GC-MS and 13C NMR study of epicuticular waxes of olive fruit(*Olea europaea*)cv. Dritta

Giovanna Vlahov*, Giuseppe Rinaldi, Paolo Del Re, Angela Alessia Giuliani
CRA-Istituto Sperimentale per la Elaiotecnica, Viale L.Petruzzi 75, 65013 Citta S.Angelo PE, (ITALY)
Tel : (39) 085-95212,(39) 085-95294; Fax(39) 085-959518
E-mail : g.vlahov@tiscali.it

ABSTRACT

The chloroform-soluble waxes of the olive fruit(*Olea europaea*) of cv. Dritta were separated by thin layer chromatography(TLC) and analyzed by gas liquid chromatography(GLC) and gas liquid chromatography-mass spectrometry(GC-MS). The classes of different compounds include apolar molecules(n-alkanes, aldehydes and benzyl esters), long chain aliphatic alcohols(C_{18}-C_{30}), triterpenic dialcohol(erythrodiol) and triterpenic acids(oleanolic and maslinic acid). A further study on the crude waxes was carried out by using high-resolution 13C nuclear magnetic resonance spectroscopy(13C NMR).   © 2008 Trade Science Inc. - INDIA

INTRODUCTION

Epicuticular waxes are found in all higher plants on the surface of leaves, seeds, flowers and fruits. The term "waxes", according to its chemical definition, is referred to esters of long-chain aliphatic fatty acids with long-chain aliphatic alcohols. However, waxes were more widely defined as a complex mixture of cyclic and aliphatic molecules of different structure, polarity, and homologue distribution[1,2]. The chemical structure of epicuticular waxes was studied in a great number of plants with an economical and nutritional interest[3-10]. The main physiological role of epicuticular waxes is to retain water by protecting the gas exchange surfaces with a waterproof membrane, and providing a variable aperture control mechanism(stoma) which could regulate the water evaporation and carbon dioxide exchange[11]. In addition , epicuticular waxes have the function of protecting plants against pathogenic organisms[12], of shielding plant organs from UV light[13], and of acting as

an allelochemical agent towards herbivores[14-15]. Moreover, epicuticular waxes regulate a wide range of plant-host interactions in the plants[16] and represent a class of molecules of nutritional and clinical interest for humans[17-19]. An increasing demand for finding even more miniaturized methods for determining quality, adulteration and typicity of olive oils, suggested to undertake a new study of the epicuticular wax profiles of the olive fruits. The study was expected to provide further compositional data to be used for a chemotaxo nomical characterization of different olive fruit cultivars.

Furthermore, 13C NMR spectroscopy was applied to measure the 13C spectrum of the epicuticular wax sample extracted from the cv Dritta olive fruits and dissolved in a mixture of deuterated chloroform and methanol as described under the experimental section. By analogy with the characterization of the triacylglycerol profiles of olive oils as a whole[20], the availability of the 13C spectrum was checked to determine the compo sitional profiles of the epicuticular waxes as they were

Full Paper

extracted from the olive fruit samples without any further chemical treatment.

EXPERIMENTAL

Reagents

All solvents and reagents used through this research were of analytical grade(Carlo Erba Reagenti, Milan, Italy). Silica gel 60 G for TLC was purchased from Merck KGaA(Darmstadt, Germany). Authentic erythrodiol, uvaol, phytol, campesterol and 1-eicosanol samples were from Sigma-Aldrich(Milan, Italy) whilst β-sitosterol, cycloartenol, α-amyrin, β-amyrin, oleanolic, ursolic and betulinic acid were from extrasynthese(Genay, France).

Wax extraction

Intact and healthy samples of olive fruits(500g) of the Dritta cultivar were collected at the end of October 2006 in the Institute olive groves. Waxes were extracted by dipping the fruits into cold chloroform for three times and gently stirring them each time for 1min. The pooled surface lipid fractions were dried over anhydrous sodium sulphate and evaporated to dryness by using a rotary evaporator. The yield of surface waxes was on average of 320mg/100g fruit weight.

