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Solid Olive Residues: Insight into Their Phenolic Composition

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Solid olive residues (SOR) are byproducts of the olive-milling process, but they have an increasing importance in the pharmaceutical industry due to their rich content of biophenols. Such compounds are studied widely for their antioxidant and antimicrobial activities, but there is a lack of information about their quantitative recovery. This research highlighted the key role played both by the selection of the cultivar and by the degree of olive fruit ripening on the phenolic content on the SOR. The extraction methods were selected to reach the best quantitative results mainly using a safe food solvent. In light of the results the Soxhlet extraction with ethanol could be proposed as preferential for a higher recovery of verbascoside and its analogues.

KEYWORDS: Solid olive residues; extraction; Soxhlet; verbascoside; phenols; HPLC-DAD/MS

INTRODUCTION

Nowadays, two main milling processes are applied to obtain extra virgin olive oil: the three-phase system, which is used widely in Italy, Greece, and other Mediterranean countries, and the two-phase system, which is mainly used in Spain. In the first case, the obtained waste is olive mill waste water and a solid olive residue (olive pomace), whereas with the latter system, only a semisolid byproduct is obtained that contains both water and solid residue. In this field, the management of these wastes from olive oil production represents an ancient problem that remains unresolved even today. Some approaches toward this problem have been reported in the literature (*1*−*3*). The principal reasons for this gap are related to (a) the production of large quantities of these wastes in a seasonal period, not more than 2−3 months during a year and (b) the considerable number of olive fruit varieties and, consequently, the extreme variability of the waste composition.

At present, only a few papers reported in the literature focus on the evaluation of the phenolic content of waste waters (*4*−*6*) and of solid olive residues (SOR) from different milling processes (*7*−*9*). Several studies, developed in the past decade, have estimated the presence of contaminants (pesticides) in the SOR (*10*, *11*), the detoxification of this residue by the use of microorganisms (*12*−*15*), or reusing SOR as metal ion adsorbent (*16*).

The present work was aimed to investigate the phenolic content in several samples of fresh SOR, mainly from the cultivar Coratina harvested in various years, with the goal of selecting the best extraction procedure to increase the yields of phenylpropanoidic derivatives in the obtained extracts. No data are available in the literature on the content of these molecules within this byproduct. Moreover, many interesting activities have been attributed to this class of compounds as reported in the literature (*17*, *18*); in particular, they act as antioxidant (*19*), neurosedative, anti-inflammatory, antiviral, and anticancer agents (*20*). Recently, it was highlighted that verbascoside and its analogues from olive fruit can consistently contribute to the intake of antioxidants in the diet (*21*). Thus, the possibility to recover an extract enriched with these bioactive compounds, obtained from a low-cost and widely available byproduct in the Mediterranean basin, was evaluated.

MATERIALS AND METHODS

Oleuropein, caffeic acid, and luteolin 7-*O*-glucoside were purchased from Extrasynthese (Geney, France), tyrosol was from Sigma-Aldrich (St. Louis, MO), and hydroxytyrosol was purchased from Cayman Chemical (SPI-BIO-Europe). Hexane, ethyl acetate, methanol, ethanol, acetonitrile, hydrochloric acid, and formic acid were purchased from Mallinckrodt Baker BV (Deventer, The Netherlands). The duolite resin was from Rohm and Haas Ltd.

All of the analyzed samples, listed in **Table 1** together with their source and their corresponding date of crushing, were obtained by a continuous three-phase system mill. Each sample was representative of a batch of ∼100 kg of SOR.

Sample Preparation. *Hydroalcoholic Extraction.* A total EtOH/acid H_2O (7:3 v/v) extract from fresh SOR was prepared as follows: 25 g of each sample was submitted to the extraction (2 × 50 mL), with a hydroalchoholic solution, EtOH/H_2O (adjusted to pH 2 by HCOOH) 7:3, v/v. The sample was left under magnetic stirring at room temperature in the dark for 2 h. Defatting with hexane (3 × 25 mL) was performed to remove the lipid fraction, and the water−alcoholic extract of each sample was analyzed by HPLC-DAD.

