UNIVERSITY OF LJUBLJANA
BIOTECHNICAL FACULTY

Špela MOŽE BORNŠEK

BIOEFFICACY OF ANTHOCYANINS FROM BILBERRIES (*VACCINIUM MYRTILLUS* L.)

DOCTORAL DISSERTATION

Ljubljana, 2012

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BIOLOŠKA UČINKOVITOST ANTOCIANOV GOZDNIH BOROVNIC (*VACCINIUM MYRTILLUS* L.)

DOKTORSKA DISERTACIJA

Ljubljana, 2012

On the basis of the Statute of University of Ljubljana, and by decisions of Senate of the Biotechnical Faculty and the decision of the University Senate, dated from July 9th 2009, the continuation to doctoral postgraduate studies of Biological and Biotechnical Sciences, field: Food Science and Technology, was approved. Prof. Veronika Abram, PhD, as the supervisor, and Assist. Prof. Andreja Vanzo, PhD, as the co-advisor, were confirmed.
Na podlagi Statuta Univerze v Ljubljani ter po sklepu Senata Biotehniške fakultete in sklepa Senata Univerze z dne, 9. 7. 2009, je bilo potrjeno, da kandidatka izpolnjuje pogoje za neposreden prehod na doktorski podiplomski študij bioloških in biotehniških znanosti ter opravljanje doktorata znanosti s področja živilstva. Za mentorico je bila imenovana prof. dr. Veronika Abram ter za somentorico doc. dr. Andreja Vanzo.

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The dissertation thesis is a result of my own research work.
Naloga je rezultat lastnega raziskovalnega dela.

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AB Berries, including bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.) have an important role in human diet. The aim of this dissertation was to assess the biological efficacy of Slovenian bilberries and blueberries. The study included a determination of phenolic content, an estimation of antioxidant potentials in the absence and presence of biologic structures (cells), anthocyanin cellular uptake and their interactions with a model of cell membrane and a membrane transport protein bilitranslocase. Determined spectrophotometrically, bilberries were richer in total phenolics and total anthocyanins than blueberries. Using LC-MS/MS, 15 anthocyanins were determined as major phenolics in both, the bilberries and blueberries. Delphinidin and cyanidin glycosides were predominant in the bilberries, malvidin glycosides in the blueberries while peonidin glycosides were the minor anthocyanidin glycosides in both berry species. By LC-MS two flavanols (epicatechin, catechin), three flavonols (quercetin, myricetin, rutin), six phenolic acids (chlorogenic, caffeic, ferulic, ellagic, gallic, *p*-coumaric) and one stilbene (*trans*-resveratrol) were determined. In both, bilberries and blueberries, phenolic acids were found to be present in high amounts while chlorogenic acid was the predominant. Statistical analysis showed that the content and the pattern of the individual phenolics can be used to identify the picking region of the Slovenian bilberries. The antioxidant activity of the bilberry extracts determined by DPPH method correlated linearly to their anthocyanin content. The intracellular antioxidant capabilities of the extracts were assessed by the CAA assay using four different cell lines (Caco-2, HepG2, EA.hy926 and A7r5) with the intention to follow the absorption of anthocyanins in the gastrointestinal tract and their distribution to the liver and blood vessels. The results showed that anthocyanins acted as powerful intracellular antioxidants even in nanomolar concentrations which were reported to be found in plasma after the oral administration. We found out that cyanidin 3-glucoside can enter into cells of liver and vascular endothelium in intact form, while in the liver cells it can also be metabolized. The spectrofluorimetrical investigation of cyanidin 3-glucoside interaction with liposomes showed that anthocyanins cannot pass cell membrane by diffusion. The CAA assay, which included an incubation of cells with bilitranslocase antibodies, showed that hepatic and endothelial cellular uptake of cyanidin 3-glucoside is carrier-mediated by bilitranslocase.

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AI Jagodičje, vključno gozdne (*Vaccinium myrtillus* L.) in ameriške borovnice (*Vaccinium corymbosum* L.), predstavlja pomemben delež v vsakodnevni prehrani človeka. Cilj doktorske disertacije je bil raziskati biološko učinkovitost gozdnih in ameriških borovnic iz Slovenije. V obeh vrstah borovnic smo določili fenolne spojine, ocenili njihov antioksidativni potencial v prisotnosti bioloških struktur (celic) in brez njih, ocenili privzem antocianinov v celice ter preučili njihove interakcije s celično membrano in transportnim proteinom bilitranslokazo. Gozdne borovnice so bile v primerjavi z ameriškimi bolj bogate s skupnimi fenolnimi spojinami in skupnimi antocianini, ki smo jih določili spektrofotometrično. S LC-MS/MS smo določili 15 antocianinov, ki so predstavljali večino vseh analiziranih fenolnih spojin. Delfinidin in cianidin glikozidi so prevladovali v gozdnih borovnicah, malvidin glikozidi v ameriških borovnicah, peonidin glikozidi pa so bili najmanj zastopani v obeh vrstah borovnic. S LC-MS smo določili tudi dva flavanola (epikatehin, katehin), tri flavonole (kvercetin, miricetin, rutin), šest fenolnih kislin (klorogenska, kavna, ferulna, elagična, galna, *p*-kumarna) in en stilben (*trans*-resveratrol). Med njimi smo v obeh vrstah borovnic našli največ fenolnih kislin, med katerimi je prevladovala klorogenska. Statistična obdelava je pokazala, da se da z identificiranimi fenolnimi spojinami zelo dobro določiti lokacijo rastišča gozdnih borovnic. Antioksidativni potencial ekstrakta iz gozdnih borovnic smo določili z DPPH metodo in ugotovili, da je le-ta linearno odvisen od koncentracije antocianinov. Intracelularni antioksidativni potencial ekstraktov iz obeh borovnic smo določili s CAA metodo s štirimi celičnimi linijami (Caco-2, HepG2, EA.hy926 in A7r5), ki predstavljajo model za študijo absorpcije antocianinov v prebavnem traktu in distribucijo v tkiva, kot so jetra in ožilje. Ugotovili smo, do so ekstrakti borovnic in njihovi antocianini zelo dobri intracelularni antioksidanti in so aktivni tudi pri nanomolarnih koncentracijah, ugotovljenih v plazmi po zaužitju. Ugotovili smo, da lahko cianidin-3-glukozid vstopi v celice jeter in žilnega endotelija v nespremenjeni obliki, a le v jetrnih lahko pride do njihovega metabolizma. Ugotovili smo tudi, da antocianini z difuzijo ne morejo prehajati skozi modelno celično membrano. Z modificirano CAA metodo, ki je vključevala inkubacijo celic z bilitranslokaznimi protitelesi, pa smo dokazali, da antocianin cianidin-3-glukozid potrebuje bilitranslokazo za prehod skozi celično membrano jetrnih in žilnih endotelijskih celic.

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ABBREVATIONS AND ACRONYMS

ABAP	2,2'-azobis(2-amidinopropane)dihydrochloride
AUC	area under the curve
A7r5	smooth muscle cell line
CAA	cellular antioxidant activity
Caco-2	heterogenous human epithelial colorectal adenocarcinoma cell line
DCF	dichlorofluorescein
DCFH	2',7'-dichlorofluorescin
DCFH-DA	2',7'-dichlorofluorescin diacetate
ΔA	difference of anisotropy
ΔP	difference of polarisation
DMSO	dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle's Medium
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPH	2,2-diphenyl-1-picrylhydrazyl
EA.hy926	human endothelial cell line
ESI	electrospray ionizator source
ESI^-	electrospray ionizator source in the negative ion mode
ESI^+	electrospray ionizator source in the positive ion mode
eUV	extruded unilamellar vesicles
FBS	fetal bovine serum
HBSS	Hanks' Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HepG2	hepatocellular carcinoma human cell line
HPLC	high performance liquid chromatography
IgG	immunoglobulin G
LC-MS	liquid chromatography - mass spectrometry
LC-MS/MS	liquid chromatography - tandem mass spectrometry
LDA	linear discriminant analysis
MAP	multiple antigen peptide (bilitranslocase antibody)
MEME	Minimal Essential Medium Eagle
MS	mass spectrometry
PBS	phosphate saline buffer
PC	phosphatidylcholine
PCA	principal component analysis
ROS	reactive oxygen species
SM	sphingomyelin
SPE	solid phase extraction
UV	ultraviolet spectral field of light
VIS	visible spectral field of light

1 INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.) is a low-growing shrub arisen from northern Europe and than spread to Asia and North America while highbush blueberry (*Vaccinium corymbosum* L.) is a native species from North America. Both, bilberry and blueberry are members of the family *Ericaceae* and genus *Vaccinium*. In Slovenia blueberries are commercially cultivated on plantations while bilberries are grown in the woods and are traditionally collected during their harvest season in June and July. Both, bilberries and blueberries are consumed in Slovenia as fresh fruits, or are processed into various foods, such as jams, syrups, alcoholic and nonalcoholic beverages, teas and sweets.

There is a worldwide interest in berries including bilberries and blueberries due to their high content of natural antioxidants such as phenolic compounds, especially anthocyanins. Beside high quantities of anthocyanins, bilberries and blueberries contain other phenolics such as flavonols, flavones, flavonones, isoflavones, tannins, phenolic acids, lignins and stilbenes. Their content varies with geographical region and cultivar.

Anthocyanins, as the most abudant phenolics in bilberries and blueberries, are part of a large and widespread group of plant phenolic constituents, known as flavonoids. They are responsible for a blue or purple color of bilberries and blueberries. As anthocyanins are common component of human daily diet, many researches have concentrated on the investigation about their biological activities and possible health benefits in protecting against some chronic diseases, including cancer, cardiovascular and neurodegenerative diseases and diabetes. Some of these biological activities and protective functions may be attributable to their high antioxidant capacities. Studies on anthocyanins antioxidant capacities revealed that they could act in scavenging free radicals and metal chelation. To evaluate the health benefits of anthocyanins in humans, the bioavailability including absorption, distribution, metabolism, and excretion must be known.

1.1 AIM OF THE STUDY

The aim of the study was to estimate the phenolic content in bilberries sampled in woods from seven different locations in Slovenia, and blueberries sampled on the plantation in Ljubljansko barje in Slovenia. Total phenolics, and total anthocyanins content were estimated spectrophotometrically while individual anthocyanins, flavanols, flavonols, phenolic acids and stilbenes were determined by liquid chromatography connected to mass spectrometry. Furthermore, the antioxidant properties, metabolism and the mechanisms of transport of anthocyanins and anthocyanidins through mammalian were studied.

The estimation of the phenolic content in Slovenian bilberries and blueberries was performed as follows:

- The total phenolics were determined spectrophotometrically by Folin-Ciocalteu method.
- The total anthocyanins were determined spectrophotometrically.
- The individual anthocyanins were determined by LC-MS/MS.
- The content of flavanols, flavonols, phenolic acids and resveratrol were determined by LC-MS.
- The examination of potential correlations with the locations of the bilberry sampling was evaluated by a statistical analyses.

Antioxidant properties were preformed as follows:

- *In vitro* antioxidant test based on radical scavenging capacity by DPPH was used to estimate antioxidant potential of bilberry extracts.
- Cellular antioxidant activity (CAA) assay was used to measure antioxidant capabilities of crude bilberry, purified bilberry, crude blueberry extracts and anthocyanin cyanidin 3-glucoside in four different cell lines (Caco-2, HepG2, EA.hy926 and A7r5). These cell lines were used with the intention to follow absorption of anthocyanins in those cells which may reflect their tissue distribution from the gastro-intestinal tract to various tissues such as liver and blood vessels. The purpose was to compare intracellular antioxidant potentials of bilberry and blueberry extracts. Further purified bilberry extract and anthocyanin cyanidin 3-glucoside were applied to confirm if they are responsible for the antioxidant activity in the tested cell lines.

The transport mechanisms of anthocyanins and anthocyanidins across the cell membrane and their metabolism were studied as follows:

- LC-MS/MS method was used to examine the accumulation of anthocyanins (e. g. cyanidin 3-glucoside) into the HepG2 and EA.hy926 cells and to identify its metabolites.
- The modified CAA assay was used to study a role of bilitranslocase in anthocyanin transport across the cell membrane by using Caco-2, EA.hy926 and A7r5 cell lines. The modified procedure of basic CAA included an incubation of cyanidin or cyanidin 3-glucoside in the presence or absence of bilitranslocase antibodies.
- The measurements of fluorescence polarisation and anisotropy in the presence of fluorophor 1,6-diphenyl-1,3,5-hexatriene (DPH) were used to investigate the interaction of cyanidin and cyanidin 3-glucoside with model membranes – liposomes. They were prepared from phosphatidylcholine (PC) and sphingomyelin (SM) as a model membrane of endothelial cells.

1.2 THE HYPOTHESIS

- Bilberries and blueberries from Slovenia are a good source of phenolics, especially anthocyanins.
- Beside high content of anthocyanins bilberries and blueberries contain other phenolic compounds, like flavanols, flavonols, phenolic acids, and stilbenes.
- Bilberries are richer in anthocyanin content than blueberries.
- The content and profile (or pattern) of anthocyanins in bilberries and blueberries could be significantly different.
- Anthocyanins from bilberries and blueberries can be taken up by mammalian cells and there they might act as antioxidants.
- Anthocyanins can enter into cells in their intact form and can be metabolised there.
- Anthocyanidins and anthocyanins can not pass the cell membrane by passive diffusion.
- The membrane transport protein bilitranslocase is involved in anthocyanidin and anthocyanin transport through a cell membrane.

2 LITERATURE REVIEW

2.1 PHENOLICS

Phenolics are a diverse class of plant secondary metabolites that are synthetised from pentose phosphate via shikimate and phenylpropanoid pathways in plants. The main dietary sources of phenolics are fruits, vegetables and beverages such as fruit juice, coffee, tea and wine. Their total intake to the human body is estimated to be about 1 g per day. Its content in fruits and vegetables depends on intrinsic (genus, species, cultivars) and extrinsic (agronomic, environmental, handling and storing) factors. They are the most widely occurring group of phytochemicals and have various functions, e.g. color of leaves, flowers and fruits, taste of food, antimicrobial function, antifungal function, insect feeding deterrence, screening from damage by solar UV radiation, chelation of toxic heavy metals and antioxidant protection from free radicals (Bravo, 1998; Balasundram et al., 2006; D'Archivio et al., 2007; Singh et al., 2008).

Structurally, they are characterized by the presence of one or more six-carbon aromatic rings and two or more hydroxyl groups, linked directly to the aromatic ring. A straightforward classification attempts to divide the broad category of phenolics into simple phenols and polyphenols, based exclusively on the number of phenol subunits present. Thus, the term "plant phenolics" encompasses simple phenols, phenolic acids, coumarins, flavonoids, stilbenes, tannins, lignans, and lignins. On the other hand, phenolics can be divided into two categories, flavonoids and non-flavonoid phenolics. Usually in the nature, non-flavonoid phenolics (phenolic acids, lignins, stilbenes) account for about one-third of the total phenolics, whereas flavonoids (anthocyanins, flavanols, flavonols, flavones, flavonones, isoflavones, and tannins) account for two-thirds (Bravo, 1998; Scalbert and Williamson, 2000; Stalikas, 2007; Stevenson and Hurst, 2007).

2.1.1 Phenolics of bilberries and blueberries

The content of phenolics in berries is affected by the degree of maturity at harvest, by the cultivar, and by the pre- and postharvest environments. Flavonoids (anthocyanins, flavanols and flavonols) and non-flavonoids (phenolic acids and stilbenes) were found in bilberries and blueberries. Commonly, the occurrence of total phenolics is determined spectrophotometrically by using Folin-Ciocalteu reagent (Folin and Ciocalteu, 1927) and is usually expressed as gallic acid equivalents. Bilberries were reported to have 525 - 890 mg/100 g fw of total phenolics while blueberries 115 - 444 mg/100 g fw. Table 1 shows the review of the total phenolics content in bilberries and blueberries.

Table 1: The literature review of total phenolics content in bilberries and blueberries.
Preglednica 1: Pregled literaturnih podatkov o vsebnosti skupnih fenolnih spojin v gozdnih in ameriških borovnicah.

TOTAL PHENOLICS	**mg/100 g fw**	**Reference**
Bilberries	525.0 ± 5.0	Prior et al., 1998
	577 - 614	Giovanelli and Buratti, 2009
	890 ± 9	Šavikin et al., 2009
Blueberries	260.9 - 347	Prior et al., 1998
	179	Ehlenfeldt and Prior, 2001
	444 ± 155	Moyer et al, 2002
	399.28 ± 149.12	Sellappan et al., 2002
	373 ± 8	Zheng et al., 2003
	115 ± 4	Taruscio et al., 2004
	190.3 - 319.3	Wang et al., 2008a
	298 – 310	Giovanelli and Buratti, 2009

Flavonoids are the largest group of phenolics. Structurally they are low molecular weight compounds consisting of fifteen carbon atoms, arranged in a C_6-C_3-C_6 configuration (Figure 1). The basic structure consists of two aromatic rings A and B, joined by a 3-carbon bridge, commonly in the form of heterocyclic central pyran ring C. The ring A is derived from the acetate/malonate pathway, while the ring B is derived from phenylalanine through shikimate pathway. Flavonoids can be divided into six subclasses, depending on the oxidation state of the ring C: flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols. Numerous flavonoids have been identified in plants due to the occurrence of many substitution patterns in which primary substituents (as hydroxyl group) can themselves be substituted (i. e. additionally glycosylated or acylated), sometimes yielding highly complex structures (Bravo, 1998; Balasundram et al., 2006; D'Archivio et al., 2007).

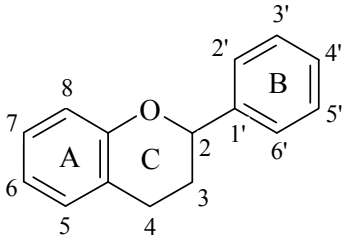
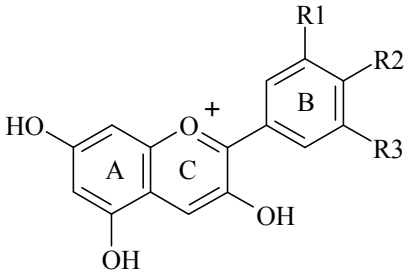


Figure 1: Basic structural formula of a flavonoid (Bravo, 1998; Balasundram et al., 2006).
Slika 1: Osnovna strukturna formula flavonoida (Bravo, 1998; Balasundram in sod., 2006).

2.1.1.1 Anthocyanins

Anthocyanins (Greek anthos = flower and kyanos = blue) are water-soluble pigments in fruits and vegetables. In plant cells, they are present in vacuoles in the form of various sized granules. Chemically, anthocyanins belong to a larger group of compounds known as

flavonoids. Anthocyanidins (aglycones) are the basic structures of anthocyanins. The anthocyanidins consist of an aromatic ring A bonded to an heterocyclic ring C that contains oxygen, which is also bonded by carbon-carbon bond to a third aromatic ring B. The anthocyanidins (aglycone forms) are highly unstable, due to that they commonly occur in glycoside forms. The sugar moiety is mainly attached at the 3-position on the ring C or at the 5, 7-position on the ring A. Glucose, galactose, arabinose, rhamnose and xylose are the most common sugars that are bonded to the anthocyanidins. In such forms they are known as anthocyanins. More than 500 different anthocyanins and 23 anthocyanidins have been found. The most common anthocyanidins in the nature are pelargonidin, peonidin, cyanidin, malvidin, petunidin and delphinidin (Figure 2) while the most widespread anthocyanin is cyanidin 3-glucoside (Manach et al., 2004; Szajdek and Borowska, 2008; Castañeda-Ovando et al., 2009).



Anthocyanidin	R1	R2	R3
Pelargonidin	H	OH	H
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Peonidin	OCH_3	OH	H
Petunidin	OCH_3	OH	OH
Malvidin	OCH_3	OH	OCH_3

Figure 2: Structural formula of anthocyanidins (Prior and Wu, 2006).
Slika 2: Strukturna formula antocianidinov (Prior in Wu, 2006).

Anthocyanins impart a pink, red, blue or purple color according to pH (Figure 3). At pH 1, the flavylium cation is the predominant species and contributes to purple and red colors. At pH between 2 and 4, the quinoidal blue species are predominant. At pH between 5 and 6 two colorless carbinol pseudobase and chalcone can be observed. At pH higher then 7, the anthocyanins are degraded. However, anthocyanins are highly unstable and susceptible to degradation. Their stability is affected not only by pH but also by solvents, temperature, concentration, oxygen, light and enzymes. Co-pigmentation is the phenomenon of anthocyanins in which the presence of accompanying compounds such as other organic compounds (e. g. flavonoids, alkaloids, amino acids, proteins, nucleotides, polysaccharides) or metallic ions form molecular or complex associations, generating a charge or an increment in the color intensity (McGhie and Walton, 2007; Castañeda-Ovando et al., 2009).

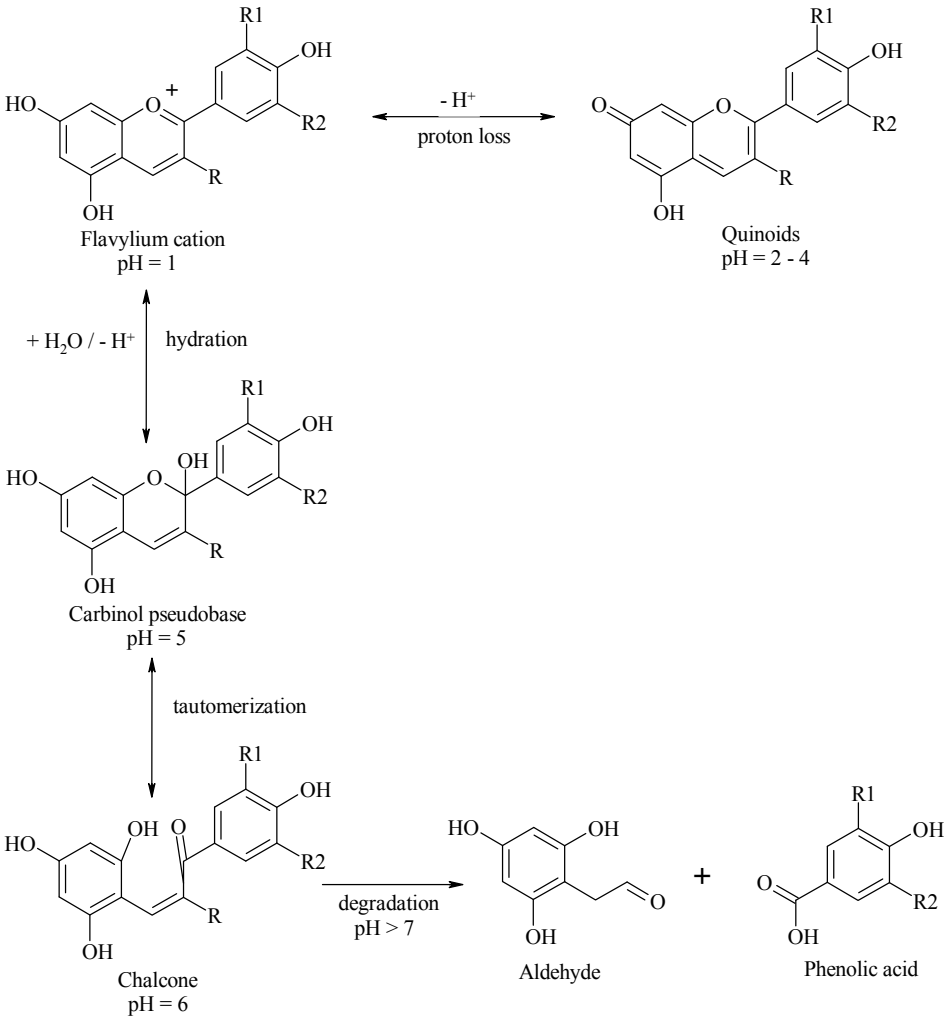


Figure 3: Anthocyanin chemical forms depending on pH and degradation reaction, where R = glycosyl, R1 and R2 = H, OH or OCH_3 (McGhie and Walton, 2007; Castañeda-Ovando et al., 2009).

Slika 3: Strukturne formule različnih oblik antocianinov v odvisnosti od pH in reakcija razgradnje, kjer je R = glikozil, R1 in R2= OH ali OCH_3 (McGhie in Walton, 2007; Castañeda-Ovando in sod., 2009).

Anthocyanins are the abundant phenolics in bilberries and blueberries. Their content in bilberries has been found to be clearly higher than in blueberries (Burdulis et al., 2009; Giovanelli and Buratti, 2009) due to anthocyanin presence in the skin and pulp of bilberry but only in the skin of blueberry (Riihinen et al., 2008). However, anthocyanins present nearly 90 % of total phenolic compounds in bilberries (Heinonen, 2007). Their content in berries is usually proportional to the color intensity and increases by fruit ripeness. The content of anthocyanins can be measured spectrophotometrically or by HPLC. Reported values of the total anthocyanins, determined spectrophotometrically, ranged from 230 to 370 mg/100 g fw in bilberries whereas from 32 to 208 mg/100 g fw in blueberries. Table 2 shows the review of total anthocyanins content, determined spectrophotometrically, in bilberries and blueberries.

Table 2: The literature review of total anthocyanins in bilberries and blueberries, determined spectrophotometrically.
Preglednica 2: Pregled literaturnih podatkov o vsebnosti skupnih antocianinov v gozdnih in ameriških borovnicah, določenih spektrofotometrično.

ANTHOCYANINS (determined spectrophotometrically)		
	mg/100 g fw	**Reference**
Bilberries	229.6 ± 12.9	Prior et al., 1998
	370	Kalt et al., 1999
	330 - 344	Giovanelli and Buratti, 2009
Blueberries	92.6 - 129.2	Prior et al., 1998
	83 - 250	Kalt et al. 1999
	95	Ehlenfeldt and Prior, 2001
	208 ± 78	Moyer et al., 2002
	84.12 ± 39.72	Sellappan et al., 2002
	182 ± 9	Zheng et al., 2003
	82 ± 4	Taruscio et al., 2004
	82.36 - 131.02	Wang et al., 2008a
	92 - 129	Giovanelli and Buratti, 2009

Anthocyanin content, determined by HPLC, ranging from 350 to 525 mg/100 g fw in bilberries, was determined in many studies, but in Finnish bilberries it was reported to be much higher, about 2129.6 mg/100 g fw. Using HPLC, blueberries were reported to have 49 - 624 mg/100 g fw of anthocyanins. Table 3 shows the results of a review of total anthocyanins, determined by HPLC, in bilberries and blueberries.

Table 3: The literature review of total anthocyanins in bilberries and blueberries determined by HPLC.
Preglednica 3: Pregled literaturnih podatkov o vsebnosti antocianinov v gozdnih in ameriških borovnicah, določenih s HPLC.

ANTHOCYANINS (determined by HPLC)		
	mg/100 g fw	**Reference**
Bilberries	360 ± 3	Nyman and Kumpulainen, 2001
	472.3 ± 13.1	Kähkönen et al., 2003
	350 - 525	Lätti et al., 2008
	2129.6	Riihinen et al., 2008
Blueberries	109.30 - 112.30	Gao and Mazza, 1994
	624.2	Riihinen et al., 2008
	49 - 423	Scalzo et al., 2008

Likewise the difference between anthocyanin profiles of bilberries and blueberries was found to be significant (Burdulis et al., 2009; Giovanelli and Buratti, 2009). Both bilberries and blueberries contained 15 anthocyanins: glucosides, galactosides and arabinosides of 5 anthocyanidins (cyanidin, petunidin, peonidin, delphinidin and malvidin) (Figure 4). Delphinidin and cyanidin glycosides were the predominant anthocyanidin glycosides in bilberries (Kalt et al., 1999; Nyman and Kumpulainen, 2001; Kähkönen et al., 2003;

Määttä-Riihinen et al., 2004; Zhang et al., 2004; Koponen et al., 2007; Lätti et al., 2008; Åkerström et al., 2009). In blueberries, some publications reported that the most abundant anthocyanidin glycosides were malvidin glycosides (Kader et al., 1996; Määttä-Riihinen et al., 2004; Brambilla et al., 2008; Wang et al., 2008a; Burdulis et al., 2009) while others concluded that the predominant were delphinidin glycosides (Kalt et al., 1999; Taruscio et al., 2004). Peonidin glycosides were the least present anthocyanidin glycosides in both bilberries and blueberries (Kader et al., 1996; Nyman and Kumpulainen, 2001; Kähkönen et al., 2003a; Määttä-Riihinen et al., 2004; Taruscio et al., 2004; Zhang et al., 2004; Koponen, et al., 2007; Brambilla et al., 2008; Lätti et al., 2008; Åkerström et al., 2009).

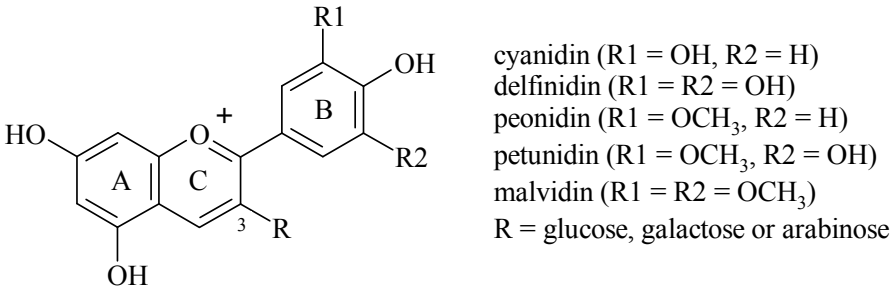


Figure 4: Structural formula of anthocyanins from bilberries and blueberries (Manach et al., 2004; Castañeda-Ovando et al., 2009).

Slika 4: Strukturne formule antocianinov gozdnih in ameriških borovnic (Manach in sod., 2004; Castañeda-Ovando in sod., 2009).

2.1.1.2 Flavanols

Flavanols or flavan-3-ols are flavonoids characterized by a saturated 3-C chain. They exist in both, the monomer and the polymer form (catechins and proanthocyanidins respectively). Four main flavanols were found in fruits. Two were hydroxylated in the 3'- and 4'- positions in the B-ring ([+]-catechin and [–]-epicatechin) (Figure 5) and two were trihydroxylated in the 3'-, 4'-, and 5'-positions ([+]-gallocatechin and [–]-epigallocatechin) (Macheix et al., 1990). Previously published reports showed 0.7 - 2.3 mg/100 g fw of catechin and 1.3 - 6.8 mg/100 g fw of epicatechin in bilberries. Meanwhile, blueberries have either 0.6 - 29.3 mg/100 g fw of catechin or it was not detected and 0.7 - 1.6 mg/100 g fw of epicatechin or it was not detected. Table 4 shows the content of flavanol in bilberries and blueberries.

Table 4: The literature review of flavanol content in bilberries and blueberries.
Preglednica 4: Pregled literaturnih podatkov o vsebnosti flavanolov v gozdnih in ameriških borovnicah.

FLAVANOLS	**mg/100 g fw**	**Reference**
Catechin		
Bilberries	1.17	Stöhr and Herrmann, 1975
	0.7	Määttä-Riihinen et al., 2004
	1.06 - 2.25	Tsanova-Savova et al., 2005
	not detected	Ehala et al., 2005
Blueberries	not detected	Arts et al., 2000
	9.87 - 29.28	Sellappan et al., 2002
	0.6	Määttä-Riihinen et al., 2004
	3.73 ± 1.52	Taruscio et al., 2004
Epicatechin		
Bilberries	1.27	Stöhr and Herrmann, 1975
	6.8	Määttä-Riihinen et al., 2004
	1.43 - 4.53	Tsanova-Savova et al., 2005
Blueberries	1.11 ± 0.10	Arts et al., 2000
	not detected	Sellappan et al., 2002
	0.7	Määttä-Riihinen et al., 2004
	1.59 ± 0.80	Taruscio et al., 2004

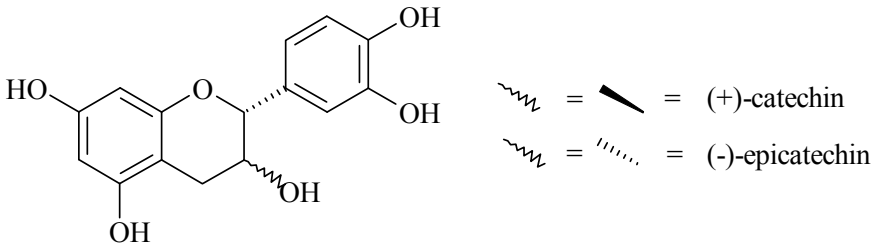


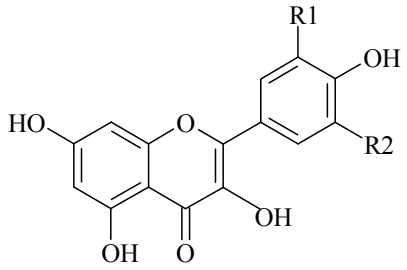
Figure 5: Structure of flavanols from bilberries and blueberries (Macheix et al., 1990).
Slika 5: Strukturni formuli flavanolov gozdnih in ameriških borovnic (Macheix in sod., 1990).

2.1.1.3 Flavonols

Flavonols are flavonoids characterized by unsaturated 3-C chain with a double bond between C-2 and C-3 and by presence of a hydroxyl group in the third position (Macheix et al., 1990). Their biosynthesis is stimulated by light, so they are accumulated in outer and aerial tissue of plants. They represent the most ubiquitous flavonoids in food (D'Archivio et al., 2007). Quercetin and myricetin were detected in both, bilberries and blueberries (Figure 6). Published values of quercetin range from 1.3 to 17.1 mg/100 g fw in bilberries and between 1.7 to 53.1 mg/100 g fw in blueberries. Myricetin varied from 1.2 to 5.0 mg/100 g fw in bilberries and from 0.8 - 8.6 mg/100 g fw in blueberries. Table 5 shows the content of flavonols in bilberries and blueberries.

Table 5: The literature review of flavonol content in bilberries and blueberries.
Preglednica 5: Pregled literaturnih podatkov o vsebnosti flavonolov v gozdnih in ameriških borovnicah.

FLAVONOLS	mg/100 g fw	Reference
Quercetin		
Bilberries	2.9 - 3.0	Häkkinen et al., 1999b
	1.7 ± 0.16	Häkkinen and Törrönen, 2000
	8.1	Määttä-Riihinen et al., 2004
	1.275 ± 0.070	Ehala et al., 2005
	17.1	Riihinen et al., 2008
Blueberries	1.7 - 2.4	Häkkinen et al., 1999b
	2.2 - 4.6	Häkkinen and Törrönen, 2000
	9.70 -14.60	Sellappan et al., 2002
	9.5	Määttä-Riihinen et al., 2004
	1.29 ± 0.14	Taruscio et al., 2004
	53.1	Riihinen et al., 2008
Myricetin		
Bilberries	1.4 - 2.1	Häkkinen et al., 1999b
	1.2 ± 0.11	Häkkinen and Törrönen, 2000
	3.1	Määttä-Riihinen et al., 2004
	5.0	Riihinen et al., 2008
Blueberries	2.3 - 2.6	Häkkinen et al., 1999b
	0.8 - 1.8	Häkkinen and Törrönen, 2000
	6.72 - 6.98	Sellappan et al., 2002
	1.7	Määttä-Riihinen et al., 2004
	8.64 ± 0.30	Taruscio et al., 2004
	3.1	Riihinen et al., 2008



Quercetin (R1 = OH, R2 = H)
Myricetin (R1 = R2 = OH)

Figure 6: Structural formulae of flavonols from bilberries and blueberries (Macheix et al., 1990).
Slika 6: Strukturne formule flavonolov gozdnih in ameriških borovnic (Macheix in sod., 1990).

2.1.1.4 Phenolic acids

The term "phenolic acids", in general, designates phenols that possess one carboxyl group. Phenolic acids consist of two subgroups, the hydroxybenzoic acids (C_6-C_1 as a basic structure) and hydroxycinnamic acids (C_6-C_3 as a basic structure). Hydroxybenzoic acids include benzoic, *p*-hydroxybenzoic, vanillic, gallic, protocatechuic, syringic, gentisic, veratric and salicylic acids while hydroxycinnamic acids include cinnamic, *o*-coumaric, *m*-

coumaric, *p*-coumaric, ferulic, sinapic and caffeic acids (Bravo, 1998; Balasundram et al., 2006; Stalikas, 2007). Various phenolic acids were found in bilberries and blueberries (Figure 7): chlorogenic, caffeic, ferulic, *p*-coumaric, gallic and ellagic acids. Chlorogenic acid is an ester formed between caffeic acid and quinic acid while ellagic acid is an ester of 2 gallic acids. It is actually the dilactone of hexahydroxydiphenic acid. The content of chlorogenic acid varies from 14.1 ± 1.3 mg/100 g fw in bilberries and from 4.3 - 126.1 mg/100 g fw in blueberries. Caffeic acid ranges from 0.3 to 10.6 mg/100 g fw in bilberries and from 0.5 to 18.2 mg/100 g fw in blueberries. Ferulic acid is reported to be from 0.3 to 2.3 mg/100 g fw in bilberries and from 3.5 to 4.4 mg/100 g fw in blueberries. The content of *p*-coumaric acid in bilberries ranged from 0.1 to 13.2 mg/100 g fw while in blueberries from 0.5 to 7.2 mg/100 g fw. Bilberries are reported to have 0.1 - 3.2 mg/100 g fw of gallic acid and from 2.0 - 4.8 mg/100 g fw in blueberries. Ellagic acid was found in both, bilberries and blueberries but only one publication reported of ellagic acid content in blueberries, ranging from 0.75 to 6.65 mg/100 g fw. Table 6 shows the content of phenolic acids in bilberries and blueberries.

