Article

Characterization and Quantification by Gas Chromatography of Free Steroids in Unsaponifiable Matter of Vegetable Oils

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O estudo sobre os esteróides livres e sua utilização na caracterização de óleos é interessante já que sua distribuição é diferente para cada óleo. Embora vários trabalhos mostrem a análise desses esteróides em óleos, um trabalho mais abrangente mostrando a presença de esteróides menos abundantes ainda é limitado. Os esteróides foram extraídos a partir da matéria insaponificável usando reação de saponificação em microescala. Após a extração os esteróides foram caracterizados por cromatografia gasosa (GC) utilizando os detectores por ionização de chama (FID) e espectrometria de massas (MS). Todos os picos cromatográficos presentes nos óleos que poderiam ser de esteróides livres foram caracterizados e quantificados. Os resultados evidenciaram grande diversidade de tipos e teores de esteróides livres. Os óleos de milho e moringa apresentaram maiores teores de esteróides em relação aos outros óleos analisados. Os óleos de babaçu, palmiste e canola foram os que apresentaram os menores teores.

The study of free steroids and their use in oil characterization is interesting because the distribution of these steroids varies from one oil to another. Although several papers analyze the most abundant steroids in oils, studies including less common steroids are scarce. Free steroids were extracted from unsaponifiable fraction using the saponification reaction adapted micro-scale. After extraction, steroids were characterized by gas chromatography (GC) using flame ionization (FID) and mass spectrometry (MS) detectors. All chromatographic peaks in the oils that could belong to free steroids were characterized and quantified. Results evidenced great diversity in types and levels of free steroids. Corn and moringa oils had higher levels of steroids than the other oils analyzed. Babassu, palm kernel and canola oils had the lowest steroid levels.

Keywords: phytosterols, saponification reaction, GC-FID, GC-MS

Introduction

The characterization of oils is of great importance for different areas of science since it makes possible to predict the properties inherent in each oil, and depending on the goal, to inform whether or not they present the expected properties. Several methods use fatty acid composition analysis to characterize oils.[1-3] However, this characterization method can be inefficient since most of the fats and oils may contain the same fatty acids.[4-5]

One group of chemical components that can be used for characterization purposes is steroids, which are formed in oils from reactions involving condensation and rearrangements of the lipid squalene.[6-8] They are present in oils along with other compounds forming unsaponifiable.[9]

Steroids are predominantly distributed in free and, to some extent, in conjugated fatty acids and glucosides.[10-13]

The so-called steroid profile, that is, the types of steroids present in a substance and their relative proportions, is not the same for different oils. This therefore serves as a fingerprint for identifying them.[14-15] In addition to providing characterization, steroid analysis can serve as a traceability parameter in identifying and detecting adulteration.[16-17] Using steroid composition, Al-Ismail *et al.* studied adulteration in olive oil, a high-quality and high-value oil, using other cheaper vegetable oils such as cottonseed, sunflower, corn and soybean oil.[18]

Among the known methods for isolating steroids, the conventional method of analysis involves saponification of the sample followed by extraction of the unsaponifiable using an organic solvent and pre-fractionation techniques such as gas chromatography (GC) for separating the steroids

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contained in this fraction.[19-21] What sets this study apart is the reduction to micro-scale of the sample saponification phase coupled with GC using medium polarity capillary columns rather than the nonpolar columns used in other methods.[22-24]

The sample saponification method is applicable only to the analysis of steroids in the free form. Saponification reaction conditions break existing connections in conjugated steroids, which can therefore not be identified by this method. [25]

In this context, considering how little is known about which free steroids are found in various oils and how they are distributed, the goal of this paper is to characterize and quantify the steroids present in these samples using a method developed for the extraction and separation of the steroids in unsaponifiable fraction.

Experimental

Reagents, solvents and reference standards

Solvents used were of HPLC grade and purchased from the Tedia Company Inc. (Fairfield, CT, USA). Reference standards of stigmasterol, cholesterol, β-sitosterol, campesterol, brassicasterol and betulin were of GC purity (greater than 99%) and were acquired from Sigma Aldrich Chemical Company (St. Louis, MO, USA). The derivatizing agent used was MSTFA [*N*-methyl-*N*-(trimethylsilyl) trifluoroacetamide] also purchased from Sigma Aldrich. All other reagents were of analytical grade.

