Anthocyanins standards (cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside)
isolation from freeze-dried açaí (Euterpe oleracea Mart.) by HPLC

Isolamento de padrões de antocianinas (cianidina-3-O-glucosídeo e cianidina-3-O-rutinosídeo) de açaí liofilizado (Euterpe oleracea Mart.) por CLAE

Ana Cristina Miranda Senna GOUVÊA¹*, Manuela Cristina Pessanha de ARAUJO², Daniel Filisberto SCHULZ³, Sidney PACHECO², Ronel Luis de Oliveira GODOY², Lourdes Maria Corrêa CABRAL²

Abstract

Availability of analytical standards is a critical aspect in developing methods for quantitative analysis of anthocyanins. The anthocyanins cyanidin-3-O-glucoside and cyanidin-3-O-rutinoside were isolated from samples of freeze-dried açaí (Euterpe oleracea Mart.), which is a round and purple well-known palm fruit in Brazil, and then used as standards. The isolation of the anthocyanins was performed by High Performance Liquid Chromatography (HPLC), using an adapted six-channel selection valve. The identification of anthocyanin pigments in açaí was based on mass spectrometric data for molecular ions and MS-MS product ions and on previous published data. After the collection procedure, standards with a high purity grade were obtained and an external standard curve of each anthocyanin was plotted.

Keywords: anthocyanins; standard; isolation; açaí; Euterpe oleracea Mart; HPLC.

Resumo

Disponibilidade de padrões analíticos é um aspecto crítico no desenvolvimento de métodos de análises quantitativas de antocianinas. Para a obtenção dos padrões isolados de antocianinas cianidina-3-O-glicosídeo e cianidina-3-O-rutinosídeo foram utilizadas amostras liofilizadas de açaí (Euterpe oleracea Mart.), que é um conhecido fruto de palmeiras no Brasil de forma arredondada e roxo. O isolamento das antocianinas foi realizado por Cromatografia Líquida de Alta Eficiência (CLAE), utilizando uma válvula seletora de seis canais. A identificação de antocianinas no açaí foi baseada em dados publicados na literatura e por espectrometria de massas. Após a coleta, padrões com um alto grau de pureza foram obtidos e uma curva para padronização externa de cada antocianina foi feita.

Palavras-chave: antocianinas; padrões; isolamento; açaí; Euterpe oleracea Mart; CLAE.

1 Introduction

Anthocyanins are mainly distributed among flowers, fruits, and vegetables and are responsible for most of the red, blue, and purple color (BROUILLARD, 1982; MALACRIDA; MOTTA, 2005). Recently, increased attention has been given to their possible health benefits in preventing chronic degenerative diseases including cancer and heart disease (DOWNHAM; COLLINS, 2000; MARTÍNEZ-FLÓREZ et al., 2002; KUSKOSKI et al., 2004; WALLE, 2004).

Measurement of anthocyanin content is critical for both research and industrial applications. Molar absorptivity in the solvent of choice is required for accurate measurement of anthocyanin content of identified pigments. To use the absorption coefficients, it is necessary to have the anthocyanins standards available. In spite of the large amounts of anthocyanins found in nature, not all of them are available on the market, and those that are commercialized are sold in small quantities with a low purity grade, besides being very expensive. The isolation of each anthocyanin is considered a problem mainly due to the difficulties of preparing crystalline anthocyanins, free from impurities, in sufficient amounts to allow reliable weighing under optimal conditions (GIUSTI; RODRÍGUEZ-SAONA; WROLSTAD, 1999). The availability of an efficient method for separation of anthocyanins, such as High Performance Liquid Chromatography (HPLC), combined with a list of absorption coefficients should simplify the quantitative estimation of individual anthocyanins (FRANCIS, 1989). A good source of anthocyanins and a practical method to collect them are also necessary to allow the isolation of those compounds in the laboratory.

Açaí is a fruit from typical Amazon palm tree Euterpe oleracea Mart., which has a great content of anthocyanins, specially cyanidin-3-glucoside and cyanidin-3-rutinoside (ARAUJO et al., 2008).

