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Journal of Chromatography A, 763 (1997) 345–352

JOURNAL OF CHROMATOGRAPHY A

Preparative separation of anthocyanins by gradient elution centrifugal partition chromatography

Jean-Hugues Renault[a,*], Philippe Thépenier[a], Monique Zèches-Hanrot[a], Louisette Le Men-Olivier[a], André Durand[b], Alain Foucault[1,b], Rodolphe Margraff[b]

[a]*Laboratoire de Pharmacognosie, URA CNRS 492, Faculté de Pharmacie, Université de Reims, 51 rue Cognac-Jay, 51096 Reims, France*

[b]*Rhône–Poulenc Rorer SA, Natural Products Department, 13 quai Jules Guesde, 94403 Vitry-sur-Seine, France*

Abstract

Centrifugal partition chromatography (CPC) allowed purification of anthocyanins from Champagne vintage by-products (*Vitis vinifera*) and from blackcurrant (*Ribes nigrum L.*). In the first case a 5-l pilot-CPC was used on a preparative multi-gram scale. The biphasic solvent system used was ethyl acetate–1-butanol–water, acidified by TFA (pH 1–3) for both gradient and isocratic normal-phase elutions. Separation selectivities which differ for cyanidin and delphinidin glycosides in CPC and cellulose-TLC using the solvent system 1-butanol–acetic acid–water (4:1:5) are discussed.

Keywords: Centrifugal partition chromatography; Preparative chromatography; Anthocyanins; Flavonoids; Glycosides

1. Introduction

Anthocyanins are water-soluble pigments responsible for the orange, pink, red, purple and blue colours of many flowers, fruits and leafs from many higher plants [1–3]. These secondary metabolites, belonging to the class of flavonoids, are glycosylated derivatives of polyhydroxylated and/or polymethoxylated 2-phenylbenzopyrilium or flavylium salts. Monoglucosides are most often encountered. Anthocyanins differ in respect to the number and location of hydroxy or methoxy groups, the nature and the number of sugars and their position on the aglycon. Moreover, sugars can be esterified by aliphatic or aromatic acids. The structures of isolated anthocyanins are shown on Fig. 1.

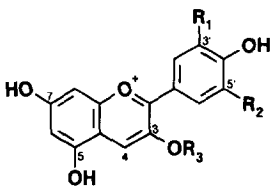
In aqueous media, the unstable anthocyanins give several pH-dependent resonance forms, diversely coloured according to the particular polyphenolic structure of their aglycon part. The most stable form is the flavylium cation which is prevailing at pH values below 2. In plants, anthocyanins are located in the vacuoles where they are stabilised by low pH (2–6.5) and a stacked supramolecular structure involving inter- or intra-copigmentation, self-association or chelation with metal ions [3]. Thus extraction and separation amenable to disrupt these stabilising forms should be carried out under mild and acidic conditions.

Their non-toxicity has been well established through millennial consumption by man of fruits like blue grapes, blackcurrants or bilberries and has been confirmed by toxicological studies on animals. Cur-

*Corresponding author.

[1]Present address: Unité de Recherche Marine 2, IFREMER, rue de l'Ile d'Yeu, BP 1049, 44037 Nantes Cedex 01, France.

PII S0021-9673(96)00880-1



Anthocyanins	R_1	R_2	R_3
Malvidin 3-glucoside	OCH_3	OCH_3	β-D-glucose
Peonidin 3-glucoside	OCH_3	H	β-D-glucose
Petunidin 3-glucoside	OCH_3	OH	β-D-glucose
Cyanidin 3-glucoside	OH	H	β-D-glucose
Cyanidin 3-rutinoside	OH	H	rutinose
Delphinidin 3-glucoside	OH	OH	β-D-glucose
Delphinidin 3-rutinoside	OH	OH	rutinose

Fig. 1. Structures of isolated anthocyanins from blue grapes and blackcurrant.

rently, they are used as complex mixtures as atoxic colouring agents and for their P-vitaminic properties in the Food and Pharmaceutical Industries [4]. Furthermore, they show promising activities as antioxidants and free radical scavengers. These activities are interesting as they may prevent degenerative diseases due to oxidative stress (heart disease, cancer, Alzheimer's, ...) [5–9].

