Combining pressurized liquid extraction and enzymatic-assisted extraction to obtain bioactive non-extractable polyphenols from sweet cherry (Prunus avium L.) pomace

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Abstract: Sweet cherry pomace is a by-product that can be a source of bioactive phenolic compounds. Usually, polyphenols have been extracted using conventional extraction methodologies. However, a significant fraction, called non-extractable polyphenols (NEPs), remains retained in the conventional extraction residues. Therefore, this work is aimed, for the first time, to investigate the release of NEPs from cherry pomace combining pressurized liquid extraction (PLE) and enzyme-assisted extraction (EAE) using Promod enzyme. A response surface methodology was employed to study the influence of temperature, time, and pH on the NEPs extraction. The response variables were the total phenolic content (TPC) measured by Folin-Ciocalteu method, total proanthocyanidin (PA) content evaluated by vanillin, DMAC, and butanol/HCl assays, and total antioxidant capacity determined by Trolox equivalent antioxidant capacity and inhibition of hydroxyl radical assays. The results indicated that PLE-EAE was more suitable and selective to obtain NEPs from sweet cherry pomace than PLE alone. In fact, the extracts obtained by PLE-EAE displayed higher TPC, PA content, and bioactivity than the extracts obtained by PLE under the same extraction conditions, and those obtained by conventional methods. Moreover, size-exclusion chromatography profiles showed that the combination of PLE and EAE enabled the recovery of NEPs with higher molecular weight than PLE without EAE treatment.

Keywords: pressurized liquid extraction; enzyme-assisted extraction; non-extractable polyphenols; proanthocyanidins; sweet cherry pomace.

1. Introduction

Phenolic compounds have been widely studied due to their potential activity as antioxidants. Vegetables, fruits, and grains are the main sources of these compounds. The processing of these products originates a significant amount of by-products containing high quantities of phenolic compounds [1]. In particular, sweet cherries are processed into different products, such as marmalades or juices, generating important amounts of by-products highlighting the pomace as the main residue [2]. Sweet cherry pomace has resulted as an undervalued source of bioactive phenolic compounds like flavonols, flavan-3-ols, anthocyanins, or hydroxycinnamic acids that present antioxidant, anticarcinogenic, and anti hypertensive capacities, among others [3]. These types of phenolic compounds have been recovered by different extraction methodologies from food by-products to be used in pharmaceutical, cosmetic, or food industries. Conventional extraction is the most used technique to obtain phenolic compounds but requires a high volume of solvents and long extraction times and provides low reproducibility and selectivity [4]. In order to mitigate these problems, advanced extraction techniques such as pressurized liquid extrac-
tion (PLE) have been used to increase the efficiency and speed to extract phenolic compounds using a lower volume of solvents resulting in a more sustainable process [5]. PLE is based on the employ of solvents at high pressure and temperature maintaining the solvent in the liquid state and increasing its penetration into the food matrix to extract bioactive compounds [6]. However, conventional and advanced extraction techniques do not allow to extract an important fraction of bioactive phenolic compounds called non-extractable polyphenols (NEPs). NEPs have been shown to possess high antioxidant, anti-hypertensive, and antidiabetic properties [7-9]. NEPs comprise different classes of phenolic compounds such as phenolics with high molecular weight or simple phenols associated with macromolecules, like proteins, in the cell wall [10]. These compounds remain in the residue obtained by conventional extraction due to their strong interactions with the cell wall.

Recovery of bioactive NEPs requires an additional treatment to break these interactions. In this sense, acid and alkaline hydrolysis are the most used treatments to release NEPs from the residue of conventional extractions [9]. Nevertheless, these hydrolysis treatments are non-specific and use aggressive extraction conditions that might change the conformation of NEPs [11]. Thus, enzymatic-assisted extraction has emerged in the last years as one of the most selective and environmentally sustainable techniques to extract NEPs. Different enzymes have been employed to release NEPs from food matrices such as pectinases, cellulases, tannases, and glucuronidases, among others [12, 13]. In the case of NEPs from sweet cherry pomace, EAE has proven to extract bioactive NEPs with higher molecular weight than acid hydrolysis. In fact, extracts obtained by EAE from sweet cherry pomace presented higher proanthocyanidin content and antioxidant capacity than the extracts obtained by acid and alkaline hydrolysis [9]. However, as far as we know, there are no studies based on the combination of PLE with EAE to release NEPs from the matrix. For instance, EAE and PLE were compared when used separately to extract bioactive compounds from lemon balm observing that extracts obtained by PLE presented higher bioactive phenolic compounds than EAE [14]. Besides, PLE has been used after EAE with the extraction residue of EAE to obtain phenolic compounds from Sargassum muticum alga, but the studies about the combination of both extraction techniques are very limited [15].

