyzed.

Peroxide Value. Evaluation of primary auto-oxidation products was carried out by the determination of the peroxide value (PV) according to the official method described in Regulation EEC 2568/91 (*19*). We measured this parameter after intervals of thermal treatment of 30 min.

Oxidative Stability of Oil Samples. The instrumental conditions were set following the analytical protocol described by Jebe et al. (*30*). To obtain the oxidative stability index, a stream of purified air (120 mL min^{-1} air flow rate) was passed through the sample (5 g) and the effluent air for the oil sample was then bubbled through a vessel containing deionized water. The effluent air contains volatile organic compounds that contain acids and other types of organic compounds formed during thermal degradation of the oils, which increased the conductivity of the water. The temperature at which this test was carried out was 110 °C. The OSI index (or OSI time) was expressed in hours ($n = 5$). The OSI time was controlled after intervals of thermal treatment of 30 min.

HPLC Analysis. The mobile phases were water with acetic acid (0.5%) (phase A) and acetonitrile (phase B), and the solvent gradient changed according to the conditions described by Carrasco-Pancorbo et al. (*7*).

The injection volume was 10 μL. The wavelengths were set at 240, 280, and 330 nm. The detection was made using MS as well, and the analyses were carried out using an electrospray (ESI) interface operating in positive mode using the following conditions: drying gas flow, 9.0 L min^{-1}; nebulizer pressure, 50 psi; gas drying temperature, 350 °C; capillary voltage, 3000 V; fragmentor voltage, 60 V; and also using an atmospheric pressure chemical ionization (APCI) interface operating in negative mode using the following conditions: drying gas flow, 9.0 L/min; nebulizer pressure, 30 psi; gas drying temperature, 350 °C; vaporizer temperature, 450 °C; capillary voltage, 3000 V; fragmentor voltage, 60 V. The polarity of the ESI and the APCI and all the parameters of the MS detector were optimized using the height of the MS signal for the phenolic compounds from the methanol/water extracts of extra-VOO as an analytical parameter.

Calibration Curves and Quantification with HPLC. To carry out the quantification with HPLC, five standard calibration curves were made using four commercial reference compounds: tyrosol, hydroxytyrosol, dopac (3,4-dihydroxyphenylacetic acid), and oleuropein (oleuropein glucoside). Tyrosol and hydroxytyrosol were quantified using the calibration curves of their corresponding commercial standards at 280 nm; other simple phenol (hydroxytyrosol-acetate) and phenolic acids were quantified using the calibration curve of dopac at 280 nm; (+)-pinoresinol, (+)-1-acetoxypinoresinol, and complex phenols or secoiridoid derivatives (decarboxymethyl oleuropein aglycon, oleuropein aglycon, and ligstroside aglycon) were quantified with the calibration curve of oleuropein obtained at 280 nm. However, the curve of oleuropein at 240 nm was used for the quantification of elenolic acid.

The "unknown" compounds which appeared after the thermal treatment in the phenolic profiles of the oil were quantified using the calibration curve of dopac at 280 nm. The quantification was carried out in all the cases using UV detection, but the MS detection was used as well in all the analyses to confirm the identity of the analytes.

The detection limit (DL), quantification limit (QL), and precision (as relative standard deviation (RSD) of the intermediate concentration value of the linear range) of this method were calculated for the studied analytes using the method proposed by Curie (*31*). Three replicates of each analyte at different concentrations were done in order to set up the calibration.

All calibration curves showed good linearity in the studied range of concentrations. All the features of the proposed method are summarized in **Table 1**.

Electrophoretic Procedure. The CE method was that previously described by Carrasco-Pancorbo et al. (*32*). CE separation was carried out on a fused silica capillary (50 μm i.d., 375 μm o.d., total length 47 cm; a detection window was created at 40 cm from the capillary inlet, by removing the polyimide coating and using a cartridge with a slide of 100 μm × 200 μm). Every time a new capillary was used, it was

