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ORIGINAL PAPER

Variations in Fatty Acids, Phospholipids and Sterols During the Seed Development of a High Oleic Sunflower Variety

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Abstract The changes in the content and composition of total fatty acids, phospholipids and sterol esters, and their fatty acids, and of free sterols and tocopherols in developing seeds of a selection of high oleic acid sunflower varieties grown in Bulgaria were examined over a period of 15th to 90th day after flowering by means of various chromatographic methods. Under the climatic and geographical conditions typical for the South-East Balkans phospholipid, sterol-, sterol ester- and tocopherol- species are formed practically completely in the first 15 days after flowering. Until the 90th day, only quantitative changes were detected to give a product with 65% oil content, 1% phospholipids, 0.3% total sterols and 0.09% tocopherols. Oleic acid is the main component in all acyl derivatives, reaching 85% of the total fatty acids while palmitic and stearic acid content is about 4% each. The product is a good quality HOSO with beneficial content of FA and good prospects as a salad and cooking oil.

Keywords High oleic sunflower oil · Fatty acids · Phospholipids · Sterols · Tocopherols · Seed developing

Introduction

The sunflower (*Helianthus annuus* L.) is one of the most important oilseed crops in the world. The nutritional quality of the sunflower oil ranks among the best vegetable oils in cultivation and is widely used as salad oil, cooking and frying oil and in margarine production. The high nutritional value of the traditional sunflower oil is due to the high level (60–75%) of the linoleic acid [1, 2]. The oil is rich in tocopherols (α-tocopherol is the main component) which are natural antioxidants preventing to some extent the autoxidation of lipids at ambient temperature. When used as frying oil (at 160–180 °C) physical and chemical degradation occur as a result of oxidative and polymerization processes [3]. In order to enhance the use of sunflower oil in culinary applications, new varieties of sunflower have been selected in which the main fatty acid in the seed oil is oleic acid (*cis*-9-18:1) [2]. Increased interest in these high oleic sunflower oils (HOSO) has been inspired by dietary recommendations for beneficial nutritional effect of high monounsaturated oils with a low content of saturated fatty acids [4]. HOSO are considered to be a valuable alternative to the hydrogenated and partially hydrogenated oils which are used in many food products (confectionery fats and oils, spray oil for fruits and cereals, salad and frying oils) and in industrial applications where high oxidative stability is required [5]. In addition to the fatty acid composition of triacylglycerols, the nutritional value of edible oils depends on the content and composition of other accompanying biologically active components such as phospholipids, sterols and tocopherols [4].

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While the lipid composition of the commercial edible sunflower oil and of some of its varieties is well known [6], the data on the changes in lipids occurring during the seed maturation are focused exclusively on total fatty acid composition [7–16] or some lipid classes [17]. Of these, only a few deal with HOSO [10, 15, 16], but with some exceptions [10] do not examine the changes in lipids during seed maturation. This information is of certain interest for the agronomic and genetic research on sunflower and sunflower cultivars.

The aim of the present work was to expand the study on HOSO by tracking the changes in phospholipids and accompanying components such as sterols, sterol esters and tocopherols, during seed maturation. The changes in the total fatty acid composition and the fatty acid composition of phospholipids and sterol esters during this period were determined as well. The work was carried out on a HOSO from the kernel of the oilseed variety "Diamant", created and cultivated in Bulgaria.

Materials and Methods

All solvents and reagents were analytical grade and were used without additional purification. Reference phospholipids and fatty acid methyl esters were purchased from Fluka Chemie GmbH (Buchs, Switzerland). Reference tocopherol isomers and individual sterols were purchased from Merck (Darmstadt, Germany). TLC plates were prepared in the laboratory using Silica gel 60 (Merck, Darmstadt, Germany).

