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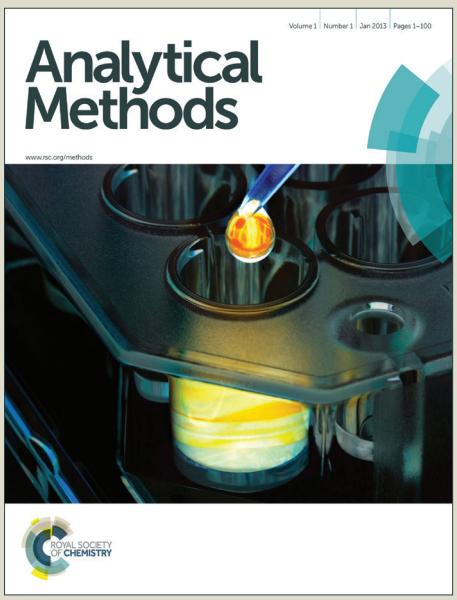

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Validation of HPLC assay for the identification and quantification of anthocyanins in black currants

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Abstract

Many publications describe separation and quantification techniques for anthocyanins. However, chromatographic conditions to optimize the separation were seldom reported. We developed a sensitive and reliable HPLC method and validated the method for anthocyanin separation, identification, and quantification. Separation was performed on a Zorbax SB C18 column. The conditions including ion pair reagent, mobile phase pH, and mobile phase flow rate were optimized to improve the separation efficiency. Tandem MS was used to identify the total 6 anthocyanins in black currant. Validation of the developed method showed good linearity, precision, repeatability, and excellent recovery. The validated method was used to quantify anthocyanins from black currant samples.

1. Introduction

Consumption of foods that contain polyphenolic compounds are associated with reduced risk of chronic and degenerative diseases in humans.[1,2] Researchers have demonstrated that polyphenols contribute significantly to antioxidant capacity in fruits and vegetables.[3] Epidemiological studies have suggested positive correlations between the consumption of foods rich in polyphenols and a reduced risk and mortality of chronic disease, such as cancers and heart disease.[4] Anthocyanins are one of the important phenolic compounds in fruits and vegetables in human diet. Anthocyanins are the glycosidic forms of anthocyanidins that produce blue, red, and purple colors in flowers and fruits. Major anthocyanins in fruits are based on the cyanidin aglycon. Anthocyanins are widely used as nutrition supplements, constituents in functional food formulations, medicines, etc.[5] Potential health benefits of anthocyanins include antioxidants, anti-inflammatory agents, and protection against cardiovascular disease.[6,7] Cyanidin 3-O-rutinoside was reported to induce a significant apoptosis of leukaemic cells as an antitumor agent.[8] Chen et al. discovered that cyanidin 3-O-glucoside and cyanidin 3-O-rutinoside inhibited in vitro invasion and motility of cancer cells.[9] Furthermore, anthocyanins can be used as natural food colorants instead of artificial pigments.[10]

Methods to characterize chemical structure and accurately quantify anthocyanin content in food are important to the food industry, especially in the study of food composition and its effect on human health. The common method in industry is to quantify total anthocyanins in raw materials using spectrophotometer at 520 nm with a controlled pH. This method can rapidly measure the total anthocyanins with milder conditions. However, this method cannot provide accurate quantification due to the variability in sample extraction media, chemical properties of anthocyanins, and molar absorptivity of anthocyanins. Another drawback of this method is that it cannot provide detailed information of individual anthocyanin.

Recently, HPLC and LC-MS has being widely used in separation and identification of anthocyanins, and to some extent, in quantification.[11-14] HPLC with both diode array detector (DAD) and MS detectors is sensitive and efficient for the separation and detection of multiple components based on UV absorbance and MS fragmentation behavior. For example, Vian et al. identified nine anthocyanins in the extracts of skin from Syrah grapes by HPLC with diode array detection, including 3-monoglucosides of delphinidin, cyanidin, petunidin, peonidin, and malvidin, and the acetylglucosides and p-coumarylglucosides of malvidin and peonidin.[15] HPLC coupled to MS is a sensitive tool to identify structure information. With tandem MS, it elucidates anthocyanins by investigating the aglycon moiety and number and types of sugars even without standards. Alberts et al. reported a rapid, high-efficiency ultra high-pressure liquid chromatography (UHPLC) procedure with tandem mass spectrometric (MS/MS) detection for the in-depth screening of wine pigments.[16] Selective detection of wine anthocyanins and derived pigments was achieved utilizing MS/MS in neutral loss scanning mode to observe the loss of dehydrated sugar moieties. By comparing the characteristic fragmentation patterns, the anthocyanins were finally identified.

