ATLANTIS PRESS

Advances in Biological Sciences Research, volume 7

1st International Symposium Innovations in Life Sciences (ISILS 2019)

Peculiarities of Use of Bentonite Clay at Solid-Phase Purification of Anthocyanins and Flavonoids from Leaves of Plants

Andrey Doronin
Institute of Pharmacy, Chemistry and Biology
Belgorod State National Research University
Belgorod, Russia
634212@bsu.edu.ru

Victor Deineka
Institute of Pharmacy, Chemistry and Biology
Belgorod State National Research University
Belgorod, Russia
deineka@bsu.edu.ru

Ludmila Deineka
Institute of Pharmacy, Chemistry and Biology
Belgorod State National Research University
Belgorod, Russia
deyneka@bsu.edu.ru

Mikhail Tretiakov
Botanial Garden
Belgorod State National Research University
Belgorod, Russia
tretyakov_m@bsu.edu.ru

Valery Tokhtar
Botanial Garden
Belgorod State National Research University
Belgorod, Russia
Tokhtar@bsu.edu.ru

Andrey Chulkov
Belgorod branch
Federal Center for Safety and Quality Assessment of Grain and Products of its Processing
Belgorod, Russia
Ach87@mail.ru

***Abstract*—Anthocyanins of red colored leaves of *Cercis canadensis* L. were found to be 3-glucosides of five common anthocyanidins - of delphinidin, cyaniding, petunidin, peonidin and malvidin with overall accumulation more than 350 mg per 100 g of FW in early summer. The content of anthocyanins gradually drops to total disappearance at the late September. Extract of anthocyanins may content a large amount of flavonoids that are not separated during solid phase extraction (SPE) on C18-silica syringe cartridges. In the paper, the ability of bentonite clay as a sorbent for SPE of anthocyanins was investigated. Non-charged flavonoids were shown to compete with anthocyanins during sorption. Meanwhile sorption ability of anthocyanins markedly exceeds that of non-charged flavonoids. The method for anthocyanins and non-charged flavonoids is proposed.**

Keywords—anthocyanins, Cercis canadensis L., leaves, SPE, flavonoids, clay

I. INTRODUCTION

Montmorillonite is a unique clay possessing cation-exchange properties due to heterovalent (Al^{3+} by Mg^{2+}) substitution in central octahedron metal-hydroxyl ions sheets, providing to layers a negative charge [1]. This property was explored to create polymer-clay composite sorbents to fix natural dyes (including anthocyanins) [2]. In the previous investigation in our laboratory [3] we proved the high potency of bentonite clays (containing namely montmorillonite) for anthocyanins sorption in acidic medium. Unfortunately, in the English version of the latter article “montmorillonite” was translated as “kaolin”, though the latter mineral is not suitable for anthocyanins adsorption at all. Later ability of bentonite clays to adsorb anthocyanins was found by another researchers [4, 5].

Eastern redbud (*Cercis canadensis* L.) is a small tree with considerable morphological diversity, including variation in plant architecture, size, and flower and leaf colors [6]. The “Forest pansy” cultivar is one of the ornamental trees growing in the Botanical Garden of Belgorod National State University. Anthocyanins of rose flowers of the plant were investigated by reversed-phase HPLC [7] and were identified as a mixture of 3-glucosides and 3,5-diglucosides of common anthocyanidins though identification seems to be not correct as may be seen by investigation of peak elution sequence and shape on the chromatogram presented in the paper. Meanwhile no information about substances responsible for *C. canadensis* leaves coloration was not found in accessible for us literature.

The latter was a reason of the current paper together with investigation of bentonite clay for anthocyanins and flavonoids separation.

II. EXPERIMENTAL

Plant material

Leaves of *Cercis canadensis* were cut from plants in Botanical Garden of Belgorod National Research University and transferred into laboratory for chemical investigations. Anthocyanins were extracted by maceration of the plant material in 0.1 M water solution of HCl overnight. Than extract was separated by paper filtration and maceration was repeated until colorless extract obtaining. All fractions of extract were combined in a volumetric flask, made up by the same solvent for further differential spectrophotometric anthocyanins determination.

