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Some important aspects of sterol analysis of vegetable oils

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Abstract. The content and composition of the sterol fraction is an important indicator of the value of vegetable oils, mainly due to the cholesterol-lowering effect of certain sterol compounds. Although many methods have been described in the literature, various steps may still cause errors during sample preparation. The present article covers some of the key steps in sterol analysis that are necessary to consider while implementing these procedures in laboratory and when dealing with different types of oils. The importance of the right solvents and the amount and concentration of the alkali solution is explained. The variation between results of analysing underivatized sterols compared to the derivatized ones is discussed. In our experiment with various rapeseed oil samples, the underivatized sterols resulted in lower values (9–11%). This outcome enables to (re)evaluate previous results. We also proposed fast and easy check-up methods for several sample preparation steps in order to avoid deviation in results and possible damage to GC apparatus.

Key words: vegetable oil, phytosterol, GC analysis.

INTRODUCTION

The determination of the content and composition of unsaponifiable material in various natural materials and food products became a very important task during the last decade [1, 2]. Knowledge about the content of sterols, the most interesting part of unsaponifiables, is needed in order to find new sources of valuable biochemicals, for various national food databases monitoring people's eating habits, for detecting adulteration of 'virgin' oils, etc. As the positive effect of sterols against cardiovascular diseases is known, they are widely used as

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active compounds in various functional foods and medicaments for the treatment and prevention of heart-related diseases. On the other hand, the sterol pattern is a valuable indicator of the quality and origin of butter and similar products [3].

Nowadays the methods of analysis should correspond to many strict requirements like high throughput, reliability, simplicity. One of the most demanding parts of analysis is sample preparation, particularly with matrices like those of vegetable oils, plant materials, and food products. There are many different procedures available for sample preparation for the determination of sterols in vegetable oils [1, 2, 4]. The most common method for analysis is gas chromatography (GC), which enables precise determination of all types of sterols. As there are differences between the characteristics of various plant oils, some problems might occur when applying universal methods commonly used by food control laboratories. The aim of the present work was to give an experiment-based detailed discussion about troubleshooting in order to avoid possible deviation in results.

EXPERIMENTAL

Equipment

The GC analysis was performed on a HP5890 instrument, using 30 m × 0.25 mm, i.d. 0.22 μm, BP-5 fused silica capillary column. Nitrogen was used as carrier gas. The oven temperature was 300 °C and the total time of a run was 20 min. Injector: 300 °C, flame-ionization detector: 340 °C. The FT-IR Spectrometer PC16 was from Perkin Elmer. All weighings were performed on analytical balances from Adam Equipment (readability 1 mg). For TLC silica plates Alugran UV 254 from Macherey-Nagel were used. For column separation, silica (70–200 mesh) from Aldrich was used.

Chemicals and raw material

Dichloromethane, hexane, cyclohexane, and ethyl ether were purchased from Aldrich and ethyl ether from Fischer Scientific (all analytical grade). Ethyl acetate and ethanol were from Rathburn (HPLC grade). Reference materials included cholesterol (Fluka, 97% purity) and stigmasterol (Sigma, 95% purity). Potassium hydroxide was from Chemapol, Checkoslovakia; manganese(II)chloride, sulphuric acid, and phosphomolybdic acid were from Reakhim, Russia. Pyridine from Fluka was distilled from CaH_2 before use. BSTFA with 1% TMCS was from Supelco, Inc.

Rapeseeds as raw material were from the trial fields of the Department of Field Crop Husbandry, Estonian University of Life Sciences (Tartu), and from the Agricultural Research Centre (Viljandi). Ten spring rapeseed varieties were used. Commercially available rapeseed oil Olivia (AS Werol Tehased, Estonia) was used to test the overall accuracy of measurement. All samples were tested for naturally occurring cholesterol content. It was below detection limit in all samples.

Saponification

Oil samples (0.5 g) were measured into a 100 mL flask. Then 0.5 mL of KOH solution (76 g in 100 mL H_2O), 4.5 mL of ethanol, and 200 μL of internal standard solution (500 mg cholesterol in 100 mL ethanol) were added. For saponification, flasks were heated under reflux for 30 min.

Liquid–liquid extraction of unsaponifiable compounds

After saponification, 10 mL of cyclohexane and 5 mL of water were added to the reaction mixture. It was shaken intensively for 5 min and then left to separate. The upper layer was collected and, after combining it with the upper layer from the second extraction, evaporated to dryness. Two extractions were performed.

