Thermal and non-thermal processing of red-fleshed apple: how are (poly)phenol composition and bioavailability affected?

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ABSTRACT

The present study evaluated the impact of different thermal (infrared-drying, hot air-drying and purée pasteurization) and non-thermal (freeze-drying) processing technologies on red-fleshed apple (poly)phenolic compounds. We further investigated the processing effect on the (poly)phenol bioavailability in a crossover postprandial study where three subjects consumed three apple products (freeze-dried snack, hot air-dried snack and pasteurized purée). (Poly)phenolic compounds present in the apple products and their biological metabolites in urine were analyzed using liquid chromatography coupled to mass spectrometry (UPLC-MS/MS). When comparing different processes, infrared-drying caused important losses in most of the apple (poly)phenolics, while hot air-drying and purée pasteurization maintained approximately 83% and 65% of total (poly)phenols compared with the freeze-dried snack, respectively. Anthocyanins in particular were degraded to a higher extent, and hot air-dried apple and pasteurized purée maintained respectively 26% and 9% compared with freeze-dried apple snack. The acute intake showed that pasteurized purée exhibited the highest (poly)phenol bioavailability, followed by hot air-drying and freeze-dried snack, highlighting the impact of processing on (poly)phenols absorption.

In conclusion, for obtaining affordable new red-fleshed apple products with enhanced (poly)phenol bioavailability, purée pasteurization and hot air-drying represent viable techniques. However, to obtain a red-fleshed apple snack with high anthocyanin content, freeze-drying is the technique that best preserves them.

KEYWORDS: anthocyanins, bioavailability, (poly)phenolic compounds, red-fleshed apple, thermal-processing, UPLC-MS/MS.
1. INTRODUCTION

Numerous *in vivo* and *in vitro* studies have demonstrated that (poly)phenol compounds exhibit a wide range of biological effects such as lowering cholesterol,\(^1\) antiproliferative activity on cancer cells,\(^2\) and reducing the risk of suffering heart diseases, asthma and type-2 diabetes,\(^3-5\) among others. However, the dietary habits of the majority of people do not guarantee an adequate intake of fruits and vegetables, and the intake of (poly)phenols is below the amounts found to have significant health effects.\(^6\)

In recent years, due to the global interest in phytochemicals and in developing functional foods that are fortified, enriched, or enhanced with improved health-promoting effects, there has been growing interest in the development of commercial red-flesh apple cultivars. These apple cultivars have been obtained by traditional breeding methods from new hybrids with red pulp.\(^7\) The flesh of these singular apple varieties has an enhanced content of anthocyanins (red colour) with high antioxidant activity and potential health-promoting effects.\(^8\) Besides, through crossbreeding programs with good-flavoured white-fleshed apples, the poor taste of the wild red-flesh varieties has been improved.\(^9\) So, these new apple varieties could make healthy eating easier and more available, thus satisfying the increasingly widespread needs for high food quality and diversity.

Since the production of apple fruit is seasonal, processing methods have been developed and applied to obtain apple derived products with shelf-stability and increase useful life while minimizing changes in the quality attributes.\(^10\) Dehydration is one of the main and oldest techniques for preserving agricultural and food products, and in recent years for producing ready-to-eat and healthy *snacks* from fresh fruit such as
apples, while retaining their nutrients and bioactive compounds and thus, being a healthier alternative to salty or sugary snacks.11,12

In the preparation of functional and ready-to-eat foods, freeze-drying method is commonly used as there is a minimum loss of flavour, aroma and bioactive compounds, with near-perfect preservation results. However, due to its expensive cost, other dehydration methods such as hot air-drying or infrared-drying are employed to produce high-quality dried fruits.11,13,14 Pasteurization of fruit purée is another commercial and common thermal treatment used to increase shelf-life of fresh fruits. In addition fruit purée can be used as an intermediate product for the production of other products such as nectars, juices with solid particles or smoothies.15-17

In most dehydration methods, such as hot air-drying or infrared-drying, vegetables are subjected to high temperatures at which highly thermosensitive and unstable (poly)phenols can be readily degraded.18-22 However, it has also been shown that food processing can induce chemical or physical modifications such as degradation or modification of cell wall polysaccharides or proteins, molecular interactions between components, and other food matrix factors that enhance (poly)phenol bioaccessibility and bioavailability during digestion.23,24

Therefore, the first aim of the present research was to evaluate the impact of four different processing conditions (infrared-drying, hot air-drying, freeze-drying and pasteurization of purée) on apple (poly)phenols stability. Secondly, we also investigated the effect of the processing conditions on the apple (poly)phenol bioavailability in a human pilot study in order to search the optimal conditions to obtain an apple snack product with a higher (poly)phenol bioavailability and, thus, enhancing its functional value.

