Phenolic Compounds of Olive Fruit: One- and Two-Dimensional Nuclear Magnetic Resonance Characterization of Nüzhenide and Its Distribution in the Constitutive Parts of Fruit

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The phenolic composition of peel, pulp, and seed of the olive fruit was studied for several Italian cultivars used for oil extraction. The seed contained a compound never previously detected in peel and in pulp. The spectroscopic characterization of this compound proved, for the first time, the presence of nüzhenide in the olive seed. Study of the phenolic composition showed that oleuropein, demethyloleuropein, and verbascoside were present in all of the constitutive parts of the fruit; by contrast, nüzhenide was exclusively present in the seeds of all the cultivars at all ripening stages studied.

Keywords: *Nüzhenide; phenols; secoiridoids; olive fruit; NMR*

INTRODUCTION

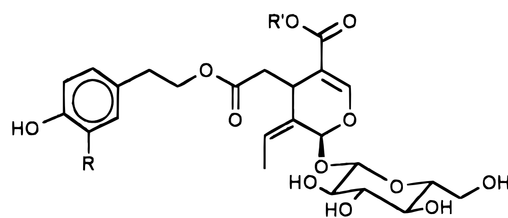
The olive fruit contains different phenolic compounds such as phenyl acids, flavonoids, and secoiridoids (Macheix et al., 1990). Flavonol glycosides such as luteolin-7-glucoside, rutin, and anthocyanins, such as cyanidin and delphinidin glycosides, are the main flavonoids in olives (Macheix et al., 1990; Brenes-Balbuena et al., 1992; Mazza and Miniati, 1993). Oleuropein, demethyloleuropein, ligstroside, and oleoside (Chart 1) are the predominant secoiridoids of fruit (Panizzi et al., 1960; Ragazzi et al., 1973; Gariboldi et al., 1986; Kuwajiama et al., 1988), whereas the verbascoside (Chart 1) is the main hydroxycinnamic derivative in olives (Andary et al., 1982). Several authors have studied the phenolic composition of olive pulp (Panizzi et al., 1960; Ragazzi et al., 1973; Amiot et al., 1986; Gariboldi et al., 1986); however, so far, the seed phenolic composition is not well-known (Maestro-Duran et al., 1994).

In this paper we report the 1D- and 2D-NMR characterization of nüzhenide (Chart 2) isolated from the olive seed and the phenolic composition of the peel, pulp, and seed of three Italian olive cultivars used for the mechanical extraction of olive oil.

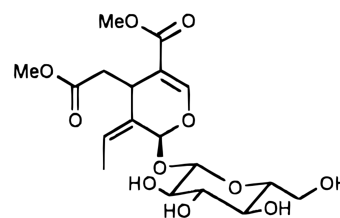
MATERIALS AND METHODS

Materials. *Sample Preparation*. Olive drupes from Coratina, Leccino, and Moraiolo cultivars harvested during the years 1995 and 1996 were used. Fruit ripening was evaluated as pigmentation index, according to the method of Pannelli et al. (1994). The olives were peeled and destoned. To inhibit enzymatic activities, peel, pulp, and stone were immediately frozen in liquid nitrogen and freeze-dried. After freeze-drying, the stones were crushed to recover the seed. Freeze-dried samples were stored at −30 °C before analysis. The distribution of the constitutive parts of olive fruit is reported in Table 1.

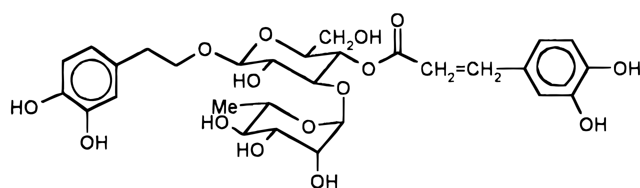
Chart 1



R = OH; R' = Me *Oleuropein*
R = OH; R' = H *Demethyloleuropein*
R = H; R' = Me *Ligstroside*



Oleoside



Verbascoside

Reference Compounds. (3,4-Dihydroxyphenyl)ethanol (3,4-DHPEA) was synthesized according to the procedure of Baraldi et al. (1983); demethyloleuropein, verbascoside, and 3,4-DHPEA-EDA were extracted, respectively, from olive fruit and virgin olive oil using procedures reported in earlier papers (Servili et al., 1998; Montedoro et al., 1993). The purity of these