Samples

The high oleic sunflower "*Diamant*" variety was grown on a farm situated in the Plovdiv region, South Bulgaria in May through September 2002. The region has a moderate continental climate with considerable reflection of the Mediterranean environment. In 2002, the average temperatures and precipitations in the region were as follows—June: 22.6 °C and 81 mm, July: 24.4 °C and 61 mm, August: 22.1 °C and 58 mm. Samples were taken randomly between the 15th and 90th day after flowering in intervals of 15 days by picking 10–20 flower heads randomly from the field (in dry days) and the whole seeds were removed by hand. From a portion of 800 g seeds a sample of 200 g was obtained by quartering. Hulls were removed, and hull and kernel were weighed separately. Specified portions of air-dried kernel were taken for lipid analysis.

Isolation of Glyceride Oil and Determination of Oil Content

A 50-g portion of kernels was subjected to Soxhlet extraction with *n*-hexane for 8 h. The solvent was partly removed in a rotary vacuum evaporator, the residue was quantitatively transferred in pre-weighed glass vessel and the rest of the solvent was removed under stream of nitrogen to a constant weight to determine the oil content.

Phospholipids

Another part (10 g) of air-dried kernels was subjected to Folch extraction according to Christie [18]. Polar lipids were isolated from the total lipids by column chromatography [18]. Briefly, the sample (100 mg) was applied on a 40 × 2 cm glass column packed with Silica gel Unisil 100–200 mesh (Clarkson Chemicals Co., USA) and eluted in sequence with chloroform (for neutral lipids, sterols and sterol esters), acetone (sterol glycosides) and with methanol to isolate phospholipids. The phospholipid classes were isolated by a modified [19] two-dimensional TLC on 20 × 20-cm glass plates pre-coated with 0.2-mm Silica gel 60 G layer (Merck) and impregnated with aqueous $(NH_4)_2SO_4$ (1 g in 100 mL water). 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). The individual phospholipids were detected and identified by spraying with specific reagents according to Christie [18]: Dragendorff test (detection of choline-containing phospholipids); Ninhydrin spray (for phospholipids with free amino groups), and Schiff'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 silica gel 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 sulfuric acid, 1:1 (by volume). The calibration curve was constructed by using a standard solution of KH_2PO_4. It was linear in the concentration range 1–130 µg/mL (as phosphorus). In each series of measurements a standard solution of KH_2PO_4 (10 µL/mL in water) was used to confirm the validity of calibration.

Sterols

Extracted HOSO (sample size of 100 mg, precisely measured) was applied to laboratory-made 20 × 20 cm glass TLC plates (ca. 1 mm thick silica gel G layer) and developed with hexane–acetone, 100:8 (by volume). Free ($R_f = 0.4$) and esterified sterols ($R_f = 0.8$) were detected under UV light by spraying the edges of each plate with 2′,7′-dichlorofluorescein, they were then scraped, transferred to small glass columns and eluted with diethyl ether.

The solvent was evaporated under a stream of nitrogen and the residue was weighed in small glass container to a constant weight. Free sterols were then subjected to GC, without derivatization. Sterol esters were hydrolyzed with ethanolic KOH [18], the free sterols were extracted with light petroleum ether and purified by TLC under the above conditions prior to the GC analysis. Sterol composition was determined on gas chromatograph equipped with 25 m × 0.25 mm × 25 μm HP5 capillary column (Agilent Technologies, Santa Clara CA, USA) and flame ionization detector. The temperature gradient was programmed from 90 °C (held for 2 min) to 290 °C at 15 °C/min then to 310 °C at 4 °C/min and held at this temperature for 10 min; the injector temperature was 300 °C and the detector temperature was 320 °C. Nitrogen was the carrier gas at a flow rate 0.8 mL/min; split 100:1. Identification was confirmed by comparison of retention times with those of a standard mixture of sterols [20].

Tocopherols

Tocopherols were determined directly in the oil by HPLC on an instrument equipped with 250 × 4 mm Nucleosil Si 50-5 column (Merck, Darmstadt, Germany) and fluorescent detector. The operating conditions were as follows: mobile phase of *n*-hexane:dioxane, 96:4 (by volume), flow rate 1 mL/min, excitation 295 nm, emission 330 nm. A 20-μL sample of a 1% solution of crude sunflower oil was injected. Tocopherols were identified by comparing the retention times with those of authentic individual tocopherol standards. The content was calculated on the base of respective peak areas in the sample versus the peak area of a standard α-tocopherol solution as shown in [21, 22].

