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In vitro assessment of the bioavailability of bioactive non-extractable polyphenols obtained by pressurized liquid extraction combined with enzymatic-assisted extraction from sweet cherry (*Prunus avium* L.) pomace

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ABSTRACT

In vitro digestion and absorption simulation processes of non-extractable polyphenols (NEPs) obtained by pressurized liquid extraction combined with enzymatic-assisted extraction with Promod enzyme (PLE-EAE) from the residue of conventional extraction of sweet cherry pomace were studied. In general, total phenolic and proanthocyanidin contents decreased in each phase of the digestion. However, the antioxidant capacity increased when the digestion process progressed. In addition, the highest total phenolic and proanthocyanidin contents and antioxidant capacity were obtained in the absorbed fraction. NEPs from PLE-EAE extract, digestive fractions, absorbed and unabsorbed fractions were analyzed by ultra-high-performance liquid chromatography coupled to electrospray ionization quadrupole Exactive-Orbitrap mass spectrometry (UHPLC-ESI-Q-Orbitrap-MS). Fifteen NEPs were identified in the intestinal fraction and five in the absorbed fraction after the digestion process. Results obtained in this study define for the first time the bioavailability of antioxidant NEPs obtained from sweet cherry pomace.

1. Introduction

There is evidence that the consumption of dietary antioxidants prevents the risk of cardiovascular diseases (Varadharaj et al., 2017). Dietary antioxidants are an extensive group of chemical compounds that include carotenoids, vitamins C and E, and polyphenols. Among them, polyphenols are the most consumed phytochemicals since they correspond to around 90% of dietary antioxidants intake (Pérez-Jiménez, Díaz-Rubio, & Saura-Calixto, 2013). In fact, they are the most studied naturally occurring antioxidants in fruits and vegetables. In particular, several studies show that red fruits present high contents of phenolic compounds, especially anthocyanins, flavonols, proanthocyanidins, phenolic acids, catechins, and isoflavones with high antioxidant capacity (Soutinho, Guiné, Jordao, & Goncalves, 2013). In the red fruits, sweet cherries have intensively been studied for their high content in antioxidant phenolic compounds associated with the prevention of degenerative diseases.

Sweet cherries are mainly consumed fresh. However, a third of the sweet cherries are processed into jams, dried forms, jellies, and juices products which are available over the year (Boriss, Brunke, & Kreith, 2006; Goncalves, Bento, Silva, & Silva, 2017). During the fruit processing, around 20–30% of fresh fruit weight constitutes by-products that consist of pomace (skin and flesh), seeds, and stones (Milea et al., 2019). These by-products cause environmental problems due to their poor biological and oxidative stability and high water activity (Milea et al., 2019). Nevertheless, these by-products present interesting biological properties being considered a significant source of natural antioxidant compounds. In fact, sweet cherry pomace has shown high phenolic content with high antioxidant capacity and anti-aging properties with a protector effect against Alzheimer's disease (Milea et al., 2019; Domínguez-Rodríguez, García, Marina, & Plaza, 2021; Domínguez-Rodríguez, Marina, Plaza, 2021). However, the evidence supporting the biological effects of phenolic compounds is limited to laboratory animals. Human trials are very limited and the results are inconclusive (Morton, Abu-Amsha Caccetta, Puddey, & Croft, 2000). Moreover, studies about sweet cherry pomace are scarce.

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Usually, phenolic compounds have been extracted by conventional or advanced extraction techniques with different extraction solvents. Nevertheless, the total phenolic content of the extracts obtained by these extraction techniques is underestimated because an important fraction called non-extractable polyphenols (NEPs) remains retained in the extraction residues. Additional treatments are necessary to release NEPs from the residue of conventional extraction. In this sense, acid and alkaline hydrolysis have been employed to obtain NEPs from different matrices. These hydrolyses produce alterations in the structure of phenolic compounds caused by the extreme pH used during the treatments (Seke et al., 2021). For this reason, enzymatic-assisted extraction (EAE) has emerged as a sustainable and more selective alternative than hydrolysis treatments to release NEPs from food matrices. Enzymes with ß-glucanase, protease, polygalacturonase, and pectin lyase activities have been used to release NEPs from sweet cherry pomace (Domínguez-Rodríguez, Marina, & Plaza, 2017). EAE has been employed combined with PLE to release NEPs from sweet cherry pomace (Domínguez-Rodríguez, García, Marina, & Plaza, 2021). However, the studies about the release of NEPs through PLE in combination with EAE are very limited.

Even though NEPs have been less studied than extractable polyphenols (EPPs), in some cases, NEPs fraction has a higher phenolic content with antioxidant capacity than EPPs fraction (Domínguez-Rodríguez, García et al., 2021; Pérez-Jiménez et al., 2013). Nevertheless, the beneficial effects of NEPs intake on humans depend on their bioaccessibility and bioavailability in the digestive tract. In this sense, Saura-Calixto, Serrano, and Goni (2007) showed that around 50% of NEPs with low molecular weight are bioaccessible in the small intestine while NEPs with medium–high molecular weight remain intact to the colon. Once in the colon, they can be bioaccessible by their release from macromolecules (proteins and carbohydrates) by the action of microbial enzymes. These enzymes break covalent bonds producing absorbable metabolites (Pérez-Jiménez et al., 2013). Though the bioavailability and absorption of NEPs have not been intensively studied.

Considering the lack of information on the bioavailability of NEPs from sweet cherry pomace, a more in-depth study is needed. Thus, this study aimed to develop an *in vitro* digestion and absorption simulation of NEPs obtained from sweet cherry pomace by PLE-EAE. The total phenolic and proanthocyanidin contents and antioxidant capacity were determined in each phase of the *in vitro* simulated digestion process and after the simulated absorption process. Furthermore, NEPs present in PLE-EAE extracts as well as the fractions obtained after the *in-vitro* oral, gastric, and intestinal processes, and the fraction got after absorption process of PLE-EAE extracts were analyzed by reversed-phase (RP)-ultra-high-performance liquid chromatography (UHPLC) coupled to electrospray ionization (ESI) quadrupole (Q) Exactive-Orbitrap mass spectrometry (MS). Therefore, the influence of the digestion process and intestinal absorption of NEPs from sweet cherry pomace on their bioavailability was studied.

2. Materials and methods

2.1. Chemicals and samples

Ethanol, acetone, and hydrochloric acid (37%) of HPLC grade were purchased from Scharlab Chemie (Barcelona, Spain). Methanol (99.99%) was from Fisher Scientific (Leicestershire, UK). Gallic acid, epicatechin, vanillin, iron(III) chloride, sodium carbonate, hydrogen peroxide, Folin-Ciocalteu reagent, 4-dimethylaminocinnamaldehyde (DMAC), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), potassium persulfate, 2,2́-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), calcium chloride, potassium chloride, monopotassium phosphate, sodium bicarbonate, sodium chloride, magnesium chloride hexahydrate, ammonium carbonate, bile extract porcine, porcine pepsin, pancreatin, and α-amylase were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Dipotassium hydrogen phosphate and sodium dihydrogen phosphate dihydrate were supplied from Merck (Darmstadt, Germany).

