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# One-step rapid extraction of phytosterols from vegetable oils

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ABSTRACT

The conditions for the extraction of phytosterols (campesterol, stigmasterol and β-sitosterol) from vegetal oils were optimized by means of response surface methodology (RSM). A $2^4$ central composite rotatable design (CCRD) was used to investigate the effects of four independent variables: sample weight (g), saponification temperature (°C), saponification time (h) and number of extractions (n). The CCRD was carried out in 27 trials, including eight axial and three central points; and the response variables were the contents of campesterol, stigmasterol, β-sitosterol and total phytosterols. The optimized conditions established by the RSM were 0.3 g of sample, saponification for 3 h at 50 °C and 4 extractions with *n*-hexane. Satisfactory values for linearity, recovery, repeatability, accuracy, precision, limits of detection (2.0–2.3 mg/100 g) and quantification (6.5–7.7 mg/100 g) were achieved. The optimized method was also validated by comparison with the official AOCS method, and the contents of stigmasterol and β-sitosterol did not show significant differences ($p > 0.05$) when determined by both methods. However, low values ($p < 0.05$) for campesterol were found when the samples were analyzed by the AOCS method. The method optimized and validated in the present work is easy to carry out, fast and accurate. The method was successfully applied to sunflower, canola, corn, soybean and olive oils, and the lowest contents of total phytosterols were found in olive oil while and the highest amounts, in corn oil.

## 1. Introduction

Phytosterols, free, esterified with fatty acids or conjugated with glycosides, are naturally found in vegetables, particularly in seeds, vegetable oils, cereals, nuts, legumes and fruits (Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000). In vegetable oils, the phytosterols occur mainly as free sterols or as steryl esters of fatty acids (Verleyen et al., 2002). Although more than 250 phytosterols have been identified and reported in the literature so far, the most commonly found in foods are campesterol, stigmasterol and β-sitosterol, the former being found in large amounts in vegetable oils such as olive oil (Piironen et al., 2000). Several beneficial effects to human health have been attributed to phytosterol consumption, such as lowering of serum cholesterol levels and prevention of cardiovascular diseases and development of cancer (Jones, Macdougll, Ntanious, & Vanstone, 1997; Kangsamaksin et al., 2017; Kritchevsky & Chen, 2005; Meguro, Hase, Otsuka, Tokimitsu, & Itakura, 2003; Moreau, Whitaker, & Hicks, 2002). Phytosterols also protect plant oils from oxidation and polymerization during thermal treatment and/or light exposure (Moreau et al., 2002); however, their amount decreases during the various stages of the refining process due the high temperature (Verleyen et al., 2002). In addition, the phytosterols have been used for identification and characterization of vegetable oils, including for the fraud detection (Lukić et al., 2013). Consequently, the interest in quantifying them in vegetable oils has grown considerably.

The determination of phytosterols in vegetal oils is based on chromatographic techniques, usually gas chromatography (Cunha, Fernandes, & Oliveira, 2006; Haddada et al., 2007; Verleyen et al., 2002; Xu et al., 2018) with flame ionization or mass spectrometer detectors; however, high performance liquid chromatography (Careri, Elviri, & Mangia, 2001; Figueiredo et al., 2018; Sun et al., 2016) may also be used. Gas chromatography coupled to mass spectrometer detector with electron or chemical ionization source allows the best resolution for identification and quantification of phytosterols. Typically, phytosterols analysis includes: (1) extraction of lipids; (2) saponification or acid hydrolysis to release free phytosterols; (3) extraction of the unsaponifiable matter; (4) separation or purification of the phytosterols by thin layer chromatography (TLC) or solid phase extraction (SPE); (5)

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derivatization of the phytosterols; and (6) analysis by capillary gas chromatography (GC).

The extraction of phytosterols from food depends on the nature of the matrix, its physical state (solid or liquid), and chemical form (free, esterified or glycosylated). Several SPE cartridges have been used for phytosterol extraction from vegetal oils and fats, for instance, neutral alumina cartridges for free and esterified phytosterols (Phillips, Ruggio, Toivo, Swank, & Simpkins, 2002); silica cartridges for free phytosterols (Xu et al., 2018; Xu et al., 2014); and $NH_2$ and $C_{18}$ cartridges for phytosterols after saponification (free plus esterified) (García-González, Velasco, Velasco, & Ruiz-Méndez, 2018; Toivo, Piironen, Kalo, & Varo, 1998). The official method of the American Oil Chemists Society (AOCS; Firestone, 2017) uses TLC to separate the sterols from interfering substances, which consists in a laborious method that takes a long time and may lead to loss of the analytes.

In this sense, the aims of the present work were: (1) to optimize the phytosterol extraction without the use of SPE cartridge or thin layer chromatography; (2) to quantify the phytosterols without derivatization by GC-FID; (3) to validate the optimized method; (4) to compare the optimized and validated method with the official AOCS method; and (5) to apply the method for the determination of phytosterols in different vegetable oils.

## 2. Material and methods

### 2.1. Samples and chemicals

One sample of a Spanish olive oil and one of corn oil were acquired at the local market (Campinas, São Paulo, Brazil) for the optimization of phytosterol extraction and validation of the optimized method for phytosterol determination. The optimized and validated method was then applied to analyze the phytosterols from 22 vegetable oils (three brands of soybean, corn, sunflower and canola oils, and 10 brands of olive oil). For each sample, three bottles (900 mL each of the soybean, corn, sunflower and canola oils and 500 mL each of the olive oil) from three batches of different expiration dates were also acquired at the local market (Campinas, São Paulo, Brazil) and the phytosterols were analyzed in triplicate.

