ELSEVIER

Food Chemistry 77 (2001) 405–411

Food Chemistry

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The occurrence in olive oil of a new class of phenolic compounds: hydroxy-isochromans

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Received 7 August 2001; received in revised form 1 October 2001; accepted 1 October 2001

Abstract

A new class of phenolic compounds, hydroxy-isochromans, was found in different samples of extra-virgin olive oil. In particular, the presence of 1-phenyl-6,7-dihydroxy-isochroman, **10** and 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochroman, **11** was demonstrated by *comparison* of the high performance liquid chromatography–mass/mass (HPLC–MS/MS) spectra of biophenolic samples from extra-virgin olive oils with those of compounds obtained by a reaction between hydroxytyrosol and the aromatic aldehydes, benzaldehyde and vanillin, respectively. © 2002 Published by Elsevier Science Ltd.

Keywords: Olive oil; Phenol fraction; Isochromans; HPLC–MS/MS

1. Introduction

Isochromans are 3,4-dihydro-1H-benzo[*c*]pyran derivatives generally present in nature as part of complex fused ring systems (Peng, Lu, & Ralph, 1999).

In a previous paper (Guiso, Marra, & Cavarischia, 2001), we reported a facile synthesis of isochroman derivatives by the acid-catalysed oxa-Pictet-Spengler reaction, between hydroxytyrosol, **1**, and a series of carbonylic compounds. This reaction gives very high yields as **1**, has a phenolic function in *para* position to the carbon on which the dihydropyran ring must be closed. This reaction occurs, even with moderate yields, at room temperature, using a mild organic acid as catalyst. It was verified that this reaction also occurs in a natural matrix, such as olive oil, catalysed by a small quantity of oleic acid (Guiso et al., 2001).

The feasibility of this reaction in bioinimetic conditions prompted us to investigate the presence of hydroxy-isochromans in extra-virgin olive oil. In fact, it is well known (Balestrieri; Bottari; Festa, &. Marini; 1988; Cartoni, Coccioli, Jasionowska, & Ramires, 2000) in this matrix, that small quantities of many carbonylic compounds occur, together with hydroxytyrosol **1**, in the presence of a mild organic acidity, caused by the oleic acid.

The phenolic alcohol **1** is present in olive fruits, both free or glycosylated, but mainly linked by an ester function to the aglycone moiety of oleuropein, **2**, (Panizzi, Scarpati, & Oriente, 1960), the abundant secoiridoid glucoside which is characteristic of the olive free. During the oil preparation, in particular in the "kneader" step, hydrolytic processes, due to the uncontrolled activity of hydrolytic enzymes (glycosidases and esterases) enhance the quantity of free **1** as well as carbonylic compounds, so favouring the co-occurrence of all compounds necessary to the formation of isochroman derivatives.

2. Materials and methods

2.1. Materials

Methanol (HPLC grade) and ammonium formate were obtained from Carlo Erba (Italy), formic acid from Fluka (Germany). All the other reagents were of analytical grade.

Ammonium formate (1 mM) was prepared by dissolving 63 mg of ammonium formate in 1000 ml of water. Ultrapure water was produced with a Pure Lab™ system (USF Elga, Germany).

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0308-8146/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0308-8146(01)00366-1

A 3 ml Sep-Pak C_{18} cartridge was obtained from Waters (Milford, MA, USA). The Sep-Pak cartridge were washed with methanol and stored in methanol.

Extra-virgin olive oils: Farchioni, Frantoio della Rocca, unbottlied oil, Trasimeno, were obtained from commercial sources, and a specimen sample was stored in the department at −20 °C.

2.2. General extraction procedure

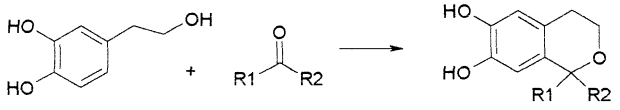
Extra-virgin olive oil (12 g) was thrice extracted by 4 ml of a solution of methanol/water (8:2). The collected alcoholic extracts (12 ml) were evaporated to 3 ml under reduced pressure and at a temperature below 35 °C to eliminate the methanol. The obtained water solution was acidified by 6 N HC1 to pH = 2.2 and passed through a C_{18} cartridge. The phenolic substances were then eluted by methanol (3.5 ml); the obtained solution was evaporated under N_2 and the residue dissolved in methanol (200 μl).

