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Isolation and Characterization of Anthocyanins from Blue-fleshed Potatoes (*Solanum tuberosum* L.)

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ABSTRACT

During the last decade anthocyanin-related research has been intensified due to the fact that a high intake of anthocyanin-rich food has been linked to health-protecting effects. Pigmented potato (*Solanum tuberosum* L.) varieties constitute an important source of anthocyanins. So far, little information concerning the metabolism, bioactivity, and bioavailability of acylated anthocyanins has been published because it is still difficult to obtain pure pigments on a preparative scale. In our studies, the pigment composition of the blue-fleshed potato cultivars 'Hermanns Blaue', 'Vitelotte', 'Shetland Black', and 'Valfi' were examined. The preparative isolation of anthocyanins was carried out by applying two different methods of countercurrent chromatography (CCC), the so-called High-Speed Countercurrent Chromatography (HSCCC) and Low Speed Rotary Countercurrent Chromatography (LSRCCC), respectively. By application of LSRCCC, HSCCC, as well as preparative HPLC it was possible to isolate and characterize the major pigments, i.e. 3-*p*-coumaroylrutinoside-5-glucosides of petunidin, malvidin, and peonidin, of the four cultivars. From the cultivar 'Hermanns Blaue' LSRCCC enabled the isolation of the major pigment petunidin-3-*p*-coumaroylrutinoside-5-glucoside on a preparative scale. Furthermore, it was possible to separate and characterize several non-acylated 3,5-diglucosides, i.e. 3-rutinoside-5-glucosides of petunidin and malvidin, acylated 3,5-diglucosides, i.e. 3-feruloylrutinoside-5-glucosides of petunidin and malvidin as well as the 3-caffeoylrutinoside-5-glucoside of petunidin, and the p-coumaric acid derivative petunidin-3-*p*-coumaroylrutinoside. Purity and identity of the so-obtained anthocyanins were controlled by HPLC-DAD, ESI-MS^n, and NMR-measurements.

Keywords: anthocyanin composition, acylated anthocyanins, High-Speed Countercurrent Chromatography, Low Speed Rotary Countercurrent Chromatography

INTRODUCTION

Potato (*Solanum tuberosum* L.) is after rice, wheat, and maize the most important staple food in the nutrition of world's population. Most potatoes belong to the species *Solanum tuberosum* and in recent years there has been an increasing interest in red- and purple-fleshed potato varieties (Andersen *et al.* 2002; Reyes *et al.* 2004, 2005). The pigments responsible for the attractive color of these potato varieties belong to the class of anthocyanins (Mazza and Miniati 1993; Clifford 2000). They are part of a very large group of plant constituents known as flavonoids and widely distributed in red, blue, and purple colored fruits and vegetables. The anthocyanin concentration in pigmented potatoes is known to vary in large ranges (Reyes *et al.* 2005; Jansen and Flamme 2006) and correlates with the degree of pigmentation in potato flesh. According to Reyes *et al.* (2005) and Lewis *et al.* (1998) certain purple and red colored potatoes contain high amounts of anthocyanins (> 100 mg per 100 g fresh weight) depending on their cultivar. **Fig. 1** shows blue-fleshed tubers of the deep-colored variety 'Vitelotte'. The identification and structure elucidation of anthocyanins in colored potatoes has been reviewed in several papers. It was found that red-fleshed potatoes predominantly contain acylated pelargonidin derivatives (Naito *et al.* 1998; Rodriguez-Saona *et al.* 1998) while blue-fleshed varieties show a complex composition of several acylated derivatives of petunidin, malvidin, and peonidin, respectively (Andersen *et al.* 1991; Fossen and Andersen 2000; Fossen *et al.* 2003; Eichhorn and Winterhalter 2005).

During the last decade research related to anthocyanins has been intensified because of their possible benefits on human health. A good correlation between the total anthocyanin content and the antioxidant level of colored tubers was found by Reyes *et al.* (2005). The antioxidant activity of potatoes depends on the flesh composition and therefore blue- and red fleshed varieties exhibit antioxidant values which are 2- to 3-fold higher than in white- or yellow-fleshed cultivars (Brown *et al.* 2003; Brown 2005; Lachman *et al.* 2008). Due to this fact, a high intake of anthocyanin-rich food has been linked to health preventive effects and reduced risks of e.g., aged-related macular degeneration (Jang *et al.* 2005), certain forms of cancer (Zhao *et al.* 2004; Cooke *et al.* 2006) or cardiovascular disorders (Mazza 2007). Although numerous studies have focused on the bio-

Fig. 1 The blue-fleshed potato variety 'Vitelotte'.

