VALIDATION OF MONOMERIC ANTHOCIANIN DETERMINATION METHOD FOR BILBERRY JUICE AND MARC EXTRACTS

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Abstract

The solid waste generated in industrial berry juice production was considered as a low cost raw material for the extraction of natural antioxidants. Berries contain phenolic compounds with high antioxidant potential, including anthocianin. Quantitative determination method for monomeric anthocianins in bilberry juice and marc was validated. An official method from Association of Analytical Communities was used to determine anthocianins in juice and marc extracts by measuring light absorption for solutions with pH values 1.0 and 4.5 at 520 nm and 700 nm. Results were expressed as cyanidin-3-glucoside equivalent, as it is the most common anthocianin pigment. Calibration curve was obtained, linearity was checked, working interval and accuracy was determined and samples were tested. Mentioned method was evaluated as appropriate for quantitative anthocianin analysis in bilberry juice and marc. Necessity of calibration curve was approved using extinction coefficient of cyanidin-3-glucoside instead. Method assures adequate precision and accuracy as well.

Key words: natural antioxidants, monomeric anthocianins, bilberry juice, bilberry marc

Introduction

Anthocyanin pigments are responsible for the red, purple and blue colours of many fruits, vegetables, cereal grains, and flowers. Food scientists study these compounds because of their obvious importance to the colour quality of fresh and processed fruits and vegetables. Interest in anthocyanin pigments has intensified because of their possible health benefits as dietary antioxidants. Over 300 structurally distinct anthocyanins have been identified in nature (Wrolstad, 2001). Anthocyanins are one class of flavonoid compounds, which are widely distributed plant polyphenols. Flavonols, flavan-3-ols, flavones, flavanones, and flavanonols are additional classes of flavonoids that differ in their oxidation state from the anthocyanins.

Anthocyanin is a glycoside composed of an aglycon named anthocyanidin and a sugar residue. The six most widespread anthocyanidins are cyanidin, delphinidin, peonidin, pelargonidin, petunidin and malvidin in decreasing order of occurrence. Anthocyanidins occur most commonly as *O*-glycosides. When anthocyanidins are coupled to sugars, anthocyanins are formed. The possible sugar residues are D-glucose, D-galactose, L-rhamnose, L-arabinose and D-xylose. These five sugars are involved in the formation of monoglycosides, in which a sugar residue almost always is located at the hydroxyl group of C-3. Diglycosides may be formed when another sugar is linked to monoglycoside at C-3 or to another hydroxyl group at C-5. More rare triglycosides may be formed similarly from diglicosides. Three linked sugars in a side chain may form linear or branched structures. Acylated anthocyanins are typically found in some plant foods such as blueberry, red onion and potato. Acylation by phenolic acid appears to be related to the stabilization of anthocyanins in the acid environment of the cell sap (Riihinen, 2005). As sugars can be coupled at different places and many different sugars are present in plants, it is clear that a very large range of anthocyanins can be formed (Food-info, 2011). While there are six common anthocyanidins, more than 540 anthocyanin pigments have been identified in nature (Anderson, Francis, 2004), with most of the structural variation coming from glycosidic substitution at the 3 and 5 positions and possible acylation of sugar residues with organic acids. Anthocyanins lend themselves to systematic identification as the component anthocyanidins, sugars and acylating acids can be liberated by acid hydrolysis and subsequently identified by chromatographic procedures. Saponification of acylated anthocyanins will produce the anthocyanin glycosides and acylating acids for subsequent identification. These methods are described in several publications (Durst, Wrolstad, 2001; Hong, Wrolstad, 1990; Wrolstad et al., 2002). Electrospray (ES-MS), tandem (MS/MS), and liquid chromatography mass spectroscopy (LC-MS) are powerful

