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Unique variability of tocopherol composition in various seed oils recovered from by-products of apple industry: Rapid and simple determination of all four homologues (α, β, γ and δ) by RP-HPLC/FLD

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ABSTRACT

The tocochromanol profile was studied in seed oils recovered from by-products of fruit industry, five dessert and seven crab apple varieties grown in Eastern Europe (Latvia). The seed oils obtained from dessert apples were characterized by higher contents of tocopherols (191.05–379.08 mg/100 g oil) when compared to seed oils recovered from crab apples (130.55–202.54 mg/100 g oil). The predominant homologues of tocopherol in all the studied samples were α and β over γ and δ. However, seed oils recovered from the apple cultivars 'Antej' and 'Beforest' had a unique profile of four tocopherol homologues (α:β:γ:δ) 91.41:80.55:72.46:79.03 and 114.55:112.84:78.69:73.00 mg/100 g oil, respectively. A single dilution of seed oils in 2-propanol facilitated the direct use samples in the DPPH assay as well as injection into the RP-HPLC system containing a PFP (pentafluorophenyl) column, which resulted in a rapid separation of all four tocopherol homologues with excellent repeatability and reproducibility.

1. Introduction

Apple (*Malus domestica* Borkh.) is one of the most popular fruit crops in the world. The global production of apple in the last five decades has increased by 444% from 17 million tons in 1961 to 76 million tons in 2011 and 2012. In Europe production of apples in 2012 amounted to 15 million tons, of which 50% were cultivated in Eastern Europe (FAOSTAT, 2013). The increased apple production raises the amount of by-products after processing. The two branches of industry that produce large amounts of apple by-products are the juice and fresh-cut fruit salad production. For instance, the apple pomace press cake obtained during juice pressing represents approximately 25% of the initial amount of fruits (Mahawar, Singh, & Jalgaonkar, 2012). Also the fresh-cut fruit salads market, which rapidly has been expanding in the last few years, generates an increasingly large volume of by-products (Buckley, Cowan, & McCarthy, 2007). Seeds are one of the valuable parts of apple fruit by-products. It was noted that the seeds in some varieties can constitute up to 0.7% of fresh apple, where the yield of oil in seeds can reach even 29.4% (Fromm, Bayha, Carle, & Kammerer, 2012).

Plant oils, especially cold-pressed, have an important role in human daily diet. They are not only a source of triglycerides and unsaturated fatty acids, but also a valuable source of biocomponents such as phenolic compounds, squalene, sterols, carotenoids, lignans and tocochromanols (Górnaś, Siger, Juhņeviča, et al., 2014; Górnaś, Siger, Pugajeva, & Segliņa, 2014; Górnaś, Siger, & Segliņa, 2013). According to Eitenmiller and Lee (2004), fats and oils are major sources of vitamin E in the daily diet. Biological components with the activity of vitamin E are classified as the most important natural antioxidants and are mainly present in plants and their products. It is assumed that the main function of tocopherols is to protect polyunsaturated fatty acids against oxidation (Ratnayake & Daun, 2004). Among four tocopherol homologues α, β, γ and δ, the α-T has the highest biological activity (Eitenmiller &

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Lee, 2004) and serves important physicochemical functions in biological membranes (Dwiecki, Górnaś, Jackowiak, Nogala-Kałucka, & Polewski, 2007; Dwiecki, Górnaś, Wilk, Nogala-Kałucka, & Polewski, 2007).

The presence of tocopherols in apple seed oils was reported previously by Arain et al. (2012) and Fromm, Bayha, Kammerer, and Carle (2012); however, only four seed oils recovered from dessert cultivars grown in South Asia (Pakistan) and six seed oils obtained from two cider and four dessert varieties grown in Western Europe (Germany), respectively, were studied. Moreover, the results reported by Arain et al. (2012) and Fromm et al. (2012) are at least inconclusive, which may be associated with different varieties of apples used in those studies, their place of cultivation as well as the used techniques for tocopherol detection. Therefore, due to the lack of information on tocopherol composition in seed oils recovered from crab apples as well as the lack of data regarding apples cultivated in Eastern Europe the present studies were undertaken to shed more light on this topic. Additionally, the present study showed that only a single dilution of seed oil in 2-propanol made it possible to use samples in the DPPH assay as well as facilitated rapid separation of all four tocopherol homologues by RP-HPLC using a PFP (pentafluorophenyl) column.

