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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[a] *, María del Carmen Pérez-Camino[a], Wenceslao Moreda[a]

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

* Corresponding author. Tel.: +34 954 611 550 (ext. 251); fax: +34 954 616 790
Postal address: Avda. Padre García Tejero 4, 41012 Sevilla - Spain
E-mail address: raquel.coca@ig.csic.es (R. B. Gómez-Coca)

## 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 utilizing 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 ionization detection for both identification and quantification of the derivatised species. Additionally, mass-spectrometry detection has been utilized 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.

Keywords: edible oils, olive oil, Solid Phase Extraction (SPE), steryl glucosides, esterified steryl glucosides, acylated steryl glucosides.

## 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 taster 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, Racette, Anderson Spearie, & Ostlund, 2009; Lin, Ma, Moreau, & Ostlund, 2011). 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, Ma, Racette, Anderson Spearie, & Ostlund, 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 summarized 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 deodorized 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 recognized 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

125 is above 0; finally, the category LOO refers to crude, virgin olive oil in which the
126 median of the defects is above 3.5.

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

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

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

140 To derivatise the hydroxyl groups, solutions consisting of
141 pyridine:hexamethyldisilazane:chlorotrimethylsilane 9:3:1 (v/v/v) were prepared
142 periodically and let to decant at 13 ºC before use. Once the samples had been
143 extracted, they were dried under a gentle flux of nitrogen, dissolved with 200 µl
144 of the aforementioned mixture, and kept for one hour at 60 ºC before taking
145 them to the gas chromatograph. Complete silylation was monitored as
146 described previously (Gómez-Coca, Pérez-Camino, & Moreda, 2012).

147

148 *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 ºC, 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, an Abencor® system and a small-quantity mill simulating commercial oil-extraction system (MC2 Ingeniería de Sistemas, Sevilla, Spain) were used. 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 ºC; 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 ionization 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 x 0.32 mm ID x 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 ºC (1 min), 50 ºC/min to 320 ºC (12 min), and 40 ºC/min to 360 ºC (19 min). The detector temperature was 360 ºC.

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 x 0.25 mm ID x 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 ºC (10 min), and 2 ºC/min to 200 ºC (10 min). The injector and detector temperatures were 210 ºC and 240 ºC, respectively.

An Ultimate 3000 LC System (Thermo Scientific, Vertex Technics S.L., Hopitalet de Llobregat, Barcelona, Spain) equipped with an Ultimate 3000 NS pump and an Ultimate 3000 NS autosampler, was also used during the analysis of the ESG. A first stage separation of the ESG was performed on a LiChrospher Si60 column (250 x 4 mm ID, 5 µm; Merk Chemicals Spain, Mollet del Vallès, Barcelona, Spain), using an isocratic mode of 80% chloroform-20% methanol for 15 min. The flow rate was 0.4 ml/min and the column temperature was kept at 30 ºC. The injection volume was 30 µl. This system was coupled to a micrOTOF II mass spectrometer paired to an APCI source (Bruker Daltonics, Bruker Corporation in Spain, Rivas-Vaciamadrid, Madrid, Spain). The APCI inlet conditions were: heated vaporization temperature 400 ºC; curtain gas 2 bar, 8 ml/min; dry temperature 200 ºC. Further separation of the individual ESG was performed on a Kinetex C18 column (50 x 2.10 mm ID, 2.6 µm; Phenomenex, Torrance, California, USA). The elution solvent was a mixture of water+1% formic acid: methanol+1% formic acid, beginning with a 50:50 solution (10 min),

199 then 15:85 (10 min) and finally 0:100 (11 min). The flow rate was 0.8 ml/min and
200 the column temperature was 30 ºC. The injection volume was 30 µl. Here the
201 APCI inlet conditions were: heated vaporization temperature 300 ºC; curtain
202 gas, 2.5 bar, 4 l/min; dry temperature 230 ºC.
203
204 *2.5. Analysis of standards*
205 *2.5.1. Purity of the commercial products*
206 According to the manufacturers, the standards for both SG and ESG were of
207 natural origin, whereas ChSG was a synthetic product.
208 The determination of the purity (and composition) of the SG mixture had
209 been done in previous work (Gómez-Coca, Pérez-Camino, & Moreda, 2012).
210 We applied the same procedure to the ChSG and ESG standards. In short, we
211 carried out thin layer chromatography (TLC) tests in chloroform:methanol:water
212 70:15:2 (v/v/v), spraying with concentrated sulfuric acid and developing at 190
213 ºC. We repeated the tests eluting with chloroform:methanol:water 80:20 (v/v),
214 and developing in a chamber containing $I_2$.
215
216 *2.5.2. Determination of the molecular composition of the individual ESG*
217 The commercially available standard for ESG consisted of a mixture of
218 esterified phytosterol glucosides. To identify those glucosides, two approaches
219 were carried out: hydrolysis of the ESG with GC-FID analysis of the fatty acids
220 and SG released, and direct analysis of the individual molecular species by
221 normal phase (NP) and reverse phase (RP) high performance liquid
222 chromatography (HPLC) with MS detection.

