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Gas chromatography screening of bioactive phytosterols from mono-cultivar olive oils

G. Sivakumar [a,*], C. Briccoli Bati [b], E. Perri [b], N. Uccella [c]

[a] *ENEA, Biotech Genomics, Casaccia, Via Anguillarese, 301 00060 Rome, Italy*
[b] *Istituto Sperimentale per la Olive coltura, C. Da Li Rocchi, 87036 Rende (CS), Italy*
[c] *CIRASAIA, Calabria University, 87036 Arcavacata, Rende (CS), Italy*

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Abstract

Phytosterols (PS) from nine samples of olive oil from *Olea europaea* L., the *Carolea, Cassanese* and *Coratina* mono-cultivars, have been analyzed by gas chromatography. *Coratina* virgin olive oil (VOO) from the month of November showed highest contents of β-sitosterol (5491 mg kg^{-1}) and Δ^5 avenasterol (1767 mg kg^{-1}). Olive pomace oil had the lowest total content of all PS, when compared to VOO. These results suggests that, PS can be an important regulatory factor for the functional quality of olive oil along the agro-industry chain from the orchard to nutraceutical.

Keywords: Olive oil; Phytosterols; Pomace; Nutraceutical

1. Introduction

Olive oil is one of the principal ingredients in the Mediterranean diet and is used in frying, roasting and seasoning, mainly for triacylglycerols, which are responsible for their nutritional and physico-chemical properties (Uccella, 2001). Some other minor lipids, such as PS, have aroused the interest of food researchers on account of their peculiar functional properties (Piironen, Linsay, Miettinen, Toivo, & Lampi, 2000). PS foods, as nutraceuticals have been introduced into the market with added free or esterified plant sterol or stanols in margarine (Maki et al., 1999). PS are structurally similar to cholesterol but with some modifications. These modifications involve the side chain and include the addition of a double bond and/or methyl or ethyl group. The most common PS are β-sitosterol, campesterol and stigmasterol. PS esters have been shown to have beneficial effects on plasma cholesterol level and have recently used as food ingredients in modern formulations. Some of the cholesterol is synthesized endogenously but some comes from the diet (Vu, Shin, Lim, & Lee, 2004). Epidemiologic and experimental investigations suggest that dietary sterols may offer protection from the most common cancers in Western societies, such as colon, breast and prostate cancer. PS mixtures rich in the five α-saturated derivative of β-sitosterol, sitostanol, have also been shown to be effective in reducing circulating cholesterol concentrations (Jones, Mac Dougall, Ntanios, & Vanstone, 1997). The role of PS and the development of innovation in the olive oil chain hold the potential for a significant effect on the producing country's economy, because medical care cost can be saved through preventive functional effects (Buttriss & Saltmarsh, 2000) and the provision of less tangible, but not less important, benefits to the consumer's quality of life.

In Italy, some of the commercially important olive cultivars are *Carolea, Cassanese* from Calabria and

* Corresponding author. Tel.: +39 06 3048 3405; fax: +39 06 3048 6044.
E-mail address: sivakumar@libero.it (G. Sivakumar).

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Coratina from Apulia. These are known in the market for the production of mono-cultivar olive oils. The objective of this investigation is to differentiate the three mono-cultivar olive oils, within the realm the PS fraction of the lipids present in the oil, which can provide an innovative contribution towards the production of functional olive oils for the prevention of degenerative diseases. The PS fraction was analyzed in virgin olive oil (VOO) from these cultivars extracted from two different months of harvesting. The olive pomace oil was also extracted from these mono-cultivars. Olive pomace is the pulpy material remaining after removing most of the oil from the olive paste. It contains fragments of skin, pulp, pieces of stone and some oil. A significant amount of oil is retained in the olive pomace during VOO extraction, because the cell walls are not properly broken in all the cells. When pomace is treated with hexane, all the oil comes out from cells, because all the cell walls are broken. Pomace must be dried to 10–20% moisture content for extraction of olive pomace oil. Higher moisture content results in poor recovery of oil. From the low moisture pomace, the oil has been extracted using hexane solvent. Olive pomace oil has a PS composition similar to VOO, but of low quantity. PS fraction, after the extraction of the oil, has been saponified and the unsaponifiable part has been separated by thin layer chromatography (TLC), and analyzed by gas chromatography (GC).

2. Materials and methods

2.1. Virgin olive oil extraction

Olea europaea L. fruits were harvested from ISOL orchard, Rende (CS), Italy in two different months (October and November 2002) from *Carolea, Cassanese* and *Coratina* cultivars. On the same day of harvest, olive oil was extracted from the above cultivars using Oliomio, Toscana, Enologica instrument, Italy. Nine samples of olive oil, six of the VOO from *Carolea, Cassanese* and *Coratina* cultivars and of pomace oil from above cultivars, were analyzed. The oils samples were stored in refrigerator until use.

