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Simple phenolic content in olive oil residues as a function of extraction systems

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Abstract

Olive oil residues were tested for their composition in simple phenolic compounds as a function of the extraction system, i.e. the three- and two-phase centrifugation systems. Phenolic compound extraction with ethyl acetate was efficient and allowed recovery of 28.8 and 42.2% of total phenols present in dry olive oil residues originating from three-phase and two-phase systems, respectively. The qualitative and quantitative HPLC analyses of the extracts showed that hydroxytyrosol and *p*-tyrosol were the most abundant phenolic compounds. *p*-coumaric, caffeic, ferulic and vanillic acids were also present. The phenolic extract from the two-phase system had the highest concentration in hydroxytyrosol (1.16% (w/w) dry residue) and the strongest antioxidant activity. Olive oil residues were confirmed as a cheap source of large amounts of natural phenolic antioxidants.

Keywords: Phenolic compounds; Olive oil residue; Extraction system; Hydroxytyrosol; *p*-tyrosol; DPPH; Antioxidant

1. Introduction

The olive oil market has recently developed, since the ''Mediterranean diet'' is widely appreciated throughout the world by consumers more attentive to both health and nutritional aspects of food. The increasing popularity of olive oil has been mainly attributed to (1) its high content of oleic acid, which may affect the plasma lipid/lipoprotein profiles (Delplanque, Jusselin, Le Roy, & Motta, 1999) and (2) its richness in phenolic compounds acting as natural antioxidants, which may contribute to the prevention of human disease (Fito et al., 2000; Saija et al., 1998; Visioli, Bellomo & Galli, 1998; Visioli & Galli, 1998).

Usually, olive oil is extracted mechanically by pressure and by a three-phase centrifugation system, which results in the production of more than 30 million m^3 of black olive mill wastewater (OMWW; Borja, Alba, & Banks, 1997; Hamdi & Ellouz, 1992). This liquid effluent has a high polluting organic load, due to a high content of organic substances, including sugars, tannins, polyphenols, polyalcohols, pectins, and lipids (Balice & Cera, 1984; D'Annibale, Crestini, Vinciguerra, & Giovannozzi Sermanni, 1998; Sayadi & Ellouz, 1995). Centrifugation, despite its high water consumption (around 0.6 m^3 per ton of olives processed), is still the most widely employed method for production of virgin olive oil, especially in countries that produce large amounts of olives in a short time (Benitez, Beltran-Heredia, Torregrosa, Acero, & Cercas, 1997). Most frequently, OMWW are pumped and discharged into evaporation ponds or directly dumped in rivers or spread on soil (Greco, Toscanoa, Cioffi, Gianfreda, & Sannino, 1999). This becomes a major environmental problem in the main olive-producing countries of the Mediterranean region, such as Italy, Spain, Greece, Tunisia and Turkey. It is known that phenolic compounds are major contributors to the toxicity and the antibacterial activity of OMWW, which limit its microbial degradability (Borja, Alba, & Banks, 1997; Capasso, Evidente, Schivo,

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Orru, Marcialis, & Cristinzio, 1995). However, these phenolic compounds possess strong antioxidant properties (Galli & Visioli, 1999), which may turn the olive oil residues into a cheap source of natural antioxidants.

Recently, the olive oil industry has adopted a new continuous centrifugation system with a two-phase decanter, which separates the virgin olive oil by recycling vegetative water of processed olives (Di Giovacchino, 1994). This technology considerably decreases the volume of plant effluent and the disposal problems (Moreno, Benitez, Melgar, Polo, Gomez, & Nogales, 2000). While the impact of the extraction system on virgin oil quality is well known (Bianchi, 1999; De Stefano, Piacquadio, Servili, Di Giovacchino, & Sciancalepore, 1999; Piacquadio, De Stefano, & Sciancalepore, 1998), information dealing with the effects of extraction processes on OMWW composition is scarce, especially on phenolic compound composition.

The objective of this work was to investigate the effects of the classical three-phase centrifugation system and the two-phase centrifugation system on the phenolic composition of olive oil residues. Simple phenolic compounds were quantified and studied with regard to their antioxidant potentialities.

