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Journal of Chromatography A, 1127 (2006) 70–75

JOURNAL OF CHROMATOGRAPHY A

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Effect of tetraalkylammonium salts on retention of betacyanins and decarboxylated betacyanins in ion-pair reversed-phase high-performance liquid chromatography

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Received 12 March 2006; received in revised form 21 May 2006; accepted 24 May 2006
Available online 21 June 2006

Abstract

The ability of tetraalkylammonium salts to act as ion-pairing agents for betacyanins and mono- as well as bidecarboxylated betacyanins in HPLC on reversed phase is reported. The results indicate substantial and various influences of tetraalkylammonium salt addition to eluent on magnitude and direction of retention time changes of the analytes. The interactions of the tetraalkylammonium cations with the accessible positive and negative charged parts of the analysed molecules at their different level and position of decarboxylation determined the possibility of ion-pair formation. A remarkable positive influence was observed for betacyanins on the basis of strongly enhanced retention of the resulted ion-pairs. In contrast, the 2,17-bidecarboxy-betacyanin retention, in most of the applied conditions, strongly decreased under the impact of the reagents. The effect of the salt concentration on direction of 17-decarboxy-betacyanin and 2,17-bidecarboxy-betacyanin retention changes strongly varied with the eluent pH. The carboxylic group at the C-2 carbon cannot interact with tetraalkylammonium cations as strongly as does the C-17 carboxylic group, leading to different effects in the absence of carboxylic group at the C-2 or C-17 carbon in the molecules.

Keywords: Tetraalkylammonium; Ion-pair; Betanin; Phyllocactin; Hylocerenin; Decarboxy-betacyanins

1. Introduction

Betacyanins are water-soluble betalain pigments derived by glycosylation of betanidin, which can be considered as a condensation product of cyclodopa with betalamic acid [1,2]. From these red–violet pigments, betanin and its C-15 isoform derived from red beet root (*Beta vulgaris* L.) are extensively used as food colorants. Most of betacyanins are 5-*O*-glucosides (Fig. 1), such as betanin, the simplest 5-*O*-glucosylated betacyanin. Further esterification of the 5-*O*-glucoside with acids such as ferulic, *p*-coumaric or malonic acid is very common [1,2]. Recent studies reported three main pigments in fruits of newly domesticated species of *Hylocereus* cacti (another potential source of betacyanins), betanin, phyllocactin and hylocerenin as well as their C-15 isoforms [3,4].

Betacyanins are known to be very sensitive to several factors including elevated temperatures [5–7]. Recent studies on thermal decarboxylation of betacyanins from red beet (*Beta vulgaris* L.) [8,9] and purple pitaya (*Hylocereus polyrhizus*) [10,11] monitored by RP-HPLC-MS/MS reported betacyanins with different decarboxylation levels. The main decarboxylation products were 2-decarboxy-, 17-decarboxy- and 2,17-bidecarboxy-betacyanins. Some of these pigments were analysed by HPLC for the first time in thermally obtained complex mixtures resulting in frequently overlapped chromatographic peaks of not yet identified compounds.

HPLC is an excellent means in the analysis of betalains [1,2,12]. The most common support is C_{18}-derivatized silica providing adequate efficiency and retention of betacyanins as well as their sufficient resolution on conventional stationary phases. Because betacyanins exist in aqueous solution in different ionisation forms at changed pH values, the use of typical acidic eluents with or without buffers is a useful factor governing their separation.

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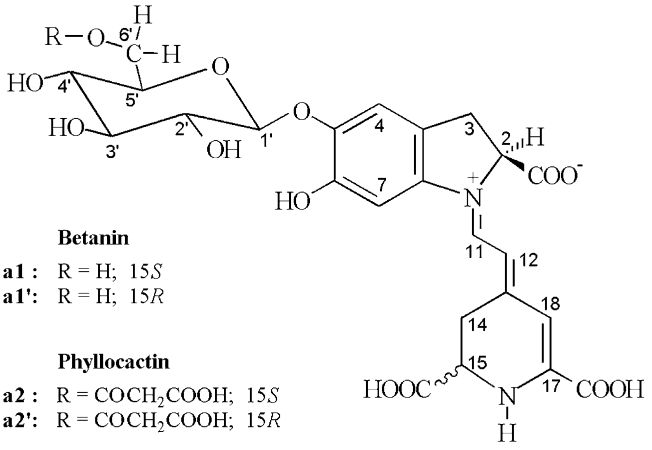
doi:10.1016/j.chroma.2006.05.072



Betanin

a1 : R = H; 15*S*
a1': R = H; 15*R*

Phyllocactin

a2 : $R = COCH_2COOH$; 15*S*
a2': $R = COCH_2COOH$; 15*R*

Hylocerenin

a3 : $R = COCH_2C(OH)(CH_3)CH_2COOH$; 15*S*
a3': $R = COCH_2C(OH)(CH_3)CH_2COOH$; 15*R*

Fig. 1. Chemical structures of studied betacyanins in this report. The betacyanin derivatives were obtained by their decarboxylation at carbon no. 2 and/or 17.

