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Free and Esterified Sterol Distribution in Four Romanian Vegetable Oil

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Abstract

The unsaponifiable lipid fraction of plant-based foods is a potential source of bioactive components such as phytosterols, triterpenoids, carotenoids, tocopherols and various hydrocarbons. The free and esterified sterol concentrations in four Romanian edible oils (corn germ, wheat germ, sweet almond and grape seed oil) were determined, including individual values for β-sitosterol, campesterol, stigmasterol, Δ5-avenasterol, sitostanol, campestanol, and cholesterol. Free and esterified sterols were separated by solid-phase extraction (SPE), saponified, and analyzed as trimethylsilyl ether derivatives using gas-chromatography (GC) with flame ionization detector (FID). Differences in total sterol content and the proportion of esterified (ES) and free sterols (FS) were evident for studied oil samples. In general, β-sitosterol was the most prevalent phytosterol, ranging in concentration from 158.3 mg/100 g in grape seed oil to 478.5 mg/100 g in corn germ oil. Only in these two vegetable oil, we identified trace amount of cholesterol (<3 mg/100g). The total sterol concentrations ranged from 199.9 mg/100g (sweet almond oil) to 745.2 mg/100 g (corn germ oil). In corn germ and wheat germ oil, the dominant form of sterols was the esterified one (60.7% ES and 55.6% ES, respectively, of total sterols). This study consolidates the view that vegetable oils are good natural sources of phytosterols. The analyses of these components provide rich information about the identity and quality of vegetable oils. The corn germ and wheat germ oils proved to be the richest sources in phytosterols, being recommended as functional oils.

Keywords: sterols, free sterols, esterified sterols, vegetable oils, GC-FID

Introduction

In vegetable oils, triglycerides are the main constituents, making up about 95-98% of the oils. The remaining nonglyceridic fraction consists of different compound classes such as sterols, tocopherols, hydrocarbons etc. Phytosterols (PS) are members of the triterpene family of natural products and make up the greatest proportion of the unsaponifiable fraction of lipids. Their chemical structure is similar to cholesterol, but differs in the side chain. PS have a double bond between C5-C6 (Δ5) and a hydroxyl group in *β*-position at C3 and if the molecule is saturated at C5-C6 the compound is referred to as a "stanol" (Fig. 1). PS can be divided into three major subgroups based on the number of methyl groups on carbon 4, two (4, 4'-dimethyl), one (4-monomethyl) and none (4-desmethyl) (Moreau *et al.,* 2002). 4-Desmethyl sterols are predominant in vegetable oils. *β*-Sitosterol usually makes up more than 50% of the desmethyl sterols. The other particularly significant sterols are stigmasterol and campesterol.

In nature, in all plant tissues sterols can be found as free sterols or as four types of conjugates: steryl fatty-acid esters (SE), hydroxycinnamate steryl esters (HSE), steryl glycosides (SG), and as acylated steryl glycosides (ASG). Vegetable oils usually contain primarily free sterols (FS), SE and little or no SG or ASG (Normen *et al.,* 2007).

Free sterols are a part of the cell wall and have important structural functions They can interact, by hydroxy group from C3 with phospholipids and proteins in the cell membranes. FS also regulate the fluidity of the cell membranes and play an important role in the temperature adaptation of the membranes (Piironen *et al.,* 2000). Phytosteryl esters are storage products in the cell and can be found in the cytosol and in droplets or vesicles (Lorentz *et al.,* 1989).

The analysis of free and esterified components of phytosterols can provide some key information concerning authenticity of the oils (Cercaci *et al.,* 2003; Mandl *et al.*, 1999).

The richest natural sources of plant sterols in the human diet are unrefined vegetable oils, seeds, nuts, legumes, and vegetables. In plant oils, the composition of phytosterols may vary due to plant species, agronomic and climatic conditions, extraction and refining procedures and the quality of the fruits or seeds.