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activity, bioavailability, and metabolism of non-acylated anthocyanins (Murkovic *et al.* 2000; Wu *et al.* 2002; Bitsch *et al.* 2004), there is only little information about *in vitro* and *in vivo* analyses of complex acylated anthocyanins, because it is very difficult to obtain these pigments on a preparative scale.

In our study, the preparative isolation of anthocyanins from blue-fleshed potato cultivars 'Hermanns Blaue, 'Vitelotte', 'Shetland Black', and 'Valfi' was carried out by application of two modern methods of countercurrent chromatography (CCC), the so-called High-Speed Countercurrent Chromatography (HSCCC) and Low Speed Rotary Countercurrent Chromatography (LSRCCC), respectively. LSRCCC and HSCCC are automated and easy to handle liquid-liquid chromatographic techniques which have been used for the preparative isolation of numerous natural products because of the gentle isolation conditions and large yields of pure compounds (Winterhalter 2007). Purity and identity of the so-obtained anthocyanins were checked by HPLC-DAD, ESI-MS^n, and NMR-measurements before submitting them for further *in vitro* and *in vivo* analyses.

MATERIALS AND METHODS

Plant material

The potato varieties 'Hermanns Blaue', 'Vitelotte', and 'Shetland Black' were supplied in 2003 by Karsten Ellenberg, Bioland Bauernhof, Barum, Germany. In the case of variety 'Valfi' the tubers were grown on a field of the agricultural area of the University of Applied Sciences Osnabrück (Germany) in 2006.

Extraction of pigments

The entire blue-fleshed potatoes were washed with cold water, dried, and cut into small pieces using a vegetable slicer. Approximately 800 grams of potato slices were blanched for 3 min with 800 mL of water. The same volume of a mixture of water/hydrochloric acid (19/1, v:v) was added, the suspension was cooled at 0°C for 3 h and stored at room temperature for at least 8 h. To remove solid material, the suspension was filtered over glass wool. The crude extract was applied onto an Amberlite XAD-7 column. The column was carefully washed with water, and the anthocyanins were eluted with a mixture of methanol/acetic acid (19/1, v:v). The eluate was concentrated *in vacuo*, dissolved in water and freeze-dried. Due to the remaining high content of polymers (e.g. starch), which hamper the subsequent fractionation by countercurrent chromatography, the obtained XAD-7 extracts were cleaned-up by precipitation. Approximately 100 mg of XAD-7 extract were dissolved in 4 mL of a mixture of methanol/water (1/1, v:v). After addition of 36 mL of a mixture of *tert*-butyl metyl ether/methanol (7/2, v:v), the precipitate was removed by filtration. The combined clear filtrates were evaporated *in vacuo* and the aqueous phase was lyophilized.

Countercurrent Chromatography (CCC)

The HSCCC separations of the blue-fleshed potato varieties 'Vitelotte', 'Shetland Black', and 'Valfi' were carried out with a high-speed model CCC-1000 (Triplecoil, diameter of tubing: 2.6 mm, total volume: 850 mL, revolution speed: 850 rpm) produced by Pharma-Tech Research Corporation (Baltimore, Maryland, USA) and *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water (2/2/1/5, v:v:v:v, acidified with 0.1% trifluoroacetic acid as solvent system. The less dense layer was acting as stationary phase, and a flow rate of 4 mL/min was delivered by a Biotronik HPLC-Pump-BT 3020 from Jasco, Groß-Umstadt, Germany). Approximately 1 g of the XAD-7 extract was injected for each run. Fractions were collected with a Pharmacia LKB super Frac fraction collector (Bromma, Schweden). The process of separation was monitored at 520 nm with a Knauer UV/Vis detector (Berlin, Germany) and the chromatogram was recorded by a plotter (BBC Goerz Metrawatt SE 120, Vienna, Austria).