The major challenge for quantification of anthocyanins using HPLC is that there are no commercial standards for most anthocyanins. However, most of anthocyanins have an aglycon backbone containing one of the anthocyanidins, including cyanidin, delphinidin, petunidin, peonidin, malvidin, and pelargonidin (Fig. 1). These anthocyanidins can be used for quantification of anthocyanins in fruits. In this work, we developed an analytical procedure to separate, identify, and quantify anthocyanins using HPLC-DAD and LC-MS/MS followed by a method validation. We optimized liquid chromatography conditions for anthocyanin separation. The validated method was utilized to separate and quantify anthocyanins in black currants.

2. Materials and methods

2.1. Samples

Four black currant extraction powders (S1-4) and one liquid black currant extract (S5) were obtained from Complete Phytochemical Solutions, LLC (Cambridge, WI). The powders were stored at room temperature and liquid extraction was stored at 4 °C. All samples were analyzed within one month of acquisition.

2.2. Chemicals and reagents

Chemical standard of cyanidin 3-O-rutinoside was purchased from Extrasynthese (Genay Cedex, France). Water and methanol of HPLC grade, trifluoroacetic acid (TFA), and formic acid (FA) were from Fisher Scientific (PA, USA).

2.3. Instrumentation

2.3.1. HPLC–DAD analysis

The HPLC system consisted of a Waters automated gradient controller, two Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector. A Waters 996 diode array detector using Waters Empower software for collecting and analyzing three-dimensional chromatograms was used to monitor the elution. 100 µL of sample was injected onto an Agilent Zorbax SB C18 column (4.6 × 150 mm, 5µm, 80Å) with a guard column (4.6 × 12.5 mm, 5µm). The solvents for elution were water (mobile phase A) and methanol (mobile phase B) containing various amounts of TFA or FA. The HPLC elution program was as follows: the first 5 min isocratic at 5% B; 5–45 min, B increased linearly from 5 to 50% followed by reconditioning of the column.

2.3.2. HPLC-MS analysis

Anthocyanin identification was conducted on an Eksigent 2D LC coupled to Thermo LTQ mass spectrometer. The sample (5 µL) was loaded on an Agilent Zorbax SB C18 column (0.3 × 150 mm, 5µm, 80Å) at a flow rate of 6 µL/min. The other separation conditions are the same as in HPLC–DAD analysis. The eluted compounds are injected into a Thermo Fisher linear ion trap (LTQ) mass spectrometer (Thermo Fisher, San Jose, CA) using electrospray ionization (ESI). The spray voltage was set at 5.0 kV and the temperature of the heated capillary was 275 °C. Nitrogen was used as the sheath (75 psi) and auxiliary (10 units) gas. The mass spectrometer was operated in a data-dependent mode in which a survey scan (*m/z* 100–1400) was followed by MS/MS scans of the top three most abundant ions with a collision energy of 35% normalized CID energy.

Dynamic exclusion was used with the following settings: exclusion list size, 250; repeat count, 2; repeat duration, 30 s; exclusion time, 180 s; exclusion window, ±0.5 Da.

2.4. Preparation of standard and sample solutions

The standard stock solution of cyanidin 3-O-rutionoside was prepared by dissolving 5 mg compound in 25 mL methanol to obtain a concentration of 200 mg/L and stored at −20 °C. A certain amount of the stock solution was transferred to 50 mL volumetric flask to prepare working solution with water to obtain appropriate concentrations of 0.1, 0.5, 1, 5, 12.5, and 25 mg/L. All sample solutions were prepared by dissolving approximate 0.1 g sample extraction in 50 mL of 50% methanol aqueous solution. The sample solutions were stored at 4 °C. The prepared sample solutions were further diluted 10 times before injection for HPLC analysis.

2.5. Validation of the HPLC method

2.5.1. Calibration curves, limit of detection (LOD), limit of quantification (LOQ)

The calibration curves were established by plotting the peak areas versus the concentration of each working solution. The LOD and LOQ were determined at S/N = 3 and 10, respectively.

2.5.2. Precision and stability

The precision of the HPLC analysis method was established by analyzing the standard solution at three concentrations of 0.2, 5, and 25 mg/L, individually. To determine the intra-day precision of standard solution, the standard solutions (0.2, 5, and 25 mg/L) were examined five times within one day. The inter-day precision was established by analyzing standard solution on four consecutive days. The precision of the assay was expressed by the RSD (%) of the replicate measurements of peak area. In order to examine the repeatability, five different sample solutions prepared from the same sample (S1) were analyzed. Stability was tested with sample solution (S1) at 0, 1, 2.5, 4, 8, and 24 h. The RSD was taken as a measurement of repeatability and stability.

