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Total phenolics, flavonoids and antioxidant activity of different apple cultivars

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The concentration of polyphenolic compounds and the antioxidant activity in apples seem to differ with cultivar, maturity storage, environmental conditions and part of the fruit. In this work, the total phenolic and flavonoid content and the antioxidant activity were measured in the whole fruit from 15 apple cultivars cultivated in Serbia. Total phenolic content (TP) was assayed by Folin-Ciocalteu method, flavonoid (TF) by colorimetric method with $AlCl_3$. Total antioxidant activity (TAA) of selected apples was determined using 2,2-azinobis(3-ethylbanzothiazoline-6-sulfonic acid) radical cation (ABTS), 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH), ferric ion reducing power (FRP) and ferric ion reducing antioxidant power (FRAP). Polyphenolic content for the whole apple was in the range of 72.80 – 217.37 mg GAE/100 g fresh weight. The apple extracts had different TAA in relation to the method applied, and the different TAA of apples can be ascribed to their TP and TF content. A ripping correlation between TP and TAA was observed using FRAP method.

Keywords: phenolics; flavonoids; antioxidant capacity; apple.

INTRODUCTION

There is strong evidence that free radicals are responsible for the damage of lipids, proteins and nucleic acids in cells [1] leading to several physiological and pathological abnormalities, such as inflammation, cardiovascular diseases and ageing. Recent studies indicate that frequent consumption of fruits is associated with lower risk of stroke and cancer [2,3]. This protective effect is related to the content of plant antioxidant microconstituents. Different fruits exhibit different antioxidant capacities according to their contents of polyphenols, vitamins C, E, carotenoids and flavonoids [4,5].

Apple consumption has been associated with reduced risk of degenerative diseases, such as cancer and cardiovascular diseases [6,7]. The association is often attributed to the polyphenolic antioxidants contained in apples which can protect the human body against oxidative stress by scavenging oxygen free radicals [8]. Apples also contain ascorbic acid but it can explain less then 0.4% of the antioxidant activity, indicating that other factors, such as phenolics, are the main contributors [9]. Many studies show that the concentration of phenolic compounds, such as flavanols and anthocyanins in apple differ with cultivar, maturity stage, environmental conditions and part of the fruits [10].

Several characterization studies of different apple parts in cultivars grown in the United States [11], Italy [10], Poland [12], Brazil [8] and Czech Republic [13] have been carried out on the basis of their phenolic profiles. However, little attention has been given to apple cultivars grown in Serbia. Therefore, the objective of this study was to determine the total phenolic content, the flavonoid content and the antioxidant capacity in the whole fruit from 15 apple cultivars grown in Serbia.

EXPERIMENTAL PROCEDURES

MATERIALS

Standards and reagents

All chemicals used were analytical reagent grade from well-reputed companies. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), iron(II) sulphate, 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 2,2'-diphenyl-1-picrylhydrazil (DPPH), Folin-Ciocalteau's reagent, gallic acid, catechin were obtained from Fluka (UK).

Raw materials

Fifteen apple cultivars: Jonathan, Gloster, Melrose, Red Delicious, Golden Delicious, Granny Smith, Sharunka, Gala, Jonagold, Idared, Braeburn, Mutsu, Chadel, Kozara and Red Chief were picked

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at commercial maturity during the 2010 harvest season in southern Serbia, and stored at -20˚C. Prior to analysis apple was thawed at room temperature, pitted and mixed in a house blender.

Extraction of Phenolics

The phenolics were extracted by using an ultrasound-assisted method [14]. Briefly, phenolics were extracted from 20 g sample using 100 ml of methanol. The mixture was sonicated for 20 min with a continual stream of nitrogen gas purging to prevent possible degeneration of phenolics, filtered through Whatman No. 2 filter paper using chilled Buchner funnel, and rinsed with 50 ml of 100% methanol. Extraction of the residue was repeated under the same conditions. The two filtrates were combined and transferred into 1l evaporating flask with additional 50 ml of 80% aqueous methanol. The solvent was evaporated in a rotary evaporator at 40˚C. The remaining phenolic concentrate was first dissolved in 50 ml of 100% methanol and was diluted to final volume of 100 ml with methanol.