Thin layer chromatography(TLC)

A crude sample(10mg) of epicuticular waxes was dissolved in 1.0ml of chloroform with a drop of methanol and applied approximately 1.5cm above the bottom of a home made plate(20cm×20cm×0.60mm film thickness). The plate was developed in a benzene:acetone solution(96:4) and visualized under UV light after spraying with an ethanolic solution of 2',7'-dichlorofluorescein. The bands corresponding to apolar components, aliphatic alcohols, triterpenic alcohols and triterpenic acids, were scraped off, extracted with diethyl ether and evaporated to dryness under a gentle stream of nitrogen. The identification of molecular classes was made by comparison of R_f with authentic samples which were detected at 120^0C for 2h by using as a visualizing reagent a 3% solution of chromium anhydride in a water:sulphuric acid 1:1.

Trimethylsilyl ether derivatives

Molecules containing free polar groups were silylated by adding 80μl of silylating reagent(pyridine: hexamethyldisilazane: trimethylchlorosilane 9:3:1) at room temperature for 30min in glass stoppered tubes and centrifugated at 3500rpm for 5min. The supernatant (0.5μl) was directly used for GLC and GC-MS analysis.

Gas liquid chromatography(GLC)

GLC analysis of triterpenoids and aliphatic alcohols was performed on a HRGC5160 gas chromatograph (Carlo Erba Reagenti, Milan, Italy) using a RTX®-5(5% diphenyl/95% dimethylsiloxane) capillary column (30m×0.32mm i.d., 0.25 μm film thickness; Restek Corporation, Bellefonte, PA, USA) in splitless mode, FID detector and injector temperatures of 290^0C and 280^0C, respectively. The aliphatic alcohols chromatogram was run by keeping the oven at 180^0C for 8 min, and then raising the temperature to 265^0C at 5^0C min^{-1} and holding at 265^0C until all the components were eluted. Triterpenoids were determined under isothermal conditions at 265^0C. GLC analysis of apolar components were performed with FID detector at 340^0C and with the oven at 70^0C for 1 min, raised to 150^0C at 25^0C min^{-1}, held at 150^0C for 3 min, raised again to 330^0C at 4^0C min^{-1} and held at 330^0C until all components were eluted.

Gas liquid chromatography-mass spectrometry (GC-MS)

GC-MS analyses were performed on a TraceGC gas-chromatograph coupled to a Polaris Q Ion Trap mass spectrometer(Thermo Finnigan, MA, USA) equipped with a RTX®-5MS capillary column 30m×0.25mm i.d., 0.25μm film thickness(Restek Corporation, Bellefonte, PA, USA). The chromato graphic conditions were as follows: injector 280^0C, transfer line 240 or 300^0C, ion source 250^0C. Full spectra(50-700amu) were recorded in the electron impact(EI) mode at 70eV.

High-resolution ^{13}C nuclear magnetic resonance spectroscopy(^{13}C NMR)

A 40mg portion of the crude epicuticular wax sample was dissolved in 1.0ml deuterated chloroform (CDCl$_3$; Sigma-Aldrich, Milan, Italy) and 50μl deuterated methanol(CD$_3$OD; Sigma-Aldrich, Milan, Italy). The ^{13}C NMR spectrum was obtained at 25^0C with a Unity Inova Narrow Bore 500 MHz spectrometer equipped with a Unix-based Sun Microsystems

workstation(Varian NMR Instruments, Palo Alto, CA). The spectrum was acquired under proton decoupling (Waltz-16 broadband decoupling), with 64 K data points and an acquisition time of 1.1s at a repetition rate determined by an interval between pulses of 2s. Signal averaging was carried out for a total time of 4h. A resolution enhancement function was applied to the free induction decay before fourier transformation to improve resolution and sensitivity of the ^{13}C resonances.

RESULTS AND DISCUSSION

The epicuticular waxes are generally obtained by dipping the intact plant organ(leaves, seeds, flowers, fruits) into chloroform or other solvents for about 1 min. After removing the solvents, the crude extract is fractionated into various classes of compounds by column chromatography[2]. This method allows large quantities of pure compounds to be obtained but it is time consuming and requires large amounts of obnoxious solvents. In the first report on the composition of cuticular lipids of olive fruit[3], a profile made up of alkanes, aldehydes, alkyl and methyl phenyl esters, triacylglycerols, aliphatic alcohols, triterpenoids and fatty acids was described. In more recent reports[4,5], the dominant classes of epicuticular waxes of olive fruit were found to be triterpenoid substances where triterpenic acids represented the dominant compounds. In the present work, we use preparative TLC to obtain separation of the different classes of compounds of the epicuticular waxes of the olive fruit(*Olea europaea*) cv. Dritta(Figure 1).