Differential spectrophotometry

Differential spectrophotometric determination of anthocyanins was performed according to widely adopted procedure [8]. Spectra were registered for plant extract

properly diluted (to escape copigmentation artefacts) by Shimadzu UV-2550 in quartz cuvettes.

Reversed-phase HPLC

Hydrochloric acid extract was subjected for purification using solid-phase extraction on Diapak C18 syringe cartridges (BioChemMack ST, Moscow) before injection into chromatograph.

Chromatographic investigations were performed on an Agilent 1200 Infinity chromatograph equipped with a diode array and mass-spectrometric detectors. A Symmetry C18, 3.5 μm 150×4.6 mm column was used for spectrophotometric detection while Kromasil 100-5C18 150×2.1 mm column was used for mass-spectrometric detection. Mobile phase was composed using formic acid (10 vol. %) in mixture of water and acetonitrile. For isocratic elution some different vol. fractions of acetonitrile were used (from 6 to 14 vol. %). For gradient elution to solvents were prepared. Solvent A was 6 vol. % of acetonitrile and 10 vol. % of formic acid in water. Solvent 30 vol. % of acetonitrile and 10 vol. % of formic acid in water. The elution program: 0 min – 0 % B; 30 min – 100 % of B; 31 min – 0 % of B and 40 min – 0 % of B. Rate of elution was 0.8 ml·min^{-1}. Mass-spectra were recorded in ESI-mode with fragmentation voltage 225 V and of negative ion scanning.

All data were obtained, saved and processed by Agilent ChemStation.

III. Results and Discussion

Anthocyanins of the leaves

Reversed-phase HPLC analysis of red leaves extracts of the plant showed that these were anthocyanins responsible for the coloration. Moreover the plant synthesize a valuable set of anthocyanins – mixture of 3-glucosides of delphinidin, petunidin and malvidin (Dp3G, Pt3G and Mv3G) of delphinidin series and of cyanidin and peonidin (Cy3G and Pn3G) of cyanidin series, Fig.1.

It should be mentioned that for purposes of compound identification in HPLC a wide set of anthocyanins' standards is necessary that are hardly available, expensive and are readily destroyed at storage. Meanwhile natural sources with constant qualitative composition of anthocyanins may be used as cheap and suitable material for storage and simple extraction and clean-up technic to prepare standard solutions.

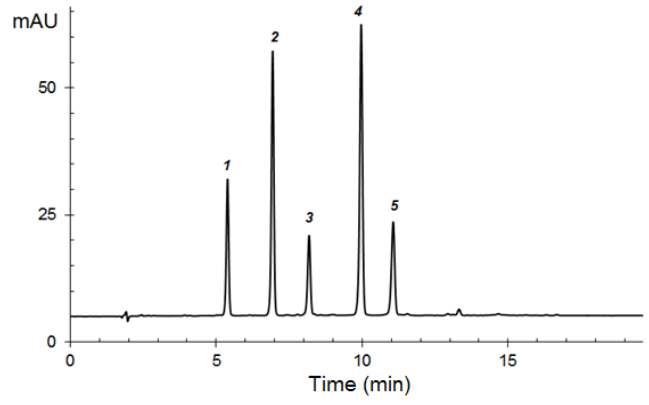

Fig.1. Separation of five main anthocyanins of *Cercis canadensis* leaves Mode of elution and conditions see text. Compounds: 1 – Dp3G; 2 – Cy3G; 3 – Pt3G; 4 – Pn3G; 5 – Mv3G.

The set of anthocyanins of this plant is more convenient than that of *Vitis vinifera* L. fruits that ordinary have one or two dominant compounds (malvidin-3glucoside or/and peonidin-3-glucoside) while the content of other members is much lower [9]. Moreover anthocyanin set of grape fruits may have anthocyanins with acylation by acetic, caffeic and *p*-coumaric acid demanding only gradient elution type in HPLC.

Variation of relative content of the five above mentioned anthocyanins may be rather broad but the set and elution order is not changed at least for leave samples that were investigated during 2018 and 2019, Table I.

From the Fig.1 it may be concluded that any 3,5-diglucosides in the samples under investigation were absent. Not to make errors for differentiation one difference in propertied of 3,5-diglucosides and 3-glucosides mast be taken into account: peaks of the former substances on the chromatograms are to be apparently broader than peaks of 3-glucosides [10].

