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Rapid large scale purification of ellagitannins from pomegranate husk, a by-product of the commercial juice industry

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Abstract

Pomegranate (*Punica granatum* L.) fruits are widely consumed fresh and in processed forms as juice, jams and wine. Pomegranate fruit husk/peel is a rich source of hydrolyzable tannins called ellagitannins (ETs). In the commercial pomegranate juice (PJ) industry, these ETs are extracted from the husk in significant quantities into the juice due to their hydrophilic properties. Pomegranate husk, a by-product of the PJ industry, is therefore an inexpensive and abundant source of ETs. Previous methods to isolate pomegranate ETs included labor intensive and time-consuming solid phase extractions by column chromatography (C-18, polyamides, cellulose, Sephadex Lipophilic LH-20, Diaion HP20) and/or use of specialized instruments such as preparative-high performance liquid chromatography (HPLC). We have used an Amberlite XAD-16 resin vacuum-aspirated column to rapidly purify an aqueous extract of pomegranate husk to afford total pomegranate tannins (TPT) in substantial yields (58–60 g TPT/kg husk; time <1 h). Using analytical HPLC and tandem LC-ES/MS, evaluation of TPT showed that it contains the major fruit husk ET, punicalagin (80–85% w/w) and ellagic acid (EA; 1.3% w/w) and unquantified amounts of punicalin and EA-glycosides (hexoside, rhamnoside and pentoside). Since pomegranate ETs are reported to show potent antioxidant, antiatherosclerotic and anticancer activities, this method can be used for the large-scale production of TPT for future in vitro and in vivo biological studies. This method is practical for industrial applications and could provide a low-cost means to use a currently underutilized food by-product to develop phytoceuticals with potential health benefits or to develop products for use in the cosmetic and food biopreservative industries.

Keywords: Pomegranate; *Punica granatum*; Ellagitannins; Punicalagin; Amberlite XAD-16

1. Introduction

Tannins are high molecular weight plant polyphenols divided into two chemically and biologically distinct groups: condensed tannins or proanthocyanidins (e.g. from tea, grapes, cranberries etc.) and hydrolyzable tannins or ellagitannins (ETs) (e.g. from raspberries, strawberries, pomegranates etc.) and gallotannins (GTs). Tannins gained original popularity in the commercial 'tanning' industry where animal hides were converted into leather by using plant extracts but have attracted much recent attention due to their numerous biological activities and implications in potential benefits to human health.

Pomegranate (*Punica granatum* L.) is grown mainly in the Near East, India, Spain (southeastern), Israel and the United States (California) and is of significant economic importance since the fruits are either consumed fresh or used commercially in the juice, jam and wine industries [1,2]. Pomegranate husk is rich in ETs such as punicalagin and its isomers [2,3-hexahydroxydiphenoyl-4,6-gallagylglucose] (**1**), as well as lesser amounts of punicalin [4,6-gallagylglucose] (**2**), gallagic acid (GA) (**3**), ellagic acid (EA) (**4**) and EA-glycosides (hexoside, pentoside, rhamnoside etc.) [1–4]. These ETs are extracted in significant levels into the juice during industrial hydrostatic processing methods of the whole fruits [1]. Commercial pomegranate juices (PJ) exhibit potent antioxidant properties which have been attributed to their high content of polyphenols including punicalagin which can reach levels >2 g/L juice, depending on the fruit cultivar and processing methods [1,2]. Tannins have also been identified as the active antiatherosclerotic compounds in PJ responsible for the ability of this juice to protect human low-density lipoprotein cholesterol from oxidation in vivo [5].

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Pomegranate peel extracts are currently used for treatment of respiratory diseases and in the preparation of tinctures, cosmetics and other therapeutic formulae [6,7]. Pomegranate extracts are also being investigated for their potential use as food biopreservatives and for the formulation of products in the nutraceutical industry [6]. Since pomegranate-derived foods are widely consumed and there have been conflicting reports regarding the toxicity of punicalagin [8–10], there is a need for in depth in vitro and in vivo studies to determine the biological properties of these compounds. Unfortunately, there are no cost-effective and rapid methods available to obtain pomegranate ETs in sufficient yields and purity required for in vitro and in vivo biological studies. Present purification methods include the use of time consuming and labor intensive columns and solid phase extractions with a variety of stationary phases (Sephadex Lipophilic LH20, polyamides, microcrystalline cellulose, C-18, Diaion HP20 etc.) and/or preparative-HPLC [1,2,11–13].

