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Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins

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Abstract

The paper reports the use of anthocyanins as natural food colorants and their potential health benefits regarding coronary heart disease and cancer prevention. The principles of countercurrent chromatography are described and several applications dealing with the isolation of anthocyanins from *Tradescantia pallida* leaves, purple corn, elderberry juice, red wine and blackberries are presented. Several hundred milligrams of pure anthocyanins were obtained within a single CCC run. Isolated pigments include monoglycosylated, acylated and highly glycosylated derivatives of anthocyanins. Purity and identity of the isolated anthocyanins were confirmed by HPLC with diode array detection, HPLC-electrospray ionization multiple mass spectrometry and nuclear magnetic resonance spectroscopy.
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Keywords: High-speed countercurrent chromatography; Anthocyanins; Health effects; Coronary heart disease; French paradox; *Tradescantia pallida*; *Zea mays*; Purple corn; *Vitis vinifera*; Red wine; *Sambucus nigra*; Elderberry; *Rubus fruticosus*; Blackberry; Antioxidant capacity; TEAC; Cyanidin-3-glucoside

1. Introduction

Anthocyanins form one of the most widespread groups of pigments throughout the plant kingdom with several hundred structurally different compounds already elucidated. Anthocyanins are responsible for most of the reddish and bluish colors found in leaves, vegetables, fruits and blossoms. Due to the growing demand for natural pigments anthocyanin enriched extracts from e.g. elderberry, red grape skins or red cabbage are widely used today as coloring agents for different types of food [1].

Anthocyanins also exhibit a range of biological activities and their intake may help to improve or at least maintain human health. One of the best known attributes of anthocyanins is the antioxidant activity, especially of the cyanidin derivatives [2–4]. In 1992, the incidence of coronary heart disease (CHD) in France was reported to be much lower compared to other nations, even though the uptake of fats in the diets were otherwise similar [5]. This phenomenon, known today as the “French Paradox”, has been explained by the higher consumption of red wine in France (red wine consumption *per capita* 1999, France: 59.9 l; USA: 7.6 l; CHD mortality of men aged 35–74 per 100,000, France: 87; USA 214) [6,7]. The antiatherosclerotic effect of red wine is considered to be linked to the antioxidant properties of anthocyanins and other polyphenolic wine constituents which may prevent oxidation of low density lipoproteins, one of the key steps in the formation of CHD.

Furthermore, trials with rabbits and rats demonstrated antiinflammatory effects of anthocyanins from bilberry (*Vaccinium myrtillus*) [8]. Kamei et al. [9] tested different classes of phenolic compounds for their ability to inhibit colon cancer cell growth *in vitro* and found the anthocyanins to be the most potent phenolics. Especially cyanidin was very effective in inhibiting cell growth at concentrations as low as 2 μg/ml, which is only 1/10 of the concentration required for the potent anticarcinogen genistein, an isoflavone. A recent trial also revealed anti-cancer activity for anthocyanins from blueberries [10].

Despite the growing knowledge about biological activities, surprisingly little is known about the bioavailability of anthocyanins and their metabolic fate in humans [11,12].

To carry out the necessary experiments about resorption and metabolism, anthocyanin preparations are needed which preferably contain only a single compound to keep the test system as simple as possible. In nature, however, anthocyanins generally occur as mixtures. In blueberries e.g. as much as 16 different anthocyanins were identified by HPLC

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[1]. Furthermore in clinical trials, large amounts of material are required and the existing HPLC methods are not suited for large-scale isolation of anthocyanins. Hence, alternative methods such as the support-free technique of countercurrent chromatography (CCC) have gained growing importance in anthocyanin separation.

CCC as an all liquid chromatographic technique operates under gentle conditions and allows non-destructive isolation even of labile natural compounds. Due to the absence of any solid stationary phase, adsorption losses are minimized and, hence a 100% sample recovery is guaranteed. CCC has been successfully applied to the isolation of e.g. carotenoids from *Gardenia jasminoides* [13], flavonol glycosides from black tea, endive and shallots [13–15], catechins and proanthocyanidins from black tea [14], isoflavones from soy flour [16] and lignans from flaxseed [17]. With the use of CCC it was also possible to isolate anthocyanins from various sources, in amounts up to several hundred milligrams of pure compounds [13,18,19].

2. Materials and methods

2.1. Chemicals

Acetonitrile was of HPLC purity, *n*-butanol and hexane were of analytical grade. Methanol and *tert*-butyl-methyl-ether (TBME) were re-distilled prior to use. Trifluoroacetic acid (TFA) was obtained from Sigma-Aldrich (Munich, Germany), deuterated NMR-solvents from Deutero (Kastellaun, Germany).

2.2. CCC Instrumentation

A CCC-1000 high-speed countercurrent chromatograph (Pharma-Tech Research Corporation, Baltimore, MD) equipped with three coils connected in series (inner diameter of tubing = 2.6 mm, total volume 850 ml) was used. Revolution speed was set to 1000 rpm. Solvent system I consisted of *n*-butanol–TBME–acetonitrile–water (2:2:1:5, v/v/v/v, acidified with 0.1% TFA). Solvent system II had the same composition but contained less TFA (0.01%). Solvent system III was *n*-butanol–TBME–acetonitrile–water (3:1:1:5, v/v/v/v, acidified with 0.1% TFA). Flow rates are specified in the description of the respective application. The less dense layer was always used as the stationary phase, therefore elution mode was head to tail. The solvent systems were delivered by a Biotronik BT 3020 HPLC pump (Jasco, Gross-Umstadt, Germany). Separation was monitored by a Knauer UV/Vis-Detector at 520 nm and chromatograms were recorded with a Knauer L250E plotter (Knauer, Berlin, Germany). Fractions were collected every 3–5 min with a Super Frac fraction collector (Pharmacia LKB, Bromma, Sweden). Typical sample loads were between 500 and 1000 mg injected through a 20 ml sample loop.

