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Concept of sequential analysis of free and conjugated phytosterols in different plant matrices

Patrick Breinhölder, Livia Mosca, Wolfgang Lindner*

Institute of Analytical Chemistry, University of Vienna, Währingerstraße 38, A-1090 Vienna, Austria

Abstract

A unique concept and method for the determination of the total plant sterol content as sum of free sterols (FS), steryl esters (SE), steryl glycosides (SG) and acylated steryl glycosides (ASG) in different plant materials (pumpkin seeds, lecithins) and phytopharmaceuticals derived thereof, was developed. For this purpose, a multidimensional sample clean-up protocol based on efficient solid-phase extraction materials was elaborated and the SG were isolated employing a novel phenyl boronic acid modified silica gel material. Along this line also a set of steryl glucosides was synthesised and employed as internal standard and for calibration in the course of quantitative analysis. Final quantification of SG was carried out with reversed-phase HPLC in combination with evaporative light scattering detection (ELSD); the ASG were determined after conversion to SG by mild alkaline hydrolysis. In order to determine the total plant sterol profile the sum of FS and SE was additionally analysed from the unsaponifiable lipid fraction by GC–FID. The yields obtained from recovery tests for the determination of SG using soya lecithin as matrix to which 2, 20 and 40 mg/g of cholesterol-β-D-glucoside was added were 99.10, 98.07 and 90.00%, and the RSDs were 4.11, 2.62 and 4.50%, respectively. Application related to the qualitative and quantitative analysis of total phytosterol profiles in different plant matrices and extracts demonstrate the validity of the method.

Keywords: Sequential analysis; Lecithin; Oil seeds; Phytosterols; Phytosteryl glycosides

1. Introduction

Phytosterols are widely distributed natural substances [1–3]. Together with phospholipids and other glycolipids, phytosterols and their derivatives are essential components of plant biomembranes [4]. They are biogenetic precursors of numerous metabolites including plant steroid hormones [5]. Along with free sterols (FS), plants also contain sterol derivatives with the 3-hydroxyl group either esterified with a long chain fatty acid (steryl esters, SE) or alternatively β-linked to the 1-position of a free monosaccharide (steryl glycosides (SG) or of an acylated monosaccharide (acylated steryl glycosides, ASG)) in which preferably the 6-position of the sugar is esterified by a fatty acid. In this context, the occurrence of e.g. steryl glycosides containing oligosaccharide moieties has been reported [1,6].

Phytosterols are frequently classified according to double bond positions in the ring skeleton (Fig. 1). Most plants contain predominantly Δ5-sterols (Fig.

*Corresponding author. Tel.: +43-1-4277-52300; fax: +43-1-513-1826.

E-mail address: wolfgang.lindner@univie.ac.at (W. Lindner).

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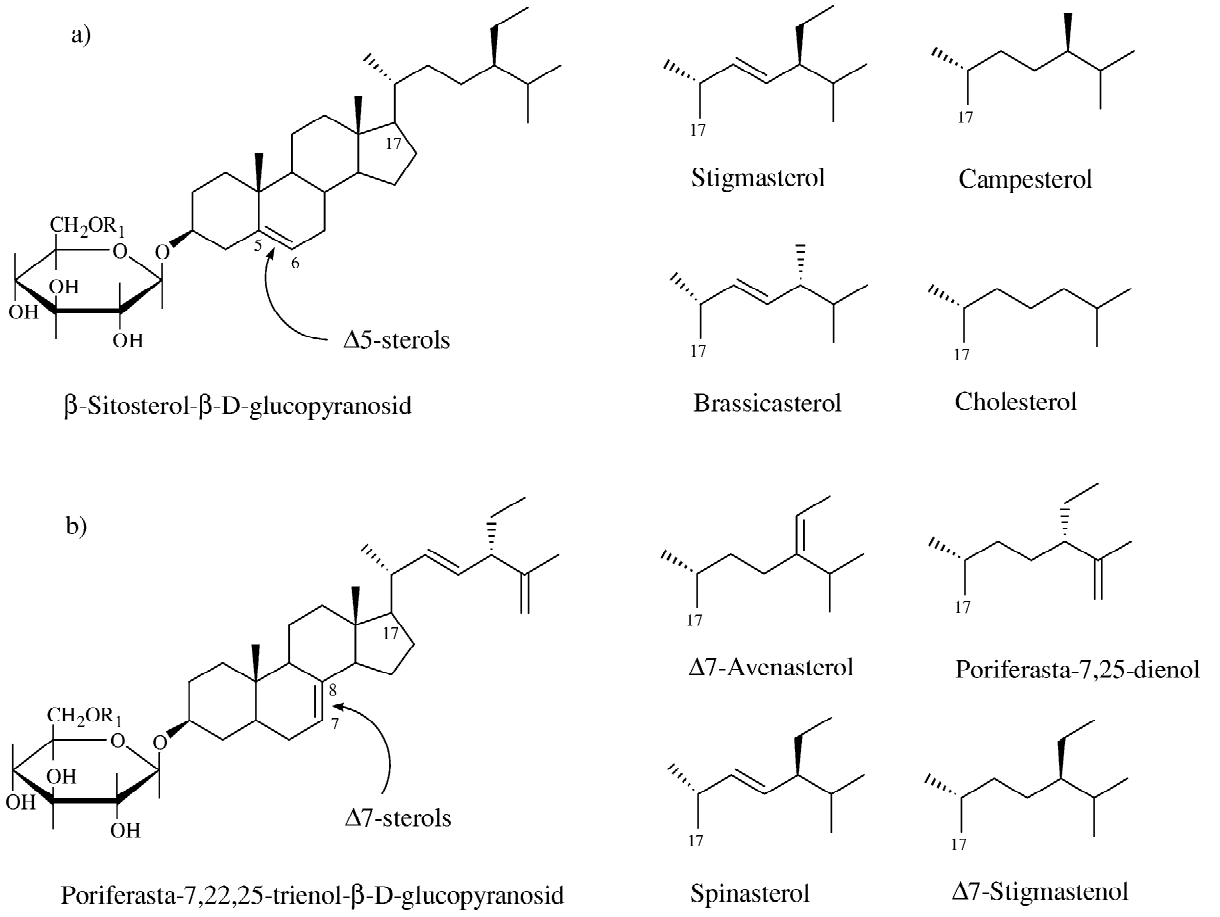

Fig. 1. Structure of Δ5- and Δ7-steryl glycosides (R_1 = H) or acylated steryl glycosides (R_1 = acyl residue). The side chain attached to carbon 17 varies between the major plant sterols. (a) Most predominant Δ5-steryl glycosides and the internal standard cholesterol-β-D-glucoside; (b) pumpkin seed specific Δ7-steryl glycosides.

1a) with only trace amounts of Δ7-sterols (Fig. 1b). β-Sitosterol, stigmasterol and campesterol are among the most common Δ5-sterols, while brassicasterol is for example specific for rape seeds.

The predominance of Δ7-sterols appears to be restricted to only a few plant families, e.g. Cucurbitaceae and Theaceae. Larger amounts, either in free or glycosidic bound form can be found in pumpkin seeds (Fig. 1b). Due to their structural resemblance with androgens, Δ7-sterols are supposed to play a role in the treatment and prophylaxis of disorders of the prostate gland and the urinary bladder [7]. Moreover sterols and their derivatives show a multitude of different pharmacological properties [7–11]. Along this line the inhibition of cholesterol absorption seems to be the best understood mechanism of action of phytosterols [11]. Miettinen et al. demonstrated significant blood cholesterol reduction in patients with mildly elevated blood cholesterol by the regular use of a specially formulated margarine that contained sitostanol (hydrogenated β-sitosterol). As a result, phytosterol-enriched food products (''functional foods'') and phytopharmaceuticals containing the oil or the extract of plants rich in phytosterols have been introduced to the food market.

