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Partial characterization of a new kind of Chilean Murtilla-like berries

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ABSTRACT

The aim of this research was to characterize a new kind of Chilean Murtilla-like berries (*Myrteola nummularia* (Poiret) Berg. Myrtaceae, called by locals as Daudapo) vs. well known Murtilla, blueberries, raspberries and black chokeberries. Polyphenols, flavonoids, flavanols and tannins and the level of antioxidant activity by ABTS, FRAP and CUPRAC radical scavenging assays of methanol extract of studied berry samples were determined and compared. It was found that the contents of the polyphenol compounds and the level of antioxidant activity in extracts of berries differ significantly ($P < 0.05$). The significantly highest contents of polyphenol compounds were in methanol extract of non-ripe Murtilla (121.31 ± 5.9 mg GAE/g for polyphenols; 14.43 ± 0.7 , 31.79 ± 1.5 , and 9.93 ± 0.3 mg CE/g for flavonoids, tannins and flavanols, respectively). Also the antioxidant activity according to ABTS, FRAP and CUPRAC was significantly highest in methanol extract for non-ripe Murtilla (878.18 ± 41.2 , 486.92 ± 23.3 and 1012.42 ± 43.2 $\mu\text{M TE/g}$, respectively). The amount of polyphenol compounds and their antioxidant activities of Murtilla berries are significantly higher than in other studied berries and are comparable with blueberries and raspberries, however, these indices in the Murtilla-like non-ripe berries were the following: 31.55 ± 1.4 mg GAE/g for polyphenols; 5.22 ± 0.3 , 12.16 ± 0.6 and 2.24 ± 0.1 mg CE/g for flavonoids, tannins and flavanols; ABTS, FRAP and CUPRAC: 244.22 ± 12.1 , 81.32 ± 3.9 and 203.83 ± 9.3 $\mu\text{M TE/g}$, respectively. The correlation between the polyphenol compounds and the antioxidant activities were relatively high. DPPH kinetic measurements were used to compare, distinguish and discriminate the antiradical activity among berry methanolic extracts by multivariate analysis. 3-D fluorescence was used as an additional tool for the characterization of the polyphenol extracts during various stages of ripening and different berries cultivars. The interaction between methanol polyphenol extracts of Murtilla-like and bovine serum albumin (BSA) showed that the new kind of berries has a strong ability, as other studied berries, to quench the intrinsic fluorescence of BSA by forming complexes.

In conclusion, for the first time these berries were analyzed and compared with widely consumed cultivars, using their polyphenols' composition, antioxidant activities and fluorescence properties. The ability of Murtilla-like berries to quench the intrinsic fluorescence of BSA and relatively high content of polyphenol compounds can be used as a new source of antioxidants.

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1. Introduction

Health-beneficial effects of fruits such as grapes have been linked to the presence of various polyphenols, including anthocyanins in these products (Jacob, Hakimuddin, Paliyath, & Fisher, 2008). Tannins in persimmon showed high antioxidant ability as well (Gu et al., 2008). Nowadays berries are intensively studied (Schreckinger, Lotton, Lila, & Gonzalez de Mejia, 2010) and it was shown that among them the strawberries possess very high bioactivity (Pineli et al., 2010; Proteggente

et al., 2002; Simirgiotis, Theoduloz, Caligari, & Schmeda-Hirschmann, 2009; Sun, Chu, Wu, & Liu, 2002). The consumption of berries increased during the last years and berries of different kinds are widely consumed in many countries (Delporte et al., 2007; Heinonen, 2007; Szajdek & Borowska, 2008), based on high contents of their bioactive compounds. And indeed, it was shown that berries contain polyphenols, including anthocyanins, phenolic acids, and tannins, as well as nutritive compounds such as carotenoids and vitamin C (Kähkönen, Hopia, & Heinonen, 2001; Pineli et al., 2010). The extractable and unextractable proanthocyanidins were determined in plant material using normal phase HPLC (Borges, Degeneve, Mullen, & Crozier, 2010; Hellström & Mattila, 2008). The amount of these substances depends on extraction procedure (Khanal, Howard, & Prior, 2009). The comparison of the extracted phenolics and

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their antioxidant activities in different solvents was studied previously (Pérez-Jiménez & Saura-Calixto, 2006; Pérez-Jiménez et al., 2008). It is shown that the research on berries has been carried out intensively. The composition of phenolic acids in several small berries grown in Northeastern Poland, namely, low-bush blueberries, black mulberries, European junberries, black currants, fruits of blue-berried honeysuckle, and blackberries, was determined (Zadernowski, Naczek, & Nesterowicz, 2005). Blackberry (*Rubus* spp.), black raspberry (*Rubus occidentalis*), blueberry (*Vaccinium corymbosum*), cranberry (i.e., the American cranberry, *Vaccinium macrocarpon*, distinct from the European cranberry, *V. oxycoccus*), red raspberry (*Rubus idaeus*) and strawberry (*Fragaria × ananassa*) are a part of diets in USA (Seeram, 2008). Seeram (2008) claims that the dietary intake of berry fruits has a positive impact on some diseases: heart health and cardiovascular disease, neurodegenerative and other diseases of aging, obesity, and also certain human cancers, such as esophageal and gastrointestinal cancers. In addition, the effects of berry consumption on symptoms of the metabolic syndrome and on human performance enhancement were also reported (Seeram, 2008). Dietary intake of berry fruits has been demonstrated to positively impact human health.

Experiments on animals and clinical investigations confirmed that consumption of berries is effective in prevention and treatment of some disease (Delporte et al., 2007; Erlund et al., 2008; McDougall, Ross, Ikeji, & Stewart, 2008; Suwalsky, Orellana, Avello, & Villena, 2007). McDougall et al. (2008) found that Rowan berry, raspberry, lingonberry, cloudberry, artichoke and strawberry extracts have showed antiproliferative effectiveness on human cervical cancer (HeLa) cells grown in microtiter plates. Erlund et al. (2008) investigated the effects of berry consumption on hemostatic function, serum lipids, and blood pressure on middle-aged subjects with cardiovascular risk factors. It was found that the berry consumption inhibited platelet function, increased significantly serum HDL-cholesterol concentrations and decreased systolic blood pressure.