In case of the blue-fleshed variety 'Hermanns Blaue', the separation was carried out by LSRCCC with a low speed countercurrent chromatograph (Prototype Pharma-Tech Research Corp., Baltimore, Maryland, USA) equipped with a single coil (diameter of tubing: 8.2 mm, total volume: 5.5 L, revolution speed 60 rpm). The solvent system consisted of a mixture of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water (2/2/1/5, v:v:v:v) acidified with 0.1% trifluoroacetic acid. The less dense layer was acting as stationary phase at a flow rate of 5 mL/min (HPLC-Pump 64, Knauer, Berlin, Germany). A 9.25 g amount of the XAD-7 extract was injected in a single run. Elution was monitored at 520 nm with a Knauer UV/Vis detector (Berlin, Germany) and recorded by using a plotter (BBC Goerz Metrawatt SE 120, Vienna, Austria). Fractions were collected with a Super Frac fraction collector (Phamacia LKB, Bromma, Sweden).

High-Performance Liquid Chromatography (HPLC)

HPLC-DAD analyses were performed on Jasco MD-910 Multiwavelength Detector (Groß-Umstadt, Germany, range of wavelength between 220 and 650 nm), equipped with Jasco DG-980-50 3-Line Degasser and Jasco LG-980-02 Ternary Gradient Unit (Groß-Umstadt, Germany), Jasco PU-980 Intelligent HPLC-Pump (Groß-Umstadt, Germany) and Borwin PDA chromatography software. Peaks were detected at 520 nm. HPLC separation was carried out on Luna RP-18 column (250 × 4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) and eluted at a flow rate of 0.5 mL/min. The samples were injected via a Rheodyne 7175 injection valve using a 20 µL sample loop.

Two solvent systems were used. Solvent system A consisted of water/acetonitrile/formic acid 87/3/10 (v:v:v) and solvent system B contained water/acetonitrile/formic acid 40/50/10 (v:v:v). The gradient was as follows: 0 min 6% B, 20 min 20% B, 35 min 40% B, 40 min 60% B, 45 min 90% B and 55 min 6% B.

High-Performance Liquid Chromatography – Electrospray Ionization Multiple Mass Spectrometry (HPLC-ESI-MS^n)

HPLC-ESI-MS^n analyses were performed on a Bruker Esquire-LC multiple ion trap mass spectrometer (Bruker Daltonik, Bremen, Germany). Mass spectral analyses were recorded under following conditions: positive ion mode, capillary voltage - 3500 V, dry gas flow 4.0 mL/min, dry temperature 300°C, nebulizer pressure 10.0 psi. Chromatographic analyses were performed using an Agilent System 1100 binary pump (Böblingen, Germany) equipped with a Rheodyne 7725i injection valve and a 20 µL loop (Techlab, Erkerode, Germany) employing the same chromatographic conditions as described above. LC conditions were controlled using a ChemStation, and mass spectrometric data acquisition was performed by Esquire NT 4.0 software (Bruker Daltonik, Bremen, Germany).

Preparative HPLC

Purifications were performed on Knauer HPLC Pump K-1001 (Berlin, Germany) equipped with a Knauer UV/Vis detector K-2600 (Berlin, Germany) and Knauer HPLC Software Eurochrom 2000. The samples were injected via a Rheodyne injection valve using a 200 µL sample loop. The separation was carried out on Luna RP-18 column (250 × 15.0 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) with a flow rate of 6.25 mL/min. Solvent system A consisted of water/acetonitrile/formic acid 87/3/10 (v:v:v) and solvent system B contained water/acetonitrile/formic acid 40/50/10 (v:v:v). Three different gradients were used. Gradients I to III processed as follows: 0 min 6% B, 20 min 20% B (gradient I); 0 min 12% B, 15 min 20% B, 20 min 30% B, 23 min 40% B (gradient II); 0 min 30% B, 15 min 40% B, 20 min 60% B (gradient III). Gradient I was used to separate non-acylated anthocyanins. Gradients II and III were used to isolate acylated pigments. Spectra were recorded at 520 nm and 280 nm, respectively.

Nuclear Magnetic Resonance Spectroscopy (NMR)

Proton- and Carbon-NMR analyses were performed on a Bruker AMX 300 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 300.13 and 75.49 MHz, respectively. All samples were dissolved in CD_3OD/CF_3COOD (19/1, v:v), and NMR data were pro-

cessed by WIN-NMR software version 6.1.0.0.

