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Application of Analytical Methods for the Determination of Bioactive Compounds in Some Berries

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Abstract Fluorometry, ESI-MS, FTIR, and radical scavenging assays were used for characterization of bioactive compounds and the levels of their antioxidant activities. Polyphenols, flavonoids, anthocyanins, and ascorbic acid and the level of antioxidant activity of water extracts of "Murtilla-like" [*Myrteola nummularia* (Poiret) Berg.], and other widely consumed berries were determined and compared. The contents of bioactive compounds and the levels of antioxidant activities in water extracts differed significantly in the investigated samples ($P<0.05$). "Murtilla-like" extracts contained polyphenols (mg GAE/g)—19.13±0.9, flavonoids, (mg CE/g)—3.12±0.1, anthocyanins (mg CGE/g)—120.23±5.4, and ascorbic acid (mg/g)—2.20±0.1; and antioxidant activities (μmolTE/g) by ABTS and CUPRAC assays were 200.55±8.7 and CUPRAC 116.76±5.7, respectively. Chemometrical processing was done on the basis of kinetic data of two variables (concentration and reaction time) by DPPH scavenging reaction. Polyphenol content highly correlated with antioxidant capacity (R^2 from 0.96 to 0.83). The quenching properties of berries were studied by the interaction of water polyphenol extracts with a small protein such as BSA by 3-D fluorescence and FTIR spectroscopy. These methods were used as additional tools for the characterization of polyphenols. Wild-grown non-investigated berries were compared with widely consumed ones, using their bioactive composition, antioxidant activities, and antiproliferative and fluorescence properties. In conclusion, the antioxidant properties of "Murtilla-like" can be used as a new source for consumption. The bioactivity of "Murtilla-like" is comparable with blueberries and raspberries. 3-D fluorescence and FTIR

This article was written in memory of my dear brother Prof. Simon Trakhtenberg, who died in November 2011, who encouraged me and our entire scientific group during all his life.

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spectroscopy can be applied as additional analytical tools for rapid estimation of the quality of food products.

Keywords Analytical methods · Berries · Bioactive compounds · Antioxidant activity

Introduction

Consumption of berries and fruits has become popular among health-conscious consumers due to the high levels of valuable antioxidants, such as plenolics (Wolfe et al. 2008). These phytochemicals include flavonoids, stilbenes, tannins, phenolic acids, and anthocyanins (Céspedes et al. 2010; Paredes-Lopez et al. 2010; You et al. 2011; Ahmad et al. 2012; Kang et al. 2012; Yadav et al. 2012). Administration of a freeze-dried powder of mulberry (*Morus alba* L.) fruit (MFP) to rats on a high-fat diet resulted in a significant decline in levels of serum and liver triglyceride, total cholesterol, serum low-density lipoprotein cholesterol, and a decrease in the atherogenic index. Oppositely, the serum high-density lipoprotein cholesterol was significantly increased (Yang et al. 2010). Berry phenolics may also act as antimicrobials which may be of help in the control of the wild spectra of pathogens, in view of recent problems associated with antibiotic resistance (Paredes-Lopez et al. 2010). Recent studies in vitro and in vivo have been improved the scientific understanding of how berries and fruits promote human health and prevent chronic illnesses such as some cancers, heart and neurodegenerative diseases (Prior et al. 2008; Seeram 2010). The purpose of some studies was to investigate and to compare the composition, stability, antioxidant and anticancer properties, and mechanisms of anthocyanin-containing berries extracts from selected cultivars and using different extraction methods. The influence of water content in the extraction system was evaluated. A 90-day stability study of the extract and a 48-h stability study of the extract in biologically relevant buffers were completed (Dai et al. 2009). Potential benefits of polyphenolic compounds from raspberry seeds of three different extracts as efficient antioxidants was studied (Gođevac et al. 2009). The use of blackberry showed also its different properties: blackberry administration minimized the toxic effects of fluoride (Hassan and Abdel-Aziz 2010). Berries contain powerful antioxidants, potential allergens, and other bioactive compounds (Battino et al. 2009). Anthocyanins are water-soluble plant pigments that have important functions in plant physiology as well as possible health effects (Valcheva-Kuzmanova et al. 2005; Wu et al. 2006). Antioxidant capacity and phenolic compounds (phenolic acids and anthocyanins) of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuckthorn were compared (Li et al. 2009). Different fractions of mature wild blackberry *Aristotelia chilensis* (Mol) Stuntz (Elaeocarpaceae) were analyzed. Cranberry was investigated as chemotherapeutic agent (Elberry et al. 2010; Cuevas-Rodriguez et al. 2010). Some wild Jamaica-grown species and the Michigan-grown *Rubus acuminatus*, *Rubus idaeus* cv. Heritage, and *Rubus idaeus* cv. Golden were analyzed for their anthocyanin contents, and lipid peroxidation, cyclooxygenase enzyme, and human tumor cell proliferation inhibitory activities. The high anthocyanin contents and biological activities of these fruits indicate that their consumption would be beneficial to health. This may be useful in the production of functional foods containing an efficacious dose of anthocyanins (Bowen-Forbes et al. 2010). The subject of different berries was investigated intensively, and it was shown in the cited literature, including the studies of Chilean berries (Céspedes et al. 2010). *Ugni molinae Turcz*, also known as "Murtilla", is a plant that grows in the south of Chile. Infusions of their leaves have long been used in traditional native herbal medicine (Rubilar et al. 2006; Suwalsky et al. 2006). The bioactivity of "Murta" ("Murtilla") was investigated by Rufino et al. (2010). It was interesting to compare different extraction procedures in some fruits and plants (Chanda and Kaneria 2012; Khoo et al. 2012). In our recent research the methanol extracts of different berries was investigated and compared (Arancibia-Avila et al. 2011). We were interested to investigate water extracts of a new kind of Chilean berry known by the name of "Myrteola" or "Murtilla-like" and to compare its composition with the widely consumed berries. The water extracts of berries are important from the point of tea consumption. To meet this aim the contents of bioactive compounds (polyphenols, flavonoids, anthocyanins, and ascorbic acid) and the level of antioxidant activities (AA) were determined and compared. In order to receive reliable data the AA was determined by three assays: cupric-reducing antioxidant capacity (CUPRAC), 1,1-diphenyl-2-picrylhydrazyl (DPPH), and 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Apak et al. 2004; Ozgen et al. 2006; Re et al. 1999). In order to compare the fluorescence properties of the extracted bioactive compounds, in vitro studies were performed by interaction of protein with flavonoids. Human serum albumin is the drug carrier's protein and serves to greatly amplify the capacity of plasma for transporting drugs. It is interesting to investigate in vitro how this protein interacts with flavonoids extracted from berry samples in order to get useful information of the properties of flavonoid–protein complex. Therefore the functional properties of a new kind of berry were studied by the interaction of water polyphenol extracts with a small protein such as bovine serum albumin (BSA) (Zhang et al. 2009). The advanced analytical methods such as 3D-FL and FTIR spectroscopy were applied in this research.

As far as we know no results of such investigations were published.

Material and Methods

Reagents

6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), 1,1-diphenyl-2-picrylhydrazyl, Folin–Ciocalteu reagent, urea, catechin, $CuCl_2 \times 2H_2O$, 2,9-dimethyl-1,10-phenanthroline (neocuproine), and lanthanum (III) chloride heptahydrate were purchased from Sigma Chemical Co., St Louis, MO, USA. All reagents were of analytical grade. Deionized and distilled water was used throughout.

