doi: 10.17113/ftb.53.04.15.4252 *scientific note*

Influence of climate, varieties and production process on tocopherols, plastochromanol-8 and pigments in flaxseed oil

Influence of climate, varieties and production on antioxidants in flaxseed oil

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Received: May 12, 2015
Accepted: June 2, 2015

Summary

The objective of this study was to compare the influence of: genotype, environmental conditions and processing methods on levels of tocochromanols carotenoids and chlorophyll after maturation and harvesting in flaxseed oil from four varieties (Altess, Biltstar, Niagara and Oliwin). Oils were produced by cold pressing of dry seeds and seeds that were heated for 30 min at 60 °C. Temperature, sunshine and rainfall were primary environmental conditions included. Grand mean for γ-tocopherol was (522±29) mg/kg of oil, for plastochromanol-8 was (305±2) mg/kg of oil and total tocochromanols (831±3) mg/kg of oil. The highest levels and strongest antioxidant activity were found with cold pressed oil from variety Biltstar. Levels of γ-tocopherol and plastochromanol-8, increased with average temperature and total sunshine during seed maturation and decreased with total rainfall during seed maturation. Fifth week after flowering was identified as maturation period with best climate conditions to achieve optimal tocochromanol content. Grand mean for carotenoids was (1.83±0.01) mg/kg of oil, and chlorophyll (0.43±0.10) mg/kg of oil. Variety Altess had the highest levels of pigments. Antioxidant activity decreased with increase of chlorophyll, while correlations with

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carotenoids were not determined. Generally, oils obtained by cold pressing had higher levels of tocochromanols but lower values of pigments than oils after seed conditioning with similar antioxidant activity. The results from this study contribute to identifying the best flaxseed variety with regards to oil production and the highest antioxidant activity and nutritive value, and provide better understanding of tocochromanol biosynthesis in regard to different climate conditions.

Key words: flaxseed oil, variety, cold pressed, conditioning, tocopherols, plastochromanol-8, carotenoids, chlorophyll, climate

Introduction

Flaxseed oils are distinguished by more than 50 % of α-linolenic acid and more than 13 % of linoleic acid which makes them very susceptible to oxidation and taste deterioration (*1*). Antioxidants help protect flaxseed oil from oxidation. The primary antioxidants in flaxseeds are tocochromanols, a group that consists of four tocopherols (α-, β-, γ-, δ-) and four tocotrienols (α-, β-, γ-, δ-). As reported in other studies, quantities of tocopherols in flaxseed oils significantly differ from 154 to 934 mg/kg of oil with the main representative γ-tocopherol (γ-T) (*1,2*). Such a big variations could be result of different harvesting locations and varieties, extraction technologies or storage time of packed flaxseed oil.

Plastochromanol-8 (PC-8) is considered as natural homologue of γ-tocotrienol and second most represented tocochromanol in flaxseed with more than 25% out of total tocochromanols (*3*). Recently, its antioxidant capacity was documented to be similar as γ-tocotrienol and 1.5 times higher than α-tocopherol (α-T) (*3*). Various plants and oilseeds have shown in their fat and oil content extreme variations with regards to tocopherols and other antioxidants, which also differed with various cultivars and geographical location (*4,5*). Herchi *et al.* (*6*) reported detailed analysis of lipid biosynthesis in flaxseed from flowering to full maturity which lasted approximately 56 days or 8 weeks. The most intensive biosynthesis of oil happened between 7th and 42nd day after flowering after which oil content slightly declined until harvesting time. In comparison with other work by Herchi *et al.* on tocochromanol biosynthesis, in flaxseed it is noticeable how these compounds follow similar path, indicating that tocochromanols have the main role as seed antioxidants (*7*). Other important antioxidants in flaxseed are carotenoids which are members of larger group of plant pigments with 750 different structures (*8*). They function as hormone precursors, colorants and essential elements in photosynthetic cycle (*8*). In some cases, specific carotenoids are essential for mammalian diets as

precursors for vitamin A synthesis. Carotenoids, having a highly conjugated double bonds, are known to act as antioxidants by trapping the hydroperoxide intermediates and stopping the chain reactions in autoxidation. Both carotenoids and tocochromanols are synthesized partially or entirely as plastidic isoprenoid during biosynthetic pathway (*8*).

Currently, the influence of climate on flaxseed oil content has not been clarified. Few studies have shown that flaxseed development, processing and variety influence the characteristics of flaxseed oil (content of tocochromanols and pigments) (*2,7,9,10*). However, these studies observed oils obtained from one harvesting year, therefore providing a skewed view of how climatic factors associate with oil characteristics. Furthermore, there is no published data that combines different varieties, production processes with climate factors and their cumulative influence on tocochromanols, and the pigment content in flaxseed oil.

Flaxseed oil production was positioned 11th in 2013 with 564 818 t of global vegetable oil production. The main producers were China, India, Germany, Canada, Italy and Egypt covering different continents and climate areas (*11*). Its long history of cultivation in different regions resulted in vast botanical flaxseed diversity. For instance, Canada and EU combined, listed 223 different flaxseed varieties (*12,13*).

Information on chemical composition of particular flaxseed varieties opens a possibility for different blends of produced oils with the aim of improving their nutritional and sensory value and oxidative stability. Similar research was done on extra virgin olive oils (*14*).