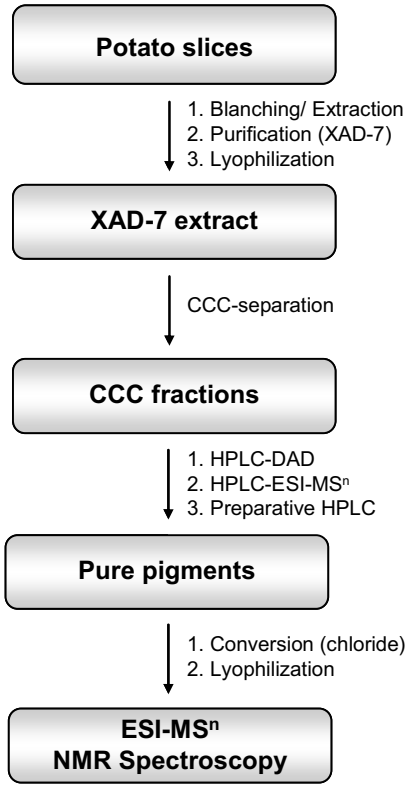

Fig. 2 Schematic presentation of key steps used for the isolation of anthocyanins from pigmented potatoes.

Purity determination of pigments

CCC fractions were checked by HPLC and HPLC-ESI-MS^n analyses. The purity was calculated as percentage peak area. The purification of CCC fractions by preparative HPLC afforded pure pigments, which were transferred into the chloride salts by adding an equimolar amount of hydrochloric acid. The freeze dried pigments were finally analyzed by ESI-MS^n and NMR-measurements. **Fig. 2** shows the schematic presentation of key steps used for the isolation of anthocyanins from pigmented potatoes.

RESULTS AND DISCUSSION

Preparative isolation by Countercurrent Chromatography (CCC)

1. 'Hermanns Blaue'

Blue-fleshed potato variety 'Hermanns Blaue' is an old potato cultivar that is grown in Germany. The tubers are large sized and oval shaped with white and blue marbled flesh. Typical for this potato variety is a small white-fleshed zone under the purple skin. The anthocyanin composition of this breeding cultivar was examined by HPLC-DAD and HPLC-ESI-MS^n analyses. One major and nine minor anthocyanins were detected at 520 nm. **Fig. 3** shows the HPLC chromatogram at 520 nm. By means of Low Speed Rotary Countercurrent Chromatography (LSRCCC) it was possible to isolate the major pigment petunidin-3-*p*-coumaroylrutinoside-5-glucoside (**5**) in large yields and good purity. A 2.5 g amount of this acylated anthocyanin with a purity of 85% could be isolated from fraction 5 in a single run. Further isolation by preparative HPLC afforded approximately 800 mg of pure pigment. **Fig. 4** shows the chromatogram of the LSRCCC separation at 520 nm. With the aid of preparative HPLC it was possible to isolate further minor pigments