Samples

Chilean "Murtilla", "Murta" *(U. molinae* Turcz) and "Myrteola" berries (*Myrtaceae*, *Myrteola nummularia* (Poiret) Berg.), Chilean and Polish blueberries (*Vaccinium corymbosum*), raspberries (*R. idaeus*), and black chokeberry (*Aronia melanocarpa*) were investigated. "*Myrteola*" *nummularia* (Poiret) Berg. *Myrtaceae*, (Daudapo) is distributed geographically from Valdivia to Magallanes. The fruit is edible. The fruits were harvested at their maturity stage and "Murtilla" and "Myrteola" berries were in two stages of ripening. "Myrteola" ripe was harvested in May 2008. "Myrteola" non-ripe was harvested in February 2010, in Chiloé. "Murtilla" non-ripe was collected in Puerto Varas, Chile, in February 2010 (Fig. 1). Arandano (blueberries) and raspberries were purchased at the local market in Chillan, Chile; and blueberries and chokeberries were purchased at the local market in Warsaw, Poland. For the investigation five replicates of five berries each were used. 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 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.

Determination of Bioactive Compounds and Antioxidant Activity

The contents of polyphenols, flavonoids, anthocyanins, and ascorbic acid in water extracts of the studied samples were determined as previously described (Gorinstein et al. 2009). Phenols were extracted from lyophilized berries with water (concentration 25 mg/ml) at room temperature twice during 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 milligrams of gallic acid equivalents (GAE) per gram of DW (Singleton et al. 1999). Flavonoids, extracted with 5 % $NaNO_2$, 10 % $AlCl_3 \times 6H_2O$ and 1 M NaOH, were measured at 510 nm. The extracts of condensed tannins (procyanidins) with 4 % methanol vanillin solution were measured at 500 nm. (+)-Catechin served as a standard for flavonoids, and the results were expressed as catechin equivalents (CE). Total ascorbic acid was determined by CUPRAC assay (Ozyurek et al. 2007) in water extract (100 mg of lyophilized sample and 5 ml of water). The absorbance of the formed bis (Nc)-copper (I) chelate was measured at 450 nm. The total anthocyanins were measured by a pH differential method. Absorbance was measured in a Beckman spectrophotometer at 510 nm and at 700 nm in buffers at pH 1.0 and 4.5, using the following equation: $A = [(A_{510}–A_{700})_{\ pH1.0}—(A_{510}–A_{700})_{\ pH4.5}]$. Results were expressed as milligrams of cyanidin-3-glucoside equivalent (CGE) per gram of DW (Cheng and Breen 1991).

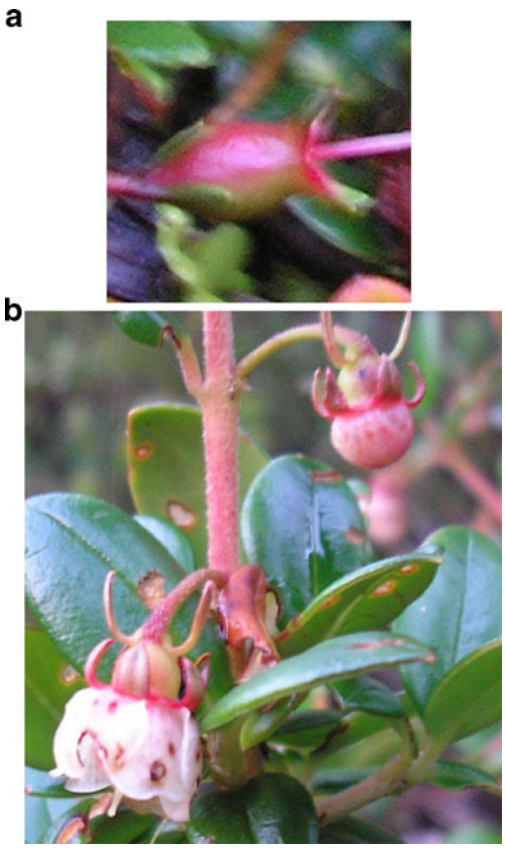

Fig. 1 Chilean berries: **a** "Myrteola", **b** "Murtilla"

The AA was determined by three 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 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 $^{\bullet+}$ radical cation was generated by the interaction of ABTS (7 mM/L) and $K_2S_2O_8$ (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. Cupric-reducing antioxidant capacity: This assay is based on utilizing the copper (II)–neocuproine [Cu (II)–Nc] reagent as the chromogenic oxidizing agent. The absorbance at 450 nm was recorded against a reagent blank (Apak et al. 2004).
3. 1,1-diphenyl-2-picrylhydrazyl antioxidant activity assay (Ozgen et al. 2006) was used for kinetic studies with extracts of different berries.

Fluorometry and Fourier Transform Infrared Spectra Studies

Two dimensional (2D-FL) and three dimensional (3D-FL) fluorescence measurements were done using a model FP-6500, Jasco Spectrofluorometer, serial N261332, Japan. Fluorescence emission spectra for all berries samples at a concentration of 0.25 mg/ml in water were taken at emission wavelength (nm) of 330, and recorded from wavelength of 265 to a wavelength of 310 nm, at emission wavelengths of 685 nm from 300 to 750 nm; and at excitation of 350 nm from 370 to 650 nm. Catechin was used as a standard. 3D-FL spectra of the investigated berries extracts were collected with subsequent scanning emission spectra from 250 to 750 nm at 1.0 nm increments by varying the excitation wavelength from 230 to 350 nm at 10 nm increments. The scanning speed was set at 1,000 nm/min for all measurements. All measurements were performed with emission mode and with intensity up to 1,000 (Wulf et al. 2005; Zhang et al. 2009). All solutions for protein interaction were prepared in 0.05 mol/L Tris–HCl buffer (pH 7.4), containing 0.1 mol/l NaCl. The final concentration of BSA was 2.0×10^{-4} mol/L. All solutions were kept in dark at 0–4 °C. The BSA was mixed with catechin. The samples were mixed in the proportion of BSA: extract=1:1. Denaturation with 2.4 M and 4.8 M urea was carried out as well. The samples after the interaction with BSA were lyophilized and subjected to FTIR.

The presence of polyphenols in the investigated berries samples and the interaction between polyphenols and bovine serum albumin was studied by Fourier transform infrared spectroscopy. A Nicolet iS 10 FT-IR Spectrometer (Thermo Scientific Instruments LLC, Madison, WI, USA), with the smart iTR$^{TM\ ATR}$ (attenuated total reflectance) accessory was used to record IR spectra (Sinelli et al. 2008).

Chemometrical Processing

Samples with different concentrations of berry water extracts (1, 2.5, 5, 10, 15, 20, and 30 mg/mL) were analyzed by DPPH antioxidant activity assay (Ozgen et al. 2006). In the kinetic studies two variables were used: the change in the concentration of the samples and the change in time of the reaction with scavenging radical: 1, 10, 30, 60, and 90 min. The DPPH data (μmol Trolox equivalent TE)/g DW) set consisted of a 25×7 matrix in which rows represent the different extract concentrations and columns the seven berry species. Basic chemometric characterization of the investigated berry extract samples according to their ability to reduce the DPPH was carried out by summary, descriptive (normal probability, box/whisker, and dot plots) statistics and multisample median testing using the statistical program Unistat® (London, UK).

Extraction of Phenolic Compounds for MS

The lyophilized samples of berries (1 g) were extracted with 100 mL of methanol/water (1:1) at room temperature and in darkness for 24 h. The extracts were filtered in a Buchner funnel. After removal of the methanol in a rotary evaporator at a temperature below 40 °C, the aqueous solution was then freeze-dried. These extracts were used for MS.

