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ORIGINAL PAPER

Analysis of Sterol Glycosides in Biodiesel and Biodiesel Precipitates

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Abstract Biodiesel is produced by the transesterification of vegetable oils with short chain alcohols, usually in the presence of an alkali catalyst. Minor components in biodiesel exist as a result of unreacted reagents, by-products, additives, and auto-oxidation products, such as water, free glycerin, bonded glycerin, free fatty acids, catalysts, residual alcohol, unsaponifiable matter (plant sterols, antioxidants, and hydrocarbons), soaps and polymers. The biodiesel properties, such as cold flow properties, acid number, cetane number, and oxidative stability are oftentimes significantly affected by these minor components. Sterol glycosides, as one of the most important minor components in biodiesel, and due to their polarities and limited solubility, can accelerate precipitate formation even at room temperature and possibly block fuel filters. In this paper, reversed phase high-performance liquid chromatography with an Evaporative Light Scattering Detector (ELSD) is evaluated for the analysis of sterol glycoside (SG) content in not only biodiesel precipitates but also in biodiesel. SG was found to be a major component in soy biodiesel precipitates and an SG peak was found in biodiesel after concentration by centrifugation.

Keywords Biodiesel · Sterol glycosides · HPLC · Biobased products · Chromatography · Lipid chemistry · Lipid analysis

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Introduction

Biodiesel is attractive as an alternative fuel mainly because it is renewable, biodegradable and environmentally friendly, and also can be manufactured from common feedstocks, such as vegetable oils and animal fats. Biodiesel is produced by the transesterification of fats and oils with an alcohol using a base catalyst. The properties of biodiesel are affected by the by-products of the transesterification reaction, such as water, free and bonded glycerides, free fatty acids, catalyst, residual alcohol, and unsaponifiable matter (plant sterols, tocopherols and hydrocarbons).

Sterols are some of the most common minor components distributed in animal fats and vegetable oils and are found in many forms, such as free sterols, acylated (sterol esters), alkylated (sterol alkyl ethers), sulfated (sterol sulfate), or linked to a glycoside moiety (sterol glycosides) which can be itself acylated (acylated sterol glycosides) [1–3]. Among the several common sterols, sterol glycosides have been found to be a major component of biodiesel precipitates [4–6]. In plant tissues and in vegetable oils, sterol glycosides occur naturally as both sterol glycosides (SG) and acylated sterol glycosides (ASG). During the transesterification process, acylated sterol glycosides can be converted into sterol glycosides due to the alkaline catalysts. Therefore, the SG concentration in biodiesel is normally higher than that in the feedstock oils. The polar SG in biodiesel may change the crystallization onset temperature and cause the formation of cloud-like agglomerates of various sizes composed of discrete 10–15 μm particles even at room temperature and at relatively low levels (35 parts per million or higher) [7].

Gas chromatography (GC) has been broadly applied to identify and quantify minor components in biodiesel due to

its relatively high sensitivity and accuracy. Gas chromatography (GC) with flame ionization detection (FID) is a test method standardized by ASTM D6584 to determine the free and total glyceride contents in biodiesel, through which the amount of free and total glyceride in the range of 0.005–0.05 and 0.05–0.5 mass% can be detected, respectively. A detailed test procedure according to ASTM D 6584 with GC-FID was reported by Ruppel and Hall [8]. Recently, a GC method for the quantitative evaluation of sterol glucoside (SG) concentrations in biodiesel precipitates was presented by Bondioli et al. [4]. However, the GC method has certain disadvantages in biodiesel analysis. First of all, due to low volatilities, most of the samples must be derivatized by silylating reagents such as *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) or *N*,*O*-bis (trimethylsilyl)trifluoroacetamide (BSTFA) before the analysis. Secondly, different internal standards are required for different feedstocks in the quantification analysis when applied to biodiesel analysis. Last, but not least, the accuracy of GC analyses is susceptible to many factors such as baseline drift, overlapping signals, and auto-oxidation of standards and samples.

