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Main Polyphenols in the Bitter Taste of Virgin Olive Oil. Structural Confirmation by On-Line High-Performance Liquid Chromatography Electrospray Ionization Mass Spectrometry

F. GUTIÉRREZ-ROSALES,* J. J. RÍOS, AND MA. L. GÓMEZ-REY

Instituto de la Grasa (CSIC), Departamento Caracterización y Calidad de los Alimentos, Avda Padre García Tejero 4, 41012 Sevilla, Spain

Twenty virgin olive oils of extra quality and different bitter intensity were submitted to sensory evaluation and to the determination of polyphenols. A linear regression analysis was carried out assuming, as an independent variable, bitter intensity perceived by tasters, as an independent variable, the concentration (mmol/kg) of dialdehydic and aldehydic forms oleuropein aglycon, and dialdehydic and aldehydic forms ligstroside aglycon. Structural confirmation of these compounds was done by online high-performance liquid chromatography-electrospray ionization-collison-induced dissociation-mass spectrometry. The results obtained demonstrate the essential role played by this compound in the bitter taste of virgin olive oil.

KEYWORDS: **Bitter taste; polyphenols; virgin olive oil; MS**

INTRODUCTION

Virgin olive oil, the only juice of the olive obtained by pressing, is one of the few oils that is consumed without any further refining process. Properly extracted from fresh ripe fruits, the oil is characteristically aromatic and slightly bitter.

Virgin olive oil is rich in phenolic compounds. These polyphenols enhance the resistance to autoxidation of the oil (*1*) and contribute to its bitter taste (*2*−*4*). Virgin olive oils with a low or moderate level of bitterness are accepted by consumers, but very bitter ones are rejected.

The intensity of the bitterness of virgin olive oil has been related with the presence of phenolic compounds derived from the hydrolysis of oleuropein, a secoiridoid glucoside characteristic of the Oleaceae (*5*, *6*). These compounds, which are partially soluble in lipids, are conferred to the virgin oil during its extraction, through an unknown process in which at least two kinds of enzymes, such as glycosidases and esterases, could be involved (*7*).

To the authors' knowledge, the bitterness of virgin olive oil has been related only with the total derivatives of oleuropein (*4*) or with the total polyphenol content (*3*). Nevertheless, the relationship between the individual hydrophilic phenols of virgin olive oil and its sensory characteristics was not clearly defined. This research was undertaken to determine the main phenolic compounds responsible for the bitterness of virgin olive oil.

MATERIALS AND METHODS

Oil Samples. Twenty virgin olive oil samples were used from the seasons 2000−2001. Half of the oil samples was obtained using dual phase decanter centrifugation for varieties Picual, Hojiblanca, and Cornicabra, and the other half was from commercial oils. All of the samples were bitter, in a range of bitterness from slight to extreme.

Extraction of Phenolic Compounds. The phenolic extracts of virgin olive oil were obtained following a previously described procedure (*8*). To a weighed sample of oil (2.5 ± 0.001 g), 0.5 mL of standard solution (4.64×10^{-2} mg/mL of *p*-hydroxyphenyl-acetic acid) was added. The solvent was evaporated in a rotatory evaporator at 40 °C under vacuum, and the oily residue was dissolved in 6 mL of hexane. A diol-bonded phase cartridge was placed in a vacuum elution apparatus and conditioned by the consecutive passing of 6 mL doses of hexane. Then, the vacuum was released to prevent drying of the column. The oil solution was applied to the column, and this was washed twice with 3 mL of hexane, which was run out of the cartridge. The sample container was washed again with 3 mL of a mixture of hexane/ethyl acetate (90:10, v/v), which was run out of the cartridge and discarded. Finally, the column was eluted with 10 mL of methanol, and the solvent was evaporated in a rotatory evaporator at room temperature under vacuum until dry. The residue was redissolved with 500 μL of methanol/water (1:1, v/v) at 4 °C. An aliquot (20 μL) of the final colorless solution was injected into the high-performance liquid chromatography (HPLC) system.

