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ORIGINAL PAPER

Changes in Fatty Acids, Tocochromanols, Carotenoids and Chlorophylls Content During Flaxseed Development

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Abstract A comparative study was performed to determine tocopherols, tocotrienols, fatty acids, and pigments content during the development of three varieties of flaxseed (H52, O116 and P129). Seed samples were collected at regular intervals from 7 to 56 days after flowering (DAF). The highest content of chlorophyll (89.72–130.5 mg/kg oil) was detected at 7 DAF. The maximum level of carotenoids (52.10–65.55 mg/kg oil) was reached at 21 DAF. During seed development, unsaturated fatty acids are the major component, reaching 85% of the total fatty acids while saturated fatty acids content were about 15%. The maximum level of γ-tocopherol (585 mg/kg oil) was reached at 42 DAF in P129 variety. These results may be useful for evaluating the flaxseed quality and determining its optimal harvest period.

Keywords Tocochromanols · Fatty acids · Flaxseed · Development · Chlorophylls · Carotenoids

Introduction

During the last decade, there has been an increasing interest in the use of flaxseed (*Linum usitatissimum* L., Linaceae) in the diet in order to improve the nutritional and health status [1]. The beneficial effects are mostly due to flaxseed lipids [1]. In flaxseed, lipids are protected against oxidation by various mechanisms, for example, the presence of antioxidants such as lignans and phenols [2]. This crop is rich in lignans with a high omega-3 fatty acid content and a relatively low glucosinolate content [3]. They also might provide low-cost renewable resource of high value-added compounds such as tocopherols and phytochemicals [4]. Both tocopherols and tocotrienols are important antioxidants in the stabilization of unsaturated fatty acids in foods and provide effective protection against oxidative stress, together with other antioxidants, in the human body [4]. Carotenoids, having a highly conjugated double bond system, are known to act as antioxidants by trapping the hydroperoxide intermediates and stopping the autoxidation chain reaction [5].

Whole seed is used in the baking of multigrain breads, biscuits, confectionery industries, and organic human consumption products [6]. Several physiological studies focused on dry matter accumulation and changes in several seed components, e.g. oil, phytosterols and aliphatic-hydrocarbons during flaxseed development [7, 8]. However, no data are available on the developmental changes in tocochromanols and pigments of flaxseed.

The aim of this study was to determine the change in tocochromanols, pigments, and fatty acids content during flaxseed development. The results could be useful for many aspect of vegetable oil production.

Materials and Methods

Reagents and Standard

Methanol (MeOH), *n*-hexane—solvents for GC analysis—were purchased from Panreac Quimica SA (Barcelona,

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Spain). Reagents of HPLC grade were purchased from E. Merck. Ethanol and cyclohexane were purchased from Scientific Limited (Northampton, UK). Chloroform ($CHCl_3$) and petroleum ether were purchased from Fisher Scientific SA (Spain). Tocochromanols and fatty acids standards were acquired from Sigma–Aldrich (Madrid, Spain).

Samples

Three varieties of flaxseeds, H52, O116 and P129, were obtained from the Institut National Recherche Agronomie Tunis (INRAT), Tunisia. The varieties of flaxseed (*L. usitatissimum* L.) were grown in restricted zones (15 m × 3 m) on the Agronomy farm of the INRAT from the middle of November 2006 until the end of June 2007. Each sample was collected at intervals after the dates of flowering. The harvest period stretched from 7 days after flowering (DAF) to 56 DAF, the time required for complete maturity.

Oil Extraction

The total lipids were extracted by the method of Folch et al. [9] modified by Bligh et al. [10]. Seeds (2.5 g) were washed with boiling water for 5 min to denature the phospholipases [11] and then crushed in a mortar with a mixture of $CHCl_3$–MeOH (2:1, V/V). The water of fixation was added and the homogenate was centrifuged at 3,000*g* for 15 min. The lower chloroform phase containing the total lipids was kept and dried in a rotary evaporator at 40 °C.

