# Influence of different extracts addition on total phenols, anthocyanin content and antioxidant activity of blackberry juice during storage

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## Summary

The aim of this study was investigation of influence of different extracts addition on total phenols, anthocyanin content, antioxidant activity and percent of polymeric colour of blackberry juice during storage of 52 days at 4 °C. Anthocyanin content of control sample (blackberry juice without extracts addition) was 149.91 mg/L. Samples with addition of extracts (olive leaf, pine bark PE 5:1, pine bark PE 95 %, green tea, red wine PE 30 %, red wine PE 4:1 and bioflavonoids had higher anthocyanin content (from 152.42 to 161.19 mg/L) in comparison to control sample. Sample with addition of bioflavonoids had the highest anthocyanin content. Samples with addition of extracts had much higher total phenol content and antioxidant activity than control sample, what was expected since extracts are rich in phenols. During storage decrease of phenols, anthocyanins and antioxidant activity occurred in higher or lesser extent, depending on extract type addition. Anthocyanin content in control sample was 119.85 mg/L. Samples with addition of bioflavonoids, olive leaf, pine bark PE 5:1 and red wine PE 4:1 had lower (from 103.44 to 118.84 mg/L), while other samples had higher (from 131.99 to 135.57 mg/L) anthocyanin content than control sample. After storage, decrease of anthocyanins was followed with increase of percent of polymeric colour, with exception of samples with addition of green tea.

*Keywords:* anthocyanins, phenols, crude extracts, polymeric colour, antioxidant activity

## Introduction

Colour is, next to texture and aroma, one of the most important quality properties of food products. Anthocyanins have a crucial role in the colour quality of many fresh and processed fruits. They are a good source of natural antioxidants, however, they are quite unstable during processing and storage (Artes, 2002; Stintzing and Carle, 2004). The temperature, pH, oxygen, and water activity are considered to be important factors influencing their stability (Tsai and Huang, 2004). It is well known that copigmentation has been suggested as a main colour stabilising mechanism in plants protecting the coloured flavylium cation from the nucleophilic attack of the water molecule (Mazza and Brouillard, 1987; Baranac et al., 1997). The attack by water converts the flavylium ion to colourless pseudobase resulting in colour loss. Formation of complex between the pigment and copigment causes a hyperchromic effect (ΔA) and a bathochromic shift (Δλ). While hypochromic effect means an increase in colour intensity, the bathochromic shift presents shift of the maximum absorbance wavelength (Chen and Hrazdina, 1981; Mazza and Miniati, 1993).

Phenolic compounds, which are present naturally in vegetables, fruits and grains exhibit wide range of physiological properties and possess the ability to reduce oxidative damage associated with many diseases, including cancer, cardiovascular diseases, cataracts, atherosclerosis, diabetes, asthma, hepatitis, liver injury, arthritis, immune deficiency diseases and ageing (Pietta et al., 1998; Lee et al., 2003; Middleton et al., 2000). The oxidative stress, defined as "the imbalance between oxidants and antioxidants in favour of the oxidants potentially leading to damage", has been suggested to be the cause of aging and various disease in humans. In modern western medicine, the balance between antioxidation and oxidation is believed to be a critical concept maintaining a healthy biological system (Davies, 2000; Tiwari, 2001; Katalinić et al., 2006).

It is possible to fortify food products with addition of extracts rich in phenolic compounds and through that increase nutritional and health-promoting value of food products. Consequently, in this study influence of addition of different plant extracts which are rich in phenolics to blackberry juice on total phenol content, anthocyanin content, antioxidant activity and formation of polymeric colour was investigated. Those parameters were also monitored during 52 days of storage at 4 °C.

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## Materials and methods

*Materials*

Blackberry was bought at local market in Osijek, Croatia and kept at –20 °C before sample preparation. Crude extracts were from Naturex (France) and obtained from Vitis d.o.o. (Croatia). Potassium chloride, sodium acetate, hydrochloric acid, methanol, sodium carbonate, sodium disulfite, Folin-Ciocalteu reagents were bought from Kemika (Zagreb, Croatia). Trolox was obtained from Sigma (Germany). 2,2'azinobis-(3-ethylbenzthiazoline-6-sulphonicacid (ABTS) and 2,2-diphenyl-1 picrilhydrazyl (DPPH) were obtained from Fluka.

