ANTHOCYANIN AND ANTHOCYANIDIN CONTENT OF ***HIGHBUSH*** BLUEBERRIES CULTIVATED IN BRAZIL

JÚLIA RIBEIRO SARKIS *
ISABEL CRISTINA TESSARO **
LIGIA DAMASCENO FERREIRA MARCZAK **

The objective of this study was to apply a HPLC methodology for qualitative and quantitative analysis of the six anthocyanidins present in fruits and to analyze the anthocyanin and anthocyanidin content of blueberries cultivated in Southern Brazil. The samples used belong to highbush blueberries (*Vaccinium corymbosum*) cultivars. Total anthocyanin content was determined by the pH differential method and an HPLC gradient elution system with C_{18} column and UV-Vis detection at 520 nm were used for separation and quantification of the anthocyanidins. Total anthocyanin content was of 128 ± 3 mg per 100 g of fresh pulp. Blueberry pulp presented 55 % of delphinidin, 8 % of cyanidin, 3 % of peonidin and 34 % of malvidin. Pelargonidin was not identified in the sample and petunidin was below the limits of quantification. The results were similar to those reported in studies using North American and European blueberries.

KEY-WORDS: Vaccinium corymbosum; ANTHOCYANIDIN PROFILE; HPLC.

* Ph.D student in the area of heat and mass transfer, Chemical Engineering Departament, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS (e-mail: julia@enq.ufrgs.br).

** Professor, Chemical Engineering Departament, UFRGS, Porto Alegre, RS (e-mail: isabel@enq.ufrgs.br; ligia@eng.ufrgs.br).

1 INTRODUCTION

Blueberries (*Vaccinium* spp.) have high anthocyanins content, presenting elevated radical-scavenging activity and being potent antioxidants (PRIOR *et al.*, 1998; ZHENG & WANG, 2002). Researches suggest that anthocyanins in fruits and vegetables, and their antioxidant capacity, contribute to lower the risk and to treat chronic and degenerative diseases (BRAVO, 1998; KOWALCZYK *et al.*, 2003; KONCZAK & ZHANG, 2004). Cumulative research shows the influence of these compounds in reversing age-related deficits such as memory and locomotion (RAMIREZ *et al.*, 2005), reducing the risk of diabetes and obesity (GRACE *et al.*, 2009; VUONG *et al.*, 2009) and inhibiting proliferation of cancer cells (KAMEI *et al.*, 1998; HAGIWARA *et al.*, 2001; SEERAM, ZHANG & NAIR, 2003). For these reasons this fruit is seen as a very appealing option to consumers interested in functional foods.

The basic structure of an anthocyanin molecule consists of glycosylated polyhydroxyl or polymethoxyl derivatives of the flavylium cation. This basic structure, with no glucose substituents, is called anthocyanidin or aglycone and can be obtained by acid hydrolysis. This process reduces the complex pattern of anthocyanins to six major anthocyanidins: delphinidin, cyanidin, pelargonidin, peonidin, petunidin and malvidin. The anthocyanidin quantification and qualification of blueberries have been reported in limited papers (NYMAN & KUMPULAINEN, 2001; YUE & XU, 2008; QUEIROZ *et al.*, 2009; OLIVEIRA *et al.*, 2010) and these studies only refer to blueberries cultivated in Europe and North America, where these fruits are originally from.

Blueberry culture is currently cultivated in Brazil, with the cultivars belonging to the groups: *rabbiteye*, *highbush* and *southern highbush.* The *highbush* group is the most important commercial variety in Europe and the United States (PAGOT & HOFFMAN, 2003; VIZZOTO & PEREIRA, 2009). Brazilian blueberry production is of approximately 60 t per year, occupying a 35 ha area, being 20 ha in the state of Rio Grande do Sul, mainly in the region of Vacaria. This region was pioneer in blueberry production in the 1990's and became a reference of this culture in Brazil (PAGOT, 2006; SANTOS *et al.*, 2007).

