Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography A, 1029 (2004) 97–101
JOURNAL OF CHROMATOGRAPHY A
www.elsevier.com/locate/chroma

Influence of perfluorinated carboxylic acids on ion-pair reversed-phase high-performance liquid chromatographic separation of betacyanins and 17-decarboxy-betacyanins

Sławomir Wybraniec [a,*], Yosef Mizrahi [b]

[a] *Department of Chemical Engineering and Technology, Institute C-1, Faculty of Analytical Chemistry, Cracow University of Technology, ul. Warszawska 24, Cracow 31-155, Poland*
[b] *Department of Life Sciences, The Institutes for Applied Research, Ben Gurion University of the Negev, P.O. Box 653, 84105 Beer-Sheva, Israel*

Received 10 July 2003; received in revised form 22 December 2003; accepted 22 December 2003

Abstract

The ability of trifluoroacetic acid, pentafluoropropionic acid and heptafluorobutyric acid to act as ion-pairing agents for betacyanins and 17-decarboxy-betacyanins during HPLC analysis on a Luna $C_{18}(2)$ reversed-phase column is reported. While the perfluorinated carboxylic acids affect the retention of both groups of compounds by changing the pH of the mobile phase, the possibility of ion-pair chromatography for 17-decarboxy-betacyanins was noticed. In order to explain the accessibility of the positive charge for the counter-anion in decarboxy-betacyanins, the mesomeric structures of the polymethine system at low pH (around a value of 1.5), when the carboxylic group in the 2 position is protonated, should be taken into consideration.

Keywords: *Hylocereus polyrhizus*; Carboxylic acids; Fluorinated carboxylic acids; Betacyanins; Decarboxy-betacyanins; Betanin; Phyllocactin; Hylocerenin; Betalains

1. Introduction

Trifluoroacetic acid (TFA) and other perfluorinated carboxylic acids are the most commonly used ion-pair reagents in the separation of peptides and proteins by ion-pair HPLC, due to their high purity, water solubility [1–4], UV transparency up to 200 nm and volatility, which makes recovery of analytes relatively easier [5].

A type of compounds exhibiting complex ionization processes comprise betacyanins, which are water soluble pigments providing the colors in a wide variety of flowers and fruits. The red–violet betacyanins and the yellow betaxanthins belong to the group of betalain pigments. Currently about 30 structures of betacyanins are known and are well documented [6,7]. Most of them are 5-*O*-glucosides (Fig. 1), such as betanin and isobetanin (the corresponding C-15 diastereoisomer), the simplest 5-*O*-glucosylated betacyanins, which are present in almost all plants containing betacyanins and are the major red–violet pigments in red beet root (*Beta vulgaris*), but 6-*O*-glucosides have also been detected [6,7].

Betanin has three carboxyl groups, of which during titration with alkali only two have $pK_a = 3.3$. The third carboxyl in the 2 position must have a lower pK_a, because the isoelectric point in betanin is at pH 1.5–2.0, as demonstrated in electrophoretic separations [8]. The quaternary nitrogen atom ($>N^+=$) with a positive charge is neutralized by the third carboxyl group in the 2 position, which gives the pigment amphoteric properties. On the other hand, with an increase of the pH to the 8.6 region, the phenol group of the pigment should partly change into phenoxide ion with negative charge [8].

Further glycosylation of the 5-*O*-glucoside is very common and also esterification with hydrocinnamic acids such as ferulic or *p*-coumaric acids, or malonic acid [6,7]. Recent research reported structural elucidation and discovery of a new betacyanin, hylocerenin, in newly domesticated species of *Hylocereus* cacti [9].

17-Decarboxy-betacyanins belong to modified betacyanin group and can be obtained by heating of acidified solutions

* Corresponding author. Tel.: +48-12-628-2707; fax: +48-12-628-2036.
E-mail address: swybran@chemia.pk.edu.pl (S. Wybraniec).

