High-Performance Liquid Chromatography Evaluation of Phenols in Virgin Olive Oil During Extraction at Laboratory and Industrial Scale

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ABSTRACT: Phenolic compounds are of fundamental importance to the quality and nutritional properties of virgin olive oils. In this paper, the high-performance liquid chromatographic analysis of simple and complex olive oil phenols in the streams generated in the two-phase extraction system was carried out using Arbequina and Picual olives. The malaxation stage reduced the concentration of orthodiphenols in oil *ca.* 50–70%, while the concentration of the nonorthodiphenols remained constant, particularly the recently identified lignans 1-acetoxypinoresinol and pinoresinol. Oxidation of orthodiphenols at laboratory scale was avoided by malaxing the paste under a nitrogen atmosphere. Phenolic compounds in the wash water used in the vertical centrifuge were also identified. Hydroxytyrosol, tyrosol, and the dialdehydic form of elenolic acid linked to hydroxytyrosol were the most representative phenols in these waters. Hence, phenolic compounds in the wash waters came from both the aqueous and the lipid phases of the decanter oily must.

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KEY WORDS: HPLC analysis, oil nitrogen, phenols, two-phase extraction system, virgin olive oil.

Virgin olive oil, unlike other vegetable oils, is consumed unrefined and, consequently, is rich in phenolic compounds. These polyphenols enhance the resistance to autoxidation of the oil (1) and contribute to its pungent and bitter taste (2). Both ortho- and nonorthodiphenols of olive oil have been shown to exert, *in vitro*, potent biological activities (3).

The most important classes of phenolic compounds in virgin olive oil include phenyl acids, phenyl alcohols, flavonoids, secoiridoids, and lignans. The main phenyl alcohols of virgin olive oil are 3,4-(dihydroxyphenyl)ethanol or hydroxytyrosol (Hy) and *p*-(hydroxyphenyl)ethanol or tyrosol (Ty). The flavonoids include luteolin and apigenin (4), and *p*-coumaric, vanillic, and ferulic acids are the most representative phenyl acids. However, the prevalent phenolic compounds in virgin olive oils are the secoiridoid derivatives, such as the dialdehydic form of elenolic acid linked to hydroxytyrosol (Hy-EDA) or tyrosol (Ty-EDA) and isomers of oleuropein aglycon (Hy-EA) or ligstroside aglycon (Ty-EA) (5,6). The most recent compounds identified in olive oil have been 4-(acetoxyethyl)-1,2-dihydroxybenzene (Hy-AC) (7) and the lignans pinoresinol and 1-acetoxypinoresinol (8).

Arbequina cultivar yields oil that contains a great amount of lignans (8) and valuable organoleptic characteristics (9). The production of Arbequina oils is rapidly expanding in Spain, although Picual oils are the most-consumed olive oils.

Nowadays, the industrial processing of olive oil in Spain includes four defined steps: crushing of fruits, malaxation of the resulting paste, separation of the oil by the two-phase decanters, and washing of the oil with tap water by using a vertical centrifuge (10,11). It has been reported that at laboratory scale the concentration of phenolic compounds in oil diminishes with increasing malaxation time (12,13), although this behavior may be different if stone mills are used to crush the fruits (14). Moreover, the amount of phenols in oil also depends on the type of extraction system and the temperature during malaxation (15,16).

The oily must obtained from the decanter centrifuge, when using the two-phase extraction system, contains impurities that must be removed by washing the oil in a vertical centrifuge with tap water. This wash water represents a new type of effluent (17), and there are no reports in the literature on the phenolic composition of this stream.

This research was undertaken with three purposes in mind. The first purpose was to compare the difference in oxidation of orthodiphenols when processing olives at laboratory and industrial scales. The second was to study the use of nitrogen during processing to avoid the oxidation reactions. The third was to evaluate the content in polyphenols, particularly the recently identified lignans 1-acetoxypinoresinol and pinoresinol, in the different streams generated during extraction of olive oil at industrial scale as well as their changes.

