DEVELOPMENT OF A QUANTITATIVE HIGH PERFORMANCE THIN LAYER CROMATOGRAPHIC METHOD FOR ANALYSIS OF DELPHINIDIN 3-GLUCOSIDE IN BERRY EXTRACTS

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The paper presents a simple and precise HPTLC method for determination of delphinine from berry extracts. Delphinidin was extracted with a mixture of methanol acidified with hydrochloric acid 25 % and separated on HPTLC silica gel 60F_{254} plates with ethyl acetate, toluene, water and formic acid 12:3:0.8:1.2 (v/v/v/v) as mobile phase within 20 minutes. Densitometric analysis of this compound was performed under white light illumination in the transmission mode and the absorbance was measured at 555 nm. Calibration data revealed good linear relationship (r = 0.99978) between peak area and concentration of delphinidin. This method can be used for rapid routine analysis based on the results obtained in this study.

Keywords: anthocyanins, delphinidin, HPTLC, berries, quantification

1. Introduction

Medicinal plants have played an essential role in the development of medicine and nutritional sciences for their potential benefit effects on human health, because they can be used as dietary supplements in functional food products [1,2]. Plants are directly used as medicines by a majority of cultures around the world (e.g. Chinese medicine and Indian medicine). A lot of medicinal, aromatic, spice and other plants contain chemical compounds which have antioxidant properties and high content of free radicals scavengers (e.g., carotenoids, polyphenols, flavones, anthocyanins, unsaturated fatty acids, vitamins, enzymes and cofactors) [3,4].

Berries are a particularly rich source of anthocyanins and polyphenols. This class of compounds contains phenolic acids, flavonoids, anthocyanins, quinines and tannins [3]. The most important among the flavonoids are the anthocyanins which are responsible for the red, orange, purple and blue colours in

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many fruits (bilberry, blueberry, cranberry, strawberry, raspberry, blackcurrant), flowers and vegetables [5-9]. The pigments are closely related with pH [10].

Anthocyanins may be used in industry like food colorants (juice, jams), in the pharmaceutical industry for capsules, in the cosmetics production (soaps and shampoos) and for manufacturing of red wine [11]. Chemically, these pigments are glycosides of 18 anthocyanidins that differ one of the other by their degree of hydroxylation and methoxylation [12]. The six important anthocyanins for nutrition are: delphinidin (3,5,7,3,4,5-hexahydroxyflavylium), cyanidin (3,5,7,3,4-pentahydroxyflavylium), malvidin (3,5,7,4-tetrahydroxy-3,5-dimethoxyflavylium), peonidin (3,5,7,4-tetrahydroxy-3-methoxyflavylium), pelargonidin (3,5,7,4-tetrahydroxyflavium) and petunidin (3,5,7,3,4-pentahydroxy-5-methoxyflavylium), some fruits also contain di-glucosides [13-16]. The chemical structure of anthocyanidins is shown in Fig. 1.

