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Phenolic compounds in virgin olive oils I. Low-wavelength quantitative determination of complex phenols by high-performance liquid chromatography under isocratic elution

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Abstract

An HPLC method that allows the determination of complex phenolic compounds at low λ (225 nm) after solid-phase extraction from virgin olive oils is reported. The separation was achieved on ODS-2, ODS-1 and C_8 columns in eluting mixtures 10^{-3} *M* H_2SO_4–CH_3CN employed with different ratios. The method was employed for the quantitative determination of phenolic compounds in oils. It allows the presence of some new unknown compounds to be shown. The preliminary spectroscopic data of these compounds, probably phenolic, are reported. The reactivity with protic solvents of the deacetoxy oleuropeine aglycon is discussed.

Keywords: Olive oil; Phenols

1. Introduction

Virgin olive oils contain several phenolic substances acknowledged as being very important [1–3]. These compounds have a high activity on the autooxidative stability of oils [4,5], and their amount could be positively related to the sensorial characteristics of oils [6–9]. Quantitative and qualitative determinations of phenolic substances in oils are therefore very important, as regards simple (compounds **6–9**) and complex (compounds **2–5**) phenols (Fig. 1).

At present the quantitative determination of the phenolic substances in oil is mainly performed by the Folin–Ciocalteau colorimetric method [6]. However this method is non-specific, and both simple and complex phenols are detected indistinctly. Furthermore the total phenol values found do not correlate with the oil autooxidation stability as evaluated by the accelerated Swift test performed with the Rancimat apparatus and with the values found by HPLC [7,8].

Several authors have developed extraction methods from oils as well as HPLC separations for the analysis of simple phenols [10–14] and complex phenolic substances [4,15–17]. Reversed-phase columns and solvent gradient elutions have been em-

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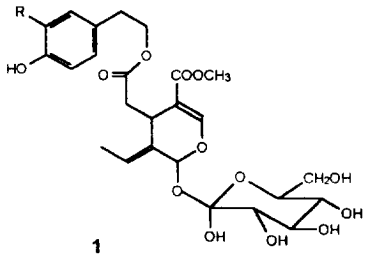
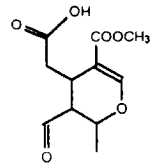
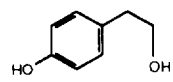


1

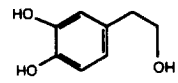
a R=OH, Oleuropeine glycoside
b R=H, Ligstroside



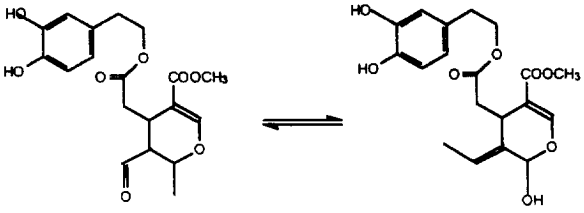
2 Elenolic acid



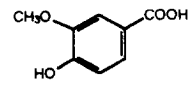
6, *p*-hydonxy phenyl ethanol



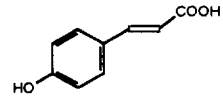
7, 3,4-dihydroxy phenyl ethanol



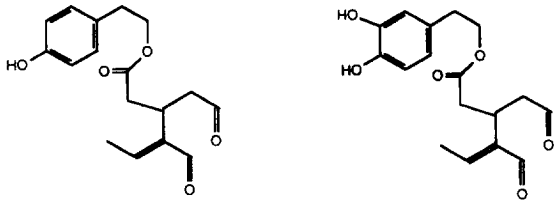
5 Oleuropeine aglycone



8, vanillic acid

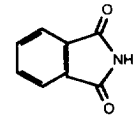


9, *p*-coumaric acid

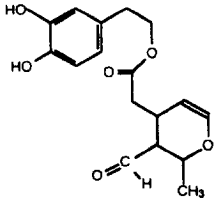


3, Dialdheydic Ligstroside derivative

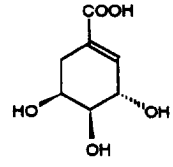
4, Dialdheidic Oleuropeine derivative



10, phthalimide



12 Deacetoxy oleuropeine aglicone (monoaldheydic form)



17, Shikimmic acid

Fig. 1. Structures of simple and complex phenols.

ployed with UV (at λ=280 nm) or amperometric detection.

