Simple and Hydrolyzable Phenolic Compounds in Virgin Olive Oil. 2. Initial Characterization of the Hydrolyzable Fraction

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This paper reports the preliminary characterization of unknown phenolic compounds in virgin olive oil, separated by HPLC using hydrolytic, chromatographic, and spectrophotometric techniques. It was possible to characterize oleuropeine aglycon and three hydrolyzable phenols containing (3,4-dihydroxyphenyl)ethanol or (*p*-hydroxyphenyl)ethanol.

INTRODUCTION

An HPLC procedure for the separation and quantitative and semiquantitative determination of some of the phenolic components present in olive oil was previously reported (Montedoro et al., 1992).

In this study we have attempted an initial identification of four unknown compounds, with phenolic-like behavior, present in virgin olive oil. Their concentrations were correlated with the colorimetric determination of the total phenols; two were also correlated with oil autoxidation stability.

MATERIALS AND METHODS

Materials. *Oils.* Virgin olive oils from Umbria, Apulia, and Liguria were used.

Standard Compounds. Oleuropeine glucoside was produced by Extrasynthèse (Z. I. Lyon-Nord, Genay, France); (*p*-hydroxyphenyl)ethanol was produced by Janssen Chemicals (Beerse, Belgium). (3,4-Dihydroxyphenyl)ethanol was synthesized in the laboratory following the Schoepf et al. (1949) procedure. Oleuropeine aglycon (Figure 1) was obtained by enzymatic hydrolysis of oleuropeine glycoside using the procedure described by Walter et al. (1973). Purification of the extract in chloroform was carried out with TLC on a 0.25-mm silica gel layer made by Whatman (London); the mobile phase consisted of a benzene/methanol/acetic acid (45:8:1 v/v) mixture.

Methods. *Column Chromatography.* The purified phenolic extract obtained according to the procedure described in the previous paper (Montedoro et al., 1992) was passed through a Sephadex LH20 column (Montedoro, 1972), 80 cm long × 2.5 cm i.d., as a precautionary measure. This was done to improve the HPLC resolution and, at the same time, to eliminate overlaying of peaks of the compounds with similar physicochemical properties.

The mobile phase was made up of methanol/water (80:20 v/v) at a flow rate of 15 mL/h. A UV detector at 278 nm was used, and 1.5 mL of phenolic extract corresponding to 5 mg of the total polyphenols, determined by the Folin–Ciocalteu reaction with respect to gallic acid, was injected (Montedoro, 1972; Montedoro et al., 1978).

The different fractions were collected, concentrated with a light spray of nitrogen, and then dissolved in methanol and used in the HPLC analysis; the analytical conditions are described below.

Hydrolysis of Phenolic Extract. To characterize the molecular structures present, the purified phenolic extracts were hydrolyzed using the methods described by Walter et al. (1973) but with the following modifications: 5 mg of phenolic extract, determined by the Folin–Ciocalteu reaction with respect to gallic acid (Montedoro, 1972; Montedoro et al., 1978); 10 mL of 1 N H_2SO_4 was added and then hydrolyzed for 30 min at 100 °C.

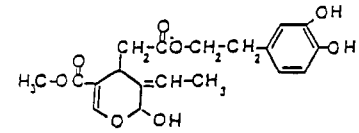

Figure 1. Oleuropeine aglycon (Walter et al., 1973).

Table I. HPLC Analysis of Main Phenolic Substances Separated with Column Chromatography Using Sephadex LH20

	fraction A,% total area	fraction B,% total area	fraction C,% total area	fraction D,% total area	total area, IU × 10^4
(3,4-dihydroxyphenyl)ethanol			62.3	37.6	749
(*p*-hydroxyphenyl)ethanol			94.1	5.9	424
elenolic acid	90.4	6.5	2.2	0.8	2289
peak 8[a]	16.8	73.6	9.6		1912
peak 9		100.0			531
peak 10	22.7	27.4	31.0	18.9	1437
peak 12			100.0		484

[a] See Figure 2.

This hydrolyzed substance was adjusted to pH 2 and then extracted with ethyl acetate (5 mL three times).

This collected extract was then passed over anhydrous Na_2SO_4, dried with a spray of nitrogen, reconstituted with methanol, and then analyzed with TLC and HPLC, together with reference compounds, as reported below.