2. Materials and methods

2.1. Standards, solvents and reagents

Standards of α, β, γ, and δ homologues of tocopherol (T) (>95% of purity) were purchased from Merck (Darmstadt, Germany). Ethanol (96%) was received from SIA Jaunpagasts Plus (Jaunpagasts, Latvia). Methanol, ethanol, 2-propanol and *n*-hexane (HPLC grade) were purchased from Sigma–Aldrich (Steinheim, Germany). The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was obtained from Fluka (Buchs, Switzerland).

2.2. Origin of apple seeds

Seeds of six crab apple cultivars and one hybrid ('Kerr', 'Kuku', 'Riku', 'Ritika', 'Ruti', 'Quaker Beauty' and K-8/9-24) were obtained from apple pomace supplied by a local producer of cider (Lejaskerzeni, Valmiera, Latvia). Seeds of five dessert apple cultivars ('Antej', 'Beforest', 'Kent', 'Sinap Orlovskij' and 'Zarja Alatau') were obtained as a by-product during the preparation of fruit salads at the processing facility at the Latvia State Institute of Fruit-Growing (LSIFG). Both dessert and crab apples were collected in September 2012 in Dobele, at the LSIFG, GPS location: N: 56°36′39″ E: 23°17′50″. Samples were prepared according to the flow scheme shown in Fig. 1. Briefly, seeds were separated from apple flesh and core, then oven-dried (5 h) in Orakas 5600 (Marlemi, Lemi, Finland) with forced hot air circulation at 55 ± 1 °C. Next, undamaged seeds were selected (~50 g) and milled with a Knifetec™ 1095 (Foss, Höganäs, Sweden) universal laboratory mill to pass through a sieve of 0.75 mm mesh size to finally obtain a powder.

2.3. Extraction of oil

For extraction of oil ground apple seeds (5 g) were supplemented with *n*-hexane (25 ml) in a centrifuge tube and vortexed on the Vortex REAX top (Heidolph, Schwabach, Germany) at 2500 rpm (1 min). Samples were subjected to ultrasound treatment in the Sonorex RK 510 H ultrasonic bath (Bandelin electronic, Berlin, Germany) (5 min, 35 °C) and centrifuged on a Centrifuge 5804 R (Eppendorf, Hamburg, Germany) (10,000*g*, 5 min, 21 °C). The supernatant was collected in a round bottom flask and the remaining solid residue was re-extracted (twice) as described above. The combined supernatants were evaporated in a Laborota 4000 vacuum rotary evaporator (Heidolph, Schwabach, Germany) at 40 °C till constant weight.

2.4. Determination of tocochromanols by RP-HPLC/FLD

Tocopherols were determined according to the method previously developed and validated by Górnaś, Siger, Czubinski, et al. (2014). Apple seed oil samples (0.2 g) were diluted in 2-propanol, made up to 10 ml and filtered through an MS® nylon syringe filter with 0.22 μm pore size (Membrane Solutions, Plano, TX, USA) directly to vials and immediately analysed on an HPLC system. The chromatographic separation was carried out on the Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of a pump (LC-10ADvp), a degasser (DGU-14A), a low pressure gradient unit (FCV-10ALvp), a system controller (SCL-10Avp), an auto injector (SIL-10AF), a column oven (CTO-10ASvp), a fluorescence detector (RF-10AXL) and a Luna PFP column (3 μm, 150 × 4.6 mm) with a guard column (4 × 3 mm) (Phenomenex, Torrance, CA, USA). The analysis was performed under the following conditions: mobile phase methanol:water (93:7; v/v); flow (1.0 ml/min); column oven temperature (40 °C); room temperature (22 ± 1 °C) and runtime (13 min). The identification and quantification were conducted in a fluorescence detector at an excitation wavelength of 295 nm and emission wavelength of 330 nm.