Overall anthocyanins accumulation determined by convenient differential spectrophometric method was found to depend upon plant stages development, Table II. At the stage of the leaves opening the accumulation was highest to protect leaves from UV-radiation excess [11]. Gradually the concentration of anthocyanins drops down; while the leaves become yellow in the late autumn.

The concentration of anthocyanins on first stage of vegetation is comparable to that for mature black currant fruits [12]. Thus, leaves of *Cercis canadensis* L. on the early stages of development are valuable and renewable source of anthocyanins as well as a source of five common 3-glucosides that may be used for anthocyanins identification

TABLE I. The relative composition of five main anthocyanins of *Cercis canadensis* L. leaves

Sample No.	Date of analysis	Mole fraction of anthocyanins, %, ± 0.5				
		Dp3G	*Cy3G*	*Pt3G*	*Pn3G*	*Mv3G*
1	28.08.2018	10.0	32.8	7.9	39.3	8.9
2	06.09.2018	20.6	25.1	12.5	26.7	15.1
3	24.10.2018	9.9	29.8	8.0	40.6	11.7
4	01.11.2018	20.6	25.1	12.3	26.9	15.1
5	27.08.2019	11.5	31.3	8.2	37.8	11.2
6	10.09.2019	21.1	31.2	11.4	25.3	11.1
7	30.09.2019	14.0	27.8	9.7	35.4	13.1
Mean value, $\overline{M}$		15.4	27.8	10.0	35.4	12.3
and S_R		5.2	3.1	2.1	6.6	2.3

TABLE II. Overall accumulation of anthocyanins in leaves of *Cercis canadensis* L. cultivar "Forest pansy"

	Months of leaves collection				
	May		*June*	*August*	
Year	***2018***	***2019***	***2018***	***2018***	***2019***
Overall content, mg* per 100g FW	365	421	202	34	40

* - calculated as cyanoodin-3-glucoside chloride equivalent.

as standard substances mixture. But at late autumn all leaves lose their red coloration indicating the decomposition of anthocyanins while for some trees autumn leads to the appearance of red color as a consequence of namely anthocyanins synthesis.

Anthocyanins and other flavonoids of the leaves

Analysis of plant sources of anthocyanins encounters upon necessity of separation of low molecular-mass substances from polymeric or oligomeric compounds that may rapidly and irreversible contaminate guard or even the main chromatographic column. The ordinary procedure of the separation implies utilization of syringe cartridges filled with C18-functionalized silica. The procedure received a special name – SPE, "solid phase extraction". In case C18-silicas retention of substances is governed by their lipophilicity. In spite of existence of charge on the anthocyanidine backbone anthocyanins possess relatively high lipophilicity approaching to that of not charged flavonoid glycosides. Namely, the latter is a reason of obtaining a mixture of anthocyanins and flavonoids instead of pure anthocyanins mixture, Fig.2.

The peaks (Fig.2) of anthocyanins (λ_{max} is in the region 515 - 525 nm) are markedly lower than peaks of uncharged flavonoids (λ_{max} is in the region 345 - 360 nm). Thus, due to SPE of leaves extract concentrate of flavonoids was obtained rather than concentrate of anthocyanins, though it may successfully used for anthocyanins determination if the chromatogram is recorded at 520 nm.

Utilization of clays for anthocyanin SPE

Bentonite clays are interesting compounds for anthocyanin sorption by ion-exchange mechanism. If other flavonoid types are not charged at the conditions of sorption the clays must be selective namely for sorption of the anthocyanins.

In the first type of experiments a sufficient quantity of clay was added to a crude leaves extract performed with 0.1 M water solution of HCl for almost exhausting anthocyanins extraction. A colorless supernatant was withdrawn by centrifugation and anthocyanins were re-extracted from clay with solution containing 30 vol. % of acetonitrile and 30 vol. % of formic acid in water. After dilution with water (1:2 by volume) to escape artefacts during HPLC chromatogram was recorded, Fig.3.