Total Chlorophyll and Carotenoids

A 1.5-g sample of flaxseed oil was fully dissolved in 5 mL cyclohexane. Chlorophyll and carotenoid were determined calorimetrically following the method of Minguez-Mosquera et al. [12]. The maximum absorption at 670 nm is related to the chlorophyll fraction and at 470 nm is related to carotenoid fraction. The values of the coefficients of specific extinction applied were $E_0 = 613$ for the pheophytin as a major component in the chlorophyll fraction and $E_0 = 2{,}000$ for lutein as a major component in the carotenoid fraction. Thus the pigment contents were calculated as follows:

$$\text{Chlorophyll (mg/kg)} = \left(A_{670} \times 10^6\right)/(613 \times 100 \times d)$$

$$\text{Carotenoid (mg/kg)} = \left(A_{470} \times 10^6\right)/(2{,}000 \times 100 \times d)$$

where A is the absorbance and d is the spectrophotometer cell thickness (1 cm). The data reported is based on oil weight (mg/kg flaxseed oil).

Gas Chromatography–Flame Ionization Detection

The quantification of fatty acids methyl esters was performed using a gas chromatography–flame ionization detection (GC–FID) apparatus. Fatty acid methyl esters were prepared by simultaneous extraction and methylation following the procedure described by Metcalfe et al. [13] modified by Lechvallier [14]. The GC system used was a thermo Finnigan Trace GC (Hewlett-Packard, Avondale, PA) equipped with a split–splitless injector, a FID detector and a Rtx-Wax capillary column ($L = 30$ m, I.D. = 0.25 mm, film thickness = 0.25 µm.). The initial column temperature was at 90 °C and programmed to increase at a rate of 10 °C/min to 240 °C and then held for 15 min. The injector and detector temperatures were at 250 °C. Helium was used as the carrier gas with a column flow of 1 mL/min. The identification of the peaks was achieved by retention times by means of comparing them with standards analyzed under the same conditions. The area under each peak was measured and the percentage expressed in regard to the total area. A typical chromatogram of fatty acids methyl ester is presented in Fig. 1.

HPLC

Tocols were obtained from the oil according to a slightly modified method of Oomah et al. [15]. The oil was homogenized in HPLC grade methanol and then the samples were centrifuged. The supernatant was removed and residue resuspended in methanol, and the homogenization and centrifugation steps were repeated. The supernatants were combined and methanol was removed under nitrogen. The dried residue was redissolved in hexane, and then placed in a 2-mL ambercript vial and stored at −20 °C until analysis. Tocols (tocopherols and tocotrienols) were analyzed by HPLC. The Varian 9010 HPLC system (Varian, Mississauga, ON) was equipped with HP1050 series auto injector. The detector used was a Shimadzu-RF 535 fluorescence detector (Shimadzu, Tokyo, Japan) with wavelengths set at 330 nm for emission and 298 nm for extinction. Tocols were separated on a normal phase column (Supelcosil-LC-Diol, 25 cm × 4.6 mm ID, 5 mm particle size, Supelco, Oakville, ON) with the mobile phase flow rate at 1.2 mL/min. The mobile phase was a mixture of *n*-hexane: isopropanol (99.4: 06, V/V). The data were integrated and analyzed using Varian Galaxie Software system (Varian, Walnut Creek, CA, USA). Standards of tocopherols α, β, γ and δ isomers (Sigma Chemical Co, St. Louis, MO) and tocotrienol α, β, γ and δ isomers (Merck, Darmstadt, Germany) were dissolved in hexane and used for identification and quantification of peaks. The amount of tocols in