2.5.3. Recovery study

The recovery study was used to evaluate the accuracy of the developed method, which was carried out by spiking known amounts of standard solution into samples before injection. The follow-up HPLC analyses were performed as described in section 2.3.2. Two aliquots of HPLC sample solutions (1 mL each) of S1 (original amount) were spiked separately with 100 μL and 50 μL of freshly prepared cyanidin 3-O-rutinoside standard solutions (50 mg/L). The recoveries were calculated as follows: recovery (%) = (amount found − original amount)/amount spiked × 100%.

3. Results and discussion

3.1. Optimization of chromatographic conditions

Since anthocyanins have complex structures, they are still challenging to accurately quantify. Therefore, the LC separation has to be carefully optimized to provide efficient separation of anthocyanins with good resolution to ensure accurate quantification. We used reversed-phase (RP) chromatographic separation due to its high sensitivity, selectivity, good performance, and relative short analysis time. Ion-pairing agents are

generally added in mobile phases for RPLC to improve peak shape, thus increase resolution. TFA and FA are typical ion-pairing agents for RPLC. With 0.1% TFA in mobile phases, anthocyanins in black currant eluted between 30 and 40 min as shown in Fig. 2. Anthocyanin1 and 2 (An1 and An2) were not base line separated. Since FA is a MS compatible additive in mobile phases, we then added FA in mobile phases instead of TFA. At 1% FA in mobile phases, anthocyanins eluted around 30 min with poor resolution and poor peak shape. With the increase of FA to 5%, anthocyanins eluted earlier with good resolution. When 10% FA was employed, anthocyanins eluted in 20 min with better resolution and peak shape. The reduced retention time and improved peak shape with the increased FA amount in mobile phase is due to the formation of neutral ion pairs of FA and positively charged anthocyanins.

As reported previously, anthocyanins exist in solutions as a mixture of four different species: flavylium cation, quinoidal base, carbinol pseudo base, and chalcone.[17,18] At pH below 3, the flavylium cation predominates in the mixture. When the pH increases, hydration of flavylium cation produces carbinol pseudo base, which can further yield the chalcone due to a thermodynamic reaction. At the same time, proton transfer involving the hydroxyl groups produces quinoidal base due to a kinetic reaction. de Villiers et al. studied these secondary reactions using chromatography.[19] Therefore, a low mobile phase pH is essential to keep flavylium species and improve chromatographic efficiency in separation of anthocyanins. As a result, separation with mobile phases containing 10% FA (pH 1.34 in mobile phase A) exhibited best performance than mobile phases containing 5% FA (pH 1.83 in mobile phase A), 1% FA (pH 2.13 in mobile phase A), and 0.1% TFA (1.95 and in mobile phase A). However, mobile phases with a very low pH will reduce column life dramatically. As a trade-off, we finally chose 5% FA as mobile phase additive.

Mobile phase flow rate also affects chromatographic separation. In order to observe the optimal separation flow rate, we constructed plate heights with the conditions obtained above. The mobile phase was the mixture of 75% mobile phase A and 25% mobile phase B, where A was 5% FA in water and B was 5% FA in methanol. Cyanindin 3-O-rutinoside was used as probe compound. This mobile phase ensured the retention factor of cyanindin 3-O-rutinoside larger than 2.5. The plate height curve is presented in Fig. 3. With the flow rate increased from 0.2 to 2 mL/min on the 4.6 mm i.d. column, the plate height increased from 13 to 36 μm. Obviously, the optimal flow rate is lower than 0.2 mL/min. The minimum plate height is approximate $2d_p$ (d_p, particle size), which is typical for RPLC. However, due to the practical considerations such as analysis time, gradient separations were performed at 1.5 mL/min on this column. Although the plate height value was 32 μm corresponding to $6d_p$ at the flow rate of 1.5 mL/min, the gradient separation provided adequate resolution for quantification of anthocyanins.

