Journal of Chromatography A, 1217 (2010) 7932–7941

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of anthocyanin pigments in *Lonicera* (*Caerulea*) extracts using chromatographic fractionation followed by microcolumn liquid chromatography-mass spectrometry

Renáta Myjavcová[a], Petr Marhol[b], Vladimír Křen[b], Vilím Šimánek[c], Jitka Ulrichová[c], Irena Palíková[c], Barbora Papoušková[a], Karel Lemr[a], Petr Bednář[a,*]

[a] Department of Analytical Chemistry, Faculty of Science, Palacký University, Tř. 17. Listopadu 1192/12, CZ 771 46 Olomouc, Czech Republic
[b] Institute of Microbiology, Center for Biocatalysis and Biotransformation, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ 142 20 Prague, Czech Republic
[c] Department of Medical Chemistry and Biochemistry, Faculty of Medicine and Dentistry, Palacký University, Hněvotínská 3, CZ 775 15 Olomouc, Czech Republic

ARTICLE INFO

Article history:
Available online 2 June 2010

Keywords:
Anthocyanin
Blueberry honeysuckle
Chromatography
Dietary supplement
Fractionation
LC/MS
Lonicera
Mass spectrometry
MS
μLC
Pyranoanthocyanin
QqTOF

ABSTRACT

Anthocyanins from the fruit *Lonicera caerulea* L. var. *kamtschatica* (blueberry honeysuckle, Caprifoliaceae) were studied via (semi)preparative chromatographic fractionation followed by MS and μLC/MS analysis. The extraction procedure was optimized with respect to analytical purposes as well as its potential use for the preparation of nutraceuticals. The highest yield of anthocyanins was obtained using acidified methanol as the extraction medium. A comparable total anthocyanin content was obtained using a mixture of methanol and acetone. However, when Lonicera anthocyanins were in contact with acetone, a condensation reaction occurred to a large extent and related 5-methylpyranoanthocyanins were found. The effect of other extraction media, including ethanol as a "green" solvent, is also discussed. The potential of two fractionation procedures for extract purification differing in their chromatographic selectivity and scale was studied (i.e. using a Sephadex LH-20 gel column and a reversed phase). Fractions obtained by both procedures were used for a detailed analysis. MS and μLC/MS^2 methods were used for monitoring anthocyanin and 5-methylpyranoderivatives content as well as identifying less common and more complex dyes (dimer of cyanidin-3-hexoside, cyanidin-ethyl-catechin-hexosides, etc.). These more complex dyes are most likely formed during fruit treatment.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

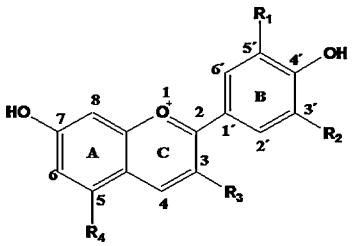
Lonicera caerulea var. *kamtschatica* (blueberry honeysuckle, Caprifoliaceae) is a fruit-bearing bush species that originated from the forests growing on the Kamchatka Peninsula in the Russian Far East. Its fruit (edible berries) are relatively unknown in North America and Europe. The fruit is oval and its color is from dark blue to purple [1–3]. The main composition of *L. caerulea* is comparable to blueberry, blackberry and blackcurrant [4,5].

L. caerulea berries are a rich source of phenolic compounds such as phenolic acids as well as anthocyanins (Fig. 1), proanthocyanidins and other flavonoids, which display potential health-promoting effects. Chemopreventive, antimicrobial, anti-adherence and antioxidant effects, among others, have been described for these compounds [6–9]. Studies on anthocyanin absorption and bioavailability have reported the absorption of intact anthocyanin glycosides [10]. This fact accounts for the key difference between the absorption of anthocyanins and other flavonoids. The potential of *L. caerulea* berries to prevent chronic diseases such as diabetes mellitus type 2, cardiovascular diseases [11] and cancer [12] seems to be above all related to their phenolic content [2,13].

It was recently reported that anthocyanins bearing vicinal hydroxyl groups in the B-ring of their anthocyanidin skeleton were associated with the potency of apoptotic induction, antioxidative and chemoprotective effects of human cells, i.e. human leukemia [14,15]. On the other hand, some articles suggest that pigments having methoxy group(s) in their molecule (e.g. malvidin) are more effective against human cancer of the stomach, colon, CNS and lung cells than other anthocyanins [16]. Peonidin-3-glucoside and cyanidin-3-glucoside also gave very good results in the regulation and reduction of the expression of the metaloproteinases (MMPs) and urokinasesurokinasis (u-PA), which are responsible for the initiation and proliferation of metastasis among the cells [17]. The ability of the *Lonicera* berry phenolic fraction to prevent and reduce

* Corresponding author. Tel.: +420 585634403; fax: +420 585634433.
E-mail address: bednar.petr@seznam.cz (P. Bednář).

0021-9673/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2010.05.058



Anthocyanidin	R_1	R_2
pelargonidin	-H	-H
cyanidin	-OH	-H
delphinidin	-OH	-OH
petunidin	-OH	$-OCH_3$
malvidin	$-OCH_3$	$-OCH_3$
peonidin	$-OCH_3$	-H

R_3, R_4 – OH, glycosyl unit(s), other compounds (i. e. another flavonoid unit, either directly linked or bridged), formation of pyranoanthocyanins (i. e. formation of a cycle from R_4 to position 4 of C ring)

Fig. 1. Structure of common anthocyanins and derived pigments.

UVA-related damage at a cellular level in human keratinocytes was described [18].

Cyanidin-3-glucoside is the principal anthocyanin of *L. caerulea* berries (comprising 83% of the anthocyanin pigments, w/w). Glucosides, rutinosides and 3,5-dihexosides of cyanidin, peonidin, delphinidin and pelargonidin were also found. Their detailed composition can be found in recent articles by Jordheim et al. [19] and our group [20]. A gold standard method used for the analysis of anthocyanins in berries is based on a solid phase extraction (SPE) sample pretreatment, followed by liquid chromatography on-line coupled with tandem mass spectrometry. Deactivated C18 stationary phases are the most common column packings. Generic mobile phases, most often water–acetonitrile mixtures sufficiently acidified by a volatile acid (below pH 2) to avoid peak broadening are used for gradient elution. There are a number of similar systems useful for reliable anthocyanin profiling. However, a strict unification of those methods does not seem to make practical sense due to the huge diversity of analyzed materials as well as long-term studies employing constant and formerly designed methods. These analytical procedures have been reviewed in many articles [21,22]. Generally, the critical step in the analysis of anthocyanins and other flavonoids is the sample pretreatment. During time many extraction procedures were designed for this purpose as can be found in a comprehensive review of Stalikas [23], for instance.

It is well known that during the life of the plant [24,25] as well as during the treatment of berries (i.e. preparation of food products) and their maturation [26–28] some more complex pigments are formed. The most frequent processes leading to complex pigments are polyglycosylation, acylation of the sugar moiety as well as the formation of more cycles and mutual condensation of several anthocyanin (flavonoid) units. Nowadays, many investigators focus their attention on their C-4/C-5 anthocyanin cycloaddition reactions with pyruvic acid, vinylphenols, vinylflavanols and many other groups leading to the formation of pyranoanthocyanins. Two of the most widely known derivates are vitisin A (anthocyanidin-3-hexoside-pyruvic acid adduct) and B (anthocyanidin-3-hexoside-vinyl adduct). Those pigments were reported for the first time in 1976 [29], and are usually studied in wine products. Some of those derivatives were, however, also found in berry juices, mainly after reaction with artificially added phenolic acids [28]. The great interest in pyranoanthocyanins stems from their higher color and pH stability compared to anthocyanins. Andersen et al. found 5-carboxypelargonidin in an acidified methanolic extract of strawberries [25]. However, it is not clear whether this pigment originates from the plant tissue itself or whether it is a product of the extraction procedure. To the best of our knowledge, no pyranoanthocyanins have been reported during the processing of Caprifoliaceae berries to date.