2. Materials and methods

2.1. Chemicals

Ethyl acetate, methanol and acetonitrile were HPLC-grade solvents from SDS (Peypin, France) and ethanol was pure grade solvent. 1,1-diphenyl-2-picrylhydrazyl (DPPH), tyrosol (>95%), caffeic acid (>98%), *p*-coumaric acid (>98%), ferulic acid (>98%), vanillic acid (>98%), vanillin (>98%) and oleuropein (>98%) were analytical-grade reagents purchased from Sigma-Aldrich Chemical Co. (St-Louis, MO, USA). Hydroxytyrosol (purity around 90%) was a gift of Dr Sayadi (Center of Biotechnology, Sfax, Tunisia).

2.2. Olive oil residues

Olive oil residues were collected during the 1999–2000 harvest season in the French Provence-Alpes-Côte-d'Azur area from olive mills. In the La Cravenco cooperative (Raphèle-les-Arles, France), olive oil was extracted using a classical three-phase centrifugation system: leafless and washed olives were crushed, kneaded and then extracted with a horizontal centrifugal three-phase decanter. This extraction process requires 60–70 l fresh water/100 kg of olives processed to separate the oil from the other two phases, wastewaters and olive husk. In the Mouries cooperative, olives were processed using a new centrifugation system with a horizontal centrifugal two-phase decanter (Ecological Decanter, Rapanelli, Italy), leading to a single wet solid residue, composed of olive pulp and husk.

Fresh olive oil residues from the three-phase system were stabilised with ethanol [final concentration 30% (v/v)] and then clarified by filtration and centrifugation (9000 rpm, 10 min). Fresh wet solid residues issued from the two-phase centrifugation system were diluted with water, stabilised with 30% (v/v) ethanol, then homogenised and clarified by hydraulic pressing. Stabilisation of the olive oil residue was found to be an essential step to prevent enzymic and non-enzymic oxidative reactions responsible for phenolic compound degradation (Perrin, 1992). The ethanol-stabilised and clarified olive oil residues recovered from both the three- and two-phase systems were kept at 4 °C in the dark. Their phenolic compositions determined by HPLC, were stable for several months under these conditions (data not shown).

2.3. pH and dry matter measurements

pH was determined in the stabilised olive oil residues. Dry matter was estimated by weighing freeze-dried samples.

2.4. Phenolic compound extraction

Stabilised and clarified olive oil residues, originating from both extraction systems, were adjusted to pH 3 with HCl and extracted with ethyl acetate (1:1, v/v) two times. The ethyl acetate phase was evaporated to dryness and the residue redissolved in methanol for subsequent analyses.

2.5. Alkaline treatment of phenolic extracts

Alkaline hydrolysis of the phenolic extracts was performed with 2 M NaOH at 35 °C for 30 min according to Saulnier, Vigouroux, & Thibault (1995). The mixture was then acidified to pH 2 with HCl and extracted twice with ethyl acetate. After evaporation, samples were dissolved in methanol and analyzed by HPLC.

2.6. Phenol content determination

The total phenol content was determined colorimetrically at 750 nm, using the Folin-Ciocalteu reagent (Folin & Ciocalteau, 1927) and expressed as gallic acid equivalents (g/l residue or g/100 g dry residue). When necessary, the samples were diluted by a suitable mixture of methanol:H_2O (1:1, v/v) and the calibration curve was established from 0 to 100 mg/l.

2.7. Antiradical activity

The antiradical properties of samples were evaluated according to Goupy, Hugues, Boivin, and Amiot (1999).

Two millilitres of a methanolic solution of the sample to be tested was added to an equal volume of a methanolic solution containing 60 μM DPPH. After 30 min in the dark, the absorbance was recorded at 517 nm and compared to the control. Antiradical activity was defined as the concentration of sample (EC_{50}) necessary to decrease the initial DPPH radical concentration by 50%. EC_{50} was expressed in mg total phenols (as gallic acid equivalents)/litre of reaction medium. All measurements were duplicated and repeated at least three times.