2.2. Saponification of the lipids (ISO 11294, 1994)

Eight hundred micro litre of α-cholesterol was taken as an internal standard and the solvent was evaporated using N_2 gas. About 8 gm of olive oil was added to it. The olive oil was treated with 80 ml of 2 M solution of potassium hydroxide in methanol–water (80:20, v/v) and the mixture was refluxed with constant stirring for 2 h. Then 80 ml of water was added and allowed over night. Next day an extraction with 3×70 ml portions of diethyl ether was carried out. The organic extract was separated and washed with equal portions of water until pH becomes neutral. Then it was dried over anhydrous sodium sulphate and filtered into a 250-ml round-bottomed flask and concentrated on a rotary evaporator under reduced pressure at room temperature to distillate the diethyl ether. The unsaponifiable fraction was dissolved in chloroform to obtain approximately a 5% (m/v) solution. Sterol esters were usually not greatly affected by bleaching and refining of the fats. For the determination of their sterol content, they were normally saponified. However, it could be shown that these compounds also offer great potential for the differentiation of oils and fats by isolating a sterol ester-rich fraction of different oils and fats by TLC prior to their determination by GC (Gordon & Griffith, 1992).

2.3. Separation of the PS from the unsaponified fraction by TLC

This solution was spotted on a KOH-impregnated silica gel TLC plate. Twenty micro litre of the internal standard solution was also spotted with the solution of the sample. The plate was developed using benzene–acetone (95:5, v/v) and subsequently dried. Then it was sprayed with the Dicloroflucinol solution and the pink bands of the sterols could be observed under UV light together with the spot of the internal standard. This band was scraped off and the sterols were dissolved in isopropyl ether and filtered through a paper filter. The solvent was evaporated under N_2 and the PS fraction was dried in an oven at 105 °C. The PS fraction was treated with the derivatizing reagent to obtain the trimetyhlsilyl (TMS) derivatives. A volume of 20 μl of reagent for each mg of sterol was added. Aliquots of 2 μl of this solution were injected into the gas chromatograph and the ratio of the peak areas of the analyte and internal standard was used as an analytical signal. The content of individual sterols was expressed as the percentage of the PS fraction obtained.

2.4. Gas chromatograph

A Hewlett-Packard 5890 II gas chromatograph equipped with a flame ionization detector, a fused silica capillary column of $30\ m \times 0.32\ mm$ coated with a 0.2 μm film of HP-5 stationary phase and a Hewlett-Packard 7673 automatic injector was used. The oven was isothermally operated at 265 °C and the injector and detector were held at 280 and 300 °C, respectively. Hydrogen was used as carrier gas at a flow rate of $0.7\ ml\ min^{-1}$ through the column with a split ratio of 1:80. Air and hydrogen with flow rates of 430 and $30\ ml\ min^{-1}$, respectively, were used for the detector, which had an auxiliary flow of $30\ ml\ min^{-1}$ of nitrogen.

2.5. Data analysis

The limit values of PS used for the quality control of extra virgin olive oil suggested by the European Union (1991) and International Olive Oil Council IOOC (2001). 12 PS, that have been analyzed in the above mentioned olive oil samples. They will be referred to as follows: cholesterol, 24-metilencholesterol, campesterol, campestanol, stigmasterol, chlerosterol, β-sitosterol, sitostanol, Δ^5 avenasterol, Δ^5 24 stigmastadienol, Δ^7 stigmastenol, Δ^7 avenasterol.

2.6. Statistical analysis

Five replicates were used in each sample. Every analysis was repeated three times. Statistical analysis was performed according to the SAS System (Version 6.21, SAS Institute Inc. Cary, NC 27513, USA). Statistical significance of the differences observed among mean values was assessed using a Duncan's multiple range test. A probability of $P \leqslant 0.05$ was considered significant.

3. Results and discussion

The PS molecule consists of two parts – a nucleus and side chain. The nucleus, like that of the steroid hormones and bile acids, is based on the four-ring cyclopentanoperhydrophen anthrene structure. In cholesterol and in PS there is a double bond in the C5–C6 position and a β-orientated hydroxyl group attached to C3. In cholestanol and phytostanols (e.g., Sistostanol), there is no C5–C6 double bond. Cholesterol has 8-carbon atoms (a C_{27} sterol). Campesterol has an extra methyl group attached to C24 producing a C_{28} sterol, sitosterol has an ethyl group in this position and is thus a C_{29} sterol. In isofucosterol (also a C_{29} sterol), the C24 substituent is an unsaturated ethylidene group. Stigmasterol has the same structure as sitosterol except that it has an additional double bond between C22 and C23, all this and much more was won by the same laborious procedures extended over the span on a human life (Christopoulou, Lazaraki, Alexiou, Synouri, & Frangiscos, 1996).

