Uniwersytet Gdański

CHEMIA UG

Department of Environmental Analysis

Instruction for laboratory course

Food safety and food control

Exercise 2

Phytostanols in functional foods: identification and quantification

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Phytostanols in functional foods: identification and quantification

1. Introduction

1.1 Phytostanols in food

Phytostanols (Fig. 1) are plant-derived compounds that are saturated analogs of more common phytosterols. They are present in small amounts in many plant-based products, including nuts, vegetable oils and vegetables [1]. Their presence in a diet lowers the low-density lipoprotein (LDL; so-called bad cholesterol) in human blood by reducing cholesterol intestinal absorption [2,3]. This phenomenon is the main reason for producing functional foods rich in lipids (margarine, yoghurt, mayonnaise, etc.) that contain elevated amounts of phytostanols. Increased intake of phytosterols and phytostanols helps to decrease the risk of cardiovascular diseases, that are among the major causes of death in Western population. Therefore, their consumption may be a simple, effective way to prevent high cholesterol levels in blood plasma. While the typical diet provides less than 0.5 g of phytosterols and phytostanols per day, the effective dose is around 2-3 g per day. This is the main reason for the growing consumption of products that are fortified with phytosterols and phytostanols. The production of phytostanols may include their separation from some phytostanol-rich vegetable oils, but mostly is based on hydrogenation of respective phytosterols, which occur much more commonly. The aim of the study is to verify the amounts of phytostanols present in the fortified margarine.

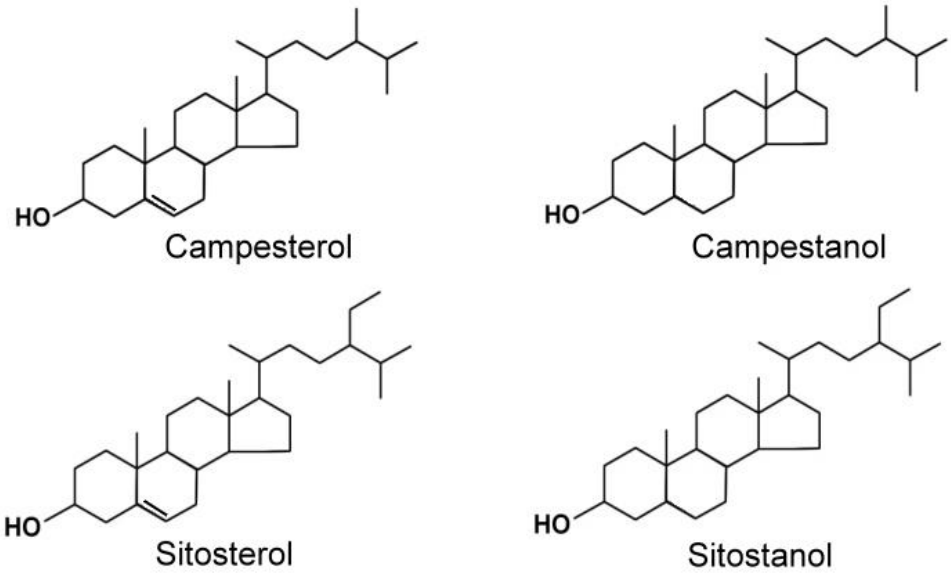

Figure. 1. Chemical structure of common phytosterols and phytostanols.

