^{1}H NMR Study of Phenolics in the Vegetation Water of Three Cultivars of *Olea europaea*: Similarities and Differences†

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In this work free phenolics and phenolic glucosides present in the water content (vegetation water, VW) of olive fruits of the three Italian cultivars (cvs.) Cipressino, Dritta, and Leccino were identified by ^{1}H NMR. The phenolic metabolite content of the three cultivars was studied at three fruit growth stages. The major phenolic compounds, common to all three cultivars, were 4-hydroxyphenylethanol (tyrosol) (**1**), 4-hydroxyphenylethanol glucoside (**2**), and oleuropein (**3**). Considerable differences in the content of compounds (**1**−**3**) were found to occur in the fruits during growth and maturation of the drupe. Glucoside (**4**), a major component of the low molecular weight solutes of Dritta and Leccino cvs., was not detected in Cipressino fruits. The quinol glucoside (**4**) was thought to derive from oxidation of the glucoside of 4-hydroxyphenylethanol (**2**). Hydrolysis of (**4**) followed by cyclization yielded the bicyclic compound halleridone (**5**).

Keywords: *Olea europaea; Oleaceae; ^{1}H NMR; free and conjugated phenolics; metabolite monitoring in olives*

INTRODUCTION

Olives are an ancient culture in the Mediterranean Basin, and it is documented how they have been processed since early times. Olives are used both for production of high-quality olive oil and as eating olives. The fruits have a triacylglycerol content in the range 15−25% by weight with a saturated−unsaturated fatty acid ratio favorable to human health. Furthermore, among vegetable oil crops, olives are the most rich in phenolic compounds concentrated in the vegetation water of the fruit that contains also large amounts of sugars (Perrin, 1992; Bianchi and Pozzi, 1994).

The water-soluble free and conjugated phenols are considered to contribute to a large extent to sensory characteristics of olives such as the astringency and the bitter taste of unripe olives and, on the other hand, of the most desirable attributes of processed table olives (Shahidi and Naczk, 1992).

These phenolic substances have been reported to improve the stability of oil against autooxidation (shelf life) and also to affect the well-known lipoxygenase pathway inhibiting oxidative attack on reactive substrates (Perrin, 1992; Montedoro and Cantarelli, 1969; Camurati et al., 1982; Chimi et al., 1988; Le Tutour and Guedon, 1992; Papadopoulos and Boskou, 1991; Gutiérrez Gonzàles-Quijano et al., 1977; Montedoro et al., 1992; Tsimidou et al., 1992a,b; Dziedzic and Hudson, 1984; Montedoro et al., 1978; Nergiz and Unal, 1991; Papadopoulos and Tsimidou, 1992; Solinas and Cichelli, 1981; Fedeli et al., 1973; Fernandez-Diez, 1971; Flath et al., 1973; Olìas et al., 1980; Guth and Grosch, 1991; Phillips and Galliard, 1978). Besides these properties, phenolics substances were proved to play also relevant physiological and pharmaceutical roles in human health (Shahidi and Naczk, 1992; Tsimidou et al., 1992a,b; Vàsquez Roncero et al., 1974a,b). A large number of records concerning total phenol content in olive oil by using different methods of analysis are available in the literature (see Table 1), but to our knowledge, only few papers have appeared on detailed analysis of low molecular weight substances present in the olive fruit vegetation water (Ragazzi and Veronese, 1967; Vàsquez Roncero et al., 1974a,b; Balice and Cera, 1984; Lo Scalzo and Scarpati, 1993; Bianchi and Pozzi, 1994).

A survey list of compounds, whose detection and presence in leaves, fruits, and oil were reported for olive plants, is shown in Table 1. More precisely, regarding olive fruits vegetation water, besides the free C6, C6−C1, C6−C2, and C6−C3 simple benzenoids and phenols, the aqueous phase was reported to contain a large amount of glucosides among which the secoiridoid oleuropein was dominant (see Table 1).

In a continuation of our investigation on the water-soluble olive fruit substances and on the "in vitro" transformation of oleuropein by β-glucosidase (Limiroli et al., 1995; Bianchi and Pozzi, 1994), ^{1}H NMR spectroscopy was used as a versatile and rapid method to determine the water-soluble substances of olives in order to widen our knowledge on the change of composition of these secondary metabolites during the ontogeny of the fruit and to understand biosynthetic relationships linking some of the observable metabolites. The compositional differences or similarities between the three varieties would suggest the presence of underlying genetic and biochemical differences which may have a phylogenetic component in phenolic compounds.

RESULTS

The qualitative distribution of the main free and conjugated phenols of the three varieties is shown in Table 2. The samples examined were obtained from about 300 g of olives. The identification and a qualitative evaluation of water-soluble metabolites present in the vegetation water of the fruits relied on ^{1}H NMR techniques. Our method of determining these complex

† This paper is dedicated to the memory of Professor Pierluigi Gariboldi.

