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High hydrostatic pressure assisted by food-grade enzymes as a sustainable approach for the development of an antioxidant ingredient

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ABSTRACT

Apple is one of the most important fruits in the world. The elaboration of apple-based products such as cider or apple juice generates around 3–4.2 million tonnes of apple by-product that are annually discarded. Apple by-product has been described as an interesting source of bioactive compounds, including polyphenols. These valuable components, once extracted, could enable the usage of the apple by-product as a natural antioxidant ingredient. The aim of the present work was to address the effect of high hydrostatic pressure (HHP), and HHP assisted by two alternative food-grade enzymes, Celluclast®, and Ultraflo®. For that purpose, the total phenolic content was measured by Folin-Ciocalteu and Fast-Blue BB assays. Furthermore, antioxidant capacity was determined by three analytical methods (DPPH, ORAC and FRAP) and the characterization of phenolic compounds was carried out by Chromatography and Mass Spectrometry analysis. Results indicated a significant increase of the total phenolic content and the antioxidant capacity after the HHP aided by Celluclast® or Ultraflo® treatment. Conversely, the exclusive application of HHP induced minor changes. Thus, the combination of HHP and the food-grade enzymes Celluclast® or Ultraflo®, has proved to be an effective strategy for the extraction of natural antioxidant compounds from the apple by-product.

1. Introduction

Apple (*Malus domestica* Borkh.) is one of the most widely cultivated fruits among the temperate regions (Velasco et al., 2010). The 2020 EU apple crop was at 10.6 million tonnes (European Commission, 2021). Apple is mainly consumed as fresh fruit and it is highly appreciated in terms of nutritional and bioactive compounds (Mignard, Beguería, Reig, Font i Forcada, & Moreno, 2021). However, around 25–30% of apple production is converted into different processed products, being apple juice and cider the main outcomes of the manufacturing procedures (Bhushan, Kalia, Sharma, Singh, & Ahuja, 2008; Cruz et al., 2018; Fernandes et al., 2019). Globally, the apple transformation process generates an average of 3.5 million tonnes of apple by-product per year. Nevertheless, apple by-product has been recognized as a source of health-promoting natural antioxidant components (Fernandes et al., 2019; Mateos-Aparicio et al., 2020). Phenolic compounds are of great interest because of their preventive effect against cardiovascular diseases, type 2 diabetes, or obesity (Fraga, Croft, Kennedy, & Tomás-Barberán, 2019). The polyphenols reported by other authors in apple by-product include flavonols, dihydrochalcones, hydroxycinnamic acids and, flavan-3-ols acids i.e., quercetin glycosylated derivatives, phloretin 2-*O*-glucoside, chlorogenic acid, and epicatechin or catechin, respectively (Rabetafika, Bchir, Blecker, & Richel, 2014). Thus, the sustainable recovery of these added-value compounds, from apple-derived wastes, may help to address the current economic and ecological challenges (Pinela et al., 2017).

The recovery of bioactive compounds from plants has been carried out over the years by classical extraction methods. These methodologies are associated with a series of disadvantages such as low selectivity or degradation of thermolabile compounds (Gligor et al., 2019). In addition, they may require the use of environmentally dangerous organic solvents (Khataei et al., 2022). Besides, a significant amount of antioxidant elements, known as insoluble bound phenolic compounds, may be covalently linked to different components of the plant cell wall including lignin, pectin, cellulose, or hemicellulose (Acosta-Estrada, Gutiérrez-Uribe, & Serna-Saldívar, 2014). Hence, alternative strategies like the utilization of food-grade enzymes and the application of green technologies, such as high hydrostatic pressure (HHP), could provide a

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solution to recover natural antioxidant compounds (Mateos-Aparicio, 2020). On the one hand, enzyme-assisted strategy enables substrate specificity extractions and increases the recovery of a large number of antioxidant bioactive compounds. It also allows the possibility of using the whole plant material for the process, and it typically requires fewer steps than conventional extraction methodologies (Gligor et al., 2019). On the other hand, HHP is a cold pasteurization technique by which high levels of pressure are transmitted to the products. This procedure inactivates microorganisms and, therefore, it extends the products shelf-life (Mateos-Aparicio, 2020). In addition, HHP has proven to increase the plant cell wall accessibility, enabling the extraction of diverse bioactive compounds, including soluble dietary fiber (De la Peña-Armada, Villanueva, & Mateos, 2020). Thus, the combination of both strategies, high pressure plus food-grade enzymes, has been successfully applied for soluble dietary fiber maximization to okara soybean by-product (Pérez-López, Mateos-Aparicio, & Rupérez, 2016; Pérez-López, Mateos-Aparicio, & Rupérez, 2017), and to apple by-product (De la Peña-Armada, Villanueva-Suárez, Molina-García, Rupérez, & Mateos-Aparicio, 2021). Furthermore, it has demonstrated high efficacy in the extraction of specific molecules such as proteins, polyphenols, and polysaccharides in red macroalgae (Suwal et al., 2019). In addition, Cascaes Teles and collaborators (Cascaes Teles et al., 2021) proved the effectiveness of HHP and enzyme-assisted extraction for phenolic compounds recovery from grape pomace. However, to the best of our knowledge, the combined strategy of HHP with Celluclast® L or Ultraflo® to release phenolic compounds from apple by-product has not been assessed before. Therefore, the aim of this study was to determine the effects of HHP (200, 400 and 600 MPa for 15 and 30 min), either assisted or not by the food-grade enzymes, Celluclast® L or Ultraflo®, on apple by-product to maximize the phenolic extraction, and to compare both enzymes activity.

2. Material and methods

2.1. Raw material

Apple by-product from "Golden Delicious" variety was supplied by Zumos Catalano- Aragoneses S.A. The by-product, consisting of apple seeds, peel, pulp, and stalks, was freeze-dried at −45 °C in the absence of light for preservation, and then, it was stored at room temperature under dry atmosphere, and darkness.

