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Development of Methods to Quantify Free and Conjugated Steroids in Fatty Matrices by HPLC−MS/MS

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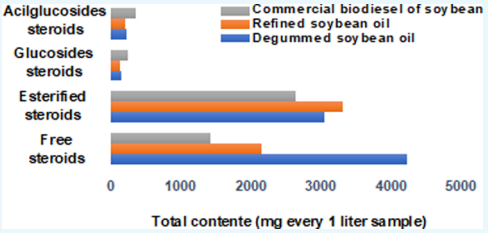
ABSTRACT: Steroids are called the "fingerprint" of oils, fats, and their derivatives. Different classes of steroids may be present in these matrices. Most of the methods developed to analyze these constituents involve the determination of free steroid content, although their conjugated forms are extremely important in determining the total composition. Thus, this article demonstrates that the coupling of sequential mass spectrometry and high-performance liquid chromatography obtained high sensitivity and high specificity of mass resolution to identify and quantify the main classes of steroids. Four methods were developed to quantify steroids free, esterified, glucosides, and acylated glucosides by internal standardization using betulin. The main validation parameters were tested and demonstrated good correlation results for the methods. The content of free steroids was the majority in all samples, whereas the content of glucoside steroids was the least abundant. The contents of free steroids quantified in the degummed soybean oil were significantly reduced in relation to the refined oil. A small amount of esterified steroids was superior in refined soybean oil than in degummed oil. Comparing the steroid content between degummed oil and biodiesel, we found that the concentration of free and esterified steroids decreases in the conversion to biodiesel, whereas the concentration of glucoside steroids increases slightly.



INTRODUCTION

Oils, fats, and their derivatives are compounds composed mainly of fatty acids. However, among their minor compounds are steroids, which are called the "fingerprint" of these matrices.[1] Phytosteroids are steroids of plant origin,[2] among which are β-sitosterol, campesterol, and stigmasterol.

Among the steroids found in oils and fats from animal origin, cholesterol is the best known.[3] In addition to steroids in free form (S), other different classes of steroids may be present, such as sterol esters (SE), when the hydroxyl group of the free steroid is esterified by a saturated or unsaturated long chain fatty acid or by phenolic acids, glucoside steroids (SG), when the hydroxyl group is attached to a monosaccharide, usually glucose, and acylated (esterified) glucoside steroids (ASG), when a fatty acid molecule is also attached to this monosaccharide.[4]

Most of the research involves determining the free steroid content, although its conjugated forms are important in determining the total composition of these constituents.[5] However, methods to determine the conjugated steroids in fatty matrices are more expensive and therefore not common in the scientific literature.[6] In addition, the content of conjugated steroids is much lower than that of free steroids.[7]

The classes of conjugated steroids, that is, SE, SG, and ASG, act as membrane components for transport and/or as signaling molecules. Some factors such as species, growth, and storage conditions cause variations in their contents, and this should be taken into account when the steroid content is evaluated and compared.[8]

The quantification of steroids using different extraction and separation techniques in different matrices and the technique capable of quantifying all classes of steroids need to be investigated. This is because the interconversion of the steroid classes can occur within the study matrix, making it difficult to understand, for example, which fatty acids the esterified steroids (ES) and ASG steroids may be linked to.[9] Another issue is the development of the analytical method because the lack of pure and certified standards for all phytosteroids and the presence of a large number of molecular species interfere with the quality of the analysis and quantification.[10]

Apparently, no single sample preparation technique is available that can be universally applied for the steroid analysis. Selection of a specific type of specimen preparation depends on the sensitivity required, the type of matrix that contain these steroids, and the efficiency of the method.[11] The coupling of high-performance liquid chromatography and sequential mass spectrometry (HPLC−MS/MS) obtained high sensitivity and high specificity for identification and quantification of lipids at low concentrations and with complex structures.[12]

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Many methods have been developed to analyze cholesterol and oxysteroids in biological samples by HPLC−MS/MS.[10,11,13−15] However, for oilseeds and derivatives, no comprehensive method for steroid analysis has been developed. Thus, considering all of the above challenges, this work aimed to develop a method to analyze each of the steroid classes, using HPLC−MS/MS.

