Forensic identification of biodiesel

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1. Introduction

Renewable biodiesels have attracted great interest as a new energy source as the costs of global oil production of petroleum, both financial and environmental have continued to rise. Biodiesel, an “alternative” diesel fuel derived from both vegetable oils and animal fats, consists mainly of alkyl esters of long-chain fatty acids (*1-4*). Biodiesel as a transportation fuel replacement has attracted world-wide interest due to its environmentally safety, non-toxicity and biodegradability (*5*).

Many analytical methods have been developed for the quality control of biodiesel production (*6, 7*). Different spectroscopic (*8, 9*) and chromatographic methods (*10, 11*) have been developed for determining or verifying the blend level of biodiesel in petrodiesel.

Generally, the feedstock source oil used to make biodiesel is largely influenced by geography and local market conditions. In Europe, rapeseed and sunflower oils are used, palm oil is common in tropical countries, and soybean oil is the major feedstock in the United States. Canola is the most abundant oilseed in Canada and is a major source of Canadian biodiesel. In addition, re-cycled cooking oils and rendered animal fats are used world-wide due to their low cost. Although the main effective components of biodiesel are the fatty acid methyl esters (FAMEs), considerable chemical variation with source stocks has been observed because various oils have been in use in different countries as raw materials for biodiesel production owing to its availability (*5*), even the genotype, growing seasons, agriculture factors, and growing locations of feedstocks have been found to affect oil content and fatty acid profiles (*12-14*).

Sterols are natural non-glyceridic compounds that occur in vegetable oils and animal fats, and can remain in biodiesel after processing due to their solubility in such fuel. Hence, the distributions of sterols in biodiesel may reflect those in the source oil(s). The compositional distributions of phytosterols in certain vegetable oils have been used for their identification, and the makers for the assessment of adulterated oils, despite being minor constituents (*15*).

In the event of an oil spill, differences in chemical distributions between

spilled product and suspect sources can be used to resolve questions of environmental impact and legal liability (*16-19*). In the case of biodiesel, however, FAMEs may be consumed in a short period following the spill, hence, samples from a contaminated area could be indistinguishable from a fossil diesel spill (*20*). Plank and Lorbeer (*21, 22*) found that free physterols can be found in FAME mixtures produced from vegetable oil by on-line LC-GC, and suggested that sterols would be useful tracers for some pure biodiesels. However, no any sterols were detected by Demello et al. (*20*) in their recent study.

The purpose of the present study is to elucidate the chemical fingerprinting of biodiesel by distinguishing fatty acid esters and sterols profiles in different biodiesels. Gas chromatography with mass selectivity detection (GC/MSD) will be employed to determine the FAMEs profile in various biodiesels: canola oil, soybean oil, and re-cycled frying oil (containing beef tallow). The fatty acid profile of the source vegetable and animal oils will be compared to that of the the FAMEs for the forensic identification of biodiesels with different feedstocks. Secondly, the free sterols in the biodiesels will be analyzed, and their potential as complementary "markers" for the forensic identification of spilled biodiesel will also be estimated.

2. Experimental

2.1 Chemicals and Materials

All solvents were of the highest purity available without further purification. Silica gel (100-200 mesh, 150 Å, pore 1.2 cm^2/g, active surface 320 m^2/g) was obtained from Sigma-Aldrich (Milwaukee, WI, USA).

FAME standards (C 8:0 to C24:0) and heptadecanoic acid methyl ester (C17:0), the internal standard, were purchased from Sigma-Aldrich (Bellefonte, PA, USA). All sterol standards (phytosterols: β-sitosterol (SI), campesterol (CA), campestanol (CAO), brassicasterol (BR), stigmasterol (ST), stigmastanol (STO), and Δ5-avenasterol (AV), and zoosterols: cholesterol (CS), and β-cholestanol (CO)), surrogate (β-estradiol-17-acetate) and internal standard (5-α-cholestane) were supplied by Sigma-Aldrich (Bellefonte, PA, USA) and Chiron (Trondheim, Norway). Silyation reagents, bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) and N-methyl-N-(trimethylsily)trifuloroacetamide, were obtained from Supleco (Bellefonte, PA, USA).