2. MATERIALS AND METHODS
2.1. Chemicals and Reagents

Pelargonidin-3-\textit{O}-glucoside, cyanidin-3-\textit{O}-glucoside, cyanidin-3-\textit{O}-galactoside, delphinidin-3-\textit{O}-glucoside, malvidin-3-\textit{O}-glucoside, hydroxytyrosol, luteolin, kaempferol, eriodictyol, quercetin, luteolin-7-\textit{O}-glucoside, kaempferol-3-\textit{O}-glucoside, quercetin-3-\textit{O}-rhamnoside, quercetin-3-\textit{O}-glucoside, isorhamnetin-3-\textit{O}-glucoside, procyanidin dimer B₂, quercetin-3-\textit{O}-rutinoside (rutin), myricetin, and phloretin-2-\textit{O}-glucoside were purchased from Extrasynthese (Genay, France). \textit{p}-Hydroxybenzoic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), \textit{p}-coumaric acid, gallic acid, caffeic acid, ferulic acid, chlorogenic acid (5-caffeoylquinic acid), naringenin, catechin, and epicatechin were acquired from Sigma-Aldrich (St. Louis, MO, USA). The vanillic acid was from Fluka (Buchs, Switzerland). Methanol (HPLC grade), acetonitrile (HPLC grade), acetic acid, and formic acid were purchased from Scharlab Chemie (Sentmenat, Catalonia, Spain). The water was of Milli-Q quality (Millipore Corp., Bedford, MA, USA).

Stock solutions of standard compounds were prepared by dissolving each compound in methanol at a concentration of 1 g/L and storing it in dark flasks at -80 °C.

2.2. Plant Material

The red-fleshed apple variety used was ‘Redlove Era’. The apples were provided by NUFRI SAT (Mollerussa, Lleida, Spain) and planted in the “La Rasa” experimental plot (La Rasa, Soria, Spain).

2.3. Non-thermal apple processing: freeze-drying

Before drying, the apples were washed, wiped with paper towels and cut into 1 cm-sized cubes. The apple cubes were frozen in liquid nitrogen and the freeze-drying
was then performed at 0.6 bar with a temperature ramp of -20 to 0°C over 25 hours, followed by a second complete vacuum drying with a temperature ramp of 0 to 20°C over 40 hours (Lyophilizer TELSTAR Lyobeta 15, Terrassa, Spain).

2.4. Thermal apple processing

2.4.1. Hot air-drying: apples were washed, wiped with paper towels and cut into 1 cm-sized cubes. The apple cubes were immediately dried in a pilot dryer composed of a cylindrical stainless-steel basket where the sample was introduced, an electric resistance of 8 kW to heat the air to the desired temperature and a Casals model MA 26 M 2H fan (Kv = 0.865 Cv) (Casals, Girona, Spain) connected to the speed variation system. The drier was equipped with a dashboard with an Eliwell thermostat (Protherm controls, Atherstone, England) to control the air temperature and speed of the fan, and a Vaisala hygrometer equipped with a HMP 125B probe (Vaisale, Vantaa, Finland) that controlled the relative humidity of the air at the entrance and exit of the drying equipment. Finally, the control for the velocity of the air entering the basket containing the apple cubes consisted of a WM-DA 4000 turbine anemometer (Pacer instruments, Keene, United States) and several valves. The apple cubes were placed in the basket and hot air without recirculating was passed through it. Drying was carried out until a constant weight (weighing the basket with the apples on an electronic balance mod. EM-60 KAM, A&D Company, Tokyo, Japan) at 60°C for 80 minutes with an air velocity of 1.5 m/s and then the temperature was increased until 70°C and maintained for 40 minutes with the same air velocity.

2.4.2. Infrared-drying: the infrared-drying process was carried out in an infrared moisture analyzer mod. IRCDi5 FIR dehydrator (Irconfort, Seville, Spain). A thin layer
of the apple cubes was placed inside an aluminium support equipped with a precision balance to record the weight. The continuous weighing of the sample allowed the drying kinetics to be determined. The infrared-drying experiments were automatically stopped when the weight of the cubes remained constant. Several tests were performed for a range of infrared-drying temperatures (35, 40, 50 and 60ºC) and the sample mass versus time was recorded. In all the cases, the product weight, initial moisture content and dry matter content of the apple cubes were used to calculate the moisture content obtained at any drying time.

After the completion of the different drying processes, the dried apple cubes were immediately transferred to airtight plastic containers and kept at -80ºC until the (poly)phenol chromatographic analysis. Prior to analysis, a fine powder of the dried apple samples was obtained with the aid of an analytical mill (A11, IKA, Germany).

2.4.3. Pasteurized apple purée: red-fleshed apples were supplied to a local company (Anela Fruits, Girona, Spain) to be processed to pasteurized purée. Briefly, apples were milled to a fine purée which was hermetically closed into sterile containers and submitted to continuous pasteurization in a tubular system (94ºC for 10 min).

