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Specific procedure for analysing steryl glucosides in olive oil

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Steryl glucosides in olive oil

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

ABBREVIATIONS

AvSG (Δ^5 -avenasteryl glucoside), BSSG (β -sitosteryl-glucoside), CC (column chromatography), CSG (campesteryl glucoside), EVOO (extra virgin olive oil), FID (flame ionization detector), FS (free sterols), GC (gas chromatography), LOD (limit of detection), LOQ (limit of quantitation), MS (mass spectrometry), OO (olive oil), ROO (refined olive oil), Rt (retention time), SD (standard deviation), SG (steryl glucosides), SPE (solid phase extraction), SSG

26 (stigmasteryl glucoside), TLC (thin layer chromatography), TMS (trimethylsilyl),
27 VOO (virgin olive oil).
28
29

30 Avoiding any kind of oil sample pretreatment, a fraction containing the steryl
31 glucosides (SG) was directly isolated by solid phase extraction. This fraction
32 was derivatised and analysed by gas chromatography in order to quantify the
33 SG content. The limit of detection of the method was 0.37 mg/kg and the
34 recovery 90%. Additionally, the identity of the SG was confirmed by mass
35 spectrometry. We applied the procedure to oil samples of different categories
36 and origins indicating that the only SG that could be quantified in olive oil was
37 β -sitosteryl glucoside, which was present at concentrations not greater than
38 3.00 mg/kg.

39

40 **Practical applications:** Olive oil is of the utmost importance from both the
41 nutritional point of view and from the economic repercussion that fraud may
42 have. Among the many parameters required by the EU in order to determine
43 olive oil characteristics, SG are completely overlooked and no specific
44 methodology has been elaborated so far. We have developed a
45 straightforward protocol for the determination of SG in olive oil. Uses of this
46 method might be to provide with both an additional identity parameter and a
47 new blend indicator. This method is fast, cheap and easy to perform. Besides,
48 it has a great potential for automation what would allow its routine application.
49 The possibility of applying the procedure to study vegetable oils used in the
50 biodiesel industry is also pointed out.

51

52 1 INTRODUCTION

53 Plant sterols are ubiquitous molecules that, from a functional point of view, are
54 classified either as free or as conjugated phytosterols. SG are phytosterol
55 conjugates where one glucose moiety binds at the C-3 position of the sterol
56 residue via an acetyl bond. Both kinds of sterols can be esterified to a fatty
57 acid giving rise to steryl esters or to acylated steryl glycosides. In the case of
58 steryl esters the fatty acid is linked at the C-3 position of the free sterol,
59 whereas in the case of the glycosides it is bound to the C-6 position of the
60 sugar moiety.

61 The richest natural sources of dietary phytosterols are edible vegetable oils
62 like those from corn, palm, rapeseed or sunflower [1]. Within this group olive
63 oil is of the utmost importance not only from the nutritional point of view but
64 also from the economic repercussion that fraud may have [2,3].

65 One of the parameters required by International Organizations in order to
66 classify olive oil is the sterol composition, since it is considered the oil's
67 analytical fingerprint [2]. According to the Official Methods on chemical
68 analysis of olive oil the composition on the different sterols must be given as a
69 percentage of *total* sterols and the content (mg/kg) as the sum of *all* of them
70 [4]. This sterol content refers only to 4-desmethylsterols and no mention is
71 done regarding the glucosylated derivatives, which are not recovered as free
72 species after the saponification process, as are free and esterified sterols.

