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Analytical Methods

Determination of the phenolic composition from Brazilian tropical fruits by UHPLC–MS/MS

Giovana A. Bataglion [a], Felipe M.A. da Silva [b], Marcos N. Eberlin [a], Hector H.F. Koolen [a,b,*]

[a] Institute of Chemistry, University of Campinas, UNICAMP, 13083-970 Campinas, SP, Brazil
[b] Departament of Chemistry, Federal University of Amazonas, UFAM, 69077-000 Manaus, AM, Brazil

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ABSTRACT

Although Brazil is the third largest fruit producer in the world, several specimens consumed are not well studied from the chemical viewpoint, especially for quantitative analysis. For this reason and the crescent employment of mass spectrometry (MS) techniques in food science we selected twenty-two phenolic compounds with important biological activities and developed an ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC–MS/MS) method using electrospray (ESI) in negative ion mode aiming their quantification in largely consumed Brazilian fruits (açaí-do-Amazonas, acerola, cashew apple, camu-camu, pineapple and taperebá). Multiple reaction monitoring (MRM) was applied and the selection of proper product ions for each transition assured high selectivity. Linearity ($0.995 < r^2 < 0.999$), limit of detection (28.85–333.3 pg/mL), limit of quantification (96.15–1111 pg/mL), inter- and intraday accuracy (>80%), precision (CV < 20%) and extraction recovery rate (>80%) were satisfactory and showed that the method provides an efficient protocol to analyze phenolic compounds in fruit pulp extracts.

1. Introduction

Tropical areas of the world display a great variety of fruit species and the interest in their consumption has been increasing in recent years (Rufino et al., 2010). The growing interest in fruit consumption brings attention to typical fruits commonly encountered in Brazilian markets, which is the third largest producer of fruits in the world (Koolen, da Silva, Gozzo, de Souza, & de Souza, 2013; Silva, Hill, Figueiredo, & Gomes, 2014). This interest in part relies on the presence of nutrients capable of preventing degenerative diseases (Silva et al., 2014). Tropical fruit pulps and their by-products have therefore become popular; being widely applied in nutraceutical supplements, dietary additives, new food and pharmaceutical products (Bataglion et al., 2014b; Rufino, Alves, Fernandes, & Brito, 2011). Fruits specimens such as açaí-do-Amazonas (*Euterpe precatoria*), acerola (*Malpighia emarginata*), cashew apple (*Anacardium occidentale*), camu-camu (*Myrciaria dubia*), pineapple (*Ananas comosus*) and taperebá (*Spondias mombin*), particularly harvested in tropical areas of Brazil (Amazonian and Northeast regions) are also rich sources of water-soluble vitamins, phytosterols and phenolic compounds (Cetó, Capdevila, Mínguez, & del Valle, 2014; Furlan, Marques, Marineli, & Maróstica Júnior, 2013; Márquez-Sillero, Cárdenas, & Valcárcel, 2013).

Phenolic compounds are attractive due to their antioxidant activity that reduces oxidative stress and prevents or delays oxidation by scavenging free radicals (Bataglion, da Silva, Eberlin, & Koolen, 2014a; Plaza et al., 2014). The use of antioxidants derived from natural resources is gaining attention due to their health benefits, which include prevention of cardiovascular diseases, inflammations, and aging-related disorders (Huang, Ou, & Prior, 2005). Polyphenols are secondary metabolites, widespread among plant species, and are the most common and important antioxidants in the human diet (Kim, Moon, Choi, Kim, & Lee, 2013; Minutolo, Amalfitano, Evidente, Frusciante, & Errico, 2013). In addition, due to their presence in various types of biomass, polyphenols have potential as additives to industrially produced products (Ekman et al., 2013).

