Identification and quantification of major derivatives of ellagic acid and antioxidant properties of thinning and ripe Spanish pomegranates

Nallely Nuncio-Jáuregui[1], Paulina Nowicka[2], Sandra Munera-Picazo[1], Francisca Hernández[3], Ángel A. Carbonell-Barrachina[1], Aneta Wojdyło[2,*]

[1] Departamento de Tecnología Agroalimentaria. Grupo Calidad y Seguridad Alimentaria. Universidad Miguel Hernández de Elche. Carretera de Beniel, km 3,2. 03312 Orihuela, Alicante, Spain.

[2] Department of Fruit and Vegetable Processing. Wrocław University of Environmental and Life Science. 37/41 Chełmońskiego Street, 51-630 Wrocław, Poland.

[3] Departamento de Producción Vegetal y Microbiología. Grupo de Fruticultura y Técnicas de Producción. Universidad Miguel Hernández de Elche. Carretera de Beniel, km 3,2. 03312 Orihuela, Alicante, Spain.

*Corresponding author: aneta.wojdylo@up.wroc.pl

ABSTRACT

Major derivatives of ellagic acid and antioxidant properties of 9 Spanish pomegranate cultivars were studied at two development stages: thinning and ripening. A total of 35 major derivatives of ellagic acid were identified by LC-PDA-QTOF/MS and quantified by UPLC-PDA methods; however, only 7 of them were found simultaneously in thinning and ripe fruits. The total content of derivatives of ellagic acid was higher in thinning fruits (3521 to 18236 mg 100 g^{-1} dry matter, dm) than in ripe fruits (608 to 2905 mg 100 g^{-1} dm). The antioxidant properties were evaluated using four methods: ABTS, DPPH, FRAP, and ORAC. Experimental values for these four methods in thinning fruits ranged from 2837 to 4453, 2127 to 2920, 3131 to 4905, and 664 to 925 mmol trolox kg^{-1}, respectively; ripe fruits had lower values of the antioxidant activities than thinning fruits, and values ranged from 1567 to 2905, 928 to 1627, 582 to 1058, and 338 to 582 mmol trolox kg^{-1}, respectively. In general, sour-sweet cultivars (PTO8 cultivar) had the highest value of derivatives of ellagic acid and antioxidant properties in pomegranates fruits. Experimental results clearly proved the potential of thinning pomegranate fruits for its use as supplement in food, pharmaceutical and cosmetics industries.

Keywords: Pomegranate, LC-MS analysis, ellagic acid, antioxidant properties.

1. Introduction

There is a major interest in the consumption of foods with health benefits (Wu et al., 2004). The human diet often comprises foods and beverages with significant amounts of phenolic compounds such as fruits, vegetables, wines and teas (Alén-Ruiz, García-Falcón, Pérez-Lamela, Martínez-Carballo, & Simal-Gándara, 2009; Komes, Horžić, Belščak, Ganić, & Vulić, 2010; Lui, 2003). Actually, food producers are increasingly interested in developing new products offering compounds that can improve health (Suarez-Jacobo, Rufer, Gervilla, Guamis, & Roig-Sagues, 2011). Pomegranate fruits are a well-known source of many valuable substances that show high antioxidant activity (Ordoudi et al., 2014; Madrigal-Carballo et al., 2009; García-Alonso, De Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2004) and might induce health benefits against cancer, cardiovascular and other health problems (Wu, Ma, & Tian, 2013; Park et al., 2009; Basu, & Penugonda, 2009).

Additionally, pomegranate peel contains a high amount of ellagic acid, ellagitannins, such as punicalin and punicalagin, as well as hexahydroxydiphenic acid (HHDP) which possess anti-inflammatory, antitumor, and apoptotic properties (Seeram, Lee, Hardy, & Heber, 2005). Therefore, the health benefits of pomegranate peel are accredited for the pharmacological activities exhibited by bioactive phytochemicals like polyphenols (Al-Rawahi et al., 2014). There has also been an increase in the use of pomegranate fruit extracts as botanical ingredients in herbal medicines and dietary supplements (Elfalleh et al., 2011).

Spain is the one of the main European pomegranate producer and its production is mainly located in the provinces of Alicante and Murcia (Melgarejo, Hernández, & Legua, 2010). Thinning is a routine farming practice, which takes place at an immature stage of the fruits, and consists of removing part of the fruits to benefit the development and quality of the remaining fruits (Melgarejo et al., 2010). This practice is carried out in the first week of June and can be repeated after 20-30 days (end of June or early July), and among 7-15 kg per tree could be removed (Melgarejo et al., 2010). After thinning, the fruits removed from the

pomegranate trees are left to spoil in the soil and farmers do not get any direct payback for this expensive farming practice, which needs specialized labor and is conducted manually. The fruits that remain in the tree continue their ripening process and experience significant changes in their physicochemical and phenolic compositions as well as antioxidant activity (Fawole & Opara, 2013; Shwartz, Glazer, Bar-Ya'akov, Matityahu, & Bar-Ilan, 2009). These changes are influenced by variety, growing region, farming practices and ripening stage of the fruit at harvest (Mirdehghan, & Rahemi, 2007).

The aim of the present study was therefore to evaluate the potential of thinning and ripe fruits from nine common Spanish pomegranate cultivars as sources of bioactive compounds, especially ellagitannins. In this way two factors will be evaluated: (i) thinning or ripe fruits, and (ii) cultivars. The identification and quantification of major derivatives of ellagic acid (MDEA) will be carried out using LC-PDA-QTOF/MS and UPLC-PDA; the antioxidant activity was evaluated using four methods, namely ABTS, DPPH, FRAP, and ORAC.

2. Materials and methods

2.1. Plant material and sample processing

Fruits of nine different cultivars of pomegranate were collected in the last week of June and beginning of September from the experimental field station of the Universidad Miguel Hernandez de Elche in the province of Alicante, Spain (02°03'50''E, 38°03'50''N, and 25 masl). This experiment shows values of two consecutive seasons (2012 and 2013). The orchard is one of the main pomegranate gene banks of the European Union and was established in 1992; hence, trees are now 20 years old. Pomegranate trees were trained to the vase-shaped system and planted at a spacing of 4 m $\times$ 3 m. They are drip irrigated, and standard cultural practices are performed (pruning, thinning, fertilization and pest control treatments).

The following cultivars were selected: (i) 3 sour cultivars [*Borde de Albatera 1* ("BA1"), *Borde de Orihuela 1* ("BO1"), *Borde de Beniel 1* ("BBE1")], (ii) 3 sour-sweet cultivars [*Piñón Tierno de Ojós 5* ("PTO5"), *Piñón Tierno de Ojós 8* ("PTO8"), *Piñón Tierno de Ojós 10* ("PTO10")], and (iii) 3 sweet cultivars [*Mollar de Elche 14* ("ME14"), *Mollar de Elche 17* ("ME17") and *Valenciana 1* ("VA1")]. After picking, all fruits were immediately transported into the laboratories of the Universidad Miguel Hernández de Elche (Orihuela, Alicante, Spain).

Thinning is conducted as a routine farming practice in the selected pomegranate orchard, generally from middle of June to the first week of July. Usually, pomegranate thinning is conducted at the stage of young fruit (Fleckinger code I; BBCH code 71); at this stage about 7-8 kg of young fruits are removed per each tree. Only fruits weighting less than 100 g or having a diameter smaller than 60 mm are removed. Following all the previously mentioned requirements, 5 fruits were selected from those removed by the routine thinning practice.

Two times for five fruits per cultivar were randomly collected (90 thinning fruits and 90 ripe fruits; 180 fruits in total). After harvest the fruits were frozen immediately and then lyophilized using a freeze drier (Christ Alpha 2-4; Braum Biotech Int., Melsungen, Germany) for 24 h and a pressure of 0.220 mbar. The samples were subsequently ground in a pestle and mortar to a fine powder and stored vacuum-packed in a freezer (-80 ºC) until analysis.

2.2. Identification of major derivatives of ellagic acid by the LC-PDA-QTOF/MS method and quantification by UPLC-PDA

Samples of pomegranate extract for analysis were prepared as previously described by Wojdyło, Oszmiański and Bielicki (2013). Identification and quantification of MDEA of pomegranate fruits extracts was carried out using an Acquity® ultra performance LC system equipped with a photodiode detector (UPLC-PDA) with binary solvent manager (Waters Corp., Milford, MA, USA) series with a mass detector G2 QTOF Micro mass spectrometer (Waters, Manchester, UK)

equipped with an electrospray ionization (ESI) source. Separations of polyphenols were carried out using a UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm; Waters Corp., Milford, MA, USA) at 30 °C, whereas the samples were maintained at 4 °C during the analysis.