HPLC Analysis. The HPLC analysis for analytical separations was performed in a Hewlett-Packard series 1100 liquid chromatographic system equipped with a diode array UV detector, and a Rheodyne injection valve (20 μL loop) A Lichrospher 100RP-18 column (4.0 mm i.d. × 250 mm; particle size 5 μm) (MercK, Darmstat, Germany), maintained at 30 °C, was used. Elution was performed at a flow rate of 1.0 mL/min, using as the mobile phase a mixture of water/phosphoric acid (99.5:0.5 v/v) (solvent A) and methanol/acetonitrile (50:50 v/v) (solvent B). The solvent gradient changed according to the following conditions: from 95% (A):5% (B) to 70% (A):30% (B) in 25 min, to 62% (A):38% (B) in 10 min, to 55% (A):45% (B) in 10 min, and to 47.5% (A):52.5% (B) in 5 min; 100% (B) was maintained for 5 min, and the run was ended. Quantification of phenols was carried out at 280 nm, and the results are expressed in millimoles per kilogram. Triplicate determinations were made.

The HPLC analysis of semipreparative chromatographic separations was performed in a Hewlett-Packard series 1100 liquid chromatographic

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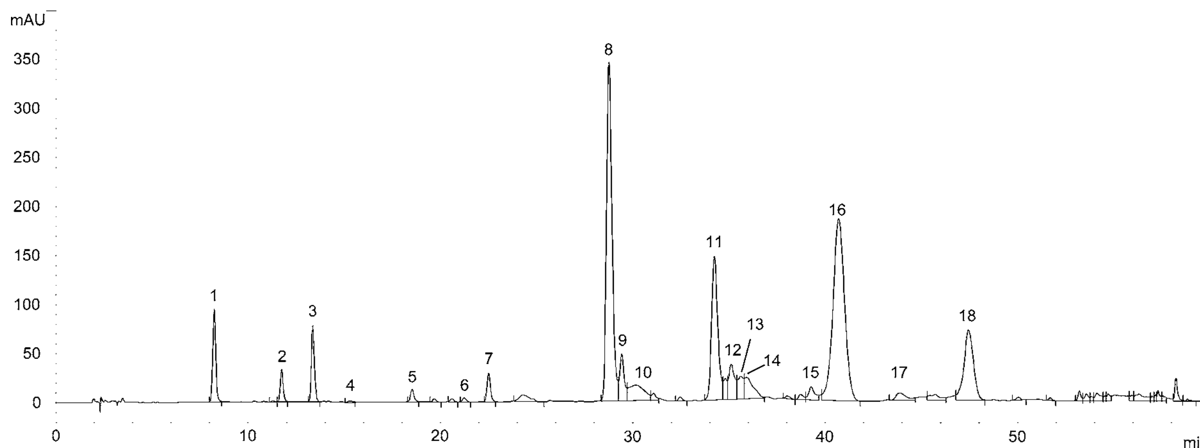

Figure 1. HPLC chromatogram of phenolic compounds isolated from Picual virgin olive oil by SPE on diol phase (detection at λ = 280 nm). Peaks: (1) hydroxityrosol, (2) tyrosol, (3) internal standard *p*-hydroxyphenylacetic, (4) vanillic acid, (5) vanillin, (6) *p*-coumaric acid, (7) hydroxitirosol acetate, (8) dialdehydic form of decarboxymethyl oleuropein aglycone, (9) tyrosyl acetate, (10) isomere aldehydic form of oluropein aglycon, (11) dialdehydic form of decarboxymethyl ligstroside aglycone, (12) pinoresinol, (13) cinnamic acid, (14) L-acetoxypinoresinol, (15) luteolin, (16) aldehydic form of oeuropein aglycon, (17) apigenin, and (18) aldedydic form of ligstroside aglycon.

Figure 2. Relationship between the content of DGO, DGL, AGO, AGL, and bitter intensity.

system equipped with a diode array UV detector, and a Rheodyne injection valve (200 μL loop) A Lichrospher 100RP-18 column (250 mm i.d. × 10 mm; particle size 5 μm) (MercK) was used. Elution was performed at a flow rate of 1.5 mL/min, using the same mobile phase and gradient as for analytical separations.