Oil samples

Oils used in this study were purchased locally in Goiânia, GO, Brazil and Marabá, PA, Brazil. Canola, cotton, palm kernel and corn oils were industrially refined; the remaining oils, crambe, moringa, soybean, sunflower, peanut, palm, babassu and castor oils, were used in their raw form.

Extraction of free steroids in unsaponifiable fraction

The saponification reaction was started by weighing 1 g of sample in a test tube and adding 15 mL ethanol and 10 mL of 50% m/v potassium hydroxide aqueous solution. The test tube was allowed to react in a previously sealed water bath at 60 °C for 60 min. After the reaction time, the reaction product contained in the test tube was transferred to a separatory funnel. The tube was washed successively with 20 mL of ethanol, 10 mL of cold water, 10 mL of hot water and 3 mL of hexane, which were all transferred to the funnel. The unsaponified matter was extracted five times with 25 mL portions of hexane. These extracts were combined and washed three times with 10 mL of 10% v/v ethanol aqueous solution. The solution was dried with anhydrous sodium sulfate and the solvent was evaporated. The sample was solubilized in 1000 μL of pyridine, of which 200 μL was transferred to a vial for analysis. To this vial a volume of 100 μL of MSTFA (derivatizing agent) was added.

Quantification by GC-FID

A chromatographic method for quantification the free steroids was developed etulin as internal standard. In quantitative analysis, 150 μL of betulin solution was added to each vial before derivatization.

The analysis of free steroids was performed on a Shimadzu GC 2010 gas chromatograph equipped with a flame ionization detector (FID) in accordance with the chromatographic method developed in this study. A DB17HT capillary column (30 m × 250 μm × 0.15 μm) was used. The injector temperature was 280 °C with splitless injection of 2 μL of sample and a linear velocity of 30 cm s^{-1}, H$_2$ 5.0 was the carrier gas with a flow rate of 40 mL min^{-1}. The oven operated initially at 200 °C. It was heated to 330 °C at a rate of 3 °C min^{-1} and was kept at this temperature for 5 min. The detector operated at a temperature of 320 °C.

Steroid content was determined by the ratio between the area and mass of internal standard and the area of identified steroids. Concentration of the different steroids present in the oil was expressed as mg steroid *per* kg of oil.

Characterization by GC-MS

The GC-MS analysis was performed on a Shimadzu GC-17A gas chromatograph directly coupled to a Shimadzu QP5050A mass spectrometer. The chromatographic column and temperature programming was similar to that described for GC-FID analysis. The injection mode was manual in this case. The carrier gas used was He 5.0 with a linear velocity of 35 cm s^{-1}. Mass spectra were obtained in electron impact mode (EI) between 40 and 600 a.m.u. using 70 eV with a quadrupole analyzer. For GC-MS analyses, the internal standard betulin was not added to the samples.

Assay recovery

The efficiency of the proposed method was evaluated by recovery assays with soybean oil. Before saponification reaction the oil was fortified by adding standard solutions (10, 2000 and 10000 mg kg^{-1}) of cholesterol and

brassicasterol. Afterwards, samples were analyzed by GC-FID.

Multivariate analysis

Principal component analysis (PCA) was used for the data analyses of steroids composition, using the software Octave 3.2.4 for Windows.

Results and Discussion

This method was developed on the basis of test tube microscale transposition of the conventional saponification reaction. This approach provides speed and ease of operation. Traditional methods, on the other hand, use reflux under heating, which requires more complex experimental apparatus than with a test tube and also require large quantities of sample and reagents.[26]

The different steroids present in oils were characterized using data obtained by chromatographic analysis with FID and MS detectors. The chromatographic method proved efficient in detecting and separating steroids since it can be applied to different types of detectors with no loss of analytical sensitivity. Figures 1 and 2 show the steroid analysis chromatograms for soybean and palm oil, respectively, examples of the excellent results obtained using this method.

The major steroids in oils, brassicasterol, campesterol, stigmasterol and β-sitosterol, were characterized by analyzing the corresponding reference standards. Other steroids characterized without reference standards in this study were labeled St. 1 to St. 16.