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Table 1. Literature Data on Free and Conjugated Phenols in Olive Fruits, Leaves, and Oils: A Survey

classes of compounds	source	references[a]
C6		
cathecol	VW,[b] WW[c]	9, 27
C6−C1		
benzoic acid	oil	16
4-hydroxybenzoic acid	oil	11, 16, 23, 25
3,4-dimethoxybenzoic acid	oil, VW	3, 16
4-hydroxy-3-methoxybenzoic acid	oil, VW	3, 6, 11, 12, 16, 23, 25, 31, 36
4-hydroxy-3,5-dimethoxybenzoic acid	oil, VW	3, 11, 16, 23, 25, 31
3,4,5-trimethoxybenzoic acid	VW	3
3,4-dihydroxybenzoic acid	VW, oil	11, 25, 27, 36
C6−C2		
4-hydroxyphenylacetic acid	oil, VW	1, 3, 17
4-hydroxy-3-methoxyphenylacetic acid	oil	1, 17
4-hydroxyphenylethanol	oil, VW, WW	1, 4, 5, 10, 15, 17, 18, 23, 27, 30−33, 35−36
3,4-dihydroxyphenylethanol	oil, VW, WW	1, 4, 6, 8, 10, 11, 15, 17, 18, 20, 25, 26, 29, 31, 33, 34−37, present work
3,4-dihydroxyphenylglycol	VW	4, present work
halleridone	VW	5, present work
C6−C3		
cinnamic acid	oil	11
2-hydroxycinnamic acid	oil	11
4-hydroxycinnamic acid	oil, olive	6, 8, 11, 12, 23, 31, 36, 37
3,4-dihydroxycinnamic acid	oil, olive, VW	1, 3, 5 4, 8, 23, 25, 29, 36, 37
4-hydroxy-3-methoxycinnamic acid	oil, VW	23
4-hydroxy-3,5-dimethoxycinnamic acid	oil	11
(C6−C2) glucosides		
dihydroxyphenylethanol glycoside	VW	37
salidroside	seeds	21
cornoside	olive	5, present work
(C6−C2; C6−C3) glucosides		
verbascoside	olive	2, 5
iridoids		
elenolic acid	oil	23
oleuropein aglycones	oil, olive	13, 20, 23, 29, 30
oleoside	leaves	14
oleuropein	oil, olive, VW, leaves	2, 4, 6, 8, 19, 20, 25, 30, 36−40, present work
nuzhenide	seeds	21
demethyloleuropein	olive	2, 5, 28, 30
ligstroside	leaves	19
oleuroside	leaves	14, 19
secoiridoids	oil, leaves	14, 24, present work

[a] (1) Akasbi et al., 1993. (2) Amiot et al., 1986. (3) Balice and Cera, 1984. (4) Bianchi and Pozzi, 1984. (5) Bianco et al., 1993. (6) Brenes-Balbuena et al., 1992. (7) Breton et al., 1987. (8) Camurati and Fedeli, 1982. (9) Capasso et al., 1992. (10) Cortesi and Fedeli, 1983. (11) Cortesi et al., 1981. (12) Evangelisti et al., 1994. (13) Fernandez-Diez, 1971. (14) Gariboldi et al., 1986. (15) Graciani Constante and Vàzquez Roncero, 1980. (16) Graciani Constante et al., 1980. (17) Graciani Constante and Vàzquez Roncero, 1981. (18) Janer del Valle and Vàzquez Roncero, 1980. (19) Kuwajima et al., 1988. (20) Lo Scalzo and Scarpati, 1993. (21) Maestro-Duràn et al., 1994. (22) Montedoro and Cantarelli, 1969. (23) Montedoro et al., 1992b. (24) Olìas et al., 1980. (25) Perrin, 1992. (26) Ragazzi and Veronese, 1973. (27) Ragazzi and Veronese, 1967. (28) Ragazzi et al., 1973. (29) Solinas et al., 1975. (30) Solinas et al., 1978. (31) Solinas and Chichelli, 1981. (32) Solinas, 1987. (33) Tsimidou et al., 1992b. (34) Vàzquez Roncero et al., 1973. (35) Vàzquez Roncero et al., 1975. (36) Vàzquez Roncero et al., 1976. (37) Vàzquez Roncero et al., 1974b. (38) Cortesi et al., 1984. (39) Goupy et al., 1991. (40) Panizzi et al., 1990. [b] VW, vegetation water. [c] WW, wastewater.

Table 2. Phenolic Compounds in the Fruits of Olive Cultivars[a]

compound	Cipressino	Dritta	Leccino
1	+	+	+
2	+	+	+
3	+	+	+
4	−	+	+
5	−	+	+
7[b]	+	+	
8[b]	+	+	
9	+	+	+
10	+	+	+

[a] +, presence; −, absence. [b] Identified only in organic extracts. Organic extract of Leccino cultivar was not analyzed.

mixtures of compounds was mainly based on chemical shifts, peak multiplicities, and scalar correlations in two-dimensional and selective excitation experiments. The distribution of the free phenols and their glucosides was obtained by ^{1}H NMR analysis of the total crude aqueous extract and also from the extracts with organic solvent. When possible, resonances were assigned by comparison of chemical shifts and multiplicities with those of standards or with data available in the literature (Montedoro et al., 1993; Lo Scalzo and Scarpati, 1993; Kuwajima et al., 1988; Gariboldi et al., 1986; Messana et al., 1984; Bianco et al., 1993; Kuwajima et al., 1993).