Sensory Analysis. The oil samples were evaluated for bitterness by 12 panel members of the Instituto de la Grasa, very familiar with oil flavor quality. A scale of 1−5 was used to determine the intensity of bitterness: 1 indicates imperceptible, 2 indicates slight, 3 indicates moderate, 4 indicates great, and 5 indicates extreme. The panelists were

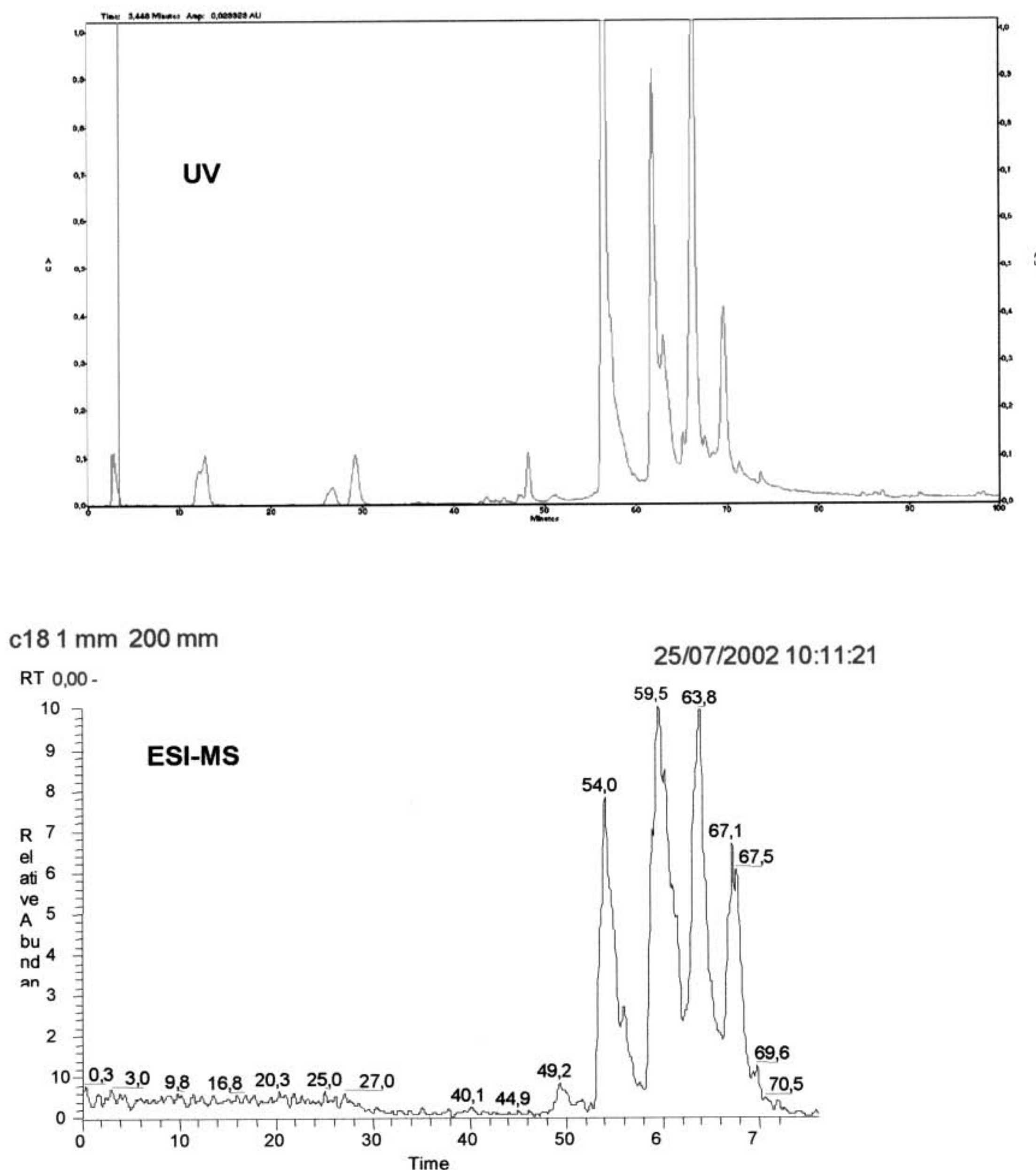

Figure 3. Chromatographic profile of 5 μL of complete extract. Above: UV signal recording; below, mass spectrometry total ion current signal.

