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Efficient Conditions of Enzyme-Assisted Extractions and Pressurized Liquids for Recovering Polyphenols with Antioxidant Capacity from Pisco Grape Pomace as a Sustainable Strategy

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Abstract: The pisco industry generates significant environmental waste, particularly grape pomace, a rich source of phenolic compounds. Emerging extraction technologies offer promising alternatives for recovering these bioactive components. This study evaluated enzyme-assisted extraction (EAE) and pressurized liquid extraction (PLE) techniques, using response surface methodology to optimize phenolic compound yield and antioxidant capacity. Specifically, a D-optimal design was applied for EAE, and a Box-Behnken design for PLE. The optimal extraction conditions for EAE were 0.75 U of tannase, 40 U of cellulase, 20 °C, and 15 minutes. For PLE, the optimal parameters were 54% ethanol, 113 °C, and three extraction cycles. These conditions yielded 38.49 mg GAE g^{-1} dw and 50.03 mg GAE g^{-1} dw of total polyphenols, and antioxidant capacities of 342.47 µmol TE g^{-1} dw and 371.00 µmol TE g^{-1} dw, respectively. The extracts obtained under optimal conditions were further characterized through chromatographic techniques to determine their phenolic profiles. Seven phenolic compounds were identified: gallic acid, catechin, epicatechin, 4-hydroxybenzoic acid, quercetin-3-rutinoside hydrate, quercetin-3-O-rhamnoside, and kaempferol. PLE extracts exhibited the highest concentration of these compounds. These findings demonstrate that recovering antioxidant-rich phenolic compounds from pisco grape pomace using innovative extraction methods is a viable strategy for obtaining functional ingredients and supporting sustainable industrial practices.

Keywords: Pisco grape pomace; efficient conditions; enzyme-assisted extraction; liquid pressurized extraction; polyphenols; antioxidant capacity

1. Introduction

Grapes are one of the most produced fruits and are essential in the world economy, with approximately 75 million tons/year. Its production is oriented to fresh consumption, juices, raisins, wine, and derivatives [1,2]. In Chile, pisco is a distilled beverage obtained from grapes, and this significant industry is concentrated in the Atacama and Coquimbo regions, with the majority of grape plantations located in the latter region [3]. The pisco production process generates a by-product known as grape pomace is mainly composed of seeds and skins and weighs about 20-30 % of the harvested grapes [4], which constitutes a significant volume of waste generated and has a strong environmental impact. This residue is composed of polyphenols and other metabolites; These polyphenols have been shown to have properties that prevent various diseases [2,5]. The solution to the problem of final disposal of this pomace is to find an appropriate technology for valorization so that it can be transformed into a product with added value for marketing.

Conventional extraction (solid-liquid) is generally used to recover polyphenols from different plant matrices. However, this process has several drawbacks as it uses various toxic solvents in high quantities, generating environmental risk, long extraction processes, and low yields [6]. Among the non-conventional methods is enzyme-assisted extraction (EAE), a more environmentally friendly

alternative strategy based on enzymes' ability to degrade complex cell wall materials, thus promoting the release of trapped compounds [7]. The cell wall of grape skins is a complex network formed by various components such as pectin, lignin, proteins, and polysaccharides. The phenols in grapes include those that bind to these latter constituents through hydrogen bonds and hydrophobic interactions [8], and enzymes could release them [9]. The action of cellulases consists of the depolymerization of cellulose into simpler fermentable sugars, and tannase hydrolyzes the ester and depside bonds of tannins, producing glucose and gallic acid. Therefore, a selective enrichment in different extract phenols is expected depending on the action of each enzyme [8]. Another type of extraction is pressurized liquid extraction (PLE), which has several advantages over other extraction methods. Among these is its mechanism that establishes that high temperatures and pressure promote greater recovery of the phenolic compounds in a short period because there is an increase in the mass transfer rate from the matrix to the solvent [10].

Emerging extraction techniques such as Enzyme-Assisted Extraction (EAE) and Pressurized Liquid Extraction (PLE) are gaining increasing attention as environmentally friendly and sustainable strategies for recovering bioactive compounds from agricultural by-products. Response Surface Methodology (RSM) offers valuable statistical and mathematical tools to develop, optimize, and improve these extraction processes. RSM enables the evaluation of the effects of independent variables on response variables through experimental designs that reduce the number of required trials. Standard designs used for optimizing the extraction of polyphenolic compounds include Central Composite Design (CCD), Box-Behnken, and D-optimal designs, among others [11,12].

The main objective of this study is to use two emerging extraction technologies (EAE and PLE) for the recovery of phenolic compounds and antioxidants from Pisco grape pomace, applying experimental design models to find the best conditions in both extractions to obtain compounds with health benefits, and thus use and valorize this plant residue as a potential source for the future development of functional foods.

2. Materials and Methods

2.1. Materials

The pisco grape pomace was obtained from Pisquera de Chile S.A., located in Ovalle, Coquimbo Region, Chile, during the 2021 harvest, corresponding to the Muscat variety.

2.2. Chemicals and Reagents

The enzymes used were Aspergillus niger cellulase (white powder, ~0.8 U/mg) (Sigma-Aldrich, St. Louis, Missouri, USA) and food grade tannase (white powder ≥100 U/g) (High-tech industrial zone, China).

Ethanol, water milli-Q, Folin Ciocalteu reagent, sodium carbonate, DPPH (2,2-Diphenyl-1-picrylhydrazyl), AAPH (2,2'-Azobis (2-methylpropionamidine dihydrochloride), fluorescein sodium salt, trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), acetonitrile, water (HPLC grade), formic acid were purchased from Merck (Darmstadt, Germany), glucose 3,5-dinitrosalicylic acid and standards of phenolic compounds gallic acid, catechin, epicatechin, 4-hydroxybenzoic, quercetin-3-rutinoside hydrate, quercetin-3-O-rhamnoside, and kaempferol) were purchased from Sigma Aldrich Co. (St. Louis, MO, US).

2.3. Preparation of the Raw Material

Once the grape pomace was received, it was dehydrated in a vacuum oven at a temperature of 60 °C with a pressure of 100 mbar (Memmert, model VO 400, Schwabach, Germany) for 390 min based on a study carried out in a previous work [13]. The sample was stored until further analysis at -18 °C.

