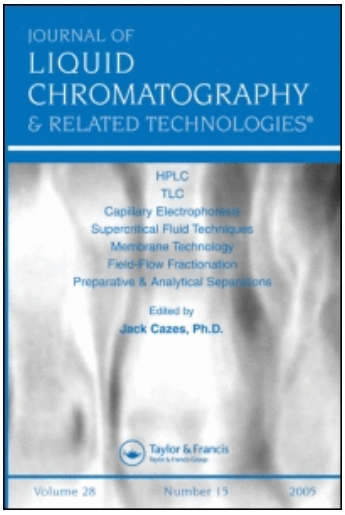

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of the Two Major Anthocyanins from Champagne Vintage By-Products by Gradient Elution Centrifugal Partition Chromatography

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To cite this Article Renault, J. H. , Thepenier, P. , Zeches-Hanrot, M. and Foucault, A. P.(1995) 'Separation of the Two Major Anthocyanins from Champagne Vintage By-Products by Gradient Elution Centrifugal Partition Chromatography', Journal of Liquid Chromatography & Related Technologies, 18: 8, 1663 — 1670

To link to this Article: DOI: 10.1080/10826079508009303

URL: http://dx.doi.org/10.1080/10826079508009303

JOURNAL OF LIQUID CHROMATOGRAPHY, 18(8), 1663-1670 (1995)

SEPARATION OF THE TWO MAJOR ANTHOCYANINS FROM CHAMPAGNE VINTAGE BY-PRODUCTS BY GRADIENT ELUTION CENTRIFUGAL PARTITION CHROMATOGRAPHY

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ABSTRACT

Centrifugal Partition Chromatography (CPC) was applied, for the first time, to the preparative scale separation of anthocyanins. The two major anthocyanins from Champagne vintage by-products, malvidin 3-glucoside and peonidin 3-glucoside, were isolated in one step, using gradient elution with the TFA-acidified ternary solvent system ethyl acetate / 1-butanol / water.

INTRODUCTION

Anthocyanins are natural water-soluble pigments which are responsible for the pink, orange, red, purple, blue... colors of flowers, fruits and vegetables [1-2]. They

are used in a complex mixture form as natural colorants and for their P-vitamin properties in food and pharmaceutical industries [3]. They have a neutralizing effect against toxic free radicals and, therefore, have a protective activity towards collagen [4].

Since the elaboration of Champagne white wines requires mostly red grapes, anthocyanins need to be expelled from wine making; within the context of agroresource valorization of Champagne-Ardenne region, it appeared interesting to further purify these anthocyanins present in vintage by- products.

Anthocyanins are polar and fragile heterosides which belong to the class of flavonoids. In aqueous media, these polyphenolic compounds lead to several resonance forms which are pH-dependent. The most stable form is the flavylium cation predominant under acid conditions (pH 1-3). Separation and isolation techniques must be carried out in mild conditions and in acidic media [2], and the main chromatographic methods to isolate these compounds [5] are paper chromatography, ion exchange chromatography on resins or Sephadex, reversed phase HPLC, and droplet counter current chromatography (DCCC) [6]. Irreversible adsorption and degradation of these polar molecules are often encountered on solid sorbents, while DCCC, which does not have these disadvantages, turns out to be time consuming and restricted in the choice of solvents .

It seemed interesting to apply, for the first time, the centrifugal partition chromatography (CPC) for quantitative purification of anthocyanosides.

We report, in this paper, our results on isolation of two major anthocyanins, malvidin 3-glucoside and peonidin 3-glucoside, from by-products of Champagne vintage (Pinot noir wine plants).

MATERIAL AND METHODS

Extraction

The by-products from Champagne vintage (1.015 kg), which contain wetted red grape skins, seeds and stalks, were collected in October, 1993, and macerated during 5

days in MeOH-AcOH (99:1 , 1.45 L). After filtration the aqueous methanolic solution was concentrated to 0.25 L under reduced pressure at 28°C. This solution was then extracted with 1-BuOH (4 x 0.2 L) saturated with water. The organic solvent was evaporated *in vacuum* at 65°C and the residue was lyophilized to give the anthocyanic extract (14.29 g; yield = 1.4 %).

Fractionation by CPC

A Series 1000 HPCPC (Sanki Eng. Limited, Nagaokakyo, Kyoto, Japan) was used [7]. It is a bench top CPC (30 x 45 x 45 cm, ≈ 60 kg); the column is a stacked circular partition disk rotor which contains 2136 channels with a total internal volume of around 240 mL. The column is connected to the injector and the detector through two high pressure rotary seals. A 4-port valve included in the series 1000 allows the HPCPC to be operated in either the descending or ascending mode. The HPCPC was connected to a solvent delivery pump Techlab economy 2/ED (Techlab, D-38173 Erkerode, Germany), supplied with solvents through a gradient generator ISCO model 2360 with preparative options (ISCO, Inc., Lincoln. NB, USA).