For this reason, this work aimed to increase the efficiency in the extraction of antioxidant NEPs from the residue of conventional extraction of sweet cherry pomace (Prunus avium L.) developing an optimum extraction method based on the combination of PLE with EAE. Promod enzyme with protease activity was selected because it was the most appropriate enzyme, based on previous researches, to extract NEPs from sweet cherry pomace [9]. A Box-Behnken experimental design was used to determine the influence of time, temperature, and solvent pH on the extraction conditions to obtain high phenolic and proanthocyanidin contents with high antioxidant capacity. Additionally, extracts performed under the optimal extraction conditions to obtain antioxidant NEPs by PLE-EAE were compared with extracts obtained under the same conditions with PLE without EAE and with conventional extraction. Furthermore, a determination of the distribution of the molecular weight of NEPs extracted by PLE-EAE and PLE was carried out by HPLC-SEC.

2. Materials and Methods

2.1. Chemicals and reagents

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, 2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), polyethylene glycol (8000 Da), polyethylene glycol (4000 Da), twin20 (1228 Da), ethylene glycol
(62 Da) and dextran (50000 Da) 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 (Leicestershire, UK). Ultrapure water (18.2 MΩ/cm) was generated with a Millipore system (Millipore, Billerica, MA, USA).

Promod 439 L enzyme was kindly donated by the company “Biocatalysts Limited” (Cardiff, UK).

2.2. Samples

Cherries belonging to the Prunus avium L. genus, Early Lory variety, and Rosaceae family were selected from La Almunia de Doñana Godina (Zaragoza, Spain) for this study. Fruits were washed, de-stemmed, de-stoned, and pressed manually to obtain the pomace. Finally, the pomace was stored at -20 ºC until its analysis.

2.3. Conventional extraction of extractable polyphenols (EPPs)

Extractable polyphenols were obtained from the cherry pomace through the method employed by Condezo-Hoyos et al. [16] with some modifications [9]. Briefly, 15 g of cherry pomace were mixed with 20 mL of methanol/water (50:50, v/v) acidified to pH 2 with 2 N of HCl for 1 h at room temperature under shaking. To obtain the supernatant, the extract was centrifuged at 2100 xg for 10 min. Then, extraction residue was re-extracted employing 20 mL of acetone/water (70:30, v/v) for 1 h at room temperature with shaking, followed by centrifugation at 2100 xg for 10 min. Subsequently, methanol and acetone supernatants were combined. Extract and extraction residue were stored at -20 ºC until the analysis of extractable polyphenols and the NEPs extraction, respectively.

2.4. Release of non-extractable polyphenols (NEPs)

An experimental design was carried out using PLE combined with EAE employing Promod 439 L enzyme to optimize the NEPs extraction from the residue of conventional extraction of sweet cherry pomace. The Box-Behnken design was selected since it is a second-order design based on three levels. To investigate the effect of time (5-40 min), temperature (60-80 °C), and pH (6-10) with 3 levels and 5 central points on the NEPs extraction, MODDE 10.1 software (Sartorius Stedim Biotech, Malmö, Sweden) was employed. A Dionex ASE 150 instrument (Thermo Fisher; Germering, Germany) was used for the extractions. Extractions were achieved in 10 mL extraction cells, which were filled with 5.5 g of the dried residue of the conventional extraction of sweet cherry pomace on were added 140 µL of Promod enzyme/g of sample. Buffer phosphate (100 mM) was selected as an extraction solvent for releasing NEPs from cherry pomace based on the results displayed in a previous study carried out by our research team [9]. This extraction solvent was sonicated for 30 min to remove dissolved oxygen. The cell was heated up for 6 min before each analysis.

