



THE DEVELOPMENT OF LARGE-SCALE TECHNIQUE FOR EXTRACTION OF ANTHOCYANINS FROM ECOLOGICALLY PURE PLANT MATERIALS

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Abstract:

The present investigation is dedicated to the development of large scale technique for isolation of anthocyanins from plant materials of Carpatian region. The main interest have been concentrated on local plant materials - Aronia melanocarpa, Sambucus nigra, Vaccinum myrtillus, Vaccinum corumbosum and Vitis vinifera. Different states of source plant materials (fresh, frozen and freeze-dried) were used and different extraction systems (water-acetone, methanol-water and ethanol-water) were investigated for discovering of optimal conditions. The final purification have carried out by reversed phase C-18 column chromatography. The anthocyanins extraction efficiency can reached up to 90% of total amount in fresh plant materials.

The content and composition of anthocyanins in obtained extracts been examined by pH-differencial spectroscopy and HPLC. It was found that major anthocyanin in was Aronia melanocarpa is cyanidine-3-galactozide (over 60 % of all anthocyanins) and the major anthocyanin in Sambucus nigra is cyanidine-3-glucozide (over 40%).

The method is time-saving, low risk of anthocyanins destruction, high sample recovery and is suitable for large-scale preparation of anthocyanins for preparation of powder form by lyophilisation.

Keywords:

anthocyanins, large-scale extraction, isolation

INTRODUCTION

Anthocyanins belongs to an unique and most studied subgroup of phenolic secondary metabolites found in plants and represents one of many classes that fall under the flavonoid group, possessing a bi-phenolic structure. The core of the anthocyanin's structure is a flavylum cation, may be represented as a C₆-C₃-C₆ skeleton with a phenolic ring fused to a pyran with an additional phenolic ring connected to the second position of the pyran. The anthocyanins can be glycosylated and the glycosides can be acylated. This composition is a base of possible functional and structural variants [1]. Approximately 400 individual anthocyanins have been identified [2, 3]. The six anthocyanidins most often can be found in plants and are named pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin.

Anthocyanins are plays a significant role in the producing of bright red, blue and purple colors of berries and fruits. Recently and importantly, they have been the focus of significant beneficial health claims due to their antioxidant capacity.

The anthocyanins contents in some plants are described in many sources [1]. The most prominent are bilberries (300 - 698), black currant (130 - 476 mg/100 g f.w.), blackberries (82.5 - 325.9 mg/100 g f.w.), blueberry (61.8 - 299.6 mg/100 g f.w.), red cabbage (322 mg/100 g f.w.), red grape (30 - 750 mg/100 g f.w.) etc.

The methods for small scale anthocyanins extraction, purification and analysis have been refined. Since anthocyanins are not stable compounds in neutral or alkaline solutions, acidic aqueous solvents have been used as extraction solvents in order to destroy cell membranes and dissolve the water-soluble pigments. Usually, HCl (1 %, 0.1 % and even 0.01 %) are chosen for acidulating the extraction solvent for small scale extraction [4].

The extraction process consists of number of steps which has to be optimized according to starting plant material, scale and expected final product. It means, that small scale extraction (for analytical project – qualitative and quantitative composition) has be done with different way as large scale extraction of anthocyanins for pharmacy or food product. The difference will be in using of different scale equipment, different price and toxicity reagents.

The focus of this work was to develop a set of techniques to extract anthocyanins from plant material and identify them.

EXPERIMENTAL

Chemicals

Ethanol, acetone, hydrochloric, oxalic, citric or siccine acids as well as adsorbents (Amberlite XAD-7, Talcum and C₁₈) were purchased from Sigma. All used chemicals were analytical grade.

Fruit Source

Fresh plant materials *Vaccinium corymbosum* L., *Vaccinium myrtillus* L., *Sambucus nigra* L., *Aronia melanocarpa* Wild. and *Vitis vitifera* L. were obtained from local farms in Eastern Slovakia. Samples were stored at temperature of -20 °C until use.

