Total Phenolics and Antioxidant Capacity Assays of Selected Fruits

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Summary

The biologically active compounds, especially fruit phenolics, are responsible for reduced risk of developing chronic diseases (cardiovascular disease, cancer, diabetes, etc.), due to their antioxidant activities. This study summarized some chemical principles of antioxidant capacity assays and the antioxidant capacity of selected fruit varieties (strawberry, sour cherry, cornelian cherry, blackthorn). We determined the contents of total phenolics (TPC), total flavonoids (TF), total non-flavonoids (TNF) and total anthocyanins (TA) in selected fruits. The content of TPC, TF and TNF in fruits was analyzed by Folin-Ciocalteu colorimetric method, while the TA content of extracts was determined by bisulphite bleaching method. Total antioxidant capacity (TAC) of selected fruits were analyzed using 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS), 2,2-diphenil-1-picrylhydrazyl radical scavenging capacity (DPPH), ferric ion reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC). Cornelian cherry had the highest content of TPC, while sour cherry cv. Marasca had the highest content of TA. TAC was the highest in cornelian cherry fruits and decreased in other fruits as follow: sour cherry, blackthorn and strawberry. The fruit extracts had different TAC in relation to the method applied, and the different TAC of fruits can be ascribed to their TPC, TF, TNF or TA content. There was a direct correlation between the TAC determined using DPPH and ORAC methods and the TA content of fruit extracts. Using FRAP method correlation was weaker, but using ABTS method correlation was not observed. The ripping correlation between TF or TNF and TAC was observed using ABTS and ORAC methods.

Key words

antioxidant capacity, phenolics, anthocyanins, fruits

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Introduction

The consumption of antioxidant-rich food (fruits, vegetables, whole grains, tea, wine etc.) might play an important role in the maintenance of health and in diseases (inflammation, cardiovascular disease, cancer and aging-related disorders) prevention (Ziegler,1991; Rimm et al., 1996; La Vecchia et al., 2001; Terry et al., 2001). Due to the complexity of food composition it is not known which dietary constituents are responsible for this association, but antioxidants appear to play a major role in the protective effect of plant foods (Gey, 1990; Gey, 1991). The main characteristic of an antioxidant is its ability to trap free radicals which may oxidize nucleic acids, proteins, lipids or DNA. Several methods were developed recently for measuring the total antioxidant capacity (TAC) of food and beverages because of the difficulty in measuring each antioxidant component separately (Wang et al., 1997; Benzie and Strain, 1999). In addition, levels of antioxidants in food do not necessarily reflect their total antioxidant capacity which also depends on the synergic and redox interactions among the different molecules present in the food (Pellegrini et al., 2003). Because different antioxidant compounds may act *in vivo* through different mechanisms, no single method can fully evaluate the TAC of foods. 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 reactions 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 apsorbance capacity (ORAC), total radical trapping antioxidant power (TRAP), β-carotene bleaching 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 colour 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-diphenil-1-picrylhydrazyl radical scavenging capacity assay (DPPH) (Huang et al., 2005). The measured antioxidant capacity of a sample depends on methodology and on free radical generator or oxidant used in the measurement (Cao et al., 1993; Halliwell and Gutteridge, 1995). Therefore, the comparison of different analytical methods constitutes a key factor in helping investigators to choose a method and to understand the result obtained using the method.

The objectives of this study were to determined the content of total phenolics (TPC), total flavonoids (TF), total non-flavonoids (TNF) and total anthocyanins (TA) of selected fruits and to compare the ABTS, DPPH, FRAP and ORAC assays for assesing the total antioxidant capacity (TAC) of phenolics extract of selected fruits.