Ethanolic Extraction. Twenty-five grams of fresh COR-03 was submitted to extraction (2 × 50 mL) with ethanol, and the sample was

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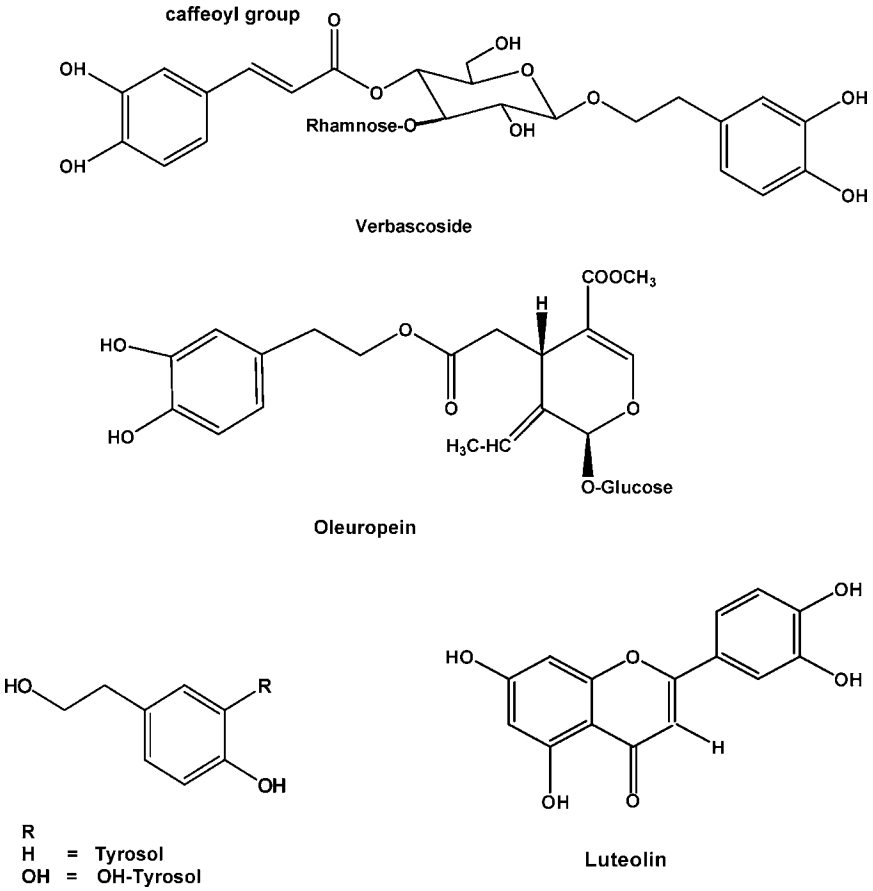


Figure 1. Chemical reference structures of compounds in the SOR samples.

Table 1. Fresh SOR Samples Analyzed

sample	origin (cultivar)	date of olive milling
RCORS1-00	Coratina	Nov 10, 1999
VOLVU2-00	mixed cultivars	Nov 21, 1999
VOL2-00	mixed cultivars	Nov 21, 1999
PER D-00	Peranzana	Nov 29, 2000
COR D-00	Coratina	Nov 29, 2000
COR1-00	Coratina	Nov 6, 2000
COR2-00	Coratina	Nov 14, 2000
COR3-00	Coratina	Dec 5, 2000
COR4-00	Coratina	Dec 13, 2000
COR-02	Coratina	Nov 5, 2002
COR-03	Coratina	Nov 22, 2003

left under magnetic stirring at room temperature in the dark for 2 h. Defatting with hexane (3 × 25 mL) was performed to remove the lipid fraction, and the water−alcoholic residue was analyzed by HPLC-DAD.

Soxhlet Extraction. The Soxhlet extraction was carried out with EtOH for 15 h with a drug/solvent ratio of 0.2 g mL^{-1}. The sample was concentrated to dryness under reduced pressure and dissolved in EtOH/H_2O (adjusted to pH 2 by HCOOH) 7:3, v/v.

Ethyl Acetate Extract (EtOAc). COR1-00 (2.2 kg) was extracted twice with 6 L of 70% $EtOH_{(aq)}$ acid by HCOOH (pH 3). The hydroalcoholic solution was concentrated under vacuum and extracted with ethyl acetate (5 × 2 L). The organic layers were pooled, concentrated under reduced pressure to dryness, and dried at 35 °C under vacuum overnight.