The evaluation of the phenolic content for different fruits consumed in Brazil has been subject of several works in the recent years (Silva et al., 2014). Such studies employ traditional methodologies based on colorimetric approaches supported by spectroscopic measurements (Alothan, Bhat, & Karim, 2009; Lim, Lim, & Tee, 2007). These methodologies constitute the initial approach for phenolic quantification and antioxidant assays (AOS). Further characterization of which compounds are respon-

* Corresponding author at: Department of Chemistry, Federal University of Amazonas, UFAM, Avda. General Rodrigo Otávio, s/n, Bairro Japiin, 69077-000 Manaus, AM, Brazil. Tel.: +55 (19)8311 1441; fax: +55 (19)3521 3023.
E-mail address: Hectorkoolen@gmail.com (H.H.F. Koolen).

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sible for the activities recorded are mainly achieved by using high performance liquid chromatography (HPLC) techniques, normally employing ultraviolet (UV) (Bystrom, Lewis, Brown, Rodriguez, & Obendorf, 2008; Mertz et al., 2009) and fluorescence (FL) (Rodríguez-Delgado, Malovaná, Pérez, Borges, & García Montelongo, 2001) as detection modes. More recently mass spectrometry (MS) has been employed in food analysis for qualitative and quantitative approaches (Biesaga & Pyrzynska, 2009; Gruz, Novák, & Strnad, 2008). The use of high performance liquid chromatography coupled to mass spectrometry (HPLC–MS) and newer methodologies based on tandem mass spectrometry experiments (MS/MS) have been gained attention due to the capacity of characterizing structurally similar compounds in complex matrices (Bazoti, Gikas, Skaltsounis, & Tsarbopoulos, 2006; Francescato, Debenedetti, Schwanz, Bassani, & Henriques, 2013; Gallart-Ayala, Moyano, & Galceran, 2008; Nagy, Redeuil, Bertholet, Steiling, & Kussmann, 2009).

The cited fruits have been extensively studied in the last years, where AOS (Silva et al., 2014), phenolic content by traditional methods (Silva et al., 2014) and exploratory analysis with chromatographic techniques (Scherer et al., 2012) have been applied, highlighting the beneficial effects of these fruits largely consumed in Brazil. Based on the existing knowledge and the crescent use of MS in food analysis the aim of our work was therefore to develop and validate a fast and sensitive method by ultra-high performance liquid chromatography coupled to tandem mass spectrometry (UHPLC–MS/MS) using the multiple reaction monitoring (MRM) mode for the determination of 22 different important phenolic compounds. This analytical approach was applied for the analysis of pulps from the açaí-do-Amazonas (*E. precatoria*) acerola (*M. emarginata*), cashew apple (*A. occidentale*), camu-camu (*M. dubia*), pineapple (*A. comosus*) and taperebá (*S. mombin*), important fruits largely consumed in Brazil.

2. Materials and methods

2.1. Standards chemicals

Standards of *p*-coumaric acid, syringic acid, homogentisic acid, vanillic acid, ferulic acid, sinapinic acid, pyrogallol, gallic acid, (+)-catechin, (−)-epicatechin, myricetin, apigenin, luteolin, caffeic acid, kaempferol, quercetin, isorhamnetin, ethyl gallate, propyl gallate, protocatechuic acid, chlorogenic acid, quinic acid and benzoic acid-d_5, were purchased from Sigma–Aldrich (St. Louis, MO, USA). The standard *trans*-resveratrol-d_4 was purchased from Cayman Chemical (Ann Arbor, MI, USA). The monosaccharides glucose, fructose, saccharose, arabinose and rhamnose were purchased from Merck Co. (Darmstadt, Germany). HPLC grade methanol was from J.T. Baker (Mexico City, D.F., Mexico) and water was purified by a Milli-Q gradient system (Millipore, Milford, MA, USA). Stock solutions of each standard compound (1 mg/mL) were prepared and stored in methanol at 4 °C. An intermediate solution containing all standard compounds (1 μg/mL) was prepared in methanol and dilutions from this solution were done at 9 different levels for calibration curves and method validation. A stock solution of internal standards (IS) of 20 μg/mL was prepared and stored in methanol and dilutions were done to reach a final concentration of 500 ng/mL in the calibration curves, which were generated in the concentrations of 20, 50, 75, 100, 200, 400, 600, 800 and 1000 ng/mL of standards. Based on structural similarities, deuterated compounds (benzoic acid-d_5 and *trans*-resveratrol-d_4) were used as IS for quantification of phenolic acids and flavonoids/catechins, respectively.

