A new method for determination of campesterol, stigmasterol and β-sitosterol in edible oils by supercritical fluid chromatography

NINGLI QI – YIJUN LIU – LIANGKUN LIAO – XIAO GONG – CHUNLIANG YANG

Summary

This study aimed to develop a rapid method for separating three major phytosterols (stigmasterol, campesterol and β-sitosterol) in edible oils by supercritical fluid chromatography using the ultra-performance convergence chromatography system. The samples were prepared by alkaline saponification followed by *n*-hexane extraction and then separated on a high strength silica C_{18} column with selectivity for bases using acetonitrile-methanol (50 : 50) as a co-solvent with a gradient elution (98 : 2 to 80 : 20) and UV-spectrophotometric detection at 210 nm. The optimized method yielded a linear calibration curve ranging from 0.07 ng·ml^{-1} to 200 ng·ml^{-1} for stigmasterol, from 0.12 ng·ml^{-1} to 200 ng·ml^{-1} for campesterol and from 0.06 ng·ml^{-1} to 200 ng·ml^{-1} for β-sitosterol. The limit of detection and limit of quantification of the phytosterols were 20–42 ng·ml^{-1} and 75–117 ng·ml^{-1}, respectively. The recovery rates ranged from 96.4 % to 101.2 % with relative standard deviations of 1.7–3.8 %, depending on the sterol type and the specific sample. The developed method is simple and sensitive, as demonstrated by its successful use for phytosterol characterization in edible oils.

Keywords

campesterol; stigmasterol; β-sitosterol; edible oils; supercritical fluid chromatography

Vegetable oils are used for cooking and frying as well as in food formulations. The vegetable oil consumption in China has markedly increased with a remarkable income growth in the last two decades. The annual consumption of vegetable oil exceeds 30 million tons in China [1], making it one of the largest importing countries in the world. The nutritional value and antioxidant characteristics of the edible plant oils have gained immense attention in terms of technology research and new product development.

Phytosterols are a group of compounds similar to cholesterol, which naturally occur in plants and differ from cholesterol in carbon side chains and/or presence or absence of a double bond. They are found in many edible plants, nuts, seeds, vegetables and edible oils. So far, 200–300 different types of phytosterols have been successfully separated and identified in botanical sources, where campesterol, stigmasterol and β-sitosterol were found to be dominant and most frequently studied [2]. Phytosterols have attracted great attention due to their nutritional properties and biological effects, for example their anti-hypocholesterolemic, anti-inflammatory, anti-oxidative and anti-tumour activities. They are now widely used in pharmaceuticals, nutritional supplements and cosmetics [3–5].

The official methods for separation and quantitative analysis of phytosterols are conventionally based on gas chromatography (GC). However, it usually requires chemical derivatization for favourable peak shape, better sensitivity and resolution, and a higher stability for labile unsaturated sterols with thermal instability [6]. In general, liquid chromatography (LC) has better operating conditions, including milder temperatures and pressure conditions for column separation, non-

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destructive analysis as well as several available detectors and has been widely used for sterol separation [5, 7, 8]. ROCCO and FANALI [9] developed a nano-LC method for the determination of stigmasterol, campesterol and β-sitosterol in extra-virgin olive oil, after sample treatment, with good sensitivity, precision and speed, the analysis taking 20 min. ZARROUK et al. [10] for the first time reported on LC with positive ion, atmospheric pressure chemical ionization and ion trap mass spectrometry, which was successfully applied to direct determination of sterols and lipids with no sample preparation and derivatization required. Seven plant sterols and stanols could be separated within 8.5 min using an ultra-high-performance LC method, with a low limit of detection (*LOD*) value of 400–600 $mg \cdot ml^{-1}$ was obtained [11]. Supercritical fluid chromatography (SFC) integrates the advantages of both ultraperformance LC and supercritical fluid separation techniques. It exhibits good performance in terms of thermal stability and volatility in GC analysis, significantly reduces the analysis time of LC separation and can also reduce organic solvent usage. As SFC is effective, less time-consuming and cost-effective in terms of separation and determination, it is extensively applied in foodomics, biopharmaceuticals, drug epidemiology and environmental monitoring [12, 13].

To date, no studies on SFC use for separation of phytosterols from food, including edible oils in China, have been reported. The present study aimed to develop a simple and rapid method for simultaneous separation of three major phytosterols, namely, campesterol, stigmasterol and β-sitosterol, using an ultra-performance convergence chromatography (UPC^2) system coupled to diode array detector (DAD). Further, it was efficiently validated and used in phytosterol analysis and characterization of edible oils that are widely consumed in South China.

