Analytical Methods

Rapid screening of phytosterols in orange juice by solid-phase microextraction on polyacrylate fibre derivatisation and gas chromatographic–mass spectrometric analysis

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1. Introduction

Sterols are important constituents of cells. A high cholesterol level in serum is a risk for coronary heart disease. Several studies showed that the regular intake of food enriched in phytosterols could lead to a hypocholesterolaemic effect (Heinemann & Axtmann, 1999; Piironen & Lindsay, 2000). Consequently lower serum cholesterol level decreases the risk of coronary disease (Sprecher & Watkins, 2003). Principal sources of phytosterols are vegetable oils and cereal products. However phytosterols are present in all vegetative cells in low quantity.

Mass spectrometry combined with gas chromatography has provided to be efficient and is commonly used to separation and identification of sterols (Wyllie & Amos, 1977), generally after derivatisation by acetylation (Pelillo & Galletti, 2000) or silylation of hydroxyl group for a better gas chromatography separation (Dutta & Normen, 1998; Gerst & Ruan, 1997; Nguyen & Bruchet, 1995). The critical step in sterol analysis is the extraction of biological samples (Johnsson & Dutta, 2006; Thanh & Vergnes, 2006). Generally, analytes are recovered from biological fluids or solids by solvent extraction using liquid–liquid or solid–liquid extraction techniques such as Soxhlet extraction, maceration, percolation and ultrasonication. These techniques are often time-consuming, require relatively large amounts of organic solvents and involve reconstitution steps (Ng & Hupe, 1998).

Solid-phase microextraction is a sample preparation technique based on the partitioning of analytes between a stationary phase coated fused silicate fibre and the sample (Theodoridis & Castellari, 2004; Sanchez-Palomo & Diaz-Maroto, 2005) and fruit aroma (Mahattanatawee & Rouseff, 2005; Ngassoum & Jirovetz, 2001). Direct immersion is used for non-volatile compounds (Cavalli & Fernandez, 2004; Riu-Aumatell & Castellari, 2004; Sanchez-Palomo & Diaz-Maroto, 2005) and fruit aroma (Mahattanatawee & Rouseff, 2005; Ngassoum & Jirovetz, 2001). Direct immersion is used for non-volatile compound analysis. During the last decade, SPME techniques have been applied to the simultaneous extraction and silylation (Stashenko & Martinez, 2004) of steroids (Okeyo & Snow, 1998; Yang & Luan, 2006) or fatty acids (Cha & Liu, 2006) in biological samples. Recently, a procedure using SPME extraction and derivatisation for determination of sterols in serum samples has been reported by Domeno & Ruiz (2005). In this work authors studied two ways of sterol extraction. Total immersion of the SPME fibre into the analyte solution followed by derivatisation with N,O-bis(trimethylsilyl)trifluoro-acetamide in headspace mode lead to low recoveries of the analytes, but simultaneous extraction and trimethylsilylation was possible on direct immersion of fibre previously coated with BSTFA.

Abbreviations: BSTFA, bis(trimethylsilyl)trifluoro-acetamide; GC/MS, chromatographic–mass spectrometric; TMS, trimethylsilyle; SPME, solid-phase microextraction; PA, polyacrylate; HS, head space; DVB/CAR/PDMS, divinylbenzene/carboxen/polydimethylsiloxane; TIC, total ion chromatograms.

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SPME fibre selection was an important element. Although different coatings may be used for extraction of non-volatile or semi-volatile compounds (Theodoridis & Koster, 2000), polyacrylate coated SPME fibre was shown to be best adapted for the extraction of sterols from serum (Domeno & Ruiz, 2005). Contrarily to DVB/CAR/PDMS-coated fibre, PA-SPME was not degraded by BSTFA reagent.

The application of SPME for analysis of phytosterols contained in fruit juices should also be possible if the phytosterols adsorption on fibre is faster than other non-volatile compounds much more abundant than sterols, like sugars, organic acids, flavourings, etc. In this paper, we report a fast and optimised SPME procedure for the screening of phytosterols contained in orange juice. At the first phase of the work, we evaluated the influences of different experimental factors on the recovery of sterols by the SPME fibre and the efficiency of BSTFA derivatisation, using standard sample solutions containing four sterols and one stanol: cholesterol, sitosterol, \( \beta \)-sitosterol and \( 5\alpha \)-cholest-7-en-3\( \beta \)-ol and sitostanol. The second part of the work was to assess the optimum conditions for screening of the phytosterols in a commercial orange juice.