Analytical grade *n*-hexane (98.5%), ethanol (99.3%) and potassium hydroxide, and chromatographic grade isopropanol (99.8%) were purchased from J.T. Baker (Phillipsburg, New Jersey, USA). The standards of dihydrocholesterol, stigmasterol, campesterol and β-sitosterol with a minimum purity of 95% were acquired from Sigma-Aldrich (Saint Louis, Missouri, USA).

### 2.2. Phytosterol analysis by GC-FID and GC–MS

#### 2.2.1. Experimental design

The extraction of phytosterols from vegetable oils was optimized by means of a $2^4$ central composite rotatable design (CCRD) and based on the method previously developed by Mazalli, Sawaya, Eberlin, and Bragagnolo (2006) for eggs. The independent variables were saponification time ($X_1$), saponification temperature ($X_2$), number of extractions ($X_3$) and sample weight ($X_4$) (Table 1), and the response variables were the contents of campesterol, stigmasterol, β-sitosterol and total phytosterol contents (sum of campesterol, stigmasterol and β-sitosterol). Table 2 shows the CCRD experimental design comprising 27 trials with 3 central points and 8 axial points that were carried out in a randon order.

#### 2.2.2. Phytosterol extraction

The oil was weighted in a glass tube and homogenized with 10 mL of 3% ethanolic KOH solution, followed by saponification in a hot water bath. After that, the sample was cooled by adding 10 mL of water and the phytosterols were extracted with *n*-hexane (10 mL each extraction). The phytosterol extract was dried under nitrogen flux, 1 mL of dihydrocholesterol solution containing 50 μg dihydrocholesterol dissolved in isopropanol was added as internal standard and 1 μL was injected into the GC-FID for quantification or GC–MS for identification. Fig. 1 shows the simplified flow diagram of phytosterol extraction.

**Table 1**
Independent variables and levels in the $2^4$ central composite rotatable design for the optimization of phytosterol extraction from vegetable oils.

| Independent variables | Levels | | | | |
|---|---|---|---|---|---|
| | −2 | −1 | 0 | +1 | +2 |
| $X_1$: saponification time (h) | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| $X_2$: saponification temperature (°C) | 30 | 40 | 50 | 60 | 70 |
| $X_3$: number of extractions | 1 | 2 | 3 | 4 | 5 |
| $X_4$: sample weight (g) | 0.3 | 0.4 | 0.5 | 0.6 | 0.7 |

#### 2.2.3. Chromatographic analysis

An HP model 6890 gas chromatograph equipped with an automatic sampler, split injector (ratio 50:1) at 250 °C, and flame ionization detector (FID) at 300 °C was used to quantify the phytosterols. Separation was achieved in a stationary phase capillary column (HP-5, 5% phenyl, 95% dimethylpolysiloxane, 30 m, 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies, Santa Clara, California, USA). The chromatographic conditions were optimized based on the method described by Schmarr, Gross, and Shibamoto (1996) as follows: programmed column temperature starting at 150 °C/1 min, rising to 300 °C at 10 °C/min and remaining at this temperature for 10 min; stripping gas helium at 1 mL/min; flame ionization detector at 300 °C; make-up gas nitrogen at 30 mL/min, hydrogen at 30 mL/min and synthetic air at 300 mL/min; injector temperature 250 °C; and injection volume 1 μL. The phytosterols were identified by comparison of the retention times of the peaks in the samples with those of the standards and by co-chromatography. Quantification was done by internal standardization using dihydrocholesterol (50 μg in isopropanol, Sigma-Aldrich, Saint Louis, Missouri, USA) as the internal standard.

The campesterol, stigmasterol and β-sitosterol were confirmed by mass spectrometry using an HP model 6890 gas chromatograph coupled to an HP model 5973 mass spectrometer. The electron impact energy was 70 eV and the chromatographic conditions were: split injector, ratio 50:1; HP-5MS capillary column (30 m, internal diameter 0.25 mm and 0.25 μm film thickness, Agilent Technologies, Santa Clara, California, USA); column temperature programmed to start at 160 °C/0.1 min, rising to 300 °C at 5 °C/min and remaining at this temperature for 10 min; stripping gas helium at 1.5 mL/min; injector initial temperature 160 °C/0.01 min, rising to 300 °C at 50 °C/min and remaining at this temperature for 1 min; source at 230 °C and quadrupole detector at 150 °C.

#### 2.2.4. Method validation

The method was validated for linearity, recovery and repeatability and the limits of detection and quantification were also determined. The linearity of the analytical curve was calculated using 5 points in the concentration range from 4 to 100 μg/mL. The recovery of the phytosterols was determined by fortifying the olive oil sample at two levels (66 and 100 mg/100 g for sitosterol, 5 and 20 mg/100 g for stigmasterol, and 7 and 25 mg/100 g for campesterol), with eight replicates at each level of addition. The repeatability and intermediate accuracy were verified by the coefficients of variation of five replicates. The detection limit was calculated as three times the standard deviation of eight replicate analyses and the quantification limit as 10 times the standard deviation of eight replicate analyses (Keith et al., 1983). The accuracy was evaluated using two parameters: repeatability (two analyses carried out on the same day) and the intermediary accuracy (two analyses carried out on different days).