Phytosterols have been studied for their hypocholesterolemic, anticarcinogenic, and other health effects (Miettinen and Vanhanen, 1994; Noakes *et al.,* 2002; Ostlund,

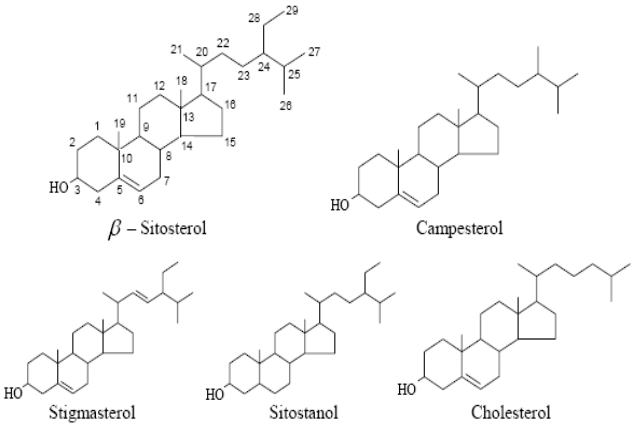

Fig. 1. The chemical structures of some common sterols: sitosterol, campesterol, stigmasterol, sitostanol (the saturated form of sitosterol) and cholesterol

2002). Several studies showed that the regular intake of food enriched in phytosterols could lead to a decrease in the low-density lipoprotein (LDL) cholesterol without a significant change in the high-density lipoprotein (HDL) cholesterol or triglycerides (Ewart *et al.,* 2002; Ntanios *et al.,* 2003). Therefore, the development of food technology has created some foods enriched with phytostanols and phytosterols. At present, several functional food product types such as yoghurts and milk with added plant sterols and stanols are available on the market (Lagarda *et al.,* 2006). One of the main objectives of industry is to identify plant matrices rich in those compounds.

In the last decade, food, pharmaceutical and cosmetic industries have shown a great interest for the corn germ, wheat germ, sweet almond and grape seed oils due to their properties.

Almonds are a rich source of nutrients and phytochemicals such as vitamin E, monounsaturated fatty acids and polyunsaturated fatty acids (Mandalari *et al.,* 2010). Consumption of sweet almonds (as snacks in the diets) or sweet almond oil has been linked to a lowered risk of cardiovascular heart disease (CHD) (Jenkins *et al.,* 2002). Almonds also showed to be more effective than wheat bran in inhibiting colon cancer cells (Davis and Iwahashi, 2001; Yang *et al.*, 2009).

Wheat germ oil is extracted from the germ of the wheat kernel, which contributes to only 2½ percent weight of the kernel. The crude oil is usually somewhat high in unsaponifiable matters (2-6%) and contain high concentrations of vitamin E, essential fatty acids, and long-chain waxy alcohols such as octacosanol (Megahad and El Kinawy, 2002).

Grape seed oil is rich in unsaturated fatty acids, such as linoleic and oleic acid. Grape seeds extracts can offer many advantages for human consumption as it also contains monomeric flavan-3-ols, phenolic acids and oligomeric proanthocyanidins, which exhibit potential antioxidant activity (Dalmolin *et al.*, 2010).

Most commercial corn oil is produced by either pressing corn germ (a fraction derived from the kernel, consisting mainly of embryos) or extraction with hexane. Linoleic acid is the predominant fatty acid in corn oil, with smaller proportions of oleic, palmitic, and linolenic acids. Although commercial corn oil contains very low levels of carotenoids and high levels of tocopherols and phytosterols (Moreau *et al.,* 2010).

Few studies on composition of free and esterified sterols in vegetable oils have been reported. Free sterols dominate in sesame, palm, soybean, olive and sunflower oils (50-80%) but, in corn oil, free sterols represent only one-third of total plant sterol (Phillips *et al*., 2002).

The objective of this work was the identification and quantification of total, free and esterified sterols as trimethylsilyl ether derivatives (TMS), of four Romanian vegetable oil (corn germ, wheat germ, sweet almond and grape seed oil) using gas-chromatography (GC) with flame ionization detector (FID).

Materials and methods

Samples

Four edible seed/ kernel oils: corn germ *(Zea mays)*, wheat germ *(Triticum vulgare*), sweet almond (*Amygdalus dulcis*) and grape seed *(Vitis vinifera)* oils were analyzed in this study. The oils were bought from a local health food store in Cluj-Napoca, Romania. Lipid standards were from Sigma-Aldrich (St. Louis, MO, and USA), Merck or Fluka (Buchs, Switzerland). All chemicals and solvents (analytical-reagent grade or HPLC grade) used were purchased from Merck (Darmstadt, Germany).

