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Solanum retroflexum Fruits: A Rich Source of Anthocyanins.

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ABSTRACT

Cultivation of Solanum retroflexum by seedling method permits to get a good harvest of fruits with average fruit yield 1.2 kg/m2. The fruits accumulate up to 700 mg of anthocyanins (expressed as cyanidin-3-glucoside chloride equivalent) per 100 g of FW. The main component of anthocyanins is petunidin-3-(p-coumaroylrutinoside)-5-glucoside accounting for almost 80 % proving the fruits to be a rich source of acylated anthocyanins. The advantage of the anthocyanins is a possibility of preparation of spray dried forms with maltodextrin as a matrix of two colors, red and blue.

Keywords: Solanum retroflexum, fruits, anthocyanins, maltodrxtrin, extraction, purification, spray drying.

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INTRODUCTION

Anthocyanins are prominent natural substances that in acidic media are in colored flavylium form being responsible for coloration of fruits, flowers, leaves and other plant parts [1]. Due to this property anthocyanins as natural compounds may substitute synthetic colorants in food and medicinal industries. The substitution is highly desirable because of possible negative influence of synthetic substances upon men health, while anthocyanins are active water soluble antioxidants revealing a series of biological activity expressions [2].

High level of anthocyanins accumulation can be detected by a dark (red, blue or even black) coloration of plant material. *Solanaceae* family includes a lot of plants that may be used as sources of anthocyanins. Though, for example, eggplant (*Solanum melongena* L.) being of a dark purple coloration is not a rich source because anthocyanins are accumulated only in the thin skin of the fruit [3]. The same is true for exotic varieties of purple tomatoes (*S. lycopersicum*) [4] and sweet pepper (*Capsicum annuum*) [5]. Still the exotic varieties of purple potato (*S. tuberosum*) are more promising sources of anthocyanins in the case of pulp dark coloration [6] as well as some *Solanacea* plants small fruits – *S. scabrum* [7], *S. stenotomun* [8] and *S. nigrum* [9].

Anthocyanins content in tubers of the best *S. tuberosum* cultivar (of Guincho Negra genotype) exceeded 1.5 % per dry weight [10].Fruits of *S. scabrum* accumulate 0.8 – 1.4 g of anthocyanins per 100 g of fresh weight [7]. The latter plant is uncommon for Russian Federation but another closely related to it species [11] *S. nigrum* is found in wild nature. But a promising large-fruited plant of the family known as “sunberry” may be of specific interest for introduction in RF. The plant produces a lot of shiny black fruits indicating an intensive anthocyanins biosynthesis: the fruits are black not only due to skin coloration, the matured fruits have dark colored pulp as well. The nature of the species of the *Solanum* genus is not clear. The systematic name of the plant is *S. retroflexum*, it is regarded to be a hybrid between *S. scabrum* and *S. villosum* (that was introduced by L. Burbank [12]). But this plant occurs naturally in Africa [13] and according to morphological and DNA markers is closely related to *S. villous,* both belonging to one of the most widespread and variable species groups of the genus *Solanum* L. centering around the type species *Solanum nigrum* L., the black nightshade.

The major anthocyanin of the *S. nigrum* fruit is known to be petunidin-3-(*p*-coumaroylrutinoside)-5-glucoside [9]. This compound is common for many plants of the genus [6 - 11] and is believed to be synthesized from delphinidin derivative by specific anthocyanin-3’-O-methyltransferase, while for production of malvidin derivatives anthocyanin-3’,5’-O-methyltransferase is necessary [14].

Information about anthocyanins of *S. retroflexum* was not found in accessible for us literature. Thus, the aim of a present paper is an estimation of the plant fruits as a source of anthocyanins for encapsulation in natural polymers matrix by spray drying method.

MATERIALS AND METHODS

Plant cultivation

Method I - the seedling method. Seeds of *S. retroflexum* were sown in the greenhouse in the first decade of April. The seedlings were planted in open ground in late May with a distance between seedlings 40-45 cm.

Method II - the sawing method. Seeds of *S. retrofllexum* were sown directly in open ground in the first decade of May.

Mature fruits were harvested starting from the second decade of August (method I) or from first decade of September (method II).

Fruits were laid for storage in the freezer.

