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Product Quality and Safety

Extraction and characterization of anthocyanin colorants from plant sources

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Abstract. *Natural pigments (and especially those of anthocyanins) are a valuable source of bioactive compounds and may be used in the production of new functional food ingredients. Furthermore, their applications in the treatment and prevention of chronic disorders are becoming more and more widespread. In the last few years consumers have focused their attention on the natural biologically active compounds as functional food ingredients, and therefore, it may be assumed that natural colorants are an alternative source of synthetic additives. The aim of the study was to determine the quantitative content of monomeric anthocyanin pigments in extracts obtained from eight plants. The total content of monomeric anthocyanin pigments was measured by a pH-differential method. The TLC analysis of the pigment extracts from the different plants showed intensive rose, red and violet stripes corresponding to the anthocyanin content. The extracts from chicory and lavender petals were unstable and their color decreased in intensity in 1 month. The analysis of the experimental data shows that the yield of pigment substances depends on a few factors: the type of plant, the preliminary treatment of the plant and the solvent that is used. The largest quantity of extracted substances in the studied plants were isolated from chokeberry (2 195.9 cyd eq mg/l), followed by blackberry (1 466.2 cyd eq mg/l) and one variety of the grapes (1 199.3 cyd eq mg/l). In the case of chokeberry, the pigment content included a large number of anthocyanins and the combination of these components was the reason for the deep red/violet color of the extract. Fresh or frozen materials are the most suitable for extraction of anthocyanin pigments. On the whole, fruit pulp yielded a larger quantity of pigments than juice. Anthocyanins are water-soluble compounds and for that reason their isolation requires water and other polar solvents. Better stabilization of color is obtained by a slight acidification of the solvent with diluted hydrochloric acid. Due to the high content of organic acids in fruits, this effect is attained in a natural way.*

Keywords: natural pigments, anthocyanins, extraction, healthy food

Introduction

Currently, pigments in various types and shapes are widely used as additives in the food industry, cosmetics, pharmaceutical industry, food stuffs, etc. (Boo et al., 2011; Lazze et al., 2004; Katsube et al., 2003). The accumulated data that many synthetic colorants cause health problems, including toxicity and carcinogenicity, led to a surge of interest in natural dyes (Puupponen-Pimia et al., 2005; Wang and Mazza, 2002.). In response to this trend, and in order to protect human health, experts are looking for ways to apply natural colorants in the manufacture of new health and functional foods, cosmetic products, etc. (Jensen et al., 2011; Kim et al., 2011; Sivakumar et al., 2011, Bener et al., 2010). Sources of natural pigments are plants, animals and microorganisms, but only some of them are available in sufficient quantities for commercial use as colorants for food and most are derived from plants (Commission Regulation (EU) 231/2012).

In general, pigments are organic or inorganic compounds that can absorb electromagnetic waves with length from 350 to 750 nm (visible light). Color is an important factor for consumers when choosing a final product, which is of great importance for the food industry (Boo et al., 2012). Plant pigments include a variety of different compounds, including anthocyanins, carotenoids and chlorophylls (Jensen et al., 2011). They can be classified into two broad groups lipid-soluble and water-soluble (Kong et al., 2003; Wrolstad, 2004). Among the most common lipid-soluble pigments are chlorophyll and carotenoids. The most common water-soluble pigments, on the other hand, are anthoxanthins, anthocyanins, betalains, etc. Anthocyanins are the most varied in composition and in color.

