Implementation of a method for the identification and quantification of phytosterols in peanut (*Arachis hypogaea* L.) by gas chromatography

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ABSTRACT
Peanut (*Arachis hypogaea* L.), a major source of oil, is the second most widely cultivated legume crop worldwide after soybean. Argentina is the largest producer of confectionery peanuts in Latin America and the seventh in the world. This legume has bioactive compounds of nutritional value, phytosterols among the most important ones. Phytosterols protect against cancer and lower cholesterol rates. The objective of this work was to adjust and refine a method to identify and quantify phytosterol content in peanut seeds by gas chromatography. The method was found to be accurate, as demonstrated through repeatability and reproducibility tests, and expressed by the coefficients of variation. No significant differences were found between the results obtained and the reference values using a confidence level of 95 % and 5 degrees of freedom. The results obtained in this work presented 44.31 mg of beta-sitosterol and 3.39 mg of stigmasterol per 100 g of roasted peanuts, in agreement with the values reported in the literature.

Key words: sterols, cholesterol, beta-sitosterol, stigmasterol, mass spectrometry

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RESUMEN
El maní (*Arachis hypogaea* L.), una importante fuente de aceite, es la segunda leguminosa más cultivada en todo el mundo después de la soja. Argentina es el mayor productor de maní confitero de América Latina y el séptimo del mundo. Esta leguminosa posee compuestos bioactivos y los fitoesteroles

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son uno de los más importantes. Los fitoesteroles protegen contra el cáncer y reducen el colesterol. El objetivo de este trabajo fue ajustar y mejorar un método para identificar y cuantificar el contenido de fitoesteroles en semillas de maní mediante cromatografía de gases. Se concluyó que el método era preciso, como lo demuestran las pruebas de repetibilidad y reproducibilidad expresadas por los coeficientes de variación. No se encontraron diferencias significativas entre los resultados obtenidos y los valores de referencia utilizando un nivel de confianza del 95 % y cinco grados de libertad. Los resultados obtenidos en este trabajo, correspondiente a 44,31 mg de beta-sitosterol y 3,39 mg de estigmasterol por 100 g de maní tostado, concuerdan con los informados en la literatura.

Palabras clave: esteroles, colesterol, beta-sitosterol, estigmasterol, espectrometría de masas

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INTRODUCTION

Peanut (*Arachis hypogaea* L.) is a legume; as such, it plays a major role in a healthy diet, sustainable food production and especially food security (Food and Agriculture Organization, 2014). It is an annual, erect herb, 15-70 cm in height, with slightly hairy stems. The fruits are pods, known as shells, containing seeds which develop underground. One to four seeds grow per shell, depending on the cultivated variety. Two variety groups are recognized: plants with either erect or procumbent growth habits. Most of the commercially cultivated varieties belong to the former group.

Argentina is the largest peanut producer in Latin America and the seventh in the world (Portal oficial del Estado argentino, 08/09/2023). Nearly 80 % of this oilseed crop is grown in the province of Córdoba, with its production playing a major role in the regional economy. The provinces of Buenos Aires and La Pampa rank second and third in production after Córdoba, followed by the provinces of San Luis, Santa Fe, Salta, Catamarca, Tucumán, Formosa and Santiago del Estero (Sistema de Información Simplificado Agrícola, 2021). Argentina is the main peanut supplier in the European market, where this product is considered the best in the world. The Netherlands is the main importer of our seeds (Casini et al., 2003; Haro et al., 2020).

Peanut is qualified as a superfood because a small serving provides a high concentration of nutrients and, therefore, beneficial health effects. This crop has a high content of healthy fats, proteins and fibers. Regarding the lipid profile, the greatest proportion corresponds to monounsaturated lipids, with a very low content of saturated fats. Moreover, peanut consumption contributes more proteins than any other nut (Cámara Argentina del Maní, 2020).

According to The Peanut Institute (2020), besides being a tasty snack, this legume presents bioactive compounds with important benefits for consumers. Bioactive compounds are nutritional components that are present in some foods and that help to prevent diseases, improve life quality of elderly people, and maintain a healthy digestive, endocrine, cardiovascular, immunological and nervous system. One of the compounds present in

peanuts is arginine, which reduces blood pressure and keeps arteries flexible. Other compounds with antioxidant properties include vitamin E, which is found in greater proportion in roasted peanuts with skin (Cámara Argentina del Maní, 2020), and resveratrol, which helps prevent growth of cancer cells. In addition, phenols, flavonoids and biotin help reduce skin aging and growth of skin cancer cells, prevent inflammatory processes, prevent the formation of blood clots, protect against diabetes and neurological disorders, and help to keep hair and skin healthy (The Peanut Institute, 2020).