Anthocyanins extraction

For determination of anthocyanin accumulation in fruits grinded plant material was mixed with 0.1 M solution of HCl in distilled water (solvent I) in ratio 1 : 100 (g/ml) and macerated overnight. Then extract was separated from solid residue by vacuum filtration through polyamide membrane filters with pores 0.45 mcm (BIOCHEMMACK, ST, Moscow, RF). The solid residue was as a rule almost colorless proving an exhaustive anthocyanin extraction.

For preparative anthocyanins extraction sample to solvent ratio was increased and for exhaustive anthocyanin extraction several successive stages were explored as well as another solvent II for extraction was used being 1 % of concentrated water HCL solution in ethanol.

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) with subsequent desorption with mixture of 30 vol.% of CH_3CN and 30 vol.% of HCOOH in water (40 vol.%) and subsequent dilution with water 1 : 2 by volume. Before sorption cartridges were washed with acetone (5 ml) and conditioned with 0.1 M HCl water solution (15 ml).

Semi-preparative anthocyanin separation

For semi-preparative petunidin-3-(*p*-coumaroylrutinoside)-5-glucoside (Pt3CoumR5G) 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: 14 vol. % of CH_3CN, 10 vol. % of HCOOH and 76 vol. % of distilled water, mobile phase flow rate was 5 ml·min^{-1}, peaks monitoring at 525 nm.

Anthocyanin hydrolysis

For partial 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.

Qualitative and quantitative determination of anthocyanins

Overall anthocyanin concentration in solutions was determined by the spectroscopic differential method [15] 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; the mobile phase: 12 vol. % of CH_3CN, 10 vol. % of HCOOH and 78 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 14 vol. % of CH_3CN, 10 vol. % of HCOOH and 76 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.

To monitor products of partial anthocyanins hydrolysis linear gradient was used starting 100 % of eluent A (8 vol.% of CH_3CN, 10 vol.% of HCOOH in water) to 100% of eluent B (18 vol.% of CH_3CN, 10 vol.% of HCOOH in water) in 20 min.

Spray drying

To prepare dried forms of anthocyanins in maltodextrine matrix solutions spray dryer EYELA SD-1000 was explored. Conditions for drying: air flow 1 m^3/min; the nebulizer spray generation at a flow rate of 1.5 L/min, pressure 120 kPa and temperature 120^oC.

Freeze drying

For anthocyanin precipitate drying taking into account low stability of the substances lyophilization was used in equipment LABCONCO FreeZone 2.5. The samples were preliminary frozen at -20^oC, the drying was completed in 6 - 7 hr at pressure 0.03 mBar and condenser temperature -50^oC.

Chemicals and equipment

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, ethanol, concentrated water HCl solution and distilled water were used for extraction and partial purification of anthocyanins.

To prepare dried forms maltodextrin DE 18-20 (SportPit, RF) was used as a matrix.

RESULTS AND DISCUSSION

Anthocyanins separation and identification

According to chromatographic profile, Fig.1 and Table 1, fruits of *S. retroflexum* are colored by a series of anthocyanins with one type of glycosylation being predominated. There are the three glycosides having acylated with *trans-p*-coumaric acid rhamnosyl moiety in 3-rutinoside-5-glucoside structures of delphinidin (Dp3CoumR5G) (peak 1), petunidin (Pt3CoumR5G) (peak 5) with a small addition of *cis*-isomer (peak 4) and malvidin (Mv3CoumR5G) (peak 7) that may be identified by mass-spectrometric (with partial fragmentation) and spectrophotometric data.

Table 1: Anthocyanins of *Solanum retroflexum* fruits

No*	Solute structure	Molecular and fragmented ions, M/z				λ_{max}, nm	Mole** fraction, %
1	Dp3CoumR5G	919.1	627.1	465.1	303	529.5	4.8
2	Pt3CafR5G	949.1	641.1	479.1	317	531.5	1.4
3	Dp3FerR5G	949.1	-	465.1	303	530.5	1.0
4	Pt3(*cis*-Coum)R5G	933.2	641.1	479.1	317	534.5	2.1
5	Pt3(*trans*-Coum)R5G	933.2	641.1	479.1	317	531.5	77.8
6	Pt3FerR5G	963.2	641.1	479.1	317	532.5	7.7
7	Mv3CoumR5G	947.2	655.2	493.1	331.1	534.0	4.9
							98.7

* - number of peak on Fig.1; ** - calculated for peak areas;

Indeed besides of the main signals with M/z corresponding to “molecular” ions, dihexosides as products of acylated rhamnosyl moiety cleavage, monohexosides and corresponding aglycone ions are detected, Table 1. Electronic spectra also support the proposition by consecutive 1.0 ÷ 1.5 nm bathochromic shifts of spectrum maxima moving from delphinidin to malvidin derivatives Fig.2, as well as bathochromic shift for pair *trans*- and *cis*-isomers of petunidin derivatives.

