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In vitro gastrointestinal digestion of pomegranate peel (*Punica granatum*) flour obtained from co-products: Changes in the antioxidant potential and bioactive compounds stability

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ABSTRACT

The effect of *in vitro* gastrointestinal digestion (GID) on the recovery, bioaccessibility and stability of polyphenolic compounds, the changes in antioxidant activity and the short chain fatty acids (SCFAs) production of pomegranate peel flour (PPF) were evaluated. The extracts obtained in each step of GID were used to determine the stability of polyphenolic profile using HPLC whilst the antioxidant properties were determined using five methodologies. The SCFAs production from PPF fermentation was also determined. At the end of GID process, the bioaccessibility of phenolic and flavonoid compounds was 35.90 and 64.02%, respectively. The polyphenolic compounds decreased after GID except that for ellagic acid which increased. GID increased the chelating activity and reducing power. However, the scavenging properties were reduced. Fermentation of PPF by colonic bacteria generated acetic, propionic and butyric acids. PPF could be used in the food industry as a potential ingredient to develop functional foods that promote health benefits.

1. Introduction

Pomegranate (*Punica granatum*) is an important fruit due to its many functional components. This fruit is, mainly, consumed in fresh; the arils contain proteins, crude fibres, vitamins, minerals, pectin, sugars and polyphenols (Viuda-Martos, Fernández-López, & Pérez-Alvarez, 2010). The arils are also used to make popular pomegranate products such as fresh juice. Once the juice has been extracted, the co-products that remain are composed of two fractions: bagasses and peel. Uses for these co-products are limited. However, due to their composition, these co-products have the potential to be used for other ends for example to obtain bioactive compounds or antioxidant dietary fibre (Viuda-Martos et al., 2011).

In the last years, special attention has been made to pomegranate non-edible parts. Nevertheless, pomegranate peel is an important source of bioactive compounds such as phenolic

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acids, flavonoids, proanthocyanidins and ellagitannins as well as ellagic acid and ellagic acid glycosides (Çam, İçyer, & Erdoğan, 2014; Devatkal, Narsaiah, & Borah, 2010) or antioxidant dietary fibre which is defined as dietary fibre rich in associated polyphenolic compounds. This product combines in a single material the physiological effects of both dietary fibre and antioxidants (Saura-Calixto, 1998). Evidence from epidemiological studies suggests that diets rich in antioxidant dietary fibre are protective against several degenerative diseases such as cancer, cardiovascular diseases, diabetes and metabolic syndrome, among others (Grooms, Ommerborn, Pham, Djoussé, & Clark, 2013; Kaczmarczyk, Miller, & Freund, 2012). In addition, antioxidant dietary fibre has shown techno-functional properties that play an important role in the digestion process and is especially beneficial from a physiological point of view (Hasnaoui, Wathelet, & Jimenez-Araujo, 2014). In this way, studies about the bioaccessibility of bioactive compounds present in foods are necessary, because a link between the differences in bioactive compounds digestibility, bioaccessibility and the food matrix composition has been suggested. The bioaccessibility can be defined as the amount of compound solubilised in the small intestine and available for subsequent absorption. This process comprises the release of compounds from food matrices and their stability under the gastrointestinal condition (Gawlik-Dziki, 2012; Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Definitive studies regarding the bioaccessibility of bioactive compounds require *in vivo* experiments with humans. However, *in vitro* methods have also proven to be useful in determining their stability under gastrointestinal conditions (Toydemir et al., 2013). The *in vitro* methods are applied to a system of simulated gastrointestinal digestion using α-amylase in mouth phase, pepsin in the gastric phase and a mixture of pancreatin and bile salts during the intestinal tract. The element diffused through a semi-permeable membrane in the intestinal phase is used as measure of the compounds bioaccessibility (Kulkarni, Acharya, Rajurkar, & Reddy, 2007). Indeed, despite limitations that constitute a static model of digestion, the evaluation of bioaccessibility by *in vitro* models can be well correlated with results from human studies and animal models (Bouayed, Hoffman, & Bohn, 2011).

To the best of our knowledge, there are no studies, in the scientific literature, regarding the stability of phenolic and flavonoid compounds and the antioxidant activity changes during *in vitro* gastrointestinal digestion of pomegranate peel flour (PPF). Thus, the effect of *in vitro* gastrointestinal digestion on (i) the recovery and bioaccessibility indexes, (ii) the stability of phenolic and flavonoid compounds, (iii) the changes in antioxidant activity and (iv) the short chain fatty acids (SCFAs) production of pomegranate peel flour (PPF) obtained from co-products of juice industry were evaluated.

2. Material and methods

2.1. *Pomegranate peel flour (PPF)*

Pomegranate (*cv*. "Mollar de Elche") peels, obtained as a co-product during pomegranate juice extraction, were supplied by a juice industry located in Murcia (Spain). The material (peel) was triturated for 40 s in a vertical cutter (Tecator 1094 Homogeneizer, Tekator, Hoganas, Sweden) to obtain uniformly sized pieces and thus increase the contact time during washing (1 L of water per kg of product). The mixture was stirred constantly and the water temperature was kept at 40° C during the 10 min washing process. The whole co-product was pressed to drain the liquid and then it was lyophilised in a Christ Alpha 2-4 lyophiliser (B. Braun Biotech, Melsungen, Germany) for 48 hours to improve the product shelf life without the addition of any chemical preservative. A grinder mill and sieves were used to obtain pomegranate peel flour (PPF) with a particle size of less than 0.417 mm. Fig. 1 showed the flowchart of the process to obtain pomegranate peel flour. PPF had total dietary fibre (TDF) content of 51.80 g/100 g sample dry weight (dw). In addition, PPF was characterised by higher amount of insoluble dietary fibre (IDF) (27.02 g/100 g dw) than soluble dietary fibre (SDF) (24.78 g/100 g dw) which indicates relevant content of celluloses, lignin and hemicelluloses in the sample (Bailina, 2014).