With regard to analysis methods related to this group of compounds, gas chromatography (GC) proved to be simple and accurate to determine free sterol compounds in oil matrices [12]. However, a

quantitative analysis of the total plant sterol content as sum of all of the steryl species (FS, SE, SG and ASG) in plant extracts is more problematic. In this context, well established chemical protocols using acidic hydrolysis [13] of the glycosides prior to GC analysis are not applicable due to the acid lability of Δ7-phytosterols [14,15]. Investigations in our laboratories have revealed that Δ7-phytosterol already decompose or isomerise after a short time of acidic hydrolysis.

Alternatively, reversed-phase HPLC in combination with UV or light scattering detection has been shown to be useful for the analysis of molecular species of steryl glycosides and acylated steryl glycosides [2,16–19]. Prior to LC separation thin-layer chromatography was predominantly applied for sample clean-up and for the separation of different steryl species. To simplify this pattern, the acylated species (SE and ASG) were converted to the corresponding non-acylated ones (FS and SG) by alkaline hydrolysis. Using HPLC in combination with UV-detection (200 nm), free sterol standards can be used to quantify steryl glycosides since the glycosidic residues do not affect the UV absorption properties of steryl glycosides at low wavelengths [16,17]. This approach has the advantage that no standard materials are required to quantify quasi indirectly the steryl glycosides. On the other hand elaboration of an accurate and precise method necessitates standards to probe the analytical concept and to proof the chemical integrity and the recovery efficiency. Furthermore, quantification of complex phytosteryl glycoside mixtures from natural sources may be critical if a large variety of different and partly unknown sterol moieties are present. Moreover, the selection of mobile phases are limited to be used at low detector wavelengths to reach highest sensitivity. To circumvent this problem steryl glycosides may also be transformed to their anthronylnitrile derivatives to enable UV detection at higher wavelengths (254 nm) [20]. Additionally the derivatisation increased detection sensitivity to determine trace amount of steryl glycosides but as any derivatisation protocol it has the risk of degradation and incomplete reaction.

Circumventing this dilemma, the use of an evaporative light scattering detector (ELSD) has the advantage that all common HPLC-solvents can be used in isocratic as well as gradient mode [18]. Due to the mass dependency of the ELSD response, standard materials, such as e.g. cholesterolglycoside, are, however, essential to set up a quantitative assay. However, beyond the detection calibration, internal standards are also essential in the course of a total analysis protocol accounting for losses during sample extraction and clean-up involving multidimensional selectivity principles.

The aim of the present work was the development of a HPLC–ELSD method to determine Δ7- and Δ5-steryl glycosides in different plant matrices avoiding chemical alteration (derivatisation) and acidic hydrolysis prior to analysis to prevent any degradation phenomena. In order to optimise the final HPLC separation and analysis of naturally occurring SG mixtures, SG of different plant matrices (pumpkin seeds, lecithins) have been isolated and examined. For sample clean-up, a multidimensional approach was elaborated (Fig. 4) to enrich the analytes and to separate them groupwise from the highly complex matrix. A key step of this sample clean-up protocol was an efficient solid-phase extraction material based on silica gel that had been modified with phenylboronic acid according to a novel preparation protocol. To simplify the method, ASG was determined after conversion to SG by mild alkaline hydrolysis with sodium methoxide. Additionally, the sum of FS and SE was determined after separation from the glycosylated sterols by GC–FID following saponification of the lipid matrix. The GC samples, however, needed to be derivatised according to standard procedures [12]. In order to create an accurate and reproducible method, a set of three different standard steryl glycosides were synthesised and tested for their applicability as internal standards to establish the recovery efficiencies, etc.

2. Experimental

2.1. Chemicals

β-Sitosterol (60% β-sitosterol, 33% campesterol) and the internal standard dihydrocholesterol (3β-hydroxy-5α-cholesterol, 95%) were purchased from Sigma–Aldrich (Deisenhofen, Germany). All solvents (methanol, acetonitrile, chloroform, ethanol, *tert*.-butyl-methyl ether (TBME) and ethyl acetate)

were of HPLC grade (Merck, Darmstadt, Germany). 1,2-Dimethoxyethane (glyme) and 1,4-dioxane were of analytical grade (Fluka Chemie, Vienna, Austria). The derivatising agent *N,O*-bis(trimethylsilyl)trifluoracetamide (BSTFA) was obtained from Sigma–Aldrich. Potassium hydroxide p.a., anhydrous sodium sulphate p.a. and silica gel 60 (particle size 0.063–0.200 mm) were purchased from Merck (Darmstadt, Germany).

2.2. Sample material

Commercially available dried pumpkin seed samples (water content<10%) were obtained from Biosonn (Graz-Raaba, Austria). These samples have been collected from various fields in the southern parts of Styria (seeds of *Cucurbita pepo* L. convar. *citrullina* I. GREB. var. *styriaca* I. GREB.) with a sample size of 200 g. A 10 g portion of these seeds were ground and an aliquot of 1 g was finally analysed. Pharmaceutical formulations referenced as pumpkin seed extract were commercially available at different pharmacies and drug stores. Lecithin samples were purchased from different companies (Lucas Meyer GmbH, Hamburg, Germany and Werba OHG, Vienna, Austria).

2.3. Standard materials

2.3.1. Synthesis of β-D-steryl glucoside

2.3.1.1. Chemicals. Cholesterol (96%), dihydrocholesterol (95%), stigmasterol (96%), silver trifluoromethane-sulfonate (silver-triflate, 99.95%) were purchased from Sigma–Aldrich (Deisenhofen, Germany). Silver carbonate was prepared freshly and dried under vacuum before use. Tetra-*O*-pivaloyl-α-D-glucopyranosyl bromide was synthesised in our laboratory according to Kunz et al. [21]. For flash chromatography, silica gel 60 (0.040–0.063 mm) was employed.

2.3.1.2. Synthesis of cholesterol-β-D-glucoside. Glucoside synthesis was accomplished according to the method of Kunz and Harreus [21]; 1.45 g (2.5 mmol) tetra-*O*-pivaloyl-α-D-glucopyranosyl bromide, 1.35 g (3.5 mmol) cholesterol, 0.83 g (3 mmol) silver carbonate, 0.05 g silver trifluoromethane-sulfonate and 5 g molecular sieve (3 Å) were stirred under argon in 30 ml dry ether for 24 h. The reaction mixture was filtered, the filtrate was evaporated to dryness and the residue was purified by silica gel flash chromatography using petroleum-ether/ethyl acetate (10:1) as mobile phase. The purified cholesterol-tetra-*O*-pivaloyl-β-D-glucoside was suspended in 50 ml methanol and 10 ml sodium methoxide (0.1 mol/l) were added. After refluxing for 24 h, the solution was cooled and diluted with 100 ml methanol. The precipitated product was filtered off, boiled with water, filtered again and dried under reduced pressure. The crude crystals were dissolved in hot pyridine and precipitated by adding water to the hot solution. After the suspension was left overnight at ca. 4 °C, the product was filtered off. The colourless crystals were washed with water and acetone, and dried over potassium hydroxide in vacuum to obtain pure cholesterol-β-D-glucoside. Dihydrocholesterol and stigmasterol were processed accordingly to obtain dihydrocholesterol-β-D-glucoside and stigmasterol-β-D-glucoside. Characterisation of standard substances was carried out using NMR (^{1}H and ^{13}C) and high resolution mass spectrometry (HRMS). Purity was also verified using HPLC in combination with ELSD and UV detection.

