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Optimization of analytical procedures for GC–MS determination of phytosterols and phytostanols in enriched milk and yoghurt

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Abstract

The aim of the present study is to contribute to a correct determination of vegetal sterol and stanols found in enriched milks and yogurts, used as functional foods, using GC–MS. The optimization of the method, specially the saponification step, as well as the corresponding analytical validation, is the main goal of this study. Saponification temperatures and times that gave the best results were 80 °C/45 min for milk, and 60 °C/90 min for yoghurt samples. KOH concentration solutions of 2.0 and 2.5 M were selected as the best saponification reagents for milk and yoghurt, respectively. It was also verified that volumes of 1500 μl and 2500 μl KOH were enough to react with 250 μl of milk and 100 μl of yoghurt. Accuracy, repeatability and intermediate precision of the method were calculated at 89.2% and 91.5%, 6.0% and 4.45%, 11.8% and 9.4%, for milk and yoghurt, respectively, while the limit of detection was determinate at 0.1 μg/ml for both matrices.

Keywords: Phytosterols; Phytostanols; GC–MS; Milk; Yoghurt

1. Introduction

Cardiovascular diseases (CVD) are, since the beginning of the last century, the main cause of death in Europe. Several risk factors for the premature development of CVD have already been identified, namely the high plasmatic cholesterol level (Jong, Plat, & Mensink, 2003; Kiechl & Willeit, 1999; Teunissen et al., 2003; Trautwein et al., 2003). Therefore, diminishing plasmatic cholesterol levels are vital for the prevention of CVD. Additionally, eating style is an essential factor in plasmatic regulation of cholesterol levels. The natural constituent compounds of some food products have the ability to reduce total plasmatic cholesterol levels as well as low density lipoproteins (LDL) levels. In animals, cholesterol is the most abundant sterol. In plants, more than 40 sterols have been identified, of which β-sitosterol, stigmasterol and campesterol are the most abundant. These phytosterols and cholesterol are all 4-desmethyl sterols that share identical ring structures. The various sterols only differ in their side chains. Surprisingly, these minor differences result in major changes in their biological function. A less abundant class of related compounds found in plants are the phytostanols (or stanols). Phytostanols are completely saturated forms of phytosterols and lack the carbon–carbon double bonds found in cholesterol and phytosterols (Hicks & Moreau, 2001; Miettinem & Gyllimg, 2004; Nguyen, 1999; Piironen, Lindsay, Miettinen, Toivo, & Lampi, 2000; Pollak, 1953). However, today's modern way of life is not compatible with the preparation and ingestion of vegetables that contain these substances. So, the development of food technology has created products that are enriched with phytosterols and phytostanols. In the beginning, because of their lipophylic properties, the referred compounds were added to fat foods, such as oils and margarines (Katan et al., 2003;

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Law, 2000; Weststrate & Meijer, 1998). Nonetheless, as the quantity of dietary fat ingested should not supply more than 30% of the total energy intake, those kinds of products did not prove to be the best option for human health (Noakes, Clifton, Doornbos, & Trautwein, 2005). Therefore, over the last few years several studies have been performed in order to determine if plant sterols and stanols, when added to low fat products eaten daily such as milk and yoghurt could prove themselves sufficient (Clifton et al., 2004; Nguyen, 1999; Noakes et al., 2005). Most studies comparing the activity of vegetal sterols and stanols conclude that their ability to decrease cholesterol is equal, although some consider that stanols are more efficient. This better efficiency is related to the fact that stanols, which have practically no absorption, remain for longer period in the intestinal lumen where they interfere continually and in a more efficient way with the absorption of cholesterol (Hicks & Moreau, 2001; Nguyen, 1999). However, the qualitative and quantitative determination of phytosterols and phytostanols, although being well documented in diet samples (Phillips, Tarragó-Trani, & Stewart, 1999) or in numerous food matrices like vegetables and vegetable oils, fruits, cereals, bread, margarines and other enriched foods (Grandgirard, Martine, Joffre, Juaneda, & Berdeaux, 2004; Johnsson & Dutta, 2006; Piironen, Toivo, & Lampi, 2002; Piironen, Toivo, Puupponen-Pimiä, & Lampi, 2003; Sivakumar, Briccoli Bati, Perri, & Uccella, 2006; Weihrauch & Gardner, 1978), is not known in milk and yoghurts.

Therefore, the aim of this study was to optimize an analytical methodology based on GC–MS. Mass detection was elected due to its properties, namely specificity, in order to identify the different compounds based on their derivatized mass spectra. This approach also provides better sensitivity when compared to other GC detectors, such as FID for instance. The present study also aims to contribute to the optimization of the saponification step, namely the concentration and volume of the saponification reagent, as well as times and temperatures of saponification. We seek to obtain a rapid, reliable, and effective method to evaluate phytosterols and phytostanols in enriched milk and yoghurts that are used as functional foods.

