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Anthocyanins of *Tagetes patula* flower petals.

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ABSTRACT

Some cultivars of *Tagetes patula* with red spots on the flower petals were found to be rich sources of anthocyanins accumulating 0.25 g (as cyanidin-3-glucoside chloride equivalent) per 100 g FW. The main components of petal anthocyanins were cyanidin-3-galloylsophoroside (60 - 90 % by peak normalization) and cyanidin-3-glucoside (5 – 40 %), though cyanidin-3-sophoroside (2 – 4 %) was also detected in all cases. The anthocyanin accumulation depends upon plant cultivar and plant maturation.
Keywords: *Tagetes patula*, anthocyanins, cyanidin-3-galloylsophoroside, cyanidin-3-glucoside, extraction.

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INTRODUCTION

Four from fifty-six known species of *Tagetes* (Marigold) genus are worldwide grown as ornamental herbaceous plants: *T. erecta* L. (African, Aztec or Mexican marigold), *T. patula* L. (French marigold), *T. lunulata* Ort. и *T. tenuifolia* Cav. [1, 2]. Meanwhile *T. erecta* has a long history of traditional medicinal use in many countries [3] currently this species is utilized for lutein production that is accumulated in flower petals as diesters formed by predominantly four saturated fatty acids from lauric to stearic [4]. The level of xanthophyll accumulation determines the petals color, raising manifolds from cultivars with pale yellow to dark orange coloration [5]. It should be mentioned that flowers of *T. patula* accumulate almost the same level of xanthophylls [5] and though the flowers are of smaller dimensions compared to flowers of *T. erecta,* the former still may be of technological value as a source of xanthophylls.

One of the most visible differences between the flowers of the two species is an existence of *T. patula* cultivars with red spots on the petals due to anthocyanins biosynthesis [5]. These anthocyanins are known to be glycosides of cyanidin origin with cyanidin-3-glucoside, Cy3G, to be one of them [5]. Fat-soluble lutein (with zeaxanthin) is widely used in poultry to improve the quality of egg products [6] as well as directly in ophthalmological preparations to withstand age-related macular degeneration [7], while anthocyanins are water-soluble antioxidants [8], also being exploring in drugs to cure eye [9].

The aim of the present paper was to determine anthocyanins composition of *T. patula* cultivars flower petals with red spots, to estimate the level of anthocyanins accumulation.

MATERIALS AND METHODS

Plant cultivation

Plants were grown by the sawing method. Seeds of *T. patula* some cultivars were sown directly in open ground in late April.

Anthocyanins extraction

For determination of anthocyanin accumulation in flower petals non-grinded plant material was transferred into 0.1 M solution of HCl in distilled water for maceration at room temperature.

Partial purification of anthocyanins

Before anthocyanins determination by reversed-phase HPLC the samples were partially purified by solid phase extraction on the reversed-phase sorbent of DIAPAC C18 cartridge (BIOCHEMMACK, ST, Moscow, RF) [10].

Semi-preparative anthocyanin separation

For semi-preparative cyanidin-3-galloylsophoroside (Cy3X, Cy3GallS) separation from partially purified extract Shimadzu LC-20 equipment with spectrophotometric detector was used. The column 10×250 mm SUPELCOSIL^{TM}C-18 at ambient temperature, mobile phase the mobile phase: 8 vol. % of CH_3CN, 10 vol. % of HCOOH and 82 vol. % of distilled water, mobile phase flow rate was 5 ml·min^{-1}, peaks monitoring at 525 nm.

Anthocyanin hydrolysis

For acid hydrolysis to prove the structure of anthocyanin the solution of separated substance was mixed with 20 % sulfuric acid solution in water (1 : 1 by volume) and the mixture was heated upon boiled water bath for different times. Then the solution was cooled and anthocyanins were prepared for HPLC analysis by solid phase extraction.

For base hydrolysis solution of separated substance was mixed with 20 % sodium hydroxide solution in water (1 : 1 by volume) and the mixture left for some minutes at room temperature. Then the mixture was

neutralized and acidified with 1 M water HCl solution and was prepared for HPLC analysis by solid phase extraction.