2.2. *Simulated in vitro gastrointestinal digestion*

In vitro gastrointestinal digestion of samples was performed according to the method described by Mills et al. (2008) with some modifications (Fig. 2). Digestion was performed by enzymes and absorption was simulated by using dialysis tubing. The method includes three different stages of digestion: Mouth, gastric and small intestine conditions. Mastication was simulated using a saliva solution prepared with 100 U/mL α-amylase (Sigma, Germany) and diluted in 1 mM $CaCl_2$, whereas 1 M $NaHCO_3$ was used to adjust pH to 6.9. The simulated saliva (1 mL) was added to 2.4 g of sample and 9 mL of water and then the mixture was stomached for 2 min. The sample solution was transferred to a glass screw topped bottles and incubated at 37 °C for 5 min. In gastric conditions, the sample was adjusted to pH 2 with HCl (6 M) and then 1 mL pepsin (Sigma) (0.108 g) dissolved in HCl (0.1 M; 10 mL) was added and incubated for 2 h in a shaking water bath at 37 °C and 50 rpm. In Small intestine conditions, the pH was increased to 7 with NaOH (6 M) and 2.5 mL pancreatin (Sigma) (80 mg) dissolved in $NaHCO_3$ (0.5 M; 10 mL) and 2.5 mL bile salt mixture (Sigma) (500 mg) dissolved in $NaHCO_3$ (0.5 M; 10 mL) were added, and the incubation was continued for another 2 h in a shaking water bath at 37 °C and 50 rpm. After this time, the sample solution was transferred to 3 kDa molecular weight cut-off dialysis tubing and dialysed overnight against NaCl (10 mM) at 37 °C to remove low molecular mass digestion products. Mechanical agitation was used to parallel peristaltic movements. At the end of the mouth, gastric and intestinal steps, the digestion mixtures were centrifuged for 12 min at 8000 *g* at 4 °C, yielding the chyme soluble fraction (CSF) and the pellet fraction (PF). Both fractions were lyophilised and stored for chromatographic analysis of the phenol composition and antioxidant activity. All enzymes solutions were prepared fresh and filter-sterilised using a 0.22 μm-membrane filter previously used. All solutions were maintained in an ice bath during the entire period of gastrointestinal digestion processes prior to gradual addition (when appropriate).

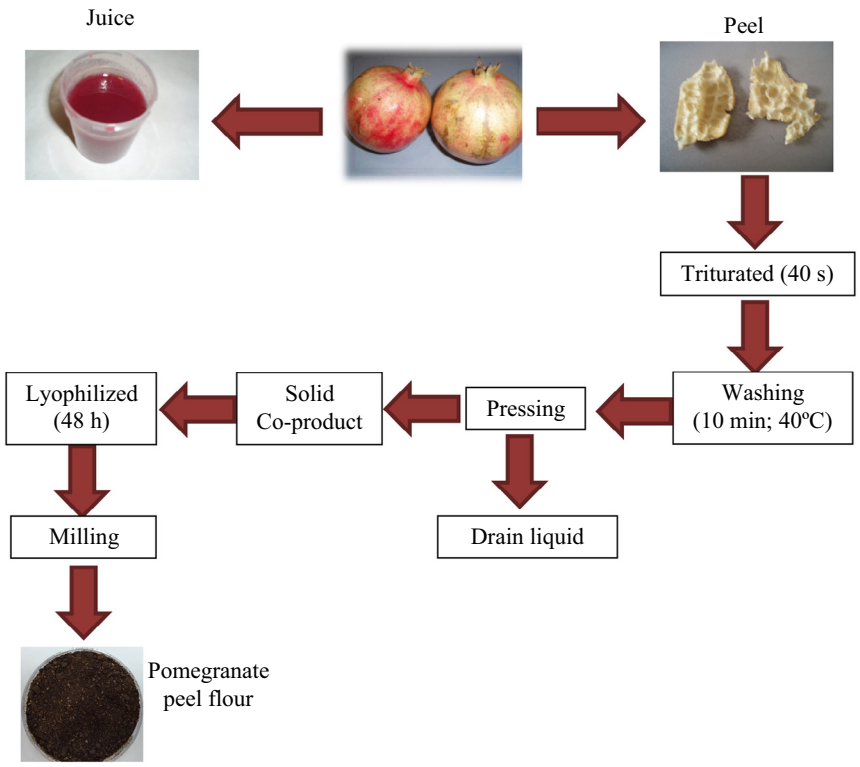


Fig. 1 – Flowchart of the process to obtain pomegranate peel flour from pomegranate peels co-product.

2.3. *Recovery index and bioaccessibility index*

To evaluate the effect of the matrix composition on the digestion of the phenolic group (phenolic acids and flavonoids) two different indexes were studied following the indications of Ortega, Macia, Romero, Reguant, and Motilva (2011): The percentage of recovery and the percentage of bioaccessibility. The percentage of recovery allows the amount of phenolic group

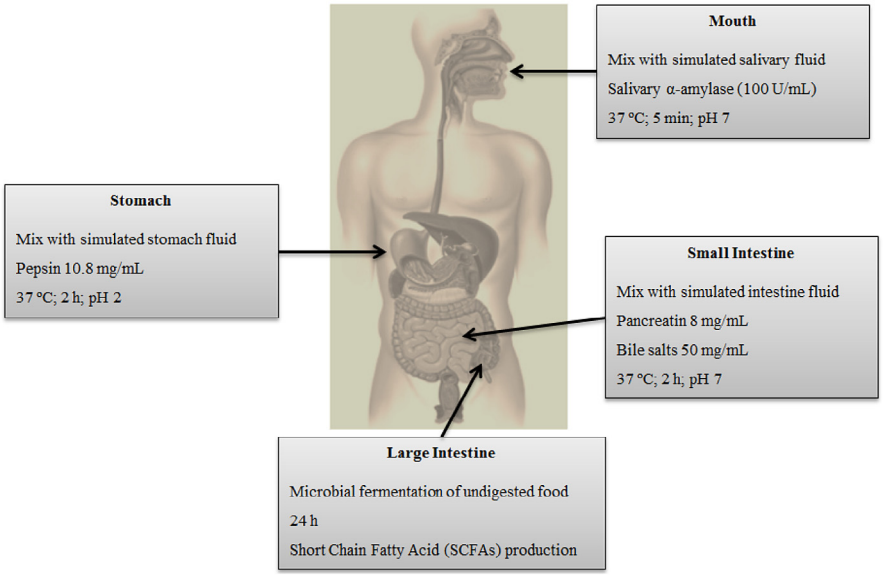


Fig. 2 – Graphic representation of the static *in vitro* gastrointestinal digestion procedure carried out with pomegranate peel flour.

present in the complete digestion (CSF and PF) after mouth, gastric and intestinal digestion of test food to be measured according to:

$$\text{Recovery index (\%)} = (PC_{DF}/PC_{TF}) \times 100$$

Where PC_{DF} is the total phenol content (mg) in the digested (CSF + PF) and PC_{TF} is the total phenol content (mg) quantified in test matrix.

For each phenol group, the bioaccessibility is defined as the percentage of polyphenolic compounds that is solubilised in CSF after intestinal dialysis step. Thus, this index defines the proportion of the polyphenolic compounds that could become available for absorption into the systematic circulation:

$$\text{Bioaccessibility index (\%)} = (PC_S/PC_{DF}) \times 100$$

where: PC_S is the total phenol content (mg) in the CSF after the intestinal dialysis step and PC_{DF} is the total phenol content (mg) in the digested sample (CSF + PF) after the intestinal step.

2.4. Total phenol content

The total phenol content (TPC) of each extract was performed using the Folin–Ciocalteu's reagent (Singleton & Rossi, 1965). Lyophilised samples were dissolved in methanol to obtain a concentration comprise between 20 and 40 mg/mL. Gallic acid (GA) was the reference standard and the results were expressed as mg GA equivalents/g sample. Each assay was carried out in triplicate.

