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Article

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Abstract: Pomegranate peels are important source of polyphenols with remarkable interest in food, pharmaceutical and cosmetic industry. An improved extraction of total polyphenolic compounds (TPC) from pomegranate peels by Solid-State Fermentation (SSF) was achieved. A Box Hunter & Hunter (BHH) followed by Central Composite Design (CCD) were performed to assess the effect of the process variables on TPC release. The statistic designs indicate that the best TPC extraction (234.85 mg GAE/gdm) by means of SSF occurs, at 42°C, 50 % moisture, pH 5.0, mineral solution (g/L): $NaNO_3$ (3.83), KH_2PO_4 (1.52), $MgSO_4$ (4.66) and KCl (1.52) at 36 h. At the best fermentation conditions, TPC (248.78±1.24 mgGAE/gdm) increased 5.96-fold than values previously reported and antioxidant activity [AA] increased 5.81-fold than the value obtained before the SSF optimization. High-valued 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 pomegranate peels valorization through the recovery of molecules with high added value with potential use in food, pharmacy and cosmetic industries; a diversification in the use of food agroindustry by-products is obtained as approach to the circular economy model through biotechnological processes.

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

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 [Kalaycıoglu and Erim, 2017]. 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 and peels [Ascacio-Valdés *et al.* 2011; Tehranifar *et al.* 2011].

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Pomegranate intake is mainly in juice, jellies, jams and liquors; however, their biological activity is highly appreciated in the food, cosmetic and pharmacy industries [Ascacio-Valdés, *et al.* 2011]. According to Kazemi *et al.* [2016] one ton of fresh pomegranate generates 669 kg of by-products and those include 78 % peel and 22 % seeds. Although, the fast increase of pomegranate cultivation limits the precise calculation of the worldwide production, in 2021 based on the 8,636.2 tons of pomegranate harvested in 2021 for Mexico, 6,736.2 tons of pomegranate peels are available for the highly valued biomolecules extraction [https://nube.siap.gob.mx/cierreagricola/]. Organic solvents are commonly used for the extraction of vegetal biomolecules; however, their use is associated with environmental pollution and with toxicological safety concerns. Implementation of emerging technologies [i.e. supercritical fluid, microwave, electric field, pressurized liquid] for biomolecules extraction improves extraction yields; however, the requirement of polar solvents and expensive extractive equipment strongly limits its applications [Makris *et al.* 2007; Martins *et al.* 2011; Cano-Lamadrid *et al.* 2022].

In contrast, fermentative or enzymatic methods for the extraction of biomolecules from agricultural by-products have been successfully used [De la Torre *et al.* 2019; Buenrostro-Figueroa *et al.* 2017; Robledo *et al.* 2008]. Optimization methods based on one-factor-at-a-time are expensive, time-consuming and interactions between processes variables are not considered [Saffarzadeh-Matin and Khosrowshahi, 2017]. Experimental designs for the study of several variables at a time, as well as their interactions are highly desirable.

This work is aimed to increase the extract concentration of Total Polyphenol Compounds (TPC) with AA from pomegranate peels by SSF, furthermore the identification of the extracted high-valuable 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) to 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

Chemical composition (protein, fat, carbohydrate, fiber, moisture and ash) was determined according to the official procedures reported by the Association of Official Analytical Chemists (AOAC, 2012). Water absorption index (WAI) and critical humidity point (CHP) were assessed according to Orzua [2009].

2.3. Microorganism

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

2.4. Solid-state fermentation (SSF)

Pomegranate peels were used as support and sole carbon source. Fermentable mass (2.8 g of dry pomegranate peels per reactor) was adjusted at 60 % moisture by adding 4.2 mL of saline solution (previously inoculated with $1x10^6$ spores/g of support) and aseptically packed in tray reactors (60x15 mm). Saline solution was prepared as follow: (g/L): $NaNO_3$ (7.65); KH_2PO_4 (3.04); $MgSO_4*7H_2O$ (1.52); KCl (1.52). The SSF was set at 30 °C for 72 h, samples were withdrawn every 12 h. The fermented extracts were obtained by addition of 10 mL of 50 mM citrate buffer (pH 5) to each try-reactor, shaken (100 rpm, 15 min) and centrifuged (3500 rpm/15 min, 4 °C). Supernatant was filtered (0.45 μM) to remove impurities and fungal debris and stored at 16 °C until further analysis.

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2.5. Analytical analysis.

