Journal of Chromatography A, 1572 (2018) 137–144

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Vulnerability of anthocyanins to the components of a thin-layer chromatographic system and comprehensive screening of anthocyanes in alimentary products

Eliza Łata, Agnieszka Fulczyk, Teresa Kowalska*, Mieczysław Sajewicz

Department of General Chemistry and Chromatography, Institute of Chemistry, University of Silesia, 9 Szkolna Street, 40-006 Katowice, Poland

ARTICLE INFO

Article history:
Received 1 July 2018
Received in revised form 9 August 2018
Accepted 19 August 2018
Available online 20 August 2018

Keywords:
Anthocyanes
TLC
Multiple development
Alimentary products
Authentication

ABSTRACT

The aim of this study was to revisit the TLC authentication of alimentary products concept based on analysis of anthocyanes with the foodstuffs of plant origin. To this effect, we used two anthocyanins (cyanin and keracyanin) and two anthocyanidins (pelargonidin and delphinidin) as phytochemical standards. The first step was to develop a novel method making use of the RP-18 F_{254s} stationary phase (which ensures mixed-mode retention mechanism with the localized adsorption on the non-bonded silanols) and acetic acid as the mobile phase component. Importantly, similar TLC systems are currently used for the analysis of anthocyanes. Individual steps of our method development enabled a deeper insight in vulnerability of anthocyanins to external conditions resulting in hydrolysis thereof. In this study, it was impossible to fully separate the products of hydrolytic degradation of the test anthocyanins in a single development run and it was only triple development which ensured distinct and symmetrically shaped chromatographic spots, further scrutinized with use of mass spectrometry. The identity of the hydrolytically split fractions was additionally studied with use of the *p*-aminobenzoic acid (PABA) test. To obtain calibration curves, triple development was employed for cyanin, keracyanin, and pelargonidin, while delphinidin was developed in one development run. The respective LOD and LOQ values were: for spot **(i)** derived from the cyanin standard, 0.005 and 0.016 μg spot^{-1}; for spot **(ii)** derived from the cyanin standard, 0.006 and 0.017 μg spot^{-1}; for spot **(i)** derived from the keracyanin standard, 0.092 and 0.274 μg spot^{-1}; for spot **(ii)** derived from the keracyanin standard, 0.035 and 0.104 μg spot^{-1}; for the pelargonidin standard, 0.013 and 0.040 μg spot^{-1}; and for the delphinidin standard, 0.036 and 0.108 μg spot^{-1}. The developed method was used to identify and quantify cyanin, keracyanin, pelargonidin and delphinidin in selected alimentary products (syrups, juices and herbal infusions), keeping in mind that the obtained numerical results were of semi-quantitative nature only.

1. Introduction

In modern world, alimentary products of all kinds undergo strict quality control for evident reasons of the consumers' safety and summarized information in form of an adequate labeling should be available for each consumer. In European Union, this issue is regulated by the Directive 2000/13/EC of the European Parliament and of the Council on the approximation of the laws of the Member States [1]. The natural first step in quality control of alimentary products is authentication thereof by selecting such biomarkers which are suitable for rapid screening and provide a unanimous answer to the question of authenticity of a given product. Many alimentary products make use of botanical raw materials (e.g., fruits, vegetables, herbs and spices), most of them containing natural pigments. Authentication of foodstuffs based on identification and quantification of natural pigments is a concept which has long gained an acceptance of the researchers and the official regulatory bodies.

Based on their distinct colors from pink and red to dark purple, natural pigments from the group of anthocyanes have attracted interest of the foodstuff analysts as the compounds which seem relatively easy to trace with use of the vibrational and fluorescence spectroscopy (hence, with use of the TLC and HPLC detectors also)

* Corresponding author.
E-mail addresses: lata.eliza@gmail.com (E. Łata), afulczyk@onet.pl (A. Fulczyk), teresa.kowalska@us.edu.pl (T. Kowalska), mieczyslaw.sajewicz@us.edu.pl (M. Sajewicz).

https://doi.org/10.1016/j.chroma.2018.08.040
0021-9673/

[2]. An interesting PhD dissertation on this subject matter was completed in 2010 by T.C. Wallace from the Ohio State University [3]. It summarizes the research on protocyanidins and anthocyanins in the foodstuffs carried out by the candidate in a number of scientific papers and three of them deal with analytical problems related to the anthocyane pigments [4–6]. Anyway, identification and quantification of anthocyanes in red fruits is by no means a novel subject matter and quite a number of papers on these issues have already been published in the past decade or more (e.g., [7–12]).

However, authentication of foodstuffs containing pink, red or purple fruits and based on quantification of anthocyanes is a challenging task, due to the recognized destructive role of the environmental factors (such as light, pH and temperature), which strongly affect this class of compounds (e.g., [13–16]). In the indicated (and other) papers, kinetics of the anthocyane color loss is thoroughly measured and reported, and vulnerability of these compounds to the environment is convincingly documented. When authenticating the foodstuffs by targeting anthocyanes and using thin-layer chromatography as a measuring tool, there is no way how to protect these pigments from an influence of stationary and mobile phase, which can contribute to partial decomposition of these important biomarkers.

