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Microcomponents of olive oil. Part II: Digalactosyldiacylglycerols from *Olea europaea*

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Two galactolipids, **1** and **2**, were isolated and identified both in olive fruits and in olive oil. They are characterized by a low polarity, despite the presence of a diglycosidic unit, giving, in water, micelles. Because of this characteristic, compounds **1** and **2** could be responsible for the stability of the light emulsion typical of freshly produced olive oil. The presence of hydrophilic *ortho*-diphenolic compounds enhances the antioxidant properties of this oil, particularly in the first period after the production. In addition, the functions present in **1** and **2** are susceptible to hydrolysis and may be easily modified in the alkaline treatment used for the olive oil refining process. For this reason, their presence in olive oil may be a very useful indication of the untreated food product.

INTRODUCTION

Olives and oil are major examples of the health food concept linked to the Mediterranean diet.

The chemical and physical characteristics of olives are strongly dependent on the cultivar, the habitat and the geographical area. In particular, they influence the quality of olive and are related to the subspecies considered, the age of the plant, the characteristics of cultivation, of climate, of habitat and general geographical area, and inorganic and organic microcomponents present in the soil. Therefore, together with chemical and environmental studies, it is necessary to make a careful examination of taxonomic characteristics of subspecies used for olive production, and of genetic maps of their subspecies. This knowledge would allow selection of suitable plants, and creation of new hybrids particularly dedicated to the production of table olives or olives for oil production.

In addition, the degree of ripening has a direct influence on the chemical composition of the olives, it being well known that green olives differ from black olives.

The investigation of molecular composition of olive fruits would allow prediction of the final microcomponents in the produced oil and therefore rational criteria for determination of quality.

It has benn already demonstrated (Bianco *et al.*, 1996) the presence of glucosides of 2(3,4-dihydroxyphenyl)ethanol, which are responsible, together with the other *o*-diphenolic derivatives present in olives (Camurati *et al.*, 1981; Montedoro *et al.*, 1992*a*,*b*, 1993; Cortesi *et al.*, 1994), for organoleptic characteristics of olive oil as well as for its well-known antioxidant properties. These compounds are transferred in the oil during the process of production and we have proposed for them the name of 'biophenols' in consideration of their biological activities.

The glycosidic non-phenolic microcomponents of olive fruits have also been investigated in order to show new molecules that are transferred in the resulting oil. They could be characteristic of olive oil and therefore they may make a specific contribution towards the organoleptic and nutritional quality of this traditional food. In fact the quality of olive oil is strongly dependent on the original components of olive fruits and organoleptic and nutritional quality and authenticity of traditional Mediterranean foods is strongly related to the nature of microcomponents and to their solubility in the oil. Olive oil is, as is well known, a fine emulsion of a generally small quantity of a polar phase, water, in a large quantity of a lipophilic phase, glycerides.

MATERIALS AND METHODS

TLC: Silica gel SiF_{254} (Merck) and RP-8 F_{254} (Merck). Spray reagents: 2 N H_2SO_4. NMR: Bruker AM 500. MS: Kratos-80. IR: Shimadzu IR-470. $[\alpha]_D$: Jasco DIP-370. HPLC: Hewlett Packard with UV detector.

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Plant material

Olea europaea leaves and fruits were hand-collected in Calabria (Italy) in 1995 at the beginning of ripening from plants without chemical treatments for 1 year. Voucher specimens are deposited in the ISOL, Istituto Sperimentale per l'Olivicoltura, Rende, Cosenza, which prepared the olive oil by cold pressing on the same day of the collection. Two subspecies were examined: subspecies 'Carolea', cultivated in the neighbouring town of Cropani, Catanzaro, and subspecies 'Ottobratica', cultivated in the neighbouring town of Rende.

Extraction and separation

Leaves and olive fruits

Plant material (about 500 g of leaves) was exhaustively extracted with EtOH at room temperature and the extract evaporated to an aqueous suspension. Charcoal (50 g) was added until negative H_2SO_4 test and the resulting suspension was layered on a Gooch funnel. Elution with water (5% and 10% EtOH) removed salts and simple sugars, whereas 30, 60, and 90% EtOH eluted glycosidic-containing components. 90, 60, 30%, EtOH fractions were worked up as follows.

Solvents were evaporated *in vacuo* and the resulting crude material was chromathographed on Si gel in *n*-BuOH saturated water. A preliminary separation of components, in relation to their polarity was also performed.