The objective of this study was to compare the influence of: genotype, environmental conditions (temperature, sunshine hours and rainfall) and processing methods on levels of carotenoids, chlorophyll and tocochromanols after maturation in flaxseed oil from four varieties (Altess, Biltstar, Niagara and Oliwin).

Materials and Methods

Flaxseed and Sampling Conditions

Three brown flaxseed varieties (Altess, Biltstar and Niagara) and one golden variety (Oliwin) were used in experiments. Cultivars were grown during 3 years period (between 2011 and 2013) on experimental field of the Faculty of Agriculture, University of Zagreb, Croatia on 120 m above sea level. All cultivars were sowed in the first half of April and seed was harvested when they reached

harvesting maturity (between mid to late July) determined by morphological markers that included pod and seed color. Experiments were set up according to the randomized complete block design in 5 repetitions. Size of a plot was 6.6 m^2. Soil of the experimental field Zagreb – Maksimir is anthropogenized eutric brown, on slightly luvic loam. The soil is weakly acid reaction in the plough layer (pH u 1M KCl = 6.29), poorly supplied with humus (2.6 %), well supplied with available phosphorus (P_2O_5 = 39.9 mg/100 g soil) and medium supplied with available potassium (K_2O = 18.7 mg/100 g soil).

Reagents

All chemicals and solvents were analytical grade, obtained from Carlo Erba Re´actifs-SdS (Chausse´e du Vexin, France). The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Tocopherols (α-, β-, γ- and δ-) were acquired from Merck KGaA (Darmstadt, Germany). Plastochromanol-8 was provided by Dr Jerzy Kruk from Jagiellonian University, Kraków, Poland.

Oil extraction

Oil was extracted within 40 days after harvesting for each cultivar by two methods: cold pressing of dry seeds (CP oil) (*15*) and seeds that were heated for 30 min at 60 °C (HP oil). Quantity of sample in both methods was 700 g of freshly grounded flaxseed. For the CP oil T≤50 °C, while for the HP flaxseeds were firstly heated in a tray at T=60 °C for 30 min with constant stirring with addition of 80 mL of water (divided in three parts; one third added at the beginning, one after 15 min and one at the end) and then immediately pressed. The CP and HP oils were pressed by laboratory screw press Komet CA/53 (Monforts and Reiners, Rheydt, Germany). Oils were filtered through a sintered glass filter (10–16 μm pore size) and stored at room temperature in dark bottles with nitrogen until analysis. All analyses were finalized in less than a month from oil production.

HPLC – tocochromanol

Tocochromanol content was determined according to ISO method 9936 (*16*) by using normal-phase HPLC analysis. Samples were prepared by dissolving 0.1 g of flaxseed oil in 10 mL n-hexane and then analyzed by HPLC equipped with a fluorescence detector and LiChroCART Silica 60 column (250 mm 9 4.6 mm, 5 l; Merck, Darmstadt, Germany). Tocochromanols were detected at 295 nm and 330 nm excitation emission wavelengths and separated by isocratic chromatography by mobile phase of 0.7 % propan-2-ol in n-hexane at 0.9 mL/min flow rate. Analyses were performed at room temperature.

Tocochromanols were quantified by standard calibration curves for α-, β-, γ- and δ-tocopherols. Plastochromanol-8 was quantified by using calibration curve from α-tocopherol.

Pigments

Pigments were determined spectrophotometrically. Total chlorophylls, expressed as pheophytin a, were determined by using the method of Pokorny *et al.* (*17*) and by measuring the absorbance of the oils against the air at 630, 670 and 710 nm. Total carotenoids were determined by measuring the absorbance of oil solution in cyclohexane at 445 nm using the BSI method (*18*). Equations 1 and 2 were used to calculate total chlorophylls and carotenoids content, respectively.

$$\text{Total chlorophylls} = 34.53\frac{A_{670} - 0.5\,(A_{630} + A_{710})}{L} \qquad /1/$$

$$\text{Total carotenoids} = \frac{383 \times A_{445}}{L \times c} \qquad /2/$$

Where A_i was absorbance at the specified wavelength, L was the thickness of the glass cell (cm) and c was the concentration (g/100 mL) of oil solution in cyclohexane.

Radical Scavenging Activity

Radical scavenging activity was defined as the ability of oil to quench the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH). This method is commonly used to determine antioxidant capacity oils. Reduction in the amount of free DPPH radical was determined by spectrophotometry with measuring the color change of the reaction solution at 515 nm and following the method described by Kalantzakis *et al.* (*19*). Briefly, 10 % (w/v) oil solution in ethyl acetate (1 mL) was added to freshly prepared 0.125 mM DPPH solution (4 mL). The reaction mixture was shaken, kept in dark for 30 min, and then the absorbance was measured against the blank solution (without DPPH radical). The DPPH concentration was calculated from calibration curve, and was used for calculating radical scavenging activity following eq. 3:

$$\%\ \text{DPPH reduction} = 100 \cdot \left(1 - \frac{[\text{DPPH}]_{30}}{[\text{DPPH}]_0}\right) \qquad /3/$$

where $[DPPH]_0$ and $[DPPH]_{30}$ are concentrations of DPPH in the control sample (t = 0 min) and in the test mixture (t=30 min of reaction).