2.3. Reference compounds

Hydroxytyrosol, **1** was prepared from 3,4-dihydroxyphenylacetic acid according to the method described (Bianco, Passacantilli, & Righi, 1988). Carbonylic com-

Table 1

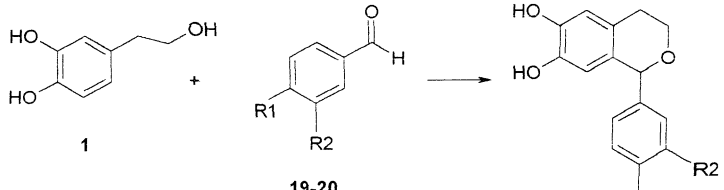
Reagents		Obtained product		
(a)				
1	Propanal, **12**	**3**	$R_1 = H$	$R_2 = CH_2CH_3$
1	Acetone, **13**	**4**	$R_1 = CH_3$	$R_2 = CH_3$
1	Butanal, **14**	**5**	$R_1 = H$	$R_2 = n$-propyl
1	3-Pentanone **15**	**6**	$R_1 = CH_2CH_3$	$R_2 = CH_2CH_3$
1	Pentanal, **16**	**7**	$R_1 = H$	$R_2 = n$-butyl
1	3-Methylbutanal **17**	**8**	$R_1 = H$	$R_2 =$ isobutyl
1	2-Hexanone **18**	**9**	$R_1 = CH_3$	$R_2 = n$-butyl



1 **12-18** **3-9**

(b)

1	Benzaldehyde, **19**	**10**	$R_1 = H$	$R_2 = H$
1	Vanillin, **20**	**11**	$R_1 = OH$	$R_2 = OCH_3$



1 **19-20** **10-11**

Table 2
MS/MS mass spectra of hydroxy-isochromans

Compounds	Precursor ion $[M–H]^-$	Product ions
1-ethyl-6,7-dihydroxy-isochroman **3**	193.2	148.0–163.2
1,1-dimethyl-6,7-dihydroxyisochroman **4**		123.0–163.2
1-propyl-6,7-dihydroxyisochroman **5**	206.9	162.0–177.2
1,1-diethyl-6,7-dihydroxyisochroman **6**	221.0	191.2–220.8
1-butyl-6,7-dihydroxyisochroman **7**		162.2–191.2
1-isobutyl-6,7-dihydroxyisochroman **8**		176.2–205.2
1-butyl-1-methyl-6,7-dihydroxyisochroman **9**	235	176.0–205.2
1-phenyl-6,7-diliydroxyisochroman **10**	241.2	193.2–211.2
1-(3′-methoxy-4′-hydroxy)phenyl-6,7-ldihydroxyisochroman **11**	286.7	242.0–257.1

pounds: propanal **12**, acetone **13**, butanal **14**, 3-pentanone **15**, pentanal **16**, 3-methylbutanal **17**, 2-hexanone **18**, benzaldehyde **19** and vanillin **20** as well as the 3,4-dihydroxyphenylacetic acid were pure products obtained from Sigma-Aldrich (Milan).

Hydroxy-isochromans (**3–11**, see Table 1a and b) were prepared according to the following general procedure. 20 mg of **1** is dissolved in methyl alcohol (1 ml) together with the selected carbonylic compound (**12–20**) in molar ratio 1:1. Successively, a catalytic amount of *p*-toluenesulfonic acid is added. The mixture is left to react at 4 °C for the required period of time (about 24 h for aldehydes and 48 h for ketones). After chromatographic control on a silica gel plate, the volume of the reaction mixture is divided into two under reduced pressure at low temperature, diluting with ethyl acetate *(25* ml). The organic layer is washed with a saturated solution of NaCI until neutrality, dried with anhydrous $Na_2 SO_4$, concentrated, and the residue purified by chromatography on silica gel, by eluting with $CHC1_3$/MeOH 9:1 (compounds **3**, **5**, **7**, **8**, **9**, **10**) and with $CHC1_3$/EtOAc 8:2 (compounds **4**, **6**, **11**).

2.4. Apparatus

A Shimadzu gradient HPLC system with two pumps LC-10 AD (Shirnadzu Kyoto, Japan), with high-pressure mixing system Gilson 811-B, was used. The injection valve was a Rheodyne model 7410 with a 5 µl sample loop.

The column (L = 250×2.1 mm i.d.) was slurry packed in the laboratory with Nucleosil ODS (5 µm) obtained from Nacherey-Nagel D-5160 Duren (Germany). A analytical guard column (L = 2 cm × 2 mm i.d.) Upchurch Scientific (Oak Harbor WA 98277-9986) was used. The elution was carried out under gradient conditions at a flow of 150 µl/min.