2. Experimental

2.1. Chemicals and materials

Cholesterol (99%), stigmastanol (95%), \( \beta \)-sitosterol (90%), stigmasterol (95%) and \( 5\alpha \)-cholest-7-en-3\( \beta \)-ol were purchased from Sigma-Aldrich (Buchs, Switzerland). Pyridine, BSTFA (10 mL \( \times \) 1 mL for GC) and acetone (>99.5 for GC) were purchased from Fluka (Buchs, Switzerland), potassium chloride was from Acros Organics (Geel, Belgium).

2.2. Sample preparation

Standard sterol solutions at different concentrations were prepared by dissolving an appropriate amount of each sterol in acetone. Standard solutions were obtained by addition of 50 \( \mu \)L of sterol solution to 8 mL of KCl 1 M containing 8 \( \mu \)L of pyridine to catalyse derivatisation.

Orange juice sample was obtained by addition to 8 mL of commercial orange juice (Jafaden, France) 0.6 g KCl and 8 \( \mu \)L pyridine.

2.3. Direct SPME and headspace derivatisation procedures

Standard solutions are placed in a 10 mL vial, sealed with a Teflon lined silicone septum. The solution was stirred by a cylindrical magnetic rotator (5 mm \( \times \) 2 mm) at 1200 t min \( ^{-1} \). SPME extraction and headspace derivatisation were performed by a manual holder with a PA-SPME fibre (85 \( \mu \)m, Supelco, Bellefonte, PA, USA). Each fibre was conditioned in the GC inlet for 2 h at 300 °C in split mode according to Supelco’s instructions.

Two different protocols were tested for extraction and derivatisation of sterols as shown in Fig. 1. In protocol A, a PA-SPME fibre was introduced in the solution containing sterols (standard solution or orange juice sample) at 65 °C for 10, 20, 30 or 60 min before
temperature programme was as follows: the initial temperature was set to 60 °C during 5 min, from 60 °C to 270 °C via a ramp of 20 °C min⁻¹, from 270 °C to 295 °C via a ramp of 1 °C min⁻¹ and hold at 295 °C for 5 min. The MS was operated in full scan mode (m/z 50–650) by electron ionisation. The MS transfer line temperature was maintained at 300 °C and ion source at 250 °C. MS processing, data acquisition and treatment were performed by Xcalibur software. NIST mass spectra database was used for MS spectra analysis.

2.5. Quantitative analysis

GC/MS calibration have been made by liquid injection (1 μL) of each sterol solution (concentration between 4 × 10⁻³ and 4 × 10⁻⁶ M). Peak area values obtained have been fitted by linear function with $R^2 > 0.99$.

3. Results and discussion

Conventional methods for analysing phytosterols contained in aqueous solutions involve liquid–liquid extraction of sample and subsequent derivatisation. Direct SPME extraction and derivatisation by BSTFA to rapid screening of sterols has been investigated. Two protocols (noted A and B) (Fig. 1) with different sterol extraction and derivatisation times have been used for sterol analysis: (i) protocol A: sterol extraction (10, 20, 30 or 60 min) before BSTFA derivatisation (30 or 60 min); (ii) protocol B: BSTFA absorption (30 or 60 min) before sterol extraction (10, 20, 30 or 60 min). Sterols recovered on the fibre were directly analysed by GC/MS.

Before application to fruit juice, direct SPME extraction and derivatisation have been studied by BSTFA with standard sterol solutions containing stigmastanol, β-sitosterol, stigmasterol, cholesterol and 5α-cholest-7-en-3β-ol (Fig. 2) at two different concentrations. Besides the first three sterols which are common phyto-sterols in fruits and vegetables, the standard mixture included the two $C_{27}$ sterols in order to observe if the double bond position ($\Delta^5$ and $\Delta^7$) have an influence in SPME adsorption kinetics. In addition stigmasteranol and stigmastanol permit to examine if the presence or absence of $\Delta^5$ double bond can modify the adsorption kinetics.

The evaluation of protocols performance was based on sterol recovery and derivatisation yield. The sterol recovery was obtained by calculation of peak areas on total ion chromatograms (TIC) of GC/MS acquisitions for each sterol, assuming all components have equal response factors. Derivatisation yields was calculated by division between the sum of peak area of sterol–TMS and the sum of total sterols (derivatized or none) recovery, assuming they have the same response factor.

