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ORIGINAL PAPER

Identification and Occurrence of Steryl Glucosides in Palm and Soy Biodiesel

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Abstract A problem of excessive sedimentation was detected in soy and palm biodiesel, preventing the product from complying with requirements on contamination/filterability. The objective of the study was to determine the nature of the sediment by different analytical techniques and to obtain data on the typical range of its components in industrially produced biodiesel samples. The sediment was investigated and the appearance of haze is linked to the presence of free steryl glucosides (FSG) above a certain concentration. This paper focuses on the original analytical approach, taking into account particular physical properties of FSG. Nuclear magnetic resonance and mass spectrometry were used as fast and reliable identification methods, without the need for a prior hydrolysis of the glucosidic bond. A GC method, including optimised sample preparation, was developed for the quantification of the FSG in biodiesel as well as in filter residues. The FSG concentrations in biodiesel produced by different processes ranged between 55 and 275 mg/kg for palm and from not detectable to 158 mg/kg for soy biodiesel.

Keywords Biodiesel · Haze · Palm · Sediment · Soy · Steryl glucosides

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Introduction

As a renewable and readily available energy resource, biodiesel is more and more finding its place as a promising alternative to the traditional petroleum fuels. The important quality of biodiesel, compared to the original vegetable oil, is that it can be used in a diesel engine without major modifications of the latter. Modern engines feature a sophisticated design involving fine openings for fuel injection, protected by dedicated filters. The content of insoluble contaminants in biodiesel is a closely monitored parameter, since an excess of them might cause operational problems in vehicles due to clogging of the engine filters.

Excessive sedimentation may occur in biodiesel well above its cloud point. This phenomenon is frequently detected in soy and palm biodiesel and induces a number of undesired consequences at both the production and quality control stages. In the beginning of crystallization a cloud of tiny particles is dispersed through the entire volume of the biodiesel. It causes a hazy appearance of the product, marked by the loss of transparency and brilliancy. As sedimentation progresses, deposits are formed on the bottom of biodiesel storage tanks. In particular cases the haze manifests itself within a short time delay after biodiesel production and at rather high temperatures (60 °C). Then

the process equipment upstream of the tank farm is affected, and frequent maintenance of fouling heat exchangers and centrifuges may be necessary. As a result, the haze impedes the product from meeting the requirements on contamination/filterability according to the biodiesel quality standards, e.g. European norm EN14214 and ASTM D6751 adopted in the US.

Recently, the problem of deposits on plugged vehicles filters was linked to the presence of free steryl glucosides (FSG) in blended fuel systems [1]. A first objective of this study was to develop a systematic and detailed analysis method for the detection and quantification of steryl glucosides in biodiesel, involving the isolation, spectrometric determination and chromatographic quantification of the compounds. Secondly, biodiesel samples were subjected to this analysis in order to confirm that steryl glucosides are indeed the main cause of haze. Sediments from storage tanks and samples of filter cake from polishing filters were examined as well in order to determine the average composition of the solid impurities.

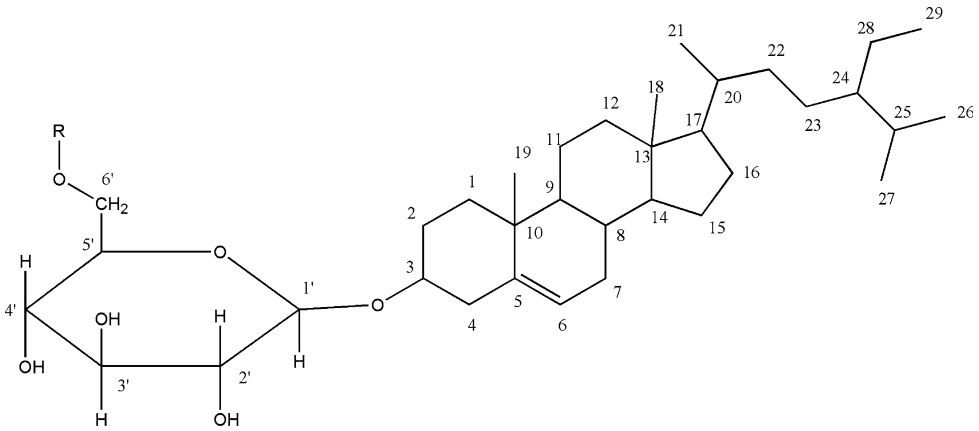
In plant tissues and in vegetable oils, steryl glucosides occur naturally in both FSG and acylated steryl glucosides (ASG) forms. In the latter, the 6-position of the sugar is esterified with a long chain fatty acid (Fig. 1). Under alkaline conditions, this ester bond between the glucose and the fatty acid is broken, and an acylated steryl glucoside is converted into its free form. Such a side reaction occurs during transesterification, resulting in an increased FSG concentration in biodiesel in comparison to their initial amount in the feedstock oil.