In total, 17 experiments were carried out in a random run order (Table S1). Total phenolic content (Folin-Ciocalteu assay), total proanthocyanidin content (DMAC, vanillin, and butanol/HCl assays), and antioxidant capacity (Trolox Equivalent Antioxidant Capacity (TEAC) and inhibition of hydroxyl radical assays) were selected as response variables.

The adequacy of fitted models between time, temperature, and pH and response variables was evaluated by analysis of variance (ANOVA). Graphical and numerical analyses based on the response surface plots and the criteria of the desirability function were employed to calculate the theoretical optimal processing conditions. Finally, experimental extractions were carried out using the theoretical optimal extraction conditions to verify the study.

Additionally, PLE without enzyme was also performed under the optimal extraction conditions achieved by the Box-Behnken experimental design.
2.5. Total phenolic content (TPC)

Folin-Ciocalteu (FC) assay was used to determine TPC according to Kosar et al. [17] with some modifications. Briefly, 600 µL of water and 50 µL of undiluted FC reagent were added to 10 µL of sample and the mixture was shaken. After 1 min, 150 µL of 2% (w/v) Na₂CO₃ and 190 µL of water were added to the mixture and were shaking. After completing the reaction at 20 °C for 2 h, the absorbance was measured at 760 nm in a Cary 8454 UV-Vis spectrophotometer (Agilent Technologies, Palo Alto, CA, USA). Finally, results were expressed as mg of gallic acid equivalents (GAE)/100 g sample.

2.6. Total proanthocyanidin content

2.6.1. DMAC assay

Total proanthocyanidin content (PA) was measured through DMAC method used by Montero et al. [18]. In order to prepare the DMAC solution, 0.1% DMAC reagent (w/v) on a mixture of ethanol/water/HCl (75:12.5:12.5, % vol) was used. The extract (140 µL) was mixed with 420 µL of DMAC solution and after 15 min at room temperature, the absorbance was read at 640 nm. Blanks with 140 µL of methanol instead of sample and control samples without DMAC solution were included. Results were expressed as mg of epicatechin/100 g sample.

2.6.2. Vanillin assay

Vanillin assay was employed according to Gu et al. [19] to measure the total PA content. Briefly, 1.7 mL of a solution of 0.5% vanillin and 4% concentrated HCl in methanol was mixed with 100 µL of extract. After 20 min at room temperature, the absorbance was read at 500 nm. The amount of PAs was expressed as mg of epicatechin/100 g sample.

2.6.3. HCl/butanol assay

HCl/butanol assay described by Pérez-Jiménez et al. [20] with some modifications was applied. 200 µL of extract were added to 800 µL of HCl/butanol (5:95, v/v). They were let to react for 1 h at 100 °C. Then, tubes were centrifuged at 2500 xg for 10 min and the supernatants were collected. Subsequently, the absorbance was measured at 555 nm and the results were expressed as mg epicatechin/100 g sample.

2.7. Antioxidant capacity determination

2.7.1. Trolox equivalent antioxidant capacity (TEAC) assay

TEAC assay was used according to Re et al. [21]. To form the ABTS radical cation (ABTS⁺), a stock solution of 7 mM ABTS was made to react with 2.45 mM potassium persulfate during 12-16 h at room temperature and under darkness. The stock solution was diluted with 5 mM phosphate buffer (pH 7.4) to form the work solution until absorbance reached values of 0.70 (±0.02) AU at 734 nm. The reaction was started by adding 10 µL of different sample concentrations to 990 µL of work solution. The bleaching of ABTS was followed at 734 nm at room temperature until completely reacted (45 min). Trolox was used as a reference standard to express the results as TEAC (Trolox equivalent antioxidant capacity) values (µmol trolox/g sample) employing four different concentrations of each extract giving a linear response between 20 and 80% comparing with the initial absorbance.