Fatty Acids

The total fatty acid (FA) composition as well as the fatty acid composition of phospholipids and sterol esters was determined by GC after transmethylation of the respective samples with 1% methanolic sulfuric acid for 2 h at 50 °C according to Christie [18]. Fatty acid methyl esters (FAME) were purified by silica gel TLC on 20 × 20 cm plates covered with 0.2-mm Silica gel 60 G layer (Merck, Darmstadt, Germany) with a mobile phase *n*-hexane:acetone 100:8 (by volume). GC was performed on a gas chromatograph equipped with a 30 m × 0.25 mm × 25 μm capillary InnoWax column (cross-linked PEG, Hewlett Packard GmbH, Austria) and a flame ionization detector. The column temperature was programmed from 165 to 240 °C at 4 °C/min and held at this temperature for 10 min; injector and detector temperatures were 260 °C. Nitrogen was the carrier gas at a flow rate 0.8 mL/min; split was 100:1. Identification was performed by comparison of retention times with those of a standard mixture of FAME subjected to GC under identical experimental conditions [20].

Statistics

All data are presented as a mean value of three separate measurements ± standard deviation (SD). The SD range depended on the component proportions and varied between 5% relative for the values above 20 and 10% relative for values below this value. SD values were determined for instrumental measurements only and do not represent the reproducibility for the whole determination since the latter includes too many analytical stages.

Results and Discussion

General Characteristics of the Oil

Major characteristics of the seeds such as: oil content, content of total phospholipids, sterols and tocopherols were determined. Additionally, the moisture content of kernels was determined. The results are shown in Table 1.

Evidently, oil accumulates rapidly in the kernels in the first 30 days—the oil content increases more than two times between the 15th and 30th day after flowering. Then the accumulation rate slows down and increases by 50% for the next 60 days. The amounts of sterols and of phospholipids remain almost constant, i.e. their formation is completed in the first 15 days of growing.

During the initial stages of development, the mass of the hull predominates over that of the kernels, causing difficulties in determining the oil content of the whole seed. Then hull mass decreases rapidly to reach 23% of the total fresh weight on the 90th day. Thus, the oil content of the whole seeds increased from 2% at the beginning of the growing period to 50% on the 90th day.

These results are close to those reported by other authors. It has been found that oil content in the kernel of the seeds increases to 46–60% on the 30–35th day after flowering and then remains constant or even decreases to 41–48% on the 45th day, while the final oil content may reach 46–64% [23]. The oil accumulation rate in the seeds is assumed to depend on the influence of ambient temperature and humidity during the ripening process [8, 16, 23–26]. The total amount of tocopherols increases gradually throughout the examined period (Table 1). The moisture content (kernels only) decreases gradually from 78 to 6% (Table 1).

Table 1 Characteristics of a high oleic sunflower oil variety grown in Bulgaria during the growing period of 2002

Characteristics	Growing period, in days after flowering[a]					
	15	30	45	60	75	90
Oil in the kernels (% dry wt)	21	45	54	61	62	66
Phospholipids in the oil (% wt)	1	1	1	1	1	1
Sterols in the oil (% wt)	0.5	0.4	0.3	0.3	0.3	0.3
Tocopherols in the oil (mg/kg)	560	572	554	675	750	869
Moisture content in the kernel (% wt)	78	58	31	4	6	6

[a] Mean of three separate determinations

Total Fatty Acid Composition

Figure 1 shows the gradual changes of the FA composition in the kernel oil. As expected, eight fatty acids (a series of even chain saturated FA 14:0–20:0; and unsaturated—16:1, *cis*-9-18:1, *cis*-9, *cis*-12-18:2, *cis*-9, *cis*-12, *cis*-15-18:3) were detected in the oil on the 15th day after flowering, with oleic acid being the main component. Substantial amounts of linolenic acid were found. The saturated palmitic (16:0) and stearic (18:0) FA were found in amounts of about 10% each. With the increasing time of development, the number of FA detected decreased to six. The quantity of oleic acid continued to increase gradually (from 52 to 85%). The content of linoleic acid, while remaining generally low, increased substantially (from 1 to 6%) and the quantities of 16:0 and 18:0 FA decreased to 3–4%. After the 30th day of seed development, the quantity of 20:0 was below 1%. The quantity of linolenic acid decreased substantially (from 22% on the 15th day to hardly detectable traces on the 90th) and palmitoleic acid was practically absent on the 90th day. In general, the unsaturation of the oil (determined as the ratio of unsaturated vs. saturated FA quantities) increased from 3 on the 15th day to 11 on the 90th day.

