1 **Original research article**

2 Fatty acid, vitamin E and sterols composition of seed oils from 3 nine different pomegranate (*Punica granatum* L.) cultivars 4 grown in Spain

5 Luana Fernandes [a], José Alberto Pereira [a], Isabel Lopéz-Cortés [b], Domingo M. Salazar [b],

6 Elsa Ramalhosa [a*] and Susana Casal [c*]

7 [a]*Mountain Research Centre (CIMO) - School of Agriculture, Polytechnic Institute of*

8 *Bragança, Campus de St[a] Apolónia, Apartado 1172, 5301-855 Bragança, Portugal*

9 [b]*Departamento de Producción Vegetal. Universidad Politécnica de Valencia. Camino*

10 *de Vera s/n, 46022 Valencia, Spain*

11 [c]*REQUIMTE/Laboratory of Bromatology and Hydrology, Faculty of Pharmacy, Porto*

12 *University, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal*

13 *To whom correspondence should be addressed. Telephone: +351-273303308. Fax:

14 +351-273325405. E-mail: elsa@ipb.pt; Telephone: +351-220428638. Fax: +351-

15 226093390. E-mail: sucasal@ff.up.pt

16 Abstract

17 The present study was conducted to determine the major bioactive lipid components of

18 the seed oils of nine pomegranate (*Punica granatum* L.) cultivars grown in Spain,

19 namely fatty acids, vitamin E and sterol compositions. The seeds yielded oil contents

20 ranging from 4.44–13.70% of dry matter and showed high contents of polyunsaturated

21 fatty acids (86.7.2-90.3%). The predominant fatty acid was 9,11,13-octadeca-trienoic

22 acid (punicic acid), a conjugated linolenic acid characteristic from pomegranate seeds,

with contents between 3523 and 10586 mg/100 g of seeds. Total tocopherol contents ranged from 135–525 mg/100 g of oil, with γ-tocopherol as the main component, and with different compositional ratios between varieties. Concerning sterols in the oil, total amounts ranged from 364–553 mg/100g, with a predominance of β-sitosterol. After performing principal component analysis, intercultivar differences were found, a potential tool for cultivar authenticity purposes. Moreover, the ingestion of pomegranate arils, with their seeds, increases their beneficial health properties.

Keywords: Pomegranate; Seed oil; Fatty acid; Tocopherol; Sterol; Carotenoid; Spanish cultivars; Biodiversity and nutrition; Cultivar difference; Food analysis; Food composition

1 Introduction

Pomegranate (*Punica granatum* L.) is an ancient fruit tree species native to Afghanistan, Iran, China and India, and has traditionally been cultivated in the Near and Middle East (Ismail et al., 2012; Jing et al., 2012). Due to its adaptation to a wide range of climate and soil conditions, the most important growing regions include Iran, Israel, USA, India, China, Turkey and Spain; Spain is the largest European exporter with a worldwide production of approximately 3×10^4 tons (Andreu-Sevilla et al., 2009). In Spain, pomegranates are grown mainly in the provinces of Alicante and Murcia (southeast Spain), due to the high temperatures (> 40 °C) in summer. The most common varieties are Mollar de Elche, Roja/White and Valenciana (Glozer & Ferguson, 2011).

Pomegranate fruit can be divided into several anatomical compartments: (1) outside peel, (2) inside peel (pellicles), and (3) arils (pulp and seeds). Arils are usually used for fresh consumption, juice, jams and jellies production, and also for developing extracts

to be used as ingredients in medicinal herb preparations and dietary supplements (Goula & Adamopoulos, 2012).

Seeds are a byproduct of the pomegranate industry, but recent reports have highlighted their potential use as a source of seed oil with beneficial health attributes. Seeds may represent up to about 20% of the total fruit weight, ranging from 9.3% to 57.5% (Al-Maiman & Ahmad, 2002; Gözlekçi et al., 2011; Habibnia et al, 2012; Tehranifar et al., 2010) depending on the variety, geographical location, growing conditions, maturity stage, etc. Pomegranate seeds have antioxidant properties (Jing et al., 2012; Pande & Akoh, 2009) and are mainly composed of fiber and lipids (Eikani, et al., 2012; Hernández et al., 2011), with an oil content varying from 12–20% (Lansky & Newman, 2007). Several studies have shown that pomegranate seed oils are good sources of polyunsaturated fatty acids, especially linoleic and punicic acid (Eikani et al, 2012; Jing et al, 2012; Liu et al., 2012), and tocopherols (Jing et al., 2012). Due to these characteristics, extraction of pomegranate seed oils should be encouraged, with potential as a source of nutrients and antioxidants with benefits to human health, reducing the risk for cardiovascular diseases and cancer (Kohno et al., 2004), alleviating menopausal symptoms (Lansky & Newman, 2007), improving immune function (Yamasaki et al., 2006) and preventing genetic disorders (Guo et al., 2007), among others.

Most of the work published in pomegranate seed oils has focused on technological issues, such as optimization of oil extraction (Ahangari & Sargolzaei, 2012; Eikani et al., 2012; Liu et al., 2009; Liu et al., 2012; Tian et al., 2013), with fewer studies on their physicochemical characterization. Even though several works have been published on fatty acids composition of pomegranate seed oils from several countries (e.g. Elfalleh et al., 2011a; Habibnia et al., 2012; Hernández et al., 2011; Jing et al., 2012), a few studies

on tocopherols, sterols and carotenoids have been published (Caligiani et al., 2010; Habibnia et al., 2012; Jing et al., 2012), particularly with characterization of pomegranate varieties. Diverse chromatographic techniques had been used in pomegranate seed oil characterization. Tocopherols have been determined by high performance liquid chromatography with photodiode array detection (HPLC-PDA) (Habibnia et al., 2012; Jing et al., 2012) or gas-chromatography (GC)–mass spectrometry (MS) (Caligiani et al., 2010). This last technique has been also used in sterol identification (Caligiani et al., 2010); however, Habibnia et al. (2012) had used thin-layer chromatography (TLC) to identify these compounds. Carotenoids have been tentatively detected by HPLC-PDA (Jing et al., 2012). Nevertheless, the only European variety studied until now in terms of these compounds was the Wonderful variety.