2.5. DPPH assay

The method consisted of a spectrophotometric measurement of the scavenging of stabilized DPPH free radical. Each of the oil samples (0.20 g) was diluted in 2-propanol (10 ml). The DPPH radical solution was prepared freshly by dissolving 0.005 g of the DPPH radicals in 250 ml of 96% ethanol and homogenized in a Sonorex RK 510 H ultrasonic bath (Bandelin electronic, Berlin, Germany) for 0.5 min. The obtained absorption of the DPPH solution was

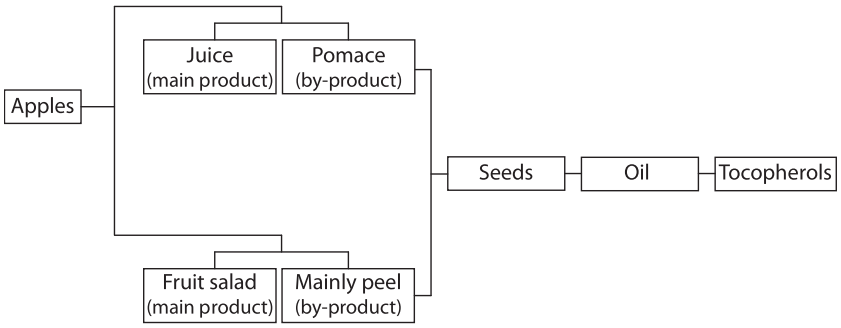

Fig. 1. Scheme of potential utilization of seeds recovered from by-products of apple industry.

0.670 ± 0.01. The reaction was initiated by mixing of the diluted oil sample in 2-propanol (0.2 ml) with the DPPH solution (3.8 ml). Absorbance was measured at λ_{max} of 517 nm in a 1650 PC spectrophotometer (Shimadzu, Kyoto, Japan) after 30 min storage in dark (experimentally determined as the stable state of reaction). The activity of the samples in scavenging DPPH radicals was calculated as follows:

$$\%\text{DPPH scavenging} = \left[\frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}}\right] \times 100$$

2.6. Statistical analysis

The results were presented as means ± standard deviation from three replicates of each experiment. The p-value < 0.05 was used to denote significant differences between mean values determined by one-way analysis of variance (ANOVA). The homogeneity of variance was checked by Levene's test. Homogeneous groups were determined by Tukey's post hoc test with the assistance of Statistica 10.0 (StatSoft, Tulsa, OK, USA) software. The multiple linear regression, analysis of variance and Pearson correlations were determined using Excel 2010 software (Microsoft, Redmond, WA, USA).

3. Results and discussion

The apple seed oils were recovered by ultrasound treatment, since conventional methods of extraction (e.g. Soxhlet) are solvent- and time-consuming. Therefore, one of the novel and promising alternative extraction technologies is ultrasound treatment. The ultrasound frequency accelerates the kinetics and increases the extraction yield; moreover, the applicability at low operating temperatures preserves thermolabile nutraceuticals against losses (Wang & Weller, 2006). Furthermore, research conducted by Cravotto et al. (2008) showed that ultrasound extraction makes it possible to obtain higher oil yields in comparison with conventional methods. In contrast, methyl ester profiles of oils extracted by ultrasound treatment and the Soxhlet method showed only minor or insignificant differences.