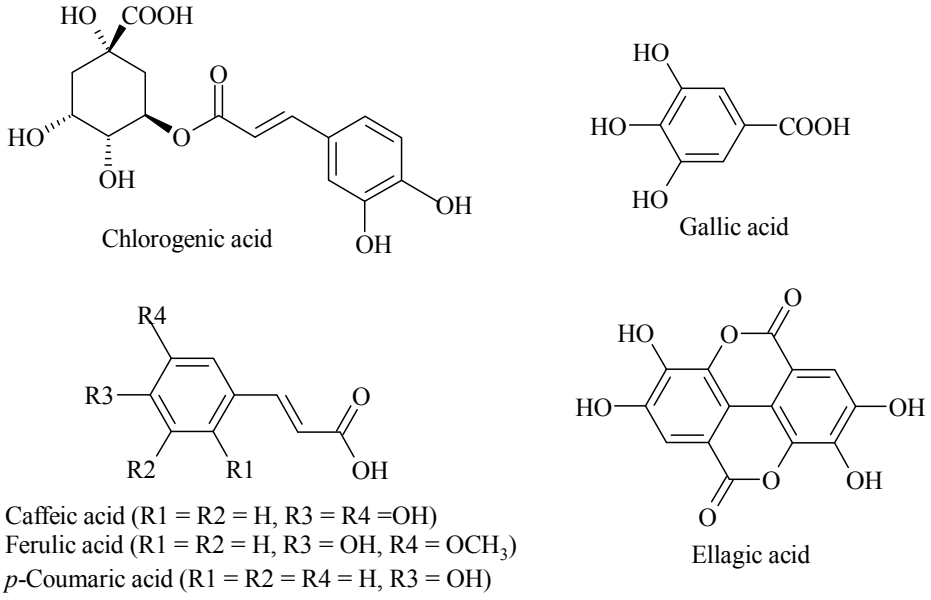


Figure 7: Structural formulas of phenolic acids from bilberries and blueberries (Macheix et al., 1990; Robards, 2003).

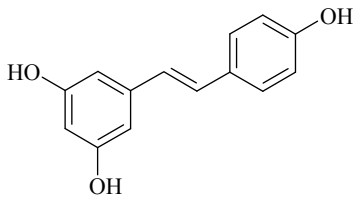
Slika 7: Strukturne formule fenolnih kislin gozdnih in ameriških borovnic (Macheix in sod., 1990; Robards, 2003).

Table 6: The literature review of phenolic acid content in bilberries and blueberries.
Preglednica 6: Pregled literaturnih podatkov o vsebnosti fenolnih kislin v gozdnih in ameriških borovnicah.

PHENOLIC ACIDS	mg/100 g fw	Reference
Chlorogenic acid		
Bilberries	not quantified	Ehala et al., 2005
	14.1 ± 1.3	Hukkanen et al., 2006
Blueberries	97.74 - 100.96	Gao and Mazza, 1994
	64.59 ± 0.93	Zheng and Wang, 2003
	25.42 ± 1.23	Zheng et al., 2003
	126.1 ± 17.3	Taruscio et al., 2004
	4.336 - 10.090	Wang et al., 2008a
	12.49 ± 0.34	Wang et al., 2008b
Caffeic acid		
Bilberries	not quantified	Ehala et al., 2005
	0.33 ± 0.02	Zadernowski et al., 2005
	9.5 - 10.6	Mattila et al., 2006
Blueberries	0.5 - 1.5	Häkkinen and Törrönen, 2000
	3.00 - 3.33	Sellappan et al., 2002
	18.2 ± 2.12	Taruscio et al., 2004
Ferulic acid		
Bilberries	2.301 ± 0.169	Ehala et al., 2005
	0.25 ± 0.02	Zadernowski et al., 2005
	1.1 - 1.2	Mattila et al., 2006
Blueberries	3.45 - 4.16	Sellappan et al., 2002
	4.39 ± 0.59	Taruscio et al., 2004
***p*-Coumaric acid**		
Bilberries	0.4 ± 0.15	Häkkinen and Törrönen, 2000
	6.5	Määttä-Riihinen et al., 2004
	0.60 ± 0.02	Ehala et al., 2005
	0.12 ± 0.01	Zadernowski et al., 2005
	6.1 - 8.1	Mattila et al., 2006
	13.2	Riihinen et al., 2008
Blueberries	not detected	Häkkinen and Törrönen, 2000
	2.40 - 7.15	Sellappan et al., 2002
	1.1	Määttä-Riihinen et al., et al., 2004
	0.45 ± 0.06	Taruscio et al., 2004
	2.6	Riihinen et al., 2008
Gallic acid		
Bilberries	0.1 - 0.2	Stöhr and Herrmann, 1975
	0.06 ± 0.01	Zadernowski et al., 2005
	1.53 - 3.2	Mattila et al., 2006
Blueberries	1.95 - 4.76	Sellappan et al., 2002
Ellagic acid		
Bilberries	quantified in %	Häkkinen et al., 1999a
Blueberries	quantified in %	Häkkinen et al., 1999a
	0.75 - 6.65	Sellappan et al., 2002

2.1.1.5 Resveratrol

Resveratrol is a naturally occurring compound belonging to a group called stilbenes and can be found in the *cis*- or *trans*- configurations and either glucosylated or not. Biologically, *cis*-resveratrol is not active while *trans*-resveratrol was recognized to be most bioactive and stable form. In several *in vitro* studies, *trans*-resveratrol has been recognized for its powerful antioxidant properties (Singh et al., 2008). Resveratrol is known as a phytoalexin, which is produced by plants in response to damage and is presented in grapes and wine, peanuts, soy and berries (bilberries, blueberries, cowberries, redcurrant, cranberries and strawberries) (Burns et al., 2002; Rimando at al., 2004; Szajdek and Borowska, 2008). *Trans*-resveratrol (Figure 8) was found in both bilberries and blueberries. They determined 0.002 - 0.678 mg/100 g fw in bilberries while in blueberries from 0.003 - 0.269 mg/100 g fw. Table 7 presents the content of *trans*-resveratrol in bilberries and blueberries.



trans-Resveratrol

Figure 8: Structural formula of *trans*-resveratrol from bilberries and blueberries (Rimando et al., 2004).
Slika 8: Strukturna formula *trans*-resveratrola gozdnih in ameriških borovnic (Rimando in sod., 2004).

Table 7: The literature review of *trans*-resveratrol content in bilberries and blueberries.
Preglednica 7: Pregled literaturnih podatkov o vsebnosti *trans*-resveratrola v gozdnih in ameriških borovnicah.

***trans*-Resveratrol**	**mg/100 g fw**	**Reference**
Bilberries	0.0016	Lyons et al., 2003
	0.678 ± 0.018	Ehala et al., 2005
Blueberries	0.0032	Lyons et al., 2003
	0.248 - 0.269	Wang et al., 2008a
	0.25 ± 0.02	Wang et al., 2008b

2.1.2 Determination of food phenolics

Food, fruits and vegetables consist of a complex mixture of phenolic compounds, and only a small number of them have been examined systematically for their phenolic content. The assays used for the analysis of phenolics can be classified as either:

- those which determine total phenolics content,
- those determining a specific group or class of phenolic compounds,
- and those determining individual phenolics.

The general analytical strategy of phenolics involves recovery of the phenolics from the samples followed by preparation of extracts, clean-up step in some cases, separation, identification and quantification of individual components (Robards, 2003; Naczk and Shahidi, 2004).

2.1.2.1 Extraction

The first step of the extract preparation procedure is milling, grinding and homogenization of the sample. Extraction is the main step for the recovery and isolation of bioactive phytochemicals from the materials, before analysis. It is influenced by chemical nature of the compounds, the extraction method, sample particle size, storage time and presence of interfering substances. The type of solvent used in the extraction depends on solubility of phenolics, degree of their polymerization, their interaction with other food constituents and formation of insoluble complexes. Extractions are performed on freeze-dried ground fruits or by maceration of the fresh fruit with the extracting solvent. Solvents commonly used for the extraction of phenolics from fruits and vegetables are following: methanol, ethanol, acetone, water, ethyl acetate, propanol, dimethylformamide or their combinations. The extracts of fruits are mixtures of different classes of phenolics and other compounds soluble in the solvents and next step may include the removal of unwanted phenolics and non-phenolic substances. Solid phase extraction (SPE) techniques and fractionations based on polarity and acidity are commonly used to remove unwanted phenolics and non-phenolic substances. The SPE is a fast and reproducible method, and fairly clean extracts can be obtained. SPE mini-cartridges with C18 reversed-phase are usually used to remove sugars from phenolics such as flavonoids, flavonols and resveratrol (Tura and Robards, 2002; Naczk and Shahidi, 2004).

2.1.2.2 Spectrophotometric analysis

The Folin-Ciocalteu assay is the most widely used procedure for determination of the total phenolics in plant and food. The method was originally described by Folin and Denis (1912) and later it was modified by Folin and Ciocalteu (1927). It is based on a chemical reduction of the Folin-Ciocalteu reagent, a mixture of tungsten and molybdenum oxides, by phenolics. The products of the metal oxide reduction have a blue color that exhibits a broad light absorption with a maximum at 760 nm. The intensity of light absorption at that wavelength is proportional to the concentration of phenolics. The disadvantage of the assay is that Folin-Ciocalteu reagent is not specific and can detect all phenolic groups found in extracts, including those in the extractable proteins. Reported values are commonly quantified as gallic equivalents using units of mg/L for extracts and liquid samples or mg/100 g fwfor food samples (Waterhouse, 2003; Naczk and Shahidi, 2004).

The content of total phenolics can be determined by spectral analysis due to the fact that they all absorb UV light at 280 nm. The problem of the method is that each class of phenolic substances has a different absorptivity (extinction coefficient) at 280 nm. Thus, the results cannot be related to any specific standard. Disparate samples are difficult to compare with this method, as they are likely to have very different compositions (Waterhouse, 2003).

2.1.2.3 Chromatographic analysis

High performance liquid chromatography (HPLC) is an analytical technique most applied for the analysis of individual phenolics. HPLC is used for separation of components of mixtures by differential migration through a column containing a microparticulate solid stationary phase. Solutes are transported through the column by a pressurized flow of liquid mobile phase and detected when they are eluted. The columns chosen for the separation of phenolics are usually reverse phase columns, composed of a C18 stationary phase, or narrow-bore columns. C18 columns usually range from 100 to 250 mm in length and with an internal diameter from 3.9 to 4.6 mm. Particle sizes are in the range of 3-10 μm. Special silica sorbents with reduced metallic residue contents and minimum residual silanol groups on the surface could positively influence peak symmetry as chromatographic resolution and the efficiency of the column are better for columns with very good free silanol group covering, end capping, or embedding. Most HPLC analyses of phenolic compounds are performed at ambient column temperature, but moderately higher temperatures between 30 and 40 °C have also been recommended. The solvents system consists of an aqueous phase and an organic phase (mainly methanol or acetonitrile). Methanol is often preferable to acetonitrile because of its nontoxic properties and the possibility of using higher percentages in the mobile phase which could protect the HPLC column. Commonly an acid is added to the solvents (water-organic mixture). Recommended pH range for the HPLC assay is pH between 2 and 4. Aqueous acidified solvents such as acetic, formic, phosphoric, and rarely perchloric acid are usually employed. Isocratic or gradient elutions are applied for analyses of phenolic compounds. The HPLC system combined with electrochemical (e. g. mass spectrometry), UV-VIS and fluorescent detectors have been widely used in food phenolics research. These combination of columns, solvents and detectors are successfully applied to a great number of phenolic compounds such as anthocyanins, flavanols and isoflavones, phenolic acids, flavonols, flavones, flavanones and also stilbenes. Phenolics can be detected by using UV-VIS detector, diode array detector (DAD) or UV-fluorescence detectors. HPLC coupled with mass spectrometer (LC-MS or LC-MS/MS) is currently the most selective analytical technique for the identification and quantification of unknown compounds from crude and partially purified samples. The LC-MS method can provide a soft-ionization process that

leads to a mass spectrum with only a few ions. Electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI) interfaces are techniques used in mass spectrometry to produce ions. They are highly sensitive and show great ionization stability. Generally, APCI can be used for relatively non-polar compounds that can undergo acid-base reactions in the gas phase, whereas ESI is more suitable for compounds that can be ionized in solution. According to most studies, for both APCI and ESI the negative mode is applied for most of the phenolics while positive mode is applied for anthocyanins. The application of tandem mass spectrometry (MS/MS) is useful for the identification of unknown compounds, including phenolics, and their quantification (Stalikas, 2007; Xing et al., 2007; Valls et al., 2009).

2.1.2.4 Anthocyanin analysis

Due to their polarity, anthocyanins are normally extracted from plant materials by using methanol, ethanol or acetone that contains small amounts of hydrochloric acid or formic acid. This solvent system destroys the cell membranes, simultaneously dissolves the anthocyanins and stabilizes them. The extraction with methanol was demonstrated to be the most efficient. These methodologies imply the co-extraction of non-phenolic substances such as sugars, organic acids and proteins requiring subsequent purification processes. SPE using C18 cartridges or Sephadex is commonly used for the purification of the crude anthocyanin extracts before further analysis (e. g. HPLC). The anthocyanins, at low pH, are bound strongly to adsorbents through their unsubstituted hydroxyl groups and are separated from unrelated compounds by using a series of solvents of increasing polarity (Kong et al., 2003; Naczk and Shahidi, 2004; Castañeda-Ovando et al., 2009).

Total anthocyanins can be determined spectrophotometrically. The pH differential method was originally described by Fuleki and Francis (1968a, 1968b). Anthocyanins reversibly change color with pH, which limits their effective use as food colorants for many applications. Absorbance measurements are taken at the wavelength of maximum absorbance at 520 nm. The difference in absorbance between the two buffer solutions is due to the anthocyanin pigments. The intensity of light absorption at that wavelength is proportional to the concentration of total anthocyanin content. Usually values are quantified as equivalents of one of anthocyanins (e. g. cyanidin 3-glucoside, malvidin 3-glucoside) using units of mg/L for extracts and liquid samples or mg/100 g fwfor food samples such as fruits and vegetables (Wrolstad et al., 2005).

Individual anthocyanins can be determined by chromatographic techniques. The characterization of a mixture of anthocyanins involves the separation, identification and quantification of each compound by HPLC-DAD, LC-MS or LC-MS/MS. Columns with

C18 stationary phase are usually used for anthocyanin separation. With these columns the elution pattern of anthocyanins is mainly dependent on the partition coefficients between the mobile phase and the stationary phase, and on the polarity of the anthocyanins. The elution order in C18 columns is normally a function of the number of hydroxyl groups and their degree of methoxylation (delphinidin < cyanidin < petunidin < pelargonidin < peonidin < malvidin). The mobile phase consists normally of acidified aqueous phase and of organic solvent (methanol or acetonitrile). With HPLC-DAD technique, the anthocyanin identification is based primarily on the UV-VIS spectrum or retention time and compared with standard compounds. The acidified aqueous mobile phase allows the complete displacement of the equilibria to the flavylium cation, thus resulting in a better resolution and a great characteristic absorbance between 515 and 540 nm, which explains the universal application of DAD detection for anthocyanins quantification. DAD detection is not sufficient to discriminate between compounds with similar spectroscopic characteristics and the lack of reference compounds for comparison makes mass spectrometry a supporting technique in anthocyanin characterization. The use of LC-MS or LC-MS/MS technique permits the separation and structural analysis of individual anthocyanins. HPLC coupled with electrospray ionization mass spectrometer (MS), especially the tandem mass spectrometer (MS/MS) can provide mass spectra of intact molecular and fragment ions. Electrospray ionization in the positive mode (ESI^+) is commonly used for the analysis of anthocyanins. ESI is known to be a soft ionization technique. Anthocyanins undergo collision-induced cleavage of the *O*-glycosidic bond producing deprotonated aglycone. The individual mass spectra showed peaks for the molecular ions, together with a fragment corresponding to aglycone, when acylation was present. An additional fragment was detected at mass/charge values corresponding to the loss of acyl moiety from the molecular ion. Peaks can be identified categorically by matching their mass spectrum and retention time with reference compounds (Kong et al., 2003; Xing et al., 2007; Valls et al., 2009).

2.2 BIOLOGICAL AND PHARMACOLOGICAL ACTIVITIES OF PHENOLICS FROM BILBERRIES AND BLUEBERRIES

The diversity of bioactive compounds such as phenolics in berries is reflected in the wide spectrum of their biological activities which have beneficial effects on human health and disease prevention. These include antioxidant, antimutagenic, anticarcinogenic, anti-inflammatory and antimicrobial activity. Beneficial effects were established in cardiovascular diseases (atherosclerosis, arterial hypertension and diabetes), ocular health, neural and cognitive brain functions as well (Zafra-Stone et al., 2007).

2.2.1 Antioxidant potential

2.2.1.1 Oxidative stress and antioxidant capacity

Our organisms are exposed to oxidation due to oxygen presence in metabolism and of existence of some physiopathological situations (cigarette smoke, air pollutants, UV radiation, high polyunsaturated fatty acids diet, stress, etc.). Oxidation is the transfer of electrons from one atom to another and the oxygen is the ultimate electron acceptor in the electron flow system that produces energy in the form of ATP. If the electron flow becomes uncoupled (transfer of unpaired single electrons), the generation of reactive oxygen radicals (ROS) arise. ROS are superoxide ($O_2^{\bullet -}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$), hydroxyl ($HO^{\bullet}$) and nitric oxide ($NO^{\bullet}$). Other ROS non-radical derivates are singlet oxygen (O_2), hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl). *In vivo* ROS are responsible for the oxidative damage of biological macromolecules (lipids, proteins, carbohydrates, DNA) what can lead to the risk of various degenerative diseases such as cancer, inflammatory diseases, cardiovascular diseases, osteoporosis, diabetes, neurodegenerative diseases and age-related functional decline. The ROS can be metabolized by series of antioxidant defences, some synthesized in the body (endogenous) and others obtained from the diet (exogenous). However, antioxidants are molecules which can donate a free electron or hydroxyl atoms to ROS. Vegetables and fruits are rich in phytochemical substances, included phenolics, nitrogenous compounds (chlorophyll derivates), tocopherol, carotenoides and ascorbic acid, which can exhibit an antioxidant activity. The purpose of antioxidants is not to remove all ROS but to control their level and to minimize oxidative damage (Diplock et al., 1998; Pietta, 2000; D'Archivio et al., 2007; Castañeda-Ovando et al., 2009).

2.2.1.2 Relationship between the structure and antioxidant activity of phenolics

Phenolics have good antioxidant activity due to their capacity to scavenge free radicals, donate hydrogen atoms or electrons and chelate metal cations (Figure 9). These abilities are determined due to their chemical structures (Balasundram et al., 2006). They have been shown to be effective antioxidants *in vitro* more than tocopherol (vitamin E) and ascorbate (vitamin C) (Rice-Evans et al., 1997). Flavonoids due to some of structural features and nature of substitutions on the ring B and on the ring C can determine following antioxidant activity:

- The degree of hydroxylation and the hydroxyl groups in the ring B can confer high stability to the aroxyl radical by electron delocalization or acts as preferred binding site for trace metals.

- The presence of hydroxyl groups at the 3'-, 4'- and 5'-positions on the ring B can enhance the antioxidant activity compared to the flavonoids with only one hydroxyl group.
- A double bound between 2- and 3-position, conjugated with the 4-oxo group or combined with a 3-OH, in ring C, can enhance the radical scavenging.
- Substitution of hydroxyl groups in ring B by methyl groups alters the redox potential, which can affect the radical scavenging capacity.
- Glycosylation reduces greatly the radical-scavenging capacity (Pietta, 2000; Balasundram et al., 2006).

The antioxidant activity of phenolic acids depends on the number and positions of the hydroxyl groups in relation to the carboxyl functional group. Monohydroxy benzoic acids with hydroxyl moiety at the *ortho*- or *para*- position to the carboxyl group show no antioxidant activity, though the same is not true for *m*-hydroxybenzoic acid. The antioxidant activity increases with increasing degree of hydroxylation but the substitution of the hydroxyl groups at the 3- and 5-position with methoxyl groups reduces the activity (Balasundram et al., 2006).

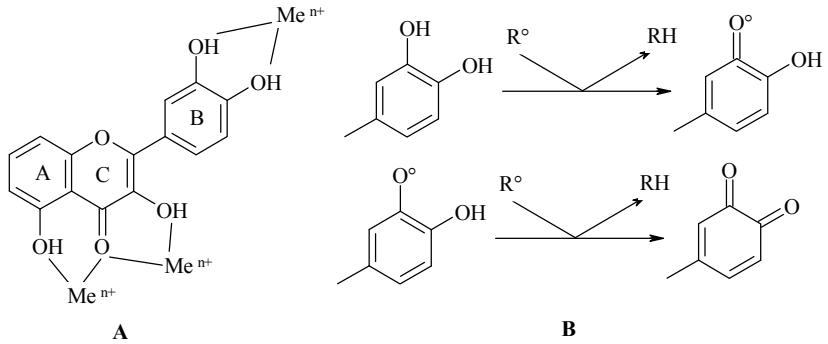


Figure 9: Binding sites for trace metals (A) and scavenging of ROS by flavonoids (B) (Pietta, 2000).
Slika 9: Mesta vezave kovinskih ionov (A) in odstranjevanje ROS s flavonoidi (B) (Pietta, 2000).

2.2.1.3 Antioxidant tests

Berries owe their antioxidant properties mainly to phenolic compounds (Szajdek and Borowska, 2008). The measurement of antioxidant activity can be determined the oxidation/reduction potentials of fruits and their phytochemicals. Several *in vitro* antioxidant tests are used, such as the oxygen radical absorbance capacity (ORAC), total radical-trapping antioxidant parameter (TRAP), Trolox equivalent antioxidant capacity (TEAC), total oxyradical scavenging capacity (TOSC), the ferric reducing/antioxidant power (FRAP) assay and radical scavenging capacity methods using DPPH or ABTS. Due to complexity of the composition of food the individual compounds have been studying, but fruit extracts showed higher antioxidant activity than many pure phenolic compounds

or vitamins (Wolfe and Liu, 2007; Szajdek and Borowska, 2008). Bilberries and blueberries are very complex in their composition. Therefore it is difficult to distinguish which compounds contribute the most to the antioxidant activity (Burdulis et al., 2009). Their antioxidant potential was performed by many antioxidant test such as DPPH, FRAP and ORAC assay. The results showed that antioxidant activity was much higher in bilberries than in blueberries (Prior et al., 1998; Giovanelli and Buratti, 2009).

DPPH radical scavenging assay

DPPH radical scavenging assay is used to evaluate the antioxidative activity of specific compounds or extracts. This method was developed nearly 50 years ago by Marsden Blois (Blois, 1958). The molecule of 1,1-diphenyl-2-picrylhydrazyl (DPPH) is characterized as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole, so that the molecules do not dimerise, as it would be the case with most other free radicals. The delocalization also intensifies the deep violet color, characterized by an absorption band in methanol solution. When a solution of DPPH is mixed with an antioxidant (AH) that can donate a hydrogen atom, then this gives rise to the reduced form with the loss of this violet color (Figure 10). The reduction of DPPH is followed by monitoring the decrease in its absorbance at a characteristic wavelength during the reaction (Molyneux, 2004).

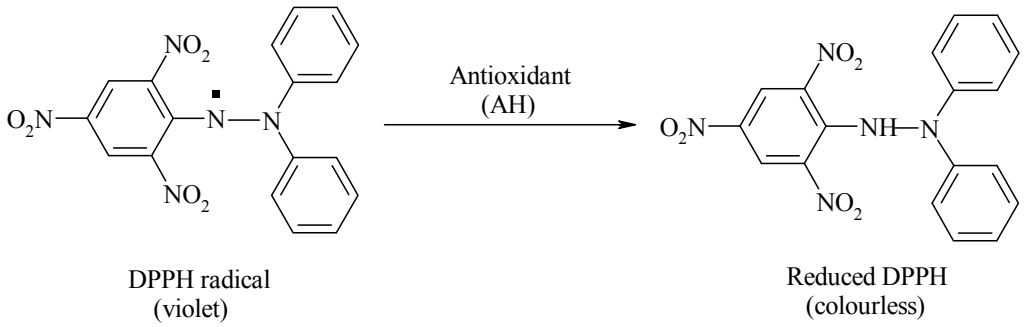


Figure 10: Reduction of DPPH (Molyneux, 2004).
Slika 10: Redukcija DPPH (Molyneux, 2004).

Cellular antioxidant activity (CAA) assay

The weakness of these antioxidant tests is that they do not reflect the cellular physiological conditions and do not consider the bioavailability and metabolism issues. Due to the deficiency of simple cell-based antioxidant activity assays for studying the bioavailability of phytochemicals, cellular antioxidant activity (CAA) assay was developed by Wolfe and Liu (2007). The CAA assay measures antioxidant capabilities of food extracts and dietary supplements in cells and can simulate some *in vivo* conditions. Its principle (Figure 11) is an inhibition of formation of a fluorescent compound dichlorofluorescein (DCF) from 2',7'-dichlorofluorescin (DCFH) in the presence of ROS generated from 2,2'-azobis(2-amidinopropane)dihydrochloride (ABAP) as a model of an acute oxidative stress.

DCFH is formed from 2',7'-dichlorofluorescin diacetate (DCFH-DA) after its absorption into the cells by esterases. Antioxidant compounds bounded on the cell membrane and/or passed through membrane into the cell quench free radicals and inhibit DCF formation (Wolfe and Liu, 2007). The screening of antioxidant potential in HepG2 cells by CAA assay showed that berries (wild blueberry, blackberry, raspberry and blueberry) and pomegranate exerted the highest antioxidant potential (Wolfe et al., 2008).

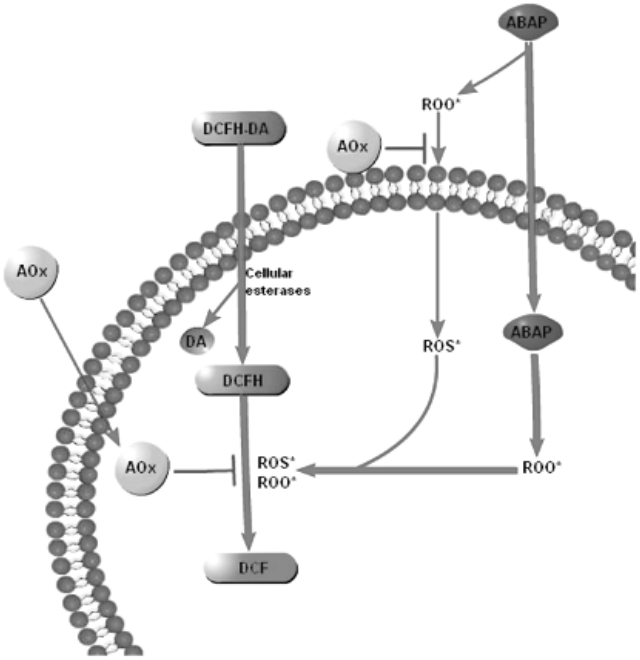


Figure 11: Principle of the cellular antioxidant activity (CAA) assay (Wolfe and Liu, 2007).
Slika 11: Princip testa celične antioksidativne aktivnosti (CAA) (Wolfe in Liu, 2007).

2.3 BIOAVAILABILITY

To determine phenolics bioactivity in the body, it is important to understand how these compounds are absorbed and where they are possibly further metabolized to biologically active or inactive metabolites. Thus biological properties and health benefits of nutrients (e. g. phenolics) depend on their bioavailability. Bioavailability can be defined as a proportion of a nutrient that is absorbed, distributed, metabolized and excreted through normal pathway or is often just characterized as plasma concentrations.
The concept of bioavailability incorporates:

- availability for the absorption or bioaccessibility (defined as the amount of an ingested compound that becomes available for absorption in the gastrointestinal tract),
- absorption,
- tissue distribution and
- bioactivity (Stahl et al., 2002).

2.3.1 Fate of anthocyanins in human body

The journey of anthocyanins through the human body can be described in the following stages: consumption, gut luminal events, absorption, distribution and metabolism. Possible pathway of dietary anthocyanins in human body is represented in Figure 12.

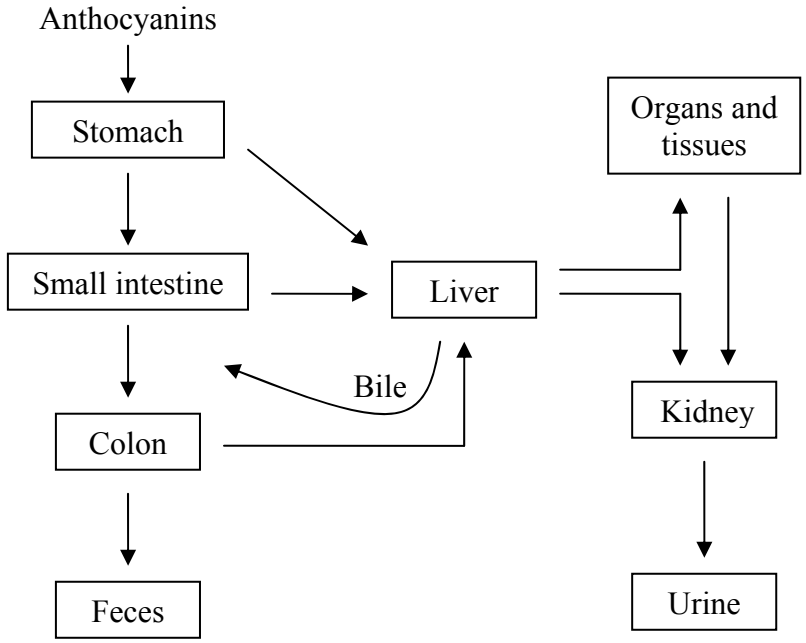


Figure 12: Possible pathway of anthocyanins in humans (Scalbert and Williamson, 2000; McGhie and Walton, 2007).
Slika 12: Možne poti antocianinov v človeškem telesu (Scalbert in Williamson, 2000; McGhie in Walton, 2007).

2.3.1.1 Gastrointestinal tract

The lack of the knowledge of anthocyanin metabolism in the gastrointestinal tract was mentioned by many authors (McGhie and Walton, 2007; Vitaglione et al., 2007; Aura, 2008; Hassimotto et al., 2008; He et al., 2009). The fate of anthocyanins in gastrointestinal tract is summarized in Table 8.

Table 8: Fate of anthocyanins through the gastrointestinal pathway (Selma et al., 2009).
Preglednica 8: Usoda antocianinov v gastrointestinalnem traktu (Selma in sod., 2009).

Part of gastrointestinal tact	Anthocyanin fate
Mouth	Deglycosylation?
Stomach	Absorption
Small intestine	Deglycosylation, degradation, absorption
Large intestine	Deglycosylation, degradation

The pathway of consumed anthocyanins starts in mouth contacting to saliva. There are no data of saliva effect to anthocyanins but some publications suggest that according to

previous studies flavonoid glycosides were hydrolyzed to corresponding aglycons (McGhie and Walton, 2007; Selma et al., 2009). In the stomach anthocyanins remain intact due to the pH about 1 and they occur in the most stable flavylium cation. Antocyanins from red wine were stable under *in vitro* gastric conditions (presence of pepsin and HCl, 37 °C) (McDougall et al., 2005) as well intact anthocyanins were detected in the rat stomach after a diet with wild mulberry (Hassimotto et al., 2008). The stomach is a site of anthocyanins absorption with active transport including transport carriers such as bilitranslocase (Passamonti et al., 2003) and sodium dependent glucose transporter (Felgines et al., 2008). Small intestine, jejunum more precisely, is considered to be a site of anthocyanins absorption (Talavéra et al., 2004) as well.

At the intestine neutral pH anthocyanins exist in equilibrium with four molecular forms (flavylium cation, quinoidal base, carbinol pseudobase and chalcone pseudobase) thus they can be easily exposed to degradation (McDougall et al., 2005). First studies showed degradation of anthocyanins from tart cherries to phenolic acids (Seeram et al., 2001). Later their degradation was demonstrated by two steps (Figure 13). The first step is deglycosylation of anthocyanins to anthocyanidin aglycon while the second step is degradation of the formed aglycon to a phenolic acid and an aldehyde (Fleschhut et al., 2006; Ávila et al., 2009).

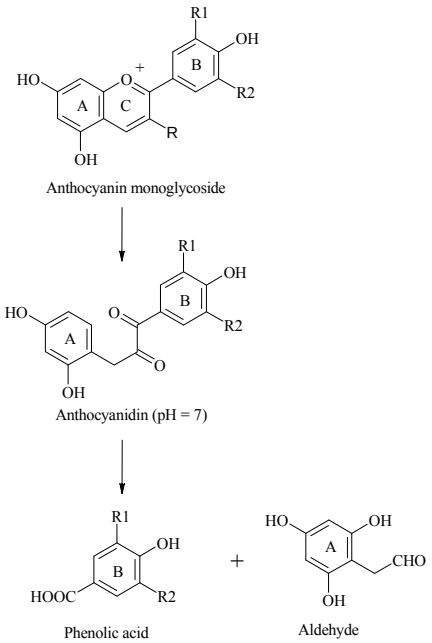


Figure 13: Anthocyanin monoglycoside degradation reactions, where R1 and R2 = H, OH or OCH_3 (Keppler and Humpf, 2005; Fleschhut et al., 2006; Selma et al., 2009).

Slika 13: Reakcije razpada monoglikozidnih antocianinov, R1 in R2 = H, OH ali OCH_3 (Keppler in Humpf, 2005; Fleschhut in sod., 2006; Selma in sod., 2009).

Deglycosylation is the cleavage of the glycosyl moiety from anthocyanins structure to form anthocyanidin aglycons. In the presence of human fecal microflora aglycons were formed

from anthocyanins (Fleschhut et al., 2006) due to the reason that intestinal bacteria possess enzymes such as α-rhamnosidase, β-glucosidase and β-glucuronidase which can catalyze the reaction (Aura et al., 2005; Ávila et al., 2009). The other study showed that aglycons can be formed under intestinal conditions at pH 7 (Fleschhut et al., 2006) as well anthocyanidins were found as deglycosylation products after anthocyanin incubation with intestinal epithelial cells (Caco-2) at 37 °C (Kay et al., 2009) and they were detected in the rat small and large intestine after a diet with wild mulberry (Hassimotto et al., 2008). However in the intestine, aglycones can be produced from anthocyanins by enzymes of gut microflora or by exposing to intestinal cell conditions.

Degradation of anthocyanidin aglycon is a breakdown of its heterocycle and cleavage of the C-ring to form a phenolic acid and an aldehyde (Keppler and Humpf, 2005) and in the intestine can be caused spontaneously or by microflora (Fleschhut et al., 2006; Forester and Waterhouse, 2008; Ávila et al., 2009). Spontaneous degradation is a consequence of the neutral pH. Therefore, anthocyanidin aglycones are observed in the chalcone form which is rather unstable and can be easily degraded (Keppler and Humpf, 2005; Fleschhut et al., 2006). Anthocyanidins were also degraded after incubation with human (Aura et al., 2005; Fleschhut et al., 2006) or pig gut microflora (Keppler and Humpf, 2005; Forester and Waterhouse, 2008) or with predominant bacteria of intestinal microflora *Bifidobacteria* and *Lactobacillus* (Ávila et al., 2009). Data showed that major degradation products of anthocyanidin aglycons were corresponding phenolic acids (Table 9), as well as some other minor still unidentified products were found (Fleschhut et al., 2006; Forester and Waterhouse, 2008; Ávila et al., 2009). Phenolic acids can be further transformed to the benzoic acids in the presence of intestinal bacteria by cleavage of hydroxyl group in the 4-position (Aura, 2005; Selma et al., 2009).

Table 9: Degradation products of anthocyanidins (Fleschhut et al., 2006; Selma et al., 2009).
Preglednica 2: Razgradni produkti antocianidinov (Fleschhut in sod., 2006; Selma in sod., 2009).

Anthocyanidin	**Corresponding phenolic acid**
Cyanidin	Protocatechuic acid
Delphinidin	Gallic acid
Pelargonidin	4-hydroxybenzoic acid
Malvidin	Syringic acid
Peonidin	Vanilic acid
Petunidin	3-*O*-methylgallic acid

The anthocyanins degradation to phenolic acid is rapid, within 2 hours (Aura et al., 2005; Keppler and Humpf, 2005). However, anthocyanin monoglycosides were degraded faster than diglycosides (Keppler and Humpf, 2005), galactosides, arabinosides, xylosides (He et al., 2005) and rutinosides. Diglycosides are firstly converted to monosaccharides and than

to an aglycon and rutinosides were firstly converted to glucosides (Aura et al., 2005; Keppler and Humpf, 2005) and then to an aglycon. Anthocyanidin aglycons were degraded the fastest, much faster than anthocyanin monoglycosides (Keppler and Humpf, 2005). Beside this anthocyanin degradation by intestinal microflora was much faster than spontaneous one (Forester and Waterhouse, 2008).

Anthocyanins that are not absorbed or degraded in the gastrointestinal tract can be excreted in intact forms. Unchanged anthocyanins were detected in human fecal samples 24 hours after blood orange juice consumption (Vitaglione et al., 2007) and fecal samples collected from rats previously fattened by chokeberries, bilberries and grapes contained unchanged anthocyanins (He et al., 2005).

2.3.1.2 Organs and tissues

After the rapid absorption in the gastrointestinal tract, the anthocyanins pass through the liver and enter the systemic circulation where they are excessively metabolized in the hepatic cells (Tsuda et al., 1999; Marczylo et al., 2009). Then they are distributed by blood to several tissues. Intact anthocyanins and/or their metabolites can be found in the blood, bile, liver, kidney, heart, brain, testes, urine, prostate and lung in rats or/and mice (Scalbert and Williamson, 2000; Passamonti et al., 2005a; Passamonti et al., 2005b; Ichiyanagi et al., 2006; McGhie and Walton, 2007; Vanzo et al., 2008; Marczylo et al., 2009; Sakakibara et al., 2009). However exceptionally fast uptake and methabolism of anthocyanins were exerted in rat kidneys and liver (Vanzo et al., 2011). The post-absorption plasma concentrations of anthocyanins were estimated to be in the range from 0.001 to 530 µg/L after 15 to 30 min of administration (Tsuda et al, 1999; Mazza et al., 2002; Ichiyanagi et al., 2005a; Ichiyanagi et al., 2005b; Ichiyanagi et al., 2006; Galvano et al., 2007; Felgines et al., 2008; Sakakibara et al., 2009). Anthocyanins are excreted as intact or/and as their metabolites by urine and faces (Felgines at al., 2002; Kay, 2006; Prior and Wu, 2006).

