

Article

Improved Extraction of High Value-Added Polyphenols from Pomegranate Peel by Solid-State Fermentation

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Abstract: Pomegranate peel is an important source of polyphenols of remarkable interest in the food, pharmaceutical and cosmetic industry. The improved extraction of total polyphenolic compounds (TPC) from pomegranate peel by solid-state fermentation (SSF) was achieved. The Box, Hunter and Hunter (BHH) followed by the central composite design (CCD) processes were performed to assess the effect of the process variables on TPC release. The statistical designs indicate that the best TPC extraction (234.85 mg GAE/gdm) by means of SSF occurs at 42 °C, 50% moisture, 5.0 pH, mineral solution (g/L): NaNO₃ (3.83), KH₂PO₄ (1.52), MgSO₄ (4.66) and KCl (1.52) at 36 h. Under the best fermentation conditions TPC (248.78 ± 1.24 mgGAE/gdm) increased 5.96-fold more than values previously reported and antioxidant activity (AA) increased 5.81-fold compared to the value obtained before the SSF optimization. High-value citric acid, α and β punicalin, α and β punicalagin, punigluconin, galloyl-HHDP hexoside and ellagic acid molecules were identified. The increased extraction of TPC by SSF provides a suitable alternative for the valorization of pomegranate peel through the recovery of molecules with high added value with potential use in the food, pharmacy and cosmetic industries; a diversification in the use of food agroindustry by-products is obtained as an approach to the circular economy model through biotechnological processes.

Keywords: pomegranate peel; polyphenols; improved extraction; high value-added; molecules identification; solid-state fermentation



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1. Introduction

Pomegranate (*Punica granatum* L.) is a native fruit from the Middle East; however, Afghanistan, Iran, India, Spain, China, Turkey, United States, South Africa, Peru, Chile and Argentina are considered the world's leading exporters [1]. Several reports on antioxidant, anti-inflammatory, antihypertensive, antineurodegenerative, immune modulatory, antiviral, antitumor, anticarcinogenic, antimicrobial and antifungal among other biological activities are related to the polyphenols found in pomegranate, either in the edible parts, seeds or peel [2,3].

Pomegranate intake is mainly through juice, jellies, jams and liquors; however, their biological activity is highly appreciated in the food, cosmetic and pharmacy industries [2]. According to Kazemi et al. [4] one ton of fresh pomegranate generates 669 kg of by-products

and those include 78% peel and 22% seeds. Although the fast increase in pomegranate cultivation limits the precise calculation of worldwide production, in 2021, based on the 8636.2 tons of pomegranate harvested in 2021 for Mexico, 6736.2 tons of pomegranate peel was available for the extraction of highly valued biomolecules [5]. Organic solvents are commonly used for the extraction of vegetal biomolecules; however, their use is associated with environmental pollution and with toxicological safety concerns. The implementation of emerging technologies (i.e., supercritical fluid, microwave, electric field, pressurized liquid) for the extraction of biomolecules improves extraction yields; however, the requirement for polar solvents and expensive extractive equipment strongly limits its applications [6–8].

In contrast, fermentative or enzymatic methods for the extraction of biomolecules from agricultural by-products have been successfully used [9–11]. Optimization methods based on one-factor-at-a-time are expensive and time-consuming and the interactions between the process variables are not considered [12]. Experimental designs for the study of several variables at a time, as well as their interactions are highly desirable.

Polyphenols are molecules that present different biological and therapeutic activities; several studies have reported their health effects. Bioactive agents such as antioxidant, anti-proliferative/anticarcinogenic, antifungal, antimicrobial, fat-lowering, anti-inflammatory, anti-aging neuroprotective, antibacterial, hypocholesterolemic, renoprotective, hepatoprotective, hypoglycaemic, antihyperglycemic and antihypertensive activities, among others, have been attributed to polyphenols [13]. Value-added “bound or insoluble” polyphenols remain attached to the agroindustry by-products [14] and these can be successfully extracted using fermentative processes. Solid state fermentation (SSF) consists of the growth of a specific microorganism in a fermentable substrate in the absence or near absence of fresh water. Due to its simplicity, SSF is preferred for the valorization of agroindustry by-products [15].

Considering the hasty increase in the consumption of pomegranate, the development of optimized eco-friendly processes to increase the extraction of high added-value compounds from pomegranate peel is highly desirable. Therefore, this work aims to increase the extract concentration of total polyphenol compounds (TPC) with AA from pomegranate peel using the environmental process of SSF; furthermore, the identification of the extracted high-value phenolic compounds is presented.

2. Materials and Methods

2.1. Raw Material

Pomegranate by-products from a local wine-making industry located in Tasquillo, Hidalgo, Mexico were dehydrated (60 °C for 24 h) and pulverized (PULVEX® Mini 100, México City, México) to a particle size of 0.85–1 mm, then stored in hermetic black polyethylene bags at room temperature (22 ± 1 °C) until use.

2.2. Physicochemical Characterization of Pomegranate By-Products

The chemical composition (protein, fat, carbohydrate, fiber, moisture and ash) was determined according to the official procedures reported by the Association of Official Analytical Chemists [16]. The water absorption index (WAI) and critical humidity point (CHP) were assessed according to [15].