Other berries are less studied (Howell, 2007; Kresty, Howell, & Baird, 2008; Rao & Snyder, 2010), therefore interest in exploring new and exotic types of berries has grown in recent years (Schreckinger et al., 2010). Composition and traditional folk medicine of berries suggest significant health benefits, but few studies to date have investigated these potentials, such as botanical descriptions, chemistry, biological activities, and commercialization of berry-producing plants from South America (Schreckinger et al., 2010). The most significant health benefits have been attributed to phenolic compounds and vitamin C, potentially protective against cardiovascular disease and cancer (Schreckinger et al., 2010). The following species from South America (*Aristotelia chilensis*, *Euterpe oleracea*, *Malpighia emarginata*, *Ugni molinae*, *Fragaria chiloensis*, *Rubus glaucus*, *Rubus adenotrichus*, and *Vaccinium floribundum*) possess a rich and diversified composition of bioactive compounds with health-promoting properties (Schreckinger et al., 2010). Among intensively studied are some Chilean berries (Speisky et al., 2008), including Murtilla (*U. molinae* Turcz.) (Ruiz et al., 2010; Shene et al., 2009; Suwalsky et al., 2007). The antiradical capacity of Murta was determined by DPPH• assay in recent report and compared with other exotic fruits (Rufino, Alves, Fernandes, & Brito, 2010).

We collected a new kind of wild growing berries which were not investigated till now, but the natives are using these berries as medicine and food. The appearance of this fruit was similar to Murtilla, therefore we called it Murtilla-like. It was of great interest to analyze for the first time Murtilla-like in order to find a place among already wide consumed samples of different berries. We decided to assess its composition vs. wide consumed Murtilla, blueberries, raspberries and black chokeberries. To meet this aim the contents of their polyphenol compounds (polyphenols, flavonoids, flavanols, and tannins) and the level of antioxidant activity (AA) were determined and compared in different stages of ripening for two kinds of Chilean berries. In order to receive reliable data of overall antioxidant activities four complementary assays: [2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) with Trolox equivalent antioxidant capacity (TEAC); Ferric-

reducing/antioxidant power (FRAP) and Cupric reducing antioxidant capacity (CUPRAC) were carried out. 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) assay was applied for kinetic measurements, and the obtained results were used for multivariate analysis. Three dimensional fluorimetry (3D-FL) was used in this study as additional analytical tool for berries characterization. It was shown below in many publications that polyphenols play protective effects against cardiovascular disease (Seeram, 2008; Delporte et al., 2007; Erlund et al., 2008; McDougall et al., 2008; Suwalsky et al., 2007). The interaction between drugs and bovine serum albumin (BSA) is important in the metabolism of drugs (Ni, Zhang, & Kokot, 2009). Such interaction between the extracted polyphenols and bovine serum albumin can provide knowledge for the use of berries in every day consumption. Therefore, the functional properties of a new kind of berry will be studied by the interaction of methanol polyphenol extracts with a small protein such as BSA, using 3D-FL (Shi et al., 2010).

As far as we know, no results of such investigations were published. Any information about the characterization of the new berries is not found.

2. Material and methods

2.1. Chemicals

6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid (Trolox), catechin, Tris, tris(hydroxymethyl)aminomethane; bovine serum albumin, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent (FCR), lanthanum (III) chloride heptahydrate, $\text{FeCl}_3 \times 6\text{H}_2\text{O}$, $\text{CuCl}_2 \times 2\text{H}_2\text{O}$, and 2,9-dimethyl-1,10-phenanthroline (neocuproine) were purchased from Sigma Chemical Co., St Louis, MO, USA. 2, 4, 6-Tripyridyl-s-triazine (TPTZ) was purchased from Fluka Chemie, Buchs, Switzerland. All reagents were of analytical grade. Deionised and distilled water was used throughout.

2.2. Samples

Chilean 'Murtilla' (*U. molinae* Turcz) and 'Murtilla-like' berries (Myrtaceae, *Myrteola nummularia* (Poir) Berg.), Chilean and Polish blueberries (*V. corymbosum*), raspberries (*R. idaeus*), and black chokeberry (*Aronia melanocarpa*) are small fruits with a special aroma which are consumed raw, and as jams, juices, canned products, confectioneries and liquors (Scheuermann et al., 2008).

Myrteola nummularia (Poir) Berg. Myrtaceae, (Daudapo) is distributed geographically from Valdivia to Magallanes (X trough XII region) and up to 1,300 m above sea level. It is also found in Juan Fernandez, Argentina, Perú, Bolivia, and Ecuador. Colombia and Venezuela associated to Sphagnum bogs. The fruit is edible, it is a white-red to pink subglobose baya, 5–8 mm of diameter with several internal seeds, shrub always green, up to 1 m height (Hoffmann, 1982; Landrum, 1988). The berries were harvested at their maturity stage and 'Murtilla' and 'Murtilla-like' berries were harvested in two stages of ripening. Daudapo (Murtilla-like) ripe was harvested in May 2008, in Quila Quemada, location: 43° 05' 10.53" S and 73° 35' 59.96" W; Daudapo (Murtilla-like) non-ripe was harvested in February 2010, in Chiloé, location: 43° 06' 17.1" S and 73° 30' 708" W (Fig. 1). Murtilla non-ripe was collected in Puerto Varas, Chile, Saltos Del Petronue, location: 41° 10' 43.4" S and 72° 26' 979" W in February 2010 and Murtilla ripe in May 2010 at the market in Puerto Mont. Arandano (blue berries) and raspberries were purchased at the local market in Chillán, Chile; and blueberries and chokeberries were purchased at the local market in Warsaw, Poland.

For the investigation were using five replicates of five berries each. Their edible parts were prepared manually without using steel knives. The prepared berries were weighed, chopped and homogenized under liquid nitrogen in a high-speed blender (Hamilton Beach Silex professional model) for 1 min. A weighed portion (50–100 g) was



Fig. 1. *Myrteola nummularia* (Poiret) Berg. Myrtaceae, a small creeping evergreen shrub with white flowers and bright red or rose edible fruits, distributed in Chile from Valdivia to Magallanes up to 1300 m. The pink to red berries are sweet with a soft aroma like roses; it is called “Daudapo” by locals. The pen (12 cm long) serves as a comparison for size.

then lyophilized for 48 h (Virtis model 10–324), and the dry weight was determined. The samples were ground to pass through a 0.5 mm sieve and stored at -20°C until the bioactive substances were analyzed.