Total Phenolic Content (TP)

Total phenolic content (TP) of the apple extracts was determined using Folin-Ciocalteau (FC) assay described by Singleton and Rossi [15]. Apple extracts (0.15 ml) were mixed with 0.5 ml of FC reagent. After standing for 5 min at room temperature 2.0 ml of (20% w/v) sodium carbonate solution were added and deionized water was added to a final volume of 10.0 ml. The solutions were mixed and allowed to stand for 1 h at room temperature. Then, the absorbance was measured at 760 nm, using a UV-visible spectrophotometer (Agilent 8453). A calibration curve was prepared, using a standard solution of gallic acid (20, 40, 60, 80 and 100 mg/l, r=0.99978). Results were expressed on fresh weight basis (fw) as mg gallic acid equivalents per 100g of sample.

Total Flavonoid Content (TF)

The total flavonoid content of apple methanol extracts was determined by a colorimetric method [16]. A suitable volume of sample was mixed with 2 ml of distilled water and subsequently with 0.3 ml of a $NaNO_2$ solution (5%). After 5 min, 3 ml of $AlCl_3$ solution (1%) was added, and the solution was allowed to stand for 5 min at room temperature. Then, 2 ml of NaOH solution (1M) was added to the mixture and water was added to a final volume of 10 ml. The mixture was thoroughly mixed and absorbance was immediately measured at 510 nm *versus* water blank. Results were expressed on a fresh weight basis as mg catechin equivalents (CE) per 100 g of sample.

ABTS Radical-Scavenging Capacity Assay

The trolox equivalent capacity test developed by Lee et al. [15] was used in this study. In brief, the ABTS radical cation ($ABTS^{\cdot+}$) solution was prepared by the reaction of 7 mM ABTS and 2.45 mM potassium persulphate, after incubation at 23°C in the dark for 12–16 h. The $ABTS^{\cdot+}$ solution was then diluted with 80% ethanol to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Solution of $ABTS^{\cdot+}$ (3.9 ml; absorbance of 0.700 ± 0.005) was added to 0.1 ml of the test sample and mixed thoroughly. The reaction mixture was allowed to stand at 23ºC for 6 min and the absorbance was immediately measured at 734 nm. The samples were diluted with 80% ethanol so as to give 20–80% reduction of the blank absorbance with 0.1 ml of sample. Reagent blank reading was taken using 0.1 ml of 80% ethanol. Standard curve was obtained by using trolox standard solution at various concentrations (ranging from 2 to 10 μM, r=0,9985) in 80% ethanol. Total antioxidant activity of apples was expressed as mmol trolox equivalents (TE) per 100 g fresh weight [17]. The experiment was carried out in triplicate.

DPPH Free Radical-Scavenging Assay

The antioxidant capacity of the apple extracts was also studied through evaluation of the free radical-scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The determination was based on the method proposed by Miliauskas *et al.* [15] and De Arcos et al. [18]. An aliquot (0.1 ml) of apple extract was mixed with 2.5 ml of 100 mM DPPH methanol solution. The mixture was thoroughly vortex-mixed and kept in the dark for 30 min and the decrease in absorbance was measured at 515 nm. A blank sample containing the same amount of methanol and DPPH solution was prepared daily and its absorbance was measured. A calibration curve was prepared using a standard solution of trolox (ranging from 2 to 10 μM). The results were expressed on a fresh weight basis as mmol trolox equivalents (TE)/100g of sample. The experiment was carried out in triplicate.

Ferric-Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was carried out according to the procedure of Benzil and Strain [19]. Briefly, a 0.1 ml apple extract was mixed with 3.0 ml of FRAP reagent. Then, the reaction mixture was incubated at 37°C for 4 min. After that, the

absorbance was measured at 593 nm against a blank that was prepared using deionized water and incubated for 1h instead of 4 min. FRAP reagent should be pre-warmed at 37°C and should always be freshly prepared by mixing 2.5 ml of a 10 mM 2,4,6-tris (1-pyridyl)-5-triazine (TPTZ) solution in 40 mM HCl with 2.5 ml of 20 mM $FeCl_3 \cdot 6H_2O$ and 25 ml of 0.3 M acetate buffer, pH 3.6. A calibration curve was prepared, using an aqueous solution of ferrous sulphate $FeSO_4 \cdot 7H_2O$ (200, 400, 600, 800 and 1000 µM, r=0,9972). FRAP values were expressed on a fresh weight basis as mmol of ferrous equivalent Fe (II) per 100 g of sample.