A more extensive usage of these polyphenolic substances requires additional chemical and pharmacological studies on pure compounds. This is a difficult task considering the problems encountered in preparative classical liquid chromatography. In order to avoid irreversible adsorption or degradation of these polar and fragile molecules on solid supports [10], we have used, for the first time, centrifugal partition chromatography (CPC) gradient elution in a preliminary report [11].

CPC is a support free liquid–liquid partition chromatographic method generally referred to as counter-current chromatography (CCC) [12,13], though there is no counter-current between the two phases: one is mobile and the other stationary as in HPLC. Modern counter-current chromatographs are available of two types, called "CCC" for the planetary motioned coiled tube type developed by Ito [12,13] and "CPC" for the type containing discrete partition cells inside a rotor [14]. The latter type was used here because viscous solvent systems containing butanol–water mixtures are better retained and because pilot CCC are not available.

In this article, we initially describe the transposition of this separation to a multi-gram scale on a 5-l pilot CPC chromatograph and show the possibility of isolating gram quantities of pure anthocyanins. Subsequently, we generalise the gradient elution with the same biphasic solvent system ethyl acetate–1-butanol–water, on a laboratory-scale, to the separation of anthocyanins from blackcurrant whose non-methoxylated aglycons are substituted on position 3 by mono- or disaccharides.

2. Experimental

2.1. Anthocyanins sources

2.1.1. From blackcurrant

A 2.5 g sample of a semi-purified blackcurrant extract was a kind gift from Professor Metche, ENSAIA, Nancy, France. Enhancement of its anthocyanic content was done by adsorption on Amberlite XAD-1600 followed by elution with methanol–0.1 *M* HCl (80:20) and gave 1 g of a prepurified extract, which was used without further purification for CPC experiments.

2.1.2. From Champagne vintage by-products

The by-products of Champagne vintage (7.5 kg), which contained wetted blue grape skins, seeds and stalks, were collected in October 1995, and macerated during 5 days in 10 l methanol–acetic acid (99:1). After filtration the aqueous methanolic solution was concentrated to 3 l under reduced pressure at 30°C to an aqueous layer which was successively extracted with chloroform (4×1 l), and with 1-butanol (6×1.5 l). The chloroform extract was discarded and the butanolic solution, containing the anthocyanins, was concentrated in vacuum at 40°C to a residual aqueous solution which was freeze-dried to give 124.5 g of crude extract. Its titre was improved by the above described method on Amberlite XAD-1600 used for blackcurrant, giving a final extract of 24.5 g which anthocyanic content was estimated by a spectroscopic measurement at 520 nm in 0.1 *M* HCl [16] using the molar absorption coefficient of 28000 from malvidin 3-glucoside [17].

2.2. CPC apparatuses

Both apparatuses used were commercial models produced by Sanki Engineering Ltd, Kyoto, Japan and distributed by EverSeiko, Tokyo, Japan.

The laboratory Model LLB-M and the pilot Model LLI-7 are both fitted with a stacked disks type rotor. The characteristics of these rotors are respectively: total volume: 230 ml and 5470 ml, total number of partition cells: 2136 and 1040, length of the cells: 15 mm and 46 mm, average centrifugal radius at mid cells: 82.5 mm and 111 mm.

The overall dimensions and masses of the centrifuges are 31×47×50 cm (47 kg) and 60×81×106 cm (300 kg).

Two Shimadzu LC8A pumps (1–150 ml/min) (Roucaire, Courtaboeuf, France) were used for solvent delivery and gradient generation.

Samples were injected from a loop through a 3725-038 Rheodyne valve or with a 500 D Isco syringe pump (Roucaire, Courtaboeuf, France).

2.3. Biphasic solvent systems

2.3.1. Isocratic system 1-butanol–acetic acid–water

The classical 1-butanol–acetic acid–water (4:1:5) was used in the normal-phase mode (upper phase mobile). The same upper phase was used for developing the TLC cellulose plates.

2.3.2. Gradient system ethyl acetate–1-butanol–water

The peculiarity of this ternary system, described elsewhere [15], are the converging tie-lines to the point "S" (Fig. 2) corresponding to the composition of the lower aqueous phase which is roughly identical for all biphasic mixtures and immiscible with the corresponding organic phases. Gradients were run in the normal-phase mode by varying linearly the composition of the organic mobile phase from point "I" corresponding to the initial mobile phase to point "F" the final mobile phase on Fig. 2.