MATERIALS AND METHODS

Olives. Fruits used in this study were of the Arbequina (Chucena, Huelva, Spain) and Picual (Villacarrillo, Jaén, Spain) cultivars. The degree of ripening, estimated by fruit color (18), was 2.0 and 4.7 for Arbequina and Picual fruits, respectively.

Olive processing at laboratory scale. Virgin olive oil was obtained by an Abencor analyzer (Comercial Abengoa, S.A., Seville, Spain), consisting of three basic elements: a mill, a thermobeater, and a pulp centrifuge (19). Milled paste was malaxed without adding water for 45 min at 30ºC under air or

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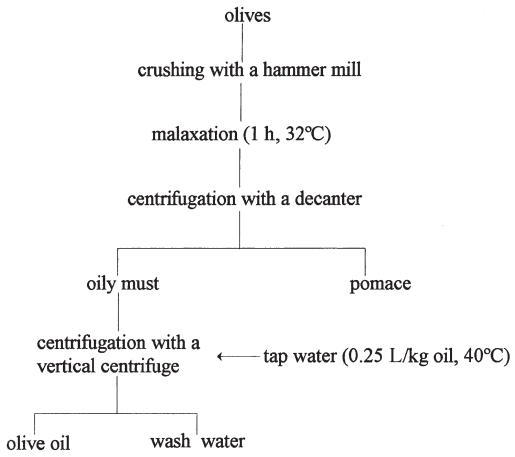

SCHEME 1

nitrogen atmosphere. Assays were run in triplicate, and oil samples were passed through paper filter before analyzing.

Olive processing at industrial scale. A two-phase continuous extraction system (Scheme 1) was employed (Pieralisi Model SC-45; Jesi, Ancona, Italy). Olives (1500 kg) were crushed by using an inox hammer mill, operating at 3000 rpm, that was equipped with a sieve with 5-mm holes. Malaxation of pastes was made in a mixer at 14 rpm and 30ºC for 1 h. Separation of the paste into oily must and pomace was performed by a two-phase centrifugal decanter working at 3500 rpm. Finally, a vertical centrifuge at 40°C, operating at 6500 rpm and fed with 0.25 L tap water/kg oily must, was used to remove the remaining solids from the must. Olive oil was also obtained from crushed paste by centrifuging the paste in a laboratory centrifuge (Sorvall RC-5, Norwalk, CT) for 10 min at 10,000 rpm.

Oils from the decanter, vertical and laboratory centrifuges were filtered through sodium sulfate before analysis.

Analysis of phenolic compounds. The phenolic extract of virgin olive oil was obtained following the procedure described elsewhere (20). Briefly, 14 g of virgin olive oil was extracted using 4 × 14 mL of methanol/water (80:20, vol/vol). After methanol had been removed, the residue was taken up in 15 mL of acetonitrile. Washings with hexane (3 × 20 mL) were performed, and the resulting acetonitrile solution was evaporated under vacuum, giving a residue that was dissolved in 1 mL of methanol.

Phenolic compounds were also analyzed in the water that accompanied the oily must and from the wash water of the vertical centrifuge. The oily must was centrifuged at 10,000 rpm for 10 min (Sorval RC-5), and the water phase was taken from the centrifuge tube using a Pasteur pipette. Water samples from both the decanter and vertical centrifuges were diluted with distilled water (1:10, vol/vol), acidified with phosphoric acid to pH 3, centrifuged, and, finally injected into the chromatograph. The phenolic content was expressed as mg phenols/kg oil, considering the humidity of the oily must that was previously determined by heating at 105ºC.

