RECOVERY OF ANTHOCYANINS FROM GRAPE POMACE EXTRACT (PINOT NOIR) USING MAGNETIC PARTICLES BASED ON POLY(VINYL ALCOHOL)

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The paper is devoted to the possibility of separating a concentrate of anthocyanin pigments from an aqueous extract (0.1% HCl) of grape pomace (Pinot noir) with the use of magnetic particles based on poly(vinyl alcohol) (M-PVA) having an average size of 1-3 microns. Due to their magnetic properties, these biologically inert, hydrophilic particles provide the possibility of fast and efficient separation from crude suspensions using an easily scalable magnet technology. The application of magnetic separation technology allows carrying out the process of isolating anthocyanins within 25 minutes. The sorption capacity of M-PVA is 4-5 times higher compared to other non-ionic sorbents (Amberlite XAD 16, Stirosorb MCDS×100), widely used for the extraction of polyphenolic compounds. In the developed process, the M-PVA particles adsorb up to 40-60% of the anthocyanins present in the initial solution. Afterwards, ethanol containing 0.1% HCl extracts 85% of the absorbed anthocyanins resulting in a purified and threefold concentrated solution if compared to the original extract. HPLC indicated that the pigment composition of the concentrate is the same as in the original extract. The efficiency of the magnetic particles does not decrease by the adsorption in turbid media and reuse is possible without special treatment.

Keywords: anthocyanins, magnetic particles, M-PVA, magnetic separation, concentration

INTRODUCTION

In recent years, the interest in using anthocyanins as a natural alternative to synthetic food colorings is constantly increasing. Anthocyanins account for the red, purple and blue colors of most fruits and vegetables. In addition to their coloring efficiency, increasing evidence suggests that anthocyanins are not only non-toxic and mutagenic, but also have a wide range of therapeutic properties. In this regard, new and effective separation methods of anthocyanins are of great interest to both food and pharmaceutical industries.

In terms of chemical structure, anthocyanins are glycosides of six anthocyanidins: pelargonidin (Pg), cyanidin (Cy), peonidin (Pn), delphinidin (Df), petunidin (Pt) and malvidin (Md), differing in number and position of CH_3O- and OH- groups in ring B (Fig. 1).[1] The molecules of the carbohydrate residue (-gluc) may be different, but the most common are glucose, rhamnose, galactose or arabinose. The carbohydrate part of the molecule can be represented as mono- and disaccharides, as well as acylated phenolic or aliphatic acids.

Because of their high reactivity, these pigments can be easily destroyed by harsh extraction conditions. The stability of anthocyanins depends on molecular structure, pH, solvent nature, temperature and the presence of ascorbic acid, sugars, metal ions and pigments in solution.[2,3] Figure 2 shows the balance between the five forms of cyanidin-3-glucoside with different color depending on the pH of the aqueous solution.[4] The intensive red color of anthocyanins is characteristic only in strongly acidic medium. In solution at a pH above 3.5, most of the pigments exist in colorless form. These physico-chemical properties of anthocyanins were first described in literature by G. M. Robinson and R. A. Robinson.[5] Under

natural conditions, at physiological pH, the color intensity of anthocyanins is not reduced as a result of intra- and intermolecular copigmentation.[6,7,8]

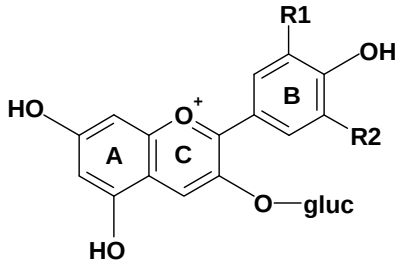

Figure 1: Structural formula of anthocyanin-3-glycosides in the form of a flavylium cation
(Pg: R_1, R_2 –H; Cy: R_1 –OH, R_2 – H; Pn: R_1 –OCH_3, R_2-H; Df: R_1 –OH, R_2 –OH; Pt: R_1-OH, R_2 -OCH_3)