Thus, on the 90th day, sunflower seeds produce oil with a high percentage of oleic acid (85%) and a low percentage of saturated FA components. Comparison with FA composition of other HOSO [6] shows that the examined variety matches the quantitative composition of typical good quality high oleic acid oils.

Sterols

The changes in the total and individual content of free sterols and sterol esters are shown on Fig. 2a–c. The amounts of free and esterified sterols are given as percentages of the total sterol content. The general pattern shows that the content of free sterols increases and that of sterol esters decreases with seed developing. A 74% fraction of the free sterols are formed in the first 15 days and their content increases to 86% by the 90th day while the content of sterol esters decreases to14.4% (Fig. 1a).

As expected, β-sitosterol was the main component in the free sterol fraction and gradually increased throughout the whole investigated period reaching 78% on the 90th day (Fig. 2b). The contents of stigmasterol and campesterol did not change significantly while the amount of Δ^5-avenasterol decreased rapidly and was only 1% of the total free

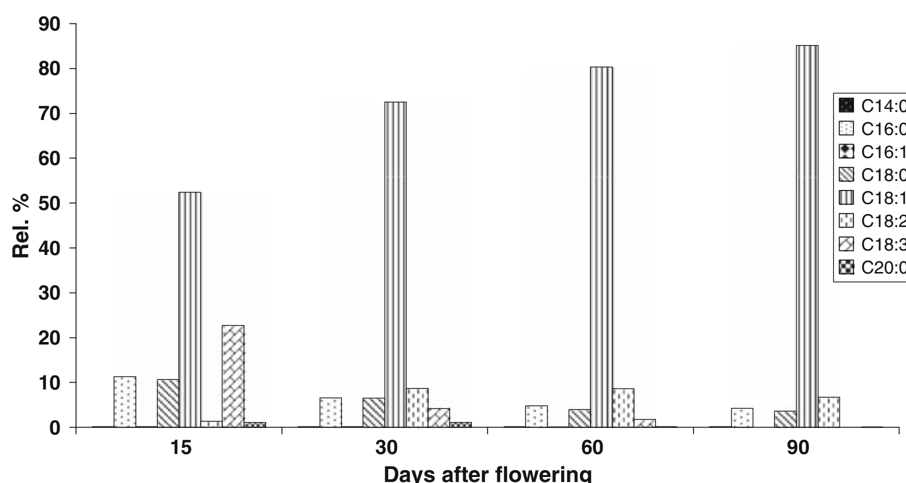

Fig. 1 Fatty acid profile of a high oleic sunflower kernel oil variety during seed development