Detection was performed with a UV/visible detector ISCO type V^4, set at 540 nm. Fractions were collected with a collector model Superfrac manufactured by Pharmacia (Pharmacia, Uppsala, Sweden). Sample injections were carried out by a Rheodyne injection valve type 7125 (Altech Associates, Inc., Deerfield, Illinois USA) through a 5 mL sample loop.

The solvent system ethyl acetate / 1-butanol / water (EtOAc/1-BuOH/H_2O) has been used for this separation. Figure 1 shows the ternary diagram corresponding to this solvent system.

The initial mobile phase was EtOAc/1-BuOH/H_2O 77/15/8 (V/V/V) (Point I, Fig 1), the final mobile phase was EtOAc/1-BuOH/H_2O 40/46/14 (V/V/V) (Point F, Fig 1) and the stationary phase was water saturated with EtOAc and 1-BuOH; all phases were acidified with trifluoroacetic acid (0.8%) . The gradient was linear with a duration of 3 hours, the flow rate being 3 ml/min in the ascending mode and the rotational speed varied between 1300 rpm and 1500 rpm. With these conditions, the volume of the

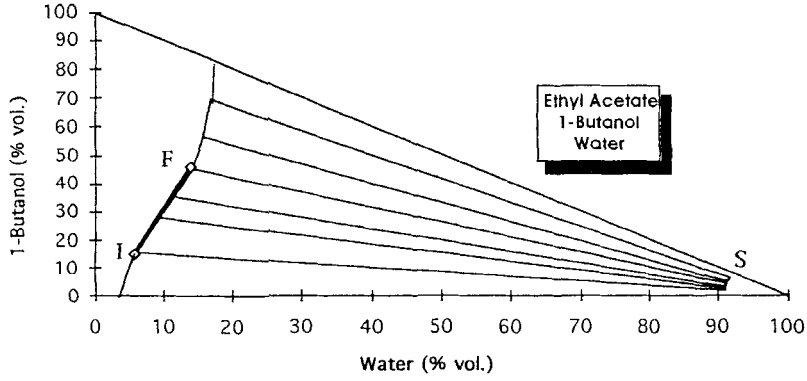

Figure 1 Ternay system ethyl acetate / 1-butanol / water
Composition of the liquid phases used to generate a gradient of butanol in ethyl acetate on a water-rich liquid stationary phase : I : initial mobile phase
F : final mobile phase
S : stationary phase

mobile phase in the CPC "column" was around 78 mL, corresponding to a retention of the stationary phase of 67%, and the pressure drop was 35 to 47 bars.

Identification of anthocyanins

Composition of the fractions was determined by TLC on cellulose F plates (Merck, Darmstadt, Germany), using a mixture of 1-BuOH/AcOH/H_2O (61:10.5: 28.5) as the mobile phase. The pure isolated products were compared by TLC with authentic samples of malvidin-3-glucoside and peonidin-3-glucoside (Extrasynthese, Genay, France). They were also analyzed by ^1H NMR spectroscopy in CD_3OD acidified with 0.3% of TFA. Spectra were recorded at 300 MHz on a Bruker AC 300 spectrometer (Bruker, Wissemburg, France) and compared with spectra obtained from commercial samples and with data found in the literature [8].

Reagents

All chemicals were analytical grade (SDS, 13124 Peypin, France)

RESULTS AND DISCUSSION

Considering the diversity of the compounds present in our extract, gradient elution turned out to be necessary to isolate anthocyanins. This method requires a biphasic ternary system, the ternary diagram of which possesses converging tie-lines. Because of the glycosidic structures of anthocyanins, we selected the biphasic solvent system EtOAc/1-BuOH/H_2O, which we used already in our laboratory for isolation of ginsenosides from *Panax quinquefolium* L. [9]. With a similar ternary system (EtOAc/2-BuOH/H_2O), Vanhaelen *et al.* obtained good fractionation of flavonoids from a commercial extract of *Ginkgo biloba* leaves [10]. Our system looks very favorable to run gradients in the normal phase mode, using the polar water rich phase as the stationary phase, and varying the polarity of the mobile phase by changing the ratio of 1-BuOH to EtOAc, while the composition of the stationary phase remains relatively constant, *i.e.* water saturated in EtOAc and 1-BuOH. [11].

Partition tests of our anthocyanic extracts in systems EtOAc/1-BuOH/H_2O of various compositions directed us to start our gradient with more polar conditions than those previously used to purify the ginsenosides from *Panax quinquefolium* L. [9]; we also acidified all phases, in order to preserve the anthocyanin stability.