2.2. HHP treatment assisted by Celluclast® L or Ultraflo® L

In order to simulate raw material conditions, the lyophilized apple by-product was hydrated (10 g/100 mL) in water before HHP –aided or not by food-grade enzymes treatments. For the HHP (200, 400 and 600 MPa) plus Celluclast® (Novozymes Spain, S.A., Pozuelo de Alarcón, Madrid, Spain) procedure the enzyme was added to the apple by-product in a ratio of 1:40 enzyme:substrate (v/w) and the analyses were performed at 50 °C for 15 or 30 min, as previously reported by De la Peña-Armada and collaborators (2020). On the other hand, the hydrated apple by-product was treated with HHP (200, 400 and 600 MPa) and Ultraflo® (Novozymes Spain, S.A., Pozuelo de Alarcón, Madrid, Spain) in the same 1:40 ratio of enzyme:substrate (v/w), for 15 or 30 min. Control was the sample at atmospheric pressure 0.1 MPa.

Apple by-product samples -without or with added enzyme-were placed in vacuum-sealed plastic bags (200 × 300 mm CRYOVAC, Ref. BB3255; Mam Envases Alimentarios, S.L., Madrid, Spain) (De la Peña-Armada, Villanueva-Villanueva, & Mateos-Aparicio, 2020). Then, HHP treatment was applied in a laboratory-scale high-pressure vessel (Stansted SFP 7100:9/2C equipment) sited in ICTAN (CSIC). After the HHP treatment, the samples were freeze-dried.

2.3. Polyphenol extraction

Freeze-dried powders (0.25 g) of apple by-product samples derived from the previous HHP treatments, were subjected to a two-step polyphenol extraction adapted from the protocol described by Saura-Calixto (Saura-Calixto, 1998). Briefly, 2.5 mL of acidified (pH = 2) mixture of methanol (Scharlab, S.L., Sentmenat, Barcelona, Spain) - distilled water 50:50 (v/v) were used for the first stage, and 2.5 mL of acetone (Scharlab, S.L., Sentmenat, Barcelona, Spain) - distilled water 70:30 (v/v) for the second one. Subsequently, extracts were filtered through 45 μm diameter syringe filters, and stored at −20 °C until future analysis.

2.4. Characterization of phenolic compounds by Chromatography and Mass Spectrometry analysis

HPLC-ESI-QTOF equipment was used for a Chromatography and Mass Spectrometry analysis in order to characterize the extracted phenolic compounds. The HPLC unit (Agilent Technologies, Waldbroon, Germany) comprised of a quaternary pump (G1311A) with integrated degasser (G1322A), an autosampler (G1367B), a thermostatted column compartment (G1316A) a diode array detector (DAD) (G1315B) and a hybrid mass spectrometer quadrupole-time of flight *via* an electrospray ionization source (ESI) with JetStream technology (Agilent Accurate Mass QTOF LC-MS, Waldbronn, Germany) in series in the same chromatographic line. The used column was a Zorbax Eclipse XDB C18 993,967-902 Agilent 150 mm × 5 μm x 4.6 mm.

The gradient elution was carried out with a binary system consisting of 0.1% formic acid Sigma (St. Louis, MO, USA) in water (solvent A) and 0.1% acetonitrile (Labscan Ltd. Dublin, Ireland) in aqueous formic acid (solvent B). The following gradient was applied at a flow rate of 1 mL/min: 0 min, 95% (A); 30 min, 70% (A); 40 min, 50% (A); 45 min, 95% (B); 50 min, 95% (B). The injection volume was 50 μL, and the column temperature was 40 °C. Mass spectra were acquired with electrospray ionization and the TOF mass analyser in negative (rutin, quercetin, ferulic acid, chlorogenic acid, gallic acid, catechin, caffeic acid, procyanidin dimer 1, procyanidin dimer 2, epicatechin, quercetin-arabinoside, quercetin-rhamnoside, quercetin-galactoside, quercetin-glucoside, coumarolyquinic and cyanidin-galactoside) and positive (cyanidin glucoside) mode, over the range m/z: 100–1000.

The quantification of the phenolic compounds was performed using the calibration curve of commercial standards (Sigma, St. Louis, MO, USA), namely, rutin, quercetin, ferulic acid, chlorogenic acid, gallic acid, catechin, caffeic acid, and cyanidin-glucoside. In addition, tentative compounds were also included, specifically, procyanidin dimer 1, procyanidin dimer 2, epicatechin, quercetin-arabinoside, quercetin-rhamnoside, quercetin-galactoside, quercetin-glucoside, coumarolyquinic acid, and cyanidin-galactoside.

2.5. Antioxidant assessment

2.5.1. Total phenolic analysis

The total phenolic content (TPC) of the extracts was analysed by two different methods. For the first procedure, Folin–Ciocalteu (FC) assay was adapted from the methodology reported by Singleton, Orthofer & Lamuela-Raventós (Singleton, Orthofer, & Lamuela-Raventós, 1999). Concisely, for total phenolic content Folin–Ciocalteu (TPC-FC) quantification, Folin-Ciocalteu reagent (15 μL) (Scharlab, S.L., Sentmenat, Barcelona, Spain) was added to gallic acid standards (0.01–0.30 mg/mL) (Sigma-Aldrich Química S.A, Madrid, Spain) and samples (15 μL). Consecutively, standards and samples, were mixed with 120 μL of distilled water and allowed to react before adding 30 μL of Na_2CO_3 4 No. eq/L, and another 120 μL of distilled water. The absorbance was measured at 750 nm on a Synergy HTX Multi-Mode microplate reader (Bio-Tek Instruments, Winooski, USA). TPC was also determined by the Fast Blue BB (FB) method, previously outlined as a novel technique by

the author Marjorie B. Medina (Medina, 2011b). Briefly, FB determination consisted of adding 15 μL of 0.1 g/100 mL Fast Blue BB diazonium dye (Sigma-Aldrich Química S.A, Madrid, Spain) to the 150 μL gallic acid standards (0.01–0.30 mg/ml) and to the samples, followed by the addition of 15 μL of 5 g/100 mL NaOH. After 90 min reaction time, the absorbance was measured at 420 nm on a Synergy™ HTX Multi-Mode microplate reader (Bio-Tek Instruments, Winooski, USA). Both methodologies, as described, were previously miniaturized and adapted to microplate reader. Results obtained for FC assay and FB procedure were expressed as milligrams of gallic acid equivalents (GAE) per gram of dry matter (D.M.).