Pomegranate samples (5 μL) were injected, and elution was completed within 22 min using a sequence of elution modes: linear gradients and isocratic. The flow rate was 0.45 mL/min. The mobile phase was composed of solvent A (4.5 % formic acid) and solvent B (100 % of acetonitrile). Elution was as follows: 0−10 min, linear gradient from 1 to 10 % B; 10−15 min, linear gradient from 10 to 17% B; than 100% B from 15 to 18 min for column washing; and reconditioning for next 4.00 min. A partial loop injection mode with a needle overfill was set up, enabling 5 μL injection volumes when a 5 μL injection loop was used. Acetonitrile (100 %) was used as a strong wash solvent and acetonitrile−water (10 %) as a weak wash solvent. Analysis was carried out using full scan, data-dependent MS scanning from *m*/*z* 100 to 1000. The mass tolerance was 0.001 Da, and the resolution was 5.000. Leucine enkephalin was used as the mass reference compound at a concentration of 500 pg/μL at a flow rate of 2 μL/min, and the $[M - H]^-$ ion at 554.2615 Da was detected over 15 min of analysis during ESI-MS accurate mass experiments, which was permanently introduced via the LockSpray channel using a Hamilton pump. The lock mass correction was ±1.000 for Mass Window. The mass spectrometer was operated in a negative ion mode and set to the base peak intensity (BPI) chromatograms and scaled to 12400 counts per second (cps) (=100 %). The optimized MS conditions were as follows: capillary voltage of 2500 V, cone voltage of 30 V, source temperature of 100 °C, desolation temperature of 300 °C, and desolation gas (nitrogen) flow rate of 300 L/h. Collision-induced fragmentation experiments were performed using argon as collision gas, with voltage ramping cycles from 0.3 to 2 V. The characterization of the single components was carried out via retention time and the accurate molecular masses. Derivatives of ellagic acid were optimized to its estimated molecular mass $[M-H]^-$ in the negative mode

before and after fragmentation. The data obtained from LC-MS were subsequently entered into MassLynx 4.0 ChromaLynx Application Manager software. On the basis of these data, the software is able to scan different samples for the characterized substances.

Quantification of MDEA was performed using UPLC-PDA; PDA spectra were measured over the wavelength range of 200−600 nm in steps of 2 nm. The runs were monitored at 320 nm. These compounds were evaluated and expressed as ellagic acid and derivatives. Retention times (R_t) and spectra were compared with those of pure standards. Identification of MDEA were based on MS/MS analysis and literature data (Fischer, Carle, & Kammerer, 2011; Calani et al., 2013). Calibration curves at concentrations ranging from 0.05 to 5 mg/mL ($R^2 \leq 0.9998$) were made from ellagic acid. All analyses were done in triplicate. Results were expressed as milligrams per 100 g dry matter (dm).

2.3. Antioxidant properties

2.3.1. ABTS, DPPH and FRAP methods

For the antioxidant activity determination, a methanol extract was prepared for each sample to be analyzed. Freeze-dried fruits (0.5 g) were mixed with 10 mL of MeOH/water (80:20 % v/v) + 1 % HCl, sonicated at 20 °C for 15 min and left for 24 h at 4 °C. Then the extract was again sonicated for 15 min, and centrifuged at 15000 *g* for 10 min.

The free scavenging activity was evaluated using the DPPH (radical 2,2-diphenyl-1-picrylhydrazyl) method as described by Brand-Williams, Cuvelier and Berset (1995), with a modification in the reaction time. Briefly, 10 µL of the supernatant were mixed with 40 µL of MeOH and added to 950 µL of DPPH solution. The mixture was shaken vigorously and placed in a dark room for 10 min. The decrease in absorbance was measured at 515 nm in UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France).

Additionally, the ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)] radical cation and ferric reducing antioxidant power (FRAP) methods were also used as described by Re, Proteggente, Pannala, Yang, and Rice-Evans (1999) and Benzie and Strain (1996) respectively. Briefly, 10 µL of the supernatant were mixed with 990 µL of ABTS or FRAP. After 10 min of reaction, the absorbance was measured at 734 nm for ABTS and 593 nm for FRAP. The absorbance was measured in UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France). Calibration curves, in the range 0.01–5.00 mmol trolox L^{-1} were used for quantification of the three methods of antioxidant activity showing good linearity ($R^2 \geq 0.998$). The analyses were run in five replications (n=5) and results were expressed as mean ± standard error and units in mmol trolox per kg dm.

2.3.2. ORAC method

The fourth method used to evaluate the antioxidant capacity of pomegranate fruits was oxygen radical absorbance capacity (ORAC), as described by Ou, Hampsch-Woodill, and Prior (2001). Briefly, each sample (0.1 mL) was diluted with phosphate (K_2HPO_4 + Na_2HPO_4) buffer solution (75 mM, pH 7.4). Later, 375 µL of sample together with 2.25 mL of fluorescein (42 nM) were added in cuvettes; buffer solution was used as blank and trolox solution (25 µM trolox) as calibration solution. Fluorescence readings were taken at 5 s and then every minute thereafter. Finally, 375 µL of freshly prepared AAPH reagent [2,2'-azobis(2-amidinopropane) dihydrochloride] (153 mM) was added in cuvettes every 5 s. The fluorescence spectrophotometer (Shimadzu, model RF-5301; Kyoto, Japan) was set up at an excitation wavelength of 493 nm and an emission wavelength of 515 nm and readings were recorded every 5 min for 40 min after the addition of AAPH. During the analysis all the cuvettes were incubated at 37 °C. The final ORAC values were calculated, in triplicate, using a regression equation between the trolox concentration and the net area under the fluorescence decay curve and final data were expressed as mmol trolox per kg dm.

213

214 2.4. Statistical analysis

215 Results are provided as the mean ± standard error of three replications. First, data
216 was subjected to one-way analysis of variance (ANOVA) and later data was also
217 subjected to Tukey's multiple-range test to compare the means. Differences were
218 considered statistically significant at $p < 0.05$. All statistical analyses were
219 performed using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD,
220 USA). The figures of ABTS, DPPH, FRAP, and ORAC data, were prepared using
221 SigmaPlot Version 11.0 (Systat Software Inc.).

222

223 **3. Results and discussion**

224 3.1. Identification of major derivatives of ellagic acid

225 Ellagic acid and its derivatives were the main class of identified and quantified
226 compounds in this particular product. The identification of MDEA in thinning and
227 ripe pomegranate fruits was carried out by LC-PDA-QTOF/MS method (**Table 1**).
228 The aim of many pomegranates studies has been the identification of the bioactive
229 compounds that correlate with health (García-Alonso et al., 2004; Sun, Chu, Wu, &
230 Liu, 2002). In this sense, it has been shown that ellagic acid has anti-
231 atherosclerotic and biological properties can be used as a preventive agent in
232 cancer treatment (El-Shitany, El-Bastawissy, & El-Desoky, 2014; Lu, Ding, & Yuan,
233 2008). High concentrations of derivatives of ellagic acid are positively correlated
234 with the high antioxidant activity of pomegranate peel extracts (Al-Rawahi et al.,
235 2014).

236 Among the 35 major derivatives of ellagic acid found in thinning and ripe
237 pomegranates (mainly hydrolyzable tannins), 7 were found in both types of fruits.
238 These seven compounds were punicalagin isomer (R_t = 1.61 min) and HHDP-
239 gallagyl-hexoside (punicalagin) (R_t = 3.52 min) had an $[M-H]^-$ at *m/z* 1083 and
240 similar MS/MS fragments (300/622/781); granatin A (R_t = 4.40 min) had an
241 $[M-H]^-$ at *m/z* 799; ellagic acid derivative (R_t = 5.32 min) had an $[M-H]^-$ at *m/z*

301; ellagitannin (R_t = 8.79 min) had an $[M-H]^-$ at *m/z* 784; granatin B (R_t = 10.54 min) had an $[M-H]^-$ at *m/z* 951; and ellagic acid derivative (R_t = 11.06 min) had an $[M-H]^-$ at *m/z* 951. Calani et al. (2013) and Fischer et al. (2011) identified those compounds in pomegranate. Hydrolyzable tannins are the most abundant antioxidant polyphenolic compounds in pomegranate (Gil, Tomás-Barberán, Hess-Pierce, Holcroft, & Kader, 2000) and include ellagitannins, such as punicalagins and punicalins (Calani et al., 2013).