The major metabolites of anthocyanins are methylated compounds, glucuronide and sulphate conjugates (Ichiyanagi et al., 2005a; Prior and Wu, 2006), while minor metabolites are hydroxylated and further methylated compounds (Vanzo et al., 2011) (Figure 14). Glucuronide conjugation is regarded as the major conjugation reaction involved in the metabolism of a flavonoid. The glucuronidation is catalysed by UDP-glucuronosyltranferases which are found in the liver, intestine and kidneys. Methylation appears to be the second most significant conjugation reaction in the metabolism of flavonoids and is driven by a group of enzymes referred to as methyltransferases. These enzymes are found in many tissues including the liver and intestine. Sulfation is also common conjugation reaction in the metabolism of flavonoids

and is catalysed by sulfotransferases, which are a small group of cytosolic enzymes widely distributed throughout the body. Sulfation is relatively costly in ATP and sulfate and it is more likely to be rapidly limited by aglycone loading than glucuronidation is. They utilize phosphoadenosine-5'-phosphosulfate as a cofactor and their known substrates include phenolics such as flavonoids (Kay, 2006).

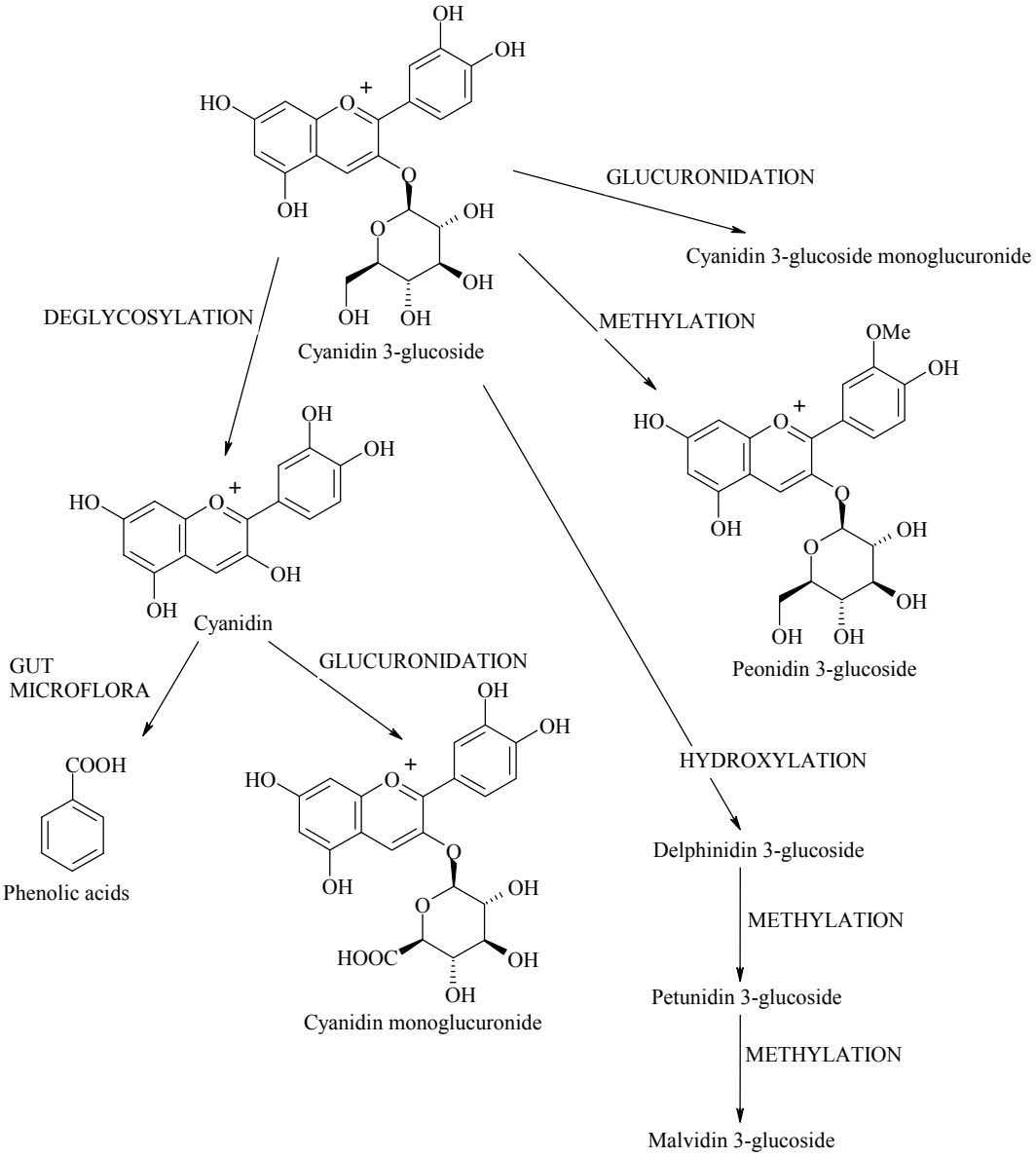


Figure 14: Metabolic pathways of anthocyanins, e. g. cyanidin 3-glucoside (Ichiyanagi et al., 2005a; Prior and Wu, 2006; Vanzo et al., 2011).

Slika 14: Metabolične poti antocianinov, primer cianidin-3-glukozida (Ichiyanagi in sod., 2005a; Prior in Wu, 2006; Vanzo in sod., 2011).

2.3.1.3 Bilitranslocase

The absorption of anthocyanins into the cells requires a specific transport mechanism. It was observed that anthocyanins can pass the cell membrane by transport proteins such as glucose transporters (Hollman et al., 1999; Mülleder et al., 2002; Faria et al., 2009) and an organic anion carrier bilitranslocase (Passamonti et al., 2003; Passamonti et al., 2009). Bilitranslocase is an organic anion membrane carrier and is responsible for the ATP-independent transport of sulfobromophtalein, bilirubin, nicotic acid and flavonoids across the cell membrane. In the Transporter Classes Data Base, bilitranslocase code is 2.A.65.1.1. Its primary structure consists of 340 amino acids and contains a motif that is highly conserved in α-chains of phycocyanins (Battiston et al., 1998). It was found in liver (Baldini et al., 1986), gastric mucosa (Battistion et al., 1999) and vascular endothelium (Maestro et al., 2009). The interaction of anthocyanins with the bilitranslocase occurs through hydrophilic moieties, such as the 3-glucosyl moiety and the ring B for the monoglucosides, through the 5-glucosyl moiety and the ring A for the diglucosides, and through either the ring A or the ring B for the anthocyanidins (Passamonti et al., 2002).

3 MATERIALS AND METHODS

3.1 DETERMINATION OF PHENOLICS

3.1.1 Bilberry and blueberry samples

Bilberries (*Vaccinium myrtillus* L.) and highbush blueberries (*Vaccinium corymbosum* L.) were used in this study. Bilberries were handpicked in woods from seven different locations of Slovenia: Ljubljana (n = 6), Pohorje (n = 6), Pokljuka (n = 3), Kranj (n = 6), Goričko (n = 5), Škofja Loka (n = 6) and Celje (n = 4). Blueberries (n = 5) were handpicked in the plantation named Kolekcijsko poskusni nasad Kmetijskega inštituta Slovenije in Drenov grič on Ljubljansko barje. The sampling points are demonstrated in Figure 15. Number of independent measurements is shown as n. The ripe bilberries and blueberries were sampled in June 2007 and immediately frozen at – 20 °C until extraction.

Figure 15: Sampling points of bilberries and blueberries in Slovenia.
Slika 15: Vzorčna mesta gozdnih in ameriških borovnic v Sloveniji.

3.1.2 Preparation of bilberry and blueberry extracts

3.1.2.1 Chemicals and material

Sep-Pak C18 cartridge, 1 g (Waters, Milford, USA); methanol and formic acid (Merck, Darmstadt, Germany); Rotavapor (Büchi, Fawil, Switzerland); Shaker EV403 (Tehtnica, Železniki, Slovenia).

3.1.2.2 Extraction

The aim was to extract phenolics from bilberry and blueberry samples. The crude bilberry and blueberry extraction procedure was as follows:

- Frozen bilberry or blueberry samples (50 g) were homogenized with 150 mL of cooled methanol and extracted 3 h by shaking in the dark at room temperature.
- Then the extracts were centrifuged at 3472 *g* for 5 min.
- The sediments remaining after removing the supernatants were mixed with 100 mL of cooled methanol and extracted again for 2 h by shaking in the dark and centrifuged at 3472 *g* for 5 min.
- After that step both supernatants of each sample were collected, saturated with nitrogen and stored at – 20 °C until analysis.

3.1.3 Spectrophotometric analysis

3.1.3.1 Chemicals and material

UV-VIS spectrophotometer Agilent 8453 (Agilent Technologies, Palo Alto, USA); Solid phase extraction (SPE) cartridge (Sep-Pak C18, 1 g; Waters, Milford, USA); Folin-Ciocalteu reagent, H_2SO_4, Na_2CO_3, HCl and ethanol (Merck, Darmstadt, Germany); cyanidin 3-glucoside (Polyphenols Laboratories, Sandnes, Norway); gallic acid (Sigma Aldrich, Steinheim, Germany). Aqueous solutions were prepared with Milli-Q water (Millipore, Bedford, USA).

3.1.3.2 Determination of total phenolics

Total phenolics were determined by the reduction of phosphotungstic and phosphomolybdic acids (Folin-Ciocalteu reagent) to blue pigments in alkaline solutions (Di Stefano and Guidoni, 1989). 2 mL of a bilberry or blueberry extract was evaporated to dryness using rotavapor, redissolved in 0.5 M H_2SO_4 and quantitatively loaded on the Sep-Pak C18 previously activated with methanol and 5 mM H_2SO_4, and the polar

substances were removed with 2 mL of 5 mM H_2SO_4. Then the phenolic compounds were eluted into a 20 mL calibrated flask, with 2 mL of methanol followed by 5 mL Milli-Q water. One milliliter of Folin-Ciocalteu reagent and after 3-4 min 4 mL of 10 % Na_2CO_3 was added and the solution was brought to 20 mL with Milli-Q water. After 90 min at room temperature the absorbance of the sample (filtered through 0.45 μm) was read at 700 nm against a blank prepared with methanol instead of the extract. Phenolic concentrations in extracts were expressed as equivalents of gallic acid and quantified as gram per 100 gram of fresh bilberries or blueberries (Rigo et al., 2000).

3.1.3.3 Determination of total anthocyanins

The method of anthocyanin analysis was modified by Glories (1984) and adjusted to Di Stefano et al. (1989). An aliquot of 2 mL of a bilberry or blueberry extract diluted with methanol (usually 4 times) was evaporated to dryness using rotavapor, redissolved in 0.5 M H_2SO_4 and quantitatively loaded on the Sep-Pak C18 previously activated with methanol and 5 mM H_2SO_4, and the polar substances were removed with 3 mL of 5 mM H_2SO_4. Then the phenolic compounds were eluted with 3 mL of methanol into a 20 mL calibrated flask, 0.1 mL of concentrated HCl added and then the volume was brought to 20 mL with ethanol/water/HCl (70:30:1). The total anthocyanins were directly quantified on the basis of maximal absorbance at 520 nm in the visible range (536-540 nm) against a blank (ethanol/water/HCl 70:30:1). The bilberry or blueberry anthocyanin content was expressed as cyanidin 3-glucoside (MW= 484.4 g/mol, ε=15500 $L \cdot mol^{-1}cm^{-1}$) and as gram per 100 gram of fresh bilberries or blueberries.

3.1.4 HPLC-MS analyses of phenolic compounds

3.1.4.1 Chemicals and material

Sep-Pak C18 cartridge, 1 g (Waters, Milford, USA); Strata-X cartridge, 60 mg (Phenomenex, Torrance, USA, 60 mg); Gemini C18 column (150 × 2.0 mm, 3 μm) coupled with Security Guard Cartridge Gemini C18 (4 × 2.0 mm) (Phenomenex, Torrance, USA); rotavapor (Büchi, Fawil, Switzerland); HPLC-DAD-MS/MS (Agilent Technologies Inc., Wilmington, USA); methanol and formic acid (HPLC grade, Merck, Darmstadt, Germany); acetonitrile (HPLC grade, J. T. Baker, Deventer, Netherlands); ammonium formate (Sigma Aldrich, Steinheim, Germany); cyanidin 3-glucoside (Polyphenols Laboratories, Sandnes, Norway); chlorogenic acid, caffeic acid, ferulic acid, *p*-coumaric acid, ellagic acid, gallic acid, catechin, myricetin, quercetin, rutin and *trans*-resveratrol (Sigma Aldrich, Steinheim, Germany). Aqueous solutions were prepared with Milli-Q water (Millipore, Bedford, USA).

3.1.4.2 HPLC-MS analyses

Individual anthocyanins were determined by LC-MS/MS technique while phenolic acids, flavanols and flavonols were determined by LC-MS technique. HPLC system consisted of Agilent 1100 binary pump (G1312A) and Autosampler (G1330B) was coupled with a Micromass Quattro Micro mass spectrometer equipped with an electrospray ionizator source (ESI). Reversed-phase HPLC separations were carried out using a Gemini C18 column (150 × 2.00 mm, 3 μm) protected by the Security Guard Cartridge Gemini C18 (4.0 × 2.0 mm). Each sample was analyzed in three replicates. Before LC-MS/MS and LC-MS analysis, both crude bilberry and blueberry extracts were cleaned-up by solid phase extraction. The anthocyanin fraction was injected as such.

3.1.4.3 LC-MS/MS analysis of individual anthocyanins

Individual anthocyanins were analyzed according to the optimized method of Lätti et al., 2008.

Clean-up of crude berry extracts: 500 μL of a berry extract was dried by rotavapor and resolubilized in 1 mL of 3 % formic acid, loaded onto a 1 g Sep-Pak C18 cartridge previously activated with 3 mL of pure methanol and 5 mL of 3 % formic acid. After washing with 6 mL of 3 % formic acid, the anthocyanins were eluted with 5 mL of pure methanol. The eluate was evaporated to dryness and dissolved in 5 mL of a mobile phase used for HPLC separation.
LC-MS/MS conditions: The mobile phase consisted of 3 % formic acid (A) and acetonitrile:methanol (85:15; v:v) (B). Anthocyanins were separated at 40 °C with the following linear gradient: 0-2 min, 7-9 % B; 2-4 min, 9-11 % B; 4-12 min, 11-12 % B; 12-13 min, 12 % B; 13-25 min, 12-13 % B; 25-40 min, 13-100 % B. The flow rate of the mobile phase was 0.250 mL/min at 0-4 min, 0.225 mL/min from 4-13 min, and 0.200 mL/min from 13-40 min. The injection volume was 10 μL. The mass spectrometer was operated in the positive ion mode (ESI^+) with the following operating parameters: capillary voltage 3.0 kV, cone voltage 20 V, extractor 5 V. The source temperature was 100 °C and the desolvation temperature was 350 °C. Cone gas flow was set to the 40 L/h, the desolvation gas flow was set to 400 L/h while the collision energy was 30 V. The transition of molecules is shown in Table 10.

Anthocyanins were identified on the basis of their retention time, MS spectra and molecular ion identification. The quantification of individual anthocyanins was calculated to the cyanidin 3-glucoside as an external standard from the spiked calibration curve in the range from 0.5 mg/L to 45 mg/L.

Table 10: The transition states of anthocyanins.
Preglednica 10: Prehodna stanja antocianinov.

Anthocyanin	M_W	MS/MS *(m/z)*
Delphinidin 3-galactoside	465	303
Delphinidin 3-glucoside	465	303
Cyanidin 3-galactoside	449	287
Delphinidin 3-arabinoside	435	303
Cyanidin 3-glucoside	449	287
Petunidin 3-galactoside	479	317
Cyanidin 3-arabinoside	419	287
Petunidin 3-glucoside	479	317
Peonidin 3-galactoside	463	301
Petunidin 3-arabinoside	449	317
Peonidin 3-glucoside	463	301
Malvidin 3-galactoside	493	331
Peonidin 3-arabinoside	433	301
Malvidin 3-glucoside	493	331
Malvidin 3-arabinoside	463	331

M_W = molecular weight

3.1.4.4 LC-MS analysis of phenolic acids, flavanols, flavonols and resveratrol

Phenolic acids, flavanols, flavonols and resveratrol were analyzed according to the optimized method of Bertoncelj et al., 2011.

Clean-up of crude berry extracts: 500 µL of a berry extract, supplemented with 500 µL of MilliQ and 3 mL of 20 mmol/L ammonium formate, was loaded on the Strata-X cartridge, 60 mg, previously conditioned with 2 mL of pure methanol and 2 mL of 20 mmol/L ammonium formate (pH=2.4). After washing with 2 mL of 15 % of methanol in 20 mmol/L ammonium formate (pH=2.4) the retained phenolic fraction was eluted with 2 mL of pure methanol. The eluate was evaporated and dissolved in 500 µL of 1 % formic acid in 50 % methanol.

LC-MS conditions: The mobile phase components were 1 % formic acid (A) and acetonitrile (B). A mobile-phase gradient was linear: 0-5 min: 10 % B; 5-50 min: 10-60 % B; 50-52 min: 60-80 % B; 52-60 min: 80 % B; 60-70 min: 80-10 % B; 70-80 min: 10 % B. The injection volume was 20 µL, the column temperature was 25 °C. The flow rate of the mobile phase was 0.2 mL/min. The mass spectrometer was operated at the following parameters: capillary voltage 3.0 kV, cone voltage 25 V, extractor 5 V. The source temperature was 100 °C and the desolvation temperature was 350 °C. Cone gas flow was set to the 50 L/h while the desolvation gas was set to 400 L/h. Phenolic acids, flavanols and flavonols were determined in the negative ion mode (ESI^-) whereas *trans*-resveratrol in the positive ion mode (ESI^+).

Phenolic acids, flavanols, flavonols and *trans*-resveratrol were identified on the basis of their retention time, MS spectra and molecular ion identification. The quantification of the compounds was calculated to the corresponding external standard from the spiked calibration curve in the range from 0.1 mg/L to 40 mg/L.

3.1.4.5 Multivariate analyses

Statistical analyses of phenolic profile of bilberries sampled from seven different locations were performed using SPSS for Windows, version 15.0, as the evaluation version (SPSS Inc., Chicago, IL). Relations between the main components were assessed by Pearson correlation coefficients. The multivariate analyses included principal component analysis (PCA) and linear discriminant analysis (LDA). Previously, the normality of the data was tested using the Shapiro-Wilk test.

3.2 ANTIOXIDANT POTENTIAL

3.2.1 DPPH radical scavenging assay

3.2.1.1 Chemicals and material

Methanol (Merck, Darmstadt, Germany); 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma Aldrich, Steinheim, Germany); Microplate Reader (BioTek Instruments, Inc., Winooski, USA).

3.2.1.2 DPPH radical scavenging assay

The antioxidant activity was determined using DPPH as a free radical measured by the modified method of Blois (1958) using Microplate Reader. 100 µL of the one hundred times diluted bilberry extract were added to 100 µL of 100 µM DPPH methanol solution. The decrease of absorbance at 544 nm was measured every 5 minutes for half an hour. The antioxidant activity of the diluted bilberry extracts was calculated as the DPPH scavenging activity (%) as follows:

$$\text{DPPH radical scavenging activity (\%)} = [(A_{control} - A_{sample})]/(A_{control})] \times 100,$$

where $A_{control}$ is the absorbance of DPPH radical + methanol; A_{sample} is the absorbance of the DPPH radical + the sample of bilberry extract.

3.2.2 Cellular antioxidant activity assay

Method of cellular antioxidant activity - CAA assay was resumed and modified by Wolfe and Liu (2007). Different compounds and solutions enriched with antioxidants (bilberry extract, blueberry extract, purified bilberry extract, anthocyanin cyanidin 3-glucoside and aglycone cyanidin) were verified on four different cell lines (Caco-2, HepG2, EA.hy926 and A7r5) by using CAA assay.

3.2.2.1 Chemicals and material

Completed Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, Steinheim, Germany) supplemented with 10 % fetal bovine serum (FBS) (EuroClone, Milano, Italy), 1 mM L-glutamine and 1 mM penicillin-streptomycin solution (EuroClone, Milano, Italy) was used as growth medium for culturing HepG2, EA.hy926 and A7r5 cell lines. Completed Minimal Essential Medium Eagle (MEME) (Sigma Aldrich, Steinheim, Germany) supplemented with 10 % fetal bovine serum (FBS) (EuroClone, Milano, Italy), 1 mM L-glutamine, 1 mM penicillin-streptomycin solution and 1 mM sodium pyruvate (EuroClone, Milano, Italy) was used as growth medium for culturing Caco-2 cell line. Solution of 0.05 % trypsin and 0.02 % EDTA in PBS (EuroClone, Milano, Italy) was used to separate cells during culturing; optical microscope (Labovert, Wetzlar, Germany); 75 cm^2 sterile polystyrene flasks (Corning Incorporation, New York, USA); laminar air flow chamber (Jouan SA, St. Herblain, France); incubator (Jouan SA, St. Herblain, France); Sep-Pak C18 cartridge, 1 g (Waters, Milford, USA); lipophilic Sephadex LH-20 (Sigma, Steinheim, Germany); methanol, formic acid and ethyl acetate (Merck, Darmstadt, Germany); thirty-milliliter columns (Lenz Laborglasinstrumente, Werthein, Germany); Rotavapor (Büchi, Fawil, Switzerland); Shaker EV403 (Tehtnica, Železniki, Slovenia).

Cell cultures: Heterogenous human epithelial colorectal adenocarcinoma cell line (Caco-2), hepatocellular carcinoma human cell line (HepG2) and human endothelial cell line (EA.hy926) were obtained from the American Type Culture Collection (Rockville, USA) whereas smooth muscle cell line (A7r5) was obtained from HPA Culture Collections (Salisbury, UK).

Phosphate Saline buffer (PBS): 200 mg/L KCl (Merck, Darmstadt, Germany); 200 mg/L KH_2PO_4 (Carlo Erba, Milano, Italy); 8000 mg/L NaCl; 1150 mg/L Na_2HPO_4 (Carlo Erba, Milan, Italy).

Bilberry and blueberry samples: Bilberries (*Vaccinium myrtillus* L.) were were handpicked in woods in Smerečje in Slovenija while highbush blueberries (*Vaccinium corymbosum* L.)

in the plantation named Kolekcijsko poskusni nasad Kmetijskega inštituta Slovenije in Drenov grič on Ljubljansko barje. The ripe bilberries and blueberries were sampled in June 2008 and immediately frozen at – 20 °C until extraction.

3.2.2.2 Extraction

Crude extracts of bilberries and blueberries were prepared as described in Chapter 3.1.2.2.

3.2.2.3 Purification of the bilberry extract

The crude bilberry extract was purified according to Youdim et al., 2002 (Figure 16). The aim of the purification of the crude bilberry extract was to obtain a fraction enriched with anthocyanins. First 2 mL of the extract was dried at 40 °C under vacuum in the rotavapor, resolubilized in Milli-Q water and then loaded onto a 1 g Sep-Pak C18 cartridge, previously activated by 3 mL of pure methanol and 5 mL of Milli-Q water. The loaded sample was washed with 5 mL of Milli-Q water and 3 mL of 15 % methanol in order to elute phenolic acids. The anthocyanins and other compounds (proanthocyanidins, flavanols and flavonols) were eluted with 3 mL of 3 % formic acid in methanol and then with 3 mL of pure methanol only. The anthocyanin containing fraction was dried and resolubilized in 5 mL of 20 % methanol. Then it was loaded on 1 g of Sephadex LH-20, previously hydrated in 5 mL of 20 % methanol for 2 h and packed into thirty-milliliter glass column. Anthocyanins and flavonols were eluted with 10 mL of 20 % methanol and 10 mL of 60 % methanol whereas proanthocyanidins remained bound on the column. The methanolic fraction (anthocyanins and flavonols) was dried and resolubilized in 3 mL of 5 % formic acid and loaded on a new Sep-Pak C18 cartridge previously activated with 3 mL of pure methanol and with 5 mL of 5 % formic acid. After washing with 5 mL of 5 % formic acid, flavonols were eluted with 5 mL of ethyl acetate and anthocyanins with 5 mL of 10 % formic acid in methanol. Anthocyanin fraction was dried by rotavapor, resolubilized in 1 mL of pure methanol and stored at – 20 °C until analysis.

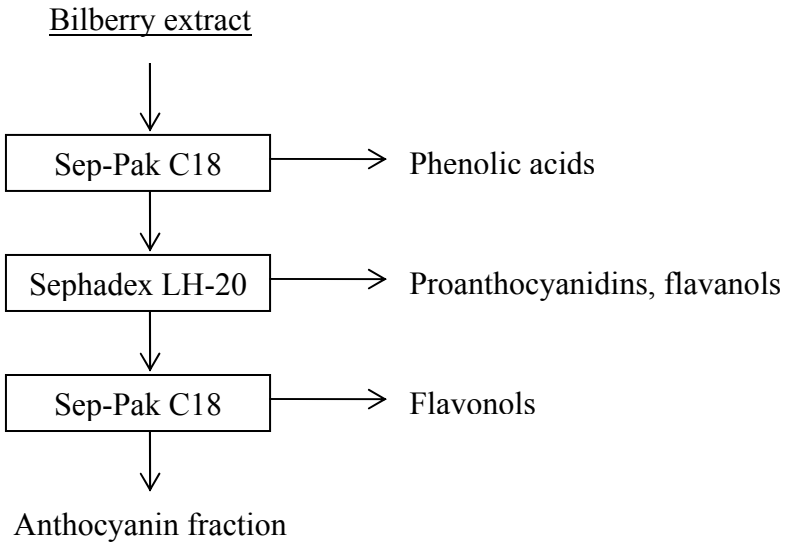


Figure 16: Simple scheme of bilberry extracts' purification to anthocyanin fraction (Youdim et al., 2002).
Slika 16: Enostavna shema čiščenja ekstrakta gozdnih borovnic do frakcije antocianinov (Youdim et al., 2002).

3.2.2.4 Cell culturing

- Stored cells at – 80 °C were rapidly thawed, passed in sterile 75 cm^2 flask and supplemented with a corresponding growth medium.
- Cells were maintained in the incubator at 37 °C in humidified atmosphere of 95 % air and 5 % carbon dioxide. The medium was changed until a monolayer was formed.
- The medium was removed from the flask using a vacuum Pasteur pipette and cells were washed with 10 ml of sterile PBS.
- 2 mL solution of 0.05 % trypsin and 0.02 % EDTA in PBS was added for few minutes at 37 °C to separate the cells.
- Then the activity of trypsin was blocked with 8 mL of the corresponding growth medium.
- Cells were counted under the microscope using hemacytometer.
- A glass coverslip was placed over both counting areas of the hemacytometer and diluted cell suspension was allowed to spread by capillary force.
- Cells of five squares within the grid lines in each area were counted (one square represented 1 mm^2 area).
- The average number of ten squares ($\overline{N}$) was calculated and multiplied by 10^4 and dilute factor (D). The result ($C\left[\frac{cells}{mL}\right]$) presents the number of cells/mL of the cell suspension and was calculated using the following equation:

$$C\left[\frac{cells}{mL}\right] = \overline{N} * 10^4 * D$$

- After determination of the cell number the cell suspension was diluted in corresponding growth medium in order to reach the final concentration and seeded in a new flask to continue culturing cells or on 96-well plate (100 µL of suspension in each well whereas the outside wells of the plate were not used) for CAA assay.

3.2.2.5 Chemicals and material for CAA assay

2',7'-Dichlorofluorescin diacetate (DCFH-DA), phlorizin, 2,2'-azobis(2-amidinopropane)dihydrochloride (ABAP) (Sigma Aldrich, Steinheim, Germany); L-glutamine (EuroClone, Milano, Italy); phosphate saline buffer (PBS); cyanidin 3-glucoside (Polyphenols Laboratories AS, Sandnes, Norway); cyanidin (Extrasynthese, Genay Cedex, France); crude bilberry extract; crude blueberry extract; purified bilberry extract (the procedure of purification is described in chapter 3.2.2.3); 96-well plate (Corning Incorporated, Lowell, USA); rotavapor (Büchi, Fawil, Switzerland); Microplate Reader (Bio-Tek Instruments, Winooski, USA); laminar air flow chamber (Jouan SA, St. Herblain, France); incubator (Jouan SA, St. Herblain, France).

Bilitranslocase antibodies: Polyclonal bilitranslocase antibodies were obtained from rabbit sera immunized with a peptide corresponding to the segment 65–75 (EDSQGQHLSSF) of the primary structure of bilitranslocase. Antibodies, also named antibody A (Passamonti et al., 2005c; Maestro et al., 2009), were purified by affinity chromatography from immune sera of rabbits, as described earlier (Battiston et al., 1998).

Hanks' Balanced Salt Solution (HBSS): 185 mg/L $CaCl_2 \times 2H_2O$; 60 mg/L KH_2PO_4; 350 mg/L $NaHCO_3$; 8000 mg/L NaCl; 47.88 mg/L Na_2HPO_4 (Carlo Erba, Milano, Italy); 100 mg/L $MgCl_2 \times 6H_2O$; 1000 mg/L glucose (Sigma, Steinheim, Germany); 100 mg/L $MgSO_4 \times 7H_2O$; 400 mg/L KCl (Merck, Darmstadt, Germany). pH was adjusted to 7.4 using 0.1 % HCl.

3.2.2.6 Development of control for CAA assay

- Cells seeded in 96-well plate at a density of 1×10^4/well (HepG2, EA.hy926, A7r5) or 2×10^4/well (Caco-2) were maintained in the incubator at 37 °C in humidified atmosphere of 95 % of air and 5 % of carbon dioxide for 24 h.
- The medium was removed by aspiration.
- Then cells were treated for one hour with 100 µL of solution supplemented with 50 µM DCFH-DA or/and 25 µM DCFH-DA, 1 mM L-glutamine and dissolved in not-

completed corresponding medium. Each concentration of each substance was repeated in 6 wells. Each plate also included 6 control and 6 blank wells treated without antioxidant substance.
- The medium was removed by aspiration and the wells were washed with 100 µL PBS.
- Then 1.2 mM, 2.4 mM, 3.6 mM and 5 mM ABAP concentrations was applied to the cells in 100 µL of HBSS. For the blank 100 µL of HBSS without ABAP was applied to cells.
- The 96-well plate was immediately placed into a Microplate Reader at 37 ºC. Emission was measured at 535 nm with excitation at 485 nm every five minutes for one hour.

3.2.2.7 CAA assay

- Cells seeded in 96-well plate at a density of 1×10^4/well (HepG2, EA.hy926, A7r5) or 2×10^4/well (Caco-2) were maintained in the incubator at 37 °C in humidified atmosphere of 95 % air and 5 % carbon dioxide for 24 hours.
- The medium was removed by aspiration.
- After this step cells were treated for one hour with 100 µL of solution supplemented with an antioxidant substance (cyanidin 3-glucoside, crude bilberry extract, purified bilberry extract or crude blueberry extract) in divergent concentrations, 50 µM DCFH-DA, 1 mM L-glutamine and dissolved in not-completed corresponding medium. Each concentration of each substance was repeated in 6 wells. Each plate also included 6 wells of control and 6 wells of blank treated without an antioxidant substance.
- After one hour of incubation solutions were removed and cells were washed twice with PBS.
- Then 5 mM ABAP was applied to cells in 100 µL of HBSS. Control wells were treated with 100 µL of HBSS enriched with ABAP whereas blank wells were applied with 100 µL of HBSS without ABAP.
- The 96-well plate was immediately placed into a Microplate Reader at 37 ºC. Emission was measured at 535 nm with excitation at 485 nm every five minutes for one hour.

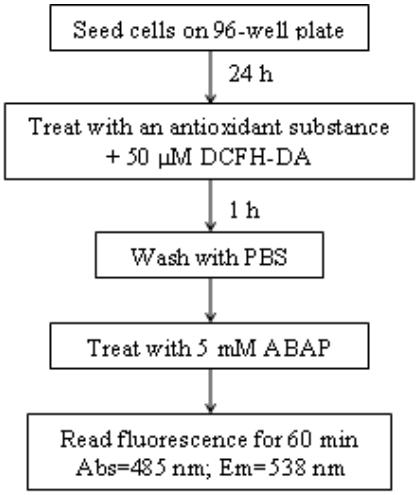


Figure 17: Procedure of the cellular antioxidant activity (CAA) assay.
Slika 17: Postopek poskusa celične antioksidativne aktivnosti (CAA).

3.2.2.8 CAA quantification

To quantify the cellular antioxidant activity the CAA units (%) were calculated as follows:

$$CAA = 100 - (\int SA/\int CA) \times 100$$

where ∫SA is the integrated area under the sample fluorescence readings, with the blank previously subtracted, versus time to the 60 min, and ∫CA is the integrated area of the control. The measurements were repeated in six repetitions.

The calculated CAA units versus anthocyanins concentration were used to create a dose-response curve of each berry extract for each cell line. From these curves, half maximal effective concentrations (EC_{50}) and CAA maximal antioxidant activity (CAA_{max}) of the anthocyanins were determined. We calculated the area under the curve (AUC) of CAA units for each extract and for each cell line versus anthocyanin concentration (up to 50 µg/L) in order to compare the antioxidant activity of the berry extracts in different cell lines.

3.2.2.9 Statistical analyses

Statistical analyses were performed using GraphPad Prism software (version 5.00, GraphPad Software, San Diego, USA). Differences between means for total phenolics and total anthocyanins in bilberries and blueberries analyzed by spectrophotometer, anthocyanin glycosides and total anthocyanins in bilberries and blueberries from different parts of Slovenia have been compared using one-way ANOVA with post-Bonferroni test. Differences between means for AUC of CAA units have been compared using one-way ANOVA with post-Bonferroni test or Student's t-test.

3.3 ANTHOCYANIN BIOAVAILABILITY

3.3.1 Cyanidin 3-glucoside uptake in HepG2 and EA.hy926 cells

Anthocyanin uptake in two different cells (HepG2 and EA.hy926) was demonstrated by incubation with the pure anthocyanin cyanidin 3-glucoside. Our aim was to examine the accumulation of cyanidin 3-glucoside into the cells and to detect metabolites by LC-MS/MS method. The procedure was as follows:

- Cells (HepG2 or EA.hy629) were cultivated in a flask to rich a monolayer (see Chapter 3.2.2.4).
- The medium was removed from cells using vacuum Pasteur pipette.
- Cells were washed with 10 mL of PBS for three times.
- 5 mL of 10 mg/L cyanidin 3-glucoside in a corresponding growth medium without phenolphthalein supplemented with 1 mM L-glutamine was placed on the cells.
- Cells were incubated for 1 hour at 37 °C in humidified atmosphere of 95 % of air and 5 % of carbon dioxide.
- Cyanidin 3-glucoside solution was removed from cells using vacuum Pasteur pipette.
- Cells were washed with an ice-cold PBS three times and dried using vacuum Pasteur pipette.
- 2 mL of trypsin were added to separate the cells.
- Then 3 mL of the corresponding growth medium without phenolphthalein supplemented with 1 mM L-glutamine was added to stop the trypsin activity.
- Separated cells were quantitatively passed into a 50 mL falcon, completed with 45 mL of ice-cold nitrogenated pure methanol and mixed well.
- Samples were centrifuged for 5 min at 3640 *g* at 4 °C and stored at – 20 °C until LC-MS/MS analysis.
- Cyanidin and its metabolites were detected by LC-MS/MS method (see Chapter 3.1.4.3).

3.3.2 Membrane transport of anthocyanins

3.3.2.1 CAA assay and bilitranslocase

Modified CAA assay was employed to demonstrate a role of bilitranslocase in anthocyanin transport into the cell using Caco-2, EA.hy926 and A7r5 cell lines. The modified procedure of basic CAA includes an incubation of antibodies rabbit immunoglobulins (IgG) and MAP (Multiple Antigen Peptide or bilitranslocase antibodies), respectively.

- Cells seeded in 96-well plate at a density of 1×10^4/well (EA.hy926, A7r5) or 2×10^4/well (Caco-2) were maintained in the incubator at 37 °C in humidified atmosphere of 95 % air and 5 % carbon dioxide for 24 h.
- The medium was removed by aspiration.
- Cells were incubated with 100 µL of solution supplemented with 1 mM L-glutamine and 0.24 µg/mL of antibodies (IgG or MAP) dissolved in not-completed corresponding medium for 30 min.
- After 30 min of incubation the medium was removed by aspiration.
- After this step cells were treated for one hour with 100 µL of solution supplemented with an antioxidant substance (cyanidin 3-glucoside, cyanidin or phlorizin) in divergent concentrations, 50 µM DCFH-DA, 1 mM L-glutamine and dissolved in not-completed corresponding medium. Each concentration of each substance was repeated in 6 wells. Each plate also included 6 wells of control and 6 wells of blank treated without an antioxidant substance.
- After one hour of incubation solutions were removed and cells were washed twice with PBS.
- Then 5 mM ABAP was applied to cells in 100 µL of HBSS. Control wells were treated with 100 µL of HBSS enriched with ABAP whereas blank wells were applied with 100 µL of HBSS without ABAP.
- The 96-well plate was immediately placed into a Microplate Reader at 37 ºC. Emission was measured at 535 nm with excitation at 485 nm every five minutes for one hour.

The quantification of the CAA units (%) was calculated as described above (see chapter 3.2.2.8). For comparison of CAA (%) in cells pre-incubated with either IgG or MAP, the integrated areas under either the concentration-dependence or the time-dependence curves (not fitted) were calculated. The standard error of each area was estimated by averaging coefficients of variation of CAA values for each concentration and time point, respectively. Differences have been compared using Student's t-test using GraphPad Prism software (version 5.00, GraphPad Software, San Diego, CA, USA).