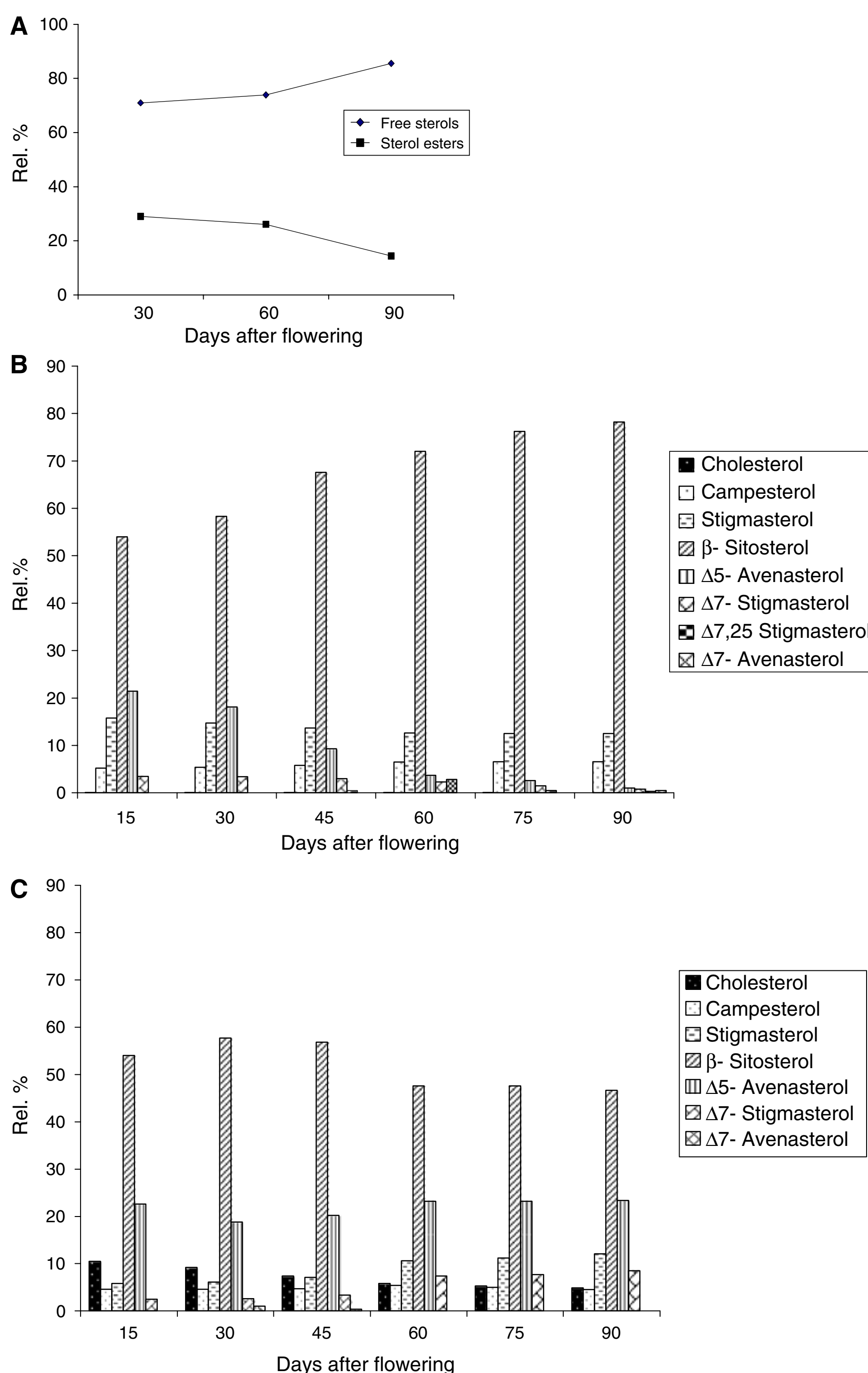

Fig. 2 Changes in sterol composition in high oleic sunflower kernel oil during seed development. **a** Free sterols versus sterol esters, **b** free sterols, **c** sterol esters

sterols on the 90th day. $\Delta^{7,25}$-Stigmasterol was detected on the 60th day and measurable amounts of Δ^7-avenasterol were detected on the 90th day only. In general, the amount of unsaturated sterols (stigmasterol, Δ^7-stigmasterol, $\Delta^{7,25}$-stigmasterol, Δ^5-avenasterol and Δ^7-avenasterol) decreased from 36 to 15% of the total content with increasing time of seed development.

The same main components were present in the sterol esters fraction (Fig. 2c). In contrast to free sterols, Δ^5-avenasterol is the second major component in the sterol esters and while the content of *β*-sitosterol slightly decreases, that of Δ^5-avenasterol slightly increases with seed development. Stigmasterol and campesterol were found in amounts close to those in free sterols.

In contrast to the free sterols, the content of unsaturated sterols in sterol esters increases during development from 28 to 44%.

Sterol esters in this HOSO variety are characterized mainly by the relatively high, gradually decreasing (10% on the 15th day to 5% on the 90th day) amount of cholesterol present. The presence of cholesterol in sterol esters is ascribed to the facts that: (1) sterol esters are the first to biosynthesize and (2) cholesterol is biosynthesized firstly in this sterol fraction and is then used as an intermediate for the synthesis of the other sterols [26].

