Anthocyanins Identification, Separation and Measurement from Cranberry, Blueberry, Bilberry, Chokeberry and Acai Berry Extracts by HPTLC

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*In this paper is presented a high performance quantitative method for the quantification of anthocyanins (mv 3-glc, cy 3-glc, pn 3-glc and dp 3-glc) from cranberry, blueberry, bilberry, chokeberry and acai berry extracts. The anthocyanins were extracted using acidified methanol (4:1) and separated on HPTLC 60 F_{254} silica gel plates with ethyl acetate, 2-butanone, water and formic acid 7/3/0.8/1.2 (v / v / v / v) as mobile phase within 30 min. The plates evaluation was made in white light transmitted after development. The correlation coefficient of the calibration curves was ≥ 0.99988 and the analysis repeatability was calculated on three applications of the same analyzed sample (≤ 3.6%). Based on the results obtained, this method can be used in routine analysis for the anthocyanins determination from the above extracts. In order to identify the unknown compounds, mass spectrometry was used. The bioactivity and antioxidant properties of anthocyanins were highlighted by the Aliivibrio fisheri bacteria and DPPH * detection.*

Keywords: cranberry extract, acai berry extract, HPTLC, anthocyanins identification.

Medicinal plants have played an essential role in the development of human culture. These plants are used in human or veterinary practice for therapeutic or prophylactic purposes due to their antioxidant qualities. Herbs are available in many forms including fresh, dried, capsules, tablets or bottled in liquid form [1-3]. Medicinal plants are, for example, a good source of antioxidant phenolic acids (caffeic acid derivatives), flavonoids (quercetin and myricetin), anthocyanidins (cyanidin and delphinidin) and flavan-3-ols (catechin) [2-5].

Anthocyanins (from greek *anthos* = flower and *kianos* = *blue)* are polyphenolic pigments responsible for the red, purple and blue shades present in fruits, vegetables, grains and as well as in products made from them. The hue and stability of these compounds is influenced by *p*H, as follows: at low pH, anthocyanin color is deep red while at a high pH the color turns into dark blue [6-11].

The anthocyanins are easily dissolved in water and alcohol but are not dissolved in fats and oils. The study of natural dyes is an area of active and extensive investigation due to increased interest for synthetic colors replacement which have adverse effects on human health. The most important property of anthocyanins is their antioxidant activity, which plays an essential role in cancer, diabetes, neuronal and cardiovascular disease prevention [12-23].

There are studies regarding the effect of anthocyanins in cancer treatments, the effect in human nutrition and its biological activity. These pigments exhibit homeostatic, hypotensive, cardioprotective and antitumoral properties [24,25]. During the last decade, TLC and HPTLC have become important analytical techniques [21-27]. High Performance Thin Layer Chromatography (HPTLC) methods present many advantages including: simple sample preparation, low operating cost, short analysis time and simultaneous analysis of several samples [1-5, 26-31].

In this paper is presented a high performance quantitative method for the quantification of anthocyanins (mv 3-glc, cy 3-glc, pn 3-glc and dp 3-glc) from cranberry, blueberry, bilberry, chokeberry and acai berry extracts.

Experimental part

Reagents

All the standards for anthocyanins used in this study were in the form of chlorides.

Cyanidin (3,30,40,5,7-pentahydroxyflavilium), malvidin (3,40,5,7-tetrahydroxy-30,50-dimethoxy flavilium), delphinidin (3,30,40,5,50,7-hexahydroxyflavilium) and peonidin (3,40,5,7-tetrahydroxy-30-methoxyflavilium) were supplied by (Germany). Pelargonidin (3,40,5,7-tetrahydroxyflavilium) and pelargonidin-3-glucoside were provided by (Germany). The dp 3-glc, mv 3-glc, cy 3-glc and pn 3-glc standards have been obtained from (Germany). Mv-3,5-diglc was purchased from (Germany).

For mobile phase, ethyl acetate (Merck, Darmstadt, Germany) preparation, 2-butanone and formic acid (Sigma-Aldrich) were used. The purified water system was purchased from Millipore (Germany). Methanol, hydrochloric acid (37% and 25%) and 2,2-diphenyl-1- picril hydrazyl radical (DPPH *) were purchased from Sigma-Aldrich.

All the reagents used for the biological tests, silica gel 60 F_{254} 20 × 10 cm HPTLC plates and potassium acetate, used for producing a controlled humidity during plates development were purchased from Merck.

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Analyzing solutions preparation

For the preparation of samples, 1 g of sample (cranberry, blueberry and bilberry extract) was individually weighed into a 10 mL volumetric flask to a four decimal analytical balance. For chokeberry and acai berry were weighed 20, respectively 500 mg.

The volumetric flasks were brought up to the mark with acidified methanol (a mixture of methanol and 25% hydrochloric acid, 4:1, v / v). The sample is stirred on an ultrasonic bath for 30 min at room temperature (heating to a higher temperature leads to the degradation of anthocyanins). Stock solutions are filtered through a 0.45 μm cellulose filter and transferred to a 1.8 mL container. The storage and preservation of the analyzing solution is done in a freezer at -20°C.