2.3. Determination of the contents of the main bioactive compounds and fluorimetry

The three-dimensional spectra (3D-FL) of methanol extracts (0.01 mg/ml) of the investigated berries were collected with subsequent scanning emission spectra from 270 to 750 nm at 1.0 nm increments by varying the excitation wavelength from 260 to 500 nm at 10 nm increments. A model FP-6500, Jasco Spectrofluorometer, serial N261332, Japan was used. The scanning speed was set at 1000 nm/min for all measurements. All measurements were performed with emission mode and with intensity up to 1000 (Yin, Li, Ding, & Wang, 2009). All solutions for protein interaction were prepared in 0.05 mol/l Tris-HCl buffer (pH 7.40), containing 0.1 mol/l NaCl. The final concentration of BSA was 1.0×10^{-6} mol/l. All solutions were kept in dark at $0-4^{\circ}\text{C}$. The samples were mixed in the properties of BSA: extract = 1:1 and BSA: extract = 1:5. Phenols were extracted from lyophilized berries with 100% methanol (concentration 25 mg/ml) at room temperature twice for 3 h. The polyphenols were determined by Folin-Ciocalteu method with measurement at 750 nm with spectrophotometer (Hewlett-Packard, model 8452A, Rockville, USA). The results were expressed as mg of gallic acid equivalents (GAE) per g DW (Singleton, Orthofer, & Lamuela-Raventos, 1999). Total flavonoid content was determined by an aluminum chloride colorimetric method (Zhishen, Mengcheng, & Jianming, 1999) with some modifications (Liu et al., 2002). Briefly, 0.25 ml of the berry sample extract was diluted with 1.25 ml of distilled water. Then 75 μl of a 5% NaNO_2 solution was added to the mixture. After 6 min, 150 μl of a 10% $\text{AlCl}_3 \times 6\text{H}_2\text{O}$ solution was added, and the mixture was allowed to stand for another 5 min. Half of a milliliter of 1 M NaOH was added, and the total was made up to 2.5 ml with distilled water. The solution was well mixed, and the absorbance was measured immediately against the prepared blank at 510 nm in comparison with the standards prepared similarly with known (+)-catechin concentrations. The results are expressed as milligrams of catechin equivalents. The total flavanols were estimated using the *p*-dimethylaminocinnamaldehyde (DMACA) method. Methanol extract of berry (0.2 mL), diluted 1:100 with MeOH, was introduced into a 1.5-mL Eppendorf tube, and 1 mL of DMACA solution (0.1% in 1 N HCl in MeOH) was added. The mixture was vortexed and allowed to react at room temperature for 10 min. The absorbance at 640 nm was then read against a blank prepared similarly without DMACA

(Arnous et al., 2001; Vivas et al., 1994). The analysis of condensed tannins (procyanidins) was carried out according to the method of Broadhurst and Jones (1978). To 50 μl of methanol extract of berry sample, 3 ml of a 4% methanol vanillin solution and 1.5 ml of concentrated hydrochloric acid were added. The mixture was allowed to stand for 15 min, and the absorption was measured at 500 nm against methanol as a blank. The amount of total condensed tannins is expressed as (+)-catechin equivalents per g of the sample. As it was mentioned previously, (+)-catechin served as a standard for flavonoids, flavanols, and tannins, and the results were expressed as catechin equivalents (CE).

2.4. Determination of antioxidant activity

The AA was determined by four complementary assays:

- (1) 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt (ABTS^{+}) method for the screening of antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids, and plasma antioxidants. The pre-formed radical monocation ABTS^{+} is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of such hydrogen-donating antioxidants. The influences of both the concentration of antioxidant and duration of reaction on the inhibition of the radical cation absorption are taken into account when determining the antioxidant activity. ABTS^{+} radical cation was generated by the interaction of ABTS (7 mM/l) and $\text{K}_2\text{S}_2\text{O}_8$ (2.45 mM/l). This solution was diluted with methanol until the absorbance in the samples reached 0.7 at 734 nm (Re et al., 1999).
- (2) Ferric-reducing/antioxidant power (FRAP) assay measures the ability of the antioxidants in the investigated samples to reduce ferric-tripiridyltriazine (Fe^{3+} -TPTZ) to a ferrous form (Fe^{2+}). FRAP reagent (2.5 ml of a 10 mM ferric-tripiridyltriazine solution in 40 mM HCl plus 2.5 ml of 20 mM $\text{FeCl}_3 \times \text{H}_2\text{O}$ and 25 ml of 0.3 M acetate buffer, pH 3.6) of 900 μl was mixed with 90 μl of distilled water and 30 μl of berry samples or methanol as the appropriate reagent blank. The absorbance was measured at 595 nm (Benzie & Strain, 1996).
- (3) Cupric reducing antioxidant capacity (CUPRAC): this assay is based on utilizing the copper (II)-neocuproine [Cu (II)-Nc] reagent as the chromogenic oxidizing agent. To the mixture of 1 ml of copper (II)-neocuproine and NH_4Ac buffer solution, acidified and non acidified methanol extracts (or standard) solution (x , in ml) and H_2O [(1.1- x) ml] were added to make the final volume of 4.1 ml. The absorbance at 450 nm was recorded against a reagent blank (Apak, Guclu, Ozyurek, & Karademir, 2004).
- (4) Scavenging free radical potentials were tested in a methanolic solution of 1, 1-Diphenyl-2-picrylhydrazyl method (DPPH). The degree of decoloration of the solution indicates the scavenging efficiency of the added substance. In its radical form, DPPH has an absorption band at 515 nm, which disappeared upon reduction by an antiradical compounds. DPPH solution (3.9 ml, 25 mg/l) in methanol was mixed with the samples extracts (0.1 ml), then the reaction progress was monitored at 515 nm until the absorbance was stable (Brand-Williams, Cuvelier, & Berset, 1995).

Samples with different concentrations of berry methanol extracts (1, 2.5, 5, 10, 15, 20 and 30 mg/ml) were analyzed by DPPH antioxidant activity assay (Ozgen, Reese, Tulio, Scheerens, & Miller, 2006). In the kinetic studies two variables were used: the change in the concentration of the applied samples and the change in reaction time of the extracts with the scavenging radical: 1, 10, 30, 60 and 90 min.

2.5. Statistical analyses

To compare, distinguish and discriminate the antiradical activity among fruit methanolic extracts multivariate analysis, employing

methods of principal component, canonical discriminant analysis and classification were performed by means of Unistat v. 5.6 (Unistat, London, United Kingdom) statistical software, taking into consideration all the experimental data obtained from DPPH kinetic measurements. The methods are designed in a way that enables the enhancement of hidden properties of the original data and allows the reduction of multi-dimensional data set to only a few dimensions, which can sufficiently explain all the original data.

To verify the statistical significance, mean \pm SD of five independent measurements were calculated. Differences between groups were tested

by two ways ANOVA. In the assessment of the antioxidant activity, Spearman correlation coefficients (R) were used. Linear regressions were also calculated. P-values of <0.05 were considered significant.

