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ENZYME-ASSISTED EXTRACTION OF BIOACTIVE NON-EXTRACTABLE POLYPHENOLS FROM SWEET CHERRY (*PRUNUS AVIUM* L.) POMACE

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ABSTRACT

Sweet cherries processing produces big amounts of wastes mainly constituted by cherry pomace that can be a source of bioactive polyphenols. However, during the extraction process, an important fraction called non-extractable polyphenols (NEPs) remains retained in the extraction residue. This work describes the development of an enzyme-assisted extraction (EAE) method to obtain NEPs from sweet cherry pomace employing three different enzymes. Box-Behnken experimental designs were employed to select the optimal conditions of extraction time, temperature, enzyme concentration, and pH. The total phenolic and proanthocyanidin contents and the antioxidant and antihypertensive capacities were measured. Optimal EAE conditions extracted higher content of proanthocyanidins and with higher bioactivity from extraction residue than alkaline and acid hydrolysis. Moreover, there were higher amounts of bioactive phenolics in the extraction residue than in the sweet cherry pomace extract. The estimation of NEPs molecular weight distribution by HPLC-SEC demonstrated that EAE extracted NEPs with high molecular weight.

Keywords: Antihypertensive capacity; antioxidant capacity; enzyme-assisted extraction; non-extractable polyphenols; proanthocyanidins; size-exclusion chromatography; sweet cherry pomace.

1. INTRODUCTION

Sweet cherries (*Prunus avium* L.) are consumed in large quantities due to their attractive color, sweetness and wealth of antioxidants and nutrients. They contain carotenoids, serotonin, melatonin and high amounts of phenolic compounds (Goncalves et al., 2019; Ballistreri et al., 2012). Due to their short life (7-10 days), sweet cherries are processed into a variety of food products such as marmalades or juices, among others (Mehmet-Yilmaz et al., 2015; Kolodziejezyk et al., 2013). The large volume of processed cherries results in significant quantities of wastes, including pomace. Thus, there has been a great interest in reusing cherry waste because it represents a potential source of high added value bioactive compounds currently underutilized. As far as we know, there are not studies about bioactive compounds in sweet cherries waste or pomace. However, it has been studied the presence of phenolic compounds with high antioxidant capacity in sour cherry pomace (*Prunus cerasus* L.) such as flavonols, flavan-3-ols, anthocyanins, hydroxycinnamic acids, and hydroxybenzoic acids (Mehmet-Yilmaz et al., 2015; Kolodziejezyk et al., 2013). These phenolic compounds have been extracted by solid-liquid extraction as well as advanced extraction techniques such as ultrasound assisted extraction, microwave assisted extraction, and supercritical carbon dioxide (Mehmet-Yilmaz et al., 2015; Kolodziejezyk et al., 2013; Demirdoven et al., 2015; Simsek et al., 2012; Wozniak et al., 2016). However, even though the advanced extraction techniques are more selective and give rise to greater extraction yields, an important fraction of polyphenols remains retained in the extraction residue. This fraction corresponds to non-extractable polyphenols (NEPs), which are high molecular weight polymeric polyphenols or individual low molecular weight phenolics linked to macromolecules (i.e. polysaccharides, proteins, …) inaccessible to solvents in the extraction due to their different interactions with the sample matrix (Pérez-Jiménez et al., 2011). NEPs with high

molecular weight are less known and they belong to proanthocyanidin group, whose monomers are flavan-3-ols, and to hydrolysable tannins, derived from gallic and ellagic acid (Domínguez-Rodríguez et al., 2017). In sweet cherry pomace, NEPs are an understudied important part of total phenolic compounds in this matrix.

The recovery process of NEPs requires acid, alkaline or enzymatic treatments of the extraction residue to release these compounds from proteins or cell wall polysaccharides (Pérez-Jiménez et al., 2009; Arranz et al., 2010). Acid and alkaline hydrolysis are the most common extraction methods employed to recover NEPs from cell wall matrices. Nonetheless, many phenolic compounds are unstable at low pH and high temperature degrading or producing structure changes on phenolics during the extraction process upon acid hydrolysis. Acid hydrolysis is efficient in breaking the glycosidic bonds but it is not appropriate for hydrolyzing ester bonds (Fazary et al., 2007). Otherwise, alkaline hydrolysis is effective in hydrolyzing both ether and ester bonds (Acosta-Estrada et al., 2014). However, acid and alkaline hydrolysis are non-specific and alter the conformation of NEPs becoming difficult to know their real structure. That is why, enzymatic hydrolysis may be an option to promote a discriminated release of NEPs because it is more selective than acid and alkaline hydrolysis (Fernández et al., 2015). Additionally, it minimizes the loss of phenolics due to extreme pH conditions and shorts extraction times (Shashidi et al., 2016; Tang et al., 2016). In spite of this, studies on the NEPs extraction from residues by enzymes are very limited. Pectinase, cellulase and tannase have been used to extract NEPs from the residue of the extraction of skins and seeds of grapes being pectinase the most effective enzyme on the release of phenolic compounds from skins while the three enzymes were effective for seeds (Fernández et al., 2015). Besides, casein protease, esterase and a commercial enzyme composed of endogalacturonase and cellulase were employed to release NEPs from apple, yellow peach and nectarine (Pérez-

Jiménez et al., 2009). Nevertheless, to our knowledge an optimization method for the extraction of NEPs by enzymes have not been described in the literature. Furthermore, NEPs from apple waste can contribute to prevent different diseases such as cancer, due to their antioxidant and antiproliferative properties, among others (Tow et al., 2011). However, the information on the content and bioactivity of NEPs in foods are still limited. Therefore, the main aim of this work was to develop an efficient extraction method based on enzyme-assisted extraction (EAE) for the recovery of NEPs with antioxidant and antihypertensive properties from sweet cherry pomace. Three different enzymes (Depol 740L, Promod 439L, and Pectinase 62L) were studied to select the suitable enzyme to extract NEPs from this matrix. Two Box-Behnken designs for each enzyme were used to select optimal extraction conditions (extraction time, temperature, pH and enzyme concentration) to reach extracts with high phenolic and proanthocyanidin contents and high antioxidant and antihypertensive capacities. Furthermore, the extracts rich in bioactive NEPs obtained under optimal extraction conditions by EAE with the three enzymes were compared with those using alkaline and acid hydrolysis. Additionally, the presence of extractable polyphenols obtained by conventional extraction method from cherry pomace was compared with the NEPs obtained by EAE, acid and alkaline hydrolysis. HPLC-SEC was employed to estimate the molecular weight distribution of the recovered NEPs and extractable polyphenols.

2. MATERIALS AND METHODS

2.1.Chemical and reagents

Ethanol, acetone, acetonitrile (99.9%), formic acid (98-100%) 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, methacrilate (8000 Da), polyethylene glycol (4000 Da), punicalagin (1084 Da), ethylene glycol (62 Da), dextran (50000 Da), iron(III) chloride, sodium carbonate, sodium hydroxide, sodium chloride, hydrogen peroxide, Folin-Ciocalteu reagent, 4-dimethylaminocinnamaldehyde (DMAC), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), potassium persulfate, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), and 2,2-diphenyl-1-picrylhydrazyl (DPPH•), 1,10-phenantroline, trifluoroacetic acid (TFA), angiotensin converting enzyme (ACE) from rabbit lung, hippuryl-histidyl-leucine, 2-[4-(2-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) and ethanolamine were obtained from Sigma-Aldrich (Saint Louis, MO, USA). Dipotassium hydrogen phosphate and sodium dihydrogen phosphate dihydrate were supplied from Merck (Darmstadt, Germany).

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

Depol 740L (36 U/mL from *Humicola* sp.), Promod 439L (220 U/mL from *Bacillus licheniformis*) and Pectinase 62L (1060 U/mL from *Aspergillus* sp.) enzymes were kindly donated by the company "Biocatalysts Limited" (Cardiff, UK).

2.2. Instrumentation

Spectrophotometric analysis to determine the total phenolic and proanthocyanidin contents and antioxidant capacities were performed using a Cary 8454 UV-Vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA).

The analysis to evaluate the antihypertensive capacity was achieved with an High-Performance Liquid Chromatography (HPLC) with a modular capillary chromatographic

system (Agilent Technologies, Palo Alto, CA, USA) equipped with a diode array detector (DAD).

The determination of NEPs molecular weight was carried out using an HPLC system 1100 from Agilent (Agilent Technologies) equipped with DAD.

2.3. Samples

Cherries belonging to the Rosaceae family, *Prunus avium* L. genus and Early Lory variety were collected in La Almunia de Doña Godina (Zaragoza, Spain) selecting ripe cherries from different trees at the end of May. The fruits were washed, de-stemmed, de-stoned, and pressed manually in order to obtain the pomace. Then, pomace was grounded in a commercial blender and stored at -20ºC until its analysis.