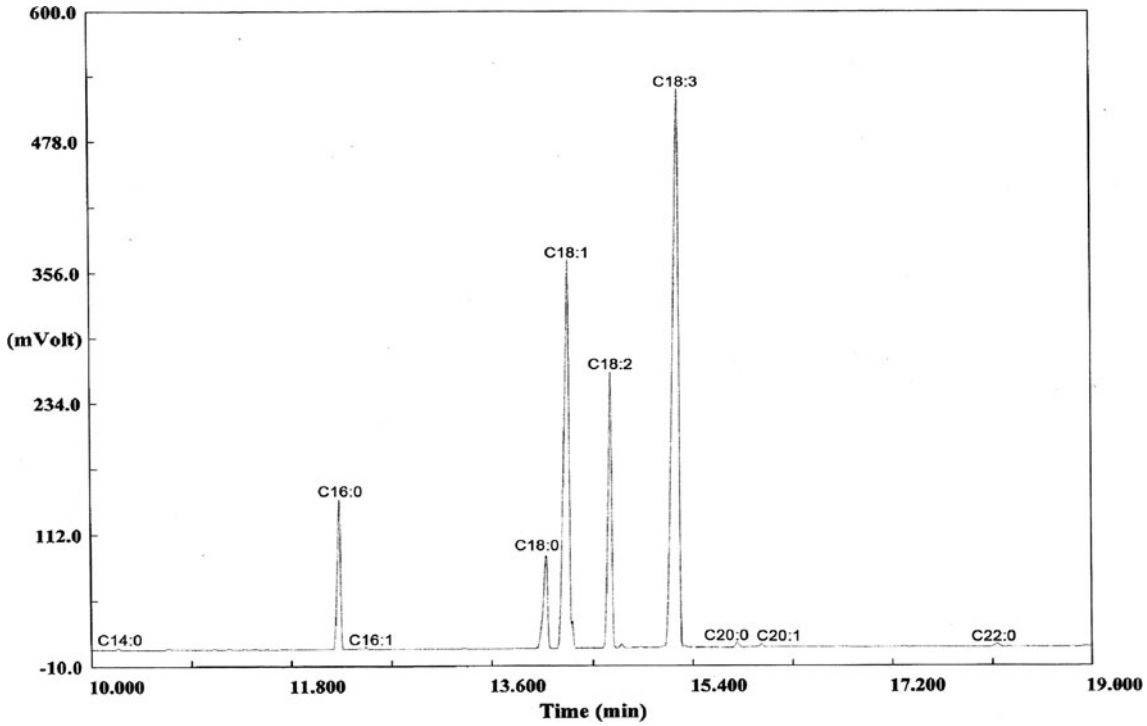

Fig. 1 Gas Chromatograph of the fatty acid methyl ester of flaxseed oil (H52 variety-at 28 DAF)

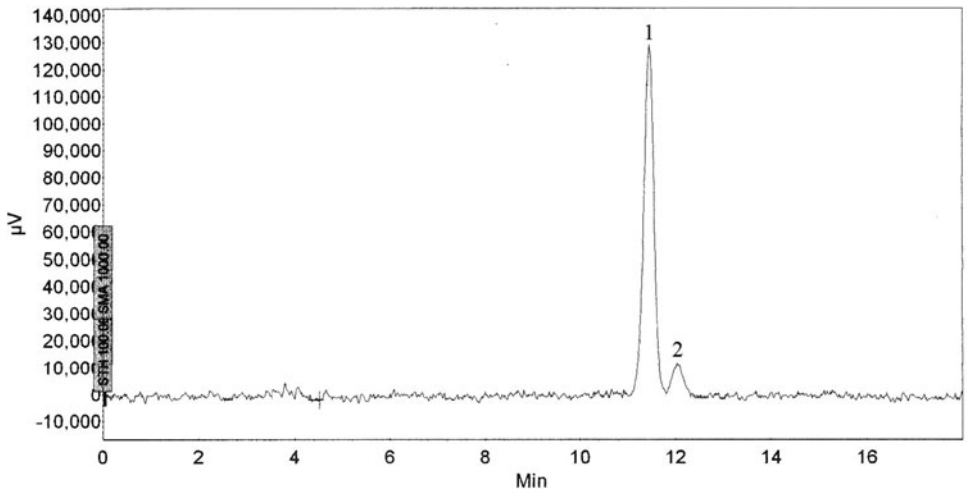

Fig. 2 HPLC chromatogram of tocopherol and tocotrienol in flaxseed oil (1: gamma-tocopherol; 2: gamma-tocotrienol) (P129 variety-at maturity (56 DAF))

the extract samples was calculated as mg tocols per 100 g oil using external calibration curves, which were obtained for each tocol isomer standard. Separation of tocochromanol is shown on Fig. 2.