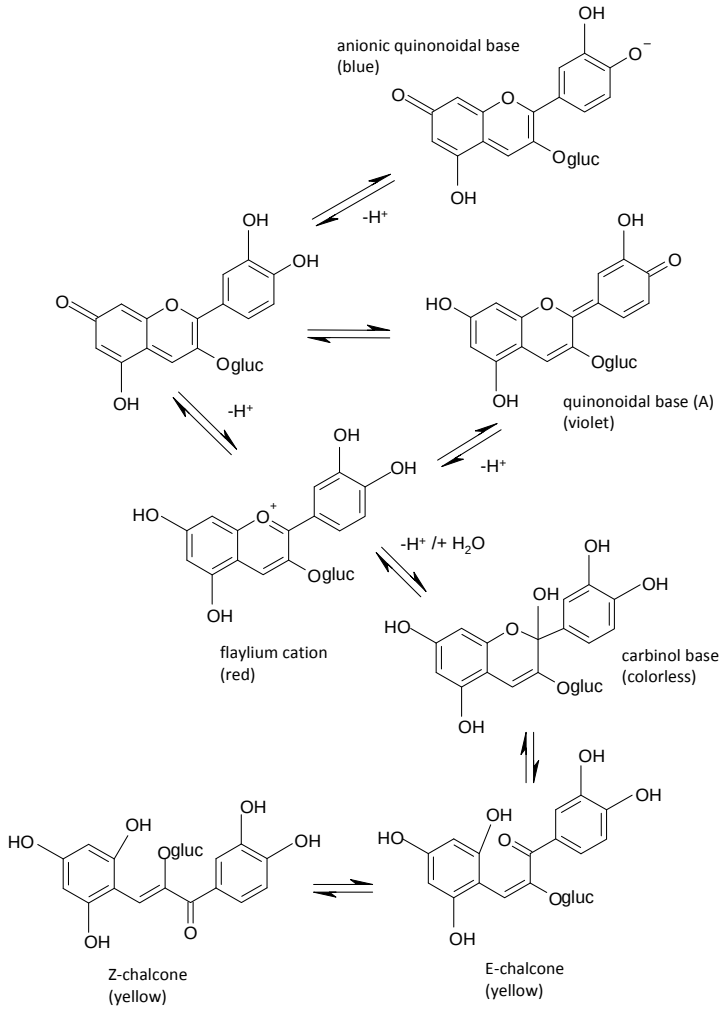

Figure 2: Equilibrium of forms with differently colored cyanidin-3-O-glycoside in aqueous solution as a function of pH

The list of natural sources of anthocyanins is extensive and includes most of the colored cultivated vegetables and fruits. From an economic point of view grape pomace is an attractive raw material, which remains as waste from the manufacture of wine in quantities of up to 10 million tons per year.[8] Grape pomace can be used not only as a soil conditioner or for obtaining

fertilizers, but also as a raw material for the recovery of numerous high-value compounds, such as grape seed oil, hydrocolloid and dietary fiber, ethanol, polyphenolic acids, flavonoids and anthocyanins.[9] Hardly surprising, the first anthocyanin extract, isolated from red grape pomace and used in the food industry – enotsianin – has been produced in Italy since 1879.[10] Nowadays, grape extract is often used for coloring pastry, dairy products, ice cream and other products.

The conventional method of obtaining the pigment from plant material is extraction. As anthocyanins are unstable in a neutral medium, acidified aqueous solutions, typically HCl (<1%), are usually used for extraction. This allows destroying the cell membrane and simultaneously extracts water-soluble pigments in the most stable form of flavylium cations (Fig. 2).[11] Nevertheless, it is possible to extract anthocyanins with other solvents containing hydrochloric acid: ethanol, methanol, n-butanol, cooled acetone, propylene glycol, and more complex systems, such as methanol/acetone/water and others.[12]

After extraction, a further concentration of the anthocyanins is needed. This can be carried out by several methods, each with its own advantages and disadvantages. Thermal concentration of selected extracts leads to loss of color due to hydrolysis and condensation reactions. An alternative is the use of selective adsorbents of different nature, which allows for the concentration and purification of biologically active compounds that are sensitive to temperature.[13] The criteria for the successful choice of sorbents do not refer only to the selectivity to the extract components, but also to the speed of sorption-desorption processes, ease of regeneration of the sorbent, and simplicity of the sorption process. That is why the development of new principles of separation of biologically active substances can lead to substantial cost savings of extractable products. This work is devoted to the process of anthocyanin concentration from aqueous solutions by the use of M-PVA adsorbent, which has magnetic properties and an average particle size of 1-3 microns. Its magnetic properties allow the manipulation of the particles by the application of an external magnetic field, which creates the conditions for a simple and easy scale-up of the concentration process. The main constituent of the particles, polyvinyl alcohol, makes their use possible for the sorption of organic compounds of different classes.[14]

EXPERIMENTAL

Materials

All reagents and solvents used were purchased from Merck (Germany) and were of analytical or high-performance liquid chromatography (HPLC) grades. Amberlite XAD 16 (Pour l'usage experimental seulement, Lot №2 7958, Rohm and Haas France S.A.), Stirosorb MCDS×100 ("A. N. Nesmeyanov" Institute of Organoelement Compounds, Russia), and M-PVA (Chemagen Biopolymer Technologie AG, Germany) were used for the adsorptive concentration of anthocyanins. Malvidin-3-O-ß-glucopyranoside (1601-1 HPLC) was used for identification with HPLC-diode-array detection (DAD).

