

# HPLC Analysis of Flavonoids and Secoiridoids in Leaves of *Ligustrum vulgare* L. (Oleaceae)

Annalisa Romani,<sup>†</sup> Patrizia Pinelli,<sup>†</sup> Nadia Mulinacci,<sup>†</sup> Franco F. Vincieri,<sup>†</sup>  
Elisabetta Gravano,<sup>‡</sup> and Massimiliano Tattini<sup>\*‡</sup>

Dipartimento di Scienze Farmaceutiche, Università di Firenze, Via Gino Capponi 9, I-50100 Florence, Italy,  
and Istituto sulla Propagazione delle Species Legnose, Consiglio Nazionale delle Ricerche, Via Ponte di  
Formicola 76, I-50018 Scandicci, Florence, Italy

Identification and quantification of flavonol glycosides and secoiridoids was carried out on leaves of *Ligustrum vulgare* L. (Oleaceae) by means of HPLC-DAD and HPLC-MS analysis. In addition to previously reported secoiridoids (oleuropein, ligustalosite A, ligustalosite B, and ligstroside) four kaempferol glycosides (kaempferol 3-*O*-glucoside 7-*O*-rhamnoside, kaempferol 3,7-*O*-dirhamnoside, kaempferol 3-*O*-rhamnoside, and kaempferol 3-*O*-glucoside) and two quercetin glycosides (quercetin 3-*O*-glucoside 7-*O*-rhamnoside and quercetin 3,7-*O*-dirhamnoside) were present in leaves of *L. vulgare* L. Although secoiridoids accounted for nearly the 76% of the total leaf polyphenols content (with ligustalosite A as the main component), kaempferol glycosides were also accumulated in the leaves of *L. vulgare* L. to a relatively high extent (23%). Contribution of quercetin derivatives was minor under our experimental conditions. Our findings suggest that flavonol glycosides may have a central role in both the ecology and the biology of *L. vulgare* L.

**Keywords:** HPLC-DAD; HPLC-MS; kaempferol glycosides; privet; quercetin glycosides; oleuropein; flavonoids

## INTRODUCTION

*Ligustrum vulgare* L., a member of the Oleaceae family distributed worldwide, largely contributes to the vegetational cover of the Mediterranean basin and consequently plays a fundamental role in the maintenance of fragile areas that are under the incipient risk of desertification (Bailey, 1963; Brosse, 1979). Extracts from different plant parts (leaves, fruits, and flowers) of *Ligustrum* spp. have been in the past used in popular medicine, and the use of the species as an ornamental plant or cut foliage has increased during recent years (Lieutaghi, 1975; Brosse, 1979).

Phytochemical characterization of the members of the Oleaceae family, including *Ligustrum* spp., has been mainly based upon the relative distribution of secoiridoids and secoiridoid glycosides in different plant parts (Inoue and Nishioka, 1972; Inoue et al., 1982; Damtoft et al., 1993; Romani et al., 1996; Romani et al., 1999a), although quantification of individual metabolites has not yet been reported. In addition to their role in ecological biochemistry (Harborne, 1993), secoiridoids have been shown to determine most of the well-known pharmacological activities of tissue extracts from Oleaceae spp., in view of their antioxidant properties (Driss et al., 1996; Romani et al., 1996; Visioli et al., 1998; Visioli et al., 1999).

Phytochemical characterization of different taxa has traditionally been based on end products of specific branch pathways of the general phenylpropanoid metabolism, especially flavonoid compounds (Williams et

al., 1991; Williams and Harborne, 1993). The role of flavonoids in both plant biology and ecology has been revisited during the recent past since flavonoid biosynthesis is strongly regulated by several environmental stimuli (Halbrock and Scheel, 1989; Dixon and Paiva, 1995; Romani et al., 1996; Romani et al., 1999b). Flavonoids play a key role in countering the detrimental effects of excess of light (as they strongly absorb in the UV region of the solar spectrum) and those deriving from pathogen attacks or woundings (Caldwell et al., 1983; Halbrock and Scheel, 1989; Dixon and Paiva, 1995). Furthermore flavonols, such as glycosides of quercetin and kaempferol, may scavenge reactive oxygen-containing species that are produced under severe stress conditions, thus protecting plant cell metabolism from oxidative damages (Husain et al., 1987; Takahama, 1988; Yamasaki et al., 1997). More recently, flavonol glycosides (including both kaempferol and quercetin glycosides) have been also shown to protect animal cell metabolism from oxidative damages and consequently may have an important role in human health (Ioku et al., 1998; Morand et al., 1998; Noroozi et al., 1998; Wang et al., 1998).