techniques for identifying anthocyanins from their discrete mass units and fragment ions (Giusti et al., 1999; Wang et al., 2003). For more complete identification, NMR can be used for sugar identification and determining the position of sugar attachment and angle of the glycosidic linkages (Giusti et al., 1998; Anderson, Fossen, 2003). Anthocyanins reversibly change colour with pH, which limits their effective use as food colourants for many applications, but also provides an easy and convenient method for measuring total pigment concentration (Giusti, Wrolstad, 2001). The described method is a modification of methods originally described by Fuleki and Francis (1968). Samples are diluted with aqueous pH 1.0 and 4.5 buffers and absorption measurements are taken at the wavelength of maximum absorption of the pH 1.0 solution. The difference in absorption between the two buffer solutions is due to the monomeric anthocyanin pigments. Polymerized anthocyanin pigments and nonenzymic browning pigments do not exhibit reversible behavior with pH, and are thus excluded from the absorption calculation. It is customary to calculate total anthocyanins using the molecular weight and molar extinction coefficient of the major anthocyanin in the sample matrix. The number of anthocyanins for which molecular extinction coefficients have been determined is limited, however (Giusti, Wrolstad, 2001). When using this procedure, extinction coefficients that have been determined in aqueous solutions should be used rather than those determined in acidic ethanol or methanol because of solvent effects (Lee et al., 2005). There is a need for a standardized method for determining total anthocyanins in commerce, since products are being marketed on the basis of their pigment content (Wrolstad et al., 2005). Association of Analytical Communities is published this pH differential method as official anthocyanin identification method called by Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colourants and Wines. The principle is that monomeric anthocyanin pigments reversibly change colour with a change in pH; the coloured oxonium form exists at pH 1.0, and the colourless hemiketal form predominates at pH 4.5. The difference in the absorption of the pigments at 520 nm is proportional to the pigment concentration. Results are expressed on a cyanidin-3-glucoside basis. Degraded anthocyanins in the polymeric form are resistant to colour change regardless of pH and are not included in the measurements because they absorb at pH 4.5 as well as pH 1.0 (AOAC, 2005).

Table 1

Flavonoid Content in Bilberries

Subclass	Flavonoid	mg $100g^{-1}$ edible portion	Min	Max	No. of samples
Anthocyanidins	Cyanidin	15.02	4.79	28.72	12
	Delphinidin	29.54	20.82	47.37	
	Malvidin	49.21	32.95	69.44	
	Peonidin	7.05	1.01	19.37	
	Petunidin	11.73	7.19	18.25	
Flavan-3-ols	(-)-Epicatechin	1.11	1.11	1.11	4
Flavonols	Myricetin	0.82	0	2.60	6
	Quercetin	3.11	1.70	7.30	7

Bilberries are one of the raw materials for dietary supplement production in medical company Silvanols Ltd. therefore it is necessary to define quality requirements for bilberry juice and marc. Juice is one of the components for dietary supplement production. For now berry marc is a waste with usage potential in the nearest future. During the storage and recycling processes anthocyanins decrease, so it is necessary to determine anthocyanin content in both – juice and marc, but not in the berries before juice squeezing.

There is Table 1 given below in order to approximately comprehend anthocyanin content in bilberry juice and marc. The *USDA Database for Flavonoids* was created as a response to the interest of scientific community in types of flavonoid compounds and their varied biological properties. According to their data bilberries contain anthocyanidins in significant quantities.

Materials and Methods

The validation of monomeric anthocyanin determination method was carried out in Silvanols Ltd. company in Riga, Latvia, 2010. The object of the validation was bilberry (*Vaccinium myrtillus)* juice and marc. Juice was obtained from previously frozen and thawed berries picked in the woods of Preili region in 2010. After the juice squeezing process berry marc was dried in hot air dryer at 70 ºC until marc moisture was <8% and grounded for 25–30 seconds in blender grinder. Then marc extracts in ethanol/water solution were prepared. Extraction kinetics study was made with 6 samples prepared from 0.1–0.12 g bilberry marc, 10 ml water and 20 ml ethanol. The samples were then extracted for 15, 30, 60, 90, 120 and 180 min on magnetic stirrer. After extraction samples were filtered with water-jet vacuum pump, residues were washed twice with purified water; filtrate was quantitatively transferred to 100 ml flask and refilled with purified water to the mark. For further experiments 4 ml extract was taken and transferred to 25 ml flask which was refilled with buffer solutions – with potassium chloride buffer to obtain pH 1.0, but sodium acetate buffer to obtain pH 4.5.

To determine other parameters in validation process – linearity, working range, accuracy, precision, repeatability and robustness – samples were prepared by the same method, but the extraction time was 2 hours constantly. There was Cyanidin-3-glucoside choride 99.58% used as a reference material to obtain cyanidin-3-glucoside spectrum and calibration curve and also in accuracy test.

The pH differential method – AOAC Official Method 2005.02 (mentioned above) which is applicable to the determination of monomeric anthocyanins in fruit juices, beverages, natural colorants, and wines within the range of 20–3000 mg L^{-1} as cyanidin-3-glucoside equivalents was the selected method for monomeric anthocyanin determination.