Anthocyanins are water-soluble polyphenol natural pigments. These substances give the flowers and fruits a variety of different colors: orange, pink, red, violet and blue (Kumar and Sinha, 2004; Gris et al., 2007; Castaneda-Ovando et al., 2009; Scotter, 2011). Anthocyanins occur in nature as glycosides of polyhydroxy or polymethoxy derivatives of 2-phenylbenzopyrylium salts, such as aglycones, known as anthocyanidins (Scotter, 2011; Scotter and Castle, 2004). The most commonly found anthocyanidins in nature are cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin (Gris et al., 2007; Castaneda-Ovando et al., 2009; Scotter, 2011). The sugar can be attached at various positions, and can be glucose, galactose, xylose, rhamnose and arabinose. Glycosylation ensures better stability in water-medium, compared to the source aglycone. The coloring of plants is rarely due to only one anthocyanin. The wide variety in coloring is determined by the number of hydroxyl groups, the degree of methoxylation, the type and number of sugar residues, their position of linking, the environmental pH etc. (Solomosi et al., 2015).

Anthocyanins can exist in different forms depending on the pH of the solution. When pH is 1.0 the dominant ion is flavylium cation (red color) and the purple and red coloring prevails. At pH 4.0 blue quinonoidal structures prevail. When there is a pH value between 5.0 and 6.0 two colorless structures - carbinol base and chalcone are observed. At pH above 7.0 anthocyanins decompose depending on

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the substitutes contained. At pH between 4.0 and 6.0 there are four structural forms of anthocyanins – flavylium cation, anhydrous quinonoidal base, colorless carbinol base and pale yellow chalcone. Changes in color of anthocyanins are more significant in an alkaline environment due to their instability (Castaneda-Ovando et al., 2009).

Apart from pH, anthocyanin color depends on the structure and the concentration of the pigment, the presence of metal ions, oxygen, enzymes, sugars, pigments and other factors. Co-pigments can be flavonoids, alkaloids, amino acids, organic acids, polysaccharides, metals, and anthocyanins. Their main role is to protect the color flavylium cation from the nucleophilic attack of the water molecules. Oxygen is an important factor affecting the degree of decomposition of anthocyanins, along with the partial opening of the ring structure. Chemical stabilization of anthocyanins is the subject of numerous modern researches due to the various possibilities for their application, their performance and use as an alternative to synthetic dyes (Gomez-Plaza et al., 2006; Castaneda-Ovando et al., 2009).

It has been found that anthocyanins show no toxic or mutagen action even at high concentrations. They can be used in food, cosmetic and pharmaceutical industry not only as substitutes for synthetic colorants but also because of the wide range of biological activities. They exhibit antioxidant properties (Kong et al., 2003; Kumar and Sinha, 2004; Gomez-Plaza et al., 2006; Gris et al., 2007; Castaneda-Ovando et al., 2009;), reduce the risk of cancer (Katsube et al., 2003; Lazze et al., 2004; Kim et al., 2012), have anti-inflammatory properties (Yuodim et al., 2002), and can modulate the immune response (Mazza and Wang, 2002).

Anthocyanin pigment content can be determined by different methods – HPLC analysis, spectrophotometric methods, etc. The pH differential method has been used extensively by food technologists and horticulturists to assess the quality of fresh and processed fruits and vegetables, fruit juices, nutraceuticals and natural colorants. This spectrophotometric method is based on the structural change of anthocyanin chromophore in different pH, 1.0 and 4.5, respectively (Lee et al., 2005). The pH-differential method is rapid, simple and economically advantageous. Lee et al. (2008) established a very good correlation between the pH differential method and HPLC when determining the amount of anthocyanins found in various samples.

The aim of the study was to determine the quantitative content of monomeric anthocyanin pigments in extracts obtained from eight plants

Material and methods

The subject of current research were eight plant materials – fruit of chokeberry (*Aronia sp*), blackberry (*Rubus fruticosus*), bilberry (*Vaccinium myrtillus*), three red grape varieties (*Vitis vinifera*) – Gamza, Muscat of Hamburg and Pinot Noir, chicory (*Cychorium intibus*) and lavender (*Lavandula angustifolia*) flowers. The flowers and fruits were collected in the summer and autumn of 2015.

