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Solid-phase extraction gas chromatography-ion trap-mass spectrometry qualitative method for evaluation of phenolic compounds in virgin olive oil and structural confirmation of oleuropein and ligstroside aglycons and their oxidation products

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Abstract

Phenolic compounds in Spanish virgin olive oil were analyzed by GC–MS after an SPE diol cartridge extraction and clean-up procedure. Posterior derivatization to trimethylsilyl (TMS) ethers using a mixture of hexamethyldisilazane:dimethylclorosilane (HMDS:DMCS) in pyridine (3:1:9) was performed. Several compounds were detected and 21 of them were identified. Free phenols such as hydroxytyrosol, tyrosol, tyrosyl and hydroxytyrosyl acetate, and aldehydic and dialdehydic forms of elenolic acid linked to tyrosol and hidroxytyrosol were the most abundant compounds. Likewise, oxidation products coming from the aldehydic and dialdehydic forms of elenolic acid, and of ligstroside and oleuropein aglycons, were detected, and their structure confirmed by other mass spectrometry technique, i.e., HPLC-APCI-MS. Individual oxidation products were isolated from an oxidized sample by preparative HPLC, converted to TMS ethers and re-analyzed by GC–MS. When necessary and for identification purposes, selective ion monitoring, namely, GC–MS-SIM, was employed. This is the first time that structures of oxidized forms are determined by GC–MS.

Keywords: Phenolic compounds; Oxidized phenols; Oxidized aglycons; Virgin olive oil; Ion trap- gas chromatography; APCI

1. Introduction

Olive phenols are a group of minor biomolecules that display important biological activities. In virgin olive oil, they are present either as free or esterified forms in different quantities although their qualitative profile is constant for a specific cultivar. Such compounds are eliminated during refining processes [1–3]. Virgin olive oil phenols provide a source of natural antioxidants because of the presence of phenolic and *o*-diphenolic structures (tyrosol and hydroxytyrosol) with free radical scavenging properties, as confirmed by the DPPH quenching test [4]. Specifically, hydroxytyrosol has been also shown to exhibit a strong antoxidant activity regarding the oxidation process of methyl linolenate [5,6].

The secoiridoid aglycons are characteristics of *Olea europeae L. Species* [7], constitute an important class of the phenolic compounds present in virgin olive oil, and are genetically associated with oleuropein and ligstroside [8], the secologanin being their precursor.

The level of phenolic compounds is a very important parameter in the evaluation of virgin olive oil quality since phenols are closely related to both the oil resistance to oxidation because of their antioxidative properties [9,10] and the typical bitter taste of olive oil [11,12]. Furthermore, some studies have shown that the amount of phenolic substances present, together with the characteristic fatty acid composition, are related to the beneficial health effects that make virgin olive oil a very valuable and appreciated dietary oil [13,14].

Therefore, it is of great relevance to assay phenols in olive oils, both qualitative and quantitatively. Nowadays,

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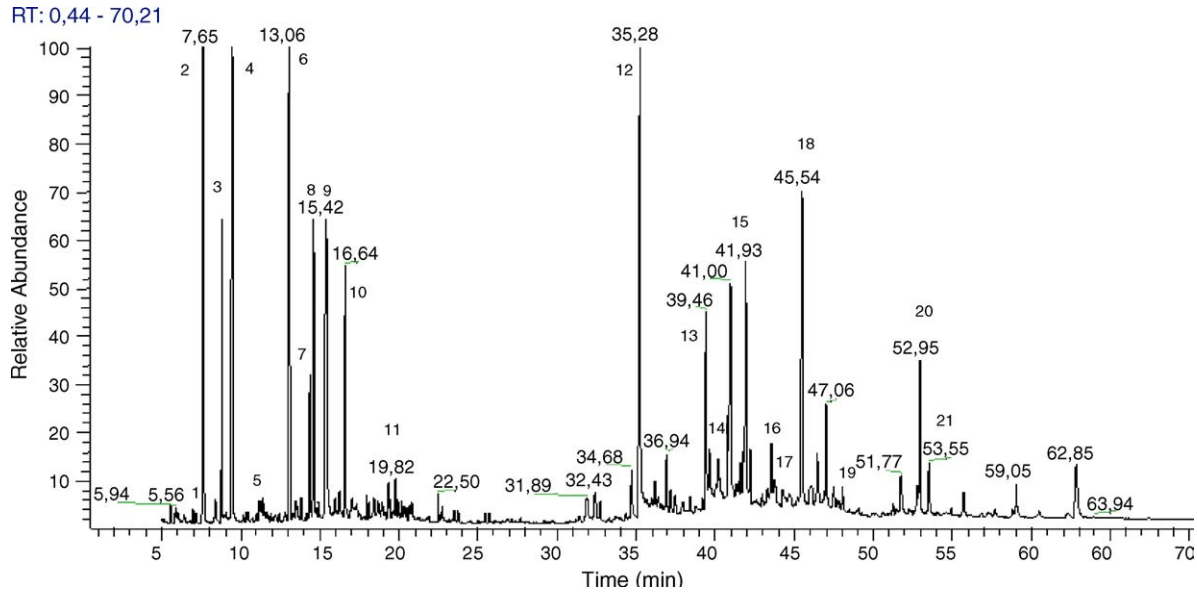

Fig. 1. GC-ion trap-MS chromatogram in EI mode of silyl derivatives of a representative SPE extract from intact virgin olive oil. Peaks: (1) cinnamic acid; (2) tyrosol; (3) tyrosyl acetate; (4) internal standard (*p*-hidroxyphenylacetic acid); (5) homovanillic acid; (6) hydroxytyrosol; (7) IS (*o*-coumaric acid); (8) hydroxytyrosyl acetate; (9) elenolic acid; (10) dihidroxy benzoic acid; (11) oxidation product of the dialdehydic form of elenolic acid; (12) dialdehydic form of decarboxymethyl ligstroside aglycone; (13) dialdehydic form of decarboxymethyl oleuropein aglycone; (14) oxidation product of the dialdehydic form of decarboxymethyl ligstroside aglycone; (15) aldehydic form of ligstroside aglycone; (16) oxidation product of the dialdehydic form of decarboxymethyl oleuropein aglycone; (17) oxidation product of the aldehydic form of ligstroside aglycone; (18) aldehydic form of oleuropein aglycone; (19) oxidation product of the aldehydic form of oleuropein aglycone; (20) pinoresinol and (21) pinoresinol acetate.

the most commonly used method for phenolic determination in virgin olive oil is based in HPLC with UV detection since, in contrast with GC, no previous derivatization is required to obtain quantitative data. However, discrimination between some phenolic compounds and their oxidation products by HPLC has not been satisfactorily achieved [15]. Scarce studies based on GC have been reported in virgin olive oil [16–18], generally showing poor results in terms of number of phenolic compounds determined and lacking information on the oxidized compounds potentially formed.