2.3. Microorganism

Lyophilized *Aspergillus niger* GH1(ENA-KP273835) fungal spores were suspended in sterile water and cultivated in PDA plates (30 °C, 5 days). For the inoculum preparation, fungal spores were harvested (Tween-80, 0.01% v/v) and counted in a Neubauer chamber [17].

2.4. Solid-State Fermentation (SSF)

Pomegranate peel was used as a support and the sole carbon source. The fermentable mass (2.8 g of dry pomegranate peel per reactor) was adjusted at 60% moisture by adding

4.2 mL of a saline solution (previously inoculated with 1×10^6 spores/g of support) and aseptically packed in tray reactors (60 L 60 W $\times$ 15 H mm). The saline solution was prepared as follows: (g/L): NaNO_3 (7.65); KH_2PO_4 (3.04); MgSO_4 (1.52); KCl (1.52). The SSF was set at 30 °C for 72 h and the samples were withdrawn every 12 h. The fermented extracts were obtained by the addition of 10 mL of 50 mM citrate buffer (pH 5) to each tray-reactor, shaken (100 rpm, 15 min) and centrifuged (3500 rpm, 15 min, 4 °C). The supernatant was filtered (0.45 μm) to remove impurities and fungal debris and stored at 16 °C until further analysis.

2.5. Analytical Analysis

Phenolic compounds on the extracts were determined by the Folin–Ciocalteu assay described in Buenrostro-Figueroa et al. [10]. The Folin–Ciocalteu (200 μL) reagent (Sigma-Aldrich®, México City, México) was added to cuvettes containing aliquots (200 μL) of either the fermented extract or standard solution, mixed and incubated (5 min). Then, 200 μL of 0.01 Na_2CO_3 was added, the samples were shaken and left for 5 min. Finally, the samples were diluted with the addition of 5 mL of distilled water. The absorbance was recorded at 730 nm in a UV-Vis spectrophotometer (UV-1800 spectrophotometer; Shimadzu®, Kyoto, Japan). Gallic acid was used as the standard; the calibration curve was plotted in a range of 0–250 $\mu\text{g}/\text{mL}$. All analyses were performed in triplicate. TPC were expressed as mg of the gallic acid equivalent (GAE)/g of dry matter (gdm).

The antioxidant activity of the extracts was evaluated based on the scavenging activity of the 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich®) free radical, as described by Meléndez et al. [18]. Briefly, a reaction mixture consisting of 7 μL of the extract and 193 μL of 60 μM DPPH in absolute methanol was analyzed on a BioTek® Microplate reader (ELx808™, Santa Clara, CA, USA) with absorbance filters for a wavelength of 520 nm. The decoloring process was recorded during 30 min of reaction. The antioxidant activity was calculated on a base of gallic acid (Sigma-Aldrich®) with a standard curve (0–200 $\mu\text{g}/\text{mL}$) and expressed as mgGAE/gdm. Control samples were prepared with methanol (100 μL). Distilled water (100 μL) was used for equipment calibration. The samples were analyzed in triplicate.

2.6. Phenolic Profile

A phenolic profile from the extracts obtained under optimized SSF conditions was analyzed using reverse-phase high-performance liquid chromatography on a Varian HPLC system equipped with an autosampler (Varian® ProStar 410, Champaign, IL, USA), ternary pump (Varian® ProStar 230I, Champaign, IL, USA) and PDA detector (Varian® ProStar 330, Champaign, IL, USA). A liquid chromatograph ion trap mass spectrometer (Varian® 500-MS IT Mass Spectrometer, Champaign, IL, USA) equipped with an electrospray ion source was used. The samples (5 μL) were injected into a Denali C_{18} column (150 mm $\times$ 2.1 mm, 3 μm , Grace®, New York, NY, USA). The oven temperature was maintained at 30 °C. The eluents were formic acid (0.2%, *v/v*; solvent A) and acetonitrile (solvent B). The following gradient was applied: initial, 3% B; 0–5 min, 9% B linear; 5–15 min, 16% B linear; 15–45 min, 50% B linear. The column was then washed and reconditioned, then the flow rate was maintained at 0.2 mL/min and elution was monitored at 245, 280, 320 and 550 nm. The whole effluent (0.2 mL/min) was injected into the source of the mass spectrometer, without splitting. All MS experiments were carried out in the negative mode $[\text{M}-\text{H}]^{-1}$. Nitrogen was used as a nebulizing gas and helium as a damping gas. The ion source parameters were a spray voltage of 5.0 kV and the capillary voltage and temperature were 90.0 V and 350 °C, respectively. Data were collected and processed using MS Workstation software (V 6.9). The samples were firstly analyzed in full scan mode acquired in the *m/z* range 50–2000. MS/MS analyses were performed.

2.7. Statistical Analysis

A two-step optimization of TPC extraction from pomegranate peel was performed. Firstly, to identify the variables with a significant effect on TPC release, seven variables

(temperature, pH, moisture, NaNO_3 , KH_2PO_4 , MgSO_4 and KCl) were evaluated using the Box–Hunter–Hunter design (BHH) (Table 1). Based on the BHH results, a central composite design (CCD) was completed to find the variable value levels for higher TPC release.