2.4. Characterization of the Dried Pomace

The proximate analysis of dried grape pomace was measured according to the methodology of the Association of Official Analytical Chemists [14]. Carbohydrates were calculated by difference. Water activity (aw) was measured at 25 °C using AQUA LAB equipment (4TE, Pullman, WA, USA). Reducing sugar content was measured according to Bailey et al. [15] and expressed as grams of glucose equivalents 100 g^{-1} dw. All measurements were performed in triplicate.

2.5. Conventional Extraction by Agitation (EA)

Conventional extraction will be carried out according to the methodology of Li et al. [16] with some modifications. One g of sample was weighed and mixed with 40 mL of 50% ethanol for 30 min in an orbital shaker (Boeco, OS-20) at 200 rpm. It was then centrifuged for 5 min at 4193 x g, and the supernatant was filtered for further analysis.

2.6. Experimental Design of Enzyme-Assisted Extraction (EAE)

A D-optimal design was used to optimize the EAE parameters and evaluate the main influences of various parameters on the total polyphenol extraction yield (Y_1) and antioxidant capacity (Y_2) of grape pomace. Four independent variables (Tannase (X_1), cellulase (X_2), temperature (X_3), and time (X_4)) with their levels (0.75-5 U; 4-40 U, 20-40 °C and 30-180 min, respectively) were considered, and the response variables were evaluated to determine the optimal extraction condition. The experimental data obtained were fitted to a second-order polynomial regression model.

2.7. Enzyme Assisted Extraction (EAE)

One g of dry pomace was weighed and mixed with 20 mL of acetate buffer (0.1 M, pH 4.8), and the dose of each enzyme was added and subjected to the process conditions delivered by the design. Then, the sample obtained in EAE was taken to a conventional extraction by shaking under conditions described by Chamorro et al. [7] with some modifications. 20 mL of absolute ethanol was added to the previous mixture and shaken for 30 min (Boeco, OS-20). It was centrifuged, filtered, and reconstituted to a final volume of 50 mL.

2.8. Experimental Design of Pressurized Liquid Extraction (PLE)

A Box Behnken design was used to optimize the PLE parameters and evaluate the main influences of various parameters on the TPC extraction yield (Y_3) and antioxidant capacity (Y_4) of grape pomace. Three independent variables (Ethanol Concentration (X_1), Temperature (X_2), and extraction cycles (X_3) with their levels (of 10-90 %; 40-120 ° C and 1-5 cycles, respectively) were considered, and the response variables were evaluated to determine the optimum extraction condition. The experimental data obtained were fitted to a second-order polynomial regression model.

2.9. Pressurized Liquid Extraction

One g of dry pomace was weighed and mixed with diatomaceous earth (1:1). It was placed in a 34 mL stainless steel cell and taken to the Accelerated Solvent Extraction (Thermo Scientific Thermo Fisher Scientific Dionex ASE 150, EE.UU), according to the experimental design. The fixed conditions of the process were 3 min according to preliminary tests, where 3-15 min were evaluated, and 103 bar of pressure, depending on the operation of the equipment. Then, the supernatant was centrifuged, filtered, and replaced with 50 mL [16].

2.10. Characterization of Optimal EAE and PLE Extracts

Total phenolic compounds (TPC) content was assessed by the Folin-Ciocalteu assay based on the method of Singleton and Rossi [17] in a multiplate reader (Perkin-Elmer, Victor TM χ3, Turku, Finland) at 750 nm. Determining antioxidant capacity by DPPH assay was performed using the method described by Grajeda-Iglesias et al. [18] in a Multiplate Reader at 510 nm. Total flavonoid

content (TFC) was determined in a spectrophotometer (Thermo Scientific, ORION AQUAMATE 8000, Madison, USA) at 415 nm [19]. The ORAC assay was performed in a 96-well microplate at an excitation wavelength of 485 nm and an emission of 535 nm [19]. Reducing sugar content was determined using dinitrosalicylic acid reagent (DNS) according to the method of Bailey et al. [15]. For the determination of the phenolic profile, the extracts were automatically injected into an HPLC system (Agilent 1200 series, Santa Clara, CA, USA) with a diode array detector (DAD) at wavelengths of 280, 310 and 370 nm, and the chromatographic conditions used were those reported by Vega-Gálvez et al. [19].

2.11. Statistical Analysis

The selected D-optimal design consisted of 29 experiments, which included four central points for calculating variance, and the Box Behnken design consisted of 17 experimental runs. For both designs, statistical regression validation was performed using ANOVA with a confidence level of 95%. The optimal conditions of the variables that maximize the response were calculated using RSM, using the Design-Expert software (test version 8.0.6, Stat Ease Inc., Minneapolis). In addition, the effect of both extractions was evaluated using an analysis of variance and the multiple-range test. Statistically significant differences were determined when the p-value was < 0.05 (Statistical Graphics Corp., Herndon, VA, USA).

3. Results and Discussion

3.1. Characterization of the Dried Pomace

Table 1 shows the proximate analysis of the vacuum-dried sample. The moisture content of the final product was 5.51 g 100 g^{-1} expressed on a dry basis and an aw of 0.3069. The water activity must be less than 0.6 to ensure the raw matrix is microbiologically stable and thus increases its shelf life. The lipid content was 7.35 g 100 g^{-1} dw, mainly attributed to grape pomace seeds. The ashes and proteins presented values of 5.83 and 12.74 g 100 g^{-1} dw, respectively. These values of the proximal analysis varied from those of other studies on grape pomace [20–23].

The total dietary fiber (TDF) content was high, at 40.94%. It should be noted that the total dietary fiber is composed of lignins, celluloses, and hemicelluloses that are linked to the insoluble dietary fiber (38.08%), the predominant fraction present in the pomace. The literature shows that the TDF in grape varieties is 35-55% [24], while in pomace in red grape varieties, a content of 16.4-58% TFD was found [25].

The total carbohydrate content was 74.07 g per 100 g^{-1} dw, with reducing sugars expressed as glucose accounting for 33.65 g per 100 g^{-1} dw. This indicates a substantial amount of simple sugars, with the remaining fraction mainly corresponding to dietary fiber. These findings align with those reported for pisco grape pomace dehydrated using different drying methods and similar grape varieties [21]. In pomace, carbohydrates can vary between 6 - 15% depending on the grape variety, agro-climatic conditions, or viticultural practices. It should be considered that these white pomaces contain a relatively high number of soluble sugars, unlike pomace from red grapes [23].

Taken together, the compositional profile characterized by high fiber, relevant sugar content, and low water activity supports not only the stability and conservation of the dried matrix but also its potential as an optimal substrate for emerging extraction technologies, aimed at the efficient recovery and valorization of its bioactive compounds.