Sample preparation for sterol analysis

Total sterols

After the addition of 5α-cholestane-3β-ol (2 mg) as an internal standard, 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.

The sterols were derivatized with N, O-bis (trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) in pyridine (Piironen *et al.*, 2003).

Free and esterified sterols

Free and esterified sterols, was separated by solid phase extraction using a glass column (inside diameter: 10 mm, length: 20 cm) with a stopcock (Teflon) at the bottom to control the solvent flow, filled with Silica gel 40 (0.063-0.200 mm) (Merck-Germany).

Four glass tubes were pre-loaded with 50 μg each 5α-cholestane and 5α- cholestane-3β-ol (0.5 ml each of 0.1 mg/ml chloroform solution evaporated to dryness). 5α-cholestane was used as internal standard for the esterified sterols, eluted from the column with this fraction and 5α-cholestane-3β-ol as internal standard for the free sterols, eluted with this last fraction (Phillips *et al.*, 2002).

Tab. 1. The elution scheme for separation of free sterols and sterol esters

Eluting solvent mixture	Volume (ml)	Lipids eluted
Petroleum ether : diethyl ether (98:2, v/v)	40	Sterol esters
Petroleum ether : diethyl ether (95:5, v/v)	60	Triacylglycerols
Petroleum ether : diethyl ether (50:50, v/v)	40	Free sterols, diacylglycerols, and residual triacylglycerols

The oil sample (300 mg) was weighed into each tube. One millilitre of diethyl ether/petroleum ether (2:8, v/v) was added to each sample and thoroughly mixed. The oil sample solutions were quantitatively transferred to the column filled with Silica gel. The elution scheme for separation of free sterols and sterol esters is presented in Tab. 1.

The sterol ester fractions were transesterified to fatty acid methyl esters (FAMEs) and sterols (Christie, 1982). The free sterol fraction was subjected also to transesterification to degrade the diacylglycerols and the residual triacylglycerols, co-eluted with free sterols. The dried organic phases which were obtained after transesterification were fractionated again by column chromatography. The FAMEs were eluted with petroleum ether: diethyl ether = 90:10(v/v) solvent mixtures and the free sterols were eluted with petroleum ether: diethyl ether = 50:50(v/v). After the fractions were collected, the solvent mixtures were evaporated. The sterols from each fraction were derivatized in the same way like for the total sterols and submitted to GC analysis too.

The experimental conditions

GC separation of sterol trimethylsilyl (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°C, 10°C/min to 300°C (hold 20 min). The injector and FID temperatures were 280°C and 300°C, respectively. The injection volume was 0.5 μl (split ratio 1:40). The carrier gas was helium.

Identification of sterols was based on comparison of their relative retention times (RRt to β-sitosterol) with data from the literature (Kalo and Kuuranne, 2001; Philips e*t al.*, 2005; Yang *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 concentrations were calculated using the aria of the internal standard peak (Li *et al.*, 2007).

Statistics

All the extractions and GC-FID analysis were made in triplicate. Dates were expressed as mean±S.D. The Student's *t*-test was used to test for statistical differences between the individual sterol compositions investigated in the two sterol fractions. Statistical differences between samples were tested using ANOVA (GraphPad Prism Version 4.0, Graph Pad Software Inc., San Diego CA). The level of significance was set at $p<0.05$.

Results and discussion

The individual and total sterol contents of the four analyzed seed/kernel oils are shown in Tab. 2.

The corn germ oil had the highest concentration of total sterols (745.21 mg/100g), and the sweet almond oil sample had the lowest sterol content (199.90 mg/100g). Maguire *et al*. (2004) and Miraliakbari *et al*. (2008) found much higher levels of total sterols in sweet almond oil (220-260 mg/100g) compared to our results. The total sterol concentrations for corn germ oil reported in this study were in good agreement with Normen *et al*. (2007) (843 mg/100g), Phillips *et al*. (2002) (680-760 mg/100g) and Verleyen *et al*. (2002) (699-900 mg/100g). There was some difference in the total sterol concentration of wheat germ and grape seed oils found in this study compared to that of Nystrom *et al.* (2007), Normen *et al.* (2007) and Crews *et al.* (2006). Content of the total unidentified sterols in the analyzed vegetable oils varied from 6.27mg/100g (in sweet almond oil) to 62.65 mg/100g (in wheat germ oil).