3.3.2.2 Interactions of cyanidin, cyanidin 3-glucoside and bilirubin with model liposome membranes

The aim of these experiments was to investigate the interaction of cyanidin, cyanidin 3-glucoside and bilirubin with model membranes – liposomes. Fluorescence polarisation and anisotropy were measured in the presence of fluorophor 1,6-diphenyl-1,3,5-hexatriene (DPH) by fluorescence spectroscopy.

Chemicals and material

Cary Eclipse Fluorescence Spectrophotometer (Varian, Mulgrave, Victoria, Australia); Mini-Extruder, phosphatidylcholine (PC), filter supports (Avanti Polar Lipids, Inc., Alabaster, Alabama, USA); Nuclepore track-etched membrane, 100 nm (Whatman, Schleicher & Schuell, Maidstone, UK); 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chem. Co., Milwaukee, USA); cyanidin (Extrasynthese, Genay Cedex, France); cyanidin 3-glucoside (Polyphenols Laboratories AS, Sandnes, Norway); chloroform (Merck, Darmstadt, Germany); 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sphingomyelin (SM), dimethyl sulfoxide (DMSO), bilirubin (Sigma, Steinheim, Germany).

Preparation of vesicles by the extrusion

Vesicles were prepared from phosphatidylcholine (PC) and sphingomyelin (SM) in a ratio 2.4:1 by extrusion. This phospholipid composition was used as a model membrane of vascular endothelial cells (EA.hy926) determined by Cansell et al. (1997). Lipid films were formed after removing chloroform from phospholipids solution using a rotavapor. Phospholipids were swollen in buffer HEPES (20 mM, pH=7) and vortexed vigorously to give multilamellar vesicles. These multilamellar liposomes were disrupted by several freeze-thaw cycles and forced through the filter which consisted of two filter supports and Nuclepore track-etched membrane with 100 nm pores. Extrusion through filters yielded to extruded unilamellar vesicles (eUV). The procedure of eUV preparation is shown in Figure 18.

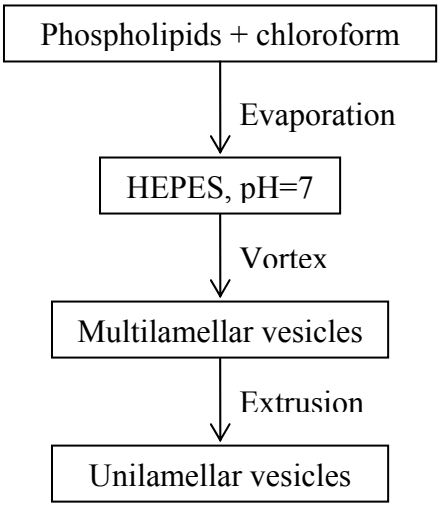


Figure 18: Procedure of extruded unilamellar vesicles (eUV) preparation.
Slika 18: Postopek priprave unilamelarnih liposomov.

Fluorescence anisotropy and polarisation measurements

The method of Rebolj et al., 2006 for fluorescence anisotropy and polarisation measurements was slightly modified. The measurements were performed at 20 °C in a 1 cm pathlength cuvette using Cary Eclipse Fluorescence Spectrophotometer. DPH was

added to the 100 µg/mL solution of eUV. The final concentration of DPH in a mixture was 0.5 µM. The DPH fluorescence anisotropy and polarisation were measured at an excitation wavelength of 358 nm with the excitation polarizer oriented in the vertical position, while vertical and horizontal components of polarized emission light were recorded through a monochromator at 410 nm. The value of G-factor (the ratio of the sensitivities of the detection system for vertically and horizontally polarized light) was determined for each sample separately. A tested compound (cyanidin, cyanidin 3-glucoside and bilirubin) was gradually added to eUV liposomes in different concentrations which were the same as in the case of the cellular antioxidant activity assay. Cyanidin and cyanidin 3-glucoside were dissolved in 20 mM HEPES, pH 7.0, and bilirubin in pure dimethyl sulfoxide (DMSO). The mixture with no anthocyanidin or anthocyanin but only HEPES or DMSO added was used as a control. Anisotropy and polarisation was measured after two minutes of shaking. The subtraction of a sample anisotropy or polarisation from a control one was expressed as a difference in anisotropy (ΔA) or polarisation (ΔP). Each compound was analyzed in three replicates. All results are expressed as a mean ± SD.

4 RESULTS AND DISCUSSION

4.1 PHENOLICS IN THE BILBERRIES AND BLUEBERRIES

4.1.1 Spectrophotometric analysis

4.1.1.1 Total phenolics

Total phenolics of bilberries and blueberries were determined spectrophotometrically by the Folin-Ciocalteu method (Di Stefano and Guidoni, 1989; Rigo et al., 2000). The calibration curve (Annex A) was based on the concentration of gallic acid in the range at 1 - 11 mg/L. The coefficient of the correlation was 0.9964.

The samples of bilberries were handpicked in woods from 7 different locations of Slovenia: Ljubljana (n = 6), Pohorje (n = 6), Pokljuka (n = 3), Kranj (n = 6), Goričko (n = 5), Škofja loka (n = 6) and Celje (n = 4) where n means a number of microlocations. Blueberries (n = 5) were handpicked from a plantation named Kolekcijsko poskusni nasad Kmetijskega inštituta Slovenije in Drenov grič on the Ljubljansko barje. The results of the total phenolic content in the bilberries and blueberries are in Figure 19. The data (mean ± SEM) were expressed as milligrams of gallic acid equivalents (GAE) per 100 g of fresh weight (fw) of berries. All data are represented in Annex B. Statistical analysis was performed using one-way ANOVA with a post-Bonferroni test. Statistically significant differences ($p < 0.05$) between the columns are marked with different letters (a, b).

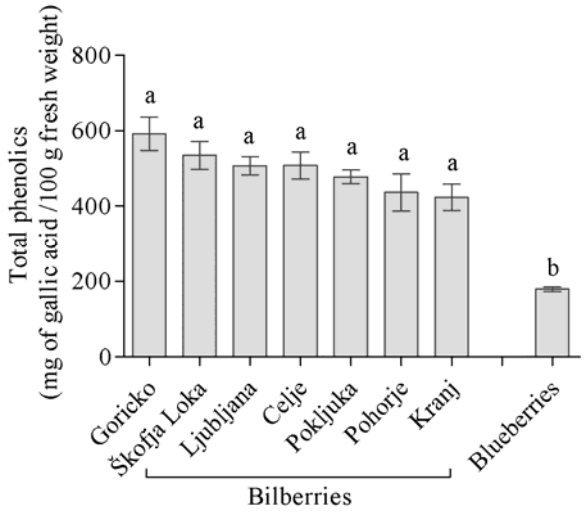


Figure 19: Content of total phenolics in Slovenian bilberries and blueberries. Statistically different differences are marked with different letters (a,b).

Slika 19: Vsebnost skupnih fenolnih spojin v gozdnih in ameriških borovnicah iz Slovenije. Statistično značilne razlike so označene z različnimi črkami (a,b).

It can be concluded concluded from Figure 19 that there were no significant differences between the total phenolics content of the bilberries sampled from different locations, but the difference between the bilberries and the blueberries was significant. The average content of total phenolics in the bilberries was 495.2 ± 16.3 mg/100 g fw whereas in the blueberries it reached 178.9 ± 5.6 mg/100 g fw. However, there were over 3-fold more total phenolics determined in the bilberries than in the blueberries. Previous reports were similar, the bilberries had 525 - 890 mg/100 g fw (Prior et al., 1998; Giovanelli and Buratti, 2009; Šavikin et al., 2009) and the blueberries 115 - 444 mg/100 g fw (Prior et al., 1998; Ehlenfeldt and Prior, 2001; Moyer et al, 2002; Sellappan et al., 2002; Zheng et al., 2003; Taruscio et al., 2004; Wang et al., 2008a; Giovanelli and Buratti, 2009). Other berries have similar amount of total phenolics. Blackberries contain 275 - 678 mg/100 g fw, raspberries 126 - 1073 mg/100 g fw, ribes 191 - 1342 mg/100 g fw (Moyer et al, 2002) and strawberries 357 - 370 mg/100 g fw (Lin and Tang, 2007). Also other fruits such as red grape (311 to 561 mg/100 g fw) (Revilla et al., 1998) and pink grapefruit (425 mg/100 g fw) (Cieślik et al., 2006) have similar content of total phenolics. The content of total phenolics in mango reaches 71 - 113 mg/100 g fw (Patthamakanokporn et al., 2008), in white grapes 95 mg/100 g fw, plum 200 mg/100 g fw, orange 217 mg/ 100 g fw, kiwi fruit 273 mg/100 g fw and in apples 132 mg/100 g fw (Cieślik et al., 2006). All these fruits are also good sources of phenolics. Larger amounts of total phenolics were found in the European elder, 1540 mg/100 g fw (Cieślik et al., 2006), whereas nectarine (57 mg/100 g fw) (Cieślik et al., 2006), banana (14 ± 0.5 mg/100 g fw) and papaya (54 ± 2.6 mg/100 g fw) contain lower amounts of total phenolics (Patthamakanokporn et al., 2008).

4.1.1.2 Analysis the total anthocyanins

The total anthocyanins of the bilberries and blueberries were determined spectrophotometrically by Glories (1984) and adjusted to Di Stefano et al. (1989). The calibration curve (Annex C) was based on the concentration of cyanidin 3-glucoside in the range from 9 - 18 mg/L. The coefficient of correlation was 0.9989.

All data are represented in Annex D. The results of the total anthocyanin content are shown in Figure 20. The data (mean ± SEM) were expressed as milligrams of cyanidin 3-glucoside equivalents per 100 g of fresh berries. Statistical analysis was performed using one-way ANOVA with a post-Bonferroni test. Statistically significant differences ($p < 0.05$) between the columns are marked with different letters (a, b).

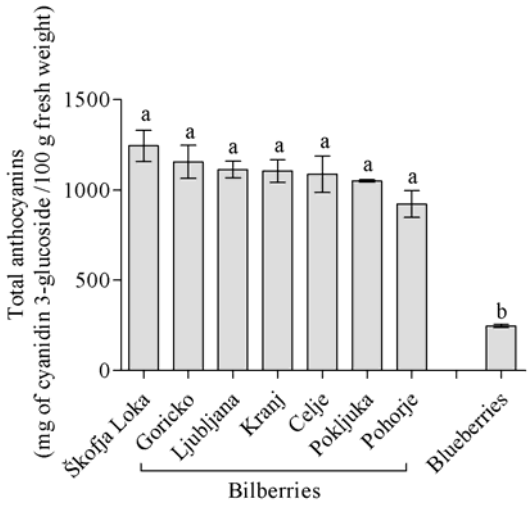


Figure 20: Contents of total anthocyanins in Slovenian bilberries and blueberries. Statistically different differences are marked with different letters (a,b).

Slika 20: Vsebnost skupnih antocianinov v gozdnih in ameriških borovnicah iz Slovenije. Statistično značilne razlike so označene z različnimi črkami (a,b).

There were no significant differences between the total anthocyanin content of the bilberries sampled on different locations. The difference between the bilberries and the blueberries was significant and correlates with their total phenolic content. The bilberries contained in average 1101.2 ± 30.7 mg total anthocyanins/100 g fw whereas the blueberries had 246.5 ± 8.9 mg/100 g fw. Thus, there were over 4-fold more total anthocyanins determined in the bilberries than in the blueberries. Reported values of the total anthocyanin content in the bilberries are much lower, ranging from 229 to 370 mg/100 g fw (Prior et al., 1998; Kalt et al., 1999; Giovanelli and Buratti, 2009) whereas they are similar for the blueberries, ranging from 82 to 250 mg/100 g fw (Prior et al., 1998; Kalt et al. 1999; Ehlenfeldt and Prior, 2001; Moyer et al., 2002; Sellappan et al., 2002; Zheng et al., 2003; Taruscio et al., 2004; Wang et al., 2008a; Giovanelli and Buratti, 2009). It looks like that the bilberries from Slovenian woods are very good source of anthocyanins. Other berries are also good sources of anthocyanins. A few examples: blackberries contain 80 - 230 mg/100 g fw of total anthocyanins, raspberries 52 - 627 mg/100 g fw, ribes 14 - 411 mg/100 g fw (Moyer et al, 2002) and cranberries 360 mg/100 g fw (Prior et al., 2001). On the other side cherries contain 2 - 297 mg of total anthocyanins /100 g fw (Gao and Mazza, 1995), red grape 4 - 111 mg/100 g fw (Revilla et al., 1998, Orak, 2007), and strawberries 20 - 42 mg/100 g fw (Klopotek et al., 2005; Zheng et al., 2007).

In conclusion consuming of about 18 g of Slovenian bilberries or 80 g of blueberries is sufficient to reach the estimated average value of the anthocyanin daily intake (180-215 mg of anthocyanins) (Hertog et al., 1993).

4.1.2 HPLC-MS analyses

4.1.2.1 LC-MS/MS analysis of individual anthocyanins

Individual anthocyanins were determined in the bilberries and blueberries by LC-MS/MS in the positive ion mode (ESI^+) and were identified on the basis of their retention times, MS spectra and molecular ion identification. Their contents were expressed as milligrams of cyanidin 3-glucoside equivalents per 100 g of fresh bilberries or blueberries according to spiked calibration curve shown in Annex E. The data were expressed as a mean ± SEM. The coefficient of correlation was more than 0.99. The number of independent measurements for the bilberries was 36 (n = 36) and 5 (n = 5) for the bilberries. The repeatability of a standard cyanidin 3-glucoside is shown in the Annex F. The limit of detection (LOD) with a signal-to-noise ratio (S/N) ≥ 3 for cyanidin 3-glucoside was 0.3×10^{-6} g/kg. The limit of quantification (LOQ) with a signal-to-noise ratio (S/N) ≥ 10 also for cyanidin 3-glucoside was 0.9×10^{-6} g/kg. Recovery of cyanidin 3-glucoside in the spiked calibration curve was 96.50 %. LC-MS/MS chromatogram of individual anthocyanins from the bilberry sample is shown in Annex G. All data are represented in Annex H.

The individual anthocyanin content in the bilberries and blueberries, determined by LC-MS/MS, is shown in Table 11. The content of individual anthocyanins were determined by LC-MS/MS, according to the optimized method of Lätti et al. (2008). The data were expressed as milligrams of cyanidin 3-glucoside equivalents per 100 g of fresh berries, according to the linear calibration curve with the correlation coefficient 0.9983, from the spiked samples. The limit of detection (LOD), the limit of quantification (LOQ) and repeatability (CV) of the standard were determined.

Table 11: The individual anthocyanin content of the bilberries and blueberries.
Preglednica 11: Vsebnost posameznih antocianinov v gozdnih in ameriških borovnicah.

Anthocyanins	**Retention time** (min)	**Bilberries** (mg/100 g)	**Blueberries** (mg/100 g)
Delphinidin 3-galactoside	10.5	167.1 ± 6.5	23.4 ± 1.4
Delphinidin 3-glucoside	11.5	169.1 ± 6.6	15.4 ± 1.4
Cyanidin 3-galactoside	12.2	122.6 ± 5.7	4.2 ± 0.2
Delphinidin 3-arabinoside	12.7	152.3 ± 6.1	24.5 ± 1.7
Cyanidin 3-glucoside	13.7	130.4 ± 6.2	2.6 ± 0.3
Petunidin 3-galactoside	14.8	50.0 ± 2.6	11.7 ± 0.8
Cyanidin 3-arabinoside	15.2	110.6 ± 4.9	3.5 ± 0.3
Petunidin 3-glucoside	16.7	101.9 ± 6.2	12.4 ± 0.8
Peonidin 3-galactoside	17.8	13.3 ± 1.2	1.8 ± 0.01
Petunidin 3-arabinoside	19.0	23.9 ± 1.5	9.3 ± 0.6
Peonidin 3-glucoside	20.8	56.7 ± 5.1	2.1 ± 0.1
Malvidin 3-galactoside	21.7	27.5 ± 3.3	34.9 ± 3.1
Peonidin 3-arabinoside	23.0	4.5 ± 0.4	1.0 ± 0.1
Malvidin 3-glucoside	24.9	67.7 ± 5.9	31.2 ± 3.4
Malvidin 3-arabinoside	28.2	12.8 ± 1.3	34.7 ± 3.7
total		1210.3 ± 111.5	212.4 ± 14.1

The bilberries were sampled in woods from seven different locations in Slovenia: Pokljuka (n = 3), Celje (n = 4), Goricko (n = 5), Skofja Loka (n = 6), Ljubljana (n = 6), Pohorje (n = 6), and Kranj (n = 6), where n means a number of microlocations. The blueberries were picked from a plantation in Ljubljana. All samples were collected at the full ripe stage in the latter part of June. Anthocyanins quantified as mg of cyanidin-3-glucoside equivalents/100 g berries fresh weight (fw). Data expressed as the mean ± SEM. Number of independent measurements: bilberries n = 36, blueberries n =5; t_R, retention time.

The sum of the individual anthocyanins (1210.3 ±111.5 mg/100 g fw) in the bilberries was about 6-fold greater than that in the blueberries (212.4±14.1 mg/100 g fw). Obtained data are in agreement with our previous spectrophotometric analysis. However, the anthocyanin content in the Slovenian bilberries were greater for ca. 3-fold than previously reported data of bilberries from Finland (350-525 mg/100 g fw) (Kähkönen, et al., 2003; Lätti et al., 2008). The variations of the anthocyanins profile and content observed in bilberries might occur upon genetic and/or pedoclimatic diversity. In our investigations (unpublished data) no genetic diversity was detected among the bilberries sampled in those growing regions. Concerning climate, Pokljuka and Pohorje belong to the mountainous growing region; Celje, Goričko and Škofja Loka belong to the hilly growing region and Ljubljana and Kranj belong to the lowland growing region. In all growing regions bilberries grow in acid soil with high organic matter. However, the higher anthocyanin levels in bilberries than in blueberries are due to anthocyanins found in both the skin and pulp of bilberries, whereas in blueberries they are only in the skin (Riihinen in sod., 2008). The skin is also where red anthocyanins are mainly found in red grapes and blackcurrants (Bakker and Timberlake, 1985) while the whole fruits of strawberries, blackberries, raspberries and elderberries are

colored because of the anthocyanins (Mullen et al., 2002; Hartmann et al., 2008; Cuevas-Rodríguez et al., 2010). As already mentioned bilberries are richer with anthocyanins than other berries such as blackcurrants, cranberries, lingonberries, raspberries and strawberries (Heinonen, 2007), while blueberries have similar content of anthocyanins as blackcurrant (Riihinen et al., 2008) but lower than blackberries (Sellappan et al., 2002), cranberries (Taruscio et al., 2004) and rowanberries (Hukkanen et al., 2006).

The contents of each of the individual anthocyanins in the bilberries and blueberries were calculated according to the five anthocyanidin classes found (delphinidin, cyanidin, petunidin, malvidin, peonidin) and expressed as percentages of the total anthocyanins (Figure 21). In the Slovenian bilberries, the delphinidin and cyanidin glycosides were predominant (>70%), as has been reported recently for Finnish (Lätti et al., 2008) and Swedish bilberries (Åkerström et al., 2009). However, the anthocyanidin glycosides of the blueberries collected in Slovenia were different when compared to the Slovenian bilberries, with their most abundant anthocyanidin glycoside as the malvidin glycosides (ca. 47%). Some published data are in agreement to ours here (Wang et al., 2008; Burdulis et al., 2009), although others are in contradiction. In blueberries from Canada and USA, for instance, only the delphinidin glycosides were predominant (Taruscio et al., 2004). However, in both of these Slovenian berries, the peonidin glycosides content was the lowest of anthocyanidin glycosides as has been seen for blueberries grown in Finland (Lätti et al., 2008). However, Slovenian bilberries and blueberries represent a good source of bioactive compounds in human nutrition.

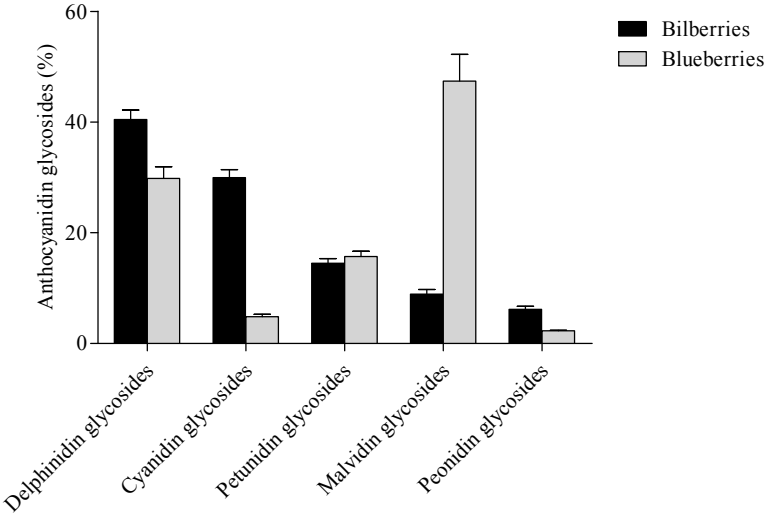


Figure 21: Content of anthocyanidin glycosides in Slovenian bilberries and blueberries. Data are expressed as the mean (% of total anthocyanidin glycosides) ± SEM of glycoside types indicated. Number of independent measurements: bilberries n = 36; blueberries, n = 5.

Slika 21: Vsebnost antocianidin glikozidov v gozdnih in ameriških borovnicah iz Slovenije. Podatki so podani kot srednja vrednost skupnih antocianidin glukozidov (%) ± SEM. Število paralelk: gozdne borovnice n = 36, ameriške borovnice n= 5.

4.1.2.2 LC-MS analysis of phenolic acids, flavanols, flavonols, flavones and resveratrol

Flavanols, flavonols, phenolic acids and resveratrol were determined in the bilberries and blueberries by LC-MS and were identified on the basis of their retention times, MS spectra and molecular ion identification. Flavanols, flavonols and phenolic acids have been determined in the negative ion mode (ESI^-) whereas *trans*-resveratrol in the positive ion mode (ESI^+). Flavanols were quantified on catechin and flavonols to each corresponding standard (myricetin, quercetin and rutin). The phenolic acids were quantified to each corresponding standard (chlorogenic acid as 3-caffeoylquinic acid, caffeic acid, ferulic acid, *p*-coumaric acid, ellagic acid and gallic acid). Resveratrol was quantified to a standard *trans*-resveratrol. The spiked and not-spiked calibration curves are shown in Annex I. The coefficients of correlation were more than 0.9. The results were expressed as milligrams of corresponding standard equivalents per 100 g of fresh bilberries or blueberries. The data were expressed as the mean ± SEM. The number of independent measurements for the bilberries was 36 (n = 36) and 5 (n = 5) for the bilberries. The limit of detection (LOD), quantification (LOQ), repeatability (CV) and recovery of the studied compounds are shown in Annex J. LC-MS chromatograms of flavanols, flavonols, phenolic acids and *trans*-resveratrol of the bilberry sample were shown in Annex K. The occurrence of flavanols, flavonols, phenolic acids and resveratrol in the bilberries and blueberries are presented in Table 12. All data are represented in Annex L.

Table 12: Content of flavanols, flavonols, phenolic acids and resveratrol in Slovenian bilberries and blueberries.

Preglednica 12: Vsebnost flavanolov, flavonolov, fenolnih kislin in resveratrola v gozdnih in ameriških borovnicah iz Slovenije.

Phenolic compounds	**M_W**	**Retention time** (min)	**Bilberries** (mg/100 g fw)	**Blueberries** (mg/100 g fw)
Flavanols[1]				
Catechin	290	9.7	0.2 ± 0.0	1.8 ± 0.1
Epicatechin	290	15.9	2.0 ± 0.2	0.5 ± 0.1
Flavonols[2]				
Quercetin	302	32.8	0.8 ± 0.0	0.1 ± 0.0
Myricetin	318	32.9	0.4 ± 0.0	nd
Rutin	610	23.7	0.2 ± 0.0	3.1 ± 0.1
Phenolic acids[3]				
Chlorogenic acid	354	10.5	23.1 ± 1.1	70.0 ± 3.4
Caffeic acid	180	13.2	0.3 ± 0.0	0.2 ± 0.0
Ferulic acid	194	26.2	0.4 ± 0.0	2.2 ± 0.2
p-Coumaric acid	164	19.7	0.3 ± 0.0	id
Gallic acid	170	3.7	6.19 ± 0.3	1.8 ± 0.2
Ellagic acid	302	24.1	1.16 ± 0.0	0.1 ± 0.0
Resveratrol[4]				
trans-Resveratrol	228	29.3	0.20 ± 0.0	0.4 ± 0.0

The bilberries were sampled in woods from seven different locations in Slovenia: Pokljuka (n = 3), Celje (n = 4), Goricko (n = 5), Skofja Loka (n = 6), Ljubljana (n = 6), Pohorje (n = 6), and Kranj (n = 6), where n means a number of microlocations. The blueberries were picked from a plantation in Ljubljana. All samples were collected at the full ripe stage in the latter part of June. M_w, molecular weight; t_R, retention time. Phenolic compounds were quantified as mg equivalents/100 g berries fresh weight (fw):

[1] to catechin or

[2] to each flavonol or

[3] to each phenolic acid or

[4] to *trans*-resveratrol.

id = identified but not quantified

nd = not detected

Data expressed as the mean ± SEM. Number of independent measurements: bilberries n = 36, blueberries n =5.

Flavanols

Catechin and epicatechin were the flavanols found in the bilberries and blueberries. Catechin reached 0.2 ± 0.0 mg/100 g fw in the bilberries while the blueberries were better source with 1.8 ± 0.1 mg/100 g fw. The bilberries had more epicatechin (2.0 ± 0.2 mg/100 g fw) than the blueberries (0.5 ± 0.1 mg/100 g fw). In comparison to the previously published data, bilberries have catechin, ranging from 0.7 to 2.3 mg/100 g fw and the content of epicatechin is similar, ranging from 1.3 to 6.8 mg/100 g fw (Stöhr and Herrmann, 1975; Määttä-Riihinen et al., 2004; Tsanova-Savova et al., 2005). Meanwhile, in the blueberries catechin ranges from 0.6 to 29.3 mg/100 g fw or it was not detected and

epicatechin varies from 0.7 - 1.6 mg/100 g fw or it was not detected (Arts et al., 2000; Sellappan et al., 2002; Määttä-Riihinen et al., 2004; Taruscio et al., 2004). Both of these flavanols have been detected in other berries, such as blackberries, cranberries, strawberries and in higher amouts in white and red grape (Tsanova-Savova et al., 2005).

Flavonols

Quercetin, myricetin and rutin were the flavonols determined in the bilberries and blueberries. The bilberries had 0.8 ± 0.0 mg quercetin /100 g fw and the blueberries only 0.1 ± 0.0 mg/100 g fw. Published values are higher in both; in bilberries, ranging from 1.3 to 17.1 mg/100 g fw (Häkkinen et al., 1999b; Häkkinen and Törrönen, 2000; Määttä-Riihinen et al., 2004; Ehala et al., 2005; Riihinen et al., 2008) and in blueberries, from 1.7 to 53.1 mg/100 g fw (Häkkinen et al., 1999b; Häkkinen and Törrönen, 2000; Sellappan et al., 2002; Määttä-Riihinen et al., 2004; Taruscio et al., 2004; Riihinen et al., 2008). Myricetin was detected only in the bilberries (0.4 ± 0.0 mg/100 g fw) although in lower amount than in previously reported data (1.2 to 5.0 mg/100 g fw) (Häkkinenet et al., 1999b; Häkkinen and Törrönen, 2000; Määttä-Riihinen et al., 2004; Riihinen et al., 2008). The blueberries did not contain myricetin. Quercetin and myricetin were detected in many other berries such as lingonberries, gooseberries, chokeberries, rowanberries, strawberries, cranberries, crowberries and currants (Häkkinen et al., 1999a). Rutin was found in both, the bilberries (0.2 ± 0.0 mg/100 g fw) and blueberries (3.1 ± 0.1 mg/100 g fw). There are no data in the literature of rutin presence in the bilberries or blueberries. The compound has been found in high amounts in buckwheat (Quettier-Eleu et al., 2000).

Hydroxycinnamic acids

Chlorogenic acid, caffeic acid, ferulic acid and *p*-coumaric acid were the hydroxycinnamic acids identified in the bilberries and blueberries. Our results showed that chlorogenic acid was the predominant phenolic acid in both, the bilberries (23.1 ± 1.0 mg/100 g fw) and the (70.0 ± 3.4 mg/100 g fw). Some other bilberries had 14.1 ± 1.3 mg chlorogenic acid/100 g fw (Hukkanen et al., 2006) or the acid was found but not quantified (Ehala et al., 2005). It was determined in the blueberries too, from 4.3 - 126.1 mg/100 g fw (Gao and Mazza, 1994; Zheng and Wang, 2003; Zheng et al., 2003; Taruscio et al., 2004; Wang et al., 2008a; Wang et al., 2008b). Caffeic acid was present in small amounts in both, the bilberries (0.3 ± 0.0 mg/100 g fw) and blueberries (0.2 ± 0.0 mg/100 g fw). Published data are ranging from 0.3 to 10.6 mg/100 g fw bilberries or it was found but not quantified (Ehala et al., 2005; Zadernowski et al., 2005; Mattila et al., 2006) and from 0.5 - 18.2 mg/100 g fw blueberries (Häkkinen and Törrönen, 2000; Sellappan et al., 2002; Taruscio et al., 2004). Ferulic acid was also present in the bilberries (0.4 ± 0.0 mg/100 g fw) and the blueberries (2.2 ± 0.2 mg/100 g fw). Other studies showed the content of ferulic acid from 0.3 to 2.3 mg/100 g for the bilberries fw (Ehala et al., 2005; Zadernowski

et al., 2005; Mattila et al., 2006) whereas 3.5 to 4.4 mg/100 g for the blueberries fw (Sellappan et al., 2002; Taruscio et al., 2004). We determined 0.3 ± 0.0 mg *p*-coumaric acid /100 g of the bilberries fw. The result is in accordance with the previously published data for the content ranging from 0.1 to 13.2 mg/100 g fw (Häkkinen and Törrönen, 2000; Määttä-Riihinen et al., 2004; Ehala et al., 2005; Zadernowski et al., 2005; Mattila et al., 2006; Riihinen et al., 2008). In our experiment *p*-coumaric acid was not quantified in the blueberries though it was identified. The result is comparable to some previous results (Häkkinen and Törrönen, 2000) while others showed the content ranging from 0.5 to 7.2 mg *p*-coumaric acid/100 g fw (Sellappan et al., 2002; Määttä-Riihinen et al., 2004; Taruscio et al., 2004; Riihinen et al., 2008).

Hydroxybenzoic acids

Gallic acid and ellagic acid were the hydroxybenzoic acids found in the bilberries and blueberries. The content of gallic acid was higher in the bilberries (6.2 ± 0.3 mg/100 g fw) than in the blueberries (1.8 ± 0.2 mg/100 g fw). In comparison to published data, the bilberries are reported to contain 0.1 - 3.2 mg/100 g fw (Stöhr and Herrmann, 1975; Zadernowski et al., 2005; Mattila et al., 2006) versus 2.0 - 4.8 mg/100 g fw in the blueberries (Sellappan et al., 2002). Ellagic acid was found in both, the bilberries (1.2 ± 0.0 mg/100 g fw) and the blueberries (0.1 ± 0.0 mg/100 g fw). It was already reported that bilberries are richer with ellagic acid than blueberries (Häkkinen et al., 1999a). So far only one publication reported the content of ellagic acid in the blueberries ranging from 0.75 to 6.65 mg/100 g fw (Sellappan et al., 2002).

Resveratrol

Trans-resveratrol was found in both, the bilberries (0.2 ± 0.0 mg/100 g fw) and the blueberries (0.4 ± 0.0 mg/100 g fw). There are not many publications of *trans*-resveratrol reporting its presence in bilberries and blueberries. They determined 0.002 - 0.678 mg t*rans*-resveratrol/100 g fw bilberries (Lyons et al., 2003; Ehala et al., 2005) and 0.003 - 0.269 mg/100 g fw blueberries (Lyons et al., 2003; Wang et al., 2008a; Wang et al., 2008b). *Trans*-resveratrol was also found in other berries such as cowberries, cranberries, strawberries and red currant (Ehala et al., 2005) and in high levels in red grapes and red wine (Careri et al., 2003).

4.1.2.3 Correlations with the locations of the bilberry sampling

PCA (principal component analysis) and LDA (linear discriminant analysis) were performed to classify the Slovenian bilberries according to the relative compositions of their anthocyanins, flavanols, flavonols, phenolic acids, and stilbenes. The 15 anthocyanins and 12 phenolic compounds were included. Their contents were above the detection limits in all of the bilberry samples analyzed. PCA was performed to provide a data structure

study over a reduced dimension, covering the maximum amounts from the information present in the basic data set. The seven principal components thus accounted for 82.97% of the variation among the bilberry samples analyzed (PC1, 30.93 %; PC2, 15.81 %; PC3, 12.94 %; PC4, 7.54 %; PC5, 6.54 %; PC6, 5.19 %; and PC7, 4.02 %). Therefore, all of the parameters were included in the LDA.

Using LDA, six parameters were selected as the most discriminating variables: cyanidin 3-arabinoside, ellagic acid, gallic acid, *p*-coumaric acid, quercetin, and cyanidin 3-glucoside. The other four parameters (catechin, chlorogenic acid, cyanidin 3-galactoside, and delphinidin 3-galactoside) also contributed significantly to the better separation of the bilberries. When the LDA was applied to the data set (36 samples, 27 variables), two discriminant functions were obtained. Function 1 explains 49.1 % of the total variance, and function 2 explains 20.7 %. The scores of the samples for these two functions are plotted in Figure 22. As can be seen, the bilberry samples were well separated according to the picking regions. They were sampled in seven different locations. The statistical analysis showed that 27 parameters (anthocyanins, flavanols, flavonols, phenolic acids, and stilbene profiles) can be used for picking-region determination of these Slovenian bilberries. This conclusion is in agreement with the recently published results of a Finnish study, in which obvious differences among the various regions in Finland were observed (Lätti et al., 2008).

It is generally recognized that content of phenolic compounds in plants depends on cultivar used, temperature, light, amount of rainfall, agro-technical conditions, and food processing. In Table 15 the content of total phenolics determined was calculated and statistically analyzed. It can be concluded that the Goričko and Škofja Loka picking regions differ significantly ($p < 0.05$) from the other five locations. Also, when picking regions were correlated to climate, the total phenolics determined in the bilberries from the lowland growing region (Ljubljana, Kranj) and from the mountainous region (Pokljuka, Pohorje) are comparable (1182 vs 1121 or 1027 vs 1198 mg equiv/100 g fw). As mentioned before, the pedoclimatic and the autochthonous growing region differences among picking locations in Slovenia are the only reasons for the different phenolics composition because no genetic variability in bilberries was noted (unpublished results). Similarly, there have been other recognized and published differences found for other fruits and vegetables that have been considered to be due to different cultivars (Quettier-Eleu et al., 2000) and/or agronomic and climate conditions (Kacjan-Maršić et al., 2010). These conditions can result in complex combinations of bioactive compounds that will generally be related to specific characteristics of the genotype and of its interaction with the environment, potentially leading to increased total phenolic content and to higher total antioxidant capacity (Capocasa et al., 2008). In other words, the positive influences of these conditions can be

used toward producing fruits with more phenolic compounds and with higher nutritional quality.

The classification of the data obtained using LDA is shown in Annex M. It can be seen that all of the picking locations of the bilberries were 100 % correctly classified. Overall, the accuracy of the placement of each sample into its corresponding group (location) was also 100 %, with none of the 36 samples misplaced. Similar statistical analysis was performed for flavonoids and other components in different types of Slovenian honey that were collected from different locations in Slovenia that also allowed the successful differentiation of their botanical origin (Bertoncelj et al., 2011).

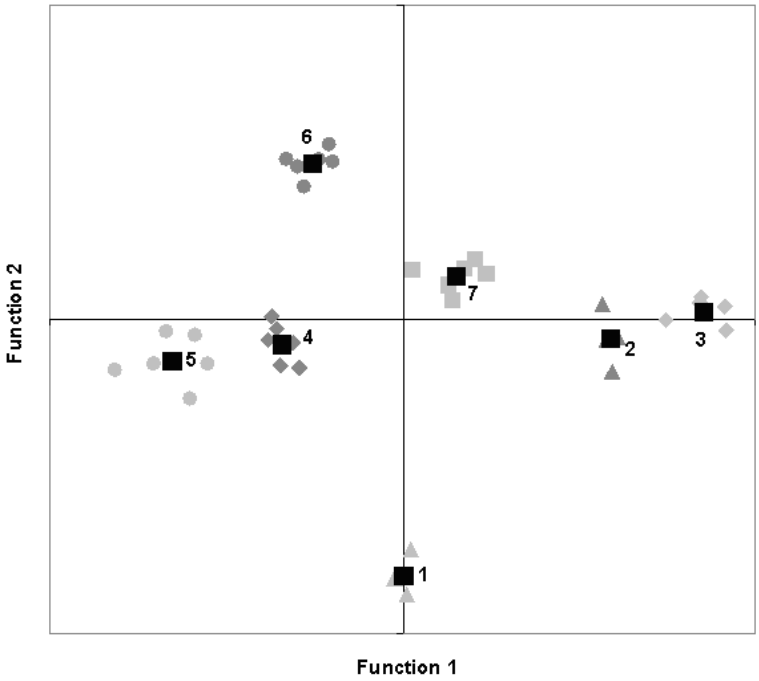


Figure 22: Linear discriminant analysis (LDA) performed using the levels of phenolics from 36 bilberry samples from seven different locations in Slovenia.
▲1, Pokljuka; ▲2, Celje; ♦3, Goricko; ♦4, Ljubljana; ●5, Kranj; ●6, Skofja Loka; ■7, Pohorje; ■, group centroid.