Fatty Acid Profile of Sterol Esters

Nine fatty acids were detected in the sterol esters fraction. In addition to the FA reported above, a small amount of lauric acid (12:0) was found in the sterol esters. Lauric acid was not detected in total FA obviously because the content was under the detection limits of the GC determination. With this exception, the qualitative FA composition of sterol esters matched that of total FA and remained practically the same during the growing period (Fig. 3). In quantitative terms, there was a tendency of increasing amounts of 18:1 and decreasing amounts of 16:0 leading in general to a slight decrease in the sum of saturated fatty acids (from 44 to 39.4%) while that of unsaturated fatty acids increased to the same extent—from 56% on the 30th day to 61% on the 90th day due, as expected, to the increase in oleic acid content.

Phospholipids

The composition of phospholipid classes did not change during the growing period and phosphatidylcholine (PC),

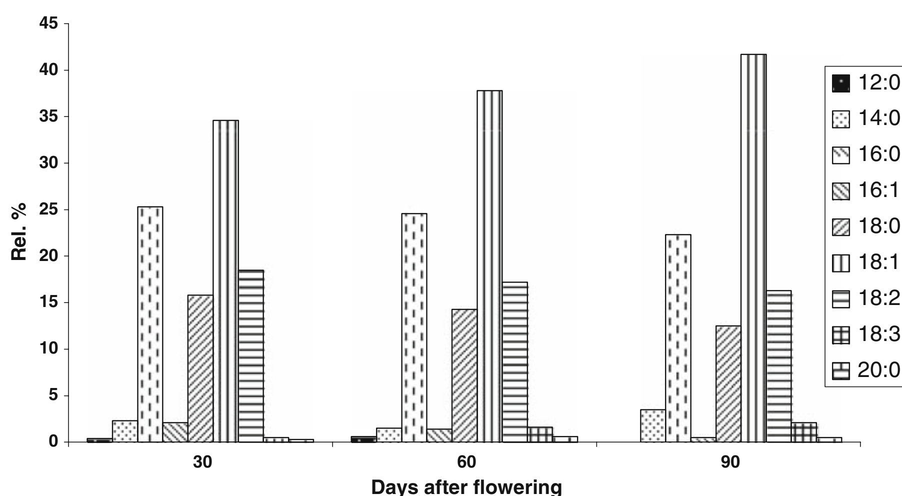

Fig. 3 Fatty acid composition of sterol esters in high oleic sunflower kernel oil during seed development