Meteorological data

Meteorological data were provided by Meteorological and Hydrological Service of Republic of Croatia for exact location (Maksimir, Zagreb, Croatia; 45°49'0'' N, 16°02'00'' E). Data for sunshine (h) and rainfall (mm) ware given as total daily values; temperature as mean daily value (°C).

Data Analysis

Descriptive statistics were used to assess the basic information about data. Dependent continuous variables were contents of: α- tocopherol, plastochromanol-8, γ-tocopherol, δ-tocopherol, total tocochromanols, chlorophyll, and carotenoids. Independent categorical variables were: harvest year, flaxseed variety and type of pressing. Continuous variables were analyzed using three-way ANOVA. Pearson's linear correlation tested relation between the pairs of continuous variables. Marginal means were compared with LSD multiple comparison tests. PCA analyses was used to calculate simultaneous association of selected climate variables (temperature, sunshine, and rainfall). Appropriateness of this approach was evaluated by Kaiser-Meyer-Olkin Measure of Sampling Adequacy that equaled to 0.7. All variables for PCA were standardized and formed factor called Climate Index. Keiser rule was used for factor retention. Factor analysis score was obtained by linear regression. The significance levels for all tests were $\alpha \leq 0.05$, while analyses were performed with IBM SPSS Statistics (v.20).

Results and Discussion

The results showed flaxseed oil as rich source of natural vitamin E that changed significantly with different climate conditions during maturation, variety, and type of oil production (Table 1).

Maturation period, climate and climate index

Flaxseeds were harvested in three consecutive years, 2011-2013, after maturation period which lasted 45, 49 and 48 days after flowering or approximately 7 weeks. For each year and full period of maturation three main climate parameters were measured, analyzed and compared. Values for climate parameters were evaluated on a weekly basis for easier comparison with previous published research on oil and tocochromanol biosynthesis in flaxseed. It can be concluded that 2012 had the highest average weekly temperature (22.9 °C) with the highest average weekly precipitation (21.6 mm), 2011 had the lowest average of total weekly sunshine (59 h) and 2013 had the lowest average weekly precipitation (1.3 mm) with the highest average of total weekly sunshine (71 h). To provide better understanding of climate influences on studied bioactive compounds we calculated climate index

based on PCA analysis with combing all annual values of temperature, sunshine and rainfall for maturation period of flaxseed starting from the moment of flowering until harvesting. Equations needed for conversion of climate index to:

Temperature = 22.56+2.32*Climate Index /4/

Sunshine = 10.05+1.58*Climate Index /5/

Rainfall = 1.92-1.36*Climate Index /6/

Correlations of climate index with individual/total tocochromanols and pigments with % of reduced DPPH are presented in Table 2. For each examined variable we calculated optimal value to determine which particular climate parameters (that are function of climate index from EQ. 4-6) have the strongest influence on antioxidative activity measured with % of reduced DPPH. Optimal value is largest or lowest mathematical extremum obtained with RSM for single mathematical function that takes into account 3 variables: (i) one of the studied variable (α- T, PC-8, γ-T, δ-T, total T, chlorophyll, and carotenoids), (ii) % DPPH, and (iii) climate index (that can be converted to temperature, sunshine, and rainfall with eq. 4-6). This number gives highest (α- T, PC-8, γ-T, total T, and carotenoids) or lowest (δ-tocopherol and chlorophyll) concentration for particular biologically active compound and answers under what combination of climate parameters such extremum will be obtained (*6,7*).

γ-tocopherol

In analyzed samples, the most abundant tocochromanol, including PC-8, was γ–T. Grand average value in the study for γ-T was (522±2) mg/kg of oil (Table 1) or 63% of total tocochromanols. Previously published results for cold pressed flaxseed oil were 800±0 mg/kg of oil and (370±0) mg/kg of oil (*20,21*). Similar average results were obtained through organic solvent extraction at (322±2), (403±42), (542±36) and (757±13) mg/kg of oil (*7,22-24*). Choo *et al.* (2007) reported much lower values for γ–T with average (124±15) mg/kg of oil (*1*). However, the authors obtained their samples from bottled oils from shops, while others analyzed freshly pressed or extracted oil. Therefore, undefined storage time from Choo *et al.* (2007) (*1*) likely decreased tocochromanols under prolonged oxidation. Other published papers on flaxseed oils from toasted flaxseeds (*9*), and pressing under elevated temperature (80-120 °C) (*10*) did not report results for tocochromanols.

Harvesting year significantly affected the amounts of γ–T where in 2011 there was the least, while in 2013 had the most of γ–T (Table 1). This can be explained by climate influence (Figure 1) where γ-T increased with average temperature and total sunshine during seed maturation. However, γ-T showed no association with the total rainfall during seed maturation (Table 2).

Changes in γ–T content and antioxidative capacity with climate index can be observed from Table 2. Climate index identified optimal yield of particular tocochromanol in relation to all climate factors of interest while retaining all of their mutual relations. Hence, Table 2 shows that increase of temperature and sunshine favors increase of γ–T with analogous increase of antioxidative capacity. On the contrary, increase of rainfall decreases the γ–T content and antioxidative capacity. All but for rainfall, relations were in agreement with Pearson's correlations from Table 2. Optimal value for γ–T is 707 mg/kg of oil at climate index = 0.1982 which converted with equations 4-6 equals to average daily temperature of 23 °C, daily sunshine of 10.4 h and average daily rainfall of 1.7 mm. This combination of calculated climate conditions was best achieved during the 5th week of flaxseed maturation. Some earlier and recent research by Herchi *et al.* (*6*), also identified 5th week of maturation as the period with maximum level of oil in flaxseed after which biosynthesis gradually slows down and slightly declines before harvesting. In their other work 5th week was associated with second most intensive increase in γ–T levels (other was 2nd week of maturation) (*7*).