We obtained four bands at R_f values of 0.85, 0.45, 0.36 and 0 in correspondence of apolar compounds, aliphatic alcohols, triterpenic alcohols and triterpenic acids, respectively.

Apolar compounds

n-Alkanes

The apolar compounds which represent 23% of the total epicuticular waxes of the olive fruits cv. Dritta, comprised n-alkanes, long-chain aldehydes and benzyl

TABLE 1 : Composition of epicuticular waxes of the olive fruit of cv. Dritta

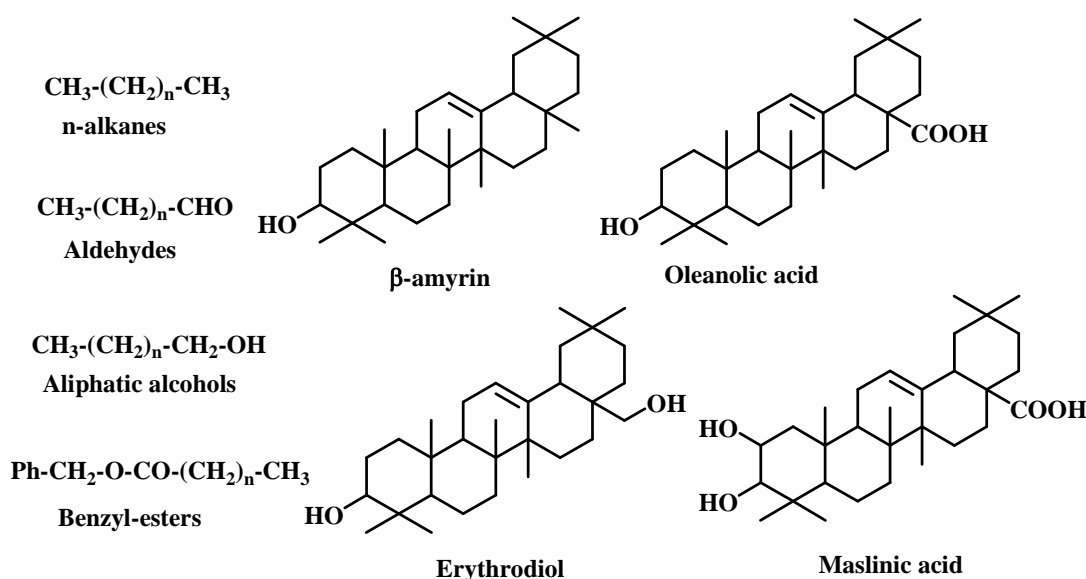| Components	% total waxes		Carbon chain lenght																	
			18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
		n-alkanes	-α	-	-	2	2	7	4	20	6	32	5	15	-	7	-	-	-	-
Apolar	23	aldehydes	-	-	-	-	3	-	8	4	36	6	30	3	10	-	-	-	-	-
		ph-esters	-	-	-	-	-	-	-	-	-	-	-	-	-	23	-	44	-	33
Aliphatic alcohols	23		1	1	1	-	18	2	25	3	31	2	14	1	1	-	-	-	-	-
Triterpenoids	54																			

α : Not detected

CH₃-(CH₂)ₙ-CH₃
n-alkanes

CH₃-(CH₂)ₙ-CHO
Aldehydes

β-amyrin

Oleanolic acid

CH₃-(CH₂)ₙ-CH₂-OH
Aliphatic alcohols

Ph-CH₂-O-CO-(CH₂)ₙ-CH₃
Benzyl-esters

Erythrodiol

Maslinic acid

Figure 1: Structural formulas of the main constituents of the epicuticular waxes of the olive fruit of cv. Dritta

Full Paper

esters(TABLE 1).

All these components were detected in a single chromatogram which is reported in figure 2. All the apolar compounds followed a bell-shaped distribution. In particular, the composition pattern of n-alkanes showed a distribution curve in which the odd homologue series was centred at the C_{27} homologue.

