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Analytical Methods

Quantitative determination of major polyphenol constituents in pomegranate products

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ABSTRACT

The anti-oxidant content and potential health benefits associated with consuming pomegranate and pomegranate-containing products has lead to increased consumer demand for this crop resulting in it becoming a high value crop. The potential health benefits and high anti-oxidant content of this fruit is attributed to the polyphenolic compounds it contains, including the ubiquitous phenolic acids, gallic acid and ellagic acid, and punicalagin A and punicalagin B, two polyphenolics unique to this fruit. A rapid HPLC–UV method targeting these four metabolites requiring minimal sample cleanup and offering run-times half as long as existing methods was established. Within day and inter-day run-to-run variability for the four metabolites ranged from 1.9% to 6.6% and 5.3% to 11.4%, respectively. Spike recovery percentages for gallic acid, punicalagin A, punicalagin B and ellagic acid were found to be 98.5%, 92.4%, 95.5%, and 96.5%, respectively. This method was applied to the evaluation of various pomegranate products, including commercial drinks, handmade juice, and marc extracts. This method may be readily used to verify the presence of pomegranate metabolites in juices, extracts, and other products.

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1. Introduction

In recent years pomegranate (*Punica granatum*) has become a high value crop due to increased consumer demand resulting from the potential health promoting benefits obtained through consuming pomegranate fruits and pomegranate containing products. Pomegranate fruits are rich in ellagitannins and much of the health promoting potential of pomegranate has been attributed to these polyphenolic compounds. Some of the main polyphenol constituents found in pomegranate include punicalagins A and B, gallic and ellagic acids (Fig. 1) (Gil, Tomas-Barberan, Hess-Pierce, Holcroft, & Kader, 2000; Pérez-Vicente, Serrano, Abellán, & García-Viguera, 2004; Seeram, Lee, Hardy, & Heber, 2005). Punicalagins are reported to possess remarkable anti-inflammatory and anti-genotoxic properties (Chen, Li, Liu, & Lin, 2000; Kulkarni, Mahal, Kapoor, & Aradhya, 2007). Whereas antiproliferative, anti-cytotoxic, antifungal and antibacterial properties have been reported for gallic acid (Fiuza et al., 2004). Ellagic acid has been shown to possess antioxidant, anticancer and anti-atherosclerotic activities (Seeram, Lee, & Heber, 2004).

In the USA, pomegranate is commercially cultivated almost exclusively in the California, USA. Of the approximate 20.5 thousand tons of pomegranate fruits produced annually, 75% of the harvest is marketed as fresh fruit and the remaining 25% is processed into juice and used in making 100% juice beverages, soft drinks, confectionary products, and in the preparation of natural red food colourants (Mishkin & Saguy, 1982). Processing one ton of fruit yields approximately 322–341 L of juice and generates about 669 kg of pomegranate marc, a by-product made up of seeds and peels. In California alone, the annual production of pomegranate marc amounts to 3.4 thousand tons. Like pomegranate juice (Gil et al., 2000), pomegranate marc has also been shown to contain high levels of polyphenols (Qu et al., 2009) and thus this material is a potential source for isolating value-added antioxidants.

Since the popularity of pomegranate containing fruit juices and related products with the general public stems from the presence of bioactivity of polyphenols, the ability to quantitate these compounds in fruits, beverages, and extracts is essential to studying their nutritional and health effects, and for proper product labelling. From a manufacturing perspective, measuring these compounds in raw and finished materials is not only important because they contribute to sensorial-organoleptic attributes of products (Tiwari, O'Donnell, Patras, & Cullen, 2008), but it is becoming increasingly more important to address growing

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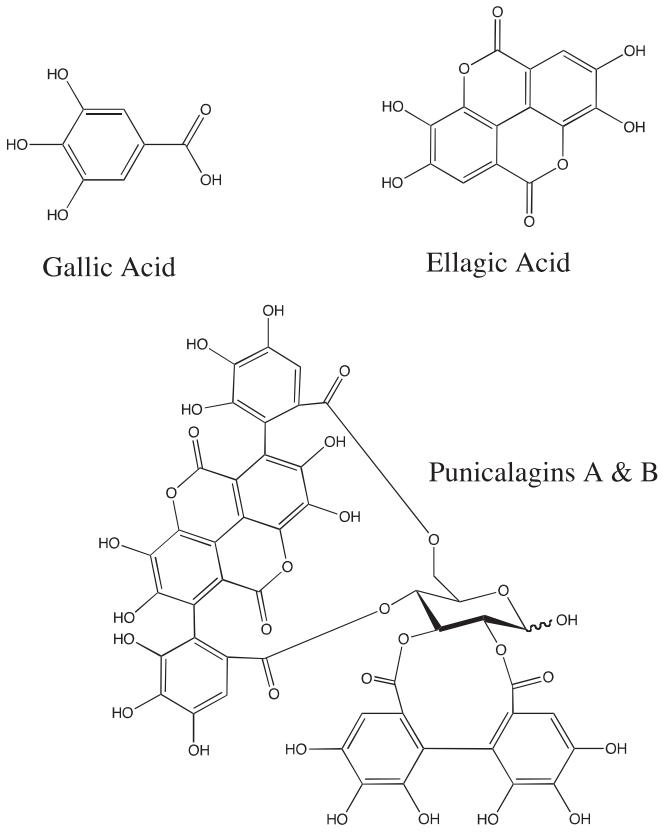

Fig. 1. Chemical structures of gallic acid (A), ellagic acid (B) and punicalagin (C).