3.2. Identification of anthocyanins in black currants

Positive ion mode was used in the MS analysis due to more sensitivity and response for investigated analytes because flavylium cations are the main species in acidic mobile phases. The identification of anthocyanins was based on mass measurement, LC-MS/MS spectra, and compared with literature published previously.[12,20] The data used for

identification of anthocyanins in black currants is given in Table 1. An example of MS and MS/MS mass spectra for anthocyanin identification were presented in Fig. 4, in which 6 anthocyanin peaks in sample S1 were separated and the corresponding chromatographic peaks were extracted (Fig. 4A) and the MS and MS/MS spectra of the 6 anthocyanins were presented (Fig. 4B and C). The measured masses are close to the theoretical values. For example, the measured molecular weight of An4 was 595.25, which could be cyanidin 3-O-rutinoside (595.52 in theory). Two major fragment ions at *m/z* 287.12 and 449.15 were detected. The ions at *m/z* 287.12 corresponded to the loss of coumaroyl-glucoside (loss *m/z* 308) and cyanidin (*m/z* 287.12) was detected. The ions at m/z 449.15 corresponded to the loss of one glucoside in the rutinoside (loss *m/z* 146) (Fig. 5). The other fragment ions and possible cleavages of the separated anthocyanins were also exhibited in Fig.5. Therefore, by comparing literatures[12] and current MS spectra, An4 can be identified as cyanidin 3-O-rutinoside. Based on all the MS and MS/MS spectra, the 6 anthocyanins (An1-6) were identified as delphinidin 3-O-glucoside, delphinidin 3-O-rutinoside, cyanidin 3-O-glucoside, cyanidin 3-O-rutinoside, petunidin 3-(6″-coumaroyl) glucoside, and peonidin 3-(6″-coumaroyl) glucoside, individually. From Fig. 4A, we can see that the ion intensity (normalized level, NL) of An5 and An6 was over 10 times lower than that of the other 4 anthocyanins. This is in accordance with the chromatogram in Fig. 2, where peak height and area of An5 and 6 were much lower than other anthocyanin peaks, which is similar to published data.[12]

3.3. Method validation

3.3.1. LOD and LOQ

The calibration curve showed good linear regression (Y=175144X-5021) with coefficient $r^2 > 0.999$ within the test ranges. The LOD (S/N = 3) and the LOQ (S/N = 10) were 0.979 and 2.968 mg/L, respectively.

3.3.2 Precision and stability

Precision was expressed via repeatability and reproducibility measurements. The intra-day variations at three concentrations of 0.2, 5, and 25 mg/L were 1.56%, 0.94%, and 0.10%, respectively. The inter-day variations at three concentrations were 0.86%, 0.68%, and 0.80%, respectively. These data demonstrated excellent reproducibility of the method. Good repeatability with RSD of 1.08% for the investigated anthocyanin was measured. The RSD of the cyanidin 3-O-rutinoside was 1.89% with 24 h in the stability study, indicating that the sample solution is stable for at least 24 h when stored at 4 °C.

3.3.3. Recovery study

Two aliquots of HPLC S1 sample solutions (1 mL each) that contained 9.52 mg/ L of cyanidin 3-O-rutinoside (original amount) were spiked separately with 100 µL (containing 0.005 mg) and 50 µL (containing 0.0025 mg) of freshly prepared cyanidin-3-rutinoside standard solution (50 mg/L). This theoretically afforded 13.2 and 11.448 mg/L cyanidin 3-O-rutinoside in resultant spiked solutions. Analysis of these solutions against the calibration curve of cyanidin 3-O-rutinoside resulted in 13.23 and 11.46 mg/L amounts of the target analyte. This indicated a spiked recovery of 100.7% and 100.5% at spike levels of 138% and 126% of the originally present cyanidin 3-O-rutinoside, respectively. The recovery study demonstrated excellent method accuracy. All these data

proved that the developed HPLC-DAD method was precise, accurate, and stable for quantitative analysis of anthocyanins in black currants.

3.4. Quantification analysis of anthocyanins

The developed and validated HPLC-DAD method was applied to quantify anthocyanins in black currants. Each sample was analyzed three times. Based on the calibration curve and the measured peak areas of each separated anthocyanins in the 5 samples, the individual concentration of anthocyanin in each black currents were obtained (Table 2). The four major anthocyanins (An1-4) contributed more than 98% of the total content of anthocyanins in black currants, which is in agreement to published data.[21] However, anthocyanin content varied greatly in different samples.

4. Conclusions

In this work, we developed an HPLC-DAD method to separate and quantify anthocyanins in black currant. The chromatographic conditions were optimized to ensure highly efficient separation to provide enough resolution for accurate quantification. The LC-MS/MS was used to identify the separated anthocyanins. The validation showed that the developed method is precise, repeatable, and accurate. Finally, the developed method was employed to quantify anthocyanins in various black currant samples. This work provided an example for anthocyanin quantification. However, the developed method could be utilized to analyze anthocyanins in more complicated samples with further optimized chromatographic conditions.

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Notes

The authors declare the following financial interest(s): Jess D. Reed and Christian G. Krueger have ownership interest in Complete Phytochemical Solutions, LLC and in full disclosure of their affiliation with this company is acknowledged in the author affiliation.