Acetonitrile, formic acid, and butanol of HPLC grade were provided from Fisher Scientific. Ultrapure water (18.2 MΩ/cm) was generated with a Millipore system (Millipore, Billerica, MA, USA).

Promod 439 L enzyme with protease activity was kindly donated by the company "Biocatalysts Limited" (Cardiff, UK).

Sweet cherries corresponding to *Prunus avium* L. genus and Early Lory variety were obtained from La Almunia de Doña Godina (Zaragoza, Spain) and washed, de-stemmed, de-stoned, and pressed manually to obtain the pomace to carry out PLE-EAE extraction.

2.2. Release of non-extractable polyphenols (NEPs) by PLE-EAE

In order to obtain NEPs, conventional extraction was carried out to obtain EPPs according to Condezo-Hoyos et al., (2014) with some modifications (Domínguez-Rodríguez, García et al., 2021; Domínguez-Rodríguez, Marina et al., 2021) from sweet cherry pomace. First, cherry pomace (15 g) was treated for 1 h at room temperature with 20 mL of methanol/water (50:50, v/v) acidified with 2 N HCl (pH 2.0) under shaking. The extract was centrifuged at 2100×*g* for 10 min to recover the supernatant. Subsequently, the extraction residue was re-extracted for 1 h at room temperature with 20 mL of acetone/water (70:30, v/v) under shaking. The centrifugation was repeated with this extract and both supernatants (methanol and acetone) were combined. The residue of this extract was used to release NEPs.

NEPs were extracted by PLE-EAE from the residue of conventional extraction using a Dionex ASE 150 instrument (Thermo Fisher, Germering, Germany) according to the optimized conditions obtained by Domínguez-Rodríguez et al. (2021b). Briefly, 5.5 g of the conventional extraction residue of sweet cherry pomace were included in 10 mL extraction cells where 140 μL of Promod enzyme/g was added. The PLE extraction was performed using 100 mM phosphate buffer at pH 10.0 (previously sonicated for 30 min) for 31 min and a temperature of 60 °C. The final volume of the extracts was 20 mL and they were stored at −20 °C until the analysis.

2.3. In vitro simulation of the human digestion process of NEPs

The *in vitro* digestion method developed by Minekus et al. (2014) was employed for this study. Simulated salivary fluid consisted of 15.1 mL of a solution of potassium chloride (0.5 M), 3.7 mL of monopotassium phosphate (0.5 M), 6.8 mL of sodium bicarbonate (1 M), 0.5 mL of magnesium chloride hexahydrate (0.15 M), and 0.06 mL of ammonium carbonate (0.5 M). PLE-EAE extract (5 mL) was mixed with 3.5 mL of salivary fluid, 0.5 mL of α-amylase (1500 U/mL) dissolved in salivary solution, 25 μL of 0.3 M $CaCl_2$, and 975 μL of H_2O. The mixture was adjusted to pH 7.0 with NaOH or HCl (1.0 M) and maintained for 2 min at 37 °C under shaking to obtain the oral fraction of the digestion process.

Oral fraction (10 mL) was mixed with 7.5 mL of simulated gastric fluid which consisted of 6.9 mL of potassium chloride (0.5 M), 0.9 mL of monopotassium phosphate (0.5 M), 12.5 mL of sodium bicarbonate (1 M), 11.8 mL of sodium chloride (2 M), 0.4 mL of magnesium chloride hexahydrate (0.15 M), and 0.5 mL of ammonium carbonate (0.5 M). Then, 1.6 mL of porcine pepsin (25000 U/mL) dissolved in the simulated gastric fluid were added to the mixture along with 5 μL of 0.3 M $CaCl_2$ and 0.2 mL 1 M HCl until reaching a pH 3.0. This solution was shaken for 2 h at 37 °C to obtain the gastric fraction, commonly called gastric chyme.

Subsequently, to obtain a digestive extract, 20 mL of gastric chyme were mixed with 11 mL of simulated intestinal fluid composed of 6.8 mL of potassium chloride (0.5 M), 0.8 mL of monopotassium phosphate (0.5 M), 42.5 mL of sodium bicarbonate (1 M), 9.6 mL of sodium chloride (2 M), and 1.1 mL of magnesium chloride hexahydrate (0.15 M). Then, 5 mL of pancreatin (800 U/mL) dissolved in the simulated intestinal fluid

were added to the mixture along with 2.5 mL of bile extract porcine (160 mM), 40 μL of $CaCl_2$ (0.3 M), 0.15 mL of NaOH (1 M) until reaching pH 7.0, and 1.31 mL of H_2O_2.

Oral, gastric, and intestinal fractions were prepared in triplicate and stored at −20 °C until analysis. The intestinal fraction which corresponds to the digestive extract (DIG) was used to carry out the cytotoxicity evaluation and the *in vitro* simulated human absorption assays.

2.4. Cell viability

To determine the possible cytotoxicity of DIG, the effect of different concentrations of DIG on cell viability was determined by the MTT assay described by Hernández-Corroto, Marina, and García (2018) using human cervical cancer cells (HeLa).

HeLa cells from the American Type Culture Collection ATCC (Rockwell, MD, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/mL), amphotericin (250 ng/mL), streptomycin (100 μg/mL), and 10% of fetal bovine serum. The cells were maintained under 37 °C, 95% of humidity, and 5% CO_2 in their culture medium. These cells were treated with 45.83, 68.75, 103.13, 206.25, and 412.5 mg/mL extract. Cell viability was calculated by the following equation:.

$$\%\ cell\ viability = \frac{Abs\ sample - Abs\ control}{Abs\ control} \times 100$$

2.5. In vitro simulated intestinal absorption of NEPs

The absorption of NEPs from PLE-EAE extracts and DIG obtained from sweet cherry pomace was evaluated according to Goncalves et al. (2019). Caco-2 cells, a human colorectal adenocarcinoma epithelial cell line, were cultured in RPMI medium supplemented with fetal bovine serum and maintained under 37 °C, 95% of humidity, and 5% CO_2 in their culture medium. The cells were seeded between 30 and 34 passages onto a culture insert on a plate of 12 chambers at a cell density of 6×10^4 cells. During 21 days, the volume of the culture medium was changed every 48 h adding 0.5 mL in the apical chamber and 1.5 mL in the basolateral chamber and the transepithelial electrical resistance (TEER) was measured by a transepithelial electrical resistance meter to monitor the integrity of the cell layer. After 21 days, when a confluent monolayer is formed (TEER value higher than 350 Ω), 0.5 mL of PLE-EAE extract and non-toxic DIG were added on cell monolayer in the apical chamber (API) and 1.5 mL of PBS in the basolateral chamber (BASO). Fractions were maintained in contact with the cell monolayer for 6 h. Finally, unabsorbed fraction corresponding to PLE-EAE and DIG extracts (PLE-EAE-API and DIG-API, respectively) and absorbed fraction corresponding to PLE-EAE and DIG extracts (PLE-EAE-BASO and DIG-BASO, respectively) were collected and stored at −20 °C until analysis.