seated in individual booths, and the oil samples (15 mL) were served at 28 °C in blue glass cups; duplicate determinations were made. The temperature of the samples was maintained during the evaluation period by setting the cups in holes drilled in heavy aluminum blocks preheated to the desired temperature. The bitterness assigned to each sample was the mean of the intensities as evaluated by the panelist.

To determine the basic taste of the extract from virgin olive oil, samples of 2.5 g of oil were extracted as described above, solvents were removed under vacuum at 30−35 °C, and the mixtures of components were taken up in 250 μL of water. Freshly prepared extracts were used in evaluation sessions. The samples were taken by five panelists using Pasteur capillary pipets. The fractions of the peaks obtained by semipreparative HPLC from the bitter extracts were prepared for evaluation of sensory characteristics as described above.

HPLC-Mass Spectrometry (MS) of Phenolic Compounds. To confirm the identity of tasted peaks, a sample extract was analyzed using a 126 pump with a 168 diode array detector (Beckman, Inc., U.S.A.) on-line with a MAT95's magnetic sector mass spectrometer (Finnigan Mat, Bremen, Germany) equipped with an ESI-II electrospray ionization (ESI) interface. A Spherisorb ODS-2 column (3 μm, 200 mm × 1 mm i.d., Tracer Analitica) at a flow rate of 40 μL/min, was used, and fused silica capillary line tubes (50 μm i.d.) were used to reduce the distance between diode array cell outlet and ESI interface inlet for minimizing MS peak broadening. The solvent composition and gradient profile were the same as for previous analytical and semipreparative procedures.

The ESI mass spectra in the positive ion mode were obtained under the following conditions: capillary temperature, 220 °C; lens, skimmer, and octapole voltages were set to get optimal response for a pattern solution of reserpine. Nitrogen at 200 Kpa was used as the sheath gas. Afterward, partial defocusing of interface parameters was done in order to generate moderate collision-induced dissociation (CID) inside the ionic transport region. Under these conditions, the spectra show enough ionic fragmentation to verify structural information from the protonated molecular ion.

Statistical Analysis. All of the determinations were carried out in triplicate. The results are expressed as mean ± standard deviation. Correlation studies were performed using CoStat 2.10 sofware (CoHort Sofwares, Berkeley, CA).

