CHANGES IN GRAPE SEED OIL DURING FERMENTATION

T. Ovcharova[1], M.Zlatanov[1]*, A. Ivanov[2]

[1]Department of Chemical Technology, University of Plovdiv "PaisiiHilendarski",
24 Tsar Assen Str.,4000 Plovdiv, Bulgaria;
E-mail: magzlat@uni-plovdiv.bg;/ temenuzhka_ovcharova@mail.bg

[2]Agricultural University, Faculty of Viticulture and Horticulture;
E-mail: ivanovangel12@yahoo.com

***Corresponding Author:**
Magdalen D Zlatanov,
Department of Chemical Technology, University of Plovdiv,
24 Tsar Assen Str., 4000, Plovdiv, Bulgaria
E-mail: magzlat@uni-plovdiv.bg

ABSTRACT

The changes of the content and composition of glyceride seed oil during fermentation of one Bulgarian grape variety (Mavroud) were examined. The content of phospholipids (from 0.9% to 0.2%), sterols (from 0.3% to 0.2%), tocopherols (from 359.8mg/kg to 232.5mg/kg) and glyceride oil (from 15.7% to 10.7%) was greatly reduced as a result of the fermentation. Oleic acid increased (from 18.7% to 21.35%)at the expense of linoleic(from 70.1% to 66.8%)in the triacylglycerols. In the fraction of sterol esters oleic and palmitic acids decreased (from 35.5% to 26.4% and from 16.4% to 11.6% respectively) at the expense of the increase of linoleic acid (from 40.7% to 59.4%). Higher quantities of oleic and linoleic acids were established in the phospholipids fraction as well. It was determined that the fermentation affected sterol esters. The main component in both sterol fractions was β-sitosterol. Cholesterol increased from 1.3% to 6.4% and brasicasterol from 1.4% to 4.3%at the expense of campesteroland β-sitosterol. No changes were detected in the fraction of free sterols. It was observed that the amounts of α-tocotrienol increased insignificantly after fermentation. The level of phosphatidylinositol decreased from 29.7% to 19.9% at the expense of increasing quantity of diphosphatidylglycerol from 4.9% to 12.6%. The acid value increased slightly afterfermentation as a result of hydrolysis processes.

Keywords: grape glyceride oil, fatty acids, phospholipids, tocopherols, sterols.

1. INTRODUCTION

Grape (*Vitisvinifera L.*), one of the largest fruit crops worldwide, is mainly used for juice and wine production. Grape pomace is the solid waste product leftover from wine and juice processing, and generally consists of pulp, skins and seeds (Ahmandi and Siahsar, 2011). Approximately 71% of the grape production in the world is used for wine, 27% as fresh fruit and 2% as dried fruit (Anonymous, 2008).Regarding this fact, grape seeds are often referred to as significant agricultural and industrial waste for obtaining glyceride oil (Lutterodt*et al*., 2011; Luque- Rodriguez *et al*., 2005; Freitas*et al*., 2008; Kim *et al.,* 2008). Grape oil is extracted from the seeds after fermentation process.The oil content of grape seeds varies between 10-20 % (Canbay and Bardakc, 2011; Cempos*et al.,* 2008) and depends on grape variety. The main interest in grape seed oil is the high content of the unsaturated fatty acids and in particular linoleic acid (63.0-73.1%) followed by oleic acid (15.4-23.0%) (Akin and Altindisti, 2011; Mattick and Rice, 1976). According to Sabir*et al*. (2012), the amount of oleic acid was higher (16.2-31.2%). Grape seed oil containother important biologically active components such as tocopherols, sterols and phospholipids. The hydrolysis and oxidation that happen during fermentation lead to significant changes in the content and composition of these biologically active components. In literature there is no information about the changes occurring after fermentation. In this connection, the aim of the present study is to determine the changes of all biological active substances of grape seed oil during fermentation.

2. MATERIALS AND METHODS

2.1 Samples. The grape seeds of investigated Bulgarian grape variety *(Mavroud)* were used as materials in the present study.They were provided bythe Agricultural University, Faculty of Viticulture and Horticulture, Plovdiv, Plovdiv region in South Bulgaria, crop 2013.Before the analyses, all seeds were air-dried at room temperature. The content of humidity was found to be 7.8% wt.