All absorption measurements were done by UV/VIS spectrophotometer Lambda 25 by Perkin Elmer, wavelength precision ±0.1 nm, bandwidth 1 nm. All experiments were made in triplicate and mean values with standard deviations are reported. A linear correlation analysis was performed with the software SPSS 14.00 for Windows in order to evaluate cyanidin-3-O-glucoside calibration curve.

Results and Discussion

Cyanidin-3-O-glucoside chloride solution at pH 1.0 has orange-red colour with absorption maximum at 520 nm, but at pH 4.5 this solution is colourless with no absorption.

Bilberry juice and marc extract solutions are orange-red at pH 1.0 with absorption maximum at 520 nm, but at pH 4.5 these solutions are in light blue colour with absorption maximum at 500–550 nm. Obtained cyanidin-3-O-glucoside calibration curve is showed in Figure 1. Using four point calibrations the correlation coefficient is 0.9999.

Monomeric anthocyanin extraction kinetics indicated in Figure 2. As we see monomeric anthocyanin extraction from bilberry marc is complete in 90 minutes, however, the recommended extraction time is 2 h.

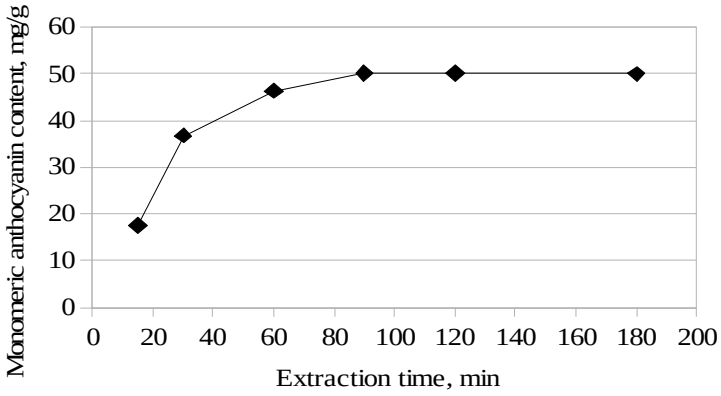

Figure 1. Cyanidin-3-O-glucoside calibration curve

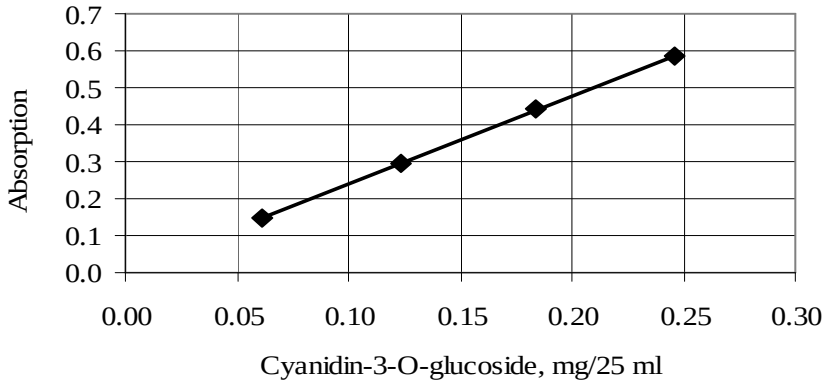

Figure 2. Monomeric anthocyanin extraction kinetics from bilberry marc

Determined working and linear ranges approved that there is no necessary to obtain calibration curve to detect monomeric anthocyanins in bilberry marc and also in the juice. Calculations instead can be done by using cyanidin-3-O-glucoside molar extinction coefficient, because there is no difference in results as represented in Table 2. Recommended bilberry marc volume for analysis is 0.1–0.12 g, but juice volume – 0.5 ml according to selected method.

Table 2

Result statistics using calibration curve and molar extinction coefficient for calculations

Parameter	**From calibration curve**	**With molar extinction coefficient**		**From calibration curve**	**With molar extinction coefficient**
	Bilberry juice			**Bilberry marc**	
Mean	513.31 mg l^{-1}	512.98 mg l^{-1}		50.59 mg g^{-1}	50.39 mg g^{-1}
Standard deviation	0.4823 mg l^{-1}	0.3801 mg l^{-1}		0.6912 mg g^{-1}	0.6848 mg g^{-1}
Relative standard deviation	0.09	0.07		1.37	1.36

Experimental data represent very high correlation between results: juice samples from 0.2–0.8 ml indicate correlation coefficient R=0.9999, but marc samples from 0.6–0.14 g – R=0.9998. Experimental data also represent a very high accuracy percentage for both analytes – juice and marc, in both cases it was close to 100%.