2.2. UHPLC–MS/MS

Analyses of phenolic compounds in tropical fruit pulp extracts were performed using an UHPLC–MS/MS 8040 (Shimadzu, Kyoto, Japan) consisted of a liquid chromatography system coupled to a triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. The chromatographic separation was performed on a Shim-pack XR-ODS III 2.2 μm, 2.0 mm i.d., 150 mm column (Shimadzu, Kyoto, Japan) using a binary mobile phase. Solvent A was water and solvent B was methanol. The gradient elution at 30 °C was as follows: 0–1 min, 5% B; 1–4 min, 5–60% (v:v) B; 4–7 min, 60–70% (v:v) B; 7–10 min, 70–100% (v:v) B; 10–10.50 min, 100% B; 10.50–11 min, 100–5% (v:v) B; 11–15 min, 5% B, at a flow rate of 0.4 mL/min. The autosampler temperature was maintained at 10 °C and the injection volume was 10 μL.

The ESI source parameters were as following: capillary voltage, 3.5 kV; heat block temperature, 300 °C; desolvation line temperature, 250 °C; drying gas flow (N_2), 20 L/min; nebulizing gas flow (N_2), 3 L/min; collision induced dissociation gas pressure (Ar), 224 kPa. For each standard, first the ESI(−)-MS/MS was collected for its $[M-H]^-$ and two of the most selective product ions were chosen for the MRM transitions using 20 ms of dwell time. Major settings of the mass spectrometer optimized for each MRM transition are shown in Table 1. Data were acquired and processed by Labsolution software (version 5.53 SP2, Shimadzu).

2.3. Fruit samples preparation

Fruit samples (açaí-do-Amazonas, acerola, cashew apple, camu-camu, pineapple and taperebá) were purchased at Adolpho Lisboa market in Manaus, a city located in the Amazon region of Brazil. The ripeness state of each fruit was carefully checked, where only samples with the proper state to be consumed were purchased (ripe fruits). The pulps were manually separated from the peels and seeds for each fruit and submitted to extraction procedure in triplicate. Benzoic acid-d_5 and *trans*-resveratrol-d_4 (IS) were added to 500 g of fruit pulp before extraction to mimic the extraction of the native compounds. The analytes of interest were then extracted with 500 mL of methanol in a glass blender. The liquid extracts were freeze-dried and an amount of 1 mg of the dried extracts was dissolved in methanol and filtered through a polyvinylidene difluoride (PVDF) membrane filter of a 0.45 μm pore size. Phenolic acids and flavonoids/catechins present in the fruit pulp extracts were quantified using benzoic acid-d_5 and *trans*-resveratrol-d_4 as IS, respectively.

2.4. Method validation

The method was validated according to the U.S. Food and Drug Administration (FDA) guidelines over three consecutive days for linearity, LOD, LOQ, inter- and intraday accuracy and precision, extraction recovery and stability.

The solutions of the phenolic compound standards prepared at 9 different concentrations were run in triplicate (intra- and inter-day) and the calibration curves were constructed by plotting the peak area ratio (analyte standard area/IS area) versus analyte standard concentration using the least-squares linear regression method and coefficient of determination (r^2). For concentration determination of each analyte in real samples (fruit pulp extracts), the peak area ratio between the analyte and its IS (benzoic acid-d_5 or *trans*-resveratrol-d_4) was used, and the concentrations were calculated using the calibration curve equation. LOD and LOQ were calculated according to equations LOD = 3 s/S and LOQ = 10 s/S, respectively, where s is the standard deviation of the blank and S is the calibration curve slope. Accuracy and precision were deter-