3. Results and discussion

3.1. Fluorimetry

Three-dimensional fluorescence spectra (Fig. 2) illustrated the elliptical shape of the contours. The x-axis represents the emission

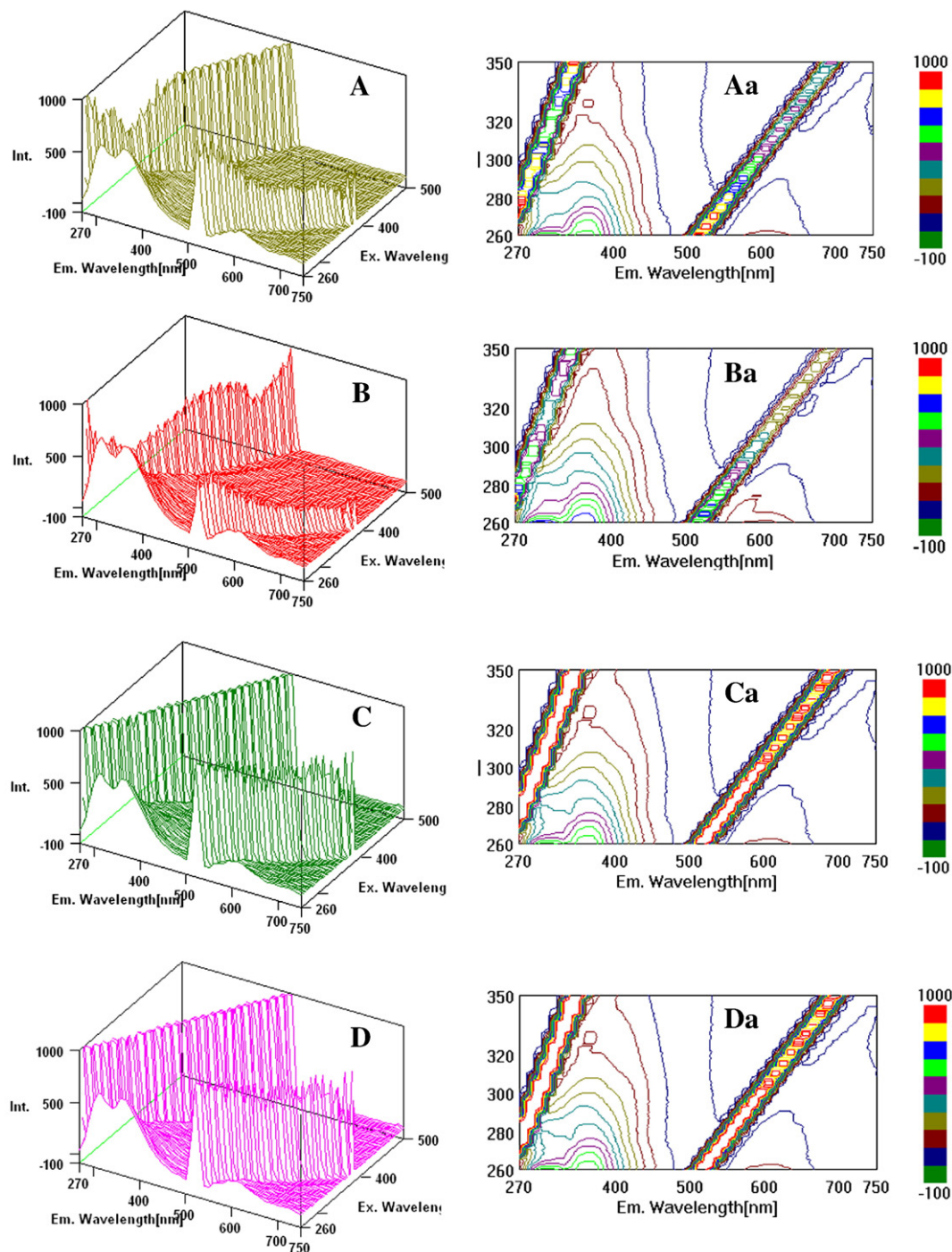


Fig. 2. Three-dimensional fluorescence (3D-FL) of methanol extracts (0.01 mg/ml) of: M-like NRMe, M-like RMe, MNRMe, RaspMe (A, B, C, and D); contour maps of M-like NRMe, M-like RMe, MNRMe, RaspMe (Aa, Ba, Ca, and Da). The 3D-FL were run emission mode and fluorescence intensity up to 1000, emission wavelengths from 270 to 750 nm and excitation wavelengths from 260 to 350 nm; scanning speed was 1000 nm/min, excitation wavelength on x-axis and fluorescence intensity on y-axis; A–D, emission wavelength on x-axis and excitation wavelength on y-axis. Abbreviations: M-likeNRMe, M-likeRMe, MNRMe, and RaspMe, Murtilla-like non-ripe methanol extract; Murtilla-like ripe methanol extract; Murtilla non-ripe methanol extract, and raspberry methanol extract, respectively.

spectra from 270 to 750 nm, while the y-axis is the excitation spectra from 260 to 350 nm, for Murtilla-like non-ripe (A, Aa); Murtilla-like ripe (B, Ba); Murtilla non-ripe (C, Ca), and raspberries (D, Da). One main peak can easily be observed at the approximate location of em/ex 360–370/260 nm nearly in all investigated samples, explaining that the nature of the extracted polyphenols with the same solvent show similar fluorescence properties (Fig. 2). There are some additional peaks for the samples, depending on the berry extract. Murtilla-like non-ripe methanol extracts (0.01 mg/ml) showed the following peaks: a big one at em/ex 360/260 nm with fluorescence intensity (FI) = 622, and a small at em/ex 610/260 nm with FI 149. Sample in the ripe stage showed the following peaks: a big one at em/ex 370/260 nm with fluorescence intensity (FI) = 652, and a small at em/ex 610/260 nm with FI 165. The difference was only in a small shift in case of the ripe sample and bigger fluorescence intensity (Fig. 2, A, Aa, B, and Ba). Murtilla non-ripe sample (Fig. 2, C, Ca) showed exactly the same peaks and at the same wavelength as the Murtilla-like non-ripe (em/ex 360/260, FI = 666; em/ex 610/260 nm with FI 168) with higher intensity than in ripe Murtilla-like berry. Raspberry showed the following peaks: a big one at em/ex 360/260 nm with fluorescence intensity (FI) = 611, and a small at em/ex 610/260 nm with FI 158 (Fig. 2, D, Da). Catechin showed the following peaks: a big one at em/ex 360/260 nm with fluorescence intensity (FI) = 628, and a small at em/ex 610/260 nm with FI 194. Aronia extracts showed the following peaks: at em/ex 360/260 nm with fluorescence intensity (FI) = 663, and a small at em/ex 610/260 nm with FI 190. Chilean blueberries showed the following peaks: a big one at em/ex 370/260 nm with fluorescence intensity (FI) = 605, and a small at em/ex 610/260 nm with FI 131. The Polish samples of blueberries showed the same peaks, but the fluorescence intensity was higher than in all samples in the main peak at em/ex 370/260 nm with fluorescence intensity (FI) = 830, and a small at em/ex 610/260 nm with FI 155.