2.8. HPLC analysis

HPLC analyses were performed at 280 nm and 30 °C on a HP model 1050 (Hewlett-Packard, Rockville, MD) equipped with a variable UV/VIS detector, a 34-position autosampler-autoinjector. Separations were achieved on a Waters (St-Quentin Yvelines, France) C-18 reversed-phase column (Symetry 3.5 μm, 4.6×100 mm). The flow rate was 0.7 ml/min. The mobile phase used was 0.01% acetic acid in water (A) versus acetonitrile (B) for a total running time of 50 min, and the gradient changed as follows: solvent B started at 5% for 2 min, then increased to 10% in 6 min, to 20% in 17 min, to 70% in 20 min and to 100% in 1 min until the end of running. The data were processed by a HP 3365 ChemStation and the quantification was performed by external standard calibration. The identity of phenolic compounds was confirmed by LC-mass spectrometry using a Perkin-Elmer ApI150EX apparatus (Perkin-Elmer Applied Biosystems, Courtaboeuf, France) equipped with an electrospray ionisation using a 20-V orifice voltage and a nitrogen nebuliser pressure of 45 psi (3.0 bar).

3. Results and discussion

3.1. Total phenol content, pH, and dry matter in olive oil residues

The stabilised and clarified olive oil residues were analysed for pH, dry matter, and total phenol content (Table 1). As a consequence of the continuous washing with water during the process (Piacquadio, De Stefano, & Sciancalepore, 1998), the dry matter of olive oil residues, originating from the three-phase system, was two-fold lower than that of residues from the two-phase system. However, total phenol contents, estimated as gallic acid equivalents of dry residue, were found to be higher for the three-phase system, i.e. 24% instead of 20.4% (w/w) for the two-phase system. It is suggested that, in the three-phase procedure, phenolic compounds would be largely extracted during the continuous washing applied to the olive paste and end up in the aqueous olive oil residues (Visioli, Vinceri, & Galli, 1995). The pH appeared to be a little higher than that reported in the literature, often found close to 5 (Martin, Borja, Garcia, & Fiestas, 1991).

3.2. Extraction of phenolic compounds

Ethyl acetate extracted low amounts of dry matter, i.e. 9.6% (w/w) dry residue for the three-phase system and 14.2% for the two-phase system, but the major part of these extracts was composed of phenolic compounds, 72.4% for the three-phase system and 60.7% for the two-phase system (Table 2). In addition, by comparing data from Tables 1 and 2, phenolic compound extraction with ethyl acetate was shown to be efficient; i.e. 28.8 and 42.2% of total phenols present in dry olive oil residues were recovered from the three-phase and two-phase systems, respectively.

Table 1
Characteristics of olive oil residues originating from the three- and two-phase systems

Parameters	Olive oil residues[a,b]	
	Three-phase system	Two-phase system
Dry matter (g/l)	30.4	62.7
Total phenol content (% (w/w) dry residue)[c]	24.0	20.4
pH	5.7	5.7

[a] Values are means of duplicate analyses and did not differ by more than 5%.
[b] Residues were previously stabilised with ethanol and clarified just after being collected (see Section 2).
[c] Determined as gallic acid equivalents by Folin–Ciocalteu method.

Table 2
Characteristics of ethyl acetate extracts of olive oil residues originating from the three- and two-phase systems

Parameters	Ethyl acetate extracts	
	Three-phase system	Two-phase system
Dry matter[a]		
g/l of residue[b]	2.9	8.9
% (w/w) of dry residue[c]	9.6	14.2
Total phenol[a,d]		
g/l of residue[b]	2.1	5.4
% (w/w) of dry extract	72.4	60.7
% (w/w) of dry residue[c]	6.9	8.6
% (w/w) of total phenols present in dry residue[c]	28.8	42.2

[a] Data are means of duplicate analyses and did not differ by more than 5%.
[b] Values are given as dry matter or total phenols recovered in the ethyl acetate extracts and corresponding to 1 l of olive oil residues.
[c] Values were calculated using data from Table 1.
[d] Determined as gallic acid equivalents by Folin–Ciocalteu method.

