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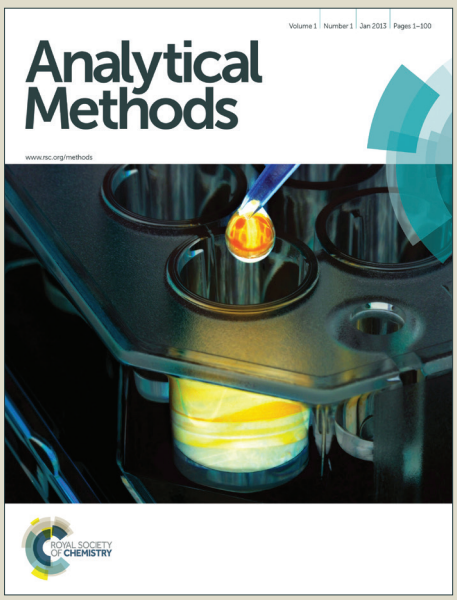

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Characterization and authentication of four important edible oils using free phytosterol profiles established by GC-GC–TOF/MS

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Abstract

Adulteration of high-price edible oils has become a focus of attention and a tough problem in the food trade and consumption all over the world. Therefore, there is a great demand for detecting oil adulteration to protect interests and rights of customers and safeguard their health. In this study, free phytosterol profiles of peanut, soybean, rapeseed, and sunflower seed oils were established by SPE–multidimensional gas chromatography coupled with time-of-flight mass spectrometry (GC-GC–TOF/MS) and employed to classify the four edible oils with the help of unsupervised (principal component analysis and hierarchical clustering analysis) and supervised (random forests) multivariate statistical methods. The results indicated that free phytosterol profiles of edible oils could help classify the four edible oils into four groups completely, and therefore could be taken as important markers of the oils studied. Moreover, a simulated data test revealed that free phytosterol profiles could also be used to detect peanut oil adulterated with 5% soybean oil, which was simulated by the Monte Carlo method.

Keywords: Free phytosterol profiles; Adulteration identification; Edible oil; GC-GC–TOF/MS; Chemometrics

Introduction

Vegetable oils play a vital role in human nutrition as the most important food in our daily life. They provide energy and nutritional components including but not limited to essential fatty acids, phytosterols, tocopherols, phenolic compounds, and vitamins[1, 2], as well as greatly affect flavor and taste of food. In edible oil consumption in China, soybean oil possesses the largest market share, followed by rapeseed, peanut, and sunflower seed oils[3]. However, due to the non-transgenic merit and pleasant flavor, the market shares of peanut, rapeseed, and sunflower seed oils have been increasing gradually though they are more expensive than soybean oil in China[3]. As the same as olive oil in western countries, these high-price oils adulterated with lower-price oils including soybean oil as a major adulterant, have become the biggest source of agricultural fraud in China and other developing countries. Therefore, reliable detection of such adulterations is in great demand.

Adulteration of edible oils has been chronically practiced for many years. Besides economic fraud, it sometimes causes potential harms or threats to the health of consumers[4]. To ensure authenticity of edible oils, a number of analytical methods have been established to detect and quantify these adulterations. The most common methods are based upon detection and quantification of one or more particular compounds, which are specific to adulterants and absent from authentic oils. Some previous studies detected adulteration of a target oil by analyzing marker(s), such as detecting olive oil adulterated with soybean, peanut, sunflower seed, corn, or sesame oil by the level of trilinolein (LLL)[5], and using specific sesamol to detect sesame oil adulterated with other oils or fats[6]. Although these methods are simple and reliable for routine detection, an obvious limitation is that not every oil/fat has its own marker, so that they are not always effective for purity tests of all edible oils. Another option is to directly analyze oils without sample pretreatment or with only organic solvent-based dilution. In this

respect, a number of methods were proposed based on fluorescence spectroscopy[7, 8], Raman spectroscopy[9, 10], Fourier transform near infrared spectroscopy[11, 12], mid-infrared spectroscopy[13, 14], nuclear magnetic resonance spectrometry[15], electronic nose[16, 17], and differential scanning calorimetry[18], as well as chemometric analysis methods such as linear discriminant analysis[11, 12], multiple linear regression[14, 18], principal component analysis[12, 17], cluster analysis[15], partial least squares[7, 8], and artificial neural networks[16]. Since the optical and electrical signal based methods use the integrated information of the whole sample but not quantitative information of some components, chemometric analysis methods are necessary to identify adulteration. Multivariate analysis could provide a higher accuracy rate for adulteration identification. However, every coin has a flip side. An optimized predictive model depends on the training samples, and is therefore hard to detect possible adulteration out of the training set. As a compromise, metabolite profiles become promising for detection of oil adulteration. Recently, some specific compounds in oil samples were taken as the target compounds, including polar compounds[19], triacylglycerols (TAGs)[20, 21], fatty acids[22, 23], or volatile compounds[24]. In the third strategy, chemometric methods were employed to select important markers and establish a discriminative model for adulterated oils and pure oils.

As characteristic and potential nutrient components of vegetable oils, phytosterols make up the largest proportion of the non-saponifiable fraction[25]. They are a group of naturally occurring substances derived from hydroxylated polycyclic isopentenoids[26]. As in many other foods, sterols occur in edible oils as free sterols and conjugated forms including steryl fatty acid esters, phenolic acid esters, steryl glycosides, and acylated steryl glycosides[27]. Generally, the analysis of plant sterols in edible oils is mainly based on the determination of the amounts of free sterols and liberated ones from steryl fatty acid esters after saponification[28, 29], or determination of the total amounts after a combination of

conjugated sterols of all types liberated by acidic and alkaline hydrolysis[30]. In contrast, the entire information of free sterols' distributions and concentrations in edible oils is rare. Investigation on free sterols in edible oils are commonly based on the isolation of this type of compound by solid phase extraction (SPE)[31-33] or preparative online/offline liquid chromatography (LC)[34, 35] and analysis by means of GC-FID[31], GC-MS[32, 33], or reversed-phase high-performance liquid chromatography equipped with an evaporative light-scattering detector (RP-HPLC-ELSD)[25]. Using these approaches, free sterols were determined in several edible oils[31-33]. In previous studies[31-33], however, there are insufficient qualitative and quantitative data on the distributions of free sterols in edible oils, and the studies mainly focused on the dominating sterols, such as β-sitosterol, campesterol, stigmasterol, brassicasterol, delta-5-avenasterol, and sitostanol. Therefore, it is necessary to obtain the entire information on the distributions and contents of free sterols in different edible oils.

As a set of important metabolism products, phytosterols exist in edible oils, the contents and compositions of which mainly depend on the plant species and also vary with agronomic, geographical, and climatic conditions and the oil processing technology[36]. Recently, phytosterol profiles were employed to characterize and classify virgin olive oils by the genetic variety or olive ripening degree[36-38]. In addition, Gázquez-Evangelista et al.[39] determined the contents of 4-desmethylstrols using offline HPLC-GC-FID and established the concentration profiles to discriminate extra virgin olive oil, pomace olive oil, sunflower seed oil, and soybean oil. However, the phytosterols determined and used for establishment of the discriminate model were based on the total amount, including the liberated ones released from the sterol esters, so that the sample preparation used in those studies included saponification, solvent extraction and concentration, and thin-layer chromatography (TLC), which were a tedious, time-consuming, and non-environmentally-friendly (requiring large amounts of organic

solvents) procedure. In contrast, information about free phytosterol profiles used to characterize, classify, and detect oil adulteration is unavailable. Therefore, the aim of this study was to develop classification and adulteration identification methods for soybean, peanut, rapeseed, and sunflower seed oils using free phytosterol profiles. Firstly, we developed a rapid and environmentally-friendly SPE method for free phytosterol extraction, and then established a GC-GC coupled with TOF/MS analysis method for phytosterol detection after trimethylsilyl (TMS) derivatization. Secondly, after qualitative and quantitative analysis of free phytosterols, unsupervised (principal component analysis (PCA) and hierarchical clustering analysis) and supervised (random forests, RF) multivariate statistical methods were used to build a classification model for the four edible oils. Thirdly, free phytosterol profiles were employed to detect peanut oil adulterated with 5% soybean oil, which was simulated by the Monte Carlo method[40].

