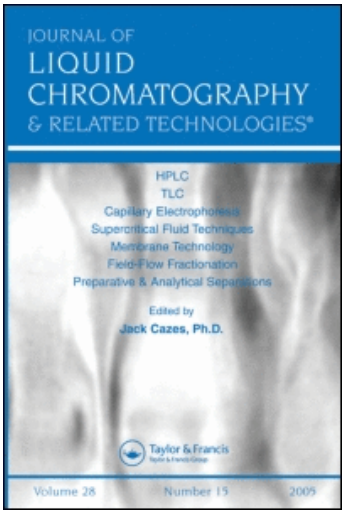

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EXTRACTIONS AND PURIFICATIONS

SEPARATION OF ACYLATED ANTHOCYANIN PIGMENTS BY HIGH SPEED CCC

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ABSTRACT

The reddish pigments of red onions (*Allium cepa*) and flowers of the tulip cultivar Los Angelos, including fragile anthocyanins with aliphatic acylation, were separated in semi-preparative scale by high speed countercurrent chromatography, HSCCC. The lower, polar phase of the biphasic solvent, *t*-butyl-methylether:*n*-butanol:acetonitrile:water (2:2:1:5 v/v) acidified with 0.01% trifluoroacetic acid, was used as mobile phase. All the five major anthocyanins in red onion, which contained the same aglycone (cyanidin), were separated from a 750 mg anthocyanin-enriched isolate. Relative to cyanidin 3-glucoside, analogous anthocyanins with additional acyl group(s) (malonyl) and sugar (glucosyl) entailed higher and lower elution volume, respectively.

The major pigments in a 430 mg anthocyanin-enriched sample from flowers of the tulip "Los Angelos" were determined to be the

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3-*O*-(6”-rhamnosylglucosides) of delphinidin, cyaniding, and pelargonidin, and the 3-*O*-[6”-(2”’-acetylrhamnosyl)glucosides] of cyanidin and pelargonidin. Acetylated anthocyanins were eluted after their non-acetylated analogues. Within each of the two groups, acetylated and non-acetylated, individual anthocyanins were separated according to their number of hydroxy-groups on the aglycone B-rings; highest number entailed lowest elution volume. All the acylated anthocyanins in the anthocyanin-enriched isolates of both red onions and tulip “Los Angelos” were nearly baseline separated.

INTRODUCTION

Anthocyanins constitute a flavonoid subclass, and are responsible for the majority of reddish to bluish colours found in plants.[1] They all consist of an aromatic C15-skeleton with hydroxy- and/or methoxy-groups in various positions. These aglycones are *in vivo* always connected to one or more *O*-glycosyl units, which themselves can be esterified with aliphatic and/or aromatic acids. More than 500 different anthocyanins have been elucidated, and the number has, in recent years, increased more than steadily along with advances in analytical techniques and increased knowledge about these polar plant pigments. Separation and purification have mainly been carried out using paper, thin-layer, and various column chromatographic techniques.[2] Most isolation procedures today incorporate high-performance liquid chromatography. However, countercurrent chromatography (CCC) techniques may provide some advantages such as high separation scale and lack of irreversible adsorption due to lack of solid stationary phase.

Droplet countercurrent chromatography (DCCC) on anthocyanins from black currant and raspberries was the first CCC technique applied on these types of pigments.[3] Later, DCCC has successfully been used on several samples, however, its major drawbacks include very long analysis time and limitations with respect to appropriate solvents. Suitable alternatives are centrifugal partition (CPC) and multilayer coil countercurrent chromatography (MLCCC). CPC has allowed preparative purification of anthocyanins from Champagne vintage by-products[4] and from black currant.[5] MLCCC has been used to isolate riccionidin A and its dimer (riccionidin B) from cell walls of the liverwort *Ricciocarpos natans*,[6] and for partial separation of anthocyanins from black currant.[7] More recently, high speed countercurrent chromatography (HSCCC) has been used to fractionate anthocyanin mixtures from black currant, black chokeberry, Roselle, and for partial fractionation of anthocyanin mixtures from red cabbage,[7] and to isolate malvidin 3-glucoside from red wine.[8]

Anthocyanins acylated with aliphatic acid(s) are rather fragile pigments, which easily degrade during the isolation procedure. The aim of this paper is mainly to show how high speed countercurrent chromatography can be used for semi-preparative isolation of anthocyanins containing various aliphatic acyl groups.

EXPERIMENTAL

Samples

Red onions, *Allium cepa,* (appr. 0.8 kg) bought at the local market were cut into pieces with a knife, and extracted four times using MeOH containing 1% trifluoroacetic acid (TFA). The concentrated and combined extracts were purified by partition against ethyl acetate before application on an Amberlite XAD-7 column.[9] A 750 mg dried sample was, thereafter, subjected to HSCCC.