It was shown that 3D-FL spectrum provides the change in the conformation of proteins (Table 1). Three peaks were observed in BSA: the first two are big and the third is small. All three peaks of BSA were quenched in presence of methanol extracts of studied berries, as well as in the presence of pure catechin as a standard. Different proportions of extracted polyphenols from the studied berries and BSA were used. The most effective was BSA: extracts = 1:5. Peak 2 was stronger than peak 1 and the intensity ration of peak 2 to peak 1 was in BSA 1.27. In catechin this ratio was 1.51, but during interaction with BSA it has diminished to 1.45. In comparison with BSA in the investigated samples this ration changed to 1.19 for raspberry, followed by 1.06 for Murtilla and 1.01 for Murtilla-like (Table 1). These results show that the fluorescence at the wavelength of peak 2 is more significantly quenched than at the wavelength of peak 1. This confirms that the

Table 1
Three-dimensional fluorescence spectral parameters of BSA and BSA interactions with polyphenols.

Fluorescence parameters	BSA	Catechin (C)	BSA + C	BSA + M-LB	BSA + M	BSA + Rasp
λ_1 em	340	320	320	360	360	340
λ_1 ex	285	280	280	290	290	285
$\Delta \lambda$	55	40	40	70	70	55
FI ₁	613	496	510	616	635	608
λ_2 em	420	420	420	420	420	420
λ_2 ex	310	310	310	310	310	310
$\Delta \lambda$	110	110	110	110	110	110
FI ₂	779	751	741	625	678	728
λ_3 em	640	620	630	630	620	620
λ_3 ex	280	280	280	280	280	280
$\Delta \lambda$	355	340	350	350	340	340
FI ₃	90	96	102	44	87	73

Abbreviations: λ_1 em, λ_2 em, and λ_3 em, wavelength (nm) of emission of peaks 1, 2 and 3; λ_1 ex, λ_2 ex and λ_3 ex, wavelength (nm) of excitation of peaks 1, 2 and 3; FI₁, FI₂, and FI₃, fluorescence intensity of peaks 1, 2, and 3; BSA, bovine serum albumin; M-LB, Murtilla-like berries, M, Murtilla; and Rasp, raspberry.

Table 2
Polyphenols compounds in methanol (Me) extract^{1,2,3}.

	POL, mg GAE/g	FLAVON, mg CE/g	FLAV, μ g CE/g	TAN, mg CE/g
M-like NRMe	31.55 ± 1.4 ^b	5.22 ± 0.3 ^c	2239.8 ± 103.1 ^c	12.16 ± 0.6 ^e
M-like RMe	15.44 ± 0.8 ^a	2.46 ± 0.1 ^b	1164.2 ± 53.3 ^b	9.64 ± 0.5 ^b
MNRMe	121.31 ± 5.9 ^e	14.43 ± 0.7 ^e	9929.3 ± 302.4 ^f	31.79 ± 1.5 ^e
MRMe	61.15 ± 3.1 ^d	7.56 ± 0.4 ^d	4940.1 ± 231.4 ^e	25.41 ± 1.2 ^d
RaspMe	14.46 ± 0.7 ^a	0.42 ± 0.02 ^a	396.4 ± 19.4 ^a	1.41 ± 0.07 ^a
AroniaMe	44.87 ± 2.2 ^c	5.65 ± 0.3 ^c	3940.1 ± 231.4 ^d	5.60 ± 0.2 ^b
ChilBlueMe	30.61 ± 1.4 ^b	4.98 ± 0.3 ^c	1582.8 ± 71.4 ^b	16.68 ± 0.8 ^c
PolBlueMe	57.96 ± 2.3 ^d	7.16 ± 0.4 ^d	4460.1 ± 219.3 ^e	21.82 ± 1.1 ^d

Abbreviation. POL, polyphenols; FLAVON, flavonoids; FLAV, flavanols; TAN, tannins; CE, catechin equivalent; GAE, gallic acid equivalent; CE, catechin equivalent; GAE, gallic acid equivalent; Blue, blueberries; ChilBlue, Chilean blue berries; PolBlue, Polish Blueberries; M, Murtilla; Rasp, raspberries; R, ripe; and NR, non-ripe.

¹ Values are means ± SD of 5 measurements.

² Values in columns for every bioactive compound with the same solvent bearing different superscript letters are significantly different (P < 0.05).

³ per g dry weight.

conformation of the BSA changes in the presence of flavonoids and extracts. Based on our results of the amount of polyphenol compounds (polyphenols, flavonoids, flavanols and tannins) in investigated samples and the results of the following reports (Borges et al., 2010; Hellström and Mattila, 2008; Khanal et al., 2009) that berries are a rich source of dietary antioxidants and proanthocyanidins play one of the major role, therefore we used catechin and epicatechin for the interaction of protein molecule such as BSA. The data with epicatechin were exactly the same, therefore these data were omitted. Our results differ from Shi et al. (2010), probably of the different antioxidant ability of the pure flavonoids and different ranges of scanning in fluorimetry which were used in a similar study. There are no publications on applications of 3D fluorescence spectra, therefore our present conclusions that 3D fluorescence can be used as an additional tool for the characterization of the polyphenol extracts during different stages of ripening and different berries cultivars correspond with the previous data (Gorinstein, Haruenkit et al., 2010; Gorinstein, Poovarodom et al., 2010).

3.2. Bioactive compounds

The results of the contents of the polyphenol compounds in all studied samples are summarized in the Table 2. As can be seen, the contents of most of the polyphenol compounds in Murtilla non-ripe methanol extract (MNRMe) are significantly higher than in other studied berries (P < 0.05). The contents of most polyphenol compounds in raspberries are comparable with the data in Murtilla-like berries. Significantly highest contents of polyphenols, flavonoids,

Table 3
The antioxidant activity of all studied berries (μ M TE/g) in methanol (Me) extract^{1,2,3}.

	ABTS	FRAP	CUPRAC
M-likeNRMe	244.22 ± 12.1 ^c	81.32 ± 3.9 ^b	203.83 ± 9.3 ^c
M-like RMe	65.35 ± 3.1 ^a	34.12 ± 1.6 ^a	92.36 ± 4.4 ^b
MNRMe	878.18 ± 41.2 ^e	486.92 ± 23.3 ^e	1012.42 ± 43.2 ^e
MRMe	405.76 ± 20.3 ^d	204.21 ± 9.9 ^d	507.89 ± 22.6 ^d
RaspMe	80.04 ± 3.7 ^a	33.98 ± 1.6 ^a	69.91 ± 3.1 ^a
AroniaMe	152.63 ± 7.9 ^b	100.81 ± 5.1 ^c	215.85 ± 10.1 ^c
ChilBlueMe	150.45 ± 7.3 ^b	67.12 ± 3.1 ^a	141.36 ± 7.1 ^b
PolBlueMe	265.92 ± 11.1 ^c	149.04 ± 9.9 ^c	265.76 ± 11.1 ^c

Abbreviation: Blue, blueberries; ChilBlue, Chilean blue berries; PolBlue, Polish Blueberries; M, Murtilla; Rasp, raspberries; R, ripe; NR, non-ripe; ABTS, 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; CUPRAC, cupric reducing antioxidant capacity; and FRAP, ferric-reducing/antioxidant power.