Regarding other derivatives of ellagic acid found exclusively in thinning (i) or ripe (ii) fruits the most abundant ones were: (i) digalloyl-HDDP-glucoside (pedunculagin II) (R_t = 3.80 min, $[M-H]^-$ at *m/z* 785) and HHDP-digalloyl-glucose (R_t = 5.89 min, $[M-H]^-$ at *m/z* 785) and (ii) ellagitannin (R_t = 2.86 min, $[M-H]^-$ at *m/z* 783) and an unknown compounds, which main characteristics were R_t = 0.63 min, and $[M-H]^-$ at *m/z* 215. These compounds have been reported by Fischer et al. (2011), Calani et al. (2013) and Sentandreu, Cerdán-Calero, and Sendra (2013) in ripe pomegranates.

3.2. Quantification of major derivatives of ellagic acid

The quantification of major derivatives of ellagic acid was conducted using UPLC-PDA detection. The effect of the ripening stage on the MDEA was evident and the values found in thinning fruits were 3 to 19 times higher than those found in ripe fruits. According to the mean values of all samples, the MDEA was about seven times higher in thinning fruits (10450 ± 1581 mg 100 g^{-1} dm) than in ripe fruits (1553 ± 270 mg 100 g^{-1} dm). The highest changes with time were found in fruits from sweet cultivars, which decreased from an initial mean value of 11734 mg 100 g^{-1} dm to as low as 833 mg 100 g^{-1} dm; this means that the ratio $MDEA_{thinning}/MDEA_{ripe}$ had a mean of 14.1. This same ratio, $MDEA_{thinning}/MDEA_{ripe}$, took values of 5.0 and 5.2 for sour and sour-sweet cultivars, respectively. Al-Rawahi et al. (2014) found 6420 mg GAE 100 g^{-1} dry solids (ds) in freeze dried pomegranate peel and Fischer et al. (2011) reported a total phenolic value of 8489

mg 100 g^{-1} dm, in peel and mesocarp of pomegranate. The differences in the phenolic content could be associated with the difference in cultivars, methods of extraction and analysis (chromatography or spectrophotometry) and environmental conditions (Al-Rawahi et al., 2014). The high amounts of bioactive compounds in thinning fruits imply the high interest of this material for industrial applications, such as enrichment or development of new products.

The factor cultivar significantly (p<0.05) affected the amount of MDEA, which ranged (i) in thinning pomegranates between 3521 and 18236 mg 100 g^{-1} dm in PTO10 and PTO8, respectively, and (ii) in ripe pomegranates between 608 and 2905 mg 100 g^{-1} dm in ME14 and PTO8, respectively. The two cultivars with the highest values of MDEA in both thinning and ripe pomegranates were PTO8 (18236 and 2905 mg 100 g^{-1} dm, respectively) and BO1 (15338 and 2415 mg 100 g^{-1} dm, respectively).

Tables 2 and **3** show that 24 and 18 major derivates of ellagic acid were found in thinning and ripe pomegranates, respectively. The 3 most abundant compounds in thinning fruits were (**Table 2**): (i) HHDP-gallagyl-hexoside (**13**): 3635 mg 100 g^{-1} dm, (ii) punicalagin isomer (**7**): 1986 mg 100 g^{-1} dm, and (iii) granatin B (**28**): 830 mg 100 g^{-1} dm; these values represented 36.4, 19.9 and 7.3% of the total concentration of MDEA. Consequently, only these 3 compounds represented more than 60% of the total concentration of MDEA in unripe fruits. In a similar way, the most abundant compound in ripe fruits was ellagitannin (**12**): 858 mg 100 g^{-1} dm (**Table 3**). This value represented 42.9 % of the total concentration of MDEA in ripe fruits.

There were 7 compounds (peaks **7**, **13**, **16**, **19**, **25**, **28** and **29**) that were present in both thinning and ripe fruits. These 7 compounds represented about 70 % of the major derivatives of ellagic acid in thinning fruits, while only 14.5 % in ripe fruits. The **Figure 1** shows the comparison of MDEA profile of thinning and ripe fruits for PTO8 cv. In this and other cv. these 7 compounds was always major in thinning than in ripe fruits. Therefore, a big portion of these 7 compounds were

transformed in ellagitannins which are the predominate compound in the MDEA profile of ripe fruits.

Flavonoids and phenolic acid are secondary metabolites produced by plants. Gallic and ellagic acids are common precursors of hydrolyzable tannins; they will be transformed via 1-*O*-galloylglucose into a wide range of complex galloylglucosides and further complex of ellagitannins. The direct synthesis of gallic acid from dehydroshikimic acid will block the shikimate pathway enzyme, 5-enolpyruvylshikimate-3-phosphate synthase, and thus will cause a reduction in the synthesis of aromatic amino acids and phenylpropanoids. In contrast, the synthesis and accumulation of gallic acid and hydrolyzable taninns are activated (Gross, 1999; Grundhöfer, Niemetza, Schilling, & Grossa, 2001).

Therefore, one of the major derivatives of ellagic acid found in thinning fruits was a punicalagin isomer (**7**), together with the gallagyl group is a part of the chemical structure of many of the phenols that are commonly found in pomegranate, such as punicalin and punicalagin derivatives (Sentandreu et al., 2013; Zahin, Ahmad, Gupta, & Aqil, 2014). The other majority compound in thinning fruits was granatin B (**28**) which forms part of type III-tannins (dehydroellagitannins) (Okuda, Yoshida, & Hatano, 2000). Granatin A and B were first identified as the major components of pomegranate leaves (Tanaka, Nonaka, & Nishioka, 1985). These types of compounds, especially ellagic acid derivatives, have been also found in camu camu, strawberries and various berries (Aaby, Mazur, Nes, & Skrede, 2012; Fracassetti, Costa, Moulay, & Tomas-Barberan, 2013; Simirgiotis, Theoduloz, Caligari, & Schmeda-Hirschmann, 2009).

Anthocyanin content was known to be affected by several parameters such as harvest maturity, storage temperature, and relative humidity (Shin, Ryu, Liu, Nock, & Watkins, 2008; Elfalleh et al., 2011). Therefore, the content of anthocyanins in thinning pomegranate fruits was very low and was not a suitable parameter to compare the amount of polyphenols among thinning and ripe pomegranate fruits.

328 Despite a great number of studies, the analysis in the content of phenolic
329 compounds (specially ellagic acid derivatives) with literature data is still inquired
330 due to different analytical methodologies and because the contents may
331 considerably vary with the pomegranate cultivar and maturity stage of
332 pomegranates (Mousavinejad, Emam-Djomeh, Rezaei, & Khodaparast, 2009;
333 Fischer et al., 2011).
334
335 3.3. ABTS, DPPH and FRAP methods
336 There are different methods for evaluating the antioxidant activity of foods. This
337 variety of methods is due to the fact that none of them by itself is able to
338 determine exactly the total antioxidant potential in a food system. For this reason,
339 the antioxidant "activity" of thinning and ripe pomegranates fruits was evaluated
340 using three different analytical methods: ABTS, DPPH and FRAP (**Figure 2**). The
341 factor "cultivar" significantly ($p<0.05$) affected the antioxidant activity of thinning
342 and ripe fruits. The mean thinning values for ABTS, DPPH, and FRAP were 3603,
343 2541, and 3977 mmol trolox kg^{-1} dm, respectively; while the values for the same
344 methods but in ripe fruits were 2177, 1245, and 683 mmol trolox kg^{-1} dm,
345 respectively. These results showed that the antioxidant activity of thinning fruits is
346 among 2-6 times higher than that of ripe fruits for all three methods (ABTS, DPPH,
347 and FRAP). In general, the highest values of antioxidant activity were found in
348 sour-sweet cultivars, especially in PTO8 cultivar. This trend is similar to that found
349 in Brazilian red cherry, where the DPPH activity decreased from 171 to 83 mmol
350 trolox kg^{-1} dm throughout the development of fruits (Celli, Pereira-Netto, & Beta,
351 2011).
352 The values obtained in the current study are quite high, especially those of
353 the ripe fruits, in comparison with those found in the literature for ripe
354 pomegranate rind, arils and juice (Calín-Sánchez et al., 2013; Mena et al., 2011;
355 García-Alonso et al., 2004). The antioxidant potential of pomegranate can be
356 affected by many factors, including maturity stage, fruit cultivar, the different

nature of the materials (solid: thinning fruits or liquid: pomegranate juice), extraction procedure and the specific method for their determination. Although results may vary substantially due to all these factors, it must be highlighted that the pomegranate is a fruit with high antioxidant potential, especially thinning fruits, which are currently wasted in the soils and no revenue at all is obtained from them.