Petals (appr. 2.0 kg) of the tulip cultivar Los Angelos were collected in the centre of Bergen during June 1999, and treated similarly as described for red onions. A 430 mg sample was subjected to HSCCC.

Authentic pigments used for structure elucidation of the anthocyanins in the tulip cultivar Los Angelos were isolated from black currant (delphindin 3-rutinoside, **6**, and cyanidin 3-rutinoside, **7**)[3] and the tulip "Queen Wilhelmina" (pelargonidin 3-rutinoside, **8**, cyanidin 3-(2'''-acetyl-rutinoside), **9**, and pelargonidin 3-(2'''-acetyl-rutinoside), **10**).[10]

High Speed Countercurrent Chromatography

The HSCCC instrument (Model CCC-1000, Pharma-Tech Research Corp., Baltimore, Maryland, USA) equipped with 3 preparative coils (tubing i.d. 2.6 mm; total volume 850 mL; revolution radius: 15 cm; beta value: 0.5 to 0.75) was used in descending mode with the biphasic solvent *t*-butyl methyl ether: *n*-butanol: acetonitrile: water (2:2:1:5 v/v/v/v) slightly acidified with 0.01% TFA. This solvent has previously been used for separation of anthocyanins from various foodstuffs.[7,8] The flow rate was 5 mL·min^{-1}, and the revolution speed of the centrifuge was 1000 rpm.

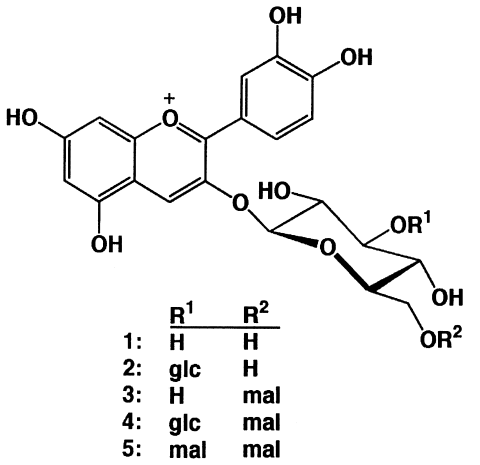
The samples were dissolved in a 1:1 mixture of the two solvent phases, and injected through an injection loop of 40 mL. The retention of the stationary phase was 46% and 56% for the red onion and tulip sample, respectively. Bleeding of stationary phase during elution was marginal. A SPD-10AV VP UV-vis detector (Shimadzu) was used at 520 nm to monitor elution, and an HP-3395

integrator (Hewlett-Packard) was utilised for plotting profiles. The fractions were collected manually.

Quantitative Determinations by HPLC

All the HSCCC fractions were examined by analytical HPLC using diode array detection for proper visualisation of the separation of individual anthocyanin in each of the two HSCCC chromatograms. Based on a linear standard curve obtained for pure cyanidin 3-galactoside, the integrated peak area was used to calculate absolute amounts of each anthocyanin in each HSCCC-fraction (see Fig. 2 and 4). We have not taken into account different molar absorptivities for the individual anthocyanins.

The HPLC results were obtained with a HP-1050 system (Hewlett-Packard) with an ODS Hypersil column (25 cm × 0.5 cm, 5 μm). Two solvents were used for elution; H_2O:HCOOH (18:1, v/v) (A) and MeOH:H_2O:HCOOH (10:8:1, v/v) (B). The elution profile consisted of a linear gradient from 10% to 100% B during the first 17 min, isocratic elution using 100% B the next 6 min, and finally, a new linear gradient back to 10% B during the last minute. The flow rate was set to 1.0 mL·min^{-1}, and aliquots of exactly 15 μL were injected. The chromatograms were monitored at 520 ± 20 nm.



	R^1	R^2
1:	H	H
2:	glc	H
3:	H	mal
4:	glc	mal
5:	mal	mal

Figure 1. Structures of anthocyanins in red onions. 1–5: The 3-glucoside, 3-(3”-glucosylglucoside), 3-(6”-malonylglucoside), 3-(3”-glucosyl-6”-malonylglucoside) and 3-(3”,6”-dimalonylglucoside) of cyanidin.

RESULTS AND DISCUSSION

After extraction, followed by partial purification of the concentrated extract by partition against ethyl acetate and column chromatography on Amberlite XAD-7 resins, the individual anthocyanins were isolated using HSCCC. For proper visualisation of the separation of individual anthocyanin in the HSCCC chromatograms (Figs. 2 and 4), the quantitative amount of each anthocyanin in each HSCCC fraction were calculated after HPLC analysis.