Ferric-Reducing Power assay (FRP)

For assessing ferric-reducing power (FRP), the assay described by Chan *et al*. [20] was adapted. Different dilutions of extracts (1 ml) were added to 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of potassium ferricyanide (1%, w/v). The mixture was incubated at 50ºC for 20 min. After trichloroacetic acid solution (2.5 ml, 10%, w/v) was added, the mixture was separated into aliquots of 2.5 ml and diluted with 2.5 ml of water. To each diluted aliquot, 0.5 ml of ferric chloride solution (0.1%, w/v) was added. After 30 min, absorbance was measured at 700 nm. FRP of extracts was expressed as mg gallic acid equivalent (GAE) per 100 g of fresh fruit. The calibration equation for gallic acid was $y = 0.31143 x + 0.01061$ (r = 0.99912), where y is the absorbance and x is the gallic acid concentration in µg/ml.

Statistical analysis

The data were reported as mean ±standard deviation (SD) for triplicate determinations. The significance of inter-group differences was determined by the analysis of variance (ANOVA). A value of $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Phenolic compounds are generally considered as a very important antioxidant source in fruits. Therefore, TP of 15 apple cultivars were examined by the Folin-Ciocalteu assay and the results are presented in Table 1. The 15 apple cultivar samples investigated exhibited considerable differences in their TP values, varying from 72.80 mg GAE/100g fw for Braeburn apple to 217.37 mg GAE/100 g fw for Kozara apple. Chadel, Idared, Red Chief and Granny Smith apples also had relatively higher TP (> 190 mg GAE/100g fw). The results were lower than the data of Imeh and Khochar [21] (300 – 535 mg GAE/100g fw), but higher than those of Valavanidis *et al*. [22] and Lachman *et al*. [13] (80–196 mg GAE/100g fw, 76–134 mg GAE/100 fw, respectively). This differences may be due to multiple reasons including genetic factors, different environmental conditions, storage of maturity, cultivar or varietals differences, growth stage, soil fertilization and part of the fruit used, amongst other factors that effect quantitive variation in these phytochemicals [23,24]. The total flavonoids (TF) content of these apples was determined. Granny Smith apple had the highest TF content (111.82 mg CE/100 g fw), followed by Golden Delicious apple (106.99 mg CE/100 g fw). The results are presented in Table 1. Correlation analysis was performed on the polyphenolic content analysis methods for the 15 apple cultivars. The correlation between TP and TF assays is at the 0.05 level. These results indicate that the flavonoids are an important phenolic group in apple fruit. Correlation coefficient is 0.790.

There are huge varieties of antioxidants contained in fruits. Therefore, measuring the antioxidant capacity of each compound separately becomes very difficult. Several methods have been developed to estimate the antioxidant capacity of different plant materials [5,25]. Usually these methods measure the ability of antioxidants, in a particular plant material, to scavenge specific radicals, by inhibiting lipid peroxidation or by chelating metal ions.

Depending upon the reaction involved, the antioxidant capacity assays can be based on hydrogen atom transfer reactions and assays based on electron transfer. Hydrogen atom transfer reaction based assays are methods in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. Those are: oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant power (TRAP), β-carotene blenching assay, inhibition of linolenic acid oxidation, and inhibition of LDL oxidation. Electron transfer based assays measure the capacity of an antioxidant in the reduction of an oxidant which changes color when reduced. Described methods include 2,2'–azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation assay (ABTS), ferric ion reducing antioxidant power assay (FRAP), 2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay (DPPH) [24,26].

In this study the total antioxidant activity of 15 apple cultivar extracts was determined using DPPH radical scavenging activity, ABTS radical cation

Table 1. Total phenol (TP) and total flavonoid (TF) contents of apple samples

Apple Cultivar	TP* mg GAE/100 g f.w.	TF* mg CE/100 g f.w.	TF/TP
1. Jonathan	134 ± 2^{h}	74.7 ± 0.9^{d}	0.55
2. Gloster	86 ± 2^{j}	53.1 ± 0.9^{g}	0.61
3. Melrose	85 ± 2^{j}	61 ± 2^{f}	0.72
4. Red Deliciose	87 ± 2^{j}	55.8 ± 0.9^{gf}	0.64
5. Golden Deliciose	180 ± 1^{f}	107 ± 2^{a}	0.59
6. Greeny Smith	197 ± 1^{e}	112 ± 3^{a}	0.57
7. Sharunka	161 ± 2^{g}	70 ± 5de	0.43
8. Gala	94 ± 2^{i}	42.0 ± 0.5^{h}	0.45
9. Jonagold	130 ± 1^{h}	59.5 ± 0.7^{gf}	0.46
10. Idared	202 ± 2^{c}	67.1 ± 0.8^{ef}	0.33
11. Braeburn	73 ± 2^{k}	37.2 ± 0.4^{h}	0.51
12. Mutsu	134 ± 2^{h}	54 ± 2^{gf}	0.40
13. Chadel	212 ± 2^{b}	82.57 ± 0.08^{c}	0.39
14. Kozara	217 ± 2^{a}	96 ± 4^{b}	0.44
15. Red Chief	200 ± 2^{d}	76 ± 2^{dec}	0.38

*) The data are reported as mean ± standard deviation (n=3); Bars with no letters in common are significantly different ($p<0.05$) in the same column.