Extraction

Ethanol extraction. Freeze plant materials were blended in home blender. Samples with weight of 1000 g plant material was mixed with double (weight to volume) volume of 20-96 % (volume to volume) ethanol-water solution acidified by 0-5 % of hydrochloric, oxalic, citric or siccine acids for 0.5-1.5 hour extraction with continuous mixing. Extracts were separated from plant material by filtration through a filter paper with vacuum suction using a Buchner funnel and water-flow pump. Plant material was mixed with fresh ethanol-water solution two more times for maximal extraction of anthocyanins. Filtrates were moved to boiling flask and ethanol was removed by rotary evaporator. Purification was carried out by mixing of 30-50 g of solid adsorbents (Amberlite XAD-7, Talcum and C₁₈) which was activated with double volumes of ethanol and than with three volumes of acidified deionized distilled. Filtrate was mixed with 50 g of adsorbent and separated from filtrate by filtration through a filter paper. Adsorbent was flashed by two volumes of acidified water with 1% citric acid (to remove water soluble compounds – colorants, sugars, organic acids etc.) and that with two volumes of ethylacetate (to remove polyphenols). Elute anthocyanin pigment was removed from solid adsorbent by extraction with acidified ethanol-water solution. Ethanol was removed by vacuum evaporation at 38°C.

Acetone extraction. 1000 g of material was macerated with same amount (weight to volume) of acetone. Filtrate was separated by vacuum suction using filter paper and Buchner funnel. Extraction of anthocyanins was carried by maceration of plant material with 70% (v/v) aqueous acetone (70 % of acetone and 30 % of 1% citric acid water solution) for 30 min. Filtrate was separated by filter paper and Buchner funnel and moved to a separatory funnel and mixed with double volume of chloroform,

mixed by turning funnel upside down a few times. Solution was stored overnight at 4°C. Aqueous phase (upper portion) was separated by boiling flask. Admixtures of acetone and chloroform were removed by rotary evaporation in vacuum at 38 °C.

Measurement of Total Anthocyanins.

Total anthocyanins were determined by the pH differential method [5]. Two dilutions of the sample, one with potassium chloride buffer, pH 1.0 and the other with sodium acetate buffer, pH 4.5, were prepared and equilibrated for 15 min. Absorbance of each dilution was measured at 700 nm (to correct for haze), against a blank cell filled with distilled water and 520 nm. The absorbance of the diluted sample (A) as follows was calculated: $A = (A_{520} - A_{700})_{pH1.0} - (A_{520} - A_{700})_{pH4.5}$. The monomeric anthocyanin pigment concentration calculate in the original sample using the following formula:

$$\text{Monomeric anthocyanin pigment (mg/liter)} = (A \cdot MW \cdot DF \cdot 1000)/(e \cdot l);$$

where *MW* is the molecular weight; *DF* is the dilution factor, and *e* is the molar absorptivity. Pathlength was 1 cm.

HPLC analysis

Final extracts were diluted in methanol acidified by formic acid (CH₃OH : acetic acid = 99 : 1) in ration 1:9 and analyzed directly on HPLC-DAD system (Dionex UltiMate 3000). Mobile phase A was water : formic acid 9:1, mobile phase B – acetonytril : water : formic acid = 5:4:1. Detection wavelength $\lambda = 520$ nm. Content of anthocyanins was determined by using of standards from Sigma.

RESULTS AND DISSCUSION

Preparation of pure anthocyanins extract involves extraction with weakly acidified alcohol/acetone solvents, followed by concentration under vacuum, purification, and separation of the pigments. The recovery procedure must avoiding any chemical modification. For instance, acylated anthocyanins are degrading in mineral acids (hydrochloric or sulfuric acids) solvents and organic acids (e.g., acetic or formic acid) are preferable. Also, anthocyanins are heat-sensitive; thus, as low as possible temperatures (below 40 °C) during extraction and concentration are recommended.

It is known, that anthocyanins are very sensitive to external effects like pH, temperature, light and chemical environment. Its instability at high temperature requests to store them at temperatures below zero degrees (not longer as 48 hours at temperature lower as +4 °C) and storage in longer time requests the temperatures below -18 °C. We have stored the fresh plant material as well as all products at -20 °C.