Statistical Analysis

Statistical analysis was performed by using the Proc ANOVA in SAS (software version 8). For each sample three measures were taken.

Results and Discussion

Changes in Chlorophyll Content During Flaxseed Development

During flaxseed development, chlorophyll accumulation followed a similar pattern in three varieties (Fig. 3). The highest change in chlorophyll content (expressed as mg/kg flaxseed oil) occurred during the early stages of seed development. Maximal chlorophyll content (89.72–130.5 mg/kg

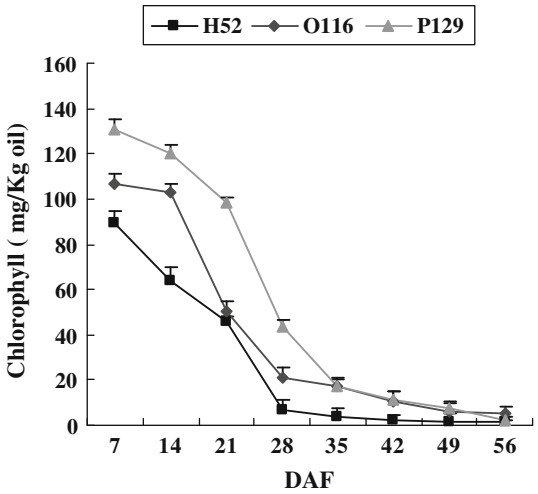

Fig. 3 Variation in total chlorophyll content (expressed in mg/kg oil) during maturation of three varieties (*vertical bars* indicate standard deviation of the means)

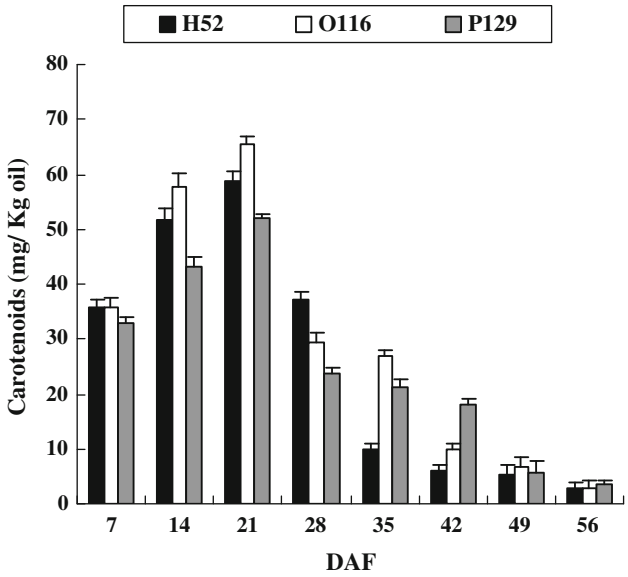

Fig. 4 Variation in total carotenoids content (expressed in mg/kg oil) during maturation of three varieties (*vertical bars* indicate standard deviation of the means)

of oil) was achieved at 7 DAF. The P129 variety consistently had a higher chlorophyll content than H52 and O116 at all stages of development. The seeds in the first stages of development had the highest levels of chlorophyll. Considering the fact that chlorophyllase is the first enzyme in the pathway of chlorophyll degradation [16], we suggested that the activity of this enzyme was high during the early stages of seed development. At maturity, the three varieties H52, O116 and P129 have a chlorophyll content of 1.14, 5.38 and 2.44 mg/kg oil, respectively. These results are in agreement with data from the literature indicating that the content of chlorophyll in flaxseed at maturity was 3.4 mg/kg oil [17]. Tuberoso et al. [17] mentioned that in many oilseeds the chlorophyll amounts range from 1.5 (in peanut oil) to 33.9 mg/kg (in olive oil).