73 Since they were first isolated back in 1913 [5], SG have been object of a
74 wide number of studies either from a biochemical point of view –where
75 different sterol moieties and (acylated) sugar residue(s) are taking into
76 account- [6] or from an analytical one [7]. More than one decade ago a

77 number of researchers were aware of the serious errors in plant sterol values
78 due to a methodology derived from that of cholesterol analysis that totally
79 overlooked the presence of SG [1]. In the present time there exist a number of
80 analysis for SG determination both in foods and in edible oils other than olive
81 oil some of which rely on the use of acid and/or alkaline hydrolysis to initially
82 release all kinds of conjugated phytosterols present in the matrix, and proceed
83 to the subsequent analysis [8-14]. One of the disadvantages of those
84 procedures is that the liberated sterols cannot be distinguished from the
85 originally FS making the specific quantitation of the former SG impossible.
86 Other scientists have avoided the initial use of hydrolysis and determined the
87 SG content of a number of samples by isolating the corresponding fraction via
88 SPE [15-18] or CC [19]. Breinhölder and co-workers developed a quite
89 selective clean-up protocol to retain specifically the glycolipids present in a
90 lipid extract. They used 500 mg-silica cartridges, eluted the glycolipids with 15
91 mL of a chloroform:methanol (2:1, v/v) blend, evaporated to dryness and
92 dissolved in dimethyl ether. Further fractionation was carried out with a non-
93 commercial phenilboronic acid (PBA)-silica cartridge [15]. The high group
94 selectivity of the PBA represents a great advantage in comparison with the
95 selectivity exhibited by the former silica cartridge [15], but it implies additional
96 cost, preparation might be time-consuming and results difficult to reproduce.

97 The same sorbent (1 g-Si) has been used in other cases to separate
98 specifically free and esterified sterols from sterol conjugates. In this case the
99 retained FS eluted with 1 mL chloroform, whereas the SG were released with
100 2 mL methanol [16], showing the partially polar nature of these molecules. The
101 toxicity of methanol does not let it be a first-choice solvent but its use might be

justified by the need of such small volume and the fact of taking advantage of the amphipathic character of the sterol conjugates.

Nyström et al. also used 500 mg silica-columns to isolate SG from the milling fractions of wheat and rye. They utilized mixtures of heptane:diethyl ether to isolate some other sterol derivatives and acetone to elute the SG. These SG were analysed as FS after acid and alkaline hydrolysis. To protect the glycosides during the acid treatment, they added 1 mL semi-skimmed milk, which obliged to carry out an extra saponification step [17]. The same methodology but with minor changes has been applied recently to identified sources of glycosylated phytosterols in the diet [18]. Overall, if saponification is involved cost increases. Besides, there might exist incomplete recoveries during postsaponification extraction.

These procedures were not of application in olive oil. One of the reasons may rely on the extremely low concentration of this kind of sterols here (below 3 mg/kg), compared to that in other kind of samples: above 18 mg/g in rape seed lecithin, or 14 mg/g in soya bean lecithin [15], around 33 and 53 mg BSSG/100 g in dietary supplements [16], from 9.2 to 17.1 mg/kg and from 6.4 to 10.3 mg/kg in the milling fractions of wheat and rye, respectively [17], or from 838.4 to 1660.4 mg/kg in mature banana plants [20]. Furthermore the lack of detail of some of the published methodology hampers the reproducibility.

Therefore the characterization of the SG fraction in olive oil requires the development of a specific protocol. It is the aim of this study to describe in detail a simple method for SG determination in olive oil. In order to save time and resources SPE has been chosen as sample preparation technique since it

is analyte specific, allows the use of small solvent volumes (compared to those needed for CC [19]), and permits to take samples directly through the cartridge. The lack of any kind of pre-treatment keeps the glucosides intact. The extracted SG may be quantified as such by GC and taken to MS for the characterization of the molecular species.

2 Materials and method

2.1 Chemicals

The mixture of SG used as standards were purchased from Matreya LLC (Pleasant Gap, Pennsylvania, USA). Pyridine, hexamethyldisilazane and chlorotrimethylsilane, iodine, and sulfuric acid were from Fluka (Buchs, Switzerland). Trichloromethane was supplied by Panreac (Montcada I Reixac, Barcelona, Spain), hexane by VWR International LLC (West Chester, Pennsylvania, USA), *tert*-butyl methyl ether by Scharlau Chemie SA (Barcelona, Spain), and methanol by Romil (Waterbeach, Cambridge, GB). All chemical reagents were analytical grade.