In a preliminary experiment (Pinelli et al., 1999) two kaempferol diglycosides were identified in *L. vulgare* L. leaves, which were not previously detected in corresponding tissues of other members of the Oleaceae family (Heimler et al., 1992; Romani et al., 1996). Furthermore it has been shown that infusions and teas from *L. vulgare* L. leaves have high antioxidant activities, as estimated by the 2,2'-diphenyl-1-picryl-hydrazyl (DPPH) method (Pinelli et al., 1999). Therefore, we decided to carry out an experiment aimed at identifying and quantifying the full spectrum of leaf polyphenols in *L. vulgare* L., and in this paper experimental data

\* To whom correspondence should be addressed. Phone: +39055 750340. Fax: +39055 755121. E-mail: tattini@ipsl.fi.cnr.it.

<sup>†</sup> Università di Firenze.

<sup>‡</sup> Consiglio Nazionale delle Ricerche.

from HPLC-DAD and HPLC-MS analysis of secoiridoids and flavonoids are reported. To our knowledge this is the first report on this topic.

## EXPERIMENTAL PROCEDURES

**Plant Material and Growing Conditions.** *Ligustrum vulgare* L. plants, selected for both leaf shape and shoot length, were grown outdoors in Florence from April to June. Plants grown in 3 L pots were supplied with Hoagland solution three times a week. The integrated photon flux density (PFD) averaged  $24.2 \mu\text{mol m}^{-2} \text{d}^{-1}$ , and the photosynthetic active radiation (PAR) at midday averaged  $800 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Plants consisted of two or three 35–40 cm long shoots and leaf area averaged  $4.5 \text{ cm}^2$ . Leaves were harvested from the medial part of each shoot from three plants per replicate on June 15th. There were three replicates for a total of nine plants. The main vein was excised from the leaf lamina, and the tissue was rapidly quenched in liquid nitrogen and stored at  $-80^\circ\text{C}$  until the start of the analysis.

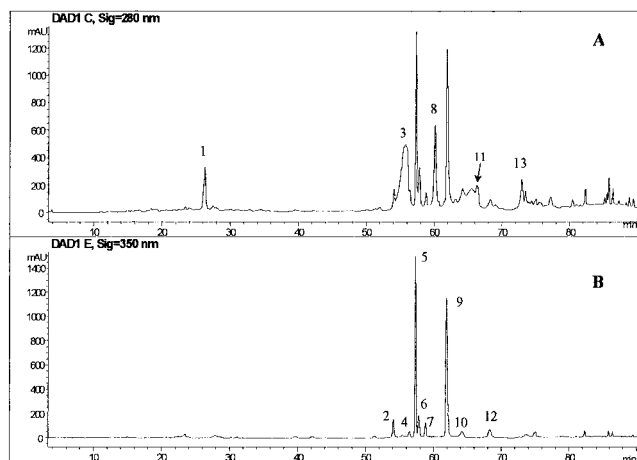
**Extraction and Purification Procedures.** Lyophilized leaf tissue (1–2 g) was extracted with  $4 \times 80 \text{ mL}$  of 70% EtOH adjusted to pH 2.5 by formic acid at room temperature similarly to the protocol of Romani et al. (1999b). In brief, the raw ethanolic extract was concentrated under vacuum (Rotavapor 144R, Büchi, Flawil, Switzerland), diluted with ultrapure water to 100 mL, and then further extracted with  $4 \times 50 \text{ mL}$  of *n*-hexane to remove lipophilic compounds. A 10 mL volume of the defatted ethanolic extract was dried under reduced pressure, diluted with 1 mL of water/methanol/acetonitrile (20:60:20 v:v:v), and used for HPLC-DAD analysis. The ethanolic extract was further purified using a liquid–solid extraction (LSE) procedure. The ethanolic solution (90 mL) was dried under vacuum, diluted to 15 mL with aqueous formic acid (pH 2), and applied to a 20 mL Extrelut cartridge (Merck, Darmstadt, Germany). Elution was carried out with (i) 200 mL of *n*-hexane, (ii) 250 mL of EtOAc, and (iii) 250 mL of pH 2 (by formic acid) MeOH as previously reported (Romani et al., 1999b). The methanol and ethyl acetate eluates were mixed and then evaporated to dryness, diluted with 1 mL of water/methanol/acetonitrile (20:60:20 v:v:v), and adjusted to pH 2 with formic acid. The samples were analyzed by HPLC-DAD and HPLC-MS.