3.2. Experimental Design of Enzyme-Assisted Extraction

To define the experimental design, preliminary tests were carried out to determine the factors and levels to be used. The effect of three enzymes, pectinase, cellulase, and tannase, applied individually at different concentrations, on the extraction yield of total polyphenols was evaluated. According to the results, cellulase and tannase increased polyphenol extraction in a concentration-

dependent manner, showing a progressive improvement in yield with increasing dosage. In contrast, pectinase (results not shown) did not significantly improve polyphenol recovery, regardless of the concentration applied. Several studies have shown a positive effect of using individual and combined enzymes [8]. The action of cellulase is because its substrate is cellulose, one of the polysaccharides found in the highest proportion in the pomace (12%) and is characterized by giving stability and rigidity to the cell wall. Therefore, its degradation allows access to the compounds trapped in its structure; and tannase can depolymerize the polyphenols present [7].

D-optimal experimental design using response surface methodology (RSM) was performed to establish the optimal conditions for maximizing total polyphenol extraction yield (TPC) and antioxidant capacity (DPPH). The effects of four independent variables, Tannase (U), Cellulase (U), Temperature (°C), and Time (min), were investigated. Table 2 shows the experimental values of the 29 runs of the design for each of the response variables evaluated.

The most reliable way to assess the quality of the adapted model is to apply the analysis of variance (ANOVA), which consists of determining the effects of several independent variables on the response variable and deciding whether these effects are significant [26].

According to the ANOVA (Table 3) analysis for TPC, the value of R^2=0.9639 and the adjusted R^2= 0.9456. The R^2 is related to the terms of the mathematical model, while the adjusted R^2 is the one that allows making decisions and relates to the quality of the fit; it also indicates that the model obtained can explain 94.56% of the variation in the TPC value [12]. The coefficient of variation (CV) is a relationship between the mean and the deviation. A low CV value indicates that there is a high reproducibility of the results obtained [23]; for this model, a CV value of 6.32% was obtained.

Analysis of the quadratic regression model showed that it was significant ($p<0.05$), while the "lack of fit" was not significant ($p>0.05$). Of the four independent variables in the model, temperature and time were significant ($p<0.05$) at the 95.0% confidence level. The model showed that the independent variable with the most significant effect on the TPC was time (X_4), with a lower p-value ($p<0.05$) and an F value of 218.54, followed by temperature (X_3), with an F value of 78.81. At the same time, tannase (X_1) and cellulase (X_2) did not significantly affect the TPC yield ($p>0.05$). A reduced quadratic model was developed since the quadratic independent variables and interactions that did not present significance in the design for the TPC data set were eliminated in the analysis and adjusted to the following equation 1:

$$\text{TPC}\ (Y_1) = 20.51 + 0.3892\ X_1 + 0.9420\ X_2 - 3.86\ X_3 - 6.71\ X_4 - 2.66\ X_3X_4 + 2.00\ X_1^2 + 8.46\ X_3^2 + 2.09\ X_4^2 \quad (1)$$

For the antioxidant capacity, the value of R^2=0.8698 and the adjusted R^2= 0.8116 presented a % CV of 6.13%. Analysis of the quadratic regression model showed that it was significant ($p<0.05$), while the "lack of fit" was not significant ($p>0.05$). Of the four independent variables in the model, temperature (X_3) and time (X_4) were significant again ($p<0.05$) at the 95.0% confidence level. The model showed that the independent variable with the greatest effect on the extraction yield was time ($p<0.05$), with an F value of 44.46, followed by temperature, with an F value of 28.05. Meanwhile, tannase and cellulase did not have significant effects on the antioxidant yield ($p>0.05$) (Table 3).

The quadratic model developed in the antioxidant capacity data set was adjusted to the following equation 2:

$$\text{Antioxidant}\ (Y_2) = 257.25 - 6.52\ X_1 + 5.00\ X_2 - 21.75X_3 - 27.49\ X_4 - 15.74\ X_1X_2 - 10.83\ X_3X_4 + 25.65\ X_3^2 + 22.05\ X_4^2 \quad (2)$$

The 3D surface graphs were obtained by keeping two variables constant and varying the other two within the experimental range. Figure 1 depicts the impacts of EAE conditions, such as enzyme type, temperature, and time, on TPC and antioxidant capacity. Figures 1-A and 1-B show that a shorter extraction time (15 min) and a lower temperature (20 °C) results in a higher TPC yield and greater antioxidant capacity (with variables constant: 40 U of cellulose and 0.75 U of tannase). It can be observed that the highest values were found at the extremes of the experimental levels; this can be attributed to the fact that the range of levels chosen was limited for this type of extraction. Considering this, experimental tests were carried out (results not shown), where temperatures of 10

°C and 60 °C were evaluated, and times of 10 min and 200 min; here, it was observed that both the content of total polyphenols (21.89-34.38 mg GAE g^{-1} dw) and antioxidant capacity decreased (221.47-264.64 µmol ET g^{-1} dw). As mentioned above, the TPC 3D surface graphs evaluating the effect of enzymes (tannase-cellulase) were not shown because it was not significant for the model. Figure 1-C shows that with a higher amount of cellulose (40 U) and a lower amount of tannase (0.75 U) the antioxidant capacity is higher (with variables constant: 20 °C and 15 min). In addition, it was found that the TPC value varied in wide ranges from 16.19-44.78 mg GAE g^{-1} dw depending on the changes in the process variables, while the antioxidant capacity varied from 226.34-369.49 µmol ET g^{-1} dw (Table 2). The optimal conditions for the determination of TPC and antioxidant capacity for EAE were 0.75 U tannase, 40 U cellulase, 20 °C temperature, and a time of 15 min, with a desirability value of 0.734, where values close to 1 indicate that the value of response is consistent with the target value.