2.5. Apple phenol bioavailability human study

2.5.1. Subjects and study design

The protocol of the study was approved by the Ethical Committee of the Human Clinical Research Unit at the Arnau Vilanova University Hospital, Lleida, Spain (Approval Number: 13/2016). In this study the kinetics of phenolic metabolites in 24 h urine in different interval times (0-2, 2-4, 4-8, and 8-24 h) were monitored in healthy
volunteers after the acute ingestion of freeze-dried apple, hot air-dried apple and pasteurized apple purée. The study was performed in a crossover design to reduce interpersonal variability. The volunteers comprised three healthy women, aged from 24 to 38 years, with body mass index between 19.4 and 25.1 kg/m². They declared no gastrointestinal alterations and reported no antibiotic use over the last months before the study. To standardize the baseline point, subjects were asked to follow a low-(poly)phenol diet during the 2 days preceding each dietary intervention and during the dietary intervention day. For this, subjects were asked to limit fruit and vegetables consumption, and to avoid cherries, strawberries, blueberries, tea, coffee, wine, beer, chocolate and all their derived products.

Each volunteer received in random order the three apple products in a crossover design with a washout period of 14 days between interventions. The amount of each administered product was: 60 g of freeze-dried apple snack, 66 g of hot-air apple snack and 500 g of apple pasteurized purée, which represented a similar phenolic dose (134±18 mg total phenols) (Supplemental Table 1).

Urine samples were collected 24 h before (basal conditions) and at the interval times of 0-2, 2-4, 4-8, and 8-24 h after the apple products intake. The volume of urine in each interval was measured and aliquots were stored at -80°C prior to the (poly)phenol chromatographic analysis.

2.6. Analysis of phenolic compounds in apple products and phenolic metabolites in urine

2.6.1. Sample Pre-treatment

The pre-treatment for the analyses of the phenolic compounds in apple products was carried out according to Bars-Cortina et al. (9) with some modifications. Briefly, 0.4
9 g of dried apple powder or 1.6 g of lyophilized purée (the lyophilization parameters were the same as those detailed in section 2.3.) was weighed and extracted with 10 mL of methanol/Milli-Q water/formic acid (79.9:20:0.1, v/v/v). The samples were vortexed for 10 min and then centrifuged at 8784 g for 10 min. The extraction was repeated three times and the supernatants were collected, combined and filtered through a 0.22 μm PVDF filter (Scharlab, Barcelona, Spain) prior to the chromatographic analysis. Samples were analyzed in triplicate.

The urine samples were pre-treated by µSPE. The micro-cartridges and their conditioning and equilibration steps were the same as reported in our previous study. In this case, 100 μL of phosphoric acid at 4% were added to 100 μL of the urine sample, and this solution was loaded into the micro-cartridge. The retained phenolic compounds were then eluted with 2 x 50 μL of methanol. Each sample was prepared in triplicate.

2.6.2. Ultra-performance Liquid Chromatography Coupled to Tandem Mass Spectrometry (UPLC-MS/MS)

Liquid chromatography analyses were carried out on an AcQuity Ultra-Performance liquid chromatograph coupled to a tandem mass spectrometer from Waters (Milford, MA, USA).

Two chromatographic methods using UPLC-MS/MS were used for the analysis of 1) anthocyanins and their metabolites, and 2) the rest of the phenolic compounds and their metabolites. In both methods, the flow rate was 0.3 mL/min, and the injection volume 2.5 μL. The UPLC-MS/MS conditions were the same used in our previous study. Briefly, for the analysis of (poly)phenolic compounds (including anthocyanins), the analytical column used was an AcQuity BEH C18 (100 mm × 2.1 mm i.d., 1.7 μm) equipped with a VanGuard PreColumn AcQuity BEH C18 (5 × 2.1 mm, 1.7 μm), also
from Waters. For the analysis of anthocyanins and their metabolites, the mobile phase was 10% acetic acid (eluent A) and acetonitrile (eluent B). The elution gradient was 0−10 min, 3−25% B; 10−10.10 min, 25−80% B; 10.10−11 min, 80% B isocratic; 11−11.10 min, 80−3% B; 11.10−12.50 min, 3% B isocratic. For the analysis of the rest of the (poly)phenolic compounds and their metabolites, the mobile phase was 0.2% acetic acid (eluent A) and acetonitrile (eluent B). The elution gradient for the analysis of these (poly)phenolic compounds was 0−5 min, 5−10% B; 5−12 min, 10−12.4% B; 12−18 min, 12.4−28% B; 18−23 min, 28−100% B; 23−25.5 min, 100% B isocratic; 25.5−27 min, 100−5% B; and 27−30 min, 5% B isocratic.