This analysis made possible to identify the retention times of the steroids present in the different oils as well as retention times relative to the internal standard betulin via GC-FID analysis. In Table 1 the retention times obtained
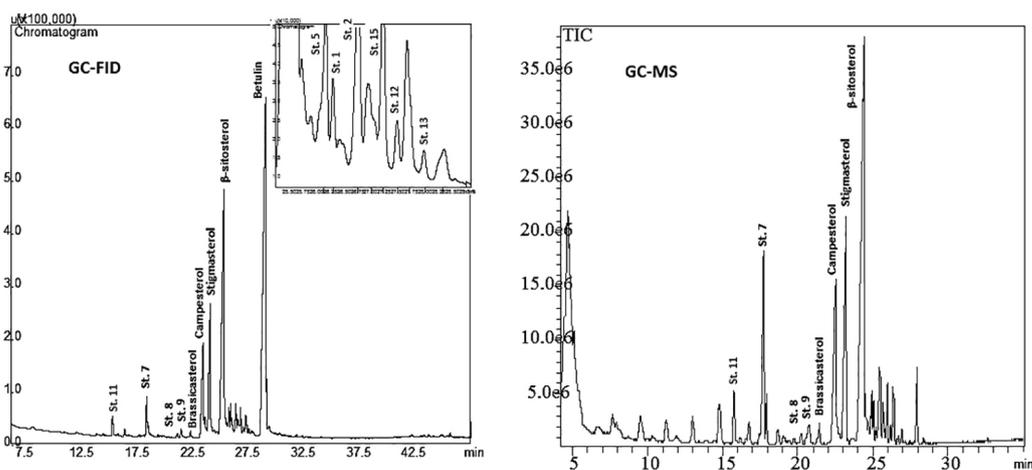

Figure 1. Chromatographic analysis by gas chromatography coupled to a flame ionization detector (GC-FID) and gas chromatography coupled to a mass detector (GC-MS) of free steroids present in soybean oil.
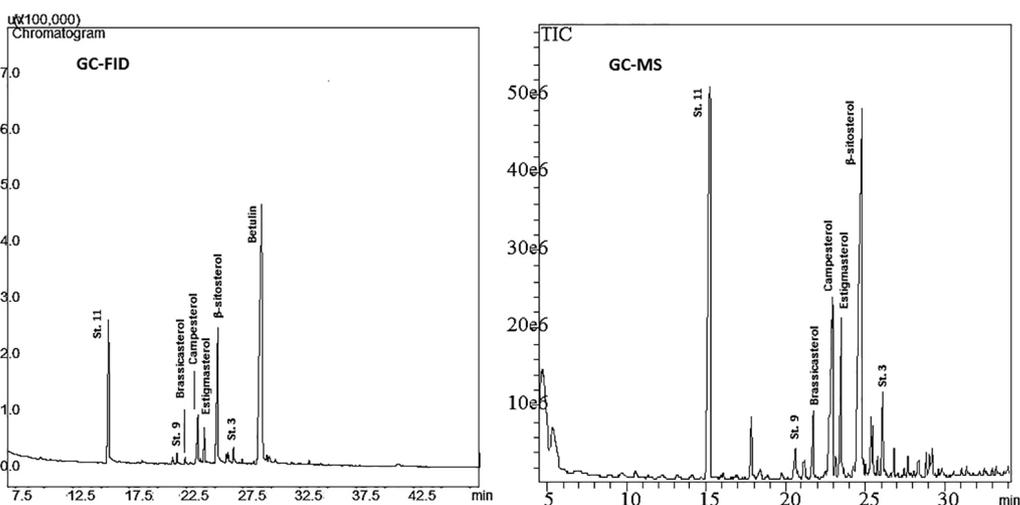

Figure 2. Chromatographic analysis by gas chromatography coupled to a flame ionization detector (GC-FID) and gas chromatography coupled to a mass detector (GC-MS) of free steroids present in palm oil.

for all steroids characterized are listed in ascending order, along with standard deviations. The steroids eluted in time periods ranging from 15.53 min to 28.16 min. The internal standard used in quantifying the steroids eluted in 29.06 min.

