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Seeds of pomegranate, tomato and grapes: An underestimated source of natural bioactive molecules and antioxidants from agri-food by-products

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Highlights

- Pomegranate, tomato and grape seeds were biochemically characterized
- Seeds were extracted by supercritical CO_2 to prepare high-value vegetable oils
- Oils were assayed for isoprenoids, fatty acids and antioxidant activity
- Seeds are useful sources of phenolics, tocochromanols and unsaturated fatty acids
- Carefully designed oil blends can meet specific dietary requirements of customers

Abstract

Pomegranate, tomato and grape seeds are quantitatively relevant agri-food by-products rich in molecules beneficial to human health. To valorize this resource, the composition and antioxidant activity of seeds and deriving supercritical CO_2 (SC-CO_2) extracted oleoresins were evaluated. Grape seeds showed the highest content of total phenolic compounds (33.9 mg GAE/g), flavonoids (15.6 mg CE/g) and condensed tannins (14.0 mg CE/g), while tomato seeds presented the highest content

of tocochromanols (159.6 µg/g). Grape seeds showed the highest total antioxidant activity (178.2 µmol TE/g), as evaluated by TEAC assay, followed by pomegranate (19.8 µmol TE/g) and tomato (9.8 µmol TE/g). Oleoresin yields obtained by SC-CO_2 extraction from the seeds ranged between 3.1 (pomegranate) to 7.8 (tomato) g oleoresin/100 g. Total tocochromanols were abundant in pomegranate (2008 µg/g) and tomato (1769 µg/g) oleoresins; a relatively low amount was instead detected in the oleoresin extracted from grape seeds (636 µg/g). Carotenoids were not detected in all oleoresins. Pomegranate oleoresin had a higher antioxidant activity than the others. Mono- and polyunsaturated fatty acids were more abundant than saturated in all oleoresins, with the highest percentage of unsaturated fatty acids detected in pomegranate seed oleoresin (~90%), mainly due to punicic acid (~70%).

Abbreviations: ABTS, 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid); CE, catechin equivalents; GAE, gallic acid equivalents; HAA, hydrophilic antioxidant activity; LAA, lipophilic antioxidant activity; MUFA, monounsaturated fatty acids; ORAC, oxygen radical absorbance capacity; PUFA, polyunsaturated fatty acids; SC-CO_2, supercritical CO_2; SFA, saturated fatty acids; TAA, total antioxidant activity; TE, trolox equivalents; TEAC, trolox equivalent antioxidant capacity; UFA, unsaturated fatty acids.

Keywords: by-products, carotenoids, fatty acids, food analysis, food composition, *Lycopersicon esculentum* L., *Punica granatum* L., supercritical fluid extraction, tocochromanols, *Vitis vinifera* L.

1. Introduction

Agri-food by-products produced during handling and processing of fruits and vegetables, including peels, seeds, leaves, bracts, stems, roots and bark, represent a major waste disposal problem for industry (Ezejiofor et al., 2014). Besides large amounts of storage (sucrose, starches, inulin, pectin like polysaccharides, etc.) and cell wall structural (cellulose, hemicelluloses and pectins)

carbohydrates, proteins and lipids, potentially useful for animal and human food supplementation and/or bioenergy production, high-value natural compounds such as carotenoids, phenols, tocochromanols, vitamins, or phytosterols can be found in most of these by-products, many of them having health-promoting properties (Kamal-Eldin and Appelqvist, 1996; Kohno et al., 2004; Lenucci et al., 2013).

Seeds represent a quantitatively abundant by-product of fruit industrial processing. They are often discarded within the so called pomace together with skins, and vascular tissues of the fruits, but can be easily recovered by separation and sifting technologies. An estimated 13-20% by weight of the grapes processed by the wine industry ends up as pomace after pressing, with seeds accounting for approximately 8-20% of the total waste depending on the grape cultivar and processing method, equivalent to 0.5-2.0 million tons per year (Schieber et al., 2001; Dwyer et al., 2014). Similarly, seeds account for approximately 60% of the total waste of tomatoes (3-6 million tons per year) and 22% of the waste (rind plus seeds) of pomegranate juice industry (Fao, 2011; Abid et al., 2017). Seeds represent the portion of the fruit with the highest concentration of bioactive molecules so that their waste represents a double loss for agri-food industry that has to face the cost of disposal and the loss of profits for their re-use and valorization. Pastrana-Bonilla et al. (2003) reported that among the different portions of grape fruit, seeds exhibit the highest antioxidant activity followed by the skin and the flesh; similarly, the seed fraction of tomato was shown to contain higher phenolic content than pulp, thus representing an important reservoir of these compounds whose bioactivity has been unequivocally established (Chandra and Ramalingam, 2011). The antiproliferative and apoptotic effects of proanthocyanidins from grape seeds on colon cancer Caco2 cells has been demonstrated (Engelbrecht et al., 2007; Kaur et al., 2009). Further, tomato and pomegranate seeds have attracted interest since their oil is rich in unsaturated fatty acids (UFA), especially linoleic acid (>50% in tomato), phytosterols and antioxidants which make it particularly suitable as edible oil (Roy et al., 1996; Eller et al., 2010). Pomegranate seed oil is a major source of punicic acid, a distinctive ω-5

trienoic fatty acid with emerging evidences for important therapeutic uses in human health including inhibition of cancer cell proliferation (Albrecht et al., 2004; Jeune et al., 2005; Aruna et al., 2016).

