GRASAS Y ACEITES, 61 (2),
ABRIL-JUNIO, 157-164, 2010,
ISSN: 0017-3495
DOI: 10.3989/gya.087309

Secoiridoids in olive seed: characterization of nüzhenide and 11-methyl oleosides by liquid chromatography with diode array and mass spectrometry

By **Sandra Silva,**[a,b,c*] **Lucília Gomes,**[a,c] **Fausto Leitão,**[d] **Maria Bronze,**[a,b,c]
Ana V Coelho [b,e] **and Luís Vilas Boas** [a,b]

[a] Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781 - 901 Oeiras, Portugal.
[b] Instituto de Tecnologia Química e Biológica, Apartado 127, 2781 - 901 Oeiras, Portugal.
[c] Faculdade de Farmácia de Lisboa, Av. das Forças Armadas 1649 - 019 Lisboa, Portugal.
[d] Instituto Nacional de Recursos Biológicos I.P., Unidade de Recursos Genéticos, Ecofisiologia e Melhoramento de Plantas, Quinta do Marquês, 2784 - 505 Oeiras, Portugal.
[e] Universidade de Évora, Departamento de Química 7000, Évora, Portugal.
(*Corresponding auhor: ssilva@ibet.pt)

RESUMEN

Secoiridoides en semillas de aceituna: caracterización de nuzenida y 11-metil oleósidos por cromatografía líquida con batería de diodo y espectrometría de masas.

Extractos de semillas de tres cultivos fueron analizados por HPLC en fase reversa con detector de diodos y espectrometría de masas. La unión del HPLC con la especrometría de masas (modo ESI) en ambas polaridades permitió la identificación de nuzenida y 11-metil oleósido de nuzenida entre otros 11-metil oleósidos. El método usado nos permitió obtener los perfiles de los compuestos fenólicos en las semillas de aceitunas y concluir que ellos son secoiridoides. La cuantificación de los secoiridoides fenólicos detectados fue también llevada a cabo usando detección por ultravioleta (λ = 240 nm) lo que permitió la comparación de las muestras. Nuzenida y el 11-metil oleósido de nuzenida fueron los principales componentes detectados en semillas de aceitunas en todos los cultivos estudiados, aunque variaciones en los componentes individuales de las semillas de aceitunas fueron verificados entre los cultivos. Los resultados también apoyan la existencia de di y tri(11-metil oleósidos) de nuzenida.

PALABRAS CLAVE: Compuestos fenólicos – Espectrometría de masas – 11-Metil oleósidos – Nuzenida – Secoiridoides - Semillas de aceituna.

SUMMARY

Secoiridoids in olive seed: characterization of nüzhenide and 11-methyl oleosides by liquid chromatography with diode array and mass spectrometry.

The seed extracts of olive tree cultivars were analyzed by reverse phase HPLC with diode array detection and mass spectrometry. HPLC hyphenation with mass spectrometry (ESI source) in both polarity modes enabled the identification in olive seeds of nüzhenide and nüzhenide 11-methyl oleoside, among other 11-methyl oleosides, by means of MS^n. The methods used allowed us to obtain olive seed profiles of phenolic components and to conclude that they are mainly secoiridoids. The quantification of detected phenolic secoiridoids was also achieved using ultraviolet detection (λ = 240 nm) which enabled comparison of the samples. Nüzhenide and nüzhenide 11-methyl oleoside were the major components detected in olive seeds of all the cultivars studied, but variations in individual components of olive seeds were verified among the cultivars. The results also support the existence of di and tri(11-methyl oleosides) of nüzhenide.

KEY-WORDS: Mass spectrometry – 11-Methyl oleosides – Nüzhenide – Olive seed – Phenolic compounds – Secoiridoids.

1. INTRODUCTION

The olive tree belongs to the genus *Olea* of the Oleaceae family with 25 genera and about 600 species (Jensen *et al.*, 2002). Members of the Oleaceae family are characterized by the presence of secoiridoids. These compounds have elenolic acid or its derivatives in their structures, figure 1. Derivatives of elenolic acid include oleosides. Oleosides are not necessarily phenolic compounds but may include a phenolic moiety as a result of esterification (Ryan *et al.*, 2002).

While phenolic acids, phenolic alcohols and flavonoids occur in many fruits and vegetables belonging to various botanical families, secoiridoids are present exclusively in plants belonging to the Oleaceae family which includes *Olea europaea* L. (Robards *et al.*, 1999; Servili and Montedoro, 2002). Oleuropein, demethyloleuropein, ligstroside and nüzhenide are the most abundant secoiridoids in olives (Servili and Montedoro, 2002) and their structures are presented in figure 1.