MS and MS/MS analyses were performed on a triple quadrupole PE-SCIEX API 365 (Perkin-Elmer Sciex Instruments Foster City, CA USA), equipped with Turboion Spray interface in negative ion mode.

The API source voltage was set at −3000 V. The orifice potential (OR) was optimised between −0.5 and −10 and the ring potential (RNG) from −100 to −200V. Nitrogen was used as a nebulising, curtain and collisional gas in the second quadrupole region. The settings for the nebuliser, curtain and collision gas were 8, 8 and 3 (arbitrary units). The collisional energy was adjusted by variation of the voltage difference between the high pressure entrance quadrupole (Qo) and the collisional cell quadrupole ($R0_2$) and was found to give highest sensitivity for these analytes at 18 eV. The vapouriser was set at 450 ° C.

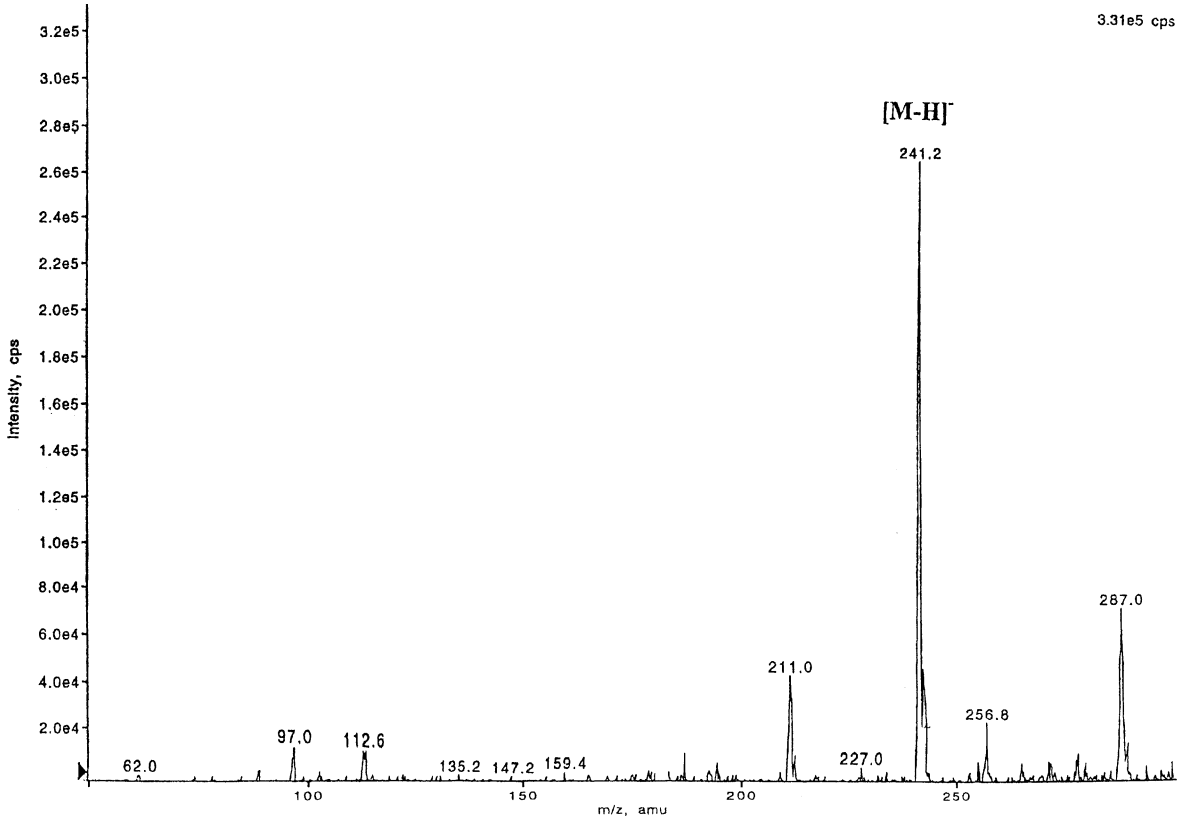


Fig. 1. Full scan atmospheric pressure ionisation/MS (API/MS) spectrum, negative ions, obtained by infusion (10 µl/min) of standard 1-phenyl-6,7-dihydroxyisochroman, **10** (20 ng/µl) in ammonium formate 1, mM. MS conditions: IS = −3000, Orifice (OR) = −2.0, Focusing ring (RNG) = −200 V.