The increasing awareness of the potential commercial value of most agri-food by-products has stimulated the exploitation of efficient extraction techniques of their bioactive compounds with undeniable environmental sustainability benefits and a more effective use of the harvested plant material (González-Paramás et al., 2004; Vági et al., 2007).

In recent years, supercritical CO_2 (SC-CO_2) extraction technology has been widely proposed to prepare food-grade oleoresins from a variety of by-products, including seeds, as an alternative to conventional extraction techniques. Being non-toxic, non-flammable, non-corrosive and highly selective, the use of CO_2 as unique extraction solvent is, in fact, safer and more environmentally friendly compared to the use of most conventional organic solvents (Reverchon and De Marco, 2006; Liu et al., 2012; Durante et al., 2012). The use of seeds as co-matrix to increase the solubility and extractability of carotenoids (especially lycopene from tomato dehydrated matrices) in SC-CO_2 and for the enrichment of the extract in bioactive compounds has been also described (Lenucci et al., 2015; Durante et al., 2016).

Thus, we present, for the first time, a comparative analysis of the main bioactive constituents from pomegranate, tomato and grape seeds, as well as of the oleoresins obtained from their extraction by SC-CO_2, including fatty acid composition and the content of tocochromanols, in order to better assess the potential of these agri-food by-products as sources of nutraceutical or functional food natural ingredients.

2. Materials and Methods

2.1. Chemicals

Tocopherols (α-, β-, δ- and γ-forms), myristic, palmitic, stearic, arachidic, margaric, palmitoleic, oleic, linoleic and linolenic acids used as standards, PBS solution (pH 7.4), 2,2'-azinobis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) diammonium salt, 6-Hydroxy-2,5,7,8-

tetramethylchromane-2-carboxylic acid (trolox), catechin, Folin & Ciocalteu's phenol reagent, sodium carbonate (Na_2CO_3), gallic acid, vanillin, sodium nitrite ($NaNO_2$), aluminium chloride hexahydrate ($AlCl_3 \cdot 6H_2O$), sodium chloride (NaCl), as well as all solvents were purchased from Sigma–Aldrich (Milan, Italy). Punicic acid and tocotrienols (α-, β-, δ- and γ-forms) were purchased from Cayman chemicals (Ann Arbor, MI, USA). Carotenoid standards were purchased from CaroteNature (Lupsingen, Switzerland). High purity carbon dioxide (99.995%) for supercritical fluid extraction was purchased from Mocavero Ossigeno (Lecce, Italy).

2.2 Plant material processing

The analyses were carried out on pomegranate (*Punica granatum* L., cultivar Dente di cavallo), tomato (*Lycopersicon esculentum* L., cultivar San Marzano) and grape (*Vitis vinifera* L., cultivar Nero di Troia) seeds, which were manually separated from the pomaces obtained from the mechanical pressing of the fruits to produce juices. All fresh fruits were grown in open-field in the province of Lecce in southern Italy (latitude 40°23'16"80 N, longitude 17°57'41"40E; decimal degrees 40.3881; 17.9615) by local farmers. Approximately 10 kg of fully ripe healthy fruits were processed into juices. For pomegranate an electrical juice squeezer (Fimar S.p.A., Rimini, Italy) was used, while tomato and grape were processed by a Kuvings CS600 cold-press juicer (NUC Electronics Co., Daegu, Korea). Approximately 0.3 Kg of seeds were collected from each pomace. Isolated seeds were vacuum-dried to constant weight at 60 °C by a Salvis Lab IC40 vacuum-drying oven (Bio Instruments S.r.l., Firenze, Italy) and then ground in a laboratory ultra-centrifugal mill (ZM200, Retsch GmbH, Haan, Germany) through 18 mesh (1 mm) sieve. These cultivars were selected since they are traditional Italian and largely used for industrial processing.

2.3 Extraction of soluble and insoluble-bound phenolic compounds

Extraction of phenols was carried out according to Adom et al. (2003). Briefly, 0.05 g of samples (three independent replicates from the same seed batch) were mixed with 1 mL of 80% (v/v) chilled

ethanol for 10 min. After centrifugation at 2500g for 10 min, the supernatant containing the soluble phenolic compounds was recovered. The extraction was repeated twice and the supernatants were combined.

The insoluble-bound phenols were extracted from the pellets resulting from the soluble phenolic extraction. The pellets were sequentially washed twice with 100% methanol (1 mL) and chloroform/methanol/water (1/1/1 v/v/v – 1.5 ml) and once with 100% acetone (1 mL). The supernatants were discarded after each extraction and centrifugation at 9000g for 7 min. The residues were then digested with 1 mL of 1 M NaOH at room temperature for 1 h with shaking under nitrogen gas. The mixtures were acidified to pH 2 with 12 M HCl and extracted three times with 500 µL of ethyl acetate. The ethyl acetate fraction was evaporated to dryness under vacuum by a rotary evaporator. Phenolic compounds were dissolved in 500 µL of 80% (v/v) ethanol.

2.3.1 Determination of total phenolic content

Total phenolic content was determined on each extract (soluble and insoluble-bound phenolic extracts) according to the method of Xu et al. (2008). Briefly, 50 □L of extract were mixed with 50 □L of Folin-Ciocalteu's phenol reagent and 450 □L distilled water. The mixture was kept at room temperature for 5 min, and then 500 □L of 7% (w/v) Na_2CO_3 added, reaching the final volume of 1250 □L with distilled water. The mixture was left at room temperature in the dark for 90 min. Absorbance was measured at 750 nm using a Beckman DU650 spectrophotometer (Beckman Coulter Ltd., High Wycombe, UK). The amount of total phenols was calculated using gallic acid as calibration standard within the range of 0-12 µg GA/100 µL in 80% ethanol. The results were expressed as mg gallic acid equivalents (GAE)/g seeds. A single technical replica was performed for each independent extract.

