Impact of an *in vitro* dynamic gastrointestinal digestion on phenolic compounds and antioxidant **capacity** of apple treated by high-pressure processing

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ABSTRACT

The aim was to evaluate the effect of an *in vitro* dynamic gastrointestinal digestion (GID) on the recovery index and bioaccessibility, the stability of phenolic compounds, and the changes in antioxidant **capacity** (AC) of apples 'Golden Delicious' treated by high-pressure processing (HPP-treated). All the phenolic compounds decreased during GID in untreated and HPP-treated apple and in consequence a decrease in the antioxidant **capacity** (DPPH[•], ABTS^{•+}, TP-FC and FRAP) and in the effect on the inhibitory capacity of lipoxygenase and xanthine oxidase (XO) activities was also observed. The dynamic-GID increased the release of quercetin-aglycone and led to low quercetin-glycosides bioaccessibility (~10%). HPP-treated compared to untreated-apple presented higher recovery index for hydroxycinnamic acids (~67% and 52%) and higher bioaccessibility for hydroxycinnamic acids (40% and 31%) and dihydrochalcones (30% and 19.5%). The bioaccessible fraction of HPP-treated apple showed a high retention of the **undigested AC**:

24% (DPPH[•]), 14% (ABTS^{•+}), 86% (TF-FC), 48% (FRAP) and the 68% and 100% of XO and LOX inhibitory capacities.

Keywords: HPP; quercetin glycosides; chlorogenic acid; LOX; XO; CAT

Industrial relevance: The consumer is willing to pay for added value in functional foods or ingredients that represent a substantial improvement in terms of quality of life and health. Therefore, functional food industries need to find valid methods to guarantee that the bioactive compounds present in the functional food or ingredient are effective and should do the biological action for which they were designed. The functional food industry relies more and more on the technology implemented in *in vitro* digestion models that simulate gastrointestinal digestion, to assess the bioaccessibility of bioactive compounds as the first step to investigate their bioavailability. The current study provides relevant information for the use dynamic gastrointestinal models instead of the more frequently employed static models to obtain more exact data about the bioaccessibility of phenolic compounds of a functional apple ingredient obtained by high-pressure processing of apples pieces with peels.

These results should provide realistic information to the functional food industry about the amounts should be consumed of a certain functional food or ingredient to achieve the promised biological effect. This information can suppose an added value to these functional foods which will revert in the economic improvement and the prestige of the food industry.

1. Introduction

The apple (*Malus domestica* Borkh.) is one of the most consumed fruit worldwide (USDA, 2019) and represent a significant source of dietary bioactive compounds (Briones-

Labarca, Venegas-Cubillos, Ortiz-Portilla, Chacana-Ojeda, & Maureira, 2011; Oszmiański, Lachowicz, & Gamsjäger, 2020). The dietary intake of apples has been associated with lower risk of cardiovascular diseases (Bondonno, Bondonno, Ward, Hodgson, & Croft, 2017), cancer, type-2-diabetes, obesity and inflammatory disorders, among others (Boyer & Liu, 2004; Tu, Chen, & Ho, 2017).

The main compounds responsible to these health-protecting effects of apple consumption are proposed to be the phenolic compounds (Serra et al., 2012) due to their antioxidant properties, their ability to neutralize free radicals and protect cells against oxidative stress (Zhang & Tsao, 2016). Moreover, phenolic compounds can exert indirect antioxidant **capacity** due to their effect protecting antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD) or glutathione peroxidase (GPX), enzymes that play an important role in cell protection breaking down reactive oxidative species (ROS) such as hydroperoxides and superoxide ions (Tsao, 2010). In addition, phenolic compounds have been related to the decrease of the activity of enzymes that produce ROS such as xanthine oxidase (XO), lipoxygenase (LOX), cyclooxygenase (COX), among others (Gawlik-Dziki, 2012).

The qualitative and quantitative phenolic composition of apples depends largely on the variety, environmental and agricultural practises, preharvest and postharvest conditions and type of processing (Boyer & Liu, 2004). The application of high-pressure processing (HPP) on plant foods such as onion, apple, fruit juices, beverages, among others, could produce an increase of the extractability of phenolic compounds due to changes in the food matrix and in the cell wall permeability (Xu, Zhu, & Yu, 2016), and in consequence their bioaccessibility could also be improved (Briones-Labarca et al., 2011). Different apple products, mainly apple juices, submitted to HPP treatments maintained or increased the amount of phenolic compounds extracted and their antioxidant **capacity**, although this

effect depend on the HPP conditions and apple variety Fernández-Jalao, Sánchez-Moreno, & De Ancos, 2019; Landl, Abadias, Sarraga, Vinas, & Picouet, 2010). HPP has been proposed as an alternative to the traditional thermal processing technologies to obtain safe, nutritive fresh-tasting plant-derive, avoiding the degradation of nutrients and bioactive compounds as well as inhibiting microbial growth and enzyme activity (Balasubramaniam, Barbosa-Cánovas, & Lelieveld, 2016).

In terms of evaluation of the biological activity of the phenolic compounds of apple products, is much more important to know the fraction potentially available for further intestinal absorption (bioaccessibility) than the quantification of these compounds in the food matrix. During the gastrointestinal digestion, the food components (included phenolic compounds) are constantly exposed to different physical (temperature), chemical (pH) and biochemical (enzymes) conditions. As consequence, the bioaccessibility, bioavailability and bioactivity of these compounds are affected. Bioavailabitliy is defined as the fraction of ingested bioactive compounds that are digested, absorbed and metabolized through normal pathways. Additionally, the bioaccessibility is the fraction which is released from the food matrix during the gastrointestinal digestion and becomes available for absorption (Carbonell-Capella et al., 2014). In general, phenolic compounds are poorly absorbed during the gastrointestinal digestion although their bioaccessibility could be influenced by their chemical structure and by other components of the food matrix, like proteins, lipids and carbohydrates, but also by the food processing (Bohn, 2014).

