Phenolic Compounds in Virgin Olive Oil. 2. Reappraisal of the Extraction, HPLC Separation, and Quantification Procedures

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The extraction procedures (solid/liquid SPE and liquid/liquid LLE) and HPLC separation and quantification methods of polyphenolic compounds have been checked in virgin olive oils in order to explain the differences in content reported in the literature. The work has been carried out on oils prepared from one cultivar and produced under the same protocol. The extraction methods are practically equivalent, but the SPE technique is more favorable because it is faster and simpler. It has been proved that the chromatographic features and the method of chemical expression of the concentrations may greatly affect the final values. Thus, under the same analytical method, the total concentration values of polyphenols of the same oil show variations from 18% to 80%, according to the formality of expression as gallic acid, caffeic acid, or tyrosol equivalents. The role of the nature and spectrophotometric features of the phenols and of the internal standard is also discussed, and it was found to be an important source of reported variation. A gradient separation with an eluent mixture acetonitrile−sulfuric acid (0.1 mol/L), detection at 225 nm, and quantitative calculation of polyphenolic compounds in oils (expressed as tyrosol equivalents, THY_{eq}) is proposed.

Keywords: *Olive oil; polyphenols; HPLC; quantification*

The phenolic compounds of *Olea europaea* Ln., commonly named polyphenols (Harborne, 1989, Figure 1), are related to the sensory and nutritional characters of virgin oils, where they play a role in its shelf life (Montedoro et al., 1993). In the past 10 years, they have been investigated by many researchers and from different viewpoints (Tsimidou, 1998 and references therein).

Here we studied the following aspects of the determination of polyphenols in oils: extraction, chromatographic separation, and quantification. Two main extraction techniques have been reported in the literature: the liquid−liquid extraction (LLE, Montedoro et al., 1992; Cortesi et al., 1995a) and solid-phase extraction (SPE; Papadopoulos and Tsimidou, 1992; Mannino et al., 1993; Pirisi et al., 1997). From the prepared extracts, the separation and routine quantitative determination have been performed by HPLC or GC. HPLC with UV detection (often with diode array detectors) has been preferred for routine analysis on virgin oils and olive extracts (Nergiz and Ünal, 1991; Tsimidou et al., 1992; Mannino et al., 1993; Montedoro et al., 1992; Cortesi et al., 1995a; Pirisi et al., 1997; Cioni et al., 1998; Esti et al., 1998; Romani et al., 1999). Nevertheless, from the data reported in the literature, it is clear that a real comparison of concentrations of polyphenols in oils is difficult. Indeed, the reported concentrations often differed in magnitude, as recently pointed out by Tsimidou (1998). This author suggests that these differences are due to the "genetic" characteristic of the olive cultivar; others think they depend on the extraction technology (e.g., two-phase decanter vs three-phase decanter; Ranalli and Angerosa, 1996), but neither agrees with other literature data.

Significant differences in polyphenol content have been reported in oils obtained from the same cultivar (Cortesi and Fedeli, 1983; Mattei, et al., 1988). Moreover, it has also been reported that "technology... does not change the magnitude of total phenol content" (Tsimidou, 1998). If the "genetic" and/or "technological" reasons are unreliable, where do the differences come from and how are they generated? Could they not derive from the different procedures of extraction, separation, and chromatographic analysis? Moreover, could they not be due to the formality of expression of the polyphenol concentration? In our opinion these hypotheses are reasonable. For instance, all extraction methods seem to be quantitative. However, to the best of our knowledge, they have never been compared starting from the same matrix. Furthermore, HPLC methods are different in their eluting mixtures, their columns, and their detection wavelength. Thus, the chromatographic response of one or all phenolic compounds, analyzed under two different conditions, cannot be comparable. Finally, the response factors (RF) of the polyphenols have generally been unknown, because the polyphenols have rarely been obtained as pure samples (few examples have been reported and they cannot apply to a routine analysis; Pirisi et al., 1997; Romani et al., 1999). Their concentrations in oils have therefore been expressed as gallic acid (GAL, i.e., Montedoro et al., 1992;), caffeic acid (CAF, i.e., Tsimodou et al., 1992), 4-hydroxyphenyl ethanol (THY, i.e., Cioni et al., 1998), or oleuropein (Esti et al., 1998). It is clear that a comparison of such data is not valid.

