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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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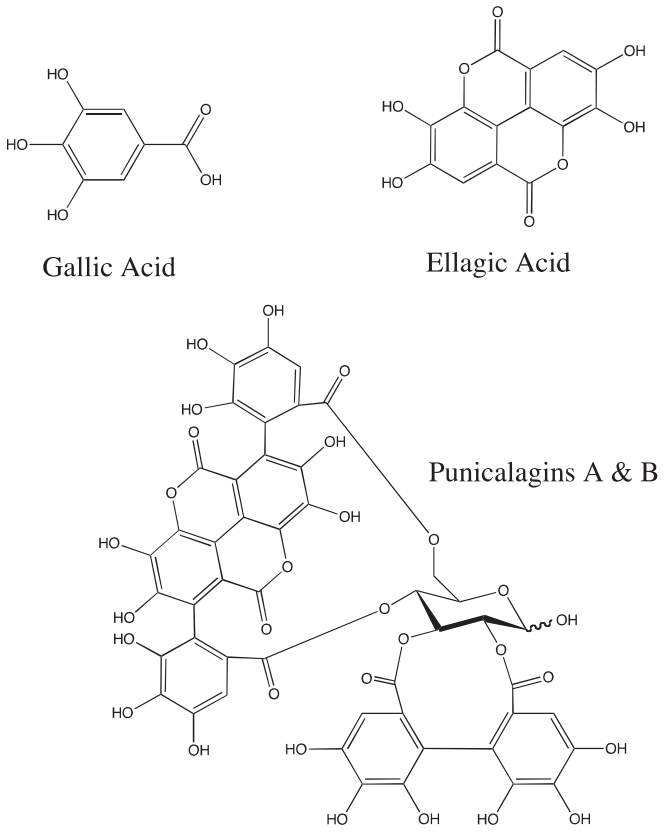

Fig. 1. Chemical structures of gallic acid (A), ellagic acid (B) and punicalagin (C).

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,

1.5–3.0 min; isocratic elution 4.5% B, 3.0–5.0 min; linear gradient from 4.5% B to 7.0% B, 5.0–6.5 min; isocratic elution 7.0% B, 6.5–8.5 min; linear gradient from 7.0% B to 25% B, 8.5–13.73 min; to 90% B, 13.73–14.39 min; to 1% B, 14.40–16.39 min. The system was maintained at a constant temperature of 30 °C and flow rate 1.8 mL/min was used. The injection volume was 10 μL. The PDA detector was set to scan from 210 to 600 nm. Retention time, UV spectrum and spiking experiments were used to verify the presence of the targeted analytes in the samples. Concentrations of the targeted analytes were based upon external calibration curves and calculated using the Quanlynx software module of Masslynx. Samples with analyte concentrations in excess of the calibration curve were diluted 4-fold with water and analysed again.

2.4. Preparation of marc extracts

Prior to the extraction, pomegranate marc was thawed at 4 °C and then dried at 40 °C using a hot air cabinet drier (CPM Wolverine Proctor LLC, Horsham, Pennsylvania, USA). Following drying, seeds were manually removed and the residual material ground to a powder with particle size less than 40-mesh using a model WBB-6 mill (Gruendler Pulverizing Co., St. Louis, Missouri, USA). Seeds were removed based on our previous results that had shown that pomegranate peel was an ideal material for extracting polyphenols (Qu et al., 2009). Extraction of the dried material was accomplished using distiled water (50:1 (w:w), water to powder) at a constant temperature of 25 °C and with the aid of stirring (1200 rpm). Extraction time was varied from 2 to 90 min. During extraction, the sample container was protected from light in order to prevent photo oxidation of the extract. The liquid extract was separated from the residue by centrifugation (3500 rpm, 20 min, 4 °C) using a Sorvall RC-3B Plus (Kendro Laboratory Products Inc., Langenselbold, Germany) and collected for analysis. Samples were analysed as described above.