In recent years, the impact of gastrointestinal digestion on the stability and bioaccessibility of phenolic compounds and on the antioxidant activities of different plant derived foods such as grapes (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010), apple (Bouayed, Deusser, & Hoffmann, 2012), fruit juices (Quan et al., 2018), wild blueberry (Correa-Betanzo et al., 2014), maqui berry (Lucas-Gonzalez et al., 2016), dried fruit

pomace (Gouw, Jung, & Zhao, 2017), leafy vegetables (Gunathilake, Ranaweera, & Rupasinghe, 2020), among others, have been extensively studied using static *in vitro* models. The static models easily simulate the physiological conditions of the human gastrointestinal digestion, usually using a single set of initial conditions (pH, concentration of enzymes, bile salts, etc.) for each part of the gastrointestinal tract. However, this simple approach is often not a realistic simulation of the more complex *in vivo* conditions, where the biochemical environment encountered is constantly changing and physical parameters such as shear and grinding forces can have a large impact on the breakdown of larger food particles and the release of nutrients. For that reason, some dynamic models have been developed in the last years to better mimic the human gastrointestinal digestion (Thuenemann, 2015).

Therefore, the aim of this study was to investigate the effect of the different phases of an *in vitro* dynamic gastrointestinal digestion model on the stability, recovery index and bioaccessibility of phenolic compounds of high-pressure processing treated apple product (HPP-treated apple). In addition, consequent changes in total antioxidant capacity of apple products during the gastrointestinal digestion determined by different methodologies were also studied. To our knowledge, there is not report available on investigating the effect of a simulated gastrointestinal digestion using a dynamic model on the stability and bioaccessibility of phenolic compounds and on the antioxidant capacity of HPP-treated apple products.

2. Materials and methods

2.1. Raw material and sample preparation

'Golden Delicious' apples produced in the Northeastern of Spain (lowland climate) were purchased from a local supermarket in Madrid, Spain. The physical and physicochemical characteristics of the apple fruits were calculated according to Fernández-Jalao et al. (2019): weight (201.52 ± 16.25 g); size (78.33 ± 1.53 mm); water content ($81.94 \pm 0.07\%$); pH (3.64 ± 0.05), soluble solids (13.18 ± 0.97 °Brix) and titratable acidity (0.13 ± 0.005 % of malic acid). Approximately 12 kg of apples were selected according to uniform colour and size, absence of external damages and stored at 4 °C. The apples were washed and cut into small pieces of about 2 cm with skin and without core. Two batches of apples cubes were separated, one group correspond to untreated apples and the other group to the samples submitted to high-pressure processing (HPP-treated apple). For each group, 200 g of apples pieces were packed into low permeability plastic bags (BB4L, Cryovac, Barcelona, Spain) and sealed with light vacuum. Untreated apples were frozen with liquid nitrogen and lyophilized at 0.1 mbar and -90 °C (Lyalfa, Telstar S.A, Barcelona, Spain). Lyophilized samples were pulverized using an ultracentrifugal grinder ZM 200 (Retsch GmbH, Haan, Germany) to obtain a fine powder (0.5 mm of particle size) that was stored at -20 °C until their analysis.

2.2. High-pressure processing (HPP)

Two bags with 200 g of apple cubes were introduced in the pressure unit vessel (two bags for each cycle) filled with pressure medium (water) and treated at 400 MPa at 35 °C during 5 min. The hydrostatic pressure unit was formed by a vessel of 1925 mL capacity and it was able to work to a maximum pressure of 800 MPa and a maximum temperature of 70 °C (High Pressure Iso-Lab System, Model FPG7100:9/2C, Stansted Fluid Power LTD., Essex, UK). The compression rate was 3 MPa/s and decompression rate

instantaneous. Pressure, time and temperature were controlled by a computer program, being constantly monitored and recorded during the process. HPP at 400 MPa/35 °C/5 min has been employed due to previous results showing that this treatment maintained or even increased the main apple phenolic compounds (Fernández-Jalao et al. 2019). HPP-treated apples were immediately frozen with liquid nitrogen, lyophilized, pulverized and maintained at -20 °C.

2.3. Characterization of untreated and HPP-treated apple products

The physicochemical, chemical, biochemical and microbiological characteristics of untreated and HPP-treated apple powders are shown in Table 1. Titratable acidity, pH, total vitamin C and ascorbic acid were determined according to Fernández-Jalao et al. (2019). Total pectin content (%) and degree of esterification (DE) (%) were determined according to Canteri-Schemin, Fertonani, Waszczynskyj, & Wosiacki (2005). Sugars and organic acids content were analysed according to Colina-Coca et al. (2014). Polyphenoloxidase (PPO), peroxidase (PPO) activity and soluble proteins were determination according to Hernández and Cano (1998). Soluble, insoluble and total fibre (AOAC 991.43, 2012) AMD total viable count (AFNOR. 1999) were also determined.

2.4. *In vitro* dynamic gastrointestinal digestion of untreated and HPP-treated apple products: recovery index and bioaccessibility

A dynamic gastrointestinal digester (DGD) was used to digest apple powder samples (untreated and HPP-treated). The DGD is a multi-compartmental, dynamic and computer-controlled system developed at AINIA Technologic Center (Valencia, Spain)

based on Villemejeane et al. (2016). The digester consists of two successive serial compartments simulating stomach and small intestine, respectively (Fig.1). Each compartment is formed by two glass units with a flexible silicone wall jacket inside. Heated water to 37 ± 1 °C is pumped through the space between the glass and silicone jacket to maintain the constant body temperature in all the system. Peristaltic movements, produced in both compartments stomach and small intestine, are simulated by alternate compression and relaxation of the flexible silicone walls following changes in the water pressure. The food received in the stomach compartment is mixed with gastric electrolytes and enzymes and is referred as digest or chyme. The stomach emptying was programmed by a slow chyme transit ($t_{1/2} = 70$ min and $\beta = 2$) and also the small intestine emptying was done by slow transit ($t_{1/2} = 160$ min and $\beta = 1.6$). The chyme transit was regulated by opening or closing the peristaltic valve pumps that connect the compartments. The whole system is computer controlled through an operator panel and programmable logic controller.