2.6. Total phenolic content (TPC)

Total phenolic content (TPC) was determined by Folin-Ciocalteu (FC) assay described by Kosar, Dorman, and Hiltunen (2005). Briefly, 600 μL of water and 50 μL of undiluted FC reagent were mixed with 10 μL of sample and the mixture was shaken for 1 min. Subsequently, 150 μL of 2% (w/v) Na_2CO_3 and 190 μL of water were added to the mixture and it was agitated. After 2 h at 20 °C, the absorbance was measured at 760 nm in a Cary 8454 UV–Vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Results were expressed as mg of gallic acid equivalents (GAE)/100 g sample.

2.7. Total proanthocyanidin content

2.7.1. DMAC assay

DMAC method was used to determine the total proanthocyanidin content (PA) according to Montero, Herrero, Ibáñez, and Cifuentes (2013). Briefly, 0.1% DMAC reagent (w/v) on a mixture of ethanol/water/HCl (75:12.5:12.5, % vol) was used as DMAC solution. DMAC solution (420 μL) was mixed with 140 μL of extract, adding 140 μL of methanol instead of sample to make the blank. The absorbance was read at 640 nm after 15 min at room temperature. Finally, results were expressed as mg of epicatechin/100 g sample.

2.7.2. Vanillin assay

Total PA content was measured by vanillin assay according to Gu et al. (2008). Briefly, 1.7 mL of a vanillin solution composed of 0.5% vanillin and 4% concentrated HCl in methanol were added to 100 μL of extract. Then, the absorbance was measured at 500 nm after 20 min at room temperature. The amount of PAs was expressed as mg epicatechin/100 g sample.

2.7.3. HCl/butanol assay

HCl/butanol assay was employed to determine the total PA content using Pérez-Jiménez, Arranz, and Saura-Calixto (2009) method. The extract (200 μL) was mixed with 800 μL of HCl/butanol (5:95, v/v). After 1 h at 100 °C, tubes were centrifuged at 2500×*g* for 10 min and the supernatants were collected. The absorbance was read at 555 nm and the results were expressed as mg epicatechin/100 g sample.

2.8. Antioxidant capacity determination

2.8.1. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay was carried out according to Re et al. (1999). ABTS stock solution was made by mixing 7 mM ABTS and 2.45 mM potassium persulfate and kept for 12–16 h at room temperature and under darkness. A working solution was prepared by diluting the stock solution with 5 mM phosphate buffer (pH 7.4) until absorbance reached values of 0.70 (±0.02) AU at 734 nm. Then, 10 μL of different sample concentrations were added to 990 μL of the working solution. Absorbance was measured at 734 nm after completing the reaction (45 min). Results were expressed as TEAC (Trolox equivalent antioxidant capacity) values (μmol trolox/g sample) using Trolox as reference standard employing four different concentrations of each extract giving a linear response between 20 and 80% compared with the initial absorbance.

2.8.2. Capacity to inhibit the formation of hydroxyl radical assay

The capacity to inhibit the formation of hydroxyl radicals was evaluated according to Ajibola, Fashakin, Fagbemi, and Aluko (2011) method. Briefly, 50 μL of 3 mM 1,10 phenanthroline in 0.1 M phosphate buffer (pH 7.4) was mixed with 50 μL 3 mM $FeSO_4$, 50 μL sample, and 50 μL 0.01% H_2O_2. Subsequently, the mixture was incubated for 1 h at 37 °C and 700 rpm. After that, the absorbance was measured at 536 nm. The results were expressed as % of hydroxyl radical formation inhibition through the following equation:.

$$\% = \frac{Abs\ sample - Abs\ blank}{Abs\ control - Abs\ blank} \times 100$$

where Abs sample is the absorbance of the sample, Abs blank is the absorbance of the buffer, and Abs control is the absorbance of the solution prepared with water instead of H_2O_2.

2.9. UHPLC-MS/MS analysis

NEPs obtained by PLE-EAE as well as oral, gastric, and intestinal extracts from the digestion process and apical (API) and basolateral (BASO) extract from simulated intestinal absorption of PLE-EAE extracts and digestive extract (DIG) were characterized using a UHPLC-MS/MS system which consisted in a Thermo UltiMate 3000 UHPLC instrument equipped with a quaternary pump coupled to a Q Exactive Pro Mass Spectrometer using a HESI-II probe at 3 kV in negative mode and 3.5 kV in positive mode (Thermo Fisher Scientific, Bremen, Germany).

Chromatographic separation was performed using a porous shell fused-core Ascentis Express C18 analytical column (150 × 2.1 mm, 2.7 μm particle size) and a guard column (0.5 cm × 2.1 mm, 2.7 μm particle size) (Supelco, Bellefonte, PA, USA). The separation was performed at a flow of 0.3 mL/min with mobile phases that consisted of (A) water with 0.5% of formic acid and (B) acetonitrile with 0.5% of formic acid using an elution gradient of 5 to 30% B (0–3 min), 30 to 95% B (3–25 min), and 95 to 5% (25–26 min). The injection volume and column temperature were 5 μL and 50 °C, respectively. The mass spectrometer operated in positive and negative modes using a data-dependent acquisition mode (DDA). MS spectra were acquired within a *m/z* range from 120 to 1500 with a resolution of 1×10^6 at 70,000 using an automatic gain control (AGC) and with a maximum ion time (IT) of 200 ms. MS/MS spectra were acquired with an AGC of 1×10^5 at a resolution of 17,500 and a maximum IT of 50 ms.

2.10. Statistical analysis

Statistical software Statgraphics Centurion version XVII (Statistical Graphics Corp, USA) was used to observe differences in TPC, PA content, and antioxidant capacity among oral, gastric, intestinal, PLE-EAE-API, PLE-EAE-BASO, DIG-API, and DIG-BASO extracts. ANOVA by Fisher's exact test allowed determining statistically significant differences ($p \leq 0.05$) between mean values for different extracts at 95% confidence level. All the analyses were carried out in triplicate for each extract.

3. Results and discussion

Fig. 1 shows the analytical process carried out in this study for the extraction of NEPs from the residue of conventional extraction of sweet cherry pomace by PLE-EAE. The bioavailability of NEPs was evaluated by *in vitro* simulated digestion and intestinal absorption process in terms of total phenolic and proanthocyanidin content and antioxidant capacity during the different phases of the digestion process and intestinal absorption. In addition, NEPs from PLE-EAE extracts and NEPs obtained after oral, gastric, and intestinal processes, as well as NEPs presented in PLE-EAE-API, PLE-EAE-BASO, DIG-API, and DIG-BASO samples obtained after simulated intestinal absorption, were identified for the first time by UHPLC-MS/MS.

