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Phytosterols Content in Vegetable Oils of Brazil: Coconut, Safflower, Linseed and Evening Primrose

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HIGHLIGHTS

- β-sitosterol was found in higher concentrations.
- Evening primrose oil presented the highest amounts of phytosterols.

Abstract: In the last years phytosterols, natural components of plants, have received more attention due to association of their consumption with reducing risk of cardiovascular diseases and cancer. There are several scientific studies about phytosterols in vegetable oils, but they are scarce in unconventional oils. The objective of this research was evaluating the content of phytosterols (β-sitosterol, stigmasterol and campesterol) in vegetable oils sold in São Paulo city, in Brazil. The analysis included cold alkaline saponification, derivatization with hexamethyldisilazane and trimethylchlorosilane reagents, and quantification by gas chromatography using flame ionization detection and internal standardization. The quality control parameters indicated that the method was suitable for analysis. Total sterols were between 272.3 mg kg^{-1} (coconut oil) to 6169.7 mg kg^{-1} (evening primrose oil). β-sitosterol was the component found in higher concentrations and evening primrose oil was the most representative in quantity of phytosterols.

Keywords: gas chromatography; plant sterols composition; unconventional oils.

INTRODUCTION

Oils and fats are good sources of nutritional energy to humans, conferring taste, consistency, and stability on food. Also take part in some cellular functions: they are structural components of the membranes, responsible for the transport and absorption of fat-soluble vitamins, precursors of hormones, and provide essential fatty acids [1]. Cold-pressed vegetable oils have these functions especially unconventional vegetable oils, like safflower, coconut, evening primrose, and linseed. They have biologically active lipids like sterols which provide health benefits, and diseases prevention [2,3].

Triacylglycerols are the main components of the vegetable oils, corresponding to 95-98 % of total lipids. The remaining percentage contains monoacylglycerols, diacylglycerols, free fatty acids, and other minor components in the unsaponifiable fraction, like sterols that are in greater quantity in this fraction [4].

Phytosterols are natural components of plants, found in free and esterified forms. More than 100 different phytosterols were identified in vegetable foods, and they have similar chemical structures with cholesterol, with suchlike biological functional [5,6].

There are several health benefits related to the phytosterols consumption, including reduction of cholesterol absorption, reduction of low-density lipoprotein (LDL), effects against inflammations and cancer [6-9].

Maki and coauthors [10] studied the inclusion of 1.8 g of phytosterols per day in patients with primary hypercholesterolemia, associated with a healthy diet, and this intake promoted favorable changes in atherogenic process, likely total cholesterol, no-HDL, and triacylglycerols. In children with hypercholesterolemia, the LDL was significantly reduced [11].

The Brazilian Society of Cardiology recognizes the impact of phytosterols on hypercholesterolemia, with excellent results in multiple controlled clinical trials [12]. According to Dumolt and Rideout [9], plant sterols may interfere with the cholesterol and triacylglycerols absorption, as well as modulating the ability in lipid-regulating genes through the activation of the liver X receptor (LXR).

Some studies have reported sterols's action suggesting reduction of cancer, with associations of β-sitosterol and stigmasterol to inhibit cancer cells in esophageal tissues, ovaries, breast, colon, and prostate [6,13].

The most common phytosterols found in food are β-sitosterol, stigmasterol, and campesterol. β-sitosterol is plenty, and others are present in lower concentrations. The main sources in vegetable foods are nuts, grains, seeds, legumes, and vegetable oils [13,14]. Sterols composition is specific for each oil category, with a characteristic relative ratio, so the sterols can be used for providing characterization and identity of oils [15].

The analysis of phytosterols generally includes several steps, as lipid extraction, alkaline saponification, derivatization, and chromatographic quantification [4]. In the AOCS official method [16], sample is saponified with alkali reagent, phytosterols are extracted with organic solvent, and compounds have been separated by thin-layer chromatography; however, this methodology spend more time and use a lot of samples and reagents, so authors have been adopted assays in smaller scales [17]. The quantification systems include liquid chromatography, using an ultraviolet-visible detector, fluorescence or mass spectrometer with atmospheric pressure chemical ionization (APCI) mode [18-20], or gas chromatography (GC) with flame ionization (FID) or mass detectors [14,15,21-23].