Material and methods

Material

Strawberry fruits (*Fragaria ananassa* Duch.) cvs. 'Maya' and 'Queen Elisa' were obtained from Institute of Pomology, Zagreb in June; sour cherry (*Prunus cerasus*) cvs. 'Marasca' and 'Cigancica' were harvested in July (cv. Marasca from Zadar and cv. Cigancica from Osjek); cornelian cherry (*Cornus mas*), two varieties-red ('CC1') and dark red ('CC2'), were harvested in September and blackthorn (*Prunus spinosa*) in October ('BT1') and in November ('BT2'). All fruit species were harvested in 2005 at maturity, and stored at -20 °C for 7 days. Before analysis fruit was thawed at room temperature, pited and mixed in house blender (Mixy, Zepter International). All chemicals were obtained from Sigma-Aldrich (St.Louis, MO).

Total phenolics, flavonoids and non-flavonoids content

Total phenolics (TPC), flavonoids (TF) and non-flavonoids (TNF) were determined using the Folin-Ciocalteu colorimetric method described by Amerine and Ough (1980) and Singleton and Rossi (1965) with some modification. Phenolics of the fruits were extracted from 10 g fresh samples using 40 ml 80% aqueous ethanol. The mixture was extracted 20 min in inert atmosphere, filtered through Whatman filter paper using a Buchner funnel. Extraction of the residue was repeated using the same conditions. The filtrates were combined and diluted to 100 ml in volumetric flask with 80% aqueous ethanol. Obtained extract was used for determination of TPC, TF and TNF. The formaldehyde precipitation was used to determine flavonoids in fruit samples (Kramling and Singleton, 1969). The content of TPC and TNF was measured as follow: 0.5 ml diluted extracts or standard solutions of gallic acid (20-500 mg L^{-1}) was added to a 50 ml volumetric flask containing 30 ml of ddH_2O, than 2.5 mililiter of Folin/Ciocalteu's reagent was added to the mixture and shaken. After 5 min, 7.5 mL of 7% Na_2CO_3 solution was added with mixing and the solution was immediately diluted to 50 mL with ddH_2O. After incubation at room temperature for two hours the optical density of the solution was measured at 760 nm. The content of flavonoids was calculated as difference between total phenolic content and non-flavonoid content. TPC, TF and TNF were expressed as mg gallic acid equivalents (GAE)/kg of fresh weight of edible part of fruits. The extract of total phenolics was used for ABTS, DPPH, FRAP and ORAC assays.

Anthocyanins

The total anthocyanin content (TA) in extract from selected fruits was determined using bisulphite bleaching method (Riberéau-Gayon & Stonestreet, 1965). Anthocyanins of the fruits were extracted from 2 g fresh samples using 2 ml of 0.1% HCl (v/v) in 96% ethanol and

40 mL 2% aqueous HCl (v/v). The mixture was centrifugated at 5500 rpm for 10 min. The obtained supernatant was used for determination of TA. The content of TA was measured as follow: 10 ml of extract was pipet into a two test tubes, than 4 mL of 15 % sodium bisulfite was added to one test tube and 4 mL of ddH_2O to the other. After 15 min incubation at room temperature the absorbance of each mixture at 520 nm was measured. The molar absorbance value for cyanidin-3,5-diglucoside was used as a standard value. Results were expressed as mg cyanidin-3,5-diglucoside equivalnets/kg of fresh weight of edible part of fruits.

Total antioxidant capacity

ABTS [2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation assay. The radical scavenging capacity of fruit extracts was evaluated against ABTS generated by chemical method according to a previously reported protocol (Miller and Rice-Evans, 1997). The method was based on the ability of antioxidant molecules to quench the long-lived ABTS, a blue-green chromophore with caracteristic absorption at 734 nm, compared with that of Trolox, a water soluble vitamin E analog. The addition of antioxidants to the performed radical cation reduces it to ABTS, determining a decolorization. Results were expressed as mmol Trolox equivalnt per kg of fresh weight of edible part of fruits.

DPPH. The free radical scavenging capacity of fruit extracts was determined according to previously reported procedure using the stable DPPH radical (Brand-Williams et al., 1995). The method was based on reduction of stable DPPH nitrogen radicals in presence of antioxidants. Results were expressed as mmol Trolox equivalnt per kg of fresh weight of edible part of fruits.