Initially acquisition parameters were optimised in ion spray mode by direct continuous pump infusion of each standard solution (20 ng/l in aqueous $HCOONH_4$ 1 mM) at a flow rate of 10μl/min in the mass spectrometer.

Data acquisitions were performed first, on each standard compound in full scan, in negative mode using the first quadrupole to choose an abundant precursor. MS/MS product ion scans were then recorded. Finally, all the analyses on standards and real samples were carried out by HPLC–MS/MS in MRM (multiple reaction monitoring) mode (sample control) monitoring the product ions selected from MS–MS spectra to obtain a high specificity and sensitivity. The mobile phase was methanol + HCOOH 2 mM and H_20 + HCOOH 2 mM in gradient elution.

At the exit of the chrornatographic column, a 1.1 M NH_3 solution, at a flow rate of 1.7μl min, was added to the mobile phase, using the syringe infusion pump. The resulting mixture (pH = 7) was then conveyed to the API (atmospheric pressure ionisation) interface of the mass spectrometer.

3. Results and discussion

On the basis of the previously reported hypothesis, we prepared, as reference compounds, a series of isochroman derivatives (compounds **3–11**, see materials and methods and Table 1a and b) by reacting **1** with carbonylic compounds **12–20**, whose presence in olive oil has been demonstrated.

The oil purification protocol was successfully used to analyse the biophenolic compounds (Bianco et al 2001; Cartoni, et al., 2000), to verify the effective presence of some of these in extra-virgin olive oils, by means of HPLC–MS/MS technique (Table 2). The presence of two isochroman derivatives, **10** and **11**, in commercial extra-virgin olive oils, was shown.

The full scan spectra of these two isochromans (1-phenyl-6,7-dihydroxy-isochroman 10 and 1-(3′-methoxy-4′-hydroxy)phenyl-6,7-dihydroxy-isochromans **11**) are reported in Fig. 1 and 2, respectively. The corresponding full scan spectra in MS/MS are reported in Figs. 3 and 4, respectively.

Fig. 5 shows the HPLC chromatogram of a standard mixture of the synthesised hydroxy-isochromans, analysed by MS/MS in negative ionisation. Fig. 6 shows the HPLC chromatogram, obtained using the same conditions, that demonstrated the presence of compounds **10** and **11** in the biophenolic mixture obtained from extra virgin olive oils by the biophenolic analysis method.