Nine biodiesel samples were obtained from domestic Canadian manufacturers. These samples include four soybean oil fuels (BSO-1, 2, 3, 4), 3 fuels derived from canola (BCO-1, 2, 3), and 2 samples of fuel from mixed waste fry tallow (BMO-1, 2).

2.2 Sample preparation

One hundred mg of each biodiesel was diluted to a concentration of 10 mg/mL in hexane. Several drops of DCM were required to completely dissolve the tallow fuels. Prior to GC/MS analysis, the stock solutions were spiked with 2 μg/mL internal standard (C17:0 FAME).

2.3 Separation of Polar Components

The biodiesels were fractionated by a preconditioned 3g-silica gel column to remove interferences for analysis. Approximately 20 mg of biodiesel spiked

with 5 µg/mL of surrogate β-esradiol-17-acetate was loaded onto the column. The column was then rinsed with 45 mL of DCM followed by 15 mL methanol. The corresponding fractions were designated as F1, and F2, respectively, where F1 fraction contained > 95% FAMEs, and F2 fraction contained all polar components.

2.4 Derivatization of Sterols

The sterol calibration curve was generated using mixtures of the sterols reference materials (see 2.1 above) of 0.2, 1.0, 5, 20, and 100 µg/mL for each compound were prepared in acetone. Aliquots of 0.25 mL of each solution were blown to dryness. TMS-ether derivatives were prepared by adding 200 µL of BSTFA with 1% TMCS to the vials. The vials were tightly capped and heated at 60^{o}C for 1 hour, and kept overnight at ambient temperature to complete the silylation reaction. Excess reagent and solvent were by evaporation and the residue was dissolved in 250 µL hexane containing 5-α-cholestane (internal standard) at 5 µg/mL.

Similarly, 250 µL of the F2 fractions (see Section 2.3) of biodiesel samples were derivatized in the same manner for GC/MS analysis.

2.5 GC/MS analysis

All analysis was carried out using a 6890 Agilent Gas Chromatograph (GC) equipped with a 5973 Mass Selectivity Detector (MSD). Samples (1 µL) were injected in splitless mode. A DB-225 column (30 m long, 0.25 mm i.d., film thickness 0.25 µm) was used for FAMES analysis. A SAC-5 column (30 m long, 0.25 mm i.d., film thickness 0.25 µm) supplied by Supleco (Bellefonte, PA, USA) was used to separate the sterol compounds. Helium was used as a carrier gas at a constant flow rate 1 mL/min. For the analysis of FAMEs, the column temperature was programmed from 50^{o}C (held for 1 min) to 185^{o}C at a rate of 7^{o}C/min (held for 10 min), and furthered increased to 230^{o}C at a rate of 15^{o}C/min, where the temperature held for 5 min. For the analysis of sterol compounds, the column temperature was programmed from 50^{o}C (held for 1 min), and increased to 275^{o}C at a rate of 15^{o}C/min, where the temperature held for 10 min. The temperature of injector, transfer line, ion source, and MS quadrapole analyzer was held constant at 280, 280, 230 and 150^{o}C, respectively. Mass spectrum was acquired in the positive electron impact mode at 70 eV by the full scan mode.

2.6. Identification and Quantification

Target analytes were identified by comparing the peak retention times with those of standards, EI mass spectra, and the standard mass spectra.

The FAME analysis was calibrated with a series of successively-diluted standard solutions using C17:0 FAME as an internal standard. Similarly, sterol mixtures were calibrated using the solutions described in section 2.4. An internal standard method was used to determine all target analytes in the present study.