0021-9673/$ – see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.chroma.2003.12.055

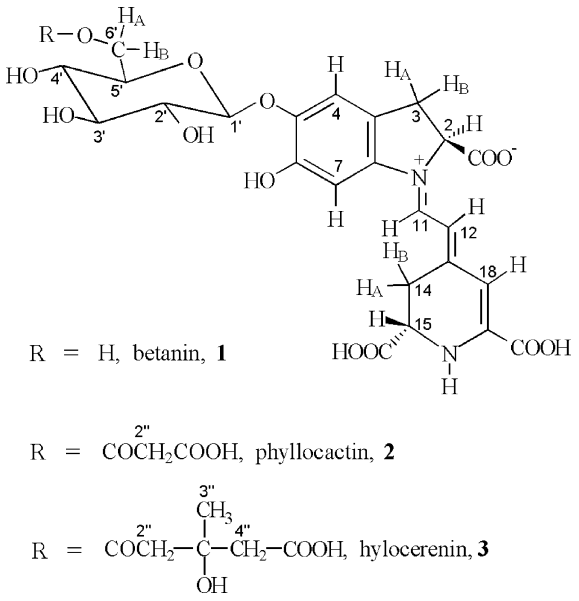

Fig. 1. Molecular structures of studied betacyanins in this report.

of betacyanins or can arise during complex processing of betacyanin samples. The decarboxylation process was described in [10–12]. The visible absorption spectrum of 17-decarboxy-betanins showed λ max at 535 nm, identical with that of betanin or isobetanin and the retention times were longer compared with the retention times of betanin or isobetanin due to their decarboxylation and formation of less polar compounds. The HPLC system applied in [13] obviously did not allow separating both stereoisomers of 17-decarboxy-betanidin. For HPLC analysis of other betacyanin decomposition products, another ion-pair system was applied with tetrabuthylammonium phosphate (pH 7.3) designed especially for the separation of betalamic acid (one of the precursors in the betacyanin biosynthesis and also decomposition product) from betanin [13].

The configuration of the C-15 epimers of betacyanins allows greater interaction with the stationary phase and therefore these compounds have a greater retention time relative to their parents [13]. A method applied for ion-pair HPLC analysis of betalains was developed using triethylamine as the ion-pair agent at pH adjusted to 4.2 [14]. The compounds separated were betaxanthins with betanin and betalamic acid. Some examples of using TFA as a modifier of the mobile phase can also be presented in betaxanthin analysis, however acting only as a factor of peak shape improvement and possessing higher acidity [15].

In this study, the ability of TFA and other perfluorinated carboxylic acids to act as ion-pairing agents with betacyanins and 17-decarboxy-betacyanins during HPLC analysis on a C_{18} column is reported. In this case ion-pairing would be possible, if the ionization of the carboxylic groups was suppressed in a highly acidic environment. It was of interest to understand the difference in the chromatographic behavior between these two closely related groups of betacyanins under ion-pair conditions and how the loss of one of the carboxylic groups determines the change in the access to the positive charge on the molecule for the counter-anions.

2. Experimental

2.1. Reagents

Sodium trifluoroacetate (NaTFA), sodium chloride, trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), heptafluorobutyric acid (HFBA) were obtained from Aldrich (Milwaukee, WI, USA). Formic acid, hydrochloric acid, HPLC-grade acetonitrile and HPLC-grade water were obtained from Merck (Darmstadt, Germany). Sodium heptafluorobutyrate (NaHFBA) was prepared by titration of HFBA with NaOH.

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 data acquisition, the software package Chromeleon 4.32 (Gynkotek Separations) was used. The column used was Luna C_{18}(2), 250 mm × 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 betacyanin and 17-decarboxy-betacyanin solution

Betacyanins from the fruit of *Hylocereus polyrhizus* were extracted and purified by gel chromatography according to the procedure described in [16]. 17-Decarboxy-betacyanins were synthesized along to a procedure similar to the one described in [10], as follows. A purified water solution of betacyanins isolated from *H. Polyrhizus* fruit was heated at 80 °C in a water bath for 40 min at pH 4.5. The resulting solution contained decarboxylation products of betanin, phyllocactin, hylocerenin and their isoforms as the main compounds showing light absorption at 548 nm, and minor amounts of unreacted substrates. The solution was then mixed with purified betacyanin solution giving the final sample for research. The HPLC retention time for each 17-decarboxy-betacyanin in the solution was checked after comparison with decarboxylation products of each purified betacyanin isolated by semipreparative HPLC. It was taken into account, that isoforms were eluted always after the natural forms of 17-decarboxy-betacyanins in the resulting pair of diastereoisomers [13]. Additionally the molecular masses of the compounds were checked by LC–MS–MS, according to procedures which can be found elsewhere [6,7,9].