2.5. Total flavonoid content

For the total flavonoid content (TFC), the method based on Blasa et al. (2005) was used. Methanolic solutions of lyophilised samples were used for the analysis. Different concentrations of rutin (8.5–170 μg/mL) were used for calibration. The results were expressed in mg rutin equivalents (RE)/g of sample as mean of three replicates.

2.6. Determination of polyphenolic compounds

Samples (20 μL) were injected into a Hewlett-Packard HPLC series 1100 instrument (Woldbronn, Germany) equipped with a diode array detector. Separations were realised on a C_{18} Teknokroma column (Mediterranea Sea_{18}, 25 × 0.4 cm, 5 μm particle size. Teknokroma, Barcelona, Spain) and the chromatograms were recorded at 280, 320, 360 or 520 nm. Phenolic compounds were analysed, in standard and sample solutions, using a gradient elution at 1 mL/min with the following gradient programme, starting with 95% A, 75% A at 20 min, 50% A at 40 min, 20% A at 50 min and 20% A at 60 min. The mobile phases were composed by formic acid in water (4.5:95.5, v/v) as solvent A and acetonitrile as solvent B. The quantitative analysis of the components was achieved with reference to authentic standards. The standards used were catechin and epicatechin, as well as caffeic, ferulic, sinapic, *p*-coumaric, ellagic, gallic, and chlorogenic acids along with ellagitannin, punicalagin, rutin, quercetin and apigenin (Extrasynthese, Genay, France). Compound identification was carried out by comparing UV absorption spectra and retention times of each compound with those of pure standards injected in the same conditions. The compounds were quantified through calibration curves of standard compounds. Ellagic acid derivatives were tentatively quantified using the calibration curves of ellagic acid. All determinations were made in triplicate.

2.7. Antioxidant activity

2.7.1. Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) of lyophilised samples digested was determined using the potassium ferricyanide-ferric chloride method (Oyaizu, 1986). The FRAP value was estimated in mg Trolox equivalents (TE)/g of sample. Each assay was carried out in triplicate.

2.7.2. Ferrous ion-chelating ability assay

Ferrous ions (Fe^{2+}) chelating activity, of lyophilised samples digested, was measured by inhibiting the formation of Fe^{2+}-ferrozine complex after treatment of test material with Fe^{2+}, following the method of Carter (1971). Results were expressed in mg EDTA equivalents/g of sample as mean of three replicates.

2.7.3. DPPH radical scavenging ability assay

The antioxidant activity of different lyophilised samples digested was measured in terms of radical scavenging ability, using the stable radical DPPH (Brand-Williams, Cuvelier, & Berset, 1995). Results were expressed in mg Trolox equivalents (TE)/g of sample as mean of three replicates.

2.7.4. Oxygen radical absorbance capacity (ORAC assay)

The oxygen radical absorbance capacity (ORAC) of different lyophilised samples was determined at different concentrations by the method of Ou, Hampsch-Woodill, and Prior (2001) using fluorescein as the "fluorescent probe" with some modifications. Briefly, 20 μL of phosphate buffer (75 mM, pH 7), Trolox or lyophilised samples at different concentrations were incubated with 120 μL of fluorescein (20 nM) at 40 °C for 10 min. The reaction was started by thermal decomposition of AAPH (19 mM in 75 mM phosphate buffer, pH 7.0) and performed at 37 °C in 96-well black plates. Fluorescence was measured immediately after the addition of AAPH at excitation of 485 nm and an emission wavelength of 535 nm for 105 min at 40 °C. The automated ORAC assay was carried out on a FLUOstar Optima fluorescence microplate reader (BMG LABTECH GmbH). The ORAC values, expressed as μg trolox equivalents per mg sample, were calculated by applying the following formula.

$$\text{Relative ORAC value} = \left(C_{Trolox} \times (AUC_{Sample} - AUC_{Blank}) \times k\right) / (AUC_{Trolox} - AUC_{Blank})$$

where C_{Trolox} is the concentration of Trolox, k is the sample dilution factor, and AUC is the area under the fluorescence decay curve of the sample, blank, and trolox, respectively.

2.7.5. *ABTS radical cation (ABTS•+) scavenging activity assay*

The $ABTS^{\bullet+}$ scavenging activity assay was determined as described by Leite et al. (2011) with some modifications. The $ABTS^{\bullet+}$ solution was produced by reacting aqueous ABTS solution (7 mM) with potassium persulfate (2.45 mM). Diluted $ABTS^{\bullet+}$ solution with an absorbance of 0.70 ± 0.02 at 734 nm was employed in the analysis. The reactions were performed by adding 990 μL of $ABTS^{\bullet+}$ solution to 10 μL of each extract solution. After 6 min of incubation at room temperature, absorbance values were measured on a spectrophotometer at 734 nm. The results were calculated based on a calibration curve of Trolox, and results were expressed as mg Trolox equivalents (TE)/g of sample.

2.8. *In vitro microbial fermentation*

2.8.1. *Faecal inocula*

The faecal samples were obtained fresh at the premises of the department from three healthy human donors, who were free of any known metabolic and gastrointestinal diseases; not taking probiotic or prebiotic supplements and had not taken any form of antibiotics 3 months prior to faecal sample donation. Faeces were collected into sterile vials, kept in an anaerobic cabinet and used within a maximum of 2 h after collection. Faecal inocula (FI) were prepared by dilution in a reduced physiological salt solution (RPS; cysteine-HCl (Merck, Darmstadt, Germany) 0.5 g/L and NaCl (Panreac, Madrid, Spain) 8.5 g/L) at a 100 g faeces/L RPS and pH 6.8, following the methodology described by Gullón et al. (2014).

2.8.2. *Fermentation media*

The nutrient base medium used in fermentations comprised of 5.0 g/L trypticase soya broth (TSB) without dextrose (BBL, Lockeysville, USA), 5.0 g/L bactopeptone (Amersham, Buckinghamshire, UK), 0.5 g/L cysteine-HCl (Merck, Darmstadt, Germany), 1.0 % (v/v) of salt solution A (100.0 g/L NH_4Cl, 10.0 g/L $MgCl_2{\cdot}6H_2O$, 10.0 g/L $CaCl_2{\cdot}2H_2O$) and trace minerals solution, 0.2% (v/v) of salt solution B (200.0 g/L $K_2HPO_4{\cdot}3H_2O$) and 0.2% (v/v) of 0.5 g/L resazurin solution, prepared in distilled water. The final pH of the medium was adjusted to 6.8. Aliquots were dispensed into airtight anaerobic serum bottles, which were sealed with aluminium caps before sterilisation by autoclave. Stock solution of Yeast Nitrogen Base (YNB) (Sigma-Aldrich Co., St. Louis, USA) was sterilised under syringe filters of 0.22 μm (Chromafils, Macherey-Nagel, Düren, Germany) into sterile airtight serum bottles.