We recently published a paper devoted to the phenolic composition of *L. caerulea* var. *kamtschatica* fruit [20]. The aim of this paper is to investigate the effect of various solvents on anthocyanin extraction, optimize their purification methods, chromatographic fractionation and analytical description based on MS and μLC/MS. Special attention was also paid to the formation of more complex pigments.

2. Materials and methods

2.1. Chemicals

Standards of anthocyanins (cyanidin-3-galactoside chloride, cyanidin-3,5-diglucoside chloride) were obtained from Carl-Roth (Karlsruhe, Germany). Methanol, ethanol, acetone, acetonitrile (HPLC-grade) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid (35%) and phosphoric acid (99.8%), both p.a. were from Lachema (Brno, Czech Republic). Trifluoroacetic acid (99%) was provided by Fluka (Buchs, Switzerland). The column for fractionation was packed with Sephadex LH-20 (Sigma, USA). Double distilled water was used for all experiments. Fresh berries of *L. caerulea* were from the 2006 and 2007 harvest at Lipník nad Bečvou (Czech Republic).

2.2. Preparation of *L. caerulea* phenolic fraction (LCPF)

Frozen fruits (4.84 kg) (from the 2006 harvest) were percolated with an aqueous solution of phosphoric acid (0.1%, v/v; 50 °C) for 14 h. The obtained primary extract was purified on a column filled with non-ionic brominated polystyrene-divinylbenzene resin Sepabeads SP 207 (mean pore size 105 Å, particle size 20–60 mesh, provided by Sigma). The column was washed with deionized water; the phenolic compounds were then eluted by ethanol and concentrated by evaporation to yield 20.3 g of the *L. caerulea* phenolic fraction (LCPF) [20].

2.3. Other extraction procedures and fractionation

The berries of *L. caerulea* (from the 2007 harvest) were stored at −25 °C and subsequently lyophilized and further stored under an Ar atmosphere at −25 °C. A voucher specimen is deposited at the Department of Medical Chemistry and Biochemistry (Olomouc, Czech Republic). The analytes were extracted from lyofilized samples of berries using 4 different solvents, under the conditions given in Table 1. The berries were manually powdered immediately before extraction was begun. Filtration (Filtrak 388) of the mixture was used between extraction portions. The combined filtrates were then dried using a rotary evaporator at 40 °C and finally lyophilized and stored at −25 °C for further experiments, referred to as crude extracts (A–G). Sum of anthocyanins represents roughly 0.1–5% of mass of extract. Fractionation procedures were implemented separately with each portion of crude extracts (A—76 mg, B—80 mg, C—78 mg). The extracts were dissolved in 500 μL of methanol or mixture methanol:acetone (1:1, v/v; extract C) and loaded into a glass column (1400 mm × 25 mm i.d.) packed with Sephadex LH-20 (Sigma, USA). Methanol at 1.45 mL/min was used as the mobile phase, fractions were collected every 8 min. The separation was monitored at 280 nm. Selected fractions were dried *in vacuo* and stored at −25 °C until μLC/MS analysis. A portion of crude extract D (94 mg) was dissolved separately in 500 μL of double distilled water and loaded into a glass column (930 mm × 25 mm i.d.) packed

Table 1
Extraction of the anthocyanins from *L. caerulea* L. berries.

Extraction solvent	Sample weight (g)[a]	Yields[b] (%)	Extraction conditions for all solvents
A: 50% Methanol/50% acetone/0.1% H_3PO_4 (v/v)	50.5	6.1	3× 200 mL of extraction solvent, each by 60 min, darkness, room temperature, Ar atmosphere
B: 100% Methanol/0.1% H_3PO_4 (v/v)	50.9	8.1	
C: 100% Acetone/0.1% H_3PO_4 (v/v)	50.9	0.7	
D: 100% Ethanol/0.1% H_3PO_4 (v/v)	49.3	5.8	
E: 80% Ethanol/20% water/0.1% H_3PO_4 (v/v/v)	50.4	9.6	
F: 80% Methanol/20% water/0.1% H_3PO_4 (v/v/v)	5.2	8.8	3× 20 mL of extraction solvent, each by 60 min, darkness, room temperature, Ar atmosphere
G: 80% Acetone/20% water/0.1% H_3PO_4 (v/v/v)	5.0	6.1	

[a] Mass of lyofilized fruits taken for extraction.
[b] Yield represents ratio of mass of extract after removing of extraction solvent and mass of fresh fruits (expressed in percents).

with Sephadex LH-20 (Sigma, USA). Ethanol 80/20 in water (v/v) at 0.5 mL/min was used as the mobile phase, fractions were collected every 16 min. The separation was monitored at 280 nm.

2.4. Analysis of selected fractions by μLC/MS

The μLC profiles were recorded using a Waters CapLC XE System (Milford, USA) with UV detection. A Gemini C18 column was used (150 mm × 0.3 mm i.d., Phenomenex, USA). The optimized mobile phase was 0.12% trifluoroacetic acid in water with 5% acetonitrile (part A) and 0.12% trifluoroacetic acid in acetonitrile (part B). The optimal gradient was 0–5 min 10% B, 5–10 min 10–20% B, 10–30 min 20–30% B, 30–40 min 30–50% B, 40–50 min 50–70% B, 50–55 min 70% B, 55–60 min 70–100% B, 60–70 min 100% B, 70–70.5 min 100–10% B, 70.5–75 min 10% B and the flow rate was kept at 5.0 μL/min.

Selected fractions were dissolved in the mobile phase (part A) and analyzed by μLC/MS. A QqTOF mass spectrometer (Q-TOF Premier, Waters Corporation) equipped with electrospray ionization (ESI) (Z-spray) was used for MS detection. The capillary voltage was kept at +3.0 kV, the temperature of the source was 120 °C and the temperature of the desolvation gas was 300 °C. The flow rate of the desolvation gas was set at 350 L/h. Quantification of cyanidin-3-glucoside was done using linear regression of calibration dependence in the range of concentration 10–100 mg/L (R^2 = 0.99). The calibration dependence obtained for cyanidin-3-glucoside was employed for all the other peaks as well.

Analysis of fractions by direct infusion of their solutions into the ion source of the mass spectrometer was performed with a flow rate of 5 μL/min, capillary voltage +2.5 kV, temperature of the source 150 °C and temperature of the desolvation gas 150 °C. The flow of the desolvation gas was 100 L/h. For all CID experiments the isolation width of the first quadrupole of QqTOF instrument was set on 1 Da.

2.5. Fragmentation experiments using ion trap (IT)

Identification of anthocyanins was supported by CID experiments using an ion trap mass spectrometer with electrospray ionization (Finnigan MAT LCQ^{TM}, Finnigan, San Jose, USA). The capillary voltage was +5.6 kV, temperature of heated capillary 200 °C. The flow of the desolvation gas was 40 arbitrary units. The identification of anthocyanins was carried out on the basis of parent ions and multistage fragmentation after collision-induced dissociation in the ion trap (MS^n). 60% normalized collision energy was applied.