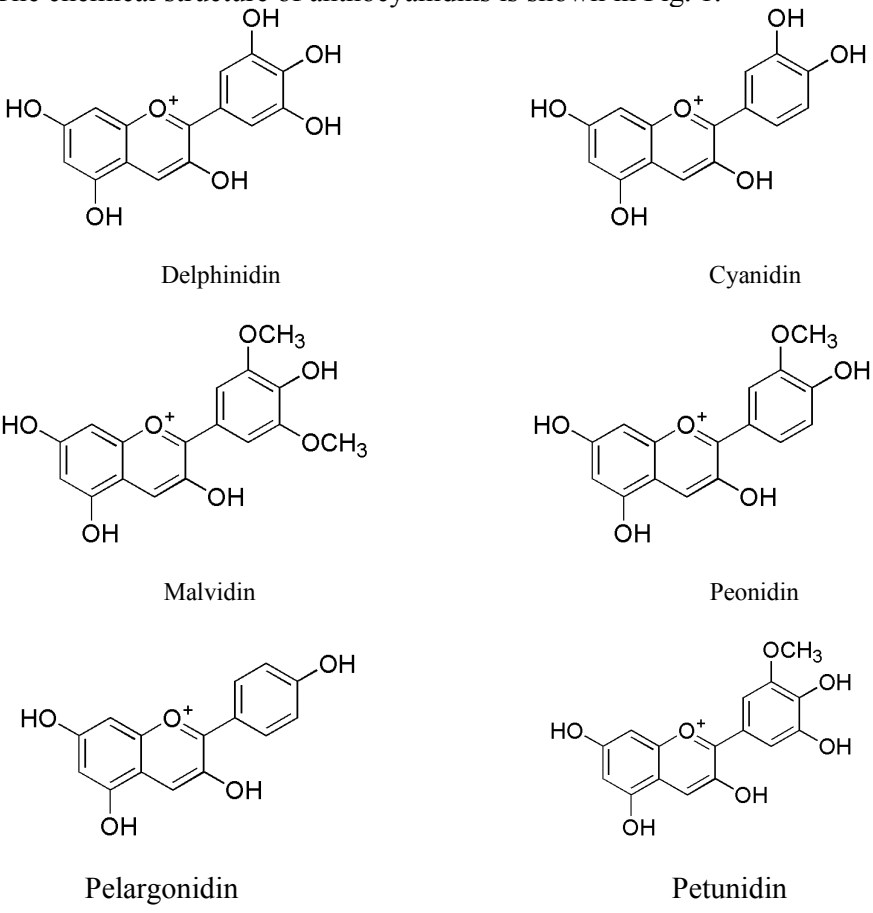

Fig.1. Chemical structure of delphinidin, cyanidin, malvidin, peonidin, pelargonidin and petunidin

Delphinidin 3-glucoside has strong antioxidant properties and also other beneficial traits like anti-inflammation and antimicrobial activities, ultraviolet light protection [17-28].

Anthocyanins are polar compounds and they are extracted from plant species using polar solvents like methanol, ethanol and water.

Blueberry fruit *(Vaccinium cyanococcus)* are native from North America. These fruits are loved for their intensive dark blue colour and for their sweet tart taste. Blueberry fruits are a good source of vitamins (A, B, C, E), minerals (potassium, manganese and magnesium) and antioxidants. The antioxidant capabilities of these fruits can be attributed to the abundance of anthocyanins. These compounds play an important role in keeping our body healthy and young. These fruits have a very high fibre content and have low amounts of saturated triglycerides, cholesterol and sodium.

Bilberry fruit (*Vaccinium myrtillus*) are native from Northern Europe. These fruits were used for centuries in medically sense and also as food in jams and pies. The majority compounds from bilberry fruits are anthocyanins, which have excellent antioxidant properties. Antioxidants are essentials to optimizing health by helping to combat the free radicals that can damage cellular structures as well as DNA. These fruits also contain vitamin C, which is another antioxidant. Bilberries were used to control blood sugar levels in people with diabetes. Anthocyanins found in this fruits may also be used for people with vision problems.

Anthocyanins quantification is nowadays mostly performed by RP-HPLC using DAD or MS (/MS) detectors. However, a standardized, quantitative HPTLC method would offer a more efficient analysis.

The aim of this study was to determine the delphinidin in berry extracts using high performance thin layer chromatographic method and to optimize a good mobile phase for separation of this compound.

2. Materials and methods

2.1 Reagents

Delphinidin, malvidin, cyanidin and peonidin chloride salts were provided by ABCR (Karlsruhe, Germany). Pelargonidin chloride and pelargonidin 3-glucoside (Cl-salt) were provided by Sigma-Aldrich (Schnelldorf, Germany). The 3-glucoside forms of delphinidin, malvidin, cyanidin, peonidin were obtained from Phytolab (Vestenbergsreuth, Germany) as HPLC purity grade chloride salts. Malvidin 3,5 diglucoside (Cl salt) was produced by Carl Roth (Karlsruhe, Germany). HPTLC plates silica gel 60 F_{254} (20 × 10 cm, Art. no. 105642.0001) were supplied by Merck (Darmstadt, Germany). For preparation of mobile phase ethyl acetate and toluene (Merck), formic acid (Fluka) were used. Ultra-pure

water was produced by Synergy System (Millipore, Schwalbach, Germany). Methanol and Hydrochloric acid 25% were purchased from Sigma and Riedel de Haën (Schnelldorf, Germany).