Recently, a GC–MS evaluation of derivatized phenolic compounds in oil extracts has been reported [18].

These methods, mainly those developed by Montedoro et al. [4], have been a very interesting innovation in the study of complex phenolic substances, and have allowed elucidation of the structures [15] of some separated compounds (Fig. 1, **2–5**).

Nevertheless, the methods have some drawbacks:

(1) Liquid–liquid extractions are laborious and during this process oxidation of phenolic substances could occur. Several authors have thus developed solid-phase extraction (SPE) methods employing anionic exchange [17] or reversed-phase cartridges [19];

(2) The UV absorbance profile of eluting mixtures employed in the literature methods (methanol and water with acetic acid) requires the detection of complex phenolic compounds only at their secondary UV maximum (λ=280 nm), whereas the main UV maximum ranged between 220 and 225 nm [15]. Therefore at λ>254 nm compounds such as **2** (max=240 nm and no absorption over 254 nm), cannot be detected simultaneously with other phenolic substances, and their quantitative analysis can have high detection limits (DL).

(3) The literature methods are lacking in the R_Fs of complex phenol. Thus the quantitation of phenolic compounds from the HPLC chromatograms could made only with the following approximations: by assuming the R_Fs to be coincident with that of *p*-hydroxy phenyl ethanol, 6 [9]; by relative response factors (RRFs) with respect to an internal standard (I.S.) [4].

Thus, both approximations gave an unreal quantitation.

Hence in this paper we describe a reversed-phase HPLC method for a better separation of phenolic substances extracted from oil by SPE with two isocratic eluting mixtures at different ratios containing 10^{-3} M H_2SO_4–CH_3CN at successive times. UV detection at 225 nm and quantitative determination were carried out using the R_F calculated in this paper.

2. Experimental

2.1. Apparatus

A chromatographic Varian 5020 pump equipped with a UV–Vis 100 variable-wavelength detector set at 225 nm (Varian, Palo Alto, CA, USA), HP 1050 Automatic injector (loop 100 μl) and an HP 3396 reporting integrator (Hewlett-Packard, Milan, Italy) have been employed. The detector output was also connected to an LC-235 diode array detector whose signals and UV spectra were processed by an LCI-100 computer integrator (Perkin-Elmer, Milan). ^{1}H NMR spectra were recorded on a computer controlled Sun 3/60 Varian 300 MHz spectrometer in $CDCl_3$ solutions.

2.2. Chemicals

Acetone, acetonitrile, benzene, cyclohexane, ethyl acetate, dioxane, *n*-hexane and methanol were HPLC Grade solvents from Carlo Erba (Milan, Italy). 4-Hydroxyphenyl ethanol (**6**, >98.5%), vanillic acid (**8**), *p*-coumaric acid (**9**) and phthalimide (**10**) (all >98%) were purchased from Aldrich (Milan), while oleuropeine glycoside (**1a**, >96%) was purchased from Extrasynthese (Lyon, France). 3,4-Dihydroxyphenylethanol (**7**) was obtained by the method of Baraldi et al. [21] and oleuropeine aglycon (**4**) by enzymatic hydrolysis of **1a** with β-glucosidase, while elenolic acid (**2**) was obtained by a Walters' hydrolysis [20] modified as follows: 250 mg of oleuropeine glycoside (**1a**) were dissolved in 50 ml of water; 50 ml of 0.5 M H_2SO_4 were added to water and the solution was heated at 40°C for 30 min and then kept at room temperature and stirred overnight; the solution was then extracted twice with 50 ml of ethyl acetate; the organic layer, dried with anhydrous Na_2SO_4 and evaporated under reduced pressure, gave a pale yellow oil identified as **2** by HPLC, UV spectrum and NMR data.

2.3. Extraction procedure of phenolic compounds from oils

A C_8 extract clean-up cartridge (500 mg, 3.5 ml, Alltech, Milan) was washed with 10 ml of *n*-hexane

and then activated with 10 ml of acetonitrile. Oil (1 g) dissolved in 10 ml of *n*-hexane was percolated into the cartridge and washed under vacuum (35 mm) with 10 ml of an *n*-hexane–cyclohexane (1:1, v/v) solution in order to remove the non-polar fraction of the oils. The phenolic compounds were eluted with 2.5 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 removed by suction, evaporated under N_2 and then recovered with 1.0 ml of water containing the internal standard (at 5.0 ppm). The extraction method gave good recoveries ranging between 70–105% (S.D.=±4–10%) as tested in oils washed according to the literature [4] and fortified by known amounts of the phenolic compounds isolated as described below.