Grape pomace (cultivar Pinot Noir, vintage 2010) originating from high-temperature short-time treatment of the red grape mash and subsequent juice release using a hose press was obtained from winegrowers from the Pfalz region, Germany. Grape skins were manually isolated from the pomace samples using a sieve. Then they were milled in a grinder (Waring HP3.75, U.S.A.). The raw material prepared in this way was kept at -35 °C until extraction.

Preparation of grape pomace extracts

For extraction, the raw material was mixed with acidified water (0.1% HCl) (2.5:1, w/w). The resulting mass was heated in a water bath at 50 °C for 2.5 hours, under constant stirring. After cooling to 18-20 °C, the extract was separated from the raw material through sieves to give a cloudy extract containing particles of plant material. A clear extract was obtained by centrifugation (Rotina 420R, Hettich, 20 min, 20 °C, spin at 4000 rpm) and filtration of the initial extract.

Concentration of grape pomace anthocyanins

In flacon tubes a fixed volume of sorbent suspension (M-PVA, 50 mg/mL) was prepared and the magnetic particles were separated from the liquid phase using a magnet and a micropipette. Afterwards, the sorbent was immersed in acidified water for 2 minutes before removing the supernatant from the sorbent. The extract of anthocyanins was added to the equilibrated sorbent and stirred (Termomixer comfort 5, Eppendor, spin = 1400 rpm) for 10 min. Then the supernatant was removed as described above. Before desorption, the sorbent was washed a few times with acidified water until the supernatant stayed uncolored. Then a fixed volume of 96% ethanol containing 0.1% HCl was added and stirred for 10 minutes. The resulting solution was separated from the magnetic particles.

Comparison of the efficiency of M-PVA magnetic particles adsorption with Stirosorb MCDS×100 and Amberlite XAD 16

Stirosorb MCDS×100 was immersed in acetone for 8 h, afterwards it was rinsed with water to complete the removal of the solvent. Before usage, Amberlite XAD 16 was immersed in 96% ethanol for 2 h, followed by washing with distilled water. To 10 mg ready-to-use sorbents, 500 µL anthocyanin extract was added. Adsorption carried out with intensive stirring for 10 minutes.

HPLC analysis

The crude pomace extracts were membrane-filtered (0.45 µm, regenerated cellulose) before HPLC analysis. HPLC analysis was conducted on a Hewlett Packard series 1100 with a DAD detector G 1315 A. The separation of anthocyanins was accomplished on a MZ-analytical Hyersil ODS column (3 µm, 100×4 mm). The direct analysis, mobile phases were B, acetonitrile and D, 1% H_3PO_4 in water. The gradient conditions were as follows: 0-20 min, 5% B; 20-27 min, 25% B; 27-45 min, 5% B. Other chromatographic conditions were the following: flow rate, 0.7 mL/min; column temperature, 45 °C; detection, 525 nm. The injection volume for all samples was of 40 µL. The total run time was 45 min.

Total anthocyanin content

The total anthocyanin content was determined using the pH differential method.[15] A UV/Vis spectrophotometer (Agilent) and 1 cm path length disposable cells were used for spectral measurements at 520 and 700 nm. Pigment content was calculated as malvidin-3-glucoside (Md-3-gluc), using a molar absorptivity (ε) of 28000 and molecular weight of 493.4. All determinations were performed in duplicate.

RESULTS AND DISCUSSION

The extraction of anthocyanins from grape pomace (Pinot noir) by 0.1% aqueous solution of hydrochloric acid resulted in a solution with a concentration of 12-16 mg/L pigments and pH 3. Under these conditions, anthocyanins are predominantly present as carbinol, an unstable form. It is known that high concentrations of pigments lead to its stabilization due to processes of self-association and co-pigmentation, even in an alkaline medium.[16]

The concentration and purification of anthocyanins can be carried out by the adsorption method.[17,18] At the beginning of our research, we examined the adsorption process of pigments from an aqueous solution by M-PVA magnetic particles. The M-PVA magnetic particles with sizes of 1-3 microns, used in the experiment, are ready-to-use, while the preparation of traditional sorbents is a time-consuming procedure taking 2-10 hours. Usually, the equilibrium in the sorbent-sorbate system establishes very slowly, from 3 to 6 hours. Figure 3 illustrates that the adsorption equilibrium of anthocyanins onto M-PVA particles occurred in less than 5 minutes. Thus, the first stage of concentration was reduced by 30-70 times. The reason for this is the highly specific surface of the microparticles, in comparison with that of commercial sorbents. The reaction kinetics can be described by a quasi-homogeneous solution model.