MATERIALS AND METHODS

Raw materials

Nine brands of edible oils, including palm oil, tea tree oil, linseed oil, blend oil, camellia oil, colza oil, soybean oil, olive oil and sunflower oil, were obtained from local retail outlets in Zhanjiang, China. They were all produced in 2016 and represented approximately 90.0 % of the vegetable oil consumption in South China.

Chemicals and reagents

The standard compounds of campesterol (99.6 %), stigmasterol (98.1 %) and β-sitosterol (98.6 %) were obtained from ANPEL Laboratory Technologies (Shanghai, China). HPLC-grade methanol and acetonitrile were obtained from Merck Chemicals (Shanghai, China). Carbon dioxide (99.9 %) was obtained from Zhanjiang Oxygen Plant (Zhanjiang, China). High-purity deionized water was prepared by a Milli-Q ultrapure purification system (Millipore, Billerica, Massachusetts, USA). All other chemicals and reagents were of analytical grade.

Standard solutions and calibration

The working standard solutions of individual sterols were prepared by dissolving the respective reference compounds separately in methanol. A linear regression equation was prepared from five increasing concentrations by diluting the stock solution in methanol. A linear relationship between peak area and concentrations (0.06–200.00 $\mu g \cdot ml^{-1}$) was obtained, and the linear regression equation for each standard was used for phytosterol quantification in the selected oil samples.

Sample preparation

The procedure for phytosterol extraction reported by BEDNER et al. [14] was slightly modified and applied to the sample preparation. Briefly, 1.0 g edible oil sample was weighed, transferred into a 50 ml saponification bottle and mixed with 2.0 $mol \cdot l^{-1}$ ethanolic potassium hydroxide (10 ml). The mixture was immediately vortexed and placed in a boiling water bath in darkness for 30 min. After saponification, the sample was cooled down in an ice-water bath, 10 ml of deionized water was added and the resulting solution was transferred into a separatory funnel. The unsaponifiable fraction was twice extracted with 10 ml of *n*-hexane and washed with distilled water until neutral pH. The extracts were combined and dried under a constant nitrogen gas flow supplied using an N-EVAP 112 nitrogen evaporator (Organomation Associates, Worcester, Massachusetts, USA), while the samples were maintained at room temperature. The residues were dissolved in HPLC-grade methanol (2.0 ml) and then filtered through a syringe nylon membrane filter (pore size 0.22 μm).

Instrument and separation conditions

Phytosterols were quantitatively and qualitatively analysed using ACQUITY UPC^2 system (Waters, Milford, Massachusetts, USA) consisting of a Diode Array Detector (DAD) detector, an autosampler, an automated back pressure regulator (ABPR), a column oven, a binary sol-

vent delivery pump and a convergence chromatography manager. The separation was carried out on an ACQUITY UPC^2 high strength silica C_{18} with selectivity for bases (HSS C_{18} SB) column (100 mm × 3.0 mm, particle size 1.7 μm; Waters). The elution process started at 98 % of A (supercritical CO_2) and 2 % of B (acetonitrile-methanol, 50:50), decreased via linear gradient to 90 % of A for the first 0.5 min, held for 3.0 min, declined linearly to 80 % of A within 2.0 min, and then was maintained for 1.0 min. The temperature inside the chromatography column was kept almost constant at 35 °C, and a flow rate of 1.2 ml·min^{-1} was used. The automated back pressure regulator (ABPR) was maintained at the range of 10.3–20.7 MPa. Absorbance was recorded at 210 nm with compensation from 260 nm to 360 nm, and the injection volume was 2.0 μl. All assays were performed at least in triplicate, and an external standard method was applied to the phytosterol quantification. Data processing was performed using Empower 3 software (Waters).

Method validation and analysis of samples

Identification of analyte compounds was based on retention time matching and co-injection with authentic standards under identical analytical conditions. The analytical method was validated for linearity, limit of detection (*LOD*) and limit of quantification (*LOQ*), precision and accuracy. Selected samples of edible oil were analysed using the optimized method.