Thus, the main objective of the present work was to characterize the main constituents present in the seed oils of nine pomegranate varieties of European origin, collected in Spain, including their fatty acid composition, vitamin E, sterol, and carotenoid content, to better assess the potential of these pomegranate seed oils to be used as nutraceuticals or functional food ingredients. Moreover, it was predicted that valuable information for cultivar selection would be also obtained.

2 Material and methods

2.1 Standards and reagents

All reagents were of analytical, chromatographic or spectroscopic grade. A certified fatty acids methyl ester (FAME) reference standard mixture (37 fatty acids from C4 to C24) from Supelco, TraceSelec (Bellefonte, PA, USA) was used, together with the internal standard (triundecanoin) and some individual fatty acid isomers, all from Sigma-Aldrich (Bellefonte, PA, USA). Tocopherols (α (≥96%), γ (≥96%), δ(≥90%) and

tocotrienols (α (≥97.0%)β (≥97.0%), γ (≥97.0%), δ (≥97.0%)) were purchased from Calbiochem (La Jolla, CA, USA) and Sigma–Aldrich (St. Louis, MO, USA) and the internal standard tocol (98%) was obtained from Matreya LLC (Pleasant Gap, PA, USA). Carotenoid standards, all-trans-β-carotene (>97%) and lutein (>90%, alfalfa), were obtained from Sigma–Aldrich (St. Louis, MO, USA). Tocopherols, β-carotene and lutein standards purity was monitored by spectrophotometry (UV-1800, Shimadzu, Japan), based on their $E^{1\%}_{1\ cm}$ values (Craft & Soares, 1992; Nesaretnam et al., 2007). All sterol standards campesterol (~65%), stigmasterol (~95%), β-sitosterol (≥97%) and sitostanol (≥95%)) were purchased from Sigma–Aldrich (St. Louis, MO, USA), as well as the internal standard dehydrocholesterol (≥95.0%). The other reagents were supplied by Merck (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, MO, USA). A Milli-Q water purification system (Millipore, Molsheim, France) was used to obtain ultrapure water (resistivity of 18.2 MΩ cm^{-1}) for quantitative analysis.

2.2 Pomegranate varieties

The pomegranates used in the present work were harvested in Elche, Alicante (Spain), between 6th to 23rd September 2012, to full ripeness. Of each variety, three lots were constituted, each with three fruits, being the pomegranates collected from different trees in the same experimental field. Each lot was analysed in triplicate. Nine cultivars were selected, namely: CG8, Cis 127, Mollar de Elche, Parfianka, Katirbasi, Valenciana, White, Wonderful 1 and Wonderful 2 (Figure 1). After harvest, the pomegranates were transported to the laboratory under refrigeration. On their arrival, pomegranates were washed with ultra-pure water (Milli-Q system) and immediately processed for their physicochemical characterization.

2.3 Physicochemical characterization

The following parameters were evaluated in the nine pomegranate cultivars: fruit weight, external peels, pellicles and arils. Seeds were also separated from the pulp and dried. In a small portion of pulp, juice was extracted and the total soluble solids content (ºBrix) determined by refractometry (Optic Ivymen System, Madrid, Spain).

2.4 Seed oils extraction

Seed oils were extracted using the procedure described by Fernandes et al. (2013). For each variety, 15 grams of seeds were crushed in a mortar with a pestle. Anhydrous sodium sulfate was added to remove moisture remains. The lipid fraction was obtained by Soxhlet extraction with petroleum ether with 0.01% BHT (2,6-di-*tert*-butyl-4-methylphenol, Sigma) to prevent oxidation for a 4 h period. The solvent was removed with a rotary evaporator RE300DB (Stuart, Stone, United Kingdom) and the samples were stored at -20 °C until analysis, closed under a nitrogen stream.

2.4.1 Fatty acids

Fatty acid methyl esters were obtained by fast cold transmethylation with methanolic potassium hydroxide 2M, according to ISO 12966-2 (2011). Fatty acids were determined by gas chromatography (Chrompack, CP-9001 model, The Netherlands) with flame ionization detection (GC-FID) as described by Malheiro et al. (2013). The gas chromatograph was equipped with a split/splitless injector system and an autosampler (Chrompack CP-9050 model). Fatty acids separation was carried out on a CP-Sil 88 column (50 m × 0.25 mm × 0.19 μm; Varian). Helium was used as carrier gas at a pressure of 120 kPa. The temperatures of the injector and detector were 250 ºC and 270 ºC, respectively. Methyl esters separation was carried out with a temperature gradient between 120 and 200 °C. The collection and processing of the data were

141 performed by the CP Maitre Chromatography Data System program, Version 2.5
142 (Chrompack International B.V.). The identification of the chromatographic peaks was
143 performed by comparing the retention time of the sample with a certified FAME mix
144 and diverse individual fatty acid methyl esters and by comparison with literature data on
145 pomegranate seed oils. For quantification of total fatty acid content in the oil, an
146 internal standard (triundecanoin) was used. The detection limit (LOD) corresponded to
147 the analyte amount for which the signal-to-noise ratio was equal to 3, and the
148 quantification limit (LOQ) corresponded to the analyte amount for which the signal-to-
149 noise ratio was equal to 10, i.e. 20 and 50 mg/100 g oil, respectively. The linearity range
150 of the FID detector was tested up to 5 mg/ml of injected FAME solutions.