Few studies have focused on the phenolic composition of olive seeds. However some phenolic compounds have been identified in olive seeds, such as salidroside, nüzhenide, hydroxytyrosol, nüzhenide 11-methyl oleoside, oleuropein, tyrosol, and demethyloleuropein (Maestro-Durán *et al.*, 1994; Servili *et al.*, 1999). Nüzhenide (nüzhenzi in chinese medicine), was for the first time fully characterized by NMR spectroscopy in 1999 (Servili *et al.*, 1999). This compound has already been

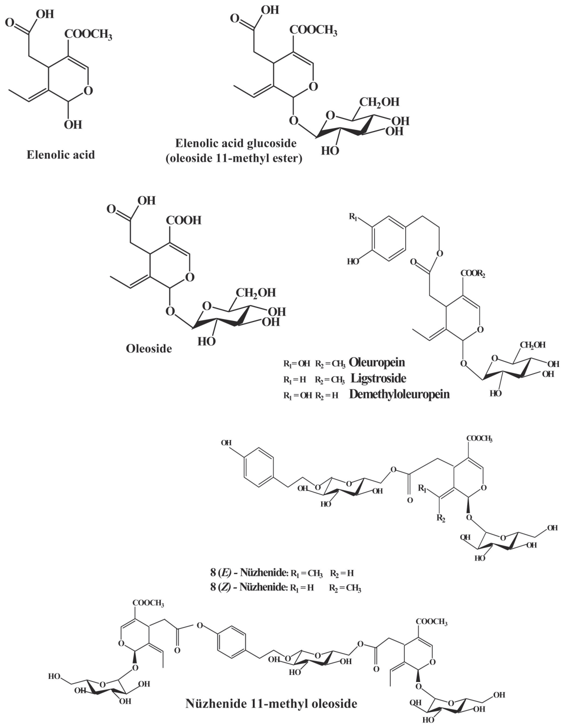

Figure 1
Chemical structures of secoiridoid compounds
found in Oleaceae

characterized by mass spectrometry using FABMS in other plants of the Oleaceae family such as *Fraxinus angustifolia* (Calis *et al*., 1996), *Ligustrum lucidum* (He *et al*., 2001) and *Syringa reticulata* (Koichi *et al*., 2002). Two isomers are described for this compound as illustrated in figure 1.

According to Maestro-Durán *et al.* (1994) the presence of nüzhenide 11-methyl oleoside in olive seeds was hypothesized, but studies on seed storage of the *Chionanthus retusus*, a plant of the Oleaceae family, enabled the confirmation of the structure of nüzhenide 11-methyl oleoside by Chien *et al.* (2004), figure 1.

As far as we know, there are no reports concerning the analysis of secoiridoids in olive seeds with liquid chromatography hyphenated with mass spectrometry. In this paper, we report the analysis of seed extracts from six portuguese olive tree cultivars from *Olea europaea* L. using HPLC with diode array detection and hyphenated with mass spectrometry (electrospray ionization in both polarity modes). This methodology enabled the characterization of nüzhenide by means of MS^n as well as the characterization of other 11-methyl oleosides present in the samples. Quantification using ultraviolet data was done in order to compare samples as to their secoiridoid composition.

2. MATERIALS AND METHODS

2.1. Reagents

Acetonitrile and methanol, HPLC grade, were from Riedel-de-Häen (Seelze, Germany). Phosphoric and formic acids were from Panreac (Barcelona, Spain). Hexane was purchased from LabScan (Dublin, Ireland) and sodium metabisulfite from BDH laboratories.

Water purified by means of Milli-Q from Millipore (Molsheim, France) unit was used.

Caffeic acid was purchased from Aldrich (Steinheim, Germany), oleuropein and tyrosol were purchased from Extrasynthese (Genay, France).

Stock solutions were prepared in a mixture of methanol:water (80:20, v/v) with a concentration of 1000 mg/L. Solutions of caffeic acid and oleuropein were prepared in concentrations of 2 and 5 mg/L, respectively for direct injection in the mass spectrometer for parameter optimization. Quantification by HPLC-DAD (240 nm) of secoiridoids detected in samples was done using a calibration curve over the 25-1000 mg/L range, with oleuropein solutions.

2.2. Samples and Sample Pre-treatment

Olive seeds of six olive tree cultivars (*Olea europaea* L.) from the region of Trás-os-Montes e Alto Douro (Portugal) were studied: “Bical”, “Cobrançosa”, “Lentisca”, “Madural”, “Santulhana” and “Verdeal Transmontana”. Olives were randomly picked at optimum ripening stage for the production of olive oil, according to their skin color, in 2002/2003 crop (January 2003).

The stones were broken in order to remove the intact seed. Seeds (1g) were extracted by solid-liquid extraction with a mixture of methanol:water (80:20, v/v), after adding 10 mL of sodium metabisulfite 2% to the samples. After three extractions (3 × 10 mL), the total extract was used for HPLC analysis. All samples were filtered through a 0.45 μm filter Acrodisc® (Pall, USA) and stored at −20 °C until analysis.

2.3. METHODS

High Performance Liquid Chromatography (HPLC) with diode array detector (DAD)

The HPLC system (Thermofinnigan) consisted of a pump, an autosampler and a diode-array detector (PDA detector). Data acquisition and remote control of the system were done by Chromquest version 4.0 (Thermofinnigan). HPLC separation was performed with a LiChrospher (Merck) C18 column (5 mm, 250 mm × 4 mm i.d. with a C18 precolumn) at 35 °C.

Samples were injected (20 μL) and eluted with an aqueous gradient (flow rate of 0.7 mL/min) prepared from a mixture of water (99.9%) and phosphoric acid (0.1%) as solvent A, water (59.9%), acetonitrile (40%) and phosphoric acid (0.1%) as solvent B. The solvent gradient started with 100% solvent A, reaching 80% after 15 minutes, 30% after 70 minutes and 0% after 85 minutes, followed by a 30 minute isocratic step and the return to initial conditions. Diode array detection was done using the following conditions: scan 200-600 nm, scan rate 1 Hz and band width of 5 nm. Chromatograms at 254, 280 and 320 nm were used and were acquired with 10 Hz rate and 11 nm band width. Chromatograms at 240 nm were obtained from the scan data.