concerns over sourcing, traceability, and adulteration that have surfaced due to the expanded demand for pomegranate materials (Zhang, Wang, Lee, Henning, & Heber, 2009). However, quantification of complex polyphenol mixture is problematic and widely used methods, such as the Folin–Ciocalteu assay often give inaccurate results. The limitations of the Folin–Ciocalteu assay due to interfering matrix components may be partially overcome by utilising HPLC or LC–MS approaches. Yet the currently available HPLC and LC–MS methods present some drawbacks, including the difficulty to quantitatively determine A and B anomers of punicalagin (Lu, Ding, & Yuan, 2008), long run times, large amount of solvent consumption and complicated pretreatments by Sephadex or resin columns (Martin, Krueger, Rodriquez, Dreher, & Reed, 2009; Seeram et al., 2005). In addition, a method to measure gallic and ellagic acids and punicalagin A and B in a single run by HPLC with UV detection is not available. Therefore, the objectives of this study were to (1) establish a rapid and efficient HPLC method to determine punicalagin A and B, ellagic acid and gallic acid, the main polyphenol constituents of pomegranate juice and marc; and (2) demonstrate the feasibility of applying this method to the evaluation of pomegranate drinks, juices and extracts.

2. Materials and methods

2.1. Materials and chemicals

Sodium hydroxide (analytical grade), and HPLC grade o-phosphoric acid (85%), methanol, and acetonitrile were purchased from Fisher Scientific Inc. (Fair Lawn, New Jersey, USA). Analytical standards of gallic and ellagic acids were obtained from Sigma–Aldrich Co. (St. Louis, Missouri, USA) and a mixture of punicalagin A and B (51.54% punicalagin A and 48.46% punicalagin B) was ordered from ChromaDex Co. (Irvine, California, USA). Water used in the HPLC analysis was deionized to $\geqslant$18.1 MΩ/cm resistance using a Barnstead NANOpure Deionization System (Dubuque, Iowa, USA) and filtered through a 0.45 μm type HA membrane filter (Millipore, Billerica, Massachusetts, USA) prior to use.

Commercial pomegranate drinks and fresh pomegranate fruit (c.v. Wonderful) were purchased from a local grocery store. Fresh pomegranate juice was prepared by hand by removing the arils from the peels and then manually squeezing them to yield juice. Pomegranate marc (c.v. Wonderful) was obtained from POM Wonderful LLC (Del Rey, California, USA). The pomegranate marc was stored at −20 °C until use.

2.2. Preparation of standards and samples

Stock solutions of punicalagin A and B (1905.36 mg/L), and gallic acid (299.40 mg/L) were prepared in MeOH and MeOH:water (1:1), respectively. Ellagic acid (570.00 mg/L) was dissolved in HPLC water in the presence of a small quantity of 1 N NaOH (0.6 mL per 100 mL water). Stock solutions were further diluted to target concentrations ranging from 0.10 to 98.20 mg/L for punicalagin A, 0.09 to 92.33 mg/L for punicalagin B, 0.03 to 29.94 mg/L for gallic acid, and 0.03 to 285.00 mg/L for ellagic acid.

Samples were clarified by centrifugation (13,000 rpm, 5 min, room temperature) using an Abbott Laboratories Model 3531 centrifuge (Abbott Park, Illinois, USA) and the resulting clarified liquid filtered through a 0.2 μm PTFE syringe filter (Millipore Corp., Billerica, Massachusetts, USA) in preparation to HPLC analysis.

2.3. Development of HPLC method

Method development experiments were conducted using a Waters HPLC system equipped with a Model 2695 Separations Module coupled to a Waters model 996 photodiode array detector (PAD) (Waters Corp., Milford, Massachusetts, USA). Chromatography was achieved using a 4.6 × 100 mm Kinetex 2.6 μm C-18 column (Phenomenex Inc., Torrance, California, USA) equipped with a KrudKatcher Ultra in-line column filter. Instrument control and data acquisition were accomplished using Masslynx (Version 4.0). Analyses were conducted at constant temperature of 30 °C using a flow rate 1.8 mL/min and a sample injection volume of 10 μL. Detector wavelengths of 270 nm for gallic acid, 254 nm for ellagic acid and 378 nm for punicalagin A and B were used. Parameters recommend by the column manufacturer for the analysis of phenolics in green tea (Phenomenex HPLC Application ID No. 18549) were used as a starting point and subsequently modified through a series of experiments directed towards optimising the separation of the targeted compounds while minimising the overall sample analysis time. Modifications to the gradient conditions and mobile phase were explored and a finalised method developed.

Performance of the finalised method also was confirmed through a series of experiments evaluating the LOD, LOQ, quantitative concentration range and quantitative equation obtained for each of the polyphenol standards. For LOD and LOQ experiments, limits were determined empirically using standards with concentrations as low as 0.03, 0.03 and 0.19 mg/L for gallic and ellagic acids, and punicalagin A and B, respectively. A pomegranate marc extract was used for evaluating within day and day-to-day variability and was also used for spike recovery experiments.

For the finalised method, a biphasic mobile phase consisting of 0.1% (v/v) H_3PO_4 in HPLC water (A) and 0.1% (v/v) H_3PO_4 in acetonitrile (B) was utilised. Prior to use, mobile phase A was filtered through a 0.45 μm HA membrane filter and B was filtered through a 0.45 μm ZapCap-CR Bottle-Top filter (Schleicher & Schuell, Keene, New Hampshire, USA). The elution conditions were as follows: isocratic elution 1% B, 0–1.5 min; linear gradient from 1% B to 4.5% B,