Slika 22: Linearna diskriminantna analiza (LDA) narejena na podlagi vsebnosti posameznih fenolnih spojin iz 36 vzorcev gozdnih borovnic vzorčenih na sedmih različnih lokacij v Sloveniji. ▲1, Pokljuka; ▲2, Celje; ♦3, Goricko; ♦4, Ljubljana; ●5, Kranj; ●6, Skofja Loka; ■7, Pohorje; ■, osrednja točka.

4.2 ANTIOXIDANT POTENTIAL OF BILBERRIES AND BLUEBERRIES

4.2.1 DPPH radical scavenging assay

Antioxidant activity of the bilberry extracts was determined using DPPH test. The influence of total phenolics and total anthocyanin content, both determined spectrophotometrically, to antioxidant activity tested with DPPH radicals is shown in Figure 23.

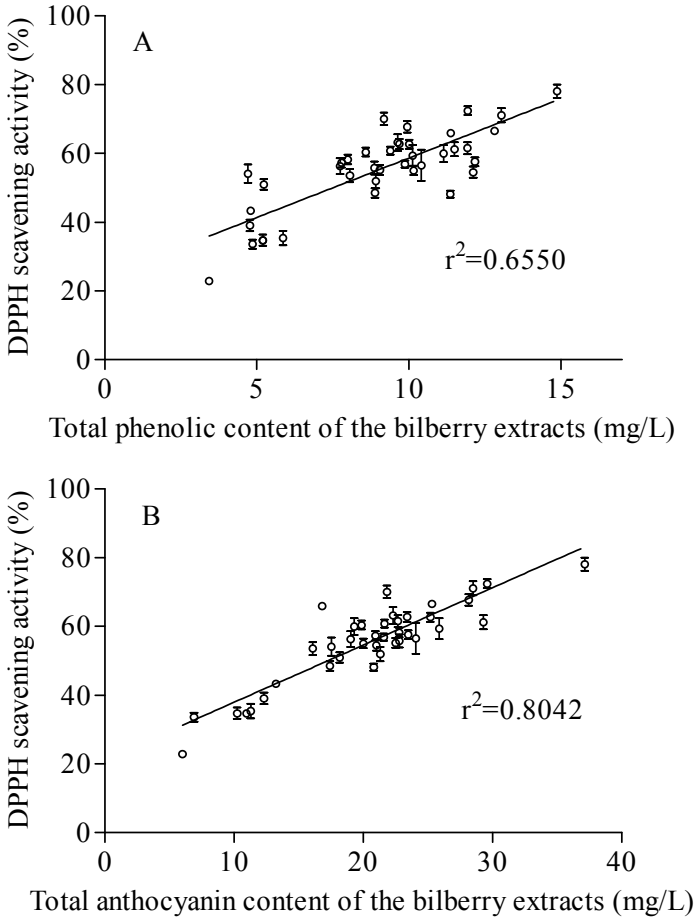


Figure 23: Influence of the total phenolics (A) and the total anthocyanin (B) content on antioxidant activity of Slovenian bilberries.

Slika 23: Vpliv vsebnosti skupnih fenolnih spojin (A) in skupnih antocianinov (B) na antioksidativno aktivnost gozdnih borovnic iz Slovenije.

The data were expressed as a mean ± SEM for n = 3. It can be seen that there is an increasing trend of DPPH scavenging activity with the increase of the total phenolics and the total anthocyanin content of the bilberry extracts. Therefore, the linear correlation of the total phenolics and the total anthocyanins versus the antioxidant activity was

determined. Though the coefficient of correlation of total phenolics versus DPPH (r^2=0.6550) is not high, our results confirm previous studies that the total phenolics content may indicate the antioxidant activity of bilberries (Moyer et al., 2002). The coefficient of correlation of the total anthocyanins versus DPPH (r^2=0.8042) was higher than the one for the total phenolics, thus the total anthocyanins were in better correlation to the antioxidant activity than the total phenolics. However, the scavenging effect of the bilberries may depend on hydrogen atom donation by a huge number of phenolic compounds especially of anthocyanins presented.

4.2.2 Cellular antioxidant activity assay

4.2.2.1 Development of control for CAA assay

The CAA assay was firstly developed by Wolfe and Liu (2007). We had to prepare controls for the CAA assay for four different cell lines (Caco-2, HepG2, EA.hy926 and A7r5) by testing concentrations of ABAP, DCFH-DA and the density of cells seeded in wells of a 96-well plate.

Different concentrations of DCFH-DA were tested on the EA.hy926 and A7r5 cells. The cells were seeded at the density of 1×10^4/well. ABAP as source of peroxyl radicals was utilized at different concentrations (1.2 mM, 2.4 mM, 3.6 mM and 5 mM). The dose-response curves of peroxyl radical-induced oxidation of DCFH-DA are shown in Figure 24.

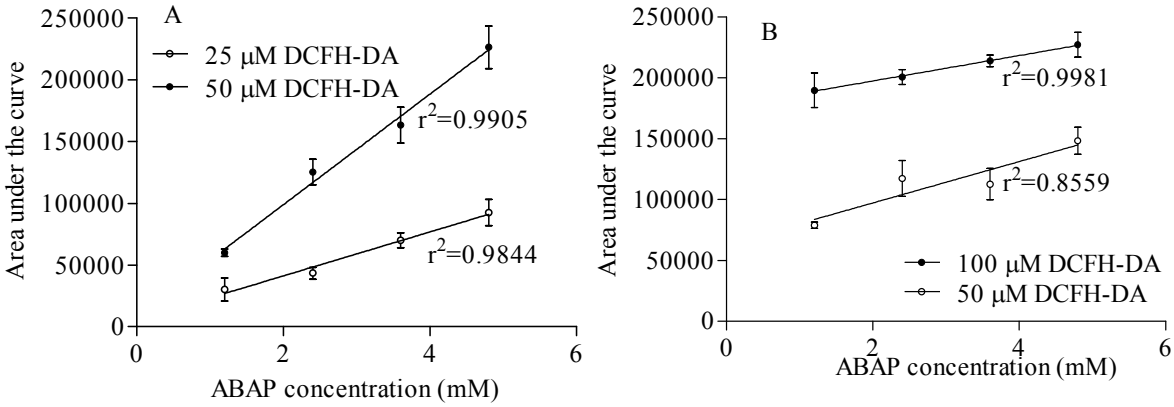


Figure 24: Dose-response curves of oxidation of DCFH-DA in EA.hy926 (A) and A7r5 (B) cells.
Slika 24: Krivulje oksidacije DCFH-DA v celicah EA.hy926 (A) in A7r5 (B).

The data were expressed as a mean ± SEM with n = 6. The liner correlation of area under the curve – AUC - versus ABAP concentration was determined. The coefficients of correlations were about 0.9. The DCFH-DA in the concentration at 50 μM was shown to

be the most appropriate. Therefore, DCFH-DA at this concentration was tested on HepG2 and Caco-2 cells as well. ABAP was utilized at different concentrations (1.2 mM, 2.4 mM, 3.6 mM and 5 mM) as well. Figure 25 represents the dose-response curves of peroxyl radical-induced oxidation of DCFH-DA in HepG2.

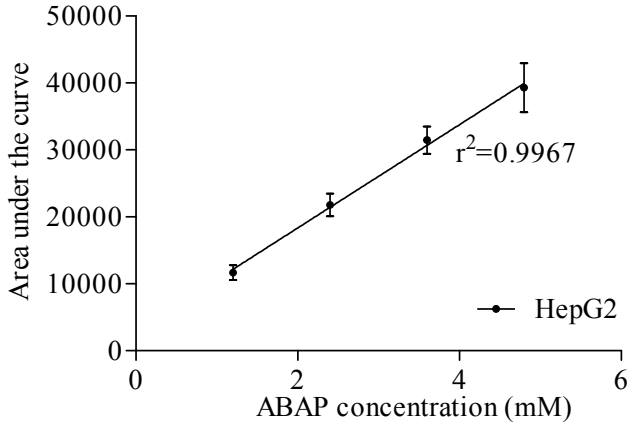


Figure 25: Dose-response curve of oxidation of DCFH-DA in HepG2 cells.
Slika 25: Krivulja oksidacije DCFH-DA v celicah HepG2.

The data were expressed as a mean ± SEM, n = 6. As well the liner correlation of area under the curve versus ABAP concentration was determined. Coefficient of correlation was more than 0.9.

Due to the fact that Caco-2 cells are smaller than the others we tested two different density of cell seedings in wells of 96-well plate. Thus, Caco-2 cells were seeded at a density of 1×10^4/well and 2×10^4/well, respectively. The DCFH-DA concentration used was 50 µM. Figure 26 presents the dose-response curves of peroxyl radical-induced oxidation of DCFH-DA in Caco-2.

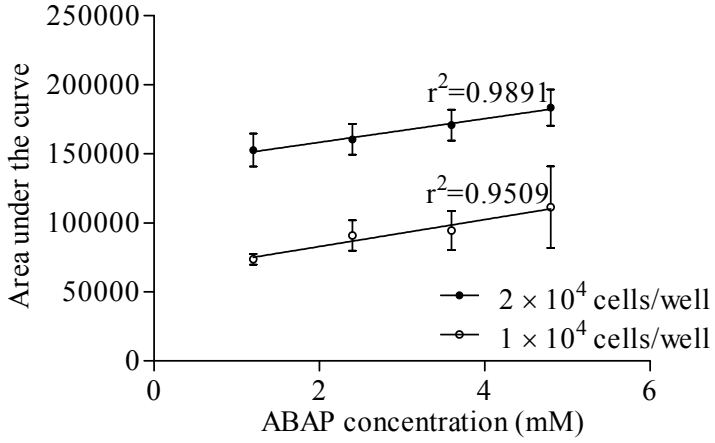


Figure 26: Dose-response curve of oxidation of DCFH-DA in Caco-2 cells at different seeded density.
Slika 26: Krivulja oksidacije DCFH-DA v celicah Caco-2, nasajenih pri različni gostoti.

The data were expressed as a mean ± SEM, n=6. The liner correlation of area under the curve versus ABAP concentration was determined, too. The coefficients of correlations were more than 0.9. The density of the cells at 2×10^4/well showed higher values of areas under the curve. Thus, we decided to seed Caco-2 cells at this density whereas the other cells (HepG2, EA.hy926 and A7r5) at 1×10^4/well.

In conclusion, for the CAA assay, due to the results above:

- HepG2, EA.hy926 and A7r5 cells were seeded at the density 1×10^4/well while Caco-2 cells at 2×10^4/well.
- The DCFH-DA concentration of 50 μM was used for all experiments.
- The ABAP concentration of 5 mM was used for all experiments.

The bilberries were sampled in woods in Smrečje while blueberries on a plantation in Ljubljansko barje at the full ripe stage in June 2008. Extracts of the bilberry and the blueberry samples were prepared and the bilberry extract only was further purified in order to obtain anthocyanins mainly. The preparation of the extracts and the purification of the bilberry extract were as described. In all extracts (crude bilberry, crude blueberry and purified bilberry), total phenolics were determined as well as the the content of individual anthocyanins and the content of phenolic acids, flavanols and flavonols. The methods are described in the chapters 3.1.3 and 3.1.4, while other details of the measurements are presented in the chapters 4.1.1 and 4.1.2.

4.2.2.2 Total phenolics in bilberry and blueberry extracts used for CAA assay

Table 13: Total phenolics in Slovenian bilberries and blueberries used in the CAA assay.
Preglednica 13: Vsebnost fenolnih spojin v gozdnih in ameriških borovnicah iz Slovenije, ki so bile uporabljene v CAA poskusu.

	Bilberries (mg/100 g fw)	Blueberries (mg/100 g fw)
Total phenolics[1]	381.5 ± 12.3	173.4 ± 3.6
Individual anthocyanins[2]		
Delphinidin 3-galactoside	149.6 ± 3.4	24.5 ± 0.5
Delphinidin 3-glucoside	149.4 ± 4.8	18.7 ± 1.5
Cyanidin 3-galactoside	104.4 ± 4.1	4.3 ± 0.2
Delphinidin 3-arabinoside	134.4 ± 5.2	27.0 ± 0.9
Cyanidin 3-glucoside	106.0 ± 4.4	3.3 ± 0.3
Petunidin 3-galactoside	43.8 ± 1.8	12.3 ± 0.5
Cyanidin 3-arabinoside	81.4 ± 3.0	4.1 ± 0.2
Petunidin 3-glucoside	97.9 ± 3.7	14.4 ± 1.0
Peonidin 3-galactoside	11.1 ± 0.2	1.8 ± 0.0
Petunidin 3-arabinoside	26.9 ± 0.3	10.9 ± 0.8
Peonidin 3-glucoside	46.7 ± 1.2	2.5 ± 0.2
Malvidin 3-galactoside	36.1 ± 0.6	46.4 ± 1.4
Peonidin 3-arabinoside	4.7 ± 0.2	1.1 ± 0.1
Malvidin 3-glucoside	110.0 ± 3.0	44.6 ± 2.7
Malvidin 3-arabinoside	20.9 ± 0.3	46.4 ± 1.9
Total anthocyanins:	1122.9 ± 27.4	262.2 ± 12.0
Phenolic acids[3]		
Chlorogenic acid	22.3 ± 0.2	65.0 ± 2.3
Caffeic acid	0.2 ± 0.0	0.2 ± 0.0
Ferulic acid	0.3 ± 0.0	2.1 ± 0.3
p-Coumaric acid	id	id
Ellagic acid	0.9 ± 0.0	0.1 ± 0.0
Gallic acid	9.9 ± 0.1	1.8 ± 0.2
Flavanols[4]		
Catechin	0.7 ± 0.0	id
Epicatechin	0.5 ± 0.0	0.4 ± 0.0
Flavonols[5]		
Myricetin	0.3 ± 0.0	nd
Quercetin	0.7 ± 0.0	0.1 ± 0.0
Rutin	0.1 ± 0.0	2.9 ± 0.2

The bilberries were sampled in woods in Smrečje while blueberries on a plantation in Ljubljansko barje at the full ripe stage in June 2008. Data expressed as the mean ± SEM. Number of independent measurements: n = 3. Phenolic compounds were quantified: [1] to gallic acid, [2] to cyanidin 3-glucoside, [3] to each phenolic acid, [4] to catechin,[5] to each flavonol (mg/100 g fresh berries (fw)). id = identified but not quantified, nd = not detected.

Total phenolics in the bilberries and the blueberries used in the CAA assay are presented in Table 13. The data are expressed as a mean ± SEM with n = 3. There were about 2-fold more total phenolics and over 4-fold more anthocyanins determined in the bilberries than in the blueberries. As we demonstrated before the difference between the content of anthocyanidin glycosides was significant (Figure 27). Delphinidin and cyanidin glycosides were predominant in the bilberries while malvidin glycosides in the blueberries. Chlorogenic acid was the main non-flavonoid in both, the bilberries and the blueberries. Its content was about 3-fold higher in the blueberries than in the bilberries.

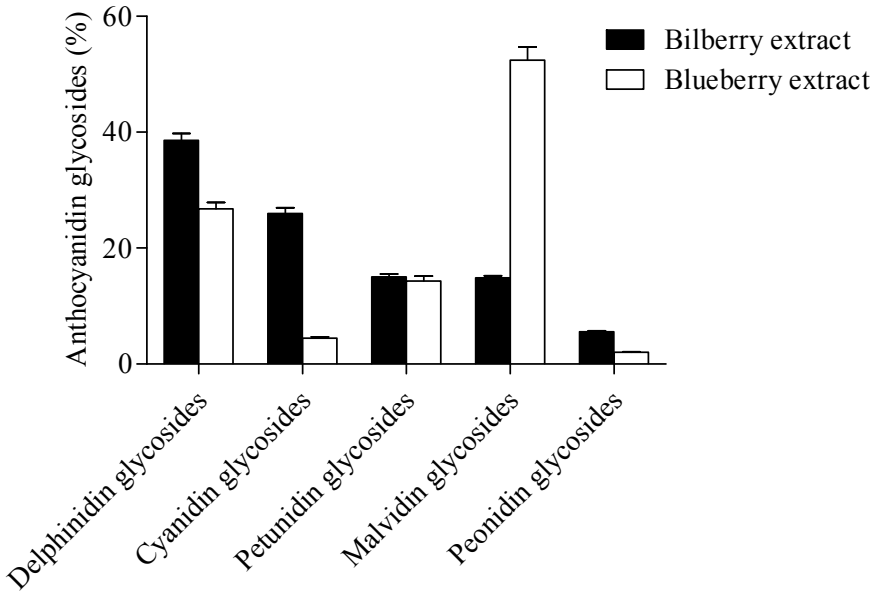


Figure 27: The content of anthocyanidin glycosides in the bilberry and blueberry extracts used in the CAA assay.
Data are expressed as the mean (% of total anthocyanidin glycosides) ± SEM of glycoside types indicated. Number of independent measurements: n = 3.

Slika 27: Vsebnost antocianidinovih glikozidov v gozdnih in ameriških borovnicah, uporabljenih v CAA poskusih.
Podatki so podani kot srednja vrednost skupnih antocianidin glukozidov (%) ± SEM. Število paralelk: n = 36.

4.2.2.3 CAA of the bilberry extract with high content of anthocyanins

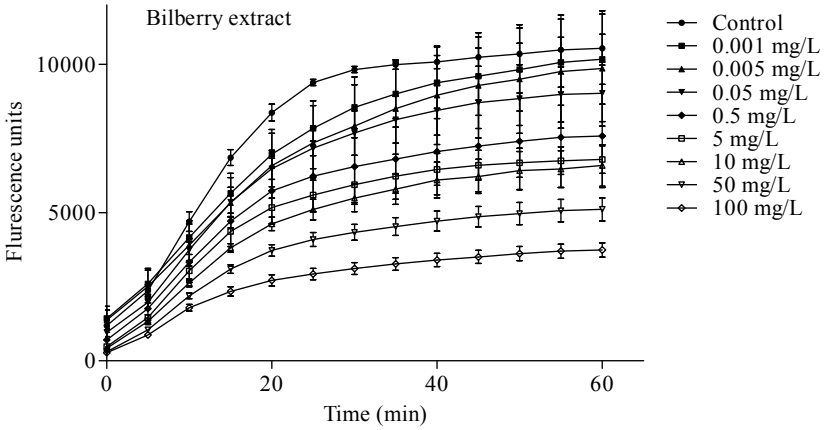


Figure 28: The radical-induced oxidation of DCFH to DCF and the inhibition of the oxidation by bilberry extract in EA.hy926 cells.

Slika 28: Oksidacija DCFH v DFC, inducirana z radikali, in njena inhibicija po dodatku ekstrakta gozdnih borovnic v celicah EA.hy926.

The radical-induced oxidation of DCFH to DCF and the inhibition of the oxidation by the bilberry extract in EA.hy926 cells employed as a model of vascular endothelium are shown in Figure 28. The curves shown in each graph are from a single experiment. The data were expressed as a mean ± SEM, n = 6. The curves perform kinetics of DCFH oxidation by peroxyl radicals generated from ABAP and the inhibition of the oxidation dependent on anthocyanins' content of the bilberry extract, performed as the increase in fluorescence. The concentrations of the anthocyanins were chosen in the range 0 as a control, 0.001 mg/L, 0.005 mg/L, 0.05 mg/L, 0.5 mg/L, 5 mg/L, 10 mg/L, 50 mg/L and 100 mg/L. The control attained the highest value of the fluorescence whereas the crude bilberry extract with the highest anthocyanins' concentration reached the lowest value of the fluorescence.

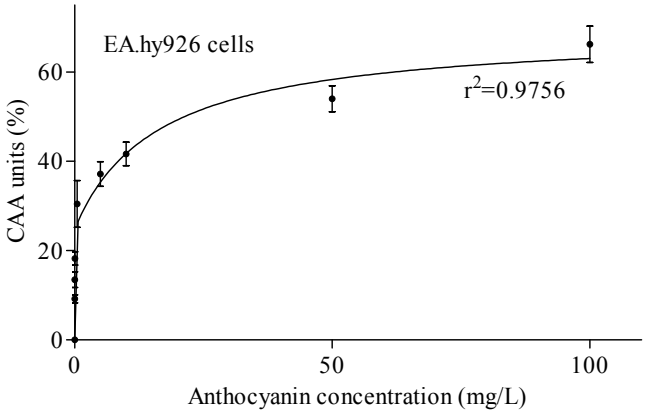


Figure 29: The dose-response curve for inhibition of DCFH oxidation by the bilberry extract in EA.hy926 cells.

Slika 29: Inhibicija oksidacije DCFH po dodatku ekstrakta gozdnih borovnic v celicah EA.hy926.

The data were expressed as a mean ± SEM, n = 6. The curve shown is from a single experiment. The dose–response curve from the ratio of the area under the curve to that of the control and the median effect curve were plotted. The plotted dose–response curve was hyperbola (Figure 29). The coefficient of correlation was more than 0.9 which correlates to previous investigations by Wolfe and Liu (2007). The concentration-dependent increase in antioxidant activity was seen only in low concentration range (<0.5 mg/L), while higher concentrations (>0.5 mg/L) kept antioxidant activity constant. This saturable activity of flavonoids was already suggested by another study employing CAA assay, where dose-response curves for blueberry extracts had hyperbolic shape with apparent saturation in higher concentrations (Wolfe and Liu, 2007). These results showed that endothelial cells have a limited intracellular capacity for antioxidant protection induced by bilberry extract.

4.2.2.4 Comparing CAA with DPPH test for high anthocyanins

The following concentrations of the bilberry anthocyanins were tested (mg/L): 0.01, 0.1, 1, 5, 10, 25, 50, 100 and 0 as the control by DPPH in order to assess an *in vitro* antioxidant activity in the absence of cells as biologic structures. Measurements were performed in triplicates. The results (Figure 30) showed that the used bilberry extract acted as an antioxidant. Moreover, DPPH scavenging activity was strongly correlated to the bilberry anthocyanins levels in a concentration-dependent manner. Under the tested conditions, we observed no saturable activity of the bilberry anthocyanins in their free radical scavenging activity. These observations are in agreement with many similar studies, where total anthocyanins concentrations of different berries were tested for their corresponding antioxidant activities by using DPPH test (Kähkönen et al., 2003).

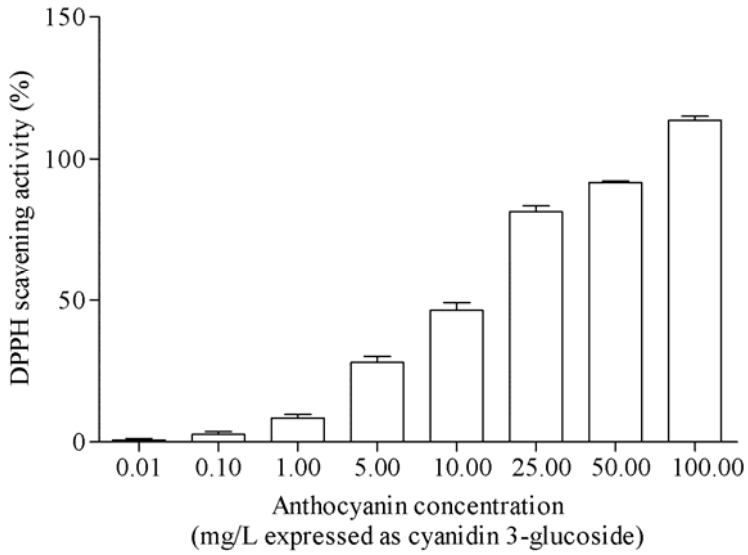


Figure 30: Antioxidant potential of the bilberry extract with high content of anthocyanins by DPPH test.
Slika 30: Antioksidativni potencial ekstrakta iz gozdnih borovnic, ki smo ga določili z DPPH testom.

4.2.2.5 CAA assay of the bilberry extracts in four different cell lines

The CAA assay was employed to estimate the antioxidant activity of the bilberries in four different cell lines (Caco-2, HepG2, EA.hy926 and A7r5). These cell lines were used with the intention to follow absorption of anthocyanins, their tissue distribution from the gastro-intestinal tract to various tissues such as the liver and blood vessels. Thus, Caco-2 cells represent the intestinal epithelium and are therefore used in studies of intestinal absorption; HepG2 cells offer a model of the liver, the main site of metabolism and disposition of xenobiotics; EA.hy926 and A7r5 are models of the vascular endothelium and musculature, respectively. The anthocyanin concentrations of the bilberry extract were following: 0 as a control, 0.001 mg/L, 0.005 mg/L, 0.01 mg/L, 0.025 mg/L, 0.05 mg/L, 1 mg/L and 5 mg/L (expressed as cyanidin 3-glucoside).

The dose-response curves for the inhibition of DCFH oxidation by the berry extracts and cyanidin 3-glucoside are shown in Figure 31. The data are expressed as a mean ± SEM, n = 6. Each curve shown is from a single experiment. As we determined above all dose-response curves had the shapes of hyperbola. Their coefficients of correlation were more than 0.9.

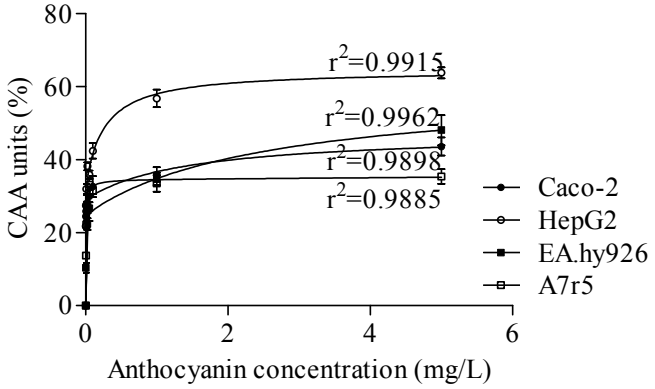


Figure 31: Dose-response curves for inhibition of DCFH oxidation by the bilberry extract in four different cell lines (Caco-2, HepG2, EA.hy926 and A7r5).

Slika 31: Krivulje inhibicije oksidacije DCFH ob dodatku ekstrakta gozdnih borovnic v štirih različnih celičnih kulturah (Caco-2, HepG2, EA.hy926 in A7r5).

The intracellular antioxidant activity of the bilberry extract was determined in all the tested cells. The antioxidant activity of several foods and antioxidants (e. g. flavonoids) have been already examined in HepG2 (Wolfe and Liu, 2007; Wolfe and Liu, 2008; Wolfe et al., 2008), but there are no publications of using other cell cultures. Due to PBS washing after the one hour cell incubation with antioxidants from the bilberry and blueberry extract, we believe that they might have passed through the membrane, entered the cells and exerted intracellular antioxidant activities.

4.2.2.6 CAA for the berry extracts with low anthocyanin content

The anthocyanin concentrations in the bilberry and blueberry extracts were lowered to 0.25 µg/L, 0.5 µg/L, 1 µg/L, 2 µg/L, 5 µg/L, 10 µg/L, 25 µg/L, 50 µg/L (expressed as cyanidin 3-glucoside) to check their antioxidant activity at low concentrations which correlate to their post-absorption plasma concentrations (Mazza et al., 2002; Felgines et al., 2008). The dose-response curves for inhibition of DCFH oxidation by the bilberry and blueberry extracts with low anthocyanin content are shown in Figure 32.

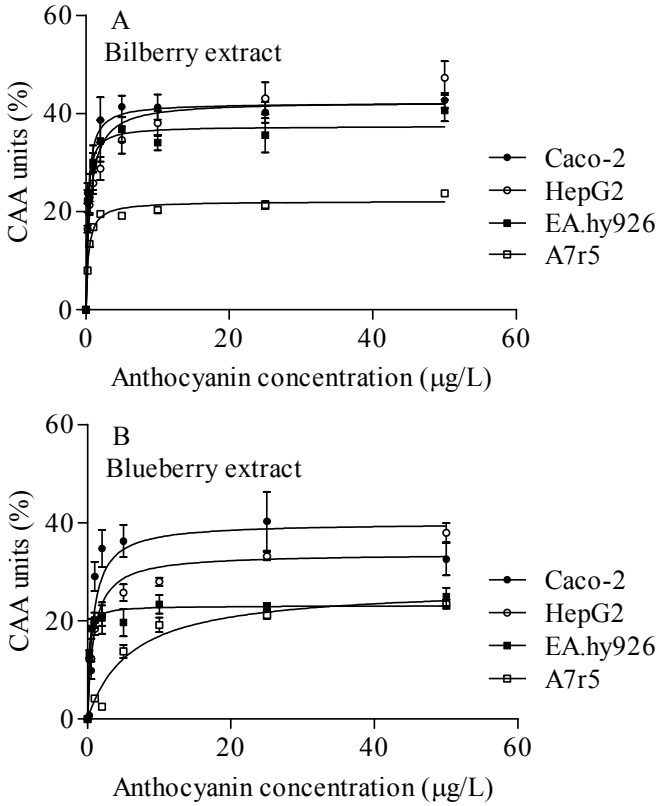


Figure 32: Dose-response curves for inhibition of DCFH oxidation by the bilberry extract (A) and the blueberry extract (B) with low anthocyanin content.

Slika 32: Krivulje inhibicije oksidacije DCFH ob dodatku ekstrakta gozdnih (A) in ameriških (B) borovnic pri nizki vsebnosti antocianinov.

The data are expressed as a mean ± SEM with n = 6. The curves shown are each from a single experiment. Graph A shows the dose-response curves for the inhibition of peroxyl radical-induced DCFH oxidation by the bilberry extract at low anthocyanins' concentrations in the four different cells. Graph B shows the dose-response curves for inhibition of peroxyl radical-induced DCFH oxidation by the blueberry extract at low anthocyanins' concentrations in the four different cells. Thus, both berry extracts exerted good intracellular antioxidant activity already at low anthocyanin content in all four tested cells.

4.2.2.7 Are anthocyanins responsible for intracellular antioxidant activity of the berry extracts

The bilberry extract was purified further to obtain anthocyanin rich fraction and to check if anthocyanins are responsible for the intracellular antioxidant activity in the tested cell lines. Total phenolics in the crude and purified bilberry extract are presented in Table 14.

Table 14: Total phenolics in the crude and purified bilberry extract preparations applaid in CAA assay.

Preglednica 14: Vsebnost fenolnih spojin v neočiščenem in očiščenem ekstraktih gozdnih borovnic, ki smo jih uporabili pri CAA metodi.

	Crude bilberry extract (µg/L)	Purified bilberry extract (µg/L)
Total phenolics[1]	19.99 ± 0.55	9.80 ± 0.32
Individual anthocyanins[2]		
Delphinidin 3-galactoside	6.66 ± 0.15	6.55 ± 0.04
Delphinidin 3-glucoside	6.65 ± 0.22	6.67 ± 0.08
Cyanidin 3-galactoside	4.64 ± 0.18	4.49 ± 0.13
Delphinidin 3-arabinoside	5.99 ± 0.23	6.02 ± 0.03
Cyanidin 3-glucoside	4.72 ± 0.23	4.66 ± 0.07
Petunidin 3-galactoside	1.95 ± 0.08	2.04 ± 0.08
Cyanidin 3-arabinoside	3.63 ± 0.14	3.63 ± 0.12
Petunidin 3-glucoside	4.36 ± 0.16	4.69 ± 0.09
Peonidin 3-galactoside	0.49 ± 0.01	0.54 ± 0.02
Petunidin 3-arabinoside	1.20 ± 0.02	1.15 ± 0.06
Peonidin 3-glucoside	2.08 ± 0.06	2.16 ± 0.07
Malvidin 3-galactoside	1.61 ± 0.03	1.60 ± 0.05
Peonidin 3-arabinoside	0.21 ± 0.01	0.20 ± 0.02
Malvidin 3-glucoside	4.90 ± 0.13	4.71 ± 0.18
Malvidin 3-arabinoside	0.94 ± 0.01	0.91 ± 0.09
Total anthocyanins:	50.00 ± 1.22	50.00 ± 1.03
Phenolic acids[3]		
Chlorogenic acid	1.00 ± 0.01	0.06 ± 0.00
Caffeic acid	0.01 ± 0.00	id
Ferulic acid	0.02 ± 0.00	0.01 ± 0.00
p-Coumaric acid	id	id
Ellagic acid	0.04 ± 0.00	0.01 ± 0.00
Gallic acid	0.44 ± 0.01	0.03 ± 0.00
Flavanols[4]		
Catechin	0.01 ± 0.00	nd
Epicatechin	0.02 ± 0.00	id
Flavonols[5]		
Myricetin	0.02 ± 0.00	id
Quercetin	0.03 ± 0.00	id
Rutin	0.01 ± 0.00	nd

The bilberries were sampled in woods in Smrečje at the full ripe stage in June 2008. Data expressed as the mean ± SEM. Number of independent measurements: n = 3. Phenolic compounds were quantified: [1] to gallic acid, [2] to cyanidin 3-glucoside, [3] to each phenolic acid, [4] to catechin, [5] to each flavonol (µg/L of extract). id = identified but not quantified, nd = not detected.

Purification resulted in essentially complete recovery of anthocyanins and complete removal of other flavonoids, such as flavanols and flavonols. Other phenolics, were extensively removed, so that their relative concentrations with respect to anthocyanins were of limited relevance. Intracellular antioxidant activity was determined with both, crude and purified bilberry extract and as well as with anthocyanin 3-glucoside.

4.2.2.8 Half maximal effect concetrations (EC_{50}) and CAA maximal antioxidant activity (CAA_{max})

A half maximal effective concentration (EC_{50}) and CAA maximal antioxidant activity (CAA_{max}) of anthocyanins for CAA was determined for all berry extracts (crude bilberry, crude blueberry and purified bilberry) and cyanidin 3-glucoside in all studied cell lines (Caco-2, HepG2, EA.hy926 and A7r5). The data are shown in Table 15.

Table 15: Half maximal effect (EC_{50}) of CAA of anthocyanin concentration (µg/L).
Preglednica 15: Koncentracija antocianinov (µg/L) CAA, ki izzove polovico maksimalnega učinka (EC_{50}).

Parameter	Cell line	Crude blueberry extract	Crude bilberry extract	Purified bilberry extract	Cyanidin 3-glucoside
EC_{50} (µg/L)	Caco-2	0.78 ±0.15 aA	0.29 ±0.02 bA	0.53 ±0.04 abA	0.27 ± 0.02 aA
	HepG2	0.88 ±0.10 aA	0.59 ±0.05 bB	0.63 ±0.03 abA	0.84 ± 0.04 abB
	Ea.hy926	0.17 ±0.02 aA	0.22 ±0.02 aA	0.59 ±0.06 bA	0.22 ± 0.14 aA
	A7r5	5.99 ±0.81 aB	0.36 ±0.02 bA	1.38 ±0.10 bB	0.36 ± 0.06 aA
CAA_{max} (%)	Caco-2	39.95 ±1.77 aA	42.14 ±0.57 aA	43.94 ±0.74 aA	36.93 ±0.42 abA
	HepG2	33.69 ±0.80 aB	42.47 ±0.80 bA	35.47 ±0.39 aB	42.48 ±0.52 bB
	Ea.hy926	23.08 ±0.03 aC	37.41 ±0.47 bB	33.99 ±0.80 cB	42.97 ±0.42 dB
	A7r5	27.01 ±1.12 aC	22.13 ±0.25 bC	19.31 ±0.30 cC	16.39 ±0.21 dC

The data were expressed as a mean ± SEM, n = 6. Statistical analysis was performed using one-way ANOVA with post-Bonferroni test. Statistically significant differences ($p < 0.05$) are marked with lowercase letters (a, b, c, d) in the same row and with uppercase letters (A, B, C) within the same column.

The EC_{50} indicates the potency of the treatment while CAA_{max} indicates the efficacy of the treatment. The EC_{50} for all the extracts and cyanidin 3-glucoside and the cell lines used was estimated to be from 0.17 to 1.38 µg of anthocyanins/L but the EC_{50} for the blueberry extract in A7r5 was much higher, valued at 5.99 µg of anthocyanins/L, showing an unexpected antioxidant potency. The CAA_{max} value was not higher than 44%, showing a limited capacity of anthocyanins to protect the cells under a high oxidative stress.

4.2.2.9 CAA of the berry extracts and cyanidin 3-glucoside

In order to compare the antioxidant activity of the berry extracts (crude bilberry, crude blueberry, purified bilberry) and cyanidin 3-glucoside in different cell lines the area under the curve of CAA units versus anthocyanins' concentration up to 50 µg/L was calculated. All these areas expressed as AUC of CAA units for all four cell lines are represented in Figure 34.