Thin-Layer Chromatography (TLC). The stationary phase used was a 0.25-mm silica gel layer from Whatman. The mobile phases were as follows: A, toluene/ethyl formate/formic acid (50:40:10 v/v); B, benzene/methanol/acetic acid (45:8:1 v/v); C, ethyl acetate/methanol/water (100:16.5:13.5 v/v); D, chloroform/ethyl acetate/formic acid (50:40:5 v/v). One hundred microliters of the phenolic extract dissolved in methanol (containing 10 mg of total phenols/mL) was applied on the layer. The different spots, separated in the layers, were scrapped, dissolved in methanol, and used in HPLC and UV analysis.

Detection of TLC spots was done by (a) spraying with Folin–Ciocalteu reagent followed by ammonia vapors; (b) spraying with *p*-toluenesulfonic acid plus vanillin and heating at 105 °C for 10 min (Montedoro and Cantarelli, 1969; Ragazzi and Veronese, 1973; Walter et al., 1973); and (c) leaving the layers in the air and evaluating the browning of the spots after 24 h of exposure at room temperature.

High-Pressure Liquid Chromatography (HPLC). The HPLC system was composed of a Varian Model 5000 chromatograph with a 150 mm × 4.6 mm C_{18} Erbasil column, coupled with a UV Varian Polychrom 9060 photodiode spectrophotometer; the eluates were detected at 278 and 239 nm at 25 °C. The flow rate was 1 mL/min; the mobile phase used was acetic acid 2% (pH 3.1) in water (A)/methanol (B). The total running time of analysis

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Table II. Acid Hydrolysis Effects on the Qualitative Composition of Phenolic Substances in Virgin Olive Oil

	low (total phenols 50–200 mg/kg)				medium (total phenols 200–500 mg/kg)				high (total phenols 500–1000 mg/kg)			
	control		after hydrolysis		control		after hydrolysis		control		after hydrolysis	
	av[a]	range	av	range	av	range	av	range	av	range	av	range
fraction 1												
(3,4-dihydroxyphenyl)ethanol	1.2[b]	0.0, 3.0	30.5[b]	2.0, 82.0	24.2[b]	3.0, 74.0	287.5	103.0, 394.4	10.7	5.0, 15.4	1024.2	666.0, 1628.5
(*p*-hydroxyphenyl)ethanol	33.4	0.7, 94.0	95.1	37.9, 173.0	61.1	0.5, 267.4	284.3	41.3, 902.0	8.7	3.2, 13.0	291.0	65.5, 359.0
elenolic acid	376.0	167.1, 661.0	690.2	172.0, 1440.2	887.5	594.3, 1322.0	2008.5	1009.2, 2930.0	1022.0	680.1, 2358.2	2346.5	1664.0, 3311.1
fraction 2												
peak 8[c]	50.4[b]	1.0, 169.5	11.1[b]	0.0, 52.1	329.5[b]	55.0, 705.1	54.7	17.0, 96.4	1440.2	852.0, 2059.1	277.8	105.0, 454.0
peak 9	32.0	10.5, 84.4	4.2	0.0, 12.5	125.6	24.3, 328.0	4.9	0.0, 12.6	255.5	318.3, 548.0	29.1	5.0, 48.2
peak 10	196.6	133.4, 359.1	29.3	0.0, 57.1	221.6	61.0, 432.2	39.6	16.0, 65.4	115.5	86.0, 146.1	15.2	0.0, 33.1
peak 12	24.1	6.0, 56.1	2.3	0.0, 6.1	146.5	92.1, 278.3	8.2	0.0, 20.1	332.1	158.2, 605.1	35.0	0.0, 114.3

[a] $n = 9$. [b] Peak area: $n \times 10^4$. [c] See Figure 2.