Tandem MS analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters, Milford, MA, USA) equipped with a Z-spray electrospray interface. Ionization was achieved using the electrospray (ESI) interface operating in the positive mode \([M−H]^+\) for the analysis of anthocyanins and in the negative mode \([M−2H]^-\) for the other compounds. The data were acquired through selected reaction monitoring (SRM). The ionization source parameters were as follows: capillary voltage, 3 kV; source temperature, 150 °C; desolvation gas temperature, 400 °C, with a flow rate of 800 L/h. Nitrogen (99.99% purity, N2LCMS nitrogen generator, Claind, Lenno, Italy) and argon (≥99.99% purity, Aphagaz, Madrid, Spain) were used as the cone and collision gases, respectively. The dwell time established for each transition was 30 ms. Data acquisition was carried out with MassLynx 4.1 software.

Due to the lack of commercial (poly)phenolic standards and their generated metabolites, some of the compounds were tentatively quantified by using the calibration curve of their precursor or of a (poly)phenolic compound with a similar structure. **Supplemental Table 2** shows the selected reaction monitoring (SRM) conditions as well as its cone voltage and collision energy used for the quantification of these compounds.
(poly)phenolic compounds. This table also shows in which (poly)phenolic standard compound, these (poly)phenolics have been quantified.

2.7. Statistical Analysis

Concentration values of the (poly)phenolic compounds and their metabolites studied were reported as means ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey’s test at a level of 0.05 were used to determine the significance of differences among the different apple processing techniques, among the urine excretion of the different (poly)phenolic families after the intake of the three red-fleshed apple products studied, and among intra- and inter-individual differences in this (poly)phenol excretion. Moreover, General Linear Model and One-way ANOVA at a level of 0.05 was used to determine the significance of the % excretion of total (poly)phenols after the intake of the three products derived from red-fleshed apple. All data were analyzed with the Minitab Statistical Software, version 17.2.1 (Minitab Inc., State College, Pennsylvania, United States).

3. RESULTS AND DISCUSSION

3.1. Effect of apple processing on the phenol stability

The aim of the present study was to explore the potential of new red-fleshed apple varieties, as anthocyanin enriched alternatives (biofortified) to common white-fleshed apples for the development of an apple snack product with an enhanced functional value. So, the first objective of this research was to evaluate the impact of different processing technologies on the (poly)phenols stability of red-fleshed apple and, therefore, to search the optimal conditions to develop an apple snack product with a
high shelf-stability maintaining at the same time a high concentration of bioactive (poly)phenols. The different products are shown in Figure 1.

A total of 26 (poly)phenols were identified in the red-fleshed apple products using UPLC-MS/MS (Table 1) and included compounds from the following 6 (poly)phenol classes (Figure 2): i) Phenolic acids, ii) Flavan-3-ols, iii) Flavonols, iv) Anthocyanins, v) Flavanones, and vi) Dihydrochalcones.

The impact of the different processes on the stability of the (poly)phenols in red-fleshed apple products was compound-dependent and some (poly)phenols were degraded more than others (Table 1). In general terms as seen in Figure 2, when comparing the different processes, infrared-drying caused important losses in most of the apple (poly)phenolics, while hot air-drying and purée pasteurization allowed the maintenance of approximately 83% and 65% of the (poly)phenols compared with the freeze-dried snack, respectively. In accordance with the literature, 5-O-caffeoylquinic acid (chlorogenic acid) (Table 1) was the most abundant phenolic acid and the main phenolic compound detected in the apple samples (representing 90%-95% of the total phenolic acids and 60-70% of the total phenolic content). The group of phenolic acids was significantly influenced by the dehydration technique used, freeze-drying and hot air-drying being the techniques that preserves them best (Figure 2). The total content of phenolic acids was 1661 mg/kg d.w. in freeze-dried apple samples, which was reduced to 1587 mg/kg d.w. in hot air-dried and followed by the values found in the pasteurized purée (1187 mg/kg d.w.), while infrared-dried samples presented the lowest amounts ranging from 474 to 690 mg/kg d.w. (Table 1).

In this study, four flavan-3-ols were detected and quantified in the apple samples. The freeze-dried samples contained the highest amounts (197 mg/kg d.w.), followed by the hot-air dried (101 mg/kg d.w.) and the pasteurized purée (80.9 mg/kg...
In accordance with Bars-Cortina et al.\(^{(9)}\), the most abundant flavan-3-ol was a epicatechin dimer followed by epicatechin and a trimer. In the infrared-dried apple samples, all flavan-3-ols were found in significantly lower concentrations and the trimer was not detected in any of the infrared temperatures studied. Compared to phenolic acids, flavan-3-ols showed higher losses due to thermal treatment with losses of approximately 49% and 59% in hot air-dried apples and pasteurized purée, respectively and between 87% and 93% in infrared-dried apples with respect to freeze-dried apples.