2.3. HPLC with diode array detection (HPLC-DAD)

A Jasco PU-980 Intelligent HPLC Pump equipped with a DG-980-50 3-Line Degasser, a LG-980-02 Ternary Gradient Unit and a MD-1510 Multiwavelength Detector was used (Jasco, Gross-Umstadt, Germany). Sample was injected via a Rheodyne 7175 injection valve (Techlab, Erkerode, Germany) equipped with a 20 μl loop. Chromatograms were recorded with Jasco-Borwin chromatography software version 1.50. Separations were carried out on a RP-18 5 μm Luna 150 mm × 4.6 mm column (Phenomenex, Aschaffenburg, Germany). Solvents were water–formic acid–acetonitrile (87/10/3, v/v/v, solvent A; 40/10/50, v/v/v, solvent B) and the flow rate was 0.8 ml/min. Linear gradient from 6 to 20% B at 0–20 min, 20 to 40% B at 20–35 min, 40 to 60% B at 35–40 min, 60 to 90% B at 40–45 min, and finally back to initial conditions.

2.4. HPLC with electrospray ionisation multiple mass spectrometry (HPLC-ESI-MS^n)

A Bruker Esquire LC-MS system was used (Bruker Daltonik, Bremen, Germany). The HPLC system consisted of a System 1100 Binary Pump G1312A (Agilent, Böblingen, Germany), a Rheodyne 7725i injection valve with a 20 μl loop (Techlab, Erkerode, Germany) and a Lichrograph L-4000 UV/Vis-Detector (Merck Hitachi, Tokyo, Japan). UV-chromatograms were recorded with a Chromatopac C-R6A integrator (Shimadzu, Kyoto, Japan), LC part of the system was controlled by ChemStation version A.06.01, MS-data was processed by esquire NT 4.0 software (Bruker Daltonik, Bremen, Germany). MS parameters: positive mode, capillary −2500 V, end plate offset −500 V, capillary exit 70 V, skim 1 20 V, skim 2 10 V, dry gas 325 °C, gas flow 11 l/min, nebulizer 60 psi.

2.5. NMR

^{1}H and ^{13}C NMR spectra were recorded on a Bruker AMX 300 spectrometer (Bruker Biospin, Rheinstetten, Germany) at 300.13 and 75.49 MHz, respectively. Anthocyanins were dissolved in a mixture of methanol-d_4-TFA-d_1 (19:1, v/v). Data was processed by WIN-NMR software version 6.1.0.0.

2.6. General procedure for anthocyanin extraction

Anthocyanin enriched extracts were isolated by solid phase extraction using Amberlite XAD-7 (Fluka, Buchs, Switzerland). A glass column (100 cm × 6 cm) was filled with the resin in a mixture of methanol–water (1:1, v/v), then thoroughly washed with methanol and finally rinsed with water. Fruit juices or liquid commercial extracts were applied directly onto the column. Red wines were diluted with an equal amount of water. Solid samples like fruits or blossoms were cut into smaller pieces and extracted with

methanol–acetic acid (19:1, v/v) in a household blender. After filtration the organic solvent was evaporated *in vacuo* and the remaining aqueous phase poured onto a XAD-7 resin column. The column was then washed with water to remove sugars, organic acids, proteins and salts. Phenolic compounds (like the anthocyanins) were retained by the resin and eluted by a mixture of methanol–acetic acid (19:1, v/v). Methanol was evaporated *in vacuo* and the aqueous phase lyophilized. Removal of non-colored polyphenols (i.e. flavanols) can be achieved by extraction of the aqueous solution with ethyl acetate, either before or after XAD-7 enrichment.

2.7. Determination of total antioxidative capacity

Total antioxidant capacity of isolated anthocyanins was determined by using the modified TEAC assay published by Re et al. [20]. The assay is based on the ability of antioxidants to quench the mono radical cation 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$). Trolox, a water-soluble vitamin E analogue, is used as reference compound with an antioxidant capacity of 1 mmol Trolox/mmol per definition.

3. Results and discussion

3.1. Countercurrent chromatography

CCC is an automated version of liquid–liquid extraction, comparable to the repeated partitioning of an analyte between two immiscible phases by vigorous mixing in a separatory funnel. Modern CCC started in 1970 with the development of the so-called droplet countercurrent chromatograph (DCCC). The DCCC apparatus consists of up to 600 vertical glass tubes (2 mm inner diameter) which are connected through thin Teflon capillaries. The tubes were first filled with one layer (the stationary phase) of a two phase solvent system and then equilibrated by pumping the other layer (the mobile phase) through the system. After sample injection partitioning between the steady stream of droplets and the surrounding stationary phase occurs, and the analytes are separated according to their partition coefficient. DCCC was successfully applied to the isolation of glycosides, e.g. glycosidic flavor precursors from grape juice and wine [21], as well as other natural compounds such as saponins, alkaloids and peptides [22].

However, the DCCC-technique also had some disadvantages: Solvent systems were limited to those being able to form droplets, a characteristic that is influenced by flow rate, surface tension and viscosity. As only low flow rates could be applied the separation time was long and leakage at the several hundred connections was also a common problem. Moreover, separation efficiency was low because of the poor mixing of the two phases.

In order to improve mixing of the two phases techniques known as rotation locular CCC (RLCCC) and gyration locular CCC (GLCCC) were developed. The real breakthrough of CCC came with the invention of the Coil Planet Centrifuge that was introduced by Ito in 1981 [23]. Today this technique is known as high-speed CCC (HSCCC).

HSCCC separation takes place in a so-called “multi-layer coil” that is made by wrapping an inert Teflon tubing around a holder in multiple layers. Consequently the technique is also known as multilayer coil CCC (MLCCC). The tubing usually has an inner diameter between 1.6 and 2.6 mm and the length can reach 160 m. Multiple coils can be connected in series to increase the total volume of the instrument and the sample capacity. During separation the coil is rotated in a planetary fashion; it rotates at 800–1000 rpm around its own ‘planetary’ axis and simultaneously around a parallel ‘solar’ axis. This planetary rotation has two effects:

1. The rotation creates a fluctuating acceleration field which enables vigorous mixing of the two phases followed by settling within the coil. In areas of the coil which are close to the center of rotation the force field is weak. As a consequence, the phases are mixed. At a further point of their orbit, when they are far away from the center of rotation, the force field becomes stronger and the two phases are separated. Alternate mixing and settling is repeated with each rotation and in this way up to 50,000 partitioning steps per hour can be achieved.
2. Rotation of the coil also enables retention of stationary phase. During rotation of a coil filled with two immiscible liquids, it can be observed that the two phases move towards opposite ends of the coil, known as head and tail. Generally the less dense phase displaces the heavier phase towards the tail, but the orientation is also known to be influenced by viscosity and interfacial tension. This phenomenon, known as hydrodynamic equilibrium, requires to choose the elution mode carefully but it also gives the analyst the freedom to select either the lighter