¹ Values are means ± SD of 5 measurements.

² Values in columns for every value of antioxidant activity bearing different superscript letters are significantly different (P < 0.05).

³ Per g dry weight.

Table 4

Correlation coefficients between polyphenols compounds and the overall antioxidants activities (AA) in methanol (Me) extract of investigated berries.

	POLxAA	FLAVONxAA	FLAVxAA	TANxAA
ABTSM _e	0.9402	0.8972	0.9011	0.7113
FRAPM _e	0.9677	0.9051	0.9443	0.6833
CUPRACM _e	0.9413	0.8890	0.9257	0.6228

Abbreviations: POLxAA, polyphenols vs. antioxidant activities; FLAVONxAA, flavonoids vs. antioxidant activities; FLAVxAA, flavanols vs. antioxidant activities; TANxAA, tannins vs. antioxidant activities; ABTS, 2, 2-Azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt; CUPRAC, cupric reducing antioxidant capacity; and FRAP, ferric-reducing/antioxidant power.

flavanols and tannins ($P < 0.05$) were in MNRMe (121.31 ± 5.9 mg GAE/g, 14.43 ± 0.7 , 9.93 ± 3.02 and 31.79 ± 1.5 mg CE/g, respectively). Our results are in correspondence with others where extraction of phenolic compounds from persimmon pulp was done with methanol acidified with 1% HCl, suggesting that high molecular weight condensed tannins are the major antioxidant composition in persimmon pulp (Gu et al., 2008). Pérez-Jiménez et al. (2008) and Pérez-Jiménez and Saura-Calixto (2006) showed as well the relationship between the value of extracted compounds and the solvents used in the procedures.

3.3. Antioxidant activity

The results of this experiment show that the significantly highest antioxidant activity ($P < 0.05$, Table 3) was in MNRMe: 878.18 ± 41.2 , 486.92 ± 23.3 and 1012.4 ± 43.2 $\mu\text{M TE/g}$ for ABTS, FRAP and CUPRAC, respectively. The high antioxidant activity correlates with high content of polyphenol compounds. So, the highest antioxidant activity was found in MNRMe, the sample with the highest content of polyphenol compounds. Also other investigators reported such similar results (Pineli et al., 2010; Poltanov et al., 2009), where the antioxidant properties of four commercial *E. officinalis* fruit extracts were analyzed in order to determine if there are any qualitative-quantitative differences. As it was mentioned, the antioxidant activity of raspberries was comparable with Murtilla-like (Table 3). As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols and other polyphenol compounds (Tables 2–4). Cluster bar chart (Fig. 3) represents the total antioxidant capacities ($\mu\text{M TE/g DW}$) analyzed for berry methanolic extracts at different concentrations and reaction times. Data showed that the quenching ability of berry methanolic extracts were comparable

with the exception of higher values of Aronia (6) and Polish blueberry (7) and markedly Murtilla non-ripe berry (3), which had the highest antioxidant capacity values.

Cluster analysis (Fig. 4) shows that Murtilla-like non-ripe berry methanolic extract (2) exhibits nearly the same DPPH antiradical activity as its co-partner Murtilla-like ripe extract (5), because of identical cluster positions seen at the distance near 10 on the dendrogram. Some analogy can be found between these methanolic extracts and extracts of Chilean blueberry (1) and raspberry (4) species as well. The lowest antiradical activity (Fig. 5) was found for the raspberry methanolic extracts ($\text{IC}_{50} > 30 \text{ mg ml}^{-1}$) and the highest value for Murtilla non-ripe extract with IC_{50} value at 1.5 mg ml^{-1} . Middle and comparable values of the DPPH antiradical activity were found for Murtilla-like ripe and non-ripe berries. Although there some significant dissimilarities in the antiradical activity of examined berries were found, the IC_{50} value does not fully explain the differences in the antioxidant activity throughout all of the experimental DPPH scavenging variables. Therefore, the effect of all descriptors (absorbance readings at: 1, 10, 30, 60, and 90 min, different fruit extract concentrations from 1 to 30 mg/ml, observed inhibition and DPPH data) on antiradical activity of methanolic berry extracts was studied by multivariate statistics.

Principal component analysis (PCA) was performed to see how all the 15 DPPH scavenging variables measured and calculated at different times and concentrations in three replications contribute to positioning of berry samples on plot of principal components (Fig. 6A). After the PCA the dimensionality of data was reduced from 15 partially correlated variables to two uncorrelated principal component 1 (PC1) and 2 (PC2) with almost only 6% loss of variation, because first two PCs accounted for 94% of the total variability. While PC1 correlates highly with the original variable of inhibition at 30 min, most significant correlations were found in PC3 and PC5 belonging to inhibition values measured at time of 1 and 10 min. Plot of these principal components (Fig. 6A) shows significant clustering of all berry methanolic extracts, but mainly the separation of Murtilla non-ripe, Murtilla-like non-ripe, Aronia and Murtilla-like ripe extracts from other berry samples is visible. A multiparametric approach of canonical discrimination analysis (CDA) was carried out in order to evaluate the influence of above mentioned DPPH antiradical parameters in the classification and differentiation of examined methanolic berry extracts according to their scavenging ability. The plot of factor score obtained is shown in Fig. 6B, where the 7 main totally separated berry species clusters can be observed. CDA based on the selected antiradical variables indicated that the first two significant canonical discriminant functions with

