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Food Chemistry 88 (2004) 225–231
Food Chemistry
www.elsevier.com/locate/foodchem

Susceptibility of lipids from different flax cultivars to peroxidation and its lowering by added antioxidants

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Received 2 October 2003; received in revised form 20 December 2003; accepted 20 December 2003

Abstract

Consumption of flax (*Linum usitatissimum*) seeds is beneficial for human health. Flax seeds, containing about 40% of oil, are the richest (among crop plants) source of polyunsaturated fatty acids (PUFA) essential in the human diet. PUFA are highly susceptible to oxidation. Thus only certain cultivars (e.g. Linola) with low linolenic acid content are suitable for commercial preparation of edible oil, which has, nevertheless, a very short shelf life. To study the factors influencing the stability of flax oil, the oil was extracted from nine flax cultivars and analyzed. Linola contained about 3% of linolenic acid while, in other analyzed cultivars, its content ranged from 52% to 73%. Instead, Linola is rich in linoleic acid (about 75%), which in other cultivars varied from 12% to 18%. The susceptibility to oxidation of extracted oil has been analyzed using two methods (measurement of conjugated dienes and thiobarbituric acid-reactive substances (TBARS) formation). Even the low linolenic acid content Linola oil was easily oxidized. The most resistant to peroxidation was the oil extracted from Abby. The potential to reduce peroxidation has been tested using natural antioxidants (β-carotene and quercetin) at concentrations ranging from 10 to 250 μM. The formation of TBARS was most efficiently reduced by 25 μM concentrations of both β-carotene and quercetin. Higher concentrations of β-carotene increased the level of TBARS. The efficiency of β-carotene and quercetin varied, depending on the analyzed cultivar, probably due to intrinsic content of antioxidants.
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Keywords: Flax oil; PUFA; Lipid peroxidation; Antioxidants; Carotene; Quercetin

1. Introduction

Flax (*Linum usitatissimum*) seeds, containing about 40% of oil, have long been used in human and animal diets and in industry as a source of oil and as the basic component or additive of various paints or polymers. Recently there has been a growing interest in the probiotic properties of flax and in its beneficial effects on coronary heart disease, some kinds of cancer and neurological and hormonal disorders (Huang & Milles, 1996; Huang & Ziboh, 2001; Simopoulos, 2002). The beneficial effects are mostly due to flax lipids. Flax oil is the richest plant source of linoleic and linolenic polyunsaturated fatty acids (PUFA), which are essential for humans since they cannot be synthesized in the organism and must be ingested in food.

Unfortunately, essential fatty acids are highly susceptible to oxidation and flax oil, therefore, has a very short shelf life. Only certain cultivars (e.g. Linola) with appropriate lipid composition are suitable for commercial preparation of edible oil (Dribnenki & Green, 1995; Green, 1986). In flax grains, lipids are protected against oxidation by various mechanisms, for example, the presence of antioxidants such as lignans (Kitts, Yuan, Wijewickreme, & Thompson, 1999). However, even after cold extraction, most of these mechanisms are no longer operative and lignans are not effectively extracted with oil. To avoid a rapid appearance of rancidity, flax oil is often supplemented with vitamin E, stored in dark glass jars and it may

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doi:10.1016/j.foodchem.2003.12.042

not be used for frying. As none of these protection methods are fully satisfactory, further improvements are sought. One approach could involve the overexpression of various natural antioxidants, such as carotenoids or flavonoids within flax grains. In addition to preventing fat rancidity, these antioxidants could increase commercial value of food products and have beneficial effects on human health. When consumed together with essential unsaturated fatty acids, they can reduce the risk of various diseases (Romieu & Trenga, 2001). Carotenoids are supposed to act as free radical-scavengers by electron transfer to their double-bond structure. In contrast, antioxidant ability of flavonoids is related to the presence of –OH groups which may directly bind free radicals and chelate metals (Halliwell & Gutteridge, 1999). Another desirable method would be to elucidate the molecular basis of lipid synthesis and to make the genetic engineering of fatty acid composition possible. Description and analysis of the differences between existing cultivars could be very helpful in designing future experiments focussed on molecular engineering of this type.

We analyzed the fatty acid composition of various flax cultivars, compared their susceptibilities to oxidation using fatty acid standards and assessed the protective effect of added antioxidants (quercetin and β-carotene), representing two natural groups (flavonoids and carotenoids), by using standards and lipids extracted from various cultivars.