The methodologies developed in this article were applied to study the composition of the main classes of steroids in oils, fats, and their main bioderivative, biodiesel. Particularly for biodiesel, such a development is very important because in recent years, the residues generated during the processing, use, and storage of biodiesel have been reported, with evidence that these residues come from byproducts and unsaponifiable compounds, mainly steroids.[16,17]

Research that cites steroids as the cause of sedimentation in biodiesel always considers steroid classes of glucosides as potentially harmful. This is because, with their greater polarity, their insolubility in biodiesel tends to occur when acylglycerides are converted to esters of fatty acids, which is intensified when biodiesel is mixed with highly apolar diesel oil.[18] Hence, the research presented in this article will provide further understanding of the composition of oils, fats, and biodiesel as well as promote the understanding of events such as the formation of lees and sediments in biodiesel.

RESULTS AND DISCUSSION

Considering the variety of steroids that exist, external standardization often becomes impractical because several analytical curves are required, and for many steroids, their standards are not even marketed.[19] Thus, the internal standardization method has been increasingly applied to study the diversity of steroids in a sample.[20]

Internal standards commonly used are steroids not present in the composition of oils and fats, such as ergosterol, or other derivatives of triterpene alcohols, such as betulin. Thus, betulin was used as an internal standard in all methods developed in this article. The concentration of these steroids is then commonly expressed in milligrams of steroid per kilogram of sample (mg kg^{-1}) or milligrams of steroid per liter of sample (mg L^{-1}).

It is necessary to show that the analysis of different classes of steroids is only possible when using a matrix without modification because the fatty acid-linked steroid classes (ES and ASG) may undergo loss of the steroid−acid bond fat.[20] We already stated that combining any or all of the methods proposed and developed was not practical. This is because the number of ions monitored is very large, and the concentration of these constituents is low.

In the mass spectra, the analysis of free steroids could not monitor the molecular ion of the analytes or the internal betulin standard, which produced intense ions corresponding to the loss of water internally in the sterol molecule. The analysis of the ES was able to monitor the molecular ion, which was connected to an ammonium (adduct) molecule, derived from the ammonium acetate added to the mobile phase. As water was present in the mobile chromatographic phase of the methods to quantify glycoside steroids SG and ASG, the hydrogen bond formed between the molecular ion of the SG and a water molecule was considered.

With the developed method, we managed to monitor particular fragments of the set of fused rings present in the steroid structure and help in structural characterization.[14] As avenasterol and stigmasterol are position isomers, the quantification of the two was given by the sum of their contents and quantified together. The same occurred in the quantification of cholesterol and lathosterol.

To quantify steroids in their different classes, calibration curves of betulin were set up in the refined soybean oil matrix, considering the range of concentration expected for each class. Thus, the linear regression method was used to determine the parameters of the quantitative analytical curve.

A single betulin calibration curve was applied to the steroid glucoside and acylated glucoside methods. Thus, the linear regression curves for the free steroids, ES, and glucoside steroids (SG and ASG) presented linear correlation coefficients (r) 0.9994, 0.9979, and 0.9989, respectively. Another parameter evaluated was the recovery test, which evaluates the precision and accuracy of the methods. The concentration obtained from betulin in each method was quantified by its recovery, considering the fortified concentration and the average concentration obtained by the result of the tests in triplicate.

The coefficient of variation (CV) and the standard deviation (SD) of the recovery trials were calculated to determine the precision and accuracy of the methods, respectively, by applying the Student's t-distribution statistical test. Table 5 provides the results obtained for the recovery for each method. All samples were analyzed by the same analyst in a short time and using the same equipment.

The results in Table 1 demonstrate that both precision and accuracy of the methods were statistically adequate.

Table 1. Analysis of Variance (ANOVA) Obtained for Each Method

	ANOVA of the regression for the free steroid method (S)				
	gl	SQ	MQ	$F_{calc.}$	$F_{tab.}$
regression	1	1.57×10^{14}	1.57×10^{14}	6.21×10^{3}	8.07
residue	7	1.77×10^{11}	2.53×10^{10}		
total	8	1.57×10^{14}			
	ANOVA of the regression for the ES				
	gl	SQ	MQ	$F_{calc.}$	$F_{tab.}$
regression	1	1.07×10^{13}	1.07×10^{13}	1.95×10^{3}	7.57
residue	8	4.38×10^{10}	5.48×10^{9}		
total	9	1.07×10^{13}			
	ANOVA of the regression for the glucosylated steroids (SG & ASG)				
	gl	SQ	MQ	$F_{calc.}$	$F_{tab.}$
regression	1	3.23×10^{8}	3.23×10^{8}	1.47×10^{3}	7.57
residue	8	1.76×10^{6}	2.19×10^{5}		
total	9	3.25×10^{8}			

