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Phytosterols as Markers in Identification of the Adulterated Pumpkin Seed Oil with Sunflower Oil

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Abstract. In food production, the quality assessment of raw materials and final products is a fundamental parameter for maintaining high quality standards. Pumpkin seed oil is rather expensive compared to other vegetable oils. Therefore, it is often adulterated by the addition of cheaper oils. In contrast to other edible oils, the content of Δ5-sterols is very low, while Δ7-sterols are dominating. The aim of this study was to develop a rapid and convenient chromatographic method for authenticity control of pumpkin seed oil using the total sterol profile determined by gas-chromatography (GC) with flame ionization detector (FID). Two pure vegetable oils, pumpkin seed and sunflower oil, processed by minimal technologies (cold pressing) in Romania and an adulterated pumpkin seed oil with 30% of sunflower oil were used as sample matrices in the method development. The determination of Δ5-sterols, especially β-sitosterol has proven to be a good possibility to detect admixture of cheap vegetable oils.

Keywords: edible plant oils, phytosterol, GC-FID analysis.

INTRODUCTION

Pumpkin seed oil pressed from the seeds of *Cucurbita pepo* L. is a popular salad oil in the southern Styrian parts of Austria, in Hungary and in Slovenia. Seeds of Cucurbitae are also commonly used phytotherapeutically in the therapy of small disorders of the prostate gland and the urinary bladder. Recent studies also showed a potential anti-inflammatory activity of pumpkin seed oil in the treatment of adjuvant arthritis in rats. Due to the laborious production process, involving the manual harvest of the seeds, this expensive oil is often adulterated by the addition of cheap vegetable oils such as sunflower or rapeseed oils. The intense dark green color of pumpkin seed oil and its characteristic smell of various pyrazines make it difficult for the consumer to recognize the admixture of even large amounts of other oils (Mandl et al.1999).

Adulteration has always been practised and it is carried out for economical purposes (e.g. to increase the bulk volume, to overevaluate a product of inferior quality or to subtract/save expensive ingredients). On the other hand, contaminations may occur accidentally, e.g. in factories, where several oils are produced or used at the same time. These cross-contaminations are usually below 1 – 2 % of the total amount.

To assess the authenticity of oils it is fundamental to know, not only the biological origin of seeds, but also the technologies applied, the fat modification techniques used and the chemical composition of the authentic oil(s) and of the potential adulterants (Kamm et al., 2001b). For plant oils, the secondary metabolites are used as markers of biological or geographical authenticity, as well for adulteration and traceability studies, representing the metabolic profile or fingerprint (Socaciu et al., 2009). Specific components in the

unsaponifiable fraction of the oil were considered as markers for adulteration. The predominant part of the unsaponifiables includes various hydrocarbons, triterpenoids, carotenoids, tocopherols and phytosterols. These minor lipid compounds have proven of interest to food analysts and the composition and/or ratios of these trace compounds can provide a "fingerprint" for edible oils (Mandl et al.1999).

For the detection of manipulation of the natural composition of the oil, different techniques were applied. Chromatographic techniques like gas chromatography and high performance liquid chromatography are dominating, but also spectroscopic methodologies were suggested (El-Hamdy et al., 1995). Different classes of chemical compounds, such as: fatty acids, hydrocarbons, triterpenoids, carotenoids tocopherpls and phytosterols, were investigated toward the potential of unambiguous discrimination between manipulated and genuine vegetable oils (Mannina et al., 1999; Rezanka et al., 1999; Younis et al., 2000).

Phytosterols and cholesterol are polycyclic steroid compounds with similar chemical structure. Both cholesterol and phytosterols occur in free and esterified forms (Moreau et al., 2002).

The most appropriate and frequently used method for the determination of total sterols content in foods and oils is the direct saponification, fallowed by the extraction of the unsaponificable residue into the nonpolar solvent and final gas chromatographic detection (Abidi, 2001).

The objective of this study was to develop a rapid and convenient chromatographic method for authenticity control of pumpkin seed oil using the total sterol profile determined by gas-chromatography (GC) with flame ionization detector (FID). Two pure vegetable oils, pumpkin seed and sunflower oil, processed by minimal technologies (cold pressing) in Romania and an adulterated pumpkin seed oil with 30% of sunflower oil were used as sample matrices in the method development.