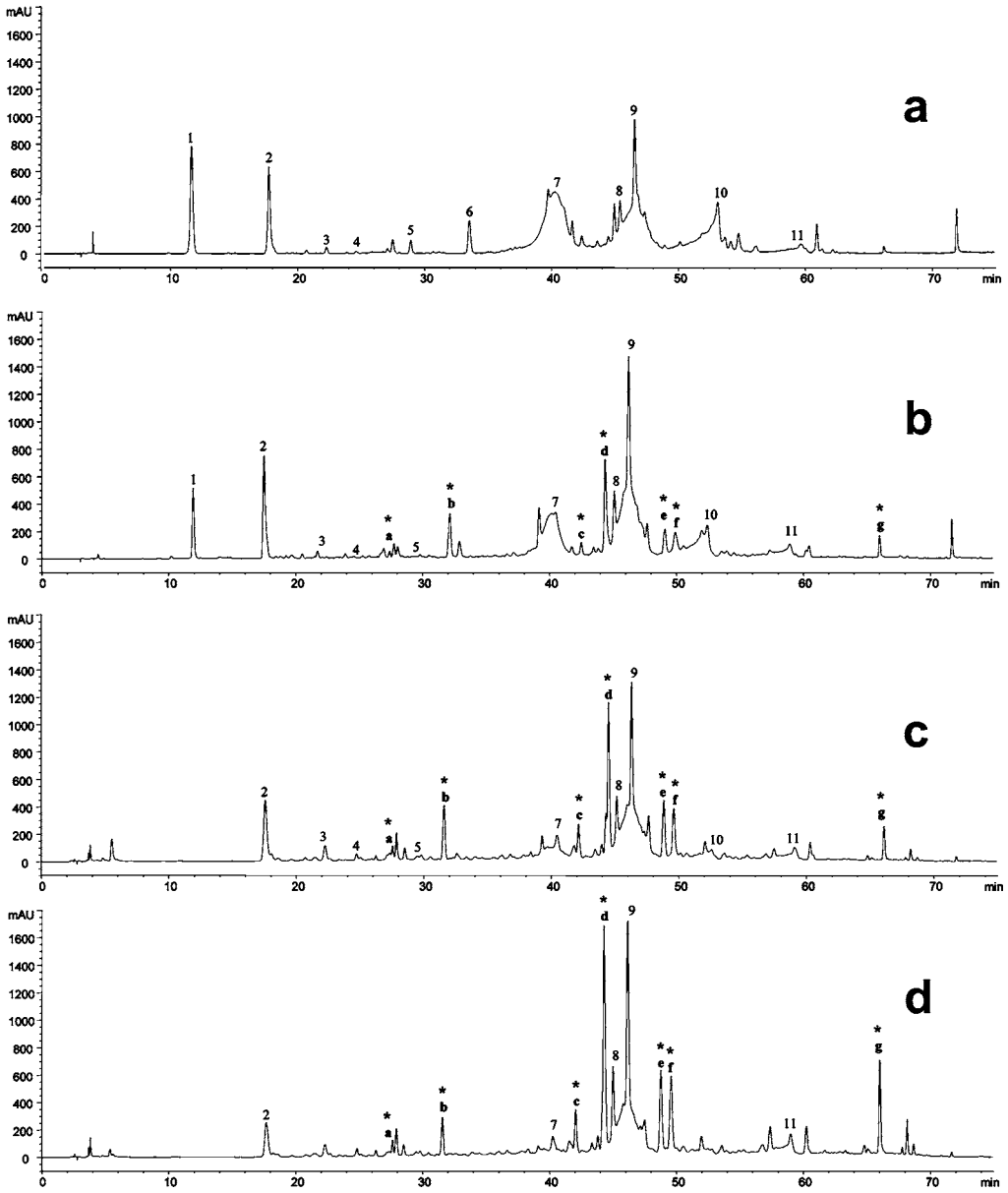

Figure 2. UV-chromatograms which show the evolution in the phenolic profile of Bertolli extra-VOO after thermal treatment at 180 °C: (**a**) Extra-virgin olive oil without treatment; (**b**) the same oil after 1 h at the mentioned temperature; (**c**) the same oil after 2 h at 180 °C; (**d**) the same oil after 3 h at the mentioned temperature. Peak identification numbers: **1**, hydroxytyrosol; **2**, tyrosol; **3**, gentisic acid; **4**, vanillic acid; **5**, sinapic acid; **6**, hydroxytyrosol acetate; **7**, decarboxymethyl-oleuropein aglycon; **8**, (+)-pinoresinol; **9**, 1-(+)-acetoxypinoresinol; **10**, oleuropein aglycon; **11**, ligstroside aglycon. Detection wavelength: 280 nm. For elenolic acid, detection was performed at 240 nm. (The "unknown compounds" that appeared after the heating are marked with an asterisk and a letter.)

preconditioned by rinsing with 0.5 M NaOH for 20 min, followed by a 5 min rinse with Milli-Q water and a 15 min rinse with buffer. For the following analyses, the capillary was rinsed with 0.1 M NaOH for 2 min, followed by a 2 min flush with Milli-Q water to ensure good repeatability. The capillary was equilibrated with the running buffer (45 mM sodium tetraborate adjusted to pH 9.3) for 3 min before each sample injection. After each analysis, the capillary tubing was rinsed for 2 min with Milli-Q water. All solutions and samples were filtered through a 0.25 μm syringe filter. The running buffer was changed after four runs.

Samples were injected hydrodynamically in the anodic end with a low-pressure mode (0.5 psi) for 3 s (1 psi = 6895 Pa). Electrophoretic separations were performed at 28 kV for 7 min, and the temperature was maintained at 22 °C. UV detection was performed in all cases at 200, 240, 280, and 330 nm.

Calibration Curves and Quantification in CE. Standard calibration graphs for lignans and complex phenols were prepared using oleuropein (oleuropein glucoside) at two wavelengths (200 and 240 nm). An external standard methodology (with oleuropein glucoside) was used for the quantification of lignans and complex phenols.

Using this methodology, time-corrected area counts are necessary for quantitative CE. Since separation is dependent on analytes migrating at different rates, the separated analytes will pass the detector at different rates. Time-corrected area counts are the product of the area counts and the velocity for each peak in the electropherogram.