In this study, we report a rapid method to prepare total pomegranate tannins (TPT) in substantial yield and purity which can be used by researchers for future in vitro and in vivo biological studies. This method has industrial applications since it can be easily scaled-up and is cost-effective since its uses an inexpensive by-product of the commercial PJ industry, fruit husk, which after pressing is currently used as cattle-feed. We also report the use of NMR spectroscopic methods and tandem LC-ES/MS in negative mode, to identify ETs in TPT. HPLC quantification studies of TPT using pure punicalagin (isolated from TPT) and EA (commercially available), for generation of standard calibration curves, are also reported herein.

2. Experimental

2.1. Reagents

All solvents were HPLC grade and purchased from Fisher Scientific Co. (Tustin, CA). Ellagic, formic and acetic acids, Sephadex Lipophilic LH-20 and Amberlite XAD-16 resins were purchased from Sigma Aldrich Co. (St. Louis, MO, USA).

2.1.1. General experimental procedures

Nuclear magnetic resonance (NMR) spectra were recorded on Bruker instruments operating at 400 MHz for ^{1}H and 150 MHz for ^{13}C. Chemical shifts for punicalagin (**1**) were recorded in acetone-d6 and are in δ (ppm) relative to the solvent peaks. HPLC-ES/MS analyses were carried out on an LCQ Classic Finnigan LC-MS/MS system (ThermoFinnigan, San Jose, CA), equipped with a HP 1100 series HPLC system consisting of an autosampler/injector, quaternary pump, column heater, and diode array detector (DAD). Data handling was carried out using Xcalibur 1.2 software (Finnigan Corp.). HPLC analyses were carried out on Waters Alliance 2690 system equipped with a photo diode array (PDA) detector (Waters Corp., Milford, MA) and data handling was with Waters Millenium v 3.02 software.

2.1.2. Purification of total pomegranate tannins (TPT)

Pomegranate fruits (Ruby River, Lot # 3127, Whole Foods Supermarket, Westwood, LA, CA, USA) were washed and cleaned to yield their husks/peels, separate from seeds and juice. The husk (1 kg, fresh weight) was percolated with water (5 L) for 1 h, squeezed by hand, then instantly blended (Waring Blender, New Hartford, CN, USA). The thick husk puree was filtered through cheesecloth and squeezed dry to yield a dark brown aqueous extract. The XAD-16 resin column was prepared for chromatography by pre-washing in methanol then pre-equilibrating in water for 12 h. The aqueous extract was divided into 200 mL portions and adsorbed onto a vacuum aspirated column of Amberlite XAD-16 resin (500 g). The optimal loading volumes of the aqueous extract varied from 200 ± 10 mL per 500 g of pre-conditioned XAD-16 resin per column. Each column was eluted with copious amounts of distilled water (4 L) until the sugary pale yellow eluate was clear in color. Remaining water was removed from the resin by vacuum aspiration and the adsorbed tannins were eluted with MeOH (2 × 400 mL) to yield a dark brown solution. The time taken to complete one cycle per column was <15 min. Methanol was removed by a Rota-vap (Buchi) in vacuo at low temperature (37 °C) to yield total pomegranate tannins (TPT) as a dark brown powder (58–60 g/kg husk). The XAD-16 column was regenerated by washing with water and can be re-used (over 100 times) in this processing method. In addition, although other low carbon alcohols such as ethanol can also be used as eluents, purification parameters were optimized for methanol due to its ease of removal in vacuo under normal laboratory-scale conditions. Efficiency of recoveries were also calculated for punicalagin (77–85%) and EA (80–95%) on the resin columns by spiking known quantities in water, re-application on the columns, collection and quantification (see Section 2.1.4.)