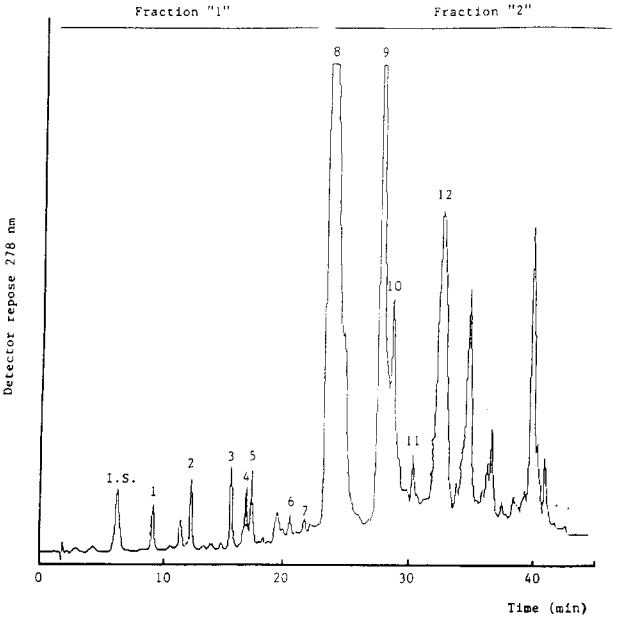

Figure 2. Fractionation of the phenolic extract of olive oil by HPLC at 278 nm. Peak numbers: (I.S.) gallic acid; (1) (3,4-dihydroxyphenyl)ethanol; (2) (*p*-hydroxyphenyl)ethanol; (3) vanillic acid; (4) caffeic acid; (5) syringic acid; (6) *p*-coumaric acid; (7) ferulic acid; (8) RT 23.50; (9) RT 27.70; (10) RT 28.40; (11) cinnamic acid; (12) RT 32.50.

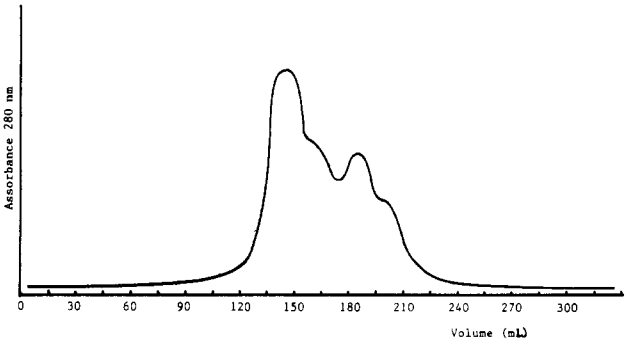

Figure 3. Gel filtration on Sephadex LH20 of the phenols present in the oil extract (see Materials and Methods). The fractions corresponding to elution volumes A (120–150 mL), B (150–165 mL), C (165–187.5 mL), and D (187.5–217.5 mL) were collected.

was 45 min, and the gradient changed as follows: 95% A/5% B for 2 min, 75% A/25% B in 8 min, 60% A/40% B in 10 min, 50% A/50% B in 10 min, and 0% A/100% B in 10 min until the end of running. Samples were dissolved in methanol, and 10 μL of this solution was injected in the column.

Table III. Final Model of Multiple Regression Analysis of Analytical Data Obtained with Acid Hydrolysis of Phenolic Substances in Virgin Olive Oil[a]

independent variable	coeff	SE	*F* ratio	*t* value	sig level
Model Fitting Results for (3,4-Dihydroxyphenyl)ethanol[b]					
constant	−26.752	51.763	106.231	−0.516	0.611
peak 8[c]	0.711	0.093	207.025	7.668	0.000
peak 12	0.960	0.411	5.435	2.331	0.030
Model Fitting Results for (*p*-Hydroxyphenyl)ethanol[d]					
constant	−83.491	21.662	153.481	−3.854	0.001
peak 9	1.363	0.082	262.440	16.545	0.000
peak 10	0.730	0.109	44.520	6.672	0.000
Model Fitting Results for Elenolic Acid[e]					
constant	226.797	165.519	41.850	1.370	0.185
peak 12	5.583	0.863		6.469	0.000

[a] Total degrees of freedom, 26. Confidence level, 95%. [b] R^2 adjusted, 0.909. SE, 146.348. $F(2.24, 0.95)$, 3.40. [c] See Table I. [d] R^2 adjusted, 0.935. SE, 43.485. $F(2.24, 0.95)$, 3.40. [e] R^2 adjusted, 0.660. SE, 468.087. $F(1.25, 0.95)$, 4.24.

The spectrophotometric evaluation of the individual compounds was obtained using the same apparatus and analytical conditions described above.

A chromatogram of virgin olive oil is shown in Figure 2.

Multiple linear regression analysis was employed using the STATGRAPHICS program (STSC Inc., 1987) using HPLC peak area numbers 8–10 and 12 in relation to the HPLC peak area increase of (3,4-dihydroxyphenyl)ethanol, (*p*-hydroxyphenyl)ethanol, and elenolic acid in the hydrolyzed phenolic extracts of 27 virgin olive oil samples.

RESULTS AND DISCUSSION

Column Chromatography. The fractions collected from the Sephadex column (Figure 3) characterized by HPLC are shown in Table I. The results indicate a certain inefficiency in the separation of the unknown compounds. In fact, three components, 8 and 10 and elenolic acid, were present in nearly all of the fractions collected.