The data in Table 1 represent the comparative characteristics of the adsorption process for 10 minutes on M-PVA magnetic particles and sorbents Stirosorb MCDS×100 and Amberlite XAD 16. At pH 3, 4-5 times more pigments adsorbed onto the M-PVA particles than on macroporous sorbents. It should be noted that the adsorption of anthocyanin pigments on sorbents Stirosorb MCDS×100 and Amberlite XAD 16 occurred with equal efficiency.

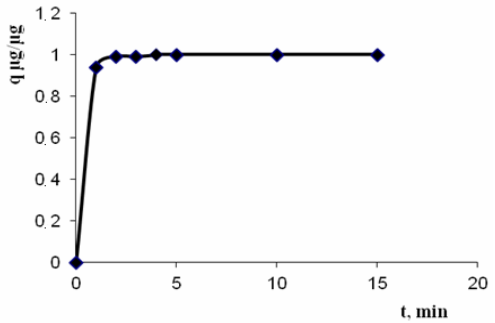

Figure 3: The kinetic curve of adsorption of anthocyanin pigments from aqueous extract onto M-PVA (pH = 3, V_1An = 0.5 mL, C_s = 20 mg/mL)

The repeated use of traditional sorbents (1-3 cycles) significantly reduced their sorption capacity. Consequently, the problem of sorbent regeneration arises, making the use of adsorption methods more complex on an industrial scale. Table 2 shows that in the case of M-PVA no decrease in sorption efficiency is observed over seven cycles.

From an aqueous extract, a sorbent concentration of 10 mg/mL adsorbs up to 40% anthocyanin pigments. One of the possible adsorption mechanisms of anthocyanin pigments onto the M-PVA is the formation of hydrogen bonds between the OH-groups on the sorbent surface and the OH-groups of the adsorbed molecules. A twofold increase the concentration of M-PVA pushes the adsorption yield up to 60% (Table 1). An increase in the adsorption capacity of the particles can be achieved not only by raising the sorbent concentration, but also by the chemical modification of the hydroxyl groups of the polymer matrix. This can be done by the introduction of various groups: anionic ($-COO^-$,$-SO^{3-}$,$-SO^{4-}$), cationic ($-NHR_2^+$,$-NR_3^+$), and hydrophobic ($-C_{18}$).[14]

The comparative HPLC analysis of the pigment composition of the extract prior and after the contact with M-PVA particles showed that long retained fractions of anthocyanins adsorbed the most intensely (Fig. 4). A 33% adsorption of the main component of the extract, malvidin-3-O-glucoside, was achieved, with a characteristic retention time of 19.5 min.

Table 1
Comparative characteristics of anthocyanin pigments adsorption from an aqueous extract using the sorbents M-PVA, Stirosorb MCDS×100 and Amberlit XAD 16

Sorbent					
M-PVA		Stirosorb MCDS×100		Amberlit XAD 16	
C_2An, mg/L	q, μg/μg	C_2 An, mg/L	q, μg/μg	C_2 An, mg/L	q, μg/μg
12±2	1.0±0.1	28±3	0.24±0.06	28±1	0.21±0.05

pH 3, V_1An = 0.5 mL, C_1An = 32.5±0.7 mg/L, C_s = 20 mg/mL, t = 10 min

Table 2
Characteristics of the concentration process using M-PVA particles without special cleaning procedure

n	C_2An, mg/L	m_2An, μg	m_sAn, μg	q, μg/μg	C_3An, mg/L	m_3An, μg
1	5.4±0.1	27.1±0.4	22.7±0.4	0.45±0.02	31±3	16±2
2	6.2±0.1	31.1±0.4	18.9±0.4	0.38±0.02	32±6	16±3
3	6.5±0.2	32.6±0.9	17.2±0.9	0.34±0.02	39±2	19±1
4	5.9±0.3	29.5±1.7	20±2	0.41±0.03	35±2	18±1
5	6.1±0.4	30.6±2.2	19±2	0.38±0.04	39±4	19±2
6	6.3±1.1	31.5±5.6	18±6	0.40±0.10	31±2	15±1
7	5.8±0.1	28.9±0.4	20.9±0.4	0.42±0.01	29.2±0.3	14.6±0.1
Average	6.0±0.3	30±1	20±1	0.40±0.02	34±3	17±4

pH 3, V_1An = 5 mL, C_1An = 10±1 mg/L, C_s = 10 mg/ml, t = 10 min

Table 3
Adsorption of anthocyanin pigments by M-PVA magnetic particles from aqueous extracts