Table 2. DPPH radical scavenging activity. ABTS radical cation activity. ferric-ion reducing antioxidant parameter-FRAP and ferric-reducing power - FRP of apple samples

Apple Cultivar	ABTS* mmol TE/100g f.w.	DPPH* mmol TE/100g f.w.	FRAP* mmol Fe/100g f.w.	FRP* mg GAE/100g f.w.
1. Jonathan	0.371 ± 0.009^{d}	0.259 ± 0.002^{a}	0.663 ± 0.017^{e}	40.440 ± 1.214^{g}
2. Gloster	0.245 ± 0.004^{f}	0.231 ± 0.003^{b}	0.460 ± 0.011^{gf}	28.670 ± 0.861^{i}
3. Melrose	0.250 ± 0.003^{f}	0.236 ± 0.003^{b}	0.432 ± 0.005^{gi}	26.590 ± 0.915^{i}
4. Red Deliciose	0.257 ± 0.003^{f}	0.240 ± 0.002^{b}	0.486 ± 0.019^{gh}	32.910 ± 0.794^{h}
5. Golden Deliciose	0.501 ± 0.009^{b}	0.266 ± 0.001^{a}	0.987 ± 0.019^{a}	53.610 ± 0.736^{e}
6. Greeny Smith	0.504 ± 0.007^{ba}	0.256 ± 0.005^{a}	1.003 ± 0.021^{a}	56.820 ± 0.828^{e}
7. Sharunka	0.314 ± 0.006^{e}	0.257 ± 0.004^{a}	0.670 ± 0.016^{ed}	75.307 ± 0.609^{c}
8. Gala	0.186 ± 0.003^{g}	0.255 ± 0.004^{a}	0.387 ± 0.007^{i}	45.296 ± 0.823^{f}
9. Jonagold	0.367 ± 0.008^{d}	0.253 ± 0.006^{a}	0.773 ± 0.015^{c}	68.782 ± 0.533^{d}
10. Idared	0.382 ± 0.009^{d}	0.256 ± 0.004^{a}	0.846 ± 0.022^{b}	74.764 ± 0.852^{bc}
11. Braeburn	0.181 ± 0.003^{g}	0.227 ± 0.004^{b}	0.319 ± 0.009^{h}	42.090 ± 0.745^{gf}
12. Mutsu	0.304 ± 0.006^{e}	0.248 ± 0.006^{a}	0.505 ± 0.014^{f}	43.008 ± 0.595^{gf}
13. Chadel	0.421 ± 0.008^{c}	0.261 ± 0.004^{a}	1.001 ± 0.012^{a}	77.100 ± 0.657^{bc}
14. Kozara	0.536 ± 0.013^{a}	0.276 ± 0.005^{a}	1.003 ± 0.017^{a}	85.510 ± 0.729^{a}
15. Red Chief	0.384 ± 0.011^{d}	0.250 ± 0.007^{a}	0.729 ± 0.015^{d}	76.246 ± 0.670^{bc}

*) The data are reported as mean ± standard deviation (n=3); Bars with no letters in common are significantly different ($p<0.05$) in the same column.

scavenging activity, FRAP ferric ion reducing antioxidant power and FRP ferric reducing power.

The DPPH radical scavenging activities of 15 apple cultivar extracts are shown in Table 2. All extract samples selected exhibited strong DPPH radical scavenging activities at the test concentration. The values of DPPH radical scavenging activities ranged from 0.227 to 0.276 mmol TE/100g fw.

Different apple cultivars were also measured and compared for the free radical scavenging activity against the ABTS radical cation. Results showed that all apple samples used in this study had significant ABTS radical cation activities (Table 2). The values of ABTS radical cation scavenging

Table 3. Correlations among apple extracts activity evaluation indices and total phenolic content.