The most used extraction technique for isolation of anthocyanins consists of following steps:

1. extraction from fresh, frozen or dry plant material by water-alcohol/acetone solvent system during 0.5-12 hours;
2. remove of organic part of solvent system (usually, by vacuum rotary evaporator);
3. purification by solid adsorbent (C₁₈, Amberlite, Talcum etc.);
4. dissolving of anthocyanins from solid adsorbent;
5. and remove of organic part of solvent system (usually, by vacuum rotary evaporator) again.

The polar character of anthocyanins makes them soluble in different polar solvents such as methanol, ethanol, acetone and water. Solvent extraction of anthocyanins is the initial step in the isolation of anthocyanins prior to quantification, purification, separation, and characterization and generally involves the use of acidified methanol, ethanol or acetone. Even though ethanol is less efficient and more difficult to eliminate later, it would be preferred for food use, because methanol and acetone/chloroform are toxic. However, solvent acidified with hydrochloric acid may hydrolyze

acylated anthocyanins, which explains why it has been overlooked in the past that many anthocyanins are acylated with aliphatic acids. To avoid or at least minimize the breakdown of acylated anthocyanins, organic acids such as acetic, citric, or tartaric acids, which are easier to eliminate during anthocyanin concentration have been preferred.

In order to find the most efficient extraction system, fruits were subjected to varying multiple extraction solvents, along with varying the types and amounts of acid used in the extraction solvent, time of extraction. The combination of this polar and hydrophobic nature makes aqueous/organic solvent mixtures the ideal solvent. Typically, the organic solvent content varies from 50% to 100% of the mixture.

We have studied the extraction efficiency for ethanol and acetone solvent. The amounts of total anthocyanins in obtained extract are shown on Figure 1. The presented values were calculated to weight of dry berries and for one cycle extraction.

It was found, the lower extraction efficiency for acetone-water solvent system into comparison to acetone-water. However, the time of processing at relatively high (room) temperature by acetone extraction experiment in 3-4 times shorter by ethanol extraction. High temperature necessarily will lead to degradation of anthocyanins.

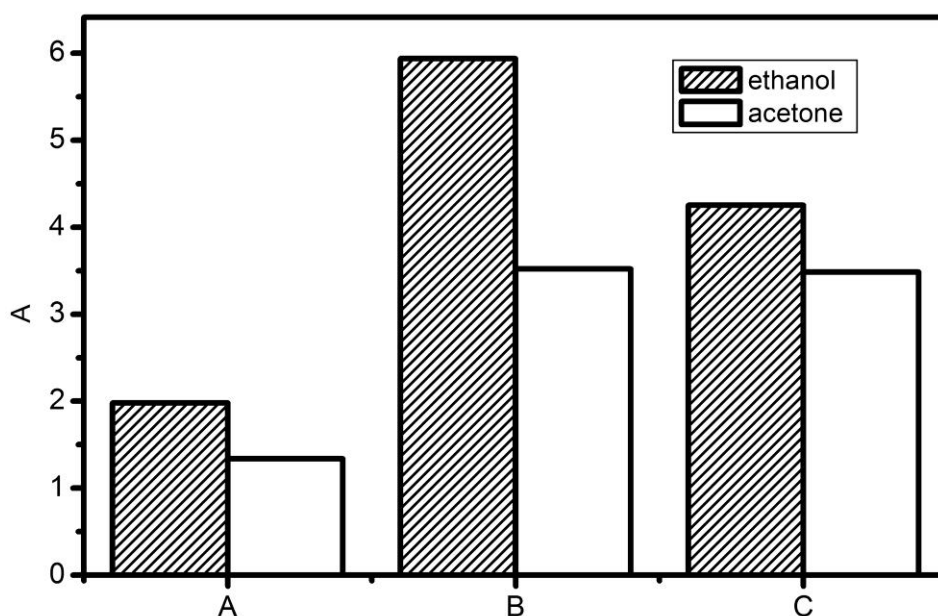


Figure 1. Percentage of total anthocyanins extracted from freeze dried fruits by different solvent systems: black – 70% ethanol-water acidified with 1% of citric acid; patterned – 70% acetone-water acidified with 1% of citric acid.