The present study was directed to improve determination of the phenolic compounds present in virgin olive oil by GC and, moreover, to gain insight into the structures of the oxidized products of elenolic acid, oleuropein and ligstroside aglycons, only determined so far by HPLC. Thus, an

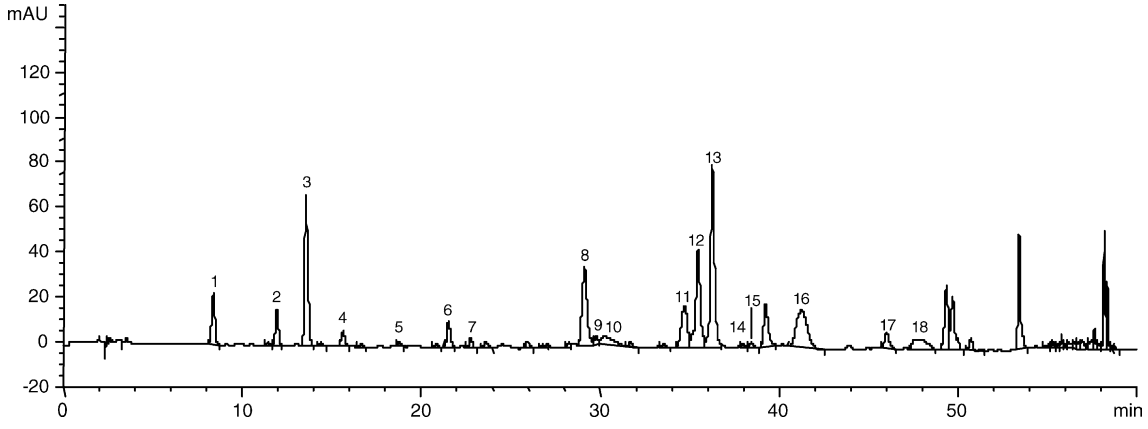

Fig. 2. HPLC chromatogram of phenolic compounds isolated from intact Picual virgin olive oil by SPE on diol phase (detection at λ = 280 nm). Peaks (1) hydroxityrosol, (2) tyrosol, (3) internal standard *p*-hydroxiphenylacetic, (4) vanillic acid, (5) vanillin, (6) *p*-coumaric acid, (7) hydroxitirosyl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

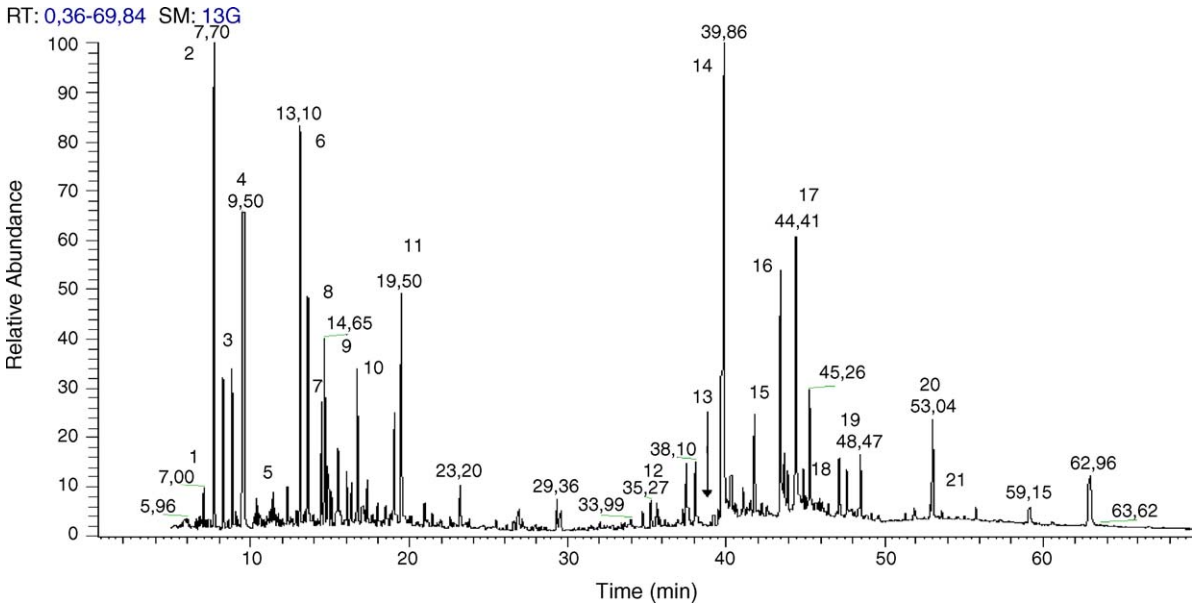

Fig. 3. GC-ion trap-MS chromatogram in EI+ mode of silyl derivatives of a representative SPE extract from oxidized virgin olive oil. For peak identification, see Fig. 1.

alternative analytical approach is proposed to monitor oxidation in commercialized oil samples and shelf-life studies.

2. Experimental

2.1. Samples

Extra virgin olive oil (Cv *Picual*, Jaén, Spain) was used for experiments. Twenty grams of original extra virgin olive oil was oxidized in a Rancimat apparatus, Model 679 (Metrohm Co., Basel, Switzerland) at 100 °C for 8 h, at 10 L/h of air flow.