Long-chain aliphatic aldehydes

The long-chain aliphatic aldehydes, which can be also found in olive oil[21], were determined in the epicuticular waxes of the olive fruit cv. Dritta. They comprised the even compounds from C_{24} to C_{30} centred at the C_{26} homologue(Figure 2). The characteristic ions $[M]^+$, $[M-18]^+$ and $[M-44]^+$ which are detected in the mass spectra of short-chain aliphatic aldehydes, were found in the mass spectra of long-chain aliphatic aldehydes but with lower intensities. The mass spectrum of hexacosanal(Figure 3) showed the molecular ion(m/z 380), the $[M-18]^+$ ion(m/z 362) due to the loss of a water molecule from the enolic form of the aldehydic function and the $[M-44]^+$ ion(m/z 336) which was obtained from a β-cleavage. Fragments of the hydrocarbon moiety of aldehydes were also determined at low m/z values. These findings were in agreement with the data reported in literature[22].

Benzyl esters

The last components of the apolar fraction were the aromatic esters formed by the esterification of benzyl alcohol with long chain fatty acids comprising even carbon numbers from C_{24} to C_{28}(Figure 2). The identification of these compounds was confirmed by their mass spectra[23]. The fragmentation of molecular ion of benzyl hexacosanoate(m/z 486), which was the major homologue, generated two ions at 91 and 108m/z arising from the benzyl alcohol moiety, and two ions at 377 and 395m/z arising from the hexacosanoic acid moiety(Figure 4). The alkyl-esters and triacylglycerols could not be detected because of their low concentration levels.

Aliphatic alcohols

The aliphatic alcohol fraction which represents 23% of the total waxy material , was shown in figure 5. The homologues from C_{18} to C_{30} followed a bell shaped distribution centred at the C_{26} homologue. Their chain
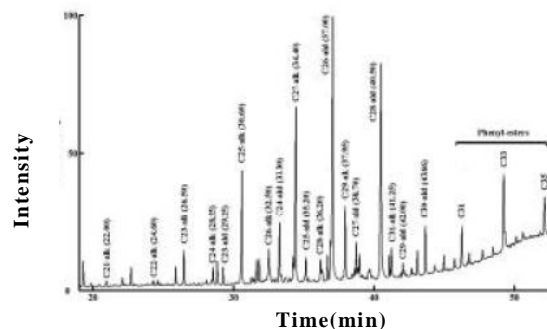

Figure 2: GC-MS chromatogram of the apolar epicuticular components of the olive fruit of cv. Dritta
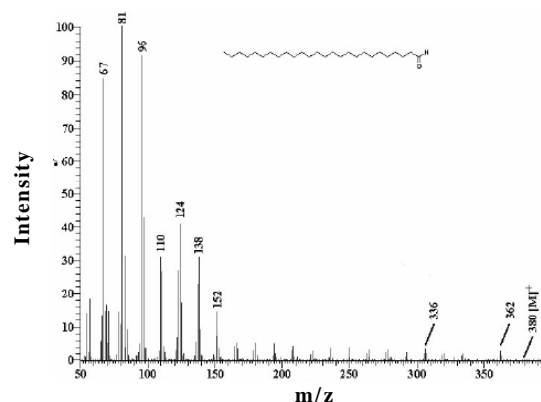

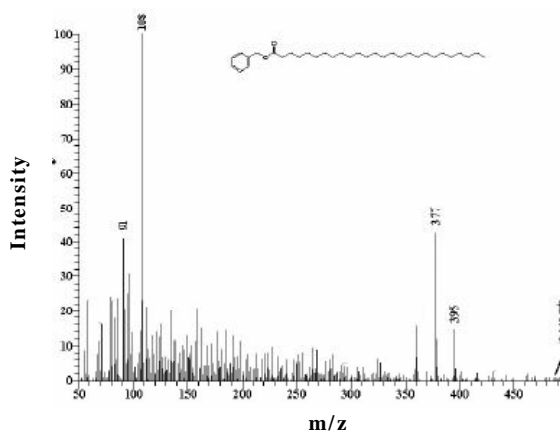**Figure 3: Mass spectrum of C_{26} aldehyde hexacosanal**

Figure 4: Mass spectrum of benzyl hexacosanoate

length patterns suggest that they are produced from the same pool of fatty acyl chains and that the aldehydes are intermediate in the reduction process to alcohols[2].