Pure compounds FSG and ASG exist as a white to off-white solid [2]. Considering the physical properties, it is important to note that both ASG and FSG are very high melting compounds. The acylated form melts around 197–200 °C, while FSG require heating to at least 252–254 °C (for sitosteryl glucoside isolated from soybean oil) [3], or even as high as 300–310 °C [4]. Pure FSG are best dissolved in pyridine, tetrahydrofuran, dimethyl sulfoxide and a mixture of chloroform/methanol (2:1). Dioxane and dimethylformamide were also reported as suitable solvents [5]. The present work has shown that when in contact with fatty acid methyl esters (FAME), FSG feature a high adsorption capacity and can retain efficiently up to nearly ten times their own mass.

During the last decades, different methods have been described for the analysis of steryl glucosides (both FSG and ASG) in various plant matrices, differing in isolation method, chromatographic separation technique (GC, HPLC, with or without derivatization) and direct or indirect analysis (without or with hydrolysis).

The isolation of FSG and ASG from the lipid extract can be done by column chromatography [6, 7], or faster and using less solvent, by solid phase extraction (SPE) alone [8–11], or combined with preparative TLC [12–15].

The FSG and ASG fractions were identified and quantified indirectly *by gas chromatography*, after acid hydrolysis of FSG and ASG into the corresponding free sterols [6]. Alternatively, *high performance liquid chromatography* with UV detection (254 nm) was applied, after derivatization to 1-anthroylnitriles, to quantify the FSG originally present and those obtained after hydrolysis of the ASG in alkaline medium [14, 15]. Murui and Siew reported the quantification of 1-anthroylnitriles of FSG with HPLC-UV and those of ASG with HPLC–fluorescence detection (Ex 370 nm, Em 470 nm) [12, 13]. SG and ASG could be determined, without any hydrolysis or derivatization, by TLC with colorimetric detection and quantification (densitometer) [7]. However, recently, HPLC combined with evaporative light scattering detector (ELSD) was applied successfully in the quantification of FSG and ASG [8, 16]. Phillips et al. [9] used GC combined with MS for the quantification of FSG (after silylation). The GC method



R = H : free steryl glucoside
R = $CH_3(CH_2)_nCO$: acylated steryl glucoside

Fig. 1 Structure of free and acylated steryl glucosides

results in a better separation between the different FSG than the HPLC method.

However, as discussed above, recently the need appeared for a method adapted to identify and quantify particularly the FSG in biodiesel samples. The few methods described recently involve the separation of the FSG from the methyl esters by SPE, followed by HPLC–ELSD [10] or GC, quantifying as silylated FSG with a standard curve of pure FSG [11].

The main interest of this study was the content of these compounds in soybean and palm oil and in the biodiesel therefrom. The content of free and ASG in crude palm and soybean oil varies greatly as reported by different research groups. Analysing seven samples of crude palm oil, Homberg and Bielefeld found 2–16 mg/kg FSG and 54–340 mg/kg ASG [7]. Similar concentration ranges were reported by Murui and Siew from analysis of eight crude palm oil samples of Malaysian origin: 8–81 mg/kg FSG and 173–352 mg/kg ASG [13]. However, much higher concentrations were detected in palm oil samples from India: 686 and 2,212 mg/kg for FSG and ASG, respectively [17]. A few data have been published about the content of these compounds in crude soybean oil: one study reported steryl glucoside content as high as 2,300 mg/kg [18], while only 300 mg/kg of FSG were found in another study [19].