Let us now check this hypothesis. First of all, we have eliminated the uncertainties due to the cultivar and to oil production conditions (milling and kneading). We

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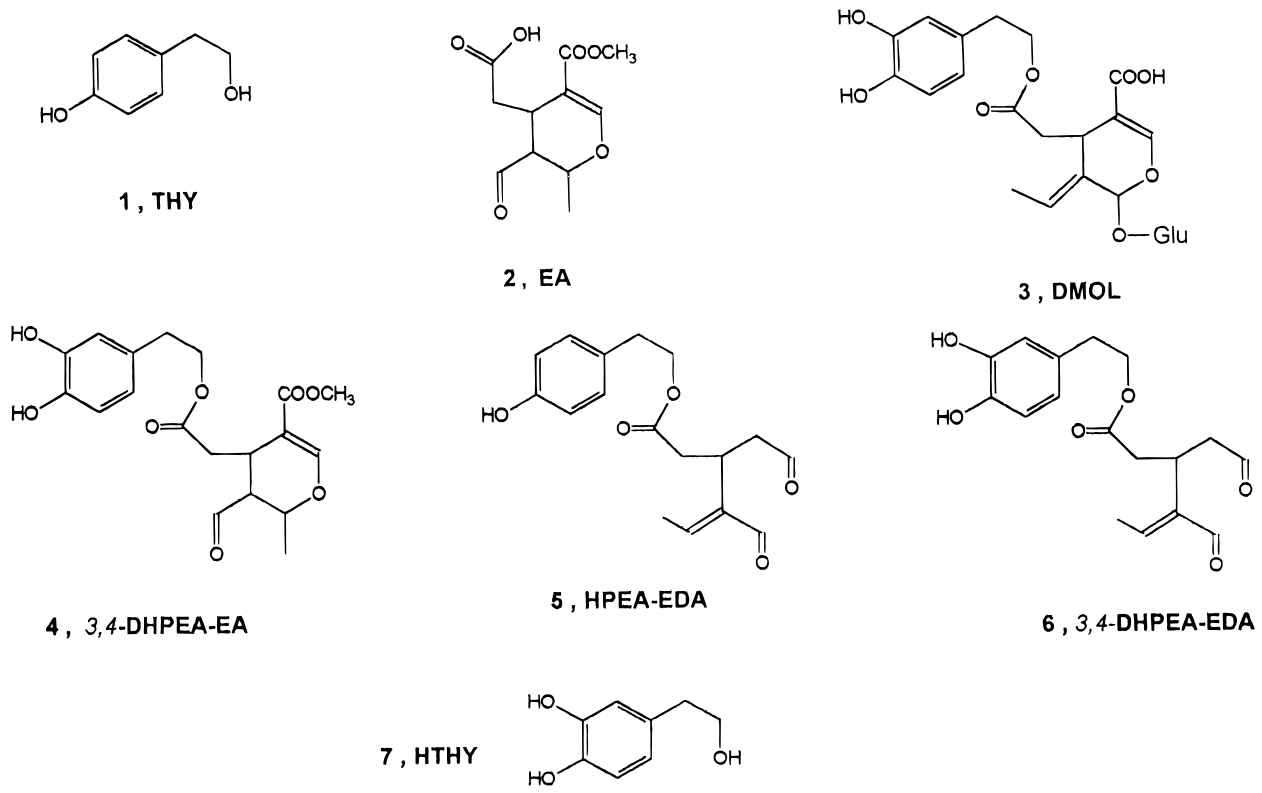

Figure 1. Structural formulas of simple and complex phenols.

have produced virgin oil according to strict, step-controlled conditions from olives harvested on the same day in the same orchard from one and the same cultivar. We then extracted the polyphenols from the oil with LLE and SPE procedures and determined their content by HPLC. We performed this analysis using our method and the method generally used in the literature (derived from the original procedure by Montedoro et al., 1992). Finally, we expressed the polyphenols concentration data using three different units, which we then compared.

