Escuela Politécnica Superior de Orihuela
Departamento de Tecnología Agroalimentaria

EFFECT OF CULTIVAR, MATURATION AND PROCESSING ON THE CHEMICAL, FUNCTIONAL AND SENSORY PROPERTIES OF POMEGRANATE FRUIT AND JUICE

Paloma Nallely Nuncio Jáuregui

Tesis Doctoral 2014

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UNIVERSIDAD MIGUEL HERNÁNDEZ DE ELCHE

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TESIS DOCTORAL

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UNIVERSITAS Miguel Hernández

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Effect of cultivar, maturation and processing on the chemical, functional and sensory properties of pomegranate fruit and juice

Tesis doctoral realizada por Paloma Nallely Nuncio Jáuregui, Ingeniera en Alimentos, en la Facultad de Ciencias Químicas de la Universidad Autónoma de San Luis Potosí y Máster Universitario en Investigación en Ciencia, Tecnología y Control de los Alimentos, en el Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández de Elche, para la obtención del grado de Doctor.

Fdo.: **Paloma Nallely Nuncio Jáuregui**

Orihuela,_____de _____de 2014

Dr. José Ramón Díaz Sánchez, Dr. Ingeniero Agrónomo, Catedrático de Escuela Universitaria y Director del Departamento de Tecnología Agroalimentaria de la Universidad Miguel Hernández de Elche,

CERTIFICA:

Que la Tesis Doctoral titulada "**Effect of cultivar, maturation and processing on the chemical, functional and sensory properties of pomegranate fruit and juice**" de la que es autora la Ingeniera en Alimentos y M. Sc. en Investigación en Ciencia, Tecnología y Control de los Alimentos **Paloma Nallely Nuncio Jáuregui** ha sido realizada bajo la dirección del **Dr. Ángel A. Carbonell Barrachina**, Catedrático de Universidad del Departamento de Tecnología Agroalimentaria y la **Dra. Francisca Hernández García**, Titular de Universidad del Departamento de Producción Vegetal, ambos de la Universidad Miguel Hernández de Elche; la considero conforme en cuanto a forma y contenido para que sea presentada para su correspondiente exposición pública.

Y para que conste a los efectos oportunos firmo el presente certificado en Orihuela a 02 de octubre de dos mil catorce.

Fdo.: Dr. José Ramón Díaz Sánchez

Dr. D. Ángel A. Carbonell Barrachina, Catedrático de Universidad del Departamento de Tecnología Agroalimentaria y la **Dra. Dña. Francisca Hernández García**, Titular de Universidad del Departamento de Producción Vegetal, ambos de la Universidad Miguel Hernández de Elche.

CERTIFICAN:

Que la Tesis Doctoral titulada "**Effect of cultivar, maturation and processing on the chemical, functional and sensory properties of pomegranate fruit and juice**" de la que es autora la Ingeniera en Alimentos y M. Sc. en Investigación en Ciencia, Tecnología y Control de los Alimentos **Paloma Nallely Nuncio Jáuregui** ha sido realizada bajo nuestra dirección y autorizamos que sea presentada para optar a la obtención del grado de Doctor por la Universidad Miguel Hernández de Elche.

Y para que conste a los efectos oportunos firmamos el presente certificado en Orihuela a 30 de septiembre de dos mil catorce.

Fdo.: Dr. Ángel A. Carbonell Barrachina   Fdo.: Dra. Francisca Hernández García

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Muchas gracias!!
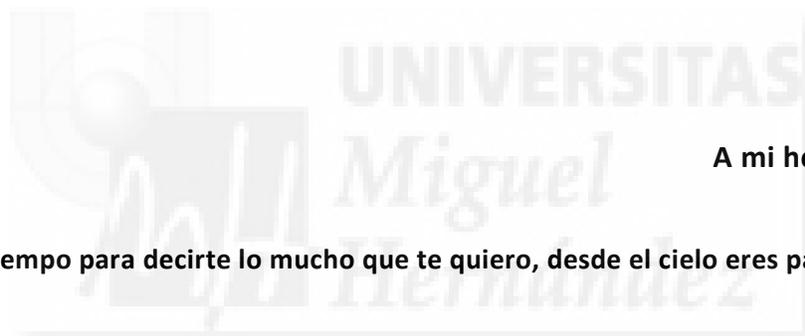
A mi hermano Daniel

Me falto tiempo para decirte lo mucho que te quiero, desde el cielo eres parte de mi vida.

INDICE

ESTRUCTURA DE LA TESIS

Para la elaboración de la presente Tesis Doctoral se ha seguido una metodología basada en la publicación de artículos de investigación y un capítulo de un libro de editorial Elsevier Inc. Con esta Tesis Doctoral se pretende obtener el título de Doctor con mención Europea, para ello en la redacción de la misma, se ha seguido la normativa vigente de la Universidad Miguel Hernández de Elche, concretamente el artículo 1.2 donde se indica: "*Que parte de la Tesis Doctoral, al menos el resumen y las conclusiones, se hayan presentado en una de las lenguas oficiales de la Unión Europea, distinta a alguna de las lenguas oficiales en España*". En este caso el idioma seleccionado es el inglés; y este idioma será empleado tanto para la redacción como para la exposición de parte de la Tesis.

La estructura de esta Tesis Doctoral consta de una breve **Introducción** en la que se incluye una revisión bibliográfica sobre los parámetros físico-químicos y de calidad, compuestos fenólicos y actividad antioxidante de la granada, así como una revisión sobre los cambios que presenta según sea el cultivar, las técnicas de cultivo y, especialmente los cambios que presenta durante las diferentes etapas de maduración y procesamiento industrial. También se incluye una breve revisión de la evaluación sensorial enfocada a la granada, ya sea para consumo en fresco o para su procesamiento industrial. Para finalizar este capítulo introductorio, se describen los cambios que se producen cuando la granada es sometida a un proceso industrial, específicamente, elaboración de zumo y los cambios en los parámetros de calidad cuándo este tipo de producto es adulterado con otros zumos en diferentes proporciones. En los dos capítulos posteriores se describen los **Objetivos** planteados y los **Materiales y Métodos** empleados para poder entender el diseño y preparación de las muestras, así como el análisis sensorial y las determinaciones físico-químicas realizadas. A continuación se recogen las **Publicaciones Científicas** publicadas y/o aceptadas para publicación que componen el núcleo de la presente Tesis Doctoral:

- La **primera publicación** recoge los resultados obtenidos al analizar los frutos de granada que provienen del aclareo. Este artículo está aceptado para publicación en la revista *Journal of Food Composition and Analysis y* en el mismo se estudia el potencial que tienen los frutos provenientes del aclareo como fuente de compuestos bioactivos. Así mismo se determinan parámetros de calidad, ácidos orgánicos, azúcares, y minerales.

- La **segunda publicación** recoge los resultados obtenidos al analizar las propiedades de calidad, color, composición físico-química, compuestos fenólicos y actividad antioxidante de los frutos de granada en tres etapas de maduración. Este artículo se publicó en la revista *Scientia Horticulturae*. Así mismo, en este artículo se estudia el efecto que puede tener la posición del fruto dentro del árbol (sol/ sombra) sobre los parámetros anteriormente mencionados.

- La **tercera publicación** hace un estudio sobre las principales diferencias sensoriales entre los frutos de 20 cultivares de granada; identificándose las variedades que son óptimas para consumir en fresco y/o para ser procesadas industrialmente. Este artículo está publicado en la revista *Food Science and Technology*.

- La **cuarta publicación** determina los ácidos orgánicos, azúcares, minerales, prolina y compuestos volátiles de un zumo comercial de granada (puro) y dos zumos potenciales para la adulteración (uva y melocotón). Este artículo está publicado en la revista *Journal of the Science of Food and Agriculture*. En este artículo se evalúan los cambios que se observan después de adulterar el zumo de granada con diferentes proporciones de zumo de uva o melocotón para establecer parámetros simples pero prácticos que puedan comprobar la autenticidad o la adulteración del zumo de granada.

- La **quinta publicación** es un capítulo del libro *Processing and Impact on Active Components in Food* de la editorial Elsevier Inc, que resume la composición del zumo de granada y el impacto que tiene el procesamiento industrial sobre los compuestos bioactivos.

El quinto capítulo recoge una **Publicación Científica en revisión:**

- La **sexta publicación** hace una comparación entre las granadas provenientes del aclareo (inmaduras) y granadas maduras. Este artículo se encuentra bajo revisión en la revista *Journal of Functional Food* y en él mismo se estudia el contenido de compuestos fenólicos y la actividad antioxidante de estos dos tipos de granada.

El siguiente capítulo corresponde con **Resultados y Discusión**, aquí se presenta un resumen global de los resultados más relevantes obtenidos en los diferentes estudios realizados y se hace una discusión general de los mismos. Finalmente, en el capítulo séptimo se recogen las **Conclusiones** generales de todos los estudios que forman parte de la presente Tesis Doctoral, mientras que en el octavo y último capítulo corresponde a la **Referencias Bibliográficas** y consultadas empleadas para la elaboración de esta memoria, sin considerar la sección de Publicaciones Científicas.

RESUMEN

En años recientes, la granada (*Punica granatum* L.) ha adquirido una amplia aceptación debido a la creciente evidencia de que su consumo está asociado con propiedades beneficiosas para la salud. Durante la maduración y procesamiento del fruto se producen cambios significativos en las propiedades fisicoquímicas, compuestos fenólicos y actividad antioxidante. Estos cambios están influenciados por el cultivar, región de cultivo, técnicas de cultivo y etapas de maduración del fruto en la cosecha.

Por lo tanto, el objetivo principal de esta tesis doctoral es evaluar la evolución en las propiedades químicas, funcionales y sensoriales de la granada durante el cultivo, estado de madurez y procesamiento industrial. Los objetivos específicos son: (i) evaluar el potencial de los frutos de granada que son retirados durante el aclareo como una fuente de compuestos bioactivos y actividad antioxidante, (ii) determinar el efecto de la posición de los frutos en el árbol en la calidad principal y parámetros físico-químicos, (iii) utilizar el análisis sensorial descriptivo para determinar la mejor opción comercial para las frutas de granada, ya sea como consumo en fresco o en la fabricación de zumo, (iv) determinar el efecto del procesamiento industrial del zumo de granada y la adulteración sobre las características físico-químicas y compuestos bioactivos.

Para el análisis se utilizaron frutos de granada, los cuales fueron recogidos durante el aclareo y a tres diferentes estados de madurez. El aclareo es una práctica agrícola que tiene lugar en una etapa inmadura de los frutos en la que se eliminan parte de los frutos para beneficiar el desarrollo y la calidad de los frutos restantes en el árbol. Así mismo, se usó zumo comercial de granada para el análisis; para simular la adulteración, el zumo comercial de granada se mezcló con zumo de uva o zumo de melocotón a diferentes concentraciones. Los parámetros de calidad en estudio incluyen, ácidos orgánicos, azúcares, prolina, minerales, compuestos fenólicos totales, punicalaginas, ácido elágico, actividad antioxidante y compuestos volátiles, así como la acidez titulable, sólidos solubles totales, índice de madurez, pH y color. En la granada que proviene del aclareo, el ácido cítrico y quínico fueron los principales ácidos orgánicos y, la glucosa y fructosa los principales azúcares. El potasio es el mineral predominante; el contenido de prolina en la primera etapa de maduración varió desde 32,2 hasta 52,1 mg L^{-1}. El contenido de polifenoles totales varió desde 190 hasta 288 g GAE kg^{-1} peso seco. La actividad antioxidante se evaluó mediante cuatro métodos,

DPPH, ABTS, FRAP y ORAC. Los valores de la actividad antioxidante en los frutos que provienen del aclareo fueron entre 2 - 6 veces más alto que en las granadas maduras. Los valores de la actividad antioxidante, acidez titulable, el contenido total de ácidos orgánicos y polifenoles totales disminuyeron con la maduración o con el procesamiento industrial. Los sólidos solubles totales, índice de madurez, contenido total de azúcares y prolina, aumentaron significativamente en los frutos de granada. La posición en el árbol sólo tuvo efecto significativo ($p < 0,05$) en las coordenadas de color externo.

Un total de 35 derivados principales del ácido elágico fueron identificados por LC-PDA-QTOF/ MS y cuantificado por el método UPLC-PDA, sin embargo, sólo 7 de ellos fueron encontrados tanto en las granadas de aclareo como en las maduras. El contenido de estos compuestos fue mayor en los frutos que provienen de aclareo que en los frutos maduros. Después de la evaluación sensorial, los resultados mostraron que el cultivar *Wonderful* fue el cultivar más apreciado por ser ácido y con notas saladas y similares al vino. Por otro lado, la mayoría de los cultivares de *Mollar* y *Valencia* resultaron ser altamente apreciados en España y se caracterizan por ser dulces presentando notas a remolacha, sabor afrutado, fermentado, y mohoso/ terroso.

En el zumo comercial de granada mezclado con zumo de uva (10, 25 y 50 %), aumentó el contenido de Ca, Mg y Fe, compuestos volátiles como el ácido acético, butirato de isoamilo, 1-hexanol y linalol y, especialmente aumentó el ácido tartárico y prolina; disminuyendo simultáneamente el contenido de K. Del mismo modo, la adición de zumo de melocotón sólo hasta el 10 % resultó en un aumento significativo ($p < 0,001$) del contenido de sacarosa y compuesto volátiles como acetato de butilo, butirato de isobutilo, acetato de bencilo y butirato de isoamilo.

La presente Tesis Doctoral muestra que los frutos de granada provenientes del aclareo (especialmente cultivares agridulce), son ricos en compuestos bioactivos, y por lo tanto, tienen un importante uso potencial en la industria alimentaria, química y farmacéutica. También describe los perfiles sensoriales de los cultivares de granada para determinar la mejor opción comercial para los frutos, ya sea para el consumo en fresco o la elaboración de zumo. Y por último, evalúa los cambios que se presentan después de adulterar el zumo de granada con diferentes concentraciones de zumos de uva o melocotón, para indicar los parámetros simples pero prácticos que comprueben la autenticidad o la adulteración de un zumo de granada.

ABSTRACT

In recent years, the pomegranate (*Punica granatum* L.) has acquired wide acceptance due to the growing evidence that consumption is associated with beneficial health properties. During fruit ripening and manufacturing there are significant changes in the physicochemical, phenolic compositions and antioxidant activity. These changes are influenced by cultivar, growing region, cultivation techniques and ripening stage of the fruit at harvest.

Thus, the main objective of this PhD Thesis is to evaluate the evolución in chemical, functional and sensory properties of pomegranate during cultivation, maturity stage and industrial processing. The specific objectives are: (i) evaluate the potential of pomegranate fruits removed during thinning as a source of bioactive compounds and antioxidant activity, (ii) determine the effect of the position of the fruits within the tree in the main quality and physicochemical parameters, (iii) use descriptive sensory analysis to determine the best commercial option for pomegranate fruits, either fresh consumption or juice manufacture, (iv) determine the effect of industrial processing and pomegranate juice adulteration on physico-chemical characteristics and bioactive compounds.

Pomegranates fruits were used for the analysis and were collected from the thinning and during three different maturity stages. Thinning is an agricultural practice which takes place at an immature stage of the fruits at which parts of the fruits are removed to benefit the development and quality of the remaining fruits on the tree. Likewise commercial pomegranate juice was used for the analysis; to simulate adulteration, commercial pure pomegranate juice was mixed with grape juice or peach juice at different concentrations. The quality parameters under study included, organic acids, sugars, proline, minerals, total phenolic compounds, punicalagins, ellagic acid, antioxidant activity and volatile compounds, as well as titratable acidity, total soluble solids, maturity index, pH and color.

In pomegranate that coming from thinning, citric and quinic acid were the main organic acids and glucose and fructose the main sugars. Potassium was the predominant mineral; the proline content in the first ripening stage ranged from 32.2 to 52.1 mg L^{-1}. Total polyphenol content ranged from 190 to 288 g GAE kg^{-1} dw. The antioxidant activity was assessed by four methods, DPPH, ABTS, FRAP and ORAC. The antioxidant activity values of thinning fruits were between 2 - 6 times higher than ripe

pomegranate fruits. The antioxidant activity values, titratable acidity, total organic acid and total polyphenols, decrease with ripening progresses or industrial processing, as well as. The total soluble solids, maturity index, total sugars content and proline, increased significantly in pomegranate fruits. The position within the tree only had significant ($p<0.05$) on external color coordinates.

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 in thinning and ripe fruits. The content of these compounds was higher in fruits that coming from thinning than in ripe fruits. After sensory evaluations, the results show that *Wonderful* cultivar was the most appreciated cultivar in by being sour and having salty and wine-like notes. On the other hand, most of M*ollar* and V*alencia* cultivars highly appreciated cultivars in Spain were characterized by being sweet and having beet, fruity-dark, fermented, and musty/earthy flavor notes.

In commercial pure pomegranate juice, mixed with grape juice (10, 25 and 50 %), increased the content of Ca, Mg and Fe, volatile compounds like acetic acid, isoamyl butyrate and 1-hexanol and linalool and especially increases of tartaric acid and proline, decreased simultaneously, the content of K. Likewise, Addition of peach juice up to 10 % only resulted in a significant ($p<0.001$) increase of the sucrose content and volatile compounds like butyl acetate, isobutyl butyrate, benzyl acetate and especially isoamyl butyrate.

This PhD Thesis shows that pomegranate thinning fruits (especially sour-sweet cultivars), are rich in bioactive compounds, and thus, have an important potential use in food, chemical and pharmaceutical industries. Also, described the sensory profiles of pomegranate cultivars to determine the best commercial option for fruits, either fresh consumption or juice manufacture. And finally, evaluated the changes observed after adulterating pomegranate juice with different concentrations of grape or peach juices, to state simple but practical parameters to check the authenticity or adulteration of pomegranate juice.

Chapter 1. Introduction

1. INTRODUCTION

1.1. Pomegranate fruit origin, description and morphology

1.1.1. Origin

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruits. It is an interesting and promising species for different world areas, because it adapts quite well to arid and semi-arid soils and dry weather conditions (Melgarejo and Salazar, 2003). It is considered native to India and Iran but nowadays is grown in many different geo-graphical regions, satisfying the nutritional and medicinal needs of various countries (Holland *et al.*, 2009). Among those countries are India, Iran, Afghanistan, U.S.A. and Mediterranean countries (Tunisia, Turkey, Egypt, Spain and Morocco). Spain is the main European pomegranate producer and its production is mainly located in the provinces of Alicante and Murcia (Melgarejo and Salazar, 2003). In recent years pomegranate has gained popularity due to its multi-functionality and nutritional value in human diet.

1.1.2. Plant description and morphology

Pomegranate tree is a tropical and sub-tropical fruit tree that belongs to *Punicaceae* family. The gene name is *Punica* L. The two best known species are: *Punica granatum* L. (edible fruits) and *Punica nana* L. (ornamental use and inedible fruits) (Melgarejo *et al.*, 2010).

The pomegranate, like any fruit tree, is a morphological and functional unit. Its external development is the answer to internal physiological conditions and environmental conditions. A fully grown tree is between 6 and 10 m tall, much-branched, more or less spiny, and extremely long-lived (Morton, 1987). Leaves are bright and the bark cracks and takes a grayish color. The flowers are large, bright, of red color, with 5-8 petals; flowering begins from May to November (Bartual Martos, 2011). Fruit development starts after flowering of the ovary, with flowering and fruit set lasting about one month (Holland *et al.*, 2009) (**Figure 1**).

Figure 1. Pomegranate tree and flower.

Pomegranate ripe fruits can be up 12 cm wide with a leathery rind and surmounted by a pointed calyx; maturing between 5-7 months from flowering (Jurenka, 2008). Require high temperatures in development and maturation and it is considered as a non-climacteric fruit because once is harvested, it does not continue maturing (even with ethylene treatment) (Bartual Martos, 2011). The interior is separated by membranous walls and white spongy tissue into compartments packed with transparent sacs filled with fleshy, juicy, red, pink or whitish pulp called arils. In each aril sac, there is one white or red, angular, soft or hard seed. The arils account for about 52-65 % of the weight of the whole fruit (Al-Said *et al.*, 2009; Holland *et al.*, 2009) (**Figure 2**).

Figure 2. Pomegranate fruit.

1.2. **Pomegranate cultivars**

This fruit is quite old and there are a large number of varieties which takes different names, but in most cases have a high similarity among them. In general, varieties are classified by their citric acid content (Melgarejo *et al.*, 2000). Citric acid is higher than malic acid in sour and sour-sweet cultivars, while concentrations of citric and malic acids are similar in sweet cultivar (Mena *et al.*, 2011; Carbonell-Barrachina *et al.*, 2012). In general, sour cultivars are red-skinned, while sweet cultivars are pinker. The color of fruits is due to pigments like anthocyanins. Likewise, it has been established a classification for Spanish cultivars based on the maturity index (MI), which is the ratio of total soluble solids and titratable acidity (TSS/TA) (Martínez *et al.*, 2006). The **Table 1** shows the classification for Spanish pomegranate cultivars based on their citric acid content and maturity index (MI).

Table 1. General classification of Spanish pomegranate cultivars.

Cultivar	TA (% citric acid)	MI
sour	2.3 – 2.7	5 - 7
sour - sweet	0.5 – 1.0	17 – 24
sweet	0.15 – 0.5	31 - 98

In Spain, there are two traditionally groups of varieties with commercial interest, *Valenciana* and M*ollar;* although various studies have demonstrated the richness and interest of other Spanish varieties (Melgarejo *et al.*, 2010).

The *Mollar* group is the most important and the most widely grown and consequently marketed in Spain and in the European Union. The fruits are characterized by their high organoleptic quality, and the harvest time is between the 25th of September and the 15th of November. In general, the fruits from the *Valenciana* group have less quality than those of the *Mollar* group. Fruit trees are significantly smaller and the harvest takes place between the 5th of August and the 20th of September. Other cultivars in Spain are *PTO* (*Piñon Tierno de Ojós*) with a sour-sweet taste and large size of its fruits. In addition, the *BA* (*Borde of Albatera*) with sour taste, are hard and have a woody portion of ~13 % (Hernández, *et al.*, 1999). The *Wonderful variety* is one of the most cultivated in the world (USA,

Israel, Greece, Chile, etc.); this cultivar has sour or sour-sweet seeds, depending on the harvest, with an attractive intense red color. In general, *Wonderful* fruits are appropriate for industrial use but not for fresh consumption. Productivity is usually medium to low and does not exceed 18,000 kg ha^{-1}. (Melgarejo *et al.*, 2010). Nowadays, the pomegranate variety can be selected according to their yield, organoleptic and taste qualities, but also, for industrial, nutritional and/or healthy interest.

1.3. Agricultural techniques

1.3.1. Pomegranate farming

When it comes to traditional irrigation, it is necessary to level the surface of the plot on which the pomegranate trees will be grown. The opening of the holes can be made 1-2 months before planting. This can be done at ground level or plateaus. Planting distances should be sufficient to ensure good lighting, allowing the fruit to fully develop their color, and allow for the completion of other regular farming practices. Thus, farmers used greater separation between rows of trees than between trees within a row: 6 x 4 m, 6 x 3 m, 5 x 3 m (Melgarejo *et al.*, 2010).

1.3.2. Irrigation

Irrigation is a "must" practice in traditional pomegranate farming in the province of Alicante, since it is an area where the average rainfall of the last decade below 300 mm; besides, the average evapotranspiration is around 1,200 mm annual. Thus, it is in an arid area, according to different climate indicators, with an additional high risk of salinization. Melgarejo *et al.* (2010) determined that the average total irrigation requirements obtained for pomegranate crops are 5,271 m^3 ha^{-1}.

1.3.3. Fertilization

There are a few scientific publications about nutrient requirements and fertilization of the pomegranate. Blumenfeld et al. (1998) indicate that in Israel the pomegranate is fertilized with 200-300 fertilizers units (UF) of N ha^{-1} and K_2O 200-300 UF.

Some general considerations are:

a) Excessive irrigation and nitrogen fertilization in spring can produce an imbalance favoring vegetation on flowering.

b) The excess nitrogen, especially if accompanied by water imbalances, may increase the cracking of the fruit before the time of maturity. It may also influence negatively in the development of color.

c) Potassium has a favorable effect in reducing fruit cracking.

1.3.4. Thinning

Thinning is an agricultural practice, which consists in reducing fruit load at immature stage and thus allowing remaining fruits to develop to their maximum size and quality (Melgarejo *et al.*, 2010). In pomegranates, as in other fruits such as peaches, apricots or loquats, this operation is performed to remove the twins, small and irregular fruits to obtain fruits with the size required by the market (Hueso *et al.*, 2003; Njoroge and Reighard, 2008; Missang *et al.*, 2011). In the Spanish pomegranate trees, this practice is conducted in the first week of June and should be repeated after 20-30 days (end of June or early July); depending on the phenological stage of the fruits at thinning, among 7-8 to 12-15 kg per tree could be removed (Melgarejo *et al.*, 2010). According to our calculations, immature fruits removed during thinning can represent a value close to 2.500.000 kg in the Alicante province. This value represents approximately 10 % of the total pomegranate production, 22311 t in 2010 (MMARM, 2010). After thinning the fruits removed from the trees are left to spoil in the soil and the farmer does not get any direct payback for this expensive (needs specialized labor) farming practice.

1.3.5. Pruning

The main aim of the pruning is to increase production, favoring the production of fruits not only in the periphery but also in the interior of the tree, improving the quality of the fruit, reducing expenses of other farming practices and facilitating their implementation (pesticides treatment, thinning and harvesting). Some considerations to keep in mind are (Melgarejo *et al.*, 2010):

a) Annual pruning should be done.
b) The pruning time matches the winter rest period (December-February).

c) The pomegranate has two main flushes, spring and summer.

d) It should remove branches which intersect and interfere with the passage of light.

e) The pruning creates a structure capable of supporting productive harvesting. The most appropriate is proven to date structure, for the Spanish varieties grown in this area, is to form the tree with three main branches of a trunk of 30-50 cm.

1.4. Pomegranate Spanish production

Spain is the main European producer and exporter (Andreu-Sevilla *et al., 2008*). Historically their cultivation was practiced mainly in the provinces of Murcia and Alicante but in recent years, probably for climate changes and extreme drought conditions, there has been a decline in the cultivated area of Murcia. The yield is 22,311 t (MMARM, 2010) mainly in the province of Alicante (98 %) in the region of Elche, Crevillente and Albatera, which reflecting socio economic importance of these areas. One of the main pomegranate gene banks of the European Union is located at the experimental field station of the Miguel Hernández University in Orihuela, Alicante, Spain (02 ° 03'50'' E, 38 ° 03'50'' N, and 25 meters above sea level).

1.5. Pomegranate processing

The pomegranate generally is consumed in fresh but there is an important part of the crop that does not have enough quality and their acceptance by consumers is quite low. In some cases, the appearance of some fruits are not appropriate for their commercialization, mainly due to defects caused by farming issues (e.g. low development of rind color), ripening (fruits maturation is not homogeneous) and physiopathies (e.g. cracking) (Melgarejo and Salazar, 2003). This part of the crop, in many cases, can potentially cause economic losses to farmers due to the costs of collection and transport; therefore, is necessary to find other commercial options within the agro-food industry for this part of crop that is not suitable for direct consumption, but it can be for industrial use. The high number of scientific papers that describing the many benefits of pomegranates is being translated into an increase in consumption of products derived from this fruit such as, pomegranate juice, jams, jellies, food supplements etc. From pomegranate is possible to obtain all kinds of primary products, such as fresh fruit, natural juices,

jams, jellies, beverages prepared from pomegranate juice as grenadine, or arils from pomegranate shelled which are processed and packed in modified atmosphere (**Figure 3**). There is also a wide range of secondary products, ranging from animal feed to extracts from the rind and other waste materials.

Fresh fruit
Natural juices
Arils packed in
modified atmosphere

Jams
Jellies
Beverages

Food-supplements
based on
pomegranate peel
extracts
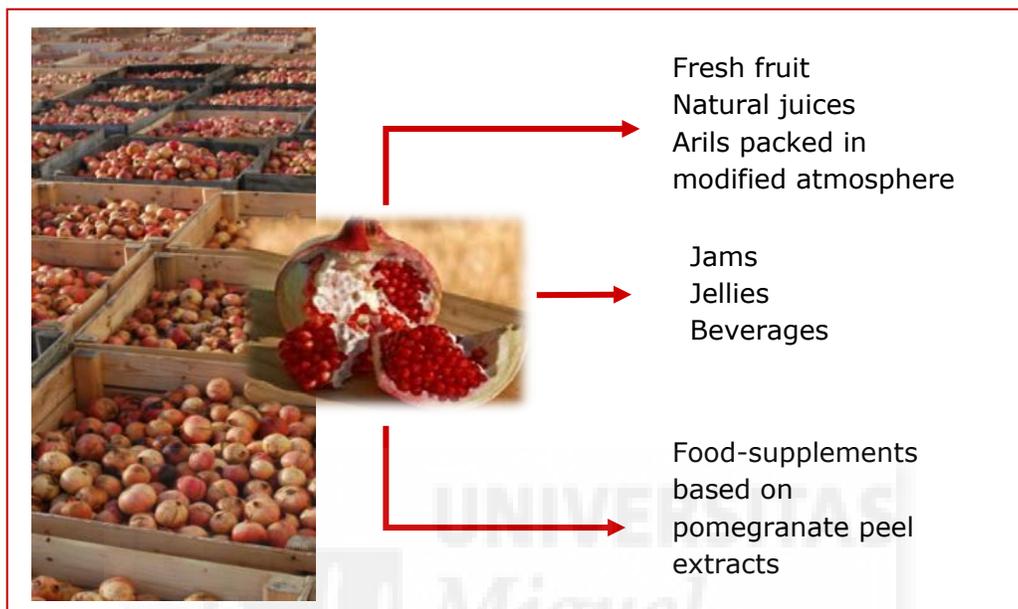
Figure 3. Products derived from pomegranate fruit.

Technological advances and changing lifestyles and consumer tastes encourage the development of new products, which will certainly contribute to a development of the sector. Some of these products are gaining popularity in both domestic and international markets, considered as a true "gourmet" product because it assures potential health benefits, as well as high consumer acceptance and a long shelf life (Andreu-Sevilla *et al.*, 2008).

In a rapidly changing society, the pomegranate juices is a perfect choice for consumers who are interested in enjoying the healthy properties of pomegranate with the advantage of finding the ready-to-eat product available in different outlets without having to manipulate the fruit. Although pomegranate juice is not used for aspects such as color (from intense garnet to brown), or loss of phenolic compounds that give a distinctive flavor, some consumers are drinking pomegranate juice by its enormous potential health benefit (Aviram *et al.*, 2004). Recently it has been found that the pomegranate juice is a preventive medicine against heart disease (Basu and Penugonda, 2009), has positive chemotherapeutic

effects against prostate cancer in humans (Malik *et al.*, 2005), helps in reducing blood pressure (Aviram *et al.*, 2001) and improves the stress-induced ischemia in patients with coronary heart disease (Sumner *et al.*, 2005).

Pomegranate rind or peel is a richer source of antioxidants than the edible arils (Li *et al.*, 2006) and could be used as a nutraceutical supplement due to its elevated content in ellagitannins and ellagic acid (Espín *et al.*, 2007). Dried and ground pomegranate rind or even its extract can be also used as an antioxidant ingredient for fruit juices and at the same time increase their vitamin C content, without significant changes in the juice sensory attributes (Navarro *et al.*, 2011).

Jellies and jams are other options for using the pomegranate. These are foods that are made from fruit juice and sugar until a semisolid or gelatinous consistency in reached. In their preparation pieces of fruit can be included. The amount of sugar should not exceed one and half times the weight of the fresh juice.

The industrial pomegranate processing is an important part which requires a thermal processing (pasteurization), which if not done properly can be reflected in a degradation of anthocyanins, significant loss of quality, organoleptic and nutritional properties and volatile compounds (Andreu-Sevilla *et al.*, 2008).

1.6. Pomegranate composition

The chemical pomegranate composition as any plant product depends among other factors, on the variety, growing area, environmental conditions, maturity degree, agricultural techniques and storage conditions (Mirdehghan and Rahemi, 2007; Viuda-Martos *et al.*, 2010). The edible part of pomegranate fruit (50 %) includes 40 % arils and 10 % seeds. In turn, the arils juice contains 85 % water, 10 % of total sugars, and 1.5 % pectin, ascorbic acid, and phenolic compounds (Viuda Martos *et al.*, 2010). The edible part of pomegranate is consumed in fresh and is also used to prepare natural juices, jellies, jams, flavored beverages and even dyes. Undoubtedly, the products obtained from the pomegranate, the most interesting and studied is the pomegranate juice (Fadavi *et al.*, 2005). **Table 2** shows the main pomegranate components in each part of the fruit.

Table 2. Pomegranate main components.

Pomegranate part	Principal components	Reference
Rind and carpelar membranes	Ellagitannis and ellagic acid[1, 2], flavonoids, condensed and hydrolysable tannins[3].	Gil *et al*. (2000)[1] Calin-Sánchez *et al*. (2012)[2] Elfalleh *et al*. (2011)[3]
Arils	Ellagitannis, ellagic acid, organic acids sugars[2], anthocyanins[4, 5].	Calin-Sánchez *et al*. (2012)[2] Jaiswal *et al*. (2010)[4] Hernandez *et al*. (1999)[5]
Juice	Ellagitannins[6], ellagic acid[7], organic acids, sugars[7, 8, 9], anthocyanins[6], minerals (especially K) [9], aminoacids[10].	Zhang *et al*. (2009)[6] Mena *et al*. (2011)[7] Carbonell *et al*. (2012)[8] AIJN (2012)[9] Lansky *et al*. (2007)[10]

Recent studies have shown that fruit cultivar and maturity status influence the antioxidant activity and other physicochemical properties of pomegranate such as total soluble solids (TSS), pH, titratable acidity (TA), organic acids, total sugars, total phenolics and anthocyanins as well as mineral elements composition (Al-Maiman and Ahmad, 2002; Opara *et al*., 2009). During pomegranate fruit maturation, significant changes in organic acids, sugars and phenolic composition have been reported by various authors (Al-Maiman and Ahmad, 2002; Poyrazoglu *et al*., 2002; Mirdehghan and Rahemi, 2007).

1.6.1 Total soluble solids, pH and titratable acidity

Total soluble solids (TSS), pH and titratable acidity (TA) are an important attributes of pomegranate juice which are used to identify the type of fruits included in a particular juice and the quality of the juice (Shwartz *et al*., 2009). The pH value of pomegranate juice determines the sour taste of juice (Al-Maiman and Ahmad, 2002). The pH of the pomegranate juice increases with maturity, reaching a maximum 3.57 at the full ripe stage. Generally, TA in pomegranate juice decreases with advancing fruit maturation but the rate of decline differs among cultivars and growing region (Shwartz *et al*., 2009). This decrease in TA values levels during fruit development coincides with the increase in sugar concentration, and it is an inherent process during ripening of pomegranate to impart the characteristic flavor to each pomegranate cultivar (Kulkarni

and Aradhya, 2005). The ratio TSS/TA, also referred to as maturity index, MI (Hernandez *et al.*, 1999), it is commonly used to define the 'taste' of pomegranate fruit during development. In general, the MI for pomegranate cultivars reaches values of 5-7 for sour cultivars, 17-24 for sour-sweet ones and 31-98 for sweet cultivars (Martínez *et al.*, 2006). The MI has been reported as one of the most reliable indicators of pomegranate fruit maturity (Fawole and Opara, 2013a), although it depends on the cultivar and climatic conditions.

1.6.2 Organic acids

Organic acids contents of pomegranate fruits depend on the cultivar (Legua *et al.*, 2000) and they are a key part of the sour-sweet balance of pomegranate fruits. The ratio of total acids content to sugars content is a determinant parameter of fruit maturity. According to the AIJN Reference Guide (2012), the values of citric and malic acids should range among 0.1-33 g L^{-1} and 0.02-3.6 g L^{-1}, respectively.

The composition and concentration of organic acids are important because of their contribution to sensory attributes and their influence on consumer perceptions of both sweetness and sourness in pomegranate fruits (Carbonell-Barrachina *et al.*, 2012). Regarding organic acids, citric, malic and oxalic acids are considered as the major organic acids in pomegranates, while tartaric, succinic and quinic acids are only usually found in minor quantities. However, the levels of these minor acids were higher in some cases and exceeded the level of those major organic acids (Poyrazoglu *et al.* 2002). One of the ripening effects is a significant decrease in organic acid content, this behavior was reported in pomegranate by Fawole and Opara (2013a) and Kulkarni and Aradhya (2005). The decrease is due to that organic acids are accumulated during fruit growth and are used as respiratory substrates in ripe fruits (Moing *et al.*, 2001).
The **Table 3** shows the average values of organic acid content in pomegranate (commercial fruit and juice).

Table 3. Pomegranate main organic acids content (g L^{-1}) in fresh fruit and commercial juice.

Product	Citric	Malic	Oxalic	Tartaric	Ascorbic	Reference
	Organic acids					
	(g L^{-1})					
Pomegranate	15.4	12.4	1.0	1.7	2.3	Carbonell et al. (2012)
	5.6	1.6	0.15	trazas	na	Melgarejo et al. (2000)
Commercial	6.8	7.2	0.5	0.2	1.5*	Carbonell et al. (2012)
juice	1.0-48.0	1.5	na[†]	na	na	AIJN (2012)

*maximum level; [†]na: not available.

1.6.3 Sugars

Fructose and glucose are the most abundant and characteristic sugars in pomegranate fruit and juice, with the ratio glucose/fructose being in the range 0.7-1.0 (Melgarejo *et al.*, 2000; Mena *et al.*, 2011). However, other studies have reported that glucose was slightly higher than fructose (Ozgen *et al.*, 2008). These differences could be related to, among other factors, fruit cultivar, climatic conditions and irrigation management (Carbonell-Barrachina *et al.*, 2012). Sucrose is not presented in all cultivars (Melgarejo *et al.*, 2000) and its content is a trace level especially in sour–sweet fruits. According to the AIJN Reference Guide (2012) the values of fructose and glucose in pomegranate juice should range among 50-100 g L^{-1} and 45-85 g L^{-1}, respectively (**Table 4**). The **Table 4** shows the average values of sugars contents in pomegranate (commercial fruit and juice).

Table 4. Sugar content (g L^{-1}) in pomegranate (fruit and commercial juice).

Product	Fructose	Glucose	Sucrose	Reference
	Sugars			
	(g L^{-1})			
Pomegranate	111	90.5	11.5	Carbonell *et al.* (2012)
	66.2	63.2	0.20	Melgarejo *et al.* (2000)
Commercial	85.8	65.4	0.00	Carbonell *et al.* (2012)
juice	45.0-100	40.0-80.0	na[†]	*AIJN* (2012)

[†]na: not available

During fruit ripening there is an increase in total sugar content (Kulkarni and Aradhya, 2005; Fawole and Opara, 2013a) this can be due to that one of the processes occurring in fruit during ripening is the hydrolysis of starch that accumulates into simple sugars in the early stages of fruit development (Shwartz *et al.*, 2009). As a result, the fruit get its sweetness and increase the amount of the two principal sugars (glucose and fructose), also affects the TSS content which increase during maturity stages.

1.6.4 Minerals

Potassium is the predominant macro-element in pomegranate arils, while iron, in general, is the predominant micro-element (Mirdehghan and Rahemi, 2007; Gozlekci *et al.*, 2011). Normally, in pomegranate fruit, the concentration of minerals in fruit parts investigated, at each maturity stage followed the order of K > Ca > Mg > Na > Fe > Zn > Cu > Mn (**Table 5**). As the fruit ripens there are significant decreases in mineral elements contents (Fawole and Opara, 2013b). The composition and concentration of mineral nutrients at fruit developmental stages have been implicated in cracking incidence in pomegranate fruit. The disorder is reported to be associated with B and Ca deficiency (Mir *et al.*, 2012).

In pomegranate juice, K is the most abundant and characteristic mineral as well (Ekşi and Özhamamcı, 2009; KFL, 2012). According to the AIJN Reference Guide (2007) the values of Ca, Mg and K in pomegranate juice should range among 5-150, 20-100, and 800-2500 mg L^{-1}, respectively.

The **Table 5** shows the average values of minerals content in pomegranate (commercial fruit and juice).

Table 5. Minerals content (mg L^{-1}) in pomegranate (fruit and commercial juice).

Product	Minerals (macro-elements)				Reference
	Ca	**Mg**	**K**	**Na**	
	(mg L^{-1})				
Pomegranate	80	30	2750	50	Mataix et al. (2009)
Commercial	5-120	20-110	1300-3000	30	AIJN (2012)
juice	30	30	2590	30	USDA (2012)
Product	Minerals (micro-elements)				Reference
	Fe	**Zn**	**Cu**	**Mn**	
	(mg L^{-1})				
Pomegranate	6.0	3.0	1.7	na†	Mataix et al. (2009)
Commercial	5.0*	5.0*	5.0*	na	AIJN (2012)
juice	3.0	1.2	0.7	na	*USDA* (2012)

*maximum level; †na: not available.

1.6.5 Proline

Water is known to play an important role in the growth and maturation of fruits (Khattab *et al.*, 2011). Due to the fact that pomegranate is mainly grown in arid and dry geographic regions, the amino acid "proline" is another parameter to consider during fruit ripening. The proline content is considered as an indicator of changes in cellular metabolism caused by abiotic factors, such as water deficit, high salinity, extreme temperatures, high concentrations of heavy metals in the soil-plant system, and high light intensity (Claussen, 2005). Proline is one of the 22 proteinogenic amino acids (proteins main components); works as a protein stabilizer, hydroxyl radical scavenger and serves as a source of energy and nitrogen (Claussen, 2005). Proline is one of the main amino acid present in citric juices, and it has been suggested as a purity index in pomegranate juice (Niedmann, 1976; Ting y Rouseff, 1979). The proline content in pomegranate fruit ranges from 30 to 93 mg L^{-1} (Halilova y Yildiz, 2009). However, proline content increases during ripening and senescence in most fruits. Currently there is not enough information in the literature on whether this parameter is affected by fruit ripening or just accumulates in plants under unfavorable environmental conditions.

1.6.6 Color

The color of pomegranates is an important factor that clearly affects market acceptance (Opara *et al.*, 2009) and it has been often associated with high fruit consumer preference and/or acceptance for different commodities. For instance in peaches and nectarines, consumers prefer full red color fruits (Crisosto *et al.*, 2003); a similar situation is expected for pomegranates. Recent studies have found that the external color of pomegranate (cv. *Mollar de Elche*) is correlated with the number of days from the beginning of its development (Manera *et al.*, 2013). During ripening, the values of *L**, *b** and Hue angle decreased while the values of *a** and chroma increased (Manera *et al.*, 2012). The growth of the fruit, its color and the chemical maturity index (ratio TSS/TA) provide farmers cheap but objective way of establishing the optimal time for fruit harvest. All these statements highlight the enormous interest in fruit colorimetric, especially at ripening. However, there is no correlation between the outer rind color and the inner arils color.

1.6.6.1 External color

Although studies have been conducted on the effects of different farming practices on the quality parameters of pomegranate, the external color of the fruit has not been studied in detail; however, fruit maturity is commonly evaluated based on the color of the fruit rind (Manera *et al.*, 2013). For instance, Manera *et al.* (2011) studied the correlation between pomegranate rind color and air temperature; these authors hypothesized that one of the parameters that could affect the color of the pomegranate fruits was the exposure to sunlight.

1.6.6.2 Internal color

The increase in the green-red coordinate, *a**, is without any doubt related to the increased biosynthesis and accumulation of anthocyanin pigments, which are responsible for the intense red color of ripe pomegranate fruits. In general, the most abundant anthocyanins are cyanidin-3, 5-diglucoside and cyanidin-3-glucoside in sour and sweet cultivars, respectively; however, the anthocyanin profile could be changed during fruit ripening (Hernández *et al.*, 1999).

1.6.7 Volatile compounds

Aroma consists of a large combination of substances that are directly responsible for the odor and flavor. Aroma compounds can be classified into chemical families as aldehydes, alcohols, ketones, esters, lactones, terpenes, etc. (Raisi *et al.*, 2008) and they can be analyzed, among other techniques, by headspace solid phase micro-extraction (HSSPME). The profile of volatile compounds reflects a rough idea of pomegranate odor and flavor; however, pomegranate fruit has low concentrations of volatile compounds, leading to low intensities of both odor and aroma (Carbonell-Barrachina *et al.*, 2012). The main volatile compounds in pomegranate can be grouped in seven chemical families (Melgarejo *et al.*, 2011):

i) monoterpenes: *α*-pinene, *β*-pinene, *β*-myrcene, *p*-cymene, limonene, and *γ* -terpinene;

ii) aldehydes: *cis*-3-hexenal, hexanal, *trans*-2-hexenal, nonanal, and decanal;

iii) monoterpenoids: fenchone, camphor, and *α*-terpineol;

iv) esters: 3-hexenyl acetate, hexyl acetate, and hexenyl butyrate;

v) alcohols: *cis*-3-hexenol and 1-hexanol;

vi) ketones: 6-methyl-5-hepten-2-one; and

vii) sesquiterpenes: *trans*-caryophyllene.