Table 1. Box–Hunter–Hunter condensed matrix used to determine the influence of independent factors (A, B, C, D, E, F and G) on TPC (mg/g) from pomegranate by-product.

Treatment	A	B	C	D	E	F	G	TPC (mg/g) *
1	−1	−1	−1	1	1	1	−1	189.93 ± 4.40^a
2	1	−1	−1	−1	−1	1	1	171.76 ± 0.66^{bc}
3	−1	1	−1	−1	1	−1	1	169.90 ± 5.14^{bc}
4	1	1	−1	1	−1	−1	−1	175.11 ± 5.55^b
5	−1	−1	1	1	−1	−1	1	127.67 ± 2.64^e
6	1	−1	1	−1	1	−1	−1	144.92 ± 8.96^d
7	−1	1	1	−1	−1	1	−1	165.07 ± 4.21^c
8	1	1	1	1	1	1	1	151.85 ± 3.04^d

Code	Factor	−1	Levels	+1
A	pH	5		6
B	Temperature (°C)	30		40
C	Moisture (%)	50		60
D	NaNO_3 (g/L)	3.83		7.65
E	KH_2PO_4 (g/L)	1.52		3.04
F	MgSO_4 (g/L)	1.52		3.04
G	KCl (g/L)	1.52		3.04

* Different letters mean no significant differences among treatments (Tukey $\alpha = 0.05$).

Three independent variables (Table 2) were coded at three value levels (−1, 0 and 1) and at two axial points (− α and α).

Table 2. Condensed matrix from CCD to optimize TPC release by *A. niger* GH1.

Treatment	X ₁	X ₂	X ₃	TPC (mgGAE/gdm) *
1	−1	−1	−1	236.84 ± 3.89^{bc}
2	−1	−1	1	223.01 ± 4.73^c
3	−1	1	−1	174.52 ± 5.41^f
4	−1	1	1	250.78 ± 4.67^a
5	1	−1	−1	222.57 ± 8.38^c
6	1	−1	1	203.02 ± 4.84^d
7	1	1	−1	206.66 ± 3.84^d
8	1	1	1	201.14 ± 8.97^{de}
9	−1.68	0	0	148.18 ± 4.54^g
10	−1.68	0	0	175.82 ± 6.45^f
11	0	−1.68	0	243.17 ± 2.83^{ab}
12	0	1.68	0	184.67 ± 2.72^f
13	0	0	−1.68	226.49 ± 1.72^c
14	0	0	1.68	186.48 ± 6.94^{ef}
15	0	0	0	233.22 ± 4.46^{bc}
16	0	0	0	235.39 ± 2.49^{bc}

Code	Factor	−1.68	−1	Levels	0	1	+1.68
X ₁	Moisture (%)	42	45		50	55	58
X ₂	Temperature (°C)	31	35		40	45	48
X ₃	MgSO_4 (g/L)	0.48	1.52		3.04	4.56	5.59

* Different letters mean no significant differences among treatments (Tukey $\alpha = 0.05$).

The experimental designs (BHH and CCD) were performed in duplicate and the samples were analyzed in triplicate. The data analysis and model building were analyzed using Statistica® 7.0 software (Stat Soft, Tulsa, OK, USA). The outcome results were visualized in a Pareto Chart with the absolute value of the magnitude of the variables' level in increasing order and compared to the minimum magnitude of statistically significant factors. For the CCD, optimal conditions were estimated by means of the regression coefficient generated for each assayed term and its combination; their significance was obtained by $\alpha = 0.05$. Then, with the empiric polynomial model, the experimental data and regression coefficients were adjusted; the regression coefficients were obtained by the multiple lineal regression equation (Equation (1)):

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=i+1}^k \beta_{ij} X_i X_j \quad (1)$$

where Y represents the predicted response (TPC expressed in GAE/gdm); X_i and X_j represent the independent variables; k is the number of variables evaluated; β_0 , β_i , β_{ii} and β_{ij} are the regression coefficient for the intercept, lineal, quadratic and interaction effect terms, respectively, and gdm means grams per dry matter.

2.8. Validation of the Model

The optimal conditions for TPC release from pomegranate by-products (temperature, pH, moisture, NaNO_3 , KH_2PO_4 , MgSO_4 and KCl) were obtained from the predictive surface response equation. Experimental and predicted values were statistically compared for the validation of the model.

3. Results

3.1. Physicochemical Characterization of Pomegranate Peel

Fermentation processes are significantly influenced by the chemical composition of the substrates. The chemical analysis (Table 3) of pomegranate by-products was analyzed to evaluate its nutritional feasibility to be used as substrate for microbial cultivation. Carbohydrates are the highest component in pomegranate peel, followed by fiber, protein, ash and fat. The physicochemical values (Table 3) of WAI and CPH indicate the suitability of the by-products as a solid support for SSF.

Table 3. Physicochemical characterization of pomegranate by-products.

Component (%)	Value *
Moisture	11.86 ± 0.05
Fat	2.64 ± 0.08
Fiber	8.81 ± 0.07
Protein	8.66 ± 0.01
Ash	4.51 ± 0.01
Carbohydrates	75.38 ± 0.18
C/N	41.51
WAI **	4.38 ± 0.48
CHP ***	10.13 ± 2.13

* gram per gram of dry sample; ** WAI: water absorption index; *** CHP: critical humidity point.