Methanol Extract from Resin (MeOH). COR1-00 (2.2 kg) was extracted twice with 6 L of 70% $EtOH_{(aq)}$ acid by HCOOH (pH 3). The hydroalcoholic layers were pooled and concentrated under vacuum, and the aqueous solution was purified by a duolite resin column, eluted with water and then with MeOH. This last fraction was concentrated under reduced pressure to dryness and dried at 35 °C under vacuum.

Apparatus. *HPLC-DAD Analysis.* All of the samples were filtered with a 0.45 μm regenerated cellulose filter before the HPLC-DAD/MS analysis. The analyses were carried out using a HP 1100L liquid chromatograph equipped with a DAD detector and managed by a HP 9000 workstation (Agilent Technologies, Palo Alto, CA). The elution method was as follows: multistep linear solvent gradient starting from 87% H_2O to 85% H_2O in 10 min; 10 min to 75% H_2O, then a plateau of 3 min to 5% H_2O in 2 min and a final plateau for 3 min. Total time of analysis was 28 min, equilibration time was 20 min, and flow rate was 0.8 mL min^{-1}. The column was a Varian Polaris C18-E (250 × 4.6 mm i.d., 5 μm) mantained at 26 °C with a precolumn of the same phase. Eluents were H_2O (pH 3.2 by HCOOH) and CH_3CN, all of HPLC grade.

HPLC-MS Analysis. The HPLC-MS analyses were performed using a HP 1100L liquid chromatograph linked to a HP 1100 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies). Spectra were recorded in negative ion mode, setting the fragmentation energy between 80 and 180 V and applying the same chromatographic conditions as described previously. The mass spectrometer operating conditions were as follows: gas temperature, 350 °C; nitrogen flow rate, 10.0 L min^{-1}; nebulizer pressure, 40 psi; quadrupole temperature, 40 °C; and capillary voltage, 3500 V.

Quantitative Analysis. Quantitative evaluation of individual phenols was performed by means of a four-point regression curve ($r^2 \geq 0.999$) using authentic external standards. The tyrosol (Tyr) and hydroxytyrosol (OH-Tyr) derivatives (**Figure 1**) were calculated at 280 nm using Tyr as reference; the secoiridoids (Ol Der.) were calculated at 280 nm using oleuropein as a standard. Verbascoside and its analogues and the other cinnamoyl derivatives were evaluated at 330 nm using caffeic acid as reference and, except for the latter compounds, a correction of molecular weight was applied. Luteolin was evaluated at 350 nm using luteolin as a pure standard.

RESULTS AND DISCUSSION

The aim of this research was to evaluate the phenolic content of several fresh SOR obtained from the milling processes of