High-performance liquid chromatography (HPLC) analysis of phenolic compounds. The HPLC system consisted of a Waters 717 plus autosampler, a Waters 600 E pump, a Waters column heater module, and a Waters 996 photodiode array detector operated with Millennium 2010 software (Waters Inc., Milford, MA). A Spherisorb ODS-2 (5 µm, 25 cm by 4.6 mm i.d.; Tecknokroma, Barcelona, Spain) column was used. Separation was achieved by elution gradient using an initial composition of 90% water (pH adjusted to 3.1 with 0.2% acetic acid) and 10% methanol. The concentration of the latter solvent was increased to 30% in 10 min and maintained for 20 min. Subsequently, the methanol percentage was raised to 40% in 10 min, maintained for 5 min, increased to 50% in 5 min, and maintained for another 5 min. Finally, the methanol percentage was increased to 60, 70, and 100% in 5-min periods. Initial conditions were reached in 15 min. A flow of 1 mL/min and a temperature of 35ºC were also used. Reference compounds were obtained as described elsewhere (7).

RESULTS AND DISCUSSION

Phenolic composition in virgin olive oils obtained at laboratory scale. Figure 1 shows the phenolic chromatograms of the Picual and Arbequina virgin olive oils, which are very different, and the identity of the phenolic compounds in olive oil (5,7,8). As reported before (7), Hy-EA peak was higher for Picual than Arbequina and, in contrast, 1-acetoxypinoresinol peak was higher for Arbequina than Picual which may be useful to distinguish between oils of these two cultivars. The ul-

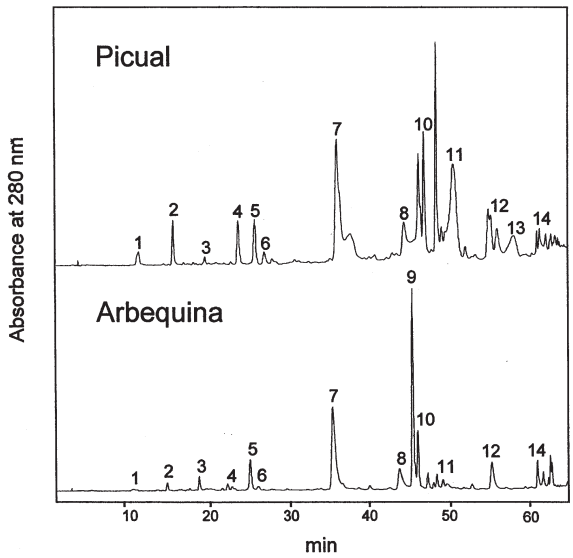

FIG. 1. High-performance liquid chromatograms of phenolic compounds in virgin olive oils of Picual and Arbequina cultivars obtained at laboratory scale. Peaks correspond to (1) hydroxytyrosol (Hy), (2) tyrosol (Ty), (3) vanillic acid, (4) vanillin, (5) 4-(acetoxyethyl)-1,2-dihydroxybenzene (Hy-AC), (6) *p*-coumaric acid, (7) dialdehydic form of elenolic acid linked to Hy (Hy-EDA), (8) dialdehydic form of elenolic acid linked to Ty (Ty-EDA), (9) 1-acetoxypinoresinol, (10) pinoresinol, (11) isomers of oleuropein aglycon (Hy-EA), (12) luteolin, (13) isomers of ligstroside aglycon (Ty-EA), and (14) apigenin.

traviolet (UV) spectrum of the peak eluting at the same retention time as 1-acetoxypinoresinol in the Picual chromatogram was different from that of standard 1-acetoxypinoresinol, and thus this peak was not quantified as 1-acetoxypinoresinol.

The amount of polyphenols in the Picual and Arbequina olive oils obtained at laboratory scale is reported in Tables 1 and 2. The differences in the total amount of phenolics were influenced by the different indexes of ripeness of the fruits since they were 2.0 and 4.7 for Arbequina and Picual, respectively.