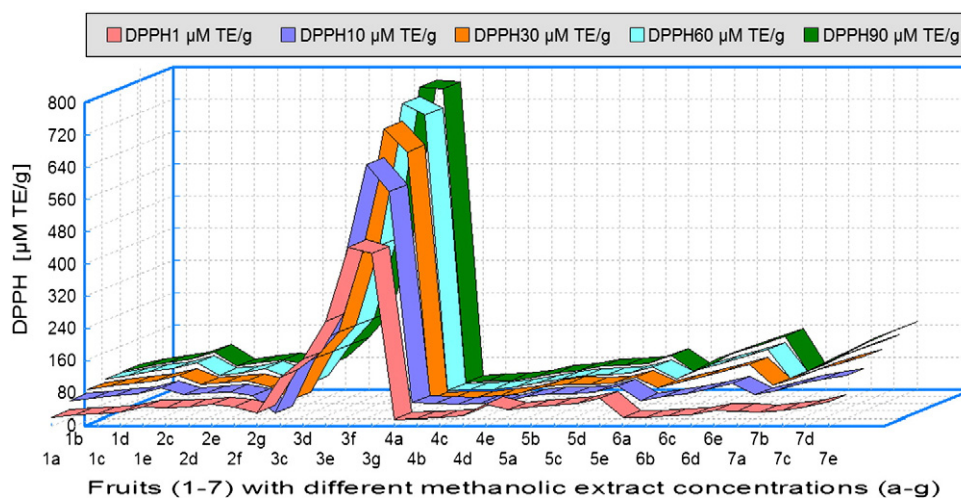


Fig. 3. Comparison of methanolic extracts' DPPH antiradical activity ($\mu\text{M TE/g DM}$) of investigated berries (1 – Chilean blueberry, 2 – Murtilla-like non-ripe, 3 – Murtilla non-ripe, 4 – raspberry, 5 – Murtilla-like ripe, 6 – Aronia, and 7 – Polish blueberry) determined at extract concentrations: a – 30, b – 20, c – 15, d – 10, e – 5, f – 2.5, and g – 1 (mg ml^{-1}) and reaction times: 1, 10, 30, 60 and 90 min. (Data are means of three replications).

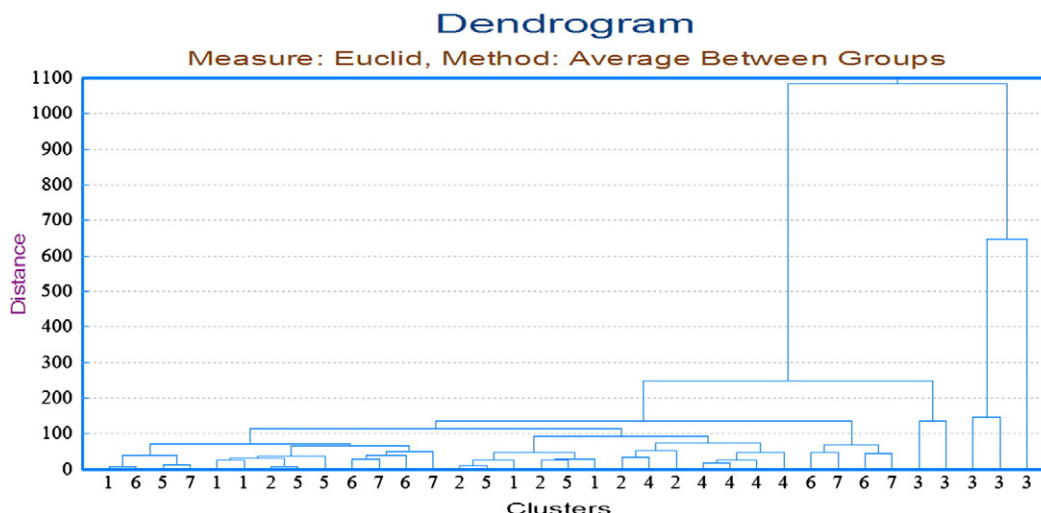


Fig. 4. Cluster analysis of berry methanolic extracts according to their DPPH scavenging activity (1 – Chilean blueberry, 2 – Murtilla-like non-ripe, 3 – Murtilla non-ripe, 4 – raspberry, 5 – Murtilla-like ripe, 6 – Aronia, and 7 – Polish blueberry; concentrations: 15, 10, 5, 2.5 and 1 mg/ml; DPPH variables were calculated from the absorbance readings at 1, 10, 30, 60 and 90 min) (Data are means of three replications).

eigenvalues >1, Wilk's lambda ~0, Chi-square test significance, $p < 0.0001$, explained 97.9% of cumulative variance (first function 89.9%). Taking into account the coefficients of canonical discriminant functions (data not presented here), the most significant contribution to discrimination in the first function was obtained from absorbance readings in time 30 min. The stepwise discrimination selected the absorbance readings in 90 and 30 min, as the most discriminant variables. Furthermore, the classification matrix gave evidence that 100% of the studied methanolic extracts were correctly classified to their fruit classes according to their DPPH scavenging ability.

As it was mentioned any description and characterization of the new kind of berries was not found in the literature, therefore the antioxidant activity of Murtilla-like was compared with other kinds of berries. The obtained results correspond with Rufino et al. (2010), where different exotic fruits were compared with murta. Relatively low values of antiradical capacity and the second-order rate constants (k^2) during the oxidation of methanol extracts by DPPH• were found. Difference in the antioxidant activities of studied berries can be explained as well that the fractionation of polyphenols is based on hydrophobicity and subsequent evaluation of antioxidant activities showed varying efficiencies in scavenging superoxide, hydroxyl and DPPH radicals (Jacob et al., 2008). Data reported in Speisky et al. (2008) showed that Chilean blueberries and raspberries were comparable with

the same kind of berries from different geographical location, and in some cases were moderately higher in levels of antioxidants. Oszmiński and Wojdyło (2005) found that in *A. melanocarpa* berries the average concentration of polyphenols (mg/100 g DW) ranged from 3729 for chokeberry juice up to 7849 in pomace and for antioxidant activities ($\mu\text{M TE}/100 \text{ g DW}$) in DPPH from 127 to 302 and ABTS from 314 to 779. The present results of polyphenols (Table 2) in methanol extract of *A. melanocarpa* berries were 4487 mg/100 g DW which were lower than shown in Oszmiński and Wojdyło (2005). This can be explained by the sample collection, growing area, storage, freeze-drying and extraction. The total content of phenolic acids, ranged from 2845.8 ± 141.0 (black mulberries) to 5418.2 ± 228.0 (blue-berried honeysuckle). The phenolic acids liberated from esters and glycosidic bonds were the major fractions of phenolic acids in the berries (Zadernowski et al., 2005). Samples of murta growing in three locations of Chile with diverse climatic conditions were extracted by ethanol/water mixtures at different ratios and the polyphenol content was assessed. Extracts containing the highest polyphenolic content were from murta plants grown nearer to the mountain ($58 \text{ mg GAE/g murta}$), subjected to extreme summer/winter-day/night temperature changes and rainy regime (Shene et al., 2009). Our data were slightly higher (Table 2, MRMe = 61.15 mg GAE/g). Extracts from leaves collected in the valley and coast contained 46 and 40 mg GAE/g murta, respectively. A mixture of 50% ethanol/water was