In general, *aldehydes* are the predominant group in pomegranate juices, followed by *monoterpenes*. Aldehydes can be related to green, grassy, and herbaceous notes, while monoterpenes can be related to pine and citrus notes; a*lcohols* and especially *esters* are related to fruity and sweet aromas (**Table 6**) (Melgarejo *et al.,* 2011; Vázquez-Araújo *et al.,* 2011a). The difference in chemical groups may have some influence on consumers' preference for pomegranate (fruit or juices) (Vázquez-Araújo *et al.*, 2011b).

Table 6. Volatile compounds found in fresh fruits and pomegranate juices.

Compound	Sensory Descriptor	Reference[†]
Hexanal	Fatty, green, grassy, powerful	1, 2, 3, 5
cis-3-Hexenal	Apple, grape, floral, green, vegetable	1, 2, 5
cis-3-Hexenol	Fresh, green grass	1-5
1-Hexanol	Mint, grass	1-5
α-Pinene	Sharp, pine	1-5
β-Pinene	Woody, pine	1-5
Limonene	Mild, citrus, sweet, orange, lemon	1-5
γ-Terpinene	Herbaceous, citrus	1-5
α-Terpineol	Fragrant, floral, lilac	1-5
4-Terpineol	Grapefruit, lemon, lime, pepper, herbaceous	2-5
β-Myrcene	Sweet, balsamic	1, 2, 5

[†]*Melgarejo et al. (2011)[1]; Calín-Sanchez et al. (2011)[2]; Vázquez-Araújo et al. (2011a)[3]; Vázquez-Araújo et al. (2011b)[4]; Carbonell-Barrachina et al. (2012)[4].*

It is expected that during juice manufacturing, the volatile composition and therefore, the functionality associated with terpenes and related chemical groups, changes as well. These changes will be mainly related to oxidation and enzymatic reactions, activated by cell rupture (Belitz *et al.*, 2009). For example, esters are significant aroma constituents of many fruits and plants and are synthesized only by intact cells, but during the processing of the plant material, esters are rapidly hydrolyzed by enzymes and the fruity aroma flattens (Belitz *et al.*, 2009). This is the main reason why the flavor of fruit juices is different from those of the fresh fruits; besides, differences are more pronounced after the application of thermal treatments, such as pasteurization.

Calín-Sánchez et al. (2011) studied the relationship among instrumental parameters of pomegranate fresh juices and overall liking of consumers. Overall liking of the juices seemed to be related to the attributes "fresh flavor" and "fresh odor", which in turn seemed to be related to the presence of some volatile compounds, mainly terpenes (*a*-pinene, *β*-pinene, *β*-myrcene, limonene, and *γ*-terpinene). During storage of pomegranate juices, the amounts of ethanol, ethyl acetate (from the esterification of ethanol) and sesquiterpenes (e.g. β-caryophyllene, α-bergamotene, and β-farnesene) significantly increased and simultaneously the consumer acceptance

decreased. In this way, the "flavor life" of pomegranate juices is often shorter than their "storage life" as describe by physico-chemical and microbiological quality parameters.

1.6.8 Phenolic compounds

Phenolic compounds are the bioactive compounds with the highest antioxidant activity and abundant in the human diet. This is a large group of compounds with aromatic rings and conjugated double bonds from which they exert their antioxidant action (Arranz *et al.*, 2010). The main compounds responsible for the antioxidant capacity of pomegranate are punicalagins, anthocyanins and ellagic acid (Gil *et al.*, 2000). However, results from Gil et al. (2000) and Tzulker et al. (2007) concluded that while punicalagins played an important role in the antioxidant capacity, anthocyanins only played a minor role. The **Figure 4** shows the most common phenolic compounds found in plant foods.
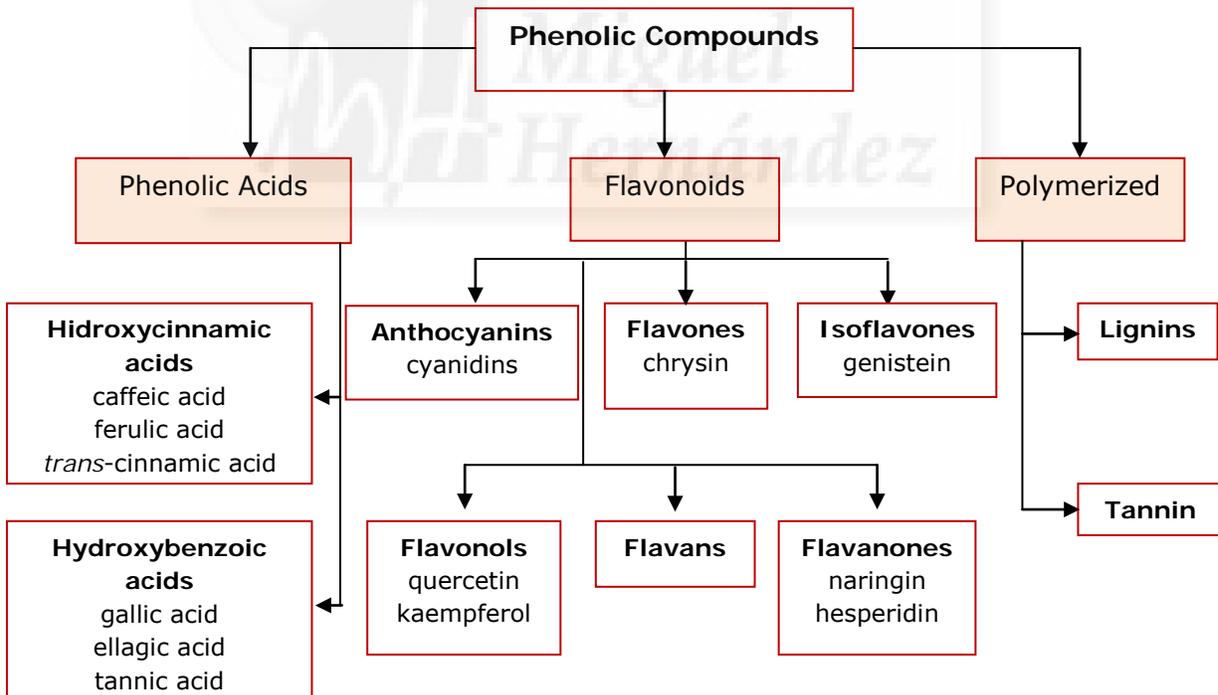
Figure 4. Most common phenolic compounds in plant foods.

Phenolic compounds are endowed with numerous biological properties and also are well-known for their ability to eliminate free radicals, inhibit lipid oxidation and induce health benefits against cancer, cardiovascular, atherosclerotic, anti-inflammatory and other health diseases (Aviram *et al.*, 2000). Besides, it has been demonstrated that there is a positive correlation between the total content of phenolic compounds and the antioxidant capacity (Wojdyło *et al.*, 2008; Wu *et al.*, 2004). Tezcan et al. (2009) reported that both hydrolysable tanning and anthocyanins from the rind increased the antioxidant capacity of commercial pomegranate juices. The **Figure 5** shows the principal phenolic compounds present in pomegranate and pomegranate based products.
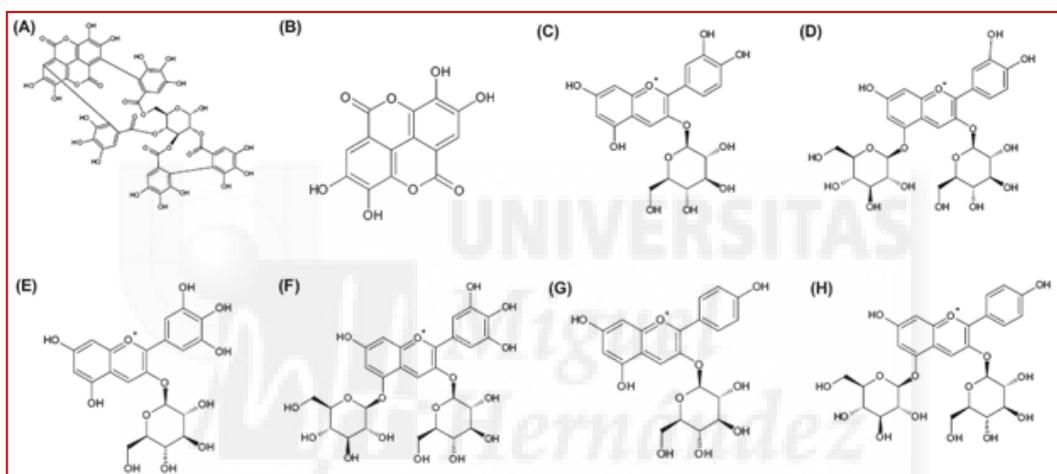
Figure 5. Principal phenolic compounds present in pomegranate: (a) punicalagin; (b) ellagic acid; (c) cyanidin 3-glucoside; (d) cyaniding 3,5-diglucoside; (e) delphinidin 3-glucoside; (f) delphinidin 3,5-diglucoside; (g) pelargonidin 3-glucoside and (h) pelargonidin 3,5 diglucoside.

1.6.8.1 Hydrolysable tannins

The classification of "hydrolysable tannins" is based on the fact that tannins can be fractionated hydrolytically into their components, for example by treatment with hot water, acids or with alkalis (Khanbabaee and Van Ree, 2001). Non-hydrolyzable oligomeric and polymeric proanthocyanidins are classified as condensed tannins. Therefore, the term 'hydrolyzable tannins' includes both the gallotannins and the ellagitannins (Khanbabaee and Van Ree, 2001). Ellagitannins are hydrolysable tannins, wherein the acid form hexahydroxydiphenic produces di-esters with sugars, typically β-D-glucose or quinic acid (Madrigal-Carballo *et al.*, 2009).

Monomeric ellagitannins structures can be oxidized inside the plants and lead to dimeric, trimeric and tetrameric structures. These polymers can be hydrolyzed in the presence of acids or bases to give ellagic acid (Häkkinen *et al.*, 2000). Punicalagins isomers (α and β) are the main ellagitannins, non-colored phenolic compounds found in pomegranate juices, and they are responsible for a high percentage of the antioxidant capacity of pomegranate (Calin-Sánchez *et al.*, 2013 arils and rind). The punicalagin content is generally higher in commercial juices than in fresh pomegranate fruit (arils) due principally to the hydrostatic pressure to crush the whole fruit to release the juice from the arils, also extracts the water-soluble ellagitannins from the rind that pass to the juice in proportion to the force used (Gil *et al.*, 2000).

1.6.8.2 Ellagic acid

Ellagic acid (EA) can be found as a free compound in pomegranate but always in a relatively small amount. EA are found more often in the form of ellagitannins, and also as C-glycoside derivatives of these acids. The main biological activity of EA is its potential anticarcinogenic activity and antiatherosclerotic biological properties. The main biological activity of EA its potential anticarcinogenic activity and antiatherosclerotic biological properties (Lu, *et al.*, 2008; El-Shitany *et al.*, 2014). EA is present in the plant vacuole, either in its free forms or as EA derivatives (Häkkinen *et al.*, 2000) and its consumed constantly in fruit, seeds, and in the foods or beverages based on fruit juices and jams, etc. (Clifford and Scalbert, 2000). Higher ellagic acid concentration are directly associated with the antioxidant activity of pomegranate peel extracts (Al-Rawahi *et al.*, 2014).

1.6.8.3 Anthocyanins

Pomegranate fruits are rich in anthocyanin pigments (Hernandez *et al.*, 1999), which are potent antioxidant flavonoids and provide pomegranate juice with its characteristic bright and intense dark red color. This red color depends on anthocyanin concentration and on the chemical structure of the individual anthocyanin (Holcroft *et al.*, 1998). The six principals anthocyanins in pomegranate are: delphinidin 3-glucoside and 3,5-diglucoside, cyanidin 3-glucoside and 3,5-diglucoside, pelargonidin 3-glucoside and 3,5-diglucoside (Gil *et al.*, 1995; Hernandez *et al.*, 1999). Generally, there is an increase in juice pigmentation during fruit ripening; in the early fruit ripening stages, delphinidin 3,5-diglucoside are the main pigment, followed by cyanidin 3,5-diglucoside;

while in the later stages, the monoglucoside derivatives cyanidin 3-glucoside (30-35 %) and delphinidin 3-glucoside (> 20 %) significantly increased and become predominant. The pelargonidin derivatives are always present in small amounts (< 5 %). The content of total anthocyanins generally decreases during juice manufacturing; this has a direct effect on the dark red color intensity.

1.7 Antioxidant activity

An antioxidant with biological function is defined as a substance that reduces or prevents oxidation of the substrate resulting in a more powerful reducing agent (Kuskoski *et al.,* 2005). Antioxidants in fruit and vegetables are of interest for many reasons; they can protect components of the food itself against oxidative damage and later they can be absorbed into the human body and could have beneficial health effects. Most of the antioxidants from vegetal sources help to strengthen the endogenous human antioxidant capacity (Prior, 2003). There are different antioxidant defense systems in the body, and they can be classified according to their nature, as *enzymatic antioxidants*, such as superoxide dismutase and catalase enzymes (SOD), glutathione peroxidase (GPx) and catalase (CAT), and *non-enzymatic antioxidants*, including vitamins such as ascorbic acid (vitamin C), α-tocopherol (vitamin E), thiols such as glutathione (GSH) or thioredoxin, carotenoids, flavonoids and other antioxidants.

Among the reactive species, it is important to mention the free radicals; which are chemical species that contain in their structure one or more unpaired electrons that can be given or taken by an adjacent molecular structure to get stabilized (Gilbert, 2000). These radicals are grouped under the name of reactive oxygen species (ROS) and nitrogen species (RNS) (**Table 7**).

Table 7. Reactive species of oxygen and nitrogen.

Radicals		
ROS	$O_2^{\bullet}$	Superoxide radical
	$^{\bullet}OH$	Hydroxyl radical
	$^{\bullet}OOH$	Hydroperoxyl radical
RNS	$NO^{\bullet}$	Nitric oxide radical

Free radicals have a dual role that can be beneficial or not for living systems (Pervaiz and Clement, 2007). At low concentrations, free radicals are necessary for the proper functioning of cells, being able to act as second messengers, stimulating cell proliferation and/or acting as mediators for the activation of the cells (Weinberg and Chandel, 2009). However, at high concentrations they are able to damage reversibly or irreversibly all types of biomolecules, including proteins, carbohydrates, and nucleic acids (Valko *et al.*, 2007). Consequently, the ROS are involved in controlling the pathogenesis of many degenerative diseases (Valko *et al.*, 2007), such as cancer, atherosclerosis, cardiovascular disease, etc. (Fearon *et al.*, 2009).

Due to the variety of oxidizing agents and different antioxidant mechanisms, nowadays there is no a universal method to evaluate the antioxidant capacity of a food (Schlesier et al., 2002). There are several methods to evaluate the antioxidant capacity in foods, including fruit and vegetables. The most widely used methods in pomegranate juices are:

(i) The **DPPH** method, which uses 2,2 diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$), a free radical that measures the ability of a compound to donate an electron (Mena *et al.*, 2012; Calín-Sánchez *et al.*, 2013).

(ii) The **FRAP** method developed to measure the ability to reduce ferric complex with the molecule tripyridyl s-triazine (TPTZ) to its ferrous form at low pH (Zaouay *et al.*, 2012).

(iii) The **ORAC** method uses fluorescein as target molecule; in this assay, 2,20-azobis(2-amidinepropane) dihydrochloride (AAPH) is used to produce peroxyl radicals that react with fluorescein. The addition of an antioxidant delays the fluorescence decay and the quantification of the antioxidant capacity is carried out from the net integrated areas under the fluorescence decay curves (Bentayeb *et al.*, 2014).

(iv) The **ABTS** method, antioxidants are added previously to the generation of the ABTS$^{+\bullet}$ radical and the inhibition in the radical formation is evaluated (Carbonell-Barrachina *et al.*, 2012; Mena *et al.*, 2012).

Using all these methods, Seeram et al. (2008) concluded that the antioxidant capacity in beverages rich in polyphenols followed the order: *pomegranate juice* > red

wine > grape juice > blueberry juice > blackberry juice =cranberry juice > orange juice = iced tea beverages = apple juice. The exact values of the antioxidant capacity depend on many factors, including cultivar, maturity index, geographical source, irrigation regime, etc. The main compounds responsible for the antioxidant capacity of pomegranate are punicalagins, anthocyanins and ellagic acid (Gil *et al.,* 2000). These compounds are well-known for their ability to eliminate free radicals and inhibit lipid oxidation. During the manufacturing of fresh and commercial pomegranate juices, if the whole fruit is pressed (arils and rind), it is expected that a large amount of phenolic compounds present in the rind migrate to the juice (Tezcan *et al.*, 2009). In this way, it is normal to find higher contents of antioxidant compounds in commercial juices than in the pomegranate arils themselves (consumed as fresh products) or freshly squeezed juices.

1.8. Functional and healthy properties

Nowadays, the number of studies on the beneficial properties of pomegranate is increasing. Due to the content in phenolic compounds, recent studies have identified healthy properties of pomegranate, such as anticarcinogenic, antiatherogenic, antioxidant, antihypertensive (Hong *et al.,* 2008; Basu and Penugonda, 2009). The phenolic compounds may be involved in the antiproliferative ability of various carcinogenic cells associated with various cancers, such as colon or prostate (Sun *et al.*, 2002). Recent research *in vitro* has shown that pomegranate extracts selectively inhibit the growth of breast, prostate, colon and lung cancer cells (Kim *et al.*, 2002; Seeram *et al.*, 2005).

Besides, pomegranate also has anti-inflammatory effect due to the high content of tannins. This anti-inflammatory action is important in the inflammatory processes during the creation of the atheroma plaque, and therefore could mediate and prevent pathological processes in the cardiovascular system such as heart attacks (Andreu-Sevilla *et al.*, 2008). Pomegranate juice seems to prevent the oxidation of LDL (Low Density Lipoprotein) in bloodstream vessels; this fact has importance in creating the atheroma and the subsequent action of related inflammatory effects. Also, pomegranate rind extract has antibacterial, anti-inflammatory and anti-allergic activities and could be considered a nutraceutical product (Panichayupakaranant *et al.*, 2010).

1.9. Sensory evaluation

Sensory analysis is a tool in the total quality control of an agro-food company, and therefore goes in the same direction in which it develops. Thus, it can be considered to be directed to the assessment, analysis and control of both the manufacturing process and the product or its markets (Sancho *et al.*, 1999).

Sensory analysis of food is intimately linked to the concept of sensory quality; their importance and methods used in their measurement and control have evolved in parallel to technological development of the food industry. Sensory analysis does not act only in the selection of premium materials, but it is also useful in the control of the manufacturing process, as well as adaptation of the product to its final market (Sancho *et al.*, 1999). Pomegranate acceptability by consumers and producers depends basically on a combination of quality external attributes such as size, shape and rind color and internal attributes such as color, total soluble solids, sugars and organic acids (Holland *et al.*, 2009). These attributes mainly depend on cultivar and maturity of the fruit. Early-harvest may impede the full development of characteristic color, taste and aroma of pomegranates, while late-harvest fruits exhibit a reduced shelf life (Kulkarni and Aradhya, 2005). Koppel and Chambers (2010) determined a sensory lexicon and the main sensory attributes of 33 commercial pomegranate juices, and found large variations among the different juices. Some of those differences, such as astringency, bitterness, or toothetch might be caused by processing (use of clarification, concentration, pasteurization, etc.), presence/absence of some preservatives, or presence/absence of added flavorings in the juices.

1.10. Current problems in the pomegranate sector

1.10.1 High demand, limited production

In recent years, consumers have been hearing about all health benefits that pomegranate possess. This has caused an increase in the demand for fresh fruits, as well as for pomegranate-based products, such as juices, jams, jellies, capsules etc.

The main pomegranate producers are: Central Asia (India, Iran, China, Pakistan, Iraq and Afghanistan), Mediterranean countries (Turkey, Syria, Egypt, Tunisia and Israel), European countries (Spain, Great Britain), and USA. Most of the

pomegranate production is intended to satisfy exporting needs; more than 50 % of the total production is exported (Melgarejo *et al., 2010*).

Traditionally, the main export destination for the Spanish pomegranates is England, followed by Holland and Germany (about 30 % of total exports). About 10-20 % is destined for the agro-food industry, manufacturing basically juices, grenadine, syrup, jellies and it is also used in the pharmaceutical industry (Ernst, 2010). The rest of the production is consumed locally in fresh. The 95 % of pomegranate production in Spain comes from Alicante and this agricultural sector involves each year about 4,000 direct and indirect jobs during the growing season. According to the Association of Producers and Distributors of Elche pomegranates, the production in reached 45,000 t, which means 3 % more than in 2012 (http://www.granadaselche.com/asociacion).

1.10.2 Juice adulteration

The adulteration of pomegranate juice is increasingly present due to various factors, such as high product demand and shortage of fruit for juice manufacturing. Other factors could be that lead to juice adulteration are: i) the need to reduce production expenses, using cheaper fruits, including low quality fruits and other fruits with similar flavor, ii) the need to mask the astringent and bitter taste characteristic of some pomegranate juices, and iii) the need to improve the pale color of some juices due to the absence or reduced amounts of anthocyanins (Zhang *et al.*, 2009). This may cause the consumer to purchase products that promise to be 100 % pure pomegranate juice when actually they are a mixture of pomegranate with other fruits.

Some methods of adulteration consist in the addition of sugar to mask the astringency of tannins and adding fruit juices deeply colored to mimic the natural color of the pomegranate juice. Adulteration of juice depends on the similarities in chemical composition, availability and price of adulterant (Pushparajah and Nicholas, 2006). Adulteration of a commercial juice can be identified if their chemical composition deviates significantly or is outside the range of a pure juice, and whether its chemical composition is outside the ranges given by guides or standards such as AIJN (Association of the Industry of Juices and Nectars from Fruits and Vegetables of the EEC) (Bakir *et al.,* 2007). For instance, organic acids profile can be used to detect adulteration of pomegranate juice with other juices (Ehling and Cole, 2011); however, the relative ratios among the acids strongly depend on the pomegranate cultivar and

the ripening stage. There is some controversy about the presence of sucrose in pomegranate juices. On one hand, authors such as Mena et al. (2011) claim that the presence of sucrose should be considered a quality parameter in freshly squeezed pomegranate juice. On the other hand, authors such as Zhang et al. (2009) conclude that detection of sucrose indicates adulteration with cane sugar or other sucrose sources.

During commercial processing pomegranate juice, sucrose should not be present due to the isomerase activity (Zhang *et al.,* 2009), while other researchers (Mena *et al.*, 2011) propose that the presence of low levels of sucrose should be considered as an indicator of juice freshness. As for potassium, the Department of Agriculture U.S.A. (USDA, 2012) indicates that in the pomegranate juice should to have a 2500 mg L^{-1}. For the amino acid proline, there are a few data on the amount that must be present in pomegranate juice and there is also a lot of discrepancy which is postulated by various authors (Niedmann, 1976; Ting and Rouseff, 1979). For instance, Zhang et al. (2009) concluded that proline contents above 25 mg L^{-1} are indicative of addition of grape products, while Hanim and Nesrin (2009) found higher proline contents in fresh pomegranate juices.

Chapter 2. Objectives

2. OBJECTIVES

2.1.1 Main objective

The aim of this Ph.D. dissertation was to evaluate the changes of the physico-chemical, functionality and sensory properties of Spanish pomegranate along their growing season and industrial processing. A second aim of this work was to identify new pomegranate co-products or wastes of interest for the food and/or pharmaceutical industries.

2.2. Specific objectives

- Evaluate the potential of pomegranate fruits removed **during thinning** as a source of bioactive compounds (organic acids, minerals, punicalagins, and ellagic acid) and the antioxidant activity as affected by the pomegranate cultivar.

- Evaluate the changes of the main morphological and physicochemical parameters **during different stages of maturation** and the effect of the position of the fruits within the tree on the main quality parameters.

- Evaluate the comparative potential of **thinning and ripe** pomegranate fruits as source of bioactive compounds.

- Describe the **sensory** profiles of Spanish pomegranate cultivars, and to use descriptive sensory analysis to determine the best commercial option for pomegranate fruits, either fresh consumption or juice processing.

- Determine the effect of industrial **processing** and pomegranate juice adulteration on physico-chemical characteristics and bioactive compounds as well as the change that occurs with pomegrante juice to be altered by mixing with other juices at different concentrations.

Chapter 2. Materials and Methods

3.   MATERIALS AND METHODS

In this section a summary of the main vegetal materials used is described together with the different methods for sample processing and the main analytical protocols and techniques used to reach the targeted objectives.

3.1.   Plant material and samples

Pomegranate fruits

Eighteen different cultivars of pomegranate were collected in one of the most important European pomegranate gene banks, which is located at the Experimental Field Station of the Universidad Miguel Hernández de Elche in the province of Alicante, Spain (02°03′50″E, 38°03′50″N, and 25 masl). The orchard was established in 1992; hence, trees are now 20 years old. Pomegranate trees were trained into a vase-shaped system and planted at a spacing of 4 m × 3 m. They are drip irrigated, and standard cultural practices are performed (pruning, thinning, fertilization and pest control treatments). Other seven commercial pomegranate fruits collected at commercial ripening in October 2011; five commercial cultivars purchased in the farmers' market of the area, and fruits from 2 commercial cultivars grown in the Canary Islands (Spain) were studied to compare with the fruits from the germplasm (**Table 8)**.

The pomegranate fruits were collected at different ripening stages:

a) Thinning: Last week of June 2013. Usually, pomegranate thinning is conducted at the stage of young fruit (Fleckinger code I; BBCH code 71), this is equivalent to 35-40 days after the trees flowered (Melgarejo *et al.*, 1997). 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. The fruits collected during thinning stages were used for the publication 1 and 6.

b) Three ripening stages since July to the beginning of October: (i) R1 small size (<70 g), green and fully unripe fruits, (ii) R2 medium size (120-250 g), light red but still unripe fruits, and (iii) R3 large size (>300 g), reddish and ripe fruits. The fruits collected during these three ripening stages were used for the publication 2 and 6.

These cultivars are showed in **Table 8**; each one of them has been classified as sweet, sour-sweet, or sour cultivar.

Table 8. Twenty five different cultivars of pomegranate fruits.

Abbreviation	Cultivar	Origin	Type
BO1	*Borde de Ojós*	UMH Germplasm Bank	Sour
BA1	*Borde de Albatera*	UMH Germplasm Bank	Sour
BBE1	*Borde de Beniel*	UMH Germplasm Bank	Sour
CRO1	*Casta del Reino*	UMH Germplasm Bank	Sweet
ME1	*Mollar de Elche*	UMH Germplasm Bank	Sweet
ME2	*Mollar de Elche*	UMH Germplasm Bank	Sweet
ME14	*Mollar de Elche*	UMH Germplasm Bank	Sweet
ME17	*Mollar de Elche*	UMH Germplasm Bank	Sweet
MA1	*Mollar de Albatera*	UMH Germplasm Bank	Sweet
MO4	*Mollar de Orihuela*	UMH Germplasm Bank	Sweet
VA1	*Valenciana de Albatera*	UMH Germplasm Bank	Sweet
VA11	*Valenciana de Albatera*	UMH Germplasm Bank	Sweet
PTO3	*Piñón Tierno de Ojós*	UMH Germplasm Bank	Sour-sweet
PTO5	*Piñón Tierno de Ojós*	UMH Germplasm Bank	Sour-sweet
PTO7	*Piñón Tierno de Ojós*	UMH Germplasm Bank	Sour-sweet
PTO8	*Piñón Tierno de Ojós*	UMH Germplasm Bank	Sour-sweet
PTO10	*Piñón Tierno de Ojós*	UMH Germplasm Bank	Sour-sweet
ADO4	*Agridulce de Ojós*	UMH Germplasm Bank	Sour-sweet
Commercial cultivars			
HIZC	*Hizcaznar*	Alicante	Sour
WOND	*Wonderful*	Alicante	Sour
M50	*Mollar de Elche*	Alicante	Sweet
VAcom	*Valenciana*	Alicante	Sweet
Mcom	*Mollar*	Alicante	Sweet
FV1	*Mollar*	Canary Island	Sweet
FV2	*Mollar*	Canary Island	Sweet

Also, the second publication contains the results about the effect of the position within the pomegranate trees; (i) East orientation: highly exposed to the sunlight ("sun"), and (ii) West orientation: poorly exposed to the sunlight ("shadow").

Commercial juice

Since Spain is one of the main producers of pomegranate juice within the European Union, a pomegranate juice prepared using the most widely grown pomegranate cultivar in Spain, *Mollar de Elche*, was selected for this study. Grape and peach juices were chosen for the adulteration of pomegranate juice. The commercial juices used were (1) pomegranate juice (PgJ) from VitalGrana (Catral, Alicante, Spain), (2) grape juice (GJ) from Premium (Murcia, Spain) and (3) peach juice (PJ) from Rostoy (Murcia, Spain). The pomegranate juice under study (VitalGrana) is prepared by mixing *Mollar de Elche* and *Wonderful* juices at a ratio of 4:1 (v/v); these two pomegranate cultivars are the most widely grown in Spain and in the USA respectively. Consequently, this pomegranate juice can be considered as representative of a high percentage of the pomegranate juices being sold in international markets. The grape and peach cultivars used for manufacturing the studied juices were *Merlot* and *Baby Gold* respectively; these two cultivars are also widely cultivated throughout the world.

Commercial juices were selected because the protocol developed in this study should be applied to control the authenticity of such juices; however, it was essential to prove that the juices were 100 % pure and no initial adulteration was found. Consequently, the commercial juices were supplied directly (October 2012) by three different juice companies with cooperation agreements with our university and research group; for instance, the Food Quality and Safety group of the Universidad Miguel Hernández de Elche has characterized all products from VitalGrana and established their nutritive, functional and sensory values and shelf-life (http://www.vitalgrana.com). As a result of all the above, we are completely sure that the juices were 100 % pure products of pomegranate, grape and peach respectively.

3.2. Sample preparation

<u>Pomegranate fruit</u>

After selecting the pomegranates, all fruits were immediately transported to the laboratory. When the pomegranates are unripe (fruits that coming from thinning), it is impossible to separate the arils from the rest of the fruit. Thus unripe pomegranates were cut in half and the following chemical parameters were analyzed on the juice obtained by manually squeezing each half of the thinning fruits total soluble solids (TSS), titratable acidity (TA), pH, and profiles of organic acids and sugars. The same chemical parameters were analyzed for ripe pomegranates, but in this case, each husk was carefully cut at the equatorial zone with a sharpened knife, and then arils were manually extracted. Chemical composition was immediately determined on the juice obtained by squeezing the arils. The juice was filtered through filter paper. After extracting the juice, fruits (rind, carpelar membranes and squeezed arils) were dried in an hot air oven (Selecta, Barcelona, Spain) at 60 °C until constant weights were reached (36 h) for mineral analysis. For antioxidant activity (AA), total polyphenols content (TPC), α-punicalagin, β-punicalagin and ellagic acid analysis, the pomegranates were immediately frozen in liquid nitrogen and later freeze dried in an Alpha 2-4 freeze drier (Christ Alpha 2-4; Osterode am Harz, Germany) for 24 h at a pressure reduction of 0.220 mbar. The temperature in the drying chamber was -25 °C while the heating plate reached 15 °C. At the end of freeze drying, the samples were powdered and packed in vacuum for analysis.

<u>Commercial juice</u>

Each commercial juice (pomegranate, grape and peach) was first analyzed without any mixing. Later, pomegranate juice was adulterated with grape or peach juice at concentrations (v/v) of 10, 25 and 50 % of grape juice and 5 and 10 % of peach juice. The maximum values of these concentrations were below the detection thresholds established by a trained sensory panel with wide expertise in sensory analyses. Thresholds were established at 55 and 12 % for grape and peach juices respectively; at these concentrations, 50 % of the panelists were able to detect a significant difference from the control sample, pure pomegranate juice. Juice blends were stored at 4 °C until 30 min before analyses, which were conducted within 1 week.

The following parameters were analyzed in pure and juice blends: organic acids, sugars, minerals (Ca, Mg, K, Na, Fe, Cu, Mn and Zn), proline and volatile composition.

3.3. Morphological parameters

In the pomegranate fruit, the next parameters were measured: maximum width or fruit diameter, FD (mm) and fruit length from calyx to base, FL (mm) using a digital caliper/caliper (model CD-15 DC; Mitutoyo (UK) Ltd, Telford, UK) with 0.01 mm accuracy; fruit weight, FW (g) using a precision weighing balance (Mettler AJ50, Goettingen, Germany) with an accuracy of 0.0001 g.

3.4. Total soluble solids, pH and total titratable acidity

In pomegranate fruits, total soluble solids (TSS) were measured with a digital Atago refractometer (model N-20; Atago, Bellevue, Wash., U.S.A.) at 20 °C with values being expressed as °Brix. The titratable acidity (TA) and pH was determined by acid-base potentiometer (877 Titrino plus, Metrohm ion analyses CH9101, Herisau, Switzerland), using 0.1 N NaOH up to pH 8.1, values were expressed as g citric acid/L. Finally, maturity index (MI), which is a ratio of TSS to TA, was also calculated for each sample. Analyses were run in three and five replications and results were expressed as g citric acid/L.

3.5. Organic acids and sugars profile

Organic acids and sugars profile were quantified according to Carbonell-Barrachina et al. (2012). The juices obtained after (i) manually squeezing the unripe fruits cut in half and (ii) by squeezing the arils of ripe fruits were diluted using ultra-high-purity deionized water (1:10) and centrifuged at 15 000 rpm for 20 min; commercial juices (pomegranate, grape and peach) were centrifuged at 10 000 rpm for 20 min (Sigma 3–18K, Osterode and Harz, Germany).

Then, 1 mL of supernatant was filtered through a 0.45 μm Millipore filter and 10 μL were injected into a Hewlett-Packard high-performance liquid chromatography (HPLC) series 1100 (Wilmington Del., U.S.A.). A column (Supelcogel TM C-610H column 30 cm × 7.8 mm) and a pre-column (Supelguard 5 cm x 4.6 mm, Supelco, Inc., Bellefonte, PA) were used for the analyses of both organic acids and sugars. The

elution buffer consisted of 0.1 % phosphoric acid and organic acid absorbance was measured at 210 nm using a diode-array detector (DAD). These same HPLC conditions (elution buffer, flow rate and column) were used for the analysis of sugars. The detection was conducted using a refractive index detector (RID). Standards of organic acids (oxalic, citric, tartaric, malic, quinic, shikimic, and fumaric acids) and sugars (glucose, fructose and sucrose) were obtained from Sigma (Poole, Dorset, UK).

Calibration curves with a concentration range between 1 and 10 g L^{-1}, were used for the quantification of organic acids and sugars, and showed good linearity ($R^2 \geq 0.999$). Analyses were run in three and five replications and results were expressed as mean ± standard error and units in g L^{-1}.

3.6. Minerals analysis

Pomegranate fruit

The dried material of pomegranate (0.5 g) was taken to the muffle furnace (Hobersal, Barcelona, Spain) model 12 PR/300 series 8B and digested at 450 °C for 6 h. Ashes were mixed with 4 mL of HCl (50 % v/v) and transferred to volumetric flask in dilutions 1:25 and 1:50 were prepared using ultra-high-purity deionized water. Samples were stored at 4 °C until analysis was performed.

Commercial juice

Pure juices and juice blends (15 mL) were digested for 2 h at a temperature below 130 ºC in a multi-place digestion block (Block Digest 20, Selecta, Barcelona, Spain) using 5 mL of 65 % HNO_3. Samples were left to cool to room temperature and then transferred to volumetric flasks. Dilutions of 1:10 and 1:50 (v/v) were prepared using ultrahigh-purity deionized water. Samples were stored at 4 ºC until analysis.

For all samples, the determination of Ca, Mg, K, Na, Cu, Fe, Mn and Zn in previously mineralized samples was performed using a Unicam Solaar 969 atomic absorption-emission spectrometer (Unicam Ltd., Cambridge, U.K.). K and Na were analyzed using atomic emission, while the rest of elements were analyzed by atomic absorption. Instruments were calibrated using certified standards. In each analytical batch, at least two reagents blanks were included to assess precision and accuracy for chemical analysis. Calibration curves, with a concentration range between 0 and 10.0 mg L^{-1} for Ca, Mg, K, and Na and between 0 and 2.0 mg L^{-1} for Fe, Cu, Mn, and Zn,

were used for the quantification of minerals, and showed good linearity ($R^2 \geq 0.997$). Analyses were run in three and five replications and results were expressed as mean ± standard error and units in mg kg^{-1} dw (pomegranate fruit) and mg L^{-1} (juice).

3.7.   Determination of proline

Proline was quantified by the colorimetric method recommended by the International Federation of Fruit Juice Producers (IFU, 2005). A solution of ninhydrin in ethylenglycol monomethyl ether (30 g/L) was prepared. Then 1 mL of juice sample (pomegranate and commercial juice), 1 mL of formic acid (98 %) and 2 mL of the ninhydrin solution were added, mixed and placed for 15 min in a bath with boiling water. After this time, 20 mL of butyl acetate (99.5 %) were added to extract the color into the organic phase. Then, the solution was filtered and dried using filter paper containing 0.2 g of anhydrous Na_2SO_4. After 15 min, the absorbance of the organic phase was measured at 509 nm in a UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France).

Calibration curves, in the range 0-50 mg L^{-1}, were used for the quantification of proline and showed good linearity ($R^2 > 0.999$). Analyses were run in triplicate and the results were expressed as mg L^{-1} .

3.8   Color (*L**, *a**, *b** parameters)

Color measurements were performed according to Manera et al. (2012), using a Minolta C-300 Chroma Meter (Minolta Corp., Osaka, Japan) coupled to a Minolta DP-301 data processor. This colorimeter uses an illuminant D_{65} and a 10° observer as references. Color was assessed according to the *Commission Internationale de l'Éclairage* (CIE) and expressed as *L**, *a**, *b**. *L** indicates lightness, taking values within the range 0–100 (black-white, respectively), and *a** and *b** are the chromatic coordinates, green-red and blue-yellow coordinates, respectively. *a** takes positives values for reddish colors and negatives for the greenish ones, whereas *b** takes positive values for yellowish colors and negative values for bluish ones. Finally, *C** is Chroma [$C^* = \sqrt{(a*^2) + (b*^2)}$], 0 is at the center of a color sphere and increases according to the distance from the center. Hue (*H**) is the angular component of the polar representation of the product color, while chroma is the radial component.

External color was measured directly in the pomegranate fruits as affected by the fruit position within the trees. For color measurement 6 fruits were used and 3 readings were taken along the 360° equatorial perimeter of each fruit; thus, color values reported were the mean of 18 readings per treatment. Internal color was measured in the juice obtained by squeezing the pomegranate arils and using the Minolta adaptor for liquid products. Internal color results (mean ± standard error) were the mean of 6 determinations for each sample.

3.9   Volatile compounds

Extraction procedure

Head space solid phase micro extraction (HS-SPME) was the method selected to study the volatile composition of the juices under analysis. After several preliminary tests to optimize the extraction system, 10 mL of juice was hermetically placed in a 50 mL vial with a polypropylene cap and a PTFE/silicone septum; the juice/headspace ratio was approximately 1:4 (v/v). A magnetic stirring bar was added together with NaCl (150 g/L) and the vial was placed in a water bath with temperature control and stirring. The vial was equilibrated for 15 min at 40 °C, and then a 50/30 μm DVB/CAR/PDMS fiber was exposed to the sample headspace for 50 min at 40 °C. This type of fiber was chosen for its high capacity to trap fruit volatile compounds (Ceva-Antunes *et al.,* 2006). A similar extraction procedure was previously carried out with tomatoes by Alonso *et al.* (2009) and with pomegranates by Melgarejo *et al.* (2011) and Vázquez-Araújo *et al.* (2011b).

After sampling, desorption of the volatile compounds from the fiber coating was carried out in the injection port of the gas chromatography/mass spectrometry (GC/MS) system for 3 min.

Chromatographic analysis

Isolation and identification of the volatile compounds were performed using a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu QP-5050A mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The GC/MS system was equipped with a TRACSIL Meta.X5 column (95 % dimethylpolysiloxane/ 5 % diphenylpolysiloxane, 60 m × 0.25 mm, 0.25 μm film thickness; Teknokroma S. Coop. C. Ltd, Barcelona, Spain). Analyses were carried out using helium as carrier gas at a

column flow rate of 0.6 mL min^{-1} in a split ratio of 1:5 and the following program: 80 °C for 0 min; increase at 3 °C min^{-1} from 80 to 210 °C and hold for 1 min; increase at 25 °C min^{-1} from 210 to 300 °C and hold for 3 min. The temperatures of the injector and detector were 230 and 300 °C respectively. Most compounds were identified using three different analytical methods, namely (i) retention indices (NIST, 2013), (ii) GC/MS retention times (authentic chemicals) and (ii) mass spectra (standards and Wiley229 spectral database). Identification was considered tentative when it was based on only mass spectral data. The volatile studies were conducted in triplicate. The concentration of each compound is expressed as % of the total arbitrary area units.

3.10. Total polyphenols content

Total polyphenols content (TPC) was quantified using Folin-Ciocalteu reagent (Singleton *et al.,* 1999). Briefly, the sample was prepared in two different ways: (i) freeze-dried fruits (0.5 g) were mixed with 10 mL of extract MeOH/water (80:20 v/v) containing 2 mM NaF and (ii) 5 mL of pomegranate juice was homogenized in 5 mL of the same extract. Then, the sample was centrifuged at 15000 rpm for 15 min. Later, 50 µL of sample were mixed with 2.5 mL of Folin-Ciocalteu reagent (1:10 v/v), 450 µL of phosphate buffer (pH 7.8) and 2 mL of sodium carbonate (75 g L^{-1}). The samples were left in a water bath at 50 °C for 5 min. Absorption was measured at 760 nm using a UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France).

Calibration curves, with a concentration range between 0 and 0.25 g GAE L^{-1}, were used for the quantification of TPC, and showed good linearity (R^2≥0.996). Analyses were run in three replications and results were expressed as mean ± standard error and units in mg GAE L^{-1} (natural pomegranate juice) and g GAE kg^{-1} dw (pomegranate fruit).

3.11. Identification and quantification of punicalagin isomers and ellagic acid

Punicalagins (α and ß) and ellagic acid contents were determined in freeze-dried fruits (0.3 g) diluted with 7 mL of MeOH/water (80:20 v/v) and 1 % acetic acid and then centrifuged at 15000 rpm for 20 min. Supernatants were filtered through a 0.45-µm Millipore filter and then injected into a Hewlett-Packard HPLC series 1200 equipped with a diode-array detector. Each sample (20 µL) was analyzed on a LiChroCART 100

RP-18 reversed-phased column (250 ×4 mm, particle size, 5 µm; Merck, Darmstadt, Germany) equipped with a pre-column C18 (LiChrospher 100 RP-18, 5 µm; Merck, Darmstadt, Germany) using a mobile phase of 1 % acetic acid in ultra-high-purity deionized water (solvent A) and 1 % acetic acid in MeOH (solvent B). Elution was performed at flow rate of 1 mL/min using a gradient starting with 1 % B for 5 min, and increasing to 60 % B at 40 min. Punicalagins and ellagic acid detection were conducted at 360 nm. For the identification of punicalagins and ellagic acid, absorption spectra and retention times were employed and compared with those obtained from the chemical standards.

Standard curves for pure punicalagins (Chengdu Biopurify Phytochemicals Ltd. Sichuan, China), with a concentration range between 0.05 and 0.80 g L^{-1}, as well as for ellagic acid (Tocris Bioscience, Ellisville, MO, USA), with a concentration range between 0.0025 to 0.0200 g L^{-1}, were used for quantification. Results for individual isomer punicalagins (α and β) and ellagic acid were expressed as mean ± standard error and units in g kg^{-1} dw. Analyses were run in five replications.

3.12. Extraction procedure for identification and quantification of phenolic compounds and antioxidant activity (DPPH, ABTS and FRAP methods)

A methanol extract was prepared with 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 15,000 rpm for 10 min. The pomegranate juice (1 mL) was diluted with 5 ml of MeOH/water (80:20 v/v) and then centrifuged at 15000 rpm for 10 min.

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

Identification and quantification of polyphenols of pomegranate fruits extracts was carried out using an Acquity ultra performance LC system equipped with a photodiode detector (PDA; UPLC) 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^{-1}. 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. Ellagic acid derivatives compound was 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 phenolic compounds 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. Retention times (R_t) and spectra were compared with those of pure standards. Calibration curves at concentrations ranging from 0.05 to

5 mg mL^{-1} (R^2 ≤ 0.9998) were made from ellagic acid. All analyses were done in triplicate. Results were expressed as milligrams per 100 g dry matter (dm).