The most common analytical technique for determination of tocochromanols is the high-performance liquid chromatography (HPLC). Due to the better repeatability, reproducibility, speed and environmental reasons the reversed-phase (RP) technique is used more often than normal-phase (NP), for instance, to check authenticity of oils (Chena et al., 2011). However, the most popular column used for RP named C18 has some disadvantage over NP columns – it cannot separate isomers β and γ. Nevertheless, RP is widely used in analytics, where the contents of tocopherol homologues β and γ are given as total β + γ. In most cases the presence and concentration of β-T in the plant raw material and food products (with some exceptions) is omitted due to its rare prevalence and low concentration. However, it is worth noting that good separation of tocopherol homologues β and γ on a RP column, named PFP (pentafluorophenyl), has been developed by Richheimer, Kent, and Bernart (1994). Nevertheless, this method has not been sufficiently popularized in the science community, perhaps due to the length of the analysis (20 min). Recently, in traditional analytical techniques such as HPLC the following factors gained in importance: speed of analysis, protection against the loss of analyte, saving of time and effort, reproducibility, as well as reducing of the environmental impact (toxic solvents and energy consumption). The highest impact to the repeatability and reproducibility of results had the preparation of samples for analysis. Czauderna and Kowalczyk (2006) observed that the saponification procedure had a negative impact on stability of tocopherols in analysed samples of milk and butter. To prevent analyte losses during sample preparation it is required to reduce all the necessary procedures before analysis to a minimum. Therefore, oil samples were only diluted in 2-propanol and directly injected into the RP-HPLC system. Precision (repeatability, reproducibility and retention time) were evaluated for seed oil samples recovered from apple cultivar 'Riku' (Table 1). The coefficients of variation (CV) were very low for the retention time (lower than 1%) and repeatability, as well as reproducibility (lower than 3%). The maintenance of constant temperature inside the laboratory was a key factor to achieve such good precision. Moreover, when compared to previous studies conducted by Richheimer et al. (1994), the time of separation was reduced from 20 to 13 min, saving time and solvent consumption during analysis. An example of a typical chromatogram of the tocopherol composition in the apple seed oil provided by RP-HPLC/FLD method is shown in Fig. 2.

Generally, in most plant oils the predominant homologues of tocopherol are γ-T and α-T, while β-T and δ-T are present in low amounts or absent (Górnaś, Siger, Juhņeviča, et al., 2014). In all studied apple seed oils four homologues of tocopherol (α, β, γ, and δ) were identified. The concentration of individual tocopherol homologues in seed oils obtained from various cultivars of apples varied. The most constant level was noted for α-T (51.40–88.70 and 91.41–114.55 mg/100 g of oil recovered from crab and dessert apple seeds, respectively). The greatest variability, especially for seed oils obtained from dessert apples, was observed for γ-T (16.71–37.03 and 2.37–78.69 mg/100 g of oil extracted from crab and dessert apple seeds, respectively) and δ-T (7.01–26.59 and 0.67–79.03 mg/100 g of oil recovered from crab and dessert apple seeds, respectively). The β-T was detected in the range of 27.92–69.49 and 70.69–124.28 mg/100 g of oil obtained from crab and dessert apple seeds, respectively (Table 1). The high concentration of β-T in crab and dessert apple seed oils is unique in the plant

Table 1
Tocopherol determination accuracy in oil recovered from seeds of apple cultivar 'Riku'.

Tocopherols	Precision								
	Retention time[a] (min)			Repeatability[b]			Reproducibility[c]		
	Mean[d]	SD[e]	CV[f] (%)	Mean	SD	CV (%)	Mean	SD	CV (%)
α-T	12.08	0.11	0.91	79.53	0.80	1.01	79.14	1.64	2.07
β-T	9.83	0.08	0.81	69.32	0.65	0.94	69.07	1.37	1.98
γ-T	10.36	0.08	0.77	27.07	0.25	0.92	26.78	0.55	2.05
δ-T	8.18	0.06	0.73	26.46	0.28	1.06	26.41	0.59	2.23

[a] n = 10.
[b] Repeatability refers to the results of independent determinations carried out on a sample by analysing five replicates of the sample on the same day.
[c] Reproducibility refers to the results of independent determinations carried out on a sample by analysing five replicates of the sample at different times.
[d] n = 5 (mg/100 g oil).
[e] SD, standard deviation.
[f] Coefficient of variation calculated as the SD divided by the mean.