3.4. ORAC determinations

The antioxidant capacity of thinning and ripe pomegranate fruits was evaluated by ORAC method. Results showed that thinning fruits have higher values than maturity pomegranate (**Figure 3**). The ORAC values ranged from 664 to 924 mmol trolox kg^{-1} dm and from 338 to 582 mmol trolox kg^{-1} dm in thinning and ripe fruits, respectively. In the literature (Wojdylo et al., 2013; Calani et al., 2013) there is a general trend in which high antioxidant activity values are positively correlated with the high values in the total phenolic content; in this particular case, the correlation among MDEA and the ORAC antioxidant capacity values was significant ($p<0.05$) and showed a correlation coefficient, R= 0.627. The low correlation between MDEA and ORAC capacity may be due to other phenolic compounds (not determined in this study) may have a higher correlation with antioxidant capacity.

There are only very few studies evaluating the antioxidant potential of fruits from different species removed during thinning. For instance Zheng, Kim, and Chung (2012) studied the changes of the antioxidant activity of *Fuji* apples from thinning to the optimal harvest time; these authors observed a decrease of as much as 98% in the antioxidant activity from thinning to ripe apples. Li et al. (2006), reported ORAC values between 100 and 350 μmol L^{-1} in pomegranate extract. Elfalleh et al. (2011) reported values between 192 and 237 mmol trolox kg^{-1} in pomegranate peel. The mean value reported by these authors (215 mmol trolox kg^{-1}) is about 2-5 times lower than that of thinning pomegranates. Similar results were obtained for pomegranate juice (25.0 mmol L^{-1}) by Seeram et al. (2008). As a comparison, the antioxidant activity of pomegranate juice is three times higher

than the red wine and green tea (Gil et al., 2000). These results are interesting because shows the richness of thinning pomegranates as a natural antioxidant (especially from sour-sweet cultivars).

The factor cultivar significantly (p<0.05) affected the ORAC antioxidant capacity. The two cultivars with the highest ORAC values in thinning (i) and ripe (ii) fruits were: (i) PTO10 (925 mmol trolox kg^{-1} dm) and PTO5 (827 mmol trolox kg^{-1} dm), and (ii) BO1 (582 mmol trolox kg^{-1} dm) and BA1 (498 mmol trolox kg^{-1} dm), respectively.

After grouping pomegranate cultivars in sour, sour-sweet and sweet, the groups with the highest ORAC value were sour-sweet (823 mmol trolox kg^{-1} dm) in thinning fruits and sour (517 mmol trolox kg^{-1} dm) in ripe fruits.

4. Conclusions

This study demonstrated that LC-PDA-QTOF/MS and UPLC-PDA are a good methodology for the identification and quantification of the major derivates of ellagic acid in pomegranate fruit. The content of the major derivatives of ellagic acid was significantly affected by the development stage of fruits. A total of 35 compounds were indentified and quantified to compare the difference among thinning and ripe pomegranate fruits; only 7 of them were found in thinning and ripe fruits and the values of the ellagic acid derivates found in thinning fruits were 3 to 19 times higher than those found in ripe fruits. Experimental results proved that thinning sour-sweet cultivars, especially PTO8 cultivar, can be considered as a good source of bioactive compounds, which are clearly reflected in high values of antioxidant properties. Furthermore, those findings seemed to make pomegranate, specially the fruits that coming from thinning, a waste product of the pomegranate industry, an attractive candidate as a nutritional supplement for its use as supplement in food, pharmaceutical and cosmetics industries.

Acknowledgments

Funding for this study was provided by the Spanish Ministry of Economy and Competitiveness through grant AGL2013-45922-C2-2-R. Besides the visit of N. Nuncio-Jáuregui to Wrocław University of Environmental and Life Science (Wrocław, Poland) was funded within the Erasmus Program (Universidad Miguel Hernández de Elche).

REFERENCES

Aaby, K., Mazur, S., Nes, A., & Skrede, G. (2012). Phenolic compounds in strawberry (*Fragaria x ananassa* Duch.) fruits: composition in 27 cultivars and changes during ripening. *Food Chemistry, 132*, 86-97.

Alén-Ruiz, F., García-Falcón, M. S., Pérez-Lamela, M. C., Martínez-Carballo, E., & Simal-Gándara, J. (2009). Influence of major polyphenols on antioxidant activity in Mencía and Brancellao red wines. *Food Chemistry, 113*, 53-60.

Al-Rawahi, A. S., Edwards, G., Al-Sibani, M., Al-Thani, G., Al-Harrasi, A. S., & Rahman, M. S. (2014). Penolic constituents of pomegranate peels (*Punica granatum* L.) cultivated in Oman. *European Journal of Medicinal Plants, 4*, 315-331.

Basu, A., & Penugonda, K. (2009). Pomegranate juice: a heart-healthy fruit juice. *Nutrition Reviews, 67*, 49-56.

Benzie, I. F. F., & Strain, J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power: The FRAP assay. *Analytical Biochemistry*, *239*, 70–76.

Brand-Williams, W., Cuvelier, M. E., & Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. LWT-International. *Journal of Food Science and Technology*, *28(1)*, 25−30.

Calani, L., Beghe, D., Mena, P., Del Rio, D., Bruni, R., Fabbri, A., Dall'asta, C., & Galaverna, G. (2013). Ultra-HPLC-MS(n) (Poly)phenolic profiling and chemometric analysis of juices from ancient Punica granatum L. Cultivars: a nontargeted approach. *Journal of Agricultural and Food Chemistry, 61*, 5600-9.

Calín-Sánchez, A., Figiel, A., Hernández, F., Melgarejo, P., Lech, K., & Carbonell-Barrachina, A. A. (2013). Chemical composition, antioxidant capacity, and

447 sensory quality of pomegranate (*Punica granatum* L.) arils and rind as
448 affected by drying method. *Food Bioprocess Technology*, *6*, 1644-1654.

449 Celli, G. B., Pereira-Netto, A. B., & Beta, T. (2011). Comparative analysis of total
450 phenolic content, antioxidant activity, and flavonoids profile of fruits from two
451 varieties of Brazilian cherry (*Eugenia uniflora* L.) throughout the fruit
452 developmental stages. *Food Research International*, *44*, 2442–2451.

453 Elfalleh, W., Tlili, N., Nasri, N., Yahia, Y., Hannachi, H., Chaira, N., Ying, M., &
454 Ferchichi, A. (2011). Antioxidant capacities of phenolic compounds and
455 tocopherols from Tunisian pomegranate (*Punica granatum*) fruits. *Journal of*
456 *Food Science, 76*, C707-13.

457 El-Shitany, N. A., El-Bastawissy, E. A., & El-Desoky, K. (2014). Ellagic acid protects
458 against carrageenan-induced acute inflammation through inhibition of nuclear
459 factor kappa B, inducible cyclooxygenase and proinflammatory cytokines and
460 enhancement of interleukin-10 via an antioxidant mechanism. *International*
461 *immunopharmacol,* doi.org/10.1016/j.intimp.2014.02.004.

462 Fawole, O. A., & Opara, U. L. (2013). Changes in physical properties, chemical and
463 elemental composition and antioxidant capacity of pomegranate (cv. Ruby)
464 fruit at five maturity stages. *Scientia Horticulturae, 150*, 37-46.

465 Fischer, U. A., Carle, R., & Kammerer, D. R. (2011). Identification and
466 quantification of phenolic compounds from pomegranate (*Punica granatum* L.)
467 peel, mesocarp, aril and differently produced juices by HPLC-DAD-ESI/MS(n).
468 *Food Chemistry, 127*, 807-21.

