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Determination of Anthocyanins of Purple Carrot Two Cultivars

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***Abstract*—Two cultivars of purple carrot 'Pupur' and 'Mayami shokoladnaya" were investigated. Anthocyanins of both cultivars was qualitatively the same – main compound was cyanidin-3-feruloylhexosyl-pentosyl-hexodide, mole fraction of the anthocyanin was approximately 72 % of the overall anthocyanin content of carrot roots of cv. "Purpur" (I) and only 49 % in in carrot roots of cv. "Mayami shokoladnaya" (II). Cyanidin-3-pentosyl-hexoside (33.8 %) was found in carrot roots of (II) and only 4 – 18 % in carrot roots of (I). The overall accumulation of anthocyanins was fond in roots of carrots was 0.111 and 0.232 g per 100 g FW for cultivars I and II, respectively.**

Keywords — anthocyanins, purple carrot, HPLC, extraction

I. INTRODUCTION

Anthocyanins belong to a wide class of the secondary plant metabolites flavonoids [1]. However, these compounds possess some outstanding properties – they may exist in solution in some pH-dependent forms [2], some of them having unique coloration especially in unique stacking systems enabling plant's fruits, flowers and some other parts coloration from orange-red to blueish-black. Preserving the antioxidant potential inherent in flavonoids anthocyanins are regarded to as prominent natural food colorants with a great health benefit [3]. For this reason, rich sources of anthocyanins are of technological interest [4].

Among the plants that are used nowadays for production of anthocyanin colorants there are purple (or even black) colored carrot roots [5-10]. However, in Russia, this type of carrots was still out of interest, and only recently, seeds of some purple-colored carrots became available in the market for the gardeners, though we could not find information about type of anthocyanins and overall anthocyanin accumulation in the roots of the carrots.

To obtain the information of anthocyanins types, their relative quantities and overall anthocyanin accumulation in roots of the two purple carrot cultivar grown and harvested in conditions of Belgorod region was the objective of the current study.

II. EXPERIMENTAL

Carrot growing

Direct seed sowing in the ground in late April in the three different experimental field site grew carrots. The carrot cultivars under investigation were F1 "Purpur" (Semena NK, Ltd, RF") and F1 "Mayami shokoladnaya" (SeDeK Ltd., RF). The crop was harvested in mid-September of 2019. Roots were transferred to laboratory for chemical investigations.

Anthocyanins extraction and spectrophotometric analysis

Small pieces of carrot were macerated in 0.1 M water solution of HCl (or another solvent) overnight. The extract was separated from residue by paper filtration and to the solid residue was a new portion of extraction solvent was added; addition was repeated until colorless extract was obtained. All extract portions were combined and overall anthocyanin accumulation was determined by differential spectrophotometric method [11], calculating the result as cyanidin-3-clucoside chloride equivalent.

Reversed-phase HPLC

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

Chromatographic studies 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 was composed from 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

Reversed-phase HPLC of purple carrot anthocyanins

The chromatograms of anthocyanins of the two cultivars of purple carrot extracts under investigation in a current paper are almost the same but different from published elsewhere [5-10], Fig.1.

There are four main compounds with surprisingly small retention. Electronic absorption spectra of peaks No.1 (not shown) and No.3 on the chromatogram of Fig.1 (518 nm) indicates it to be for the derivative of cyanidin [12], Fig.2. The two some strongly adsorbed compound have maxima of the bands shifted batochromically to 530 and 528 nm, correspondingly.

