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Application of high performance thin layer chromatography method for ophthalmological preparations containing anthocyanins fractions

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Abstract: The aim of the investigation was to elaborate a fast, easy, and cheap method of analysis of ophthalmological preparations containing anthocyanins fractions. A high performance thin layer chromatography (HPTLC) was applied; the chromatographic conditions were determined experimentally. Eight preparations with extracts of *Vaccinium myrtillus* L. and *Aronia melanocarpa* L. as reference material were analyzed. In the optimal chromatographic system, with methyl tert-butyl ether as the modifier of mobile phase, the separation of anthocyanins fractions was very good. The mobile phase enables separation of natural anthocyanins fractions from synthetic pigments (e.g., methylene blue).

Key words: HPTLC, densitometry, anthocyanins, ophtalmological preparations

INTRODUCTION

Medical preparations with anthocyanins extracts are frequently used in ophtalmological treatment. Recent clinical investigations conducted in the European Union have shown that Strix component containing anthocyanins helps in accommodation of the eye after changing the light, strengthens capillaries and improves blood circulation in the eye [1]. It stimulates the flow of nutritious components in the eye, feeds mucous membranes of the eye, and has anti-inflammatory activity. Additionally, it relives the symptoms of irritation and tiredness of the eyes and improves visual functions, as well as having antioxidant activity. *Vaccinium myrtillus* L., through stimulating the blood circulation, improves night vision.

It very often happens that in comparison to structurally identified medicinal substances, the biopharmaceutical quality of preparations with plant material is not properly characterized. Plant preparations are chemically complicated, very complex mixtures of compounds obtained from plants. It is not always known that only one substance is responsible for the therapeutic effect in the mixture of active compounds isolated from plants. The basic problem in the analysis of plant extracts is the separation of the compounds.

Separation of anthocyanins is a difficult problem in many aspects. Mobile phase composition ought to be optimized [2]. The mentioned plant material contains groups of anthocyanins which have very similar structures. They belong to the large group of phenolic compounds and have antioxidant activity [3]. They are analyzed by several methods such as: HPTLC [4], LC, LC-MS [5], capillary zone electrophoresis (CZE)[6], micellar electrokinetic chromatography (MEKC) [7]. The cost of these methods is very high because of complicated equipment. Very often, the main compound is accompanied by substances present in smaller amounts – even in trace amounts. The instability of these compounds must also be taken into consideration, and many factors may lead to decomposition.

In the process of creating a new medicine (after it registration) it is necessary to carry out quality investigations to demonstrate the repeatability of the production process for each series of preparation. Control tests also demonstrate whether storage conditions influence the quality of the medicine.

The objective of our research was to elaborate a cheap and fast method of anthocyanins control in ophthalmological preparations. Investigations were focused on the selection of the best chromatographic conditions (in HPTLC) for anthocyanins separation of *Vaccinum myrtillus* L. and *Aronia melanocarpa* L. anthocyanins.

METHODS AND MATERIALS

Solvents used in the study (analytical grade) were purchased from Merck (Darmstad, Germany). All ophthalmologic medicines (Table 1) were purchased from a public pharmacy in Lublin, Poland, and are generally available.

Aronia melanocarpa L. fruits were collected in Dys near Lublin, Poland, in 2005. *Vaccinium myrtillus* L. fruits were collected in the Motycz Leśny forest near Lublin.

The amounts of anthocyanins in the preparations: Strix, Bilberin, Klarin Activ, Pro-wzrok, Vizik, Vitavision and Aronox, are shown in Table 2. Three tablets of preparation were crushed in a porcelain mortar with a pestle. Tablet mass of each preparation was placed in a flask and extracted for 30 minutes with 10 mL of mixture of methanol with 25% hydrochloric acid (9:1 v/v) at 22°C. Subsequently, each mixture

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Table 1 Characteristic of ophthalmological preparations