The linearity range was evaluated by plotting the relative peak area of phytosterol versus the relative concentration. The correlation coefficient (R^2) was calculated for linearity evaluation. The lowest concentrations at the signal-to-noise ratio of 3 and 9 were used for *LOD* and *LOQ* determination, respectively. The intraday (repeatability) and interday (reproducibility) precisions were determined by measuring individual sterol concentrations in six replicates during a single day and in three replicates on five consecutive days, respectively. Precision was calculated using the relative standard deviation (*RSD*). Accuracy was expressed as recovery of each phytosterol. Briefly, 1 g soybean oil sample was carefully weighed and extracted, and then spiked with moderate concentrations of a standard (0.8–1.2 mg·ml^{-1}), representing approximately 80.0 %, 100.0 % and 120.0 % of the original contents of phytosterols in the oil sample. The recovery value was calculated by comparing the content determined with that of the added. All procedures were performed in triplicate and the injection was performed in duplicate.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

Chromatographic conditions were optimized for good separation according to the following steps. First, the column screening experiment using four kinds of packing particles with different selectivity was conducted. Second, different co-solvents on the capacity ratio (retention volume, bandwidth, and resolution) were examined to enhance resolution. Finally, the effects of gradient program, flow rate, ABPR and temperature of column on sensitivity, resolution and separation time were evaluated.

The columns Tours Diol, Ethylene-Bridged Hybrid (BEH), BEH 2-ethylpyridine (BEH 2-EP; (all 100 mm × 3.0 mm, particle size 1.7 μm), and HSS C18 SB (150 mm × 3.0 mm, particle size 1.8 μm; all from Waters) were investigated. Although various stationary phases and several gradient programs were tested, at least two sterols were not separated using the columns except for HSS C18 SB, on which the standard sterols were fully separated. Therefore, further experiments were performed using the HSS C18 SB column, which enabled high selectivity and high resolution. Different co-solvents (methanol, acetonitrile, *n*-hexane, and isopropanol) and/or ratios were evaluated to enhance the separation of the three phytosterols. The results showed that the best peak shape and resolution were achieved when CO_2 and co-solvent B (acetonitrile-methanol, 50:50) mixture was used, with a linear gradient elution mode. Besides, the highest theoretical plate number of the analytes was obtained by this mobile phase. Therefore, it was selected as the optimal co-solvent.

The gradient elution was optimized, the optimal program starting at 98 % of supercritical CO_2 (A) and 2 % of acetonitrile-methanol (50:50) (B), decreased via linear gradient elution to 90 % of A for the first 0.5 min, maintained for 3.0 min, declined linearly to 80 % of A in 2.0 min, held for 1.0 min and finally returned to the initial conditions for column equilibration within 0.5 min.

The analytes were fully separated, the peak resolutions between the main peaks ranging from 1.4 to 1.5. ABPR could change the eluting power of supercritical carbon dioxide by altering the density and viscosity of the confluent phase, which was directly related to dissolution [15]. In this study, ABPR at 13.8 MPa showed the best separation performance and was applied to the analysis of phytosterols, the flow rate and column temperature being maintained at 1.2 ml·min^{-1} and 35 °C, respectively. High resolution with values greater

than 1.4 were achieved in the optimized chromatographic conditions.

Method validation

For validation, the linearity of calibration curves was observed under the optimized analytical conditions at the injection of increasing concentrations of individual working solutions of each analyte. The equations of the calibration curves were obtained by least-squares linear regression method, and the linearity was obtained with correlation coefficients higher than 0.9996 within concentration ranges of 0.06–200 ng·ml^{-1}, 0.07–200 ng·ml^{-1} and 0.15–200 ng·ml^{-1} for campesterol, stigmasterol and β-sitosterol, respectively (Tab. 1).

The precision of an analytical procedure expresses the closeness of agreement or the degree of scatter between a series of measurements obtained from multiple sampling of the same homogenous sample under aforementioned optimized conditions. In the present work, low *RSD* values of repeatability (0.3 %, 0.3 % and 0.4 %) and reproducibility (1.0 %, 1.0 % and 1.3 %) were obtained (Tab. 2) using the SFC method for campesterol, stigmasterol and β-sitosterol analysis, while corresponding *RSD* values ranging from 3.6 % to 43.9 % for repeatability and from 0.0 % to 43.9 % for reproducibility had been reported by Sorenson et al. [6] using the GC procedure.

The accuracy of the SFC procedure was evaluated by combining the closeness of agreement between the conventional true and assigned values. As shown in Tab. 2, the recovery of campesterol, stigmasterol and β-sitosterol was 98.5 %, 96.4 % and 101.2 %, respectively, reflecting a lower matrix effect on the derivatization efficiency, which was consistent with the acceptable values reported (99.8 %, 111.0 % and 111.0 %) in the aforementioned AOAC official method [16]. It was also better than the results from a previous study using HPLC with higher recovery rates (> 80 %) and lower *RSD* values (< 4.2 %) [17].