Blueberry culture has adapted well to Southern Brazil weather, representing an interesting alternative for exportation and national consumption (PAGOT, 2006; SANTOS *et al*., 2007). The objective of this study was to apply a High Performance Liquid Chromatography (HPLC) methodology for qualitative and quantitative analysis of the six anthocyanidins present in fruits and to analyze the anthocyanin and anthocyanidin content of blueberries (*Vaccinium corymbosum*) cultivated in the Southern Brazil.

2 MATERIAL AND METHODS

Highbush blueberries (*Vaccinium corymbosum*) from the region of Vacaria, in Southern Brazil were used in the experiments. The fruits belong to two different cultivars, being a mixture of Bluecorp and Darrow. The samples, previously cleaned and frozen, were bought from Italbraz Company (Vacaria, Brazil) and kept at -18 °C until analysis. A blueberry pulp was elaborated by grinding the blueberries and diluting the samples to adjust the total solids content to 16 %. The pulp presented pH value of 3.18 ± 0.01 and a soluble solids content (°Brix) of 13.0 ± 0.5.

Standards of cyanidin, delphinidin, peonidin, petunidin, malvidin and pelargonidin were purchased from Sigma Aldrich (St. Louis, United States). HPLC grade solvents including acetonitrile, methanol, *o*-phosphoric acid, acetic acid, and chloridric acid were obtained from Vetec (Duque de Caxias, Brazil). All experiments described in this work were performed at the Chemical Engineering Department of the Federal University of Rio Grande do Sul (BRAZIL).

2.1 SAMPLE PREPARATION

The anthocyanins were extracted from a 2 g sample with 20 mL of acidified methanol (0.01 %, v/v HCl) by homogenizing for 1 h in a shaker (Marconi, Brazil). The sample was centrifuged for 20 min at 4 C and 4757 *g* and the supernatant was collected. At this stage, the solids were in a pale brown color. The flasks containing the samples were flushed with Nitrogen for 2 min before storage. All sample preparation procedures were performed avoiding light and temperatures above 20 °C in order to prevent degradation of the pigments (DURST & WROLSTAD, 2001).

2.2 DETERMINATION OF TOTAL ANTHOCYANIN CONTENT

The methanolic extract, as described above, was used in the determination of the total anthocyanin content by the pH differential method (GIUSTI & WROLSTAD, 2001; LEE, DURST & WROLSTAD, 2005). Samples were diluted in pH 1.0 and pH 4.5 buffers, and the absorbance was measured at 510 and 700 nm in a Spectrophotometer (*Pro-Analise*, UV-Visible 1600, Brazil). Anthocyanin content was expressed based on cyanidin-3-glucoside with molar extinction coefficient of 26.900 L/mol·cm and molar weight of 449.2 g/mol. Analysis was performed in duplicate and the resultant values were expressed in terms of mg of anthocyanin per 100 g of fresh pulp.

2.3 ACID HYDROLYSIS

Acid hydrolysis was performed according to the methodology of Rodriguez-Saona and Wrolstad (2001) with the modifications proposed by Lima *et al*. (2006). The methanolic solution, prepared as described in section 2.1, was used to hydrolyze the anthocyanins to aglycons, adding 3 mL of sample solution to a 10 mL of a 2 mol/L HCl solution. The flask was wrapped in aluminum foil, flushed with Nitrogen for 2 min and immersed in boiling water for 1 h. After hydrolysis, the samples were cooled in an ice bath in the dark for 10 min prior to use.

The hydrolyzed extract was passed through a sorbent C_{18} solid phase extraction (SPE) cartridge (Waters Associates, Milford, United States) which was previously activated with acidified methanol (0.01 %, v/v HCl) followed by 0.01 % (v/v) aqueous HCl. Anthocyanidins were adsorbed onto the cartridge and water soluble compounds were washed off. The pigments were eluted using acidified methanol (0.01%, v/v HCl) which was subsequently evaporated using Nitrogen flux until a more concentrated sample was obtained. The sample solution was filtered through a 0.45 µm membrane filter (Milipore) and 20 µL were injected for HPLC analysis.