2.4. Conventional extraction of extractable polyphenols

The extraction of extractable polyphenols was carried out as previously described to obtain these compounds from different peel fruits such as apple, banana, kiwi, among others (Condezo-Hoyos et al., 2014; Zurita et al., 2012; Taha et al., 2012). Briefly, the extraction of cherry pomace (15 g) was achieved with 20 mL of methanol/water (50:50, v/v, pH 2.0) acidified with 2 N HCl during 1 h at room temperature with shaking. The extract was centrifuged at 2100x*g* for 10 min in order to obtain the supernatant. Extraction residue was re-extracted with 20 mL of acetone/water (70:30, v/v) for 1 h at room temperature with shaking, followed by a centrifugation at 2100x*g* for 10 min. Finally, both supernatants, methanol and acetone, were combined. Samples were prepared in triplicate and the cherry pomace extracts were stored at -20ºC until their analysis. In addition, the extraction residue was employed to carry out the extraction of NEPs.

2.5. Extraction of non-extractable polyphenols

2.4.1. Enzyme-assisted extraction (EAE)

163 The optimization of NEPs extraction with three different enzymes was achieved in two
164 sequential experimental designs for each enzyme (Depol 740L (Depol), Promod 439L
165 (Promod) and Pectinase 62L (Pectinase) enzymes). Box-Behnken design was selected
166 since it is a second order design based on three levels. MODDE 10.1 software (Sartorius
167 Stedim Biotech, Malmö, Sweden) was employed to investigate the effect of 4 factors
168 (enzyme concentration, time, temperature and pH) on the NEPs extraction from residues
169 obtained after conventional extraction of extractable polyphenols of sweet cherries
170 pomace. A Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 750 rpm was
171 used in all experiments to carry out the EAE, as well as sodium phosphate buffer (100
172 mM) and a ratio solid to liquid of 0.38 g/mL. After the extractions, the extracts were
173 centrifuged at 15000x*g* for 10 min at 4°C and supernatants were recovered to a final
174 volume of 1 mL.
175 Firstly, a Box-Behnken design was employed for screening purposes with each enzyme
176 (Design N1). In this design, the effects of the extraction temperature (30-70°C), time (30-
177 300 min), enzyme concentration (1-120 µL/g), and pH (3.0-8.0) with 3 levels and 5
178 central points were investigated. In total, 29 experiments for each enzyme design were
179 carried out in a random run order (**Table S1**). The response variables were total phenolic
180 content (Folin-Ciocalteu method) and total proanthocyanidin content (DMAC, vanillin,
181 and butanol/HCl assays). This experimental design N1 allowed to select more closed
182 ranges of the experimental factors in order to get more efficient and precise optimal
183 extraction conditions in a second one (Design N2).
184 Secondly, a Box-Behnken design N2 was carried out based on the ranges close to the
185 optimal extraction conditions obtained in the design N1 for each enzyme. In this sense,
186 three levels for each variable were tested following the ranges 5, 22.5, and 40 min for
187 extraction time, 60, 70, and 80°C for extraction temperature, and pH of 6.0, 8.0, and 10.0

for the three enzymes. The enzyme concentration ranges employed were 40, 65, and 90 µL/g for Depol, 90, 115, and 140 µL/g for Promod, and 0.50, 25.25, and 50.00 µL/g for Pectinase. 5 central points were achieved. In total, 29 experiments for each enzyme design were carried out in a random run order (**Table S2**). The response variables were the results obtained by Folin-Ciocalteu, DMAC, vanillin, butanol/HCl, DPPH, TEAC, capacity to inhibit the formation of hydroxyl radical, and Angiotensin Converting Enzyme (ACE) inhibition assays.

Analysis of variance (ANOVA) was employed to evaluate the adequacy of fitted models settled between enzyme concentration, temperature, time, and pH and the different responses. The theoretical optimal processing conditions were calculated by using graphical and numerical analysis based on the criteria of the desirability function and the response surface plots. Repetitions in triplicate of the extractions obtained by EAE with Depol, Promod and Pectinase enzymes under the theoretical optimal extraction conditions found with the experimental design N2 were conducted experimentally to verify the study.

2.4.2. Acid hydrolysis

Acid hydrolysis based on Hartzfeld et al. (2002) method, which was used to determine hydrolysable tannins, was applied. Briefly, the extraction residue (0.38 g) was treated with 1 mL of methanol/H_2SO_4 (90:10, v/v) for 20 h at 85°C in a thermoreactor (Spectroquant TR420, Merck, Germany). Then, the extracts were centrifuged at 3000x*g* for 10 min, and the supernatants were collected. After two washing with distilled water, the final volume was taken up to 2 mL. Subsequently, 200 µL of ethanolamine were added in agitation and pH was adjusted to 5.5 with a pH meter using a pH sensitive microelectrode (Metrohm pH Meter 744, Herisau, Switzerland). All extractions were conducted in triplicate.

213 **2.4.3. Alkaline hydrolysis**

214 Alkaline extraction was carried out according to Arranz et al. (2010) for the extraction of
215 polyphenols in cereals. Briefly, extraction residue (9.38 g) was treated with 25 mL of
216 NaOH (2 M) for 4 h at room temperature. Then, the mixture was neutralized with an
217 appropriate amount of hydrochloric acid (pH 3.0). All extractions were conducted in
218 triplicate.

219 **2.6. Total phenolic content (TPC)**

220 In order to determine the total phenolic content, the Folin-Ciocalteu (FC) method based
221 on the protocol by Kosar et al. (2005) with different modifications, was applied (Plaza et
222 al., 2017). Results were compared with a gallic acid calibration curve (0.05-0.90 mg/mL)
223 prepared equally and expressed as mg of gallic acid equivalents (GAE)/g sample.

224 **2.7. Total proanthocyanidin content**

225 **2.6.1. DMAC assay**

226 DMAC method was applied according to Montero et al. (2013). Results were compared
227 to an epicatechin calibration curve (0.001-0.02 mg/mL) prepared equally. The results
228 were expressed as mg of epicatechin/100 g sample.

229 **2.6.2. Vanillin assay**

230 The vanillin assay described by Gu et al. (2008) was employed to measure the total
231 proanthocyanidin (PA) content. The PA content was expressed as mg epicatechin/100 g
232 sample which was calculated from a standard curve (0.5-0.025 mg/mL) prepared at the
233 same time.

234 **2.6.3. Butanol/HCl assay**

235 Butanol/HCl assay based on the protocol by Pérez-Jiménez et al. (2009) was used. The
236 PA content was expressed as mg epicatechin/100 g sample which was obtained through
237 a standard curve (1-0.025 mg/mL).

2.7. Antioxidant capacity determination

2.7.1. Trolox equivalent antioxidant capacity assay (TEAC)

TEAC assay was applied according to Re et al. (1999) with some modifications (Plaza et al., 2013). Standard curve was obtained employing Trolox as reference standard to express the results as TEAC values (μmol Trolox/g sample). The TEAC values were obtained from four different concentrations of each extract giving a linear response between 20 and 80% comparing with the initial absorbance.

2.7.2. DPPH radical scavenging assay

The DPPH method described by Brand-Williams et al. (1995) with some modifications was employed (Plaza et al., 2013). The DPPH-methanol solution was used as a reference. The DPPH remaining in the reaction medium was calculated from a calibration curve. In order to obtain the concentration to decrease the initial DPPH concentration by 50% (EC_{50}), the percentage of remaining DPPH was plotted on a graph against the sample concentration. Thereby, a greater EC_{50} implies less antioxidant capacity in extracts.

2.7.3. Capacity to inhibit the formation of hydroxyl radical assay

The capacity to inhibit the formation of hydroxyl radicals was performed according to Ajibola et al. (2011) with some modifications (Hernández-Corroto et al., 2018). The results were expressed as % of hydroxyl radical formation inhibition using the following equation:

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

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 H_2O_2.

2.8. Antihypertensive capacity

ACE inhibition was used to determine antihypertensive capacity from cherry pomace following the Geng et al. (2010) method with some modifications. Briefly, 10 μL of ACE

enzyme (0.05 U/mL), 17.5 µL of 500 mM HEPES buffer (pH 8.3) with 300 mM NaCl and 5 µL of tripeptide HHL (hippuryl-histidyl- leucine) (1.3 mg/mL) were mixed with 2.5 µL of sample. Then, the mixture was incubated during 4.30 h at 37°C and 750 rpm, and the reaction was stopped by adding 50 µL of cold acetonitrile (-20°C). Hippuric acid (HA) formed by the hydrolysis of HHL by the action of ACE enzyme was measured with an HPLC-DAD using a Chromolith Performance RP-C18 endcapped column (100×4.6 mm) from Merck (Darmstadt, Germany). Mobile phases consisted of water with 0.025% (v/v) of trifluoroacetic acid (TFA) (mobile phase A) and acetonitrile with 0.025% (v/v) of TFA (mobile phase B). Separation was carried out in a linear gradient as follow: 5 to 85% B (0-6 min); 85 to 95% B (6-18 min); 95% B (18-19 min) and 95 to 5% B. The injection volume, flow rate, and column temperature were 10 µL, 1 ml/min, and 25°C, respectively. Detection was made at 228 nm. Captopril was used as positive control. Results were calculated for the extracts obtained in the design of experiments (Design N2) as percentage of ACE inhibition using the following equation:

$$\%\ ACE\ inhibition\ = \frac{A\ control - A\ sample}{A\ control} \times 100$$

where *A control* is the area under the peak of HA in the control and *A sample* is the area under the peak of HA in the sample.