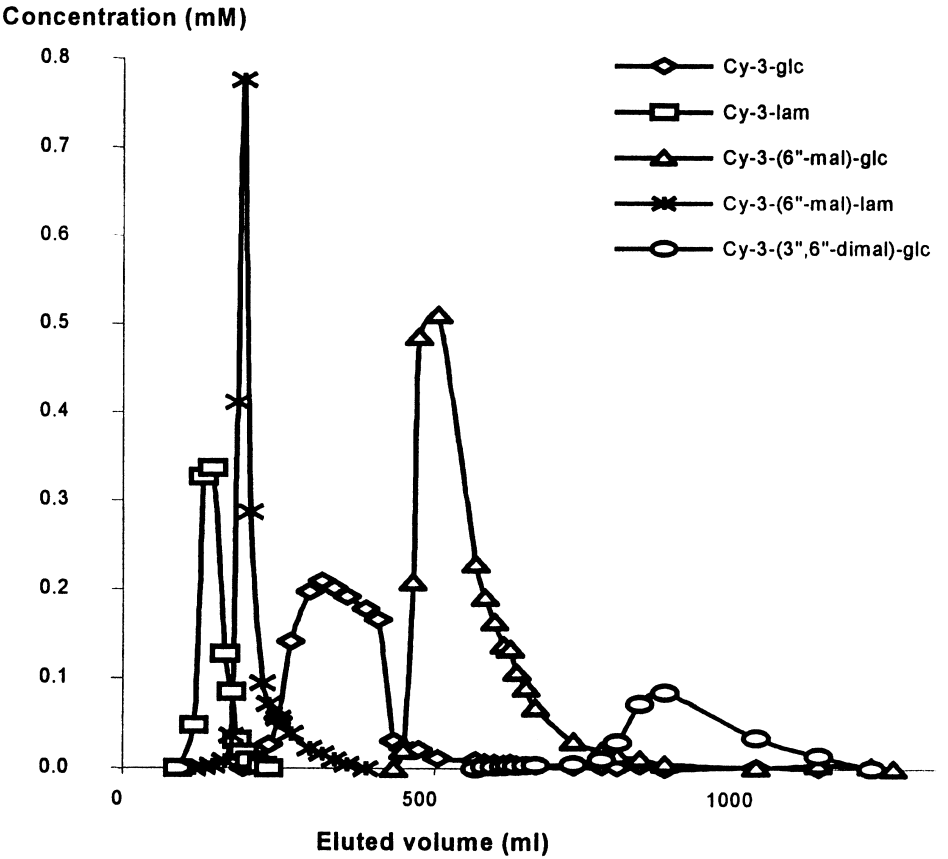


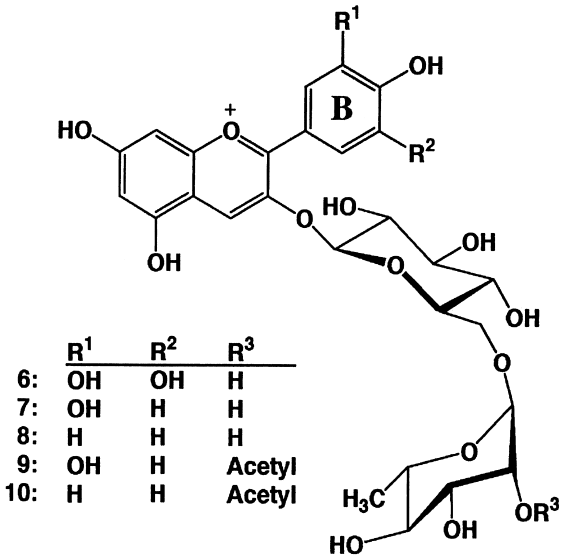
Figure 2. HSCCC separation of the five major anthocyanins in a partly purified sample (750 mg) from red onion. Cy = cyanidin; glc = glucoside; lam = 3-glucosylglucoside; mal = malonyl. HSCCC machine of 850 mL volume, 1000 rpm, descending mode, aqueous mobile phase, 5 mL/min, liquid system t-butyl methyl ether /n-butanol/ acetonitrile/ water (2/2/1/5 v/v/v/v) acidified with 0.01% TFA. Injection volume: 40 mL. UV 220 nm detection. Elution volume represents the volume of mobile phase leaving the column.

Table 1. Retention Times (t_R), Visible Maxima and the Ratio of the Absorbency at 440nm and Visible Maximum for the Five Pigments in the Tulip "Los Angelos" Recorded Online During HPLC-Analysis

Compound	t_R (min)	λ_{max} (nm)	A_{440}/A_{max} (%)
6	12.88	529	26
7	14.22	522	29
8	15.70	506	43
9	17.24	524	28
10	18.66	507	40

Red Onion

The five major anthocyanins (**1**–**5**) in red onion are based on the same aglycone, cyanidin.[11] They differ by number of malonyl and glucosyl moieties (Fig. 1). As shown in Fig. 2, all these pigments were separated by HSCCC applied on an 750 mg Amberlite XAD-7 isolate. Cyanidin 3-laminariobioside (**2**) was



	R^1	R^2	R^3
6:	OH	OH	H
7:	OH	H	H
8:	H	H	H
9:	OH	H	Acetyl
10:	H	H	Acetyl

Figure 3. Structures of anthocyanins from the tulip cultivar Los Angelos. 6–8: The 3-rutinosides of delphindin, cyanidin and pelargonidin. 9–10: The 3-(2'''-acetyl-rutinosides) of cyanidin and pelargonidin.

eluted before cyanidin 3-glucoside (**1**), which contains one glucosyl unit less than the former. However, both of these two non-acylated pigments are eluted prior to their malonylated analogues (**3**–**5**).