In crude biodiesel, before any washing procedure was applied, FSG content of 115 and 75 mg/kg was found for samples prepared from palm and soybean oils, respectively [11]. In finished biodiesel from once-refined and refined-bleached soybean oil, the amount of FSG is slightly lower: 78 and 64 mg/kg [5]. Ringwald and FutureFuel Company detected varying contents of FSG in biodiesel produced from different refining grades of the same soybean oil: 272 mg/kg from crude, 54 mg/kg from degummed and 190 mg/kg from refined. The same study reported 141 mg/kg of FSG in biodiesel from palm oil [10]. As quantitative data is rather scarce, the third aim of this study was to provide more analytical data.

Experimental Procedures

Sample Preparation: Biodiesel

Samples were collected from biodiesel processes based on different technologies processing fully refined palm and soybean oil. A sample of 150–250 ml was distilled in a lab-deodorizer at 180 °C, 3 mbar and 1% direct steam injection for 60 min. Under these conditions, the FAME were stripped and recovered in the distillate, while the heavier components (including partial glycerides, sterols, tocopherols and also steryl glucosides) were concentrated in the pitch. Betulin (Sigma-Aldrich, >98%) and tricaprin (Fluka, purity >99%) were used as internal standards for the quantification of FSG. Solutions of both internal standards in pyridine were added to the pitch to achieve a concentration of about 6 mg internal standard/g pitch. The distillation glassware was carefully rinsed with pyridine until it was quantitatively dissolved. An aliquot of the solution was taken for silylation and GC analysis as described below.

Sample Preparation: Filter Cake

The procedure was different for the impurities in their concentrated form, such as residue collected after filtration of biodiesel between storage tanks through a polishing bag filter. The method for a filter residue was based on the insolubility of steryl glucosides in many organic solvents. It consists of a multiple step extraction of the sample with hexane, acetone, methyl-ethyl ketone and iso-octane, gradually removing FAME, free and bound glycerine, sterols and steryl esters as soluble fractions. The extraction was followed by a vacuum filtration through a glass microfibre filter Whatman GF/C, leaving the FSG on the filter.

Analytical Methods

Gas Chromatographic Analysis

A sample (0.5–1 ml of the pitch solution in pyridine or 0.1 g/ml of filter cake dissolved in pyridine) was silylated at 75 °C for 30 min with 1 ml of *N*,*O*-Bis(trimethylsilyl) trifluoroacetamide containing 1% trimethylchlorosilane (Sigma-Aldrich). The derivatized sample was further dissolved in 8 ml of heptane, and 1 µl of the solution was injected on-column in the gas chromatograph (HP 5890, Series II) equipped with a 100% polydimethylsiloxane column (15 m, 0.25 mm id, 1 µm film). The following temperature program was applied: initial oven temperature 50 °C, held for 3 min, then increased at 15 °C/min to 200 °C, and at 3°C/min to 290°C, which was held for 10 min, and finally the temperature was increased at a rate of 10 °C/min to 360 °C, which was maintained for 10 min. Peak identification, based on retention time, was accomplished by using purchased standards of steryl glucosides (Matreya, 98+%). The quantification of FSG was made on the basis of a response factor (1.050 ± 0.002) to the internal standard, betulin.

NMR

A Jeol JNM-EX 300 nuclear magnetic resonance (NMR) spectrometer was used. The ^{1}H-NMR spectra were

recorded at 300 MHz. The samples were dissolved in deuterated dimethyl sulfoxide (DMSO-D6) or in a mixture of deuterated chloroform and fully deuterated methanol ($CDCl_3/CD_3OD$) (2:1). All solvents were from Acros and 99.5 or more atom % D. The ^{1}H-NMR spectra of the biodiesel haze and of the solids obtained by consecutive extraction steps, were compared with those of pure methyl esters (haze-free biodiesel) and with standards of pure glucose (Acros, D(+)-glucose, 99+%, anhydrous, HPLC grade), steryl glucosides (Matreya, 98+%), free sterol (cholesterol, Aldrich, 99%) and acylated sterol (cholesteryl stearate, Acros, 96+%).