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Table 1. Distribution of the Constitutive Parts of Olive Fruit (Grams per 100 g of Dry Weight of Olive)

olive	pigmentation index	peel	pulp	stone	seed
			1995 Harvest		
Moraiolo	2.38	4.35 ± 0.57[a]	50.44 ± 0.69	45.20 ± 0.97	5.81 ± 0.12
Leccino	2.59	5.83 ± 0.65	42.17 ± 0.45	51.99 ± 1.08	7.58 ± 0.25
Coratina	2.48	6.14 ± 0.53	56.68 ± 0.98	37.19 ± 1.01	2.78 ± 0.13
			1996 Harvest		
Coratina	0.40	4.97 ± 0.55	54.01 ± 0.97	41.49 ± 1.03	3.09 ± 0.11
Coratina	2.67	5.94 ± 0.52	54.68 ± 0.99	39.39 ± 1.07	2.78 ± 0.15
Coratina	3.21	6.36 ± 0.28	56.15 ± 0.60	37.53 ± 0.87	2.99 ± 0.07

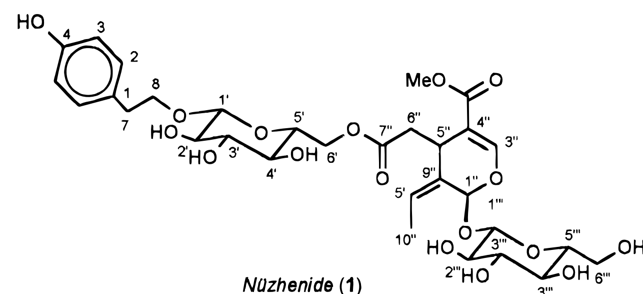
[a] Mean ($n = 4$) ± standard deviation.

Table 2. ^{1}H and ^{13}C NMR Data

	^{1}H[a]	^{1}H[b]	^{13}C[a]	^{13}C[b]		^{1}H[a]	^{1}H[b]	^{13}C[a]	^{13}C[b]
1			129.3	129.4	1″	5.87 s	5.95 s	93.8	94.1
2	6.77 d	6.73 d	130.0	129.8	3″	7.55 s	7.51 s	153.4	154.2
	($^{3}J_{2,3}$ = 8.6)	($^{3}J_{2,3}$ = 8.5)			4″			108.4	108.4
3	7.13 d	7.04 d	115.0	115.4	5″	4.01 dd	4.02 dd	30.7	30.8
	($^{3}J_{2,3}$ = 8.5)	($^{3}J_{2,3}$ = 8.5)				($^{3}J_{5'',6''a}$ = 9.2;	($^{3}J_{5'',6''a}$ = 8.6;		
4			155.3	155.9		$^{3}J_{5'',6''b}$ = 4.6)	$^{3}J_{5'',6''b}$ = 4.8)		
7	2.85 t	2.85 m	34.9	35.6	6″a	2.51 dd	2.52 dd	40.0	40.5
	($^{3}J_{7,8a}$ = 7.2)	($^{3}J_{7,8a}$ = 3.2;				($^{2}J_{6''a,6''b}$ = 14.1;	($^{2}J_{6''a,6''b}$ = 14.2;		
		$^{3}J_{7,8b}$ = 7.1)				$^{3}J_{6''a,5''}$ = 9.2)	$^{3}J_{6''a,5''}$ = 8.8)		
8a	3.97 dt	3.92 m	70.6	71.2	6″b	2.81 dd	2.76 dd		
	($^{2}J_{8a,8b}$ = 9.8;	($^{2}J_{8a,8b}$ = 8.3;				($^{2}J_{6''a,6''b}$ = 14.1;	($^{2}J_{6''a,6''b}$ = 14.2;		
	$^{3}J_{7,8a}$=7.2)	$^{3}J_{7,8a}$ = 3.2)				$^{3}J_{6''b,5''}$ = 4.6)	$^{3}J_{6''b,5''}$ = 4.9)		
8b	3.70 dt	3.69 dt			7″			171.8	171.8
	partially buried	partially buried						171.3	
	under COO*Me*	under COO*Me*			8″	6.11 qd	6.12 qd	123.6	124.0
1′	4.31 d	4.31 d	102.8	103.5		($^{3}J_{8'',10''}$ = 6.9;	($^{3}J_{8'',10''}$ = 6.7;		
	($^{3}J_{1',2'}$ = 7.8)	($^{3}J_{1',2'}$ = 7.8)				$^{4}J_{8'',1''}$ = 0.7)	$^{4}J_{8'',1''}$ = 0.9)		
2′	3.15 dd	3.26 dd	73.5	73.9	9″			129.9	129.5
	($^{3}J_{1',2'}$ = 8.1;	($^{3}J_{1',2'}$ = 7.9;			10″	1.77 dd	1.75 dd	12.8	13.1
	$^{3}J_{2',3'}$ = 8.7)	$^{3}J_{2',3'}$ = 8.9)				($^{3}J_{10'',8''}$ = 7.1;	($^{3}J_{10'',8''}$ = 7.1;		
3′	3.37 m	3.47[c]	76.5	77.0		$^{5}J_{10'',1''}$ = 1.3)	$^{5}J_{10'',1''}$ = 1.5)		
	buried under				COO*Me*	3.72 s	3.70 s	51.0	51.1
	CD_2HCN				*C*OOMe			166.7	167.4
4′	3.37[c]	3.38[c]	70.2	70.5	1‴	4.80 d	4.83 d	99.4	99.8
5′	3.51[c]	3.47 m	73.4	74.1		($^{3}J_{1''',2'''}$ = 7.8)	($^{3}J_{1''',2'''}$ = 7.7)		
6′a	4.26 d	4.34 dd	63.4	64.2	2‴	3.35 dd	3.35 dd	73.2	73.7
	($^{3}J_{6',5'}$ = 3.6)	($^{2}J_{6a',6b'}$ = 11.8;				buried under			
		$^{3}J_{6a',5'}$ = 2.2)				CD_2HCN			
6′b		4.24 dd			3‴	3.42 m	3.38[c]	76.6	77.4
		($^{2}J_{6a',6b'}$ = 11.9;			4‴	3.36[c]	3.38[c]	69.9	70.6
		$^{3}J_{6b',5'}$ = 5.7)			5‴	3.35[c]	3.38[c]	76.5	77.0
					6‴a	3.95[c]	3.97[c]	61.6	61.8
					6‴b	3.72[c]	3.71[c]		