HPLC coupled with Electrospray (ESI) Mass Spectrometry (MS)

The mass spectrometry system was an LCQ ion trap mass spectrometer (Thermofinnigan) equipped with an ESI source and run by Xcalibur (Thermofinnigan) version 1.3 software.

The HPLC conditions mentioned above were used in the HPLC/MS assays but the solvents were prepared replacing phosphoric acid (0.1%) by formic acid (0.5%), and the injection volume was 50 μL.

The following conditions were used in the ESI experiments: ion spray voltage, 4 kV (positive mode), −4.5 kV (negative mode); temperature of the heated capillary, 350 °C. Nitrogen was used as sheath and auxiliary gases. Their flow rate was 80 and 20 arbitrary units, respectively.

HPLC/MS was performed using an acquisition two segment method in full scan mode: 0-45 min, from *m/z* 130 to 2000; 45-115 min, from *m/z* 250 to 2000. The tune methods of each segment were adjusted after analyses of the individual standard solutions of caffeic acid (molecular mass 180) and oleuropein (molecular mass 540) by direct injection in the mass spectrometer. The choice of those standards was related with the sample compound molecular masses and the retention time in the gradient program used.

All the fragmentation experiments were done with 40% collision energy, since this value was suitable for oleuropein, a typical secoiridoid compound, analysed by direct injection in the mass spectrometer.

3. RESULTS AND DISCUSSION

3.1. Identification of secoiridoids in olive seed

Samples were analysed by HPLC using mass spectrometry with electrospray as the ionisation source, in negative and positive polarity modes. However, negative ionization mode enabled higher signal to noise ratios than positive mode for all the detected compounds.

The total ion current and UV chromatographic profiles obtained for the several seed extracts analyzed were similar, although differences in concentrations were observed for the cultivars studied. The seed extract of the “Cobrançosa” cultivar was chosen to show the profile of TIC (negative mode) and 240 nm chromatograms, figure 2, because it was the cultivar with the highest concentrations of phenolic compounds in fruit (Silva, 2004). A value of 240 nm was chosen due the characteristic absorbance of secoiridoids at this wavelength (Cardoso *et al.*, 2005).

The similarity between the total ion chromatogram (TIC) obtained and the chromatographic profile at 240 nm, confirms that the compounds detected in the “Cobrançosa” seed extract are ionizable in the conditions used. The ions *(m/z)* detected in TIC chromatogram are presented in table 1. The presence of several adducts was observed: formate in negative ionization mode, due to the presence of formic acid in the mobile phase; and sodium and ammonium adducts in positive mode. Compound identification was done by the search of the main $[M - H]^-$ or $[M + Na]^+$ ions together with the interpretation of its collision-induced dissociation fragments. However, the conditions used did not enable fragmentation of all parent ions selected. MS^2 data are also presented in table 1.

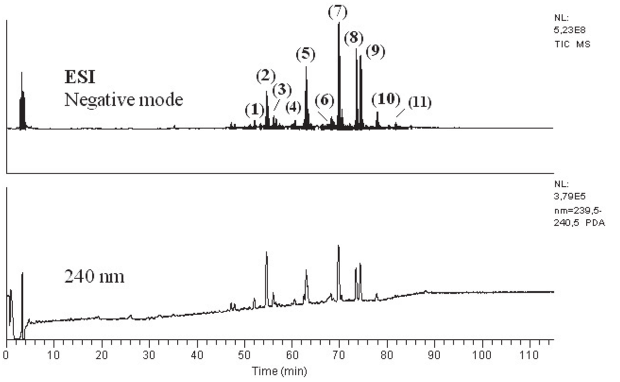

Figure 2
Chromatograms of “Cobrançosa” cultivar seed extract: TIC and absorbance at 240 nm; the MS data corresponding to the chromatographic peaks is presented in table 1.

Nüzhenide and nüzhenide 11-methyl oleoside were detected in samples and represent compounds that have already been identified in *Olea europaea* L. seeds. However, compounds with molecular mass 716, 772, 1102, 1458, 1488 and 1844 were not yet described for this plant, and a contribution for their characterization is reported in the present study.

Maestro-Durán *et al.* (1994) referred that hydrolyses of seed components of *Olea europaea* L. originated tyrosol, elenolic acid and glucose. According to this author, tyrosol glucoside known as salidroside is the unique exception.