**Table 2**
$2^4$ central composite rotatable design for the optimization of phytosterol extraction after saponification (free plus esterified) from vegetable oils and response variables.

| Independent variables | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Trial[a] | $X_1$ | $X_2$ | $X_3$ | $X_4$ | β-sitosterol | Campesterol | Stigmasterol | Total Phytosterol |
| 1 | −1 | −1 | −1 | −1 | 125.64 | 11.15 | 7.88 | 144.67 |
| 2 | −1 | −1 | −1 | +1 | 106.07 | 8.57 | 5.64 | 120.28 |
| 3 | −1 | −1 | +1 | −1 | 128.22 | 6.69 | 5.29 | 140.20 |
| 4 | −1 | −1 | +1 | +1 | 125.18 | 11.24 | 8.12 | 144.54 |
| 5 | −1 | +1 | −1 | −1 | 125.48 | 10.76 | 7.98 | 144.22 |
| 6 | −1 | +1 | −1 | +1 | 114.76 | 9.21 | 6.38 | 130.35 |
| 7 | −1 | +1 | +1 | −1 | 138.94 | 14.49 | 12.22 | 165.65 |
| 8 | −1 | +1 | +1 | +1 | 131.30 | 12.40 | 9.08 | 152.78 |
| 9 | +1 | −1 | −1 | −1 | 132.50 | 10.82 | 7.48 | 150.80 |
| 10 | +1 | −1 | −1 | +1 | 102.61 | 9.12 | 5.79 | 117.52 |
| 11 | +1 | −1 | +1 | −1 | 158.33 | 13.98 | 10.41 | 182.72 |
| 12 | +1 | −1 | +1 | +1 | 139.27 | 13.21 | 9.02 | 161.50 |
| 13 | +1 | +1 | −1 | −1 | 130.29 | 12.03 | 8.97 | 151.29 |
| 14 | +1 | +1 | −1 | +1 | 120.48 | 10.01 | 6.92 | 137.41 |
| 15 | +1 | +1 | +1 | −1 | 138.31 | 12.39 | 8.98 | 159.68 |
| 16 | +1 | +1 | +1 | +1 | 133.78 | 11.01 | 7.37 | 152.16 |
| 17 | −2 | 0 | 0 | 0 | 125.80 | 10.92 | 7.65 | 144.37 |
| 18 | +2 | 0 | 0 | 0 | 131.88 | 12.03 | 8.50 | 152.41 |
| 19 | 0 | −2 | 0 | 0 | 131.90 | 11.21 | 7.79 | 150.90 |
| 20 | 0 | +2 | 0 | 0 | 127.41 | 12.91 | 10.07 | 150.39 |
| 21 | 0 | 0 | −2 | 0 | 87.41 | 7.77 | 6.00 | 101.18 |
| 22 | 0 | 0 | +2 | 0 | 148.17 | 11.14 | 7.69 | 167.00 |
| 23 | 0 | 0 | 0 | −2 | 139.91 | 14.22 | 11.06 | 165.19 |
| 24 | 0 | 0 | 0 | +2 | 120.70 | 10.07 | 6.86 | 137.63 |
| 25 | 0 | 0 | 0 | 0 | 128.72 | 11.74 | 8.80 | 149.26 |
| 26 | 0 | 0 | 0 | 0 | 122.91 | 9.93 | 7.50 | 140.34 |
| 27 | 0 | 0 | 0 | 0 | 127.88 | 11.71 | 8.43 | 148.02 |

[a] The assays were performed in duplicates in random order; $X_1$: saponification time (h); $X_2$: saponification temperature (°C); $X_3$: number of extractions; $X_4$: sample weight (g). Levels for the independent variables are shown in Table 1.Total Phytosterol = sum of campesterol, stigmasterol and β-sitosterol.

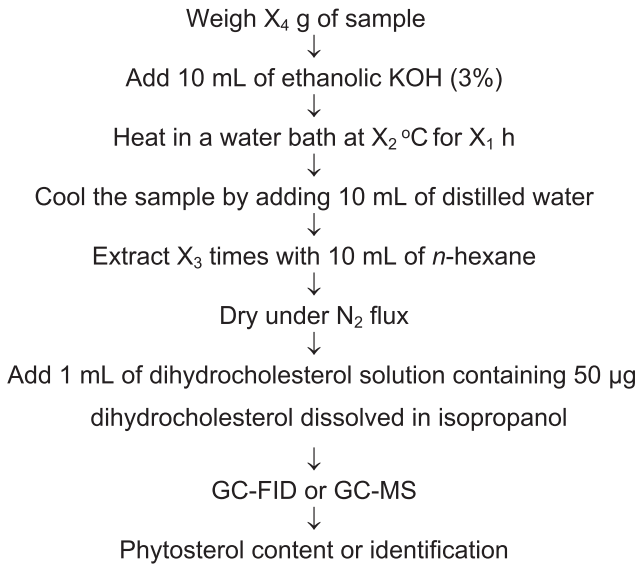


**Fig. 1.** Flow diagram for the analysis of phytosterols from vegetable oils. Legend: $X_1$ (1.0–3.0 h), $X_2$ (30–70 °C), $X_3$ (1–5 extraction), and $X_4$ (0.3–0.7 g) see Tables 1 and 2.

**Table 3**
Regression coefficients for the predictive model of β-sitosterol, campesterol, stigmasterol and total phytosterols (sum of β-sitosterol, campesterol and stigmasterol).

| | β-sitosterol | Campesterol | Stigmasterol | Total Phytosterol |
|---|---|---|---|---|
| Means | 126.50* | 11.13* | 8.24* | 145.87* |
| $X_1$ (L) | 6.01* | 0.86 | 0.34 | 7.21 |
| $X_1$ (Q) | 1.68 | 0.10 | −0.16 | 1.62 |
| $X_2$ (L) | 0.54 | 0.91 | 1.07 | 2.52 |
| $X_2$ (Q) | 2.09 | 0.39 | 0.26 | 2.75 |
| $X_3$ (L) | 21.41* | 1.71* | 1.40* | 24.53* |
| $X_3$ (Q) | −3.84 | −0.91 | −0.78 | −5.52 |
| $X_4$ (L) | −11.89* | −1.32 | −1.61* | −14.82* |
| $X_4$ (Q) | 2.42 | 0.43 | 0.28 | 3.14 |
| $X_1X_2$ | −4.40 | −1.36 | −1.15 | −6.91 |
| $X_1X_3$ | 4,01 | 0.43 | −0.03 | 4.42 |
| $X_1X_4$ | −2.79 | −0.52 | −0.32 | −3.64 |
| $X_2X_3$ | −4.11 | 0.35 | 0.17 | −3.59 |
| $X_2X_4$ | 4.86 | −0.81 | −0.74 | 3.30 |
| $X_3X_4$ | 4.46 | 1.02 | 0.53 | 6.02 |

$X_1$: saponification time (h); $X_2$: saponification temperature (°C); $X_3$: number of extractions; $X_4$: sample weight (g); L - linear, Q - quadratic, * statistically significant ($p < 0.05$).
Total Phytosterol = sum of campesterol, stigmasterol and β-sitosterol.

