THE PROFILE OF POLYPHENOLS AND ANTIOXIDANT PROPERTIES OF SELECTED APPLE CULTIVARS GROWN IN POLAND

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A B S T R A C T

The aim of the study was to determine the polyphenol profile and antioxidant activity of eleven apple cultivars harvested from an orchard in Garlica Murowana (Poland). 'Antonowka', 'Red Boskoop', and 'Winter Goldparmine' had the highest antioxidant properties of all the cultivars studied. The profile of polyphenols was different in individual cultivars. In all the studied fruits, though, it was shown that (-)epicatechin, procyanidin B2, and chlorogenic acid essentially outnumbered other compounds quantitatively. The apple cultivars recommended for manufacturing apple preserves are presented.

Key words: apple cultivars, antioxidant capacity, polyphenols

INTRODUCTION

Apples, together with oranges, bananas, grapefruits and grapes, are the top five consumed fruits in the world. In 2008, the annual apple harvest in Poland was estimated at about 2.83 million tonnes, and they constituted about ¾ of the total fruit production. Apples can be consumed fresh, but they are also a valuable raw material for processing into apple juice, concentrates, canned, frozen, dried, and stewed fruits, jellies, purée and cider. The percentage of apples used for processing increased in Poland from 50% in the 1980's to about 70% in the 1990's, and to 80% at the beginning of the new century (Brzozowski, 2009). Poland is one of the world leaders in production and export of apple juice concentrate.

Many phytochemicals exert a positive impact on human health. Polyphenols are secondary metabolites of plants. Phenolic acids and flavonoids make up the most important and numerous classes of polyphenols. Their abundant quantities were identified in, among others, green tea, soya beans, as well as in grapes and other fruits (Manach et al., 2004). Apples contain many flavonoids and phenolic acids. Owing to their widespread availability and relatively low price, apples are a key element in our everyday diet. As for Poland, they are the most frequently eaten fruits available throughout the whole year, and eaten in different forms (fresh fruits, dried apples, purée and pastes, juices, ciders, wines, and compotes). In the Polish diet, apples constitute one of the basic sources of antioxidants.

Fruits and vegetables are rich in polyphenols and other antioxidants. However, a range of technological and culinary processing procedures including boiling, pasteurization, or even just peeling and trimming, devoid them, to a large degree, of their most valuable pro-healthy components (Ewald et al., 1999; Aherne and O'Brien, 2002; van der Sluis et al., 2002). It should be highlighted, that the effect of processing on polyphenols depends on the kind of fruit and technological parameters of processing (Nicoli et al., 1999; Duda-Chodak et al., 2009; Tarko et al., 2009a).

Epidemiological studies have linked the consumption of apples with reduced risk of some cancers, cardiovascular disease, asthma, and diabetes. *In vitro* studies show that apples have strong antioxidant activity, inhibit cancer cell proliferation, decrease lipid oxidation, and lower cholesterol (Boyer and Liu, 2004).

Polyphenols are also involved in the quality characteristics of fresh fruits and its processed products. They influence texture, color (mainly anthocyanins) and taste, e.g. bitterness and astringency (Awad et al., 2001) as well as the susceptibility to darkening. Hence, some apple cultivars are more suitable for industrial processing while others should be consumed fresh.

Apple fruits contain strong antioxidants such as catechin, chlorogenic acid, phloridzin, anthocyanins, and quercetin (Podsędek et al., 2000; Boyer and Liu, 2004). The concentration of individual phenolic compounds depends on the species and cultivar of a fruit (Robards et al., 1999; Podsędek et al., 2000; Markowski and Płocharski, 2006). However, it is difficult to compare values obtained from different studies performed in different conditions. It is because the polyphenol concentration depends also on the fruit ripening degree, vegetation season, cultivation methods, soil and climatic conditions, and insolation degree (Kondo et al., 2002; McGhie et al., 2005; Podsędek et al., 2000; van der Sluis et al., 2001). Storage time and conditions (van der Sluis et al., 2003) as well as fruit processing parameters (Markowski and Płocharski, 2006; Satora et al., 2008) influence the level of antioxidants in the final foodstuff.

The aim of the study was to determine the antioxidant activity and to analyze the composition of selected polyphenols in several apple varieties cultivated in Poland. The studied apple varieties are those that are commonly available to the food processing industry and to the retail trade. All apples originated from the same orchard and were harvested the same year. Therefore, the impact of other factors on the analyzed parameters can be excluded (e.g. soil and climatic condition, insolation, cultivation methods and vegetation season).