Thus, peak areas and migration velocity (cm/min) were used to calculate the time-corrected area counts to compensate for the differences in migration velocities among oleuropein glucoside, lignans, and complex phenols.

Lig Agl (a), Pin, Ac Pin, Ol Agl (a) + DOA (a), DOA (b), Lig Agl (b), Ol Agl (b), EA (a), Ol Agl (c) + Lig Agl (c) + DOA (c) + EA (b + c), and DOA (d) + EA (d) were quantified with the data obtained for the calibration curve of oleuropein obtained at 200 nm. However,

Table 3. Results of the Analysis of Bertolli Extra-Virgin Olive Oil Samples before and after Thermal Treatment (HPLC)[a]

analyte[b]	Bertolli	Bertolli, 30 min	Bertolli, 1h	Bertolli, 90 min	Bertolli, 2 h	Bertolli, 150 min	Bertolli, 3 h
HYTY[c]	9.83	3.79	2.57	2.26	0.84	0.73	nd
TY[d]	17.32	13.87	10.04	8.86	8.85	8.62	4.66
gentisic[e]	3.10	3.05	3.01	2.59	2.36	2.04	nd
vanillic[e]	3.28	3.16	3.09	2.49	2.14	1.98	nd
sinapic[e]	4.39	3.98	3.19	2.31	1.37	nd	nd
HYTY Ac[e]	1.66	1.58	1.38	0.86	0.64	0.37	0.36
EA[f]	197.17	86.14	61.63	26.24	24.70	19.03	12.27
DOA[g]	109.84	78.72	62.82	49.59	24.99	14.19	5.51
Pin[g]	22.61	22.58	22.30	20.47	19.52	11.58	10.23
Ac Pin[g]	122.51	118.88	104.62	88.72	53.41	48.21	46.16
Ol Agl[g]	58.91	43.99	31.45	16.51	8.66	4.15	3.68
Lig Agl[g]	7.31	7.08	6.91	6.62	6.27	5.96	5.53
"unknown" a[h]	nd	0.28 (1.41)	0.32 (1.44)	0.36 (1.66)	0.67 (3.15)	0.87 (4.11)	0.95 (4.49)
"unknown" b[h]	nd	2.05 (10.87)	2.55 (12.15)	3.05 (15.82)	3.11 (14.80)	3.83 (18.27)	4.34 (20.51)
"unknown" c[h]	nd	1.18 (5.55)	1.22 (5.81)	1.23 (6.38)	1.80 (8.57)	1.98 (9.45)	2.08 (9.83)
"unknown" d[h]	nd	4.50 (21.17)	5.70 (27.14)	6.35 (32.94)	7.83 (37.28)	8.43 (40.23)	9.18 (43.38)
"unknown" e[h]	nd	1.65 (7.76)	1.95 (9.28)	2.31 (10.89)	3.37 (16.04)	3.75 (17.90)	4.28 (20.23)
"unknown" f[h]	nd	1.67 (7.85)	1.82 (8.66)	2.10 (9.90)	3.34 (15.90)	3.24 (15.47)	3.88 (18.34)
"unknown" g[h]	nd	1.34 (6.30)	1.56 (7.42)	1.76 (8.30)	1.89 (9.00)	3.54 (16.90)	4.37 (20.66)

[a] Quantification of the individual components ($n = 7$) (value $= \bar{X}$) (in all the cases, SD was lower than 5% of the mean value). mg/kg = mg of analyte/kg of olive oil. nd = nondetected. [b] HYTY, hydroxytyrosol; TY, tyrosol; gentisic, gentisic acid; vanillic, vanillic acid; sinapic, sinapic acid; HYTY-Ac, hydroxytyrosol-acetate; EA, elenolic acid; DOA, decarboxymethyl oleuropein aglycon; Pin, (+)-pinoresinol; Ac Pin, 1-(+)-acetoxypinoresinol; Ol Agl, oleuropein aglycon; Lig Agl, ligstroside aglycon. [c] Quantified with a calibration curve of hydroxytyrosol at $\lambda = 280$ nm. [d] Quantified with a calibration curve of tyrosol at $\lambda = 280$ nm. [e] Quantified with a calibration curve of dopac at $\lambda = 280$ nm. [f] Quantified with a calibration curve of oleuropein glucoside at $\lambda = 240$ nm. [g] Quantified with a calibration curve of oleuropein glucoside at $\lambda = 280$ nm. [h] Values without parentheses were quantified with a calibration curve of dopac at $\lambda = 280$ nm, while values in parentheses were quantified with a calibration curve of oleuropein glucoside at $\lambda = 280$ nm.

Table 4. Retention Times, Absorption Maxima, and the Fragmentation Patterns Using the Two Interfaces for the Unknown Compounds Found in the Chromatographic Profiles after the Thermal Treatment

analyte	t_r (min)	λ_{max} (nm)	major fragments, APCI negative	major fragments, ESI positive
unknown a	27.41	282	nondetectable in APCI neg	249.0/475.2
unknown b	32.15	235/280	261.1	221.1/181.1
unknown c	42.51	226/282	227.1/309.1/319.2/ 85.1	321.1/361.2/241.3/121.1
unknown d	44.40	244/276	319.1/181.1	343.0/500.1/121.1
unknown e	49.09	280	125.2/169.0/85.1/173.1/329.2	221.2/261.1/349.2
unknown f	49.92	280	329.2/349.1	315.1/349.2/373.2 /239.1 /221.1/121.1
unknown g	66.01	288	373.2/232.51/197.2	nondetectable in ESI positive

for the quantification of Lig Agl (d) + Ol Agl (d) + EA (e), the curve of oleuropein at 240 nm was used.