*Blackberry juice preparation*

Blackberry juice was prepared by fruit pressing through cheese cloth and centrifuged, shortly heated at 90 °C for enzyme inactivation. Samples of juice were prepared without and with addition of selected extracts (0.1 %), namely olive leaf, green tea, pine bark PE 95 %, pine bark PE 5:1, red wine PE 30 %, red wine PE 4:1 and bioflavonoids. After preparation, samples were kept in dark at 4 °C for 24 hours for stabilization and after that all measurements were conducted. Samples were stored for 52 days at 4 °C and all measurements were conducted.

*Determination of total phenol content*

The Folin-Ciocalteu method (Ough and Amerine, 1988) was used to determine total phenol content. Gallic acid was used as standard to produce the calibration curve. Total phenol content was expressed in g of gallic acid equivalents (GAE)/L of sample. Measurements were done in duplicates.

*Determination of antioxidant activity*

Antioxidant activity was determined using ABTS and DPPH method. The ABTS assay followed the method of Arnao et al. (2001) with some modifications. The results were expressed in mmol trolox equivalents (TE)/100 mL of sample. Additional dilution was needed if the ABTS value was over the linear range of the standard curve.
For DPPH assay 0.2 mL of the sample was diluted with methanol and 1 mL of DPPH solution was added. After 15 minutes absorbance was read at 517 nm. The results were expressed in mmol trolox equivalents (TE)/100 mL of sample. Additional dilution was needed if the DPPH value was over the linear range of the standard curve. Measurements were done in duplicates.

*Determination of monomeric anthocyanins*

Determination of monomeric anthocyanins was conducted by pH-differential method (Giusti and Wrolstad, 2001). Total monomeric anthocyanins were expressed as cyanidin-3-glucoside. Sample absorbance was read against a blank cell containing distilled water using spectrophotometer (Jenway 6300 Spectrophotometer). The absorbance (A) of the sample was then calculated according the following formula:

$$A = (A_{\lambda vis} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\ max} - A_{700})_{pH\ 4.5}$$

where $A_{\lambda vis\ max}$ was wavelength at which maximal absorbance of samples was achieved.

The monomeric anthocyanin pigment content in the sample was calculated according the following formula:

$$\text{Anthocyanin content (mg/L)} = (A \times MW \times DF \times 1000) / (\varepsilon \times l)$$

where DF was dilution factor, MW cyanidin-3-glucoside molecular weight (449.2) and ε molar absorptivity (26,900). Measurements were done in duplicates.

*Determination of percent of polymeric colour*

Percent of polymeric colour was determined using the method described by Giusti and Wrolstad (2001). For analysis, 0.2 mL of sodium bisulfite was added to 2.8 mL diluted sample and 0.2 mL of water was added to 2.8 mL diluted sample. After equilibrating for 15 min, samples were evaluated at $\lambda_{vis\ max}$, 700 and 420 nm. Colour density was calculated using the control sample according to the following formula:

$$\text{Colour density} = [(A_{420} - A_{700}) + (A_{\lambda vis\ max} - A_{700})] \times DF$$

Polymeric colour was determined using the bisulfite-bleached sample using the following formula:

$$\text{Polymeric colour} = [(A_{420} - A_{700}) + (A_{\lambda vis\ max} - A_{700})] \times DF$$

Percent polymeric colour was calculated using the formula:

$$\%\ PC = (\text{polymeric colour/colour density}) \times 100$$

*Statistical analysis*

Anthocyanin content was analyzed by the analysis of variance (ANOVA) and Fisher's least significant difference (LSD) with significance defined at $P<0.05$. All statistical analyses were carried out using the software

program STATISTICA 8 (StatSoft, Inc, USA). The results were expressed as means ± standard deviation.

## Results and discussion

*Total phenol content*

In Table 1 results of total phenol content of blackberry juice are presented. Total phenol content in control sample was 0.52 g GAE/L. All samples with addition of extracts had much higher values, from 1.13 to 1.59 g GAE/L, with blackberry juice with addition of pine bark 95 % having the highest total phenol content. Used extracts are rich in phenols thus that tendency was expected (Kopjar et al., 2009a). During storage decrease of total phenol occurred. Control sample had the lowest total phenol content, 0.38 g GAE/L, while all other samples had higher values, from 0.71 to 1.13 g GAE/L.

