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Preliminary studies of tocochromanol content in seeds of open pollinated and hybrid varieties of winter rape

Wstępne badania zawartości tokochromanoli w nasionach odmian populacyjnych i mieszańcowych rzepaku ozimego

Key words: rapeseed, open pollinated and hybrid varieties, homologous tocopherols, HPLC

The objective of this study was determination of natural antioxidants, such as tocochromanols, in seeds of fifteen varieties of winter rape registered by COORU in the years 2002–2003. There were open pollinated varieties: Polish – Batory and Bazyl and foreign – Rasmus, Digger, Capio, Cazek, Californium and Spencer NL as well as Polish composite hybrids Kaszub and Mazur and foreign restored hybrids Kronos, Baldur, Extrem and Titan. Materials from the first reproduction – K_1 – were supplied by seed producing company OBROL. All studied rapeseeds came from the crop of 2004. Tocochromanol composition of new varieties was compared to that of varieties already cultivated – Kana, Lirajet, Lisek and Buffalo, which had already been studied earlier and results were published in 2003. The material under investigation included also the seeds of winter turnip rape (*B. rapa*) Lufowy variety and winter false flax *Camelina sativa* Przybrodzka II variety harvested in 2003.

The qualitative identification and quantitative determination of homologous tocopherols were carried out by HPLC after saponification of samples and extraction of unsaponifiable substances. Tocopherol separation was performed with the use of Waters HPLC system equipment with LiChrosorb Si 60 column. The mobile phase was *n*-hexane and *1,4* dioxane. Tocopherols were monitored by a fluorimetric detector (295, 330 nm). Contents of individual tocopherol homologues were calculated on the basis of calibration curves for pure forms of these compounds.

Results obtained in this study indicated that total tocochromanol content in the majority of rapeseed varieties was above 300 mg/kg d.m of seeds. The greatest tocopherols content was determined in two rapeseed varieties – restored hybrids Kronos and Titan (above 400 mg/kg d.m). Each of four homologous tocopherols was identified in all investigated winter rapeseed varieties. α- and γ-tocopherols occurred in greatest quantities. The calculated ratio of α- to γ-tocopherol content ranged from 0.8 to 1.10 for open pollinated varieties of rapeseed, and it was higher for the restored hybrids (1.32). Among the open pollinated varieties of winter rapeseed the seeds of Lisek variety were richest in tocopherols (383 mg/kg d.m.). Tocopherol content was also higher when compared to the 2000–2003 crops of this variety.

Słowa kluczowe: nasiona rzepaku, odmiany populacyjne i mieszańcowe, homologiczne tokoferole, HPLC

W pracy badano zawartość natywnych przeciwutleniaczy, jakimi są tokochromanole, w nasionach 15 odmian rzepaku ozimego, zarejestrowanych przez COBORU w latach 2000–2003. Badano polskie

odmiany populacyjne Batory i Bazyl oraz zagraniczne Rasmus, Digger, Capio, Cazek, Californium i Spencer NL, polskie mieszańce złożone Kaszub i Mazur oraz zagraniczne mieszańce zrestorowane Kronos, Baldur, Extrem i Titan. Materiał siewny pochodził z pierwszego rozmnożenia K_1 i został zakupiony w przedsiębiorstwie nasiennym OBROL. Do badań włączono także nasiona rzepaku starszych odmian (Kana, Lirajet, Lisek i Buffalo). Wszystkie badane nasiona pochodziły ze zbiorów w 2004 r. Analizowano również nasiona rzepiku ozimego odmiany Ludowy i lnianki ozimej odmiany Przybrodzka II (namnożone przez nas w 2003 roku). Zawartość homologicznych tokoferoli oznaczano techniką HPLC, po uprzednim zmydleniu nasion i ekstracji eterem etylowym. Do rozdziału stosowano kolumnę LiChrosorb Si 60 (250 × 4,6 mm; 5 μm). Fazę ruchomą stanowił *n*-heksan z *1,4* dioksanem (97 : 3 v/v) o szybkości przepływu 1,5 cm^3/min. Stosowano detektor fluorymetryczny (295, 330 nm). Zawartość poszczególnych homologów tokoferoli obliczono na podstawie krzywych kalibracji wykonanych za pomocą próbek wzorcowych tokoferoli.