The extraction of plant material was conducted with various extraction solvents: water-ethanol solutions at a concentration from 50 to 95% v/v, with or without acidification. The mixtures were placed in an ultrasonic bath (Model 7652 Ultrasonic System) at room temperature and sonicated for 20 min.

The residual moisture content of the plant raw materials was measured with Sartorius Thermo Control balance YTC 01L.

Qualitative analysis and identification of anthocyanins in pigment extracts were carried out using thin layer chromatography (TLC) according to the method of Krüger et al. (2013). To a 1 ml sample of each extract were added 9 ml of 0.5% hydrochloric acid in 96% ethanol, stirred for about 5 min and centrifuged for 5 min at 3000 rpm. The supernatant was collected and stored in the dark. The samples were sprayed as 10.0 mm bands on TLC plates of silica gel 60 F254 (Kieselgel 60 F_{254}, Merck). Sample volumes ranged between 40 and 60µl. Development was performed with a mixture of ethyl acetate, n-butanol, formic acid, water (70:30:12:8; v/v/v/v). The migration distance was 10 cm from the lower edge of the plate. After development, the plate was dried at ambient temperature and observed in visible light.

Quantitative determination of total monomeric anthocyanins in extracts was carried out with the pH-differential method used by Lee et al. (2005). Test samples were diluted in 50 ml volumetric flasks. The appropriate dilution factor was determined by diluting the test portion with pH 1.0 buffer, until absorbance at 520 nm was within the linear range of the spectrophotometer. Using this dilution factor, 2 dilutions of the test sample were prepared, one with pH 1.0 (KCl 0.025M) buffer and the other with pH 4.5 (sodium acetate, 0.4M) buffer. The absorbance of the test portion diluted with pH 1.0 buffer and pH 4.5 buffer was determined at both 520 and 700 nm.

The anthocyanin pigment concentration, expressed as cyanidin-3-glucoside equivalents, was calculated as follows:

$$Anthocyanin\ pigment\ (cyd\ eq\ mg/l) = \frac{A \,.\, Mw \,.\, DF \,.\, 10^3}{\varepsilon \,.\, l}$$

where A = (A520nm – A 700nm) pH 1.0 – (A520nm – A700nm) pH 4.5; Mw = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in D; l = pathlength in cm; ε = 26900 molar extinction coefficient, for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

The statistical processing of the data was done with the help of Microsoft Excel 2013. The data represent mean ± standard deviation (SD) of the three independent experiments. The data were analyzed by one-way ANOVA. Differences were considered statistically significant when the p level was less than 0.01.

Results and discussion

The following fruits were selected for extraction of anthocyanin pigments: blackberry, bilberry, chokeberry and three grape varieties (Table 1). Two variants from every kind of fruit were studied – juice and pulp. Further extraction from chicory and lavender flowers was conducted. The raw materials were previously refined into small pieces or pressed to separate the juice from the pulp then the dry matter content (g/100g) in each of the samples was determined (Table 1). Because of the high moisture content of the samples, extraction was carried out with hydro module 1:5 (by weight of the dry matter in fruits).

In order to choose an appropriate solvent, a preliminary experiment was made with 4 different concentrations of ethanol – 50, 70, 80 and 95% (v/v), with and without acidification. In samples with acidification, hydrochloric acid was added to water-ethanol mixture at a concentration of 0.1%. In this test the fruit of chokeberry (pulp) was selected as plant material. The results of the quantitative content of anthocyanin pigments in each of the variants are presented in Table 2. The highest yield of anthocyanins was obtained for variants 4 and 8 (extraction with 95% ethanol). For this

Table 1. Characteristic of plant raw materials used

Plant material	Variant	Dry matter		Color of the resulting extract
		g/100g	±SD	
Grape variety "Gamza"	juice	28.20	0.92	Dark-Violet
	pulp	34.10	0.90	Dark-Violet
Grape variety "Muscat of Hamburg"	juice	22.67	2.15	Violet
	pulp	26.65	1.64	Violet
Grape variety "Pinot Noir"	juice	26.55	1.78	Violet
	pulp	33.67	3.05	Violet
Chokeberry	juice	18.66	1.49	Violet-Red
	pulp	30.70	4.56	Violet-Red
Bilberry	juice	12.38	1.35	Violet
	pulp	26.77	3.11	Violet
Blackberry	juice	12.38	2.36	Dark-Red
	pulp	23.90	4.00	Dark-Red