151 ### *2.4.2 Vitamin E*

152 Tocols were evaluated following the international standard ISO 9936 (2006), with some
153 modifications as described by Casal et al. (2010). Briefly, tocochromanols were
154 separated on a HPLC chromatograph (Jasco, Tokyo, Japan) equipped with a pump (PU-
155 980 model), mixing chamber (HG 980-30) and an autosampler (AS2057 Plus model).
156 The detection was performed by the fluorescence detector FP2020 Plus model at 290
157 nm (excitation) and 330 nm (emission) wavelengths. The tocopherols and tocotrienols
158 separation was performed on a normal phase silica Supelcosil LC-SI (Supelco) column
159 (250 mm × 3.0 mm × 3 μm), using hexane:dioxane (97:3 v/v) mixture as eluent (1.2
160 mL/min) at ambient temperature. The quantification was performed using the internal
161 standard method (tocol).For analysis, an accurate oil amount was weight (≈30mg), the
162 internal standard added, dissolved in *n*-hexane, centrifuged (Heraeus Sepatech,
163 Germany) at 4,000 g and transferred to the injection vials. A LOD and LOQ of 1 and 3
164 mg/100 g oil, respectively, were achieved for the global method, with a linearity from 2
165 to 100 μg/ml of injected solution.

2.4.3 Carotenoids

Carotenoids were tentatively analyzed simultaneously with tocopherols, using a diode array detector (Jasco, PU-980 model, Tokyo, Japan) connected in series with the fluorescence detector, as mentioned by Casal et al. (2001) and Panfili et al. (2004). The chromatograms were analyzed at 450 nm, with tentative identification performed by retention time and spectra comparison with those of carotenoid standards, namely, all-*trans*-β-carotene and lutein. The LOD and LOQ were equal to 0.1 and 0.3 mg/100g oil, respectively, but all samples were below these limits.

2.4.4 Sterols

A 150 mg amount of each pomegranate seed oil was accurately weighed into a clean tube and mixed with 100 μL of dehydrocholesterol solution (2mg/mL in *n*-hexane; internal standard) and 400 μL of *n*-hexane, following the procedure of Cunha et al. (2006). This mixture was loaded onto a silica SPE column (1 g; Tecnokroma, Spain), conditioned previously with 5 mL of *n*-hexane (twice). Three 500 μL portion of *n*-hexane were used to transfer the sample solution to the SPE column. Elution was performed with 5 mL of *n*-hexane/ethyl acetate (90:10, v/v), followed with more 2.5 mL. Then, 5 mL of ethanol/diethyl ether/*n*-hexane (50:25:25, v/v/v) were added twice to the SPE column. After solvent evaporation under nitrogen stream of the combined extracts (60 ºC), 2.5 mL of KOH 1 M in 96% ethanol was added. The solution was heated for 30 min at 70 ºC. Afterwards, 5 mL of water, 5 mL of diethyl ether (twice), 2mL of KOH 0.5 M and 4 mL of KCl 0.88% (w/v) were added and centrifuged at 4,000 g for 7 min. The aqueous phase was withdrawn and the organic fraction was dried over anhydrous sodium sulfate. The solution was evaporated under a gentle nitrogen stream at 60 ºC. Derivatization was performed with 100 μL of *N,O*-Bis(trimethylsilyl)trifluoroacetamide in 1% trimethylchlorosilane at 70 ºC for 20 min.

Samples were analyzed by GC–FID Thermo Finnigan (Milan, Italy) using a DB-5MS column (30 m × 0.25 mm × 0.25μm; J&W Scientific, USA), with a temperature program from 250 ºC to 300 ºC. Helium (Gasin, Portugal) was used as carrier gas at an internal pressure of 100 kPa. Chromatographic parameters and quantification was based on ISO 12228 (1999), with a LOD and LOQ of 1 and 2.5 mg/100 g oil, respectively.

2.5 Statistical analysis

The Statistic SPSS software, version 18.0 (SPSS Inc., Chicago, IL, USA), was used for the statistical treatment of the data. The influence of the cultivar over fatty acid, vitamin E and sterol compositions was evaluated using the one-way analysis of variance (ANOVA) ($p<0.05$), followed by the Tukey HSD post hoc test, when variances of the groups were identical. On the other hand, when variances were not identical, the Games-Howell test coupled with Welch's statistic was applied. The variance homogeneity was evaluated by Levene's test.

Principal component analysis (PCA) was also performed for the results of fatty acids, tocopherols and sterols of the studied pomegranate cultivars. The PCA score plot was used to differentiate pomegranate cultivars through their chemical compositions.

3 Results and discussion

3.1 Physicochemical characterization of pomegranates

The fruits were initially characterized by weighting their constitutive parts, namely outer peel, pellicle and arils, and these latter for the seeds weight and total soluble solids content (TSS) of the arils juice. Significant differences ($p<0.05$) were observed between the nine cultivars (Table 1). The average of fruit weights varied between 185 g and 439 g for the cultivars CG8 and Parfianka, respectively. When sized by weight and

following the classification of "size code" described by the Codex Alimentarius Commission (2013), the pomegranates of the nine cultivars were classified on the A to E size codes, for which the pomegranate weights must be greater than or equal to 501 g and between 191 and 250 g, respectively (Supplementary Material, Table S1). Mollar de Elche variety presented the highest percentage of the heaviest fruits (40% on A size code), followed by the Parfianka and White cultivars with 75.0 and 42.9% in B size code, respectively. On the other hand, the Katirbasi cultivar presented 20.0% of the fruits classified in E size code, corresponding to the lightest fruits. The other cultivars presented intermediate size codes.

The outer peel (Table 1) represented 30.6 to 49.6% for the Valenciana and CG8 cultivars, respectively. The pellicles corresponded to much lower percentages, ranging from 1.1 to 2.0% for the Mollar de Elche and CG8 cultivars, respectively. Finally, the arils represented 45.6 to 65.8% of the whole fruit weight for the CG8 and Valenciana cultivars, respectively.

Regarding pomegranate seeds, their relative percentage in the whole fruit weight varied between 3.7 and 7.9% for the Wonderful 1 and Valenciana cultivars, respectively. However, when evaluating the percentage of seeds in the edible part (arils), values between 8.8 and 14.4% were determined for the Mollar de Elche and Wonderful 2, respectively. The lowest percentage of seeds in the arils observed for the Mollar de Elche explains why this cultivar is preferred by both consumers and the pomegranate juice industry. On the other hand, the Wonderful 2 cultivar (14.4%), followed by the CG8 (13.8%), showed the highest ratios of seeds per edible part and therefore have the highest potential for the pharmaceutical, cosmetic and food industries that wish to extract pomegranate seed oils. Nevertheless, the total soluble solids content of the CG8

cultivar juice was one of the highest (18.7 ºBrix), whereas the juice of the Wonderful 2 cultivar (16.7 ºBrix) was the third lowest, showing the potential of the former to be consumed in fresh or used in juice production.