2.1.3. Purification of punicalagin (**1**) for quantification studies of TPT

Due to the unavailability of a commercial standard of punicalagin, a Sephadex Lipophilic LH-20 resin column was used to isolate the pure compound from TPT as follows. TPT (300 mg) was adsorbed onto a Sephadex-LH-20 column that was pre-equilibrated with H_2O:MeOH (8:2, v/v) and eluted with increasing amounts of MeOH. LC-ES/MS analyses revealed that the 50% MeOH fraction was enriched in two compounds, both corresponding to the molecular weight for punicalagin ($M - H$ m/z 1083). This fraction (273 mg) was evaporated in vacuo and re-chromatographed, this time pre-equilibrating the column with EtOH. Elution with increasing amounts of H_2O and Me_2CO to EtOH:H_2O:Me_2CO (6:3:1, v/v/v) and finally EtOH:Me_2CO (1:1, v/v) yielded a fraction which was evaporated in vacuo to afford a yel-

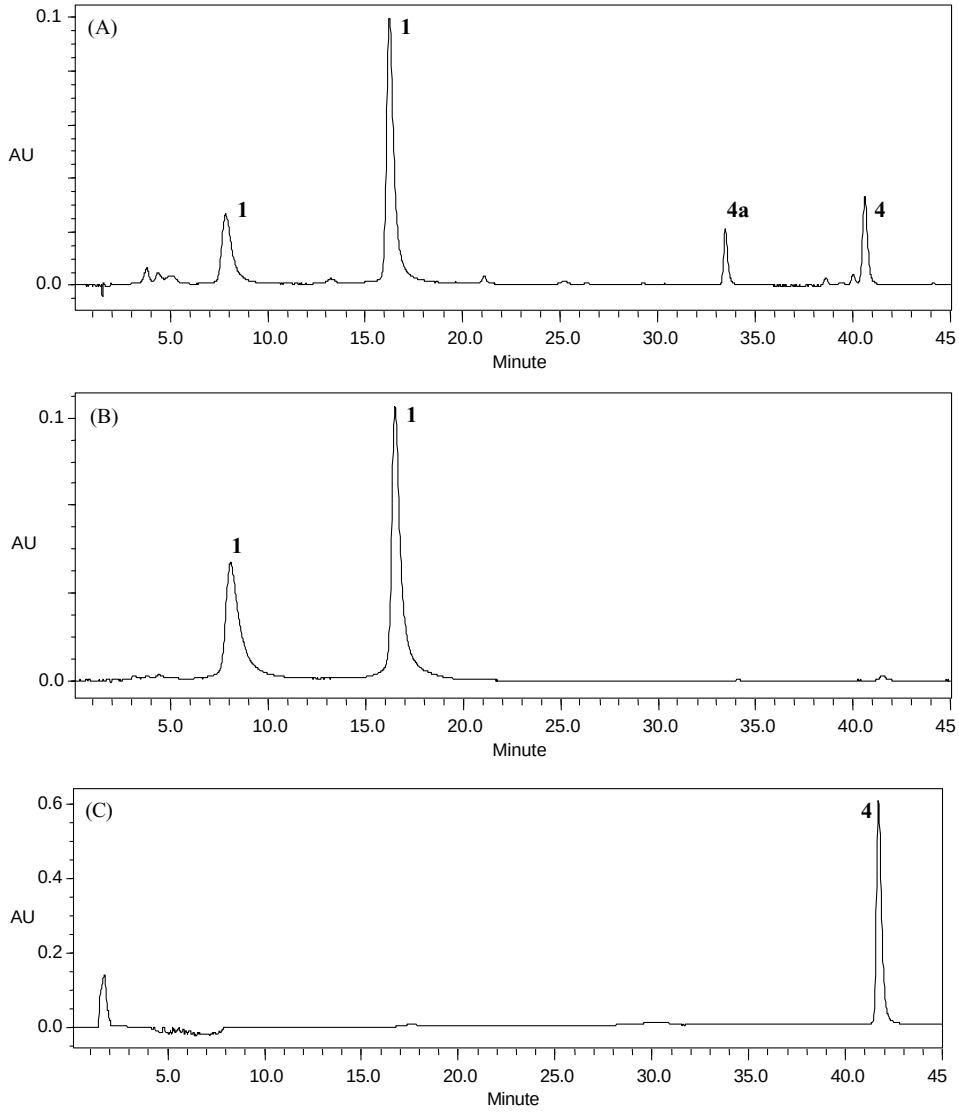

Fig. 1. (A) HPLC chromatogram of total pomegranate tannins (TPT) purified from fruit husk. Peaks were identified by comparison of their retention times with isolated pure standards of: punicalagin anomers (**1**) and commercial standard of ellagic acid (EA) (**4**), corresponding to $M-H$ *m*/*z* 1083 and $M-H$ *m*/*z* 301 in LCMS, respectively. Peak 4a was identified as EA-hexoside based on its ion at $M-H$ *m*/*z* 463 as previously reported [1]. (B) HPLC chromatogram of punicalagin anomers isolated from TPT. C: HPLC chromatogram of commercially available EA. HPLC-UV detection wavelengths for quantification of punicalagins and EA in TPT were 378 and 366 nm, respectively.