Codex Stan 210 establishes the quality and identity standards of coconut and safflower oils, including phytosterols, but this reference does not report parameters to evening primrose and linseed oils [24]. Furthermore, studies about the phytosterols content in unconventional oils are limited in the literature. As the survey of this data could be important, especially as a quality parameter for these products, the objective of this work was to evaluate a method for the quantification the phytosterols, β-sitosterol, stigmasterol, and campesterol, in vegetable oils and to measure the content in coconut, safflower, evening primrose and linseed oils commercialized in São Paulo city, Brazil.

MATERIAL AND METHODS

Samples

Four kinds of cold-pressed vegetable oils were studied, with different brands and lots, 15 of coconut oils (*Cocos nucifera* L.), 9 of safflower oils (*Carthamus tinctorius* L.), 3 of evening primrose oils (*Oenothera biennis* L.), and 12 of linseed oils (*Linum usitatissimum* L.), totaling 39 samples. Oils used in this study were purchased from local market and drugstores in São Paulo city, Brazil.

Chemicals

Potassium hydroxide (KOH) was analytical grade. The solvents were HPLC grade: n-hexane, and ethanol (Merck, Darmstadt, Germany). Deionized water was obtained with the Milli-Q purification system (Millipore, Bedford, MA, USA). The derivatizing reagent, hexamethyldisilazane:trimethylchlorosilane (3:1) (Sylon HT Kit) was obtained from Supelco (PN 3-3046; Bellefonte, PA, USA).

The standards were obtained from Sigma-Aldrich (St. Louis, MO, USA): stigmasterol (PN S2424; purity ~95 %), β-sitosterol (PN S1270; purity ~95 %), campesterol (PN C5157; purity ~65 %), and 5α-cholestane (PN C8003; purity ~97 %; internal standard).

Individual solutions of phytosterols were prepared in n-hexane with a concentration of 400 μg mL^{-1} (campesterol and stigmasterol) and 1000 μg mL^{-1} (β-sitosterol). So, a working solution with three phytosterols was prepared in n-hexane, with a concentration of 200 μg mL^{-1} (campesterol and stigmasterol) and 500

μg mL^{-1} (β-sitosterol), and used to analytical curve preparation. The 5α-cholestane was prepared in n-hexane (concentration 200 μg mL^{-1}), and used as internal standard (IS).

Phytosterols analysis

The method was based on Saldanha and coauthors [21], with some modification (internal standardization using 5α-cholestane).

Saponification and extraction of unsaponifiable material of phytosterols

150 mg of the oil was weight in a 50 mL centrifuge tube and 100 μL of IS solution and 10 mL of 15 % KOH ethanolic solution were added. The tube was sealed and the saponification was performed for 22 h in the dark at room temperature. Then, 10 mL of deionized water and 10 mL of n-hexane were added and vigorously mixing on a vortex. After separation of phases, the n-hexane was removed to another tube. The procedure was repeated three times (totaling 40 mL of n-hexane), and the combined fractions were evaporated under nitrogen stream until dryness [25].

Phytosterol derivatization

The dried unsaponified material was derivatized with 100 μL of silylating agent (Sylon HT Kit) for 2 h in the dark at room temperature. After that, the reagent was evaporated under nitrogen stream and the residue was dissolved in 1.0 mL of n-hexane. The solution was subjected to centrifugation at 5,000 rpm for 5 min, and the upper phase was then transferred into vials suitable for GC-FID analysis [26].

Chromatographic conditions in GC-FID

A GC-2010 gas chromatograph equipped with a split/splitless injector and a flame ionization detector (Shimadzu, Kyoto, Japan) was used with a capillary silica column VF-5ms (30 m x 0.25 mm x 0.25 μm; Varian) under programmed conditions: injector temperature 290 ºC, detector temperature 350 ºC, hydrogen as carrier gas with a constant flow of 1.0 mL min^{-1}, injection volume 1 μL, split 1:20, oven temperature: 230-264 ºC at 2 ºC min^{-1}, 264 ºC for 5 min, 264-275 ºC at 1 ºC min^{-1}, 275 ºC for 6 min.