The interpretation of full mass spectra, obtained by HPLC/MS, for several TIC detected peaks in samples suggested the presence in seed extracts of mono, di and tri(methyl oleosides) of nüzhenide, since there was a successive difference of 386 mass units with increasing retention time, in negative and positive modes. Therefore the ions detected in samples at higher *m/z* values than nüzhenide 11-methyl oleoside could correspond to secoiridoid compounds with more units of 11-methyl-oleoside. The detected ions, in negative mode, at *m/z* 685 (nüzhenide), 1071 (nüzhenide 11-methyl oleoside), 1457 - nüzhenide di(11-methyl oleoside) - and 1843 - nüzhenide tri(11-methyl oleoside) elucidate the referred mass difference. Moreover, another group of compounds showing the same 386 mass difference was detected in both polarity modes but starting with a compound with 716 mass units (more 30 mass units than nüzhenide). The ions detected, in negative mode,

Table 1
Chromatographic peaks of "Cobrançosa" cultivar seed extract and results obtained by MS

		Main negative ions (*m/z*)		**Main positive ions (*m/z*)**		
Peak number	**Retention time (min)**	**$[M-H]^-$ (*MS² fragments*)**	**$[M+HCOO]^-$**	**$[M+NH_4]^+$**	**$[M+Na]^+$ (*MS² fragments*)**	**Identification**
1	52.1	715 *(385, 451, 553)*	761*	734	739* *(545, 577)*	compound A[1]
2	54.6	685 *(299, 403, 421, 453, 523)*	731*	704*	709 *(515, 547)*	Nüz. [a]
3	56.1	715 (329, 385, 451, 483, 553)	761*	734	739* *(545, 577)*	isomer of compound A[1]
4	60.7	685 *(299, 403, 421, 453, 523)*	731*	704*	709 *(515, 547)*	Nüz. [a]
5	63.1	1071	1117*	1090*	1095	Nüz. 11-Me-oleoside[b]
		1101	1147	1120	1125	11-Me oleoside of compound A[1]
		771 (223, 315, 385, 403, 547, 609, 669, 701, 739)	817	790	795	compound B[1]
6	68.4	1071	1117*	1090*	1095 *(901, 933)*	Nüz. 11-Me oleoside[b]
7	69.9	1071	1117*	1090*	1095	Nüz 11-Me oleoside
		1101	1147	1120	1125	11-Me oleoside compound A isomer[1]
8	73.6	1071	1117	1090	1095 *(901, 933)*	Nüz 11-Me oleoside[b]
		1457	1503*	1476*	1481 *(933, 1287, 1319)*	Nüz. di(11-methyl oleoside)[c]
9	74.6	1457 1487	1503* 1532	1476* 1506	1481 *(933, 1287, 1319)* 1511	
10	78.0	1457 1843 *(685, 771, 1071)*	1503 1889*	1476* 1862	1481 1867 *(1319, 1672, 1704)*	Nüz. di(11-Me oleoside)[c] Nüz. tri(11-Me oleoside)[c]
11	81.8	1457 1843	1503* 1889	1476* 1862	1481 *(933, 1287, 1319)* 1867 *(1319, 1672, 1704)*	Nüz. di(11-Me oleoside)[c] Nüz. tri(11-Me oleoside)[c]

* Most intense ion detected in mass spectrum. Identification of compounds based on the following: [1]possible identification discussed in this paper; [a] retention time and DAD spectrum consistent with respective phenolic group; MS data consistent with molecular mass; MS fragmentation consistent with literature data (Servili *et al.*, 1999); [b] retention time and DAD spectrum consistent with respective phenolic group; MS data consistent with molecular mass referred to in the literature (Maestro-Durán *et al.*, 1994); [c]retention time and DAD spectrum consistent with respective phenolic group; MS data consistent with molecular mass. Nüz. Di(11-Me oleoside) in peaks 10 and 11 is provably a product of fragmentation in ion source.

at *m/z* 715, 1101 and 1487 have more 30 mass units than the corresponding group of compounds related with nüzhenide, as previously mentioned.

Nüzhenide

The ions detected in the mass spectrum in the negative mode of 54.6 min chromatographic peak (peak 2) at *m/z* 685 and 731 are shown in figure 3. The ion at m/z 731, detected in full MS spectrum, is a formic acid adduct, $[M+HCOO]^-$, of nüzhenide (molecular mass 686). The MS² spectrum obtained by fragmentation of the ion m/z 685 is also shown in the same figure: the *m/z* values obtained for the fragment ions were 299, 403, 421, 453 and 523. The fragmentation of the $[M-H]^-$ ion of nüzhenide at *m/z* 685 yields the corresponding aglycone with *m/z* 523, by neutral loss of 162 mass units. The ion at *m/z* 299 is formed by the loss of 11-methyl oleoside unit. The ions at *m/z* 421 and 453 are fragments of ion *m/z* 523. The proposed scheme for the MS² fragments referred to is presented in figure 4. The presence of the *m/z* 403 fragment may be explained by a neutral loss of 18 mass units, equivalent to a molecule of water, from ion at *m/z* 421. It is worth noting that the analysis of the same seed extract by HPLC/MS in negative mode using atmospheric pressure chemical ionisation (APCI) as the ion source resulted in the same fragmentation pattern for this compound, supporting the suggested identification (Silva, 2004).