2.6. Semipreparative high performance liquid chromatography

A Knauer semipreparative liquid chromatograph (Berlin, Germany) equipped with a UV/VIS DAD detector (operating in a wavelength range 190–510 nm) and Gemini C18 column (150 mm × 10 mm i.d., Phenomenex) was used for further fractionation of the LCPF and isolation of particular dyes. The mobile phase was identical to that in the μLC system, the gradient was 0–15 min 5% B, 15–25 min 5–10% B, 25–35 min 10–20% B, 35–40 min 20–50% B, 40–55 min 50–100% B, 55–56 100–5% B, 56–60 min 5% B, at flow rate 5.0 mL/min. 100 μL of extract was injected (full loop). The fractions obtained were lyophilized and kept frozen before μLC/MS analysis (−80 °C).

2.7. Calculation of partition coefficient (C log P)

The calculated values were obtained using the software ChemBioDraw Ultra (Cambridge Soft). Calculations were done for β-D-glucopyranosylated anthocyanins. Specific algorithms for calculating log *P* (*C* log *P*) from fragment-based methods were developed by the Medicinal Chemistry Project and BioByte. The results produced by the software were kindly provided by Department of Organic Chemistry, Palacký University, Olomouc, Czech Republic.

3. Results and discussion

3.1. Extraction procedure

The proper choice of an extraction solvent is a key variable in the efficient release of colored material from fruit. From the viewpoint of the food and pharmaceutical industry the selection of extraction medium is often restricted by biosafety demands and the content of residual solvent(s) is one of most critical parameters in the quality of the final product [30,31]. Table 2 shows the differences in the extraction efficiency of various extractions with respect to anthocyanins (all extraction media discussed below were acidified using 0.1% H_3PO_4, v/v, as given in Table 1). The highest yield of common anthocyanins from fruits was obtained using acidified methanol (sum of anthocyanins in Table 2). This contributes to the known fact that this extraction solvent is the most efficient medium for a broad range of matrices [32,33]. A lower yield of identified dyes was obtained when water was added to the methanolic extraction medium. Substituting ethanol for methanol in the extraction medium significantly decreased the amount of extracted anthocyanins. When water, being a more polar solvent, is mixed with ethanol, the extraction was improved. Some authors also reported the usefulness of acidified ethanol–water solutions [34]. Although the extraction of dyes using a water–ethanolic medium is less efficient than a methanolic medium, the decrease in anthocyanin content is not by an order of magnitude. Ethanol and its mixtures with water have a special use in the food and pharmaceutical industry, since they fulfill health requirements. When *Lonicera* fruits are extracted with an 80:20 ethanol:water mixture, 0.037% (w/w) of the anthocyanins are released (this is roughly 15% of the amount extracted by acidified methanol). This can be acceptable for many industrial purposes once the water content is optimized more precisely. Several authors reported that

Table 2
Amount of anthocyanins in extracts of *L. caerulea* L. fruits[a,b].

	m/z	(50:50) methanol: acetone (A)	100% methanol (B)	80% methanol (F)	100% acetone (C)	80% acetone (G)	100% ethanol (D)	80% ethanol (E)
Anthocyanin								
Cyanidin-3-glucoside	449	0.117	0.192	0.120	0.024	0.073	0.003	0.017
Peonidin-3-glucoside	463	0.018	0.026	0.023	0	0.009	0.002	0.007
Pelargonidin-3-glucoside	433	0.002	0.008	0.011	0	0	0	0
Cyanidin-3-rutinoside	595	0.012	0.013	0.015	0	0.005	0.002	0.006
Peonidin-3-rutinoside	609	0.006	0.008	0	0	0	0	0
Cyanidin-3,5-dihexoside	611	0.008	0.007	0	0	0	0.002	0.007
Peonidin-3,5-dihexoside	625	0	0	0	0	0	0	~0.0001
Sum of anthocyanins		0.161	0.253	0.169	0.024	0.088	0.009	0.037
Pyranoanthocyanin								
5-Methylpyranocyanidin-3-glucoside	487	0.085	0	0	0	0.013	0	0
5-Methylpyranopeonidin-3-glucoside	501	0.015	0	0	0	0	0	0
Sum of pyranoanthocyanins		0.1	0	0	0	0.013	0	0
Sum of all identified dyes		0.261	0.253	0.169	0.024	0.101	0.009	0.037

All fractions were measured in triplicate; relative standard deviation ranges from 0.0 to 13.6%.
[a] All extraction media contain 0.1% (v/v) phosphoric acid.
[b] Content is given as % (w/w) related to fresh fruits.

acetone is a better extraction solvent for anthocyanins than light alcohols [35,36]. It was stated that acetone as an extraction solvent enables a more reproducible extraction, a lower temperature for sample concentration and it avoids possible problems with pectins (the treatment of strawberries). Mixtures of acetone–water were also used [37]. Acetone or its mixture with water exhibited a substantially lower extraction efficiency for *Lonicera* berries than methanolic mixtures. On the other hand, our results suggest that the extraction efficiency of a 50:50 methanol:acetone mixture is much higher (comparable with an 80:20 methanol:water mixture). However, a proportion of the anthocyanins are converted into the corresponding 5-methylpyranoanthocyanins during the contact with acetone. If we consider the sum of the anthocyanins and 5-methylpyranoanthocyanins, the extraction yield of the methanol–acetone mixture is comparable with methanol. The identity of the pyranoderivatives is discussed below.

The amount of all identified anthocyanins obtained by extraction using acidified methanol roughly corresponds with the amount found in the LCPF obtained from fruits harvested one year earlier (relative difference represents 20.2%) [20]. This agreement suggests that both extraction methods can be used for the long-term analytical monitoring of anthocyanins during the treatment of the fruit. This data conformity was achieved aside from the neglect of response factors and quantification of all major dyes (listed in Table 2) using regression equation obtained for cyanidin-3-glucoside.

3.2. Purification and fractionation procedure

In order to isolate the anthocyanins from crude extracts, it was necessary to implement a purification (fractionation) step. In the preparative-scale fractionation of anthocyanins and related compounds there are several suitable stationary phases: octadecyl silica gel, Amberlite XAD-7 (nonionic macroporous polymer of poly(methyl methacrylate)), Toyopearl gel (hydroxylated methacrylic polymer resin) or Sephadex LH-20 (hydroxypropylated cross-linked dextran) [38–41]. An alternative method to the large-scale isolation of anthocyanins (up to 1000 mg of crude extract in single run) is countercurrent chromatography (CCC), which can be described as an automated version of liquid–liquid extraction [42]. In our study, low-pressure LC on Sephadex LH-20 was used to fractionate the crude extracts. Sephadex LH-20 not only swells in solvents of weak and medium polarity but also in strongly polar ones (e.g. methanol). The exclusion limit at maximum swelling was 4000, i.e. it is suitable for the fractionation of anthocyanins. Details of the fractionation procedure can be found in Section 2. Fig. 2 describes the process of fractionation using various solvents (UV detection, $\lambda = 280$ nm).

Four main fractions were obtained during the fractionation of the acetone–methanol solution (Fig. 2A). The first peak contained the majority of the pyranoanthocyanins. Anthocyanins with two sugar units eluted in the closest smaller peak but their separation was not complete. Anthocyanins with one sugar unit eluted in the last two peaks. In the chromatogram of the methanolic extract, the peak of pyranoanthocyanins was clearly missing (Fig. 2B). The elution order of the other dyes corresponded with that observed in the chromatogram of the acetone–methanol extract, however, the resolution of peaks 3 and 4 was improved. The same elution order was observed during the fractionation of acetone (Fig. 2C) and ethanolic (Fig. 2D) extracts. However, the separation and peak shapes, especially when ethanol was used, were deteriorated and their elution times shifted. This can be explained by the compression of the polar stationary phase during contact with the less polar solvent. The chromatogram of the acetone extract is also complicated by some other co-extracted impurities (especially peak 3). These experiments indicated that the extraction medium composition is the key parameter not only for the extraction itself but also for efficient chromatographic fractionation (and consequently the purity of the final product). Methanol was used as the mobile phase for the fractionation of the methanolic, methanol–acetone and acetone extracts and the ethanol:water (80:20, v/v) mixture for the fractionation of the ethanolic extract (to avoid contact with methanol) as described in Section 2.