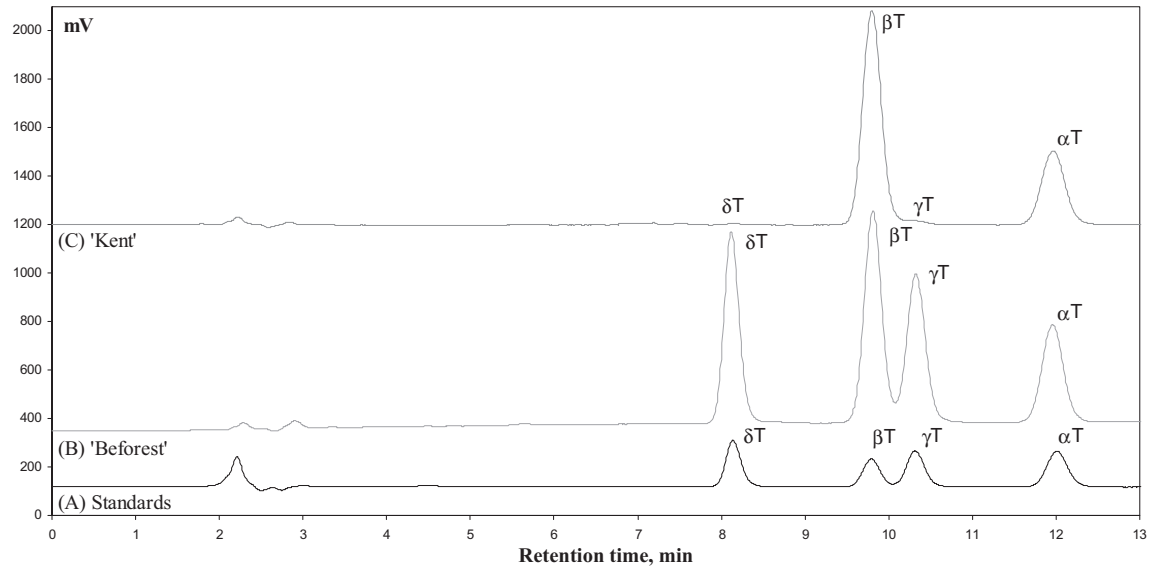

Fig. 2. RP-HPLC/FLD chromatograms of tocopherols separated on a Luna PFP column. A, standards; B and C, seed oils recovered from two apple cultivars: B, 'Beforest'; C, 'Kent'.