2.2. Materials and reference compounds

All reagents were of analytical reagent grade. Acetonitrile, methanol and acetic acid were of HPLC grade (Romil Ltd., Cambridge, UK). Hexamethyldisilazane (HMDS), dimethylchlorosilane (DMCS) and pyridine were purchased from Supelco (Bellefonte, PA). Caffeic, *o*- and *p*-coumaric, vanillic acid, sinapic acid, protocatechuic acid,

Table 1
List of peaks identified

Peak	Compound	Rt	MW (TMSiO)
1	Cinamic acid	6.92	220
2	Tyrosol	7.65	138
3	Tirosyl acetate	8.81	180
4	*p*-Hydroxyphenyl acetic acid (IS)	9.47	296
5	Homovanillyc acid	11.37	168
6	Hydroxytyrosol	13.06	370
7	*o*-cumaric aid	14.54	308
8	Hidroxytyrosyl acetate	14.66	340
9	Dialdehydic form of elenolic acid	15.42	314
10	Dihidroxybenzoic acid	16.64	370
11	Oxidation product of the dialdehydic form of elenolic acid	19.52	402
12	Dialdehydic form of decarboxymethyl ligstroside aglycone	35.28	376
13	Dialdehydic form of decarboxymethyl oleuropein aglycone	39.46	464
14	Oxidation product of the dialdehydic form of decarboxymethyl ligstroside aglycone	39.86	464
15	Aldehydic form of ligstroside aglycone	41.93	434
16	Oxidation product of the dialdehydic form of decarboxymethyl oleuropein aglycone	43.55	552
17	Oxidation product of the aldehydic form of ligstroside aglycone	44.41	522
18	Aldehydic form of oleuropein aglycone	45.54	522
19	Oxidation product of the aldehydic form of oleuropein aglycone	47.45	610
20	Pinoresinol	52.95	502
21	Acetoxipinoresinol	53.55	560

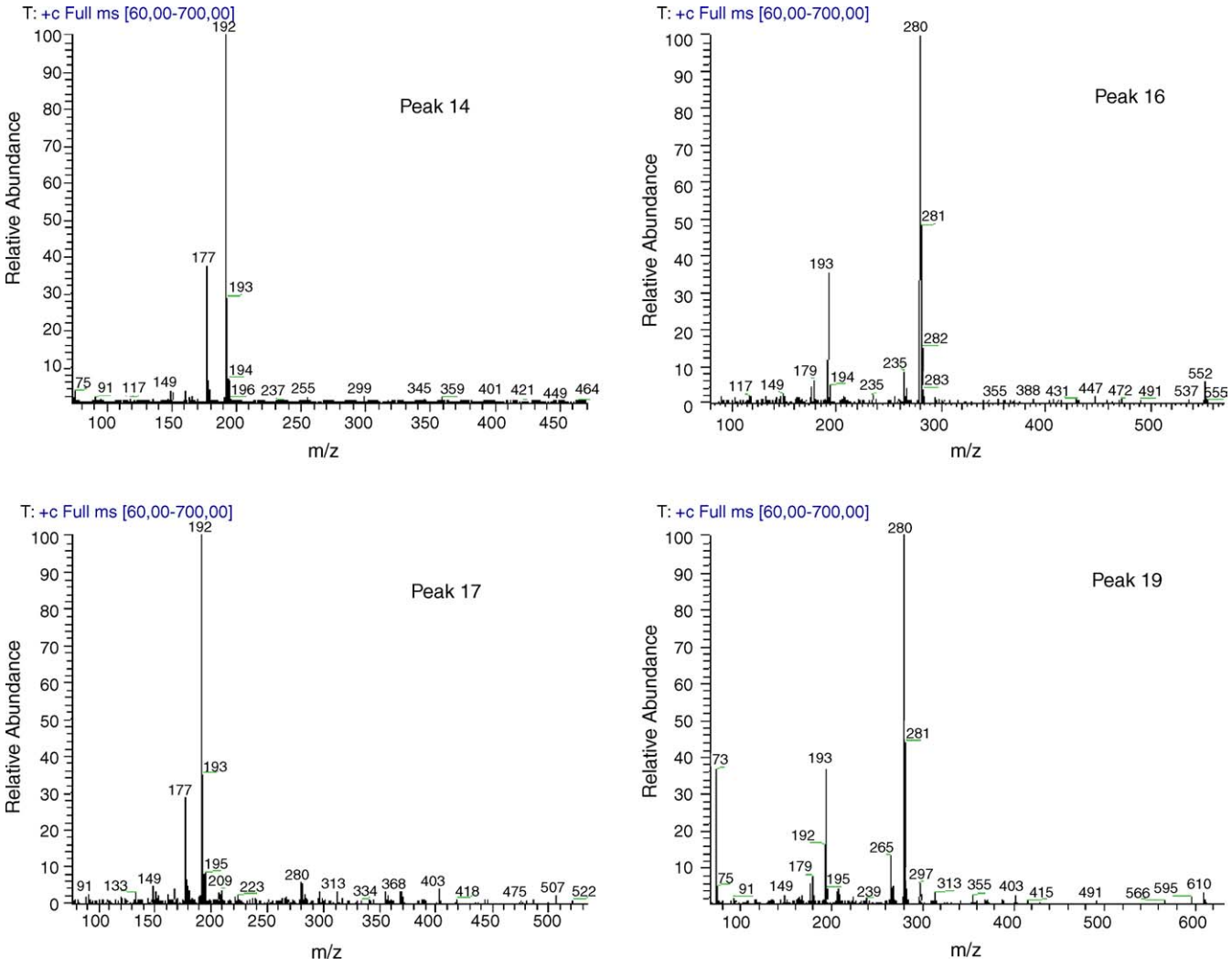

Fig. 4. EI mass spectra of oxidation products of: peak 14, dialdehydic form of decarboxymethyl ligstroside aglycone; peak 16, dialdehydic form of decarboxymethyl oleuropein aglycone; peak 17, aldehydic form of ligstroside aglycone; peak 19, aldehydic form of oleuropein aglycone.

p-hidroxybenzoic acid, gallic acid, ferulic acid, vanillin, luteolin, apigenin, tyrosol 2-(4-hydroxyphenyl)ethanol were from Janssen Chemical Co. (Beerse, Belgium); hydroxytyrosol 2-(3,4-dihydroxyphenyl)ethanol, hydroxytyrosyl acetate 2-(3,4-dihydroxyphenyl)ethyl acetate and tyrosyl acetate 2-(4-hydroxyphenyl)ethyl acetate were kindly provided by the Organic Chemistry Department of the University of Seville, Spain.