It is also known, that the extraction efficiency of methanol was 23% more efficient than ethanol and 73% more efficient than water. [R.P. Metivier et al. J. Food Science. 1980, 45:1099-1100.]. This solvent has not a highest extraction efficiency but fits to aim of current project – obtaining of pharmaceutically and/or food grade of quality. However, in this study we were concentrated on generally accepted to be safe ethanol. It is why we have tried to avoid contacts with toxic compounds.



We have analyzed the optimal ethanol-water ratio for maximal extraction efficiency from *Vaccinium myrtillus* L.. The efficiency of anthocyanins extraction was displayed with value A (absorbance) which is a difference between absorbance at 520 nm (proportional total anthocyanins and other colorants) and absorbance at 700 nm (non-anthocyanin's colorants) Figure 2.

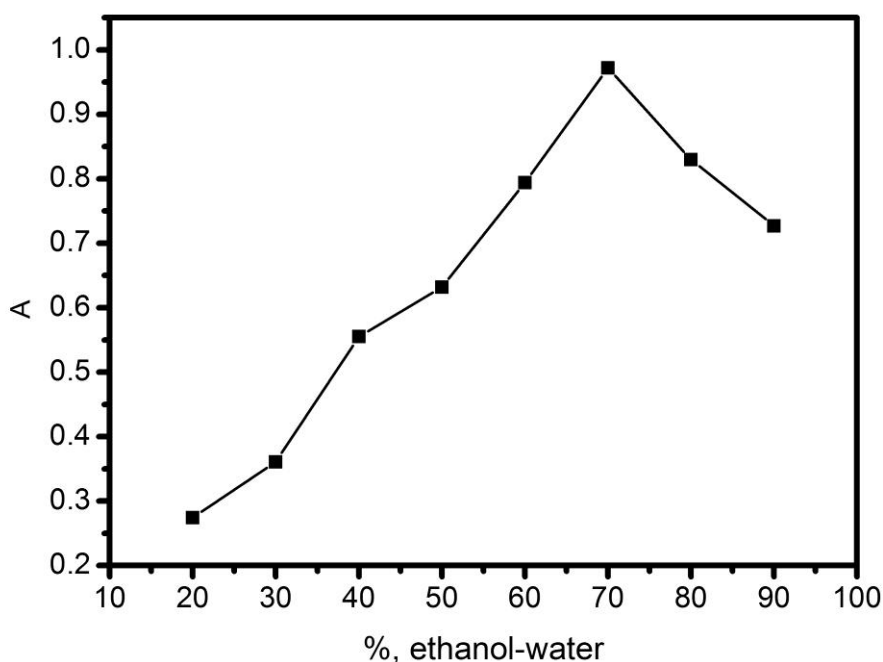


Figure 2. Dependence of absorbance A from concentration of ethanol-water (average of three experiments).

From this picture one can see, that optimal solvent system for extraction of anthocyanins observed to be 70 % ethanol and 30 % water (volume to volume). This solvent system was used for other plants.

The flavylium cation is stable at high solution acidity (low pH) when using acidified extraction solvents. However, the instability of anthocyanins, especially with pendant acyl groups, should be considered when mineral acids are used [6]. Therefore, very low amounts of weak acids may be justifiable in stabilizing the anthocyanin compound in solution without degradation. This parameter we have investigated for searching of an optimal acidification of solvent system. According to the above-mentioned, we have considered organic acids as acidifying agents. Figure 3 shows the efficiency of using of different non-volatile organic acids (5 % weight) in comparison with 1 % HCl. The concentration of hydrochloric acid was five times lower because of its high dissociation coefficient and relatively few acid is necessary to produce low pH.

From Fig. 3, we can see that the most efficient extraction of anthocyanins can be done by using oxalic acid. However, high intake of oxalic acid to the human body can support formation of kidney stones. It is why we choose citric acid for acidification of the solvent system. We have examined the 70 % ethanol solution with concentrations of citric acid in 0.1; 0.5, 1, 2 and 5 %. It was found that extraction efficiency reaches its maximum already at 1 % and does not change at higher concentrations of citric acid.