Tyrosol was quantified using the calibration curve of its commercial standard at 200 nm; hydroxytyrosol was quantified with the calibration curve of its commercially available standard at the same wavelength as that for the other simple phenol (200 nm). Peak areas were used for the quantification of these compounds versus the corresponding commercial standards.

The phenolic acids and the "unknown" compounds which appeared after the thermal treatment in the phenolic profiles of the oil were quantified using the calibration curve of dopac at 200 nm, taking into account the time-corrected area counts versus time.

The detection limit (DL), quantification limit (QL), and precision (as relative standard deviation (RSD) of the intermediate concentration value of the linear range) of this method were calculated for the studied analytes using the method proposed by Curie (*31*). Three replicates of each analyte at different concentrations were done in order to set up the calibration.

All calibration curves showed good linearity in the studied range of concentrations. The calibration plots indicate good correlation between peak areas and analyte concentrations in the case of tyrosol and hydroxytyrosol, and between time-corrected area counts and analyte concentrations for dopac and oleuropein; regression coefficients were higher than 0.992. All the features of the proposed method are summarized in **Table 2**.

RESULTS AND DISCUSSION

Peroxide Value. In the case of the Bertolli commercial extra-virgin olive oil, the peroxide value without any thermal treatment was 10.54 mequiv of O_2 kg^{-1} ($n = 5$); then we measured this parameter as commented before after intervals of thermal treatment of 30 min, as can be seen in **Figure 1a**. It can be observed that the peroxide values during this study changed in the range of 6.26−10.79 mequiv of O_2 kg^{-1}; at the beginning a decrease was observed in this parameter (from 0 to 1 h), it remained almost stable during the next 90 min, and finally, it was possible to observe an increase of this value after 3 h of heating at 180 °C. The peroxides are primary oxidation products, and therefore, in the secondary phase, secondary products are generated.

Oxidative Stability of Oil Samples. To determine the oxidative stability of extra-VOOs (OSI), the conductivity was measured as a function of time. The trend of the oxidation process can be evaluated by the determination of secondary oxidation products by this test, since volatile oxidized products such as formic acid are absorbed in the deionized water, thus enhancing the conductivity of the solution. This test involves the measurement of an induction period, which is defined as the time during which the olive oil's natural resistance to

Table 5. Results of the Analysis of Bertolli Extra-Virgin Olive Oil Samples before and after Thermal Treatment (CE)[a]

	mg of analyte/kg of olive oil						
analyte[b]	Bertolli	Bertolli 30 min	Bertolli 1h	Bertolli 90 min	Bertolli 2 h	Bertolli 150 min	Bertolli 3 h
Lig Agl (a)[d]	43.50	42.86	42.20	26.42	23.89	23.64	6.89
TY[c]	18.26	13.60	11.47	9.68	9.61	8.18	5.91
Pin[d]	19.98	19.65	19.22	17.98	17.21	10.19	8.91
Ac Pin[d]	118.12	114.34	100.34	85.78	51.65	46.63	44.60
Ol Agl (a) + DOA (a)[d]	11.44	9.81	7.50	3.45	2.55	1.87	nd
DOA (b)[d]	66.78	48.38	31.39	11.01	6.27	5.46	nd
Lig Agl (b)[d]	6.43	5.49	4.92	1.62	nd	nd	nd
Ol Agl (b)[d]	5.04	4.55	3.95	3.73	2.98	2.86	1.20
EA (a)[d]	4.33	3.56	2.84	1.28	nd	nd	nd
Ol Agl (c) + Lig Agl (c) + DOA (c) + EA(b, c)[d]	37.03	31.23	23.47	8.66	5.20	3.87	3.18
HYTY[f]	8.27	4.11	2.89	2.50	0.89	0.73	0.58
DOA (d) + EA (d)[d]	14.56	11.10	9.50	8.65	8.15	8.05	nd
EA (e) + Lig Agl (d) + Ol Agl (d)[e]	122.04	63.54	57.32	15.86	15.29	11.78	7.60
4-HFA[g]	2.62	2.34	2.18	nd	nd	nd	nd
sinapic[g]	4.45	4.01	3.15	2.26	1.32	nd	nd
gentisic[g]	3.14	3.12	3.10	2.68	2.32	2.09	nd
"unknown"[d]	nd	54.91	71.91	83.82	92.95	108.96	116.89
"unknown" [g]	nd	21.60	28.29	33.06	36.71	43.11	46.24