2. Materials and methods

2.1. Standards and reagents

Cholesterol, dihydrocholesterol, desmosterol, campesterol, lathosterol, stigmasterol, β-sitosterol, dihydrobrassicasterol, stigmastanol and cholestane, which was used as an internal standard, were all acquired from Sigma (Madrid, Spain). Reagents used in the saponification, extraction and derivatization steps were potassium hydroxide, absolute ethanol, *n*-hexane (Merck, Darmstad, Germany), water for chromatography, obtained by a Milli-Q system from Millipore (Bedford, MA, USA), and N-O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS) (Supelco, Sintra, Portugal).

2.2. Gases

Nitrogen and helium gases were supplied by Sogafer (Coimbra, Portugal).

2.3. Materials

For other steps of the analytical methodology, a Bandelin Sonorex RK100 ultrasound (Bandelin, Berlin, Germany), vortex, heating block with nitrogen evaporation system (Reagente 5, Porto, Portugal), a Hewlett–Packard (HP) chromatographic system (Soquimica, Lisbon, Portugal) controlled by an HP Vectra VL2 4/50 computer, an automatic HP6890 injector and a GC HP5890 Série II equipped with an HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), connected to an HP5972 mass detector, were used.

2.4. Standards preparation

Stock solutions of cholestane, cholesterol, dihydrocholesterol, desmosterol, campesterol, lathosterol, stigmasterol, dihydrobrassicasterol, β-sitosterol and stigmastanol were prepared in ethanol, with a concentration of 2 mg/ml. Work solutions were made at concentrations of 80 μg/ml, also in ethanol, by diluting stock solutions. The internal standard work solution, cholestane, was prepared with a concentration of 50 μg/ml. The temperature of all the solutions was maintained between 2 and 8 °C in a refrigerator.

2.5. Samples

The matrices used for the analytical methodology optimization were Bécel Pro-Active® milk and strawberry yoghurt samples. For validation of the methodology parameters, phytosterol- and phytostanol-free samples with a fat content similar to the reference milk and yoghurt samples were used.

2.6. Sample preparation

Original packages were conveniently homogenized and then 5 mL from each sample, milk or yoghurt, were put into centrifuge tubes. 250 μl, in the case of milk, and 100 μl for yoghurts, were used as the sample aliquots. 50 μl of internal standard and KOH solution in 90% ethanol were added and the saponification procedure occurred at different temperatures and times. After saponification, the unsaponifiable fraction was extracted with 1 mL of *n*-hexane. Then, 250 μl of *n*-hexane solution was transferred to a derivatization vial and was evaporated to dryness through a nitrogen stream at 60 °C. The dry residue was derivatized with 50 μl of BSTFA:TMCS (99:1), at 60 °C for 30 min (Kinter, Herold, Hundley, Wills, & Savory, 1988; Kuksis, 2001; Yamaga et al., 2002).

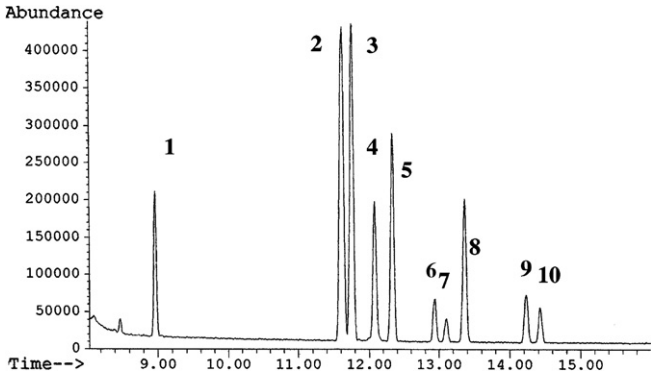

Fig. 1. Chromatogram of standard mixture. (Peak identification: 1 – cholestane, 2 – cholesterol, 3 – dihydrocholesterol, 4 – desmosterol, 5 – lathosterol, 6 – campesterol, 7 – dihydrobrassicasterol, 8 – stigmasterol, 9 – β-sitosterol, 10 – stigmastanol.)

2.7. Chromatography

Phytosterol and phytostanol determination was made via injection in splitless mode, in 1 min, with 2.0 μl of the derivatized sample at an injection temperature of 250 °C. Helium was used as the mobile phase at a pressure of 10 psi at the head of the column. The column initial temperature, 200 °C, was kept for 1 min after which it was increased 20 °C/min until it achieved a temperature of 300 °C, which was maintained for 10 min (Kinter et al., 1988). The detector temperature was set at 280 °C and the data was obtained in scan mode by electron impact ionization. Results were evaluated comparing the ratios obtained between the campesterol and the internal standard peak areas of each chromatogram. The observation of Fig. 1 demonstrates that the developed methodology can accurately determine phytosterols and phytostanols in milks and yoghurts through the chromatographic evaluation of the trimethylsilyl derivatives obtained using optimal experimental conditions. The fact that the obtained peaks were individualized also reveals the good selectivity of the method.