[a] In acetonitrile-d_3. [b] In ethanol-d_6. [c] Chemical shift derived from the ^{1}H,^{13}C-inverse correlations.

Chart 2



substances was tested by HPLC, and the chemical structures were verified by NMR.

The oleuropein glucoside was obtained from Extrasynthèse Co. (Genay, France), (*p*-hydroxyphenyl)ethanol (*p*-HPEA) from Janssen Chemical Co. (Beerse, Belgium), luteolin-7-glycoside from Roth Co. (Karlsruhe, Germany), and rutin from BDH Co. (Poole, U.K.).

Extraction and Separation of Phenolic Compounds from Peel, Pulp, and Seeds of Olive Fruit and Destoned Olives. Fifty milliliters of 80% methanol and 20 mg g^{-1} sodium diethyldithiocarbamate (DIECA) at −25 °C were added to the peel (2 g), the pulp (5 g), and the seed (5 g) of olive fruit. The mixture was homogenized in an Omni-mixer (Sorvall) for 30 s at 16000*g* and filtered through filter paper in a Büchner funnel. The extraction was repeated six times. The MeOH extract was evaporated under vacuum in a nitrogen flow at 35 °C, and the aqueous extract was obtained (Figure 1).

Solid-phase extraction (SPE) was used to separate the phenols from the water extract: 2 mL was put in a High-load C_{18} cartridge (Alltech Co., Milano, Italy), and the phenolic compounds were eluted with methanol (600 mL).

High-Performance Liquid Chromatography (HPLC). The HPLC system was a Varian 9010 chromatograph with a 150 mm × 4.6 mm C_{18} Inertsil ODS-3 column (Alltech Co.) coupled to a Varian Polychrom 9065 UV diode array detector. The samples were dissolved in methanol, and a sample loop of 20 L capacity was used. The mobile phase was 0.2% acetic acid (pH 3.1) in water (A)/methanol (B) at a flow rate of 1.5 mL/min. The total running time was 55 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, maintained for 5 min, return to initial conditions in 10 min; the total running time was 55 min.