Preparation of Vaccinum myrtillus L.	Package serial number	Producer
STRIX	148768	Ferrosan A/S, Sydmarken 5, 2860 Soeborg, Denmark
BILBERIN	010205	HASCO-LEK SA, Żmigrodzka 242E, 51-131 Wrocław
KLARIN ACTIV	0050704	FARMAPOL Sp.z o.o., Św.Wojciech 29, Poznań
PRO-WZROK	010405	HASCO-LEK SA, Żmigrodzka 242E 51-131 Wrocław
VIZIK	506015	NP Pharma SP. z o.o., Podstoczysko 30, 07-300 Ostrów Maz.
VITAVISION	MFG310105L C5A3060	WALMARK, a.s., Oldrichovice 44, 739 61 Trinec, Czech Republic
Preparation of *Aronia melanocarpa* L.	Package serial number	Producer
ARONOX	030502	AGROPHARM S.A., Starościańska 33, 95-080 Tuszyn

Table 2 Composition of anthocyanins preparations

Preparation of *Vaccinum Myrtillus* L.	Amount of *Vaccinum myrtillus* L. extract [mg]	Amount of dry essence [mg]	Amount of anthocyanins [mg]	Mass of tablet/ capsule [mg]
STRIX	178	82	12.36	450
BILBERIN	Not given	200	50 (25%)	Not
KLARIN ACTIV	250	40	Not given	370
PRO-WZROK	Not given	20	Not given	Not
VIZIK	150	40	Not given	540
VITAVISION	1000	10	Not given	840
Preparation of *Aronia melanocarpa* L.	Amount of *Aronia melanocarpa* L. extract	Amount of dry essence [mg]	Amount of anthocyanins [mg]	Mass of tablet
ARONOX	60% polyphenol compounds	Not given	102	850

was filtered. Collected filtrates were stored in a refrigerator at the temperature of 4°C. Samples for investigation were taken each time from these extracts.

Extracts of fresh fruits (5 g) were prepared using 5 mL of a mixture of methanol and 25% hydrochloric acid (9:1 v/v).

Juices from *Vaccinum myrtillus* L. (5 g) and *Aronia melanocarpa* L. (5 g) were prepared in quartz crucibles. Fresh berries were squeezed out , using a pestle. The juices obtained (10 mL) were stored in a freezer at the temperature of -15°C.

The optimal composition of eluent was determined experimentally after several preliminary experiments. High performance thin layer chromatography (HPTLC) was conducted using HPTLC silica gel plates (10×20 cm) (Merck, Germany). All chromatographic experiments were carried out in Teflon DS chambers with mobile phase reservoir/injector for gradient elution (Chromdes, Lublin, Poland) [8]. Extracts were applied useing a 20 µL Hamilton syringe on a plate with areas of 3-4 mm zones. 15 µL of each extract was applied. The distance of development was 8.5 cm. Five-step gradient elution of extracts was performed with use of a mobile phase composed

Table 3 Composition of mobile phases in gradient elution on HPTLC plates (MTBE – methyl tert-butyl ether used as a modifier)

Solution A			
3 mL toluene 5 mL acetonitril 1 mL water 1 mL formic acid 0.4 mL n-butanol 0.4 mL 2-propanol	Step of gradient elution	Composition of mobile phase	Distance of development
	1	10 mL A solution	1.5 cm
	2	10 mL A solution + 0.5 mL MTBE	1.5 cm
	3	10 mL A solution + 1.0 mL MTBE	1.5 cm
	4	10 mL A solution + 1.5 mL MTBE	1.5 cm
	5	10 mL A solution + 2.0 mL MTBE	1.5 cm

of: methyl tert-butyl ether, toluene, acetonitrile, water, formic acid, n-butanol and 2-propanol. Each step was developed at a distance of 1.5 cm, without drying the plate. The programme of gradient continuous elution is shown in Table 3.

Densitograms were obtained using Desaga CD-60 densitometer (Heidelberg, Germany) controlled by a Pentium computer with Windows Softwere ProQuant. Linear scans were obtained with slit dimensions of 0.1 mm×0.4 mm. Measurements were carried out at 470 nm.

RESULTS AND DISCUSSION

Stepwise gradient elution enables separation of complex samples containing fractions with a wide retention range [9, 10]. In anthocyanins analysis this must be carried out continuously and without drying the plate because of the instability of anthocyanins. Methyl tert-butyl ether was selected as the modifier. In each step of elution, the eluent strength increases [11].

During preparation of extracts, mechanical grinders were not used because of metal parts whose ions accelerate the decomposition of anthocyanins.