2. Materials and methods

2.1. Plant material

Flax seed cultivars (fibre flax: Voronezski (Russia); linseed: Jenny, Opal, Szafir, (Poland); La Estanzuela E, La Estanzuela AR, La Estanzuela 117 (Uruguay); Abby (Great Britain); Linola (Canada)), were obtained from The Flax and Hemp Collection of the Institute of Natural Fibres, Poland (Rutkowska-Krause & Silska, 2002).

2.2. Lipid isolation from flax seeds by a modified method of Allen and Good (1971)

Flax seeds (1 g) were ground in a mortar with 1 ml of water. The homogenate was suspended in 2 ml of methanol and 4 ml of chloroform. Then, 3.5 ml of 0.9% NaCl was added, gently mixed and left to settle for 24 h at room temperature (RT). The lower chloroform phase was collected and extraction was repeated. Following chloroform evaporation on a rotary vacuum evaporator, lipids were resuspended in a chloroform/methanol mixture (1:2 v/v) and stored at −20 °C. For each analyzed cultivar, seed lipids were extracted at least twice.

2.3. Chromatographic determination of lipid contents

The fatty acid composition of the total fat was examined by gas chromatography. Methyl esters of the fatty acids (FAMEs) were obtained by esterification of fat samples by a modified method of Prescha, Swiedrych, Biernat, and Szopa (2001). 50 mg of lipids was saponified at 70 °C for 1 h with 1 ml of 2 M KOH in 75% aqueous methanol. The unsaponifiable material was extracted twice with petroleum ether and then discarded. The potassium salts of the fatty acids were treated with 1 ml of 2 M HCl in water for 30 min at 70 °C. After the addition of saturated sodium chloride solution, the fatty acids were extracted twice with 1 ml portions of hexane. The fatty acids were then esterified with 1 ml of 0.5 M KOH for 30 min at 70 °C and then with 1 ml of 1.25 M HCl for 30 min (both solutions in anhydrous methanol). The FAMEs were extracted with hexane as described previously. The methyl ester mixture was separated on a CP-Sil 88 Chrompack capillary column (50 m × 0.25 mm). Helium was used as carrier gas and the separation was carried out at a temperature programmed to rise from 150 °C (for 6 min) to 235 °C at a rate of 6 °C per minute; film thickness was 0.2 μm. Particular fatty acids were identified, using a flame ionisation detector, by comparison with external standards (linolenic, linoleic, oleic, and stearic acid, Sigma–Aldrich, Poland).

2.4. Determination of conjugated dienes

Conjugated diene concentration was determined spectrophotometrically according to Recknagel and Glende (1984). Oil samples (1 μl) were oxidized at 140 °C for up to 40 min with or without 25 μM β-carotene or quercetin (Sigma–Aldrich, Poland). β-Carotene was dissolved in cyclohexane and quercetin in methanol. Under these experimental conditions most samples after heat treatment had to be diluted for OD_{234} determination.

2.5. TBARS determination

The level of thiobarbituric acid-reactive substances (TBARS) in the samples was determined according to Aust (1994). Oil samples (1 μl) were oxidized at 140 °C for up to 40 min, with or without 25 μM β-carotene or quercetin. Two ml of reagent A (15% trichloroacetic acid and 0.37% thiobarbituric acid in 0.25 M HCl) were added and the mixture was thoroughly blended. Test tubes containing the samples were stoppered with glass marbles, heated at 100 °C for 15 min, cooled under running tap water and centrifuged for 10 min at 2000*g*. Sample absorbance was measured at 535 nm on a Cecil CE-2020 spectrophotometer against a reference blank containing the TBA reagent.