Hydrolysis of Phenolic Extract. The fact that these components were present in fresh oil with elevated autoxidation stability, as reported in the previous paper (Montedoro et al., 1992), led to the hypothesis that these compounds contained functional groups with antioxidizing properties. On this basis an initial characterization by hydrolysis was attempted, and the following data emerged:

Acid hydrolysis (27 samples), as summarized in Table II, shows a marked reduction in the concentrations of compounds corresponding to peaks 8–10 and 12 and an increase in (3,4-dihydroxyphenyl)ethanol, (*p*-hydroxyphenyl)ethanol, and elenolic acid.

Table IV. Physical and Chemical Properties of Unidentified Peaks of Phenolic Substances in Fraction 2 and Standard Compounds

	HPLC exam[a] RT, min	TLC exam[b] A	B	C	D	sp reactions air ox.	Folin	PTSA + V[c]	spectrometry max1;2, nm	min, nm	max1/ min	max2/ min
standard compounds												
(3,4-dihydroxyphenyl)ethanol	9.27	0.41	0.27	0.81	0.45	brown	blue	orange	278	249		10.35
(*p*-hydroxyphenyl)ethanol	12.50	0.52	0.37	0.86	0.64	neg[d]	blue	neg	220;273	244	50.26	11.64
oleuropeine glycoside	26.46	0.05	0.10	0.52	0.04	brown	blue	yellow	229;278	268	6.69	1.29
oleuropeine aglycon	32.50	0.55	0.40	0.86	0.62	brown	blue	yellow	229;278	268	5.62	1.25
fraction 2												
peak 8[e]	23.50	0.49	0.33	0.83	0.56	brown	blue	yellow	224;278	258	20.89	3.00
peak 9	27.70	0.52	0.43	0.85	0.70	brown	blue	neg	229;273	258	21.11	1.78
peak 10	28.40								229;278	254	15.36	3.99
peak 12	32.50	0.54	0.40	0.87	0.63	brown	blue	yellow	229;278	268	5.62	1.25

[a] Measured at 278 nm. [b] A, toluene/ethyl formate/formic acid (50/40/10). B, benzene/methanol/acetic acid (45/8/1). C, ethyl acetate/methanol/water (100/16.5/13.5). D, chloroform/ethyl acetate/formic acid (50/40/15). [c] PTSA + V = *p*-toluenesulfonic acid + vanillin. [d] Negative reaction. [e] See Figure 2.

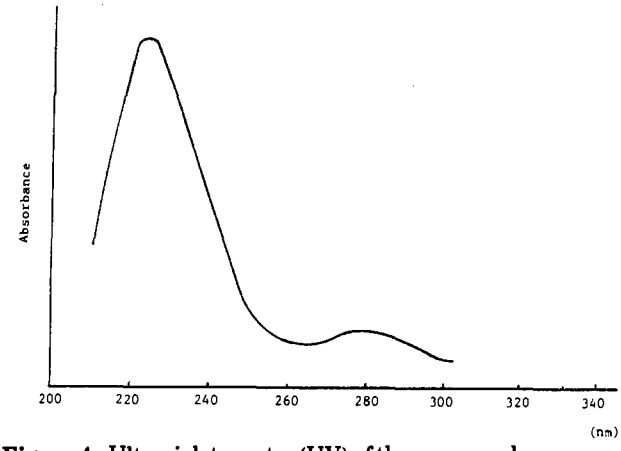

Figure 4. Ultraviolet spectra (UV) of the compound corresponding to peak 8 (RT 23.50) obtained in 2% acetic acid in water/methanol during the HPLC analysis.

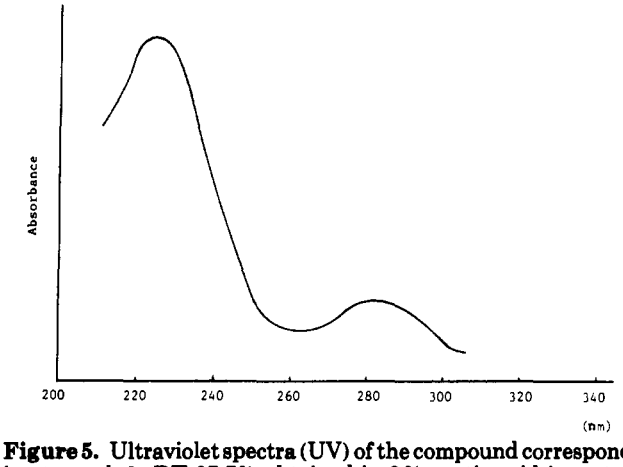

Figure 5. Ultraviolet spectra (UV) of the compound corresponding to peak 9 (RT 27.70) obtained in 2% acetic acid in water/methanol during the HPLC analysis.