Materials and methods

Oil samples

Edible plant oils used in this study consist of oils pressed in the laboratory and commercially available refined oils. To ensure that the selected oil samples could represent the actual status of commercially available peanut, soybean, rapeseed, and sunflower seed oils, we adhere to the following sampling rules: (a) with respect to four types of oil seeds selected for laboratory pressing, each type of sample should be planted in large amounts in the main producing areas of China; (b) the commercially available four types of refined oils should be provided by a large edible oil company dominating the Chinese oil market. The detailed information about 20 hulled peanut (*Arachis hypogaea* L.) seeds, 19 soybeans (*Glycine max* (L.) Merr.), 40 rapeseeds (*Brassica campestris* L.), and 19 hulled sunflower (*Helianthus annuus* L.) seeds are shown in Supplementary Material Table S1. The commercially

available refined edible oils (including 6 peanut oils, 8 soybean oils, 7 rapeseed oils, and 6 sunflower seed oils) were purchased at the local market and stored in darkness at 4°C for further analysis. According to the labels, the peanuts, rapeseeds, and sunflower seeds used for oil processing are non-genetically modified organism (GMO) materials, whereas the soybeans are of the GMO material.

Before squeezing, the sunflower seeds and peanut seeds were hulled manually, and then the four types of oil seeds (dehulled peanuts, soybeans, rapeseeds, and dehulled sunflower seeds) were dried at 60°C for 4 h in a thermostat oven. To obtain laboratory pressed oils, these seeds were squeezed using a TEN GUARD oil pressing machine (TZC-0502, made in China). In each round of squeezing, 100 g of oil seeds (pre-fragmentized peanuts, pre-fragmentized soybeans, rapeseeds, or dehulled sunflower seeds) was loaded to the hopper, and then performed at direct squeezing mode. In these conditions, the oils obtained from peanuts, rapeseeds, and sunflower seeds were about 30 mL, while the oils obtained from soybeans were about 15 to 20 mL. Finally, the raw oils obtained were centrifuged (2306 × *g* for 10 min) to separate non-oil fractions from the oil phase, and the purified oils were loaded into 10 mL brown sample bottles fully, and capped tightly, then stored in darkness at 4°C. All of these oil samples were analyzed within one month. During the processing, the machine was cleaned thoroughly when squeezing of each sample was finished.

Reagents and solvents

Cholesterol (3β-cholest-5-en-3-ol, with purity of 99%), brassicasterol ([24S]-24-Methyl cholesta-5,22-dien-3β-ol, of the analytical standard), campesterol ([24R]-24-Methyl cholest-5-en-3β-ol, with purity of 98%, but shown to contain 35% dihydrobrassicasterol by ^{13}C-NMR), stigmasterol ([24S]-24-Ethyl cholesta-5,22-dien-3β-ol, with purity of 95%), and β-sitosterol ([24R]-24-Ethyl cholest-5-en-3β-ol, with purity ≥ 97%) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cholestanol (5α-cholestan-3β-ol, of the analytical grade) and N-Methyl-N-trimethylsilylheptafluorobutyramide (MSHFBA) were purchased from Sigma-Aldrich, Chemie Gmbh (Steinheim, Germany). 1-methylimidazole was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Normal hexane (HPLC grade), diethyl ether (analytical grade), and anhydrous sodium sulfate (analytical grade) were purchased from Merck (Darmstadt, Germany), and Sep-Pak cartridges (0.5 g Silica) were obtained from Dikma Technologies Inc. (Beijing, China).

Free sterol purification by solid-phase extraction (SPE)

About 1.0 g anhydrous Na_2SO_4 was loaded onto the Sep-Pak cartridge. Fifty milligrams of edible oil were added with 20 μg cholestanol, which was used as an internal standard (IS). The edible oil was dissolved in 5 mL n-hexane and then loaded onto the cartridge, which was first equilibrated with 10 mL n-hexane, at the flow rate of 1.2 mL/min, while the effluent was discarded. Triglycerides on the cartridge were then washed off with 10 mL mixture of n-hexane/ethyl ether (95:5, v/v) at the flow rate of 1.2 mL/min. Finally, free phytosterols were eluted with 10 mL n-hexane/ethyl ether mixture (80:20, v/v) at the flow rate of 1.5 mL/min.

Derivatization procedure

Trimethylsilyl ether derivatives of sterols were prepared according to ISO 12228:1999[41]. The final 10 mL eluted fractions containing phytosterols were rotary-evaporated under vacuum at 50°C to approximately 1 mL, and then the concentrated solution was transferred to a reaction vial, which was dried by a gentle nitrogen flow and then added with 100 μL N-Methyl-N-trimethylsilylheptafluorobutyramide/1-methylimidazole (95:5, v/v) mixture. After that, the vial was sealed and heated at 105°C for 15 minutes and then cooled to room temperature for

GC-TOF/MS analysis.

In-house GC-GC analytical conditions

As for multidimensional GC–TOF/MS, a LECO Corporation Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA) equipped with an Agilent 7890A GC, which contained a primary oven and a separate secondary oven (Agilent Technologies, Santa Clara, CA, USA) was used for GC-GC analysis. The column set consisted of two columns: one was 30 m DB-5ms (0.25 mm I.D. × 0.25 μm film thickness, Phenyl Arylene polymer, Agilent Technologies), and the other was an Rxi-17Sil MS with dimensions of 2 m × 0.15 mm I.D. × 0.15 μm film thickness, similar to 50% phenyl/50% dimethylpolysiloxane (Silarylene), (Restek U.S.). The injection volume was 1 μL in split mode at a ratio of 20:1, with the injector temperature being 320°C. Helium was used as the carrier gas at a constant flow rate of 0.7 mL/min. The primary oven temperature program was that 180°C was held for 1 min and then increased to 300°C at a rate of 4°C/min, with the final temperature held for 13 min; and the secondary oven followed the primary oven with a lead of 10°C. The modulator temperature offset and transfer line temperature were 25°C and 300°C, respectively. The mass spectrometer was operated at an acquisition rate of 10 spectra/s and scanned from 50 *m*/*z* to 550 *m*/*z*. No mass spectrum was collected during the solvent delay for the first 10 min of each run. The detector voltage was set to 1750 V, the electron energy was -70 V, and the ion source temperature was kept at 250°C. The data was processed using LECO Corp's Chromatography TOF software version 4.43.3.0 optimized for Pegasus 4D.

Qualitative and quantitative analysis

For qualitative analysis, sterols with available standards (cholestanol, brassicasterol, campesterol,

stigmasterol, and β-sitosterol) were identified by comparing their retention times (RTs) and mass spectra (MS) with the standards. The peaks were also confirmed with the NIST mass spectral library. Moreover, β-amyrin and other sterols (24-methylene-cholesterol, campestanol, delta-7-campesterol, delta-5,23-stigmastadienol, sitostanol, delta-5-avenasterol, and delta-7-stigmastenol) in oils without available commercial standards were identified by comparing their MS data with the NIST MS library, which were also referred to in the relevant literature[41]. The relative retention times (RRTs) and characteristic fragments of TMS-sterols were provided in Table 1.