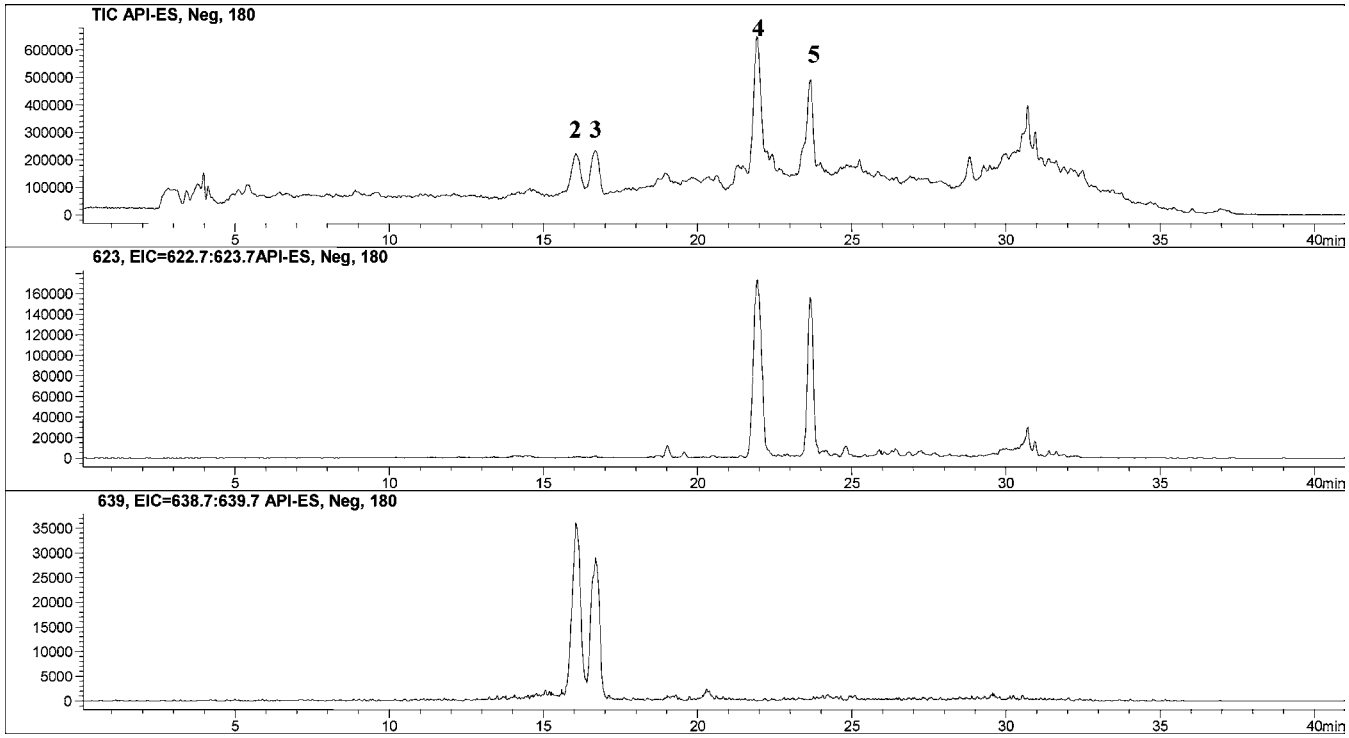


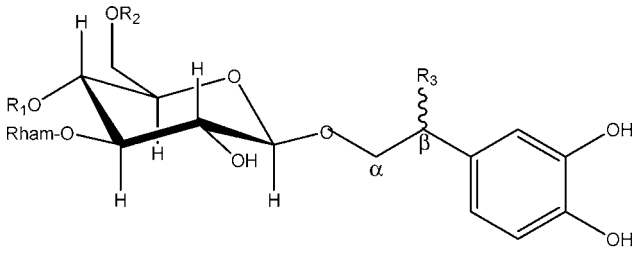
Figure 2. TIC and EI at *m*/*z* 623 and 639 for the COR-03 from 70% $EtOH_{(aq)}$. Peaks: 2 and 3, diastereoisomers of β-hydroxyacteoside; 4, verbascoside; 5, isoacteoside.

both single and mixed cultivar olive batches. **Table 1** shows all of the analyzed SOR samples with indications regarding their origin and the dates of milling processes. The comparison among all of the samples was first carried out by applying the same hydroalcoholic extractive procedure. Our previous works, focused on the extraction of the minor polar compounds from olive fruit (*8*), from extra virgin olive oils (*9*, *22*), and from olive oil waste waters (*6*, *17*) using the same acidic extractive solution, highlighted the chemical stability of the compounds in the applied experimental conditions.

MS Analyses. To identify the phenolic compounds, the extracts were analyzed by HPLC-DAD/MS, and the results were compared with previous findings obtained from the investigations of olive oil waste waters (*6*), olive fruit (*8*), and extra virgin olive oil (*9*, *22*). This approach was specifically applied to characterize the fraction of the cultivars Coratina and Peranzana, mainly constituted by verbascoside and other minor components with analogous structure. This technique was a very diagnostic tool for the identification of this class of compounds, as clearly shown by the comparison of the total ion current and EI profiles at *m*/*z* 623 and 639 obtained for the hydroalcoholic extract from COR-03 (**Figure 2**).

Figure 3 reports the general chemical structure of the main phenylpropanoidic derivatives identified in this work, in which are evidenced the groups linked to the central glucose and the different bonding positions.