2.9. *In vitro colonic fermentation*

YNB solution and pomegranate lyophilised residue obtained after enzymatic digestion were aseptically added to the anaerobic serum bottles with nutrient base medium before inoculation to achieve a final concentration of 5.0 g of YNB/L and 10.0 g of extract/L. The serum bottles were inoculated with faecal slurry dilution to a final concentration of 2% (v/v) and incubated at 37 °C for 24 h without shaking. All additions and inoculations were carried out inside an anaerobic cabinet (5% H_2, 10% CO_2 and 85% N_2). Samples were collected 24 h of fermentation, cultures were centrifuged at 16,000 *g* for 10 min, and supernatants were collected for analysis of short chain fatty acids (SCFA) using high performance liquid chromatography (HPLC).

2.9.1. *Determination of fermentation products in cell-free supernatants*

Supernatants from the anaerobic culture tubes inoculated with FI were filtered through 0.20 μm cellulose acetate membranes. Aliquots of the filtered samples were assayed for organic acids (acetic, propionic and butyric acids) using an Agilent 1200 series HPLC instrument with a UV-Vis Diode Array Detector (Agilent, Germany). Twenty microlitres of sample were injected in an Aminex HPX-87H column (from BioRad, Hercules, CA) using sulphuric acid (0.003 M) as mobile phase, operating flow rate of 0.6 mL/min. Samples were run at 50 °C. Standards of organic acids (acetic, butyric, propionic, fumaric and succinic acids) were obtained from Sigma (Poole, Dorset, UK). Peaks were identified by comparison with retention time of the standards and were quantified by regression formula obtained with the standards.

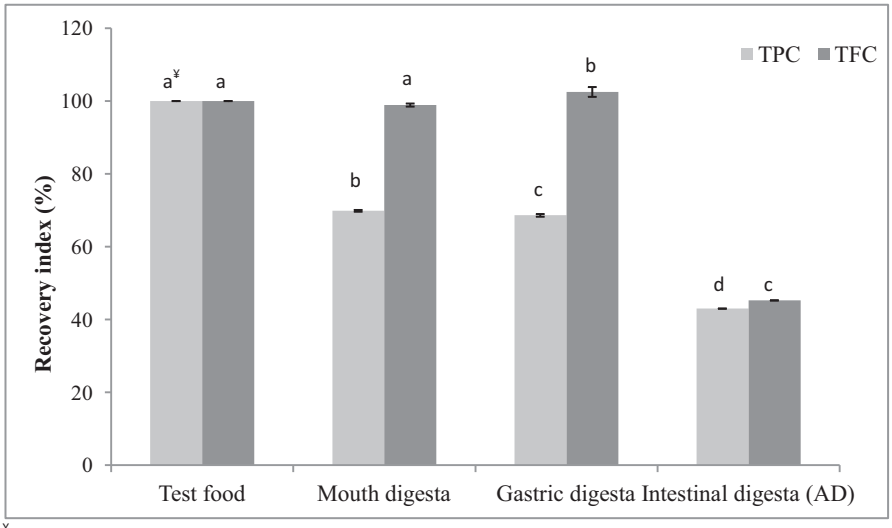
2.10. *Statistical assay*

Statistical analysis and comparisons among means were carried out using the Statistical Package SPSS 19.0 (SPSS Inc., Chicago, IL, USA). All experiments were carried out in triplicate and data were reported as mean ± standard deviation. The differences of mean values among concentration of bioactive compounds or antioxidant activity and that obtained in the different steps of the *in vitro* gastrointestinal digestion were analysed by one-way analysis of variance (ANOVA). The Tukey's post hoc test was applied for comparisons of means; differences were considered significant at $p < 0.05$. Correlation analysis was performed between phenolic compounds (phenolic acids and flavonoids) contents and antioxidant activities of extracts using Pearson correlation analysis.

3. Results and discussion

3.1. *Recovery index and bioaccessibility index*

Fig. 3 showed the total phenolic (TP) and total flavonoid (TF) recovery index after mouth, gastric and intestinal steps of pomegranate peel flour. The values for test matrix were obtained using a methanol extraction and these values were assumed as 100% of TPC or TFC of sample. The mouth step had a deep effect ($p < 0.05$) in TP recovery since the percentage of phenolic compounds recuperated was 69.85%. However, the TF recovery was not affected ($p > 0.05$). Again, gastric digestion decreased the TP recovery ($p < 0.05$) with reference to test food and the mouth step while the TF recovery increased ($p < 0.05$) both test matrix and mouth. The flavonoids released from the test matrix after gastric digestion could be due to the breakdown of these compounds to proteins, fibre or sugar residues. In this way, Saura-Calixto, Serrano, and Goñi (2007) informed that flavonoids linked to high molecular weight compounds,



¥For the same determination (TPC or TFC) bars with same lower case letter are not significantly different (p>0.05) according to Tukey's Multiple Range Test.

Fig. 3 – Recovery index of total phenolic content and total flavonoids content after the simulated gastro-intestinal digestion (Mouth, gastric and intestinal (after dialysis (AD) digestion steps) of pomegranate peel flour (PPF) obtained from juice co-products.

such as proteins and carbohydrates, may be released by digestive enzyme action, leading to a significant increase in their concentrations after gastric digestion. At the end of intestinal step, both TP and TF recovery were profoundly affected ($p < 0.05$) by the recovery index of 43.00 and 45.26% respectively. This observation is in agreement with the work carried out by Ortega et al. (2011) in which TP and TF recovery index of washed carob flour decreased after intestinal step. Li, Deng, Liu, Loewen, and Tsao (2014) also detected that the TP and TF recovery index decreased after intestinal digestion of purple tomato. Argyri, Komaitis, and Kapsokefalou (2006) observed that solubility and availability of phenolic compounds (phenolic acids and flavonoids) are influenced by pH conditions and interaction with dietary constituents, such as iron, fibre or proteins.

To exert bioactivity, phenolic acids and flavonoids first must be bioaccessible, i.e. released from the food matrix and solubilised as mentioned in Bouayed et al. (2011). The bioaccessibility of phenolic and flavonoid compounds in pomegranate peel flour at the end of intestinal digestion was 35.90 and 64.02% respectively. These values suggest that several changes in phenolic and flavonoid compounds like modification of chemical structure, increased or reduced solubility or interaction with other compounds might happen during the gastrointestinal digestion of pomegranate peel flour, which influence the bioaccessibility. Thus, Helal, Tagliazucchi, Verzelloni, and Conte (2014) obtained a bioaccessibility of 79.8% of total phenolic compounds in cinnamon beverage. Ortega et al. (2011) informed that the bioaccessibility, after intestinal digestion, of phenolic and flavonoids compounds were 81 and 65% respectively. Rodríguez-Roque, Rojas-Graü, Elez-Martínez, and Martín-Belloso (2013) reported that the total flavonoids present in soymilk showed a bioaccessibility of 16%, whereas total phenolic acids were not bioaccessible after gastrointestinal digestion.