2.2 Samples, sample preparation, and preparation of standard solutions

From local markets the following oils were purchased: blended and varietals EVOO (arbequina, hojiblanca, manzanilla and picuda cv.), and olive oils designated as 'mild OO' or 'intense OO' by the manufacturer. Directly from producers we got samples of: coratina EVOO and arauco EVOO, chemically refined olive oil, chemically refined olive-pomace oil, 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-

Crude-oil physical refining consists of degumming, bleaching and steam stripping in order to remove free fatty acids, odor and volatile organic compounds, all in one step.

Producers also provided us with samples of raw and refined oils other than olive oils: walnut, corn, soya bean and sunflower oils.

Raw avocado oil was purchased in the market.

Samples were prepared just before the analysis and consisted of a solution of 2 g oil in 4 mL chloroform.

Stock solutions of SG were prepared by dissolving the standard mixture in a chloroform:methanol 2:1, v/v, blend. The concentration of the reference mixtures varied between 100 µg/mL and 0.31 µg/mL.

In order 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 and then silylated with 200 µL of the aforementioned mixture. They were left thirty minutes at room temperature before taking them to the gas chromatograph. Complete silylation was monitored chromatographing the same samples (one from each olive oil category, namely EVOO, lampante OO, and ROO) once a week, checking the absence of 'double' peaks or any other change in the chromatograms.

2.3 Instrumentation

GC analyses were carried out with an Agilent 6890N Gas Chromatograph (Agilent Technologies, Santa Clara, California) equipped with an Agilent

7683B Automatic Liquid Sampler and FID. Acquisition of data was done 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 μ m film; Teknokroma, Sant Cugat del Vallés, Barcelona, Spain), 1.0 μ L injection volume, hydrogen carrier gas at 3 mL/min and ECP cool on-column injection. The oven temperature program was: 80 $^{\circ}$ C (1 min), 40 $^{\circ}$ C/min to 120 $^{\circ}$ C (2 min), and 10 $^{\circ}$ C/min to 320 $^{\circ}$ C (18 min). The detector temperature was 340 $^{\circ}$ C.

GC-MS determinations of SG were carried out with a Finnigan Trace Gas Chromatograph 2000 coupled to a Polaris Q Iontrap with electron impact ionization. The conditions were as follows: DB5-MS column (5% diphenyl-95% dimethylpolysiloxane; 30 m, 0.25 mm ID, 0.25 μ m film; Bacterius Ltd., Houston, Texas, USA), 2.0 μ L injection volume, helium carrier gas at 1.05 mL/min, split ratio 1:10 and injector temperature 300 $^{\circ}$ C. The oven temperature program followed the sequence: 285 $^{\circ}$ C (0.5 min), 2 $^{\circ}$ C/min to 325 $^{\circ}$ C (12 min). The transfer line temperature was 290 $^{\circ}$ C and the electron energy 70 eV. The use of vacuum diminished the risk of condensation in the transfer line. The scan range was 35-900 amu at 1.4 scan/s.

2.4 Analysis of the commercial mixture

The commercially available standard used in this study consisted of a mixture of four SG. According to the literature [16] CSG, SSG, BSSG and AvSG were to be present. They were eluted after the named order from the GC column under the conditions described in the corresponding Section. We used TLC analysis to double-check the purity reported by the manufacturer (98+%). We

carried out TLC tests in chloroform:methanol:water 70:15:2, v/v/v, and after air-drying the plate we sprayed it with concentrated sulfuric acid and developed it at 190 °C. Besides, we also eluted with chloroform:methanol:water 80:20, v/v, and developed in a chamber containing I₂. The composition was determined by 10 doubled runs of GC analysis of the TMS ether derivatives of the standards at two different concentrations (0.6 and 9.8 mg SG 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 SG.