Next experiment we carried out to find an optimal extraction time. In solid-liquid extraction, the concentration of each compound reaches equilibrium between the solid and the solvent. This equilibrium can be further characterized by a compound's diffusivity, solubility and mass transfer. Optimization of the solid-liquid extraction can be done through manipulation of these attributes.

Natural products have to be grinded to reduce the particle size and allows the solute to reach the solvent quicker, increasing the diffusivity and improves the homogeneity of the sample.

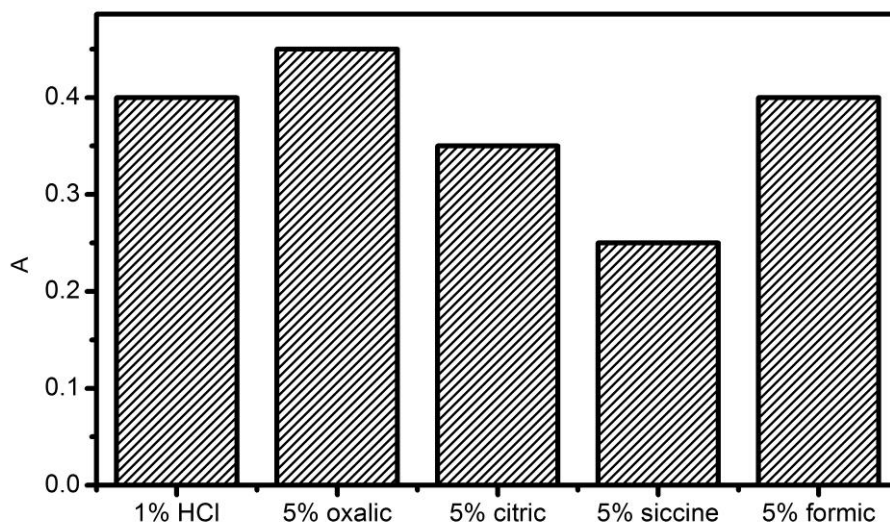


Figure 3. Extraction efficiency (by absorbance A) for different organic acids (average of three experiments).

In the same time, we want to know the minimal time of extraction to minimize the time of exposition at high temperatures and minimize the degradation of anthocyanins. We have carried out the extraction experiments at the same conditions but different time of extraction. Results are presented of Figure 3.

From Fig. 3, we can see that equilibrium between solid and liquid phases comes already after 20 min for within 70 % ethanol-water 1% citric acid. To be on safe side (difference in grinding, plant material etc.), we declare that 30 min is optimal time for extraction experiment.

Sonication of the solid-liquid extraction reported to improve on the extraction efficiency. We have carried out the series of extraction experiments and have observed that ultrasound assistance have not shown significant increase of the extraction efficiency by using the frozen plant material. .

The next experiments were carried out to find out the number of extractions which has to be done with same plant sample to extract the main part of anthocyanins. Total amount of anthocyanins was determined by pH-differential spectrophotometry. We have observed that first extraction of anthocyanins from *Vaccinium myrtillus* L. gives 0.31 % of the fresh plant weight. The second extraction gives other 0.14 % and the third extraction give other 0.07 % of fresh plant weight. Solution after forth extractions have contained a trace amounts of anthocyanins. Totally, after three extractions we have obtained anthocyanins in amount of near 0.52 % of fresh plant weight (expected 0.300-0.698 % wt.).

Extraction experiments at optimal conditions were carried for following plant materials: *Vaccinium myrtillus* L., *Vaccinium corymbosum* L., *Sambucus nigra* L., *Aronia melanocarpa* Wild. The amounts of obtained anthocyanins presented in Table 1.