Blueberry and bilberry extract stock solutions were diluted at 1:20 and 1:500 with acidified methanol. The *p*H of the test solutions was 1. The final concentration of the analyzing solution was 100 mg / mL (cranberry), 5 mg / mL (blueberry), 0.2 mg / mL (bilberry), 2 mg / mL (chokeberry), 50 mg / mL (acai berry).

Reference solution preparation

For the preparation of anthocyanins stock solutions, 11 mg of each were individually dissolved in a 10 mL of 37% hydrochloric acid (0.6 mL) and methanol (120mL) mixture.

For the preparation of dp 3-glc, mv 3-glc, cy 3-glc, pn 3-glc (each 1.1 mg/10 mL), pg 3-glc (4.3 mg / mL) and mv-3, 5-diglc (1.6 mg/10 mL) stock solutions were individually dissolved in acidified methanol. For the preparation of standards mixture (1.1 mL), 20 μL dp, mv and cy, and 10 μL of pn and pg, 150 μL mv - 3,5-diglc, 250 μL of dp-3-glc, mv-3-glc and cy-3-glc, 110 μL of pn-3-glc and 10 μL of pg-3-glc were pipetted together in a container. The storage and preservation is made in the freezer at -20° C.

The optimization of mobile phase corresponding to anthocyanins separation

For the separation of anthocyanins (mv 3-glc, cy 3-glc, dp 3-glc, pn 3-glc, pg 3-glc and mv 3, 5-diglc) from cranberry, blueberry, bilberry, chokeberry and acai berry extracts, as mobile phase a mixture of ethyl acetate -2-butanone-water-formic acid in different volumetric ratios was tested (fig. 1). As volumetric ratios were tested 7/3/1/1, 7/3/1.2/0.8 and 7/3/0.8/1.

The best separation was achieved with ethyl acetate-2-butanone-water-formic acid, 7/3/0.8/1. For plates evaluation the mixture of standards was tested. Four different volumes of this mixture have been applied (4, 8, 12 and 16 μL).

Working procedure

After the preparation of samples and reference solution, the circuit traveled through by the sample and the syringe are washed with methanol (dilution solvent). In order to apply the samples a 25 μL syringe was used. This operation is performed automatically in order to remove any existing foreign trace which can contaminate the samples.

After washing the circuit, the vessels in which are analyzed the samples are placed in the autosampler, and the plate in its compartment. The vessels position in the autosampler must be identical to the one from the software. The method and sequence are created in the winCATS device software and then is started the application of samples and standards.

The sample application is done automatically using ATS4 (Camag) at a distance of 8 mm from the lower edge of the plate. This distance is called the starting line. The distance

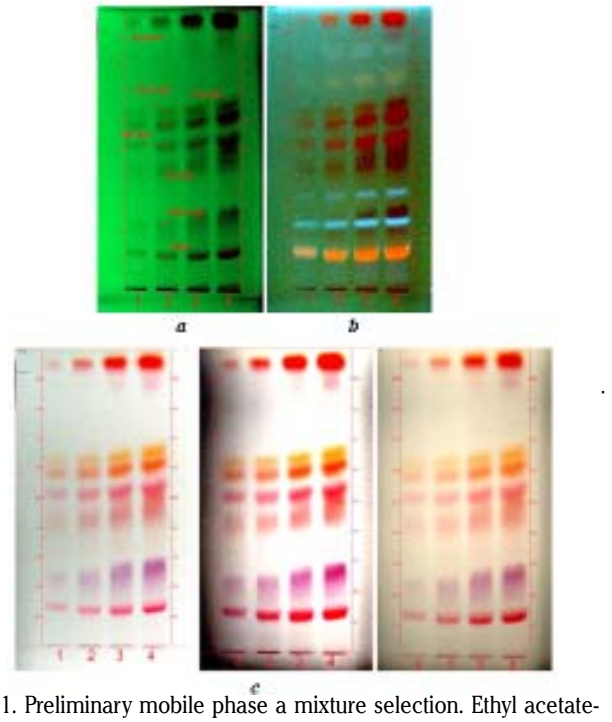


Fig. 1. Preliminary mobile phase a mixture selection. Ethyl acetate-2-butanone-water-formic acid (10:1.1:1.1:2, *v/v/v/v*: a) evaluation in UV light at 254 nm; b) evaluation in UV light at 366 nm; c) evaluation in white light (reflected, reflected transmitted, transmitted)

left - right (edges) for a plate with 13 applications was 30 mm. The distance between applications was 11.6 mm. The samples were applied in the form of 8 mm strips. The sample application rate was 150nL/s. After the application of samples, the plate is submerged automatically in ADC2 (Camag), which was previously washed with methanol. The plate is dried for 4 min in a stream of cold air, while the humidity of the development room becomes optimal, 25 ± 2%. The adjustment of the humidity is done using potassium acetate saturated solution (260 g per 100 g water).