3.14. Antioxidant activity

3.14.1 DPPH, ABTS and FRAP methods

The free scavenging activity was evaluated using the DPPH (radical 2,2-diphenyl-1-picrylhydrazyl) method as described by Brand-Williams et al. (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).

The ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation and ferric reducing antioxidant power (FRAP) methods were also employed according to Re et al. (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 for DPPH, ABTS and FRAP methods were in the range 0.01–5.00 mmol Trolox L^{-1} were used for the quantification of the three methods of antioxidant activity showing good linearity (R^2≥0.998). The analyses were run in three and five replications and results were expressed as mean ± standard error and units in mmol Trolox L^{-1} and mmol Trolox kg^{-1} dw.

3.14.2 ORAC method

The antioxidant activity by Oxygen Radical Absorbance Capacity (ORAC) method was evaluated according to Ou et al. (2001) . Briefly, each sample (0.1 mL) was diluted with phosphate (K$_2$HPO$_4$ + Na$_2$HPO$_4$) 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 kg^{-1} dry matter (dm).

3.15. Sensory analysis

The descriptive sensory analysis was conducted by four highly trained panelists from the Sensory Analysis Center (Manhattan, KS). Each of the panelists had more than 1000 h of testing experience with a variety of food products. For the current study, the panelists received further orientation on fresh and processed pomegranates. The panelists travelled from Kansas (USA) to Spain to conduct the study.

The samples (pomegranate arils) were served into odor-free, disposable 90 mL covered plastic cups, (Sweetheart Cup Co., Inc., Owings Mills, MD) for the evaluation. All samples were served at room temperature. For each sample, the panel evaluated 5 subsamples (A, B, C, D, and E) coded with the three digits of the sample and a letter (e.g. sample: 997a, 997b, 997c, 997d and 997e). Unsalted crackers, cheese, and distillated water were used to clean palates between samples.

Ten sessions of 2 h were held for the samples evaluation. Two samples (a total of 10 subsamples) were evaluated per session. The panel started working with the lexicon reported by Koppel and Chambers (2010) for pomegranate juices, but some attributes, definitions and/or references were removed, included, and/or adapted to pomegranate fruit evaluation. A modified consensus profile method, which uses a numerical scale where 0 represents none and 15 extremely strong with 0.5 increments, was used (Adhikari *et al.*, 2011; Talavera-Bianchi *et al.*, 2010; Koppel and Chambers, 2010). The panelists independently scored each subsample and also provided a "representative score" for each sample (not the average, but the score they considered representative for that singular sample/cultivar). The testing room was at ~21 °C; the illumination was a combination of natural and non-natural (fluorescent) light.

3.16. **Statistical analysis**

One-way analysis of variance (ANOVA) and multiple-range tests were used for comparison of the pomegranate fruit and juices results. The method used to discriminate among the means (Multiple Range Test) was the Tukey's procedure. Differences were considered statistically significant at $p \leq 0.05$. Statistical analyses were performed using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD). Principal component analysis (PCA) was carried out using the Unscrambler Version 9.7 (Camo Software, Oslo, Norway). Figures on antioxidant activity (ABTS, DPPH, FRAP and ORAC) data was carried out using SigmaPlot Version 11.0 (Systat Software Inc.).

Sensory data (using the 5 subsamples as replicas) were subjected to statistical analysis using SPSS® (version 12.0; SPSS Inc., Chicago, Ill.), for analysis of variance (ANOVA) and Tukey's honestly significant differences (HSD) for post-hoc mean separation. Principal Components Analysis (PCA) was used for the data analysis on the consensus profiles in order to study patterns, if any, among cultivars. Only flavor and mouthfeel attributes were used for the analysis. Representative scores were used for this analysis, avoiding the use of attributes which appeared in single fruits (subsamples) but were not typical of the cultivar. Also, the Statistical Analysis System version 8.2 (SAS, Cary, NC, 2001) was used for clustering the samples and for the correlation analysis, using Pearson correlation coefficients. Clustering of the samples was done by using the CLUSTER procedure (Ward's Minimum Variance Cluster Analysis). The number of clusters was set according to the eigenvalues of the correlation matrix (>1). Again, only flavor and mouthfeel representative scores were used for the clustering analysis of the samples.

Chapter 4. Publications

PUBLICATION 1

Bioactive compound composition of pomegranate fruits removed during thinning

Nallely Nuncio-Jáuregui, Sandra Munera-Picazo, Ángel Calín-Sánchez, Aneta Wojdyło, Francisca Hernández, Ángel A. Carbonell-Barrachina

Accepted Manuscript

Title: Bioactive compound composition of pomegranate fruits removed during thinning

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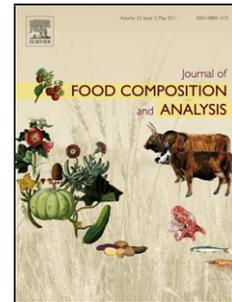
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1   **HIGHLIGHTS**

2

3   • Punicalagin content is similar in thinning pomegranate fruits than in mature rind

4   • Polyphenol content in thinning pomegranate fruits is higher than mature fruits

5   • Bioactive compounds are affected by cultivar in thinning pomegranate fruits

6   • Thinning fruits, up to now a waste, are a potential source of bioactive compounds

7

1

7   **Original research article**

8   **Bioactive compound composition of pomegranate fruits removed**
9   **during thinning**

10   **Running title:** Pomegranate fruits thinning

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23   **Abstract**

24   Thinning consists of reducing fruit load at immature stage and thus allowing remaining fruits

25   to develop to their maximum size and quality. The waste material produced during this

26   farming practice was characterised in 9 pomegranate cultivars, by evaluating: weight, size,

27   maturity index, pH, organic acids and sugars profiles, contents of minerals, punicalagin, and

2

Nuncio-Jáuregui

28 ellagic acid, total polyphenols and antioxidant activity. Citric and quinic were the main

29 organic acids. Potassium was the predominant mineral, reaching up to 11 g kg^{-1} dry weight

30 (dw). Total polyphenol content ranged from 777 to 1660 g GAE kg^{-1} dw, α-punicalagin from

31 101 to 195 and β-punicalagin from 80.1 to 111 g kg^{-1} dw. The antioxidant activity was

32 assessed by three methods and its values varied from 2923 to 4486 for ABTS, from 3153 to

33 4685 for FRAP, and from 2075 to 2934 mmol Trolox kg^{-1} dw for DPPH. Pomegranate

34 thinning fruits, especially sour-sweet cultivars, are rich in bioactive compounds, with a

35 potential use in the food, chemical and pharmaceutical industries.

36 **Keywords:** Antioxidant activity; Minerals; Organic acids; *Punica granatum*; Punicalagins;

37 Total polyphenols; Horticultural practices and nutrition; Food composition; Food analysis

1 Introduction

39 Pomegranate fruits grow in warm climates and require high temperatures for ripening. South-

40 eastern Spain offers optimal conditions to produce good quality fruits (Hernandez et al.,

41 2012). Spain is the greatest European pomegranate producer (FAO, 2013) and its production

42 is mainly located in the province of Alicante (92%), in particular the cities of Elche,

43 Crevillente and Albatera (Melgarejo et al., 2010).

44 Fruit trees often set more fruit than they can adequately support and develop. Excessive fruit

45 compete among each other for carbohydrates and remain small. This competition can also

46 weaken the tree and make it more susceptible to pests and sun damage. Besides, less crowded

47 fruit receive more sunlight and the fruit colour and flavour can be improved (Ingels et al.,

48 2001). Thinning is an agricultural practice, which takes place at an immature stage of the

49 fruits, at which a proportion of the fruits is removed to benefit the development and quality of

50 the remaining fruits (Melgarejo et al., 2010). In pomegranates, as in other fruits, such as

3

51   peaches, apricots or loquats, this operation is performed to remove small and irregular fruit

52   but mainly to obtain fruits with the size and quality required by the market (Melgarejo et al.,

53   2010; Missang et al., 2011; Njoroge and Reighard, 2008; Hueso et al., 2003). In the Spanish

54   pomegranate trees, this practice is conducted in the first week of June and should be repeated

55   after 20–30 days (end of June or early July) (Melgarejo et al., 2010). Depending on the

56   phenological stage of the fruits at thinning, from 7–8 to 12–15 kg per tree could be removed.

57   According to our calculations, immature fruits removed during thinning in the Alicante

58   province can reach a weight close to 2500 t. This value represents approximately 10% of the

59   total pomegranate production, 22311 t in 2010 (MARM, 2010). After thinning the fruits

60   removed from the trees are left to spoil in the soil and the farmer does not get any direct

61   payment for this expensive (labour-intensive) farming practice.

62   Pomegranates are a well-known source of many valuable substances, such as organic acids,

63   hydrolysable tannins and phenolic compounds (Gil et al., 2000; Poyrazoglu et al., 2002; Mena

64   et al., 2011), all of which show high antioxidant activity (García-Alonso et al., 2004) and

65   provide health benefits against cancer, cardiovascular and other health diseases (Aviram and

66   Dornfeld, 2001; Sumner et al., 2005; Malik et al., 2005; Basu and Penugonda, 2009). In

67   recent years, this relationship between health and pomegranate has created a great demand for

68   pomegranate-based products (juices, jams, etc.).

69   On the other hand, there are no scientific data describing the chemical composition of

70   pomegranate fruits removed during thinning (hereafter, "pomegranate thinning fruits") as a

71   source of bioactive compounds. Therefore, it is important to know the exact composition

72   (total soluble solids, titratable acidity, pH, contents of organic acids, sugars, macro- (Ca, Mg,

73   K, and Na) and micro-elements (Fe, Zn, Cu, and Mn), punicalagins and ellagic acid, total

74   polyphenol content and antioxidant activity) of the pomegranate thinning fruits to evaluate

75   their possible application or use in both the food and pharmaceutical industries. Thus, the aim

4

76   of the present study was to evaluate the potential of pomegranate fruits removed during

77   thinning as a source of bioactive compounds (organic acids, minerals, punicalagins, and

78   ellagic acid) and the antioxidant activity as affected by the pomegranate cultivar. Nine

79   cultivars were evaluated and represented sour, sour-sweet and sweet pomegranate fruits.

80   ## 2   Materials and methods

81   ### 2.1   Plant material and sample processing

82   Fruits of nine different cultivars of pomegranate were collected in the last week of June 2013

83   in one of the most important European pomegranate gene banks, which is located at the

84   experimental field station of the Miguel Hernandez University in the province of Alicante,

85   Spain (02°03'50''E, 38°03'50''N, and 25 masl). The orchard was established in 1992; hence

86   the trees are now 20 years old. Pomegranate trees were trained to the vase-shaped system and

87   planted at a spacing of 4 m × 3 m. They were drip irrigated, and standard cultural practices

88   were performed (pruning, thinning, fertilisation and pest control treatments).

89   The following cultivars were selected: 3 sour cultivars [*Borde de Albatera 1* ("BA1"), *Borde*

90   *de Orihuela 1* ("BO1"), *Borde de Beniel 1* ("BBE1")], 3 sour-sweet cultivars [*Piñón Tierno*

91   *de Ojós 5* ("PTO5"), *Piñón Tierno de Ojós 8* ("PTO8"), *Piñón Tierno de Ojós 10*

92   ("PTO10")], and 3 sweet cultivars [*Mollar de Elche 14* ("ME14"), *Mollar de Elche 17*

93   ("ME17") and *Valenciana 1* ("VA1")].

94   Thinning is conducted as a routine farming practice, generally from middle of June to the first

95   week of July. Usually, pomegranate thinning is conducted at the stage of young fruit

96   (Fleckinger code I; BBCH code 71), this is equivalent to 35–40 days after the trees flowered

97   (Melgarejo et al., 1997). At this stage about 7–8 kg of young fruits are removed from each

98   tree; only fruits weighing less than 100 g or having a diameter smaller than 60 mm are

5

99   removed. Following all the previously mentioned requirements, 10 fruits were selected from

100   those removed by the routine thinning practice; each fruit was considered a single replicate.

101   **2.2   Morphological parameters**

102   After selecting 10 fruits per cultivar, all fruits were transported to the laboratory and analyses

103   were performed immediately. For each fruit, the following parameters were measured:

104   maximum width or fruit diameter, FD (mm), and fruit length from calyx to base, FL (mm),

105   using a digital calliper/calliper (model CD-15 DC; Mitutoyo (UK) Ltd, Telford, UK) with

106   0.01 mm accuracy; fruit weight, FW (g) using a precision weighing balance (Mettler AJ50,

107   Goettingen, Germany) with an accuracy of 0.0001 g. Morphological parameters were

108   measured in five replications.

109   At this stage it is impossible to separate the arils from the rest of the fruit. Thus five unripe

110   pomegranates were cut in half and the following chemical parameters were analysed on the

111   juice obtained by manually squeezing each half of the thinning fruits: total soluble solids

112   (TSS), titratable acidity (TA), pH, and profiles of organic acids and sugars. After extracting

113   the juice, fruits (rind, carpelar membranes and squeezed arils) were dried in a hot-air oven

114   (Selecta, Barcelona, Spain) at 60 °C until constant weights were reached (36 h) for mineral

115   analysis. The other five pomegranates were immediately frozen in liquid nitrogen and later

116   freeze-dried in an Alpha 2-4 freeze drier (Alpha 2-4; Christ, Osterode am Harz, Germany) for

117   24 h at a pressure reduction of 0.220 mbar. The temperature in the drying chamber was -25 °C

118   while the heating plate reached 15 °C. At the end of freeze drying, the samples were

119   powdered and packed under vacuum. Then antioxidant activity (AA), total polyphenol

120   content (TPC), α-punicalagin, β-punicalagin and ellagic acid were analysed.

6

121 **2.3   Total soluble solids, pH and total titratable acidity**

122   Total soluble solids (TSS) were measured with a digital Atago refractometer (model N-20;

123   Atago, Bellevue, WA) at 20 °C with values being expressed as °Brix. The titratable acidity

124   (TA) and pH were determined by acid-base potentiometer (877 Titrino plus; Metrohm ion

125   analyses CH9101, Herisau, Switzerland), using 0.1 N NaOH up to pH 8.1; values were

126   expressed as g citric acid L^{-1}. Finally, maturity index (MI), which is a ratio of TSS to TA, was

127   also calculated for each sample. Analyses were run in five replications ($n = 5$).

128   **2.4   Organic acids and sugars**

129   Organic acids and sugars profile were quantified according to Carbonell-Barrachina et al.

130   (2012). The juices obtained after manually squeezing the immature fruits cut in half were

131   diluted using ultra-high-purity deionized water (1:10) and centrifuged at 15000 rpm for 20

132   min (Sigma 3–18K; Sigma. Osterode am Harz, Germany). Then, 1 mL of supernatant was

133   filtered through a 0.45-μm Millipore filter and 10 μL were injected into a Hewlett-Packard

134   (Wilmington DE). Series 1100 high-performance liquid chromatograph (HPLC). A column

135   (Supelcogel TM C-610H column 30 cm × 7.8 mm) and a pre-column (Supelguard 5 cm × 4.6

136   mm; Supelco, Bellefonte, PA) were used for the analyses of both organic acids and sugars.

137   The elution buffer consisted of 0.1% phosphoric acid and organic acid absorbance was

138   measured at 210 nm using a diode-array detector (DAD). These same HPLC conditions

139   (elution buffer, flow rate and column) were used for the analysis of sugars. The detection was

140   conducted using a refractive index detector (RID). Standards of organic acids (oxalic, citric,

141   tartaric, malic, quinic, shikimic, and fumaric acids) and sugars (glucose, fructose and sucrose)

142   were obtained from Sigma (St Louis, MO). Calibration curves, with a concentration range

143   between 1 and 10 g L^{-1}, were used for the quantification of organic acids and sugars, and

144   showed good linearity ($r^2 \geq 0.999$). Analyses were run in five replications and results were

145   expressed as mean ± standard error in g L^{-1}.

7

146   **2.5   Minerals**

147   Dried pomegranate (0.5 g) was taken to a muffle furnace (Hobersal, Barcelona, Spain) model

148   12 PR/300 series 8B and digested at 450 °C for 6 h. Ashes were mixed with 4 mL of HCl (50

149   % v/v) and transferred to a volumetric flask in dilutions 1:25 and 1:50, prepared using ultra-

150   high-purity deionized water. Samples were stored at 4 °C until analysis was performed.

151   Determination of Ca, Mg, K, Na, Cu, Fe, Mn and Zn in previously mineralised samples was

152   performed using a Unicam Solaar 969 atomic absorption-emission spectrometer (Unicam

153   Ltd., Cambridge, U.K.). K and Na were analysed using atomic emission, while the other

154   elements were analysed by atomic absorption.

155   Instruments were calibrated using certified standards. In each analytical batch, at least

156   two reagent blanks were included to assess precision and accuracy for chemical analysis.

157   Calibration curves, with a concentration range between 0 and 10.0 mg L^{-1} for Ca, Mg, K, and

158   Na and between 0 and 2.0 mg L^{-1} for Fe, Cu, Mn, and Zn, were used for the quantification of

159   minerals, and showed good linearity ($r^2 \geq 0.997$). Analyses were run in five replications ($n =$

160   5) and results were expressed as mean ± standard error in units of mg kg^{-1} dw.

161   **2.6   Total polyphenol content**

162   Total polyphenol content (TPC) was quantified using Folin-Ciocalteu reagent (Singleton et al.

163   1999). Briefly, freeze-dried fruits (0.5 g) were mixed with 10 mL of MeOH/water (80:20 v/v)

164   containing 2 mM NaF and then centrifuged at 15000 rpm for 15 min. Later, 50 μL of sample

165   were mixed with 2.5 mL of Folin-Ciocalteu reagent (1:10 v/v), 450 μL of phosphate buffer

166   (pH 7.8) and 2 mL of sodium carbonate (75 g L^{-1}). The samples were left in a water bath at 50

167   °C for 5 min.

8

168   Absorption was measured at 760 nm using a UV-Vis Uvikon XS spectrophotometer (Bio-Tek

169   Instruments, Saint Quentin Yvelines, France). Calibration curves, with a concentration range

170   between 0 and 0.25 g GAE L^{-1}, were used for the quantification of TPC, and showed good

171   linearity ($r^2 \geq 0.996$). Analyses were run in five replications ($n = 5$) and results were

172   expressed as mean ± standard error and units in g GAE kg^{-1} dw.

2.7   Punicalagin isomers and ellagic acid

174   Punicalagins (α and ß) and ellagic acid contents were determined in freeze-dried fruits (0.3 g)

175   diluted with 7 mL of MeOH/water (80:20 v/v) and 1% acetic acid and then centrifuged at

176   15000 rpm for 20 min. Supernatants were filtered through a 0.45-μm Millipore filter and then

177   injected into a Hewlett-Packard series 1200 HPLC equipped with a diode-array detector. Each

178   sample (20 μL) was analysed on a LiChroCART 100 RP-18 reversed-phased column (250×4

179   mm, particle size, 5 μm; Merck, Darmstadt, Germany) equipped with a C18 pre-column

180   (LiChrospher 100 RP-18, 5 μm; Merck, Darmstadt, Germany) using a mobile phase of 1%

181   acetic acid in ultra-high-purity deionised water (solvent A) and 1% acetic acid in MeOH

182   (solvent B). Elution was performed at flow rate of 1 mL min^{-1} using a gradient starting with

183   1% B for 5 min, and increasing to 60% B at 40 min. Punicalagins and ellagic acid detection

184   was conducted at 360 nm. For the identification of punicalagins and ellagic acid, absorption

185   spectra and retention times were employed and compared with those obtained from chemical

186   standards. Standard curves for pure punicalagins (Chengdu Biopurify Phytochemicals Ltd.

187   Sichuan, China), with a concentration range between 0.05 and 0.80 g L^{-1}, as well as for

188   ellagic acid (Tocris Bioscience, Ellisville, MO), with a concentration range between 0.0025 to

189   0.0200 g L^{-1}, were used for quantification. Results for individual isomer punicalagins (α and

190   β) and ellagic acid were expressed as mean ± standard error and units in g kg^{-1} dw. Analyses

191   were run in five replications ($n = 5$).

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192   **2.8   Antioxidant Activity (DPPH, ABTS and FRAP methods)**

193   For the antioxidant activity determination, a methanol extract was prepared with each sample

194   to be analysed. Freeze-dried fruits (0.5 g) were mixed with 10 mL of MeOH/water (80:20 v/v)

195   + 1 % HCl, sonicated at 20 °C for 15 min and left for 24 h at 4 °C. Then the extract was again

196   sonicated for 15 min, and centrifuged at 15,000 rpm for 10 min.

197   The radical scavenging activity was evaluated using the DPPH radical (2,2-diphenyl-1-

198   picrylhydrazyl) method, as described by Brand-Williams et al. (1995) with a modification in

199   the reaction time. Briefly, 10 μL of the supernatant were mixed with 40 μL of MeOH and

200   added to 950 μL of DPPH solution. The mixture was shaken vigorously and placed in a dark

201   room for 10 min. The decrease in absorbance was measured at 515 nm using a UV-Vis

202   Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France).

203   Additionally, the ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)] radical cation

204   and ferric reducing antioxidant power (FRAP) methods were also employed, according to Re

205   et al.(1999), and Benzie and Strain (1996) respectively. Briefly, 10 μL of the supernatant were

206   mixed with 990 μL of ABTS or FRAP. After 10 min of reaction, the absorbance was

207   measured at 734 nm for ABTS and 593 nm for FRAP. The absorbance was measured by UV-

208   Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France).

209   Calibration curves, in the range 0.01–5.00 mmol Trolox L^{-1} were used for the quantification

210   of the three methods of antioxidant activity showing good linearity ($r^2 \geq 0.998$). The analyses

211   were run in five replications ($n = 5$) and results were expressed as mean ± standard error and

212   units in mmol Trolox kg^{-1} dw.

213   **2.9   Statistical analyses**

214   One-way analysis of variance (ANOVA) and multiple-range tests were used for comparison

215   of the pomegranate thinning results. The method used to discriminate among the means

216   (Multiple Range Test) was Tukey's procedure. Significance was defined at $p \leq 0.05$.

217   Statistical analyses were performed using StatGraphics Plus 5.0 software (Manugistics, Inc.,

218   Rockville, MD). Principal component analysis (PCA) was carried out using Unscrambler

219   Version 9.7 (Camo Software, Oslo, Norway).

3   Results and discussion

3.1   Morphological parameters

222   The main morphological characteristics of fruits removed during pomegranate thinning are

223   described in **Table 1**; the factor "cultivar" significantly ($p < 0.05$) affected all three

224   parameters under study. Fruit weight ranged from 46.1 to 65.1 g, fruit diameter from 43.8 to

225   50.3 mm and fruit length from 40.0 to 46.9 mm. Although all fruits were collected at the same

226   time in the last week of June, in general the sour cultivars (BA1, BO1, BBE1) showed the

227   highest values for weight, diameter and length, followed by sweet cultivars (ME14, ME17,

228   VA1) and sour-sweet cultivars (PTO5, PTO8, PTO10). This fact suggests that the weight of

229   fruits during thinning depends on cultivar, and provides valuable information for farmers

230   and/or processing industries.

231   The values obtained in morphological characteristics (fruit weight, fruit diameter and fruit

232   length) were lower than those found by other authors that studied the pomegranate from the

233   first maturity stage (Fawole & Opara, 2013a; Al-Maiman & Ahmad, 2002); however, these

234   differences could be due to: (i) different pomegranate cultivars, and (ii) different climatic

235   conditions.

236   The water content of each thinning fruit is also included in **Table 1** to allow conversion of

237   data expressed on a dry weight basis to values in fresh weigh basis.

11

238   **3.2   Total soluble solids, pH, total titratable acidity and maturity index**

239   The values of TSS, TA, MI and pH of the juice obtained by manually squeezing fruits of nine

240   pomegranate cultivars at thinning are shown in **Table 2**. The factor "cultivar" significantly

241   affected ($p < 0.001$) all four parameters. However, the factor "cultivar type" (sour, sour-sweet

242   and sweet) did not significantly affect TSS (10.3, 9.83, and 10.4 ºBrix, respectively). The

243   highest TA content was 35.5 g L^{-1} in sour cultivars, followed by 7.52 g L^{-1} in sour-sweet

244   cultivars and finally 6.22 g L^{-1} in sweet cultivars. In a recent study it was reported that TSS

245   and TA values were 15.7 ºBrix and 9.6 g L^{-1} citric acid in ripe pomegranate fruits (Nuncio-

246   Jáuregui et al., 2014a). In this particular study, the MI were 2.95, 13.35 and 15.26 for sour,

247   sour-sweet and sweet cultivars, respectively **(Table 2)**. However, these values should be taken

248   with caution as only trace levels of sugars were detected by HPLC and thus the TSS did not

249   represent sugars but other water-soluble compounds. As a result, it is not fully appropriate to

250   compare TSS or MI values with those of ripe fruits or juices prepared using this type of fruit.

251   **3.3   Organic acids and sugars**

252   The pomegranate removed during thinning only contained trace levels of sugars. With

253   ripening, starch in the fruit is degraded to simple sugars, while a simultaneous decrease in the

254   organic acids and acidity is observed (Nuncio-Jáuregui et al., 2014a; Biale & Young, 1981).

255   **Table 3** shows the organic acids profiles of the juice obtained by manually squeezing thinning

256   fruits; the factor "cultivar" significantly ($p < 0.001$) affected all three acids found. Citric and

257   quinic acids predominated over malic acid in all cultivars; quinic acid predominated in most

258   sour-sweet and sweet thinning fruits, while citric acid only predominated in sour BA1 and

259   sour-sweet PTO8 fruits. Fruits from the sour cultivars had the highest value of total acids,

260   63.6 g L^{-1}, followed by the sour-sweet, 32.0 g L^{-1}, and the sweet, 19.4 g L^{-1}. In general, citric

261   acid is considered as the main acid in ripe pomegranate (Melgarejo et al., 2000), while malic

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262 acid could be considered, in general, as the most abundant acid in thinning pomegranate

263 fruits. The total content of organic acids found in thinning fruits (38.4 g L^{-1}, mean value for

264 all samples) was higher than that previously reported in ripe fruits (mean of 18.4 g L^{-1})

265 (Nuncio-Jáuregui et al., 2014a). These authors found total acids contents of 28.5, 17.3, and

266 9.6 g L^{-1}, in ripe sour, sour-sweet and sweet Spanish pomegranates, respectively.

3.4 Mineral content

268 The minerals contents in immature pomegranate fruits are shown in **Table 4**; it is important to

269 highlight that the material analysed included pomegranate rind, carpelar membranes and arils,

270 and not only arils as usually is done when focusing in the edible portion of pomegranates. The

271 data clearly showed that potassium (K) was the predominant macro-element in all cultivars,

272 while zinc (Zn) was the predominant micro-element in the majority of the cultivars, although

273 both copper (Cu) and iron (Fe), presented also relatively high contents. Previous studies in

274 ripe fruits, reported that K and Fe were the most abundant macro- and micro-elements,

275 respectively (Gozlekci et al., 2011; Mirdehghan & Rahemi, 2007). As maturation progresses,

276 there are significant decreases in mineral contents (Fawole & Opara, 2013b). In the current

277 study, the mineral contents were higher than those reported by Fawole and Opara (2013a),

278 and Al-Maiman and Ahmad (2002), who reported the highest minerals values at the first

279 maturity stage. This variation could be attributed to differences in cultivar, plant nutrition,

280 climate and soil conditions (Hamurcu et al., 2011), but are mainly linked to the different

281 nature of the material under analysis; most of the literature references report data on edible

282 arils, while in this study data on non-edible whole immature fruits are being reported.

283 The sour-sweet fruits had the highest contents of Ca and Mg, while sweet fruits had the

284 highest contents of Fe and Zn; no clear trends were found for the other minerals.

13

285   According to Nuncio-Jáuregui et al. (2014b) the contents of the macronutrients (Ca, Mg, K,

286   and Na) and micro-nutrients (Fe, Zn, Cu, and Mn) in pure pomegranate juice were: 25.3, 27.3,

287   2492, and 29.5 mg L^{-1}, and 1.03, 1.28, 0.41, and 0.35 mg L^{-1}, respectively. The contents

288   found in thinning fruits were significantly much higher than the normal values found in edible

289   arils and pure juice, making this material very interesting as a mineral supplement. The mean

290   values of the contents of Ca, Mg, K, Na, Fe, Zn, Cu, and Mn in immature thinning fruits

291   were: 226, 439, 10171, 253, 5.86, 7.51, 6.12, and 3.06 mg kg^{-1}, respectively, making these

292   values about 8–9 times higher than those from pure juice. For instance, the content of the

293   most abundant element, K, in thinning fruits was 10171 mg kg^{-1}, which is about 4 times

294   higher than the K content in pure pomegranate juice, while the contents of Mg and Cu were

295   about 15 times higher in thinning material than in juice. The high content of some nutrients in

296   the dried material from the thinning pomegranates is important and could be used for instance

297   to enrich pomegranate or other fruit juices similarly to the enrichment reported by Vázquez-

298   Araújo et al. (2011) with pomegranate albedo and carpelar membranes homogenate.

299   **3.5   Total polyphenol content (TPC)**

300   Total polyphenol contents in thinning fruits, including rind, carpelar membranes and

301   pomegranate arils, are presented in **Table 5.** Significant differences ($p < 0.001$) were

302   observed among cultivars, with TPC values ranging from 777 to 1660 g GAE kg^{-1} dw, and

303   with sour-sweet cultivars showing the highest values.

304   Pomegranate wastes (rind and carpelar membranes) are a richer source of antioxidants than

305   the edible arils (Li et al., 2006). Calín-Sánchez et al. (2013) evaluated the total polyphenols of

306   mature arils and rind of pomegranate dried using different methods, and concluded that the

307   highest TPC were found in freeze-dried rind (118 mg GAE g^{-1} dw). A similar trend is found

308   in several stone fruits, in which the skin has higher TPC than the edible flesh; for instance,

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309 Tomás-Barberán et al. (2001) reported that the skins of nectarines, peaches and plums contain

310 higher amounts of phenols, anthocyanins and flavonols than pulp. The mean TPC found in

311 immature thinning fruits, 1130 g GAE kg^{-1} dw is higher than any value previously reported in

312 pomegranate juice but even higher than in pomegranate rind (Calín-Sánchez et al., 2013). For

313 instance, this value is about 10 times higher than that of mature pomegranate rind or about

314 250–750 times higher than that of pomegranate juice.

315 As ripening progresses, total polyphenol content decreases; this trend can be attributed to

316 changes such as hydrolysis of glycosides, the oxidation of phenols by polyphenol oxidases

317 and polymerisation of free phenols (Remorini et al., 2008). The high content of TPC in

318 thinning pomegranates can significantly contribute to the use of this material as a source of

319 natural antioxidants.

320   **3.6 Punicalagin isomers and ellagic acid (EA)**

321 In whole thinning fruits the content of α-punicalagin ranged from 101 to 195 g kg^{-1} dw, β-

322 punicalagin from 80.1 to 111 g kg^{-1} dw, and EA from 1.96 to 3.00 g kg^{-1} dw (**Table 5**). In

323 general, the sour-sweet cultivars, especially PTO5, showed the highest values of these three

324 bioactive compounds. The results showed that α-punicalagin was more abundant than β-

325 punicalagin, as previously reported by Calín-Sánchez et al. (2013); in this way, the ratio α-

326 punicalagin/β-punicalagin took values of ~1.7.

327 The most abundant of the polyphenolic compound in pomegranate is punicalagin; punicalagin

328 together with ellagic acid are potent antioxidants, anticancer and have anti- atherosclerotic

329 biological properties (Lu et al., 2008). Furthermore, ellagic acid has shown to be effective as

330 an inhibitor of lipid peroxidation (Häkkinen et al., 2000; Seeram et al., 2005). However, the

331 contents of ellagic acid are significantly lower than those of punicalagins (**Table 5**) in this

332 particular material. The contribution of punicalagins and ellagic acid to the total antioxidant

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333   activity of pomegranate represents almost 87% of the total activity (Gil et al., 2000).

334   Pomegranate rind is a richer source of punicalagins and ellagic acid than arils (Seeram et al.,

335   2005) and even higher when the fruits are at the beginning of their growing cycle (Kulkarni &

336   Aradhya, 2005). The contents of α- and β-punicalagins and ellagic acid found in immature

337   thinning pomegranate fruits (means of 150, 88.3 and 2.59 g kg^{-1} dw, respectively) were

338   similar to those previously reported by Calín-Sánchez et al. (2013) in rind of mature

339   pomegranate fruits cv. Mollar de Elche (139, 143, and 2.49 g kg^{-1} dw, respectively).

340   ### 3.7   Antioxidant activity (AA)

341   There are different methods for evaluating the AA of foods. This variety of methods is due to

342   the fact that none of them is able to determine exactly the total antioxidant capacity of a

343   product. The measured AA of a sample depends on methodology and on free radical

344   generator or oxidant in the measurement (Cao et al., 1993). Electron-transfer-based assays

345   (ABTS, FRAP and DPPH) measure the capacity of an antioxidant in the reduction of an

346   oxidant which changes colour when reduced. However, there are differences among them; for

347   instance, ABTS measures both hydrophilic and lipophilic AA, while DPPH only considers

348   lipophilic compounds (Kuskosksi et al., 2005). For this reason, the antioxidant activity of

349   thinning fruits was evaluated using three different analytical methods: ABTS, DPPH, and

350   FRAP (**Fig. 1**). The factor "cultivar" significantly ($p < 0.05$) affected the antioxidant activity

351   of thinning fruits. The AA values ranged from 2923 to 4486 mmol Trolox kg^{-1} dw for ABTS,

352   from 3153 to 4685 mmol Trolox kg^{-1} dw for FRAP, and finally from 2075 to 2934 mmol

353   Trolox kg^{-1} dw for DPPH. In general and agreeing with the TPC trend, the highest values

354   were found in sour-sweet cultivars, especially in PTO8 and PTO5 cultivars. The differences

355   in AA among pomegranate varieties could be primarily attributed to their different contents of

356   polyphenols.

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357   These values are quite high in comparison with the 6.5 mmol Trolox L^{-1} reported by Nuncio-

358   Jáuregui et al. (2014a) in ripe Spanish pomegranate fruits; these authors quantified AA using

359   the DPPH method. However, the different natures of the compared materials (solid: thinning

360   fruits and liquid: pomegranate juice) must be highlighted. Pomegranate rind had the highest

361   antioxidant activity compared with peel, carpelar membrane and arils measured using these

362   different methods (Calín-Sánchez et al., 2013; Murthy et al., 2002). Using the DPPH method,

363   Calín-Sánchez et al. (2013) reported values of 45.1 and 1.2 mg Trolox equivalents g^{-1} dw for

364   rind and arils of mature Spanish pomegranates. It can be concluded that the AA of thinning

365   pomegranates was about 11–26 times higher than that of the rind of ripe pomegranates.

366   ### 3.8   Principal component analysis (PCA)

367   With the aim of enabling a better and simple visual interpretation of the results, a PCA was

368   conducted (**Fig. 2**). PCA1 and PCA2 explained 65.85% of the variability of the samples. The

369   first group of cultivars (positive PCA1 and negative PCA2) included BBE1 and PTO8

370   samples; it was characterised by simultaneous high levels of Cu, Mn and FRAP-AA. The

371   second group (positive PCA1 and PCA2) included two more cultivars, ME14 and ME17, and

372   was characterised by high levels of Mg, Fe, Zn, pH, TPC and DPPH-AA. The third group of

373   cultivars (negative PCA1 and positive PCA2) included PTO10, PTO5 and VA1 and was

374   defined by high levels of Ca, K, MI , malic acid, α-punicalagin, β-punicalagin, TSS and

375   ABTS-AA. The fourth and last group (negative PCA1 and PCA2) included the cultivars BO1

376   and BA1, and was defined by high contents of Na, ellagic acid, quinic acid, citric acid and

377   TA.

378   ## 4   Conclusions

379   Pomegranate trees require thinning to allow the remaining fruits to develop to their maximum

380   size and quality without reduction of tree vigour. In this study, the pomegranate fruits

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381   removed during routine thinning of trees from 9 Spanish cultivars were fully characterised.

382   Only small fruits (weight < 65.1 g, fruit diameter < 50.3 mm and fruit length < 46.9 mm) are

383   removed during thinning. The titratable acidity ranged from 5.57 to 38.7 g citric acid L^{-}-^{1}, and

384   the most abundant organic acids were quinic and citric acids with total concentration of acids

385   being as high as 65.3 g L^{-}-^{1}. Pomegranate thinning fruits are rich in K (mean content of 10171

386   mg kg^{-}-^{1} dw) and Zn (7.5 mg kg^{-}-^{1} dw). The TPC of thinning fruits is high: 1130 g GAE kg–-

387   1 dw compared to any other previously studied product or co-product, including dry

388   rind/husk. The high values of TPC are linked with: (i) high contents of both isomers of

389   punicalagin α and β, mean values of 151 and 88 g kg^{-1} dw, respectively; and (ii) high mean

390   values of antioxidant capacity as measured by three different assays: ABTS: 3591, FRAP:

391   3893, and DPPH: 2487 mmol Trolox kg^{-1} dw.

392   In summary, pomegranate thinning fruits are a good source of bioactive compounds (quinic

393   and citric acids, K, Zn, (- and (-punicalagin) with high antioxidant capacity. This observation

394   holds especially true in fruits from sour-sweet cultivars, such as PTO5 and PTO10. This

395   composition makes this material interesting for the food, pharmaceutical or chemical

396   industries as well as being an extra source of income for the farmers. As a simple example,

397   dry thinning pomegranates are appropriatecould be used to enrich fruit juices, poor in

398   nutrients such as K and Zn, and with low antioxidant capacity.

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522 **Figure captions**

523 **Fig. 1. Antioxidant activity, AA (mmol Trolox kg^{-1} dw) in fruits removed during pomegranate**
524 **thinning.**

525 **Fig. 2. Principal component analysis of the main morphological, physicochemical and chemical**
526 **parameters of fruits removed during pomegranate thinning.**

527

25

527 **Figure 1**. Antioxidant activity, AA (mmol Trolox kg^{-1} dw) in fruits removed during

528 pomegranate thinning.
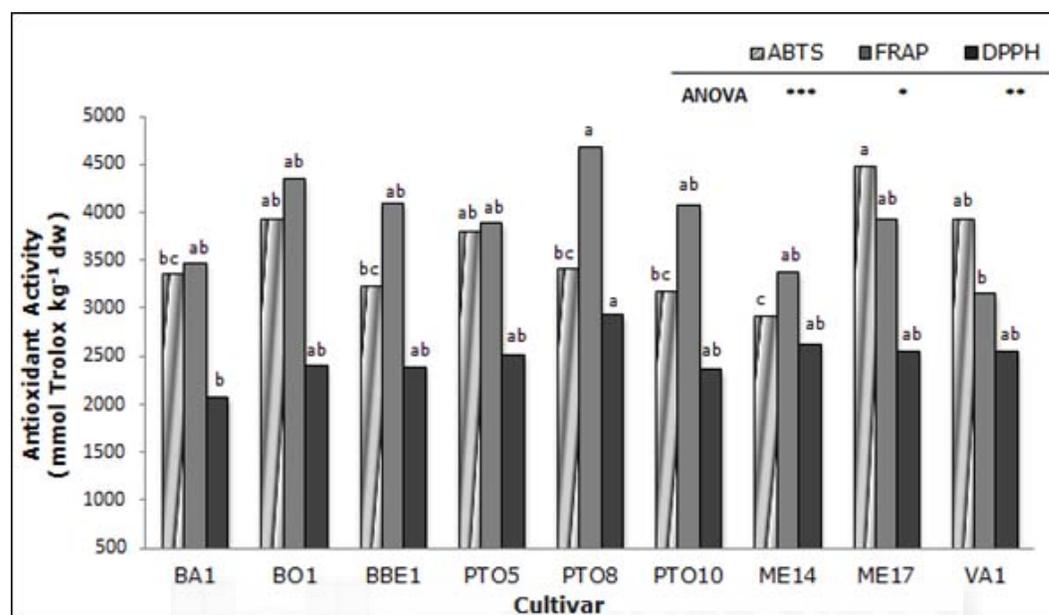
529 *The values represented in the bars are the means of 5 replications. Bars with the same letter,*

530 *for each of the AA assays, were not statistically different according to Tukey's multiple range*

531 *test ($p < 0.05$); *, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively.*

532

26

532　　　　**Figure 2**. Principal component analysis of the main morphological, physicochemical and

533　　　　　　　　chemical parameters of fruits removed during pomegranate thinning.
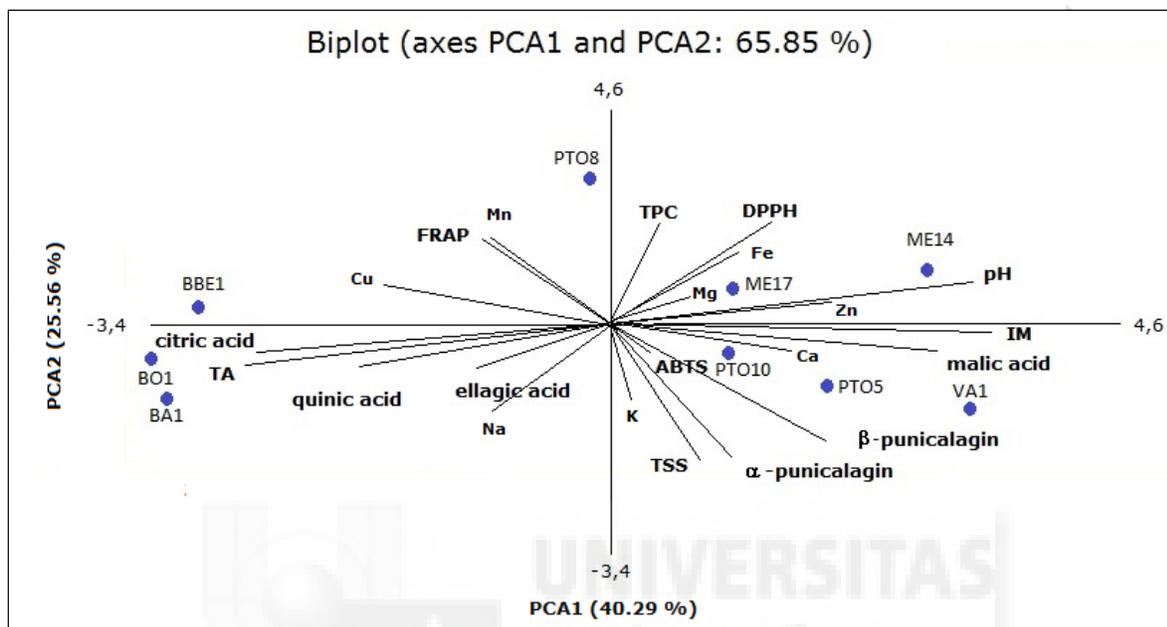
534
535

535   **Table 1.** Main physical properties of immature fruits removed during pomegranate thinning:

536   (i) fruit weight, FW (g), (ii) fruit diameter, FD (mm) and (iii) fruit length, FL (mm): from

537   calyx to base, and (iv) water content, WC (g water 100 g^{-1} fresh weight).

Cultivar	Type	FW (g)	FD (mm)	FL (mm)	WC (g water 100 g^{-1} fw)
BA1	Sour	$64.8^{†} \pm 5.9a^{‡}$	$50.3 \pm 1.8a$	$43.8 \pm 1.4ab$	65.7 ± 4.6 ab
BO1		$65.1 \pm 3.1a$	$49.7 \pm 1.1ab$	$44.3 \pm 1.0ab$	66.3 ± 1.3 ab
BBE1		$60.7 \pm 1.4ab$	$47.5 \pm 0.7abc$	$42.5 \pm 0.7ab$	65.2 ± 3.3 b
PTO5	Sour-sweet	$59.9 \pm 2.3ab$	$48.4 \pm 0.6abc$	$41.4 \pm 0.5b$	66.6 ± 3.3 ab
PTO8		$46.1 \pm 5.9b$	$43.8 \pm 1.9c$	$40.0 \pm 1.4b$	65.5 ± 4.6 b
PTO10		$54.1 \pm 1.8ab$	$44.5 \pm 0.7bc$	$46.9 \pm 1.5a$	65.1 ± 1.3 b
ME14	Sweet	$59.1 \pm 2.2ab$	$48.7 \pm 0.6abc$	$41.4 \pm 0.7b$	64.0 ± 3.2 c
ME17		$56.8 \pm 3.7ab$	$47.6 \pm 1.2abc$	$40.1 \pm 1.0b$	71.3 ± 3.0 a
VA1		$56.3 \pm 2.8ab$	$46.9 \pm 0.9abc$	$41.7 \pm 0.7b$	67.3 ± 1.3 b
ANOVA		*	**	***	*

538

539   *† Values are the mean of 10 replications (± standard error). ‡ Values followed by the same*

540   *letter within the same column were not statistically different according to Tukey's multiple*

541   *range test (p < 0.05); *, **, and ***, significant at p< 0.05, 0.01, and 0.001, respectively.*

542

28

542   **Table 2.** Main quality physicochemical parameters of juice from manually squeezed whole

543   immature fruits removed during pomegranate thinning: (i) total soluble solids, TSS (°Brix),

544   (ii) titratable acidity, TA (g L^{-1} citric acid), (iii) maturity index, MI, and (iv) pH.