Tulip "Los Angelos"

Based on UV-vis spectroscopic data and co-chromatography (Table 1) with authentic pigments,[3,10] the identities of the major pigments in the tulip culivar Los Angelos were determined to be the 3-*O*-(6"-rhamnosylglucosides) of delphinidin (**6**), cyanidin (**7**), pelargonidin (**8**), and the 3-*O*-[6"-(2"'-acetylrhamnosyl)glucosides] of cyanidin (**9**) and pelargonidin (**10**) (Fig. 3). The two latter pigments have previously been identified only in flowers of two other tulip cultivars.[10]

The HSCCC separation of the anthocyanins in 430 mg of an Amberlite XAD-7 isolate of "Los Angelos" is shown in Figure 4. By using the most polar

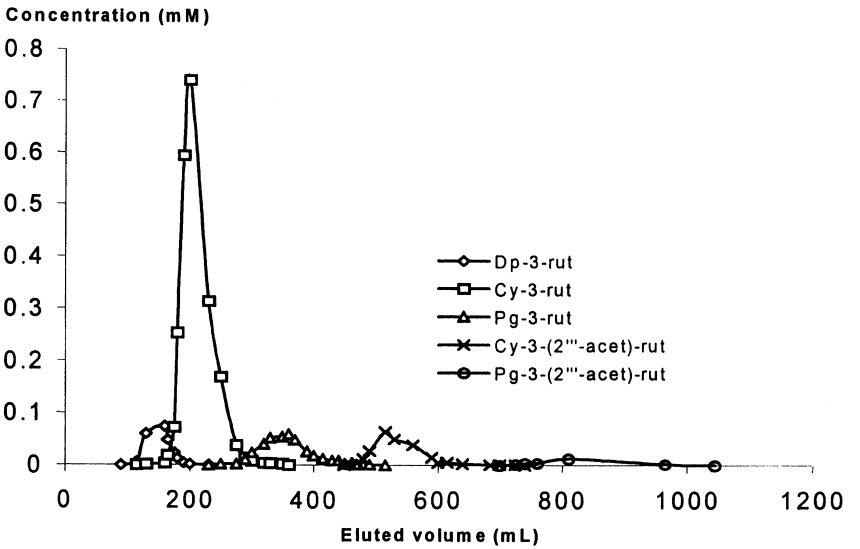


Figure 4. HSCCC separation of anthocyanins in a partly purified sample (430 mg) from the tulip cultivar Los Angelos. Dp = delphinidin; Cy = cyanidin; Pg = pelargonidin; rut = 6-rhamnosylglucoside; acet = acetyl. HSCCC machine of 850 mL volume, 1000 rpm, descending mode, aqueous mobile phase, 5 mL/min, liquid system t-butyl methyl ether /n-butanol/ acetonitrile/ water (2/2/1/5 v/v/v/v) acidified with 0.01% TFA. Injection volume: 40 mL. UV 220 nm detection. Elution volume represents the volume of mobile phase leaving the column.

phase of the solvent as mobile phase, the anthocyanidin 3-rutinosides (**6**–**8**) were eluted before their acetylated analogues (**9**-**10**). Within each of these two groups, acetylated and non-acetylated, individual anthocyanins were separated according to their number of hydroxy-groups on the aglycone B-rings. The highest number (three in delphinidin) entailed the lowest elution volume (Fig. 4). Thus, all the major anthocyanins were separated in one HSCCC step.

CONCLUSION

A centrifugal field is used for the immobilisation of the liquid stationary phase during HSCCC analysis, while impregnation of this phase on solid support is applied in most other chromatographic techniques used for separation of anthocyanins. Other beneficial parameters with HSCCC are, in this context, fast chromatographic speed and relatively low solvent consumption.

With respect to fragile anthocyanins acylated with aliphatic acids, such as those of red onions and tulip "Los Angelos," HSCCC, thus, suppresses the risks of pigment degradation and irreversible adsorption. Nearly baseline separation of all the acylated anthocyanins in anthocyanin-enriched isolates of both red onions and tulip "Los Angelos" was achieved in semi-preparative scale (around 500 mg). In our opinion, this scale may be increased considerably without too many obstacles.

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