Polyphenolic Content in Five Tuscany Cultivars of *Olea europaea* L.

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In this study polyphenolic compounds extracted from olive fruits of five registered cultivars were analyzed. A solid−liquid extraction (LSE) procedure with Extrelut cartridge (diatomaceous earth) using different eluents was developed to obtain polyphenolic compounds. HPLC-DAD and HPLC-MS methods were applied for the quali-quantitative analysis of each fraction obtained from LSE. The results of this work show that the LSE procedure with diatomaceous earth cartridge supplies a rapid and reproducible fractioning method able to obtain a quantitative recovery of all compounds and to collect fractions directly analyzed by HPLC. A comparison among different cultivars shows significant quantitative differences in some polyphenols, such as verbascoside, anthocyanic compounds, and oleuropein derivatives.

Keywords: *Polyphenolic compounds; olive fruit; anthocyanins; oleuropein; verbascoside; solid−liquid extraction; HPLC-DAD; HPLC-MS*

INTRODUCTION

Polyphenolic compounds influence the sensorial properties of olive fruits and virgin olive oils and are important markers for studying fruit characteristics of different cultivars and for controlling oil production processes. Their antioxidant effects are also correlated to the high stability of virgin olive oil against thermoxidation and autoxidation processes over time. Some authors (Perrin, 1992; Ciappellano et al., 1994; Baldioli et al., 1996) have compared antioxidant activity in some olea polyphenols and synthetic derivatives, concluding that caffeic acid and hydroxytyrosol showed stronger activity than BHT.

Polyphenols of olea fruits show both autoprotective and nutritional−therapeutic effects.

They play an important role in human nutrition as preventative agents against several diseases. The antiatherogenic effect of an olive polyphenolic extract protecting the LDL against oxidative modifications was recently evidenced (Visioli et al., 1994). Ficarra and others have also shown hypocholesterolemic and hypoglycemic activity of oleuropein (Ficarra et al., 1991; Le Tutor et al., 1992; Driss et al., 1996). Further study suggested that dietary intake of olive oil polyphenols may lower the risk of reactive oxygen metabolite-mediated diseases such as some gastrointestinal diseases and atherosclerosis (Manna et al., 1997).

Activities leading to enzymatic inhibition have been investigated for caffeic acid glycoside esters (Andary, 1993). Verbascoside particularly inhibits the activity of 5-lypoxygenase and protein kinase C, which are involved in some pathological processes.

The aim of the present investigation was to evaluate the polyphenolic content in different olive cultivars. The total polyphenols amounts of olives are very different due to several factors, that is, type of cultivar, growing conditions, and time of ripening (Vlahov, 1992; Esti et al., 1998).

Polyphenolic compounds from five different cultivars registered in Tuscan germplasm (Cimato et al., 1997) were analyzed by HPLC-DAD. A quantitative evaluation of each compound was performed to obtain better knowledge of the olive polyphenolic profile.

Table 1. Weights (Grams) of 100 Olives of Each Cultivar[a]

	Ci	Cu	Ro	Gr	Fr
olives	233	138	272	249	188
pulps	176	97	215	195	135
pulp/stone	3.1	2.4	3.8	3.6	2.5
moisture %	50.6	46.2	47.6	52.2	47.3
longitudinal diameter[b]	13.3	13.4	16.0	16.2	14.7
trasversal diameter[b]	7.0	7.2	8.3	7.5	6.4

[a] Means of three determinations. [b] Data obtained from Cimato et al. (1997).

EXPERIMENTAL PROCEDURES

Sample Preparation. In the spring of 1992, all of the mother plants were cloned and used to establish a germplasm nursery in Follonica (Tuscan coastal area).

The following Tuscan cultivars were considered: Frantoio (Fr), Rossellino (Ro), Ciliegino (Ci), Cuoricino (Cu), and Grossolana (Gr). Except for Frantoio, the cultivars have never before been analyzed.

The olive fruits were harvested in November at technological ripening, immediately frozen in liquid nitrogen, and stored at −80 °C.

In Table 1 several agronomical parameters, as well as the average of three different samples relative to the weight of 100 olives chosen among 4 kg of fruits and the corresponding pulp, are reported for each cultivar.