2.7.2. Capacity to inhibit the formation of hydroxyl radical assay

The capacity to inhibit the formation of hydroxyl radicals was measured using the Ajibola et al. [22] method. 50 µL of 3 mM of 1,10 phenanthroline in 0.1 M of phosphate buffer (pH 7.4) was added to 50 µL of 3 mM FeSO₄ in water, 50 µL of sample and 50 µL of 0.01% H₂O₂. Then, the mixture was incubated for 1 h at 37 °C and 700 rpm. The capacity to inhibit the formation of hydroxyl radicals was obtained by measuring the absorbance
at 536 nm. The results were expressed as % of hydroxyl radical formation inhibition through the following equation:

\[
\% = \frac{\text{Abs sample} - \text{Abs blank}}{\text{Abs control} - \text{Abs blank}} \times 100
\]

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 H₂O₂.

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

In order to determine the phenolic profile of NEPs obtained by PLE with Promod enzyme and PLE without enzyme under the optimal extraction conditions, size-exclusion chromatography (SEC) was performed using an 1100 HPLC-DAD system from Agilent (Agilent Technologies, Santa Clara, CA, USA). Separation was carried out on a SEC column (PolySep-GFC-P2000, 300 x 7.8 mm, Phenomenex, Torrance, CA, USA) with a fractionation range of 100 Da-10 KDa. Separation conditions were applied according to Domínguez-Rodríguez et al. [9], where 100% water was used in isocratic mode at 0.3 mL/min as mobile phase for 60 min with a column temperature of 25 °C and an injection volume of 20 µL. Twenty microliters of extract were injected. The detection wavelength employed was 280 nm. Polyethylene glycol (8000 Da), polyethylene glycol (4000 Da), twin20 (1228 Da), and ethylene glycol (62 Da) standards were used for molecular weight calibration of the SEC column. The calibration curve obtained plotting molecular weight (MW) as a function of retention time (min) was employed to determine the MW of the extracted NEPs. Responses obtained were expressed by a linear equation (\(y = -0.0028 x + 37.043\)) with an \(R^2\) determination coefficient value of 0.9122. The void volume was determined with dextran (50000 Da).

2.9. Statistical analysis

Statistical software Statgraphics Centurion version XVII (Statistical Graphics Corp, USA) was used to observe differences in TPC, PA contents, and antioxidant capacity between PLE with Promod enzyme extracts, PLE without enzyme extracts, and conventional extraction extracts. ANOVA by Fisher’s exact test allowed to determine 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

This work describes for the first time the use and optimization of the combination of two environmentally sustainable extraction methodologies based on PLE with EAE to release NEPs from the residue of conventional extraction of sweet cherry pomace.

3.1. Optimization of NEPs extraction from cherry pomace extraction residue by PLE combined with EAE

The extraction of NEPs was carried out from the residue obtained by the conventional extraction of polyphenols from sweet cherry pomace. Promod enzyme was selected because it was the most efficient in the extraction of bioactive NEPs from cherry pomace compared with Pectinase and Depol enzymes in a previous work performed by our research group. Other conditions consisted of the use of a phosphate buffer (100 mM) and an enzyme concentration of 140 µL of Promod enzyme/g of sample. This enzyme was added to the residue of conventional extraction before starting the PLE process. Promod enzyme presents protease and polygalacturonase activities and allows to modify the functionality of the proteins as well as to solubilize proteins and their aggregates improving the release of NEPs from the cell wall of the matrix and aggregates. The protease activity enables to break the proteic tonoplast surrounding cellular vacuoles contributing to the
release of phenolic compounds contained in them. On the other hand, the polygalacturonase activity enables to disrupt α-1,4-glycosidic bonds on polygalacturonic acid of pectins degrading the pectic chain and releasing phenolic compounds that interact with carboxyl and hydroxyl groups of pectin [23, 24].