2.5.2. Antioxidant capacity

Apple by-product extracts were subjected to DPPH-free radical scavenging activity, Ferric Reducing Antioxidant Power (FRAP), and Oxygen Radical Absorbance Capacity (ORAC) assays to assess their antioxidant capacity. All measurements were developed on a Synergy™ HTX Multi-Mode microplate reader (Bio-Tek Instruments, Winooski, USA). DPPH assessment was carried out according to the methodology described by Karadag, Ozcelik, & Saner (Karadag, Ozcelik, & Saner, 2009). Briefly, 280 μL of 100 μmol/L DPPH solution (Sigma-Aldrich Química S.A, Madrid, Spain) were added to 20 μL of the Trolox standards (0.01–0.9 mmol/L) (Sigma-Aldrich Química S.A, Madrid, Spain) and samples. After 30 min of incubation, the absorbance was measured at 517 nm, free radical scavenging activity was calculated, and results were reported in terms EC50 (concentration required to obtain a 50% antioxidant effect) (Chen, Bertin, & Froldi, 2013).

Regarding FRAP methodology, 9 μL of Trolox standards (0.1–0.9 mmol/L) (Sigma-Aldrich Química S.A, Madrid, Spain) and samples were combined with 26 μL of distilled water and with 265 μL of FRAP reagent. FRAP reagent was freshly prepared as follows: 25 mL Acetic/acetate buffer (Scharlab, S.L., Sentmenat, Barcelona, Spain) 0.3 mol/L pH 3.6, 2.5 mL TPTZ (Sigma-Aldrich Química S.A, Madrid, Spain) solution 10 mmol/L, and 2.5 mL of Iron (III) chloride (Sigma-Aldrich Química S.A, Madrid, Spain) solution 0.03 mol/L. Furthermore, it was warmed at 37 °C. Absorbance reading were carried out at 595 nm after 30 min of incubation and results were expressed as millimoles of Trolox equivalents (TE) per gram of dry matter (mmol TE/g D.M.).

Oxygen radical absorbance capacity (ORAC) was carried out according to Serra, Duarte, Bronze, & Duarte (Serra, Duarte, Bronze, & Duarte, 2011), using a microplate fluorescent reader (Synergy™ HTXMulti-Mode, BioTek, Winooski, VT, USA). Antioxidant scavenging ability of the different compounds of the sample was evaluated. The method measured the activity against peroxyl radicals (ROO·) generated from AAPH (Sigma-Aldrich Química S.A, Madrid, Spain) using disodium fluorescein (Merck, Darmstadt, Alemania). Results of antioxidant capacity were expressed as micromoles of Trolox equivalents per gram of dry matter (μmol TE/g D.M.).

2.6. Statistical analysis

Descriptive statistics of the data was determined, and the differences within groups were studied by one-way analysis of variance (ANOVA) and post-hoc Ryan-Einot-Gabriel-Welsch F. The analysis was performed using the Statistical Package for the Social Sciences (SPSS). Furthermore, principal component analysis (PCA) was employed to study the effects of the different variables (time, pressure, and enzyme presence or absence) on the antioxidant capacity, and polyphenols concentration of apple by-product samples.

3. Results

3.1. Characterization of phenolic compounds by Chromatography and Mass Spectrometry analysis

Phenolic compounds determined by HPLC-ESI-QTOF after HHP, HHP assisted by Celluclast® enzyme, and HHP assisted by Ultraflo® enzyme, are presented in Fig. 1. The polyphenolic content increased for all the samples after the treatments as compared to control. The major identified component for all the samples were chlorogenic acid, followed by quercetin-galactoside and quercetin-rhamnoside. Results for apple by-product samples treated by HHP revealed an increase in the TPC which appeared to be related with the increase of the applied pressure (Fig. 1A). Thus, samples subjected to 600 MPa achieved the highest concentration of the studied polyphenols, followed by the 400 MPa treated samples, and 200 MPa treated samples. In addition, the greatest enhancement occurred for catechin compound increasing more than 10-fold after 600 MPa treatment. However, high pressure assisted by Celluclast® enzyme procedure enabled the highest recovery of polyphenols raising the TPC from 234.48 μg/g of dry matter to an average value of 739.65 μg/g of dry matter after the treatment (Fig. 1B). Quercetin compound increased by 9.6-times after the application of 200 MPa pressure for 15 min combined with Celluclast® enzyme. Furthermore, the enhancement of catechin and procyanidin dimer 1 components was also notable raising 10.7-fold and 5.2-fold, respectively. Regarding, high pressure assisted by Ultraflo® procedure, results indicated an unequal increase of the polyphenolic content related to the length of the pressure treatment applied (Fig. 1C). Thus, the studied polyphenols were enhanced in 2.7-times on average after the application of high pressure (200, 400 or 600 MPa) assisted by Ultraflo® for 15 min, while the phenolic concentration was 2.4-times higher for the high pressure (200, 400 or 600 MPa) assisted by Ultraflo® for 30 min treatment as compared to control values. Caffeic acid, which was not possible to quantify in the non-treated apple by-product, achieved for the high pressure (200, 400 or 600 MPa) plus Ultraflo® enzyme samples values of more than 7 μg/g of dry matter. In addition, quercetin reached average values of 19.64 μg/g of dry matter after the 15 min of combined treatment, whereas the concentration was less than 1.6 μg/g of dry matter for 30 min procedure.

