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Isolation of punicalagin from *Punica granatum* rind extract using mass-directed semi-preparative ESI-AP single quadrupole LC-MS

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Abstract

We are living in a era of alarming increases in microbial resistance to currently available antibiotics, and there is a growing need for new pharmaceutical products to treat infectious diseases. The pomegranate is an edible fruit that has virucidal and antimicrobial activities which is primarily attributable to the high concentration of hydrolysable tannins. Punicalagin, a high molecular weight tannin (1084.7), accounts for approximately 70% of the total and is concentrated in the fruit exocarp (rind). It is the focus of much research, although it is prohibitively expensive to purchase which presents an obstacle to further exploitation and development. Here we describe a method for the isolation of punicalagin from pomegranate rind extract and total pomegranate tannins using an Agilent preparative mass-directed LC-MS single quadrupole ESI-API system, where the ionization was set as negative and the mass signal acquired in Single Ion Monitoring. Thanks to the automatic fraction collector, the method can be used in automatic mode and is capable of producing punicalagin with >95% purity.

Keywords: Punica Granatum, Pomegranate Rind Extract, TPT, Punicalagin, LC-MS

44 1. Introduction

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We are currently living in an era of alarming and unprecedented increases in microbial resistance to our current arsenal of antibiotic drugs, and no new drugs have come onto the market in 30 years or so. New products are thus urgently needed [1, 2]. In addition, there is a clear predilection by patients for medicines that are of natural origin compared to synthetic drugs [3, 4].

The fruit of *Punica granatum* L. (pomegranate) has been used as a healthy and healing fruit for many years. It has long been used in Middle Eastern and Chinese medicine for its antioxidant, anti-inflammatory [5] bactericidal [6] and virucidal [7] properties. The phytochemicals constituents of pomegranates include many chemical species including sugars, flavonoids, anthocyanin, tannins, [8] and can be obtained from most parts of the fruit but the exocarp, or rind, has been shown to contain the highest concentration of polyphenols. A major class of compounds is the hydrolysable ellagitannins of which punicalagin (2,3-(S)-hexahydroxydiphenoyl-4,6-(S,S)-gallagyl-D-glucose) constitutes up to 70% [Figure 1]. It is a large molecule (MW 1084.71) and is comprised of 3 moieties: gallagic acid and ellagic acid linked via a glucose unit. The molecule naturally adopts one of two anomeric forms, α and β , depending on whether the glucose is in the boat or chair conformation, and the two exist in a 1:2 ratio under standard conditions.

Ongoing research shows that punicalagin is primarily responsible for antimicrobial, anti-inflammatory and virucidal properties of PRE. The microbicidal activities of PRE and punicalagin have been shown to undergo significant potentiation when co-administered with metal ions, eg Zn (II) [9, 10]. Punicalagin therefore has potential as a therapeutic agent as outlined; however, it is very expensive to purchase (eg £217 for 10 milligrams from one major supplier) which presents a barrier to further development, as substantial quantities are required to carry out the necessary pre-clinical drug development work.

PRE is typically prepared from pomegranate rinds by extraction into boiling water [19] followed by centrifugation and filtration. The non-tannin components of PRE can be removed by column chromatography to yield concentrated total pomegranate tannins (TPT), for example using Amberlite XAD-16 resin. Punicalagin may then be isolated by column chromatography using a Sephadex LH-20 column which entails a complex gradient elution [11] or by countercurrent chromatography [12]. The methods typically used to isolate punicalagin thus require numerous time-consuming laboratory steps and lengthy sample preparation.

Given the need for new antimicrobial molecules has arisen over recent years, fast and reproducible methods to isolate significant amounts of antimicrobial molecules has the potential to expedite the development of new therapeutic products. Mass-directed purification is a type of preparative LCMS in which the instrument may be set to collect column eluate upon detecting a pre-selected mass or mass range, once the chromatographic conditions have been established. These sophisticated instruments are available from a number of manufacturers including Waters and Shimadzu, although we have in our laboratory an Agilent 1260 Infinity Automated LC/MS Purification System [13]. In the current work we describe a method for the isolation of high-purity punicalagin using this system.