[a] Quantification of the individual components ($n = 7$) (value $= \bar{X}$) (in all the cases, SD was lower than 5% of the mean value). nd = nondetected. [b] Lig Agl, ligstroside aglycon; TY, tyrosol; Pin, (+)-pinoresinol; Ac Pin, 1-(+)-acetoxypinoresinol; DOA, decarboxymethyl oleuropein aglycon; Ol Agl, oleuropein aglycon; EA, elenolic acid; 4-HFA, 4-hydroxyphenylacetic acid; sinapic, sinapic acid; gentisic, gentisic acid. [c] Quantified with a calibration curve of tyrosol at λ=200 nm. [d] Quantified with a calibration curve of Oleuropein glucoside at λ=200 nm. (Time corrected area counts vs concentration). [e] Quantified with a calibration curve of Oleuropein glucoside at λ=240 nm. (Time corrected area counts vs concentration). [f] Quantified with a calibration curve of hydroxytyrosol at λ=200 nm. [g] Quantified with a calibration curve of dopac at λ=200 nm. (Time corrected area counts vs concentration).

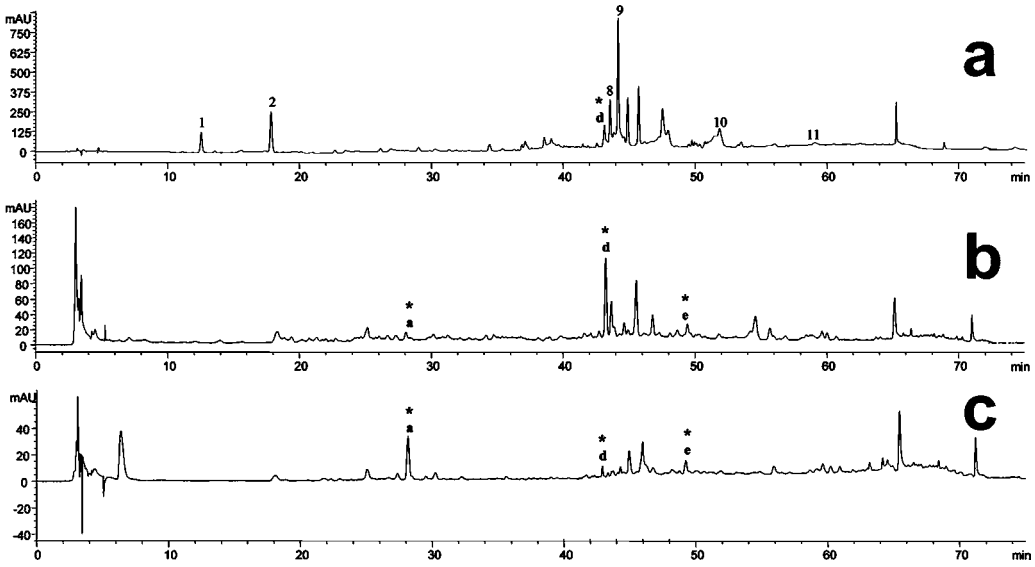

Figure 3. HPLC–UV analyses of three refined olive oils looking for the unknown compounds detected in the extra-VOO subjected to thermal treatment: (**a**) a commercial refined olive oil; (**b**) a crude refined olive oil after the bleaching phase; (**c**) a crude refined olive oil before the bleaching phase. Peak identification numbers as in **Figure 2**. Detection wavelength: 280 nm (injection volume of the three examples: 50 μL).

oxidation, due to the presence of naturally occurring antioxidants, inhibits the oxidation.

In **Figure 1b**, it is possible to observe the values of the media of five determinations for the induction time of an olive oil without any treatment and of the same oil after thermal treatment (different interval time). The trend is clear: the induction time decreased with longer heating times. During the first hour, we can see that the induction time was practically constant, a fact which could be explained because the phenolic compounds are one of the main groups of compounds acting as primary antioxidants to inhibit oxidation in virgin olive oil, and they mainly act as chain breakers by donating a radical hydrogen to alkylperoxyl radicals formed during the initiation step of lipid oxidation. After the first hour, they were not active anymore.

HPLC Analyses. All components decreased in concentration with an increase in the duration of the thermal treatment, although the rate of loss depended on chemical structure and antioxidant activity (see **Figure 2**).

When Bertolli commercial extra-virgin olive oil was studied, we observed that HYTY, in the first step of the thermal treatment (180 °C during 30 min), was reduced from 9.83 to 3.79 mg kg^{-1} (in a 61.5%), and then it continued to decrease during the heating (see **Table 3**). After 3 h it was impossible to detect this compound with the HPLC method in the polar extract of this olive oil. The behavior of TY was totally different, as it only decreased in a percentage of 19.92 after 30 min. The amount of TY which remained in the olive oil after the thermal treatment of 90, 120, and 150 min was practically the same; this evidence