3.2. Antioxidant assessment

TPC and antioxidant capacity results are presented in Table 1, Table 2, and Table 3. FC methodology for TPC quantification exhibited, in general, a slight increase for HHP assisted by food grade enzymes treated samples ($P < 0.05$) as compared to the control. Overall, HHP assisted by Celluclast® enzyme samples reached the greatest values (9.11–10.62 mg GAE/g D.M.) for TPC-FC followed by samples treated by HHP assisted by Ultraflo®. Conversely, TPC by FB (TPC-FB) assay only revealed a significant increase ($P < 0.05$) for the HHP assisted by Celluclast® samples (Table 2) as compared to the control. Regarding the antioxidant capacity assays, results for DPPH method revealed that the lower EC50 values were achieved after HHP and HHP plus Celluclast® enzyme treatment as compared to the control. Specifically, for HHP assisted by Celluclast®, EC50 data increased in line with the pressure rise (Table 2). In addition, for HHP plus Ultraflo® samples, EC50 results reached the highest values of all treated samples (Table 3). Besides, in data obtained by FRAP assay for HHP assisted by Celluclast® no significant differences ($P < 0.05$) were detected as compared to the control (Table 2). However, a significant increase ($P < 0.05$) of the values obtained for FRAP assay appeared for HHP samples non-assisted and assisted by Utraflo® (Table 1, Table 3), reaching in the latter case the highest values for the 15 min treated samples. Results for ORAC determination showed a significant increase ($P < 0.05$) of the values for all HHP assisted by food grade enzymes treated samples. HHP assisted by Celluclast®, and HHP assisted by Ultraflo® treatments enhanced in more than one third the ORAC results as compared to the control. No significant differences were detected among the HHP plus Ultraflo® samples. For HHP plus Celluclast® treated samples it was possible to detect an increase of the antioxidant capacity measured by ORAC assay when higher levels of pressure were applied.

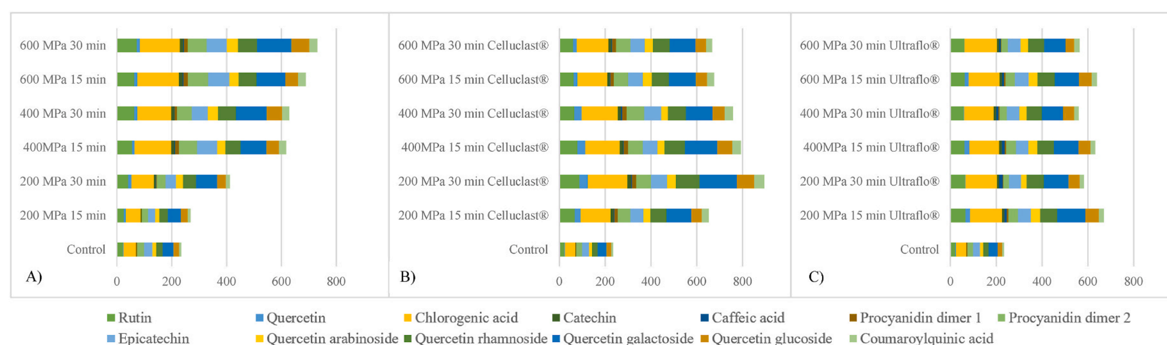

Fig. 1. Graphical distribution of phenolic composition of apple by-product after HHP (A), HHP assisted by Celluclast® enzyme (B), and assisted by Ultraflo® enzyme (C) expressed in μg/g of dry matter.

Table 1
Multifunctional antioxidant capacity of apple by-product subjected to HHP-treatment.

Sample	Time (min)	TPC-FC [a] (mg GAE/g D.M.)	TPC-FB [b] (mg GAE/g D.M.)	DPPH (EC50)	FRAP [c] (mmol TE/g D. M.)	ORAC [d] (μmol TE/g D. M.)
Control 0.1 MPa		8.62 ± 0.34^{a}	25.03 ± 1.57^{a}	10.76 ± 0.34^{d}	54.73 ± 2.23^{ab}	37.92 ± 3.92 ^{a}
200 MPa	15	9.36 ± 0.54 ^{a}	25.03 ± 0.39^{a}	9.51 ± 0.19^{c}	58.01 ± 2.93^{bc}	40.10 ± 3.19 ^{a}
	30	9.73 ± 0.52 ^{a}	25.98 ± 0.17^{a}	10.62 ± 0.31^{d}	58.11 ± 1.41^{c}	36.52 ± 1.23 ^{a}
400 MPa	15	9.72 ± 0.49 ^{a}	25.23 ± 0.83^{a}	8.64 ± 0.23^{ab}	58.02 ± 2.53^{c}	34.13 ± 3.71 ^{a}
	30	9.95 ± 0.78 ^{a}	24.83 ± 1.04^{a}	8.68 ± 0.13^{b}	55.75 ± 2.13^{ab}	36.03 ± 3.69 ^{a}
600 MPa	15	9.29 ± 0.59 ^{a}	25.56 ± 0.15^{a}	8.02 ± 0.13^{a}	58.76 ± 1.66^{c}	41.71 ± 3.25 ^{a}
	30	9.08 ± 0.45 ^{a}	24.96 ± 1.51^{a}	8.92 ± 0.02^{b}	52.81 ± 2.49^{a}	40.87 ± 3.38 ^{a}

Mean values of triplicate analyses ± standard deviation. Means with different letters in each column differ significantly ($P < 0.05$).
[a] Total phenolic content Folin-Ciocalteu.
[b] Total phenolic content Fast-Blue BB.
[c] Ferric Reducing Antioxidant Power.
[d] Oxygen Radical Absorbance Capacity.

Table 2
Multifunctional antioxidant capacity of apple by-product subjected to HHP-treatment assisted by Celluclast® enzyme.