Fractions containing digalactosyl-diacylglycerols were successively chromatographed on Si gel in CH_2Cl_2/MeOH 8:2, affording a faction containing semipurified mixture of **1** and **2**.

Separation of pure **1** and **2** was obtained by semi-preparative HPLC in μ-bondopack C_{18} using MeOH/H_2O gradient as eluent: 100 mg of **1** and 30 mg of **2** were obtained, both as colourless viscous materials.

Compound **1**-$[\alpha]_D^{25} = +7°$ (MeOH, c = 0.1). UV (MeOH): $\lambda_{max} = 205$ nm (log = 1). IR ($CHCl_3$): ν_{max} 3600. 3500, 1650, 1100, 1020 cm^{-1}. ^{1}H-NMR-(CD_3OD): 0.98 (3H, t, J = 7.5, CH_3), 2.85 (4H, c), 2.35 (2H, d) 2.10 (4H, a, b) 1.60 (2H, g), 1.35 (8H, e), 5.3–5.5 (7H, f, H-2), 4.45, 4.23 (2H-3, AB system, J = 3.0, 12.0) 4.2, 4.9 (H-1′, H-1″), 4.0–3.4 (12H, galactose protons), ^{13}C-NMR (CD_3OD),: δ 175.3, 175.0 (COOR), 133.0, 131.4, 129.5, 129.2, 128.5 (f), 14.0 (CH_3), 21.8 (a), 26.3, 26.7(c), 26.8 (b), 28.5 (g), 30.4, 30.5, 30.6, 31.0 (e), 35.2, 35.4 (d), 63.1 (3), 64.3 (1), 68.1 (6'), 69.0 (6″), 71.4 (2), 70.3, 70.5 (3′ 3″), 71.4, 71.7 (4′ 4″), 72.7, 72.8 (2′ 2″), 74.9, 75.0 (5′ 5″), 105.6, 101.0 (1′ 1″).

Compound **2** $[\alpha]_D^{25} = +6.5$ (MeOH, c = 0,1). UV (MeOH) $\lambda_{max} = 205$ nm (log = 1). IR ($CDCl_3$) ν_{max} 3550, 3400, 1640, 1120, 1100, 1040 cm^{-1}. ^{1}H-NMR-(CD_3OD): δ: 0.72 (3H, t, J = 7.5, CH_3 oleic), 0.77 (3H, t, J = 7.5, CH_3 linolenic), 1.05 (CH_2), 1.15 (CH_2), 1.41 (CH_2 α-ester), 1.90 (CH_2 α-double bonds), 2.13 (CH_2 α-double bonds), 2.63 (CH_2 α-double bonds linolenic), 5.1, 5.2 (olefinic protons, H-2), 4.0, 4.2 (2H-3, AB system, J = 3.0, 12.0) 4., 4.3 (H-1′, H-1″), 3.7–3.3 (12H, galactose protons), ^{13}C-NMR (CD_3OD),: (175.3, 175.0 (COOR), 135.0, 131.2, 131.3, 131.0, 130.4.(f), 16.7 (CH_3), 25.9 (a), 28.2, 28.6, 28.7 (c), 30.3 (b), 28.5 (g), 32.3, 32.6, 32.7, 32.9 (e), 35.3, 37.0 (d), 64.9 (3), 66.2 (1), 70.0 (6′ 6″), 72.4 (2), 73.2, 73.6 (3′ 3″), 74.4, 74.5 (4′ 4″), 74.6, 74.7 (2′ 2″), 76.7, 76.8 (5′ 5″), 102.6, 107.4 (1′ 1″).

A similar work-up was used with olive fruits. Starting from 250 g of fresh plant material, 50 mg of **1** and 20 mg of **2** were obtained.

Olive oil

Olive oil (10 ml) was diluted in EtOAc (100 ml) and CH_2Cl_2 (100 ml). Water was added by shaking until an approximate volume of 1–2 ml was separated. The mixture was shaken for 1 min and left to stand for 5 min for a duration of 1 h; water was then separated and directly analysed by HPLC, using compounds **1** and **2** as references. In 10 ml of olive oil, 1.5 mg of **1** and 1.0 mg of **2** were isolated.