The frozen pulp of each sample was ground and then extracted with 4 × 400 mL of 80% EtOH with 2% sodium bisulfite. The raw ethanolic extracts were concentrated under reduced pressure (Rotavapor 144R, Büchi, Switzerland) and rinsed with acid water (pH 2.5 by formic acid) to a final volume of 250 mL. For each cultivar 50 mL of the aqueous solution

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was then extracted more times with *n*-hexane to completely remove lypophilic compounds, and the aqueous phase was concentrated (T = 26 °C) to 20 mL.

Liquid−Solid Extraction (LSE). For each sample the defatted aqueous solution (20 mL) was placed on the Extrelut cartridge of 20 mL (Merck, Darmstadt, Germany). Twenty minutes after deposition the following elution steps were performed: (1) *n*-hexane; (2) EtOAc; (3) acid MeOH (pH 2.5 by HCOOH). All elution steps were carried out up to colorless eluate using 250−300 mL of solvents 1 and 2, whereas for acid methanol 150−300 mL was usually applied.

A thin-layer chromatography (TLC) control of the EtOAc eluate and the disappearance of the red color on the cartridge were, respectively, applied to verify the exhaustive extraction at steps 2 and 3. One milliliter of the EtOAc eluate was concentrated to 100 μL and applied on a silica gel TLC plate (Merck). The absence of spots was indicative for stopping the elution.

The EtOAc and MeOH fractions were concentrated at dryness under reduced pressure and then analyzed by HPLC-DAD and HPLC-MS.

Analytical Techniques and Equipment. HPLC-DAD analysis was performed on an HP 1090L liquid chromatograph equipped with an HP 1040A DAD detector and managed by an HP 9000 workstation (all from Hewlett-Packard, Palo Alto, CA).

The column was a 4.6 × 250 mm LiChrosorb RP18 (5 μm) (Merck) maintained at 26 °C. The eluent was H_2O (adjusted to pH 3.2 by H_3PO_4)/CH_3CN. A four-step linear solvent gradient was used, starting from 100% H_2O up to 100% CH_3-CN, during a 106 min period, at a flow rate of 1 mL min^{-1}, as previously reported (Romani et al., 1996), and UV−vis spectra were recorded in the 190−450 nm range; chromatograms were acquired at 254, 280, 310, 330, and 360 nm.

The anthocyanic fractions were analyzed by HPLC using the following conditions: Aquapore RP 300 column (Browlee Laboratory, Santa Clara, CA), 7 μm, 250 × 4.6 mm, fitted with a precolumn (30 mm) of the same phase. Mobil phases: solution A, H_2O/HCOOH 9:1; solution B, H_2O/HCOOH/MeOH/CH_3CN 45:10:22.5:22.5 in a three-step linear gradient. For each compound the HPLC-DAD quantitative analysis was performed with calibration curves obtained using pure standards and/or isolated compounds. Four-point calibration curves were used, and the regression coefficients were in the 0.987−1.00 range. The experimental findings represent the average of three different measurements.

HPLC-MS analyses by thermospray mode were carried out according to the method given in a previous paper (Romani et al., 1996).

The anthocyanic HPLC-MS analysis by API ion spray interface was carried according to experimental conditions previously reported (Baldi et al., 1995a).

Centrifuge preparative chromatography (C-TLC) was performed by a Chromatotron 3924, Harrison Research (CT), equipped with a fraction collector (ISCO Retrivier 500). Two hundred and thirty fractions were collected and checked by HPTLC, HPLC-DAD, and HPLC-MS. The experimental conditions have previously been described (Baldi et al., 1995a; Romani et al., 1996).

All of the pure standards were purchased from Extrasynthese S.A. (Lyon, Nord-Genay, France): thyrosol, vanillic acid, oleuropein, rutin, luteolin 7-*O*-glucoside, luteolin, apigenin 7-*O*-glucoside, apigenin 7-*O*-rutinoside, homoorientin, cyanidin 3-*O*-rutinoside, and cyanidin 3-*O*-glucoside. Compounds were identified by comparing their t_R and UV-vis and MS spectra with those of reference standards.