On the other hand, peanuts have phytosterols, which consist of lipid components whose contents vary with genetic variability and environmental conditions. Among these, beta-sitosterol, campesterol and stigmasterol are found in greatest proportion. Phytosterols have a chemical structure very similar to that of cholesterol. For this reason, when both molecules are ingested, phytosterols displace cholesterol, favoring the reduction of bad cholesterol (LDL) levels without altering the values of good cholesterol (HDL) (Alimentos Argentinos, 2013).

As stated by the Comisión Nacional de Alimentos (2021), a daily intake of 1 to 3 g of phytosterols has been found to reduce cholesterol absorption. These sterols are not absorbed in the body, meaning that they act at the moment of consumption and are then excreted. For this reason, the consumption of these foods is recommended during or after the most important meals to reduce the blood cholesterol levels. Thus, phytosterols from phytosterol-enriched foods lower the absorption of dietary cholesterol, reducing the possibilities of cardiac diseases (Administración Nacional de Medicamentos, Alimentos y Tecnología Médica, n.d.).

Alimentos Argentinos (2013) explains the benefits of consuming phytosterols in a diet containing cholesterol as follows: To be absorbed by the organism, cholesterol must be part of micelles along with other lipids. Then, cells in the intestine absorb the micelles. Since phytosterols are similar to cholesterol, they displace the cholesterol in the micelle; thus, cholesterol cannot enter the micelle and remains free in the intestinal lumen.

When micelle containing phytosterols reaches the intestine cells, it is poorly absorbed, and the remaining phytosterols are also excreted through feces. Another additional benefit of phytosterols is that the small fraction of cholesterol that entered the micelle or was present endogenously in the intestinal cell cannot enter the bloodstream because they stimulate the efflux back to the intestine lumen.

The cholesterol present in the intestine lumen, either exogenous or endogenous, is excreted through feces because it is not part of the micelle (Alimentos Argentinos, 2013).

Studies demonstrated that in mice fed a phytosterol-rich diet, prostate tumor was reduced by more than 40 % (The Peanut Institute, 2020). In addition, Awad et al. (2000), who confirmed and extended these studies, found that beta-sitosterol has an inhibitory effect on tumor growth and stimulates cell apoptosis in colon, prostate and breast cancer.

Phytosterols consumed by healthy people help to improve the functioning of the organism, but they are not indicated in the treatment of diseases.

The study of the quality of lipids, specifically phytosterols, involves complex methodologies. González et al. (2018) determined phytosterols in seed oils using gas chromatography (GC) coupled to a double mass spectrometer (MS/MS), Awad et al. (2000) used GC with flame detector and Zhou et al. (2019) used GC-MS. This last methodology was chosen because it was simpler, lower cost, faster and because the supplies and equipments were available.

The general objective of this work was to implement a method to identify and quantify phytosterols in peanuts by gas chromatography. The specific aims were to determine the repeatability of the method in order to evaluate the minimum variability of the analytical process; to establish the reproducibility of the method to assess the maximum variability of the analytical process; and to measure the bias to estimate the trueness of the method.

MATERIALS AND METHODS

The study was conducted at the Laboratorio de Calidad de Alimentos, Suelos y Agua (LCASyA), Estación Experimental INTA Pergamino, Argentina, using a representative sample of peanut kernels, Runner variety, harvested at physiological maturity in the province of Córdoba.

Phytosterols were identified and quantified using the technique of Zhou et al. (2019), published at the US National Library of Medicine. Whole peanut kernels were ground and the lipid fraction was extracted with n-hexane. After extraction, the phytosterols were derivatized using N, O-bis(trimethylsilyl)trifluoroacetamide and pyridine as catalyst. Finally, 1 µl of the derivatized sample was injected.

Instrumental analysis

The equipment used was Perkin Elmer (PE) Clarus 500 gas chromatograph (GC) coupled with PE SQ8 mass spectrometric detector (MS). The capillary column used was PE Elite 5 (30 m × 0.25 mm ID × 0.25 µm df). The carrier gas used was helium at a constant flow of 1 ml/min. The GC oven temperature was initially 180 °C, held for three minutes; then increased to 275 °C at 8 °C/min and held at 275 °C for 16.40 min. The data were processed with the software TurboMass (version 6.1.0.1963). Phytosterols were identified by comparing the specific mass spectra with those of the National Institute of Standards and Technology Software (NIST, 2023), which was installed in the equipment.