As an alternative to GC, high performance liquid chromatographic (HPLC) methods have been developed for analyzing transesterification reaction mixtures [9–12] because of advantages such as no derivatization of samples, shorter analysis times, and direct applicability to most biodiesel fuels and all neutral lipid classes. The early literature related to biodiesel analysis with HPLC [10] used an isocratic solvent system (chloroform with an ethanol content of 0.6%) on a cyano-modified silica column coupled to two GPC columns with density detection to detect mono-, di- and tri-glycerides as well as methyl esters. The method can be used for monitoring conversion degree of the transesterification reaction. A recent paper [13] proposed a binary gradient method using non-aqueous reverse phase HPLC with a UV detector to analyze the monoglycerides (MGs), fatty acid methyl esters (FAMEs), diglycerides (DGs) and triglycerides (TGs) in biodiesel mixtures. There are also several other publications [14–16] which describe the application of HPLC in the monitoring of biodiesel products and production process. Qualitative and quantitative analysis with these HPLC methods were provided without saponification and off-line pre-separation.

Though HPLC has many advantages over GC, the analysis of sterols in biodiesel by HPLC is still problematic because sterols such as cholesterol and related compounds cannot be separated very well from fatty acid methyl esters [17]. Also because of the relatively low concentrations in biodiesel and relatively low response of SG with HPLC compared to GC techniques, it is a great challenge to directly detect the SG content in biodiesel by HPLC without precipitation and extraction. In 2007, Ringwald [18] collected the biodiesel residue from fuel filters and analyzed it by a LC method with a silica column and an Evaporative Light Scattering Detector (ELSD). The isolation of SG from the residue was done by solid phase extraction (SPE) prior to the analysis. More recently, SG content has been reported to be separated from various commercial biodiesel precipitates by HPLC coupled with different detectors [6]. After precipitation from the turbid liquids, no further purification process was performed before the normal-phase or reversed-phase HPLC. Calibration curves were reported for both ELSD and UV detectors. However, there were no further attempts to recover SG from biodiesel and determine the detection limit of SG in liquid biodiesel by these methods. In summary, all previous studies have shown that the analysis of this class of compounds in biodiesel directly by HPLC is not as successful as for biodiesel precipitates.

The main objective of this work is to apply reversed phase HPLC-ELSD for the identification and quantification of sterol glycosides in biodiesel. Compared with previous HPLC methods, there are two major improvements with this new study. Firstly, a high carbon load C18 column, an alternative to normal C18, which has a higher sample load capacity, is used. With the higher sample load capacity, biodiesel with low SG concentration could be injected in larger amounts and without further separation. Furthermore, the high carbon load makes the column more non-polar and, therefore, the most retentive of the reversed phases, providing good resolution of non-polar and polar compounds and allowing for higher organic solvent in the mobile phase which contributes to greater sensitivity in the LC–MS application. The second improvement of this study is to quantify the SG content in biodiesel with an HPLC-ELSD method after a simple centrifugation process. FTIR was also used to analyze the similarities and differences among SG, SBO B100, and SBO B100 precipitates before the HPLC analysis.

Materials

Soy oil based biodiesel (B100) was obtained from Wacker Oil Co. (Manchester, MI). The biodiesel precipitates were contributed by REG (Renewable Energy Group Inc., Ames, IA). The sterol glycosides standard (98+%) was acquired from Matreya (Pleasant Gap, PA). HPLC-grade methanol and methylene chloride were purchased from Fisher Scientific (Pittsburgh, NJ). The sterol glycoside standard and all of the biodiesel precipitates were dissolved in MeOH/CH_2Cl_2 (1:2, v/v). The precipitates were purified with various solvents by REG (Renewable Energy Group Inc., Ames, IA) and verified to be clean by FTIR in the ester and

soap region before being sent to our lab. In order to obtain a higher concentration of SG in the oil, 3 g of the B100 was centrifuged in a 5-mL centrifuge tube at 5,000*g* and ambient temperature for 15 min using an Eppendorf Centrifuge 5804 R with a fixed-angle Rotor A-4-44 (Eppendorf North America, Inc., Westbury, NY). After centrifugation, the clear oil sample became turbid because the SG precipitated out. All of the solutions were filtered through the Whatman filter with 125 mm diameter and the stock solutions were stored in a refrigerator at 4 °C. Before use, standard working solutions were prepared by diluting appropriate amounts of the stock solution in MeOH/CH_2Cl_2 (1:2, v/v).