Changes in Carotenoids Content during Flaxseed Development

Figure 4 shows that carotenoids content varies according to the growth stage. The patterns of carotenoids accumulation were the same in the three varieties. From 7 to 21 DAF there was an increase in the amount of carotenoids. Actually, carotenoids are essential photoprotectants in green tissues [18]. Cardini [19] reported an increase in carotenoids during the ripening of apples due to the accumulation of newly synthesized carotenoids in postharvest ripening. The highest levels of carotenoids in each variety were detected at 21 DAF (52.1–65.55 mg/kg oil). At this stage, these lipid components could be used for economic and food industrial exploitation. From 21 DAF to complete maturity, a dramatic decrease was observed. The decrease in carotenoids could be explained by their conversion into other lipids compounds. Gross [20] and Okombi et al. [21] reported the general decrease trend of the total carotenoids in maturing date and kiwi fruits, respectively. The reduction was due not only to partial degradation of carotenoids but also probably due to isomerization. At all stages of development, O116 variety exhibited much higher quantities of carotenoids than did the other varieties. At maturity, the three varieties H52, O116 and P129 had carotenoids contents of 2.93, 2.75 and 3.63 mg/kg oil, respectively. Similar contents in flaxseed oil have been reported by Tuberoso et al. [17].

Changes in Major Fatty Acid Composition During Flaxseed Development

Changes in fatty acids are of special importance to oil quality. Major fatty acids composition of flaxseed oils are presented in Table 1. An important effect of variety on the major FA content (expressed as per cent of total FA), during development. The differences in FA amount probably reflect the metabolic behavior of each variety, such as the amounts and activities of enzymes catalyzing the various fatty acids synthetic processes. At maturity (56 DAF), flaxseed oil contained 59–69% polyunsaturated fatty acids (PUFA), mainly linolenic acid (C18:3) (38–54%). The results are in broad agreement with those described in reference [17] where it was reported that the percentage of linolenic acid was found in the range of (51%). The highest level of C18:3 suggests Δ15 desaturase activity. PUFA are essential in the human diet and lower the risk of diseases related to cholesterol oxidation [22]. The level of C18:1 in

flaxseed oils was above 20% of total fatty acids. Higher amounts of unsaturated fatty acids (64–89%) were detected during flaxseed development. Saturated fatty acids were found in the range of 20–30%. The PUFA/SFA ratio was irregular during seed development. The highest PUFA/SFA ratio was reached at 56 DAF for O116 and P129 varieties and at 28 DAF for H52 variety.

Tocochromanol Accumulation During Flaxseed Development

γ-Tocopherol was the only and major isomer detected in these varieties. γ-Tocopherol accumulation followed different patterns in the three varieties (Fig. 5). The synthesis of γ-tocopherol started from 7 DAF, with a maximum of 585 mg/kg oil at 42 DAF for P129 variety. According to Hashim et al. [23], accumulation of γ-tocopherol in seeds is responsible for seed stability. γ-Tocopherol content increased during the early stages of seed development. Considering the fact that "2,3-dimethyl-5-phytylbenzoquinol specific translocase" catalyzes the conversion of 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) to yield γ-tocopherol [24], we suggested that 2,3-dimethyl-5-phytylbenzoquinol specific translocase activity was high during the early stages of seed development. It was reported that the high unsaturation degree of fatty acids in flaxseed oil was responsible for the higher γ-tocopherol content [25]. By using statistical analyses of data from