Cholesterol-β-D-glucoside: Yield: 1.9 g (85%), purity>99% (HPLC), MP 262–263 °C.

^{13}C NMR (100 MHz, pyridine-d_5): δ=12.16, 19.11, 19.60, 21.47, 22.85, 23.10, 24.31, 24.66, 28.41, 28.67, 30.45, 32.24, 32.36, 36.20, 36.66, 37.11, 37.67, 39.53, 39.90, 40.15, 42.66, 50.55, 56.54, 57.01, 63.05, 71.91, 75.53, 78.29, 78.67, 78.80, 102.78, 122.10, 141.10.

HRMS calculated for $C_{33}H_{56}O_6$ (M^+): 548.4077, found 548.4093.

Dihydrocholesterol-β-D-glucoside: Yield: 1.0 g (70%), Purity>99% (HPLC), MP 256–258 °C.

^{13}C NMR (100 MHz, pyridine-d_5): δ=12.42, 12.49, 19.07, 21.63, 22.85, 23.10, 24.31, 24.61, 28.41, 28.69, 29.22, 30.20, 32.45, 35.03, 35.78, 35.91, 36.20, 36.64, 37.41, 39.90, 40.41, 42.95, 44.80, 54.66, 56.69, 56.74, 63.15, 72.01, 75.56, 77.36, 78.73, 78.85, 102.32.

HRMS calculated for $C_{33}H_{58}O_6$ (M^+): 550.4233, found 550.4218.

Stigmasterol-β-D-glucoside: Yield: 1.1 g (73%), Purity>96% (HPLC), MP 297–298 °C.

^{13}C NMR (100 MHz, pyridine-d_5): δ=12.17, 12.54, 19.21, 19.44, 21.29, 21.31, 21.50, 24.57, 25.72, 29.33, 30.29, 32.09, 32.19, 36.97, 37.51, 39.38, 39.86, 40.80, 42.39, 50.39, 51.45, 56.11, 56.96, 62.89, 71.75, 75.38, 78.12, 78.51, 78.65, 102.61, 121.93, 129.50, 138.85, 140.95.

HRMS calculated for $C_{35}H_{58}O_6$ (M^+): 574.4233, found 574.4251.

2.3.2. Isolation of phytosteryl glycosides from pumpkin seeds and lecithin

A total lipid fraction was generated by exhaustive extraction of ground pumpkin seeds by stirring or shaking the suspension for 12 h using a mixture of chloroform/methanol (2:1, v/v). The extraction was repeated with further portions of extraction solvent until the extract remains colourless (200 g seeds were extracted three times with 500 ml extraction solvent). This protocol replaces the Soxhlet extraction, which puts some thermic stress on the sample. The combined extracts were evaporated under reduced pressure and the residue was refluxed with a potassium hydroxide solution (20 g KOH in 100 ml methanol/water, 88:12, v/v). For this saponification step, 5 ml of the potassium hydroxide solution per g extract was employed. After saponification for 12 h, silica gel was added until the whole solution was adsorbed and the batch was dried under reduced pressure at 40 °C. The loaded silica was filled in a chromatography column and was washed with chloroform until the solvent became colourless. The remaining glycolipids were eluted with acetone and the fractions containing SG (monitored by TLC [2]: silica gel, solvent system: $CHCl_2$/MeOH/H_2O 85:15:0.5 v/v/v, visualisation: Anisaldehyde-reagent: 0.5 ml anisaldehyde, 85 ml MeOH, 10 ml sulphuric acid conc., 10 ml glacial acetic acid, RF: SG 0.35, ASG 0.55, FS 0.70, SE 0.90) were combined and evaporated. The residue was resuspended in chloroform and further cleaned on a silica gel column using a stepwise elution with chloroform and chloroform/methanol mixtures (19+1, 18+2). The fractions containing steryl glycosides (monitored by TLC, as described above) were combined and evaporated. The crystals obtained were recrystallized in pyridine/water, as described in Section 2.3.1, to obtain the Δ7-phytosteryl glycoside mixture from pumpkin seeds. The Δ5-phytosteryl glycosides from soya lecithin were isolated and purified accordingly, but without performing the first lipid extraction step.

2.3.3. Isolation of phytosterols from different vegetable oils

The vegetable oils (pumpkin seed oil, soya oil and rape seed oil, respectively) were directly filtered through a plug of silica gel at ambient pressure (0.1 g silica/g oil). Then the column was washed with hexane (or heptane) to remove the remaining triglyceride fraction. The adsorbed phytosterols were then eluted with hexane containing 10–20% of TBME. The free sterol containing fractions (monitored by TLC, as described above) were combined and evaporated. The remaining crude material was recrystallised in heptane at −20 °C to obtain the phytosteryl mixtures used as reference materials.

2.4. Synthesis of the boronic acid substituted silica gel (PBA-cartridges)

The phenylboronic acid (PBA) solid-phase extraction (SPE) material, based on a 15 μm silica material and a immobilised phenyl boronic acid derivative was synthesised in our laboratory. The material exhibited a high loading level of phenyl boronic acid groups of about 500 μmol per g. A 50 mg portion of this material was filled into 1 ml polypropylene extraction tubes, fitted with a polyethylene frit. The thus self-made PBA-SPE-cartridges were used for solid-phase extraction and purification of steryl glycosides. Details of the synthesis and the characterisation of this novel boronic acid phase will be published elsewhere [22].

2.5. Instrumentation and chromatography

2.5.1. High-performance liquid chromatography (HPLC)

The HPLC system consisted of a Merck-Hitachi-L6200A pump, a Merck-Hitachi-L7200 autosampler (Merck, Darmstadt, Germany) and a Sedex 55 evaporative light scattering detector (Sedere, France). Separation of steryl glycosides was achieved on a Hypersil BDS RP18 column (5 μm, 250×4 mm, Hewlett-Packard, Waldbronn, Germany) with metha-

nol/acetonitrile/water (75:15:10, v/v/v) as mobile phase at a flow-rate of 1 ml/min and at a temperature of 35 °C; 30 min after the injection (injection volume: 50 μl) the column was washed with methanol for 5 min and re-equilibrated for 10 min. The ELS detector was adjusted to 40 °C and run with a nitrogen pressure of 1.8 bar.

HPLC–MS analyses were performed on a PESciex API365 LC–MS–MS system (Perkin-Elmer Sciex Instruments, Thornhill, Ont., Canada) equipped with an electrospray ionisation interface (ESI) and an Agilent 1100 LC-system. Chromatographic conditions were used as described above. To improve the ionisation efficiency and stability, 10 mmol ammonium acetate was added to the mobile phase. The ESI interface was used in the positive ion mode at an ionspray voltage of 5000 V. Scan range was set from 250 to 1000 at a step size of 0.1 and a scan duration of 4 s. Most of the MS parameters were optimised with a syringe pump at a flow-rate of 5 μl/min of a stock solution of stigmasterol-β-D-glucoside.

