THE INFLUENCE OF MATURITY STAGE AND EXTRACTION SOLVENTS ON PHENOLIC CONTENT AND ANTIOXIDANT ACTIVITY OF THREE SWEET CHERRY CULTIVARS

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Abstract

The effects of two extracting solvents (70% acetone and 70% ethanol) and maturity stage (semi ripe and ripe) on the phenolic content and antioxidant activity of fruits of three sweet cherry cultivars (Burlat, New Star and Peter) were investigated. Results showed that extraction solvent did not have significant effects on total phenolics (TP), tannins (TT) and flavonoids (TF) content and antioxidant activity (1,2-diphenyl-2-picryl-hydrazyl (DPPH) assay, ferric-reducing antioxidant power (FRAP) assay, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) assay, total antioxidant activity (TAA) by phosphomolybedenum complex formation method and reducing power (RP) assay) in dried fruits of sweet cherry. The results did not showed significant changes in phenolic content and antioxidant capacity of fruits during the ripening**.** Among the investigated sweet cherry fruits, Peter cultivar contained the highest amounts of all groups of phenolics, followed by Burlat and New Star. TP in fruits ranged from 10.90 (ripe New Star, ethanol extract) to 28.92 (semi ripe Peter, acetone extract) mg gallic acid equivalents (GAE)/g dry weight (DW). The highest amount of TF in fruits was detected in ethanol extract of semi ripe Peter cultivar (12.97 mg quercetin equivalents (QE)/g DW), while the lowest content was found in ethanol extracts of semi ripe New Star cultivar (7.80 mg QE/g DW). The examined cultivars possess a high antioxidant capacity, and all measured phenolic groups were highly correlated with performed antioxidant assays. The antioxidant activity values obtained with DPPH in the dry fruits (ranging from 7.68 to 13.29 mg trolox equivalents (TE)/ g DW) were comparable to those obtained with FRAP 3.69 to 13.28 mg TE/g DW).

Key words: antioxidant capacity, phenolics, sweet cherry

Reactive oxygen species (ROS), including free radicals and other reactive oxygen molecules, are normally formed during metabolic processes, and are considered to have very important roles in cell signaling and homeostasis. ROS are in more reactive state than molecular oxygen. The formation of superoxide radicals ($O_2^{\cdot -}$) is a well-known example of ROS generation during normal respiration. Further reactions may lead to formation of hydroxyl radicals ($OH^{\cdot}$), most reactive species in chemistry, especially in the presence of metal ions. The formation of ROS is seen at high rates in many diseases, including diabetes, cancer, cardiovascular diseases, and other neurodegenerative disorders (Gill S.S. and Tuteja N., 2010; Karuppanapandian T. et al, 2011; Jajic, I. *et al*, 2015).

The high antioxidant capacity of polyphenol components could contribute to health benefits by acting to ameliorate the detrimental effects of ROS generated through oxygen metabolism in the human body. Polyphenols could act as chain-breaking agents preventing the ROS from instigating free radical cascades that could damage cell components like membranes and DNA (Grassmann J. *et al*, 2002). However, this simple and attractive precept is not generally tenable and high antioxidant capacity *in vitro* does not automatically translate into *in vivo* effectiveness. Different polyphenols have different stabilities, bioavailability and therefore potential effectiveness (Ozcan T. *et al*, 2014).

Cherries are among the coloured fruits that are a very popular and important part of food consumed in Europe and other parts of the world. Sweet cherry fruits have been collected and consumed simply because of their sweetness and taste aspects (Crisosto C.H. *et al*, 2003). Recently, the awareness of the fruit health compounds has directed consumer preferences more to the nutritional, antioxidant and physiological qualities of sweet cherry fruits, based on the level and composition of various bioactive substances such

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as minerals, vitamins and phenolic compounds (Ferretti G. *et al*, 2010; Delgado J. *et al*, 2012).

Many aspects such as genetics, environmental factors and agricultural practices affect the pre-harvest quality of sweet cherries. Total polyphenol content can vary hugely between varieties and under different growing conditions (Faniadis D. *et al*, 2010). Maturity stage is also an important factor that influences the compositional quality of fruit cultivars. Several structural, biochemical and physiological modifications take place at different maturity stages and these changes determine the final composition and quality during ripening (Gonçalves B. *et al* 2007; Kállay E. *et al* 2008). For extraction of phenolic compounds from plant material different solvent systems have been used and their efficiency varies (Melicháčová S. *et al* 2010).