RESULTS AND DISCUSSION

The LSE procedure using a diatomaceous earth cartridge supplies a rapid and reproducible fractioning method able to obtain a quantitative recovery of all compounds. Complete recovery was checked using simulated mixtures at different concentrations of oleuropein, rutin, and cyanidin 3-*O*-rutinoside. The fractions collected can be directly analyzed by HPLC-DAD and HPLC-MS. Using this method, it was possible to divide the total raw extract (aqueous residue) into two main fractions: the anthocyanic compounds in acid MeOH and the other polyphenols in EtOAc solution. This simple procedure makes it possible to prepare and analyze several samples in a short time. It was possible, using a small quantity of solvent, to detect and quantify several subclasses of polyphenols, such as phenolic acids, oleuropein derivatives, flavons, flavonol glycosides, and anthocyanin derivatives.

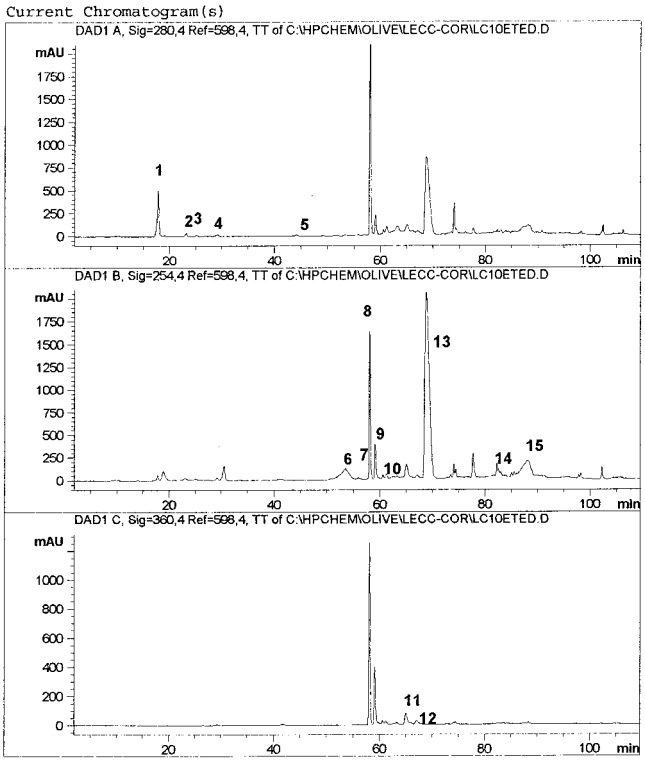

Figure 1. Chromatographic profile of EtOAc fraction from Extrelut at 280, 254, and 360 nm. Peaks: 1 = hydroxytyrosol; 2 = tyrosol; 3 = vanillic acid; 4 = *p*-coumaric acid; 5 = vanillin; 6 = demethyloleuropein; 7 = homoorientin; 8 = verbascoside; 9 = rutin; 10 = luteolin 7-*O*-glucoside; 11 = apigenin 7-*O*-rutinoside; 12 = apigenin 7-*O*-glucoside; 13 = oleuropein; 14 = luteolin; 15 = oleuropein aglycon.

In Figure 1 the chromatograms obtained from the LSE EtOAc extract of Ciliegino olives, registered at 280, 254, and 360 nm, are reported. In Figure 2 the HPLC profiles of the methanolic fraction from cv. Frantoio and cv. Rossellino are shown.

To isolate several compounds, also present in trace amounts, the fractions obtained from LSE were fractionated by silica phase centrifuge-TLC (C-TLC). Particularly, homoorientin (luteolin 6-*C*-glucoside), up to now undetected in olive, was collected and analyzed by HPLC-DAD and HPLC-MS. Identification was performed by comparing t_R and UV and MS spectra with the authentic standard data.

Standards were not available for the following compounds: hydroxytyrosol, verbascoside, demethyloleuropein, and oleuropein aglycon. The identification of these compounds was performed by comparing the HPLC-DAD and HPLC-MS profiles of specific C-TLC fractions with the hydrolyzed products of oleuropein standard (Baldi et al., 1992; Romani et al., 1996).