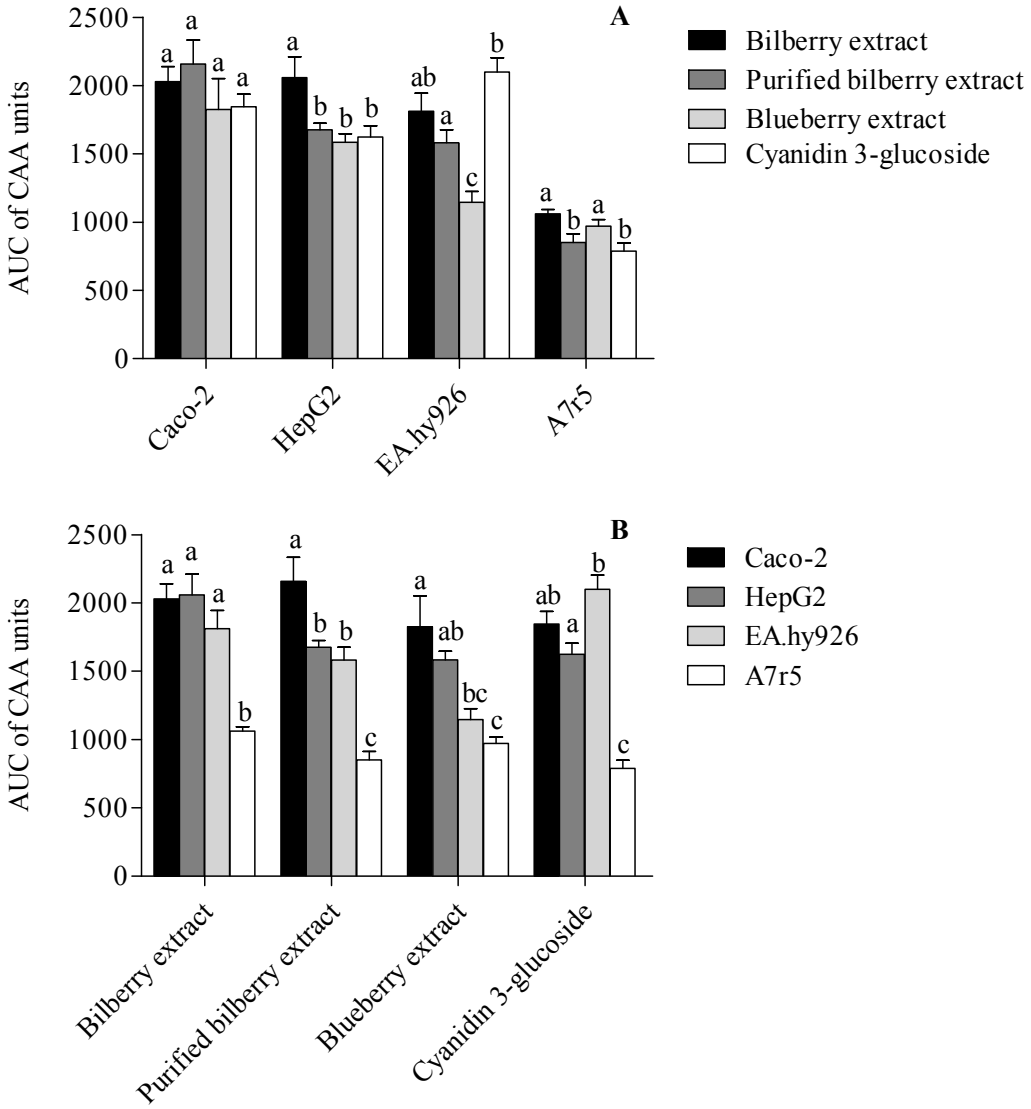


Figure 33: AUC of CAA units (%) of all berry extracts and cyanidin 3-glucoside for all cell lines. Graph A shows AUC of CAA units of different cell lines while graph B AUC of CAA units of the extracts.

Slika 33: AUC CAA (%) za vse ekstrakte in cianidin-3-glukozid za vse celične linije. Graf A predstavlja AUC od CAA glede na celične linije, graf B pa glede na ekstrakte.

The data were expressed as a mean ± SEM, n = 6. All data are represented in Annex E. Graph A shows AUC of CAA units of different cell lines. Graph B shows AUC of CAA units of the extracts. Statistical analysis was performed using one-way ANOVA with post-Bonferroni test. Statistically significant differences ($p < 0.05$) are marked with different letters (a, b, c) in each group of the columns.

AUC of CAA units for all four cell lines is presented in Figure 33A. We did not notice any significant difference between the AUC of all berry extracts and cyanidin 3-glucoside in the Caco-2 cells. Statistical analysis of other measurements showed a significant difference among the HepG2, EA.hy926, and A7r5 cells. The crude bilberry extract had about 10 – 30 % higher AUC than the crude blueberry extract and showed the highest antioxidant activity in all studied cell lines. According to the literature, the antioxidant activity of several fruits have been already examined by CAA assay in HepG2 cells (Wolfe and Liu, 2007; Wolfe et al., 2008). They found that extracts from berries and pomegranate have higher CAA in a comparison to other common fruits such as plum, apple, red grape, kiwifruit, mango, pineapple, orange, lemon, peach, pear, nectarine, banana, watermelon etc.

To confirm if the anthocyanins are responsible for the antioxidant activity in cells, the purified bilberry extract was compared to the crude bilberry extract. The AUC of the crude bilberry extract was only about 8 % higher than the purified bilberry extract in HepG2, EA.hy926 and A7r5 cells while there was no difference in the Caco-2 cells. Cyanidin 3-glucoside exerted good antioxidant activity in all cells, comparable to the extracts. Therefore, the anthocyanins in the bilberry and the blueberry extract could be mainly responsible for the antioxidant activity. Likewise, antioxidant activity of individual anthocyanins, which were determined in our extracts, has been confirmed by several other *in vitro* antioxidant assays (Prior et al., 1998; Kähkönen and Heinonen, 2003). However, the crude bilberry's extract exerted higher cellular antioxidant activity than the blueberry one. The difference could be attributed to the anthocyanins the extracts contain and to their *in vitro* antioxidant activity. Individual antioxidant activity was determined by several *in vitro* tests (Prior et al., 1998; Giovanelli and Buratti, 2008). In our extracts delphinidin and cyanidin glycosides were predominant in the bilberry extract whereas malvidin glycosides dominated in the blueberry one. It was also found that delphinidin, cyanidin and their glucosides have higher antioxidant activity than malvidin and its glycosides (Kähkönen and Heinonen, 2003). Based on these, the higher antioxidant activity of the bilberry extract is then well explained.

Four different cell lines (Caco-2, HepG2, EA.hy926 and A7r5) were used with the intention to follow absorption of anthocyanins in those cells which may reflect their tissue

distribution from the gastrointestinal tract to various tissues such as liver and blood vessels and to determine their CAA. A comparison of the AUC of CAA units versus a different berry extract and cyanidin 3-glucoside for each cell line is shown in Figure 33B. The highest AUC was determined in the Caco-2 cells and the lowest in the A7r5 cells. Therefore, the Caco-2 cells were also the best protected, EA.hy926 and HepG2 cells were protected less but similarly whereas A7r5 cells were the least protected cells by the antioxidants from the berry extracts, especially anthocyanins, from the berry extracts. Thus, our results suggested that anthocyanins passed the cell membrane and exerted their antioxidant activity effect inside the cell.

4.3 ANTHOCYANIN BIOAVAILABILITY

4.3.1 Cyanidin 3-glucoside uptake in HepG2 and EA.hy926 cells

Anthocyanin uptake in two different cells (HepG2 and EA.hy926) was demonstrated by incubation the cells with the pure compound cyanidin 3-glucoside that is one of anthocyanins occurred in the bilberries and the blueberries. Cyanidin 3-glucoside and some metabolites were detected in the cells (Table 16).

Table 16: Cyanidin 3-glucoside and its metabolites detected in HepG2 and EA.hy926 cells.
Preglednica 16: Cianidin-3-glukozid in njegovi metaboliti določeni v HepG2 and EA.hy926 celicah.

Compound	HepG2 cells	EA.hy926 cells
Cyanidin 3-glucoside	detected	detected
Peonidin 3-glucoside	detected	not detected
Delphinidin 3-glucoside	detected	not detected

Intact cyanidin 3-glucoside was found in both HepG2 and EA.hy926 cells while its metabolites peonidin 3-glucoside and delphinidin 3-glucoside in HepG2 cells only (Figure 35 and Figure 36). HepG2 cells represent hepatic cells which are the basic metabolic cells and likewise the liver play a major role in the metabolism in human body. Apparently cyanidin 3-glucoside converted to peonidin 3-glycoside inside HepG2 cells. This metabolic step called methylation has been published many times (Ichiyanagi et al., 2005a; Ichiyanagi et al., 2005b; Ichiyanagi et al., 2006, Tsuda et al., 1999). Minor metabolic transformation of peonidin 3-glycoside to delphinidin 3-glucoside by hydroxylation of C-5' of the B ring has been described recently (Vanzo et al., 2011) therefore peonidin 3-glycoside hydroxylated to delphinidin 3-glucoside. Therefore, we concluded that anthocyanins can enter cells in intact form and can be metabolized there.

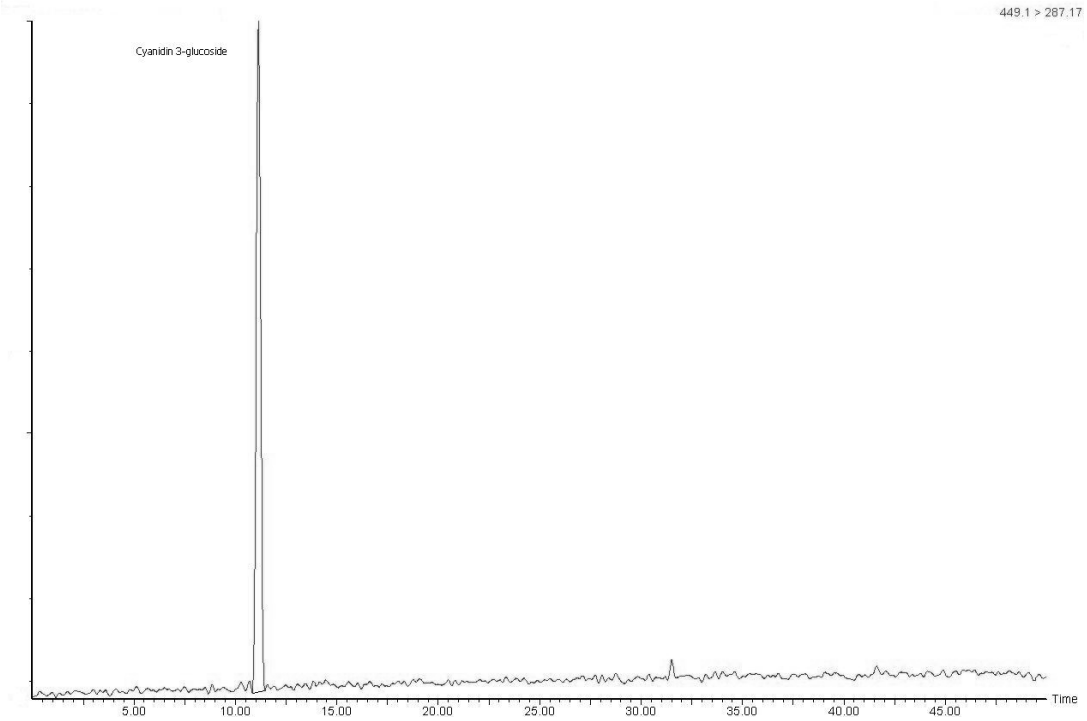


Figure 34: LC-MS/MS chromatogram of cyanidin 3-glucoside detected in EA.hy926 cell line.

Slika 34: LC-MS/MS kromatogram cianidin-3-glukozida, ki smo ga določili v celicah EA.hy926.

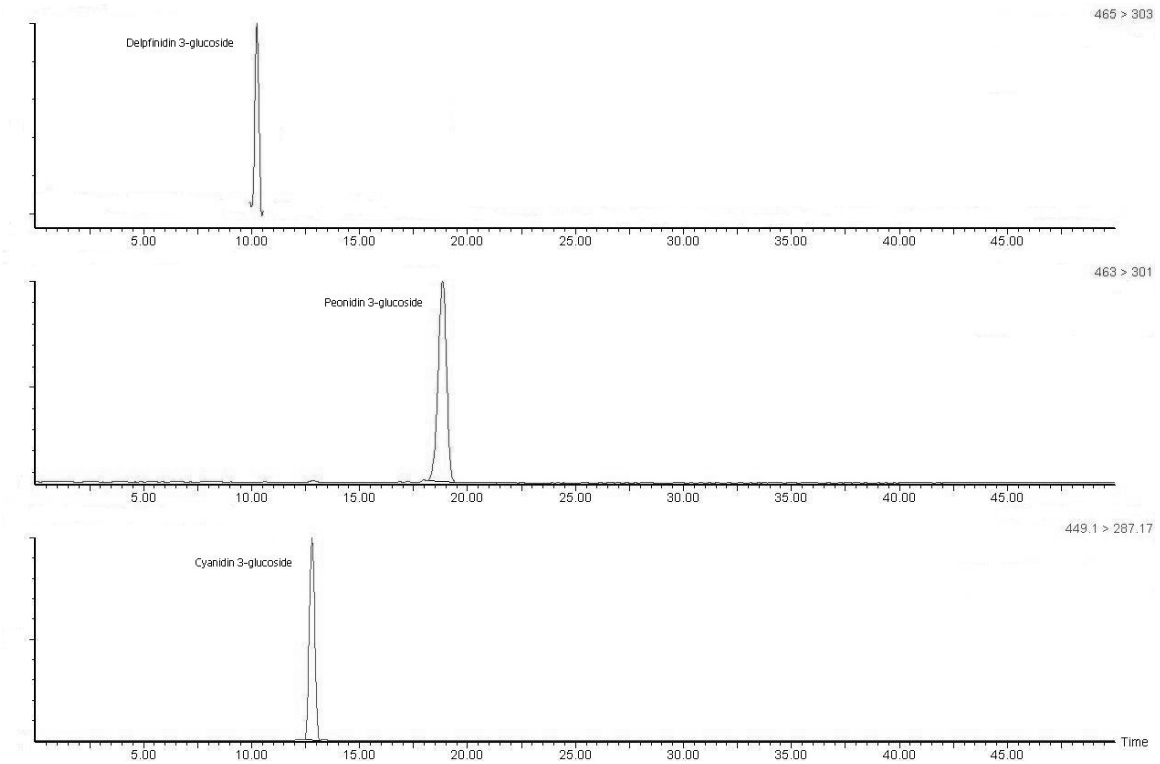


Figure 35: LC-MS/MS chromatogram of delphinidin 3-glucoside, peonidin 3-glucoside and cyanidin 3-glucoside detected in HepG2 cells.

Slika 35: LC-MS/MS kromatogram delfinidin-3-glukozida, peonidin-3-glukozida in cianidin-3-glukozida, ki smo jih določili v celicah HepG2.

4.3.2 Membrane transport of anthocyanins

4.3.2.1 CAA assay and bilitranslocase

Modified CAA assay was employed to demonstrate a role of bilitranslocase in anthocyanin transport into the cell using Caco-2, EA.hy926 and A7r5 cell lines and involved an incubation of antibodies rabbit immunoglobulins (IgG) and Multiple Antigen Peptide or bilitranslocase antibodies (MAP), respectively.

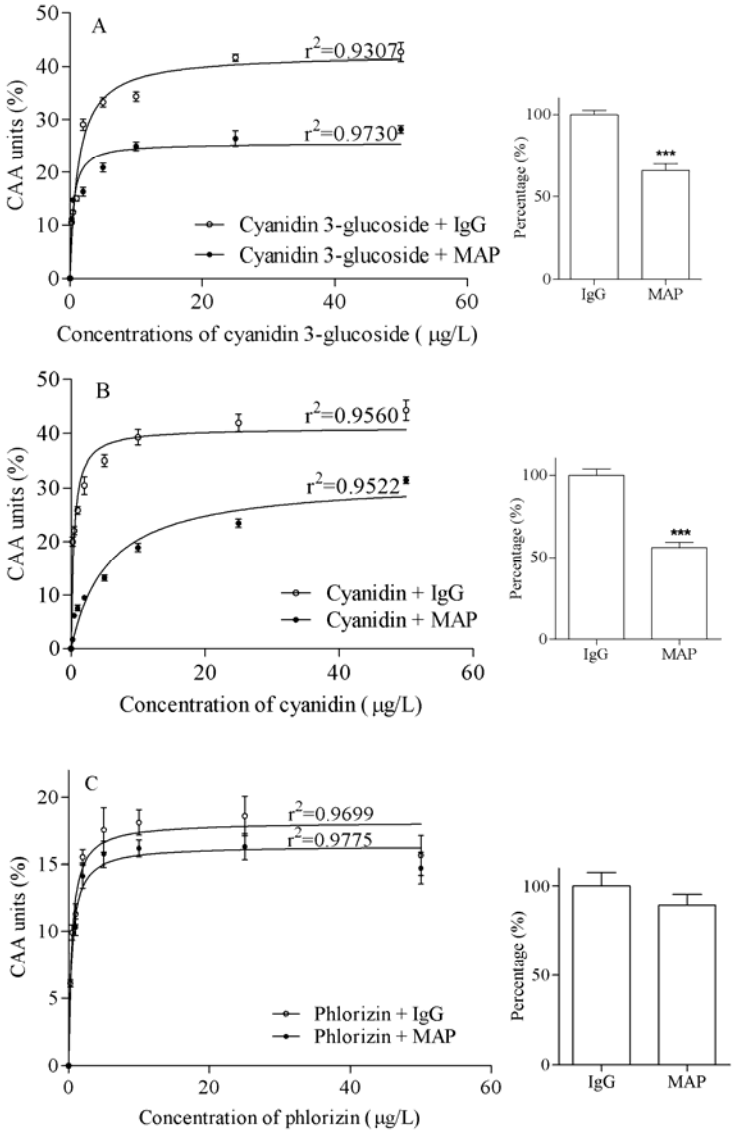


Figure 36: CAA of cyanidin 3-glucoside (A), cyanidin (B) and phlorizin (C) in Caco-2 cells in the absence or in the presence of specific bilitranslocase antibodies.

Slika 36: CAA cianidin-3-glukozida (A), cianidina (B) in florizina (C) v celicah Caco-2 ob prisotnosti ali odsotnosti specifičnih protiteles za bilitranslokazo.

Dose-response curves for the inhibition of peroxyl radical-induced DCFH oxidation by cyanidin 3-glucoside, cyanidin and phlorizin in Caco-2 cell line pre-incubated with antibodies IgG and MAP are shown in Figure 36. The curves shown were each from a single experiment. The data were expressed as a mean ± SEM, n = 6. The insets reports show percentage (%) of AUC of cyanidin 3-glucose, cyanidin and phlorizin in Caco-2 cell line pre-incubated with antibodies IgG and MAP. Differences have been compared using Student's t-test. The results were considered to be significant when *** $p<0.0001$.

Our results showed the decrease of CAA of both cyanidin 3-glucoside and cyanidin in Caco-2 cells in the presence of MAP in comparison to IgG. The decline of CAA was valued to about 34 % for cyanidin 3-glucoside and to about 43 % for cyanidin. Thus, both are suggested to be transported from the medium into the cells by bilitranslocase. Previously reported data also showed the decrease of cellular uptake of anthocyanins such as malvidin 3-glucoside by using bilitranslocase antibodies (Passamonti et al., 2005b). It is known that the absorption of anthocyanins occurs in gastrointestinal tract (Morazzoni et al., 1991; Passamonti et al., 2003; Ichiyanagi et al., 2006; Matuschek et al., 2006) and the bilitranslocase was found there (Battistion et al., 1999).

As a negative control, phlorizin, a glycosylated flavonoid dihydrochalcone, was tested in order to see if a compound that is not a substrate of bilitranslocase can nevertheless act as an intracellular antioxidant. As we expected the results did not show significant decrease of CAA of phlorizin in Caco-2 cells in the presence of MAP in comparison to IgG. Phlorizin showed low antioxidant activity and might have been transported by the other transporters but not by bilitranslocase.

Dose-response curves for the inhibition of peroxyl radical-induced DCFH oxidation by cyanidin 3-glucoside in EA.hy926 and A7r5 cell line pre-incubated with antibodies IgG and MAP are shown in Figure 37. The curves shown were each from a single experiment. The data were expressed as a mean ± SEM, n = 6. The insets reports show percentage (%) of AUC of cyanidin 3-glucose in EA.hy926 cell line pre-incubated with antibodies IgG and MAP. Differences have been compared using Student's t-test. Results were considered to be significant when ** $p<0.01$ and *** $p<0.0001$.

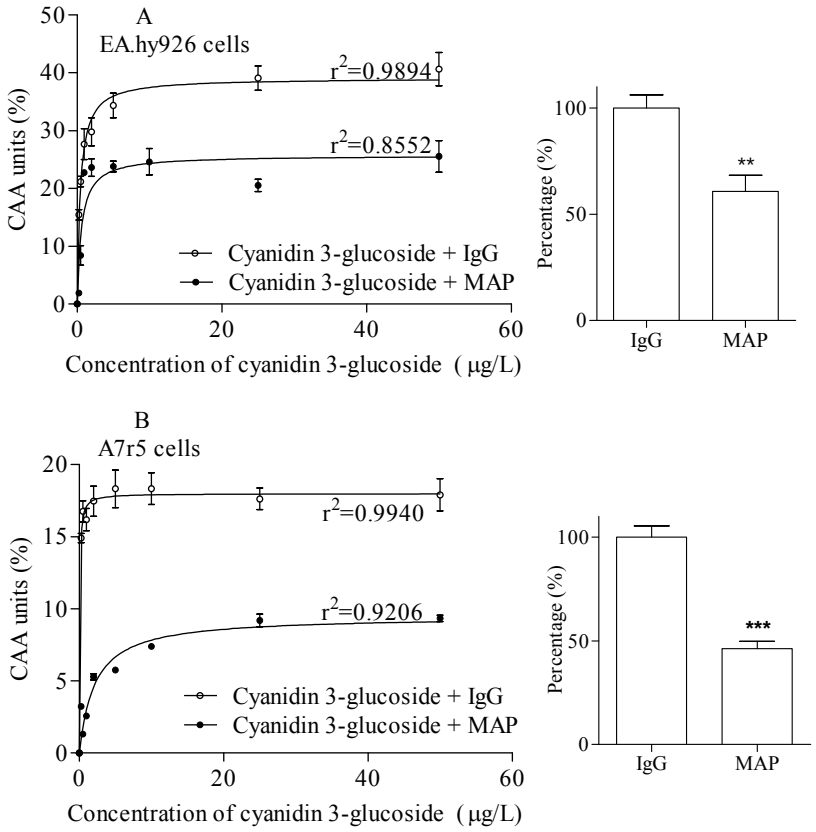


Figure 37: CAA of cyanidin 3-glucoside in EA.hy926 (A) and A7r5 (B) cells in the absence or in the presence of specific bilitranslocase antibodies.

Slika 38: CAA cianidin-3-glukozida v celicah EA.hy926 (A) in A7r5 (B) ob prisotnosti ali odsotnosti specifičnih protiteles za bilitranslokazo.

Our results showed the decrease of CAA of cyanidin 3-glucoside in both EA.hy926 and A7r5 cells in the presence of MAP in comparison to IgG. The decline of CAA was valued to about 39 % in EA.hy926 cells and to about 54 % in A7r5. Bilitranslocase was found in vascular endothelium (Maestro et al., 2009). Thus, cyanidin 3-glucoside might be transported from the medium (e. g. blood) into the cells by bilitranslocase.

4.3.2.2 Interactions of cyanidin, cyanidin, cyanidin 3-glucoside and bilirubin with model liposome membranes

The aim of these experiments was to investigate the interaction of cyanidin, cyanidin 3-glucoside and bilirubin with model membranes (liposomes) and to check if the tested compounds can enter model cell membrane by passive diffusion. Vesicles were prepared from phosphatidylcholine (PC) and sphingomyelin (SM) in a ratio 2.4:1 by extrusion. This phospholipid composition was used as a model membrane of vascular endothelial cells (EA.hy926). The results are shown in Figure 39.

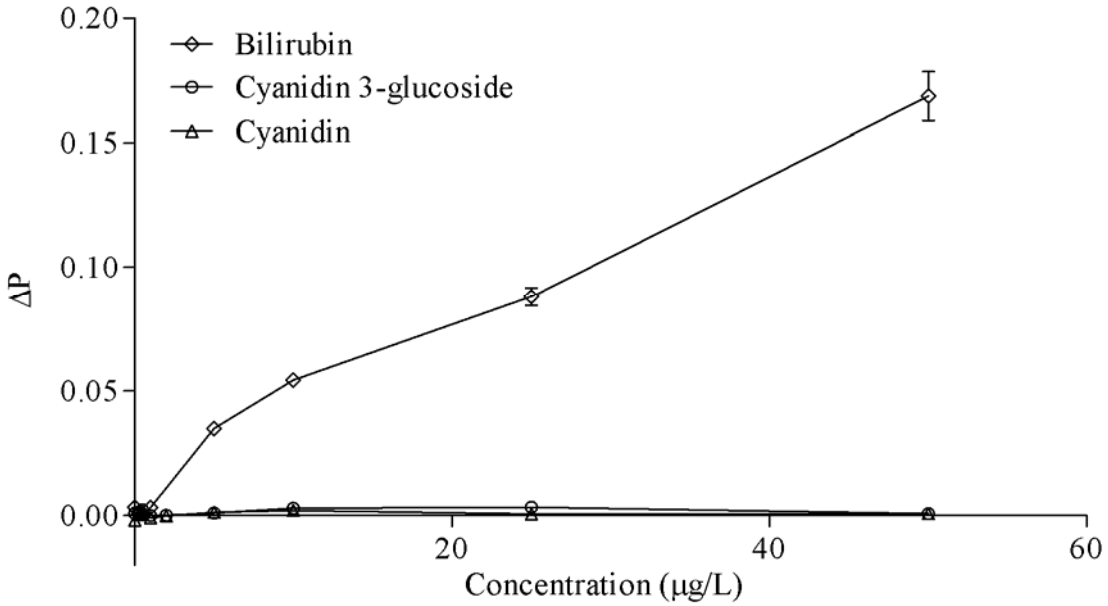


Figure 38: The difference of polarization (ΔP) in extruded liposomes in the presence of cyanidin, cyanidin 3-glucoside and bilirubin.

Slika 38: Razlika polarizacije (ΔP) v ekstrudiranih liposomih v prisotnosti cianidina, cianidin-3-glukozida in biliverdina.

The data were expressed as a mean ± SEM, n = 3. When cyanidin and cyanidin 3-glucosides were added to the eUV liposomes prepared from PC:SM in a ratio 2.4:1 only a very small difference in polarisation (ΔP) was noticed. The highest ΔP for cyanidin 3-glucoside was 0.0006 at the 25 μg/L. It looks like that cyanidin and cyanidin 3-glucoside did not enter a membrane. Both compounds have at the used pH a positive charge and the eUV membrane composed from phosphocholine and sphingomyelin also have a positive charge. Due to the electrostatic repulsive forces formed between these components small differences in ΔP clearly reflect such behavior as presented earlier for some other flavonoids (Poklar Ulrih et al., 2010). We can conclude that cyanidin 3-glucoside and cyanidin cannot pass through the cell membrane by diffusion.

In the case of bilirubin, a substrate of bilitranslocase (Passamonti et al., 2005d), the changes in the ΔP were significantly higher. We think that bilirubin interacted with phospholipids PC and SM on the surface of the eUV membrane and remained adsorbed on the surface of a eUV membrane. Both phospholipids carry positive charge and bilirubin two negative ones due to two ionized carboxyl groups. So bilirubin can bind to the surface of a phospholipid bilayer of these model membranes by electrostatic attractive forces. On the other side it was shown that bilirubin is transported through the endothelial membrane with a special membrane bilitranslocase transporter (Tramer et al, unpublished data).

5 CONCLUSIONS

- Slovenian bilberries (*Vaccinium myrtillus* L.) and blueberries (*Vaccinium corymbosum* L.) are good source of phenolics (anthocyanins, flavanols, flavonols, phenolic acids and *trans*-resveratrol).
- Bilberries contain more total phenolics and total anthocyanins than blueberries.
- Anthocyanins were found as the main phenolics in both, bilberries and blueberries.
- Both, the bilberries and the blueberries contain 15 anthocyanins: 3 glycosides (glucosides, galactosides and arabinosides) of 5 anthocyanidins (cyanidin, petunidin, peonidin, delphinidin and malvidin).
- The difference between the bilberry and the blueberry anthocyanidin profiles was significant. Delphinidin and cyanidin glycosides were predominant in the bilberries while malvidin glycosides in the blueberries. Peonidin glycosides were the least presented anthocyanidin glycosides in both berries.
- Chlorogenic acid was the abundant phenolic acid in both berry species.
- Flavonol rutin was found in both, the bilberries and the blueberries, for the most first time.
- All 27 phenolic compounds (anthocyanins, flavanols, flavonols, phenolic acids and stilbenes) can be used to identify the picking location of the bilberries.
- The bilberry extract acted as an antioxidant. Its antioxidant activity determined by DPPH test was linearly correlated to its anthocyanin content.
- The bilberry and the blueberry extracts expressed their intracellular antioxidant activity at very low anthocyanin concentrations in the tested mammalian cells (Caco-2, HepG2, EA.hy926 and A7r5).
- The bilberry extract exerted higher intracellular antioxidant potential than the blueberry extract.
- Anthocyanins from the bilberry and the blueberry extract could be mainly responsible for the intracellular antioxidant activity.
- The Caco-2 cells were the best protected, EA.hy926 and HepG2 cells were protected similarly whereas A7r5 cells were the least protected cells by the antioxidants from the berry extracts.
- Anthocyanins (e. g. cyanidin 3-glucoside) can enter into cells of liver and vascular endothelium in intact form, while in the liver cells they can also be metabolized.
- Anthocyanins (e. g. cyanidin 3-glucoside) and anthocyanidins (e. g. cyanidin) cannot pass through the cell membrane by diffusion.
- Anthocyanins (e. g. cyanidin 3-glucoside) and anthocyanidins (e. g. cyanidin) can be absorbed in cells of gastrointestinal tract and vascular endothelium by an organic anion membrane carrier bilitranslocase.

6 SUMMARY (POVZETEK)

6.1 SUMMARY

Fruits have an important role in everyday human diet. In Slovenia bilberries (*Vaccinium myrtillus* L.) are traditionally collected during the harvest season in June and July. They are supposed to be an important source of phenolic compounds, particularly of anthocyanins. Blueberries (*Vaccinium corymbosum* L.) although cultivated on plantations are an important source of phenolic compounds as well. Until recently there were no reports of phenolic compound content and of composition in bilberries and blueberries grown in Slovenia. One of the aims of our study was to determine qualitatively and quantitatively phenolic compounds in Slovenian bilberries and blueberries and to investigate if phenolic compounds can be correlated to picking locations of the bilberries. Another purpose of the study was to assess antioxidant potential of the collected bilberries and blueberries *in vitro* in extracts and in different mammalian cell lines. The involvement of bilitranslocase, a known transport protein for anthocyanins was tested for selected anthocyanins and for bilberry and blueberriy extracts on four cell lines. Furthermore, using an enthotelium cell membrane model we have shown that cyanidin 3-glucoside and cyanidin, both hydrophilic and bulky molecules, cannot pass the cell membrane by passive diffusion.

Samples of bilberries were handpicked in woods from seven different locations in Slovenia (Ljubljana, Pohorje, Pokljuka, Kranj, Goričko, Škofja Loka and Celje) while blueberries were handpicked from a plantation on Ljubljansko barje. The content of total phenolics and total anthocyanins was determined spectrophotometrically. The content of total phenolics and total anthocyanins was higher in the bilberries than in the blueberries for about 3-fold and 4-fold, respectively. The content of individual anthocyanins was determined by LC-MS/MS while flavanols, flavonols, phenolic acids and resveratrol in the bilberries and blueberries were determined by LC-MS. The anthocyanins content in the bilberries was 1210.3 ± 111.5 mg/100 g fw and in the blueberries 212.4 ± 14.1 mg/100 g fw. Both, the bilberries and the blueberries contained 15 anthocyanins: glucosides, galactosides and arabinosides of 5 anthocyanidins (cyanidin, petunidin, peonidin, delphinidin and malvidin). However, the pattern of individual anthocyanins was significantly different between them. Delphinidin and cyanidin glycosides were predominant in the bilberries, malvidin glycosides in the blueberries while peonidin glycosides were the minor anthocyanidin glycosides in both, the bilberries and the blueberries. The two flavanols (epicatechin and catechin) were found in both of the berries. The three flavonols revealed in the present study were quercetin, myricetin and rutin. Quercetin was found in both of the berries, myricetin only in the bilberries while rutin was found in both of the berries for the first time. Phenolic acids were present in high amounts. The predominant phenolic acid in both

of the berries was chlorogenic acid. Caffeic, ferulic, ellagic and gallic acids were all detected in both of the berries, as well as *p*-coumaric acid, although this was quantified only in the bilberries. Stilbenes were also detected in the bilberries and the blueberries, but only *trans*-resveratrol was found in both. Multivariate statistical analysis was used to examine potential correlations with the locations of the bilberry sampling. Results showed that the content of 27 individual phenolics (anthocyanins, flavanols, flavonols, phenolic acids and stilbenes) can be used to identify the picking location of the Slovenian bilberries.

The antioxidant activity of bilberry extracts from different picking locations was determined *in vitro* by DPPH method. All methanolic extracts exhibited significant antioxidant activity which was linearly correlated to the anthocyanin content.

The CAA assay is able to measure antioxidant activity of food extracts in cells. It can simulate *in vivo* conditions and it was used to measure intracellular antioxidant activity of bilberry and blueberry extract and also their anthocyanins. Four different cell lines were used: human epithelial colorectal adenocarcinoma (Caco-2), human hepatocellular carcinoma (HepG2), human endothelial (EA.hy926) and rat smooth muscle (A7r5). These cell lines were used to model the absorption of anthocyanins from the gastrointestinal tract to various tissues such as the liver and blood vessel endothelium. Obtained results showed that the bilberry and the blueberry anthocyanins, expressed their intracellular antioxidant activity at very low concentrations, ranged from 0.25 to 50 µg/L. The obtained concentrations are in the range of plasma anthocyanins concentrations determined after the oral administration. The bilberry anthocyanin extract exerted higher intracellular antioxidant activity than the blueberry. The Caco-2 cells were the best protected, EA.hy926 and HepG2 cells were protected less but similarly whereas A7r5 cells were the least protected cells by the anthocyanins in the berry extracts. The results confirmed that anthocyanins are the main antioxidants in the bilberries and the blueberries.

Using LC-MS/MS method the anthocyanin cellular uptake was investigated. The standard anthocyanin used was cyanidin 3-glucoside and the investigated cells were EA.hy926 and HepG2 in order to simulate anthocyanin absorption in vascular endothelial and hepatic cells. Intact cyanidin 3-glucoside was found in both EA.hy926 and HepG2 cells while its *O*-methylated and hydroxylated metabolites: peonidin 3-glucoside and delphinidin 3-glucoside were found only in the HepG2 cells.

The interactions of cyanidin 3-glucoside and its aglycon cyanidin with liposomes were investigated by spectroscopic fluorescence measuring difference in polarization (ΔP). Liposomes were prepared from phosphatidylcholine and sphingomyelin in a molar ratio 2.4:1 and served as a model of vascular endothelial cell membrane. The results

showed that cyanidin 3-glucoside and cyanidin cannot pass the model membranes. The obtained results showed that the tested compounds with a relatively high molecular mass and prone to form hydrogen bonds were unable to cross biological membranes by passive (i.e. not carrier-mediated) diffusion. Thus, anthocyanin uptake into cells would require a carrier-mediated transport. An organic anion membrane carrier bilitranslocase was a suggested transporter for anthocyanins into different mammalian cells. The modified CAA assay, which included an incubation of cells with bilitranslocase antibodies, confirmed that cyanidin 3-glucoside can be transported into the Caco-2, EA.hy926 and A7r5 cells by bilitranslocase.

6.2 POVZETEK

Znano je, da uravnotežena prehrana, ki vključuje tudi sadje in zelenjavo, potencialno ugodno deluje na zdravje ljudi zaradi naravnih antioksidantov. Borovnice spadajo v sam vrh po vsebnosti antioksidantov, saj vsebujejo veliko fenolnih spojin, zlasti antocianinov. V Sloveniji je nabiranje in vključevanje borovnic v vsakodnevno prehrano precej razširjeno, zlasti v poletnih mesecih, v času njihove polne zrelosti. Ker do datuma prijave teme doktorske disertacije, podatkov o vsebnosti fenolnih spojin v slovenskih borovnicah še ni bilo, je bil naš namen identificirati in kvantitativno določiti fenolne spojine v gozdnih borovnicah (*Vaccinium myrtillus* L.) in v ameriških borovnicah (*Vaccinium corymbosum* L.). Zanimalo nas je tudi, kako različne regije oziroma rastišča vplivajo na sestavo fenolih spojin v borovnicah.

Namen disertacije je bil tudi razširiti raziskavo na biološko dostopnost antocianinov v organizmu. Posebej nas je zanimal prehod iz prebavnega trakta in distribucija v nekatera tkiva in celice, saj je to področje, ki je zaenkrat le malo raziskano in je ključno v odgovoru na to ali človeški organizem sprejema antioksidante iz hrane in v kakšni meri. Poleg tega nas je zanimalo ali potrebujejo antocianini za prehod skozi celično membrano transportni protein ali lahko prehajajo s pasivno difuzijo. Pri tem smo se osredotočili predvsem na transportni protein bilitranslokazo. Zanimalo nas je tudi ali se antioksidativna aktivnost odraža tudi znotraj celic in če da, kolikšna koncentracija je potrebna za njihovo antioksidativno delovanje. S temi poskusi smo hoteli preveriti učinkovitost izbranih antioksidantov iz borovnic tudi na celičnem nivoju.

Vsebnost fenolnih spojin v gozdnih in ameriških borovnicah

Vzorce gozdnih borovnic smo nabrali na sedmih divjih rastiščih po različnih regijah v okolici Ljubljane, na Pohorju, na Pokljuki, v Kranju, na Goričkem, v Škofji Loki in v okolici Celja. Ameriške borovnice so bile vzgojene na plantažnem rastišču na Drenovem

griču na Ljubljanskem barju. Iz nabranih vzorcev borovnic smo z ustreznimi organskimi topili pripravili ekstrakte.

Vsebnost skupnih fenolnih spojin in skupnih antocianinov

Vsebnost skupnih fenolnih spojin in skupnih antocianinov smo določili spektrofotometrično in sicer smo skupne fenolne spojine določili po metodi Folin-Ciocalteu in jih izrazili kot mg galne kisline na 100 g svežih borovnic. Povprečna vrednost skupnih fenolnih spojin v gozdnih borovnicah je bila 5,0 mg/100 g, v gojenih ameriških borovnicah pa je bila za 64 % nižja in je znašala 1,8 mg/100 g. Vsebnost skupnih antocianinov smo merili pri absorpcijskem vrhu med 536 in 542 nm pri nizki pH vrednosti. Rezultate smo izrazili kot g cianidin-3-glukozida na kg svežih borovnic. Povprečna vrednost antocianinov v gozdnih borovnicah je bila 11,00 mg/100 g, v ameriških borovnicah pa 2,5 mg/100 g, kar je za 77 % manj. Iz podatkov lahko zaključimo, da so gozdne borovnice boljši vir skupnih fenolnih spojin in skupnih antocianinov v primerjavi z ameriškimi borovnicami. Z zaužitjem 18 g slovenskih gozdnih borovnic ali z zaužitjem približno 80 g gojenih ameriških borovnic dosežemo povprečno vrednost dnevnega vnosa antocianinov v telo, ki je bila ocenjena na 180-215 mg (Hertog in sod., 1993).