2.5.2. Gas chromatography

A Hewlett-Packard 5890-series II gas chromatograph equipped with either a flame ionisation detector (FID) or a mass selective detector (MSD) 5972 was used (Hewlett-Packard, Waldbronn, Germany). A sample volume of 1 μl of the reaction solution was injected using a HP 7673 A autosampler with a Hamilton 10-μl syringe. GC separations were carried out on a fused-silica capillary column (30 m×0.25 mm×0.25 μm) coated with a 0.25-μm layer of crosslinked 75% dimethyl-35% diphenyl polysiloxane (HP-35) (Hewlett-Packard, Waldbronn, Germany). The injector was heated at 260 °C and was used in the splitless mode. The FID-detector was heated at 290 °C. The column temperature was programmed from 220 °C (held for 1 min) to 290 °C at 5 °C/min and held at 290 °C for 10 min. Helium 5.0 was used as carrier gas at 17 p.s.i. column headpressure in the constant pressure mode. Trimethylsilyl ether derivatives of the sterols were identified by comparison of their retention time and by their mass spectra. For GC–MSD, the transfer line heater was set at 280 °C and the ionisation energy was 70 eV in the electron impact mode. Spectra were scanned within the mass range m/z 50–500.

2.6. Analytical procedure

2.6.1. Standard solutions

Stock solution of stigmasterol-β-D-glucoside and cholesterol-β-D-glucoside was prepared in chloroform/methanol (2:1, v/v) at 1 mg/ml. Stock solution of β-sitosterol (60% β-sitosterol, 33% campesterol) was prepared in methylene chloride at a concentration of 5 mg/ml. Internal standard solution of dihydrocholesterol was prepared in the same solvent at a concentration of 2 mg/ml.

2.6.2. Sample extraction procedure

A 0.5 to 1 g portion of ground seeds or pulverised tablets (pharmaceutical formulations, containing pumpkin seed extracts) were weighed into screw-capped glass tubes. Then, 250 μl of the internal standard solution of cholesterol-β-D-glucoside and 150 μl of the internal standard solution of dihydrocholesterol were added. The sample was extracted with 10 ml of a mixture of chloroform/methanol (2:1, v/v) by ultrasonication for 15 min and shaking for 12 h at room temperature. After centrifugation, the remaining pellet was reextracted for 1 h using 5 ml of fresh extraction solvent. The combined extracts were evaporated under reduced pressure at 40 °C and worked up as described below.

Lecithin could be used directly without further extraction step because it was already completely soluble in the solvents used. A 0.025 g portion of lecithin was weighed into screw-capped glass tubes and the internal standard solutions were added as described above. Solvents of standard solutions were evaporated under nitrogen before starting with the sample clean-up procedure.

2.6.3. Sample preparation and clean-up protocol

2.6.3.1. Non-acylated steryl glycosides (SG). The seed extracts and lecithin samples spiked with the internal standards (see Section 2.6.2) were dissolved in 2–3 ml chloroform and applied on a silica gel glass column (14 cm length and 1 cm I.D.) containing 0.5 g silica material (silica gel 60, particle size 0.063–0.200 mm). The loaded column was washed successively with 5 ml chloroform and 5 ml TBME. The chloroform and TBME eluates were combined and stored at 4 °C. This non polar lipid fraction was used for determination of FS and SE by

GC–FID (see Section 2.6.3.3). The glycolipid fraction was eluted with 15 ml of a mixture of chloroform/methanol (2:1, v/v) and the solvent was evaporated at reduced pressure at 40 °C. The residue was dissolved in 1 ml glyme containing 10% of triethylamine. (If the residue was not completely soluble the turbid solution was centrifuged.) The clear solution was applied to a PBA-solid-phase extraction cartridge (PBA-silica, see Section 2.4) pre-conditioned with 1 ml glyme containing 10% triethylamine. The column was washed with 0.5 ml of the glyme–triethylamine mixture and two times with 0.5 ml of pure glyme. SG was eluted with a total of 3 ml of methanol containing 1 mmol sorbitol. The eluate was evaporated to dryness under nitrogen. The residue was finally dissolved in 1 ml dioxane/water (4:1, v/v) and a 50 μl aliquot was analysed by HPLC–ELSD.

2.6.3.2. Total steryl glycoside (sum of SG and ASG). ASG were converted to SG by mild alkaline hydrolysis using a freshly prepared 0.1 mol/l sodium methoxide solution: 1 ml of the sodium methoxide solution was added to the spiked extracts or lecithin samples. The reaction mixtures were incubated at 50 °C for 60 min. Afterwards, 4 ml of water, 0.5 ml of a saturated sodium chloride solution and 5 ml of chloroform were added. SG (sum of original SG and SG released from ASG) were extracted into the organic phase by shaking vigorously for 1 min. To achieve a better phase separation, finally 0.5 ml ethanol was added and the mixture was centrifuged for 30 min. The aqueous phase was discarded and the organic layer was diluted with 2 ml methanol and evaporated to dryness under reduced pressure at 40 °C. The residue was dissolved in 1 ml glyme (10% triethylamine) and solid-phase extraction on PBA-cartridges was performed as described above (see Section 2.6.3.1).

2.6.3.3. Free sterols and steryl esters (sum of FS and SE). Determination of FS and SE was carried out according to a modified method of Mandl et al. [12]: Accordingly the chloroform and TBME eluates (see Section 2.6.3.1) were combined and the solvents were removed partly under reduced pressure at 40 °C. The remaining solution was transferred into a screw-capped glass tube and the solvent was evaporated by a stream of nitrogen. After saponification of the residue with 1 ml of a methanolic potassium hydroxide solution (20 g KOH in methanol/water, 88:12, v/v) for 45 min at 70 °C, the unsaponifiable lipid fraction was isolated and purified using a silica gel column of 14 cm length and 1 cm I.D. filled with 0.5 g anhydrous sodium sulphate and 0.2 g of silica gel. The saponified mixture adsorbed on 1 g of silica gel was transferred to this column. The unsaponifiable fraction was eluted with 10 ml of a mixture of *tert*.-butylmethyl ether (TBME) and ethyl acetate (1:1, v/v). Then, 1.5 ml of the eluate was transferred to a 2 ml glass vial and the solvent was removed under nitrogen. The residue was dissolved in 50 μl of a mixture of BSTFA and dry pyridine (2:1, v/v) in 400 μl of TBME [12]. This reaction mixture was kept in a sealed vial at 70 °C for 2 h. A 1-μl aliquot of this reaction mixture was subjected to GC-analysis.

2.6.4. Calibration, repeatability, recovery and limit of quantification (LOQ)

Quantification of SG was performed via calibration curves using the stigmasterol-β-D-glucoside stock solution. The area of each SG-peak was referred to the area obtained from the peak of the internal standard (cholesterol-β-D-glucoside). Calibration was performed over the range 0.01–1.6 mg/ml (10 calibration points). It was assumed that the ELSD response factor obtained for stigmasterol-β-D-glucoside could be adopted linearly for all steryl glycoside compounds.

The sum of FS and SE was determined using the β-sitosterol stock solution (60% β-sitosterol, 33% campesterol). The area of each peak was referred to the area obtained from the peak of the internal standard solution (dihydrocholesterol). Calibration was performed over the range of 0.03–6.5 mg/ml (10 calibration points) for β-sitosterol and of 0.01–3.5 mg/ml (10 calibration points) for campesterol. FID response factors of the Δ7-phytosterols could not be determined due to the lack of standard material. However, due to the structural homology of all the sterols measured, the same FID response was assumed, which lead also to simplification, and to apply the β-sitosterol calibration curve also for the quantification of the Δ7-phytosterols and the campesterol calibration curve also for brassicasterol.

The limit of quantification (LOQ) for FS (GC–FID) as well as for SG (HPLC–ELSD) was set at the

lowest standard concentration of the calibration curve, which had a signal-to-noise (S/N) level >10.

Recovery of the method was established at low, medium and high concentration levels. Soya lecithin was used as matrix to be spiked with cholesterol-β-D-glucoside and dihydrocholesterol and all data points were made in triplicate.