Recently, a strong effect of anionic counteranions of trifluoroacetic acid and other perfluorinated carboxylic acids on retention of betacyanins and decarboxylated betacyanins was shown [13]. While the perfluorinated carboxylic acids increased the retention of the compounds by lowering the pH of the mobile phase, the possibility of ion-pair chromatography for decarboxylated betacyanins was noticed.

In this study, the ability of tetrabutylammonium salt to influence the retention of betacyanins and decarboxylated betacyanins is reported. Recently, this agent was successfully applied in separation of two C-15 stereoisomers of 2,17-bidecarboxy-betanin, which were completely unresolved in RP-HPLC mode [9]. A method applied for ion-pair HPLC analysis of other betalains (betaxanthins, betanin and betalamic acid) was developed using triethylamine as the ion-pair agent at pH adjusted to 4.2 [14]. For HPLC analysis of other betacyanin decomposition products, another ion-pair system was applied with tetrabutyl ammonium phosphate (pH 7.3) designed especially for the separation of betalamic acid from betanin [7].

2. Experimental

2.1. Reagents

Tetrabutylammonium bromide (TBA), tetrapropylammonium bromide (TPA), tetraethylammonium bromide (TEA) and sodium hydroxide were obtained from Aldrich (Milwaukee, WI, USA). Formic acid, HPLC-grade acetonitrile and HPLC-grade water were obtained from Merck (Darmstadt, Germany).

2.2. Apparatus

A Gynkotek HPLC system with UVD170S, Gynkotek HPLC Pump Series P580 and thermostat (Gynkotek Separations, H.I. Ambacht, The Netherlands) was used for the chromatographic analysis. For the data acquisition, the software package Chromeleon 4.32 (Gynkotek Separations) was used. The column used was Luna C18(2), 250 × 3 mm I.D., particle diameter 5 μm protected by a guard column (Phenomenex, Torrance, CA, USA). The ODS material in the column was made from high-purity silica particles.

2.3. Preparation of analyte solutions

The solutions of betacyanins (betanin, phyllocactin, hylocerenin and their C-15 stereoisomers) from extracts of commercially available fruits of *Hylocereus polyrhizus* and their decarboxylated derivatives (2-mono-, 17-mono-, and 2,17-bidecarboxy-betacyanins as well as their C-15 stereoisomers) were prepared by procedures described in [11]. The solutions of each analyte group were used alone or in mixtures with other groups. The HPLC elution order of all pigments was compared with the data from [11] or with retention of isolated compounds by semipreparative HPLC as well as it was established by mass spectrometric analysis [11].

2.4. Chromatographic system

For the separation of the analytes the following gradient system was used: 97% A with 3% B at 0 min; gradient to 80% A with 20% B at 30 min. Solvent A, buffer (HCOOH/NaCOOH, 20 mM); Solvent B, acetonitrile. In each experiment, the modifier (TBA, TPA or TEA) of different mass was added to the mobile phase to obtain concentration in the range of 0.01–11 mM. The pH of the solvent A ranged between 2.3 and 6.7. The injection volume was 10 μL, and the flow rate was 0.5 mL/min. Detection in the UV–vis detector was performed at $\lambda = 538$, 533 and 505 nm. The column was thermostated at 35 °C.

3. Results and discussion

Recent reports established the HPLC elution order of the analysed pigments [9,11]. It is well known, that the configuration of betacyanin C-15 isoforms allows greater interaction with the stationary phase and, therefore, these compounds have a greater retention time relative to their parents [1,2]. Similar elution order was confirmed for 17-decarboxy-betacyanins, however, it was reversed for 2-decarboxy-betacyanins [9,11], indicating different chromatographic mode of separation resulting from the loss of the second chirality center at C-2 and the lack of the ionic interaction between the positively charged quaternary nitrogen ($>N^+=$) and the ionised carboxyl in the 2 position. Hitherto, the elution order of 2,17-bidecarboxy-betacyanins has not been known, therefore, it was formally assumed, that 2,17-bidecarboxy-betacyanins eluted later than their corresponding C-15 isoforms [11]. Our very recent study supported the proposed elution pattern (in preparation [15]), which was the same as the elution order of 2-decarboxy-betacyanins.

As a result of betacyanin heating in their ethanolic and aqueous solutions three pigment groups differing in decarboxylation position (Table 1) and absorption maximum λ_{max} (538, 533, 505 and 507 nm for betacyanins, 2-mono-, 17-mono-