Table 1. Absolute elution times and elution times in relation to the internal standard of steroids identified using the developed method

Steroid	Retention time[a]	
	Absolute / min	Relative betulin
St. 11	15.53 ± 0.09	0.53 ± 0.00
St. 4	17.86 ± 0.07	0.61 ± 0.00
St. 6	18.97 ± 0.26	0.65 ± 0.01
St. 7	18.97 ± 0.24	0.65 ± 0.01
St. 14	20.65 ± 0.19	0.71 ± 0.01
St. 8	21.63 ± 0.56	0.74 ± 0.02
St. 9	21.93 ± 0.14	0.75 ± 0.00
Brassicasterol	22.52 ± 0.41	0.77 ± 0.01
Campesterol	22.82 ± 0.46	0.79 ± 0.02
St. 16	24.01 ± 0.01	0.83 ± 0.00
Stigmasterol	24.30 ± 0.28	0.84 ± 0.01
β-sitosterol	25.78 ± 0.26	0.89 ± 0.01
St. 5	26.11 ± 0.26	0.90 ± 0.01
St. 1	26.67 ± 0.15	0.92 ± 0.01
St. 2	26.87 ± 0.11	0.92 ± 0.00
St. 3	27.27 ± 0.32	0.94 ± 0.01
St. 15	27.32 ± 0.14	0.94 ± 0.00
St. 12	27.54 ± 0.15	0.95 ± 0.01
St. 10	27.56 ± 0.07	0.95 ± 0.00
St. 13	28.16 ± 0.03	0.97 ± 0.00

[a]Retention time ± standard deviations.

GC-MS in electron impact mode was used to obtain mass spectra for each characterized steroid. Table 2 shows the main fragmentation standards for these steroids derivatized with MSTFA: (1) the loss of a methyl group from the derivatization structure connected to the steroid molecule; (2) the loss of the entire structure of the derivatizer connected to the steroid molecule; (3) the loss of the steroid's entire angular side chain.[27]

Therefore, derivatized steroids easily lose a methyl cluster ($M^{+}-15$) under ionization, generating the first fragmentation ion. Fragment $M^{+}-90$ has already sustained the loss of methyl together with the hydroxytrimethylsilane (TMSOH) cluster. Another fragmentation ion, characteristic of most steroids, is formed with the elimination of the entire side chain, leaving only the set of the four rings represented by the fragment m/z 255. Other important fragments relating to ring cleavage in the steroid molecule are $M^{+}-129$, m/z 213 and m/z 173. Spectra of steroids derivatized using MSTFA substituents have fragmentation ions typical of m/z 73 for the $Si(CH_3)_3$ cluster of the derivatizing molecule.[28-30]

Steroids St. 1 to St. 16 did not have their structures elucidated since it is known that steroids may appear as structural isomers or stereoisomers. For this reason, any proposed structure may not be correct and may not include all possible isomeric configurations.[31]

Recovery assays were performed to evaluate the precision and accuracy of the developed method. This assay was performed using cholesterol standard solutions, which do not naturally occur in vegetable oils, and brassicasterol standard solutions, present in many vegetable oils. Results are shown in Table 3.

For the study of precision, the results obtained by the assay recovery were expressed by coefficients of variation (CV). The lowest CV (5.36%) was found for the recovery of cholesterol, 10 mg kg^{-1}. The highest CV (18.76%) was found for the recovery of brassicasterol, 10 mg kg^{-1}. Coefficients of variation values were considered suitable for the developed method. The values are in agreement with those obtained for steroids analysis in literature.[32,33]

Good recoveries were observed for both fortification levels, with typical values ranging from 77.32 to 110.74%. To confirm the accuracy of method, recoveries were tested using Student's t-test, being established as a null hypothesis (H0) the recovery of 100% of standard solutions. The null hypothesis is acceptable when the absolute value of t_{calc} does not exceed the value of t_{tab}. The t-test was applied with 99% confidence and n–1 degrees of freedom. Thus, one cannot affirm the existence of significant differences between the recovery obtained and expected value (100%), except for the recovery of cholesterol 10000 mg kg^{-1}, which was not satisfactory.