low amorphous powder (34 mg). This was identified as a mixture of α- and β-punicalagin anomers by ^{1}H and ^{13}C NMR spectral data which were consistent with literature reports [9,12,13]. It is noteworthy that the Sephadex LH-20 column does not represent a rapid method to produce TPT but was used to isolate small amounts of an analytically pure sample of punicalagin for NMR and quantification purposes.

2.1.4. HPLC conditions for analyses and quantification

All samples (50 μL injection volume) were filtered (0.22 μm) and analyzed on a Novapak (Waters Corp.) C-18 column, 150 mm × 3.9 mm i.d., 5 μm. The mobile phase, solvent A (2% CH_3COOH/H_2O) and solvent B (2% aqueous CH_3COOH/MeOH) was used under linear gradient conditions starting with 99% A in B for 5 min to 40% A in B over 40 min, hold time, 5 min with a flow rate of 1.0 mL/min. All compounds were detected at 254 nm, and at 378 nm (punicalagins) and 366 nm (EA) for quantification. Pure isolated punicalagin (1) (2 mg) was accurately weighed and dissolved in H_2O:MeOH (1:1,v/v) then serially diluted to afford 0.20, 0.10, 0.05 and 0.025 mg/mL concentrations, respectively. EA (4) (1 mg) was dissolved in DMSO and serially diluted to afford 0.02, 0.01, 0.005 and 0.0025 mg/mL concentrations, respectively. Each sample was injected in duplicate and concentrations were determined from the peak area by using the equation for linear regression obtained from the calibration curves. TPT (1 mg) was dissolved in H_2O:MeOH (1:1, v/v), and the mean area percentages of the punicalagin (two individual peaks corresponding

