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Changes in squalene and sterols associated with olive maturation

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ABSTRACT

Squalene and sterols are biologically active compounds of great relevance for olive oil quality. The objective of this research was to study the dynamics of squalene and sterols accumulation during olive maturation. Fruits of cultivars 'Picual' and 'Arbequina' and two breeding selections derived from crosses between them were harvested monthly from September to December in Córdoba, Spain. Squalene and sterols contents were measured directly in olive flesh by gas chromatography. The fresh fruit weight, oil content, and ripening index were also determined. Oil content reached a maximum in November (19.4%) and maintained similar levels in December (19.8%). Squalene content in the fruit flesh increased significantly from September (4102 mg kg^{-1}) to November (4673 mg kg^{-1}), showing no significant difference between November and December. Sterol content increased significantly every harvest date, from 723 mg kg^{-1} in September to 1188 mg kg^{-1} in December, though no significant differences were observed between November and December in three out of four genotypes. The concentration of β-sitosterol was reduced from 87.3% in September to 75.9% in November, whereas Δ^5-avenasterol concentration concomitantly increased from 7.2% in September to 18.6% in November, remaining unaltered in December. As only very small differences were found for oil, squalene and sterols contents between samples collected in November and December, it can be concluded that early harvest in November will not affect the fruit and oil composition for squalene and sterols.

1. Introduction

Virgin olive oil is produced from olive fruits by physical extraction. It is regarded by consumers around the world as one of the healthiest vegetable oils, as there is a large body of evidence for the role of olive oil in disease prevention, mainly in relation to cardiovascular disease (Covas, 2007; Pérez-Jiménez, Ruano, Pérez-Martínez, López-Segura, & López-Miranda, 2007). Recent studies also point to the chemoprotective action of olive oil constituents against cancer development (Kampa, Pelekanou, Notas, & Castanas, 2009; Psaltopoulou, Kosti, Haidopoulos, Dimopoulos, & Panagiotakos, 2011). Beneficial health effects of olive oil are attributed to its high content of monounsaturated oleic acid as well as the presence of a myriad of biologically active minor components, which include a broad range of phenolic compounds, squalene, tocopherols, and sterols (Boskou, 2009; Pérez-Jiménez et al., 2007).

Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is a naturally occurring terpenoid hydrocarbon produced by all plants and animals including humans, where it is one of the main components of skin surface lipids (Huang, Lin, & Fang, 2009). It is widely used by the cosmetic industry as well as in the pharmaceutical industry for preparation vaccine and drug delivery emulsions (Fox, 2009). The main source of squalene has been traditionally shark liver oil, though vegetable sources are becoming increasingly important. Olive oil is one of the richest sources of squalene, which accounts for more than 50% of the unsaponifiable matter (Boskou, 2009). Squalene content in virgin olive oil typically ranges from 200 to 7500 mg kg^{-1} (Boskou, 2009) though values up to 12,000 mg kg^{-1} have been reported in virgin olive oils (Lanzón, Albi, Cert, & Gracián, 1994). A high influence of the cultivar on this trait has been reported (Manzi, Panfili, Esti, & Pizzoferrato, 1998). Squalene plays a major role in olive oil quality, as a number of studies have demonstrated a significant role of squalene in cancer prevention (Huang et al., 2009).

Phytosterols or plant sterols are natural constituents of plants with similar chemical structure and biological function as mammalian cholesterol (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). Because of their chemical similarity, dietary phytosterols reduce intestinal absorption of cholesterol and subsequently serum cholesterol levels (Plat & Mensink, 2005). Phytosterols and derived products are widely used as ingredients of functional foods (Bacchetti, Masciangelo, Bicchiega, Bertoli, & Ferretti, 2011). Vegetable oils and oil-based products are the richest dietary sources of phytosterols, followed by cereal grains, cereal-based products, and nuts (Piironen et al., 2000). Virgin olive oil has moderate sterol content, typically between 1000 and 2000 mg kg^{-1}, compared to larger contents is seed oils such as soybean (2290 to 4590 mg kg^{-1}), sunflower (3740 to 7250 mg kg^{-1}), rapeseed (4130 to 9790 mg kg^{-1}) and corn (8090 to 15,570 mg kg^{-1}). The sterol fraction in olive oil is largely dominated by β-sitosterol, which typically accounts for 75 to 90% of the sterols, and to a lesser extent by