The peaks were identified by their absolute and relative Rt which were the result of eight injections of standard solutions at two different concentrations (0.6 and 9.8 mg/kg).

To control the robustness and reproducibility, and to compensate for minor shifts in absolute Rt as a result of chromatographic variability, Rt windows were set. We established the Rt windows for each target analyte by multiple GC injections of spiked samples at different concentrations (0.49, 1.25, 2.13, 2.49, and 4.23 mg/kg). The absolute Rt for each analyte was measured to three decimal places.

Additionally, we carried out GC-MS tests to confirm the identity of the glucosides.

2.5 SG extraction

A SPE procedure under negative pressure was used to isolate the SG. A 1 g silica cartridge (Varian, Inc., Harbor City, CA) was conditioned with 5 mL *tert*-butyl methyl ether, 5 mL hexane, 5 mL chloroform, and then 10 mL of a newly

prepared blend consisting of chloroform:methanol 4:1, v/v. The sample was then quantitatively transferred to the cartridge using 1 mL chloroform to wash the walls of the container. The loaded cartridge was washed with 10 mL hexane, 10 mL *tert*-butyl methyl ether, and 10 mL chloroform, applied one after the other. The adsorbed SG were then eluted with 20 mL of the chloroform:methanol 4:1, v/v, solution, divided in four portions of 5 mL each to make sure there were no losses on the stationary phase. They were derivatized and chromatographed using cool on-column injection.

2.6 Sensitivity and method validation

Recovery tests to assess the reproducibility of the method, and trials to establish the LOD and the LOQ were performed using in-house 'blank' oil labeled as ROO (oil comprising exclusively olive oils that have undergone refining). Crude oil chemical refining consists of degumming –for the removal of phospholipids-, neutralization with hot caustic soda –for the removal of free fatty acids-, bleaching, and deodorization. Beforehand, a number of aliquot-parts of this oil were taken through the whole SPE process and then chromatographed to make sure no peaks appeared within the Rt windows of the respective SG (Fig. 1a).

The sensitivity was determined by spiking a number of samples of ROO with SG standard solutions at increasingly lower concentrations. The minimum concentration of SG that could be measured and reported with an acceptable confidence that the analyte concentration was higher than zero had to be established. In order to do this concentration values that produced sharp,

symmetrical analyte peaks with a signal-to-noise ratio of at least 2 and with no tailing or shoulders were accepted.

To provide with recovery values fifteen more samples of ROO were spiked with the SG standard mixture at five different concentrations (0.49, 1.25, 2.13, 2.49, and 4.23 mg BSSG/kg), bracketing the values found so far in samples of VOO.

2.7 SG quantification

GC-MS analysis showed that the commercially available standards consisted of the same SG expected in real samples (Fig. 1b-d). This non-availability of commercial internal standards obliged to use external standardization during the quantification stage.

The calibration curves were carried out by progressively diluting one SG standard solution. The final derivatised volumes were of 500 μ L. These critical volumes were measured with a calibrated 200-1000 μ L precision pipette (-1.00 μ L systematic error). The curves consisted of five points that encompassed the range from 0.006 to 0.098 μ g SG mixture, corresponding to 0.6 to 9.8 mg SG mixture/kg oil in real samples. Absolute amounts instead of concentrations have been chosen in order to simplify data handling. We have prepared one specific calibration curve for each SG.

Separately prepared standard solutions of SG at two different concentrations (9.8 μ g/mL and 19.6 μ g/mL, corresponding to 1.0 and 2.0 mg SG mixture/kg oil in real samples) were also analysed in each GC run to check the suitability of quantitation by the external calibration curve.

3 Results and discussion

3.1 Analysis of the commercial mixture

Routine analysis of standard mixtures stored at -20 °C and at room temperature kept showing the same quantitative and qualitative results. The same holds for standard prepared samples (2 g oil in 4 mL chloroform), proving that no special conditions have to be followed during the analysis. The fact that the analytes were stable during storage conditions (-20 °C), at room temperature, throughout the method preparation phase, and in the standard prepared sample, demonstrated the ruggedness of the method.