2.2 Reagents: All reagents are with analytical grade of purity (p. a).

2.3 Isolation of Glyceride oil. The sample of grape seeds was milled and then extracted in Soxhlet apparatus with hexane. The time required to complete extraction was 8 h (ISO 659, 2009). By means of rotary evaporator the solvent was removed and its remaining amount was evaporated under a stream of nitrogen. The oil content was determined by weight.

2.4 Fatty acids. The fatty acid composition of triacylglycerols, phospholipids and sterol esters of the oil was determined by gas chromatography (GC). The fatty acids were converted to their methyl esters (FAME) after transmethylationat 50^0 C with sulfuric acid in methanol as catalyst (Christie, 2003). Fatty acid methyl esters (FAME) were purified by thin-layer chromatography (TLC) on 20x20 cm plates covered with 0.2 mm Silica gel 60 G layer (Merck, Darmstadt, Germany) with mobile phase n-hexane:diethyl ether 97:3 (by volume). The received FAME were stored at -15^0 C. The samples were analyzed by gas chromatography. GC was performed on a HP 5890 (Hewlett Packard GmbH, Austria) equipped with flame ionization detector (FID),60 m x 0.25 mm capillary DB-23 column (Agilent Technologies, Santa Clara CA, USA).The column temperature was programmed from 130^{o}C (1 min) to 170^{o}C at 6.5 ^{o}C/min; at 3^{o}C/min to 215^{o}C (9 min), at 40^{o}C/min to 230^{o}C (1 min); injector and detector temperatures were 270^{o}C and 280^{o}C respectively. Hydrogen was the carrier gas at a flow rate 0.8 mL/min; split was 100:1. The fatty acids were identified by comparison with standard mixture of FAME (37 component standard mixture provided by Agilent Technology). Each of the determinations was repeated three times.

2.5.Sterols. The total oil sample(sample size of 2-2.5 g, precisely measured) was applied on several 20 cm x 20 cm glass plates with 0.5 mm thick silica gel 60 G layer(*Merck*, Darmstadt, Germany) and developed with hexane:diethyl ether: acetic acid (80:20:1) (by volume). Free (R_f=0.4) sterols were detected by spraying the edges of the plate with methanol, then were scraped, transferred to small glass columns and eluted with chloroform. The solvent was removed by rotary evaporator. Free sterols were subjected to gas chromatography without derivatization. Sterol esterswereсaponificated with ethanolic KOH (Christie, 2003) and the obtained free sterols were separated with n-hexane and then purified by TLC on 0.2N NaOH silica gel 60 G plates, mobile phase hexane: diethyl ether (1:1). Then sterol composition was established by GC with HP 5890 (Hewlett Packard GmbH, Austria) provided with 30 m x 0.25 mm DB-5 column. The temperature gradient ranged from 90^{o}C (2 min) to 290^{o}C (15min) and then increased to 310 ^{o}C at 4 ^{o}C/min (hold 10 min). The injector and detector temperatures were 300 ^{o}C and 320 ^{o}C respectively. Hydrogen was used as a carrier gas at 0.8 mL/min velocity. Sterols were identified by comparison ofthe retention times with these of a standard mixture of sterols (Zlatanov *et. al.*, 2009).

2.6. Tocopherols. The analysis of tocopherol composition was performed with a high performance liquid chromatography (HPLC) (ISO 9936, 2006). The samples of oils were weighed (0.0400-0.0500g) and dissolved in n-hexane (2.0 %) and aliquot of 20 µL of this solution was injected directly into the chromatograph. The analysis was carried out at room temperature on a Merck-Hitachi (Merck, Darmstadt, Germany) HPLC equipped with a 250 x 4 mm Nucleosil Si 50-5 column (Macherey-Nagel, Germany) and fluorescent detector (Merck-Hitachi F 1000) set at the emission at 330 nm and excitation at 295 nm. Tocopherols were eluted with mobile phase hexane: dioxane 96:4, at a flow rate 1 ml/min. They were identified according to order of elution and by comparing their retention times with those of (mixture of pure α, β, γ, δ- tocopherol) standard (Merck Darmstadt, Germany). The amount of tocopherols in the oil was calculated as the sum of tocopherol components; total content was expressed as mg/kg.