2.4 Extraction of flavonoids and condensed tannins

To 0.1 g of sample seeds (three independent replicates from the same batch) 1.5 mL of 100% methanol were added. The mixture was then shaken at 4°C for 16 h, centrifuged at 9000g for 10 min and the supernatant was recovered.

2.4.1 Determination of total flavonoid content

The total flavonoid content was determined as described by Zhishen et al. (1999). 50 □L of extract were diluted with distilled water to a final volume of 500 □L and 30 □L of 5% $NaNO_2$ (w/v) were added. After 5 min, 60 □L of 10% $AlCl_3$ were added, followed, after further 6 min, by 200 □L of 1M NaOH and 210 □L of distilled water. The absorbance was read at 510 nm in a Beckman DU650 spectrophotometer. The linear calibration curve was from 0 to 400 □g catechin/mL in 100% methanol. The results were expressed as mg catechin equivalents (CE)/g seeds. A single technical replica was performed for each independent extract.

2.4.2 Determination of condensed tannins

Condensed tannins were quantified according to Broadhurst and Jones (1978). 100 µL of extract, contained in a test tube covered with aluminum foil, were mixed with 600 µL of 4% vanillin–methanol solution (w/v) and then with 300 µL of hydrochloric acid. The mixture was allowed to stand for 15 min at 20 °C in the dark. The absorbance of the mixture was measured at 500 nm in a Beckman DU650 spectrophotometer. The linear calibration curve was from 3.9 to 250 □g catechin/mL in 100% methanol. The results were expressed as mg catechin equivalents (CE)/g seeds. A single technical replica was performed for each independent extract.

2.5 Extraction and determination of tocochromanols and carotenoids in the seeds

Extraction and quali-quantitative analyses of tocochromanols and carotenoids from seeds were carried out by the method of Fraser et al. (2000), slightly modified. In brief, aliquots (1.0 g) of milled seeds (three independent replicates from the same batch) were extracted with 3 mL of 100%

methanol under constant stirring for 5 min at 4 °C. Subsequently, 3 mL of 50 mM Tris HCl buffer (pH 7.5) containing 1 M NaCl were added. Samples were vigorously stirred for further 10 min at 4 °C. Chloroform (8 mL) was added to the mixture and incubated on ice for 10 min. A clear partition was formed by centrifugation at 3000 g for 5 min at 4°C. The hypo-phase was removed and the aqueous phase re-extracted with chloroform (8 mL). The pooled chloroform extracts were taken to dryness by a rotary evaporator. Dried residues were stored under an atmosphere of nitrogen at -20°C prior to HPLC. HPLC analyses were performed using an Agilent 1100 Series HPLC system (Agilent Technologies, Waldbronn, Germany) equipped with a reverse-phase C30 column (5 µm, 250 x 4.6 mm) (YMC Inc., Wilmington, NC, USA). The mobile phases were: methanol (A), 0.2% ammonium acetate aqueous solution/methanol (20/80, v/v) (B) and tert-methyl butyl ether (C). The gradient elution was as follows: 0 min, 95% A and 5% B; 0–12 min, 80% A, 5% B and 15% C; 12–42 min, 30% A, 5% B and 65% C; 42–60 min, 30% A, 5% B and 65% C; 60–62 min, 95% A, and 5% B. The column was re-equilibrated for 10 min between runs. The flow rate was 1.0 mL/min and the column temperature was maintained at 25 °C. The injection volume was 10 µL. Absorbance was registered by diode array at wavelengths of 475 nm for carotenoids and 290 nm for tocochromanols. Peaks were identified by comparing their retention times and UV–Vis spectra to those of authentic isoprenoid standards. The limit of detection was 0.4 mAU, typically in the 2-10 ng/g range per compound. A single technical replica was performed for each independent extract.

2.6 Assay for hydrophilic and lipophilic antioxidant activity

Hydrophilic and lipophilic antioxidants were sequentially extracted from 0.1 g of each sample seeds (three independent replicates from the same batch) with 100% methanol and 100% acetone, respectively, at 4°C under constant shaking (300 rpm) for 20 h. Samples were centrifuged at 8800 g for 7 min. Supernatants were recovered and used for antioxidant activity assay.

Hydrophilic antioxidant activity (HAA) and lipophilic antioxidant activity (LAA) were measured by the TEAC (trolox equivalent antioxidant capacity) assay as described by Re et al. (1999), using the ABTS discoloration method.

Ten microliters were mixed with 1.0 mL of $ABTS^{\cdot+}$ solution and the absorbance decrease was measured at 734 nm in a Beckman DU650 spectrophotometer. Methanol and acetone were used as blank for hydrophilic and lipophilic extracts respectively. The linear calibration curves were from 0 to 15 □M trolox for both hydrophilic and lipophilic antioxidant activities. The antioxidant activity of the samples was calculated, on the basis of the inhibition exerted by standard trolox concentrations at 734 nm, inhibition time being fixed at 10 min. Total antioxidant activity (TAA) was calculated as the sum of HAA and LAA. Results were expressed as µmol trolox equivalents (TE)/g seeds. A single technical replica was performed for each independent extract.