Once the enzyme was added, extraction processes were carried out according to Box Behnken’s experimental design to optimize the influence of time (5-40 min), temperature (60-80 °C), and solvent pH (6-10) on six response variables (FC, DMAC, vanillin, butanol/HCl, TEAC, and hydroxyl radical assays). The time, temperature, and pH ranges to carry out the experimental design were established taken into account the results obtained by Domínguez-Rodríguez et al. [9] and according to the enzyme specifications. The 17 experiments established by Box Behnken design with their respective TPC, total PA content, and antioxidant capacity values are summarized in Table S1. Table S2 shows the analysis of variance, goodness of fit, and the adequacy of the model. For instance, it can be observed that the regression of the model of Promod enzyme could explain a satisfactory developed model with a range from 81.6 to 97.1% of the results obtained by FC, DMAC, vanillin, butanol/HCl, TEAC, and hydroxyl radical assays. In addition, the standard error of the regression model expressed as relative standard deviation (RSD) was below 6.01% in all assays. Besides, ANOVA analysis showed an adequate regression model for the responses of vanillin, butanol/HCl, TEAC, and inhibition of hydroxyl radical assays since it presented a p-value for the regression lower than 0.05. However, the regression model was not adequate for FC and DMAC assays since they showed p-values of 0.073 and 0.167, respectively. Additionally, all responses displayed an adequate p-value for the lack-of-fit test, presenting values higher than 0.05 (Table S2).

Furthermore, the ANOVA test was used to determine the main variables (time, temperature, and/or pH) that can affect the response factors with a significant effect (p-value < 0.05) (see Table S2). The time followed by the temperature were the variables that presented less significant effects on the different responses. That is why, as can be seen in Figure 1, to observe the effects of variables (time and pH) on the five responses, the counterplots were fixed at the optimum extraction temperature for obtaining the highest response values (60 °C). The TPC values increased at high pH (p-value < 0.05) while the temperature and time did not have a significant effect (p-value > 0.05) (Figure 1A and Table S2). The extraction time, temperature, and pH did not present a significant effect on the extraction of PAs when these compounds were measured by DMAC and vanillin assays (p-value > 0.05) (Figures 1B and 1C and Table S2). However, when the PA content was determined by butanol/HCl assay, the pH presented a negative effect on their extraction (p-value < 0.05). Therefore, when a high pH was employed in the extraction of PAs, the PA content decreased (Figure 1D). Concerning antioxidant capacity, the time presented a negative effect while temperature and pH showed a positive impact on the TEAC value (see Table S2). In this sense, Figure 1E shows that the antioxidant capacity measured by TEAC assay was higher at lower extraction times but at higher temperatures and pH values (p-value < 0.05). As can be seen in Table S2, the positive and negative effects of temperature and pH, respectively, were observed on antioxidant capacity measured by the inhibition of hydroxyl radical assay. In fact, Figure 1F shows that high temperatures and low pH allowed to obtain extracts with a high capacity to inhibit the formation of hydroxyl radicals.
Figure 1. Contour plots showing the effect of time (min) and pH at the optimum extraction temperature (60 °C) on the TPC (Folin-Ciocalteu method, mg GAE/g sample), total PA content (DMAC, vanillin, and butanol/HCl assays, mg epicatechin/100 g sample), and total antioxidant capacity (TEAC (µmol Trolox/g sample), and capacity to inhibit the formation of hydroxyl radical (% inhibition) methods) from PLE-EAE extracts.
Therefore, the optimal extraction conditions to obtain the highest TPC, PA content, and antioxidant capacity from cherry pomace extraction residue were an extraction temperature of 60 °C for 31 min at pH 10. These optimal extraction conditions were employed to release antioxidant NEPs from cherry pomace extraction residue. Table 1 shows the theoretical values of TPC, total PA content (DMAC, vanillin, and butanol/HCl assays), and total antioxidant capacity (TEAC and percentage of inhibition of hydroxyl radical formation assays) that were predicted under the optimal extraction conditions. As can be seen in Table 1, experimental values of TPC, PA content, and antioxidant capacity were within the range of theoretical values obtained by Box-Behnken design and closer to optimum value excepting the total PA content values obtained by vanillin and butanol/HCl assays which were closer to the upper theoretical value. This means that the predictive model from experimental design allowed to attain a good prediction to release antioxidant NEPs from cherry pomace extraction residue by PLE in combination with EAE with Promod enzyme.