2.4. HPLC semipreparative isolation of phenolic compounds

The semipreparative isolation of complex phenols was performed starting from 50 g of oils extracted as above. The extracts were concentrated to 15 ml in acetonitrile solution and 250 μl were injected in an Econosil C_8 (250×10.0 mm I.D., Alltech) or an RP-18 (250 mm×10.0 mm I.D., Merck, Darmstadt, Germany) eluted at the flow-rate of 3.5 ml/min with CH_3CN–H_3O^+ mixtures at ratios 66:34 or 90:10. The fractions were collected starting from 1/4 of the peak height uphill to 3/4 downhill. After recovery from the eluent by extraction with an equal volume of ethyl acetate (twice), and evaporation of the organic layer, dried over Na_2SO_4, the compounds were further purified on preparative silica gel plates (thickness 0.25 cm, C. Erba, Milan) eluted with a mixture of benzene–acetone–dioxane–methanol in the ratio 9:1:1:0.5 (v/v). The compounds were recovered from silica by a 30-min sonication of the suspension in an appropriate amount of ethyl acetate.

2.5. Chromatography

ODS-1, ODS-2, C_8, C_1, CN and Phenyl analytical columns (Spherisorb, 250×4.6 mm I.D., 3 μm, Waddinxveen, Netherlands) were employed with a 1.0 cm guard cartridge (C_{18}, 10 μm). The columns were eluted at a flow-rate of 1.0 ml/min for a total running time of 40 min. The mobile phase was a mixture of A (10^{-3} *M* H_2SO_4) and B (CH_3CN). The elution profile was: from $t=0$ to $t=11.9$ min, A–B (85:15, v/v); from $t=12$ to $t=35$ min, A–B (66:34, v/v); from $t=35.1$ to $t=40$ min, A–B (85:15, v/v).

The relative retention times (RRT) of compounds **2–9** and unknown compounds (possibly phenolic derivatives) **11**, **12**, **14** and **16** are reported in Table 1. Separations on C_1 and CN columns were also made with different mobile phases, but they were inadequate.

2.6. R_F calculation of phenols

The response factors (R_F) for the phenols were calculated from the injection of 100 μl of aqueous solution. Analytical standard solutions were employed for the simple phenols **6** and **8**. R_F calculation of some complex phenols was carried out in the following two ways: the R_F of compounds 2, 5, and 7 were calculated on standard solutions obtained as above, while for each of the compounds **3**, **4**, and unknowns **11** and **12** (see Fig. 3) we collected 50 ml from the semipreparative HPLC separation. The eluate was divided into five aliquots of 10 ml and each was extracted twice with 10 ml of $CHCl_3$. The chloroformic extracts were concentrated to small volume, dried under anhydrous Na_2SO_4, and evaporated to a gentle stream of N_2 into pre-weighted vials. Once the solvent evaporated, the vials were

Table 1
Relative retention time (±0.02) of separated compounds from the same oil on reversed-phase (250×4.6 mm with C_{18} 10.0 mm guard cartridge) columns

Peak n[a]	ODS-2[b]	ODS-1[b]	C-8[b]	Phenyl[b]
7	0.31	0.35	0.35	0.43
11	0.36	0.39	0.42	0.57
6	0.45	0.49	0.51	0.61
9	0.70	n.d.[c]	n.d.	n.d.
2	1.14	1.29	1.27	1.26
12	1.18	1.38	1.38	n.d.
5	1.22	1.41	1.40	2.07
3	1.34	1.55	1.55	2.40
4	1.44	1.63	1.62	2.49
14	1.77	1.90	1.92	2.94
16	1.82	n.d.	n.d.	n.d.

[a] See Fig. 1.
[b] For eluent mixtures see Section 2.5.
[c] n.d.=not detected.