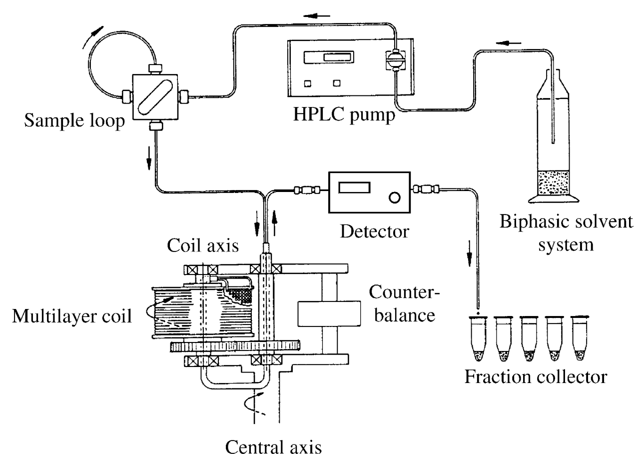

Fig. 1. Instrumental setup of a single coil HSCCC [24], modified. Solvents are delivered by an HPLC pump, sample is injected via sample loop. Separation takes place in a multilayer coil and is monitored by UV-Vis detection. Fractions are collected with a fraction collector.

or the heavier layer as mobile or stationary phase. When the heavier phase is selected as the mobile phase, the proper elution mode is head to tail and the mobile phase is introduced from the head of the system. By choosing the lighter phase as the mobile phase the elution order of the compounds is reversed, the correct elution mode is tail to head and the mobile phase is pumped into the tail of the system.

The first step before performing a separation is the selection of a suitable two phase solvent system by measuring the partition coefficients of the target compounds. This can be done in a simple test tube experiment. A solvent system is prepared by equilibrating the solvents in a separatory funnel to make sure that both phases are saturated with each other. Two milliliters of each upper and lower phase are mixed with a few milligrams of the sample in a test tube. Settling of the two phases should not take longer then 30 s. A longer settling time will reduce retention of stationary phase and consequently reduce the separation efficiency. Measuring of the concentration of the analytes in both phases is done by spectrophotometry, HPLC, GC or TLC. The partition coefficient is calculated by dividing the concentration of the target compound in the stationary phase by the concentration in the mobile phase. Suitable values are in the range of 0.5 and 1. A lower value means the compound is barely soluble in the stationary phase and therefore elutes very early and probably not well separated. A higher value means the compound is soluble mostly in the stationary phase and the separation will take too long.

After determination of the correct elution mode the coil is loaded with stationary phase, i.e. the lighter phase in head to tail mode. Solvents are delivered by an HPLC pump. The sample is dissolved in equal amounts of both phases and then injected via a sample loop. After injection, rotation of the system is started immediately accompanied by pumping of the mobile phase at the optimum flow rate which depends on total volume of the coil and the individual separation problem. During the first stage of separation, a certain amount of stationary phase will be displaced until the hydrodynamic equilibrium is reached. From then on only mobile phase will elute from the system. In the case of anthocyanins, the separation can be monitored by a UV/Vis-Detector and the separated compounds will be collected by a fraction collector. The basic instrumental setup of a single coil HSCCC is shown in Fig. 1 [24].

One possibility to increase the throughput of HSCCC is the use of pressure resistant stainless steel coils with larger diameters which allow higher flow rates. However, the additional weight due to the stainless steel tubing and increase in total volume may cause mechanical problems. In addition, hazards related to the high-speed rotation of heavy weights in a centrifugal force field should not be left unattended [25].

A different approach is used in low-speed rotary CCC (LSRCCC). In LSRCCC a cylindrical column rotates slowly around a single axis. Du et al. [26] showed that the use of special convoluted tubing (e.g. with 8.5 mm inner diameter) enabled sufficient retention of stationary phase at a rotational speed of only 5–100 rpm. In first experiments with an instrument equipped with a 10 l column, Du et al. separated 150 g of a crude tea extract and obtained 40 g of the anticancer compound epigallocatechin gallate. Separation time was 72 h, but due to the slow rotation (21 rpm) the system can be operated without supervision during the separation process. The instrument is readily scaled up by using longer columns and/or by increasing the inner diameter of the convoluted tubing. Instruments that are suitable for separation in the kg-scale are under development in our laboratory.

4. Application of HSCCC

4.1. Isolation of anthocyanins from purple heart (*Tradescantia pallida Rose*)

T. pallida, also known as purple heart, is a plant with dark purple leaves native to eastern Mexico. For its anthocyanin mixture, an excellent stability has been reported [27] and the structure of the major anthocyanin was determined to be 3-*O*-[6-*O*-[2,5-di-*O*-(*E*)-α-L-arabinofuranosyl]-β-D-glucopyranosyl]-7,3′-di-*O*-[6-*O*-(*E*)-ferulyl-β-D-glucopyranosyl] cyanidin [28]. Leaves from *T. pallida* were collected in the botanical garden of our university and extracted with methanol–acetic acid (19:1, v/v). After removal of the organic solvent, chlorophyll was removed from the aqueous residue by repeated washing with pentane before the solution was applied to the XAD-7 column. Colorless polyphenols were removed post-column by extraction with ethyl acetate. HSCCC separation was achieved using solvent system I at a flow rate of 5.0 ml/min. The CCC chromatogram as well as the structure of the major isolated pigment is displayed in Fig. 2.

4.2. Isolation of anthocyanins from purple corn (*Zea mays L.*)

Maíz morado (purple corn) is a pigmented variety of *Z. mays* L. originating from Peru and Bolivia where the traditional purple drink ‘Chicha Morada’ is prepared by boiling the corn with pineapple and quince peel, cinnamon and cloves. Maíz morado is also used in the production of purple tortilla chips and the extracts are applied as food colorants.

There are several reports about the anthocyanin composition in various parts of the plant. A summary can be found in [1,29]. The 3-*O*-glucosides of cyanidin, peonidin and pelargonidin as well as a coumaroylated cyanidin-3-galactoside have been reported to occur in seed coats and cobs [30–33]. Cyanidin-3-(6″-malonylglucoside) and cyanidin-3-dimalonylglucoside have been identified only in reddened maize leaves [29,34].