469 Fracassetti, D., Costa, C., Moulay, L., & Tomas-Barberan, F. A. (2013). Ellagic acid
470 derivatives, ellagitannins, proanthocyanidins and other phenolics, vitamin C
471 and antioxidant capacity of two powder products from camu-camu fruit
472 (*Myrciaria dubia*). *Food Chemistry, 139*, 578-88.

García-Alonso, M., De Pascual-Teresa, S., Santos-Buelga, C., & Rivas-Gonzalo, J. C. (2004). Evaluation of the antioxidant properties of fruits. *Food Chemistry, 84*, 13-18.

Gil, M. I., Tomás-Barberán, 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*, 4581-4589.

Gross, G. (1999). Biosynthesis of hydrolyzable tannins. In: Pinto, B (Ed.), Comprehensive Natural Products Chemistry. Carbohydrates and Their Derivatives Including Tannins, Cellulose and Related Lignins 3. *Elsevier, Amsterdam, 3*, 799-826.

Grundhöfer, P., Niemetza, R., Schilling, G., & Grossa, G. G. (2001). Biosynthesis and subcellular distribution of hydrolyzable tannins. *Phytochemistry 57*, 915-927.

Komes, D., Horžić, D., Belščak, A., Ganić, K. K., & Vulić, I. (2010). Green tea preparation and its influence on the content of bioactive compounds. *Journal of Food Research International, 43*, 167-176.

Li, Y., Guo, C., Yang, J., Wei, J., Xu, J., & Cheng, S. (2006). Evaluation of antioxidant properties of pomegranate peel extract in comparison with pomegranate pulp extract. *Food Chemistry, 96*, 254-260.

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

Lui, R. H. (2003). Health benefits of fruit and vegetables are from additive and synergistic combinations of phytochemicals. The *American Journal of Clinical Nutrition, 78*, 517S-20S.

498 Madrigal-Carballo, S., Rodriguez, G., Krueger, C.G., Dreher, M., & Reed, J.D.
499 (2009). Pomegranate (*Punica granatum*) supplements: authenticity,
500 antioxidant and polyphenol composition. *Journal of Functional Foods*, 1, 324-
501 329.

502 Melgarejo, P., Hernández, F., & Legua, P. (2010). El Granado. Proceedings of I
503 Jornadas Nacionales sobre el Granado: Producción, Economía,
504 Industrialización, Alimentación y Salud. Universidad Miguel Hernández de
505 Elche, Departamento de Producción Vegetal y Microbiología: Elche (Alicante),
506 Spain,. 36-37.

507 Mena, P., García-Viguera, C., Navarro-Rico, J., Moreno, D. A., Bartual, J., Saura,
508 D., & Martí, N. (2011). Phytochemical characterisation for industrial use of
509 pomegranate (*Punica granatum* L.) cultivars grown in Spain. *Journal of the*
510 *Science of Food and Agriculture*, *91*, 1893–1906.

511 Mirdehghan, S. H., & Rahemi, M. (2007). Seasonal changes of mineral nutrients
512 and phenolics in pomegranate (*Punica granatum* L.) fruit. *Scientia*
513 *Horticulturae, 111*, 120-127.

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

518 Okuda, T., Yoshida, T., & Hatano, T. (2000). Correlation of oxidative transformation
519 of hydrolyzable tannins and plant evolution. *Phytochemistry*, *55*, 513-529.

520 Ordoudi, S.A., Mantzouridou, F., Daftsiou, E., Malo, C., Hatzidimitriou, E., Nenadis,
521 N., & Tsimidou, M.Z. (2014). Pomegranate juice functional constituents after
522 alcoholic and acetic acid fermentation. *Journal of Functional Foods*, 8, 161-
523 168.

524 Ou, B., Hampsch-Woodill, M., & Prior, R. L. (2001). Development and Validation of
525 an Improved Oxygen Radical Absorbance Capacity Assay Using Fluorescein as
526 the Fluorescent Probe. *Journal of Agricultural and Food Chemistry, 49*, 4619-
527 4626.

528 Park, J.E., Kim, J.Y., Kim, J., Kim, Y.J., Kim, M.J., Kwon, S.W., & Kwon, O. (2014).
529 Pomegranate vinegar beverage reduces visceral fat accumulation in
530 association with AMPK activation in overweight women: a double-blind,
531 randomized, and placebo-controlled trial. *Journal of Functional Foods*, 8, 274-
532 281.

533 Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C.
534 (1999). Antioxidant activity applying an improved ABTS radical cation
535 decolorization assay. *Free Radical Biology and Medicine*, *26*, 1231–1237.

536 Seeram, N. P., Aviram, M., Zhang, Y., Henning, S. M., Feng, L., Dreher, M., &
537 Heber, D. (2008). Comparison of antioxidant potency of commonly consumed
538 polyphenol-rich beverages in the United States. *Journal of Agricultural and*
539 *Food Chemistry, 56*, 1415-1422.

540 Seeram, N., Lee, R., Hardy, M., & Heber, D. (2005). Rapid large scale purification
541 of ellagitannins from pomegranate husk, a by-product of the commercial juice
542 industry. *Separation and Purification Technology, 41*, 49–55.

543 Sentandreu, E., Cerdán-Calero, M., & Sendra, J. M. (2013). Phenolic profile
544 characterization of pomegranate (*Punica granatum*) juice by high-
545 performance liquid chromatography with diode array detection coupled to an
546 electrospray ion trap mass analyzer. *Journal of Food Composition and*
547 *Analysis, 30*, 32-40.

548 Shin, Y., Ryu, J. A., Liu, R. H., Nock, J. F., & Watkins, C. B. (2008). Harvest
549 maturity, storage temperature and relative humidity affect fruit quality,

550 antioxidant contents and activity, and inhibition of cell proliferation of
551 strawberry fruit. *Postharvest Biology Technology, 49(2*), 201–9.

552 Shwartz, E., Glazer, I., Bar-Ya'akov, I., Matityahu, I., Bar-Ilan, I., Holland, D., &
553 Amir, R. (2009). Changes in chemical constituents during the maturation and
554 ripening of two commercially important pomegranate accessions. *Food*
555 *Chemistry, 115*, 965-973.

556 Simirgiotis, M. J., Theoduloz, C., Caligari, P. D. S., & Schmeda-Hirschmann, G.
557 (2009). Comparison of phenolic composition and antioxidant properties of two
558 native Chilean and one domestic strawberry genotypes. *Food Chemistry, 113*,
559 377-385.

560 Suarez-Jacobo, A., Rufer, C. E., Gervilla, R., Guamis, B., Roig-Sagues, A. X., &
561 Saldo, J. (2011). Influence of ultra-high pressure homogenisation on
562 antioxidant capacity, polyphenol and vitamin content of clear apple juice.
563 *Food Chemistry, 127*, 447-54.

564 Sun, J., Chu, W. F., Wu, X., & Liu, R. H. (2002). Antioxidant and Antiproliferative
565 Activities of Common Fruits. *Journal of Agricultural and Food Chemistry, 50*,
566 7449−7454.

567 Tanaka, T., Nonaka, G., & Nishioka, I. (1985). Punicafolin, an ellagitannin from the
568 leaves of Punica granatum. *Phytochemistry, 24*, 2075-2078.

569 Wojdyło, A., Oszmiański, J., & Bielicki, P. (2013). Polyphenolic composition,
570 antioxidant activity, and polyphenol oxidase (PPO) activity of quince (*Cydonia*
571 *oblonga* Miller) varieties. *Journal of Agriculture and Food Chemistry, 61*,
572 2762-7272.

573 Wu, D., Ma, X., & Tian, W. (2013). Pomegranate husk extract, punicalagin and
574 ellagic acid inhibit fatty acid synthase and adipogenesis of 3T3-L1 adipocyte.
575 *Journal of Functional Foods*, 5, 633-641.

576 Wu, X., Gu, L., Holden, J., Haytowitz, D. B., Gebhardt, S. E., Beecher, G., & Prior,
577 R. L. (2004). Development of a database for total antioxidant capacity in
578 foods: a preliminary study. *Journal of Food Composition and Analysis, 17*,
579 407-422.

580 Zahin, M., Ahmad, I., Gupta, R. C., & Aqil, F. (2014). Punicalagin and ellagic acid
581 demonstrate antimutagenic activity and inhibition of benzo[a]pyrene induced
582 DNA adducts. *BioMed Research International, 2014*, 1-10.