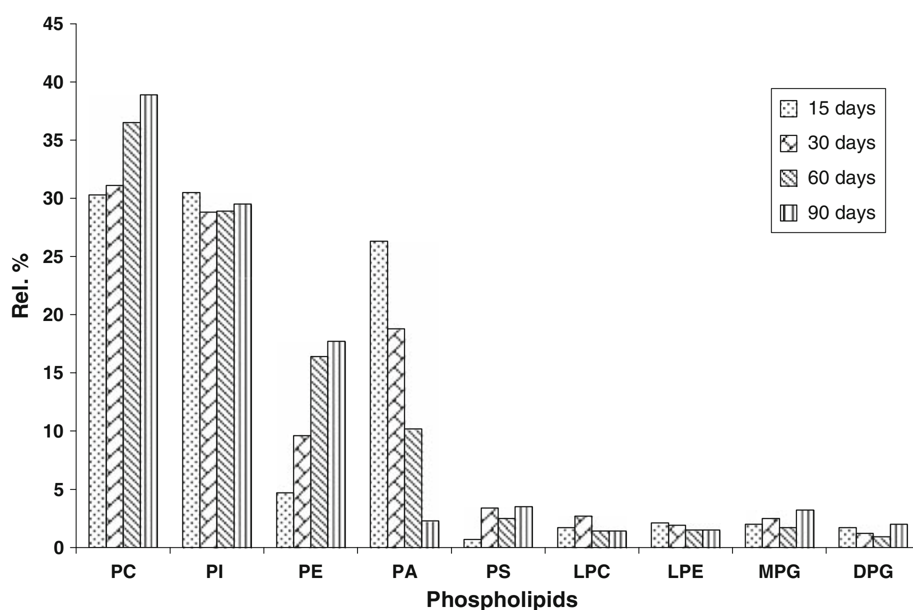

Fig. 4 Phospholipid composition in high oleic sunflower kernel oil during seed development. *PC* Phosphatidylcholine, *PI* phosphatidylinositol, *PE* phosphatidylethanolamin, *PA* phosphatidic acids, *PS* phosphatidylserine, *LPC* lysophosphatidylcholine, *LPE* lysophosphatidylethanolamin, *MPG* monophosphatidylglycerol, *DPG* diphosphatidylglycerol