Our results were similar to those described for Spanish cultivars by Martínez et al. (2006), who found TSS between 12.4 ºBrix for the "Mollar de Elche 14" and 16.3 ºBrix for the "Piñón tierno of Ojós 7" cultivars, by Melgarejo et al. (2011) with 14.3 to 15.8 ºBrix for the "CRO2" and "ME2" Spanish cultivars; or by Mena et al. (2011), with values ranging from 13.7 to 17.6 ºBrix. Our results are slightly higher than those presented by Tehranifar et al. (2010) for the "Agha Mandali Save" and "Torsh Shavar Ferdows" cultivars of Iran with values between 11.4 and 15.1 ºBrix, but are similar to those obtained by Gözlekçi et al. (2011) for the "Asinar" and "Cekirdeksiz-IV" Turkish cultivars (13.9 to 15.0 ºBrix), Zaouay et al. (2012) for the "Jerbi1" and "Mezzi2" Tunisian cultivars (14.3 to 16.3 ºBrix), Legua et al. (2012) for the "Grenade Jaune" and "Bouâadime" Moroccan cultivars (15.2 to 17.6 ºBrix), and Zarei et al. (2010) for the "Shirin-e-Bihaste" and "Rabbab-e-Fars" Iranian cultivars (15.77 to 19.56 ºBrix).

3.2 Pomegranate seed oil identification

3.2.1 Total oil content

The oil contents, expressed in percentage of seed weight, are reported in Table 2. The highest lipid content (13.70%) was obtained for the Katirbasi cultivar, followed by CG8 (12.04%), three times higher than the Valenciana cultivar, with a mean of 4.44%. Our lipid range was lower than those referred by Pande and Akoh (2009) who obtained values between 18.1% and 21.5% for the R19 and North varieties, respectively, Elfalleh et al. (2011a), with 5.98% (Mezzi 2) to 21.58% (Rafrafi), Kýralan et al. (2009) of 13.95% (Eksilik) to 24.13% (Fellahyemez), and Fadavi et al. (2006) of 6.6% (Syah) to

19.3% (Syahdane Shahvar Kan). On the other hand, our range was identical to that reported by Melgarejo and Artes (2000) of 6.2% to 12.2% for the Piñón Tierno de Ojos - PTO4 and Piñonenca de Blanca - PB1 varieties, respectively. Moreover, our maximums were identical to those found by Jing et al. (2012) for the Suanshiliu and Sanbaitian varieties of 11.4 and 14.8%, respectively. When comparing our results obtained for the Valenciana and Mollar de Elche varieties with those reported by Hernandez et al. (2011) and Melgarejo and Artés (2000), lower results were obtained in the present work. In fact, Hernandez et al. (2011) reported 6.9% and 8.1% for the Valenciana (VA1) and Mollar de Elche (ME16) varieties, respectively, and Melgarejo and Artés (2000) of 9.0% and 10.1% for the Mollar de Elche ME3 and ME1 clones, respectively. These results may be due to the existence of variability between clones of the same cultivar or the edaphoclimatic conditions.

3.2.2 Fatty acid composition

The fatty acid composition of the seed oils extracted from the nine pomegranate cultivars are presented in Table 2, reported on a seed basis for a real perception of their potentialities. It consisted mainly on 9,11,13-octadeca-trienoic acid (C18:3(9,11.13)) (punicic acid), identified by comparison with published data on pomegranate seed oils (Eikani et al. 2012; Favadi et al. 2006; Kýralan et al. 2009; Pande & Akoh, 2009), followed in much smaller quantities by the *cis, cis*-9,12-octadecadienoic acid acid (C18:2 (9,12)), *cis*-9-octadecenoic acid acid (C18:1(9)) and hexadecanoic acid (C16:0). Other minor fatty acids were detected but only 10 were identified. An example of fatty acids chromatogram (White cultivar) is presented as Supplementary Material (Figure S1). In general terms, our results were similar to those reported by other authors who indicated punicic acid as the most abundant fatty acid in pomegranate seed oils.

Punicic acid ranged between 3523 and 10586 mg/100g of seed. In terms of percentage, it ranged from 77.3% to 83.6% of total fatty acids. These results are in accordance with previous reports, once Pande and Akoh (2009) obtained values for this fatty acid between 78.3 and 83.4% for pomegranate seed oils of "North" and "R19" cultivars; Hernández et al. (2011) between 66.7 and 79.2% for "Mollar de Elche 16" and "Borde de Albatera" cultivars; Jing et al. (2012) from 73.4 to 78.8% for "Tianhongdan" and "Jingpitian" cultivars; Kýralan et al. (2009) between 70.4 and 76.2% for Turkish cultivars; Favadi et al. (2006) from 31.8 and 84.5% for "Tabestani" and "Gorche Shahvar Yazdi" cultivars; and Eikani et al. (2012) between 69.8 and 81.7% for Siahdaneh Shirazi pomegranate seed oil after using different extraction methods, with the highest values obtained using the Soxhlet method, also adopted in the present study. On contrary, punicic acid contents in the nine Spanish pomegranate cultivars were higher than the "Mezzi2" and "Jebali3" cultivars studied by Elfalleh et al. (2011a) (12.4 - 55.4%, respectively) and to the values reported by Liu et al. (2009, 2012) that varied between 59.3 to 61.0% and 55.5 to 61.9% for pomegranate seeds obtained from Huiyuan Juice Company (Xinjiang, China), respectively, after applying different extraction conditions.