The initial mobile phase ethyl acetate–1-butanol–water (77:15:8), the final mobile phase ethyl acetate–1-butanol–water (40:46:14) and the stationary aqueous phase ethyl acetate–1-butanol–water (5:5:90) were prepared separately. Preparation of the mobile phases by mixing the 3 solvents in these proportions gives, in each case, the separation of about 1% (v/v) aqueous phase. This ensures their equilibration toward the aqueous phase. Each phase was acidified with 0.2% trifluoroacetic acid.

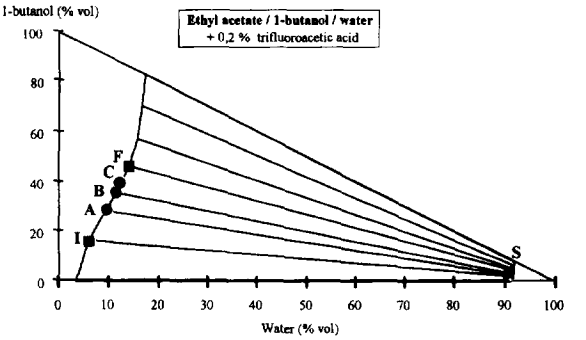


Fig. 2. Ternary diagram for the biphasic system ethyl acetate–1-butanol–water. S: stationary aqueous phase (5:5:90). I,F: initial and final mobile organic phases used in gradient elution, (77:15:8) and (40:46:14), respectively. A, B and C: mobile phases used in second stages isocratic elutions, (62:28:10), (53:35:12) and (48:40:12), respectively.

Three other mobile phases (named A, B and C on Fig. 2) have been selected and used isocratically for second stage runs. Their respective compositions were (62:28:10), (53:35:12) and (48:40:12).

2.4. Injection and CPC operating procedure

2.4.1. Separation of anthocyanins from blackcurrant

The rotor was first entirely filled with the stationary phase while rotating at 100 rpm. After injection of the 1-g extract dissolved in 10 ml stationary phase, speed was increased to 1400 rpm, and the initial mobile phase I was pumped into the column in ascending mode at a flow-rate of 3 ml/min for 30 min. The mobile phase displaced 25% stationary phase and therefore the retention of the stationary phase is 75%. Then a linear gradient to 50% final mobile phase F was performed in 50 min. Next, elution remained 30 min isocratic before a second linear gradient to 70% F in 60 min was established. After 30 min elution at 70% F a third linear gradient reached 100% F in 120 min. Retention of the stationary phase remained constant at 75% throughout the gradient duration avoiding bleeding. Frac-

tions of 9 ml were collected every 3 min. The back-pressure was 37 bars. Detection was performed at 540 nm with a UV/Visible detector ISCO type V^4.

2.4.2. Separation of anthocyanins from blue grapes on the 5-l pilot CPC

Injection and elution procedures were as above. The 24.5-g extract was dissolved in 500 ml stationary phase, the rotation speed was 1140 rpm and the flow-rate 60 ml/min resulting in 30 bar back-pressure. The gradient profile was: 30 min 100% I, from 100% I to 50% I / 50% F in 90 min, 30 min 50% I / 50% F, to 100% F in 60 min and 100% F for 90 min.

Fractions of 600 ml were collected every 10 min. All fractions having the characteristic bright colours of the anthocyanins were recovered between 40 and 290 min elution. The liquids remaining inside the rotor contained no anthocyanins.

2.4.3. Characterisation of the anthocyanins

All fractions were checked by TLC on cellulose F plates (E. Merck, Darmstadt, Germany) developed with the upper phase from 1-butanol–acetic acid–water (4:1:5). Malvidin 3-glucoside, peonidin 3-glucoside, cyanidin 3-glucoside and cyanidin 3-rutinoside, used as standards, were purchased from Extrasynthèse (Gemey, France)

The fractions of interest and all isolated compounds were analysed by ^{1}H-NMR spectroscopy in $C^2H_3O^2H$ acidified with 0.3% TFA. Spectra were recorded at 300 MHz on an AC 300 Bruker spectrometer (Wissembourg, France). In the case of the preparative separation of the anthocyanins from blue grapes, integration of the H-4 protons was used for identification and estimation of the anthocyanins from each fraction. The reconstructed chromatogram is shown on Fig. 3.