MATERIAL AND METHODS

Chemicals. Diammonium salt of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic) acid (ABTS diammonium salt); 2,2'-diphenyl-1-picrylhydrazyl (DPPH); (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox); a phosphate buffer saline (PBS): 0.01 M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride; pH 7.4 at a temperature of 25 °C; and the enzymes: β-glucosidase, β-xylosidase, β-galactosidase and β-hesperidase, as well as HPLC standards, were purchased from the SIGMA-Aldrich Company (Steinheim, Germany). Potassium iodide, potassium persulfate ($K_2S_2O_8$) and methanol (analytical grade) were obtained from the POCh Company, and a 96% ethanol from the Chem-Pur Company.

Investigated materials. Eleven local apple cultivars originated from an experimental orchard, run by the University of Agriculture in Krakow, in Garlica Murowana near Krakow were used in the investigation. There were: 'Antonowka', 'Gala Mast', 'Gloster', 'Golden Delicious', 'Idared', 'Jonagold', 'Cox's Orange Pippin', 'Ligol', 'Red Boskoop', 'Šampion', and 'Winter Goldparmine'. The Starch Index method was used to determine the harvest maturity. The representative sample consisted of 8 ripe fruits of each cultivar picked from the central axes of four different trees. Seed cores were cut out and then the apples were cut into fine pieces and lyophilized. They were stored at -20 °C until analysis.

Starch Index. Each fruit was cut in half, and the cut surface was immersed in a test solution (10 g of potassium iodide and 2.5 g of iodine crystals dissolved in 1 l of water). Using a starch index chart (Cowgill et al., 2007), a starch index value (1-9) was assigned to each fruit, where 1 = total surface stained and 9 = no stain. Apples with scores of 4, 5, and 6 were considered mature (of harvest maturity). Fruits measuring 7 or above were of consumption maturity and to be marketed immediately.

Methanol extraction. A portion of the lyophilized sample was placed in a container of the laboratory mill (WŻ-1, Poland) and ground (2 × 12 seconds). An amount of 25 ml methanol was poured over 0.500 g of ground lyophilisate and mixed for 2 h by magnetic stirrer at 500 rpm (Wigo, Poland). The whole mixture was centrifuged (MPW–350R, Poland) for 10 minutes (1467 × g,

20 °C), and the supernatants were collected into capped test-tubes. The methanol extracts were then stored in a freezer (-20 °C) until the analysis.

Assessment of the antioxidant activity

ABTS assay. The antioxidant activity was assayed following the protocol presented in our previous study (Tarko et al., 2009a). Briefly, the $ABTS^{+}$ radical was generated during a chemical reaction between the 7 mM aqueous solution of diammonium salt of the 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic)-acid and the 2.45 mM potassium persulfate. The solution was kept at room temperature and in darkness throughout the night in order to complete the reaction, and to stabilize the ABTS cation-radical. To investigate extracts, a concentrated solution of the radical was diluted by a phosphate buffer (PBS), pH 7.4, to obtain a final absorbance A = 0.70 ±0.02 ($ABTS_{0.7}$) at 734 nm.

An amount of 100 µl of the 15-fold diluted sample investigated or of the synthetic vitamin E (Trolox) solutions (their concentration rates ranging from 0 to 10 mg/100 ml) was added to 1 ml $ABTS_{0.7}$. The absorbance was then measured in the 6^{th} minute when mixing was completed. The antioxidant capacity of the extracts under study was calculated using a standard curve drawn up for solutions of Trolox and expressed as mg of Trolox equivalents per 100 g of fresh weight. All determinations were performed in triplicate.

DPPH assay. In the determination procedure using the DPPH method, antioxidants present in the sample investigated, reduced stable DPPH nitrogen radical, thus decreasing absorbance measured at a wavelength of 515 nm. The scavenging capacity of the DPPH radical was assessed on the basis of method described by Schlesier et al. (2002). 200 µl of the extract analyzed (diluted 10-fold with a re-distilled water) or Trolox solutions (their concentrations ranging from 0 to 2.5 mg/100 ml) was added to 800 µl of a 225 µM ethanolic solution of DPPH and, then, the rate of absorbance disappearance was measured in the 10^{th} minute after mixing of reagents in a cuvette. The antioxidant capacity of methanol extracts was calculated using a standard curve developed for Trolox, and expressed as mg of Trolox equivalents/100 g of fresh weight. All determinations were performed in triplicate.

