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Detection of adulteration of pumpkin seed oil by analysis of content and composition of specific Δ7-phytosterols

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Abstract A simple and accurate method for the determination of phytosterols by capillary gas chromatography was developed for the analysis of the seeds and oil of the pumpkin *Cucurbita pepo* L., the naked seed variety growing in the southern Styrian parts of Austria. After extraction of the oil and saponification, the remaining unsaponifiable material was isolated and purified using silica gel columns. For greater volatility, the sterols were derivatised to trimethylsilylethers, and gas chromatography was performed on a column of high polarity, allowing separation of the Δ5-sterols from the Δ7-sterols and thus yielding a typical chromatographical pattern. Quantification of the phytosterols using 5α-cholestan or cholesterol as internal standards led to a method of high accuracy. Comparison of the chromatographic pattern of the phytosterol fraction of pumpkin seed oil with those of other vegetable oil samples permitted the use of this method to detect adulteration of pumpkin seed oil.

Key words Pumpkin seed oil · Oil adulteration · Δ7-Phytosterols · Sample clean-up procedure · Gas chromatography

Introduction

Pumpkin seed oil pressed from the seeds of *Cucurbita pepo* L. convar. *citrullina I.GREB*. var. *styriaca I.GREB*. is a popular salad oil in the southern Styrian parts of Austria, in Hungary and in Slovenia. Seeds of Cucurbitae are also commonly used phytotherapeutically in the therapy of small disorders of the prostate gland and the urinary bladder [1]. Recent studies also showed a potential anti-inflammatory activity of pumpkin seed oil in the treatment of adjuvant arthritis in rats [2].

Due to the laborious production process, involving the manual harvest of the seeds, this expensive oil is often adulterated by the addition of cheap vegetable oils such as sunflower or rape-seed oils. The intense dark green colour of pumpkin seed oil and its characteristic smell of various pyrazines [3] make it difficult for the consumer to recognise the admixture of even large amounts of other oils. In general, efforts have been made to detect adulteration of oils by their tocopherol patterns [4] and also by the fatty acid contents of the oils [5]. Recent studies on the variability of the tocopherol content in pumpkin seeds and their oil revealed high γ-tocopherol values but lower α-tocopherol values with a broad range of variation, whilst β- and δ-tocopherols were detected only at low levels [6], making it difficult to detect admixtures. As dominant fatty acids, ca. 10% palmitic (C_{16}), ca. 5% stearic (C_{18}), ca. 35% oleic ($C_{18:1}$) and ca. 50% linoleic ($C_{18:2}$) acids were found in the seeds of the most frequently used breeding lines, Wies 371 and Gleisdorf, and the oils thereof; the levels of other fatty acids were below 0.5% [5, 7, 8]. As the fatty acid composition may be similar to that of other vegetable oils and may also depend on the climate and area of cultivation, detection of the adulteration of pumpkin seed oil via the fatty acid pattern can be critical. However, rape-seed oil admixture is incontrovertibly detected through erucic acid, which is not normally present in pumpkin seed oil.

Consequently, as a complementary technique, specific components in the unsaponifiable fraction of the oil were considered as markers for adulteration. The predominant part of the unsaponifiables includes various hydrocarbons, triterpenoids, carotenoids, tocopherols and phytosterols. These minor lipid compounds have proven of interest to food analysts and the compo-

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sition and/or ratios of these trace compounds can provide a "fingerprint" for edible oils [9–11]. In contrast to other vegetable oils with Δ5-sterols (β-sitosterol, campesterol, stigmasterol) as major components, pumpkin seed oil contains specific Δ7-phytosterols (Fig. 1) [12], together with their glycosides, which have been suggested to be active inhibitors of the enzyme 5α-reductase, due to their conformative similarity to testosterone [1]. The concentration of dihydrotestosterone in the prostate gland can thus be reduced using these compounds, followed by an improvement of the typical symptoms in the early stages of prostate hypertrophy. A simple but unreliable thin layer chromatographic method based on these compounds has been established by Sauter et al. [13] in order to detect adulteration of pumpkin seed oil. Starting from their results an accurate method for the determination of the *Cucurbita*-type Δ7-phytosterols by gas chromatography (GC) was developed.