3.1. Sterol recovery and derivatisation yield evolution with fibre exposure time

First, the recovery and derivatisation yield of sterols was studied for each protocol (A and B) using the standard solution containing

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Recoveries quantity of individual sterol standard with fibre immersion different time using protocol A and B. The concentration of sterols in aqueous solution are the same : 6.25 × 10⁻⁶ M.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol A</td>
<td></td>
</tr>
<tr>
<td>Sterol immersion time (min)</td>
<td>10</td>
</tr>
<tr>
<td>Cholesterol–TMS (mol)</td>
<td>4.08E−11</td>
</tr>
<tr>
<td>Stigmasterol–TMS (mol)</td>
<td>2.11E−11</td>
</tr>
<tr>
<td>Sitosterol–TMS (mol)</td>
<td>3.84E−11</td>
</tr>
<tr>
<td>Stigmastanol–TMS (mol)</td>
<td>7.36E−11</td>
</tr>
<tr>
<td>CholesterolD7–TMS (mol)</td>
<td>2.97E−11</td>
</tr>
</tbody>
</table>
equal concentrations ($2.5 \times 10^{-6}$ M) of the five sterol standards, in function of the fibre exposure time. The results reported in Fig. 3a show that the protocol A, compared to the protocol B, led to a poor recovery of sterols from the aqueous solution. Even for prolonged exposure times up to 60 min, the sterol recovery remained poor. The derivatisation yield was 100% with protocol A after exposition of the fibre to BSTFA for 30 min. With an aqueous solution containing standard sterols in higher concentration ($6.25 \times 10^{-5}$ M), protocol A leads to higher recoveries but it is then necessary to apply a fibre exposure to BSTFA for 60 min. to complete derivatisation (Fig. 3b).

For protocol B, BSTFA was first absorbed for 30 min on the fibre before immersion in the sterol solution for 10–60 min. In all cases the recovery of sterols obtained with protocol B was much higher than with protocol A, but the derivatisation was complete only for an immersion time of 10 min (Fig. 3a and b). For longer sterol exposure times the derivatisation yield decrease with sterol immersion time. Similar results were obtained when BSTFA absorption duration was increased to 60 min. These results can be explained by the competition of three types of reaction likely to occur during fibre exposition to the aqueous solution, i.e., BSTFA hydrolysis, sterol–TMS hydrolysis and trimethylsilylation of sterols. Actually, if a SPME PA fibre coated with BSTFA is immersed for 24 h in pure water before exposure to sterol solution, the derivatisation yield was 0%. This result confirms that sterol–TMS can be hydrolysed during fibre immersion in sterol solution. Although none of the two protocols allows a quantitative sterol adsorption on the fibre, the protocol B leads to a higher recovery and a complete derivatisation for a short fibre immersion time (10 min).

The goal of this work was to establish the potential of the SPME technique to a rapid determination of the distribution of sterols contained in an aqueous food matrix. Thereby it was essential to verify if there was discrimination in the absorption kinetics of sterols with different structures. The quantitative distributions of the five sterols–TMS (based on the peak areas in the total ion chromatograms) for protocols A and B in function of the fibre immersion into the standard sterol solution at the same concentration $6.25 \times 10^{-6}$ M have been reported on Table 1. For short (10 or 20 min) immersion times, differences of the recovery of standard sterols are obvious, more particularly for the protocol A. However, for longer exposure time (30 or 60 min), these differences are considerably reduced. The above results show that protocol A or protocol B cannot be used for quantitative analysis. Nevertheless these protocols can be used to quickly obtain the composition of sterols present in aqueous solutions.

### Table 2

Recoveries quantity (mole) of individual sterol standard with fibre immersion different time using protocol A ($b$-sitosterol concentration $6.25 \times 10^{-6}$ M) and B ($b$-sitosterol concentration $6.25 \times 10^{-5}$ M) for different proportion of sterols ($b$-sitosterol:stigmasterol:cholesterol (100:2:1 M ratios)).