Table 2
HPLC-APCI-MS fragmentation pattern of peaks 1 to 8 in Fig. 5

HPLC	$[M+H]^+$	Ions from $[M+H]^+$ by common neutral losses			Asiggnated compound
		$[H_2O]$	[Acidic group]	[Phenolic group]	
Peak 1	321	303	137	167	Dialdehydic form of decarboxymethyl oleuropein aglycone
Peak 2	337	319	137	183	Oxidation product of the dialdehydic form of decarboxymethyl oleuropein aglycone
Peak 3	305	287	121	167	Dialdehydic form of decarboxymethyl ligstroside aglycone
Peak 4	321	303	121	183	Oxidation product of the dialdehydic form of decarboxymethyl ligstroside aglycone
Peak 5	395	377	137	241	Oxidation product of the aldehydic form of oleuropein aglycone
Peak 6	379	361	137	225	Aldehydic form of oleoropein aglycone
Peak 7	379	361	121	241	Oxidation product of the aldehydic form of ligstroside aglycone
Peak 8	363	345	121	225	Aldehydic form of ligstroside aglycone

2.3. Extraction and derivatization of phenolic compounds

The phenolic extracts were obtained following the procedure of Mateos et al. [19]. Briefly, 2.5 ± 0.001 g of oil were weighed and 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 oil residue was dissolved in 6 mL hexane. A diol-bonded phase SPE cartridge (Supelco, Bellefonte, PA) was placed in a vacuum elution apparatus and conditioned with 6 mL of hexane. Then, vacuum was released to prevent the column from drying. The oil solution was then applied to the column, which was subsequently washed twice with 3 mL of hexane and once with 3 mL of the a mixture of hexane/ethyl acetate (90:10, v/v). Finally, the retained fraction was eluted with 10 mL of methanol and evaporated in a rotatory evaporator at room temperature under vacuum. The dry residue was redissolved in 500 μL of methanol/water (1:1, v/v) at 4 °C, and used directly for HPLC analysis. For GC–MS experiments, the residue was evaporated to dryness under a nitrogen stream and derivatized to his TMS ethers with 100 μL of a mixture of HMDS:DMCS in pyridine (3:1:9). For analyses, a mixture of two phenolic extracts obtained by two separate extractions from 2.5 g oil were used and eight samples of intact and oxidized virgin olive oil were extracted.

2.4. Preparative HPLC analysis

Isolation of individual oxidation products from the aldehydic and dialdehydic forms of oleuropein and ligstroside aglycons was carried out by a set of five sucessive injections of each SPE phenolic extract in the HPLC system described in point 2.6. Four fractions were manually collected, checked again, concentrated under nitrogen stream and converted into silyl derivatives for GC–MS analysis.

2.5. GC–MS and GC-SIM-MS instrumental analysis

The GC-ion trap-MS experiments were performed using a Trace GC2000 gas chromatograph coupled to a GCQ/Polaris ion trap mass spectrometer (ThermoFinnigan, Austin, TX, USA) equipped with an AS2000 autosampler operating in full scan mode and in selective ion monitoring (SIM) mode only for identification purposes. The column used was a Zebron ZB-5 ms (Phenomenex, Torrance, CA, USA) fused silica capillary column (30 m long × 0.25 mm I.D. × 0.25 film thickness). The oven temperature was programmed as follow: the initial temperature was held for 5 min at 150 °C and then from 150 to 295 °C at 3 °C/min and maintained for 18 min. Injector temperature was set at 300 °C. Carrier gas was helium at 1 mL/min in constant flow mode.

The MS operating conditions were the following: ion source and transfer line temperatures 200 and 290 °C,