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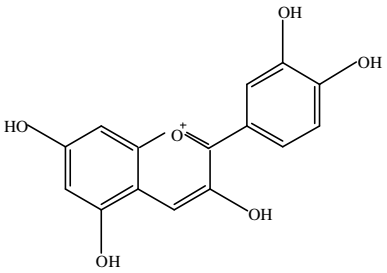
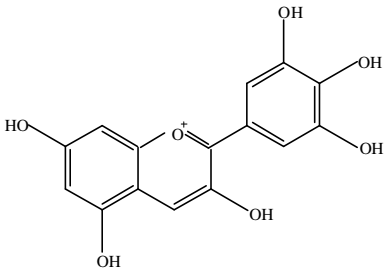
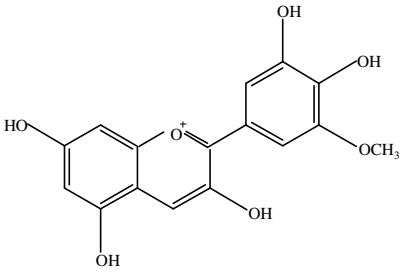


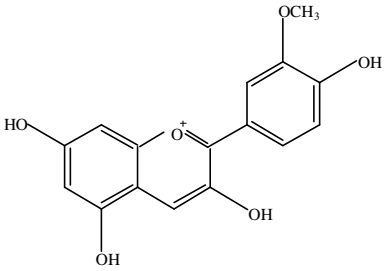
Cyanidin



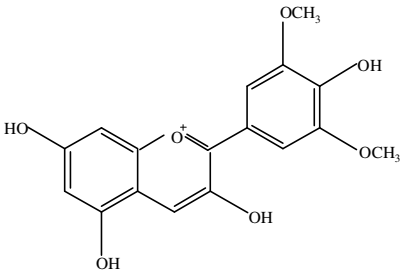
Delphinidin



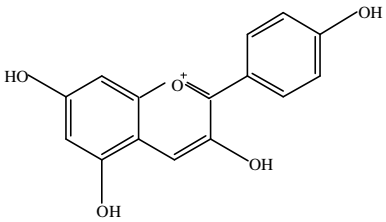
Petunidin



Peonidin



Malvidin



Pelargonidin

Fig. 1 Structures of anthocyanidins

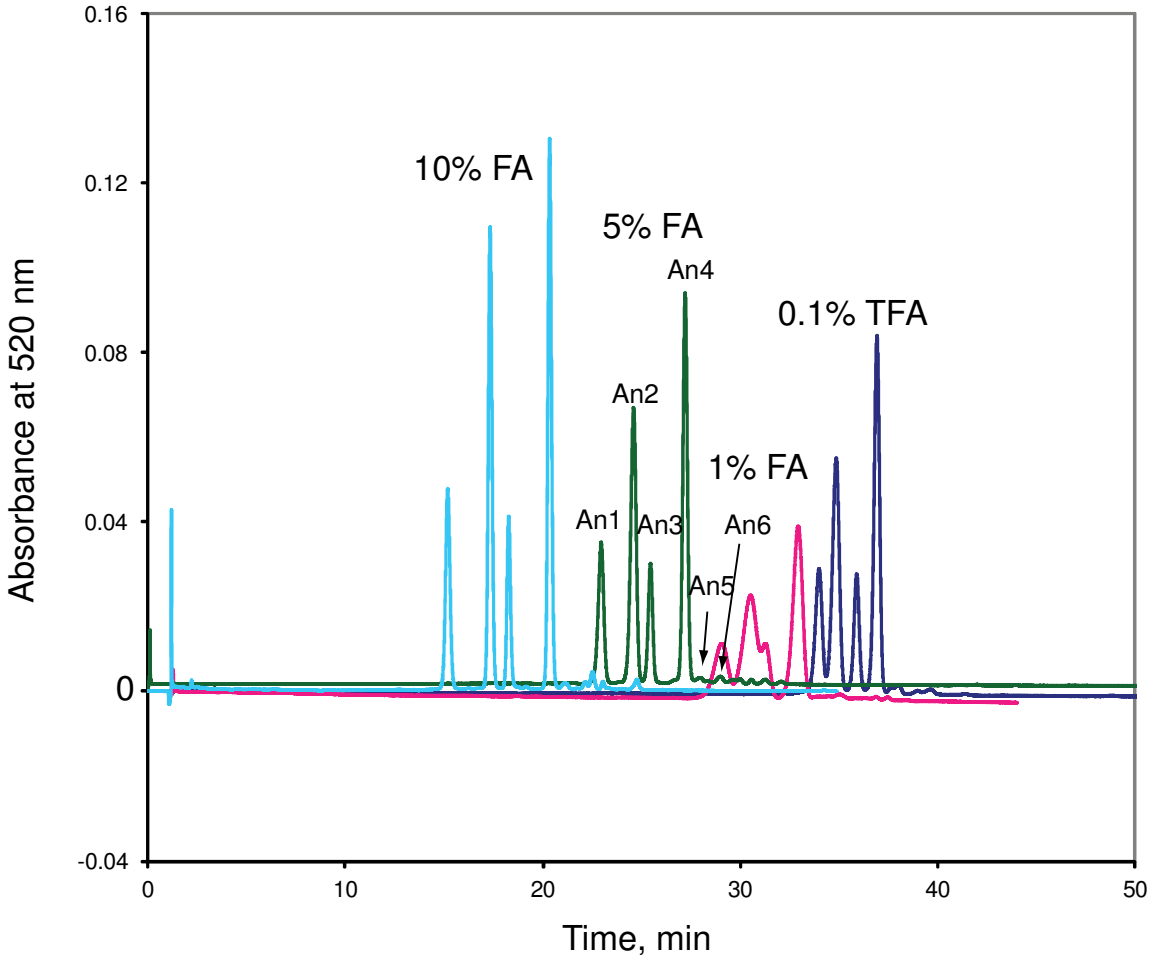


Fig. 2 LC-DAD chromatograms at 520 nm for the separation of anthocyanins with various ion-pair additives in mobile phases. An1-6 correspond to anthocyanin 1-6. Separation conditions are in section 2.3.1.