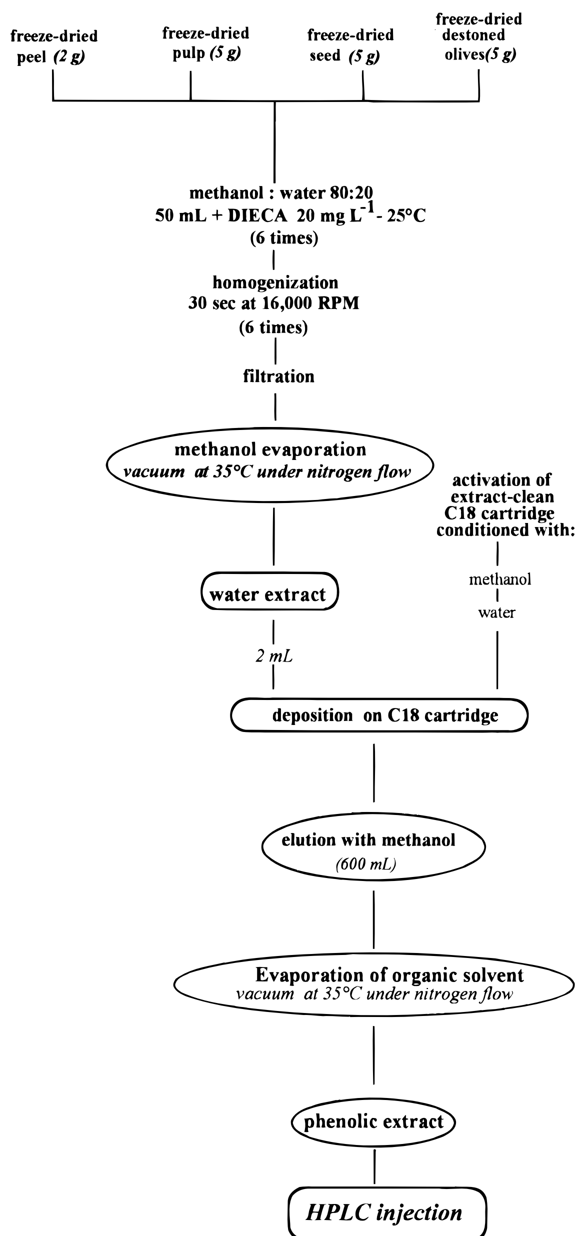


Figure 1. Scheme for extraction of phenolics from destoned olives, peel, pulp, and seeds of olive fruit.

Preparative HPLC. A Varian liquid chromatograph model 5000 equipped with a 500 mm × 9.4 mm Whatman Partisil 10 ODS-2 semipreparative column, coupled to a Varian Polychorom 9065 diode array detector, was used. The phenolic extract, obtained as reported above from cv. Coratina seeds, was injected in the column using a 1 mL sample loop. The peak corresponding to retention time (RT) 23.70 min was recovered using a Gilson model 201 fraction collector using as mobile phase mixtures of solution A (0.2% acetic acid, pH 3.1) and methanol (B) (flow rate = 5.6 mL/m). The total running time of the analysis was 60 min, and the gradient changed as follows: 80% A/20% B at time 0 min, 60% A/40% B realized in 20 min, maintained for 20 min, 0% A/100% B in 5 min, maintained for 10 min, return to 80% A/20% B in 5 min.

For each injection an amount of phenolic extract corresponding to ~10 mg of total phenols, expressed as 3,4-dihydroxyphenylethanol equivalents, determined by the Folin–Ciocalteu reaction (Montedoro et al., 1992), was injected in the column and the eluate monitored at 278 nm. The fractions containing the recovered substance were pooled and concentrated according to the procedure reported previously (Montedoro et al., 1993).

NMR. The NMR spectra were recorded on Bruker DRX 400 and DRX 500 spectrometers, operating at 400.13 and 500.13 MHz for ^{1}H and at 100.62 and 125.77 MHz for ^{13}C, using TMS as external standard. About 20 mg of sample was dissolved in 0.6 mL of deuteriated solvent. ^{1}H, ^{13}C{^{1}H}, ^{13}C DEPT-90 and -135, ^{1}H COSYdqf, ^{1}H COSYgs, ^{1}H NOESY phase sensitive, ^{1}H–^{13}C CORRgs, and ^{1}H–^{13}C CORRgs long-range were performed (Sanders et al., 1987). All of the experiments were performed using standard Bruker programs, and most of the acquisition parameters were used as suggested by the programs. The temperature of the probe was 300 K. The ^{1}H NOESY phase sensitive spectrum was obtained with a mixing time of 800 ms. The ^{1}H–^{13}C CORRgs and ^{1}H–^{13}C CORRgs long-range experiments were performed using 3.3 and 50 ms, respectively, as delays for the evolution of coupling constants.

The structure was first achieved by recording the ^{13}C NMR spectrum that gives the total number of carbons of the molecule, successively sorted by DEPT experiments into quaternary, CH, CH_2, and CH_3 groups. However, the complete assignments of all resonances were derived from the bidimensional experiments and the crucial indications from the ^{1}H–^{13}C CORRgs long-range experiments.

RESULTS AND DISCUSSION

NMR Characterization of Peak RT 23.70 min. The HPLC chromatograms of the peel, pulp, and seed of Coratina cultivar are shown in Figure 2. An undetected compound with an RT of 23.70 min was detected exclusively in the seed. This compound (Chart 2) was identified by the ^{1}H and ^{13}C NMR data (Table 2) using acetonitrile-d_3 and ethanol-d_6 as solvents. The assignment of the protons and carbons relative to the four fragments of compound **1** (elenolic acid, *p*-HPEA, and two β-glucoside groups) was easily achieved from the 1D- and 2D-NMR experiments and from the comparison with the NMR data of similar compounds (Montedoro et al., 1993). Signals of protons belonging to the two β-glucosidic moieties, namely 3′-5′ and 2‴-5‴, were very close in the monodimensional ^{1}H NMR spectrum, so the assignments were taken from ^{1}H–^{13}C correlation experiments. Furthermore, the connections among the four fragments of compound **1** were derived from ^{1}H–^{13}C CORRgs long-range experiment. Indeed, the isolated spin of the sugar moiety indicated as C1‴–C6‴ showed diagnostic cross-peaks between the anomeric carbon of this glucose moiety and the proton linked to carbon 1 of elenolic acid (namely, C-1″) and between the anomeric proton of the glucose moiety (namely, H-1‴) and C-1″. No cross-peaks between signals of this glucose with other glucose units or the *p*-HPEA moiety were present.