Variety Biltstar had the highest amounts of γ-T, while lowest amounts were detected for Altess and Oliwin, and if flaxseed was pressed after conditioning, it had higher content of γ-T (Table 1).

In conclusion, our results for γ-T are similar to other literature findings and show that increased content of γ-T was favored by increased warmth, sunshine and conditioning of flaxseeds before oil extraction and follows biosynthesis of oil published in previous researches (*6*).

Plastochromanol-8

Second most represented tocochromanol in our samples was PC-8. Our grand average value was (305±2) mg/kg of oil (Table 1) or 37% of all tocochromanols. Varieties Altess and Biltstar had the highest amounts of PC-8 and cold pressed oils had more PC-8 in comparison to oils from conditioned seeds (Table 1). PC-8 is considered to be a natural homologue of γ-tocotrienol that contains longer side chain (*3*). Apart from flaxseed, where it is abundantly represented, it can be found in smaller quantities in variety of oilseeds like rapeseed, mustard, soybean or camelina (*25*). Recent studies from Ciftici *et al.* (2012) reported PC-8 values of (191±12) mg/kg of oil or 26% of all tocochromanols (*23*). Results from Gruszka and Kruk (2007) reported PC-8 with average value of (217±5) mg/kg of oil or between 46% of all tocochromanols (*25*).

Similarly to γ-T, content of PC-8 was highest in 2013 (Table 1), when it strongly increased with average temperature and total sunshine during seed maturation (Figure 1) while moderately decreased with rainfall during seed maturation (confirmed by significant correlations from Table 2). These results imply that biosynthesis of two major tocochromanols in flaxseed oil is highly associated with climate.

As documented by Kruk *et al.* (2014) in *Arabidopsis* leaves, PC-8 rises from 38% under low-light and up to 50% under high-light conditions (*26*). PC-8 showed higher values in cold pressed flaxseed oil (Table 1).

Results on Table 2 show that increase of temperature and sunshine favors increase of PC-8 with analogous increase of antioxidative capacity. On the contrary, increase of rainfall decreases the PC-8 content and antioxidative capacity (all but % of reduced DPPH correlations were confirmed by Table 2). Optimal value for PC-8 was 392 mg/kg of oil at climate index = 0.1982. As with γ-T, climate index conversion yielded average daily temperature of 23 °C, daily sunshine of 10.4 h and average daily rainfall of 1.7 mm, all achieved during the 5th week of flaxseed maturation. Herchi *et al.* (*6*) have also identified 5th week of maturation with second most intensive increase in PC-8 levels (other was 3rd week of maturation) (*7*).

α-tocopherol

Amounts of α-tocopherol (α-T) where much lower in comparison to other major tocochromanols. The grand mean value in our samples was (3±1) mg/kg of oil (Table 1) or 0.4% of total tocochromanols. Harvesting year significantly affected amounts of α-T and, as with other tocochromanols, we detected the year 2013 to have significantly highest amounts of this tocochromanol (Table 1). Variety Oliwin had the highest amounts of α-T, while Altess, Biltstar, and Niagara had similar lowest amounts of this compound. Levels of α-T increased with average temperature during seed maturation and decreased with mm of rainfall during seed maturation (Table 2). Type of pressing had no significant effect on levels of α-T (Table 1). Accordant with our result, reported average levels of α-T in flaxseed oils obtained from organic solvent extraction were much lower than other tocochromanols and were from (6±1) to (16±0) mg/kg of oil (*2,7,22,23*), however there was one exception with 40 mg/kg of oil (*20*). Similar high variations between values for α-T were found with shea butter (*4*) and camelina oil samples (*27*) influenced by variety and region of harvesting.

Results in Table 2 show that increase of temperature and sunshine favors increase of α-T with analogous increase of antioxidative capacity. On the contrary, increase of rainfall decreases the α-T content and antioxidative capacity (Table 2). Similar to other tocochromanols, optimal value for α-T was 9 mg/kg of oil with climate index = 0.1982 (average daily temperature=23 °C, daily sunshine=10.4 h and average daily rainfall=1.7 mm detected at 5th week of flaxseed maturation). In research on α-T in soybean large increase was noted under higher temperature and drought combined with decrease in γ- and δ-T (*28*). From biosynthetic pathway presented in paper by Mene-Saffrane and DellaPenna we can see that 2-methyl-6-phytyl-benzoquinol (MPBQ) is a shared substrate for synthesis both γ-T and

δ-T (*29*). It appears that higher temperature and lack of rainfall forces plants to go one step further in synthesis of α-T to prevent oxidative damage under stressed conditions. In response to high light conditions, wild type plants, accumulate only α –tocopherolquinol, product of α-T oxidation (*29*). Based on this we can hypothesize that α-T is an antioxidant which is synthesized in flaxseed mostly in extreme conditions while its excessive levels added to preserve flaxseed oil can act as prooxidants as shown in research by Bravi et. al (*30*).