EXPERIMENTAL PROCEDURES

Chemicals. Acetonitrile, cyclohexane, *n*-hexane, and methanol were HPLC-grade solvents from Rhône-Poulenc Ltd. (Manchester, U.K.). Sulfuric acid (0.5 mol/L), Na_2SO_4, and H_3PO_4 (95%) were analytical-grade reagents purchased from Carlo Erba (Milan, Italy), whereas oleuropein glycoside (OLG, >96%) and quercetin (>98%) were from Extrasynthèse (Genay, Lyon, France). Tyrosol (THY, >98%), caffeic (CAF, >98%), and gallic acids (GAL, >98%) were purchased from Aldrich (Milan), carbaryl (1-naphthyl methyl carbamate, employed as an internal standard, I.S.) from Ehrenstorfer (Augsburg, Germany), and SPE cartridges C_{18} (0.5 g) from Alltech (Milan). Water was distilled twice and purified through a MilliQ apparatus (Millipore Italia, Milan). Analytical standards of hydroxy tyrosol (HTHY), oleuropein aglycone (elenolic acid linked to hydroxy tyrosol, 3,4-DHPEA-EA), and elenolic acid (EA) were prepared as described elsewhere (Pirisi et al., 1997).

Apparatus. A Varian 5020 pump equipped with a UV−Vis 100 variable wavelength detector set at 225 and 280 nm (Varian, Palo Alto, CA), an HP 1050 automatic injector (loop volumes, see Chromatography section), and an HP 3396 reporting integrator (Hewlett-Packard, Milan, Italy) were employed. The detector output was also connected to another diode array detector (LC-235, Perkin-Elmer, Milan) whose signals and UV peak spectra were processed by an LCI-100 computer integrator (Perkin-Elmer).

Chromatography. Elution on ODS-2 analytical columns (Spherisorb, 250 × 4.6 mm i.d., 3 μm, Waddinxveen, The Netherlands) with a 1.0 cm guard cartridge (C_{18}, 10 μm) was performed under the conditions named as methods 1a, 1b, and 2. Methods 1a and 1b were an improvement of our original method (Pirisi et al., 1997), whereas method 2 is the method employed by many other researchers and was derived from the original method of Montedoro et al. (1992). The methods were as follows.

Method 1: mixture of A (H_2SO_4 (10^{-3} M)) and B (CH_3CN) at a flow rate of 1.0 mL min^{-1}. The elution profile (%) was as follows: $t = 0$ min, A = 85 and B = 15, $t = 35$ min, A = 34 and B = 66; $t = 35.1-40$ min, A = 85 and B = 15. Method 1a was with the detector set at 225 nm and a loop of 20 μL, and method 1b was with the detector set at 280 and a loop of 40 μL.

Method 2: mixture of H_2O with 0.5% of H_3PO_4 (A) and 50:50 CH_3CN/CH_3OH (v/v, B) at 1.2 mL min^{-1} with the following elution profile (%): $t = 0$, A = 96 and B = 4; $t = 1$ min, A = 96 and B = 4; $t = 26$ min, A = 70 and B = 30; $t = 36$ min, A = 40 and B = 60; $t = 66$ min, A = 2 and B = 98; $t = 70$ min, A = 96 and B = 4; $t = 80$ min, A = 96 and B = 4. The detector was at 280 nm; injection volume = 10 μL (concentrated samples 20×) or 40 μL (samples concentrated 5×), see below.

The identification of peaks in the chromatograms was made by comparing their relative retention times (rrt) from carbaryl as an internal standard (I.S.) with those of standard samples (if available) or according to literature data (Montedoro et al., 1992; Cortesi et al., 1995a; Pirisi et al., 1997; Esti et al., 1998) and by UV-DAD spectra of eluted peaks.