3.3. Phenolic extract analysis

Both phenolic extracts exhibited similar chromatographic profiles (Fig. 1). Series of simple phenols were identified by HPLC in the olive oil residues by comparing retention times and mass spectra with standards (Fig. 2). Results confirmed the efficiency of ethyl acetate for recovering low-weight molecular phenolic compounds from olive oil residues (Visioli et al. 1999).

The negative ion spectra of hydroxytyrosol and *p*-tyrosol, showed molecular ions at m/z 153 and 137, respectively (data not shown). In contrast, for all the other compounds detected, namely, caffeic acid, *p*-coumaric acid, ferulic acid, vanillic acid, and vanillin, the protonated and nonprotonated molecular ion peaks were observed in the positive and negative ion spectra, respectively.

Hydroxytyrosol and *p*-tyrosol were the major compounds detected. The values for the two-phase extract were significantly higher (1.4-fold) than those obtained for the three-phase extract (Fig. 2A, C). In addition, vanillin and various phenolic acids, such as caffeic acid, *p*-coumaric acid, ferulic acid, vanillic acid, were quantified as a function of the process (Fig. 2B, D). Their amounts ranged from 2 to 30 mg/100 g of dry residue, depending on the molecule and the type of system. The highest values were obtained with the phenolic extract issued from the two-phase system, except for vanillin. The extraction system modified quantitatively, but not qualitatively, the phenolic composition of the residues. Similarly, it was already reported that these extraction systems did not qualitatively alter the phenolic composition of olive oil, but affected their concentrations (De Stefano et al., 1999; Montedoro, Servili, Baldioli, & Miniati, 1992). Other phenolic acids, such as protocatechuic acid, veratric acid, syringic acid, cinnamic acid or *p*-hydroxyphenylacetic acid, found in OMWW by Balice and Cera (1984) and Lafont et al. (1999), were not detected in the ethyl acetate extracts.

Oleuropein, an ester of elenolic acid and hydroxytyrosol, found by Visioli et al. (1995) as a major compound of OMWW, was not detected in the studied residues. Olive fruit polyphenols, generally associated with glucosides by ester or ether linkages, are possibly degraded during the oil extraction process (Perrin, 1992). This was probably the case with oleuropein in the studied samples, since abundant contents in hydroxytyrosol were mainly recovered in olive oil residues.

In order to partly characterise the structure of the phenolic compounds recovered by ethyl acetate extraction, alkaline hydrolysis was performed. While no variation was observed in the quantities of hydroxytyrosol