The use of a slightly polar stationary phase, as recommended in the literature [16,19,21], provided good resolution of the peaks allowing almost baseline separation of the different TMS ether derivatives.

The relative R_t values kept constant all over the study. These values, with three times their corresponding SD, were: BSSG/CSG, 1.051 ± 0.003 ; BSSG/SSG, 1.035 ± 0.003 ; BSSG/AvSG, 0.993 ± 0.0001 .

The R_t windows ($R_t \pm 3SD$) for CSG, SSG, and BSSG were, respectively, 31.888 ± 0.120 min, 32.389 ± 0.102 min, and 33.473 ± 0.132 min. The analogous value for AvSG had to be estimated on the bases of the corresponding BSSG R_t and was calculated to be 33.710 ± 0.123 min.

Small changes in parameters such as detector temperature or temperature of the sample did not affect the results. This evidences the robustness of the method.

GC-MS confirmed the identity of CSG, SSG and BSSG. In the cases of SSG and BSSG mass spectra identical to those published by Laine and Elbein [22] were obtained whereas for CSG our results were supported by those

obtained by Phillips and co-workers [16]. Fig. 2 shows the mass spectra of the TMS ethers of CSG, SSG, and BSSG, respectively. Although, the corresponding molecular ions do not appear, the respective mass spectra showed ion fragments with masses 147, 204, 217, 331 and 361. All these masses correspond to glucose ion fragments after cleavage of the C-O glycosidic bond [22,23]. We also observed characteristic ions for stigmasterol and sitosterol residues at m/e 395 and 397 [22] and at m/e 383 for campesterol [16], after cleavage of the C-O bond. Mass 395 together with the equivalent GC peak, associated to that of BSSG, support the possible presence of the glucoside of Δ^5 -avenasterol. This is in good agreement with the evidences showed previously in the literature [16].

The quantitative composition of the standard mixture was carried out as explained in the corresponding Section. TLC analysis reported that the standard mixture contained 98% minimum total SG. After checking that the mixture contained CSG, SSG and BSSG, and assuming also the presence of AvSG, the relative amount of each of them turned out to be 25.82% (0.48% Relative SD), 13.35% (1.16% Relative SD), 58.42% (0.15% Relative SD) and 2.41% (8.92% Relative SD), respectively. Thereafter, the concentration of each glucoside in the standard solutions was calculated taking into account its relative quantity together with the 98% purity value. These values were utilized to build the calibration curves.

3.2 SG extraction

If we considered olive oil analysis, SG would belong to a wide group of substances contained in what is called 'minor compounds'. The determination

of this group of compounds has proven to be a valuable tool in oil characterization and efforts have been made to systematize its analysis in both edible and non-edible matrices [15-17,19]. Traditionally this determination involves the extraction of the unsaponifiable matter, its fractionation and later analysis following a specific protocol [24]. All of them share the pass through GC as a last step for their evaluation [4].

Our aim when choosing SPE as a first stage in the analysis of SG was saving resources, since just the extraction of the unsaponifiable matter is time-consuming, requires considerable volumes of solvents, and there might be losses during the extraction stage [4]. The same holds for CC.

The different phases in a SPE protocol are clearly defined [25] but, as we explained before, the published procedures have not worked when applied to olive oil. One further reason might be the complexity of olive oil, where just the unsaponifiable matter, a fraction that accounts for 0.5 to 1.5% of the oil [26], includes a large amount of different minor compounds [24,26].

A second consideration related to VOO concerns the extremely small quantity of SG expected in comparison with those present in other raw materials [15,16,19,21,27], which obliges to use relatively high quantities of samples (2 g oil) to detect the analyte within a certain confidence interval.

Apart from dealing with olive oil's distinctive properties, one has to overcome obstacles characteristic of the method like solvent evaporation, non-constant flow rates, loss of analyte, or co-elution of undesirable compounds.