For quantitative analysis, unsaturated TMS-sterols were quantified based on an IS. Considering the low level of some free phytosterols in vegetable oils, we selected 5 different fragmentation ions (*m/z*) to calculate the peak areas of different TMS-sterols, expecting to obtain relatively high abundance of ionic fragments (*m/z*). Each group of the 5 fragmentation ions was specific to the corresponding TMS-sterol and had similar response abundance in mass spectra. The groups of fragmentation ions used for different phytosterols are shown in Table 1. The internal calibration curve was obtained using regression between the ratio of the peak areas of the standard to the IS (cholestanol) and the concentration of the standard sterol, and each calibration point was analyzed in triplicate. To quantify the phytosterols with available standards, six levels of standard solutions (each level containing 20 μg cholestanol used as an IS) were prepared and used as data points for calibration curves. Specifically, brassicasterol was set at 0.1, 1, 5, 10, 20, and 30 μg/100 μL, campesterol and stigmasterol were both set at 1, 5, 10, 20, 30, and 40 μg/100 μL, and β-sitosterol at 10, 20, 40, 80, 120, and 140 μg/100 μL. In order to quantify β-amyrin, and the free phytosterols found in the samples (24-methylene-cholesterol, delta-7-campesterol, delta-5,23-stigmastadienol, delta-5-avenasterol, and delta-7-stigmastenol), which were not available as commercial standards, a new calibration curve of

stigmasterol was used to estimate their contents with eight concentration points set at 0.05, 0.1, 0.5, 1, 2, 4, 8, and 16 μg/100 μL (each level added with 20 μg cholestanol). The other saturated free phytosterols (campestanol and sitostanol) were also quantified as their TMS-derivatives using a response factor (*Rf*) of 1.0 relative to the IS cholestanol, owing to their structural resemblance to cholestanol. The concentration of each free phytosterol in edible oils was expressed as mg/100 g of oil, and their contents in each oil sample were determined for three independent replicates, and the mean values were used in further data elaboration.

Validation of the method

To validate the method, a blank edible oil sample that does not contain free phytosterols should be obtained first. According to the SPE procedure in the free sterol purification section of this study, five hundred milligrams of peanut oil (dissolved in 5 mL n-hexane) were processed by SPE from the beginning to the washing step, but instead, the 5 mL sample loading solution and 10 mL washing solution were collected using a test tube which contained triglycerides. Then, the collected effluent was combined and concentrated to 5 mL, which was used as a new loading solution. After that, a new SPE cartridge was also used to perform the operation in the same way, and the loading and washing effluent was collected again. Later, the third and the fourth SPE processing were performed and the loading and washing effluent was collected once again. The finally collected fraction (15 mL) containing triglycerides was rotary-evaporated under vacuum at 50°C to approximately 3 mL, and then the concentrated solution was transferred to a brown sample vial, which was dried by a gentle nitrogen flow. The obtained oil was subjected to qualitative analysis once a day for a total of 7 days, and no free phytosterol was detected, so that it could be used as a blank oil and was stored in darkness at 4°C.

The limit of detection (LOD) and lower limit of quantification (LLOQ) of the selected phytosterol were determined based on the signal-to-noise approach following standard procedures and criteria[42]. According to the natural concentrations of different free phytosterols in the four edible oils, different concentration ranges of each standard phytosterol used for calibration curve establishment were selected, as described in the "Qualitative and quantitative analysis" section of this study, namely 0.1-30 μg/100 μL for brassicasterol, 1-40 μg/100 μL for campesterol and stigmasterol, 10-140 μg/100 μL for β-sitosterol, and 0.05-16 μg/100 μL of standard stigmasterol for the calculation of β-amyrin and the other five unsaturated phytosterols. Each concentration point was performed in triplicate for regression analysis. The GC-GC–TOF/MS responses were linear over the measured concentration ranges with the coefficients of determination (R^2) greater than 0.9987. The repeatability (within-day precision) and recovery rate of the method were confirmed by quality control (QC) samples, which were obtained by spiking blank oil samples with selected standard phytosterols at low, middle, and high concentrations relative to the calibration range with each level performed in triplicate. To assess the stability of the method, a specified QC sample was used for routine check in triplicate once in each day of analysis. The reproducibility of the method in terms of inter-laboratory precision was not assessed.

Statistical analysis

The absolute concentrations of 11 phytosterols and β-amyrin were employed to construct the data matrix. Data preprocessing (Pareto scaling), clustering (PCA and hierarchical clustering analysis), and classification (RF) were conducted by a metabolomic data analysis tool MetaboAnalyst 2.0[43, 44]. Data handling was performed on a Pentium 4 personal computer, and data simulation of adulterated oils was implemented in Matlab 2011a for Windows (The Mathworks, Natick, MA).

Results and discussion

SPE separation and GC-GC–TOF/MS analysis

Sample preparation in our study does not involve alkaline saponification and acid hydrolysis, indicating that it does not recover sterol esters and sterol glucosides but only contains free forms. However, as described in ISO 12228:1999[41], the procedure used for the isolation of phytosterols from vegetable oils included saponification, extraction of the unsaponifiable matter by aluminium oxide column, and TLC. And the contents of individual sterol determined were in total amounts including free sterols and liberated ones from steryl fatty acid ester. Based on SPE, a facile method was developed for the separation of free phytosterols from edible oils. Free sterols were eluted with a mixture of n-hexane/ethyl ether (80:20, v/v) and separated from triglycerides, steryl esters, steryl glycosides, and tocopherols. By comparing the SPE method with the isolation procedure of sterols reported in ISO 12228:1999[41], it could be found that the SPE method was more rapid, convenient, and organic solvent-saving. In the eluted fractions containing free phytosterols, several other constituents were detected. The preliminary analysis by GC-GC–TOF/MS indicated the presence of monoglycerides, diglycerides, and free fatty acids, and the result was consistent with that reported by Esche et al.[45]. However, full structural elucidation has not been performed. Although monoglycerides, diglycerides, and free fatty acids exist, separation and detection of free phytosterols by GC-GC–TOF/MS was not interfered because the contents of these non-target compounds were in a relatively low level and did not occur in the region of free phytosterols.

GC-GC separation was achieved using a 30 m non-polar Phenyl Arylene polymer capillary column connected to a 2 m medium polar (similar to 50% phenyl/50% dimethyl polysiloxane) capillary column, which was proven suitable for the separation of TMS-phytosterols (Fig. 1). By using GC-GC, better

separation of these TMS-sterols could be achieved compared to a single fused-silica column (30 m × 0.25 mm I.D. × 0.25 μm film thickness) coated with 5% phenyl methyl silicone reported by Toledano et al.[34], and better separation could also be obtained in the region of TMS-24-methylene-cholesterol, TMS-campesterol, and TMS-campestanol compared to a single 50 m SE-54 column (0.25 mm I.D. × 0.10 μm film thickness) reported in ISO 12228:1999[41]. Individual TMS-sterols was identified on the basis of RTTs and mass spectral data (Table 1). In this study, we focused on the distributions and concentrations of 11 free sterols (see Table 1) and a sterol-like compound (β-amyrin) in four edible oils, rather than limited to the investigation of the dominating ones such as campesterol, stigmasterol, β-sitosterol, brassicasterol, delta-5-avenasterol, and sitostanol reported in other literatures[31-33]. We intended to establish whole free sterol profiles to classify oils and detect oil adulteration.

(Fig. 1)

(Table 1)

The recoveries, within-day precisions, LODs, and LLOQs of the used IS and representative free phytosterols were presented in Table 2. The recoveries of the IS and selected phytosterols spiked at three different levels (low, middle, and high concentrations) after SPE and GC-GC analysis were ≥ 90% with a qualified within-day precision (relative standard deviation) ranging from 1.3% to 19.2%, which indicated good accuracy and repeatability. The reproducibility in terms of the inter-laboratory precision of the approach was not assessed.

(Table 2)

Determination and quantification of free phytosterols in four edible vegetable oils

To ensure the stability of the approach performed in routine sample analysis, a specified QC sample was obtained by spiking blank oil samples with selected free brassicasterol, campesterol,

stigmasterol, and β-sitosterol at the concentrations of 30, 40, 40, 140 mg/100 g oil, respectively, and the experiment was performed in triplicate once on each day of the analysis. During the whole stage of sample analysis, the relative standard deviations (n = 3) of the determined amounts of brassicasterol, campesterol, stigmasterol, and β-sitosterol were less than 3%, with the recoveries ranging from 94% to 98%.