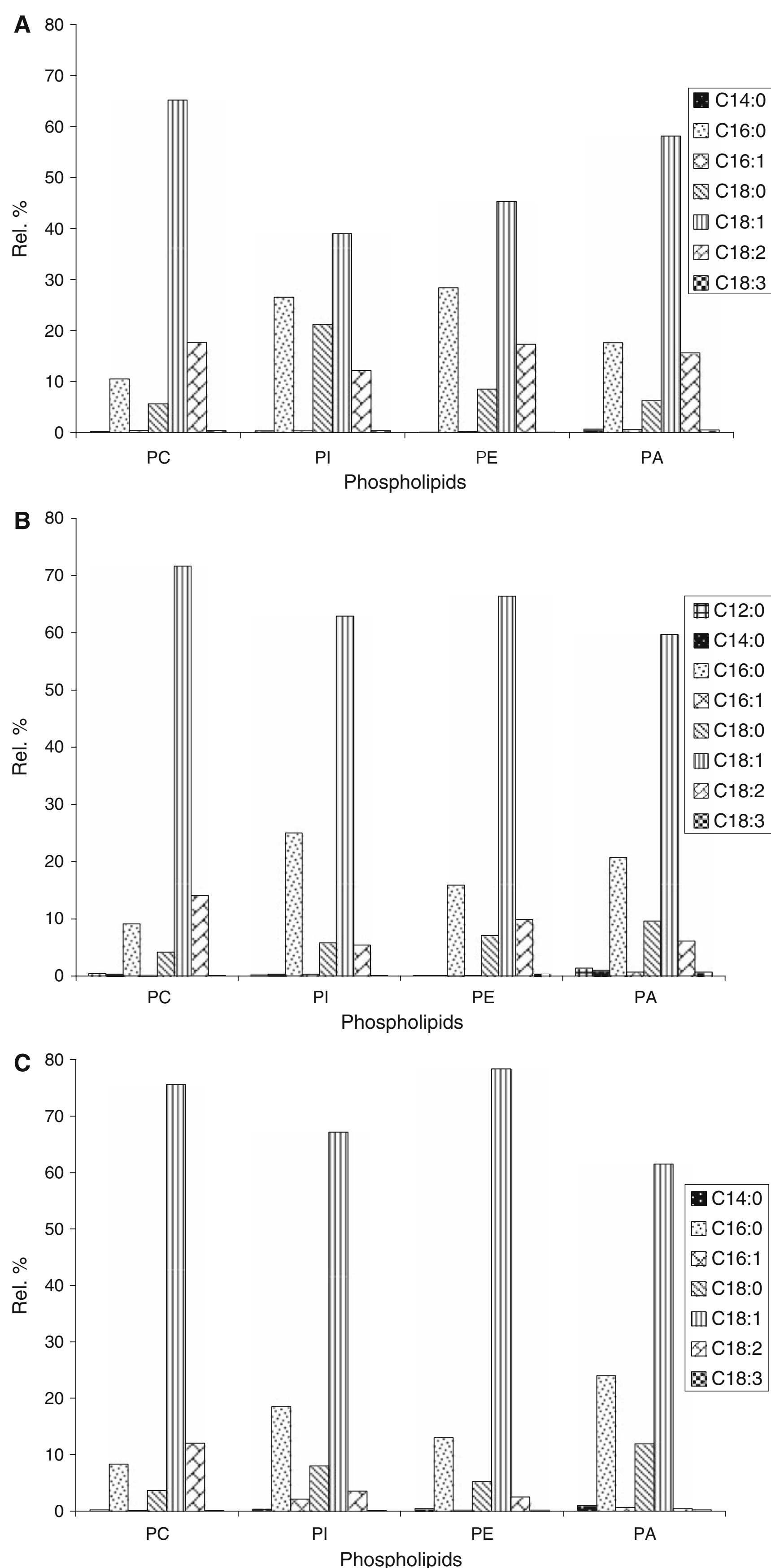

Fig. 5 Fatty acid composition of the main phospholipids in high oleic sunflower kernel oil during seed development. **a** 30th day, **b** 60th day, **c** 90th day after flowering. For abbreviations see Fig. 4

phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidic acid (PA), phosphatidylserine (PS), lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), monophosphatidylglycerol (MPG), diphosphatidylglycerol (DPG), were detected over the whole examination period (Fig. 4). PC, PI and PE are the main components. Since PA is the first to be biosynthesized and is a precursor for the biosynthesis of PC, PI and PE [27], as expected, its amount is higher in the early stages of the seed development and then decreases gradually (from 25% on the 15th day down to 2% on the 90th day). Accordingly, the amounts of PC and PE increase from 31 to 39% and from 5 to 18%, respectively, while the amount of PI remains the same. The other phospholipid species are in insignificant quantities which practically do not change during growing.

Fatty Acid Profile of Phospholipids

The fatty acid composition of the main phospholipid classes (PC, PI, PE and PA) is presented in Fig. 5a–c. Eight fatty acids were determined and in qualitative terms they matched the composition of the sterol esters (Fig. 3). Icosanoic fatty acid (20:0) was not detected in phospholipids. In qualitative terms, the composition of FA did not change during the growing period with the exception of 12:0 which was detected in traces on the 60th day only. The respective quantities changed with the percentage of saturated fatty acids decreasing and that of unsaturated fatty acids increasing due to the substantial increase in the amount of oleic acid (while the amount of linoleic acid decreased). Note that while FA composition of PC hardly changes throughout the investigated period, a major increase of oleic acid content between the 30th and the 60th day of seed developing occurs in PE and PI.

Tocopherols

The total amount of tocopherols determined is in agreement with the data reported in the literature [6]. Only saturated tocopherols were detected in tocopherol fraction (Table 2). Of these α-tocopherol dominated by far. β-, γ- and δ-tocopherols were found in insignificant and decreasing amounts.

Table 2 Changes in the tocopherol composition of high oleic sunflower oil during the growing period (%)

Tocopherols[a]	Growing period, days after flowering					
	15	30	45	60	75	90
α-Tocopherol	93	93	96	98	98	98
β-Tocopherol	4	3.0	3	2	2	2
γ-Tocopherol	3	2	1	<0.5	<0.5	<0.5
δ-Tocopherol	Traces	Traces	Traces	Traces	Traces	Traces

[a] Mean of three separate measurements

Conclusion

It is evident from the results presented in Tables 1 and 2 and Fig. 2b, c that the qualitative composition of phospholipids, sterols, sterol esters and tocopherols is largely fixed in the first 15 days after flowering. Until the 90th day only quantitative changes are detected as biosynthesis of these lipid species continues. The amounts of the earlier formed lipid precursors decrease while those of products increase. The change in the total fatty acid composition demonstrates the gradual formation of oleic acid in the HOSO variety examined. The changes in the fatty acid composition of phospholipids and of sterol esters correspond to the different phases of their biosynthesis. The product is a good quality oil with a beneficial FA content and has good prospects as a salad and cooking oil.

The information about the changes in the oil composition during the growing period of this high oleic sunflower allowed us to estimate the rate of formation of the main biologically active lipid components: fatty acids, phospholipid, sterol and tocopherol species. The information was obtained using a specific HOSO variety but it is of general interest for agronomic and genetic research helping to determine the optimal harvest period and the best balance between the lipid classes and their fatty acid composition in regions with similar climatic conditions.

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