223 Hydrolysis of the ESG was achieved according the following procedure: 3 ml
224 of a 100 µg commercial ESG/ml solution were evaporated to dryness under
225 nitrogen flux. We added 3 ml of a 0.119 M $CH_3NaO$ solution, and incubated for
226 one hour at 50 ºC. Next we added 3 ml of heptane and shook cautiously. After
227 phase separation, the upper layer was evaporated until a 200 µl volume was
228 reached. The unbound fatty acids as fatty acid methyl esters (FAME's) were
229 chromatographed according to the conditions given in Section 2.4.
230 Isolation of the resulting SG was carried out following the procedure
231 published by Breinhölder and coworkers (Breinhölder, Mosca, & Lindner, 2002),
232 with some modifications. After removing the upper layer, to the lower layer we
233 added 4 ml water, 0.5 ml saturated sodium chloride solution and 5 ml
234 chloroform. The mixture was shaken vigorously for one minute, 0.5 ml ethanol
235 was added and centrifuged at 2000 rpm for 10 minutes. Immediately afterwards
236 the lower phase was evaporated to dryness under nitrogen flux. We
237 chromatographed the trimethyl silyl (TMS) ether derivatives of the SG (Section
238 2.2) according to the conditions given in Section 2.4.
239 In parallel we also chromatographed an ESG solution identical to the starting
240 one (3 ml of a 100 µg/ml solution evaporated and derivatised) by means of
241 control.
242 HPLC-MS analysis was carried out as described in Section 2.4, by direct
243 injection of the ESG standard solutions.
244
245 *2.5.3. Composition of the ESG mixture*
246 The composition of the ESG mixture was determined by 13 doubled runs of
247 GC analysis (Section 2.4) of the TMS ether derivatives of the standard at three

different concentrations (10, 20, and 25 mg commercial mixture/kg oil). The relative amount of each peak was calculated on the basis of the corresponding area as a percentage of the area equivalent to the total ESG.

The ESG peaks were identified by their absolute and relative retention time (Rt), which were the result of fourteen injections of standard solutions at three different concentrations (3.92, 7.84, and 9.80 mg ESG/kg oil). The changes in the chromatographic conditions, with respect to those applied previously (Gómez-Coca, Pérez-Camino, & Moreda, 2012), made us also recalculate the absolute and relative Rt of SG. In this case, the values given are the result of seven injections of standard solutions at two different concentrations (9.80 and 24.50 mg SG/kg). The absolute Rt was always measured to three decimal places.

The Rt window for each target analyte was established by multiple GC injections of the blank sample (Section 2.7) spiked with ChSG, SG and ESG standard solutions (4.90 mg ChSG/kg; 2.45, 4.90, 7.35, 9.80, and 24.50 mg SG/kg, and 3.92, 7.84, 9.80, 11.76, and 15.68 mg ESG/kg) to compensate for shifts in absolute Rt as a result of chromatographic variability.

*2.6. Evaluation of the method*

Recovery tests to assess the reproducibility of the method, and trials to establish the limit of detection (LOD) and the limit of quantitation (LOQ) were performed using in-house blank oil labelled as *refined olive-pomace oil* (oil comprising exclusively olive-pomace oils that have undergone classical refining), which had showed no chromatographic peaks within the Rt windows of any of the glucosides under study.

The sensitivity (the minimum concentration of analyte that could be measured and reported with an acceptable confidence that it was higher than zero) was determined by spiking six samples of refined olive-pomace oil with SG and ESG standard solutions at increasingly lower concentrations. The accepted concentration values were those that produced sharp, symmetrical analyte peaks with a signal-to-noise ratio of at least 2 and with no tailing or shoulders.

To obtain recovery values, fourteen more samples of refined olive-pomace oil were spiked with the standard mixtures at different concentrations (1.43, and 2.86 mg β-sitosteryl glucoside/kg, and 4.13, and 8.26 mg esterified β-sitosteryl glucoside/kg).