2.4. Chromatographic system

For the separation of the analytes on the Luna $C_{18}(2)$ column, the following gradient system was used: A–B (94:6) at 0 min; gradient to A–B (83:17) at 50 min.

Solvent A is water, solvent B is acetonitrile. In each experiment modifiers of different concentrations were added to the mobile phase, i.e.,

1. TFA, PFPA or HFBA (mobile phase with 250 mM HCOOH).
2. NaTFA or NaHFBA salts (solvent A was a buffer of pH 1.7 (HCl/NaCl)).

The ranges of concentration are given in the corresponding Figures in the results and discussion section. The injection volume was 10 μl, and the flow rate was 0.5 ml/min. UV-Vis detection was generally performed at 548 nm (with additional wavelengths of 480 and 290 nm). The columns were thermostated at 35 °C.

3. Results and discussion

The influence of perfluorinated carboxylic acids added to the mobile phase containing 250 mM HCOOH on retention of 17-decarboxy-betanin on Luna $C_{18}(2)$ column is presented in Fig. 2. The effect of the PFPA and HFBA concentration on the retention of the analytes is more pronounced than that of TFA, for HFBA being the highest, along the order of acidity as follows: TFA < PFPA < HFBA [17]. The results obtained with HFBA for all the examined compounds are presented in Fig. 3. The names and numbers of the analysed compounds are included in Table 1. The rate of the increase at the beginning is more pronounced for decarboxy-betacyanins for which the retention is markedly increasing in the range of PDFA or HFBA concentration ca. 0–5 mM. In the upper concentration range, the effect looks similar for betacyanins and decarboxy-betacyanins.

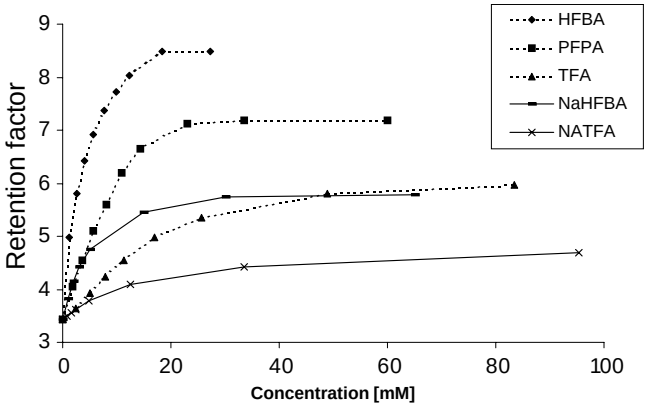

Fig. 2. Comparison of the retention factors of 17-decarboxy-betanin on the Luna $C_{18}(2)$ column in dependence on the concentration of perfluorinated carboxylic acids in the mobile phase containing 250 mM HCOOH and on the concentration of their salts at pH 1.7.

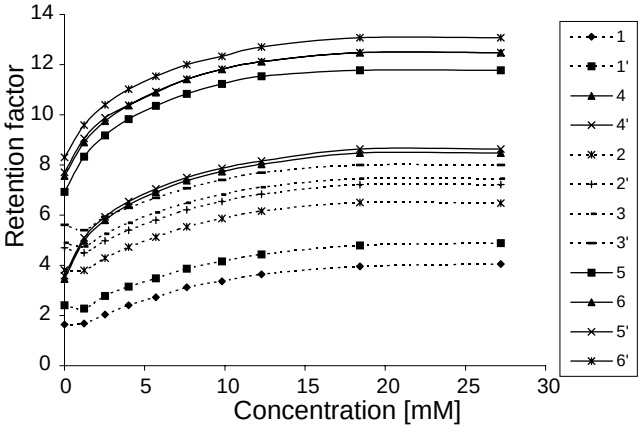

Fig. 3. Retention factors of betacyanins and 17-decarboxy-betacyanins on the Luna $C_{18}(2)$ column in dependence on the HFBA concentration in the mobile phase with 250 mM HCOOH.

However, at the beginning betacyanins exhibit a decrease of retention, while decarboxy-betacyanins act in the opposite way. This decrease of retention deserves further research.