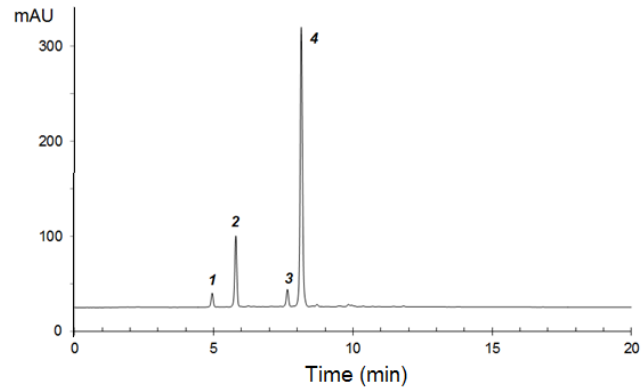

Fig.1. Separation of anthocyanins of purple carrot extract. Gradient elution mode, 0.8 ml/min, 515 nm

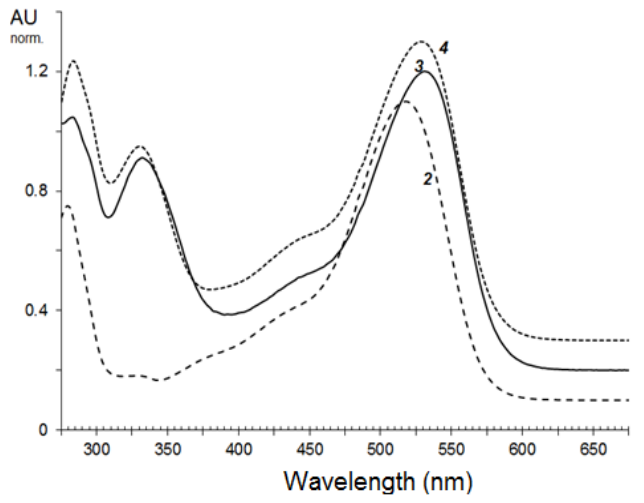

Fig.2. Electronic absorption spectra of purple carrot anthocyanins. Numbers of spectra correspond to numbers of substances on chromatogram in Fig.1.

The shift may be the consequence of cyanidin derivatives acylation with substituted cinnamic acids, the proposition is confirmed by appearance of local maxima at 330 nm. These maxima of absorption reveal the presence in the anthocyanins' structure substituted cinnamic acids chromophore groups [12].

The degree of glycosylation of compounds No.1 and No.2 may be predicted by analysis of separation maps [13] taking into account the slopes of trend lines of solute relative retention *vs* that of cyanidin-3-glucoside, Cy3G, Fig.3. Retention of cyanidin-3-glucoside (Cy3G) was determined from anthocyanis mixture of *Aronia melanocarpa* L. fruits extracts [14].

Trend lines for the solutes are:

- for solute No.1:

$$\log k(1) = 1.258 \cdot \log k(\text{Cy3G}) - 0.468. \quad (1)$$

- for solute No.2:

$$\log k(2) = 1.069 \cdot \log k(\text{Cy3G}) - 0.202. \quad (2)$$

The slope of the first trade line indicates the existence of tree molecules of monoses in position No.3 of cyanidin, while the slope for solute No.2 indicates the presence of only two molecules of monoses.

To confirm the above proposition and to determine the type of cinnamic acids acylated the anthocyanins No.3 and No.4 (Fig.1) mass-spectra were registered and analyzed, Fig.4. Fragmentation of native ions revealed all substances to be derivatives of cyanidin (m/z = 287.0), that is in full agreement with literature data [5 - 10]. The substance No.1 is cyanidin-3-pentosylhexosylhexoside (m/z = 743). The substance No.2 is cyanidin-3-pentosylhexoside (m/z = 581); the substance No.3 is cyanidin-3-pentosylhexosylhexoside acylated with synapic acid (m/z = 949); the substance No.4 is cyanidin-3-pentosylhexosylhexoside acylated with ferulic acid (m/z = 919).