Table 1. Theoretical and experimental values of TPC (Folin-Ciocalteu method), total PA content (DMAC, vanillin, and butanol/HCl assays), and antioxidant capacity (DPPH and TEAC methods) obtained under the optimal PLE-EAE conditions as well as experimental values obtained by PLE under the optimal PLE-EAE conditions but without enzyme. a,b Letters show the significant differences among extraction methods of NEPs (p ≤ 0.05).

| Optimal EAE conditions | Theoretical values | Experimental values |
|------------------------|--------------------|---------------------|
|                        | Optimum value      | Lower   | Upper   | PLE with Promod enzyme | PLE without enzyme |
| TPC (mg GAE/100 g sample) | 72.1               | 57.7    | 86.5    | 75 ± 8<sup>a</sup>      | 14 ± 1<sup>b</sup> |
| DMAC (mg epicatechin/100 g sample) | 1.04               | 0.65    | 1.44    | 0.97 ± 0.07<sup>a</sup> | 0.24 ± 0.03<sup>b</sup> |
| Vanillin (mg epicatechin/100 g sample) | 66.2               | 53.8    | 78.6    | 76 ± 8<sup>a</sup>      | 30 ± 8<sup>b</sup> |
| Butanol/HCl (mg epicatechin/100 g sample) | 13.2               | 2.4     | 24.2    | 20 ± 2<sup>a</sup>      | 11.6 ± 0.9<sup>b</sup> |
| TEAC (µmol Trolox/g sample) | 0.0056             | 0.0039  | 0.0074  | 0.0051 ± 0.0006<sup>a</sup> | 0.0027 ± 0.0008<sup>b</sup> |
| Hydroxyl radical assay (% of hydroxyl radical inhibition) | 22.8               | 10.8    | 34.9    | 20 ± 4<sup>a</sup>      | 10 ± 2<sup>b</sup> |

3.2. Comparison of PLE combined with EAE and PLE alone to release NEPs from cherry pomace extraction residue

The efficiency of the combination of both advanced extraction techniques, PLE and EAE with Promod enzyme, for the recovery of NEPs from the extraction residue of sweet cherry pomace (obtained after EPPs conventional extraction) was next evaluated. For that purpose, PLE without EAE was carried out under the optimal extraction conditions achieved by Box-Behnken experimental design (60 °C for 31 min at pH 10) and results were compared to the obtained when using simultaneously PLE and EAE.

As can be seen in Figure 2, results obtained for the EPPs extract obtained by a conventional method, and NEPs extract by PLE with and without enzyme showed statistically significant differences. As can be observed, conventional EPPs extract presented the lowest TPC and PA content values obtained by Folin-Ciocalteu, DMAC, and vanillin assays. However, PA content using butanol/HCl assay was higher in EPPs conventional extract than in PLE-EAE and PLE extracts (Figure 2.B). This fact may be because each ass
has a different reaction mechanism since butanol/HCl is more specific to determine polymeric polyphenols while DMAC and vanillin assay react with monomeric polyphenols [9].

On the other hand, TPC, PA content obtained by vanillin, DMAC, and butanol/HCl assays and antioxidant capacity measured by TEAC and percentage of inhibition of hydroxyl radical assays showed higher values in PLE combined with EAE with Promod enzyme extract than PLE without EAE extracts.

![Figure 2](image_url)

**Figure 2.** Experimental values of A) TPC (Folin-Ciocalteu method), B) total PA content (DMAC, vanillin, and butanol/HCl assays), and antioxidant capacity by C) inhibition of hydroxyl radical and D) TEAC assays obtained under the optimal PLE-EAE conditions and PLE without enzyme from the extraction residue of sweet cherry pomace and EPPs conventional extraction from sweet cherry pomace. a,b Letters show the significant differences among extraction methods of NEPs (p ≤ 0.05).

Despite PLE is an advanced extraction methodology that increases the extraction yields and reproducibility compared with conventional extraction techniques, this extraction methodology alone produced an incomplete extraction of NEPs. Possibly, this inefficient extraction could be due to the strong interactions of NEPs with the matrix. Different compounds of the food matrix can interact with NEPs being inaccessible to pressurized liquids or conventional extraction techniques [25]. However, the combination of PLE with EAE allowed releasing higher PA content than extracts obtained with PLE without EAE. In this sense, the protease and polygalacturonase activity of Promod enzyme could have released NEPs from the cell wall of the residue of the extraction of sweet cherry pomace. In fact, protease activity promotes the breakage of ester linkages with carboxylic groups in proteins and polygalacturonase activity makes possible the disruption of hydroxyl groups of pectins. The overall effect is the increase in the release of NEPs.

