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Novel thin-layer chromatographic method of screening the anthocyanes containing alimentary products and precautions taken at the method development step

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

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ABSTRACT

The purpose of this study was to develop a novel and cost-effective thin-layer chromatographic method (TLC) using cellulose powder as stationary phase for authentication of the selected fruit-based alimentary products and targeting anthocyanes as the authenticity markers. Our method outperformed the HPTLC method earlier developed by another research team using silica gel as stationary phase. It was demonstrated that due to a limited chemical stability of anthocyanes, employing them as authenticity markers is burdened with a non-negligible uncertainty risk. Hydrolytic split of the glycosides into the aglycone and carbohydrate moieties can lead to a confusing multiplication of chromatographic bands and therefore it is advisable to use for the authentication purposes a limited set of well selected and stable enough anthocyane markers. Cyanin chloride, keracyanin chloride, pelargonidin chloride and delphinidin chloride were selected as the external standards and for the development of the calibration curves. The TLC-obtained LOD and LOQ values were 0.025 and 0.075 μg spot^{-1} for cyanin, 0.055 and 0.166 μg spot^{-1} for keracyanin, 0.047 and 0.140 μg spot^{-1} for pelargonidin, and 0.171 and 0.513 μg spot^{-1} for delphinidin, respectively. The analogous HPTLC-obtained LOD and LOQ values were 0.107 and 0.321 μg spot^{-1} for cyanin, 0.189 and 0.566 μg spot^{-1} for keracyanin, and 0.161 and 0.484 μg spot^{-1} for pelargonidin, respectively. Delphinidin was not detectable with use of the HPTLC method. Consequently, quantification of anthocyanes in the alimentary products carried out with use of TLC allowed identification of more target compounds and in a higher number of alimentary products than it was possible with use of HPTLC, apparently due to the LOD levels by one magnitude order lower for TLC than HPTLC.

1. Introduction

Phytochemicals are the secondary plant metabolites responsible for the metabolic pathways and biosynthesis. Phenolic compounds make a group of the secondary plant metabolites and one of these groups are flavonoids, further divided into the subgroups of anthocyanins, flavanols, flavanones, flavones, flavonols, flavanonols, and isoflavones [1]. Anthocyanins are a class of plant pigments, being the so-called natural non-food vegetable substances soluble in water [2]. They appear in plants as a connection of an aglycone part of a compound known as anthocyanidin and a sugar (which is mostly glucose, but it can also be rhamnose, galactose, xylose, rutinose, neohesperidose, etc.) [3]. Furthermore, anthocyanins can appear as mono-, di-, or even trisaccharides [2]. Not only the presence and the type of sugar determines structural differences among anthocyanins, but also the substituents in positions R1 and R2 of the aglycone part of the compound (structural scheme is given in Fig. 1) [3]. Natural anthocyanin pigments appear mostly in fruits and flower petals, but they can also be found in leaves, shoots and roots. Their colors can range from orange through various shades of red and violet to black [1,2,4–6]. The rich color palette of anthocyanins can be used for dying of certain food products (like the fruit concentrates, fruit and vegetable juices, jams, wines etc. [7]). Another reason for an enrichment of foods with anthocyanins is to enhance their pro-health properties and in the first instance, their antioxidant potential. The presence of anthocyanins in a daily diet helps counteract many health problems, e.g., cardiovascular diseases, liver dysfunctions, hormone-dependent diseases, or vision disorders [3,8–10]. Anthocyanins have been reported as active food ingredients in cancer prevention, owing to their ability to reduce cancer cell proliferation and to inhibit tumor forma-

* 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).

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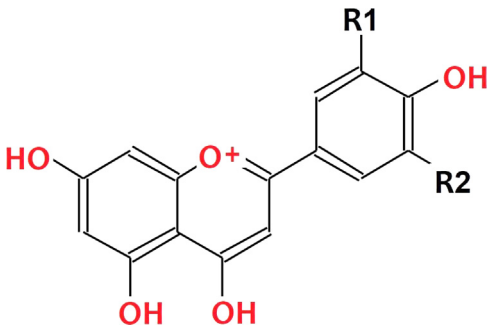

Fig. 1. Selected examples of the anthocyanin aglycones, i.e., anthocyanidins: R1 = OH, R2 = H, cyanidin; R1 = H, R2 = H, pelargonidin; R1 = OCH_3, R2 = OCH_3, malvidin; R1 = OH, R2 = OH, delphinidin.

tion [11]. Equally important are their anti-allergic, antibacterial, anti-inflammatory and antiviral effects [1,3]. Last not least, anthocyanins have a capacity to effectively chelate metals, in the first instance iron and copper [1,12–14].