Under the employed experimental conditions, all oil samples used in this study were analyzed, the content of each free phytosterol in every sample was expressed as the mean value calculated from independent triplicate analyses, and the values were used for further data elaboration. According to the data of free sterols detected in each sample, the contents of free phytosterols representing each oil type were calculated and expressed as the mean value of the free phytosterols in the samples of the same type (where it could be determined and quantified). The detailed data is presented in Table 3. The contents of the found free sterols were also compared with those reported in other literatures**.** Lechner et al.[31] reported the contents of the main free sterols (β-sitosterol, campesterol, stigmasterol, brassicasterol, delta-5-avenasterol, and delta-7-stigmastenol) in rapeseed, sunflower seed, and soybean oils, which were comparable to our results except the contents of campesterol in the three oils. Their results were approximately twice those found in this study. Another difference was that the contents of delta-5-avenasterol and delta-7-stimastenol in sunflower seed oil were 13.1 mg/100 g and 79 mg/100 g, respectively, compared to 21.6 mg/100 g and 21.9 mg/100g found in this study. However, in a research reported by Phillips et al.[32], the contents of free β-sitosterol in rapeseed, soybean, peanut, and sunflower seed oils and those of free delta-5-avenasterol in soybean and sunflower seed oils were lower than in our study, while the levels of other free sterols (campesterol, stigmasterol, brassicasterol, sitostanol, and campestanol) in the four edible oils were in agreement with the data determined in this

study. The observed difference could be attributed to the influences of several factors, such as different genetic varieties, climates, irrigation systems, and locations of the cultivars. As it can be seen in Table 3, the main free phytosterols found in the four types of oils were β-sitosterol, stigmasterol, campesterol, and delta-5-avenasterol, but for brassicasterol, which was specific to rapeseed oil and had a very high level of 42.8 ± 12.0 mg/100 g, was trivial in the other three types of oils. In the contrary, the content of stigmasterol in rapeseed oil was very low (3.0 ± 1.5 mg/100 g), compared with that found in the other three oils. As for saturated free sterols, the results showed that the sitostanol content in soybean oil was higher than that found in the others. Free sitostanol was not detected in rapeseed or sunflower seed oil, and its content was in trace level in some peanut oil samples (found in 16 samples out of the total 20 samples). With regard to free campestanol, similar results were found. Therefore, they could be used as specific markers to detect soybean oil, with which adulteration occurs in the other three oils. In Table 3, it is shown that the level of delta-7-stigmastenol could also be used to discriminate the four edible oil types and detect adulteration, since it was determined as 6.8 ± 2.3 mg/100 g in soybean oil and 21.9 ± 8.8 mg/100 g in sunflower seed oil, but not detected in the other two oils.

(Table 3)

Exploratory data analysis

After determination and quantification of free phytosterols in the four edible oils, the data matrix of the phytosterol contents was preprocessed by generalized log transformation and Pareto scaling (mean-centered and divided by the square root of the standard deviation of each variable). Firstly, principal component analysis (PCA) and hierarchical clustering analysis (HCA) were employed to screen the sampling clusters and variable distributions in the four groups. The score plot obtained from PCA in Fig. 2 shows that the four edible oils are clearly classified into four groups, among which the

free phytosterol profiles of peanut and soybean oils are similar, while rapeseed and sunflower seed oils are far from peanut and soybean oils.

(Fig. 2)

To investigate variable distributions in the four groups, the heat map of free phytosterol profiles of the four edible oils was illustrated. In the heat map, the similarity measure was the Euclidean distance, while the clustering algorithm was Ward's linkage by clustering to minimize the sum of squares of any two clusters. As shown in Fig. 3, the similar cluster analysis results were obtained by PCA. More importantly, we could find the variable distributions in the four groups from this heat map as follows: (a) brassicasterol is the marker phytosterol of rapeseed oil; (2) campestanol and sitostanol are markers of soybean oil; (3) sunflower seed and soybean oils have high contents of delta-7-stigmastenol and delta-7-campesterol; and (4) peanut oil possesses a relatively low level of total free phytosterols, which are in line with the reported results[31, 32].

(Fig. 3)

Classification of four edible oils by random forests

After exploratory data analysis, we found that the four edible oils could be clearly classified into four groups. Among them, rapeseed oil has a relatively high content of free brassicasterol, while soybean oil possesses a relatively high content of free stigmasterol, which are in good agreement with previously reported data[30-32, 46]. To build a classification model for the four edible oils, an effective supervised multivariate statistical method of random forests (RF) was used. Random forests are a multitude of tree predictors combined in such a way that each tree depends on the values of a random vector sampled independently, with the same distribution for all the trees in the forest[47]. The sample proximity matrix derived from these training trees is generated to collect similarity information of the samples for

sample classification. Class prediction is based on the majority vote of the ensemble. Compared with other supervised multivariate statistical methods such as partial least squares-discriminant analysis (PLS-DA) and support vector machine (SVM), RF can be employed for multi-class classification. Furthermore, the RF classifier needs to optimize only one parameter among a number of classification trees, which is relatively insensitive to the predictive effect. In this study, the number of classification trees is set to 500. During tree construction, about one third of the samples are left out of the bootstrap samples. This out-of-bag (OOB) data is then used as a test sample to obtain an unbiased estimate of the classification error (OOB error). As the results show, the five errors decrease to zero after less than 20 trees, and the OOB error equals 0 in the final classification model. The element (i, and j) of the proximity matrix produced by random forest is the fraction of trees in which elements i and j fall in the same terminal node[47]. Therefore, the proximity matrix could be used to identify the structure in data. Multidimensional scaling of proximity matrix is usually employed to illustrate the proximity matrix in low dimensional space. As shown in Fig. 4, we can easily find that the edible oil samples could be classified into four classes. Meanwhile, the oil samples in the same class locate at very small region.

(Fig. 4)

(Fig. 5)

Moreover, Random forests could provide a measure for variable importance. Fig. 5 shows the contribution of each variable to oil classification. According to the mean decrease, the stigmasterol, β-amyrin, delta-7-stigmastenol, brassicasterol, and delta-7-campesterol are five important free phytosterols for classification of the four edible oils, among which stigmasterol is a phytosterol with a high content in the four edible oils and therefore an important marker. Using this marker, we can

completely classify the four edible oils into four groups, among which soybean oil has the highest content of stigmasterol and rapeseed oil possesses the lowest level. Meanwhile, free delta-7-stigmastenol and delta-7-campesterol could be employed to differentiate peanut oil from sunflower seed oil. To validate the classification model, 27 commercial edible oil samples (including 6 peanut oils, 8 soybean oils, 7 rapeseed oils, and 6 sunflower seed oils) were employed as a test set. The results indicate that all these refined oils could be correctly identified. Thus, free phytosterols are important markers of edible oils. In this study, free phytosterol profiles could correctly classify the four edible oils into four groups with the help of random forests. Moreover, since phytosterols are important nutrient components, the phytosterol profiles could also be employed to evaluate the quality and grade of edible oils[27].

Adulteration identification by free phytosterol profiles

Though free phytosterol profiles could be used to completely classify the four edible oils into four groups, a more significant issue remained to be resolved for adulteration identification is whether adulterated oils could be differentiated from pure oils. Therefore, to test whether free phytosterol profiles could identify adulteration, two types of edible oil samples of soybean and peanut oils with similar free phytosterol profiles were selected as an example. Since there is no chemical reaction occurring on phytosterols in adulteration, 20 adulterated peanut oil samples were simulated by the Monte Carlo method[40]. In detail, the simulation procedures of adulterated peanut oils were as follows: (1) randomly selected one peanut and one soybean sample, respectively; (2) free phytosterols composition of the blended oil is sum of the free phytosterols of 5% soybean and 95% peanut oils; (3) repeated the steps of (1)-(2) for 20 times. The discriminative model was built for pure and adulterated peanut oils by partial least squares-discriminant analysis (PLS-DA) after generalized log

transformation and Pareto scaling. As shown in Fig. 6, adulterated peanut oils are significantly different from pure peanut oils. The cross-validation results indicate that adulterated peanut oils could be identified.