world, as there are only a few well-known sources where β-T is the dominant tocopherol, e.g. coffee (Górnaś, Siger, Polewski, Pugajeva, & Waśkiewicz, 2014), as well as bran and germ of wheat (Eitenmiller & Lee, 2004). Four homologues of tocopherol were also detected in seed oils recovered from four cultivars of dessert and two of cider apples cultivated in Western Europe (Germany) (Fromm et al., 2012), whereas only α-T and β-T were found in seed oils obtained from four cultivars of dessert apples grown in South Asia (Pakistan) (Arain et al., 2012). The lack of γ-T and δ-T may be explained by fact that Arain et al. (2012) used the GC–MS method for tocopherol determination. The GC separation of tocopherols is difficult due to their high and relatively close boiling points. To reduce the boiling point and also to avoid decomposition of tocopherols during GC analysis the derivatisation procedure needs typically to be performed. It means transformation of the hydroxyl groups of tocopherols to trimethylsilyl forms, resulting in lower boiling points. However, to obtain high yields of derivatives the conditions of derivatisation are critical and high variability may occur. Therefore, the GC method is not the best tool for determination of tocochromanols (Xu, 2005). The α-T was the primary tocopherol in both crab and dessert apple seed oils, with only one exception of cv. 'Kent', in which a higher concentration of β-T when compared to α-T was detected. Dominance of α-T over β-T, approximately four times, was also reported by Arain et al. (2012). The opposite results were found by Fromm et al. (2012), where the β-T was the dominant tocopherol, except for one variety, in which α-T predominated. The lowest concentrations among all tocopherol homologues were found for γ-T and δ-T, especially in cv. 'Kent' (2.37 and 0.67 mg/100 g of oil, respectively) and 'Sinap Orlovskij' (9.22 and 5.80 mg/100 g of oil, respectively). For the same apple cultivars the lowest total tocopherol content among all the tested dessert apples was recorded. Low amounts of γ-T and δ-T in apple seed oils were also detected by Fromm et al. (2012); however, in the present study, except for the above mentioned two cultivars, the concentrations of forms γ-T and δ-T were approximately 10 times higher. Additionally, in seed oils recovered from the apple cultivars 'Antej' and 'Beforest' a unique composition of tocochromanols was found, characterized by high concentrations and almost equal amounts of all four tocopherol homologues (α:β:γ:δ) 91.41:80.55:72.46:79.03 and 114.55:112.84:78.69:73.00 mg/100 g oil, respectively. Such a tocopherol profile was not found before in any of studied conventional and unconventional oils. The content of all tocopherol homologues in crab apple seed oils was lower than in dessert apple seed oils (average, Table 2). Total tocopherol concentration in crab apple seed oils was significantly lower in comparison with dessert apple seed oils, with the exception of the sample originating from cv. 'Riku'. The amount of tocochromanols in seed oil recovered from crab apples (130.55–202.54 mg/100 g of oil) was approximately two times lower than that obtained from dessert apples (191.05–379.08 mg/100 g of oil). Fromm et al. (2012) reported about 20% higher contents of tocopherols in seed oils recovered from cider apples than obtained from the dessert apples. However, those findings were based solely on research conducted on two cider and four dessert apple cultivar seed oils. The current study was conducted on seven crab and five dessert apple varieties. It needs to be stressed that concentrations of tocopherols detected in the present study were 1.5–2 times and 2 up to almost 5 times higher for crab and dessert apple seed oils, respectively, in comparison with the results obtained by Fromm et al. (2012). Moreover, apple seeds in present study were dried conventionally by dry air, while in a study of Fromm et al. (2012) it was by freeze drying to protect against loss of bio-components during drying.

The obtained results showed a great diversity of tocopherol contents and composition in oils recovered from seeds of various apple cultivars. In the current study, the effects of the environment as well as harvest year can be omitted because all analysed apple samples were harvested in the year 2012 at the same location. As it was reported by Beardsell, Francis, and Ridley (2002), the composition and contents of tocochromanols in the plant material are mostly influenced by its origin and genotype. A crucial role is also played by climate and soil, in which the plant is grown, and the year of harvest (Alasalvar et al., 2003). In a study by Dolde, Vlahakis, and Hazebrock (1999) it was shown that the tocopherol composition in oilseeds (e.g. sunflower, soybean or canola) is highly dependent on environmental conditions, particularly the temperature, and much less on the genotype. This finding was in contrast to results obtained in apple seed oils, where a great variation was observed among cultivars, especially in the case of dessert apples (Table 2).

The calculated weight ratios of four tocopherols (α-T:β-T:γ-T:δ-T, average) in crab and dessert apple seed oils were 4.1:2.7:1.6:1 and 2.6:2.5:1.1:1, respectively. The ratio between homologous α and β observed by Fromm et al. (2012) in seed oils recovered from

Table 2
Content of tocopherols in dessert and crab apple seed oils mg/100 g oil.