HPLC Conditions

The HPLC analysis was conducted using a PerkinElmer Series 200 with an Altech 3300 Evaporative Light Scattering Detector (ELSD) and a high carbon load reversed phase column—Altech C18-HL (250 × 4.6 mm i.d., 5 μm) with guard column (7.5 × 4.6 mm i.d., 5 μm) as the stationary phase. Mobile phase solvents were methylene chloride (Phase A) and methanol (Phase B). The samples were analyzed with a gradient of CH_2Cl_2/MeOH at a flow rate of 1 mL/min. The column temperature was set to 25 °C and the injection volume was 20 μL. Two gradient conditions were evaluated for the analysis. After 15 min equilibrium at 0% (A):100% (B), the first gradient condition was: 0% (A):100% (B) maintained for 10 min and then 0% (A):100% (B) to 50% (A):50% (B) in 10 min; in the following 4 min, 50% (A):50% (B) to 75% (A): 25% (B), and back to 100% (B) within 1 min, then the run was finished. However, with this method, the separation of methyl stearate (C18:0) and SG was not satisfactory as shown in Fig. 1a. Thus, the HPLC condition was optimized to the gradient condition illustrated in Table 1. With this HPLC method, good separation of methyl stearate (C18:0) and SG was obtained (Fig. 1b).

Table 1 Gradient condition of the HPLC method

Step	Time (min)	Flow rate (mL/min)	A (%)	B (%)
Equilibrium	15	1	0	100
1	5	0.5	15	85
2	17	1	25	75
3	5	1	50	50
4	3	1	70	30
5	5	1	70	30

Results and Discussion

FTIR Spectra

Figure 2 shows FTIR spectra obtained from the sterol glycosides (SG) standard, SBO B100, and SBO B100 precipitates. The typical C=O stretching band of the methyl ester usually appears at 1,750 ± 50 cm^{-1}. Both SBO B100 and PBO B100 (palm oil based biodiesel) show a strong peak in this range. An –O–H stretching band around 3,400 cm^{-1} in the spectrum of the SBO B100 precipitates indicates the presence of hydroperoxyl and hydroxyl groups. The spectrum of the sterol glycosides standard in Fig. 2 shows the similar –O–H stretching band and fingerprint area as that of the SBO B100 precipitates. In the spectra of both SG standard and SBO B100 precipitates, the strongest peak in the area of 1,300–1,000 cm^{-1} is due to the C–O moiety. Also finger print areas and the strong absorptions of the two spectra caused by CH_3 and CH_2 vibrations are similar. Therefore, from the IR spectra, it can be concluded that the major component of the precipitates from REG is SG, which is consistent with the HPLC results discussed later.

HPLC Calibration and Analysis

The retention time of SG was 14.6 min with the second gradient method. The lowest concentration of detection for SG standard was about 0.005 mg/mL. Therefore, standard