The elution order and separation of anthocyanins into several groups in accordance with their structure cannot be explained simply. We propose that it is a combination of hydrophilic interactions and an increased permeation of smaller molecules into the stationary phase. No tabulated parameter describing the hydrophobicity of pyranoderivatives was found in the literature. However a rough idea of the polarity differences among the studied dyes can be obtained from approximative calculations of the partition coefficients ($C\log P_{ow}$) of cyanidin derivatives. The value reaches +1.8 for 5-methylpyranocyanidin-3-glucoside; 0.0 for cyanidin-3-glucoside and −1.6 for cyanidin-3,5-diglucoside. The contribution

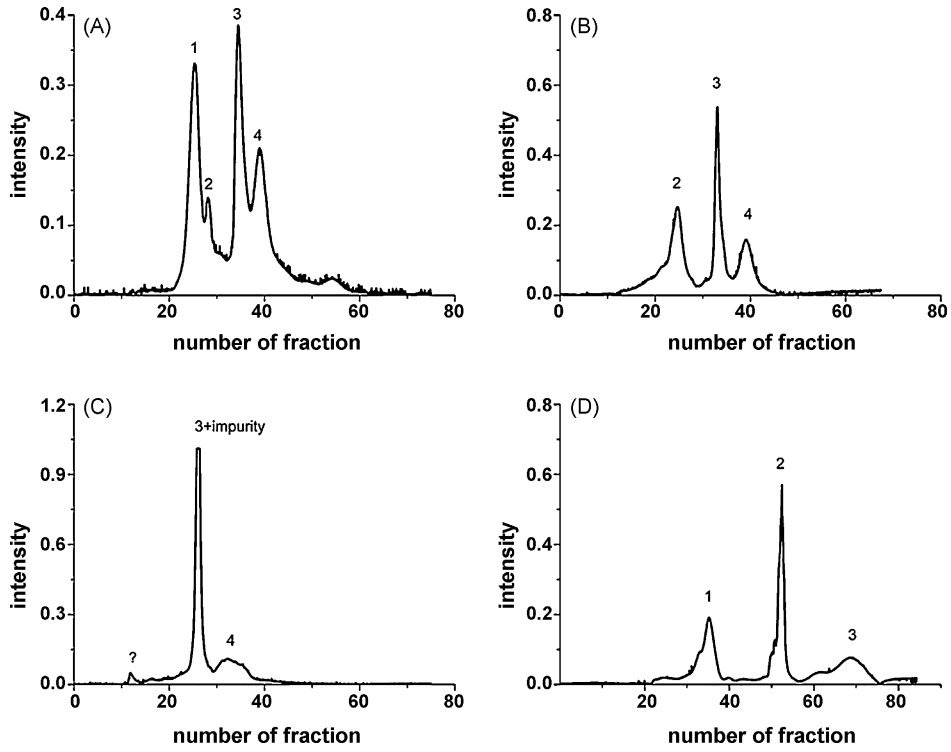


Fig. 2. Chromatograms of Lonicera crude extracts fractionation on Sephadex LH-20 (A—acetone–methanol mixture; B—methanol; C—acetone; D—ethanol; λ = 280 nm).

of the 4-ring and methyl group of the pyranoderivative to the decrease in polarity is apparently significant and it could explain the shortening of its elution time with respect to the other cyanidin derivatives.

As has already been mentioned, a reversed phase is often used for fractionation of phytochemicals from plant extracts. We studied a combination of the prepurification of crude Lonicera extract using non-ionic polystyrene-divinylbenzene resin Sepabeads SP 207 (preparation of the LCPF, for details see Section 2) with subsequent high performance semipreparative fractionation using a reversed phase resistant against acidic mobile phases. Such an approach enabled pure fractions of many dyes to be retrieved, as can be seen from the chromatogram in Fig. 3. Several mono- and diglycosylated anthocyanins, pyranoanthocyanins and some more complex pigments were identified in these fractions (Table 3).

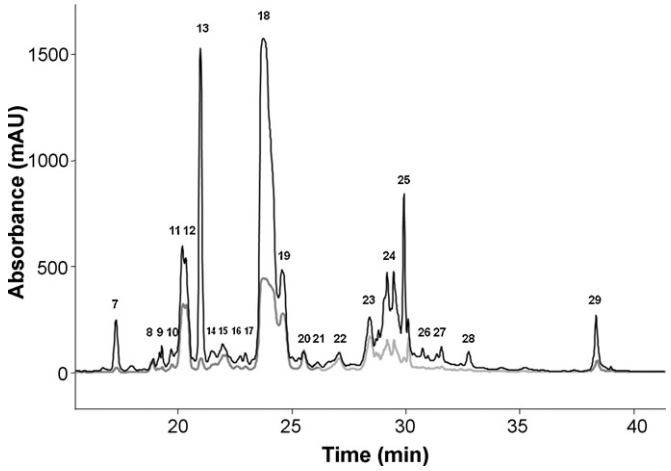


Fig. 3. Semipreparative fractionation of *L. caerulea* phenolic fraction (LCPF) on reverse phase (black—λ = 280 nm; grey—λ = 505 nm). number assigned to each peak corresponds with the number of fraction in Table 3.

The identification of those derivatives is discussed below. The second fractionation approach (due to the column dimensions used, 150 mm × 10 mm i.d.) is more suited to the isolation of individual dyes to make their identification easier and study their properties than to the industrial-scale processing of Lonicera fruit (preparative purification). Scaling-up to a preparative level is of course possible, however, as far as the expected very high price of an appropriate preparative column is concerned, it is probably not acceptable for industrial purposes and exceeds the scope of this paper. The elution order corresponds with the decreasing polarity of the separated dyes. The injection of 100 μL (1 mg of extract) of purified extract into the column did not cause overloading of the column and good separation was observed.

3.3. Identification of extracted dyes

Crude extracts as well as fractions obtained by both fractionation procedures (Sephadex and reversed phase) were analyzed using ESI-MS and μLC/ESI-MS^2. The identification of anthocyanins was based on exact mass measurement, study of fragmentation after isolation of the parent ion and its collision-induced dissociation in the collision cell. Further valuable information was obtained from the comparison of retention times and UV/vis spectra recorded using a DAD detector. Common anthocyanins are listed in Table 2. It can be seen that cyanidin-3-hexoside is a dominant dye in Lonicera extracts. Some other cyanidin derivatives as well as peonidin and pelargonidin glycosides were also identified.

A relatively intensive dye with m/z 487.1224 was observed in fraction 1 of the methanol–acetone extract (fractionation on Sephadex LH-20) as can be seen in Fig. 4. The fragment at m/z 325.0703 was observed in related MS^2 spectra. The process of its formation corresponds with the cleavage of dehydrated hexose ($\Delta m/z = 162.0528$). Further fragmentation of the aglycon was studied by ion trap mass spectrometry. The ion with m/z 325 fragments via consequent losses of carbon monooxide ($\Delta m/z = 28$) and water ($\Delta m/z = 18$). Based on this information, the dye corresponds with

Table 3
Identified anthocyanin pigments in *L. caerulea* phenolic fraction (LCPF).