Regarding flavonols, the most abundant were those derived from quercetin, in accordance with the literature,\(^{(26)}\) being quercetin-rhamnoside the most abundant in all the apple products. Apart from these, other flavanols, such as dihydroquercetin and quercetin arabinoside were detected in all samples (Table 1). Quercetin was only detected in the hot air-dried and pasteurized purée samples. Furthermore, quercetin glucoside and dihydrokaempferol glucoside were not detected in the infrared-dried samples. As in the previous (poly)phenolic groups, the treatments that best preserved flavonol group, when comparing with the non-thermal freeze-drying method, were hot air-drying and purée pasteurization.

Regarding anthocyanins, the most distinctive (poly)phenolics in red-fleshed apple cultivars, cyanidin-3-\(O\)-galactoside and cyaniding arabinoside were detected in all the apple samples (Table 1). According to the literature,\(^{(9,27,29)}\) cyanidin-3-\(O\)-galactoside was the most abundant anthocyanin detected and quantified in the red-flesh apple samples (around 90% of the total anthocyanin content). Concerning the impact of the different treatments, results showed that anthocyanins were degraded to a higher extend compared to other (poly)phenolic compounds. Results showed that freeze-drying is the technique that statistically best preserves these compounds (285 mg/kg d.w) followed...
by hot air-drying (73.2 mg/kg d.w.) and purée pasteurization (26.2 mg/kg d.w.). In the
infrared-dried samples, these compounds were degraded almost completely with
observed losses of > 98% when comparing with freeze-drying.

Other groups of (poly)phenols influenced by the processing technique applied
were the flavanones and dihydrochalcones (Table 1). In the flavanone group, only
naringenin-glucoside and eriodictyol-hexoside were detected as reported in the literature
26 and the processes that statistically best preserved them were freeze-drying and purée
pasteurization with total values of 15.7 and 15.2 mg/kg d.w., respectively. Within the
group of the dihydrochalcones, phloretin-xylosil-glucoside was the predominant
compound. At a general level, this (poly)phenol group is the least affected by the apple
transformation, and the total values of non-thermal (freeze-drying), and some thermal
(hot air-drying, infrared-drying at 40ºC and pasteurized purée) treatments show no
statistically significant differences. Our results are in accordance with a previous
study,30 confirming that apple dihydrochalcones are more stable against the application
of high temperatures than other (poly)phenol classes, such as flavan-3-ols and
anthocyanins.

Considering the freeze-dried samples as reference, infrared-drying (at all
temperatures) caused significant (p < 0.05) losses in most of the apple (poly)phenols,
while (poly)phenol losses by hot air-drying and by the purée pasteurization were
considerably lower. This fact is probably a consequence of the intense time/temperature
treatment applied in the infrared processes and to a lesser extend in hot air-drying or in
the production of the pasteurized purée, in which the treatment at high temperatures
lasts only a few minutes. Thermal treatment can cause severe degradation of
(poly)phenolic compounds as it is well known that these compounds are temperature
sensitive.18-22 This fact is also reflected in infrared-dried samples where those subjected
to higher temperatures (50°C and 60°C) showed the greatest phenol degradation/losses. Moreover, the losses could also be due to the presence of oxygen in the case of infrared-drying and hot air-drying producing an oxidative degradation of the (poly)phenols and consequently the browning of the apple samples (Figure 1). (Poly)phenols are the desirable substrates of oxidoreductive enzymes such as phenoloxidases, whose main function is to oxidized phenols.\textsuperscript{18,28} These enzymes catalyze the oxidation of $\alpha$-diphenols into quinones, which polymerize to form brown melanin pigment.\textsuperscript{31} In freeze-dried samples, these reactions do not occur because the freezing and subsequent sublimation of water under vacuum conditions prevents the action of these enzymes and consequently the browning of the samples.\textsuperscript{32} However, although this process efficiently preserves bioactives, freeze-drying costs can be 2-5 times higher than hot air-drying in order to achieve the same final moisture content. This fact justifies the need to find alternatives for the production of economically affordable healthy food for everyone.\textsuperscript{33} In this sense, when comparing the different dehydration techniques for preparing healthy apple snacks preserving the content of (poly)phenols (Figure 2), infrared-drying showed high losses of 57-74% depending on the temperature. On the other hand, hot air-drying and purée pasteurization allow the maintenance of 83% and 65% respectively of (poly)phenols quantified in the freeze-dried snacks and may be good alternatives to the costly freeze-drying technique.

3.2. Effect of apple processing on the phenolic bioavailability: human pilot study

The second aim of the present work was to study how apple processing impacts on the release and absorption of (poly)phenols present in the apple products and determine the optimal processing technique that improves (poly)phenol bioavailability.
For this, the apple products with the highest (poly)phenolic contents (freeze-dried apples, hot air-dried apples and pasteurized apple purée) were chosen to conduct a crossover acute intervention study with three subjects. The amounts of each product administered to the subjects were selected to match as best as possible the total content of phenolic and the contents of the different phenolic subclasses (Supplemental Table 1).