Extract	C_2An, mg/L	q, μg/μg	Adsorbed pigments, %
clear	7.0±0.8	0.63±0.08	46
cloudy	7.5±0.7	0.53±0.07	38

pH = 3, V_1An = 100 mL, C_1An = 13±1 mg/L, Cs = 10 mg/mL

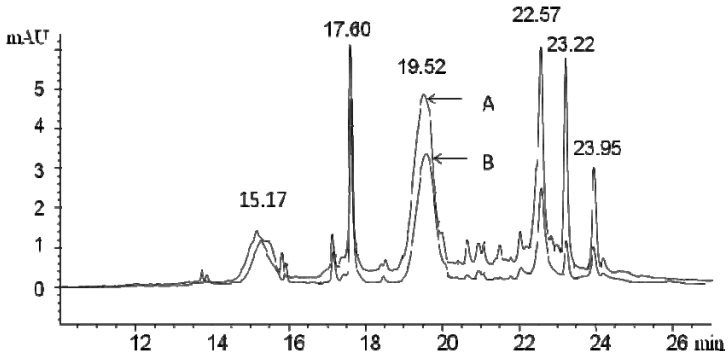

Figure 4: HPLC profiles of anthocyanins in aqueous extract (0.1% HCl) (525 nm), A – before adsorption, B – after adsorption onto M-PVA

For desorption of anthocyanins, acidulated ethanol (0.1% HCl) was used. The degree of extraction of the adsorbed pigments was of 85%. In the resulting ethanol solution, the concentration of pigments was three times higher than in the original aqueous extract. The choice of 96% ethanol as eluent is preferable to different aqueous-alcohol solutions, as it allows carrying out further concentration of the solution under mild conditions, with a primary allocation of a large part of the solvent for reuse. As a result, we obtained a more concentrated solution, which could be used as a food colorant or as a biologically active food additive. The qualitative pigment composition of the concentrate was equivalent to that of the original extract, which was established by HPLC.

It is known that in order to avoid clogging of chromatographic columns, the extraction of target molecules is carried out only from clear solutions. Therefore, additional steps of centrifugation and filtration of the extracts are necessary. Consequently, the ability to extract molecules from turbid media is especially important when working with natural extracts. The data presented in Table 3 indicate that the efficiency of molecule adsorption by the M-PVA magnetic particles does not significantly depend on the purity of the solution, containing the anthocyanins.

CONCLUSIONS

The method developed here of anthocyanin concentration from aqueous extracts using M-PVA magnetic particles with sizes of 1-3 microns exhibits high performance with respect to speed and simplicity. The paper demonstrates the advantages of M-PVA magnetic particles for the extraction of bioflavonoids on an industrial scale in comparison with conventional polymeric sorbents, like Stirosorb MCDS×100 and Amberlite XAD 16. First, the rate of sorption-desorption is 30-70 times higher and the duration of the separation of the ethanol concentrate is only about 25 minutes. Second, M-PVA particles can be reused within the process and can be applied in clear as well as in turbid media, without reducing the efficiency of the process. Using acidulated 96% ethanol for desorption, a solution is obtained having a concentration of anthocyanin pigments 3-4 times higher than the original extract. The removal of ethanol from this elution could be carried out under mild conditions. This provides an even more concentrated solution, which can be used as a food colorant or as a biologically active food additive.

ABBREVIATIONS

V_1An – volume of the extract (mL)
C_1An – concentration of anthocyanins in the initial solution (mg/L)
C_2An – concentration of anthocyanins in the water solution after adsorption (mg/L)
m_2An – mass of pigments in aqueous solution after adsorption (μg)
m_sAn – mass of adsorbed pigments (μg)
C_3An – concentration of anthocyanins in 0.5 mL of ethanol concentrate (mg/L)
m_3An – mass of pigments in 0.5 mL of ethanol concentrate (μg)
C_s – concentration of the adsorbent (mg/mL)
q – the adsorbate concentration per unit of mass of adsorbent (mg/mg)
t – contact time adsorbate with adsorbent (min)

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