Two compounds, more polar than verbascoside, were identified as two diasteroisomers of β-hydroxyacteoside (MW 640), with the presence of a hydroxyl group in the β-position of the 3,4-dihydroxyphenylethanol as the only difference with respect to verbascoside. **Figure 4** reports a typical fragmentation pattern obtained for these molecules showing, in addition to the molecular ion $[M - H]^-$, the ion species with *m*/*z* 621, due to the loss of a water molecule and the typical fragmentation pattern of a verbascoside derivative as reported in the literature (*23*). The presence of such structures has been described



Compounds	R_1	R_2	R_3
β-OH acteoside	caffeic acid	H	OH
isoacteoside	H	caffeic acid	H

Figure 3. General chemical structures of the identified verbascoside derivatives.

previously in another Oleaceae plant, *Forsitya viridissima* L. (*24*), but, to our knowledge, this is the first report of their presence in *Olea europaea* L.

Comparison of the UV−vis and MS spectra also confirmed the presence of isoacteoside, an isobaric isomer of verbascoside (MW 624), with the caffeoyl molecule linked to C_6 of the central glucose. This compound has been isolated recently in the olive fruit of Coratina (*21*).

Table 2 summarizes the ion species and their relative abundances obtained for these molecules by applying the HPLC-API/ES analysis in negative ionization mode.

Finding of Different Extractive Procedures. Among the considered cultivars, the hydroalcoholic extraction yields, expressed with respect to the fresh SOR, were quite similar in almost all of the samples ranging between 6.6 and 8.8 (**Table 3a**), with the only exception being VOLVU2-00, which gave the lowest value (5.9). As shown in **Table 3b**, when extractions different from the hydroalcoholic one, such as purification procedures of the hydroalcoholic extract, were applied, similar yields were obtained.

Table 2. Ion Species with Their Relative Abundances of the Identified Phenylpropanoidic Compounds, Obtained in Negative Ionization Mode and with Fragmentor 180

compound	ion species (relative abundances, %)
verbascoside	623 (87%) $[M-H]^-$; 461 (33%) $[M-H-162]^-$; 161 (100%) [caffeic acid – $H_2O]^-$; 179 (5%) [caffeic acid – $H]^-$
isoverbascoside	623 (100%) $[M-H]^-$; 461 (30%) $[M-H-162]^-$; 161 (73%) [caffeic acid – $H_2O]^-$; 179 (4%) [caffeic acid – $H]^-$
β-hydroxyacteoside diastereoisomers	639 (100%) $[M-H]^-$; 621 (38%) $[M-H-18]^-$; 459 (12%) $[M-H-18-162]^-$; 179 (35%) [caffeic acid – $H]^-$; 161 (80%) [caffeic acid – $H_2O]^-$

Table 3. Yields of the Dried Extracts[a]

(a) Hydroalcoholic Extraction

	RCORS1-00	VOLVU2-00	VOL2-00	COR1-00	COR2-00	COR3-00	COR4-00	CORD-00	PERD-00	COR-02	COR-03
yield (%)	6.6	5.9	7.6	7.5	8.3	7.6	8.8	7.5	8.0	8.1	7.8

(b) Other Procedures (See Materials and Methods)

	EtOAc (COR1-00)	MeOH (COR1-00)	EtOH (COR-03)	Soxhlet (COR-03)
yield (%)	6.7	5.9	6.5	8.2

[a] Data expressed with respect to the fresh weight of the SOR.

Table 4. Quantitative Results from the Hydroalcoholic Extraction (70% $EtOH_{(aq)}$)[a]

compound	RCORS1-00	VOLVU2-00	VOL2-00	COR1-00	COR2-00	COR3-00	COR4-00	CORD-00	PERD-00	COR-02	COR-03
Σ tyrosol + hydroxytyrosol	1.04	0.84	0.67	1.10	1.18	0.82	1.23	1.14	1.82	0.97	nq[b]
Σ oleuropein derivatives				0.08	0.08	0.06	0.10	0.19	0.14	nq	nq
caffeic acid	nq	nq	nq	nq	0.01	0.01	0.04	nq	nq	0.20	0.03
verbascoside	nq	nq	nq	3.90	3.30	1.03	1.10	1.40	0.61	0.15	4.15
Σ analogous verbascoside				0.88	0.69	0.42	0.70	0.97	0.51	0.13	1.03
Σ cinnamoyl derivatives	0.47	0.16	0.23	0.25	0.20	0.14	0.19	0.26	0.36	0.60	0.70
luteolin	0.16	0.23	0.19	0.02	0.03	0.03	0.06	0.32	0.08	nq	nq
total phenols	1.67	1.23	1.09	6.23	5.49	2.51	3.42	4.28	3.52	2.05	5.91

[a] Results expressed as milligrams per gram of fresh SOR. All of the data are a mean of three determinations with a percentage RSD below 5%. [b] Nonquantifiable compound due to low signal/noise ratio and/or low peak resolution.