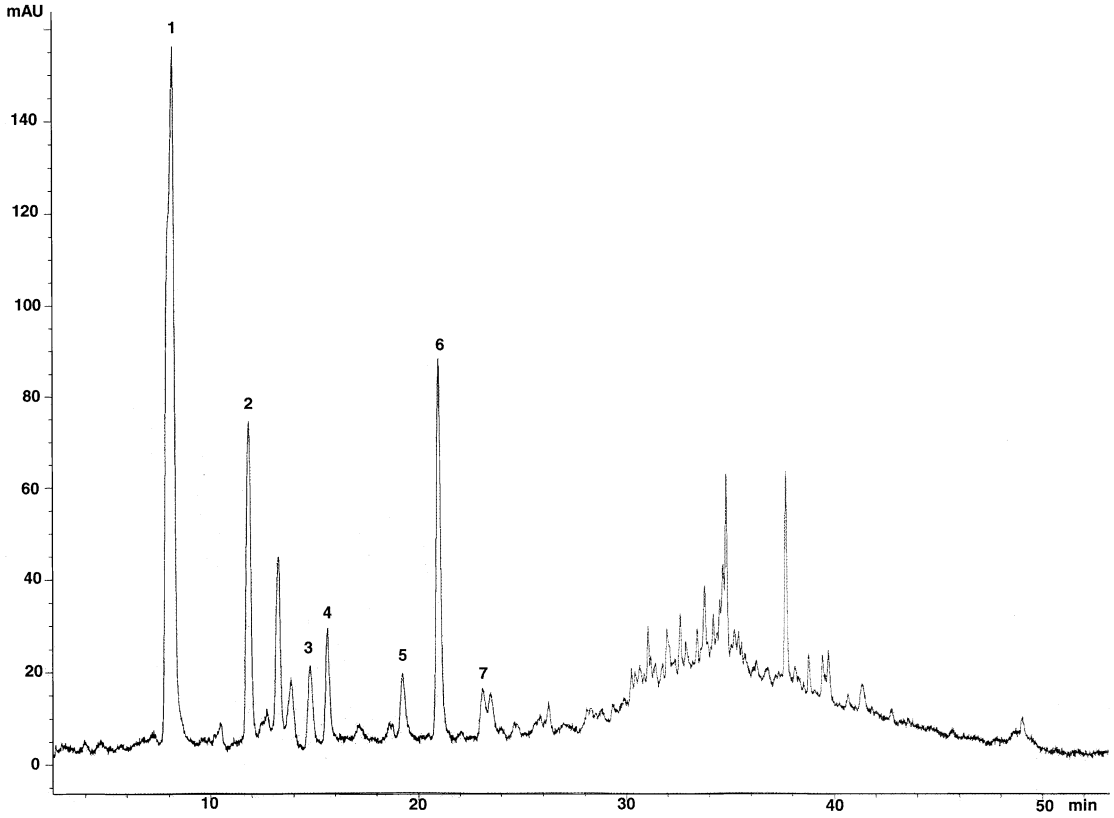

Fig. 1. HPLC chromatogram of phenolic extracts of olive oil residues originating from the three-phase system. For the extract from the two-phase system, the chromatogram was similar. Peak identities: 1, hydroxytyrosol; 2, *p*-tyrosol; 3, vanillic acid; 4, caffeic acid; 5, vanillin; 6, *p*-coumaric acid; 7, ferulic acid.

recovered after alkaline hydrolysis, the release of tyrosol, *p*-coumaric and caffeic acids was observed (Fig. 2). These results suggest that esterified linkages occurred with most simple phenolic molecules, except for hydroxytyrosol, which is supposed to be easily released during the extraction processes.

3.4. Antioxidant activities on phenolic extract

Phenolic extracts, originating from the three- and two-phase systems, were tested for their antioxidant activity (Table 3), using the stable free radical DPPH as test (Goupy et al., 1999). The two-phase extract exhibited