3.1. Total phenolic and proanthocyanidin contents and antioxidant capacity of digestive extracts

To determine the bioavailability of NEPs obtained by PLE-EAE from the conventional extraction residue of sweet cherry pomace, an *in vitro* simulated digestion process was carried out. PLE-EAE extract was obtained under the optimized extraction conditions achieved in a previous work of our research group (Domínguez-Rodríguez et al., 2021b). Under these conditions, this extract presented high content of antioxidant NEPs compared with the PLE extract obtained without enzyme using the same extraction conditions and the conventional extract. The PLE-EAE extract was employed to develop the *in vitro* simulated digestion process (5.5 g/20 mL). The total phenolic and PA contents were evaluated in each phase of the *in vitro* digestion process (oral, gastric, and intestinal phases).

As can be seen in Table 1, total phenolic and PA contents suffered changes during the digestion process. The oral sample showed the highest TPC value compared with the rest of the digestion phases but without difference with the gastric sample. Mostly TPC values decreased when the digestion process progressed. This behavior may be attributed to the effect of enzymes that are implied in each digestive process, as well as the pH of digestive conditions (Laib, Kehal, Haddad, Boudjemia, & Barkat, 2020). Besides, Hamauzu and Suwannachot (2019) observed that NEPs from persimmon presented a strong bile acid-binding

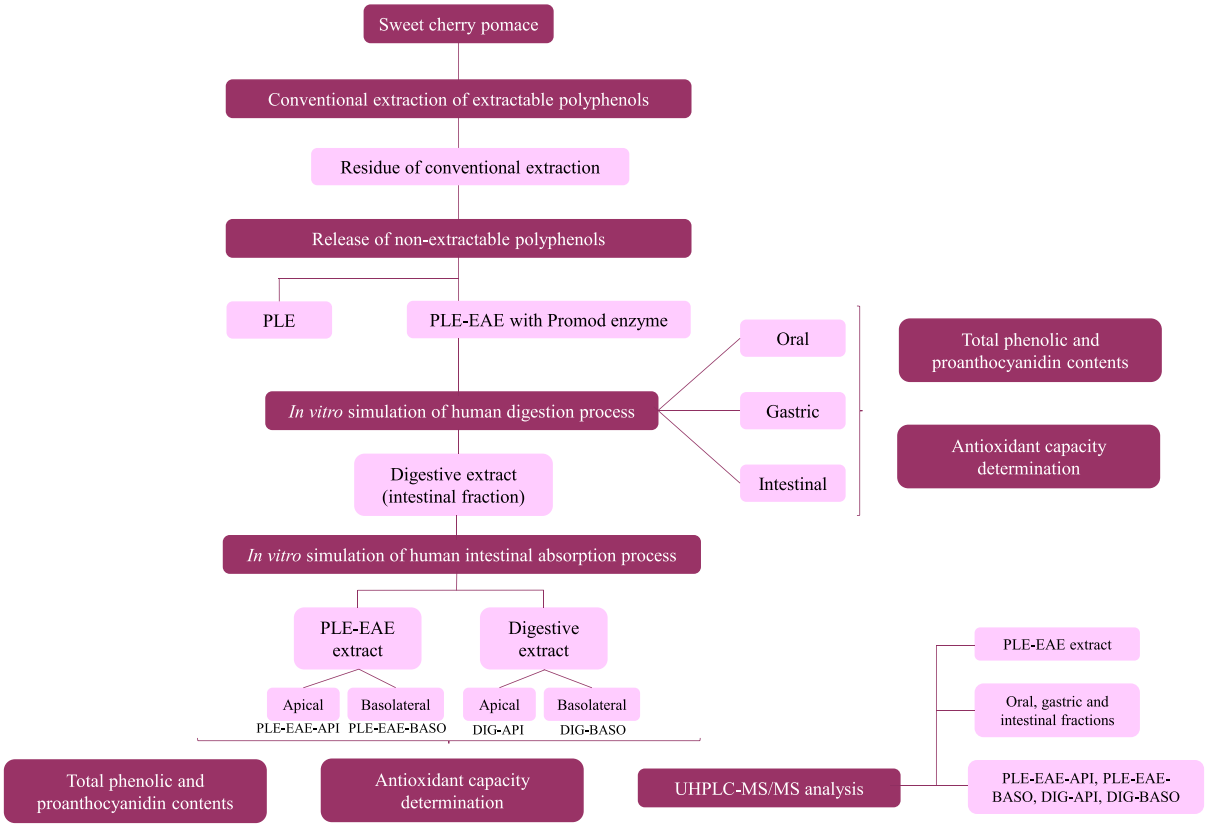

Fig. 1. Procedure to extract NEPs from sweet cherry pomace, determine their bioavailability through *in vitro* simulated digestion and absorption process and evaluate the antioxidant capacity and their total phenolic and proanthocyanidin content as well as to characterize NEPs by UHPLC-MS/MS.

Table 1
TPC (Folin-Ciocalteu method), total PA content (DMAC, vanillin, and butanol/HCl assays), and antioxidant capacity (TEAC and inhibition of hydroxyl radical assays) obtained in each phase of the digestive process (oral, gastric, and intestinal phases) from PLE-EAE extracts of sweet cherry pomace.

	Oral	Gastric	Intestinal
Folin-Ciocalteu (mg GAE/100 g sample)	61 ± 10^{a}	53 ± 10^{a}	44 ± 5^{b}
DMAC (mg epicatechin/100 g sample)	0.56 ± 0.04^{b}	0.7 ± 0.1^{a}	0.52 ± 0.08^{b}
Vanillin (mg epicatechin/100 g sample)	87 ± 3^{a}	35 ± 4^{b}	28 ± 5^{b}
Butanol/HCl (mg epicatechin/100 g sample)	14 ± 3^{c}	47 ± 7^{a}	20 ± 5^{b}
TEAC (nmol Trolox/g sample)	1.9938 ± 0.0005^{b}	1.72 ± 0.06^{c}	3.69 ± 0.07^{a}
Hydroxyl radical assay (% of hydroxyl radical inhibition)	12 ± 2^{b}	13 ± 1^{b}	26 ± 3^{a}

[a,b,c] Letters show significant differences among digestive phases ($p \leq 0.05$).

producing a weakening of the reducing power of phenolic hydroxyl. This could explain the decrease in TPC values during the intestinal digestion process. This effect was also observed in the determination of the total PA content using vanillin assay although statistical differences ($p \leq 0.05$) between the gastric and intestinal samples were not observed. In the oral phase, α-amylase enzyme was added to simulate the chemical conditions in the oral cavity. This enzyme promotes the hydrolysis of α-1,4-glucans releasing PAs attached to their structure (Shori, 2020). Nevertheless, the gastric sample showed higher PA content than oral and intestinal samples using DMAC assay. The low pH in the stomach degrades oligomers to smaller units (Tarko, Duda-Chodak, & Zajac, 2013). As a result of this degradation, a higher monomers content can be determined by DMAC assay because this method is more specific to establish monomeric PAs (Domínguez-Rodríguez et al., 2017). Although vanillin assay measures also monomeric PAs, this method is less specific than DMAC because pH conditions in the gastric phase could influence the analysis results (de la Rosa, Alvarez-Parrilla, & González-Aguilar, 2010). Besides, differences in total PA content between DMAC and vanillin assays may be due to the different reaction mechanisms of each assay. Vanillin reacts with the flavonoid ring at the 6- or 8-position while DMAC reacts with free meta-oriented hydroxyl groups and with single bonds at the 2,3-positions (de la Rosa et al., 2010).