2.7 SC-CO_2 extraction

SC-CO_2 extraction was carried out using a laboratory apparatus (Spe-ed SFE system, Applied Separations, Allentown, PA, USA), fitted with a 25 mL stainless-steel extraction vessel (ø = 1 cm; h = 25 cm). For each extraction (three for sample), 25 g of the fed material (seeds) were packed into the vessel and extracted dynamically until oil spillage has been observed (typically 2h for both pomegranate and tomato seeds and 1 h for grape seeds). The carbon dioxide flow rate was kept constant at 4 mL/min. The other operative parameters were 35 MPa and 60 °C for pressure and temperature, respectively. The extraction conditions were chosen on the basis of existing literature data (Roy et al., 1996; Beveridge et al., 2005; Vági et al., 2007; Liu et al., 2012). The extraction yield was expressed as the ratio between the weight of extracted oleoresin and the weight of seeds loaded in the extractor vessel. The extracted oleoresins were stored, under enriched CO_2 atmosphere and protected from light, at -20 °C until further analyses.

2.8 Determination of tocochromanols and carotenoids in the oleoresins

Aliquots (0.1 g) of each oleoresin were dissolved in 1 mL of ethyl acetate, filtered through a 0.45 µm syringe filter (Millipore Corporation, Billerica, MA, USA) and immediately analyzed by HPLC (Puspitasari-Nienaber et al., 2002). Quali-quantitative analyses of tocochromanols and carotenoids were carried out as described in section 2.5.

2.9 Antioxidant activity of oleoresins

Aliquots of each oleoresin were assayed by the TEAC and ORAC (oxygen radical absorbance capacity) methods to determine the TAA.

TEAC was performed according to Re et al. (1999) with some modifications as reported in Laus et al. (2017). The aqueous solution of ABTS radical cation was diluted with ethanol. Measurements were carried out in a final volume of 1.1 mL; four different amounts (ranging from 20 to 100 µL) of a proper dilution (1:50 or 1:100) in ethanol of oleoresins were analysed in duplicate. The (%) decrease of absorbance measured at 734 nm after 4 min incubation of sample was calculated with respect to the uninhibited radical cation solution.

ORAC measurements were performed as described by Ou et al. (2001), properly modified as in Soccio et al. (2016) using a CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany). Fluorescence intensity decay due to 3',6'-dihydroxyspiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one (fluorescein) oxidation by peroxyl radicals generated by 2,2'-azobis(2-amidinopropane) (AAPH) thermal decomposition was monitored at 37 °C at excitation and emission wavelengths of 483 nm (bandwidth 14 nm) and 530 nm (bandwidth 30 nm), respectively. Four different amounts (ranging from 20 to 50 µL) of a proper dilution (1:5000) in ethanol of oleoresins were analysed in triplicate. In the assay mixture (final volume of each well: 0.2 mL) a fixed concentration of ethanol was maintained. The difference was calculated between the area under the fluorescence decay kinetic curve (area under curve, AUC) of sample and the AUC of the blank.

For both methods, antioxidant activity was calculated using a dose-response curve obtained by using trolox and expressed as µmol trolox equivalents (TE)/g oleoresin. A single technical replica was performed for each extract.

2.10 Sample preparation for fatty acid analysis

Fatty acid derivatization was carried out according to the Deutsche Einheitsmethoden zur Untersuchung von Fetten (DGF) C-VI 11a method (Lange, 2000). A metanolic solution (3 mL) of 0.5 M NaOH was added to 0.1 g of each oleoresin. The mixture was incubated at 100°C for 5 min in a water bath to dissolve lipids. After cooling at room temperature, 2.0 mL of boron trifluoride-methanol solution (14% w/v) were added and the sample incubated at 100°C for 30 min in a water bath and then rapidly cooled in an ice bath before the addition of 1 mL of hexane for extraction. The sample was vigorously stirred for 30 s before the addition of 1 mL of a 0.6% (w/v) sodium chloride solution. The esterified sample was placed in a refrigerator for better phase separation. After collecting the supernatant 1.0 mL of hexane was added and sample stirred. The supernatant was collected and added to the previous fraction. The sample was concentrated to a final volume of 1.0 mL for GC-MS analysis.

2.10.1 Fatty acid analysis by GC–MS

The analyses were performed on the Agilent 5977A Series GC/MSD System (Stevens Creek Blvd. Santa Clara, CA 95051, USA) as described by Durante et al. (2016). Compounds were separated on DB-WAX column (60 m, 0.25 mm i.d., 0.25 mm film thickness, Agilent).The GC parameters were as follows: the temperature of the column was 50 °C after injection for 1 minute, then programmed at 25 °C/min to 200 °C, at 3 °C/min to 230 °C and maintained at constant temperature of 230 °C for 23 min. Split injection was conducted with a split ratio of 5:1, the flow-rate was 1.0 mL/min, carrier gas used was 99.999% pure helium, the injector temperature was 250 °C and the column head pressure was 40 psi for 0.4 min, constant pressure at 20 psi. The MS detection conditions were as

follows: transfer line temperature 250°C, mode Scan, source and quadrupole temperature 230°C and 150°C respectively, scanning method of acquisition, ranging from 46 to 500, for mass/charge (m/z) was optimized. Spectrum data were collected at 0.5 s intervals. Solvent cut time was set at 2 min and 40 min retention time sufficient for separating all the fatty acids. Compounds were identified by using online NIST-library spectra and published MS data. Moreover, fatty acids standard were used to confirm MS data. A single technical replica was performed for each independent extract.