Triterpenoids

All the compounds of the triterpenoid fraction which represents more than half of the total epicuticular waxes(54%)(TABLE 2), belong to the β-amyrin series
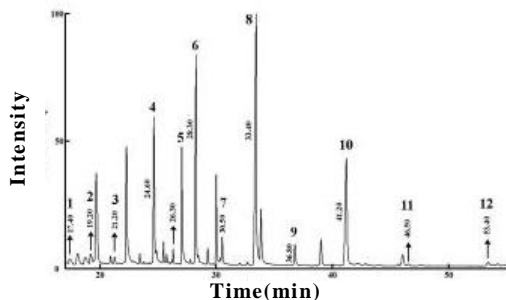
Figure 5: GC-MS chromatogram of the aliphatic alcohols of the epicuticular waxes of the olive fruit of cv. Dritta(1 to 12: C_{18} to C_{30})
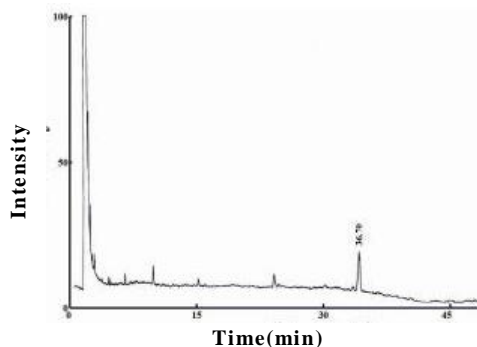

Figure 6: GC chromatogram of the TLC band with R_f=0.36 of the epicuticular waxes of the olive fruit of cv. Dritta
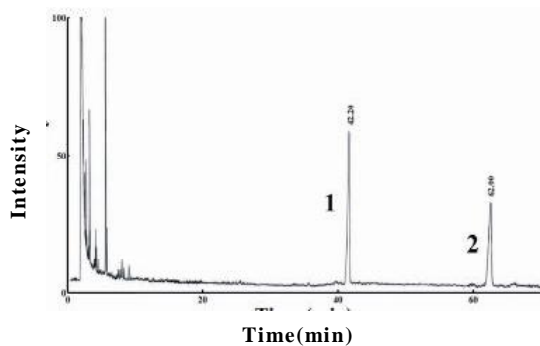

Figure 7: GC chromatogram of the TLC band with R_f=0 of the epicuticular waxes of the olive fruit of cv. Dritta(1: Oleanolic acid; 2: Maslinic acid)

at different oxidation levels(Figure 1).

The TLC band with R_f=0.36 contains only erythrodiol(4%)(Figure 6) whereas the TLC band with R_f=0 was made up of a mixture of oleanolic(52%) and maslinic acids(44%)(Figure 7).

The structures of these compounds were assigned by using GC-MS spectrometry and confirmed by means of the data reported in literature and by comparison of their mass spectra to those of authentic samples[4,24].

TABLE 2 : Composition of triterpenoid molecules of epicuticular waxes of the olive fruit of cv Dritta

Compounds	Composition(%)
Triterpenic dialcohols	
Erithrodiol	4
Triterpenic acids	
Oleanolic acid	52
Maslinic acid	44

TABLE 3 : Diagnostic ions and relative intensities of the major components within each epicuticular wax class of olive fruit of cv. Dritta

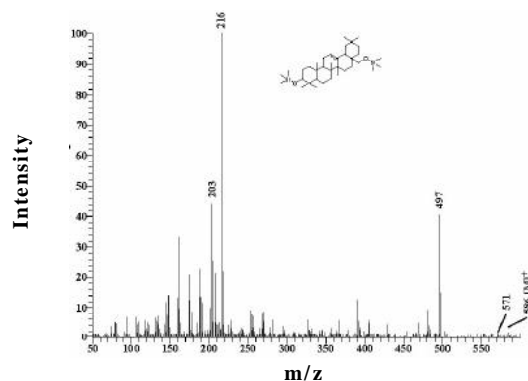| Carbon assignments	Oleanolic acid δ, ppm	Maslinic acid δ, ppm
C-28	181.008	180.978
C-13	143.697	143.749
C-12	122.182	121.999
C-5	55.105	55.142
C-3	78.757	83.385
C-2	-	68.480