3. Results and Discussion

3.1 FAMEs composition in biodiesesl

The FAME distributions in selected biodiesels can be seen in Figure 1. For the canola oil samples, as the relative FAME abundances were: cis-9, C18:1 >

cis-9,12, C18:2 > cis-6,9,12, C18:3 > cis-11, C18:1 > C16:0 > C18:0. No significant differences in abundances were observed for all biodiesels from same feedstocks, across different production batches. All canola samples were obtained from the same supplier. Similarly, for the soy-based biodiesels, the most abundant FAMEs are cis-9,12, C18:2, followed by cis-9 > C18:1 > C16:0 > cis-9,12,15 C18:3 > C18:0. No significant differences in FAMEs abundances were observed among different batches, either.

For the two biodiesel samples made from tallow/waste fry oil, the FAMEs distribution form high to low is ordered as: cis-9, C18:1 > cis-9, 12-C18:2 or C18:0 > cis-11, C18:1 > cis-6, 9, 12, C18:3. The FAME compositions of the waste oil products are more complex than those from vegetable sources, having many low molecular-weight FAMEs. Noted that both iso-branched before anteiso-branched FAMEs were identified in these samples, typical of natural waxes originating from animals (*23, 24*). The most abundant fatty acid of tallow is cis-9, C18:1, followed by C16:0, and C18:0. The main fatty acids of canola oil are cis-9, C18:1, cis-9, 12-18:2, cis-6, 9, 12, C18:3, and C16:0. It appears, therefore, that the feedstocks of the two waste fry samples may be a mixture of beef tallow and canola oil.

3.2 Distinguishing Biodiesels by Feedstock

Significant differences were observed for biodiesels from different feedstocks, because the different fatty acid composition of the lipid feedstocks resulted in correspondingly different FAME composition. In Figure 2, the fatty-acid compositions of several vegetable and animal sources are compared with the corresponding biodiesel FAMEs distributions in the measured fuel samples.

For feedstocks of canola oil, soybean oil, and the mixture of animal fats and re-cycled waste vegetable oils, FAMEs with 16 and 18 carbons were the main constitutes for all biodiesels. However, cis-9, C18:1 for the soy oils; cis-9, 12-C18:2, for the canola oils; and cis-9, C18:1 for the waste fry oils were, respectively, the most dominant FAME components.

From Figures 1 and 2, the BSOs can be easily distinguished from the other two feedstocks by the high relative abundance of cis-9, 12-C18:2. However, the difference between canola and fry feedstock biodiesels is based only on the most abundant cis-9, C18:1. Fortunately, the biodiesels can be easily distinguished by the proportions of saturated, monounsaturated, and polyunsaturated fatty acids in the original feedstocks. The total saturated, monounsaturated, and diunsaturated FAMEs in each feed stock were calculated. The total saturated FAMEs in BMO was as high as 30%, those of the BCOs, and BSOs were about 5%, and 20%, respectively. Similarly, the total mono-unsaturated FAMEs was 60 to 80% in canola fuels, the total diunsaturated composed up to 65% of the soy-based fuels. The high content of saturated FAMEs in the waste fry sources can be ascribed to beef tallow, where the saturated fatty acids can be up to 40% of the total, but are less than 10% in canola oil, and less than 20% in most vegetable oils (*5, 25*).

3.3 Sterols in biodiesel

The phytosterols β-sitosterol (SI), campesterol (CA), campestanol (CAO), brassicasterol (BR), stigmasterol (ST), stigmastanol (STO), and Δ5-avenasterol (AV), and zoosterols including cholesterol (CS), and

β-cholestanol (CO) were identified in the biodiesel samples, and their presence confirmed by comparison with certified standards and/or literature mass spectra. Distributions of these sterols in canola, soy and waste fry oil biodiesels are shown in Figure 3.

Most abundant in the soy, BSO, samples were SI (1230 µg/g), CA (1130 µg/g) and ST (540 µg/g), with STO, AV, CS, and CAO present in concentrations ranging from 10 to 30 µg/g. However BSO-3 was notable for it's near complete lack of sterol content, even though it's FAME distribution confirms its origin from soy oil.

In the canola oils, BCO, most abundant were SI (1650 µg/g) and CA (1930 µg/g), followed by BA (420 µg/g), AV (160 µg/g), with CS being present in low quantities.