Qualitative and quantitative determination of anthocyanins

Anthocyanin concentration in extracts was determined by the spectroscopic differential method [11] as a cyanidin-3-glucoside chloride equivalent.

For analytical HPLC Agilent Infinity 1200 equipment with diode array (DAD) and MS (6130 Quadrupole LC/MS) detectors were explored. Chromatographic column: 4.6×150 mm Symmetry®C18, 3.5 mcm; the mobile phase: 8 (or 6) vol. % of CH_3CN, 10 vol. % of HCOOH and 82 (or 84) vol. % of distilled water, mobile phase flow rate was 0.8 ml·min^{-1}. The electronic spectra of the anthocyanin peaks were recorded in DAD cell with a range step 0.50 nm. Mass spectra were recorded at positive ESI-mode when column 2.1×150 mm Kromasil 100-3.5C18 was used with mobile phase 8 vol. % of CH_3CN, 10 vol. % of HCOOH and 72 vol. % of distilled water, flow rate was 150 mcl·min^{-1}. Fragmentor voltage of 100 V was applied to get molecular ions and 150 or 200 V was applied to get fragmented ions including anthocyanidins.

Chemicals

The mobile phases for HPLC were composed of distilled water, acetonitrile (Super Gradient, LABSCAN), and reagent grade formic acid (SPECTR-CHEM Ltd, RF). Before exploitation mobile phase was filtered through polyamide membrane filters with pores 0.45 mcm and degassed under vacuum.

Reagent grade acetone, reagent grade HCl water solution and distilled water were used for extraction and partial purification of anthocyanins.

RESULTS AND DISCUSSION

Anthocyanins separation and identification

Chromatographic consideration

The cultivars for the current investigation were chosen due to red spots on the flowers petals typical for anthocyanins biosynthesis, while simultaneous biosynthesis of xanthophyll provides the orange to yellow color of petals background. It was found also that with the maturation flower size increases while the color intensity of red spots is reduced.

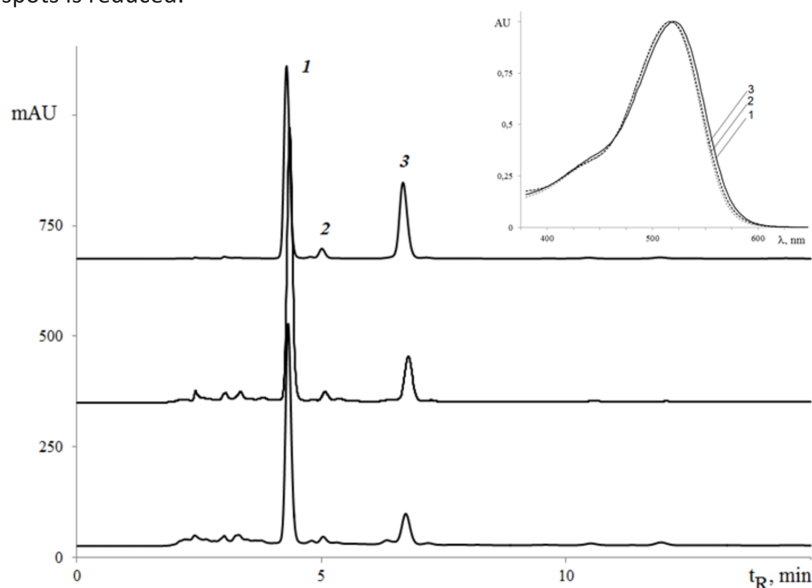

Figure 1: Separation of anthocyanins of three *T. patula* cultivars flower petals extracts with inserted electronic spectra
Column: 150×4.6 mm Symmetry C18, 5 mcm; mobile phase: 5 vol. % CH_3CN, 10 vol. % HCOOH and 82 vol. % distilled water, 0.8 ml/min. Detector λ = 515 nm. Compounds: 1 – Cy3X, 2 – Cy3S, 3 – Cy3G.