**Table 1.** Total phenol content (g GAE/L) of blackberry juice without and with addition of extracts during 52 days of storage at 4 °C

| Sample | Total phenol content (g GAE/L) | |
|---|---|---|
| | Storage (days) | |
| | "0" | 52 days |
| BJ | 0.52±0.039$^{a}$ | 0.38±0.005$^{a}$ |
| BJ + olive leaf | 1.13±0.099$^{b}$ | 0.77±0.011$^{c}$ |
| BJ + pine bark PE 95 % | 1.59±0.088$^{e}$ | 1.13±0.004$^{e}$ |
| BJ + green tea | 1.57±0.011$^{e}$ | 1.03±0.010$^{d}$ |
| BJ + pine bark PE 5:1 | 1.17±0.094$^{b,c}$ | 0.72±0.023$^{b}$ |
| BJ + red wine PE 30 % | 1.35±0.024$^{d}$ | 1.02±0.041$^{d}$ |
| BJ + red wine PE 4:1 | 1.19±0.037$^{c}$ | 0.75±0.048$^{c}$ |
| BJ + bioflavonoides | 1.14±0.050$^{b}$ | 0.71±0.010$^{b}$ |

BJ – blackberry juice
Values in the same column with different superscripts (a-e) are significantly different (P<0.05) by analysis of variance (ANOVA) and Fisher's least significant difference (LSD).

*Antioxidant activity*

Results of determination of antioxidant activity of blackberry juice without and with addition of extracts during storage at 4 °C are presented in Table 2. The lowest antioxidant activity had control sample (0.46 and 0.52 mmol TE/100 mL, determined by DPPH and ABTS method, respectively), while samples with addition of extracts had higher antioxidant activity since extracts are rich in phenolics. The highest antioxidant activity had samples with green tea. (1.21 and 1.38 mmol TE/100 mL, determined by DPPH and ABTS method, respectively). During storage decrease of antioxidant activity occurred, in higher or lesser extent. In some samples there was very low decrease of antioxidant activity, probably due to formation of Maillard reaction products (Yilmaz and Toledo, 2005), or the formation of anthocyanin polymers (Brownmiller et al., 2008). Results of antioxidant activity determination by ABTS were higher than results obtained by DPPH method. Depending on applied method for determination of antioxidant activity different results could be obtained since different agents were used for determination of antioxidant activity. Various responses of various phenolic compounds in different assays (different agents used) can be obtained depending on the number of phenolic groups they have (Singleton and Rossi, 1965). Kopjar et al. (2009a) reported antioxidant activity of water plant extracts using different methods showing that same extract reacted differently with different reagents depending on used method.

**Table 2.** Antioxidant activity (mmol TE/100 mL) of blackberry juice without and with addition of extracts during 52 days of storage at 4 °C

| Sample | Antioxidant activity (mmol TE/100 mL) | | | |
|---|---|---|---|---|
| | DPPH method | | ABTS method | |
| | "0" | 52 days | "0" | 52 days |
| BJ | 0.46±0.010$^{a}$ | 0.39±0.014$^{a}$ | 0.52±0.004$^{a}$ | 0.49±0.003$^{a}$ |
| BJ + olive leaf | 0.88±0.016$^{b}$ | 0.81±0.052$^{b}$ | 1.05±0.024$^{b}$ | 1.01±0.010$^{b}$ |
| BJ + pine bark PE 95 % | 1.07±0.004$^{c}$ | 1.01±0.013$^{c}$ | 1.28±0.006$^{c}$ | 1.15±0.010$^{c}$ |
| BJ + green tea | 1.21±0.073$^{d}$ | 1.06±0.021$^{c}$ | 1.38±0.004$^{d}$ | 1.27±0.004$^{d}$ |
| BJ + pine bark PE 5:1 | 1.00±0.006$^{c,e}$ | 0.78±0.021$^{d}$ | 1.15±0.004$^{e}$ | 0.94±0.004$^{e}$ |
| BJ + red wine PE 30 % | 1.04±0.038$^{c}$ | 0.99±0.038$^{c}$ | 1.23±0.006$^{c}$ | 1.22±0.006$^{d}$ |
| BJ + red wine PE 4:1 | 0.95±0.008$^{e}$ | 0.81±0.014$^{b}$ | 1.15±0.017$^{e}$ | 1.06±0.026$^{b}$ |
| BJ + bioflavonoides | 1.01±0.042$^{c}$ | 0.71±0.010$^{d}$ | 1.23±0.034$^{c}$ | 1.05±0.003$^{b}$ |

BJ – blackberry juice
Values in the same column with different superscripts (a-e) are significantly different (P<0.05) by analysis of variance (ANOVA) and Fisher's least significant difference (LSD).