MATERIALS AND METHODS

Sampling and Reagents

Two pure oils, pumpkin seed oil and sunflower oil, processed by minimal technologies (cold pressing, not rafined) in Romania and an adulterated pumpkin seed oil with 30% of sunflower oil were selected for analysis. Lipid standards were from Sigma-Aldrich (St. Louis, MO, USA), Merck or Fluka (Buchs, Switzerland). All solvents (analytical-reagent grade or HPLC grade) used were purchased from Merck (Darmstadt, Germany).

Sample preparation for total sterol analysis

After the addition of 5α-cholestane-3β-ol (2 mg) as an internal standard, the oils (1.5 ±0.01 g) were saponified by refluxing in 70 ml of a 1M KOH ethanol/water (8:2, v/v) solution for 1 h. The refluxed mixture was then transferred into a separatory funnel, and the reflux bottle was washed with 10 ml of water. The unsaponifiables (the total sterols) in the combined solution were then extracted two times: firstly with 15 ml of petroleum ether and secondly with 15 ml of diethyl ether. The ether phase was combined, washed three times with 20 ml 5% NaCl solution, and dried with sodium sulfate overnight. The ether phase was filtered into a evaporation bottle and after was evaporated to dryness (using rotavapor). The residue was transferred in a vial with petroleum ether and stored until derivatisation process.

Derivatization of the sterols was made with N,O bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) in pyridine (Piironen V., et al., 2003).

Instrumentation, conditions of analysis and identification

The GC separation of sterol TMS ethers was performed using an Rtx-5 fused silica capillary column (5% phenyl / 95% dimethylpolysiloxane, 30 m x 0.25mm i.d., film thickness 0.25 µm; Restek Corporation, Bellefonte, PA, USA). A SHIMADZU GC-17-A gas-chromatograph equipped with a flame ionization detector (FID) was used. The temperature program was: 5 min at 200 ^{o}C, 10 ^{o}C / min to 300 ^{o}C (hold 20 min).The injection volume was 0.5 µl (split ratio 1:40). The carrier gas was helium.

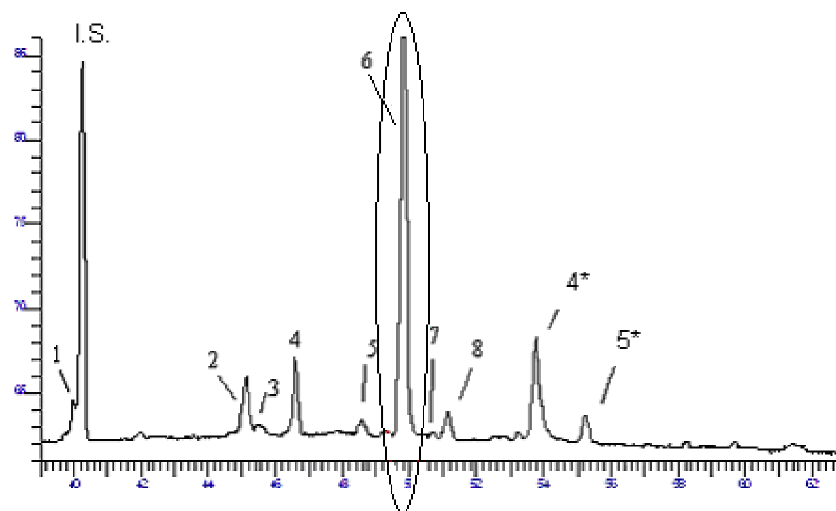
The identification of sterols was based on comparison of their retention times (R_t) with data from the literature (Phillips et al., 2005; Kalo et al., 2001). A mixture of sterol standards (sitosterol 95%, campesterol 98%, stigmasterol 95% and sitostanol 96.7%; Sigma Chemical Co) was studied in the same conditions and the retention times (R_t) were used to assist the peak identification. The sterol concentration were calculated using the aria of the internal standard peak.