*2.7. Extraction procedure and steryl glucoside quantification*

The SPE procedure developed previously to isolate SG (Gómez-Coca, Pérez-Camino, & Moreda, 2012) was utilized, with some modifications. The cartridges (ExtraBond Si-1 g; Scharlab S.L., Barcelona, Spain) were conditioned with 5 ml *tert*-butyl methyl ether, 5 ml hexane, 5 ml chloroform, and 10 ml of a newly prepared blend consisting of chloroform:methanol 4:1 (v/v). Samples were then quantitatively transferred to the cartridges using 1 ml chloroform to wash the walls of the containers. The loaded cartridges were washed with 10 ml hexane, 10 ml *tert*-butyl methyl ether and 10 ml chloroform, applied one after the other. The adsorbed SG (including the internal standard) and ESG were eluted with 5 ml of the chloroform:methanol 4:1 (v/v) solution. The procedure was carried out under negative pressure (1 in.Hg). The eluted

fractions were evaporated to dryness under nitrogen flux, derivatised and chromatographed using cool on-column injection.

The quantitative evaluation of the SG was carried out using ChSG as internal standard. The response coefficient for ChSG equalled 1. Once the areas of the ChSG and SG peaks had been determined using the integrator, we calculated the concentration of each individual SG in mg/kg oil as follows:

$$SG\ x = A_x \cdot m_{IS} \cdot 1000/A_{IS} \cdot m_S$$

Where:

$A_x$ is the peak area for steryl glucoside x,

$A_{IS}$ is the area of the ChSG peak,

$m_{IS}$ is the mass of the ChSG added, in milligrams,

$m_S$ is the mass of the sample used for the determination, in grams.

Regarding the ESG, the composition of the commercially available standard was a mixture of the same ESG expected in the real samples, therefore confirming the need to use external standardization during the quantification stage. The calibration curves were carried out by progressively diluting one ESG standard solution. The final derivatised volumes were of 500 µl. These critical volumes were measured with a calibrated 200-1000 µl precision pipette (-1.00 µl systematic error). The curves consisted of three points that encompassed the range from 15.36 to 59.09 mg ESG mixture/kg corresponding, i.e. to 3.17 to 12.20 mg esterified β-sitosteryl glucoside/kg. One specific calibration curve was prepared for each ESG.

Two separately prepared standard solutions of ESG (corresponding to 3.92 and to 7.84 mg ESG/kg) were also analysed in each GC run to check the suitability of quantitation by the external calibration curve (data not shown).

## 3. Results and discussion

### *3.1. Analysis of standards*

#### *3.1.1 Purity of the commercial products*

TLC analysis reported that the ChSG standard contained 98% ChSG minimum, whereas in the case of the ESG commercial mixture, analysis showed only 39% purity, which was in agreement with the results observed during the chromatography analysis (data not shown).

#### *3.1.2 Determination of the molecular composition of the individual ESG*

ESG are bulky molecules whose complexity deserves some considerations. From a structural point of view one can split the moiety in two parts, an SG fragment and a fatty-acyl residue. According to the manufacturer, the ESG mixture purchased was based on β-sitosteryl (6'-*O*-palmitoyl)-β-D-glucoside, and its origin was natural (from soybean extract). Therefore it was reasonable to assume that not only β-sitosteryl (6'-*O*-palmitoyl)-β-D-glucoside was to be expected in the commercial product. This was supported by observing not only one, but up to three (even four), GC-peaks (Figure 1). If one analyses the sterol composition and content of soybean oil (Moreda, Pérez Camino, & Cert, 2004), the following desmethylsterols are mainly present: β-sitosterol (35-72%), campesterol (16-34%) and stigmasterol (10-28%). There may also be small quantities of Δ7-stigmastenol (1-5.2%), Δ7-avenasterol (1-5%), and Δ5-avenasterol (1-4%). When these sterols carry a sugar residue attached at the C3 position, the order of elution from a GC column is the same as when free sterols are injected (Gómez-Coca, Pérez-Camino, & Moreda, 2012). However,

in the case of ESG, one could expect some changes depending on the fatty-acyl residue esterifying the C6 position. On the other hand, the fatty acid content of soybean oil consist of 50-62% linoleic acid (C18:2), 17-26% oleic acid (C18:1), 8-13% palmitic acid (C16:0), 4-10% linolenic acid (C18:3), and 3-5% stearic acid (C18:0) (Zamora, & Hidalgo, 2004), thus the number of possible ESG combinations.

After hydrolysing the ESG present in the standard mixtures, it was clear that under the working conditions, the molecules broke at the ester bond formed between the fatty acid at C6 position and the sugar residue. Actually, GC-results showed four unique SG peaks and four major fatty acid signals.