	DPPH	ABTS	FRAP	FRP	TP
DPPH	1	0.801	0.806	0.706	0.798
ABTS		1	0.917	0.640	0.880
FRAP			1	0.730	0.914
FRP				1	0.813
TP					1

activities of the 15 apple samples were in the range 0.181 – 0.536 mmol TE/100 g fw. The highest and lower ABTS radical cation scavenging activities among the apple samples studied were found in Kozara and Braeburn apples, respectively. It is important to mention that the TAA values of the same apple obtained by the ABTS assay were consistently higher that those obtained by the DPPH assay. The same phenomena were found in recent studies on antioxidant activity of selected fruits by Dragovic-Uzelac *et al*. [24], and that of red fruit juices by Bermudez-Soto and Tomas-Barberan [27]. Different reaction kinetics between phenol and ABTS radical cation or DPPH radical over a similar range of concentrations might lead to the different results from two methods [28]. Actually, the ABTS radical cation scavenging activity also reflects hydrogen-donating ability.

The FRAP assay involves a single electron reduction of the $Fe(TPTZ)_2(III)$ complex (pale yellow) to the $Fe(RPTZ)_2(II)$ complex (blue) by single electron donor species/antioxidants. The FRAP assay is one of the most simple, rapid, inexpensive tests and is very useful for routine analysis. FRAP assay is developed as a direct test of the total antioxidant power of a sample. The antioxidant activity of apple extracts using FRAP assay is shown in Table 2. The ferric reducing power of apple extracts tested in this investigation ranged from 0.319 mmol Fe^{2+}/100 g fw in the case of Braeburn apple to 1.003 mmol Fe^{2+}/100 g fw in the case of Kozara and Granny Smith apples.

As shown in Table 2, there are significant variations in reducing power (FRP) for the different apple samples. The reducing power of 15 apple cultivars tested in this investigation ranged from 26.59 mg GAE/100 g fw (Melrose apple) to 85.51 mg GAE/100 g fw (Kozara apple). The results were partly different from those obtained in the assays mentioned above, and this might be due to the different reaction mechanisms of the antioxidant evaluation assays. Reducing power is generally associated with antioxidant activity and may serve as a significant measure of the antioxidant activity. Compounds with reducing power indicate that they could reduce oxidized intermediates of lipid peroxidation processes and act as primary or secondary antioxidants.

Correlations among apple extract activity assays and total phenolic contents

The Pearson product moment correlation coefficients calculated from four different apple extracts antioxidant activity assays and total phenolic contents are shown in Table 3. Significant positive correlations between the four antioxidant activity assays for apple extracts were observed (ranging from 0.640 to 0.917, $p < 0.05$), especially between FRAP ferric ion reducing antioxidant power and ABTS radical cation scavenging activity suggesting that overall antioxidant activity evaluation results for 15 apple samples using four assays were consistent although these assays involved different reaction mechanisms. The measured antioxidant activity of an apple sample depends on the methodology and on the free radical generator or oxidant used in the measurement. As for correlations between antioxidant activity assays and TP, significant ($p<0.05$) positive correlations with FRAP ferric ion reducing antioxidant power, ABTS radical cation scavenging activity and relatively good positive correlation with reducing power and DPPH reducing scavenging activity were obtained.

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ОБЩИ ФЕНОЛИ, ФЛАВОНОИДИ И АНТИОКСИДАНТНА АКТИВНОСТ НА РАЗЛИЧНИ ЯБЪЛКОВИ СОРТОВЕ

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(Резюме)

Изглежда, че концентрацията на полифенолните съединения и антиоксидантната активност на ябълките зависи от сорта, зреенето при съхранение, условията на околната среда и отчасти от плода. В настоящата работа са определени общото съдържание на феноли, флавоноиди и антиоксидантната активност на цели плодове от 15 сорта ябълки, отглеждани в Сърбия. Общото съдържание на феноли (TP) е анализирано по метода на Folin-Ciocalteu; общите флавоноиди (TF)- колориметрично с $AlCl_3$. Общата антиоксидантна активност (TAA) на избраните ябълки бе определяна с 2,2-азино-бис(3-етилбензотиазолин-6-сулфонова киселина) като радикал-катион (ABTS), 2,2-дифенил-1-пикрилхидразил радикал-премахващ капацитету (DPPH), редукционната способност спрямо ферийони (FRP) and антиоксидантна способност спрямо ферийони (FRAP). Съдържанието на полифеноли за целите плодове бе в границите на 72.80 – 217.37 mg GAE/100 g fw. Ябълковите екстракти имат различна TAA във връзка с прилагания метод, а тези разлики в TAA може да се отдадат на съдържанието на TP и TF. С помощта на метода FRAP е намерена между зреенето и съдържанието на TP и TAA.