Sample	Time (min)	TPC-FC [a] (mg GAE/g D. M.)	TPC-FB [b] (mg GAE/g D.M.)	DPPH (EC50)	FRAP [c] (mmol TE/g D. M.)	ORAC [d] (μmol TE/g D. M.)
Control 0.1 MPa		8.62 ± 0.34^{a}	25.03 ± 1.57^{a}	10.76 ± 0.34^{d}	54.73 ± 2.23^{a}	37.92 ± 3.92^{a}
200 MPa	15	10.62 ± 0.46^{d}	46.81 ± 2.88^{b}	7.11 ± 0.13^{a}	56.97 ± 2.12^{a}	55.15 ± 6.17^{bc}
	30	10.17 ± 0.39^{cd}	45.64 ± 4.73^{b}	7.31 ± 0.15^{a}	54.38 ± 2.32^{a}	50.56 ± 1.76^{bc}
400 MPa	15	9.33 ± 0.14^{abc}	44.54 ± 3.20^{b}	8.51 ± 0.17^{b}	54.11 ± 0.99^{a}	54.75 ± 7.21^{bc}
	30	10.07 ± 0.55^{cd}	47.05 ± 2.89^{b}	8.51 ± 0.12^{b}	56.56 ± 2.61^{a}	54.30 ± 10.18^{bc}
600 MPa	15	9.11 ± 0.28^{ab}	44.54 ± 0.59^{b}	9.30 ± 0.13^{c}	54.37 ± 1.07^{a}	56.99 ± 6.18^{bc}
	30	9.92 ± 0.53^{bcd}	44.22 ± 4.02^{b}	9.43 ± 0.13^{c}	56.40 ± 3.08^{a}	62.29 ± 7.76^{c}

Mean values of triplicate analyses ± standard deviation. Means with different letters in each column differ significantly ($P < 0.05$).
[a] Total phenolic content Folin-Ciocalteu.
[b] Total phenolic content Fast-Blue BB.
[c] Ferric Reducing Antioxidant Power.
[d] Oxygen Radical Absorbance Capacity.

3.3. *Principal components analysis (PCA)*

The PCA was performed in order to transform the antioxidant assessment data set, into a reduced number of independent variables named as principal components. Data included the results of the TPC determinations (FC and FB) and antioxidant capacity methodologies (FRAP, DPPH and ORAC) undertaken on the apple by-product samples. The two main principal components of the analysis could explain 70.2% of total variance, being for the principal component 1 a 48.5% and for the principal component 2 a 24.3%. Additionally, PCA revealed a possible cluster aggrupation within the analysed treated apple by-product samples, Cluster number one consisted of the control and the HHP treated samples, cluster number two was composed by HHP plus Ultraflo® treated samples, while cluster number three comprised HHP plus Celluclast® treated samples (Fig. 2). In addition, cluster one was associated to a low value for TPC-FB and ORAC assays. Cluster two was also associated to a low value for TPC-FB, but it was possible to observe a relation with a high value of FRAP, DPPH and ORAC analysis. Regarding cluster three, data for TPC-FB, ORAC and TPC-FC were higher than the global mean, whereas DPPH and FRAP methodologies exhibited lower results as compared to the average values. Furthermore, supplementary variables were displayed through a correlation figure to improve the results understanding (Fig. 3). These supplementary variables were the additional polyphenol concentrations detected in the samples analysed by Chromatography and Mass Spectrometry methodology. Thus, cluster number one was negatively related to the polyphenol concentration of quercetin-arabinoside, coumaroyl-quinic acid, quercetin-glucoside, quercetin, catechin, quercetin-galactoside, rutin, chlorogenic acid, caffeic acid, quercetin-rhamnoside, while cluster number three exhibited opposite results, including high values for the concentration of nine out of the twelve studied polyphenols (coumaroylquinic acid, catechin, rutin, chlorogenic acid, quercetin, or epicatechin among others). Regarding cluster two, it showed a greater concentration of caffeic acid than clusters one and three.

4. Discussion

The primary objective of the selected procedures was to improve the comprehension of the phytochemical composition of apple by-product in both, the raw material, as well as the treated samples. The HPLC-ESI-QTOF equipment enabled a broad characterization of the phenolic compounds in the apple by-product. Identification was performed according to their relative retention times (Fig. 4) and with the information previously found in the literature. Regarding the plant antioxidant assessment, it appears to be complicated, as no single assay or combination of assays is certainly optimal due to the wide diversity of

Table 3
Multifunctional antioxidant capacity of apple by-product subjected to HHP-treatment assisted by Ultraflo® enzyme.

Sample	Time (min)	TPC-FC [a] (mg GAE/g D.M.)	TPC-FB [b] (mg GAE/g D.M.)	DPPH (EC50)	FRAP [c] (mmol TE/g D. M.)	ORAC [d] (μmol TE/g D. M.)
Control 0.1 MPa		8.62 ± 0.34^{a}	25.03 ± 1.57^{c}	10.76 ± 0.34^{cd}	54.73 ± 2.23^{a}	37.92 ± 3.92^{a}
200 MPa	15	10.76 ± 0.47^{c}	18.17 ± 0.85^{a}	10.11 ± 0.18^{ab}	60.30 ± 3.08^{b}	53.40 ± 6.57^{b}
	30	8.78 ± 0.28^{a}	19.46 ± 2.83^{ab}	10.50 ± 0,18^{bcd}	56.50 ± 1.44^{a}	54.96 ± 3.48^{b}
400 MPa	15	9.39 ± 0.32^{b}	19.05 ± 1.36^{a}	10.25 ± 0.17^{ab}	64.10 ± 2.36^{c}	53.27 ± 6.82^{b}
	30	9.27 ± 0.26^{b}	22.73 ± 3.35^{bc}	10.37 ± 0.10^{abc}	60.42 ± 3.06^{b}	52.20 ± 5.98^{b}
600 MPa	15	9.01 ± 0.21^{ab}	25.74 ± 0.97^{c}	9.93 ± 0.09^{a}	65.58 ± 4.16^{c}	51.92 ± 2.05^{b}
	30	9.08 ± 0.31^{ab}	23.08 ± 1.94^{bc}	10.93 ± 0.09^{d}	59.99 ± 3.31^{b}	56.79 ± 6.70^{b}

Mean values of triplicate analyses ± standard deviation. Means with different letters in each column differ significantly (*P < 0.05*).
[a] Total phenolic content Folin-Ciocalteu.
[b] Total phenolic content Fast-Blue BB.
[c] Ferric Reducing Antioxidant Power.
[d] Oxygen Radical Absorbance Capacity.

chemical compounds that may be present (Power, Jakeman, & Fitzgerald, 2013; Tan & Lim, 2015). Hence, for a more detailed understanding of the antioxidant compounds involved in plant-based matrices, more than one feature should be considered (Číž et al., 2010). For that purpose, five methodologies were chosen, two of them with the aim of quantifying the TPC (FC and FB), while for the remaining three, the objective was to determine the antioxidant capacity of the studied samples. The presented assays were based on different mechanisms. FC, FB, DPPH and FRAP were based on electron transfer, whereas ORAC assay was based on hydrogen atom transfer was. The lack of specificity of FC assay for polyphenolic compounds determination (Tan & Lim, 2015) was the main reason for exploring two methodologies. Divergence among results for antioxidant assessments has been detected by numerous authors and interpreted as dependent on the performed assay (Medina, 2011a; Morales-Soto et al., 2014; Ou, Hampsch-Woodill, & Flanagan, 2002; Tan & Lim, 2015).