3. Results

3.1. Lipid composition of flax cultivars

Fatty acid composition was analyzed in lipids extracted from grains of nine flax cultivars (Voronezski, Jenny, La Estanzuela AR, La Estanzuela E, La Estanzuela 117, Opal, Szafir, Abby, Linola). Seeds of all the analyzed cultivars contained about 40% oil and the lipid composition of flax oil was cultivar-dependent (Table 1). The unique feature of flax is the accumulation of large amounts of linolenic acid, the final product of three desaturation steps (Fig. 1), in their seeds. Apart from Linola, containing 3.3%, it was the most abundant fatty acid and its amount in cultivars tested varied from 52.1% (Jenny) to 73% (La Estanzuela 117). The content of linolenic acid negatively correlated with all its precursors (Table 2). The next two most abundant fatty acids were oleic and linoleic. The content of linoleic acid was less variable than that of oleic acid, which varied from 6.4% in La Estanzuela 117 to 22.0% in Voronezski. Surprisingly, in contrast to the relationship between linolenic acid and its precursors, there was a high positive correlation ($r = 0.83$) between oleic and palmitic acid contents. The least abundant among all the analyzed fatty acids was the arachidic acid (20:0) whose content seemed to correlate positively ($r = 0.40$) with the oleic acid content and negatively ($r = -0.45$) with the linolenic acid content. Similar correlation was observed for other cultivars (data not shown).

3.2. Optimization of antioxidant concentrations

The first stage in lipid peroxidation is an abstraction of hydrogen from a molecule of PUFA and formation of conjugated dienes. This process can be quantified spectrophotometrically. The formation of conjugated dienes was measured in oil extracted from the Linola cultivar (Fig. 2). Determination of lipid oxidation stability was performed according to international standards (ISO 6886:1996, Animal and vegetable fats and oils – determination of oxidation stability. Accelerated oxidation

Table 1
Variation in oil lipid composition of the investigated flax cultivars

	16:0	18:0	18:1	18:2	18:3	20:0
Cultivar/acid	Palmitic	Stearic	Oleic	Linoleic	Linolenic	Arachidic
Voroezski	6.11	4.6	22.0	14.9	52.2	0.24
Jenny	6.91	5.7	19.7	14.2	52.1	0.36
La Estanzuela AR	6.28	4.1	17.7	14.1	57.5	0.46
La Estanzuela E	5.47	nd	13.5	12.2	68.8	nd
La Estanzuela 117	0.04	4.45	6.48	15.5	73.4	0.16
Opal	5.43	3.73	18.2	15.3	56.1	1.22
Szafir	5.19	3.28	14.1	11.9	65.4	0.11
Abby	5.99	3.4	13.3	18.2	58.9	0.11
Linola	6.28	1.91	13.5	74.5	3.27	0.28
Average	5.21	3.65	15.7	14.5	60.5	0.33

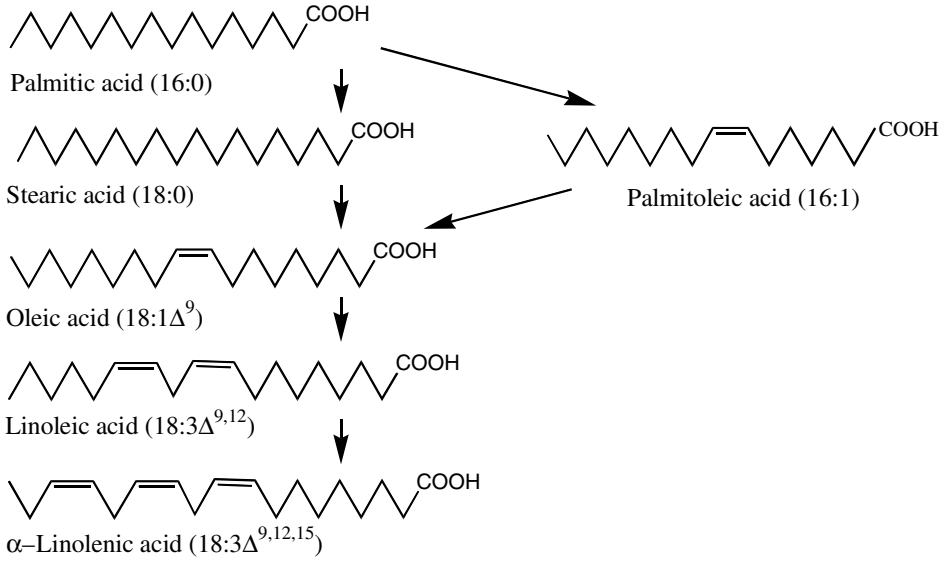

Fig. 1. Possible biosynthetic pathways of major flax fatty acids. Adapted from (Napier, 2002; Voelker & Kinney, 2001).