Table 1 Major fatty acids composition (% GC area) during flaxseed development

Stages (DAF)	C16:0	C18:0	C18:1	C18:2	C18:3	ΣSFA[a]	ΣUFA[b]	ΣPUFA[c]	ΣPUFA/ΣSFA[d]
H52									
7	10.54 ± 0.47	5.15 ± 0.10	19.68 ± 0.75	18.03 ± 0.11	45.77 ± 0.34	15.69 ± 0.42	83.48 ± 0.24	63.80 ± 0.36	4.07 ± 0.15
14	9.79 ± 0.94	5.01 ± 0.10	19.73 ± 0.34	17.11 ± 0.34	47.61 ± 0.33	14.80 ± 0.19	84.45 ± 0.47	64.72 ± 0.48	4.37 ± 0.26
21	15.40 ± 0.30	5.23 ± 0.11	46.07 ± 0.60	11.88 ± 0.39	14.99 ± 0.41	20.63 ± 0.15	72.94 ± 0.42	26.87 ± 0.47	1.30 ± 0.10
28	6.50 ± 0.39	5.23 ± 0.59	26.97 ± 1.24	13.86 ± 0.63	46.79 ± 0.53	11.73 ± 0.34	87.62 ± 0.51	60.65 ± 0.61	5.17 ± 0.16
35	10.27 ± 0.20	7.94 ± 0.89	42.49 ± 0.26	13.60 ± 0.52	25.81 ± 0.55	18.21 ± 0.26	81.90 ± 0.25	39.41 ± 0.45	2.16 ± 0.10
42	8.91 ± 0.68	7.02 ± 0.17	44.49 ± 0.65	13.16 ± 0.10	24.39 ± 0.45	15.93 ± 0.42	82.04 ± 0.35	37.55 ± 0.33	2.36 ± 0.13
49	8.21 ± 0.18	7.02 ± 0.71	41.85 ± 0.80	13.41 ± 0.39	27.90 ± 0.16	15.23 ± 0.16	83.16 ± 0.21	41.31 ± 0.42	2.71 ± 0.47
56	9.36 ± 0.41	7.31 ± 0.17	24.08 ± 0.75	21.04 ± 0.31	37.19 ± 0.84	16.67 ± 0.31	82.31 ± 0.74	58.23 ± 0.70	3.49 ± 0.23
O116									
7	21.50 ± 0.28	8.81 ± 0.70	34.56 ± 0.27	15.22 ± 0.21	18.02 ± 0.33	30.31 ± 0.51	67.80 ± 0.29	33.24 ± 0.39	1.10 ± 0.12
14	24.51 ± 0.18	10.31 ± 0.24	36.19 ± 0.32	13.80 ± 0.17	13.56 ± 0.41	34.82 ± 0.66	63.55 ± 0.53	27.36 ± 0.31	0.78 ± 0.10
21	7.53 ± 0.17	4.90 ± 0.33	21.43 ± 0.42	13.76 ± 0.34	51.78 ± 0.35	12.43 ± 0.21	86.97 ± 0.22	65.54 ± 0.23	5.27 ± 0.33
28	6.47 ± 0.37	5.75 ± 0.47	24.59 ± 0.45	12.57 ± 0.37	49.94 ± 0.15	12.22 ± 0.31	87.10 ± 0.11	62.51 ± 0.41	5.11 ± 0.21
35	15.22 ± 0.51	12.62 ± 0.28	53.03 ± 0.97	9.17 ± 0.39	8.49 ± 0.61	27.84 ± 0.17	70.69 ± 0.40	17.66 ± 0.62	0.63 ± 0.10
42	5.84 ± 0.14	5.01 ± 0.18	26.92 ± 0.82	13.59 ± 0.10	48.03 ± 0.19	10.85 ± 0.23	88.54 ± 0.20	61.62 ± 0.14	5.68 ± 0.22
49	8.46 ± 0.42	7.62 ± 0.25	36.83 ± 0.18	13.99 ± 1.03	32.17 ± 1.21	16.08 ± 0.36	82.99 ± 0.83	46.16 ± 1.13	2.87 ± 0.12
56	5.80 ± 0.24	4.72 ± 0.83	19.46 ± 0.22	15.01 ± 0.50	53.80 ± 0.83	10.52 ± 0.61	88.27 ± 0.71	68.81 ± 0.61	6.54 ± 0.73
P129									
7	11.57 ± 0.58	4.36 ± 0.19	18.90 ± 0.71	18.99 ± 0.20	45.31 ± 0.26	15.93 ± 0.37	83.20 ± 0.22	64.30 ± 0.24	4.03 ± 0.38
14	15.71 ± 0.69	8.48 ± 0.17	32.90 ± 0.61	16.75 ± 0.10	24.95 ± 0.34	24.19 ± 0.43	74.60 ± 0.36	41.70 ± 0.27	1.72 ± 0.19
21	8.40 ± 0.33	5.19 ± 0.10	22.35 ± 0.10	15.76 ± 0.91	47.58 ± 0.40	13.59 ± 0.24	85.69 ± 0.75	63.34 ± 0.67	4.66 ± 0.10
28	16.45 ± 0.24	12.26 ± 0.83	51.39 ± 0.67	8.91 ± 0.23	9.62 ± 0.37	28.71 ± 0.17	69.92 ± 0.29	18.53 ± 0.32	0.64 ± 0.10
35	10.37 ± 0.22	9.05 ± 0.10	44.40 ± 0.26	12.49 ± 0.46	22.47 ± 0.39	19.42 ± 0.18	79.36 ± 0.76	34.96 ± 0.41	1.80 ± 0.11
42	6.28 ± 0.20	5.88 ± 0.77	29.60 ± 0.14	14.72 ± 0.21	42.77 ± 0.98	12.16 ± 0.49	87.09 ± 0.99	57.49 ± 0.64	4.73 ± 0.22
49	6.55 ± 0.28	5.87 ± 0.48	29.97 ± 1.08	15.83 ± 0.37	41.05 ± 0.71	12.42 ± 0.34	86.85 ± 0.82	56.88 ± 0.41	4.58 ± 0.14
56	6.17 ± 0.21	6.48 ± 0.40	24.22 ± 0.63	19.13 ± 0.44	42.94 ± 0.12	12.65 ± 0.52	86.29 ± 0.16	62.07 ± 0.32	4.90 ± 0.30