2.2 Plate pretreatment

Plate pre-treatment can be performed for a whole plate package in advance. HPTLC silica gel plates were washed by pre-development with methanol. The cleaned plate is dried (and activated) on the TLC Plate Heater III (CAMAG, Muttenz, Switzerland) at 120°C for 20 min. The last step is necessary to completely remove all traces of the washing solvent. In a desiccator, the active plate is cooled down to room temperature and equilibrated with the relative humidity of the laboratory atmosphere. For temporary storage, the pre-washed plate was wrapped in aluminium foil.

2.3 Standard solution

As stock solutions (1.1 mg/mL) of delphinidin, malvidin, cyanidin, peonidin, pelargonidin, chloride salts were dissolved individual in a mixture of 0.6 mL of hydrochloric acid concentrate 37% and 120 mL of methanol (1100 ng/µL). The 3-glucoside forms of delphinidin, malvidin, cyanidin, peonidin, (1.1 mg/10 mL), pelargonidin (4.3 mg/mL) and malvidin 3,5-diglicoside (1.6 mg/10 mL) chloride salts were dissolved individual in the same mixture. For application we prepared a mixture of this stock solutions (1000 µL): 20 µL of delphinidin chloride, 20 µL of malvidin chloride, 20 µL cyanidin chloride, 10 µL of peonidin chloride, 10 µL of pelargonidin chloride, 150 µL of malvidin 3,5-diglicoside chloride salt, 250 µL of delphinidin 3-glicoside chloride salt 250 µL of malvidin 3-glicoside chloride salt, 250 µL of cyanidin 3-glicoside chloride salt, 110 µL of peonidin 3-glicoside chloride salt and 10 µL of pelargonidin 3-glicoside chloride salt.

2.4 Origin of samples

Bilberry (*Vaccinium myrtillus*) and Blueberry (*Vaccinium cyanococcus*) extracts were originated from Europe and were purchased from BerryPharma (Leichligen, Germany) and Huisong Pharmaceuticals (Zhejiang, China) respectively.

2.5 Extraction of samples

The anthocyanins were extracted from frozen berries using ethanol or a 50% aqueous ethanol solution at 40-60 ºC without acid, during 12 h.

2.6 Application

The solutions were sprayed as band (8 mm) with the Automatic TLC Sampler 4 (ATS4, CAMAG) with 25 µL syringe allowing 13 tracks to be applied on one HPTLC plate of 20 × 10 cm (distance between bands 11.6 mm, distance from lower edge 8 mm, distance from the left side 30 mm). For four-point-calibration, volumes of 3, 5, 7.5, and 10 µL of the standard solution were applied on the plate (70 - 280 ng/band for standard-mix) over a calibration range of 1:4. From sample solutions were applied in volumes of 5 µL for cranberry extract, 6 µL for blueberry extract and for 7 µL bilberry extract each were sprayed-on as starting zone. A constant application rate of 150 nL/s was used. For routine analysis each sample was applied three times on the plate.

2.7 Thin layer chromatography

Chromatography was performed on 20 × 10 cm silica gel 60 F_{254} (Merck) and development was performed in the Automated Developing Chamber 2 (CAMAG) at a relative air humidity of 25 ± 2 % with a mixture of ethyl acetate, toluene, water and formic acid (12:3:0.8:1.2, *v/v/v/v*). For a standardized separation, the plate activity was controlled for 4 minutes using a saturated potassium acetate solution (257.6g/100g H_2O) filled in the humidity control unit. The migration distance was 70 mm from the lower plate edge and the migration time was 20 min. After the development the plate was dried in a stream of cold air followed for 3 min.

2.8 Documentation

Plate images were documented by TLC Visualizer Documentation System (CAMAG) consisting a powerful high-resolution 12 bit CCD digital camera with outstanding linearity. All images were captured with an exposure time of 30 ms under white light illumination in the transmission mode. Data obtained was processed with winCATS software, version 1.4.7.2018 (CAMAG)

2.9 Quantification by densitometry

Densitometric evaluation was performed by TLC Scanner 4 (CAMAG) via absorbance measurement (slit size 6 mm x 0.2 mm, scanning speed 20 mm/s) and integrated by peak height. The source of radiation was a D2&W (deuterium and halogen tungsten) lamp. The delphinidin absorbance was measured at 555 nm.