2.4 HPLC

The HPLC analysis was performed on a PerkinElmer chromatograph system (series 200) equipped with a quaternary pump, an UV-Vis detector and a column oven (PerkinElmer, series 200). Separation was conducted on a C_{18} reversed-phase, 5 µm (250 x 4.6 mm i.d., PerkinElmer) column coupled to a C_{18}, 5 µm (15 x 3.2 mm i.d., PerkinElmer) guard column. The injection volume was 20 µL, the detection was done at the wavelength of 520 nm and the temperature and flow rate were controlled at 30 °C and 1 mL/min, respectively. Gradient elution was performed according to Durst and Wrolstad (2001) using as eluent A 100 % acetonitrile and as eluent B 10 % acetic acid/5 % acetonitrile/1 % phosphoric acid (v/v/v) in Mili-Q water. The modified gradient elution program was performed as follows: linear gradient from 5 % A and 95 % B to 20 % A and 80 % B, 0 – 20 min; linear gradient from 20 % A and 80 % B to 5 % A and 95 % B, 20 – 25 min; post-time 1 min before next injection.

2.5 IDENTIFICATION AND QUANTIFICATION

Identification of anthocyanidins was carried out by comparing the HPLC retention times for

the sample and for the standards. The quantification was accomplished by using external calibration method with the concentrations being calculated using the corresponding anthocyanidin standard calibration curves. These curves were obtained by plotting the peack area *versus* the concentration of the standard. The mean retention times (t_R) and the standard deviations (SD) were calculated using the values obtained from the calibration curves and are presented in Table 1. The standard solutions were prepared by dissolving the standards in mili-Q water (1000 mg/L) and diluting to obtain solutions with different concentrations. All solutions were measured in triplicate.

TABLE 1 – RETENTION TIMES (t_R), LIMITS OF DETECTION (LOD) AND QUANTIFICATION (LOQ) FOR PELARGONIDIN, DELPHINIDIN, CYANIDIN, PETUNIDIN, PEONIDIN AND MALVIDIN

Anthocyanidin	t_R (min)		LOD (mg/100 g)	LOQ (mg/100 g)	R^2
	Mean	SD			
Pelargonidin	3.4	0.1	0.55	1.84	0.998
Delphinidin	5.4	0.3	0.32	1.10	0.999
Cyanidin	8.5	0.2	0.34	1.13	0.998
Petunidin	10.5	0.2	0.27	0.89	0.999
Peonidin	14.9	0.1	0.12	0.39	0.999
Malvidin	15.5	0.3	0.66	2.20	0.998

Calibration curves were satisfactory, being the peak areas of the anthocyanidins in the blueberry extract within the linear range of the curves. The concentration of anthocyanidins was further corrected according to blueberry total solids and expressed as milligrams of anthocyanidin per 100 g of fresh matter (FM). Analysis was performed in duplicate.

The four pointed calibration curves constructed were linear (correlation coefficients > 0.997) in the ranges of: 10 – 90 mg/L for pelargonidin, 50 – 250 mg/L for delphinidin, 10 – 50 mg/L for cyanidin, 5 – 60 mg/L for petunidin and peonidin and 50 – 100 mg/L for malvidin. The linearity of the curves was determined using the least squares method, obtaining the angular and linear coefficients of each curve and the standard deviations of these parameters. The limits of detection (LOD) and of quantification (LOQ) for the 6 anthocyanidins analyzed are defined as 3 and 10 times, respectively, the ratio of the standard deviation of the linear coefficient to the angular coefficient of the calibration curves (SKOOG, DONALD & HOLLER 2004). The values obtained for these limits are presented in Table 1.