3.2. *Stability of polyphenolic compounds present in pomegranate peel flour during simulated in vitro gastrointestinal digestion*

The HPLC analysis of the PPF (Fig. 4) showed a total of seven phenolic compounds identified as ellagic acid or ellagic acid derivatives (four peaks), ellagitannins (two peaks) and punicalagin (one peak). Catechin, epicatechin, caffeic, ferulic, sinapic, p-coumaric, gallic and chlorogenic acids as well as rutin, quercetin and apigenin were not found in all analysed samples. Scientific literature reported that these compounds are components of pomegranate peel but they are present at low concentration (Akhtar, Ismail, Fraternale, & Sestili, 2015; Mosele, Macià, Romero, Motilva, & Rubió, 2015). Probably, the process of obtaining the flour causes these compounds to degrade or be removed from the matrix, and only the compounds present at high concentrations were detected. Table 1 showed that punicalagin was found with the highest ($p < 0.05$) concentration (2850.21 mg/100 g of PPF) followed by ellagitannin (2) and ellagic acid (66.05 and 65.28 mg/100 g of PPF, respectively). With the aim of analysing the stability of each individual compounds, during the simulated *in vitro* gastrointestinal digestion, their concentration in the pellet fraction (PF) and in the chyme soluble fraction (CSF) was measured after each step of the process (mouth, gastric and intestinal steps). In the mouth step (Table 1), all the phenolic compounds detected increased the concentration ($p < 0.05$) with respect to the initial concentration which showed the release of phenolic compounds present in PPF matrix. Again, punicalagin, ellagitannin and ellagic acid, respectively, were found in the highest ($p > 0.05$) concentration. The phenolic compounds detected were found in higher concentration in the pellet fraction than chyme soluble fraction. Anyway, the presence of phenolic compounds in CSF indicates the solubilisation of these compounds. The enzyme

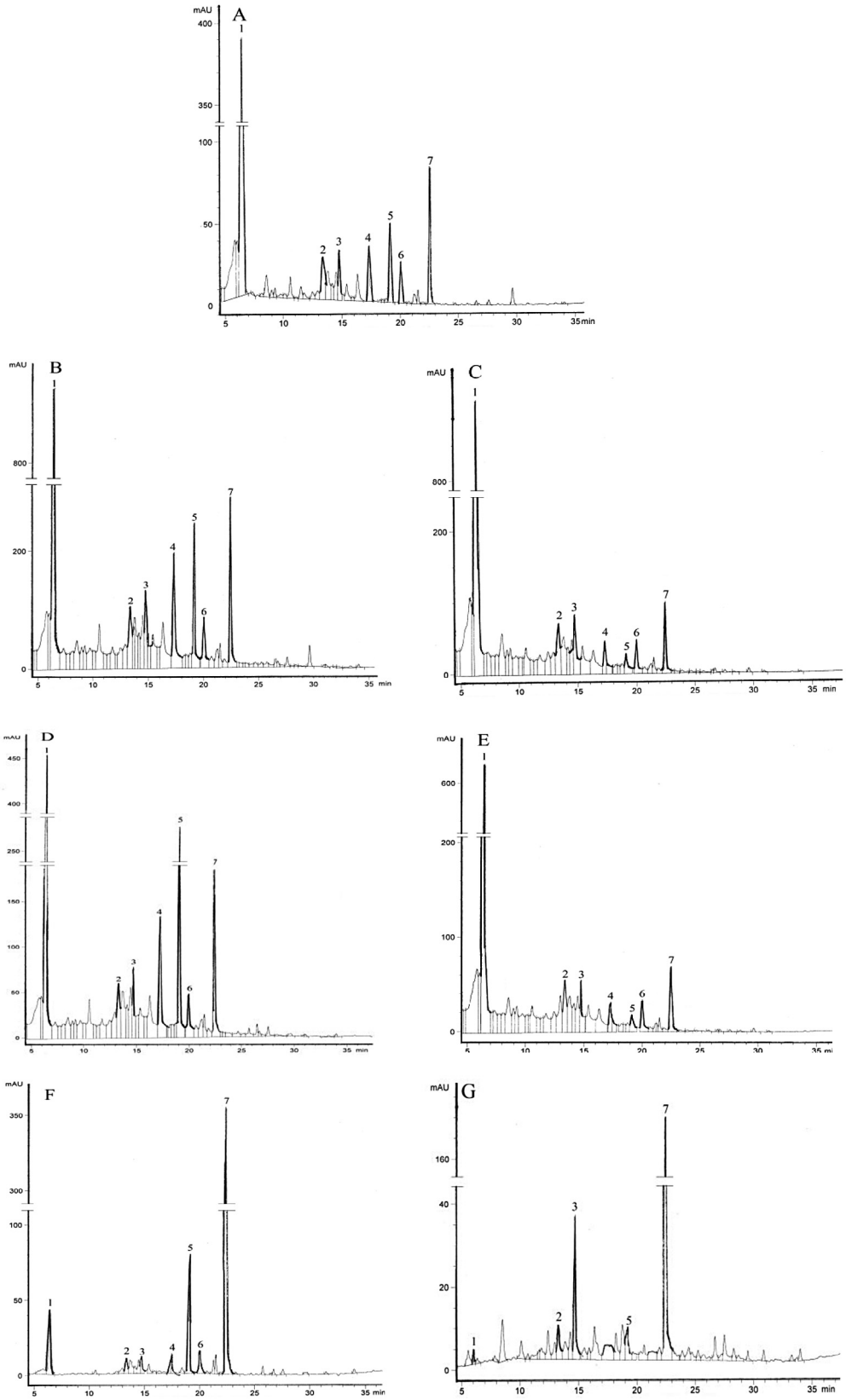


Fig. 4 – Representative HPLC chromatograms of: (A) PPF matrix; (B) Pellet fraction of PPF after mouth step; (C) Chyme soluble fraction of PPF after the mouth step; (D) Pellet fraction of PPF after gastric step; (E) Chyme soluble fraction of PPF after the gastric step; (F) Pellet fraction of PPF after the intestinal step; (G) Chyme soluble fraction of PPF after the intestinal step. Numbers refer to the main peaks identified: (1) Punicalagin; (2) Ellagitannin; (3) Ellagic acid derivate; (4) Ellagitannin; (5) Ellagic acid derivate; (6) Ellagic acid derívate; (7) ellagic acid.

Table 1 – Polyphenolic concentration obtained from the two fractions (pellet fraction (PF) and chyme soluble fraction (CSF)) after the simulated gastrointestinal digestion (Mouth, gastric and intestinal steps) of pomegranate peel flour (PPF) obtained from juice co-products.