MS Analysis A mass spectrometer, a TSQ Quantum Access Max (Thermo Fisher Scientific, Basel, Switzerland) was used. Analytes were ionized by electrospray ionoization in positive mode. Vaporizer temperature was kept at 100 °C. Settings for the ion source were as follows: spray voltage 3,000 V, sheath gas pressure 35 AU; ion sweep gas pressure 0 AU; auxiliary gas pressure at 30 AU; capillary temperature at 200 °C, and skimmer offset 0 V (Gómez-Romero et al. 2011).

MTT Assay

Anticancer activity of water extracts of the studied berries on human cancer cell lines (Calu-6 for human pulmonary carcinoma and SNU-601 for human gastric carcinoma) were measured using MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. The cell lines were purchased from Korean Cell Line Bank (KCLB) for MTT assay. Cells were grown in RPMI-1640 medium at 37 °C under 5 % CO_2 in a humidified incubator. Cells were harvested, counted (3×10^4 cells/mL), and transferred into a 96-well plate, and incubated for 24 h prior to the addition of test compounds. Serial dilutions of test samples were prepared by dissolving compounds in water followed by dilution with RPMI-1640 medium to give final concentration at 25, 50, 100, 200, 400, and 800 and 1,000 μg mL^{-1}. Stock solutions of samples were prepared for cell lines at 90 μL and samples

at 10 μL, and incubated for 72 h. MTT solution at 5 mg mL^{-1} was dissolved in 1 mL of phosphate buffer solution, and 10 μL of it was added to each of the 96 wells. The wells were wrapped with aluminum foil and incubated at 37 °C for 4 h. The solution in each well containing media, unbound MTT, and dead cells were removed by suction and 150 μL of DMSO was added to each well. The plates were then shaken and optical density was recorded using a micro plate reader at 540 nm. Distilled water was used as positive control and DMSO as solvent control. Controls and samples were assayed in duplicate for each concentration and replicated three times for each cell line. The cytotoxicity was obtained by comparing the absorbance between the samples and the control (Heo et al. 2007).

Statistical Analyses

To verify the statistical significance, mean ± 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.

Results

Bioactive Compounds

The results of the determination of the contents of the bioactive compounds in all studied samples are summarized in Fig. 2a. As can be seen, the significant highest content ($P<0.05$) of polyphenols, flavonoids, anthocyanins, and ascorbic acid was in "Murtilla" non-ripe sample (84.81±3.9 mg GAE/g, 11.47±0.6 mg CE/g, 16.7±0.9 mg CGE/g, and 9.12±0.4 mg/g, respectively, Fig. 2a and b). Only the content of anthocyanins (Fig. 2b) was significantly higher ($P<0.05$) in blueberries from Poland (323.2±16.1 mg CGE/g). The following order of the value of polyphenols was obtained (Fig. 2a): "Murtilla" non-ripe (MNR) > *Aronia* (ARON) > Polish blueberry (POLBB) > Chilean blueberry (CHBB) > "Murtilla-like" non-ripe (M-LNR) > raspberry (RASB) > "Murtilla-like" ripe (M-LR).

Antioxidant Activity

The results of the determination of the level of antioxidant activity of all studied samples are shown in the Fig. 2c. As can be seen, the AA of Murtilla non-ripe as determined by ABTS and CUPRAC assays was 620.74±30 and 600.52±27 μmol TE/g, respectively) was significantly higher than in other studied berries ($P<0.05$). The antioxidant activity of blueberries was higher than that of raspberries, and comparable with AA of "Murtilla" non-ripe (Fig. 2c). As was calculated, a very good correlation was found between the antioxidant activity and the contents of total polyphenols and other bioactive compounds (R^2 from 0.96 to 0.83) in water extracts. Flavonoids showed lower correlation. The correlation between the antioxidant activity and ascorbic acid (Fig. 1c and b) was lower than with polyphenols (R^2 from 0.84 to 0.50).

Anticancer Activity

It was observed that the percentage of proliferativity of the water extracts of berries samples on two cell lines (Fig. 2d, Calu-6 for human pulmonary carcinoma and Fig. 2e, SNU-601 for human gastric carcinoma) were different. The proliferativity (%) for concentrations of 1,500 μg/mL for water extracts of "Murtilla" on Calu-6 and SNU-601 were 41.76 and 42.12 %, respectively, and for "Murtilla-like" were 73.43 % and 71.23 % on Calu-6 and SNU-601, showing the higher antiproliferative activity of "Murtilla" in comparison with all other samples. Our investigation showed that antioxidant activity of the studied samples was correlated with their antiproliferative activity directly: the highest antioxidant activity was matching the highest antiproliferative activity.

Fluorometric Data

Fluorometric data showed the characterization of bioactive compounds in different berries with their specific fluorescence intensity and the location of the main peak and its shift. In addition the quenching ability of bioactive compounds in extracts was compared with pure catechin by the interaction with BSA in the presence of urea. The 3-D FL was used to determine the peak situation and the picture of the full peak. The 2-D FL was used for the determination of the fluorescence properties and for the change in the fluorescence intensity.

In three-dimensional fluorescence spectra and contour maps of berries one main peak can easily be observed in water extracts at the location of λ em/ex 340/275 nm in "Murtilla-like" non-ripe with fluorescence intensity (FI) of 680 and the average second peak at em/ex 430/310 nm with FI=480; and one very small peak at em/ex 620/280 nm with FI 80 (Fig. 3a). "Murtilla-like" ripe (Fig. 3b) showed nearly the same two peaks at em/ex 330/280 nm with intensity of 507; and the second peak at em/ex 420/310 nm with FI=400; one very small peak at em/ex 620/280 nm with FI 60. The difference was only in a small shift in the case of the ripe sample and higher fluorescence intensity of "Murtilla" non-ripe sample. "Murtilla" non-ripe (Fig. 3c) showed only one main peak at λ em/ex 420/320 nm with FI=800; raspberry (Fig. 3d) showed the following peaks: an average one at λ em/ex 290/280 nm with FI=200, and a small one at

