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Isoprenoids composition and colour to differentiate virgin olive oils from a specific mill

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ABSTRACT

The objective of this work was verify if, based on the concentration of some key isoprenoids and the colour, olive oils from a specific mill could be differentiated from those from other mills by linear discriminant analysis. The isoprenoids studied were β-carotene, lutein, α-tocopherol, β-sitosterol, pheophytin *a* and squalene, which are isoprenoids of interest in food science and nutrition. 88% of correct classification was obtained by linear discriminant analysis and, therefore, a good differentiation was achieved. This fact can reveal the impact of the conditions of elaboration of each particular mill on the isoprenoid content of the olive oil and therefore on its colour, which is a characteristic of foods with a high effect on consumers.

1. Introduction

The nutritional quality of olive oil concerns increasingly to both consumers and health systems. Olive oil consumption has been associated with a lower risk of developing diseases like myocardial infarction, atherosclerosis and certain cancers, among others (Fitó, De la Torre, & Covas, 2007; Pérez-Jiménez, Ruano, Pérez-Martínez, López-Segura, & López-Miranda, 2007). Until recently, most of the protective effects were associated almost exclusively to the high proportion of unsaturated fatty acids (Pérez-Jiménez et al., 2007). However, in recent years it is becoming increasingly evident the importance that minor constituents of the unsaponifiable fraction, such as isoprenoids and phenolic compounds, have on the benefits associated with olive oil consumption (Covas et al., 2006). Within the isoprenoid fraction, there are a series of compounds like α-tocopherol, β-sitosterol, squalene, carotenoids and chlorophylls, which are of great importance. Indeed, altogether, they are related to the nutritional and sensory quality of the product.

Tocopherols exhibit vitamin E activity. They are considered potent lipophilic antioxidants that can protect membrane lipids from oxidation, although non-antioxidant biological actions of these compounds have also been postulated (Azzi, 2007). They are commonly found in vegetable oils, fats and some vegetables like broccoli, celery and tomatoes (Monge-Rojas & Campos, 2011). The main tocopherol in virgin olive oil (VOO) is α-tocopherol (Haddam et al., 2014). Among phytosterols, the most abundant in olive oil is β-Sitosterol (Haddam et al., 2014). It has a recognized effect on lowering cholesterol concentrations by interfering in the absorption of cholesterol in the intestinal tract (Trautwein et al., 2003). Some studies suggest that the high concentration of squalene in olive oil could be one of the factors contributing to the anti-carcinogenic effect associated to the intake of it (Reddy & Couvreur, 2009). The major olive oil carotenoids are β-carotene and lutein (Giuffrida, Salvo, Salvo, Cossignani, & Dugo, 2011). β-carotene is a provitamin A carotenoid and continues eliciting interest as a health-promoting carotenoid. The study of the health benefits of lutein is particularly important for its roles in the macula lutea and the brain (Johnson, 2004; Krinsky & Johnson, 2005). During the extraction of olive oil, chlorophylls lose the coordinated magnesium and are transformed into the respective pheophytin derivatives, which are consequently the major chlorophyll pigments in them (Giuffrida et al., 2011). Unlike carotenoids, there are not many studies on the possible health benefits of chlorophylls (Moyano, Heredia, & Meléndez-Martínez, 2010).

The colour of foods is an attribute closely related to their

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acceptability or rejection by consumers, and therefore is very important in economic terms. Generally, the consumers associate the observed colours with possible aromas and tastes of the product (Moyano et al., 2010). The characteristic colour of an olive oil depends on the concentration and the ratio of the different pigments. These are classified in chlorophyll pigments, responsible for greenish hues, and carotenoids, responsible for yellow hues (Giuffrida et al., 2011). Autooxidation of olive oil results in losses of pigments with the concomitant decolouration (Aparicio-Ruiz & Gandul-Rojas, 2014).

In this study, both some key isoprenoids and the colour of different VOO marketed in Andalusia were determined. The main objective was to evaluate if the concentration of these isoprenoids and the colour parameters can be useful to differentiate the samples from a specific mill (SM) from those from other mills (OM).

2. Materials and methods

2.1. Reagents and standards

HPLC-grade reagents, i.e. methanol (MeOH) and methyl *tert*-butyl ether (MTBE), and α-tocopherol, β-sitosterol, β-carotene, squalene, chlorophyll *a and b* standards were supplied by Sigma-Aldrich (Steinheim, Germany). Pheophytin *a* and *b* were obtained from chlorophylls *a and b* respectively by acidification with 0.1 mol/L methanolic hydrochloric acid (Sievers & Hynnienen, 1977). Lutein was isolated from a saponified extract of green leaves by open column chromatography according to recommended procedures (Rodríguez-Amaya, 2001, chap. 3).

2.2. Samples

Thirty samples of extra virgin olive oil (EVOO) produced in different parts of Andalusia were analysed. Fifteen of them (OM) were EVOO corresponding to common brands retailed in Spain and were purchased at a local supermarket. The rest (SM) were EVOO obtained with different olive varieties in an oil mill located in Palma del Rio (Oleopalma S.A.T., Palma del Río, Córdoba, Spain) from 2015 harvest. SM samples were obtained at an industrial scale. They were taken from the deposits from where the samples that are eventually bottled and marketed are kept. Both, OM and SM sets includes samples from different olive cultivars and geographical origins, within Andalucía. However, while OM were processed in different mills, the SM set includes oils obtained according to the same technological procedures. Information on the olive variety, geographic origin and type of packing as well as the companies of both groups of samples is summarised in Supplementary Table 1. Although in most cases the conditions of production of olive oil, i.e. temperature, extraction time, etc., were not indicated, it seems sensible to assume that these conditions vary between the different mills.