To quantify the studied phytosterols, a calibration curve was performed with external standards of 5alpha-Cholestan-3beta-ol, stigmasterol and Beta-sitosterol, with a coefficient of determination (R^2) higher than 0.98.

Verification of the method

The quality of the results was verified by evaluating the two components of the method accuracy: precision and trueness. Precision was evaluated using repeatability and reproducibility.

To evaluate method repeatability, ten determinations of a single sample (study material) were performed on the same day by the same analyst, with the same equipment and under the same environmental and operational conditions. Repeatability was calculated using the coefficient of variation.

To evaluate reproducibility of the method, ten determinations of the same sample (study material) were performed on different days, by different analysts and using different reagents. The same equipment was used, under identical environmental and operational conditions. Reproducibility was calculated using the coefficient of variation.

According to Instituto de Salud Pública de Chile (2010), the criterion of acceptability of precision (repeatability and reproducibility) can be carried out through the Horwitz coefficient of variation (CVh). This value is compared with the experimentally obtained coefficient of variation value (CV).

The formula for repeatability

$CVh = 2(^{1-0.5 \log c})/2$

Where:

CVh: Horwitz coefficient of variation (percentage)

c: concentration of the analyte expressed in power of 10

The formula for reproducibility

$CVh = 2*(2(^{1-0.5 \log c}))/3$

Where:

CVh: Horwitz coefficient of variation (percentage)

c: concentration of the analyte expressed in power of 10 in both tests the CV must be <CVh.

Trueness is the agreement between a mean value of the determinations and the theoretical value; it was evaluated using the bias method (Instituto de Salud Pública de Chile, 2010).

For the bias method, a calibration curve was performed with external standards and the R^2 was verified, which was not lower than 0.98. Then the mean point of the curve was calculated six times and the bias was calculated using the formula: S = (X - Xa), where: S: bias; X: mean value of the readings; Xa: assigned value, certified value of the reference material or expected value.

Statistical analysis

To assess the behavior of the method with respect to the bias obtained and the quantification method, the student's t test was used to compare measure pairs (Instituto de Salud Pública de Chile, 2010).

To calculate the student's t test, the calculated t value and the critical t value were used.

The critical t value was obtained from tables and the calculated t value, using the following formula:

$t\ calc = (xa-x)/(S^* \sqrt{n})$

Where: t calc: observed or calculated t; X: mean value of the readings obtained from the experiment; Xa: assigned value, certified value of the reference material or expected value; S: standard deviation of the readings; n: number of readings.

RESULTS AND DISCUSSION

Repeatability test and reproducibility test

Table 1 shows the results of the repeatability test: the coefficient of variation (CV) was 0.26 for stigmasterol and 0.06 for beta-sitosterol; these values correspond to the same variability among determinations. Table 2 shows the results of the reproducibility test: the coefficient of variation (CV) was 0.30 for stigmasterol and 0.06 for beta-sitosterol; these values correspond to the same variability of the analytical procedure. Using the Horwitz coefficient of variation (CVh) formula, the

following results were obtained:

Repeatability:

Stigmasterol: CV (0.30) is lower than CV*h* (5.88).

Beta-Sitosterol: CV (0.06) is lower than CV*h* (5.48).

Reproducibility:

Stigmasterol: CV (0.30) is lower than CV*h* (7.85).

Beta-Sitosterol: CV (0.06) is lower than CV*h* (5.97).

The CVs of the repeatability and reproducibility tests for both compounds are acceptable according to the Horwitz coefficient of variation method.

Table 1. Trials involved in the repeatability analysis

Sample	Stigmasterol	Beta-sitosterol
Trial 1	2.46	21.55
Trial 2	1.57	20.34
Trial 3	1.21	23.22
Trial 4	1.60	22.40
Trial 5	1.64	22.21
Trial 6	1.10	19.90
Trial 7	1.12	19.82
Trial 8	1.90	20.21
Trial 9	2.01	21.29
Trial 10	1.95	22.50
Mean	**1.66**	**21.34**
Standard deviation	**0.44**	**1.22**
CV	**0.26**	**0.06**

Table 2. Trials involved in the reproducibility test

Sample	Stigmasterol	Beta-sitosterol
Trial 1	1.67	22.50
Trial 2	1.65	22.18
Trial 3	1.07	19.95
Trial 4	1.16	19.88
Trial 5	1.66	22.56
Trial 6	1.97	22.35
Trial 7	2.46	21.55
Trial 8	1.57	20.34
Trial 9	1.21	23.22
Trial 10	2.57	20.62
Mean	**1.70**	**21.52**
Standard deviation	**0.51**	**1.22**
CV	**0.30**	**0.06**

Trueness test

Table 3 shows the results of the bias method obtained for six trials. Finally, a student's t test was performed to evaluate the statistical differences of the method.