3. Results and discussion

3.1. Development and finalisation of HPLC method

Many of the published methods for examining the metabolites found in pomegranate have focused on determining ellagic and gallic acids, or the punicalagins A and B, but not all four of the metabolites in a single HPLC analysis. This divided approach has largely resulted from the differences in the chromatographic properties of these four compounds when applied to C-18 stationary phases. Gallic and ellagic acids represent the extremes in terms of retention and are relatively simple to separate if the oligomeric hydrolyzable polyphenols that elute in between them are ignored. In contrast, punicalagins A and B are part of the oligomeric hydrolyzable polyphenols that elute in the between the extremes and require elongated chromatographic runs focused on that region to resolve. We selected a 2.6 μm Kinetex C-18 column with the hope that the additional resolution afforded by a smaller particle size would facilitate resolution of the target compounds, while minimising sample run time.

We initially employed the aggressive parameters recommend by the column manufacturer for the HPLC–UV analysis of phenolics in green tea to the analysis of a pomegranate marc extract, but quickly learned that the suggested chromatographic conditions did not adequately resolve the targeted compounds and resulted in gallic acid eluting in the column void volume. Since our objective was to minimise or eliminate any pretreatment steps we elected to modify the chromatographic conditions, including the overall run time, slope of the gradient, and phosphoric acid composition of the mobile phase. In the course of our method development studies we found that addition of phosphoric acid and minimising the initial organic content of the mobile phase were essential to optimising the retention of gallic acid. Ellagic acid was strongly retained on the Kinetex C18 stationary phase regardless of the acid content and required an acetonitrile content in excess of 65% in the mobile phase in order to be eluted in a timely fashion. Modifications to the slope of the gradient between the two extremes were made towards optimising the resolution of punicalagins A and B from the other matrix components found in the extract. The finalised method was capable of resolving the targeted polyphenols in a runtime less than 17 min (Fig. 2A–D). Fig. 2B–D shows chromatograms extracted at individual wavelengths and demonstrate that base line resolution of the targeted compounds was achieved.

The retention times of gallic acid, punicalagin A, punicalagin B and ellagic acid were 2.45, 7.61, 10.20, and 13.41 min, respectively, which were in agreement with polarity sequences of four constituents but significantly shorter than currently reported retention times. For example, Lu et al. (2008) reported that the retention times of punicalagin A and punicalagin B were 17 and 20 min, respectively for a method with a total run time of 40 min. For another method, the retention times of punicalagin A, punicalagin B and ellagic acid were 18, 22 and 37 min, respectively (Seeram et al., 2004). Based on the present results, the presented method requires less than half the analysis time of commonly used methods.

For gallic acid, punicalagin A, punicalagin B and ellagic acid, LOD values were determined to be 0.12, 1.53, 1.44, and 0.22 mg/L, and LOQ values were 0.23, 3.07, 2.89, and 0.45 mg/L (Table 1). Equations and R^2 values for the relationship between concentration and UV response for each of the analytes are shown in Table 1. Within day and inter-day run-to-run variability was evaluated using a randomly selected pomegranate marc extract and results were reported in terms of %CV. Within day %CV values of gallic acid, punicalagin A, punicalagin B and ellagic acid ranged from 2.3% to 6.1%, 2.9% to 5.8%, 2.6% to 6.6%, and from 1.9% to 6.2%, respectively. Inter-day %CV values were 5.3%, 10.2%, 11.4%, and 6.8% for gallic acid, punicalagin A, punicalagin B and ellagic acid, 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.

3.2. Application of HPLC method

Fig. 3A–E shows chromatograms resulting from the analysis of various pomegranate products, including commercial drinks and handmade juice. Concentrations of the targeted metabolites found in the samples are listed in Table 2. There is a high degree of similarity between the chromatograms obtained for the 100% pomegranate juice drinks (POM Wonderful (Fig. 3C), All Natural (Fig. 3D), and Langers 100% (Fig. 3E)) and pomegranate marc extract (Fig. 2A). Whereas the chromatograms resulting from the fresh hand-squeezed juice (Fig. 3A) and pomegranate containing drink (Fig. 3B, Honest Ade Pomegranate Blue) were dominated by different peaks and contained minimal concentrations of the targeted phenolics (Table 2).