All data were collected by the *GC Solution* software (Shimadzu). The compounds were identified by comparing the retention times with the individual standards. Internal standardization was employed to quantification, by the ratio between the concentration and peak areas of phytosterols and IS. The concentration was expressed as mg kg^{-1} of oil.

Quality control of phytosterol method

Tests were performed to select 5α-cholestane as IS and parameters were evaluated for methodology verification. Limits of detection (LOD) and quantification (LOQ) were calculated for each phytosterol considering the signal-to-noise ratio of 3:1 and 10:1, respectively. The linearity of the standard curve was checked through square correlation coefficients (r^2) at triplicate determinations of six different concentrations of each sterol. Precision and accuracy were evaluated with a reference sample of the International Olive Oil Council (IOC), with the following composition: 90 % lampante olive oil and 10 % palm olein (COI CHEM/2015, Proficiency Testing, Madrid, Spain).

RESULTS

Methodology verification – Results of quality control

Figure 1 shows a chromatogram for a commercial evening primrose oil and phytosterol standards. Coefficients related to the verification of the method are in Table 1. To check the linearity of the method, an analytical curve for each phytosterol was prepared in six different concentrations: campesterol, and stigmasterol (range 5.0 to 200.0 μg mL^{-1}), and β-sitosterol (range 12.5 to 500 μg mL^{-1}), by the correlation between phytosterol concentrations and IS concentration versus areas of phytosterols and area of the IS. The angular and linear coefficients were estimated by regression using the simple least squares method and are presented in Table 1. Good linearity was found, with r^2 values upper than 0.998. The LOD and LOQ values indicating that the method was sensitive to the suggested proposal (Table 1).