The repeatability of the method was tested by analysing six aliquots of the same lecithin (soya lecithin) sample. For this purpose a soya lecithin stock solution was prepared in chloroform at 0.025 mg/ml and 1 ml aliquots were analysed.

3. Results and discussion

3.1. Standard reference materials and HPLC analysis of steryl glycosides

In analysis of natural compounds, where complex sample matrices are common, there is frequently a lack of standard reference material to be used for quantitative analysis. However, suitable standard compounds may be necessary to guarantee reliable method development and stable method performance in terms of high sample clean-up recovery rates, accurate quantification and sufficient chromatographic separation prior to final detection to avoid biased results. The generation of suitable standards was, therefore, a crucial prerequisite for the present method, as no such compounds were available.

The appropriately selected standard steryl glucosides cholesterol-, dihydrocholesterol- and stigmasterol-β-D-glucoside were obtained in good purity ($>95\%$) and yields (70–85%) by a two-step synthesis from free sterols and tetra-*O*-pivaloyl-α-D-glucopyranosyl bromide. In addition, procedures for the isolation and purification of sterols and their glycosides from different plant lipid matrices were elaborated. In this context, a rapid and simple procedure for the isolation of free sterols from vegetable oils, employing silica gel column chromatography, was established. Furthermore, steryl glycosides were isolated from soya lecithin and from pumpkin seeds to obtain the soya specific Δ5-phytosteryl- and the pumpkin specific Δ7-phytosteryl glycosides. Both approaches resulted in high quantities of pure Δ5- and Δ7-phytosteryl glycoside reference materials as shown by HPLC with ELS-detection (Fig. 2).

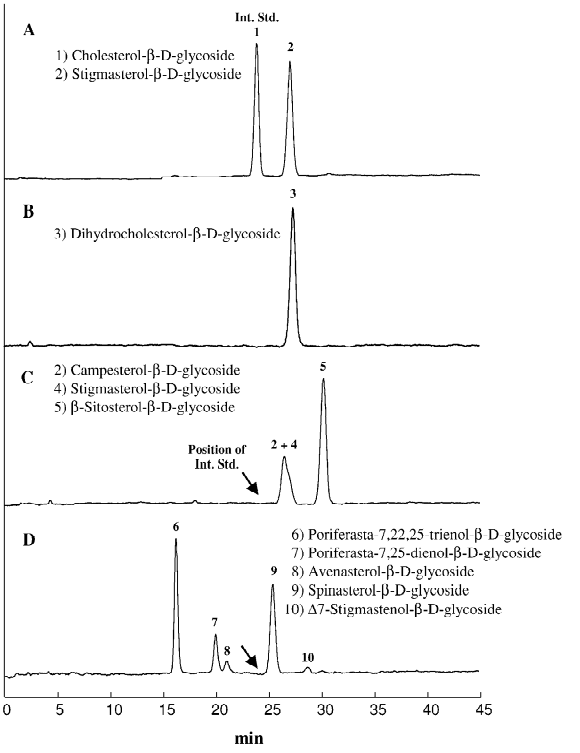

Fig. 2. HPLC–ELSD chromatograms of synthesised and isolated standard steryl glycosides (isocratic conditions MeOH/ACN/H_2O 75:15:10, 1 ml/min); (a) and (b) synthesised steryl glucosides; (c) main soya Δ5-steryl glycosides; (d) pumpkin seed specific Δ7-steryl glycosides.

HPLC separation efficiency was optimised with the mixture of pumpkin seed Δ7-phytosteryl glycosides (Fig. 2D). The Δ7-phytosteryl glycosides were well separated using an octadecyl column and isocratic conditions with MeOH/ACN/water (75:15:10, v/v/v). HPLC–MS data revealed that no other steryl glycoside compounds occurred within this steryl glycoside mixture. Under these conditions the Δ5-phytosteryl glycosides from soya-lecithin (Fig. 2C) could not be completely separated, since stigmasterol- and campesterol glycoside coeluted. Complete separation of these compounds was only achieved with gradient elution of MeOH/water (data not shown) or ACN/water mixtures [15]. Furthermore, HPLC evaluation revealed that cholesterol-β-D-

glucoside (Fig. 2A) was applicable as internal standard for both the Δ7- as well as the Δ5-phytosteryl glycoside-mixtures. Dihydrocholesterol-β-D-glucoside (Fig. 2B) was not suitable as internal standard since it coeluted with natural occurring stigmasterol glycoside.

The elution sequence and peak assignment of phytosteryl glycoside mixtures was elaborated by comparing the results obtained for steryl glycosides with the elution pattern of the corresponding free sterols and by HPLC–MS-analysis. Steryl glycosides separated using reversed-phase HPLC on the basis of the structure of the sterol moiety in a similar order to that of free sterols [19]. For identification, the individual HPLC-peaks of free sterols were collected and also analysed by GC–MS as the respective trimethylsilyl ethers. The obtained mass spectra data of the silylated phytosterols were identical with those reported in the literature [2,12]. Complementary, the isolated phytosteryl glycoside-mixtures were analysed by HPLC–MS not only to proof the elution order but also to proof peak purity of the worked up samples. By HPLC, the steryl glycosides eluted faster than the corresponding free sterols but the elution sequence remained similar.

3.2. Sample extraction procedure

The extraction efficiency of ground pumpkin seeds, which stands for various oil seeds, by five different solvent systems like acetone, chloroform, glyme, methanol and chloroform–methanol (2:1, v/v) was investigated. Quantitative analysis of the analyte amounts in the extracts proved that for FS all solvents or solvent mixtures, except methanol, exhibited good extraction yields. For SG the results were somewhat different since the extraction efficiency was strongly dependent on the solvents, whereby a chloroform–methanol mixture and glyme were found to be best suited to extract these compounds with high yields. Finally, a chloroform–methanol mixture (2:1, v/v) was selected, since this medium exhibited best extraction efficiency for both the non-glycosylated lipid fraction and the glycolipid fraction.

The physical properties of plant materials under study and the structure of sterols (i.e. FS, SE, SG, ASG) need to be carefully considered when a suitable extraction method is chosen. However, it was recognised that there can be sterols in some tissues which are more difficult to extract [2,3]. The lecithin samples examined were completely soluble in most of the solvents used and therefore the sample solution could be directly subjected to the SPE column. The examined pharmaceutical formulations were based on sample extracts of to us unknown protocols; they were powdered prior to our extraction procedure outlined in the analysis protocol. The pumpkin seed samples were ground before solvent extraction. However, it should be considered that in some cases a more effective extraction can be obtained by homogenisation of the seed material in the extraction solvent, e.g. by Ultra-Turrax.

3.3. Sample preparation and clean-up procedure

The usefulness of a boronic acid solid-phase extraction material for the isolation and purification of various molecular species containing 1,2- and 1,3-bis-hydroxyl groups has been shown previously [23,24]. The high group selectivity of these solid-phase extraction (SPE) materials is based on a well-known reversible reaction between preferably *cis*-configured hydroxyl groups and boronic acid (Fig. 3), whereby the reversible formation of the trigonal boronate esters is carried out under basic conditions.