LOD and *LOQ* were measured by analysing a series of standard working solutions. *LOD* was defined as the lowest quantity of each phytosterol that produced a signal at least three times the average, the values being approximately 27 ng·ml^{-1}, 42 ng·ml^{-1} and 20 ng·ml^{-1} of stigmasterol, campesterol and β-sitosterol, respectively. These values were remarkably lower compared to 0.78 μg·ml^{-1}, 0.78 μg·ml^{-1} and 0.40 μg·ml^{-1} reported in previous studies [9, 11]. *LOQ* was defined as the minimum content of each phytosterol that could be quantitatively measured in the edible oil product, and it was 75 μg·kg^{-1}, 117 μg·kg^{-1} and 66 μg·kg^{-1} for stigmasterol, campesterol and β-sitosterol, respectively. *LOQ* values of stigmasterol and β-sitosteol obtained in the present study were obviously lower than the values of approximately 3 g·kg^{-1} and 10 mg·kg^{-1}, respectively, obtained by Yuan et al. [8] and Rocco and Fanali [9].

In the system suitability test, both peak fronting and tailing were observed for all components when tested at a higher level. The distorted peaks had a low tailing factor (0.60–0.64) but remained within the linear range and did not affect the chromatographic resolution significantly. The low tailing factor indicated that the tailing slope of the peak was sharper than the leading slope.

Tab. 1. Parameters of the method for determination of phytosterols.

Phytosterols	*RT* [min]	Calibration curve	R^2	Linear range [mg·ml^{-1}]	Resolution
Stigmasterol	5.81	$y = 457894x + 5087.7$	0.9998	0.07–200.00	–
Campesterol	6.03	$y = 177693x + 7158.3$	0.9996	0.12–200.00	1.40
β-Sitosterol	6.26	$y = 65106x + 7717.5$	0.9998	0.06–200.00	1.42

In calibration curve $y = mx + b$, y is the integrated peak area and x is the concentration.
RT – retention time, *R* – correlation coefficient.

Tab. 2. Sensitivity, precision and accuracy of the developed method for detection of phytosterols.

Phytosterol	*LOD* [ng·ml^{-1}]	*LOQ* [ng·ml^{-1}]	Repeatability	Reproducibility	Recovery	*RSD*
Stigmasterol	27	75	0.3	1.0	98.5	2.0
Campesterol	42	117	0.3	1.0	96.4	3.8
β-Sitosterol	20	66	0.4	1.3	101.2	1.7

Sensitivity is presented as *LOD* and *LOQ*, precision is expressed as repeatability and reproducibility with *RSD*, and accuracy was evaluated by the recovery test.
LOD – limit of detection, *LOQ* – limit of quantification, *RSD* – relative standard deviation.

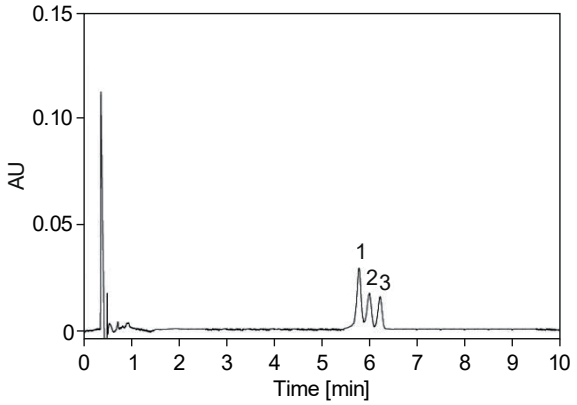

Fig. 1. Separation of a standard mixture of phytosterols.

Peak identities: 1 – stigmasterol, 2 – campesterol, 3 – β-sitosterol.

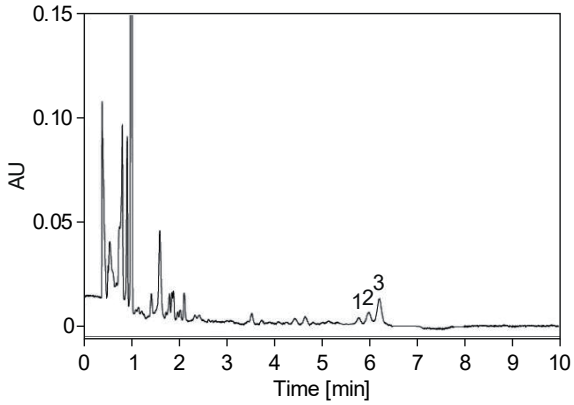

Fig. 2. Analysis of phytosterols in a colza oil sample.