Methods developed for the low-level analysis should have an acceptable CV of up to 20% for the method to be considered accurate, depending on the complexity of the sample. To evaluate the accuracy of the methods by Student's t-distribution, the value of t-distribution t_{calc} should be less than or equal to that of t-distribution tab, with reliability of 95%.[21]

The exact concentration of the internal standard was determined by the analytical curves and the linear relationship between the concentrations of the internal standard. Each quantified steroid was established by taking into account the volume of the standard added and the volume of the sample tested.[21] In addition, the correction factor for each steroid was calculated, taking into account the ratio of the molar masses of the internal standard and the respective steroid in question. The

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Table 2. Quantitative Data for All Classes of Steroids in the Matrices Study

retention time (min)	free steroids (S)	mass (mg) every 1 L sample		
		degummed soybean oil	refined soybean oil	commercial biodiesel of soybean
6.75	β-sitosterol	2520.00 ± 0.21	1121 ± 0.21	814.38 ± 0.21
6.12	avenasterol + stigmasterol	730.69 ± 0.22	409.13 ± 0.22	229.09 ± 0.22
4.28	brassicasterol	60.22 ± 0.22	0.00 ± 0.22	10.43 ± 0.22
6.03	campesterol	900.78 ± 0.22	610.49 ± 0.22	370.20 ± 0.22
6.77	stigmastanol	16.30 ± 0.21	6.81 ± 0.21	4.09 ± 0.21
	total content	4227.09 ± 0.22	2147.43 ± 0.22	1428.19 ± 0.22
retention time (min)	ES	mass (mg) every 1 L sample		
		degummed soybean oil	refined soybean oil	commercial biodiesel of soybean
3.55	β-sitosterol—C 14:0	0.00 ± 0.52	0.00 ± 0.52	72.80 ± 0.52
3.57	β-sitosterol—C 16:0	381.95 ± 0.52	124.70 ± 0.52	155.15 ± 0.52
3.59	β-sitosterol—C 18:0	194.00 ± 0.52	182.37 ± 0.52	133.13 ± 0.52
3.58	β-sitosterol—C 18:1	549.58 ± 0.52	1009.63 ± 0.52	308.73 ± 0.52
3.58	β-sitosterol—C 18:2	528.39 ± 0.52	448.11 ± 0.52	230.05 ± 0.52
3.57	β-sitosterol—C 18:3	114.74 ± 0.52	197.94 ± 0.52	308.24 ± 0.52
3.56	β-sitosterol—C 20:0	125.43 ± 0.52	0.00 ± 0.52	230.70 ± 0.52
3.59	β-sitosterol—C 22:0	0.00 ± 0.52	0.00 ± 0.52	122.99 ± 0.52
	total β-sitosterol esterified	1894.09 ± 0.52	1962.75 ± 0.52	1561.79 ± 0.52
3.11	avenasterol + stigmasterol-C16:0	209.97 ± 0.52	128.42 ± 0.52	118.79 ± 0.52
	total avenasterol + stigmasterol esterified	209.97 ± 0.52	128.42 ± 0.52	118.79 ± 0.52
2.78	campesterol—C 14:0	0.00 ± 0.52	0.00 ± 0.52	16.80 ± 0.52
2.80	campesterol—C 16:0	121.84 ± 0.52	24.22 ± 0.52	58.18 ± 0.52
2.77	campesterol—C 16:1	94.00 ± 0.52	82.37 ± 0.52	71.39 ± 0.52
2.79	campesterol—C 18:0	126.32 ± 0.52	409.63 ± 0.52	208.73 ± 0.52
2.79	campesterol—C 18:1	23.87 ± 0.52	216.59 ± 0.52	28.41 ± 0.52
2.80	campesterol—C 18:2	230.51 ± 0.52	83.59 ± 0.52	213.07 ± 0.52
2.81	campesterol—C 18:3	18.00 ± 0.52	118.87 ± 0.52	31.18 ± 0.52
2.80	campesterol—C 20:0	22.44 ± 0.52	0.00 ± 0.52	13.93 ± 0.52
2.81	campesterol—C 22:0	0.00 ± 0.52	0.00 ± 0.52	9.27 ± 0.52
	total campesterol esterified	636.98 ± 0.52	935.27 ± 0.52	717.76 ± 0.52
2.87	stigmastanol—C 14:0	0.00 ± 0.52	18.58 ± 0.52	0.00 ± 0.52
2.89	stigmastanol—C 16:0	23.86 ± 0.52	0.00 ± 0.52	0.00 ± 0.52
2.90	stigmastanol—C 18:1	46.31 ± 0.52	27.31 ± 0.52	0.00 ± 0.52
2.91	stigmastanol—C 18:2	54.48 ± 0.52	41.72 ± 0.52	33.19 ± 0.52
2.90	stigmastanol—C 18:3	0.00 ± 0.52	52.19 ± 0.52	0.00 ± 0.52
	total stigmastanol esterified	124.65 ± 0.52	139.80 ± 0.52	33.19 ± 0.52
2.57	lanosterol—C 14:0	0.00 ± 0.52	0.00 ± 0.52	9.16 ± 0.52
2.58	lanosterol—C 16:0	0.00 ± 0.52	20.06 ± 0.52	25.52 ± 0.52
2.57	lanosterol—C 16:1	0.00 ± 0.52	0.00 ± 0.52	10.41 ± 0.52
2.58	lanosterol—C 18:0	25.11 ± 0.52	0.00 ± 0.52	29.69 ± 0.52
2.60	lanosterol—C 18:1	67.44 ± 0.52	48.37 ± 0.52	33.49 ± 0.52
2.59	lanosterol—C 18:2	63.45 ± 0.52	34.13 ± 0.52	41.29 ± 0.52
2.59	lanosterol—C 18:3	0.00 ± 0.52	39.81 ± 0.52	22.83 ± 0.52
2.61	lanosterol—C 20:0	26.65 ± 0.52	0.00 ± 0.52	20.32 ± 0.52
2.60	lanosterol—C 22:0	0.00 ± 0.52	0.00 ± 0.52	8.61 ± 0.52
	total lanosterol esterifieds	182.65 ± 0.52	142.37 ± 0.52	201.32 ± 0.52
	total content	3048.34 ± 0.52	3308.61 ± 0.52	2632.06 ± 0.52
retention time (min)	glucosides steroids (SG)	mass (mg) every 1 L sample		
		degummed soybean oil	refined soybean oil	commercial biodiesel of soybean
13.91	β-sitosterol—gluc.	74.24 ± 0.76	61.96 ± 0.76	103.41 ± 0.76
12.90	avenasterol—gluc. + stigmasterol—gluc.	00.00 ± 0.76	00.00 ± 0.76	35.26 ± 0.76
12.80	campesterol—gluc.	36.31 ± 0.76	37.84 ± 0.76	37.05 ± 0.76
17.40	stigmastanol—gluc.	50.46 ± 0.76	37.14 ± 0.76	32.03 ± 0.76
10.59	lanosterol—gluc.	00.00 ± 0.76	00.00 ± 0.76	37.09 ± 0.76
	total content	161.01 ± 0.76	136.94 ± 0.76	244.84 ± 0.76
retention time (min)	acylated glucosides steroids (ASG)	mass (mg) every 1 L sample		
		degummed soybean oil	refined soybean oil	commercial biodiesel of soybean
16.48	β-sitosterol—C 16:1—gluc.	00.00 ± 0.76	00.00 ± 0.76	55.25 ± 0.76
16.49	β-sitosterol—C 18:1—gluc.	58.70 ± 0.76	63.32 ± 0.76	65.69 ± 0.76