Phenolic compound	PPF (100 g)	Mouth Step			Gastric step			Intestinal step		
		PF	CSF	Total	PF	CSF	Total	PF	CSF	Total
Punicalagin	2850.21 ± 3.1^{cA}	1790.21 ± 4.1	1180.20 ± 3.1	2970.41 ± 3.2^{aA}	1340.49 ± 3.1	1435.82 ± 2.2	2776.31 ± 3.1^{bA}	124.63 ± 1.1	2.39 ± 0.1	127.02 ± 1.1^{dB}
Ellagitannin	57.83 ± 0.1^{bC}	39.83 ± 0.1	19.50 ± 0.1	59.33 ± 0.1^{aD}	32.60 ± 0.1	22.52 ± 0.1	55.12 ± 0.1^{cE}	4.62 ± 0.1	3.25 ± 0.1	7.87 ± 0.1^{dD}
Ellagic acid derivative	29.59 ± 0.1^{cE}	22.81 ± 0.1	7.73 ± 0.1	30.54 ± 0.1^{aF}	21.58 ± 0.1	10.98 ± 0.1	32.56 ± 0.1^{bF}	3.00 ± 0.1	1.99 ± 0.1	4.99 ± 0.1^{dE}
Ellagitannin	66.05 ± 0.1^{bB}	63.05 ± 0.1	7.94 ± 0.1	70.99 ± 0.4^{aB}	61.54 ± 0.1	9.01 ± 0.1	70.55 ± 0.4^{aC}	2.18 ± 0.1	0.00 ± 0.1	2.18 ± 0.1^{cF}
Ellagic acid derivative	52.36 ± 0.1^{cD}	49.11 ± 0.1	5.57 ± 0.1	54.68 ± 0.1^{bE}	77.61 ± 0.1	6.21 ± 0.1	83.82 ± 0.1^{aB}	19.55 ± 0.1	2.27 ± 0.1	21.82 ± 0.1^{dC}
Ellagic acid derivative	29.32 ± 0.1^{bE}	23.71 ± 0.1	7.53 ± 0.1	31.24 ± 0.2^{aG}	22.14 ± 0.1	9.04 ± 0.1	31.18 ± 0.1^{aG}	4.96 ± 0.1	0.00 ± 0.1	4.96 ± 0.1^{cE}
ellagic acid	65.28 ± 0.9^{aB}	57.27 ± 0.1	11.42 ± 0.1	68.68 ± 0.2^{bC}	50.21 ± 0.1	13.20 ± 0.1	63.41 ± 0.1^{dD}	102.22 ± 0.1	36.63 ± 0.1	138.85 ± 0.1^{aA}

Values expressed as mg of each compound per 100 g of product.
For the same phenolic compound, values in the same row followed with same lower case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.
For the same digestion step, values in the same column followed with same upper case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.

activity or agitation conditions could facilitate the breakage of large molecules as high molecular weight phenols which initially may be insoluble. In gastric step (Table 1), punicalagin, ellagitannin and ellagic acid were slightly ($p < 0.05$) reduced (2.59, 4.68 and 2.86%, respectively) with respect to initial values. On the other hand, other phenolic compounds detected had an increment ($p < 0.05$) of their concentration in reference to initial values. The behaviour of phenolic compounds under gastric conditions was contradictory. Therefore, the phenolic acids showed low stability in agreement with the results reported by Kamiloglu and Capanoglu (2013) or Mosele et al. (2015) whilst flavonoids and tannins were more stable under the same conditions (Bouayed, Deußer, Hoffmann, & Bohn, 2012). Except for punicalagin, the phenolic compounds concentration was higher ($p < 0.05$) in PF than CSF. It should be noted that, as mentioned in Chandrasekara and Shahidi (2012), the phenolic compounds released in the gastric step may be absorbed and they may have some local antioxidant effects in the small intestine as well due to their solubility in the digest.

At the end of the intestinal step (Table 1), a drastic reduction of polyphenolic compounds was found. The results obtained are similar to those reported by Ortega et al. (2011) who showed that after the intestinal digestion step, important losses of phenolic compounds were found. As mentioned above, all the compounds detected were reduced ($p < 0.05$) with percentage of reduction between 58.32 and 96.3% by reference to initial concentration, except the ellagic acid, which increased ($p < 0.05$) by 112.69% with reference to initial values. The increased concentration of ellagic acid after intestinal digestion might be due to this compound being bound to proteins or fibre in the original matrix and as a result of enzymatic digestion was then released from these structures. In this sense, Mosele et al. (2015) reported that ellagic acid concentration increased after gastrointestinal incubation due to liberation from the complex phenols present in the food matrix. It is important to notice that in this step, the phenolic compounds detected were found in higher concentration in the pellet fraction than in chyme soluble fraction.

3.3. *Antioxidant activities of gastrointestinal digest*

The antioxidant activity of fruit products are linked to the phenolic acids and flavonoids content. However, the antioxidant properties of these compounds might change due to the chemical transformations during the gastrointestinal digestion. In addition, since the antioxidant activity of foodstuff is determined by multiple reaction characteristics and different mechanisms, it is necessary to combine more than one method in order to determine the *in vitro* antioxidant capacity of foods. In addition, each method only provides an estimate of antioxidant capacity that is subjective to its conditions and reagents (Ma et al., 2011; Viuda-Martos, Ruiz-Navajas, Sánchez-Zapata, Fernández-López, & Pérez-Álvarez, 2010). Therefore, in this work to evaluate the changes produced by the gastrointestinal digestion of PPF extracts, the antioxidant activity was determined using five different methodologies.

Table 2 showed the ferrous ion chelating activity (FIC) of digested PPF. The mouth step increased ($p < 0.05$) the FIC values by 57.14% with reference to initial values. The PF showed a slight decrease ($p > 0.05$) with respect to PPF values whilst that of the

Table 2 – Antioxidant properties of the two fractions (pellet fraction (PF) and chyme soluble fraction (CSF)) after the simulated gastro-intestinal digestion (Mouth, gastric and intestinal steps) of pomegranate peel flour (PPF) obtained from juice co-products.