The humidity at the beginning of the analysis was 29% and at the end was 23.7%. After drying, the plate is immersed in 10 mL of mobile phase (ethyl acetate /2-butanone – water - formic acid). The migration distance was 70 mm, and the migration time was 30 min. After the development, the plate was dried for 3 min in a stream of cold air and evaluated in transmitted white light using the TLC Visualizer (Camag) equipment. The exposure time in the transmitted white light was 30 ms. The spectrum registration is done by means of a TLC scanner 4 (Camag) at a 520 nm wavelength for mv 3-glc and pn 3-glc, at 530 nm for cy 3-glc and at 555nm for dp 3-glc using D2 & W (deuterium and tungsten) lamp (fig. 2 and 3). The obtained data were processed using winCATS software, 1.4.7.2018 version from Camag.

At the end, the plates are covered with a piece of glass with the same size as the plate and are wraped in aluminum foil.

Results and discussions

The quantification of anthocyanins by means of HPLC

The presence of anthocyanins in the analyzed samples was confirmed by applying the same sample in triplicate. As it can be seen from the chromatograms below (fig. 4 and 5), in the cranberry, chokeberry and acai berry extract dp 3-glc has not been identified. In the blueberry and bilberry extract is present. In none of the studied extracts was not found mv 3,5 diglc or pg 3-glc .

As it can be seen from the chromatogram shown below (fig. 6), in the cranberry sample (the first 2 applications)