The profile of the released SG corresponded to that obtained previously in the analysis of the SG standards (Gómez-Coca, Pérez-Camino, & Moreda, 2012) therefore, we concluded that the sterol residues of the ESG commercial mixture were campesterol, stigmasterol, β-sitosterol and Δ5-avenasterol. On the other hand, the relative proportions of the four SG released after hydrolysis were the same, within the error limits, to those of the four ESG showed in the gas chromatograms; that is ca. 25%, 20%, 52% and 3% for (esterified) campesteryl glucoside ((E)CSG), (esterified) stigmasteryl glucoside ((E)SSG), (esterified) β-sitosteryl glucoside ((E)BSSG), and esterified Δ5-avenasteryl glucoside ((E)AvSG), respectively. This indicates that the ESG elute in the same order as the free sterols and SG, and that there is no influence (beyond that of increasing the temperature of evaporation) of the attached C6-fatty acid under the applied GC conditions.

Regarding the analysis of the fatty-acyl residues, four fatty acids were predominantly present: linoleic acid (30.44 ± 0.22%), palmitic acid (28.76 ±

372 0.05%), stearic acid (12.40 ± 0.34%) and oleic acid (12.96 ± 0.39%). Small
373 amounts of linolenic acid and of behenic acid (C22:0) were also detected.
374 These results are the average of three independent measures.

375 To confirm the identity of the four GC peaks corresponding to the esterified
376 steryl glucosides in the standard mixture, a 39.19 µg ESG/ml solution was taken
377 to both NP and RP HPLC-MS, where all spectra were acquired by APCI in the
378 positive ion mode (Section 2.4).

379 In NP HPLC-MS the predominant ions in the mass spectra were $[M\text{-}H_2O+H]^+$.
380 In these spectra five major ions (those with intensity higher than $5{\cdot}10^{-5}$) could be
381 identified, which were tentatively assigned to: β-sitosteryl (6'-*O*-linoleoyl)-β-D-
382 glucoside, (*m/z* = 821), β-sitosteryl (6'-*O*-palmitoyl)-β-D-glucoside (*m/z* = 797),
383 stigmasteryl (6'-*O*-linoleoyl)-β-D-glucoside (*m/z* = 819), campesteryl (6'-*O*-
384 linoleoyl)-β-D-glucoside (*m/z* = 807) and stigmasteryl (6'-*O*-stearoyl)-β-D-
385 glucoside (*m/z* = 823). The ions with intensities between $5{\cdot}10^{-5}$ and $2.5{\cdot}10^{-5}$
386 were: campesteryl (6'-*O*-palmitoyl)-β-D-glucoside (*m/z* = 783), stigmasteryl (6'-
387 *O*-palmitoyl)-β-D-glucoside (*m/z* = 795), β-sitosteryl (6'-*O*-stearoyl)-β-D-
388 glucoside, (*m/z* = 821), and campesteryl (6'-*O*-oleoyl)-β-D-glucoside (*m/z* =
389 807). The presence of Δ5-avenasteryl (6'-*O*-palmitoyl)-β-D-glucoside (*m/z* =
390 795) and of Δ5-avenasteryl (6'-*O*-oleoyl)-β-D-glucoside (*m/z* = 821) could be, so
391 far, neither confirmed nor rejected. These ions have been given in decreasing
392 order of signal intensity.

393 In RP HPLC-MS we scanned the $[M+H_2O]^+$ and the $[\text{aglycone-}H_2O+H]^+$ ions
394 from those ESG which were to be present according to the preliminary results.
395 Those ESG may consist by different combinations between CSG, SSG and
396 BSSG, and stearic acid, oleic acid and linoleic acid. We assumed the presence

of EAvSG, since this has the same molecular weight as the corresponding ESSG. This analysis showed that our commercial standard had the following composition for the ECSG: 7.0% campesteryl (6'-*O*-stearoyl)-β-D-glucoside (*m/z* = 818 and *m/z* = 383, respectively), 10.9% campesteryl (6'-*O*-oleoyl)-β-D-glucoside (*m/z* = 844 and *m/z* = 383, respectively) and 7.4% campesteryl (6'-*O*-linoleoyl)-β-D-glucoside (*m/z* = 842 and *m/z* = 383, respectively); for the ESSG (and EAvSG): 7.8% stigmasteryl (6'-*O*-stearoyl)-β-D-glucoside (*m/z* = 830 and *m/z* = 395, respectively), 4.7% stigmasteryl (6'-*O*-oleoyl)-β-D-glucoside (*m/z* = 856 and *m/z* = 395, respectively), and 11.7% stigmasteryl (6'-*O*-linoleoyl)-β-D-glucoside (*m/z* = 854 and *m/z* = 395, respectively); finally, the EBSSG is a mixture of: 15.8% β-sitosteryl (6'-*O*-stearoyl)-β-D-glucoside (*m/z* = 832 and *m/z* = 397, respectively), 3.4% β-sitosteryl (6'-*O*-oleoyl)-β-D-glucoside (*m/z* = 858 and *m/z* = 397, respectively), and 31.2% β-sitosteryl (6'-*O*-linoleoyl)-β-D-glucoside (*m/z* = 856 and *m/z* = 397, respectively).