A number of analytical methods for the analysis of phytosterols in vegetable oils have already been published. Normally, these method protocols comprise saponification (with potassium hydroxide) or transesterification of the triglycerides [9, 10] followed by the extraction of the unsaponifiable material from the saponified fatty acids by liquid-phase separation with cyclohexane [14] or hexane [15], thin-layer chromatography on silica-gel plates [16, 17], on-line liquid-gas chromatography [18, 19] or, alternatively, off-line supercritical fluid chromatography [20], subsequent derivatisation to form trimethylsilylethers and final analysis on a nonpolar GC capillary column [9–21]. However, these columns are not convenient for the analysis of the phytosterol pattern of pumpkin seed oil as β-sitosterol (1) and spinasterol (2) (Fig. 1) appear as one peak in the chromatogram. This has already been shown in the structure-retention relationship studies of sterols and triterpene alcohols in GC on a glass capillary column (OV-1) by Itoh et al. [22]. By switching to a GC capillary column of intermediate to high polarity (with 35% diphenyl polysiloxane) the compounds of interest can be chromatographically separated, thus allowing the exact quantification of the Δ5- as well as of the Δ7-phytosterols of pumpkin seed oil in one run. In addition to this improvement of the chromatographic separation of the phytosterols of interest, this contribution describes a new sample preparation method simplifying the removal of the interfering triglycerides by the use of silica gel columns. Subsequently, this method was used to establish the average sterol content for a representative number of pumpkin seed samples leading to a set of data enabling the detection of adulteration of pumpkin seed oil via a "finger printing" and multiple component approach.

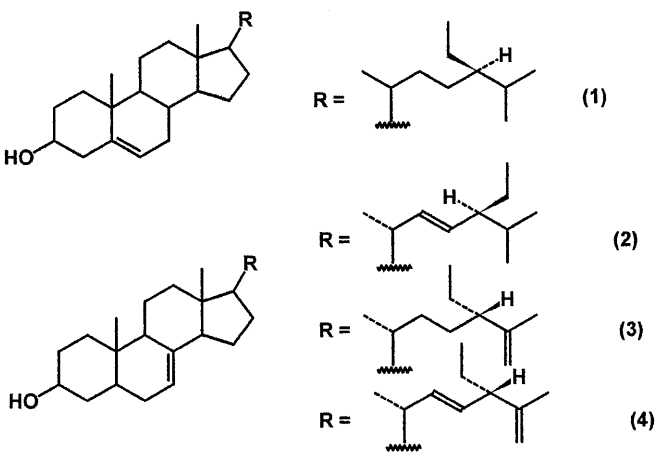

Fig. 1 Structures of the Δ5-phytosterol, β-sitosterol (*1*) and the Δ7-phytosterols, spinasterol (*2*), stigmasta-7,25-dienol (*3*), and stigmasta-7,22,25-trienol (*4*)

Materials and methods

Chemicals and solvents

Standard materials β-sitosterol (60% purity, containing 33% campesterol) and stigmasterol (96%), and internal standards cholesterol (96%) and 5α-cholestane (98.3%) and 2,6-di-*tert*-butyl-4-methylphenol (BHT) were obtained from Sigma (Vienna, Austria) as was the derivatising reagent *N,O*-bis(trimethylsilyl)trifluoracetamide (BSTFA). All solvents (petroleum benzene, *tert* butylmethyl ether and ethyl acetate) were of analytical grade from Merck (Darmstadt, Germany). Silica gel 60 no. 7734 (particle size 0.063–0.200 mm, 70–230 mesh ASTM), anhydrous sodium sulphate p.a. and potassium hydroxide p.a. were purchased from Merck. An artificial oil neutral for pharmaceutical purposes as fatty matrix for calibration had to be used, as all vegetable oils contain small amounts of phytosterols.

Sample material and sample preparation

Pumpkin seed samples originated from various fields in the southern parts of Styria. Oil samples were commercially available at different local shops and markets.