When the data are evaluated from a quantitative point of view (**Table 4**), great differences among the samples can be seen. The total concentration of the phenolic compounds ranged between 1.1 and 6.23 mg g^{-1} of fresh SOR, and also the phenolic distribution among the different chemical classes was very different. These variations are probably due to several factors: type of cultivar, ripening degree, different milling process, and pedoclimatic factors.

The amounts of simple phenols (Tyr + OH-Tyr) were quite similar within each sample, with only the SOR from the cultivar Peranzana of harvest 2000 showing a little higher content.

Analogously, also the content of derivatives of oleuropein, caffeic acid, and luteolin showed very low content in all of the samples, whereas slightly higher amounts, between 0.14 and 0.7 mg g^{-1} of fresh SOR, were observed for the cinnamoyl derivatives.

The greatest differences among the samples were observed for verbascoside, which ranged between 0.15 and 4.15 mg g^{-1}, and for its less abundant analogues. The latter compounds were found only in the fresh SOR from cultivars Coratina and Peranzana. Such data agree with a previous investigation carried out on brined Coratina olives that showed verbascoside and isoverbascoside, along with simple phenols, as the major components present in the fruits from this cultivar (*21*).

According to previous papers (*8*, *25*−*27*), verbascoside or acteoside could be proposed as a marker for differentiating the cultivars. Moreover, in agreement also with previous observations, our data suggest that verbascoside could be used as a tool to differentiate olives of the same cultivar but at different

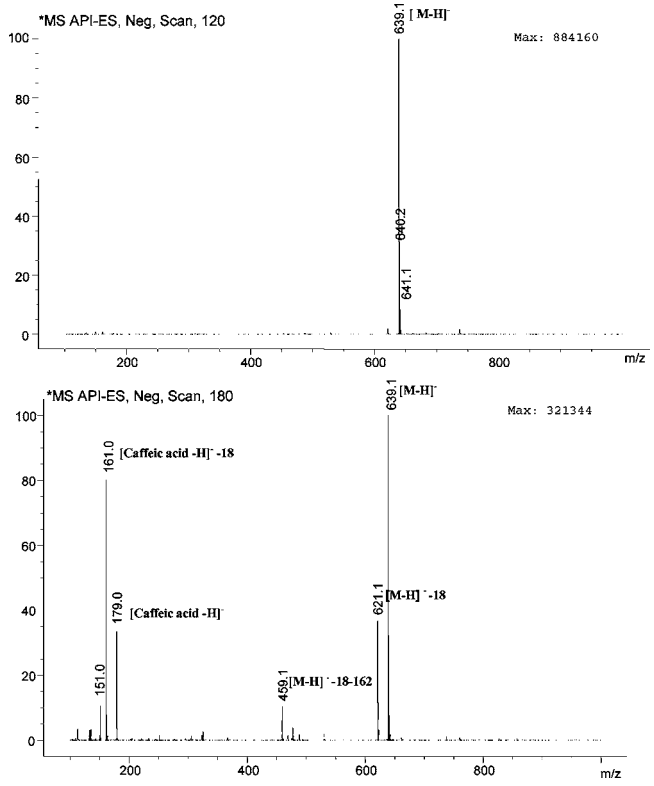


Figure 4. MS spectra in negative ionization mode of diastereoisomers of β-hydroxyacteoside.