Figures 1 and 2 show NMR spectra of Dritta and Cipressino aqueous extracts, respectively. Table 3 reports the resonances observed in the spectra of extracts of Cipressino, Dritta, and Leccino olive cultivars. Resonances are detailed with splitting pattern and chemical shifts and assigned to the appropriate molecular moiety. In contrast to many previous reports, our analyses failed to detect compounds of C6−C3 class (e.g., cinnamic acid) (see Table 1). Verbascoside, a caffeyl glucoside reported to be an ortho diphenolic compound accompanying the generally dominant oleuropein in a number of olive cultivars, was not found in the extracts examined (Amiot et al., 1986, Bianco et al., 1993). The substance, if present, may have been removed from the aqueous extracts by the browning

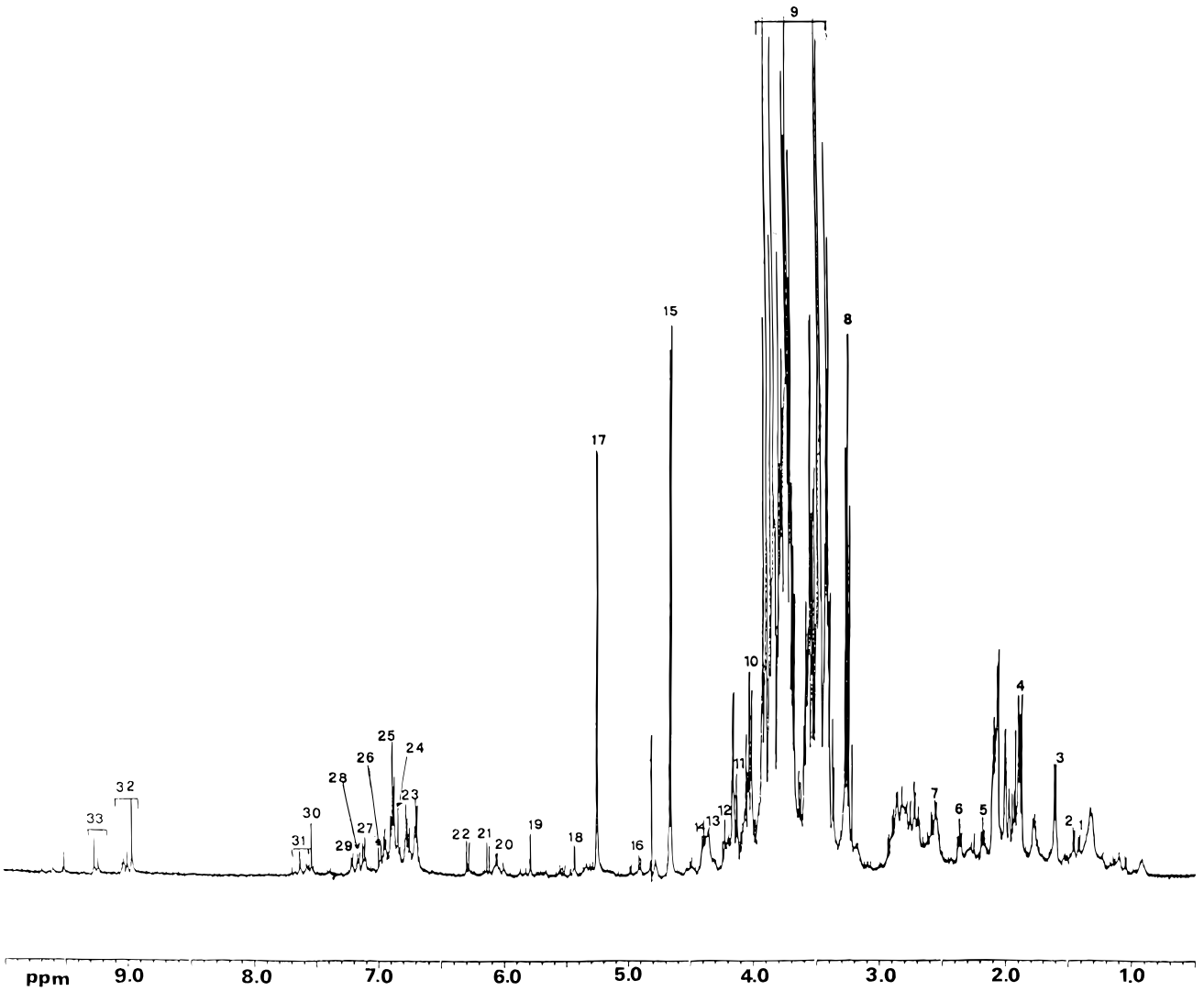


Figure 1. Vegetation water extract of olive fruits of Dritta cultivar collected in August: 500 MHz ^{1}H NMR spectrum in D_2O. Peak numbering refers to the first column of Table 3.

enzymatic process of olives as previously suggested in the literature (Goupy et al. (1991) and references therein reported).

In agreement with data previously reported (Bianchi and Pozzi, 1994), the sugar compositions of the three varieties were essentially the same: glucose was the predominant sugar present, the remainder being made up of fructose, sucrose, myoinositol, and mannitol. Significant differences were observed in the qualitative and quantitative distribution of simple C6−C2 phenolics, according to the variety and the growth stage of the olive fruit (Bianchi and Pozzi, 1994).