In vitro gastrointestinal digestion process was performed on 37.5 g of freeze-dried and powdered apple (untreated and HPP-treated). The experiments began combining the apple powder with artificial saliva contained 9600 units of α -amylase, 30 mL of citrate buffer (pH 6), and 100 mL of electrolyte solution (NaCl, CaCl₂ and NaHCO₃). Milli-Q water was added to the mixture up to final weight of 300 g. This mixture was homogenized for two minutes and bubbled with nitrogen gas for ten minutes and then introduced in the gastric compartment representing the stomach, and the gastrointestinal digestion was started. Previously to digestion process, 10 mL gastric secretions (hydrochloric acid, gastric electrolytes solution, gastric lipase and pepsin) and 60 mL of intestinal secretions (sodium bicarbonate, intestinal electrolytes solution, pancreatin, bile and trypsin) were added to the stomach and small intestine compartments, respectively. The pH is continuously controlled in the stomach (from pH 5 to pH 2) and small intestinal (from pH 6.5 to pH 7)

compartments by secretions of 1 M hydrochloric acid and 1 M sodium bicarbonate, respectively. Thus, the volume, the pH of the chyme and the digestive secretions introduced in each compartment are computer-controlled. The total *in vitro* dynamic gastrointestinal digestion was performed during 360 min (from 0 to 120 min in the stomach and from 0 to 360 min in the small intestine) at 37 °C in absence of light and under anaerobic conditions by injecting nitrogen gas in the system.

To monitor the release of individual polyphenols from apple products at different stages of digestion, aliquots from the artificial saliva treatment (oral phase), gastric digest at 120 min and intestinal digest from 0 to 360 min were separated and acidified to pH 2. Then, these aliquots were frozen and stored at -20 °C until its analysis. The rest of the intestinal digest at pH 2 was centrifuged (Sigma Laboratory Centrifuge at 6K15) at 3890 g at 4 °C for 60 min and the supernatant were separated. This supernatant represents the bioaccessible fraction (BF) or soluble fraction according to procedure described in different studies (Tagliazucchi et al., 2010). Centrifugation has been recognized as valid systems to determine de compounds available for intestinal absorption (Alegría, Garcia-Llatas, & Cilla, 2015). The bioaccessible fraction was immediately frozen and stored at -20 °C until its analysis. *In vitro* digestion of each sample was done in duplicate.

To evaluate the effect of food matrix (untreated and HPP-treated) and the different gastrointestinal phases on the phenolic compounds, two different indexes were studied: the recovery index (%) (by group of phenolic compounds) and bioaccessibility (%) (by groups and by individual phenolic compounds).

Recovery index (RIX) (%) allows to measure the amount of total phenolic compounds of the same group (flavonols, flavan-3-ols, hydroxycinnamic acids and chalcones) present in

the digest of the oral, gastric and intestinal phase (Ortega, Macia, Romero, Reguant, & Motilva, 2011).

$$\text{Recovery index (DIX) (\%)} = (\text{TPCd} / \text{TPCnd}) \times 100$$

TPCd = Concentration of total phenolic content of a family in the digest of each digestion phase

TPCnd = Concentration of total phenolic content of a family in non-digested sample

Bioaccessibility defined as the portion of phenolic compounds (PC) that is released from the food matrix into the gastrointestinal tract and thus become available for intestinal absorption was determined in the bioaccessible fraction (BF):

$$\text{Bioaccessibility (\%)} = (\text{PC}_{\text{BF}} / \text{PCnd}) \times 100$$

PC_{BF} = Concentration of a phenolic compound in the bioaccessible fraction

PCnd = Concentration of a phenolic compound in non-digested sample

2.5. Determination of phenolic compounds by HPLC-DAD-ESI-MS-QTOF

Samples untreated and HPP-treated apple powder samples before digestion (0.5 g) were extracted using a methanol/water solution (80:20, v/v) according to the procedure described by Fernández-Jalao et al. (2019).

The separation, identification and quantification of phenolic compounds by HPLC-DAD and HPLC-ESI-MS-QTOF was carried out according to the methodology described by Fernández-Jalao et al. (2019).

2.6. Antioxidant capacity analysis

The antioxidant **capacity** of apple products at different stages of gastrointestinal digestion was determined in the hydro-methanolic phenolic extracts obtained for analysing phenolic compounds.

2.6.1. Antioxidant **capacity** by DPPH[•], ABTS^{•+}, FRAP and Folin-Ciocalteu assays

Four different methods were employed according to Fernández-Jalao et al. (2017): 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) scavenging assay, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) radical cation (ABTS^{•+}) scavenging assay, ferric reducing antioxidant power (FRAP) assay, and Folin-Ciocalteu method for total phenols (TP-FC). The results were expressed as μmol trolox equivalents per gram of dry weight ($\mu\text{mol TE/g dw}$) for FRAP, DPPH[•] and ABTS^{•+} assays and mg of gallic acid equivalents per gram of dry weight (mg GAE/ g dw) for TP-FC. All samples were run in quadruplicate.