Extraction of the oil seeds. Pumpkin seeds (50 g) were ground with a mill and 5 g of this granulate was mixed with 5 g anhydrous sodium sulphate, 20 mg BHT and 50 ml petroleum benzene. As this sample preparation technique can also be used for the analysis of organochlorine pesticides [23], samples may be dosed with stock solutions of pentachlorobenzene and Mirex as internal standards. The suspension of the seed granulate in petroleum benzene was ultrasonicated for 1 min followed by shaking of the mixture for 12 h in order to facilitate extraction of the total vegetable oil from the seeds. The average oil content of pumpkin seeds was between 45% and 55%, determined after evaporation of the solvent.

Saponification and clean-up procedure of the oil-seed extract and/or the respective vegetable oil. To 0.5 g vegetable oil or 0.5 g evaporated pumpkin seed oil extract, 250 µl of the internal standard solution (5 mg ml^{-1} cholesterol or 2 mg ml^{-1} 5α-cholestane, both in dichloromethane) and 1 ml of a potassium hydroxide solution (20 g KOH in 88 ml methanol with 12 ml deionised water to limit the transesterification to methylesters) were added. This mixture was saponified for 45 min at 70 °C in a 10-ml screw-capped reaction tube. The end of the reaction was indicated by the clearing of the two-phasic oil-methanol/water system.

A glass column (20 cm length and 1.5 cm i.d.) with a glasswool frit at the bottom was dry-filled with 1 g anhydrous sodium sul-

phate as the lowest layer and 1 g silica gel as a second layer. The total saponified oil mixture adsorbed to 3 g silica gel by rotary evaporation gave the third layer of this sandwich-type sample preparation column. The unsaponifiable material was eluted from the silica column with 20 ml of a mixture of *tert* butylmethyl ether and ethyl acetate (1:1, v/v), while the potassium salts of the fatty acids were retained on the column. From the total extract collected (about 15 ml) a volume of 1.5 ml was subjected to the derivatisation/silylation reaction.

Derivatisation to trimethylsilyl ethers. The extract (1.5 ml) was transferred to a 2 ml vial, and the solvent removed by a stream of nitrogen. To the residue, 50 μl of a mixture consisting of BSTFA and dry pyridine (2:1, v/v) and 400 μl of *tert* butylmethyl ether was added. The sealed vial was heated at 70 °C for 2 h and 1 μl of this mixture was injected onto the GC column.

Capillary GC

Analyses were carried out on a Hewlett Packard HP 5890 A gas chromatograph equipped with a flame ionisation detector (FID) or an HP 5972 GC-MSD as a mass-specific detector. A sample volume of 1 μl was injected using an HP 7673 A autosampler with a Hamilton 10 μl syringe connected to a capillary inlet with a glass liner in the splitless mode. The column was a fused silica capillary column (30 m length and 0.25 mm i.d.), coated with a 0.25 μm layer of crosslinked 65% dimethyl-35% diphenyl polysiloxane (HP 35), purchased from Hewlett Packard (Vienna, Austria). The injector and detector were heated at 300 °C. The oven temperature was isothermal at 280 °C with a 45 min run time. Helium 5 was used as a carrier gas at 16 psi (110 kPa) column head pressure, corresponding to a flow rate of 1.1 ml min^{-1}. GC control and data processing were performed using an HP Chemstation with Win 95 software.

For GC-MS analysis, the transfer line was heated to 280 °C, and ionisation energy was 70 eV in the electron impact mode. The spectra were scanned within the range *m/z* 60–500.

Quantification of the phytosterols and calibration

Quantification of the phytosterol trimethylsilyl ethers was performed via calibration curves using a neutral oil as fatty matrix spiked with β-sitosterol, campesterol and stigmasterol. The area of each phytosterol peak was referred to the area of the peak of the internal standard. GC-MS analyses were quantified at *m/z* 129 (corresponding to fragment C1-C3 + TMSO). Instead of 5α-cholestane, bearing no free hydroxylic functionality, cholesterol had to be chosen as internal standard, although vegetable oils are reported to possibly contain minimal amounts of this sterol [21]. For further studies the use of saturated α-cholestanol as internal standard may be an option.