Fatty acids detected: C16:0 (palmitic), C18:0 (stearic), C18:1 (oleic), C18:2 (linoleic), C18:3 (linolenic)

% GC area, mean of three measurements

[a] Sum of major saturated fatty acids

[b] Sum of major unsaturated fatty acids

[c] Sum of major polyunsaturated fatty acids

[d] Polyunsaturated fatty acids to saturated fatty acids ratio

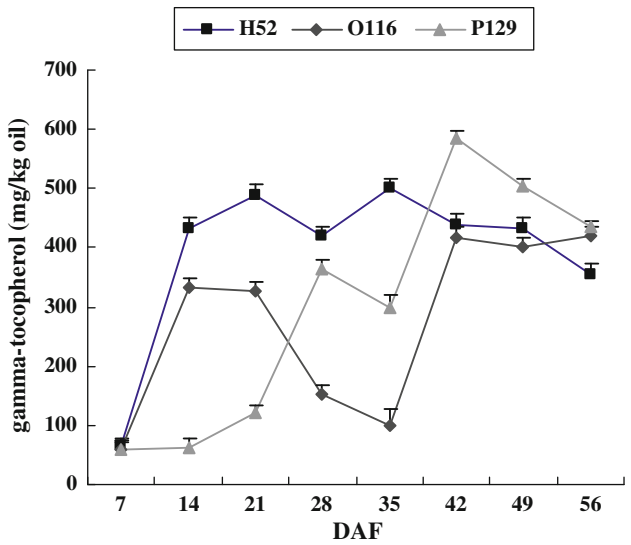

Fig. 5 Variation in gamma-tocopherol content (expressed in mg/kg oil) during maturation of three varieties (*vertical bars* indicate standard deviation of the means)