For each analysis, the relative standard deviation (RSD) was determined using the least squares method in order to determine the accuracy of the methodology.

3 RESULTS AND DISCUSSION

Total anthocyanin content was determined using the pH differential method. The average anthocyanin content obtained in the experiments was 128 ± 3 mg/100 g of fresh-frozen blueberry pulp with 16 % solids content. The error between duplicates was 1.26 %. The value is within the range of 93.1 to 235.4 mg of anthocyanins/100 g of fresh matter reported by Prior *et al*. (1998) who

studied several different cultivars of *V. corymbosum* and also in good agreement with previously reported values of 120 mg/100 g fresh weight (ZHENG & WANG, 2002); 99.9 mg/100 g (SKREDE, WROLSTAD & DURST, 2000a) and between 116 and 224 mg/100 g (YOU *et al.*, 2011). All results mentioned above were obtained applying the pH differential method.

After acid hydrolysis, the anthocyanidin glycoside pattern was reduced to anthocyanidins. Figure 1 presents a chromatogram from the HPLC analysis where five well-separated and defined peaks can be observed. Peaks 1 to 5 refer to delphinidin, cyanidin, petunidin, peonidin and malvidin, respectively; these anthocyanidins were identified through comparison of the retention times. Elution order was in agreement with literature (NYMAN & KUMPULAINEN, 2001; LIMA *et al.*, 2006; OLIVEIRA *et al.*, 2010) with the exception of pelargonidin which presented a lower retention time than expected. Nonetheless, previous studies (SKREDE, WROLSTAD & DURST, 2000a; LEE, DURST & WROLSTAD, 2002; YUE e XU, 2008; YOU *et al.*, 2011) did not found peaks associated with pelargonidin when analyzing blueberry samples.