3. Results and discussion

Saponification, as it is known, is the fundamental step in this type of analysis because it allows the transformation of liposoluble triacylglycerols into water soluble compounds, facilitating the posterior extraction of phytosterols and phytostanols by an apolar solvent that can then be determined as free compounds (Toivo, Phillips, Lampi, & Piironen, 2001). Thus, an evaluation of concentration and volume of the saponification reagent, as well as saponification temperature and times for milk and yoghurt was performed in order to optimize the methodology.

3.1. Saponification reagent concentration

The evaluation of the saponification reagent concentration was made with 5 milk and 5 yoghurt samples. To each sample, an ethanolic KOH solution was added in concentrations of 0.5, 1.0, 1.5, 2.0 and 2.5 M with the proportion of 1:1 (matrix:saponification reagent). The saponification temperature and times were kept at 60 °C/60 min. Fig. 2 shows data obtained with referred parameters.

The best results, in the case of milk, were obtained when the 2.0 M KOH solution was used. For yoghurt, the data obtained show that campesterol/cholestane ratios raise with increasing KOH concentration. A concentration of 2.5 M KOH satisfied necessary requirements to be chosen for yoghurt saponification.

3.2. Saponification reagent volume

To study the quantity of the saponification reagent required, 5 samples each of the matrices were used and 500, 1000, 1500, 2000 and 2500 μl of KOH were added to the 2.5 and 2.0 M concentrations for milk and yoghurt, respectively. Saponification temperature and times were maintained at 60 °C/60 min.

Obtained results are presented in Fig. 3. For milk, the best values occurred when 2500 μl of KOH were added. However, no phase separation occurred in the centrifuge tubes where this volume was added, when 1 ml of *n*-hexane

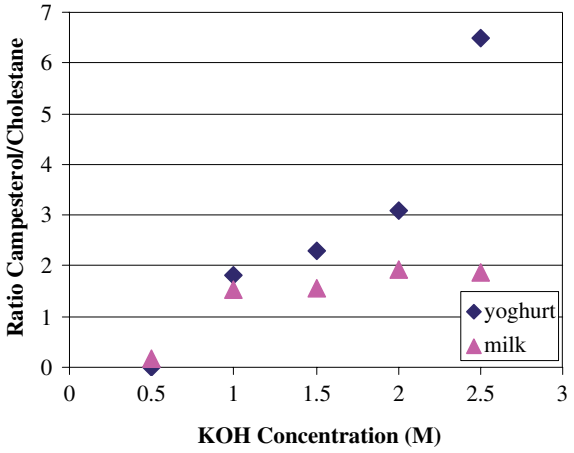

Fig. 2. Peak area ratio between campesterol and cholestane at different KOH concentration levels.

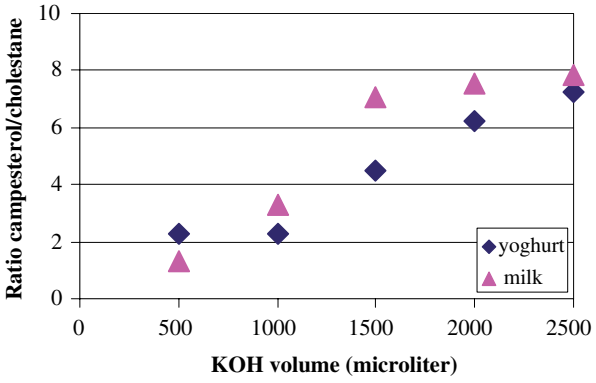

Fig. 3. Peak area ratio between campesterol and cholestane at different KOH volumes.

was joined for phytosterols and phytostanols extraction. A supplementary addition of 5 ml of water was necessary to obtain correct phase separation. Water addition allowed the dissolution of the saponificated compounds that are water soluble, facilitating the extraction of the unsaponifiable fraction by *n*-hexane. Similar results were obtained with the extraction of the 2000 μl KOH solution.

The results obtained with 1500 μl of saponification reagent were similar to those obtained with 2500 μl. As it was not necessary to add water, which facilitates the procedure and diminishes the errors, a volume of 1500 μl was chosen.

For yoghurt, 2500 μl of saponification reagent was used because it was the maximum volume that could be contained in the centrifuge tubes during saponification with the heating process used.