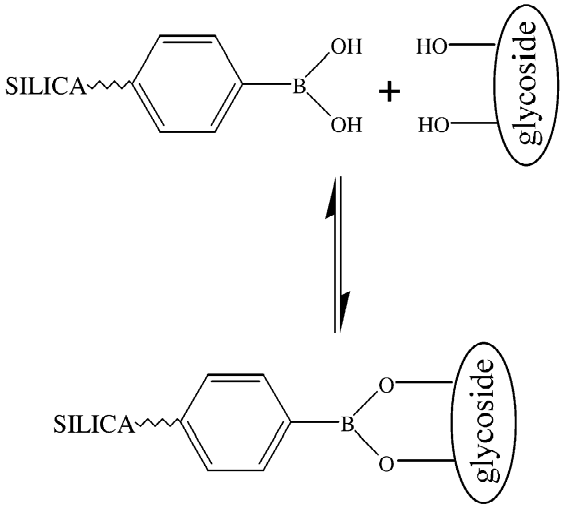

Fig. 3. Scheme of complex formation between immobilised phenylboronic acid and 1,2- or 1,3-diol compounds.

The cyclic esters can be cleaved either by a switch to acidic pH values or, alternatively, by adding agents which effectively compete for the boronate binding site. This efficient, highly selective and reversible retention mechanism is active in both aqueous and non aqueous media. Compounds, like e.g. sugars, nucleotides, catecholamines [23,24] but also water insoluble compounds like brassinosteroids [25] have

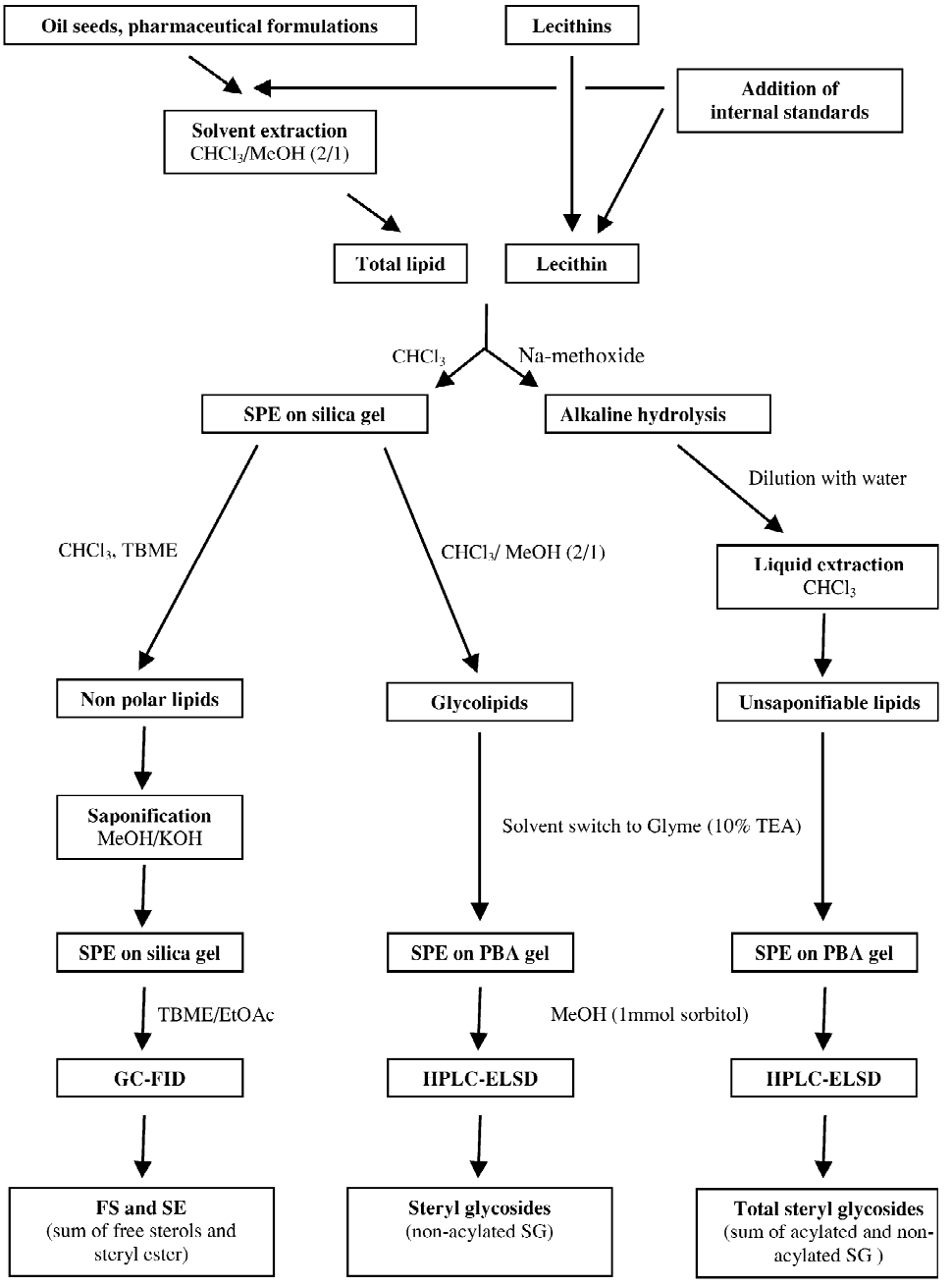

Fig. 4. Multidimensional sample preparation and analysis scheme.

been successfully isolated with this approach. In the present analysis concept, as outlined in Fig. 4, the PBA-SPE approach proved to be a key step to separate efficiently glycolipids from the non-glycosylated matrix compounds. This was carried out by sample loading onto the PBA-phase. A solution of 10% of triethylamine in glyme proved to be a most efficient solvent for retention and purification followed by 3 ml methanol containing 1 mmol sorbitol as cleaving eluent, achieving recovery rates between 97 and 100% for this step. Methanol without any additive resulted in an incomplete analyte elution with recovery rates below 80% or in very large elution volumes. Acidic eluent conditions had to be avoided due to the acid lability of the Δ7-phytosterols.

To determine all individual sterol species as the free sterols (FS), steryl ester (SE), steryl glycosides (SG) and acylated steryl glycosides (ASG), a multidimensional sample clean-up protocol was employed (Fig. 4). Prior to PBA-SPE to determine non-acylated SG a further SPE fractionation step on a silica gel column was needed. By this step, the total lipid extracts of oil seeds and the lecithin samples, respectively, were fractionated into a non polar lipid fraction containing FS and SE and a glycolipid fraction containing non-acylated SG. Furthermore, the ASG content was determined in a second ex-