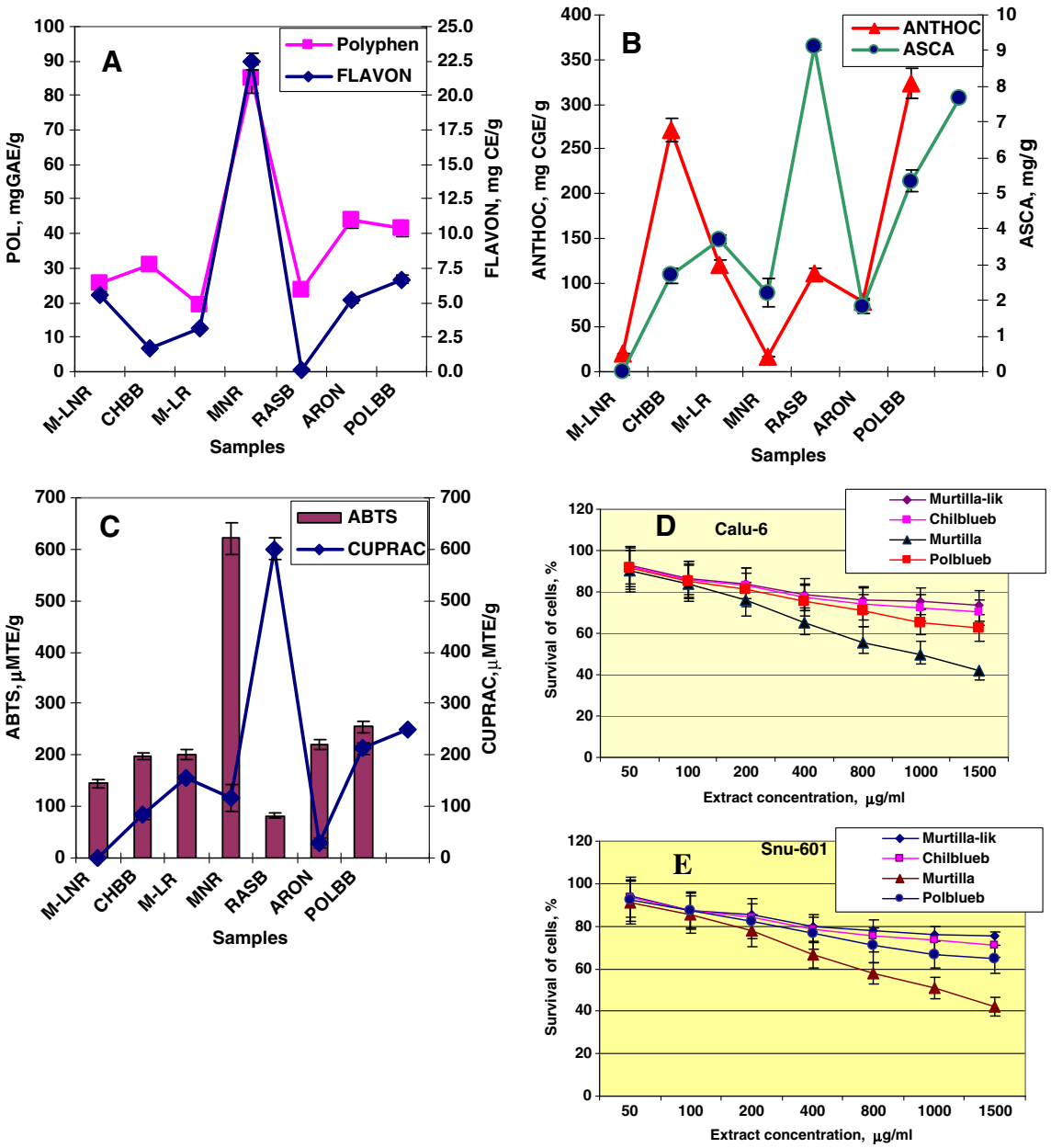

Fig. 2 **a** Total polyphenols (*Polyphen*, mg GAE/g) and flavonoids (*FLAVON*, mg CE/g); **b** anthocyanins (*ANTHOC*, mg CGE/g) and ascorbic acid (*ASCA*, mg/g); **c** antioxidant activities (µMTE/g) by *ABTS* and *CUPRAC* in the following berries: "Murtilla-like" non-ripe (*M-LNR*), Chilean blueberry (*CHBB*), "Murtilla-like" ripe (*M-LR*), "Murtilla" non-ripe (*MNR*), raspberry (*RASB*), *Aronia* (*ARON*), Polish blueberry (*POLBB*). *Abbreviations*: *GAE* gallic acid equivalent, *CE* catechin equivalent, *CGE* cyanidin-3-glucoside equivalent, *ABTS* 2, 2-azino-bis (3-ethyl-benzothiazoline-6-sulfonic acid) diamonium salt, *CUPRAC* cupric-reducing antioxidant capacity, *TE* trolox equivalent. The survival of cells (%) of human cancer cells of the **d** Calu-6 and **e** SNU-601 in the presence of water extracts of "Murtilla-like" (*Murtilla-lik*); Chilean blueberries (*Chilblueb*); "Murtilla", Polish blueberries (*Polblueb*). Each point represents the mean±SD (n=6)

em/ex 620/280 nm with FI 78. Chilean blueberries (Fig. 3e) showed one peak at λ em/ex 420/325 nm with FI=468, and a small one at λ em/ex 640/270 nm with FI=27. *Aronia* (Fig. 3f) showed one big peak at λ em/ex 420/310 nm with FI=580, and Polish blueberries (Fig. 3g) - two peaks: one small at λ em/ex 380/275 nm with FI=11, and another bigger one at λ em/ex 400/330 nm. There are not too many applications of 3D fluorescence spectra, therefore our present conclusions that 3-D 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 et al. 2010). The interaction between BSA, urea, catechin, and berry extract is shown in Fig. 4 by the changing of fluorescence intensity and shift of the main peak. Two different concentrations of urea were used: 2.4 M at 37 °C during 1 h

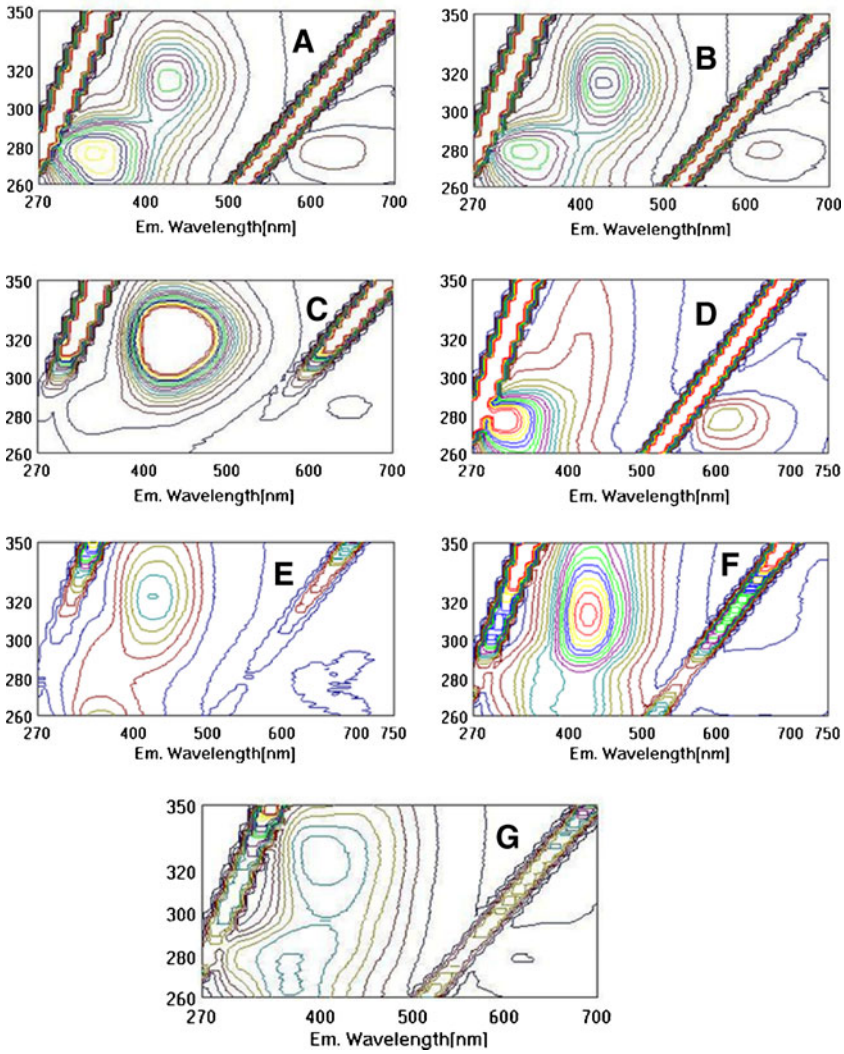

Fig. 3 Contour maps in three-dimensional fluorescence of water extracts (2.5 mg/mL) of "Murtilla-like" non-ripe, "Murtilla-like" ripe, "Murtilla" non-ripe, raspberry, Chilean blueberries, *Aronia*, Polish blueberries (**a**–**g**). The 3D-FL were run emission mode and fluorescence intensity up to 1,000, emission wavelengths from 270 to 750 nm and excitation wavelengths from 260 to 350 nm; scanning speed was 1,000 nm/min, emission wavelength on *x*-axis and excitation wavelength on *y*-axis

and the FI of BSA decreased from 878 to 605 (Fig. 4a and c). Oppositely at 4.8 M urea at the same conditions of time and temperature the FI of BSA decreased till 97, nearly full denaturation (Fig. 4e). Partly the same binding was obtained with 2.4 M urea and addition of catechin (Fig. 4b) and water extract of "Murtilla" non-ripe (Fig. 4c). The binding of catechin was higher (Fig. 4d, FI=646) than under the same conditions of the extract of "Murtilla" non-ripe (Fig. 4f, FI= 731.2). The main peak has changed in the region of λex/em of 225–230/335 nm.