Our study showed that the Wonderful 2 cultivar presented the highest punicic acid content in the oil (83.6%); however, Katirbasi cultivar presented the highest concentration in the seeds, 10586 mg/100g. When evaluated by fruit, taking into account the fruit mean weight and seeds proportion, Katirbasi presented the most elevated punicic amounts per fruit, with an average of 1.5 g, followed by Wonderful 2 with 1.2 g. Punicic acid is a conjugated linolenic acid (CL*n*A), claimed to present anti-carcinogenic activity, including interference with tumor cell cycle and pharmacological invasion, as well as angiogenesis (Kohno et al., 2004; Lansky & Newman, 2007). In

311 addition, punicic acid is a known inhibitor of prostaglandin biosynthesis and it might
312 ultimately inhibit skin cancer promotion (Hora et al., 2003). Therefore, seed oils of the
313 Katirbasi cultivar could potentially serve as a dietary source for CL*n*A for reducing
314 risks of cancer, as well as be a source of compounds that improve skin condition with
315 potential as a topical chemopreventive agent against skin cancer.

316 When considering the overall fatty acids composition it was found that the pomegranate
317 seed oils followed the order: PUFA > SFA > MUFA, comparable to the reported by
318 Jing et al. (2012). Differences among cultivars were observed, with values of PUFA
319 ranging from 88.1 to 90.3% for Katirbasi and Cis 127 cultivars, respectively; SFA
320 between 6.1 and 7.4% for CG8 and Mollar de Elche cultivars; and MUFA from 3.9 to
321 6.3% for White and Wonderful 2 cultivars. In our study, the unsaturated fatty acids
322 (∑Unsat = MUFA+PUFA) accounted for 92.6 (White) to 95.2% (Cis 127) of total fatty
323 acids. Our range was smaller than the reported by Melgarejo and Artés (2000) of 73.35
324 (Mollar Orihuela-MO6) to 95.84% (Piñón Tierno of Ojós-PTO4). However, these
325 authors when studying a similar Mollar de Elche cultivar obtained slightly lower values
326 (90.16 and 91.37% for ME1 and ME3, respectively) than our (94.3%). Also, Hernández
327 et al. (2011) obtained lower values of ΣUnsat for the Mollar de Elche-ME16 variety
328 (80.41%) than our (94.3%), while similar results were obtained for the Valenciana –
329 VA1 cultivar (91.03%).

330 The ratio SFA/(PUFA+MUFA) ranged between 0.065 (CG8) and 0.079 (Mollar de
331 Elche), similar to those obtained by Hernández et al. (2011), of 0.057 for the
332 “Valenciana 1” cultivar and 0.077 for both “Mollar de Elche 16” and “Piñón Tierno de
333 Ojós 8” cultivars, as well as the minimum value reported by Elfalleh et al. (2011a) of
334 0.071 for the “Jebali3” variety but well below the maximum of 0.395 reported for the

335 "Sichuan2" cultivar. Melgarejo and Artés (2000) also reported a higher range for the
336 saturated/unsaturated ratio that varied between 0.04 (Mollar de Orihuela - MO16) and
337 0.35 (Piñón Tierno de Ojós - PTO4). Due to the high proportion of unsaturated fatty
338 acids, the pomegranate seed oils of the nine cultivars studied in the present work are
339 highly recommended for human consumption, having a fatty acid profile more favorable
340 than other vegetable oils.

341 ***3.2.3 Tocopherol and carotenoid composition***

342 The vitamin E composition of seed oils of the nine pomegranate cultivars studied in the
343 present work is described in Table 3. The nine cultivars differed in α-, γ- and δ- and
344 total tocopherol contents (Table 3). Chromatograms of standards and of one sample are
345 presented as Supplementary Material, Figure S2. γ-tocopherol was the most abundant,
346 followed by α-tocopherol and δ-tocopherol. Tocotrienols were not detected. Total
347 tocopherol contents in the oil ranged from 135.3 to 524.6 mg/100g for the White and
348 Wonderful 2 cultivars, respectively. γ-Tocopherol, clearly the main tocopherol in all the
349 samples analyzed, ranged from 123.0 to 449.7 mg/100 g of oil for the White and
350 Parfianka cultivars, respectively, comparable to those described by Liu et al. (2012) for
351 pomegranate seed oils extracted after applying different conditions in which the values
352 ranged between 120.6 and 672.6 mg/100 g oil. Nevertheless our results were different to
353 those described by Pande and Akoh (2009), who referred higher α-tocopherol values
354 (between 161.2 and 173.7 mg/100g) than ours (7.3 to 16.6 mg/100g), and Jing et al.
355 (2012) who reported the following order: δ-tocopherol > α-tocopherol > γ-tocopherol
356 for seed oils of Chinese pomegranates, as well as Elfalleh et al. (2011b) who indicated
357 the order: α-tocopherol > γ-tocopherol > δ-tocopherol for seed oils of Tunisian
358 pomegranates, or Caligiani et al. (2010), with higher amounts of β-tocopherol in all the

359 samples analyzed. This is clearly the chemical class with the highest variability between
360 cultivars and authors, opening the field for possible misidentification in some cases.

361 Nevertheless, it was found that the seed oils of the White cultivar contained
362 significantly lower γ- and δ-tocopherol amounts than the other cultivars, showing also
363 the lowest total and α- tocopherol contents.

364 Carotenoids were not detected in any oil samples, suggesting that pomegranate seeds
365 are a low-carotenoid fruit (chromatogram of a sample is presented as Supplementary
366 Material, Figure S3). It confirms the previous observation reported by Jing et al. (2012).