(Fig. 6)

Highly accurate identification of adulterated peanut oils depends on selective components of adulterants. As described in the sections of "Exploratory data analysis" and "Classification of four edible oils by random forests", four edible oils have selective phytosterols. Therefore, free phytosterol profiles could be employed to identify adulteration between the four types of oils. In contrast, without clear chemical information of components, an optimized predictive model of spectroscopy based method depends on the training samples and is therefore hard to detect possible adulteration out of the training set. Compared with spectroscopy based methods, the method developed in this study is based on the profiles of a group of important metabolic compounds, and could characterize the four target edible oils as well as effectively detect the the oils adulteration in a larger sample scale.

Conclusion

In this study, a simple and rapid SPE method has been developed for separating free sterols from edible oils, and their silylation derivatives have been analyzed by GC-GC–TOF/MS, leading to a good separation resolution. Under the employed experimental conditions, free phytosterol profiles of four types of edible oils were established by GC-GC–TOF/MS and employed to classify these oils with the help of multivariate statistical methods. The results indicated that the free phytosterol profiles of the four edible oils could completely and correctly classify the oils into four groups, and therefore could be taken as effective markers for identification of the studied oils. Meanwhile, stigmasterol, delta-7-stigmastenol, delta-7-campesterol, brassicasterol, and β-amyrin were found as important

phytosterols for classification of the four edible oils. Using the classification model, 27 commercial edible oil samples (including 6 peanut oils, 8 soybean oils, 7 rapeseed oils, and 6 sunflower seed oils) could be correctly identified. Moreover, a simulated data test indicated that free phytosterol profiles could be used to detect peanut oil adulterated with 5% soybean oil, which was simulated by the Monte Carlo method.

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1. A. Cert, W. Moreda and M. C. Pérez-Camino, *J. Chromatogr. A*, 2000, **881**, 131-148.
2. S. O. Yeboah, Y. C. Mitei, J. C. Ngila, L. Wessjohann and J. Schmidt, *Food Res. Int.*, 2012, **47**, 259-266.
3. Y. Wang, *China Oils and Fats*, 2010, **35**, 1-5.
4. Q. Zhang, C. Liu, Z. Sun, X. Hu, Q. Shen and J. Wu, *Food Chem.*, 2012, **132**, 1607-1613.
5. S. C. Cunha and M. B. P. P. Oliveira, *Food Chem.*, 2006, **95**, 518-524.
6. A. K. Shukla and R. P. Singh, *J. Oleo Sci.*, 2005, **54**, 543-544.
7. K. Poulli, G. Mousdis and C. Georgiou, *Anal. Bioanal. Chem.*, 2006, **386**, 1571-1575.
8. K. I. Poulli, G. A. Mousdis and C. A. Georgiou, *Food Chem.*, 2007, **105**, 369-375.
9. E. C. López-Díez, G. Bianchi and R. Goodacre, *J. Agric. Food Chem.*, 2003, **51**, 6145-6150.

10. M.-Q. Zou, X.-F. Zhang, X.-H. Qi, H.-L. Ma, Y. Dong, C.-W. Liu, X. Guo and H. Wang, *J. Agric. Food Chem.*, 2009, **57**, 6001-6006.

11. B. F. Ozen, I. Weiss and L. J. Mauer, *J. Agric. Food Chem.*, 2003, **51**, 5871-5876.

12. A. Rohman and Y. B. C. Man, *Food Res. Int.*, 2010, **43**, 886-892.

13. A. Rohman and Y. B. Che Man, *Food Chem.*, 2011, **129**, 583-588.

14. N. Sinelli, M. Casale, V. Di Egidio, P. Oliveri, D. Bassi, D. Tura and E. Casiraghi, *Food Res. Int.*, 2010, **43**, 2126-2131.

15. G. Fragaki, A. Spyros, G. Siragakis, E. Salivaras and P. Dais, *J. Agric. Food Chem.*, 2005, **53**, 2810-2816.

16. Z. Hai and J. Wang, *Eur. J. Lipid Sci. Technol.*, 2006, **108**, 116-124.

17. A. M. Marina, Y. B. Che Man and I. Amin, *J. Am. Oil Chem. Soc.*, 2010, **87**, 263-270.

18. T. S. T. Mansor, Y. B. Che Man and M. Shuhaimi, *J. Am. Oil Chem. Soc.*, 2012, **89**, 485-496.

19. R. R. Catharino, R. Haddad, L. G. Cabrini, I. B. S. Cunha, A. C. H. F. Sawaya and M. N. Eberlin, *Anal. Chem.*, 2005, **77**, 7429-7433.

20. J. O. Lay, R. Liyanage, B. Durham and J. Brooks, *Rapid Commun. Mass Spectrom.*, 2006, **20**, 952-958.

21. D.-S. Lee, E.-S. Lee, H.-J. Kim, S.-O. Kim and K. Kim, *Anal. Chim. Acta*, 2001, **429**, 321-330.

22. D.-S. Lee, B.-S. Noh, S.-Y. Bae and K. Kim, *Anal. Chim. Acta*, 1998, **358**, 163-175.

23. Y. W. Park, P.-S. Chang and J. Lee, *Food Chem.*, 2010, **123**, 377-383.

24. F. Zhao, J. Liu, X. Wang, P. Li, W. Zhang and Q. Zhang, *Eur. J. Lipid Sci. Technol.*, 2013, **115**, 337-347.

25. M. J. Lagarda, G. García-Llatas and R. Farré, *J. Pharm. Biomed. Anal.*, 2006, **41**, 1486-1496.

26. S. L. Abidi, *J. Chromatogr. A*, 2001, **935**, 173-201.

27. R. A. Moreau, B. D. Whitaker and K. B. Hicks, *Prog. Lipid Res.*, 2002, **41**, 457-500.

28. T. T. Thanh, M.-F. Vergnes, J. Kaloustian, T. F. El-Moselhy, M.-J. Amiot-Carlin and H. Portugal, *J. Sci. Food Agric.*, 2006, **86**, 220-225.

29. Y. C. Mitei, J. C. Ngila, S. O. Yeboah, L. Wessjohann and J. Schmidt, *J. Am. Oil Chem. Soc.*, 2009, **86**, 617-625.

30. L. Normén, L. Ellegård, H. Brants, P. Dutta and H. Andersson, *J. Food Compos. Anal.*, 2007, **20**, 193-201.

31. M. Lechner, B. Reiter and E. Lorbeer, *J. Chromatogr. A*, 1999, **857**, 231-238.

32. K. M. Phillips, D. M. Ruggio, J. I. Toivo, M. A. Swank and A. H. Simpkins, *J. Food Compos. Anal.*, 2002, **15**, 123-142.

33. S. S. Cunha, J. O. Fernandes and M. B. P. P. Oliveira, *J. Chromatogr. A*, 2006, **1128**, 220-227.

34. R. M. Toledano, J. M. Cortés, Á. Rubio-Moraga, J. Villén and A. Vázquez, *Food Chem.*, 2012, **135**, 610-615.

35. R. Esche, B. Scholz and K.-H. Engel, *J. Agric. Food Chem.*, 2013, **61**, 10932-10939.

36. M. Lukić, I. Lukić, M. Krapac, B. Sladonja and V. Piližota, *Food Chem.*, 2013, **136**, 251-258.

37. M. J. Lerma-García, E. F. Simó-Alfonso, A. Méndez, J. L. Lliberia and J. M. Herrero-Martínez, *Food Res. Int.*, 2011, **44**, 103-108.