MS

Mass spectra were obtained after direct injection for the pure FSG standard and for the solid residue of the filter cake, isolated from soy biodiesel haze. A Thermo Finnigan MAT95XP-TRAP Mass Spectrometer was used. In each mode the mass resolution was 1,000. Samples were dissolved in pyridine (Acros, 99.5%, water <50 mg/kg, extra dry over molecular sieve) and in a mixture of chloroform/methanol (2:1) (LC–MS grade, BioSolve) in concentrations ranging from 0.08 till 0.5 mg/ml. Analyses were done in *electron impact mode* (direct injection probe, 15–70 eV; source temperature 200 °C, scanning from 100 till 700 *m/z*, at 2 s per decade), *electrospray ionization* (ESI) {loop injection, positive mode, spray voltage 3 kV, heated capillary temperature 250 °C, mobile phase 50 µl/min [MeOH/H_2O (50/50), with 0.5% formic acid], scanning from 300 till 900 *m/z*, at 3 s per decade} and in *atmospheric pressure chemical ionization* [loop injection, positive mode, vaporizer temperature 300 °C, Corona current 5 µA, heated capillary 200 °C and flow 250 µl/min MeOH/H_2O (50/50), sheath gas: nitrogen, 4 bar; mass scanning from 100 till 800 *m/z*, at 2 s per decade].

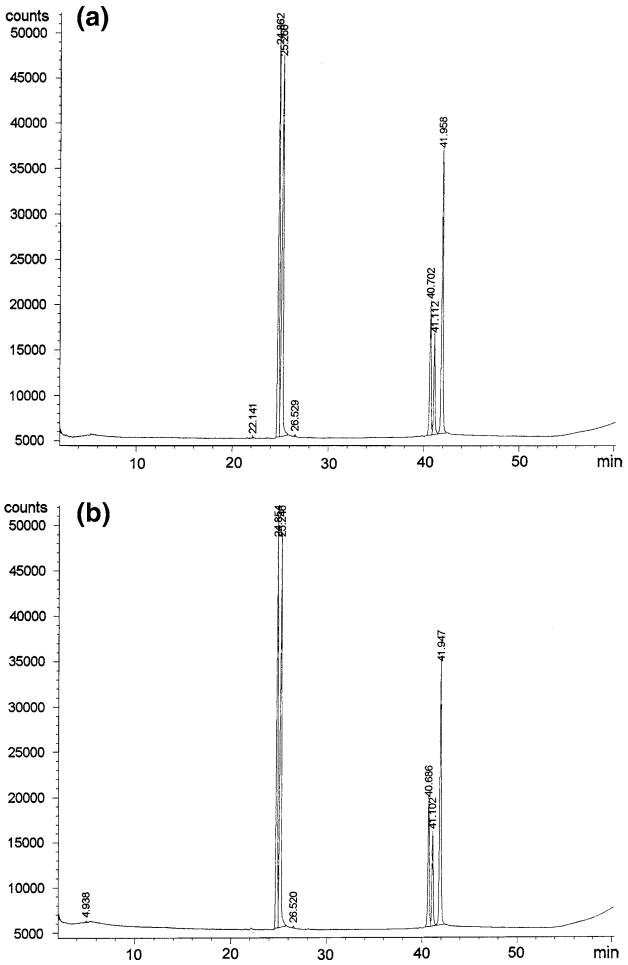


Fig. 2 GC-chromatograms of: **a** Standard of steryl glucosides (Matreya) consisting of campesteryl glucoside (RT 40.7 min), stigmasteryl glucoside (RT 41.1 min) and *β*-sitosteryl glucoside (RT 41.9 min); **b** Filter residue from soy biodiesel purified by multi-step extraction. In both chromatograms: standards betulin (RT 24.8 min) and tricaprin (RT 25.2 min)

Results and Discussion

General Haze Composition

The analysis of numerous samples revealed that hazy biodiesel contains minor quantities of steryl glucosides, some bound glycerin (in the form of partial glycerides and triglycerides) and free sterols. Additionally, soaps, free glycerine, sodium citrate and inorganic impurities can be present. Quantitatively, the relative proportions of the components depend on the concentration of the haze. In biodiesel, steryl glucosides are present in the mg/kg range, and the maximum value found in the studied samples is below 300 mg/kg. For the filter residue, the content of steryl glucosides varies from nearly 10 to 40%, with the major component being always FAME. No significant accumulation of bound glycerine (mono-, di- and triglycerides) in filter cake samples was found, which is explained by the high filtration temperature of 55 °C.