We wszystkich nasionach rzepaku, rzepiku i lnianki zidentyfikowano cztery homologiczne tokoferole α-, β-, γ- i δ-T. Całkowita ilość tokoferoli w nowych odmianach populacyjnych rzepaku kształtowała się od 295 mg/kg s.m. (Batory 2001 r.) do ponad 397 mg/kg s.m. (Digger 2003 r.). We wszystkich badanych odmianach rzepaku dominowały homologi α- i γ–tokoferolu. W nasionach był identyfikowany w niewielkich ilościach β- i δ-tokoferol. Obliczony stosunek zawartości α- do γ-tokoferolu kształtował się dla odmian populacyjnych rzepaku ozimego na poziomie 0,8 do 1,10. W odmianach mieszańcowych zrestorowanych był znacznie wyższy i sięgał wartości 1,32. W badanych nasionach odmian populacyjnych rzepaku ozimego największą zawartość oznaczono dla odmiany Lisek (383,0 mg/kg s.m.), znacznie większą w porównaniu do ilości tokochromanoli oznaczonych w nasionach tej samej odmiany ze zbiorów wcześniejszych (2000–2003 r.).

Introduction

The seeds of double low quality (00) rapeseed cultivated in Poland are a valuable material for production of plant oils and fats. Oils obtained from rapeseed are characterized by high content of triacyloglycerols — a source of polyunsaturated fatty acids (PUFAs). Rapeseed oil contains about 20% of linoleic acid ($C_{18:2}$ n-6) and about 10% of linolenic acid ($C_{18:3}$, n-3) necessary in human diet (Mikołajczyk et al. 1999). On the other hand, high level of unsaturated fatty acids (90%) can influence decrease of the oxidative stability of oil and other products containing this oil. Free radicals and also peroxides and other products of their conversion are formed during oxidation, which can cause many civilization diseases (Korczak et al. 1999, Cadenas and Davies 2000, Wang and Quinn 1999). By combining modern biotechnology methods with classical plant breeding novel varieties of rapeseed were generated having specific properties and chemical composition (Leckband et al. 2002). The new geneticaly modified varieties of rapeseed are characterized by reduced levels of e.g. linolenic acid (Murphy and Mithen 1995, Jourdren at al. 1996, Mikołajczyk et al. 1999, Spasibionek et al. 2000, Hom 2004). New methods of genetic modification can give new rapeseed varieties having higher level of native oxidation inhibitors such as tocopherols (Goffman and Becker 1998, 2001, 2002; Marwede et al. 2004, 2005). Tocopherols are natural antioxidants in plant oils. All tocopherols are amphipathic molecules

having polar chroman rings and hydrophobic prenyl chain (Hofius and Sonnewald 2003).

The main biochemical function of tocopherols is protection of polyunsaturated fatty acids against peroxidation (Kamal-Eldin and Appelqvist 1996). The strongest biological activity is displayed by α-tocopherol whereas γ-tocopherol has the strongest antioxidant activity (Goffman and Becker 2001, Nogala-Kałucka et al. 2003). Homologous tocopherols are synthesised by plants and photosynthetic organisms (Cahoon and Coughlan 2004, Sadre et al. 2001). Formation of four tocopherols results from the reactions of cyclization and methylation of their aromatic precursor — homogentisic acid (Janiszowska 1986). Four homologous tocopherols occur in rapeseed and other photosynthetic plants (Nogala-Kalucka et. al. 2002). Among the tocopherols, α- (α-T) and γ- (γ-T) are the predominant forms. β- (β-T) and δ-tocopherols (δ-T) are identified in small (δ-T) or trace amounts (β-T) (Nogala-Kalucka et al. 2002).