367 ***3.2.4 Sterol composition***

368 Even though more peaks were detected, only four were individually identified and
369 quantified on the basis of retention time comparison with authentic standard and
370 literature data, and the internal standard amount, corresponding tocampesterol,
371 stigmasterol, β-sitosterol and sitostanol. The others, corresponding to a minor fraction
372 of the formers, were quantified and included in the class called "Others". Table 4 shows
373 the sterol composition and total sterol contents of the pomegranate seed oils
374 (chromatogram of a sample is presented as Supplementary Material, Figure S4). In
375 relation to the sterol relative composition, all seed oils followed the order: β-sitosterol >
376 campesterol > sitostanol ≈ stigmasterol, which is consistent with the results published
377 by Habibnia et al. (2012) after analyzing five Iranian seed oils or Caligiani et al. (2010)
378 when studying seed oils of the Wonderful and Dente di cavallo varieties. On contrary
379 our results were slightly different to those described by Pande and Akoh (2009) who
380 found the following order: β-sitosterol > stigmasterol > campesterol > brassicasterol.
381 However, the last compound was not detected in Crab and Cranberry cultivars

382 Regarding total sterol contents, Mollar de Elche cultivar had the highest concentration
383 (552.7 mg/100 g seed oil) whereas the Parfianka cultivar had the lowest content (363.6
384 mg/100 g). Habibnia et al. (2012) also determined total sterol contents between 523.9
385 and 575.8 mg/100 g for seed oils of "Red Seed Ardestani" and "Rizdavar's Dorpaye"
386 cultivars, respectively. Concerning sterol composition, Pande and Akoh (2009) obtained
387 similar β-sitosterol contents, between 243.5 (North) and 345.8 mg/100g (Crab).
388 Nevertheless, those authors found higher stigmasterol contents (27.8 mg/100g for CVG
389 Eve cultivar to 46.3 mg/100g for Crab cultivar) and lower campesterol concentrations,
390 between 17.9 and 39.3 mg/100g for the R26 and North cultivars, respectively, than our
391 values. Caligiani et al. (2010) when studying seed oils of pomegranates from different
392 sources, including one commercial seed oil and oil extracted from the seeds of two
393 varieties, namely Wonderful and Dente di cavallo , found that campesterol ranged from
394 107.3 (commercial) and 57.9 (Dente di cavallo) mg/100g, stigmasterol varied between
395 18.2 (Wonderful) and 30.4 (commercial) mg/100g, and β-sitosterol between 414.0
396 (Wonderful) and 806.9 (commercial) mg/100g; some of the results are identical to ours.

397 When quantified per fruit, in the interest of possible seed reuse, Katirbasi had the
398 highest amount of sterols (8.1 mg), while White and Wonderful1 had the lowest (3.8
399 mg).

400 Phytosterols are plant sterols with biologic functions similar to those of mammalian
401 cholesterol; however, due to small differences in their chemical structure, they are much
402 less absorbed (2–5%) than cholesterol (56%) (Martins et al., 2013). Moreover, they also
403 inhibit the absorption of intestinal cholesterol including recirculating endogenous biliary
404 cholesterol, a key step in cholesterol elimination (Ostlund, 2002). So, phytosterols have

been proposed as lipid-lowering agents (Martins et al., 2013), that can reduce the risk of certain types of cancer as well (Lagarda et al, 2006; Moreau et al., 2002).

3.2.5 Principal component analysis

Principal component analysis (PCA) was applied to observe any possible clusters within the analyzed pomegranate samples. The scores of the first two principal components for the nine pomegranate cultivars are presented in Figure 2. The first two principal components took into account 93.5% (PC1 = 55.0% and PC2 = 38.5%, respectively) of the total variation. PC1 was highly contributed by γ-tocopherol, δ-tocopherol, α-tocopherol and MUFA (%). PC2 was mainly correlated positively to SFA (%), PUFA (%) and sterols. Two cultivars could be separated from the others, namely the Mollar de Elche and White. In the PC2, the Mollar de Elche cultivar had positive scores due to its high percentage of SFA, PUFA and sterols, two important health attributes. White cultivar had negative scores on PC1 because it has the lowest percentage of MUFA and α-tocopherol, γ-tocopherol, δ- tocopherol contents. Concerning these results, selection of pomegranate seed oils with specific quality characteristics for industry can be carried out, valorizing this by-product. Moreover, this statistical method may be used in pomegranate cultivars identification, bringing economic advantages because it might be used as a tool to detect falsifications in pomegranate cultivars sold in the market.

4 Conclusions

The present study demonstrated that the nine pomegranate cultivars grown in Spain presented different physicochemical characteristics that may be important in distinguishing pomegranate cultivars with respect to future and potential use. Some cultivars showed characteristics more in line with consumer's preference, such as size, percentage of edible part and total soluble solids content, in particular Mollar de Elche.

On the other hand, all cultivars have seed oils high in PUFA, mainly punicic acid that has interesting health properties. Katirbasi cultivar presented the highest content of punicic acid per seed weight fruit. Wonderful 2 had the highest total tocopherols content that, together with a high ratio of seeds in the arils and low total soluble solids content, make it interesting for seed by-products exploration as well. Concerning sterols, the seed oils of Mollar de Elche presented the highest content of these compounds. In summary, this study might provide valuable information for pomegranate cultivar selection and for developing value-added pomegranate seed oils based products, such as nutraceuticals or functional food ingredients.

Supplementary Material

Table S1; Figures S1–S4.

Acknowledgements

Authors are grateful to POCTEP - Programa de Cooperação Transfronteiriça Espanha - Portugal for financial support (Project "RED/AGROTEC – Experimentation network and transfer for development of agricultural and agro industrial sectors between Spain and Portugal) as well as to the European Union (FEDER funds through COMPETE) and National Funds (FCT, Fundação para a Ciência e Tecnologia) through project Pest-C/EQB/LA0006/2013.

References

Ahangari, B., Sargolzaei, J. (2012). Extraction of pomegranate seed oil using subcritical propane and supercritical carbon dioxide. *Theoretical Foundations of Chemical Engineering*, *46*, 258-265.

Al-Maiman, S. A., Ahmad, D. (2002). Changes in physical and chemical properties during pomegranate (*Punica granatum* L.) fruit maturation. *Food Chemistry*, *76*, 437-441.

Andreu Sevilla, A. J., Signes-Pastor, A. J., Carbonell-Barrachina, A. A. (2009). La granada: Producción, Composición y Propiedades Beneficiosas para la Salud. *Informe de la Universidad Miguel Hernández*, 1-4.

Caligiani, A., Bonzanini, F., Palla, G., Cirlini, M., Bruni, R. (2010). Characterization of a Potential Nutraceutical Ingredient: Pomegranate (*Punica granatum* L.) Seed Oil Unsaponifiable Fraction. *Plant Foods for Human Nutrition*, *65*, 277–283.