*Monomeric anthocyanin content and percent of polymeric colour*

Results of determination of monomeric anthocyanins are presented in Table 3. Anthocyanin content of control sample (blackberry juice without extracts addition) was 149.91 mg/L. Samples with addition of extracts had higher anthocyanin content in comparison to control sample. Anthocyanin content in samples with addition of extract was from 152.42 to 161.19 mg/L. Sample with addition of bioflavonoids extract had the highest anthocyanin content. During 52 days of storage at 4 °C degradation of anthocynins occurred. Since extracts are rich in phenols, copigmentation effect was most likely reason for this increase of anthocyanin content in samples with addition of extracts. It is well known that phenolic compounds act as copigments and through copigmentation effect improve anthocyanin stability. The attack by water converts the flavylium ion to colourless pseudobase resulting in colour loss. Several factors influence copigmentation, among which copigment type is the most important one (Mazza and Brouillard, 1990; Dimitrović-Marković et al., 2000; Bąkowska et al., 2003). Blackberry juice without addition of extracts had $A_{max}$ at 510 nm, while samples with addition of extracts had $A_{max}$ at higher λ. The difference in increase of λ (Δλ), i.e. bathochromic shift for different extracts was different. Δλ were 3 nm for pine bark PE 5:1 and bioflavonoides, and 2 nm for all other extracts. In red current juice increases of λ was 3 nm for catechol and 4-methyl catechol, 4 nm for gallic acid and 5 nm for catechin and chlorogenic acid (Kopjar and Piližota, 2009; Kopjar et al., 2009b). Mollov et al. (2007) found out that addition of polyphenolic copigments extracted from distilled rose petals to strawberry anthocyanins caused Δλ of 6 nm. Bąkowska et al. (2003) reported that shift of λ of cyanidin were 21.4, 14.9, 5.5, 3.8 and 2.5 nm after addition of flavones of *Scutellaria baicalensis* Georgi, quercetin-5´-sulphonic acid, sodium salt of morin-5´-sulphonic acid, tannic acid and chlorogenic acid, respectively. Also they reported that absorbance i.e. hyperchromic effect increased with addition of copigments in higher or lesser extent. Copigmentation phenomenon is not always predictable, and it is not well understood how different factors enhance or reduce this phenomenon. The structure of the anthocyanin aglycone seems to significantly affect rate and degree of copigmentation (Mazzaracchio et al., 2004), probably by influencing the degree of intramolecular copigmentation, as well as the hydration efficiency of the pyranic ring. Other important factors that influence the degree of copigmentation include pH, ionic strength of solution, temperature and pigment to copigment molar ratio (Davies and Mazza, 1993).

After storage, anthocyanin content of control sample was 119.85 mg/L. In samples with addition of olive leaf, pine bark PE 5:1, red wine PE 4:1 and bioflavonoides extracts, anthocyanin content was lower than in control sample, from 103.44 to 118.84 mg/L. In other samples, juices with addition of pine bark PE 95 %, green tea and red wine PE 30 %, anthocyanin content was higher than in control sample, from 131.99 to 135.57 mg/L, with sample with addition of pine bark PE 95 % having the highest anthocyanin content after storage.

Values of polymeric colour are represented in Table 3. Control sample had 31.91 % of polymeric colour. With addition of extracts that value increased ranging from 32.76 to 38.42 %, with an exception of sample with addition of green tea (31.9 %). During storage percent of polymeric colour increased, except in the case of samples with addition pine bark PE 95 % and red wine PE 30 %. It is possible to make correlation between anthocyanin content and % of polymeric colour after storage. With increased anthocyanin content lower % of polymeric colour was observed, with exception of sample with addition of green tea extract.

The $A440/A_{vis\ max}$ ratio values were calculated for each sample since it can be used as indicator of substitution in C-3 position of flavilium ring (Giusti et al., 1999; Longo and Vasapollo, 2005; Longo et al., 2005). After preparation there was no big difference in that ratio between samples no meter of addition of extracts (Table 3). After storage, increase of ratio was observed in all samples but the lowest increase was observed in samples with addition of pine bark PE 95 % and red wine PE 30 %. This change can be indicator of change in anthocyanin structure, thus we can assume that the smallest change of anthocyanin structure was observed in samples with addition of pine bark PE 95 % and red wine PE 30 %. Those two samples were also the samples with the highest anthocyanin content after storage. It can be observed that after storage samples with addition of pine bark PE 95 % and red wine PE 30 % had the highest anthocyanin content, the lowest percent of polymeric colour and the lowest ratio $A440/A_{vis\ max}$ indicating that there was slight polymerization observed in those samples, and that there was slight change in anthocyanin structure (substitution in C-3 position of flavilium ring). Sample with addition of green tea showed different tendency. In that sample high anthocyanin content was observed, but also there was high percent of polymeric colour and high change of ratio $A440/A_{vis\ max}$.