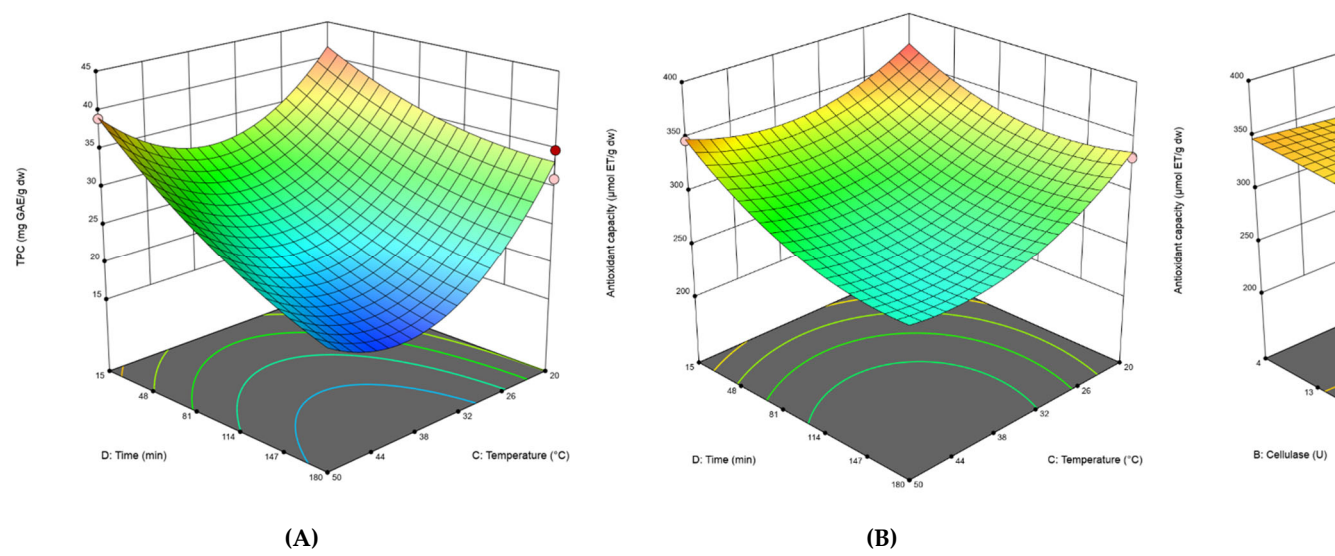**(A)**   **(B)**

Figure 1. 3D surface graphs from Enzyme Assisted Extraction (EAE). A) Total polyphenol content (TPC) (Time-Temperature); B) Antioxidant c (Tannase-Cellulase). On the color scale: blue (low values), and red (high values).

3.3. Experimental Design of Pressurized Liquid Extraction (PLE)

According to preliminary experiments and a literature search based on studies that have used PLE to extract different compounds from grapes [16,27,28], the experimental design was defined considering the effects of three independent variables: ethanol concentration, temperature, and extraction cycles, evaluating the phenolic content and the antioxidant capacity. Table 4 shows the experimental values of the 17 design runs for each of the response variables evaluated. A quadratic model was developed for the total polyphenols and antioxidant capacity data set. In the ANOVA analysis for TPC, the value of $R^2=0.9889$, the adjusted $R^2= 0.9747$, and the CV (%) was 6.37 (Table 5). A quadratic model was developed, considering the interactions and quadratic independent variables, and the analysis was adjusted to the following equation 3:

$$TPC\ (Y_3) = 43.27 + 11.17X_1 + 7.40X_2 + 0.8588X_3 - 3.39X_1X_2 - 1.12X_1X_3 - 0.07X_2X_3 - 17.81X_1^2 + 0.8715X_2^2 - 3.20X_3^2 \ (3)$$

The antioxidant capacity, the value of $R^2=0.9777$ and, the adjusted $R^2= 0.9490$ and presented a CV (%) of 8.82. The quadratic model developed in the antioxidant capacity data set was adjusted to the following equation 4:

$$Antioxidant\ (Y_4) = 298.81 + 63.69X_1 + 24.59X_2 + 1.86X_3 - 29.91X_1X_2 - 8.01X_1X_3 - 21.08X_2X_3 - 98.51X_1^2 + 25.27X_2^2 - 16.16X_3^2 \ (4)$$

Analysis of the quadratic regression model showed that it was significant ($p<0.05$), while the "lack of fit" was not significant ($p>0.05$). The model showed that the independent variables with the most significant effect on TPC yield were ethanol concentration (X_1), with an F value of 215.09, and temperature (X_2), with an F value of 94.47 ($p<0.05$), while in antioxidant capacity, the independent variables with the most significant effect were ethanol concentration (X_1) ($p<0.05$), with an F value of 114.05. The extraction cycle variable (X_3) for both response variables was not significant ($p>0.05$).

The 3D surface graphs were obtained by keeping one variable constant and varying the other two within the experimental range. Figure 2 shows the impacts of PLE conditions, such as ethanol concentration, temperature, and extraction cycles, on TPC and antioxidant capacity.
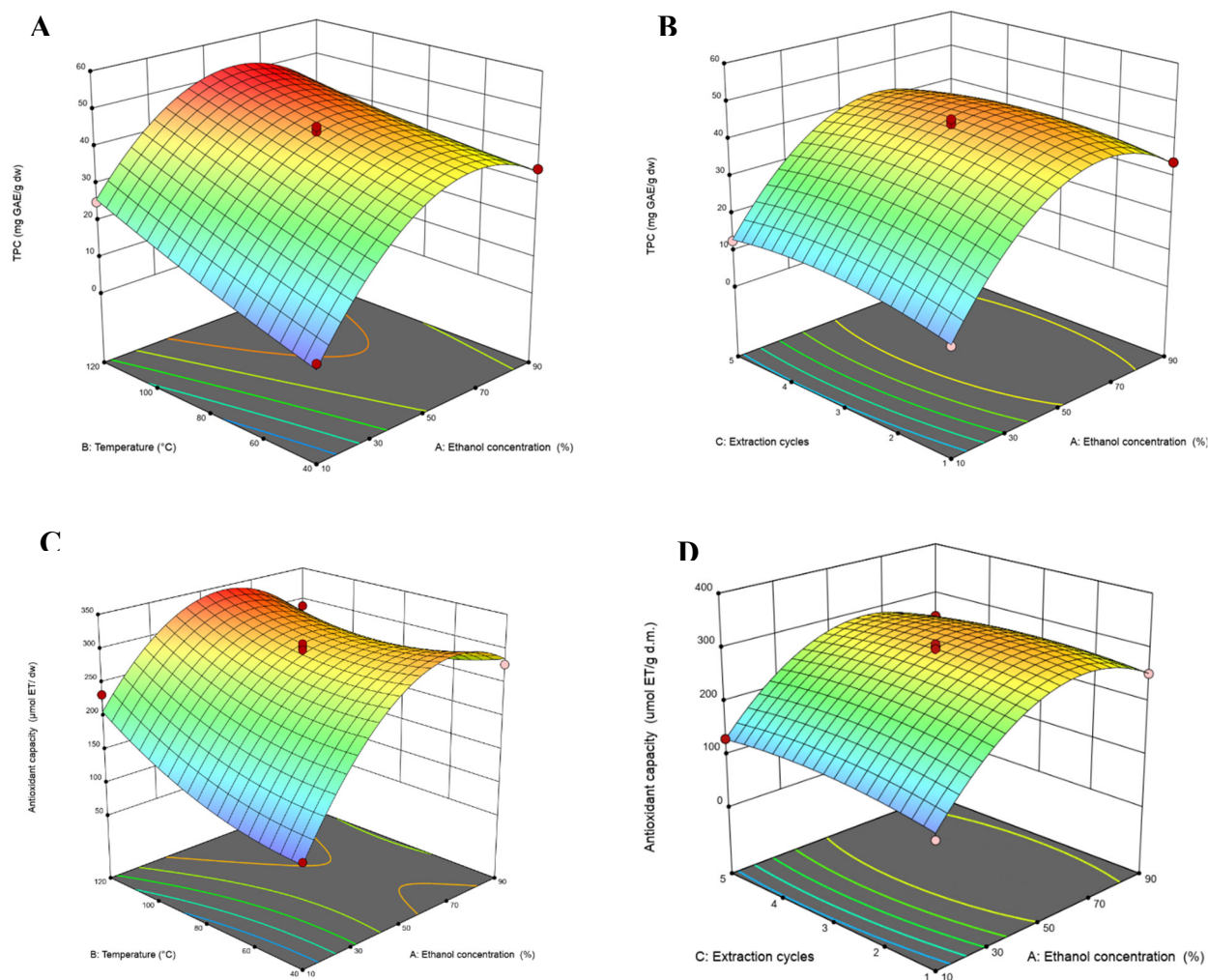
Figure 2. 3D surface graphs from Pressurized Liquids Extraction (PLE). A) Total polyphenol content (TPC)(Ethanol concentration-temperature); B) Total polyphenol content (TPC) (Ethanol concentration-extraction cycles); C) Antioxidant capacity (Ethanol concentration-temperature), and D) Antioxidant capacity (Ethanol concentration-extraction cycles). On the color scale: blue (low values) and red (high values).