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Additionally not only products of acylation of petunidin-3-rutinoside-5-glucoside by two isomeric *trans* (the main peak 5) and *cis p*-coumaric acids (peak 4), but also the substitution of the acylating acid by ferulic acid: (peak 3) – delphinidin-3-(feroylrutinoside)-5-glucoside (Dp3FerR5G) and (peak 6) – petunidin-3-(feroylrutinoside)-5-glucoside (Pt3FerR5G) as well as by caffeic acid: (peak 2) – petunidin-3-(caffeoylrutinoside)-5-glucoside (Pet3CafR5G) are found in the mixture.

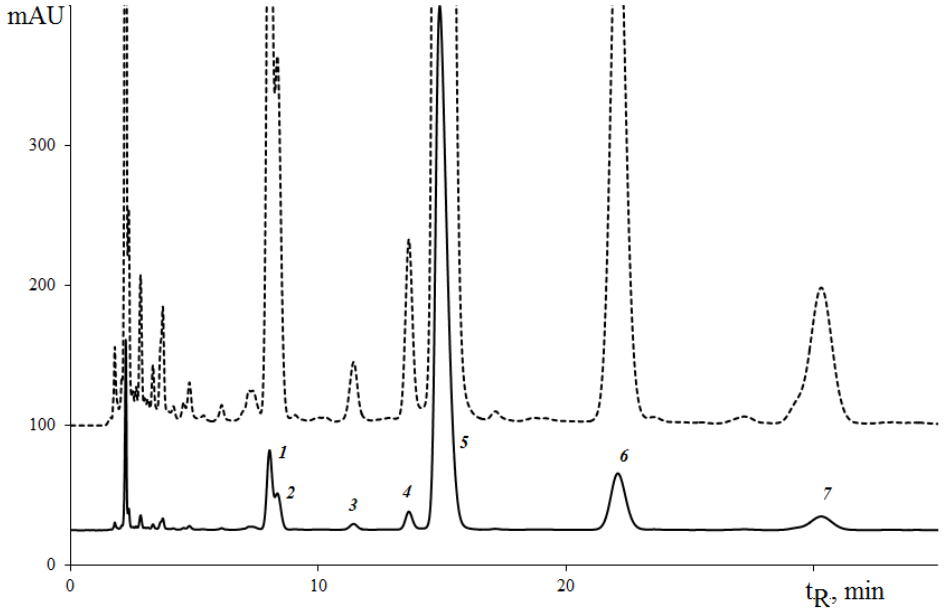

Figure 1: Separation of *Solanum retroflexum* fruit anthocyanins

Column: 150×4.6 mm Symmetry C18, 5 mcm; mobile phase: 10 vol.% of CH_3CN, 10 vol.% of HCOOH in water, 0.8 ml/min; 515 nm. 1 – Dp3CoumR5G; 2 – Pt3CaffR5G; 3 – Dp3FerR5G; 4 – Pt3(*cis*-Coum)R5G; 5 – Pt3(*trans*-Coum)R5G; 6 – Pt3FerR5G; 7 – Mv3CoumR5G.

The structure of the main component Pt3(*trans*-Coum)R5G (or simply Pt3CoumR5G) was confirmed by HPLC analysis of substances formed during partial hydrolysis of the semi-preparatively isolated substance. The possible products of acidic hydrolysis are summarized in the reaction schema on the Fig.3, and are found on the chromatogram, Fig.4. The results support the known fact about relative stability of ester bond compared to glycosidic bond towards acidic hydrolysis [16], because of a small yield of products formed by ester bond hydrolysis (Pt3Rut5Glu and Pt3Rut), not shown in Fig.1.