Vsebnost posameznih antocianinov

Vsebnost posameznih antocianinov v gozdnih in ameriških borovnicah smo določili s tekočinsko kromatografijo in masno spektrometrijo (LC-MS/MS), s katero smo identificirali in kvantitativno ovrednotili posamezne antocianine. Rezultate smo izrazili kot cianidin-3-glukozid v miligramih na 100 gramov svežih borovnic glede na umeritveno krivuljo s standardnim dodatkom in s korelacijskim koeficientom 0,9983. Določili smo mejo detekcije, mejo kvantifikacije in ponovljivost metode. Rezultati analize so prikazani v preglednici 1. Vsebnost posameznih antocianinov v gozdnih borovnicah (1210,3 ± 111,5 mg/100 g) je bila 6-krat večja od njihove vsebnosti v ameriških borovnicah (212,4 ± 14,1 mg/100 g), kar sovpada s spektofotometričnimi analizami skupnih antocianinov. V obeh vrstah borovnic smo določili 15 antocianinov, ki imajo na osnovno strukturo antocianidinov (cianidin, delfinidin, peonidin, petunidin in malvidin) vezan po en monosaharid (glukozo ali galaktozo ali arabinozo). V primerjavi z objavljenimi rezultati so bile naše slovenske gozdne borovnice bogatejše z antocianini kot gozdne borovnice iz drugih evropskih držav kot sta Italija (Giovanelli in Buratti, 2009) in Finska (Kähkönen in sod., 2003). Gozdne borovnice so boljši vir antocianinov v primerjavi od večine jagodičevja kot so črni ribez, brusnice, maline in jagode (Heinonen, 2007). Ameriške borovnice pa vsebujejo podobno količino antocianinov kot robide (Sellappan in

sod., 2002), črni ribez (Riihinen in sod., 2008) vendar manj kot brusnice (Taruscio in sod., 2004) in jerebika (Hukkanen in sod., 2006).

Vsebnost in profil glikozidov antocianidinov v gozdnih borovnicah se značilno razlikuje od ameriških borovnic. V gozdnih borovnicah so prevladovali delfinidin in cianidin glikozidi (> 70 %). Enako so ugotovili tudi pri finskih (Lätti in sod., 2008) in švedskih gozdnih borovnicah (Åkerström in sod. 2009). V ameriških pa so prevladovali malvidin glikozidi (ca. 47 %). Enake vrednosti so našli tudi nekateri drugi avtorji (Wang in sod., 2008, Burdulis in sod., 2009), vendar so analize pokazale, da so v borovnicah v Kanadi in ZDA prevladovali delfinidin glikozidi (Taruscio in sod., 2004). Pri obeh vrstah borovnic iz Slovenije pa so bili peonidin glikozidi najmanj zastopani, kar je enako kot so ugotovili v ameriških borovnicah s Finske (Lätti in sod., 2008).

Razlike v vsebnosti in profilu antocianinov lahko pripišemo genetski in/ali pedoklimatski raznolikosti. Glede na še ne objavljene rezultate se po genetski sestavi gozdne borovnice iz sedmih različnih regij vzorčenja ne razlikujejo. Glede na podnebje, sta Pokljuka in Pohorje na gorskem rastnem področju, Celje, Goričko in Škofja Loka na hribovitem rastnem področju, Ljubljana in Kranj pa na nižinskem rastnem področju. V vseh rastnih področjih pa borovnice uspevajo v kislih tleh bogatih z organskimi snovmi. Antocianini so v gozdnih borovnicah prisotni tako v kožici kot v mesu jagod, v ameriških pa le v kožici (Riihinen in sod., 2008). Za primerjavo se antocianini nahajajo le v kožici rdečega grozdja in črnega ribeza (Bakker in Timberlake, 1985) in v celotnem sadežu jagode, robide, maline in bezgove jagode (Mullen in sod., 2002; Hartmann in sod., 2008; Cuevas-Rodríguez in sod., 2010).

Vsebnost posameznih flavanolov, flavonolov, fenolnih kislin in stilbena

Vsebnost posameznih flavanolov, flavonolov, fenolnih kislin in stilbena smo prav tako določili s tekočinsko kromatografijo in masno spektrometrijo (LC-MS). Rezultate smo izrazili kot miligram posameznega standarda na 100 gramov svežih borovnic glede na umeritvene krivulje s standardnim dodatkom in s korelacijskim koeficientom ≥ 0,99. Flavanole smo kvantificirali kot katehin, flavonole pa na posamezen standard (miricetin, kvercetin in rutin). Prav tako smo na posamezne standarde kvantificirali fenolne kisline (klorogenska, kavna, ferulna, *p*-kumarna, elagična in galna kislina). Stilben smo izrazili na *trans*-resveratrol. Vsem standardom smo določili mejo detekcije, mejo kvantifikacije in ponovljivost metode. Rezultati so predstavljeni v preglednici 2.

V gozdnih in ameriških borovnicah smo našli dva flavanola. Vendar je bila vsebnost epikatehina (2,0 mg/100 g) večja v gozdnih borovnicah, vsebnost katehina pa v ameriških

borovnicah (1,8 mg/100 g). Ta dva flavanola so predhodno že našli v obeh vrstah borovnic (Taruscio in sod., 2004) in tudi v robidah (Sellappan in sod., 2002) in brusnicah (Taruscio in sod., 2004).

Kvercetin, miricetin in rutin so flavonoli, ki smo jih določili v borovnicah. Kvercetin smo našli v obeh vrstah borovnic, medtem ko je bil miricetin prisoten le v gozdnih borovnicah. Glede na predhodne raziskave naše borovnice vsebujejo manj kvercetina (Riihinen in sod., 2008). Rutin, katerega vsebuje veliko ajda (Quettier-Eleu in sod., 2000), smo po do sedaj znanih podatkih v obeh vrstah borovnic našli prvič. Ameriške borovnice (3,1 mg/100 g) so ga vsebovale 15-krat več kot gozdne (0,2 mg/100 g).

Vsebnost fenolnih kislin je bila v ameriških in gozdnih borovnicah večja od flavanolov in flavonolov. Klorogenske kisline je bilo največ v obeh vrstah borovnic, vendar je bila njena vsebnost 3-krat večja v ameriških borovnicah (70,0 mg/100 g) kot v gozdnih (23,1 mg/100 g). Kavno, ferulno, elagično, galno in *p*-kumarno kislino smo našli v obeh vrstah borovnic, vendar je v ameriških borovnicah nismo mogli kvantitativno ovrednotiti. O vsebnosti fenolnih kislin v gozdnih in ameriških borovnicah so poročali že številni avtorji (Sellappan in sod., 2002; Ehala in sod., 2005; Hukkanen in sod., 2006; Wang in sod., 2008).

Stilben, ki smo ga našli v gozdnih in ameriških borovnicah, je bil *trans*-resveratrol. V literaturi ni veliko podatkov o vsebnosti *trans*-resveratrola v borovnicah. Slovenske gozdne borovnice so vsebovale 0,2 mg *trans*-resveratrola /100 g borovnic, kar sovpada z že objavljenimi podatki (Ehala in sod., 2005). Ameriške borovnice pa so vsebovale *trans*-resveratrola 0,4 mg/100 g, kar je manj v primerjavi s predhodno objavljenimi podatki (Wang in sod., 2008). *Trans*-resveratrol lahko najdemo tudi v ostalem jagodičevju kot so brusnice, jagode in rdeči ribez (Ehala in sod., 2005), v velikih količinah pa ga vsebujejo rdeče grozdje in rdeče vino.

Multivariantna analiza

S statistično analizo (multivariantno analizo) smo ocenili morebitne korelacije med vzorčnimi lokacijami gozdnih borovnic. Rezultati so pokazali, da vsebnost 27 posameznih fenolnih spojin (antocianini, flavanoli, flavonoli, fenolne kisline in stilbeni) lahko opredeljuje lokacijo vzorčenja slovenskih gozdnih borovnic in da se da z identificiranimi fenolnimi spojinami zelo dobro določiti tudi rastišče gozdnih borovnic in da rastišče pomembno vpliva tudi na sestavo fenolnih spojin v gozdnih borovnicah.

Zaključimo lahko, da prevladujejo antocianini v gozdnih in ameriških borovnicah. Kljub temu pa so gozdne in ameriške borovnice ravno tako dober vir ostalih fenolnih spojin kot so fenolne kisline, flavanoli, flavonoli in *trans*-resveratrol.

Antioksidativni potencial gozdnih in ameriških borovnic

DPPH test

DPPH test je standardna metoda za določanje antioksidativnega potenciala spojin v *in vitro* pogojih. Metoda temelji na merjenju absorbance vijolično obarvane raztopine 2,2-difenil-1-pikrilhidrazil radikala (DPPH) pri valovni dolžini 517 nm. Ob dodatku antioksidantov pride do redukcije DPPH in posledično do razbarvanja, kar privede do zmanjšanja absorbance. S pomočjo DPPH testa, ki ga je prvi razvil Blois (1985) smo določili antioksidativni potencial ekstraktom gozdnih borovnic z naslednjimi vsebnostmi antocianinov (izraženi kot mg cianidin-3-glukozid/L ekstrakta): 0,01; 0,1; 1; 5; 10; 25 in 50. Rezultati so pokazali, da je prečiščen ekstrakt gozdnih borovnic dober antioksidant in da je njegova antioksidativna aktivnost linearno odvisna od koncentracije antocianinov. Pri danih koncentracijah ni prišlo do nasičenja. Te ugotovitve sovpadajo s predhodno objavljenimi rezultati merjenja antioksidativnih aktivnosti nekaterega jagodičevja z DPPH testom (Kähkönen in sod., 2003).

CAA metoda

CAA (Cellular Antioxidant Activity) metoda, ki sta jo nedavno razvila Wolfe in Liu (2007), omogoča določanje antioksidativnaga potenciala ekstraktov živil in prehranskih dodatkov v notranjosti celic. Princip metode temelji na merjenju spremembe fluorescence intracelularnega barvila zaradi peroksilnih radikalov, ki so nastali kot posledica oksidativnega stresa, povzročenega z dodatkom spojine ABAP (2,2'-azobis (2-amidinopropan)dihidroklorid). Ob dodatku ekstrakta živil se fluorescenca zmanjša zaradi manjšega števila peroksilnih radikalov, ki so predhodno reagirali z antioksidanti iz živil. S CAA metodo smo ekstraktom iz gozdnih in ameriških borovnic določili antioksidativne sposobnosti znotraj različnih vrst celic.

Ekstraktu iz gozdnih borovnic smo določili znotrajcelični antioksidativni potencial s CAA metodo pri enakih koncentracijah antocianinov (izražene kot mg cianidin-3-glukozid/L ekstrakta): 0,01; 0,1; 1; 5; 10; 25 in 50, kot pri DPPH testu. Uporabili smo EA.hy926 celice kot model žilnega endotelija, saj smo želeli raziskati vpliv antocianinov iz gozdnih borovnic na zaščito ožilja pred oksidativnim stresom. Meritve so pokazale očitno zmanjšanje spremembe florescence znotraj celic v odvisnosti od časa. Izračunali smo

površine pod dobljenimi krivuljami in rezultate podali kot CAA vrednosti (izraženih v odstotkih antioksidativne aktivnosti) glede na koncentracije antocianinov v ekstraktu. Dobili smo krivuljo v obliki hiperbole. V nasprotju z rezultati DPPH testa smo pri krivulji CAA vrednosti v odvisnosti od koncentracije antocianinov zaznali nasičenje. Antioksidativna aktivnost se je povečevala pri nizkih koncentracijah antocianinov (<0.5 mg/L), medtem ko je bila konstantna pri visokih koncentracijah (>0.5 mg/L). Da je pri tej krivulji prišlo do nasičenja so opazili tudi pri študiju intracelularne antioksidativne aktivnosti ameriških borovnic (Wolfe in Liu, 2007). Zaključimo lahko, da imajo antociani iz gozdnih borovnic dober intracelularni antioksidativni potencial in zmožnost zaščititi celice pred oksidativnim stresom in da se s povečanjem koncentracije antocianinov ta vrednost več ne povečuje.

S CAA metodo smo določili intracelularni antioksidativni potencial gozdnih in ameriških borovnic tudi pri zelo nizkih koncentracijah antocianinov. Uporabili smo štiri različne celične linije: celice humanega adenokarcinoma debelega črevesa (Caco-2), humane jetrne celice (HepG2), humane celice endotelija žil (EA.hy926) in podganje celice gladke mišične žil (A7r5), z namenom slediti absorpciji in distribuciji antocianinov od prebavnega trakta do organov in tkiv kot so jetra in krvne žile. Torej, Caco-2 celice ponazarjajo celice črevesnega epitelija in se zato uporabljajo pri študijah gastrointestinalne absorpcije, HepG2 celice služijo kot model jetrnih celic in so model za študije absorpcije spojin v jetra, EA.hy926 in A7r5 celice pa služijo kot model celic žilnega endotelija in celic žilnega gladkega mišičevja. Ekstrakte smo, z namenom pridobiti predvsem antocianine, predhodno očistili po že objavljenem postopku (Youdim in sod., 2002). Koncentracije antocianinov (izražene kot μg cianidin-3-glukozida/L ekstrakta), ki smo jih uporabili, so bile sledeče: 0,25; 0,5; 1; 2; 5; 10; 25 in 50. Take koncentracije antocianinov so v krvi našli po peroralnem doziranju (Mazza in sod., 2002; Felgines in sod., 2008). Meritve so pokazale dobro antioksidativno aktivnost obeh ekstraktov v vseh vrstah celic. Prav tako smo iz dobljenih krivulj izračunali CAA vrednosti (izraženih v odstotkih antioksidativne aktivnosti) glede na koncentracije antocianinov v ekstraktih in ponovno smo dobili krivulje v obliki hiperbol, ki je pri večini dosegla maksimalno vrednost pri koncentraciji 5 μg/L. Določili smo tudi koncentracijo pri polovični antioksidativni aktivnosti (EC_{50}) in maksimalno antioksidativno aktivnost (CAA_{max}). Vrednosti EC_{50} so bile <1μg/L, CAA_{max} pa pod 40 %. Torej je znotrajcelična antioksidativna aktivnost antocianov iz borovničevih ekstraktov omejena, kar kaže le na delno zaščito celic. Da smo lahko primerjali antioksidativno aktivnost obeh ekstraktov v štirih različnih celicah, smo nato izračunali površine pod dobljenimi krivuljami (hiperbolami), jih izrazili kot AUC in jih statistično obdelali. Pri Caco-2 celicah razlik med AUC vrednostmi med ekstraktoma gozdnih in ameriških borovnic ni bilo. Pri HepG2 in EA.hy926 je imel ekstrakt iz gozdnih borovnic okoli 10-30 % večji AUC kot ekstrakt iz ameriških borovnic. Največji AUC smo določili

pri Caco-2 celicah, najnižjega pa pri A7r5. To lahko pripišemo ali različni prisotnosti transportnih proteinov v omenjenih celicah ali različni znotrajcelični antioksidativni kapaciteti, ki jo sprožajo molekule iz ekstraktov. Sklepamo lahko, da antioksidanti iz borovničevih ekstraktov najbolj zaščitniško delujejo v Caco-2 celicah, manj, vendar podobno v EA.hy926 in HepG2 celicah ter najslabše v A7r5 celicah. S to študijo smo prvi pokazali, da imajo ekstrakti iz gozdnih in ameriških borovnic dober antioksidativni potencial pri zelo nizkih koncentracijah antocianinov v celicah iz različnih tkiv. Ker antocianini predstavljajo večino fenolnih spojin tako v gozdnih kot ameriških borovnicah, lahko zaključimo, da so le-ti odgovorni za njihovo antioksidativno aktivnost. Izkazalo se je, da je imel ekstrakt iz gozdnih borovnic boljši antioksidacijski potencial kot ekstrakt iz ameriških borovnic pri vseh testiranih celicah. Do enake ugotovitve so predhodno prišli tudi z *in vitro* antioksidativnimi testi (Giovanelli in Buratti, 2009). To razliko lahko pripišemo različnemu profilu antocianinov. Delfinidin in cianidin glikozidi so prevladovali v ekstraktu iz gozdnih borovnic, malvidin glikozidi pa v ekstraktu iz ameriških borovnic. Znano je, da so delfinidin in cianidin in njuni glukozidi boljši antioksidanti kot malvidin in njegovi glukozidi (Kähkönen and Heinonen, 2003).

Biorazpoložljivost antocianinov

Privzem antocianinov v celice

Privzem antocianinov v celice smo raziskali z metodo LC-MS/MS. Kot standard smo uporabili antocianin cianidin 3-glukozid in celični liniji HepG2 ter EA.hy926, saj le-ti dobro simulirajo absorpcijo antocianinov v celice gastrointestinalnega trakta in žilnega endotelija. Ugotovili smo, da cianidin-3-glukozid lahko vstopa v celice jeter in celice žilnega endotelija v nespremenjeni obliki, v jetrnih celicah pa nato lahko pride do nastanka metabolitov, saj smo v njih identificirali peonidin-3-glukozid in delfinidin-3-glukozid.

Transport antocianinov skozi celično membrano

S prilagojeno CAA metodo smo ocenili vlogo transportnega proteina bilitranslokaza pri prehajanju antocianinov skozi celično membrano. V že obstoječo metodo smo vključili predinkubacijo celic z dvema vrstama protiteles: zajčjih imunoglobulinov (IgG) in bilitranslokaznih protiteles Multipli Antigen Peptidov (MAP). Protitelesa MAP se vežejo na bilitranslokazo in zato blokirajo prehod molekul skozi celično membrano, IgG protitelesca pa so bila uporabljena kot negativna kontrola. Celično linijo EA.hy926 smo uporabili kot model žilnega endotelija. Testirali smo cianidin-3-glukozid, florizin in katehin pa sta bili kontroli. Uporabili smo naslednje koncentracije vseh treh spojin (nM): 0; 0,5; 1; 2; 4; 10; 20; 50; 100. Preučili smo tudi kinetiko intracelularne antioksidativne

aktivnosti cianidin-3-glukozida, tako da smo na celice, ki smo jih predhodno inkubirali s protitelesi, nanesli 1 nM cianidin-3-glukozid v različnih časovnih intervalih (10; 5; 4; 3; 2; 1; 0,167 min).

Pri oceni intracelularnega antioksidativnega potenciala cianidin-3-glikozida smo ponovno izračunali površine pod krivuljami in jih izrazili kot CAA vrednosti v odvisnosti od koncentracije antioksidantov. Rezultati so pokazali, da je antioksidativna aktivnost cianidin-3-glikozida omejena, ker pride do nasičenja. Vrednost EC_{50} je bila 0,9 nM, CAA_{max} pa 0,39±0,008 nM. Po inkubaciji s bilitranslokaznimi protitelesi se je CAA_{max} zmanjšal na 0,26±0,02 nM. Torej, ob prisotnosti bilitanslokaznih protiteles je bil antioksidativni potencial cianidin-3-glikozida v notranjosti celic manjši kot v prisotnosti imunoglobulinov, ki se ne vežejo na bilitranlokazo. Takšen rezultat nakazuje, da je bilitranslokaza membranski protein, ki vpliva na celično absorpcijo cianidin 3-glukozida. Enako je bilo pri poskusu preučevanja časovne kinetike antioksidativnega potenciala cianidin-3-glukozida. S tem poskusom smo pokazali, da je prehod antocianinov preko celične membrane zelo hiter, saj smo antioksidativno aktivnost zaznali že po 0,167 min inkubacije. Nadaljnji testi z florizinom in katehinom so pokazali, da je florizin podobno dober antioksidant kot cianidin-3-glikozid, katehin pa je pokazal zelo nizko intracelularno antioksidativno aktivnost. Pri poskusu z bilitranslokazo ni bilo razlik v antioksidativni aktivnosti od poskusov z imunoglobulini. Sklepamo lahko, da transport tako florizina kot katehina skozi celično membrano ni odvisen od bilitranslokaze. Obratno pa velja za cianidin-3-glikozid, za katerega smo potrdili, da ima bilitranslokaza pomembno vlogo pri njegovem prehajanju skozi celično membrano endotelijskih celic.

Interakcije antocianinov z liposomi smo preučevali z metodo, ki temelji na merjenju spremembe fluorescenčne polarizacijske intenzitete (ΔP) fluorofora 1,6-difenil-1,3,5-heksatriena (DPH) ob dodajanju antocianina k mešanici lipidnih liposomov, ki služijo kot model celične membrane. Liposome smo pripravili s pomočjo ekstruzije z dodatkom pufra HEPES pri pH 7 iz posameznih fosfolipidov, fosfatidilholina (PC) in sfingomielina (SM) v razmerju 2,4:1, ki služi kot model celične membrane žilnih endotelijskih celic (Cansell in sod., 1997). Pripravljenim liposomom smo dodali DPH in merili ΔP ob dodatku cianidin-3-glukozida in bilirubina kot kontrole pri različnih koncentracijah. Sprememb nismo zaznali pri cianidin-3-glukozidu. Pri bilirubinu pa smo opazili naraščanje enako kot pri holesterolu in flavonoidu kemferolu, o čemer poročajo Poklar Ulrih in sod. (2010). Sklepamo lahko, da cianidin-3-glukozid nima sposobnosti prehajati preko izbrane celične membrane s pasivnim transportom (difuzijo). Torej skozi celično membrano prehaja s pomočjo transportnega proteina.

Sklepi

- Gozdne in ameriške borovnice iz Slovenije so dober vir fenolnih spojin (antocianinov, flavanolov, flavonolov, fenolnih kislin in *trans*-resveratrola)
- Gozdne borovnice vsebujejo več skupnih fenolnih spojin in skupnih antocianinov kot ameriške borovnice.
- Antocianini so glavni predstavniki fenolnih spojin v gozdnih in ameriških borovnicah.
- Obe vrsti borovnic vsebujeta 15 antocianinov: po 3 glikozide (glukozidi, galaktozidi in arabinozidi) 5 antocianidinov (cianidin, petunidin, peonidin, delfinidin in malvidin).
- Profila antocianidin glikozidov se med obema vrstama borovnic razlikujeta. Prevladujoči antocianidin glikozidi v gozdnih borovnicah so bili glikozidi delfinidina in cianidina, v ameriških pa glikozidi malvidina. Peonidin glikozidi so dosegli najnižje vrednosti pri obeh vrstah borovnic.
- Klorogenske kisline je bilo med fenolnimi kislinami največ v obeh vrstah borovnic.
- Prvič smo v obeh vrstah borovnic identificirali flavonol rutin.
- Lokacijo rastišča gozdnih borovnic lahko zelo dobro določimo z vsebnostjo 27 fenolnih spojin (antocianinov, flavanolov, flavonolov, fenolnih kislin in stilbena).
- Ekstrakt iz gozdnih borovnic ima antioksidativne lastnosti. Njegova antioksidativna aktivnost, ki smo jo določili DPPH testom, je linearno odvisna od njegove vsebnosti antocianinov.
- Ekstrakta iz gozdnih in ameriški borovnic sta dobra antioksidanta pri zelo nizkih koncentracijah antocianinov in sta aktivna tudi znotraj celic sesalcev (Caco-2, HepG2, EA.hy926 in A7r5).
- Ekstrakt iz gozdnih borovnic ima boljši antioksidativni potencial znotraj celic kot ekstrakt iz ameriških borovnic.
- Znotrajcelično antioksidativno aktivnost gozdnih in ameriških borovnic pripisujemo antocianinom.
- Antioksidanti iz obeh vrstah borovnic so najbolj zaščitniško delovali v Caco-2 celicah, manj, vendar podobno v EA.hy926 in HepG2 celicah, najmanj pa v A7r5 celicah.
- Antocianini (npr. cianidin-3-glukozid) lahko vstopijo v celice jeter in žilnega endotelija v nespremenjeni obliki, v jetrnih celicah pa se lahko pretvorijo v metabolite, kot sta hidroksiliran in metiliran produkt cianidin-3-glukozida.
- Antocianini (npr. cianidin-3-glukozid) in antocianidini (npr. cianidin) skozi celično membrano ne morajo prehajati z difuzijo.
- Antocianini (npr. cianidin-3-glukozid) in antocianidini (npr. cianidin) se v celice prebavnega trakta in žilnega endotelija absorbirajo s pomočjo membranskega prenašalca bilitranslokaze.

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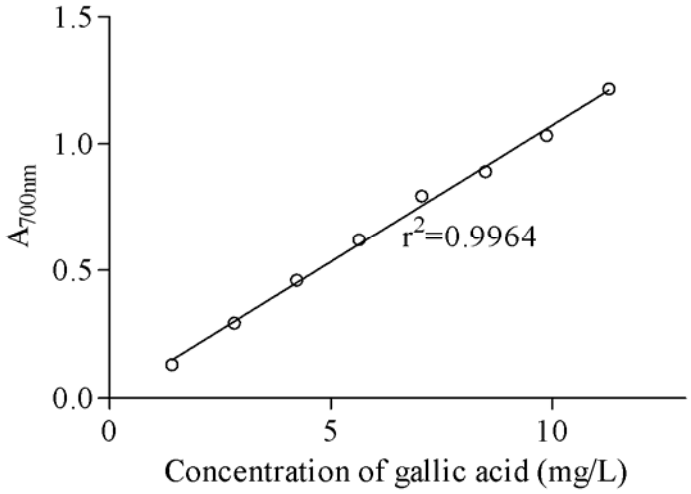
ANNEXES



Annex A: Calibration curve for quantification of total phenolics in bilberries and blueberries.

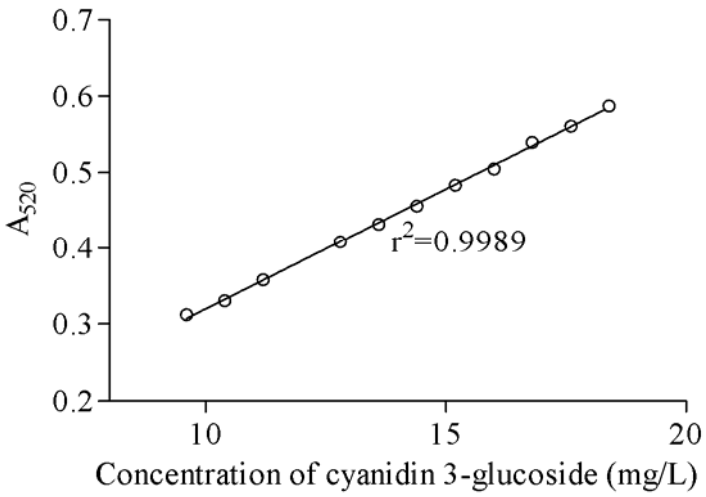
Priloga A: Umeritvena krivulja za kvantifikacijo skupnih fenolnih spojin v gozdnih in ameriških borovnicah.

Annex B: Total phenolics in Slovenian bilberries and blueberries.

Priloga B: Vsebnost skupnih fenolnih spojin v gozdnih in ameriških borovnicah iz Slovenije.

Sample	Mass (g)	Absorbance	Total phenolic (mg/100 g fw)	Average (mg/100 g)	SEM (mg/100 g)
Pokljuka 1	50.23	49.220	482.11	477.85	17.72
Pokljuka 2	50.10	51.545	506.19		
Pokljuka 3	50.01	45.259	445.26		
Celje 1	50.10	58.390	573.41	507.83	35.86
Celje 2	50.24	45.957	450.06		
Celje 3	50.11	57.672	566.25		
Celje 4	50.25	45.104	441.62		
Goričko 1	50.27	61.823	605.07	591.23	43.99
Goričko 2	50.14	61.526	603.73		
Goričko 3	17.06	34.801	501.82		
Goričko 4	50.10	75.546	741.89		
Goričko 5	44.00	45.043	503.66		
Ljubljana 1	50.09	46.584	457.56	507.10	24.79
Ljubljana 2	50.05	51.399	505.26		
Ljubljana 3	50.50	50.130	488.40		
Ljubljana 4	27.84	52.774	466.32		
Ljubljana 5	20.77	52.777	625.09		
Ljubljana 6	50.02	50.829	499.96		
Kranj 1	50.11	40.601	398.64	423.09	34.95
Kranj 2	50.05	39.547	388.75		
Kranj 3	29.88	24.315	400.37		
Kranj 4	50.02	60.616	596.27		
Kranj 5	32.34	48.417	368.29		
Kranj 6	50.06	39.300	386.25		
Škofja Loka 1	30.80	53.060	423.79	534.47	37.07
Škofja Loka 2	50.19	50.485	494.89		
Škofja Loka 3	50.13	65.100	638.92		
Škofja Loka 4	50.06	52.880	519.72		
Škofja Loka 5	50.19	48.939	479.74		
Škofja Loka 6	50.16	66.244	649.76		
Pohorje 1	50.12	47.764	234.44	436.12	49.55
Pohorje 2	50.06	40.952	402.48		
Pohorje 3	28.35	49.309	427.87		
Pohorje 4	50.06	57.754	567.62		
Pohorje 5	50.02	56.550	556.23		
Pohorje 6	50.09	43.584	428.10		
Blueberry 1	50.06	36.102	177.41	178.88	5.56
Blueberry 2	50.14	38.114	187.00		
Blueberry 3	50.01	35.993	177.05		
Blueberry 4	50.00	39.193	192.83		
Blueberry 5	50.78	33.055	160.13		

The data are quantified as gallic acid.



Annex C: Calibration curve for quantification of total anthocyanins in the bilberries and blueberries.

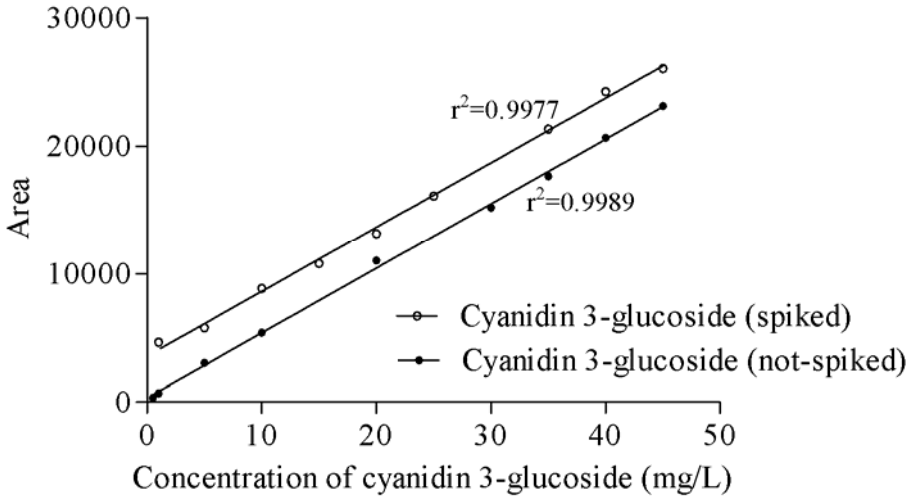
Priloga C: Umeritvena krivulja za kvantifikacijo skupnih antocianinov v gozdnih in ameriških borovnicah.

Annex D: Total anthocyanins in Slovenian bilberries and blueberries.

Priloga D: Vsebnost skupnih antocianinov v gozdnih in ameriških borovnicah iz Slovenije.

Sample	Mass (g)	Absorbance$_{520\ nm}$	Total anthocyanins (mg/100 g fw)	Average (mg/100 g)	SEM (mg/100 g)
Pokljuka 1	50.23	0.428	1065.05	1052.34	6.38
Pokljuka 2	50.10	0.419	1045.06		
Pokljuka 3	50.01	0.419	1046.92		
Celje 1	50.10	0.552	1377.72	1089.03	99.71
Celje 2	50.24	0.425	1058.12		
Celje 3	50.11	0.397	989.95		
Celje 4	50.25	0.374	930.35		
Goričko 1	50.27	0.470	1169.11	1157.34	91.14
Goričko 2	50.14	0.393	980.60		
Goričko 3	17.06	0.252	923.32		
Goričko 4	50.10	0.550	1371.08		
Goričko 5	44.00	0.473	1342.59		
Ljubljana 1	50.09	0.462	1153.80	1114.21	46.71
Ljubljana 2	50.05	0.513	1281.47		
Ljubljana 3	50.50	0.444	1097.85		
Ljubljana 4	27.84	0.419	939.83		
Ljubljana 5	20.77	0.351	1055.67		
Ljubljana 6	50.02	0.463	1156.66		
Kranj 1	50.11	0.404	1007.73	1106.37	61.82
Kranj 2	50.05	0.446	1113.24		
Kranj 3	29.88	0.534	1116.68		
Kranj 4	50.02	0.551	1377.36		
Kranj 5	32.34	0.481	929.79		
Kranj 6	50.06	0.438	1093.44		
Škofja Loka 1	30.80	0.693	1406.55	1245.13	86.09
Škofja Loka 2	50.19	0.577	1437.44		
Škofja Loka 3	50.13	0.519	1294.41		
Škofja Loka 4	50.06	0.371	927.36		
Škofja Loka 5	50.19	0.418	1040.82		
Škofja Loka 6	50.16	0.547	1364.21		
Pohorje 1	50.12	0.427	1065.77	924.75	74.16
Pohorje 2	50.06	0.402	1003.75		
Pohorje 3	28.35	0.277	610.10		
Pohorje 4	50.06	0.329	820.42		
Pohorje 5	50.02	0.383	956.59		
Pohorje 6	50.09	0.438	1091.91		
Blueberry 1	50.06	0.375	234.36	246.53	8.94
Blueberry 2	50.14	0.368	229.64		
Blueberry 3	50.01	0.379	236.60		
Blueberry 4	50.00	0.406	253.72		
Blueberry 5	50.78	0.452	278.36		

The data are quantified as cyanidin 3-glucoside.



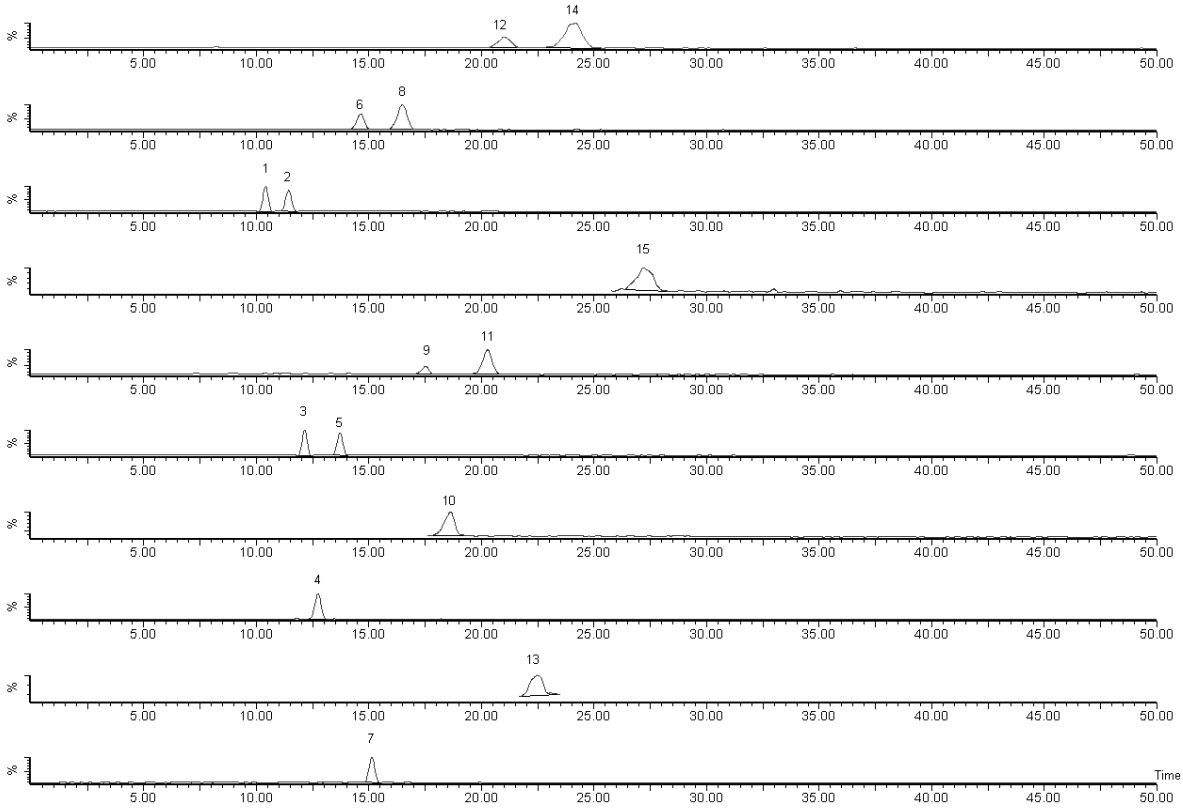
Annex E: Spiked and not-spiked calibration curves of a standard cyanidin 3-glucoside for quantification of individual anthocyanins in the bilberries and blueberries by LC-MS/MS.

Priloga E: Umeritvena krivulja brez in s standardnim dodatkom cianidin 3-glukozida za kvantifikacijo posameznih antocianinov v gozdnih in ameriških borovnicah z LC-MS/MS.

Annex F: Repeatability of LC-MS/MS method for the standard cyanidin 3-glucoside.

Priloga F: Ponovljivost LC-MS/MS metode za standard cianidin 3-glukozid.

Injection	Area	Retention time (min)
1	3950.80	11.78
2	3730.40	11.82
3	3465.60	11.81
4	3061.00	11.76
5	3842.60	11.78
6	3230.20	11.76
7	3225.30	11.79
8	3687.70	11.72
9	3393.20	11.74
average	3509.64	11.77
SD	308.55	0.03
CV (%)	8.79	0.26



Annex G: LC-MS/MS chromatogram of individual anthocyanins from the bilberries sampled in Smrečje.