Qualitative and quantitative analysis of polyphenols. The polyphenols in the apples investigated were determined by the HPLC method described earlier (Tarko et al., 2009b). 2 g of apple lyophilisate was extracted three times with 80% aqueous solution of methanol in order to obtain 50 ml of extract (ultrasonic bath, 40 kHz, BAS-10, BAS, Warsaw, Poland, 15 minutes). The extracts were then filtered through a Schott G4 funnel and centrifuged (10 minutes, 14,000 rpm).

The extracts were analysed by high performance liquid chromatography (HPLC), on a Merck-Hitachi

L-7455 apparatus with a diode array detector (DAD). Separation was performed on a Synergi Fusion RP-80A 150×4.6 mm (4 μm) Phenomenex column (Torrance, CA, USA) thermostated at 30 °C. The mobile phase consisted of 2.5% acetic acid (solution A) and acetonitrile (solution B), applied in a gradient changing linearly from 0% B to 25% B during 36 minutes. The column was then washed with the pure solution A. The flow of the liquid phase was 1 ml/min, and the detection was conducted at four wavelengths: 280 nm (flavanols), 320 nm (phenolic acids), 360 nm (flavonols) and 520 nm (anthocyanins).

In order to identify the compounds, retention times of the compounds under analysis and standard compounds were compared. In addition, enzymatic hydrolysis of flavonol glycosides and cyanidin glycosides in a citrate buffer solution (citric acid and sodium citrate, pH 5) was performed for identification. The disappearance of single peaks in the chromatogram and formation of the corresponding aglycones was observed using HPLC after 1-hour incubation at 38 °C with a specific enzyme: β-glucosidase, β-xylosidase, β-galactosidase and β-hesperidase. The calibration curves were made from (–)epicatechin, (+)catechin, phloridzin, chlorogenic acid, isoquercitrin, and cyanidin-3-glucoside as standards. Procyanidin B2, C1, and B1 used as standards were obtained by the method of Oszmiański and Bourzeix (1995). For those analytes where no standard was available, standards of the same family were used; thus quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-arabinoside, quercetin-3-xyloside and quercetin-3-rhamnoside were quantified as quercetin-3-glucoside (isoquercitrin); phloretin-2-glucoside as phloridzin; *p*-coumaric derivative as *p*-coumaric acid; and caffeic acid derivatives as chlorogenic acid. Results were expressed as mg/100 g of fresh weight. The assays were performed in duplicate. The standard error of the method was determined in preliminary determinations and was below 10%.

Statistical analysis. The results were shown as an arithmetic mean (± standard deviation) of three independent determinations. A single-factor Analysis of Variance test (ANOVA) with a *post hoc* Tukey test was applied to perform a statistical analysis. A Kolmogorov-Smirnov test was applied to examine the normality of distribution. The correlation was evaluated by Pearson analysis. Differences were considered to be signiffi-cant at $p < 0.05$.

RESULTS

There was a considerable diversity of the capacity to scavenge free radicals depending on the cultivar studied (Fig. 1). 'Antonowka', 'Red Boskoop', and 'Winter Goldparmine had the highest antioxidant properties of all the analyzed cultivars. The results of antioxidant activity assays obtained with the use of $ABTS^{\cdot+}$ radical were on the average five times higher compared to the results of assays performed using a DPPH radical (266-593 comparing 53-105 mg Trolox equivalents/100 g of fresh weight).