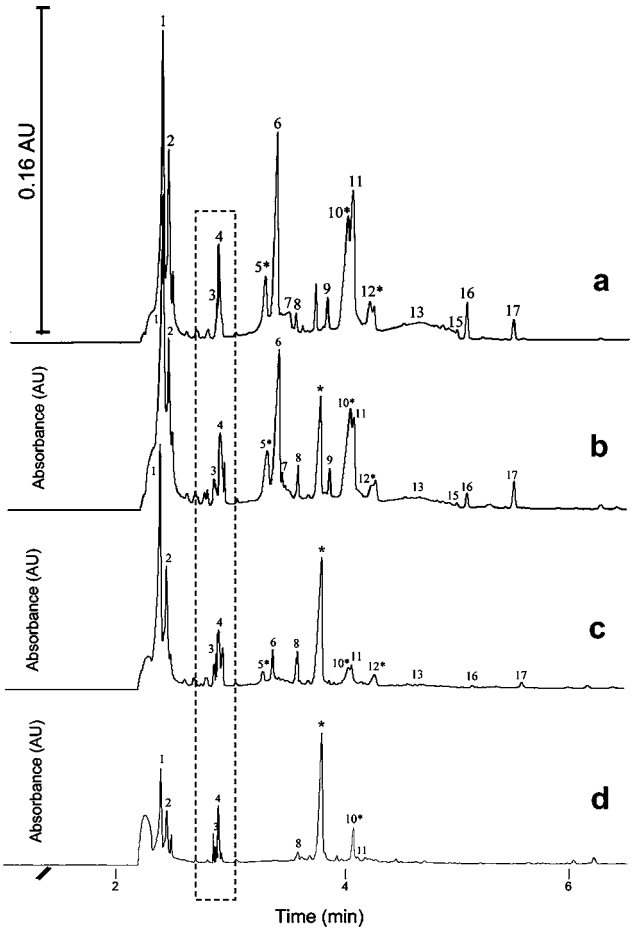

Figure 4. Electropherograms obtained for (**a**) Bertolli extra-virgin olive oil without treatment; (**b**) the same oil after 1 h at the mentioned temperature; (**c**) the same oil after 2 h at 180 °C; (**d**) the same oil after 3 h at the mentioned temperature. Peak identification numbers: **1**, Lig Agl (**a**); **2**, TY; **3**, Pin; **4**, Ac Pin; **5**, Ol Agl (**a**) + DOA (**a**); **6**, DOA (**b**); **7**, Lig Agl (**b**); **8**, Ol Agl (**b**); **9**, EA (**a**); **10**, Ol Agl (**c**) + Lig Agl (**c**) + DOA (**c**) + EA (**b**, **c**); **11**, HYTY; **12**, DOA (**d**) + EA (**d**); **13**, EA (**e**); **14**, *trans*-cinnamic acid; **15**, 4-hydroxyphenylacetic acid; **16**, sinapic acid; **17**, gentisic acid. Detection wavelength: 200 nm. *Peaks overlapped. (The "unknown compound" that appeared after the heating that can be detected using the CE method is marked with an asterisk.)

is in accord with the results of Cheikhousman et al. (*9*). Moreover, other authors (*7*) determined the lower antioxidant activity or even pro-oxidant effect of this compound in olive oil during an accelerated oxidation test. After 3 h at 180 °C, the concentration of TY determined in the extract was almost 30% of the initial quantity.

HYTY-Ac is a compound closely related to HYTY; however, it was more resistent to these treatments than HYTY. The determined concentration of this compound in Bertolli olive oil without treatment was 1.66 mg kg^{-1}, and after heating the olive oil during 3 h, we found 0.36 mg kg^{-1}.

The decrease of EA was very drastic, since the 56% (approximately) of the initial concentration disappeared after the first step. After 2 h it was possible to find only 12.5% of the initial concentration, and after 3 h, 6.2%.

Regarding individual secoiridoid compounds studied by using this HPLC method, we can distinguish two families: the secoiridoids which are HYTY derivatives and those that derive from TY. The tendencies for DOA and Ol Agl were very similar, decreasing more or less in the same proportion until the final step. After 3 h at 180 °C, the percentages of the initial amount of these compounds present in Bertolli olive oil were 5.0 and 6.3%, respectively. However, Lig Agl seemed to be one of the compounds more resistant to this kind of thermal treatment. These results are in good agreement with the data found in the bibliography, where, in general, when olive oil and other vegetable oils are subjected to thermal treatments, hydroxytyrosol derivatives are the first antioxidants that are lost during thermal oxidation and tyrosol derivatives seem to be the most stable compounds (*9*). This means that hydroxytyrosol derivatives are the first compounds to be oxidized, providing therefore oxidative stability to the oil (*7*, *23*, *25*, *26*). This observed trend is also consistent with the high antioxidant activity of hydroxytyrosol and its secoiridoid derivatives in virgin olive oil, as compared to those compounds belonging to the TY family (*6*), since antioxidants act by reacting rapidly with lipid radicals and are thereby consumed.

Furthermore, the levels of hydroxytyrosol and its derivatives in virgin olive oil have been reported to correlate well with the oxidative stability of the oil, as determined by the Rancimat method (*33*). In this case, after 1 h of treatment, 73.8, 42.8, and 46.6% of the initial amounts of HYTY, DOA, and Ol Agl, respectively, had been consumed, and after 2 h, 91.5, 77.3, and 85.3%. This fact can explain the shape of the graph which represents the OSI results (**Figure 1b**).