3.2. Kinetics of TPC Extraction and AA

The kinetics of metabolite production provide a quick sign of the suitability of the substrate and culture conditions to obtain the desired metabolites. The kinetics (as described in Section 2.4) of TPC release and AA by *Aspergillus niger* GH1 are shown in Figure 1. Despite the fact that TPC release starts at the beginning of the process, the higher increase occurs from 24–36 h, attaining a value of 106.56 mgGAE/gdm at 36 h. A similar pattern was observed for AA, which showed the highest activity (7.95 mg GAE/gdm) at 36 h. Data

from the extraction kinetics were used to estimate the Pearson correlation coefficient and point out the relationship between TPC and AA. The value obtained was 0.86 ($p < 0.01$), indicating a significant positive correlation; that is, if the TPC value increases, the AA also increases. For this reason, only the TPC value was considered to optimize and validate the SSF model.

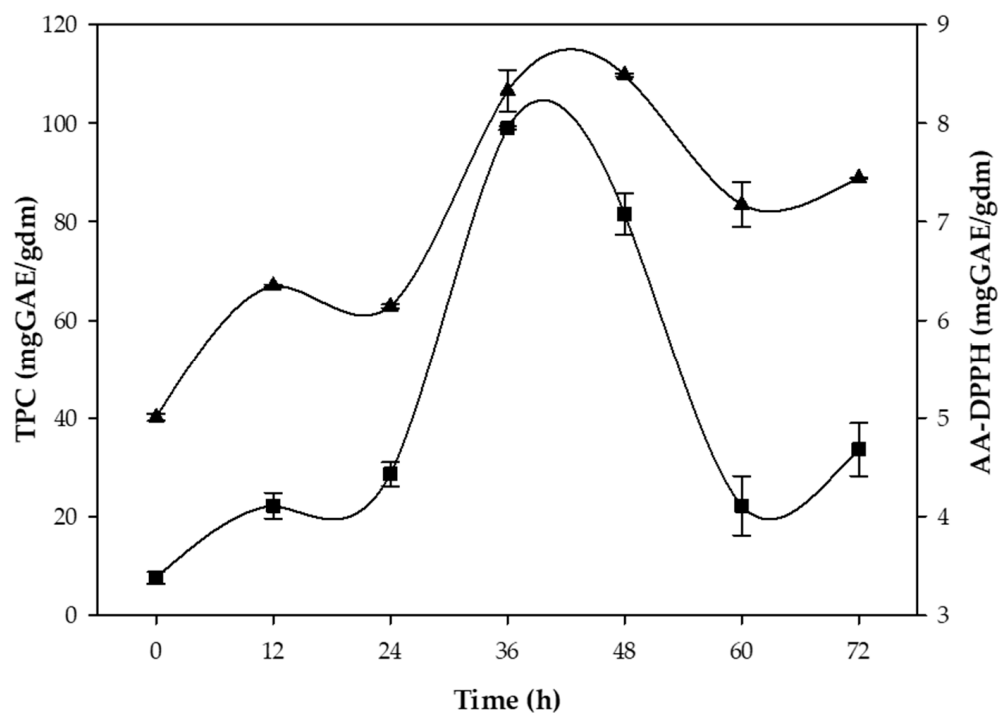


Figure 1. Kinetics of total polyphenols compounds (▲) and antioxidant activity (■) from fermented pomegranate peel.

3.3. Significant Factors for TPC Recovery by SSF

To identify the variables with a significant effect on TPC release by *A. niger* GH1, seven variables (temperature, pH, moisture, NaNO_3 , KH_2PO_4 , MgSO_4 and KCl) were evaluated using the Box–Hunter–Hunter design (BHH) (Table 1). The maximum TPC release (189.93 mgGAE/gdm) was achieved in treatment one, while the minimum TPC release (127.67 mgGAE/gdm) was observed in treatment five. To identify the influence of each selected factor on TPC release, a Pareto chart was plotted (Figure 2). The factors whose values exceeded the dotted line (moisture, MgSO_4 , KCl and temperature) have a significant effect ($\alpha = 0.05$) on TPC production. MgSO_4 and temperature have a positive effect; as their level value increases, the response value also increases. In contrast, moisture and KCl have a negative effect on the response variable; thus, with any increase in these variables, TPC extraction decreases. Therefore, lower moisture and KCl levels should be used to increase TPC release. The NaNO_3 , KH_2PO_4 and pH variables had no significant impact ($\alpha = 0.05$) on TPC extraction by SSF, thus were not considered in further studies.