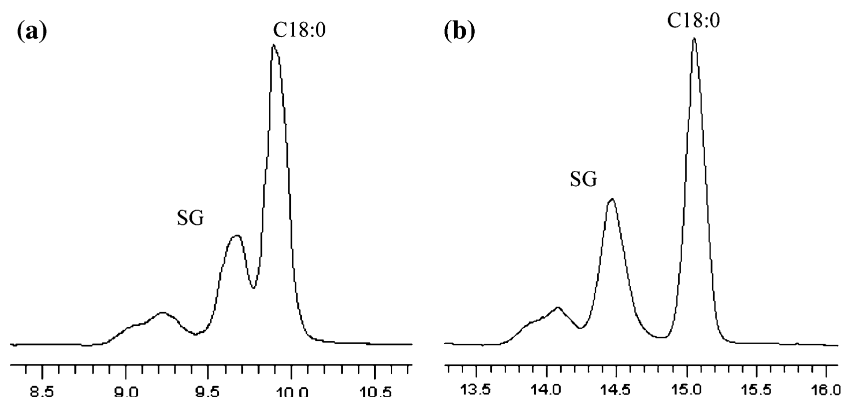

Fig. 1 HPLC separation of methyl stearate and SG under two gradient conditions: (**a**) first gradient condition; (**b**) second gradient condition

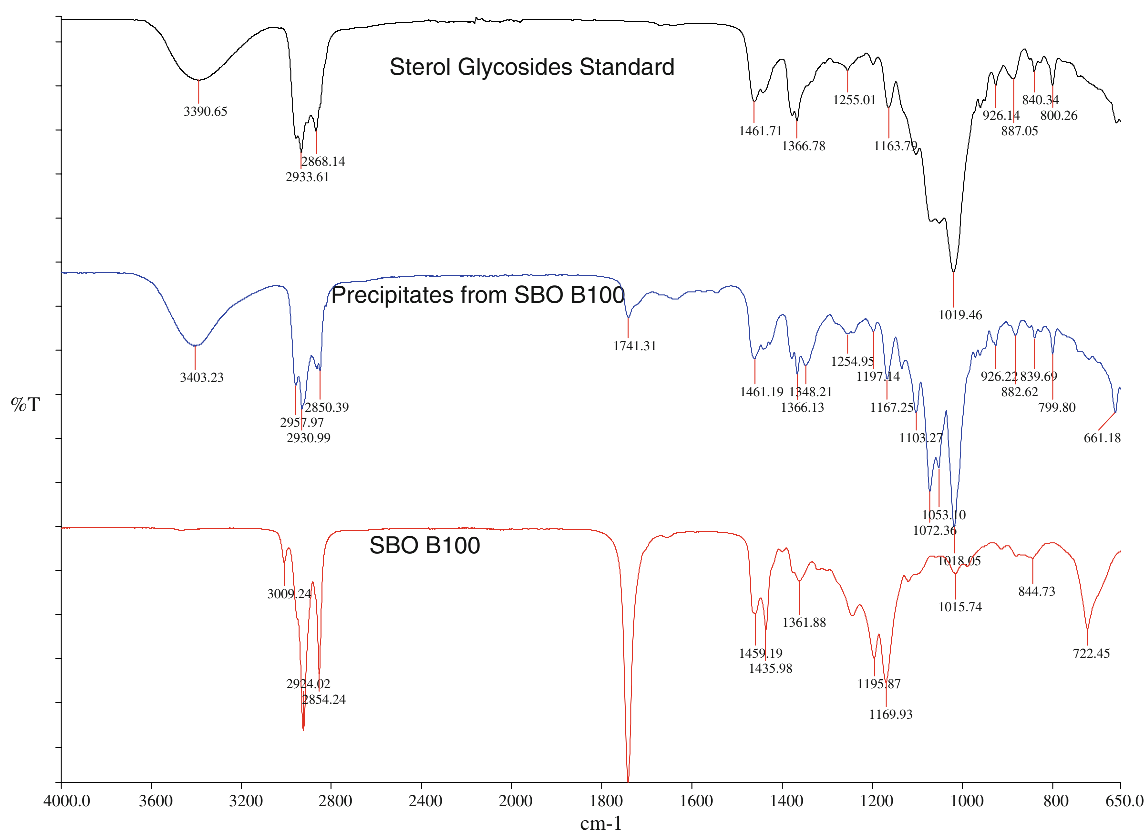

Fig. 2 FTIR spectra of sterol glycosides (SG) standard, SBO B100 and SBO B100 precipitates. IR spectrum of the precipitates from SBO B100 is similar to that of the SG standard. From the spectra, it can be concluded that the major component of precipitates is SG