2.7. Phospholipids. The air-dried seeds were subjected to Folch extraction according Christie (Christie, 2003). Thus obtained phospholipids were purified with 20cm^{3}refrigerated acetone and the solution stayed about one hour at -18 ^{o}C.Then acetone was decanted for the removing of other impurities and phospholipids were dissolved in 10 cm^3 chloroform. The phospholipid classes were isolated by two-dimensional thin-layer chromatography. The sample from 0.1 cm^3 was poured out drop by drop on 20 x 20 cm glass plates with 0.2 mm Silica gel 60 G layer impregnated with aqueous $(NH_4)_2SO_4$ (1%).In the first direction the plate was developed with chloroform:methanol:ammonia, 65:25:5 (by volume) and in the second –with chloroform:methanol:ammonia:acetic acid:water, 50:20:10:10:5 (by volume) (Zlatanov *et. al.*,2009). The individual phospholipids weredetected and identified by spraying with specific reagents according to Christie (*6*): Dragendorff test (detection of choline-containing phospholipids); Ninhydrin spray (for phospholipids with free amino groups), and Shiff's reagent (for inositol containing phospholipids). Additional identification was performed by comparing the respective R_f values with those of authentic commercial standards subjected to silicagel TLC under identical experimental conditions. The quantification was carried out spectrophotometrically against a standard curve by measuring the phosphorous content at 700 nm after scraping the respective phospholipid spot and mineralization of the substance with a mixture of perchloric acid and sulphuric acid, 1:1 by volume.The calibration curve was constructed by using a standard solution of KH_2PO_4(1-130 µg/ml as phosphorus)(Beshkov and Ivanova, 1972).

2.8 Statistical analysis. All the analyses were made in triplicate. Statistical differences between samples were tested by one-way analysis of variance (ANOVA) using SAS version 8.1 (SAS Institute, Cary, NC, USA). Data were expressed as mean ± SD. The level of significance was set at $p<0.05$.

3. RESULTS AND DISCUSSION

Data about the changes of oil content in the seeds and general composition of glyceride oil are presented in Table 1.

Table 1

Changes of physico-chemical indexes and composition of seed oil during fermentation

№	Compounds	Values	
		before	after
1.	Oil content, %	15.7± 0.5	10.7± 0.2
2.	Phospholipids, %	0.9 ± 0.04	0.2 ± 0.01
3.	Sterols, %	0.3 ± 0.01	0.2 ± 0.01
	Free	93.5	94.1
	Esterified	6.5	5.9
4.	Tocopherols, mg/kg	359.8± 10.8	232.5± 9.3
5.	Wet in seeds, %	7.8± 0.2	8.5± 0.2
6.	Acidity value, mg KOH/g	2.1 ± 0.1	2.4 ± 0.1
7.	Peroxide value, meqO_2/kg	18.2 ± 0.7	17.2 ± 0.5

*Means ± SD of three determination

Changes of physico-chemical indexes and composition of seed oil during fermentation*

The investigated seeds contain relatively low amounts of glyceride oil. These values were similar to data reported about some varieties of foreign origin (Canbay and Bardakc, 2011; Compos*et al.,* 2008; Schuster, 1992). The data clearly shows that the oil content in the seeds and the content of biologically active compounds considerably decreased as result of fermentation. Especially high variation was observed in the quantity of phospholipids whose level decreased more than four times. Total content of tocopherols decreased also significantly. The ratio between free and esterified sterols remainded practically constant. Regarding physico-chemical values it is striking that acidity increased insignificantly. This fact can be explained with hydrolysis processes which occur during the fermentation. There is a slightchange inthe peroxide value because at the time of fermentation there is carbon dioxide and no conditions for oxidative processes. On the other hand, a part of them can dissolve in the wine.

The changes of fatty acid composition of the oil are given in Table 2.