From a commercial water-soluble extract from Maíz morado cobs the anthocyanin fraction was isolated by ads-

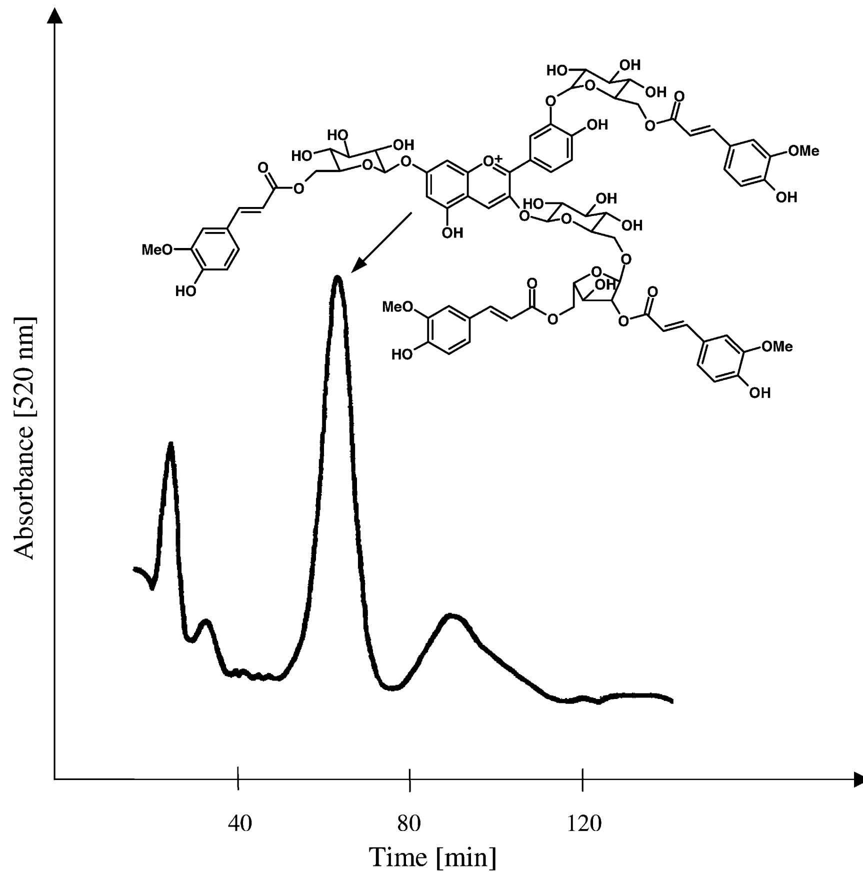

Fig. 2. HSCCC isolation of 3-*O*-[6-*O*-[2,5-di-*O*-(*E*)-α-L-arabinofuranosyl]-β-D-glucopyranosyl]-7,3′-di-*O*-[6-*O*-(*E*)-ferulyl-β-D-glucopyranosyl]cyanidin from leaves of *T. pallida*. Solvent system *n*-butanol–TBME–acetonitrile–water (2:2:1:5, v/v/v/v, acidified with 0.1% TFA), flow rate 5.0 ml/min, detection at 520 nm.

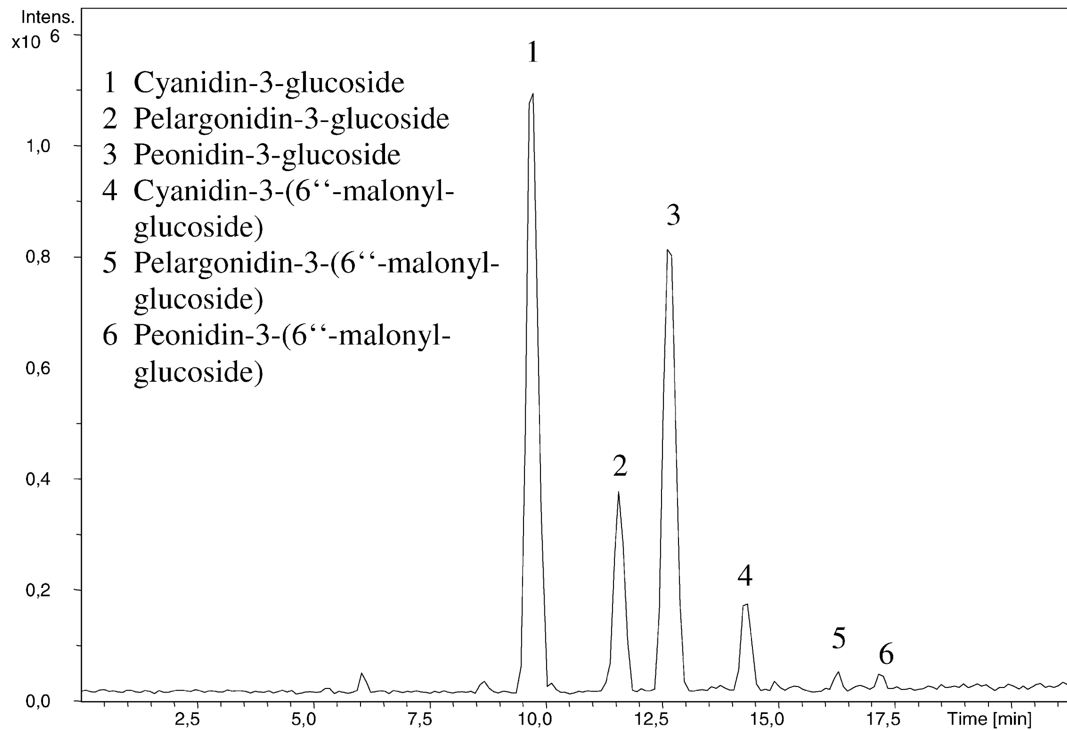

Fig. 3. HPLC-ESI-MS^n chromatogram of an anthocyanin XAD-7 extract from Maíz morado (cf. text for experimental conditions).