The aim of this study was to determine the content of phenolic compounds and antioxidant activity in fruits and stalks of three sweet cherry cultivars extracted at different maturity stages with two different extraction solvents (70% acetone and 70% ethanol).

MATERIAL AND METHOD

Plant material. Fruits of sweet cherry cultivars were collected in 2015 from the productive orchard "Sloga" Kać in vicinity of Novi Sad, Serbia. Fruits of three red-coloured sweet cherry cultivars (Burlat, New Star and Peter) were included in this study. Cherry fruits were picked at two maturity stages: seven days before commercial maturity and at commercial maturity, on the basis of fruit colour. Approximately 2 kg of fruits per cultivar was harvested from the trees. The fruits were selected according to uniformity of size, shape and colour and then transported to the laboratory for analysis.

Extraction of bioactive compounds. Edible parts of fresh fruits were dried in an vacuum oven (50°C) to the constant weight. Dry fruit material (1 g per sample) was ground to a fine powder and extracted with 70% aqueous ethanol or acetone solution (50 mL) by sonication for 20 minutes in an ultrasonic bath at ambient temperature. The extracts were rapidly vacuum-filtered through a sintered glass funnel and kept refrigerated until assayed.

Determination of phenolic compounds. The total phenolic (TP) content was determined using a Folin-Ciocalteu colorimetric method (Nagavani and Raghava Rao, 2010) and the results were expressed as milligrams of gallic acid equivalents per 1 g dry fruit weight (mg GAE/g DW). Total tannins (TT) content was determined by the Folin-Ciocalteu procedure, after removal of tannins by their adsorption on insoluble matrix PVPP (polyvinylpolypyrrolidone). Calculated values were subtracted from total phenolics content, and total tannin contents were expressed as milligrams of quercetin equivalents (QE) per 1 gram of dry fruit weight (FW). The total flavonoid (TF) content was determined spectrophotometrically with 3 mL of 2% $AlCl_3$ solution (Saha *et al,* 2013). The amount of flavonoids was calculated as a quercetin equivalent (QE) from the calibration curve of quercetin standard solutions and expressed as milligrams of quercetin per 1 gram of FW.

Measurement of antioxidant activity. Scavenging of free radicals was tested in a DPPH (2,2-diphenyl-1-picrylhydrazyl) acetone solution (Lai and Lim, 2011). The degree of decoloration of solution indicates the scavenging efficiency of the substance added. Ferric-reducing antioxidant power (FRAP) assay was carried out according to the procedure described in the literature (Valentão *et al,* 2002). The ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) assay was based on a method developed by Re *et al* (1999). Acetone solution of known trolox concentrations were used for calibration and the results were expressed as mg trolox equivalents per g of dry fruit weight (mg TE/g DW) for all three assays. The total antioxidant activity of plant extracts were evaluated by phosphomolybdenum method as reported by Kalaskar and Surana (2014) and results were expressed as mg of butylated hydroxytoluene equivalents per gram of dry fruit weight (mg BHTE/g DW). A reducing power assay (total reduction capacity) was performed by method of Saha *et al* (2013). The standard curve was constructed using different concentrations of trolox, and the results were expressed as mg trolox equivalents per gram of dry fruit weight (mg TE/g DW).The superoxide free radical scavenging activity was carried out by NBT (nitroblue tetrazolium) test (Kalaskar and Surana, 2014). The percent inhibition of superoxide anion generated was calculated using the following formula:

Scavenging activity (%) = (1 - absorbance of sample/absorbance of control) x 100

Statistical analysis. Results were expressed as mean of determinations of 3 independent samples made in triplicates. Statistical significance was tested by analysis of variance followed by comparison of means by Duncan's multiple range test ($P<0.05$) calculated using STATISTICA for Windows version 12.0 (StatSoft, Tulsa, OK, USA). Stepwise multiple regression analyses were used to determine correlation among variables.