2.3. Isoprenoid analysis

2.3.1. Extraction and saponification

The samples were extracted in triplicate by using the protocol described by Mínguez-Mosquera, Gandul-Rojas, and Gallardo-Guerrero (1992) with some modifications. The method is based on a liquid-liquid distribution so that there is a partition of the compounds between *N*,*N*-dimethylformamide (DMF) and hexane. In the hexane phase, which is subsequently saponified, triglycerides, carotenes (mainly β-carotene), squalene and β-sitosterol are retained and in the DMF phase, xanthophylls, tocopherols and chlorophyll pigments are extracted.

Three-gram aliquots of the EVOO were dissolved directly in 30 mL of DMF saturated with $MgCO_3$ and treated with five successive 10 mL portions of hexane. The mixtures were centrifuged at 3000 rpm for 5 min at 4 °C. The two phases were transferred to different separatory funnels.

Eighty mL of Na_2SO_4 solution (2 g/100 mL in water at approximately 0 °C), 15 mL of hexane and 15 mL of diethyl ether were added to the separatory funnel containing the DMF phase. This solution was shaken and kept approximately 20 min. Then, the lower aqueous phase was discarded. Afterward, the ether phase was washed three times with the Na_2SO_4 solution and was concentrated to dryness in a rotary evaporator at 30 °C.

The hexane phase was saponified by adding 20 mL of methanolic KOH (35 g/100 mL) and 30 mL diethyl ether. The reaction mixture was maintained with mechanical shake overnight, in darkness and at room temperature. Then, the organic phase was washed with water and a solution of 10 g/100 mL NaCl until neutrality of the waste water. Finally, the remaining water was removed with the Na_2SO_4 solution. The organic phase was concentrated to dryness by rotary evaporation at 30 °C.

The two extracts obtained from each sample (DMF and hexane extracts) were kept in the freezer under a nitrogen atmosphere. Prior to injection in the HPLC system, the two extracts were re-dissolved in 300 μL of ethyl acetate and filtered through a nylon membrane (13 mm × 0.45 μm) (Billerica, MA, USA). The injection volume was 20-μL. In samples where the concentrations of squalene and β-sitosterol were out of the linearity range of the detector, the hexane phase was further diluted with approximately 1.5 mL of ethyl acetate and 20 μL were injected.

2.3.2. Qualitative and quantitative analysis

The analysis of isoprenoids was carried out by High Performance Liquid Chromatography (HPLC) with external calibration. The concentrations of the standards were determined spectrophotometrically using the specific extinction coefficients in ethanol (Britton, 1995). To prepare the stock solutions the standards were dissolved in ethyl acetate. HPLC analyses were carried out on an Agilent Technologies 1100 system (Agilent Technologies, Palo Alto, CA, USA). A C_{30} YMC column (3 μm, 150 cm × 4.6 mm) (Wilmington, NC, USA) kept at 20 °C and a flow rate of 1 mL/min were used. The diode array detector was set at 450, 410, 290, 215 and 210 nm for the detection of carotenoids, pheophytins, tocopherols, squalene and β-sitosterol, respectively. The gradient was: 0 min: 85% MeOH +15% MTBE; 20 min: 68% MeOH +32% MTBE; 25 min: 85% MeOH +15% MTBE. MeOH contained a small proportion of ammonium acetate (0.1 g/100 mL) in order to protect the carotenoids during the chromatographic analysis and to improve the recovery of them from the column.

The identification of the isoprenoids was made by comparison of their spectroscopic and chromatographic features with those of standards. For chlorophyll pigments and derivatives, the characteristic peak ratio was that between the absorbance of the Soret band (I) and the absorption maximum in the red region (II). For carotenoids, the height of the largest wavelength absorption band (III) was expressed as a percentage of that of the middle absorption band (II) (Rodríguez-Amaya, 2001, chap. 3). To identify the *cis* isomers of carotenoids the hypsochromic shifts of the absorption maxima relative to those of the corresponding all-*trans* isomers and the intensity of the *cis* peak (measured as D_B/D_{II}) have been taken into account (Rodríguez-Amaya, 2001, chap. 3).

2.4. Colour measurement

Colour measurements were made on a Hewlett Packard 8453 UV–Vis diode array spectrophotometer (Palo Alto, CA). Samples, without dilution, were filtered through paper filter. The entire visible spectra of the filtered samples in 5-mm pathlength quartz cuvettes were recorded. n-Hexane was used as blank reference due to its transparency and lipophilicity (Moyano, Meléndez-Martínez, Alba, & Heredia, 2008a). The colour parameters under CIE Illuminant D65 and 1964 Standard Colourimetric Observer were obtained by means of the software CromaLab© (Heredia, Álvarez, González-Miret, & Ramírez, 2004).

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