The most intense ions in positive mode mass spectrum, for 54.6 min chromatographic peak (peak 2) were detected at *m/z* 704 and 709. The ion at *m/z* 709, detected in full MS spectrum, is a sodium adduct, $[M+Na]^+$, of nüzhenide. The MS² spectrum obtained by fragmentation of ion *m/z* 709 showed the main *m/z* values at 515 and 547. The fragmentation of $[M+Na]^+$ adduct yields the corresponding aglycone at *m/z* 547, by neutral loss of 162 mass units. The *m/z* 515 ion is formed by the loss of 32 mass units from fragment at *m/z* 547, following the same fragmentation pattern proposed for ion *m/z* 421 in negative mode (figure 4). The ion at *m/z* 704, detected in full MS spectrum, was more intense than the ion detected for the

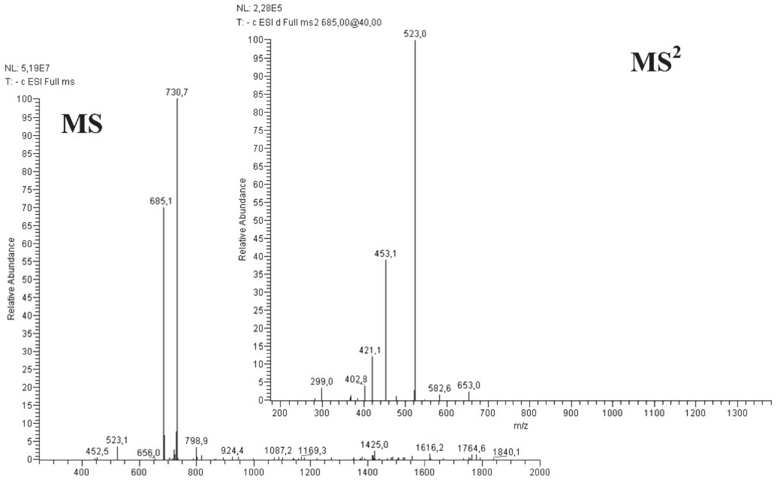

Figure 3
Mass spectra of 54.6 min TIC peak (nüzhenide) in negative mode: full MS and MS^2 of precursor ion *m/z* 685.

sodium adduct of nüzhenide (*m/z* 709), which could indicate an adduct formation with nüzhenide. Analyzing the peak purity of this chromatographic peak with the diode array data, we concluded that it had 99% purity. Besides this observation, the correspondent full MS spectra in negative mode did not show any co-elution of other compounds. Therefore, this ion corresponds to a nüzhenide adduct and it is suspected that it could be an adduct with a compound of 18 mass units, probably the ammonium adduct $[M + NH4]^+$.

Detection of the same adduct using APCI as the ionisation source, in positive mode, also confirms those observations (Silva, 2004). The presence of ammonium adducts without the presence of NH_3 or NH_4^+ in the mobile phase may be explained by a decomposition product of acetonitrile in the ion source or trace contaminant in the components of the mobile phase.

Nüzhenide (*m/z* 685) was detected at two retention times, 54.6 and 60.7 min (peaks 2 and 4, table 1), with the same fragmentation pattern which could indicate the presence of two isomers for this compound, as reported in the literature for *Ligustrum lucidum* (He *et al.*, 2001) and *Syringa reticulate* (Koichi *et al.*, 2002).

Nüzhenide 11-methyl oleoside

This compound was detected in negative and positive modes. Since it has a molecular mass of 1072 the deprotonated ion, $[M-H]^-$, in the mass spectrum in negative mode was observed at *m/z* 1071. The positive ionization mode enabled the detection of the ion *m/z* 1095, corresponding to the sodium adduct of the compound, and the ammonium adduct at *m/z* 1090. Nüzhenide 11-methyl oleoside has more 386 mass units than nüzhenide corresponding to an addition of one unit of 11-methyl oleoside to the latter molecule. The detection of this compound at four retention times could correspond to the presence of isomers.

MS^2 fragmentation of this compound was not achieved in negative mode. However, fragmentation of the corresponding sodium adduct was obtained, in positive mode, yielding the *m/z* fragments: 901 and 933. The loss of the glucose moiety results in the ion detected at *m/z* 933 and the further loss of 32 mass units (CH_3OH group) from the elenolic unit yielding the *m/z* 901 ion.

Nüzhenide di and tri(11-methyl oleosides)

An analysis of the total ion current chromatograms obtained allowed for the detection of ions with higher molecular mass than nüzhenide 11-methyl oleoside. Examples of such ions were detected in negative mode, in the deprotonated form, at *m/z* 1457 and 1843, and the corresponding ammonium adducts in positive mode at *m/z* 1476, 1861 and sodium adducts 1481 and 1866, respectively.

Analysis by MS^2 of the supposed nüzhenide di(11-methyl oleoside) was only successful in the positive mode using the sodium adduct as the parent ion (*m/z* 1481). The *m/z* fragments obtained were 933, 1287 and 1319. The loss of a glucose moiety yields the ion detected at *m/z* 1319, and the successive loss of 32 mass units gives the fragment at *m/z* 1287, as previous referred to for nüzhenide and nüzhenide 11-methyl oleoside. The ion at *m/z* 933 could be explained by the loss of 548 mass units (elenolic acid plus two glucose units).

MS^2 of the proposed nüzhenide tri(11-methyl oleoside) was achieved in positive and negative modes. The *m/z* fragments obtained in positive mode (parent ion at *m/z* 1866) followed a fragmentation pattern similar to that previously explained for the di(11-methyl oleoside) form: loss of the glucose moiety (*m/z* 1704), glucose and methanol (*m/z* 1672) and the elenolic moiety and two glucose units (*m/z* 1320). The *m/z* fragments obtained in negative mode were 685, 771 and 1071. Fragments at *m/z* 685 and 1071 correspond to ions of nüzhenide and nüzhenide 11-methyl oleoside, respectively. Fragments detected at *m/z* 771 could be obtained from *m/z* 1071 by the loss of 300 mass units (tyrosol and elenolic acid moiety).