Moreover, the concentration required for the 50% inhibition of ACE activity (IC_{50}) was calculated for the extracts obtained under the optimal conditions by EAE and the extracts performed by conventional extraction and acid and alkaline hydrolysis. The percentage of ACE inhibition corresponding to four sample dilutions was plotted against the sample concentration. Then, IC_{50} value was reached by interpolation at 50% of ACE activity.

2.9. Determination of NEPs molecular weight from cherry pomace extracts by high performance liquid size-exclusion chromatography (HPLC-SEC)

To obtain an estimation of the molecular weight range of NEPs from cherry pomace extracts, SEC was carried out using an HPLC-DAD. 20 µL of the extracts obtained by conventional extraction, acid, alkaline and enzymatic hydrolysis were injected using a size exclusion chromatography (SEC) column (PolySep-GFC-P2000, 300x7.8 mm, Phenomenex, Torrance, CA, USA) with a fractionation range of 100 Da-10 KDa. Elution was carried out in isocratic mode at 0.3 mL/min for 60 min with water as mobile phase. The column compartment was thermostated at 25ºC. The detection wavelength used was 280 nm. Methacrylate (8000 Da), polyethylene glycol (4000 Da), punicalagin (1084 Da) and ethylene glycol (62 Da) standards were used for molecular weight calibration of the SEC column. The calibration curve of molecular weight with the four standards with specific molecular weights was obtained by plotting Log MW (molecular weight) as a function of retention time (min). Responses obtained were expressed by a linear equation ($Y = -0.0857X + 5.1644$) with a R^2 determination coefficient value of 0.98572, which indicated a good linear retention between both variables. The void volume was determined with dextran (50000 Da).

2.10. Statistical analysis

In order to compare the total phenolic and PA content and antioxidant and antihypertensive capacity of the extracts obtained by conventional extraction, acid hydrolysis, alkaline hydrolysis, and EAE with Depol, Promod and Pectinase enzymes, the statistical software Statgraphics Centurion version XVII (Statistical Graphics Corp, USA) was used. ANOVA by Fisher´s exact test allowed to determine statistical 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

This work describes for the first time the development and optimization of a new environmentally sustainable extraction method based on EAE for obtaining NEPs from sweet cherry pomace. **Fig 1** shows the process followed to carry out the extraction and characterization of NEPs from cherry pomace.

3.1. Optimization of the extraction of NEPs by enzyme-assisted extraction

EAE was carried out on the residues from cherry pomace obtained after their extraction of extractable polyphenols by conventional extraction method.

Depol, Promod and Pectinase enzymes with ß-glucanase, protease and polygalacturonase and pectin lyase activities, respectively, were chosen in order to reach extracts with great bioactive NEPs content. Depol has the ability to release phenolic acids from waste material, while Promod is useful for modification of protein functionality and solubilizing proteins and their aggregates, and Pectinase is used to improve the yield from pressed fruit pulps by breaking down pectin.

In order to select the most suitable composition of the extraction buffer for recovering NEPs from cherry pomace, 100 mM sodium acetate, 100 mM sodium phosphate, and 100 mM tris-maleate buffers were tested for each enzyme, keeping constant the sample to solvent ratio (0.38 g sample/mL buffer), pH (7.0), enzyme concentration (120 µL/g of sample residue), extraction time (5 h), and temperature (55ºC). Total phenolic (FC assay) and total PA (DMAC assay) contents were measured. As can be observed in **Table S3**, the total phenolic and PA contents were higher with 100 mM sodium phosphate buffer using the three enzymes. Then, sodium phosphate buffer (100 mM) and the extraction conditions described above were employed to achieve the optimization of sample to solvent ratio (0.15, 0.25, 0.38, and 0.50 g sample/mL sodium phosphate buffer) (**Table S4**). 0.38 and 0.50 g sample/mL sodium phosphate buffer allowed the recovery of higher phenolic and PA contents than 0.15 and 0.25 g sample/mL sodium phosphate buffer.

337 Therefore, 100 mM sodium phosphate buffer and 0.38 g sample/mL were picked out to
338 perform all the extractions.
339 Once the composition of the extraction buffer and the ratio of solid to solvent were
340 selected for the extraction of NEPs from cherry pomace extraction residue, Box-Behnken
341 experimental design was utilized to optimize the influence of enzyme concentration (1.0,
342 60.5, and 120.0 μL/g sample), pH (3.0, 5.5, and 8.0), extraction time (30, 165, and 300
343 min), and temperature (30, 50, and 70°C) on four response variables (FC, DMAC,
344 vanillin, and butanol/HCl assays) (**Design N1**). **Table S1** shows the 29 experiments
345 established by the experimental design for each enzyme. Three of these experiments were
346 replicated at the central point. Additionally, **Table S1** also shows the TPC and total PA
347 content of the extracts obtained in the 29 experiments for each enzyme. The results of the
348 analysis of variance, goodness of fit, and the adequacy of the model are summarized in
349 **Table S5**. **Table S5** shows the coefficients of the established multiple linear regression.
350 The regression models of Depol, Promod, and Pectinase enzymes could explain the
351 ranges of 59.1-72.9%, 57.4-85.6%, and 57.4-85.6%, respectively, of the results variability
352 obtained by FC, DMAC, vanillin, and butanol/HCl assays. Moreover, the standard error
353 (expressed as relative standard deviation (RSD)) of the regression model was below 7.3
354 for the three employed enzymes. Additionally, ANOVA was employed to evaluate the
355 adequacy of the regression model and results were also included in **Table S5**. The
356 regression models were not considered adequate since the *p*-value for the regression test
357 was higher than 0.05 in most of the responses with the three enzymes; however, the *p*-
358 value for the lack-of-fit test was adequate because in most of the responses was higher
359 than 0.05 (see **Table S5**). Moreover, as can be seen in **Table S6,** the optimal extraction
360 conditions to recover phenolic compounds and PAs were found on the limit of the tested
361 ranges of enzyme concentration, time, temperature and pH. Therefore, a second design

362 of experiments was needed with a more precise range of the parameters in order to
363 optimize the extraction of NEPs with high biological capacities from extraction residues
364 of cherry pomace (Design N2).
365 In order to carry out the second experimental design (Design N2), a Box-Behnken design
366 with a total of 29 experiments was selected as in the first experimental design (Design
367 N1). The extraction parameters to optimize the NEPs recovery from extraction residues
368 of cherry pomace were chosen based on the results of Design N1 (see **Table S2),** which
369 were extraction times of 5.0, 22.5, and 40.0 min, temperatures of 60, 70, and 80°C, pH
370 6.0, 8.0 and 10.0, and enzyme concentration of 40, 65 and 90 µL of Depol enzyme/g of
371 sample, 90, 115 and 140 µL of Promod enzyme/g of sample, and 0.5, 25.25 and 50 µL of
372 Pectinase enzyme/g of sample. **Table S2** shows the 29 experiments established by the
373 experimental design for each enzyme and the results obtained in the eight response
374 variables selected (total phenolic content (FC assay), total PA content (DMAC, vanillin,
375 and butanol/HCl assays), total antioxidant capacity (DPPH, TEAC, and capacity to inhibit
376 the formation of hydroxyl radical assays), and antihypertensive capacity (ACE inhibition
377 method)).
378 An empirical relationship expressed by a second-order polynomial equation with
379 interaction terms was fitted between the experimental results obtained from experimental
380 design and the input variables by applying multiple linear regression. The fitted model
381 showed a total explained variance for Depol, Promod, and Pectinase enzymes of 90.4-
382 98.5%, 82.1-97.4%, and 81.8-99.0%, respectively (see Table S7). These values indicate
383 that the developed model, Design N2, was satisfactory. Moreover, the standard error
384 (expressed as relative standard deviation (RSD)) of the regression model was below 6.5,
385 8.7, and 5.3 for Depol, Promod, and Pectinase enzymes, respectively. Additionally,
386 ANOVA was employed to evaluate the adequacy of the regression model and results. In

Depol enzyme, both regression models were considered adequate since the *p*-value for the regression test was lower than 0.05 (except for the TEAC assay that presented a *p*-value of 0.134) and the *p*-value for the lack-of fit test was higher than 0.05. In Promod enzyme, all the responses presented an adequate *p*-value for the regression test and for the lack-of-fit except TEAC assay that has a *p*-value for the regression test > 0.05 (0.08) and a *p*-value for the lack-of fit test < 0.05 (0.026) and the antihypertensive capacity presented a *p*-value for the regression test > 0.05 (0.162); however, the *p*-value for the lack-of fit test was higher than 0.05 (0.396). In Pectinase enzyme, all the responses presented an adequate *p*-value for the regression test and for the lack-of-fit except antihypertensive capacity that showed a *p*-value for the regression test > 0.05 (0.125); however, the *p*-value for the lack-of fit test was higher than 0.05 (0.475) (**Table S7**).