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Δ^5-avenasterol, which usually ranges between 5% and 20% though larger concentrations up to 36% have been reported for some cultivars (Boskou, Blekas, & Tsimidou, 2006). For virgin olive oil, the EU regulation establishes a minimum sterol content of 1000 mg kg^{-1} and a minimum concentration of 93% of apparent β-sitosterol, which includes both β-sitosterol and Δ^5-avenasterol together with other minor sterols (European Comission, 2002). The concentration of Δ^5-avenasterol is important for olive oil quality, as the presence of an ethylidene group in this sterol confers exceptional antioxidant properties at high temperatures (Rossell, 2001). Total sterol content and profile in olive oil largely depends upon the cultivar, environmental conditions, stage of maturity of olives, and extraction method (Aparicio & Luna, 2002).

Understanding how biologically-active components accumulate during olive development is important to determine the optimal harvest time. The accumulation of squalene has been scarcely studied. Sakouhi, Herchi, Sbei, Absalon, and Boukhchina (2011) characterized squalene accumulation in olive oil extracted from olives harvested from 21 to 38 weeks after flowering. In relation to sterols, several studies have evaluated the evolution of the sterol profile during olive development, concluding that there is a reduction in the concentration of β-sitosterol and a concomitant increase in the concentration of Δ^5-avenasterol during olive ripening (Ajana, El Antari, & Hafidi, 1998; Aparicio & Luna, 2002; Frega & Lercker, 1986; Gutiérrez, Jiménez, Ruíz, & Albi, 1999; Vekiari et al., 2010). As these studies were conducted on the extracted oils, changes in sterol profile associated with olive maturation have been explained on the basis of a reduction in the proportion of stone in olive weight during ripening, as the oil from stones has a high concentration of β-sitosterol but a low concentration of Δ^5-avenasterol (Aparicio & Luna, 2002). The objective of the present research was to study the evolution of sterols and squalene during olive ripening by analysing the compounds directly on olive flesh from olives of four cultivars.

2. Materials and methods

2.1. Plant materials and cultivation

Two Spanish cultivars, 'Picual' and 'Arbequina', and two selections derived from crosses between them were used. Selection 1 is derived from 'Arbequina' × 'Picual' cross, whereas Selection 2 is derived from 'Picual' × 'Arbequina' cross. The cultivars 'Picual' and 'Arbequina' were selected because they show large differences for overall oil composition, including sterol profile (Wiesman, 2009). The two selections come from crosses carried out in 1998/99 and were selected from the initial population according to the procedure followed in the breeding program, mainly on the basis of their early crop (short juvenile period) and high oil content (León, Rallo, Del Río, & Martín, 2004). After vegetative propagation, a comparative field trial was established in open field in June 2007 at 6 × 5 m spacing. Trees were trained as single trunk vase with 3–4 main branches and minimal pruning was carried out to allow early bearing. Around 0.5 kg fruits per tree were collected once a month, from September to December 2010, in three trees per genotype. Fruits were collected from different tree areas to be representative of tree variation.

2.2. Analysis of fruit traits

The ripening index of samples was determined based on color changes of peel and pulp according to the procedure described by Frías et al. (1991). Fruit fresh samples were weighted and then dried in a forced-air oven at 105 °C for 42 h to determine fruit weight and moisture content. Oil content of dried samples was recorded by NMR Minispec NMS100 (Bruker Optik GmbH, Ettlingen, Germany) and afterwards expressed as percentage on fresh weight basis.