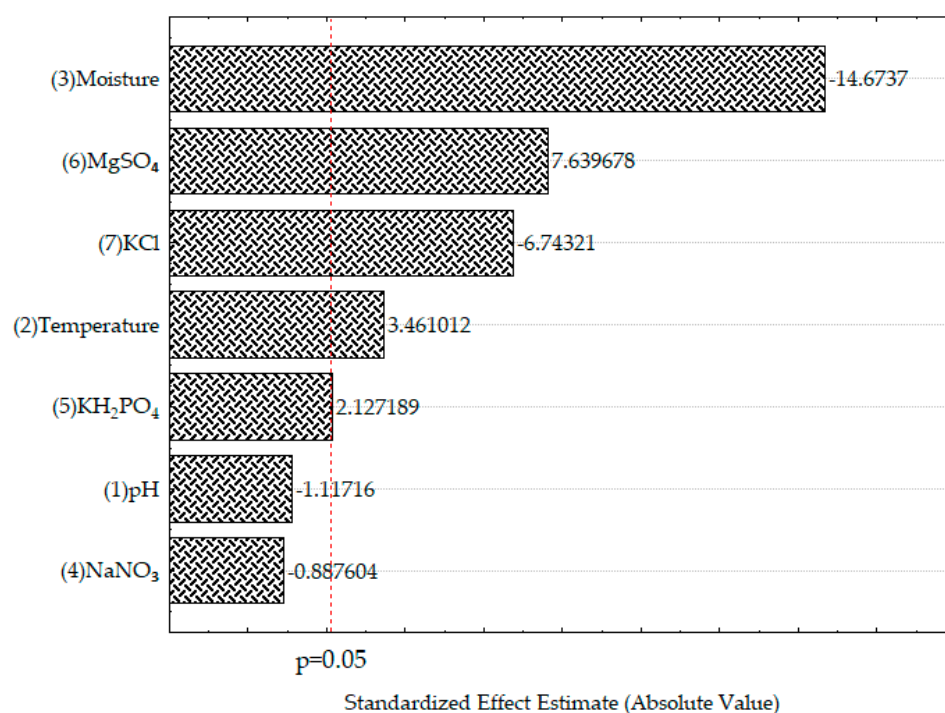


Figure 2. Pareto chart: significative independent variables on TPC recovery with *A. niger* GH1.

3.4. Optimization of the Culture Conditions for the Release of TPC

An experimental design was established to evaluate the influence of temperature, moisture and MgSO₄ levels on maximal TPC release from pomegranate by-products by SSF. Table 2 shows the values and coded levels of the variables studied in the CCD, as well as TPC release. According to the CCD, the maximum TPC release was achieved in treatment 4, attaining 250.78 mgGAE/gdm, while the minimum TPC release was observed in treatment 9 (148.18 mgGAE/gdm). The regression coefficients in linear (L) and quadratic (Q) effects and the interaction between factors were obtained from the CCD analysis. The linear term of temperature was significant in the process at a level of $\alpha < 0.05$. For quadratic terms, only moisture was significant. The interaction between moisture and MgSO₄, as well as the interaction between temperature and MgSO₄, were also significant ($\alpha \leq 0.05$). The quadratic effect of temperature and MgSO₄, the linear effect of MgSO₄, as well as the interaction between moisture and temperature were not significant ($\alpha \leq 0.05$). To determine the influence of the resulting significant variables and find the ideal process conditions for TPC release by SSF from pomegranate by-products, contour plots were constructed (Figure 3). The tendency to obtain higher TPC release was observed at central-low moisture (Figure 3a,b), central-high temperature (Figure 3a) and high MgSO₄ values (Figure 3b,c), respectively. To optimize the selected factors (moisture, temperature and MgSO₄) over the TPC release, a second-order polynomial model (Equation (1)), experimental data and regression coefficients were obtained by multiple linear regressions (Equation (2)) as follows:

$$TPC = 231.98 - 20.17X_1^2 - 11.03X_2 - 10.93X_1X_2 + 13.01X_2X_3 \quad (2)$$

Under ideal conditions, the simplified model (Equation (2)) predicted a maximal yield of TPC_{max} = 234.85 mgGAE/gdm ($R^2 = 0.95$). In order to validate the model, another experiment design was established (in triplicate) at the following values: temperature 42 °C, moisture 50%, pH 5, NaNO₃ 3.83 g/L, KH₂PO₄ 1.52 g/L, MgSO₄ 4.66 g/L and KCl 1.52 g/L. The experimental value obtained was 248.78 ± 1.24 mgGAE/gdm, this value is 5% higher than predicted but within the model error of significance.