Cultivar	Type	TSS	TA	MI	pH
		(°Brix)	(g citric acid L^{-1})		
BA1	Sour	9.85† ± 0.05d‡	38.7 ± 0.1a	2.55 ± 0.01h	3.47 ± 0.01i
BO1		10.4 ± 0.1c	36.8 ± 0.1b	2.83 ± 0.01h	3.56 ± 0.04h
BBE1		10.7 ± 0.1b	31.1 ± 0.1c	3.47 ± 0.02g	4.27 ± 0.01g
PTO5	Sour-sweet	11.3 ± 0.1a	6.95 ± 0.01g	16.3 ± 0.1c	7.34 ± 0.01b
PTO8		8.20 ± 0.01e	8.59 ± 0.01d	9.55 ± 0.01f	7.02 ± 0.01e
PTO10		10.0 ± 0.1d	7.03 ± 0.02f	14.2 ± 0.1d	7.15 ± 0.01d
ME14	Sweet	10.3 ± 0.1c	5.57 ± 0.01i	18.5 ± 0.1b	7.46 ± 0.01a
ME17		9.75 ± 0.05d	7.42 ± 0.01e	13.1 ± 0.1e	6.44 ± 0.01f
VA1		11.2 ± 0.1a	5.67 ± 0.01h	19.8 ± 0.1a	7.23 ± 0.01c
ANOVA		***	***	***	***

545   † *Values are the mean of 5 replications (± standard error).* ‡ *Values followed by the same*

546   *letter within the same column were not statistically different according to Tukey's multiple*

547   *range test (p < 0.05); *** significant at p<0.001.*

548

548   **Table 3.** Organic acids contents (g L^{-1}) of juice from manually squeezed whole immature

549   fruits removed during pomegranate thinning.

Cultivar	Type	Citric	Malic	Quinic	Total Acids
		(g L^{-1})			
BA1	Sour	39.2† ± 0.2a‡	0.15 ± 0.01e	21.1 ± 1.6cd	60.5 ± 1.5a
BO1		32.3 ± 0.8b	0.18 ± 0.01e	32.8 ± 0.8b	65.3 ± 0.1a
BBE1		22.5 ± 0.5c	0.19 ± 0.01e	42.3 ± 2.3a	65.0 ± 2.9a
PTO5	Sour-sweet	11.1 ± 0.1e	0.28 ± 0.01d	25.3 ± 0.4c	36.7 ± 0.3b
PTO8		14.7 ± 0.1d	0.24 ± 0.01d	13.9 ± 0.16ef	28.9 ± 0.16cd
PTO10		13.7 ± 0.4d	0.40 ± 0.01b	16.4 ± 1.4def	30.5 ± 1.4bc
ME14	Sweet	4.03 ± 0.21g	0.34 ± 0.01c	19.1 ± 1.1cde	23.5 ± 1.2de
ME17		3.77 ± 0.01g	0.24 ± 0.01d	12.2 ± 0.6f	16.2 ± 0.6f
VA1		5.57 ± 0.43f	0.54 ± 0.01a	12.5 ± 0.1ef	18.6 ± 0.1ef
ANOVA		***	***	***	***

550   † *Values are the mean of 5 replications (± standard error).* ‡ *Values followed by the same*

551   *letter within the same column were not statistically different according to Tukey's multiple*

552   *range test (p < 0.05); *** significant at p<0.001.*

553

553   **Table 4.** Minerals contents (mg kg^{-1} dw) in immature fruits removed during pomegranate

554   thinning.

Cultivar	Type	Mineral (macro-elements)			
		Ca	Mg	K	Na
		(mg kg^{-1} dw)			
BA1	Sour	180[†] ± 2	430 ± 4ab[‡]	10737 ± 10cd	275 ± 1
BO1		176 ± 13	282 ± 3b	11082 ± 7bc	297 ± 1
BBE1		207 ± 3	395 ± 3ab	8427 ± 7f	263 ± 2
PTO5	Sour-sweet	329 ± 11	548 ± 3a	10651 ± 8cd	239 ± 2
PTO8		291 ± 7	508 ± 3a	9757 ± 5e	225 ± 2
PTO10		183 ± 9	382 ± 7ab	10306 ± 7de	235 ± 2
ME14	Sweet	240 ± 5	373 ± 4ab	7076 ± 8g	251 ± 1
ME17		241 ± 5	512 ± 2a	11974 ± 3a	238 ± 2
VA1		184 ± 7	519 ± 6a	11526 ± 7ab	283 ± 2
ANOVA		NS	**	***	NS
		Mineral (micro-elements)			
Cultivar		Fe	Zn	Cu	Mn
		(mg kg^{-1} dw)			
BA1	Sour	5.10 ± 1.30bc	8.26 ± 0.72ab	6.26 ± 0.01bc	2.93 ± 0.20abc
BO1		4.70 ± 0.50bc	6.40 ± 1.11ab	6.80 ± 0.31abc	2.96 ± 0.22abc
BBE1		4.60 ± 0.51bc	6.96 ± 0.25ab	7.76 ± 0.12a	4.20 ± 0.42a
PTO5	Sour-sweet	3.73 ± 0.80bc	6.40 ± 0.36ab	6.76 ± 0.52abc	2.76 ± 0.30bc
PTO8		5.86 ± 1.52bc	7.56 ± 0.86ab	7.13 ± 0.31ab	3.76 ± 0.31b
PTO10		2.51 ± 0.81c	6.66 ± 0.51ab	6.60 ± 0.13abc	2.96 ± 0.21abc
ME14	Sweet	7.86 ± 0.12ab	6.76 ± 0.37ab	3.60 ± 0.10f	2.60 ± 0.30bc
ME17		11.0 ± 0.1a	9.16 ± 0.86ab	4.70 ± 0.14ef	2.90 ± 0.01ab
VA1		7.40 ± 1.62abc	9.40 ± 0.15a	5.50 ± 0.16cd	2.43 ± 0.22c
ANOVA		***	*	***	**

555   *† Values are the mean of 5 replications (± standard error). ‡ Values followed by the same*

556   *letter within the same column were not statistically different according to Tukey's multiple*

557   *range test (p < 0.05); NS= not significant; *, **, and ***, significant at p< 0.05, 0.01, and*

558   *0.001, respectively.*

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559 **Table 5.** Contents of phenolic bioactive compounds in immature fruits removed during

560 pomegranate thinning: (i) total polyphenols content, TPC (g GAE kg^{-1} dw), and (ii) α-

561 punicalagin, β-punicalagin, and ellagic acid (g kg^{-1} dw).

Cultivar	Type	TPC	α -Punicalagin	β-Punicalagin	Ellagic acid
		(g GAE kg^{-1} dw)	(g kg^{-1} dw)		
BA1	Sour	829† ± 1d‡	156 ± 10bc	83.0 ± 1.6de	2.73 ± 0.12ab
BO1		1167 ± 3bc	151 ± 2bc	83.1 ± 0.7de	3.00 ± 0.07a
BBE1		949 ± 5cd	137 ± 3c	80.1 ± 0.8e	2.35 ± 0.04c
PTO5	Sour-sweet	1441 ± 1ab	195 ± 5a	111 ± 1a	2.84 ± 0.02a
PTO8		1660 ± 4a	101 ± 3d	64.5 ± 3.2f	2.45 ± 0.06bc
PTO10		1206 ± 8bc	155 ± 3bc	94.3 ± 1.0bc	2.83 ± 0.07a
ME14	Sweet	1205 ± 3bc	146 ± 8bc	86.7 ± 1.0cde	1.96 ± 0.04d
ME17		935 ± 4cd	138 ± 7c	89.3 ± 2.0cd	2.79 ± 0.06ab
VA1		777 ± 5d	175 ± 2ab	103 ± 1.1ab	2.38 ± 0.04c
ANOVA		***	***	***	***

562 † *Values are the mean of 5 replications (± standard error).* ‡ *Values followed by the same*

563 *letter within the same column were not statistically different according to Tukey's multiple*

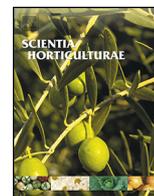564 *range test (p < 0.05); ***, significant at p < 0.001.*

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PUBLICATION 2

Changes in quality parameters, proline, antioxidant activity and color of pomegranate (*Punica granatum* L.) as affected by fruit position within tree, cultivar and ripening stage

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Changes in quality parameters, proline, antioxidant activity and color of pomegranate (*Punica granatum* L.) as affected by fruit position within tree, cultivar and ripening stage

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ARTICLE INFO

ABSTRACT

In recent years, the pomegranate (*Punica granatum* L.) has acquired wide acceptance due to the growing evidence that consumption is associated with beneficial health properties. The present study was conducted to study the effect of the position of the fruits within the tree in the main quality parameters (total soluble solids, titratable acidity, pH and maturity index), the profiles of organic acid, sugars, the amino acid proline, total phenolic compounds, antioxidant activity, and the external and internal color. Analyses were performed on three Spanish pomegranate cultivars: *Mollar de Elche* ("ME14"), *Borde de Albatera* ("BA1") y *Piñon Tierno de Ojós* ("PTO5") at three ripening stages. The results showed that the position within the tree had no significant effects on total soluble solids (TSS), the titratable acidity (TA), maturity index (MI), pH, organic acids, sugars profiles, proline, antioxidant activity (AA) and total phenolic compounds (TP); however, it significantly ($p < 0.05$) affected data on external color coordinates. External color showed a simultaneous increase in the values of a^* and C^* along with decreases in b^* and H^*; this contributed to the production of characteristic garnet color of pomegranate fruits and has high importance in deciding the appropriate harvest time. This study provides useful information about the quality parameters, proline, phenolic compound, antioxidant activity and color of three different type of pomegranate during three ripening stages. It also shows that the fruits exposed to sunlight have similar chemical composition to those fruits exposed to shade except in external color.

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1. Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest known edible fruit. It is an interesting and promising crop for different world areas due to its easy and good adaption to different weather conditions. Spain is the one of the main European pomegranate producer and its production is mainly located in the provinces of Alicante and Murcia (Melgarejo and Salazar, 2003). Pomegranate is a good source of hydrolysable tannins, anthocyanins, and minerals such as potassium (Gil et al., 2000; Hernández, 1999).

During fruit ripening there are significant changes in the physicochemical and phenolic compositions and antioxidant activity (Fawole and Opara, 2013; Schwartz et al., 2009). These changes are influenced by variety, growing region, cultivation techniques and ripening stage of the fruit at harvest (Mirdehghan and Rahemi, 2007). The pomegranate is a fruit that requires high temperatures

during development and ripening (Bartual, 2011); its full-ripening is between 5 and 7 months after flowering. At the optimum stage of ripening, there should be adequate contents of total soluble solids (TSS), titratable acidity (TA), pH, sugars, organic acids, total phenolics, anthocyanins, minerals, and appropriate color characteristics; all of these characteristics lead to high quality fruits (Fawole and Opara, 2013; Schwartz et al., 2009). Therefore, it is important that pomegranate fruits are harvested at their proper ripening stage, because at this point fruits will have their highest potential with respect to nutritional, functional and sensory properties. An early harvest of pomegranates will prevent full development of color and flavor, while a late harvest will lead to fruits with reduced shelf life and increased disease susceptibility (Schwartz et al., 2009).

Color has been often associated with high fruit consumer preference and/or acceptance for different commodities. For instance in peaches and nectarines, consumers prefer full red color fruit (Crisosto et al., 2003); a similar situation is expected for pomegranates. Recent studies have found that the external color of pomegranate (cv. *Mollar de Elche*) is correlated with the number of days from the beginning of its development (Manera et al.,

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2013). The growth of the fruit, its color and the chemical maturity index (ratio TSS/TA) allows farmers a cheap and objective way to establish the optimal moment for fruit harvest (Manera et al., 2013). All these statements highlight the enormous interest in fruit colorimetry, especially ripening. Nowadays, researchers are trying to establish relationships among different environmental parameters and pomegranate color. For instance, Manera et al. (2011) studied the correlation between pomegranate rind color and air temperature. One of the parameters that could affect the color of the pomegranate fruits is the exposure to sunlight; the effects of this factor on the main pomegranate quality parameters will be evaluated in this study.

Due to the fact that pomegranate is mainly grown in arid and dry geographic regions, the amino acid "proline" is another parameter to consider during fruit ripening. The proline content is considered as an indicator of changes in cellular metabolism caused by abiotic factors, such as water deficit, high salinity, extreme temperatures, high concentrations of heavy metals in the soil-plant system, and high light intensity (Claussen, 2005). Proline works as a protein stabilizer, hydroxyl radical scavenger and serves as a source of energy and nitrogen (Claussen, 2005). Currently there is not enough information in the literature on whether this parameter is affected by fruit ripening or just accumulates in plants under unfavorable environmental conditions.

Therefore, the aim of this study was to study the effect of the position of the fruits within the tree on the main quality parameters (total soluble solids, titratable acidity, and pH), the profiles of bioactive compounds such as sugars, organic acids and the amino acid proline, phenolic compound, the antioxidant activity, and the external and internal color of fruits from three Spanish pomegranate cultivars (sweet: "ME14", sour–sweet: "PTO5", and sour: "BA1") and at three ripening stages.

2. Materials and methods

2.1. Plant material and sample processing

Three different cultivars of pomegranate were selected: *Mollar de Elche* ("ME14"), *Piñón Tierno de Ojós* ("PTO5"), and *Borde de Albatera* ("BA1"); each one of them represented a different type of pomegranate: sweet, sour–sweet, and sour, respectively. Fruits were picked and evaluated at three different ripening stages: (i) R1 small size (<70 g), green and fully unripe fruits, (ii) R2 medium size (120–250 g), light red but still unripe fruits, and (iii) R3 large size (>300 g), reddish and ripe fruits. Based on previous studies and conclusions by Martínez et al. (2006), fruits harvest was conducted from the 2nd week of September to the last week of October 2012. Two different positions within the pomegranate trees were studied: (i) East orientation: highly exposed to the sunlight (from now on "sun"), and (ii) West orientation: poorly exposed to the sunlight (from now on "shadow").

The cultivar "ME14" was selected from the population variety Mollar de Elche (ME), which is the most widely cultivated and consequently marketed in Spain and in the European Union (Martínez et al., 2006). The cultivar "PTO5" was chosen because of the sour–sweet taste and large size of its fruits. In addition, the "BA1" cultivar was selected because its edible arils have sour taste, are hard and have a woody portion of ∼13% (Hernández, 1999).

The selected plant material belongs to one of the main pomegranate gene banks of the European Union, which is located at the experimental field station of the Miguel Hernandez University in Orihuela, Alicante, Spain (02°03′50″E, 38°03′50″N, and 25 meters above sea level). The orchard 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 × 3 m. They are drip irrigated, and standard cultural practices are performed (pruning, thinning, fertilization and pest control treatments). Three trees were selected for each cultivar, and 15 fruits per cultivar (5 fruits per tree) were picked according to fruit position within the tree, cultivar, and ripening stage. After picking, fruits were immediately transported to the laboratory. Each husk was carefully cut at the equatorial zone with a sharpened knife, and then arils were manually extracted. Chemical composition was immediately determined on the juice obtained by squeezing the arils. The juice was filtered through filter paper. The following physico-chemical parameters were analyzed: total soluble solids (TSS), titratable acidity (TA), pH, organic acids profile, sugars profile, proline content, total polyphenols content (TP), antioxidant activity (AA) and CIE$L^*a^*b^*$ color (external and internal). Analyses were run, at least, in triplicate in each one of the three pomegranate cultivars and at the three ripening stages.

2.2. Quality parameters

2.2.1. Total soluble solids, pH and total titratable acidity

Total soluble solids (TSS) were measured with a digital Atago refractometer (model N-20; Atago, Bellevue, Wash., U.S.A.) at 20 °C with values being expressed as °Brix. The *titratable acidity* (TA) and pH was determined by acid-base potentiometer (877 Titrino plus, Metrohm ion analyses CH9101, Herisau, Switzerland), using 0.1 N NaOH up to pH 8.1, values were expressed as g L^{-1} of citric acid. Finally, maturity index (MI), which is a ratio of TSS to TA, was also calculated for each sample. Results (mean ± standard error) were the mean of 3 determinations.

2.2.2. Analysis of organic acids and sugars

Organic acids and sugars were quantified according to Carbonell-Barrachina et al. (2012). The juices obtained by squeezing the arils were centrifuged at 15,000 rpm for 20 min (Sigma 3-18K, Osterode and Harz, Germany). 1 mL of supernatant was filtered through a 0.45 μm *Millipore* filter and injected into a Hewlett-Packard HPLC series 1100 (Wilmington Del., U.S.A.). The elution buffer consisted of 0.1% phosphoric acid with a flow rate of 0.5 mL min^{-1}. Organic acids were isolated using a Supelco column (SupelcogelTM C-610H column 30 cm × 7.8 mm) and Supelguard (5 cm × 4.6 mm, Supelco, Inc., Bellefonte, PA) and absorbance was measured at 210 nm using a diode-array detector (DAD). These same HPLC conditions (elution buffer, flow rate and column) were used for the analysis of sugars. The detection was conducted using a refractive index detector (RID). Standards of organic acids (citric, quinic, tartaric, ascorbic, succinic, fumaric, shikimic and malic acids) and sugars (glucose, fructose and sucrose) were obtained from Sigma (Poole, Dorset, UK). Calibration curves, obtained by triplicate injection of standard solutions, were used for quantification purposes and showed good linearity ($R^2 > 0.999$).

2.3. Determination of proline

Proline was quantified by the colorimetric method recommended by the International Federation of Fruit Juice Producers (IFU, 2005). A solution of ninhydrin in ethylenglycol monomethyl ether (30 g L^{-1}) was prepared. 1 mL of juice sample, 1 mL of formic acid (98%) and 2 mL of the ninhydrin solution were added, mixed and placed for 15 min in a bath with boiling water. After this time, 20 mL of butyl acetate (99.5%) were added to extract the color into the organic phase. Then, the solution was filtered and dried using filter paper containing 0.2 g of anhydrous Na$_2$SO$_4$. After 15 min, the absorbance of the organic phase was measured at 509 nm in a UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France). Calibration curves, in the

range 0–50 mg L^{-1}, were used for the quantification of proline and showed good linearity ($R^2 > 0.999$).

2.4. Antioxidant activity and total polyphenols content

The free radical scavenging activity was evaluated using by the DPPH (radical 2,2-diphenyl-1-picrylhydrazyl) method as described by Calín-Sánchez et al. (2013). Briefly, each pomegranate juice was diluted with MeOH (1:5), and then centrifuged at 15,000 rpm for 10 min. 10 μL of the supernatant were mixed with 40 μL of MeOH and added to 950 μL of a 0.094-mM DPPH solution. After 50 min of reaction, the absorbance was measured at 515 nm using a UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France). The proline analysis was run in triplicate and results (mean ± standard error) were expressed as mM Trolox.

Total polyphenols content (TP) was quantified using Folin-Ciocalteu reagent (Calín-Sánchez et al., 2013; Singleton et al., 1999). Briefly, for each sample, 5 mL of juice was homogenized in 5 mL of MeOH/water (80:20 v/v) plus 2 mM NaF and then centrifuged at 15,000 rpm for 15 min at 4 °C. Later, 50 μL of sample were mixed with 2.5 mL of Folin-Ciocalteu reagent (1:10 v/v), 450 μL of phosphate buffer (pH 7.8) and 2 mL of sodium carbonate (75 g L^{-1}). The samples were left in a water bath at 50 °C for 5 min. Then, absorption was measured at 760 nm using a UV-Vis Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France). Results (mean ± standard error) were expressed as mg gallic acid L^{-1} of juice.

2.5. Instrumental color of fruit

Color measurements were performed according to Manera et al. (2012), using a Minolta C-300 Chroma Meter (Minolta Corp., Osaka, Japan) coupled to a Minolta DP-301 data processor. This colorimeter uses an illuminant D$_{65}$ and a 10° observer as references. Color was assessed according to the *Commission Internationale de l'Éclairage* (CIE) and expressed as L^*, a^*, b^*. L^* indicates lightness, taking values within the range 0–100 (black–white, respectively), and a^* and b^* are the chromatic coordinates, green–red and blue–yellow coordinates, respectively. a^* takes positives values for reddish colors and negatives for the greenish ones, whereas b^* takes positive values for yellowish colors and negative values for bluish ones. Finally, C^* is Chroma [$C^* = \sqrt{(a*^2) + (b*^2)}$], 0 is at the center of a color sphere and increases according to the distance from the center. Hue (H^*) is the angular component of the polar representation of the product color, while chroma is the radial component.

External color was measured directly in the pomegranate fruits as affected by the fruit position within the trees. For color measurement 6 fruits were used and 3 readings were taken along the 360° equatorial perimeter of each fruit; thus, color values reported were the mean of 18 readings per treatment.

Internal color was measured in the juice obtained by squeezing the pomegranate arils and using the Minolta adaptor for liquid products. Internal color results (mean ± standard error) were the mean of 6 determinations for each sample.

2.6. Statistical analyses

Data from the analyses of pomegranate fresh fruit and juices obtained by squeezing the arils were first examined by three-way (factors: fruit position within the tree, cultivar and ripening stage) analysis of variance (ANOVA) for mean comparison. However and after checking that the first factor, fruit position, only affected significantly the external color of fruits, data was again examined using two-way (factors: cultivar and ripening stage) ANOVA (Tables 1–3) and color data was presented separately for the sun

and shadow positions (Table 4). Later, the method used to discriminate among the means (multiple range test) was Tukey's procedure. Data significance was defined at $p \leq 0.05$. Statistical analyses were performed using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD).

3. Results and discussion

The factor "position within the tree" had no significant effects on total soluble solids (TSS), titratable acidity (TA), maturity index (MI), pH (Table 1), organic acids and sugars profiles (Table 2), proline, antioxidant activity (AA) and total phenolic compounds (TP) (Table 3); however, it significantly ($p < 0.05$) affected data on external color coordinates (Table 4). Therefore, in the case of Tables 1–3, mean values of the fruits from the two positions studied (East = sun and West = shadow) are presented. In Tables 4 and 5, data for each position is presented and data for each of these two positions have been analyzed using the factors: (i) cultivar and (ii) ripening stage, as done for the other quality parameters.

3.1. Total soluble solids, pH and total titratable acidity

The results of TSS, TA, MI and pH of the three different pomegranate cultivars and stages of ripening are shown in Table 1. With regards to the pomegranate cultivars, significant differences ($p < 0.05$) were found in each of these parameters. Throughout the development of fruit, the highest TA content was 22.94 g L^{-1} for "BA1" (sour cultivar), followed by 5.61 g L^{-1} for "PTO5" (sour–sweet cultivar) and 2.38 g L^{-1} for "ME14" (sweet cultivar). There was a positive correlation between sourness and titratable acidity; the higher the sourness, the higher the titratable acidity. Koppel and Chambers (2010) studied the flavor profiles of pomegranate juices marketed in the USA and concluded that juices from sour or sour–sweet cultivars have a more complex and consumer attractive profile than sweet cultivars. This parameter together with the TSS content determines the fruit maturity index (TSS/TA) which is responsible for the taste and flavor of pomegranate (Tehranifar et al., 2010). The values of pH, TSS and MI for these varieties of pomegranate were similar to those reported by Calín-Sánchez et al. (2011) and Hernández et al. (1999). With respect to the ripening stages of the fruit, the pH seemed to increase but differences were not significant ($p > 0.05$). During the three stages of ripening, TSS significantly increased from 14.87 to 15.73 °Brix while TA decreased from 25.1 to 21.4, 6.4 to 5.2, and from 2.5 to 2.3 g citric acid L^{-1} in sour, sour–sweet and sweet cultivars, respectively. Several authors have reported a significant increase in the content of TSS during pomegranate ripening (Schwartz et al., 2009; Kulkarni and Aradhya, 2005). This increase may be due to an increase in starch hydrolysis as the fruit ripens (Fawole and Opara, 2013). As a result of changes in the content of TSS and TA, the MI increased from 6.2 to 7.8, 23.0 to 28.3, and from 58.1 to 70.0 in sour, sour–sweet and sweet cultivars, respectively. In general, the MI for pomegranate cultivars reached values of 5–7 for sour, 17–24 for sour–sweet and 31–98 for sweet cultivars (Martínez et al., 2006). The MI has been reported as one of the most reliable indicators of pomegranate fruit maturity (Fawole and Opara, 2013), although it depends on the cultivar and climatic conditions (Kulkarni and Aradhya, 2005; Schwartz et al., 2009).

3.2. Organic acids and sugars

The results showed significant differences ($p < 0.05$) in the organic acids profiles of pomegranate fruits as affected by cultivar and ripening stage (Table 2). In general, experimental results from this study agreed with those previously obtained by Melgarejo et al. (2000).

Table 1
Quality physicochemical parameters [total soluble solids (TSS), titratable acidity (TA) (g L^{-1} citric acid), pH and maturity index (MI)] of fruits from three pomegranate cultivars and at three ripening stages.

Cultivar		Ripening	TSS (°Brix)	TA (g L^{-1} citric acid)	MI	pH
BA1	Sour	R1	15.43[†] ± 0.17	25.10 ± 0.67	6.16 ± 0.22	3.76 ± 0.23
		R2	15.90 ± 0.36	22.38 ± 1.62	7.20 ± 0.41	3.81 ± 0.03
		R3	16.53 ± 0.43	21.35 ± 1.40	7.84 ± 0.72	3.55 ± 0.06
PTO5	Sour–sweet	R1	14.57 ± 0.09	6.38 ± 0.37	22.98 ± 1.36	4.97 ± 0.58
		R2	14.53 ± 0.32	5.24 ± 0.17	27.80 ± 1.36	5.88 ± 0.01
		R3	14.80 ± 0.25	5.23 ± 0.07	28.34 ± 0.80	5.42 ± 0.13
ME14	Sweet	R1	14.60 ± 0.26	2.52 ± 0.13	58.12 ± 3.54	4.50 ± 0.04
		R2	15.40 ± 0.21	2.35 ± 0.03	65.64 ± 3.20	4.54 ± 0.08
		R3	15.87 ± 0.07	2.29 ± 0.08	70.05 ± 1.27	4.57 ± 0.06
			TSS (°Brix)	TA (g L^{-1} citric acid)	MI	pH
ANOVA[¶]						
Cultivar			***	***	***	***
Ripening stage			*	*	***	NS
Tukey's multiple range test						
Cultivar						
Sour			15.95[‡] a	22.94 a	7.06 c	3.70 c
Sour–sweet			14.63 c	5.61 b	26.37 b	5.42 a
Sweet			15.28 b	2.38 c	64.60 a	4.53 b
Ripening stage						
R1			14.87 b	11.33 a	29.08 b	4.41
R2			15.28 ab	9.98 ab	33.54 a	4.51
R3			15.73 a	9.61 b	35.41 a	4.74

[†] Values are the mean of 6 replications (±standard error): 3 sun + 3 shadow replicates.
[‡] Values followed by the same letter, within the same variation source, were not statistically different according to Tukey's multiple range test.
[¶] NS, not significant *F* ratio (*p* < 0.05).
*, **, and ***, significant at *p* < 0.05, 0.01, and 0.001, respectively.

Table 2
Organic acids and sugars contents (g L^{-1}) in fruits from three pomegranate cultivars and at three ripening stages.

Cultivar		Ripening	Citric	Malic	Quinic	Total acids	Glucose	Fructose	Total sugars
			(g L^{-1})						
BA1	Sour	R1	28.8[†] ± 0.1	2.4 ± 0.01	6.8 ± 0.09	39.1 ± 0.1	45.3 ± 0.1	52.6 ± 0.2	97.9 ± 0.2
		R2	22.0 ± 0.2	2.2 ± 0.01	6.2 ± 0.06	31.5 ± 0.1	47.7 ± 0.1	53.6 ± 0.1	101.2 ± 0.1
		R3	20.4 ± 0.1	1.7 ± 0.01	5.2 ± 0.02	28.5 ± 0.1	51.3 ± 0.1	56.6 ± 0.1	107.9 ± 0.4
PTO5	Sour–sweet	R1	5.6 ± 0.02	2.3 ± 0.01	13.0 ± 0.1	21.0 ± 0.1	46.4 ± 0.1	59.3 ± 0.1	105.7 ± 0.1
		R2	5.0 ± 0.01	1.9 ± 0.01	12.0 ± 0.1	18.1 ± 0.1	53.2 ± 0.1	68.0 ± 0.1	121.1 ± 0.1
		R3	4.8 ± 0.01	1.6 ± 0.01	10.0 ± 0.1	17.3 ± 0.1	57.2 ± 0.1	69.2 ± 0.1	126.5 ± 0.1
ME14	Sweet	R1	1.4 ± 0.01	1.8 ± 0.01	8.6 ± 0.06	11.8 ± 0.1	52.6 ± 0.1	66.1 ± 0.3	118.7 ± 0.3
		R2	1.3 ± 0.05	1.5 ± 0.01	8.5 ± 0.03	11.3 ± 0.1	54.3 ± 0.1	70.4 ± 0.1	124.6 ± 0.1
		R3	1.1 ± 0.05	1.4 ± 0.01	7.1 ± 0.03	9.6 ± 0.03	60.1 ± 0.1	73.4 ± 0.4	133.4 ± 0.5
			Citric	Malic	Quinic	Total acids	Glucose	Fructose	Total sugars
			(g L^{-1})						
ANOVA[¶]									
Cultivar			***	**	***	***	***	***	***
Ripening stage			*	***	**	***	***	***	***
Tukey's multiple range test									
Cultivar									
Sour			24.7[‡] a	2.3 a	6.0 c	33.0 a	48.1 c	54.2 b	102.3 c
Sour–sweet			5.1 b	2.0 ab	11.7 a	18.8 b	52.2 b	65.5 a	117.7 b
Sweet			1.2 c	1.6 b	8.0 b	10.8 c	55.6 a	69.9 a	125.5 a
Ripening stage									
R1			12.1 a	2.3 a	9.4 a	23.9 a	48.1 c	59.3 b	107.4 c
R2			9.8 ab	1.9 b	8.6 ab	20.3 b	51.7 b	64.0 ab	115.6 b
R3			9.1 b	1.6 b	7.5 b	18.4 b	56.2 a	66.4 a	122.6 a

[†] Values are the mean of 6 replications (±standard error): 3 sun + 3 shadow replicates.
[‡] Values followed by the same letter, within the same variation source, were not statistically different according to Tukey's multiple range test.
[¶] N.S., not significant *F* ratio (*p* < 0.05).
*, **, and ***, significant at *p* < 0.05, 0.01, and 0.001, respectively.

Table 3
Proline contents (mg L^{-1}), antioxidant activity (AA) (mmol L^{-1} Trolox) and total phenolic compound (TP, mg GAE L^{-1}) in fruits from three pomegranate cultivars and at three ripening stages.

Cultivar		Ripening	Proline (mg L^{-1})	AA (mmol L^{-1} Trolox)	TP (mg GAE L^{-1})
BA1	Sour	R1	47.9[†] ± 3.3	8.63 ± 0.52	4210 ± 13
		R2	55.1 ± 3.2	7.87 ± 0.94	4154 ± 9
		R3	77.9 ± 3.4	6.35 ± 0.34	3876 ± 5
PTO5	Sour–sweet	R1	52.1 ± 4.0	8.07 ± 0.56	3458 ± 6
		R2	65.2 ± 3.9	7.49 ± 0.37	3307 ± 1
		R3	88.6 ± 3.5	6.61 ± 0.21	3295 ± 6
ME14	Sweet	R1	32.2 ± 1.9	7.00 ± 0.25	3725 ± 2
		R2	47.5 ± 3.2	6.53 ± 0.23	3261 ± 4
		R3	84.7 ± 2.8	6.65 ± 0.06	2674 ± 5
			Proline (mg L^{-1})	AA (mmol L^{-1} Trolox)	TP (mg GAE L^{-1})
ANOVA[¶]					
Cultivar			**	NS	***
Ripening stage			***	**	**
Tukey's multiple range test					
Cultivar					
Sour			60.3[‡] b	7.61	4065 a
Sour–sweet			68.6 a	7.38	3354 b
Sweet			54.3 b	6.72	3222 b
Ripening stage					
R1			44.1 c	7.90 a	3783 a
R2			56.0 b	7.29 ab	3576 ab
R3			83.7 a	6.53 b	3282 b

[†] Values are the mean of 6 replications (±standard error): 3 sun + 3 shadow replicates.
[‡] Values followed by the same letter, within the same variation source, were not statistically different according to Tukey's multiple range test.
[¶] N.S., not significant F ratio ($p < 0.05$).
*, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively.

Citric acid predominated over malic acid in sour (BA1) and sour–sweet (PTO5) cultivars, while the concentrations of citric and malic acid were similar in the sweet cultivar (Carbonell-Barrachina et al., 2012; Mena et al., 2011).

Glucose and fructose were the most abundant sugars found in these pomegranate cultivars. Fructose concentration was greater than glucose during the fruits ripening, with the ratio glucose/fructose taking values of approximately 0.8. Similar profiles were previously described in other cultivars (Melgarejo et al., 2000; Schwartz et al., 2009; Tezcan et al., 2009). However, the cultivar and/or the agro-climatic effect were evident in other studies in which the glucose content was higher than that of fructose (Özgen et al., 2008; Çam et al., 2009a). As the ripening progressed, the total organic acid content decreased from 23.9 to 18.4 g L^{-1} (citric + malic + quinic acids) while the total sugar content increased from 107 to 123 g L^{-1} (glucose + fructose); this was the expected behavior (Fawole and Opara, 2013; Kulkarni and Aradhya, 2005).

3.3. Proline

The proline content was significantly ($p < 0.05$) affected by both the pomegranate cultivar and the ripening stage (Table 3). Throughout the development of the fruit, "PTO5" cultivar (sour–sweet cv) presented the highest value 68.6 mg L^{-1}, followed by "BA1" (sour cv) 60.3 mg L^{-1} and "ME14" (sweet cv) 54.3 mg L^{-1}. As the ripening stage progressed, the proline content increased significantly (44.1–83.7 mg L^{-1}). Proline content increases during ripening and senescence in most fruits (Burroughs, 1970). Halilova and Yildiz (2009) in their study of the effects of climate change on the accumulation of proline in pomegranate, reported values of 30 mg L^{-1} in 2007 and 93 mg L^{-1} in 2008; these authors concluded that in warm and dry years, the proline accumulation increases. A wide variation has found in the content of proline in pomegranate; Velioglu et al. (1997) reported a value of 7.70 mg L^{-1} but Unal et al. (1995) reported a value of 23 mg L^{-1}.

Water is known to play an important role in the growth and maturation of fruits (Goñi et al., 2007; Khattab et al., 2011). The results showed that there is a correlation between the contents of sugars and proline, because as the fruit is maturing the availability and water supply is lower, which causes the sugars to concentrate and the proline to increase. Goñi et al. (2007), in his study on the changes in the water content during the maturation of the cherimoya, found that in addition to the accumulation of sugars, there was an accumulation of proline; this amino acid represented up to 74% of the total content of free amino acids in ripe cherimoya fruit.

3.4. Antioxidant activity

In this particular study, the AA was not affected by pomegranate cultivar, and only a minor decrease (from 7.90 to 6.53 mmol L^{-1} Trolox) was observed as the fruits ripened (Table 3). However, other researchers found that AA was influenced by the cultivar (Martínez et al., 2012; Tehranifar et al., 2010). Factors such as pomegranate genotypes and sample extraction protocols might certainly account for the divergence observed. Moreover, pomegranate antioxidant activity fluctuated depending on the fruit portion processed. Tzulker et al. (2007) reported that homogenates from the whole fruit exhibited an antioxidant activity of approximately 20 times higher than those from arils juice.

Reported AA values in the literature range from 6 to 15 mmol L^{-1} Trolox using the DPPH method (Seeram et al., 2008; Mena et al., 2011); results from the current study were within this interval. The behavior of the antioxidant activity during pomegranate ripening was previously reported by Kulkarni and Aradhya (2005); they reported a decrease of 13% in the AA of pomegranate arils between 20 and 60 days of fruit development. This decrease was explained by a reduction in the total phenolic content (Kulkarni

Table 4

External color coordinates in fruits from three pomegranate cultivars and at three ripening stages as affected by their position within the tree (East or "sun" and West or "shadow").

Cultivar	Ripening	Sun					Shadow				
		L^*	a^*	b^*	C^*	H^*	L^*	a^*	b^*	C^*	H^*
BA1	Sour R1	57.51† ± 2.48	11.45 ± 2.82	36.19 ± 1.81	38.92 ± 1.42	72.07 ± 4.54	65.70 ± 0.93	6.69 ± 1.65	42.27 ± 0.95	43.03 ± 1.05	81.22 ± 2.11
	R2	60.35 ± 1.39	14.60 ± 2.83	36.59 ± 1.37	40.25 ± 1.21	68.55 ± 4.24	67.63 ± 1.41	8.17 ± 1.66	41.33 ± 0.68	42.39 ± 0.70	78.93 ± 2.26
	R3	60.16 ± 1.72	23.16 ± 1.87	36.18 ± 1.36	43.35 ± 1.09	57.45 ± 2.75	69.32 ± 1.49	11.31 ± 2.08	43.66 ± 0.98	45.53 ± 0.61	75.39 ± 2.82
PTO5	Sour–sweet R1	60.90 ± 1.29	6.85 ± 3.26	32.92 ± 1.15	34.86 ± 1.18	78.36 ± 5.25	66.70 ± 1.54	3.41 ± 2.30	37.43 ± 1.25	38.19 ± 1.03	84.53 ± 3.66
	R2	62.01 ± 1.20	6.05 ± 2.43	33.31 ± 1.05	34.52 ± 1.16	79.80 ± 3.82	67.00 ± 0.73	4.96 ± 1.39	38.32 ± 0.55	38.85 ± 0.44	82.54 ± 2.10
	R3	57.33 ± 1.10	19.12 ± 4.00	29.04 ± 1.24	36.49 ± 1.48	58.12 ± 6.26	67.83 ± 2.32	15.03 ± 4.25	35.94 ± 1.73	40.99 ± 0.84	67.92 ± 6.34
ME14	Sweet R1	69.95 ± 2.43	14.03 ± 2.59	37.65 ± 1.12	40.83 ± 1.13	69.88 ± 3.64	67.76 ± 3.03	6.34 ± 2.38	36.72 ± 1.60	37.81 ± 1.75	81.02 ± 3.50
	R2	69.65 ± 2.03	16.27 ± 2.17	36.83 ± 0.76	40.73 ± 0.74	66.43 ± 3.10	67.60 ± 2.41	8.47 ± 2.96	36.29 ± 1.10	38.12 ± 1.37	77.86 ± 4.43
	R3	59.79 ± 1.73	30.51 ± 2.11	29.91 ± 1.40	43.16 ± 1.35	44.80 ± 2.91	68.91 ± 2.16	10.59 ± 2.66	36.71 ± 1.18	39.04 ± 0.62	73.73 ± 4.15
		L^*	a^*	b^*	C^*	H^*	L^*	a^*	b^*	C^*	H^*
		Sun					Shadow				
ANOVA¶											
Cultivar		***	***	***	***	**	NS	NS	***	***	NS
Ripening stage		**	***	***	**	***	NS	**	NS	*	**
Tukey's multiple range test											
Cultivar											
Sour		59.33‡ b	16.40 a	36.32 a	40.83 b	66.02 ab	67.55	8.72	42.42 a	43.65 a	78.51
Sour–sweet		60.08 b	10.67 b	31.75 b	35.28 b	72.09 a	67.17	7.80	37.22 b	39.34 b	78.32
Sweet		66.46 a	20.27 a	34.79 a	41.57 a	60.37 b	68.08	8.47	36.57 b	38.32 b	77.53
Ripening stage											
R1		64.00 a	10.75 b	35.58 a	38.20 b	73.43 a	66.72	5.48 b	38.80	39.67 b	82.25 a
R2		62.78 ab	12.30 b	35.57 a	38.49 b	71.59 b	67.40	7.19 b	38.64	39.78 b	79.77 a
R3		54.09 b	24.26 a	31.70 b	40.99 a	53.45 b	68.68	12.31 a	38.76	41.85 a	72.34 b

† Values are the mean of 18 replications (±standard error).

‡ Values followed by the same letter, within the same variation source, were not statistically different according to Tukey's multiple range test.

¶ N.S., not significant F ratio ($p < 0.05$).

*, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively.

Table 5
Internal color coordinates in three pomegranate varieties at three maturity stages, depending as affected by their position within the tree (East or "sun" and West or "shadow").

Cultivar		Ripening	Sun					Shadow				
			L^*	a^*	b^*	C^*	H^*	L^*	a^*	b^*	C^*	H^*
BA1	Sour	R1	37.97[†] ± 0.25	2.24 ± 0.1	−1.39 ± 0.02	2.63 ± 0.01	328 ± 1	36.55 ± 0.14	4.64 ± 0.02	−2.15 ± 0.09	5.12 ± 0.05	335 ± 1
		R2	36.31 ± 0.01	3.98 ± 0.02	−1.19 ± 0.01	4.15 ± 0.03	343 ± 1	36.06 ± 0.33	3.79 ± 0.17	−1.00 ± 0.19	3.92 ± 0.21	345 ± 2
		R3	37.37 ± 0.09	5.71 ± 0.09	−2.56 ± 0.01	6.25 ± 0.09	336 ± 1	37.00 ± 0.05	5.36 ± 0.07	−2.44 ± 0.06	5.89 ± 0.04	336 ± 1
PTO5	Sour–sweet	R1	35.91 ± 0.02	1.46 ± 0.02	−0.24 ± 0.04	1.48 ± 0.02	351 ± 1	35.40 ± 0.12	1.65 ± 0.05	−0.21 ± 0.04	1.66 ± 0.05	353 ± 1
		R2	36.02 ± 0.11	0.70 ± 0.07	0.80 ± 0.02	1.06 ± 0.06	357 ± 2	35.13 ± 0.02	1.13 ± 0.02	0.55 ± 0.04	1.25 ± 0.01	352 ± 2
		R3	38.11 ± 0.20	1.71 ± 0.03	−0.99 ± 0.04	1.97 ± 0.01	330 ± 2	36.09 ± 0.71	3.36 ± 0.13	−1.06 ± 0.05	3.52 ± 0.14	343 ± 1
ME14	Sweet	R1	37.61 ± 0.14	−0.37 ± 0.01	0.78 ± 0.06	0.86 ± 0.04	116 ± 2	37.53 ± 0.09	−0.22 ± 0.03	0.52 ± 0.02	0.57 ± 0.01	113 ± 3
		R2	36.76 ± 0.25	0.50 ± 0.01	−0.07 ± 0.04	0.50 ± 0.01	352 ± 5	35.91 ± 0.06	1.52 ± 0.02	−0.40 ± 0.01	1.57 ± 0.02	345 ± 1
		R3	38.31 ± 0.04	2.97 ± 0.01	−1.96 ± 0.03	3.56 ± 0.01	327 ± 1	37.94 ± 0.03	2.99 ± 0.01	−2.05 ± 0.01	3.62 ± 0.01	326 ± 1

ANOVA[¶]

	Sun					Shadow				
	L^*	a^*	b^*	C^*	H^*	L^*	a^*	b^*	C^*	H^*
Cultivar	NS	***	***	***	***	***	***	***	***	*
Ripening stage	**	***	***	***	***	NS	***	***	***	NS

Tukey's multiple range test

	Sun			Shadow		
	L^*	C^*	H^*	L^*	C^*	H^*
Cultivar						
Sour	37.21[‡]	4.34 a	336	36.53 a	4.97 a	339 a
Sour–sweet	36.67	1.50 b	346	35.53 b	2.14 b	349 a
Sweet	37.55	1.63 b	265	37.12 a	1.91 b	261 b
Ripening stage						
R1	37.15 ab	1.65 b	265	36.49	2.44 b	267
R2	36.36 b	1.90 b	351	35.69	2.24 b	348
R3	37.92 a	3.92 a	331	37.01	4.34 a	335

[†] Values are the mean of 6 replications (±standard error).
[‡] Values followed by the same letter, within the same variation source, were not statistically different according to Tukey's multiple range test.
[¶] N.S. = not significant F ratio ($p < 0.05$).
*, **, and ***, significant at $p < 0.05$, 0.01, and 0.001, respectively.

and Aradhya, 2005). Similarly, Fawole and Opara (2013) found a significant decrease in antioxidant activity of pomegranate juice at different maturation stages, a decrease of 67.8% and 66.4% for DPPH and FRAP respectively; they concluded that the reduction was associated with a decrease of polyphenols (Gil et al., 2000). In pomegranates, as well as in many other fruits, there is a general correlation between total phenolic content and antioxidant activity (Özgen et al., 2008; Gil et al., 2000).