3. Results and discussion

3.1 Chromatographic method

Thin layer chromatographic method to be used for quantification of delphinidin should be able to resolve the presence of this compound in extract.

The anthocyanins were separated on the HPTLC plate silica gel 60 F_{254} (fluorescence indicator) prewashed with methanol and heated at 120 ºC for 20 minutes, for remove all traces of the washing solvent. For optimization, various combinations of mobile phases were used to obtain best resolution. Initially, mixtures of ethyl acetate, toluene, water and formic acid were investigated for their suitability as mobile phase. A ratio of 12:3:0.8:1.2 (*v/v/v/v*) separated best up to a migration distance of 70 mm the 3-glucoside form of delphinidin. With this mobile phase delphinidin, peonidin and pelargonidin were very good separated, but we couldn't separate cyanidin and malvidin 3-glucoside from the samples. The delphinidin compound is the violet spot from the samples. The hR_F (retention factor) of this compound are 33. We can see very easy that we have delphinidin in blueberry and bilberry samples (the presence of violet spot), but in cranberry samples this compound is not detected (the colour of the spot is reddish, and hR_F are a little bit higher). The separation is presented in Figure 2.

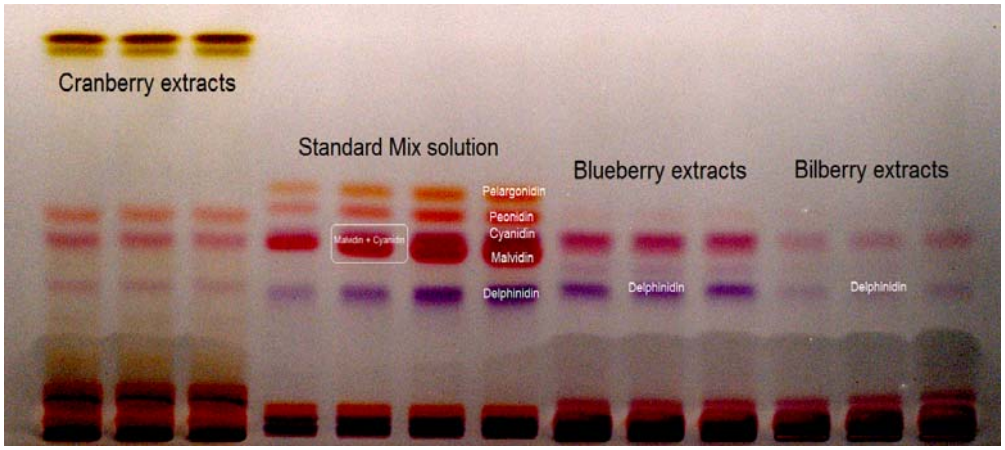

Fig. 2. Separation of anthocyanins from berry extracts - documentation under white light illumination in transmission mode

After chromatography, the HPTLC plate was dried in a stream of cold air for 3 min. Vis-spectra recorded confirmed the optimal measurement wavelength of 555 for delphinidin 3-glicoside. On one plate, the same sample was applied three times.

3.2 Sample preparation

One gram (1 g) from each sample of extract were weighed intro 10 ml dark volumetric flasks fitted with a ground-glass stopper and sonicated in water bath with 10 ml of methanol/hydrochloric acid 25 % (4:1) for 30 minutes at room temperature. The resultant solution was filtered through 0.45 μm pore-size

cellulose filters and stored at -20 ºC. The pH of the solution was around 1 and the pH of the standard solution was 2.

3.3 Quantification

After development with ethyl acetate, toluene, water, formic acid and absorbance measurement at 555 nm (Figure 3), quantification was performed by peak height evaluation.