The chromatogram resulting from the analysis of handmade juice from pomegranate arils showed two high response peaks appearing at 10.11 and 11.44 min. Evaluating the UV–VIS spectrum of these peaks, we tentatively identified the peak at 11.44 min as cyanidin 3,5-diglucoside (λ_{max} 517, 279 nm), but were unable to assign an identity to the other peak (10.11 min, λ_{max} 265 nm). Analysis of the chromatogram at 510 nm (data not shown) also revealed the presence of other anthocyanins, but additional efforts to identify these compounds were not undertaken because it was outside of the scope of our current objectives. Most reports found

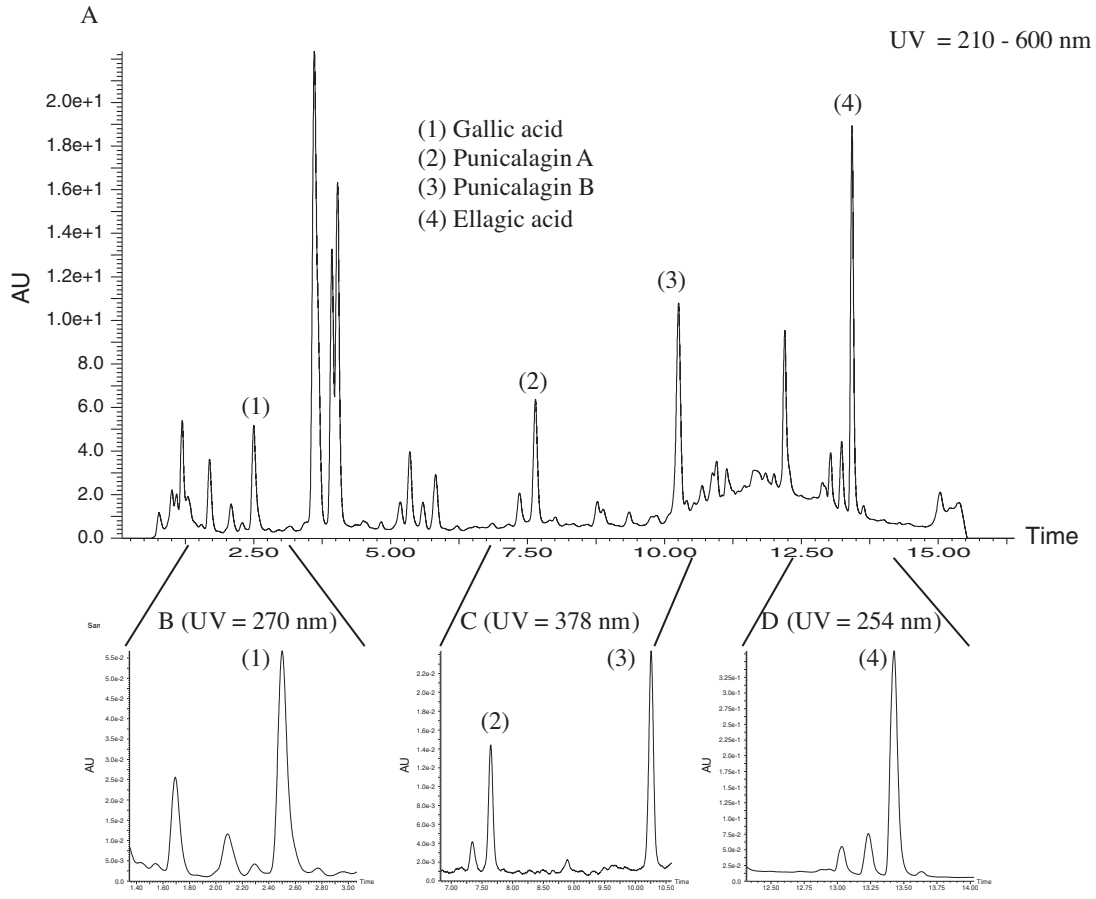

Fig. 2. Chromatograms of pomegranate marc extract demonstrating resolution of the targeted metabolites. Fig. 2A shows signal from diode array detector, whereas Fig. 2B–D shows signal extracted at three different wavelengths specific for the targeted metabolite.

Table 1
Retention times, wavelengths (λ), limits of detection (LOD), limits of quantitation (LOQ), quantitative concentration ranges used during analysis and equations and R^2 values.