3.5. Total phenolic compounds

The total phenolic content was significantly affected by both the cultivar and ripening stage (Table 3). The sour cultivar "BA1" showed the highest value 4065 mg GAE L^{-1}, followed by the sour–sweet cultivar "PTO5" 3354 mg GAE L^{-1} and the sweet cultivar "ME14" 3222 mg GAE L^{-1}. These experimental values agreed with those reported by Mena et al. (2011) in pomegranate varieties grown in Spain (range 1500–4500 mg GAE L^{-1}). Furthermore, Gil et al. (2000) reported TP concentrations of 2117 and 2566 mg L^{-1} for pomegranate juice from fresh arils and a commercial pomegranate juice, respectively. Nevertheless, the broad interval range of TP concentrations must obey to differences among cultivars (genotypes), growing seasons, farming practices, and determination assays (Tehranifar et al., 2010; Çam et al., 2009b).

As the ripening stage progressed, TP content significantly decreased from 3783 to 3282 mg GAE L^{-1}. Schwartz et al. (2009) reported a decrease in the content of phenolic compounds during fruit ripening from 3.9 to 1.9 mM. Similarly, Fawole and Opara (2013) reported a decrease in TP content from 1052 to 483 mg GAE 100 mL^{-1}). The decrease in the total phenolic content is attributed to the oxidation of polyphenols by polyphenol oxidase present during fruit ripening (Fawole and Opara, 2013; Kulkarni and Aradhya, 2005; Schwartz et al., 2009).

3.6. External color

Although many studies have been conducted on the effects of different farming practices on the quality parameters of pomegranate, the external color of the fruit has not been studied in detail; however, fruit maturity is commonly evaluated based on the color of the fruit peel (Manera et al., 2013).

The color of pomegranates is an important factor that clearly affects market acceptance and consumer preference (Opara et al., 2009). Table 4 shows the values of the external CIE$L^*a^*b^*$ color coordinates of pomegranate fruits at two different positions within the trees: (i) East, having a higher exposure to the sunlight and called from now on "sun oriented fruits" and (ii) West, having less exposure to the sunlight and been called from now on "shadow oriented fruits". In general, sun-fruits had lower values of lightness, L^*, implying darker colors, and simultaneously higher values of the green–red coordinate, a^*, and lower values of the blue–yellow coordinate, b^*. This combination of low values of L^* and b^* and high of a^* led to intense garnet (combination of red and blue tones) color, typical of pomegranate products, of the sun oriented fruits.

During ripening, L^*, b^* and Hue angle decreased while a^* and chroma increased. This same behavior was reported by Manera et al. (2012) in pomegranate rind harvested at the beginning of September. As the value of a^* increases and the value of L^* decreases steadily, the green color of pomegranate rind is replaced by the red color. A simultaneous increase in the values of a^* and C^* along with decreases in b^* and H^* contributes to the production of the characteristic garnet color of pomegranate fruits.

The reported effects of the position within the tree on the external color of pomegranate fruits are of high importance because external color is a key parameter is deciding the appropriate harvest time. A mixture of fruits from both orientations should be collected to take harvest decisions, because selecting fruits from just one orientation could lead to wrong picking dates.

3.7. Internal color

Even though, both cultivar and ripening stage significantly affected the internal color of pomegranate fruits (Table 5), the factor "position with the tree" showed no important effects on this particular quality parameter. The fact that the factor "fruit position within the tree" affected external color but not internal color seems to imply that external quality attributes are more susceptible to environmental changes than internal attributes. In this way, Fawole and Opara (2013) reported that color development occurs before in the husk than in arils.

Finally, it must be mentioned that ripening only caused significant ($p < 0.05$) increases of a^* and C^* in fruits from both positions within the tree; however, no clear effects were observed in L^* or b^*. The increase in the green–red coordinate, a^*, is without any doubt related to the increased biosynthesis and accumulation of anthocyanin pigments, which are responsible for the intense red color of ripe pomegranate fruits. In general, the most abundant anthocyanin are cyanidin-3,5-diglucoside cyanidin-3-glucoside in sour and sweet cultivars, respectively (D'Aquino et al., 2010). However, Hernández et al. (1999) that the anthocyanin profile changed during fruit ripening. These authors concluded that in the early fruit-ripening stages, delphinidin-3,5-diglucoside was the main pigment, followed by cyaniding-3,5-diglucoside; however, in later stages, the monoglucoside derivatives cyaniding-3-glucoside and delphinidin-3-glucoside increased considerably.

4. Conclusions

The position of pomegranate within the tree had no significant effect on chemical parameters, organic acids, sugars profile, proline, phenolic compounds and antioxidant activity of three pomegranate varieties grown in Spain at three ripening stages, however, if there was significant effect on the external color; this provides information on the decisive and appropriate harvest time. The third ripening stage (fruit weight > 300 g) was the optimal for a fruit with a balance between sugar and organic acid content, as well as internal and external red color characteristic of the pomegranate. However, the highest polyphenol content and antioxidant activity were reached at the second ripening stage (fruit weight 120–250 g).

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PUBLICATION 3

Physicochemical and descriptive sensory characterization of Spanish pomegranates: aptitudes for processing and fresh consumption

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Original article

Physicochemical and descriptive sensory characterization of Spanish pomegranates: aptitudes for processing and fresh consumption

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Summary   Pomegranate fruit and its products are being widely promoted to consumers as healthy alternatives for their daily diet. The aim was to study the main sensory differences among twenty pomegranate cultivars, determining which cultivars have particular flavour or texture notes that could make them more appropriate for fresh consumption or processing. Four clusters grouped all samples, two of them included sour cultivars and two of them included sweet and sweet-sour cultivars. Cluster 1 (sour) was characterized by having floral, apple, and grape flavour notes and also producing a tongue numbing sensation. Cluster 2 (sour), which included the *Wonderful* cultivar, had samples with wine-like attributes. Clusters 3 and 4 (sweet and sour-sweet) were characterized by having overall sweet, pear and grape notes for cluster 3, and beet, fruity-dark, fermented, musty and woody flavour for cluster 4.

Introduction

Pomegranate fruit and its juices are being widely promoted to consumers as one of the new 'superfoods', capable of addressing a huge variety of health disorders (Johanningsmeier & Harris, 2011). The new popularity of this fruit is shown in the large number of publications including biological, chemical and technological studies in which pomegranate and its properties are the aim of the research. Some scientific publications have shown that pomegranate and its juices have anti-atherogenic, antioxidant and antihypertensive effects (e.g. Rettig *et al.*, 2008; Saruwatari *et al.*, 2008; Basu & Penugonda, 2009); consequently, the promotion of the fruit seems justified. These beneficial health effects are in general associated with the phenolic content (Viuda-Martos *et al.*, 2010; Johanningsmeier & Harris, 2011).

Some authors (Tehranifar *et al.*, 2010; Calín-Sánchez *et al.*, 2011) have shown that diverse pomegranate cultivars produce juices with different total phenolic contents and antioxidant activities. Vázquez-Araújo *et al.* (2011a) reported that maceration of pomegranate (cultivar *Wonderful*) juice with pomegranate albedo homogenate resulted in increased total phenolic content but in a sensory profile comparable to that of the original juice. Borochov-Neori *et al.* (2009) reported that pomegranate antioxidant activity and sensory quality were not linked, and both parameters were dependent on cultivar and climatic conditions during fruit maturation and ripening. Those authors studied eleven Israeli pomegranate cultivars grown in the southern Araya Valley, but only studied the generic sensory properties 'quality' and 'colour'.

Koppel & Chambers (2010) studied thirty-three commercial pomegranate juices and developed a sensory lexicon to describe the main sensory attributes of these products. Vázquez-Araújo *et al.* (2011b) used this lexicon to study the sensory characteristics of commercial and freshly squeezed pomegranate juices

and also to establish relationships among sensory attributes and volatile composition. Koppel & Chambers (2010) grouped the thirty-three studied juice samples in five clusters characterized by having distinctive main sensory attributes. Belonging to each cluster depended on: (i) fruit cultivar, (ii) growing conditions and/or (iii) processing conditions. Calín-Sánchez *et al.* (2011) studied the volatile composition of nine Spanish pomegranate cultivars, grown in the same area (southern Spain) and weather. These authors reported significant differences in consumer acceptance of the freshly squeezed juices from nine pomegranate cultivars. Consumers liked the best the sweetness and fresh pomegranate odour of *Mollar* cultivars. Even though no descriptive analysis of the samples was conducted, it was hypothesized that these differences in consumers' liking were linked to different intensities of the main sensory attributes of the studied juices.

Nowadays, pomegranate is mainly cultivated in Iran, Afghanistan, India, Mediterranean countries (Tunisia, Turkey, Egypt, Spain and Morocco), USA, China, Japan and Russia (Carbonell-Barrachina *et al.*, 2012). Spain is the main producer and exporter of pomegranates in the European Union, with an official yield of 22 311 t in 2010 (MAGRAMA, 2010).

Although scattered information about sensory properties of Spanish pomegranate fruits as affected by cultivar was noted, no in-depth information was found in the scientific literature. Most data about pomegranate fruit or juices available either use nonspecific sensory attributes or are not related back to the fruit cultivar or to the specific manufacturing conditions. The main objectives of this study were the following: (i) to describe the sensory profiles of a large number of Spanish pomegranate cultivars, and (ii) to use descriptive sensory analysis to determine the best commercial option for pomegranate fruits, either fresh consumption or juice manufacture. This information will be of high value for farmers and food manufacturers to know, which cultivars could be the most appropriate to develop new pomegranate-based products.

Materials and methods

Samples

Fruits of thirteen cultivars were collected from one of the main European Union pomegranate germplasm banks, located at the experimental field station of Miguel Hernández University (UMH) in Orihuela (Alicante, eastern Spain). Also, fruits from five commercial cultivars purchased in the farmers' market of the area, and fruits from two commercial cultivars grown in the Canary Islands (Spain) were studied to compare with the fruits from the germplasm. Cultivars and codification for the study can be seen in Table 1.

Table 1 Cultivar names, origin and type of the pomegranate under study

Abbreviation	Cultivar	Origin	Type
VA11	*Valenciana de Albatera*	UMH Germplasm Bank	Sweet
VA1	*Valenciana de Albatera*	UMH Germplasm Bank	Sweet
CRO1	*Casta del Reino*	UMH Germplasm Bank	Sweet
ME1	*Mollar de Elche*	UMH Germplasm Bank	Sweet
ME2	*Mollar de Elche*	UMH Germplasm Bank	Sweet
ME14	*Mollar de Elche*	UMH Germplasm Bank	Sweet
MA1	*Mollar de Albatera*	UMH Germplasm Bank	Sweet
MO4	*Mollar de Orihuela*	UMH Germplasm Bank	Sweet
PTO3	*Piñón Tierno de Ojós*	UMH Germplasm Bank	Sweet
PTO7	*Piñón Tierno de Ojós*	UMH Germplasm Bank	Sour-sweet
ADO4	*Agridulce de Ojós*	UMH Germplasm Bank	Sour-sweet
BO1	*Borde de Ojós*	UMH Germplasm Bank	Sour
BA1	*Borde de Albatera*	UMH Germplasm Bank	Sour
HIZC	*Hizcaznar*	Commercial, Alicante	Sour
WOND	*Wonderful*	Commercial, Alicante	Sour
M50	*Mollar de Elche*	Commercial, Alicante	Sweet
VAcom	*Valenciana*	Commercial, Alicante	Sweet
Mcom	*Mollar*	Commercial, Alicante	Sweet
FV1	*Mollar*	Commercial, Canary Island	Sweet
FV2	*Mollar*	Commercial, Canary Island	Sweet

Approximately 80% of the pomegranate grown in Spain belong to the cultivar *Mollar de Elche*, but because of the problems of its colour (low intensity due to low content of anthocyanins; Mena *et al.*, 2011) during processing Spanish farmers are introducing the cultivar *Wonderful* in their farms. Consequently, *Mollar de Elche* and *Wonderful* fruits represent more than 90% of the total Spanish production of pomegranates. The reason for including all other cultivars in this study is because it is believed that some of them may have interesting sensory attributes that deserve attention.

All samples were collected at commercial ripening (October 2011). Over fifteen fruits (avoiding injured fruits and looking for similar ripening characteristics: external colour of fruits and total soluble solid content) were randomly harvested or purchased. Once in the laboratory, five homogeneous fruits were selected and the arils of each fruit were manually extracted and coded as 'subsamples A, B, C, D and E'. Fruits were kept under refrigeration (~5 °C) until analysis.

Physico-chemical analyses

To conduct the physico-chemical analyses, a portion of the arils of three fruits per sample (three

subsamples: A, B and C) were juiced using a domestic blender, Braun Citromatic (Braun, Madrid, Spain).

Total soluble solid content (TSS) was measured with a digital Atago refractometer (model N-20; Atago, Bellevue, WA, USA) at 20 °C with values being expressed as ºBrix. Titratable acidity (TA) was determined by acid-base potentiometer (NaOH, 0.1 N up to pH 8.1) and expressed as gram per litre of malic acid. Juice pH was measured by a Crison pH-meter (model MicropH 2001; Barcelona, Spain). Maturity index (MI), which is a ratio of TSS to TA, was calculated for each sample. Colour was determined in juices using the CIE$L^*a^*b^*$ system and a Minolta colorimeter CR200 model with D65 illuminant (Minolta camera Co., Osaka, Japan). As mentioned, all the analyses were run in triplicate (one fruit × three trees) to ensure accuracy and results were expressed as mean ± standard error.

Sensory analysis

Four highly trained panelists from the Sensory Analysis Center (Manhattan, KS, USA) participated in this study. Each of the panelists had more than 1000 h of testing experience with a variety of food products. For the current study, the panelists received further orientation on fresh and processed pomegranates. The panelists travelled to Spain to conduct the study.

The samples (pomegranate arils) were served into odour-free, disposable 90 mL covered plastic cups, (Sweetheart Cup Co., Inc., Owings Mills, MD, USA) for the evaluation. Half cup filled with pomegranate arils (approximately 40–50 g) was served to each panelist; additional sample was available if the panelists requested it. All samples were served at room temperature. For each sample, the panel evaluated five subsamples (A, B, C, D and E) coded with the three digits of the sample and a letter (e.g. sample: 997a, 997b, 997c, 997d and 997e). Unsalted crackers and distillated water were used to clean palates between samples.

Ten sessions of 2 h were held for the samples evaluation. Two samples (a total of ten subsamples) were evaluated per session. The panel started working with the lexicon reported by Koppel & Chambers (2010) for pomegranate juices, but some attributes, definitions and/or references were removed, included and/or adapted to pomegranate fruit evaluation. Attributes and definitions used for the present study are shown in Table 2.

A modified consensus profile method, which uses a numerical scale where 0 represents none and fifteen extremely strong with 0.5 increments, was used (Koppel & Chambers, 2010; Talavera-Bianchi *et al.*, 2010; Adhikari *et al.*, 2011). The panelists independently scored each subsample and also provided a 'representative score' for each sample (not the average, but most repeated value). The testing room was at ~21 °C; the illumination was a combination of natural and non-natural (fluorescent) light.

Data analyses

All physico-chemical (three replications) and sensory data (five replications) were subjected to statistical analysis using SPSS® (version 12.0; SPSS Inc., Chicago, IL, USA.), for analysis of variance (ANOVA) and Tukey's honestly significant differences (HSD) for *post hoc* mean separation. Principal Components Analysis (PCA) was used for the data analysis on the consensus profiles to study patterns, if any, among cultivars. Only flavour and mouthfeel attributes were used for the analysis. Representative scores were used for this analysis, avoiding the use of attributes which appeared in single fruits (subsamples) but were not typical of the cultivar. Unscrambler version 9.7 (Camo Software, Oslo, Norway) was used to conduct PCA.

Also, the Statistical Analysis System version 8.2 (SAS, Cary, NC, USA, 2001) was used for clustering the samples and for the correlation analysis, using Pearson's correlation coefficients. Clustering of the samples was done by using the CLUSTER procedure (Ward's Minimum Variance Cluster Analysis). The number of clusters was set according to the eigenvalues of the correlation matrix (>1). Again, only flavour and mouthfeel representative scores were used for the clustering analysis of the samples.

Results and discussion

Physico-chemical analyses

Table 3 shows the main physico-chemical differences among pomegranate cultivars. In general, significant differences were found among samples in the studied parameters. The parameter showing the largest variations was titratable acidity, with four samples (corresponding to BO1, BA1, WOND and HIZC cultivars) presenting values of malic acid equivalents higher than 16 g L^{-1} juice, two samples had values close to 8–9 g malic acid equivalents per litre juice (PTO7 and ADO4), while the rest of the samples presented values around 2–3 g malic acid equivalents per litre juice. The pH values ranged from 3.3 up to 4.9 and were similar to those previously reported by other authors (e.g. Mena *et al.*, 2011; Calín-Sánchez *et al.*, 2011), with lower values being associated with the highest values of titratable acidity, as expected. On the other hand, the TSS contents were similar in all studied samples, although statistical significant differences were found; all cultivars had values over 12 ºBrix, minimum threshold required for commercial use of the fruits (Zaouay *et al.*, 2012), and ranged from 14.6 in sample

Table 2 Colour, flavour, mouthfeel and texture attributes and definitions used in the study

Attribute	Definition	Reference (flavour)*
Colour	Intensity of the arils colour (garnet)	Dutch Boy paint sample 10144 = 5.0 Porter Paints paint sample 6030-7 = 10.0
Fruity	A general term used to describe the sweet, floral, fruity aromatics associated with a variety of fruits	Diluted Welch's white grape juice (1:1) = 5.0
Pomegranate	Sour, sweet, fruity aromatics that may be somewhat dark, musty/earthy with an astringent mouthfeel. These aromatics are reminiscent of a combination of Concord grape, cranberries and other berries such as blackberries, cherries, currants and raspberries. There are also vegetable notes of beets and carrots	VitalGrana pomegranate juice = 5.5
Apple	Sweet, light, fruity, somewhat floral aromatic commonly associated with apple juice and apples	Mott's 100% Apple Juice = 8.5
Pear	Sweet, slightly musty, floral, honey/caramel-like, fruity aromatic associated with ripe pears	Jumax Pear Nectar (can) = 7.5
Fruity-dark	Sweet, brown honey/caramel-like aromatics commonly associated with dark fruits such as raisins and prunes that have been cooked	Mixture of Sun Maid raisins, dried Ocean Spray cranberries and of Sun Maid prunes in water = 5.0
Grape	Sweet, brown, fruity, musty aromatics commonly associated with grapes	Welch's Concord Grape Juice = 9.5
Berry	Sweet, sour, sometimes dark aromatics associated with a variety of berries such as blackberries, cherries, currants raspberries etc., excluding cranberries	Diluted Welch's White Grape Juice (1:1) = 5.0 Blackwell Red Currant Jelly = 8.5
Cranberry	Sweet, fruity, slightly sour and sharp aromatics commonly associated with cranberries	Ocean Spray Dried cranberries = 9.0 Ocean Spray cranberry juice = 7.5
Cherry	Sour, fruity, slightly bitter aromatics commonly associated with cherries	RW Knudsen Cherry Juice diluted (1:2) = 4.0
Floral	Sweet, light, slightly perfume impression associated with flowers	Welch's White Grape Juice diluted (1:1) = 5.0
Beet	Damp, musty/earthy, slightly sweet aromatics commonly associated with beets	Diluted juice of Kroger Sliced beets (1:2) = 4.0
Carrot	Aromatics commonly associated with canned, cooked carrots	Del Monte Sliced Canned Carrots = 7.0
Brown sweet	Rich full-bodied medium brown sweet aromatics	C&H Golden Brown Sugar = 8
Candy-like	Sweet, non-natural aromatic usually found in candy products such as Jell-O and Kool-Aid	Jell-O Strawberry powder = 7.5
Fermented	Aromatics associated with ripe/overripe fruit; can be somewhat sweet, sour, browned, musty and fruity	Private Selection Cooking Wine = 10.0 (aroma)
Green-Viney	Green aromatic associated with green vegetables and newly cut vines and stems; characterized by increased bitter and musty/earthy character	Trans-2-hexen-1-ol 5000 ppm (in propylene glycol) = 4.0 (aroma)
Molasses	Dark caramelized top notes that are slightly sharp and acrid and characteristic of molasses	Grandmas Molasses = 6.5
Musty/Earthy	Humus-like aromatics that may or may not include damp soil, decaying vegetation or cellar-like characteristics	Diluted juice of Kroger Sliced beets = 7.0
Sweet overall	Perception of the combination of sweet taste, sweet aromatics, caramelized, brown sugar, honey and maple	Welch's White Grape Juice diluted (1:1) = 4.0
Vinegar	Sour, astringent, slightly pungent aromatics associated with vinegar	Heinz Vinegar diluted (1:20) = 7.0
Wine-like	Sharp, pungent, somewhat fruity alcohol-like aromatics associated with red wine	Regina Cooking Wine = 10.0 (aroma)
Woody	Aromatics associated with dry freshly cut wood	Forster Craft Stick = 7.5 (aroma)
Sweet	Fundamental taste factor of which sucrose is typical	2% Sucrose Solution = 2.0 4% Sucrose Solution = 4.0
Salt	Fundamental taste factor of which sodium chloride is typical	0.15% NaCl Solution = 1.5
Sour	Fundamental taste factor of which citric acid is typical	0.05% Citric Acid Solution = 3.5 0.08% Citric Acid Solution = 5.0
Bitter	Fundamental taste factor of which caffeine or quinine is typical	0.020% Caffeine Solution = 3.5 0.035% Caffeine Solution = 5.0
Astringent	Dry puckering mouthfeel associated with an alum solution	0.05% Alum Solution = 2.5
Toothetch	Sensation of abrasion and drying of the surface of the teeth	Welch's Grape Juice diluted (1:1) = 6.0
Chalky Mouthfeel	Dry, powdery sensation. Can be on mouth and/or teeth	1 g corn starch dissolved in 100 mL water = 3.0

Table 2 (*Continued*)

Attribute	Definition	Reference (flavour)*
Tongue tingle	Feeling of an increased sensation on the tongue that may be due to intense carbonation or other causes. Evaluate during first 3–5 s after sample is placed in the mouth	7-Up = 8.5
Tongue numb	Loss of sensation on tongue evaluated after swallowing the sample	7-Up = 5.5
Throat burn	The chemical feeling factor described as a burning sensation perceived in the throat and mouth surfaces	Heinz White Vinegar (1:12) = 8.0
Arils peel firmness	The degree of force required in the initial bite of a seed with the incisors until it ruptures or erupts. Testing technique: take a seed between the incisors and then bite down evenly. Evaluate the force required to rupture the peel covering the seed	Canned grapes = 8.5
Seed hardness	The degree of force required in the initial bite of a seed with the molars until it deforms or compresses. Testing technique: take a seed between the molars and then bite evenly. Evaluate the force required to compress and deform the seed	Sunflower seeds = 4.0

*References' preparation can be seen in Koppel & Chambers (2010).

Table 3 Physico-chemical and sensory colour characteristics of the samples

Sample	TSS (ºBrix)	TA (g malic acid per litre)	pH	Maturity index	Colour *L**	*a**	*b**	C	h	Sensory score
VA11	16.63 abcd*	2.37 d	4.67 ab	70.9 a	32.43 abcd	4.32 cdefg	−1.96 h	4.74 bcde	336.7	5.4 ghij
VA1	15.97 abcd	2.87 d	4.37 abcd	55.7 cd	29.89 def	4.15 cdefg	−0.98 abcdef	4.26 cde	346.7	8.0 cde
CRO1	16.73 abc	2.62 d	4.61 abc	64.1 abc	32.41 abcd	4.51 cdefg	−1.46 efdg	4.75 bcde	342.0	6.5 efghi
ME1	16.27 abcd	2.61 d	4.74 ab	62.5 abc	30.33 cdef	3.85 efg	−1.01 abcdefg	3.98 cde	345.2	10.4 ab
ME2	15.37 bcd	2.64 d	4.72 ab	58.4 bcd	31.24 abcdef	4.24 cdefg	−1.10 bcdefgh	4.39 bcde	345.5	6.9 efg
ME14	16.03 abcd	2.74 d	4.84 a	58.7 abc	33.41 ab	4.26 cdefg	−0.77 abcde	4.34 bcde	350.2	4.7 ij
MA1	16.67 abcd	2.68 d	4.85 a	62.9 abc	30.71 bcdef	6.16 ab	−1.91 gh	6.45 a	342.8	7.6 de
MO4	17.10 ab	2.73 d	4.75 ab	62.9 abc	29.92 def	5.62 abc	−1.58 efgh	5.84 ab	344.3	9.1 bcd
PTO3	14.63 cd	2.70 d	4.77 a	54.5 cd	32.83 abc	4.12 defg	−0.20 a	4.13 cde	237.4	3.8 j
PTO7	14.60 d	9.51 cd	3.99 bcde	15.4 e	29.97 def	5.47 abcd	−0.13 a	5.47 abc	358.7	9.8 abc
ADO4	15.23 bcd	8.29 d	3.68 de	18.6 e	30.75 abcdef	5.06 abcde	−0.23 ab	5.07 abcd	357.4	7.4 def
BO1	16.47 abcd	21.64 ab	3.27 e	7.9 e	30.76 abcdef	6.28 ab	−0.37 abc	6.33 a	236.8	5.6 fghij
BA1	16.80 ab	19.30 ab	3.42 e	9.3 e	31.53 abcde	6.36 a	−0.88 abcdef	6.42 a	352.2	4.9 hij
HIZC	17.77 a	16.58 bc	3.52 e	11.3 e	29.01 ef	4.06 defg	−0.38 abc	4.08 cde	354.5	11.6 a
WOND	17.07 ab	25.96 a	3.28 e	6.9 e	28.56 f	3.44 fg	−0.49 abcd	3.48 e	351.0	10.5 ab
M50	16.63 abcd	3.22 d	4.50 abc	51.7 d	33.54 a	3.35 g	−1.16 cdefgh	3.55 de	341.4	4.7 ij
VAcom	15.40 bcd	2.26 d	4.56 abc	68.5 ab	31.79 abcde	4.47 cdefg	−1.61 efgh	4.77 bcde	339.9	6.9 efg
Mcom	16.23 abcd	2.92 d	4.76 ab	55.8 cd	32.15 abcd	4.66 cdefg	−1.68 fgh	4.95 abcde	340.3	5.6 fghij
FV1	17.07 ab	3.00 d	3.88 cde	56.9 cd	30.18 cdef	4.87 bcdef	−1.00 abcdef	4.97 abcde	348.4	6.6 efgh
FV2	17.33 ab	3.26 d	3.87 cde	53.3 d	30.36 cdef	5.15 abcde	−1.34 defgh	5.32 abc	345.5	6.5 efghi

*Mean of three replications. Values followed by the different letter, in the same column, were significantly different ($P < 0.05$), according to the Tukey's honestly significant differences (HSD).

PTO7 to 17.8 in sample HIZC. The Maturity Index (MI), calculated as the ratio TSS (ºBrix):TA (g malic acid equivalents 100 mL juice), has been used as a classification parameter for pomegranate fruits (Martínez *et al.*, 2006): (i) sweet varieties, MI = 31–98, (ii) sour-sweet varieties, MI = 17–24, and (iii) sour varieties = 5–7. Following this classification, as expected, most of the cultivars used in the present study corresponded to sweet varieties, because most of the fruits under study are intended for fresh consumption. Only the commercial Wonderful cultivar had an MI lower

than 7 (6.9 ºBrix:g malic acid 100 mL juice). In general, cultivars Borde and Hizcaznar (samples BO1, BA1, and HIZC) are considered sour pomegranate varieties, but results of the resent study showed MI values slightly higher than previously described. Samples PTO7 and ADO4 presented MI values belonging to the sour-sweet group (15–19).

Data on CIE*L*a*b** coordinates (Table 3) showed that differences among the colour of the pomegranate samples under study were statistically different, but these differences were not large; for instance, *L** values

ranged between 28.6 and 33.5, with <5 units of differences. In the same way, differences for a^* and b^* were even lower, 3 and 1.8 units, respectively. However, the trained sensory panel was able to detect larger differences in the colour intensity of the arils, with sensory colour scores ranging from 3.8 (PTO3) and 11.6 (HIZC). These data resulted in a significant negative relationship between L^* and sensory colour (Pearson's correlation coefficient -0.838 with $\rho = 0.05$).

Sensory analysis

Tables 4 and 5 show the scores of the flavour, taste and mouthfeel attributes, which received higher punctuations in the pomegranate cultivars (average of five subsamples): fruity, pomegranate, apple, pear, grape, berry, cranberry, cherry, floral, green-viney, sweet overall, woody, sweet, sour, bitter, astringent, toothetch and throat burn. As shown, statistically significant differences were found for all these attributes; however, the difference between the maximum and minimum scores was only equal or above two units in few attributes: fruity, pomegranate, cranberry, woody, sour, bitter, astringent, toothetch and throat burn.

Figure 1 shows the sensory seed hardness of the samples under study. Pomegranate cultivars can be classified depending on the hardness of the seeds in: (i) hard, (ii) semisoft and (iii) soft (Melgarejo *et al.*, 2000). Softness of the seed, large fruit size, thin and coloured skin, and abundant juice are considered among the desirable characteristics in pomegranate breeding programs (Zamani *et al.*, 2010; Mansour *et al.*, 2011). Pomegranate seeds, so-called arils, have two main parts: the testa, which is the fleshy soft coat, and the tegmen (woody part), with a woody consistency and which determines the hardness of the arils (Melgarejo *et al.*, 2000). In general, hard cultivars are not appropriate for fresh consumption because of their seeds hardness; thus, this sensory attribute largely determines the initial fresh eating quality of the fruit. Seed hardness ranged from 3.2 (sample ME2) to 10.6 (sample BA1). In general, sour samples (BO1, BA1, WOND and HIZC) had higher seed hardness than the sweet or sour-sweet samples. Mollar is a Spanish term related with softness, so most of *Mollar* varieties had low seed hardness scores, as expected from their cultivar name; ME14, FV1 and FV2 were exceptions to this general rule.

Cluster analyses showed four distinctive clusters, which grouped the twenty pomegranate samples (Table 6). Clusters obtained using the pomegranate sensory descriptions were completely different from the ones reported by Koppel & Chambers (2010) in processed pomegranate juices. These authors found five

Table 4 Flavour attributes which received scores equal or higher than 2.0 for at least one of the pomegranate cultivars

Sample	Fruity	Pomeg.	Apple	Pear	Grape	Berry	Cranberry	Cherry	Floral	Green-viney	Sweet Overall	Woody
VA11	5.0 efghi*	5.1 fgh	2.3 bcde	2.0 ab	2.0 bc	1.2 bcde	1.6 ef	1.2 cdef	4.2 bcdef	1.5 abc	4.3 abcde	0.9 cdefg
VA1	4.2 i	4.3 h	2.1 cde	1.5 bc	1.6 cde	1.0 cde	1.4 f	1.1 defgh	3.7 f	1.7 abc	3.6 ef	1.6 abc
CRO1	4.0 efghi	4.6 efg	1.7 cde	1.6 abc	1.4 cde	0.8 cde	1.3 ef	0.7 fgh	3.3 ef	1.0 cd	3.7 abcd	1.4 abc
ME1	4.6 hi	5.1 fgh	2.5 abc	2.0 ab	1.7 cde	1.3 bcd	1.5 ef	1.0 efgh	3.8 f	1.1 bcd	4.5 abc	2.2 a
ME2	4.6 hi	5.2 fgh	2.1 cde	2.0 ab	1.9 c	1.0 cde	1.3 f	0.8 fgh	4.1 cdef	1.2 bcd	4.5 abc	2.0 ab
ME14	4.4 hi	5.4 efgh	2.2 bcde	1.8 abc	1.2 e	1.2 bcde	1.3 f	1.0 efgh	4.0 def	1.4 abcd	3.8 cdef	1.3 bcde
MA1	4.7 ghi	5.2 fgh	1.9 de	1.6 abc	1.7 cde	1.0 cde	1.3 f	0.6 gh	3.7 f	1.2 bcd	3.9 bcdef	1.5 abc
MO4	4.4 hi	4.8 gh	1.9 de	1.6 abc	1.6 cde	0.8 de	1.0 f	0.4 h	3.6 f	1.0 cd	3.7 def	1.4 abcd
PTO3	4.4 hi	4.9 gh	1.8 e	1.8 abc	1.3 de	1.2 bcde	1.1 f	0.6 gh	3.9 ef	0.7 d	3.8 cdef	1.4 abcd
PTO7	5.9 abcd	7.9 b	2.7 ab	2.1 a	2.1 bc	1.6 abc	2.7 cd	2.1 ab	5.1 a	1.5 abc	4.3 abcde	0.4 fgh
ADO4	6.1 ab	7.2 bcd	2.4 abcd	2.0 ab	2.0 bc	1.6 abc	2.2 de	1.8 abcd	5.0 ab	1.3 bcd	4.8 a	1.0 cdefg
BO1	6.0 abc	8.0 b	2.9 a	1.9 abc	2.1 bc	1.6 abc	3.3 bc	2.2 ab	4.9 abc	1.8 ab	4.5 abc	n.d.[†] h
BA1	5.2 cdefg	7.4 bc	2.1 cde	1.5 bc	1.8 cd	1.1 cde	2.8 cd	1.6 abcde	3.9 ef	2.1 a	3.6 ef	0.8 cdefgh
HIZC	6.6 a	11.0 a	2.1 cde	1.5 bc	2.0 bc	2.1 a	3.7 b	1.9 abc	4.2 bcdef	2.1 a	3.6 ef	1.5 abc
WOND	5.8 abcde	10.8 a	1.9 de	1.4 c	1.8 cd	1.8 ab	4.7 a	2.3 a	4.1 cdef	2.1 a	3.5 f	0.6 defgh
M50	5.1 defgh	5.5 efg	1.9 de	2.1 a	2.8 a	0.6 e	1.2 f	1.2 cdef	4.8 abcd	1.3 bcd	4.6 ab	0.5 efgh
VAcom	4.8 fghi	5.9 efg	2.0 cde	1.6 abc	2.5 ab	1.2 bcde	1.7 ef	1.0 efgh	4.7 abcde	1.5 abc	4.3 abcde	0.2 gh
Mcom	4.9 fghi	5.6 efg	2.1 cde	1.9 abc	1.8 cd	1.0 cde	1.4 f	1.3 cdef	4.1 cdef	1.7 abc	4.3 abcde	0.8 cdefgh
FV1	5.6 bcdef	6.4 cde	2.1 bcde	2.0 ab	2.0 bc	1.4 bcd	1.6 ef	1.5 bcdef	4.4 abcdef	1.6 abc	4.1 abcdef	1.2 bcdef
FV2	4.7 ghi	5.2 def	1.8 cde	1.5 abc	1.7 bc	1.3 bcd	1.3 ef	1.1 cdef	3.5 f	1.4 abc	3.4 bcdef	1.1 bcdef

n.d., not detected.

*Mean of five replications. Values followed by the different letter, in the same column, were significantly different ($P < 0.05$), according to the Tukey's honestly significant differences (HSD).

[†]Standard error was <0.1 for all data values.

Table 5 Basic tastes and mouthfeels which received scores equal or higher than 2.0 for at least one of the pomegranate cultivars

Sample	Sweet	Sour	Bitter	Astringent	Toothetch	Throat burn
VA11	3.3 abcde*	3.2 def	3.2 bc	3.2 bc	1.5 bcde	n.d. c
VA1	3.0 de	3.1 def	3.1 bc	2.8 bcd	1.3 bcde	n.d. c
CRO1	3.2 bcde	2.2 fg	2.1 ef	1.6 f	0.6 ef	n.d. c
ME1	3.9 a	2.5 efg	2.7 cdef	1.9 ef	n.d.[†] g	n.d. c
ME2	3.5 abcde	2.6 efg	2.6 cdef	1.6 f	n.d. g	n.d c
ME14	3.4 abcde	2.3 fg	2.3 def	1.6 f	0.2 fg	n.d. c
MA1	3.3 abcde	2.0 g	2.0 f	1.5 f	n.d. g	n.d. c
MO4	3.2 bcde	2.3 fg	2.2 ef	1.5 f	n.d. g	n.d. c
PTO3	3.3 abcde	2.4 efg	2.5 cdef	1.7 f	n.d. g	n.d. c
PTO7	3.5 abcde	4.4 c	3.0 bcd	3.6 b	2.0 ab	0.8 bc
ADO4	3.9 a	3.1 def	2.7 cdef	2.7 cde	1.2 cde	0.2 c
BO1	3.7 a	5.5 ab	3.2 bc	4.7 a	2.4 a	2.0 b
BA1	3.0 de	4.0 cd	3.1 bc	2.7 cde	1.4 bcde	1.2 ab
HIZC	3.1 cde	4.6 bc	3.5 b	3.3 bc	1.9 abc	1.6 a
WOND	2.9 e	6.2 a	4.3 a	5.4 a	2.4 a	1.6 a
M50	3.9 a	3.1 def	2.6 cdef	2.3 def	0.8 ef	n.d. c
VAcom	3.4 abcde	3.1 def	2.9 bcde	2.6 cde	1.5 bcde	n.d. c
Mcom	3.6 abcd	3.3 de	2.7 cdef	2.8 bcd	1.0 de	n.d. c
FV1	3.2 bcde	2.9 efg	2.8 bcde	2.8 bcd	1.4 bcde	n.d. c
FV2	2.6 e	2.2 fg	2.4 cdef	2.4 cde	1.3 bcde	n.d. c

n.d., not detected.

*Mean of five replications. Values followed by the different letter, in the same column, were significantly different ($P < 0.05$), according to the Tukey's honestly significant differences (HSD).
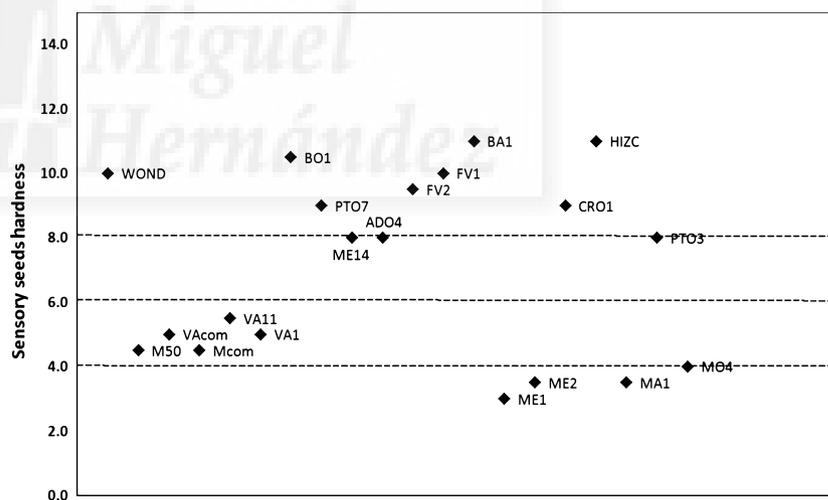
[†]Standard error was <0.1 for all data values.

Figure 1 Texture scores (average of five subsamples) for twenty pomegranate cultivar samples. Numerical 15 points scale with 0.5 intervals.

clusters characterized by the following attributes: (i) berry, dark-fruity, toothetch mouthfeel, (ii) grape, cranberry, wine-like, (iii) fermented, toothetch mouthfeel, (iv) brown colour, musty/earthy, and (v) candy-like, sweet overall. Some of those attributes were not even detected by the sensory panel when testing the fresh pomegranate samples of the present study (e.g. candy-like, brown colour) and those attributes were clearly related with the processing of the fruits to manufacture the juices.

Cluster 1 of the present study was represented by a single sample, a sour cultivar (BO1), which belonged to the UMH germplasm bank. Figure 2 shows the PCA map for the flavour and mouthfeel attributes of the samples: PC1 and PC2 explained 58% of the variation of the samples; this low explanation value could be linked with the fact that a high number of the pomegranate cultivars studied have different names but are genetically linked with sensory properties being relatively close. As shown in the map, the BO1 sample

**Table 6** Cluster analysis results of pomegranate juices (semipartial *r*-squared <0.05)

Cluster	Samples	Differential attributes
1	BO1	Sour, floral, apple, grape, tongue numb
2	BA1, WOND, HIZC	Sour, salty, wine-like, cranberry, bitter, vinegar
3	M50, VAcom, PTO7, ADO4	Sweet, sweet overall, pear, grape
4	MO4, MA1, PTO3, VA1, FV1, FV2, ME1, VA11, ME14, CRO1, Mcom, ME2	Sweet, beet, fruity-dark, fermented, musty, woody flavour

was characterized by having higher grape, apple and floral flavour notes than all other samples, and also producing a tongue numbing mouthfeel. This mouthfeel was slightly present in all subsamples of BO1 (scores ~1 in the 15 points scale), but absent in all other samples, including other sour cultivars, such as BA1, HIZC or WOND. More cultivars should be studied to confirm whether this particular cluster includes other cultivars or these characteristics are only specific for the BO1 variety. This is an ornamental cultivar, with large and acid fruits, hard seeds and with deep red juices, which showed a distinctive character when studying its genetic trait (Melgarejo *et al.*, 2009). The high levels of fruity and floral characteristics could make this cultivar of interest in further breeding programs.

Cluster 2 was composed by samples BA1, WOND and HIZC; three sour samples characterized by having salty and wine-like. Sample BA1 was characterized by being especially salty, reaching an average score of 3.0

(all other samples had 1.0 or 1.5 in the 15 points scale). Samples HIZC and WOND were commercial samples obtained from local growers. Wonderful is the most appreciated cultivar in the US, and it is characterized by having sour over sweet taste (Dafny-Yalin *et al.*, 2010). Vázquez-Araújo *et al.* (2011a) reported thirteen main sensory attributes to describe fresh pomegranate juices made with Wonderful cultivar: fruity, berry, cranberry, fruity-dark, floral, musty/earthy, sweet overall, sweet, sour, bitter, astringent, toothetch and metallic. However, the attributes: carrot, beet and woody, only obtained scores below two in the Wonderful fruits grown in Spain. Consequently, the effect of different soil and/or growing conditions (e.g. irrigation patterns) seemed to have an important role in the development of the sensory profile of pomegranate fruits. The study conducted by Vázquez-Araújo *et al.* (2011a) used just Wonderful juice samples and did not find the wine-like and salty notes predominating in the current study; it was also possible that some of the attributes characteristics of commercial juices masked the presence of these two attributes.

In general, as shown in Fig. 1, sour cultivars had higher fruity, pomegranate, green, cherry, berry, bitter, vinegar and cranberry notes. Mouthfeelings, such as astringency, throat burn, tongue tingle and toothetch, were associated with sourness and probably with the chemical composition of the fruit. Melgarejo *et al.* (2011) studied the volatile composition of nine pomegranate cultivars, including sweet, sour-sweet and sour cultivars. Their results showed the presence of some aldehydes, such as nonanal, hexanal, decanal or Z-3-hexenal in sour cultivars (*Borde de Ojós* and *Borde de Albatera*). The presence of aldehydes, together with high concentration of organic acids, might be related
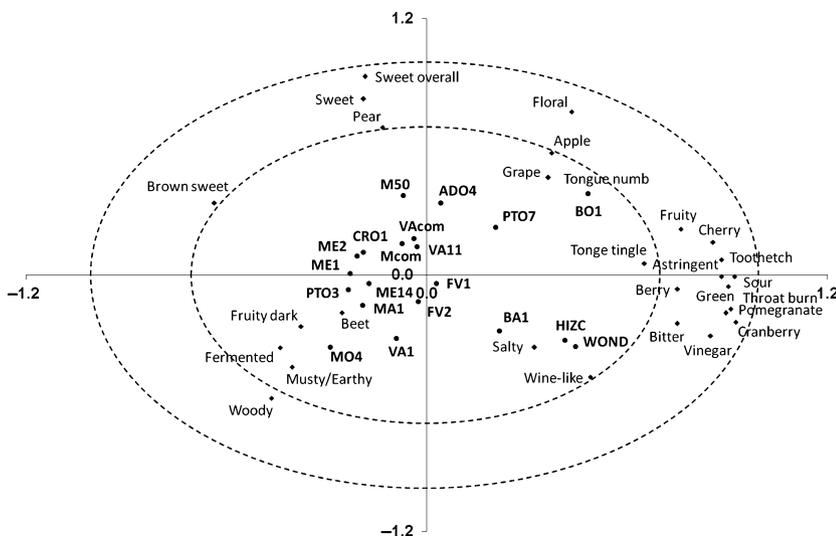
X explanation (PC1, PC2): 42%, 17%

Figure 2 PCA map showing representative scores (only flavour and mouthfeels) of juice samples. Samples abbreviations are indicated in bold font.

to the throat burn and the other mouthfeels detected in sour pomegranate cultivars.