3. Results and discussion

3.1. Scaling-up

Published isolation techniques of anthocyanins, with the exception of gel permeation, are mainly typically liquid–liquid partition techniques utilising a solid support for the immobilisation of the liquid stationary phase such as cellulose, ODS silica gel, polystyrene–divinylbenzene (PS–DVB) resins or none for droplet countercurrent chromatography (DCCC) [18,19].

CPC is very similar in many respects to DCCC, but has no solvent systems limitation and has become as rapid and performant as HPLC and similarly can be scaled-up to pilot and production levels.

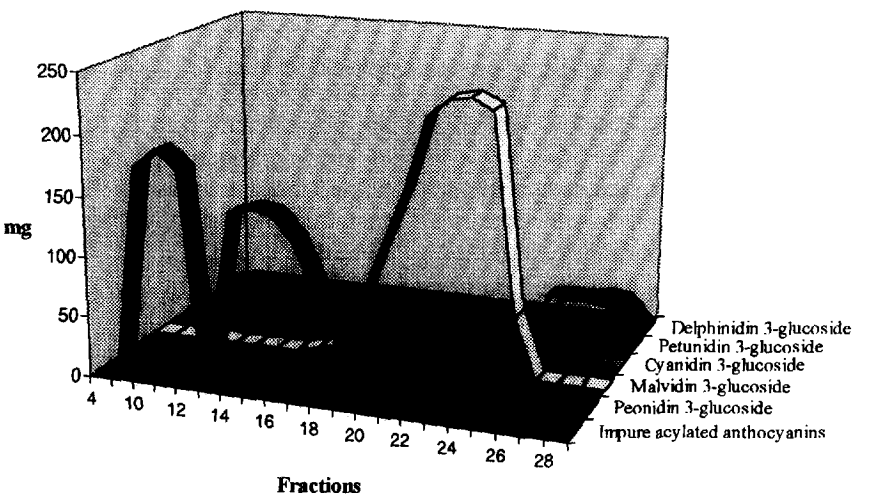


Fig. 3. Elution profile of anthocyanins from blue grapes in a 5-l CPC gradient run of a 24.5 g extract containing an estimated total amount of 2.8 g anthocyanins. The flow-rate was 60 ml/min at 1140 rpm and fractions were collected every 10 min. The content of each fraction was quantified by integration of the H-4 protons from ^{1}H-NMR spectra recorded in $C^2H_3O^2H$ at 300 MHz.

The use of a centrifugal field for the immobilisation of the liquid stationary phase instead of its impregnation on a solid support suppresses not only most of the risks of sample denaturation, but allows the use of any solvent at any pH and overall modifies drastically the ratio of the volume of the stationary phase to the volume of the mobile phase inside the rotor. As a consequence of the latter point CPC needs on average 10 times less solvents than HPLC [20] and is, in our opinion, tailor made for production-scale chromatography.

Scaling-up, generally a complex procedure in HPLC, is made easy for CPC. The construction of the 5-l model was designed to generate the same plate number (about 500) and back-pressure as the laboratory model. Hence, extrapolation can be done by using the factor 20, corresponding to the rotor volume ratio, for calculating the sample size and flow-rate. The speed may be slightly reduced to compensate for the larger rotor radius (see experimental) and to get the same centrifugal field of about 150 *g*.

Starting from 7.5 kg of wetted blue grapes marc (skins, seeds and stalks), we obtained 24.5 g of an extract containing an estimated 2.80 g anthocyanins (11.42%) which needed 500 ml stationary phase for solubilisation.

Although this sample size is more than 20-fold the previous one used at a laboratory-scale: 0.6 g in 5 ml [11] it was judged suitable for a 5-l CPC run because this chromatograph usually accommodates feeds of 100 to 150 g in up to 1.2 l.

Following injection, the retention of the stationary phase was 70%, similar to laboratory-scale experiment, but dropped to 54% after completion of the run. The high viscosity of the feed (monoglycosylated anthocyanins are sparingly soluble in water) is presumed to be responsible for the bleeding which could probably be avoided by injecting in a larger volume.