For subsequent TLC check-up hexane/ethyl acetate mixture (6 : 1.5 v/v) was used as eluent. Samples from different extraction mixtures were applied to the silica plates. For visualizing the TLC plates, two agents were equally successfully used: (a) 100 mg $MnCl_2$ 4 H_2O + 30 mL H_2O + 30 mL CH_3OH + 1 mL conc. H_2SO_4 and (b) 0.5% phosphomolybdic acid in ethanol.

Extraction of unsaponifiable compounds on silica column

The glass column (20 cm long, 1.5 cm i.d.) was first filled with 1 g of sodium sulphate and then 1 g of silica. The previously hydrolysed mixture (ca 5 mL) was loaded onto 3 g of silica, evaporated to dryness, and transferred into the column as the third layer. For the eluation of unsaponifiable matter the column was flushed with 20 mL mixture of ethyl ether and ethyl acetate (1 : 1).

Derivatization procedure

For derivatization 200 μL of pyridine and 200 μl of BSTFA containing 1% TMCS were added to the evaporated extract in the septum-capped vials. The closed vials were transferred to a sand bath previously heated to 70 °C. The temperature was maintained for 30 min.

RESULTS AND DISCUSSION

Quality assessment of analysis

The sterol analysis was performed on the basis of three main sterols present in rapeseed oil in order to indicate the key points of the present experiment. For regular analysis of the sterol pattern in vegetable oil also stanols and other minor sterols should be quantified.

The limit of detection (LOD, S/N ratio >3) and the limit of quantification (LOQ, S/N ratio >10) were determined on the basis of sitosterol. The LOD of the GC signal of sitosterol was 5 μg/mL and LOQ was fixed at 62 μg/mL.

For determining the deviation of results in time, the sterol content of a commercially available rapeseed oil Olivia was analysed over a 4-month period (once a week). Three main sterols quantified showed only a slight deviation. The average sitosterol content was 396.9 mg/100 g oil (relative standard deviation 1.09%), the average campesterol content was 315 mg/100 g oil and 81.1 mg for brassicasterol, and the relative standard deviations were 1.15% and 1.17%, respectively.

Also the liquid–liquid extraction procedure was observed and optimized (with 3 different samples). The internal standard was added to the sample after extraction procedures and compared with the ones where it had been added before. The extraction efficacy of 90–95% and proportional carryover of major sterols was achieved with 2 extractions.

Alkaline hydrolysis

For the determination of the total amount of sterols the material has to be hydrolysed. Alkaline hydrolysis is generally used for vegetable oils but as different plant matrices contain sterol glucosides, also acid hydrolysis is needed [1]. For the alkaline hydrolysis of vegetable oils excess of KOH and NaOH in different solvents (MeOH, EtOH, H_2O) in different concentrations in excess have been used. According to the literature, also the reaction times and temperatures vary in a wide range [1, 2, 4–7]. Considering the saponification value, which can be determined using titration, it is possible to find appropriate conditions of hydrolysis for every type of oil sample. An important detail is the solvent added together with the internal standard to the sample before hydrolysis. Using chloromethanes as good solvents for sterols is not always the best choice. It is well known that trichloromethane can react with alkalis and give addition products to double bonds present in oils [8]. As we determined by titration, also tetrachloromethane is reacting with alkali in the saponification mixture (2.6 M) at 70 °C, decreasing its total amount as well as the rate of hydrolysis of ester moieties. According to the conditions (0.5 mL of rapeseed oil in 4.5 mL of EtOH, 0.5 mL of 10 M KOH, and 200 μL of the internal standard solution in tetrachloromethane added) used, ~20% of KOH was consumed for the reaction with CCl_4. Accordingly, due to the loss of alkali the hydrolysis was not complete causing reduction of the total sterol contents by 15–20%. The use of $CHCl_3$ gave similar results. Despite its relatively low boiling point, only dichloromethane can be used among chlorinated methanes without any problems.

Extraction of the unsaponifiable matter

After the hydrolysis of the oil sample the unsaponifiable material has to be separated from the mixture of fatty acid alkali salts, glycerol, excess of alkali, and solvent used. The classical way is to use liquid–liquid extraction with a certain solvent. This method is sensitive to the composition of the solvent mixture used for extraction. An incorrect composition can invoke the formation

of foam or just disturb the separation and decrease the yield of carryover. In order to prevent low carryover coefficients when dealing with different oils, we used thin layer chromatographic (TLC) analysis. The method is fast, cheap, and reliable. The visual detection limit of cholesterol on a TLC plate by phosphomolybdic acid is about 0.1 μg, which is more than enough for this type of analysis. By using TLC it is possible to determine how many times the sample should be extracted. This type of testing is very useful when dealing with new sample matrices or in general testing of the reproducibility in time.