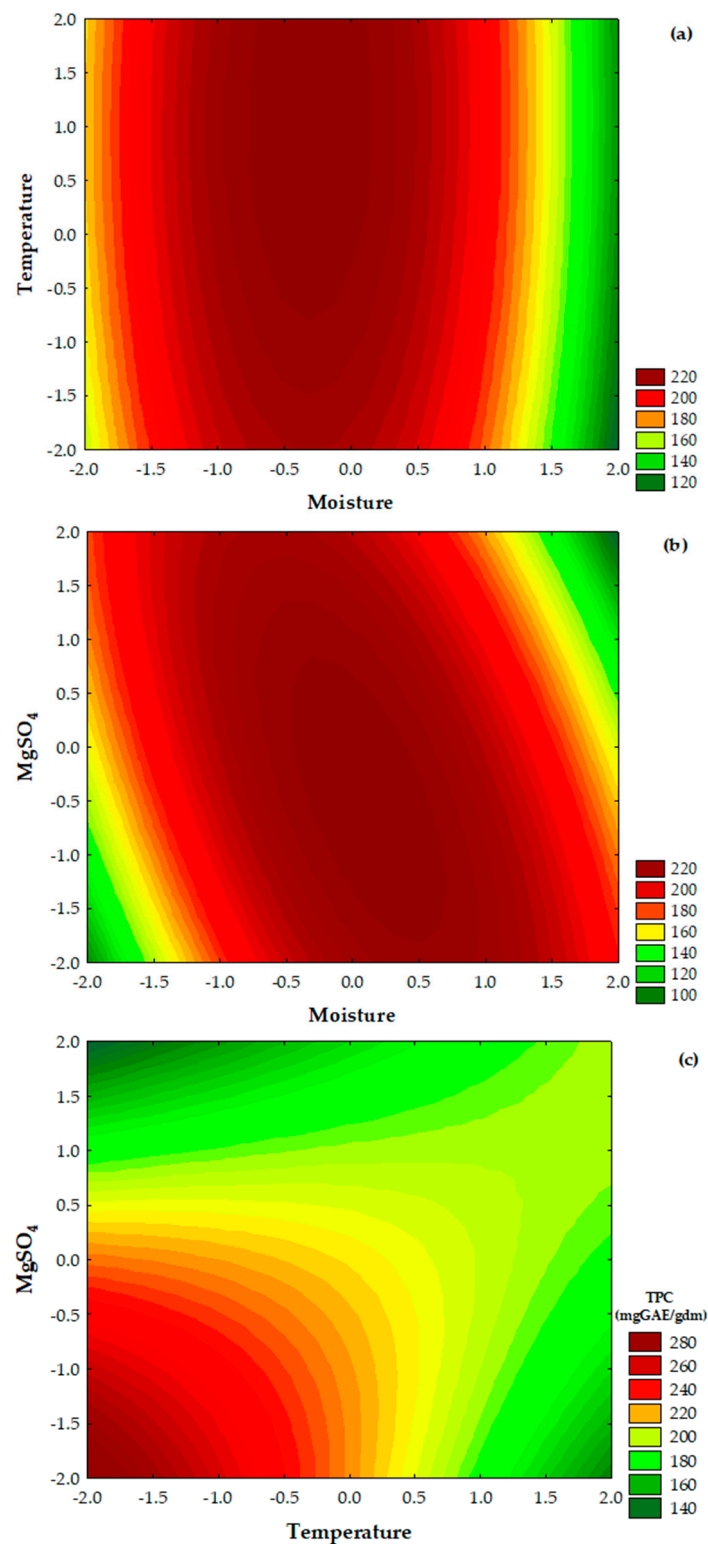


Figure 3. Combined effect of (a) moisture and temperature; (b) moisture and MgSO₄ and (c) temperature and MgSO₄ on TPC recovery by *A. niger* GH1.

3.5. Identification of Phenolic Compounds

TPC extracts obtained under optimal SSF culture conditions were characterized by HPLC-MS. A total of eight phenolic compounds were identified with the mass spectrum (Figure 4) matched as follows: compound 1 at m/z 191 and matched to 2-Hydroxypropane-1,2,3-tricarboxylic acid or citric acid. Compounds 2 and 3 have a molecular ion at m/z 781 at

two elution times (17.8 and 21.38 min), corresponding to 4,6-gallagyl-glucoside or punicalin isomers (namely α and β anomers). Furthermore, compounds 4 and 5 (m/z 1083) were identified as punicalagin (2,3-HHDP-4,6-gallagylglucoside) isomers (30.9 and 32.95 min of elution time). Compounds 6 (m/z 801.2) and 7 ($[M-H]^-$ m/z 633) were identified as digalloyl-HHDP-gluconic acid (punigluconin) and galloyl-HHDP-(hexoside or corilagin), respectively. Finally, compound 8 corresponds to 2,3,7,8-tetrahydroxy-chromen [5,4,3-cde] chromene-5,10-dione or ellagic acid (m/z 300.9).

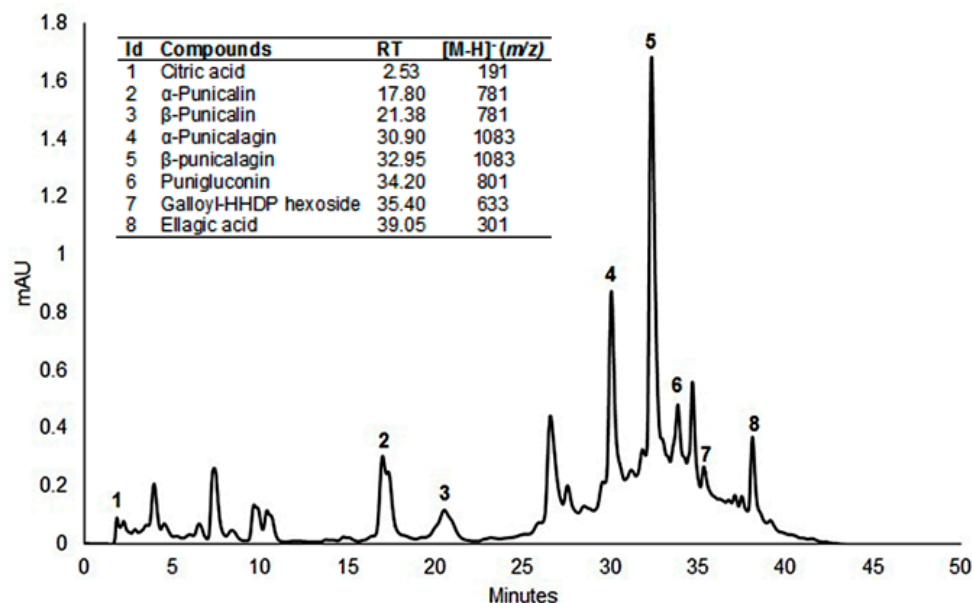


Figure 4. HPLC-MS chromatogram of TPC extracted from pomegranate peel by *A. niger* GH1.