Compounds with molecular mass 716, 1102 and 1488

A compound with molecular mass 716 (compound A) was detected in chromatographic peaks 1 and 3. The detection in negative mode of the parent ion at *m/z* 715, in peak 3, gave the following fragments by means of MS^2: 329, 385, 451, 483 and 553. This fragmentation pattern is similar to the scheme proposed for nüzhenide, in negative mode (with a difference of 30 mass units) for fragments 329, 451 and 553. Fragment 385 was also detected in MS^2 spectra for peaks 1 and 3 and corresponds to the detection of the 11-methyl oleoside unit. Fragments 451 and 553 were also detected in the MS^2 spectrum of peak 1.

The fragmentation pattern obtained for peak 3 suggests that the 30 mass units, higher than *m/z* for nüzhenide, included in the tyrosol glucoside unit but available data are not sufficient to ascertain the structure of this compound. We shall assume that a glucose unit is present in this compound and therefore the 30 mass unit group is located at the tyrosol unit.

Possible compounds with 168 molecular mass could be methoxytyrosol or 3,4-dihydroxyphenylacetic acid. As the retention time of these unknown compounds is shorter than those of the nüzhenide isomers it is possible to suggest that this compound may contain a dihydroxyphenylacetic unit. Further experimental work could support this hipothesis.

Compounds with molecular mass 1102 and 1488 were also detected in samples and could correspond to mono and di(11-methyl oleosides) of the compound with molecular mass 716, since they have a difference of 386 mass units .

Compound with molecular mass 772

This compound, named in table 1 as compound B, was detected co-eluting with other seed components in chromatographic peak 5. MS^2 from parent ion, in negative mode, at *m/z* 771 yielded the following *m/z* fragments: 223, 315, 385, 403, 547, 609, 669, 701 and 739. The loss of a glucose moiety results in fragment at *m/z* 609. While the loss of the 11-methyl oleoside unit yields *m/z* 385, the further loss of 70 mass units yield ion at *m/z* 315. Fragments at *m/z* 223 and 403 correspond to ions of elenolic acid, after a loss of one molecule of water, and 11-methyl oleoside respectively. Ion 547 is formed after a loss of 224 mass units. These fragments together with ions detected at *m/z* 701 and 739 are consistent with a di(11-methyl oleoside) form, suggesting that this compound does not have a phenolic moiety. Each 11-methyl oleoside unit has 404 mass units, therefore a di(11-methyl oleoside), has 790 mass units and should be detected in negative mode as a deprotonated ion at *m/z* 789. However, that compound was detected as an ion at *m/z* 771 (negative mode) which could correspond to the di(11-methyl oleoside) after a loss of water (18 mass units) in the ion source.

3.2. Quantification of secoiridoids in olive seed by HPLC

Nüzhenide was the most prominent compound detected in seed extracts of the cultivars studied when chromatographic profiles were compared at 280 nm, figure 5. According to Servili *et al.* (1999) nüzhenide is located mainly in olive seed and is the main phenolic compound in this part of the fruit, which is also in accordance with other results obtained concerning the analysis of phenolic compounds in fruits and seeds of portuguese olive tree cultivars by HPLC/DAD/MS using APCI as the ionization source (Silva, 2004).

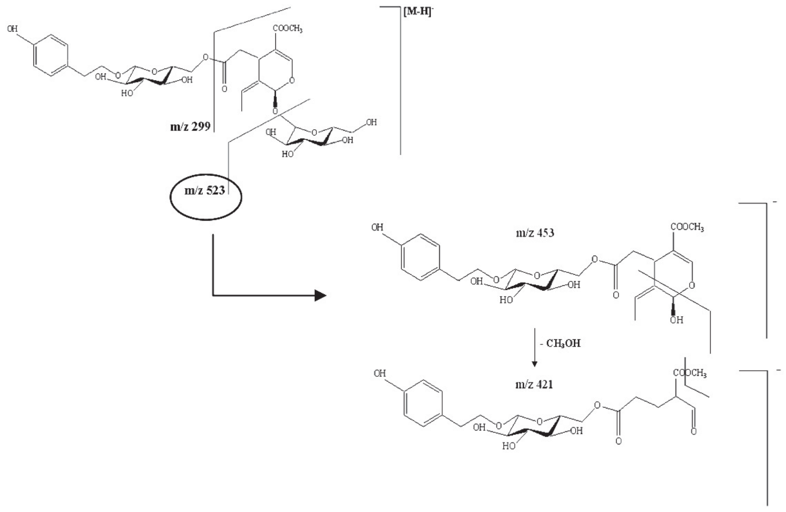

Figure 4
Fragmentation scheme for ions obtained by MS^2 of precursor ion *m/z* 685.