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Table 2. continued

retention time (min)	acylated glucosides steroids (ASG)	mass (mg) every 1 L sample		
		degummed soybean oil	refined soybean oil	commercial biodiesel of soybean
16.49	β-sitosterol—C 18:2—gluc.	62.05 ± 0.76	57.02 ± 0.76	68.43 ± 0.76
16.50	β-sitosterol—C 18:3—gluc.	94.85 ± 0.76	91.87 ± 0.76	134.70 ± 0.76
	total β-sitosterol esterified	215.60 ± 0.76	212.21 ± 0.76	324.07 ± 0.76
17.45	campesterol—C 20:0—gluc.	00.00 ± 0.76	00.00 ± 0.76	11.70 ± 0.76
	total campesterol esterified	00.00 ± 0.76	00.00 ± 0.76	11.70 ± 0.76
20.09	stigmastanol—C 18:1	00.00 ± 0.76	00.00 ± 0.76	8.27 ± 0.76
20.07	stigmastanol—C 18:2	00.00 ± 0.76	00.00 ± 0.76	10.06 ± 0.76
20.10	stigmastanol—C 18:3	6.39 ± 0.76	7.11 ± 0.76	6.02 ± 0.76
	total stigmastanol esterified	6.39 ± 0.76	7.11 ± 0.76	24.35 ± 0.76
	total content	221.90 ± 0.76	219.32 ± 0.76	360.12 ± 0.76

results are expressed in milligrams (mg) of steroid per liter (L) of the sample (Table 2).