From a quantitative point of view, HPLC revealed an increase of all the quantified polyphenols after the application of the different treatments. After the HHP process, the quantified polyphenols gradually increased as the treatment was more intense, in terms of time and pressure. Particularly, epicatechin and procyanidin dimer 2 amounts raised from 26.31 to 22.28 μg/g of dry matter under 200 MPa pressure for 15 min to 72.81 and 69.22 μg/g of dry matter under 600 MPa pressure for 30 min respectively (Fig. 1). Regarding the antioxidant assessment (Table 1), HHP did not caused a significant increase of the TPC measured by FC nor by FB methodology. In addition, HHP samples constituted the cluster number one in the principal component analysis, which was associated with a limited value for TPC-FB (Fig. 2), and also with overall lower values of the quantified polyphenols as compared to the other treatments (Fig. 3). The substantive variation of the TPC values measured by FC and FB may be due to the interference of non-phenolic antioxidants and reducing compounds, such as ascorbic acid, glucose, and fructose, with the FC reagent (Avalos-Llano, Martín-Belloso, & Soliva-Fortuny, 2018; Lester, Lewers, Medina, & Saftner, 2012). In addition, values for TPC-FC method are akin to Li and collaborators work of polyphenols in apple pomace (W. Li, Yang, et al., 2020). DPPH values seemed to be directly related with HPLC-ESI-QTOF polyphenolic data. The samples treated with 200 MPa pressure exhibited the lowest content in polyphenol compounds, and the highest EC50 (Table 1).

The highest values for polyphenolic content appeared after the application of HHP plus Celluclast® enzyme (Fig. 1). The greatest enhancement, as compared to the HHP and HHP assisted by Ultraflo® enzyme, occurred for quercetin, procyanidin dimer 1 and coumaroylquinic acid. Thus, the average increase for the mentioned compounds was 10.4-fold, 6.2-fold and 4.6-fold, respectively, as compared to the control. Results are consistent with the data presented in Table 1, Tables 2 and 3, which evidenced that the highest TPC measured by FB methodology, and by FC assay, was reached by the HHP plus Celluclast® apple by-product samples. Furthermore, the principal component analysis determined the association between a high content of polyphenol

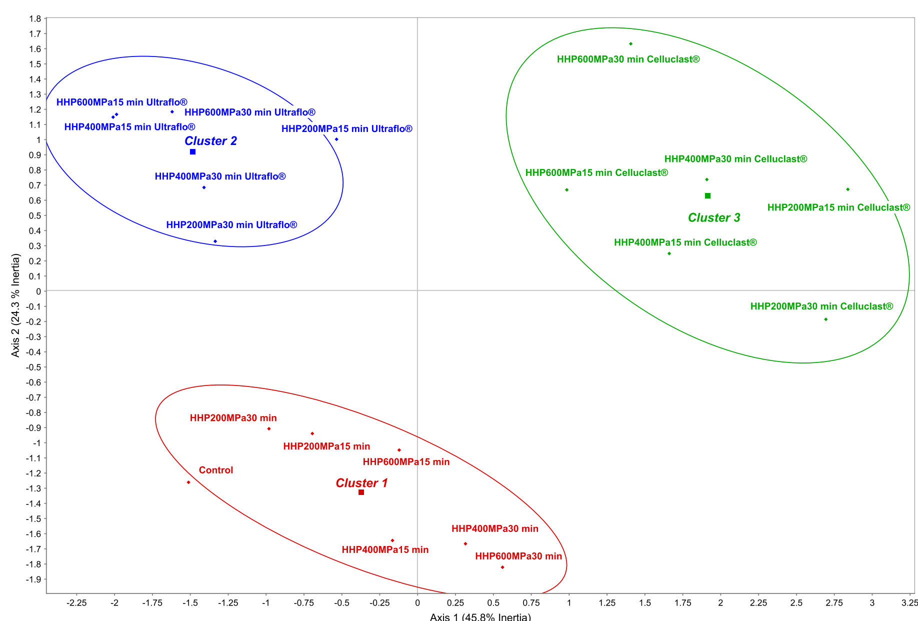

Fig. 2. Cluster plot analysis. The identified clusters presented using the antioxidant capacity discriminant scores. Red, blue, and green colours represent clusters 1, 2, and 3, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