Cluster 3 was composed by two sour-sweet (PTO7 and ADO4) and two commercial samples (Mcom and VAcom). Representative flavour notes of this cluster were the following: sweetness, sweet overall and pear (Fig. 1). This cluster can be defined as a transition group between the sour samples of clusters 1 and 2 and the sweet samples of the 4th and final cluster.

Cluster 4 included twelve samples: 8 *Mollar* samples, 2 *Valencianas*, 1 *Piñón Tierno de Ojós*, and 1 *Casta del Reino*, all sweet cultivars. Calín-Sánchez *et al.* (2011) and Melgarejo *et al.* (2011) reported that *Mollar* samples were the most liked when conducting consumers studies in which pomegranate cultivars, grown in the same UMH germplasm collection that the ones of the present study, were tested. Results of the present study indicated that these cultivars, most of them belonging to cluster 4, had beet, fruity-dark, fermented, musty and woody flavour notes. Being grown in the Spanish peninsula or in the Canary Islands did not change the main flavour and mouthfeel characteristics of the *Mollar* cultivar pomegranates, because samples FV1 and FV2 were also included in cluster 4 with most of the Mollar samples.

Best market options

The most important quality attributes for pomegranate fruits aimed for fresh consumption are the following: large size (not studied here), intense colour of skin (not studied here), intense colour of arils, high sweetness and soft seeds (Martínez *et al.*, 2012; Melgarejo *et al.*, 2012). Several cultivars evaluated in the present study did not match those requirements, some of them because of being sour or sour-sweet cultivars (BA1, HIZC, BO1 and WOND, and PTO7 and ADO4, respectively), and some because of having hard seeds or unsuitable arils colour (CRO1, ME14, FV1 and FV2). Consequently, nine out of the twenty pomegranate cultivars have appropriate sensory attributes for their commercialization as fresh products (soft seeds and high sweetness); these cultivars are the following: ME1, ME2, MA1, MO4, VA1, VA11, M50, Mcom and VAcom.

Very intense colour of arils is a key requirement for juice manufacturing because the heat treatments involved in the processing will drastically reduce the colour of the juice (Mena *et al.*, 2013). The samples that received a colour intensity score close or above 8.0 matched this requirement, according to authors' professional experience on industrial pomegranate processing: HIZC, WOND, ME1, PTO7, MO4 and VA1.

Even though FV1 and FV2 are actually being marketed as fresh products and labelled as *Mollar* fruits, the present study proved that their high seed hardness precludes them from belonging to the *Mollar* varietal group. Therefore, these two cultivars are better suited for juice manufacturing than for fresh consumption.

Carbonell-Barrachina *et al.* (2012) studied the potential of Spanish sour-sweet cultivars for the juice industry and concluded that they contributed with positive attributes (colour and fresh pomegranate flavour) to the sensory profile of pomegranate juices and to the overall liking of consumers. Therefore, fruits from cultivars ADO4, BO1 and BA1 would be better suited for juice manufacture. Depending on the market requirements and needs, these sour-sweet or sour fruits could be mixed with sweet fruits until getting the desired equilibrium of sour and sweet tastes.

Due to their relatively high seed hardness (between 8.0 and 9.0), fruits from sweet cultivars PTO3, ME14 and CRO1 are appropriate for mixing with sour-sweet or sour fruits to obtain equilibrated juices. Mixing of appropriate ratios of sweet, sour-sweet and sour fruits will make possible to adjust the sweetness and sourness of juices according to the consumers' requirements and needs.

Conclusions

Physico-chemical and sensory differences were found among the twenty pomegranate cultivars. Although physico-chemical characteristics could be used to classify pomegranate cultivars in sweet, sour-sweet or sour cultivars, the use of descriptive sensory analysis allowed a more precise classification based on a more detailed and complete set of data. Twenty-eight flavour and mouthfeel attributes were used to create different clusters and classify the pomegranate cultivars. Four clusters were created, two of which grouped sour cultivars and two of which grouped sour-sweet and sweet cultivars. *Wonderful*, the most appreciated cultivar in the USA, was characterized by being sour and having salty and wine-like notes (cluster 2). On the other hand, most of *Mollar* and *Valencia,* highly appreciated cultivars in Spain, were characterized by being sweet and having beet, fruity-dark, fermented and musty/earthy flavour notes. Using the generated information during descriptive sensory analyses of fruits from the twenty pomegranates under investigation, it can be concluded that: (i) VA11, ME2, MA1, M50, VAcom and Mcom are appropriate for fresh consumption, (ii) CRO1, ME14, PTO3, PTO7, ADO4, BO1, BA1, HIZC, WOND, FV1 and FV2 are appropriate for juice manufacturing, and finally (iii) VA1, ME1 and MO4 could be used for both fresh consumption and juice manufacturing.

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PUBLICATION 4

Pomegranate juice adulteration by the addition of grape or peach juices

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Pomegranate juice adulteration by addition of grape or peach juices

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Abstract

BACKGROUND: Pomegranate juice has gained a high reputation for its health properties and consequently is now a highly demanded product. However, owing to the limited production and high price of fresh pomegranates, adulteration of pomegranate juice seems to be happening. Hence it is imperative to establish criteria for detecting adulteration.

RESULTS: Addition of grape juice significantly increased the contents of Ca, Mg and Fe and especially tartaric acid and proline and simultaneously decreased the content of K. Addition of peach juice up to 10% (v/v) only resulted in a significant increase in sucrose content. Regarding the volatile composition, adulteration of pomegranate juice with grape juice resulted in significant increases in acetic acid, isoamyl butyrate and especially 1-hexanol and linalool, while adulteration with peach juice resulted in significant increases in butyl acetate, isobutyl butyrate, benzyl acetate and especially isoamyl butyrate.

CONCLUSION: The control protocols used in this study can serve as a basis for identification of pomegranate juice adulteration. It is important to highlight that it is necessary to simultaneously analyze and have results from several parameters to conclude that a particular pomegranate juice has been adulterated by mixing with another fruit juice.
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Keywords: authentication; organic acids; potassium; proline; *Punica granatum*; volatile composition

INTRODUCTION

Pomegranate (*Punica granatum* L.) products are gaining acceptance among consumers mainly because of their health benefits[1] but also because of organoleptic properties such as their attractive appearance and color.[2] Recently, pomegranate juice has been recommended as a preventive treatment for coronary heart disease.[3] It can also contribute favorably (1) to improve chemotherapeutic effects on human prostate cancer,[4] (2) to significantly reduce blood pressure[5] and (3) to improve induced stress of myocardial ischemia in patients with coronary artery disease.[6] However, there is controversy about which compounds (punicalagins, punicalins, urolithins, etc.) exert the beneficial health effects in the human body.

Consequently, pomegranate-based products have gained a high reputation and are being marketed as high-quality or gourmet food items. Most of these products, especially juices, claim to be 100% natural, not from concentrate, thus ensuring the greatest health benefits as well as elevated consumer acceptance.[7] Commercial pomegranate juices are a good solution for persons interested in consuming healthy products throughout the year.

On the other hand, industrial processing may have negative effects on the functionality and sensory quality of pomegranate juice; these negative effects are often associated with heat treatments, which mainly lead to loss of anthocyanins and volatile compounds.[8,9] In this way, pasteurization, the most popular heat treatment, may result, if not controlled and optimized, in significant changes in aroma profile and significant color degradation.[10]

Nowadays, adulteration of pomegranate juice has been detected owing to various factors such as high product demand, high price, short harvest season and shortage of production in some regions. Mixing with other juices is also done to compensate the negative effects of low-quality raw materials and/or processing. In this way, some companies may intentionally add other fruit juices to compensate for (1) the typical intense astringency of juice prepared with carpellar membranes or with extended maceration of the juice with the fruit rind or peel and (2) the pale brown color of the juice caused by the loss of anthocyanins during pasteurization.[11] If this happens, consumers purchase products that promise more than they actually offer. The most typical or detected adulteration methods are (1) addition of sugars or sweet juices, e.g. peach juice, to mask the astringency of tannins, (2) addition of a low volume of lemon juice to mask the intense sweetness of some pomegranate cultivars, e.g. 'Mollar de Elche', (3) addition of fruit juices with deep and intense red color, e.g. grape or raspberry juice, and (4) addition of cheap and widely available juices, e.g. grape, peach or pear juice.

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The juices used to adulterate pomegranate juice should be readily available, cheap and with a chemical composition, color and volatile profile similar to those of pomegranate.[11,12] In Mediterranean countries such as Spain, grape and peach juices may be viable alternatives to adulterate pomegranate juice owing to their high sugar contents,[11] which can mask the intense sourness of several pomegranate cultivars, including 'Wonderful'. Besides, red grapes will also improve the juice color.

An adulterated pomegranate juice can be identified if its chemical composition differs significantly from or is outside the normality range of a pure juice. However, there is much controversy about which parameters or indicators should be used to control the authenticity of pomegranate juice. For instance, some authors[11] claim that *sucrose* should not be present in commercial pomegranate juices owing to isomerase activity, while other researchers[13] propose that the presence of low levels of sucrose should be considered as an indicator of juice freshness. Another issue of discrepancy is the content of *proline*, which is postulated by various authors[14,15] as an indicator of juice purity. For instance, Zhang *et al.*[11] concluded that proline contents above 25 mg L^{-1} are indicative of addition of grape products, while Hanim and Nesrin[16] found higher proline contents in fresh pomegranate juices.

Therefore the objectives of this study were (1) to determine the main quality characteristics (organic acids, sugars, minerals, proline and volatile compounds) of pure pomegranate juice and two potential juices for adulteration, namely grape and peach juices, (2) to evaluate the changes observed after adulterating pomegranate juice with different concentrations of grape or peach juice and (3) to establish simple but practical parameters to check the authenticity or adulteration of pomegranate juice.

MATERIALS AND METHODS

Samples and experimental design

Since Spain is one of the main producers of pomegranate juice within the European Union, a pomegranate juice prepared using the most widely grown pomegranate cultivar in Spain, 'Mollar de Elche', was selected for this study. Grape and peach juices were chosen for the adulteration of pomegranate juice. The commercial juices used were (1) pomegranate juice (PgJ) from VitalGrana (Catral, Alicante, Spain), (2) grape juice (GJ) from Premium (Murcia, Spain) and (3) peach juice (PJ) from Rostoy (Murcia, Spain).

Commercial juices were selected because the protocol developed in this study should be applied to control the authenticity of such juices; however, it was essential to prove that the juices were 100% pure and no initial adulteration was found. Consequently, the commercial juices were supplied directly (October 2012) by three different juice companies with cooperation agreements with our university and research group; for instance, the Food Quality and Safety group of Miguel Hernández University has characterized all products from VitalGrana and established their nutritive, functional and sensory values and shelf-life (http://www.vitalgrana.com). Besides, completely similar pomegranate juices (cultivars, farming practices, weather conditions, etc.) were used by our research group in previous studies on this juice.[2,17–19] As a result of all the above, we are completely sure that the juices were 100% pure products of pomegranate, grape and peach respectively.

The pomegranate juice under study (VitalGrana) is prepared by mixing 'Mollar de Elche' and 'Wonderful' juices at a ratio of 4:1 (v/v); these two pomegranate cultivars are the most widely grown in Spain and in the USA respectively. Consequently, this pomegranate juice can be considered as representative of a high percentage of the pomegranate juices being sold in international markets. The grape and peach cultivars used for manufacturing the studied juices were 'Merlot' and 'Baby Gold' respectively; these two cultivars are also widely cultivated throughout the world.

Each juice (five bottles of 1 L each from three different batches) was first analyzed without any mixing. Later, pomegranate juice was adulterated with grape or peach juice at concentrations (v/v) of 10, 25 and 50% of grape juice and 5 and 10% of peach juice. The maximum values of these concentrations were below the detection thresholds established by a trained sensory panel with wide expertise in sensory analyses.[17] Thresholds were established at 55 and 12% for grape and peach juices respectively; at these concentrations, 50% of the panelists were able to detect a significant difference from the control sample, pure pomegranate juice. Juice blends were stored at 4 °C until 30 min before analyses, which were conducted within 1 week. The following parameters were analyzed in pure and juice blends: organic acids, sugars, minerals (Ca, Mg, K, Na, Fe, Cu, Mn and Zn), proline and volatile composition. Juices were prepared in triplicate and all analyses were run in triplicate.

Physicochemical analysis

Analysis of organic acids and sugars

Organic acids and sugars were quantified according to Carbonell-Barrachina *et al.*[17] Juices were centrifuged at 10 000 × *g* for 20 min. Then 1 mL of supernatant was filtered through a 0.45 µm Millipore filter and injected into a Hewlett-Packard Series 1100 (Wilmington, Del, USA) high-performance liquid chromatography (HPLC) system. The elution buffer was 1 g L^{-1} phosphoric acid at a flow rate of 0.5 mL min^{-1}. Organic acids were isolated using a Supelcogel™ C-610H column (30 cm × 7.8 mm) with a Supelguard column (5 cm × 4.6 mm) (Supelco, Bellefonte, PA, USA). The absorbance at 210 nm was measured using a diode array detector (DAD). The same HPLC conditions (elution buffer, flow rate and column) were used for the analysis of sugars. Detection was conducted using a refractive index detector (RID). Standards of organic acids (citric, tartaric and malic acids) and sugars (glucose, fructose and sucrose) were obtained from Sigma (Poole, UK). Calibration curves, obtained by triplicate injection of standard solutions, were used for quantification purposes and showed good linearity (regression coefficients (R^2) ≥ 0.999).

Mineral analysis

Pure juices and juice blends (15 mL) were digested for 2 h at a temperature below 130 °C in a multi-place digestion block (Block Digest 20, Selecta, Barcelona, Spain) using 5 mL of 65% HNO$_3$.[20] Samples were left to cool to room temperature and then transferred to volumetric flasks. Dilutions of 1:10 and 1:50 (v/v) were prepared using ultrahigh-purity deionized water. Samples were stored at 4 °C until analysis.

Determination of Ca, Mg, K, Na, Cu, Fe, Mn and Zn in previously mineralized samples was performed using a Solaar 969 atomic absorption–emission spectrometer (Unicam Ltd, Cambridge, UK). K and Na were analyzed by atomic emission, while the other elements were analyzed by atomic absorption.

Instruments were calibrated using certified standards. In each analytical batch, at least two reagents blanks, one certified reference material (CRM) and one spike were included to assess precision and accuracy for chemical analysis. The CRM selected for the current experiment was GBW07603 (bush, branches and

leaves); this material is produced by the Institute of Geophysical and Geochemical Exploration of China and was selected because the juices under analysis have significant amounts of solid vegetal material. Calibration curves were used for the quantification of minerals and showed good linearity ($R^2 \geq 0.997$). Analyses were run in triplicate.

Determination of proline
Proline was quantified by a colorimetric method recommended by the IFU.[21] A solution of ninhydrin in ethylene glycol monomethyl ether ($30\,g\,L^{-1}$) was prepared. Then 1 mL of juice sample, 1 mL of formic acid (98%) and 2 mL of the ninhydrin solution were mixed and placed in a boiling water bath, ensuring that the water level completely covered the solution. After 15 min, 20 mL of butyl acetate (99.5%) was added to extract the color into the organic phase. The solution was then filtered and dried using filter paper containing 0.2 g of anhydrous sodium sulfate. After 15 min, the absorbance of the organic phase at 509 nm was measured in a Uvikon XS UV–visible spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France). Calibration curves in the range $0-50\,mg\,L^{-1}$ were used for the quantification of proline and showed good linearity ($R^2 \geq 0.995$). Analyses were run in triplicate.

Volatile compounds
Extraction procedure
Headspace solid phase microextraction (HS-SPME) was the method selected to study the volatile composition of the juices under analysis. After several preliminary tests to optimize the extraction system, 10 mL of juice was hermetically placed in a 50 mL vial with a polypropylene cap and a PTFE/silicone septum; the juice/headspace ratio was approximately 1:4 (v/v). A magnetic stirring bar was added together with NaCl ($150\,g\,L^{-1}$) and the vial was placed in a water bath with temperature control and stirring. The vial was equilibrated for 15 min at 40 °C, then a 50/30 μm DVB/CAR/PDMS fiber was exposed to the sample headspace for 50 min at 40 °C. This type of fiber was chosen for its high capacity to trap fruit volatile compounds.[22] A similar extraction procedure was previously carried out with tomatoes by Alonso et al.[23] and with pomegranates by Melgarejo et al.[18] and Vázquez-Araújo et al.[24] After sampling, desorption of the volatile compounds from the fiber coating was carried out in the injection port of the gas chromatography/mass spectrometry (GC/MS) system for 3 min.

Chromatographic analysis
Isolation and identification of the volatile compounds were performed using a Shimadzu GC-17A gas chromatograph coupled with a Shimadzu QP-5050A mass spectrometer (Shimadzu Corporation, Kyoto, Japan). The GC/MS system was equipped with a TRACSIL Meta.X5 column (95% dimethylpolysiloxane/5% diphenylpolysiloxane, $60\,m \times 0.25\,mm$, 0.25 μm film thickness; Teknokroma S. Coop. C. Ltd, Barcelona, Spain). Analyses were carried out using helium as carrier gas at a column flow rate of $0.6\,mL\,min^{-1}$ in a split ratio of 1:5 and the following program: 80 °C for 0 min; increase at 3 °C min^{-1} from 80 to 210 °C and hold for 1 min; increase at 25 °C min^{-1} from 210 to 300 °C and hold for 3 min. The temperatures of the injector and detector were 230 and 300 °C respectively.

Most compounds were identified using three different analytical methods, namely (1) retention indices,[25] (2) GC/MS retention times (authentic chemicals) and (3) mass spectra (standards and Wiley229 spectral database). Identification was considered

tentative when it was based on only mass spectral data. The volatile studies were conducted in triplicate. The concentration of each compound is expressed as % of the total arbitrary area units.

Statistical analysis
Data from the juice analyses were examined by analysis of variance (ANOVA) and Tukey's multiple range test using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD, USA). Significance was defined at $P \leq 0.05$.

RESULTS AND DISCUSSION
Organic acids
Table 1 summarizes the contents of the main organic acids (citric, tartaric and malic acids) in pomegranate, grape and peach juices and their mixtures. In general, citric and malic acids are the most abundant and characteristic acids in pomegranate juice, with their ratios depending basically on the pomegranate cultivar.[13,17] According to the AIJN *Reference Guide for Pomegranate Juice*,[26] the values of citric and malic acids should be in the ranges 0.1–33 and 0.02–3.6 $g\,L^{-1}$ respectively. In the pomegranate juice used for the present study, the concentrations of the two main compounds were quiet similar, with citric acid at 3.23 $g\,L^{-1}$ and malic acid at 2.61 $g\,L^{-1}$. In general, the contents of citric acid are much higher than those of malic acid in sour and sour–sweet cultivars, while the concentrations of citric and malic acids are similar in sweet cultivars.[13,17,27,28] It is important to mention that tartaric acid was present in the studied pomegranate juice, but only at trace level. The concentrations of organic acids found in the selected pomegranate juice agreed well with the AIJN reference values.

On the other hand, the organic acid profile of peach juice was similar in the compounds present, but their concentrations were significantly lower.[29] Finally, malic and tartaric acids were the most abundant organic acids in grape juice.

The organic acid profile can be used to detect adulteration of pomegranate juice with other juices;[30] however, the relative ratios among the acids depend strongly on the pomegranate cultivar and ripening stage.[31,32] Addition of grape products to pomegranate

Table 1. Organic acid contents in pure commercial pomegranate, grape and peach juices and blended juices (pomegranate + grape or peach)

Juice	Organic acid ($g\,L^{-1}$)[a]		
	Citric acid	Tartaric acid	Malic acid
Pomegranate (PgJ)	$3.23 \pm 0.17a$	Trace	$2.61 \pm 0.41a$
Grape (GJ)	Trace	$2.71 \pm 0.03a$	$2.53 \pm 0.12a$
Peach (PJ)	$0.61 \pm 0.01d$	Trace	$1.19 \pm 0.02b$
Blended juices			
PgJ + GJ 10%	$3.00 \pm 0.03a$	$0.30 \pm 0.01d$	$2.58 \pm 0.01a$
PgJ + GJ 25%	$2.65 \pm 0.04b$	$0.55 \pm 0.01c$	$2.49 \pm 0.01a$
PgJ + GJ 50%	$1.65 \pm 0.02c$	$1.05 \pm 0.03b$	$2.45 \pm 0.03a$
PgJ + PJ 5%	$3.05 \pm 0.02a$	Trace	$2.48 \pm 0.02a$
PgJ + PJ 10%	$2.68 \pm 0.04b$	Trace	$2.36 \pm 0.04a$
ANOVA[b]	***	***	*

[a] Values are mean ± standard error of three replications. Means followed by the same letter within a column are not statistically different according to Tukey's multiple range test.
[b] Significance of F ratio: * $P < 0.05$; *** $P < 0.001$.

juice will result in measurable concentrations of tartaric acid, as suggested by Zhang *et al.*;[11] at the same time, the content of citric acid will be drastically reduced.[33] Mato *et al.*[34] concluded that the grape juice is characterized by its elevated concentration of tartaric acid, ranging from 2.3 to 3.5 g L^{-1} and representing more than 50% of the total acids found in this juice. Adulteration of pomegranate juice with peach juice will be difficult to determine based only on the organic acid profile, because the concentrations of citric and malic acids decreased only in very low proportions.

Sugars

Table 2 shows the contents of the main sugars (fructose, glucose and sucrose) in pomegranate, grape and peach juices and their mixtures. In general, fructose and glucose are the most abundant and characteristic sugars in pomegranate juice, with the glucose/fructose ratio being in the range 0.7–1.0.[11,13] According to the AIJN *Reference Guide for Pomegranate Juice*,[26] the values of fructose and glucose should be in the ranges 50–100 and 45–85 g L^{-1} respectively. In the pomegranate juice used for the present study, the predominant compound was fructose (70.8 g L^{-1}), followed by glucose (54.2 g L^{-1}), with a glucose/fructose ratio of 0.77; these concentrations agreed well with the AIJN reference values. It is important to mention that sucrose was present in the studied pomegranate juice, but only at trace level. There is some controversy about the presence of sucrose in pomegranate juices. On the one hand, authors such as Mena *et al.*[13] claimed that the presence of sucrose should be considered a quality parameter in freshly squeezed pomegranate juice. On the other hand, authors such as Zhang *et al.*[11] concluded that detection of sucrose indicates adulteration with cane sugar or other sucrose sources.

The sugar profile of the grape juice is very similar to that of pomegranate juice; however, the profile of the peach juice is completely different, with sucrose predominating (70.1 g L^{-1}) and fructose only present at trace level. The peach data agreed quite well with previous results of Versari *et al.*,[29] who reported a sucrose content of 73 g L^{-1}.

No significant changes were observed in the sugar profile after addition of grape juice to the pomegranate juice; however, the

content of sucrose increased significantly after addition of peach juice.

Mineral elements

Certified values for Ca (%), Mg (%), K (%), Cu (mg kg^{-1}), Fe (mg kg^{-1}), Mn (mg kg^{-1}) and Zn (mg kg^{-1}) were 1.81 ± 0.07, 0.65 ± 0.03, 1.38 ± 0.04, 274 ± 10, 9.3 ± 0.5, 45 ± 2 and 37 ± 1 respectively, while the measured values for these elements were 1.80 ± 0.05, 0.66 ± 0.03, 1.40 ± 0.05, 275 ± 8, 9.4 ± 0.4, 48 ± 5 and 35 ± 3 respectively. These results clearly proved the goodness of the digestion and quantification protocols.

Table 3 reports the contents of essential mineral elements (Ca, Mg, K, Na, Fe, Cu, Mn and Zn) in pomegranate, grape and peach juices and their mixtures. In general, K is the most abundant and characteristic mineral in pomegranate juice.[27,35] According to the AIJN *Reference Guide for Pomegranate Juice*,[26] the values of Ca, Mg and K should be in the ranges 5–150, 20–100 and 800–2500 mg L^{-1} respectively. In the pomegranate juice used for the present study, the values of Ca (25.3 mg L^{-1}) and Mg (27.3 mg L^{-1}) were in the lower sections of these ranges, while the K content (2492 mg L^{-1}) was in the upper section. Besides, the content of Na (29.5 mg L^{-1}) should be below 100 mg L^{-1}.[26] Regarding the microelements and generally, the contents of Fe (1.03 mg L^{-1}) and Zn (1.28 mg L^{-1}) are higher than those of Cu (0.41 mg L^{-1}) and Mn (0.35 mg L^{-1}), and all their contents are always below 5.0 mg L^{-1}.[26]

Adulteration of pomegranate juice by mixing with other juices can result in dilution of the most abundant mineral (K) and enrichment of some of the less abundant minerals (Fe, Cu and Mn). However, changes in most of these elements are difficult to link with adulteration because of their wide natural range in pomegranate as a result of differences in cultivars, maturation stages, soils, etc. For instance, the natural range of Ca in pomegranate juices is 5–150 mg L^{-1}, which makes it almost impossible to detect adulteration using Ca as an indicator. Consequently, K is the key mineral to be controlled.

In this specific study, addition of grape juice significantly ($P < 0.05$) increased the contents of Ca, Mg, Fe, Cu and Mn and significantly ($P < 0.05$) decreased the K content. However, the increases in Ca and Mg were mainly due to the fact that the selected pomegranate juice was low in these two elements. On the other hand, mixing with peach juice only increased the content of Mg and decreased that of K.

The K contents in the juices under study were 2492, 806 and 1002 mg L^{-1} in pomegranate, grape and peach juices respectively; these contents agreed well with those reported by the USDA,[36] namely 2590, 900 and 970 mg L^{-1} respectively. Zhang *et al.*[11] initially established a minimum value of 1800 mg L^{-1} for the K content in pomegranate juices. However, after considering that lower-K-containing pomegranate varieties are known, they reduced this minimum threshold to a value of 1300 mg L^{-1}. These authors concluded that low K should be used to classify a juice as non-authentic only when combined with other atypical criteria.

According to the current results, any juice with K content lower than 2000 mg L^{-1} is highly suspicious of being adulterated. However, as stated previously, a low K content alone is not enough to conclude that a pomegranate juice is not a pure or authentic pomegranate product.

Proline

The proline content was significantly affected by the type of juice (Table 4). Grape juice presented the highest proline content

Juice	Fructose	Glucose	Sucrose
Table 2. Sugar contents in pure commercial pomegranate, grape and peach juices and blended juices (pomegranate + grape or peach)			
	Sugar (g L^{-1})[a]		
Pomegranate (PgJ)	$70.8 \pm 0.5a$	$54.2 \pm 1.6\,cd$	Trace
Grape (GJ)	$66.0 \pm 0.8b$	$66.5 \pm 0.9a$	Trace
Peach (PJ)	$0.1 \pm 0.1d$	$6.6 \pm 0.1e$	$70.1, \pm 0.1a$
Blended juices			
PgJ + GJ 10%	$70.3 \pm 0.6a$	$54.6 \pm 0.1\,cd$	Trace
PgJ + GJ 25%	$67.3 \pm 0.9b$	$57.3 \pm 0.3c$	Trace
PgJ + GJ 50%	$66.2 \pm 0.6bc$	$60.9 \pm 0.3b$	Trace
PgJ + PJ 5%	$66.2 \pm 0.1bc$	$55.3 \pm 0.4\,cd$	$2.78, \pm 0.06c$
PgJ + PJ 10%	$64.3 \pm 0.1c$	$53.6 \pm 0.6d$	$4.88, \pm 0.03b$
ANOVA[b]	**	***	***

[a] Values are mean $\pm$ standard error of three replications. Means followed by the same letter within a column are not statistically different according to Tukey's multiple range test.
[b] Significance of *F* ratio: ** $P < 0.01$; *** $P < 0.001$.

Table 3. Mineral contents in pure commercial pomegranate, grape and peach juices and blended juices (pomegranate + grape or peach)

Juice	Mineral macroelement (mg L^{-1})[a]			
	Ca	Mg	K	Na
Pomegranate (PgJ)	25.3 ± 1.5d	27.3 ± 1.0e	2492 ± 1a	29.5 ± 0.3ab
Grape (GJ)	73.0 ± 0.1a	96.9 ± 0.2a	806 ± 2f	33.5 ± 2.3a
Peach (PJ)	37.3 ± 4.4c	95.9 ± 2.1a	1002 ± 4e	22.1 ± 2.7c
Blended juices				
PgJ + GJ 10%	33.2 ± 1.1c	34.8 ± 0.7d	2449 ± 5a	29.3 ± 0.2ab
PgJ + GJ 25%	38.9 ± 0.1c	43.7 ± 0.8c	2142 ± 5c	30.6 ± 0.2a
PgJ + GJ 50%	48.3 ± 1.1b	73.4 ± 0.6b	1779 ± 5d	32.3 ± 0.4a
PgJ + PJ 5%	23.9 ± 1.1d	32.8 ± 2.9d	2336 ± 4b	28.9 ± 0.2b
PgJ + PJ 10%	25.2 ± 2.2d	35.8 ± 1.3d	2226 ± 6b	26.7 ± 0.2bc
ANOVA[b]	***	***	***	*

Juice	Mineral microelement (mg L^{-1})[a]			
	Fe	Zn	Cu	Mn
Pomegranate (PgJ)	1.03 ± 0.12d	1.28 ± 0.02a	0.41 ± 0.01 cd	0.35 ± 0.01e
Grape (GJ)	4.85 ± 0.04a	0.59 ± 0.02d	0.75 ± 0.01a	1.11 ± 0.01a
Peach (PJ)	0.31 ± 0.02e	0.41 ± 0.01e	0.14 ± 0.01e	0.06 ± 0.01f
Blended juices				
PgJ + GJ 10%	1.22 ± 0.06d	0.96 ± 0.01b	0.44 ± 0.01 cd	0.46 ± 0.01d
PgJ + GJ 25%	1.64 ± 0.06c	0.87 ± 0.01c	0.47 ± 0.01bc	0.58 ± 0.01c
PgJ + GJ 50%	3.33 ± 0.01b	0.75 ± 0.01c	0.52 ± 0.02b	0.76 ± 0.01b
PgJ + PJ 5%	0.98 ± 0.01d	0.97 ± 0.07b	0.40 ± 0.01 cd	0.37 ± 0.01e
PgJ + PJ 10%	0.96 ± 0.03d	0.95 ± 0.01b	0.37 ± 0.01d	0.34 ± 0.01e
ANOVA[b]	**	***	*	***

[a] Values are mean ± standard error of three replications. Means followed by the same letter within a column are not statistically different according to Tukey's multiple range test.
[b] Significance of F ratio: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 4. Proline content in pure commercial pomegranate, grape and peach juices and blended juices (pomegranate + grape or peach)

Juice	Proline (mg L^{-1})[a]
Pomegranate (PgJ)	251 ± 15d
Grape (GJ)	1032 ± 12a
Peach (PJ)	182 ± 18f
Blended juices	
PgJ + GJ 10%	320 ± 15d
PgJ + GJ 25%	446 ± 14c
PgJ + GJ 50%	639 ± 16b
PgJ + PJ 5%	223 ± 16e
PgJ + PJ 10%	212 ± 15ef
ANOVA[b]	***

[a] Values are mean ± standard error of three replications. Means followed by the same letter are not statistically different according to Tukey's multiple range test.
[b] Significance of F ratio: *** $P < 0.001$.

(1032 mg L^{-1}), followed by pomegranate juice (251 mg L^{-1}) and peach juice (182 mg L^{-1}).

Gorsel et al.[37] reported a proline value of 1007 mg L^{-1} in grape juice, similar to that of the present study. Later, the AIJN *Reference Guide for Grape and Peach Juices*[38] reported a maximum value for the proline content of 1400 mg L^{-1}.

As expected, adulteration of pomegranate juice with grape juice at concentrations of 10, 25 and 50% led to a significant increase in proline content to levels of 320, 446 and 639 mg L^{-1} respectively, while adulteration with peach juice at concentrations of 5 and 10% led to a significant decrease in proline content to levels of 223 and 212 mg L^{-1} respectively (Table 4).

The controversy regarding the normal or maximum content of proline in pomegranate juice is important.

On the one hand, Zhang et al.[11] reported that one method of adulteration of pomegranate juice was 'addition of grape juice and grape skin color as detected by elevated levels of malic acid, proline, tartaric acid, grape anthocyanins, or other non pomegranate anthocyanins'. These authors concluded that the presence of the amino acid proline at >25 mg L^{-1} is indicative of added grape products. However, they did not provide any reference describing proline contents in fresh pomegranate fruits at different maturation stages, or in juices from other pomegranate cultivars, or in other types of juice.

On the other hand, Ting and Rouseff[14] found that the proline content increased with the maturation stage of orange, and this observation resulted in proline increasing from 600 to 1530 mg L^{-1} in frozen concentrated Florida orange juice. Recent studies within the Food Quality and Safety group of Miguel Hernández University (data not published) evaluated the effects of the maturation stage on the characterization of pomegranate juices from different cultivars and found a clear positive relationship between the maturation stage and the proline content. For instance, in 'Mollar de Elche' juices, proline increased from 32 to 84 mg L^{-1}. Hanin and Nesrin[16] studied the effect of climate change on the proline content in three cultivars of pomegranate. These authors concluded that hot and dry seasons resulted in higher contents of proline; for instance, 2008 was hotter and drier than 2007 and this fact resulted in a significant increase in proline from 30 to 93 mg L^{-1}.

Summarizing this section, it can be stated that addition of grape juice or grape products to pomegranate juice will result in important increases in proline content. However, the maximum level should be set at values of about 250–300 mg L^{-1} to avoid claiming false adulteration of juices that are certainly pure pomegranate juices.

Volatile composition

Before starting to discuss the obtained results, it is important to highlight that the volatile composition of pomegranate juices is a parameter that can be affected by factors such as pomegranate cultivar and agronomic and environmental conditions. However, the trends and relationships found in this study are of high importance.

The volatile compounds found in pomegranate juice can be grouped into nine chemical families: (1) *alcohols*, including ethanol, *cis*-3-hexenol, 1-hexanol and 2-ethyl-1-hexanol; (2) *esters*, e.g. ethyl acetate and isoamyl butyrate; (3) *terpenes*, including α-pinene, β-pinene and limonene; (4) *aldehydes*, pentanal, hexanal, etc., (5) *terpenoids*, with terpinene-4-ol and α-terpineol predominating; (6) *hydrocarbons*, including dodecane and tetradecane; (7) *acids*, acetic and 2-methylbutyric acids; (8) *sulfur compounds*, dimethyl disulfide; (9) *ketones*, 2-heptanone.

Alcohols (41.4%) and esters (27.3%) were the predominant groups in the headspace of pomegranate juice, followed by

Table 5. Concentrations of volatile compounds found in commercially available pure pomegranate (PgJ) and pure grape (GJ) juices and their blends

Compound	ANOVA[a]	Retention index		Concentration (% of total arbitrary area units)[c]				
		Exp.	Lit.[b]	PgJ	PgJ + GJ 10%	PgJ + GJ 25%	PgJ + GJ 50%	GJ
Alcohols								
Ethanol	***	477	482	10.0c	10.8c	11.7bc	13.4a	16.6a
Isoamyl alcohol	**	723	727	0.64c	1.06c	1.69bc	2.80b	4.85a
cis-3-Hexenol	**	863	858	4.87a	4.38a	3.65ab	2.44b	
1-Hexanol	***	873	869	14.4d	15.6cd	17.3bc	20.3b	25.9a
1-Octen-3-ol	**	993	984	1.65a	1.58a	1.37ab	1.09b	0.52c
2-Ethyl-1-hexanol	***	1015	1025	9.88a	8.79ab	7.41b	4.94c	
Phenethyl alcohol	**	1143	1137		0.10d	0.21c	0.43b	0.85a
Esters								
Ethyl acetate	***	600	608	23.9a	22.3ab	20.0b	16.1c	8.41d
Ethyl butyrate	NS	800	801		0.03	0.07	0.18	0.29
Butyl acetate	NS	806	813	0.32	0.29	0.24	0.16	
Methyl hexanoate	NS	922	927		0.02	0.02	0.04	0.06
Isobutyl butyrate	*	955	958	0.18d	0.24c	0.33bc	0.48b	0.77a
Hexyl acetate	**	1016	1023		0.16d	0.41c	0.82b	1.63a
Isoamyl butyrate	***	1061	1061	2.50a	2.88c	3.45bc	4.40b	6.30a
Benzyl acetate	**	1172	1164		0.08c	0.21c	0.43b	0.84a
Ethyl octanoate	NS	1204	1200	0.44	0.44	0.41	0.39	0.30
Terpenes								
α-Pinene	NS	945	940	0.10	0.10	0.11	0.13	0.13
Myrcene	NS	996	985		0.03	0.04	0.09	0.17
β-Pinene	NS	997	987	0.48	0.43	0.36	0.26	
α-Terpinene	NS	1029	1023	0.16	0.14	0.12	0.10	
p-Cymene	NS	1038	1030	0.56	0.55	0.55	0.55	0.50
Limonene	NS	1043	1039	10.4	10.3	10.2	10.2	9.84
γ-Terpinene	NS	1071	1066	1.32	1.32	1.28	1.26	1.15
Terpinolene	NS	1101	1092	0.12	0.15	0.09	0.06	
trans-α-Bergamotene[d]	NS	1457	1446	0.20	0.18	0.15	0.10	
Aldehydes								
Pentanal	NS	669	680	0.33	0.39	0.46	0.59	0.83
Hexanal	***	801	801	6.22a	5.86a	5.32ab	4.45b	2.61c
Furfural	NS	837	833	0.28	0.38	0.54	0.80	1.30
trans-2-Hexenal	NS	854	855	0.61	0.58	0.49	0.39	0.13
Heptanal	NS	905	898	0.30	0.27	0.23	0.15	
2-Heptenal	NS	935	946	0.15	0.14	0.11	0.08	
Octanal	NS	1010	1005		0.03	0.04	0.10	0.17
Nonanal	**	1115	1102	1.37a	1.31a	1.17ab	0.97b	0.56c
cis-2-Nonenal	*	1122	1121	1.82a	1.59a	1.37ab	0.92 g	
Benzaldehyde	*	979	970	0.28c	0.37c	0.45bc	0.61b	0.94a
Decanal	*	1221	1216	0.53a	0.48a	0.40a	0.27b	
Terpenoids								
1,8-Cineole	NS	1049	1038	0.10	0.09	0.08	0.07	
cis-Linalool oxide[d]	NS	1082	1074		0.04	0.07	0.15	0.29
trans-Linalool oxide[d]	NS	1099	1093		0.01	0.02	0.04	0.07
Linalool	***	1110	1101	1.35c	1.87c	2.65bc	3.94b	6.53a
Terpinen-4-ol	***	1202	1192	0.87a	0.80a	0.68ab	0.50b	0.12c
α-Terpineol	NS	1209	1216	2.39	2.39	2.34	2.31	2.20
Hydrocarbons								
Dodecane	NS	1204	1200	0.16	0.17	0.14	0.13	0.09
Tetradecane	NS	1401	1400	0.35	0.37	0.34	0.33	0.30
Hexadecane	*	1601	1600		0.03b	0.05b	0.10b	0.18a
Acids								
Acetic acid	***	624	628	0.44c	0.85c	1.41bc	2.48b	4.33a
2-Methylbutyric acid	NS	831	840	0.05	0.08	0.04	0.04	

Table 5. *Continued*

| Compound | ANOVA[a] | Retention index | | Concentration (% of total arbitrary area units)[c] | | | | |
		Exp.	Lit.[b]	PgJ	PgJ + GJ 10%	PgJ + GJ 25%	PgJ + GJ 50%	GJ
Sulfur compounds								
Dimethyl disulfide	NS	734	727	0.24	0.24	0.18	0.12	
Ketones								
2-Heptanone	NS	889	891	0.11	0.11	0.08	0.07	
Lactones								
γ-Valerolactone	NS	938	950		0.02	0.03	0.08	0.12
γ-Decalactone	**	1478	1471		0.03b	0.04b	0.09b	0.16a

[a] Significance of F ratio: NS, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. [b] NIST.[25] [c] Values are mean ± standard error of three replications. Means followed by the same letter within a row are not statistically different according to Tukey's multiple range test. [d] Compound tentatively identified (comparison with Wiley229 spectral database).

terpenes (13.3%) and aldehydes (11.9%). This profile of volatile compounds can be considered as typical of sweet pomegranate cultivars.[17] In general, *alcohols* and especially *esters* are related to fruity and sweet aromas, while *aldehydes* can be related to green, grassy and herbaceous notes and *terpenes* can be related to pine and citrus notes.[18,39]

A total of 39 compounds were isolated and identified in pure pomegranate juice by the HS-SPME technique (Tables 5 and 6), while 51 and 56 compounds were found in mixtures of pomegranate plus grape and peach juices respectively. These compounds were isolated and identified by the HS-SPME technique; this analytical procedure has been used previously by our research group to study the volatile composition of Spanish tomatoes[23] and pomegranates.[18,24] Six compounds had concentrations above 5%: ethyl acetate (23.9%), 1-hexanol (14.4%), limonene (10.4%), ethanol (10.0%), 2-ethyl-1-hexanol (9.9%) and hexanal (6.2%). All these compounds have been described previously in pomegranate juices by other authors[2,7,18,24,39] and consequently are typical of pomegranate products. Even though 2-ethyl-1-hexanol is sometimes considered an artifact from plastics, it has been reported previously in fresh pomegranate juices;[40] thus it is kept in the list of compounds studied.

Adulteration of pomegranate juice with grape juice is one of the most logical options, considering its low price and similar sugar, organic acid, sensory and volatile profiles. In general, grape juice was also dominated by alcohols (47.8%) and esters (18.6%), but terpenoids played a more important role than in pomegranate juice (Table 5). Adulteration of pomegranate juice with up to 50% of grape juice resulted in significant increases in acetic acid, isoamyl alcohol, isoamyl butyrate and especially 1-hexanol (up to concentrations of ~25%) and linalool (~6%). On the contrary, some compounds such as ethyl acetate, hexanal, *cis*-3-hexenol, 2-ethyl-1-hexanol and terpinene-4-ol decreased in concentration after addition of grape juice to pomegranate juice. Some compounds from the grape juice were not found in the pomegranate juice and therefore relatively high concentrations could be considered a sign of adulteration; these compounds included myrcene, hexyl acetate, linalool oxides, benzyl acetate and γ-decalactone. If only a few compounds could be controlled, the presence of *linalool* at $\geq$3% or its derivatives such as *linalool oxides* at >0.10% could be used as an indicator of adulteration of pomegranate juice with grape products.

Adulteration of pomegranate juice with peach juice could be an important option, considering its low price and the fact that its high sweetness and intense fruity flavor could be useful in improving the too intense sourness and flat flavor of some pomegranate juices. In general, peach juice was clearly dominated by esters (83.2%) and terpenes (8.8%), with alcohols and aldehydes playing a minor role compared with pomegranate juice (Table 6). Adulteration of pomegranate juice with up to 10% of peach juice resulted in significant increases in butyl acetate, isobutyl butyrate, benzyl acetate and especially isoamyl butyrate (up to concentrations of ~40%). On the contrary, some compounds such as ethyl acetate, hexanal, *cis*-3-hexenol, 1-hexanol, 2-ethyl-1-hexanol, terpinene-4-ol, and α-terpineol decreased in concentration after addition of peach juice to pomegranate juice. Some compounds from the peach juice were not found in the pomegranate juice and therefore relatively high concentrations could be considered a sign of adulteration; these compounds included ethyl butyrate, isovaleric acid, *cis*-3-hexenyl formate, benzyl acetate, γ-decalactone and especially isoamyl acetate (>25%) and hexyl acetate (>4.3%). If only a few compounds could be controlled, the presence of *isoamyl acetate* and/or *hexyl acetate* and the simultaneous presence of high concentrations of *esters* (>35%) could be used as indicators of adulteration of pomegranate juice with peach products. The presence of lactones such as γ-*decalactone* could also be a good indicator of adulteration with peach juice.

CONCLUSIONS

The control protocols used in this study can serve as a basis for pomegranate juice authentication. It is important to highlight that it is necessary to simultaneously analyze and have results from several parameters to conclude that a particular pomegranate juice has been adulterated by mixing with another fruit juice. The main parameters for the detection of adulterated pomegranate juice with grape juice were (1) decrease in K (<2000 mg L^{-1}), (2) increases in proline (>250 mg L^{-1}) and tartaric acid (>1.0 mg L^{-1}) and (3) the presence of volatile compounds such as linalool (>3%) and linalool oxide (>0.10%). The main parameters for the detection of adulterated pomegranate juice with peach juice were (1) high sucrose concentration, (2) the presence of isoamyl acetate and/or hexyl acetate and (3) the simultaneous presence of high concentrations of esters (>35%) and lactones.