Qualitative Identification of FSG

The GC-chromatogram of an unextracted residue, scrubbed from a polishing filter after filtration of a soy biodiesel, indicated that the material consisted mainly of methyl esters (80%). Traces of free sterols and bound glycerine were present. The remaining 20% of the chromatographic area consisted of peaks eluting in the region of steryl esters. As it was suspected that this group of compounds was responsible for the turbidity of the biodiesel, it was unlikely that they were indeed steryl esters, regarding the poor

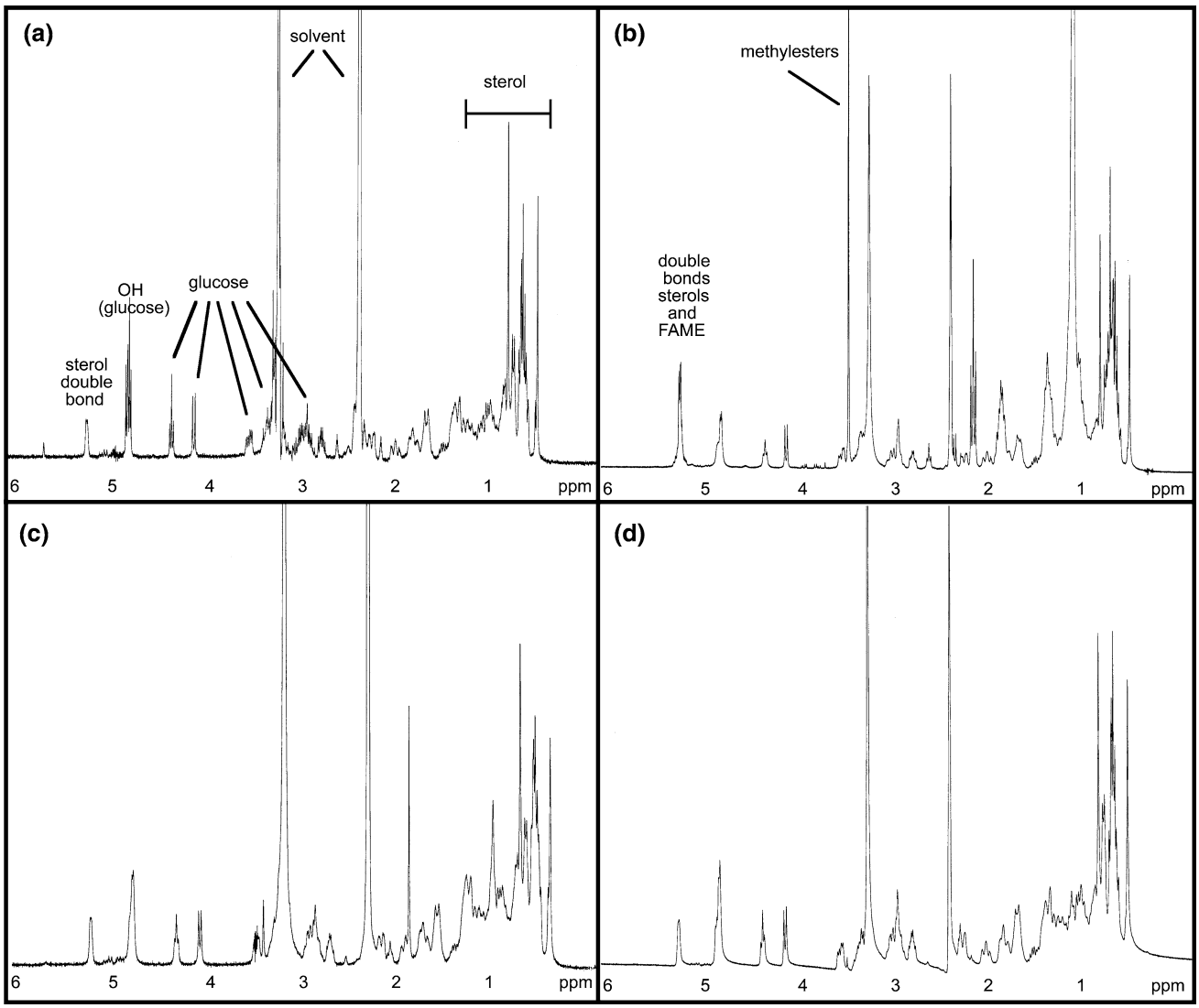


Fig. 3 Identification of free steryl glucosides with ^{1}H NMR (solvent: DMSO-D6): **a** Standard of steryl glucosides (Matreya); **b** Unextracted filter residue from soy biodiesel; **c, d** Filter residue from soy and palm biodiesel (respectively) purified by multi-step extraction