δ-tocopherol

Δ-tocopherol (δ-T) was detected in the least amounts of all tocochromanols. The grand mean value in our samples was 0.4 mg/kg of oil or 0.05% of total tocochromanols (Table 1). Presence of δ-T in analyzed oils was found only in 2011, or in the first year of harvesting. Only one sample of oil produced after conditioning from variety Altess had 1±0.1 mg/kg of oil of δ-T (Table 1). Previous results from organic solvent extraction reported quantities at (5±1), (19±0) and (23±0) mg/kg of oil (*2,23,24*).

Contrary to other tocochromanols, δ-T decreased under higher sunshine and rainfall during seed maturation but showed no association with average temperature during seed maturation (Table 2). Britz and Kremer (2002) reported decrease of δ-T under influence of higher temperature during maturation of soybeans (*28*). Correlation between γ-T, PC-8 and δ-T showed significant increase of γ-T/PC-8 with decrease in δ-T (Table 2). Pathways for biosynthesis of all tocochromanols was widely investigated in *Arabidopsis* plants and is initiated by the conversion of *p*-hydroxyphenylpyruvic acid (HPP) into homogentisic acid (HGA). Condensation of HGA and phytyl-PP forms 2-methyl-6-phytyl-benzoquinol (MPBQ), the committed intermediate of all tocochromanols. From HGA biosynthesis takes two parallel pathways, one forming PC-8 and other branching on sub pathways for δ-T and γ-T which can explain why there is inverse correlation between PC-8/γ-T vs. δ-T (*29*).

Results in Table 2 show that decrease of temperature, rainfall and sunshine favors increase of δ-T with analogous decrease of antioxidative capacity. Accordingly, relation for sunshine and rainfall were detected in Table 2 but not for temperature and antioxidative capacity (likely due to overall small quantities of δ–T in the study). Optimal value for δ-T would be 1 mg/kg of oil at climate index = -0.15 which converted with equations 4-6 equals to average daily temperature of 22 °C, daily sunshine of 9.8 h and average daily rainfall of 2.1 mm. Combination of calculated climate conditions was best achieved during first two weeks of flaxseed maturation. This results possibly identifies δ-T as an important antioxidant during earlier stages of seed development or during lower temperatures while its biosynthetic pathway is not active during later phases when this tocopherol is lost.

Total tocochromanols

The grand average value in our samples for total tocochromanols was (831±3) mg/kg of oil (Table 1). Total tocochromanols differed with harvesting year in range from (727±5) to (956±5) mg/kg of oil. The year 2011 had the least, while 2013 had the highest amounts of total tocochromanols (Table 1). Amounts of total tocochromanols strongly increased with average temperature and sunshine with no association with the mm of the rainfall during seed maturation (Table 2). This result is aligned with literature findings where it is shown that increased temperature favors increase of total tocochromanols during seed maturation at canola, soybean, oats and shea kernels (*4*). Average value for total tocochromanols in cold pressed flaxseed oils were reported from (182±11) mg/kg of oil (*1*) to (840±15) mg/kg of oil (*20*). As stated with γ-T, low levels of total tocochromanols in samples analyzed by Choo *et al.* (2007) (*1*) probably appeared because of oxidation during prolonged storage time in shops. Oils obtained through organic solvent extraction had values at (347±3), (534±95), (747±48), (794±13), (934±2) mg/kg of oil (*2,7,22-24*).

Table 2 shows that increase of temperature and sunshine favors increase of total tocochromanols with analogous increase of antioxidative capacity. On the contrary, increase of rainfall decreases the tocochromanol content and antioxidative capacity. Relations for sunshine and temperature were detected in Table 2 but not those for rainfall and antioxidative activity. This relations might not be detected due to inclusion of both, positively and negatively correlated tocols in total tocochromanols with climate and antioxidative activity. Optimal value for total tocochromanol would be 1104 mg/kg at climate index = 0.1982 (converted to temperature 23 °C, 10.4h sunshine, and rainfall of 1.7 mm, achieved at 5th week of flaxseed maturation). Variety Biltstar had the highest amounts of total tocochromanols, while lowest amounts were detected in Oliwin. Cold pressing yielded more of this group of compounds in comparison to oil from conditioned seeds (Table 1).

Carotenoids

The grand average value for carotenoids was (1.83±0.01) mg/kg of oil (Table 1). Depending on the harvesting year, carotenoids ranged from (1.39±0.01) to (2.40±0.01) mg/kg of oil (Table 1). Similar results were previously published and varied from (0.7±0.10) to (3.10±0.46) mg/kg of oil (*7,31*). As with majority of tocochromanols, year 2013 had highest amounts of carotenoids. Average temperature and sunshine during seed maturation significantly correlated with increase in carotenoids (Table 2). This was expected as carotenoids accumulated in plant seeds are important for protection of triacylglycerols, unsaturated lipids, membranes, and phenol quinones from photooxidation (*7*). Variety Altess had the highest, and Oliwin had the lowest concentrations of carotenoids (Table 1). If flaxseeds

were conditioned their oil had more of carotenoids. The higher carotenoid content in oils from conditioned seed may be partially explained by the prolonged contact time of the oil released during conditioning with the rest of the seed, which may improve pigment extraction into the oil.