Standard	Retention time (min)	λ (nm)	LOD (mg/L)	LOQ (mg/L)	Concentration range (mg/L)	Equation $y = mx + b$	R^2
Gallic acid	2.45	270	0.12	0.23	0.94–29.94	$317.6x - 40.75$	0.997
Punicalagin A	7.61	378	1.53	3.07	3.07–98.20	$46.71x - 40.74$	0.993
Punicalagin B	10.20	378	1.44	2.89	2.89–92.33	$82.30x - 30.83$	0.994
Ellagic acid	13.41	254	0.22	0.45	7.13–114.00	$938.34x - 3546.9$	0.978

in the literature for the analysis of pomegranate have focused on evaluating juices produced by pressing intact or peeled fruits (Mousavinejad, Emam-Djomeh, Rezaei, & Khodaparast, 2009), residual husks and rinds (Zhou, Wu, Li, Zhang, & Hu, 2008) or extracts produced from the same (Martin et al., 2009; Zhang et al., 2009). There have also been a limited number of reports detailing the HPLC–UV (Gil et al., 2000) or LC–MS (Fischer, Carle, & Kammerer, 2011) analysis of juices produced from arils alone. In these instances, anthocyanins were identified as the most abundant secondary metabolites present. Other metabolites including ellagic acid (2.1–15.3 mg/L) and punicalagins (A + B, 4.1–22.8 mg/L) were also present, but at concentrations much lower than those found for anthocyanins. Thus the results we observed for our handmade juice (Table 2) prepared from arils were similar to those previously reported for aril-derived juices, and were obtained with a sample runtimes that were less than half as long as the methods of Gil et al. (2000) and Fischer et al. (2011).

As it can be seen from Fig. 3C–E and Table 2, all three of the 100% pomegranate drinks (POM Wonderful, All Natural and Langers 100% pomegranate juices) had relatively high concentrations of gallic acid, punicalagin A, punicalagin B and ellagic acid when compared to the handmade juice. This observation is not surprising considering that commercial manufacturers produce pomegranate juice by pressing intact fruit, and it is during the pressing process that the majority of phenolics are extracted as the result of the rind and husk tissues being bathed with the acidic fruit juice yielded for the crushed arils. Gil et al. (2000) in their analysis of a commercial juice reported punicalagin A and B concentrations of 421.3 and 838.5 mg/L, respectively, whereas Fischer et al. (2011) reported a combined punicalagin A and B concentration of 564.5 mg/L for a juice prepared using a press, and Gonzalez-Molina, Moreno, and Garcia-Viguera (2009) reported even lower juice concentrations of punicalagin A (*ca* 40 mg/L) and B (*ca* 90 mg/L) in juice produced from a Spanish cultivar using a pilot-scale press (Gonzalez-Molina et al., 2009). These same authors also reported ellagic acid concentrations of 37.9, 7.2, and *ca* 15.2 mg/L, respectively, and another report by Mousavinejad et al. (2009) reported ellagic acid concentrations ranging from 7 to 160 mg/L for Iranian cultivars. For our sample set, we found that All Natural 100% pomegranate juice had the highest gallic acid content (72.95 mg/L), Langers 100%