Adulteration of various different alimentary products in order to enhance their esthetic qualities and hence, their commercial value has been a long established malpractice [10,15,16]. For the sake of example, one can mention falsification of juices or drinks (claimed as prepared from natural fruit or vegetable extracts) with synthetic dyes [17,18]. Thus, there is a need to develop a simple and cost-effective thin-layer chromatographic method to control the commercial fruit syrups, nectars, juices and drinks for possible adulteration with synthetic colorants. On the other hand, anthocyanins are well known for being strongly affected by such factors, as temperature, pH, light and oxygen, which can stimulate oxidation or splitting of the covalent bonds. It has been experimentally proved that anthocyanin pigments can easily transform to aglycones by hydrolytic splitting of the sugar moiety, to the chalcones by opening of the pyrylium ring, or to the coumarin glucosides by further transformation of the chalcone moiety. Such structural modifications result in color changes of the anthocyanin pigments, or even in a complete disappearance of coloration [19–23]. Hence, an assessment of anthocyanins in alimentary products should be understood as having a semi-quantitative importance only.

In the most recent literature on the application of the high-performance thin-layer chromatography (HPTLC) with use of silica gel as stationary phase to the analysis of the anthocyanin pigments, the authors have developed a protocol for the separation and quantification of eleven anthocyanins, which was further applied to the identification and quantification of these compounds in the selected alimentary products [2,4,5,24]. Thus it was the aim of this study to develop a novel thin-layer chromatography method (TLC) with use of cellulose powder as stationary phase, outperforming that proposed in papers [4,5], in order to screen the common fruit syrups, nectars, juices and drinks for possible adulteration with synthetic colorants. To this effect, we targeted a limited number of the two common anthocyanins (cyanin and keracyanin) and the two common anthocyanidins (pelargonidin and delphinidin) in these preparations, knowing that the most popular anthocyanins present in the common fruit-based foods are sugar connections with cyanidin, delphinidin, malvidin, and pelargonidin. Adequate examples are given in Table 1, where a selection of common fruits is listed with the corresponding anthocyanin pigments found therein [2,7]. Similar data are provided in Table 3 from paper [25]. We also frankly report on necessary precautions which had to be taken into account when developing our novel TLC method, resulting from sensitivity of anthocyanes to light and temperature, and from their tendency to undergo hydrolytic splitting to the aglycone and carbohydrate moiety in the chromatographic system.

2. Experimental

2.1. Reagents and standards

The following reagents of analytical purity were used in this study: methanol, glacial acetic acid and *n*-butanol (all purchased from PPH POCH, Gliwice, Poland), the 38% hydrochloric acid (Chempur, Piekary Śląskie, Poland), ethyl acetate (PPH POCH), 2-butanone (Chempur) and the 88% formic acid (PPH POCH). Water was de-ionized and double-distilled by means of the Elix Advantage model, Millipore System (Millipore, Molsheim, France).

The employed phytochemical standards of analytical purity 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). For all experiments, methanol solutions of the standards were used. They were prepared at the concentrations of 0.1 $\mu g\,mL^{-1}$ (cyanin, keracyanin, and pelargonidin) and 0.4 $\mu g\,mL^{-1}$ (delphinidin). The prepared solutions were stored in a freezer at −20 °C.

2.2. Alimentary products

As alimentary products, we used eighteen randomly selected fruit preparations which were juices, syrups, nectar, and the non-carbonated drinks. Some of these products were home-made and the others originated from the local discount stores and pharmacies. According to the manufacturer's declaration, chokeberries, raspberries, blackcurrants, plums or grapes were the substrates used for the production of the respective products. According to the literature, the presence of the employed test anthocyanes could be expected in the investigated food samples (Table 1). All preparations were liquids and for the purpose of the analysis they were pre-treated according to one and the same procedure. From the originally sealed alimentary products stored at room temperature, the 10-mL sample aliquots were collected. Then 30 mL methanol and 0.4 mL hydrochloric acid was added to them to avoid degradation of anthocyanins that might be present in the samples [19]. Tightly closed samples were then stored in refrigerator at +6 °C.

2.3. Chromatographic examination of stability of the anthocyane standard solutions

Stability of the anthocyane standards in the methanol solution was examined in the course of three weeks, in the one-week intervals. For this purpose, the 6-μL aliquots of the freshly prepared delphinidin, cyanin, keracyanin, and pelargonidin solutions in methanol were spotted onto the cellulose-precoated TLC plates and the chromatograms were developed with use of the mobile phase glacial acetic acid + water + *n*-butanol, 16:19:65 (*v/v/v*) (according to the procedure described in detail in the forthcoming section). Then the same standard solutions kept in the refrigerator at the temperature of +6 °C were spotted onto the chromatographic plates after 7, 14, and 21 days sample storage period and the respective chromatograms were developed. The results obtained are given in Table 2 with the signal intensity expressed in the percentage scale (signal intensity of the freshly prepared solutions was assumed as equal to 100%).

2.4. Thin-layer chromatography (TLC) with cellulose powder as stationary phase

Thin-layer chromatographic analyses were carried out with use of the TLC plates pre-coated with the cellulose powder (20 cm × 20 cm, cat. # 1.05716; Merck, Darmstadt, Germany). The plates were developed in the mobile phase glacial acetic acid + water + *n*-

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