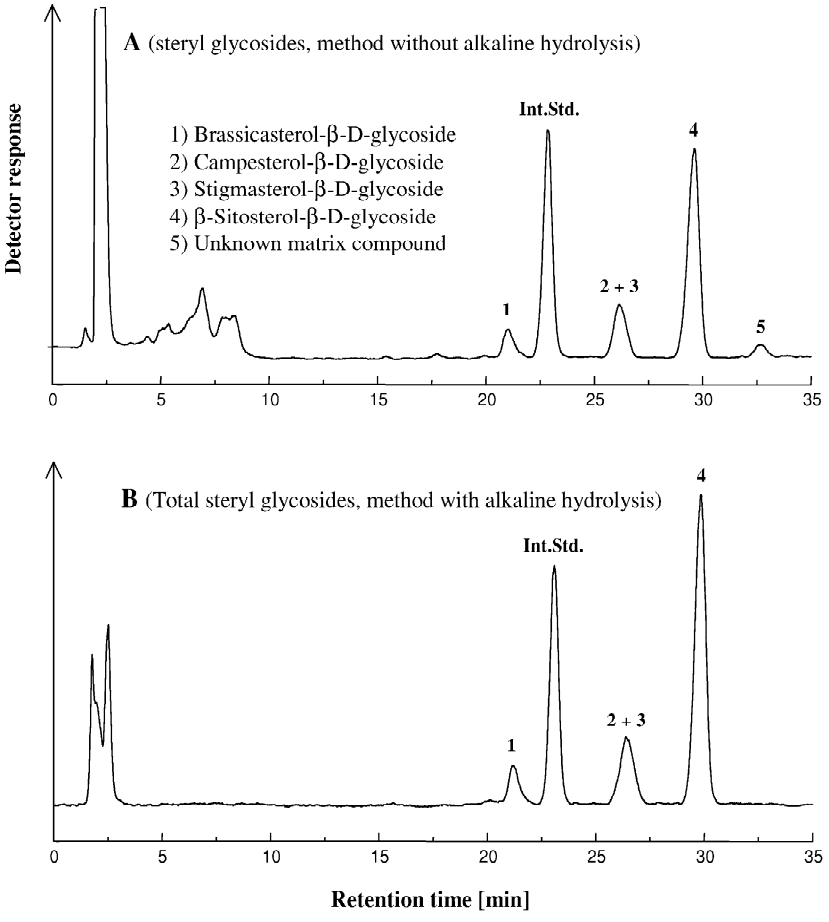

Fig. 5. HPLC–ELSD chromatograms of rape seed lecithin spiked with 0.25 mg/ml cholesterol-β-D-glucoside (injection volume 50 μl): (A) Determination of non-acylated steryl glycosides (method without alkaline hydrolysis). (B) Determination of total steryl glycosides (method with alkaline hydrolysis).

Table 1
Recovery for the whole sample clean-up procedure in spiked soya lecithin ($n=3$)

Method	Concentration (mg/g)	Recovery (%)
Steryl glycosides (SG)	2.0	99.10±4.11
	20.0	98.07±2.62
	40.0	90.00±4.50
Total steryl glycosides (SG+ASG)	2.0	89.10±1.67
	20.0	84.53±2.11
	40.0	86.30±1.20
Sum of FS and SE	1.0	91.27±2.58
	10.0	88.83±1.27
	20.0	86.13±1.83

perimental approach starting with a chemical conversion of the ASG into SG by mild alkaline hydrolysis. Afterwards, the unsaponifiable lipid including the total SG (sum of original SG and SG released from ASG) was extracted and subjected to PBA-SPE to determine total steryl glycoside as the sum of non-acylated and acylated steryl glycosides. As an example of this broadly applicable sample preparation and clean-up protocol, Fig. 5 shows the HPLC–ELSD chromatograms obtained from rape seed lecithin spiked with cholesterol-β-D-glucoside as internal standard.

3.4. Recovery, calibration and repeatability of the method

Recovery experiments for the whole sample clean-up procedure were carried out using soya lecithin as matrix spiked with internal standard solutions of SG and FS. As listed in Table 1, sample clean-up procedures for the isolation of SG and ASG as well as of FS showed excellent recoveries around 85–100%.

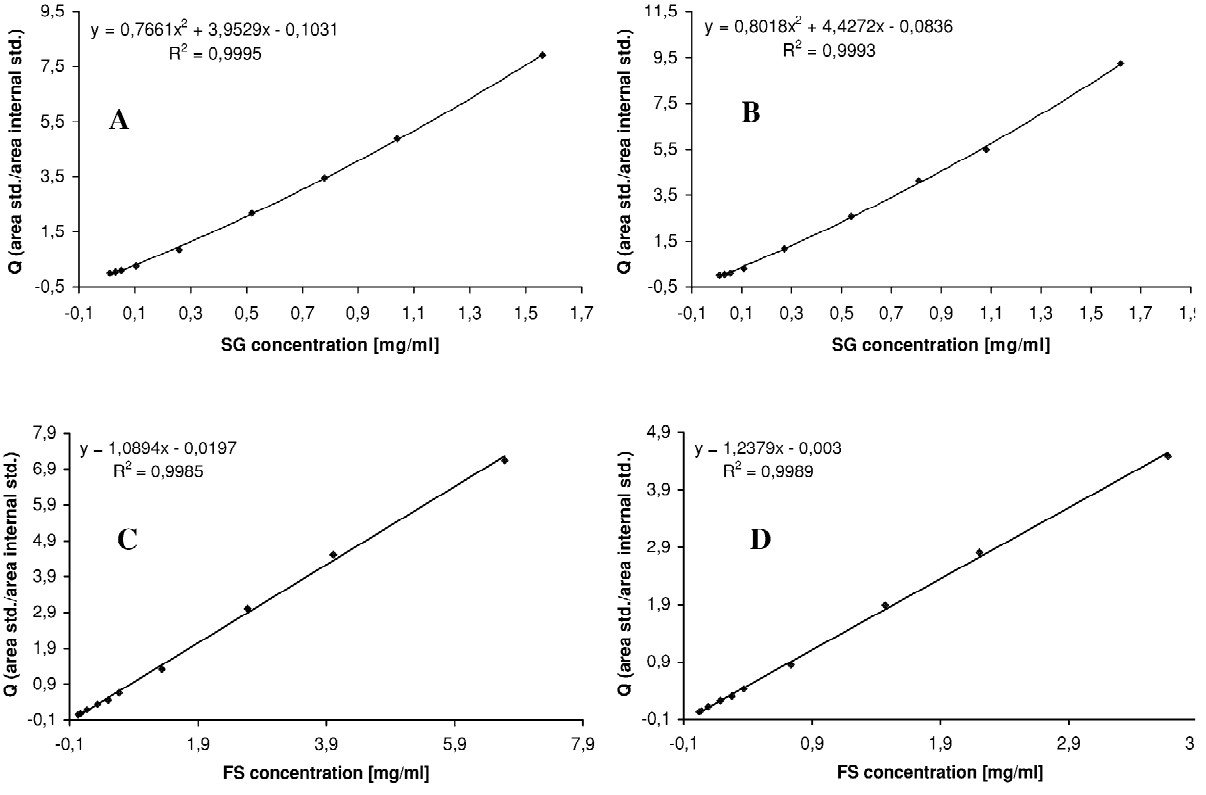

Fig. 6. Calibration curves and their equations for: (a) SG (method without alkaline hydrolysis), obtained by HPLC–ELSD; (b) Total steryl glycosides (method with alkaline hydrolysis), obtained by HPLC–ELSD and for the sum of FS and SE; (c) β-sitosterol and (d) campesterol, obtained by GC–FID.

Calibration curves for steryl glycosides (Fig. 6a) and total steryl glycosides (Fig. 6b) obtained by ELSD detection were not linear, especially at lower concentration levels but which is common for this detector system. Calibration data were established by appropriate curve-fitting calculations with correlation coefficients for the regression being 0.9993 or greater. The calibration curves obtained for free sterols exemplified for β-sitosterol (Fig. 6c) and campesterol (Fig. 6d) analysis by GC–FID showed excellent linearity.

Repeatability of the method was tested by analysing six aliquots of the same soya lecithin sample (Table 2) and was found to be sufficient (RSD values are in the range of 1.8–4.7%) for this type of analysis.

3.5. Application of the presented method

In order to demonstrate the utility of this method, several plant matrices (e.g. lecithins, pumpkin seeds, pharmaceutical formulations containing plant extracts) were analysed.

Consistent with the literature [18] a high content of sterolic compounds could be determined in lecithin (Table 2). In particular, the content of glycosylated sterols (SG and ASG), in the range of 3–28 mg/g was very high. In this context lecithins were also used in the present work to isolate higher quantities of Δ5-phytosteryl glycosides.