RESULTS AND DISCUSSION

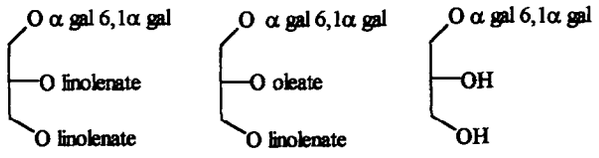
The sugars fraction of olive leaves was just examined and the sugars present determined (Romani *et al.*, 1994). Our attention was focused to the glycosidic fraction present in olives and eventually also in the aqueous phase of olive oil. Separation was performed using the so-called 'charcoal method', which allowed complete separation of the glycosidic fraction from the predominant fat material (which constituted of glycerides of saturated and unsaturated fat acids occurring in olives).

Examination of the glycosidic fraction allowed us to isolate two glycosides belonging to the class of digalactosyl-diacylglycerols, compounds **1** and **2**, which resulted in α1,6-digalactosyl derivatives of diglycerides of highly unsaturated fat acids. Galactolipids of *Olea europaea* have not been well characterized (Mancha, 1974; Vioque and Mancha, 1973; Catalano and Sciancalepore, 1976, 1975*a,b*), even if they are widespread components of plant membranes and especially of photosynthetic tissues (Bergqvist and Herslor, 1995).

Compound **1** is the α-1,6-digalactosyl derivative of the 1,2-diglyceride of linolenic acid.

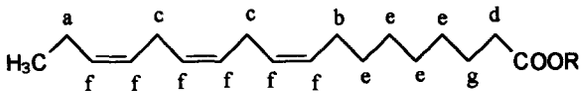
The structure of compound **1** was determined by chemical and spectroscopic methods.

The nature of the fatty acid moiety was deduced, besides spectroscopic considerations, by alkaline hydrolysis which allowed us to isolate linolenic acid, **3**



1 **2** **5**

as the sole product. Its structure was definitively determined by transforming into its methyl ester, **4**, whose structure was determined by GC-MS technique.



3 R=H linolenic acid
4 R=CH_3 methyl linolenate

The same experiment allowed the isolation of a hydrosoluble compound, **5**, which was found to be composed by two units of galactose and one of glycerol. Both galactose and glycerol were identified after acid hydrolysis and successive HPLC comparison with authentic samples.

The site of glycosylation (primary or secondary alcoholic function of glycerol) was determined by evidencing the characteristic glycosidation shift for the primary alcoholic function of glycerol in ^{13}C-NMR spectra of **1** and of its hydrolysis derivative, 5. The configuration of anomeric centres of galactose was determined by ^{1}H- and ^{13}C-NMR data, which indicate an α configuration for both centres.

The structure of compound **2** is very similar to that of **1**, from which it differs by the nature of one of the fatty acids.

In fact the alkaline hydrolysis afforded compound **5** together with a unit of oleic and a unit of linolenic acid, both identified by transforming in their methyl-esters and successive GC-MS analysis.

The linkage of oleic acid at the secondary hydroxyl of glycerol was proposed on the basis of biogenetic considerations. In fact, in an asymmetric glyceride, the less unsaturated fatty acid generally esterifies the secondary function.

The presence of galactolipides of polyunsaturated fatty acids **1** and **2** in the olive is in agreement with the sequential desaturation of oleate to linolenate via linoleate recently demonstrated for the biosynthesis of glycerolipids in *Olea* (Zarrouk *et al.*, 1990).

A modified procedure (see Experimental) was applied in order to ascertain the presence of **1** and **2** in olive oil. HPLC analysis has shown the presence of both digalactosyl-diacylglycerols under investigation.

Compounds **1** and **2** are characterized by a low polarity, despite the presence of a diglycosidic unit. They show low solubility in water, giving micelles, and these behave like non-ionic tensioactive solutes. This can be observed by NMR in different solvents. In particular the ^{13}C-NMR spectrum in D_2O reveals a broadening of glycosidic signals due to a higher relaxation time.

Because of these physico-chemical characteristics, compounds like **1** and **2** could be responsible for the formation of the light emulsion formed in the initial stage of olive oil production. This allows an increase of transfer of hydrophilic derivatives ('biophenols'; Bianco *et al.*, 1996) into the olive oil, so enhancing the antioxidant properties characteristic of this oil, particularly in the early stages immediately after production.

In addition, the molecular structure of **1** and **2**, which are both esters, are sensitive to alkaline conditions and could easily be modified in the alkaline treatment used for the olive oil refining process. For this reason their presence in olive oil may be a very useful indication of untreated food product.

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