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Protocol A</th>
<th>Protocol B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol–TMS</td>
<td>$1.23E-11$</td>
<td>$3.57E-11$</td>
</tr>
<tr>
<td>Stigmasterol–TMS</td>
<td>$1.70E-11$</td>
<td>$3.68E-11$</td>
</tr>
<tr>
<td>Sitosterol–TMS</td>
<td>$1.72E-09$</td>
<td>$3.13E-09$</td>
</tr>
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**Fig. 4.** TIC chromatograms from SPME–GC/MS of solutions containing $10^{-6}$ M $b$-sitosterol, $10^{-5}$ M cholesterol and $2.10^{-5}$ M stigmasterol with protocol A (a) and protocol B (b). RT: retention time (min); MA: peak area.
Fig. 5. TIC chromatograms from SPME–GC/MS of orange juice. (a) With protocol A (fibre immersion 60 min and BSTFA derivatisation 30 min and (b) with protocol B (BSTFA exposure 30 min and fibre immersion 10 min). (c) Sterol MS spectra obtained with protocol B.
Fruits and vegetables contain generally one major phytosterol (β-sitosterol or stigmasterol) and some other minor phytosterols. In order to verify if minor or sterols trace are correctly recovered in the presence of major ones, two experiments were performed with a standard solution containing different proportions of three sterols (β-sitosterol:stigmasterol:cholesterol (100:2:1 M ratios)). The first experience was accomplished using protocol A with fibre immersion into standard sterol solution during 60 min and BSTFA exposure time of 60 min. The second one was accomplished using protocol B with BSTFA exposure time of 30 min before immersion of the fibre inside standard solution during 10 min. GC/MS results from these two experiments are shown as TIC chromatograms in Fig. 4a and b respectively. β-sitosterol–TMS (RT = 33.2 min), cholesteryl–TMS (RT = 28.9 min) and stigmasterol–TMS (RT = 31.7 min) are easily identifiable from their mass spectra and peak areas and quantity of sterol recovery (Table 2) are reasonably in accord with their relative amounts. The value of peak area (MA) shows that sterol–TMS recovery with protocol B is better than protocol A. The detection limit of protocol A can be conceded under 10⁻⁸ M with described experimental setup.

### 3.2. Application for sterol analysis contained in orange juice

Presence of β-sitosterol (major), campstereol and stigmastereol (minor) in orange juice was previously shown by liquid–liquid extraction (Ng & Hupe, 1998). Two experiments were performed by SPME extraction and BSTFA derivatisation to verify if this technique can be applied for recovery of phytosterols present in commercial orange juices. Orange juice samples were composed by 8 mL of commercial orange juice to which we added KCl (0.6 g) and pyridin (50 µL). Protocol A was applied with immersion time of 60 min into orange juice sample before BSTFA exposure time of 60 min (Fig. 5a) and protocol B with BSTFA exposure time of 30 min before immersion of fibre inside orange juice sample during 10 min (Fig. 5b).

The GC/MS TIC chromatogram obtained by protocol A (Fig. 5a) did not show peaks for campstereol and stigmastereol and, although the mass spectrum showed its presence, an intense peak (RT = 33.26) covered β-sitosterol–TMS peak. The GC/MS result obtained from experiment 2 (Fig. 5b) was very different. Peaks for phytosterols (β-sitosterol–TMS (RT = 33.36 min), campstereol–TMS (RT = 31.20 min) and stigmastereol–TMS (RT = 31.79 min)) can be observed, as confirmed by their mass spectra (Fig. 5c).

Orange juice is a complex mixture containing different compounds (as example sugars, vitamins or proteins) that could be absorbed on SPME-PA fibre. The results show that non-steroidal compounds proportion with retention times similar to that of sterols–TMS was much lower with protocol B than with protocol A. There results permit to conclude that coating of the PA-SPME fibre by BSTFA prior to sterol extraction increase the affinity of the fibre for sterols relative to non-steroidal compounds present in the sample matrix.

### 4. Conclusion

In this work, we have studied two different SPME protocols for sterol extraction and BSTFA trimethylsilylation from model aqueous solutions and orange juice. Evaluation of these protocols for model solution containing sterol has been studied by two parameters: derivatisation yield, sterol recovery and the possibility to obtain quantitative information. On one hand the results show that protocol A give a higher derivatisation yield (100%) but a lower sterol recovery than protocol B. Nevertheless protocol B with 10 min immersion in sterol solution gave interesting results because as protocol the derivatisation yield obtained was 100%. But the different protocols cannot be use to quantitative analysis. However, the protocol B, which implies headspace coating (30 min) of the fibre with BSTFA reagent prior to simultaneous extraction (10 min) and derivatisation of sterols is particularly interesting for a rapid screening of phytosterols in aqueous food matrixes. Besides the rapidity, this method presents the advantages over other analytical techniques for sterol analysis in terms of simplicity, no organic solvent consumption and possibility of easy automation.

### References