Differences in the evaporation rates of the solvents were not significant. Flow rate was controlled working under vacuum. During the conditioning and selective washing processes flow rates were from 5.0 to 9.5 mL/min. The

sample solution passed slowly through the sorbent, generally at 1.2 mL/min, never exceeding 2.0 mL/min. Finally analyte elution took place at 10 mL/min. Faster or slower rates in any of the steps resulted in poorer recoveries.

Extraction of the SG from the samples was carried out by selective retention of the analyte. SG are molecules with a bulky hydrophobic part (the sterol fragment) and a significant hydrophilic portion (the sugar residue). The fact of avoiding any pre-treatment of the samples, and therefore keeping a non-polar matrix, allowed the use of a hydrophilic sorbent. Normal phase cartridges selectively retained polar compounds, present at very low concentrations.

To make the distribution constant of the SG between the sorbent and the matrix optimal and get a more favourable partition ratio (better recoveries) several solvents and solvent combinations were tested according to the nature of both the interferences and the compounds of interest. Owing to their lipidic origin most of the interferences were easily eluted with non-polar organic solvents like hexane and *tert*-butyl methyl ether (originally diethyl ether). Chloroform, the sample solvent, acted also as washing solvent. Due to the hydrophilic nature of the sugar residue, selective elution of the SG required a solvent with slightly higher dielectric constant. Different solvent mixtures were tried and a chloroform:methanol 4:1, v/v, solution presented the optimal strength. More environmentally friendly solvents like acetone or ethyl acetate did not allow recover the SG fraction.

3.3 Sensitivity and method validation

In olive oil, the lowest concentration of analyte detectable to fulfil a number of predetermined conditions (LOD) was determined to be at 0.37 mg/kg. Hundred

percent of the samples ($n = 6$) spiked with this amount of BSSG gave signals within the acceptance criteria although the fact of being working at the analytical low level made us accept relatively small and broad peaks. Lower concentrations failed to meet acceptance criteria and produced peaks that couldn't be clearly distinguishable from background or 'blank' even if one could tentatively identify them as BSSG on the bases of their retention time.

Normally the LOQ is set at a higher concentration than the LOD. According to the statistical method it is ten times the standard deviation above the mean blank value thus presenting a greater probability that a value at the LOQ is real and not just a random fluctuation of the blank reading [28]. At the same time, the empirical LOQ is defined as the lowest concentration at which the acceptance criteria are met and the quantitative value is within $\pm 20\%$ of the target concentration [29]. According to our results on EVOO the lowest concentration to be expected was around 1.4 mg/kg BSSG. Applying the aforementioned reasoning the LOQ should be at least of 1.1 mg/kg BSSG. We set such LOQ at 0.49 mg/kg ($n = 7$) bracketing also the concentrations expected for olive oil of lower quality.

The global recovery of the method was assessed with 21 samples of ROO spiked with SG standard solution at five different BSSG concentrations (0.49 mg/kg, 1.25 mg/kg, 2.13 mg/kg, 2.49 mg/kg, and 4.23 mg/kg). The recovery of BSSG in olive oil was determined to be $90.13 \pm 6\%$ (Table 1). All values were in agreement with AOAC guidelines according to which concentrations of the order of magnitude of ppm, require recovery limits between 80% and 115% [30].

The separately chromatographed SG standard solutions, analysed in each GC run, resulted in a deviation of (8%) from the expected BSSG concentration, indicating acceptable quantitation by the external calibration curve.

3.4 SG quantification

In order to test the utility of this method several olive oil matrices were analysed and their BSSG content quantified according to its calibration curve ($y = 25728,38x - 2.56$). In line with what we thought, very low content of SG were found. In all of them, the SG composition consisted exclusively of BSSG, which was confirmed through MS too. Although in some cases the presence of AvSG is suspected, the signal was too low to be clearly distinguishable from the background noise. We found no trace of CSG, neither of SSG. Under standard conditions VOO has high β -sitosterol content, mainly followed by campesterol and stigmasterol [24] and, even if a certain correspondence between the SG content of a given species and its free sterol composition may be expected [6], it has been proven otherwise [18], supporting our results.