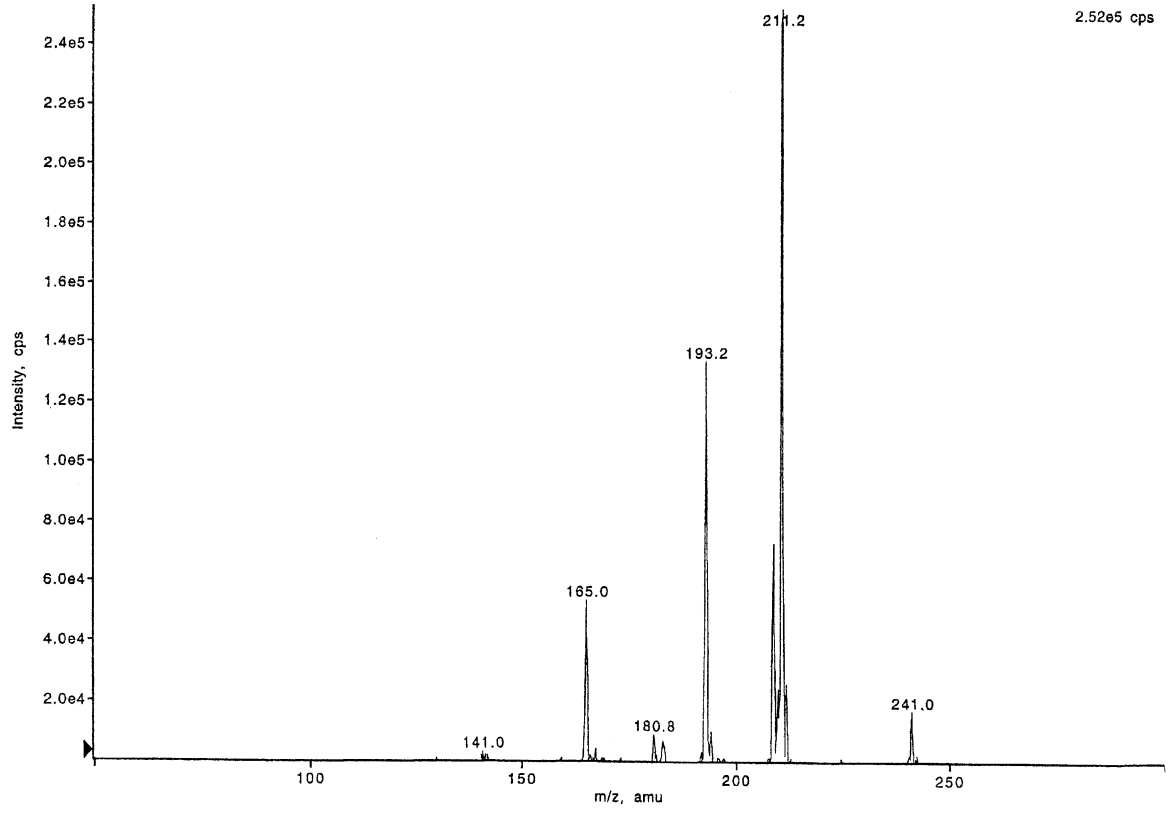


Fig. 2. Full scan atmospherric pressure ionisation (API/MS) spectrum, negative ions, obtained by infusion (10 μl/min) of standard 1-(3′-methoxy-4-hydroxyphenyl-6, 7-dihydroxyisochroman, **11** (20 ng/μl) in ammonium formate 1 mM. MS conditions: Ion spray voltage (IS) = −3000 V, Orifice (OR) = −2.0 V, Focusing ring (RNG) = −200 V.