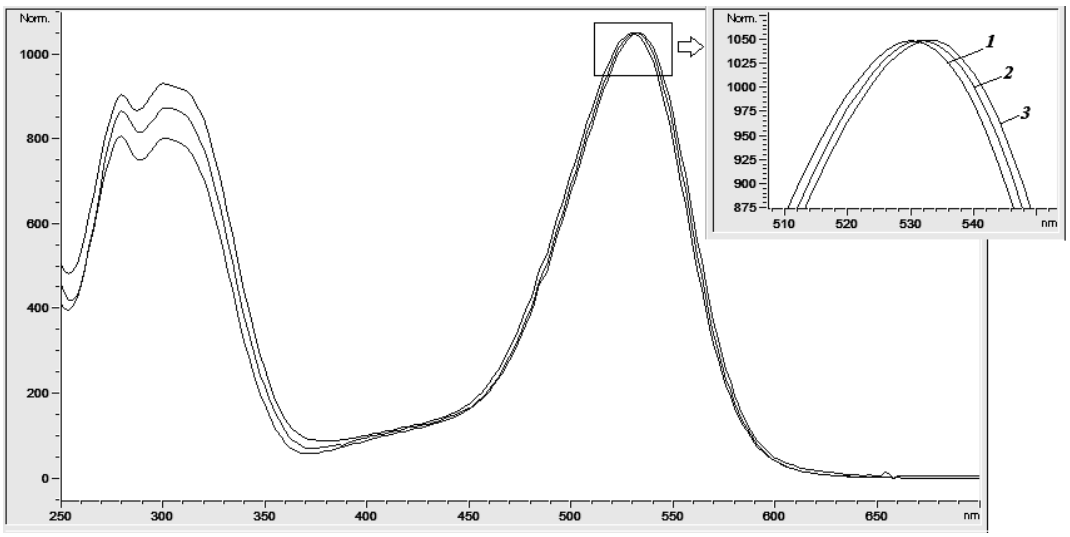

Figure 2: The shift of electronic spectra in series of the same delphinidin – petunidin – malvidin derivatives Solvent 10 vol.% of CH_3CN, 10 vol.% of HCOOH in water. Solutes: 1 – Dp3CoumR5G; 2 – Pt3CoumR5G; 3 – Mv3CoumR5G.

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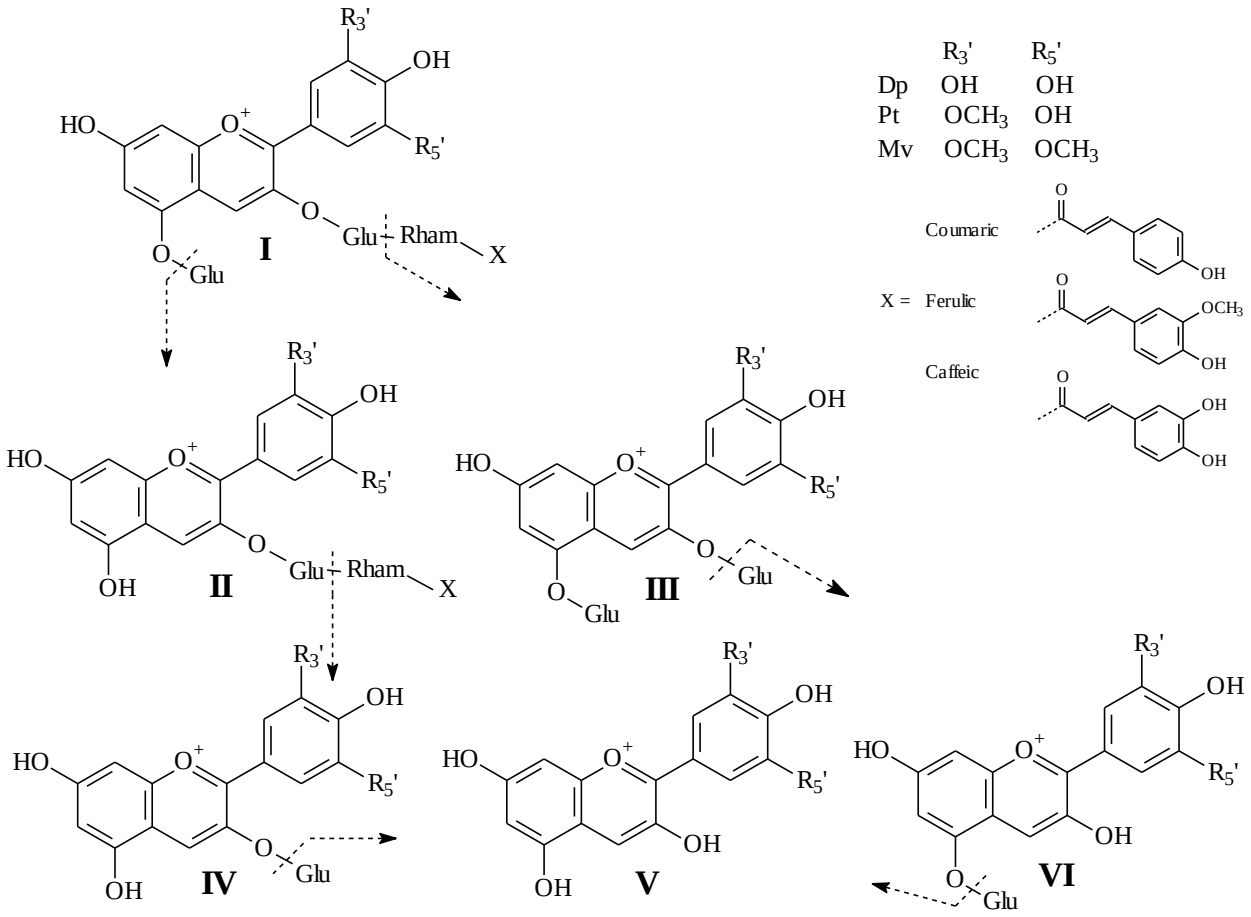