In Table 2 we can see that increase of temperature and sunshine favors increase of carotenoids with analogous increase of antioxidative capacity. On the contrary, increase of rainfall decreases carotenoid content and antioxidative capacity. These relation were further confirmed (except for rainfall) by Pearson's correlations from Table 2. Optimal value for carotenoids was 2.57 mg/kg of oil at climate index = 0.1982 which converted with equations 4-6 equals to temperature of 23 °C, sunshine of 10.4h, and rainfall of 1.7 mm. This combination of calculated climate conditions was best achieved during the 5th week of flaxseed maturation.

Chlorophyll

The chlorophyll grand average value was (0.44±0.10) mg/kg of oil (Table 1). Mean chlorophyll levels varied with year from (0.24±0.01) to (0.63±0.01) mg/kg of oil, which is less than previously reported from (2.99±0.17) to (3.40±0.10) mg/kg of oil (*7*,*31*). Again, we found in year 2013 the highest content of chlorophyll, while variety Altess had the highest levels of this pigment (Table 1). Levels of chlorophyll slightly increased with average temperature and slightly decreased with rainfall during seed maturation (Table 2). DellaPenna and Pogson (*8*) reported inverse correlations between chlorophyll and tocopherols but our results found different relation with PC-8. Positive correlation between levels of PC-8, carotenoids and chlorophyll with increase in sunshine singles out these compounds as important molecules in seeds during high UV conditions (Table 2). Type of pressing influenced levels of chlorophyll, where conditioning of seeds yielded more of this pigment (Table 1).

DPPH radical scavenging activity of flaxseed oils

For measuring antioxidant activity in oils we determined free radical scavenging activity. The average grand value for DPPH in all samples was (62±1) % (Table 1). All produced oils, with regards to harvesting year had values between (59±0) and (66±0) % of reduced DPPH (Table 1). Oils from variety Biltstar had the highest, and Altess the lowest scavenging activity. Cold pressed oils and ones from conditioned seeds showed very similar antioxidant activity (Table 1). The strongest correlation between % of reduced DPPH and tocochromanols was shown with γ-T (Table 2), with increased concentration of γ-T in oil antioxidant activity strongly increased. Contrarily, α-T and chlorophyll decreased as antioxidant activity increased (Table 2). Variety Biltstar had highest levels of γ-T while oils from varieties Altess and Olwin were lowest with γ-T and values for % of reduced DPPH. Δ-T was more active during colder periods of seed maturation and could be connected with antioxidant

activity in earlier phases of seed development. Contrary to some other reports (*3*), the level of PC-8 did not show significant correlations with antioxidant activity.

Conclusions

Comparative results on four flaxseed varieties distinguished cold pressed oil from variety Biltstar as the best source of natural tocochromanols with strongest antioxidant activity. Along with choice of variety significant influence of climate during seed development was noted. Cold pressed oils had higher levels of total tocochromanols but showed similar antioxidant activity.

The main representative from group of tocols, γ-T, was most abundant in variety Biltstar and had strong positive association with average temperature and total sunshine, and negative association with total rainfall during seed maturation. PC-8, characteristic tocochromanol for flaxseed oil showed similar relation with climatic influences but did not show significance in relation to antioxidant activity. Further research is needed to provide the best explanations about possible protective role of PC-8 in flaxseed oil.

Content of α-T and δ-T was very low while δ-T was the only tocol which had negative association both with average temperature and sunshine during seed maturation. Variety Altess had the highest levels of pigments. While levels of carotenoids had no significant influence, chlorophyll content had strong negative correlation with antioxidant activity.

Acknowledgements

The present study was supported by the Ministry of Science, Education and Sports of the Republic of Croatia (project no. 058-0580696-0704).

References

1. Choo WS, Birch J, Dufour JP. Physicochemical and quality characteristics of cold-pressed flaxseed oils. J Food Compos Anal. 2007;20: 202-11. 10.1016/j.jfca.2006.12.002.

2. Oomah BD, Kenaschuk EO, Mazza G. Tocopherols in flaxseed. J Agr Food Chem. 1997;45: 2076-80. 10.1021/jf960735g.

3. Siger A, Kachlicki P, Czubinski J, Polcyn D, Dwiecki K, Nogala-Kalucka M. Isolation and purification of plastochromanol-8 for HPLC quantitative determinations. Eur J Lipid Sci Tech. 2014;116: 413-22. 10.1002/ejlt.201300297.

4. Maranz S and Wiesman Z. Influence of climate on the tocopherol content of shea butter. J Agr Food Chem. 2004;52: 2934-37. 10.1021/jf035194r.

5. Tura D, Gigliotti C, Pedo S, Failla O, Bassi D, Serraiocco A. Influence of cultivar and site of cultivation on levels of lipophilic and hydrophilic antioxidants in virgin olive oils (Olea Europea L.) and correlations with oxidative stability. Sci Hortic-Amsterdam. 2007;112: 108-19. 10.1016/j.scienta.2006.12.036.

6. Herchi W, Bouali I, Bahashwan S, Rochut S, Boukhchin S, Kallel H, Pepe C. Changes in phospholipid composition, protein content and chemical properties of flaxseed oil during development. Plant Physiol Bioch. 2012;54: 1-5. 10.1016/j.plaphy.2012.02.008.

7. Herchi W, Sakouhi F, Boukhchina S, Kallel H, Pepe C. Changes in Fatty Acids, Tocochromanols, Carotenoids and Chlorophylls Content During Flaxseed Development. J Am Oil Chem Soc. 2011;88: 1011-17. 10.1007/s11746-010-1750-3.