Additionally, the extracts displayed by PLE-EAE and PLE presented TPC values lower than the extracts obtained by conventional extraction, alkaline, and acid hydrolysis and EAE with Promod, Depol, and Pectinase enzymes extracts obtained from sweet cherry pomace by Domínguez-Rodríguez et al. [9]. By contrast, PLE-EAE and PLE extracts showed higher polymeric non-extractable PA content than acid and alkaline hydrolysis [9]. These results indicated that the differences in TPC and PA contents between these studies may be due to the influence of climatic differences in each vintage.
Additionally, antioxidant capacity results measured by the percentage of inhibition of hydroxyl radical assay showed that the EPPs extracts obtained by conventional extraction presented lower antioxidant capacity than the NEPs extracts attained by PLE-EAE and PLE extract (see Figure 2.C). Nevertheless, TEAC values for EPPs conventional extracts showed higher antioxidant capacity than PLE-EAE and PLE extracts (Figure 2.D). The differences between antioxidant methods may be due to the different reaction mechanisms of both antioxidant assays. In fact, in TEAC assay a radical (ABTS) is employed that is not generated in the human body while in the percentage of inhibition of hydroxyl radical assay one of the most relevant radicals generated in our body is used and, thus, the percentage of inhibition of hydroxyl radical assay could provide an approximation of the antioxidant effect of the extracts on the organism.

Miron et al. [14] observed that a combination of different enzymes resulted in higher extraction yields but lower antioxidant capacity than PLE. In fact, PLE-EAE and PLE extracts presented higher non-extractable PA content but lower antioxidant capacity than EAE with Promod enzyme extracts without PLE from sweet cherry pomace [9]. However, these results cannot be strictly compared because the sweet cherry pomaces from this work and the previous one correspond to different vintage and the initial composition of phenolic compounds was different in both pomaces. PLE displayed a positive effect on the extraction of NEPs since in combination with EAE showed higher antioxidant capacity than PLE alone in TEAC and inhibition of hydroxyl radical assays. As can be observed, different results were obtained depending on the extraction method and analytical assay employed due to different compounds are extracted and interact depending on the reaction mechanism of each assay. In general, results showed that NEPs remain retained in the residue of the conventional extraction of sweet cherry pomace and the combination of PLE-EAE enhances the access to release these compounds from the food matrix.

3.3. Determination of molecular weight of NEPs by Size Exclusion Chromatography

In order to have an estimation of the molecular weight of NEPs present in PLE-EAE and PLE extracts from sweet cherry pomace residue (obtained after EPPs conventional extraction), HPLC-SEC analysis was carried out.

As can be observed in Figure 3, the chromatographic analysis showed higher signal intensity in the extracts obtained only by PLE than in that from PLE-EAE.
The molecular weight distribution in the main peaks of NEPs extracted with <2000Da and 2000-6000 Da was similar in both chromatograms obtained from the PLE-EAE and PLE extracts, notwithstanding low molecular weight compounds, were more abundant in PLE than PLE-EAE extracts (Figure 3). However, PLE-EAE presented higher peak areas which corresponded to high molecular weight compounds. In fact, PLE-EAE extract exhibited a higher area of compounds with a molecular weight from 2000 Da to >8000 Da than PLE extract highlighting compounds with a molecular weight >8000 Da that showed an area of 2145 ± 70 in PLE-EAE extract (see Table 2). By contrast, PLE extract presented a higher area of compounds with a molecular weight lower than 2000 Da than PLE-EAE extract. Compounds with this molecular weight were predominant in both extracts obtained by PLE and PLE-EAE with an area of 15580 ± 284 and 8219 ± 49, respectively (Table 2).

Table 2. HPLC-SEC estimation of molecular weight distribution at 280 nm (expressed as peak area) of NEPs from extraction residues of sweet cherry pomace obtained by PLE-EAE and PLE.

| Extraction method          | > 8000Da | 8000-6000 Da | 6000-2000Da | < 2000 Da |
|----------------------------|----------|--------------|-------------|-----------|
| PLE with Promod enzyme     | 2145 ± 70| 1604 ± 183   | 1760 ± 38   | 8219 ± 49 |
| PLE without enzyme         | 811 ± 400| 1376 ± 223   | 569 ± 49    | 15580 ± 284|
In this study, PLE-EAE allowed to recover extracts with higher total peak area for NEPs than conventional extraction and acid hydrolysis from sweet cherry pomace carried out in a previous work by our research group [9], although, as it was commented above, samples analyzed proceeded from different vintage and, therefore, results are not comparable.