Figure 2 shows the CPC chromatogram, recorded at 540 nm, obtained after injection of 600 mg of the anthocyanin extract (in 5 mL of stationary phase). 100 x 9 mL fractions were collected and further analyzed by TLC. Identical fractions 54 to 68 were pooled, evaporated to dryness, and then analyzed by ^1H NMR : they contained 15 mg (2.5 % of the extract) of pure peonidin 3-glucoside. Similarly, we found that fractions 71 to 89 yielded 20 mg (3.33% of the extract) of pure malvidin 3-glucoside. The intermediate fractions (69-70, 2 mg, 0.33%) contained a mixture of these two compounds. Thus, we obtained 35 mg of pure anthocyanins, which corresponds to 0.81 % of the starting material (grape skins, seeds and stalks). The remains (563 mg) was a mixture of minor anthocyanins, and other compounds such as sugars, flavonoids and tannins.

The observed elution sequence is in conformity with the normal phase elution mode which was used. Peonidin 3-glucoside, which does not have a methoxyl group in 3' position (Fig 2) is less polar than malvidin 3-glucoside, and is eluted first. The same order of elution is also observed on TLC plates.

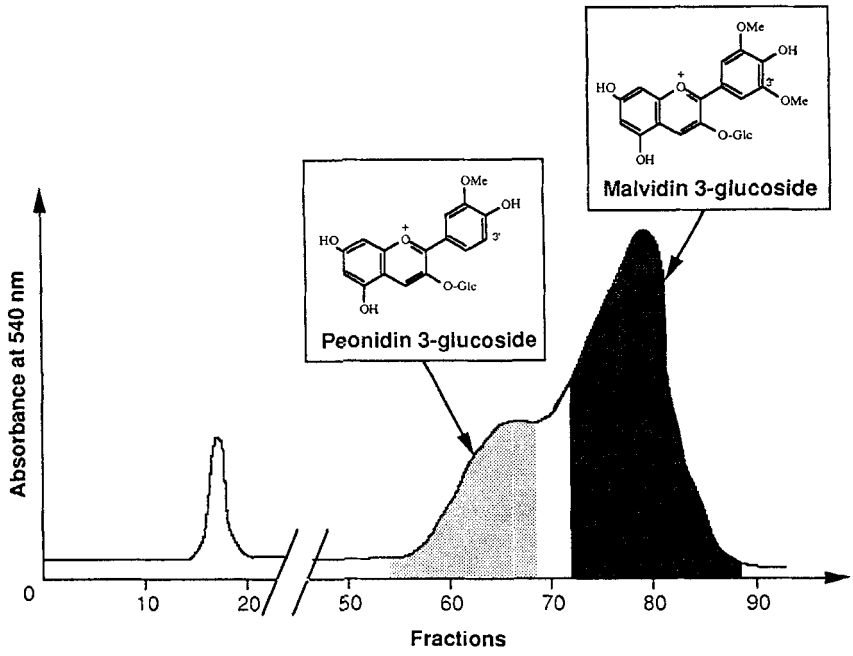

Figure 2 CPC chromatogram of anthocyanins from Champagne vintage by-products. gradient elution with the ethyl acetate / 1-butanol / water system; stationary phase : water saturated with ethyl acetate and butanol (S on Fig.1);all phases were acidified with 0.8% of trifluoroacetic acid.
Gradient duration : 3 hours.
Ascending mode, flow rate : 3 mL/min.
Rotational speed : 1300-1500 rpm; back pressure : 35 to 47 bars.
Volume of the mobile phase in the HPCPC : 78 mL.
Detection UV/Vis at 540 nm.
Sample : 650 mg of a lyophilized extract of anthocyanins in 5 mL of stationary phase.

CONCLUSION

Centrifugal Partition Chromatography is an effective tool for the separation and purification of biologically active polar and fragile molecules, extracted from plants. These kind of compounds can hardly be isolated by conventional column chromatography because of strong adsorption and decomposition. Moreover, the introduction of certain innovations like the use of mobile phase gradient offers more

possibilities. The two major anthocyanins from vintage by products have been isolated, in a pure state and with good yields. As anthocyanins are generally used in a complex mixture form in food and pharmaceutical industries, it seems interesting to get pure products in largest quantities. In this perspective we think of transposing our method on an apparatus with a larger capacity.

ACKNOWLEDGMENTS

We express our gratitude to Professor L. Le Men-Olivier for fruitful discussions on the subject. We thank Dr. J. Cazes for linguistic advice.

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Received: November 15, 1994
Accepted: December 1, 1994