Casal, S., Macedo, B., Oliveira, M.B.P.P. (2001). Simultaneous determination of retinol, β-carotene and α-tocopherol in adipose tissue by high-performance liquid chromatography. *Journal of Chromatography B, 763*, 1–8.

Casal, S., Malheiro, R., Sendas, A., Oliveira, B. P. P., Pereira, J. A. (2010). Olive oil stability under deep-frying conditions. *Food and Chemical Toxicology, 48*, 2972–2979.

Craft, N. E., Wise, S. A., Soares, J.H. (1992). Optimization of an isocratic high-performance liquid chromatographic separation of carotenoids. *Journal of Chromatography A, 589*,1–2.

Codex Alimentarius Commission (2013). *Report of the 17th Session of the Codex Committee on fresh fruits and vegetables* (pp. 29). Mexico City, Mexico: Codex Alimentarius Commission.

472 Cunha, S. S., Fernandes, J. O., Oliveira, M. B. P. P. (2006). Quantification of free and
473 esterified sterols in Portuguese olive oils by solid-phase extraction and gas
474 chromatography–mass spectrometry. *Journal of Chromatography A, 1128*, 220–
475 227.

476 Eikani, M. H., Golmohammad, F., Homami, S. S. (2012). Extraction of pomegranate
477 (*Punica granatum* L.*)* seed oil using superheated hexane. *Food and Bioproducts*
478 *Processing*, *90*, 32-36.

479 Elfalleh, W., Ying, M., Nasri, N., Sheng-Hua, H., Guasmi, F., Ferchichi, A. (2011a).
480 Fatty acids from Tunisian and Chinese pomegranate (*Punica granatum L*.) seeds.
481 *International Journal of Food Sciences and Nutrition*, *62*, 200-206.

482 Elfalleh, W., Tlili, N., Nasri, N., Yahia, Y., Hannachi, H., Chaira, N., Ying, M.,
483 Ferchichi, A. (2011b). Antioxidant Capacities of Phenolic Compounds and
484 Tocopherols from Tunisian Pomegranate (*Punica granatum*) Fruits. *Journal of*
485 *Food Science*, *76*, 707-713.

486 Fadavi, A., Barzegar, M., Azizi, M. H. (2006). Determination of fatty acids and total
487 lipid content in oilseed of 25 pomegranates varieties grown in Iran. *Journal of*
488 *Food Composition and Analysis*, *19*, 676–680.

489 Glozer, K., Ferguson, L. (2011). *Pomegranate Production in Afghanistan*. Davis, USA:
490 Mark Bell.

491 Goula, A. M., Adamopoulos, K. G. (2012). A method for pomegranate seed application
492 in food industries: seed oil encapsulation. *Food and Bioproducts Processing, 90,*
493 639-652.

494 Gözlekçi, Ş., Saraçoğlu, O., Onursal, E., Özgen M. (2011). Total phenolic distribution
495 of juice, peel and seed extracts of four pomegranate cultivars. *Pharmacognosy*
496 *Magazine*, *7*, 161-164.

497 Guo, S., Deng, Q., Xiao, J., Xie, B., Sun, Z. (2007). Evaluation of antioxidant activity
498 and preventing DNA damage effect of pomegranate extracts by
499 chemiluminescence method. *Journal of Agricultural and Food Chemistry*, *55*,
500 3134-3140.

501 Habibnia, M., Ghavami, M., Ansaripour, M., Vosough, S. (2012). Chemical evaluation
502 of oils extracted from five different varieties of Iranian Pomegranate seeds.
503 *Journal of Food Biosciences and Technology*, *2*, 35-40.

504 Hernández, F., Melgarejo, P., Martínez, J. J., Martínez, R., Legua, P. (2011). Fatty acid
505 composition of seed oils from important Spanish pomegranate cultivars. *Journal*
506 *of Food Biosciences and Technology*, *2*, 35-40.

507 Hora, J. J., Maydew, E. R., Lansky, E. P., Dwivedi, C. (2003). Chemopreventive Effects
508 of Pomegranate Seed Oil on Skin Tumor Development in CD_1 Mice. *Journal of*
509 *Medicinal Food*, *6*, 157-161.

510 Ismail, T., Sestili, P., Akhtar, S. (2012). Pomegranate peel and fruit extracts: A review
511 of potential anti-inflammatory and anti-infective effects. *Journal of*
512 *Ethnopharmacology*, *143*, 397-405.

513 ISO 9936 (2006). Animal and vegetable fats and oils—determination of tocopherol and
514 tocotrienol contents by high-performance liquid chromatography.

515 ISO 12966-2 (2011). Animal and vegetable fats and oils -- Gas chromatography of fatty
516 acid methyl esters -- Part 2: Preparation of methyl esters of fatty acids.

517 ISO 12228 1999). Animal and vegetable fats and oils -- Determination of individual and
518 total sterols contents -- Gas chromatographic method.

519 Jing, P., Ye, T., Shi, H., Sheng, Y., Slavin, M., Gao, B., Liu, L., Yu, L. (2012).
520 Antioxidant properties and phytochemical composition of China-grown
521 pomegranate seeds. *Food Chemistry*, *132*, 1457-1464.

522 Kohno, H., Suzuki, R., Yasui, Y., Hosokawa, M., Miyashita K., Tanaka, T. (2004).
523 Pomegranate seed oil rich in conjugated linolenic acid suppresses chemically
524 induced colon carcinogenesis in rats. *Cancer Science*, *95*, 481–486.

525 Kýralan, M., Golukcu. M., Tokgoz, H. (2009). Oil and Conjugated Linolenic Acid
526 Contents of Seeds from Important Pomegranate Cultivars (*Punica granatum* L.)
527 Grown in Turkey. *Journal of the American Oil Chemists' Society*, *86*,985–990

528 Lagarda, M. J., García-Llatas, G., Farré, R. (2006). Analysis of phytosterols in foods.
529 *Journal of Pharmaceutical and Biomedical Analysis*, *41*, 1486–1496.

530 Lansky, E. P., Newman, R. A. (2007). *Punica granatum* (pomegranate) and its potential
531 for prevention and treatment of inflammation and cancer. *Journal of*
532 *Ethnopharmacology*, *109*, 177–206.