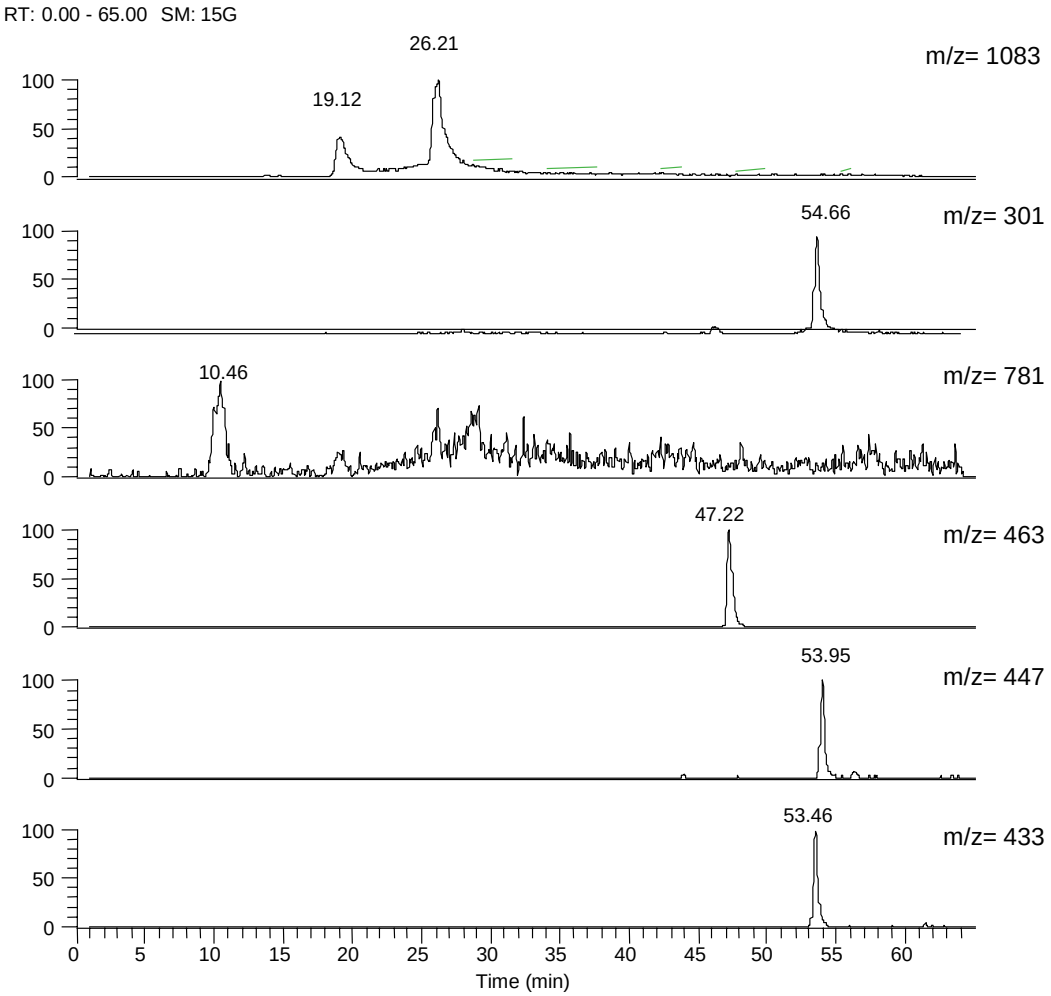

Fig. 2. LC-ES/MS spectra of total pomegranate tannins (TPT) showing extracted ion chromatograms of punicalagin anomers, ($M-H$) m/z 1083; EA, $M-H$ m/z 301; punicalin, $M-H$ m/z 781; EA-hexoside, $M-H$ m/z 463; EA-rhamnoside, $M-H$ m/z 447; EA-pentoside, $M-H$ m/z 433; as previously reported [1]. Spectra were obtained by electrospray ionization (ESI) in negative mode acquiring ions between 120 and 1500 amu.

to α- and β-anomers) and EA peaks were used for quantification.

2.1.5. LC-ES/MS analyses

Conditions for detection of tannins were as follows: Column, Symmetery C-18, 100 mm × 2.1 i.d., 3.5 μm, (Waters Corp., Milford, MA); solvent: A 2% HCOOH/H_2O, B 2% HCOOH/MeOH; gradient% A in B: initial: 99%, 30 min: 80%, 45 min: 60%, 60 min: 5%; run time 60 min; flow rate 0.15 mL/min; injection volume 20 μL; MS parameters: Ionization mode, electron spray (ES) negative mode; scan range: 120–1500 amu; scan rate: 1 scan/s; cone voltage: 17 eV, source temperature: 275 °C. Peak identities were obtained by matching their molecular ions ($M-H^+$) obtained by ES/MS and tandem MS with the expected theoretical molecular weights from literature data as: punicalagin (**1**) (m/z 1083); punicalin (**2**) (m/z 781; M-hexahydroxydiphenoyl moiety); gallagic acid (**3**) (m/z 601) and EA (**4**) (m/z 301) [1,2,9]. EA-glycosides correlated with literature [1,2] and were identified as EA-hexoside (m/z 463), EA-rhamnoside (m/z 447) and EA-pentoside (m/z 433).

3. Results and discussion

Our chromatographic method utilizing Amberlite XAD-16 resin column can be accomplished quickly and produces a high yield of purified tannins (time <1 h; 58–60 g TPT/kg husk) from fruit husk, a by-product of the commercial industry. The use of husk as starting material to produce TPT for biological tests is relevant since in the processing of commercial PJ, significant quantities of ETs are extracted from the fruit husk into PJ due to their hydrophilic properties when the fruits are hydrostatically pressed. Although commercial PJ contains a wide range of polyphenols including hydrolyzable tannins (ETs and GTs), and anthocyanins and other flavonoids, the ETs, GTs, EA and EA-glycosides predominate in content

[1]. Also, punicalagin is the major polyphenol present in PJ.

This method is cost-effective since it utilizes water and low carbon alcohols as eluants allowing organic solvents to be collected and recycled in subsequent resin columns to obtain the TPT product. In addition, Amberlite XAD-16 resin can be regenerated and reused for subsequent columns. Previous published methods used alternative stationary phases (e.g. Sephadex Lipophilic LH 20, polyamides, microcrystalline cellulose, C-18 and Diaion HP20) to purify pomegranate tannins which were time consuming and labor intensive. These methods used multiple solvent systems as eluants, afforded low yields of tannins and sometimes required the use of specialized instruments such as preparative- or semiprep-scale HPLC coupled to sensitive UV detectors [1,2,9,11–13].