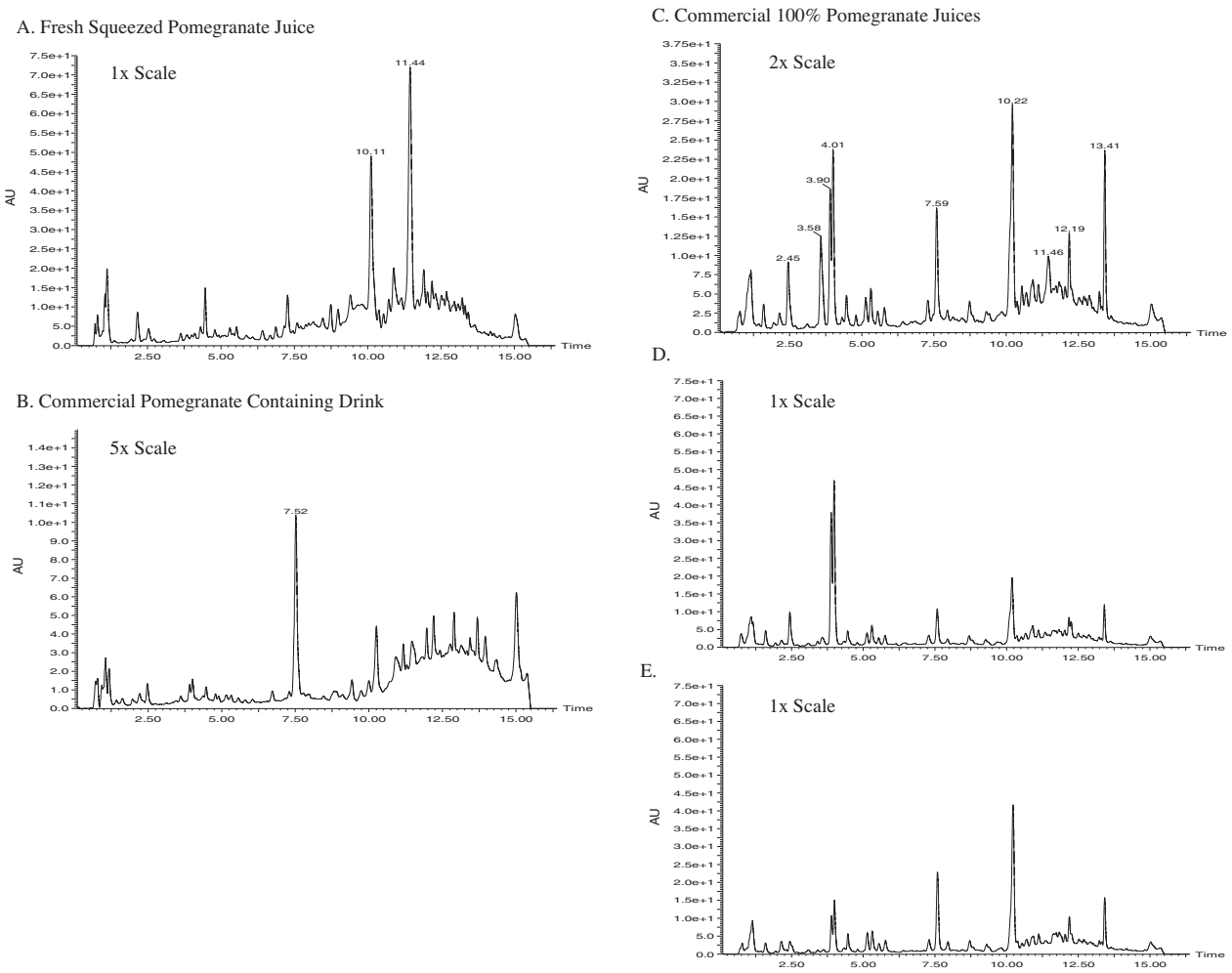

Fig. 3. Chromatograms of handmade pomegranate juice (A), Honest Ade Pomegranate Blue (B), POM Wonderful 100% pomegranate juice (C), All Natural 100% pomegranate juice (D), and Langers 100% pomegranate juice (E). Handmade juice was prepared from arils. Magnification of individual chromatograms is listed in the upper left-hand corner for each sample.

Table 2
Concentrations of gallic acid, punicalagin A, punicalagin B and ellagic acid of pomegranate products.[a]

Sample	Gallic acid concentration (mg/L)	Punicalagin A concentration (mg/L)	Punicalagin B concentration (mg/L)	Ellagic acid concentration (mg/L)
POM Wonderful 100% pomegranate juice	69.55 ± 0.60	151.92 ± 4.33	179.22 ± 6.01	113.01 ± 3.32
All Natural 100% pomegranate juice	72.95 ± 1.45	100.97 ± 1.56	123.29 ± 1.09	50.84 ± 0.44
Langers 100% pomegranate juice	30.14 ± 0.96	237.78 ± 8.02	278.06 ± 6.68	72.06 ± 1.09
Honest Ade Pomegranate Blue	2.60 ± 0.04	2.57 ± 0.03[b]	4.58 ± 0.23	0.72 ± 0.06
Handmade pomegranate juice	5.20 ± 0.10	4.67 ± 0.08	3.54 ± 0.09	2.37 ± 0.08
Pomegranate peel extract	32.62 ± 1.36	40.09 ± 2.70	43.18 ± 4.62	98.13 ± 5.45

[a] All data are recorded as means ± SD.
[b] The value is lower than LOQ but higher than LOD.

pomegranate juice gave the highest punicalagin A and B contents (237.78 and 278.06 mg/L, respectively), and POM Wonderful 100% pomegranate juice had the highest ellagic acid content (113.01 mg/L). Other than the concentration of Punicalagin B being consistently higher than the concentration of Punicalagin A, there were no apparent correlations between the concentrations of the metabolites. The variation between the samples is likely due to differences in manufacturing methods or in the varieties used by the manufacturers.

