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On the glucoside analysis: Simultaneous determination of free and esterified steryl glucosides in olive oil. Detailed analysis of standards as compulsory first step

Raquel B. Gómez-Coca *, María del Carmen Pérez-Camino, Wenceslao Moreda

Instituto de la Grasa – CSIC, Avda. Padre García Tejero 4, E-41012 Sevilla, Spain

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ABSTRACT

This work covers two important gaps in the field of micronutrient databases: herein we describe a short and easy protocol that allows the analysis of both free and esterified steryl gulcosides in olive oil. By utilising accurate quantitative methods we achieve a better understanding of olive oil composition and health promoting properties. The procedure consists of isolating the fraction of interest through solid phase extraction, and using gas chromatography–flame ionisation detection for both identification and quantification of the derivatised species. Additionally, mass-spectrometry detection has been utilised for confirming the identity of the individual esterified steryl glucosides in some cases.

The method's limit of detection has been set at 0.37 mg/kg for each free steryl glucoside and 0.20 mg/kg for each esterified steryl glucoside, whereas the recoveries are around 96% and 77%, respectively.

Finally, we provide a complete analysis of the commercial standard for esterified steryl glucosides, since such information was not yet available.

1. Introduction

Plant glycolipids form a group of compounds consisting mainly of steryl glucosides, sphingoglycolipids, and glyceroglycolipids (Sugawara & Miyazawa, 1999). Those steryl glucosides can be found either as free molecules or as esterified compounds (Grille, Zaslawski, Thiele, Plat, & Warnecke, 2010).

Free steryl glucosides (SG) are phytosterol conjugates where one glucose moiety binds at the C3 position of the sterol residue via an acetyl bond. When this kind of sterol is esterified to a fatty acid at the C6 position of the sugar moiety, it gives rise to esterified (also named as acylated) steryl glucosides (ESG).

The positive effects of plant sterols regarding their cholesterol lowering ability have been known for almost more than sixty years and this drove the use of free sterol solutions for the treatment of pathologies related to elevated cholesterol in blood (Pollak, 1953). Later on margarine spreads, cheese, chocolates, etc. – much faster than the former pharmaceutical formulations – were manufactured as functional foods where the added free sterols (FS) had been substituted by esterified sterols (ES) by means of increasing their solubility in the lipidic matrices (MacKay & Jones, 2011). Interestingly, the observed positive effects were not only due to the added phytosterols, but also due to sterols naturally occurring in the food (Ostlund, 2002).

Concerning phytosterol conjugates (SG and ESG), the lack of knowledge about steryl glucosides and their metabolism in humans has resulted in them being cast aside in the field of food enrichment. However, between 2009 and 2011 Lin and co-workers demonstrated the potential health benefits of glucosylated phytosterols both in their free and esterified forms (Lin, Ma, Moreau, & Ostlund, 2011; Lin, Ma, Racette, Anderson Spearie, & Ostlund, 2009). For instance, reductions of around 37% in cholesterol absorption were observed in the gut of human subjects thanks to the action of SG; this decrease was comparable to 30% obtained with ES (Lin et al., 2009).

Although it is well known that the richest natural sources of dietary phytosterols are edible vegetable oils, like those from corn, palm, rapeseed or sunflower (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000), relatively little information is available regarding their glycosylated sterol content. In this sense, Nyström et al. published detailed data on the content and sterol composition of glycosylated sterols in more than forty plant matrices. This was to facilitate comparisons of the potential contribution of different foods as sources of steryl glucosides when included in the diet (Nyström, Schär, & Lampi, 2012).

Regarding the methods of analysis, research on this field has been constant since ipuranol – the first steryl glucoside relative – was isolated in 1908 (Power & Salway, 1913). We have

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* Corresponding author. Tel.: +34 954 611 550x251; fax: +34 954 616 790.

E-mail addresses: raquel.coca@ig.csic.es, raquel.coca@hotmail.es (R.B. Gómez-Coca).

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summarised the progression of such work in a previous publication, where we have described the development of a specific protocol for the characterization of the SG fraction in olive oil (Gómez-Coca, Pérez-Camino, & Moreda, 2012).

The aim of this work is to cover two important gaps: to provide a short and easy protocol that allows the analysis of both SG and ESG directly, and to provide a methodology that allows greater understanding of the chemical compositions of olive oil and other edible oils. Besides, it will contribute to set up nutrient databases of specific bioactive compounds, present in edible matrices.

2. Experimental

2.1. Reagents

The internal standard cholesterol β-D-glucoside (ChSG) was purchased from Sigma–Aldrich Co. LLC (St. Louis, Missouri, USA). The commercial mixtures of ESG and SG used as references were purchased from Matreya LLC (Pleasant Gap, Pennsylvania, USA). Chlorotrimethylsilane, hexamethyldisilazane, iodine, pyridine, and sulfuric acid were from Fluka (Buchs, Switzerland). Ethanol and methanol were supplied by Romil Ltd. (Waterbeach, Cambridge, GB), formic acid, heptane, and hexane by VWR International, LLC (West Chester, Pennsylvania, USA), sodium chloride and trichloromethane by Panreac (Montcada I Reixac, Barcelona, Spain), sodium methoxide by Acros Organics (Thermo Fisher Scientific, Geel, Belgium), and *tert*-butyl methyl ether by Scharlau Chemie SA (Barcelona, Spain). Talc was from Talcoliva (Boñar León, Spain). All chemical reagents were analytical grade.