Fraction[a]	MS	Accuracy of mass measurement (ppm)	MS^2	Name
9	611.1553	9.6	449, 287	Cyanidin-3,5-dihexoside [19,20]
10	611.1602	1.6	449, 287	Cyanidin-3,5-dihexoside [19,20]
	737.1677	5.6	575, 423, 287	Cyanidin-3-hexoside-catechin [25]
11	611.1609	0.5	449, 287	Cyanidin-3,5-dihexoside [19,20]
12	611.1610	0.3	449, 287	Cyanidin-3,5-hexoside [19,20]
	625.1767	0.3	463, 301	Peonidin-3,5-dihexoside [20,25]
13	773.2135	0.6	627, 465, 303	Quercetin-methylpentoside-dihexoside
18	449.1083	0.2	287	Cyanidin-3-hexoside [20,25]
	897.2111	2.5	449, 287	Dimer of cyanidin-3-hexoside
	487.1224	3.3	325	5-Methylpyranocyanidin-3-hexoside
	595.1663	0	449, 287	Cyanidin-3-rutinoside [20]
19	595.1578	14.2	449, 287	Cyanidin-pentosylhexoside[b] [20,25]
23	463.1243	0.6	301	Peonidin-3-hexoside [20,25]
	609.1810	0.2	301	Peonidin-3-rutinoside [19,20]
	897.2019	7.8	735, 573	Dimer of cyanidin-3-hexoside
24	765.2038	0.9	603, 475, 313, 287	Cyanidin-3-hexoside-ethyl-catechin
	897.2119	3.3	735, 573, 287	Dimer of cyanidin-hexoside
	597.1470	2.3	465, 303	Quercetin-pentoside-hexoside
	911.2513	10.6	749, 621, 313, 287	Cyanidin-methylpentoside-hexoside-ethyl-catechin
	465.1122	19.1	303	Quercetin-3-hexoside[b] [2]
	595.1540	20.6	449, 287	Cyanidin-pentosylhexoside[b] [20,25]
	611.1613	0.2	465, 303	Quercetin-3-rutinoside [2]
25	465.1032	0.2	303	Quercetin-3-hexoside [2]
	611.1613	0.2	465, 303	Quercetin-3-rutinoside (rutin) [2]

[a] The number of fraction corresponding with the number of peak in Fig 3.
[b] Suggested structures.

5-methylpyranocyanidin-3-hexoside (mass difference: −3.3 ppm). Similarly, an ion with m/z 501.1401 was observed (Fig. 5). The first fragmentation step corresponds with the cleavage of a sugar moiety. During the fragmentation of the aglycon (MS^3, IT) the cleavage of a methyl group was observed ($\Delta m/z = 15$) as well as the loss of carbon monooxide and water, which suggests the presence of one methoxygroup in the anthocyanidin skeleton. This ion can be ascribed to 5-methylpyranopeonidin-3-hexoside (mass difference 0.8 ppm). During detailed investigation of MS^2 spectrum of 5-methylpyranocyanidin-3-hexoside a fragment at m/z 254.0591 was found. This ion can be explained by loss of hexose, two losses of carbon monooxide and consequent cleavage of methyl radical ($\Delta m/z = 15$) from pyrano-ring. This fragmentation pattern was unambiguously confirmed by targeted MS^3 (487 → 325→; inset in Fig. 4) and MS^4 (487 → 325 → 269→) experiments using ion trap. Those experiments show that the cleavage of methyl radical can occur solely from the pyrano-ring and thus the process is characteristic for 5-methylpyranoanthocyanins. The difference of measured mass from the theoretical value calculated for the proposed radical cation (elemental composition $C_{15}H_{10}O_4^{+\cdot}$) is 4.7 ppm showing a good agreement with our presumptions. Detailed insight into the fragmentation of 5-methylpyranopeonidin-3-hexoside (Fig. 5, bottom spectrum) disclosed fragmentation pathway leading to ion at m/z 253.0509 which is analogous structure to the fragment discussed above for 5-methylpyranocyanidin-3-hexoside (difference of measured mass from the theoretical one is 3.1 ppm in this case). As it was already mentioned the fragmentation pathway of related aglycon starts by the cleavage of methyl radical

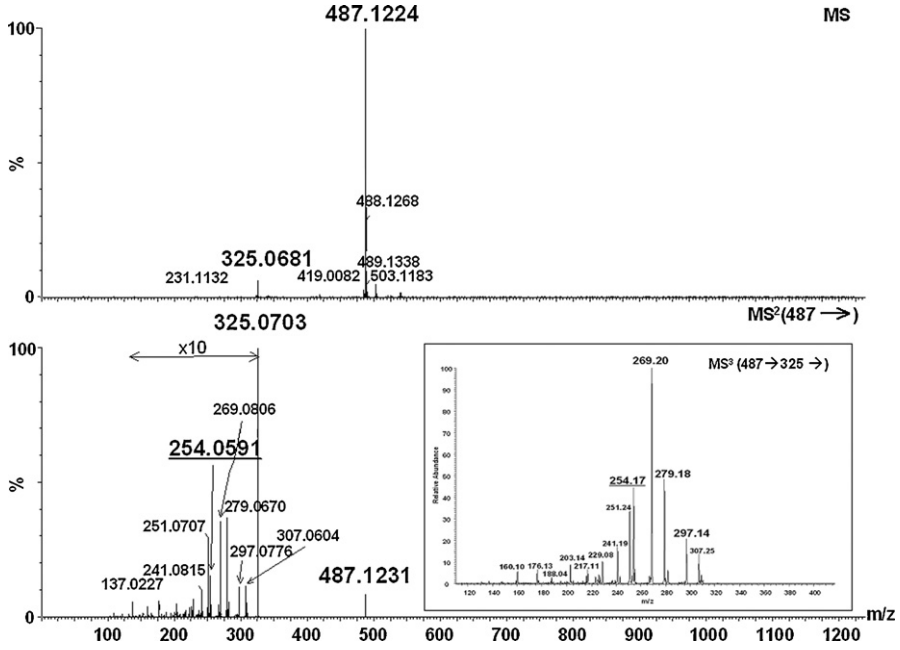


Fig. 4. ESI-MS spectra of 5-methylpyranocyanidin-3-hexoside (bottom spectrum—MS^2; fragmentation of aglycon was zoomed 10×; inset in bottom spectrum—IT-MS^3).

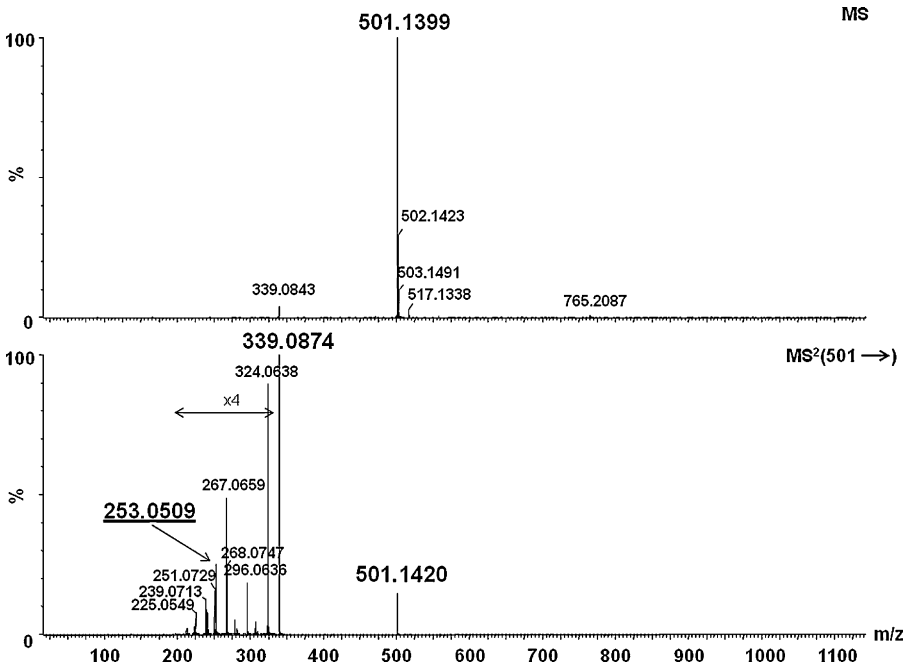


Fig. 5. ESI-MS spectra of 5-methylpyranopeonidin-3-hexoside. (bottom spectrum—MS^2; fragmentation of aglycon was zoomed 4×)