8. DellaPenna D and Pogson BJ. Vitamin synthesis in plants: Tocopherols and carotenoids. Annu. Rev. Plant Biol. 2006;57: 711-38. 10.1146/annurev.arplant.56.032604.144301.

9. Epaminondas PS, Araujo KLGV, Nascimento JA, Silva MCD, Rosenhaim R, Soledade LEB, Queiroz N, Souza AL, Santos IMG, Souza AG. Influence of toasting and the seed variety on the physico-chemical and thermo-oxidative characteristics of the flaxseed oil. J Therm Anal Calorim. 2011;106: 545-50. 10.1007/s10973-011-1731-2

10. Mridula D, Barnwal P, Singh KK. Screw pressing performance of whole and dehulled flaxseed and some physico-chemical characteristics of flaxseed oil. J Food Sci Tech Mys. 2015;52: 1498-506. 10.1007/s13197-013-1132-6.

11. FAOSTAT Statistics Division, Food and Agriculture Organization of the United Nations, Rome, Italy; http://faostat.fao.org.

12. Flax, Oilseed-List of varieties which are registered in Canada, Canadian Food Inspection Agency, Ottawa, ON, Canada; http://www.inspection.gc.ca.

13. Plant variety catalogues & databases, European Commission, Bruxelles, BE; http://ec.europa.eu/food/plant/propagation/catalogues/database/public/index.cfm?event=RunSearch.

14. Reboredo-Rodriguez P, Gonzalez-Barreiro C, Cancho-Grande B, Fregapane G, Salvador MD, Simal-Gandara J. Characterisation of extra virgin olive oils from Galician autochthonous varieties and their co-crushings with Arbequina and Picual cv. Food Chem. 2015;176: 493-503.

15. Food and Agriculture Organization of the United Nations and World Health Organization, *Codex Standard for Named Vegetable Oils*, 2011, FAO/WHO: Rome.

16. International Organization for Standardization (ISO), *Animal and vegetable fats and oils - Determination of tocopherol and tocotrienol contents by high-performance liquid chromatography*, 2006: Geneva.

17. Pokorny J, Kalinova L, Dysseler P. Determination of Chlorophyll Pigments in Crude Vegetable-Oils - Results of a Collaborative Study and the Standardized Method (Technical Report). Pure Appl Chem. 1995;67: 1781-87. http://dx.doi.org/10.1351/pac199567101781.

18. British Standards Illustrations, *Determination of carotene in vegetable oils*, in *Methods of analysis of fats and fatty oils. Other methods*1977: London.

19. Kalantzakis G, Blekas G, Pegklidou K, Boskou D. Stability and radical-scavenging activity of heated olive oil and other vegetable oils. Eur J Lipid Sci Tech. 2006;108: 329-35. 10.1002/ejlt.200500314.

20. Abuzaytoun R and Shahidi F. Oxidative stability of flax and hemp oils. J Am Oil Chem Soc. 2006;83: 855-61. 10.1007/s11746-006-5037-7.

21. Teh SS and Birch J. Physicochemical and quality characteristics of cold-pressed hemp, flax and canola seed oils. J Food Compos Anal. 2013;30: 26-31. 10.1016/j.jfca.2013.01.004

22. Bozan B and Temelli F. Chemical composition and oxidative stability of flax, safflower and poppy seed and seed oils. Bioresour. Technol. 2008;99: 6354-59. 10.1016/j.biortech.2007.12.009.

23. Ciftci ON, Przybylski R, Rudzinska M. Lipid components of flax, perilla, and chia seeds. Eur J Lipid Sci Tech. 2012;114: 794-800. 10.1002/ejlt.201100207.

24. Khattab RY and Zeitoun MA. Quality evaluation of flaxseed oil obtained by different extraction techniques. Lwt-Food Sci Technol. 2013;53: 338-45. 10.1016/j.lwt.2013.01.004.

25. Gruszka J and Kruk J. RP-LC for determination of plastochromanol, tocotrienols and tocopherols in plant oils. Chromatographia. 2007;66: 909-13. 10.1365/s10337-007-0416-2.

26. Kruk J, Szymanska R, Cela J, Munne-Bosch S. Plastochromanol-8: Fifty years of research. Phytochemistry. 2014;108: 9-16. 10.1016/j.phytochem.2014.09.011.

27. Zubr J and Matthaus B. Effects of growth conditions on fatty acids and tocopherols in Camelina sativa oil. Ind Crop Prod. 2002;15: 155-62. 10.1016/S0926-6690(01)00106-6.

28. Britz SJ and Kremer DF. Warm temperatures or drought during seed maturation increase free alpha-tocopherol in seeds of soybean (Glycine max [L.] Merr.). J Agr Food Chem. 2002;50: 6058-63. 10.1021/jf0200016.

29. Mene-Saffrane L and DellaPenna D. Biosynthesis, regulation and functions of tocochromanols in plants. Plant Physiol Bioch. 2010;48: 301-09. 10.1016/j.plaphy.2009.11.004.

30. Bravi E, Perretti G, Marconi O, Patrizi E, Fantozzi P. Secoisolariciresinol diglucoside determination in flaxseed (Linum usitatissimum L.) oil and application to a shelf life study. Food Chem. 2011;126: 1553-58. 10.1016/j.foodchem.2010.11.169.