Table 2
Fatty acid composition of triacylglycerols

Fatty acids, %	Values	
	before	after
Lauric($C_{12:0}$)	0.4 ± 0.02	0.1 ± 0.01
Myristic ($C_{14:0}$)	0.1 ± 0.01	0.1 ± 0
Myristoleic ($C_{14:1}$)	-	0.1 ± 0
Pentadecanoic($C_{15:0}$)	0.1 ± 0	0.1 ± 0
Palmitic($C_{16:0}$)	8.8 ± 0.3	9.3 ± 0.4
Palmitoleic($C_{16:1}$)	0.2 ± 0.01	0.1 ± 0.01
Margarinic ($C_{17:0}$)	0.1 ± 0.01	0.1 ± 0
Stearic ($C_{18:0}$)	1.0 ± 0.04	1.3 ± 0.1
Oleic ($C_{18:1}$)	18.7 ± 0.7	21.3 ± 1.1
Linoleic ($C_{18:2}$)	70.1 ± 1.4	66.8 ± 2.7
Linolenic($C_{18:3}$)	0.2 ± 0.01	0.2 ± 0.01
Arahinic($C_{20:0}$)	0.1 ± 0	0.2 ±0.01
Gadoleic ($C_{20:1}$)	0.1 ± 0	0.1 ± 0
Behenic ($C_{22:0}$)	0.1 ± 0	0.2 ± 0.01
SFA	10.7	11.4
MUFA	19.0	21.6
PUFA	70.3	67.0

*Means ± SD of three determinations
SFA- saturated fatty acids, MUFA- monounsaturated fatty acids, PUFA- polyunsaturated fatty acids

Fatty acid composition of triacylglycerols*

Linoleic acid (18:2) was found to be the main component in triacylglycerols (70.1%), followed by oleic (18.7%) and palmitic (8.8%) acids. These results are very close to those described by Akin and Altindisti, 2011.Other fatty acids present in small quantities were stearic, palmitoleic and linolenic. Grape seed oil was poor in linolenic acid and this is favorable to vegetable oils because exactly this fatty acid leads to unpleasant taste and odour in oil.

Results in table 2 show that oleic acid increased slightly from 18.7% to 21.3% after fermentation. This increase in oleic acid is at the expense of the decrease of linoleic acid from 70.1% to 66.8%. The amount of the other fatty acids remained unchanged.

The ratio SFA:USFA also changed significantly at the expense of the decreasing level of PUFA.

Table 3
Composition of free and esterified sterols

Sterols	Values			
	before fermentation		after fermentation	
	free	esterified	free	esterified
Cholesterol	0.5 ± 0.01	1.3 ± 0.1	0.6 ± 0.01	6.4 ± 0.2
Brasicasterol	2.4 ± 0.1	1.4 ± 0.1	1.9 ± 0.04	4.3 ± 0.2
Campesterol	20.0 ± 0.8	14.0 ± 0.3	20.0 ± 0.6	7.1 ± 0.1
Stigmasterol	3.3 ± 0.1	6.9 ± 0.3	2.5 ± 0.1	5.0 ± 0.2
Δ^7-Campesterol	1.7 ± 0.1	2.4 ± 0.1	2.0 ± 0.1	3.5 ± 0.1
β-Sitosterol	70.4 ± 1.4	70.9 ± 2.8	71.0 ± 2.1	61.6 ± 1.2
Δ^5-Avenasterol	0.5 ± 0.02	1.4 ± 0.03	0.4 ± 0.01	4.3 ± 0.1
Δ^7-Avenasterol	0.6 ± 0.02	0.8 ± 0.03	0.4 ± 0.02	4.3 ± 0.2
Δ^{7-}Stigmasterol	0.6 ± 0.02	0.9 ± 0.04	0.7 ± 0.01	3.5 ± 0.1

*Means ± SD of three determinations

Composition of free and esterified sterols*

Sterol composition of investigated oils is presented in Table 3. The qualitative sterol composition in both samples was found to be same. The main components in both sterol fractions were β-sitosterol, campesteroland stigmasterol. There was no practical change in the composition of free sterols before and after fermentation. This picture is not the same at the fraction of sterol esters. The amounts of cholesterol (from 1.3% to 6.4%), brasicasterol (from 1.4 to 4.3%), Δ^5-avenasterol (from 1.4% to 4.3%), Δ^7-avenasterol (from 0.8% to 4.3%) and Δ^7-stigmasterol (from 0.9% to 3.5%) increased at the expense of the decreasing quantities of campesterol (from 14.0% to 7.1%) and β-sitosterol (from 70.9% to 61.6%). Some sterols and sterol esters were undergoing transformation; therefore there was reduction of the total sterols (from 0.3% to 0.2%-Table 1).

The changes of fatty acid composition of sterol esters were presented in Table 4.