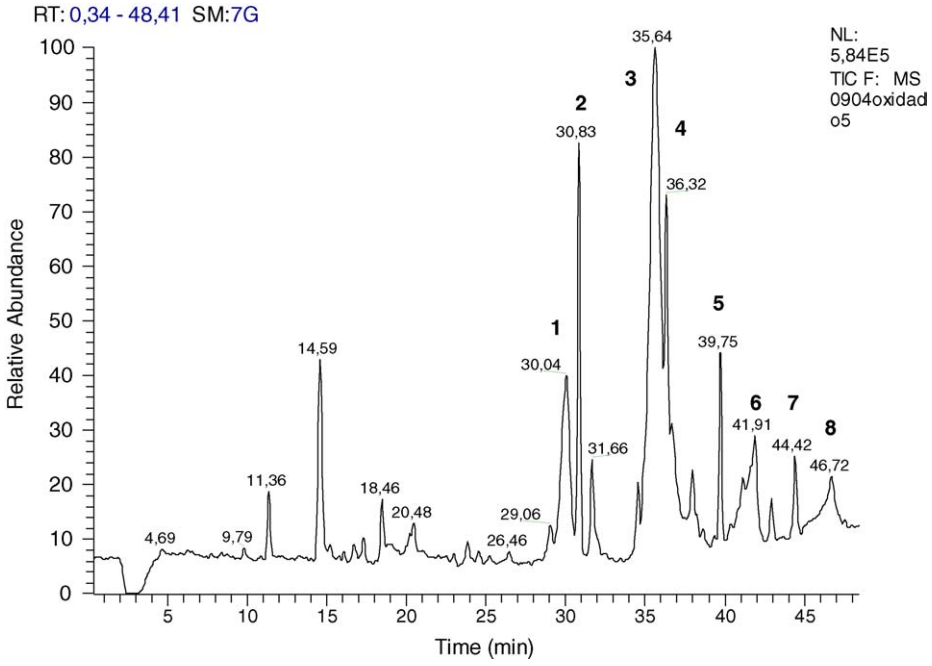

Fig. 5. On-line HPLC-APCI-MS chromatogram of phenolic compounds present in a oxidized virgin olive oil: (1) dialdehydic form of oleuropein aglycone, (2) oxidation product of the dialdehydic form of decarboxymethyl ligstroside aglycone; (3) dialdehydic form of decarboxymethyl ligstroside aglycone (4) oxidation product of the dialdehydic form of decarboxymethyl oleuropein aglycone; (5) oxidation product of the aldehydic form of oleuropein aglycone; (6) aldehydic form of oleuropein aglycone; (7) oxidation product of the aldehydic form of ligstroside aglycone; (8) aldehydic form of ligstroside aglycone.

respectively. The instrument was tuned in EI positive mode using perfluorotributylamine (FC-43) according to manufacturer's recommendations in order to achieve the maximum sensitivity. Parameters such as automatic gain control (AGC) and multiplier (1150 V, 10E5 gain) were set by automatic tuning. The electron energy was 70 eV and the emission current 250 μA.

For GC-ion trap-MS in SIM mode experiments, optimized parameter of buffer gas was set to 0.3 mL/min helium. Samples were analyzed as TMS ether derivatives. As already mentioned, phenolic extracts were evaporated to dryness under a nitrogen stream and immediately derivatized with 100 μL of a mixture of HMDS:DMCS in pyridine. Aliquotes of 3 μL were injected on split mode. Xcalibur version 1.4

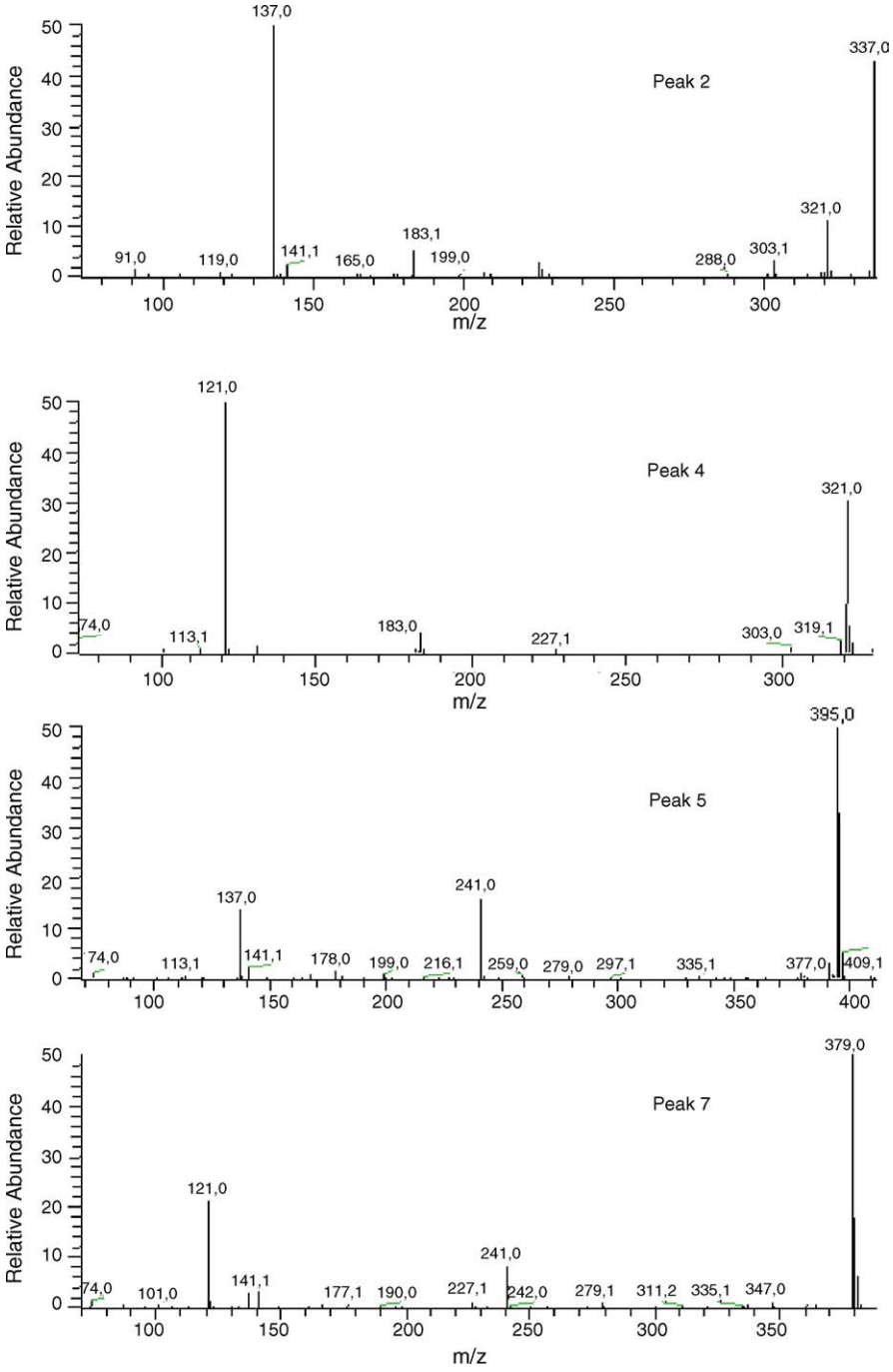

Fig. 6. EI mass spectra of oxidation products: peak 2, dialdehydic form of decarboxymethyl ligstroside aglycone; peak 4, dialdehydic form of decarboxymethyl oleuropein aglycone; peak 5, aldehydic form of ligstroside aglycone; peak 7, aldehydic form of oleuropein aglycone.

software was used for data acquisition and processing of the results.