The student's t test was calculated for stigmasterol:

t calc = (xa-x) / (S* √n)

Where: t calc: observed or calculated t; X = 4.29; Xa = 4 (value obtained from the midpoint of the calibration curve for stigmasterol according to Awad et al. [2000]); S: standard deviation of the readings; n: number of readings.

t calc = (4-4.29) / (0.36* √6) = 0.327

t critical = 2.571 (Instituto de Salud Pública de Chile, 2010)

To explore possible differences between the value of the analytic experiment and the reference value, the calculated t value (0.327) was determined, which was lower than the critical t value (2.571). This result indicates the lack of significant differences between the obtained values for 95 % confidence with 5 degrees of freedom.

The student's t test was calculated for beta-sitosterol:

t calc = (xa-x) / (S* √n)

Where: t calc: observed or calculated t; X = 39.31; Xa = 40 (value obtained from the midpoint of the calibration curve for beta-sitosterol according to Awad et al. [2000]); S: standard deviation of the readings; n: number of readings.

t calc = (40-39.31) / (2.59* √6) = 0.109

t critical = 2.571 (Instituto de Salud Pública de Chile, 2010)

To explore possible differences between the value of the analytic experiment and the reference value, the calculated t value was determined (0.109), which was lower than the critical t value (2.571). This result indicates the lack of significant differences between the values obtained for 95 % confidence with 5 degrees of freedom.

Based on the results, we conclude that the method used to identify and quantify these phytosterols in peanuts have an acceptable trueness.

Identification test

Figure 1 and 2 show compounds following the criteria established by NIST Library: the compound that had a retention or elution time of 23,30 min was identified as alfa-cholestanol; the compound that had a retention or elution time of 26,92 was identified as stigmasterol; the compound that had a retention or elution time of 28,71 was identified as beta-sitosterol.

Table 3. Trials involved in the bias method

Sample	Results (ppm)		Bias	
	Stigmasterol	Beta-Sitosterol	Stigmasterol	Beta-Sitosterol
Trial 1	4.30	38.70	0.27	1.35
Trial 2	4.40	38.90	0.37	1.13
Trial 3	4.60	40.60	0.65	0.56
Trial 4	3.83	37.47	0.17	2.53
Trial 5	3.92	36.49	0.08	3.51
Trial 6	4.68	43.79	0.68	3.79
Mean	4.29	39.31	0.29	0.69
Standard deviation	0.36	2.59	0.36	2.59
n	6	6		

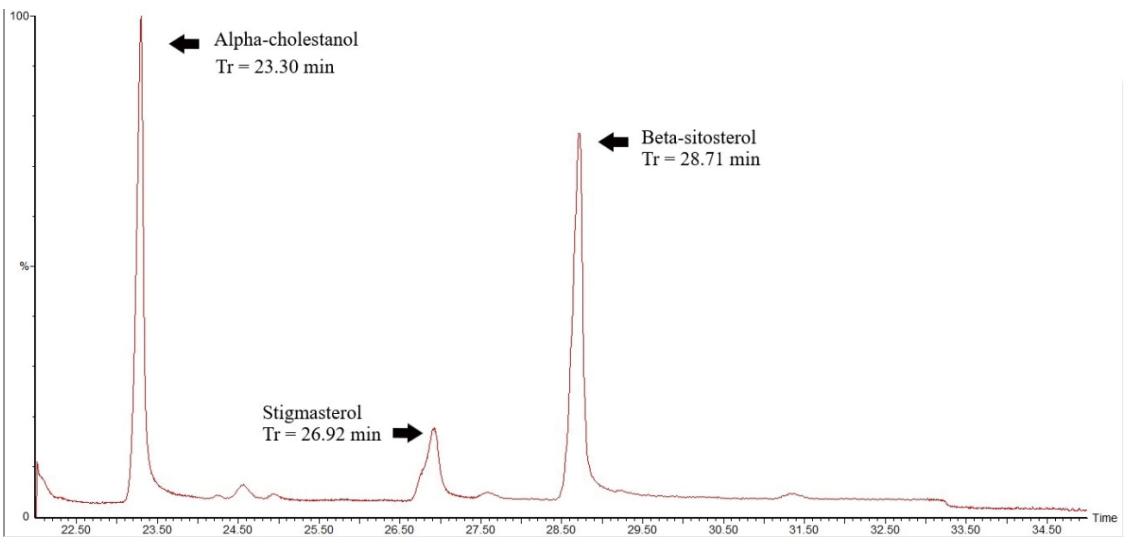

Figure 1. (Pattern) Identification of the compound alfa-cholestanol, stigmasterol and beta-sitosterol