Figure 3: Structure of main anthocyanins of *Solanum retroflexum* fruits and hydrolysis products of the main anthocyanin

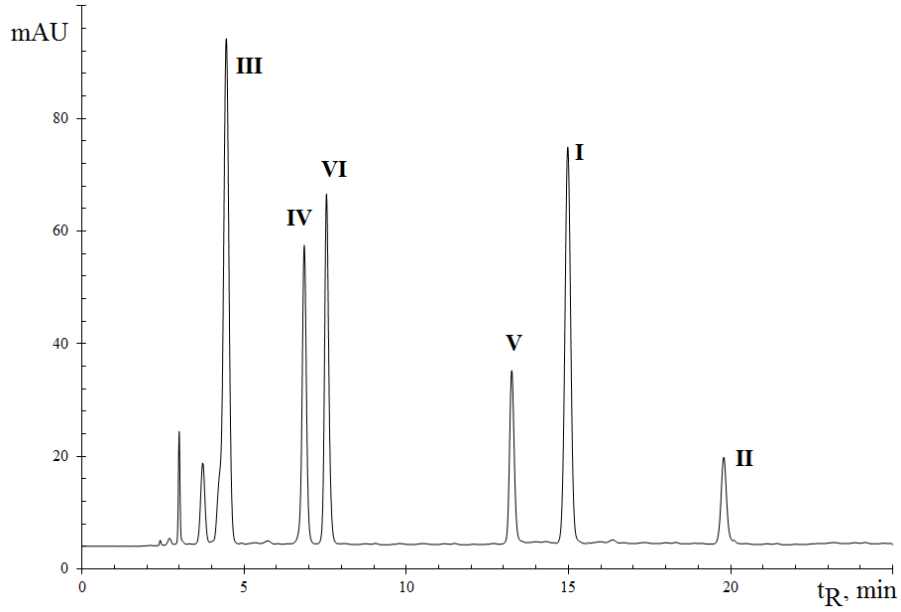

Figure 4: Separation of Pt3CoumR5G acidic hydrolysis compounds
Peaks numbering see Fig.3. For gradient elution conditions see Experimental section.