583 Zheng, H. Z., Kim, Y. I., & Chung, S. K. (2012). A profile of physicochemical and
584 antioxidant changes during fruit growth for the utilisation of unripe apples.
585 *Food Chemistry, 131*, 106-110.

Table 1. LC-QTOF/MS Analysis of the major derivatives of ellagic acid in thinning and ripe pomegranate fruits from nine Spanish pomegranate cultivars.

Peak	Compound	R_t (min)	λ_{max} (nm)	MS $[M-H]^-$ (m/z)	MS/MS $[M-H]^-$ (m/z)	Fruits	
						Thinning	Ripe
1	Unknown	0.63	248	215	179/145/135/132	-	+
2	Galloyl- HHDP-hexoside	0.90	264/377	633	275/259/169	+	-
3	Galloyl-glucoside	1.03	261/376	331	271/169/143/125	+	-
4	HHDP-gallagyl-hexoside (punicalagin)	1.04	257/377	1083	611/331/146	-	+
5	Gallolyl-HHDP-glucoside	1.29	260	633	275/249/149	+	-
6	bis-HHDP-glucoside (pedunculagin I)	1.35	243	783	481/300/275	+	-
7	Punicalagin isomer	1.61	257/377	1083	781/622/300	+	+
8	Ellagitannin	2.35	252/373	933	631/450/300/275	+	-
9	HHDP-gallagyl-hexoside (punicalagin)	2.37	252/371	352	262/235/190/162/146	-	+
10	Ellagic acid derivative	2.68	255/376	1085	907/783/300	+	-
11	Ellagitannin	2.73	243	783	481/300/275	+	-
12	Ellagitannin	2.86	242	783	481/300/275/146	-	+
13	HHDP-gallagyl-hexoside (punicalagin)	3.52	257/378	1083	781/745/622/300	+	+
14	Digalloyl-HDDP-glucoside (pedunculagin II)	3.80	271	785	483/300	+	-
15	Bis-HHDP-glucose-isomer	3.82	236	785	300/275	-	+
16	Granatin A	4.40	268	799	781/479/300/273	+	+
17	Ellagic acid derivative	4.75	255/375	1085	479/300/273	+	-
18	Granatin A	4.83	263	799	300/272	-	+
19	Ellagic acid derivative	5.32	253	301	275/217/169	+	+
20	Unknown	5.81	256	801	362/352/218/190	-	+
21	HHDP-digalloyl-glucoside	5.89	254	785	300/275/169	+	-

592 **Table 1.** Continuation.

Peak	Compound	R_t (min)	λ_{max} (nm)	MS $[M-H]^-$ (m/z)	MS/MS $[M-H]^-$ (m/z)	Fruits	
						Thinning	Ripe
22	Ellagitannin	6.21	272	784	482/419/300/275/249	+	-
23	Galloyl-HHDP-glucoside	7.80	263	633	463/300/275	-	+
24	Bis-HHDP-glucose-isomer	8.56	270	784	300/275/169	+	-
25	Ellagitannin	8.79	268	784	627/300/275/169	+	+
26	Ellagitannin	9.01	270	784	617/300/275/169	+	-
27	Ellagic acid derivative	10.38	276	937	613/300	+	-
28	Granatin B	10.54	274	951	933/765/300/273	+	+
29	Ellagic acid derivative	11.06	275	951	907/787/635/300	+	+
30	Ellagic acid derivative	11.37	213/252/361	433	352/300/160/146	-	+
31	Ellagic acid rhamnoside	11.57	252/361	447	352/262/160/146	-	+
32	Dpd-trihexoside	12.10	276	787	617/465/169	+	-
33	Punicalagin-like	12.30	254	1109	352/146	-	+
34	HHDP-trigalloyl-glucose	13.18	275	937	767/465/300/169	+	-
35	Pentagalloyl hexose	13.71	278	939	769/169	+	-

593

594

595 **Table 2**. Major derivatives of ellagic acid (mg 100 g^{-1} dm) in thinning fruits from nine Spanish pomegranate cultivars.

(Peak)	Compound	Cultivars								
		BA1	**BO1**	**BBE1**	**PTO5**	**PTO8**	**PTO10**	**ME14**	**ME17**	**VA1**
(2)	Galloyl- HHDP-hexoside	194[†]±5 d[‡]	357±11 b	87.5±3.5 de	127±1 e	475±4 a	64.4±1.3 f	245±1 cd	279±5 c	162±7 d
(3)	Galloyl-glucose	126±4 c	189±2 b	65.7±4.5 e	98.6±0.1 d	194±3 ab	39.4±0.5 f	198±2 ab	208±1 a	183±2 b
(5)	Galloyl- HHDP-glucoside	148±1 e	253±2 bc	70.0±1.8 g	102±1 f	197±3 d	50.0±1.1 g	274±1a	268±9 ab	230±3 c
(6)	Bis-HHDP-glucoside	289±4 d	493±6 a	196±3 e	185±1 e	421±2 cd	92.8±1.3 f	417±2 bc	460±7 ab	369±7 c
(7)	Punicalagin isomer	1866±1 d	2899±3 a	1009±9 e	1742±1 d	2264±1 c	746±1 f	2396±2 bc	2523±7 b	2433 ±4 bc
(8)	Ellagitannin	117±4 e	211±2 d	26.2±1.0 f	131±1 e	405±5 a	114±2 e	426±2 a	359±6 b	253±4 c
(10)	Ellagic acid derivative	163±5 b	194±7 ab	41.1±2.7 d	94.3±8.6 c	223±1 a	32.6±0.5 d	103±1 c	154±5 b	71.7±0.4 c
(11)	Ellagitannin	238±2 c	299±3 ab	152±10 d	111±1 d	259±4 bc	55.6±1.3 f	244±1 c	339±2 a	230±1 c
(13)	HHDP-gallagyl-hexoside	3140±7 d	5296±6 a	1831± 6 e	3231±1 d	4038±1 c	1352±8 f	4344±8 c	4734±4 b	4749±6 b
(14)	Digalloyl-HDDP-glucoside	286±2 de	528±5 b	94.0±3.7 f	249±1 e	904±1 a	93.4±2.1 f	356±1 cd	580±2 b	332±2 c
(16)	Granatin A	65.0±2.2 e	247±3 b	85.2±0.1 e	168±1 d	372±1 a	34.0±0.5 f	163±4 d	230±1 bc	218±4 c
(17)	Ellagic acid derivative	275±2 c	438±4 b	128±3 d	279±2 c	787±2 a	173±4 d	405±1 b	403±4 b	254±1 c
(19)	Ellagic acid derivative	150±1 d	271±2 b	72.1±1.8 f	93.7±0.5 e	320±5 a	44.7±1.0 g	148±1 e	195±7 c	145±1 d
(21)	HHDP-digalloyl-glucose	348±3 d	657±6 a	189±7 e	224±2 e	636±1 a	105±2 f	427±2 c	504±8 b	419±7 c
(22)	Ellagitannin	237±2 d	468±5 b	72.1±5.0 f	148±1 e	895±4 a	67.0±3.1 f	189±2 de	338±4 c	121±3 e
(24)	Bis-HHDP-glucose-isomer	182±1 c	278±3 b	43.5±3.0 ef	54.0±0.1 e	532±3 a	18.2±0.4 f	62.6±0.1 e	109±4 d	45.0±0.2 e
(25)	Ellagitannin	302±4 c	503±6 b	123±8 f	139±1 f	775±5 a	55.6±1.6 g	235±2 de	287±1 cd	190±2 d
(26)	Ellagitannin	48.0±1.6 d	102±1 b	40.7±1.6 de	32.6±0.3 e	120±1 a	17.0±0.3 f	100±1 b	104±1 b	66.0±1.3 c
(27)	Ellagic acid derivative	125±1 c	259±2 b	47.3±3.0 e	85.5±0.5 d	625±1 a	42.0±0.9 e	105±1 cd	112±4 cd	37.0±0.2 e
(28)	Granatin B	708±2 c	1225±2 b	351±4 ef	521±1 d	2967±2 a	284±8 f	615±2 c	460±2 d	337±4 e
(29)	Ellagic acid derivative	23.0±0.8 d	52.7±0.6 b	8.31±0.58 e	29.4±0.9 c	159±1 a	10.7±0.2 e	22.4±0.1 d	25.6±0.1 cd	9.00±0.10 e
(32)	Dpd-trihexoside	34.4±1.1 cd	61.6±0.7 b	13.0±0.9 e	28.6±0.1de	196±1 a	16.7±0.5 e	48.0±0.3 bc	57.1±0.2 b	13.6±0. cd
(34)	HDP-trigalloyl-glucose	19.4±0.6 c	31.7±0.4 b	7.80±0.54 ef	11.9±0.1 d	160±1 a	6.58±0.20 f	21.0±0.1 c	20.3±0.1 c	8.67±0.15 de
(35)	Pentagalloyl hexose	19.0±0.5 bc	21.1±0.2 bc	6.03±0.24 d	9.61±0.09 d	313±4 a	5.75±0.11 d	13.6±0.4 cd	24.0 ±0.4 b	1.40±0.03 d
	TOTAL	**9101**	**15338**	**4763**	**7896**	**18236**	**3521**	**11554**	**12773**	**10876**