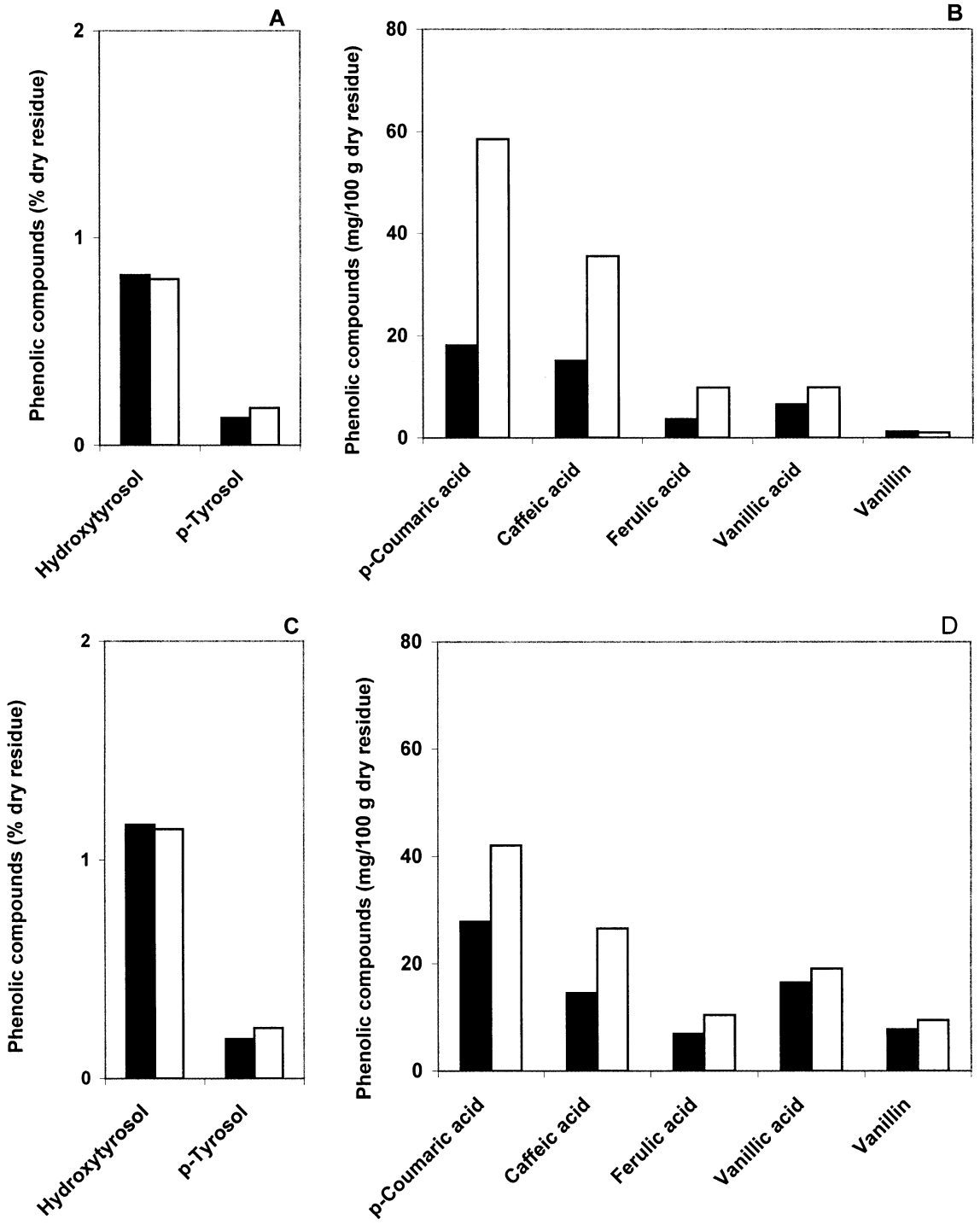

Fig. 2. Quantification of simple phenolic components identified in phenolic extracts of olive oil residues originating from the three-phase (A and B) and the two-phase (C and D) systems, before (■) and after hydrolysis (□).

Table 3
Antiradical activities using DPPH test

Phenolic extracts	EC^{a}_{50}
Three-phase system	5.27
Two-phase system	2.61

[a] Antiradical activity was defined as the concentration of sample (EC_{50}) necessary to decrease the initial DPPH radical concentration by 50%; values are means of triplicate analyses that did not differ by more than 5%.

the highest antiradical activity, with a value 2-fold greater than that of the three-phase extract. This result was attributed to the highest concentrations of antioxidant phenolic compounds, such as hydroxytyrosol, in the two-phase extract. Hydroxytyrosol is an orthodiphenol compound and was shown to exert a potent antioxidant activity (Galli & Visioli, 1999; Manna, Galetti, Cucciolla, Montedoro, & Zappia, 1999). It would thus contribute to the potential biological properties of olive oil (Ryan & Robarts, 1998) and olive oil waste waters (Visioli et al., 1995).

4. Conclusion

For the first time, the olive oil residues were tested for their composition in simple phenolic compounds as a function of extraction systems, i.e. the three-phase centrifugation system, generating large volumes of olive mill wastewaters and the "ecologically attractive" two-phase system, which produces good-quality olive oil without water addition. Ethyl acetate was confirmed to be a suitable solvent for recovering simple phenolic compounds from olive mill residues. This solvent is very selective for low and medium molecular weight phenolic molecules. The high antioxidant potential of the phenolic extracts was related to their high contents of hydroxytyrosol.

This work confirms the interest of olive oil residues, especially those issued from the two-phase olive oil extraction system, as a cheap source of natural antioxidant phenolic compounds, in concentrations 100-fold higher than in olive oil.

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