2.6.2. Effects on xanthine oxidase, lipoxygenase and catalase activity

Inhibition of xanthine oxidase (XO) activity. The XO activity with xanthine as the substrate was measured spectrophotometrically at 290 nm, based on the procedure described by Gawlik-Dziki (2014) with modifications. The reaction mixture consisted of 1.8 mL of 50 mM phosphate buffer (pH 7.5), 0.1 mL of the tested sample (or buffer for the blank), 1.0 mL of 150 mM xanthine solution. After preincubation of the mixture at 25 °C for 10 min, the reaction was initiated by adding 0.1 mL of xanthine oxidase solution (0.1 U/mL in 50 mM phosphate buffer). XO inhibitory activity was expressed as the percentage inhibition of XO by the test samples, calculated as:

$$\% \text{ Inhibition enzyme activity} = 1 - \frac{\Delta A_{290} \text{ nm/min test sample}}{\Delta A_{290} \text{ nm/min blank}} \times 100$$

Where $\Delta A_{290} \text{ nm/min test sample}$ is the linear change in absorbance per minute of the test sample, and $\Delta A_{290} \text{ nm/min blank}$ is the linear change in absorbance per minute of the blank.

Inhibitory activity was expressed as IC₅₀ defined as the amount of sample that caused 50 % of enzyme inhibition (mg of dry weight).

Inhibition of lipoxygenase (LOX) activity. LOX activity was determined spectrophotometrically at 25 °C by measuring the absorbance at 234 nm according to the procedure previously described (Gawlik-Dziki, 2014) with some modifications. Briefly, the reaction mixture contained 1.4 mL of borate buffer 0.2 M (pH 9), 0.1 mL of enzyme solution (34160 U/ml) and 0.1 mL of the tested sample (or buffer for the blank). After preincubation at 30 °C during 15 min, the reaction was initiated by adding 1.4 mL of a solution 0.5 mM of linoleic acid. One unit of LOX activity was defined as an increase in absorbance per minute at 234 nm. Inhibitory activity was expressed as IC₅₀ defined as the amount of sample that caused 50 % of enzyme inhibition (mg of dry weight).

Influence on catalase (CAT) activity. The CAT activity was determined as described by Gawlik-Dziki (2012). Decomposition of hydrogen peroxide (H₂O₂) at 25 °C was monitored by measuring absorbance at 240 nm. Reduction in absorbance is directly proportional to the enzyme activity. The reaction mixture consisted of 2.8 mL of 0.02 M H₂O₂ in 0.05 M phosphate buffer (pH 7), adjusted to obtain absorbance in the range 0.525–0.550 nm and 0.1 mL of the tested sample. After addition 0.1 mL of enzyme solution (100 U/mL) (or buffer for the blank), the absorbance was monitored at 240 nm in regular time intervals (30 s) during 5 min. The catalase activity was expressed as mmol of H₂O₂ consumed per min.

The residual catalase activity was expressed according to the following equation:

$$\% \text{Catalase activity} = \frac{\Delta A_{240} \text{ nm/min test sample}}{\Delta A_{240} \text{ nm/min blank}} \times 100$$

$\Delta A_{240} \text{ nm test sample}$ = Increased absorption per minute of the test sample (slope)

$\Delta A_{240} \text{ nm blank}$ = Increased absorption per minute of the blank (slope)

The effect of test sample on the CAT activity was expressed as IC₅₀ defined as the amount of sample necessary to preserve 50% of catalase activity (mg of dry weight).

2.7. Statistical analysis

All data are presented as mean $\pm$ standard deviation of two replicates obtained in at least two separate experiments (n=4). One-way ANOVA was conducted by the Tamhane's T2 (equal variances not assumed) and Tukey's b (equal variances assumed) *post hoc* tests. Student's t-test was used to compare pairs of means and determine statistical significance at the $p < 0.05$ level. The correlations within variables were examined by Pearson's r correlation. All analyses were performed by using the IBM SPSS Statistics 25 Core System (SPSS Inc., an IBM Company, USA).

3. Results and discussion

3.2. Effect of different phases of an *in vitro* dynamic gastrointestinal digestion on individual apple phenolic compounds

Twenty-two compounds belonging to four different phenolic families (flavonols, hydroxycinnamic acids, flavan-3-ols and dihydrochalcones) have been separated and tentatively identified by HPLC-ESI-MS-QTOF from untreated and HPP-treated apple products (Table 2).

In order to determine the influence of an *in vitro* dynamic gastrointestinal digestion (GID) on the stability and bioaccessibility of phenolic compounds from an apple product treated by high-pressure processing (HPP), the concentration of flavonols,

hydroxycinnamic acids, flavan-3-ols and dihydrochalcones was monitored after the different phases, i.e. oral phase (OP), gastric digestion phase (GDP), intestinal digestion phase (IDP) and bioaccessible fraction (BF). Similar study has been done with the untreated apple product (Table 3).

3.2.1. Flavonols

The main flavonols identified in both untreated apple and HPP-treated apple are shown in Table 2. This flavonols profile was in accordance with that described for 'Golden Delicious' apples (Ribas-Agusti, Martin-Belloso, Soliva-Fortuny, & Elez-Martinez, 2018), being the majority quercetin-3-*O*-rhamnoside (Q-rhamnoside) and quercetin-3-*O*-galactoside (Q-galactoside) that represent 32% and 24% of the total flavonols, respectively (Table 3). HPP (400 MPa/35 °C/5 min) increased significantly ($p<0.05$) between 20-30% the amount of individual and total flavonols extracted in comparison with the untreated apple (Table 2). HPP could have caused the disruption of cell wall favouring the release of phenolic compounds, as the increase of total pectin and esterification degree could indicate (Table 1) (Xu et al., 2016). Similar HPP treatment (400 MPa/25 °C/5 min) also increased the total flavonol content (10.7%) in onion (Fernández-Jalao et al., 2017).