solutions of sterol glycosides with concentrations ranging from 0.005 to 0.1 mg/mL were prepared for calibration. Figure 3 depicts the chromatograms of sterol glycoside standards with four concentrations including 0.1, 0.04, 0.025 and 0.01 mg/mL. With careful examination of the chromatograms, there are three peaks (of which 2 co-eluted as a peak with a shoulder and a third one was clearly separated) of SG can be observed in Fig. 3. The peaks can be attributed to three steryl glycosides, namely campesteryl 3-ß-D-glucopyranoside, stigmasteryl 3-ß-D-glucopyranoside and sitosteryl 3-ß-D-glucopyranoside [19]. It can be seen that with decreasing concentration, the first two peaks decreased and almost disappeared at the low concentration of 0.01 mg/mL. In order to calculate the amount of SG in very low concentrations for which there was no detectable first peak, the calibration was based on the area of the third peak. Figure 4 shows the calibration curve of the SG based on HPLC. Because of the nonlinear concentration response of the ELSD detector [20], the parameters of the calibration curves were obtained by fitting the experimental data points to a cubic polynomial, resulting in the fit equation: $y = 2E + 09x^3 - 1E + 08x^2 + 2E + 07x$, where y is the peak area (mV min) and x represents the analyte concentration (mg/mL).

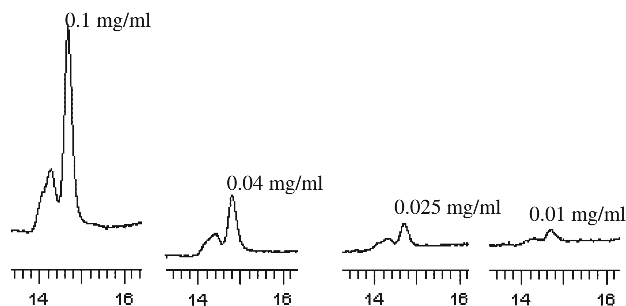

Fig. 3 HPLC chromatogram of sterol glycosides standards with concentrations of 0.1, 0.04, 0.025 and 0.01 mg/mL. It can be seen that with the decreasing concentrations, the first peak of the SG was decreasing and almost disappeared at low concentration of 0.01 mg/mL

For accuracy validation, the SG solution (0.10 mg/mL) was mixed with B100 (5.95 mg/mL) at different ratios to obtain solutions of known concentration as listed in Table 2. The recoveries shown in the table, range from 75 to 99%. With the decreasing SG concentration, the recovery decreases. Figure 5 depicts the chromatogram of the sample with 1.01% SG in B100.

In the subsequent analysis, sterol glycosides in B100 and the precipitates were determined. Figure 6 shows the

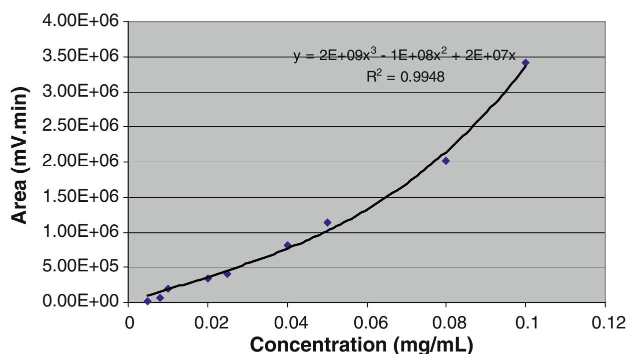

Fig. 4 the calibration curve of the SG. The parameters of the calibration curves were obtained by fitting the experimental data points to the polynomial equation$y = 2E + 09x^3 - 1E + 08x^2 + 2E + 07x$, where y is the peak area (mV min) and x represents the analyte concentration (mg/mL). Calibration concentration ranges from 0.005 to 0.1 mg/mL