Figures 2-A and 2-C show that the yield of total phenolic compounds (TPC) and antioxidant capacity increase significantly when using temperatures between 80 and 120 °C and ethanol concentrations between 50 and 80%, with three extraction cycles kept constant. These temperature and solvent concentration ranges are consistent with previous studies, in which similar conditions enhanced the extraction efficiency of phenolic compounds, attributed to improved solubility and greater release of phenolic metabolites from the plant matrix [29–31]. It is observed that the red region in Figures 2-B and 2D are similar because there were no significant differences concerning the extraction cycles and at an ethanol concentration region (with variable constant: 80 °C). In addition, it was found that the TPC value varied in wide ranges from 5.84-50.21 mg GAE g^{-1} dw, while the antioxidant capacity varied from 98.33-347.14 µmol ET g^{-1} dw (Table 4). Some studies in PLE have reported that extraction cycles did not show significance in obtaining TPC, using longer extraction times [16,27].

The optimum conditions for extracting total polyphenols and antioxidant capacity for PLE were 54% ethanol, 113 °C, and three extraction cycles with a desirability value of 0.937, where values close to 1 indicate that the response value is consistent with the target value.

3.4. Validation of Enzymatic and Pressurized Liquid Extractions

To verify the reliability of the models of both extractions, experiments were carried out under optimal conditions, in which the experimental values were compared with the predicted values to obtain the percentage error (Table 6). The percentage errors for EAE were 7.34% and 7.57% for TPC content and antioxidant capacity, respectively. In the case of PLE, they were 1.24% and 11.80% for TPC and antioxidant capacity, respectively. It is important to mention that these variations can be attributed to the samples' heterogeneity due to their composition (seeds and skin). The errors between the predicted and experimental values indicate that it can be extrapolated that the regression models obtained by RSM could predict the extraction yields of TPC and antioxidant capacity.

3.5. Characterization of Optimal Extracts

Table 7 presents the characterization of the optimal extracts obtained by EAE and PLE. The content of total polyphenols, total flavonoids, antioxidant capacity (using DPPH and ORAC assays), sugars, and phenol profile were evaluated.

The optimized extracts presented higher amounts of phenolic compounds and antioxidant capacity than by agitation. Compared with EA, the EAE extract increased 31% TPC and antioxidant capacity by 2% and 8% for DPPH and ORAC, respectively. Meanwhile, PLE stood out over agitation extraction in that the PLE extract increased 70 % TPC and antioxidant capacity by 12% and 24% for DPPH and ORAC, respectively. Both extracts showed significant differences between them ($p<0.05$).

EA showed a polyphenol content of 29.46 ± 0.37 mg GAE g^{-1} dw, flavonoids 48.04 ± 0.75 mg QE g^{-1} dw, and antioxidant capacity values of 329.67 ± 5.39 and 1549.17 ± 94.63 µmol ET g^{-1} dw, by DPPH and ORAC respectively (data not shown).

The sugars in EAE and PLE increased by 20% compared to EA (217.03 mg glucose g^{-1} dw). The sugar content was determined considering that grape skin contains large amounts of hemicellulosic sugars, which increase when hydrolyzed due to the release of xylose and glucose monomers [25].

To evaluate the distribution of polyphenolic compounds within the different fractions of grape pomace, the optimal extraction conditions obtained in this study were applied separately to the seed and skin components (data not shown). EAE in seeds had a TPC content of 58.48 mg GAE g^{-1} dw, while skin had 8.24 mg GAE g^{-1} dw. Values for antioxidant capacity by DPPH were 401.30 µmol ET g^{-1} dw and 171.86 µmol ET g^{-1} dw for seeds and skin, respectively. For PLE, TPC content was 67.73 mg GAE g^{-1} dw in seeds and 22.44 mg GAE g^{-1} dw in skin. The values for antioxidant capacity by DPPH obtained were 399.21 µmol ET g^{-1} dw in seeds and 171.86 µmol ET g^{-1} dw in skin. Notably, in all grape varieties, the highest concentration of polyphenolic compounds is present in the seeds, which are correlated with the antioxidant capacity. In addition, 70% of the extractable compounds are from the seeds, while 28-35% are from the skin [25]. The TPC values obtained in this work were higher than those obtained in other grape EAE and PLE studies. EAE variations can be attributed to the type of enzyme, the enzyme-substrate ratio, longer incubation times, and higher temperatures [7,32,33]. In PLE, the type of grape, higher temperature, and longer extraction times used can be attributed [6,16].