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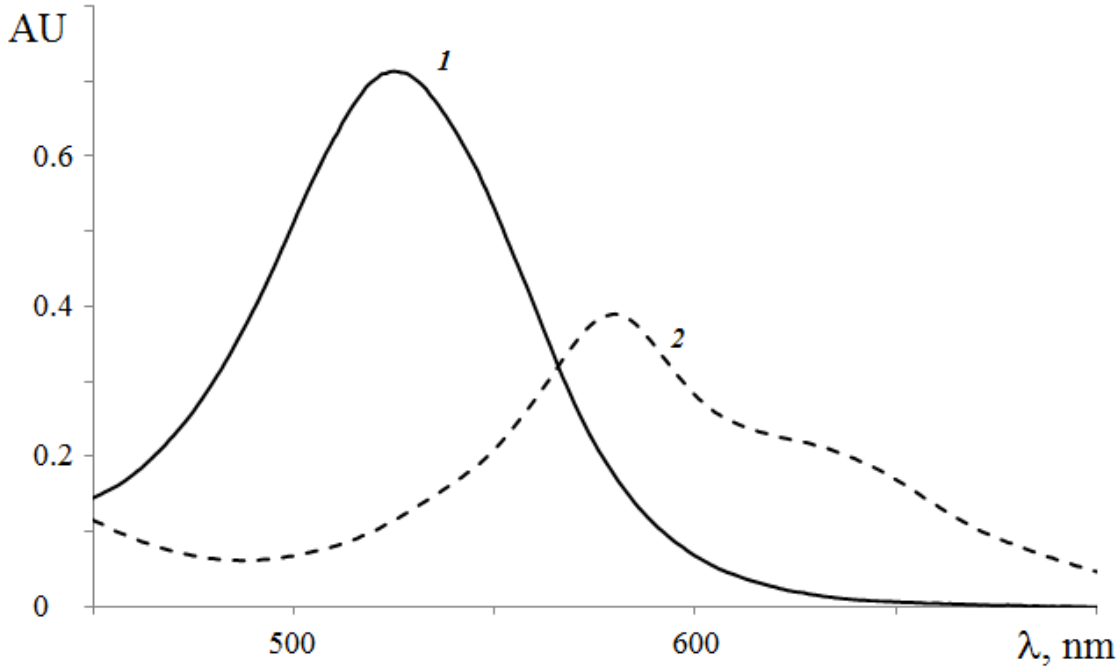

Figure 5: Electronic spectra of *Solanum retreflexum* fruit anthocyanins at pH=1 (1) and pH=8 (2) in water solution

Anthocyanins extraction and quantification

Fruits of "sunberry" become black long before maturing but for synthesis of anthocyanins in the pulp being initially green much more time is necessary. So, fully ripe fruit has dark coloration all over the fruit.

To determine the level of anthocyanin accumulation the whole uncut fruits were macerated in solvent I in ratio (0.5 – 1.5 g) : 100 ml overnight. The procedure was chosen to minimize pectin and other polymeric substances extraction and to convert all anthocyanin forms into flavylium one. It should be emphasized that the recommended [15] time of anthocyanin solution with pH = 1 equilibration (from 15 min to 1 hr.) may be not sufficient. These are slowly equilibrated transformations of some anthocyanin forms (existing in solutions with pH > 1) into colored flavylium one [17] that need much more time to establish euillibrium. The time depends upon state of anthocyanins in initial material. Thus to achieve almost constant optical density of solution with pH = 1 of precipitated anthocyanins (see down) in solvent I 24 hr. is necessary.

The results of anthocyanins determination in ripe "sunberry" fruits are given in Table 1.

The data indicate that for the exhausting anthocyanin extraction as a rule three stages must be applied while during the first stage the yield of anthocyanins is very high reaching 78 – 98 %, and the third stage may be excluded. According to overall anthocyanin accumulation in region 450 – 700 mg per 100 g of FW of the "sunberry" fruits are rich sources of anthocyanins being comparable with the other edible fruits. The plant advantage is the possibility of automatized harvesting after autumn leaf fall when remains "naked" stems with a plenty of black fruits.

During two years of investigation of the plant introduction in Belgorod State University Botanical garden it has been found that seedling method of cultivation is much more effective than direct sowing of seeds into the soil. The plant height may exceed 1.5 m; flowering period starts in June and continues till first September frosts, fruit diameter riches 2 cm. The average fruit yield is 1.2 kg/m^2.

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Fruits storage and anthocyanins extraction

Harvested fruits were stored in freezer (-25°C). During the storage meanwhile the concentration of anthocyanins was found to drop significantly, Table 3. Although in our experience anthocyanins of the fruits of *Aronia michurinii* are even less stable, the anthocyanins become lost almost completely during the same storage period.