Different oil content was obtained for the three cultivars (data not shown). To separate the unsaponifiable fraction, the olive oil was treated with a potassium hydroxide solution to transform the esters into potassium salts that were soluble in water. Other biomolecules, like the PS, did not react and could be extracted into non-polar solvents like ethyl ether. From this organic extract, the fraction of the PS was separated by TLC. KOH-impregnated silica gel TLC plates were used. Fatty acids were retained at the origin line and the PS band could be well separated, with mixtures of benzene–acetone (95:5, v/v). Hydrogen was used as a carrier gas because it allowed better resolution of some chromatographic peaks. The first peak of interest corresponded to cholesterol, which was followed by 24-metilencholesterol, campesterol, campestanol, stigmasterol, chlerosterol, β-sitosterol, sitostanol, Δ^5 avenasterol, Δ^5 24 stigmastadienol, Δ^7 stigmastenol and Δ^7 avenasterol (data not shown). Table 1 shows the contents (mg kg^{-1}) for PS of olive oil samples. The main compounds were β-sitosterol and Δ^5 avenasterol. *Coratina* VOO extracted from November 2002 showed the highest in β-sitosterol and Δ^5 avenasterol values (5491 and 1767, respectively), whereas, VOO extracted from October 2002 (3854 and 599) and pomace oil extracted from November 2002 (3832 and 652) recorded the lowest.

Table 1
Phytosterols content (mg kg^{-1}) of olive oil from Italy mono-cultivars

Name of the sterols	Virgin olive oil extracted from October 2002			Virgin olive oil extracted from November 2002			Pomace oil extracted from November 2002		
	Carolea	*Coratina*	*Cassanese*	*Carolea*	*Coratina*	*Cassanese*	*Carolea*	*Coratina*	*Cassanese*
Cholesterol	15	33	7	26	28	6	16	14	27
Brassicasterol	0	0	0	0	0	0	0	0	0
24-Metilencholesterol	13 c[A]	28 b	25 b	49 a	43 a	9 d	19 c	27 b	14 c
Campesterol	87 d	160 b	102 c	144 b	172 a	73 d	64 d	154 b	98 c
Campestanol	23 c	18 d	10 e	32 b	54 a	17 d	21 c	14 e	16 d
Stigmasterol	213 d	559 a	326 b	265 c	332 b	219 d	228 c	216 d	242 c
Δ^7 Campesterol	0	0	0	0	0	0	0	0	0
$\Delta^{5,23}$ Stigmastadienol	0	0	0	0	0	0	0	0	0
Chlerosterol	29 d	58 c	443 a	72 b	79 b	32 d	50 c	49 c	80 b
β-Sitosterol	3153 d	3854 c	3383 d	4639 b	5491 a	3951 c	2687 e	3832 c	2156 f
Sitostanol	185 e	536 a	212 d	219 d	279 c	303 b	182 e	193 d	314 b
Δ^5 Avenasterol	1072 c	599 f	684 e	1699 b	1767 a	1551 b	950 d	652 e	739 d
Δ^5, 24 Stigmastadienol	16 c	22 b	12 c	52 a	55 a	20 b	23 b	8 d	13 c
Δ^7 Stigmastenol	13 c	24 b	33 a	16 c	30 a	20 d	8 e	17 c	32 a
Δ^7 Avenasterol	15 c	31 b	5 e	46 a	31 b	17 c	14 c	10 d	17 c

[A] Mean separation within columns by Duncan's multiple range test, $P < 0.05$.

Carolea VOO, β-sitosterol content was less when compared *Coratina* but higher than *Cassanese* (4640 and 3951) and pomace oil *Carolea* and *Cassanese* (2687 and 2156), respectively. The highest PS levels found corresponded to β-sitosterol, followed by Δ^5 avenasterol, characteristic of the VOO in the pulp of the olive (Cornforth, 2002). These two major PS were strongly and positively correlated, and there was a clear differentiation into three groups or varieties. Regarding Δ^5 avenasterol content, *Carolea* VOO extracted from October 2002 showed the highest value (1699) when compared to November 2002 extracted VOO, but in *Cassanese* a vice versa was observed. When compared to campesterol, the stigmasterol content showed the highest of all the samples. The remarkable similarity between the three varieties was that the VOO from November month showed highest contents of campestanol, β-sitosterol Δ^5 avenasterol and Δ^5, 24 stigmastadienol when compared to October month extracted VOO. Pomace oil had the lowest total content of all PS when compared to VOO. With respect to ripening stage, there was a tendency for the PS content to decline. This had also been noted by other workers (Casas, Bueno, García, & Cano, 2004; Gutiérrez, Jimenez, Ruíz, & Albi, 1999). This was understood as being due to the synthesis of the sterols in the early stages of development of the fruit in the November month and as ripening progressed, the sterols become diluted and more oils were produced.

In conclusion, *Coratina* VOO extracted from November month contained higher amount of β-sitosterol than other mono-cultivar extracted oil. This olive PS could be the naturally enriched functional foods for food industry, as a novel ingredient to reduce the levels of ''bad'' serum low-density-lipoprotein cholesterol in consumers. Thus, a functional olive oil could be best produced from the appropriate selection of mono-cultivar olive oil such as, the *Coratina* one, or by the skilful blending of different cultivars via the ulivage process before milling.

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