EAE and PLE, compared with ultrasound and microwave extraction, which are also emerging techniques applied to grape pomace and seeds of red and white grape varieties, have obtained similar values in TPC and lower values in TFC and antioxidant capacity by DPPH [34]. Although studies have been conducted on optimizing EAE y PLE in grape pomace for wine production, work has yet to be reported using enzymes in pisco grape pomace. PLE proves to be more efficient in increasing the content of the compounds of interest.

The correlation between TPC and antioxidant capacity was evaluated for the EAE and PLE extracts. The EAE extract showed an $R^2 = 0.8017$, and the PLE extract an $R^2 = 0.8827$; these values indicate a high correlation between both variables studied.

3.6. Phenol Profile of Optimum Extracts

Phenolic compounds are natural antioxidants that control or prevent metabolic syndrome and various chronic diseases. They also possess various pharmacological activities, such as antimicrobial, inflammatory, and anticarcinogenic properties [16,25,35].

Figure 3 compares the profile of phenolic compounds present in the optimal EAE and PLE extracts. The phenolic compounds identified in both optimum extractions were gallic acid, catechin, epicatechin, 4-hydroxybenzoic acid, quercetin-3-rutinoside hydrate, quercetin-3-O-rhamnoside, and kaempferol.

The PLE extract showed the highest concentration of phenolic compounds compared to EAE. Quercetin-3-rutinoside hydrate was the compound in the highest concentration, with a value of approximately 3 mg g^{-1} dw (Table 7). It was followed by catechin and epicatechin with values of 0.3-0.7 mg g^{-1} dw, respectively. Due to the advantages of PLE already mentioned, and the added thermal energy, this extraction contributes to breaking the matrix bonds and favors the diffusion of some specific polyphenols, such as phenolic acids, flavonols, and flavonols [6].

EAE and PLE influenced the phenolic recovery of grape pomace extracts. Chromatographic analysis (HPLC-DAD) identified more individual phenolic compounds than EA. According to a previous study by Poblete et al. [13] using EA and comparing them with the optimal extracts, these increases in concentration and the presence of two new phenols, quercetin-3-O-rhamnoside (0.09-0.12 mg g^{-1} dw) and kaempferol (0.04-0.05 mg g^{-1} dw), was also identified and quantified. Most of the compounds found belong to the flavonol group.

Individual phenolic compounds were also evaluated in seeds and skins. In the seeds, the EAE extract was found to have a higher concentration of catechin and epicatechin (0.45-1.45 mg g^{-1} dw) than the PLE extract (0.36-1.26 mg g^{-1} dw). In the skin, the EAE extract had a higher concentration of quercetin-3-rutinoside hydrate (2.59 mg g^{-1} dw) than the PLE extract (2.42 mg g^{-1} dw). Among the compounds in grape seeds are tannins, also called proanthocyanidins (flavonols). When hydrolyzed, their main monomers are obtained, such as catechin, epicatechin, epigallocatechin, and epicatechin-3-O-gallate [36]. In PLE, high temperatures favor the release of these monomers in the extracts [6].

Some studies in grapes and grape pomace using EAE and PLE reported the presence of gallic acid, pyrogallol, catechin, epicatechin, caffeic acid, rutin, quercetin, and kaempferol. These studies reported that gallic acid was one of the predominant compounds; rutin and quercetin were found in higher concentrations, while catechin and epicatechin were found in lower concentrations, mainly in white grapes. However, the concentrations of these compounds are different compared to this study.

Among the bioactive properties of the phenolic compounds found, gallic acid is one of the most important hydroxybenzoic acids found in grapes; it can be found in the skin, pulp, or seeds but is more abundant in the skin and has a high antioxidant capacity. Catechins and epicatechins have cholesterol-lowering properties and reduce blood pressure. Rutin, quercetin, and kaempferol possess potent antioxidant capacity, playing a role in protecting against cardiovascular disease. These last two compounds belonging to the flavonols group are mainly present in grapes and grape pomace as aglycones and in glycosylated form [25,37].

Table 1. Proximal composition of vacuum dried (VAC) grape pomace at 60 °C.

Parameters	VAC 60 °C		
[1]Moisture	5.51	±	0.26
[2]Fat	7.35	±	0.24
[2]Ash	5.83	±	0.09
[2]Crude protein	12.74	±	0.54
[2]Insoluble dietary fiber, IDF	38.08	±	4.04
[2]Soluble dietary fiber, SDF	2.86	±	0.18
[2]Total dietary fiber, TDF	40.94	±	3.86
[3]Total Carbohydrates	74.07	±	0.48
[4]Reducing sugar content	33.65	±	1.09
[5]Water activity, a_w	0.3069	±	0.0066

[1] Mean value ± Standard deviation (SD) was expressed in g 100 g $^{-1}$ sample (%). [2] Mean values ± SD and the data are expressed as g 100 g^{-1} dw. [3] Total Carbohydrates (TC) by difference: TC = 100 - (% Moisture + % Crude protein + % Fat + % Ash). [4] Reducing sugar content is expressed as g glucose 100 g^{-1} dw. [5] Dimensionless.

Table 2. Experimental D-optimal design for EAE of pisco grape pomace yield of extract polyphenols, and the antioxidant capacity.

	Variables				Response	
Run	X_1 Tannase (U)	X_2 Cellulase (U)	X_3 Temperature (°C)	X_4 Time (min)	Y_1 TPC (mg GAE g^{-1} dw)	Y_2 Antioxidant capacity (µmol TE g^{-1} dw)
1	3.99	4	50	15	35.61	327.04
2	0.75	40	20	180	31.17	332.94
3	0.75	4	20	15	38.96	300.68
4	10	4	20	88.43	33.8	329.88
5	10	4	50	180	19.47	260.06
6	5.38	22	35	97.5	20.28	253.04
7	3.99	4	20	180	31.68	320.17
8	10	40	33.05	180	23.23	252.69
9	10	40	50	88.43	30.21	258.26
10	10	24.16	20	180	30.11	289.96
11	7.60	40	20	15	40.09	320.78
12	0.75	40	20	180	34.92	331.81
13	0.75	4	20	15	39.79	331.40
14	1.49	40	28.64	15	44.78	369.49
15	10	24.52	50	15	38.15	299.94
16	3.96	40	50	180	16.19	238.86
17	10	4	32.75	15	31.15	304.45
18	10	4	50	180	19.98	239.59
19	0.75	4	33.5	105.75	19.57	247.00
20	0.75	4	50	180	18.17	243.97
21	0.75	23.98	36.65	180	30.07	259.49
22	7.60	40	20	15	42.42	359.75
23	5.38	22	35	97.5	19.18	226.34
24	0.75	40	50	15	38.97	347.46
25	3.99	4	20	180	31.53	295.15
26	0.75	23.44	20	90.9	35.50	318.41
27	0.75	20.15	49.50	88.94	30.21	268.40

Response values are the mean of three replicates. TPC: Total polyphenol content.