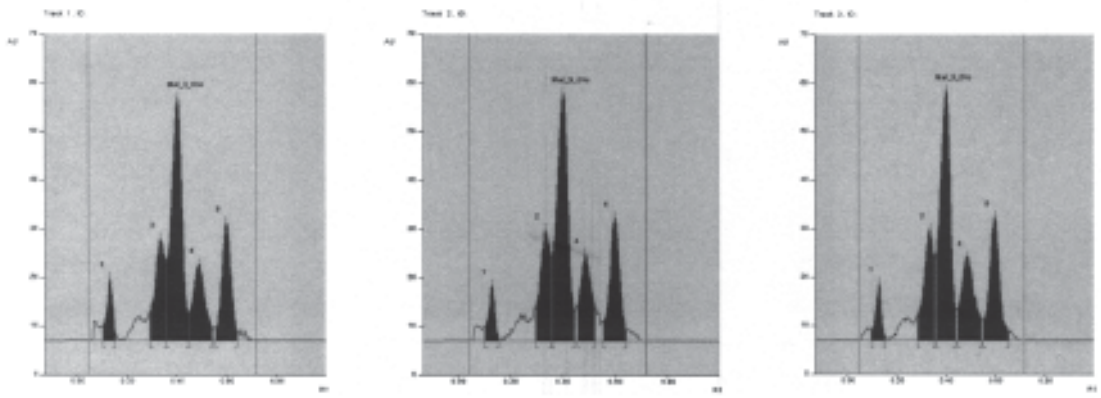


Fig. 2. The HPTLC chromatograms of malvidin in carnberry extract

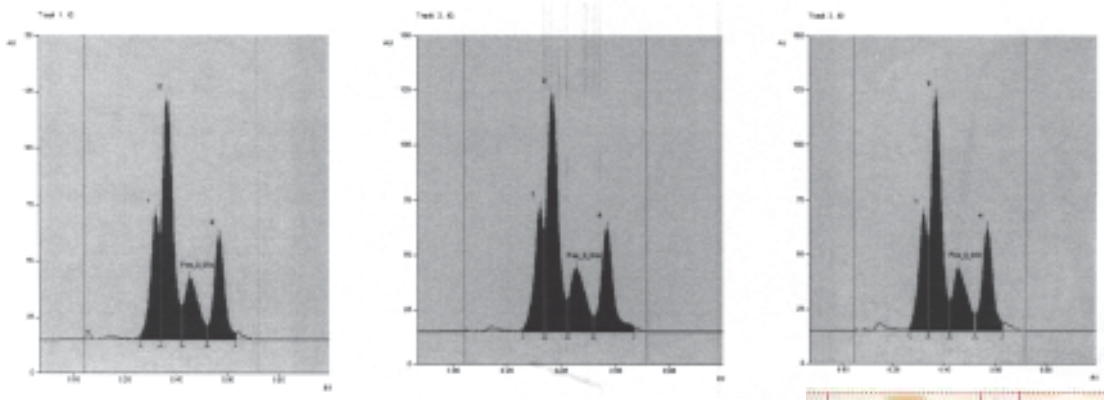


Fig. 3. The HPTLC chromatograms of peonidin in carnberry extract

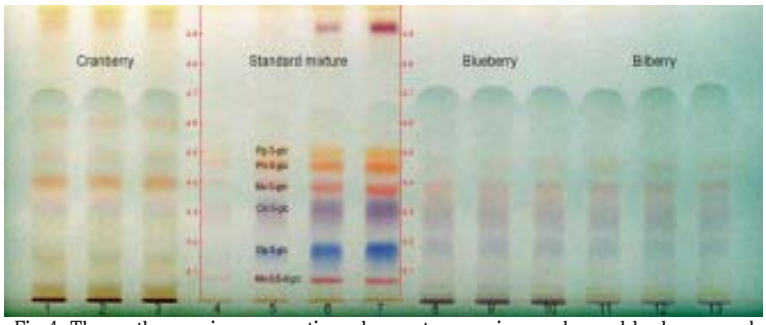


Fig.4. The anthocyanins separation chromatogram in cranberry blueberry and bilberry extracts

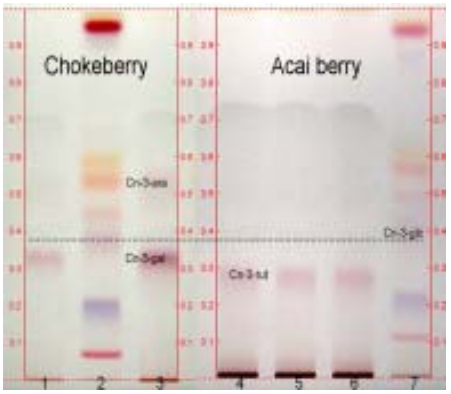


Fig.5. The anthocyanins separation chromatogram in chokeberry and acai berry extracts

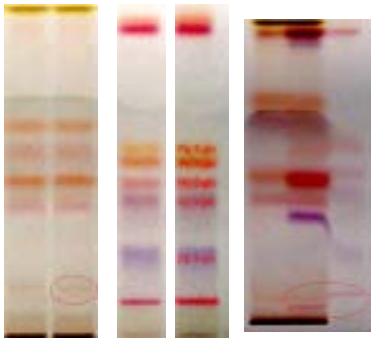


Fig.6. The identification of unknown compounds by the "Overlaping" method

besides the identified anthocyanins there is a spot that at first glance would seem to be mv 3.5diglc.

The identification of this compound was made by the "Overlaping" method. This method consists in applying equal volumes of sample and standard (6µL) in the form of a 15 mm strip.

The sample application is made at 20 mm from the right side of the plate (20 + 15 mm band size = 35 mm the distance at which ends the sample application) and the standard application starts at 27.5 mm from the right side of the plate and ends at 42.5 mm.

On the portion of 7.5 mm both sample and standard are present. The plate follows the same steps as is in the case of the quantification. After carefully examining the plate is observed that mv 3,5-diglc is not present in the cranberry sample (fig. 7).

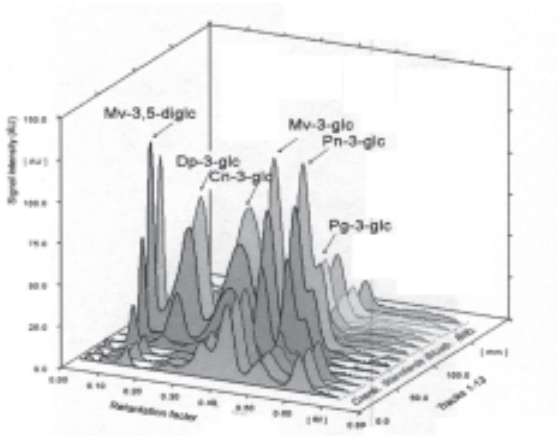


Fig. 7. The 3D chromatogram of the anthocyanins present in the cranberry, blueberry and billbery extracts

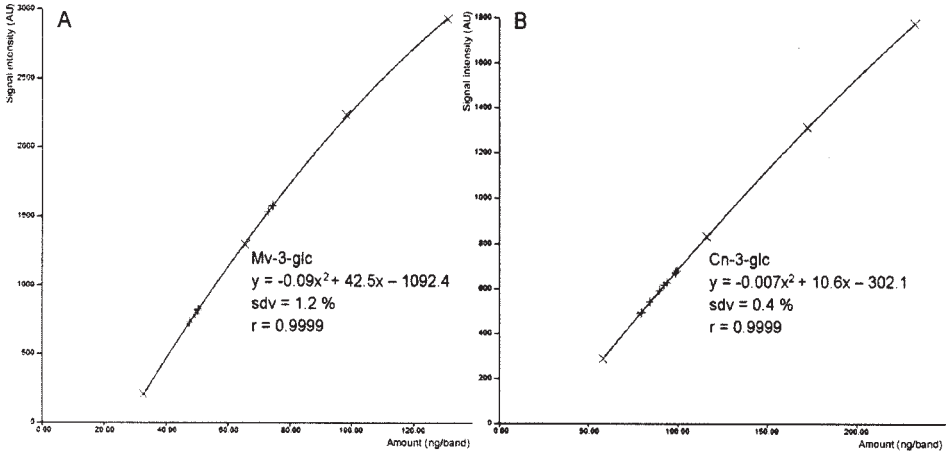


Fig. 8. Polynomial and linear calibration curves characterizing the anthocyanins present in cranberry, blueberry and billbery extracts, A – polynomial curve, B – linear curve