Quantitative data obtained by GC-FID for concentration of steroids (mg kg^{-1}) in oils are shown in Table 4. A large variation between different types of steroids in the samples could be observed. Results show that the samples of babassu, palm kernel and canola oils had the lowest free steroid concentrations. Cottonseed, peanut, sunflower, soybean, crambe, palm and castor oil had intermediate steroid levels which were close to each other. Corn and moringa oils had the highest steroid content. Since a large number of free steroids were characterized and quantified, their levels were somewhat higher than those obtained in other studies.[15,30-31]

As expected, β-sitosterol was the most abundant steroid in all samples.[31] Of the oils analyzed, soybean oil displayed the greatest diversity in steroid types. Oils such as canola,

Table 2. Mass fragments obtained from the spectrum of each steroid

Steroid	Mass fragments (*m/z*) obtained in SCAN mode from steroids derivatized with MSTFA				
	M$^+$	M$^+$−15	M$^+$−90	M$^+$−105	M$^+$−129
β-sitosterol	486	471	396	381	357
Campesterol	472	457	382	367	343
Brassicasterol	470	455	380	365	341
Stigmasterol	484	469	394	379	355
St. 1	484	469	394	nd	355
St. 2	486	471	396	381	357
St. 3	484	469	394	379	355
St. 4	498	483	nd	393	nd
St. 5	499	484	nd	394	nd
St. 6	471	456	nd	366	nd
St. 7	467	452	377	nd	338
St. 8	467	452	nd	nd	338
St. 9	500	485	nd	395	nd
St. 10	484	469	nd	379	nd
St. 11	471	456	nd	366	nd
St. 12	483	nd	393	378	nd
St. 13	483	nd	393	378	nd
St. 14	484	nd	394	379	nd
St. 15	483	468	393	378	nd
St. 16	472	457	382	nd	343

M$^+$: molecular ion; M$^+$−15: molecular ion−15 *m/z*, and so on; nd: not detected.

Table 3. Assay recoveries for steroids

Steroid	Fortification level / (mg kg^{-1})	n	Mean recovery / %	SD	CV / %	t$_{calc}$	t$_{tab}$
	10	5	109.33	5.86	5.36	3.56	
Cholesterol	2000	5	84.63	15.01	17.74	−2.29	
	10000	5	78.12	9.22	11.80	−5.31	t$_{99\%}$ = 4.60
	10	5	85.22	15.99	18.76	−2.07	
Brassicasterol	2000	5	110.74	12.87	11.62	1.86	
	10000	5	77.32	12.68	16.40	−4.00	

SD: standard deviation; CV: coefficient of variation; t$_{calc}$: value t-distribution experimentally estimed with 99% confiance; t$_{tab}$: value t-distribution theory with 99% de confiance.

corn and soybean oil contain characteristic steroids which facilitate their characterization when compared with mixtures of other oils.

It is important to mention that during the development of seeds, they are exposed to several factors that may affect their properties and characteristics. Genotypes and environmental conditions are among the main ones. In relation to environmental conditions, soil properties, cultivation's site, precipitation's amount and temperature, as well as agricultural practices (irrigation and fertilization) are important.[30] Another factor that influences the characteristics of seeds is industrial processing. Refining and degumming can lead reduction of some components

important of seeds. It was shown that such factors can affect levels, but not the composition of steroids in seeds. In this sense the composition of steroids in oil is considered a fingerprint.[34]

The observations about steroid composition in Table 5 may facilitate oil characterization by mixing with any of the oils analyzed in this paper.

Multivariate analysis

Using quantitative data for free steroids in various oils, a chemometric evaluation was made to correlate the types and levels of steroids present in them. For this, a model based on

Table 4. Free steroid levels quantified in the samples[a] Retention time ± standard deviation