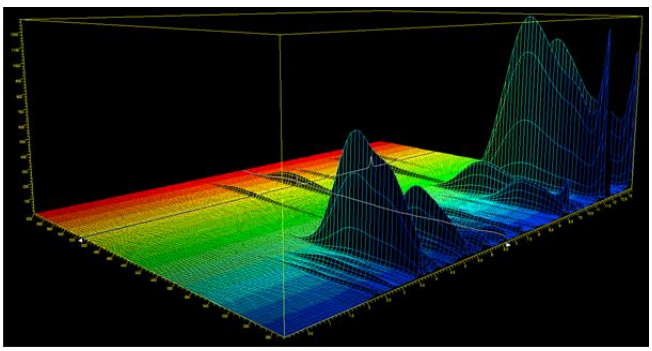

Fig.2. 3D-chromatogramm of *Cercis canadensis* leave extract after SPE on C18 cartridges

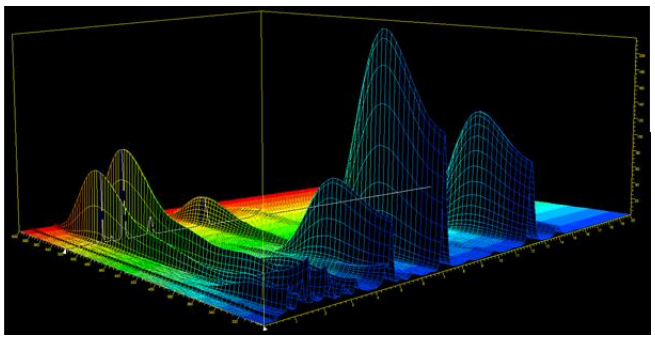

Fig.3. 3D-chromatogramm of *Cercis canadensis* leave extract after SPE on a bentonite clay at full anthocyanins sorption from extract

Analysis of resulting 3D-chromatogramm indicates that the relative fraction of non-charged flavonoids towards anthocyanins was dropped almost ten-folds when compared with the previous type of purification. Still great fraction of flavonoids in re-extract is unexpected.

Sorption of organic substances alters the clay surface properties [13], giving it the hydrophobicity. Thus, the pre-sorption of anthocyanins may be the reason of subsequent sorption of uncharged flavonoids.

In the second type of experiments, the same crude extract mixed with appropriate quantity of clay for anthocyanins sorption. After the system equilibration (half-an-hour with shaking), the supernatant No.1 was separated by centrifugation and another portion of clay was added. Than supernatant No.2 was obtained. Three extracts were subjected to HPLC and the chromatograms obtained af detection at 355 nm are presented in Fig.4.

The obtained results indicate that the concentration of flavonoids is reduced not only during anthocyanins sorption, but also at consecutive treatment with clay. Thus, flavonoids also are also adsorbed on the clay surface from acidified water solutions. The absence of influence of acidification on the flavonoid sorption on clay was proved by the third type of experiments utilizing solution at pH = 4.

Meanwhile, the obtained results indicate much stronger adsorption of anthocyanins on the clay compared to uncharged flavonoids. In this case the effectivity of

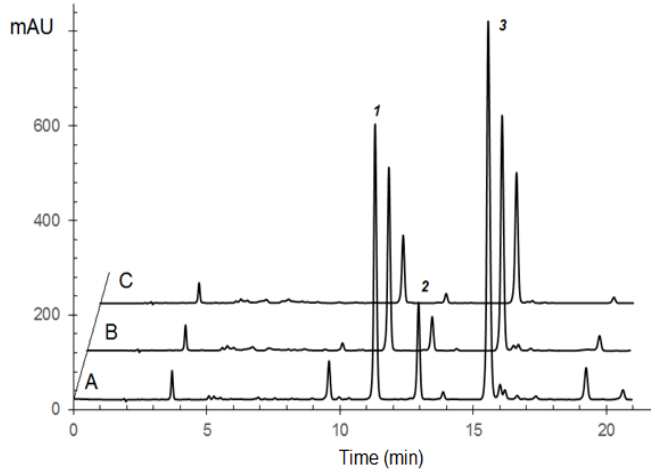

Fig.4. Separation of flavonoids in crude extract and in the two consecutive supernatants after sorption on clay

anthocyanins purification can be increased by sorption at high equilibrium concentration of anthocyanins. The proposition was confirmed in the fourth type of experiments, where it was found, that in the case of partial anthocyanins sorption on the clay the concentration of flavonoids in supernatants is raising due to a partial capture of water during sorption of anthocyanins.