In June, the phenolic composition of the crude water extracts from olive fruits of Dritta and Leccino cvs. contained almost the same phenolic substances: cornoside (**4**) (quinol glucoside) and free and conjugated tyrosol (**1**, **2**). The cornoside ^{1}H NMR spectrum showed two doublets (10 Hz splitting) at 6.28 (Figure 3B(iii) and peaks 22 in Figure 1) and 7.08 ppm (peaks 27 in Figure 1), characteristic of cis olefinic protons in α and β position to a carbonyl group, respectively, a triplet at 2.2 ppm (peak 5 in Figure 1) due to the methylene group in position 7, and a doublet at 4.4 ppm due to an anomeric proton. Resonances from protons of the methylene group in position 8 were not visible owing to sugar signal overlap. Presence and identification of free tyrosol (**1**) in the extracts were assured through enrichment with standard tyrosol (Figure 3A(i)). Aromatic H2 and H6 signals show a larger downfield shift in the free (7.22 ppm) than in the conjugated tyrosol (7.15 ppm).

The extract of olive fruits of Cipressino cv. collected in the same season, June period, was characterized by the complete absence of cornoside (**4**) (Chart 1). The aromatic and olefinic region of the spectrum (not reported) showed the signals characteristic of free (**1**) and conjugated (**2**) tyrosol and a multiplet at 6.1 ppm (lower field resonance next to peak 20), whose splitting was reminiscent of the olefinic $=CH-CH_3$ iridoid moiety of an iridoid oleuropein-like molecular structure. The NMR resonances shown in the spectrum do not allow assignment unambigously of the spin system to a molecular structure of the type of ligstroside (**3a**) or isoligustroside (**6**) found in other Oleaceae plants (He et al., 1994).

The characteristic oleuropein signals were not visible in the June olive extracts, as can be seen in Figure 3B-(ii) for the aqueous extracts of Dritta cv. This means that, in the early season, oleuropein was either absent or present in too low an amount to be detected. However oleuropein resonances became readily observable in the August olive extracts, as observed in Figure 3C-(ii).

In addition to the basic phenological varietal differences among the three cultivars, marked differences