FRAP (ferric reducing antioxidant power) assay. The FRAP assay was conducted according to Benzie and Strain (1996). The method was based on the reduction of the Fe3+ TPTZ complex to the ferous form at low pH. This reduction is monitored by measuring the absorption change at 595 nm. FRAP values were obtained by comparing the absorption change in the test mixture with those obtained from increasing concentration of Fe3+ and were expressed as mmol of Fe2+ equivalents per kg of fresh weight of edible part of fruits.

ORAC (oxygen radical absorbance capacity assay). The original method of Cao co-woker (1993), with a few modifications, was used. The final reaction mixture for the assay (3 mL) was prepared as follows: 2.25 mL 0.04 μM fluorescein sodium salt in 0.075 M sodium phosphate buffer, pH 7.0, and 0.375 mL diluted samples or 25 μM Trolox. The control was 0.075 M sodium phosphate buffer. The reaction mixtures were incubated for 30 min at 37 °C followed by reaction initiation with 0.375 mL 152 mM AAPH. Fluorescence was read every minute up to a value zero at 485 nm excitation, 520 nm emission. Results were expressed as mmol Trolox equivalnt per kg of fresh weight of edible part of fruits.

Statistical analysis.

Statistical analysis was done using the Statistica version 7.0. Differences between means were analysed by ANOVA test ($p<0.05$).

Results and discussion

The results of this study demonstrated the contents of total phenolics (TPC), flavonoids (TF), non-flavonoids (TNF) and anthocyanins (TA), as well as total antioxidant capacity (TAC) of selected cultivated (strawberry, sour cherry) and wild (blackthorn, cornelian cherry) fruits. The contents of TPC, TF and TNF in the samples were analyzed by colorimetry with the use of Folin-Ciocalteu reagent. Results are expressed as mg of gallic acid equivalents per kg of fresh weight of edible part of fruit (Table 1).

The TPC content was the highest in cornelian cherry fruits of both varieties (3055.29 and 2095.65 mg kg^{-1} f.w.), followed by sour cherry, cv. Marasca (1447.95), blackthorn 'BT2', sour cherry cv. Cigancica, blackthorn 'BT1', strawberry 'Maya' and finally strawberry 'Queen Elisa'. Cornelian cherry had much higher TPC content than any of other fruits studied. Approximately two times higher than those in sour cherry cv. Marasca, four to five times higher than those in sour cherry cv. Cigancica and in blackthorn fruits (Table 1). The highest content of TF was determined in cornelian cherry fruits followed by sour cherry cv. Marasca. Compared to amounts of TF, TNF were presented in lower amounts in all investigated fruits. TA content determined by bisulphite bleaching method was the highest in sour cherry cv. Marasca, followed by cornelian fruit ('CC2' and 'CC1') fruits. The amounts of TA in other investigated fruits was even nine times lower than in cv. Marasca. The TAC of selected fruits was evaluated using three different assays (ABTS, DPPH, ORAC), whereas using FRAP method antioxidant activity was determined (Table 2).

The fruit extracts had different TAC in relation to the method applied; thus the same item often ranked differently depending on the assay. The different antioxidant capacity of fruits can be ascribed to their TPC, TF, TNF or TA content. The high TAC value assigned to cornelian cherry fruits and sour cherry both cultivars, when analyzed by the ORAC (175.19, 119.16 and 128.68 mmol TE/kg f.w.), and DPPH (39.89, 33.41 and 43.05 mmol TE/kg f.w) assays. Results of ORAC assay TAC for sour cherry fruits are higher compare to results presented in literature (Chaovanalikit and Wrolstad, 2004). Pentelidis and coworker (2006) presented TAC for cornelian cherry fruits

Table 1.
Total phenolics, flavonoids, non-flavonoids and anthocyanins of extracts obtained from different cultivated and wild fruits[a]