3.2.1. Effect of apple processing on phenolic compounds bioavailability

A total of 59 phenol metabolites were identified and quantified in urine samples after the intake of the three red-fleshed apple derived products, including derivatives of benzoic acids, phenylpropionic acids, phenylvalerolactones and catechols (major groups) (Figure 3 a, b, c and Supplemental Table 3), and anthocyanins, flavan-3-ols, dihydrochalcones and hydroxycinnamic acids (minor groups) (Figure 3 d, e, f and Supplemental Table 3). The phenolic metabolites were mainly phase-II sulfated, glucuronidated and/or methylated conjugates of parent compounds present in the apples as well as of microbial catabolites resulting from colonic degradation (Supplemental Table 4). Excretion kinetics for each of these (poly)phenolic groups expressed as µmol/h are shown in Supplemental Figure 1 and Supplemental Figure 2.

Our data show intra-individual, inter-individual and processing-derived differences among the three products. Large inter-individual differences in urine metabolite concentration were found between the three volunteers, with highest excretion found in volunteer 1 (V1) followed by volunteer 3 (V3) and volunteer 2 (V2) (Figure 3 and Supplemental Table 3). This trend was repeated in each of the three apple products (Supplemental Table 3) and for almost all phenolic groups (except for anthocyanins which are discussed separately).
Regarding the processing effect, pasteurized purée showed significantly higher total (poly)phenol bioavailability in all three volunteers, followed by the hot air-dried red-fleshed apple and finally, the freeze-dried red-fleshed apple showed the lowest. Figure 4 shows the % excretion of (poly)phenols in urine, which have been calculated as the average between the three volunteers comparing the sum of (poly)phenols ingested and the sum of the moles excreted in 24 h urine. This was observed for almost all (poly)phenolic groups except for anthocyanin and flavan-3-ols derivatives. It is of interest to note that although freeze-drying is the method that best preserves (poly)phenolic compounds during processing, it was the product with the lowest % bioavailability, while pasteurized purée, with the highest losses during processing, showed the highest % bioavailability.

The results obtained corroborate the notion that the apple processing could enhance (poly)phenols bioavailability. Chemical structure, concentration and matrix interactions are the three basic pillars that govern bioaccessibility and bioavailability of (poly)phenolic compounds from fruits. It has been shown that food processing can influence and alter all these three factors.\textsuperscript{34} Regarding thermal processing, it has been reported that it can promote the (poly)phenol release disrupting intracellular barriers, thus modulating (poly)phenol bioaccessibility for example in tomatoes,\textsuperscript{24} in which it was found that human plasma levels of naringenin and chlorogenic acid, increased several times after the intake of cooked tomatoes compared with levels observed after consumption of fresh tomatoes. This fact was also verified in a recent study\textsuperscript{35} in which authors observed that after the administration of similar doses of flavan-3-ols through three apple products (phenolic extract, raw apple or apple purée) in hypercholesterolemic pigs, higher number of genes were modulated by purée than raw apples, which suggests that the processing of apples into purée increased the
bioavailability of some phytochemicals, as flavan-3-ols, that could contribute to the postprandial nutrigenomic response.

Apart from this, it has also been shown that liquid foods possess a lower viscosity and pass through the stomach more rapidly than solid food. This is due to a higher water content, and typically lower content in proteins or complex carbohydrates that may bind to (poly)phenols. Several studies have reported rapid absorption of (poly)phenols from liquid foods such as coffee or apple products. This latter study reported 3-fold higher quercetin plasma concentration after consumption of apple sauce than after vacuum-impregnated apple chips or apple peel extract capsules. Moreover, in this study the different treatments also resulted in a high inter-individual variability of all plasma pharmacokinetic parameter after equal intake of quercetin equivalents of the apple product types.

The high inter- and intra-individual variation in the response to (poly)phenolic intake, which in many cases leads to contradictory results in human trials, could result from intrinsic aspects such as genetics, age, sex and physiological or pathological states, in addition to the matrix effect already mentioned. For example, it has been shown that genetic polymorphisms between individuals affect the efficacy of bioactive compounds, since they differentially affect genes that encode enzymes involved in the metabolism of these compounds.