**Identification and Quantification of Individual Polyphenols.** Identity of individual polyphenols was carried out using their retention times and combined with spectroscopic and spectrophotometric data. UV–vis data of the separated compounds were recorded by a diode array detector (DAD) coupled to an HPLC system. Plant extracted components were compared with synthetic or natural product standards. The pure standards (oleuropein, kaempferol 3-*O*-glucoside, quercetin 3-*O*-rhamnoside) were purchased from Extrasynthese SA (Lyon, Nord-Genay, France). The isolated compounds (kaempferol 3-*O*-glucoside 7-*O*-rhamnoside; kaempferol 3,7-*O*-dirhamnoside; quercetin 3-*O*-glucoside 7-*O*-rhamnoside) were extracted and purified from *Sedum telephium* L. leaves as previously reported (Mulinacci et al., 1995).

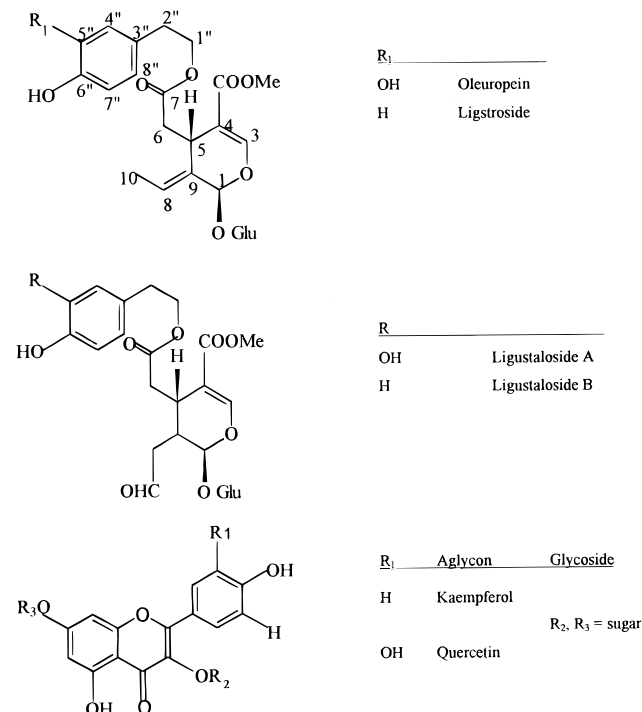
In most cases, confirmation of individual polyphenols was based on positive- and negative-ion API electrospray LC-MS data.

Individual polyphenols were quantified by a four-point regression curve ( $r^2 \geq 0.99$ ) on the basis of authentic standards. Flavonoids were calibrated at 350 nm using kaempferol 3-*O*-glucoside and quercetin 3-*O*-rhamnoside as reference compounds for kaempferol and quercetin derivatives, respectively. Secoiridoids were calibrated and quantified at 280 nm using oleuropein as the reference. The weight content of flavonols and secoiridoids in leaves of *L. vulgare* L. was calculated after correcting for the specific molecular weights.

**Analytical Techniques and Equipment.** HPLC-DAD analysis was performed on an HP 1090L Series II liquid chromatograph equipped with a diode array detector (HP 1040) and managed by an HP 9000 workstation (all from Hewlett-Packard, Palo Alto, CA).



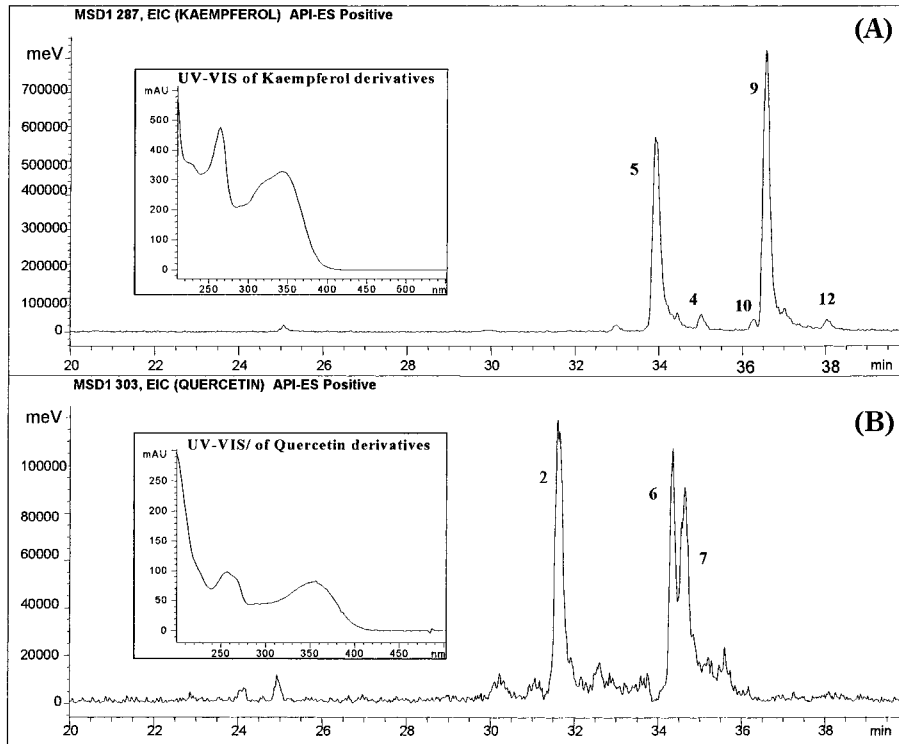
**Figure 1.** Chromatographic profile of EtOH extract of *L. vulgare* L. leaves acquired by HPLC-DAD at absorption maxima of secoiridoids (280 nm, A) and flavonols (350 nm, B), respectively. For HPLC run conditions, see Experimental Procedures.