Lampante olive oil possesses the highest content in SG of all analysed olive oil samples, followed by EVOO (blend or varietal), intense OO, and mild OO (Table 2). This is in good agreement with the amount of VOO present in each sample: the higher the VOO/ROO ratio (intense vs. mild), the higher the SG content. Results also supported our decision of using chemically refining olive oil as reference matrix instead of physically refined olive oil, since it is clear the complete absence of SG in the first case whereas physical refining still

respects some of the SG content as shown by the analysis of both deodorized and decolorized oils.

The method has also been applied to oil matrices other than olive oil. Samples of raw oils presented SG content much higher than their refined counterparts. Those were 2.68 ± 0.10 , 9.48 ± 0.34 , 20.23 ± 0.17 , and 28.77 ± 3.9 mg/kg (three times the standard deviation is given), in the cases of avocado, walnut, sunflower, and soya bean oils, respectively. These quantities were due to the presence of BSSG, except for soya bean oils, where all four SG (CSG, SSG, BSSG, and AvSG) could be detected and quantified. Therefore the given number comes from the sum of all of them. The gas chromatograms of corn oil samples presented many interfering peaks, making the quantification impossible.

Except for refined corn oils, where the BSSG amounted to 0.56 ± 0.01 mg/kg (3SD), in all other cases the refining process made the SG concentration drop below the method's LOQ (0.49 mg/kg), although BSSG was always detected.

4. Additional remarks

The presence of SG is not just to be considered in edible matrices. The biodiesel industry has been dealing with problems caused by these compounds ever since the beginning. Biodiesels are liquids produced from vegetable oil or animal fat through a trans-esterification process. Sometimes a white insoluble product appears in manufacturing facilities, transport vessels, storage tanks [32], and biodiesel-finished samples [27,33], causing problems such as fuel filter plugging which provokes the interruption of fuel flow towards

the engine [27,32,33]. The composition of those insoluble 'flakes' depends on the nature of the feedstock and in some cases SG can be the major cause of precipitate formation [32-35] reaching concentrations above 150 mg/kg [27,36].

The reported values vary a great deal from one research group to the other pointing out the need of standardizing procedures.

In some cases the authors take the sample directly into the final GC vial, using pyridine to homogenize it and adding –the internal standard and- the silylation reagent [21,34]. Others carried out some kind of sample purification. This sample pre-treatment is advisable to remove or at least to reduce interfering compounds of the matrix. Isolation of SG has been achieved via CC in silica gel. After column conditioning with a blend of petroleum ether:diethyl ether (87:13) and loading, different matrix interferences, could be discarded by using a series of solvents of increasing polarity. Finally phytosterol conjugates were eluted with acetone and methanol. According to the authors CC was chosen over SPE in order to increase the quantity of the extract collected [36]. The length of time and quantity of solvent required in each CC run (at least 24 hours and 1 L eluent) justified the optimization of a SPE protocol.

In this case, direct purification of soya bean and palm oil biodiesel has been done using a 500 mg silica micro-cartridge. The samples dissolved in chloroform, were washed with methyl *tert*-butyl ether and the polar fraction (supposedly containing SG) eluted with a blend of chloroform:methanol, 2:1 [27].

5 Conclusion

A simple method for SG determination in olive oil has been elaborated. This new procedure is short, fast, cheap, and specifically developed for olive oil. According to the results shown by this preliminary study, this new procedure might be used for further olive oil characterization and it might provide with one more control parameter in olive oil assessment.

Unlike other vegetable oils, the only SG present in olive oil is that corresponding to β -sitosterol therefore, the presence of any other SG in olive oil might be considered as fraudulent-blend indicator.