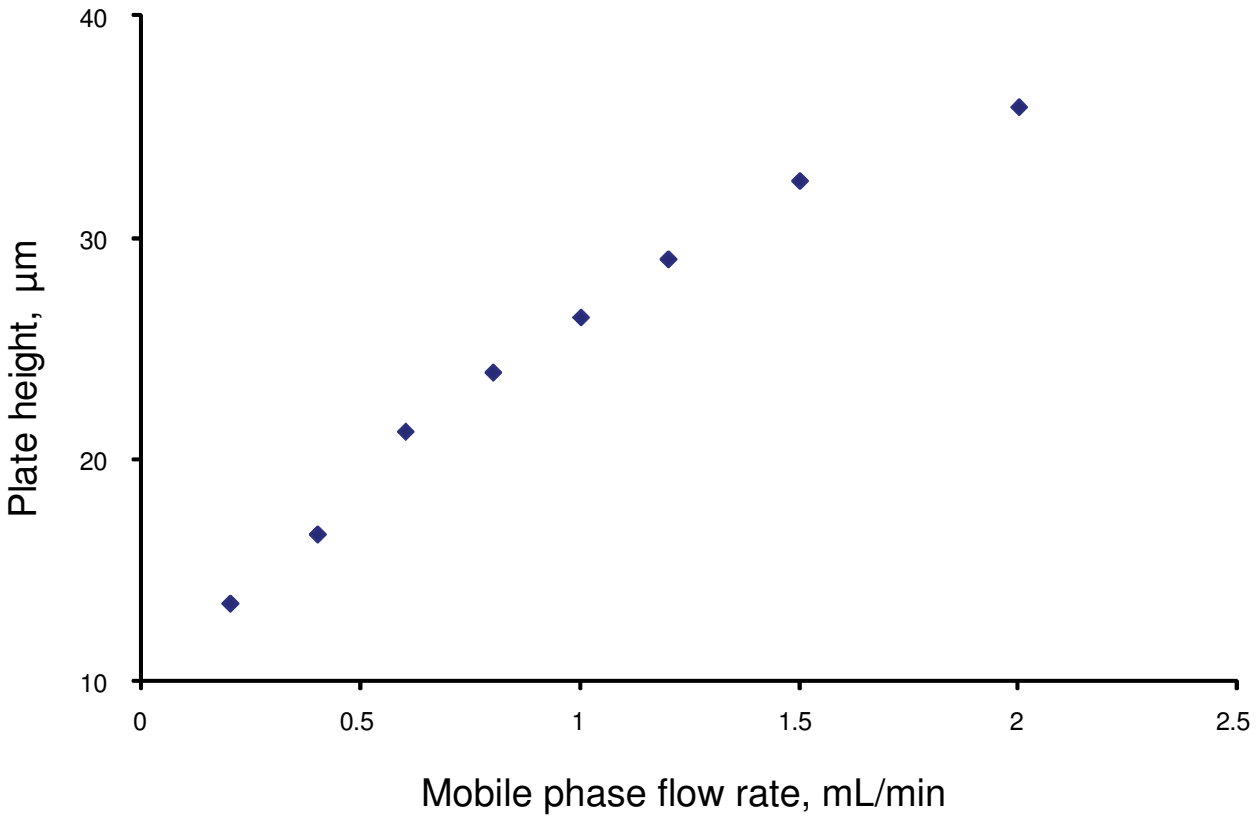


Fig. 3 Experimental van Deemter curve for cyanidin 3-O-rutinoside

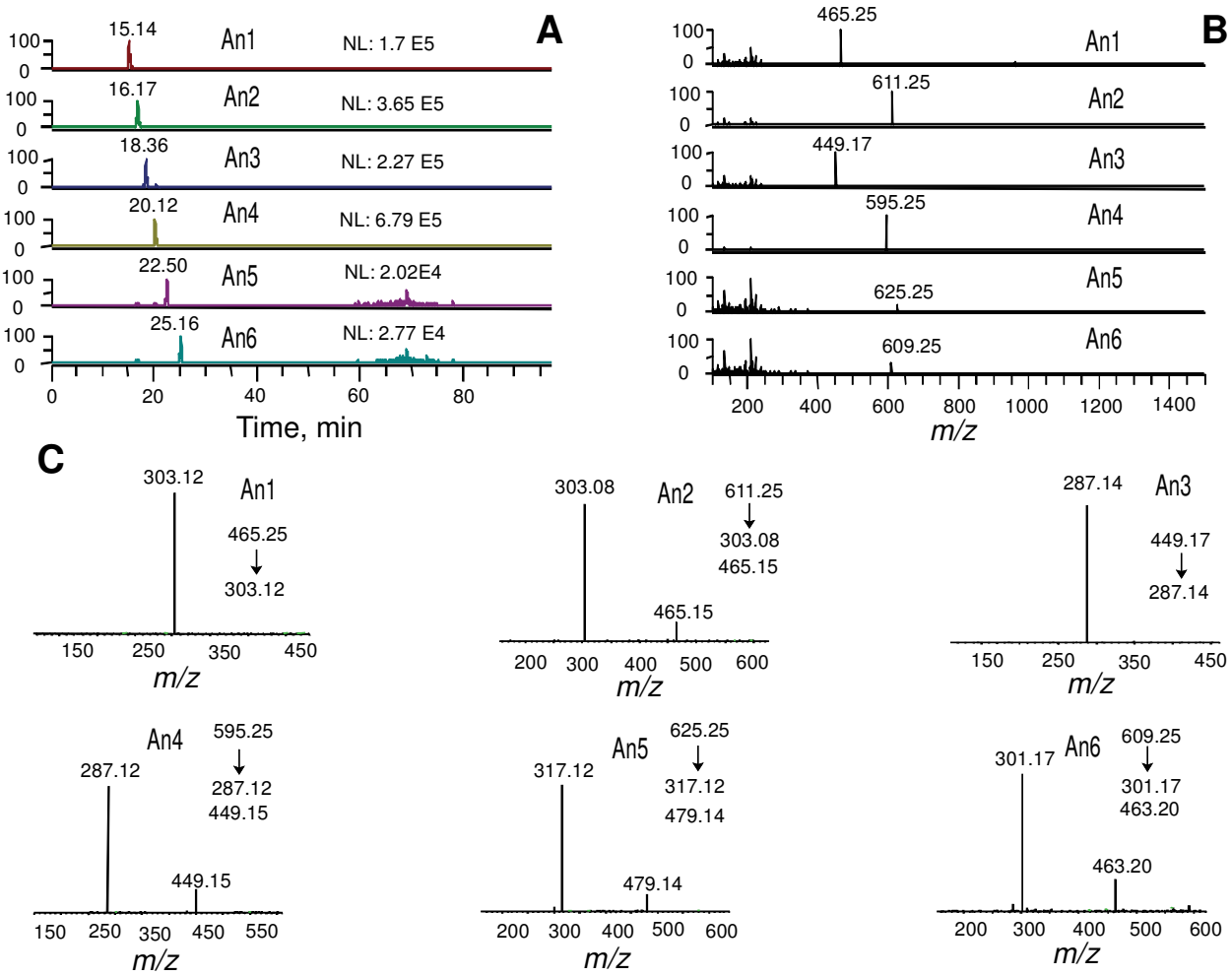


Fig. 4 Typical extracted peaks of separated anthocyanins 1-6 in sample 1 (A) and corresponding MS (B) and MS/MS spectra (C)