Table 2 Accuracy validation of the HPLC analytic method for SG in biodiesel

Sample	SG Concentration (mg/mL in Solution)	SG Concentration (wt% in B100)	Recovery (%)
1	0.003	0.05	75
2	0.006	0.1	78
3	0.01	0.2	82
4	0.02	0.4	88
5	0.03	0.55	93
6	0.04	1.01	99

The recoveries range from 75 to 99%. Samples with 1.01% (wt% in B100) showed the best recovery than the others. With the decreasing SG concentration, the recovery decreases

chromatogram of the biodiesel precipitates. Using this method the levels of SG in this precipitate sample were estimated to be 91.1% (wt/wt), SD = 0.01. Figure 7 shows the chromatogram of B100 before and after centrifugation. However, no SG peak was detected in the Wacker B100 sample before centrifugation because of the low SG concentration. In order to obtain a higher concentration of SG in B100, the sample was concentrated by centrifugation and white SG particles precipitated out. Then the bottom part (around five volume percentage) of the concentrated sample was taken and stirred to form a turbid phase. It can be seen from Fig. 7 that a small but obvious SG peak appears right before C18:0 in the turbid B100 sample which has concentrated SG composition. In the turbid sample, sterol glycosides were identified with an average weight concentration of 592 ppm. Because the turbid phase is roughly five volume percent of the original sample, it can be estimated that the SG concentration in the original Wacker B100 sample was about 30 ppm. Distilled soy oil based B100 was also analyzed with and without addition of SG to verify the SG position in the biodiesel chromatogram.

Conclusion

In this paper we have presented a study on the direct determination of the level of sterol glycosides in biodiesel by reversed phase HPLC with an ELSD. The method