Figure 8: Mass spectrum of the trimethylsilyl ether derivative of erythrodiol
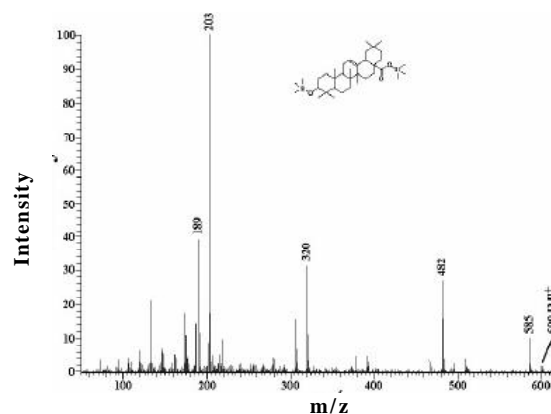

Figure 9: Mass spectrum of the trimethylsilyl ether derivative of oleanolic acid

The mass spectra of erythrodiol, oleanolic and maslinic acids, reported in figures 8,9 and 10 respectively, evidenced the fragments corresponding to the loss of
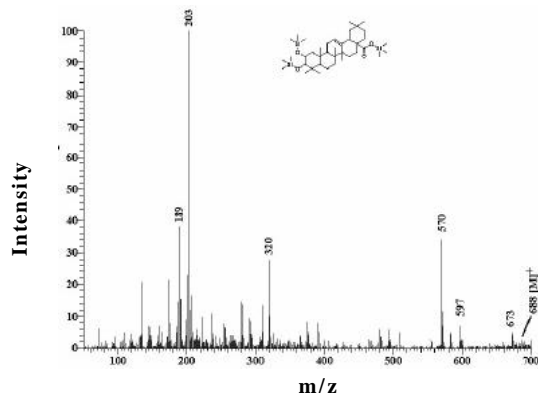
Full Paper

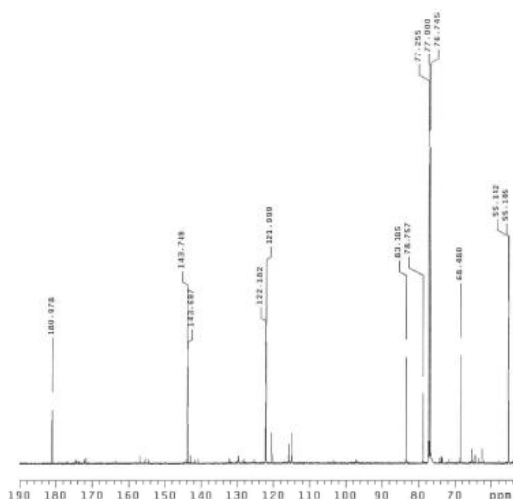**Figure 10: Mass spectrum of the trimethylsilyl ether derivative of maslinic acid**

Figure 11: ¹³C NMR spectrum where the olefinic and hydroxyl carbons of triterpenic acids resonance

the methyl and the trimethylsilyl groups from the molecular ions. Moreover, triterpenoid molecules which contain a Δ^{12}-double bond undergo a retro-Diels-Alder reaction that causes a cleavage in the five ring skeleton. These fragments were 203 and 320 m/z in triterpenic acids and 203, 216 and 306 m/z in erythrodiol[24].

High-resolution ¹³C nuclear magnetic resonance spectroscopy(¹³C NMR)

The high resolution ¹³C NMR spectrum of an epicuticular wax sample extracted from the olive fruits cv. Dritta, was run and the frequency range where the olefinic and the hydroxyl carbons resonated, was examined. The resonance assignments were made by HETCOR experiment for ¹³C-¹H shift correlation. HETCOR assured all the ¹³C resolution advantages of

a ¹³C-detected sequence as compared to the ¹H-detected ¹³C-¹H shift correlation sequences such as HMBC and HMQC which showed undoubted significant sensitivity advantages. The sensitivity didn't represent a problem in the present work because crude wax samples were directly used for NMR spectroscopy. The ¹³C frequency range from 55 to 190 ppm(Figure 11) evidenced that the olefinic and the hydroxyl carbons of the oleanolic and maslinic acids were fully resolved.