The samples of degummed and refined soybean oils in addition to 100% soybean biodiesel were chosen to verify the possible influence of oil processing on the composition of the major classes of steroids, as some authors claim.[22] We decided to use degummed soybean oil, not totally "crude" soybean oil, because the degumming stage is a basic step in the processing of oils and fats, prior to their use.[22]

Table 6 shows the junction of all steroids that were quantified in the four methods developed. The steroids without a detectable signal were not entered as quantifiable in this table. Those that showed a signal, but in intensity below the quantifiable by the analytical curve, were reported as 0.00 ± SD.

The amounts of each steroid in the ES and ASG classes in the matrices followed the abundance of the fatty acids in them, that is, the fatty acids bound to the steroidal structures are usually the most abundant in these samples.[23] It is important to note that the commercial biodiesel used was not derived from the used soybean oil samples, and the refined soybean oil was also not derived from the degummed soybean oil, which does not permit a reliable comparison of the steroid contents between the samples.

Nevertheless, some comments will be made regarding such contents. The results showed that in the three samples studied, the content of free steroids (S) was higher, followed by the contents of ES and ASG, and the lowest content was that of SG. The quantification in degummed soybean oil compared to the refined soybean oil found that the content of free steroids is approximately 51% higher in degummed oil. This was expected because the refining process ends up eliminating some of these compounds, especially when the alkaline neutralization step is used during refining.[24]

The content of ES was higher in refined oil, with 8% more than that obtained in degummed oil. Some refining methods replace the alkaline stage with acid during the process, and this acid step can also help to increase the content of ES because the high acidity of the medium leads to the formation of SE.[23]

The comparison between the contents of the SG classes found similar concentrations in the degummed and refined oil. The samples of refined and degummed oil have lower SG and ASG contents than the analyzed biodiesel sample; this can be explained by the highly nonpolar behavior of these matrices and richness in triacylglycerides.

Comparison of the steroid content between the degummed oil and the biodiesel (since this type of oil is the most used for its production) found that the concentration of free and ES is usually reduced in the conversion to biodiesel, whereas the concentrations of SG and ASG increase.

Again, this can be explained by the reactional conditions of the transesterification process (used in most industrial biodiesel production). The transesterification process increases the contents of SG and ASG by converting S to SG and ES and ASG. As it is a polar environment, under alkaline conditions, this interconversion can occur more intensely and quickly.

For biodiesel, the concentration of SG is detrimental to the sedimentation problems, causing tribulations in the use and storage of the biodiesel.[16] SG are a class that includes saponins and glucoalkaloids derived from secondary plant metabolism and characterized by the formation of lather, presenting surfactant properties.[16] However, the presence of ASG occurs because of the reaction of SG with the fatty acids in the matrix, such as the one that occurs between S and ES.[17]

The process of biodiesel production ends up eliminating part of the unsaponifiable matter constituting oils, and the refining process decreases it.[18] Thus, the amount of steroids still present will depend on how biodiesel is produced and purified, among other factors. The abundance of SG is evidence that the oil refining process cannot completely eliminate the SG and ASG contents.[17]

Haupt et al.[25] state that the presence of SG at levels above 100 ppm affects the quality of biodiesel in relation to sedimentation. Thus, considering the biodiesel analyzed here, which presented an SG concentration of 244.84 ± 0.76 mg L^{-1}, such biodiesel could generate sedimentation problems. Because of the reaction conditions, the concentration of SG is increased when the biodiesel is produced. However, the free steroid content is reduced in a substantial proportion during the oil refining process.

Thus, the production process of biodiesel from a chemically refined primary raw material could control the reduction of the classes of conjugated steroids because an interconversion between free and conjugated steroids is known. This is to support the problem of sedimentation not only in relation to steroids but also in relation to other contaminants from the unsaponifiable matter.[26]

Thus, knowing the steps of the chemical refining process would lead to significant losses or modifications in the composition of these minor compounds.[24] The degumming stage with water, for example, is cited by some researchers as being able to effectively remove steroid glycosides (SG and ASG) to an undetectable level, but the industrial refining process used increases the cost of biodiesel production.[24,27]

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CONCLUSIONS

The methodologies developed in this article, using internal standardized quantification, were comprehensive in the analysis of all classes of steroids in the matrices of oils, fats, and derivatives. The quantification of steroids in the samples studied here found that the chemical refining stage in raw materials for production biodiesel helps to eliminate a large part of the free steroid content, the most abundant class, thus reducing the chances of interconversion of free steroids in SG, ASG, and ES.