Sample	Tocopherol content (mg/100 g oil)				
	α-T	β-T	γ-T	δ-T	Total T
Crab apple seed oils					
K-8/9-24	68.51 ± 0.79^{c}	27.92 ± 0.18^{a}	27.11 ± 0.31^{f}	7.01 ± 0.14^{c}	130.55 ± 1.42^{a}
Kerr	71.26 ± 0.86^{d}	53.83 ± 0.58^{d}	19.61 ± 0.32^{d}	13.94 ± 0.20^{d}	158.64 ± 1.96^{c}
Kuku	65.18 ± 0.82^{b}	52.34 ± 0.65^{d}	16.71 ± 0.35^{c}	14.57 ± 0.07^{d,e}	148.80 ± 1.89^{b}
Quaker Beauty	88.70 ± 1.05^{f}	37.35 ± 1.06^{b}	35.16 ± 0.29^{g}	14.95 ± 0.13^{e}	176.16 ± 2.53^{d}
Riku	79.49 ± 0.78^{e}	69.49 ± 0.56^{e}	26.97 ± 0.21^{f}	26.59 ± 0.28^{h}	202.54 ± 1.83^{f}
Ritika	51.40 ± 0.58^{a}	37.54 ± 0.44^{b}	37.03 ± 0.26^{h}	24.83 ± 0.21^{g}	150.80 ± 1.49^{b}
Ruti	67.99 ± 0.63^{c}	47.71 ± 0.53^{c}	21.45 ± 0.19^{e}	17.03 ± 0.17^{f}	154.18 ± 1.52^{b,c}
Average	70.36 ± 11.66	46.60 ± 13.71	26.29 ± 7.69	16.99 ± 6.74	160.24 ± 23.03
Dessert apple seed oils					
Antej	91.41 ± 0.91^{f}	80.55 ± 0.61^{f}	72.46 ± 0.60^{j}	79.03 ± 0.57^{k}	323.45 ± 2.69^{i}
Beforest	114.55 ± 1.02^{i}	112.84 ± 0.69^{h}	78.69 ± 0.47^{k}	73.00 ± 0.44^{j}	379.08 ± 2.62^{j}
Kent	98.35 ± 0.93^{g}	124.28 ± 0.87^{i}	2.37 ± 0.11^{a}	0.67 ± 0.04^{a}	225.67 ± 1.95^{g}
Sinap Orlovskij	105.34 ± 1.17^{h}	70.69 ± 0.61^{e}	9.22 ± 0.20^{b}	5.80 ± 0.16^{b}	191.05 ± 2.14^{e}
Zarja Alatau	105.90 ± 1.31^{h}	99.50 ± 0.91^{g}	41.45 ± 0.61^{i}	36.50 ± 0.55^{i}	283.35 ± 3.38^{h}
Average	103.11 ± 8.71	97.57 ± 22.15	40.84 ± 35.05	39.00 ± 36.52	280.52 ± 75.14

Different letters in the same column indicate statistically significant differences at $p < 0.05$. T, tocopherol.

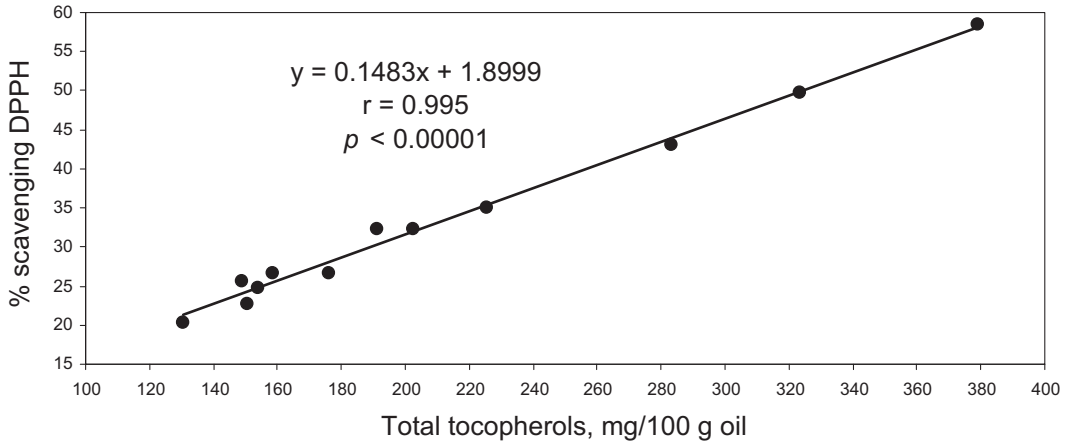

Fig. 3. Correlation between total tocopherol content (mg/100 g oil) and the radical-scavenging activity (%) of tested apple seed oils.

dessert and cider apple cultivars was approximately 1:1. In the current study the same weight ratio between α-T and β-T, as well as γ-T and δ-T was confirmed; however, this phenomenon was observed only in dessert apple seed oils. For crab apple seed oils the calculated weight ratios between α-T and β-T as well as γ-T and δ-T were approximately 1.5:1. The phenomena described above had some exceptions, especially for cultivars 'Kent' and 'Sinap Orlovskij'. The weight ratios between all tocopherol homologues in the near future could be useful tools as markers for identification of apple seed oil origin.