The method developed and optimized for the determination of the phytosterol contents in vegetable oils was also compared with the official AOCS method (method Ch 6–91, Firestone, 2017). Briefly, the AOCS method is composed of the following steps: extraction of the unsaponifiable matter, separation of the sterolic fraction by TLC, extraction of the sterols and their quantification by GC. The chromatographic conditions were the same as those described in Section 2.2.3 for the quantification of phytosterols.

The recovery trials were carried out aiming at verifying possible losses of analytes during the analytical procedure, since the procedure has several steps. The standards of β-sitosterol and stigmasterol were added to the vegetable oil samples (sunflower, canola, corn and soybean) at the same concentrations described for the method validation. For the olive oil samples, two concentrations of sitosterol (64 and 96 mg/100 g) and stigmasterol (6 and 38 mg/100 g) were added. All spiked samples were analyzed in 5 repetitions.

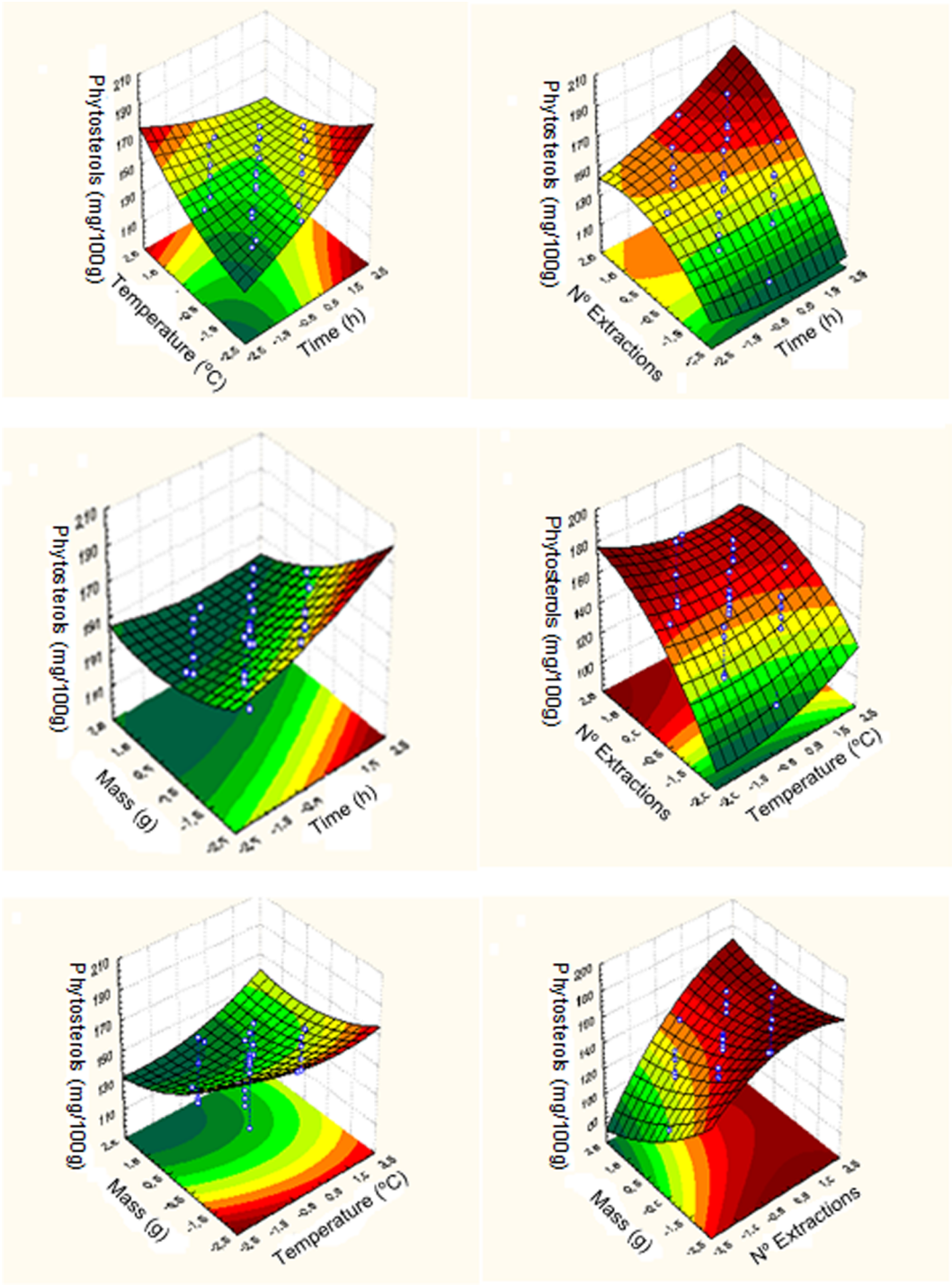


**Fig. 2.** Response surfaces for the $2^4$ complete central rotational design considering the total phytosterols (sum of campesterol, stigmasterol and β-sitosterol) as the response variable.