Introducing new winter rapeseed varieties with modified seeds quality contributed to continuation of earlier studies. The aim of these studies is determination of the quality and quantity of tocopherol contents in new winter rapeseed varieties (open pollinated varieties and composite hybrids) in comparison to the varieties already examined.

Material and methods

Seed samples of fifteen varieties of winter rapeseed registered by COBORU in the years 2002–2003 were investigated. There were open pollinated varieties: Polish — Batory and Bazyl and foreign — Rasmus, Digger, Capio, Cazek, Californium and Spencer NL as well as Polish composite hybrids (F_{1z}) Kaszub and Mazur and foreign restored hybrids (F_1) Kronos, Baldur, Extrem and Titan. Materials from first reproduction — K_1 — was supplied by the seed producing company OBROL. Four older varieties: Kana, Lirajet, Lisek and Buffalo were also investigated. Seeds of all studied varieties came from the crop of 2004, and were collected during growing period from the experimental fields of Poznań Agricultural University (Przybroda). The material under investigation included also the seeds of winter turnip rape Ludowy variety and winter false flax (*Camelina sativa*) Przybrodzka II variety — reproduced in 2003 by the authors at the experimental station Przybroda.

Tocopherols determination

In order to determine the tocopherol content, samples of rapeseeds were saponified using 60% KOH. After saponification the unsaponifiable substances were extracted three times using the peroxide free diethyl ether. Then, the ether was

distilled off and the residue was dissolved in n-hexane. The qualitative identification and quantitative determination of homologous tocopherols were carried out by HPLC (Gogolewski et al. 2000, Nogala-Kałucka et al. 2002). The tocopherols were analysed on the Waters HPLC system equipment with LiChrosorb Si 60 column (250 × 4.6 mm; 5 μm) and precolumn LiChrospher Si 60. The mobile phase was *n*-hexane and *1,4* dioxane. The tocopherols were monitored by a fluorescence detector (295, 330 nm). Contents of individual tocopherol homologoues were calculated on the basis of calibration curves made for pure forms of these compounds. Standards of α-, β-, γ- and δ-tocopherol (α-T, γ-T, β-T, δ-T) (99%) were purchased from Merck (Darmstadt, Germany). Two seed samples (3 g) were drawn from each variety and all analyses were repeated three times.

Statistical analysis

The results obtained were subjected to statistical analysis. One-factor analysis of variance and post-hoc Tukey's tests were carried out with the statistical package program Statistica (version 6.0) for the significance level $\alpha = 0.05$.

Results and discussion

The new hybrid varieties of winter rapeseed have been of interest since 1995. Their cultivation constantly increases, because the yields are high and giving rape seeds of good quality. Breeding of new varieties results in obtaining larger amount of pods with well developed seeds. The seeds are characterised by improved technological quality — higher level of fat (above 38%) and protein (above 19%) (Wójtowicz et al. 2003). The level of native antioxidant in seeds of new varieties of rapeseed has not been tested untill now. Therefore, following current research trends in genetic modification, in breeding and in cultivation of new varieties of rapeseed, determination of tocochromanol content in selected varieties of rapeseed took into account the studies which had already been published (Nogala-Kałucka et al. 2002, 2003).

In this study alkaline hydrolysis and chromatographic method (normal-phase high-performance liquid chromatography with fluorescence detection – NP-HPLC-FLD) for determination of all forms of tocopherols were used. They are also commonly used by other researchers (Gogolewski et al. 2000, Nogala-Kałucka et al. 2002, Ryynänen et al. 2004). There are three major sample preparation approaches for food samples: simple dilution of oil samples in organic solvent, direct extraction of the vitamins with organic solvents, and extraction of the vitamins after saponification (Eitenmiller and Landen 1999, Ryynänen et al. 2004). Cereals, seeds and nuts are crushed to fine particles before sample preparation. Saponification is a general term referring to alkaline hydrolysis. Usually, KOH