2.11 Statistical analysis

Results are presented as the mean value ± standard deviation of three independent replicate experiments (n = 3). Statistical analysis was based on a one-way ANOVA test. Tukey's post hoc method was applied to establish significant differences between means ($p<0.05$). Correlations were calculated using Pearson's correlation coefficient (*r*). All statistical comparisons were performed using SigmaStat version 11.0 software (Systat Software Inc., Chicago, IL, USA).

3. Results and discussion

Pomegranate, tomato and grape seeds, recovered from the pomace remaining after mechanical pressing of the fresh fruits to produce juices, were assayed for the composition of the main antioxidants and antioxidant activity. The measured amounts of phenols (soluble, insoluble-bound and total), total flavonoids and condensed tannins (oligomeric proanthocyanidins), as well as the profiles of tocochromanols (tocopherols and tocotrienols) and carotenoids of seeds are reported in Table 1, together with their HAA, LAA and TAA values evaluated by the TEAC assay.

In plant cells, phenolic compounds exist in both soluble and insoluble-bound forms. Soluble phenols, mainly compartmentalized within the vacuoles, are solvent extractable, while most insoluble-bound phenols are covalently conjugated to the plant matrix mainly by ester bonds with polymeric constituents of the cell walls, including structural polysaccharides and proteins, and are,

for this reason, insoluble in water or aqueous/organic solvents mixtures (Shahidi and Yeo, 2016). In all assayed samples, soluble phenols largely exceeded the insoluble-bound forms contributing for 64.5, 90.9 and 68.7% to the total phenol content of pomegranate, tomato and grape seeds, respectively. Grape seeds showed the highest amount of soluble, insoluble-bound and, obviously, total phenols (22.3, 11.6 and 33.9 mg GAE/g, respectively) followed, with a discrepancy of at least an order of magnitude, by pomegranate (2.0, 1.1 and 3.1 mg GAE/g) and tomato (1.0, 0.1 and 1.1 mg GAE/g). As far as total phenols are concerned, the obtained amounts are very close, or fall within, the ranges of value indicated in literature for grape (1.4-154.6 mg GAE/g) (Hao et al., 2009; Ky et al., 2014), pomegranate (3.4-11.8 mg GAE/g) (Elfalleh et al., 2012; Ambigaipalan et al., 2016) and tomato (0.11-0.94 mg GAE/g) (Toor and Savage, 2005; Chandra and Ramalingam, 2011; Chandra et al., 2012; Ilahy et al., 2016) seeds, while, to the best of our knowledge, no literature data distinguish between soluble and insoluble-bound phenols in grape and tomato seeds, though values similar to those we established have been reported by Ambigaipalan et al. (2016) in seeds of pomegranate ripe fruits grown in California (2.77 and 0.62 mg GAE/g for soluble and insoluble-bound phenols, respectively).

Grape seeds also showed the highest amount of total flavonoids (15.6 mg CE/g) and condensed tannins (14.0 mg CE/g), followed by tomato and pomegranate seeds which revealed a similar amount of total flavonoids (~4.6 mg CE/g) but significantly differed for condensed tannins (3.4 and 5.6 mg CE/g, respectively). Samavardhana et al. (2015) reported comparable concentrations of total flavonoids (12.7-18.0 mg CE/g) from grape seeds (Black queen cultivar, grown in Thailand) obtained from a red vinification process after 4 weeks of maceration. While higher amounts (107.1-111.8 mg CE/g) were reported for seeds of the same cultivar from juice production, indicating that agri-food processing strongly affects the biochemical composition of the resulting by-products and, hence, their potential usage.

With respect to lipophilic antioxidants, tomato seeds showed the highest concentration of total tocochromanols (159.6 µg/g), followed by pomegranate (73.6 µg/g) and grape (27.1 µg/g).

Extremely low amounts (~0.5 µg/g) of total carotenoids were, instead, detected only in grape and tomato seeds. Accordingly, concentrations of carotenoids (mainly lutein and zeaxanthin xanthophylls) in µg-range per 100 g seeds were reported in the seeds of two varieties (Waltingers Cocktail and Red Currant) of tomato grown in Germany by Westphal et al. (2014). Differences in the qualitative profiles of tocochromanols and carotenoids were also evidenced between seed types (Table 1). In pomegranate and tomato γ-tocopherol was the predominant form, contributing to approx. 63 and 91% of total tocochromanols, respectively. In grape seeds, α-tocopherol was the only form of tocopherol detected, comprising up to 47% of tocochromanols. The β- and δ-forms of tocopherols and tocotrienols have not been detected in all assayed seeds.

In all assayed seeds, HAA was considerably higher than LAA (Table 1). Grape showed the highest level of HAA (160.2 µmol TE/g) and LAA (18 µmol TE/g), followed by pomegranate (14.6 and 5.2 µmol T.E./g, respectively) and tomato (8.8 and 1.0 µmol T.E./g). A significant positive correlation between TAA or HAA and soluble phenols [r=1.000 (p= 0.007), r=1.000 (p= 0.005), respectively], insoluble-bound phenols [r=1.000 (p=0.016), r=0.999 (p=0.029), respectively] and total flavonoids [r=0.999 (p= 0.043); r=0.999 (p=0.032), respectively] was found (Table 2), suggesting that these compounds strongly contribute to the antioxidant activity of seeds. These findings agree with previous reports of the positive correlation between antioxidant activity and the total content of polyphenols in grape and pomegranate seed extracts (Bakkalbasi et al., 2005; Guendez et al., 2005; Anahita et al., 2015). No significant correlation was, instead, evidenced between LAA and total carotenoids or tocochromanols; this could be due to the different solvents used in the extraction methods and suggests that other lipophilic compounds may contribute mostly to LAA. Unexpectedly, significant positive correlation [r=0.999 (p=0.026)] was found between LAA and condensed tannins.