Table 1
HPLC-ESI-MS^n data and relative composition calculated from the peak areas obtained from the HPLC chromatogram at 520 nm of the anthocyanin enriched extract from Maíz morado

Pigment	t_R (min)	$[M^+]$ (*m/z*)	MS/MS (*m/z*)	Relative composition (%)
Cyanidin-3-glucoside	9.7	449	287	50.8
Pelargonidin-3-glucoside	11.6	433	271	6.6
Peonidin-3-glucoside	12.6	463	301	16.2
Cyanidin-3-(6″-malonylglucoside)	14.2	535	287, 449	18.5
Pelargonidin-3-(6″-malonylglucoside)	16.2	519	271, 433	2.8
Peonidin-3-(6″-malonylglucoside)	17.2	549	301, 463	5.1

orption on XAD-7. HPLC-ESI-MS^n analysis of the enriched extract (Fig. 3) showed the presence of four major pigments, which were identified as the 3-glucosides of cyanidin, pelargonidin and peonidin as well as cyanidin-3-(6″-malonylglucoside). Two later eluting pigments have been tentatively identified as pelargonidin-3-(6″-malonylglucoside) and peonidin-3-(6″-malonylglucoside). The relative anthocyanin composition of the extract calculated from the peak areas obtained at 520 nm is summarized together with HPLC-ESI-MS^n data in Table 1. Dimalonylated monoglucosides of cyanidin and peonidin could also be detected with HPLC-ESI-MS^n in trace amounts.

The extract was separated by HSCCC using solvent system II. In order to avoid degradation of the labile malonylated anthocyanins, the solvents were only slightly acidified with 0.01% TFA. Upon CCC (cf. Fig. 4) three fractions were collected. The first one contained pure cyanidin-3-glucoside. The second fraction consisted of a mixture of the four major compounds, however, a strong enrichment (66%) of cyanidin-3-(6″-malonylglucoside) was apparent. In the third fraction, peonidin-3-(6″-malonylglucoside) was the major anthocyanin (56%). Preparative HPLC was required for further purification of the malonylated anthocyanins. To the best of our knowledge, this is the first report of cyanidin and peonidin 3-(6″-malonylglucoside) in maize cobs. Both pigments have earlier been identified in maize flowers and leaves. The occurrence of a malonylated pelargonidin monoglycoside—tentatively identified as pelargonidin-3-(6″-malonylglucoside)—is reported for the first time.

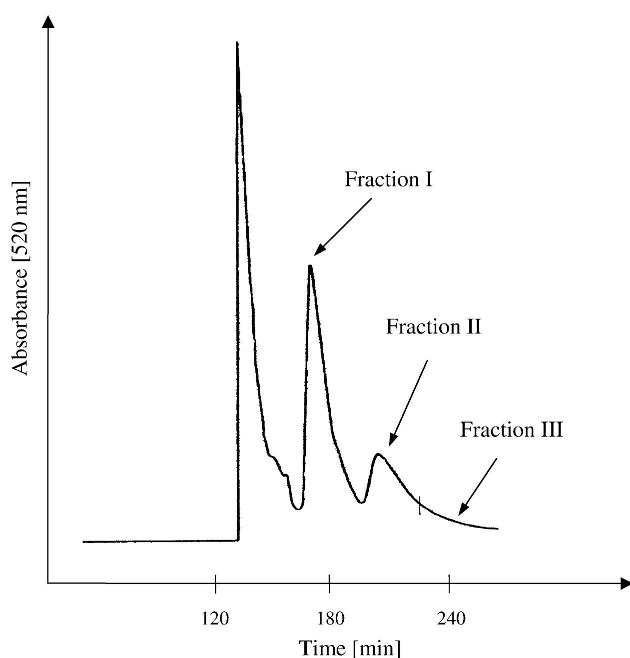

Fig. 4. HSCCC separation of an anthocyanin XAD-7 extract from Maíz morado. Solvent system *n*-butanol–TBME–acetonitrile–water (2:2:1:5, v/v/v/v, acidified with 0.01% TFA), flow rate 5.0 ml/min, detection at 520 nm. Fraction I contains pure cyanidin-3-glucoside, fractions II and III are strongly enriched in cyanidin- and peonidin-3-(6″-malonylglucoside), respectively.

4.3. Isolation of anthocyanins from elderberries (*Sambucus nigra L.*)

Elderberry (*S. nigra* L.) is a widespread tree indigenous to temperate and continental zones of Europe belonging to the family *Caprifoliaceae*. The black berries are often used to prepare wines, juices and jams [35,36]. Elderberries show beneficial effects against a variety of diseases, e.g. colds, fever and tonsillitis [36]. Due to the high amount of anthocyanins, elderberries have often been used as natural food colorant [37]. The major pigments of *S. nigra* L. were identified as cyanidin-3-glucoside and cyanidin-3-sambubioside (sambubiose = β-D-glucopyranosyl-(2 → 1)-β-D-xylopyranoside). Minor pigments were characterized as cyanidin-3,5-diglucoside and cyanidin-3-sambubioside-5-glucoside [1].

From a commercially available elderberry juice, cyanidin-3-glucoside and 3-sambubioside were obtained in pure

Table 2
Antioxidant capacity of elderberry derived pigments determined by the TEAC assay

Pigment	Antioxidant capacity	
	μmol Trolox/mg	mmol Trolox/mmol
Cyanidin-3-glucoside	4.1	1.9
Cyanidin-3-sambubioside	2.8	1.7
Cyanidin-3,5-diglucoside	1.0	0.7
Cyanidin-3-sambubioside-5-glucoside	1.4	0.8

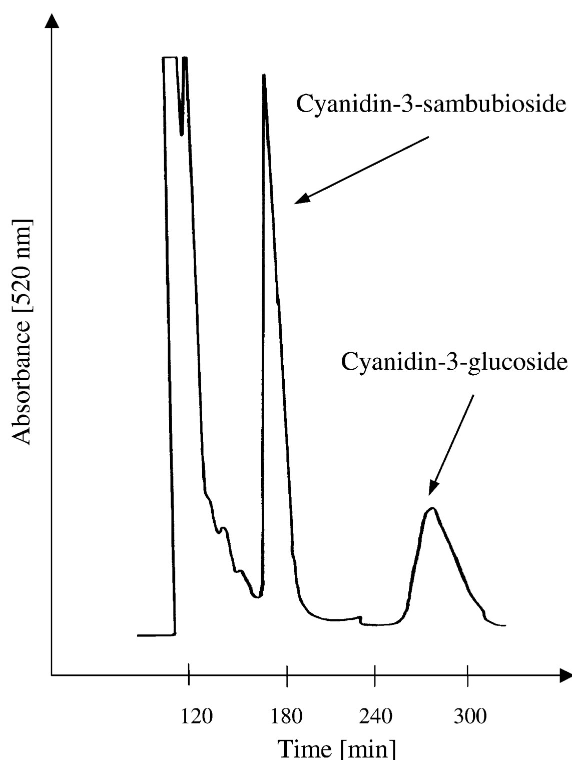

Fig. 5. HSCCC separation of the major anthocyanins from elderberry juice. Solvent system *n*-butanol–TBME–acetonitrile–water (3:1:1:5, v/v/v/v, acidified with 0.1% TFA), flow rate 5.0 ml/min, detection at 520 nm.