is used, although NaOH is specified by some procedures. Hydrolysis results in cleavage of the ester bounds of triacyloglycerols, phospholipids and sterols; it destroys pigments and disrupts the sample matrix which facilitates vitamin extraction (Eitenmiller and Landen 1999, Wrolstad 2002). Tocopherols are relatively unstable in alkaline conditions, and care must be taken to avoid their destruction. They are protected by using antioxidants (pyrogallol or ascorbic acid), flushing the saponification vessel with nitrogen and working under subdued light (Eitenmiller and Landen 1999, Eitenmiller and Lee 2004). After saponification, the digest is diluted with water or 1% NaCl to inhibit emulsion formation and is extracted with diethylether, petroleum ether, hexane or ethyl acetate in hexane, or other solvent mixtures. The solvent is used to retrieve unsaponifiable components into the organic phase. Solvent and aqueous phases are separated after centrifugation or a setting period. The unsaponifiable components, including tocopherols and tocotrienols in sample solution, are extracted into the solvent layer. Most water-soluble interfering substances, including proteins, sugars, and fibers, remain in the waste (aqueous layer) (Shin and Godber 1994).

Tocopherols were separated by reversed-phase thin-layer chromatography (C_{18} RP-HPTLC), normal-phase high-performance liquid chromatography (NP-HPLC), reversed-phase high-performance liquid chromatography (C_{18} RP-HPLC), and gas chromatography (GC). Separation using C_{18} RP-HPLC does not separate β- and γ-tocopherols. (Pyka and Sliwiok 2001). GC separation of tocopherols is difficult to achieve. To decrease their boiling points and to avoid decomposition of tocopherols at the high temperature of analysis that must be applied in the GC method, a derivatization reaction is used to transform the hydroxyl groups of tocopherol to trimethylsilyl (TMS) derivatives, which results in lower boiling points. However, the conditions of derivatization are very critical for obtaining high yields of derivatives, and high variability may occur between duplicates (Lercker and Rodriguez-Estrada 2000, Wrolstad 2002). Vitamin E analysis is most commonly conducted with NP-HPLC-FLD. A fluorescence detector is preferred to an ultraviolet detector in analysing tocopherols and tocotrienols in complex food matrices because of its specificity and sensitivity (Eitenmiller and Landen 1999, Abidi 2000, Ryynänen et al. 2004).

Two Polish composite hybrid varieties Kaszub and Mazur (2001) were among the investigated fifteen seed samples of rapeseed varieties registered by COBORU in the years 2000–2003. The remaining were Polish and foreign open pollinated varieties. The variability in tocopherol contents in the investigated rapeseed samples is presented in Table 1. In all samples of rape seeds, turnip rape and false flax seeds four homologous tocopherols α-, γ-, β- and δ- were identified. The total tocopherols content in new open pollinated varieties ranged from 295 mg/kg dry matter (Batory 2001) to above 397 mg/kg seed d.m. (Digger 2003).

Table 1

Tocopherol content in investigated winter rape seeds – open pollinated, composite hybrids and restored hybrids, also in winter turnip and winter camelina — *Zawartość homologicznych tokoferoli w nasionach rzepaku ozimego – odmian populacyjnych, mieszańców złożonych i zrestorowanych oraz rzepiku i lnianki ozimej*