Pomegranate, tomato and grape seeds were subjected to extraction with SC-CO_2 in equivalent operative conditions (350 MPa, 65 °C, 4 mL/min CO_2 flow) and the obtained oleoresins were

evaluated for extraction yield, composition of tocochromanols and TAA (Table 3). In this case TAA was assayed by both TEAC and ORAC methods.

The highest oleoresin yield was obtained from tomato seeds (7.8 g oleoresin/100 g), it was 2.2- and 2.5-fold higher than yields obtained from grape (3.5 g oleoresin/100 g) and pomegranate (3.1 g oleoresin/100 g) seeds, respectively, which did not exhibit statistically significant differences ($p<0.001$) from each other. Fruit seeds are a potential source of edible oils (Górnaś et al., 2015) so that pomegranate, tomato and grape seeds have already been effectively extracted by SC-CO_2 with yields largely varying in dependence of genotypes and operative conditions. Comparing the oil yields obtained from seed of several grape varieties by different extraction methods, Freitas (2007) reported a range between 0.56% and 7.9% with SC-CO_2. With respect to tomato seeds Sabio et al. (2003) reported a maximum oil yield of 17.3% at 80°C and 55.2 MPa. A 10% oil yield was reported for SC-CO_2 extracted pomegranate seeds by Liu et al. (2012) in operative conditions similar to those we used in the present study, although the amount of oil present in the seeds of pomegranate fruits grown all over the globe was as low as 6% up to 24% by weight depending on genotype and several other environmental, agronomical and technological factors (Aruna et al., 2016).

Tocochromanols are important antioxidants of oleoresins, whose oxidative stability is mostly based on these compounds (Kamal-Eldin and Appelqvist, 1996). Pomegranate oleoresin was characterized by the highest amount of total tocochromanols (2008 µg/g), followed by tomato (1769 µg/g) and grape (636 µg/g) (Table 3), confirming that grape oleoresin can be ascribed to the group of oils with low tocochromanol concentration according to Górnas et al. (2015). The quali-quantitative characterization of tocochromanols evidenced considerable differences between oleoresins. In pomegranate and grape oleoresins, tocopherol and tocotrienol forms were both detected, while tomato seed oleoresin was characterized by the presence of only tocopherols. According to Gliszczynska-Swiglo and Sikorska (2004), neither β- nor δ- and γ-tocopherol forms were detected in grape seed oil. γ-Tocopherol was the dominant form detected in pomegranate and tomato seed oleoresins (1270 and 1621 µg/g, respectively), while α-tocopherol, the most biologically active

form of vitamin E, was present only in tomato and grape oleoresins (148 and 290 μg/g oleoresin, respectively). Among tocotrienols, in pomegranate, we observed the presence only of the α form, although the presence of β-tocotrienol in a range between 12.5-52.1 μg/g of oil was reported by Verardo et al. (2014) in the oil extracted with organic solvents (chloroform/methanol solution, 1/1 v/v) from the seeds of 17 cultivars of pomegranate from different regions. In grape seeds, the γ-tocotrienol form (181 μg/g oleoresin) was more abundant than α-tocotrienol (155 μg/g oleoresin), in agreement to previous reports (Beveridge et al., 2005).

No carotenoids were detected in all SC-CO_2 extracted oleoresins (data not shown) indicating that extraction method can significantly affect oil composition.

The highest TAA was obtained in the oleoresin extracted from pomegranate seeds with both TEAC and ORAC assays (19.1 and 828 μmol TE/g, respectively) which resulted much higher than those of tomato (4.5 and 579 μmol TE/g, respectively) and grape (3.4 and 158 μmol TE/g, respectively) oleoresins (Table 3), thus highlighting the ability of pomegranate oleoresin to quench radical cations and scavenging peroxy-radicals.

The fatty acid composition revealed significant qualitative and quantitative differences between the assayed oleoresins (Table 4). In general, palmitic, stearic, oleic and linoleic acids were detected in all samples. Pomegranate, grape and tomato oleoresins were characterized by the highest content of polyunsaturated fatty acids (PUFA, 85.9%), monounsaturated fatty acids (MUFA, 29.6%) and saturated fatty acids (SFA, 27.9%), respectively. Linoleic acid was the most abundant fatty acid in tomato (44.8%) and grape (46.5%) seed oleoresins, followed by oleic (23.1–28.9%) and palmitic (18.8–14.6%) acids, in agreement with previous reports (Lazos et al., 1998, Lutterodt et al., 2011). The lowest MUFA/PUFA and SFA/UFA ratios were found in pomegranate seed oleoresin suggesting it may potentially contribute more significantly to health promotion than the other oleoresins as proposed by Siriwardhana et al. (2012). The use of pomegranate seeds in the food industry has received an unexpected attention due to the presence of a large amount of lipophilic

molecules with high biological activity including tocochromanols and peculiar fatty acids (Melo et al., 2014).

In terms of individual fatty acids, pomegranate seed oleoresin presents high percentages of punicic acid (~70%). Our results were similar to those reported by other authors indicating punicic acid as the most abundant fatty acid in pomegranate seed oil (Elbandy and Ashoush, 2012; Jing et al., 2012; Liu et al., 2012; Fernandes et al., 2015). Richard et al. (2008) reported that unsaturated fatty acids can act as antioxidants depending on their degree of unsaturation; indeed we found significant positive correlation ($p<0.05$) between TAA assayed by the TEAC method and PUFA (r=0.999) (Table 5).