1.2 Identification and quantification of phytostanols

In most cases, phytostanols are present in foods in form of their esters with long-chain fatty acids. They need to be extracted from the original matrix, but it is impossible to isolate them from other lipids on this stage. Next step involves alkaline hydrolysis (saponification) to break down ester bonds and to obtain free phytostanols. On this stage, it becomes possible to at least partially remove free fatty acids, which are usually the main fraction components, from the compounds of interest. Finally, derivatization is usually applied, in most cases by synthesizing trimethylsilyl derivatives (TMSi), which are more volatile than parent compounds. The most convenient analytical technique for differentiation between very similar phytosterols and phytostanols is gas chromatography with flame ionization detector (GC-FID) or with mass spectrometry (GC-MS) [4,5]. The latter also allows to directly identify phytostanols based on mass spectra obtained. A typical gas chromatogram obtained for low-level phytosterol and high-level phytostanol mixture is given in Fig. 2. Standard non-polar stationary phases (e.g. 95%

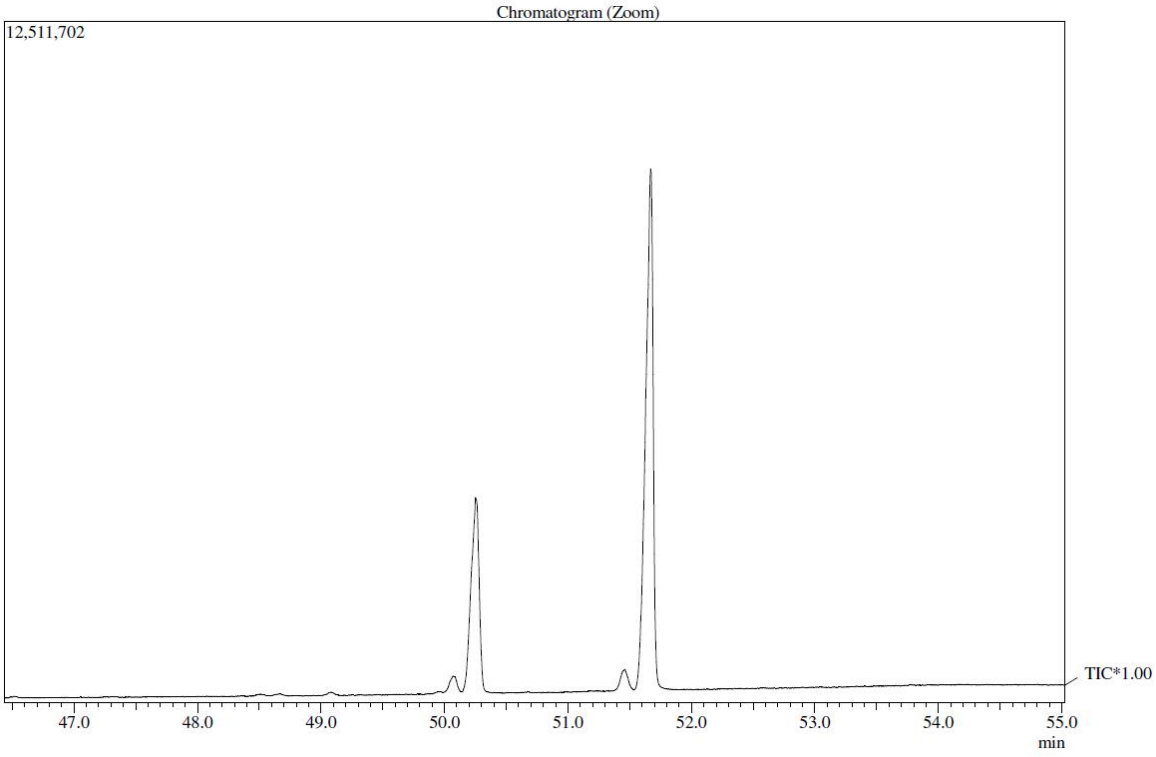

Figure. 2. Gas chromatogram from the analysis of a mixture of phytosterols and phytostanols in form of respective TMSi derivatives. Large peaks are attributed to phytostanols, the small ones – to phytosterols. The elution order is caused by the difference in polarity between unsaturated phytosterols (more polar) and saturated phytostanols (less polar).