In agreement with the DMAC assay, the gastric extract also showed a higher PA content than oral and intestinal samples using butanol/HCl assay (see Table 1). Butanol/HCl assay is based on the depolymerization of polymers under acid conditions and the conversion of the monomers to anthocyanidins which are quantified (Shay, Trofymow, & Constabel, 2017). The results indicated that probably the acid conditions of the gastric phase caused a conversion of the monomers extracted to anthocyanidins increasing the PA content in butanol/HCl assay. However, these compounds were not detected in the intestinal sample. This explains that during the digestive process, monomers could have been degraded by the low pH or by the change of pH in the intestinal phase. Besides, these compounds can suffer transformations into different structural forms undetectable by spectrophotometric methods (Lucas-González, Viuda-Martos, Pérez-Alvarez, & Fernández-López, 2018).

On the other hand, the intestinal sample attained the highest antioxidant capacity in both assays (TEAC and % inhibition of hydroxyl radical assays) (see Table 1). The antioxidant capacity increased by 50% from oral to intestinal sample. These results suggested that during the intestinal phase phenolic compounds suffer different transformations. In fact, several researchers reported that the antioxidant capacity of phenolic compounds increases after the digestion process in some cases. This effect has been associated with the pH conditions in the intestinal phase (pH 7.0) which causes a significant increase in the antioxidant capacity attributed to the deprotonation of hydroxyl moieties on the aromatic rings of polyphenols. The changes in the pH of the medium from the gastric to the intestinal phase may induce the ionization of hydroxyl groups of polyphenols increasing their antioxidant capacity (Tagliazucchi, Verzelloni, Bertolini, & Conte, 2010). Conversely, degradation of phenolic compounds during the oral phase was observed and the oral sample showed a decrease in the antioxidant capacity concerning the PLE-EAE extract. This decrease in the antioxidant capacity of the extract in the oral phase could be caused by the characteristic precipitation of proanthocyanidins when they interact with oral proteins and mucopolysaccharides (Ding, Morozova, Scampicchio, & Ferrentino, 2020).

3.2. Determination of total phenolic and proanthocyanidin contents and antioxidant capacity after simulated intestinal absorption

Fig. 2 shows the cytotoxic effect of the DIG sample obtained from PLE-EAE extracts of sweet cherry pomace at six different concentrations (412.5–45.83 mg/mL) on HeLa cells. As can be observed, cell viability was not significantly altered ($p \geq 0.05$) excepting the digestive sample at a concentration of 45.83 mg/mL which presented a reduction of 30 % of cell viability. In addition, an increase above 100% was observed at 412.5 mg/mL as well as with a concentration of 206.25 mg/mL. These results indicated that high concentrations of the DIG sample altered the normal growth of the cell as well as the use of 68.75 and 45.83 mg/mL sample. For this reason, a concentration of 103.13 mg/mL of the DIG sample was selected as the most adequate to simulate the intestinal absorption process by Caco-2 cell lines. This concentration, along with 51.56 mg/mL of digestive extract, did not show a variation in the cell viability of HeLa cells (100% cell viability). In addition, PLE-EAE extract was evaluated to know the influence of the digestive process on the bioavailability of NEPs.

Apical (API) and basolateral (BASO) fractions were collected to determine, respectively, the unabsorbed and absorbed total phenolic and proanthocyanidin contents across the intestinal barrier. As can be observed in Table 2, results showed that NEPs were degraded during the digestive process. After simulated intestinal absorption, the PLE-EAE extract showed higher total phenolic and proanthocyanidin contents than DIG extract except for PA content measured by butanol/HCl assay where digestive extract presented a higher PA content.

In addition, higher TPC values were obtained for the DIG-BASO fraction than in the DIG-API fraction. Conversely, PLE-EAE-API fraction presented higher TPC values than PLE-EAE-BASO fraction. In agreement with Ou and Gu (2014), butanol/HCl assay showed the highest polymeric PA contents in the apical fraction. Besides, PLE-EAE-API fraction presented higher polymeric PA content than DIG-API fraction in butanol/HCl assay remaining unabsorbed. These results can explain that polymeric polyphenols were converted to bioavailable monomeric polyphenols during the digestion process (Ou & Gu, 2014).

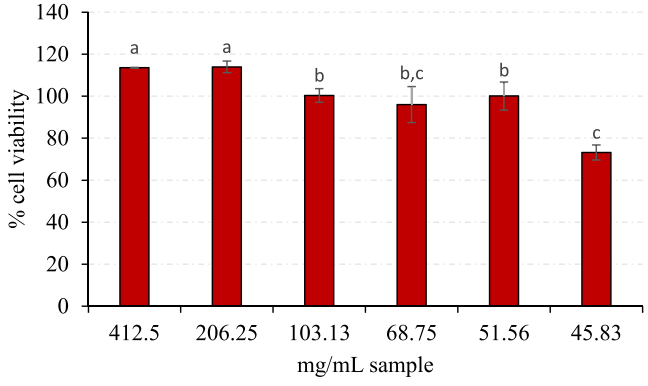

Fig. 2. Cell viability of the digestive extract at different concentrations (412.50–45.83 mg/mL) in HeLa cells. Different letters denote statistically significant differences among concentrations ($p \leq 0.05$).

Table 2
TPC (Folin-Ciocalteu method), total PA content (DMAC, vanillin, and butanol/HCl assays), and antioxidant capacity (TEAC and inhibition of hydroxyl radical assays) obtained in apical and basolateral fractions after simulated intestinal absorption process of PLE-EAE extract and digestive extract of sweet cherry pomace.