Chromatograms of anthocyanin extracts of some samples are presented on the Fig.1. The two main compounds were found in every case with one tiny component. Electronic and mass-spectra of the solutes as well as retention parameters in reversed-phase chromatography are summarized in Table 1.

The simplest anthocyanin Cy3G was identified by comparison of the retention with one of the black currant anthocyanin component, Fig.2. To escape mistakes according to our experience comparison of solute retentions in at least of two different mobile phase compositions is necessary and nearly sufficient at least for anthocyanins. Moreover there is no need to use expensive and unstable during storage anthocyanin standards for the comparison if proper plant sources are easily available. Indeed according to our experience [12] anthocyanin composition of *Ribes nigrum* skin of any cultivar is qualitatively constant with the same sequence of elution in any compositions of mobile phases. Thus it is a rather chip source of a reference anthocyanin sample due to the availability of the fruit in store all year round and simple procedure of extraction and partial clean-up of anthocyanins. The assignment was confirmed by spectral analysis.

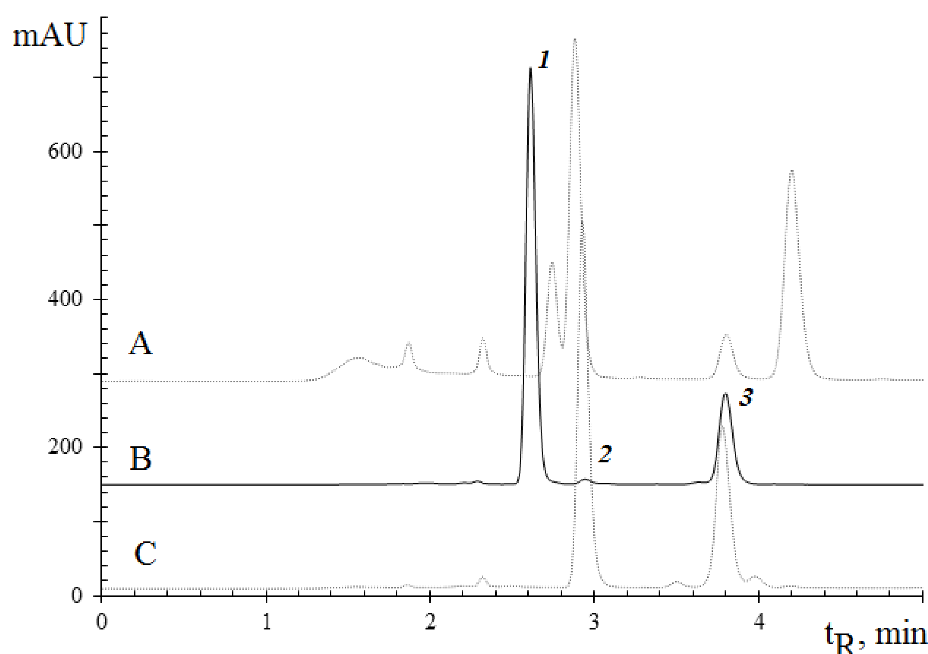

Figure 2: Separation of anthocyanins of *T. patula* L. (B) flower petals and fruit extracts of *Ribes nigrum* L. (A) and *Rubus idaeus* L. (C)

Column: 150×4.6 mm Symmetry C18, 3.5 mcm; mobile phase: 10 vol. % CH_3CN, 10 vol. % HCOOH and 80 vol. % distilled water, 0.8 ml/min. Detector λ = 515 nm. Compounds: 1 – Cy3X, 2 – Cy3S, 3 – Cy3G.