FID response factors of the Δ7-phytosterol trimethylsilyl ethers could not be determined due to the lack of standard material, so the FID response was taken to be one for all compounds. Achieved concentration values can not, therefore, be regarded as absolute. Nevertheless, calibration plots showed excellent linearity as confirmed by correlation coefficients (Table 1) and reproducibility of the method.

Table 1 Calibration data for Δ5-phytosterols in neutral oil

Compound	Correlation coefficient	Slope	Intercept
Campesterol	0.999	0.4937	0.0271
Stigmasterol	0.999	0.3387	0.0498
β-Sitosterol	0.999	0.5025	0.0298

Results and discussion

The described sample preparation and pre-concentration protocol enabled the complete separation of the minor components of vegetable oils, including the phytosterols from the triglycerides and fatty acids, thus preventing deterioration of sensitive GC detectors and/or capillary columns and revealing false positive results by various non-controlled chromatographic interferences. By means of the self-filled silica gel columns, the potassium salts of the fatty acids were retained while the unsaponifiables were quantitatively eluted with the solvent mixture chosen. The derivatisation step which followed had the advantage of providing sharp peaks at lower oven temperatures, although pyridine had to be included in the derivatizing reagent as a catalyst. Figure 2 shows a typical chromatogram of a pumpkin seed oil sample on a non-polar column (100% dimethylsilicon). Peaks could be identified by comparison with the literature [22, 24] and by GC-MS data; trimethylsilyl ethers of spinasterol and β-sitosterol appear as one peak in the chromatogram (Fig. 2, peak 1). As exact quantification required separation of Δ5- from Δ7-phytosterols, a column of higher polarity and therefore different selectivity was chosen. This separation is depicted in Figure 3, and although peak 3 represents a mixture of Δ7-stigmastenol and Δ7,25-stigmastadienol, as confirmed by the MS-Data (Fig. 4), the desired resolution for the main components had been achieved. The precision of the method was tested by analysing six aliquots of the same oil sample (Table 2) and found to be good, except for the added value of Δ7-stigmastenol and Δ7, 25-stigmastadienol (RSD = 5.1%). If exact quantification is desired for all compounds, this can be achieved with an additional injection of the sample on a non-polar GC column, e.g. HP1, as presented in Fig. 2, where baseline separation of Δ7-stigmastenol and Δ7, 25-stigmastadienol is possible.

Finally, the method was applied to the analysis of the oil extracts of 147 different pumpkin seed samples, the results of which are summarised in Table 3. The same spectrum of sterol compounds could be observed in all samples and despite the deviations of the individual values the ratios of the single components to each other were identical. Analyses of 19 genuine pumpkin seed oil samples obtained through pressing led to the same results, although slight differences in the absolute phytosterol content could be found, each phytosterol compound had the same ratios to the others as with the oil extracts from the seeds. It has to be observed that no response factors could be calculated due to the lack of suitable standard substances and that sterol glycosides (possibly 50% of the sterol content [25]) were not considered as they can not be extracted from the seeds and are not covered by this sample pre-treatment protocol. Subsequently, other vegetable oils, such as sunflower-seed oil, rape-seed oil and soya bean oil were also analysed, demonstrating the broad applicability of