2.6. HPLC–MS analysis

The HPLC analyses of phenolic extracts were performed in a Beckman Gold system using a 126 pump with a 168 diode array detector (Beckman, Inc., USA) on-line with a MAT95's magnetic sector mass spectrometer (Finnigan Mat, Bremen, Germany) equipped with an APCI ionization interface. A Lichrospher 100RP-18 column (4.6 mm I.D. × 250 mm; particle size 3 μm) (MercK, Darmstad, Germany), maintained at 30 °C, was used and Rheodyne injection valve (200 μL loop). Elution was performed at flow rate of 1.0 mL/min, using as mobile phase a mixture of water/acetic acid (99.9:0.1, v/v) (solvent A) and methanol/acetonitrile/acetic acid (50:50:1, v/v) (solvent B). The solvent gradient changed according to the following conditions: from 95% (A): 5% (B) to 45% (A): 55% (B) in 45 min, to 100% (B) in 10 min; 100% (B) was maintained for 10 min. Elution profile of phenols was carried out at 240 and 280 nm simultaneously. A split postcolumn of a 25 % of the column flow was introduced in the APCI interface.

The APCI mass spectra, in the positive-ion mode, were obtained under the following conditions: capillary temperature of 220 °C; lens, skimmer, and octapole voltages were set to get optimal response for a pattern solution of gramicidine. Nitrogen at 150 K Pa was used as sheath gas. Afterwards, partial defocusing of interface parameters was done in order to generate moderate collision-induced dissociation inside the ionic transport region. Under these conditions, the spectra showed enough ionic fragmentation to confirm or verify structural information from the protonated molecular ion.

3. Results and discussion

Figs. 1 and 2 show the GC and HPLC profiles, respectively, of a representative phenolic extract obtained from the intact olive oil sample by the procedure described above. As can

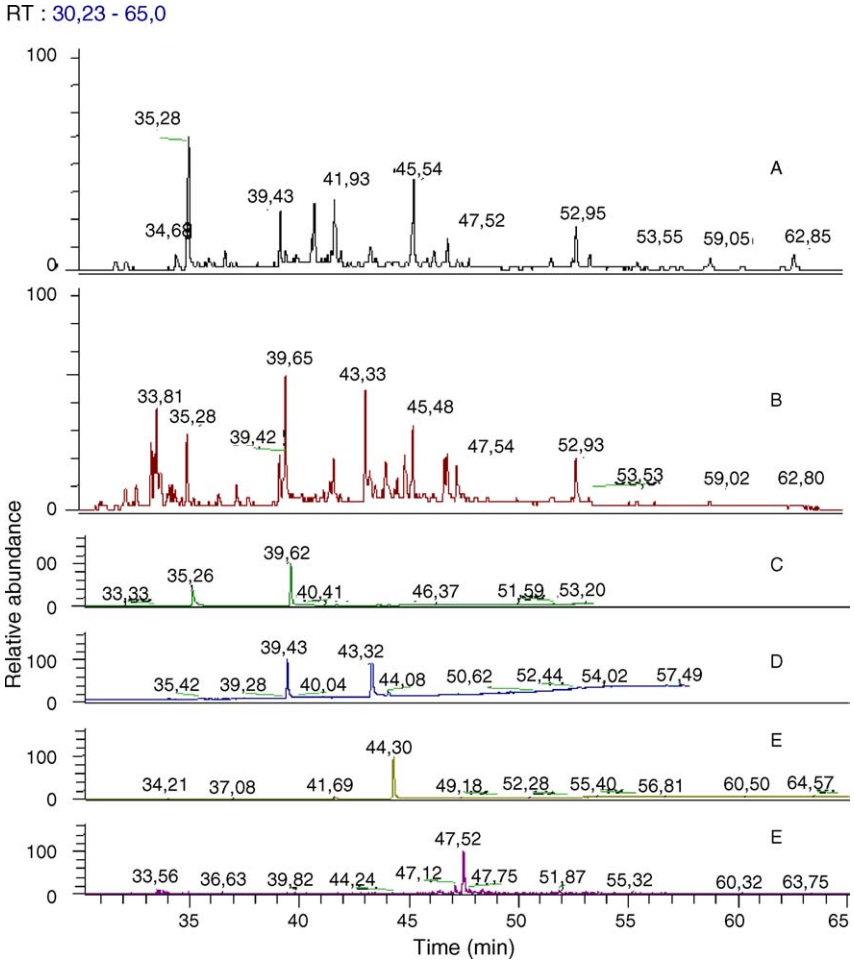

Fig. 7. Expanded plots of the second part of GC–MS chromatograms of: (A), intact virgin olive oil; (B), oxidized virgin olive oil; (C), oxidation product of the dialdehydic form of decarboxymethyl ligstroside aglycone (Rt = 39.62 min); (D), oxidation product of the dialdehydic form of decarboxymethyl oleuropein aglycone (Rt = 43.32 min), partially contaminated with the intact dialdehydic form of decarboxymethyl oleuropein aglycone (Rt = 39.43 min)); (E), oxidation product of the aldehydic form of ligstroside aglycone (Rt = 44.30 min); (F), oxidation product of the aldehydic form of oleuropein aglycone (Rt = 47.52 min).

be observed in Fig. 1, the first part of the GC chromatogram (retention times from 5 to 30 min) shows successful separation of the free phenolic compounds and higher number of compounds than those detected by HPLC (Fig. 2). The second part of the chromatogram (from 30 to 70 min retention time), although appearing more complicated because of the presence of a great number of peaks, allowed identification of most of them. Therefore, the proposed methodology enabled not only to obtain a quite clean extract but also good separation of a great number of phenolic compounds, as compared to results reported by other researchers [16–18]. An initial tentative peak identification is summarized in Table 1.