Olives and Oil Extraction. About 2400 kg of olives cv. "Frantoio" were collected on Nov 30, 1998, in a 15-year-old orchard near Florence, Italy. They were milled into 6 lots (each of 400 kg) by a mobile knife crusher with a 6 mm stoker. Each lot was milled in 9 min. Three lots were milled at 30 Hz (S1) and three at 60 Hz (S2). The pulps were kneaded for 20 min at 28 ± 2 °C and then extracted in a centrifugal decanter at 28 ± 2 °C. The oils were separated from the resulting oily must by vertical centrifuge. The main parameters of the oils are collected in Table 1.

Table 1. Chemical Parameters of the Oils[a]

oil	acidity (% oleic acid)	peroxides (mg of O_2/kg)	vitamin E (mg/kg)	K_{232}	K_{270}	ΔK	% oleic acid	% linoleic acid	% linolenic acid	yield (%)
S1	0.15 ± 0.02	6.72 ± 0.08	218 ± 0.09	1.774 ± 0.152	0.157 ± 0.08	−0.001	76.58 ± 2.05	6.50 ± 0.30	0.60 ± 0.07	14.8
S2	0.20 ± 0.02	6.23 ± 0.05	224 ± 0.07	1.784 ± 0.164	0.179 ± 0.10	−0.001	76.45 ± 1.38	6.67 ± 0.22	0.63 ± 0.02	16.5

[a] $n = 3$; all parameters are determined according to the Directive UE 2568/91.

Extraction of Phenolic Compounds from Oils. Two different methods were employed.

SPE Extraction. A C_8 extract clean-up cartridge (500 mg, 3.5 mL, Alltech) was washed with 10 mL of *n*-hexane and then conditioned with 10 mL of acetonitrile. One gram of oil, dissolved in 10 mL of *n*-hexane, was percolated into the cartridge and washed under vacuum (35 mm) with 10 mL of a solution of *n*-hexane/cyclohexane (1:1, v/v) in order to remove the nonpolar fraction of the oils. The polyphenols were eluted with 10 mL of CH_3CN at a flow rate of 1.0 mL/min. The eluate was kept overnight at −25 °C to precipitate the oil droplets. The solvent was concentrated to 1.0 mL under reduced pressure and then evaporated under N_2 and recovered with CH_3OH containing the I.S. (at 25.0 mg kg^{-1}). When the sample was injected under the chromatographic conditions of methods 1a and 1b, it was dissolved in 1.0 mL of CH_3OH. Using the chromatographic conditions of method 2, the residue was dissolved in 400 mL of CH_3OH (concentration factor 5×) containing the I.S. (25 mg kg^{-1}). After elution into the cartridge, polyphenols were not retained significantly, as confirmed by the HPLC analysis of the extracts of two further elutions (each using 10.0 mL of CH_3CN).

LLE Extraction. Two grams of oil was weighted in a centrifuge tube and added with 1.0 mL of *n*-hexane and 2.0 mL of CH_3OH/H_2O (v/v, 60/40). The mixture was stirred for 2 min in a vortex apparatus, and the tube was centrifuged at 3000 rpm. The methanol layer was separated and the extraction repeated twice. The extracts were combined and washed twice with 2 mL of *n*-hexane. The *n*-hexane was discarded, and the methanolic solutions were evaporated to dryness under reduced pressure and low temperature (<35 °C). Under the conditions of methods 1a and 1b, the samples were dissolved and injected as reported in the SPE Extraction section. When the samples were analyzed by the chromatographic conditions of method 2, the residue was dissolved in 100 (concentration factor 20×) or 400 μL (concentration factor 5×) of CH_3OH containing the I.S. (25 mg kg^{-1}).