Assay	PPF	Mouth step		Gastric Step		Intestinal Step	
		PF	CSF	PF	CSF	PF	CSF
FIC	0.35 ± 0.01^{c}	0.33 ± 0.02^{cA}	0.22 ± 0.00^{dB}	0.33 ± 0.02^{cA}	0.22 ± 0.00^{dB}	0.43 ± 0.01^{bB}	0.50 ± 0.00^{aA}
FRAP	52.83 ± 0.22^{b}	48.10 ± 0.56^{cA}	30.94 ± 0.65^{eB}	72.65 ± 0.20^{aA}	51.80 ± 0.15^{bB}	36.93 ± 0.07^{dA}	20.54 ± 0.17^{fB}
ABTS	41.24 ± 1.71^{d}	50.05 ± 2.18^{cA}	40.76 ± 0.36^{dB}	77.81 ± 0.84^{aA}	66.12 ± 2.16^{bB}	5.38 ± 0.41^{eA}	0.86 ± 0.01^{fB}
DPPH	52.36 ± 0.12^{c}	48.31 ± 0.26^{dA}	31.60 ± 0.13^{eB}	55.03 ± 0.20^{bB}	77.93 ± 0.99^{aA}	1.66 ± 0.30^{fA}	0.33 ± 0.09^{gB}
ORAC	156.23 ± 1.24^{c}	138.11 ± 0.99^{dA}	125.22 ± 1.89^{eB}	199.58 ± 1.57^{aA}	183.22 ± 1.78^{bB}	12.44 ± 0.04^{fA}	6.38 ± 0.75^{gB}

PF: Pellet fraction; CSF: Chyme Soluble Fraction.
For the same antioxidant assay, values in the same row followed with same lower case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.
For the same digestion step, values in the same row followed with same upper case letter are not significantly different ($p > 0.05$) according to Tukey's Multiple Range Test.
FIC: expressed as mg EDTA equivalents/g; FRAP: expressed as mg Trolox equivalents/g; ABTS: expressed as mg Trolox equivalents/g; DPPH: expressed as mg Trolox equivalents/g; ORAC: expressed as μg Trolox equivalents/mg.

CSF increase showed values of 0.22 mg EDTA equivalents/g. These values suggest that several compounds with chelating activity had been solubilised. It is important to notice that the FIC values were higher ($p < 0.05$) in PF than CSF. In the gastric step, the FIC values obtained were the same ($p > 0.05$) with those obtained in the mouth step which means that the pH conditions and enzymes that act in the gastric step do not affect the PPF matrix. In the intestinal step, the chelating activity increased by 167.71% with respect to initial values ($p < 0.05$). The CSF showed higher ($p < 0.05$) FIC values than PF. The results obtained are in agreement with You, Zhao, Regenstein, and Ren (2010) who informed that chelating activity of loach protein hydrolysates increased after intestinal digestion. In the same way, Chandrasekara and Shahidi (2012) also informed that chelating activity of millet grain increased significantly after the intestinal step. This increase in FIC values could be due to the liberation of several compounds like: phenolic acids, flavonoids or other constituents, such as ascorbates, reducing carbohydrates, tocopherols, carotenoids, or pigments which might contribute to the chelating activity of digested PPF.

As regard to Ferric Reducing Power (FRAP), the results of this study (Table 2) showed that mouth digested extract had higher ($p < 0.05$) FRAP values than their corresponding PPF initial extract. The FRAP values of PF were higher ($p < 0.05$) than CSF values. However, the FRAP values obtained to CSF indicated that several bioactive compounds had been solubilised in this fraction. In the gastric step, the FRAP values increased ($p < 0.05$) by 135% with respect to PPF initial extracts and by 57.45% with respect to the mouth step. Again the PF fraction showed higher values ($p < 0.05$) than CSF fraction. In the intestinal step, the FRAP values increased by 8.78% with respect to initial values ($p < 0.05$). Nevertheless, the reducing powder was reduced by 53.82% with respect to the gastric step. These results suggest that the bioactive compounds present in PPF matrix could be released at acid pH values or with enzymatic treatments which hydrolyse starch and proteins in gastric steps; however a change in pH conditions reduced their reducing power.

In this work, the scavenging potential of PPF polyphenolic compounds, towards DPPH and ABTS radicals (Table 2), during the different steps of *in vitro* digestion, was assessed. In the mouth step, both assays showed the same comportment. The ABTS values of mouth digested extract increased by 120% with respect to PPF initial extract ($p < 0.05$) whilst the DPPH values increased by 52.61% ($p < 0.05$) with respect to PPF initial extract. In both assays, the PF values were higher ($p < 0.05$) than the CSF values. In the gastric step, DPPH inhibition increased ($p < 0.05$) by 153.93% while the ABTS inhibition augmented ($p < 0.05$) by 249.05% with respect to PPF initial extract. Several authors informed that the gastric step has a strong impact on antioxidant activity determined with ABTS and especially with DPPH assays. Thus, Rodríguez-Roque et al. (2013) mentioned that gastric digestion of soymilk increased the DPPH inhibition to 30%. In the same way, Chandrasekara and Shahidi (2012) also informed that DPPH scavenging activity of millet extract increased significantly after gastric digestion. This increment in antioxidant activity might be related with a higher released of bioactive compounds, with scavenging properties, from PPF matrix due to the acidic conditions of gastric digestion. Therefore, Baublis, Decker, and Clydesdale (2000) informed that the acidic environments of gastric digestion altered the activity, composition and concentration of water-soluble, low-molecular-weight antioxidants of breakfast cereals. In intestinal step, the scavenging activity decreased by 84.86% with respect to initial values ($p < 0.05$) in ABTS assay whereas in DPPH assay it decreased by 96.20% with respect to initial values ($p < 0.05$). These results are in agreement with Correa-Betanzo et al. (2014) who reported that DPPH scavenging activity of blueberry extracts decreased over 50% after intestinal digestion.

With regard to ORAC assay (Table 2), again, the mouth step increased ($p < 0.05$) the antioxidant activity of PPF by 68.55% compared to initial values. The ORAC values of PF were higher ($p < 0.05$) than CSF values. As occurred with DPPH and ABTS assays, gastric digestion increased significantly ($p < 0.05$) the ORAC values. In this step, the antioxidant activity increased by 145.02% with respect to initial values. However, in the intestinal digestion ORAC values decreased ($p < 0.05$) significantly (87.95%) with reference to initial values.

During the digestion process, antioxidants probably undergo modifications which increase their reactivity, especially due to the changes in pH, as the pH of a substance is known to affect the racemisation of molecules creating enantiomers with different biological reactivity (Wootton-Beard, Moran, & Ryan,

Table 3 – Coefficient values of correlation between the TPC or TFC and antioxidant activity measure with five different methodologies under different steps of gastrointestinal digestion.