2. Experimental part

2.1. Extraction and hydrolysis of phytostanol esters from the margarine

No special extraction procedure is needed. The specified amount of margarine (ca. 10-15 mg) is placed in a 4 ml screw-capped chromatographic vial. Next, the solution of internal standard (ester of cholesterol and octadecanoic acid) is added in the amount similar to the expected content of phytostanols in the sample. Then, 1 ml of a 0.5 M KOH solution in methanol is added and the mixture is placed in heating block at 90 ºC for 1 hour. The mixture is then cooled down, and 1 ml of distilled water is added. Free phytostanols are then extracted as specified in paragraph 2.2.

2.2. Extraction of free phytostanols

Hydrolysate is transferred to a separatory funnel, dissolved in distilled water (5 ml) and 5 ml of a 0.5 M KOH solution in methanol is added. Next, 10 ml of a mixture of diethyl ether – hexane (1:1) is added. The mixture is shaken for 1 min, and the funnel is left for several minutes to obtain the phase separation. If phase separation is not obtained, adding 1 g of NaCl can resolve the problem. Upper phase is then collected to a flat-bottom flask, and the extraction procedure is repeated by using another portion of the diethyl ether – hexane (1:1) mixture. Organic phases are pooled and dried using a portion of an anhydrous Na_2SO_4. Finally, mixed organic phases are filtered through another portion of anhydrous Na_2SO_4 to a round-bottom flask. The extract is then evaporated to dryness under reduced pressure, dissolved in 1 ml of hexane and transferred to a 2 ml screw-capped vial. The solvent is evaporated under the stream of nitrogen.

2.3. Derivatization

The components of the extracts obtained and the standards of phytostanols should be transformed to respective volatile, trimethylsilyl derivatives using BSTFA/TMCS silylating mixture (99:1) (Figure 3). For this purpose, add silylating mixture (100 µl) to each sample and heat them for 30 minutes at 90 °C.

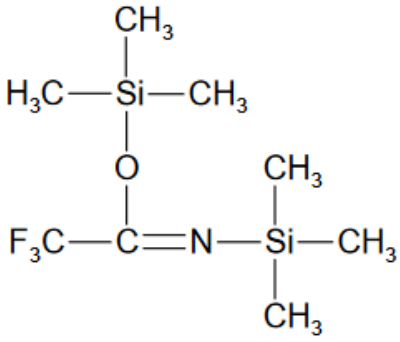

Figure 3. Chemical structure of *N,O-bis(trimethylsilyl)trifluoroacetamide* (BSTFA)

2.4. Detection and quantitative analysis of phytostanols using GC-FID and GC-MS techniques

Perform GC analysis of each sample using conditions presented below:

Gas chromatograph with FID detector (Shimadzu GC-2010)

- Zebron ZB-5 30 m capillary column, 0.25 mm internal diameter, 0.25 µm stationary phase film thickness,
- Temperature programme: 180 - 320 °C, ramp 4°C min^{-1}, 5 min at 320 °C
- Injector temperature: 320 °C,
- Detector temperature: 320 °C,
- Split ratio: 1:20,
- Mobile phase: argon, flow: 1.2 ml min^{-1},
- Gas pressure: air: 350 kPa, hydrogen: 35 kPa

Perform quantitative analysis basing on peak areas of phytostanols and of the internal standard (cholesterol liberated from the respective ester). Re-calculate the results, considering that phytostanols are present in the product in form of respective esters (we assume that with 18:0 fatty acid). Give the final result in mg per 100 g of the product.

Additional GC-MS will be performed to fully identify phytostanols and internal standard according to the instructor's guidance.

3. Report

- Present experimental part in the form of the scheme, considering the conditions of the analyses performed;
- Identify phytostanols in extracts basing on retention times from GC analysis with comparison to retention times of standards;
- Confirm the identification basing on the results from GC-MS analyses
- Compare the results with producer's claim about the phytostanol content in the product.

4. Topics to study

- The basic information about phytostanols;
- Lipids and their hydrolysis;
- Gas chromatography and its working principles;
- Retention parameters (definitions);
- Derivatization; trimethylsilyl derivatives

5. Literature

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