### 2.3. Statistical analysis

The data from the CCRD were analyzed using the Statistica software (release 5.5, STATSOFT Inc, 2000) to obtain a quadratic polynomial model (Eq. (1)) and the response surfaces.

$$Y = B_0 + B_1X_1 + B_2X_2 + B_3X_3 + B_4X_4 + B_{11}X^2 + B_{22}X^2 + B_{33}X^2 + B_{44}X^2 + B_{12}X_1X_2 + B_{13}X_1X_3 + B_{14}X_1X_4 + B_{23}X_2X_3 + B_{24}X_2X_4 + B_{34}X_3X_4. \quad (1)$$

where $X_1$ = saponification time (h); $X_2$ = saponification temperature (°C); $X_3$ = number of extractions; $X_4$ = sample weight (g); Y = contents of sterols.

The contents of phytosterols were submitted to analysis of variance (ANOVA) using the Statistica software (release 5.5, STATSOFT Inc, 2000), to verify differences between the amounts of phytosterols among the different vegetable oils.

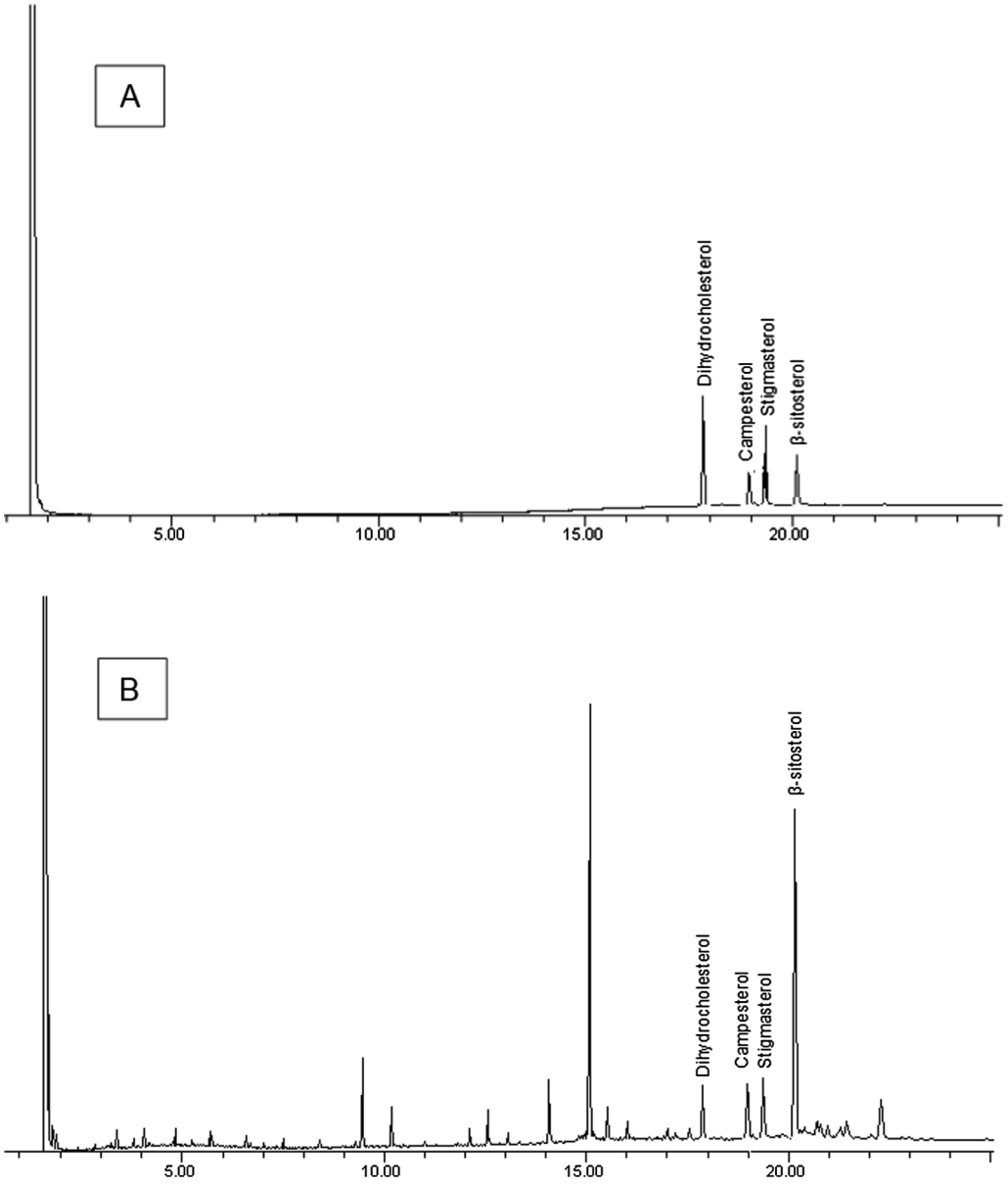


**Fig. 3.** Chromatograms obtained by GC-FID of phytosterol standards (A) and from vegetal oil (B). Peak identification: campesterol (rt = 18.98 min), stigmasterol (rt = 19.41 min), β-sitosterol (rt = 20.14 min), and dihydrocholesterol (internal standard, rt = 17.87 min). Stationary phase capillary column (HP-5, 5% phenyl, 95% dimethylpolysiloxane, 30 m, 0.25 mm i.d., 0.25 μm film).