TPT purified from fruit husk was evaluated by analytical HPLC (Fig. 1A) and tandem LC-ES/MS (Figs. 2 and 3). Compound identities were confirmed by LC-ES/MS analyses where two ions at $M-H$ m/z 1083 accounted for the α- and β-anomers of punicalagin (**1**) as previously reported [1,2,9]. The minor compounds in TPT were EA (**4**) ($M-H$ m/z 301), punicalin (**2**) ($M-H$ m/z 781) and EA-glycosides (hexoside, $M-H$ m/z 463; rhamnoside, $M-H$ m/z 447 and

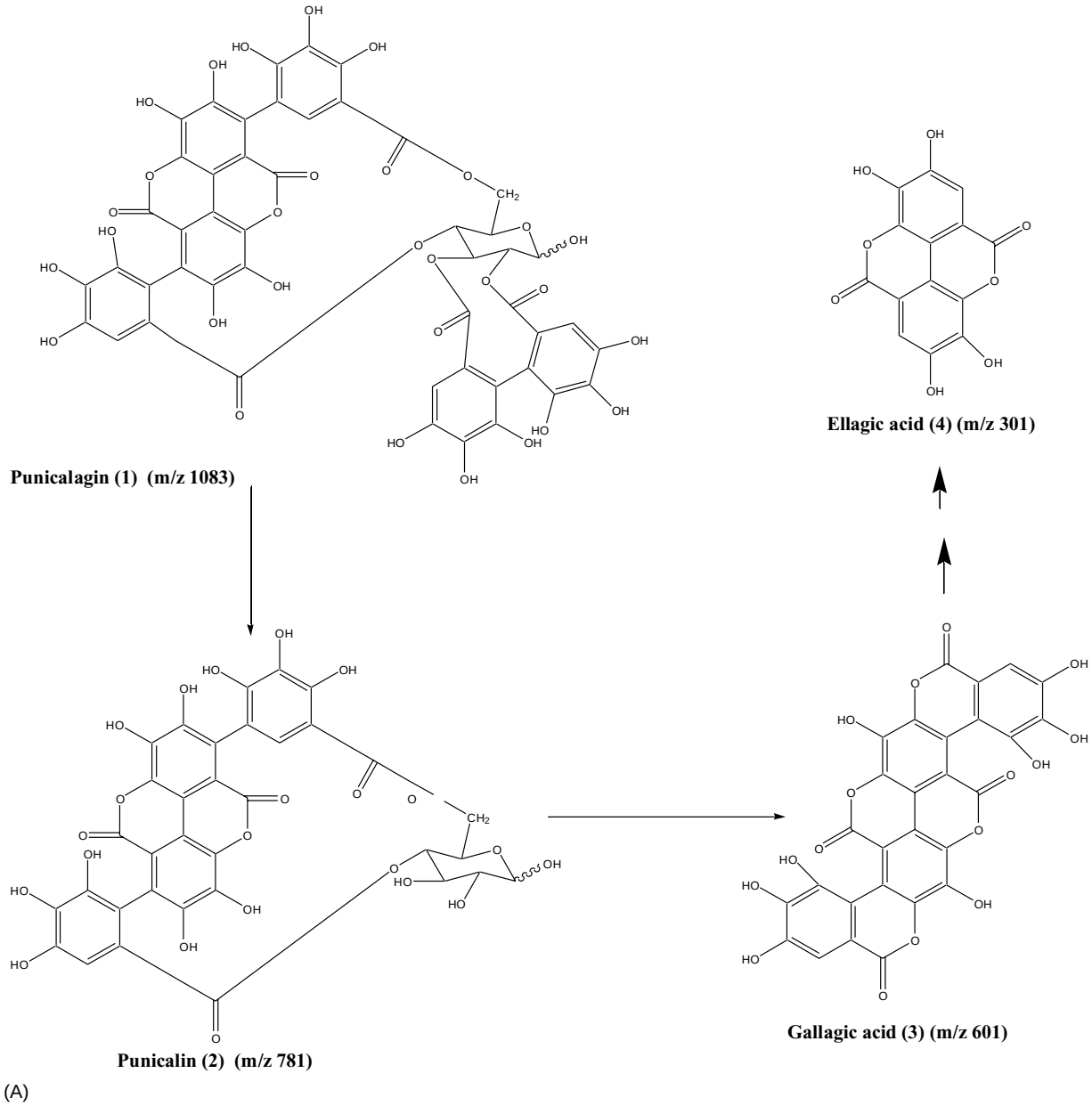