In order to obtain a prove of the ion-pair effect exhibited for betacyanins or decarboxy-betacyanins, the next set of experiments was performed with NaTFA and NaHFBA salts, thus eliminating the influence of the mobile phase pH decrease which was otherwise in parallel with the counter-anion concentration increase in the case of the acids. The analyses were performed in a buffer of pH 1.7 (HCl/NaCl), for which enough separation and peak shapes of the analytes were already achieved to notice the subsequent changes in retention after the addition of NaTFA or NaHFBA. The experimental dependencies of 17-decarboxy-betanin retention on the counter-anion concentration are shown in Fig. 2.

There is a noticeable influence of the NaHFBA derived counter-anion concentration on the retention of decarboxy-betacyanins, indicating the possibility of ion-pair interactions with these compounds. The ability of NaTFA derived counter-anions to act as ion-pairing agent is lower, but still visible very well. On the chromatograms (Fig. 4) the peaks of the pair of 17-decarboxy-betanin and its isoform are moving through isohylocerenin, finally being separated from it. The fact that the ability of acting as ion-pair agent increases with an increase of the carbon chain of the perfluorinated carboxylic acids was already frequently noticed [1].

Table 1
Names and numbers of the pigments analysed in this study

Compound	Natural forms	Isoforms
Betanin	**1**	**1′**
Phyllocactin	**2**	**2′**
Hylocerenin	**3**	**3′**
17-Decarboxy-betanin	**4**	**4′**
17-Decarboxy-phyllocactin	**5**	**5′**
17-Decarboxy-hylocerenin	**6**	**6′**

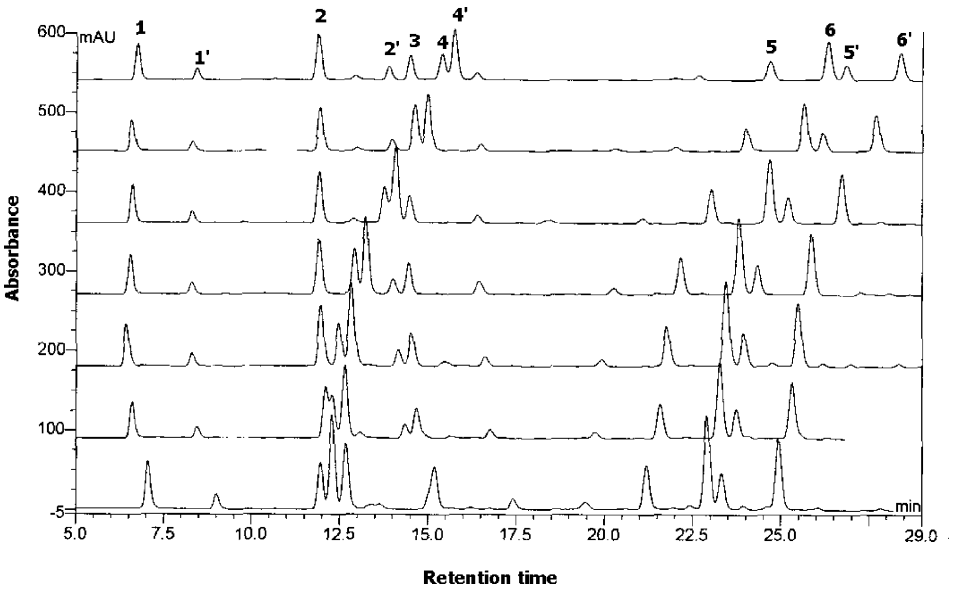

Fig. 4. Chromatograms of the analytes obtained on the Luna $C_{18}(2)$ column with increasing NaTFA concentration in the mobile phase at pH 1.7. Concentrations (mM) (starting from the bottom chromatogram): 0; 1.6; 2.5; 4.8; 13; 34; 95.

Major effects on the analyte retention are observed at low counter-anion concentration and are related to the influence of the counter-anion of the acidic modifier on the analyte solvation, and should be independent on the mobile phase pH, if complete protonation of the analyte is achieved and the positive charge of the compound is exposed. All the effects observed indicate, that there is a possibility of ion-pair chromatography for decarboxy-betacyanins in the presence of perfluorinated carboxylic acids in the mobile phase.