	PLE-EAE-API	PLE-EAE-BASO	DIG-API	DIG-BASO
Folin (mg GAE/100 g sample)	23.8 ± 0.1[b]	21.7 ± 0.5[c]	14.5 ± 0.2[d]	27.5 ± 0.2[a]
DMAC (mg epicatechin/100 g sample)	0.157 ± 0.001[c]	0.289 ± 0.006[a]	0.098 ± 0.002[d]	0.270 ± 0.003[b]
Vanillin (mg epicatechin/100 g sample)	17 ± 2[b]	23 ± 2[a]	14 ± 3[b]	22.7 ± 0.1[a]
Butanol/HCl (mg epicatechin/100 g sample)	15.6 ± 0.3[a]	4.56 ± 0.04[b]	17.13 ± 3.07[a]	3.69 ± 0.04[b]
TEAC (nmol Trolox/g sample)	2.94 ± 0.07[b]	4.27 ± 0.03[a]	2.170 ± 0.002[d]	2.79 ± 0.02[c]
Hydroxyl radical assay (% of hydroxyl radical inhibition)	9.4 ± 1.8[b]	34.9 ± 0.4[a]	7.3 ± 0.1[c]	5.1 ± 0.4[d]

[a,b,c,d] Letters show significant differences among apical and basolateral fractions ($p \leq 0.05$).

In fact, higher monomeric PA contents were observed as bioavailable NEPs in basolateral fraction than apical fraction through DMAC and vanillin assays. These results agree with those of Saura-Calixto et al. (2007) who observed that around 50% of hydrolyzable phenolics are bioaccessible in the intestine; however, polymeric polyphenols remain unabsorbed in the intestine.

On the other hand, Table 2 shows that the antioxidant capacity of the extract was affected by the digestive process. For instance, PLE-EAE-BASO fraction presented twice more antioxidant capacity than DIG-BASO fraction in TEAC assay, as well as thirty times more antioxidant capacity in the inhibition of hydroxyl radical assay. Moreover, bioavailable NEPs fraction (basolateral) presented the highest antioxidant capacity in TEAC assay. However, the unabsorbed fraction (DIG-API) showed a higher % of hydroxyl radical inhibition than the DIG-BASO fraction.

The use of different antioxidant assays may provide greater knowledge about the antioxidant capacity of the extracts against different radicals. In this sense, TEAC assay allowed obtaining the antioxidant capacity of the extracts against a synthetic radical (ABTS), while hydroxyl radical assay provided a closer approximation of the antioxidant effects of the extracts in our body because it evaluates the inhibition of one of the most potent reactive species in the biological system.

3.3. *Identification of NEPs present in PLE-EAE extract, digestive, and absorbed and unabsorbed fractions by UHPLC-MS/MS*

Table 3 summarizes the identification of NEPs obtained by PLE-EAE. The effect of the *in vitro* simulated digestion process in each digestion phase (oral, gastric, and intestinal phases) of PLE-EAE extract was evaluated by the identification of NEPs through UHPLC-MS/MS. Additionally, the PLE-EAE extract without previous digestion was submitted to the simulated intestinal absorption to determine the influence of the digestion process on the absorption of NEPs obtained by PLE-EAE. The apical and basolateral fractions of this extract (PLE-EAE-API, PLE-EAE-BASO) and DIG-API and DIG-BASO were compared by UHPLC-MS/MS analysis. A total of 22 compounds were identified in the PLE-EAE extract and its digestive fractions as well as the fractions collected from transepithelial absorption. The highest number of detected NEPs was found in PLE-EAE extract in which 18 different NEPs were determined.

Phenolic acids were the predominant phenolic group found in PLE-EAE extract, DIG sample, and absorbed and unabsorbed fractions (12 compounds). As can be observed in Table 3, quinic acid (number 3, Fig. 3A) and ferulic acid (number 8, Fig. 3B) with molecular ions at *m/z* 191.0551 and 193.0495 $[M-H]^-$, respectively, were observed in PLE-EAE extract, DIG sample, and all absorbed and unabsorbed fractions. These compounds were not degraded during the digestive process and remained partially bioavailable as they are present in basolateral fractions. Nevertheless, they were not completely absorbed under the experimental intestinal absorption conditions employed in this study. Quinic acid presented fragment ions at *m/z* 173 $[\text{quinic acid-H-H}_2\text{O}]^-$ and *m/z* 93 [phenol moiety]$^-$. Quinic acid is usually found as a product of hydrolysis due to phenolic compounds are frequently present in fruits as esters of quinic acids (Lara et al., 2020). In fact, caffeoylquinic acid (number 7) with a molecular ion at *m/z* 353.0881 (fragment ions at *m/z* 191 [quinic acid-H]$^-$, *m/z* 179 [caffeoyl-H]$^-$, *m/z* 161 $[\text{caffeoyl-H-H}_2\text{O}]^-$, and *m/z* 135 $[\text{caffeoyl-H-CO}_2]^-$) and coumaroylquinic acid (number 10, Fig. 3C) with a molecular ion at *m/z* 337.0931 (fragment ions at *m/z* 191 [quinic acid-H]$^-$, *m/z* 163 [coumaric acid-H]-, and *m/z* 119 $[\text{coumaric acid-H-CO}_2]^-$) were identified in digestive fractions (see Table 3). However, these compounds were not absorbed by the transepithelial intestinal barrier since they were not observed in the DIG-BASO fraction. Several researchers reported that caffeoylquinic acid and coumaroylquinic acid are poorly absorbed in the intestine (Dupas, Baglieri, Ordonaud, Tomé, & Maillard, 2006; Mortelé et al., 2021). Interestingly, hydroxybenzoic acid (number 13) was extracted by PLE-EAE, remaining intact during the digestive process (because it was present in all digestive fractions). This compound was incompletely absorbed in the intestine under the *in vitro* experimental intestinal absorption conditions used in this study. Thus, it was detected in both apical and basolateral fractions with a molecular ion at *m/z* 137.0231 $[M-H]^-$ and fragment ion at *m/z* 119 $[\text{hydroxybenzoic acid-H-H}_2\text{O}]^-$ and 93 $[\text{hydroxybenzoic acid-H-CO}_2]^-$ (see Table 3). On the contrary, gallic acid (number 6) was identified with a molecular ion at *m/z* 169.0131 $[M-H]^-$ and a fragment ion at *m/z* 125 $[\text{gallic acid-H-CO}_2]^-$ which was lost in the gastric phase of digestion because it only was detected in oral fraction. Gallic acid is susceptible to degradation by pH and/or the enzymes from the digestive process. However, the concentration of gallic acid after the digestion process depends on the food matrix (Ydjedd et al., 2017). For instance, the high content of fiber and sugars acts as a protective barrier to gallic acid degradation (Ortega, Macià, Romero, Reguant, & Motilva, 2011).

In general, a total of five NEPs were absorbed by *in vitro* simulated transepithelial absorption that corresponded to phenolic acids. Even though the studies about NEPs digestion are very scarce, the available information suggests that around 50% of hydrolyzable phenolics can be directly absorbed (Pérez-Jiménez et al., 2013). Nevertheless, most NEPs that are not bioavailable reach the large intestine without any transformation. Once they are in the colon can suffer different transformations by the microbiota fermentation or by the action of intestinal enzymes resulting in absorbable metabolites (Pérez-Jiménez et al., 2013).