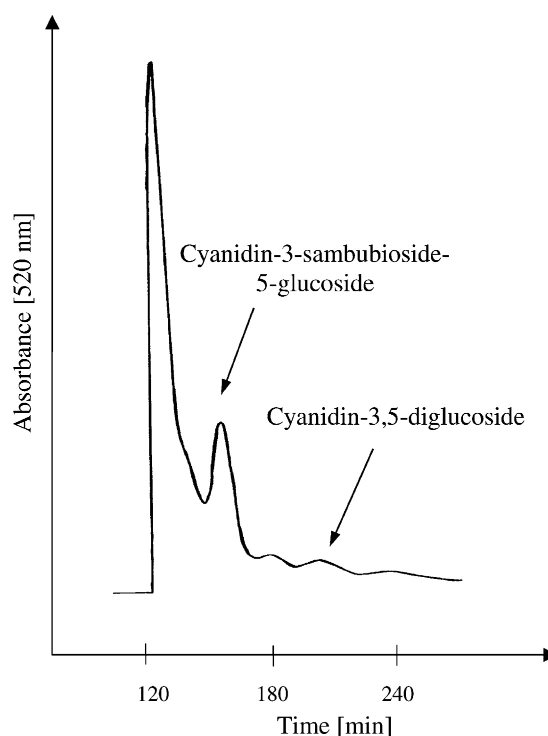

Fig. 6. HSCCC separation of the minor anthocyanins from elderberry juice. Solvent system *n*-butanol–TBME–acetonitrile–water (3:1:1:5, v/v/v/v, acidified with 0.1% TFA), flow rate 2.5 ml/min, detection at 520 nm.

form after XAD-7 enrichment and CCC separation with solvent system III at a flow rate of 5.0 ml/min (Fig. 5). The first fraction contained a mixture of the two minor pigments which were subsequently purified with the same solvent system by simply performing a second CCC separation at a lower flow rate of 2.5 ml/min (Fig. 6). Purity and identity of the obtained anthocyanins were confirmed by HPLC-DAD, HPLC-ESI-MS^n and ^1H NMR.

Total antioxidant capacity of the isolated anthocyanins was determined using the TEAC assay (Table 2). Values ranged from 0.7 to 1.9 mmol Trolox equivalents per mmol and 4.1 to 1.0 mmol Trolox per milligram, respectively. Cyanidin-3-glucoside showed the highest antioxidative capacity among the elderberry derived pigments being almost twice as effective as Trolox.

4.4. Isolation of anthocyanins from red wine

Grapes native to Europe belong to the species *Vitis vinifera* L. ssp. vinifera and contain the 3-glucosides of malvidin, peonidin, petunidin, delphinidin and cyanidin with malvidin-3-glucoside or 'oenin' usually being the major pigment. Most varieties, except for Pinot Noir, also contain a certain amount of anthocyanins acylated with acetic, caffeic or *p*-coumaric acid. 3,5-Diglucosylated pigments are present only in trace amounts in European cultivars, but

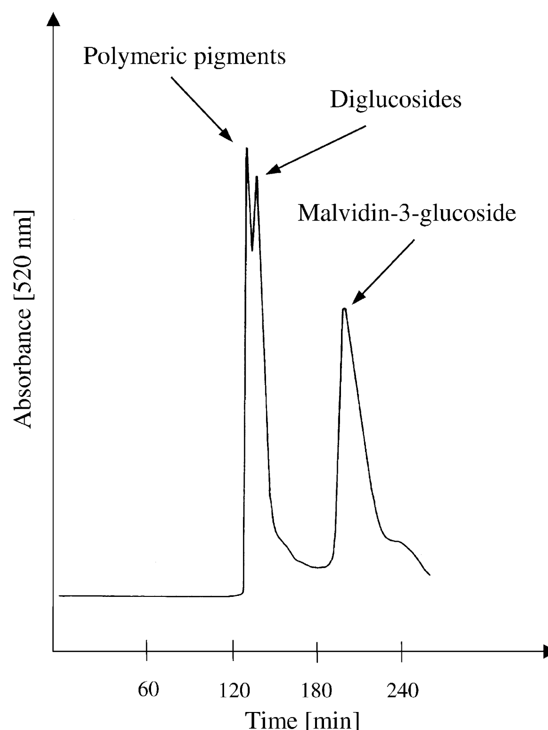

Fig. 7. HSCCC separation of anthocyanins from red wine. Solvent system *n*-butanol–TBME–acetonitrile–water (2:2:1:5, v/v/v/v, acidified with 0.1% TFA), flow rate 3.7 ml/min, detection at 520 nm. After injection of 700 mg XAD-7 extract 70 mg of the diglucoside mixture and 70 mg malvidin-3-glucoside were obtained.

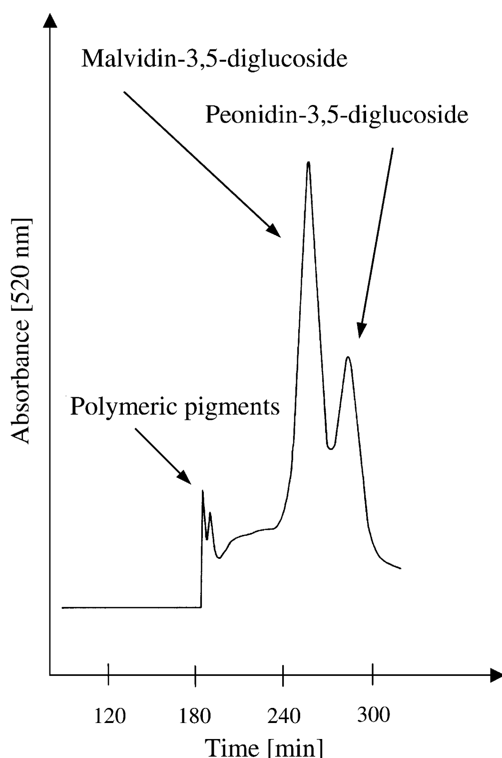

Fig. 8. HSCCC separation of the diglucoside fraction from red wine. Solvent system *n*-butanol–TBME–acetonitrile–water (3:1:1:5, v/v/v/v, acidified with 0.1% TFA), flow rate 2.0 ml/min, detection at 520 nm. Malvidin- and peonidin-3,5-diglucoside were obtained in a ratio of approx. 6:4.