Table 6. Concentrations of volatile compounds found in commercially available pure pomegranate (PgJ) and pure peach (PJ) juices and their blends

Compound	ANOVA[a]	Retention index Exp.	Retention index Lit.[b]	Concentration (% of total arbitrary area units)[c] PgJ	PgJ + PJ 5%	PgJ + PJ 10%	PJ
Alcohols							
Ethanol	***	477	482	10.0a	9.56a	9.08a	0.83b
Isoamyl alcohol	NS	723	727	0.64	0.62	0.61	0.36
cis-3-Hexenol	***	863	858	4.87a	4.57a	4.41a	0.32b
1-Hexanol	**	873	869	14.4a	13.8a	13.2a	1.99b
1-Octen-3-ol	NS	993	984	1.65	1.59	1.49	
2-Ethyl-1-hexanol	NS	1015	1025	9.88	9.39	8.89	
Esters							
Ethyl acetate	***	600	608	23.9a	22.6a	21.7a	2.05b
Isobutyl acetate	NS	756	758		Trace	Trace	0.03
Ethyl butyrate	*	800	801		0.02b	0.02b	0.21a
Butyl acetate	**	806	813	0.32b	0.39b	0.46b	1.65a
Isoamyl acetate	***	874	876		1.29c	2.55b	25.4a
Isobutyl butyrate	***	955	958	0.18c	0.45bc	0.73b	5.53a
3-Hexen-1-ol acetate	**	1010	1005		0.04b	0.08b	0.83a
Hexyl acetate	***	1016	1023		0.24b	0.43b	4.33a
2-Methylbutyl isobutyrate[d]	NS	1025	1014		Trace	Trace	0.04
Isoamyl butyrate	***	1061	1061	2.50c	4.38bc	6.26b	40.1a
2-Propenyl hexanoate	*	1084	1080		0.02b	0.01b	0.10a
Ethyl heptanoate	NS	1100	1108		0.01	0.01	0.07
Pentyl butyrate[d]	*	1110	1091		0.03b	0.06b	0.61a
Benzyl acetate	***	1172	1164		0.09b	0.18b	1.75a
Hexyl butyrate	NS	1194	1192		0.01	Trace	0.02
Ethyl octanoate	NS	1198	1200	0.44	0.44	0.42	0.27
Terpenes							
α-Pinene	NS	945	940	0.10	0.11	0.10	0.06
β-Pinene	NS	997	987	0.48	0.46	0.44	0.12
α-Terpinene	NS	1029	1023	0.16	0.15	0.14	
p-Cymene	NS	1038	1030	0.56	0.56	0.55	0.51
Limonene	**	1043	1039	10.4a	10.2a	10.1a	7.44b
γ-Terpinene	*	1071	1066	1.32a	1.29a	1.26a	0.76b
Terpinolene	NS	1101	1092	0.12	0.13	0.11	
trans-*α*-Bergamotene[d]	NS	1457	1446	0.20	0.20	0.18	
Aldehydes							
Pentanal	NS	669	680	0.33	0.33	0.32	0.23
Hexanal	***	801	801	6.22a	5.82a	5.62a	0.19b
Furfural	NS	837	833	0.28	0.28	0.27	0.21
trans-2-Hexenal	*	854	855	0.61a	0.58a	0.55a	0.06b
Heptanal	NS	905	898	0.30	0.30	0.27	
2-Heptenal	NS	935	946	0.15	0.16	0.14	
Benzaldehyde	NS	979	970	0.28	0.29	0.29	0.38
Nonanal	**	1115	1102	1.37a	1.31a	1.26a	0.24b
cis-2-Nonenal	NS	1122	1121	1.82	1.74	1.64	
Decanal	NS	1221	1216	0.53	0.52	0.48	
Terpenoids							
1,8-Cineole	NS	1049	1038	0.10	0.12	0.09	
Linalool	NS	1107	1107	1.35	1.38	1.41	1.94
Terpinen-4-ol	NS	1202	1192	0.87	0.83	0.78	
α-Terpineol	**	1209	1216	2.39a	2.29a	2.19a	0.45b
Hydrocarbons							
Dodecane	NS	1202	1200	0.16	0.17	0.15	0.02
Tetradecane	NS	1401	1400	0.35	0.36	0.32	0.06
Hexadecane	NS	1601	1600		Trace	0.01	0.06
Acids							
Acetic acid	NS	624	628	0.44	0.46	0.43	0.35
Isovaleric acid	NS	824	830		0.01	0.01	0.07

Table 6. *Continued*

Compound	ANOVA[a]	Retention index		Concentration (% of total arbitrary area units)[c]			
		Exp.	Lit.[b]	PgJ	PgJ + PJ 5%	PgJ + PJ 10%	PJ
2-Methylbutyric acid	NS	831	840	0.05	0.06	0.05	
Sulfur compounds							
Dimethyl disulfide	NS	734	727	0.24	0.26	0.22	
Ketones							
2-Heptanone	NS	889	891	0.11	0.10	0.10	
6-Methyl-5-hepten-2-one	NS	993	987		Trace	0.01	0.06
Lactones							
γ-Valerolactone[d]	NS	938	943		Trace	Trace	0.04
δ-Valerolactone[d]	NS	987	958		Trace	Trace	0.03
γ-Decalactone	**	1478	1471		0.01b	0.03b	0.28a

[a] Significance of F ratio: NS, not significant ($P > 0.05$); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. [b] NIST.[25] [c] Values are mean ± standard error of three replications. Means followed by the same letter within a row are not statistically different according to Tukey's multiple range test. [d] Compound tentatively identified (comparison with Wiley229 spectral database).

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PUBLICATION 5

Processing pomegranates for juice and impact on bioactive components

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Chapter 5. Publication under review

PUBLICATION 6

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

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

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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 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.

HIGHLIGHTS

- Ripening stage has an effect on the major derivatives of ellagic acid content.

- Major derivatives of ellagic acid content in thinning pomegranates is higher than ripe fruits.

- Thinning fruits, especially sour-sweet cultivar, are a potential source of bioactive compounds.

- Thinning fruits are interesting for industrial applications or development of new products.

1 **Identification and quantification of major derivatives of ellagic acid and**

2 **antioxidant properties of thinning and ripe Spanish pomegranates**

3

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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} 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

Nowadays, modern society has developed a major interest in the consumption of foods with health benefits (Wu, Gu, Holden, Haytowitz, Gebhardt, 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 (García-Alonso, De Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2004) and might induce health benefits against cancer, cardiovascular and other health diseases (Basu, & Penugonda, 2009).

Additionally the pomegranate peel contain significant amounts 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, Edwards, Al-Sibani, Al-Thani, Al-Harrasi, et al., 2014). Also, there has been an increase in the use of pomegranate fruit extracts as botanical ingredients in herbal medicines and dietary supplements (Elfalleh, Tlili, Nasri, Yahia, Hannachi, 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).

Therefore the aim of the present study was to evaluate the potential of thinning and ripe fruits from nine common Spanish pomegranate cultivars as source 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: 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 × 3 m. They are drip irrigated, and

98   standard cultural practices are performed (pruning, thinning, fertilization and pest

99   control treatments).

100   The following cultivars were selected: (i) 3 sour cultivars [*Borde de Albatera 1*

101   ("BA1"), *Borde de Orihuela 1* ("BO1"), *Borde de Beniel 1* ("BBE1")], (ii) 3 sour-

102   sweet cultivars [*Piñón Tierno de Ojós 5* ("PTO5")*, Piñón Tierno de Ojós 8* ("PTO8"),

103   *Piñón Tierno de Ojós 10* ("PTO10")], and (iii) 3 sweet cultivars [*Mollar de Elche 14*

104   ("ME14"), *Mollar de Elche 17* ("ME17") and *Valenciana 1* ("VA1")]. After picking, all

105   fruits were immediately transported into the laboratories of the Universidad Miguel

106   Hernández de Elche (Orihuela, Alicante, Spain).

107   Thinning is conducted as a routine farming practice in the selected

108   pomegranate orchard, generally from middle of June to the first week of July.

109   Usually, pomegranate thinning is conducted at the stage of young fruit (Fleckinger

110   code I; BBCH code 71); at this stage about 7-8 kg of young fruits are removed per

111   each tree. Only fruits weighting less than 100 g or having a diameter smaller than

112   60 mm are removed. Following all the previous mentioned requirements, 5 fruits

113   were selected from those removed by the routine thinning practice.

114   Two times for five fruits per cultivar were randomly collected (90 thinning

115   fruits and 90 ripe fruits; 180 fruits in total). After harvest the fruits were frozen

116   immediately and then lyophilized using a freeze drier (Christ Alpha 2-4; Braum

117   Biotech Int., Melsungen, Germany) for 24 h and a pressure of 0.220 mbar. The

118   samples were subsequently ground in a pestle and mortar to a fine powder and

119   stored vacuum-packed in a freezer (-80 ºC) until analysis.

120

121   2.2. Identification of major derivatives of ellagic acid by the LC-PDA-QTOF/MS

122   method and quantification by UPLC-PDA

123   Pomegranate extract samples for the analysis were prepared as previously

124   described by Wojdyło, Oszmiański & Bielicki, (2013). Identification and

125   quantification of MDEA of pomegranate fruits extracts was carried out using an

126   Acquity ultra performance LC system equipped with a photodiode detector (UPLC-

127 PDA) with binary solvent manager (Waters Corp., Milford, MA, USA) series with a

128 mass detector G2 QTOF Micro mass spectrometer (Waters, Manchester, UK)

129 equipped with an electrospray ionization (ESI) source. Separations of polyphenols

130 were carried out using a UPLC BEH C18 column (1.7 µm, 2.1 × 100 mm; Waters

131 Corp., Milford, MA, USA) at 30 °C, whereas the samples were maintained at 4 °C

132 during the analysis.

133 Pomegranate samples (5 µL) were injected, and elution was completed within

134 22 min using a sequence of elution modes: linear gradients and isocratic. The flow

135 rate was 0.45 mL/min. The mobile phase was composed of solvent A (4.5 % formic

136 acid) and solvent B (100 % of acetonitrile). Elution was as follows: 0−10 min,

137 linear gradient from 1 to 10 % B; 10−15 min, linear gradient from 10 to 17% B;

138 than 100% B from 15 to 18 min for column washing; and reconditioning for next

139 4.00 min. A partial loop injection mode with a needle overfill was set up, enabling 5

140 µL injection volumes when a 5 µL injection loop was used. Acetonitrile (100 %) was

141 used as a strong wash solvent and acetonitrile−water (10 %) as a weak wash

142 solvent. Analysis was carried out using full scan, data-dependent MS scanning from

143 m/z 100 to 1000. The mass tolerance was 0.001 Da, and the resolution was 5.000.

144 Leucine enkephalin was used as the mass reference compound at a concentration of

145 500 pg/µL at a flow rate of 2 µL/min, and the [M − H]⁻ ion at 554.2615 Da was

146 detected over 15 min of analysis during ESI-MS accurate mass experiments, which

147 was permanently introduced via the LockSpray channel using a Hamilton pump. The

148 lock mass correction was ±1.000 for Mass Window. The mass spectrometer was

149 operated in a negative ion mode and set to the base peak intensity (BPI)

150 chromatograms and scaled to 12400 counts per second (cps) (=100 %). The

151 optimized MS conditions were as follows: capillary voltage of 2500 V, cone voltage

152 of 30 V, source temperature of 100 °C, desolation temperature of 300 °C, and

153 desolation gas (nitrogen) flow rate of 300 L/h. Collision-induced fragmentation

154 experiments were performed using argon as collision gas, with voltage ramping

155 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, Beghe, Mena, Del Rio,

Bruni 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

15,000 rpm 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 &

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

184 decrease in absorbance was measured at 515 nm in UV-Vis Uvikon XS

185 spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines, France).

186   Additionally, the ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)]

187 radical cation and ferric reducing antioxidant power (FRAP) methods were also used

188 as described by Re, Proteggente, Pannala, Yang, & Rice-Evans, (1999) and Benzie

189 & Strain, (1996) respectively. Briefly, 10 µL of the supernatant were mixed with

190 990 µL of ABTS or FRAP. After 10 min of reaction, the absorbance was measured at

191 734 nm for ABTS and 593 nm for FRAP. The absorbance was measured in UV-Vis

192 Uvikon XS spectrophotometer (Bio-Tek Instruments, Saint Quentin Yvelines,

193 France). Calibration curves, in the range 0.01–5.00 mmol Trolox L^{-1} were used for

194 the quantification of the three methods of antioxidant activity showing good

195 linearity ($R^2 \geq 0.998$). The analyses were run in five replications (n=5) and results

196 were expressed as mean ± standard error and units in mmol Trolox per kg dry

197 matter (dm).

198 2.3.2. ORAC method

199 The fourth method used to evaluate the antioxidant capacity of pomegranate fruits

200 was Oxygen Radical Absorbance Capacity (ORAC), as described by Ou, Hampsch-

201 Woodill, & Prior (2001). Briefly, each sample (0.1 mL) was diluted with phosphate

202 (K_2HPO_4 + Na_2HPO_4) buffer solution (75 mM, pH 7.4). Later, 375 µL of sample

203 together with 2.25 mL of fluorescein (42 nM) were added in cuvettes; buffer

204 solution was used as blank and Trolox solution (25 µM Trolox) as calibration

205 solution. Fluorescence readings were taken at 5 s and then every minute thereafter.

206 Finally, 375 µL of freshly prepared AAPH reagent [2,2'-azobis(2-amidinopropane)

207 dihydrochloride] (153 mM) was added in cuvettes every 5 s. The fluorescence

208 spectrophotometer (Shimadzu, model RF-5301; Kyoto, Japan) was set up at an

209 excitation wavelength of 493 nm and an emission wavelength of 515 nm and

210 readings were recorded every 5 min for 40 min after the addition of AAPH. During

211 the analysis all the cuvettes were incubated at 37 °C. The final ORAC values were

212 calculated, in triplicate, using a regression equation between the Trolox

213 concentration and the net area under the fluorescence decay curve and final data

214 were expressed as mmol Trolox per kg dry matter (dm).

215

216 2.4. Statistical analysis

217 Results are provided as the mean ± standard error of three replications. First, data

218 was subjected to one-way analysis of variance (ANOVA) and later data was also

219 subjected to Tukey's multiple-range test to compare the means. Differences were

220 considered statistically significant at $p < 0.05$. All statistical analyses were

221 performed using StatGraphics Plus 5.0 software (Manugistics, Inc., Rockville, MD).

222 The figures of ABTS, DPPH, FRAP, and ORAC data, were prepared using SigmaPlot

223 Version 11.0 (Systat Software Inc.).

224

3. Results and discussion

226 3.1. Identification of major derivatives of ellagic acid

227 Ellagic acid and its derivatives were the main class of identified and quantified

228 compounds in this particular product. The identification of MDEA in thinning and

229 ripe pomegranate fruits was carried out by LC-PDA-QTOF/MS method (**Table 1**).

230 The aim of many pomegranates studies has been the identification of the bioactive

231 compounds that correlate with health (García-Alonso et al., 2004; Sun, Chu, Wu, &

232 Liu, 2002). In this sense, it has been shown that ellagic acid has anti-

233 atherosclerotic and biological properties can be used as a preventive agent in

234 cancer treatment (El-Shitany, El-Bastawissy, & El-Desoky, 2014; Lu, Ding, & Yuan,

235 2008). High concentrations of derivatives of ellagic acid are positively correlated

236 with the high antioxidant activity of pomegranate peel extracts (Al-Rawahi et al.,

237 2014).

238 Among the 35 major derivatives of ellagic acid found in thinning and ripe

239 pomegranates (mainly hydrolyzable tannins), 7 were found in both types of fruits.

240 These seven compounds were: punicalagin isomer (R_t = 1.61 min) and HHDP-

241 gallagyl-hexoside (punicalagin) (R_t = 3.52 min) had an [M−H]⁻ at m/z 1083 and

242   similar MS/MS fragments (300/622/781); granatin A (R_t = 4.40 min) had an

243   [M−H]⁻ at *m/z* 799; ellagic acid derivative (R_t = 5.32 min) had an [M−H]⁻ at *m/z*

244   301; ellagitannin (R_t = 8.79 min) had an [M−H]⁻ at *m/z* 784; granatin B (R_t =

245   10.54 min) had an [M−H]⁻ at *m/z* 951; and ellagic acid derivative (R_t = 11.06 min)

246   had an [M−H]⁻ at *m/z* 951. Calani et al. (2013) and Fischer et al. (2011) identified

247   those compounds in pomegranate. Hydrolyzable tannins are the most abundant

248   antioxidant polyphenolic compounds in pomegranate (Gil, Tomás-Barberán, Hess-

249   Pierce, Holcroft, & Kader, 2000) and include ellagitannins, such as punicalagins and

250   punicalins (Calani et al., 2013).

251   Regarding other derivatives of ellagic acid found exclusively in thinning (i) or

252   ripe (ii) fruits the most abundant ones were: (i) digalloyl-HDDP-glucoside

253   (pedunculagin II) (R_t = 3.80 min, [M−H]⁻ at *m/z* 785) and HHDP-digalloyl-glucose

254   (R_t = 5.89 min, [M−H]⁻ at *m/z* 785) and (ii) ellagitannin (R_t = 2.86 min, [M−H]⁻ at

255   *m/z* 783) and an unknown compounds, which main characteristics were R_t = 0.63

256   min, and [M−H]⁻ at *m/z* 215. These compounds have been reported by Fischer et

257   al. (2011), Calani et al. (2013) and Sentandreu, Cerdán-Calero, & Sendra (2013) in

258   ripe pomegranates.

259

260   3.2. Quantification of major derivatives of ellagic acid

261   The quantification of major derivatives of ellagic acid was conducted using UPLC-

262   PDA detection. The effect of the ripening stage on the MDEA was evident and the

263   values found in thinning fruits were 3 to 19 times higher than those found in ripe

264   fruits. According to the mean values of all samples, the MDEA was about seven

265   times higher in thinning fruits (10450 ± 1581 mg 100 g⁻¹ dm) than in ripe fruits

266   (1553 ± 270 mg 100 g⁻¹ dm). The highest changes with time were found in fruits

267   from sweet cultivars, which decreased from an initial mean value of 11734 mg 100

268   g⁻¹ dm to as low as 833 mg 100 g⁻¹ dm; this means that the ratio

269   MDEA$_{thinning}$/MDEA$_{ripe}$ had a mean of 14.1. This same ratio, MDEA$_{thinning}$/MDEA$_{ripe}$,

270   took values of 5.0 and 5.2 for sour and sour-sweet cultivars, respectively. Al-

271 Rawahi et al. (2014) found 6420 mg GAE 100 g^{-1} dry solids (ds) in freeze dried

272 pomegranate peel and Fischer et al. (2011) reported a total phenolic value of 8489

273 mg 100 g^{-1} dm, in peel and mesocarp of pomegranate. The differences in the

274 phenolic content could be associated with the difference in cultivars, methods of

275 extraction and analysis (chromatography or spectrophotometry) and environmental

276 conditions (Al-Rawahi et al., 2014). The high amounts of bioactive compounds in

277 thinning fruits imply the high interest of this material for industrial applications,

278 such as enrichment or development of new products.

279 The factor cultivar significantly ($p<0.05$) affected the amount of MDEA, which

280 ranged (i) in thinning pomegranates between 3521 and 18236 mg 100 g^{-1} dm in

281 PTO10 and PTO8, respectively, and (ii) in ripe pomegranates between 608 and

282 2905 mg 100 g^{-1} dm in ME14 and PTO8, respectively. The two cultivars with the

283 highest values of MDEA in both thinning and ripe pomegranates were PTO8 (18236

284 and 2905 mg 100 g^{-1} dm, respectively) and BO1 (15338 and 2415 mg 100 g^{-1} dm,

285 respectively).

286 Tables 2 and 3 show that 24 and 18 major derivates of ellagic acid were

287 found in thinning and ripe pomegranates, respectively. The 3 most abundant

288 compounds in thinning fruits were (Table 2): (i) HHDP-gallagyl-hexoside (13):

289 3635 mg 100 g^{-1} dm, (ii) punicalagin isomer (7): 1986 mg 100 g^{-1} dm, and (iii)

290 granatin B (28): 830 mg 100 g^{-1} dm; these values represented 36.4, 19.9 and

291 7.3% of the total concentration of MDEA. Consequently, only these 3 compounds

292 represented more than 60% of the total concentration of MDEA in unripe fruits. In a

293 similar way, the most abundant compound in ripe fruits was ellagitannin (12):

294 858 mg 100 g^{-1} dm (Table 3). This value represented 42.9 % of the total

295 concentration of MDEA in ripe fruits.

296 There were 7 compounds (peaks 7, 13, 16, 19, 25, 28 and 29) that were

297 present in both thinning and ripe fruits. These 7 compounds represented about 70

298 % of the major derivatives of ellagic acid in thinning fruits, while only 14.5 % in

299 ripe fruits. The Figure 1 shows the comparison of MDEA profile of thinning and ripe

300 fruits for PTO8 cv. In this and other cv. these 7 compounds was always major in

301 thinning than in ripe fruits. Therefore, a big portion of these 7 compounds were

302 transformed in ellagitannins which are the predominate compound in the MDEA

303 profile of ripe fruits.

304 Flavonoids and phenolic acid are secondary metabolites produced by plants.

305 Gallic and ellagic acids are common precursors of hydrolyzable tannins; they will be

306 transformed via 1-*O*-galloylglucose into a wide range of complex galloylglucosides

307 and further complex of ellagitannins. The direct synthesis of gallic acid from

308 dehydroshikimic acid will block the shikimate pathway enzyme, 5-

309 enolpyruvylshikimate-3-phosphate synthase, and thus will cause a reduction in the

310 synthesis of aromatic amino acids and phenylpropanoids. In contrast, the synthesis

311 and accumulation of gallic acid and hydrolyzable taninns are activated (Gross,

312 1999; Grundhöfer, Niemetza, Schilling, & Grossa, 2001).

313 Therefore, one of the major derivatives of ellagic acid found in thinning fruits

314 was a punicalagin isomer (**7**), together with the gallagyl group is a part of the

315 chemical structure of many of the phenols that are commonly found in

316 pomegranate, such as punicalin and punicalagin derivatives (Sentandreu et al.,

317 2013; Zahin, Ahmad, Gupta, & Aqil, 2014). The other majority compound in

318 thinning fruits was granatin B (**28**) which forms part of type III-tannins

319 (dehydroellagitannins) (Okuda, Yoshida, & Hatano, 2000). Granatin A and B were

320 first identified as the major components of pomegranate leaves (Tanaka, Nonaka, &

321 Nishioka, 1985). These types of compounds, especially ellagic acid derivatives,

322 have been also found in camu camu, strawberries and various berries (Aaby,

323 Mazur, Nes, & Skrede, 2012; Fracassetti, Costa, Moulay, & Tomas-Barberan, 2013;

324 Simirgiotis, Theoduloz, Caligari, & Schmeda-Hirschmann, 2009).

325 Anthocyanin content was known to be affected by several parameters such as

326 harvest maturity, storage temperature, and relative humidity (Shin, Ryu, Liu, Nock,

327 & Watkins, 2008; Elfalleh et al., 2011). Therefore, the content of anthocyanins in

328 thinning pomegranate fruits was very low and was not a suitable parameter to

329 compare the amount of polyphenols among thinning and ripe pomegranate fruits.

330   Despite a great number of studies, the analysis in the content of phenolic

331 compounds (specially ellagic acid derivatives) with literature data is still inquired

332 due to different analytical methodologies and because the contents may

333 considerably vary with the pomegranate cultivar and maturity stage of

334 pomegranates (Mousavinejad, Emam-Djomeh, Rezaei, & Khodaparast, 2009;

335 Fischer et al., 2011).

336

337 3.3. ABTS, DPPH and FRAP methods

338 There are different methods for evaluating the antioxidant activity of foods. This

339 variety of methods is due to the fact that none of them by itself is able to

340 determine exactly the total antioxidant potential in a food system. For this reason,

341 the antioxidant "activity" of thinning and ripe pomegranates fruits was evaluated

342 using three different analytical methods: ABTS, DPPH and FRAP (**Figure 2**). The

343 factor "cultivar" significantly ($p<0.05$) affected the antioxidant activity of thinning

344 and ripe fruits. The mean thinning values for ABTS, DPPH, and FRAP were 3603,

345 2541, and 3977 mmol Trolox kg^{-1} dm, respectively; while the values for the same

346 methods but in ripe fruits were 2177, 1245, and 683 mmol Trolox kg^{-1} dm,

347 respectively. These results showed that the antioxidant activity of thinning fruits is

348 among 2-6 times higher than that of ripe fruits for all three methods (ABTS, DPPH,

349 and FRAP). In general, the highest values of antioxidant activity were found in

350 sour-sweet cultivars, especially in PTO8 cultivar. This trend is similar to that found

351 in Brazilian red cherry, where the DPPH activity decreased from 171 to 83 mmol

352 Trolox kg^{-1} dm throughout the development of fruits (Celli, Pereira-Netto, & Beta,

353 2011).

354   The values obtained in the current study are quite high, especially those of

355 the ripe fruits, in comparison with those found in the literature for ripe

356 pomegranate rind, arils and juice (Calín-Sánchez, Figiel, Hernández, Melgarejo,

Lech, et al., 2013; Mena, García-Viguera, Navarro-Rico, Moreno, Bartual, et al.,

358 2011; García-Alonso et al., 2004). The antioxidant potential of pomegranate can be

359 affected by many factors, including maturity stage, fruit cultivar, the different

360 nature of the materials (solid: thinning fruits or liquid: pomegranate juice),

361 extraction procedure and the specific method for their determination. Although

362 results may vary substantially due to all these factors, it must be highlighted that

363 the pomegranate is a fruit with high antioxidant potential, especially thinning fruits,

364 which are currently wasted in the soils and no revenue at all is obtained from them.

365 3.4. ORAC determinations

366 The antioxidant capacity of thinning and ripe pomegranate fruits was evaluated by

367 ORAC method. Results showed that thinning fruits have higher values than maturity

368 pomegranate (**Figure 3**). The ORAC values ranged from 664 to 924 mmol Trolox

369 kg^{-1} dm and from 338 to 582 mmol Trolox kg^{-1} dm in thinning and ripe fruits,

370 respectively. In the literature (Wojdylo et al., 2013; Calani et al., 2013) there is a

371 general trend in which high antioxidant activity values are positively correlated with

372 the high values in the total phenolic content; in this particular case, the correlation

373 among MDEA and the ORAC antioxidant capacity values was significant ($p<0.05$)

374 and showed a correlation coefficient, R= 0.627. The low correlation between MDEA

375 and ORAC capacity may be due to other phenolic compounds (not determined in

376 this study) may have a higher correlation with antioxidant capacity.

377 There are only very few studies evaluating the antioxidant potential of fruits

378 from different species removed during thinning. For instance Zheng, Kim, & Chung

379 (2012) studied the changes of the antioxidant activity of *Fuji* apples from thinning

380 to the optimal harvest time; these authors observed a decrease of as much as 98%

381 in the antioxidant activity from thinning to ripe apples. Li, Guo, Yang, Wei, Xu et al.

382 (2006), reported ORAC values between 100 and 350 µmol L^{-1} in pomegranate

383 extract. Elfalleh et al. (2011) reported values between 192 and 237 mmol Trolox

384 kg^{-1} in pomegranate peel. The mean value reported by these authors (215 mmol

385 Trolox kg^{-1}) is about 2-5 times lower than that of thinning pomegranates. Similar

386 results were obtained for pomegranate juice (25.0 mmol L^{-1}) by Seeram, Aviram,

387 Zhang, Henning, Feng et al. (2008). As a comparison, the antioxidant activity of

388 pomegranate juice is three times higher than the red wine and green tea (Gil et al.,

389 2000). These results are interesting because shows the richness of thinning

390 pomegranates as a natural antioxidant (especially from sour-sweet cultivars).

391 The factor cultivar significantly ($p<0.05$) affected the ORAC antioxidant

392 capacity. The two cultivars with the highest ORAC values in thinning (i) and ripe (ii)

393 fruits were: (i) PTO10 (925 mmol Trolox kg^{-1} dm) and PTO5 (827 mmol Trolox kg^{-1}

394 dm), and (ii) BO1 (582 mmol Trolox kg^{-1} dm) and BA1 (498 mmol Trolox kg^{-1} dm),

395 respectively.

396 After grouping pomegranate cultivars in sour, sour-sweet and sweet, the

397 groups with the highest ORAC value were sour-sweet (823 mmol Trolox kg^{-1} dm) in

398 thinning fruits and sour (517 mmol Trolox kg^{-1} dm) in ripe fruits.

399

4. Conclusions

401 This study demonstrated that LC-PDA-QTOF/MS and UPLC-PDA are a good

402 methodology for the identification and quantification of the major derivates of

403 ellagic acid in pomegranate fruit. The content of the major derivatives of ellagic

404 acid was significantly affected by the development stage of fruits. A total of 35

405 compounds were indentified and quantified to compare the difference among

406 thinning and ripe pomegranate fruits; only 7 of them were found in thinning and

407 ripe fruits and the values of the ellagic acid derivates found in thinning fruits were 3

408 to 19 times higher than those found in ripe fruits. Experimental results proved that

409 thinning sour-sweet cultivars, especially PTO8 cultivar, can be considered as a good

410 source of bioactive compounds, which are clearly reflected in high values of

411 antioxidant properties. Furthermore, those findings seemed to make pomegranate,

412 specially the fruits that coming from thinning, a waste product of the pomegranate

413 industry, an attractive candidate as a nutritional supplement for its use as

414 supplement in food, pharmaceutical and cosmetics industries.

415

416 **Acknowledgments**

422

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572

573

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	+	-

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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	+	-

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
(7)	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
(8)	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
(9)	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
(10)	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
(12)	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
(13)	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
(14)	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
(15)	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
(16)	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
(18)	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
(19)	Granatin A	65.0±2.2 e	247±3 b	85.±0.1 e	168±1 d	372±1 a	34.0±0.5 f	163±4 d	230±1 bc	218±4 c
(20)	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
(21)	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
(22)	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
(23)	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**

[†] *Values are the mean of 3 replications (± standard error).* [‡] *Values followed by different letters (a, b, c, etc.) within the same row are statistically different according to Tukey's multiple range tests (p < 0.05). All were significant at p<0.001.*

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 g	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

[†] *Values are the mean of 3 replications (± standard error).* [‡] *Values followed by different letters within the same row are statistically different according to Tukey's multiple range test (p < 0.05).*
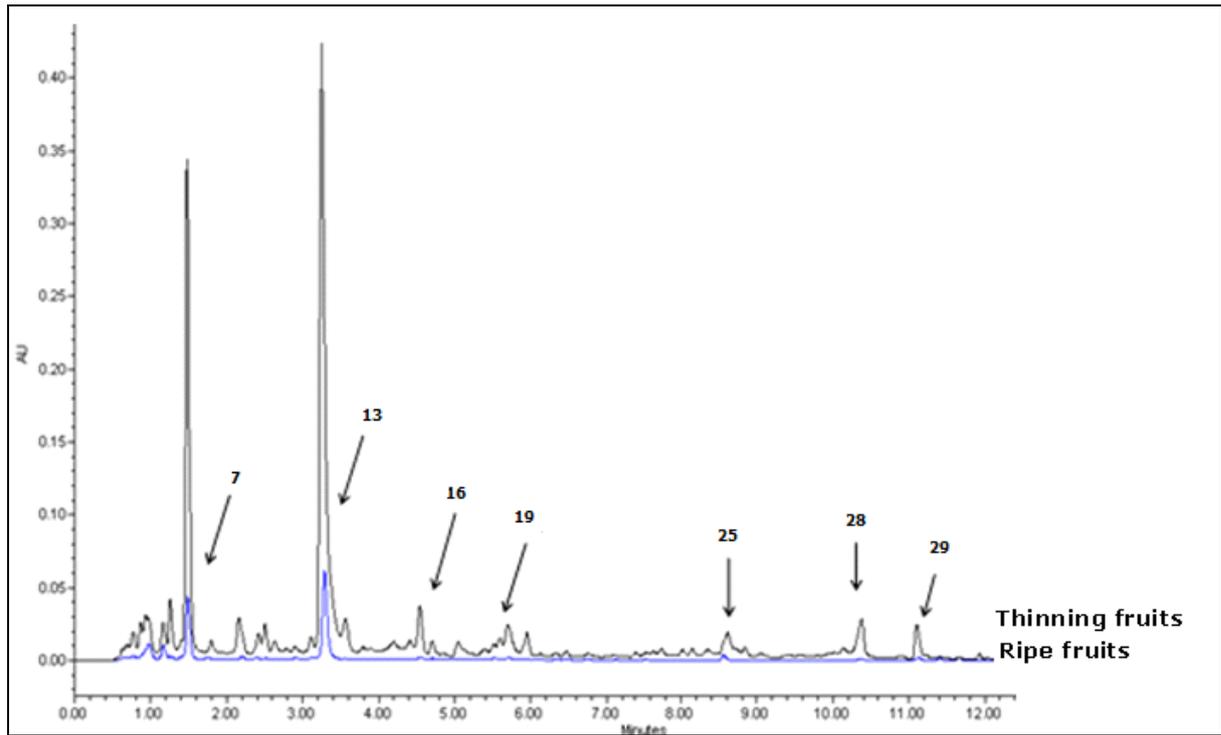
Figure 1. Comparative chromatogram of thinning and ripe pomegranate fruits (PTO8 cv.). Peaks: **7**, punicalagin isomer; **13**, HHDP-gallagyl-hexoside (punicalagin); **16**, granatin A; **19**, ellagic acid derivative; **25**, ellagitannin; **28**, granatin B; **29**, ellagic acid derivative.
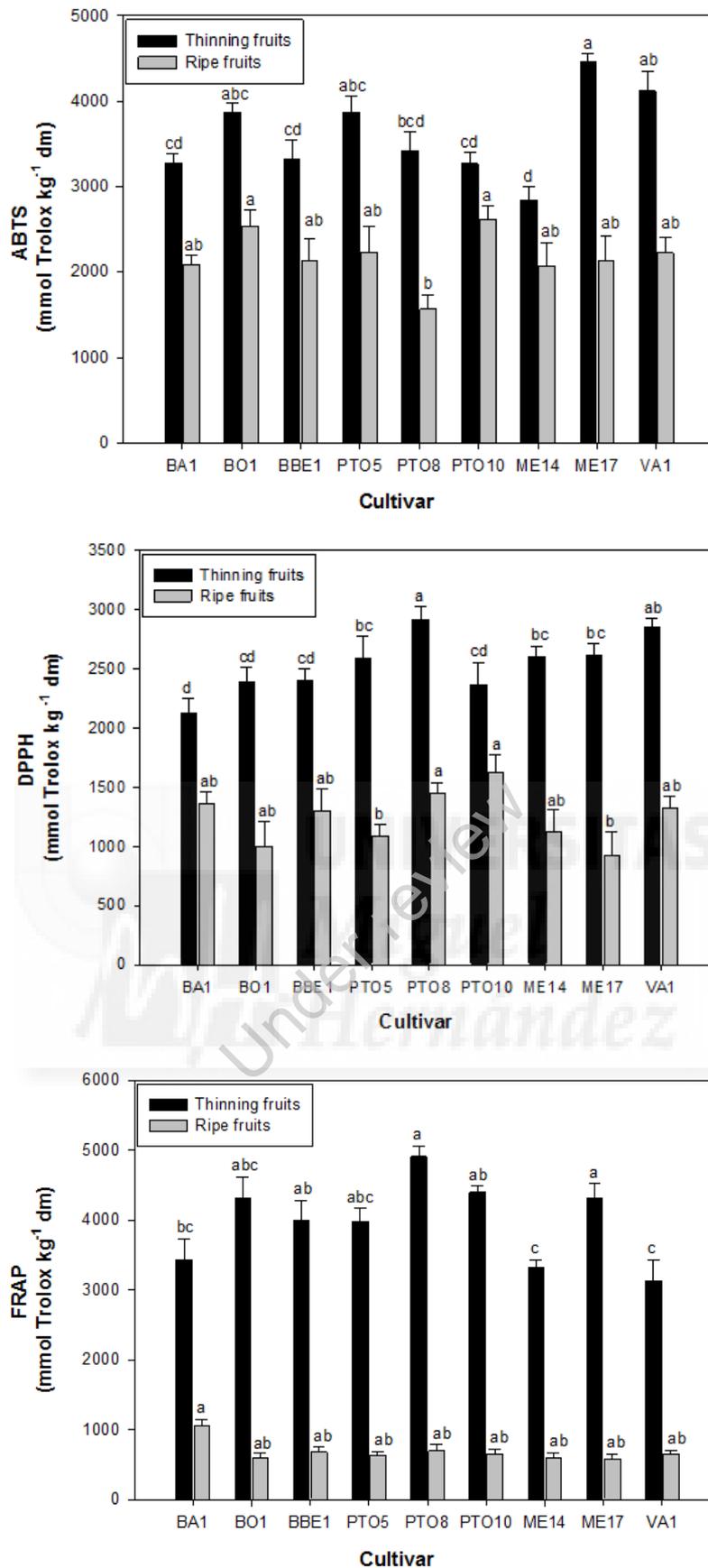
Figure 2. ABTS, DPPH and FRAP activity of thinning and ripe pomegranate fruits (mmol Trolox kg^{-1} dm) correspond to the standard deviation of three replicates. Bars with the same letter, for each development stage (thinning or ripe), were not statistically different according to Tukey's multiple range test ($p < 0.05$).

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98
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100
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Figure 3. ORAC capacity 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, for each development stage (thinning or ripe), were not statistically different according to Tukey's multiple range test (p < 0.05).*

Chapter 6. Results and Discussion

6.   RESULTS AND DISCUSSION

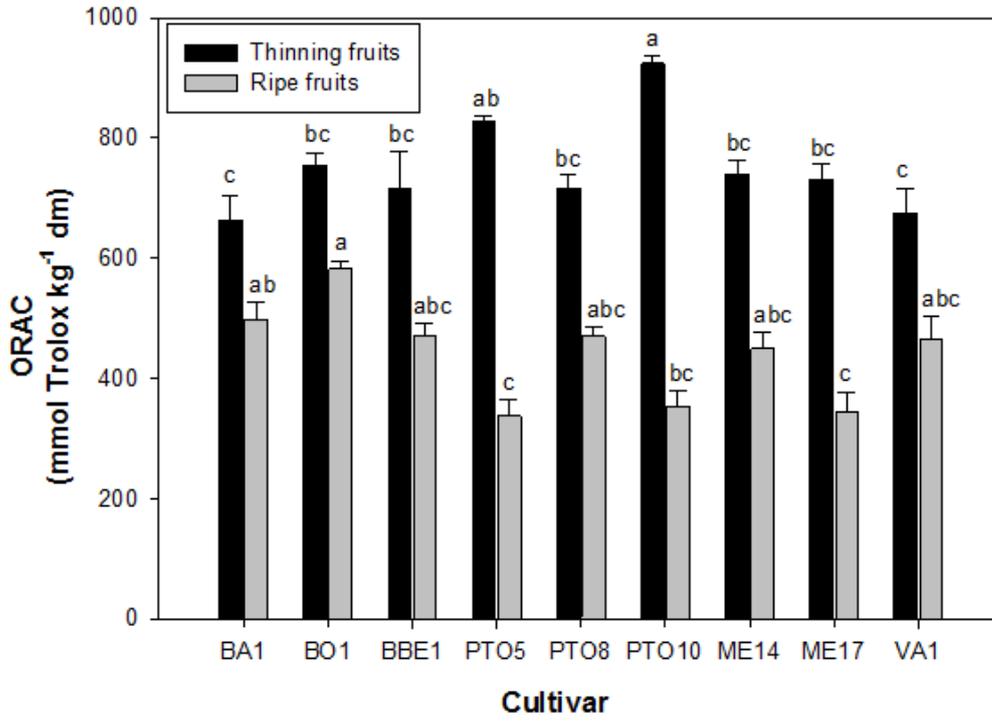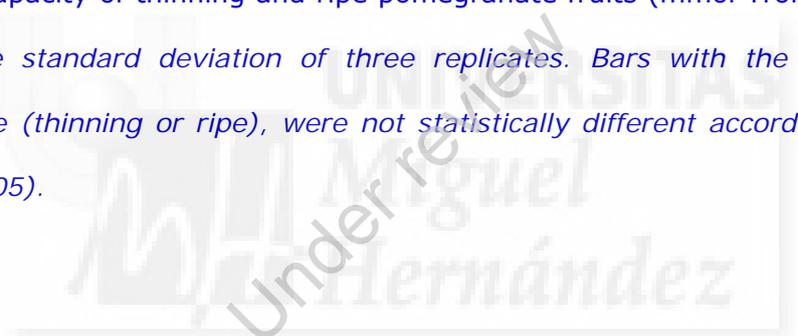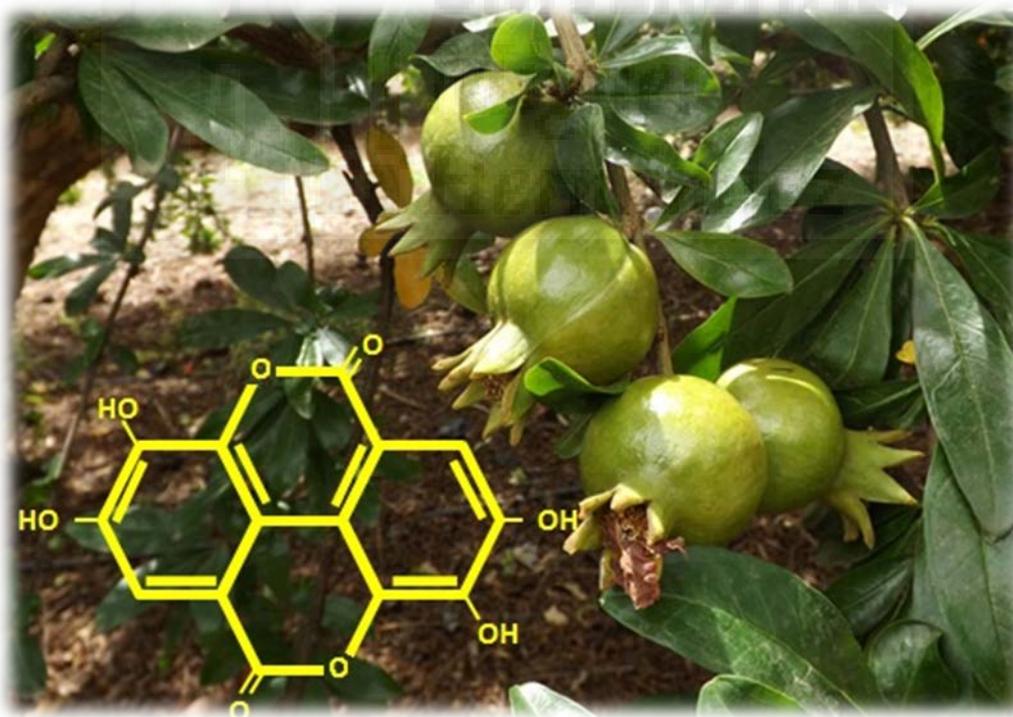During fruit ripening there are significant changes in the physico-chemical, and phenolic compositions, sensory quality as well as antioxidant. These changes are influenced by variety, cultivar type (sour, sweet-sour and sweet), growing region, farming practices and ripening stage of the fruit at harvest (Viuda-Martos *et al.*, 2010; Fawole and Opara, 2013). The current Dissertation describes in detail the physico-chemical and phenolic compositions, the sensory attributes, the antioxidant properties and their changes during development and processing of pomegranate fruits. Besides, the main quality characteristics of pure pomegranate juice and two juices (grape and peach) potentially used in its adulteration were evaluated and a protocol to identify the adulteration was developed. Finally, the phenolic composition and antioxidant properties of pomegranate thinning fruits were studied. It is important to highlight that in thinning fruits, the material analyzed included pomegranate rind, carpelar membranes and arils, and not only arils as usually done when focusing in the edible portion of pomegranates.

Tables 9-11 and **Figure 6** show the changes of: (i) the chemical parameters, organic acids and total sugars (**Table 9**), (ii) proline (**Table 10**), (iii) total phenolic compounds (**Figure 6**), and (iv) antioxidant activity (**Table 11**), of three different pomegranate varieties [one of each type (BA1: sour, PTO5: sour-sweet and ME14: sweet], at different stages of maturity [(0) *thinning,* (R1) *ripening 1*, (R2) *ripening 2* and (R3) *ripening 3*].