38. T. Galeano Diaz, I. Durán Merás, J. Sánchez Casas and M. F. Alexandre Franco, *Food Control*, 2005, **16**, 339-347.

39. D. Gázquez-Evangelista, E. Pérez-Castaño, M. Sánchez-Viñas and M. G. Bagur-González, *Food Analytical Methods*, 2014, **7**, 912-925.

40. D. P. Kroese, T. Taimre and Z. I. Botev, in *Handbook of Monte Carlo Methods*, John Wiley & Sons, Inc., 2011, ch6, pp. 225-280.

41. ISO 12228: 1999, Animal and vegeitable fats and oils - Determination of individual and total sterols contents - Gas chromatographic method.

42. ICH. Q2. B, International Conference on Harmonization (ICH), Validation of analytical methods: methodology, 1996.

43. J. Xia, R. Mandal, I. V. Sinelnikov, D. Broadhurst and D. S. Wishart, *Nucleic Acids Res.*, 2012, **40**, W127-W133.

44. J. Xia, N. Psychogios, N. Young and D. S. Wishart, *Nucleic Acids Res.*, 2009, **37**, W652-W660.

45. R. Esche, A. Barnsteiner, B. Scholz and K.-H. Engel, *J. Agric. Food Chem.*, 2012, **60**, 5330-5339.

46. S. L. Abidi, *J. Chromatogr. A*, 2004, **1059**, 199-208.

47. L. Breiman, *Machine Learning*, 2001, **45**, 5-32.

Figure titles

Fig. 1. GC-GC–TOF/MS chromatogram of silylated free sterols extracted from soybean oil (Num. 5) by SPE. For analytical conditions see the section of "Materials and methods ". Peak identified: the peak number correlates to Table 1; peak 2, 7 and13 were obtained by using selective ions: 255 + 341 + 365 + 380 + 470, 472, and 486 (*m/z*), respectively.

Fig. 2. Score plot obtained from PCA using data of four types of edible oils. The explained variances are shown in brackets.

Fig. 3. Heat map of phytosterol profiles of four types of edible oils.

Fig. 4. The classical multidimensional scaling of the proximity matrix of the four types of edible oils.

Fig. 5. Significant features identified by random forests.

Fig. 6. Score plot obtained by PLS. The explained variances are shown in brackets.

Table captions

Table S1 Detailed information of four types of oil seeds used in this study

Table 1 GC-GC–TOF/MS results [a] of the trimethylsilyl sterol ethers

Table 2 LODs, LLOQs, Within-day Precisions and Recoveries of Selected Phytosterol Derivatives

Table 3 Free phytosterol contents [a] in four types of edible oils

Table 1 GC-GC–TOF/MS results [a] of the trimethylsilyl sterol ethers

TMS-sterols	RRT [b]	qualitative ionic fragments (*m/z*)	group of quantitative ionic fragments (*m/z*)
(1) TMS-cholestanol (IS)	1.000	147, 215, 230, 305, 355, 370, 445, 460	215 + 305 + 355 + 445 + 460
(2) TMS-brassicasterol	1.014	129, 213, 255, 341, 365, 380, 470	255 + 341 + 365 + 380 + 470
(3) TMS-24-methylene-cholesterol	1.047	129, 213, 253, 296, 371, 386, 445,470	253 + 296 + 371 + 386 + 470
(4) TMS-campesterol	1.052	129, 213, 255, 343, 367, 382, 457, 472	255 + 343 + 367 + 382 + 472
(5) TMS-campestanol	1.059	129, 215, 255, 305, 343, 367, 382, 474	215 + 305 + 343 + 382 + 474
(6) TMS-stigmasterol	1.067	129, 213, 255, 343, 355, 379, 394, 484	255 + 355 + 379 + 394 + 484
(7) TMS-delta-7-campesterol	1.090	147, 213, 255, 303, 367, 382, 457, 472	255 + 367 + 382 + 457 + 472
(8) TMS-delta-5,23-stigmastadienol	1.094	129, 213, 253, 355, 379, 394, 469, 484	255 + 355 + 379 + 394 + 484
(9) TMS-β-sitosterol	1.106	129, 213, 255, 303, 357, 381, 396, 486	255 + 357 + 381 + 396 + 486
(10) TMS-sitostanol	1.114	215, 257, 305, 383, 398, 431, 473, 488	215 + 305 + 383 + 473 + 488
(11) TMS-delta-5-avenasterol	1.115	215, 257, 281, 296, 355, 386, 484	257 + 281 + 296 + 386 + 484
(12) TMS-β-amyrin	1.121	190, 203, 218, 257, 279, 393, 498	218 + 257 + 279 + 393 + 498
(13) TMS-delta-7-stigmastenol	1.148	213, 255, 357, 381, 396, 471, 486	255 + 357 + 381 + 471 + 486

[a] The results were obtained by analyzing TMS-sterols derived from soybean oil sample (Num.5) using GC-GC–TOF/MS. [b] RRT: relative retention time based on cholestanol = 1.000

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Table 2 LODs, LLOQs, Within-day Precisions and Recoveries of Selected Phytosterol Derivatives

Type of Standard	Blank sample	Spiking amount (mg/100g)	Within-day Precision (RSD, %, n = 3)	Recovery [a] (%)	LOD [b] (mg/100g)	LLOQ [b] (mg/100g)
		0.1	16.4	93.3 ± 15.3		
Cholestanol	ND[c]	20	2.6	95.4 ± 2.5	0.03	0.07
		40	1.3	97.6 ± 1.2		
		0.2	7.9	96.7 ± 7.6		
Brassicasterol	ND	30	2.4	96.2 ± 2.3	0.04	0.08
		60	1.7	97.2 ± 1.7		
		2	4.8	103.7 ± 5.0		
Campesterol	ND	40	1.9	95.4 ± 1.8	0.04	0.08
		80	2.0	98.2 ± 1.9		
		0.1	19.2	90.0 ± 17.3		
Stigmasterol	ND	40	1.4	96.5 ± 1.3	0.04	0.08
		80	2.5	98.9 ± 2.5		

[a] Values represent the mean ± standard deviation (n = 3). [b] Limit of detection (LOD) and lower limit of quantification (LLOD) were expressed as mg/100g of spiked blank oil sample, and obtained by GC-GC–TOF/MS analysis after SPE isolation. [c] ND means not detected.

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Table 3 Free phytosterol contents [a] in four types of edible oils

Oil type	Peanut oil	Soybean oil	Rapeseed oil	Sunflower seed oil
Brassicasterol	1.2 ± 0.4 (n[c] = 3)	0.8 ± 0.1 (n[c]=19)	42.8 ± 12.0 (n[c]=40)	0.6 ± 0.1 (n[c]=2)
24-methylene-Cholesterol	0.2 ± 0.1 (n[c] = 4)	0.7 ± 0.2 (n[c]=17)	ND	0.4 ± 0.2 (n[c]=10)
Campesterol	12.4 ± 3.1 (n[c] = 20)	39.8 ± 6.5 (n[c]=19)	51.7 ± 11.1 (n[c]=40)	14.3 ± 3.0 (n[c]=19)
Campestanol	0.2 ± 0.1 (n[c] = 15)	1.3 ± 0.3 (n[c]=19)	0.5 ± 0.4 (n[c]=3)	0.2 ± 0.1 (n[c]=4)
Stigmasterol	17.9 ± 4.2 (n[c] = 20)	74.4 ± 9.7 (n[c]=19)	3.0 ± 1.5 (n[c]=40)	25.0 ±5.6 (n[c]=19)
delta-7-Campesterol	ND [b]	0.6 ± 0.2 (n[c]=19)	0.2 ± 0.1 (n[c]=4)	1.1 ± 0.5 (n[c]=19)
delta-5,23-Stigmastadienol	1.0 ± 0.2 (n[c] = 20)	1.4 ± 0.3 (n[c]=19)	1.6 ± 0.5 (n[c]=40)	2.3 ± 0.5 (n[c]=19)
β-Sitosterol	118.5 ± 29.0 (n[c] = 20)	183.4 ± 21.5 (n[c]=19)	212.5 ± 38.4 (n[c]=40)	213.8 ± 36.4 (n[c]=19)
Sitostanol	0.9 ± 0.3 (n[c] = 16)	3.7 ± 0.8 (n[c]=19)	ND	ND
delta-5-Avenasterol	19.8 ± 8.5 (n[c] = 20)	8.0 ± 4.9 (n[c]=19)	5.3 ± 4.6 (n[c]=40)	21.6 ± 11.3 (n[c]=19)
β-Amyrin	3.3 ± 1.5 (n[c] = 20)	4.4 ± 1.1 (n[c]=19)	0.3 ±0.1 (n[c]=21)	1.9 ± 0.5 (n[c]=19)
delta-7-Stigmastenol	ND	6.8 ± 2.3 (n[c]=19)	ND	21.9 ± 8.8 (n[c]=19)