**Table 4**
Standard curve, correlation coefficient, precision, recovery, limits of detection and quantification for phytosterol analysis by GC-FID.

| Standard | Standard curve | Correlation coefficient ($R^2$) | Within-day precision (RSD%, n = 5) | Between-day precision (RSD%, n = 5) | Spiking amount (mg/100 g) | Recovery* (%) | LOD (mg/100 g) | LOQ (mg/100 g) |
|---|---|---|---|---|---|---|---|---|
| Campesterol | y = 0.0174x-0.0088 | 0.9998 | 3.7 | 3.4 | 7 | 96.1 (5.9) | 2.3 | 7.7 |
| | | | | | 25 | 97.1 (5.3) | | |
| Stigmasterol | y = 0.0198x-0.0213 | 0.9996 | 5.4 | 1.0 | 5 | 101.0 (4.2) | 2.0 | 6.5 |
| | | | | | 20 | 103.6 (2.8) | | |
| β-Sitosterol | y = 0.0199x-0.0229 | 0.9996 | 0.6 | 1.8 | 66 | 96.4 (2.9) | 2.2 | 7.2 |
| | | | | | 100 | 97.5 (0.7) | | |

* Mean (standard deviation) of eight determinations.

## 3. Results and discussion

### 3.1. Optimization and validation of the phytosterol extraction

Table 2 shows the results of the CCRD considering the response variables: contents of β-sitosterol, campesterol, stigmasterol and total phytosterols, while the effects and interactions among the independent variables on the phytosterol contents are presented in Table 3. The saponification time ($X_1$) showed a positive signal, meaning that the longer the saponification time, the higher the content of phytosterols,

**Table 5**
Relative intensities of the principal ions of phytosterols obtained by mass spectroscopy (MS).

| Molecular ion (*m/z*) Compound | 400 | 289 | 213 | 255 | 271 | 412 | 414 | 329 | 303 |
|---|---|---|---|---|---|---|---|---|---|
| Campesterol | 86 | 99 | 100 | | | | | | |
| Stigmasterol | | | | 100 | 83 | 48 | | | |
| β-Sitosterol | | | | | | | 85 | 92 | 100 |

Molecular mass: campesterol = 400.69; stigmasterol = 412.70; β-sitosterol = 414.72.

being significant only for β-sitosterol. The number of extractions ($X_3$) also showed a positive signal, meaning that a larger number of extractions tended to result in a higher extraction of phytosterols, and was the only variable significant for all the tested responses. The sample weight ($X_4$) presented negative signal, meaning that a higher amount of sample mass tended to result in smaller contents of phytosterols, being significant to all the responses except to campesterol. The saponification temperature ($X_2$) as well as all the interactions among the independent variables were not significant ($p > 0.05$) for any of the response variables. Thus, based on these results and on the response surface (Fig. 2) analysis, the optimal conditions for the phytosterol extraction were established as: saponification for 3 h ($X_1$) at 50 °C ($X_2$) using 0.3 g of oil ($X_4$) and 4 extractions with 10 mL of *n*-hexane totalizing 40 mL ($X_3$).

To verify the validity of the predictive model (Eq. (1) and Table 3), variance analysis were carried out (Supplementary Tables 1, 2, 3 and 4) and the results were shown to be valid.

The determination of the phytosterols by GC-FID allowed for the detection and quantification of the campesterol, stigmasterol and β-sitosterol. The chromatograms showed good separation and resolution of the phytosterol peaks, campesterol (rt = 18.98 min), stigmasterol (rt = 19.41 min) and β-sitosterol (rt = 20.14 min), allowing for the easy identification of the compounds by comparison with the retention time of the standard solutions (Fig. 3). The analytical curves of the phytosterols were linear in the concentration range between 4 and 100 μg/mL, with correlation coefficients above 0.9996 (Table 4). The repeatability and intermediate accuracy were adequate with variation coefficients below 6% (Table 4). Recovery of the spiked phytosterols at two levels varied from 96 to 105% for olive oil. The detection and quantification limits by GC-FID were for campesterol (2.3 and 7.7 mg/100 g, respectively), stigmasterol (2.0 and 6.5 mg/100 g, respectively) and for β-sitosterol (2.2 and 7.2 mg/100 g, respectively) (Table 4). By using comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry for the analysis of the same phytosterols in vegetable oils, Xu et al. (2018) found lower limits of detection and quantification and recovery levels than in the present study, however, the within-day precision was similar, except for β-sitosterol, which was lower in the present study.

The identification of the campesterol, stigmasterol and β-sitosterol was confirmed by mass spectrometry. Table 5 shows the main ions observed in the spectra of the phytosterols. The phytosterol fragmentation studies found in the literature were all carried out after derivatization of the phytosterols with trimethylsilyl ether (TMS) and therefore could not be used for comparison (Bortolomeazzi, Zan, Pizzale, & Conte, 1999; Dutta, 2002; Menéndez-Carreño, Garcia-Herreros, Astiasarán, & Ansorena, 2008; Xu et al., 2018; Zhang et al., 2008). Thus, the identification of these compounds in the samples was carried out by comparison of the mass spectra obtained for the samples with those of authentic standards of campesterol, stigmasterol and β-sitosterol.