The decrease of the intensity of the main peak of BSA with berry extract was about 16.7 % in comparison with catechin of 26.4 %. Other berry samples showed the decrease from 15 to 8 %. The decrease in the fluorescence intensity is the indicator of the quenching of berries extracts in interaction with BSA.

Fourier Transform Infrared Spectra Studies

The FTIR spectra of BSA and catechin (Fig. 5a, first line from the top) were compared with BSA and "Murtilla" non-ripe (Fig. 5a, second line from the top) and BSA (Fig. 5a, third line from the top). The amide I and amide II peaks of BSA (Fig. 5a, third line from the top) were shifted from 1,548 to 1,544 cm^{-1} and from 1,650 to 1,627 cm^{-1} upon interaction with catechin (Fig. 5a, first line from the top) and to 1,552 and 1,630 cm^{-1} upon interaction with "Murtilla" non-ripe extract (Fig. 6a, second line from the top). The FTIR wave numbers of catechin (Fig. 5a, third line from the top) shows broad phenolic OH band centered around 3,183 cm^{-1}, characteristic –CO stretching at 1,650 cm^{-1} aromatic bending and stretching around 1,040 and 1,650 cm^{-1}, –OH phenolic bending around 1,205 and 1,393 cm^{-1}. The FTIR spectra of BSA (Fig. 5b, third line from the top) were compared with BSA–urea (Fig. 5b, third line from the top). The amide I and amide II peaks of BSA (Fig. 5b, third line from the top) disappeared under denaturation with urea and with urea and addition of "Murtilla" non-ripe extract (Fig. 5b, second line from the top). The phenolic OH corresponding to catechin appeared around 3,400 cm^{-1} for the catechin–BSA complex was at

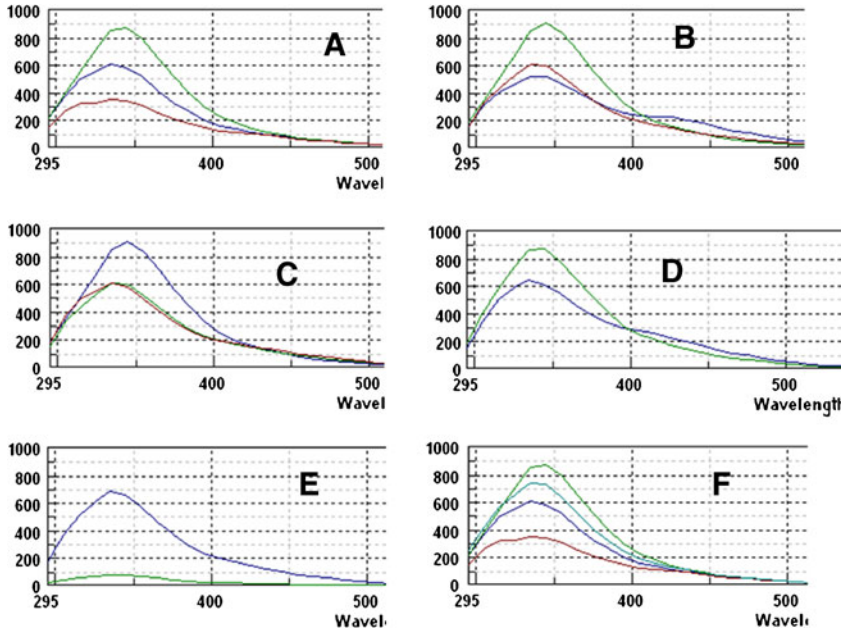

Fig. 4 Two-dimensional fluorescence spectra illustrate the interaction between BSA, catechin, urea, and water extracts of studied berries. **a** change in the fluorescence intensity as a result of binding affinity: 0.0132 μM BSA [*upper line* with fluorescence intensity of 877.8]; 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*middle line* with FI=605); 0.0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 37 °C (*lower line* with FI=341); **b** 0.0132 μM BSA (*upper line* with FI of 877.8]; 0.0132 μM BSA+2.4 M urea during 1 h at 37 °C (*middle line* with FI=600); 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of "Murtilla" non-ripe during 1 h at 37 °C (*lower line* with FI=525); **c** 0.0132 μM BSA (*upper line* with FI of 900), 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*middle line* with FI=605.3); 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of "Murtilla" non-ripe (*lower line* with FI=604.2) during 1 h at 25 °C; **d** 0.0132 μM BSA (*upper line* with FI of 878), 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*lower line* with FI=646); **e** 0.0132 μM BSA+4.8 M urea at 0 time (*upper line* with FI of 686.4), 0.0132 μM BSA+4.8 M urea during 1 h at 25 °C (*lower line* with FI of 97); **f** 0.0132 μM BSA (*first line from the top* with FI=878), 0.0132 μM BSA+50 μg/ml of water extract of "Murtilla" non-ripe at 0 h time (*second line from the top* with FI=731.2), 0.0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 25 °C (*third line from the top* with FI=600); .0132 μM BSA+2.4 M urea+30 μM catechin during 1 h at 37 °C (*fourth line from the top* with FI=341). Fluorescence intensities are on *y*-axis and emission wavelengths on *x*-axis (*Wavel*, *Wavelength)*

3,188 cm^{-1}. Matching between the peaks in the range from 4,000 to 400 cm^{-1} between (BSA + urea + "Murtilla" non-ripe)/(BSA + urea)=99.8 %; (BSA + catechin)/(BSA + "Murtilla" non-ripe)=98.05 %; (BSA + catechin)/BSA=47.38 %; and (BSA + "Murtilla" non-ripe)/BSA=48 % (Fig. 5a and b).

Chemometrical Processing

Chemometrical processing is an additional method to show the similarities and the differences in the investigated berries based on their bioactive compounds. The comparison of the DPPH antiradical activity (μmol TE/g DW) of investigated berries is shown in the Fig. 6a, where the highest values were in *Aronia* and "Murtilla" non-ripe. In order to exactly compare the quenching ability of the examined berries, the half maximal inhibitory concentration (IC_{50}), which is the concentration of the extract that inhibited DPPH free radical by 50 %, was calculated for a widely used scavenging reaction time of 30 min shown in Fig. 6b. The lower the IC_{50} value, the higher the radical-scavenging activity of the berries. By comparing the IC_{50} value of the berries water extracts, we found that the highest radical scavenging effect was observed in "Murtilla" non-ripe and *Aronia* berries with IC_{50} of about 6 mg ml^{-1}. The potency of radical scavenging effect of these two extracts was about ten times greater than in raspberry extract with the lowest antiradical activity. The scavenging activity of the extracts in decreasing order was: *Aronia* > "Murtilla" non-ripe > "Murtilla-like" ripe > blueberry (Chile) > "Murtilla-like" non-ripe ≥ blueberry (Poland) and raspberry (Fig. 6b). After the PCA, the dimensionality of data was reduced from 15 measured, calculated, and partially correlated original variables to the new set of uncorrelated variables—principal components, from which first two components accounted for 91.3 % of the total variability. These new variables highly correlate with the original antiradical descriptors of absorbance reading and inhibition at 60 min in the first principal component (PC1) and DPPH scavenging activity (μmol TE/g DW) at 60 and 90 min in the second PC. Plot of these PCs (Fig. 6c) shows not very strong clustering tendency among all berry water extracts according to scavenging ability data, but some similarities between fruit groups are evident. Clusters of water extracts of *Aronia* and "Murtilla" non-ripe fruits, both