[a] Phytosterol contents were expressed as mean value ± standard deviation, mg/100g of plant oil. [b] ND means not detected. [c] n respects the number of oil sample in which the content of corresponding free sterol could be quantified.

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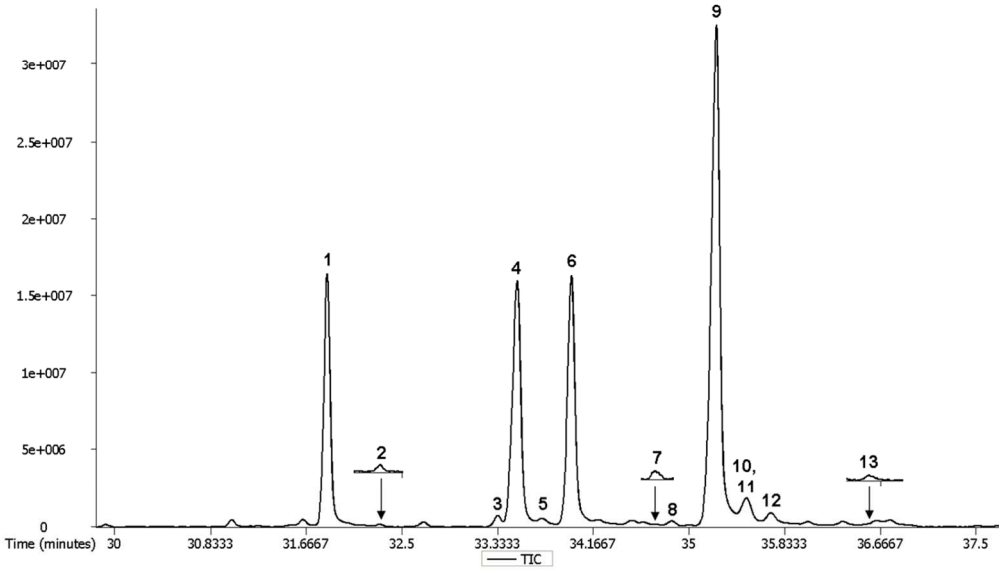
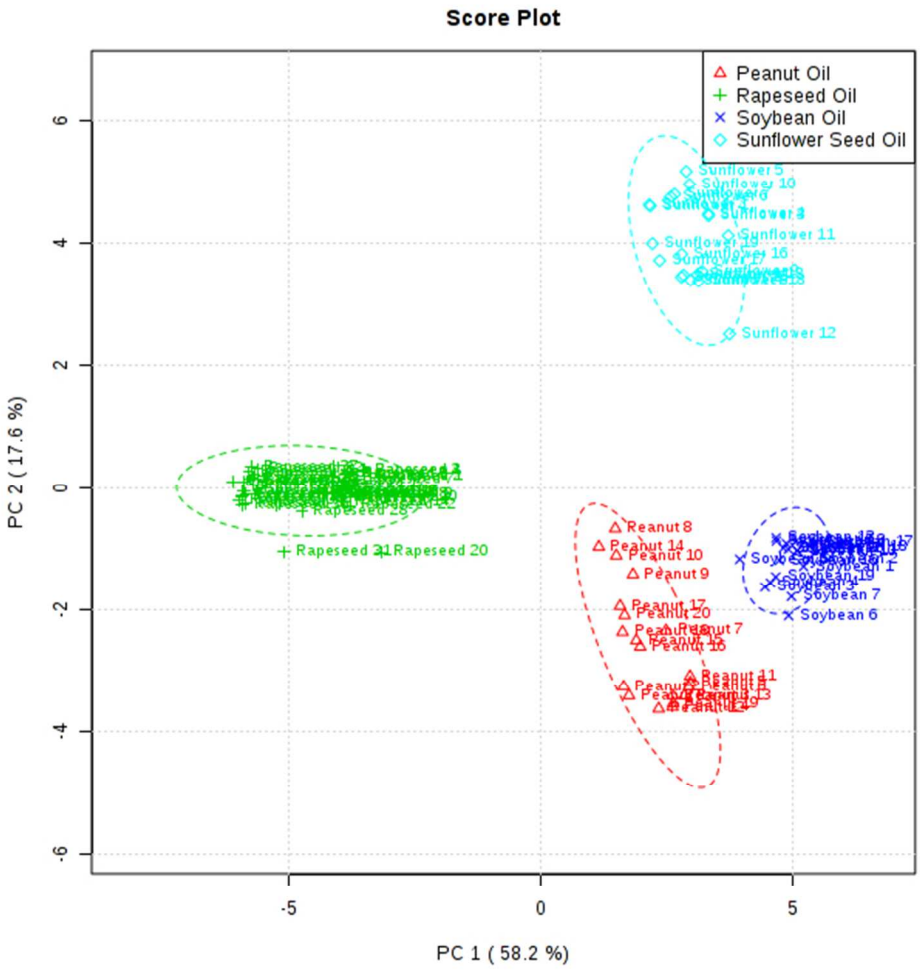
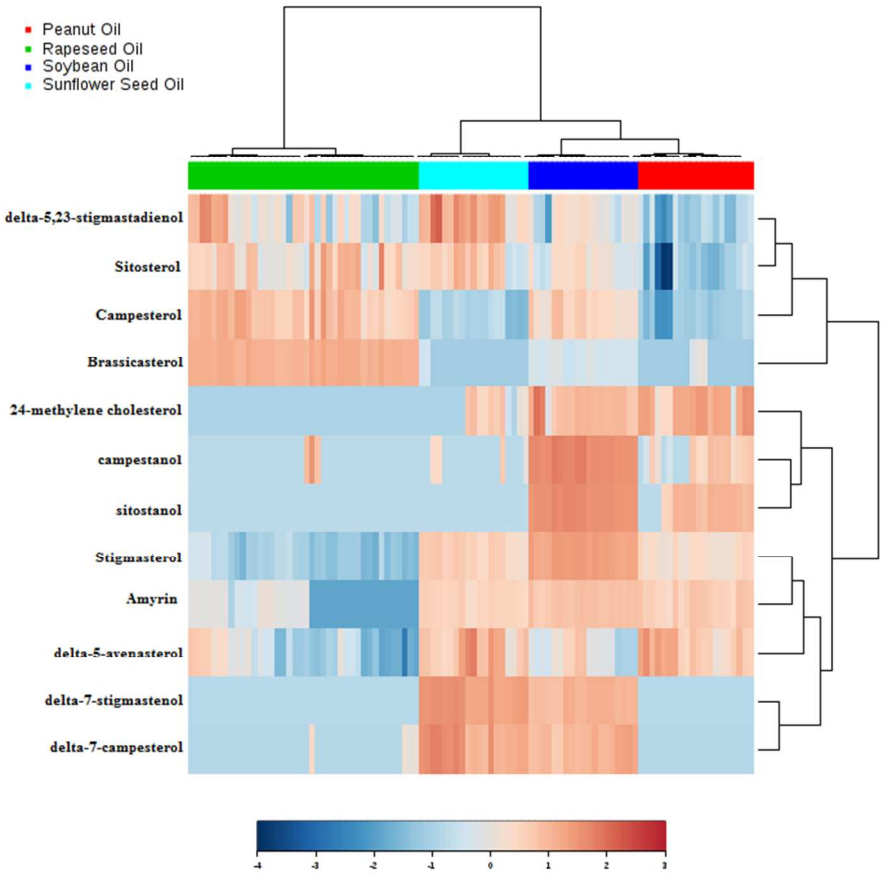