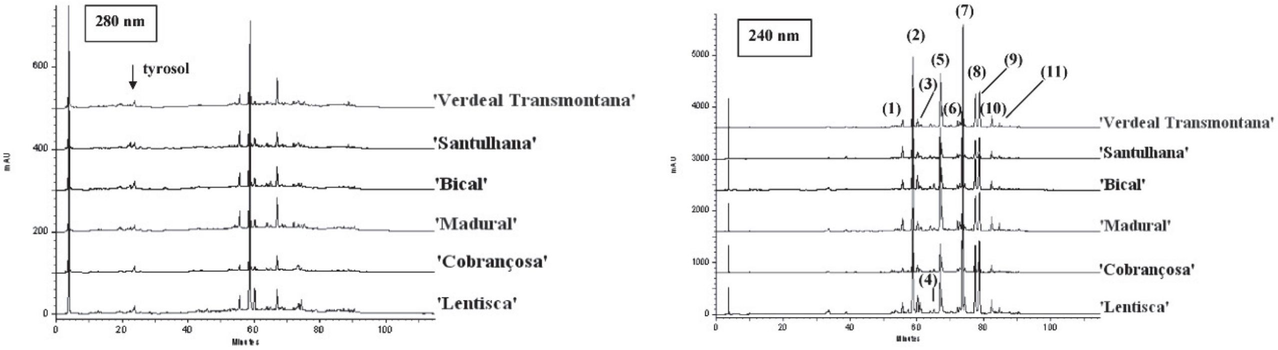

Figure 5
Chromatograms of seed extracts at 240 nm and 280 nm; the numbers in figure correspond to the peaks identified in table 1.

The most adequate wavelength for detection in HPLC is dependent not only on the class of phenolic compound but also on the particular group of such classes. The 280 nm wavelength is used as a compromise for the detection of phenolic compounds, since it shows considerably more detail than detection at other wavelengths in the range of 250 to 360 nm (Ryan *et al.*, 1999). Since secoiridoid derivatives have characteristic absorbance at 240 nm (Cardoso *et al.*, 2005), chromatographic profiles of the seed extracts were compared at 240 nm and 280 nm, figure 5. The chromatograms obtained differed mostly in the relative abundance of the various peak-forming compounds detected at 240 nm. Nüzhenide is not the highest peak, since other compounds are present in several chromatograms such as peak 7, figure 5, identified with mass spectrometry as nüzhenide 11-methyl oleoside. The results achieved combined with the observation of molecular absorption spectra (200-600 nm range) enabled us to conclude that the phenolic composition of the olive seed was mainly secoiridoid type with the exception of tyrosol marked in figure 5 (chromatogram at 280 nm) as a small peak at 22 min retention time. The identification of tyrosol was done by comparison with the respective standard, and the detection of this compound in olive seed is in accordance with published data (Maestro-Durán *et al.*, 1994; Servili *et al.*, 1999).

Additional work indicated that seed extracts had high total antioxidant activities and low total phenolic contents (Silva, 2004). These observations, together with the identifications achieved in this work, lead to the quantification of those compounds in the samples of different cultivars. The quantification of secoiridoids detected in olive seed extracts of the cultivars studied, at 240 nm, was done using a linear calibration curve obtained using standard solutions of oleuropein.

The calibration curve, concerning peak area, yielded the following equation: $y = 1.29x\ 10^5 x - 5.69x10^6\ (r^2 = 0.991)$. The estimated results (expressed as oleuropein) obtained for samples of different cultivars are shown in figure 6. For the cultivars studied, nüzhenide and nüzhenide 11-methyl oleoside were the most concentrated compounds present in the respective seeds. 'Lentisca' cultivar presented values, expressed in fresh matter, of 12,2 g/kg of nüzhenide and 16,1 g/kg of nüzhenide 11-methyl oleoside, as presented in figure 6.

Isomers of nüzhenide (peaks 2 and 4) and respective mono 11-methyl oleoside (peak 7) and di(11-methyl oleoside) (peak 9) account for more than 65% of the total peak areas in the chromatograms.

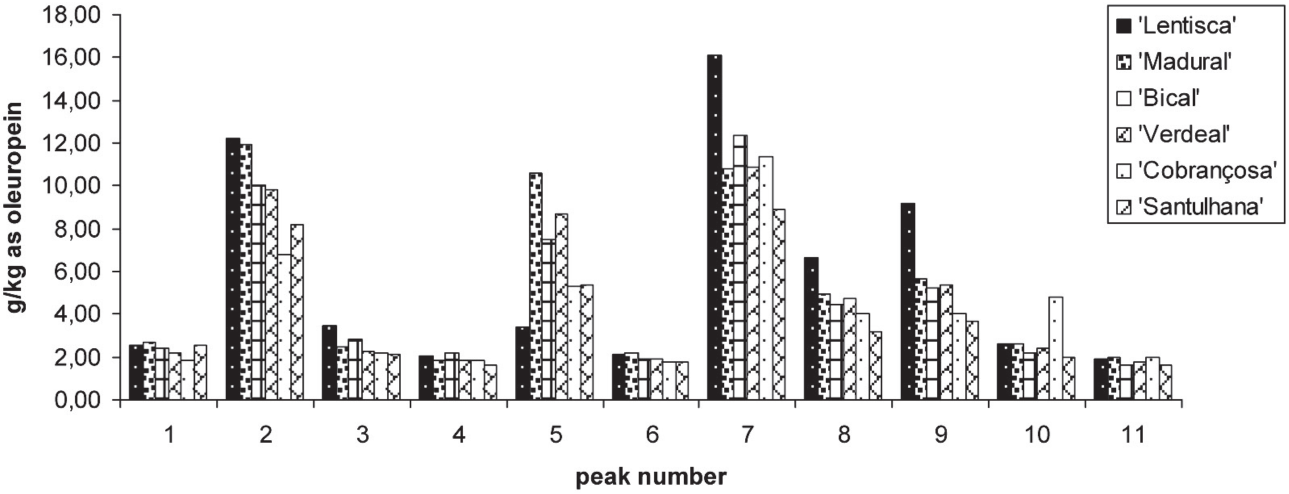

Figure 6
Quantification of detected secoiridoids, as oleuropein, at 240 nm in the olive seeds of six cultivars.