Table 2
Correlation coefficients (r) between amounts of fatty acids in flax cultivar

Palmitic acid	−0.02	0.83	−0.11	−0.78	0.16
	Stearic acid	0.3	0.38	−0.53	0.29
		Oleic acid	−0.1	−0.92	0.4
			Linoleic acid	−0.25	0.18
				Linolenic acid	−0.45
					Arachidic acid

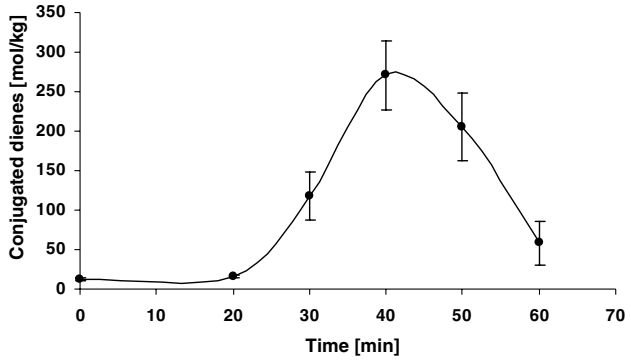

Fig. 2. Time course of formation of conjugated dienes in Linola flax oil. Crude oil extracted from Linola cultivar seeds was heated for different time periods up to 60 min at 140 °C. Data are mean conjugated dienes levels from six repetitions (two independent lipid extractions) measured spectrophotometrically at $\lambda = 234$ nm ± confidence intervals ($\alpha = 0.05$).

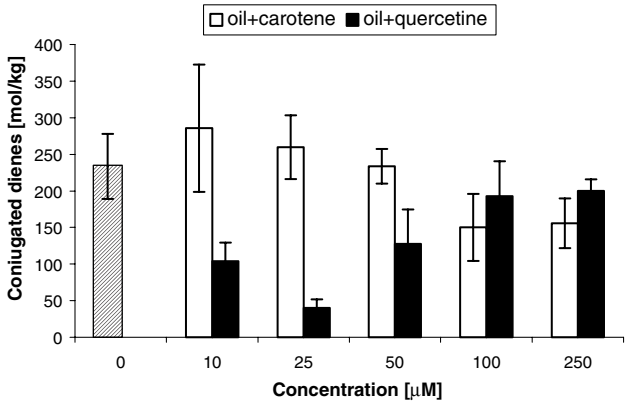

Fig. 3. Formation of conjugated dienes in the presence of β-carotene and quercetin in Linola flax oil. Crude oil extracted from Linola seeds alone, or supplemented with 25 μM β-carotene or quercetin was heated for 40 min at 140 °C. Data are mean conjugated dienes levels from six repetitions (two independent lipid extractions) measured spectrophotometrically at $\lambda = 234$ nm ± confidence intervals ($\alpha = 0.05$).

test.) at elevated temperature. We evaluated kinetics of conjugated diene formation at 140 °C. Measured optical density reached a maximum after 40 min of heating and then decreased as a result of subsequent peroxidation reactions, beginning with the attachment of oxygen to the diene molecule. The final step of lipid peroxidation is the formation of malondialdehyde (MDA) or similar substances that can be determined through their reaction with thiobarbituric acid (TBA).

The peroxidation process could be slowed by the presence of antioxidants. Since high concentrations of antioxidants may result in pro-oxidative activity, the effect of the addition of β-carotene or quercetin at concentrations ranging from 10 to 250 μM, on the levels of both conjugated dienes and TBARS was tested in Linola oil (Figs. 3 and 4). With both assays, quercetin exhibited the highest antioxidative activity at 25 μM. The potential of β-carotene to stop diene formation increased when the concentration of the antioxidant increased from 10 to 100 μM. In contrast, as β-carotene concentration increased from 10 to 25 μM, the amount of measured TBARS decreased and then increased, indicating that β-carotene itself was the source of TBARS. Thus, 25 μM concentrations of both antioxidants were used in further experiments.