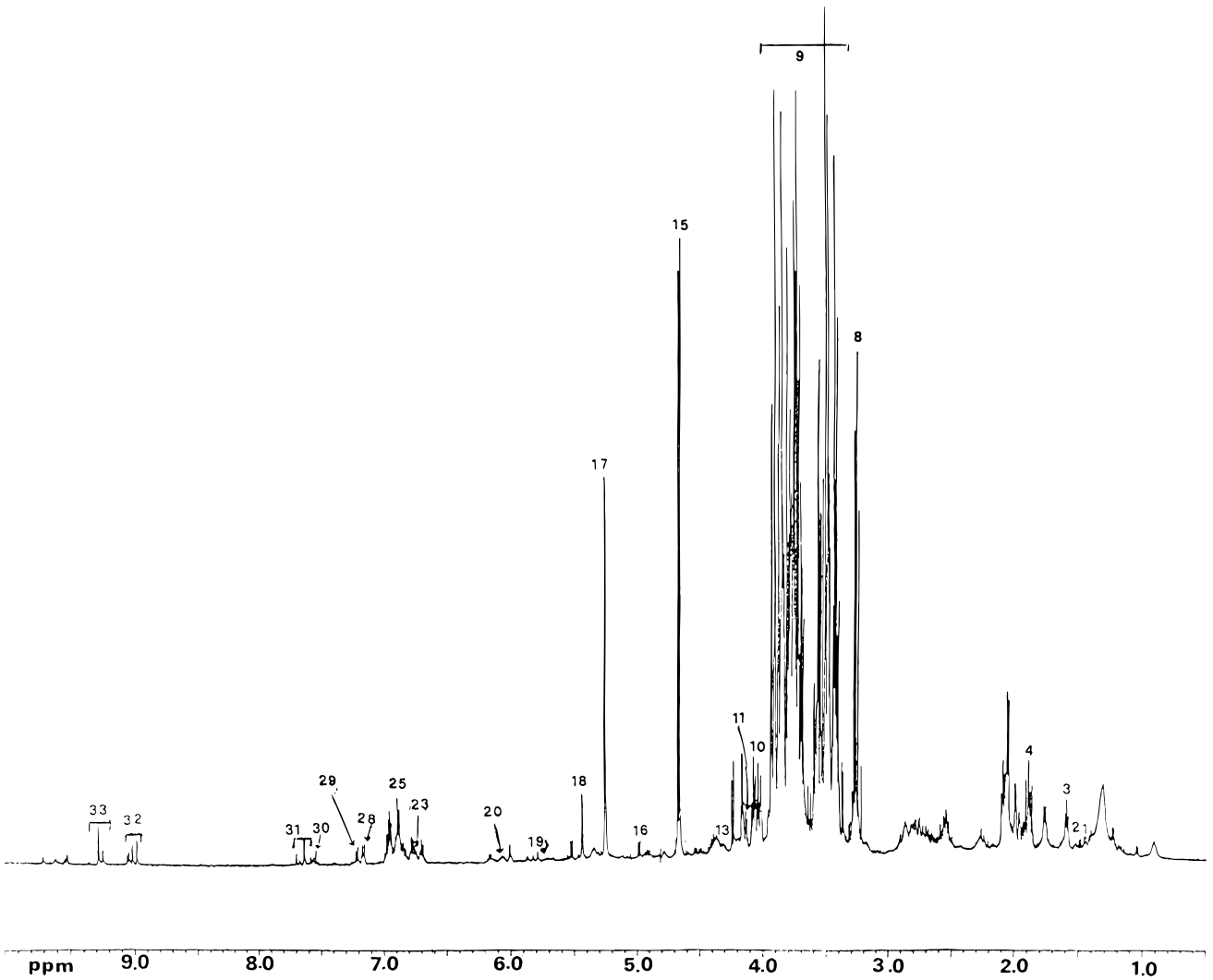


Figure 2. Vegetation water extract of olive fruits of Cipressino cultivar collected in August: 500 MHz ^{1}H NMR spectrum in D_2O. Peak numbering refers to the first column of Table 3.

were also observed in the extract compositions of the three varieties at the stages of growth considered.

Phenolic compositions altered steadily with the olive fruit development, the most notable changes affecting the simple phenolics and their derivatives of the three cultivars. In fact, in the June extracts of the Cipressino cv. the conjugated tyrosol (**2**) was prevalent with respect to the free form (**1**) (Figure 3B(i)), while the two forms were present almost at the same ratio in the August extracts (Figure 3C(i)). Cornoside (**4**), dominant in the June extracts of the Dritta (Figure 3B(iii)) and Leccino cvs., was greatly reduced in olives harvested in August (Figure 3C(iii)) and October. Apparently, and as expected, the decrease of this glucoside is accompanied by an increase of the bicyclic quinol halleridone (**5**) (Compare Figure 3B(iii), C(iii)). According to ^{1}H NMR assignments previously reported in the literature, the halleridone (**5**) could be distinguished from cornoside (**4**) by the signals of the two protons on the double bond conjugated to a carbonyl group, i.e., a 10.3 Hz doublet at 6.12 ppm (Figure 3B,C(iii) and peak 21 in Figure 1) for H3 and a double doublet at 6.98 ppm (peak 26 in Figure 1) for H2 which showed the 10.3 Hz splitting due to the coupling with H3 and a 1.3 Hz splitting due to the long-range W coupling with H6. (Messana et al., 1984; Bianco et al., 1993; Kuwajima et al., 1993).

In order to obtain a partial but useful separation of the classes of compounds comprising the vegetation water residues, the aqueous solutions were extracted with ethyl acetate. This extraction permitted a fairly good separation of phenolics from sugars and polyols that remained in the aqueous medium.

The NMR spectra of the organic extracts rich in phenolic compounds allowed complete identification of the signals of the following compounds: 3,4-dihydroxyphenylethanol (**7**), 3,4-dihydroxyphenylglycol (**8**), and two out of four possible diastereomeric (at carbons 8, 9) secoiridoids (**9**). The resonances of oleuropein glucosidic moiety, hardly distinguishable in the spectra of aqueous extracts due to overlap with the most intense signals from sugars, gave well-resolved spectra, which could be readily assigned.

3,4-Dihydroxyphenylglycol (**8**) was recently described for the first time as a component of the olive vegetation waters and identified by GC−MS (Bianchi and Pozzi, 1994). In this work, the presence of the latter metabolite is confirmed by ^{1}H NMR. In spectra recorded in acetone-d_6 the CH_2(OH)CH(OH)− group of 3,4-dihydroxyphenylglycol is an ABX spin system (A and B indicating the CH_2 protons and X the CH proton, respectively) with resonances at 3.46 ppm for A, 3.54 ppm for B, and 4.54 ppm for X. The aromatic signals of H2 and H6 are visible, respectively, at 6.88 and 6.70 ppm. On the contrary, H5 signal is not easily identifiable, being in a very complex and crowded region of the spectrum.

Table 3. ^{1}H NMR Data Assignments for Metabolites Present in the Vegetation Waters of Olive Cultivars Dritta, Leccino, and Cipressino (500 MHz, D_2O)