Table 1

Content of total phenolics, tannins and flavonoids in extracts of sweet cherry fruits

			Phenolic compounds		
Cultivar	Maturity stage	Extraction solvent	Total phenolics[1]	Total tannins[1]	Total flavoinids[1]
Burlat	Semi ripe	70% ethanol	$22.51^{a} \pm 1.98$	$4.34^{a} \pm 0.48$	$0.983^{a} \pm 0.054$
		70% acetone	$22.51^{a} \pm 1.04$	$4.02^{a} \pm 0.11$	$0.968^{a} \pm 0.071$
	Ripe	70% ethanol	$16.58^{c} \pm 0.51$	$2.96^{b} \pm 0.09$	$0.857^{a} \pm 0.055$
		70% acetone	$18.01^{c} \pm 1.54$	$2.87^{b} \pm 0.36$	$1.011^{b} \pm 0.027$
New Star	Semi ripe	70% ethanol	$11.92^{d} \pm 0.34$	$1.88^{c} \pm 0.12$	$0.780^{c} \pm 0.025$
		70% acetone	$12.28^{d} \pm 0.40$	$1.70^{c} \pm 0.26$	$1.037^{a,d} \pm 0.036$
	Ripe	70% ethanol	$10.90^{d} \pm 0.42$	$1.92^{c} \pm 0.14$	$1.047^{d} \pm 0.036$
		70% acetone	$10.95^{d} \pm 1.07$	$2.04^{c} \pm 0.31$	$1.018^{a,d} \pm 0.042$
Peter	Semi ripe	70% ethanol	$25.35^{a,b} \pm 0.57$	$4.93^{d} \pm 0.51$	$1.297^{e} \pm 0.085$
		70% acetone	$28.92^{b} \pm 0.38$	$5.08^{d} \pm 0.22$	$1.131^{d,e} \pm 0.061$
	Ripe	70% ethanol	$23.62^{a} \pm 1.03$	$4.58^{a,d} \pm 0.56$	$1.070^{d} \pm 0.083$
		70% acetone	$25.92^{a,b} \pm 1.50$	$4.60^{a,d} \pm 0.40$	$0.846^{a} \pm 0.098$

[1]mg quercetin equivalents (QE)/g dry weight
[a-e] values without the same superscript within each row differ significantly ($P< 0.05$)

RESULTS AND DISCUSSIONS

The present study was conducted to determine the phenolic compounds and antioxidant activity in fruit of three different sweet cherry cultivars at different maturity stages. Basically, phenolic content and antioxidant activity in all three cultivars varies irregularly when maturity progressed.

Table 2

Antioxidant activity (DPPH, ABTS and FRAP assays) in extracts of sweet cherry fruits

			Antioxidant test		
Cultivar	Maturity stage	Extraction solvent	DPPH[1]	ABTS[1]	FRAP[1]
Burlat	Semi ripe	70% ethanol	$9.14^{a,b} \pm 0.63$	$8.29^{a} \pm 0.94$	$6.98^{a} \pm 0.26$
		70% acetone	$8.58^{a,f} \pm 0.21$	$8.78^{a} \pm 0.40$	$7.36^{a} \pm 0.24$
	Ripe	70% ethanol	$9.42^{a,b} \pm 0.64$	$5.95^{b} \pm 0.44$	$5.21^{b} \pm 0.08$
		70% acetone	$9.85^{b} \pm 0.46$	$5.69^{b,c} \pm 0.16$	$7.23^{a} \pm 1.14$
New Star	Semi ripe	70% ethanol	$7.72^{c} \pm 0.28$	$4.78^{c,d} \pm 0.48$	$5.19^{b} \pm 0.18$
		70% acetone	$7.68^{c} \pm 0.41$	$4.08^{d} \pm 0.16$	$5.59^{b} \pm 0.22$
	Ripe	70% ethanol	$10.16^{b,d} \pm 0.43$	$5.64^{b,c} \pm 0.37$	$3.69^{c} \pm 0.42$
		70% acetone	$10.70^{d} \pm 0.30$	$5.13^{b,c} \pm 0.20$	$3.78^{c} \pm 0.28$
Peter	Semi ripe	70% ethanol	$12.82^{e} \pm 0.28$	$8.96^{a} \pm 0.27$	$11.20^{d,e} \pm 0.83$
		70% acetone	$13.29^{e} \pm 0.49$	$9.09^{a} \pm 1.46$	13.29e ± 0.47
	Ripe	70% ethanol	$7.49^{f} \pm 0.49$	$8.46^{a} \pm 0.73$	$10.61^{d} \pm 0.63$
		70% acetone	$7.23^{f} \pm 1.09$	$8.21^{a} \pm 0.79$	$11.71^{d,e} \pm 0.43$