solubility of the haze in biodiesel and in various organic solvents. On the other hand, it is known that soybean oil contains a large amount of ASG. Thus, the hypothesis was that the peaks eluting in the region of steryl esters were FSG, partially originally present, and more of them formed from ASG during transesterification. In Fig. 2 the chromatogram of the stepwise purified filter residue (b) is compared with the one obtained for the purchased steryl glucoside standard (a). A remarkable similarity can be observed between the two GC chromatograms: each features three peaks in about the same relative amounts. In palm and soybean oil and biodiesel, the sterol moiety of steryl glucosides consists mainly of β-sitosterol, campesterol and stigmasterol. The residue sample was then spiked with 5% of the FSG standard (2.5 mg/ml pyridine), confirming the exact match of the sample with the three steryl glucosides present in the standard.

NMR

Considering the poor solubility of the haze the choice of NMR-solvent was an important issue. Pure $CDCl_3$ could not be used but it was possible to dissolve the samples completely in DMSO-D6, by placing them in an ultrasonic bath for 2–4 h, or in a mixture of $CDCl_3/CD_3OD$ (2:1).

In the NMR spectrum of a pure cholesterol standard, the protons in the rings give signals as complex multiplets between 0.46 and 2.50 ppm. In Δ5-sterols the proton on carbon C6 (cfr. Fig. 1) is represented in the NMR spectrum as a signal at 5.34–5.36 ppm. The same integration value is