4. Discussion

A culture media is a mixture of nutrients that, in adequate concentrations and under optimal physical conditions, allows the growth and metabolic processes of the desired microorganisms. The obtained results (Table 3) are similar to those reported by Bhol et al. [19] in terms of fat ($2.37 \pm 0.15\%$) and protein (8.03 ± 0.21). However, differences were observed in terms of ash ($0.67 \pm 0.02\%$), fiber ($4.80 \pm 0.10\%$) and carbohydrate ($46.21 \pm 0.11\%$) content, while the C/N value (65) was lower than that reported by Ben-Ali et al. [20]. A carbon-to-nitrogen (C/N) ratio is the relationship between the mass of carbon to the mass of nitrogen present in any substance. the C/N ratio is highly important for the regulation of the metabolic pathway, either for biomass or secondary metabolites production. Then, the C/N ratio must be established according to the main product of interest [21]. Carbon and nitrogen content and consequently the C/N ratio can be adjusted by adding any source of carbon or nitrogen; however, any excess of these compounds might result in toxicity and affect fungal growth and enzyme production, then, the enzymatic breakdown of the cell wall of the substrate and the subsequent release of polyphenols is reduced [22]. The difference in the values of the chemical components is due to factors related to the variety of pomegranate used, the geographic location of the crop, the irrigation conditions and other environmental and technological factors.

The water absorption index (WAI) and critical humidity point (CHP) are physicochemical properties with a relevant importance in terms of materials to be used as a substrate-support (S-S) in SSF. WAI is related to the hydroxyl groups present on a substrate fiber, which allows additional water interaction through hydrogen bonding [23], then the WAI value indicates the amount of water that can be absorbed by the S-S. The best materials for SSF are those with a high WAI, since the moisture content of these materials can be modified to the required values either for microorganism growth or for bioprocess convenience. Pomegranate by-products presented a WAI of 4.38 g/g; similar WAI values were reported for creosote bush leaves [15], candelilla stalks [24,25] and grape by-products [26],

with all of them reported as good S-Ss for SSF. The CHP is the amount of water linked to the support macromolecules and represents the water that cannot be used with the microbe for their metabolic processes. A high CHP value represents a high amount of water bound to the material, which can select the type of strain able to grow over the substrate, meaning that materials with a low CHP are preferred in SSF [23]. The CHP value obtained for pomegranate by-products was 10.13%, this value is lower than those reported for agroindustry by-products, such coconut husk (16%), orange peel (40%), lemon peel (28%), apple pomace (35%) and grape (53%) [15,25,26]. Based on their physicochemical characterization, pomegranate by-products are suitable to be used as an S-S for SSF.

The kinetics of metabolite production provides a quick look at microbial growth, the suitability of the substrate and culture conditions, the maximal production time and the process yield. Lower TPC extraction values when using conventional methods or commercial enzymes have been reported [27]. In contrast, in SSF, different enzymes, such as amylases, pectinases, xylanases, proteases, β -glucosidase, tannase and ellagitannase are simultaneously produced, then the sum of the different enzymatic activities increases the release of phenolic compounds as a result of the breakdown of the links between polyphenols moieties and other macromolecules, so that the amount of TPC released and AA is increased in a short time [28,29]. Accordingly, for TPC kinetics, the time was set at 36 h for the following experiments.

The positive effect of MgSO_4 on TPC release is explained by the fact that magnesium is related to the growth of hyphae in *A. niger*; the increase in the sporulation rate ensures an efficient enzyme synthesis, increasing the nutrient availability and, in consequence, the microbial biomass proliferation [30]. Sepúlveda et al. [31] reported that the increase in MgSO_4 levels promotes a major ellagic acid accumulation from pomegranate husk powder by *A. niger* GH1 in SSF. Temperature directly affects the fungal metabolism; consequently, it may affect either the microbial growth or the enzyme production rate, thus impacting TPC release. Most studies related to growth and enzyme production for *A. niger* GH1 are performed at 30 °C [17,32]. In this study, temperature had a positive effect (Figure 2), showing good TPC release from 30 °C. Different microbial species have different needs for specific moisture content to support their growth and the production of metabolites; in this study, moisture had a negative effect on TPC release. Therefore, fermentation processes require the close control of water content, as it affects the adequate level of nutrients and oxygen transport. A small deviation from the optimal moisture values may decrease the production of enzymes and, consequently, affects the release of the product of interest [33].