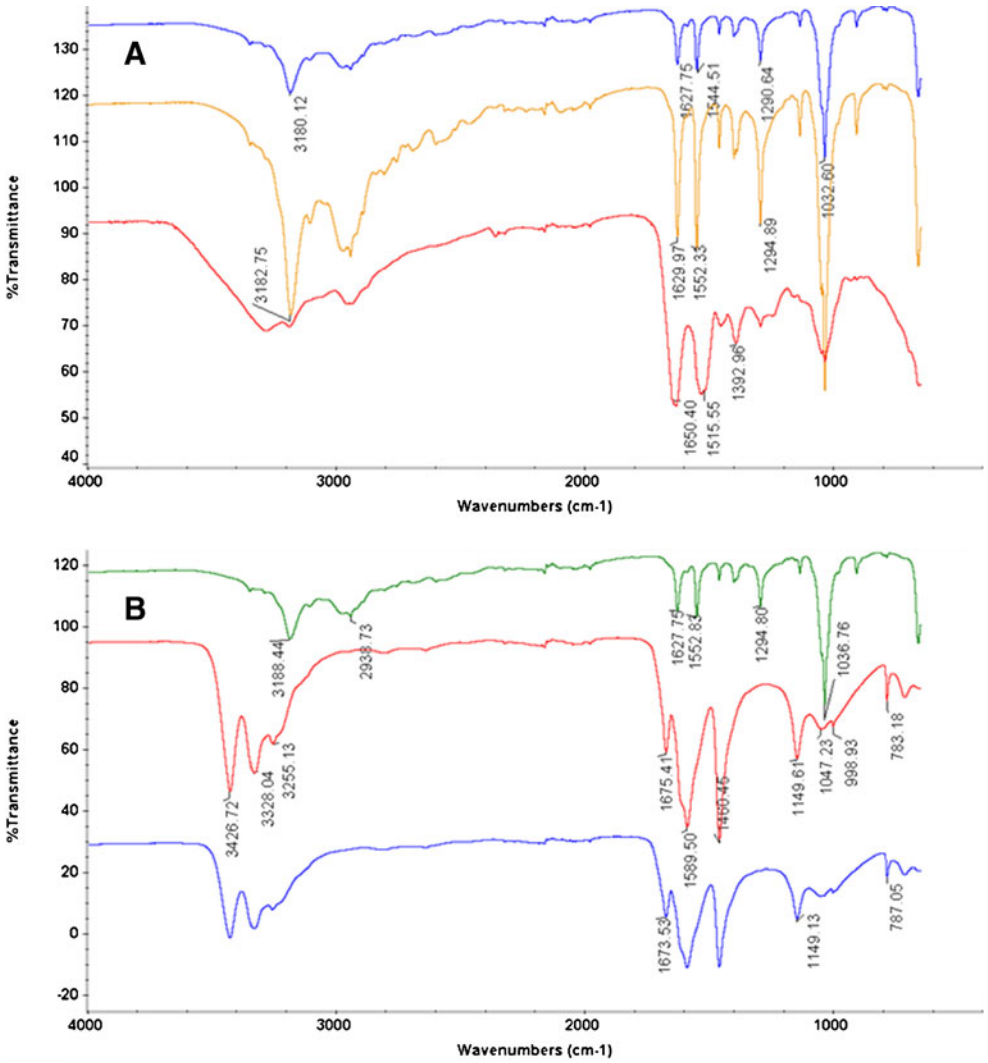

Fig. 5 Infrared study of FTIR spectra of **a** 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*upper line from the top*), 0.0132 μM BSA+50 μg/ml of water extract of "Murtilla" non-ripe at 0 h time (*second line from the top*), 0.0132 μM BSA (*third line from the top*); **b** 0.0132 μM BSA+30 μM catechin during 1 h at 25 °C (*upper line from the top*), 0.0132 μM BSA+2.4 M urea+50 μg/ml of water extract of "Murtilla" non-ripe during 1 h at 25 °C (*second line from the top*), 0.0132 μM BSA+2.4 M urea during 1 h at 25 °C (*third line from the top*)

with the relatively very high antiradical activity are well separated from "Murtilla-like" ripe and rest fruits as well as from raspberries with the lowest antioxidant activity. A multiparametric approach of canonical discrimination analysis (CDA) was carried out in order to evaluate the influence of all DPPH antiradical parameters in the classification and differentiation of examined water fruit extracts according to their scavenging ability. Main seven fruit species were totally and correctly separated into relevant clusters. CDA based on the selected antiradical variables indicated that the first two significant canonical discriminant functions with eigenvalues > 1, Wilk's lambda ~ 0, and Chi-square test significance $P<0.0001$ explained 98.9 % of cumulative variance (first function 93.8 %). 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 and inhibition value in reaction time 30 min and in the second function absorbance reading and inhibition in time 60 min. The stepwise discrimination found the DPPH antiradical activity after 1 min of reaction time as the most discriminant variables. Furthermore, the classification matrix gave evidence that the studied water extracts were correct, with 100 % success rate, classified to their fruit classes according to their DPPH scavenging ability.

Mass Spectra Data

The spectrum shows the main *m*/*z* peaks found in berries (Fig. 7a–d) in water fraction with relative abundance (RA, %) from 20 to 100 %. The main peak was about 192–193, which mostly belongs to ferulic acid (Gómez-Romero et al. 2011). The RA of the obtained peaks showed the difference in the amount of polyphenol compounds in these samples.

Discussion

It was of great interest to compare "Murtilla-like" with "Murtilla" in order to find out if the "Murtilla-like"