RESULTS AND DISCUSSION

First, it was necessary to state how the concentrations of polyphenols could be expressed. We chose to express them as tyrosol equivalents (THY_{eq}) and then to compare them with those calculated as gallic acid equivalents (GAL_{eq}) and caffeic acid equivalents (CAF_{eq}). The calculation was made by response factors (RF) obtained from standard curves constructed by plotting peak area ratios of standard solutions in CH_3OH each containing THY, GAL, CAF (25, 50, 100 ppm) and the internal standard (I.S.) carbaryl (25, 50, 100 ppm). This pesticide was selected among other chemicals as the internal standard because it's signal does not interfere with those of polyphenols in either of the gradients 1 or 2**.** Moreover, it shows good absorbance at 225 and 280 nm. The standard curve had a good correlation ranging between −0.9998 and −0.9896.

Second, it was advisable to state *which* phenols we wanted to measure. It is well-known and accepted (Tsimidou, 1998 and references therein) that the role played in oils by the "simple" polyphenols (THY, HTHY) differs from that of "complex" polyphenols (3,4-DHPEA-EA, HPEA-EDA, 3,4-DHPEA-EDA, DMOL, Figure 1). The differences refer to their antioxidative properties and to their influence on the sensory features of oils. The concentrations of "simple" and "complex" polyphenols should therefore be measured and reported separately. Consequently, here we used the following partition convention.

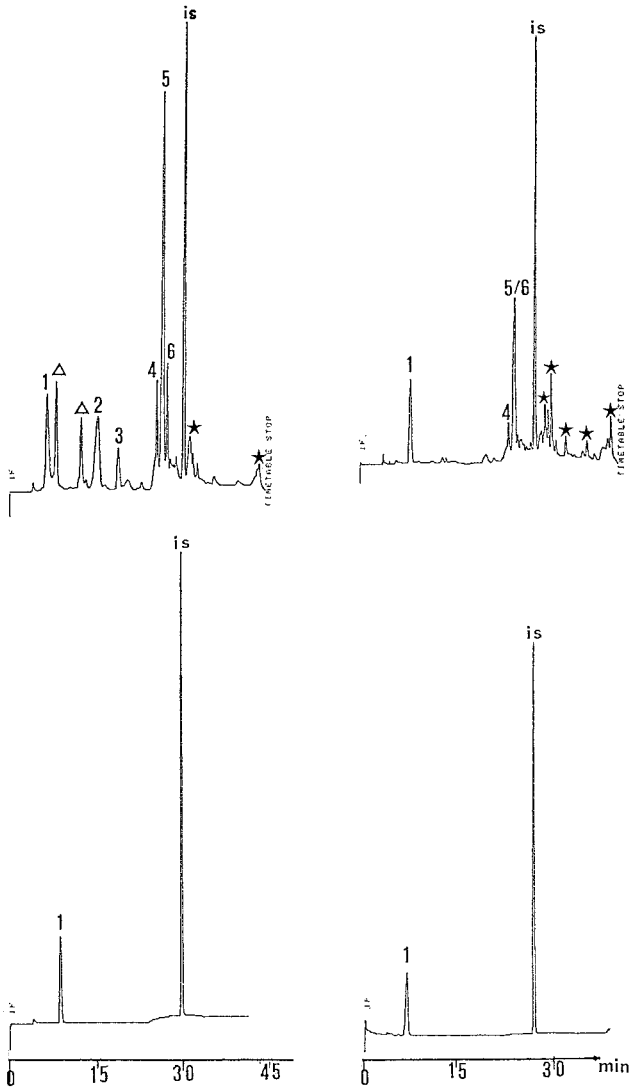

Figure 2. Chromatograms of the oil (top) and the blank with THY and I.S. (bottom). Left 1a; right 1b. Peaks: 1 = THY; 2 = EA; 3 = DMOL; 4 = 3,4-DHPEA-EA; 5 = HPEA-EDA; 6 = 3,4-DHPEA-EDA. (△) Unknown compounds of rrt < 0.50. (★) Unknown flavonoids of rrt > 1.

Total polyphenols (TP): summation of concentrations of all simple and complex, known and unknown, polyphenols reported in the chromatograms (see Figures 2 and 3).

Complex polyphenols (CP): summation of concentrations of compounds with the structures reported in Figure 1, plus the concentrations of flavonoids (rrt > 1), if detected.