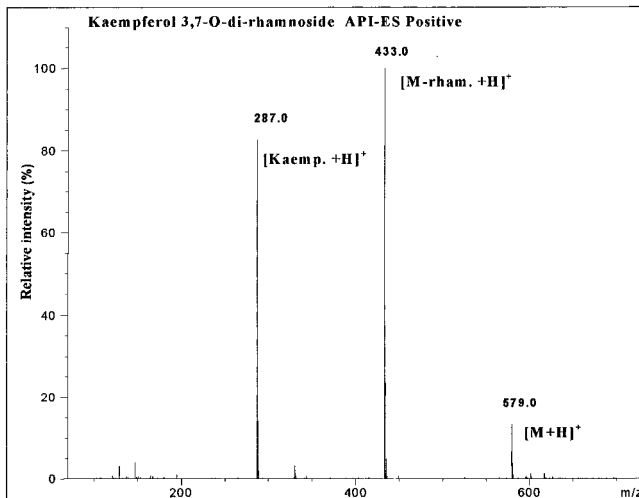
**Figure 2.** Chemical structures of secoiridoids, kaempferol, and quercetin derivatives, detected in leaves of *L. vulgare* L.

The column was a  $4.6 \times 250 \text{ mm}$  LiChrosorb RP<sub>18</sub> ( $5 \mu\text{m}$ ) (Merck) maintained at  $26^\circ\text{C}$  and equipped with a  $4 \times 10 \text{ mm}$  LiChrosorb RP<sub>18</sub> precolumn. The eluent was H<sub>2</sub>O (adjusted to pH 3.2 by H<sub>3</sub>PO<sub>4</sub>)/CH<sub>3</sub>CN. A seven-step linear gradient solvent system was used, starting from 100% H<sub>2</sub>O to 100% CH<sub>3</sub>CN, during a 106 min period, at a flow rate of  $1.0 \text{ mL min}^{-1}$  (Romani et al., 1996). UV–vis spectra were recorded in the range 190–450 nm, and chromatograms were acquired at 254, 280, 310, 330, and 350 nm.

HPLC-MS analysis was performed by using a 67 min elution program as previously described for the analysis of polyphenols in leaves of *Myrtus communis* L. (Romani et al., 1999b). In this case, the HPLC-DAD was interfaced with an HP 1100 MSD API-electrospray (Hewlett-Packard, Palo Alto, CA) operating in both negative and positive ionization mode at the following conditions: gas temperature  $350^\circ\text{C}$ ; nitrogen flow rate  $10.0 \text{ L min}^{-1}$ ; nebulizer pressure 40 psi; quadrupole temperature  $30^\circ\text{C}$ ; capillary voltage 3500 V. The mass spectrometer operated at 80–180 eV.



**Figure 3.** Extracted ion current chromatograms of the fractions from LSE acquired from HPLC-MS operating in positive ionization mode at 120 eV; (A) chromatogram recorded at  $m/z$  of kaempferol aglycone fragment ( $[M + H]^+ = 287$ ); (B) chromatogram recorded at  $m/z$  of quercetin aglycone fragment ( $[M + H]^+ = 303$ ). UV-vis spectra of both kaempferol and quercetin glycosides are also reported.

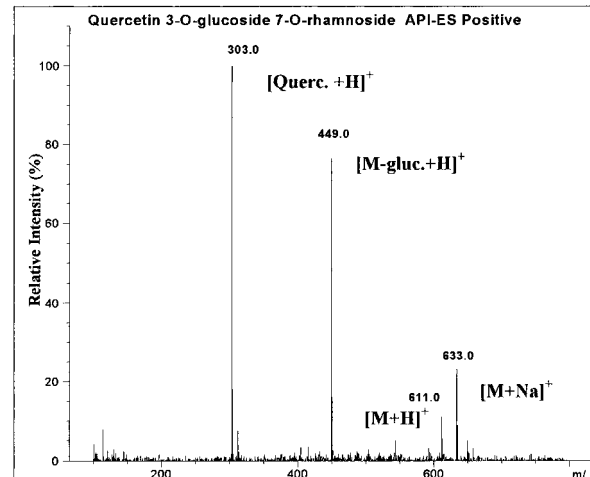


**Figure 4.** Positive ion mass spectrum of kaempferol 3,7-*O*-dirhamnoside acquired during the API-electrospray HPLC-MS analysis at the following operating conditions: gas temperature 350 °C; nitrogen flow rate 10.0 L min<sup>-1</sup>; nebulizer pressure 40 psi; quadrupole temperature 30 °C; capillary voltage 3500 V. Mass spectra were recorded in the range 0–700 amu.