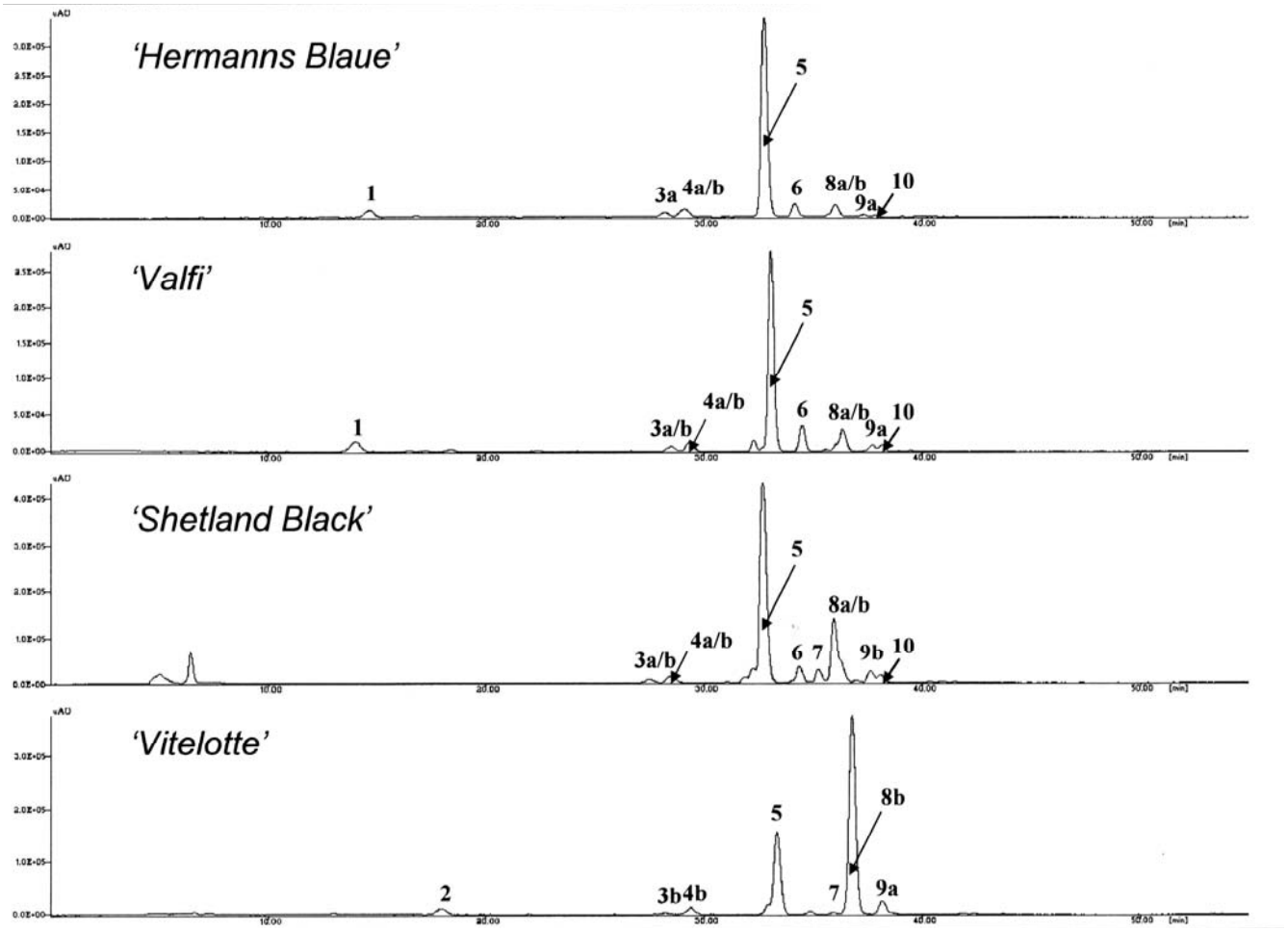

Fig. 3 HPLC chromatograms of the anthocyanin-enriched XAD-7 extracts of the blue-fleshed cultivars 'Hermanns Blaue', 'Valfi', 'Shetland Black', and 'Vitelotte' at 520 nm.

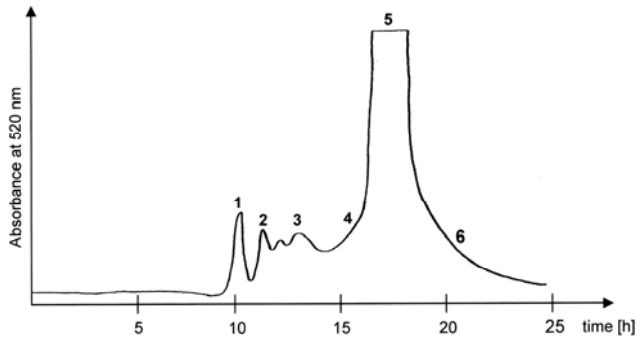

Fig. 4 LSRCCC separation of the cultivar 'Hermanns Blaue' detected at 520 nm.