[1]mg trolox equivalents (TE)/g dry weight
[a-f] values without the same superscript within each row differ significantly ($P< 0.05$)

In sweet cherry fruits the ripening process is related to a change from the initial green colour into red, with degradation of chlorophyll and accumulation of different phenolic compounds. The typical colour of sweet cherry fruits is due to the presence of water-soluble phenolic compounds (Ferretti G. *et al*, 2010). Content of total phenolics, tannins and flavonoids of the fruit extracts from

selected sweet cherry cultivars are presented in table 1. In this study, the Peter sweet cherry cultivar had the highest content of total phenolics and tannins in fruits, followed by Burlat cultivar. Fruits of New Star cultivar had a 50% less of this biomolecules in fruits than other two. The tested sweet cherry fruits had a TP range of 10.90 (cv. New Star, ripe, ethanol extract) up to 28.92 (cv. Peter, semi ripe, acetone extract) mg QE/g DW. The TP content values were significantly different among different sweet cherry genotypes. Our results are in according with results of other researcher (Pérez-Sánchez R. *et al*, 2010, 2013; Mahmood T. *et al*, 2013).

Significant variability exists among the examinated sweet cherry genotypes, regarding their content in TT, ranging from 1.70 (cv. New Star, semi ripe, acetone extract) up to 5.08 (cv. Peter, semi ripe, acetone extract) mg QE/g DW. Fruits from the cultivars that are abundant in TP content also contained more TT. Tannins are widely distributed in the plant kingdom. The biochemical activities of tannins range from beneficial antioxidants to damaging prooxidants and toxins.The concentration of tannins depend on environmental condition, maturity stage and plant genotype (Barbehenn R.V. and Constabel C.P., 2011). Tannins markedly affect the flavor and the astrigancy of fruits (Bernalte M.J. *et al,* 2003). Solvent used for extraction had no effect on quantity of extracted TP or TT.

Flavonoids are a wide group of plant secondary metabolites, occurring in all parts of the plants. They have a variety of functions in plant biochemistry and physiology, acting as antimicrobials, antioxidants, UV protectors, photoreceptors, and also play a important role in nitrogen fixation. Flavonoids have been described as health-promoting agents as well (Karabin *et al*, 2015). The range of TF in all tested sweet cherry fruits ranged between 0.780 (cv. New Star, semi ripe, ethanol extract) and 1.297 (cv. Peter, semi ripe, ethanol extract) mg QE/g DW. Although there is statistically significant difference in TF content among different samples we did not find any obvious pattern in it. Genotype, maturity stage and extraction solvent did not have any influence on TF content in sweet cherry dry fruits of selected cultivars.

The antioxidant activity of plant extracts may vary with assay performed. Therefore, a single assay could be inadequate (Yen, G.C. *et al*, 2005). For this reason, we checked antioxidant activities of different extracts of sweet cherry fruits with six different assays. Antioxidant activities measured in three different extracts obtained using DPPH, ABTS and FRAP assays are presented in table 2. The antioxidant activity of extracts from sweet cherry fruits as measured by DPPH assay

Table 3

Total antioxidant activity (TAA), total reduction capacity (TRC) and scavenger activity of $O_2^{\cdot -}$ radicals (NBT test) of sweet cherry fruit extracts

			Antioxidant test		
Cultivar	Maturity stage	Extraction solvent	NBT[1]	TRC[2]	TAA[3]
Burlat	Semi ripe	70% ethanol	52.3^{a} ± 4.0	14.75^{a,e} ± 0.24	35.86^{a} ± 0.90
		70% acetone	43.1^{b} ± 2.9	14.54^{a} ± 0.28	38.99^{a} ± 2.27
	Ripe	70% ethanol	43.8^{b} ± 2.1	11.40^{b,c} ± 0.59	37.31^{a} ± 0.79
		70% acetone	39.3^{b,d} ± 4.8	12.49^{b} ± 0.61	48.01^{b} ± 2.85
New Star	Semi ripe	70% ethanol	29.7^{c} ± 5.7	11.49^{b,c} ± 0.39	29.94^{c} ± 1.47
		70% acetone	32.4^{c,d} ± 2.6	10.41^{c,d} ± 0.07	32.78^{c} ± 1.58
	Ripe	70% ethanol	37.8^{c,d} ± 2.6	10.78^{c,d} ± 0.48	28.78^{c} ± 1.01
		70% acetone	41.6^{b} ± 3.8	10.11^{d} ± 0.03	31.63^{c} ± 0.35
Peter	Semi ripe	70% ethanol	53.5^{a} ± 1.5	14.44^{a} ± 0.26	36.13^{a} ± 0.89
		70% acetone	52.5^{a} ± 1.7	17.69^{e,f} ± 0.61	43.34^{d} ± 0.96
	Ripe	70% ethanol	51.8^{a} ± 2.2	16.45^{e} ± 0.57	36.90^{a} ± 0.50
		70% acetone	48.3^{a} ± 4.0	18.36^{f} ± 0.74	43.26^{d} ± 0.95