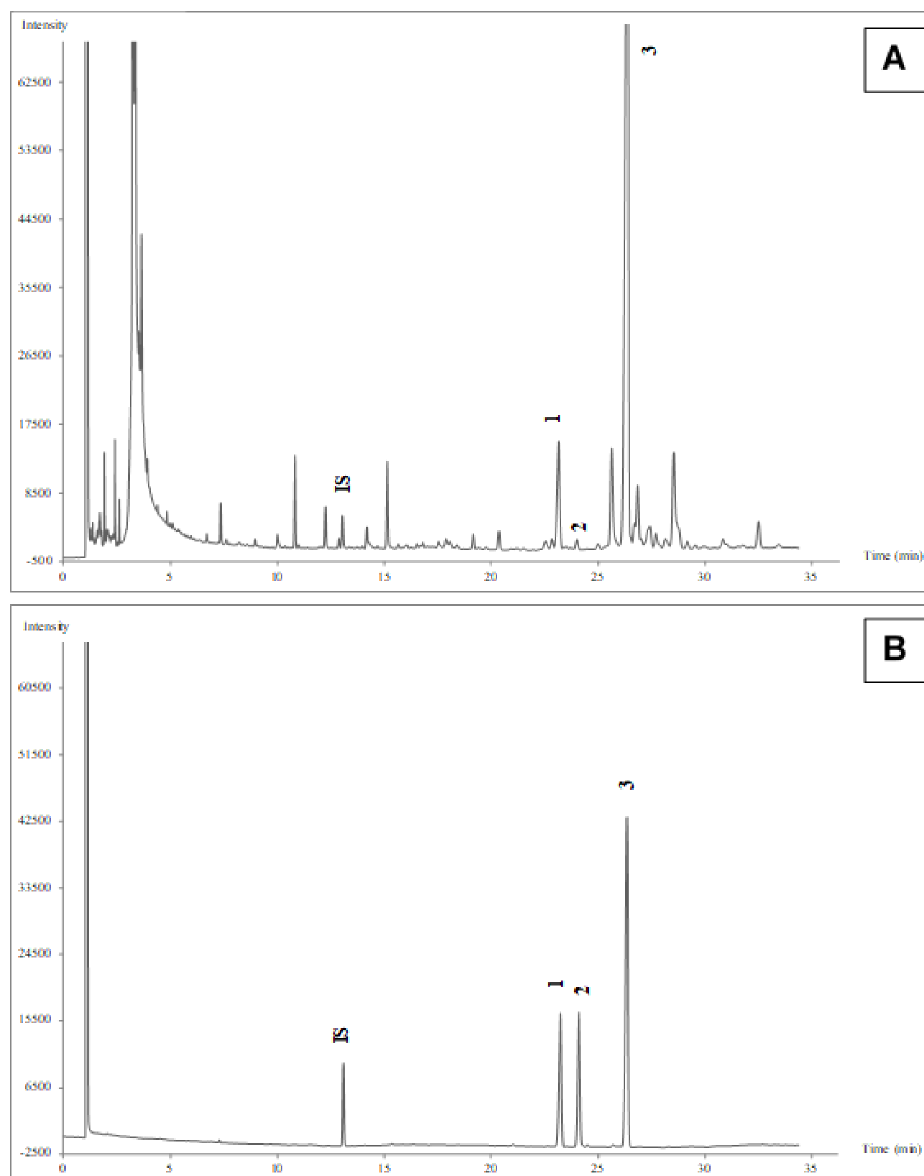

Figure 1. Chromatograms obtained by GC-FID analysis (A) sample of evening primrose oil. IS: 5α-cholestane; 1 - campesterol; 2 - stigmasterol; 3 - β-sitosterol; (B) mixture of phytosterols standards. IS: 5α-cholestane (30 µg mL^{-1}); 1 - campesterol (100 µg mL^{-1}); 2 - stigmasterol (100 µg mL^{-1}); 3 - β-sitosterol (250 µg mL^{-1}).

The performance of the method was evaluated with a reference sample sent by IOC. Results were in % of total phytosterols (4.52, 1.53, and 85.66 %, respectively for campesterol, stigmasterol and β-sitosterol) and were calculated in mg kg^{-1} (unit measured), considering the total analytes presented in the report (1284 mg kg^{-1}). The precision was adequate, with RSD < 10 %, and the mean values were within the acceptable range for each component (Table 1).

Table 1. Coefficients related to the verification of the method for phytosterols at different concentrations.

	Campesterol	Stigmasterol	β-sitosterol
Retention time (min)	23.1	24.0	26.2
Inclination (a)	0.9436	0.8946	0.9783
Intercept (b)	-0.0846	-0.0658	-0.1992
r^2	0.9989	0.9994	0.9992
Limit of detection (mg kg^{-1})	10.0	10.0	10.0
Limit of quantification (mg kg^{-1})	30.0	30.0	30.0
IOC sample (mg kg^{-1})[a]	60.5 ± 0.9	20.7 ± 1.3	1098.3 ± 3.4
Recovery (%)[b]	104.2	104.8	99.9

Values expressed as mean and standard deviation of triplicates; [a]Reference sample from International Olive Oil Council (IOC) sent in 2015. Ranges acceptable: 54.6-61.5 mg kg^{-1} for campesterol, 16.6-22.9 mg kg^{-1} for stigmasterol, and 1091.0-1108.7 mg kg^{-1} for β-sitosterol; [b]Recoveries calculated using the means of IOC ranges.

Analysis of phytosterols in commercial samples

The phytosterols analyses were carried out after the confirmation of oil identity by the fatty acid profile, described in Silva and coauthors [2]. Table 2 presents the results of phytosterols (campesterol, stigmasterol, and β-sitosterol) in unconventional vegetable oils.

Table 2. Phytosterols content (mg kg^{-1}) in vegetable oils.