IV. CONCLUSIONS

Red colored leaves of *Cercis canadensis* L. are valuable source of a set of 3-glucosides of the five common anthocyanidins - of delphinidin, cyanidin, petunidin, peonidin and malvidin.

The overall accumulation of anthocyanins depends upon time of collection, being more than 350 mg per 100 g of FW in early summer and lowering to almost total absence in the late September.

During solid phase extraction on bentonite clays the sorption ability of anthocyanins exceeds that for flavonoids, a procedure for separation of anthocyanins and non-charged flavonoids is proposed. Meanwhile at solid phase extraction on C-18 silica the separation is hardly possible.

REFERENCES

[1] V.V. Krupskaya, S.V. Zakusin, E.A. Tyupina, O.V. Dorzhieva, A.P. Zhukhlistov, P.E. Belousov., M.N. Timofeeva, "Experimental Study of Montmorillonite Structure and Transformation of Its Properties under Treatment with Inorganic Acid Solutions," Minerals, vol. 7, 49, 2017.

[2] T.J. Lopes, O.H. Gonçalves, R.B. Barros, F.C.R. Capelli, R.A.F. Machado, M.G.N. Quadri, M.B. Quadri, "Adsorption of Natural Dyes on Clay Fixed on Polymers", Brazil. Arch. Biol. Technol. vol. 48, Special n., pp. 275-280, 2005.

[3] L.A. Deineka, A.N. Chulkov, I.I. Saenko, V.I. Deineka, "Regularities in Adsorption of Anthocyanins by Natural Clays", Russ. J. Appl. Chem., vol. 82, No. 5, pp. 794−800, 2009.

[4] L. Soldatkina, V. Novotna, "Removal of anthocyanins from aqueous berry extracts by adsorption on bentonite: Factorial design analysis", Adsorption Sci. Technol., vol. 35(9–10), pp. 866–877, 2017.

[5] T.J. Lopes, M.G.N. Quadri, M.B. Quadri, "Recovery of anthocyanins from red cabbage using sandy porous medium enriched with clay", Appl. Clay Sci., vol. 37, pp. 97–106, 2007.

[6] D.J. Roberts, D.J. Werner, P.A. Wadl, R.N. Trigiano, "Inheritance and allelism of morphological traits in eastern redbud (*Cercis canadensis* L.)", Hortic. Res. vol. 2, 15049; 2015.

[7] P. Perkins-Veazie, G. Ma, D. Werner, "Anthocyanin Pigments in Redbud (Cercis spp) Flowers". J. Hortic. Sci. Res., vol. 1(1), pp. 13-18, 2017.

[8] J. Lee, R.W. Durst, R.E. Wrolstad, "Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential Method: Collaborative Study", J. AOAC Intern. vol. 88, No. 5, pp. 1269-1278, 2005.

[9] G. Mazza, F.J. Francis, "Anthocyanins in grapes and grape products" Crit. Rev. Food Sci. Nutr., vol. 35(4), pp. 341-371, Jul. 1995.

[10] S.L. Makarevitch., A.N. Chulkov, V.I. Deineka, M.O. Kostenko, L.A. Deineka, V.K. Tokhtar, "Determination of anthocyanins in fruits of some species of grapes by HPLC", Sorption and chromatohgraphic processes, vol. 14 (6), pp. 1024-1031, 2014 [In Russian].

[11] Y. Manetas, A. Drinia, Y. Petropoulou, "High Contents of Anthocyanins in Young Leaves are Correlated with Low Pools of Xanthophyll Cycle Components and Low Risk of Photoinhibition", Photosynthetica, vol. 40 (3), pp 349–354, September 2002.

[12] T. Siksnianas, V. Stanys, A. Sasnauskas, P. Viskelisand, M. Rubinskiene. "Fruit Quality and Processing Potential in Five New Blackcurrant Cultivars", J. Fruit Ornam. Plant Res. vol. 14 (Suppl. 2), pp. 265-271, 2006.

[13] Z.Z. Zhang, L.D. Sparks, N.C. Scrivner, "Sorption and desorption of quaternary amine cations on clays", Environ. Sci. Technol., vol. 27(8), pp. 1625-1631, 1993.