The method developed and validated for the determination of the phytosterols in vegetable oils was also compared with the official AOCS method (Firestone, 2017). Since this method includes the steps of

**Table 7**
Contents of phytosterols after saponification (free plus esterified) (mg/100 g) found in the vegetable oils.

| *Oil* | *Campesterol* | *Stigmasterol* | *β-Sitosterol* |
|---|---|---|---|
| **Sunflower**[a] | | | |
| Brand S | 54.30 (3.69)$^{A}$ | 32.42 (1.08)$^{A}$ | 211.33 (10.84)$^{A}$ |
| Brand L | 66.55 (5.42)$^{B}$ | 32.67 (1.58)$^{A}$ | 213.41 (7.17)$^{A}$ |
| Brand C | 60.45 (8.96)$^{AB}$ | 27.02 (3.40)$^{B}$ | 183.33 (16.75)$^{B}$ |
| **Canola**[a] | | | |
| Brand S | 314.70 (17.88)$^{A}$ | ≤ 6.5[c] | 387.12 (32.40)$^{A}$ |
| Brand L | 316.55 (13.39)$^{A}$ | 6.66 (2.94)$^{B}$ | 367.91 (7.90)$^{A}$ |
| Brand C | 209.23 (40.31)$^{B}$ | 13.70 (0.58)$^{A}$ | 264.06 (49.86)$^{B}$ |
| **Corn**[a] | | | |
| Brand S | 231.51 (11.82)$^{A}$ | 50.71 (3.68)$^{A}$ | 522.82 (32.14)$^{A}$ |
| Brand L | 239.75 (30.50)$^{A}$ | 56.03 (8.20)$^{A}$ | 540.72 (49.83)$^{A}$ |
| Brand C | 243.69 (33.04)$^{A}$ | 51.03 (3.55)$^{A}$ | 455.35 (37.93)$^{B}$ |
| **Soybean**[a] | | | |
| Brand SY | 84.94 (12.37)$^{A}$ | 61.39 (8.85)$^{AB}$ | 155.49 (12.61)$^{A}$ |
| Brand L | 92.00 (7.22)$^{A}$ | 55.25 (3.56)$^{B}$ | 158.24 (24.52)$^{A}$ |
| Brand SD | 91.44 (2.58)$^{A}$ | 62.16 (3.35)$^{A}$ | 159.37 (2.40)$^{A}$ |
| **Olive oil**[b] | | | |
| Brand M | 34.46 (6.57)$^{A}$ | 22.56 (6.36)$^{A}$ | 172.36 (48.31)$^{A}$ |
| Brand FF | 33.66 (5.42)$^{A}$ | 26.32 (1.57)$^{A}$ | 206.17 (31.82)$^{A}$ |
| Brand CB | 7.93 (1.06)$^{A}$ | ≤ 6.5[c] | 133.99 (8.83)$^{A}$ |
| Brand CT | 8.56 (1.16)$^{C}$ | 11.59 (1.04)$^{B}$ | 259.46 (26.80)$^{A}$ |
| Brand AD | 19.78 (4.58)$^{B}$ | ND | 127.74 (6.37)$^{A}$ |
| **Extra virgin olive oil**[b] | | | |
| Brand LE | 9.17 (0.82)$^{B}$ | ≤ 6.5[c] | 121.95 (9.71)$^{C}$ |
| Brand G | 9.40 (1.43)$^{B}$ | ≤ 6.5[c] | 125.04 (5.58)$^{C}$ |
| Brand BO | 8.15 (0.22)$^{B}$ | ND | 139.54 (3.99)$^{BC}$ |
| Brand BU | 41.93 (4.87)$^{A}$ | 29.56 (2.92) | 159.06 (25.50)$^{A}$ |
| Brand AN | 14.29 (2.35)$^{B}$ | ≤ 6.5[c] | 159.97 (18.06)$^{AB}$ |

ND = Not detected (limit of detection = 2.0 mg/100 g).
Values for each phytosterol, for each oil, in the same column with the same letter are not significantly different at the 5% level.
[a] Mean and estimate of the standard deviation of 27 analyses (3 brands from 3 batches analyzed in triplicate).
[b] Mean and estimate of the standard deviation of 6 analyses (3 batches in triplicate).
[c] Limit of quantification.

**Table 6**
Contents of phytosterols after saponification (free plus esterified) (mg/100 g) in the vegetable oils.

| Vegetable oil | Campesterol | | Stigmasterol | | β-Sitosterol | |
|---|---|---|---|---|---|---|
| | Validated method M (SD) [a] | AOCS M (SD) | Validated method M (SD) | AOCS M (SD) | Validated method M (SD) | AOCS M (SD) |
| Sunflower | 65.73 (6.67) | 30.60 (1.38)[b] | 32.33 (3.61) | 26.00 (3.21) | 207.72 (7.74) | 229.43 (17.15) |
| Canola | 270.79 (93.5) | 146.38 (44.62)[b] | 8.77 (4.28) | 6.49 (2.66) | 338.99 (107.34) | 316.38 (99.28) |
| Corn | 219.02 (3.51) | 118.97 (7.98)[b] | 56.72 (5.21) | 47.10 (4.27) | 540.62 (34.52) | 577.58 (37.58) |
| Soybean | 96.70 (3.15) | 50.12 (2.73)[b] | 62.81 (7.45) | 54.08 (0.81) | 174.89 (15.67) | 161.73 (42.57) |
| Olive | 14.31(9.55) | 4.87 (1.85)[b] | 7.40 (4.82) | 1.89 (1.43) | 185.61 (93.47) | 172.26 (74.67) |

Values for each phytosterol in the same line with the same letter are not significantly different at the 5% level.
Validated method: method developed and validated in the present study. AOCS: official AOCS method (Firestone, 2017).
[a] Mean and estimate of the standard deviation of 6 analyses (3 brands in duplicate).

extraction of the unsaponifiable matter and separation of the sterolic matter by TLC, recovery trials were carried out aiming at verifying possible analyte losses due to these steps. The recovery rates for the two levels of β-sitosterol (66 and 100 mg/100 g) and stigmasterol (5 and 20 mg/100 g) in olive oil were low for β-sitosterol (50 ± 13 and 70 ± 26%) and high for stigmasterol (106 ± 1 and 114 ± 4%). The recovery of stigmasterol was higher than 100% for both levels of standard addition. However, for β-sitosterol, which is the phytosterol present in larger amounts in vegetable oils, the recovery rates were low, which could be related to possible losses due to the numerous steps carried out during the extraction, especially the isolation of the sterols by thin layer chromatography. The high values of the standard deviations (13 and 26%) confirmed the occurrence of an elevated variation between the determinations.