Different procedures have been developed for the different classes of steroids, and it was not possible to combine them; however, the elimination of pretreatment steps of the sample minimizes this inconvenience, bringing greater reliability in relation to the quantitative determinations of these constituents. In addition, the methods developed have rapid analysis times, with easy quantification and statistical reliability, corresponding to an appropriate analytical solution to analyze free and conjugated steroids in oils, fats, and derivatives.

EXPERIMENTAL SECTION

The methodologies developed in this article were based on the work of Wewer et al.[15] where free, esterified, glycoside, and acyl glycoside steroids were analyzed via Q-time of flight-MS/MS operated in the positive mode through direct nanospray infusion applied to a nonoleaginous vegetable.

Reagents, Standards, and Samples. LC−MS grade solvents from J.T. Baker (USA) were used. Reference standards used for β-sitosterol, cholesterol palmitate, and betulin with purities greater than 99% were purchased from Sigma-Aldrich (USA); β-sitosterol glucoside (purity > 98%) was purchased from Clearsynth (India), and β-sitosterol glucoside palmitate (purity > 95%) was purchased from BOC Sciences (USA). The samples analyzed were degummed soybean oil obtained from an oil industry, commercial refined soybean oil, and soybean biodiesel (ANP-BRAZIL), prepared by collecting 1 mL of each sample and solubilizing them in dichloromethane in a 10 mL volumetric flask. The reference standards were also solubilized in dichloromethane.

Methods to Analyze Steroids by HPLC-MS/MS. A triple quadrupole mass sequential MS/MS (SCIEX—USA) system coupled to the HPLC (Agilent) system was used. The ionization source was the chemical ionization at atmospheric pressure (APCI) using the multiple reaction monitoring (MRM) experiment. The heating interface was activated, and the average collision frequency was applied. The gas pressure at the ion source was 45 psi. An Agilent model 1260 HPLC system containing a quaternary pump and a thermostated sampler with an automatic syringe was used as also a thermostatized column compartment. Chromatographic separations were performed using a Poroshell 120 C-18 column (50 mm × 4.6 mm × 2.7 μm). The entire HPLC−MS/MS system was controlled by Analyst software 1.5.2.

Previous knowledge about the pattern of steroid fragmentation as well as information from scientific works helped to assemble the MRM experiment in this research.[13,16,17] The reference standards β-sitosterol, cholesterol palmitate, β-sitosterol-glucoside, and β-sitosterol glucoside palmitate were used as the basis to optimize the experimental parameters in the mass spectrometer for the class of free steroids, glucosides, and acylated glycosides, respectively. Thus, with the knowledge about the fragmentation characteristic of these patterns, the fragmentation of the other possible steroids of each class could be estimated. The particularities of each method will be described in detail below.

Method for Free Steroids. From the knowledge about the characteristic fragments from β-sitosterol, it was possible to estimate the fragmentation of nine other free steroids: Δ5-avenasterol, brassicasterol, campesterol, cholesterol, desmosterol, stigmastanol, stigmasterol, lanosterol, and lathosterol. The spectral scanning range used was m/z 300−450. The temperature of the ionization source was maintained at 400 °C, the collision gas pressure was 12 psi, and the applied misting current was 3 μA. Table 3 presents the individual optimization parameters as well as the monitored ions.

Table 3. Ions Monitored without MRM Experiment to Analyze Free and Significant Steroids

	parameters for each steroid					
steroids	monitored transition (m/z)		CE (V)	DP (V)	collision cell input potential (V)	collision cell exit potential (V)
β-sitosterol	397.5	161.2	23.0	49.0	16.0	4.0
avenasterol	395.5	213.1	40.0	77.0	19.0	2.5
brassicasterol	381.5	119.2	25.5	41.0	20.0	3.2
campesterol	383.5	147.2	35.0	45.0	15.0	2.4
cholesterol	369.5	147.2	45.0	35.0	21.0	5.0
desmosterol	367.5	147.1	21.0	46.0	22.0	6.0
stigmastanol	399.5	135.1	20.0	45.0	22.0	5.0
stigmasterol	395.4	105.2	35.0	45.0	15.0	3.0
lanosterol	409.5	133.3	35.0	45.0	15.0	3.0
lathosterol	369.1	273.0	30.0	40.0	20.0	3.0

In the chromatograph, the temperature in the sampler tray was adjusted to 25 °C, and the flow rate of the mobile phase was 1000 μL min^{-1} with a sample injection of 5 μL. The composition of the mobile phase entailed a gradient of acetonitrile (90%) and dichloromethane (10%) for 6 min, the percentage being changed to 85:15% (acetonitrile/dichloromethane) up to minute 7, and later to 75:25% (acetonitrile/dichloromethane) until minute 11. The period from 11 to 15 min is to the optimal time for rebalancing the mobile phase for the subsequent sample.