peak	δ	multiplicity	signal assignment	compound	olive cultivar[a]
1	1.44	d[b]	H10	**9**	Cip., Drit., Lec.
2	1.48	d[b]	H10	**9**	Cip., Drit., Lec.
3	1.65	d	H10	**3**	Cip., Drit., Lec.
4	1.85−1.92	several d	CH_3−C=C(C)−CHO	**10**	Cip., Drit., Lec.
5	2.2	t	H7	**4**	Drit., Lec.
6	2.48	2 dd	H6a	**9** and/or **10**	Drit., Lec.
7	2.58	dd	H6a	**3**	Cip., Drit., Lec.
8	3.25	n.d.	H2	β-glucose	
9	3.4−3.9	several signals		glucose, fructose, mannitol, sucrose	Cip., Drit., Lec.
10	4.05	n.d.		fructose	Cip., Drit., Lec.
11	4.15	n.d.		fructose	Cip., Drit., Lec.
12	4.22	m	H1′a	**3**	Cip., Drit., Lec.
13	4.38	m	H1′b	**3**	Cip., Drit., Lec.
14	4.4	d	H anomeric	**4**	Drit., Lec.
15	4.68	d	H1	β-glucose	Cip., Drit., Lec.
16	4.92	d	H anomeric	**3**	Cip., Drit., Lec.
17	5.28	d	H1	α-glucose	Cip., Drit., Lec.
18	5.44	d	H1	sucrose	Cip., Drit., Lec.
19	5.78	s	H1	**3**	Cip., Drit., Lec.
20	6.05	q	H8	**3**	Cip., Drit., Lec.
21	6.12	d	H3	**5**	Drit., Lec.
22	6.28	d	H3−H5	**4**	Drit., Lec.
23	6.75	dd	H8′	**3**	Cip., Drit., Lec.
24	6.84	d	H4′	**3**	Cip., Drit., Lec.
25	6.88	d	H7′	**3**	Cip., Drit., Lec.
26	6.98	dd	H2	**5**	Cip., Drit., Lec.
27	7.08	d	H2−H6	**4**	Drit., Lec.
28	7.15	d	H2−H6	**2**	Cip., Drit., Lec.
29	7.22	d	H2−H6	**1**	Cip., Drit., Lec.
30	7.52	s	H3	**3**	Cip., Drit., Lec.
31	7.56−7.68	several s	H3 −(C)C=C*H*−O−	**9**	Cip., Drit., Lec.
32	8.92−9.04	several d	−(C)C=C−C*H*O	**10**	Cip., Drit., Lec.
33	9.22, 9.25	2d[b]	H1	**9**	Cip., Drit., Lec.

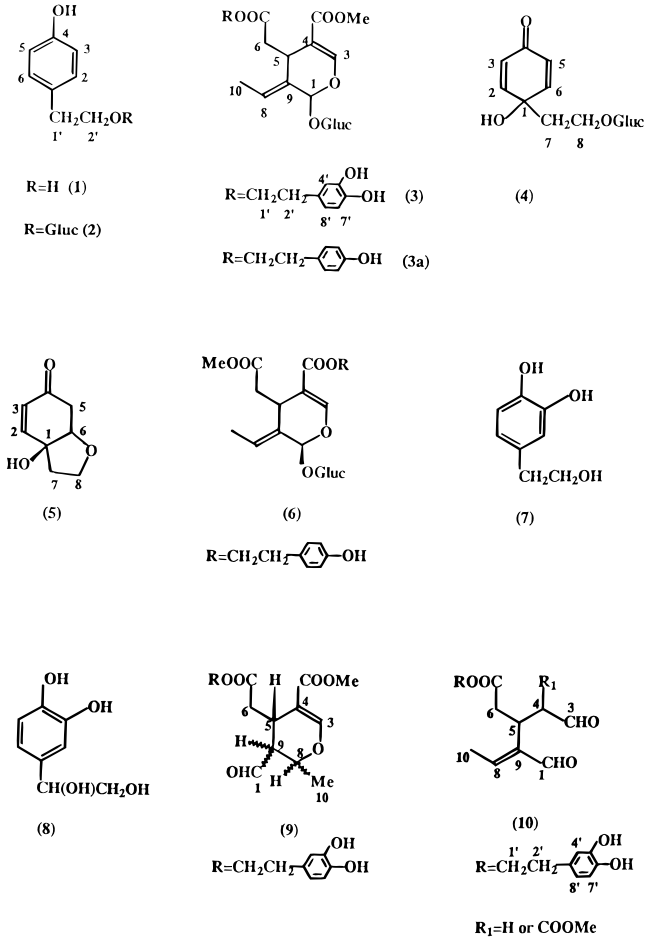
[a] Cip., Cipressino; Drit., Dritta; Lec., Leccino. [b] The doublets correspond to the presence of two out of the four possible diastereomeric (at carbons 8−9) secoiridoids.

While in the aqueous extracts of Dritta cv. collected in August both halleridone (**5**) and cornoside (**4**) are present (Figure 3C(iii)), in the corresponding organic extract only halleridone (**5**) is present. This is probably due to its lower polarity with respect to that of cornoside (**4**). In organic solvent the H2 signal of halleridone (**5**) is at 6.80 ppm with partial overlapping of other signals. The assignment of this signal to halleridone (**5**) was made by one-dimensional selective excitation experiments. Figure 4a shows an expanded region of the spectrum of Dritta extracts in acetone-d_6. Figure 4b shows the same region of the spectrum in a one-dimensional COSY experiment. The doublet at 5.85 ppm was excited with a Gaussian pulse. The excitation caused a total transfer of magnetization from the excited doublet to the coupled spin which appears as an antiphase doublet. In this way it was possible to identify the exact chemical shift of H2 and also to assign this signal to the H2 proton of halleridone (**5**). In fact, as mentioned above, H2 of halleridone is expected to be a double doublet.

DISCUSSION

The different compositions found for the phenolic solutes of the three olive varieties studied and their changes with the development of the fruits emphasize the complexity of the biochemical processes controlling the formation of these water-soluble compounds present in olive fruits. The contrasting distribution between the phenolics and quinol substances in the vegetation water of olives studied represents a major varietal difference. Such clear uncovered differences were not reported in the numerous previous analyses of olive vegetation waters.