For anthocyanins extraction maceration was used in a proper extraction solution after fruit thin cutting with blender till visual homogenization. For the extraction two methods were investigated.

Method I. Extraction with solvent II. The advantage of the method is the possibility to escape pectic substances extraction that may interfere with the forthcoming procedures of filtration and purification.

The anthocyanins yield (the data in the Table 2 refers to experiments with 6 months fruits storage) at the first stage of extraction becomes constant for solvent to solid ratios more than 5 : 1 (ml to g). The maximum of anthocyanins concentration was found to be 1.65 g/l but for the further operations anthocyanins concentration of 0.27 g/l is preferable because of the maximum yield. The extract is easily separated from residue by filtration through the paper filter under vacuum. The extract is diluted with solvent I (1 : 1 vol.) and before extract partial purification ethanol is withdrawn in vacuum rotary evaporator system.

For partial solid phase extractions series of three consecutive columns (with total volume of 200 cm^3) filled with polymeric sorbent (Sepabeads SP825/L) were used. The column after activation (with 100 ml of ethanol) and conditioning (with 200 ml of 0.1 M water HCl solution) were used to sorb anthocyanins from the concentrate. The liquid flow was created by sparse pressure in receiving vessel (Bunsen flask). After sorbent saturation the columns were washed with 50 ml of 0.1 M water HCl solution and anthocyanins were desorbed by solution II.

The resulting dark colored concentrate (with 5 – 7 g/l of anthocyanins) was diluted with solvent I in ratio 1 : 1 (vol.) and ethanol was withdrawn in vacuum rotary evaporator system. The resulted extract was transferred into refrigerator for storage. After some days of the concentrate storage dark precipitate was formed and the concentration of anthocyanins dropped to ~ 3.2 g/l.

The precipitate was freeze dried to get a powder with molar absorptivity (ε) 27520 ± 70 $l \cdot mol^{-1} \cdot cm^{-1}$ (n = 2) calculated as petunidin-3-(*p*-coumaroylrutinoside)-5-glucoside chloride.

Method II. Extraction with solvent I. The method was slightly less efficient than method I resulting in lower anthocyanins concentration and yield, Table 2.

Table 2: Extraction of anthocyanins for the determination of overall accumulation

No	Sample (g) : solvent I (ml) ratio	Concentration of 1 stage macerate, g*/l	Anthocyanins yield, g / 100 g FW, at extraction stages			
			1	2	3	summ
1	1 : 100	0.0428	0.357	0.091	0.008	0.456
2	1 : 100	0.0684	0.670	0.024	0.001	0.695
3	0.5 : 100	0.0220	0.611	0.038	0.001	0.650
4	1 : 100	0.0364	0.391	0.048	0.028	0.467
5	1.5 : 100	0.1050	0.661	0.007		0.668
					Mean value	0.587

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Table 3: Extraction of anthocyanins for preparative purposes

Solvent (ml) to sample (g) ratio	Concentration of macerate, g*/l, mean value and range (n=4)	Anthocyanins yield, g / 100 g FW
Extraction by 1 % HCl in ethanol		
1.25 : 1	1.65 ± 0.07	205.8 ± 9.0
2.5 : 1	0.98 ± 0.01	245.6 ± 3.0
5 : 1	0.55 ± 0.02	275.7 ± 8.8
10 : 1	0.27 ± 0.03	272.3 ± 25.8
Extraction by 1 M HCl water solution		
1.25 : 1	1.08 ± 0.07	135.3 ± 0.1
2.5 : 1	0.76 ± 0.02	188.9 ± 5.5
5 : 1	0.48 ± 0.02	240.5 ± 8.1
10 : 1	0.26 ± 0.01	257.7 ± 8.2

Preparation of dried compositions of anthocyanins with maltodextrin

Maltodextrine seems to be the most popular matrix to prepare compositions with anthocyanins [18]. So some forms with different anthocyanins concentrations were prepared by spray drying of anthocyanin concentrate in witch calculated amount of dry maltodextrine was dissolved. The resulting product has intense (depending upon anthocyanins concentration, Table 4) red color with tiny bluish shadow.