from different CCC-fractions. The fractions 1 and 2 afforded 18 mg of the non-acylated petunidin derivative petunidin-3-rutinoside-5-glucoside (**1**). Approximately 26 mg of petunidin-3-caffeoylrutinoside-5-glucoside (**4a**) could be isolated from fraction 4. The minor pigments petunidin-3-feruloylrutinoside-5-glucoside (**6**) and petunidin-3-*p*-coumaroylrutinoside (**10**) as well as the malvidin derivative malvidin-3-*p*-coumaroylrutinoside-5-glucoside (**8b**) coelute in fraction 5 and were separated in yields ranging between 16.5 and 17.7 mg. Furthermore, the three minor pigments delphinidin-3-*p*-coumaroylrutinoside-5-glucoside (**4b**), peonidin-3-*p*-coumaroylrutinoside-5-glucoside (**8a**), and malvidin-3-feruloylrutinoside-5-glucoside (**9a**) could be found in different CCC fractions and were characterized by HPLC-ESI-MS^n. The identity of peak **3a**, an unknown petunidin derivative which shows the same molecular ion (*m/z* 933) and fragmentation pattern (*m/z* 771, 479, and 317) as the major pigment petunidin-3-*p*-coumaroylrutinoside-5-glucoside (**5**), is still under investigation. Alcalde-Eon *et al.* (2004) found the same unknown petunidin derivative in pinta bocca (*Solanum stenotomum*) tubers and proposed different substitution patterns of the sugar moieties.

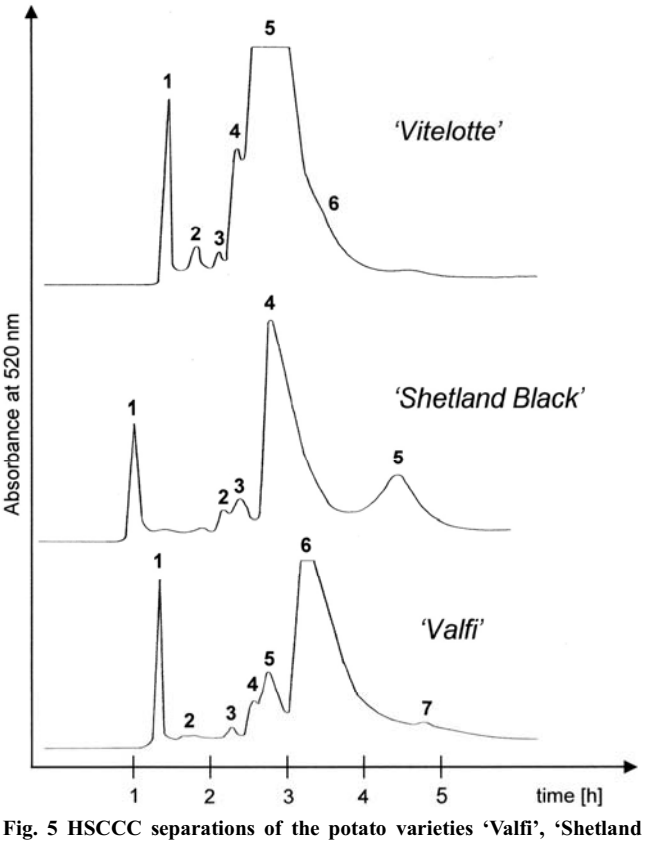

Fig. 5 HSCCC separations of the potato varieties 'Valfi', 'Shetland Black', and 'Vitelotte' monitored at 520 nm.

2. 'Valfi'

The cultivar 'Valfi' is a blue-fleshed potato that originates from Czech Republic. The tubers are medium sized, oval shaped with blue-purple skin and blue-purple marbled flesh. The HPLC chromatogram of the XAD-7 isolate of this variety is presented in **Fig. 3**. Chromatographic analyses show one major anthocyanin and ten minor pigments. In case of the variety 'Valfi', the separation was carried out by HSCCC and seven fractions as well as the coil residue were obtained (**Fig. 5**). The anthocyanin composition of each fraction was analyzed by HPLC-DAD and HPLC-ESI-MS^n. Fraction 1 (42 mg) contains the non-acylated 3,5-diglycoside petunidin-3-rutinoside-5-glucoside (**1**) in a purity of 79%. The major compound of fraction 4 (24 mg) was identified as petunidin-3-caffeoylrutinoside-5-glucoside (**4a**). Approximately 3.5 mg of this anthocyanin could be isolated from this fraction by preparative HPLC. CCC fraction 5 contains mainly the acylated petunidin derivative petunidin-3-feruloylrutinoside-5-glucoside (66%) (**6**). The major anthocyanin fraction 6 (284 mg) eluted after 3 hours and contains the major pigment of the cultivar 'Valfi' petunidin-3-*p*-coumaroylrutinoside-5-glucoside (**5**) in a purity of 74%. Furthermore, the acylated anthocyanins **3a**, **3b**, **4b**, **8a**, **8b**, **9a**, and **10** were found as minor pigments in different CCC fractions and could be characterized by HPLC-ESI-MS^n analyses.