Antioxidant assay	Mouth step		Gastric step		Intestinal step	
	TPC	TFC	TPC	TFC	TPC	TFC
DPPH	0.995	1.00	1.00	0.997	0.970	0.981
FRAP	0.997	0.999	0.999	0.997	1.00	0.999
FIC	0.999	0.999	0.991	0.982	–0.993	–0.998
ABTS	0.989	0.975	0.985	0.989	0.994	0.999
ORAC	0.997	1.00	1.00	0.997	1.00	0.999

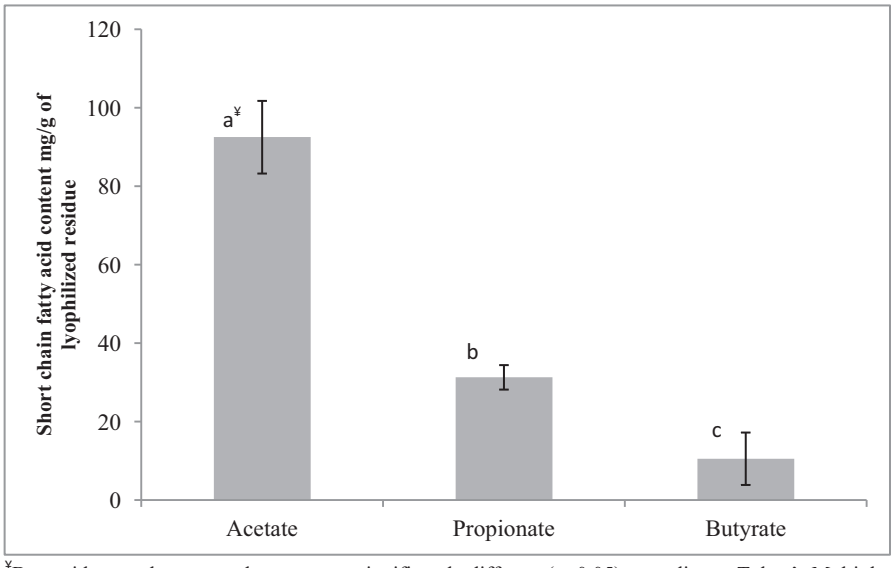
2011). Thus, antioxidant compounds would be more reactive particularly at acidic pH (as occurs in gastric digestion) and less reactive at pH close to neutrality (as occurs in intestinal digestion) (Wootton-Beard et al., 2011).

In scientific literature there are several works which reported that correlation between polyphenolic compounds and antioxidant activity occurs which indicated that these compounds largely contributed to the antioxidant properties (Chandrasekara & Shahidi, 2012; Chaves-López et al., 2015; Ma et al., 2011). Table 3 showed the coefficient values of correlation achieved between the TPC, TFC and antioxidant activity measured with five different methodologies under different steps of gastrointestinal digestion. In the mouth and the gastric steps a strong and positive correlation ($r > 0.980$) was found between TPC or TFC and the antioxidant activity obtained with DPPH, FRAP, FIC, ABTS and ORAC assays. As regards intestinal step, again a strong and positive correlation ($r > 0.970$) was obtained among TPC or TFC and the antioxidant activity obtained with DPPH, FRAP, ABTS and ORAC. In this step, a negative correlation was achieved for FIC assay which suggested that the phenolic compounds capable of chelating metals have degraded in the earlier stages of digestion process and the metal chelating activity is due to other compounds as mentioned above. These results are in concordance with several studies which reported a high correlation between polyphenolic compounds and antioxidant activity. Thus, Carbonell-Capella, Buniowska, Esteve, and Frígola (2015) found high correlation coefficients between the total phenolic content and antioxidant activities of beverages added with stevia and subjected to *in vitro* digestion. In the same way, Chen et al. (2014) informed that there is a strong correlation between the antioxidant activity and the TPC of 33 fruits after *in vitro* digestion process.

Moreover, in all digestion steps, a strong correlation ($r > 0.975$) was found for the DPPH-FRAP, DPPH-ABTS and FRAP-ABTS. These positive correlations were expected because the assays are based on the same principle, i.e. single electron transfer as mentioned in Huang, Sun, Lou, Li, and Ye (2014). In the same way, high correlations were obtained in all digestion steps between DPPH, ABTS and FRAP assays and ORAC and FIC assays ($r > 0.970$). These results are contradictory with Huang et al. (2014) who informed that there was a low correlation between DPPH, ABTS and FRAP assays and ORAC. However, Carbonell-Capella et al. (2015) found high correlation was found between ABTS-ORAC and DPPH-ORAC assays.

3.4. *Short chain fatty acids production*

Pomegranate peel flour (PPF) obtained as co-product from juice extraction could be considered a good source of dietary fibre. *In vitro* fermentation of the digested fraction of PPF by colonic microflora was determined and the production of short chain fatty acids (mainly of acetic, propionic and butyric acids), at the end of the fermentation period (24 h) was analysed. In this work, the total content of SCFAs produced (Fig. 5), which includes acetic, propionic and butyric acids was 134.42 mg/g sample. Acetic acid (92.53 mg/g sample) was the main SCFAs produced ($p < 0.05$) in the PPF fermentation by colonic



¥Bars with same lower case letter are not significantly different ($p>0.05$) according to Tukey's Multiple Range Test.

Fig. 5 – Short Chain Fatty Acids (acetic, propionic and butyric acids) produced in the *in vitro* microbial fermentation of pomegranate peel flour after gastrointestinal digestion.

microflora, followed by propionic (31.32 mg/g sample) and butyric (10.57 mg/g sample) acids.

To the best of our knowledge, there are no studies where the SCFAs production at the end of colonic fermentation of fibre obtained from agro-industrial co-products was determined. Nevertheless, SCFAs obtained from *in vitro* fermentation of digested fraction of several foods had been analysed. Thus, Anson et al. (2011) reported the production of acetic (27–41 mmol), propionic (13–23 mmol) or butyric (11–15 mmol) in the 24 h colonic fermentation of several wheat breads. In the same way Chandrasekara and Shahidi (2012) showed that the total content of SCFA produced (mainly acetic, propionic and butyric acids) ranged from 25 to 70 g/100 g of insoluble residue (dw) of millet grains.

In the last few decades, it has been hypothesised that SCFAs might play a key role in the prevention and treatment of several diseases such as metabolic syndrome, bowel disorders and cancer (Donohoe et al., 2011). On the other hand, SCFA modulate different processes including cell proliferation and differentiation, hormones secretion (e.g., leptin and peptide YY) and activation of immune/inflammatory responses (Vinolo et al., 2011; Zaibi et al., 2010). In addition to their important role as fuel for intestinal epithelial cells, SCFAs modulate different processes in the gastrointestinal tract such as electrolyte and water absorption (Vinolo, Rodrigues, Nachbar, & Curi, 2011). However, it should be borne in mind that excessive SCFAs concentrations might induce adverse effects on gastrointestinal and colonic motility and sensitivity in certain diseases such as inflammatory bowel disease and gastro-oesophageal reflux disease (Cherbut, 2003).

4. Conclusions

This work demonstrates that the polyphenolic compounds present in pomegranate peel flour are released in the different gastrointestinal digestion steps and they are bioaccessible to exert bioactivity after their absorption, mainly as antioxidant compounds for preventing oxidative stress diseases. In addition, the short chain fatty acid production also contributes to improve the health effects.

Although digestion process of PPF exerts a reduction on the polyphenolic concentration and the antioxidant properties, this co-product could be used in the food industry as potential ingredient to develop functional food that promotes health benefits.

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