*3.1.3 Composition of the ESG mixture*

From HPLC-MS results we could only be sure of the presence of three ESG (ECSG, ESSG and EBSSG)whereas the GC-FID analysis might show four peaks, which we identified as ECSG, ESSG, EBSSG and EAvSG based on the results obtained with the SG standard mixture (Figure 1). The proportions (taking into account 39% purity) were: 9.97% (2.03% Relative SD), 7.09% (8.46% Relative SD), 20.64% (7.49% Relative SD) and 1.49% (10.37% Relative SD), respectively. These quantities were utilized to calculate the concentration of each glucoside in the standard solutions and to build the calibration curves.

421 The relative Rt values were kept constant all over the study. Those values
422 and the Rt windows for both SG and ESG are shown in Table1 and Table 2,
423 respectively.
424
425 *3.2. Evaluation of the method*
426 In previous work we proved that, in olive oil, the lowest concentration of
427 BSSG detectable to fulfil a number of predetermined conditions was 0.37 mg/kg
428 (Gómez-Coca, Pérez-Camino, & Moreda, 2012). Consequently we focused on
429 BSSG as it was the only SG present in olive oil, but the same limit has proven
430 to be true for CSG and SSG (present in other edible oils such as soybean),
431 which was to be expected since all three different SG should have the same
432 response factor. In this way, hundred per cent of the samples (n = 6) spiked
433 with such SG concentration gave signals within the acceptance criteria. In the
434 cases of the ESG, LOD was 0.20 mg/kg for the three species tested (ECSG,
435 ESSG, and EBSSG). That concentration always (n = 6) gave signals clearly
436 distinguishable from the background.
437 We set the empirical LOQ for the ESG at 0.37 mg/kg. According to the
438 definition of empirical LOQ (Armbruster, Tillman, & Hubbs, 1994), 0.37 mg/kg
439 will be the lowest concentration at which the acceptance criteria are met, and
440 the lowest analyte concentration (0.46 mg/kg) is (at the minimum) 20% above
441 such limit. Following the same reasoning, the LOQ for SG was established at
442 0.49 mg/kg (Gómez-Coca, Pérez-Camino, & Moreda, 2012).
443 The global recovery of the method was assessed with fourteen samples of
444 refined olive-pomace oil spiked with SG (Table 3) and ESG (Table 4) standard
445 solutions at different concentrations. In the cases of SG there was an improved

recovery with respect to that obtained previously: 96.33 ± 2% (3SD) vs. 90.13 ± 6% (3SD) (Gómez-Coca, Pérez-Camino, & Moreda, 2012), probably due to the optimization of the extraction procedure and the use of internal standardization. However, recoveries of the ESG do not reach 80%, the actual value being 76.58 ± 0.21% (3SD). This recovery was independent of the presence of the SG in the media, and it did not seem related to incomplete silylation.

*3.3. Extraction procedure and steryl glucoside quantification*

The procedure for isolating SG from olive oil samples had been previously developed. Initially the method had been applied to a small number of olive oil samples of different categories and origin, the conclusion reached was that the only SG present was BSSG, whose concentration in olive oil was never higher than 3 mg/kg. It was also clear that in olive oil, there was no correspondence between the FS profile and its SG composition (Gómez-Coca, Pérez-Camino, & Moreda, 2012), as Nyström and colleagues had pointed out in the cases of other matrices (Nyström, Schär, & Lampi, 2012).

In olive oil, the FS profile and content is of the upmost importance since both are species-characteristic. The lack of a direct and clear relationship between FS composition and SG content is of the utmost importance, as this kind of oil is constantly under surveillance due to the economic repercussions that fraud may cause.

A second point of interest was the presence of those SG in which the C6 of the glucose moiety had been esterified to a fatty acid. If there was not parallelism between FS and SG contents and compositions, why should one expect such relationship between any of them and the ESG profile? Besides,

was such profile specific of a particular cultivar or olive oil category? If so, were the predominant defects in the lowest categories related to a specific steryl glucoside composition? How were they affected during the refining processes? And, finally, were SG and ESG affected in the same way?

In order to answer all these questions we analysed the steryl glucoside content of olive oil samples from different origins and cultivars, classified under different categories and treated with two different refining processes.