Simple polyphenols (SP): summation of concentrations of THY, HTHY, EA, and unknown compounds with rrt < 0.55 (in methods 1a and 1b) or <0.90 (in method 2).

Table 2. Polyphenol[a] Concentrations (mg of kg^{-1} ± SD[b]) Found with Different Extraction Methods

	SPE			LLE		
oil	TP	CP	SP	TP	CP	SP
S1	120.4 ± 8.0	93.1 ± 6.9	27.3 ± 7.3	123.3 ± 12	86.8 ± 11	36.5 ± 5.6
S2	98.1 ± 6.0	79.8 ± 5.0	18.3 ± 3.4	100.6 ± 9.0	75.4 ± 5.2	25.2 ± 3.3
120	112.6 ± 8.5	57.9 ± 5.5	54.7 ± 4.2	105.4 ± 12	64.4 ± 3.6	41.0 ± 7.0
121	180.4 ± 7.8	85.6 ± 4.2	94.8 ± 8.9	170.9 ± 9.5	80.4 ± 7.8	90.5 ± 6.6
124	155.8 ± 6.2	90.0 ± 4.8	65.8 ± 5.5	151.6 ± 3.5	70.6 ± 5.8	81.0 ± 4.2

[a] As THY_{eq} under chromatographic conditions 1a. [b] $n = 4$.

Table 3. Polyphenol[a] Concentrations (mg of kg^{-1} ± SD[b]) Found in Oil S1[c] under Different Chromatographic Methods

method								
1a			1b			2		
TP	CP	SP	TP	CP	SP	TP	CP	SP
120.4 ± 8.9	93.1 ± 6.9	27.3 ± 5.0	98.5 ± 8.3	42.7 ± 3.9	55.8 ± 4.6	92.7 ± 8.8	25.2 ± 4.5	67.5 ± 3.5

[a] As THY_{eq}. [b] $n = 4$. [c] Extracted by SPE.

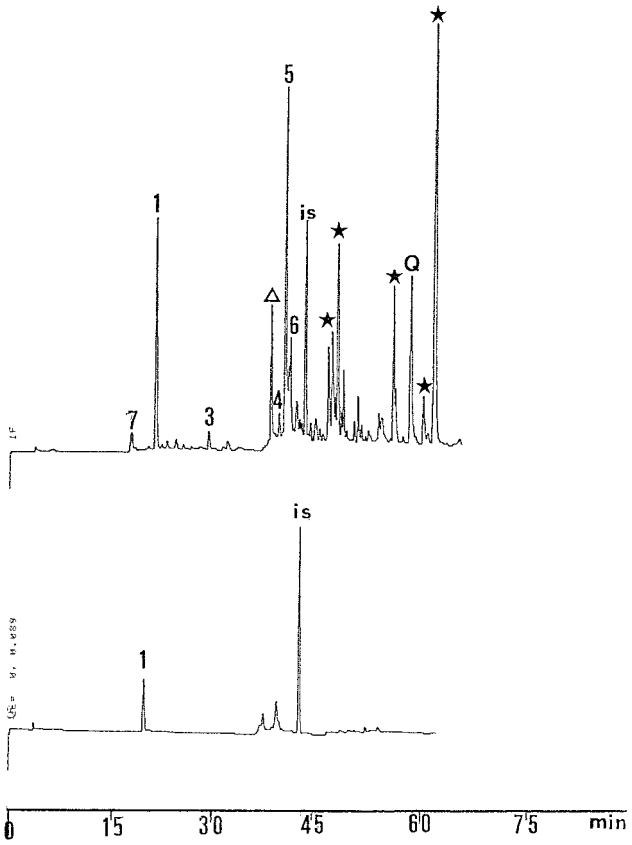

Figure 3. Chromatogram of the oil (top) and the blank with THY and I.S. (bottom) under conditions 2. Peaks: 1 = THY; 3 = DMOL; 4 = 3,4-DHPEA-EA; 5 = HPEA-EDA; 6 = 3,4-DHPEA-EDA; 7 = HTHY. (△) Unknown compounds of rrt < 0.90. (★) Unknown flavonoids of rrt > 1. Q = quercetin.