ANOVA was used to assess the main terms affecting the responses; **Table S7** shows that depending on the enzyme and the response, different variables have significant effect (*p*-value < 0.05). For instance, the effect of extraction time and enzyme concentration at a fixed pH of 10.0 and an extraction temperature of 70°C for Promod, Depol, and Pectinase enzymes on the eight different responses is shown as counter plots in **Fig 2**. In order to display the counter plots, the extraction temperature and pH were fixed at 70°C and 10.0, respectively, because these conditions were the optimal with the three enzymes to obtain the highest values in all responses. According to **Fig 2A**, in Promod enzyme, TPC increased at high enzyme concentration and time (*p*-value<0.05). In order to obtain high total PA content with DMAC and vanillin assays short extraction times (*p*-value<0.05) were necessary but the enzyme concentration did not have significant effect (*p*-value>0.05). However, the enzyme concentration had significant effect on butanol/HCl assay which increased the PA content at higher concentrations (*p*-value<0.05). Extraction time had significant negative and positive effect in DPPH and TEAC assays, respectively,

412 while neither extraction time and enzyme concentration presented significant effect on
413 the capacity to inhibit the formation of hydroxyl radical assays and in the antihypertensive
414 capacity (p-value>0.05) (**Fig 2A** and **Table S7**). In Depol enzyme, **Fig 2B** shows that the
415 extraction time had a significant effect on the extraction of PAs (p-value<0.05) while the
416 concentration of enzyme did not have effect. The extraction of PAs increased with longer
417 extraction times using DMAC and vanillin assays but the opposite behavior was observed
418 in butanol/HCl assay. However, as can be observed in **Fig 2B**, the extraction time and the
419 concentration of enzyme did not have a clear effect on the extraction of phenolic
420 compounds and on the antioxidant capacity of the extracts with the exception of the assay
421 to measure the capacity to inhibit the formation of hydroxyl radical where the antioxidant
422 capacity increased to lower enzyme concentrations. Thus, the antihypertensive capacity
423 was higher at longer extraction times and lower enzyme concentration (p-value<0.05)
424 (**Fig 2B** and **Table S7**). Finally, about Pectinase enzyme, **Fig 2C** enables to observe that
425 the extraction time had a positive effect on the extraction of phenolics and PAs with
426 vanillin and butanol/HCl assays increasing their extraction at longer extraction times (p-
427 value<0.05). Nevertheless, high enzyme concentration was needed to obtain higher
428 antioxidant capacity with DPPH and capacity to inhibit the formation of hydroxyl radical
429 assays while in the last assay also required longer extraction times to exhibit an increment
430 in the antioxidant capacity (p-value<0.05) (**Fig 2C** and **Table S7**).
431 Moreover, **Table 1** shows that the optimal extraction conditions to obtain the highest
432 content of phenolic compounds and PAs extracts, and the total antioxidant and
433 antihypertensive capacities from cherry pomace extraction residue were different for each
434 enzyme. The optimal extraction temperature (70°C) and pH (10.0) was the same for the
435 three enzymes. Nevertheless, the optimal extraction time was 40 min for Depol and
436 Promod enzymes while 18.4 min was for Pectinase enzyme (**Table 1**). Regarding to the

437 enzyme concentration, different concentrations according to the enzyme were needed to
438 obtain the optimal conditions being 140 µL of Promod/g of sample, 90 µL of Depol/g of
439 sample, and 2 µL of Pectinase/g of sample.
440 The theoretical optimal extraction conditions to obtain extracts with the highest content
441 of phenolic compounds and PAs, and the total antioxidant and antihypertensive capacities
442 from cherry pomace extraction residue from each enzyme described in **Table 1** were used
443 to carry out the experimental EAE. **Tables 1** and **2** show the theoretical optimal values
444 that should be reached under the optimal EAE conditions for each enzyme and the
445 experimental results obtained using the optimal EAE conditions for each enzyme,
446 respectively. TPC for all enzymes were within the range of predictive model obtained
447 from experimental design, except for Promod enzyme which experimental value was
448 higher than the theoretical one. On the other hand, the experimental values of total PA
449 content obtained using DMAC, vanillin and butanol/HCl assays were within the range of
450 predictive model, excepting Depol and Promod enzymes for DMAC and vanillin assays,
451 respectively, in which the experimental values were lower than theoretical values. Also,
452 the experimental result of Depol enzyme in butanol/HCl assay was higher than theoretical
453 one. Regarding to the antioxidant capacity, the experimental results were within the range
454 of the predictive model for DPPH, TEAC and capacity to inhibit the formation of
455 hydroxyl radical assays (**Tables 1** and **2**). In contrast, the experimental antihypertensive
456 capacity results obtained showed lower % ACE inhibition than theoretical ones (49.8 ±
457 0.3% and 44.8 ± 7.3% of ACE inhibition for Promod and Pectinase enzymes,
458 respectively) excepting Depol enzyme (45.7 ± 5.9% of ACE inhibition) for which its
459 experimental value was in the range of predictive model (see **Table 1**). In general, the
460 predictive model from experimental design allowed to obtain a good prediction to extract

461 bioactive NEPs by EAE with the three enzymes, Promod, Depol and Pectinase, from
462 extraction residue of sweet cherry pomace.

463 **3.2. Comparison of enzyme assisted extraction with acid and alkaline hydrolysis to**
464 **recover NEPs from cherry pomace extraction residue and with the conventional**
465 **extraction to obtain extractable polyphenols from cherry pomace**

466 The extracts collected by the optimal EAE conditions with Depol, Promod, and Pectinase
467 enzymes to reach the highest content of NEPs and antioxidant and antihypertensive
468 capacities were compared with the extracts obtained by alkaline and acid hydrolysis of
469 sweet cherry pomace extraction residue (see **Fig 1**). Furthermore, the enzymatic, alkaline
470 and acid extractions from sweet cherry pomace extraction residue were also compared
471 with the ones of extractable polyphenols from sweet cherry pomace obtained by
472 conventional extraction method (**Fig 1**).

473 **3.2.1. Total phenolic content**

474 **Table 2** shows that the richest extracts in terms of phenolic content were the extracts
475 obtained by acid hydrolysis (1.87 ± 0.05 mg GAE/g of extraction residue) and with
476 Promod enzyme (1.75 ± 0.20 mg GAE/g of extraction residue) followed by alkaline
477 hydrolysis (1.46 ± 0.20 mg GAE/g of extraction residue) and enzymatic hydrolysis with
478 Depol enzyme (1.33 ± 0.13 mg GAE/g of extraction residue). However, the EAE with
479 Pectinase enzyme showed the lowest content of the enzymatic extractions (1.11 ± 0.13
480 mg GAE/g of extraction residue).

481 Compared to the extractions carried out to cherry pomace extraction residue for the
482 recovery of NEPs, the conventional extraction technique performed in cherry pomace for
483 the extraction of extractable phenolic compounds showed the lowest TPC value (0.38 ±
484 0.01 mg GAE/g of sample) (**Table 2**). The results affirmed that after the extraction of
485 extractable phenolic compounds from cherry pomace, there are still phenolic compounds

in the extraction residue of cherry pomace. Furthermore, the TPC was 4-5 times higher in the extraction residue than in the extracts from cherry pomace. Accordingly, acid, alkaline or enzymatic treatment after conventional extraction increased the recovery of phenolic compounds remained in the cherry pomace after getting the extractable phenolic compounds. These results agreed with those observed in several fruit peels for which the total phenolic content of NEPs extracted using acid hydrolysis was higher than the extractable polyphenols content (Pérez-Jiménez et al., 2015; Pérez-Jiménez et al., 2018).