## RESULT AND DISCUSSION

**Identification and Quantitation of Individual Polyphenols.** Generally, polyphenols were identified by HPLC-DAD and HPLC-MS analysis, based on retention times and UV-vis spectral and mass spectral matches.

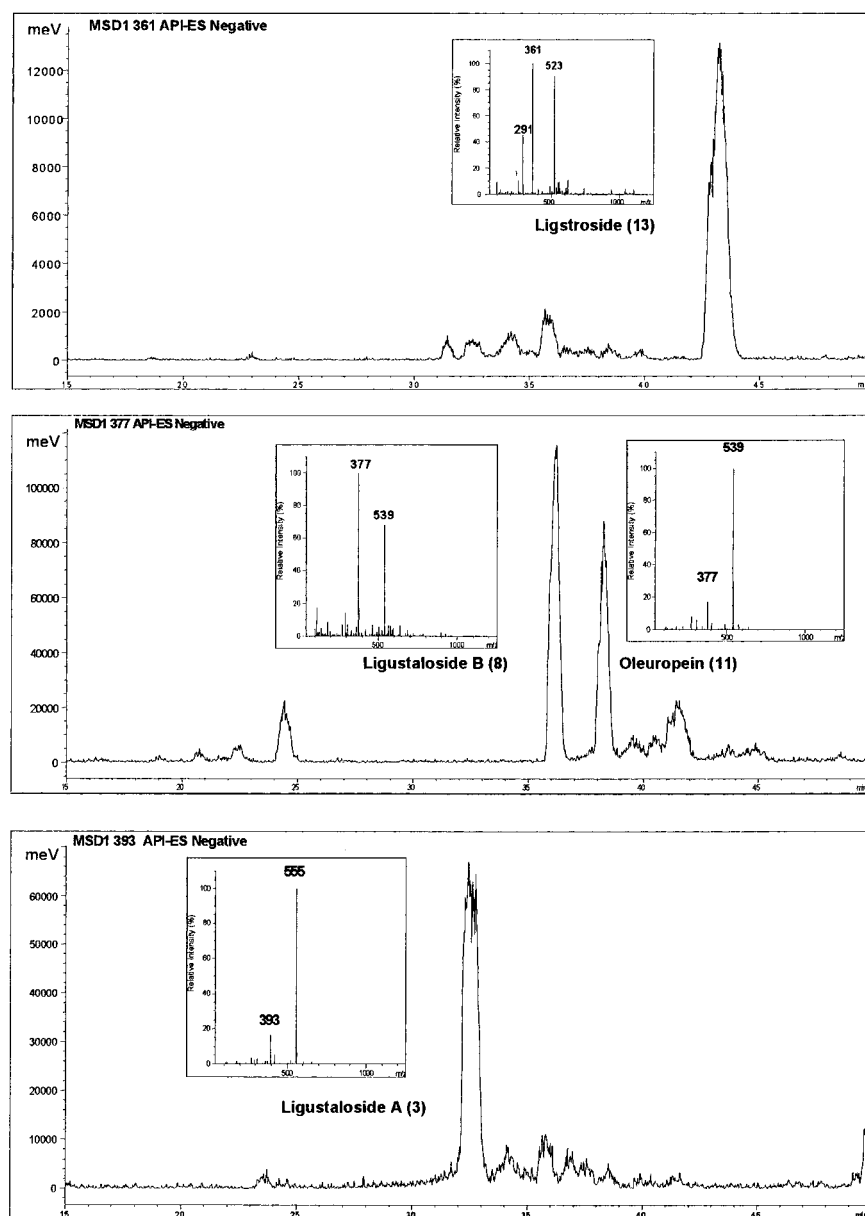
HPLC separations of EtOH-extracted components from *Ligustrum vulgare* L. were detected by UV at two different wavelengths. Differences in chromatographic profiles indicate the presence of components with dif-



**Figure 5.** Positive ion mass spectrum of quercetin 3-*O*-glucoside 7-*O*-rhamnoside acquired during the API-electrospray HPLC-MS analysis. Operating conditions are as in Figure 4.

ferent UV absorption maximum (Figure 1). Chromatograms were recorded at the relative absorption maxima of secoiridoids (280 nm) and flavonols (350 nm). Identified components are shown in Figure 2.