Chart 1



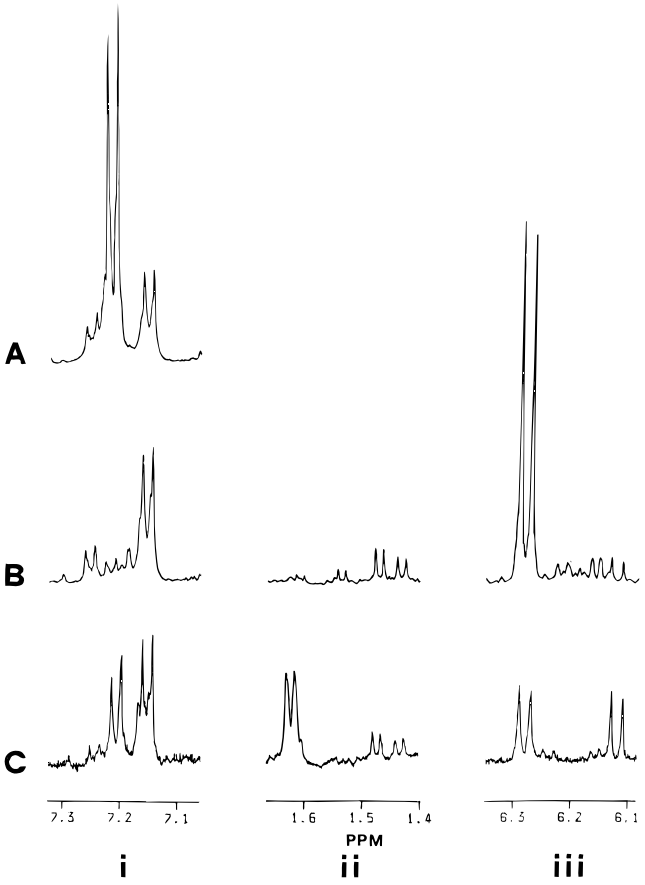


Figure 3. (A) (i) Fruits of Cipressino cv. collected in June: the same aromatic region as part B (i) after enrichment with tyrosol. Vegetation water extracts of olive fruits collected in June (B) and August (C): selected regions of 500 MHz ^{1}H NMR spectra in D_2O. (i) Cipressino cv.: aromatic region of free (**1**) and conjugate (**2**) tyrosol (H2-H6 at 7.22 ppm for (**1**) and 7.15 ppm for (**2**)). (ii) Dritta cv.: methyl region of oleuropein (**3**) (H10 at 1.65 ppm). (iii) Dritta cv.: olefinic region of cornoside (**4**) (H3, H5 at 6.28 ppm) and halleridone (**5**) (H3 at 6.12 ppm).

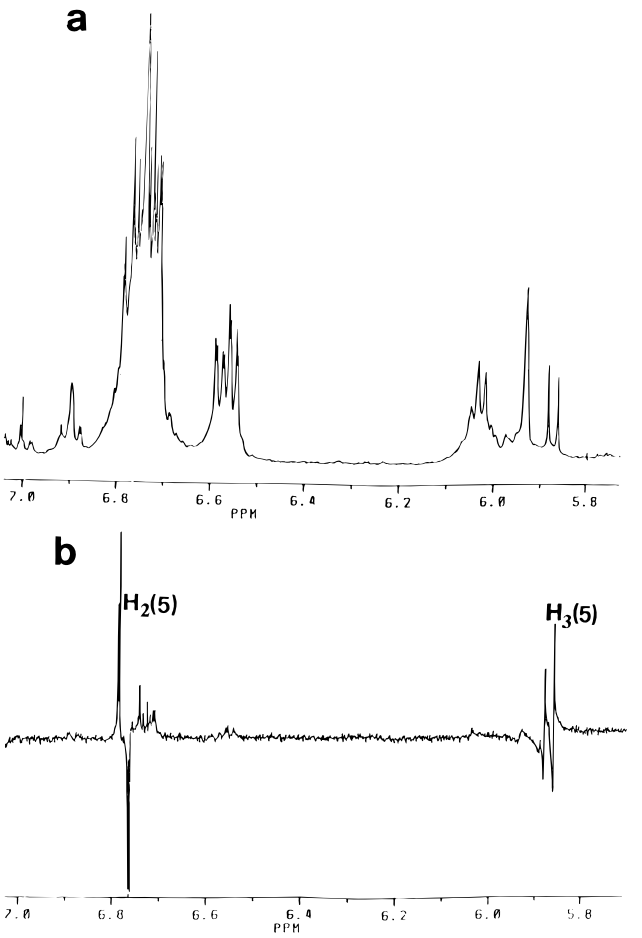


Figure 4. Vegetation water extract of olive fruits of Dritta cv. collected in August: (a) a region of 500 MHz ^{1}H NMR spectrum in acetone-d_6; (b) the same region of the spectrum as appears in a selective excitation experiment. Semiselective excitation was performed using a Gaussian-shaped pulse. The 90° soft pulse was 80 ms with an external attenuation of 42 dB.