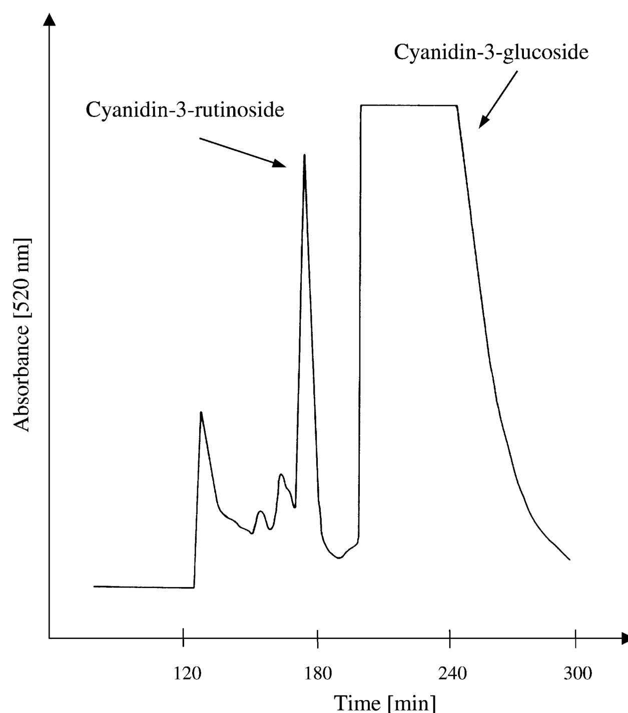

Fig. 9. HSCCC isolation of cyanidin-3-rutinoside and cyanidin-3-glucoside from wild growing blackberries. Solvent system *n*-butanol–TBME–acetonitrile–water (2:2:1:5, v/v/v/v, acidified with 0.1% TFA), flow rate 3.0 ml/min, detection at 520 nm. Injection of 800 mg XAD-7 extract yielded 330 mg cyanidin-3-glucoside in 97% purity and 40 mg cyanidin-3-rutinoside.

can be found in American grapes and their hybrids with *V. vinifera*.

An XAD-7 extract was produced from a Californian red wine made from the varieties Ruby Cabernet, Centurion and Zinfandel. The first separation was performed using solvent system I at a flow rate of 3.7 ml/min and the result is shown in Fig. 7. Polar polymeric pigments, formed by intermolecular condensation of anthocyanins or by reaction of anthocyanins with other polyphenols, elute first. Not much is known about the structure of these pigments, but they contribute significantly to the overall color of aged wines and are also responsible for the developing brownish tint. The polymeric fraction is followed immediately by a peak containing a mixture of two diglucosides. The major pigment, malvidin-3-glucoside, is collected next, and finally acetylated, caffeoylated and coumaroylated peonidin and malvidin derivatives are obtained. These latter pigments require further purification by another solvent system, i.e. ethyl acetate–water (1:1, v/v, acidified with 0.1% TFA). In order to separate the diglucosides from each other, a more polar solvent system is needed. This is achieved by changing the composition towards a higher content of *n*-butanol and less TBME. Separation of malvidin-3,5-diglucoside and peonidin-3,5-diglucoside using solvent system III at a flow rate of 2.0 ml/min is shown in Fig. 8.

4.5. Isolation of anthocyanins from blackberries (Rubus fruticosus L. agg.)

R. fruticosus L. agg. is a designation used to denote the aggregate of blackberries from the *Moriferi* subsection of the genus *Rubus*. Blackberries from this subsection are grown in Europe and eastern North America. Blackberry juice was used medicinally until the 16th century against infections of the mouth or eyes [38,39]. Regardless of the wide diversity of blackberries their major anthocyanins have always been identified as cyanidin-3-glucoside and cyanidin-3-rutinoside [1].

Wild growing blackberries were collected in the Braunschweig region. An anthocyanin extract was prepared following the procedure for fresh fruits described in the experimental section and separated by using solvent system I at a flow rate of 3.0 ml/min. Up to 800 mg of the extract were separated in a single run (Fig. 9), yielding 40 mg (5%) cyanidin-3-rutinoside and 330 mg (41%) cyanidin-3-glucoside in 97% purity. Identity of both compounds was confirmed by HPLC-ESI-MS^n, ^1H and ^{13}C NMR in thorough comparison with literature data [40]. NMR-spectra of the isolated cyanidin-3-β-D-glucoside are shown in Figs. 10 and 11.