Furthermore, the three-bond couplings between the signals attributable to the protons linked to C-6′ and C-5′ of the glucose moiety and C-7″ of the elenolic moiety (Figure 3), together with the correlations between the anomeric proton H-1′ and the C-8 of the *p*-HPEA unit, led to the unambiguous assignments of the sequence of the four moieties of compound **1**. Further confirmation of structure **1** came from the observation of "contacts" between 8 and 1′ protons and between 1″ and 1‴ protons in the ^{1}H NOESY phase sensitive spectrum.

Compound **1**, known as nüzhenide, has been previously reported from *Ligustrum lucidum* fruits (nüzhenzi in Chinese medicine) (Inouye et al., 1972), from *Fraxinus americana* seeds (LaLonde et al., 1975), and from *Syringa vulgaris* and has been hypothesized in olive fruit seeds (Maestro-Duran et al., 1994). However, this is the first time that this compound has been isolated and fully characterized by NMR spectroscopy from the seed of olive fruit. We revisited and completed such a

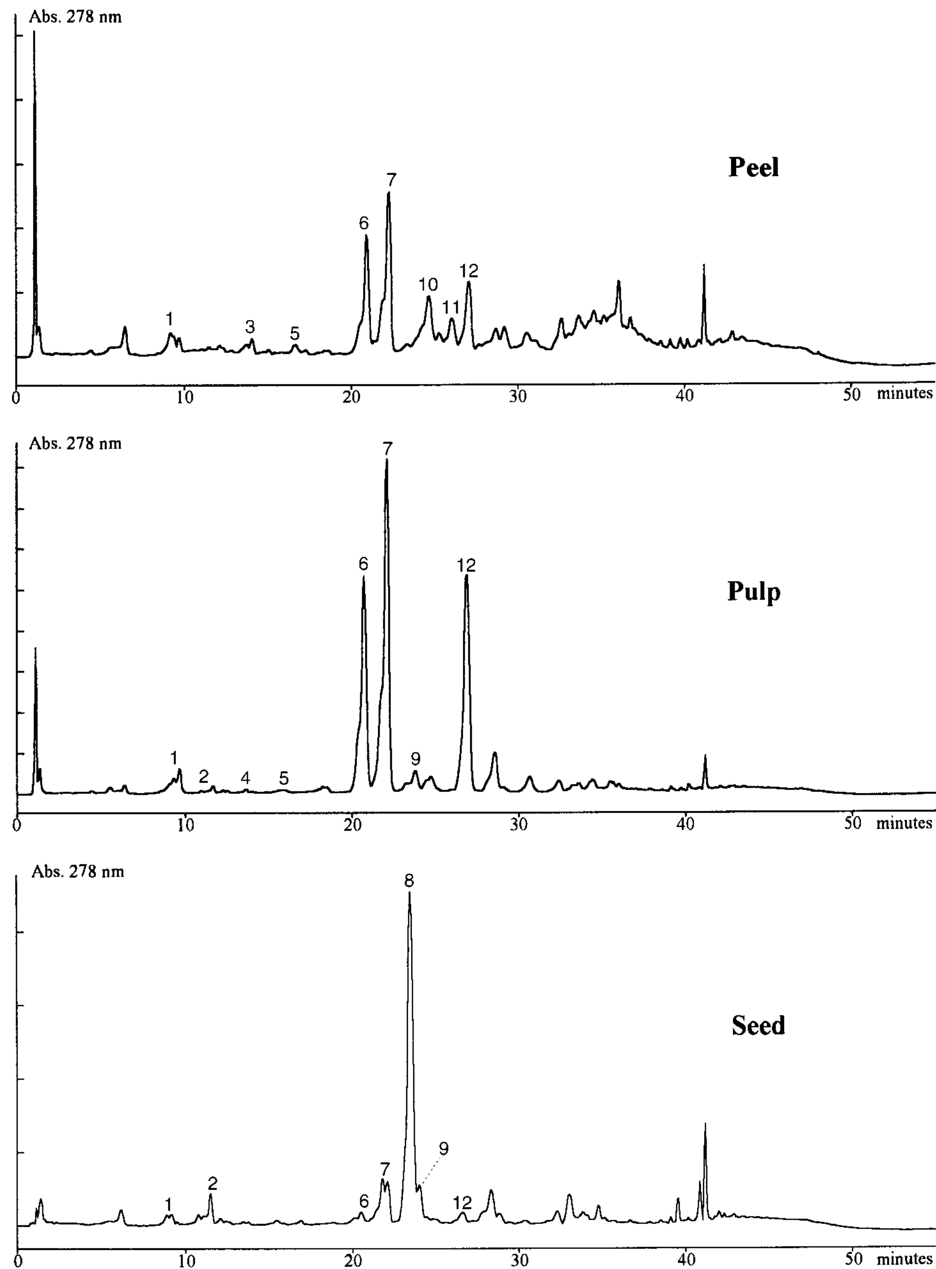


Figure 2. HPLC chromatograms of peel, pulp, and seed of olive fruit. Peak identification (RT, min): 1, 3,4-DHPEA (9.45); 2, *p*-HPEA (11.80); 3, *p*-hydroxybenzoic acid (13.60); 4, vanillic acid (15.30); 5, caffeic acid (16.30); 6, demethyoleuropein (20.80); 7, verbascoside (22.06); 8, peak RT 23.70 (23.70); 9, 3,4-DHPEA-EDA (24.10); 10, luteolin-7-glycoside (24.95); 11, rutin (25.80); 12, oleuropein glucoside (26.90).

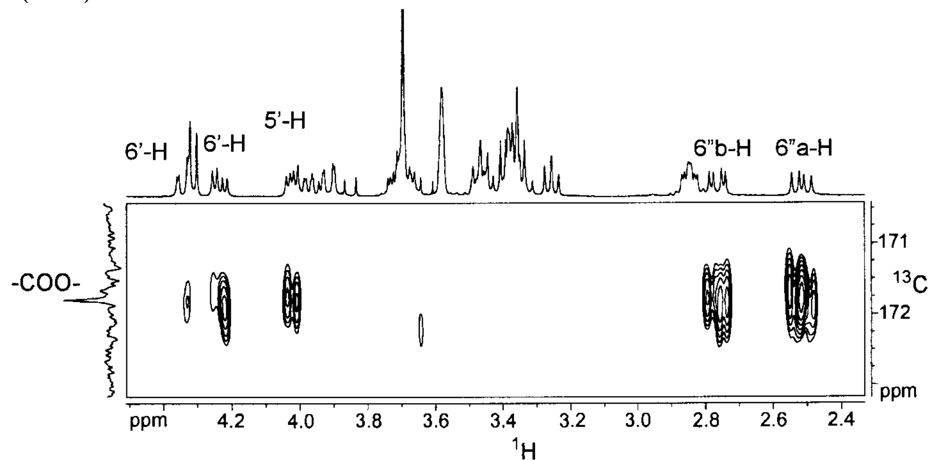


Figure 3. Section of $^1H-^{13}C$ CORRgs long-range experiment showing the correlations between the −COO− group and the 5′ and 6′ protons of the *β*-glucoside group and the 6″ protons of the elenolic acid fragment.