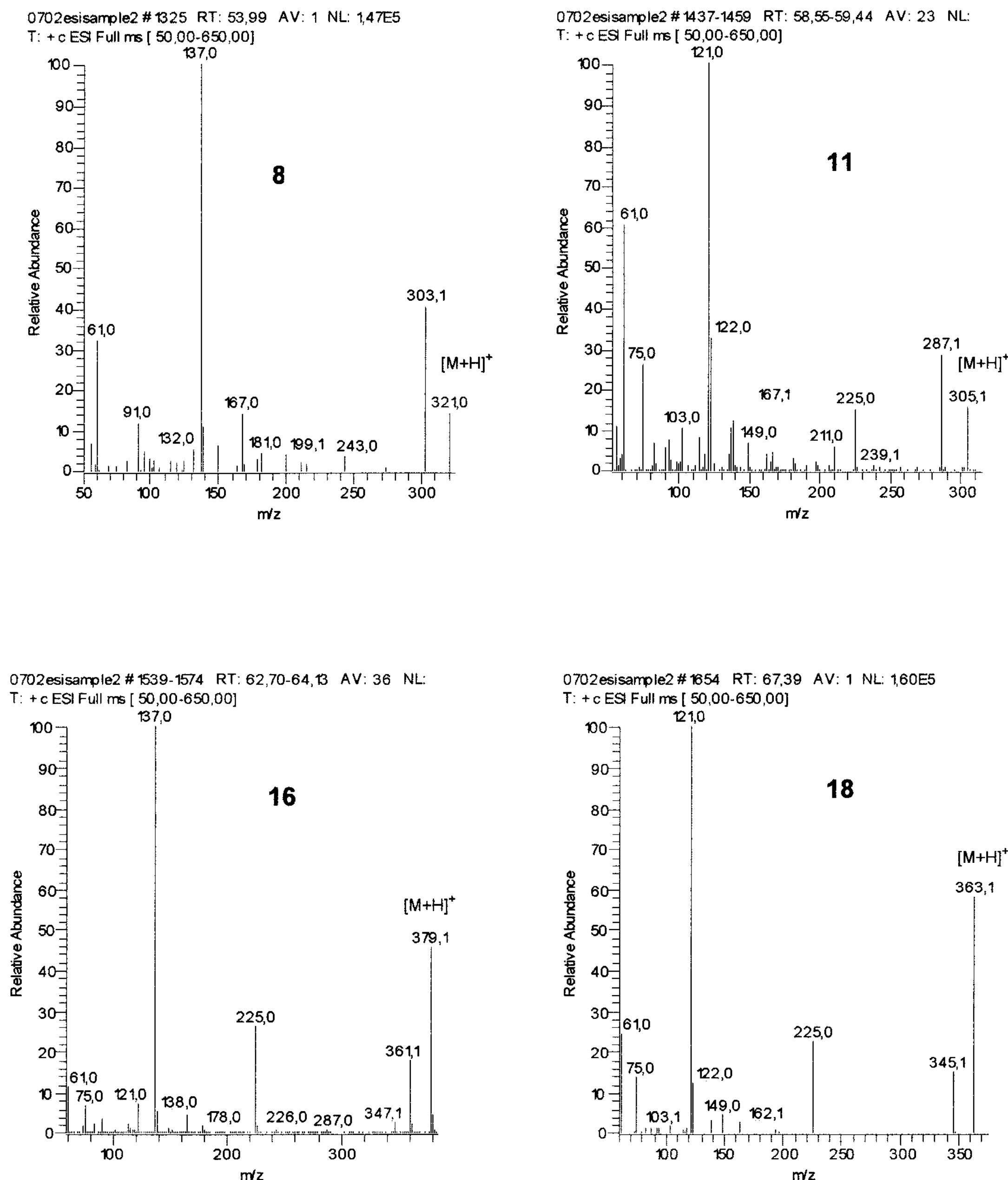

Figure 4. ESI-CID-MS of DGO, DGL, AGO, and AGL.

RESULTS AND DISCUSSION

Sensorially, all of the samples examined were "extra" grade. Direct observation of the intensities of the bitter attribute detected by the panelists shows that the oils studied were characterized by intensities ranging from slight to extreme. The sensation of bitterness is due to the interaction between polar molecules and the lipid portion of the taste membrane (*9*).

The concentration of the phenolic contents of the oils differs, depending on the genetic contribution. It is generally accepted that the stimuli mainly responsible for the attributes bitter and spicy are tyrosol, hydroxytyrosol, and their respectives aglycones, arising from glycosides occurring naturally in olive fruits (*10*, *11*). The intensity of bitterness has been related with the sum of the contents of two secoiridoid derivatives of hydroxytyrosol (*4*).

Figure 1 shows the chromatogram of the phenolic compounds of one of the oils studied. The major peaks were for the dialdehydic forms of the aglycones of oleuropein (DGO) and ligstroside (DGL) (peaks 8 and 11) and the aldehydic forms of the aglycones of oleuropein (AGO) and ligstroside (AGL) (peaks 16 and 18), respectively.