Table 2
R_F^a, detection limits (DL, mg/kg)[b] of simple[c], and complex[d] phenols and unknown compounds on ODS-2 column at different λ for the injection of 100 μl of oil extract solution

Compound	225 nm		240 nm		280 nm	
	R_F	DL	R_F	DL	R_F	DL
2	9.0±0.4	0.02	6.8±0.9	0.01	110±15	0.3
5	8.5±0.2	0.01	17±1.5	0.03	44±5	0.08
3	8.5±0.2	0.04	17±1.5	0.1	85±8	0.5
4	2.5±0.5	0.01	5.2±0.8	0.05	25±7	0.1
6	43±3	0.2	355±18	1.5	164±10	0.8
7	25±2	0.1	150±3	0.6	70±0.5	0.3
8	15±2	0.2	81±2	1.0	57±5	0.8
9	16±3	0.3	70±3	1.5	18±4	0.3
12	50±3	2.0	120±8	4.0	200±10	6.0
17	20±3	0.4	140±5	3.0	n.d.	n.d.

[a] 10^3 unity of $\frac{\text{peak height}}{\text{mg/kg}}$. One peak unity is 0.125 V on the integrator.
[b] Calculated as the ratio signal of compound/noise of a blank >3.
[c] From standard solutions.
[d] From oil extracts.
[f] For eluent mixture and conditions see Section 2.

weighted again and the medium weight ±S.D. (mg) of each compound was determined. This amount was employed to calculate the concentration (in ppm) of aqueous solutions (plus I.S.), assuming density=1. Then 100 μl of the solutions were injected individually and the titles of **3**, **4**, **11** and **12** were calculated from their chromatographic peaks. The identity of the compounds was checked by NMR analysis [4]. Good linearity was achieved in the ranges 1–100 and 100–300 ppm (all r^2>0.980). The obtained R_Fs are shown in Table 2.

3. Results and discussion

By preliminary experiments we found that eluting mixtures $10^{-3}M$ H_2SO_4–CH_3CN (85:15, v/v) allow the detection of simple phenols at λ=225 nm with a good separation. At the ratio of 70:30, a fairly good separation was obtained, but the simple phenol signals overlap. A gradient solvent program starting from 85% of 10^{-3} M H_2SO_4 to reach 70% in 20 min showed a bad separation of complex phenols. Because the two mixtures (85:15 and 70:30) show the same UV absorption background, we used the first eluent ratio for up to 12 min and subsequently the ratio 66:34. This last ratio shows the same UV absorption background of the 70:30 ratio, and was found to be better particularly in the separation of peaks **2**, **12** and **5**. Table 1 shows the RRT (internal standard phthalimide) of simple and complex phenols, and of unknown compounds (maybe of a phenolic nature), on different columns (ODS-2, ODS-1, C_8 and Phenyl). The RRTs of the complex phenolic compounds appear to be very similar in all columns employed. A good separation was achieved mainly on octadecyl and octyl columns, while the best separation was on ODS-2 columns. Fig. 2 reports a typical oil extract chromatogram obtained on ODS-2 columns at λ=225 nm under these eluting conditions compared with a blank obtained from a washed oil [4]. The elution order of the known complex phenols appears to be the same as reported in the literature [4,16,17,19]. Identification of peaks was checked by the DAD by comparing the spectra with those of standards isolated from oils as reported above. In Table 2 are reported the R_Fs and DLs of the separated compounds and from these data the following findings can be emphasized: (1) at 225 nm the response of simple and complex phenols is 3–14 fold higher than at 280; (2) at 240 nm this response was from 2–16 fold higher (the latter value is referred only to elenolic acid **2**); (3) at 225 nm new

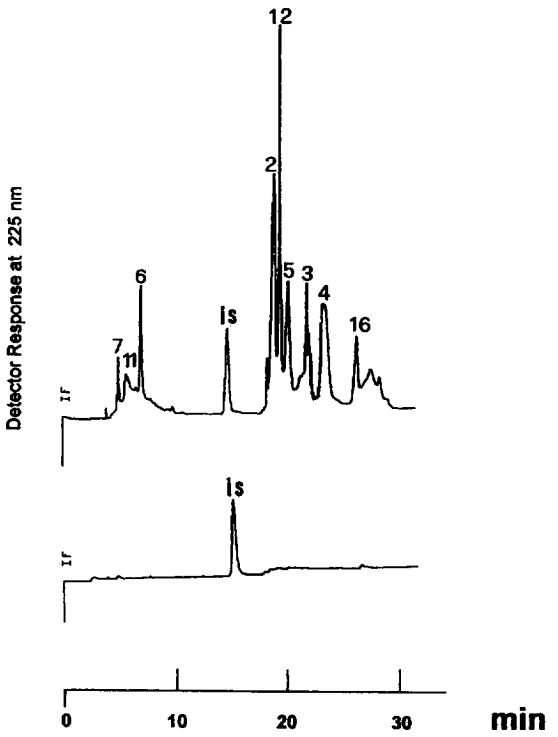


Fig. 2. Chromatogram of the separation on an ODS-2 column of an oil extract. Peaks numbers and formulas of Fig. 1 are coincident. Concentrations found (mg/kg): **7**=4.5; **11**=not determined; **6**=5.2; **2**=70.5; **12**=27.0; **5**=30.6; **3**=29.8; **4**=82.1; **16**=not determined.

signals of unknown compounds (maybe phenolic) were detected (Nos. **11**, **12**, **13**, **14**, **15**, **16**).