3. 'Shetland Black'

The tubers of the variety 'Shetland Black', native to the Shetland Islands, are small sized, round shaped and deep-blue skinned. The flesh of this cultivar is yellow with a small purple vascular ring. The HPLC chromatogram of the XAD-7 extract shows two major and nine minor pigments (**Fig. 3**). By means of HSCCC 5 fractions were obtained (**Fig. 5**). The major fraction 4 (210 mg) consisted of a mixture of different acylated anthocyanins. Approximately 10 mg of malvidin-3-*p*-coumaroylrutinoside-5-glucoside (**8b**) were isolated by preparative HPLC from this fraction. From fraction 5 (100 mg) purification by preparative HPLC afforded 20 mg of petunidin-3-*p*-coumaroylrutinoside-5-glucoside (**5**) and 12 mg of peonidin-3-*p*-coumaroylrutinoside-5-glucoside (**8a**). Additional minor pigments (i.e. **3a**, **3b**, **4a**, **4b**, **6**, **7**, **9b**, and **10**) were identified by HPLC-DAD and HPLC-ESI-MS^n analyses in different CCC fractions.

4. 'Vitelotte'

The breeding cultivar 'Vitelotte' is an old French gourmet variety with deep blue skin and violet flesh which was cultivated in the middle of the 19th century. The form and the taste of this ancient potato variety are very uncommon. The tubers are medium sized, oval shaped and contain many deep eyes. They have a nutty flavor and smell like chestnut. Also the anthocyanin composition of this variety is very unusual (cf. **Fig. 3**). Two major pigments and five minor anthocyanins were detected at 520 nm. The HSCCC separation of the XAD-7 lyophilisate yielded six fractions (**Fig. 5**). Fraction 1 contains mainly the non-acylated malvidin derivative malvidin-3-rutinoside-5-glucoside (**2**). Approximately 10 mg of this compound could be isolated by preparative HPLC. The pigments petunidin-3-*p*-coumaroylrutinoside-5-glucoside (**5**), malvidin-3-*p*-coumaroylrutinoside-5-glucoside (**8b**), and malvidin-3-feruloylrutinoside-5-glucoside (**9a**) coelute in CCC fraction 4 and were separated by preparative HPLC in yields ranging between 7 and 20 mg. The major anthocyanin fraction 5 (327 mg) eluted after 2.5 hours and contains the acylated malvidin derivative malvidin-3-*p*-coumaroylrutinoside-5-glucoside (**8b**) in good purity (85%). The pigments **3b**, **4b**, and **7** could be identified by HPLC-ESI-MS^n analyses in different CCC fractions as minor compounds.

Table 1 Mass spectrometric properties of anthocyanins found in the blue-fleshed potato varieties 'Hermanns Blaue', 'Valfi', 'Shetland Black', and 'Vitelotte'.

Compound		$[M]^+$ (*m/z*)	MS/MS (*m/z*)
1	petunidin-3-rutinoside-5-glucoside[a]	787	317, 479, 625
2	malvidin-3-rutinoside-5-glucoside[a]	801	331, 493, 639
3a	petunidin derivative[b]	933	317, 479, 771
3b	malvidin-3-rutinoside[b]	639	331, 493
4a	petunidin-3-caffeoylrutinoside-5-glucoside[a]	949	317, 479, 787
4b	delphinidin-3-*p*-coumaroylrutinoside-5-glucoside[b]	919	303, 465, 757
5	petunidin-3-*p*-coumaroylrutinoside-5-glucoside[a]	933	317, 479, 771
6	petunidin-3-feruloylrutinoside-5-glucoside[a]	963	317, 479, 801
7	pelargonidin-3-*p*-coumaroylrutinoside-5-glucoside[b]	887	271, 433, 725
8a	peonidin-3-*p*-coumaroylrutinoside-5-glucoside[a]	917	301, 463, 755
8b	malvidin-3-*p*-coumaroylrutinoside-5-glucoside[a]	947	331, 493, 785
9a	malvidin-3-feruloylrutinoside-5-glucoside[a]	977	331, 493, 815
9b	peonidin-3-feruloylrutinoside-5-glucoside[b]	947	301, 463,785
10	petunidin-3-*p*-coumaroylrutinoside[a]	771	317, 479