Statistics

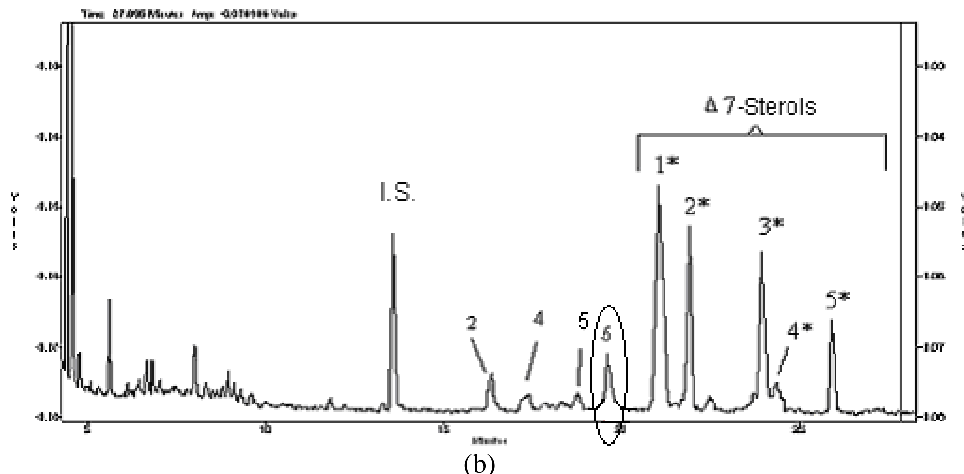
All the extractions and GC-FID analysis were made in triplicate. Sampling results were analyzed using ANOVA (GraphPad Prism Version 4.0, Graph Pad Software Inc., San Diego CA) and were expressed as mean ± standard deviation (m±SD).

RESULTS AND DISCUSSION

The GC-FID chromatograms obtained after analysis of total sterol fractions of the studied pure oils are presented in Fig.1.



(a)



(b)

Fig.1.GC-FID chromatograms of total sterols from sunflower (a) and pumpkin seed (b) oils;
The peaks identification :I.S.: 5α-cholestan-3β-ol; 1: Cholesterol; 2: Campesterol; 3: Campestanol; 4: Stigmasterol; 6: β-sitosterol; 7: Sitostanol; 8: Δ5-avenasterol; 5: Δ7- campesterol (identification tentative, SR EN ISO 12228 (2001)); The Δ 7 phytosterols from the pumpkin seed oil (identification tentative, Wenzl et al. (2002)):1*: Spinasterol; 2*: Δ 7,22,25-stigmastatrienol; 3*: Δ 7,25-stigmastadienol; 4*: Δ 7-stigmastenol; 5*: Δ 7- Avenasterol

Total sterol concentrations of sunflower (unrefined) oil, pumpkin seed oil, and adulterated pumpkin seed oil with 30% sunflower oil after direct saponification are presented in Tab. 1.

Tab. 1

Total sterol concentrations (mg/100g oil) of sunflower (unrefined) oil, pumpkin seed oil, and adulterated pumpkin seed oil with 30% sunflower oil

The saponified oil samples								
			Sunflower (unrefined) oil		**Pumpkin seed oil**		**Adulterated Pumpkin seed oil with sunflower oil**	
Total sterols (mg/100g)								
Peak nr	RRt	Sterols	$\overline{m}$	DS	$\overline{m}$	DS	$\overline{m}$	DS
1.	0.80	Colesterol	0.80	0.05	-	-	-	-
2.	0.90	Campesterol	33.19	0.87	4.29	0.08	*14.51*	*0.32*
3.	0.91	Campestanol	3.39	0.16	-	-	*0.81*	*0.08*
4.	0.94	Stigmasterol	29.17	0.87	1.72	0.05	*10.83*	*0.38*
6.	1	β-sitosterol	250.20	0.88	27.58	0.64	*102.22*	*1.21*
7.	1.02	Sitostanol	2.50	0.18	-	-	*0.75*	*0.15*
8.	1.03	Δ5-avenasterol	22.49	0.57	-	-	*7.53*	*0.98*
Total 1			**341.74**	**3.58**	**33.59**	**0.77**	**136.65**	**3.12**
Other sterols (Δ7-sterols)								
5.	0.98	Sterol 5	8.33	0.52	9.22	0.22	*8.91*	*0.28*
1*	1.10	Sterol 1*	-	-	87.30	0.16	*86.53*	*0.56*
2*	1.12	Sterol 2*	-	-	84.32	0.09	*85.33*	*0.48*
3*	1.21	Sterol 3*	-	-	73.56	0.27	*72.88*	*0.38*
4*	1.23	Sterol 4*	39.56	0.79	5.92	0.11	*15.82*	*0.18*
5*	1.33	Sterol 5*	21.83	0.63	32.31	0.10	*29.11*	*0.26*
Total 2			**69.72**	**1.94**	**292.63**	**0.95**	**298.58**	**2.14**
Total (1+2)			**411.46**	**5.52**	**326.22**	**1.72**	**435.23**	**5.26**

Abbreviations: Peak nr., the peak number of the sterol from the cromatograms (Fig.1); RRt, Retention times relative to β-Sitosterol TMS ether, using an RTX-5, 30 m column ($RR_t = R_t$ (min.) of sterol/ R_t (min.) of β-Sitosterol); $\overline{m}$, mean value of three determination ; DS, standard deviation; Total 1, sum of the identified sterol concentrations after direct saponification of the oil samples ; Total 2, sum of the Δ7-sterol concentrations after direct saponification of the oil samples ; Total (1+2), sum between Total 1 and Total 2.