Dp 3-glc has a Rf of 0.19, cy 3-glc of 0.34, mv 3-glc of 0.40 and pn 3-glc of 0.46. The compounds present in the acai berry extract, cy cy 3-gal and cy 3-ara have a Rf of 0.32 and respectively 0.54. Cy 3-rut present in the extract of acai berry has a Rf of 0.28. The anthocyanins calibration curves (fig. 8) were characterized both by polynomial equations (mv 3-glc, cy 3-glc and pn 3-gl) and by linear equations (dp 3-glc).

The characteristics of the calibration curves are presented in table 1.

The anthocyanin content calculation procedure in the analyzed samples

From the calculations made by winCATS software, a good repeatability of the three applications was obtained. The repeatability was ≤ 3.6%.

In order to achieve the calibration curve for cy 3 - glc were applied four different volumes from the mixture of standards: 2 μL of this mixture represents 58.11 ng / μL, 4μL represents 116.22 ng / vL, 6 μL represents 172.33 ng / μL, 8μL represents 232.44 ng / μL.

In order to achieve the calibration curve for mv 3-glc were applied four different volumes from the mixture of standards: 2μL of this mixture represents 32.80 ng / μL, 4μL represents 65.59 ng / μL, 6 μL represents 98.39 ng /μL, 8μL represents 131.19 ng / μL.

In order to achieve the calibration curve for pn 3-glc were applied four different volumes from the mixture of standards : 2 μL of this mixture represents 232.32 ng / μL, 4μL represents 44.64 ng / μL, 6 μL represents 65.95 ng /μL, 8μL represents 89.97 ng / μL.

In order to achieve the calibration curve for dp 3-glc were applied four different volumes from the mixture of standards : 2μL of this mixture represents 50.72 ng / μL, 4μL represents 101.44 ng / μL, 6 μL represents 152.16 ng / μL, 8μL represents 202.89 ng / μL.

The software calculates the amount of cy, mv, pn and dp expressed in ng / band. In order to express the results in percentages the quantity expressed in ng / band is divided with the applied volume from each sample and then to the concentration of each solution applied.

After this calculation, the amount of anthocyanin is expressed in ppm (mg / kg). With the help of the relation 1 ppm = 0.0001%, the amount is expressed in percents. The amount of mv 3-glc, cy 3-glc, pn 3-glc and dp 3-glc is shown in table 2.

The predominant anthocyanins in the chokeberry extract were identified as being cy 3-gal and cy 3-ara, and the ones in the acai berry extract as being cy 3-rut and cy 3-glc. The anthocyanins present in those 2 extracts were evaluated as being cy 3-glc since the colour spots present in the chromatogram showed the same colour as the one of cy-3-glc.

In order to achieve the calibration curve for 3-cy glc four different volumes from the standards mixture were applied as follows: 4 μL of this mixture represents 116.22 ng / μL, 8 μL represents 232.44 ng / μL, 12 μL represents 348.66 ng / μL and 16 μL represents 464.88 ng / μL.

Compound	Polynomial/linear calibration (evaluation by height / area)	Calibration domain (ng/band)	Correlation coefficient	Relative standard deviation (sdv, %)
Dp 3-glc	Y_A = 6.09x - 170.69 Y_A = - 0.007x2 + 10.56x - 302.09	50-210	0.99995	0.60
Cy 3-glc	$Y_{\hat{I}}$ = - 0.004x2 + 1.59x - 36.65 $Y_{\hat{I}}$ = - 0.005x2 + 1.80x - 15.29	50-250	0.99999	0.36
Mv 3-glc	Y_A = - 0.029x2 + 27.699x – 690.480	30-140	0.99955	3.39
Pn 3-glc	Y_A = 6.09x - 170.69	20-90	0.99884	4.46
Cy-3-glc chokeberry	Y_A = - 0.007x2 + 10.56x - 302.09	50-240	0.99988	1.47
Cy-3-glc acai berry	$Y_{\hat{I}}$ = - 0.004x2 + 1.59x - 36.65	50-240	0.99949	2.99

Table 1
CHARACTERISTICS OF THE CALIBRATION CURVES USED FOR ANTHOCYANINS DETERMINATION

The cy 3-glc amount of acai berry and chokeberry extract evaluated as cyanidin 3-glucoside is shown in table 3.

From the above tables it can be seen that the richest extract in anthocyanin is bilberry, followed by chokeberry, blueberry, acai berry and cranberry.

Identification of unknown compounds from chokeberry and acai berry extracts by mass spectrometry

HPLC-ESI MS spectra were directly recorded using TLC MS interface (Camag), equipped with an oval elution head (2x4 mm).