GC-GC–TOF/MS chromatogram of silylated free sterols extracted from soybean oil (Num. 5) by SPE. For analytical conditions see the section of "Materials and methods ". Peak identified: the peak number correlates to Table 1; peak 2, 7 and13 were obtained by using selective ions: 255 + 341 + 365 + 380 + 470, 472, and 486 (m/z), respectively.
113x71mm (300 x 300 DPI)



Score plot obtained from PCA using data of four types of edible oils. The explained variances are shown in brackets.
171x171mm (96 x 96 DPI)



Heat map of phytosterol profiles of four types of edible oils.
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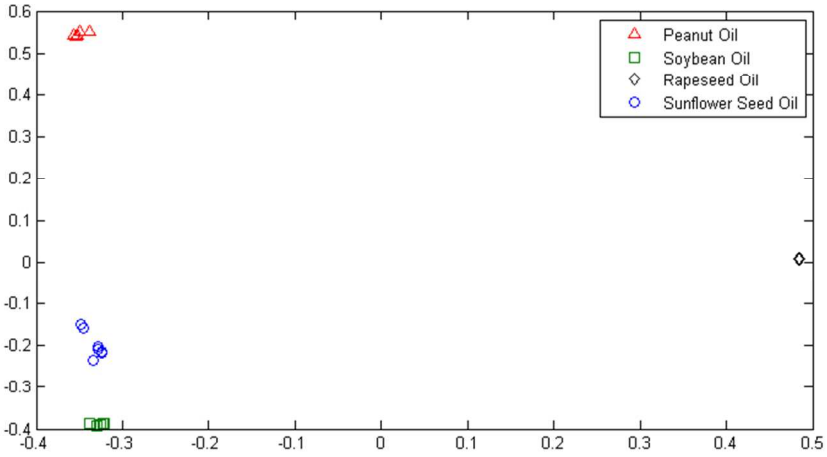

The classical multidimensional scaling of the proximity matrix of the four types of edible oils.
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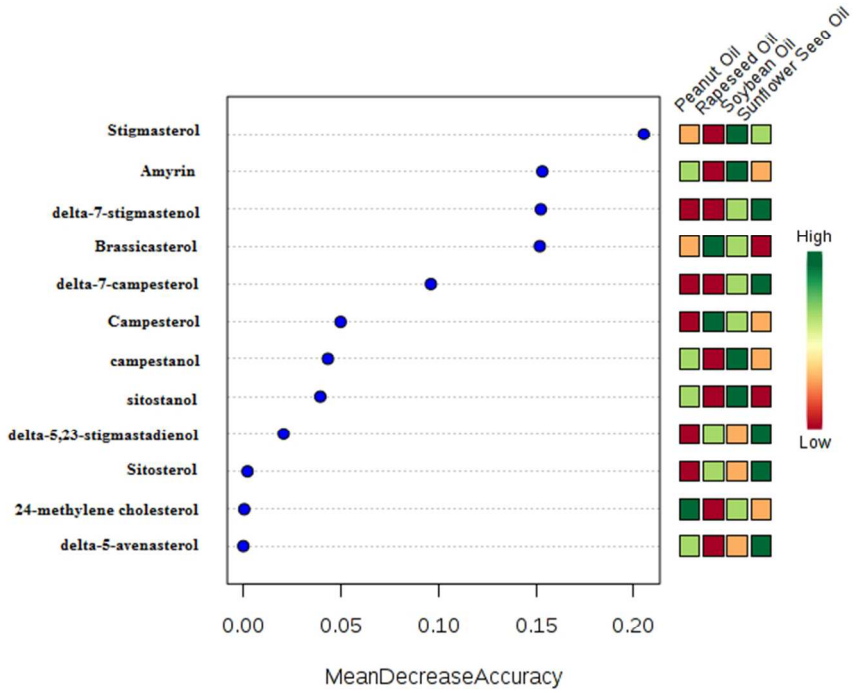
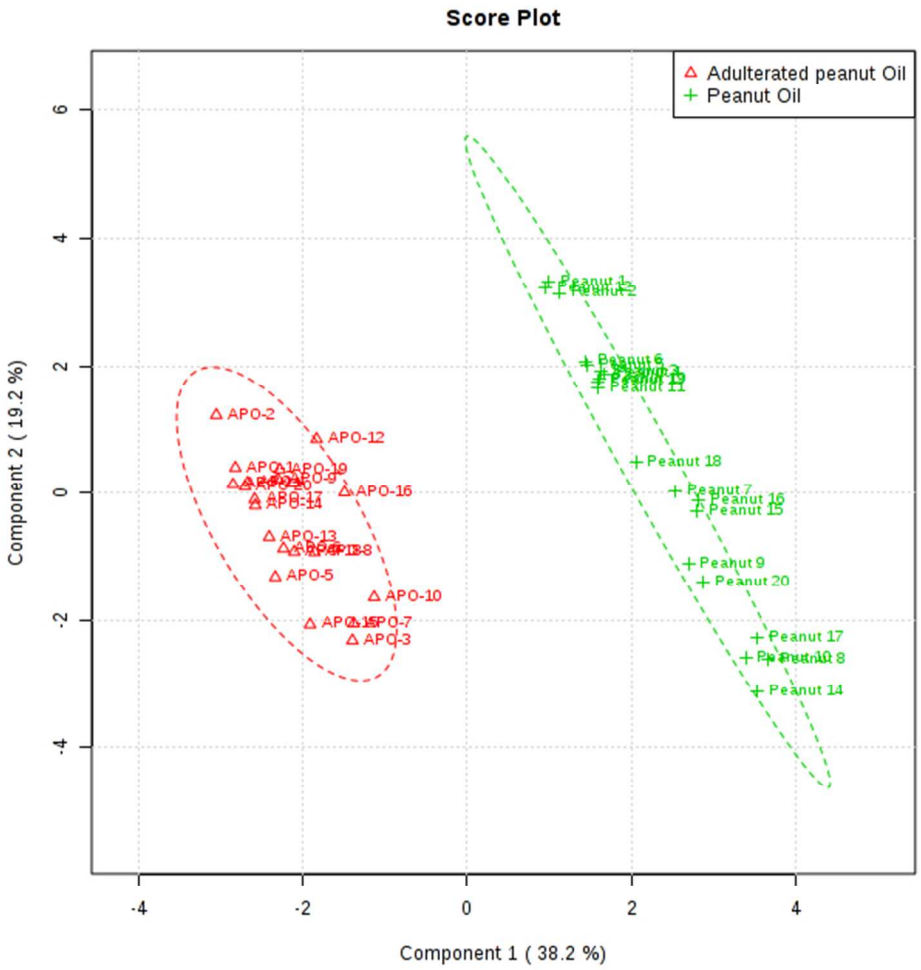


Significant features identified by random forests.
192x150mm (96 x 96 DPI)



Score plot obtained by PLS. The explained variances are shown in brackets.
171x171mm (96 x 96 DPI)