In general, the release of individual and total flavonols in comparison with their concentration in the apple product prior to digestion, decreased stepwise from oral phase (OP) to intestinal digestion phase (IDP) in both apple products (untreated and HPP-treated) (Table 3). The flavonol content in OP of the HPP-treated apple decreased between 51.4% (Q-3-galactoside) and 33.4% (Q-3-rutinoside) compared to non-digested apple (Table 3). However, the decrease of flavonols in the OP of untreated apple was lower than in HPP-treated apple, ranged between 43% (Q-3-glucoside) and 7% (Q-3-rutinoside) compared to apple prior digestion. This different behaviour found between both products could be related with the increase of soluble fiber observed in the HPP-treated apple (Table 1)

caused by the treatment that could hinder the release of quercetin glycosides in the OP. The importance of salivary digestion for determining the bioaccessibility of phenolic compounds has been highlighted (Tenore, Campiglia, Ritieni, & Novellino, 2013). In general, polyphenols rich in hydroxyl groups could bind with protein such as albumins from saliva, resulting in complexes that reduce phenolic compounds release in the OP (Bohn, 2014).

The concentration of individual and total flavonols in the gastric digestion phase (GDP) was similar ($p < 0.05$) to those found in the OP of both, untreated apple and HPP-treated apple (Table 3). In general, most of apple quercetin glycosides were resistant to the acid hydrolysis occur in the GDP and reached the small intestine intact (Bouayed et al., 2012; Correa-Betanzo et al., 2014). However, an increase of 1.5 and 2-times of quercetin aglycone concentration compared to OP has been observed in both apple products (untreated and HPP-treated), respectively, suggesting that in acid conditions of the GDP, hydrolysis of glycosidic bonds occurs. It is remarkable that the OP of HPP-treated apple presented 1.5 times higher quercetin content than in OP of untreated apple suggesting that HPP could promote the cleavage of Q-glycosides and/or the release of these compounds from the matrix in this phase. Similar result was found for untreated onion and HPP-treated onion (400 MPa/25 °C/5 min) also subjected to a dynamic GID where the Q content in the GDP was 1.8 and 2.2 times higher, respectively than in the OP (Fernández-Jalao et al., 2017).

The transition from acidic gastric to the mild alkaline intestinal digestion phase (IDP) conditions decreased significantly ($p < 0.05$) ~ 40% the amount of total flavonols compared to the non-digested samples (untreated and HPP-treated). Thus, the content of the main flavonols in the ID of HPP-treated apple, Q-galactoside and Q-rhamnoside decreased 55.5% and 51%, respectively, compared to apple prior digestion (Table 3). This

result agrees with the deglycosylation of Q-glycosides during the intestinal digestion phase (IDP) detected in studies *in vivo* but also *in vitro* (Wang et al., 2016). In fact, the concentration of Q found in the IDP of both products (untreated and HPP-treated) was 2.8 and 2 times higher than in the GDP, respectively. Previous *in vitro* gastrointestinal digestion studies with apple products did not detect Q-aglycone neither in the gastric nor in the intestinal phase perhaps due to these studies were carried out with a static GID model (Bouayed et al., 2012) meanwhile in the present study has been employed a dynamic GID model that better simulated the physiological and biochemical conditions of a gastrointestinal digestion (Thuenemann, 2015).

The majority of the Q-glycosides found in the IDP were also detected in the bioaccessible fraction (BF) albeit at lower concentrations. This drastic reduction of flavonols at the end of the digestion compared to non-digested samples has been observed after the GID of different fruits (Lucas-González et al., 2016). The bioaccessibility (%) of Q-3-galactoside, Q-3-glucoside, Q-3-arabinoside, Q-3-xyloside and Q-3-rhamnoside was higher in the BF of the untreated-apple (15%, 13%, 7%, 11% and 8%, respectively) than the HPP-treated apple (9%, 9%, 5%, and 8% respectively). Q-aglycone presented higher bioaccessibility than its glycosides, 50% in HPP-treated apple and 44% in the untreated apple. The bioaccessibility of apple Q-glycosides determined in the current study using a dynamic GID model was significantly lower than those found by Bouayed et al. (2012) with a static GID model (i.e, 46% for Q-3-galactoside, 62% for Q-3-rhamnoside and 110% for Q-glucoside). These differences could be due to the different GID model employed and procedure to obtain the bioaccessible fractions. Other important dietary source of quercetin derivatives as onion showed higher bioaccessibility for Q-glucosides than HPP-treated apples (average 51%) determined by using the same dynamic GID system (Fernández-Jalao et al., 2017). The different bioaccessibilities for Q-glycosides found in onion and

apple could be explained by the different matrix composition (potential interaction with pectins and fiber in apple products) that could be the cause of the different rheological behaviour observed during the GID of these two products (Herranz et al., 2019).

3.2.2. Hydroxycinnamic acids

Hydroxycinnamic acids (HA) are the second most abundant family of phenolic compounds of the apple products studied since it represents 21% of the total phenolic compounds. Chlorogenic acid (CGA) was the major HA representing about 83% of total HA in both, untreated apple and HPP-treated apple. The decrease of total HA (13%) in the HPP-treated apple in comparison with untreated apple could be due to the remaining activity of polyphenoloxidase (PPO) (Table 1).

The concentration of individual and total HA decreased ($p<0.05$) stepwise from oral phase (OP) to intestinal digestion phase (IDP) in both apple products (untreated and HPP-treated) (Table 3). The content of CGA in the OP was significantly reduced (40% and 23%) compared to the non-digested apple products (untreated and HPP-treated). Similar behaviour was observed for minor HA such as neochlorogenic, cryptochlorogenic and coumaroyl quinic acids in both apple products. In other products rich in HA such as artichoke (D'Antuono, Garbetta, Linsalata, Minervini, & Cardinali, 2015), the recovery index of CGA (35%) in the OP employing a static GID model was significantly lower than in the present study (60%), perhaps due to differences in the food matrix composition and/or in the type of GID model employed.