3.3. Saponification temperature

Study of the saponification temperature was carried out with five different temperatures: 20, 40, 60, 80 and 100 °C. A time period of 60 min and previously optimized KOH concentrations and volumes for milk and yoghurt, respectively, were also used.

From observation of Fig. 4, the saponification temperatures that yielded the best results were 80 °C and 60 °C, respectively, for milk and for yoghurt.

3.4. Saponification time

After temperature optimization, an evaluation of the best saponification time period was carried out. Time periods of 45, 60, 75, 90 and 105 min were used with temperatures of 80 °C for milk and 60 °C for yoghurt. KOH volumes and concentrations of 2500 μl/2.0 M and 1500 μl/2.5 M, respectively, were used for milk and yoghurt.

The obtained results can be observed in Fig. 5. It was verified that, for milk, the time exposure that gave the best results was 45 min, while for the yoghurt, the best saponification time period was 90 min.

3.5. Method validation

The validation procedure was realized through the determination of linearity, accuracy, repeatability, intermediary precision and detection limit. For the calculation of these parameters, phytosterol- and phytostanol-free samples were used. These samples had a fat content similar to the milk and yoghurt samples used as the functional foods. Three different volumes of the standard mixture

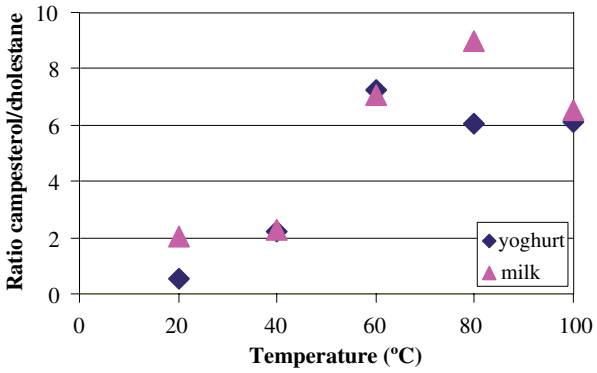

Fig. 4. Peak area ratio between campesterol and cholestane at different saponification temperatures.

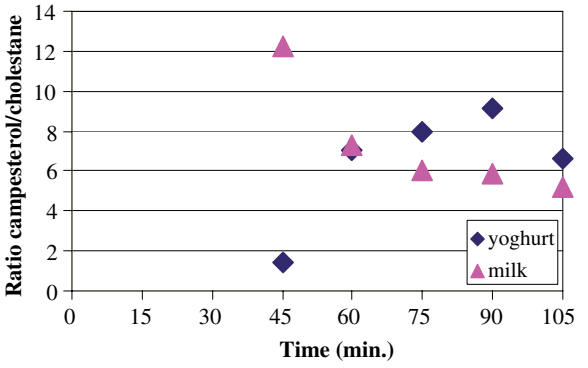

Fig. 5. Peak area ratio between campesterol and cholestane at different saponification times.

were added at 80 μg/ml (500, 750, and 1000 μl). This step was then repeated 5 times.

The presented data are relative to campesterol whose quantification was made with the help of a calibration curve obtained through the ratios between the peak areas of campesterol and the respective peak areas of the internal standard, cholestane. Table 1 shows the obtained data that match with the reference values accepted for this kind of determination and methodology (Lino and Silveira, 2001; AOAC, FAO, IAEA & IUPAC, 1999).

4. Conclusion

Development, optimization and validation of an analytical methodology for the determination of plant sterols and stanols (namely stigmastanol elected by dairy industries) used as a functional food in milk and yoghurts, were the main objective of this study. Therefore it was verified that the best results for saponification temperature and times

Table 1
Validation data

	Recovery (%)	Intra-assay CV (%)	Inter-assay CV (%)	Detection limit (μg/ml)	Linearity
Milk ($n = 5$)	89.2	6.0	11.8	0.1	0.992
Yoghurt ($n = 5$)	91.5	4.4	9.4	0.1	0.989

were 80 °C/45 min in the case of milk and 60 °C/90 min in the case of yoghurt. Ethanolic solutions of KOH with concentrations of 2.0 and 2.5 M were selected as the best saponification reagents for milk and yoghurt, respectively. It was also possible to verify that volumes of 1500 μl and 2500 μl were sufficient to saponify 250 μl of milk and 100 μl of yogurt. These results indicate that the developed methodology for evaluating phytosterol and phytostanol levels in milks and yoghurts used as functional foods is adequate, simple, fast, selective and sensitive enough to quantify the studied compounds either in the free or esterified forms. On the other hand, this method has proven to have even more advantages permitting its application in the determination of other plant sterols and stanols in the studied matrices, which allows it to be used regularly in food quality control laboratories.

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