Moreover, only small amounts of ingested (poly)phenols are absorbed in the upper gastrointestinal tract (GIT), while most compounds reach the lower GIT unmodified, where they undergo extensive metabolism mediated by the colonic microbiota. Thus, a key role is played by the gut microbiota, which may modify the structure of (poly)phenols, releasing lower molecular weight colonic catabolites that can be absorbed more easily. Each human has a unique gut microbiota that changes
throughout life, and the environment, diet and lifestyle, all influence the microbiome.\textsuperscript{41,42} In this sense, the existence of metabotypes (metabolic phenotype with specific gut microbiome-derived metabolites that characterize the metabolism of the parent compound) in the production of phenolic metabolites has been discussed almost exclusively in recent years. It was observed that the benefits associated with the ingestion of foods rich in ellagitannins, such as pomegranate and walnuts may be related to specific metabotypes.\textsuperscript{43-45} In our study, most of the apple (poly)phenol metabolites are of colonic origin while only a minority were parent compounds and their phase II conjugates from upper GIT absorption (Supplemental Figure 3 and Supplemental Table 4). Furthermore, despite having only three volunteers in our study, “low” (V2) and “high” (V3) excretors can be observed. It should be stressed that although V2 showed in all three products the lowest (poly)phenol bioavailability, this was greatly improved after the ingestion of apple purée.

Supplemental Figure 3 shows the representation of the phenolic groups detected after each ingestion for each volunteer, separated into those absorbed in the upper GIT (dihydrochalcone, flavan-3-ol, and anthocyanin parent compounds and their phase II metabolites) and in the lower GIT (simple phenolic acids). The major groups of upper GIT absorption were dihydrochalcone and flavan-3-ol derivatives while phenylpropionic acid derivatives were the major ones among the metabolites that are absorbed in the lower GIT. Similar results were observed in our previous study in which ten volunteers ingested 80g of freeze-dried red-fleshed apple snack.\textsuperscript{25}

It is of note that in the present study we observed intra-individual differences in the proportions of the different metabolite groups after the ingestion of the three apple products. If the observed differences depend on the apple processing or, more probable, on intrinsic aspects of each individual should be the focus of further studies.
The inter-individual differences are also shown here, since for V1 and V3 the (poly)phenolic groups that are absorbed in the upper GIT (dihydrochalcones, flavan-3-ols and anthocyanins) are greater in the freeze-dried snack, while for V2 it is greater in the hot air-dried red-fleshed snack (Supplemental Figure 3). Interestingly, in no case was the bioavailability of the (poly)phenols absorbed in the upper GIT greater after ingesting the purée as might be expected, since in the solid apple matrix of the freeze-dried snack and the hot air-dried snack these (poly)phenolics are bound to cell walls by covalent bonds between (poly)phenolics and polysaccharides possibly restricting bioavailability in the small intestine. Besides, genetic variation between individuals for enzymes involved in the absorption and metabolism of these groups in the gut epithelium and/or liver may result in large differences in the expression of a functional enzyme which might explain the observed inter-individual differences.

Regarding the metabolites found after the ingestion of the three apple products, the majority were sulphate and methyl-sulphate conjugates representing between 61%-83% and 7-27% of the total (poly)phenol metabolites detected, respectively (Figure 5 and Supplemental Table 4). The rest of the (poly)phenol metabolites (glucuronide, methyl, glycine, sulphate-glucuronide and methyl-glucuronide conjugates, and free acids/parent compounds) varied considerable between volunteers and between the type of the ingested apple product showing again great inter- and intra-individual differences. The differences in human subjects’ genetics regarding digestive enzymes, intestinal transporters, phase I and II metabolism or tissue carriers, and also differences in gut microbiota composition and functionality affecting the catabolism of the not absorbed (poly)phenols in the small intestine being responsible of the differences observed.

Although it is a preliminary study, our bioavailability results show large differences in the concentrations of metabolites (inter-individual differences) as well as
in the metabolic profile depending on the type of product ingested for each person (intra-individual differences), which could result in different effects on health. This fact justifies the need to carry out future studies with a greater number of volunteers to be able to address these differences. Nevertheless, an important limitation of this study is the lack of some of the authentic standards to quantify more accurately the concentrations of some (poly)phenol metabolites and thus the real bioavailability % of red-fleshed apple products.

3.2.2. Effect of apple processing on anthocyanin bioavailability

In this study, the effect of apple processing on anthocyanin bioavailability was of special interest since this phenolic group is the most characteristic of the red-fleshed apple cultivars and is not found in the pulp of any common white-fleshed apple variety. This (poly)phenol group was however, the most affected by the processing treatments (Table 1) and, consequently, different amounts of anthocyanins were consumed (2, 4 and 16 mg) after the acute intake of pasteurized purée, hot air-dried and freeze-dried apple products, respectively (Supplemental Table 1). The results showed (contrary to what happens for the other (poly)phenolic groups, Figure 4) that a higher average excretion in urine of anthocyanins was observed after the intake of hot air-dried apple (0.07%) while for the freeze-dried apple and pasteurized purée it was 0.04% and 0.03%, respectively (data not shown). These excretion rates have been calculated as the average of the three volunteers and comparing the total ingested anthocyanins with the total anthocyanins and their phase II metabolites excreted in 24 h-urine. It should be noted that these observed differences are very small and probably, if more volunteers had participated in this study, the differences would had shown no significant differences between products. Moreover, although the bioavailability of anthocyanins is very low
compared to other (poly)phenolic groups, most of these compounds pass to the colon
where they are degraded by the microbiota to simpler phenolic acids that are common to
other (poly)phenol groups and once reabsorbed they contribute to the pool of circulating
phenolic metabolites in the body.\textsuperscript{48,51}