The transition from the OP to gastric digestion phase (GDP) practically did not affect the CGA content compared to the OP in both apple products (untreated and HPP-treated) but the other HA were almost completely degraded (Table 3). The stability of CGA under GDP conditions has been evidenced by other authors (D'Antuono et al., 2015).

Also, the transition from the gastric to the alkaline intestinal environment (ID) did not affect the CGA in both apple (untreated and HPP-treated) compared to GDP (Table 3). This result agrees with those obtained during the simulated GID of chokeberry juice (Bermúdez-Soto, Tomas-Barberan, & Garcia-Conesa, 2007). Other authors observed a significant increase of CGA acid in the IDP compare to GDP assuming that the alkaline conditions of ID favoured its extraction (D'Antuono et al., 2015; Barba et al., 2017).

The bioaccessible fraction (BF) showed a significant decrease of 20% and 39% of CGA concentration in the untreated and HPP-treated apple, respectively, compared to the ID (Table 3). According to these results, the bioaccessibility of CGA in HPP-treated apple (48%) was higher than in the untreated apple (37%). This high CGA bioaccessibility was also observed in other plant foods such as artichoke heads (D'Antuono et al., 2015). A drastic decrease of ~77% of coumaroyl quinic acid in the BF in both apple products was observed resulting in a low bioaccessibility (~4.5%).

3.2.3. Flavan-3-ols

Flavan-3-ols (F3O) were present in monomeric (catechin and epicatechin) and oligomeric forms (proanthocyanidins). F3O was the major family of phenolic compounds representing a 67% and 72% of the total phenolic compounds of untreated apple and HPP-treated apple, respectively (Table 3).

HPP significantly increased the concentration of catechin, procyanidin-B2 and the dimer form (9.5%, 4% and 65%, respectively), meanwhile the content of procyanidin-B1, trimer and tetramer were maintained ($p<0.05$) (Table 3). Only epicatechin concentration decreased 4% ($p<0.05$) after HPP. In consequence, HPP significantly ($p<0.05$) increased, approximately 3%, the total F3O content extracted in apple (Table 3).

All the F3O identified in the initial apple products (untreated and HPP-treated) were also found in the oral phase (OP) but in significant ($p<0.05$) lower concentrations. The decrease of the concentration of procyanidin-B2, epicatechin, trimer and tetramer was lesser in the HPP-treated apple (71%, 47%, 52% and 56%, respectively) than in the untreated apple (75%, 94%, 72% and 81%, respectively) compared to the apple products prior to digestion. These results agree with those previously observed in other apple products submitted to simulated oral phase digestion conditions (Tenore et al., 2013).

The strong acidic conditions of gastric digestion phase (GDP) produced a significant decrease ($p<0.05$) of the concentration of main F3O (procyanidin-B2 and epicatechin) respect to OP that was higher in the HPP-treated apple (81% and 67%, respectively) than in the untreated apple (55% and 67%, respectively) (Table 3). Some authors have pointed out the instability of procyanidins during the gastric digestion where are degraded to epicatechin (Zhu et al., 2002).

The intestinal digestion phase (IDP) produced a significant ($p<0.05$) reduction of the F3O in comparison with GDP (Table 3). Epicatechin concentration diminished 76% and 66% in the IDP of untreated apple and HPP-treated apple, respectively, compared to GDP. Procyanidin-B2 was not found in the IDP on untreated apple but similar concentration than in GDP for of HPP-treated apple (Table 3). This drastic reduction of F3O could be related to the behaviour of phenolic compounds under alkaline conditions that could experiment reactions of auto-oxidation, polymerization, transformation or complexes formation with proteins, fibre and/or metal ions (Bouayed et al., 2012; Zhu et al., 2002).

3.2.4. Dihydrochalcones

Dihydrochalcones group is one of the most specific compounds of apples and represented about 4% of the total phenolic compounds of the apple products (untreated and HPP-treated). Two phloretin derivatives were identified and quantified, phloretin-2'-xyloglucoside and phloretin-hexoside (phloridzin), the latter being the majority since it represents the 74% of the total dihydrochalcones. This data was in agreement to the results obtained from different apple cultivars previously published (Jakobek et al., 2013). HPP did not significantly ($p < 0.05$) affect phloridzin but slightly decreased (9%) the content of phloretin-2'-xyloglucoside. Similar results were previously reported for apple juice treated by ultra-high-pressure homogenization (100-300 MPa at 4 °C and 20 °C) (Suarez-Jacobo et al., 2011).

All the dihydrochalcones identified in the initial apple products (untreated and HPP-treated) were also found in the oral phase (OP). Phloretin-2'-xyloglucoside and phloridzin content decreased ~58% and 66%, respectively, in the OP of HPP-treated apple. Other authors have observed higher decrease for phloridzin (90%) in the OP of an *in vitro* static GID of 'Golden Delicious' apple (Tenore et al., 2013).

The GDP decreased (~37%) the phloretin-2'-xyloglucoside in both apple products (untreated and HPP-treated) compared to OP. However, phloridzin showed similar concentration than in OP of untreated apple and a decrease of 28% in HPP-treated apple compared to OP. The stability of phloridzin after the acidic conditions of GDP has been previously reported. The results of the current study did not agree with other studies that employed a static model for simulate GID where an increase of 6% and 11.5% of phloridzin concentration in GDP compared to OP has been evidenced (Bouayed et al., 2012; Tenore et al., 2013).