Peak identities: 1 – stigmasterol, 2 – campesterol, 3 – β-sitosterol.

Compared with the existing chromatography approaches, the SFC procedure based on UPC2 system has the shortest analysis time (less than 7 min), with high resolution, tailing factor, theoretical plates and capacity (data not shown).

Analysis of phytosterols in selected oil samples

To evaluate the applicability of the developed method to analysis of edible oil, nine brands of commercial vegetable oil widely consumed in South China were purchased from commercial suppliers and analysed under the aforementioned conditions. Fig. 1 and Fig. 2 provide characteristic chromatograms of the standard mixture and of the saponified sample of colza oil, respectively. The contents of three major phytosterols, namely, stigmasterol, campesterol and β-sitosterol in the selected oil samples are summarized in Tab. 3. Colza oil had the highest content of total sterols (stigmasterol, campesterol and β-sitosterol; 6.78 g·kg^{-1}), followed by olive oil (2.25 g·kg^{-1}), while palm fruit oil sample had the lowest sterol content (0.29 g·kg^{-1}). As expected, β-sitosterol was the dominant sterol and campesterol was present at a lower level in all samples, which was consistent with results of previous studies [7, 18]. The sterol proportions of colza oil and sunflower oil in this study agreed remarkably well with those determined using HPLC by YANG et al. [19]. The sterol composition and content in linseed oil were consistent with those reported by WANG et al. [20], β-sitosterol (1.42 g·kg^{-1}) being the only sterol found in camellia oil sample. However, a discrepancy in sterol distribution was observed for olive

Tab. 3. Identification and relative contents of phytosterols in edible oil samples from the market.

Food items	Brand/Supplier	Stigmasterol [g·kg^{-1}]	Campesterol [g·kg^{-1}]	β-Sitosterol [g·kg^{-1}]	Total sterols [g·kg^{-1}]
Tea tree oil (crude)	Ganjiang (Nanchang, China)	–	–	0.89 ± 0.21	0.89
Linseed oil (cold-pressed)	Knife (Shenzheng, China)	0.45 ± 0.18	0.20 ± 0.31	0.55 ± 0.04	1.20
Blend oil (expeller-pressed)	Dinghuang (Jiangmen, China)	0.40 ± 0.27	0.31 ± 0.22	0.72 ± 0.53	1.43
Palm fruit oil (refined)	Julong (Tianjin, China)	0.09 ± 0.11	–	0.20 ± 0.18	0.29
Camellia oil (hydrogenated)	Golden Dragon Fish (Qinhuangdao, China)	–	–	1.42 ± 0.27	1.42
Olive oil (extra virgin)	Olivoilŕ (Tianjin, China)	0.16 ± 0.12	–	2.09 ± 0.13	2.25
Colza oil (expeller-pressed)	Longevity Flower (Binzhou, China)	2.16 ± 0.13	1.40 ± 0.41	3.22 ± 0.17	6.78
Sunflower oil (crude)	Mighty (Shanghai, China)	0.12 ± 0.10	0.09 ± 0.12	0.26 ± 0.10	0.57
Soybean oil (hydrogenated)	Yingma (Zhongshan, China)	–	–	0.46 ± 0.09	0.46

The concentration of each phytosterol is reported as grams of phytosterols per kilogram of oil samples. The edible oil samples were analysed in triplicate and reported as mean ± standard deviation.

oil, soybean oil and blend oil between the present study and other studies, which might have been due to the differences in the raw materials, storage conditions, refining process as well as the extraction and analysis methods used in different studies [18, 21]. In China, phytosterols are considered as important indicators in the quality evaluation of vegetable oil products. No mandatory regulation of sterols from the hygienic aspect are operational for edible oils.

CONCLUSIONS

The developed SFC method using an UPC^2 system was suitable for quantification of major phytosterols in edible vegetable oils, namely, stigmasterol, campesterol and β-sitosterol. The chromatographic separation could be completed within 7 min, which represents an obvious improvement in efficiency of the analysis. The accuracy of the developed method conformed with the overall recovery rates, ranging from 96.4 % to 101.2 %, and reproducibility with the *RSD* values lower than 1.4 %. With regard to the matrix effect, the components such as aliphatic alcohols, tocopherols and hydrocarbons may be present in the unsaponified fractions, in particular those eluted before 5.5 min, which should be studied in the future. Distribution of sterols in the edible oil samples was affected by not only raw material sources, types and refining processes but also by sample preparation and assay methods. The developed SFC method provided an available and alternative approach for the qualitative and quantitative analysis of phytosterols in edible vegetable oils

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