**Table 3.** Anthocyanin content (AC), percent of polymeric colour (PC) and ratio A440/$A_{vis\ max}$ of blackberry juice without and with addition of extracts during 52 days of storage at 4 °C

| Sample | Storage (days) | | | | | |
|---|---|---|---|---|---|---|
| | 0 | | | 52 | | |
| | AC (mg/L) | PC (%) | A440/$A_{vis\ max}$ | AC (mg/L) | PC (%) | A440/$A_{vis\ max}$ |
| BJ | 149.91±0.86[a] | 31.91 | 0.764 | 119.85±0.35[a] | 40.04 | 1.001 |
| BJ + olive leaf | 154.55±0.35[b] | 32.76 | 0.748 | 114.21±0.18[b] | 41.33 | 1.078 |
| BJ + pine bark PE 95% | 152.66±0.18[c] | 34.27 | 0.777 | 135.57±0.53[c] | 35.34 | 0.822 |
| BJ + green tea | 152.29±0.35[c] | 31.90 | 0.741 | 131.99±0.18[d] | 43.08 | 1.050 |
| BJ + pine bark PE 5:1 | 152.42±0.18[c] | 38.42 | 0.799 | 112.21±0.53[b] | 43.30 | 1.065 |
| BJ + red wine PE 30 % | 153.55±0.35[b] | 34.25 | 0.757 | 133.45±0.35[e] | 34.98 | 0.828 |
| BJ + red wine PE 4:1 | 158.56±0.71[e] | 32.99 | 0.754 | 118.84±0.35[a] | 40.04 | 0.991 |
| BJ + bioflavonoides | 161.19±0.53[f] | 34.39 | 0.766 | 103.44±0.18[f] | 44.45 | 1.081 |

BJ – blackberry juice
Values in the same column with different superscripts (a-f) are significantly different ($P<0.05$) by analysis of variance (ANOVA) and Fisher's least significant difference (LSD).

Probably different mechanism was involved in anthocyanin retention in sample with addition of green tea, pine bark PE 95 % and red wine PE 30 %. In samples with pine bark PE 95 % and red wine PE 30 % addition there was slight polymerisation and change in structure at C-3 position in flavilium ring involved, while in sample with green tea addition polymerisation and change in structure probably caused retention of anthocyanins.

In all other samples, the losses of monomeric anthocyanins were most likely due to the formation of anthocyanin polymers during storage. Several authors reported the same tendency. Brownmiller et al. (2008) investigated processing and storage effect on monomeric anthocyanins and percent of polymeric colour of processed blueberry products and observed that the losses of monomeric anthocyanins were most likely due to the formation of anthocyanin polymers during the juice processing. Hager et al. (2008) investigated processing and storage effect on monomeric anthocyanins and percent of polymeric colour of processed black raspberry products. During storage anthocyanin content decreased while polymeric colour increased. Anthocyanin losses were also accompanied by increased percent polymeric colour values in raspberry pulp stored at different temperatures (Ochoa et al., 1999). Chaovanalikit and Wrolstad (2004) also observed that with decrease of anthocyanin content increase of polymeric colour occurred during storage of fresh and processed cherries. Potential mechanism for polymerization involves condensation reactions of anthocyanins with other phenolic compounds, including flavan-3-ols or polyflavan-3-ols that can be mediated by acetaldehyde (Es-Safi et al., 1999) and furfural (Es-Safi et al., 2000), or via direct anthocyanin-tannin reactions (Reed et al., 2005; Remy et al., 2000; Brownmiller et al., 2008; Hager et al., 2008).

## Conclusions

In this study influence of different extracts on total phenol content, anthocyanin content, antioxidant activity and formation of polymeric colour of blackberry juice was investigated. After preparation of samples increase of anthocyanin content was observed when extract were added, probably due to copigmentation effect. During storage degradation of phenols and anthocyanins occurred. Loss of anthocyanins was followed by formation of polymeric colour, with exception of sample with addition of green tea. That sample had higher anthocyanin content than control sample, but also high percent of polymeric colour. This research could be benefit from nutritional point, but also from the technological point of view. These findings could be useful for development of functional food products and improvement of nutritional and health-promoting values of food products, since food products with higher anthocyanins and phenolic compounds content could be more effective against oxidative stress in human body.

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