In comparison with these results, the content of the specific Δ7-phytosteryl glycosides detected in pumpkin seeds (Table 3) was about 0.3 mg/g. Moreover, there were no acylated steryl glycosides detected.

The pharmaceutical formulations referenced as pumpkin seed extracts examined in this work offered a 10- to 20-fold enrichment of pumpkin seed ingredients. (Formulation A offers for instance a 20-fold enrichment of pumpkin seed ingredients thus 1 g

Table 2
Sterol content of different commercially available lecithins

Compound	RRT*	Soya bean[a] lecithin (mg/g)	RSD** $n=6$ (%)	Soya bean[b] lecithin (mg/g)	Rape seed[c] lecithin (mg/g)
FS (FS+SE)					
Brassicasterol	1.04	n.d.	–	n.d.	1.46
Campesterol	1.10	1.44	1.9	1.69	3.22
Stigmasterol	1.14	1.56	2.1	1.75	<LOQ
β-Sitosterol	1.20	3.34	1.8	4.26	5.33
Δ5-Avenasterol	1.25	<LOQ	–	<LOQ	<LOQ
SG					
Brassicasterol	0.92	n.d.	–	n.d	2.41
Campesterol+stigmasterol	1.14	3.76	3.7	3.57	4.14
β-Sitosterol	1.30	9.72	3.1	7.98	12.17
Δ5-Avenasterol	0.94	0.82	4.2	1.07	n.d.
ASG					
Brassicasterol		n.d.	–	n.d	<LOQ
Campesterol+stigmasterol		6.75	4.7	8.68	0.60
β-Sitosterol		12.17	3.5	19.27	2.21
Δ5-Avenasterol		0.19	3.9	0.04	n.d.

n.d., not detected; LOQ, limit of quantification.

*Values are relative to the internal standards; FS, dihydrocholesterol (analysed as the trimethylsilyl ether by GC–FID); SG, cholesterol-β-D-glucoside (analysed as the free steryl glycoside by HPLC–ELSD).

**RSD values of the repeatability test for soya bean lecithin a ($n=6$).

[a] Soya bean lecithin, Lucas Meyer GmbH, Hamburg, Germany (average value, $n=6$).

[b] Soya bean lecithin, Lucas Meyer GmbH, Hamburg, Germany.

[c] Rape seed lecithin, Werba OHG, Vienna, Austria.

Table 3
Sterol content of pumpkin seeds and different pharmaceutical formulations containing pumpkin seed extracts

Compound	RRT*	Pumpkin seed[a] (mg/g)	Pharmaceutical formulation A[b] (mg/g)	Pharmaceutical formulation B[c] (mg/g)
FS (FS+SE)				
Brassicasterol	1.04	n.d.	<LOQ	<LOQ
Campesterol	1.10	0.04	0.07	0.03
Stigmasterol	1.14	<LOQ	<LOQ	<LOQ
β-Sitosterol	1.20	0.11	0.15	0.05
Spinasterol	1.22	0.51	0.19	0.04
Poriferasta-7,22,25-trienol	1.25	0.48	0.18	0.02
Poriferasta-7,25-dienol+ Δ7-Stigmastenol	1.30	0.53	0.14	0.02
Avenasterol	1.36	0.22	0.10	0.01
SG				
Poriferasta-7,22,25-trienol	0.70	0.08	0.23	0.09
Poriferasta-7,25-dienol	0.86	0.06	0.14	0.07
Avenasterol	0.91	0.05	0.15	0.07
Spinasterol	1.09	0.07	0.20	0.10
Δ7-Stigmastenol	1.23	<LOQ	n.d.	n.d.
ASG				
Poriferasta-7,22,25-trienol		n.d.	n.d.	n.d.
Poriferasta-7,25-dienol		n.d.	n.d.	n.d.
Avenasterol		n.d.	n.d.	n.d.
Spinasterol		n.d.	n.d.	n.d.
Δ7-Stigmastenol		n.d.	n.d.	n.d.

n.d., not detected; LOQ, Limit of quantification.
*Values are relative to the internal standards; FS, dihydrocholesterol (analysed as the trimethylsilyl ether by GC–FID); SG, cholesterol-β-D-glucoside (analysed as the free steryl glycoside by HPLC–ELSD).
[a] Average content for three different pumpkin seed samples from different agricultural fields.
[b] Dr Böhm (Extr.sem.Cucurbitae peponis styriacae sicc. 20:1, 20-fold enrichment of pumpkin seed ingredients) 500 mg, Apomedica, Graz, Austria.
[c] Apozema (Extr.sem.Cucurbitae peponis styriacae sicc. 10:1) 100 mg, Apomedica, Graz, Austria.

formulation corresponds to 20 g of pumpkin seeds.) From that we expected also an enrichment of free and conjugated sterols but the contents measured were unexpectedly low. The free sterol content (sum of FS and SE) was distinctively lower in both formulations as in authentic pumpkin seed samples and it was remarkable that brassicasterol, a Δ5-sterol specific for e.g. rape seeds, could be detected in this formulation. Similar quantitative results were obtained for steryl glycosides because no 10- to 20-fold enrichment was detected. The content of steryl glycosides in formulation A was about 0.8 mg/g (two- to threefold enrichment) and in formulation B about 0.3 mg/g (no enrichment).

Furthermore, the composition ratios of Δ5- and Δ7-sterols for pumpkin seeds and the formulations were clearly different. Pumpkin seed oils [12] and the examined pumpkin seeds showed a relative content of Δ5-sterols of up to 10% referring to the total sterol content. For the investigated formulations this content ranged between 25 and 50%.

From these quantitative and qualitative results obtained, we cannot distinguish whether or not the different contents on active ingredients related to the

specific phytosterols and phytosteryl glycosides, are the consequence of different plant extraction conditions or different species of pumpkin seeds or of the admixture of other plant extracts, e.g. rape seed oil. However, no brassicasterol glycoside was found which indicates that only rape seed oil could be mixed with the pumpkin seed extract. Considering the different polarity of the extractands (FS and SG) and the relative ratios of the analytes to each other may also include that the extracts of the formulations are based on extracts of the remaining oil press cake which is richer in SG than in FS which are in the pumpkin seed oil.

In the present study, we refer results to the naked seed variety of pumpkin seed (*Cucurbita pepo* L. convar. *citrullina* I.GREB. var. *styriaca* I.GREB.) which is particularly rich in oil content (between 40 and 45% of dry mass) and may also be in phytosterol content. Other pumpkin seed species may contain much less free and conjugated phytosterols.

However, in the text of the pharmaceutical formulations it is claimed that they offer a 10- to 20-fold enrichment of highly active pumpkin seed ingredients without specifying them and without quantitative data. For phytosterols and their glycosides, being claimed as the pharmacologically active ingredients, it seems that the enrichment factors specified cannot be corroborated.

4. Conclusion

In order to be able to determine the total plant sterol content (sum of FS, SE, SG and ASG) in different plant matrices a multidimensional sample preparation protocol based on efficient solid-phase extraction steps has been developed. HPLC analysis in combination with ELS-detection has been shown to be a useful technique in analysis of molecular species of steryl glycosides. A method for the isolation and purification of phytosterols and their glycosides was developed. Additionally a set of steryl glucosides was synthesised. Due to the implementation of this standard material quantitative data can also be generated which offers the possibility to analyse in the future more precisely the steryl glycoside content in different plant materials and extracts thereof.

The present method and sample preparation concept is very general and open to allow qualitative and quantitative analysis of phytosterols and their conjugates in diverse oil seeds, lecithin, pharmaceutical formulations containing plant extracts but also of other sample matrices such as for example, root extracts, etc.

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