81 **2. Materials and methods**

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83 *2.1 Materials*

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85 Amberlite XAD-16 (Alfa Aesar), LCMS-grade water and methanol were purchased from Fisher Scientific,
86 Loughborough, UK. Pomegranates, of Spanish origin, were purchased from a local store.

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90 *2.2 Preparation of Pomegranate Rind Extract (PRE)*

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92 The rinds from five pomegranates were cut in small pieces of approx. 2cm and blended in deionized water,
93 25% w/v, before being boiled for 10 min and transferred to 50mL tubes and centrifuged 7 times at 5840rpm
94 for 30 min on each occasion. The supernatant was then vacuum filtered through 0.45µm nylon membranes
95 (Whatman, UK) to yield a clear dark yellow-orange solution which was then freeze-dried and stored at -20°C
96 until required [14].

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99 *2.3 Preparation of Total Pomegranate Tannins (TPT) from PRE*

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101 Based on a previously reported method [11] a glass column was slurry-packed with 50g of Amberlite XAD-
102 16 resin in degassed and deionized water. This was then washed with methanol, followed by deionized
103 water, and then was left overnight to equilibrate. A 2cm layer of sand was added to the top and the bottom.
104 Freeze-dried PRE was reconstituted in deionized water at a concentration of 200 mg/mL and loaded onto the
105 column. Firstly, the water-soluble components were washed out using a copious amount of water, then the
106 TPT fraction was eluted after washing with 70mL of methanol and collected. This solution was then rotary
107 evaporated using a Buchi instrument with the temperature set at 40°C and the vacuum at 50mbar. The TPT
108 product was stored at 2-4 °C until further use. Analysis of the tannin-free fraction (section 2.4) confirmed that
109 no tannins were present (data not shown).

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111 *2.4 Analytical HPLC analysis of TPT*

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113 The TPT was analysed using an Agilent 1100 system fitted with a Kinetex C18 150 x 4.6mm 5µm 100Å RP
114 column (Phenomenex, Macclesfield, UK). Based on a previous method [9] a gradient mobile phase was used
115 composed of: solvent A H₂O + 0.1%TFA and solvent B: MeOH + 0.1%TFA. The gradient timetable
116 started with 5%B, 0-3 min 10%B, 3-15 min 20%B, 15-20 min 40%B, 20-25 min 60%B, 25-27 min 5%B, 27-

117 35 min 5%B. A 1 mg/mL sample in deionized H₂O was prepared, the injection volume was 20 μL and the
118 detection was performed at a wavelength of 258 nm with a UV detector.

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121 2.5. Semi-preparative Isolation of Punicalagin using an LC-MS system

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123 The isolation was performed using a modular semi-preparative LC-MS ESI-AP single quadrupole system with
124 automatic fraction collector (Agilent Technologies, Stockport, UK). Waters X-Select CSH C18 OBD 5μm
125 130Å columns were used: firstly a 50 x 10mm scout column which was used to set up a reliable extraction
126 method, then a 250 x 19mm semi-preparative column. The solvents used were H₂O (channel A) and MeOH
127 (channel B); no mobile phase acidification was used. The separation was performed in Single Ion Monitoring
128 (SIM) negative ionization mode, with a capillary voltage of 4000V, drying gas temperature of 350°C
129 and gas nebulizer pressure of 32 psi [15]. The threshold of the automatic fraction collector was set at 2000.

130

131 The gradient was scaled from the analytical method to be reproducible with these columns. Firstly, the scout
132 column method was converted from the analytical one, and a run was performed to check the reliability of the
133 separation. The sample was 100mg/mL in H₂O and 300μL were injected. Further runs were carried out to
134 control the consistency of the separations. The gradient used with this column started at t₀ with 5%B, 0-0.38
135 min 10%B, 0.38-1.80 min 20%B, 1.80-2.39 min 40%B, 2.39-2.98 min 60%B, 2.98-3.22 min 5%B, 3.22-4.16
136 min 5%B. Although even with this column was possible to isolate punicalagin, only low amounts were
137 collected, so the method evolved to the big semi-preparative column. The gradient for this column started
138 with 5%B, 0-7.77 min 10%B, 7.77-33.36 min 20%B, 33.36-44.02 min 40%B, 44.02-54.69 min 60%B, 54.69-
139 58.95 min 5%B, 58.95-76.01 min 5%B. This sample load was 225mg in 1.5mL H₂O. The flow rate for both
140 the columns was 20mL/min. Both manual and automatic collection were performed. As already stated above,
141 the threshold for the automatic collection was set above 2000. The samples obtained were then dried to yield
142 the purified punicalagin; firstly, the methanolic fraction was evaporated using a Buchi rotary evaporator (50
143 mbar, 50 °C), then the remaining solvent (aqueous) was removed by freeze-drying.