Identification of peaks present in the first part of the GC chromatogram (peaks 1–10) has been mainly carried out by GC-ion trap-MS by using reference commercial products of free phenolic compounds, except in the case of the oxidation product of the dialdehydic form of elenolic acid, which structure was deduced from mass spectra fragmentation.

Mass spectra of most of peaks encountered in the second part of chromatogram (retention times from 30 to 70 min) had a strong base peak at *m*/*z* 192 or *m*/*z* 280. These ions corresponded to characteristic fragmentation of β-phenyl ethyl esters in a McLafferty rearrangement [20,21] of TMS derivatives from oleuropein and ligstroside aglycons, and were very useful for interpretation purposes. Thus, the presence of those base peaks allowed unambiguous structure assignments referred to presence of tyrosol or hidroxytyrosol as phenol terminal linked to elenolic acid. Even when their molecular ion was minimal or absent, identification of normal intact TMS derivatives from ligstroside and oleuropein was not difficult (peaks 12, 13, 15 and 18). Confirmation was achieved through the TMS derivatives of the fractions collected by semipreparative HPLC from phenolic extracts obtained in a previous work [12], which had been stored at −80 °C.

Peaks numbered as 14, 16, 17 and 19, despite being minimal, showed small but clear molecular ions at *m*/*z* 464, 552, 522 and 610, respectively. Such molecular weight data could correspond to oxidation products of ligstroside and oleuropein aglycons derivatives with excellent resolution and quantification possibilities. Structures of this compounds

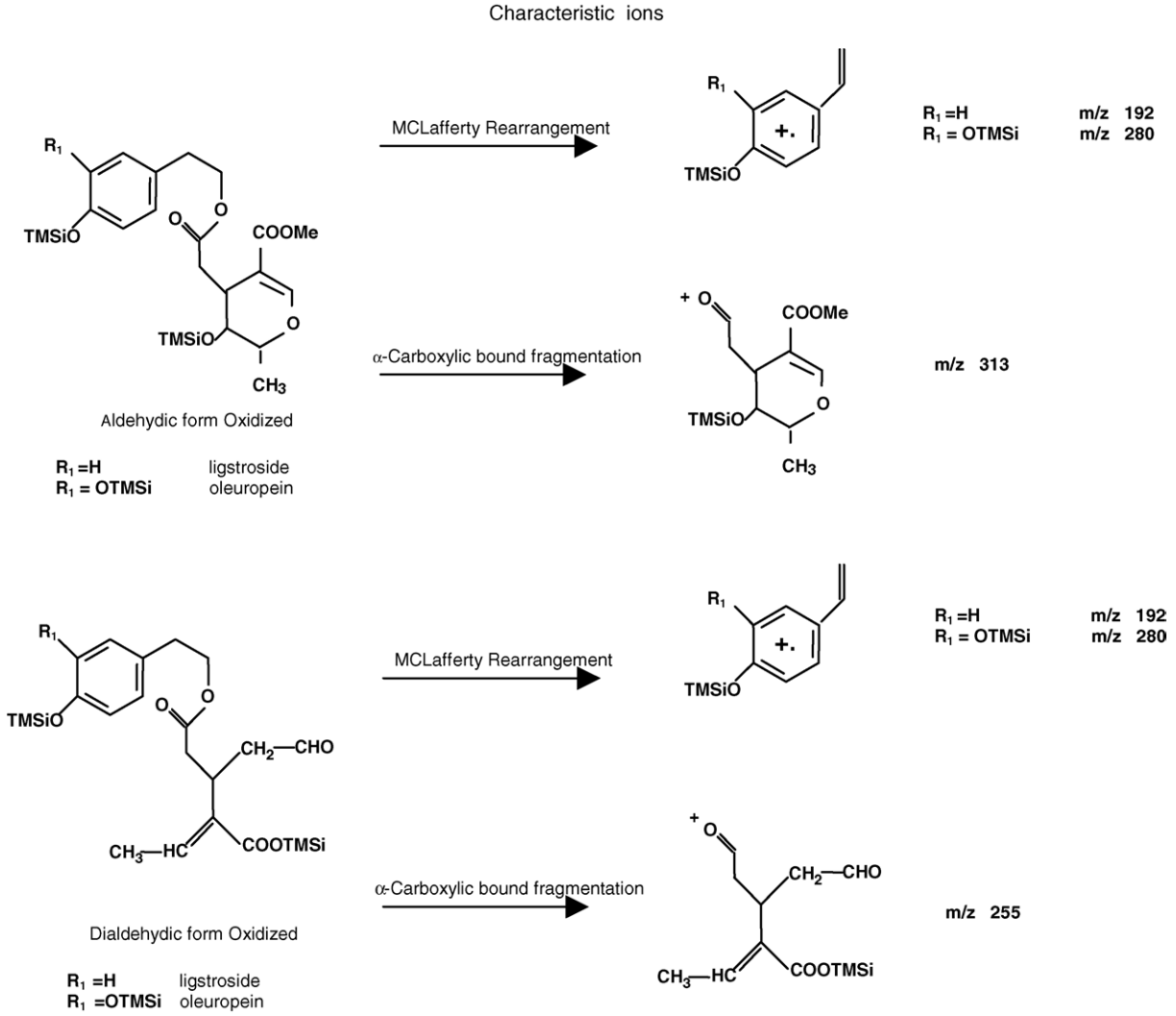

Fig. 8. McLafferty Rearrangement and α-carboxylic bound fragmentation in linked phenolic compounds.

have been so far determined by HPLC–MS [15] while determination by GC–MS has been approached in the present work for the first time.

In order to increase knowledge of these compounds, a phenolic SPE extract of oxidized virgin olive oil was converted to TMS derivatives and analyzed by GC–ion trap-MS. The chromatographic profile is shown in Fig. 3. Comparison with intact virgin GC–MS profile showed that the oxidation treatment led to an important relative decrease of peaks 9, 12, 13, 15 and 18, along with a relative increase of peaks 11, 14, 16, 17 and 19, hence referred to as oxidation products of the dialdehydic form of elenolic acid (peak 11), and oxidation products of ligstroside aglycones (peaks 14 and 17) and oleuropein aglycones (peaks 16 and 19). EI mass spectra of this latter series of compounds is shown in Fig. 4.