3.2.2. Total proanthocyanidin content

In order to measure the content of high molecular polymeric polyphenols that remain in the extraction residue of cherry pomace, the total content of PAs was determined using three spectrophotometric assays such as DMAC, vanillin, and butanol/HCl. These three different methods are not comparable with the absolute polymeric polyphenols content because they use monomeric compounds as reference standards for calibration. In fact, a lack of appropriate standards and interferences from other sample components, such as anthocyanins and extraction solvents, can lead to over- or under-estimation of PA content (Domínguez-Rodríguez et al., 2017). In order to have a broad information about PAs present in the extracts, it is needed to employ a combination of total PA determination methods because each one presents different reaction mechanism. For instance, DMAC reagent reacts with flavonoids with a single bond at the 2,3-positions of the C-ring and with free meta-oriented hydroxyl groups, while vanillin reagent reacts with the flavonoid ring at the 6 or 8 positions being the sensitivity of these two methods against polymeric and monomeric compounds different. On the other hand, butanol/HCl assay produces the oxidative depolymerization of PAs to anthocyanidins (Gardana et al., 2019; Domínguez-Rodríguez et al., 2017; Sun et al., 1998).

As can be observed in **Table 2,** using DMAC and vanillin assays, acid and alkaline hydrolysis from extraction residue of cherry pomace were more effective for the extraction of PAs than the EAE with the three enzymes. However, in butanol/HCl assay, the extracts submitted to EAE showed higher total PA content than acid and alkaline hydrolysis. It could be due to the fact that large polymeric compounds may not be detected with as much sensitivity as monomeric compounds with the DMAC reagent and vanillin assay is very similar to DMAC assay even less sensitive (Gardana et al., 2019; Sun et al., 1998). Nevertheless, in butanol/HCl assay, the PAs are converted into anthocyanidins by the presence of HCl and it is the most employed method to measure the total PA content. For instance, butanol/HCl assay has been applied to measure de PA content of NEPs from several fruit peels (such as apple, banana, grape, peach, pear, kiwi, among others) (Pérez-Jiménez et al., 2015; Pérez-Jiménez et al., 2018). The results displayed in **Table 2** in this study could suggest that the extracts obtained by EAE presented higher concentration of polymeric-PAs that the ones achieved by acid and alkaline hydrolyses.

According to **Table 2,** the extracts got from cherry pomace extraction residue showed around 4-10 times more PA content in the three different assays than the extracts from cherry pomace achieved by conventional extraction technique. Then, many of the phenolic compounds that remained on the extraction residue of cherry pomace were polymeric PAs which are not taken into account when conventional extraction techniques are used because they are not extracted.

3.2.3. Antioxidant capacity

DPPH, TEAC and hydroxyl radical scavenging *in vitro* assays were used to evaluate the antioxidant capacity of extracts. The use of three different antioxidant methods may provide a broader knowledge of the chemical composition of the extracts as well as their diverse capacities against different radicals.

535 The results obtained using these methods are summarized in **Table 2** showing that the
536 three assays gave different results. In DPPH assay, the extracts of extractable polyphenols
537 obtained by conventional extraction technique and the NEPs extracts recovered by the
538 extraction with Promod enzyme showed the highest antioxidant capacity with EC_{50} values
539 of 756 ± 36 and 788 ± 36 μg sample/mL, respectively (**Table 2**). EAE with Depol and
540 Pectinase enzymes generated less active extracts than Promod enzyme with EC_{50} value
541 of 815 ± 38 and 873 ± 67 μg sample/mL. In contrast, alkaline and acid hydrolysis showed
542 the less active extracts (958 ± 91 μg/mL and 1312 ± 20 μg/mL, respectively). However,
543 opposite behavior was observed in the extractable polyphenols from peach that showed
544 lower antioxidant capacity for scavenging DPPH radicals than the NEPs obtained by acid
545 hydrolysis (Hui et al. 2019).
546 According to TEAC assay, the extracts obtained by EAE with Depol and Promod
547 enzymes displayed the best antioxidant capacity with TEAC values of 14.3 ± 0.6 and 14.8
548 ± 1.6 μmol Trolox/g of extraction residue of cherry pomace, respectively. Also, the
549 extraction by acid hydrolysis (7.3 ± 0.7 μmol Trolox/g of extraction residue of cherry
550 pomace) presented higher TEAC value than the EAE with Pectinase enzyme and the
551 extraction with alkaline hydrolysis (5.2 ± 0.2 and 4.2 ± 0.3 μmol Trolox/g of extraction
552 residue of cherry pomace, respectively). However, the extract obtained by conventional
553 extraction of extractable polyphenols showed the lowest antioxidant capacity (2.6 ± 0.1
554 μmol Trolox/g of cherry pomace) (**Table 2**). Other studies in which the extraction of
555 NEPs was not optimized, reported that the antioxidant capacity (evaluated by TEAC and
556 DPPH methods) of conventional extracts of sour cherry was higher than that obtained by
557 alkaline and enzymatic (α-amilase, protease, and pectinase) hydrolysis (Nemes et al.
558 2018).

559 On the other hand, the capacity to inhibit the formation of hydroxyl radical assay could
560 show the effectiveness to inhibit the hydroxyl radical which is very important to protect
561 the human body cells (Gangwar et al., 2014; Ma et al., 2010). Results from **Table 2**
562 demonstrated that the inhibition of the hydroxyl radical formation from the extraction
563 residue of cherry pomace depended on the extraction method. For instance, the extracts
564 obtained by Pectinase enzyme were the most active extracts with an inhibition of the
565 radical formation of 94.6 ± 0.2% followed by Promod enzyme with 79.9 ± 0.2% and
566 Depol with 66.5 ± 0.3%. The alkaline and acid hydrolysis also inhibited the formation of
567 hydroxyl radical (17.6 ± 0.4% and 12.2 ± 0.8%, respectively). Furthermore, the
568 extractable polyphenols obtained by a conventional extraction method exhibited the
569 lowest inhibition of the formation of hydroxyl radicals (5 ± 1%).
570 The differences among the results obtained by the different antioxidant assays used in this
571 work may be due to the different mechanisms of action of radicals. For instance, DPPH
572 and TEAC assays are based on the electron transfer reaction while the capacity of
573 formation of hydroxyl radical is a hydrogen atom transfer based assay. Therefore,
574 depending on the extraction method employed, different types of antioxidant NEPs may
575 be recovered being more active with a specific kind of radical and therefore, a direct
576 correlation was not observed among the antioxidant assays.
577 In general, results obtained demonstrated that NEPs have higher antioxidant capacity than
578 extractable polyphenols obtained by conventional extraction method. Also, the EAE were
579 more efficient than acid and alkaline hydrolysis to recover antioxidant NEPs from the
580 extraction residue of sweet cherry pomace. Therefore, antioxidant phenolic compounds
581 were underestimated when the extractable polyphenols were obtained.

582 **3.2.4. Antihypertensive capacity**

583 Antihypertensive capacity of extractable phenolics and NEPs from cherry pomace was
584 measured through ACE inhibition capacity for the first time. **Table 2** shows the extract
585 concentrations required for the 50% inhibition of ACE activity (IC_{50}). Thus, the extracts
586 with the highest antihypertensive capacity are the ones with the lowest IC_{50} values. As
587 can be seen in **Table 2**, the extracts obtained by EAE with Depol enzyme was the most
588 active with an IC_{50} value of 0.06 ± 0.02 g of extraction residue of cherry pomace/mL
589 followed by Promod and acid hydrolysis with IC_{50} values of 0.075 ± 0.001 and 0.08 ±
590 0.01 g of extraction residue of cherry pomace/mL, respectively. NEPs extracts obtained
591 by alkaline hydrolysis and extractable phenolics obtained by conventional extraction
592 method displayed lower IC_{50} values (0.10 ± 0.02 g of extraction residue of cherry
593 pomace/mL and 0.105 ± 0.006 g of cherry pomace/mL), but the lowest active extract was
594 the one obtained by EAE with Pectinase enzyme (IC_{50} value of 0.14 ± 0.03 g of extraction
595 residue of cherry pomace/mL).
596 Therefore, NEPs from extraction residue of cherry pomace obtained by EAE with Promod
597 and Pectinase enzyme had higher antihypertensive capacity than extractable polyphenols
598 from cherry pomace and NEPs obtained by alkaline and acid hydrolysis from extraction
599 residue of cherry pomace. In general, NEPs from cherry pomace had higher
600 antihypertensive capacity than extractable phenolic compounds. However, the
601 antihypertensive capacity of NEPs depended on the extraction method employed. The
602 differences in the antihypertensive capacity of the extracts might be due to their
603 composition and the degree of polymerization of the extracted NEPs (Eriz et al., 2011).
604 **3.3. Estimation of molecular weight distribution of NEPs and extractable**
605 **polyphenols extracted from cherry pomace extraction residues and cherry pomace,**
606 **respectively, by HPLC-SEC**