The fact that anthocyanins were more bioavailable in the solid matrices (hot air-
dried and freeze-dried) than in the pureé, may be due to the fact that these compounds
are very unstable and more easily degraded. Thus, in the solid matrix, they could remain
more attached to the fiber that may stabilize them or offer protection against further
reactions until the site of absorption is reached. Our results could be in agreement with a
previous study reporting that when raspberry extract was digested \textit{in vitro} with
foodstuffs (bread, breakfast cereals or ice cream) higher proportions of anthocyanins
were bioaccessible compared to the extract digested alone.\textsuperscript{52}

Finally, \textbf{Supplemental Figure 2} shows the urine excretion kinetics of
anthocyanins after the apple products intake expressed as total nmols of anthocyanins
and their phase II metabolites excreted per hour. The higher concentration observed in
the freeze-dried format is due to the fact that the anthocyanin dose administered with
this product was higher. In all cases, similar kinetics were observed with a maximum
excretion between 2-4 hours, which is in agreement with previous \textit{in vivo} studies
reporting that anthocyanins are absorbed in the stomach and the small intestine with
rapid detection of intact anthocyanin glycosides in urine and plasma within 30 to 60 min
of ingestion.\textsuperscript{48,51}

\section{4. CONCLUSIONS}

Our findings revealed that, considering the freeze-drying as a reference
technology to preserve food bioactives, infrared-drying at all temperatures caused
significant losses in all the red-fleshed apple (poly)phenols, while (poly)phenol losses
by hot air-drying and the purée pasteurization were considerably lower. Anthocyanins in
particular were degraded to a higher extend after all thermal processing technologies.
So, we conclude that for obtaining red-fleshed apple products affordable for the
consumer, hot air-drying and purée pasteurization represent interesting technologies to
obtain apple products with a high shelf-stability maintaining at the same time a high
concentration of bioactive (poly)phenols. However, to obtain a product with the highest
anthocyanin content, the extra cost of freeze-drying would have to be assumed.

Results obtained from the human postprandial crossover study showed that when
comparing the total ingested (poly)phenol dose and the urinary excreted amount, the
pasteurized apple purée proved to be the processing with the highest bioavailability,
followed by the hot air-dried apple and the freeze-dried apple. Further, a great intra- and
inter-individual variability between the metabolites was found, which highlights the
importance of characterizing the metabotypes in future studies.

The present study is a proof of concept to select the most appropriate apple
processing to preserve the apple (poly)phenolic compounds and provides further
evidence on how food processing plays a significant role in the bioavailability of
(poly)phenols, which is a step forward towards the design of healthier foods.

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REFERENCES


Figure Captions

Figure 1. Different red-fleshed apple products used in this study obtained by: a) freeze-drying, b) hot air-drying, c) infrared-drying d) or by a process of purée pasteurization.

Figure 2. Impact of the different thermal and non-thermal processes on the (poly)phenolic groups in red-fleshed apple products. The letters express statistically significant differences between the content of the total (poly)phenol classes content among the processing technologies ($p < 0.05$).

Figure 3. Total major and minor metabolite excretion after intake of freeze-dried apple, hot air-dried apple and apple pasteurized purée. Data expressed as mean values ± standard deviation. Different lowercase letters: indicates differences between volunteers in excretion of total major or minor metabolites after the intake of freeze-dried red-fleshed apple. Different capital letters: indicates differences between volunteers in excretion of total major or minor metabolites after the intake of hot air-dried red-fleshed apple. Different numbers: indicates differences between volunteers in excretion of total major or minor metabolites after the intake of red-fleshed apple pasteurized purée. The symbols *, +, and # indicate differences between the 3 intakes for the same volunteer (One-way ANOVA, Tukey’s test between all means, $p < 0.05$).

Figure 4. % Excretion in urine of total (poly)phenols after the intake of freeze-dried red-fleshed apple, hot air-dried red-fleshed apple and red-fleshed apple pasteurized purée (n=3). This % was calculated as the ratio of total moles of excreted (poly)phenolic metabolites with respect to the total moles of ingested phenolic
compounds. Different letters indicate significant differences in excretion between products (General Linear Model, and One-way ANOVA, $p < 0.05$).

**Figure 5.** Schematic representation (% of each group over the total) of the main phase II metabolites, free acids and parent compounds found in urine in each volunteer after the intake of freeze-dried red-fleshed apple, hot air-dried red-fleshed apple and red-fleshed apple pasteurized purée.