The chromatographic profile reported in Figure 1B revealed the occurrence of trace amounts of several flavonol derivatives together with two main components, thus making the identification and quantification of trace amounts of individual polyphenols difficult. Therefore the relative concentrations of minor flavonols in leaves of *L. vulgare* were increased by using the above-mentioned LSE procedure and the complete characterization of both secoiridoids and flavonols was performed by both HPLC-DAD and HPLC-MS analysis, with the



**Figure 6.** Extracted ion current chromatograms of the fractions from LSE acquired from HPLC-MS operating in negative ionization mode at 120 eV. Chromatograms were recorded at  $m/z$  361, 377, and 393 corresponding to  $m/z$  of aglycone of ligstroside (A), ligustaloside B and oleuropein (B), and ligustaloside A (C), respectively. Negative ion mass spectra were acquired by API-electrospray HPLC-MS operating as reported in Figure 4.

latter operating in positive and negative ion mode with modulated fragmentation energy.

Extracted ion chromatograms of the MeOH-EtOAc eluates from LSE, recorded in positive ion mode at  $m/z$  of kaempferol aglycone ( $[M + H]^+ = 287$ ) and  $m/z$  of quercetin aglycone ( $[M + H]^+ = 303$ ), are reported in Figure 3. The chromatographic profiles and the UV-vis spectra revealed the presence of five kaempferol and three quercetin derivatives in leaf mesophyll of *L. vulgare* L. (list given in Table 1).

Identification of both kaempferol and quercetin derivatives was carried out by comparison of mass spectra of separated compounds with those of pure standards isolated from *Sedum telephium* L. leaves (Mulinacci et al., 1995). As an example we report in Figure 4 the mass spectrum of kaempferol 3,7-*O*-dirhamnoside, the flavonol detected in the highest concentration in *L. vulgare* L. leaves. Three fragment ions were recorded, at  $m/z$  579, 433, and 287, corresponding to the quasi-molecular

ion  $[M + H]^+$  and those corresponding to the loss of rhamnose  $[M - 146]^+$  and kaempferol aglycone  $[M - 292]^+$ , respectively. Analogously we report in Figure 5 the mass spectrum of the most abundant quercetin glycoside detected in *L. vulgare* L. leaves, namely quercetin 7-*O*-glucoside 3-*O*-rhamnoside. The signals at  $m/z$  633, 611, 449, 303 corresponded to the adduct with sodium  $[M + Na]^+$ , to the quasi-molecular ion  $[M + H]^+$ , to the fragment after the loss of glucose  $[M - 162]^+$ , and that after the loss of rhamnose  $[M - 308]^+$ , respectively.

Secoiridoids revealed by HPLC-DAD analysis were also identified (list given in Table 1) by using extracted-ion profiles from HPLC-MS and by relative mass spectra. We extracted relative from total ion current at  $m/z$  361, 377, and 393 (Figure 6) corresponding to the  $m/z$  of aglycones of (i) ligstroside (361), (ii) ligustaloside B and oleuropein (377), and (iii) ligustaloside A (393). Mass spectra of individual compounds were also ac-

**Table 1. Relative Amounts of Polyphenols Detected in Leaves of *Ligustrum vulgare* L. Collected during June<sup>a</sup>**

polyphenol	mg g <sup>-1</sup> of dry weight
kaempferol 3- <i>O</i> -rhamnoside (12)	0.30 ± 0.03
kaempferol 3- <i>O</i> -glucoside (4)	0.10 ± 0.02
kaempferol 3- <i>O</i> -glucoside 7- <i>O</i> -rhamnoside (5)	5.50 ± 0.49
kaempferol 3,7-dirhamnoside (9)	11.97 ± 0.67
kaempferol glycoside (10)	0.09 ± 0.01
quercetin 3- <i>O</i> -glucoside 7- <i>O</i> -rhamnoside (2)	0.35 ± 0.08
quercetin 3,7-dirhamnoside (6)	0.32 ± 0.08
quercetin glycoside (7)	0.17 ± 0.07
ligstroside (13)	4.08 ± 0.54
oleuropein (11)	4.34 ± 0.32
ligustalosite B (8)	6.60 ± 1.01
ligustalosite A (3)	40.64 ± 2.87
secoiridoid derivative (1)	2.92 ± 0.32

<sup>a</sup> Quantitation was performed for each polyphenol using a 4-point regression curve ( $r^2 \geq 0.99$ ) through the use of authentic standards as described under Experimental Procedures. The unidentified secoiridoid, kaempferol, and quercetin derivatives were calibrated at 280 or 350 nm using oleuropein, kaempferol 3-*O*-glucoside, and quercetin 3-*O*-rhamnoside as reference compounds, respectively. <sup>b</sup> Means ± SD of three replicates.

quired in the negative ion mode (Figure 6) and were consistent with the presence of above-mentioned secoiridoids which have been previously detected in other *Ligustrum* spp. as well as in other members of the Oleaceae family (Inoue et al., 1982; Willems, 1988; Damtoft et al., 1993; Romani et al., 1996; Romani et al., 1999a; Pinelli et al., 1999).