On the other hand, it seems to be very attractive to separate the sterol fraction from the mixture of fatty acid alkali salts, glycerol, excess of alkali, and solvent by using column chromatography separation with principles of solid phase extraction (SPE). Some examples can be found in the literature where silica [9, 10] and alumina [11] were used as sorbents. As we discovered in our experiment, if large excess of alkali used to ensure complete hydrolysis remains in the mixture after saponification, it will deactivate the silica and the separated unsaponifiable matter fraction will be contaminated with fatty acid salts. We introduced Fourier transform infrared spectroscopy (FTIR) as a fast detection method for separated products to investigate the extraction procedure described in [10]. Thus, taking into consideration the average saponification value of the oil sample, we found that 6-fold excess of alkali (1 mL of 10 M KOH solution/0.5 g of oil and 4 g of silica in our case) gave a strong carbonyl signal at 1720 cm^{-1}.

After the reduction of the quantity of alkali to a more optimal amount (0.5 mL of KOH, 3-fold excess), the absorption of carbonyl group disappeared in the spectra of the extraction product (Fig. 1). We suggest that similar proportions of starting materials would result in analogous effects. Moreover, at the injection of a sample contaminated with fatty acid alkali salts into a GC instrument the injection liner will be contaminated very quickly and the data of the analysed components will be affected dramatically.

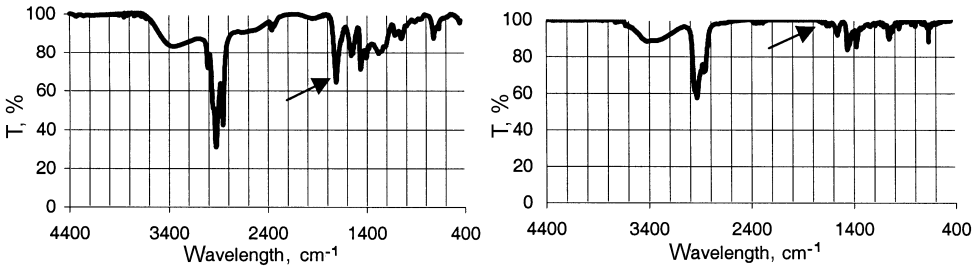

Fig. 1. The IR spectrum of the unsaponifiable fraction contaminated (*left*) and not contaminated with fatty acid salts (*right*) after extraction.

Underivatized versus derivatized sterols

For purposes of better volatilizing the hydroxyl-containing compounds, enhancing component resolution, and stabilizing thermally labile unsaturated sterols, the title compounds are most commonly analysed as their trimethylsilyl derivatives [4]. In the literature [12, 13] also analysis of sterols in unsaponifiable matter without derivatizing them, with the benefits from shorter sample preparation time and smaller costs of chemicals, has been described. In our experiment we determined the sterol content of oil samples from 10 spring rapeseed varieties and investigated the effect of derivatization (Table 1). As a result we found that underivatized sterols yielded a 6.0–8.7% smaller total sterol content compared to the derivatized ones. Derivatization influenced mainly the sitosterol content (9.4–11.8% increase) as it evaporates last among the major sterols in the injector and thus its content declines the most when analysed without derivatization.

The results obtained give a general overview of the differences of these two methods and help to evaluate experiments previously performed without the derivatization step in sample preparation.

Depending on the equipment and exact conditions of the GC analysis used, the variation in the outcome may of course differ. However, we observed that the deviation between parallels was about the same. This indicates that the difference in results between these methods in similar conditions is stable. Therefore, analysing sterols without a derivatization procedure gives also valuable and comparable information about oil samples.