Fig. 3. (A) Scheme showing chemical structures of: punicalagins (**1**), punicalin (**2**), gallagic acid (GA) (**3**) and ellagic acid (EA) (**4**) related to their mass spectral information. (B) MS-MS spectra by direct infusion of punicalagin ($M-H$ m/z 1083) shows the subsequent fragment ions of punicalin ($M-H$ m/z 781) and then gallagic acid ($M-H$ m/z 601).

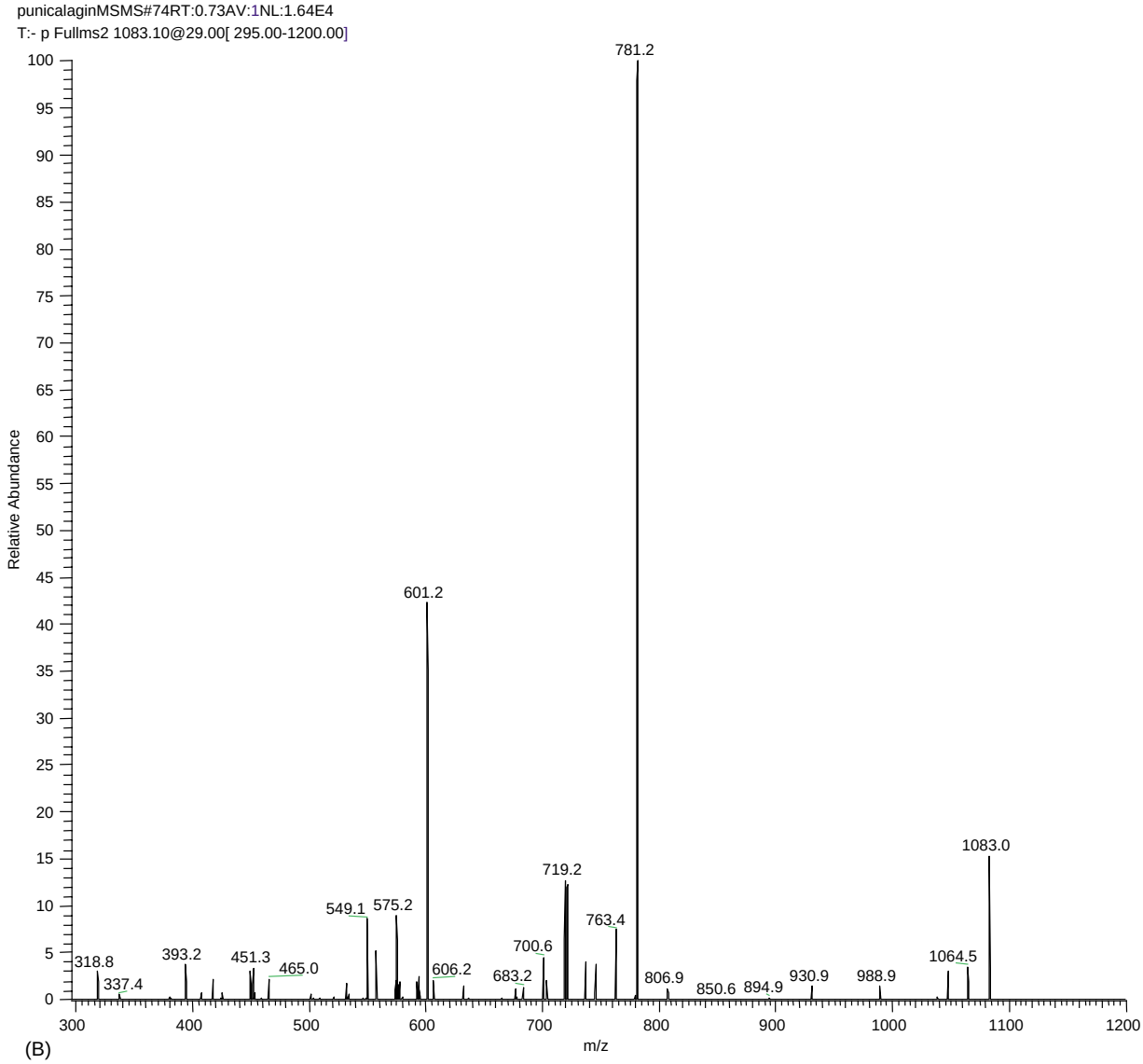

Fig. 3. (*Continued*).

pentoside, $M-H$ m/z 433) corresponding to the previous reports [1,2] (Fig. 2).