In the fry oils, BMOs, the most abundant sterol component is CS (2500 µg/g), followed by CA (250 µg/g), SI (217 µg/g), and low levels of BR, ST ,STO, and AV.

Many different sterols may be present in plant species (over 200 have been characterized), and the amounts and relative proportions of all are dependent on the plant species. The relative abundances of sterols in vegetable oils are CA, SI, and followed by ST, and AV (*26*). Also, fully saturated sterols (STO, and CAO) are generally less abundant than their unsaturated counterparts, ST and CA (*27*).

The results shown in Figure 3 are reasonable considering the complex methylation processing to produce biodiesel from origin oils. Sterols may be removed or isomerized during refining or deodorization, and reactive oxygen species, UV light, chemical catalysts, and enzymatic reactions will lead to the oxidation of sterols (*28, 29*). However, it is useful to compare the sterol profiles before and after esterificaton. The average sterol concentrations for each feedstock type were normalized to the total concentration of all phytosterols (SI+CA+CAO+BR+ST+STO+AV) and were compared with corresponding literature values (Table 1). Note that no literature values were available for phytosterols in beef tallow.

Table 1. Relative abundance (%) of free sterols to total phytosterols

Sterols	***BCO***	***Canola (12)***	***BSO***	***Soy (30)***	***BMO***
BR	10	5-13	0	-	5
CA	46	25-39	38	25	45
ST	0.2	0.2-1.0	18	23	3.9
SI	39	45-58	41	49	40
AV	3.7	2.5-6.6	1.1	1.4	3.0

The major phytosterol profiles for each biodiesel were similar to those for the corresponding feedstock oil. Note also that the phytosterol profiles of the BMOs fuels are similar to those from canola oil, further supporting the presence of canola oil in the waste fry source.

Therefore, while the transformation process from oil or fat to biodiesel may affect the absolute amount of free sterols before and after esterification, the relative abundances of the phytosterols in the biodiesels appears to be directly related to the distribution profiles of the source feedstock oils.

Phytosterols are abundant in the biodiesels from vegetable oils, while cholesterol was the main sterol component in the biodiesels from animal source-conting waste fry oil source. Cholesterol is principally synthesized by animals, therefore, this significant difference distinguish such fuels from vegetable sources.

Although SI and CA both found in the biodiesels from vegetable oils (i.e. soybean and canola oils), BR was only found to be significant in BCOs. BR concentration is often used to determine the presence of rapeseed or canola oils in other oils (*15*), because it is one of the major sterols present in rapeseed and canola, and is also unique to these oils. Similarly, BR can be selected as an indicator to reveal the source of rapeseed or canola oil source for biodiesel.

While, ST was the third most abundant sterol in the BSOs, but it was found in very low abundance in the BCOs. This difference may also be used as a marker for positively identifying the presence of soy oil in the biodiesel feedstock.

The free phytosterol profiles in biodiesels from different source oils have significant differences. These differences, combined with the FAME profiles in specific biodiesels, could be used for the forensic identification of spilled biofuels.

4. Conclusions

In the present study, the profile of fatty acid esters and free sterols for several biodiesels from different feedstocks including vegetable oils, and animal fats have been identified and quantified. The main conclusions are described as follows:

1) FAMEs are the majority components for all biodiesels examined. The main fatty acid ester profiles ranged from C16 to C18, which depending on the feedstocks. The most abundant FAMES were cis-9, C18:1, and cis-9, 12, C18:2 in canola and soy sources, respectively. The FAMEs distribution of waste fry biodiesels was more complex than biodiesels from pure vegetable oils.

2) The sums of saturated, mono-unsaturated and di-unsaturated fatty acids esters were found to elucidate differences between canola, soy and animal fats source biofuels. The amounts of saturated FAMES in animal fat biofuels were far higher than those from botanic sources.

3) Free sterols were found to have significant potential as alternative biomarkers to trace the source of biodiesels. Further, the free sterol profiles from vegetable oils were found to be strongly related to those in the corresponding biodiesels. It was found that free sterol profiles can be acted as accessorial parameters to forensic identify the biodiesel source by combining with fatty acid profiles.