According to GC fingerprint we noticed some qualitative and quantitative differences between sterol compositions of studied oils.

Qualitative differences

Comparison of the chromatographic pattern of the phytosterol fraction of pumpkin seed oil with those of other vegetable oil samples permitted to detect adulteration of pumpkin seed oil.

Most of plant oils, like sunflower oil, contain predominantly Δ5-sterols with only trace amounts of Δ7-sterols. β-Sitosterol, stigmasterol and campesterol are among the most common Δ5-sterols.

The predominance of Δ7-sterols (free or glycosidic bound form) appears to be restricted to only a few plant families, e.g. *Cucurbitaceae* and *Theaceae* (Breinholder at al., 2002).The pumpkin seed oil can be identified by the presence of larger amounts, of Δ7-sterols.

The total sterol profile of adulterated pumpkin seed oil with sunflower oil was not so different than the pure pumpkin seed oil sterol's profile. Only the peaks nr. 4 and 6 (Fig.1), which was corresponding to stigmasterol and β-Sitosterol becames bigger after the addition of sunflower oil.

Quantitative differences

The total sterol content was higher in sunflower oil (411 mg/100g oil) than in pumpkin seed oil (326 mg/100g oil) (see Table1). The sunflower oil contain higher quantities of Δ5-sterols, namely β-sitosterol (250 mg/100g oil) (peak nr. 6), campesterol (33 mg/100g oil) (peak nr. 2), and stigmasterol (29 mg/100g oil) (peak nr. 4), comparing with the pumpkin seed oil.

In sunflower oil the ratios (between concentrations) of peaks (6)/ (4) and (2)/ (4) were 8.6 and 1.1 respectively. In pumpkin seed oil the ratios of peaks (6)/ (4) and (2)/ (4) were 15.7 and 2.5 respectively. After the sunflower oil was added to pumpkin seed oil, we observed that the sterol amount in this admixture increased considerable (435 mg /100g adulterated pumpkin seed oil) (Tab. 1) and the ratios of peaks (6)/ (4) and (2)/ (4) are closer of sunflower oil (Fig.2.).

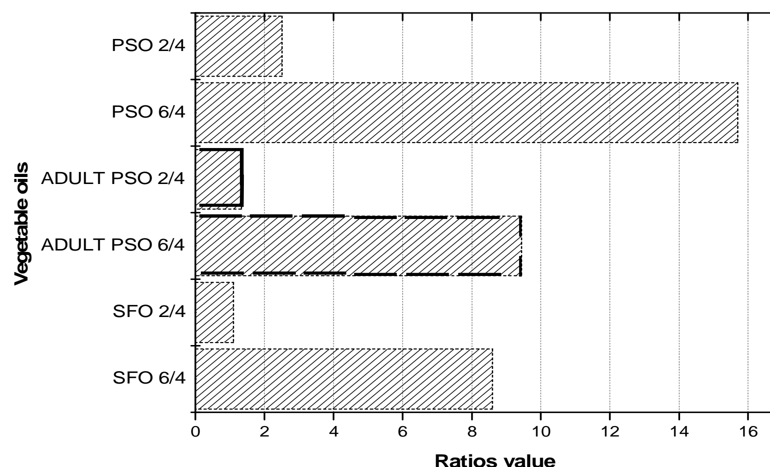


Fig.2. The ratios value of peaks (6)/ (4) and (2)/ (4)

CONCLUSIONS

In case of pumpkin seed oil, the adulterated samples can be distinguished from pure oil by their phytosterol composition. In contrast to most edible oils, pumpkin seed oil contains very low amounts of Δ5-sterols like stigmasterol (5,22-cholestadien-24β methyl- 3β-ol), campesterol (24α-ethylcholest-5-en-3β-ol) or β-sitosterol (24α-ethylcholeste- 5-en-3β-ol), while the main phytosterol content consists of Δ7-sterols. Therefore, concentration of Δ5-sterols can be considered a quantitative authenticity marker for the pure pumpkin seed oil. The concentrations of β-sitosterol, stigmasterol and campesterol as well their ratios can identify the adulteration of pure pumpkin seed oil with cheaper sunflower oil.

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