For the main peak we were not able to find analogue between anthocyanin samples of our laboratory collection. But according to mass-spectrum as well as to electronic spectrum (Table 1) this compound has a complex (dihexoside acylated with gallic acid) radical attached to position 3 of cyanidin background, Cy3X. Indeed, electronic spectra of Cy3G and Cy3X has a small band at 430 nm reviling the anthocyanins to have glycosidic moieties only in position 3, while 4.5 nm bathochromic shift of band maximum, indicates the longer chain of side radical for Cy3X in comparison to Cy3G. We may only believe that the dihexoside is of sophoroside structure because of existence of cyanidin-3-sophoroside, Cy3S, in small quantities in all extracts examined in the current investigation (Fig.1, Table 1). The Cy3S structure was elucidated by retention comparison with one component of red raspberry anthocyanin extracts [13].

In case of anthocyanins one more method to primary peak assignment is possible. The approach assumes to analyze the separation map according to relative retention analysis [14]. The equation of relative retention taking Cy3G as a reference solute must be determined for at least two mobile phase compositions and isocratic elution mode for each case: the concentration of HCOOH remains constant (10 vol.%) while concentration of CH_3CN to be taken from 6 to 10 vol.% in distilled water, Fig.4:

$$\log k(i) = a \cdot \log k(\text{Cy3G}) + b, \qquad (1)$$

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where logarithms of solute *i* capacity factors are calculated using uracil as a “dead” volume marker.

In the case of anthocyanins based upon the same aglicon for side radical attached to position 3 parameter *a* increases by 0.08 – 0.10 units for each added carbohydrate radical [15], Table 1. For Cy3X *a* = 1.175 indicates attachment of three molecules while for Cy3S *a* = 1.095 proving the solute to be diglycoside. On Fig.3 trend line for cyanidin-3-galloylglucoside (*a* = 1.089) separated from red maple leaves of “Crymson king” cultivar [16] is given to prove the input of hexoside to be nearly the same as of galloyl moiety upon parameter *a*.

Table 1: Anthocyanin types parameters

No.	Type of anthocyanin[a]	λ_{max}[b], nm	Mass-spectra signals[c], m/Z	Parameters of eq.1	
				a	*b*
1	Cy3X	520.5	763.1, (287.1)	1.175	-0.408
2	Cy3S	515.5	611.1, (287.1)	1.095	-0.233
3	Cy3G	516.0	449.1, (287.1)	1	0
4	Cy3GalG	519.5	601.1, (287.1)	1.089	0.197

[a] – abbreviations see in text;
[b] - in the mobile phase 10 vol.% of HCOOH, 8 vol.% of CH_3CN and 82 vol.% of water;
[c] - fragmentor voltage 200 V.