Table 6 shows the phytosterol contents (campesterol, stigmasterol and β-sitosterol) obtained for five vegetable oils (sunflower, canola, corn, soybean and olive) by the method optimized and validated in the present study and by the AOCS official method. **Supplementary** Table 5 shows the results obtained for each brand of oil by both methods. A significant difference ($p < 0.05$) was found between the campesterol content of all samples (sunflower, corn, canola, soybean and olive) analyzed by the two methods, and the results obtained by the method developed and optimized in the present study were always higher than those obtained by the AOCS method. On the other hand, the values for β-sitosterol and stigmasterol obtained by both methods did not show any significant difference ($p > 0.05$) for any of the analyzed vegetable oils. Thus, we can infer that the preparative step by TLC to separate the sterols can cause loss of the components.

### *3.2. Application of the optimized and validated method to vegetable oils*

Since the method optimized and validated in the present study was shown to be more efficient than the AOCS method, all the samples were analyzed by this method and the results are shown in Table 7. A significant difference was observed for the contents of phytosterols after saponification (free plus esterified) in the three analyzed brands of sunflower and canola oil. For corn oil, only β-sitosterol content of brand C differed significantly from the others, while for soybean oil, a significant difference was observed for the contents of stigmasterol among all the analyzed brands. The content of campesterol varied from 54 mg/100 g in sunflower oil to 317 mg/100 g in canola oil. The contents of stigmasterol varied from 7 mg/100 g in canola oil to 62 mg/100 g in soybean oil. The variation in the content of β-sitosterol was from 156 mg/100 g in soybean oil to 540 mg/100 g in corn oil. Among the analyzed samples, some batches presented high values for the estimated standard deviations (% CV > 10) demonstrating variation in the results. The variability in the content of phytosterols, both between batches of the same brand and between different brands, could be related to difficulties faced by the industries to maintain uniformity in the process, and also to the use of vegetables (raw material) acquired from diverse suppliers and/or regions with different edaphoclimatic conditions to obtain the respective oils. Haddada et al. (2007) analyzed 6 varieties of olive oil and found significantly different values for the phytosterols, demonstrating the great variability between cultivars, attributed exclusively to genetic factors.

The olive oils (olive and extra virgin olive) presented lower amounts of campesterol than the other vegetable oils, varying from 7.93 to 41.93 mg/100 g. β-Sitosterol content varied from 121.95 to 259.46 mg/100 g, and were more close to the values found for soybean oils (155–159 mg/100 g). On the other hand, stigmasterol content in the olive oils varied from not-detected (detection limit = 2.0 mg/100 g) to 29.56 mg/100 g, which were lower than those found in sunflower, corn and soybean oils.

Considering the average phytosterol contents found in the different vegetable oils, the highest values for campesterol were found in canola oil (289 mg/100 g) followed by corn oil (238 mg/100 g). Soybean oil presented the highest values for stigmasterol (60 mg/100 g) and olive oil the lowest (≤ 2.0 mg/100 g; limit of detection). β-Sitosterol content varied from 141 mg/100 g in extra virgin olive oil to 513 mg/100 g in corn oil. For all the analyzed vegetable oils, β-sitosterol was the phytosterol found in the highest concentrations, while stigmasterol showed the lowest values. Comparing the results of the present study for sunflower oil with the literature data (results expressed as phytosterols after saponification or total methylation, i.e., free plus esterified), the present results were higher considering the campesterol contents, similar for stigmasterol and lower for β-sitosterol than those found by Garcia-Gonçalves et al. (2018). On the other hand, Verleyen et al. (2002) found lower levels of campesterol, higher of β-sitosterol but similar of stigmasterol than in the present study for sunflower oil. For soybean oil, the campesterol, stigmasterol and β-sitosterol contents found in the present work were higher than the results obtained by Garcia-Gonçalves et al. (2018). However, the results obtained by Verleyen et al. (2002) in soybean oil were lower for campesterol, higher for β-sitosterol and similar for stigmasterol. The variation in the composition of phytosterol of the different vegetal oil is probably due to the different varieties of the vegetal (sunflower, corn, canola, soybean and olive) employed and the different process techniques applied in the extraction process.

## 4. Conclusion

The extraction procedure optimized in the present study by means of response surface methodology for the determination of the campesterol, β-sitosterol and stigmasterol in vegetable oils was easy to carry out, quite convenient and much less laborious than methods previous published in the literature. The optimal conditions were established as sample mass of 0.3 g, saponification for 3 h at 50 °C and 4 extractions with *n*-hexane. The results for the phytosterol in vegetal oils have shown that the introduced analytical changes provided reliable results in terms of accuracy. Compared to the standard AOCS method, the results did not show significant differences ($p > 0.05$) for stigmasterol and β-sitosterol, but significant differences ($p < 0.05$) were found for campesterol. Considering the vegetable oils analyzed in the present study, the lowest phytosterol content was found in the olive oils and the highest in the corn oils, there being considerable variation between the batches analyzed.

## CRediT authorship contribution statement

**Claudia Aparecida Silva Almeida:** Formal analysis, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing. **Sueli Regina Baggio:** Formal analysis, Methodology, Validation, Investigation, Writing - original draft, Writing - review & editing. **Lilian Regina Barros Mariutti:** Conceptualization, Data curation, Writing - original draft, Writing - review & editing. **Neura Bragagnolo:** Supervision, Funding acquisition, Investigation, Conceptualization, Data curation, Writing - original draft, Writing - review & editing.

## Declaration of Competing Interest

The authors have no conflicts of interest.

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## Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.108891.

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