Fruits	Total phenolics, mg GAE/kg f.w.	Total flavonoids, mg GAE/kg f.w.	Total non-flavonoids, mg GAE/kg f.w.	Total anthocyanins, mg/kg f.w.
Cultivated				
Strawberry, cv. Maya	276.90±21.15	167.45±15.55	109.45±10.05	171.89±10.87
Strawberry, cv. Queen Elisa	285.45±15.18	77.40±7.19	208.05±20.65	120.23±15.22
Sour cherry, cv. Marasca	1447.95±64.48	1240.51±96.74	207.44±19.78	1129.24±95.85
Sour cherry, cv. Cigancica	780.20±7.55	585.90±12.72	194.30±21.15	545.95±10.70
Wild				
Blackthorn BT1	546.56±45.42	436.90±25.54	109.66±12.05	305.04±26.87
Blackthorn BT2	858.75±55.75	656.10±50.26	202.65±20.18	497.43±34.55
Cornelian cherry CC1	2095.65±155.75	1827.75±115.57	267.90±25.22	954.60±55.46
Cornelian cherry CC2	3055.29±205.84	2343.31±233.05	711.98±65.70	1072,29±95.45

[a]Data presented are means of two replicates from two measurements of two extractions ±SE.

Table 2. Total antioxidant activity (FRAP) and capacities of extracts obtained from different cultivated and wild fruits[a]

Fruits	FRAP, mmol Fe^{2+}/kg f.w.	Total antioxidant capacity, mmol Trolox equivalent/kg f.w.		
		DPPH	ABTS	ORAC
Cultivated				
Strawberry, cv. Maya	7.68±0.55	8.29±0.50	26.92±2.18	47.14±3.22
Strawberry, cv. Queen Elisa	9.73±1.05	11.15±1.12	30.06±2.56	49.36±2.25
Sour cherry, cv. Marasca	32.53±2.15	43.05±2.55	45.36±3.05	128.68±5.18
Sour cherry, cv. Cigancica	24.04±1.55	32.18±2.85	40.57±4.25	75.15±5.85
Wild				
Blackthorn BT1	16.55±1.20	24.40±1.95	37.60±2.25	68.76±4.15
Blackthorn BT2	17.95±1.55	28.15±0.85	38.56±3.36	89.39±4.87
Cornelian cherry CC1	18.04±1.95	33.41±2.15	29.48±3.05	119.16±7.15
Cornelian cherry CC2	25.09±2.05	39.89±3.05	36.51±2.05	175.91±8.22

[a]Data presented are means of two replicates from two measurements of two extractions ±SE.

cv. Vermio assigned using FRAP method (83.9 mmol ascorbic acid equivalents/kg dry weight). There is not other data in literature about TAC in cornelian cherry fruits as well as in blackthorn fruits. The TAC values in investigated strawberry cultivars, Maya and Queen Elisa were higher compare to data from literature (Wang & Lin, 2000; Wang et al., 2002), but it is not surprising because we were not compare same cultivars. The response characteirstics of contetnt of TPC and TA in the ABTS, DPPH, FRAP and ORAC assays are shown in Figure 1, A,B.