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146 3. Results and discussions

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148 As can be seen in the analytical HPLC chromatogram [Figure 2], the two anomers of punicalagin were well
149 separated in the preparative LCMS method described. The two main peaks α and β punicalagin eluted with a
150 different concentration of solvent B and always tend to an approximate ratio of 1:2 ratio. The peak at the end
151 can be identified as ellagic acid, one of the major building blocks of punicalagin. The analysis of the TPT
152 was performed to prove the presence of punicalagin, so it was possible to proceed with the purification.
153 Because of the differences in the stationary phase between the preparative and the analytical columns, the

154 chromatogram of the preparative HPLC doesn't appear well separated, but thanks to the mass spectrometric
155 collection triggering, it was still possible to isolate highly pure punicalagin. Figure 2 shows the plot of the run
156 with the small scout column. The vertical lines show the windows in which the punicalagin (alpha the first
157 and beta the second) was collected; the ratio of the two isomers was found also in the mass chromatogram.

158 The analytical resolution of punicalagin utilises acidification, typically by trifluoroacetic acid (TFA)
159 [9]. In LCMS the preferred acidifying reagent is formic acid as, compared to TFA, it tends not to leave behind
160 residual molecules. However, we found that when the punicalagin fractions were evaporated to dryness, it was
161 discovered that nearly all the punicalagin had degraded. This was attributed to the increasing acidity that
162 accompanied drying as the acid was also increasing in concentration. Consequently, it was decided to omit
163 altogether the formic acid to ensure greater stability of the punicalain, albeit at the expense of superior
164 chromatographic resolution.

165 The semi-preparative column report [Figure 3] shows a different HPLC chromatogram and a similar
166 SIM chromatogram, probably due to the different sample loading in the two columns: in the small scout column
167 it was closer to the optimal loading. In this case, even though both the little signals in the mass spectrum were
168 slightly above 2000, the only fraction collected was the high peak at 22 min. For completeness, each fraction
169 collected was re-run through the preparative LC-MS [Figure 4] to check the purity of the punicalagin, and all
170 the signals detected were approximately the same: the differences represented the different grade of purity
171 (average of >95% pure). These tests were performed after 1 hour.

172 The purity of the collected punicalagin product was determined by analytical HPLC [9]. Figure 5
173 shows that 95% of the integrated areas (compared to the total areas) was accounted for by the punicalagin
174 anomer peaks. Interestingly, we attempted to collect individual anomers separately, by programming the
175 instrument to collect at times corresponding to α and β punicalagin accordingly - it would be of interest to
176 determine if the anomers give rise to differing biological (eg anti-microbial, anti-inflammatory) activities.
177 However, even though collections were seemingly successful using the acid-free elution, it was noted that
178 each 'purified' anomer reverted to the 1:2 ratio after drying, with β -punicalagin is the more abundant anomer
179 at neutral pH [16].

180

181 4. Conclusions

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183 A rapid, reliable and automatable method to isolate punicalagin from TPT was developed using the 1260
184 Infinity Automated LC/MS purification system. This facilitating the development work in order to bring to
185 market new punicalagin-based antimicrobial products at a time of great need whilst avoiding large expenses
186 to purchase it from suppliers. Work is ongoing to purify punicalagin directly from PRE, thus avoiding the prior
187 concentration of TPT through Amberlite. However, the TPT separation step is not onerous, and does provide
188 a material that is freely soluble in methanol, useful for easy injection into the LCMS.

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265 Figures:

- 266 1. Chemical structure of punicalagin
- 267 2. HPLC chromatogram of PRE
- 268 3. LC-MS report of TPT using the Scout column
- 269 4. LC-MS report of TPT using the Semi-preparative column
- 270 5. LC-MS report of collected pure punicalagin