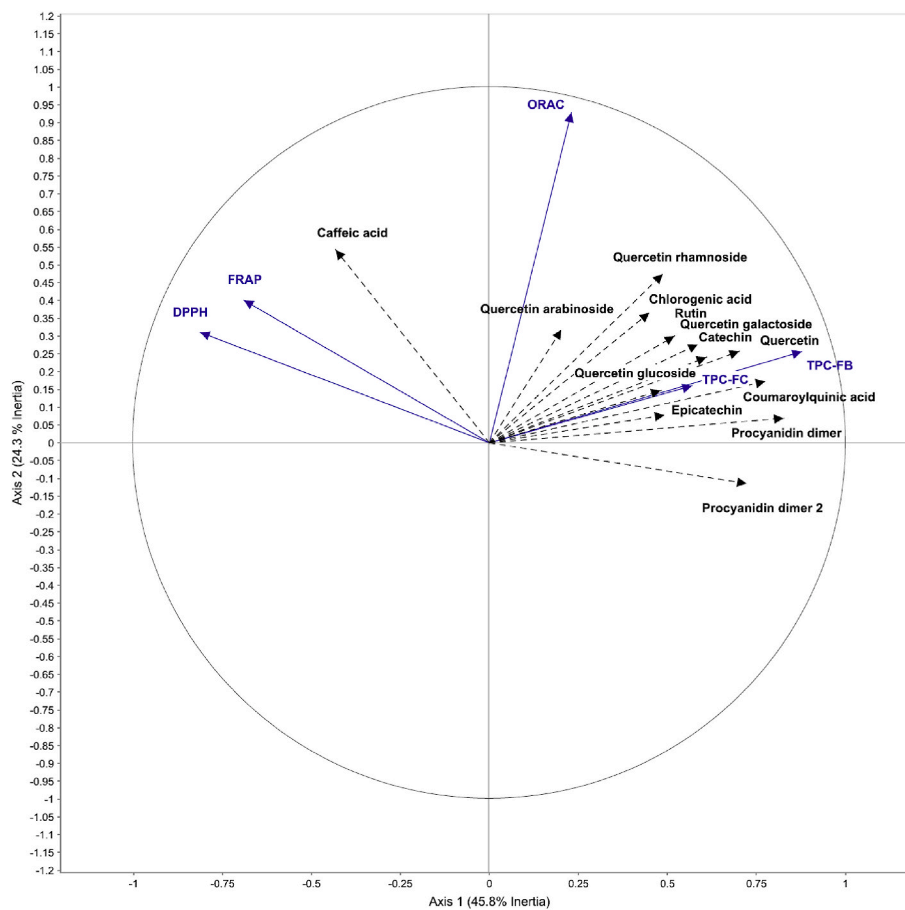

Fig. 3. Principal Component Analysis correlation circle of antioxidant capacities (blue coloured) values and phenolic (black coloured) compounds projected on principal components PC1 and PC2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

compounds and high TPC, measured by FB and FC methods (Fig. 3). However, an important difference in TPC when employing one or another method was noted. TPC-FB revealed an increase of the phenolic amount after the high pressure assisted by Celluclast® treatment was performed, while the values of the FC assay did not raise correspondingly. Data are in accordance with those previously reported by Medina (Medina, 2011b) in which tea samples exhibited about 2–3 times greater phenolic content when measured by the FB procedure than with the FC method. Moreover, apple by-product samples exhibited an increase of the oxygen radical absorbance capacity after high pressure (200, 400 or 600 MPa) plus Celluclast®, and high pressure (200, 400 or 600 MPa) plus Ultraflo® treatments. Results are in accordance to previously reported by Li and collaborators (2020) for apple pomace in which a correlation between the raise of the values for ORAC and the increase of the TPC existed (Y. Li, Yang, et al., 2020).

HHP assisted by Ultraflo enzyme® increased the quantity of caffeic acid in the apple by-product samples as compared to HHP, and HHP assisted by Celluclast®. Besides, HPLC-ESI-QTOF revealed an increase of certain compounds, namely, quercetin, procyanidin dimer 1, procyanidin dimer 2, quercetin-arabinoside, and quercetin-glucoside, which were greater after 15 min treatments than after 30 min treatments (Fig. 1). These results are in agreement with data obtained for FRAP antioxidant capacity methodology, which achieved the highest values for the samples treated during the shortest period of time as compared to the 30 min treatments (Table 3). Furthermore, a similar pattern can be encountered in DPPH antioxidant capacity assay, while for TPC-FB the behaviour was opposite (Table 3). In addition, the principal component analysis revealed that HHP plus Ultraflo® enzyme samples were related with a high value of FRAP, DPPH, and ORAC, and low values of TPC-FB (Fig. 3). Regarding TPC-FC, 400 MPa and 600 MPa samples values remained stable after the different length treatments, while 200 MPa samples exhibited an increase of the phenolic content after 15 min treatment followed by a decrease after 30 min treatment. Similar results were reported by Cascaes, Teles et al. (Cascaes Teles et al., 2021), for grape pomace, in which, the authors observed that for enzyme-assisted extraction concomitantly applied with 200 MPa, and 50 MPa HHP, the total phenolic compounds had a gradual increase up to 10 min and a reduction after this time period.

The presented outcomes revealed the improvement of TPC when both procedures, high pressure and Celluclast® or Ultraflo® enzyme treatment, were performed simultaneously on the apple by-product, an increase of the polyphenolic content and antioxidant capacity was promoted. Thereby, the development of a potential antioxidant ingredient through the application of HHP assisted by food-grade enzymes has been proved. Polyphenols, which activity has been recognized as effective against the negative effects of reactive oxygen species, could be beneficial for human health preventing diseases that are related to oxidative stress. However, aiming the formulation of future functional foods, it is essential to further study this potential ingredient.

High pressure procedure has been described as a positive influence for bioactive compounds extraction yield. Nevertheless, the effect of pressure for antioxidant compounds extraction, maintaining, increasing or decreasing the amount of these substances, differs according to the food matrices and conditions applied (Casquete et al., 2015; Keenan, Rößle, Gormley, Butler, & Brunton, 2012; Saikaew, Lertrat, Meenune, & Tangwongchai, 2018; Xi, Shen, Li, & Zhang, 2011). Furthermore, as previously reported by (Jakobek & Matić, 2019) polyphenols can bind onto dietary fibers surface modifying their bioaccessibility. For instance,