Method for ES. As the matrices of oils, fats, and their derivatives are rich in fatty acids, numerous ES can be formed.[15] Therefore, it was assumed that the formation of ES would occur from the monitored free steroids, each of which could form a different steroid ester with the main fatty acids of the soybean, being myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), linolenic acid (C18:3), arachidic acid (C20:0), and behenic acid (C22:0).

From this, the MRM experiment was set up, and the presence of 90 different ES was monitored. The spectral scanning range was m/z 600−800. The temperature of the ionization source was maintained at 430 °C, the pressure of the collision gas was 10 psi, and the applied misting current was 3 μA. The declustering potential (DP), entrance potential (EP), collision cell input potential (CEP), and collision cell exit potential (CXP) were 35, 8, 25, and 5 V, respectively. Collision energy (CE) was maintained at 25 eV. Table 4 presents the individual optimization parameters of the transition as well as the ions monitored in the method.

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ACS Omega 2020, 5, 12270−12277

Table 4. Ions Monitored in the MRM Experiment to Analyze ES and Optimized Parameters

	β-sitosterol		avenasterol		brassicasterol		campesterol		cholesterol	
fatty acid	Q1	Q3	Q1	Q3	Q1	Q3	Q1	Q3	Q1	Q3
C 14:0	642.6	397.5	640.6	395.5	626.6	381.5	628.6	383.5	614.6	369.5
C 16:0	670.6		668.6		654.6		656.6		642.6	
C 16:1	668.6		666.6		652.6		654.6		640.6	
C 18:0	698.6		696.6		682.6		684.6		670.6	
C 18:1	696.6		694.6		680.6		682.6		668.6	
C 18:2	694.6		692.6		678.6		680.6		666.6	
C 18:3	692.6		690.6		676.6		678.6		664.6	
C 20:0	726.6		724.6		710.6		712.6		698.6	
C 22:0	754.6		752.6		738.6		740.6		726.6	
	desmosterol		stigmastanol		stigmasterol		lanosterol		lathosterol	
fatty acid	Q1	Q3	Q1	Q3	Q1	Q3	Q1	Q3	Q1	Q3
C 14:0	612.6	367.5	644.6	399.5	640.1	395.4	654.6	409.5	614.1	369.1
C 16:0	640.6		672.6		668.1		682.6		642.1	
C 16:1	638.6		670.6		666.1		680.6		640.1	
C 18:0	668.6		700.6		696.1		710.6		670.1	
C 18:1	666.6		698.6		694.1		708.6		668.1	
C 18:2	664.6		696.6		692.1		706.6		666.1	
C 18:3	662.6		694.6		690.1		704.6		664.1	
C 20:0	696.6		728.6		724.1		738.6		698.1	
C 22:0	724.6		756.6		752.1		766.6		726.1	

Table 5. Ions Monitored in the MRM Experiment to Analyze SG and Optimized Parameters

transitions	β-sitosterol	avenasterol	brassicasterol	campesterol	cholesterol
Q1	594.6	592.6	578.6	580.6	566.6
Q3	397.5	395.5	381.5	383.5	369.5
transitions	desmosterol	stigmastanol	stigmasterol	lanosterol	lathosterol
Q1	564.6	596.6	592.1	606.6	566.1
Q3	367.5	399.5	395.4	409.5	369.1

Table 6. Ions Monitored in the MRM Experiment to Analyze ES and Optimized Parameters