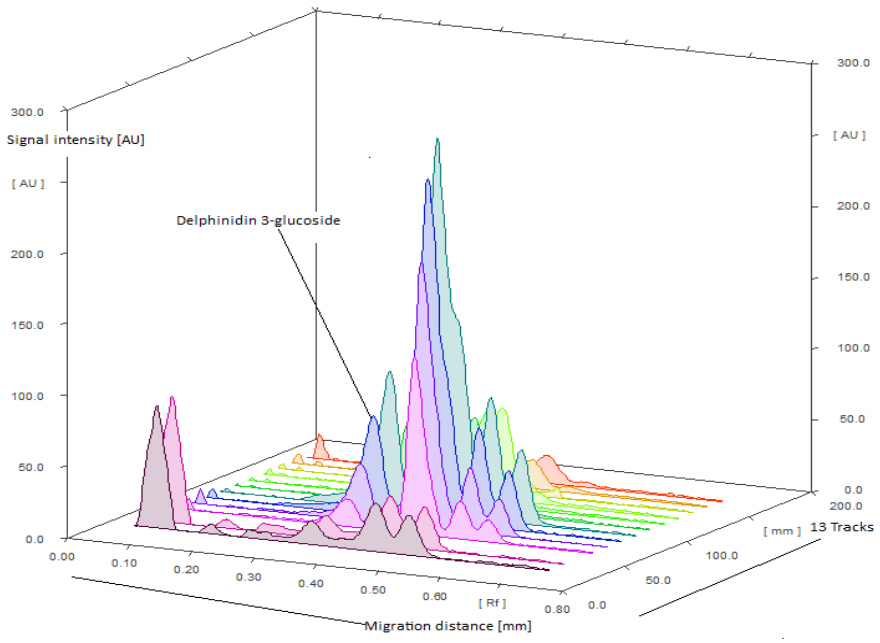

Fig.2. Substance windows of anthocyanins standards and samples of all 13 tracks on one plate (20 cm x 10 cm) - absorption scan at 555 nm.

For example, a 4-point calibration in the range from 70-280 ng/band of standard mixture was suited to analyze a wide range of anthocyanins finding in berry samples. Starting with the limit of quantification, the calibration curve for delphinidin (Figure 4) showed good performance characteristics, coefficient of correlation r = 0.99978, relative standard deviation *%RSD* = 1.6 %, by peak height, in linear regression (Figure 4).

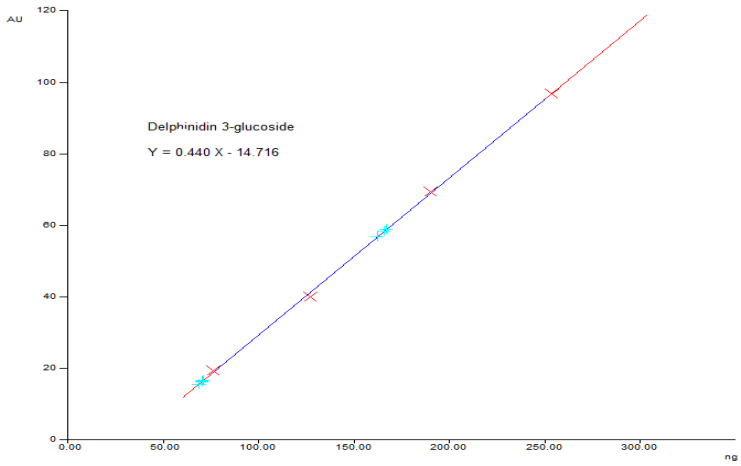

Fig. 4. Linear calibration, of delphinidin 3-glicoside via peak height by absorption at 555 nm with correlation coefficient of 0.99978

The performance data of the standardized HPTLC method for analysis of delphynidin in berry extracts is shown in tab 1.

Table 1

Performance data of HPTLC method for the determination of delphinidin in berry extracts

Samples	hR_F	V applied (µL)	Concentration of solution(g/mL)	Concentration of delphinidin(ng/band)	Concentration of delphinidin (%)
Cranberry extract	n.d.	-	-	-	-
Blueberry extract	33	6	0.005	166.19	0.55
Bilberry extract	33	7	0.0002	69.89	5

4. Conclusion

A standardized and precise HPTLC method has been shown for reliable quantitation of delphinidin in various berries. Use of silica gel HPTLC plates with ethyl acetate, toluene, water and formic acid 12:3:0.8:1.2 *(v/v/v/v)* as mobile phase enabled good separation of delphinidin. The correlation coefficients were ≥ 0.99978 and repeatability of the sample analysis (n=3) was ≤ 1.56 %. Statistical analysis proves that method is repeatable and selective for analysis. The rapidness, high throughput and cost-effectiveness of planar chromatography were advantageous and it was demonstrated that HPTLC is a fully compliant quantitative alternative to HPLC analysis.

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