Precision and repeatability also was determined. Precision was characterized by maximal relative standard deviation, which was 0.30% for juice sample measurements and 1.88% for

marc extract measurements, but repeatability was also expressed with standard deviation, that was 0.27% for juice sample measurements and 1.80% for marc extract measurements. Method robustness measurements indicated that the method precision was not exceeded in both cases.

Conclusions

1. Validation process approved that the method is suitable for monomeric anthocyanin determination in bilberry juice and marc extracts.
2. It was approved that it is not necessary to obtain calibration curve to calculate test results as it is enough to use cyanidin-3-O-glucoside molar extinction coefficient for calculations in both cases – juice and marc extracts.
3. Selected method provided adequate precision for monomeric anthocyanin content determination. Maximum relative standard deviation does not exceed 1.88% for marc extract and 0.30% for juice measurements.

References

1. Anderson, O. M., Fossen, T. (2003). Characterization of anthocyanins by NMR. In R. E. Wrolstad (Ed.), *Current Protocols in Food Analytical Chemistry*. New York: Wiley.
2. Anderson, O. M., Francis, G. W. (2004). Techniques of pigment identification. *Annual Plant Reviews – Plant Pigments and Their Manipulation*, 14, pp. 293–341.
3. AOAC (2005). Official Method 2005.02 Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines. *Journal of AOAC International*, 88, pp. 1269.
4. Durst, R. W., Wrolstad, R. E. (2001). Unit F1.3.1-13. Separation and characterization of anthocyanins by HPLC. In R. E.Wrolstad (Ed.), *Current Protocols in Food Analytical Chemistry*. NewYork: Wiley.
5. Food-info, Wageningen University, The Netherlands // Anthocyanins and anthocyanidins. Source: http://www.food-info.net/uk/colour/anthocyanin.htm; resource used on 06.03.2011.
6. Giusti, M. M., Wrolstad, R. E. (2001). Unit F1.2.1-13. Anthocyanins. Characterization and measurement with UV-visible spectroscopy. In R. E. Wrolstad (Ed.), *Current Protocols in Food Analytical Chemistry*. New York: Wiley.
7. Fuleki, T., Francis, F. J. (1968). Quantitative methods for anthocyanins. 1. Extraction and determination of total anthocyanin in cranberries. *Journal of Food Science*, 33, pp. 72–77.
8. Giusti, M. M., Ghanadan, H., Wrolstad, R. E. (1998). Elucidation of the structure and conformation of red radish (Raphanus sativus) anthocyanins using one- and two-dimensional nuclear magnetic resonance techniques. *Journal of Agricultural and Food Chemistry*, 56, pp. 4858–4863.
9. Giusti, M. M., Rodriguez-Saona, L. E., Griffin, D., Wrolstad, R. E. (1999). Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization. *Journal of Agricultural and Food Chemistry*, 47, pp. 4657–4664.
10. Hong, V., Wrolstad, R. E. (1990). Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *Journal of Agricultural and Food Chemistry*, 38, pp. 708–715.
11. Lee, J., Durst, R.W., Wrolstad, R.E. (2005). Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: Collaborative Study. Accepted for publication in the *Journal of the Association of Official Analytical Chemists International*.
12. Riihinen K. (2005). Phenolic Compounds in Berries. *Doctoral dissertation*, Institute of Applied Biotechnology Food and Health Research Centre, Department of Clinical Nutrition, University of Kuopio, Kuopio.
13. Ronald E. Wrolstad // The Possible Health Benefits of Anthocyanin Pigments and Polyphenolics. 2001. Source: http://lpi.oregonstate.edu/ss01/anthocyanin.html; resource used on 27.02.2011.
14. Wang, H., Race, E. J., Shrikhande, A. J. (2003). Characterization of anthocyanins in grape juices by ion trap liquid chromatographmass spectrometry. *Journal of Agricultural and Food Chemistry*, 51, pp. 1839–1844.
15. Wrolstad, R.E., Durst, R.W., Lee, J. (2005). Tracking color and pigment changes in anthocyanin products. *Trends in Food Science & Technology*, 16, pp. 423–428.
16. Wrolstad, R.E., Durst, R.W., Giusti, M.M., & Rodriguez-Saona, L.E. (2002). Analysis of anthocyanins in nutraceuticals. In: *Quality Management of Nutraceuticals*. (Chp. 4, pp 42-62), ACS Symposium Series No. 803, Washington DC: American Chemical Society.