Flax oil rancidity is the result of many complex reactions within a mixture of various oil components. An important factor influencing oil stability should be fatty

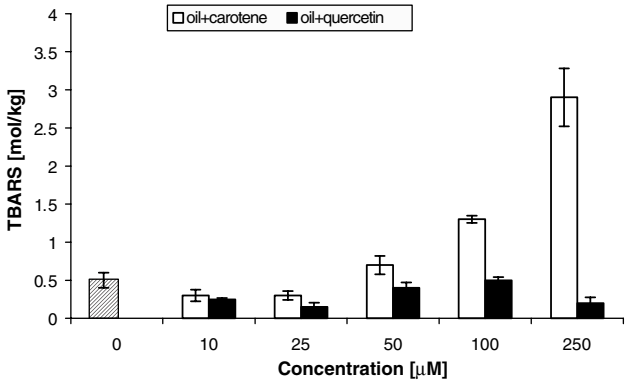

Fig. 4. TBARS formation in Linola flax oil in the presence of β-carotene or quercetin. Crude oil extracted from Linola seeds alone or supplemented with 25 μM β-carotene or quercetin was heated for 40 min at 140 °C. Data are mean TBARS levels from six repetitions (two independent lipid extractions) measured spectrophotometrically at $\lambda = 535$ nm ± confidence intervals ($\alpha = 0.05$).

acids (FA) composition. As shown with lipid standards (Fig. 5), susceptibility to conjugated diene formation is determined by the number of unsaturated bonds in the FA molecule. A similar result was previously obtained by Krasowska et al. (2002). Conjugated dienes were not detected (background OD level) in heated stearic acid.

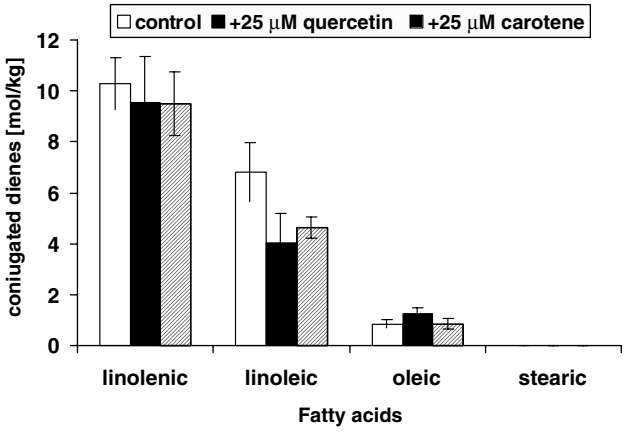

Fig. 5. Formation of conjugated dienes in the presence of β-carotene and quercetin in FA standards. Fatty acids standards alone, or supplemented with 25 μM β-carotene or quercetin were heated for 40 min at 140 °C. Data are mean conjugated dienes levels (four repetitions) measured spectrophotometrically at $\lambda = 234$ nm ± confidence intervals ($\alpha = 0.05$).

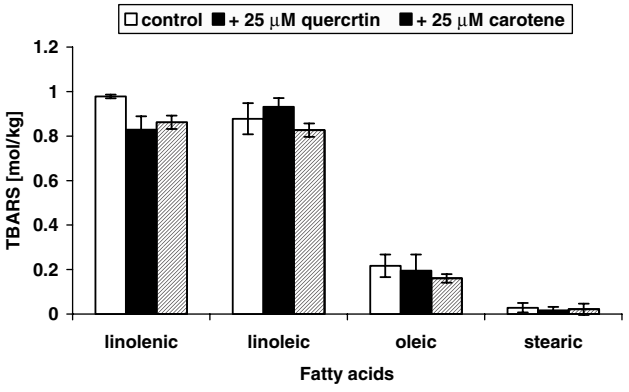

Fig. 6. TBARS formation in FA standards in the presence of β-carotene or quercetin. Fatty acids alone or supplemented with 25 μM β-carotene or quercetin were heated for 40 min at 140 °C. Data are mean TBARS levels (four repetitions) measured spectrophotometrically at $\lambda = 535$ nm ± confidence intervals ($\alpha = 0.05$).

Antioxidants significantly reduced dienes formation only in linoleic acid. Peroxidation of lipid standards, measured by TBARS, is also dependent on the number of unsaturated bonds (Fig. 6). In contrast to the diene formation, levels of TBARS, significantly higher than the background, were observed in heated stearic acid. Similar amounts of TBARS were formed in linoleic and linolenic acid. Antioxidants significantly reduced TBARS formation only in the linolenic acid.