Total sterol contents in all four analyzed Romanian vegetable oils were higher than that in extra virgin olive oil, having a median value of 160 mg/100g (Phillips *et al*., 2002) and lower than those in rapeseed oil (800 mg/100g; Schwartz *et al.*, 2008). Only in corn germ and wheat germ oils total sterol concentrations were higher than in sun-

Tab. 2. Total sterol concentrations (mg·100g $^{-1}$ ± SD)[A] of sweet almond, grape seed, corn germ and wheat germ oils, after direct saponification

The saponified oil samples					
		Sweet almond oil	Grape seed oil	Corn germ oil	Wheat germ oil
RRt	Total sterols (mg/100g)	Mean ± SD (n=3)	Mean ± SD (n=3)	Mean ± SD (n=3)	Mean ± SD (n=3)
0.80	Cholesterol	-	2.83 ± 0.03 a	1.77 ± 0.02b	-
0.90	Campesterol	6.27 ± 0.04d	25.77 ± 0.25 c	129.40 ± 0.50a	83.47 ± 0.30b
0.91	Campestanol	3.81 ± 0.03c	3.24 ± 0.04d	10.48 ± 0.12b	14.33 ± 0.15a
0.94	Stigmasterol	5.95 ± 0.03d	23.53 ± 0.48 b	56.51 ± 0.26a	7.25 ± 0.05c
1	β-sitosterol	152.70 ± 0.41d	158.30 ± 0.47c	478.50 ± 0.75a	248.40 ± 0.45b
1.02	Sitostanol	4.85 ± 0.03d	6.84 ± 0.04 c	15.43 ± 0.20a	8.26 ± 0.05b
1.03	Δ5-avenasterol	20.05 ± 0.18b	18.62 ± 0.24c	37.76 ± 0.24a	18.35 ± 0.06c
	Other sterols	6.27 ± 0.07c	61.97 ± 0.57 a	15.36 ± 0.15b	62.65 ± 0.09a
	Total	199.9 ± 0.79d	301.10 ± 2.12 c	745.21 ± 2.24a	442.71 ± 1.15b

[A] Each value is a mean ± standard deviation (SD) of a triplicate analysis performed on different samples.
a, b, c, d Different letters in the same row mean significant differences ($p<0.05$) using ANOVA "Tukey's Multiple Comparison Test".
RRt, Retention times relative to β-Sitosterol TMS ether (Rt =49.80 min.), using RTX-5, 30 m column (RRt =Rt (min.) of sterol/ Rt (min.) of β-Sitosterol). Other sterols- are the unidentified sterols (RRt ≥ 1.08)

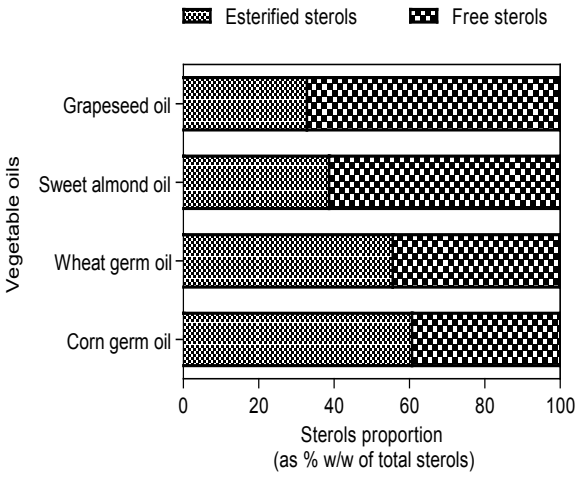

Fig. 2. Sterol composition of edible oils: total free and total esterified sterols as percents of total sterols

flower oil (expeller-pressed) (350 mg/100g) (Phillips *et al*., 2002).

The proportion of esterified and free sterols varied widely. Fig. 2 illustrates the sterol composition of the analyzed vegetable oils in order of increasing proportion of esterified sterols.