from the B-ring and formation of radical cation. Characteristic fragment rising after the cleavage of methyl radical from the pyrano-ring has one unit lesser *m*/*z* value compared to fragment of 5-methylpyranocyanidin-3-hexoside. However, the ion trap experiments done with 5-methylpyranopeonidin-3-hexoside provided only weak MS^3 and MS^4 spectra due to its lower concentration in all studied samples compared to 5-methylpyranocyanidin-3-hexoside. The structures of both dyes (aglycons) can be seen in Fig. 6.

Table 2 shows that both 5-methylpyranoderivatives were mainly found in the methanol–acetone extracts. Recently, Lu and Foo [37] also observed a significant anthocyanin reaction with aqueous acetone and the formation of 5-methylpyranoanthocyanidins. These authors did not observe such anthocyanins in other extraction solvents and our experiments are in agreement with their data (5-methylpyranoderivatives were only found in extracts with a methanol–acetone or water–acetone mixture). Interestingly, the above-mentioned pyranoanthocyanins were not found in the acetone extract. This fact could be related to a poor extraction of native anthocyanins itself and consequently their low content available for cyclization. Another explanation can also be a different reactivity in polar protic solvents such as methanol. If the transition state is stabilized by polar protic solvents the reaction

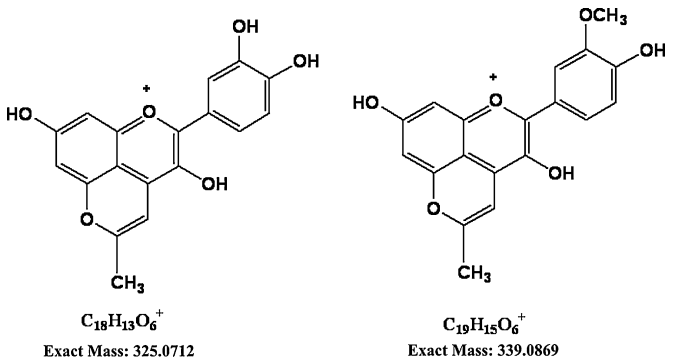


Fig. 6. Structures of identified 5-methylpyranoanthocyanidins.

may proceed faster in such a medium and more of this product will be obtained. Andersen et al. found 5-carboxypyranopelargonidin in the methanolic extract of strawberries [25]. However, its origin was not discussed in detail and it is not clear whether the dye is formed during the fruit's growth or after its harvest (i.e. before or during extraction, when contact with pyruvic acid as the cyclization agent cannot be precluded).

Other anthocyanin dyes were identified by μLC/MS^2 after a more efficient purification and fractionation of the LCPF with a reverse phase (Fig. 3). Proposed structures together with the most abundant fragments obtained after their isolation in the first quadrupole and CID in the collision cell, as well as the differences between their measured and exact masses are listed in Table 3. Anthocyanins bearing two sugar units were found in the first fractions. Simple anthocyanins with one sugar unit were eluted (not fully resolved) in fraction 18, which was the most colored. Later eluted fractions were comprised of pigments glycosylated with rutinose. In accordance with our predictions, derivates of cyanidin eluted from the reverse phase before related derivatives of peonidin. In fractions obtained after fractionation of LCPF (fraction 18, see Table 3 and Fig. 3), the 5-methylpyranocyanidin-3-hexoside (formed from very high content of cyanidin-3-hexoside) was found and confirmed by exact mass measurement and fragmentation study regardless the fact that the fraction was not in contact with acetone. This indicates that the formation of 5-methylpyranoanthocyanins in a fruit and/or in a prepared extract by an alternative way should not be precluded. Since the content of peonidin-3-hexoside (fraction 23) is much lower than the content of cyanidin-3-hexoside, the content of 5-methylpyranopeonidin-3-hexoside is much lower than 5-methylpyranocyanidin-3-hexoside and it is below the detection limit in LCPF fractions.

In addition, several derivates of quercetin were also found in the LCPF fraction (Fig. 3 and Table 3). Some of the identified polyphenols (or related isomeric structures) are reported in Lonicera fruits for the first time (cyanidin-3-hexoside-ethyl-catechin, cyanidin-methylpentoside-hexoside-ethyl-catechin, quercetin-methylpentoside-dihexoside, quercetin-pentoside-hexoside). Fig. 7 (upper spectrum) shows the MS^2 spectrum of cyanidin-3-hexoside-ethyl-catechin (*m*/*z* 765.2025; mass differ-

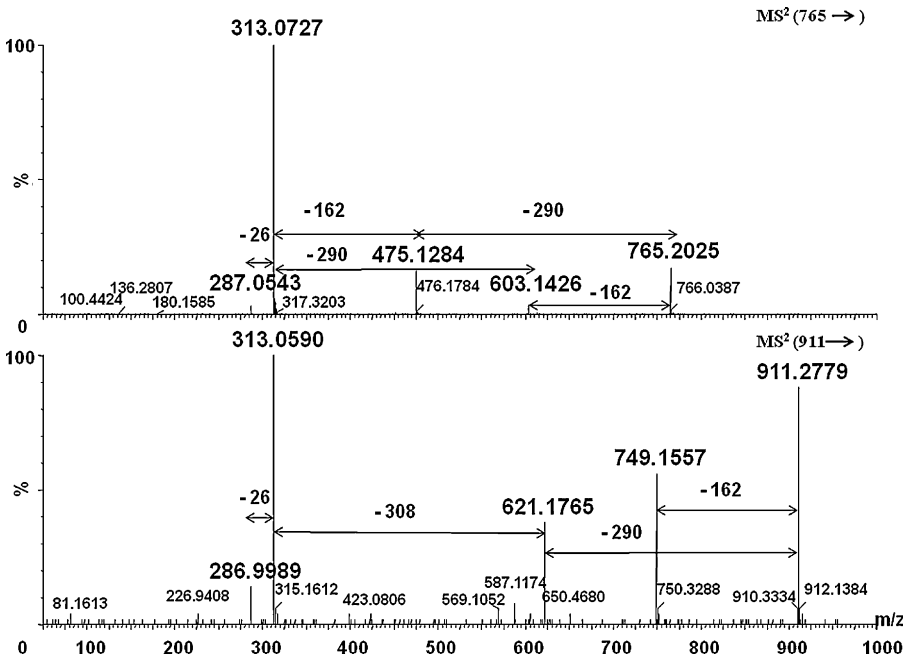


Fig. 7. ESI-MS^2 spectra of cyanidin-3-hexoside-ethyl-catechin (m/z 765) and cyanidin-methylpentoside-hexoside-ethyl-catechin (m/z 911).