In our previous paper [17], we introduced a novel TLC method of screening the anthocyanes containing alimentary products, which outperformed the existing HPTLC methods (providing the respective LOD and LOQ values by one magnitude order lower than those presented in [18,19]). In that paper, we pointed out to certain drawbacks with the analysis of anthocyanes and among the indicated drawbacks, partial hydrolysis of anthocyanins to anthocyanidins was reported. As a result of this process, on a chromatogram of a given anthocyanin two bands were observed instead of one, that with the lower R_F value belonging to an original glycoside and the other one with the higher R_F value belonging to a split off aglycone. Apparently, the presence of two chromatographic bands originating from one glycoside is confusing and it jeopardizes final analytical result both in terms of identification and quantification. This observation was explicitly confirmed by experimental results and conclusions drawn by the authors of paper [20]. Partial hydrolysis of cyanin and keracyanin reported in our previous paper [17] yielded in relatively low amounts of the split-off cyanidin, basically due to an absence of the acidic groups in the purified microcrystalline cellulose and hence, to the low chromatographic activity of the employed cellulose adsorbent. However, an important message resulting from that report and addressing the community of foodstuff analysts was to use a limited number of comprehensively selected anthocyanin standards for fast screening and authentication of alimentary products, in order to limit the number of possible confusions and mistakes.

Keeping in mind the warning message originating from our previous research, this time we decided to revisit the issue of screening and authenticating the anthocyanes containing alimentary products by developing a novel TLC method making use of the RP-18 stationary phase, which ensures a mixed-mode retention mechanism with localized adsorption of the analytes on the non-bonded silanol groups which might actively contribute (along with the acidic components of mobile phases) to partial hydrolysis of anthocyanins to the respective anthocyanidins. In paper [21], the authors reported on the blurred chromatographic spots of anthocyanins in a number of chromatographic systems employing either silica gel or the chemically bonded stationary phases (RP-18 and NH_2), which gave us a hint that this blurring might possibly result from an incomplete resolution of the anthocyanin glycosides from either their split off aglycons, or from the glycosides partially depleted of the sugar moieties. Thus the results provided in papers [17,21] spurred us to performing a detailed scrutiny of the anthocyanin behaviour in the RP-18-based thin-layer chromatographic systems.

2. Experimental

2.1. Reagents and standards

Acetonitrile (Chemsolve, Łódź, Poland) and methanol (Sigma-Aldrich, St. Louis, MO, USA) used in this study were of HPLC grade. Glacial acetic acid (Chempur, Piekary Śląskie, Poland) and hydrochloric acid (PPH POCH, Gliwice, Poland) were of analytical grade. De-ionized and double-distilled water was obtained from the Elix Advantage Model Millipore System (Millipore, Molsheim, France).

Phytochemical standards of analytical purity used in the experiments were cyanin chloride, keracyanin chloride and pelargonidin chloride (purchased from Sigma-Aldrich, St. Louis, MO, USA), and delphinidin chloride (purchased from Cayman Chemicals, Ann Arbor, MI, USA). Chemical structures of the respective aglycons are given in Fig. 1 (with cyanin and keracyanin sharing the same aglycone, cyanidin). Working samples of phytochemical standards were prepared in methanol at the concentrations of 0.01 mg mL^{-1} (cyanin, keracyanin, and pelargonidin) and 0.02 mg mL^{-1} (delphinidin), and stored in a freezer at −20 °C.

2.2. Alimentary products

As alimentary products, we choose juices, syrups and herbal materials declared by the respective manufacturers as 100% natural. These were preparations made of blackcurrant (juice), blueberry (juice), blackberry (syrup), chokeberry (juice), elderberry (juice), flower petals of rose (syrup) and hibiscus (infusion, 25 g dried petals in 200 mL water). Juices and syrups were collected from the sealed packages in the aliquots of 50 mL, then 0.2 mL hydrochloric acid (35–38 %) was added to each sample in order to avoid possible degradation of anthocyanins (as recommended in [23]) and finally, the samples were tightly closed and stored in refrigerator at +6 °C, ready for the analysis. Hibiscus infusion was prepared prior to the analysis.

2.3. Triple and single development modes for quantification of anthocyanes in the RP-18-based TLC systems

All thin-layer chromatographic analyses were carried out with use of the commercial RP-18 F_{254s} pre-coated TLC plates (10 cm × 20 cm, cat. # 115423; Merck, Darmstadt, Germany).

The cyanin, keracyanin and pelagonidin standards underwent triple development in the consecutive mobile phases. First development was carried out with mobile phase I (acetonitrile + methanol + glacial acetic acid, 16:4:0.15 (*v/v/v*)) to the distance of 90 mm from the lower plate edge. Second development was carried out with mobile phase II (methanol + glacial acetic acid, 20:0.15 (*v/v*)) to the distance of 70 mm from the lower plate edge. Third development was carried out with mobile phase III (methanol + glacial acetic acid, 20:0.45 (*v/v*)) to the distance of 60 mm from the lower plate edge. Each phytochemical standard was spotted onto the chromatographic plate 10 mm above the lower plate edge and each development was preceded by presaturation of chromatographic chamber with mobile phase for the period of 20 min. After each chromatographic run, the plates were dried in ambient air for 20 min.

The delphinidin standard was spotted onto the chromatographic plate 10 mm above the lower plate edge and it underwent single development to the distance of 90 mm from the lower plate edge with use of mobile phase composed of hydrochloric acid + glacial acetic acid + water, 9:46:90 (*v/v/v*) [22]. Pre-saturation of chromatographic chamber with mobile phase lasted 20 min and after development, the plates were dried for 20 min in ambient air.