607 The molecular weight distribution of NEPs extracted by alkaline and acid hydrolysis and
608 EAE were determined by HPLC-SEC to know the influence of the technique and
609 conditions of extraction on the molecular weight distribution of the NEPs recoveries from
610 sweet cherry pomace. For this purpose, a method based on HPLC-SEC was tuned. For
611 instance, two different size exclusion chromatography columns were tested, a PolySep-
612 GFC-P2000 (300 x 7.8 mm) and a PolySep-GFC-P1000 (300 x 7.8 mm) (Phenomenex,
613 Torrance, CA, USA) with a fractionation range of 100 Da-10 KDa and 20 Da-3 KDa,
614 respectively. PolySep-GFC-P2000 column was selected because compounds with
615 molecular weight higher than 3 KDa were observed in the samples. Mobile phases
616 consisting of 100% water, 50:50 (v/v) and 30:70 (v/v) of water and acetonitrile were
617 assayed in isocratic mode. 100% water was chosen as mobile phase because better
618 separation of NEPs was observed between NEPs with low molecular weight and high
619 molecular weight. Flow rates of 0.2 and 0.3 mL/min were compared but a flow rate of 0.2
620 mL/min showed a poor separation of standards used for molecular weight calibration of
621 the SEC column. Then, a flow rate of 0.3 mL/min was employed for further experiments.
622 **Fig 3** displays the selected chromatograms obtained for the extracts reached from EAE
623 with Depol, Promod and Pectinase enzymes under the optimal extraction conditions, as
624 well as the ones performed with acid and alkaline hydrolysis of extraction residue of
625 sweet cherry pomace and the conventional extraction of extractable polyphenols of sweet
626 cherry pomace using the best separation conditions for HPLC-SEC. The data clearly
627 demonstrated that phenolic compounds were separated according to their molecular
628 weight in an analysis time of around 50 min. In addition, the molecular weight distribution
629 of the NEPs obtained in the different extracts, their total peak areas and their relative area
630 contributions (expressed as percentage of normalized areas) are summarized in **Table 3**.
631 As can be seen in **Fig 3** and **Table 3**, results showed that EAE extracts recovered mainly

632 NEPs with molecular weight higher than 1500 Da. For instance, the extracts reached with
633 Promod enzyme presented the highest peak area of compounds between 1500 and 3000
634 Da representing around 58% of total peak area, while the compounds of 3000-5000 Da
635 meant the around 42% of total peak area. Moreover, Depol enzyme allowed to extract
636 higher amount of NEPs with a molecular weight between 3000 and 5000 Da (64% of total
637 peak area) followed by NEPs between 1500 and 3000 Da (34% of total peak area).
638 However, the extracts of Pectinase enzyme exhibited the NEPs with the highest peak
639 areas of compounds >1500 Da representing the ones with molecular weight of 3000-5000
640 Da around 85% of total peak area. In EAE extracts, the compounds with molecular weight
641 <1500 Da meant less than 2% of total peak area (see **Table 3** and **Fig 3**).
642 On the other hand, acid hydrolysis allowed the extraction of NEPs with molecular weight
643 of 1500-3000 Da (92% of total peak area) while the alkaline hydrolysis recovered
644 compounds with molecular weight of 3000-5000 Da (92% of total peak area).
645 Compounds with molecular weight of 500-1000 Da and 1000-1500 Da were observed in
646 the extracts obtained by acid hydrolysis representing 4% and 3% of total peak area,
647 respectively (see **Table 3** and **Fig 3**).
648 The extractable polyphenols obtained from sweet cherry pomace by the conventional
649 extraction method presented the highest area of compounds with molecular weigh
650 between 500 and 1000 Da being the 12% of total peak area. The 60% and 28% of total
651 peak areas were the compounds with molecular weight of 1500-3000 Da and 300-5000
652 Da, respectively, but these total peak areas were very low compared with NEPs
653 extractions (see **Table 3**).
654 Therefore, the molecular weight distribution of the NEPs extracted by EAE with pectinase
655 was similar to the distribution with alkaline hydrolysis. Both extracts displayed similar
656 total phenolic and proanthocyanidins (butanol/HCl assay), and total antioxidant (DPPH

and TEAC assays) and antihypertensive capacities, being usually slightly higher in the extracts obtained by alkaline hydrolysis (**Table 2**). Ones of the most active extracts were those obtained by EAE with Promod and Depol and both extracts presented NEPs with similar molecular weight distribution and the total area of these peaks were similar in both extracts (**Tables 2** and **3**). Nevertheless, the extracts coming from acid hydrolysis presented the lowest peak areas of NEPs with molecular weight of 3000-5000 Da but the extracts presented antioxidant and antihypertensive capacity. Moreover, the extracts with the extractable polyphenols showed the lowest peak area of phenolics with molecular weight >1500 Da but they had the highest peak area of compounds with 500-1000 Da. These extracts were the ones with the lowest total phenolic and PA contents although they presented high antioxidant capacity with DPPH method and antihypertensive capacity (**Table 2**).

4. CONCLUSIONS

The present work proposes efficient extraction methods based on EAE with three different enzymes, Depol, Promod and Pectinase, to extract bioactive NEPs from the extraction residue of sweet cherry pomace (*Prunus avium* L.). The use of two Box-Behnken experimental designs allowed to study the influence of extraction time, temperature, enzyme concentration, and pH on the recovery of NEPs from this matrix for the first time. Optimal extraction conditions to extract NEPs by EAE with Depol, Promod and Pectinase enzymes were obtained at a temperature of 70°C and a pH of 10.0. However, the optimal extraction time was 40 min for Depol and Promod enzymes and 18.4 min for Pectinase enzyme while the optimum enzyme concentration was 140 µL of Promod/g of sample, 90 µL of Depol/g of sample, and 2 µL of Pectinase/g of sample. In general, EAE with Promod enzyme followed by EAE with Depol enzyme were more

682 efficient than the EAE with Pectinase enzyme reaching extracts with higher TPC, total
683 PA content, antioxidant and antihypertensive capacities. The optimal EAE methods were
684 suitable to extract NEPs from the extraction residue of sweet cherry pomace having higher
685 content of PAs and bioactivity than the extraction by alkaline and acid hydrolysis. In
686 addition, this work brought forward that after the extraction of phenolic compounds by a
687 conventional extraction method, there are higher amount of bioactive phenolics, which
688 usually are not taken into account, in the extraction residue than in the initial extract. The
689 estimation of the molecular weight distribution of the recovered NEPs and extractable
690 polyphenols by HPLC-SEC showed that EAE extracted NEPs with higher molecular
691 weight than conventional extraction method and acid hydrolysis. Nevertheless, alkaline
692 hydrolysis presented similar molecular weight distribution of NEPs than EAE with
693 Pectinase, being these extracts, the ones with the greatest peak areas of NEPs with high
694 molecular weight ranging from 3000 to 5000 Da.

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702 **CONFLICT OF INTEREST**

703 The authors declare that they have no conflict of interest.

704

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862

FIGURE CAPTIONS

Figure 1. Procedure employed to carry out the extraction and characterization of NEPs from sweet cherry pomace.

Figure 2. Contour plots showing the effect of time (min) and enzyme concentration (μL/g) at the optimum extraction temperature (70°C) and pH (10.0) on the TPC (Folin Ciocalteu method, mg GAE/g sample), total PA content (DMAC, vanillin and butanol/HCl assays, mg epicatechin/100 g sample), total antioxidant capacity (DPPH (EC_{50}, μg/mL sample), TEAC (μmol Trolox/g sample), and capacity to inhibit the formation of hydroxyl radical (% inhibition) methods) and antihypertensive capacity (% ACE inhibition) from extracts obtained by EAE with Promod (A), Depol (B), and Pectinase (C) enzymes.

Figure 3. HPLC-SEC chromatograms profiles of the extracts achieved by EAE with Promod enzyme (A), Depol enzyme (B), and Pectinase enzyme (C); and acid (D) and alkaline (E) hydrolysis from sweet cherry pomace extraction residues, as well as, of the extracts obtained by a conventional extraction method (F) from sweet cherry pomace.

881 **Table 1.** Optimal conditions (enzyme concentration, extraction time, temperature and pH) to obtain bioactive NEPs from the extraction residue of
882 sweet cherry pomace by EAE with Depol, Promod and Pectinase enzymes. Also, theoretical values of TPC (Folin-Ciocalteu method), total PA
883 content (DMAC, vanillin, and butanol/HCl assays), antioxidant capacity (DPPH, TEAC, and capacity to inhibit the formation of hydroxyl radical
884 methods) and antihypertensive capacity (ACE inhibition method) obtained under the optimal EAE conditions.