The third carboxylic group in the 2 position must have a low pK_a not only for betacyanins (according to the data from the electrophoretic separations [8]) but also for decarboxy-betacyanins, because these two groups differ from each other only by the presence of another distant carboxylic group. It seems, that the positive charge on the nitrogen is sterically shielded by the three carboxylic groups of the chromophore part of betacyanins and is unshielded when the distant group (at C-17) is not present. Therefore, in order to explain the accessibility of the positive charge for the counter-anion in 17-decarboxy-betacyanins, the mesomeric structures of the polymethine system at low pH (around a value of 1.5), when the carboxylic group in the 2 position is protonated, should be taken into consideration [18]. In this case no neutralization of the nitrogen $N-1$ takes place and a second mesomeric structure of the molecule can be drawn as for 17-decarboxy-betanin. The change of the structure should be responsible for the color change of the resulting compound, i.e. for the shift of the absorbance maximum, leading to the diminishing of the HPLC peak signals in the UV-Vis detector, which was observed in chromatograms of more concentrated acids. The relevant studies of this phenomenon are underway.

4. Conclusions

The addition of small quantities of perfluorinated carboxylic acids to already prepared HPLC mobile phases can be another controlling factor of the retention of complex mixtures of betacyanins and modified betacyanins in samples of biological origin.

While the perfluorinated carboxylic acids increased the retention of the both groups of compounds by lowering the pH of the mobile phase, the possibility of ion-pair chromatography for 17-decarboxy-betacyanins was noticed. The presence of mesomeric structures of the polymethine system at low pH, when the carboxylic group in the 2 position is protonated, would explain the accessibility of the positive charge for the counter-anion in decarboxy-betacyanins.

Acknowledgements

This study was financed in part by Foundation for Supporting of Polish Pharmacy and Medicine Development at Polpharma S.A. Pharmaceutical Plant in the frame of research project No. 015/2002.

References

[1] K.N. Petritis, P. Chaimbault, C. Elfakir, M. Dreux, J. Chromatogr. A 833 (1999) 147.
[2] H.J. Chaves-das-Neves, Z. Braya-Morais, Anal. Quim. 93 (1997) 98.
[3] H.J. Chaves-das-Neves, Z. Braya-Morais, J. High Resolut. Chromatogr. 20 (1997) 115.
[4] R.G.J. Van Leuken, G.T.C. Kwakkenbos, A.L.L. Duchateau, J. Chromatogr. 647 (1993) 131.
[5] J.D. Pearson, M.C. McCroskey, J. Chromatogr. A 746 (1996) 277.

[6] D. Strack, W. Steglich, V. Wray, in: P.M. Dey, J.B. Harborne, P.G. Waterman (Eds.), Methods in Plant Biochemistry, vol. 8, Academic Press, London, 1993, p. 421.
[7] D. Strack, T. Vogt, W. Schliemann, Phytochemistry 62 (2003) 247.
[8] T. Nilsson, Lantbrukshoegsk. Ann. 36 (1970) 179.
[9] S. Wybraniec, I. Platzner, S. Geresh, H.E. Gottlieb, M. Haimberg, M. Mogilnitzki, Y. Mizrahi, Phytochemistry 58 (2001) 1209.
[10] L. Minale, S. Piattelli, Rend. Accad. Sci. Fis. Mat. 32 (1965) 165.
[11] S.J. Schwartz, J.H. von Elbe, Z. Lebensm, Unters. Forsch. 176 (1983) 448.
[12] E. Dunkelblum, H.E. Miller, A.S. Dreiding, Helv. Chim. Acta 55 (1972) 642.
[13] S.J. Schwartz, J.H. von Elbe, J. Agric. Food Chem. 28 (1980) 540.
[14] G.F. Trezzini, J.P. Zryd, Phytochemistry 30 (1991) 1901.
[15] F.C. Stintzing, A. Schrieber, R. Carle, J. Agric. Food Chem. 50 (2002) 2302.
[16] S. Wybraniec, Y. Mizrahi, J. Agric. Food Chem. 50 (2002) 6086.
[17] T. Abe, H. Baba, I. Soloshonok, K. Tanaka, J. Chromatogr. A 884 (2000) 93.
[18] G. Ponterini, Chem. Phys. 216 (1997) 193.