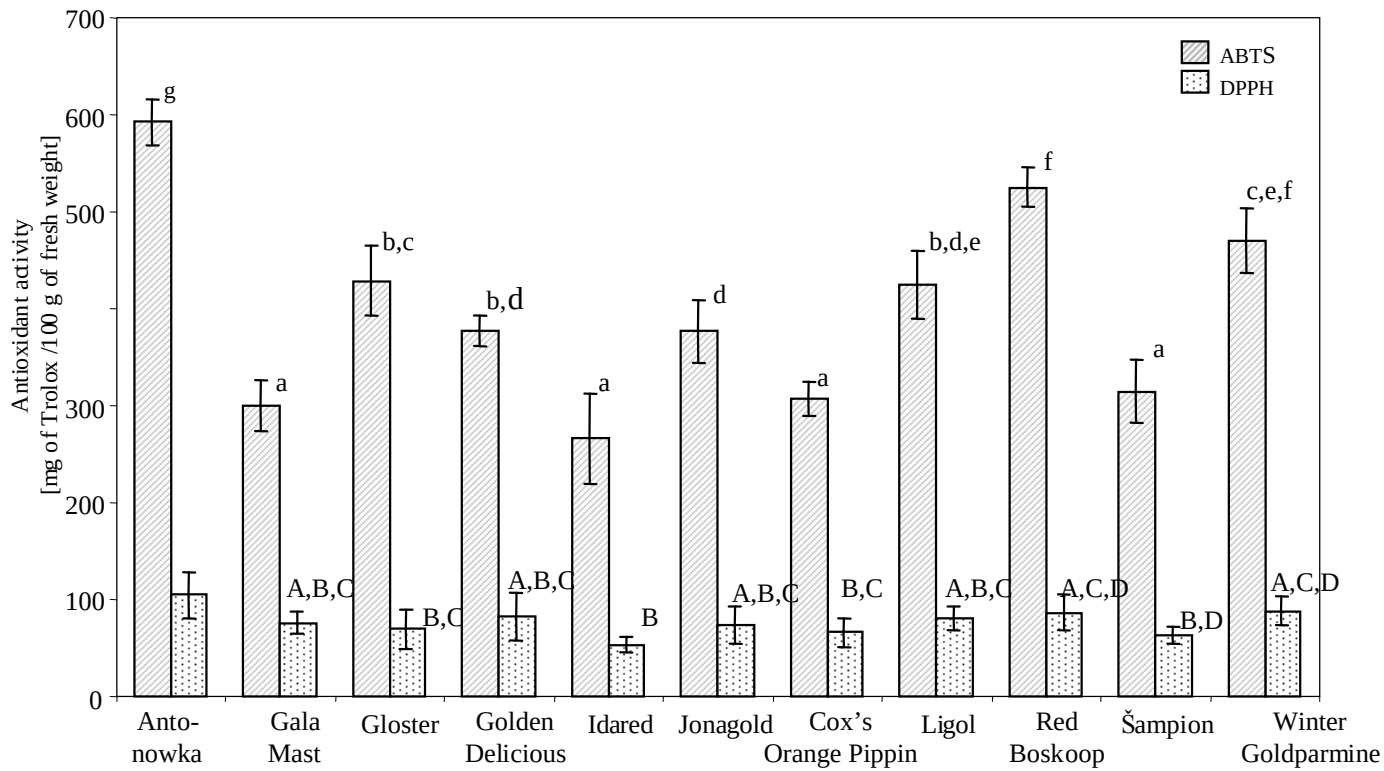

Figure 1. Antioxidant activity of the selected apple cultivars (mean value ± SD, n = 3)

Table 1. Content of polyphenol compounds in the selected apple cultivars (mg/100 g of fresh weight)

		Anto-nowka	Gala Mast	Gloster	Golden Delicious	Idared	Jonagold	Cox's Orange Pippin	Ligol	Red Boskoop	Šampion	Winter Gold-parmine
Chlorogenic acid		39.61	8.82	8.76	9.79	12.37	8.75	3.05	25.85	25.53	2.74	17.59
p-Coumaryl-quinic acid		3.11	0.79	0.68	0.91	0.55	0.47	0.73	0.57	0.96	0.74	0.91
(+)Catechin		1.30	0.13	1.22	0.49	1.32	0.11	0.24	0.95	0.88	1.72	2.44
(-)Epicatechin		12.94	5.48	5.23	5.08	2.91	5.80	8.45	9.04	10.36	10.93	6.18
Procyanidin B2		8.02	4.31	6.12	6.49	3.89	4.49	6.73	11.19	12.50	5.32	6.87
Procyanidin B1		1.58	1.24	0.46	0.52	0.64	0.47	1.45	0.57	1.45	1.35	1.91
Procyanidin C1		2.82	2.49	2.49	2.67	1.26	2.61	2.97	5.43	5.04	2.47	2.65
Phloretin xyloglucoside		1.23	1.43	0.67	1.09	0.11	1.57	1.03	1.56	1.56	0.65	6.10
Phloridzin		2.09	0.94	2.08	2.27	2.05	1.77	1.00	1.32	4.96	1.01	5.75
Quercetin glycosides	rutinoside	0.19	0.10	0.23	0.18	0.05	0.02	0.05	0.30	0.01	0.00	0.07
	galactoside	3.24	1.01	3.38	3.61	1.50	2.08	1.23	1.40	0.31	1.88	1.92
	glucoside	0.44	0.47	0.50	0.67	0.17	0.28	0.20	0.40	0.06	0.39	1.09
	xyloside	0.75	0.60	1.29	0.95	0.62	0.90	0.29	0.49	0.26	0.54	0.66
	fructorhamnoside	1.96	1.14	NA	NA	NA	2.13	0.85	NA	NA	1.31	NA
	rhamnoside	0.79	0.61	0.99	1.41	0.57	1.89	0.27	2.38	0.26	0.74	0.24
	arabinoside	NA	NA	2.57	1.96	1.58	NA	NA	0.84	0.39	NA	1.21
Cyanidin-3-galactoside		NA	NA	0.85	0.00	0.38	NA	NA	0.54	0.11	NA	0.07
Total		**80.07**	**33.00**	**37.52**	**38.09**	**29.98**	**33.32**	**28.53**	**62.80**	**64.65**	**31.78**	**55.66**