In wheat germ and corn germ oils esterified sterols constituted 55.60-60.70% of total sterols. On the other hand, in the grapeseed and sweet almond oils free sterols predominated, with esterified sterols representing only 32.80-38.70% of total sterols. High free sterol contents were also found in soybean and extra virgin olive oils (about 75% of total sterols) (Phillips *et al*., 2002).

As shown in Tab. 2, the amount of individual phytosterol components can vary significantly ($P<0.05$) depending on the oil type. The results listed in this table agreed remarkably well with the reported dates in literature (Harrabi *et al.,* 2008; Mandalari *et al.,* 2010; Normen *et al.,* 2007; Pardo *et al.,* 2009; Philips *et al.,* 2005).

Tab. 3. Content of free and esterified sterols (mg·100g $^{-1}$ ± SD)[A] in sweet almond and grapeseed oil

Sterol concentrations (mg/100 g oil)					
		Sweet almond oil		Grapeseed oil	
		ES	FS	ES	FS
RRt	Sterols	Mean ± SD (n=3)	Mean ± SD (n=3)	Mean ± SD (n=3)	Mean ± SD (n=3)
0.80	Colesterol	-	-	0.81 ± 0.01 b	2.03 ± 0.06 a***
0.90	Campesterol	3.15 ± 0.05 a**	2.92 ± 0.05 b	6.28 ± 0.09 b	18.26 ± 0.21 a***
0.91	Campestanol	1.81 ± 0.04 a	1.75 ± 0.04 a	1.42 ± 0.03 a*	1.34 ± 0.03 b
0.94	Stigmasterol	2.75 ± 0.03 b	3.15 ± 0.03 a***	6.73 ± 0.04 b	15.37 ± 0.09 a***
1	β-sitosterol	58.46 ± 0.44 b	95.63 ± 0.46 a***	68.73 ± 0.50 b	88.83 ± 0.85 a***
1.02	Sitostanol	-	4.29 ± 0.06	3.19 ± 0.09 a	3.36 ± 0.04 a
1.03	Δ5-avenasterol	5.83 ± 0.05 b	13.87 ± 0.08 a***	5.79 ± 0.08 b	12.59 ± 0.31 a***
	Other sterols	4.95 ± 0.07	-	1.87 ± 0.10 b	53.23 ± 0.29 a***
	Total	76.95 ± 0.68 b	121.61 ± 0.72 a***	94.82 ± 0.94 b	195.01 ± 1.88 a***

Tab. 4. Content of free and esterified sterols (mg 100g $^{-1}$ ± SD)A in wheat germ and corn germ oil

Sterol concentrations (mg/100 g oil)					
		Wheat germ oil		Corn germ oil	
		ES	FS	ES	FS
RRt	Sterols	Mean ± SD(n=3)	Mean ± SD (n=3)	Mean ± SD (n=3)	Mean ± SD (n=3)
0.80	Colesterol	-	-	0.89 ± 0.03 a**	0.72 ± 0.03 b
0.90	Campesterol	53.34 ± 0.15 a***	28.16 ± 0.35 b	70.42 ± 0.54 a***	58.52 ± 0.50 b
0.91	Campestanol	9.30 ± 0.10 a***	4.11 ± 0.11 b	4.90 ± 0.05 a	4.93 ± 0.15 a
0.94	Stigmasterol	5.24 ± 0.04 a***	2.80 ± 0.03 b	34.05 ± 0.18 a***	19.86 ± 0.21 b
1	β-sitosterol	115.70 ± 0.46 b	135.23 ± 0.51 a***	293.44 ± 0.57 a***	183.46 ± 0.54 b
1.02	Sitostanol	4.84 ± 0.04 a***	3.49 ± 0.03 b	9.15 ± 0.23 a***	4.58 ± 0.26 b
1.03	Δ5-avenasterol	0.87 ± 0.02 b	16.65 ± 0.32 a***	25.20 ± 0.40 a***	12.10 ± 0.26 b
	Other sterols	53.73 ± 0.06 a***	3.82 ± 0.02 b	8.30 ± 0.10 a***	4.91 ± 0.03 b
	Total	243.02 ± 0.87 a***	194.26 ± 1.37 b	446.35 ± 2.10 a***	289.08 ± 1.98 b