Table 4
The fatty acid composition of sterol esters*

Fatty acids, %	Mavroud	Mavroud during fermentation
Lauric($C_{12:0}$)	0.3 ± 0.01	0.3 ± 0.01
Myristic ($C_{14:0}$)	1.3 ± 0.1	0.3 ± 0.01
Pentadecanoic($C_{15:0}$)	0.3 ± 0.01	0.1 ± 0
Palmitic($C_{16:0}$)	16.4 ± 0.7	11.6 ± 0.3
Palmitoleic($C_{16:1}$)	0.2 ± 0.01	0.1 ± 0
Margarinic ($C_{17:0}$)	0.2 ± 0	0.1 ± 0
Stearic ($C_{18:0}$)	3.5 ± 0.1	0.3 ± 0.01

Oleic ($C_{18:1}$)	35.5 ± 1.1	26.4 ± 0.5
Linoleic ($C_{18:2}$)	40.7 ± 1.2	59.4± 2.4
Linolenic($C_{18:3}$)	0.3 ± 0.01	0.1 ± 0
Arahinic($C_{20:0}$)	0.8 ± 0.03	0.2 ± 0.01
Gadoleic ($C_{20:1}$)	0.3 ± 0.01	0.6 ± 0.03
Behenic ($C_{22:0}$)	0.2 ± 0.01	0.3 ±0.01

*Means ± SD of three determinations

The fatty acid composition of sterol esters*

The main unsaturated fatty acids in all sterol ester fractions were linoleic acid followed by oleic acid. In contrast to triacylglycerols, the ratio between them was approximately equal. Palmitic and stearic acids were the main representatives of saturated fatty acids. The results clearly show that the amount of saturated palmitic, staeric and monounsaturated oleic acids significantly decreased after fermentation at the expense of the increasing content of linoleic acid (from 40.7% to 59.4% respectively); this leads to a higher degree of desaturation than in triacylglycerols.

Table 5 presents the variation of individual phospholipid composition.

Phospholipids, %	before fermentation	after fermentation
Phosphatidylcholine	22.0 ± 0.7	21.4 ± 0.4
Phosphatidylinositol	29.7 ± 0.9	19.9 ± 0.4
Phosphatidylethanolamine	10.2 ± 0.4	13.3 ± 0.3
Phosphatidic acids	12.1 ± 0.5	14.4 ± 0.6
Phosphatidylserine	1.8 ± 0.1	n.d
Lysophosphatidylcholine	4.2 ± 0.1	5.9 ± 0.1
Lysophosphatidylethanolamine	12.6 ± 0.4	12.5 ± 0.4
Diphosphatidylglycerol	4.9 ± 0.1	12.6 ± 0.4
Sphingomyelin	2.5 ± 0.1	n.d

*Means ± SD of three determinations

n.d- not detected

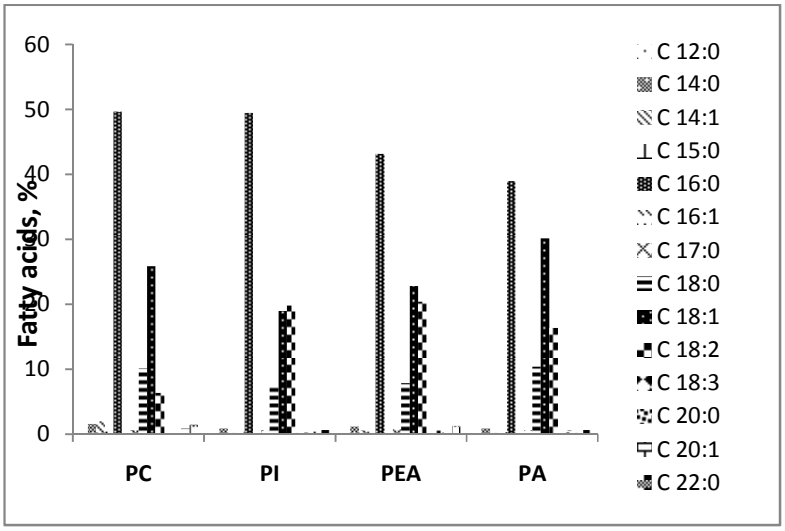
Individual phospholipid composition*

The predominant phospholipids were phosphatidylcholine and phosphatidylinositol. In comparison with other vegetable oils an unusual high content of phosphatidic acids was established. It was noted that phosphatidylinositol decreased significantly at the expense of the relative increase of diphosphatidylglycerol. The quantity of the other phospholipids remained practically unchanged during fermentation.