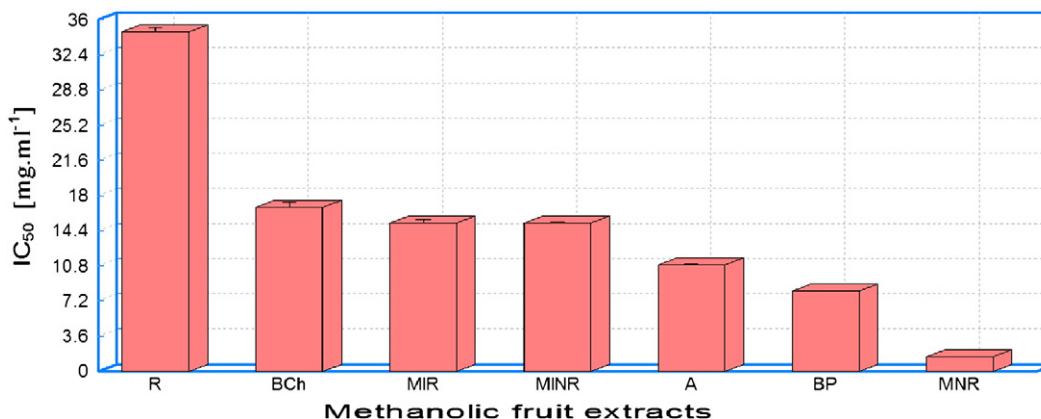


Fig. 5. IC₅₀ (mg ml^{-1}) values of DPPH-radical scavenging activity in the methanolic extract of berries. Lower IC₅₀ indicates higher antiradical activity. Extracts: R – raspberry, BCh – Blueberry Chile, MIR – Murtilla-like ripe, MINR – Murtilla like non-ripe, A – Aronia, BP – Blueberry Poland, and MNR – Murtilla non-ripe. All the data were performed in triplicates ($n = 3$) and measured for 30 min.

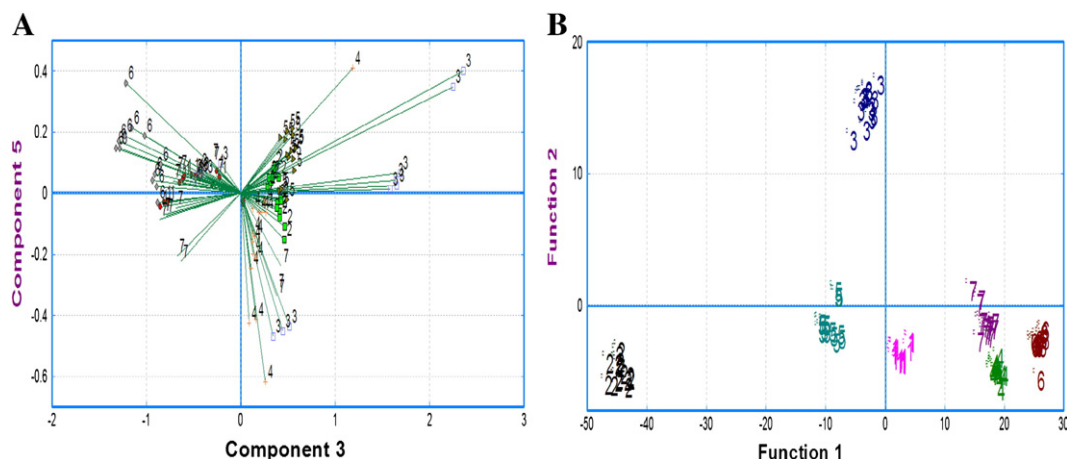


Fig. 6. Separation of the berry methanolic extracts (1 – Blueberry Chile, 2 – Murtilla-like non-ripe, 3 – Murtilla non-ripe, 4 – raspberry, 5 – Murtilla-like ripe, 6 – Aronia, and 7 – Blueberry Poland) by the principal component (A) and canonical discriminant (B) analysis of DPPH scavenging activity parameters.

the most efficient in extracting polyphenols, showing pure solvents—both water and ethanol a lower extraction capacity (Shene et al., 2009). Pérez-Jiménez et al. (2008) and Pérez-Jiménez and Saura-Calixto (2006) determined the effect of the sample solvent in the antioxidant capacity in the antioxidant capacity of catechin:gallic acid solutions and foods in different solvents (water, methanol/water, methanol, and acetone/water) measured by the four most widely used procedures (ABTS, FRAP, DPPH and ORAC). Our data of blueberries and raspberries are in agreement with Borges et al. (2010), where the antioxidant activity of blueberries and raspberries was determined by FRAP, but the extraction of the polyphenols in each experiment is different and the solvent had another polarity. Therefore the antioxidant activity by FRAP of raspberry was about 3 times lower and of blueberries was equal to the results of blueberries collected in Chile in our experiment (Table 3). In our recent research (Gorinstein, Poovarodom, et al., 2010), it was shown that the highest polyphenols and their antioxidant activity was determined in methanol extracts. Schreckinger et al. (2010) reported that the total soluble phenolic content and the antioxidant capacity in murta were 882 mg of GAE/100 g FW (46.92 mg GAE/g DW) and 1200 mg TE/100 g FW (255.06 μ M TE/g DW), and our data were 61.15 mg GAE/g DW (Table 1) and 231.65 (Table 2). Also others reported a variety of bioactivity of Murtilla from different locations (Concepción, Loncoche, Valdivia) with mean values of total phenols and antioxidant activity in the same range as our reported data. The variation in the values depends on the area of growing, the extraction procedure, ripeness, berries collection, and other conditions.

In conclusion, partial characterization of Murtilla-like berries for the first time was done by comparison with other intensively studied berries. The polyphenols content of Chilean Murtilla berries is significantly higher than of other studied samples, however this index in the Murtilla-like berries is comparable with blue and raspberries. The correlation between the polyphenols and other compounds and the antioxidant activities was high in methanol extract. Seven methanolic extracts of widely used berries and new kind berry were investigated for antiradical activity by kinetic studies. The highest ability to scavenge free DPPH radical was found for Murtilla non-ripe methanolic extract. Average and very similar quenching capacity was demonstrated by the Murtilla-like ripe and Murtilla-like non-ripe berry species according to the IC50 values. A wide-ranging multivariate statistics using the all antioxidant variables resulted in significant differences in antiradical activity between all of examined berry extracts. 3-D fluorescence was used as an additional tool for the characterization of the polyphenol extracts during different stages of ripening and various berries cultivars. The properties of polyphenol methanol extracts of Murtilla-like berry showed the ability to quench BSA by forming the complexes between proteins and flavonoids. The antioxidant properties of Murtilla-like berries (*Myrteola*) can be used as a new source of natural polyphenols.

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