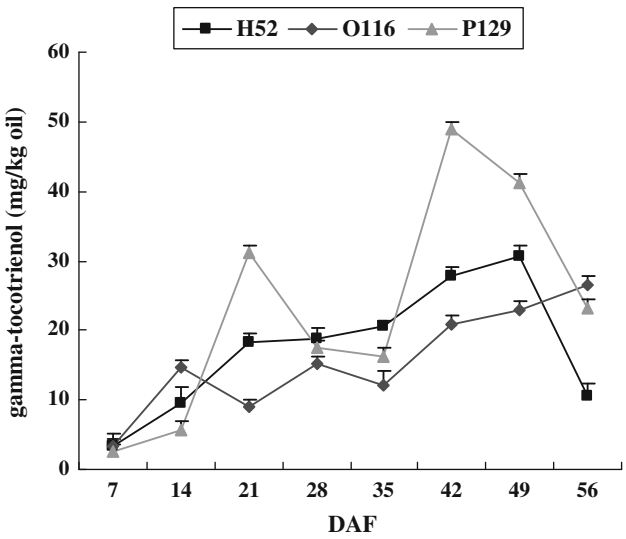

Fig. 6 Variation in gamma-tocotrienol content (expressed in mg/kg oil) during maturation of three varieties (*vertical bars* indicate standard deviation of the means)

vegetable oils, Kamal-Eldin et al. [26] suggested a positive correlation between C18:3 and γ-tocopherol. Kumar et al. [27] reported that intensive tocopherol formation was associated with increased biosynthesis of soybean oil, indicating thereby the role of seed tocopherols as antioxidants. At complete maturity, the γ-tocopherol content was 355, 420 and 434.4 mg/kg oil for H52, O116 and P129, respectively. Quantitative composition of tocopherols was similar to those reported in the literature [15, 25].

The amount of γ-tocotrienol content followed a similar trend in H52 and O116 and the highest level (30.6–26.4 mg/kg oil) was observed at 49 and 56 DAF, respectively (Fig. 6). However, the highest level of this tocotrienol isomer (48.8 mg/kg oil) was observed at 42 DAF in variety P129. Flaxseed contained small amount of γ-tocotrienol in fully mature seeds, which represents 10.5, 26.4 and 23.3 mg/kg oil for H52, O116 and P129, respectively. The amount of γ-tocotrienol increased steadily during the early stages of seed development. This could be explained by an important activity of "tocopherol cyclase" that catalyzes the conversion of 2,3-dimethyl-5-geranylgeranyl-1,4-benzoquinone (DMGGBQ) to γ-tocotrienol [28]. The content of γ-tocotrienol in flaxseed oil investigated in this study was in accordance with previously reported results [15, 25]. The patterns of tocochromanols accumulation for the three varieties were different. Bauernfeind [28] reported that the amount of tocochromanols in fruits and vegetables is affected by species, variety, maturity, growing conditions (weather, growing season, intensity of sunlight, and soil state), uneven distribution of tocochromanols, and time of harvesting. The total amount of tocochromanols increased to a maximum at 35 DAF for H52 (520.9 mg/kg oil) and at 42 DAF for P129 (633.8 mg/kg oil) and at 56 DAF for O116 (446.4 mg/kg oil) varieties. Thus, these dates are the best moment to exploit the maximum level of these high value-added compounds in flaxseed.

Conclusion

Flaxseed oil seems to be a potential source of essential fatty acids and lipid-soluble bioactivities. The high linolenic acid content makes the oil nutritionally valuable. Tocochromanols, at the level estimated, may be of nutritional importance in the application of the seed oil. The quantitative and qualitative characterizations of these phytochemical components seem to be useful for many aspects of vegetable oil production, including detection of adulteration and it is of general interest for agronomic researcher since quantitative information can be used to determine the optimal harvest period.

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