3.3. Peroxidation of oil from different cultivars

Lipid peroxidation in the oil from flax cultivars under investigation was determined, as for FA standards, by assaying the levels of both conjugated dienes (Fig. 7) and TBARS (Fig. 8). Surprisingly, Linola oil with the

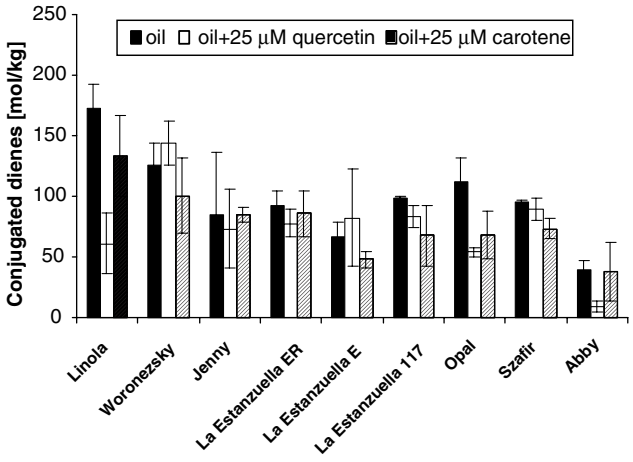

Fig. 7. Formation of conjugated dienes in oils from different flax cultivars in the presence of quercetin or β-carotene. Crude oil extracted from seeds of different cultivars alone or supplemented with 25 μM β-carotene or quercetin was heated for 40 min at 140 °C. Data are mean conjugated dienes levels from six repetitions (two independent lipid extractions) measured spectrophotometrically at $\lambda = 234$ nm ± confidence intervals ($\alpha = 0.05$).

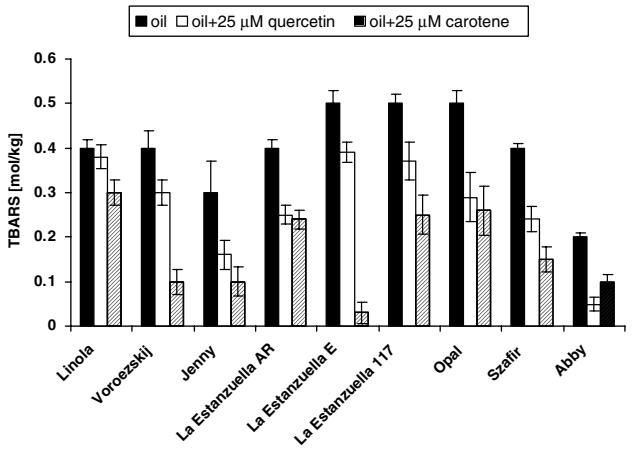

Fig. 8. Formation of TBARS oil extracted from different flax cultivars in the presence of quercetin or β-carotene. Crude oil extracted from different cultivars alone or supplemented with 25 μM β-carotene or quercetin was heated for 40 min at 140 °C. Data are mean TBARS levels from six repetitions (two independent lipid extractions) measured spectrophotometrically at $\lambda = 535$ nm ± confidence intervals ($\alpha = 0.05$).

lowest content of linolenic acid was not the most peroxidation-resistant, the lowest peroxidation being found in Abby oil. Generally, there was no clear relationship between the linolenic acid content and the magnitude of peroxidation. Quercetin significantly reduced the formation of conjugated diene in the oil from Linola, La Estanzuella 117, Opal and Abby, whereas β-carotene was most effective in oil from La Estanzuella 117, Opal and Szafir.

In all cases, β-carotene caused a strong decrease in TBARS formation whereas quercetin had a smaller effect and caused no perceptible suppression of TBARS formation in Linola oil.