Nevertheless, the elution profile remained apparently unaffected and found to be very similar to the one from the laboratory-scale experiment.

This profile (Fig. 3) shows that the two major anthocyanins namely peonidin 3-glucoside and malvidin 3-glucoside could be obtained pure (resp. 402 mg in fractions 13–15 and 682 mg in fractions 22–24) in one step. Additional 235 mg of peonidin 3-glucoside and 523 mg of malvidin 3-glucoside were recovered from fractions 16–21 (in which they co-eluted) by a second CPC run on the laboratory model using the mobile phase of composition "A" (Fig. 2) for isocratic elution. More malvidin 3-glucoside (51 mg) was separated from cyanidin 3-glucoside (5 mg), a mixture of cyanidin 3-glucoside petunidin 3-glucoside and delphinidin 3-glucoside (64 mg) by another laboratory-scale CPC run of fraction 25 using isocratic elution with system "B" (Fig. 2). Fractions 26–28 (181 mg) corresponded to a mixture of petunidin 3-glucoside and delphinidin 3-glucoside co-eluted with this chromatographic system. Fractions 9–12 contained less polar acylated anthocyanins. No attempts were made to fractionate by CPC these less polar components which can be conveniently purified by C_{18} RP-HPLC. The combined lots amounted 1.256 g for malvidin and 0.637 g for peonidin (see Table 1)

A total amount of 2.14 g anthocyanins was obtained in 0.028% yield from 7.5 kg marc. With the exception of the low cyanidin content, the relative abundance of the 5 constituents is similar to that reported in the literature [21]. The discrepancy can result from an heterogeneity of the plant material which was a blend of several vineyards.

3.2. Blackcurrant

Similarly gradient elution from I to F, but at a laboratory-scale gave 4 anthocyanins (Fig. 4). These four compounds result from a combination between

Table 1
Anthocyanins from blue grapes

Anthocyanins	Amount isolated in grams (relative %)	Literature[a] relative %
Peonidin 3-glucoside	0.637 (29.8)	21.1
Malvidin 3-glucoside	1.256 (58.6)	64.3
Cyanidin 3-glucoside	0.011[b] (0.5)	3.22
Petunidin 3-glucoside	0.147[b] (6.8)	5.99
Delphinidin 3-glucoside	0.092[b] (4.3)	5.35
Total	2.143 (100)	100

Amounts of identified compounds were from a 24.5 g extract containing an estimated amount of 2.8 g total anthocyanins. Acylated anthocyanins were not quantified.

[a] From [21].

[b] Estimated by respective integrations of the H-4 on ^{1}H-NMR spectra.

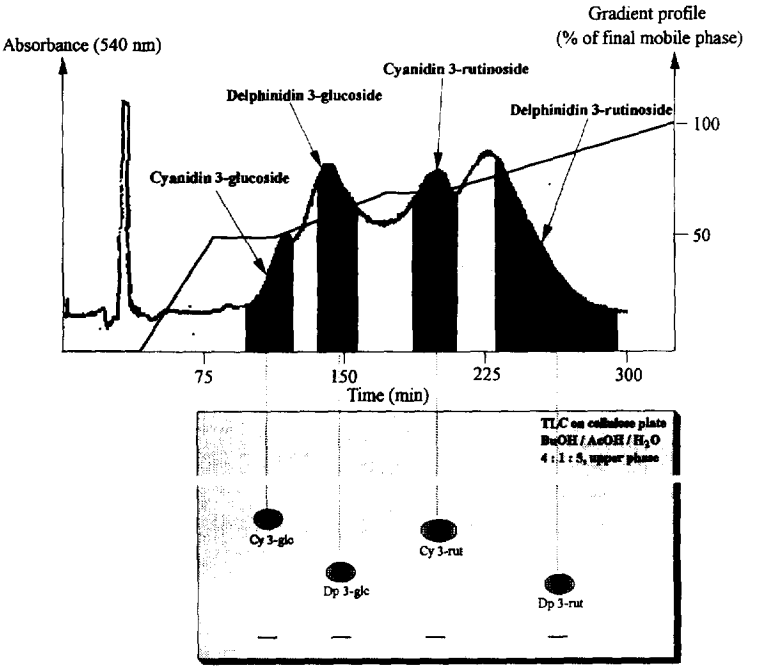


Fig. 4. CPC separation of four anthocyanins from blackcurrant in a laboratory-scale gradient run. The flow-rate was 3 ml/min at 1400 rpm. The gradient profile and a TLC of the four compounds are shown.