In the present work, the compounds cited above were isolated using preparative HPLC and (after evaporation of the solvent) redissolved in water and tasted to evaluate the intensity of bitterness. The results of mean intensity obtained were the following: peak 8, 4.9 $\pm$ 0.2; peak 11, 4.30 $\pm$ 0.4; peak 16, 3.2 $\pm$ 0.5; and peak 18, 1.9 $\pm$ 0.3. It is thus concluded that the peaks corresponding to the dialdehydic (DGO) and aldehydic (AGO) forms of decarboxymethyl oleuropein aglycone and the dialdehydic form of decarboxymethyl ligstroside aglycone (DGL) are those mainly responsible for the bitter taste of virgin olive oil. This is the first time that these results have been obtained.

The results of the quantitative analysis of polyphenols in the samples indicated that the greater the intensity of bitterness for the sample, the greater was the content of DGO, AGO, and DGL. A strong correlation was observed between the content of DGO, DGL, and AGO and bitterness intensity (**Figure 2**),

Table 1

	$[M + H]^+$	$[M + H - H_2O]^+$	B^+	C^+
peak 8	321	303	167	137
peak 11	305	287	167	121
peak 16	379	361	225	137
peak 18	363	345	225	121

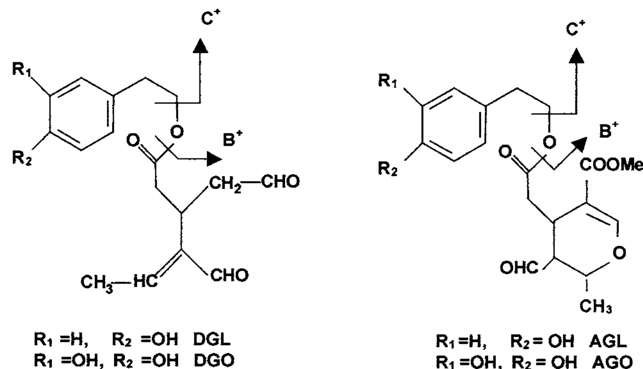

Figure 5. Fragmentation pathway of DGO, DGL, AGO, and AGL.

with very significant correlation coefficients ($P_{r=0} \subseteq 0.001$) for DGO and DGL, and ($P_{r=0} \subseteq 0.01$) for AGO, thereby confirming the results given in the previous paragraph.

Structural confirmation of the four major taste peaks was performed using online HPLC-ESI-CID-MS. Although these compounds have been identified by various authors (*12−15*), this is the first time that they have been identified simultaneously by on-line HPLC-UV-ESI-MS.

ESI and APcI are soft ionization methods, and their use has extended the applicability of MS to any type of molecule, regardless of its volatility and molecular mass. Moreover, the combined use of multistage ion analyzers coupled with various MS ionization methods allows the structural determination of complex mixtures without any previous derivatization step. In the same way, the use of CID inside the ionic transport region provides enough structural information to deduce or verify the compound structure.

An injection of 5 μL of methanolic extract into the system described above gave the chromatograms shown in **Figure 3**. The good coincidence of retention time allowed easy assignment of the UV peak elution to the MS ionic profile.

ESI mass spectra of the four majority peaks, numbered as 8, 11, 16, and 18 for coincidence with analytical elution profile, are shown in **Figure 4**, and they are coincident with the previously isolated and tasted peaks. The peaks correspond respectively with the dialdehydic form of oleuropein aglycon (8), the dialdehydic form of ligstroside aglycon (11), the aldehydic form of oleuropein aglycon (16), and the aldehydic form of ligstroside aglycon (18).

Product ions were obtained by CID in the ionic transport region by modifying lens and tube voltage by 20 V from the optimal focusing setpoints. Fragmentation patterns for all of the compounds described can be deduced (**Table 1**).

All of them showed a moderate-to-high protonated molecular ion $[M + H]^+$ and ions generated from neutral losses. Major ions, generated by neutral losses from the protonated molecular ion $[M + H]^+$, are common to all of the compounds: water elimination $[M + H - H_2O]^+$, loss of esterifying phenol $[B]^+$, and loss of aldehydic or dialdehydic acid $[C]^+$, respectively, following the fragmentation pathway scheme shown in **Figure 5**.

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