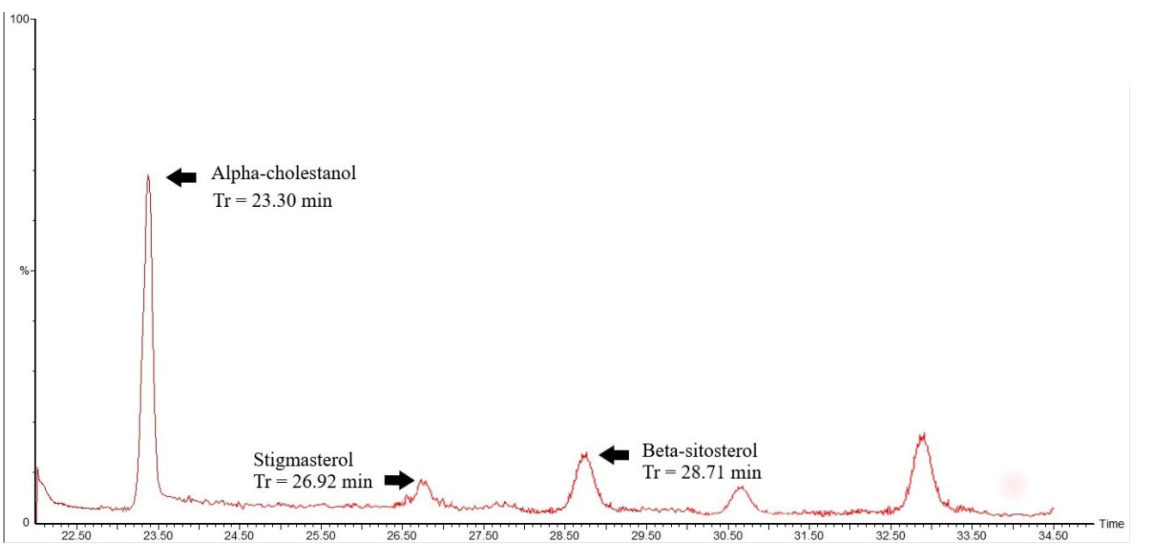

Figure 2. (Sample) Identification of the compound alfa-cholestanol, stigmasterol and beta-sitosterol

Quantification test

Phytosterol concentration was obtained from the dry weight of roasted peanut seeds, which presented 44.31 mg of beta-sitosterol and 3.39 mg of stigmasterol per 100 g of seed. The results reported by Awad et al. (2000) were 47.2 mg of beta-sitosterol and 7.7 mg of stigmasterol per 100 g of seed.

The student's t was calculated to verify possible significant differences between our values and values in the literature.

The student's t was calculated for the beta-sitosterol compound:

t calc = (47.2-44.31)/ (0.16* √9) = 1.017

t critical = 2.262 (Instituto de Salud Pública de Chile, 2010)

For beta-sitosterol, the calculated t value was determined (1.017), which was lower than the critical t value (2.262); this result indicates the lack of significant differences between the obtained values for 95 % confidence with 5 degrees of freedom.

The student's t was calculated for stigmasterol:

t calc = (7.7-3.39)/ (1.02* √9) = 2.177

t critical = 2.262 (Instituto de Salud Pública de Chile, 2010)

For stigmasterol, the t calculated (2.177) was lower than the critical t value (2.262), indicating the lack of significant differences between the obtained values for 95 % confidence with 5 degrees of freedom.

Therefore, the comparison between the values obtained with those reported by Awad et al. (2000) show no significant differences for beta-sitosterol and stigmasterol in peanut seeds. The slightly lower values obtained in this work can be attributed to the genetic variety of the seed used and the environment where it was cultivated.

CONCLUSION

The implementation of the CG-MS technique introduced a novel approach for the identification and quantification of beta-sitosterol and stigmasterol in peanuts for LCASyA Laboratory. The repeatability and reproducibility tests presented the expected CV values; therefore, the variation of the method between determinations is reliable. The veracity test showed that working with a confidence level of 95 % and 5 degrees of freedom there are no significant differences, therefore it is confirmed that the methodology is acceptable.

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