Despite the molecular weight distribution was similar between extracts, PLE-EAE extracts showed higher total phenolic and proanthocyanidin content as well as higher bioactivity than PLE extracts. For this reason, results suggested that NEPs with higher molecular weight provided higher bioactivity to the extracts.

5. Conclusions

This work proposes for the first time an efficient extraction method based on the combination of PLE with EAE using Promod enzyme with protease and polygalacturonase activities to achieve the extraction of NEPs from sweet cherry pomace. Box-Behnken experimental design allowed to study the influence of temperature, time, and solvent pH on the release of NEPs from the residue obtained in the EPPs extraction from cherry pomace by a conventional extraction method. The optimal extraction conditions to obtain NEPs using PLE-EAE with Promod enzyme were a temperature of 60 °C for 31 min at pH 10.0. In addition, PLE-EAE extract was compared in terms of TPC, PA content, and antioxidant capacity with PLE extracts obtained under the same optimal extraction conditions but without the addition of enzymes, and with EPPs conventional extract. In general, PLE-EAE was the most efficient extraction technique to release NEPs from sweet cherry pomace. This extract presented higher TPC, PA content, and antioxidant capacity than conventional and PLE extracts excepting polymeric PA content and antioxidant capacity measured by TEAC assay which presented higher values in the conventional EPPs extract. The estimation of the molecular weight distribution showed that PLE-EAE presented higher peak area of compounds with high molecular weight than PLE extract. For all these reasons, PLE-EAE can be recommended as an environmentally sustainable method to release antioxidant NEPs from sweet cherry pomace.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Table S1: Experimental design obtained by Box-Behnken and experimental results obtained under the designed conditions by PLE combined with EAE with Promod enzyme from extraction residue of conventional extraction of sweet cherry pomace using Folin-Ciocalteu (mg GAE/100 g sample), DMAC (mg epicatechin/100 g sample), vanillin (mg epicatechin/100 g sample), butanol/HCl (mg epicatechin/100 g sample), TEAC (µmol Trolox/g sample) and the capacity to inhibit the formation of hydroxyl radical (% of hydroxyl radical inhibition) assays response factors. Table S2. Coefficients of the multiple linear regression model from the experimental design for PLE combined with EAE with Promod enzyme that the best fitted responses (Folin-Ciocalteu, DMAC, vanillin, butanol/HCl, TEAC, and inhibition of hydroxyl radical assays) with the extraction parameters (t: time, T: temperature, and p: pH) and the analysis of variance (ANOVA).

Author Contributions: Conceptualization, M.P. and M.L.M.; methodology, G.D.R., M.P., M.L.M.; software, G.D.R.; validation, G.D.R.; formal analysis, G.D.R. and M.P.; investigation, G.D.R.; resources, M.L.M., M.C.G., M.P.; data curation, G.D.R. and M.P.; writing—original draft preparation, G.D.R. and M.P.; writing—review and editing, M.P., M.L.M., M.C.G.; visualization, G.D.R., M.C.G., M.P. and M.L.M.; supervision, M.P. and M.L.M.; project administration, M.L.M., M.C.G., M.P.; funding acquisition, M.L.M., M.C.G., M.P. All authors have read and agreed to the published version of the manuscript.

Funding: Authors thank financial support from the Comunidad de Madrid (Spain) and European funding from FSE and FEDER Programs (research project S2018/BAA-4393, AVANSECAL-II-CM) and the Comunidad de Madrid and the University of Alcalá (research project CM/JIN/2019-033, SOSBIO), M.C.G. and M.P. thank the Spanish Ministry of Science and Innovation (project PID2020-114891RB-100).

Acknowledgments: G.D.R. thanks the University of Alcalá for her predoctoral FPI contract.
Conflicts of Interest: The authors declare no conflict of interest.

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