2.2. Samples and standard solutions

Samples of blended olive oils were purchased from local markets. These samples belonged to the extra virgin olive oil (EVOO) and virgin olive oil (VOO) categories, and were named as EVOO blend I to VI, and VOO blend I to V, respectively. Varietal EVOO (arauco, arbequina, coratina, cornicabra, hojiblanca, and picual cv.) was obtained directly from producers, who also provided us with chemically refined olive oil, chemically refined olive–pomace oils, and oils at different stages of a physical refining process – lampante (crude, virgin olive oil not fit for consumption without further processing), bleached and deodorised olive oils. Varietal EVOO of blanqueta, frantoio, karolla, uovo di pichone, and verdial cultivars were directly prepared in the laboratory using an Abencor® system (Section 2.3).

Blended EVOO, blended VOO, and five out of the eight samples of lampante olive oil (LOO) analysed in this study were classified accordingly by a panel of tasters from the Instituto de la Grasa in Seville, Spain. This institution is recognised by the IOC (International Olive Council, 2004), and the panel followed the methodology on sensory analysis of olive oil provided by IOC (International Olive Council, 2011) and EU Regulation (European Commission Regulation, 1991) and also applied in other studies (Gómez-Coca, Moreda, & Pérez-Camino, 2012). The oil was graded by comparing the median value of the defects and the median for the fruity attribute with reference ranges. According to the European reference ranges (European Commission Regulation, 1991) the category EVOO refers to crude, virgin olive oil in which the median of the defects is 0, and the median of the fruity attribute is above 0; the category VOO refers to crude, virgin olive oil in which the median of the defects is above 0 but not higher than 3.5 and the median of the fruity attribute is above 0; finally, the category LOO refers to crude, virgin olive oil in which the median of the defects is above 3.5.

Crude oil chemical refining consists of degumming (for the removal of phospholipids), neutralisation with hot caustic soda (for the removal of free fatty acids), bleaching, and deodorisation. Crude oil physical refining consists of degumming, bleaching, and steam stripping to remove free fatty acids, odour, and volatile organic compounds, all in one step.

Stock solutions of ESG were prepared by dissolving the standard mixture in chloroform. Stocks solutions of SG and ChSG were made with a chloroform:methanol 2:1 (v/v) blend. In all cases the concentration was 100 μg/mL.

Samples were prepared just before the analysis in the following way: 100 μL of ChSG stock solution were introduced into a 25 mL flask and evaporated to dryness in a current of nitrogen. Then 2 g of oil were weighed into the same flask and dissolved in 4 mL chloroform.

To derivatise the hydroxyl groups, solutions consisting of pyridine:hexamethyldisilazane:chlorotrimethylsilane 9:3:1 (v/v/v) were prepared periodically and let to decant at 13 °C before use. Once the samples had been extracted, they were dried under a gentle flux of nitrogen, dissolved with 200 μL of the aforementioned mixture, and kept for 1 h at 60 °C before taking them to the gas chromatograph. Complete silylation was monitored as described previously (Gómez-Coca, Pérez-Camino, et al., 2012).

2.3. Oil extraction

Olive fruits were obtained from an irrigated orchard (drip irrigation) in the southern part of Spain, under optimal cultivation parameters. In this region winters are mild, January being the coldest month with temperatures between 5 and 16 °C, and 179 sunshine hours. Precipitations vary from 400 to 800 mm per year, and they concentrate from October to April, with December being the wettest month (95 mm). The olive plantation was in classical frame with 300 trees per hectare. Fruits from five different cultivars (blanqueta, frantoio, karolla, uovo di pichone, and verdial) were hand-picked in January, 2012. To assure maximum quality oil was extracted using an Abencor® system and a small-quantity mill simulating commercial oil-extraction (MC2 Ingeniería de Sistemas, Sevilla, Spain). The olives were crushed with an Abencor® hammer mill equipped with a 4 mm sieve. 700 g of paste were processed using the system's malaxer and centrifuge with the conditions: malaxation temperature, 30 °C; malaxation time, 20 min, plus 10 min after water addition; amount of distilled water added to the paste, 100 mL; amount of talc added, 40 g. The mixtures were left to decant before proceeding to oil separation.

2.4. Instrumentation

Gas chromatography (GC) analyses of the sterol glucosides were carried out with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an Agilent 7683B Automatic Liquid Sampler and a flame ionisation detector (FID). Data was acquired with the Agilent ChemStation for GC System program. The conditions for the GC assays were: TRB-5HT column (5% diphenyl-95% dimethylpolysiloxane; 30 m × 0.32 mm ID × 0.10 μm film; Teknokroma, Sant Cugat del Vallés, Barcelona, Spain), 1.0 μL injection volume, hydrogen carrier gas at 3 mL/min and ECP cool on-column injection. The oven temperature program was: 80 °C (1 min), 50 °C/min to 320 °C (12 min), and 40 °C/min to 360 °C (19 min). The detector temperature was 360 °C.

GC analyses of the fatty acids at the C6 position of the ESG were carried out under the following conditions: SP2380 column (poly 90% biscyanopropyl/10% cyanopropylphenyl siloxane), 60 m × 0.25 mm ID × 0.20 μm film (Sigma–Aldrich Co. LLC, St. Louis, Missouri, USA), 4.0 μL injection volume and split injection. The oven temperature program was: 170 °C (10 min), and