Table 4: Anthocyanin content in two types of dried forms

No.	Anthocyanin concentration in dried form, %		The loss of anthocyanins, relative %
	Theoretically	Practically, mean for n=2	
Red forms			
1	1	0.89 ± 0.01	11.0
2	5	4.17 ± 0.02	16.6
3	10	6.68 ± 0.03	33.2
Blue forms			
3	1	0.59 ± 0.05	49
5	5	1.55 ± 0.04	69
6	10	3.05 ± 0.08	70

It becomes obvious that the loss of anthocyanins at spray drying is not insignificant. But the resulting products are readily dissolvable in water to form colored solutions without formation of any precipitate.

The bright red color of the composition is worthy of special consideration. Indeed liquid solutions with the same anthocyanins concentrations are nearly black due to copigmentation. Thus in our cases we are dealing with composition in witch sugar cycles of maltodextrin disturb direct intermolecular stacking of flavylium ions preserving the initial color of the latter. So the usage of definition of the forms as microencapsulated [18] (that means incorporation into maltodextrin matrix of small drops of anthocyanins that must have dark color due to intense stacking) is not correct. By the way, freeze drying demanding significantly more time to complete drying (that may be suitable for the ions stacking rearrangement) usually results in more dark coloration of the product with the same concentration of anthocyanins.

The useful advantage of “sunberry” anthocyanins is red color (of acid solution in water) turning into a blue one in near neutral solutions due to formations of quinoidal forms, Fig.5. This phenomenon permits to prepare not only traditional red colored dry anthocyanin compositional forms but also blue colored materials. The only change in the method of producing such a form includes addition to the initial anthocyanin

concentrate of sodium hydroxide solution to change pH (to 6 - 8) to achieve solution blue coloration before addition of maltodextrin. The results of spray drying in this case are given in Table 4.

The results indicate that the losses of anthocyanins at spray drying for blue forms are significantly greater than in the case of red forms. It may be explained by known less stability of anthocyanins in form other than flavylium one. But the product is also readily dissolvable in water to form clear solutions.

CONCLUSIONS

Cultivation of *Solanum retroflexum* by seedling method permits to get a good harvest of fruits being a rich source of acylated anthocyanins that may be an be obtained in the spray dried forms of two colors, red and blue.

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REFERENCES

[1] Raghvendra, Sharma V, Shakya A, et al. Internat J Pharm Sci Rev Res 2011; 6: 28–33.
[2] McGhie TK, Walton MC. Mol Nutr Food Res 2007; 51: 702 – 713.
[3] Azuma K, Ohyama AK, Ippoushi K, et al. J Agric Food Chem 2008, 56: 10154–10159.
[4] Mes PJ, Boches P, Myers R. J Amer Soc Hort Sci2008; 133: 262–269.
[5] Sadilova E, Stintzing FC, Carle R. Z Naturforsch 2006; 61c: 527–535.
[6] Eichhorn S, Winterhalter P. Food Res Internat 2005; 38: 943–948.
[7] Lehmann C, Biela C, Töpfl S, et al. Euphytica 2007; 158: 189–199.
[8] Cardoso LC, Serrano CM, Quintero ET, et al. Molecules 2013; 18: 3137–3151.
[9] Saito N, Hotta R, Imai K, et al. Proceed. Japan Acad 1965;41: 593–598.
[10] Andre CM, Oufir M, Guignard C, et al. J Agric Food Chem 2007; 55: 10839–10849.
[11] Poczai P, Taller J, Szabó I. Plant Syst Evol 2008; 275: 59–67.
[12] Schilling EE. System. Botany 1981; 6: 172–185.
[13] Jacoby A, Labuschagne MT, Viljoen CD. Euphytica 2003; 132: 109–113.
[14] Provenzano S, Spelt C, Hosokawa S, et al. Plant Physiol 2014; 165: 962–977.
[15] Giusti MM, Wrolstad RE. Current protocols in food analytical chemistry. New York: John Wiley & Sons. 2001, F1.2.1– 1.2.13.
[16] Okumura H, Kitazawa N, Wada S, et al. J Oleo Sci 2011; 60: 313–320.
[17] Pina F, Petrov V, Laia CAT. Dyes Pigments 2012; 92: 877–889.
[18] Özkan G, Bilek SE. Internat J Nutr Food Sci 2014; 3: 145–156.