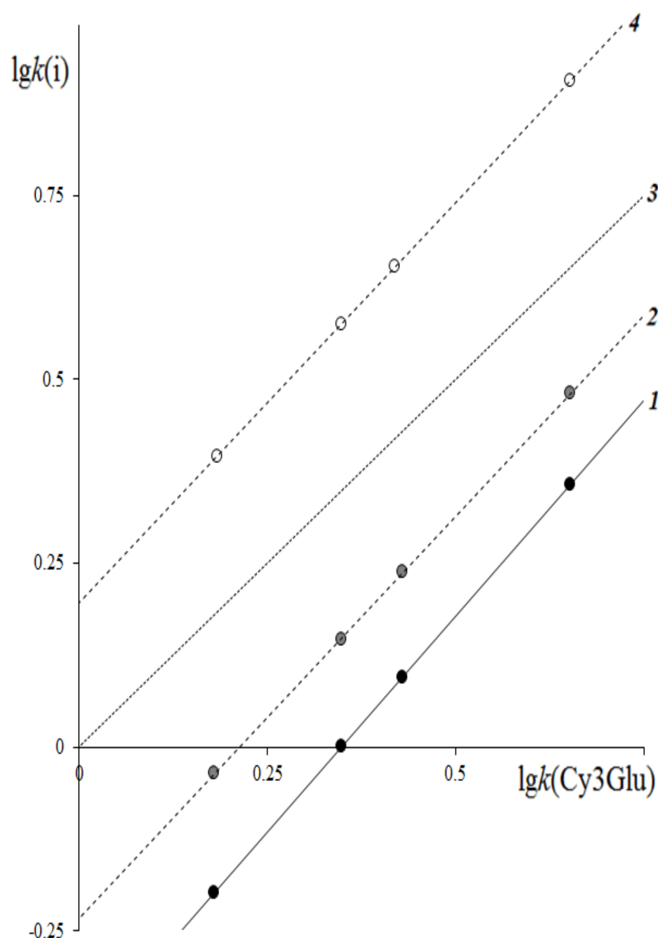

Figure 3: Separation map of some cyanidin derivatives
Column: 150×4.6 mm Symmetry C18, 3.5 mcm; mobile phase: X vol. % CH_3CN, 10 vol. % HCOOH and (90-X) vol. % H_2O.
Compounds: 1 – Cy3X, 2 – Cy3S, 3 – Cy3G, 4 – Cy3GallG.

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Acid-base hydrolysis approach

The compound Cy3X is found in nature for the first time. To elucidate a structure of Cy3X the hydrolysis technique was explored. During acidic hydrolysis the two main compounds were formed, Cy3G and aglicon, Cy, Fig.4. The acidic medium is favorable to break glycosidic linkages but not the ester bonds. For anthocyanin based upon cyanidin with two hexoside molecules and one gallic acid radical attached to position 3 it is possible to draw some structures without specifying the position of connection between them. But the only sequence Cy-G-G-Gall atches the results. In the structure the first carbohydrate molecule is attached to position 3 of cyanidin, the second carbohydrate molecule is attached to the first carbohydrate molecule, finally the latter is acylated with gallic acid. This proposition was also confirmed by the base hydrolysis, with Cy3S being the main hydrolysis product. Thus solute Cy3X is cyanidin-3-galloylsophoroside, with gallic acid radical, connected to still unknown position of the second glucose moiety. The type of the linkage may be determined only by NMR-investigation not available for us.

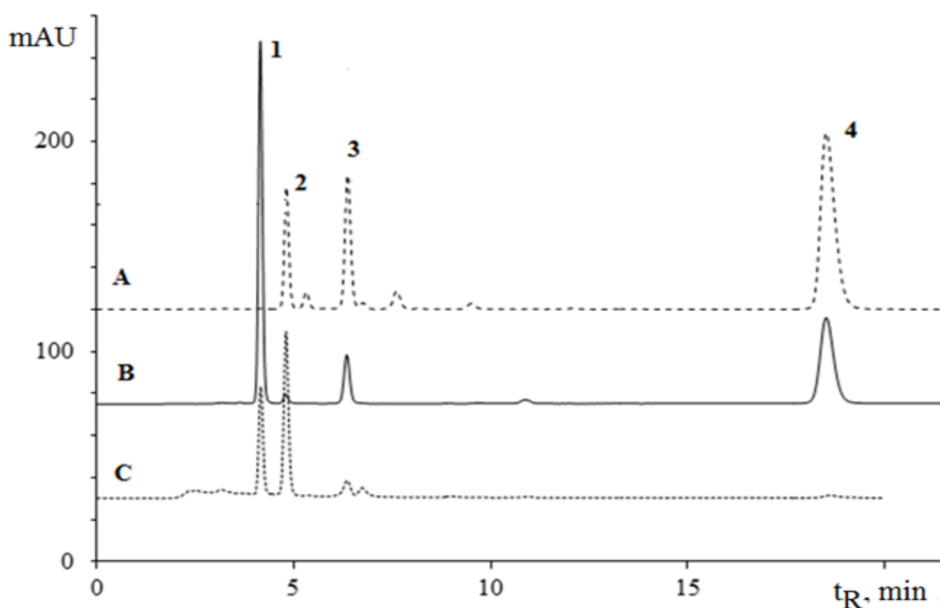

Figure 4: Separation of anthocyanins of *T. patula* L. (B) flower petals and Cy3X partial hydrolysis products in acidic (A) and alkaline (C) media

Column: 150×4.6 mm Symmetry C18, 3.5 mcm; mobile phase: 8 vol. % CH_3CN, 10 vol. % HCOOH and 82 vol. % distilled water, 0.8 ml/min. Detector λ = 515 nm. Compounds: 1 – Cy3X, 2 – Cy3S, 3 – Cy3G, 4 – Cy.