Only sparse and scarce reports are in fact available in the literature concerning ontogenic variations of phenolics in olives, and almost all of them are related to oleuropein (**3**) (Montedoro et al., 1993; Gariboldi et al., 1986). For the latter substance a general observation was that its decrease was balanced by an equivalent compensatory increase in 3,4-dihydroxyphenylethanol (hydroxytyrosol) (**7**).

Our study of Dritta and Leccino solutes has shown a strict relationship between cornoside (**4**) and halleridone (**5**), the latter formally stemming from the former via hydrolysis of the glucoside linkage (Scheme 1).

The seasonal fluctuation of the phenolic compounds can be viewed as a biogenetically controlled phytochemical activity of the plant.

In Dritta and Leccino cvs., in theory, oxidation may occur either on free 4-hydroxyphenylethanol (**1**) or on its glucoside (**2**) affording quinol **4a** or **4**, respectively. While quinol glucoside (**4**) is stable and isolable, free quinol (**4a**) was not detected probably because of its tautomerization to the more stable cyclic form (**5**) of halleridone.

The suggested interrelationships are illustrated in Scheme 1, in which the key step is seen to be the hydroxylation at the aromatic position 1 of **1** or **2** followed by reactions leading through cyclization to halleridone (**5**).

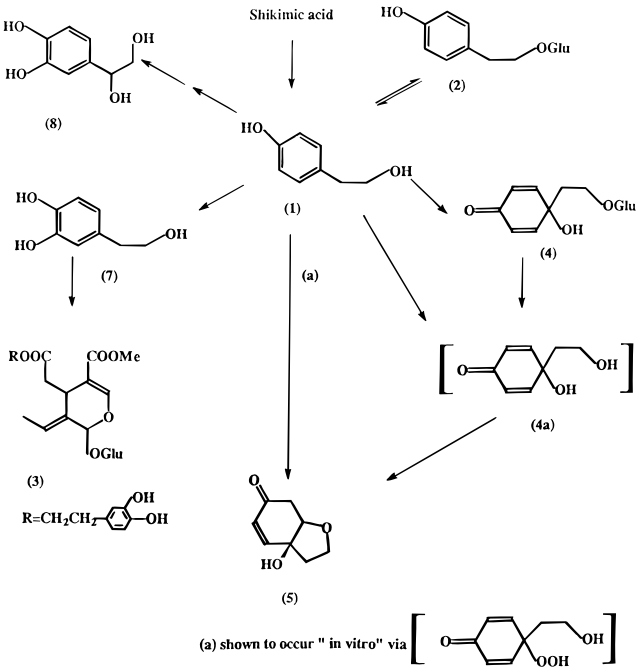
The contrast between the oxidation pattern observed for 4-hydroxyphenylethanol (**1**) or its glucoside (**2**) in Dritta and Leccino cvs. compared to the stability of these phenols in Cipressino cv. suggests that the latter olive variety is lacking in enzyme systems responsible for the oxygen insertion into the aromatic ring of (**1**) or (**2**); alternatively, oxidation is prevented by a mechanism not yet identified.

A synthesis of halleridone (**5**) from 4-hydroxyphenylethanol has been successfully accomplished in vitro (Breton et al., 1987).

In conclusion it should be stressed that, although limited to only three varieties, the results of the present work have shown that the qualitative composition of the vegetation water solutes may be used for studies aided at olive variety classification. The occurrence of substances involved in redox processes is worthy of further comments, since these constituents may play various roles in the industrial processes of transformation of olives into oil and table olives, respectively.

The results of our study should draw the cautious attention of researchers working in the field on the relevant discrepancies between the limited number of substances found in the vegetation waters of the olives studied and the large number of compounds reported in the literature to be present in olive oil.

Scheme 1. Synthesis of Phenolics and Quinols in Olives



EXPERIMENTAL PROCEDURES

Extracts were obtained from olive fruits (about 300 g) of Cipressino, Dritta, and Leccino cvs. collected in June, August, and October 1992 from trees growing in the Istituto Sperimentale per l'Elaiotecnica olive groves. Olives, after dipping in $CHCl_3$ for 1 min at room temperature, to remove epicuticular waxes, were triturated by Ultra-Turrax. The homogenates were macerated in H_2O (250 mL) for 1 h. The aqueous solutions, after filtration, were evaporated under vacuum to obtain the crude extracts. A part of the extracts, without further purification, was dissolved in perdeuterated water in NMR tube, to record spectra. Another part of the initial aqueous mixtures was successively extracted with ethyl acetate and used to record spectra in organic solvent. The solvent used was acetone-d_6.

^1H NMR spectra were recorded on a Bruker AM 500 spectrometer controlled by an Aspect 3000 computer and equipped with a selective excitation unit, suitable for creating Gaussian-shaped, low-power pulses (Brueschweiler et al., 1987; Bauer et al., 1984).

Spectra were recorded in D_2O or acetone-d_6 purchased from Merck. Chemical shifts were referred to sodium trimethylsilyl-[2,2,3,3,$^2H_4$]sulfonate (TSP) in water and to tetramethylsilane (TMS) in acetone.

The chemical shifts for the various components of the extracts were in agreement with those previously published in the literature. For available substances, comparison was made with authentic samples.

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