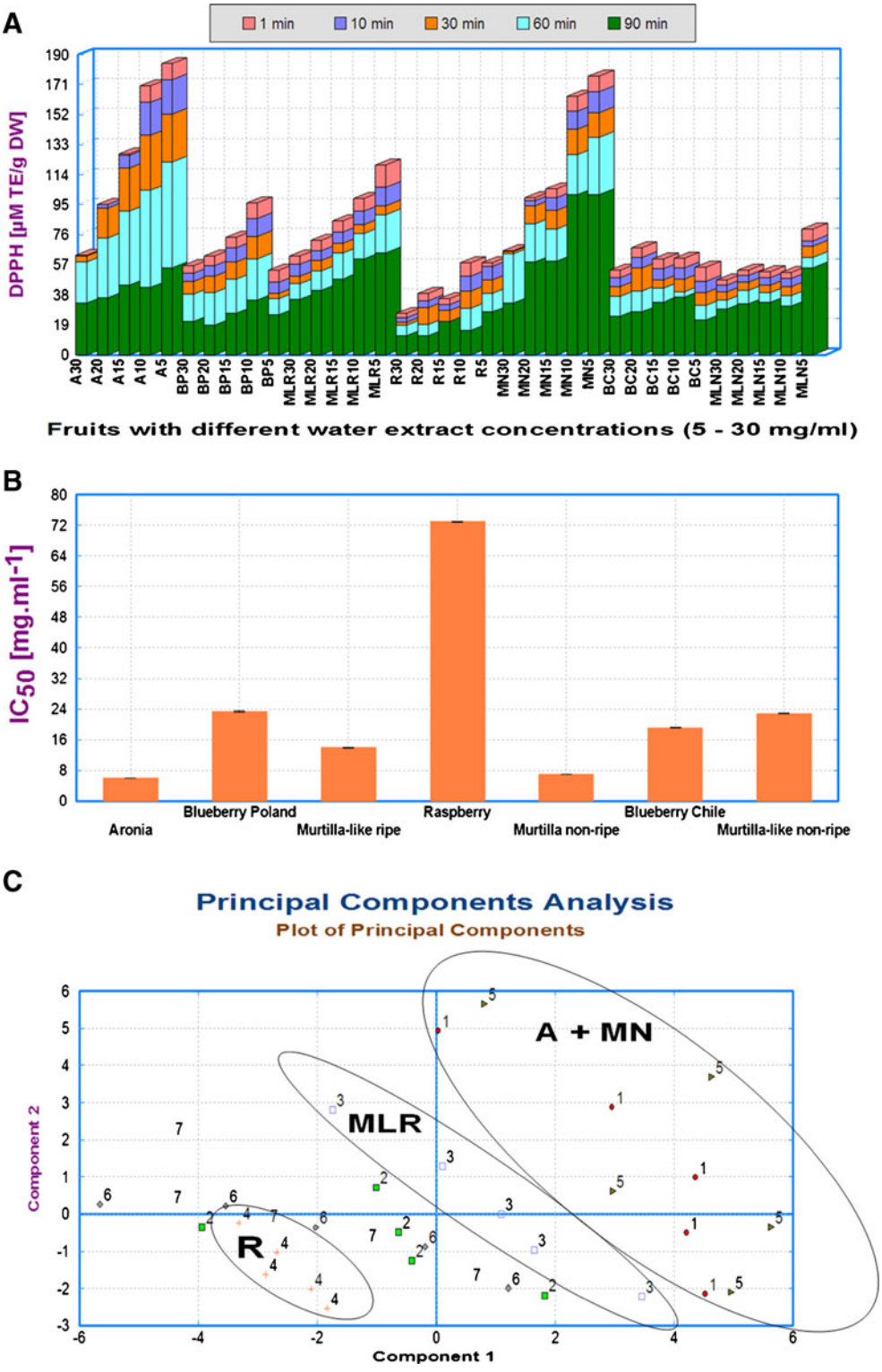

Fig. 6 **a** Overlap bar chart comparing the water extracts by DPPH antiradical activity (µM TE/g DW) of investigated berries (*A Aronia*, *BP* blueberry Poland, *MLR* "Murtilla-like" ripe, *R* raspberry, *MN* "Murtilla" non-ripe, *BC* blueberry Chile, *MLN* "Murtilla-like" non-ripe) according to reaction time contribution at 1, 10, 30, 60, and 90 min. **b** IC_{50} bar chart of DPPH-radical scavenging activity in the water extract of berries. The lower the IC_{50} values the higher antiradical activity. Data were performed in triplicates (n=3) for a reaction time of 30 min, and in the range of extract concentration was from 5 to 30 mg ml^{-1}. **c** Differentiation of the berry water extracts by the principal component analysis. Score plot on the first two components of the DPPH scavenging parameters (*1 Aronia* (*A*), *2* blueberry Poland, *3* "Murtilla-like" ripe (*MLR*), *4* raspberry (*R*), *5* "Murtilla" non-ripe (*MN*), *6* blueberry Chile, *7* "Murtilla-like" non-ripe); extract concentrations: 30, 20, 15, 10, and 5 mg ml^{-1}; reaction times: 1, 10, 30, 60, and 90 min)

bioactivity is on the same level as of original "Murtilla". Therefore, the contents of the bioactive compounds and AA were determined and compared with the widely consumed blueberries, red raspberries, and chokeberries. A number of reviewed articles showed that the main bioactive compounds determining the nutritional quality of berries are polyphenols, anthocyanins, and flavonoids (Battino et al. 2009; Bowen-Forbes et al. 2010; Cuevas-Rodriguez et al. 2010; Dai et al. 2009). As was declared in "Results", the contents of bioactive compounds (polyphenols, flavonoids, anthocyanins, and ascorbic acid) in water extracts was determined and compared, and the significantly highest were

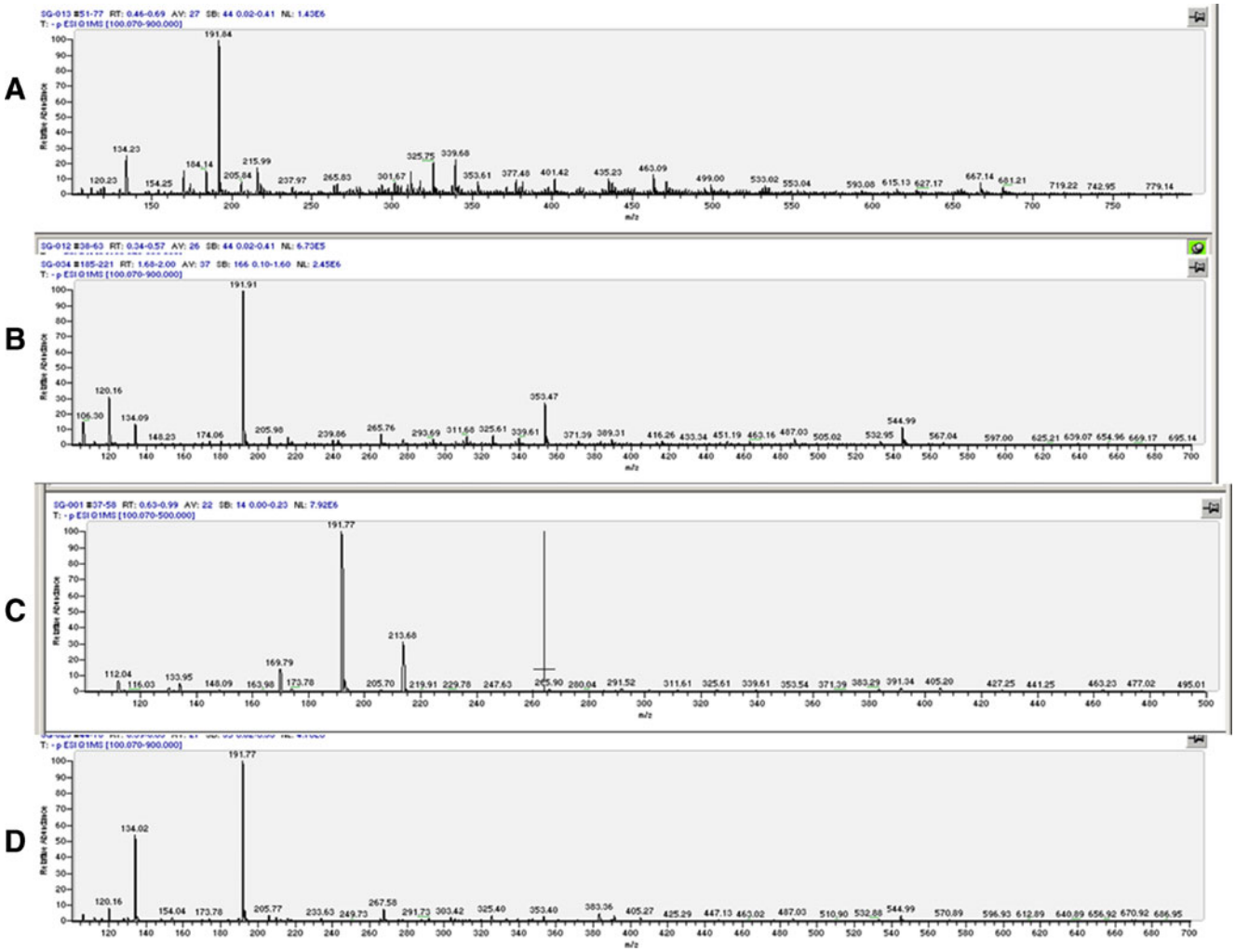

Fig. 7 ESI-MS spectra of water fractions of the following berries: **a**, **b**, **c**, **d** "Murtilla-like" non-ripe, Chilean blueberry, "Murtilla" non-ripe, Polish blueberry, respectively, in negative ion mode