Peak identification: 1, delphinidin 3-galactoside; 2, delphinidin 3-glucoside; 3, cyanidin 3-galactoside; 4, delphinidin 3-arabinoside; 5, cyanidin 3-glucoside; 6, petunidin 3-galactoside; 7, cyanidin 3-arabinoside; 8, petunidin 3-glucoside; 9, peonidin 3-galactoside; 10, petunidin 3-arabinoside; 11, peonidin 3-glucoside; 12, malvidin 3-galactoside; 13, peonidin 3-arabinoside; 14, malvidin 3-glucoside; 14, malvidin 3-arabinoside.

Priloga G: LC-MS/MS kromatogram posameznih antocianinov v vzorcu gozdnih borovnic iz Smrečja.

Identifikacija pikov: 1, delfinidin-3-galaktozid; 2, delfinidin-3-glukozid; 3, cianidin-3-galaktozid; 4, delfinidin-3-arabinozid; 5, cianidin-3-glukozid; 6, petunidin-3-galaktozid; 7, cianidin-3-arabinozid; 8, petunidin-3-glukozid; 9, peonidin-3-galaktozid; 10, petunidin-3-arabinozid; 11, peonidin-3-glukozid; 12, malvidin-3-galaktozid; 13, peonidin-3-arabinozid; 14, malvidin-3-glukozid; 14, malvidin-3-arabinozid.

Annex H: Individual anthocyanins (mg/100 g fw) in Slovenian bilberries and blueberries.

Priloga H: Vsebnosti posameznih antocianinov (mg/100 g sm) v gozdnih in ameriških borovnicah iz Slovenije.

Samlpe	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	TOTAL
Pokljuka 1	185.09	178.31	101.00	167.13	105.23	41.11	98.51	86.90	4.79	18.05	21.07	12.61	1.87	30.30	5.60	1057.58
Pokljuka 2	158.72	167.68	88.27	159.91	105.80	36.84	96.53	53.07	2.64	10.43	21.69	9.12	0.88	21.83	4.41	937.82
Pokljuka 3	131.71	137.68	91.84	142.11	110.01	41.64	96.66	94.75	6.03	22.08	26.74	10.70	2.92	27.89	6.90	949.67
Celje 1	125.59	152.36	144.30	102.06	198.96	46.00	126.43	124.11	35.04	24.74	154.09	24.21	7.26	58.79	9.76	1333.72
Celje 2	138.47	128.93	92.70	121.24	100.85	43.41	90.11	52.06	15.00	14.85	35.33	17.41	4.53	92.45	14.88	962.22
Celje 3	156.49	183.77	74.09	146.18	91.93	45.89	72.99	95.34	3.30	13.65	25.02	11.68	1.32	40.10	7.96	969.71
Celje 4	115.69	118.06	68.52	115.06	86.75	42.28	84.69	93.88	7.96	16.18	27.86	14.88	1.82	23.82	6.67	824.13
Goričko 1	155.44	185.37	92.48	134.77	121.65	51.73	84.44	124.96	14.85	18.35	36.26	20.49	1.80	38.12	5.22	1085.93
Goričko 2	103.32	97.40	108.15	85.95	115.69	36.14	100.76	49.14	14.03	17.98	79.43	26.73	3.00	26.09	9.09	872.90
Goričko 3	206.83	234.29	223.61	186.77	257.13	62.90	211.89	168.56	21.61	20.96	83.74	29.10	12.01	74.31	12.38	1806.09
Goričko 4	213.04	196.27	133.01	178.49	132.46	91.76	115.68	167.69	21.06	27.08	111.68	122.33	6.04	136.29	31.67	1684.56
Goričko 5	210.00	117.55	167.44	117.10	101.87	88.32	96.23	84.48	24.66	16.72	33.41	43.46	3.63	29.53	5.68	1140.10
Ljubljana 1	227.03	231.32	147.56	202.56	165.11	38.94	142.94	144.96	14.65	41.51	90.29	36.58	4.24	102.56	10.73	1600.99
Ljubljana 2	127.20	150.25	109.34	136.45	133.64	44.09	102.78	66.07	22.38	27.26	96.88	16.38	3.56	84.87	9.75	1130.90
Ljubljana 3	148.59	145.93	111.58	128.82	117.05	48.38	103.49	98.15	12.76	12.00	47.43	8.53	2.13	71.52	6.75	1063.09
Ljubljana 4	141.32	133.31	89.06	128.86	83.71	38.36	93.48	34.53	8.62	11.42	16.08	11.63	2.98	33.08	9.36	835.79
Ljubljana 5	164.00	177.82	98.65	180.83	99.32	60.68	97.08	70.38	7.10	29.52	53.92	17.61	3.77	47.71	11.93	1120.34
Ljubljana 6	180.49	187.36	106.47	157.46	120.84	61.41	96.84	73.04	8.15	17.85	39.50	26.32	2.45	41.04	7.29	1126.50
Kranj 1	122.62	136.48	101.82	117.05	121.28	41.24	109.19	95.53	9.54	16.40	65.07	20.48	2.51	41.07	4.96	1005.25
Kranj 2	119.20	122.71	130.71	110.33	139.53	23.31	117.02	84.05	11.54	19.83	42.50	12.02	4.35	26.38	6.11	969.58
Kranj 3	128.98	124.91	114.43	118.06	125.47	47.46	114.73	61.18	14.02	14.27	75.85	19.84	6.19	45.49	7.49	1018.36
Kranj 4	189.93	179.84	172.96	176.32	183.27	65.67	164.56	121.79	12.45	32.25	93.78	33.68	6.04	58.69	12.54	1503.78
Kranj 5	138.11	121.85	114.72	129.74	114.87	45.52	112.04	78.64	9.65	23.76	30.69	15.48	2.75	24.64	5.64	968.10
Kranj 6	148.56	157.13	90.16	138.11	99.50	45.83	91.54	102.97	6.91	26.03	33.00	30.75	5.41	69.75	10.47	1056.10
Škofja Loka 1	210.43	219.13	162.15	202.89	185.26	74.15	157.39	166.01	26.28	31.03	58.03	23.35	6.09	98.49	12.84	1633.55
Škofja Loka 2	227.28	227.67	135.25	222.09	143.26	74.93	133.00	164.39	21.88	27.83	88.55	41.74	7.67	97.91	19.66	1633.13
Škofja Loka 3	240.99	233.46	180.80	208.79	180.21	36.94	141.41	149.07	13.88	36.69	79.17	28.81	3.58	66.39	11.46	1611.63
Škofja Loka 4	223.64	214.99	122.26	212.94	121.97	66.28	113.56	145.81	12.72	44.59	57.17	54.87	8.64	147.46	37.99	1584.91
Škofja Loka 5	189.36	188.63	126.06	190.07	121.76	51.24	114.22	112.92	11.08	33.94	52.39	37.74	6.14	108.41	25.23	1369.20

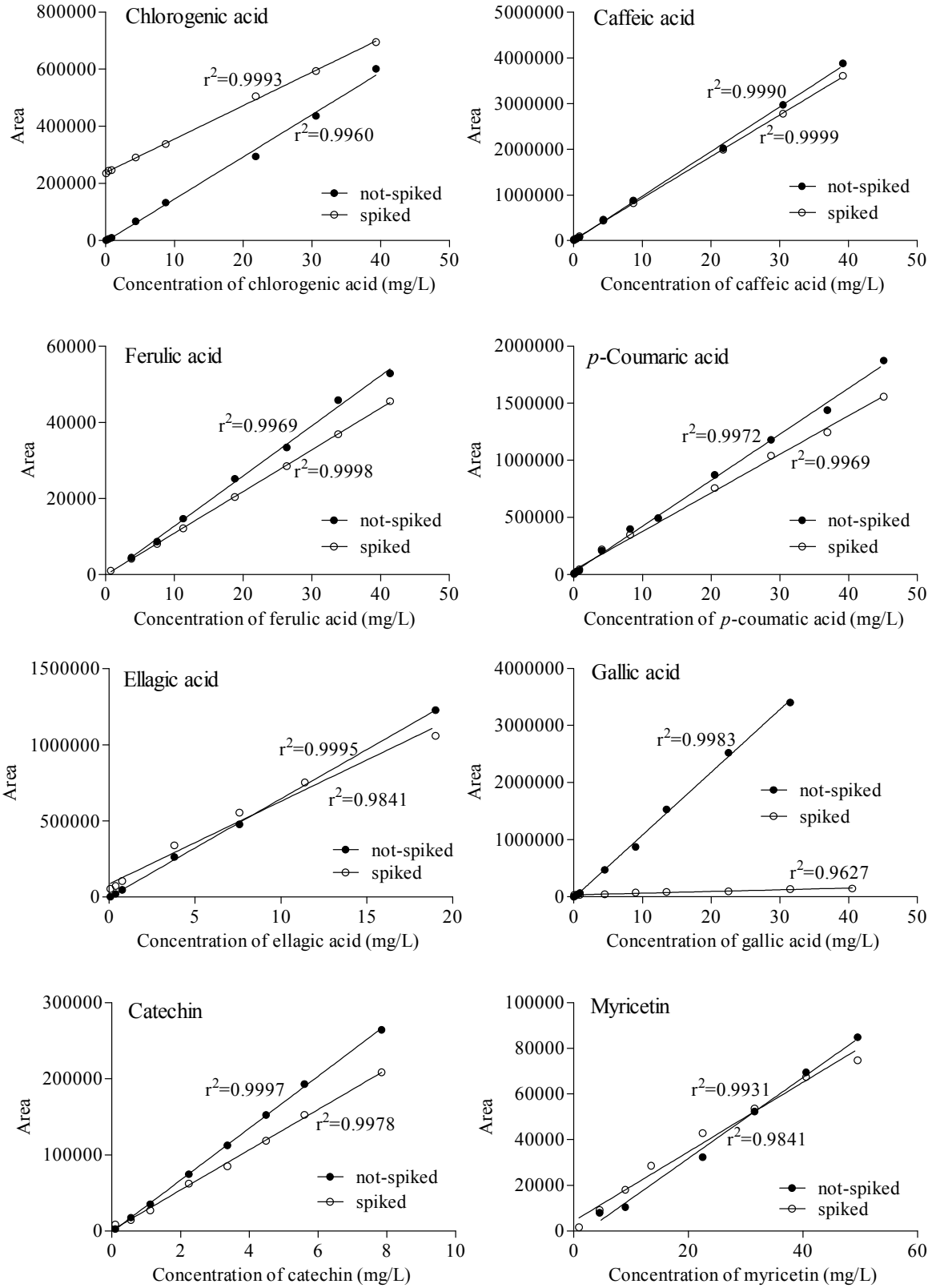
to be continued...

Continuation of Annex H: Data of the content of individual anthocyanins (mg/100 g fw) in Slovenian bilberries and blueberries.

Samlpe	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	TOTAL
Škofja Loka 6	223.83	238.38	177.02	205.95	175.03	65.28	140.34	149.67	18.13	37.96	80.31	48.13	7.79	130.82	24.96	1723.60
Pohorje 1	128.34	149.21	115.34	128.40	139.67	36.37	116.08	95.12	13.56	28.28	59.06	24.75	6.91	85.82	17.71	1144.59
Pohorje 2	124.84	126.81	103.16	114.52	96.68	34.01	77.96	70.27	10.48	24.41	40.21	26.51	5.97	78.39	19.36	953.59
Pohorje 3	206.42	213.28	173.40	191.53	166.19	53.32	139.12	124.99	13.58	34.57	62.84	33.78	5.04	117.44	21.97	1557.46
Pohorje 4	181.50	168.45	107.02	160.58	95.54	40.88	67.36	78.71	6.05	20.57	23.50	21.27	2.26	66.50	12.03	1052.22
Pohorje 5	158.32	154.27	126.37	133.31	125.22	40.81	85.35	83.56	13.43	22.91	53.13	32.28	5.01	101.01	18.72	1153.71
Pohorje 6	164.93	186.80	110.47	129.33	110.87	36.91	76.51	100.28	9.57	24.08	44.49	24.96	3.60	90.52	14.77	1128.11
AVERAGE	*167.12*	*169.10*	*122.56*	*152.27*	*130.38*	*50.00*	*110.64*	*101.86*	*13.32*	*23.89*	*56.67*	*27.51*	*4.50*	*67.65*	*12,78*	*1210.25*
SEM	*27.85*	*28.18*	*20.43*	*25.38*	*21.73*	*8.33*	*18.44*	*16.98*	*2.22*	*3.98*	*9.45*	*4.58*	*0.75*	*11.28*	*2,13*	*111.53*
Blueberry 1	24.47	18.65	4.28	27.03	3.34	12.29	4.08	14.37	1.80	10.90	2.48	46.43	1.07	44.59	46.43	262.22
Blueberry 2	27.47	18.78	4.89	28.70	2.96	13.80	4.24	14.10	1.80	10.03	2.01	33.13	0.89	29.15	31.33	223.27
Blueberry 3	20.61	12.38	4.26	21.82	2.35	10.60	3.04	11.11	1.71	9.14	2.00	28.66	0.87	25.31	27.65	181.51
Blueberry 4	19.94	12.96	3.48	19.45	1.57	9.45	2.43	10.56	1.64	7.76	2.00	34.75	0.83	29.62	40.08	196.54
Blueberry 5	24.27	14.06	4.23	25.82	2.78	12.09	3.56	11.74	1.85	8.61	2.16	31.39	1.10	27.14	27.77	198.57
AVERAGE	*23.35*	*15.37*	*4.23*	*24.56*	*2.60*	*11.65*	*3.47*	*12.38*	*1.76*	*9.29*	*2.13*	*34.87*	*0.95*	*31.16*	*34.65*	*212.42*
SEM	*1.38*	*1.39*	*0.22*	*1.71*	*0.30*	*0.75*	*0.33*	*0.78*	*0.04*	*0.55*	*0.09*	*3.06*	*0.06*	*3.44*	*3.71*	*14.14*

The units of all data are mg of cyanidin 3-glucoside /100 g fwfresh weight.

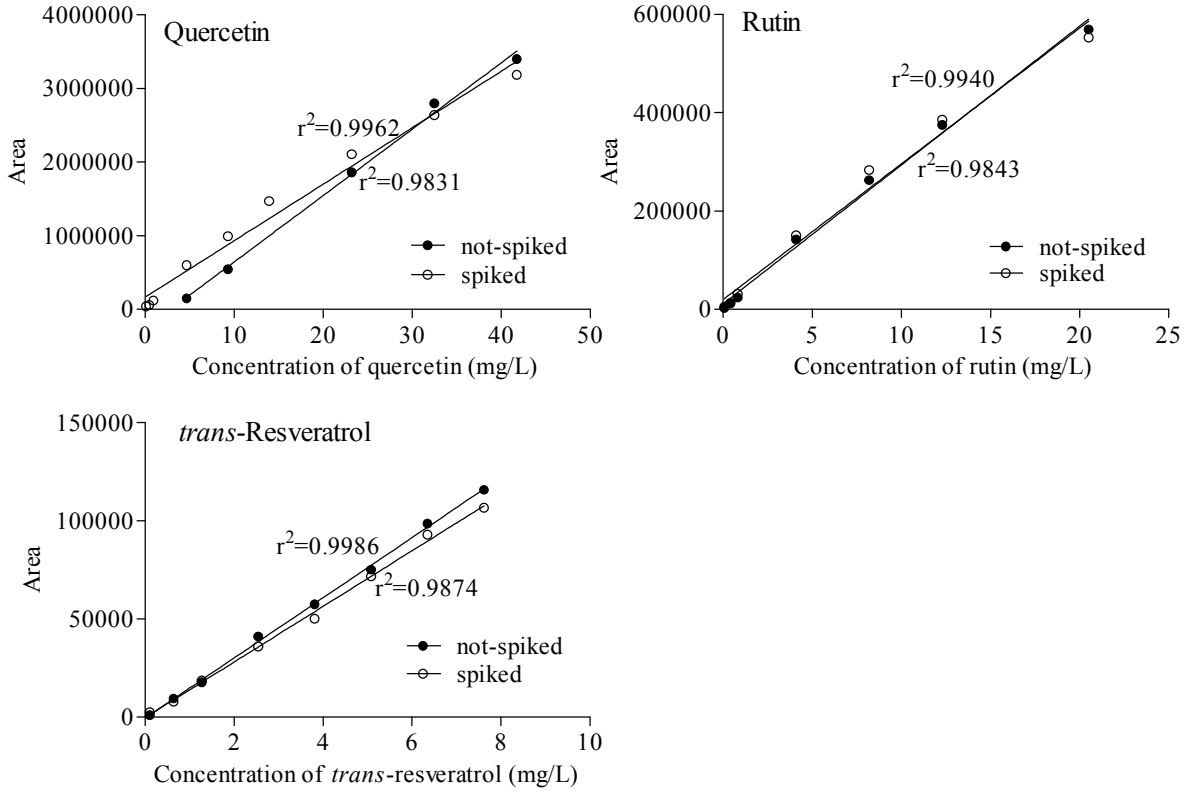
The number meaning: 1, delphinidin 3-galactoside; 2, delphinidin 3-glucoside; 3, cyanidin 3-galactoside; 4, delphinidin 3-arabinoside; 5, cyanidin 3-glucoside; 6, petunidin 3-galactoside; 7, cyanidin 3-arabinoside; 8, petunidin 3-glucoside; 9, peonidin 3-galactoside; 10, petunidin 3-arabinoside; 11, peonidin 3-glucoside; 12, malvidin 3-galactoside; 13, peonidin 3-arabinoside; 14, malvidin 3-glucoside; 14, malvidin 3-arabinoside.



Annex I: Calibration curves (not-spiked and spiked) of chlorogenic, caffeic, ferulic, *p*-coumaric, ellagic and gallic acid, catechin, myricetin, quercetin, rutin and *trans*-resveratrol.

Priloga I: Umeritvene krivulje (brez in s standardnim dodatkom) klorogenske, kavne, ferulne, *p*-kumarne, elagične in galne kisline, katehina, miricetina, kvercetina, rutina in *trans*-resveratrola.

to be continued...

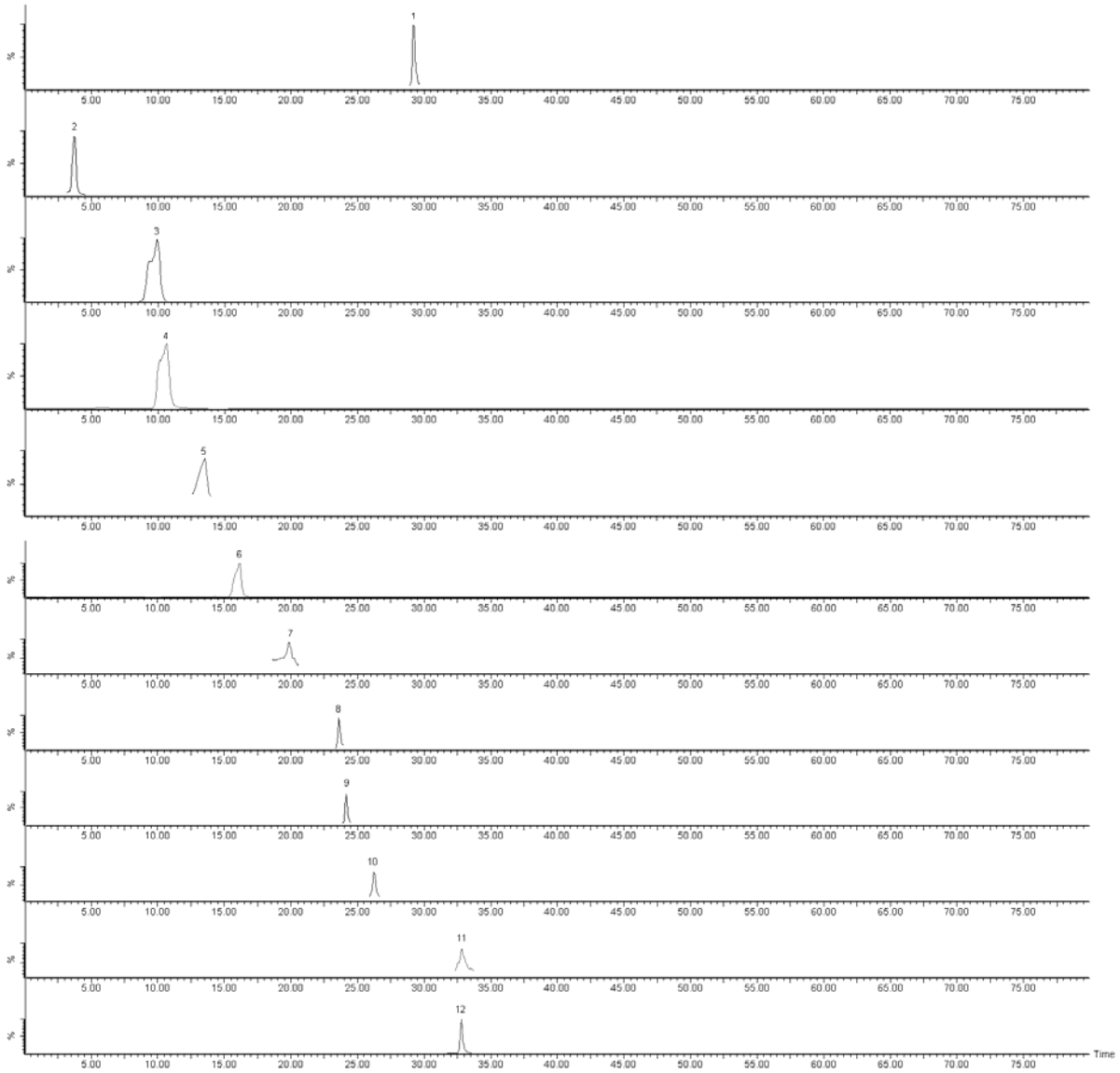


Continuation of Annex I: Calibration curves (not-spiked and spiked) of chlorogenic, caffeic, ferulic, *p*-coumaric, ellagic and gallic acid, catechin, myricetin, quercetin, rutin and *trans*-resveratrol.

Annex J: LC-MS characteristics for quantification of standards.
Priloga J: LC-MS karakteristike kvantifikacije standardov.

Standard	LOQ (10^{-6} g/kg)	LOD (10^{-6} g/kg)	CV (%)	Recovery (%)
Chlorogenic acid	9.6	2.9	1.2	79.74
Caffeic acid	6.0	1.8	1.7	93.35
Ferulic acid	22.5	6.8	2.3	83.38
p-Coumaric acid	9.6	2.9	2.6	82.55
Ellagic acid	5.6	1.7	1.3	88.51
Gallic acid	6.0	0.2	1.8	2.71
Catechin	12.8	3.9	1.1	78.04
Myricetin	13.5	4.1	2.8	90.95
Quercetin	1.1	0.3	0.8	93.69
Rutin	0.4	0.1	1.5	95.15
trans-Resveratrol	3.2	0.1	2.5	92.87

LOD and LOQ were determined from signal-to-noise ratio by our experiments
LOQ, limit of quantification with signal-to-noise ratio ≥10
LOD, limit of detection with signal-to-noise ratio ≥3
CV, repeatability of standards



Annex K: LC-MS chromatogram of phenolic acids, flavanols, flavonols and resveratrol from the bilberries sampled in Smrečje.
Peak identification: 1, trans-resveratrol; 2, gallic acid; 3, catechin; 4, chlorogenic acid; 5, caffeic acid; 6, epicatechin; 7, p-coumaric acid; 8, rutin; 9, ellagic acid; 10, ferulic acid; 11, myricetin; 12, quercetin.

Priloga K: LC-MS kromatogram fenolnih kislin, flavanolov, flavonolov in resveratrola vzorca gozdnih borovnic iz Smrečja.
Identifikacija pikov: 1, *trans*-resveratrol; 2, galna kislina; 3, katehin; 4, klorogenska kislina; 5, kavna kislina; 6, epikatehin; 7, *p*-kumarna kislina; 8, rutin; 9, elagična kislina; 10, ferulna kislina; 11, miricetin; 12, kvercetin.

Annex L: Content of phenolic acids, flavanols, flavonols and resveratrol (mg/100 g fw) in Slovenian bilberries and blueberries.
Priloga L: Vsebnost fenolnih kislin, flavanolov, flavonolov in resveratrola (mg/100 g fw) v gozdnih in ameriških borovnicah iz Slovenije.

Sample	1	2	3	4	5	6	7	8	9	10	11	12
Pokljuka 1	0.48	0.06	1.81	0.67	0.22	22.97	0.39	0.65	0.07	11.19	0.91	0.28
Pokljuka 2	0.48	4.16	0.84	0.27	0.14	33.78	0.62	0.85	0.23	6.88	0.75	0.50
Pokljuka 3	0.08	4.01	0.78	0.34	0.11	31.45	0.28	0.85	0.25	7.34	0.84	0.18
Celje 1	0.05	1.25	0.91	0.44	1.38	28.18	0.18	0.48	0.24	4.78	1.28	0.22
Celje 2	0.05	1.63	0.62	0.39	0.09	20.56	0.25	0.45	0.36	5.44	0.86	0.18
Celje 3	0.03	1.62	0.58	0.34	0.10	16.91	0.24	0.48	0.23	5.41	0.75	0.11
Celje 4	0.03	0.80	0.65	0.30	0.08	18.43	0.22	0.38	0.30	5.03	0.75	0.21
Goričko 1	0.04	0.97	0.62	0.35	0.23	19.61	0.19	0.44	0.23	6.56	0.88	0.14
Goričko 2	0.05	1.23	0.78	0.48	0.07	20.98	0.25	0.48	0.30	3.68	1.13	0.11
Goričko 3	0.29	2.60	1.09	0.85	0.07	26.71	0.47	0.00	0.67	7.09	1.69	0.17
Goričko 4	0.11	0.63	0.68	0.42	0.16	23.40	0.21	0.45	0.20	7.26	1.02	0.21
Goričko 5	0.26	0.76	0.75	0.33	0.07	23.41	0.13	0.37	0.23	5.30	1.12	0.16
Ljubljana 1	0.05	1.03	0.81	0.37	0.11	14.17	0.17	0.35	0.25	6.84	1.13	0.21
Ljubljana 2	0.08	4.29	0.81	0.37	0.06	22.11	0.12	0.34	0.15	7.15	1.19	0.16
Ljubljana 3	0.08	1.96	0.58	0.39	0.11	18.20	0.11	0.00	0.21	5.91	1.07	0.17
Ljubljana 4	0.08	1.61	0.71	0.56	0.07	20.94	0.26	0.00	0.47	7.12	1.35	0.17
Ljubljana 5	0.15	1.99	0.86	0.00	0.09	40.03	0.40	1.20	0.49	10.73	1.46	0.20
Ljubljana 6	0.05	1.38	0.64	0.35	0.15	17.72	0.14	0.00	0.20	7.33	1.07	0.11
Kranj 1	0.15	1.76	0.71	0.35	0.11	25.71	0.14	0.41	0.17	5.47	1.30	0.14
Kranj 2	0.09	1.23	0.78	0.49	0.09	16.30	0.15	0.00	0.14	4.30	1.49	0.15
Kranj 3	0.18	2.32	0.85	0.49	0.12	26.07	0.20	0.00	0.27	5.71	1.68	0.12
Kranj 4	0.22	3.29	0.84	0.39	0.15	29.08	0.14	0.42	0.20	5.99	1.56	0.19
Kranj 5	0.14	1.90	0.76	0.49	0.06	23.22	0.21	0.00	0.23	5.44	1.43	0.18
Kranj 6	0.08	2.09	0.50	0.35	0.10	20.13	0.18	0.36	0.17	5.46	1.00	0.13
Škofja Loka 1	0.14	1.02	0.84	0.43	0.10	18.91	0.15	0.00	0.30	6.84	1.69	0.16

to be continued...

Continuation of Annex L: Content of phenolic acids, flavanols, flavonols and resveratrol (mg/100 g fw) in Slovenian bilberries and blueberries.

Sample	1	2	3	4	5	6	7	8	9	10	11	12
Škofja Loka 2	0.20	1.49	0.60	0.30	0.12	23.70	0.10	0.38	0.20	6.37	1.13	0.14
Škofja Loka 3	0.17	1.71	0.69	0.38	0.14	28.02	0.16	0.42	0.18	6.02	1.26	0.29
Škofja Loka 4	0.12	2.34	0.50	0.29	0.13	20.59	0.13	0.45	0.17	6.46	0.92	0.16
Škofja Loka 5	0.15	1.76	0.61	0.30	0.10	24.91	0.14	0.45	0.18	5.74	1.00	0.17
Škofja Loka 6	0.06	1.58	0.70	0.34	0.12	31.73	0.14	0.50	0.15	5.80	1.50	0.13
Pohorje 1	0.10	2.00	0.69	0.44	0.10	10.90	0.27	0.00	0.25	4.41	1.13	0.18
Pohorje 2	0.09	1.64	0.57	0.40	0.08	16.62	0.33	0.59	0.25	4.29	1.01	0.20
Pohorje 3	0.29	5.04	0.76	0.00	0.09	22.53	0.70	0.56	0.30	5.61	1.26	0.42
Pohorje 4	0.70	5.11	0.85	0.41	0.09	32.16	0.74	0.75	0.24	7.33	0.93	0.50
Pohorje 5	0.11	2.49	0.73	0.40	0.13	17.50	0.32	0.45	0.22	5.05	1.18	0.27
Pohorje 6	0.08	2.01	0.62	0.30	0.13	21.13	0.33	0.34	0.23	5.67	1.01	0.22
AVERAGE	0.15	2.02	0.75	0.42	0.15	23.05	0.25	0.43	0.25	1.16	6.19	0.20
SEM	0.02	0.20	0.04	0.02	0.04	1.01	0.03	0.04	0.02	0.04	0.25	0.02
Blueberry 1	1.61	0.38	0.09	0.00	2.66	60.61	0.18	1.74	0.05	1.97	0.07	0.38
Blueberry 2	1.96	0.54	0.08	0.00	3.34	75.14	0.19	2.34	0.05	2.25	0.08	0.46
Blueberry 3	1.44	0.36	0.08	0.00	3.18	68.51	0.15	1.99	0.04	1.40	0.07	0.36
Blueberry 4	2.17	0.60	0.08	0.00	3.21	79.94	0.24	2.43	0.04	1.57	0.06	0.46
Blueberry 5	1.69	0.38	0.09	0.00	2.96	65.73	0.16	2.65	0.05	1.92	0.07	0.40
AVERAGE	1.77	0.45	0.08	0.00	3.07	69.99	0.19	2.23	0.05	1.82	0.07	0.41
SEM	0.13	0.05	0.00	0.00	0.12	3.42	0.02	0.16	0.00	0.15	0.00	0.02

The units of all data are mg/100 g fwand quantified on corresponding standards.

The number meaning: 1, catechin; 2, epicatechin; 3, quercetin; 4, myricetin; 5, rutin; 6, chlorogenic acid; 7, caffeic acid, 8, ferulic acid; 9, *p*-coumaric acid; 10, gallic acid; 11, ellagic acid; 12, *trans*-resveratrol.

Annex M: Quantitative analysis of individual phenolics of Slovenian bilberries.
Priloga M: Kvantitativna analiza posameznih fenolnih spojin gozdnih borovnic iz Slovenije.

Parameter	Region						
	Pokljuka (n=3)	Celje (n=4)	Goricko (n=5)	Ljubljana (n=6)	Kranj (n=6)	Skofja Loka (n=6)	Pohorje (n=6)
delphinidin 3-galactoside	158.5 ±26.7	134.1 ±17.6	177.7 ±47.9	164.8 ±35.6	141.2 ±26.1	219.3 ±17.6	160.7 ±31.2
delphinidin 3-glucoside	161.2 ±21.1	145.8 ±29.1	166.2 ±57.0	171.0 ±35.9	140.5 ±23.4	220.4 ±17.8	166.5 ±30.4
cyanidin 3-galactoside	93.7 ±6.6	94.9 ±34.5	144.9 ±52.3	110.4 ±20.0	120.8 ±29.0	150.6 ±26.0	122.6 ±26.1
delphinidin 3-arabinoside	156.4 ±12.9	121.1 ±18.5	140.6 ±42.3	155.8 ±30.5	131.6 ±24.1	207.1 ±10.7	143.0 ±28.2
cyanidin 3-glucoside	107.0 ±2.6	119.6 ±53.2	145.8 ±63.2	120.0 ±28.2	130.7 ±28.9	154.6 ±29.3	122.4 ±27.4
petunidin 3-galactoside	39.9 ±2.6	44.4 ± 1.9	66.2 ±23.8	48.6 ±10.3	44.8 ±13.6	61.5 ±14.7	40.4 ±6.9
cyanidin 3-arabinoside	97.2 ±1.1	93.6 ±23.1	121.8 ±51.6	106.1 ±18.5	118.2 ±24.5	133.3 ±17.0	93.7 ±27.8
petunidin 3-glucoside	78.2 ±22.2	91.3 ±29.7	119.0 ±52.3	81.2 ±37.3	90.7 ±21.0	148.0 ±19.1	92.2 ±19.4
peonidin 3-galactoside	4.6 ±1.7	15.3 ±14.0	19.2 ±4.6	12.3 ±5.8	10.7 ±2.5	17.3 ±5.9	11.1 ±3.0
petunidin 3-arabinoside	16.9 ±5.9	17.4 ±5.0	20.2 ±4.1	23.3 ±11.7	22.1 ±6.6	35.3 ±5.9	25.8 ±5.0
peonidin 3-glucoside	23.2 ±3.1	60.6 ±62.5	68.9 ±33.5	57.4 ±30.9	56.8 ±25.5	69.3 ±15.2	47.2 ±14.4
malvidin 3-galactoside	10.8 ±1.8	17.1 ±5.3	48.4 ±42.2	19.5 ±10.3	22.0 ±8.5	39.1 ±11.8	27.3 ±4.8
peonidin 3-arabinoside	1.9 ±1.0	3.7 ±2.7	5.3 ±4.1	3.2 ±0.8	4.5±1.6	6.7 ±1.8	4.8 ±1.7
malvidin 3-glucoside	26.7 ±4.4	53.8 ±29.5	60.9 ±46.3	63.5 ±27.3	44.3 ±17.7	108.3 ±28.3	90.0 ±17.8
malvidin 3-arabinoside	5.6 ±1.3	9.8 ±3.6	12.8 ±10.9	9.3 ±2.0	7.9±3.0	22.2 ±9.8	17.4 ±3.5
catechin	0.3 ±0.2	0.0 ±0.0	0.2 ±0.1	0.1 ±0.0	0.1 ±0.1	0.1 ±0.1	0.2 ±0.2
epicatechin	2.7 ±2.3	1.3 ±0.4	1.2 ±0.8	2.0 ±1.2	2.1 ±0.7	1.7 ±0.4	3.1 ±1.6
quercetin	1.2 ±0.6	0.7 ±0.2	0.8 ±0.2	0.73±0.1	0.7 ±0.1	0.7 ±0.1	0.7 ±0.1
myricetin	0.4 ±0.2	0.4 ±0.1	0.5 ±0.2	0.5 ±0.2	0.4 ±0.1	0.3 ±0.1	0.4 ±0.1
rutin	0.2 ±0.1	0.4 ±0.7	0.1 ±0.1	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0	0.1 ±0.0
chlorogenic acid	29.7 ±5.2	21.0 ±5.0	22.8 ±2.7	22.2 ±9.2	23.4 ±4.6	24.6 ±4.7	20.1 ±7.2
caffeic acid	0.4 ±0.2	0.2 ±0.0	0.3 ±0.1	0.2 ±0.1	0.2 ±0.0	0.1 ±0.0	0.5 ±0.2
ferulic acid	0.8 ±0.1	0.5 ±0.1	0.4 ±0.2	0.5 ±0.4	0.2 ±0.2	0.4 ±0.2	0.5 ±0.3
p-coumaric acid	0.2 ±0.1	0.3 ±0.3	0.3 ±0.2	0.3 ±0.2	0.2 ±0.1	0.2 ±0.1	0.3 ±0.0
ellagic acid	0.8 ±0.1	0.9 ±0.3	1.2 ±0.3	1.2 ±0.2	1.4 ±0.2	1.3 ±0.3	1.1 ±0.1
gallic acid	8.5 ±2.4	5.2 ±0.3	6.0 ±1.5	7.5 ±1.7	5.4 ±0.6	6.2 ±0.4	5.4 ±1.1
trans-resveratrol	0.3 ±0.2	0.2 ±0.1	0.2 ±0.0	0.2 ±0.0	0.2 ±0.0	0.2 ±0.1	0.3 ±0.1
Total phenolics determined	1027±62^{b}	1054±224^{b}	1352±409^{ab}	1182±247^{b}	1121±211^{b}	1629±120^{a}	1198±209^{b}

[1]Compounds quantified as mg equivalents/100 g fwberries fresh weight (fw), according to the corresponding standard; data are means ±SEM; n - number of replications in a region; ^{a,b}different letters in a superscript differ significantly at $p < 0.05$.

Annex N: AUC of CAA units (%) of the all berry extracts and cyanidin 3-glucoside for all cell lines.
Priloga N: AUC CAA (%) za vse ekstrakte in cianidin-3-glukozid in za vse celične linije.

Cell line	Crude bilberry extract (AUC)	Purified bilberry extract (AUC)	Crude blueberry extract (AUC)	Cyanidin 3-glucoside (AUC)
Caco-2	2030 ± 109 aA	2159 ± 174 aA	1826 ± 225 aA	1845 ± 93 aAB
HepG2	2059 ± 152 aA	1677 ± 47 bB	1585 ± 62 bAB	1624 ± 82 bA
EA.hy926	1812 ± 134 abA	1582 ± 95 aB	1146 ± 79 cBC	2099 ± 106 bB
A7r5	1063 ± 30 aB	851 ± 62 bC	973 ± 46 abC	788 ± 60 bC

Annex O: Published scientific papers (Može et al., 2011; Žiberna et al., 2010).

Priloga O: Objavljena znanstvena članka (Može in sod., 2011; Žiberna in sod., 2010).