It is important to highlight the behavior found when another family of phenolic compounds (lignans) present in olive oil was studied; considering the concentration of Pin, it was really stable during the first 2 h, since the amount of this compound only changed from 90.57 to 78.16 mg kg^{-1}. The decrease was a little bit sharpened when the thermal treatment took more than 2 h, and after 3 h, we could still determine, in the polar extracts of Bertolli olive oil, concentrations which represented 5.3% of the initial quantity. Ac Pin was stable during the first hour of heating (approximately), then the decrease was faster, and finally, after 3 h 37.7% of the initial quantity remained in the oil. Lignans were shown to be very resistant to thermal treatment in the studies carried out by Brenes et al. (*8*), while in another study, of Gómez-Alonso et al. (*24*) using another kind of frying, both the content of the lignans and the elenolic acid fell drastically in the first frying operation. The results obtained in the research carried out by Brenes et al. seem to be in agreement with those published by Carrasco-Pancorbo et al. (*7*), who demonstrated the lignans show a low antioxidant activity in comparison with the antioxidant activity shown by other phenolic compounds, such as HYTY, DOA, or Ol Alg.

"Unknown" Compounds Detected in the Chromatographic Profiles and Their Presence in Other Oils. When the chromatographic profiles shown in **Figure 2** are observed, it is also possible to see that there are several peaks (seven peaks in particular) which are higher when the thermal treatment is longer, which means there are several unknown peaks that appear in the chromatogram after the treatment. Their retention time, absorption maxima, and fragmentation patterns using the two interfaces are shown in **Table 4**. We also made their quantification: the results are summarized in **Table 3**.

Then, we analyzed a commercial refined olive oil and a crude refined olive oil after and before the bleaching phase. To get a refined oil, thermal treatments are employed, so we thought it could be possible to find the "unknown" compounds in the polar fraction of these oils. In **Figure 3**, the HPLC analyses of these three oils are shown. One of the unknown compounds was found in the commercial refined olive oil, and three were detected in the crude refined olive oils after and before the bleaching phase. In the profile of the commercial refined olive oil, six of the

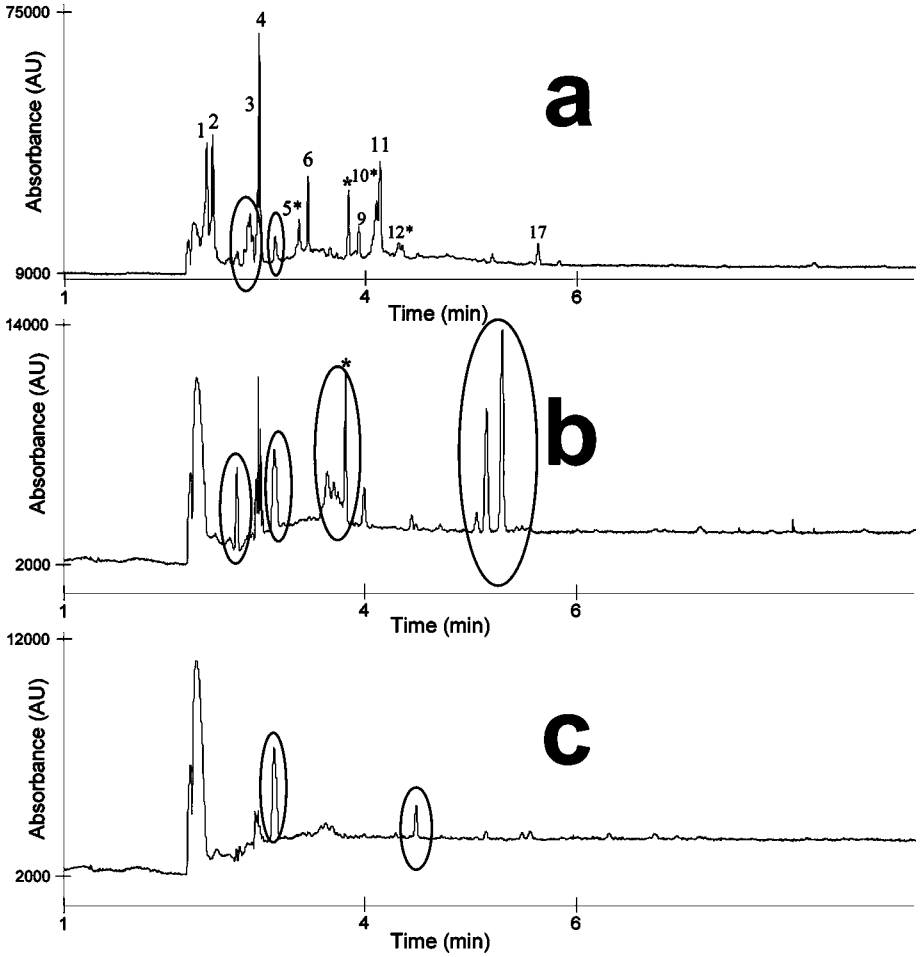

Figure 5. CE profiles of three refined olive oils used as applications of the methods looking for the unknown compounds detected in the extra-VOO subjected to thermal treatment: (**a**) a commercial refined olive oil; (**b**) a crude refined olive oil after the bleaching phase; (**c**) a crude refined olive oil before the bleaching phase. Peak identification numbers as in **Figure 4**. Detection wavelength: 200 nm (injection time of the three examples: 9 s).

eight phenolic compounds present in a normal extra-VOO were found, which means that the treatment in this case (for the refined olive oil) was probably softer than that in the other two oils under study (crude refined olive oil after and before the bleaching phase), as the alterations in the phenolic profile were not really marked.