Identification of three minor polyphenols, a secoiridoid derivative, a kaempferol glucoside, and a quercetin glucoside, detected in leaves of *L. vulgare*, has yet to be made.

**Polyphenol Composition of *L. vulgare* Leaves.** Leaf polyphenols of *L. vulgare* L. strongly discriminate this member from other members of the Oleaceae family. Kaempferol glycosides have not previously been detected in leaf tissue of either *Olea europaea* or *Phillyrea* spp. (Baldi et al., 1995; Romani et al., 1996), whereas they represent 96% of the total flavonoids in *L. vulgare* L. leaves. In contrast, flavones, such as apigenin and luteolin glycosides, the main leaf flavonoids detected in other Oleaceae spp., did not occur in *L. vulgare* L. leaves (Heimler et al., 1992; Baldi et al., 1995; Tattini et al., in press). Quercetin derivatives have previously been reported to occur in leaves of both olive and phillyrea, but diglycosides of quercetin only occur in *L. vulgare* L. leaves (Heimler et al., 1992; Baldi et al., 1995; Romani et al., 1996). Secoiridoids also strongly differentiate *L. vulgare* L. from the other members of the Oleaceae family, except for oleuropein that has been ubiquitously found in all the members of the Oleaceae family (Damtoft et al., 1993; Baldi et al., 1995; Romani et al., 1996).

In addition to the well-known antioxidant properties of secoiridoids, both kaempferol and quercetin glycosides have also been shown to protect animal cell metabolism from oxidative damage and also act as antitumor and anti-HIV agents (Ioku et al., 1998; Morand et al., 1998; Noroozi et al., 1998; Wang et al., 1998). Therefore in addition to its well-known ecological function (Bailey, 1963; Brosse, 1979), *L. vulgare* L. may also have an interesting role in human health care.

#### LITERATURE CITED

Bailey, L. H. *The standard cyclopedia of horticulture*. The MacMillan Co.: New York, 1963.