NA – a non-assayed compound

The maximum error of the measurements was 10%

The HPLC analysis demonstrated that the following apple cultivars contained the highest amounts of polyphenols: 'Antonowka', 'Ligol', 'Red Boskoop' and 'Winter Goldparmine'. The profile of polyphenol compounds varied in individual cultivars, still, a feature shared by all the fruits investigated was a significant quantitative domination of the three following substances: chlorogenic acid, (-)epicatechin, and procyanidin B2 (Tab. 1). Moreover, the high content of polyphenols was positively correlated with the antioxidant activity (ABTS *vs*. polyphenols content: R^2 = 0.90; DPPH *vs*. total polyphenols content: R^2 = 0.80). When particular phenolic compounds were analyzed the highest correlation was observed between chlorogenic acid level and antioxidant capacity measured by ABTS assay (R^2 = 0.95). Flavanol level also correlated with antioxidant activity (R^2 = 0.85 both in ABTS and DPPH method).

DISCUSSION

In the case of the present study, it was possible to eliminate the impact of soil, method of fertilizing, and climatic conditions on apples because all the fruits selected for the research were grown exclusively in one orchard. Additionally, it was possible to reduce the influence of exposure to the sun because fruit samples were collected from 4 different trees and from different tree branches, both exposed to sun and hidden in the shadow. Based on the results achieved, it can be supposed that the antioxidant activity of apples depends, to a large extent, on the apple cultivar. Furthermore, it should be emphasized that the antioxidant activity rates as achieved in the experiments with an $ABTS^{\cdot+}$ radical were on the average five times higher than the respective rates obtained with a DPPH radical (Fig. 1). This is attributed to the dissimilar nature of the two radicals applied, since they enable the determination of hydrophobic antioxidant substances only (as in the case of DPPH) or of hydrophilic and hydrophobic (as in the case of $ABTS^{\cdot+}$).

Among the apple cultivars examined, the highest antioxidant activity was found in 'Antonowka', followed by 'Red Boskoop', 'Winter Goldparmine', and 'Ligol'. Those cultivars are highly suitable for direct consumption owing to their excellent pro-health features. Their industrial utilization, in particular the production of light coloured juices, is limited because their flesh, and juices made from them, show a tendency to quickly darken (own observations). Addition of ascorbic acid can prevent this undesirable process. The results are confirmed by high polyphenol content. All four cultivars had high level of chlorogenic acid (33-53% of total polyphenols). Additionally, 'Red Boskoop' and 'Ligol' were characterized by a particularly high level of flavanols (47 and 43% of total polyphenols, respectively), mainly (-)epicatechin and procyanidin B2 (Tab. 1).

Procyanidins show a high capacity for free radical scavenging (Lotito

and Frei, 2004). Lu and Foo (2000) accomplished research aimed at the assessment of the capacity to scavenge DPPH radical. In their study, they segregated polyphenols in the following order: quercetin glycosides > procyanidins > chlorogenic acid > phloridzin. They also demonstrated that procyanidins (especially trimers) were up to 30 times stronger antioxidants, than vitamins C and E. So, the presence of procyanidins in apples seems to be desirable. However, procyanidins associate with proteins and polysaccharides in cell walls; this fact is considered to be the chief reason why apple juices become turbid and show settlings when stored (Renard et al., 2001). On the other hand, the chlorogenic acid in apples is oxidized by a polyphenol oxidase to its o-quinone (Muñoz et al., 2007), and the latter one reacts with other polyphenols and forms yellow and brown dyes which induce the undesirable darkening process of apple preserves.