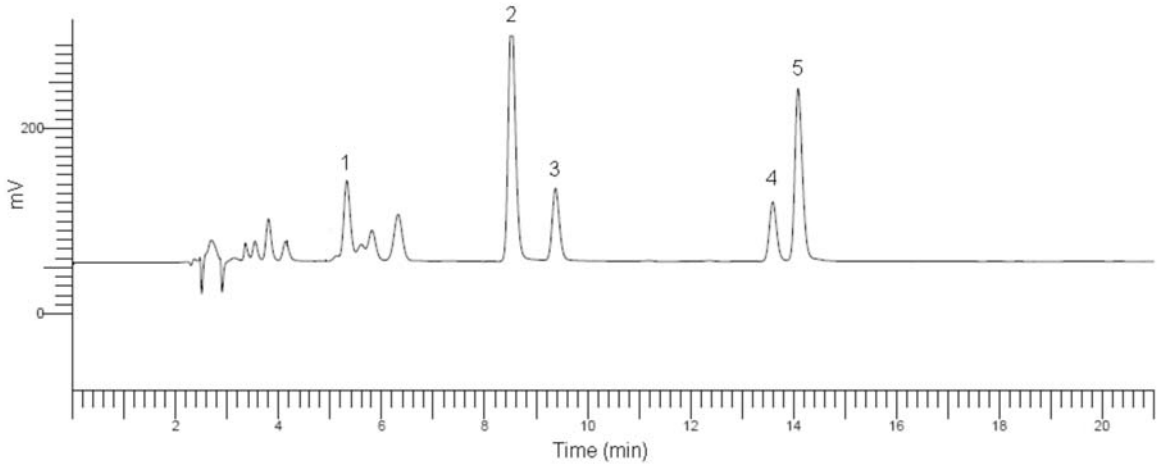

FIGURE 1 – CHROMATOGRAM OF BLUEBERRY PULP

Anthocyanidins were quantified using the calibration curves; the results for delphinidin, cyanidin, peonidin and malvidin and the standard deviation for the duplicate are presented in Table 2. The total anthocyanins in Brazilian blueberry were estimated as a sum of these four compounds. Petunidin was not quantified because its content was under the LOQ. RSD values were below 5 % for all analysis. The anthocyanin content evaluated using HPLC was 143.6 mg/100 g of fresh matter. It must be pointed out that these values are, probably, underestimated, considering the limitations of the extraction methods (SEERAM, 2008). However, the total anthocyanins content obtained from this analysis is in agreement with research evaluating anthocyanin content in blueberries using HPLC (GAO & MAZZA, 1994; KALT & DUFOUR, 1997; MOYER *et al.*, 2001). The total anthocyanin content was higher for the HPLC analysis when compared to pH differential method, which was also observed by Oliveira *et al*. (2010) and Wu *et al*. (2004).

The anthocyanidin composition shows the predominance of delphinidin with respect to the other anthocyanidins, followed by malvidin, cyanidin and peonidin, in this order. This result was in agreement with the study performed by Kalt *et al*. (1999) who evaluated several different cultivars of *highbush* blueberries and found that delphinidin was the major constituent among all anthocyanidins. Malvidin, however, appeared as the main anthocyanidin in the researches of Oliveira *et al*. (2010) and Skrede *et al*. (2000b) using *V. corymbosum* cultivars.

As a final point, it is important to emphasize that this research presents preliminary results regarding the anthocyanins content in Brazilian blueberries. Further studies, with other cultivars and

from different regions, should be performed in order to have a broader knowledge of the blueberry culture in Brazil.

TABLE 2 – ANTHOCYANIN CONTENT IN BRAZILIAN BLUEBERRIES

Anthocyanidin	Peak no.	Anthocyanin concentration (mg/100 g FM)		Relative proportion (%)
		Mean	SD	
Delphinidin	1	78.9	0.5	54.93
Cyanidin	2	11.9	0.4	8.31
Peonidin	4	4.6	0.2	3.18
Malvidin	5	48.2	0.4	33.57
Total	-	144	2	100

4 CONCLUSION

The HPLC method used in this work was considered satisfactory for anthocyanidin quantification and qualification, being possible to determine the percentage of each anthocyanidin in Brazilian blueberries.

The analysis showed delphinidin as the principal anthocyanidin, followed by malvidin, cyanidin and peonidin; the results were similar to the ones obtained in studies using North American and European blueberries.

RESUMO

TEOR DE ANTOCIANINAS E ANTOCIANIDINAS EM MIRTILOS *HIGHBUSH* CULTIVADOS NO BRASIL

O objetivo deste trabalho foi aplicar metodologia de Cromatografia a Líquido de Alta Eficiência (CLAE) para análise qualitativa e quantitativa das seis antocianidinas identificadas em frutas e avaliar o teor de antocianinas e antocianidinas em mirtilos highbush (*Vaccinium corymbosum*) cultivados no sul do Brasil. O teor total de antocianinas foi determinado pelo método do pH diferencial, utilizando-se sistema de CLAE com coluna C_{18} e detector UV-Visível no comprimento de onda de 520 nm para separação e quantificação das antocianidinas. O teor total de antocianinas foi de 128 ± 3 mg por 100 g de amostra em base úmida. Os resultados mostraram que a polpa de mirtilo apresentou, em média, 55 % de delfinidina, 8 % de cianidina, 3 % de peonidina e 34 % de malvidina. A pelargonidina não foi identificada na amostra e a petunidina ficou abaixo no limite de quantificação. Os resultados mostraram-se similares aos encontrados em pesquisas utilizando mirtilos provenientes da América do Norte e da Europa.

PALAVRAS-CHAVE: Vaccinium corymbosum; PERFIL DE ANTOCIANIDINAS; CLAE.

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ACKNOWLEDGEMENTS

The authors acknowledge the financial support received from CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), as a scholarship to the first author, and from CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) within the PRODOC project.