ence −0.8 ppm). Loss of dehydrated hexose ($\Delta m/z = 162$) and neutral molecule of catechin ($\Delta m/z = 290$) can be clearly seen. Consequent fragmentation leads to dominant fragment ion at m/z 313.0727 which corresponds with a vinyl cyanidin (elemental composition $C_{17}H_{13}O_6^+$; mass difference 4.8 ppm). The proposed structure is confirmed by a further loss of vinyl group ($\Delta m/z = 26$) and formation of cyanidin (elemental composition $C_{15}H_{11}O_6^+$; mass difference −4.5 ppm). By analogy, another bridged cyanidin glycoside can be considered. In bottom MS^2 spectrum an ion at m/z 911.2779 (mass difference 18.5 ppm) can be seen. This dye can be ascribed to cyanidin-methylpentoside-hexoside-ethyl-catechin. The ion fragments via loss of dehydrated hexose ($\Delta m/z = 162$), dehydrated methylpentosylhexose ($\Delta m/z = 308$), neutral molecule of catechin ($\Delta m/z = 290$) and formation of vinyl cyanidin and cyanidin itself. This fragmentation pattern confirms the proposed structure. Fig. 8 shows the MS^2 spectrum of quercetin-methylpentoside-dihexoside (m/z 773.2520; upper spectrum) and quercetin-pentoside-hexoside (m/z 597.4384; bottom spectrum). The losses of dehydrated methylpentose or pentose and hexose(s) ($\Delta m/z = 132$, 146 and 162, respectively) are obvious. Quercetin aglycon is obtained as dominant fragment (mass differences −0.3 and 11.2 ppm, respectively are achieved). The derivatives of quercetin are isomeric with delphinidin analogues which could be alternatively considered as well. However, the comparison of their retention times with the other found polyphenols indicates rather the presence of quercetin derivatives.

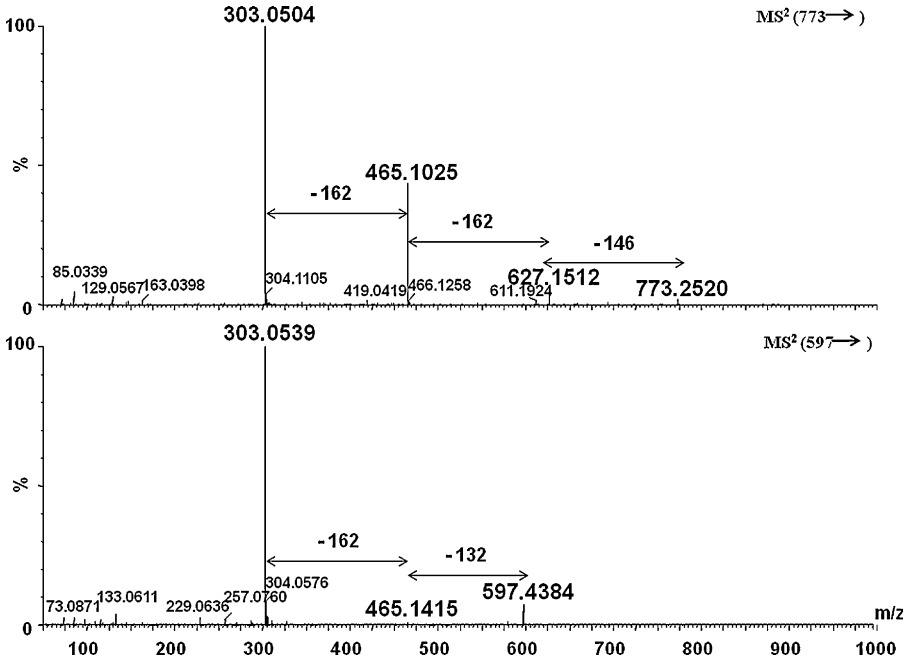


Fig. 8. ESI-MS^2 spectra of quercetin-methylpentoside-dihexoside (m/z 773) and MS^2 of quercetin-pentoside-hexoside (m/z 597).

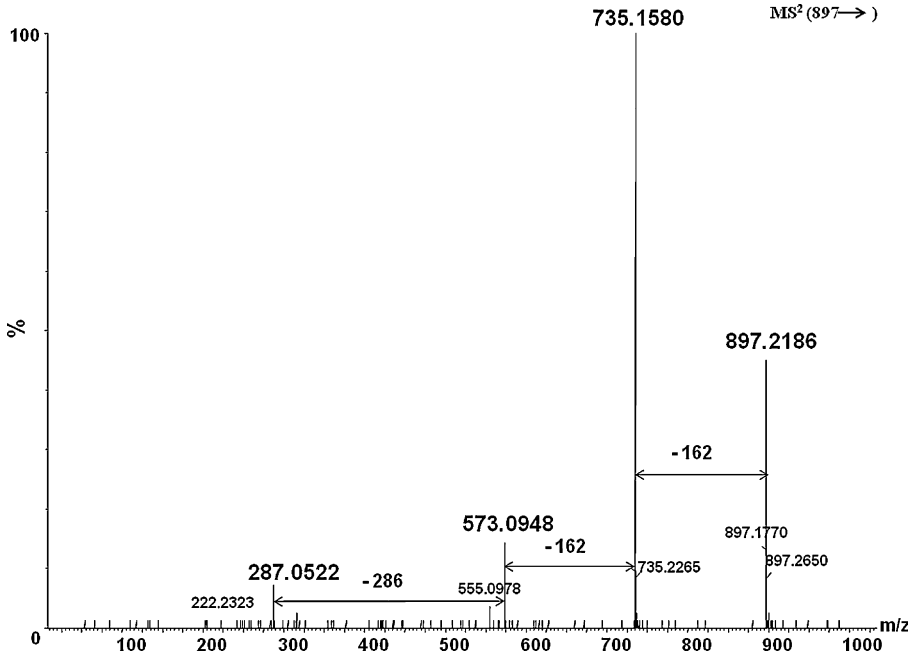


Fig. 9. ESI-MS^2 spectrum of dimer of cyanidin-3-hexoside (m/z 897).

Fig. 9 shows the collision spectrum obtained by direct infusion of colored fraction 24, isolation of an ion at m/z 897.2186 in the first quadrupole and CID in the collision cell. Although the obtained MS^2 spectra of those peaks are of a lower quality, the consequent losses of two dehydrated hexoses ($\Delta m/z = 162$) as well as the cleavage of two anthocyanidin units ($\Delta m/z = 286$) were observed. The structure of the dye corresponds with a dimer of cyanidin-3-hexoside (the differences in the measured masses of the corresponding fragments from those calculated are less than 15 ppm). An ion with the same nominal mass (m/z 897.2089) was observed in fraction 18, which contained a high concentration of cyanidin-3-hexoside. We presuppose that this ion is formed by the oxidation of cyanidin-3-hexoside to dimer in the ion source of the mass spectrometer. An independent μLC/MS analysis of purified Lonicera extract identified three separated ions with m/z 897.2 (data not shown). One of them had a retention time and peak shape identical to cyanidin-3-hexoside. This ion confirms the production of the dimer in the ion source and it serves as a good example of a pitfall stemming from sample transformation during analysis and subsequent possible misinterpretation of MS data.