Varieties *Odmiany*	Tocopherol content — *Zawartość tokoferoli* [mg/kg d.m.]					Ratio *Współczynnik* α-T / γ-T
	α-T	β-T	γ-T	δ-T	sum —*suma*	
Open pollinated — *Odmiany populacyjne*						
Kana	154,6 ± 4,9 ^{c,d}	2,2 ± 0,2 ^{a}	163,1 ± 4,6 ^{f,g}	6,8 ± 0,2 ^{a,b,c}	326,6 ± 9,9 ^{d,e,f}	0,95^{e,f}
Batory	146,8 ± 3,4 ^{c}	5,3 ± 0,1 ^{e,f}	134,3 ± 2,7 ^{a,b}	8,8 ± 0,1 ^{e,f,g}	295,1 ± 6,3 ^{b,c}	1,09^{i,j,k}
Bazyl	173,8 ± 3,5 ^{f,g,h}	6,1 ± 0,2 ^{g}	170,9 ± 13,2 ^{g,h}	9,0 ± 0,7 ^{f,g}	359,8 ± 17,7 ^{h,i}	1,02^{g,h}
Lirajet	175,2 ± 3,6 ^{g,h}	2,2 ± 0,2 ^{a}	162.0 ± 5,2 ^{e,f,g}	6,4 ± 0,1 ^{a,b}	345,8 ± 9,1 ^{f,g,h}	1,08^{i,j}
Lisek	180,4 ± 0,2 ^{h}	2,1 ± 0,1 ^{a}	194,5 ± 0,7 ^{j,k,l}	5,9 ± 0,1 ^{a}	382,9 ± 1,1 ^{i,j}	0,93^{d,e}
Cazek	157,4 ± 1,9 ^{c,d,e}	4,4 ± 0,2 ^{b,c}	147,3 ± 0,7 ^{b,c,d}	6,1 ± 0,1 ^{a}	315,3 ± 2,8 ^{c,d,e}	1,07^{h,i}
Digger	206,7 ± 7,7 ^{i,j}	4,4 ± 0,2 ^{b,c}	179,9 ± 6,6 ^{h,i}	6,4 ± 0,6 ^{a,b}	397,4 ± 15,2 ^{j,k}	1,15^{k}
Capio	164,1 ± 1,7 ^{d,e,f,g}	6,9 ± 0,3 ^{h}	164,7 ± 1,7 ^{f,g}	8,7 ± 0,5 ^{e,f,g}	344,5 ± 4,7 ^{f,g,h}	1,00^{f,g}
Contact	160,1 ± 2,7 ^{d,e}	4,4 ± 0,1 ^{b,c}	186,6 ± 1,9 ^{i,j,k,l}	8,1 ± 0,7 ^{d,e,f}	359,3 ± 5,5 ^{g,h,i}	0,86^{c}
Californium	167,8 ± 9,3 ^{e,f,g,h}	4,5 ± 0,1 ^{b,c,d}	152,4 ± 7,5 ^{c,d,e,f}	7,7 ± 0,2 ^{c,d,e}	332,5 ± 17,1 ^{e,f,g}	1,10^{i,j,k}
Rasmus	159,8 ± 1,3 ^{d,e}	4,6 ± 0,1 ^{b,c,d}	150,8 ± 5,1 ^{c,d,e,f}	9,4 ± 0,1 ^{g}	324,7 ± 6,5 ^{d,e,f}	1,06^{h,i}
Spencer NL	160,9 ± 1,5 ^{d,e}	6,1 ± 0,2 ^{g}	184,0 ± 0,4 ^{h,i,j,k}	12,3 ± 0,1 ^{h}	363,3 ± 2,2 ^{h,i}	0,87^{c,d}
Composite (F_{1z}) — *Odmiany mieszańcowe złożone*						
Kaszub	120,3 ± 3,1 ^{b}	4,2 ± 0,1 ^{b}	146,5 ± 1,4^{b,c}	8,8 ± 0,1 ^{e,f,g}	279,8 ± 4,7 ^{a,b}	0,82^{c}
Mazur	159,0 ± 1,9 ^{c,d,e}	4,8 ± 0,1 ^{c,d,e}	161,8 ± 1,2 ^{d,e,f,g}	9,1 ± 0,2 ^{f,g}	341,0 ± 3,6 ^{e,f,g,h}	0,98 ^{e,f,g}