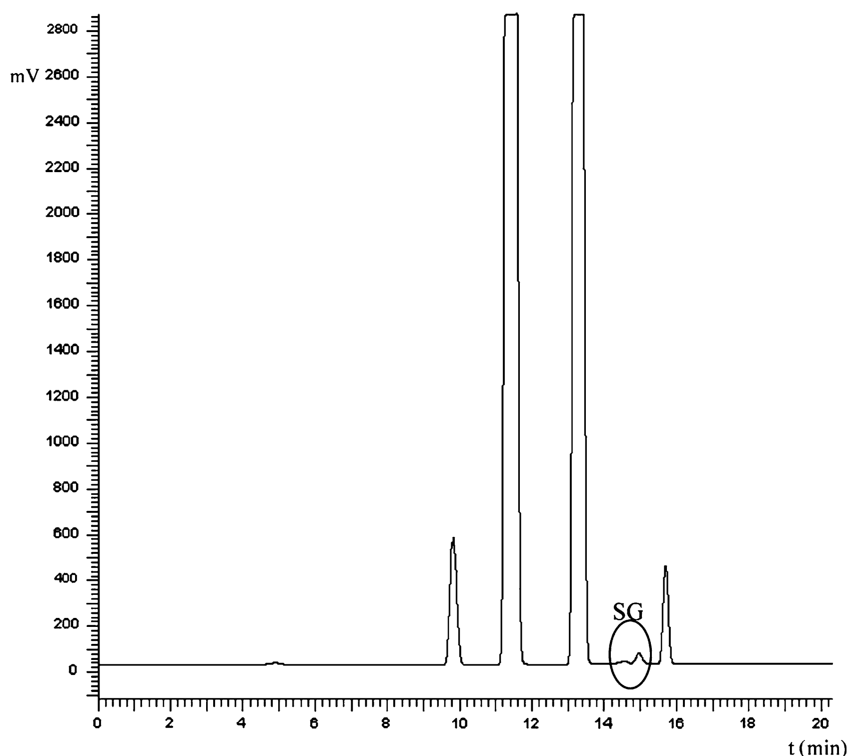

Fig. 5 HPLC chromatograms of the sample with 1.01% SG in B100

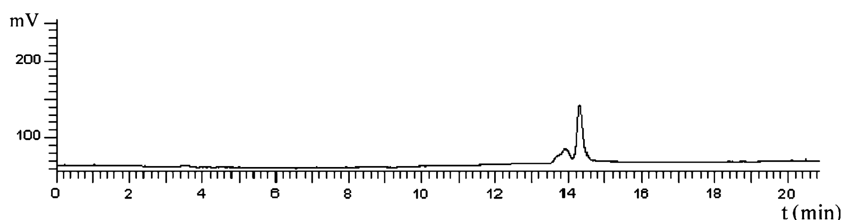

Fig. 6 HPLC chromatogram of the biodiesel precipitates. In the sample, sterol glycosides were identified with an average level of 91.13% (wt/wt), SD = 0.0099

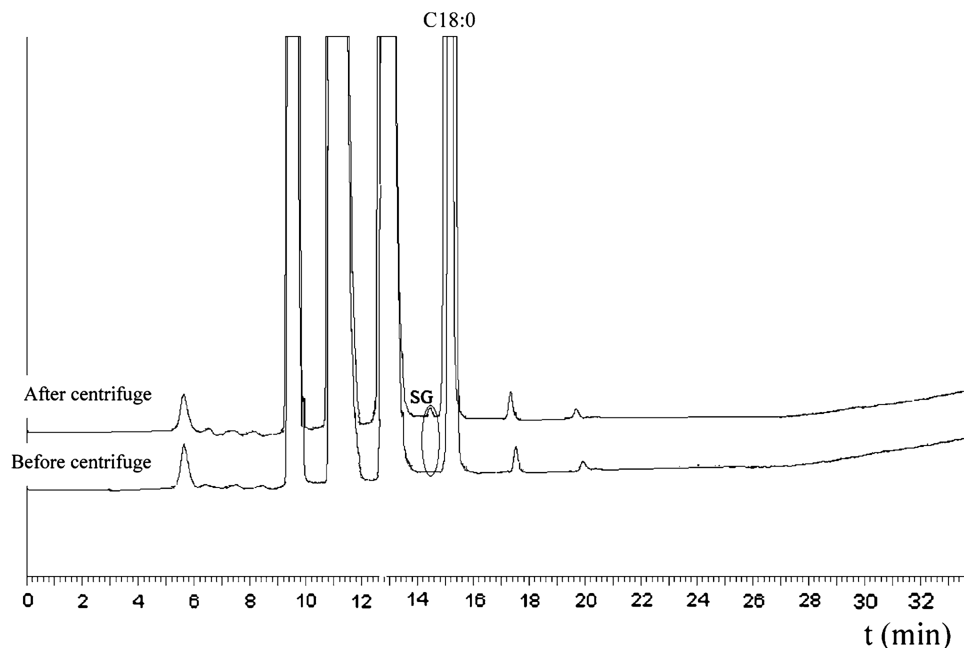

Fig. 7 HPLC chromatogram of B100 before and after centrifuge. No SG peak was detected in the original Wacker B100 sample because of the low SG concentration. After the centrifuge process, it can be seen that a small but obvious SG peak appears right before C18:0 in the B100 sample with concentrated SG. In the sample, sterol glycosides were identified with an average weight concentration of 592 ppm

allows the detection of concentration levels of sterol glycosides down to around 0.01 mg/mL in the solvent. Analysis of B100 with concentrated sterol glycosides showed that sterol glycosides could be separated from methyl ester peaks and quantified without separation when the amount is above the level of the detection limit. The HPLC method offers the advantage that it is a rapid method that can analyze sterol glycosides in biodiesel just after a simple centrifugation process. From the weight or volume percentage of the concentrated part, the SG concentration in the original sample can be calculated from the one in the concentrated sample. The limitation of this method is that it is only applicable for SG concentrations in biodiesel which are higher than or equal to 30 ppm, not below this value. The centrifugation step can be studied more rigorously in order to meet the analysis requirement of lower amount of SG in samples of biodiesel.

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