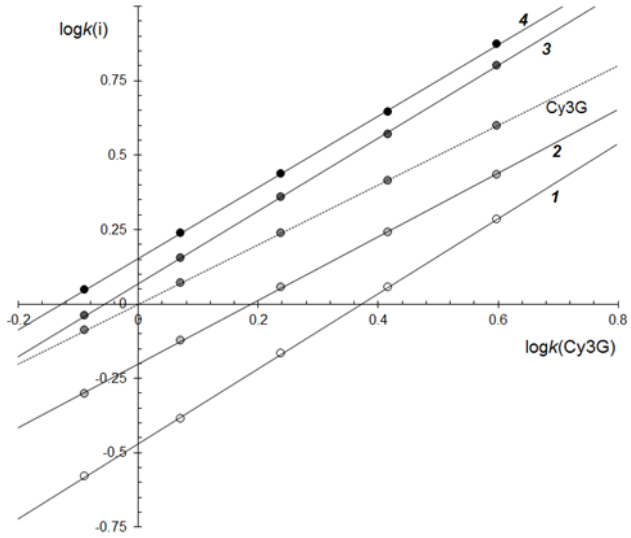

Fig.3. Separation map for anthocyanins of purple carrot roots

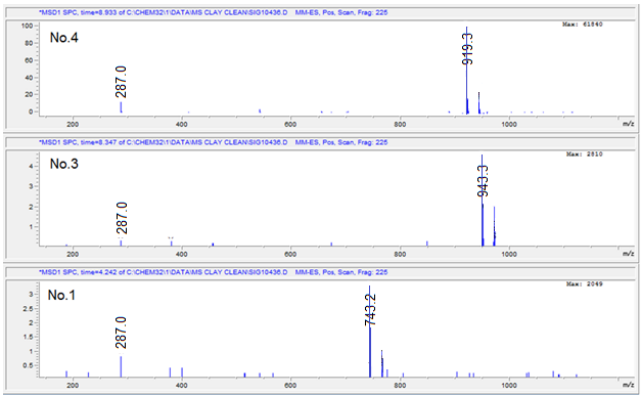

Fig.4. Mass spectra of compound No.1, No.3 and No.4 on Fig.1

Thus, both acylated anthocyanins have triglycoside moieties. The slopes for the solutes (1.220 for solute No.3 and 1.196 for the solute No.4) on the separation map correspond to namely triglycosides, thus acylation in this case do not affects the slope of solute relative retention.

The obtained results are do not completely match the results published in paper [5], where the non acylated compounds were the same – cyanidin-3-xylosyl-glycosyl-glucoside, cyanidin-3-xylosyl-glucoside. Though among acylated compounds the most abundant (33.65 %) and the most strongly retained compound was identified as cyanidin-3-xylosyl-glycosyl-glucoside acylated with ferulic acid just as in current paper. However, before this compound cyanidin-3-xylosilglycosiglucoside acylated with coumaric acid being the second abandoned (29.85 %) was found being totally absent in current case. Before these compounds relatively small peak (3.24%) of cyanidin-3-xylosyl-glucosyl-glucoside acylated with sinapic acid was detected, that is in coincidence with results of current investigation. In the paper [6] the same series of anthocyanins was identified and the position of attachment of monoses and cinnamic acid substituents was pointed out. Briefly, position 3 of cyanidin is glycosylated with galactose; xylose is attached to galactose in position 2″, while glucose is attached into position 6″. Acylation by cinnamic acids occurs in the position 6‴ of glucosyl moiety in the cases of sinapic, Fig.5, and ferulic acids, while for acylation with *p*-coumaric acid another position of glucosyl moiety is occupied - 4‴, though in another paper [7] the position of acylation is pointed out as 6‴. In the papers [8 - 10], similar results were obtained but concentration of the component acylated with *p*-coumaric acid was comparable with that acylated with sinapic acid.