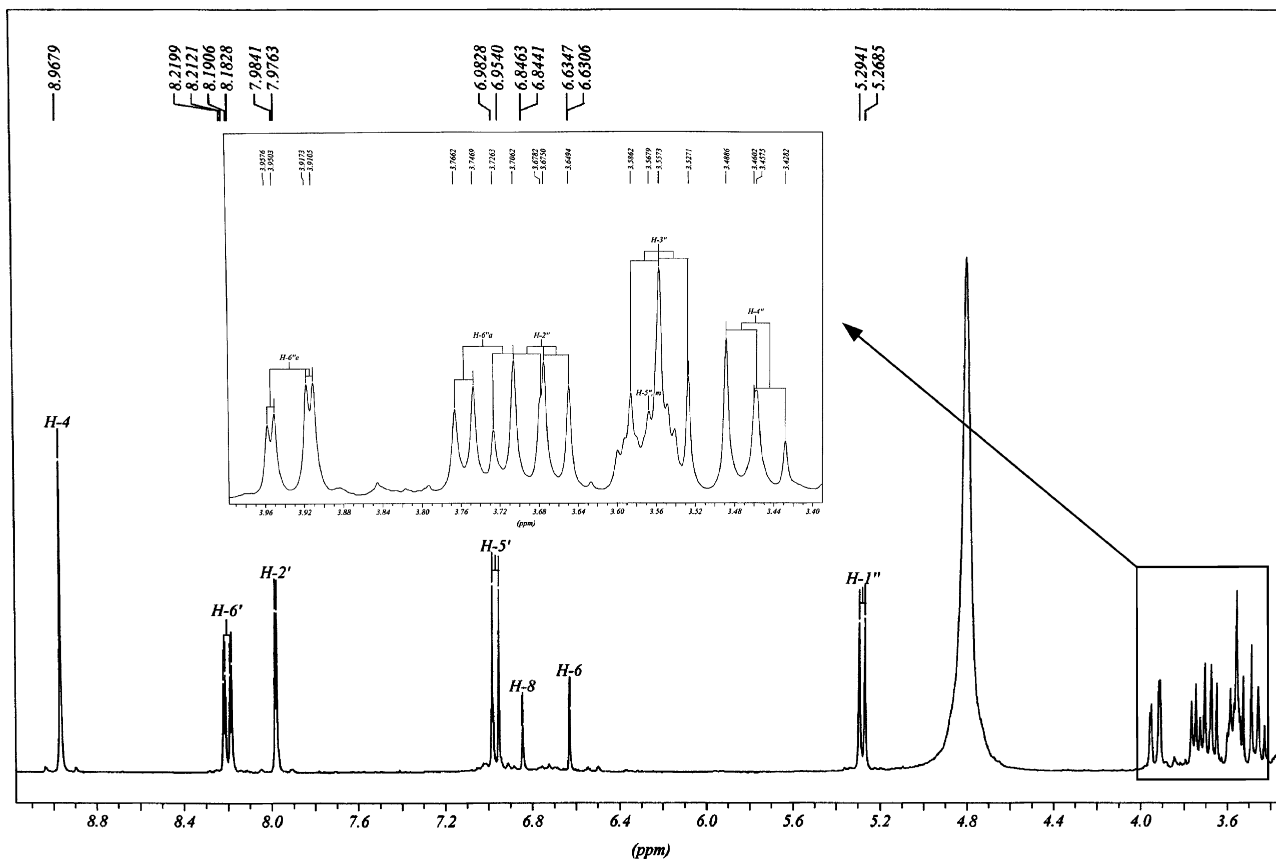

Fig. 10. ^{1}H NMR spectrum of cyanidin-3-β-D-glucoside isolated from blackberry measured at 300 MHz in methanol-d_4-TFA-d_1 (19:1, v/v).

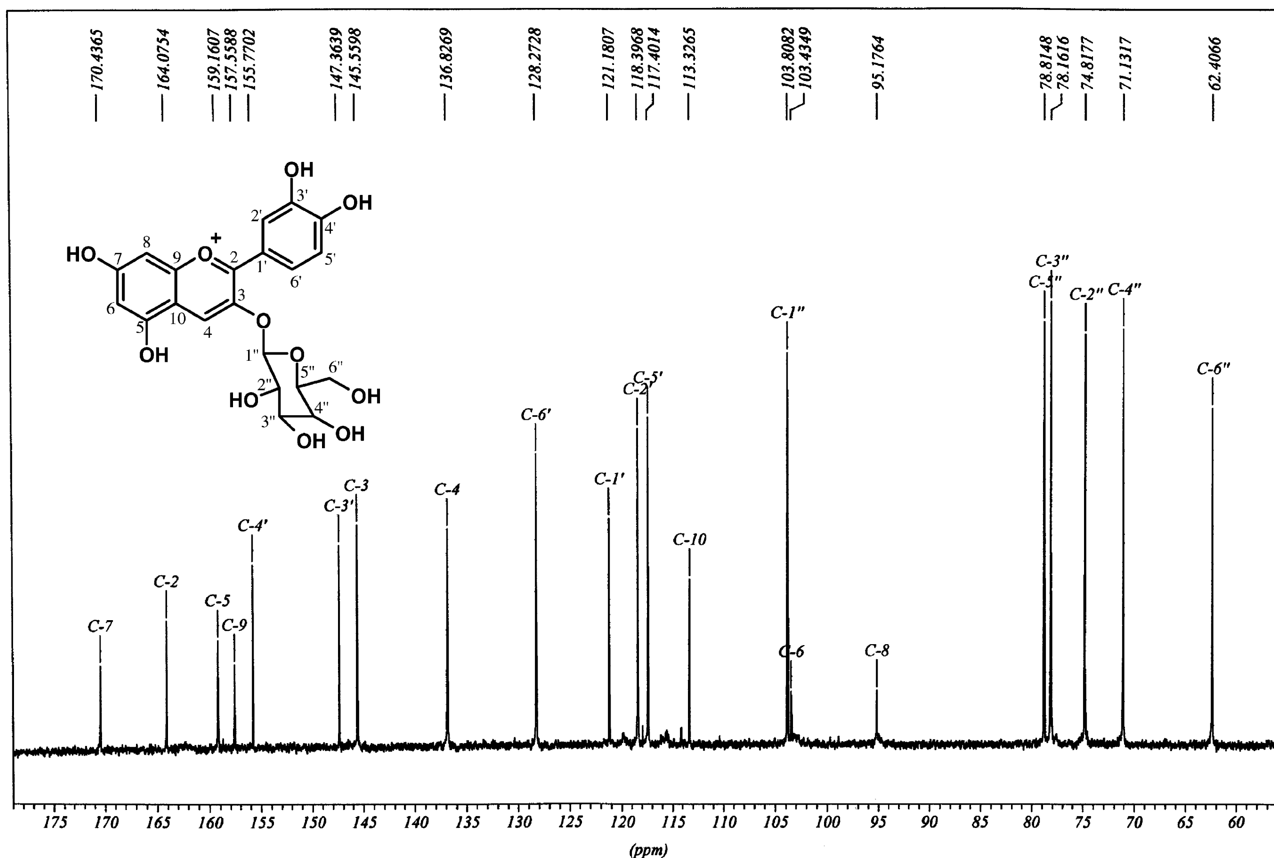

Fig. 11. ^{13}C NMR spectrum of cyanidin-3-β-D-glucoside isolated from blackberry measured at 75 MHz in methanol-d_4-TFA-d_1 (19:1, v/v).

5. Conclusions

The potential of HSCCC for the large-scale isolation of pure anthocyanins from various natural sources has been demonstrated. Blackberries proved to be an outstanding source for the isolation of cyanidin-3-glucoside, an anthocyanin with potent antioxidative and anticarcinogenic properties. From elderberries, a series of cyanidin derivatives has been obtained on a preparative scale. From Maíz morado, pelargonidin-3-(6″-malonylglucoside) could be isolated for the first time. In addition, cyanidin and peonidin-3-(6″-malonylglucoside) were also identified. Compared to preparative HPLC, HSCCC offers several advantages: the sample load is significantly higher and separation requires only cheap solvents instead of expensive solid phase columns. The gentle operating conditions of HSCCC, especially the lack of active surfaces, ensure an isolation even of labile compounds.

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