Having established this, we compared the chosen extraction procedures and chromatographic methods.

Comparison of Extraction Procedures. By preliminary experiments, method 1a was found to be faster and more sensitive than either method 1b or 2. We therefore checked under method 1a whether the SPE and LLE procedures would give a different content of polyphenols in oils S1 and S2. Four replicates of each oil were prepared (four SPE and four LLE). Each replicate was analyzed at least three times. The data are shown in Table 2, and no significant differences have been observed between the TP, CP, and SP concentrations obtained with SPE and LLE. The CV ranges between 4.5% and 11%. Similar results were also found on three oils obtained from farmers of Sardinia.

Comparison of Chromatographic Methods. From a separation viewpoint, methods 1 and 2 amount to the same result with a good separation of all polyphenols (see Figures 2 and 3). Some differences occur between the methods.

Qualitative Differences. These differences are mainly due to the wavelength employed. At 225 nm, HTHY can be detected only if its concentration exceeds 50 ppm. This phenol, instead, is easily detected at 280 nm. EA was detectable at 225 nm but not at 280 nm. DMOL showed a low response at 280 nm and was easily eluted by method 2. The compounds with rrt > 1 (flavonoids and flavonol glycosides) are best detected at 280 nm and with method 2. Quercetin is also detected at 225 nm, if its concentration exceeds 25 ppm.

Quantitative Differences. The quantitative data obtained under the different chromatographic conditions were compared as follows: method 1a vs method 1b and method 1b vs method 2.

Method 1a vs Method 1b. The concentrations of TP and CP found in the oil S1 extracted by SPE are reported in Table 3 under the conditions of methods 1a and 1b (the same results were achieved with LLE extraction and in the oil S2, data not shown). It was clear that the values of the TP concentrations were higher at 225 nm compared to that at 280 nm by a rao ≅ 1.2. This ratio increases to up ≅2.2 when we consider the CP.

This difference stems from the ratio between the responses of each phenolic compound (Rcmp) and the I.S. (Ris) at the two wavelengths. While the ratios THY/I.S. were very similar at both wavelengths (0.084 at 225 nm; 0.079 at 280 nm), the ratios of each phenolic compound and the I.S. differed at 225 and 280 nm (Table 4). They were always higher at 225 nm. The ratio between the wavelengths $(Rcmp/Ris)_{225}/(Rcmp/Ris)_{280}$ was found to be as follows: 7.31 for DMOL, 14.58 for 3,4-DHPEA-EA, 1.20 for 3,4-DHPEA-EDA, and 6.60 for HPEA-EDA. This means that the *same compound* at the *same concentration* gave a very different response under the two methods, with a higher response at 225 nm. Consequently, since the concentrations were always expressed as THY_{eq}, the calculation at 280 nm always gave lower values. It is necessary to point out that, according to the respective concentrations of compounds 3,4-DHPEA-EDA and HPEA-EDA in oil,

Table 4. Responses of Single Polyphenol Compared to the I.S. under the Different Chromatographic Conditions

	method:	1a		1b		2	
peak no.[a]	compound	rrt	Rcmp/Ris[b]	rrt	Rcmp/Ris[b]	rrt	Rcmp/Ris[b]
1	THY	0.253	0.084	0.253	0.079	0.446	0.121
3	DMOL	0.625	0.117	0.625	0.016	0.709	0.184
4	3,4-DHPEA-EA	0.849	0.525	0.849	0.036	0.913	0.119
5	HPEA-EDA	0.862	0.337	0.862	0.051	0.925	0.139
6	3,4-DHPEA-EDA	0.874	0.416	0.874	0.500	0.950	1.646

[a] See Figures 2 and 3. [b] Ratio of integration areas; $n = 4$.