The objective of this research was to isolate anthocyanins analytical standards with high purity grade using an adapted fraction collector (Rheodyne® six-channel selection valve) in order to plot external standards curves using absorption and quantify other samples with cyanidin-3-glucoside and cyanidin-3-rutinoside contents, thus solving the measurement problems previously mentioned.
2 Material and methods

2.1 Solvents

High-Performance Liquid Chromatography (HPLC) grade formic acid and methanol were purchased from Tedia (USA). Ultrapure water was obtained from Milli-Q Gradient 10A System.

2.2 Sample

The freeze-dried açaí was supplied by Embrapa CPATU at Belém, PA, Brazil and was stored at −18 °C until extraction and analysis.

2.3 Sample preparation

From each standard solution with known concentrations, 8 dilutions were done, and the anthocyanins external standard curves were plotted.

3 Results and discussion

The HPLC chromatogram obtained from freeze-dried açaí shows peaks with good resolution and magnitude (Figure 1 and Table 1) allowing the isolation of each anthocyanin by the collection of the respective peaks using an adapted Rheodyne® six-channel selection valve connected to the HPLC system.

The isolated anthocyanins (Figure 2 and 3) showed a high purity grade: cyanidin-3-glucoside = 98.9% and cyanidin-3-rutinoside = 97.2%. The procedure adopted during the standard collection changing back the selecting valve to the discharge

| Peak | Anthocyanin            | Retention time (minute) |
|------|------------------------|-------------------------|
| 1    | cyanidin-3-glucoside   | 17.8                    |
| 2    | cyanidin-3-rutinoside  | 22.2                    |

The quantification of each isolated standard was performed on a Shimadzu® UV1800 spectrophotometer using the Beer-Lambert Law (A = e.b.c), at 520 nm. 100 µL of each concentrated standard were dried under N₂ flow and resuspended in 2 mL of a solution, in which the molar absorptivity is known for the solution 1% HCl in methanol (ε = 34300 L.mol⁻¹.cm⁻¹), and for the cyanidin-3-rutinoside, it is known for the solution 1% HCl in water (ε = 28840 L.mol⁻¹.cm⁻¹) (GIUSTI, RODRIGUEZ-SAONA, WROSTAD, 1999; BRITO et al., 2007; COHEN, ALVES, 2006). From each standard solution with known concentrations, 8 dilutions were done, and the anthocyanins external standard curves were plotted.
position after partial elution, allowed the anthocyanin collection without interference. The external standard curves plotted for each one of the two anthocyanins had a good squared correlation coefficient ($r^2$): cyanidin-3-glucoside = 0.999658 and cyanidin-3-rutinoside = 0.999687 (Figure 4 and 5). The anthocyanins contents of an extract obtained from the same freeze-dried açaí sample, following a method used before (Brito et al., 2007), were determined from these curves (Table 2).

There are studies, in which the content of anthocyanins is estimated by the pH-differential method. Although it is a practical and not expensive method, it does not allow the quantification of isolated anthocyanins. Its results relate the total anthocyanins content, generally expressed in cyanidin-3-O-glucoside equivalent. In addition, there is a lack of uniformity in the values of absorptivity reported for this method, which can result in wrong content calculation (Wrolstad; Durst; Lee, 2005).

As show in Table 3, the molecular ion and its fragments were used to confirm the identity of the anthocyanins isolated. The first anthocyanin isolated, retention time 17.8 minutes, showed a molecular ion m/z 449, suggesting the presence of cyanidin-3-O-glucoside, which was confirmed by the fragment ion m/z 287, which corresponds to aglycone cyanidin (Figure 6).

| Anthocyanin       | mg 100 g$^{-1}$ |
|-------------------|-----------------|
| cyanidin-3-glucoside | 35.29 ± 0.12 |
| cyanidin-3-rutinoside | 58.73 ± 0.22 |

Table 3. Identification of anthocyanins isolated from freeze-dried açaí.

| Peak | [M]$^+$ (m/z) | MS-MS (m/z) |
|------|--------------|------------|
| 1    | 449          | 287        |
| 2    | 595          | 449/287    |

Figure 4. External standard curve of isolated cyanidin-3-O-glucoside ($Y = 4.03 \times 10^7 X + 2.81 \times 10^6$).