The transition from acidic to alkaline conditions in the IDP decreased 72% and 55% the phloretin-2'-xyloglucoside content in the untreated apple and HPP-treated apple, respectively, compared to GDP. However, the concentration of phloridzin in both products (untreated and HPP-treated) increased 3 and 2.5 times, respectively, compared to GDP. Similar results were referenced by other authors that found a phloridzin increase of 65% (Bouayed et al., 2012) and 43% (Tenore et al., 2013) in the IDP of 'Golden Delicious' apples. The exposure to phenolic compounds to the mild alkaline found in the IDP could promote several changes in the structure and chemical properties of determined apple phenolic compounds that would allow them to transform into chalcones (Quan et al., 2018).

The dihydrochalcones identified in the IDP were also detected in the bioaccessible fraction (BF) but at a significant lower concentration (~ 80%) than in the IDP (Table 3). Thus, the bioaccessibility of phloridzin was 32% in HPP-apple and 26% in the untreated apple. Greater phloridzin bioaccessibility (96%) were reported by Bouayed et al. (2012) in 'Golden Delicious' apple submitted to a static GID model.

3.2.5. Recovery index (RIX) and bioaccessibility of total flavonols, total hydroxycinnamic acids, total flava-3-ols and total chalcones

In general, the more abundant family of phenolic compounds in the different phases of a dynamic GID model (OP, GDP and IDP) were flavan-3-ols (F3O) followed by hydroxycinnamic acids, flavonols and chalcones (Table 3). However, in the bioaccessible fraction (untreated and HPP-treated), the main phenolic compounds were hydroxycinnamic acids (235 and 264 µg/g dw) and flavonols (30.19 and 29.05 µg/g dw) (Table 3).

In general, the recovery index (RIX) of total flavonols and total flavan-3-ols were higher in the GID phases of untreated apple than in the HPP-treated apple (Table 4).

Contrary to this result, total hydroxycinnamic acids in HPP-treated apple product showed higher RIX than untreated apple (Table 4). For total chalcones, the recovery index of OP and GD was similar for both products (untreated and HPP-treated). It is remarkable the high RIX for dihydrochalcones in the IDP that could be related with the increased content of these compounds in this phase.

Regarding bioaccessibility (untreated and HPP-treated), total hydroxycinnamic acids presented the higher bioaccessibility (31.10–39.78%) followed by dihydrochalcones (19.49–29.66%), flavonols (11.58–8.58%) and total phenolic compounds (9.44–10.87%). It is remarkable that HPP-treated apple products presented higher bioaccessibility than the untreated apple for total hydroxycinnamic acids, total dihydrochalcones and total phenolic compounds (Table 4).

3.3. Antioxidant capacity

Phenolic compounds are antioxidants present in plant derived foods able to neutralize the excess of reactive oxygen and/or nitrogen species produced as consequence of an unbalance with the antioxidant defences, although the exact mechanisms of action of polyphenols in the human body have not been decisively proven, but there is strong evidence that some targets such as nitric oxide metabolism and oxidative enzymes could be related with the health benefits of these compounds (Williamson, 2017).

3.3.1. Antioxidant capacity determined by DPPH[•], ABTS^{•+}, FRAP and TP-FC assays

The changes of antioxidant capacity measured by DPPH[•], ABTS^{•+}, FRAP and TP-FC during the *in vitro* dynamic gastrointestinal digestion (GID) were evaluated (Table 5). Before digestion, apples treated by HPP (400 MPa/35 °C/5 min) showed a decrease

($p < 0.05$) of the antioxidant capacity (AC) determined by DPPH[•] (16%), ABTS^{•+} (25%) and FRAP (35%) methodologies as well as Folin-Ciocalteu method for total phenolic compounds (TP-FC) (14%) compared to untreated apple (Table 5).

In general, GID caused a progressive decrease of AA (by the four methodologies) from the oral phase to the bioaccessible fraction that coincides with the decreasing of the concentration of total phenolic compounds in the different GID phases (Table 3). In general, the AA values were better preserved in the HPP-treated apple than in untreated apple during GID. Thus, in the gastric phase, the HPP-treated apple retained better the AA values (~52% in DPPH[•] and ABTS^{•+} assays and ~82% determined by FRAP and TP-FC assays) than the untreated apple (~39% in DPPH[•] and ABTS^{•+} assays and ~58% by FRAP and TP-FC assays) compared to apples prior digestion. It is remarkable the significant ($p < 0.05$) increase of 2.3 and 1.6 times in the reducing capacity values determined by TP-FC and FRAP, respectively, in the IDP of HPP-treated apple in comparison to gastric phase (Table 5). These increases of AA values could be related with the increase of total dihydrochalcones concentration observed in the GDP (Tables 3).

The bioaccessible fraction (BF) showed lowest AA values of the digestion phases (Table 5) in consonance with its lowest total phenolic content (Table 3). Also, the bioaccessible fraction (BF) of HPP-treated apple showed better retention of the AA values (14% in ABTS^{•+}, 24% in DPPH[•], 48% in FRAP and 86% in TP-FC) than the untreated apple (9.4% in ABTS^{•+}, 14% in DPPH[•], 28% in FRAP and 81% in TP-FC) (Table 5) compared to non-digested products. As the main phenolic compounds in the bioaccessible fraction were hydroxycinnamic acids (mainly chlorogenic acid) and flavonols (mainly Q-glycosides) (Table 3) these compounds could be the responsible of the AA of the BF instead of procyanidins and epicatechin (missing in the BF) as some authors suggested in non-digested apple products (Lee, Kim, Kim, Lee, & Lee, 2003). These results agree with

previous studies that showed large decreased of AA after GID in fruit juices (Bermúdez-Soto et al., 2007) and in maqui fruit (Lucas-González et al., 2016), but they do not agree with the results of Wootton-Beard et al. (2011) that indicated a relatively stability vegetable juice antioxidant **capacity** during GID, and with the results of Gawlic-Dziki (2012) that found an increase of AA after a GID of different vegetables. Significant positive correlations were found between total phenolic compounds determined by HPLC (TP-HPLC) and DPPH• (R=0.893, $p<0.01$), ABTS•⁺ (R=0.931, $p<0.01$) and FRAP (R=0.542, $p<0.01$) in HPP-treated apple (Table 6). For untreated apple, the correlation between TP-HPLC and AA (DPPH•, ABTS•⁺ and FRAP) were positive and similar (R=0.954, R= 0.974 and R=0.872, $p<0.01$) than in HPP-treated apple.