Optimal EAE conditions	**Promod enzyme**				**Depol enzyme**				**Pectinase enzyme**			
	Enz. Conc. (µL/g)	**Time (min)**	**Temp. (°C)**	**pH**	**Enz. Conc. (µL/g)**	**Time (min)**	**Temp. (°C)**	**pH**	**Enz. Conc. (µL/g)**	**Time (min)**	**Temp. (°C)**	**pH**
	90	40	70	10.0	140	40	70	10.0	2	18.4	70	10.0
Theoretical values	**Optimum value**	**Lower**	**Upper**		**Optimum value**	**Lower**	**Upper**		**Optimum value**	**Lower**	**Upper**	
Folin-Ciocalteu method (mg GAE/g sample)	2.8	2.6	3.1		1.1	0.9	1.3		1.1	0.9	1.3	
DMAC assay (mg epicatechin/100 g sample)	0.03	-0.1	0.2		0.2	0.1	0.3		0.09	-0.03	0.2	
Vanillin assay (mg epicatechin/100 g sample)	57.3	36	78.6		42.3	26.3	58.3		20.8	16.6	25	
Butanol/HCl assay (mg epicatechin/100 g sample)	43.8	22.4	85.4		20.1	13.2	27		33.9	25.9	41.9	
DPPH method (EC_{50}, µg/mL sample)	110.3	70.5	1501.2		963.3	485.3	1441.8		573.9	493.0	6547.7	
TEAC method (µmol Trolox/g sample)	59.4	11.6	107.1		28.3	4.0	52.9		62.1	-7.2	131.4	
Capacity to inhibit formation hydroxyl radical (% inhibition)	93.2	78.7	107.7		97.9	79.1	116.6		9.3	0.8	17.7	
Antihypertensive capacity (IC_{5O} g of extraction residue/mL)	0.18	0.12	0.25		0.16	0.06	0.26		0.21	0.17	0.24	

885 **Table 2.** Total phenolic content (Folin Ciocalteu method), total PA content (DMAC, Vanillin and Butanol/HCl assays), total antioxidant capacity
886 (DPPH, TEAC, and capacity to inhibit the formation of hydroxyl radical assays), and antihypertensive capacity (ACE inhibition method) obtained
887 by EAE with Promod, Depol, and Pectinase enzymes, and acid and alkaline hydrolysis from the extraction residue of sweet cherry pomace and by
888 a conventional extraction method from sweet cherry pomace. [a,b,c,d,e,f] *Letters show the significant differences among the extraction methods of NEPs*
889 *($p \leq 0.05$).*

Extraction method	TPC (mg GAE/g sample)	DMAC (mg epicatechin/100 g sample)	Vanillin (mg epicatechin/100 g sample)	Butanol/HCl (mg epicatechin/100 g sample)	DPPH (EC_{50} µg/mL sample)	TEAC (µmol Trolox/g sample)	Hydroxyl radical (% of hydroxyl radical inhibition)	IC_{5O} values of antihypertensive capacity (g of extraction residue/mL)
Promod	1.7 ± 0.2^{a}	0.06 ± 0.01^{d}	28.2 ± 0.1^{c}	43 ± 3^{a}	788 ± 36^{a}	14.3 ± 0.6^{a}	79.9 ± 0.2^{b}	0.075 ± 0.001^{b}
Depol	1.3 ± 0.1^{b}	0.08 ± 0.01^{c}	26.8 ± 0.5^{d}	40 ± 4^{b}	815 ± 38^{b}	14.8 ± 1.6^{a}	66.5 ± 0.3^{b}	0.06 ± 0.02^{a}
Pectinase	1.1 ± 0.1^{c}	0.036 ± 0.001^{e}	23 ± 1^{e}	29.2 ± 0.9^{c}	873 ± 67^{b}	5.2 ± 0.2^{c}	94.6 ± 0.2^{a}	0.13 ± 0.03^{d}
Acid hydrolysis	1.87 ± 0.05^{a}	0.15 ± 0.03^{a}	30.6 ± 0.4^{b}	18.7 ± 0.5^{d}	1311 ± 20^{d}	7.3 ± 0.7^{b}	12.2 ± 0.8^{c}	0.08 ± 0.01^{b}
Alkaline hydrolysis	1.5 ± 0.2^{b}	0.10 ± 0.01^{b}	82 ± 2^{a}	15 ± 2^{e}	958 ± 91^{c}	4.2 ± 0.3^{c}	17.6 ± 0.4^{c}	0.10 ± 0.02^{bc}
Conventional extraction	0.38 ± 0.01^{d}	0.02 ± 0.01^{e}	2.6 ± 0.8^{f}	3.9 ± 0.2^{f}	755 ± 36^{a}	2.6 ± 0.1^{d}	5 ± 1^{d}	0.105 ± 0.006^{c}

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Table 3. HPLC-SEC estimation of molecular weight distribution at 280 nm (expressed as peak area, normalized area (%) and total peak area) of NEPs and extractable polyphenols recovered from extraction residues of sweet cherry pomace (by EAE with Promod, Depol, and Pectinase enzymes, and acid and alkaline hydrolysis) and sweet cherry pomace (by conventional extraction method), respectively.

Extraction method	500-1000 Da		1000-1500 Da		1500-3000 Da		3000-5000 Da		Total Peak area (mAU*s)
	Peak area (mAU*s)	Area (%)	Peak area (mAU*s)	Area (%)	Peak area (mAU*s)	Area (%)	Peak area (mAU*s)	Area (%)	
Promod enzyme	99 ± 3	0.66	--	--	8612 ± 164	57.64	6230 ± 153	45.70	14941 ± 314
Depol enzyme	304 ± 9	1.86	--	--	5589 ± 132	34.19	10452 ± 90	63.95	16345 ± 52
Pectinase enzyme	--	--	89 ± 10	0.24	5675 ± 76	15.20	31580 ± 216	84.57	37344 ± 150
Acid hydrolysis	267 ± 40	3.74	188 ± 6	2.64	6550 ± 252	91.81	129 ± 9	1.81	7136 ± 296
Alkaline hydrolysis	--	--	--	--	2471 ± 211	8.44	26803 ± 241	91.56	29274 ± 1516
Conventional extraction	342 ± 28	11.90	--	--	1716 ± 18	59.73	815 ± 20	28.37	2873 ± 10

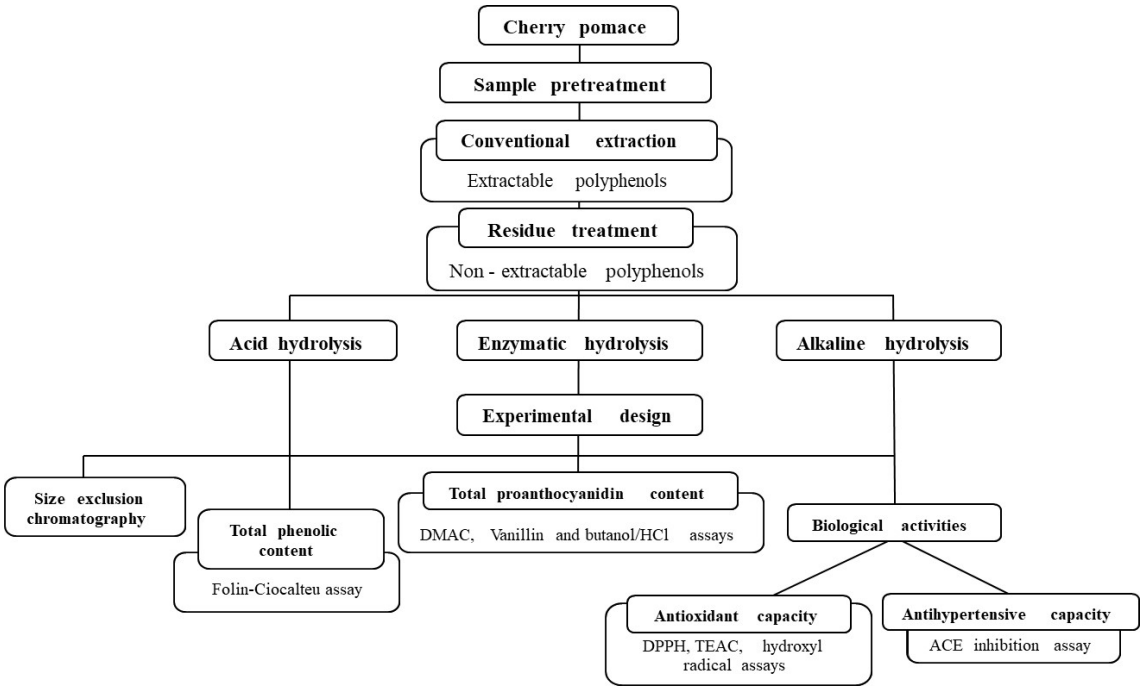
Cherry pomace
Sample pretreatment
Conventional extraction
Extractable polyphenols
Residue treatment
Non - extractable polyphenols
Acid hydrolysis
Enzymatic hydrolysis
Alkaline hydrolysis
Experimental design
Size exclusion chromatography
Total phenolic content
Folin-Ciocalteu assay
Total proanthocyanidin content
DMAC, Vanillin and butanol/HCl assays
Biological activities
Antioxidant capacity
DPPH, TEAC, hydroxyl radical assays
Antihypertensive capacity
ACE inhibition assay

Figure 1.