[1]% of inhibition of superoxide anion generated

[2]mg trolox equivalents (TE)/g dry weight

[3]mg butylated hydroxytoluene equivalents (BHTE)/g dry weight

[a-f] values without the same superscript within each row differ significantly ($P< 0.05$)

ranged from 7.23 mg TE/g DW (cv. Peter, ripe, acetone extract) to 13.29 mg TE/g DW (cv. Peter, semi ripe, acetone extract). Differences for the ABTS radical cation scavenging capacities of each sample was recorded in this study. Among various samples acetone extract of cv. Peter (semi ripe, acetone extract) possessed the highest ABTS radical scavenging activity (9.09 mg TE/g DW), while acetone extract of cv. New Star (semi ripe) showed the lowest ABTS radical scavenging activity (4.08 mg TE/g DW). FRAP test has shown that fruits of sweet cherry have the significant reduction potential. Extracts of semi ripe sweet cherry fruits demonstrated higher scavenging activities then ripe samples. Our investigation shows that the FRAP method is independent of the extraction solvent polarity.

Results of total antioxidant activity, total reduction capacity, inhibition of superoxide anion ($O_2 \cdot^-$) radical scavenging activity are shown in table 3. The phosphomolybdenum assay is quantitative method to evlauate fat and water soluble antioxidant activity (total antioxidant activity), in which transforming of Mo(VI) into more stable Mo(V) non-reactive products occurs (Kalaskar and Surana, 2014). Transformation of Fe^{3+} to Fe^{2+} in the presence of sweet cherry fruits extracts was performed to measure the total reductive capability. The total antioxidant activity and total reduction capacity in all tested genotypes has been similar. The lowest bioactivity was measured in extracts of New Star cultivar, while the total antioxidant activity of extracts of Burlat and Peter cultivars found to be highest. Acetone and ethanol extracts of fruits of Peter cultivar expressed the highest scavenging activity for superoxide radicals, while New Star extracts possessed the lowest scavenging activity for these radicals.

There were statistically significant correlation between TP content and TT content (r=0.914), as well as, between TP content and antioxidant capacity measured with some, but not all, assays (DPPH: r=0.534; ABTS: r=0.811; FRAP: r=0.928; NBT: r=0.860; total antioxidant activity: r=0.517; total reduction capacity: r=0.409). In this study, no statistically significant correlation was observed between antioxidant activity and TF content in sweet cherry fruits. Positive correlation between amount of phenolic compounds in samples of different plant origin and antioxidant capacity is supported by work of other researchers (Medić-Pap S. *et al,* 2014, 2015).

This comprehensive study clearly demonstrates the variability among cherry cultivars for total phenolics, flavonoid and tannins content, which is also reflected in their antioxidant activities. The results of this study were in good agreement with previous reports of various scientists (Faniadis D. *et al,* 2010; Ferretti G. *et al,* 2010; Prvulović D., *et al* 2012; Ognjanov V. *et al,* 2016). In general, this study suggests those sweet cherries are an excellent source of antioxidants as health-improving compounds in human diet.

CONCLUSIONS

The results of the present investigation revealed that phenolic compound contents and antioxidant capacity of extracts of sweet cherry fruits are significantly affected by genotype and maturity stage, but not by the solvent system used for the extraction process. Data on phenolic compounds investigated in this study, as well as the antioxidant capacity of extracts of sweet cherry fruits of different cultivars could be valuable to the food industry for selection of genotypes rich in nutriceuticals and could be also valuable for sweet cherry producers in order to increase the biological value of the commercial products.

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