Concentration of orthodiphenols in oils drastically diminished during malaxation of the paste under air (Table 1). The concentrations in oil of Hy, Hy-EDA, Hy-EA, luteolin, and the recently identified Hy-Ac decreased *ca.* 30–80%, which is in agreement with previous reports (13,15). In contrast, the concentrations of nonorthodiphenols only diminished *ca.* 17% for both cultivars although this reduction depended on the type of polyphenol. Thus, the concentrations of Ty and Ty-EA increased slightly, while there was no clear trend for Ty-EDA and apigenin. The concentrations of the lignans 1-acetoxypinoresinol and pinoresinol diminished slightly for both cultivars.

There is no widely accepted explanation for the decrease of phenolics in oils during malaxation of the paste under air, although it has been suggested that certain enzymes such as polyphenol oxidase and peroxidase may play an important role in this phenomenon (13,15). Orthodiphenols may be oxidized owing to both enzymatic and chemical reactions at the pH of the paste (between 5 and 6) although it could be supposed that enzymatic oxidation is more rapid than chemical. Polyphenol oxidase and peroxidase can also catalyze oxidation reactions of nonorthodiphenols and, therefore, the slight reduction of these compounds during malaxation may also be due to enzymatic reactions.

TABLE 1
Phenol Composition (mg/kg) of Virgin Olive Oils Obtained from Arbequina Olives by the Abencor Laboratory System[a]

Phenolic compound	P	M + A	M + N
Hy	1.2 (0.4)[b]	0.4 (0.2)	1.3 (0.1)
Ty	1.0 (0.1)	1.7 (0.2)	1.3 (0.1)
Vanillic acid	1.1 (0.2)	0.8 (0.2)	1.1 (0.1)
Vanillin	0.4 (0.2)	0.4 (0.1)	0.5 (0.1)
Hy-AC	78.8 (35.4)	10.8 (7.6)	111.8 (13.6)
p-Coumaric acid	0.3 (0.1)	0.2 (0.1)	0.3 (0.1)
Hy-EDA	255.9 (41.6)	25.4 (18.4)	293.1 (33.4)
Ty-EDA	15.0 (2.3)	8.5 (1.7)	15.3 (1.8)
1-Acetoxypinoresinol	81.5 (5.5)	77.3 (4.0)	78.6 (3.0)
Pinoresinol	65.2 (14.3)	46.2 (6.7)	64.3 (3.1)
Hy-EA	12.4 (1.9)	2.9 (1.3)	20.1 (4.5)
Luteolin	8.4 (1.2)	7.8 (1.2)	8.2 (0.8)
Apigenin	2.0 (0.2)	1.7 (0.2)	1.8 (0.2)
Nonorthodiphenols	166.5 (15.5)	136.8 (8.0)	163.2 (4.7)
Orthodiphenols[d]	356.7 (54.7)	47.3 (20.0)	434.5 (36.4)

[a]Hy, hydroxytyrosol; Ty, tyrosol; Hy-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; Ty-EDA, dialdehydic form of elenolic acid linked to tyrosol; Hy-EA, oleuropein aglycon; Hy-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene. Oils were from paste without malaxation (P), malaxed paste under air (M + A), and malaxed paste under nitrogen (M + N).
[b]Standard deviation (n = 3).
[c]Sum of Ty, vanillic acid, vanillin, *p*-coumaric acid, Ty-EDA, 1-acetoxypinoresinol, pinoresinol, and apigenin.
[d]Sum of Hy, Hy-AC, Hy-EDA, Hy-EA, and luteolin.