Restored (F_1) — *Odmiany mieszańcowe zrestorowane*						
Buffalo	195,5 ± 0,6 i	4,5 ± 0,1 b,c,d	148,4 ± 5,8 b,c,d,e	7,7 ± 0,6 c,d,e	356,1 ± 6,5 g,h,i	1,32 m
Kronos	202,3 ± 8,7 i,j	5,0 ± 0,3 d,e,f	199,9 ± 7,6 l	8,7 ± 0,6 e,f,g	415,9 ± 17,2 k	1,01 g,h
Baldur	177,4 ± 0,3 h	4,7 ± 0,4 b,c,d	157,0 ± 1,4 c,d,e,f,g	8,7 ± 0,5 e,f,g	347,8 ± 2,6 f,g,h	1,13 j,k
Extrem	161,6 ± 5,7 d,e,f	4,2 ± 0,2 b	128,7 ± 1,6 a	7,5 ± 0,2 b,c,d	302,0 ± 7,7 b,c,d	1,25 l
Titan	210,1 ± 1,4 j	5,4 ± 0,2 f	198,5 ± 0,3 k,l	9,1 ± 0,0 f,g	423,1 ± 2,0 k	1,06 h,i
Others — *Inne*						
Rzepik ozimy Ludowy	127,1 ± 0,3 b	2,1 ± 0,1 a	180,0 ± 0,2 h,i,j	6,8 ± 0,1 a,b,c	316,1 ± 0,7 c,d,e	0,71 b
Lnianka ozima Przybrodzka II	11,6 ± 0,1 a	2,4 ± 0,0 a	244,7 ± 0,1 m	6,5 ± 0,1 a,b	265,3 ± 0,3 a	0,05 a

* values followed by different letters are statistically significant at $\alpha = 0.05$
wartości oznaczone różnymi literami różnią się statystycznie istotnie przy α = 0,05

In all analysed samples of rape seeds α- and γ-tocopherols occurred in greatest and usually equivalent quantities. The amount of α tocopherol was between 120 mg/kg d.m in the composite hybrid Kaszub 2001 and 200 mg/kg d.m. in the restored hybrid Titan (Table 1). These varieties contained similar amounts of γ-tocopherol. The restored hybrids — varieties Kronos and Titan — were characterized by the highest total content of tocohromanols — above 415 mg/kg d.m. Among the investigated seed samples of open pollinated rapeseed varieties two were richest in vitamin E-active compounds. Lisek variety (registered in 1998) contained 380 mg/kg d.m. of these compounds. In 2004 the tocopherol content in this variety and also in Lirajet (registered in 1994) and Buffalo (registered in 1999) was higher in comparison to the tocopherol content determined in evaluated seed samples of these varieties from cultivations in 2001 and 2002 (Nogala-Kałucka et al. 2003). The ratio of the α- / γ-tocopherols content was calculated for all tested rape seed samples (Tables 1). This ratio is a parameter pointing to the increase of the antioxidant content as the effect of genetic modification of rape in many papers (Goffman and Becker 2001, 2002; Marwede et al. 2004, 2005).

The calculated ratio was greater than 1.0 in eight among twelve investigated open pollinated varieties of winter rapeseed and in four varieties it was a little lower (Table 1). In all seed samples of foreign restored hybrids the calculated ratio was above 1.0 and for Buffalo and Extrem it was 1,3. For Polish composite hybrids this parameter was below 1.0 (Mazur 0.98 and Kaszub 0.82). These varieties contained smaller amounts of α- than γ-tocopherol.

In the investigated samples of turnip rape seeds the ratio of the α- to γ-tocopherols content was smaller (0.71). The seeds of winter false flax Przybrodzka variety contained small amount of α-tocopherol as compared to higher amount of γ-tocopherol, so the ratio was only 0.05. The calculated ratio of the α- to γ-tocopherols content in seed for all tested rapeseed varieties was similar to the values published by Goffman and Becker (2001, 2002).

Conclusions

- Four homologous tocopherols were identified in seed samples of all investigated winter rapeseed varieties and also in turnip rape and false flax seeds. α- and γ-tocopherols occurred in seeds in greatest and similar quantities.
- The total tocochromanol content in the majority of rapeseed varieties was above 300 mg/kg d.m. The greatest tocopherol content was found in two rapeseed varieties — restored hybrids Kronos and Titan (above 400 mg/kg d.m).
- The calculated ratio of the α- / γ-tocopherol content ranged from 0.8 to 1.10 for examined open pollinated varieties of rapeseed.
- For the restored hybrid varieties the ratio of the α- / γ-tocopherol content was higher and ranged from 1.01 to 1.32.

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