Table 1. Comparison of oil sterol content of spring rapeseed varieties with and without the derivatization procedure in sample preparation

Variety	Sterol content, mg/100 g oil							
	Sitosterol	Sitosterol–trimethylsilyl ether	Campesterol	Campesterol–trimethylsilyl ether	Brassicasterol	Brassicasterol–trimethylsilyl ether	Total	Total–trimethylsilyl ether
Olga	256.0 ± 7.9	290.3 ± 7.2	166.0 ± 5.6	175.3 ± 2.1	84.7 ± 3.1	82.3 ± 1.2	506.7	547.9
Natan	341.0 ± 4.6	384.3 ± 4.0	192.7 ± 1.5	205.7 ± 4.2	69.7 ± 2.1	71.0 ± 1.0	603.4	661.0
Hunter	382.0 ± 6.6	425.6 ± 9.1	258.7 ± 5.1	268.8 ± 5.9	97.3 ± 1.5	99.2 ± 2.5	738.0	793.6
Licolly	364.2 ± 6.8	402.2 ± 4.0	269.2 ± 4.5	274.0 ± 2.0	96.2 ± 1.9	96.2 ± 1.6	729.6	772.4
Liaison	350.3 ± 4.0	396.3 ± 5.2	250.2 ± 2.3	254.6 ± 2.9	98.2 ± 1.6	99.1 ± 0.4	698.7	750.0
Heros	354.7 ± 6.1	396.3 ± 7.5	232.0 ± 4.6	238.3 ± 4.7	94.0 ± 1.4	92.0 ± 3.5	680.7	726.6
Quantum	342.9 ± 7.4	378.3 ± 1.8	241.9 ± 3.4	244.7 ± 0.6	73.1 ± 2.2	76.5 ± 1.7	657.9	699.5
Lara	350.1 ± 5.5	391.0 ± 8.0	214.0 ± 2.3	223.6 ± 3.2	100.8 ± 1.7	102.1 ± 2.4	664.9	716.7
Maskot	386.3 ± 3.2	433.0 ± 9.0	281.7 ± 5.5	291.7 ± 3.8	120.7 ± 2.5	120.3 ± 2.3	788.7	845.0
Sponsor	306.1 ± 0.7	346.3 ± 3.9	214.6 ± 0.1	220.7 ± 2.8	83.0 ± 1.6	85.5 ± 1.2	603.7	652.5
Increase, %		10.7		3.3		0.8		6.9

CONCLUSIONS

During the present research we found several factors that could cause problems in vegetable oil sterol analysis not explained before. Fast, cheap, and easy-to-use check-up methods were proposed in order to avoid and/or prevent these problems. Some of these have been described before in the context of sterol analysis but together with awareness of the possible sources of errors they form a knowledge basis that is vital while implementing a new method in laboratory or when analysing oils of different origin and properties.

So far it has been the general opinion that suitable conditions for alkaline hydrolysis vary in a wide range. We found, on the contrary, that the suitable amount and concentration of alkaline solution for hydrolysis of vegetable oils must be followed more precisely than hitherto. Furthermore, application of IR spectroscopy as a detection method of artefacts in an oil sample before GC analysis proved to be very useful in order to avoid errors and possible harmful effects to the expensive GC instruments. Comparison of the results of the analysis of derivatized and underivatized sterols gives new information about this important aspect of sample treatment in vegetable oil analysis. A stable difference in results (9–11% sitosterol) was observed. The data compared in our experiment enable more precise interpretation of previous articles where sterols have been analysed without derivatization.

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Mõningaid taimsete õlide steroolide analüüsi olulisi aspekte

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Steroolide sisaldus ja kompositsioon on taimsete õlide väärtuse hindamisel oluline näitaja: seda peamiselt nende komponentide omaduse tõttu alandada veres kolesteroolitaset. Kuigi kirjanduses on steroolide määramiseks hulgaliselt erinevaid meetodeid, võivad mõned proovitöötluse etapid põhjustada määramisvigu. Artiklis on käsitletud tähtsamaid steroolianalüüsi nüansse, mida on eriti oluline arvestada uute meetodite rakendamisel või erineva keemilise koostisega õlide analüüsimisel laboris. On selgitatud sobivate lahustite ja leeliselahuse õige kontsentratsiooni valikukriteeriume. Samuti on käsitletud derivatiseeritud (silüülitud) ja derivatiseerimata steroolide analüüsitulemuste erinevusi. Ilmneb, et derivatiseeritud steroolide gaaskromatograafilisel analüüsil on saadud 9–11% suuremad väärtused. See tulemus võimaldab anda hinnangut mitmetele varasematele steroolide sisalduse määramise andmetele kirjanduses. Veel on esitatud kiired ning lihtsad kontrollmeetodid, vältimaks analüüsitulemuste triivi ja ka võimalikke kahjustusi gaaskromatograafi süsteemidele.