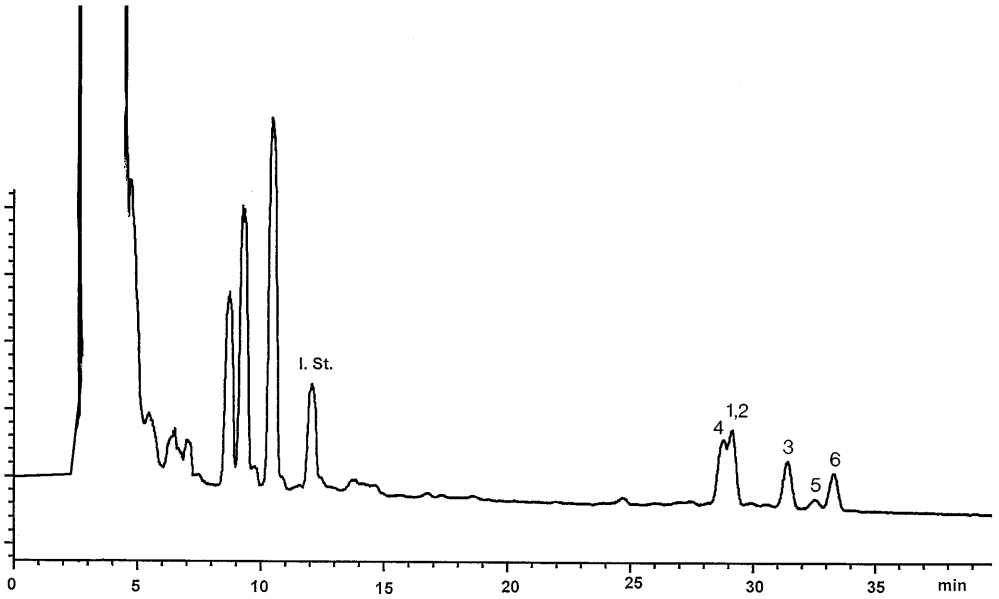

Fig. 2 GC-FID chromatogram of pumpkin seed oil on a non-polar GC capillary column (HP 1, 25 m length and 0.32 mm i.d., 0.52 μm film thickness), for conditions see Materials and methods. I. St. Internal standard, *1* β-sitosterol, *2* spinasterol, *3* stigmasta-7,25-dienol, *4* stigmasta-7,22,25-trienol, *5* Δ7-stigmastenol, *6* Δ7-avenasterol

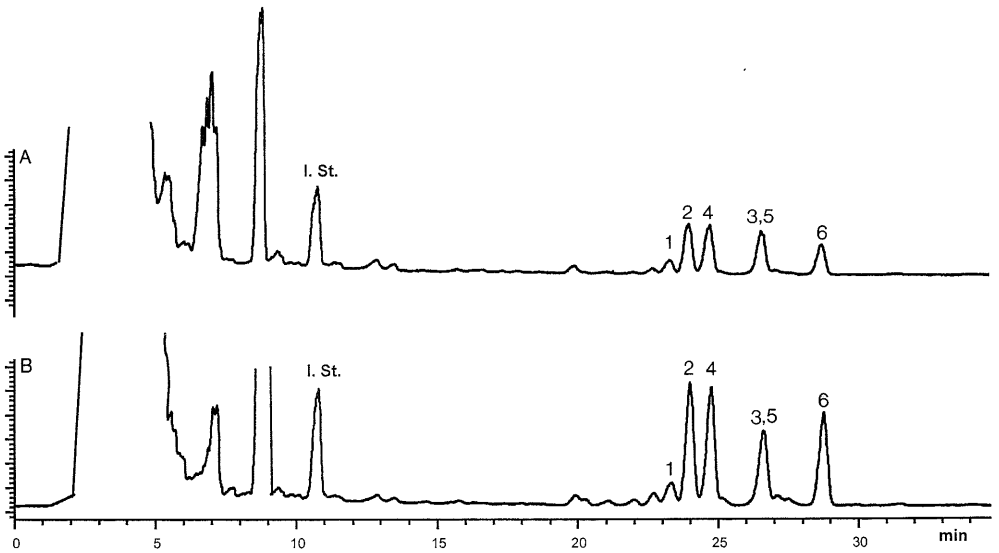

Fig. 3 GC-FID chromatograms of **A** pumpkin seed oil and **B** extract from pumpkin seeds on a GC column of intermediate polarity (HP 35). For exact conditions see Materials and methods; peaks numbered according to Fig. 2. 1. *St* Internal Standard

this method (Fig. 5). As expected, β-sitosterol was the main sterol compound in these oils, accompanied by smaller amounts of campesterol and stigmasterol, resulting in other but also typical chromatographic patterns like the one for pumpkin seed oil. This fact allows the identification of the respective oil either by the existence of single peaks (e.g. brassicasterol in rape-seed oil and Δ7-phytosterols in pumpkin seed oil) and/or by the ratios of β-sitosterol versus campesterol and versus stigmasterol.