533 Legua, P., Melgarejo, P., Abdelmajid, H., Martínez, J.J., Martínez, R., Ilham, H.,
534 Hafida, H., Hernández, F. (2012). Total phenols and antioxidant capacity in 10
535 Moroccan pomegranate varieties. *Journal of Food Science*, *71*, 115-120.

536 Liu, G., Xu, X., Gong, Y., He, L., Gao, Y. (2012). Effects of supercritical CO_2
537 extraction parameters on chemical composition and free radical-scavenging
538 activity of pomegranate (*Punica granatum* L.) seed oil. *Food and Bioproducts*
539 *Processing*, *90*, 573-578.

540 Liu, G., Xu, X., Hao, Q., Gao, Y. (2009). Supercritical CO_2 extraction optimization of
541 pomegranate (*Punica granatum.* L.) seed oil using response surface methodology.
542 *LWT - Food Science and Technology*, *42*, 1491-1495.

543 Luana, F., Casal, S., Rebeca, C., Pereira, J. A., Ramalhosa, E. (2013). Seed oils of ten
544 traditional Portuguese grape varieties with interesting chemical and antioxidant
545 properties. *Food Research International, 50,* 161–166.

546 Malheiro, R., Casal, S., Teixeira, H., Bento, A., Pereira, J.A. (2013). Effect of olive
547 leaves addition during the extraction process of overmature fruits on olive oil
548 quality. *Food and Bioprocess Technology*, *6*, 509–521.

549 Martínez, J. J., Melgarejo, P., Hernández, F., Salazar, D. M., Martínez, R. (2006). Seed
550 characterization of five new pomegranate (*Punica granatum* L.) varieties.
551 *Scientia Horticulturae, 110*, 241-246.

552 Martins, C. M., Fonseca, F. A., Ballus, C. A., Figueiredo-Neto, A. M., Meinhart, A. D.,
553 Godoy, H. T., Izar, M. C. (2013). Common sources and composition of
554 phytosterols and their estimated intake by the population in the city of São Paulo,
555 Brazil. *Nutrition*, *29*, 865–871.

556 Melgarejo, P., & Artés, F. (2000). Total lipid content and fatty acid composition of
557 oilseed from lesser known sweet pomegranate clones. *Journal of the Science of*
558 *Food and Agriculture*, *80*, 1452-1454.

559 Melgarejo, P., Calín-Sánchez, Á., Vázquez-Araújo, L., Hernández, F., Martínez, J. J.,
560 Legua, P., Carbonell-Barrachina, A. A. (2011). Volatile Composition of
561 Pomegranates from 9 Spanish Cultivars using Headspace Solid Phase
562 Microextraction. *Journal of Food Science, 76*, 114-120.

563 Mena, P., García-Viguera, C., Navarro-Rico, J., Moreno, D. A., Bartual, J., Saurab, D.,
564 Mar, M. (2011). Phytochemical characterisation for industrial use of pomegranate
565 (*Punica granatum* L.) cultivars grown in Spain. *Journal of the Science of Food*
566 *and Agriculture, 91*, 1893–1906.

567 Moreau, R. A., Whitaker, B. D., Hicks, K. B. (2002). Phytosterols, phytostanols, and
568 their conjugates in foods: structural diversity, quantitative analysis, and health-
569 promoting uses. *Progress in Lipid Research, 41*, 457–500.

570 Nesaretnam, K., Yew, W. W., & Wahid, M. B. (2007). Tocotrienols and cancer:
571 Beyond antioxidant activity. *European Journal of Lipid Science and Technology,*
572 *109*, 445–452.

573 Ostlund, R. JR. (2002). Phytosterols in human nutrition. *Annual Review of Nutrition, 22*,
574 533-49.

575 Pande, G., Akoh, C. C. (2009). Antioxidant capacity and lipid characterization of six
576 Georgia-grown pomegranate cultivars. *Journal of Agricultural and Food*
577 *Chemistry*, *57*, 9427-9436.

578 Panfili, G., Fratianni, A., and Irano, M. (2004) Improved Normal-Phase High-
579 Performance Liquid Chromatography Procedure for the Determination of
580 Carotenoids in Cereals. *Journal of Agricultural and Food Chemistry*, 52, 6373-
581 6377.

582 Tehranifar, A., Zarei, M., Nemati, Z., Esfandiyari, B., Vazifeshenas M. R. (2010).
583 Investigation of physico-chemical properties and antioxidant activity of twenty
584 Iranian pomegranate (*Punica granatum* L.) cultivars. *Scientia Horticulturae, 126*,
585 180-185.

586 Tian, Y., Xu, Z., Zheng, B., Lo, Y. M. (2013). Optimization of ultrasonic-assisted
587 extraction of pomegranate (*Punica granatum* L.) seed oil. *Ultrasonics*
588 *Sonochemistry*, *20*, 202-208.

589 Yamasaki, M., Kitagawa, T., Koyanagi, N., Chujo, H., Maeda, H., Kohno-Murase, J.,
590 Imamura, J., Tachibana, H., Yamada, K., (2006). Dietary effect of pomegranate
591 seed oil on immune function and lipid metabolism in mice. *Nutrition*, *22*, 54–59.

592 Zaouay, F., Mena, P., Garcia-Viguera, C., Mars, M. (2012). Antioxidant activity and
593 physico-chemical properties of Tunisian grown pomegranate (*Punica granatum*
594 L.) cultivars. *Industrial Crops and Products*, *40*, 81-89.

595 Zarei, M., Azizi, M., Bashiri-Sadr, Z. (2010). Studies on physico-chemical properties
596 and bioactive compounds of six pomegranate cultivars grown in Iran. *Journal of*
597 *Food Technology*, *8*, 112-117.

598

599 **Figure captions**

600 **Fig. 1. The nine pomegranate cultivars studied in the present work.**

601 **Fig. 2. Principal component analysis plot of data from fatty acid, tocopherols and sterol**
602 **contents of nine pomegranate cultivars.**

603