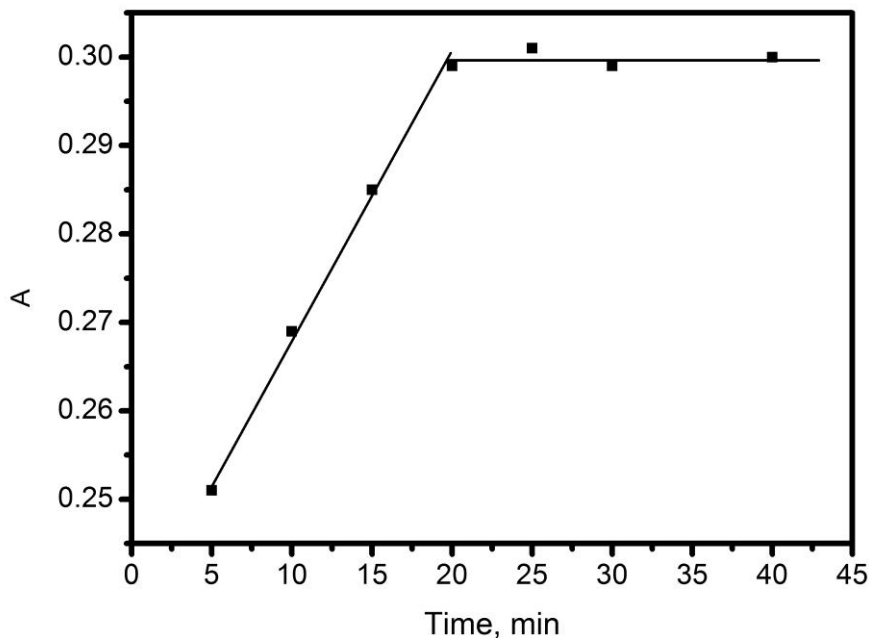


Figure 3. Dependence of absorbance A from extraction time within 70 % ethanol-water 1% citric acid (average of three experiments).

Table 1. Amount of total anthcyanins observed in plant materials by three cycle extraction with 70 % ethanol-water 1 % citric acid solvent.

Weght % to fresh plant material	<i>Vaccinium myrtillus</i> L.	<i>Vaccinium corymbosum</i> L.	<i>Sambucus nigra</i> L.	<i>Aronia melanocarpa</i> Wild.
Literature [1]	0.300 – 0.698		0.664 – 1.816	0.869
Experimental	0.52	0.62	0.98	0,65

It was found that major anthocyanin in was Aronia melanocarpa is cyanidine-3-galactozide (over 60 % of total anthocyanins) and the major anthocyanin in Sambucus nigra is cyanidine-3-glucozide (over 40%) (Table 2).

Table 2. Anthocyanins content in extracts.

Plant material	Anthocyanin	X, mg/ml (HPLC-DAD)	SX	Total anthocyanins, mg/ml (pH-differ. photometry)
Aronia melanocarpa	cyanidin-3-galactozide	3.44	0.34	7.77
	cyanidin-3-glucozide	0.34	0.04	
Sambucus nigra	cyanidin-3,5-diglucozide	0.47	0.03	13.69
	cyanidin-3-glucozide	5.76	0.44	

Natural products contain numerous other matrix components, such as sugars, phenols, organic acids, proteins, salts and other flavonoids that will be extracted along with anthocyanins during solid-liquid extraction. Additionally, liquid-liquid extraction and solid phase extraction can be used to further purify a solution containing anthocyanins. For solid phase extraction, due to the polar and hydrophobic functionality of anthocyanins, a number of different column chemistry packing can be used. Many studies use C₁₈, but others have used alumina, insoluble polyvinylpyrrolidone (PVP), Sephadex, Toyopearl, polyamide and ion-exchange resins [7].

Using of C₁₈ reported to be optimal but this adsorbent is very expensive. It is acceptable to use C₁₈ cartridge for small amounts of anthocyanins (few mg). However, for large scale extraction, we have to find more accessible adsorbent. In this study, we have used adsorbent Amberlite XAD-7 which was reported as optimal in several studies. The purification by solid phase adsorbent observed to be most complicate step in the obtaining of pure anthocyanins. We propose to solve the problem by search of alternative adsorbents.

From point of view the purity of final product, the use of acetone-water system seems to be optimal. The point is that solubility of secondary compounds except anthocyanins (polyphenoles, sugars, water soluble colorants) is much lower in acetone-water in comparison to alcohol-water systems. However, this technique requests the use the cancirogenic chloroform for remove an acetone. This can be improved by search the alternative compound for extraction of acetone (for instance, n-hexane, cyclohexane etc.).



Finally, we have carried out the optimization of most significant parameters for extraction and purification of anthocyanins for their isolation from plant material accessible in Eastern Slovakia.

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