		Campesterol	**Stigmasterol**	**β-sitosterol**	**Total**
Coconut (n=15)	Mean	48.3	109.3	267.8	425.4
	Minimum	29.9	74.5	167.9	272.3
	Maximum	62.8	136.5	337.6	535.8
Safflower (n=9)	Mean	244.3	158.1	1020.8	1423.2
	Minimum	198.8	136.4	856.9	1192.1
	Maximum	382.3	222.3	1431.2	2035.7
Evening primrose (n=3)	Mean	531.4	43.2	5450.2	6024.8
	Minimum	512.1	35.1	5183.4	5742.3
	Maximum	552.0	47.7	5604.6	6169.7
Linseed (n=12)	Mean	624.1	176.6	1472.8	2273.4
	Minimum	481.0	128.2	1109.4	1725.7
	Maximum	825.4	234.4	2012.9	3072.6

Mean values (n=2); Total: sum of stigmasterol, campesterol, and β-sitosterol.

Among the oils studied, β-sitosterol was the component found in higher concentrations, ranging from 167.9 to 337.6 mg kg^{-1} in coconut oils, from 856.9 to 1431.2 mg kg^{-1} in safflower, 5183.4 to 5604.6 mg kg^{-1} of evening primrose, and 1109.4 to 2012.9 mg kg^{-1} in linseed oils. The phytosterol with lower concentration in safflower, linseed, and evening primrose oils was stigmasterol, while in coconut oils it was campesterol.

DISCUSSION

The choice of 5α-cholestane as internal standard was made after tests performed. This IS was sufficiently separated from other compounds of interest and the retention time (13.1 min) was adequate for the analysis. According to Islam and coauthors [6], four IS are more commonly used for phytosterol analysis: 5α-cholestane, 5α-cholestan-3β-ol, 5β-cholestan-3α-ol, and betulin. The first compound that eluted was campesterol, followed by stigmasterol, and β-sitosterol (Figure 1).

For coconut oils, Martins and coauthors [8] reported ranges for stigmasterol and campesterol between 143.4-144.8 mg kg^{-1} and 57.1-58.6 mg kg^{-1}. These results were similar to those found in this study (respectively 74.5-136.5 mg kg^{-1} and 29.9-62.8 mg kg^{-1}). The levels in safflower oils were similar to those described by Firestone [27]: stigmasterol: 136-254 mg kg^{-1}, campesterol: 193-344 mg kg^{-1}, and β-sitosterol: 842-1320 mg kg^{-1}. In linseed oils, the total percentage for stigmasterol, campesterol, and β-sitosterol were respectively 8, 27, and 65 %, similar to those found by Tańska and coauthors [28]. The mean value for β-sitosterol (1472.8 mg kg^{-1}) was very similar to results by Szterk and coauthors [22] (1625 mg kg^{-1}).

Evening primrose oils were considered the best source of phytosterols, with an average value 6024.8 mg kg^{-1}. β-sitosterol was the principal phytosterol (5450.2 mg kg^{-1}), followed by campesterol (531.4 mg kg^{-1}), and stigmasterol (43.2 mg kg^{-1}). Szterk and coauthors [22] found values of 6458 mg kg^{-1} for β-sitosterol and 65 mg kg^{-1} for stigmasterol, similar to our data. So, among the oils evaluated, the evening primrose was the most representative in the total quantity of phytosterols.

Figure 2 presents phytosterols results in eight different brands and lots. Results show a high variation in phytosterols levels among different lots of the same brand. 44 % of the evaluated brands presented equal lots in all parameters evaluated. The brands that the concentrations presented statistically similar values were C (coconut oil), D (safflower oil), B and F (linseed oils). Unconventional oils usually are cold-pressed and have high aggregate value. By presenting higher prices, they may be auspicious to adulteration with the intention of potential profit. Generally, other oils with lower nutritional and commercial value are added, which can result in economic losses and reduced health benefits [2,29]. Thus, the evaluation of sterol may have consequences for quality control and authentication, since each vegetable oil has unique sterol composition [30].