The changes of the fatty acid composition of the individual phospholipids during fermentation are presented in figure 1 and figure 2.

Figure 1

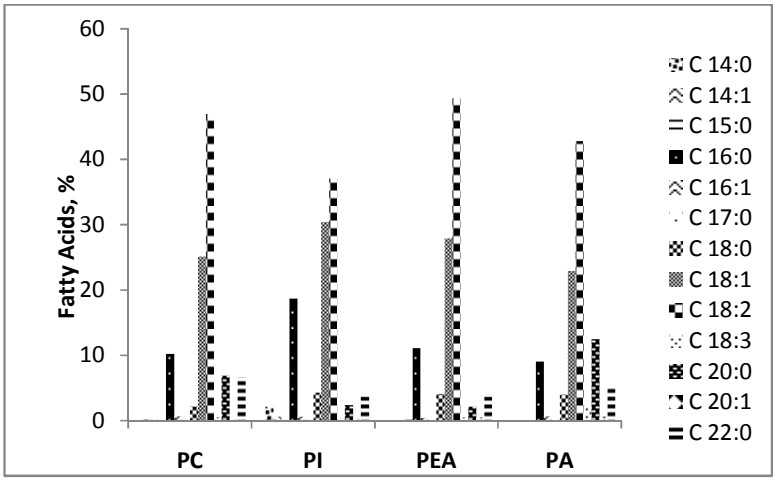
Fatty acid composition of individual phospholipid classes before fermentation



PC- Phosphatidylcholine; PI- Phosphatidylinositol; PEA- Phosphatidylethanolamine; PA- Phosphatidic acids

Figure 2

Fatty acid composition of individual phospholipid classes after fermentation



PC- Phosphatidylcholine; PI- Phosphatidylinositol; PEA- Phosphatidylethanolamine; PA- Phosphatidic acids

In comparison with fatty acid profil of triacylglycerols the content of saturated fatty acids was several times higher (palmitic from 8.8 % v/s 39.0-49.7 % in separate phospholipids, stearic 1.0 % v/s 7.3-10.4 %). The level of oleic acid in the phospholipid fraction was found to be also higher (19.0-30.2 %). These higher values were at the expense of the lower quantities of linoleic acid in individual phospholipids (70.1% in triacylglycerols v/s 6.5-20.4 % in phospholipids).

Significant differences were established between the two separate samples. It was observed that after fermentation the amounts of palmitic and stearic acids greatly decreased in all phospholipid classes (from 39.0-49.7% to 9.0-18.7 % and from 7.3%-10.4% to 2.2-4.3%). This reduction was due to the increase of the polyunsaturated linoleic acid (from 6.4-20.4% to 37.1-49.4%). The level of oleic acid remained constant in

both stages with the exception of phosphatidylinositol whose quantity increased (from 19.0% to 30.4%, respectively).These data suggest that fermentation brought desaturation and degradation of saturated fatty acid.

The variation of individual tocopherols in investigated grape seed oil is reported in table 6.

Table 6
Tocopherol content of grape seed oil*

Tocopherols,%	Values	
	before	after
α-т	36.7 ± 1.5	34.9 ± 1.4
α-3-т	56.5 ± 1.1	61.4 ± 1.2
γ-т	6.8 ± 0.1	3.7 ± 0.1
γ-3-т	tr.	tr.

Tocopherol content of grape seed oil*

The main components in tocopherols fraction were found to be α-tocopherol and α-tocotrienol, followed by γ-tocopherol. This picture is different from the data reported earlier by Sabir*et al*., 2012 and Kraujalyte*et al*., 2011,who anonced that α-tocopherol predominated. The qualitative composition in each sample was found to be similar. There was a negligible increase ofα-3-tocotrienol at the expense of α-tocopherol and γ-tocopherol after fermentation; the greatest amount was that of α-3-tocotrienol, followed by α-tocopherol.

4. CONCLUSION

The present study demonstrated that the changes of physico-chemical indexes vary insignificantly. During fermentation there was a decrease of sizable glyceride oil and the main biologically activecomponents. The fatty acid composition of the separate lipid classes: triacylglycerols, sterols and phospholipids varies significantly, with the quantity of polyunsaturated fatty acid. Increasing individual composition of phospholipids and sterol esters changed considerably, while in free sterols and tocopherols no differences were detected.

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