Concentration values and ratios of each sterol compound for all 147 pumpkin seed samples were summar-

Table 2 Reproducibility of the method for pumpkin seed oil

Compound	Average (μg ml^{-1})	RSD (%, $n=8$)
β-Sitosterol	84.6	1.9
Spinasterol	447.8	3.7
Δ7,22,25-Stigmastatrienol	427.5	4.3
Σ Δ7-Stigmastenol + Δ7, 25-Stigmastadienol	395.4	5.1
Δ7-Avenasterol	230.1	3.5

Table 3 Average sterol content for 147 different pumpkin seed samples

Compound	Average (μg ml^{-1})	Minimum (μg ml^{-1})	Maximum (μg ml^{-1})
β-Sitosterol	58	8	120
Spinasterol	300	150	450
Δ7, 22, 25-Stigmastatrienol	326	72	504
Σ Δ7-Stigmastenol + Δ7, 25-Stigmastadienol	310	54	1032
Δ7-Avenasterol	164	54	280

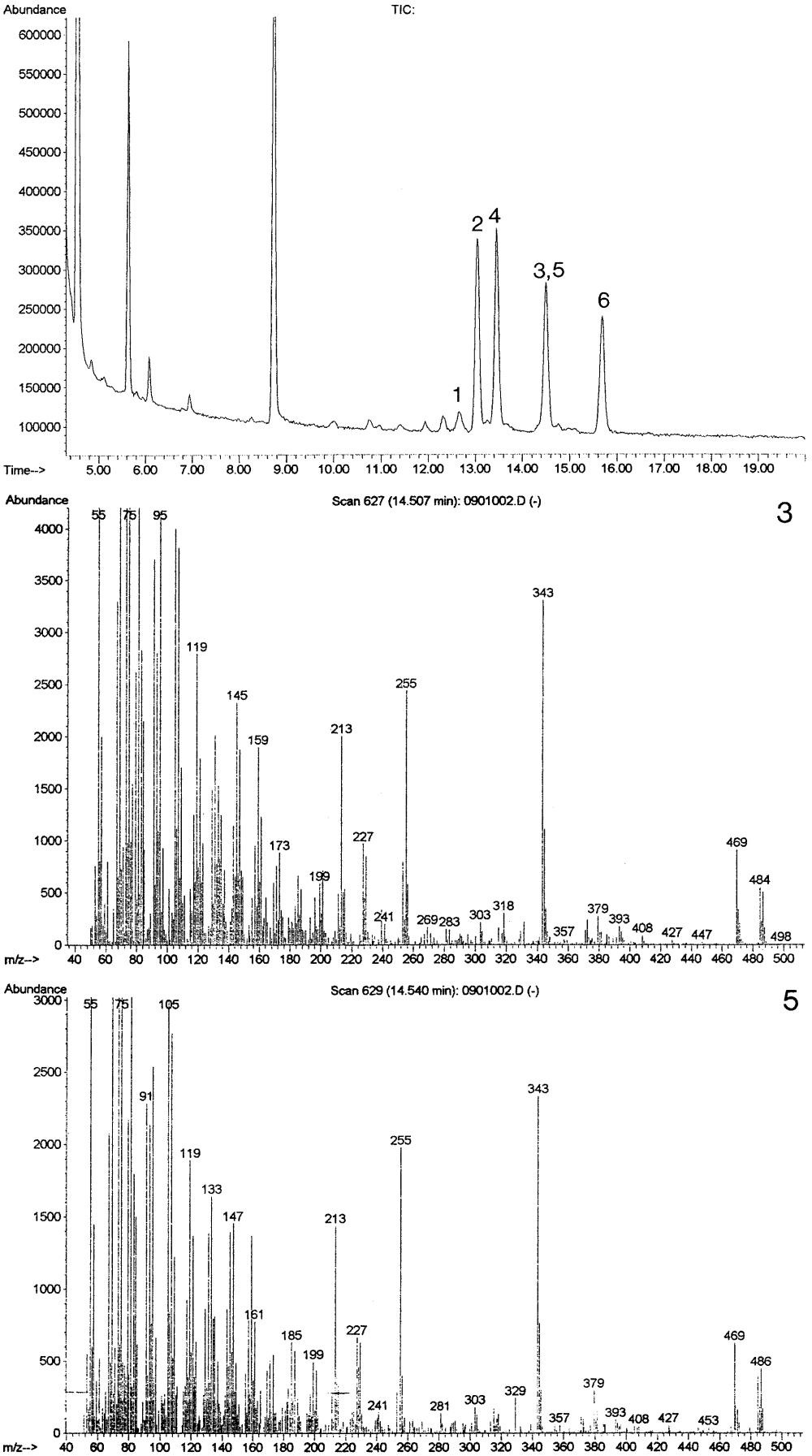

Fig. 4 GC-MS chromatogram of pumpkin seed oil and MS spectra of peaks 3 and 5 on a GC column of intermediate polarity (HP 35). For conditions see Materials and methods, peaks numbered according to Fig. 2

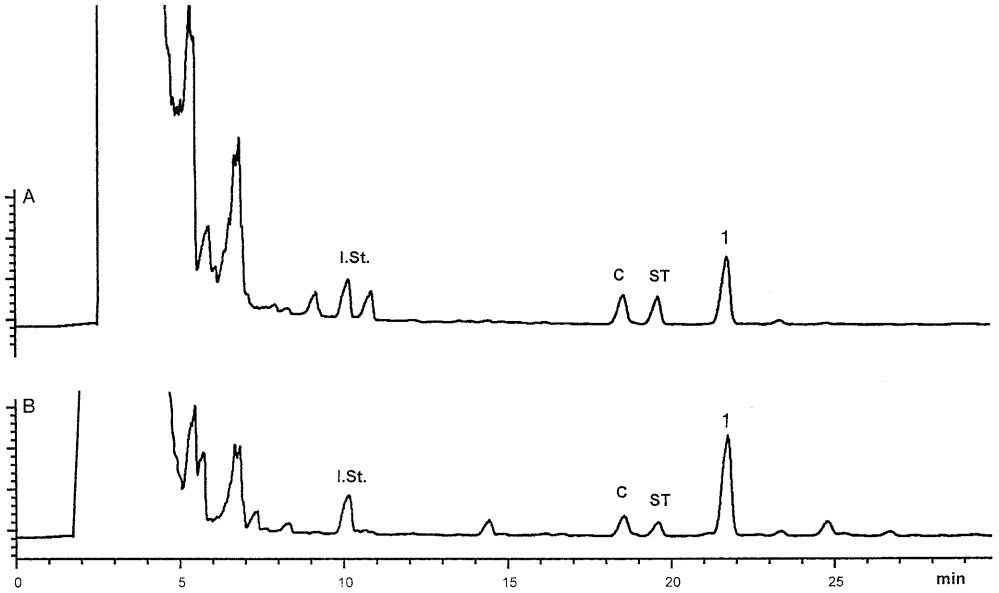

Fig. 5 GC-FID chromatogram of **A** soybean oil and **B** sunflower-seed oil on a GC column of intermediate polarity (HP 35). For conditions see Materials and methods. *I. St* Internal standard, *C* campesterol, *ST* stigmasterol

ised in a cluster analysis (Fig. 6). With the exception of 6 samples (Fig. 6 lower left quadrant) – probably due to different breeding lines – all extracted oil samples as well as the so-called genuine cold-pressed pumpkin seed oils exactly fitted into the statistical pattern, thus permitting conclusions about the purity of pumpkin seed oil via its sterol components. In order to demonstrate the usefulness of the method, pumpkin seed oil was spiked with increasing amounts (0.5–10%, v/v) of sunflower-seed oil, rape-seed oil and soy bean oil, frequently used as adulterants. Admixtures of these oils were easily detected from as low as 2%, through increased concentrations of campesterol and β-sitosterol in the chromatogram and the resulting shifts of the sterol concentrations and their ratios in the statistical evaluation. Additional evidence for adulteration or identification of the admixture was provided by other phytosterols, such as brassicasterol (found in rape-seed oil) or stigmasterol, that are not present in pumpkin seed oil. Finally, six pumpkin seed oil samples that had not been clearly identified as pure or adultered via their fatty acid pattern, were analysed, and their phytosterol con-