The interface was coupled to a mass spectrometer with a single qvadrupole (CMS expression, Advion, Ithaca, NY).

Methanol was used as an elution solvent at a flow rate of 100 μ / min (pump HP 1100, Agilent, Waldbronn, Germany). The MS system was continuously operated. For the ionisation in the positive mode, the detector parameters are: ESI voltage was set to 4 kV, the capillary voltage was set at 184 V, the source voltage was set to 40 V and the dynamic voltage of the source was 25 V. The gas pressure was 60 psig and the flow rate was 4 L / min.

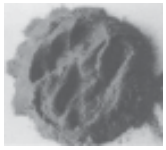
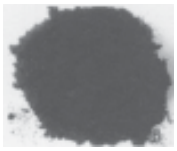
The extract solution concentration	Applied volume (μL)	Content (ng/band)	Content (%)	Repeatability (*%RSD*, n = 3)
Cranberry (100 mg/mL) Cy 3-glc 	3	P_1 = 99.50 P_2 = 98.66 P_3 = 98.45 Average = 98.87	P_1 = 0.0332 P_2 = 0.0329 P_3 = 0.0328 Average= 0.0330	0.56
Mv 3-glc	1.5	P_1 = 64.29 P_2 = 65.21 P_3 = 65.69 Average = 65.06	P_1 = 0.0429 P_2 = 0.0435 P_3 = 0.0438 Average= 0.0434	1.09
Pn 3-glc	3	P_1 = 25.81 P_2 = 26.90 P_3 = 26.81 Average = 26.51	P_1 = 0.0086 P_2 = 0.0090 P_3 = 0.0286 Average = 0.0089	2.29
Blueberry (5 mg/mL) Dp 3-glc 	2	P_1 = 121.09 P_2 = 128.98 P_3 = 124.36 Average = 124.36	P_1 = 1.2109 P_2 = 1.2898 P_3 = 1.2436 Average= 1.2481	3.17
Cy 3-glc	3	P_1 = 93.68 P_2 = 92.38 P_3 = 89.72 Average = 91.93	P_1 = 0.6245 P_2 = 0.6159 P_3 = 0.5981 Average= 0.0283	2.19
Mv 3-glc	2	P_1 = 47.98 P_2 = 47.28 P_3 = 46.04 Average = 47.10	P_1 = 0.4798 P_2 = 0.4728 P_3 = 0.4604 Average= 0.4710	2.08
Pn 3-glc	3	P_1 = 25.88 P_2 = 26.36 P_3 = 26.33 Average = 26.19	P_1 = 0.1725 P_2 = 0.1757 P_3 = 0.1755 Average= 0.1746	1.02
Bilberry (0.2 mg/mL) Dp 3-glc	2	P_1 = 117.25 P_2 = 115.18	P_1 = 29.3125 P_2 = 28.7950	2.60

Table 2
ANTHOCYANIN QUANTIFICATION IN CRANBERRY, BLUEBERRY, BILBERRY, CHOKEBERRY AND ACAI BERRY EXTRACTS

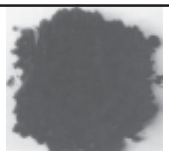
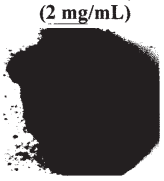
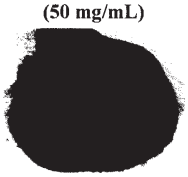
		P_3 = 121.22 Average = 117.88	P_3 = 30.3050 Average=29.4708	
Cy 3-glc	3	P_1 = 84.49 P_2 = 79.12 P_3 = 79.59 Average = 81.06	P_1 = 14.0817 P_2 = 13.1867 P_3 = 13.2650 Average=13.5111	3.67
Mv 3-glc	2	P_1 = 47.30 P_2 = 47.24 P_3 = 47.80 Average = 47.45	P_1 = 14.0817 P_2 = 13.1867 P_3 = 13.2650 Average=13.5111	0.64
Pn 3-glc	3	P_1 = 27.66 P_2 = 27.14 P_3 = 28.34 Average = 27.71	P_1 = 4.6100 P_2 = 4.5233 P_3 = 4.7233 Average = 4.6188	3.60
Extract solution concentration	**Applied volume (µL)**	**Content (ng/band)**	**Content (%)**	**Repeatability (*%RSD*, n = 3)**
Chokeberry (2 mg/mL) 	0.4	P_1 = 122.35 P_2 = 125.34 P_3 = 125.56 Average = 124.42	P_1 = 15.2938 P_2 = 15.6675 P_3 = 15.6950 Average = 15.5521	1.44
Acai berry (50 mg/mL) 	2	P_1 = 138.56 P_2 = 141.85 P_3 = 139.68 Average = 140.03	P_1 = 0.1386 P_2 = 0.1419 P_3 = 0.1397 Average = 0.1400	1.2

Table 3
CY 3-GLC QUANTIFICATION IN ACAI BERRY AND CHOKEBERRY EXTRACT

The capillary and the gas source temperature were set at 250°C each. The evaluation and processing were performed with the Advion Mass Express 1.1.22.15 software.