3.1. Oil analysis

In the 25 Sardinian oils examined, **11** (RRT=0.37), **12** (RRT=1.18) and **16** (RRT=1.82) were found most abundantly. Compounds **11** and **12** were found in all 25 oils, whatever the cultivar used in their production, while **16** was found particularly in the oils obtained from the "Bianca di Cagliari" cultivar. The UV profile of **11** is similar to that of elenolic acid **2** except for a downshift of 7 nm in its maximum. Its chromatographic and spectroscopic features agrees neither with those of known simple phenols usually found in oils [9], nor with those of the unusually shikimic acid **17** (RRT=0.25), whose presence could occur in olive oil. Compound **16** showed its UV maximum at 229 nm, and its RRT suggests an apolar structure. The UV spectrum of peak no. **12** coincides with the spectrum of a phenolic compound (diode array detector analysis, purity index 1.1) for which the structure reported in Fig. 3 was proposed by LC–MS analysis [22]. Signal **12** shows a very interesting behaviour. When the oil extract was recovered with water, **12** disappeared rapidly from the chromatograms according to a pseudo first order kinetics up to two half-lives ($t_{1/2}$= 105 min, r^2 =0.995); contemporaneously peak 4 was observed to increase. In contrast, the concentration of **12** (peak height vs. internal standard height) was found constant for up to 36 h at room temperature in extracts recovered with aprotic dipolar solvents (e.g., tetrahydrofurane, acetone, dioxane) or with protic solvents with pK_a values larger than water (e.g., methanol). This behaviour agrees with the equilibrium between these linked phenolic compounds proposed by Angerosa et al. [18], and is consistent with the disappearance of **12** during TLC purification of the compounds previously separated by HPLC. Isomerization to **4** is probably due to the acidity of silica. The same behaviour was found in standard water solutions of **12**. Therefore, isomerization from **12** to **4** could occur with the hypothetical behaviour shown in Fig. 3.

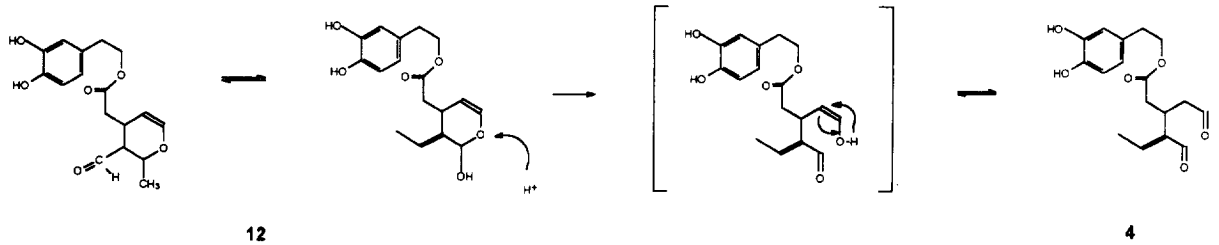


Fig. 3. Proposed behaviour for the isomerization of compound **12**.

4. Conclusions

The proposed method allows a fast and simple separation of phenolic compounds from oil extracts, starting from low amounts of oil (1.0 g). The low λ employed allows quantitative determinations of complex and simple phenols at concentrations from 2–16 fold lower than those of the HPLC methods reported in the literature. Moreover, phenolic substances with different absorbance maxima (e.g., elenolic acid **2** and peak **11**) non-detectable together with simple or complex phenols by the literature methods, were determined simultaneously. Finally, the presence of unknown compounds, probably of a phenolic structure (**11**, **16**), was shown. Further studies on the identification of these unknown compounds by NMR, GC–MS and LC–MS, on their influence on the stability of oils, and on the influence of technological parameters on the presence of these compounds in olive oil, are in progress.

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