	β-sitosterol		avenasterol		brassicasterol		campesterol		cholesterol	
fatty acid	Q1	Q3	Q1	Q3	Q1		Q1	Q3	Q1	Q3
C 14:0	804.6	397.5	802.6	395.5	788.6	381.5	790.6	383.5	776.6	369.5
C 16:0	832.6		830.6		816.6		818.6		804.6	
C 16:1	830.6		828.6		814.6		816.6		802.6	
C 18:0	860.6		858.6		844.6		846.6		832.6	
C 18:1	858.6		856.6		842.6		844.6		830.6	
C 18:2	856.6		854.6		840.6		842.6		828.6	
C 18:3	854.6		852.6		838.6		840.6		826.6	
C 20:0	888.6		886.6		872.6		874.6		860.6	
C 22:0	916.6		914.6		900.6		902.6		888.6	
	desmosterol		stigmastanol		stigmasterol		lanosterol		lathosterol	
fatty acid	Q1	Q3	Q1	Q3	Q1		Q1	Q3	Q1	Q3
C 14:0	774.6	367.5	806.6	399.5	802.1	395.4	816.6	409.5	776.1	369.1
C 16:0	802.6		834.6		830.1		844.6		804.1	
C 16:1	800.6		832.6		828.1		842.6		802.1	
C 18:0	830.6		862.6		858.1		872.6		832.1	
C 18:1	828.6		860.6		856.1		870.6		830.1	
C 18:2	826.6		858.6		854.1		868.6		828.1	
C 18:3	824.6		856.6		852.1		866.6		826.1	
C 20:0	858.6		890.6		886.1		900.6		860.1	
C 22:0	886.6		918.6		914.1		928.6		888.1	

In the chromatograph, the temperature of the sampler tray was adjusted to 25 °C, and the flow rate of the mobile phase was 1000 μL min^{-1} with a sample injection volume of 10 μL. The composition of the mobile phase consisted of isocratic mixing of methanol and chloroform (6:4) with addition of 200 mmol L^{-1} of ammonium acetate. The analysis time was 10 min, with the

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ACS Omega 2020, 5, 12270−12277

rebalance time of 5 min using 100% methanol, and 15 min of total analysis. Because of the addition of ammonium acetate to the mobile phase, the HPLC quaternary pump system was washed periodically with water to avoid saline deposits.

Method for SG. Knowledge of the β-sitosterol-glucoside fragmentation pattern made it possible to estimate the fragmentation profile of the other nine steroid glucosides, each linked to a glucose molecule ($C_6H_{12}O_6$). The spectral scanning range was m/z 500−650. The temperature of the ionization source was maintained at 350 °C, the pressure of the collision gas was 10 psi, and the applied misting current was 3 μA. For the MRM experiment, the following potentials were applied: DP of 40 V, EP of 9 V, CEP of 35 V, and CXP of 3 V. The CE was maintained at 30 V. Table 5 provides the individual optimization parameters of the transition as well as the ions monitored in the method.

In the chromatograph, the temperature of the sampler tray was adjusted to 30 °C, and the flow rate of the mobile phase was 800 μL min^{-1} with a sample injection volume of 10 μL. The composition of the mobile phase consisted of a methanol gradient with 1% acetonitrile (85%) and water with 1% acetonitrile (15%) for 15 min, the percentage being changed to 100% methanol until minute 18. The period from 18 to 20 min is the optimum time for the reequilibration of the mobile phase for the subsequent sample analysis. The chromatographic method is based on the work developed by Rozenberg et al.[12]

Method for ASG. From the fragmentation pattern of β-sitosterol glucoside palmitate, the same possible ES were monitored, considering the binding of a glucose molecule ($C_6H_{12}O_6$) between the steroid and the fatty acid in question. A total of 90 different steroids glucosides were monitored, and the spectral scanning range was m/z 750−950. The mass parameters were the same as those applied in the SG method. For EP, 10 V was applied, and for CEP, 35 V. Table 6 provides the individual optimization parameters of the transition as well as the ions monitored in the method.

In the chromatograph, the experimental conditions are similar to the SG analysis method, with only the mobile phase gradient and the analysis time changed. The flow of the mobile phase was 1200 μL min^{-1} with a sample injection volume of 10 μL. The composition of the mobile phase consisted of a gradient of methanol (85%) and water (15%) for 5 min, the percentage being changed to 100% methanol up to 25 min; 1% acetonitrile was added to each solvent from the mobile phase. The optimal time to rebalance the mobile phase for the subsequent sample was 25 to 30 min.

Quantification of Steroids. In each developed method, a calibration curve of the betulin standard was created within the refined soybean oil matrix. Betulin is a derivative of terpenoids as well as steroids and is therefore widely used as an internal standard for this class of compounds, with a molecular structure similar to steroids.[18] Thus, for each class of steroids, the ions of interest of betulin were inserted into the calibration curve, which can determine the area of each concentration. For greater reliability of the methods, the main validation parameters were evaluated considering the internal betulin standard.

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Notes

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