Table 5. Phenolic Content in Extracts Obtained by Different Methods Applied to COR1-00 and COR-03 Samples[a]

	mg/g of dried extract					
	COR1-00			COR-03		
	EtOAc (%)	MeOH (%)	EtOH/H_2O (%)	EtOH/H_2O (%)	EtOH (%)	Soxhlet (%)
Σ tyrosol + hydroxytyrosol	2.4	1.9	14.7	nq[b]	nq	nq
verbascoside	79 (51.5)	91.2 (59.7)	51.4 (63.4)	53.5 (70.3)	59.0 (69.7)	86.4 (59.2)
Σ analogous verbascoside	21.2 (13.9)	24 (15.7)	11.8 (14.5)	13.2 (17.3)	14.5 (17.1)	46.7 (32.9)
caffeic acid	nq	nq	nq	0.4	0.35	0.3
Σ cinnamoyl derivatives	50.7 (33.1)	36 (23.5)	3.6 (4.4)	8.97 (11.8)	10.7 (12.6)	8.6 (6.1)
total phenols	153.3	153.1	81.5	76.1	84.6	142

[a] All data are a mean of three determinations with a percentage RSD below 5%. The numbers in parentheses indicate the percent with respect to the total phenols. [b] Nonquantifiable compound due to low signal/noise ratio and/or low peak resolution.

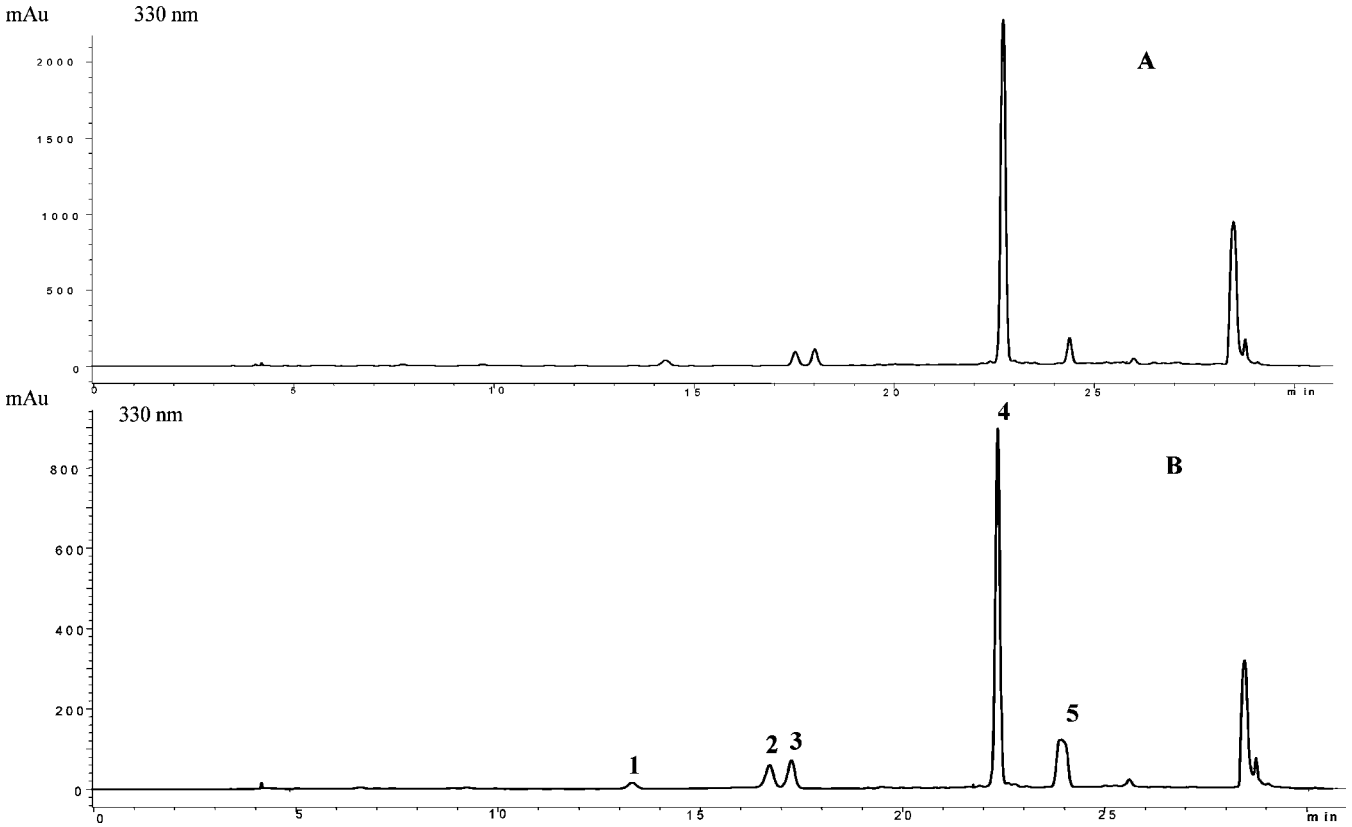


Figure 5. Comparison of the HPLC-DAD profiles at 330 nm of the COR-03 extract obtained from (**A**) Soxhlet and (**B**) 70% $EtOH_{(aq)}$. Peaks: 1, caffeic acid; 2 and 3, diastereoisomers of β-hydroxyacteoside; 4, verbascoside; 5, isoacteoside.