Table 2. Content of anthocyanin pigments in extracts of chokeberry obtained by varying the concentration of ethanol. The results are presented as means ± SD (n = 3)

№	Solvents	cyd eq (mg/l)	±SD
1	Ethanol 50%	1639.8a	14.3
2	Ethanol 50% + 0.1% HCl	1630.5b	21.1
3	Ethanol 70%	2685.2a	36.7
4	Ethanol 70% + 0.1% HCl	2693.7b	29.6
5	Ethanol 80%	2721.9a	37.2
6	Ethanol 80% + 0.1% HCl	2728.2b	41,3
7	Ethanol 95%	2885.6a	27.3
8	Ethanol 95% + 0.1% HCl	2881.9b	35.7

*Identical letter indicated significant differences between means ($p < 0.01$)

type of material (fresh fruit), the addition of ethanol with high concentration promotes better penetration of the solvent into the plant matrix and better extraction of the pigments. There were no significant differences between the versions with and without acidification. All other extractions of anthocyanins from fruit (juice and pulp) were carried out with 95% ethanol without acidification and in the ratio of 1:5 (by weight of the dry matter in fruits). Extraction of lavender petals and chicory was held with 80% ethanol, acidified with hydrochloric acid 0.1%. The time of the process was 48 h at room temperature.

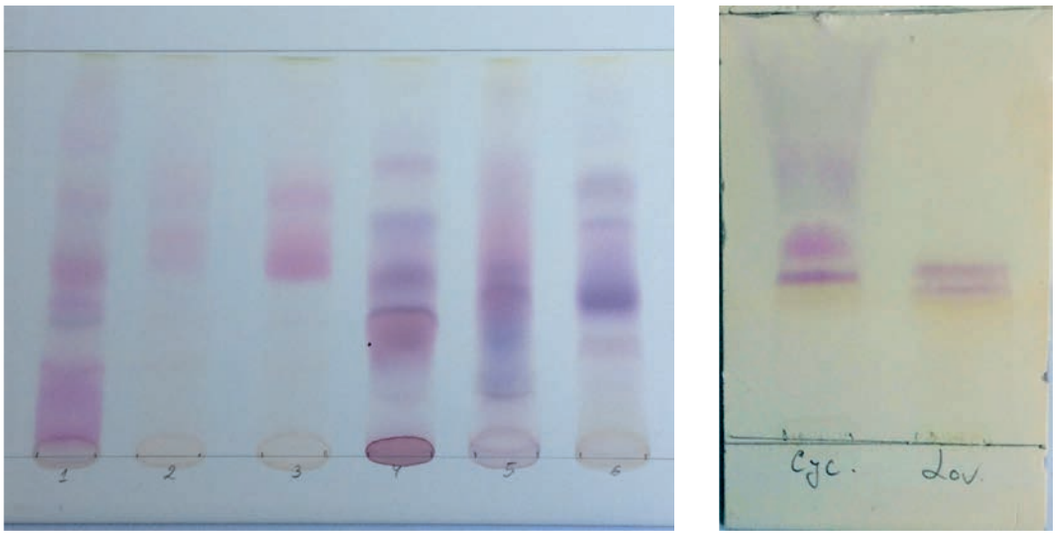
The results of the TLC analysis of the fruit extracts showed the presence of a multitude of colored fractions (Figure 1a). In almost all samples were observed pink, red and violet bands corresponding to the anthocyanins. The largest number of fractions was observed in the extracts of the first variety of grape (Gamza) and chokeberry. These samples also showed the highest intensity of colors. The samples of lavender showed the presence of three bands of different colors (from yellow-red to violet). The extracts of chicory got good separation of four anthocyanin fractions (Figure 1b). Norbaek et al. (2002) also isolated four anthocyanin pigments from chicory petals, which identify as different glycosides of delphinidin.