2.3. Analysis of sterols and squalene

Twelve fruits were randomly chosen from each sample of around 0.5 kg fruits collected from individual trees. They were stored at −80 °C shortly after harvest and lyophilized. After lyophilization, the stones were removed and the flesh was milled in a laboratory ball mill. The samples were then stored at −20 °C till analysis, usually within 48–72 h. Sterols and squalene contents in olive flesh samples were analysed by GC of the unsaponifiable fraction following silylation, without preliminary thin-layer chromatography fractionation. This approach has been used for the analysis of sterols in rape seeds (Amar, Becker, & Möllers, 2008; Gül & eker, 2006), sunflower seeds (Fernández-Cuesta, Aguirre-González, Ruiz-Méndez, & Velasco, 2012; Fernández-Cuesta, Nabloussi, Fernández-Martínez, & Velasco, 2012), and olive oil (Gül & eker, 2006), as well as both sterols and squalene in pumpkin seeds (Martínez-Aguilar, Martínez-Yero, Córdova-López, Valdivié-Navarro, & Estarrón-Espinosa, 2011) and olive oil (Giacometti, 2001). Two-hundred milligram of ground olive flesh were placed in 10-mL propylene tubes. Two-hundred microliter of internal standard solutions prepared by dissolving 5α-cholestan-3β-ol (Cat. No. D6128, Sigma-Aldrich, St. Louis, MO, USA) and squalene (Cat. No. S3626, Sigma-Aldrich), respectively in hexane-ethanol (3:2) solutions at a concentration of 0.1% were added. Alkaline hydrolysis was performed by adding 2 mL of a solution of potassium hydroxide dissolved in ethanol at a concentration of 2%. After vortexing, the tubes were left in a water bath at 80 °C for 15 min. The unsaponifiable was extracted by vortexing with 1 mL hexane and 1.5 mL water. The upper hexane layer was transferred to 2-mL glass vials that were maintained in an oven at 37.5 °C overnight. Fifty microliter hexane and 50 μL silylating mixture composed of pyridine:hexamethyldisilazane:trimethylchlorosilane 9:3:1 by vol (Cat. No. 355650.0922, Panreac Química, Barcelona, Spain) were added to the dried pellets and the vials were left at room temperature for 15 min. The solution was transferred to 2-mL vials containing 200 μL inserts and centrifuged at 4,000 rpm for 10 min. The vials were capped and conserved at −20 °C until analysis, usually within 24 h of preparation. Gas chromatographic analyses were performed on a Perkin Elmer Clarus 600 GC (Perkin Elmer Inc, Waltham, MA, USA) equipped with a ZB-5 capillary column (id = 0.25 mm, length = 30 m, film thickness = 0.10 μm; Phenomenex, Torrance, CA, USA) using hydrogen as carrier gas at a pressure of 125 KPa. Split injector and flame ionization detector were maintained at 320 °C. The oven thermal regime was the following: initial temperature of 240 °C was increased at 5 °C min^{-1} to final temperature of 265 °C and held for 10 min. Total analytical time was 15 min. Total phytosterol content

Table 1
Analysis of variance (F values) for squalene and sterol contents, concentrations of β-sitosterol and Δ^5-avenasterol, fresh fruit weight, oil content, and ripening index (RI) in four olive cultivars grown at Córdoba, Spain, harvested at four dates from September to December 2011.

Source of variation	DF[a]	Squalene (mg kg^{-1})	Sterols (mg kg^{-1})	β-sitosterol (%)	Δ^5-avenasterol (%)	Fruit wt (g)	Oil (%)	RI
Genotype (G)	3	204.9^{**b}	51.7^{**}	160.9^{**}	137.4^{**}	247.7^{**}	62.6^{**}	1.8^{ns}
Date (D)	3	14.1^{**}	116.3^{**}	129.1^{**}	138.3^{**}	87.1^{**}	163.2^{**}	125.4^{**}
G x D	9	0.2^{ns}	2.3^{*}	9.1^{**}	13.1^{**}	6.8^{**}	2.4^{*}	0.3^{ns}

[a] DF, degrees of freedom.
[b] **,*: significant at the 1% and 5% probability levels, respectively; ns: not significant.

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