Quantification studies on TPT were conducted using isolated punicalagin anomers (**1**) (Fig. 1B) whose NMR and MS-MS spectral analyses confirmed its identity [2,9,12] and a commercially available sample of EA (**4**) (Fig. 1C). TPT contains 80–85% w/w of punicalagin anomers and 1.3% w/w EA. The ^{13}C NMR spectrum of the punicalagin isolated from TPT, revealed the two anomeric carbons at δ90.2 and δ94.4 corresponding to α- and β-punicalagin, respectively [9]. Punicalagin [2,3-(*S*)-hexahydroxydiphenoyl-4,6-(*S*,*S*)-gallagyl-D-glucose] can be considered to have a hexahydroxydiphenoyl moiety attached to gallagylglucose (punicalin). MS-MS studies of punicalagin (**1**) at $M-H$ m/z 1083 showed ions corresponding to punicalin (**2**) at $M-H$ m/z 781, GA (**3**) at $M-H$ m/z 601 and EA (**4**) at $M-H$ m/z 301 as shown in Fig. 3A and B.

4. Conclusion

In conclusion, we have developed a rapid new method to isolate large amounts of total pomegranate tannins (TPT) from fruit husk which contains high levels of punicalagin, the major pomegranate tannin that is present in PJ, and hence consumed by humans. Given the biological properties of pomegranate tannins as potent antioxidants and their effects on diseases such as atherosclerosis and cancer, this method could allow investigators access to large quantities of TPT for its potential use in appropriate in vivo models for health related studies. In addition, it would be feasible to scale-up this method which may be practical for industrial applications. This could provide a low-cost means to use a currently underutilized food by-product to develop commercial products including new nutritional products with potential health benefits.

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References

[1] M.I. Gil, F.A. Tomas-Barberan, B. Hess-Pierce, D.M. Holcroft, A.A. Kader, J. Agric. Food Chem. 48 (2000) 4581.
[2] B. Cerda, J.J. Ceron, F.A. Tomas-Barberan, J.C. Espin, J. Agric. Food Chem. 51 (2003) 3493.
[3] B. Cerda, R. Llorach, J.J. Ceron, J.C. Espin, F.A. Tomas-Barberan, Eur. J. Nutr. 42 (2003) 18.
[4] T. Okuda, in: O. Hajime (Ed.), Proceedings of the International Conference on Food Factors: Chemistry and Cancer Prevention, Springer Publishers, Japan, 1995, p. 280.
[5] M. Aviram, L. Dornfield, Atheroclerosis (2001) 195.
[6] P.S. Negi, G.K. Jayaprakasha, B.S. Jena, Food Chem. 80 (2003) 393.
[7] A. Vidal, A. Fallarero, B. Pena, A. Medina, B. Gra, F. Rivera, Y. Gutierrez, P. Vuorela, J. Ethnopharm. 89 (2003) 295.
[8] A. Scalbert, C. Morand, C. Manach, C. Cemesy, Biomed. Pharmacother. 56 (2002) 276.
[9] A.J. Doig, D.H. Williams, P.B. Oelrichs, L. Baczynskyj, J. Chem. Soc., Perkin Trans. 1 (1990) 2317.
[10] L.J. Filippich, J. Zhu, M.T. Asalami, Res. Vet. Sci. 50 (1991) 170.
[11] S.A.A. El-Toumy, H.W. Rauwald, Phytochemistry 61 (2002) 971.
[12] T. Tanaka, G.-I. Nonaka, I. Nishioka, Chem. Pharm. Bull. 34 (1986) 650.
[13] T. Tanaka, G.-I. Nonaka, I. Nishioka, Chem Pharm. Bull. 34 (1986) 656.