Besides gallic and ellagic acids and punicalagin A and B, there were also several other well responding peaks (labelled in Fig. 3C; 3.58, 3.90, 4.01, 11.46, 12.19 min) in the chromatograms of the 100% juices samples and pomegranate marc extract. Based upon their UV–VIS spectra, relative retention times, and available literature, these peaks were tentatively identified as follows. The peak at 12.19 min is likely an ellagic acid derivative (λ_{max} 358, 253 nm). The peak eluting at 11.46 min was assigned the identity of cyanidin 3,5-diglucoside (λ_{max} 517, 279 nm), as was done for

the hand made juice sample. The overall contribution of anthocyanins to the chromatographic profiles of the commercial juice samples is small, but not unexpected since pomegranate anthocyanins have been shown to readily degrade during processing and storage (Alighourchi & Barzegar, 2009; Pérez-Vicente et al., 2004). The three remaining peaks eluting from 3.58 to 4.01 min were tentatively identified as punicalin isomers (λ_{max} 378 nm). The relative intensities of these three peaks among the commercial juice samples varied, indicative of differences in manufacturing processes or the cultivars used. In contrast, the most similar chromatographic profiles were shared between the POM Wonderful 100% juice (Fig. 3C) and pomegranate marc extract, two products that were produced by the same manufacturer using the same pomegranate variety (c.v. Wonderful).

Fig. 3B is the chromatogram resulting from the analysis of a pomegranate containing commercial beverage (Honest Ade Pomegranate Blue). Although the name of the beverage suggests a high content of pomegranate juice, the labelling provided by the manufacturer indicates that the product contains a total juice content of 10%, of which pomegranate juice concentrate is listed second following grape juice concentrate. The fact that punicalagin A and B were detected supports that the product contains pomegranate, however, given that the punicalagin A and B concentrations found in the 100% juices ranged from 100.97 to 237.78 mg/L and 123.29 to 278.06 mg/L, respectively, the actual pomegranate content in this beverage amounts to no more than 2%. Unlike the chromatograms for the 100% juice samples, the most predominate feature in this chromatogram was a peak that eluted at 7.52 min. The UV–VIS spectrum of this peak (λ_{max} 328, 300 sh, 247 nm) closely resembles the spectrum of caffeic acid, suggesting that the peak is a caffeoyl ester or chlorogenic acid type derivative.

The ability to detect adulteration or to verify the authenticity of raw and processed agricultural products is a growing concerning among manufacturers and consumers (Martin et al. (2009)). Pomegranate containing products are not immune to these concerns and a recent analysis of 27 products labelled as containing pomegranate by HPLC (Zhang et al., 2009), for pomegranate phenolics including punicalagin A and B, and ellagic acid, revealed that less than 20% of the tested products possessed the expected HPLC profile, and five of the tested products contained no detectable levels of pomegranate metabolites. The remaining products contained the prerequisite pomegranate metabolites, but at concentrations that were overshadowed by excessive ellagic acid levels, to which the authors concluded were the result of adulteration to effect a higher antioxidant content in the products. In their report, Zhang et al. (2009) concluded that punicalagins A and B and ellagic acid, along with punicalin, could function as sentinel metabolites for verifying the inclusion of pomegranate in commercial products. The method we have developed readily separates these sentinel metabolites and we believe that this method provides a simple, rapid and effective means to evaluate samples for the presence of pomegranate metabolites.