The chemical shifts were reported in TABLE 3. These results, even if they refer to the triterpenic acids which are quantitatively the most representative wax components, confirmed that ¹³C NMR spectroscopy could be applied to characterize the compositional profiles of olive fruit waxes and determine the peculiarity of the different olive cultivars.

ACKNOWLEDGMENT

REFERENCES

[1]   W.Barthlott, C.Neinhuis, D.Cutler, F.Ditsch, I. Meusel, I.Theisen, H.Wilhelmi; Bot.J.Linn.Soc., **126**, 237-260 **(1998)**.

[2]   G.Bianchi; Plant waxes In 'Waxes: Chemistry, Molecular Biology and Functions', R.J.Hamilton (ed.); The Oily Press, Dundee, Scotland, 175-222 **(1995)**.

[3]   G.Bianchi, C.Murelli, G.Vlahov; Phytochemistry, **31**, 3503-3506 **(1992)**.

[4]   G.Bianchi, N.Pozzi, G.Vlahov; Phytochemistry, **37**, 205-207 **(1994)**.

[5]   G.Bianchi, G.Vlahov; Fat Sci.Technol., **96**, 72-77 **(1994)**.

[6]   G.Bianchi, G.Vlahov, C.Anglani, C.Murelli; Phyto chemistry, **32**, 49-52 **(1993)**.

[7]   D.W.Griffiths, G.W.Robertson, T.Shepard, A.N. EBirch; S.C.Gordon, J.A.T.A.Woodford; Phyto chemistry, **55**, 111-116 **(2000)**.

[8]   P.G.Gulz, R.W.Scora, E.Muller, F.J.Marner; J.Agric. Food Chem., **35**, 716-720 **(1987)**.

[9]   R.Jetter, S.Schäffer, M.Riederer; Plant Cell Env., **23**, 619-628 **(2000)**.

[10] B.M.Szafranek, E.E.Synak; Phytochemistry, **67**, 80-90 **(2006)**.

[11] G.Kerstiens; J.Exp.Bot., **47**, 1313-1832 **(1996)**.

[12] F.Gniwotta, G.Vogg, V.Gartmann, T.L.W.Carver, M. Riederer, R.Jetter; Plant Physiol., **139**, 519-530 **(2005)**.

[13] T.A.Day, T.C.Vogelmann, E.H.DeLucia; Oecologia, **92**, 513-519 **(1992)**.

[14] D.Cervantes, S.D.Eigenbrode, H.Ding, N.Bosque-Perez; J.Chem.Ecol., **28**, 193-210 **(2000)**.

[15] S.D.Eigenbrode, K.E.Espelie; Annu.Rev.Entomol., **40**, 171-194 **(1995)**.

[16] J.Mann; Metabolites derived from mevalonate: isoprenoids, In 'Secondary Metabolism', 2nd edition, Clarendon Press, Oxford, NY, 138 **(1987)**.

[17] M.C.Ramirez-Tortosa, G.Urbano, M.Lopez-Jurado, T.Nestares, M.C.Gomez, A.Mir, E.Ros, J.Mataix, A.Gil; J.Nutr., **129**, 2177-2183 **(1999)**.

[18] M.N.Vissers, P.L.Zock, G.W.Meijer, M.B.Katan; Am.J.Clin.Nutr., **72**, 1510-5 **(2000)**.

[19] J.L.Hargrove, P.Greenspan, D.K.Hartle; Exp.Biol. Med., **229**, 215-226 **(2004)**.

[20] G.Vlahov; Magn.Reson.Chem., **36**, 359-362 **(1998)**.

[21] M.C.Perez-Camino, W.Moreda, R.Mateos, A.Cert; J.Chromatogr.A., **983**, 283-288 **(2003)**.

[22] K.Christiansen, V.Mahadevan, C.V.Viswanathan, R.T.Holman; Lipids, **4**, 421-427 **(1969)**.

[23] G.Bianchi, A.Tava, G.Vlahov, N.Pozzi; J.Am.Oil Chem.Soc., **71**, 365-369 **(1994)**.

[24] M.Burnouf-Radosevich, N.E.Delfel, R.England; Phytochemistry, **24**, 2063-2066 **(1985)**.