Table 2. Polyphenolic Compounds (Milligrams per Kilogram of Olives) in Different Cultivars[a]

	Ci	Cu	Ro	Gr	Fr
hydroxytyrosol	566.84 ± 28	1049.50 ± 56	4133.00 ± 41	1812.20 ± 51	1694.00 ± 78
tyrosol	101.32 ± 2.3	189.49 ± 4.9	413.13 ± 14	292.75 ± 13	1186.00 ± 92
vanillic acid	6.17 ± 0.8		1.94 ± 0.06	6.45 ± 0.5	2.38 ± 0.1
demethyloleuropein	142.57 ± 4.1	104.95 ± 1.2	13.30 ± 0.3	87.78 ± 5.1	600.30 ± 17
oleuropein	2406.2 ± 67.6	1557.90 ± 60	35.83 ± 0.1	1136.20 ± 10	590.50 ± 58
oleuropein aglycon	1311.9 ± 25.7	1555.80 ± 90	24.19 ± 1.8	1990.60 ± 45	685.20 ± 23
rutin	211.43 ± 3.3	272.96 ± 11	161.43 ± 5.4	146.89 ± 11	111.20 ± 15
luteolin 7-*O*-glucoside	129.13 ± 4.3	69.00 ± 4.8	46.60 ± 2.8	4.75 ± 0.9	60.10 ± 2.1
luteolin	14.00 ± 0.6	11.40 ± 0.9	47.92 ± 2.7	1.04 ± 0.03	29.94 ± 4.9
apigenin 7-*O*-glucoside	40.55 ± 1.6	32.23 ± 2.0	12.67 ± 0.7	29.31 ± 0.7	6.20 ± 0.3
apigenin 7-*O*-rutinoside	17.66 ± 0.4	7.90 ± 0.3	12.84 ± 1.9	3.90 ± 0.4	12.80 ± 1.8
homoorientin	5.64 ± 0.1	3.64 ± 0.3	0.37± 0.02	0.53 ± 0.05	1.05 ± 0.06
verbascoside	3202.10 ± 42	939.50 ± 60	551.24 ± 36.4	161.37 ± 2.8	216.20 ± 11
cyanidin 3-*O*-glucoside	282.8 ± 3.5	85.43 ± 1.8	881.72 ± 4.8	65.00 ± 1.7	52.33 ± 1.3
cyanidin 3-*O*-rutinoside	948.00 ± 16.9	399.99 ± 6.6	3205.71 ± 38.8	248.29 ± 4.1	307.33 ± 7.4
total acylated and/or glycoside anthocyans	370.65 ± 15.5	tr[b]	288.10 ± 9.5	252.08 ± 12.2	263.48 ± 13.1

[a] Means ± SD of three determinations. [b] Traces.

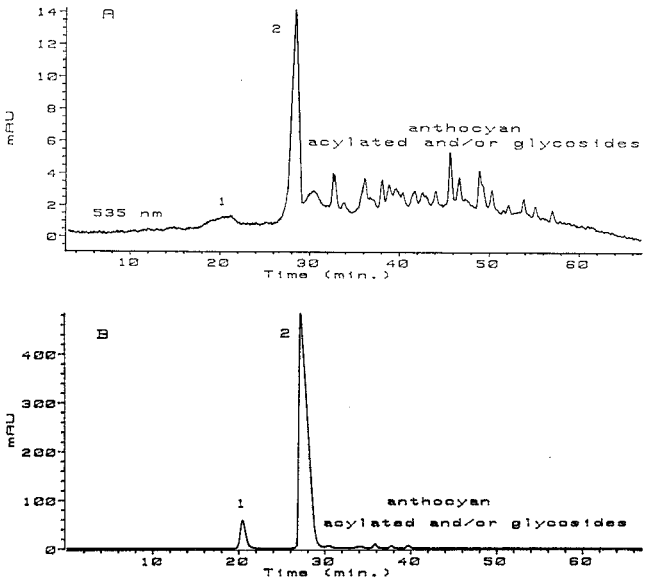

Figure 2. Chromatographic profile, at 535 nm, of the anthocyanic compounds: (A) methanolic fraction from Extrelut cartridge of cv. Cuoricino; (B) methanolic fraction from Extrelut cartridge of cv. Rossellino. Peaks: 1 = cyanidin 3-*O*-glucoside; 2 = cyanidin 3-*O*-rutinoside and anthocyan acylated and/or glycosides.