6.1.   Total soluble solids (TSS), titratable acidity (TA), maturity index (MI) and pH

Table 9 shows the results obtained in terms of TSS, TA, MI and pH in the juice obtained by squeezing the arils or whole fruits (thinning) from thinning (0) to commercial ripening stage (R1-R3). The factor "cultivar" significantly affected ($p<0.001$) these four parameters. In thinning fruits, the TSS values for cultivar type (sour, sour-sweet and sweet) were 9.9, 11.3 and 10.3 ºBrix, respectively. During the fruit ripening (R3), TSS significantly increased until 16.5, 14.8 and 15.87 ºBrix in sour, sour-sweet and sweet, respectively. The highest TA content was 38.7 g L^{-1} in thinning sour cultivar (BA1), followed by 6.95 g L^{-1} in sour-sweet cultivar (PTO5) and finally 5.57 g L^{-1} in sweet cultivar (ME14). With ripening TA decreased from 25.1 to 21.3, from 6.95 to 5.23, and from 2.52 to 2.29 g L^{-1} citric acid in sour, sour-sweet and sweet

cultivars, respectively. These two parameters determine the fruit MI (TSS/TA). The values of TSS, MI and pH for these cultivars of pomegranate were similar to those reported by Hernández et al. (1999) and Calín-Sánchez et al. (2011). In thinning fruits, the MI values should be taken with precaution as only trace levels of sugars were detected by HPLC and thus the TSS did not represent sugars but other water soluble compounds. As result, it is not fully appropriate to compare TSS or MI values with those of ripe fruits or pomegranate juice. With respect to the ripening stages of the fruit, the pH generally decreased as ripening progressed.

6.2. Organic acids and sugars profile

The results obtained for organic acids and sugars profile of pomegranate fruits, showed significant differences ($p<0.05$) as affected by "cultivar" and "ripening stage". In general, citric and malic acid, and glucose and fructose, are considered as the main organic acids and sugars in pomegranate fruits and juice (Melgarejo *et al.*, 2000; Mena *et al.*, 2011; Carbonell-Barrachina *et al.*, 2012). The total content of organic acids were 60.5, 36.7, 23.5 g L^{-1} in thinning fruits and 28.5, 17.3, and 9.6 g L^{-1} in ripe (R3) fruits from sour (BA1), sour-sweet (PTO5), and sweet (ME14) pomegranates, respectively.

The pomegranate removed during thinning only contained trace levels of sugars. With the progress of ripening, the starch content of the fruit is degraded and becomes simple sugars, while a simultaneous decrease in the organic acids and acidity is observed (Biale and Young, 1981). The fructose concentration was higher than that of glucose during fruit ripening, with the ratio glucose/fructose taking values of approximately 0.8. Similar profiles were previously described in other pomegranate cultivars (Schwartz *et al.*, 2009; Tezcan *et al.*, 2009). As the ripening progressed, the total sugar content increased until 107, 126 and 133 g L^{-1} in ripe (R3) fruits from sour, sour-sweet, and sweet cultivars, respectively (**Table 9**). The results about organic acids and sugars profiles in pomegranates are fully described in publications 1, and 2.

Table 9. Physico-chemical parameters, organic acids and sugars profiles in three pomegranate cultivars, one of each type

(BA1: sour, PTO5: sour-sweet and ME14: sweet), from thinning (0) to commercial stage (R3).

Cultivar	Type	Ripening Stage	TSS (°Brix)	TA (g L⁻¹ citric acid)	MI	pH	Citric acid	Malic acid	Quinic acid	Total acids	Glucose	Fructose	Total sugars
											(g L⁻¹)		
BA1	Sour	0	9.85	38.7	2.55	3.47	39.2	0.15	21.1	60.5	nd	nd	nd
		R1	15.4	25.1	6.16	3.76	28.8	2.40	6.80	39.1	45.3	52.6	97.9
		R2	15.9	22.4	7.20	3.81	22.0	2.20	6.20	31.5	47.7	53.6	101
		R3	16.5	21.3	7.84	3.55	20.4	1.70	5.20	28.5	51.3	56.6	107
PTO5	Sour-sweet	0	11.3	6.95	16.3	7.34	11.1	0.28	25.3	36.7	nd	nd	nd
		R1	14.6	6.38	23.0	4.97	5.60	2.30	13.0	21.0	46.4	59.3	105
		R2	14.5	5.24	27.8	5.88	5.01	1.90	12.0	18.1	53.2	68.0	121
		R3	14.8	5.23	28.3	5.42	4.80	1.60	10.0	17.3	57.2	69.2	126
ME14	Sweet	0	10.3	5.57	18.5	7.46	4.03	0.34	19.1	23.5	nd	nd	nd
		R1	14.6	2.52	58.1	4.50	1.41	1.80	8.60	11.8	52.6	66.1	118
		R2	15.40	2.35	65.6	4.54	1.30	1.50	8.50	11.3	54.3	70.4	124
		R3	15.87	2.29	70.0	4.57	1.10	1.40	7.10	9.60	60.1	73.4	133

6.3. Minerals analysis

The minerals contents were measure in thinning fruits and pomegranate juice. The content found in thinning fruits was significantly much higher than the normal values found in edible arils and pure juice, making this material very interesting as a mineral supplement. The mean values of the contents of Ca, Mg, K, Na, Fe, Zn, Cu, and Mn in immature thinning fruits were: 226, 439, 10171, 253, 5.86, 7.51, 6.12, and 3.06 mg kg^{-1}, respectively. The values of thinning fruits were about 8-9 times higher than those of pomegranate juice.

Potassium (K) was the predominant macro-element in all pomegranate cultivars, while zinc (Zn) was the predominant micro-element, although both copper (Cu) and iron (Fe), presented also relatively high contents. Previous studies on ripe pomegranate fruits, reported that K and Fe were the most abundant macro- and micro-element, respectively (Mirdehghan and Rahemi, 2007; Ekşi and Özhamamcı, 2009; Gozlekci *et al.*, 2011). As the fruit maturation progresses, there are significant decreases in mineral element contents (Fawole and Opara, 2013 b). The sour-sweet fruits presented the highest contents of Ca and Mg, while sweet fruits presented the highest contents of Fe and Zn; no clear trends were found for the rest of minerals. These results can be seen in more details in publication 1.

6.4. Proline

The proline content was significantly ($p<0.05$) affected by both, the pomegranate "cultivar" and "ripening stage" (**Table 10**). Throughout the development of the fruit (R1 to R3), PTO5 cultivar presented the highest proline values, ranging from 52.1 to 88.6 mg L^{-1}, followed by BA1 from 47.9 to 77.9 mg L^{-1}, and ME14 from 32.2 to 84.7 mg L^{-1}. The data shows that along the ripening process, the proline content increased significantly in pomegranate fruits. Halilova and Yildiz (2009) studied the effect of the climate change on the proline content in three cultivars of pomegranate. These authors concluded that hot and dry seasons resulted in higher contents of proline, for instance, 2008 was hotter and drier than 2007 and this fact resulted in a significant increased proline from 30 to 93 mg L^{-1}. The results about proline in pomegranate fruits can be seen in publication 2.

Table 10. Proline contents in three different pomegranate varieties, one of each type (BA1: sour, PTO5: sour-sweet and ME14: sweet), and at three ripening stages.

Cultivar	Type	Ripening Stage	Proline (mg L^{-1})
BA1	Sour	R1	47.9
		R2	55.1
		R3	77.9
PTO5	Sour-sweet	R1	52.1
		R2	65.2
		R3	88.6
ME14	Sweet	R1	32.2
		R2	47.5
		R3	84.7

6.5. Total polyphenols content (TPC) in pomegranate fruit

The TPC was significantly affected ($p<0.001$) by "cultivar" and "ripening stage" (**Figure 6**). The TPC in thinning fruits, which included rind, carpelar membranes and arils, had values ranging from 190 to 258 g GAE kg^{-1} dw. The sour-sweet cultivars showed the highest values. Pomegranate wastes (rind and carpelar membranes) are a richer source of antioxidants than the edible arils (Li *et al.*, 2006). Calín-Sánchez et al. (2013) evaluated the total polyphenols in mature arils and rind of fresh pomegranate. These authors reported that the TPC found in fresh rind was 125 g GAE kg^{-1} dw, while the TPC in fresh arils was 7.57 g GAE kg^{-1} dw. These values clearly showed that the highest amounts of phenolic compounds are found in pomegranate rind. The mean TPC found in immature thinning fruits (223 g GAE kg^{-1} dw) is about 2 times higher than that of ripe pomegranate rind.

At the other three ripening stages (R1 to R3), the TPC was quantified in the juice obtained by manually squeezing the arils. The sour cultivar (BA1) showed the highest value 4.06 g GAE L^{-1}, followed by the sour-sweet cultivar (PTO5) 3.35 mg GAE L^{-1} and the sweet cultivar (ME14) 3.22 g GAE L^{-1}. These experimental values agreed with those reported by Mena et al. (2011) in Spanish pomegranate varieties (range 1.5-4.5 g GAE L^{-1}). As the ripening stage progressed, TPC significantly decreased probably because of the oxidation of polyphenols by polyphenol oxidase present during fruit

ripening (Schwartz *et al.*, 2009; Fawole and Opara, 2013 b), and polymerization of free phenols (Remorini *et al.*, 2008). Nevertheless, the broad interval range of TP concentrations must obey to differences among cultivars (genotypes), growing seasons, farming practices, and determination assays (Tehranifar *et al.*, 2010). The results about TPC in thinning and ripe pomegranate fruits can be seen in detail in publications 1 and 2.
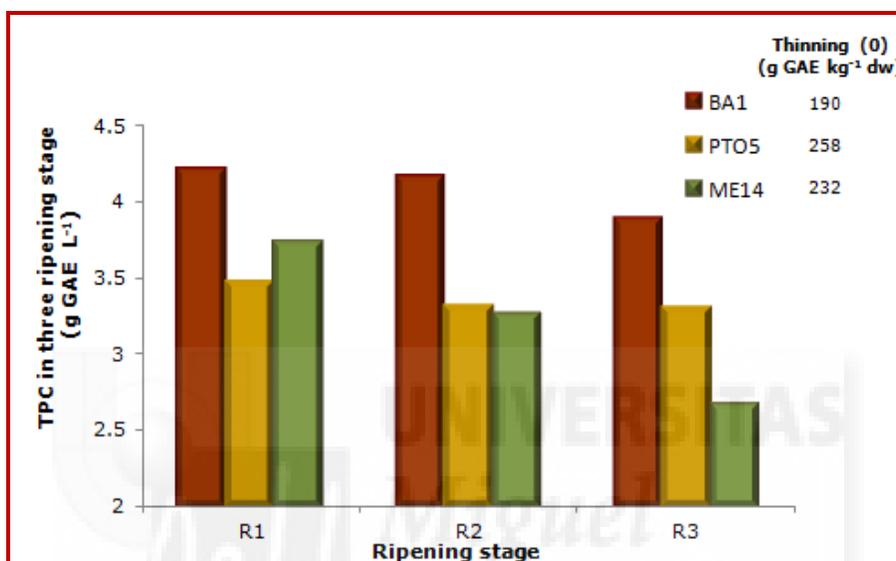
Figure 6. Total polyphenols content (TPC) in three different pomegranate cultivars (BA1: sour, PTO5: sour-sweet and ME14: sweet), in thinning (0; whole fruit) and at three ripening stages (R1, R2, R3; pomegranate arils juice).

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

The identification of major derivatives of ellagic acid (MDEA) in thinning and ripe pomegranate fruits was carried out using LC-PDA-QTOF/MS. Ellagic acid and its derivative compounds was the main class of identified and quantified compounds in this particular product.

Among the 35 MDEA found in thinning and ripe pomegranates (mainly hydrolysable tannins), only 7 were found in both types of fruits. These 7 compounds were: punicalagin isomer (R_t=1.61 min) and HHDP-gallagyl-hexoside (punicalagin; R_t=3.52 min) had an [M−H]⁻ at m/z 1083 and similar MS/MS fragments

(300/622/781); granatin A (R_t=4.40 min) had an [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 (**Figure 7**; peaks **7**, **13**, **16**, **19**, **25**, **28** and **29**, respectively). Calani et al. (2013) and Al-Rawahi et al. (2014) also identified those compounds in pomegranate. These 7 compounds represented about 70 % of the MDEA in thinning fruits, while only 14.5 % in ripe fruits. **Figure 7** shows the comparison of the MDEA profiles in thinning and ripe fruits for fruits of cultivar PTO8. In most of the cultivars, the contents of these 7 compounds were higher in thinning than in ripe fruits.

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). These compounds have been previously reported by Fischer et al. (2011) and Calani et al. (2013) in ripe pomegranates.
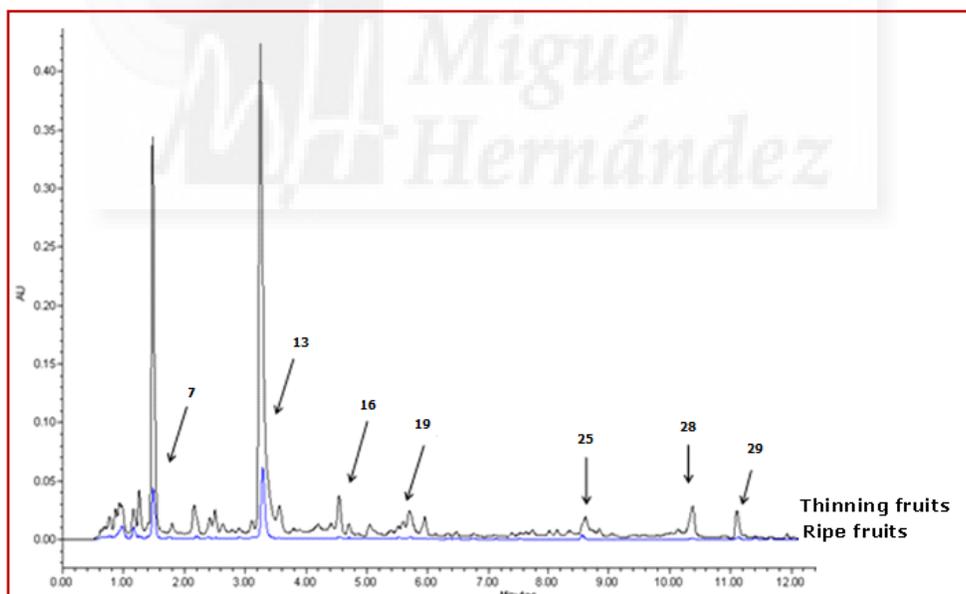

Figure 7. Comparative chromatogram of thinning and ripe pomegranates (PTO8). [*Peaks: **7**, punicalagin isomer; **13**, HHDP-gallagyl-hexoside (punicalagin); **16**, granatin A; **19**, ellagic acid derivative; **25**, ellagitannin; **28**, granatin B; **29**, ellagic acid derivative*].

The quantification of MDEA was conducted using UPLC-PDA detection. According to the mean values of all samples, the MDEA was about 7 times higher in thinning fruits (10451 ± 1581 mg 100 g^{-1} dm) than in ripe fruits (1553 ± 270 mg 100 g^{-1} dm). Al-Rawahi et al. (2014) found 6420 mg GAE 100 g^{-1} dm 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 TPC content could be due to differences in cultivars, methods of extraction and analysis (chromatography or spectrophotometry) and environmental conditions (Al-Rawahi *et al.*, 2014). Therefore, the factor "cultivar" significantly ($p<0.05$) affected the amount of MDEA; the two cultivars with the highest values of MDEA, in both thinning and ripe pomegranates, were PTO8 and BO1. Punicalagin isomer (**7**) was one of the major derivatives of ellagic acid found in thinning fruits and 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 *et al.*, 2014). The results about MDEA in thinning and ripe pomegranate fruits can be fully seen in publication 6.

6.7. Identification and quantification of punicalagin isomers and ellagic acid

In whole thinning fruits the content of: (i) α-punicalagin ranged from 101 to 195 g kg^{-1} dw, (ii) β-punicalagin from 80.1 to 111 g kg^{-1} dw, and (iii) ellagic acid from 1.96 to 3.00 g kg^{-1} dw. In general, the sour-sweet cultivars showed the highest values of these three bioactive compounds, especially PTO5. The contents of punicalagin isomers and ellagic acid found in immature thinning pomegranate fruits (150, 88.3 and 2.59 g kg^{-1} of dw α- and β-punicalagins and ellagic acid, respectively), were similar to those previously reported by Calín-Sánchez et al. (2013) in rind of mature pomegranate fruits cv. *Mollar de Elche* (139, 143, and 2.49 g kg^{-1} of dw, α- and β-punicalagins and ellagic acid, respectively); the ratio α-punicalagin/β-punicalagin took values of ~1.7. The most abundant compound in pomegranate was punicalagin; punicalagins together with ellagic acid are potent antioxidants, anticancer and have anti-atherosclerotic biological properties (Lu *et al.*, 2008). The results about identification and quantification of punicalagin isomers and ellagic acid in thinning pomegranate fruits can be fully seen in publication 1.

6.8. Antioxidant activity (AA)

The AA in pomegranate fruits was evaluated at different: (i) ripening stages (thinning and ripe R1 to R3), (ii) plant material (whole fruit and only juice obtained by squeezing the arils) and (iii) quantified using different methods (DPPH, ABTS, FRAP and ORAC). This variety of methods is due to the fact that none of the AA methods is able to determine exactly the total antioxidant capacity of a product.

In thinning and ripe fruits the factor "cultivar" significantly ($p<0.05$) affected the AA. For thinning fruits, he values ranged from 2923 to 4486 mmol Trolox kg^{-1} dw for ABTS, from 3153 to 4685 mmol Trolox kg^{-1} dw for FRAP, and finally from 2075 to 2934 mmol Trolox kg^{-1} dw for DPPH (**Figure 8**). In general and agreeing with the TPC trend, the highest values were found in sour-sweet cultivars, especially in PTO8 and PTO5 cultivars (publication 1). The differences in AA among pomegranate cultivars could be preliminarily attributed to their different polyphenols contents.

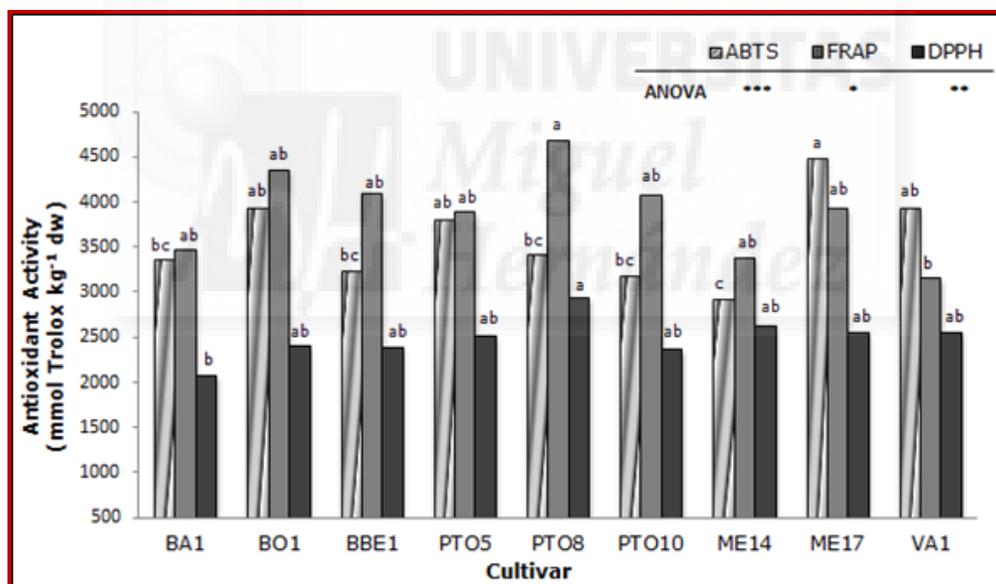**Figure 8**. Antioxidant activity (mmol Trolox kg^{-1} dw) in pomegranate fruits at the thinning stage.

The mean values for ABTS, DPPH, and FRAP methods were: 2177, 1245, and 683 mmol Trolox kg^{-1} dm, respectively. These results showed that the antioxidant activity of thinning fruits is among 2-6 times higher than that of ripe fruits for all studied methods, ABTS, DPPH, and FRAP (publication 6).

During fruit ripening, the values of AA in the juice obtained by squeezing the arils decreased from 7.90 to 6.53 mmol Trolox L^{-1}. This decrease can be explained by a reduction in the total phenolic content. Fawole and Opara (2013 b) found a significant decrease in antioxidant activity of pomegranate juice at different maturation stages, a decrease of 67.8% and 66.4% for DPPH and FRAP, respectively; they concluded that the reduction was associated with a decrease of polyphenols (Gil *et al.*, 2000). The AA values reported in the literature for pomegranate juice ranged from 6 to 15 mmol Trolox L^{-1} using the DPPH method (Mena *et al.*, 2011); therefore, the results were within this interval (publication 2). Comparing the ORAC data found in thinning and ripe fruits, it can be concluded that thinning fruits have higher values than those of ripe pomegranates. The ORAC values ranged from 664 to 925 and from 338 to 582 mmol Trolox kg^{-1} dm in thinning and ripe fruits, respectively (publication 6) (**Figure 9**).
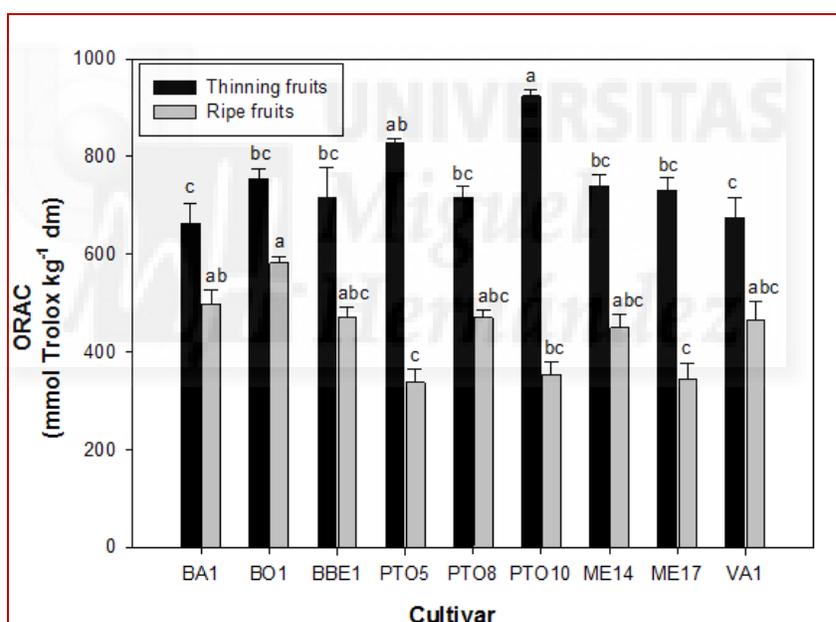
Figure 9. Antioxidant activity (ORAC method) of the studied pomegranate fruits (mmol Trolox kg^{-1} dm).

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}) and PTO5 (827 mmol Trolox kg^{-1}); and, (ii) BO1 (582 mmol Trolox kg^{-1}) and BA1 (498 mmol Trolox kg^{-1}), respectively. The results about AA in thinning and ripe pomegranate fruit can be fully seen in publications 1, 2, 5 and 6.

The antioxidant activity/capacity of pomegranate can be 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. As an example, Calín-Sánchez et al. (2013), using the DPPH method, reported values of 180 mmol Trolox kg^{-1} dw for rind of ripe commercial Spanish pomegranates. However, in publication 6, it can be observed that the mean values for the antioxidant activity (DPPH) in thinning and ripe Spanish pomegranates were 2541 and 1245 mmol Trolox kg^{-1} dw, respectively.

Table 12 shows a comparison of the antioxidant activity of pomegranate fruit (natural and commercial juice), quince juice, Brazilian cherry fruits, red wine, and green tea. For instance, this data shows that the antioxidant activities of commercial pomegranate juice are about 2 and 3 times higher than those of red wine and green tea, respectively (Gil *et al.*, 2000; Mena *et al.*, 2011). These results are interesting because they clearly show the richness of thinning pomegranates as a natural antioxidant.

Table 12. Antioxidant activity of pomegranate fruit, natural and commercial pomegranate juice, quince juice, Brazilian cherry, red wine and green tea.

Fruit material	Antioxidant Activity
Pomegranate; *Piñón Tierno de Ojós* (whole thinning fruit)	2934 mmol Trolox kg^{-1} (dw)[1]
Pomegranate; *Piñón Tierno de Ojós* (whole ripe fruit)	1087 mmol Trolox kg^{-1} (dw)[2]
Pomegranate; *Mollar Elche* (rind ripe fruit)	180 mmol Trolox kg^{-1} (dw)[3]
Quince; *Uspiech* (juice ripe fruit)	108 mmol Trolox L^{-1} [4]
Brazilian cherry; *Red*, *E. Uniflora* L. (immature pulp)	171 mmol Trolox kg^{-1} dw [5]
Brazilian cherry; Red, *E. Uniflora* L. (mature pulp)	83 mmol Trolox kg^{-1} dw [5]
Pomegranate; *Wonderful* (natural juice)	6-15 mmol Trolox L^{-1}[6]
Pomegranate commercial juice	18-20 TEAC [7]
Green Tea	6 TEAC [7]
Red wine	8 TEAC [7]

[1] *Nuncio-Jauregui et al., 2014;* [2] *Nuncio-Jauregui et al., (publication 6, under review);* [3] *Calín-Sánchez et al., 2013;* [4] ***Wojdyło et al., 2014;*** [5] *Celli et al., 20011;* [6] *Mena et al., 2011;* [7] *Gil et al., 2000.*

6.9. Volatile compounds

The volatile composition of pomegranate juices is a parameter that can be affected by factors such as pomegranate cultivar and agronomic and environmental conditions. However, the trends and relationships found in this study are of high importance. A total of 39 compounds were isolated and identified in pure pomegranate juice by the HS-SPME technique.

The volatile compounds found in pomegranate juice can be grouped into 9 chemical families: (1) *alcohols*, including ethanol, *cis*-3-hexenol, 1-hexanol and 2-ethyl-1-hexanol; (2) *esters*, e.g. ethyl acetate and isoamyl butyrate; (3) *terpenes*, including *α*-pinene, *β*-pinene and limonene; (4) *aldehydes*, pentanal, hexanal, etc.; (5) *terpenoids*, with terpinene-4-ol and *α*-terpineol; (6) *hydrocarbons*, including dodecane and tetradecane; (7) *acids*, acetic and 2-methylbutyric acids; (8) *sulfur compounds*, dimethyl disulfide; (9) *ketones*, 2-heptanone. Alcohols (41.4 %) and (27.3 %) were the predominant groups in the headspace of pomegranate juice, followed by terpenes (13.3 %) and aldehydes (11.9 %). This profile of volatile compounds can be considered as typical of sweet pomegranate cultivars (Carbonell-Barrachina *et al.*, 2012). In general, *alcohols* and especially *esters* are related to fruity and sweet aromas, while *aldehydes* can be related to green, grassy and herbaceous notes and *terpenes* can be related to pine and citrus notes (Vázquez-Araújo *et al.*, 2010). Six compounds had concentrations above 5 %: ethyl acetate (23.9 %), 1-hexanol (14.4 %), limonene (10.4 %), ethanol (10.0 %), 2-ethyl-1-hexanol (9.9 %) and hexanal (6.2 %). All these compounds have been previously described in pomegranate juices by other authors (Andreu-Sevilla *et al.*, 2008; Calín-Sánchez *et al.*, 2011; Vázquez-Araújo *et al.*, 2011) and consequently are typical of pomegranate products. The results about volatile compound in pomegranate juice can be fully seen in publications 4.

6.10. Sensory analysis

A total of 20 pomegranate cultivars were analyzed. Thirteen pomegranate cultivars were collected from the germplasm bank located at the experimental field station of Miguel Hernández University (UMH) in Orihuela (Alicante, eastern Spain). Also, fruits from 5 commercial cultivars purchased in the farmers' market of the area, and fruits from 2 commercial cultivars grown in the Canary Islands (Spain) were studied to compare results with those of thee fruits from the UMH germplasm bank (**Table 13**).

Table 13. Abbreviation and codification of the pomegranates under study.

Abbreviation	Code	Abbreviation	Code	Abbreviation	Code	Abbreviation	Code
VA11	150	**ME14**	135	**ADO4**	298	**M50**	371
VA1	274	**MA1**	571	**BO1**	997	**VA**$_{com}$	845
CRO1	703	**MO4**	634	**BA1**	459	**M**$_{com}$	096
ME1	686	**PTO3**	388	**HIZC**	819	**FV1**	747
ME2	050	**PTO7**	312	**WOND**	420	**FV2**[j]	516

The attributes used for the present study were: color, fruity, pomegranate, apple, pear, grape, berry, cranberry, cherry, floral, green-viney, sweet overall, woody, sweet, sour, bitter, astringent, toothetch, and throat burn. Statistically significant differences were found for all these attributes. **Figure 10** shows the PCA map for the flavor and mouthfeel attributes of the samples: PC1 and PC2 explained 58 % of the variation of the samples. As shown in the map, sample 997 (*BO1*) was characterized by having higher grape, apple, and floral flavor notes than all other samples, and also producing a tongue numbing mouthfeel. This mouthfeel was slightly present in all subsamples of *BO1* but absent in all other samples, including other sour cultivars, such as *BA1*. The high levels of fruity and floral characteristics could make this cultivar of interest in further breeding programs.

Pomegranate cultivars can be classified depending on the hardness of the seeds in: (i) hard, (ii) semi-soft, and (iii) soft (Melgarejo *et al.*, 2000). In general, hard cultivars are not appropriate for fresh consumption because of their seeds hardness. The sour samples (*BO1*, *BA1*, *WOND*, and *HIZC*) had higher seed hardness than the sweet or sour-sweet samples. *Mollar* is a Spanish term related with softness, so most of *Mollar* varieties had low seed hardness scores, as expected from their cultivar name; ME14, FV1 and FV2 were exceptions to this general rule.
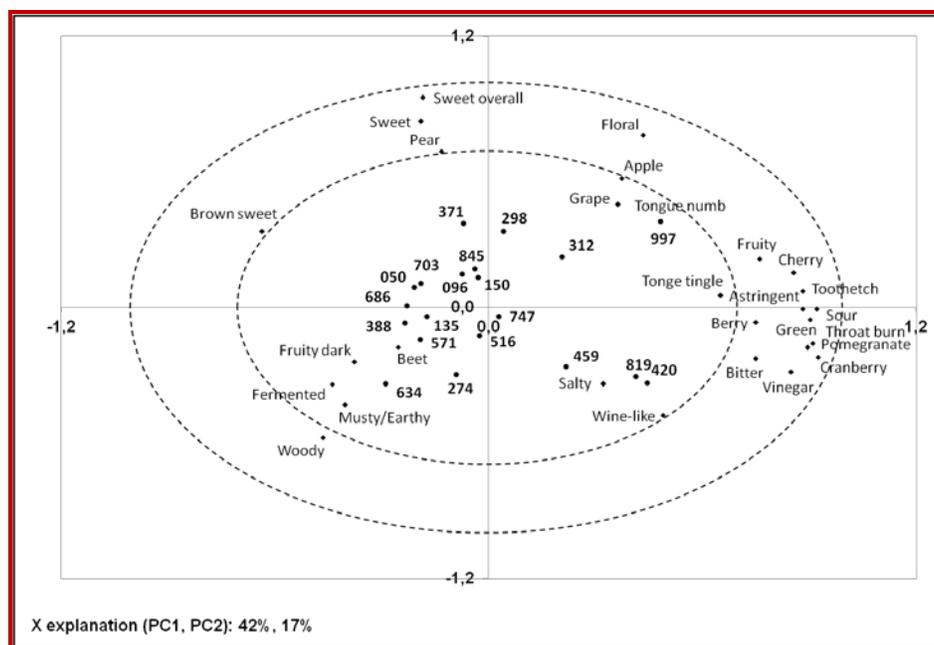
Figure 10. PCA map showing representative scores (only flavor and mouthfeels) of juice samples. Codes of samples are indicated in bold font.

Best market options

The most important quality attributes for pomegranate fruits aimed for *fresh consumption* are: large size, intense skin color, intense aril color, high sweetness, and soft seeds. This clearly excludes cultivars with hard seeds, the sour cultivars (*BA1*, *HIZC*, *BO1* and *WOND*), the sour-sweet cultivars (*PTO7* and *ADO4*), but also some sweet cultivars (*CRO1*, *ME14*, *FV1*, and *FV2*). Consequently, 9 out of the 20 pomegranate cultivars have appropriate sensory attributes for their commercialization as fresh products (soft seeds and high sweetness); these cultivars are: *ME1*, *ME2*, *MA1*, *MO4*, *VA1*, *VA11*, *M50*, *Mcom*, and *VAcom*. Intense color of arils is a key requirement for *juice manufacturing* because the heat treatments involved in the processing will drastically reduce the color of the juice. This requirement is fulfilled by samples with intense color; this is 6 out of 20 cultivars: *HIZC*, *WOND*, *ME1*, *PTO7*, *MO4*, and *VA1*. Depending on the market requirements and needs, the sour-sweet or sour fruits could be mixed with sweet fruits until getting the desired equilibrium of sour and sweet tastes. The results about sensory analysis in pomegranate fruit can be fully seen in publication 3.

6.11. **Changes in quality parameters, proline, antioxidant activity and color of pomegranate as affected by fruit position within tree**

The position within the tree had no significant effects on total soluble solids (TSS), the titratable acidity (TA), maturity index (MI), pH, organic acids, sugars profiles, proline, antioxidant activity (AA) and total phenolic compounds (TP); however, it significantly ($p<0.05$) affected data on external color coordinates. The results about the changes in all these parameters of pomegranate fruit as affected by fruit position within tree can be seen in publication 2.

6.11.1. External and internal color

The external CIE*L*a*b** color coordinates of pomegranate fruits was measure at two different positions within the trees: (i) *East*, having a higher exposure to the sunlight, and (ii) *West*, having less exposure to the sunlight. In general, sun-fruits (East) had lower values of lightness, *L**, implying darker colors, and simultaneously higher values of the green-red coordinate, *a**, and lower values of the blue-yellow coordinate, *b**. This combination of low values of *L** and *b** and high of *a** led to intense garnet (combination of red and blue tones) color, typical of pomegranate products, of the sun oriented fruits. During ripening, *L**, *b** and Hue angle decreased while *a** and chroma increased. This same behavior was reported by Manera *et al.* (2012) in pomegranate rind harvested at the beginning of September.

The fact that the factor "fruit position within the tree" affected external color but not internal color seems to imply that external quality attributes are more susceptible to environmental changes than internal attributes. In this way, Fawole and Opara (2013) reported that color development occurs before in the husk than in arils.

6.12. **Composition and adulteration of commercial pomegranate juice**

Adulteration of pomegranate juice with grape juice is one of the most logical options, considering its low price and similar sugar, organic acid, sensory and volatile profiles. Adulteration of pomegranate juice with peach juice could be another important option, considering its low price and the fact that its high sweetness and intense fruity flavor could be useful in improving the sometimes too intense sourness and flat flavor of some pomegranate juices.

6.12.1 Organic acids and sugars profile

The organic acids profile described in commercial pomegranate juice contained 3.23 and 2.61 g L^{-1} of citric and malic acids, respectively. These concentrations agreed well with the AIJN Reference Guide (2012). The ranges admitted by this guide are: 0.1-33 g L^{-1} and 0.02-3.6 g L^{-1}, for citric and malic acids, respectively. Tartaric acid was present in the studied pomegranate juice, but only at trace level. In the pomegranate juice used for the present study, the predominant sugar was fructose (70.8 g L^{-1}), followed by glucose (54.2 g L^{-1}); being the ratio glucose/fructose 0.77. According to the AIJN Reference Guide (2012) the values of fructose and glucose should range among 50-100 g L^{-1} and 45-85 g L^{-1}, respectively; with a ratio glucose/fructose being in the range 0.7-1.0 (Mena *et al.*, 2011; Zhang *et al.*, 2009).

Addition of grape products to the pomegranate juice will result in measurable concentrations of tartaric acid, as suggested by Zhang et al. (2009); at the same time, the content of citric acid will be drastically reduced (Soyer *et al.*, 2003). Mato et al. (2007) stated that grape juice is characterized by its elevated concentration of tartaric acid representing more than 50 % of the total acids found in this juice. Adulteration of pomegranate juice with peach juice will be difficult to determine because the organic acids profile is similar and only concentrations will slightly decreased. No significant changes were observed in the sugar profile after addition of grape juice to the pomegranate juice; however, the content of sucrose significantly increased after addition of peach juice. The results about organic acids and sugars profiles in commercial pomegranate juice can be seen in publication 4.

6.12.2 Mineral analysis

The contents of the macronutrients (Ca, Mg, K, and Na) and micro-nutrients (Fe, Zn, Cu, and Mn) in pure commercial pomegranate juice were: 25.3, 27.3, 2492, and 29.5 mg L^{-1}, and 1.03, 1.28, 0.41, and 0.35 mg L^{-1}, respectively. Adulteration of pomegranate juice by mixing with other juices can result in dilution of the most abundant mineral, K.

In this specific study, the addition of grape juice significantly ($p<0.05$) increased the contents of Ca, Mg, Fe, Cu and Mn and significantly ($p<0.05$) decreased the K content. On the other hand, mixing pomegranate juice with peach juice only increased the content of Mg and decreased that of K. The K content were 2492, 806 and 1002

mg L^{-1} in pomegranate, grape and peach juices, respectively; these contents agreed well with those reported by the USDA (2013), who reported values of 2590, 900 and 970 mg L^{-1}, respectively. According to the current results, any pomegranate juice with K content lower than 2000 mg L^{-1} is highly suspicious of being adulterated. The results about minerals in commercial pomegranate juice can be seen in publication 4.

6.12.3 Proline

The proline content was significantly affected by the type of juice. The grape juice presented the highest proline content, 1032 mg L^{-1}, followed by pomegranate juice (251 mg L^{-1}), and peach juice presenting the lowest value (182 mg L^{-1}). The AIJN (2005) in their Reference Guide for grape and peach juices reported a maximum value for the proline content of 1400 mg L^{-1}.

As expected, an adulteration of pomegranate juice with grape juice at concentrations of 10, 25 and 50 % implied a significant increase in the proline content at levels of 320, 446, and 639 mg L^{-1}, respectively. However, the mixing of pomegranate juice with peach juice at concentrations of 5 and 10 % led to a significant decrease of the proline content to levels of 223 and 212 mg L^{-1}, respectively. It can be stated that the addition of grape juice or products to pomegranate juice will result in important increases of the proline content. The results about proline in commercial pomegranate juice can be seen in publication 4.

6.12.4. Volatile compounds

As discussed in section 6.9., a total of 39 compounds were identified in pure pomegranate juice by the HS-SPME technique. These compounds were grouped into 9 chemical families: alcohols, esters, terpenes, aldehydes, terpenoids, hydrocarbons and acids. In general, grape juice was also dominated by alcohols (47.8 %) and esters (18.6 %). Adulteration of pomegranate juice with up to 50 % of grape juice resulted in significant increases in acetic acid, isoamyl alcohol, isoamyl butyrate and especially 1-hexanol (up to concentrations of~25 %) and linalool (~6 %). On the contrary, compounds such as ethyl acetate, hexanal, *cis*-3-hexenol, 2-ethyl-1-hexanol and terpinene-4-ol decreased after addition of grape juice to pomegranate juice. Some compounds from the grape juice were not found in the pomegranate juice and therefore relatively high concentrations could be considered a sign of adulteration; these compounds included myrcene, hexyl acetate, linalool oxides, benzyl acetate and *γ*-decalactone.

In general, peach juice was clearly dominated by esters (83.2 %) and terpenes (8.8 %), with alcohols and aldehydes playing a minor role compared with pomegranate juice. Adulteration of pomegranate juice with up to 10 % of peach juice resulted in significant increases in butyl acetate, isobutyl butyrate, benzyl acetate and especially isoamyl butyrate (up to concentrations of ~40 %). On the contrary, some compounds such as ethyl acetate, hexanal, *cis*-3-hexenol, 1-hexanol, 2-ethyl-1-hexanol, terpinene-4-ol, and *a*-terpineol decreased in concentration after addition of peach juice to pomegranate juice. Some compounds from the peach juice were not found in the pomegranate juice and therefore relatively high concentrations could be considered a sign of adulteration; these compounds included ethyl butyrate, isovaleric acid, *cis*-3-hexenyl formate, benzyl acetate, γ-decalactone and especially isoamyl acetate (>25 %) and hexyl acetate (>4.3 %). The presence of lactones such as *γ-decalactone* could also be a good indicator of adulteration with peach juice. The results about volatile compounds in commercial pomegranate juice can be fully seen in publication 4.

Chapter 7. Conclusions

7.   **CONCLUSIONS**

Thinning fruits

1.   In thinning fruits, the content of organic acids and total polyphenols content was 2-3 times higher than that of ripe pomegranates. Also, the antioxidant activity of thinning pomegranates was about 2-6 times higher than that of ripe pomegranates.

2.   In general, the group of cultivars with the highest values of α-punicalagin, β-punicalagin, ellagic acid, antioxidant activity and proline was sour-sweet.

3.   Potassium (K) was the predominant macro-element in all pomegranate cultivars, while zinc (Zn) was the predominant micro-element. In general, the minerals contents found in thinning fruits were higher than those normally found in edible arils and pure juice, making this material interesting as a mineral supplement.

4.   Pomegranate wastes (thinning fruits) are a good source of bioactive compounds, making this material interesting for food, pharmaceutical or chemical industries as well as an extra source of income for the farmers.

Pomegranate ripening stage and position within the tree

5.   As the ripening stage progressed, the total organic acids, total phenolic content and antioxidant activity significantly decreased; while the total sugar and proline content increased. The physico-chemical parameters, total phenolic content and antioxidant activity was significantly affected by the cultivar and ripening stage.

6.   The position of pomegranate within the tree had no significant effect on chemical parameters, organic acids, sugars profile, proline, phenolic compounds and antioxidant activity of three pomegranate varieties grown in Spain at three ripening stages. However, the position within the tree had significant effect on the external color.

Comparison among contents of major derivatives of ellagic acid in thinning and ripe pomegranates

7. Thirty-five major derivatives of ellagic acid were found in thinning and ripe pomegranates (mainly hydrolysable tannins), with 7 of them found simultaneously in both types of fruits: punicalagin isomer, HHDP-gallagyl-hexoside (punicalagin), granatin A, ellagic acid, ellagitannin, granatin B, and ellagic acid derivative.

8. The total content of major derivatives of ellagic acid was about 7 times higher in thinning than in ripe fruits.

Sensory characterization of pomegranates for processing and fresh consumption

9. Most of the *Mollar* and *Valenciana* pomegranate cultivars have appropriate sensory attributes for their commercialization as **fresh products** (soft seeds and high sweetness) and *Wonderful* cultivar is appropriate for **juice manufacturing** for its intense arils color.

Pomegranate juice adulteration

10. The main parameters for the detection of adulterated pomegranate juice with grape juice were: decrease of potassium (K), increases of proline and tartaric acid, and the presence of volatile compounds such as linalool and linalool oxide.

11. An increase in the sucrose concentration, the presence of isoamyl acetate and/or hexyl acetate, and the simultaneous presence of high concentrations of esters and lactones could be considered as an indicator of pomegranate juice adulteration with peach juice.

12. It is important to highlight that it is necessary to simultaneously analyze and have results from several parameters to conclude that a particular pomegranate juice is adulterated by mixing with other fruit juice.

Chapter 8. References
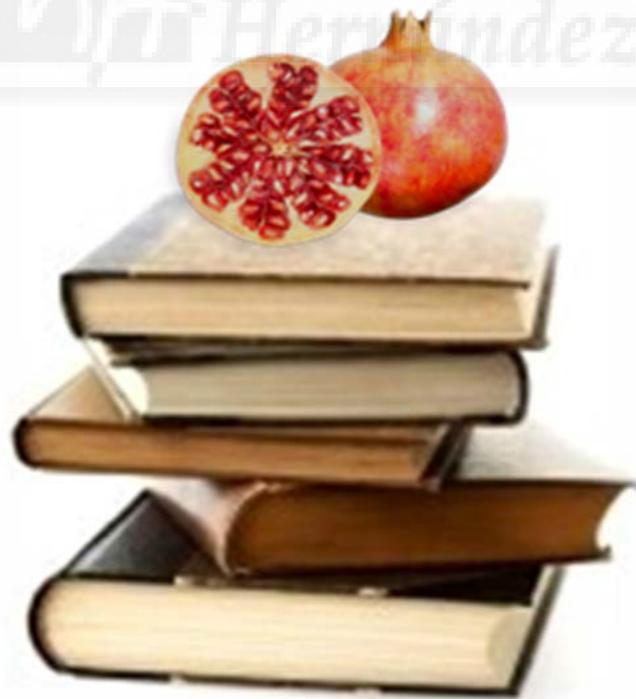
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