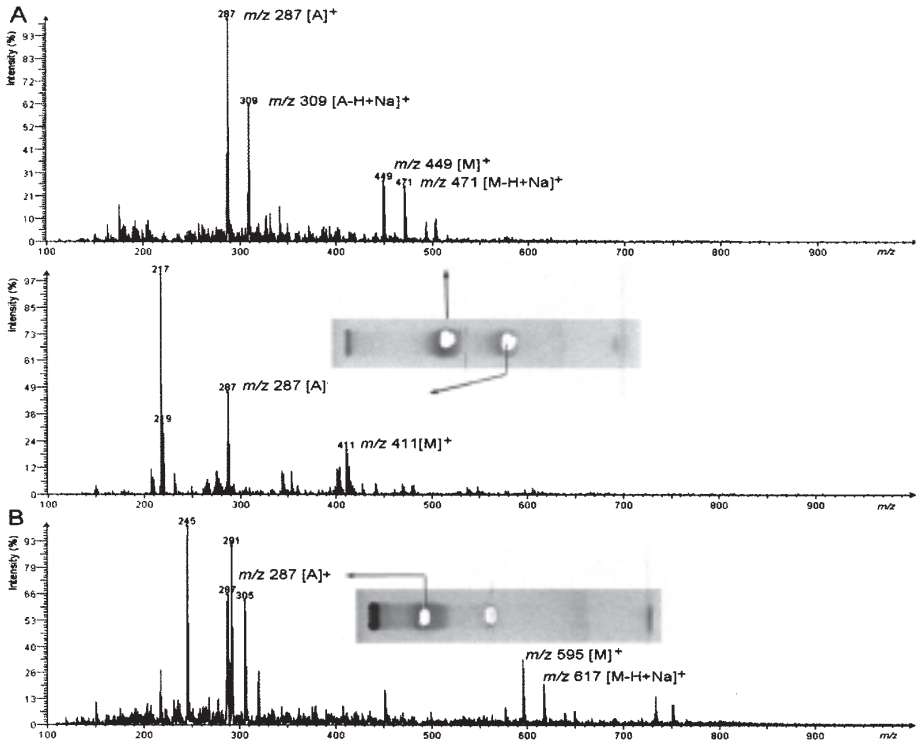


Fig. 9. Chokeberry and acai berry extracts identification by means of HPTLC-ESI-MS

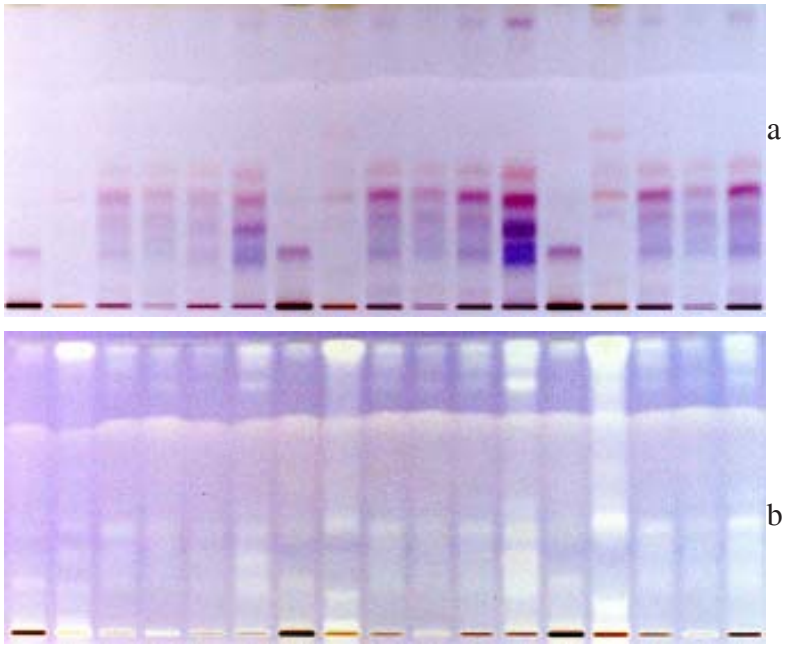


Fig. 10. DPPH* detection before DPPH* derivatization (a) and after DPPH* derivatization (b):1, 7, 13 applications– acai berry samples (50 mg/mL); 2, 8, 14 applications – cranberry samples (100 mg/mL); 3, 9, 15 applications – blueberry samples (5 mg/mL); 5, 11, 17 applications – blueberry samples (10 mg/mL); 4, 10, 16 applications – bilberry samples (0.2 mg/mL); 6, 12 applications – bilberry samples (2 mg/mL)

In the chokeberry extract, two unknown anthocyanins that are having the same color as that of the cyanidin were identified by positive ion mass spectrometry.