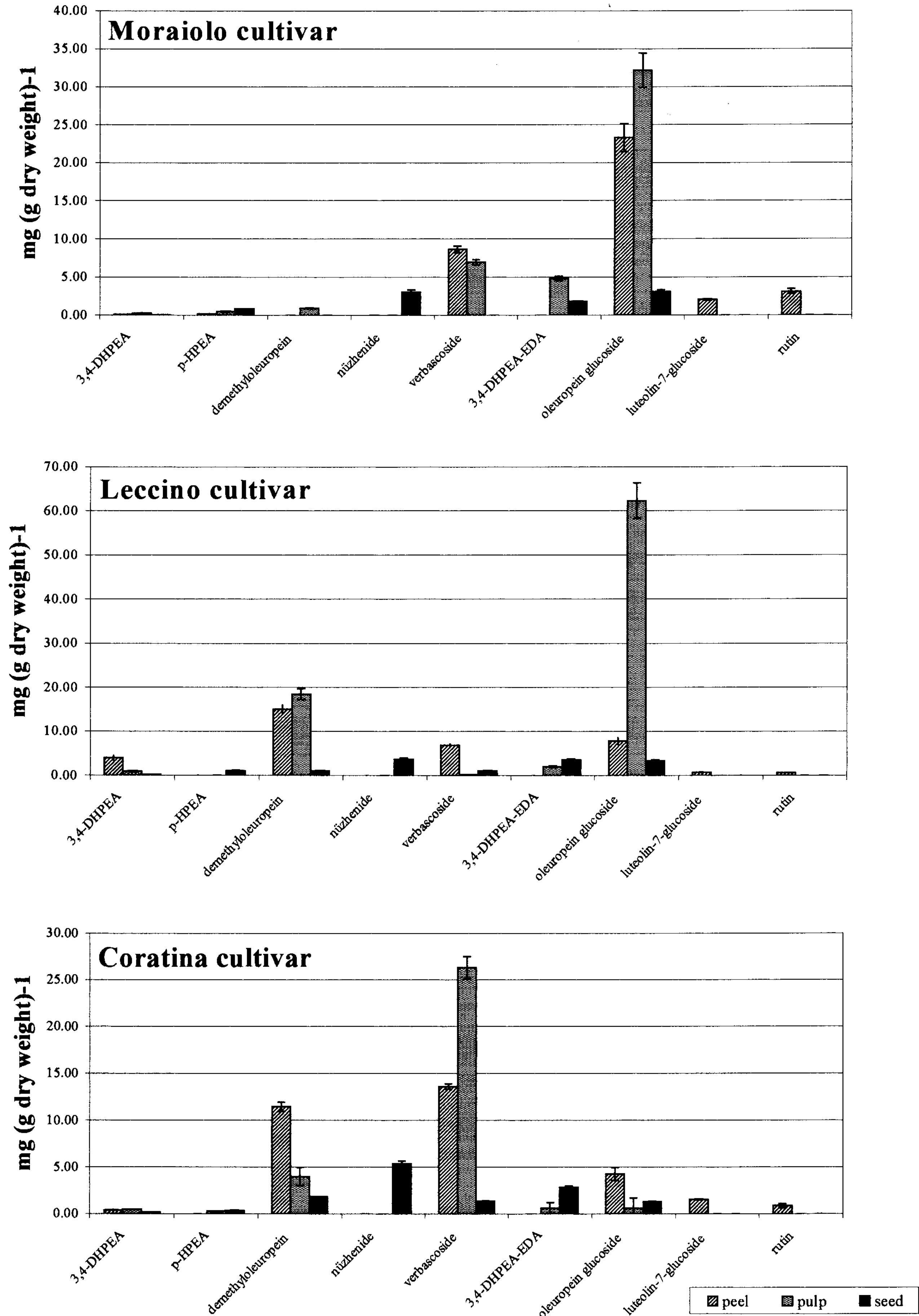


Figure 4. Phenolic composition of the constitutive parts of fruit in Moraiolo, Leccino, and Coratina cultivars evaluated at the same ripening stage. Data are expressed as the mean ($n = 3$) ± standard.