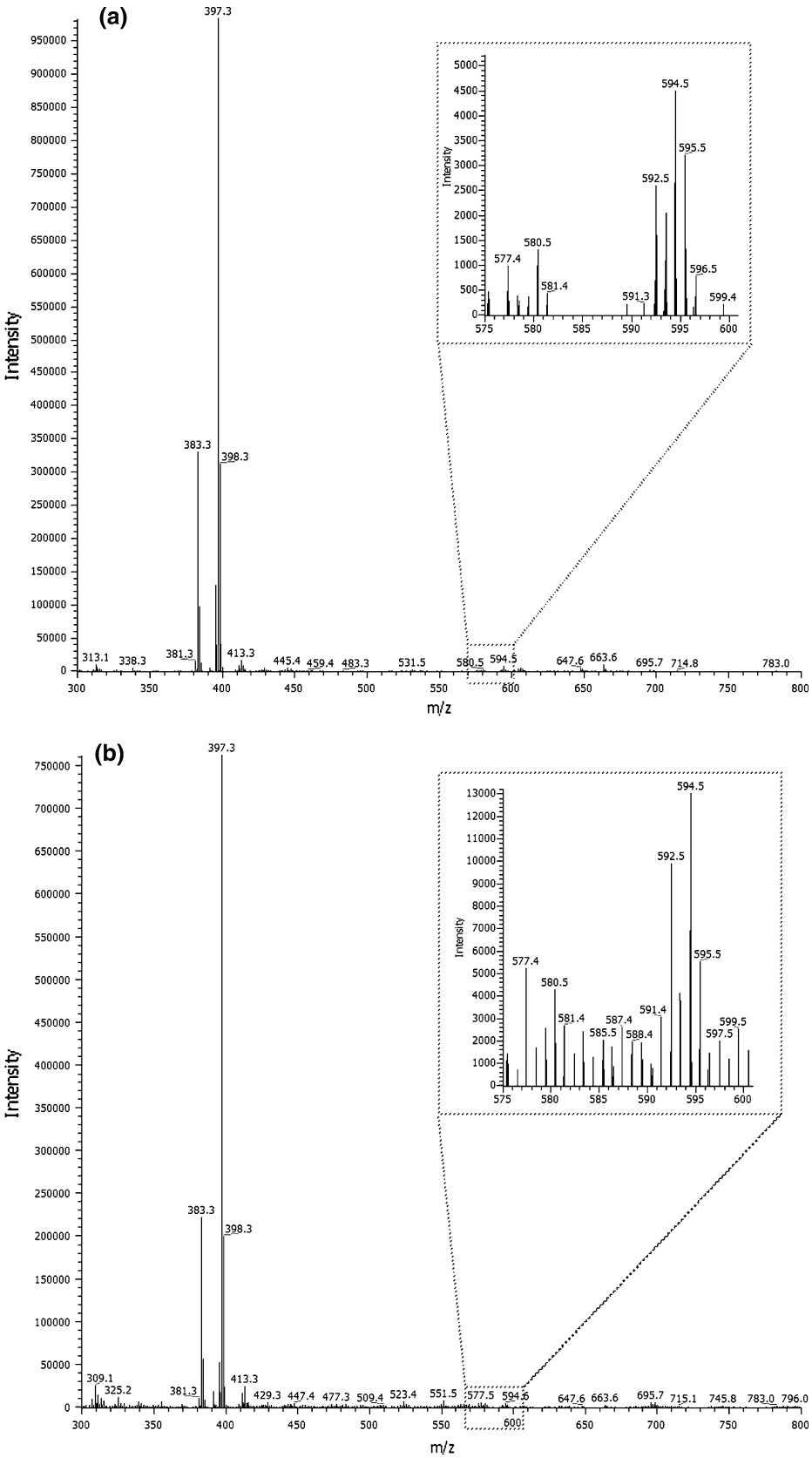


Fig. 4 Identification of free steryl glucosides with MS: Large spectrum: scan 300–800 *m/z*; insert: 575–600 *m/z*, **a** Standard (Matreya), **b** Filter residue from soy biodiesel purified by multi-step extraction

Table 1 Validation of the analytical method: concentration of biodiesel samples via distillation followed by quantification of free steryl glucosides with GC

	Test 1		Test 2		Test 3	
	Biodiesel A[a] from RBD PO		Biodiesel B[b] from RBD PO		Biodiesel C from SBO	
	Original	+250 mg/kg FSG	Original	+250 mg/kg FSG	Original	+250 mg/kg FSG
Pitch (%)	1.5	1.4	1.6	1.9	2.4	3.7
SG added (g)	0	0.0392	0	0.0501	0	0.0611
SG found (ppm)	62	297	57	331	36	289
SG found (g)	0.0093	0.0438	0.0114	0.0663	0.0094	0.0707
Recovery (%)	–	90.3	–	107.1	–	100.3

RBD PO refined, bleached, deodorized palm oil

SBO soybean oil

[a] Biodiesel produced on a lab scale

[b] Industrial biodiesel sample from a storage tank

Table 2 Quantification of free steryl glucosides in industrial and lab-scale biodiesel samples

Sample number	Feedstock oil	Biodiesel appearance		Distillation pitch (%)	SG (ppm)
		At 25 °C	At 60 °C		
Industrial samples					
1	RBD palm	Hazy	Hazy	1.3	215
2	RBD palm	Hazy	Hazy	1.3	276
3	RBD palm	Hazy	Hazy	2.0	160
4	RBD palm	Hazy	Hazy	1.6	57
5	RBD soy	Hazy	Hazy	2.2	n.d.
6	RBD soy	Hazy	Hazy	3.0	n.d.
7	RBD soy	Hazy	Hazy	2.8	158
8	RBD soy	Hazy	Hazy	2.5	60
9	Rapeseed (60%) + soy	Hazy	Hazy	2.7	<15
10	Rapeseed (60%) + soy	Hazy	Hazy	2.4	36
Produced on lab-scale					
11	Chemically neutralized PO	Clear	Clear	1.4	<15
12	RBD PO	Hazy	Hazy	1.5	62

n.d. Not detected

<15—below quantification limit

found for a multiplet at 3.50–3.53 ppm, corresponding to the proton on the carbon (C3) with the OH group. This signal shifts when the sterol is bound to a fatty acid (steryl ester) or to a sugar (ether bond). Thus, in the NMR spectrum of cholesteryl stearate (in $CDCl_3$), the multiplet was found at 4.60–4.63 ppm, while all other signals had the same chemical shifts as in cholesterol.

In the steryl glucoside standard (Fig. 3a), additional multiplets are found at 2.9–3.5 ppm, and 4.45 ppm, resulting from the sugar moiety which is bound via a 1'-3 bond to the sterol. Moreover, the proton on carbon C1' of the glucose shows a clear doublet at 4.20–4.23 ppm. This signal for glucose has the same integration value as the signal for the proton in the sterol on carbon 6 (5.3 ppm), because in the standard for each glucose one sterol is present.