[a,b]Mean values with different letters within a row for different fractions (ES and FS) of same vegetable oils are significantly different, *p<0.05; ** p<0.01; *** p<0.001. Student's t-test. [A] Each value is a mean ± standard deviation (SD) of a triplicate analysis performed on different samples. RRt, Retention times relative to β-Sitosterol TMS ether (Rt =49.80 min.), using RTX-5, 30 m column (RRt =Rt (min.) of sterol/ Rt (min.) of β-Sitosterol) *Other sterols*- are the unidentified sterols (RRt ≥ 1.08)

The predominant phytosterols in all vegetable oils were β-sitosterol and campesterol (Fig. 3), excepting the sweet almond oil, where the concentration of campesterol was significantly lower (6.27 mg/100g) (P< 0.05) than in the other four oils (Tab. 2).

High β-sitosterol content was also found in the majority of vegetable oils, such as olive oil, peanut oil, sunflower oil, soybean oil and rapeseed oil, in which the mean relative contents were 84%, 68%, 62%, 55% and 52% of total sterols, respectively (Phillips *et al.,* 2002; Normen *et al.,* 2007).

Δ^5-avenasterol was present in all samples in small percents (Fig. 3) (10,03% sweet almond oil; 6.18% in grape seed oil, 5.07% in corn germ oil and 4,14% in wheat germ oil) but corn germ oil contained significantly more (P< 0.05) than other oils (37.76 mg/100g).

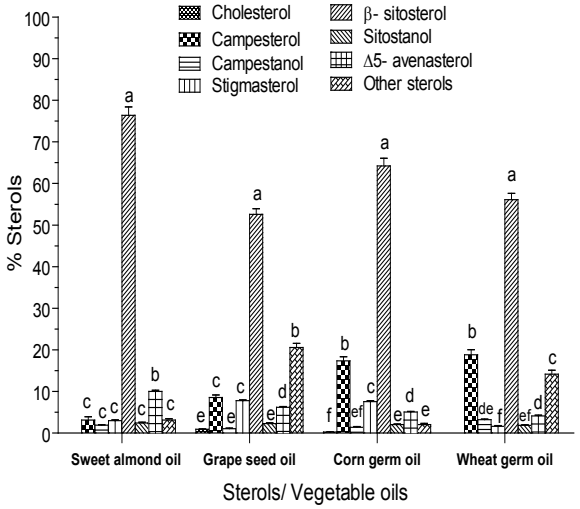

Fig. 3. The proportions (as % w/w of total sterols) of sterols in studied vegetable oils. [a, b, c, d, e, f] Different letters in the same group mean significant differences (p<0.05) using ANOVA "Tukey's Multiple Comparison Test". Horizontal bars depict the SD of the replicates

A negligible amount of the cholesterol (<3 mg/100 g) was observed in two of analyzed oils: 0.94% of total sterols of grape seed oil and 0.24% of total sterols of corn germ oil (Fig. 3).

The quantitative and qualitative composition of the free and esterified sterols is given in Tab. 3 and Tab. 4.

β-Sitosterol predominates in all the sterol fractions. β- sitosterol and Δ5-avenasterol were predominately free in sweet almond, grapeseed and wheat germ oils and esterified in corn germ oil (Tab. 3 and Tab. 4). In three of studied oils (sweet almond, wheat germ and corn germ oils), most of the campesterol was esterified. The saturated sterols (campestanol and sitostanol) were mainly esterified only in wheat germ and corn germ oils. Cholesterol is predominantly in free form in grape seed oil and in esterified form in corn germ oil.

Conclusions

The results of this work offer valuable information for food technologists in order to provide phytosterol enriched diets. This study consolidates the view that vegetable oils are good natural sources of phytosterols. The corn germ and wheat germ oils proved to be the richest sources in (total) phytosterols and in esterified form of the sterols, being recommended as functional oils. Knowledge of proportion of esterified sterols (readily soluble in oil-water mixtures) from the total sterols of vegetable oils is important for the food industry, because these forms of the sterols can be incorporated into a growing spectrum of functional foods such as margarines and salad dressings.

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