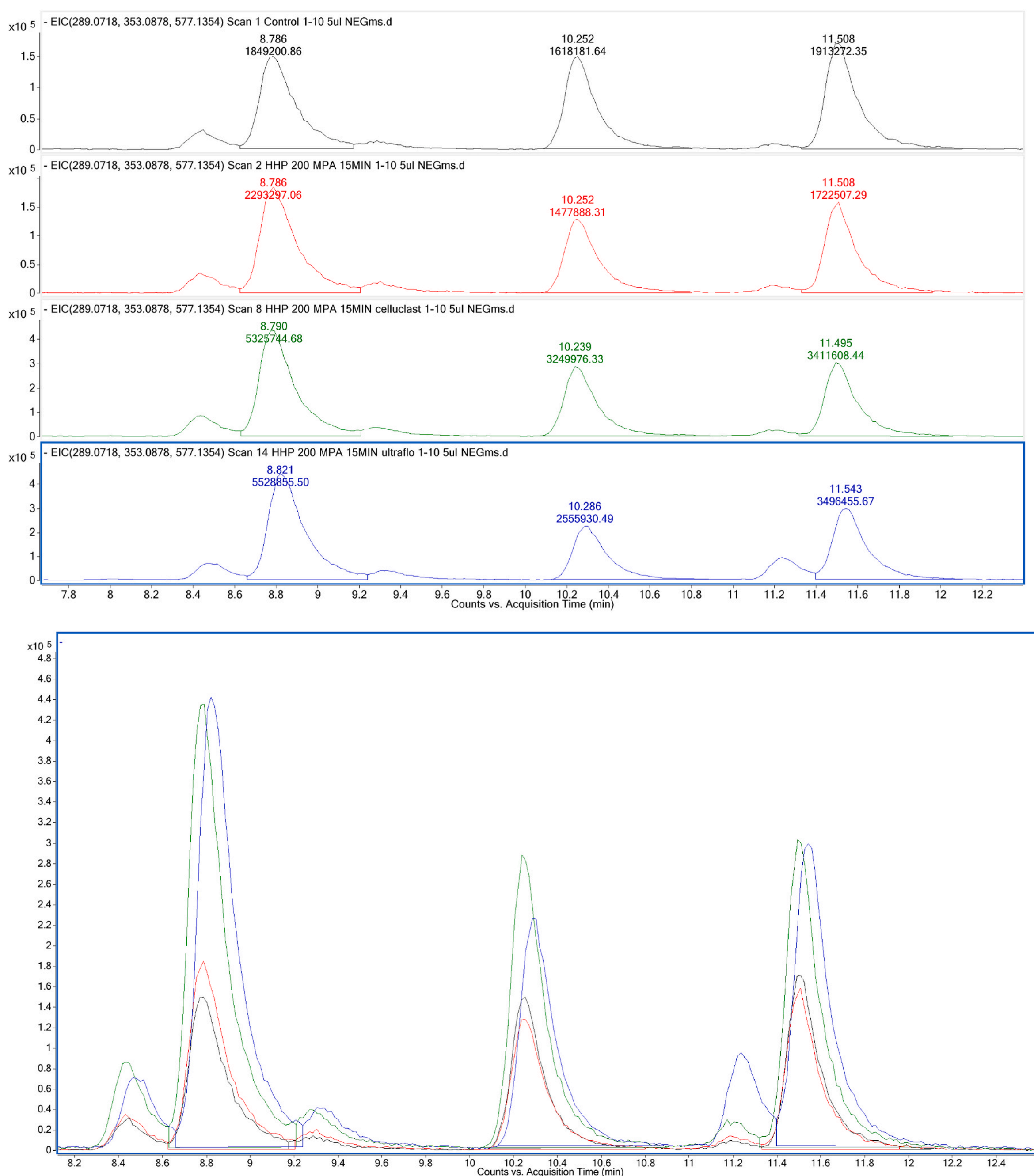

Fig. 4. HHP-treatment at 200 MPa for 15 min. Chromatogram from MS-extraction (negative mode) of the main polyphenols in apple by-product: Catechin (RT = 8.4), Chlorogenic acid (RT = 8.8), Procyanidin dimer B2 (RT = 10.2) and Epicatechin (RT = 11.5). EIC = extracted ion current.

pectins are a complex group of polyssacharides which interact with polyphenols. In the same way, cellulose represents an important dietary fiber surface in which polyphenols are linked. Therefore, the cell-wall matrix disruption by enzymatic hydrolysis has demonstrated to enable the release of phenolics compounds, increasing the extraction yield (Antunes-Ricardo et al., 2018). Pinelo and collaborators (Pinelo, Zornoza, & Meyer, 2008) reported that Celluclast® assisted extraction favours the release of the phenolic components on apple skin, therefore increasing the antioxidant capacity of the sample. Likewise, Ultraflo® L commercial enzyme preparation, has demonstrated to degrade lignocellulosic materials due to its enzymatic activity, including β-glucanase, cellulase, xylanase, or arabinase activity, which has been related to the release of phenolic compounds (Moore, Cheng, Su, & Yu, 2006; Valério et al., 2021). Recent publications have described the effect of HHP and HHP plus Celluclast® enzyme over the apple by-product revealing an increase of the cell wall matrix accessibility (De la Peña-Armada et al., 2021). Thus, the increase of the polyphenol concentration and the antioxidant capacity in the treated samples may be explained due to an increase in the polyphenol accessibility.

5. Conclusion

This work presents the first findings regarding phenolic compounds of HHP-assisted extraction from apple by-product. The application of HHP over the apple by-product matrix increased the polyphenolic concentration and the antioxidant capacity of the treated samples. The combined treatment of HHP and the food-grade enzymes Celluclast® or Ultraflo® significantly improved the extraction of phenolic compounds.

This enhancement may be due to an increase of the enzymes accessibility into the plant cell wall, and consequently leading to the polyphenols release from the vegetable cell matrix. More specifically, HHP assisted by Celluclast® enzyme enhanced the overall polyphenols quantity while HHP plus Ultraflo® enzyme mainly raised the content of caffeic acid. Hence, the suggested combined strategy could be a sustainable method to improve bioactive antioxidant compounds in the apple by-product and therefore, a novel approach for the development of a new natural active ingredient.

CRediT authorship contribution statement

R. De la Peña Armada: Formal analysis, Investigation, Methodology, Writing – original draft. **P. Rupérez:** Methodology, Validation, Writing – review & editing, Funding acquisition. **M.J. Villanueva-Suarez:** Conceptualization, Methodology, Validation. **I. Mateos-Aparicio:** Conceptualization, Methodology, Validation, Writing – review & editing, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113968.

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