Determination of the overall anthocyanins accumulation

For the determination of anthocyanins extraction of the compounds is the first stage of the procedure. It is well known that anthocyanins exist exclusively in flavylium form only in highly acidic solutions [15] and that these conditions provide the highest stability of these substances. Meanwhile in some publications the water with neutral medium is proposed for the extraction [16]. It seems to be non-correct procedure because according to our experience pH jump into basic conditions leads to a loss of some 10 - 20 % anthocyanins when the pH of solution was returned to 1. On the other hand, anthocyanins acylated with cinnamic acid derivatives at pH rise not convert to colorless pseudobase

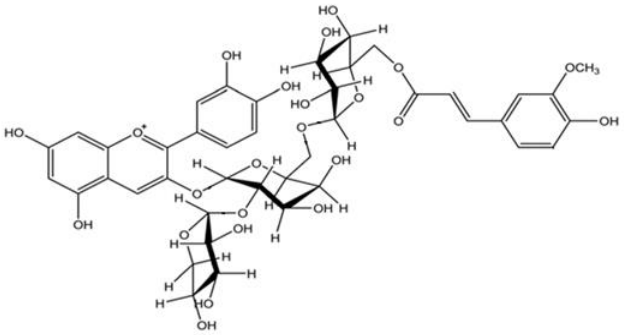

Fig.5. Structure of cyanidin 3-O-(2"-xylosyl-6"-(6"'-sinapoyl-glucosyl)-galactoside).

form but rather to quinonoidal form and it may influence the stability of anthocyanins as well as here extraction yield.

That is the reason of our experiments by extraction procedure. We set the task of research extraction effectivity for four different solvents; 1) 0.1 M water solution of HCl (pH = 1); 2) 0.01 M water solution of HCl (pH = 2); 3) 0.001 M water solution of HCl (pH = 3); 4) distilled water (pH = 5), Table I.

Samples of carrot roots (having acylated anthocyanins) and *Aronia mitschurinii* fruits (with non acylated anthocyanins) were extracted in porcelain mortar successively by portions of the solvent. The portions were transferred into volumetric flask; the volume was made up by the solvent. Portions of the extract were diluted with 0.1 M water solution of HCL and anthocyanin concentration was determined by simplified spectrophotometric method (without measurement of absorption at pH 4.5 because of formation of colored quinonoid form instead of pseudobase).

According to the obtained data pH increase leads to reduction of extraction efficiency. Thus for the further investigation only 0.1 M water solution of HCL was used. The overall accumulation of anthocyanins was fond in roots of carrots was 0.111 and 0.232 g per 100 g FW for cultivars I and II, respectively.

TABLE I. QUANTITY OF ANTHOCYANINS OBTAINED IN SEASON 2018

№	Solvent for extraction	Purple carrot		Aronia	
		*content**	*%*	*content**	*%*
1	0.1 M	0.226		0.597	
2		0.225		0.571	
Mean value		0.225	100	0.584	100
3	0.01 M	0.190		0.556	
4		0.177		0.550	
Mean value		0.183	81.4	0.553	94.7
5	0.001 M	0.147		0.267	
6		0.154		0.279	
Mean value		0.151	66.8	0.273	46.8
7	H_2O	0.114		0.240	
8		0.133		0.275	
Mean value		0.124	54.9	0.258	44.2

IV. CONCLUSIONS

The two cultivars of purple carrot 'Purpur' and 'Mayami chokoladnaya" were found to be rich sources of anthocyanins: the overall accumulation of anthocyanins was fond in roots of carrots was 0.111 and 0.232 g per 100 g FW for cultivars I and II, respectively.

Anthocyanins of both cultivars were qualitatively the same – the main compound was cyanidin-3-feruloylhexosyl-pentosyl-hexodide, mole fraction of the anthocyanin was approximately 72 % in carrot roots of cv. "Purpur" (I) and only 49 % in in carrot roots of cv. "Mayami chokoladnaya" (II). Cyanidin-3-pentosyl-hexoside (33.8 %) was found in carrot roots of (II) and only 4 – 18 % in carrot roots of (I).

REFERENCES

[1] Y. Tanaka, N. Sasaki, A. Ohmiya, "Biosynthesis of plant pigments: anthocyanins. betalains and carotenoids", Plant J., vol. 54, pp. 733–749, 2008.