Table 3. Analysis of variance of the regression parameters of the total polyphenols yield and the antioxidant capacity of EAE of the pisco grape pomace.

Response	Y_1:TPC					Y_2: Antioxidant capacity				
Source	Sum of Squares	df	Mean Square	F-value	p-value	Sum of Squares	df	Mean Square	F-value	p-value
Model	1537.99	8	192.25	53.48	< 0.0001	38917.57	8	4864.7	15	< 0.0001
X_1-Tannase	2.49	1	2.49	0.6939	0.4171	786.84	1	786.84	2.43	0.1367
X_2-Cellulase	16.05	1	16.05	4.46	0.0507	479.19	1	479.19	1.48	0.2398
X_3-Temperature	283.34	1	283.34	78.81	< 0.0001	9094.98	1	9094.98	28.05	< 0.0001
X_4-Time	785.68	1	785.68	218.54	< 0.0001	14415.47	1	14415.47	44.46	< 0.0001
X_1X_2	-	-	-	-	-	3326.98	1	3326.98	10.26	0.0049
X_3X_4	107.12	1	107.12	29.8	< 0.0001	1760.49	1	1760.49	5.43	0.0316
X_1^2	17.05	1	17.05	4.74	0.0447	-	-	-	-	-
X_3^2	228.65	1	228.65	63.6	< 0.0001	2981.37	1	2981.37	9.19	0.0072
X_4^2	18.19	1	18.19	5.06	0.0389	2259.98	1	2259.98	6.97	0.0166
Residual	57.52	16	3.6			5836.62	18	324.26		
Lack of Fit	46.68	10	4.67	2.58	0.1287	3725.84	12	310.49	0.8826	0.5997
Pure Error	10.84	6	1.81			2110.78	6	351.8		
Cor Total	1595.51	24				44754.19	26			
Fit Statistics										
R^2	0.9639					0.8698				
R^2 Adjusted	0.9456					0.8116				
R^2 Predicted	0.9085					0.7067				
CV (%)	6.32					6.13				

TPC: Total polyphenol content; p-values less than 0.05 indicate model terms are significant.

Table 4. Box Behnken experimental design for PLE of pisco grape pomace, polyphenolic yield of the extract, and antioxidant capacity.

	Variables			Response	
Run	X_1 Ethanol concentration (%)	X_2 Temperature (°C)	X_3 Extraction cycles	Y_1 TPC (mg GAE g^{-1} dw)	Y_2 Antioxidant capacity (µmol TE g^{-1} dw)
1	90	120	3	40.05	289.10
2	90	80	1	34.07	253.40
3	50	80	3	41.85	304.27
4	50	40	1	31.53	277.81
5	50	80	3	44.18	301.35
6	50	80	3	39.90	308.08
7	90	40	3	34.19	277.74
8	90	80	5	33.54	253.94
9	10	80	5	12.70	130.90
10	50	40	5	33.38	310.86
11	10	40	3	5.84	102.22
12	10	80	1	8.72	98.33
13	50	120	5	50.21	295.86
14	50	80	3	45.49	298.83
15	50	120	1	48.64	347.14
16	50	80	3	44.94	281.54
17	10	120	3	25.25	233.23

Response values are the mean of three replicates. TPC: Total polyphenol content.

Table 5. Analysis of variance of the regression parameters of the total polyphenols yield and the antioxidant capacity of PLE of the pisco grape pomace.

Response	Y_1: TPC					Y_2: Antioxidant capacity				
Source	Sum of Squares	df	Mean Square	F-value	p-value	Sum of Squares	df	Mean Square	F-value	p-value
Model	2901.32	9	322.37	69.5	< 0.0001	87221.11	9	9691.23	34.06	< 0.0001
X_1-Ethanol concentration	997.7	1	997.7	215.09	< 0.0001	32448.78	1	32448.78	114.05	< 0.0001
X_2-Temperature	438.23	1	438.23	94.47	< 0.0001	4836.36	1	4836.36	17	0.0044
X_3-Extraction cycles	5.9	1	5.9	1.27	0.2966	27.68	1	27.68	0.0973	0.7642
$X_1 X_2$	45.9	1	45.9	9.9	0.0162	3579.03	1	3579.03	12.58	0.0094
$X_1 X_3$	5.09	1	5.09	1.1	0.3299	256.48	1	256.48	0.9014	0.374
$X_2 X_3$	0.0196	1	0.0196	0.0042	0.95	1777.89	1	1777.89	6.25	0.041
X_1^2	1335.71	1	1335.71	287.96	< 0.0001	40858.42	1	40858.42	143.6	< 0.0001
X_2^2	3.2	1	3.2	0.6894	0.4338	2688.04	1	2688.04	9.45	0.018
X_3^2	43.21	1	43.21	9.32	0.0185	1100	1	1100	3.87	0.09
Residual	32.47	7	4.64			1991.65	7	284.52		
Lack of Fit	10.55	3	3.52	0.6419	0.6272	1571.2	3	523.73	4.98	0.0774
Pure Error	21.92	4	5.48			420.45	4	105.11		
Cor Total	2933.79	16				89212.76	16			
Fit Statistics										
R^2	0.9889					0.9777				
R^2 Adjusted	0.9747					0.9490				
R^2 Predicted	0.9308					0.7180				
CV (%)	6.37					8.82				

TPC: Total polyphenol content; p-values less than 0.05 indicate model terms are significant.

Table 6. Validation of enzymatic and pressurized liquid extractions.