3.3. Water-based extraction of pomegranate marc

Several reports have shown that the residual pomegranate materials generated as byproducts of juice production contain high levels of phenolic antioxidants, and for some materials the punicalagin content makes up 80–85% of total on a dry weight basis (Poyrazoglu, Gokmen, & Artk, 2002; Seeram et al., 2005). Thus pomegranate marc is poised as an ideal material for the extraction of pomegranate antioxidant compounds. Results from our evaluation of the influence of extraction time (2, 10, 20, 30, 60, and 90 min) on the concentration of the targeted metabolites are shown in Fig. 4. The concentrations of gallic acid and ellagic acid both rapidly increased almost linearly within the first 20 min,

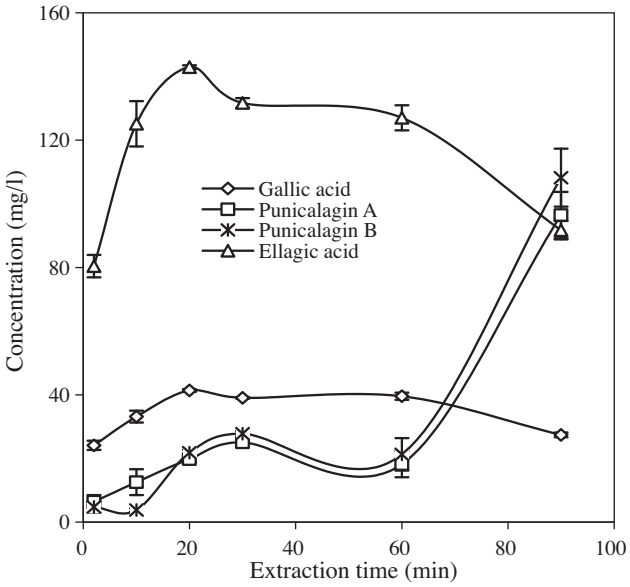

Fig. 4. Concentrations of gallic and ellagic acids, and punicalagin A and B in pomegranate peel extracts under different extraction times.

and then displayed a slow decrease from 20 to 90 min. Whereas, the concentrations of punicalagin A and B rapidly increased in the first 30 min, maintained a plateau from 30 to 60 min, and then showed a sharp increase from 60 to 90 min. The initial concentration increases observed could have been due to an enhancement in the solubility and diffusion coefficients for the polyphenol constituents resulting from the ability of these compounds to self-acidify the extraction solution based upon their weak acid properties (Coulson, Richardson, Backhurst, & Harker, 1991). The decrease in gallic acid and ellagic acid concentrations from 60 to 90 min might be due to these two acids forming conjugates, or perhaps punicalagins, which can also be used to explain the increased concentrations of punicalagin A and B (Gonzalez-Molina et al., 2009).

If pomegranate marc is to be used for the isolation of the organic acids (gallic and ellagic acids), we would recommended an extraction time of 20 min be used. If punicalagin A and B are the desired products, the preferred extraction time should be longer than 60 min. Regardless of the target, pomegranate marc, a by-product of pomegranate juice processing, appears to be a potential source for isolating value-added phenolic compounds. However, caution should be taken in establishing precise extraction parameters in order to obtain a consistent product.

4. Conclusions

A rapid and efficient HPLC method to determine the levels of the polyphenolics punicalagin A, punicalagin B, ellagic acid and gallic acid in pomegranate juice and pomegranate containing products was developed. This method displays good linearity, good daily reproducibility, a high recovery rate, and has low limits of detection (LOD) and quantification (LOQ). Compared to existing methods, this method offers significant improvements in sample throughput and facilitates the quantitative determination of four major polyphenolics in a single run. This method was applied to investigating the influence of extraction time on the water-based extraction of pomegranate marc. We found that shorter extraction times (<20 min) yielded extracts rich in gallic acid and ellagic acid, whereas longer times (>60 min) resulted in extracts with higher punicalagin levels. In the future, it may also be possible to use this

method to verify the presence of pomegranate metabolites in products, or to detect adulteration.

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References

Alighourchi, H., & Barzegar, M. (2009). Some physicochemical characteristics and degradation kinetic of anthocyanin of reconstituted pomegranate juice during storage. *Journal of Food Engineering, 90*(2), 179–185.

Chen, P. S., Li, J. H., Liu, T. Y., & Lin, T. C. (2000). Folk medicine *Terminalia catappa* and its major tannin component, punicalagin, are effective against bleomycin-induced genotoxicity in Chinese hamster ovary cells. *Cancer Letters, 152*(2), 115–122.

Coulson, J. M., Richardson, J. F., Backhurst, J. R., & Harker, J. H. (1991). *Particle Technology and Separation Processes* (4th ed.). Oxford: Butterworth-Heinemann E-Publishing Inc., pp. 385.