3.3.2. Effect on xanthine oxidase, lipoxygenase and catalase activity

Antioxidant enzymes such as catalase (CAT), which converts the cytotoxic H₂O₂ into molecular oxygen and water, are involved in the cell defence mechanisms of the organisms against oxidative damage. Other enzymes such as lipoxygenase (LOX) and xanthine oxidase (XO) catalyse reactions that generate free radicals and reactive oxygen species that contribute to oxidative damage that might play a role in the inflammation, ageing process and development of tumours (Gawli-Dziki, 2014). In the present study, the effect of an *in vitro* dynamic gastrointestinal digestion (GID) on the capacity of the apple powders (untreated and HPP-treated) to inhibit, activate or protect the activity of these enzymes has been evaluated and the results showed in Figure 2.

The effect of the GID of apple products on the xanthine oxidase (XO) activity was showed in Figure 2A. Between the non-digested products, the HPP-treated apple presented lower (12%) XO inhibitory capacity than untreated apple. In comparison to non-digested samples (untreated and HPP-treated), the digested products in the OP, GDP and BF decreased

significantly their capacity for inhibiting XO activity (Figure 2A). These results coincided with the decrease of the concentration of total phenolic compounds determined by HPLC (flavonols, flavanols, hydroxycinnamic acids and dihydrochalcones) that also occurred in these GID phases (Table 3). Only the digests of the intestinal phase showed higher XO inhibitory capacity than non-digested apples that agree with the high recovery index of total dihydrochalcones (mainly phloridzin) (150.94% and 90.97%) observed in this phase (Table 4). The BF presented the lowest XO inhibitory capacity of all the GID phases which coincides with the lowest total phenolic content (Table 3). The BF retained the 83% (untreated) and the 68% (HPP-treated) of the XO inhibitory capacity of the non-digested apple. These results do not agree with others that observed that plant products rich in quercetin glycosides as onion presented significant XO inhibitory activity (Nile, Nile, Keum, & Sharma, 2017).

In contrast, the gastrointestinal digestion phases (OP, GDP and IDP) presented higher lipooxygenase (LOX) inhibitory activity than the non-digested apples (Figure 2B). These results agree with other studies where GID of different vegetables and spices increased the extraction of potentially bioaccessible phenolic compounds with higher XO and LOX inhibitory capacities than the samples prior to digestion (Gawlik-Dziki, 2012, 2014). However, the BF of untreated apple presented the lowest LOX inhibitory capacity of all the GID phases although the HPP-treated apple BF maintained that found in the non-digested apple (Table 3).

The capacity to preserve the CAT activity by the samples studied was showed in Figure 2C. In the non-digested products, HPP-treated apple presented better activity for preserving CAT activity than untreated apple. However, the GID phases (OP and GDP) had lower CAT preserving activity than the samples prior digestion that agree with lower phenolic compounds content in this GID phases compared to non-digested samples. However, the

IDP showed better CAT preserving activity than the samples prior digestion (Figure 2C) that indicate the release of CAT activators compounds able to pass to the bioaccessible fraction. In general, HPP treatment did not modify the effect of untreated apple on the XO, LOX and CAT activity.

4. Conclusions

This is the first time that a dynamic model for simulate the human gastrointestinal digestion (GID) has been employed to study the stability and bioaccessibility of the main phenolic compounds and antioxidant **capacity** of apples treated by high-pressure processing throughout the different phases of the simulated digestion. The stability of phenolic compounds studied (flavonols, hydroxycinnamic acids, flavan-3-ols and dihydrochalcones) is profoundly affected during GID due to their concentration decreased stepwise from oral phase (OP) to bioaccessible fraction (BF) in both apple products (untreated and HPP-treated) although flava-3-ols was the most affected. This destruction of apple phenolic compounds during the different GID phases was also reflected in the loss of antioxidant properties such as the capacity of quenching free radicals (DPPH[•], ABTS^{•+}), the reducing capacity (FRAP and TP-FC) and the capacity for inhibiting lipoxxygenase (LOX) and xanthine oxidase (XO) activities. However, the capacity for protecting catalase activity (CAT) increased during the gastric and intestinal phases. The use of a dynamic GID model increased the release of quercetin aglycone and led to low total quercetin-glycosides bioaccessibility (~10%) compared to those described for static GID model (~50%). The influence of high-pressure processing could be reflected in the fact that HPP-treated apple compared to untreated apple presented higher recovery index for hydroxycinnamic acids (from ~52% to 67%) and higher bioaccessibility for

hydroxycinnamic acids (from 31% to 40%), dihydrochalcones (from 19.5% to 30%) and total phenolic compounds (TF-HPLC) (from 9% to 11%). Also, antioxidant capacity (AC) values were better preserved in the HPP-treated apple during digestion phases. In contrast, the positive effect of HPP increasing the extraction of flavonols (30%) was not reflected during GID due to lower bioaccessibility was observed in HPP-treated compared to untreated apple (8.58% and 11.58%, respectively). In contrast, the bioaccessible fraction of HPP-treated apple retained the 86% (TF-FC) and 48% (FRAP) of the reducing capacity, 24% (DPPH) and 14% (ABTS) of the radical quenching capacity, 68% and 100% of the XO and LOX inhibitory capacity and increased 9 times the CAT preserving activity of the non-digested apple.

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