Extraction	Optimal conditions		TPC (mg GAE g^{-1} dw)			Antioxidant capacity (µmol ET g^{-1} dw)		
			Value Predicted	Value Experimental	Error percentage (%) *	Value Predicted	Value Experimental	Error percentage (%) *
EAE	Tannase (U)	0.75	41.51	38.49	7.34	370.52	342.47	7.57
	cellulase (U)	40						
	Time (min)	15						
	Temperature (°C)	20						
PLE	Ethanol concentration (%)	54	50.66	50.03	1.24	331.84	371.00	11.80
	Temperature (°C)	113						
	Extraction cycles	3						

* The error percentage (%) was calculated by comparing the experimental value to the predicted value. EAE: Enzyme-assisted extraction; PLE: Extraction by pressurized liquids; TPC: Total polyphenols content.

Table 7. Characterization of optimal extracts.

Parameters	Extraction methods					
	EAE			PLE		
TPC (mg GAE g^{-1} dw)*	38.49	±	0.99[b]	50.03	±	0.58[a]
TFC (mg QE g^{-1} dw)	51.78	±	1.62[b]	75.48	±	2.12[a]
DPPH (µmol TE g^{-1} dw)*	342..47	±	1.29[b]	371.00	±	8.89[a]
ORAC (µmol TE g^{-1} dw)	1687.47	±	4.66[b]	1931.39	±	90.26[a]
Sugars (mg Glucose g^{-1} dw)	262.58	±	1.83[b]	266.79	±	0.02[a]
Phenolic compounds (mg g^{-1} dw)						
Gallic acid	0.14	±	0.01[b]	0.23	±	0.01[a]
Catechin	0.37	±	0.02[b]	0.69	±	0.00[a]
Epicatechin	0.40	±	0.01[b]	0.61	±	0.02[a]
4-Hydroxybenzoic	NQ			0.19	±	0.00[a]
Rutin	2.31	±	0.06[b]	2.88	±	0.03[a]
Quercetin	0.09	±	0.00[b]	0.12	±	0.02[a]
Kaempferol	0.05	±	0.00[b]	0.04	±	0.00[a]

Values with different letters in the same row are significantly different ($p < 0.05$). TPC: Total polyphenols content; TFC: Total flavonoid content. EAE: Enzyme-assisted extraction; PLE: Extraction by pressurized liquids. NQ: Not quantifiable.* Optimal validation values.
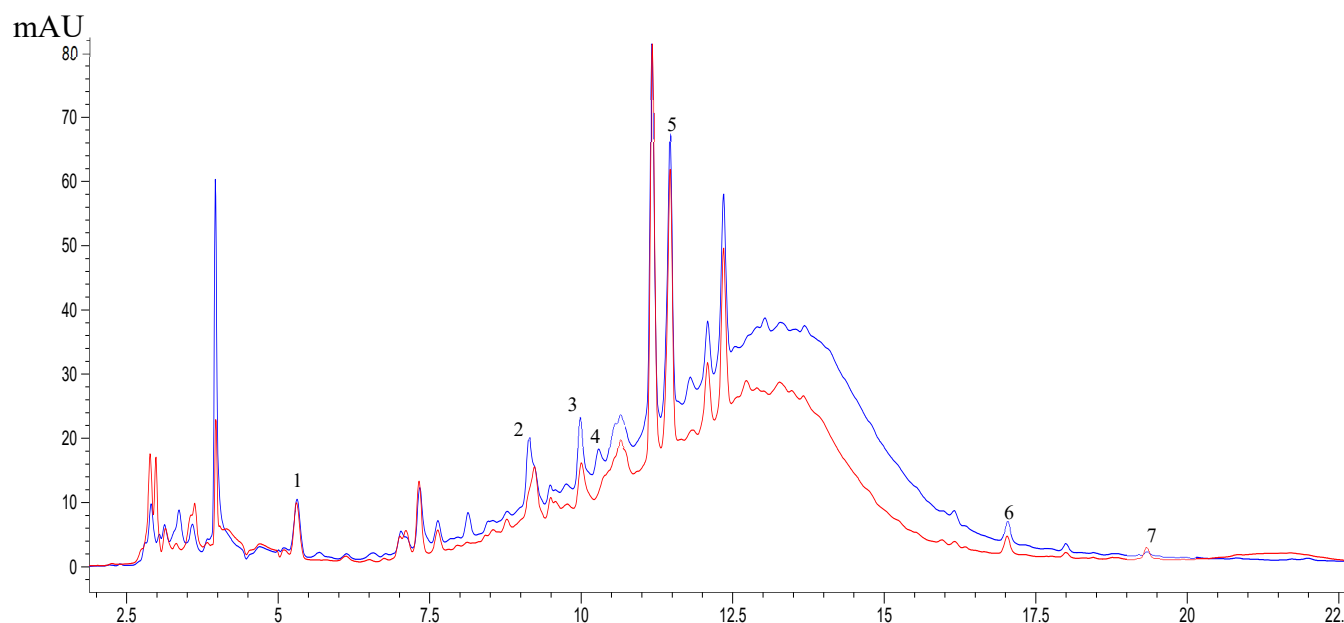
Figure 3. Profile of phenolic compounds from pisco grape pomace (1) Gallic acid, (2) Catechin, (3) Epicatechin, (4) 4-Hydroxybenzoic acid, (5) Quercetin-3-rutinoside hydrate, (6) Quercetin-3-O-rhamnoside, (7) Kaempferol. Comparison of the profile of phenolic compounds of pisco grape pomace enzyme-assisted extraction (Red) and pressurized liquid extraction (Blue).

4. Conclusions

The experimental design of the emerging extraction processes presented in this work allowed an increase in the extraction yield of bioactive compounds such as polyphenols and flavonoids with antioxidant properties compared to a conventional shaking technique. The optimal condition of EAE was 40 U cellulose, 0.75 U tannase, 20 °C, and 15 min. Compared with EA, EAE increased the polyphenol yield and antioxidant capacity by 31% and 3%, respectively. The optimal conditions of PLE were 54% ethanol, 113 °C, and three extraction cycles, increasing the polyphenol yield and antioxidant capacity by 70% and 3%, respectively, compared to EA. PLE showed the highest total polyphenol content and antioxidant capacity among both optimal extracts. Seven phenolic compounds were found in EAE and PLE: gallic acid, catechin, epicatechin, 4-hydroxybenzoic acid, quercetin-3-rutinoside hydrate, quercetin-3-O-rhamnoside, and kaempferol, with rutin predominating, followed by catechin and epicatechin. The phenolic compounds found in grape pomace from pisco production extracted by emerging techniques is the first step to be considered as an alternative in high value-added products, such as new food ingredients, nutraceuticals, sustainable packaging, and others, contributing to sustainability and generating a promising solution to develop products with benefits for human health.

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