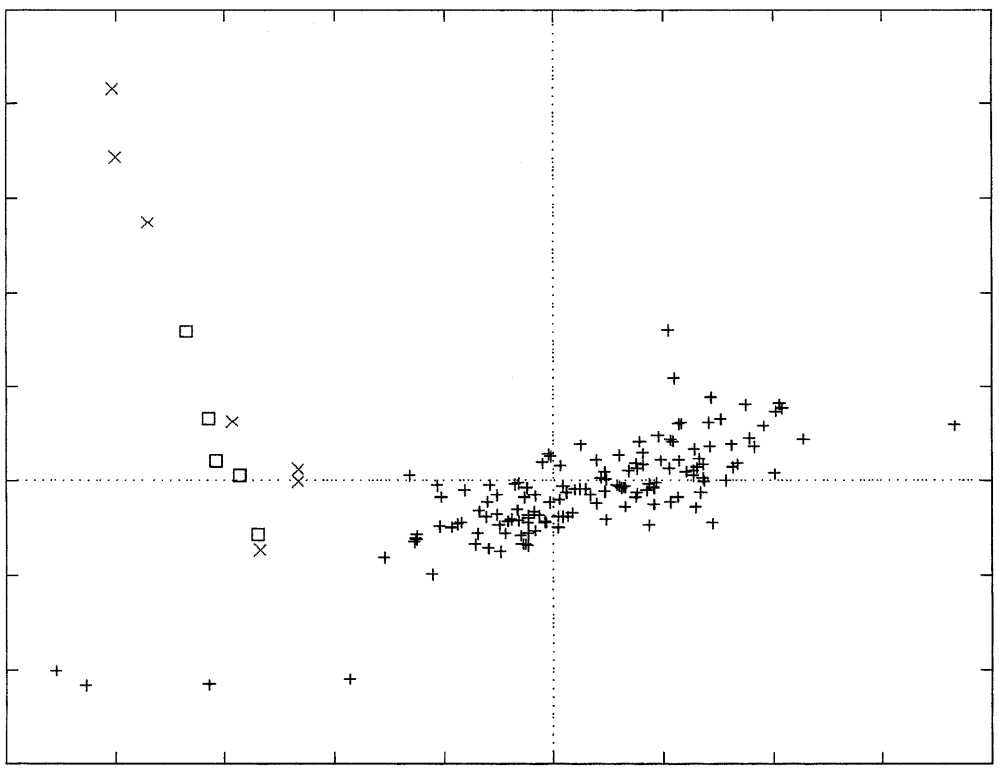

Fig. 6 Statistical evaluation (single link method of clustering) of the fingerprinting and multiple component approach. + Oil extract from pumpkin seeds and pure pumpkin seed oil, □ and × adulterated oils

centrations were integrated in the statistical analysis. Five samples clearly fitted into the area of the self-made adulterations, whilst the other sample was identified as pure via its sterol pattern.

In conclusion, a simple and reliable analysis procedure for the determination of both compositions and contents of phytosterols in vegetable oils and oil seeds has been established. Sample concentration and removal of the fatty acids was reduced to a one-step procedure employing a simple silica gel column purification. Additional information was gained through GC-MS data analysis. A further outcome of this general sample work-up protocol is the possibility of using the extracts for the well-established method for determination of organochlorine pesticides (e.g. various DDTs and HCHs) described by Filek and Lindner [23]. Based on the analysis of 147 extracted oil samples of seeds of *Cucurbita pepo* revealing a statistically settled distinct pattern of species-dependent phytosterols, detection of adulterated pumpkin seed oil at even low concentrations (starting at 2% for admixture of sunflower-seed oil) was made possible and the method may also allow the identification of the admixed vegetable oil via the sterol pattern. Although vegetable oil adulteration is preferentially detected by the main components, the triglycerides and via the fatty acid composition, the qualitative and quantitative analysis of minor compounds as phytosterols may lead to additional and complementary information, especially for identifying the sources of admixture.

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