2 aglycons: cyanidin and delphinidin and 2 sugars: β-D-glucose and a disaccharide: rutinose (α-L-rhamnopyranosyl-(1>6)-β-D-glucopyranoside). They eluted in 2 groups: the glucosides preceding the rutinosides, as expected for a normal-phase elution mode. The same was true inside each group where the more polar delphinidins, sharing one phenolic hydroxyl more than the cyanidins eluted late. The selectivity of the separation was improved through gradient optimisation. Pure fractions were isolated for all the 4 components: 9 mg of cyanidin 3-glucoside from fractions 32–45, 9 mg of delphinidin 3-glucoside from fractions 46–53, 9 mg of cyanidin 3-rutinoside from fractions 65–70 and 15 mg of delphinidin 3- rutinoside from fractions 77–95. Other fractions, in which co-elution occurred, were submitted to subsequent isocratic CPC runs with mobile phases A, B and C. Fractions 42–45 (3 mg) were submitted to a second CPC run using the mobile phase "A" (Fig. 2) for isocratic elution to give 2 mg of cyanidin 3-glucoside and 1 mg of delphinidin 3-glucoside. In the same manner, but with the mobile phase "B" (Fig. 2), fractions 54–64 (10 mg) led 4 mg of delphinidin 3-glucoside and 6 mg of cyanidin 3-rutinoside. Finally, fractions 71–76 (8 mg) gave 4 mg of cyanidin 3-rutinoside and 4 mg of delphinidin 3-rutinoside using the mobile phase "C" (Fig. 2). The results are summarised on Table 2.

Interestingly enough the elution pattern on TLC using cellulose plates and the upper organic phase from 1-butanol–acetic acid–water (4:1:5) was modi-

Table 2
Anthocyanins from blackcurrant

Anthocyanins	Amount isolated in mg (relative %)	Literature[a] relative %
Cyanidin 3-glucoside	11 (17.5)	17
Delphinidin 3-glucoside	14 (22.2)	13
Cyanidin 3-rutinoside	19 (30.1)	35
Delphinidin 3-rutinoside	19 (30.1)	30
Total:	63 (100)	100

[a] According to [22].

Table 3
TLC on cellulose of anthocyanins from blackcurrant

Anthocyanins	$R_F \times 100$
Cyanidin 3-glucoside	38
Cyanidin 3-rutinoside	34
Delphinidin 3-glucoside	26
Delphinidin 3-rutinoside	23

Eluent: 1-butanol–acetic acid–water (4:1:5) (upper phase).

fied: again 2 groups were separated but this time according to the nature of the aglycons: with cyanidins having higher R_F. Inside each group normal-phase elution was respected too: the less polar glucosides eluted at higher R_F (Table 3).

Thus, the nature of the aglycon played a major role for selectivity on TLC, while the polarity of the sugars dominated in CPC. The observed elution order in CPC remained unaffected in a CPC run using the biphasic system 1-butanol–acetic acid–water (4:1:5) in the normal-phase mode and isocratic elution. This system does not allow gradient elution because of the parallel orientation of its tie-lines and proved to be less efficient with the same blackcurrant extract.

It is obvious that partition coefficients K are quite different for mono- and disaccharides, the latter partitioning more preferentially in the aqueous phase then the former. This was the case for CPC. The different behaviour on TLC can be explained by an interaction with cellulose probably by hydrogen bonding, favoured by the acid media and the difference in polarity of cycles B which are respectively pyrocatechol- and pyrogallol-type in cyanidin and delphinidin.

4. Conclusion

It has been shown that gram-quantities of pure anthocyanins can easily be obtained in excellent yields by gradient elution CPC.

The method is less time- and solvent-consuming than previously described procedures. Interestingly enough, it has been shown that cellulose is not such an inert material for partition chromatography, though the reported phenomenon may result from the exceptionally high acidity of the pyrogallol-type subunit of delphinidin.

Acknowledgments

We thank the Région Champagne–Ardenne for financial support.

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