Anthocyanins quantification

Overall accumulation of anthocyanins was determined by differential spectrophotometric method [11], though for most samples determination of optical density at pH = 4.5 was not necessary because of low content of polymeric anthocyanins. The absence of polymeric anthocyanins may be directly detected during reverse-phase HLPC due to the absence of broad peak with bathochromic sift of peak maximum of electronic spectrum, starting near dead volume on the chromatograms.

Though the determination has some particularities connected with botanical structure of flowers. In the *T. patula* the predominant yellow color is produced by the accumulation of carotenoid pigments in the ray flowers. These water-insoluble pigments are located in chromoplasts, which pack the cytoplasm at the tip of one of the surface cells. The red spots in the ray flowers appear due to the accumulation of anthocyanins in the vacuoles of these surface cells. If for the anthocyanins extraction by acidified water to use chopped samples simultaneous xanthophyll extraction in the form of fine colloid solution occurs interfering anthocyanins determination. That is why we used petals maceration without mechanical destruction of the cell membranes. Maceration was performed overnight at room temperature, but at least three stages of maceration are necessary for exhausting anthocyanins extraction, Table 2, because of very slow anthocyanins diffusion processes through undestroyed cell membranes. Three day lasting maceration leads to the same results leaving orange petal remnants without red sports of anthocyanins.

The HPLC investigations revealed the mole fraction of Cy3X, as determined by peak area normalization, to be much greater than in previous investigations [5], but it was found to depend upon stage of maturation increasing with time up to nearly 90 %, Table 3.

Table 2: Effectivity of anthocyanins extraction for consecutive macerations

Sample No	Degree of extraction, %, for consecutive macerations				Total, g / 100 g FW
	1	2	3	4	
1	25.5	31.6	42.0	0.9	0.214
2	24.4	31.1	42.0	2.5	0.161
3	24.3	24.2	39.4	12.1	0.198
4	35.9	34.9	27.6	1.6	0.177

Table 3: Anthocyanins composition of *T. patula* flower petals (*n* = 3)

№	Mole fraction by peak area normalization, %			c**, г / 100 г свежего материала
	Cy3GallS (Cy3X)	Cy3S	Cy3G	
Flowers harvested in 2015 (unknown varieties)				
1	89.5	2.5	3.7	0.211 ± 0.021
2	84.2	3.0	5.7	0.321 ± 0.010
3	79.9	2.4	13.9	0.306 ± 0.009
4	63.1	2.1	13.0	0.356 ± 0.020
5	57.6	3.1	34.2	0.293 ± 0.022
6	55.2	2.8	37.9	0.433 ± 0.035
7	51.1	4.4	40.0	0.263 ± 0.042
Flowers harvested in 2016				
Variety “Carmen”				
8*	73.8	1.2	22.8	0.133
9*	83.4	0.9	14.5	0.056
10	84.5	1.2	4.9	0.080 ± 0.009
11	83.9	1.4	7.3	0.073 ± 0.021
12				0.180 ± 0.030
Variety “Bolero”				
13	89.0	1.7	4.9	0.088 ± 0.008
Variety “Black Velvet”				
14	75.1	1.3	21.7	0.113 ± 0.009

* - *n* = 1.

CONCLUSIONS

Cultivars of *Tagetes patula* with red spots on the flower petals may be utilized as sources of xanthophylls as well as that of anthocyanins. The main component the anthocyanins is unique cyanidin-3-galloylsophoroside (60 - 90 % by peak normalization) the other components being cyanidin-3-glucoside (5 – 40 %) and cyanidin-3-sophoroside (2 – 4 %). The anthocyanin accumulation depends upon plant cultivar and plant maturation and may exceed 0.25 g per 100 g FW (as cyanidin-3-glucoside chloride equivalent).

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