TABLE 2
Phenol Composition (mg/kg) of Virgin Olive Oils Obtained from Picual Olives by the Abencor Laboratory System

Phenolic compound[a]	P	M + A	M + N
Hy	1.1 (0.1)[b]	1.0 (0.1)	1.3 (0.1)
Ty	2.9 (0.1)	3.1 (0.2)	3.1 (0.1)
Vanillic acid	0.2 (0.2)	0.2 (0.2)	0.2 (0.1)
Vanillin	0.4 (0.1)	0.4 (0.1)	0.6 (0.1)
Hy-AC	14.0 (0.1)	6.0 (0.7)	12.3 (0.8)
p-Coumaric acid	0.3 (0.1)	0.2 (0.1)	0.2 (0.1)
Hy-EDA	89.4 (2.3)	56.3 (3.0)	78.7 (2.4)
Ty-EDA	7.3 (0.3)	9.1 (0.3)	9.7 (0.7)
Pinoresinol	47.3 (5.6)	33.8 (0.4)	33.1 (1.3)
Hy-EA	105.3 (2.4)	86.0 (5.7)	116.9 (15.3)
Luteolin	2.2 (0.1)	1.7 (0.1)	1.8 (0.1)
Ty-EA	7.3 (0.1)	8.3 (0.4)	8.9 (1.1)
Apigenin	0.4 (0.1)	0.4 (0.1)	0.5 (0.1)
Nonorthodiphenols[c]	66.1 (5.6)	55.5 (0.7)	56.3 (1.9)
Orthodiphenols[d]	212.0 (3.3)	151.0 (6.5)	211.0 (15.5)

[a]Ty-EA, ligstroside aglycon; for other abbreviations see Table 1.
[b]Standard deviation (n = 3).
[c]Sum of Ty, vanillic acid, vanillin, *p*-coumaric acid, Ty-EDA, 1-acetoxypinoresinol, pinoresinol, and apigenin.
[d]Sum of Hy, Hy-AC, Hy-EDA, Hy-EA, and luteolin.

The ratio between the surface in contact with air and mass of the paste in the mixer must also be important regarding oxygen diffusion. Therefore, differences between oxidation at laboratory and industrial scales must be expected since this ratio is higher for laboratory than industrial assays.

When pastes were malaxed under a nitrogen atmosphere, the phenolic concentration of oils obtained from only crushed or malaxed pastes was not statistically different. Obviously, a nitrogen atmosphere did not allow the oxidation reactions that happened under air. A nitrogen atmosphere during malaxation may be a good way to avoid oxidizing phenolics during this step although industrial assays should be carried out. However, the industrial use of nitrogen during olive oil processing may have some drawbacks. First, nitrogen should be employed not only during the malaxation step but also during crushing. If oxygen is not eliminated from paste during crushing, orthodiphenols may be oxidized, even if the paste is malaxed under nitrogen. Second, the mixer should be sealed; otherwise a continuous flow of nitrogen in the mixer should be maintained. Third, from a sensory point of view, an increase of polyphenols in oils must also enhance the bitter taste of some olive oils (2).

Phenolic composition of oils and waters obtained during industrial extraction of virgin olive oil. Unlike the slight decrease in nonorthodiphenols during malaxation under air obtained at lab scale, concentration of nonorthodiphenols remained statistically constant in oil after malaxation of the paste at industrial scale (Tables 3 and 4). The concentrations in oil of lignans and tyrosol derivatives did not change as a consequence of malaxation. In contrast, all orthodiphenols were oxidized during malaxation of the paste, and their concentrations diminished *ca.* 50–70%, as also occurred in the laboratory assays.

TABLE 3
Phenol Composition (mg/kg) of Olive Oils and Waters Obtained from the Two-Phase Extraction Process of Arbequina Virgin Olive Oil