The mass signal from m / z 449 $[M]^+$ in the first spot (R_F 0.32) corresponds to cn-3-gal. The mass signal from m/z 411 $[M]^+$ in the second spot (R_F 0,54) corresponds to cn-3-ara. The cyanidin was confirmed by aglycon mass m / z 287 $[A]^+$, which was visible in both spots. This was also found in acai berry. The mass signal at m / z 595 [M] +, can be identified as being cn 3-rut (fig, 9).

DPPH detection*

Aliivibrio fisheri bacteria detection

In the figures below (fig. 11) are shown the anthocyanins bioactivity present in the cranberry, blueberry and bilberry extracts, by *Vibrio fischeri* bacteria immersion.

For the detection of these compounds different volumes from the samples and standards mixture were applied. From the cranberry (fig. 11 A) samples were applied volumes of 15, 20 and 30 μL, from the blueberry samples were applied volumes of 30, 40 and 50 μL, from the bilberry samples (fig. 11 B) were applied volumes of 60, 70, 80 and 90 μL and from standard mixture were applied the following volumes: 15, 20, 25, 30, 35, 40 μL.

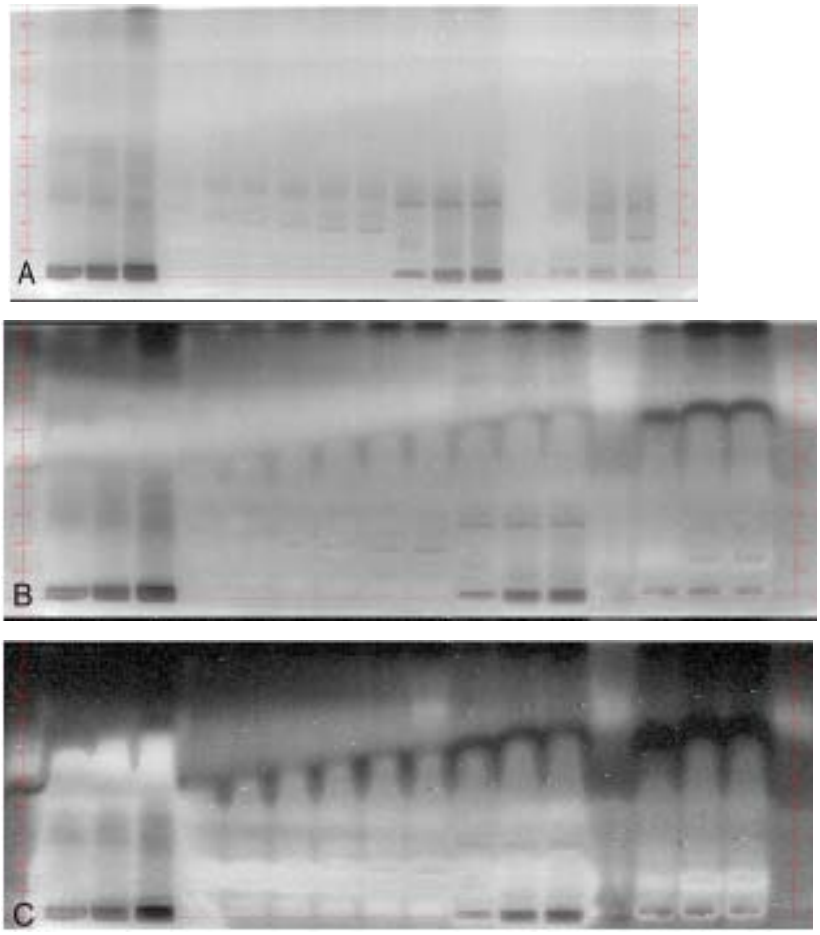


Fig. 11. Bioluminescence detection in cranberry, blueberry and bilberry extracts at: A) 1 min after immersion, B) and C) 30 min after immersion

The solutions applied from the samples were too diluted and the detection of their bioactivity was not a conclusive. The bioluminescence was monitored for 30 min after immersion (fig. 11 C). The first 3 applications are from cranberry extract (15, 20, 30 μL), the next 6 applications are from standards mixture (15, 20, 25, 30, 40μL), the next 3 applications are from blueberry extract (30, 40, 50 μL), and the last 4 are from bilberry extract (60, 70, 80, 90 μL).

Conclusions

For the first time the most important anthocyanins (dp 3-glc, cy 3-glc, mv3-glc, pn 3-glc, pg 3-glc and mv 3,5-diglc) were identified and quantified by thin layer chromatography using a single mobile phase.

By coupling the thin-layer chromatography with mass spectrometry (TLC / MS) was confirmed the presence of cyanidin in chokeberry and acai berry extracts.

The anthocyanins present bioactivity (detection with *Aliivibriofisheri* bacteria) and antioxidant properties (DPPH detected *).

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