In summary, this study confirms that seeds produced by the food industry are valuable by-products useful as source of hydrophilic and lipophilic antioxidants, as well as edible oils rich in specific bio-molecules with potential beneficial physiological activities. The results revealed a considerable diversity in the content of phenols, flavonoids, condensed tannins, tocochromanols and carotenoids as well as the antioxidant activities of the examined samples. Grape seeds represent an important source of phenols, mainly monomeric compounds such as catechins, epicatechin, epicatechin-3-O-gallate, gallic acid, quercetin, rutin, caffeic acid, resveratrol and oligomeric procyanidins (Kallithraka et al., 1995; Nawaz et al., 2006; Hao et al., 2009; Butkhup et al., 2010; Li et al., 2011; Farhadi et al., 2016), while tomato and pomegranate seeds are valuable sources of tocochromanols and punicic acid, respectively. In the tested operative conditions, SC-CO_2 extraction of tomato seeds gave the highest oleoresin yield, followed by grape and pomegranate. Pomegranate oleoresin contains significant amounts of γ-tocopherol and α-tocotrienol; tomato oleoresin is, instead, characterized by the exclusive presence of tocopherols (γ- and α-forms). Pomegranate oleoresin is also an interesting source of the ω-5 trienoic punic acid. Furthermore, the differences observed in the chemical composition of the oleoresins, especially in SFA, MUFA and PUFA content, suggest that carefully designed seed blends can result in nutritionally balanced oils with optimal fatty acid composition and increased levels of tocochromanols, useful as high quality ingredients in the

formulation of novel foods and/or food supplements to satisfy specific dietary/health customer needs.

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Table 1. Biochemical composition of pomegranate, tomato and grape seeds. Values represent the mean ± standard deviation of three independent replicates (n = 3). Data were submitted to one-way analysis of variance (ANOVA), differences among groups were detected using multiple comparison procedures (Tukey post hoc test, $P < 0.05$), different letters denote significant differences among samples.

	Seeds		
	Pomegranate	**Tomato**	**Grape**
Phenols (mg GAE/g)			
Soluble phenols	2.0±0.2^{b}	1.0±0.1^{c}	22.3±0.9^{a}
Insoluble-bound phenols	1.1±0.1^{b}	0.1±0.0^{c}	11.6±0.1^{a}
Total	**3.1±0.3^{b}**	**1.1±0.1^{c}**	**33.9±1.1^{a}**
Total flavonoids (mg CE/g)	**4.5±0.1^{b}**	**4.7±0.1^{b}**	**15.6±0.3^{a}**
Condensed tannins (mg CE/g)	**5.6±0.1^{b}**	**3.4±0.1^{c}**	**14.0±0.6^{a}**
Tocochromanols (µg/g)			
α-tocopherol	Nd	14.3±0.5^{a}	12.8±0.7^{b}
β-tocopherol	Nd	Nd	nd
δ-tocopherol	Nd	Nd	nd
γ-tocopherol	46.3±0.4^{b}	145.3±5.8^{a}	nd
α-tocotrienol	27.3±1.5^{a}	Nd	6.6±0.5^{b}
β-tocotrienol	Nd	Nd	Nd
δ-tocotrienol	Nd	Nd	Nd
γ-tocotrienol	Nd	Nd	7.7±0.6
Total	**73.6±1.9^{b}**	**159.6±6.3^{a}**	**27.1±1.8^{c}**
Carotenoids (µg/g)			
Lutein	Nd	0.14±0.01^{b}	0.21±0.02^{a}
α-Carotene	Nd	0.03±0.01^{b}	0.09±0.01^{a}
β-Carotene	Nd	0.20±0.02^{a}	0.15±0.01^{b}
Zeaxhantin	Nd	0.11±0.01^{a}	0.03±0.01^{b}
β-cryptoxanthin	Nd	0.03±0.01^{b}	0.11±0.03^{a}
Lycopene	Nd	Nd	Nd
Total	**-**	**0.51±0.06^{a}**	**0.59±0.08^{a}**
HAA (µmol TE/g)	**14.6±1.4^{b}**	**8.8±0.1^{c}**	**160.2±1.3^{a}**
LAA (µmol TE/g)	**5.2±0.6^{b}**	**1.0±0.1^{c}**	**18.0±1.4^{a}**
TAA (µmol TE/g)	**19.8±2.0^{b}**	**9.8±0.2^{c}**	**178.2±2.7^{a}**

GAE: Gallic acid equivalents; CE: catechin equivalents; TE: Trolox equivalents; HAA: hydrophilic antioxidant activity; LAA: lipophilic antioxidant activity; TAA: total antioxidant activity; nd: not detected. LOD of tocochromanols and carotenoids= 0.010 µg/g.