Phenolic composition	Oil from crushed paste	Decanter oil	Water of the decanter oil[b]	Centrifuge oil	Centrifuge water[b]
Hy	1.2 (0.1)[a]	1.2 (0.2)	2.4	0.6 (0.1)	2.6 (0.7)
Ty	0.9 (0.2)	3.0 (1.0)	0.2	1.6 (0.1)	0.9 (0.2)
Vanillic acid	2.0 (0.1)	1.2 (0.1)	0.1	0.9 (0.1)	0.5 (0.1)
Vanillin	0.4 (0.2)	0.2 (0.1)	ND[c]	0.2 (0.1)	0.1 (0.1)
Hy-AC	56.5 (0.4)	31.2 (0.1)	0.5	30.8 (3.6)	3.3 (0.5)
p-Coumaric acid	0.2 (0.1)	0.2 (0.1)	ND	0.2 (0.1)	0.1 (0.1)
Hy-EDA	267.5 (18.0)	155.1 (16.8)	3.7	142.4 (12.0)	22.5 (7.2)
Ty-EDA	15.0 (1.8)	18.5 (1.5)	ND	15.7 (1.4)	0.8 (0.2)
1-Acetoxypinoresinol	97.2 (2.8)	98.5 (1.2)	0.1	96.0 (3.1)	3.0 (0.2)
Pinoresinol	55.1 (14.0)	59.5 (1.1)	0.1	65.3 (10.6)	2.0 (0.2)
Hy-EA	14.6 (0.1)	9.8 (2.1)	ND	8.6 (0.8)	ND
Luteolin	8.3 (0.2)	5.2 (0.1)	ND	6.1 (0.2)	ND
Apigenin	2.0 (0.1)	1.5 (0.1)	ND	1.5 (0.2)	ND
Nonorthodiphenols	172.8 (14.4)	182.6 (2.4)	0.5	181.4 (11.1)	7.4 (0.4)
Orthodiphenols	348.1 (18.0)	202.3 (16.9)	6.6	188.5 (12.6)	28.4 (7.3)

[a]Standard deviation ($n = 2$).
[b]Expressed as mg phenols in water/kg oil.
[c]ND, not detected. For other abbreviations see Table 1.

The two-phase extraction system requires removal of impurities from the oily must obtained from the decanter centrifuge, and this step is carried out in the vertical centrifuges by adding tap water (10,11). The water that accompanied the oil in the oily must was 0.4% (w/w), and the phenolic composition of this low amount of water is reflected in Tables 3 and 4. The concentration of phenols in this water was high, but when expressed as mg phenols in water/kg of oil must it did not represent a high amount compared to the amount of phenols in the lipid phase. Thus, there may be only 2–3% of the total phenols in the oily must. Hydrophilic phenols such as Hy and Ty were the most representative phenolic compounds in these liquids, although Hy-EDA was also found in a high concentration.

Figure 2 shows the chromatographic profile of the phenolic compounds in the wash water used for cleaning both Arbequina and Picual oily musts in the vertical centrifuge. Most of the phenolic compounds found in olive oils (Fig. 1) were also present in the wash waters, except for the less polar ones, which elute at retention time greater than 50 min. The prevalent phenolic compound in the wash waters for both cultivars was Hy-EDA. Hydrophilic phenols such as Hy and Ty were also found in high proportion in the wash waters. In contrast, lignans, Ty-EDA, and Hy-EA were detected in low amount compared with their concentrations in the decanter oils. Considering the amount of phenolic compounds in the wash wasters (expressed as mg/kg of oil) (Tables 3 and 4), apparently the wash water used in the vertical centrifuge removed all the phenols from the water that accompanied the oil in the oily must as well as the phenols from the decanter oil.

It has been reported that the temperature of the wash water can influence the extraction yield (10). The usual processing

TABLE 4
Phenol Composition (mg/kg) of Olive Oils and Waters Obtained from the Two-Phase Extraction Process of Picual Virgin Olive Oil