31. Tuberoso CIG, Kowalczyk A, Sarritzu E, Cabras P. Determination of antioxidant compounds and antioxidant activity in commercial oilseeds for food use. Food Chem. 2007;103: 1494-501. 10.1016/j.foodchem.2006.08.014.

Table 1. Composition of tocochromanols and pigments (Mean±SE in mg/kg) in flaxseed oil from three harvesting years, four varieties and two types of pressing

Table 2. Correlation coefficients between tocochromanols, pigments and % of reduced DPPH with climate factors during seed maturation

Table 1. Composition of tocochromanols and pigments (Mean±SE in mg/kg) in flaxseed oil from three harvesting years, four varieties and two types of pressing

	α- T	PC-8	γ-T	δ-T	Total T	Chlorophyll	Carotenoids	% DPPH
Harvesting year								
2011	$(1 \pm 1)^a$	$(265 \pm 3)^a$	$(463 \pm 4)^a$	(1 ± 0.1)	$(732 \pm 5)^a$	$(0.43 \pm 0.01)^a$	$(1.39 \pm 0.01)^a$	$(59 \pm 0)^a$
2012	$(1 \pm 1)^a$	$(282 \pm 3)^b$	$(526 \pm 4)^b$	tr	$(809 \pm 5)^b$	$(0.24 \pm 0.01)^b$	$(1.69 \pm 0.01)^b$	$(66 \pm 0)^b$
2013	$(8 \pm 1)^b$	$(367 \pm 3)^c$	$(575 \pm 4)^c$	tr	$(951 \pm 5)^c$	$(0.63 \pm 0.01)^c$	$(2.39 \pm 0.01)^c$	$(60 \pm 0)^a$
Variety								
Altess	$(3 \pm 1)^a$	$(314 \pm 3)^a$	$(486 \pm 4)^a$	$(1 \pm 0.1)^a$	$(804 \pm 6)^a$	$(0.86 \pm 0.02)^a$	$(2.78 \pm 0.02)^a$	$(59 \pm 0)^a$
Biltstar	$(1 \pm 1)^a$	$(322 \pm 3)^a$	$(612 \pm 4)^b$	tr	$(936 \pm 6)^b$	$(0.23 \pm 0.02)^b$	$(1.74 \pm 0.02)^b$	$(66 \pm 0)^b$
Niagara	$(2 \pm 1)^a$	$(295 \pm 3)^b$	$(509 \pm 4)^c$	tr	$(807 \pm 6)^a$	$(0.34 \pm 0.02)^c$	$(1.76 \pm 0.02)^b$	$(63 \pm 0)^c$
Oliwin	$(7 \pm 1)^b$	$(288 \pm 3)^b$	$(480 \pm 49^a$	tr	$(776 \pm 6)^c$	$(0.31 \pm 0.02)^c$	$(1.02 \pm 0.02)^c$	$(58 \pm 0)^d$
Type of pressing								
Cold Pressed	$(3 \pm 1)^a$	$(318 \pm 2)^a$	$(518\pm3)^a$	tr	$(840 \pm 4)^a$	$(0.35 \pm 0.01)^a$	$(1.78 \pm 0.01)^a$	$(61 \pm 0)^a$
Conditioning 30min/60° C	$(4 \pm 1)^a$	$(292 \pm 2)^b$	$(525\pm3)^b$	$(1 \pm 0.1)^b$	$(821 \pm 4)^b$	$(0.52 \pm 0.01)^b$	$(1.87 \pm 0.01)^b$	$(62 \pm 0)^b$
Grand mean**	(3 ± 1)	(305 ± 2)	(522 ± 2)	(0.44 ± 0.04)	(801 ± 3)	(0.43 ± 0.10)	(1.83 ± 0.01)	(62 ± 1)

When used in the columns, different superscripts indicate significant differences ($p < 0.05$); tr trace (<0.1) in statistical analysis was set to 0.
** Total average per column

Table 2. Correlation coefficients between tocochromanols, pigments and % of reduced DPPH with climate factors from flowering to harvesting

	α-T	PC-8	γ-T	δ-T	Total T	Chlorophyll	Carotenoids	Climate Index	Temperature/ °C	Sunshine/ h	Rainfall/ mm	DPPH/%
α-T	1	0.36*	0.08	-0.13	0.28	0.05	0.03	0.48*	0.48*	0.21	-0.31*	-0.32*
PC-8		1	0.61*	-0.41*	0.88*	0.33*	0.51*	0.70*	0.70*	0.52*	-0.32*	-0.11
γ-T			1	-0.44*	0.91*	-0.11	0.24	0.39*	0.39*	0.57*	0.01	0.50*
δ-T				1	-0.47*	0.13	0.08	-0.12	-0.12	-0.53*	-0.32*	-0.13
Total T					1	0.09	0.40*	0.61*	0.61*	0.61*	-0.17	0.23
Chlorophyll						1	0.61*	0.38*	0.38*	0.02	-0.35*	-0.51*
Carotenoids							1	0.41*	0.41*	0.38*	-0.14	-0.07
Climate Index								1	0.90*	0.28*	-0.76*	-0.44*
Temperature/°C									1	0.96*	-0.39*	-0.44*
Sunshine/h										1	-0.13	0.32
Rainfall/mm											1	0.63*
DPPH/%												1

*. Correlation is significant at the 0.05 level (2-tailed)