Confirmation of correct GC–MS peak assignation for oxidation products was carried out by performing several additional experiments.

The first one consisted on verification of molecular weight of either intact and oxidized oleuropein and ligstroside aglycons products by an on-line HPLC-APCI-MS analysis of the phenolic extract from the oxidized sample. For illustration, Fig. 5 shows a representative HPLC–MS chromatographic profile, and Table 2 lists the main ions from peaks 1 to 8, as numbered in Fig. 5.

Mass spectra of intact and oxidation products of oleuropein and ligstroside aglycons showed a common fragmentation pattern: a medium to high molecular ion intensity and neutral losses of H_2O, an acidic group and phenolic-linked group. Moreover, ions at *m*/*z* 167, 183, 225 and 241, originated by a neutral loss of phenol linked to the oxidation product of elenolic acid, are characteristic of the presence of an acidic group on the aglycone moiety as a consequence of the oxidation process. Intact APCI mass spectra of the oxidation products is shown in Fig. 6 and they are coincident with those described previously by Rovellini and Cortesi [15].

In a second experiment, and in order to verify GC–MS retention time assignments, four fractions were manually collected by preparative HPLC, corresponding to elution times of peaks numbered as 2 (Rt: 30.8 min); 4, (Rt: 36.34 min); 5 (Rt: 39.75 min) and 7 (Rt: 44.32 min) in Fig. 5. These fractions were converted to their TMS ethers and injected in the GC-ion trap-MS system.

Expanded plots of the second part of the GC–MS chromatograms of intact virgin olive oil, oxidized virgin olive oil and derivatized collected fractions are shown in Fig. 7. In some cases, the fractions collected showed, depending on the efficacy of the collection cutting, contamination with compounds at close elution times.

Finally, and as was pointed before, McLafferty rearrangement of β-phenyl ethyl esters is the main process of fragmentation. Characteristic cleavage ions of the oxidation products of oleuropein and ligstroside aglycons as TMS derivatives is summarized in Fig. 8. In the case of oxidation products, characteristic ions at *m*/*z* 255 for dialdehydic forms and at *m*/*z* 313 for aldehydic forms, coming from an α-cleavage at the carboxyl group with neutral loss elimination, must be present and they can be used as quite selective ions.

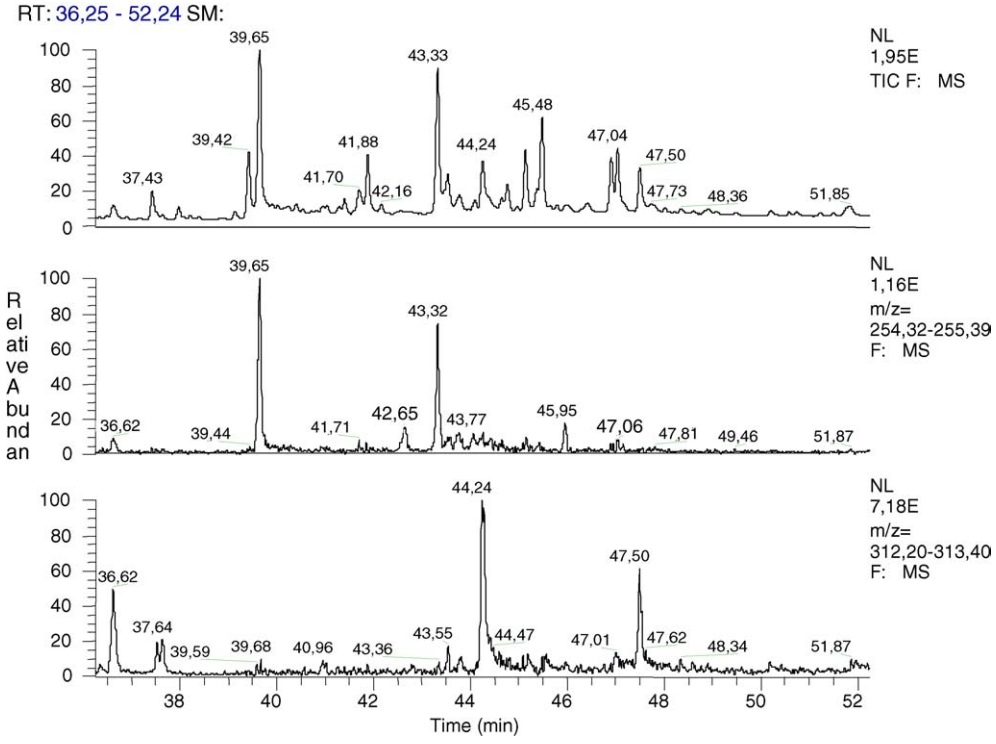

Fig. 9. GC-ion trap-SIM-MS expanded plot from 30 to 50 min for selective ions at *m*/*z* 255 and *m*/*z* 313.

Confirmation of this hypothesis was achieved by injection of a SPE phenolic extract from the oxidized sample in GC-ion trap-SIM-MS mode. The ions selected for the experiment were those coming from the α-carboxylic bound fragmentation at *m*/*z* 255 and 313. The main two couples of peaks, at Rt = 39.65 and 43.32, and Rt = 44.26 and 45.71, respectively, agreed in retention times with the dialdehydic forms of decarboxymethyl ligstroside and oleuropein aglycons, respectively (Fig. 9).

4. Conclusions

The analytical method based on GC described here can be proposed as a good alternative to the methods based on HPLC since it offers clear advantages for determination and potential quantitation of phenolic compounds in virgin olive oil and, specifically, provides a satisfactory resolution of those compounds with phenolic groups linked to aglycons. Furthermore, the method here described improves possibilities for quantitative evaluation of oxidation products of linked phenolic forms, as compared to HPLC, and offers potential applications to measure the oxidation degree of olive oils. In this context, studies are currently underway in our laboratory.

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