Six flavonols were not found in basolateral fractions and therefore were not absorbed. In particular, rutin (number 15, Fig. 3D) was detected in PLE-EAE extract, as well as in each phase of the digestion process. This flavonol had a molecular ion at *m/z* 609.1455 $[M-H]^-$ and fragment ions at *m/z* 301 $[M-H-162-146\ \text{Da}]^-$ characteristic from the loss of a neutral glucosyl residue and a rhamnosyl residue and *m/z* 271 and 255 derived from the product ion scan of quercetin derivatives (Fu et al., 2016). However, this compound was retained in the apical fraction of the absorption process. Kaempferol-rutinoside (number 19, Fig. 3E) which was identified with a molecular ion at *m/z* 593.1514 $[M-H]^-$ and fragment ions at *m/z* 285 that correspond to its aglycone, 255 $[M-H-CH_2O]^-$, and 227 $[M-H-2CH_2O]^-$. These fragments led to the aglycone identification as kaempferol in the apical fraction (see Table 3) (Kumar, Singh, & Kumar, 2017; Li et al., 2016).

In addition, the isoflavone daidzein (number 21) was detected with a molecular ion at *m/z* 253.0578 $[M-H]^-$ and fragment ions at *m/z* 163 $[M-H-H_2O-CO_2-CO]^-$ and the characteristic fragment ion at *m/z* 134 (Chen, Zhao, Plummer, Tang, & Games, 2005; Hong et al., 2011).

Table 3
Mass spectra data of non-extractable polyphenols identified in PLE-EAE extract from sweet cherry pomace and in their respective digestive phases (oral, gastric, intestinal) and unabsorbed (PLE-EAE-API and DIG-API) and absorbed (PLE-EAE-BASO and DIG-BASO) fractions through the transepithelial barrier by UHPLC-ESI-Q-Orbitrap-MS and MS/MS.

Number	Compound	Rt (min)	Molecular formula	Error (ppm)	Measured mass [M−H]⁻	Monoisotopic mass	Main fragments MS/MS ions (*m*/*z*)	PLE-EAE	Oral	Gastric	Intestinal	PLE-EAE-API	PLE-EAE-BASO	DIG-API	DIG-BASO
1	Dehydroquinic acid	1.04	$C_7H_{10}O_6$	−4.55	189.0469	190.0477	164.0338, 145.9404, 120.0442, 115.9198, 101.9507					+	+	+	+
2	Protocatechuic acid	1.52	$C_7H_5O_4$	−1.9	153.0253	154.0266	135.3131, 109.0280, 72.1831, 64.9442	+	+	+		+	+	+	+
3	Quinic acid	1.77	$C_7H_{11}O_6$	0.44	191.0551	192.0634	173.0438, 93.0331	+	+	+	+	+	+	+	+
4	Caffeic acid hexoside	1.84	$C_{15}H_{17}O_9$	2.39	341.0875	342.0951	179.0340, 135.0438	+	+	+	+	+			
5	Vanillic acid hexoside	1.92	$C_{14}H_{17}O_9$	2.66	329.0876	330.0951	121.0646	+	+	+	+				
6	Gallic acid	2.85	$C_7H_5O_5$	0.2	169.0131	170.0215	125.0231, 103.0385	+	+						
7	Caffeoylquinic acid	2.88	$C_{16}H_{17}O_9$	3.95	353.0881	354.0951	191.0552, 179.0340, 161.0232, 135.0438	+	+	+	+	+	+		
8	Ferulic acid	2.98	$C_{10}H_9O_4$	−0.42	193.0495	194.0579	164.8946, 146.9223, 130.9387, 117.0332, 102.9322, 74.0054	+	+	+	+	+	+	+	+
9	*p*-Coumaric acid-hexoside	3.35	$C_{15}H_{17}O_8$	3.29	325.0929	326.1002	163.0390, 119.0489	+	+	+	+	+	+		
10	Coumaroylquinic acid	3.48	$C_{16}H_{17}O_8$	3.9	337.0931	338.1001	191.0552, 163.0389, 119.0488	+	+	+	+	+	+	+	
11	Ellagic acid	4.13	$C_{14}H_5O_8$	2.54	300.9987	302.0063	201.0178, 185.0236	+							
12	Ethyl caffeate	4.61	$C_{11}H_{12}O_4$	4.61	207.073	208.0736	179.9352, 162.9819, 127.8689, 103.9187, 87.9238						+		
13	Hydroxybenzoic acid	5.18	$C_7H_5O_3$	−1.71	137.0231	138.0317	119.0125, 93.0330, 81.0331	+	+	+	+			+	+
14	(*Epi*)catechin	2.76	$C_{15}H_{13}O_6$	3.64	289.0717	290.0790	245.0816, 151.0387, 109.0281	+	+	+	+		+		
15	Rutin	2.79	$C_{27}H_{29}O_{16}$	0.82	609.1455	610.1534	301.0345, 300.0276, 271.1681, 255.0293, 178.9977, 121.0284	+	+	+	+	+		+	
16	Quercetin-glucoside-rutinoside	3.64	$C_{33}H_{39}O_{21}$	0.13	771.1979	772.2062	609.1464, 463.0911, 301.0352, 300.0276, 178.9975		+	+		+	+		
17	Kaempferol glucoside	4.02	$C_{21}H_{19}O_{11}$	3.36	447.0937	448.1006	285.0402, 284.0325, 255.0294, 227.0341, 151.0017		+		+	+	+		
18	Quercetin-glucoside	4.24	$C_{21}H_{19}O_{12}$	1.11	463.0876	464.0955	300.0275, 271.0252, 178.1976, 151.0021	+	+	+	+	+			
19	Kaempferol rutinoside	4.28	$C_{27}H_{29}O_{15}$	2.14	593.1514	594.1585	327.0515, 285.0404, 227.0344, 255.0298, 151.0026	+	+	+	+	+		+	
20	Taxifolin	4.29	$C_{15}H_{11}O_7$	3.72	303.0511	304.0583	285.0404, 241.0505, 175.0387, 153.0181, 125.0230	+	+	+	+				
21	Daidzein	5.54	$C_{15}H_9O_4$	−0.25	253.0578	254.0579	163.9279, 134.8934, 103.9189, 99.9243	+						+	
22	Pelargonidin-malonylglucoside	19.63	$C_{24}H_{23}O_{13}$	−3.69	518.1056	519.1138	103.9190, 131.7540, 146.9375, 162.9506, 188.9023, 271.9749, 385.2591, 415.2352, 429.2484, 508.8663	+			+		+	+	