As it is the case of other of other edible oils [31], refining processes affects strongly the SG content of olive oil even if a mild process at low temperature is utilized. Our results may suggest that both bleaching and alkali neutralization are responsible for the breakage of the glycosidic bond and therefore the reduction in the SG content seen in refined olive oils.

Finally, it seems that each olive oil category presents specific BSSG content, which indicates that this parameter might be considered as a possible differentiating factor.

The method can also be applied to oils other than olive oil.

After setting out the profile and minimum concentration of SG in olive oils of different varieties, origins and maturation index, all oils with SG different to those determined, or with BSSG concentrations out f the expected range, could be suspicious of blend with other vegetable oils. This shows the potential use of the method to detect adulterations, although specific measures in blended oils are needed. Presently further studies in this line are being carried out.

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507

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LEGENDS

Figure 1. GC-FID chromatograms for steryl glucosides in (a) chemically refined olive oil, (b) spiked chemically refined olive oil (2.13 mg BSSG/kg) (c) extra virgin olive oil, and (d) lampante olive oil. These chromatograms have been obtained after analysing the samples according to the proposed method. 1. Campesteryl glucoside, 2. Stigmasteryl glucoside, 3. β -Sitosteryl glucoside, 4. (presumptive) Δ^5 -Avenasteryl glucoside.

Figure 2. Mass spectra for trimethylsilyl ether derivatives of (a) campesteryl glucoside (CSG), (b) stigmasteryl glucoside (SSG), and (c) β -sitosteryl glucoside (BSSG).

Table 1. Assessment of the recovery of the β -sitosteryl glucoside (BSSG) determination in olive oil. Samples of chemically refined olive oil were spiked with standard solutions at the concentrations indicated in each case.

		Sample + 4.23 mg/kg BSSG	Sample + 2.49 mg/kg BSSG	Sample + 2.13 mg/kg BSSG	Sample + 1.25 mg/kg BSSG	Sample + 0.49 mg/kg BSSG
BSSG [mg/kg]	Trial 1	3.77	2.07	2.06	1.11	0.48
	Trial 2	3.73	2.02	1.90	1.11	0.51 [#]
	Trial 3	3.81	-	1.87	1.07	0.48
	Trial 4	-	-	-	1.13	0.46
	Trial 5	-	-	-	1.19	0.47
	Trial 6	-	-	-	1.11	0.48
	Trial 7	-	-	-	-	0.50 [#]
Mean value [mg/kg]		3.77	2.05	1.94	1.12	0.48
SD		0.04	0.03	0.15	0.04	0.02
RSD [%]		1.06	1.55	7.73	3.58	3.86
Recovery [%]		89.48	82.33	91.30	89.60	97.96

[#] Recoveries of 104% and 102% (Trials 2 and 7, 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% [30]

Table 2. β -sitosteryl glucoside (BSSG) content in mg/kg of olive oils from different origins. Three times the standard deviation is also given.

Oil	BSSG [mg/kg]	3SD (n)[§]
EVOO (blend)	1.83	0.21 (5)
EVOO var. Arbequina	1.86	0.06 (3)
EVOO var. Hojiblanca	1.65	0.21 (3)
EVOO var. Manzanilla	1.51	0.12 (3)
EVOO var. Picuda	2.18	0.09 (3)
EVOO var. Arauco	1.66	0.15 (3)
EVOO var. Coratina	1.69	0.06 (3)
Lampante Olive Oil	2.73	0.27 (5)
Deodorized Olive Oil	0.49	0.03 (5)
Decolorized Olive Oil	0.46	0.03 (5)
Refined olive-pomace oil	0.00	0.00 (3)
Chemically Refined Olive Oil	0.00	0.00 (5)
Intense Olive Oil	1.42	0.09 (4)
Mild Olive Oil	0.79	0.15 (4)

[§]n = single measurements of *different* oil samples of the same category

632 Figure 1, Gómez-Coca et al.

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635 b)

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638 c)

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641 d)

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Figure 2, Gómez-Coca et al.

a)

b)

c)