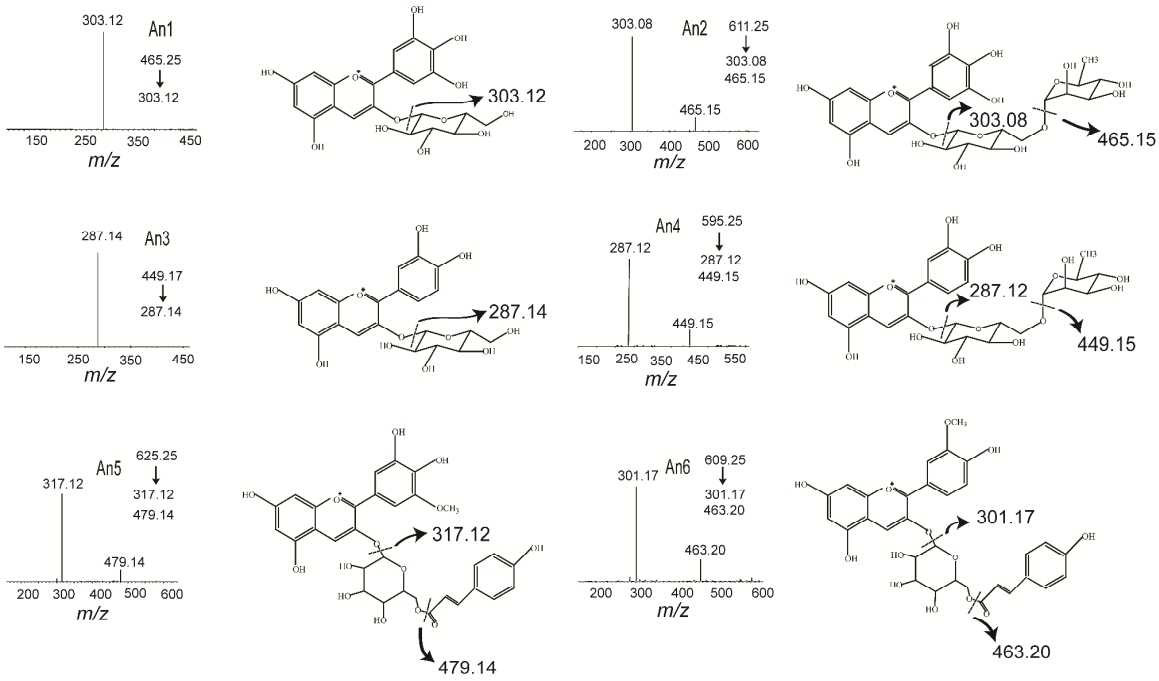


Fig. 5 Fragment ions and cleavages of the separated anthocyanins

Table 1 6 anthocyanins identified in black currants

Peak	ESI MS		Molecular formula	MS/MS (*m/z*)	MS/MS(*m/z*) from literature 12
	Measured mass	Calculated mass			
An1	465.25	465.38	$C_{21}H_{21}O_{12}$	303.12	303
An2	611.25	611.16	$C_{27}H_{31}O_{16}$	303.08 465.15	303 465
An3	449.17	449.11	$C_{21}H_{20}O_{11}$	287.14	287
An4	595.25	595.52	$C_{27}H_{31}O_{15}$	287.12 449.15	287 449
An5	625.25	625.55	$C_{31}H_{29}O_{14}$	317.12 479.14	317
An6	609.25	609.55	$C_{31}H_{29}O_{13}$	301.17 463.20	301

Table 2 Content (mg/g) of the 6 anthocyanins in 5 black currant samples (n=3)

Sample No.	An1, Delphinidin 3-O-glucoside	An2, Delphinidin 3-O-rutinoside	An3, Cyanidin 3-O-glucoside	An4, Cyanidin 3-O-rutinoside	An5, Petunidin 3-(6′-coumaroyl) glucoside	An6, Peonidin 3-(6′-coumaroyl) glucoside
1	77.55 ± 1.88	143.10 ± 2.39	34.83 ± 0.51	114.53 ± 1.24	2.27 ± 0.05	1.46 ± 0.02
2	51.33 ± 0.30	134.88 ± 1.15	24.15 ± 0.24	114.18 ± 2.15	2.21 ± 0.06	1.89 ± 0.06
3	62.60 ± 3.85	126.69 ± 6.94	126.69 ± 6.94	85.03 ± 4.78	1.63 ± 0.07	1.02 ± 0.01
4	74.63 ± 1.86	135.67 ± 3.03	38.92 ± 0.80	123.18 ± 3.26	2.48 ± 0.03	2.32 ± 0.08
5	25.64 ± 0.49	46.23 ± 0.85	17.44 ± 0.29	54.98 ± 1.06	1.18 ± 0.11	1.28 ± 0.26