Fischer, U. A., Carle, R., & Kammerer, D. R. (2011). Identification and quantification of phenolic compounds from pomegranate (*Punica granatum* L.) peel, mesocarp, aril and differently produced juices by HPLC–DAD–ESI/MSn. *Food Chemistry, 127*(2), 807–821.

Fiuza, S. M., Gomes, C., Teixeira, L. J., Girão da Cruz, M. T., Cordeiro, M. N., Milhazes, N., et al. (2004). Phenolic acid derivatives with potential anticancer properties – a structure-activity relationship study. Part 1: Methyl, propyl and octyl esters of caffeic and gallic acids. *Bioorganic & Medicinal Chemistry, 12*(13), 3581–3589.

Gil, M. I., Tomas-Barberan, F. A., Hess-Pierce, B., Holcroft, D. M., & Kader, A. A. (2000). Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *Journal of Agricultural and Food Chemistry, 48*(10), 4581–4589.

Gonzalez-Molina, E., Moreno, D. A., & Garcia-Viguera, C. (2009). A new drink rich in healthy bioactives combining lemon and pomegranate juices. *Food Chemistry, 115*(4), 1364–1372.

Kulkarni, A. P., Mahal, H. S., Kapoor, S., & Aradhya, S. M. (2007). In vitro studies on the binding, antioxidant, and cytotoxic actions of punicalagin. *Journal of Agricultural and Food Chemistry, 55*(4), 1491–1500.

Lu, J., Ding, K., & Yuan, Q. (2008). Determination of punicalagin isomers in pomegranate husk. *Chromatographia, 68*(3–4), 303–306.

Martin, K. R., Krueger, C. G., Rodriquez, G., Dreher, M., & Reed, J. D. (2009). Development of a novel pomegranate standard and new method for the quantitative measurement of pomegranate polyphenols. *Journal of the Science of Food and Agriculture, 89*(1), 157–162.

Mishkin, M., & Saguy, I. (1982). Thermal stability of pomegranate juice. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A, 175*(6), 410–412.

Mousavinejad, G., Emam-Djomeh, Z., Rezaei, K., & Khodaparast, M. H. H. (2009). Identification and quantification of phenolic compounds and their effects on antioxidant activity in pomegranate juices of eight Iranian cultivars. *Food Chemistry, 115*(4), 1274–1278.

Pérez-Vicente, A., Serrano, P., Abellán, P., & García-Viguera, C. (2004). Influence of packaging material on pomegranate juice colour and bioactive compounds, during storage. *Journal of the Science of Food and Agriculture, 84*(7), 639–644.

Poyrazoglu, E., Gokmen, V., & Artk, N. (2002). Organic acids and phenolic compounds in pomegranates (*Punica granatum* L.) grown in Turkey. *Journal of Food Composition and Analysis, 15*(5), 567–575.

Qu, W. J., Pan, Z. L., Zhang, R. H., Ma, H. L., Chen, X. G., Zhu, B. N., et al. (2009). Integrated extraction and anaerobic digestion process for recovery of nutraceuticals and biogas from pomegranate marc. *Transactions of the ASABE, 52*, 1997–2006.

Seeram, N. P., Lee, R., Hardy, M., & Heber, D. (2005). *Rapid large scale purification of ellagitannins from pomegranate husk a by-product of the commercial juice industry*. Elsevier Science BV.

Seeram, N. P, Lee, R., & Heber, D. (2004). *Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (Punica granatum L.) juice*. Elsevier Science BV.

Tiwari, B. K., O'Donnell, C. P., Patras, A., & Cullen, P. J. (2008). Anthocyanin and ascorbic acid degradation in sonicated strawberry juice. *Journal of Agricultural and Food Chemistry, 56*(21), 10071–10077.

Zhang, Y., Wang, D., Lee, R. P., Henning, S. M., & Heber, D. (2009). Absence of pomegranate ellagitannins in the majority of commercial pomegranate extracts: Implications for standardization and quality control. *Journal of Agricultural and Food Chemistry, 57*(16), 7395–7400.

Zhou, B., Wu, Z., Li, X., Zhang, J., & Hu, X. (2008). Analysis of ellagic acid in pomegranate rinds by capillary electrophoresis and high-performance liquid chromatography. *Phytochemical Analysis, 19*(1), 86–89.