CE Analyses. The analyses of the extracts of extra-virgin olive oil with and without thermal treatment were carried out by CE as well. As commented before, all the components decreased in concentration with an increase in the duration of the thermal treatment, although the rate of loss depended on chemical structure and antioxidant activity (see **Figure 4**).

Taking into account the compounds which are quantified in terms of their commercial standard (tyrosol and hydroxytyrosol), we can say that the results obtained by both techniques are in good agreement (see **Table 5**). Considering the lignans (Pin and Ac Pin), it was also possible to observe very similar concentrations determined by HPLC and CE. In the lignans zone, considerable changes took place, since after the treatment this area appeared as one of the richest, containing a lot of separated compounds.

Differences can be found when the results for the secoiridoids are studied, but the cause of these apparent discrepancies could be that CE is able to separate several isoforms of the secoiridoids (*32*). So, the secoiridoids that appear in the HPLC profiles as one peak can be separated into several peaks with the CE method.

Besides these compounds, the CE method was able to detect and determine several phenolic acids that appear within 7 min of analysis (4-HFA and sinapinic and gentisic acids). The chromatographic method used was not able to detect any phenolic acid after 3 h of treatment at high temperature.

"Unknown" Compounds Detected in the Electrophoretic Profiles and Their Presence in Other Oils. Apparently, for controlling new peaks which appear in the phenolic profile of the extra-virgin olive oil under study, HPLC seemed to be more appropriate, since in the electrophoretic data only one unknown peak could be detected with clarity without taking into account the lignans area.

As mentioned in the HPLC section, we proceeded to analyze a commercial refined olive oil and a crude refined olive oil after and before the bleaching phase. The results can be observed in **Figure 5**, and the differences in the profiles of the three applications were noticeable. Different areas are indicated with a round in the figure; in these zones there were not compounds in the profile of an extra-VOO without treatment. In the olive oils (a) and (b), several peaks were detected before and after the zone of the lignans, although in (c) only the peak which appears after the Ac Pin was detected. Moreover, in the crude refined olive oil obtained after the bleaching phase, several peaks were detected just before the unknown peak found in the extra-VOO after thermal treatment, which was also detected. This unknown peak detected also in the Bertolli extra-VOO after the thermal treatment was found in the commercial refined olive

oil. The crude refined olive oil obtained after the bleaching phase (b) had in its composition two other compounds which appeared in the electropherogram after 5 min; they were the highest peaks in the profile and were not found in the other two refined oils. Another compound with a migration time of 4.53 min was found when the analyses of the crude refined olive oil obtained before the bleaching phase were studied; it was not detected in the other two oils under study.

Changes in the phenolic extracts obtained from extra-virgin olive oil caused by thermal treatments were controlled by HPLC−UV and HPLC−MS and by CE−UV−vis. To our knowledge, it is the first time in which these phenolic compounds are quantified individually after the treatment by both techniques. The results obtained for the two separative techniques were in good agreement and confirmed other data previously published. Furthermore, the CE method was able to determine three phenolic acids and to separate several isoforms of the secoiridoids which appeared as one peak in the chromatograms.

HYTY, EA, DOA, and Ol Agl reduced their concentration with the thermal treatment more quickly than other phenolic compounds present in olive oil. HYTY-Ac and Lig Agl were shown to be quite resistant to this kind of treatment. The behavior of lignans could be outstanding, as they belong to the family most resistant to thermal treatments.

The peroxide values found during the study varied in the range of 6.26−10.79 mequiv of O_2 kg^{-1}, observing a little increase after 3 h of treatment; we can say that the peroxides are primary products of the oxidation process; that means that, in the secondary phase of the oxidation process, other secondary products will appear. The trend for the results of the OSI test was clear, since the induction time decreased when the heating was longer.

Moreover, several new unknown compounds were detected and quantified in the phenolic fraction of the oil, and their most relevant characteristics were determined. We studied several refined olive oils, looking for the unknown compounds detected in the extra-VOO subjected to thermal oxidation, and several of them were found.

ABBREVIATIONS USED

Ac Pin, 1-(+)-acetoxypinoresinol; DL, detection limit; DOA, decarboxylated oleuropein aglycon; EA, elenolic acid; Gentisic, gentisic acid; 4-HFA, 4-hydroxyphenylacetic acid; HYTY, hydroxytyrosol; HYTY-Ac, hydroxytyrosol-acetate; Lig Agl, ligstroside aglycon; Ol Agl, oleuropein aglycon; Pin, (+)-pinoresinol; PV, peroxide value; QL, quantification limit; Sinapic, sinapic acid; TY, tyrosol; Vanillic, vanillic acid; VOO, virgin olive oil.

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