Figure 5. External standard curve of isolated cyanidin-3-O-rutinoside ($Y = 3.76 \times 10^7 X + 1.75 \times 10^6$).

Figure 6. MS-MS spectra of cyanidin-3-O-glucoside.

Figure 7. MS-MS spectra of cyanidin-3-O-rutinoside.
The second anthocyanin isolated, retention time 22.2 minutes, showed a molecular ion at m/z 595 suggesting the presence of cyanidin-3-O-rutinoside. The ion of m/z 449 showed the loss of one molecule of rhamnoside and ion of m/z 287 confirmed the presence of aglycone cyanidin (Figure 7).

4 Conclusion

The selection valve adapted as a fraction collector can be considered a successful innovation since it was possible to isolate the analytical standards.

This practical and reliable method can be used to isolate other anthocyanins from different samples thus allowing the characterization of more fruits and vegetables.

References

ARAUJO, M. C. P. et al. Adaptação de um método por Cromatografia Líquida de Alta Eficiência para determinação de antocianinas em suco de açaí (Euterpe oleracea Mart.). In: CONGRESSO LATINO AMERICANO DE CROMATOGRAFIA E TÉCNICAS AFINS, 12., 2008, Florianópolis. Anais... Florianópolis, 2008.

BRITO, E. S. et al. Anthocyanins present in selected tropical fruits: acerola, jambolão, jussara, and guajiru. Journal of Agricultural and Food Chemistry, v. 55, p. 9389-9394, 2007.

BROUILLARD, R. Chemical structure of anthocyanins. In: MARKAKIS, P. (Ed.). Anthocyanins as Food Colors. New York: Academic Press, 1982. p. 1-40.

COHEN, K. O.; ALVES, S. M. Açai. Embrapa Amazônia Oriental, 2006. Sistemas de Produção, v. 4. Disponível em: <http://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Acai/SistemaProducaoAcai_2ed/index.htm>. Acesso em: 12 fev. 2008.

DOWNHAM, A.; COLLINS, P. Colouring our foods in the last and next millennium. International Journal of Food Science & Technology, v. 35, n. 1, p. 5-22, 2000. PMid:21848869. http://dx.doi.org/10.1046/j.1365-2621.2000.00373.x

FRANCIS, F. J. Food Colorants: anthocyanins, Crit. Rev. Food Science Nutrition, v. 28, n. 4, p. 273-314, 1989. PMid:2690857. http://dx.doi.org/10.1080/10408398909527503

GIUSTI, M. M.; RODRÍGUEZ-SAONA, L. E.; WROLSTAD, R. E. Molar absorptivity and Color characteristics of Acylated and non-Acylated Pelargonidin-Based Anthocyanins. Journal Agriculture Food Chemistry, v. 47, p. 4631-4637, 1999. PMid:10552862. http://dx.doi.org/10.1021/jf981271k

KUSKOSKI, E. M. et al. Actividad antioxidante de pigmentos antociánicos. Ciência e Tecnologia de Alimentos, v. 24, n. 4, p. 691-693, 2004. http://dx.doi.org/10.1590/S0101-20612004000400036

MALACRIDA, R. C.; MOTTA, S. Compostos fenólicos totais e antocianinas em suco de uva. Ciência e Tecnologia de Alimentos, v. 24, n. 4, p. 691-693, 2004. http://dx.doi.org/10.1590/S0101-20612004000400036

MARTÍNEZ-FLÓREZ, S. et al. Los flavonóides: propriedades y acciones antioxidantes. Nutritión Hospitalaria, v. 17, n. 6, p. 271-278, 2002. PMid:12514919.

WALLE, T. Serial review: flavonoids and isoflavones (phytoestrogens): absorption, metabolism, and bioactivity. Free Radical Biology & Medicine, v. 36, n. 7, p. 829-837, 2004. PMid:15019968. http://dx.doi.org/10.1016/j.freeradbiomed.2004.01.002

WROLSTAD, R. E.; DURST, R. W.; LEE, J. Tracking color and pigments changes in anthocyanins products. Trends in Food Science and Technology, v. 16, p. 423, 2005. PMid:21299575. http://dx.doi.org/10.1016/j.tifs.2005.03.019