Phenolic composition[a]	Oil from crushed paste	Decanter oil	Water of the decanter oil[a]	Centrifuge oil	Centrifuge water[b]
Hy	1.3 (0.2)[c]	0.5 (0.1)	3.1	0.7 (0.1)	1.3 (0.1)
Ty	2.7 (0.3)	3.7 (0.2)	0.3	2.3 (0.1)	0.7 (0.1)
Vanillic acid	0.2 (0.1)	0.2 (0.1)	0.1	0.2 (0.1)	0.1 (0.1)
Vanillin	0.3 (0.1)	0.4 (0.1)	ND[c]	0.4 (0.1)	0.1 (0.1)
Hy-AC	27.3 (2.5)	14.7 (0.6)	ND	13.3 (0.6)	0.9 (0.1)
p-Coumaric acid	0.2 (0.1)	0.2 (0.1)	ND	0.2 (0.1)	ND
Hy-EDA	147.9 (9.6)	41.8 (1.8)	1.4	40.7 (1.3)	7.6 (0.5)
Ty-EDA	4.6 (0.9)	7.8 (0.7)	ND	6.5 (0.3)	0.5 (0.1)
Pinoresinol	40.8 (6.6)	39.5 (0.9)	ND	36.4 (0.6)	0.7 (0.1)
Hy-EA	127.1 (19.5)	43.9 (3.6)	ND	43.8 (2.3)	2.7 (0.2)
Luteolin	1.8 (0.3)	0.7 (0.2)	ND	1.1 (0.3)	ND
Ty-EA	4.3 (1.3)	3.2 (0.9)	ND	3.3 (0.7)	ND
Apigenin	2.0 (0.1)	0.3 (0.1)	ND	0.3 (0.1)	ND
Nonorthodiphenols	53.5 (6.8)	55.3 (1.5)	0.4	49.6 (1.0)	2.1 (0.2)
Orthodiphenols	305.4 (21.9)	101.6 (7.9)	4.5	99.6 (2.7)	12.5 (0.6)

[a]For abbreviations see Tables 1–3.
[b]Expressed as mg phenols in water/kg oil.
[c]Standard deviation ($n = 2$).

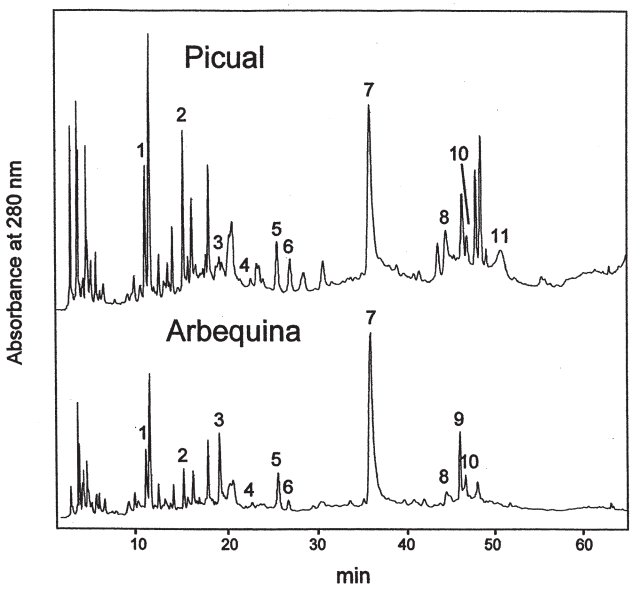

FIG. 2. High-performance liquid chromatograms of phenolic compounds in the wash waters. See Figure 1 for identification of peaks.

procedures for virgin olive oil in Spain imply the use of wash water at 40ºC because higher temperatures may lead to off-odors. Working at this temperature, we found that *ca.* 13% of orthodiphenols was removed from the oily must, while for nonorthodiphenols the comparable value was only *ca.* 4%. These figures were much lower than those reported recently (10), although in this case the determination of phenols was done by colorimetric means.

Wash waters from the vertical centrifuge and wash waters from washing the fruits before crushing are the only two wastewaters generated in the new two-phase extraction system. Wash waters from the vertical centrifuge contained a relative low amount of phenols (between 50 and 100 mg/L) compared to polyphenols in the vegetation waters generated in the three-phase extraction process. Thus, the activated sludge treatment of these streams has been carried out successfully (17).

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