- Baldi, A.; Romani, A.; Tatti, S.; Mulinacci, N.; Vincieri, F. F. HPLC analysis of polyphenolic compounds present in leaves of *Olea europaea* L. (cv. Leccino). In *Polyphenols 94*; Rouillard, R., Jay, M., Scalbert, A., Eds.; INRA Editions: Paris, 1995.
- Brosse, J. Atlas of shrubs and lianes; Bordas Editions: Paris, 1979.
- Caldwell, M. M.; Robberecht, R.; Flint, S. D. Internal filters: prospect for UV-acclimation in higher plants. *Physiol. Plant.* **1983**, *58*, 445–450.
- Damtoft, S.; Franzzyk, H.; Jonsen, S. R. Biosynthesis of secoiridoid glycosides in *Oleaceae*. *Phytochemistry* **1993**, *34*, 1291–1299.
- Dixon, R.; Paiva, N. L. Stress-induced phenylpropanoid metabolism. *Plant Cell* **1995**, *7*, 1085–1097.
- Driss, F.; Duranthon, V.; Viard, V. Biological effects of polyphenolic compounds of olive. *Rev. Fr. Corps Gras* **1996**, *3*, 448–451.
- Halbrock, K.; Scheel, D. Physiology and molecular biology of phenylpropanoid metabolism. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1989**, *40*, 347–369.
- Harborne, J. *Introduction to ecological biochemistry*. Academic Press: London, 1993.
- Heimler, D.; Pieroni, A.; Tattini, M.; Cimato, A. Determination of flavonoids, flavonoid glycosides and biflavonoids in *Olea europaea* L. leaves. *Chromatographia* **1992**, *33*, 369–373.
- Husain, S. R.; Cillard, P.; Cillard, P. Hydroxyl radical scavenging activity of flavonoids. *Phytochemistry* **1987**, *26*, 2489–2491.
- Inouye, H.; Nishioka, T. Biosynthesis of iridoid and secoiridoid glucosides in *Ligustrum lucidum* and *Ligustrum japonicum*. XIX. *Tetrahedron* **1972**, *28*, 4231–4237.
- Inoue, K.; Nishioka, T.; Tanahashi, T.; Inouye, H. Three secoiridoid glucosides from *Ligustrum japonicum*. *Phytochemistry* **1982**, *21*, 2305–2311.
- Ioku, K.; Pongpiriyadacha, Y.; Konishi, Y.; Takei, Y.; Nakatani, N.; Terao, J. Beta-glucosidase activity in the rat small intestine toward quercetin monoglycosides. *Biosci. Biotechnol. Biochem.* **1998**, *62*, 1428–1431.
- Lieutaghi, P. The book of trees and shrubs; Rizzoli Editore: Milano, Italy, 1975.
- Morand, C.; Crespy, V.; Manach, C.; Besson, C.; Demigne, C.; Remesy, C. Plasma metabolites of quercetin and their antioxidant properties. *Am. J. Physiol.* **1998**, *275*, 212–219.
- Mulinacci, N.; Vincieri, F. F.; Baldi, A.; Bambagiotti-Alberti, M.; Sendl, A.; Wagner, H. Flavonol glycosides from *Sedum telephium* subspecies maximum leaves. *Phytochemistry* **1995**, *38*, 531–533.
- Noroozi, M.; Angerson, W. J.; Lean, M. E. Effects of flavonoids and vitamin C on oxidative DNA damage to human lymphocytes. *Am. J. Clin. Nutr.* **1998**, *67*, 1210–1218.
- Pinelli, P.; Romani, A.; Mulinacci, N.; Vincieri, F. F.; Tattini, M. HPLC analysis of polyphenols from privet (*Ligustrum vulgare* L.) leaves. In *Polyphenols 98*; Bouillard, R., Jay, M., Scalbert, A., Eds.; INRA Editions: Paris, 1999.
- Romani, A.; Baldi, A.; Mulinacci, N.; Vincieri, F. F.; Tattini, M. Extraction and identification procedures of polyphenolic compounds and carbohydrates in phillyrea (*Phillyrea angustifolia* L.) leaves. *Chromatographia* **1996**, *42*, 571–577.
- Romani, A.; Pinelli, P.; Mulinacci, N.; Vincieri, F. F.; Cimato, A. Polyphenolic content in five tuscan cultivars of *Olea europaea* L. *J. Agric. Food Chem.* **1999a**, *47*, 964–967.
- Romani, A.; Pinelli, P.; Mulinacci, N.; Vincieri, F. F.; Tattini, M. Identification and quantification of polyphenols in leaves of *Myrtus communis* L. *Chromatographia* **1999b**, *49*, 17–20.
- Takahama, U. Hydrogen peroxide-dependent oxidation of flavonoids and hydroxycinnamic acid derivatives in epidermal and guard cells of *Tradescantia virginiana* L. *Plant Cell Physiol.* **1988**, *29*, 475–481.
- Tattini, M.; Gravano, E.; Pinelli, P.; Mulinacci, N.; Romani, A. Flavonoids accumulate in leaves and glandular trichomes of *Phillyrea latifolia* exposed to excess solar radiation. *New Phytol.*, in press.

- Visioli, F.; Bellosta, S.; Galli, C. Oleuropein, the bitter principle of olives, enhances nitric oxide production by mouse macrophages. *Life Sci.* **1998**, *62*, 541–546.
- Visioli, F.; Romani, A.; Mulinacci, N.; Zarini, S.; Conte, D.; Vincieri, F. F.; Galli, C. Antioxidant and other biological activities of olive mill wastewaters. *J. Agric. Food Chem.* **1999**, *47*, 3397–3401.
- Wang, H. K.; Xia, Y.; Yang, Z. Y.; Natschke, S. L.; Lee, K. H. Recent advances in the discovery and development of flavonoids and their analogues as antitumor and anti-HIV agents. *Adv. Exp. Med. Biol.* **1998**, *439*, 191–225.
- Willems, M. Quantitative evaluation of secoiridoid glucosides from the fruit of *Ligustrum vulgare* by HPLC. *Planta Med.* **1988**, *54*, 66–68.
- Williams, C. A.; Harborne, J. B.; De Menezes, N. L. The utility of leaf flavonoids as taxonomic markers in the subfamily and generic classification of the *Velloziaceae*. *Biochem. Syst. Ecol.* **1991**, *19*, 483–495.
- Williams, C. A.; Harborne, J. B. Flavone and flavonol glycosides. In *The Flavonoids: advances in research since 1986*; Harborne, J. B., Ed.; Chapman & Hall: London, 1993.
- Yamasaki, H.; Sakihama, Y.; Ikehara, N. Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H<sub>2</sub>O<sub>2</sub>. *Plant Physiol.* **1997**, *115*, 1405–1412.

Received for review December 6, 1999. Revised manuscript received June 8, 2000. Accepted June 9, 2000.

JF9913256