596 [†] *Values are the mean of 3 replications (± standard error).* [‡] *Values followed by* different letters (a, b, c, etc.) *within the same row are*

597 *statistically different according to Tukey's multiple range tests ($p < 0.05$). All were significant at $p<0.001$.*

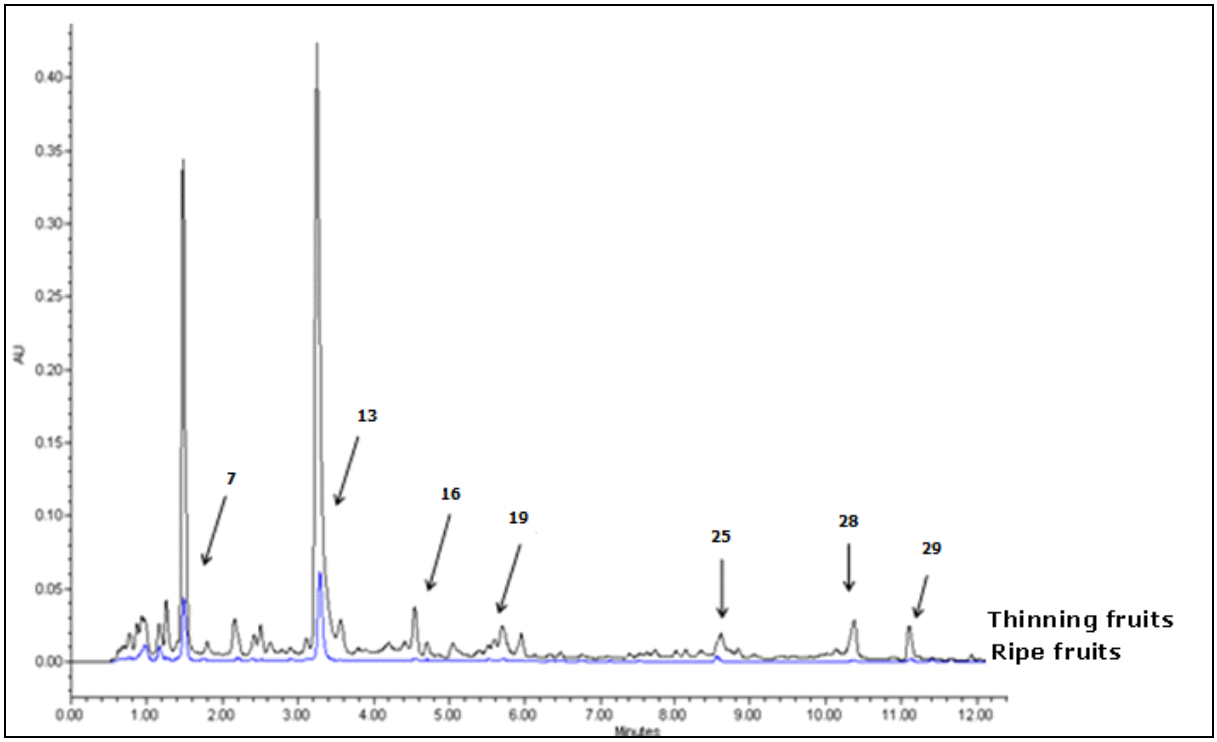
598

599 **Table 3**. Major derivatives of ellagic acid (mg 100 g^{-1} dm) in ripe fruits from nine Spanish pomegranate cultivars.

(Peak)	Compound	Cultivars								
		BA1	**BO1**	**BBE1**	**PTO5**	**PTO8**	**PTO10**	**ME14**	**ME17**	**VA1**
(1)	Unknow	149[†] ±4 b [‡]	205±4 a	67.1±0.1 de	45.4±0.4 f	146±8 b	128±1 c	43.7±0.1 f	77.6±0.9 d	52.2±5.2 ef
(4)	HHDP-gallagyl-hexoside	90.2±2.7 b	93.6±0.3 b	48.8±0.8 d	33.2±0.1 e	105±1 a	56.0±0.6 c	20.8±0.2 g	27.8±0.1 f	27.9±0.6 f
(7)	Punicalagin isomer	30.5±0.9 c	51.8±0.1 b	22.8±0.4 e	17.0±0.1 f	60.0±0.5 a	51.8±0.6 b	19.2±0.6 f	27.0±0.3 d	18.6±0.5 f
(9)	HHDP-gallagyl-hexoside	57.0±1.7 d	91.2±0.3 b	50.1±0.8 e	33.7±0.1 f	113±1 a	71.7±0.8 c	12.9±0.1 h	27.7±0.1 g	25.5±0.2 g
(12)	Ellagitannin	1264±8 b	1273±2 b	710±9 d	485±4 e	1440±1 a	1017±6 c	366±1 f	645±7 d	520±4 e
(13)	HHDP-gallagyl-hexoside	47.5±1.4 c	76.0 ±6.3 ab	44.7±0.5 c	40.3±3.6 cd	85.0±1.7 a	65.8±2.7 b	17.5±0.1 e	28.3±0.9 de	27.0±0.1 e
(15)	Bis-HHDP-glucose-isomer	57.0 ±1.7c	95.2±0.3 b	45.3±0.7 d	37.6±0.1 e	129±1 a	98.9±0.3 b	24.8±0.1 h	33.3±1.0 f	29.0±0.1 g
(16)	Granatin A	29.0±0.1 de	45.8±0.1 b	30.0±0.5 d	27.4±0.1 e	70.5±0.6 a	43.1±0.5 c	11.4±0.1 g	18.5±0.1 f	20.4±0.4 f
(18)	Granatin A	50.0±0.3 d	63.2±1.1 b	40.0±1.1 e	28.3±0.2 f	85.3±0.7 a	59.4±0.2 c	15.2±0.1 g	26.0±0.8 f	26.8±0.1 f
(19)	Ellagic acid derivative	88.3±2.7 c	89.3±0.2 c	42.5±0.7 e	35.1±0.1 f	123±1 a	104±1 b	28.4±0.2 g	37.0±0.1 f	48.0±0.4 d
(20)	Unknow	26.4±0.1 b	20.6±0.4 d	23.0±0.6 c	20.8±0.1 d	34.4±0.3 a	20.6±0.1 d	7.05±0.01 g	8.90±0.27 f	10.9±0.1 e
(23)	Galloyl-HHDP-glucoside	14.0**±**0.1 c	24.3±0.4 b	9.30±0.26 f	11.8±0.1 d	45.4±0.4 a	23.6±0.1 b	5.84±0.01 g	10.5±0.3 e	8.33±0.02 f
(25)	Ellagitannin	36.1±0.1 c	43.0±0.7b	27.0±0.4 d	19.2±0.2 e	61.5±0.1 a	37.6±0.2 c	11.0±0.3 f	18.6±0.2 e	16.9±0.5 e
(28)	Granatin B	85.0±2.6 d	116±1 b	37.5±0.6 e	32.0±0.2 e	293±3 a	98.9±2.1 c	11.0±0.1 f	15.0±0.1 f	16.6±0.3 f
(29)	Ellagic acid derivative	46.6±1.4 b	61.0±0.2 a	7.09±0.11 e	5.44±0.01 e	46.2±0.4 b	28.4±0.1 c	4.83±0.01 e	11.1±0.3 d	7.28±0.02 e
(30)	Ellagic acid derivative	22.0±0.7 b	25.4±0.1 a	5.86±0.09 d	1.64±0.01 f	21.7±0.8 b	14.4±0.1 c	3.55±0.02 e	7.28±0.06 d	4.07±0.09 e
(31)	Ellagic acid rhamnoside	23.0±0.7 b	24.6±0.4 a	5.06±0.07 e	1.48±0.01 g	22.3±0.1 b	14.0±0.1 c	3.73±0.02 ef	7.18±0.06 d	3.64±0.04 f
(33)	Punicalagin like	8.16±0.05 c	17.0±0.3 b	3.40±0.04 e	3.20±0.02 e	22.4±0.2 a	4.87±0.01 d	1.10±0.01 g	1.89±0.06 f	2.00±0.01 f
	TOTAL	**2124**	**2415**	**1219**	**878**	**2905**	**1938**	**608**	**1028**	**865**