[2] P. Trouillas, J.C. Sancho-García, V. De Freitas, J. Gierschner, M. Otyepka, O. Dangles, "Stabilizing and Modulating Color by Copigmentation: Insights from Theory and Experiment", Chem. Rev., vol. 116, pp. 4937−4982, 2016.

[3] H.E. Khoo, A. Azlan, S.T. Tang, S.M. Lim, "Anthocyanidins and anthocyanins: colored pigments as food. pharmaceutical ingredients. and the potential health benefits", Food Nutr. Res., vol. 61(1), 1361779, 2017.

[4] F. Delgado-Vargas, A.R. Jimenez, O. Paredes-Lopez, "Natural pigments: Carotenoids. anthocyanins. and betalains: Characteristics. biosynthesis. processing. and stability", Crit. Rev. Food Sci. Nutr., vol. 40(3), pp. 173–289, 2000.

[5] M.T.M Assous, M.M. Abdel-Hady, G.M. Medany, "Evaluation of red pigment extracted from purple carrots and its utilization as antioxidant and natural food colorants", Ann. Agric. Sci., vol. 59(1), pp. 1–7, 2014.

[6] A. Tsutsumi, Y. Horikoshi, T. Fushimi, A. Saito, R. Koizumi, Y. Fujii, Q.Q. Hu, Y. Hirota, K. Aizawa, N. Osakabe. "Acylated anthocyanins derived from purple carrot (*Daucus carota* L.) induce elevation of blood flow in rat cremaster arteriole", Food Funct., vol. 10, pp. 1726–1735, 2019.

[7] D. Kammerer, R. Carle, A. Schieber, "Quantification of anthocyanins in black carrot extracts (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) and evaluation of their color properties", Eur. Food Res. Technol., vol. 219, pp. 479–486, 2004.

[8] G. Barba-Espín, S. Glied, C. Crocoll, T. Dzhanfezova, B. Joernsgaard, F. Okkels, H. Lütken, R. Müller, "Foliar-applied ethephon enhances the content of anthocyanin of black carrot roots (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.)", BMC Plant Biol. vol. 17, 70, 2017.

[9] E. Agcam, A. Akyıldız, V.M. Balasubramaniam, "Optimization of anthocyanins extraction from black carrot pomace with thermosonication", Food Chem., vol. 237, pp. 461–470, 2017.

[10] P. Garcia-Herrera, M.-L. Perez-Rodrıguez, T. Aguilera-Delgado, M.-J. Labari-Reyes, B. Olmedilla-Alonso, M. Camara, S. de Pascual-Teresa, "Anthocyanin profile of red fruits and black carrot juices. purees and concentrates by HPLC-DAD-ESI/MS", Intern. J. Food Sci. Technol., vol. 51, pp. 2290–2300, 2016.

[11] 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.

[12] J.B. Harborne, "Spectral methods of characterizing anthocyanins", Biochem. J., vol. 70(1), pp. 22–28. Sep. 1958.

[13] V.I. Deineka, "Chromatographic separation map and incremental relationships in the method of relative analysis of retention under HPLC conditions", Russ. J. Phys. Chem. vol. 80, pp. 429-434, 2006.

[14] J. Oszmianski, J.C. Sapis, "Anthocyanins in fruits of *Aronia melanocarpa* (Chokeberry)", J. Food Sci. vol. 53(4), pp. 1241-1242, 1988.

[15] O. Dangles, J.-A. Fenger, "The Chemical Reactivity of Anthocyanins and Its Consequences in Food Science and Nutrition", Molecules, vol. 23, 1970, 2018.

[16] N. Aryanti, A. Nafiunisa, D.H. Wardhani, "Conventional and ultrasound-assisted extraction of anthocyanin from red and purple roselle (*Hibiscus sabdariffa* L.) calyces and characterization of its anthocyanin powder", Intern. Food Res. J., vol. 26(2), pp. 529-535, April 2019.