4. Discussion

Plant seeds contain lipids in the form of triacyl glycerols whose molecules consist of glycerol attached to FA. Their biosynthesis was recently reviewed by (Napier, 2002; Voelker & Kinney, 2001). According to the current knowledge, most of the plant fatty acids arise in plastids and usually contain up to 16–18 carbon atoms in their aliphatic chains. They are subsequently transported to cytosol as saturated FA or, after the modification by specific desaturase, as monounsaturated FA. Further modifications (desaturations) resulting in the formation of PUFA (polyunsaturated fatty acids, such as linoleic and linolenic acids) take place in cytosol (Fig. 1). It is supposed that oleic acid is synthesized mainly through stearic acid. However, an alternative pathway has been described in some plants that involves desaturation of palmitic acid and its subsequent elongation. PUFA from flax oil are essential for human diet and lower the risk of diseases related to cholesterol oxidation. Consumption of oleic, linoleic and linolenic acids lowers the level of LDL in human blood. Unfortunately, flax oil with high PUFA content is readily oxidized and thus has a minor role in human diet. As seen from studies of the peroxidation of lipid standards (Figs. 5 and 6), an increasing number of unsaturated C=C bonds increases the susceptibility to oxidation. The significantly more measured dienes in oleic acid compared to stearic acid is notable, as theoretically only PUFA would yield conjugated dienes. However similar results have previously been observed, showing that susceptibility to conjugated diene formation is determined by the number of unsaturated bonds in the FA molecule (Krasowska et al., 2002). This observation could theoretically be explained by formation of the second C=C double bond during oxidation of oleic acid, as in the reaction catalysed by desaturase. The most abundant FA in flax oil is linolenic acid, with three C=C double bonds. Its level highly negatively correlates with the content of oleic acid, suggesting that the rate-limiting step in synthesis of linolenic acid is the transport of oleic acid from plastids to the cytoplasm. Linola cultivar was developed to make linseed oil edible (Dribnenki & Green, 1995). Breeding efforts resulted in the reduction of linolenic acid level from over 50 to less than 4% while the content of linoleic acid rose from about 15% to 75%, suggesting that the activity of Δ^{15}-desaturase was affected. This results in a relatively high total amount of PUFA compared to other cultivars, which could be the reason for the observed high sensitivity to oxidation. Since the total PUFA amount is high and relatively stable in all the analyzed cultivars, future cultivar improvements should concentrate on reducing the total PUFA content while leaving oleic acid as the main component, as in olive oil. Also, the ratio of linoleic to linolenic acid should be taken into account. It is postulated to be important for human health, with an optimum of about 1:1 (Simopoulos, 2002). None of the analyzed cultivars were close to this value, as this ratio was 0.04 in Linola and ranged from 3.7 to 4.7 in high-PUFA cultivars.

Oil can be, to some extent, protected against oxidation by antioxidants. Our results show that neither of the two added antioxidants, quercetin or β-carotene, seems to be highly efficient in preventing the formation of conjugated dienes. They appear to act in subsequent steps of peroxidation, reacting with peroxy radicals and/or stopping the propagation phase of the chain reaction. In the tested cultivars, the amount of destroyed dienes does not strongly correlate with the TBARS. This could result from different kinetics of dienes appearing in various cultivars. However, neither quercetin nor β-carotene were significantly affected by heating time, when the maximum of dienes was measured (results not shown). Accordingly, both antioxidants strongly suppress the formation of the end-products of lipid peroxidation, TBARS, in most cultivars. Oil from cultivars containing high amounts of PUFA (La Estanzuela 117, La Estanzuela E) was the most highly oxidized. However, the correlation between PUFA content in oil and the extent of peroxidation was not as strong as one would expect from the results obtained with lipid standards. To a certain extent this could be explained by the fact that similar levels of TBARS were observed in linolenic and linoleic FA standards. However, there must be other factors, apart from the overall PUFA content, that influence oil stability. One of the factors could be the amounts of various indigenous antioxidants, which could also affect the antioxidative potency of the externally added quercetin and β-carotene. These two substances differ in the mechanism of radical trapping and in physico-chemical properties and the type and content of intrinsic antioxidants could contribute to the difference in their effectiveness. Cultivars, such as La Estanzuella E, could have a high content of flavonoid-like antioxidants in contrast to, e.g., Abby wherein quercetin is more effective than β-carotene. Measurement of TBARS, such as by MDA, is often criticized because of its nonspecificity, since TBARS may arise by different processes from a variety of target compounds. As shown, higher β-carotene concentrations gave rise to increased TBARS levels, conceivably as the result of a pro-oxidative action of high amounts of β-carotene or its degradation to TBARS. On the other hand, the observed results of peroxidation, measured by conjugated dienes and TBARS, support our conclusions on the re-

lationship between the oil PUFA content and susceptibility to oxidation.

An intriguing observation is the high ($r = 0.83$) positive correlation between the amount of oleic and palmitic acid in contrast to the negative correlation between linoleic acid and all its precursors. An interesting hypothesis explaining this observation could be the synthesis of oleic acid through palmitoleic acid (Fig. 1).

Acknowledgements

The authors thank Marcin Biskupski for the FA determination in nine flax oil cultivars. This work was supported by Grants No. 5PO6A 02319 and 6P06A 03720 from the Polish State Committee for Scientific Research (KBN).

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