It was established for all 7 investigated ophthalmological medicines that the reference material (comparative) will be the extract (in methanol and hydrochloric acid, 9:1, v/v) of fresh berries of *Vaccinum myrtillus* L. and *Aronia melanocarpa* L. Comparison of densitograms of separated anthocyanins fractions from samples of medicines with samples of berries extracts are shown in Figures 3-9. As it can be seen in the chromatograms and densitograms that a close similarity was observed in the quality of the medicines: Srix (Fig. 1), Bilberin (Fig. 2) and Klarin Active with lutein (Fig. 3) to the samples of reference material. The number of separated anthocyanins fractions in the preparations was higher than in the reference material. From the height of peaks of separated anthocyanins fractions from ophtalmology medicines: Prowzrok (Fig. 4), Vizik (Fig. 5) and Vitavision (Fig. 6), it appears that in the second group of investigated preparations the amounts of anthocyanins were lower compared to those previously described. An additional peak was present in samples of the medicines Vizik and Vitavision – the peak resulted from an added synthetic pigment (methylene blue) [12]. The densitogram of *Aronia melanocarpa* L. anthocyanins fractions is presented in Fig. 7.

The result of our investigations was the elaboration of a new chromatographic system to control any falsifications of ophthalmological preparations. As shown in Figs. 5 and 6, the added pigment (peak No. 5b) had a high R_F (0,8) value.

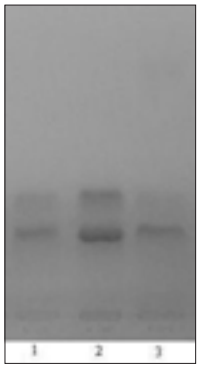

Figure 1 Documentation in form of photograph: HPTLC plate with *Aronia melanocarpa* L. fructus metanolic extracts. (1 – *Aronia melanocarpa* L. juice, 2 – *Aronia melanocarpa* L. fructus metanolic extract, 3 – Aronox).

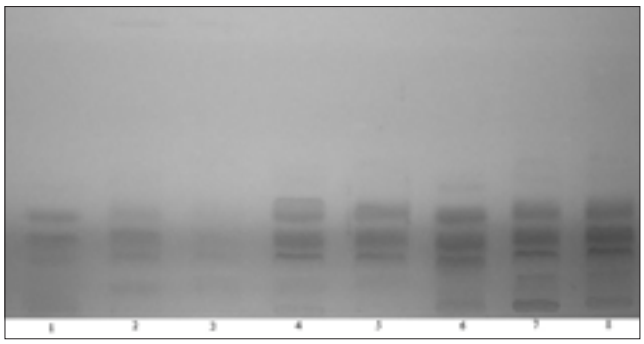

Figure 2 Documentation in form of photograph: HPTLC plate with preparations of *Vaccinium myrtillus* L. fructus extract. (1 – Pro-wzrok, 2 – Vitavision, 3 – Vizik, 4 – *Vaccinium myrtillus* L. juice, 5 – *Vaccinium myrtillus* L. fructus methanolic extract, 6 – Bilberin, 7 – Klarin Active with lutein, 8 – Strix).

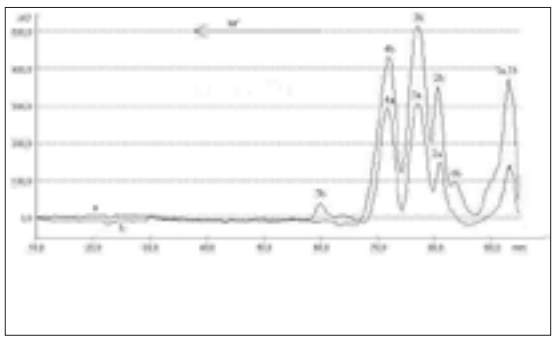

Figure 3 Densitograms of *Vaccinium myrtillus* L. fructus methanolic extract (a) and Strix preparation (b).

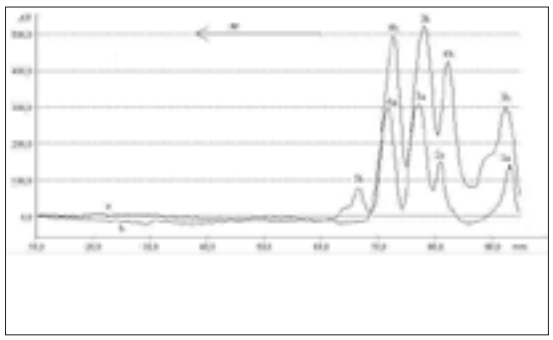

Figure 4 Densitograms of *Vaccinium myrtillus* L. fructus methanolic extract (a) and Bilberin preparation (b).