The apple cultivars 'Idared', 'Gala Mast' and 'Cox's Orange Pippin' showed the lowest capacity to neutralize free radicals. The flesh of these apple cultivars darkened slowly or did not darken at all. Thus, apples of these cultivars could be recommended for manufacturing apple preserves, provided that the other technological parameters are appropriate. It was also proved that those apple cultivars contained a low amount of procyanidins and chlorogenic acid (Tab. 1). The browning rate of apples is a very important factor in the fruit industry and the browning tendencies certainly differ between particular apples cultivars (Kuczyński, 1995; Kuczyński, 2006).

During the production of clear apple juices many components of high antioxidant potential are lost (Markowski and Płocharski, 2006). Procyanidins are removed during the clarification process of clear apple juices. Moreover, (-)epicatechin and procyanidins are oxidized and form high molecular polymers that are absorbed by gelatine and also removed from juice during clarification. The quercetin derivatives are present mainly in the peel. These derivatives are taken away with pomace after juice pressing. In cloudy juices there is no clarifying stage, so the dark products of oxidation are not removed from the juice. Therefore, production should be conducted in conditions that prevent oxidation and darkening of polyphenols. The main way of preventing these phenomena is by obtaining cloudy juice in a neutral gas atmosphere (nitrogen) and adding ascorbic acid to the apple pulp.

The content of polyphenol compounds in apples has been previously analyzed by, among other scientists, Boyer and Liu (2004), Podsędek et al. (2000), and Robards et al. (1999). The results achieved by them point out catechin, epicatechin, chlorogenic acid and procyanidins to be the key polyphenols in apples. Yet, in the apple cultivars investigated in this study, the catechin did not play any essential role. In the majority of the examined cultivars the (-)epicatechin and its dimmers (pro-

cyanidins) play a crucial role and constitute from 36% (in the 'Winter Goldparmine' cultivar) to up to 69% of the total polyphenols analyzed ('Šampion'). Only in the 'Antonowka' and 'Idared' cultivars, phenolic acids (mainly chlorogenic acid) dominate among polyphenol compounds (53 and 43%, respectively). In other cultivars, the phenolic acids are the second subclass of polyphenols and constitute between 10 and 42%. Flavonols, represented by quercetin glycosides, are an important part in polyphenols from 'Gloster' (23%), 'Golden Delicious' (23%), 'Jonagold' (22%), and 'Šampion' (15%). Analysis of different cultivars from various geographical areas can be the main cause of differences between the results.

CONCLUSIONS

On the basis of the apple cultivars examined, the research confirmed a significant diversity in the polyphenol contents and in the antioxidant activity related to them. The polyphenol profile is characteristic for the cultivar and should be taken into account when an apple cultivar is selected for processing. The same apple phytochemicals can be both desirable and undesirable; it all depends on the desired final product. Not only technological parameters should be taken into account. The pro-health properties of particular cultivars are also important. Some of those valuable compounds are lost in the production process, but they can be recovered from waste. For example, apple pomace left after clear apple juice production is a rich source of valuable antioxidants: phloridzin, chlorogenic acid, epicatechin, quercetin glucosides, and procyanidins (Lu and Foo, 2000). All of the above could be recovered and added back to clear apple juice to improve its nutritional value.

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PROFIL POLIFENOLI I WŁAŚCIWOŚCI ANTYOKSYDACYJNE WYBRANYCH ODMIAN JABŁEK UPRAWIANYCH W POLSCE

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STRESZCZENIE

Celem badań było określenie profilu związków fenolowych i właściwości przeciwutleniających jabłek w zależności od odmiany. Do doświadczeń wybrano jedenaście odmian jabłek uprawianych w Polsce, które zostały zebrane w jednym sadzie (Garlica Murowana koło Krakowa). Największą aktywność antyoksydacyjną wśród badanych odmian wykazywały owoce odmian 'Antonówka', 'Red Boskoop' oraz 'Złota Reneta'. Profil związków polifenolowych różnił się w poszczególnych odmianach, jednak cechą wspólną wszystkich badanych odmian była znacząca przewaga ilościowa 3 substancji: (-)epikatechiny, procyjanidyny B2 oraz kwasu chlorogenowego. W pracy zasugerowano odmiany rekomendowane do przetwórstwa.

Słowa kluczowe: odmiany jabłek, właściwości antyoksydacyjne, polifenole