Taking into account the estimated concentrations present in figure 6, there is a strong correlation *($r > 0.9$)* between nüzhenide 11-methyl oleoside (peak 7) with nüzhenide di(11-methyl oleosides) in peaks 8 and 9. The compound with molecular mass 716 (peak 3) is also strongly correlated with peaks 7, 8 and 9.

Servili *et al.* (1999) reported that there was no evidence of variation of nüzhenide with the maturation stage of fruits; therefore, the differences observed for the samples included in this study may be due to the cultivar or to the influence of climatic conditions.

4. CONCLUSIONS

The described data contribute for a better understanding of the phenolic composition in olive seeds. The results achieved by HPLC confirmed that the phenolic composition of the olive seed was mainly the secoiridoid type: nüzhenide and nüzhenide 11-methyl oleoside are the main phenolic compounds in olive seed. Nüzhenide was characterized by HPLC with mass spectrometry by means of MS^2 using electrospray ionization. In addition, there is an indication of the presence of secoiridoid compounds with higher molecular mass than nüzhenide 11-methyl oleoside suggesting the presence of nüzhenide di and tri(11-methyl oleosides) in seeds. Another family of secoiridoids characterized by molecular mass 716 and corresponding 11-methyl oleosides was found in samples but further work will be necessary in order to elucidate the structures of those compounds.

ACKNOWLEDGEMENTS

The authors thank Manuel dos Santos (Escola Superior Agrária de Bragança) for the collection of fruits used in this work.

REFERENCES

Calis I, Hosny M, Lahloub MF. 1996. A secoiridoid glucoside from Fraxinus Angustifolia. *Phytochemistry* **41**, 1557-1562.

Cardoso SM, Guyot S, Marnet N, Lopes-da-Silva J A, Renard CMGC, Coimbra MA. 2005. Characterization of phenolic extracts from olive pulp and olive pomace by electrospray mass spectrometry. *J Sci. of Food Agric.* **85**, 21-32.

Chien CT, Ling-Long KH, Shen YC, Zhang R, Chen SY, Yang JC, Pharis RP. 2004. Storage behaviour of *Chionanthus retusus* seed and asynchronous development of the radicle and shoot apex during germination in relation to germination inhibitors, including abscisic acis and four phenolic glucosides. *Plant Cell Physiol.* **45**, 1158-1167.

Jensen SR, Franzyk H, Wallander E. 2002. Chemotaxonomy of the Oleaceae: iridoids as taxonomic markers. *Phytochemistry* **60**, 213-231.

Maestro-Durán R, Cabello LR, Gutiérrez RV and Roncero VA. 1994. Glucósidos fenólicos amargos de las semillas del olivo (Olea europaea). *Grasas y Aceites* **45**, 332-335.

He ZD, Dong H, Xu HX, Ye WC, Sun HD, But PPH. 2001. Secoiridoid constituents from the fruits of *Ligustrum lucidum*. *Phytochemistry* **56**, 327-330.

Koichi M, Kaneko A, Hosogai T, Kakuda R, Yaoita Y, Kikuchi M. 2002. Studies on the constituents of *Syringa* species. X. Five new iridoid glycosides from the leaves of *Syringa reticulate* (Blume) Hara. *Chem. Pharm. Bull.* **50**, 493-497.

Robards K, Prenzler PD, Tucke G, Swatsitang P, Glover W. 1999. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* **66**, 401-436.

Ryan D, Robards K and Lavee S. 1999. Determination of phenolic compounds in olives by reversed-phase chromatography and mass spectrometry. *J. Chromatogr.* A **832**, 87-96.

Ryan D, Antolovich M, Prenzler P, Robards K and Lavee S. 2002. Biotransformations of phenolic compounds in *Olea europaea* L. *Sci. Hortic.* **92**, 147-176.

Servili M, Baldioli M, Selvagini R, Macchioni A and Montedoro G. 1999. Phenolic compounds of olive fruit: one- and two-dimensional nuclear magnetic resonance characterization of nüzhenide and its distribution in the constitutive parts of fruit. *J. Agric. Food Chem.* **47**, 12-18.

Servili M and Montedoro G. 2002. Contribution of phenolic compounds in virgin olive oil quality. *European Journal of Lipid Science and Technology* **104**, 602-613.

Silva S. 2004. *Phenolic compounds in samples of olive tree cultivars* (*Olea europaea L.).* M.Sc. Thesis, Faculty of Pharmacy, Lisbon University, Lisbon, Portugal.

Recibido: 3/8/09
Aceptado: 6/10/09