grades of ripening (*27*). Nevertheless, to the best of our knowledge, scant information has been available in the literature on this topic up to now. From findings in **Table 4**, the amount of verbascoside in the fresh SOR greatly decreased from early (COR1-00) to later milling dates (COR4-00). Moreover, it is known that in the extra virgin olive oils, the highest phenolic contents are obtained from fruit at a low degree of ripening. Within the considered samples the very low content in verbascoside and its analogues of the COR-02 can be correlated with previous evidence obtained for corresponding extra virgin olive oils (*22*). In fact, the fruits harvested in southern Italy in 2002 were characterized by olives that were partially damaged by *Bactrocerae oleae.* This factor reduced the total phenols in the oils and, as expected, also in the corresponding SOR sample.

Moreover, phenols contained in SOR belong to different classes with a large range of physicochemical properties, making the selective extraction a difficult task. To recover the main phenolic compounds quantitatively, various types of extraction procedures on fresh SOR were carried out. To evaluate the efficiency of these extractive procedures, the two richest SOR samples with a phenylpropanoidic content of >70% of the total phenols (**Table 4**) were chosen as reference material. Because it is known that the stability of many phenols is pH dependent and alkaline conditions lead to oxidation and/or polymerization, all of the procedures were carried out in neutral or acidic media (*28*).

First, purification steps were applied on the hydroalcoholic extract from COR1-00 followed by a liquid/liquid extraction with ethyl acetate or fractionation by the use of an ion-exchange resin. In the first case the use of the organic solvent increased the amount of more lipophilic compounds with respect to the methanol elution from the ion-exchange resin (**Table 5**). As expected, the applied purification steps were able to improve the total phenolic content with respect to the hydroalcoholic extract. Nevertheless, the data for the COR1-00 sample (**Table 5**) suggest that the hydroalcoholic extraction is slightly selective for the phenylpropanoidic compounds with respect to the other two time-consuming methods. In addition, it is well-known that ethanol is one of the few solvents that fulfills the requirements for safe use in food preparation. Consequently, to consider the

possible application of the described procedure in the alimentary field, other experiments were performed using only ethanol as organic solvent. Due to the unavailability of fresh SOR after some years, these two latter extractions were not applied to COR1-00, but rather a raw material of very similar phenolic composition, COR-03 (**Table 4**), was chosen. It must be emphasized that the data in **Table 5**, even if obtained from different time extraction methods, came from the application of exhaustive extractions.

From the data for sample COR-03 two main results can be highlighted: the total phenolic content varied widely, ranging from 76.1 to 142 mg g^{-1} of dried extract, and the Soxhlet extraction was more efficient in terms of quantitative yields. This latter result, together with the qualitative HPLC profiles (**Figure 5**), indirectly confirms the chemical stability of these phenols when submitted to a hot treatment (~80 °C) with EtOH for several hours.

In light of our results, the first part of this research pointed out the richest sources of phenolic compounds among the considered samples and the key role played both by the selection of the cultivar and by the degree of olive fruit ripening. In the second part of this work extraction methods were selected not only to reach the best quantitative results from an analytical point of view but also from the perspective of a possible scale-up development using mainly a food-safe solvent. From our findings, the Soxhlet extraction with ethanol could be proposed as preferential for a higher recovery of verbascoside and its analogues

This study contributes to increasing the knowledge of this widely available waste in the Mediterranean basin and opens new perspectives for the recovery of valuable byproducts deriving from the milling process.

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NOTE ADDED AFTER ASAP PUBLICATION

The milling processes to obtain extra virgin olive oil were incorrectly named in the original posting of October 13, 2005. These names in the first paragraph of the Introduction have been corrected in the posting of October 25, 2005.

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