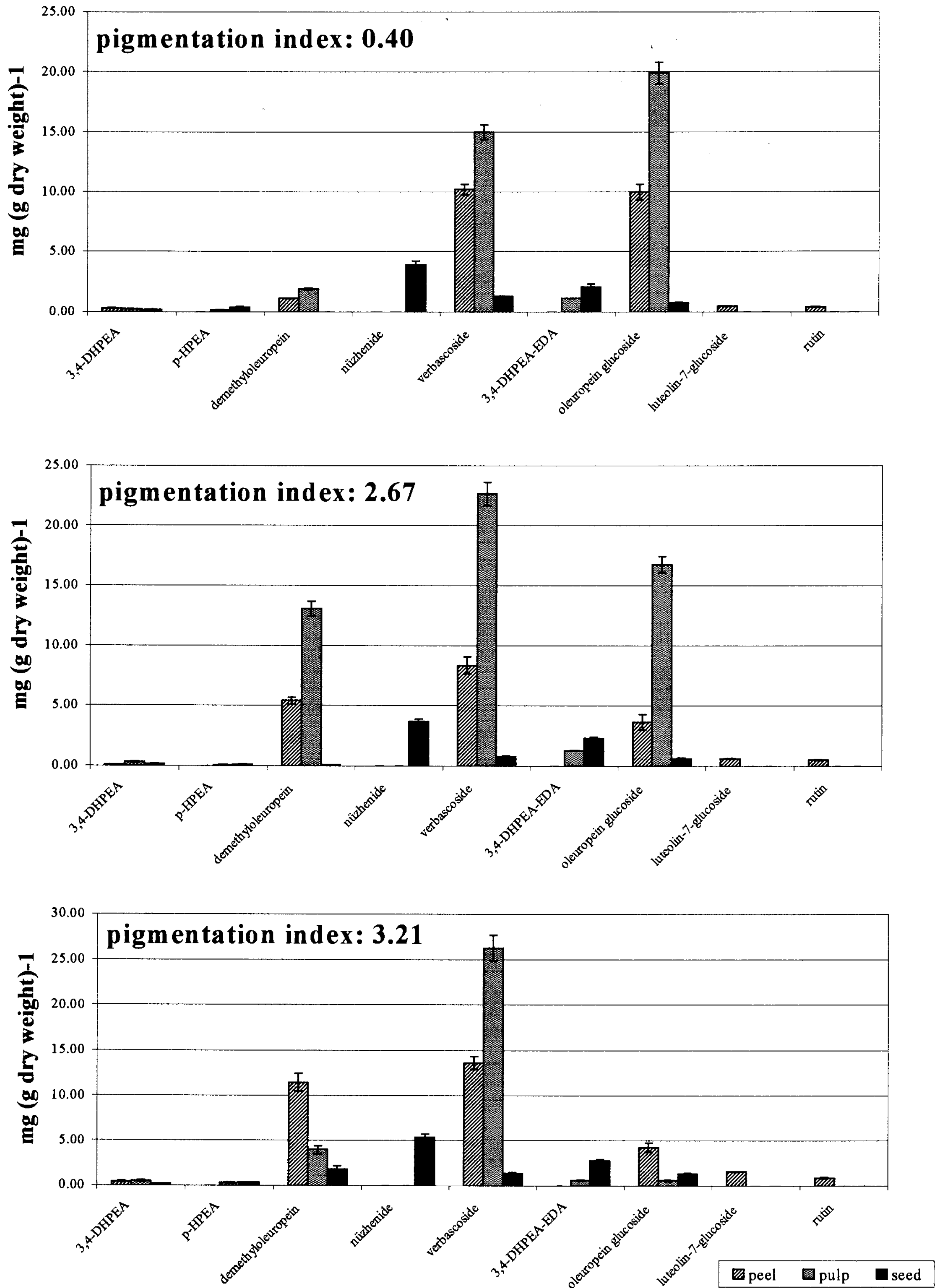


Figure 5. Phenolic composition of the constitutive parts of Coratina cultivar evaluated at three ripening steps. Data are expressed as the mean ($n = 3$) ± standard.

characterization for two main reasons: (a) at the beginning we did not realize that our compound was compound **1** and, consequently, the assignment was done ab initio; (b) the utilization of modern 1D- and 2D-NMR techniques based on the inverse detection and gradients makes possible the easy and reliable assignment and connectivity of different fragments. As confirmation of point a we performed all NMR experiments in two different solvents because the conclusions obtained in acetonitrile were not completely satisfactory. In particular, owing to the apparent presence of two $-COO^-$ groups, the connectivity of the four fragments was not clear. Another difference between the NMR parameters in the two solvents was that in ethanol-d_6 the 6′ protons were not equivalent, whereas in acetonitrile-d_3 they were. There is probably a conformational isomerism that is affected by the nature of the solvent that makes the structure of compound **1** slightly solvent dependent.

Distribution of Phenolic Compounds in Peel, Pulp, and Seed of Moraiolo, Leccino, and Coratina Cultivars and Evolution during Ripening. The results reported in Figure 4 show that luteolin-7-glucoside and rutin were present exclusively in the peel. By contrast, oleuropein and demethyloleuropein were found in all of the constitutive parts of the olive fruit, with the highest concentrations being detected in the pulp. Verbascoside was more concentrated in the pulp of Coratina than in Moraiolo and Leccino [21.20 mg (g of dry weight (dw))$^{-1}$, 6.90 mg (g of dw)$^{-1}$, and 0.23 mg (g of dw)$^{-1}$, respectively].

The phenolic composition of the seed was very interesting, and as shown in Figure 4, the concentrations of oleuropein, demethyloleuropein, 3,4-DHPEA, and *p*-HPEA were low. Nüzhenide was the most concentrated phenolic compound in seed [3.05 mg (g of dw)$^{-1}$, 3.69 mg (g of dw)$^{-1}$, and 3.81 mg (g of dw)$^{-1}$, respectively, for Moraiolo, Leccino, and Coratina cultivars) and was detected exclusively in this part of the fruit in all of the cultivars studied.

Oleuropein decreased in the peel and pulp during ripening while demethyloleuropein and verbascoside increased in pulp (Figure 5). The concentration of nüzhenide in the seed was not significantly affected by fruit ripening.

In the peel, luteolin-7-glucoside and rutin did not change during ripening, and similar results were observed for the phenyl alcohols such as *p*-HPEA and 3,4-DHPEA also present in peel and in pulp.

Conclusions. This is the first report on the isolation and spectral characterization of nüzhenide from olive seeds. The study of the distribution of phenolic compounds in the constitutive parts of the olive fruit shows that oleuropein glucoside, demethyloleuropein, and verbascoside were present in all of the constitutive parts of the olive fruit, whereas luteolin-7-glucoside and rutin were present only in the peel of all cultivars during all of the ripening steps studied. Nüzhenide was the most concentrated phenolic compound in the seed and was detected exclusively in this part of the fruit.

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