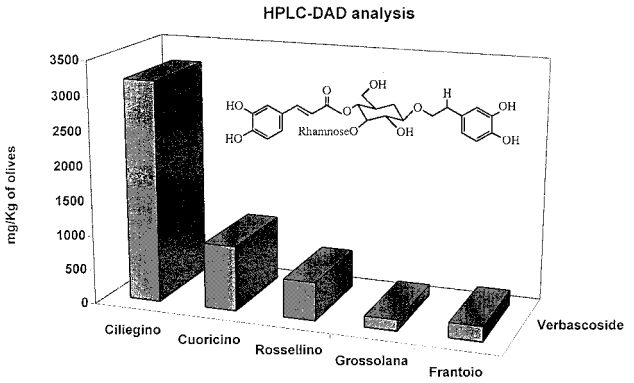

Figure 3. Verbascoside amounts (milligrams per kilogram of olives) evaluated by HPLC-DAD.

The list of polyphenolic and anthocyanic derivatives detected and calibrated in each cultivar is reported in Table 2.

"*The olive tree germplasm in Tuscany*" (Cimato et al., 1997) reports Ci olives as a slow and regular-ripening cultivar with fruits the color of red wine and a low oil yield; Cu olives having slow ripening, low oil yield with peculiar organoleptic characteristic, and black-violet fruits at harvesting; Fr olives as a gradual slow-ripening cultivar with valuable, sapid, and fragrant oil; Gr olives as a slow-ripening cultivar, having fruits used for both quality oil and salad oil production; and Ro olives as an almost precocious-ripening cultivar with a good productivity and red-wine fruits.

The olive samples show a very different polyphenolic content (Table 2) that could be related to their agronomic characteristics. Oleuropein and its aglycon are present in a very large amount in Ci, Cu, and Gr olives, described as slow-ripening cultivars, whereas Ro olive, a precocious-ripening cultivar, shows the lowest content of these compounds. Conversely, the hydroxytyrosol, which can be obtained from hydrolyzed oleuropein, is in the greatest amount in Ro olives, whereas the content is low in Ci olives. Probably at the date of olive collection the grade of ripening was higher for Ro than for Ci olives.

Interesting differences were also in evidence for the total flavonol content. Ci and Cu olives show the highest content of rutin, luteolin 7-*O*-glucoside, and apigenin 7-*O*-glucoside. Among the detected compounds particular attention should be directed to verbascoside. In fact, the presence of this compound is very diversified among the cultivars, and the largest amount was observed in Ci olives (Figure 3). As evidenced by its chemical structure, it is possible to hypothesize a correlation between the biosynthesis of verbascoside and the metabolic pathway of the oligosaccharides. These considerable differences in verbascoside concentration could be induced to propose this compound as a chemotaxonomical marker of different cultivars harvested at the same date.

Significant quali-quantitative differences were also observed in anthocyanic content. Ci and Ro olives were the richest cultivars in the two main anthocyanins, cyanidin 3-*O*-glucoside and 3-*O*-rutinoside. Fr and Gr olives contain the lowest amounts of these anthocyans; conversely, several minor components, presumably corresponding to acylated and glycoside anthocyans, were observed in these samples (Figure 2).

In Table 3 the ratio between the two main anthocyanins and the acylated and/or glycoside derivatives (*R*) is reported. This value could be an interesting parameter able to better differentiate specific cultivars. Moreover, the presence of acylated and/or glycoside anthocyanins, even in traces, could represent a useful tool for

Table 3. Ratio Values of Anthocyanic Compounds Evaluated by HPLC-DAD in Different Cultivars[a]

	Ci	Cu	Ro	Gr	Fr
R	3.33	2.52	14.19	1.24	1.36

[a] Means of three determinations.

a phytochemical characterization of the cultivars. To identify and quantify these minor anthocyans, further investigations are in progress.

The data obtained from this study may represent only a momentary averaged phenolic composition that, however, provides the basis for further work.

CONCLUSIONS

The data presented in this paper seem to indicate, despite the limited number of cultivars examined, that polyphenol composition might represent a useful contribution to biochemical characterization of olive fruit varieties.

Moreover, the fruit of some cultivars could be interesting sources of single active compounds. High amounts of oleuropein and verbascoside have been found in Ci olive, whereas Ro olive is the richest in hydroxytyrosol and cyanidin 3-*O*-rutinoside.

These findings could be used for controlling production processes and correlating the oil sensorial characteristics to the polyphenolic pattern.

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