4. Conclusion

The process of extracting anthocyanin pigments from the fruit of *L. caerulea* var. *kamtschatica* was studied. The highest yield of these compounds was obtained using acidified methanol. This result confirms the overall suitability of methanolic extraction media for the treatment of various fruits. A high extraction efficiency was also reached using a mixture of methanol:acetone:phosphoric acid (50:50:0.1, v/v/v), however during this process a considerable amount of 5-methylpyranoanthocyanins was formed due to condensation with the acetone. It was found that extraction medium strongly affects the properties of Sephadex LH-20 when used as the stationary phase for the preparative fractionation of extracts. Suitable chromatographic performance was achieved using methanolic media. Less polar solvents (i.e. ethanol and acetone) caused a phase compression as well as longer and less reproducible retention. This phase allowed fractionation into three groups (5-methylpyranoanthocyanins, diglycosylated and monoglycosylated anthocyanins). The retention mechanism seems to be a combination of hydrophilic interactions and an increased permeation of smaller molecules into the stationary phase compared to bigger ones. A combination of prepurification on Sepabeads SP 207 and semipreparative separation on the reversed phase produced relatively pure fractions of particular dyes. Besides native Lonicera anthocyanins, several less common pigments were found in these fractions using μLC/MS and MS^2 experiments. Based on precise mass measurement and the study of collision spectra, 5-methylpyranoanthocyanins, dimers of cyanidin-3-hexoside and compounds containing an anthocyanin and catechin unit connected via ethyl bridge were identified in extracts of Vacciniaceae and Caprifoliaceae berries for the first time.

Acknowledgements

The support of Ministry of Trade and Commerce (FT-TA3/024) and Ministry of Education, Youth and Sports, Czech Republic (MSM6198959216 and AV0Z50200510) is gratefully acknowledged. We thank to Miroslav Chovanec from Lipnik nad Becvou for growing the *L. caerulea* and to Assoc. Prof. Jan Hlaváč from the Department of Organic Chemistry, Palacký University for the calculation of partition coefficients.

References

[1] J. Matuskovic, T. Pokorna, M. Antalikova, Acta Horticult. Regiotect. 2 (2006) 44.
[2] A. Chaovanalikit, M.W. Thompson, R.E. Wrolstad, J. Agric. Food Chem. 52 (2004) 848.
[3] M.N. Plekhanova, Acta Hortic. 538 (2000) 159.
[4] M. Thompson, A. Chaovanalikit, Acta Hortic. 626 (2003) 65.
[5] R.A. Moyer, K.E. Hummer, C.E. Finn, B. Frei, R.E. Wrolstad, J. Agric. Food Chem. 50 (2002) 519.
[6] J. Kong, Phytochemistry 64 (2003) 923.
[7] M.P. Kähkönen, M. Heinonen, J. Agric. Food Chem. 51 (2003) 628.
[8] E. Kowalczyk, P. Krzesiñski, M. Kura, B. Szmigiel, J. Baszczyk, Pol. J. Pharmacol. 55 (2003) 699.
[9] I. Palíková, K. Valentová, I. Oborná, J. Ulrichová, J. Agric. Food Chem. 57 (2009) 6584.
[10] T.K. McGhie, M.C. Walton, Mol. Nutr. Food Res. 51 (2007) 702.
[11] A. Mauray, D. Milenkovic, C. Besson, N. Caccia, C. Morand, F. Michel, A. Mazur, A. Scalbert, C. Felgines, J. Agric. Food Chem. 57 (2009) 11106.

[12] M.I. Gruia, E. Oprea, I. Gruia, V. Negoita, I.C. Farcasanu, Molecules 13 (2008) 1195.
[13] I. Švarcová, J. Heinrich, K. Valentová, Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub. 151 (2007) 163.
[14] D.X. Hou, T. Yanagita, T. Uto, S. Masuzaki, M. Fujii, Biochem. Pharmacol. 70 (2005) 417.
[15] D.X. Hou, T. Ose, S. Lin, K. Harazono, I. Imamura, M. Kubo, T. Uto, N. Tarahara, M. Yoshimoto, M. Fujii, Int. J. Oncol. 23 (2003) 705.
[16] Y.J. Zang, S.K. Vareed, M.G. Nair, Life Sci. 76 (2005) 1465.
[17] P. Chen, W. Kuo, C. Chiang, H. Chiou, Y. Hsieh, S. Chu, Chem. Biol. Interact. 163 (2006) 218.
[18] A. Svobodová, J. Rambousková, D. Walterová, J. Vostálová, Arch. Dermatol. Res. 300 (2008) 225.
[19] M. Jordheim, N.H. Giske, Ø.M. Andersen, Biochem. Syst. Ecol. 35 (2007) 153.
[20] I. Palíková, J. Heinrich, P. Bednář, P. Marhol, V. Křen, L. Cvak, K. Valentová, F. Růžička, V. Holá, M. Kolář, V. Šimánek, J. Ulrichová, J. Agric. Food Chem. 56 (2008) 11883.
[21] C.T. da Costa, D. Horton, S.A. Margolis, J. Chromatogr. A 881 (2000) 403.
[22] J. Valls, S. Millán, M. Pilar Martí, E. Borràs, L. Arola, J. Chromatogr. A 1216 (2009) 7143.
[23] C.D. Stalikas, J. Sep. Sci. 30 (2007) 3268.
[24] I. Revilla, S. Perez-Magarin, M.L. Gonzalez-SanJose, S. Beltra, J. Chromatogr. A 847 (1999) 83.
[25] O.M. Andersen, T. Fossen, K. Torskangerpoll, A. Fossen, U. Hauge, Phytochemistry 65 (2004) 405.
[26] C. Alcalde-Eon, M.T. Escribano-Bailón, C. Santos Buelga, J.C. Rivas-Gonzalo, Anal. Chim. Acta 563 (2006) 238.
[27] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutounet, Phytochemistry 47 (1997) 1401.
[28] M.J. Rein, V. Ollilainen, M. Vahermo, J. Yli-Kauhaluoma, M. Heinonen, Eur. Food Res. Technol. 220 (2005) 239.
[29] C.F. Timberlake, P. Bridle, Am. J. Enol. Viticult. 27 (1976) 97.
[30] O. Boutin, E. Badens, J. Food Eng. 92 (2009) 396.
[31] R. Otero, G. Carrera, J.F. Dulsat, J.L. Fabregas, J. Claramunt, J. Chromatogr. A 1057 (2004) 193.
[32] L. Longo, G. Vasapollo, J. Agric. Food Chem. 53 (2005) 475.
[33] P. Bhandari, N. Kumar, A.P. Gupta, B. Singh, V.K. Kaul, J. Sep. Sci. 30 (2007) 2092.
[34] J. Oliveira, V. Fernandes, C. Miranda, C. Santos-Buelga, A. Silva, V. de Freitas, N. Mateus, J. Agric. Food Chem. 54 (2006) 6894.
[35] C. Garcia-Viguera, P. Zafrilla, F.A. Tomas-Barberan, Phytochem. Anal. 9 (1998) 274.
[36] P. Jing, M.M. Guisti, J. Food Sci. 72 (2007) 363.
[37] Y. Lu, Y. Foo, Tetrahedron Lett. 42 (2001) 1371.
[38] M.P. Kähkönen, J. Heinämäki, V. Ollilainen, M. Heinonen, J. Sci. Food Agric. 83 (2003) 1403.
[39] J. He, C. Santos-Buelga, N. Mateus, V. de Freitas, J. Chromatogr. A 1134 (2006) 215.
[40] M.M. Giusti, L.E. Rodriguez-Saona, D. Griffin, R.E. Wrolstad, J. Agric. Food Chem. 47 (1999) 4657.
[41] A.M. González-Paramaĭs, F. Lopes da Silva, P. Martín-López, G. Marcz-Pop, S. González-Manzano, C. Alcalde-Eon, J.J. Pérez-Alonso, M.T. Escribano-Bailón, J.C. Rivas-Gonzalo, C. Santos-Buelga, Food Chem. 94 (2006) 428.
[42] M. Schwarz, S. Hillebrand, S. Habben, A. Degenhardt, P. Winterhalter, Biochem. Eng. J. 14 (2003) 179.