There was a direct correlation between the TAC determined using ORAC and DPPH methods and the TA content of fruit extracts ($r=0.89$ and 0.88, $p<0.05$), using FRAP method correlation was weaker ($r=0.78$, $p<0.05$), but using ABTS method correlation was not observed ($r=0.5$, $p<0.05$). Remarkable correlation between TPC and TAC determined using ABTS and ORAC ($r= 0.99$ and 0.84, $p<0.05$) methods was observed, whereas TAC determined using other methods was not in correlation with TPC. The ripping correlation between TF or TNF and TAC was observed by using ABTS and ORAC methods. The week correlation between the ORAC and FRAP or ABTS assay was observed in our investigation. It is not unusually because mentioned methods use different principles. ORAC is inhibition method – a sample is added to a free radical generating system, the inhibition of the free radical action is measured and this inhibition is related to the antioxidant capacity of samples (Cao and Prior, 1998). The ABTS method is based on the ability of antioxidant molecules to quench the long-lived ABTS, a blue-green chromophore (Miller & Rice-Evans,1997), but unfortunately the specificity of this assay in measuring TAC of a sample to direct quench free radicals is not always guaranted (Strube et al., 1997). The FRAP assays evaluate the chain-breaking antioxidant potential (Ghiselli et al., 1995) and the reducing power of the sample (Benzie & Strain, 1999), respectively. The DPPH method is based on reduction of stable DPPH nitrogen radicals in presence of antioxidants (Huang et al., 2005). Moreover, even for values obtained from the same assay, a comparison with the data in the literature was problematic due to the large variability within the food item and to the lack of standardization of the assays.

Conclusions

These results suggest that the investigated fruits contain high contents of different group of polyphenols, which have potent antioxidant capacity. Generally, the healthiest

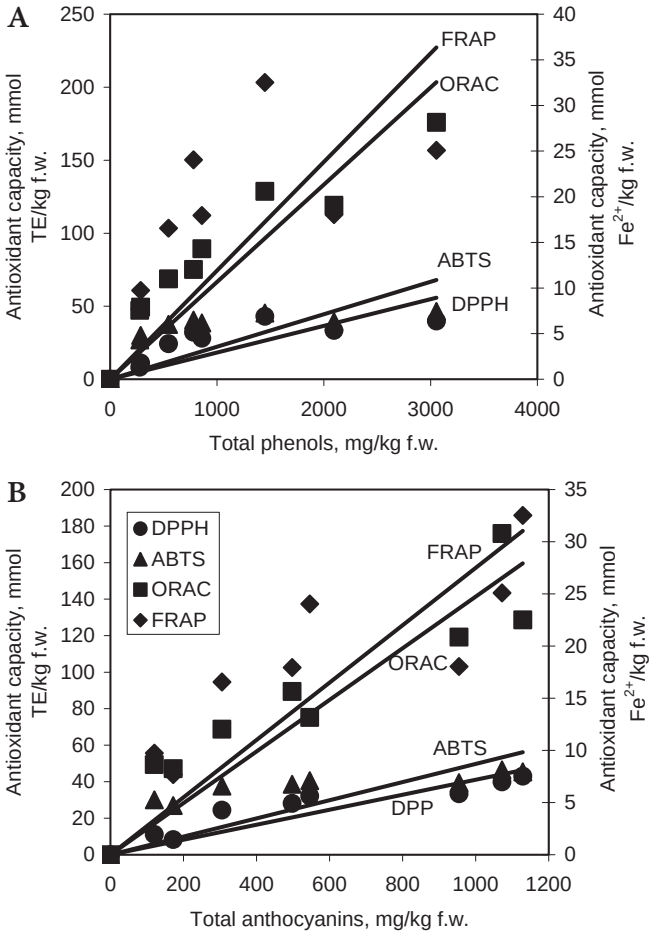

Figure 1.
Response to the contetnt of total phenolics (A) and total anthocyanins (B) in the ABTS, DPPH, FRAP and ORAC assays. (A) ABTS r=0.99; DPPH r=0.52; FRAP r=0.28; ORAC r=0.84 . (B) ABTS r=0.50; DPPH r=0.88; FRAP r=0.78; ORAC r=0.89. Data presented are means of two replicates from two measurements of two extractions ± SE, ($p<0.05$)

fruits are cornelian cherry and sour cherry cv. Marasca. The ORAC assay has good specificity and responds to numerous antioxidants (phenolics, anthocyanins etc.), but method is relatively expensive. In all investigated samples DPPH and ABTS methods were shown as suitable, simple and relatively cheap method used for determination of TAC in comparison to others. Though FRAP method is also simply and cheap, but they were not enough confident for determination in this investigation.

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