Our experimental data showed good repeatability of the results of the analyses of pigment extracts from the studied plant raw materials (Table 3). The levels of relative standard deviation (RSD) ranged from 0.89% to 7.22%. The content of anthocyanins in the extracts of chicory and lavender was 32.50 and 9.89 eq cyd (mg/l), significantly lower than other plant raw materials. In addition, for this type of extracts it is characteristic that they are very unstable even

Table 3. Total monomeric anthocyanin content in different extracts. The results are presented as means ± SD (n = 4)

Plant raw material	Extract variant					
	Juice			Pulp		
	cyd eq (mg/l)	±SD	RSD	cyd eq (mg/l)	±SD	RSD
Grape variety “Gamza”	31.73	2.29	7.22	1199.30	52.0	4.33
Grape variety “Muscat of Hamburg”	5.37	0.32	5.96	269.94	14.85	5.50
Grape variety “Pinot Noir”	0.75	0.05	6.65	271.36	18.10	6.67
Chokeberry	403.83	19.64	4.86	2195.90	60.70	2.77
Bilberry	306.56	3.86	1.26	1466.20	57.80	3.95
Blackberry	650.42	5.80	0.89	784.85	12.50	1.59
Chicory	-	-		32.50	2.51	7.12
Lavender	-	-		9.89	0.76	7.22

* Statistical significance was determined by One-Way ANOVA; significant differences between means ($p < 0.001$)



a b

Figure 1. Results of the TLC analysis of pigment extracts of various plant material.
a) water-ethanol extracts of fruits: grapes (1 – Gamza, 2 – Muscat Hamburg, 3 – Pinot Noir)
4 – chokeberry, 5 – bilberry, 6 – blackberry, b) water-ethanol extracts of petals: chicory and lavender

after acidification. For these reasons, the chicory and the lavender are not suitable raw material for obtaining anthocyanins pigments. From the fruit juices, the largest amount of anthocyanins pigments was found in blackberry (650.42 cyd eq mg/l) and chokeberry (403.83 cyd eq mg/l). The content of anthocyanins in grape juice was negligible in all three varieties. The extracts obtained from fruit pomace were with much higher levels of anthocyanins. In chokeberry and bilberry, they reached 2195.90 and 1466.20 cyd eq mg/l, respectively. From the grape varieties, the largest quantity of anthocyanin pigments was determined in Gamza (1199.30 cyd eq mg/l). Gamza is a dark-skinned variety of grape used for red wine. It has long history and is popular in Bulgaria and Hungary (where it is known as Kadarka).

The analysis of the trial data showed that extraction of pigments depends on several factors:

- Type of plant material – from the raw materials the greater amount of anthocyanins are isolated from chokeberry, followed by bilberry and grape variety "Gamza". `
- Pretreatment of raw material – fresh or frozen raw materials are the most suitable for the extraction of anthocyanins. Generally, the fruit pulp gives much higher yield than the pigments in fruit juice. This trend is mostly observed in the three red grape varieties, where freshly squeezed juice contains almost no anthocyanins, unlike the pulp. Only in the blackberry, the quantity of pigments in the juice is consistent with that in the pulp.
- Solvent used – anthocyanins are water-soluble compounds and therefore water and other polar solvents are adequate for their isolation. Better stabilization of the color is obtained with acidified ethanol. Due to the high content of organic acids, in fruits this effect is achieved in a natural way.