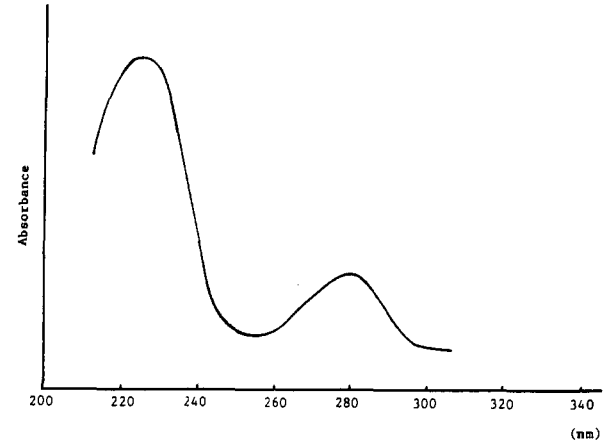

Figure 6. Ultraviolet spectra (UV) of the compound corresponding to peak 10 (RT 28.40) obtained in 2% acetic acid in water/methanol during the HPLC analysis.

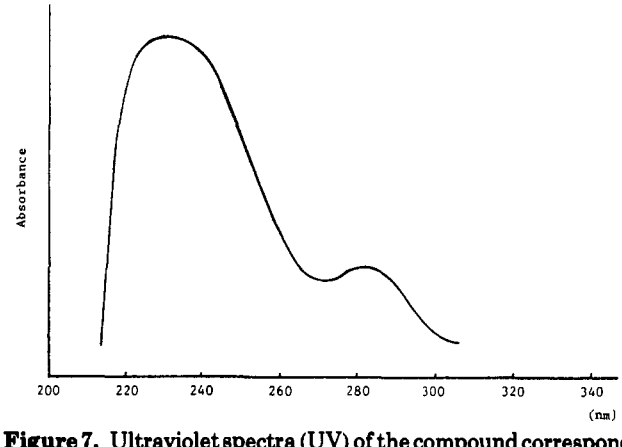

Figure 7. Ultraviolet spectra (UV) of the compound corresponding to peak 12 (RT 32.50) obtained in 2% acetic acid in water/methanol during the HPLC analysis.

The statistical analysis applied to the results of acid hydrolysis shows a positive correlation for peaks 8 and 12 with (3,4-dihydroxyphenyl)ethanol, peaks 9 and 10 with (*p*-hydroxyphenyl)ethanol, and peak 12 with elenolic acid (Table III).

Chromatographic and UV Evaluation. RT, TLC, and UV evaluations of peaks 8–10 and 12, in relation to standard compounds, are summarized in Table IV and Figures 4–7.

In relation to data reported it is possible to hypothesize that peak 12 is oleuropeine aglycon.

This consideration agrees with the hydrolytic evaluation and the data reported in the literature (Ragazzi and Veronese, 1973; Walter et al., 1973; Vazquez Roncero et al., 1974; Solinas et al., 1975; Cortesi and Fedeli, 1983).

Conclusions. Overall examination of the results leads to some considerations:

The compounds relative to peaks 8–10 and 12 in the HPLC chromatogram have a complex phenolic nature, because of their hydrolytic, chromatographic, and spectrophotometric behavior.

In particular, the hydrolysis of peaks 8 and 12 has been correlated with (3,4-dihydroxyphenyl)ethanol, peaks 9 and 10 have been correlated with the (*p*-hydroxyphenyl)eth-

anol, and peak 12 has also been correlated with the increase in elenolic acid in the hydrolyzed extracts.

In relation to hydrolytic, chromatographic, and spectrophotometric evaluations peak 12 is hypothesized to be oleuropeine aglycon.

The correlation with peaks 8 and 12 and the increase in (3,4-dihydroxyphenyl)ethanol in the hydrolyzed extracts justify the high autoxidation stability of virgin olive oil containing those compounds, because of the high antioxidation activity of (3,4-dihydroxyphenyl)ethanol (Chimi et al., 1988; Servili and Montedoro, 1989).

Work is continuing on the definitive characterization of peaks 8–10 and 12 by HPLC preparative separation and their identification by means of NMR and GC–MS.

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