Table 5 shows the results of the analysis. In the case of the SG, figures are due to BSSG, although the presence of AvSG is also possible. However, the same is not true for the ESG counterparts. Mass spectrometry results confirmed that in most of the samples, the only ESG present was that corresponding to β-sitosterol (possibly with Δ5-avenasterol derivatives), but in some cases (e.g. VOO cv. uovo di pichone) ECSG and ESSG may also contribute to the total ESG content. When considering mono-varietal oils, that is those oils made just from olives from one cultivar, the content of SG in EVOO ranged from 1.68 ± 0.08 mg/kg to 2.95 ± 0.14 mg/kg, and in VOO from 1.05 ± 0.01 mg/kg to 2.14 ± 0.10 mg/kg, meaning that they are almost the same within the error limits. As far as the ESG are concern, the concentration ranges are from 2.54 ± 0.04 mg/kg to 9.20 ± 0.36 mg/kg for EVOO, and from 3.14 ± 0.06 mg/kg to 11.95 ± 2.75 mg/kg for VOO, showing their similarity.

As far as the blends are concerned, the glucoside content depends on the oils of origin.

Interestingly, there is no relationship between the SG content and the ESG concentration. In this way, for instance, the oil with the lowest SG concentration

is not the one with the lowest ESG content, and vice versa. The ESG:SG ratio is not always above 1, although it seems to be the usual trend.

Among all samples analysed, those of LOO exhibited (again) the highest SG contents, although we could not find a relationship with the different defect.

Finally, we found the effect of the physical refining processes, whose main difference with the classical (chemical) refining is that there is no treatment with hot caustic soda. The sample labelled as 'LOO III' had been subjected to deodorization and bleaching by the manufacturer. After analysing the steryl glucosides in each of the stages, we saw the drastic effect that physical refining has on SG, whereas such an effect is much softer on the ESG. Actually, there is a decrease of around 88% in the case of SG and of 18-28% in the ESG. However, the effect of the chemical treatment is drastic in all cases, making all glucosides disappear. From this data it was clear to us that it is the acidic media created during the decolourization step that is responsible for the acetyl bond breakage, and therefore for the SG decrease. There seems to be a protective effect of the fatty-acyl residue at the C6 position of the ESG over the acetyl bond, probably due to specific molecule folding, although more studies in this area must be done.

## 4. Conclusion

The steryl glucoside composition and content may be utilized as a further chemical characterization parameter in olive oil. These glucoside compositions may help explain in more detail, the beneficial health properties of olive oil.

The only free steryl glucoside clearly present in olive oil is that derived from β-sitosterol: BSSG, whereas in the cases of ESG, other glucosides may contribute to the global content.

There is no direct relationship between the FS profile and the composition and content of SG and of ESG. The FS profile does not reflect the ESG composition.

There is no relationship between olive oil category and origin, and the ESG profile.

Refining process, especially classical refining, destroys ESG.

There are still some unanswered questions:

a) Is there any relationship between the ESG composition and content of olive oil, and any of the other legislated parameters? (e.g. free fatty acid, wax content, etc.)

b) How is it possible that the acetyl bond between the sterol moiety and the sugar residue gets hydrolysed under *in vitro* conditions but resists the extreme media of the gastro-intestinal tract? It is clear that studies on steryl glucoside metabolism remains a gap in this field.

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607

608 **LEGEND**

609 **Figure 1.** GC-FID chromatograms for steryl glucosides in spiked chemically
610 refined olive pomace oil. This chromatogram has been obtained after analysing
611 the sample according to the proposed method.
612 I. S. Internal standard Cholesterol glucoside, 1. Campesteryl glucoside, 2.
613 Stigmasteryl glucoside, 3. β-Sitosteryl glucoside, 4. (presumptive) $\Delta^5$-
614 Avenasteryl glucoside, 5. Esterified campesteryl glucoside, 6. Esterified
615 stigmasteryl glucoside, 7. Esterified β-sitosteryl glucoside, 8. (presumptive)
616 Esterified $\Delta^5$-avenasteryl glucoside.

617 Table 1. Relative retention time (Rt) values of cholesterol glucoside (ChSG),
618 campesteryl glucoside (CSG), stigmasteryl glucoside (SSG), and Δ5-
619 avenasteryl glucoside (AvSG), with respect to β-sitosteryl glucoside (BSSG),
620 with three times their SD. The corresponding Rt windows (Rt ± 3SD) are also
621 given.

| | ChSG | CSG | SSG | BSSG | AvSG |
|---|---|---|---|---|---|
| Relative Rt | 1.201 ± 0.002 | 1.095 ± 0.002 | 1.064 ± 0.001 | - | 0.990 ± 0.002 |
| Rt window [min] | 12.274 ± 0.260 | 13.926 ± 0.100 | 14.338 ± 0.100 | 15.244 ± 0.102 | 15.423 ± 0.080 |