Steroid	Free steroid in oils / (mg kg⁻¹)											
	Babassu	Canola	Castor	Corn	Cotton	Crambe	Moringa	Palm	Palm kernel	Peanut	Soybean	Sunflower
β-Sitosterol	380	1420	1360	9680	2590	1740	3390	1510	750	2110	1680	2190
Campesterol	100	750	230	2120	180	710	1380	530	90	440	530	280
Stigmasterol	60	60	520	530	20	50	1680	250	100	170	610	270
Brassicasterol	10	190	0	0	80	340	20	40	30	10	20	0
St. 1	110	80	610	650	170	170	440	0	20	320	50	90
St. 2	0	40	0	320	0	0	0	0	0	30	190	190
St. 3	290	70	0	510	0	230	160	120	110	120	0	340
St. 4	0	0	0	30	0	0	0	0	0	0	0	0
St. 5	0	0	80	0	0	0	0	0	0	0	40	0
St. 6	0	0	0	50	0	0	0	0	0	0	0	0
St. 7	10	10	150	0	190	130	60	0	0	50	140	0
St. 8	0	0	0	0	80	0	30	0	10	10	20	0
St. 9	0	0	0	80	130	0	30	80	0	20	20	0
St. 10	0	0	0	70	30	0	30	0	0	0	0	0
St. 11	20	0	330	40	0	40	0	1330	20	110	90	90
St. 12	0	0	0	0	0	0	0	0	0	0	20	0
St. 13	0	0	0	0	0	0	0	0	0	0	20	0
St. 14	0	10	0	0	0	0	0	0	0	0	0	0
St. 15	0	20	0	100	0	0	0	0	0	0	60	0
St. 16	0	0	0	0	0	70	180	0	0	0	0	0
Total[a]	980 ± 42.23	2650 ± 84.05	3280 ± 28.81	14180 ± 98.39	3470 ± 81.32	3480 ± 63.24	7400 ± 77.03	3860 ± 35.36	1130 ± 56.87	3390 ± 70.71	3490 ± 93.14	3450 ± 21.27

[a]Retention time ± standard deviation

Table 5. Data about steroid composition

Identified steroid	Consideration
β-sitosterol	Were identified in all oils
Campesterol	
Stigmasterol	
Brassicasterol	Sunflower, castor and corn oil can be distinguished from the other samples by their absence
St. 1	Palm oil can be distinguished from the other samples by their absence
St. 2	Canola, peanut, sunflower, soybean and corn oil can be distinguished from other samples by their presence
St. 3	Cotton, soybean and castor oil can be distinguished from other samples by their absence
St. 4	Present only in corn oil
St. 5	Soybean and castor oil can be distinguished from other samples by their presence
St. 6	Present only in corn oil
St. 7	Sunflower, palm kernel, palm and corn oil can be distinguished from the other samples by their absence
St. 8	Cotton, peanut, moringa, soybean and palm kernel oil can be distinguished from the other samples by their presence
St. 9	Cotton, peanut, moringa, soybean, palm and corn oil can be distinguished from the other samples by their presence
St. 10	Cotton, moringa and corn oil can be distinguished from the other samples by their presence
St. 11	Cotton, canola and moringa oil can be distinguished from the other samples by their absence
St. 12	Present only in soybean oil
St. 13	Present only in soybean oil
St. 14	Present only in canola oil
St. 15	Canola, soybean and corn oil can be distinguished from the other samples by their presence
St. 16	Moringa and crambe oil can be identified from the other samples by their presence

principal component analysis (PCA) was used to show the relative importance of each steroid in the discrimination.[35]

Using quantitative data for free steroids in various oils, a chemometric evaluation was made to correlate the types and levels of steroids present in them.Thus, a model based on principal component analysis (PCA) was used to show the relative importance of each steroid in the discrimination.[35]

Based onpreprocessed autoscaled data, two models of PDA that could together explain the discrimination of oils in theircomposition steroids were developed. It was established that only two principal components are sufficient for the discriminationfor each model. The resulting principal component projections are shown in Figure3.
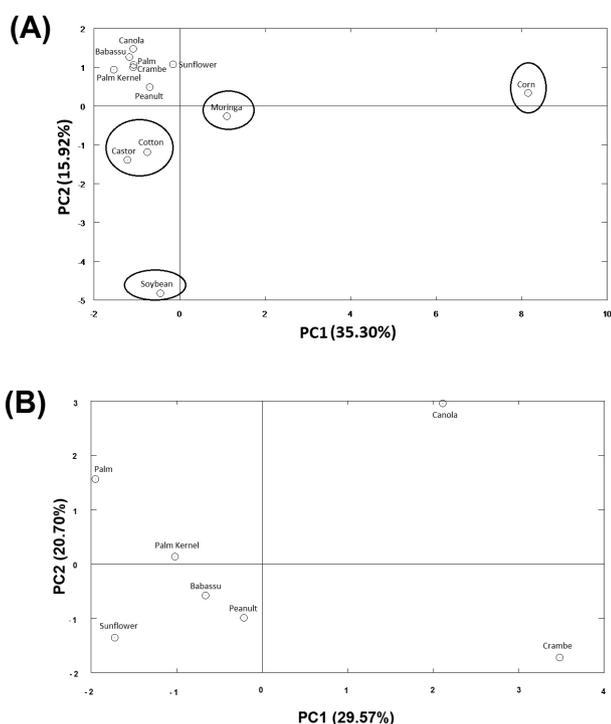