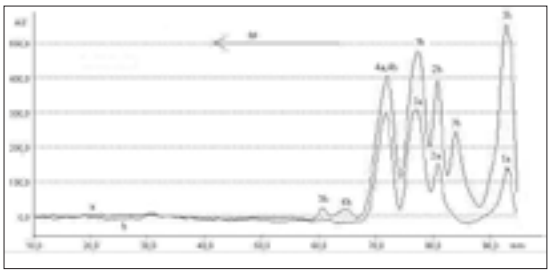

Figure 5 Densitograms of *Vaccinium myrtillus* L. fructus methanolic extract (a) and Klarin Active with lutein preparation (b).

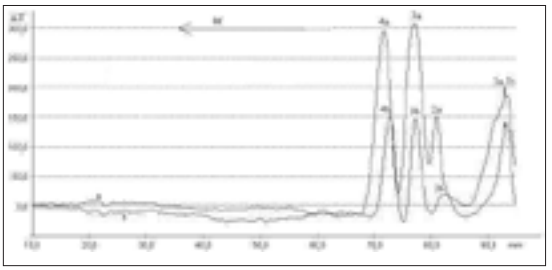

Figure 6 Densitograms of *Vaccinium myrtillus* L. fructus methanolic extract (a) and Pro-wzrok preparation (b).

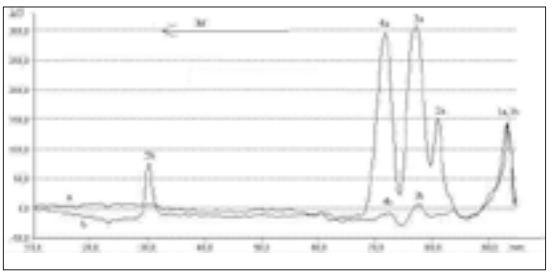

Figure 7 Densitograms of *Vaccinium myrtillus* L. fructus methanolic extract (a) and Vizik preparation (b). Peak No 5b is syntetic pigment.

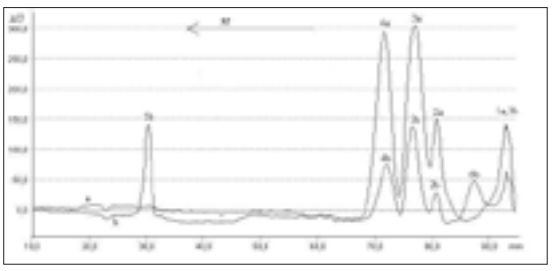

Figure 8 Densitograms of *Vaccinium myrtillus* L. fructus methanolic extract (a) and Vitavision preparation (b). Peak No 5b is syntetic pigment.

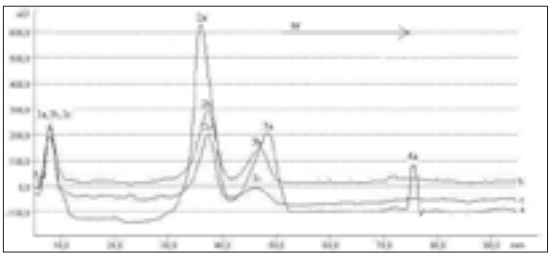

Figure 9 Densitograms of *Aronia melanocarpa* L. fructus methanolic extract (a), *Aronia melanocarpa* L. fructus juice (b) and Aronox preparation (c).

CONCLUSION

The instability of anthocyanins compounds structure under the influence of temperature, direct sunlight, and solvents is a main problem in analytical investigations of anthocyanins. In the method described in our study (HPTLC with multiple gradient continuous elution), satisfactory separation of anthocyanins fractions of plant materials and medical preparations with the mentioned plants was obtained. This method enables the detection of false pigments and the checking of the quality of medicine. The analyzed extracts and preparations retain the stability of the structure in the proposed chromatographic system. The mobile phase was used for the first time in anthocyanins fractions analysis. Well-separated zones, without tailing, were obtained.

Elaboration of this new method of separation of a natural mixture of anthocyanins enables the detection of synthetic pigments in plant preparations. This method can also be used in fast qualitative analysis of anthocyanins preparations.

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