622

623 Table 2. Relative retention time (Rt) values of cholesterol glucoside (ChSG),
624 esterified campesteryl glucoside (ECSG), esterified stigmasteryl glucoside
625 (ESSG), and esterified Δ5-avenasteryl glucoside (EAvSG), with respect to
626 esterified β-sitosteryl glucoside (EBSSG), with three times their SD. The
627 corresponding Rt windows (Rt ± 3SD) are also given.

| | ChSG | ECSG | ESSG | EBSSG | EAvSG |
|---|---|---|---|---|---|
| Relative Rt | 2.342 ± 0.011 | 1.052 ± 0.001 | 1.037 ± 0.001 | - | 0.993 ± 0.001 |
| Rt window [min] | 12.274 ± 0.260 | 28.127 ± 0.100 | 28.535 ± 0.100 | 29.533 ± 0.135 | 29.703 ± 0.114 |

628

629 Table 3. Assessment of the recovery of β-sitosteryl glucoside (BSSG) in olive
630 oil. Samples of chemically refined olive-pomace oil were spiked with standard
631 solutions at the concentrations indicated in each case.

| | | Sample + 1.43 mg/kg BSSG | Sample + 2.86 mg/kg BSSG |
|---|---|---|---|
| BSSG [mg/kg] | Trial 1 | 1.43 | 2.72 |
| | Trial 2 | 1.51[#] | 2.79 |
| | Trial 3 | 1.30 | 2.81 |
| | Trial 4 | 1.39 | - |
| | Trial 5 | 1.47[#] | - |
| | Trial 6 | 1.28 | - |
| | Trial 7 | 1.39 | - |
| | Trial 8 | 1.21 | |
| Mean value [mg/kg] | | 1.37 | 2.77 |
| SD | | 0.01 | 0.01 |
| RSD [%] | | 0.73 | 0.36 |
| Recovery [%] | | 95.80 | 96.85 |

[#] Recoveries of 107% and 103% (Trials 2 and 5, respectively) are within the AOAC accepted limits according to which concentrations of the order of magnitude of ppm must show recovery values between 80% and 115% (AOAC, 1998)

Table 4. Assessment of the recovery of esterified β-sitosteryl glucosides (EBSSG) in olive oil. Samples of chemically refined olive-pomace oil were spiked with standard solutions at the concentrations indicated in each case.

| | | Sample + 4.13 mg/kg EBSSG | Sample + 8.26 mg/kg EBSSG |
|---|---|---|---|
| EBSSG [mg/kg] | Trial 1 | 3.12 | 6.30 |
| | Trial 2 | 3.13 | 6.32 |
| | Trial 3 | 3.30 | 6.33 |
| | Trial 4 | 3.23 | 6.30 |
| | Trial 5 | 3.20 | |
| | Trial 6 | 3.03 | - |
| | Trial 7 | 2.55 | - |
| | Trial 8 | 3.40 | - |
| | Trial 9 | 3.38 | - |
| | Trial 10 | 3.38 | |
| Mean value [mg/kg] | | 3.17 | 2.77 |
| SD | | 0.06 | 0.01 |
| RSD [%] | | 1.89 | 0.36 |
| Recovery [%] | | 76.76 | 76.39 |

Table 5. Steryl glucoside (SG) and esterified steryl glucoside (ESG) contents in mg/kg of olive oil from different cultivars and categories (extra virgin, virgin, and lampante olive oils: EVOO, VOO, and LOO, respectively). Three times the standard deviation is also given.