Figure 3. Principal component score plotsof oil discrimination by analysis of steroids. (A) and (B) illustratethe two models PCA developed to discriminate oils.

Model shown in Figure 3A hasan excellentdiscrimination of corn, moringa, soybean, castor, and cotton oils.The first two principal components account for 51.22% of the total variance for data in this model.

By loadings was realized that discrimination occurred by the influence of steroids: β-sitosterol, campesterol, St. 1, St. 2 , St. 3 , St. 4 , St. 5, St. 7, St.10, St. 12, and St.15. An important fact is that most significant steroids in discrimination of this model are present in higher levels in oil. Only the peculiar steroids St. 4 (corn) and St. 12 (soybean) were significant in the PCA, so the amount has influenced more than the types of steroids for that model.

The discrimination of other oils was based on the model illustrated in Figure 3B. To develop this model oils already detailed in Figure 3A were excluded from the data matrix. The first two principal components account for 50.27% of total variance for data in this model.

The loadings that contributed to discrimination of oils in this model were campesterol, stigmasterol, brassicasterol, St. 3, St. 9, St. 11, St. 15, and St. 16. The St. 15 and St. 16 are peculiar steroids that contributed to discrimination by canola and crambe, respectively. It was noticed that in this model the types of steroids were relevantin the model in Figure 3B.Palm and palm kernel despite being two different oils belonging to same oilseed, could also be well discriminated by PCA.

These results demonstrate the ability to identify the composition of different oils steroids by PCA model. This criterion of elimination was employed considering both levels as well as the types of steroids identified in oils analyzed.

Conclusions

This study has presented an efficient method for analyzing free steroids in the unsaponifiable fraction of oils. The developed method presented proper precision and accuracy. Therefore, a quality parameter was included in the method for the analysis of vegetable oils. The analyses indicate the existence of characteristic steroids and show that each oil has its own peculiar distribution. This strategy makes feasible to characterize oils by means of free steroid analysis data. This study complements previous ones since a greater variety of oils were analyzed and a larger number of steroids were characterized, since most previous studies reported the presence of only the most important and most abundant steroids. The oils analyzed in this study were correctly discriminated by PCA analysis, proving that this is an important tool in the identification of different oils according to their steroids composition.

Supplementary Information

Supplementary information (Figures S1-S16) is available free of charge at http://jbcs.sbq.org.br as PDF file.

Acknowledgments

References

1. Spangenberg, J. E.; Macko, S. A.; Hunziker, J.; *J. Agric. Food Chem.* **1998**, *46*, 4179.

2. Butinar, B.; Bucar, M. M.; Valencic, V.; Raspor, P.; *J. Agric. Food Chem.* **2010**, *58*, 5227.

3. Simionato, J. I.; Garcia, J. C.; Santos, G. T.; Oliveira, C. C.; Visentainer, J. V.; Souza, N. E.*; J. Braz. Chem. Soc.* **2010**, *21*, 520.