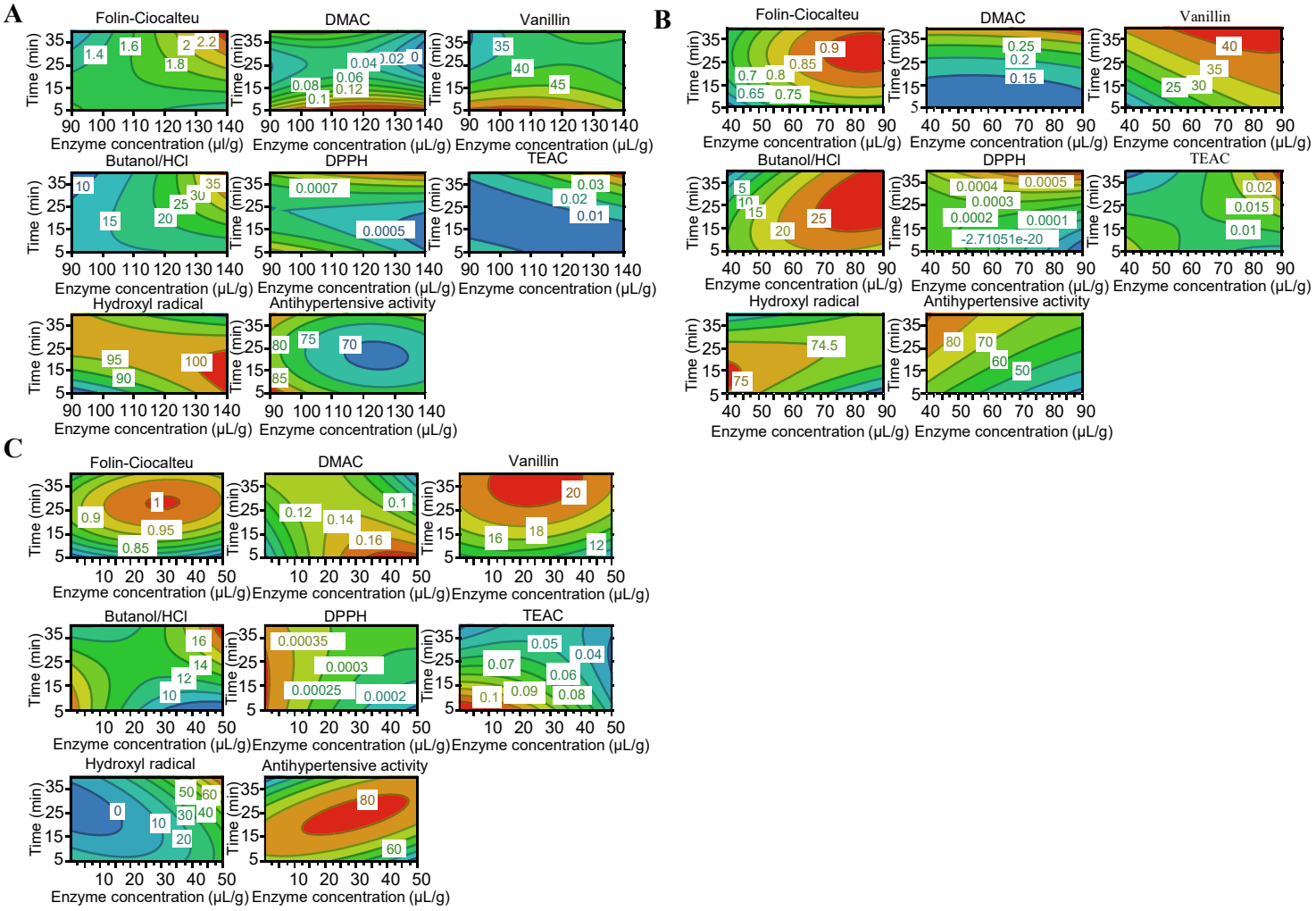
A
Folin-Ciocalteu
DMAC
Vanillin
Butanol/HCl
DPPH
TEAC
Hydroxyl radical
Antihypertensive activity
Time (min)
Enzyme concentration (µl/g)
B
Folin-Ciocalteu
DMAC
Vanillin
Butanol/HCl
DPPH
TEAC
Hydroxyl radical
Antihypertensive activity
Time (min)
Enzyme concentration (µL/g)
C
Folin-Ciocalteu
DMAC
Vanillin
Butanol/HCl
DPPH
TEAC
Hydroxyl radical
Antihypertensive activity
Time (min)
Enzyme concentration (µL/g)

Figure 2.

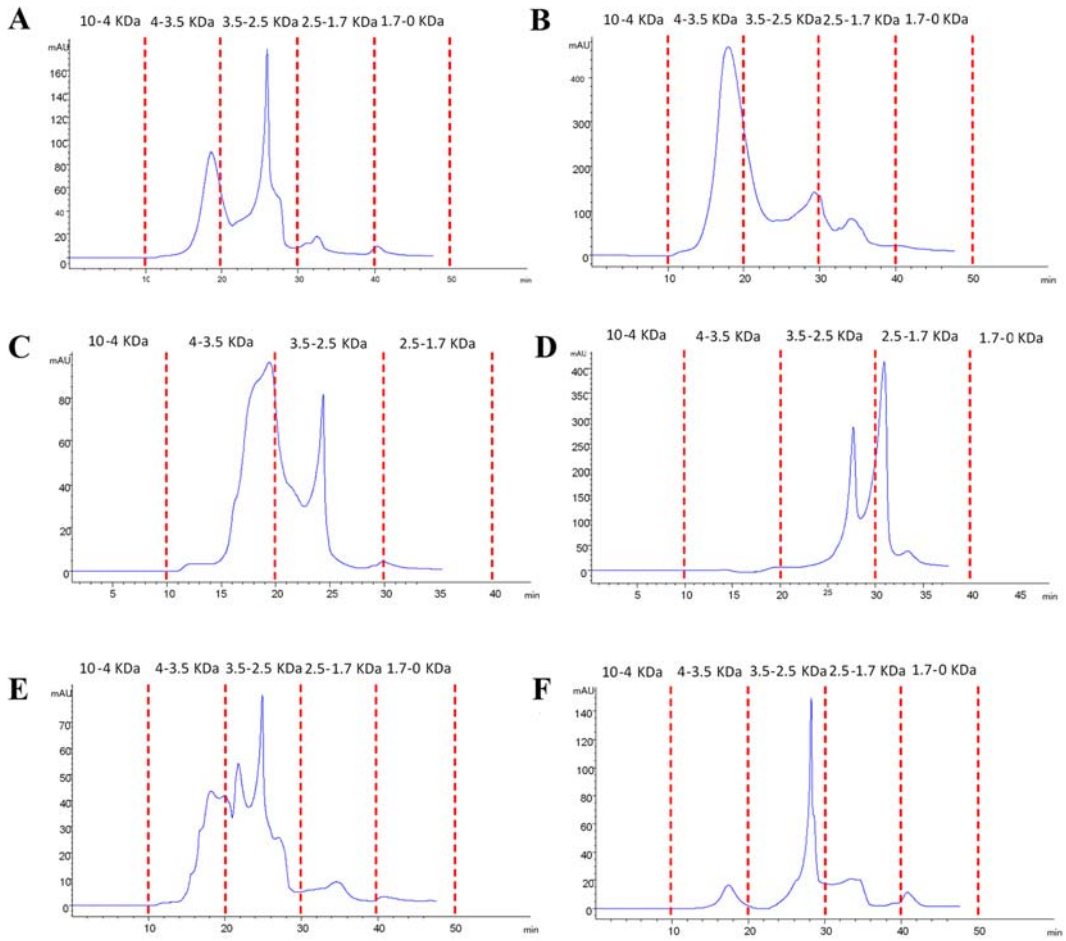
A
10-4 KDa
4-3.5 KDa
3.5-2.5 KDa
2.5-1.7 KDa
1.7-0 KDa
mAU
min
B
10-4 KDa
4-3.5 KDa
3.5-2.5 KDa
2.5-1.7 KDa
1.7-0 KDa
mAU
min
C
10-4 KDa
4-3.5 KDa
3.5-2.5 KDa
2.5-1.7 KDa
mAU
min
D
10-4 KDa
4-3.5 KDa
3.5-2.5 KDa
2.5-1.7 KDa
1.7-0 KDa
mAU
min
E
10-4 KDa
4-3.5 KDa
3.5-2.5 KDa
2.5-1.7 KDa
1.7-0 KDa
mAU
min
F
10-4 KDa
4-3.5 KDa
3.5-2.5 KDa
2.5-1.7 KDa
1.7-0 KDa
mAU
min

Figure 3.