The addition of KCl exhibited a negative effect on TPC release (Table 1 and Figure 2). Potassium ions may trigger a conformational transition when binding to a distant protein enzyme site, promoting suitable conformational changes in the active site [34]. However, experimental results (Table 1, Figure 2) show that when the concentration of KCl used was high (3.04 g/L) the possible positive effect of the K^+ was reversed, causing a decrease in the enzymatic activity required for TPC release, therefore KCl was set at a lower level (1.52 g/L). Then, the variables of MgSO_4 , temperature and moisture were further considered in the CCD to optimize TPC release from pomegranate by-products by SSF.

Based on the above results, the optimized process conditions (CCD) satisfactorily defined TPC release by SSF as a 36 h process. Robledo et al. [11] reported TPC recovery of 6.3 and 4.6 mg/gdm from pomegranate peel with *A. niger* GH1 and *A. niger* PSH, respectively. Ascacio-Valdés et al. [28] reported TPC production of 42.02 mg/g for the fungal biodegradation of punicalin previously recovered and purified from pomegranate peel used as a carbon source. The TPC released from pomegranate in the present work is 24–80% higher than the values previously reported [11,28]. In addition, optimal SSF conditions provided an increase of 5.81 in the AA of the extract (46.40 ± 0.04 mgGAE/gdm) compared with the value before the optimization (7.98 ± 0.06 mgGAE/gdm) for a 36 h process. The increase in AA is attributed to the amount and type of the phenolic compounds released. The obtained results show the suitability of SSF to obtain TPC with AA from by-products over chemical synthesis processes or by the use of commercial enzymes.

The identification of phenolic compounds (Figure 4) starts with a compound signal (compound 1) at m/z 191 which is matched to 2-Hydroxypropane-1,2,3-tricarboxylic acid or citric acid. Citric acid has been reported as the main organic acid found in pomegranate wine, juice and peel [1,35]. Compounds 2 and 3 had a molecular ion of m/z 781 at two elution times (17.8 and 21.38 min) corresponding to 4,6-gallagyl-glucoside or punicalin isomers (namely α and β anomers); both molecules are considered to be intermediate compounds during the biodegradation of ellagitannins [1,28]. Furthermore, compounds 4 and 5 (m/z 1083) were identified as punicalagin (2,3-HHDP-4,6-gallagylglucoside) isomers (30.9 and 32.95 min of elution time), the main phenolic compound found in pomegranate [33,36–38]. Punicalagin is considered a key precursor in the degradation of pomegranate ellagitannins and it is a determinant molecule for the induction of fungal *ellagitannase* production by SSF [39]. Compounds 6 (m/z 801.2) and 7([M-H][−] m/z 633) were identified as digalloyl-HHDP-gluconic acid (punigluconin) and galloyl-HHDP-(hexoside or corilagin), respectively, both hydrolysable tannins previously found in pomegranate juice [40] and seeds [41]. Finally, compound 8 corresponds to 2,3,7,8-tetrahydroxy-chromen [5,4,3-cde] chromene-5,10-dione or ellagic acid (m/z 300.9). There are no reports about the identification of phenolic compounds obtained from solid fermented pomegranate by-products; however, Ascacio-Valdés et al. [39] suggested the complete biodegradation pathway of ellagitannins by the SSF of ellagitannins previously extracted from pomegranate by-products by *A. niger* GH1. The same authors reported that punicalin, gallic acid and ellagic acid were obtained from punicalagin, identifying the intermediate molecules and the immediate precursor of ellagic acid. In this study, gallic acid was not detected at the final process time (36 h). Fischer et al. [38] reported the identification and quantification of phenolic compounds from pomegranate peel, mesocarp, aril and differently produced juices; however, they did not report citric acid, punicalin isomers (α and β) or punicalagin. Li et al. [20] reported gallic acid, punicalagin- α , punicalagin- β , catechin, chlorogenic acid, epicatechin, rutin and ellagic acid as eight characteristics of the chemical fingerprint of polyphenols extracted from pomegranate peel, but they did not find punicalin (α and β), citric acid punigluconin or galloyl HHDP hexoside. According to Gumienna et al. [42], differences among the formed bioactive compounds are explained by the reactions of polymerization, condensation, oxidation, hydrolysis, enzyme activity and molecule interactions. Furthermore, different phenolic profiles may be obtained depending on the microbial strain (fungi, yeast or bacteria), and the enzymes that they may produce, even when using the same substrate and fermentation process.

The identified polyphenol molecules have different biological activities with a wide number of possible applications in the food, pharmacy and cosmetics industries [2], which, when obtained from by-products, are considered as a high added-value product [43]. Bearing in mind that all pomegranate peel samples were treated using the described process and up to 248 kg of TPC per ton dm of pomegranate peel could be obtained, and considering that in Mexico 5937.28 tons dm of pomegranate peel were generated in 2021, then 1,472,445.44 kg of valuable TPC may be obtained from dry pomegranate peel. Considering the commercial price of ellagic acid and punicalagin is 94 USD/50 mg and 494.70 USD/10 mg, respectively (Sigma-Aldrich®), the SSF extraction process may be quite profitable in terms of industrial interests. The improved biotechnological extraction process has an impact foremost on the recovery of high-value molecules from pomegranate peel, providing higher TPC and AA values in a short time process. The recovered molecules are of great interest in the food, pharmacy and cosmetic industries, and at the same time diversification in the use of agroindustry by-products is obtained, thus approaching the highly desired circular economy model.

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