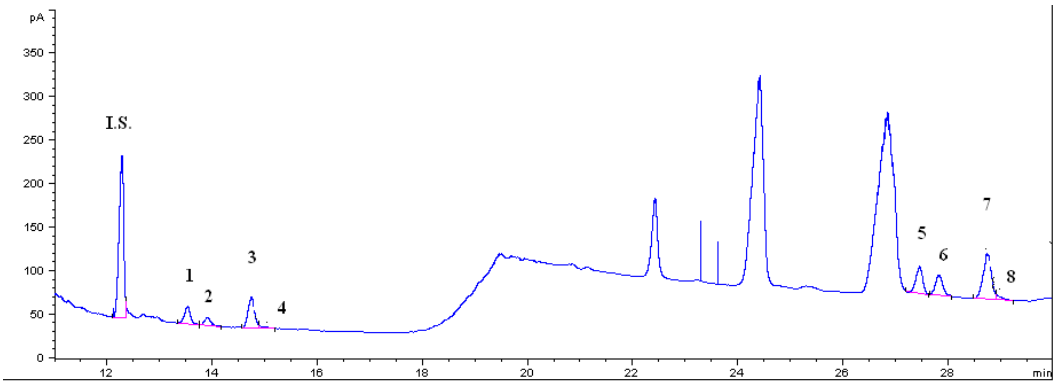
| Oil | SG [mg/kg] | 3SD (*n*)[§] | ESG [mg/kg] | 3SD (*n*)[§] |
|---|---|---|---|---|
| EVOO blend I | 1.77 | 0.06 (2) | 6.16 | 0.29 (2) |
| EVOO blend II | 2.06 | 0.01 (3) | 2.73 | 0.15 (3) |
| EVOO blend III | 1.38 | 0.04 (3) | 4.28 | 0.01 (2) |
| EVOO blend IV | 1.91 | 0.01 (3) | 5.16 | 0.09 (3) |
| EVOO blend V | 1.28 | 0.03 (4) | 3.64 | 0.30 (3) |
| EVOO blend VI | 2.08 | 0.01 (2) | 4.94 | 0.03 (2) |
| EVOO cv. Arauco | 1.68 | 0.08 (2) | 4.90 | 0.33 (2) |
| EVOO cv. Arbequina | 2.15 | 0.02 (2) | 9.20 | 0.36 (2) |
| EVOO cv. Coratina | 1.73 | 0.04 (2) | 2.54 | 0.04 (2) |
| EVOO cv. Cornicabra | 2.95 | 0.14 (2) | 2.67 | 0.19 (3) |
| EVOO cv. Hojiblanca | 1.88 | 0.01 (2) | 3.79 | 0.01 (2) |
| EVOO cv. Picual | 1.97 | 0.01 (2) | 2.78 | 0.01 (2) |
| VOO blend I | 2.60 | 0.03 (3) | 4.17 | 0.30 (2) |
| VOO blend II | 2.37 | 0.04 (2) | 3.09 | 0.01 (2) |
| VOO blend III | 1.67 | 0.01 (2) | 2.42 | 0.01 (2) |
| VOO blend IV | 0.90 | 0.01 (2) | 1.91 | 0.01 (2) |
| VOO blend V | 2.09 | 0.07 (3) | 5.87 | 0.91 (3) |
| VOO cv. Blanqueta | 1.34 | 0.01 (2) | 9.63 | 0.71 (2) |
| VOO cv. Frantoio | 1.47 | 0.01 (2) | 4.32 | 0.07 (2) |
| VOO cv. Karolla | 2.14 | 0.10 (4) | 3.14 | 0.06 (4) |
| VOO cv. Uovo di Pichone | 1.96 | 0.04 (3) | 11.29 | 2.75 (2) |
| VOO cv. Verdial | 1.05 | 0.01 (2) | 4.34 | 0.13 (2) |
| LOO I | 2.12 | 0.25 (2) | 3.44 | 0.39 (2) |
| LOO II | 2.23 | 0.01 (2) | 8.50 | 1.35 (2) |
| LOO III | 3.23 | 0.01 (3) | 6.40 | 1.02 (3) |
| Deodorized OO III | 0.36 | 0.01 (2) | 5.24 | 0.28 (2) |
| Decolorized OO III | 0.39 | 0.01 (2) | 4.64 | 0.31 (2) |
| LOO (brine: 3.5)[#] | 3.66 | 0.06 (4) | 1.61 | 0.19 (2) |
| LOO (burnt: 7.0) [#] | 1.95 | 0.01 (2) | 1.19 | 0.05 (2) |
| LOO (muddy sediment: 7.4) [#] | 1.52 | 0.04 (2) | 2.05 | 0.02 (2) |
| LOO (rancid: 4.2) [#] | 2.08 | 0.03 (3) | 2.36 | 0.05 (3) |
| LOO (winey: 4.5) [#] | 2.76 | 0.02 (3) | 5.40 | 0.01 (2) |
| Refined olive-pomace oil | 0.00 | 0.00 (3) | 0.00 | 0.00 (3) |
| Chemically Refined Olive Oil | 0.00 | 0.00 (5) | 0.00 | 0.00 (5) |

[§] *n* = number of independent measurements

[#] The main negative attributes are named according to the IOC vocabulary for sensory analysis (IOC, 2011)

632 Figure 1, Gómez-Coca et al.

633




634

635 **Highlights**

636 **Olive oil's free and esterified steryl glucosides can be analyzed**

637 **simultaneously**

638

639 **Commercial standards of esterified steryl glucosides were analysed by**

640 **HPLC-MS in detail**

641

642 **Free sterol composition and steryl glucoside profiles are independent**

643 **from each other**

644

645 **Olive oil (esterified) steryl glucoside content isn't cultivar- neither quality-**

646 **dependent**

647

648