4. Andrikopoulos, N. K.; Giannakis, I. G.; Tzamtzis, V.; *J. Chromatogr. Sci.* **2001**, *39*, 137.

5. Hlongwane, C.; Delves, I. G. L.; Wan, W.; Ayorinde, F. O.; *Rapid Commun. Mass Spectrom.* **2001**, 15, 2027.

6. Bloch, K.; *Nobel Lecture* **1964**, 79.

7. Piironen, V.; Lindsay, D. G.; Miettinen, T. A.; Toivo, J.; Lampi, A.-M.; *J. Sci. Food Agric.* **2000**, *80*, 939.

8. Bach, T. J.; *Lipids* **1986**, *21*, 82.

9. Schwartz, D. P.; *J. Am. Oil Chem. Soc.* **1988**, 65, 246.

10. Breinholder, P.; Mosca, L.; Lindner, W.; *J. Chromatogr. B* **2002**, 777, 67.

11. Cordeiro, P. J. M.; Vilegas, J. H. Y.; Lanças, F. M.; *J. Braz. Chem. Soc.* **1999**, *10*, 523.

12. Schrick, K.; Shiva, S.; Arpin, J. C.; Delimont, N.; Isaac, G.; Tamura, P.; Welti, R.; *Lipids* **2011**, *47*, 185.

13. Hang, J.; Dussault, P.; *Steroids* **2010**, *75*, 879.

14. Zhang, X.; Julien-David, D.; Miesch, M.; Geoffroy, P.; Raul, F.; Roussi, S.; Aoudé-Werner, D.; Marchioni, E. *Steroids* **2005**, *70*, 896.

15. Choong, Y. M.; Lin, H. J.; Chen, C. W.; Wang, M. L.; *J. Food Drug. Anal.* **1999**,7, 279.

16. Alonso, L. J.; Fontecha, L. L.; Juárez, M.; *J. Am. Oil Chem. Soc*. **1997**, *74*, 131.

17. Gutierrez, F. I.; Albi, V. M. A.; *J. Agric. Food Chem*. **2000**, *48*, 1106.

18. Al-Ismail, K. M.; Alsaed, A. K.; Ahmad, R.; Al-Dabbas, M.; *Food Chem.* **2010**, *121*, 1255.

19. Plank, C.; Lorbeer; *J. Chromatogr. A* **1994**, *683*, 95.

20. Phillips, K. M.; Ruggio, D. M.; Ashraf-Khorassani, M.; *J. Food Lipids* **2005**, *12*, 124.

21. Cunha, S. S.; Fernandes, J. O.; Oliveira, M. B. P. P.; *J. Chromatogr. A* 2006, *22*, 220.

22. Makin, H. L. J.; Honour, J. W.; Shackleton, C. H. L.; Griffiths W. J.; *Steroid Analysis*; Springer: New York, 2010, ch. 3.

23. Ferreira, H. E. C. S.; Elliott, W. H.; *J. Chromatogr. Biomed.* **1991**, *562*, 697.

24. Horning, E. C.; Haahti, E. O. A.; Vandenheuvel, W. J. A.; *J. Am. Oil Chem. Soc.* **1961**, *38*, 625.

25. Bhawani, S.; Sulaiman, O.; Hashim, R.; Mohamad, M. N.; *Trop. J. Pharm. Res.* **2010**, *9*, 301.

26. Brooks, C. J. W.; *Phil. Trans. R. Soc. A* **1979**, *293*, 53.

27. Cheng, M. T.; Barbalas, M. P.; Pegues, R. F.; McLafferty, F. W.; *J. Am. Oil Chem. Soc.* **1983**, *105*, 1510.

28. Kalo, P.; Kuuranne, T.; *J. Chromatogr. A*. **2001**, *935*, 237.

29. Kasal, A.; Budesinsky, M.; Griffiths, W. J.*; Steroid Analysis*; Springer: New York, 2010, ch. 2.

30. Soupas, L.; Juntunen, L.; Säynäjoki, S.; Lampi, A. M.; Piironen, V.; *J. Am. Oil Chem. Soc.* **2004**, *81*, 135.

31. Verleyen, M. T.; Verhe, K. F. R.; Huyghebaert, D. A.; Greyt, W. D.; *J. Am. Oil Chem. Soc.* **2002**, *79*, 117.

32. Lechner, M.; Reiter, B.; Lorbeer, E.; *J. Chromatogr. A* **1999**, *857*, 231.

33. Frega, N.; Bocci, B.; Lecker, G.; *J. Am. Oil Chem. Soc.* **1992**, *69*, 447.

34. Yamaya, A.; Endo, Y.; Fujimoto, K.; Kitamura, K.; *Food Chem.* **2007**, *102*, 1071.

35. Flores, I. S.; Godinho, M. S.; de Oliveira, A. E.; Alcantara, G. B.; Monteiro, M. R.; Menezes, S. M. C.; Lião, L. M.; *Fuel* **2012**, *99*, 40.