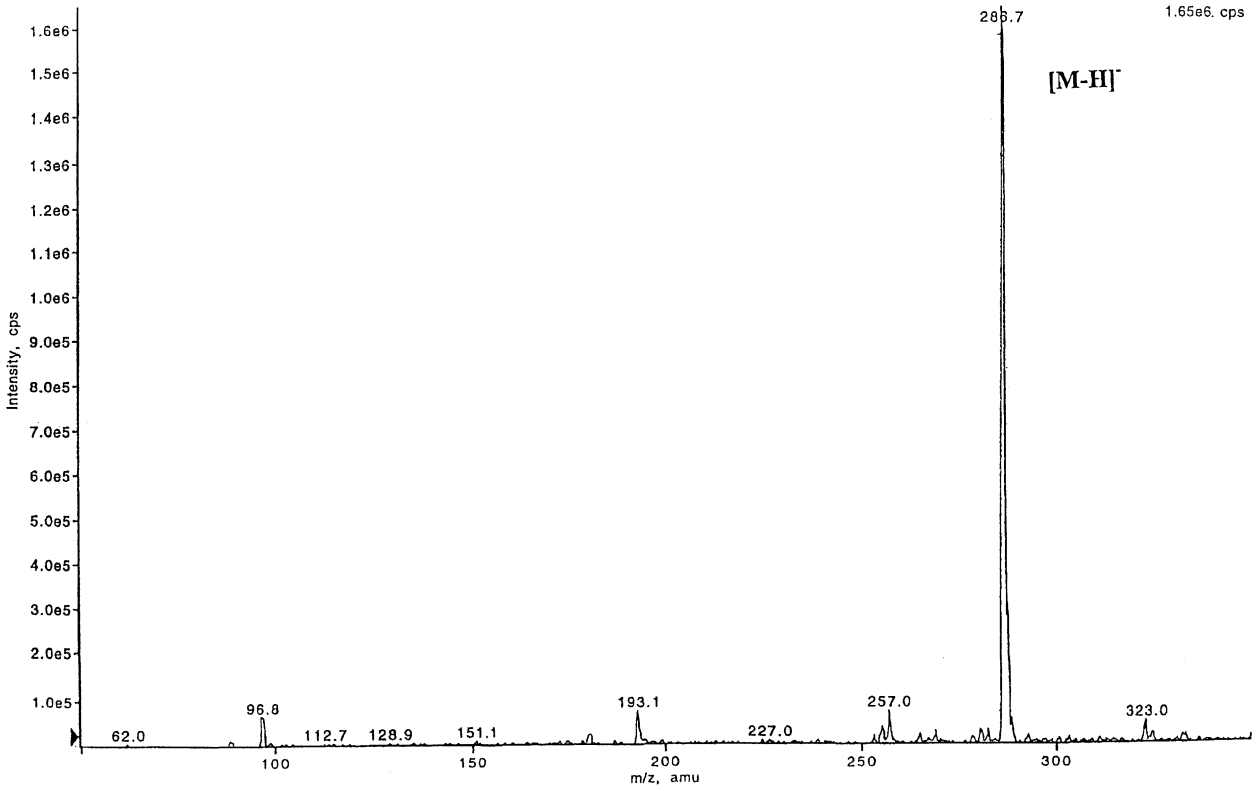


Fig. 3. Full scan Mass/Mass (MS/MS) spectrum of standard 1-phenyl-6,7-dihydroxyisochroman, **10**. Precursor ion m/z 193.2, Collision cell quadrupole (RO_2) = 30 V, Collision cell quadrupole (RO_2) = 45 V, Last resolving quadrupole (RO_3) = 33 V. The standard was infused as indicated in Fig. 1.

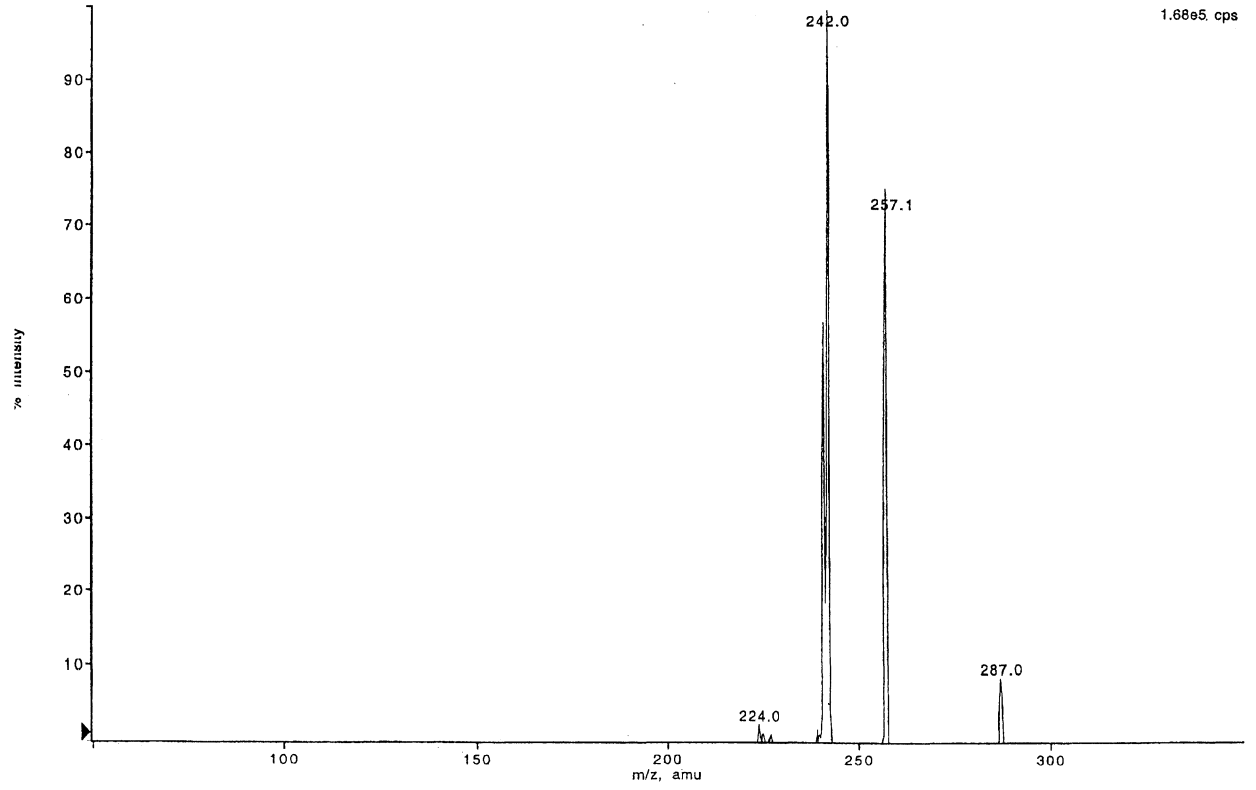


Fig. 4. Full scan Mass/Mass (MS/MS) spectrum of standard 1-(3′-methoxy-4-hydroxy)phenyl-6,7-dihydroxyisochroman **11**. Precursor ion m/z = 286.7, Collision cell quadrupole (RO_2) = 30 V, Collision cell quadrupole (RO_2) = 45 V, Last resolving quadrupole (RO_3) = 33 V.