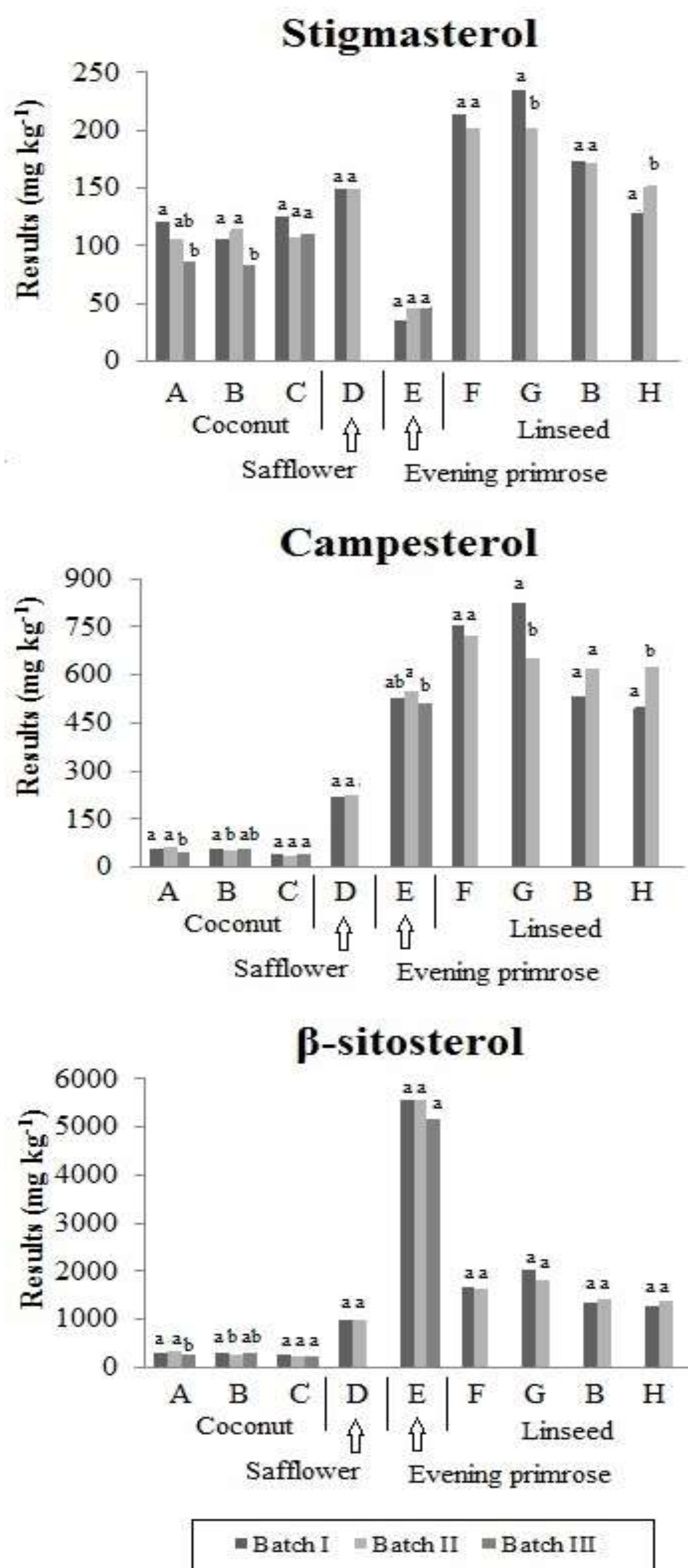

Figure 2. Phytosterols levels in different batches and brands of vegetable oils. Different letters in a brand indicate a significant difference between batches (Student t-test, $p < 0.05$).

CONCLUSION

The method applied could be considered suitable for quantitative analysis of phytosterols since it was linear within the range of concentrations investigated, and presented sensitivity, precision, and accuracy. β-sitosterol was the principal component, found in higher concentrations. The phytosterol with lower results was stigmasterol for safflower, linseed, and evening primrose oils, while for coconut oils was campesterol. Evening primrose was the most representative oil in the total phytosterols amounts (6024.8 mg kg^{-1}), with 90 % β-sitosterol contribution. A statistical evaluation of different brands shows that four oils presented similar

values for all components. The results generated in this study can be used as a phytosterols database for vegetable oils from unconventional sources since information about these oils is limited.

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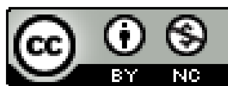

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