Very high levels of tocopherols in seed oils recovered from apples were unusual among plant oils. In terms of the total content of tocopherols, in comparison with commercial popular oils such as rapeseed, sunflower, olive, flaxseed or soybean (624.6; 634.4; 216.8; 588.5 and 1797.6 mg/kg, respectively) as well as less common oil sources such as grapeseed or pumpkin (142.6 and 508.1 mg/kg, respectively) (Tuberoso, Kowalczyk, Sarritzu, & Cabras, 2007), or unconventional plants such as Japanese quince (742.6 mg/kg) (Górnaś, Siger, & Segliņa, 2013; Górnaś, Siger, Juhņeviča, et al., 2014), oils obtained from apple seeds contained the largest amount of tocopherols. Therefore, apple seeds are a very promising source of oil recovered from fruit industry by-products and it could be included in our daily diet.

The DPPH assay is one of the oldest methods used to determine the antioxidant capacity of study samples, consisting in the determination of scavenging capacity of DPPH free radicals by antioxidants present in the analysed sample. Moreover, to date this method has had many modifications and improvements (Abderrahim, Arribas, Gonzalez, & Condezo-Hoyos, 2013). In the current study the proposed improvement of the DPPH assay for oil samples is designed to use solvents with a lower boiling point, toxicity and higher environmental friendliness. Traditionally, the method is based on dissolving of DPPH radicals and samples in methanol. However, oil is not soluble in methanol, therefore previously Rossi, Alamprese, and Ratti (2007) applied ethyl acetate. In the present study 2-propanol was selected due to the higher boiling point in comparison with ethyl acetate. For dissolving of DPPH radicals, instead of methanol (traditional) or ethyl acetate (for oil samples) ethanol was used. The 2-propanol and ethanol solvents have lower toxicity and are more environment-friendly when compared to methanol and ethyl acetate. The introduced modification contributed to a greater reproducibility of measurements (data not shown). The results of scavenging capacity assay with the use of DPPH radicals demonstrated a significance correlation ($r = 0.995$, $p < 0.00001$) between the total content of tocopherols in apple seed oils and scavenging of the DPPH radicals (Fig. 3). Such a high dependence indicates total domination of tocopherols as antioxidants in apple seed oils, despite the fact that the apple seed oils contain also carotenoids, as it was reported by Fromm et al. (2012). A significant correlation between the total content of

tocochromanols in refined vegetable oils and radical-scavenging capacity of DPPH radicals ($r = 0.941$) was found also by Rossi et al. (2007).

4. Conclusions

The present study shows that seed oils recovered from apples cultivated in Eastern Europe are very rich sources of tocopherols, mainly α-T and β-T. Nevertheless, high variation of tocopherol concentrations may be expected in seed oils obtained from different varieties of apples, especially for γ-T and δ-T. The seed oils recovered from crab apples when compared to dessert apples, seem to be more stable in terms of the expected content of individual tocopherol homologues; however, at lower concentrations. Apple seed oils, due to their high content of tocopherols and in view of an increasing customers' interest in natural products rich in health-promoting ingredients, could be utilized as promising unconventional and at the same time natural sources of vitamin E. Moreover, appropriate management of apple seeds generated by the fruit industry may ensure significant economic and environmental benefits.

The implemented rapid and simple method, with excellent repeatability and reproducibility, for separation of all tocopherol homologues (α, β, γ, and δ) in apple seed oils with the use of RP-HPLC/FLD and the PFP column seems to be a powerful tool which could be used to improve the existing methods for the first screening of various oils to verify their authenticity using tocopherols as markers.

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