[a] Structure identification based on ESI-MS^n analysis and NMR data.
[b] Structure identification based on ESI-MS^n analysis.
Numbering according to **Fig. 3**.

Comparison of the four cultivars

A comparison of the anthocyanin composition of the blue-fleshed potato varieties 'Hermanns Blaue' and 'Valfi' show remarkable agreements. Both cultivars contain only one petunidin-based major pigment **5**. 'Shetland Black' was found to contain only acylated anthocyanins. The anthocyanin profile of this cultivar is dominated by the acylated petunidin derivative **5** and the peonidin analogue **8a**. The low anthocyanin content of this variety is due to the absence of anthocyanins in the flesh. Furthermore, the deep-colored breeding cultivar 'Vitelotte' shows differences in form, taste, and anthocyanin composition. This variety contains two major pigments, i.e. the petunidin-based pigment **5** as well as the malvidin derivative **8b**. The analysis of the anthocyanin profile of the four blue-fleshed potato varieties demonstrate the complexity of the anthocyanin composition of pigmented potatoes. With the help of CCC prefractionation and HPLC purification it was possible to characterize various potato pigments with nearly the same retention time. **Table 1** summarizes the mass spectrometric properties of characterized anthocyanins and **Fig. 6** shows the structures of the main pigments **5**, **8a**, and **8b** (3-*p*-coumaroylrutinoside-5-glucosides of petunidin, peonidin, and malvidin, respectively).

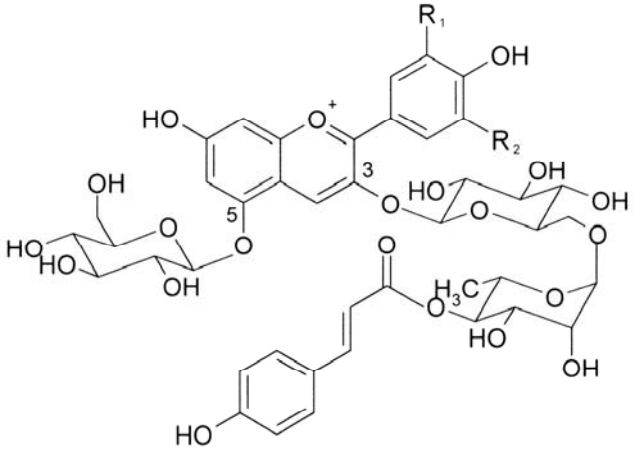

Fig. 6 Structures of major anthocyanins isolated from blue-fleshed potato varieties: petunidin-3-*p*-coumaroylrutinoside-5-glucoside (**5**) (R_1 = OCH_3, R_2 = OH), peonidin-3-*p*-coumaroylrutinoside-5-glucoside (**8a**) (R_1 = OCH_3, R_2 = H), malvidin-3-*p*-coumaroylrutinoside-5-glucoside (**8b**) (R_1 = OCH_3, R_2 = OCH_3).

CONCLUSION

With the aid of countercurrent chromatography two non-acylated (**1** and **2**) and seven acylated pigments (**4a**, **5**, **6**, **8a**, **8b**, **9a** and **10**) could be obtained and characterized from the four blue-fleshed potato cultivars under investigation. The structures of the isolated pigments were elucidated by ^{1}H- and ^{13}C-NMR spectroscopy and compared with literature data (Slimestad *et al.* 1999; Fossen and Andersen 2000; Fossen *et al.* 2003). By means of Low Speed Rotary Countercurrent Chromatography it was possible to obtain a major pigment of blue-colored potatoes (i.e. petunidin-coumaroylrutinoside-5-glucoside (**5**) from 'Hermanns Blaue') on a preparative scale and good purity in a single run. Further large-scale separations of different blue- and red-fleshed potato varieties are still under investigation.

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