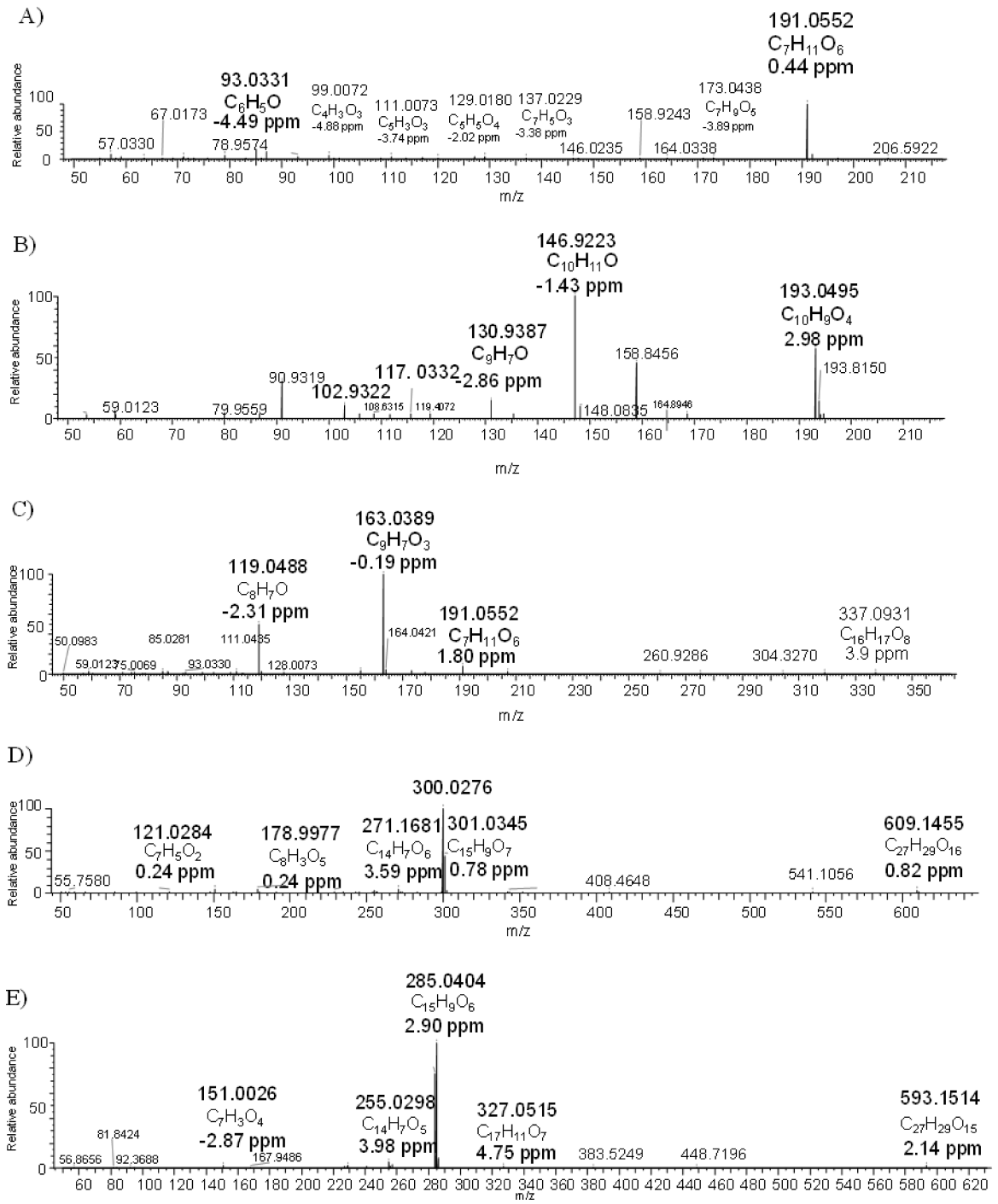

Fig. 3. MS/MS fragmentation pattern of the most representative phenolic compounds found in PLE-EAE extract, their respective digestive phases (oral, gastric, intestinal) and unabsorbed (PLE-EAE-API and DIG-API) and absorbed (PLE-EAE-BASO and DIG-BASO) fractions through the transepithelial barrier from sweet cherry pomace: A) quinic acid, B) ferulic acid, C) coumaroylquinic acid, D) rutin, and E) kaempferol-rutinoside.

Although this compound was not detected in the digestive fractions, it was identified in the DIG-API fraction. This result suggests that daidzein was released during the PLE-EAE extraction at very low concentrations, being able to be identified only in the DIG-API fraction.

Finally, pelargonidin-malonylglucoside (number 22) with a molecular ion at *m/z* 518.1056 $[M-H]^-$ was the unique anthocyanin detected in sweet cherry pomace extracts in this study, with a characteristic fragment ion at *m/z* 271 $[M-H-\text{malonylglucoside}]^-$ which represents pelargonidin (see Table 3) (Diretto, Jin, Capell, Zhu, & Gomez-Gomez, 2019). This compound was released from the residue of conventional extraction through PLE-EAE and digested but was not absorbed by the transepithelial intestine barrier since it remained retained in the DIG-API fraction. However, this anthocyanin was observed in the PLE-EAE-BASO fraction. Carrillo, Kamiloglu, Grootaert, Camp, and Hendrickx (2020) observed that pelargonidin-malonylglucoside from black carrot was not affected by the digestion process being bioaccessible. The digestion and absorption of anthocyanidins may be affected by the composition of the food matrix because they could be attached to fiber or other components of the matrix that influence their absorption. Nevertheless, anthocyanin's intestinal absorption has been estimated to be around 3–4% or less although the absorption of these compounds is not completely known (Kosinska-Cagnazzo, Diering, Prim, & Andlauer,

2015).

As can be observed, different NEPs were recovered from PLE-EAE extract. In addition, the digestion process caused a loss of NEPs, decreasing their bioavailability during simulated intestinal absorption process. In fact, a higher number of bioavailable NEPs were observed from PLE-EAE extract without being submitted to the simulated digestion process than in the digestive fraction. Therefore, UHPLC-MS/MS allowed elucidating what types of NEPs are bioavailable to be absorbed by our organism and their contributions to our biological system.

4. Conclusions

The results of this work showed that bioactive bioavailable NEPs remain retained in the residue of conventional extraction and were released by PLE-EAE with Promod enzyme. Digestion of PLE-EAE extracts caused a decrease in TPC and PA contents when the digestion process progressed except to vanillin and butanol/HCl assays where an increase of PA content was observed in the gastric phase. In addition, an increase in the antioxidant capacity was observed during the digestive process. A total of 15 NEPs were identified by UHPLC-MS/MS in the intestinal fraction from which five antioxidant NEPs were absorbed by the simulated intestinal epithelium. Moreover, higher TPC and monomeric PA values, as well as antioxidant capacity measured by inhibition of hydroxyl radical assay, were found in the basolateral fraction than in the apical fraction from the simulated intestinal absorption process.

The results obtained in this work provide greater knowledge about the possible association between NEPs intake from sweet cherry pomace and antioxidant effects in our body.

CRediT authorship contribution statement

Gloria Domínguez-Rodríguez: Validation, Formal analysis, Writing – review & editing. **María Luisa Marina:** Conceptualization, Methodology, Resources, Supervision, Funding acquisition, Project administration, Writing – review & editing. **Merichel Plaza:** Conceptualization, Methodology, Resources, Supervision, Funding acquisition, Project administration, Writing – review & editing.

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.

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