Table 2. Pearson's correlation coefficient (r) and related significance between the main bioactive molecule classes and antioxidant activities of seeds. n (sample size) = 27

	SP	**IBP**	**TF**	**TCT**	**TTc**	**TCr**	**HAA**	**LAA**	**TAA**
SP	*								
IBP	0.999^{*}	*							
TF	0.998^{*}	0.996^{ns}	*						
TCT	0.988^{ns}	0.993^{ns}	0.977^{ns}	*					
TTc	-0.795^{ns}	-0.816^{ns}	-0.758^{ns}	-0.879^{ns}	*				
TCr	0.571^{ns}	0.540^{ns}	0.617^{ns}	0.436^{ns}	0.045^{ns}	*			
HAA	1.000^{**}	0.999^{*}	0.999*	0.987^{ns}	-0.790^{ns}	0.577^{ns}	*		
LAA	0.980^{ns}	0.987^{ns}	0.968^{ns}	0.999^{*}	-0.898^{ns}	0.398^{ns}	0.979^{ns}	*	
TAA	1.000^{**}	1.000^{*}	0.999^{*}	0.990^{ns}	-0.801^{ns}	0.561^{ns}	1.000^{*}	0.983^{ns}	*

^{ns}Not significant ($P \geq 0.05$); $^{*}P<0.05$; $^{**}P<0.01$. SP: soluble phenols; IBP: insoluble-bound phenols; TF: total flavonoids; TCT: total condensed tannins; TTc: total tocochromanols; TCr: total carotenoids; HAA: hydrophilic antioxidant activity; LAA: lipophilic antioxidant activity; TAA: total antioxidant activity.

Table 3. Extraction yield, total antioxidant activity (TAA) and tocochromanols composition of oleoresins obtained from pomegranate, tomato and grape seeds by supercritical CO_2. Values in table represent the mean ± standard deviation of three independent replicates (n = 3). Data were submitted to one-way analysis of variance (ANOVA), differences among groups were detected using multiple comparison procedures (Tukey post hoc test, $P < 0.05$), different letters denote significant differences among samples.

	Supercritical CO_2 **extracted oleoresins**		
	Pomegranate	**Tomato**	**Grape**
Extraction yield (g oleoresin/100 g seeds)	**3.1±0.1^{b}**	**7.8±0.3^{a}**	**3.5±0.2^{b}**
Tocochromanols (µg/g oleoresin)			
α-tocopherol	nd	148±1^{b}	290±1^{a}
β-tocopherol	nd	nd	nd
δ-tocopherol	nd	nd	nd
γ-tocopherol	1270±11^{b}	1621±75^{a}	nd
α-tocotrienol	739±3^{a}	nd	155±2^{b}
β-tocotrienol	nd	nd	nd
δ-tocotrienol	nd	nd	nd
γ-tocotrienol	nd	nd	181±3
Total	**2008±11^{a}**	**1769±8^{b}**	**636±5^{c}**
TAA			
TEAC (µmol TE/g oleoresin)	19.1±1.3^{a}	4.5±0.4^{b}	3.4±0.1^{b}
ORAC (µmol TE/g oleoresin)	828±18^{a}	579±20^{b}	158±25^{c}

TEAC: Trolox equivalent antioxidant capacity; ORAC: Oxygen radical absorbance capacity; TE: Trolox equivalents; nd: not detected. LOD of tocochromanols = 0.010 µg/g.

Table 4. Fatty acid composition of oleoresins obtained from pomegranate, tomato and grape seeds by supercritical CO_2. Data, expressed on a percentage basis, are the mean ± standard deviation of three independent replicates (n = 3).

	Supercritical CO_2 **extracted oleoresins**		
	Pomegranate	**Tomato**	**Grape**
	% of total fatty acids		
Myristic acid (C14:0)	nd	0.59±0.01	0.29±0.02
Palmitic acid (C16:0)	3.6±0.8	18.8±0.4	14.6±0.2
Palmitoleic acid (C16:1)	nd	0.5±0.1	0.7±0.3
Margaric acid (C17:0)	Nd	0.6±0.1	nd
Stearic acid (C18:0)	2.4±0.3	7.4±0.2	7.6±0.4
Oleic acid (C18:1 n-9)	8.1±0.2	23.1±0.5	28.9±0.2
Linoleic acid (C18:2 n-6)	6.4±0.8	44.8±1.4	46.5±0.4
Linolenic acid (C18:3 n-3)	nd	3.7±0.7	0.88±0.03
Punicic acid (C18:3 n-5)	69.7±0.1	nd	nd
Arachidic acid (C20:0)	nd	0.51±0.01	0.53±0.01
Others C18:3 n-3 isomers	9.8±0.2	Nd	Nd
SFA	6.0±1.1	27.9±0.7	23.0±0.6
MUFA	8.1±0.2	23.6±0.6	29.6±0.5
PUFA	85.9±1.1	48.5±2.1	47.4±0.4
MUFA/PUFA	0.09	0.49	0.62
SFA/UFA	0.06	0.39	0.29

SFA: Saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; UFA: unsaturated fatty acids; nd: not detected. LOD of fatty acids = 0.01%.

Table 5. Pearson's correlation coefficient (r) and related significance between the main bioactive molecule classes and antioxidant activities of oleoresins obtained from pomegranate, tomato and grape seeds by supercritical CO_2. n (sample size) = 18.

	TAA_{TEAC}	**TAA_{ORAC}**	**TTc**	**SFA**	**MUFA**	**PUFA**
TAA_{TEAC}	*					
TAA_{ORAC}	0.821^{ns}	*				
TTc	0.682^{ns}	0.977^{ns}	*			
SFA	-0.962^{ns}	-0.633^{ns}	-0.455^{ns}	*		
MUFA	-0.978^{ns}	-0.922^{ns}	-0.820^{ns}	0.883^{ns}	*	
PUFA	0.999^{*}	0.799^{ns}	0.654^{ns}	-0.971^{ns}	-0.969^{ns}	*

^{ns}Not significant ($p \geq 0.05$); $^{*}P<0.05$. TEAC: Trolox equivalent antioxidant capacity; ORAC: Oxygen radical absorbance capacity; TAA: total antioxidant activity; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.