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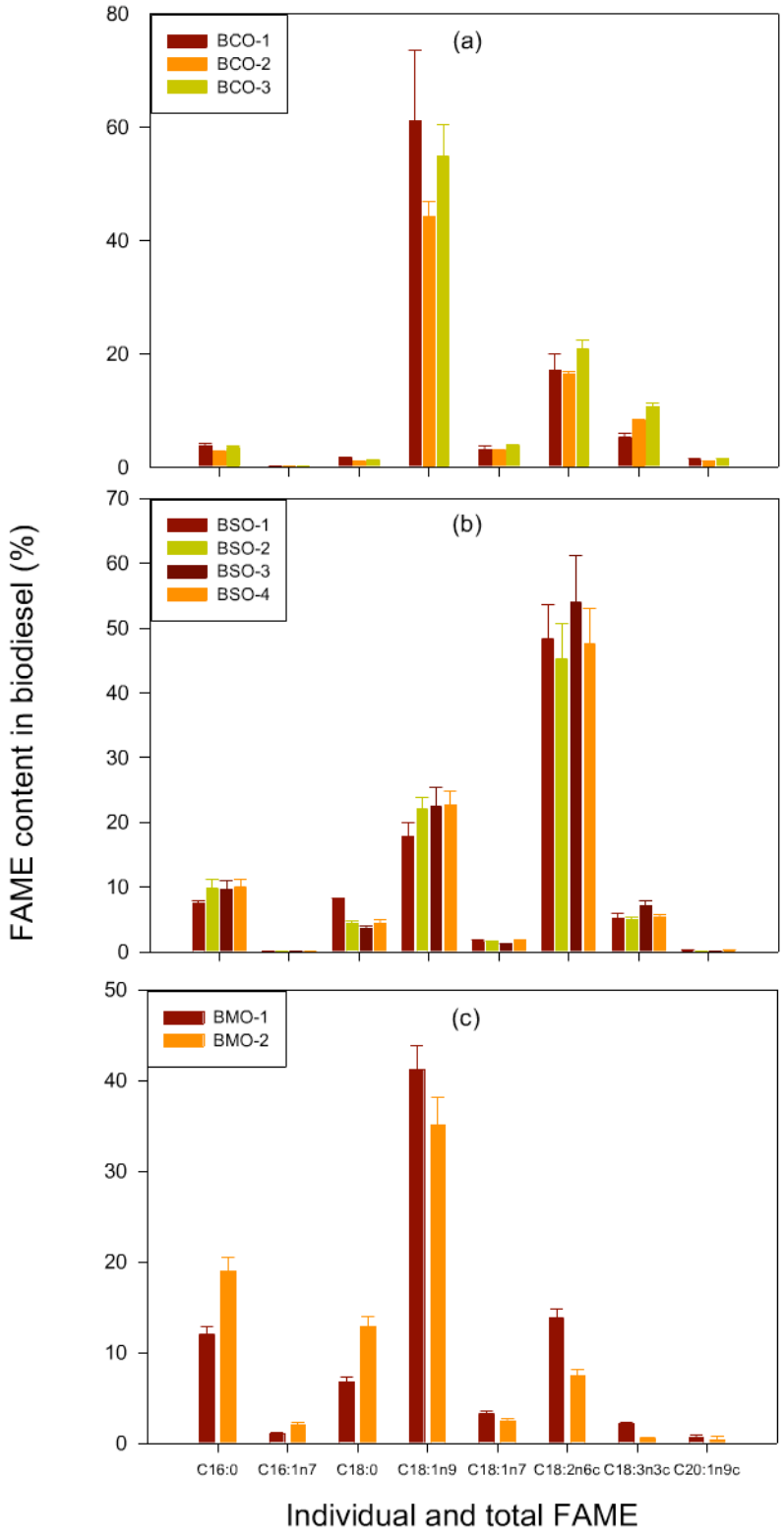

Figure. 1 Comparison of FAME Distribution of Biodiesels from Three Commonly-used Feedstocks