Table 5. Comparison of Concentrations of Polyphenols Found in Oil S1, Analyzed under Methods 1a and 1b, and Expressed with Different Formalities

	PF (mg of kg^{-1})[a]					
formality	TP	Δ[b]	CP	Δ[b]	SP	Δ[b]
			Method 1a			
THY_{eq}	120.4		93.1		27.3	
GAL_{eq}	85.0	−29	61.0	−35	24.0	−12
CAF_{eq}	98.9	−18	71.0	−24	27.9	0
			Method 1b			
THY_{eq}	98.5		42.7		55.8	
GAL_{eq}	37.0	−62	28.1	−34	8.9	−84
CAF_{eq}	19.3	−80	14.7	−65	4.6	−92

[a] $n = 4$; CV = 4.5 ± 11%. [b] Percent difference from THY_{eq}.

their peaks could collapse under method 1b conditions (Figure 2).

Method 1b vs Method 2. From the data in Table 3, it was clear that the concentrations of TP found under methods 1b and 2 were of the same order of magnitude. The CP concentrations were higher under method 1b by a factor ≅1.7. The ratio $(Rcmp/Ris)_{280}^{1b}/(Rcmp/Ris)_{280}^{2}$ was 0.087 for DMOL, 0.366 for HPEA-EDA, 0.304 for 3,4-DHPEA-EDA, and 0.119 for 3,4-DHPEA-EA (Table 4). Since the response of the I.S. under method 2 was higher, the THY/I.S. ratios were more favorable to method 1b. Therefore, the concentrations were higher in method 1b compared to method 2.

Comparison of the Concentrations Expressed with Different Units. The concentrations of TP, CP, and SP found under methods 1a and 1b, expressed as GAL_{eq} or CAF_{eq}, were always lower than those expressed as THY_{eq} (Table 5). The percentage differences at 225 nm ranged from 12 to 35 and at 280 nm from 34 to 92. These are due to the lower response compared to the I.S. of GAL and CAF as opposed to THY. The difference in concentration values is variable. Under method 1a, in the case of SP the concentration difference is negligible, in the case of TP and CP it is significantly higher than that of CV, though of the same magnitude. Under method 1b, all the differences in PF concentrations are dramatically great.

CONCLUSIONS

We can draw the following conclusions: (1) The extraction procedure of polyphenols from the oils can be carried out indifferently by SPE and LLE methods since they are quantitatively similar. The choice between these methods is related to the time needed to perform them. The SPE method requires one-half the time of the LLE method. Moreover, in LLE the separation step of the aqueous CH_3OH/ *n*-hexane layers is very critical. If not performed carefully, this step could give dramatically different results, as found in our laboratory with four inexperienced operators. In this case the TP concentration data could reach a CV of 20 ± 35% (data not shown).

(2) Method 2 is unfavorable because it is longer and more expensive. Moreover, the sample must be concentrated, with all the difficulties and mistakes generated by such a practice.

(3) The data found here could explain why the polyphenol concentrations reported in the literature are often incommensurate. The formality of expression, the different chromatographic conditions (e.g., the composition of the eluting mixture and the wavelength), and the spectrophotometric features of the reference phenol and of the I.S. dramatically affect the calculation of the polyphenol concentration of the same oil.

Therefore, to express the concentrations of polyphenols in oils, method 1a is preferred because it is faster and more sensitive, especially when the concentrations are expressed as $THY_{eq.}$ The above partition convention of polyphenols into TP, CP, and SP could also be adopted.

Furthermore, the TP vs CP and CP vs SP comparisons can be employed as a measurement of the age of the oil. It is well-known that SP concentrations are higher when the oil is aged or artificially oxidized (Cioni et al., 1998; Montedoro et al., 1993).

In this way it should be possible to perform "...a collaborative study using the same analytical method to ensure that differences in magnitude..." *should* "...depend mainly on the variety" (Tsimidou, 1998).

Before such a study can be made, we think it is necessary to study, in detail, the influence of changes in milling conditions on the polyphenol content in oils. Indeed, preliminary data on oils produced from olives of the same cultivar, but processed with different millers, show large differences in the polyphenol content (obviously detected by the same analytical method) and in sensory features (Mugelli et al., 1998). A similar result was reported previously (Servili et al., 1994). A study to verify these data is now in progress.

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