Conclusion

By pH-differential method, the quantitative content of anthocyanins in eight plant extracts was determined: fruits of chokeberry, blackberry, bilberry, grape (three varieties) and petals of chicory and lavender. It was found that the most suitable raw material for obtaining the anthocyanin pigments are pulp of chokeberry, bilberry and red grape variety Gamza. In this way byproducts from the production of fruit juices and wines could be utilized. Fruit pomace represents a cheap and accessible source for obtaining natural color additives for the food industry.

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The manuscript should be structured as follows: Title, Names of authors and affiliation address, Abstract, List of keywords, Introduction, Material and methods,Results, Discussion, Conclusion, Acknowledgements (if any), References, Tables, Figures.

The title needs to be as concise and informative about the nature of research. It should be written with small letter /bold, 14/ without any abbreviations.

Names and affiliation of authors The names of the authors should be presented from the initials of first names followed by the family names. The complete address and name of the institution should be stated next. The affiliation of authors are designated by different signs. For the author who is going to be corresponding by the editorial board and readers, an E-mail address and telephone number should be presented as footnote on the first page. Corresponding author is indicated with *.

Abstract should be not more than 350 words. It should be clearly stated what new findings have been made in the course of research. Abbreviations and references to authors are inadmissible in the summary. It should be understandable without having read the paper and should be in one paragraph.

Keywords: Up to maximum of 5 keywords should be selected not repeating the title but giving the essence of study.

The introduction must answer the following questions: What is known and what is new on the studied issue? What necessitated the research problem, described in the paper? What is your hypothesis and goal ?

Material and methods: The objects of research, organization of experiments, chemical analyses, statistical and other methods and conditions applied for the experiments should be described in detail. A criterion of sufficient information is to be possible for others to repeat the experiment in order to verify results.

Results are presented in understandable tables and figures, accompanied by the statistical parameters needed for the evaluation. Data from tables and figures should not be repeated in the text. **Tables** should be as simple and as few as possible. Each table should have its own explanatory title and to be typed on a separate page. They should be outside the main body of the text and an indication should be given where it should be inserted.

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Discussion: The objective of this section is to indicate the scientific significance of the study. By comparing the results and conclusions of other scientists the contribution of the study for expanding or modifying existing knowledge is pointed out clearly and convincingly to the reader.

Conclusion: The most important consequences for the science and practice resulting from the conducted research should be summarized in a few sentences. The conclusions shouldn't be numbered and no new paragraphs be used. Contributions are the core of conclusions.

References:

In the text, references should be cited as follows: single author: Sandberg (2002); two authors: Andersson and Georges (2004); more than two authors: Andersson et al.(2003). When several references are cited simultaneously, they should be ranked by chronological order e.g.: (Sandberg, 2002; Andersson et al., 2003; Andersson and Georges, 2004).

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Books: Author(s) surname and initials, year. Title. Edition, name of publisher, place of publication. Example:

Oldenbroek JK, 1999. Genebanks and the conservation of farm animal genetic resources, Second edition. DLO Institute for Animal Science and Health, Netherlands.

Book chapter or conference proceedings: Author(s) surname and initials, year. Title. In: Title of the book or of the proceedings followed by the editor(s), volume, pages. Name of publisher, place of publication. Example:

Mauff G, Pulverer G, Operkuch W, Hummel K and Hidden C, 1995. C3-variants and diverse phenotypes of unconverted and converted C3. In: Provides of the Biological Fluids (ed. H. Peters), vol. 22, 143-165, Pergamon Press. Oxford, UK.

Todorov N and Mitev J, 1995. Effect of level of feeding during dry period, and body condition score on reproductive performance in dairy cows,IXth International Conference on Production Diseases in Farm Animals, September 11–14, Berlin, Germany.

Thesis:

Hristova D, 2013. Investigation on genetic diversity in local sheep breeds using DNA markers. Thesis for PhD, Trakia University, Stara Zagora, Bulgaria, (Bg).

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Studies performed on experimental animals should be carried out according to internationally recognized guidelines for animal welfare. That should be clearly described in the respective section “Material and methods”.

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