Table 3
Determination of hydroxy-isochromans in commercial extra-virgin olive oils

Virgin olive oil samples	1-(3′-methoxy-4′-hydroxy) phenyl-6,7-dihydroxyisochroman **11** ng/kg	1-phenyl-6,7-dihydroxyisochroman **10** ng/kg
Frantoio della Rocca	20	260
Farchioni	392	7.9
Unbottled	249	14.9
Trasimeno	114	1400

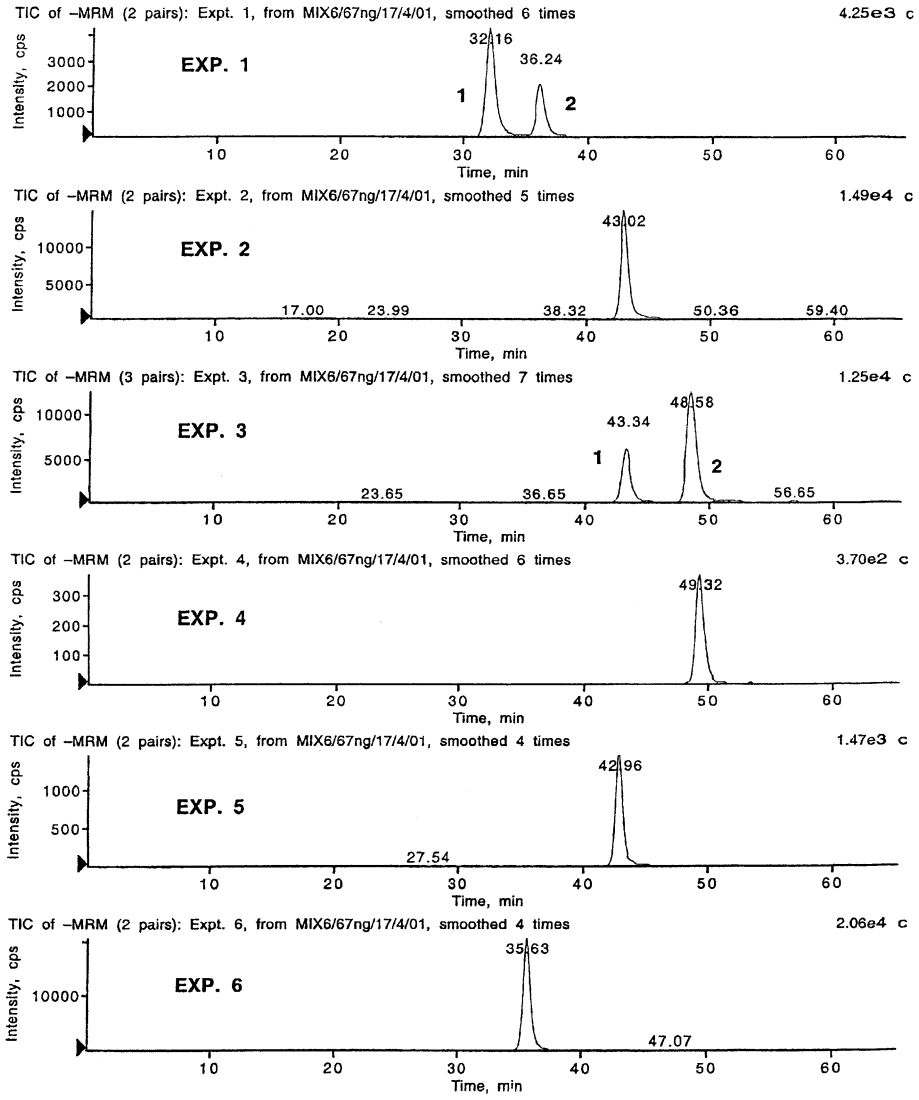


Fig. 5. Multiple reaction monitoring (MRM) (sample control) chrornatogram in negative ionisation of standard mixture of isochromans. Experiment 1: Peak I: compound **4**, Peak 2: compound **3**; Experiment 2: compound **6**; Experiment 3: Peak 1: compound **7**, Peak 2: compound **8** and compound **6**. Experiment 4: compound **9**. Experiment 5: compound **10**. Experiment 6 compound **11**. Column C18 (250×2.1mm), flow-rate 150 μl/min; mobile phase: A = 2 mM HCOOH in Methanol. B = 2 mM HCOOH in distilled water; gradient: 0 min 5% A; 50 min 70% A; 60 min 100%; 65 min. 100% A.