in water extract of non-ripe "Murtilla". Also the antioxidant activity according to ABTS and CUPRAC was significantly higher in water extract of non-ripe "Murtilla". Our results were in agreement with others, showing that water extracts of blackberries contain high amounts of anthocyanins (Dai et al. 2009). The results show promising perspectives for the exploitation of non-traditional tropical fruit species with considerable levels of nutrients and antioxidant capacity. Our data add valuable information to current knowledge of the nutritional properties of tropical fruits, such as the considerable antioxidant capacity found for acerola—*Malpighia emarginata* and camu-camu—*Myrciaria dubia* (ABTS, DPPH, and FRAP) and for puçá-preto—*Mouriri pusa* (all methods). "Murtilla" in comparison with other 18 non-traditional tropical fruits from Brazil has an average value of antioxidants (Rufino et al. 2010). For dry matter the order observed was: bacuri > carnauba > yellow mombin > java plum > umbu > cashew apple > mangaba > assai > murta > gurguri > puçá-coroa-de-frade > uvaia > nance > jaboticaba > jussara > puçá-preto > acerola > camu-camu. When evaluated by the ABTS method, our fruits ranged from 6.3 to 153 μmol TE/g FW and from 16.4 to 1,237 μmol TE/g DW. FRAP values were 11.8–279 and 16.1–2,502 μmol $FeSO_4$/g, respectively. Our data are in agreement with these results. The order of increasing antioxidant capacity, measured by the ABTS method, was: umbu < yellow mombin < carnauba < cashew apple < mangaba < assaí < uvaia < java plum < gurguri < jaboticaba < puçá-coroa-de-frade < murta (Peña-Neira et al. 2007). Vitamin C of "Murta" was 181 mg/100 g FW (6.98 mg/g DW) which is approximately equal to our results (Fig. 2b). The anthocyanins were about 143 mg/100 g FW (5.52 mg/g DW) and this number is lower than our results (Fig. 2b). The polyphenols in "Murtilla" were 20.55 mg GAE/g DW and this number is lower than our results (Fig. 2a). The antioxidant activity (μmol TE/g DW) by ABTS was about 166. A positive and significant correlation was found in this study between vitamin C-extractable polyphenols and ABTS (R^2=

0.70). Polyphenols and DPPH results expressed as antioxidant concentrations corresponding to 50 % scavenging activity were negatively and significantly correlated (R^2=0.72; P<0.05); this is due to the fact that the DPPH method yields inversely proportional results. There was also a positive and significant correlation of polyphenols (P<0.05) and ABTS (R^2=0.92) assay (Rufino et al. 2010). These data are in agreement with our results. Our results correspond also with the research approach of Wu et al. (2006), where concentrations of total anthocyanins varied considerably from 0.7 to 1,480 mg/100 g FW in gooseberry ('Careless' variety) and chokeberry, respectively. Total phenolic content and total anthocyanin content of four berry fruits (strawberry, Saskatoon berry, raspberry, and wild blueberry), chokecherry, and seabuckthorn ranged from 22.83 to 131.88 g/kg and 3.51 to 13.13 g/kg, respectively, which corresponds with our results. Our data can be comparable with another report (Cuevas-Rodriguez et al. 2010), where the proanthocyanidins (condensed tannins) were present in the blackberry fruits. The average anthocyanin concentration was 49.2 mg/g in the commercial cultivar Tupy, while in the wild genotypes and the breeding line, the range was 361.3–494.9 mg/g (cyanidin 3-*O*-glucoside equivalent). The proanthocyanidin concentration varied widely among wild genotypes (417.5–1,343.6 mg/g CE). Comparison of different fractions of water extracts from of wild blackberry *A. chilensis* (Mol) Stuntz (Elaeocarpaceae), corresponded with our results. Wu et al. (2006) showed that in chokeberry the amount of anthocyanins was 1,480 mg/100 g FW (52.54 mg/g DW), for red raspberry- 92.1 mg/100 g FW (6.48 mg/g DW). Also other authors reported similar results. So, Ruiz et al. (2010) found the highest total polyphenol content in maqui, followed by calafate and "Murtilla". Reported high anthocyanin content in calafate berries berries (17.81±0.98 μmol g^{-1}) are comparable with those indices found in maqui (17.88±1.15 μmol g^{-1}). The AA of "Murtilla-like", blueberries and red raspberries was comparable. Also other reported different AA data in different cultivars harvested in different seasons (Ruiz et al. 2010). According to these authors the means of AA for calafate, maqui, and Murtilla were 74.4±15.9, 88.1±21.5, and 11.7±2.3 μmol TE/g FW, respectively. Seeram (2010) discussed also that phytonutrients ranged from fat-soluble/lipophilic to water-soluble/hydrophilic compounds. Conclusions in the report of Elberry et al. (2010) are in line with our results about the high antioxidant activity of berries. Our results are in accordance with You et al. (2011), where four Rabbiteye blueberry cultivars (Powderblue, Climax, Tifblue, and Woodward) grown organically and conventionally were compared regarding their chemical profiles and antioxidant capacity in terms of total phenolic content, total anthocyanin content, and antioxidant values by ABTS, DPPH, FRAP, and CUPRAC. Total phenolics, flavonoids, and anthocyanins (mg/g FW) were in blueberry 261–585, 50, and 25–495 and in raspberry - 121, 6, and 99; antioxidant activity (μmol Trolox/g FW) for blueberry 14 by ABTS and 25.3 - by DPPH assays (Li et al. 2009). The result from this study indicated that blueberries had very high ORAC values, and higher antioxidant capacity than other selected fruits and vegetables (Wulf et al. 2005). The comparison of the results of different solvents in Dabai fruit parts (methanol, ethanol, ethyl acetate, acetone, and water) and total phenolics, total flavonoids, total anthocyanins, and antioxidant capacity (ABTS $^{\bullet+}$ and FRAP assays) were in accordance with our data (Khoo et al. 2012). The acetone extract had maximum phenol and flavonoid content and showed best DPPH free radical scavenging activity and reducing capacity assessment. Ethyl acetate extract showed best superoxide radical scavenging activity, while aqueous extract showed best hydroxyl radical scavenging activity (Chanda and Kaneria 2012).

In conclusion, the bioactivity of Chilean "Murtilla" berries is significantly higher than the bioactivity of other studied samples; however, this index in the "Murtilla-like" berries is comparable with blueberries and raspberries. The antiproliferative properties of the investigated samples are in correlation with the antioxidant activity. 3-D fluorescence and FTIR spectroscopy was used as an additional tool for the characterization of the polyphenol extracts during different stages of ripening and in different berries cultivars. It is a necessity of discovering new plant breeding and genetic studies of berries with the expression of compounds for human health. The analytical methods used in this study can be applied for any of the food analysis.

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