In the soy biodiesel haze, mainly methyl esters were found (Fig. 3b). However after the consecutive extraction steps, the final solids had an NMR spectrum corresponding to the steryl glucoside standard (in both DMSO-D6 and $CDCl_3/CD_3OD$), including the exact integrations for the proton in the sterol and the proton on carbon 1 in the glucose. This means not only that the steryl glucoside structure is present in the isolated solids, but also that it has a high purity. This supports the observations made by the GC analysis, where the chromatogram of the isolated solids corresponded exactly with the one of the FSG standard.

MS

The mass spectrometric data were obtained for the purified filter residue, by direct injection. Considering the peaks observed in the GC analysis, the three main steryl glucosides expected in the samples were the FSG of campesterol, stigmasterol and β-sitosterol. The molecular mass of those FSG is respectively 562.4, 574.4 and 576.4. The free sterols have molecular masses of 400.4, 412,4 and 414.4, respectively. For the electron impact ionization (EI) the sample is directly injected, solvent is evaporated and compounds are ionized. In the atmospheric pressure electrospray ionization (ESI) and the atmospheric pressure chemical ionization (APCI) the sample is injected into a

loop, and transferred with the help of a solvent, containing ions, into the ionization chamber, where all solvents are evaporated and the sample is ionized. Using the atmospheric pressure ESI, no ions were observed. It could be due to the rather soft ionization parameters characteristic for this method. On the other hand, the EI (spectrum is not shown) was only partly successful. The characteristic ions for β-sitosterol (m/z = 414.4 (M^+), 396.4, 255.2) were observed, which showed the presence sterols. But the EI method was too destructive for the detection of steryl glucosides, because even in the commercial standard not any molecular ions of FSG (*m/z* 576.4, 574.4, 562.4) were observed. Finally, using APCI in positive mode, the steryl glucosides could be identified. In Fig. 4a the MS results for the FSG standard are presented, while Fig. 4b shows the mass spectrum of the filter residue from soy biodiesel purified by multi-step extraction. The H^+ adducts of characteristic sterol fragments (*m/z*, 383.3, 395.3 and 397.3 for campesterol, stigmasterol and β-sitosterol) were the main ions observed in both standard and sample (large Fig. 4a, b). Moreover, the molecular ions of the NH_4^+ adducts of the three steryl glucosides, with m/z 594.5, 592.5 and 580.5 respectively, were in this case clearly present as well, in both the standard and the sample (inserts in Fig. 4a, b).

Quantification of FSG

Gas Chromatographic Method Validation

The method was validated for quantitative determination of steryl glucosides by adding a known amount of the FSG standard (250 mg/kg) to the samples before distilling the biodiesel (Table 1). The concentration factor achieved by distillation varies between 40 and 50% which means that the final yield of the pitch is about 2–2.5 wt.% of the initial biodiesel sample. The detection limit was found to be below 15 mg/kg. The method features a high recovery (>99%) of the added standard with standard deviation below 10% between the three tests, and good repeatability between the duplicate measurements (±5%).

In the industrial samples, the concentration of FSG lies in the range from 55 to 275 mg/kg for palm biodiesel, and from not detectable to 158 mg/kg for soy biodiesel. The lowest value corresponds to a palm biodiesel after it has been filtered through a bag filter with pore size 10 μm. For samples prepared on the lab scale from palm oil, concentration of FSG is between 15 and 62 mg/kg (Table 2). An unambiguous correlation between the minimum concentration of FSG and presence of haze in biodiesel was not observed. Therefore, the concentration of FSG is an important, but not the only parameter that determines whether these compounds remain in solution or precipitate.

In filter cake FSG compose almost half of the sticky paste material retained with bag polishing filters obtained from palm biodiesel plants (45–50%). In the case of soy biodiesel, the portion of FSG in the filter residue is less, 12–25%. For both biodiesel types the remaining major fraction is FAME, indicating the important adsorption capacity of FSG towards methyl esters. The content of bound glycerine in these samples meets the specifications for biodiesel; in particular no accumulation of monoglycerides was observed.

In this work, NMR and MS conditions were established to identify FSG in biodiesel. A new method for sample preparation prior to the quantitative GC analysis of these compounds was developed, consisting of a distillation of the methyl esters.

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