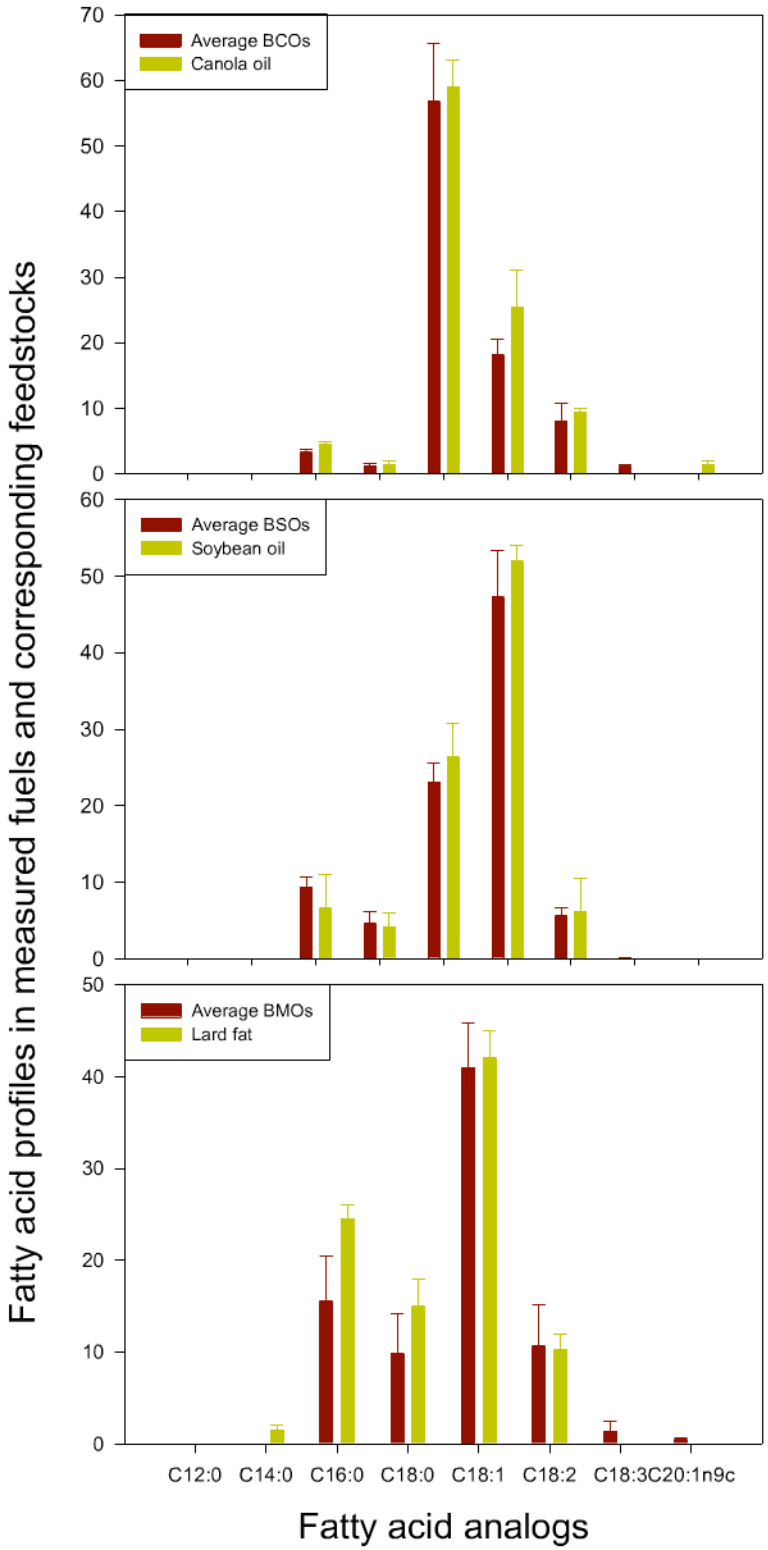

Figure 2 Comparison of Relative Abundances of Fatty Acids in Feedstock oils and FAMEs in Corresponding Biodiesels