The quantities of the earlier hydroxy-isochromans, which were found in different samples of virgin olive oil, are reported in Table 3.

The earlier data clearly demonstrate the presence of this new group of compounds in the olive oil. Hydroxy-isochromans must now be considered in the list of olive oil biophenols and their contribution to the anti-oxidising activity of this fraction is a matter of study. Now it will be necessary to investigate whether hydroxy-isochrornans are natural compounds, present in olives, or

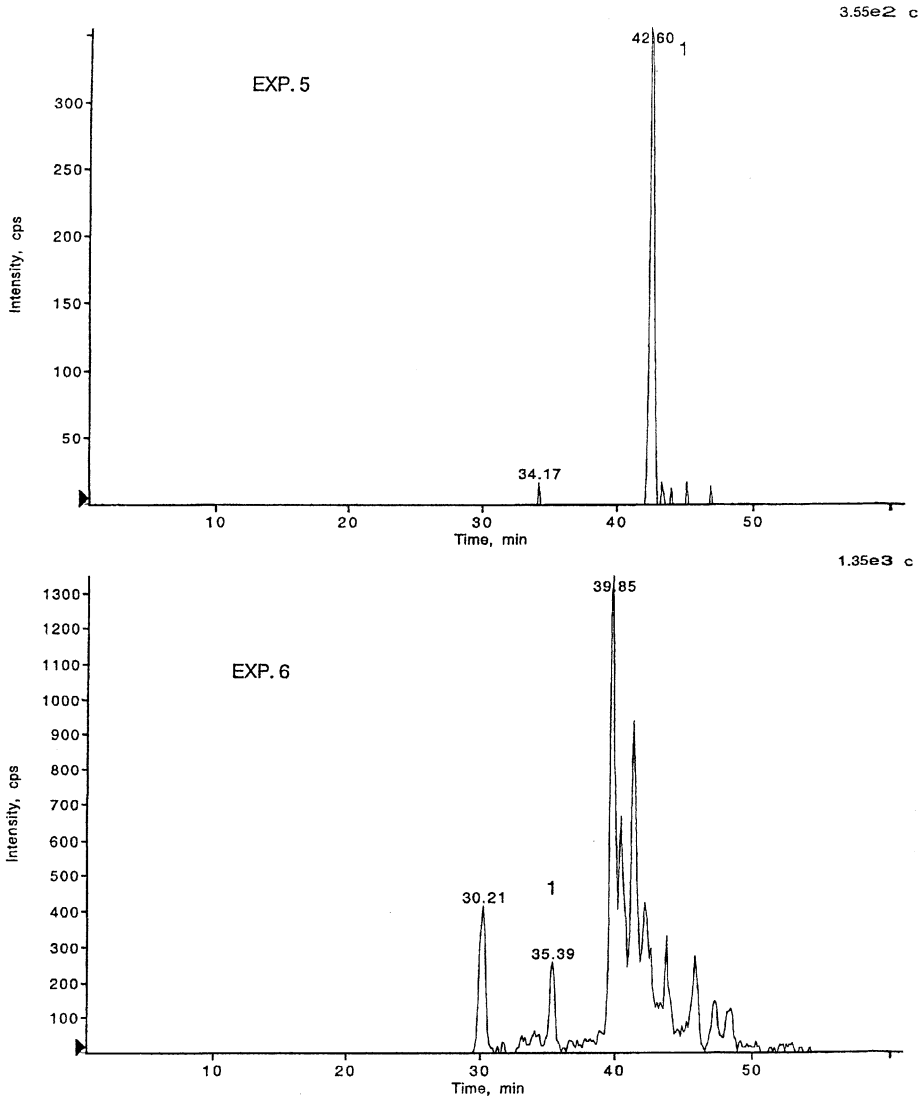


Fig. 6. Multiple reaction monitoring (MRM) (sample control) chromatogram in negative ionisation of an extra-virgin oil for the analysis of biophenolic compounds (Cartoni et al., 2000). Experiment 5: compound **10**. Experiment 6: compound **11**. HPLC conditions as in Fig. 5.

whether they are also formed during the technological "kneader" process or during the subsequent storage of oil. In the latter case, the presence of hydroxy-isochromans could be a marker to establish the age of the oil, even if their easily predictable high anti-oxidant power may decrease their abundance with time.

In this context we are testing the presence of isochromans in fresh olives, as well as in the corresponding oil, in relation to the degree of ripeness olives and to the storage time of the oil.

Acknowledgements

Financial support from MURST is acknowledged.

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