600 [†] *Values are the mean of 3 replications (± standard error).* [‡] *Values followed by* different letters within the same row are statistically

601 different a*ccording to Tukey's multiple range test ($p < 0.05$).*

602 

603 **Figure 1**. Comparative chromatogram of thinning and ripe pomegranate fruits (PTO8 cv.). Peaks: **7**,
604 punicalagin isomer; **13**, HHDP-gallagyl-hexoside (punicalagin); **16**, granatin A; **19**, ellagic acid
605 derivative; **25**, ellagitannin; **28**, granatin B; **29**, ellagic acid derivative.

606

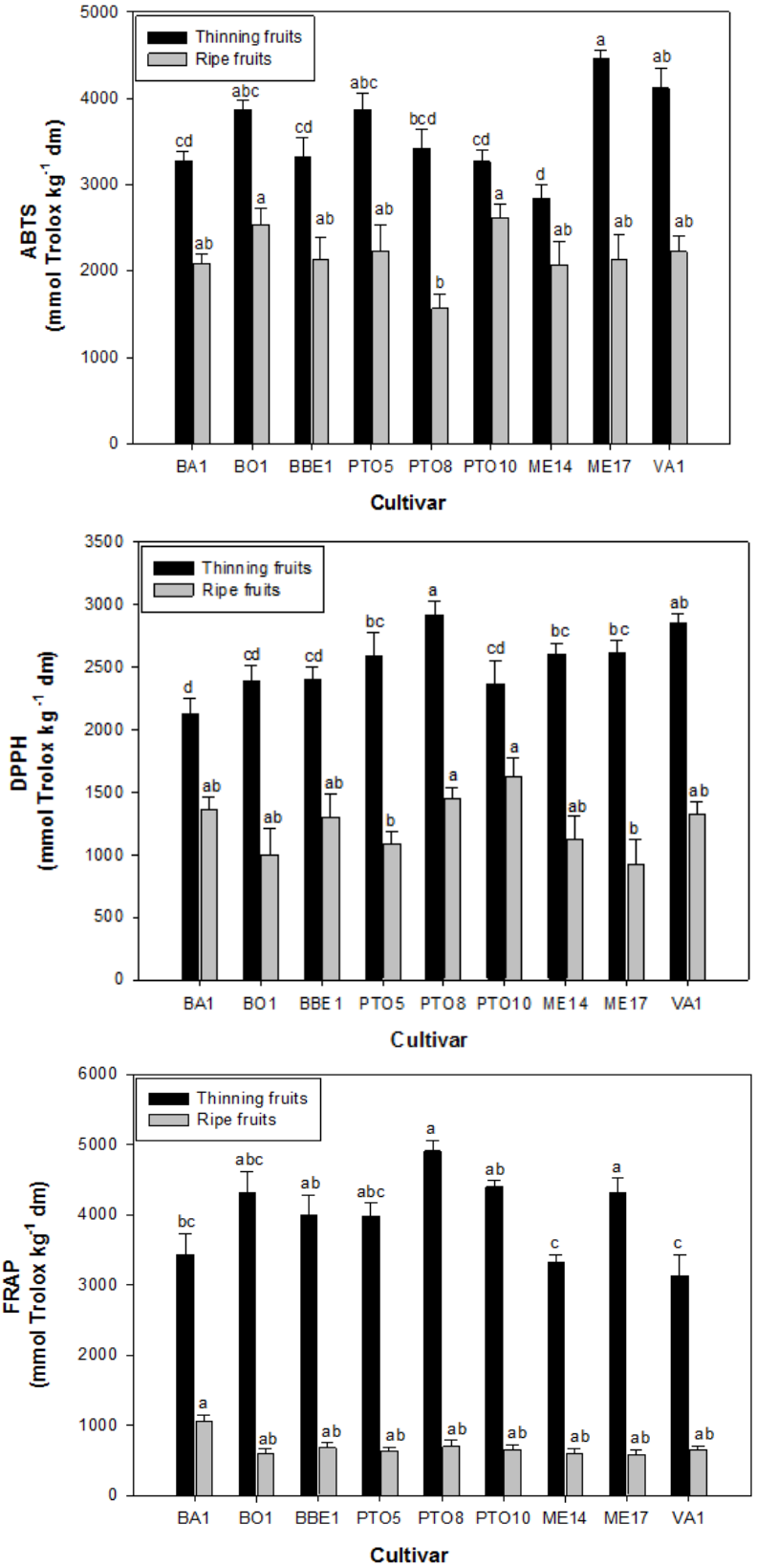
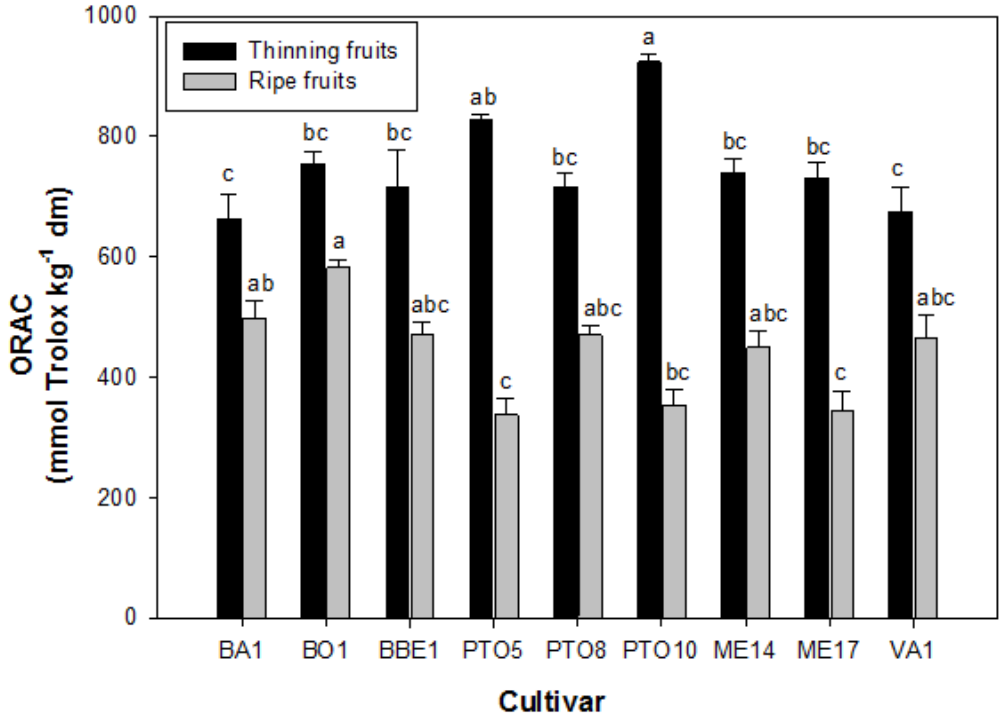


Figure 2. ABTS, DPPH and FRAP activity of thinning and ripe pomegranate fruits (mmol trolox kg^{-1} dm). *Error bars correspond to the standard deviation of three replicates. Bars with the same letter,*

612 *for each development stage (thinning or ripe), were not statistically different according to Tukey's*
613 *multiple range test ($p < 0.05$).*

614



615

616 **Figure 3**. ORAC capacity of thinning and ripe pomegranate fruits (mmol trolox kg^{-1} dm). *Error bars*
617 *correspond to the standard deviation of three replicates. Bars with the same letter, for each*
618 *development stage (thinning or ripe), were not statistically different according to Tukey's multiple*
619 *range test ($p < 0.05$).*