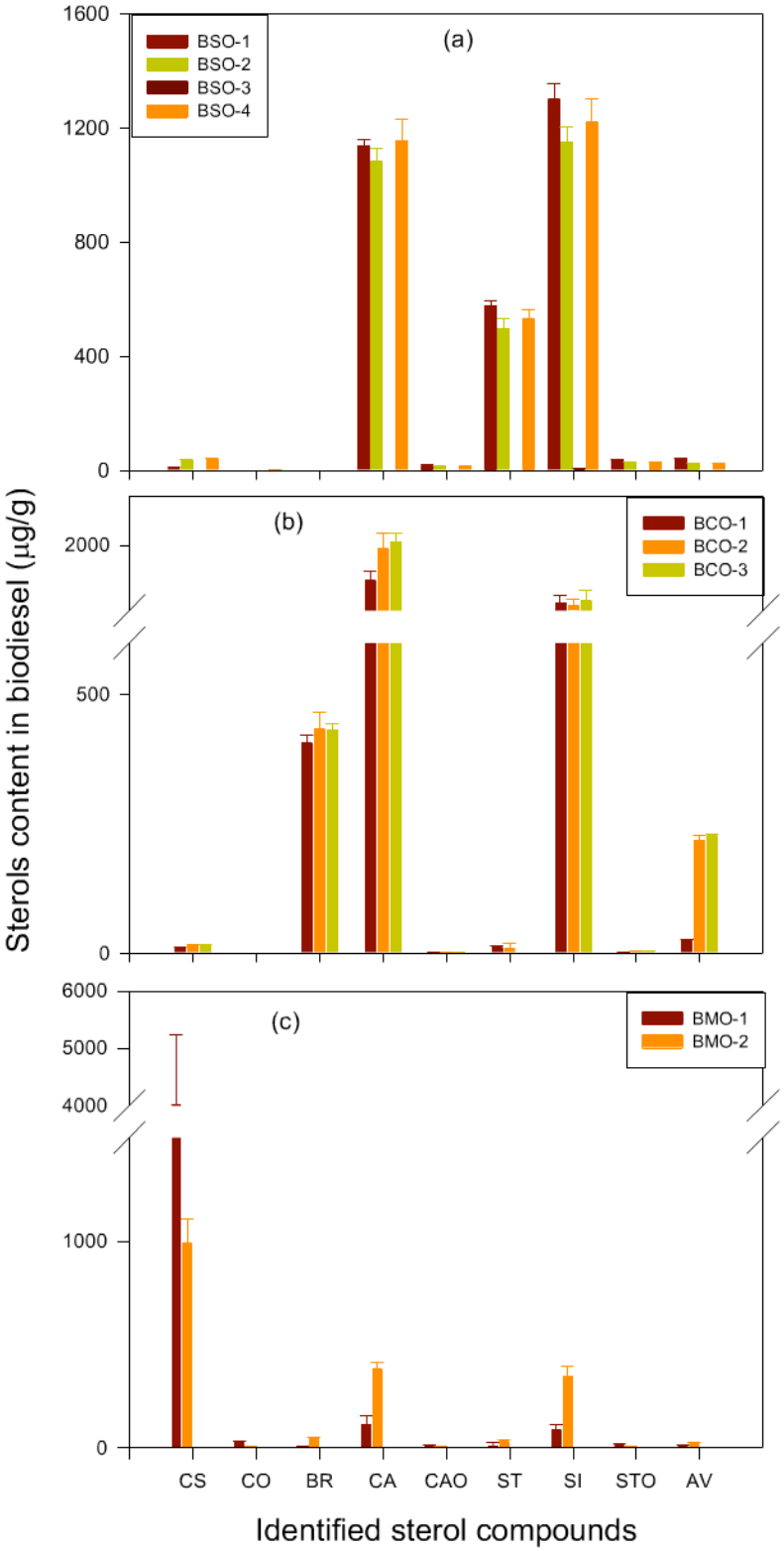

Figure. 3 Sterol Abundances in Selected Biodiesels.