Phenolic compounds on the extracts were determined by the Folin-Ciocalteu assay described in Buenrostro-Figueroa et al. [2017]. Folin-Ciocalteu (200 µL) reagent (Sigma-Aldrich®) was added to cuvettes containing aliquots (200 µL) of either fermented extract or standard solution, mixed and incubated (5 min). Then, 200 µL of 0.01 Na2CO3 were added, samples were shaken and left for 5 min. Finally, samples were diluted by addition of 5 mL of distilled water. Absorbance was recorded at 730 nm in a UV-Vis spectrophotometer (UV-1800 spectrophotometer; Shimadzu®, Kyoto, Japan). Gallic acid was used as standard; calibration curve was plotted in a range of 0-250 µg/mL. All analyses were performed in triplicate, TPC were expressed as mg of gallic acid equivalent (GAE)/g of dry matter (gdm).

Antioxidant activity of the extracts was evaluated based on the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma-Aldrich®) free radical, as described by Meléndez et al. [2014]. Reaction mixture, consisting of 7 µL of extract and 193 µL of 60 µM DPPH in absolute methanol, were analysed on a BioTek® Microplate reader (ELx808™, Vermont, USA) with absorbance filters for a wavelength of 520 nm. Decolouring process was recorded during 30 min of reaction. Antioxidant activity was calculated on a base of gallic acid (Sigma-Aldrich®) standard curve (0-200 µg/mL) and expressed as mgGAE/gdm. Control samples were prepared with methanol (100 µL); distilled water (100 µL) was used for equipment calibration. Samples were analysed in triplicates.

2.6. Phenolic profile

Phenolic profile from the extracts obtained under optimized SSF conditions was analysed by reverse phase-high performance liquid chromatography on a Varian HPLC system equipped with an autosampler (Varian® ProStar 410, USA), ternary pump (Varian® ProStar 230I, USA) and PDA detector (Varian® ProStar 330, USA). A liquid chromatograph ion trap mass spectrometer (Varian® 500-MS IT Mass Spectrometer, USA) equipped with electrospray ion source was used. Samples (5 µL) were injected into a Denali C_{18} column (150 mm × 2.1 mm, 3 µm, Grace, 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 follow 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 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 [M-H]-1. Nitrogen was used as nebulizing gas and helium as damping gas. The ion source parameters were spray voltage 5.0 kV and, capillary voltage and temperature were 90.0 V and 350 °C, respectively. Data were collected and processed using MS Workstation software (V 6.9). Samples were firstly analysed in full scan mode acquired in the m/z range 50–2000. MS/MS analyses were performed

2.7. Statistical analysis

Two-step optimization of TPC extraction from pomegranate peels was performed. Firstly, to identify the variables with significant effect on the TPC release, seven variables (temperature, pH, moisture, $NaNO_3$, KH_2PO_4, $MgSO_4$ and KCl) were evaluated by a Box-Hunter-Hunter design (BHH) (Tab. 1). Based on the BHH results, a central composite design (CCD) was completed to find the variable value levels for the 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. *There are no significant differences among letters (Tukey α=0.05).

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}

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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	Levels	
		-1	+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 α=0.05).

Three independent variables (Tab. 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 the 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	Levels				
		-1.68	-1	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

Experimental designs (BHH and CCD) were performed in duplicate and samples analysed in triplicated. Data analysis and model building were analysed by Statistica® 7.0 software (Stat Soft, Tulsa, OK, USA). The outcome results were visualized in a Pareto Chart and 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 α=0.05. Then, with the empiric polynomial model, experimental data and regression

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coefficients were adjusted, regression coefficients were obtained by the multiple lineal regression equation (Eq. 1):

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

Where Y represents the predicted response (TPC expressed in GAE/gdm); Xi and XJ represent the independent variables; k is the number of variables evaluated; β0, βi, βii and βij 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

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 validation of the model.

3. Results

3.1. Physicochemical characterization of pomegranate peels

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

Table 3. Physicochemical characterization of pomegranate by-products.

Component (%)	Value
Moisture	11.86±0.05
Fat	2.64±0.08
Fibre	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

WAI: Water absorption index
CHP: Critical humidity point
*gram per gram of dry sample

3.2. Kinetics of TPC extraction and AA

Kinetics of metabolite production provide a quick-sign of the suitability of the substrate and culture conditions on the obtention of the desired metabolites. Kinetics of TPC release and AA by *Aspergillus niger* GH1is shown in Fig. 1. Despite that the TPC release starts since the beginning of the process, the higher increase occurs from 24 – 36 h, attaining the value of 106.56 mgGAE/gdm at 36 h. The similar pattern was observed for AA showing the highest activity (7.95 mg GAE/gdm) at 36 h.

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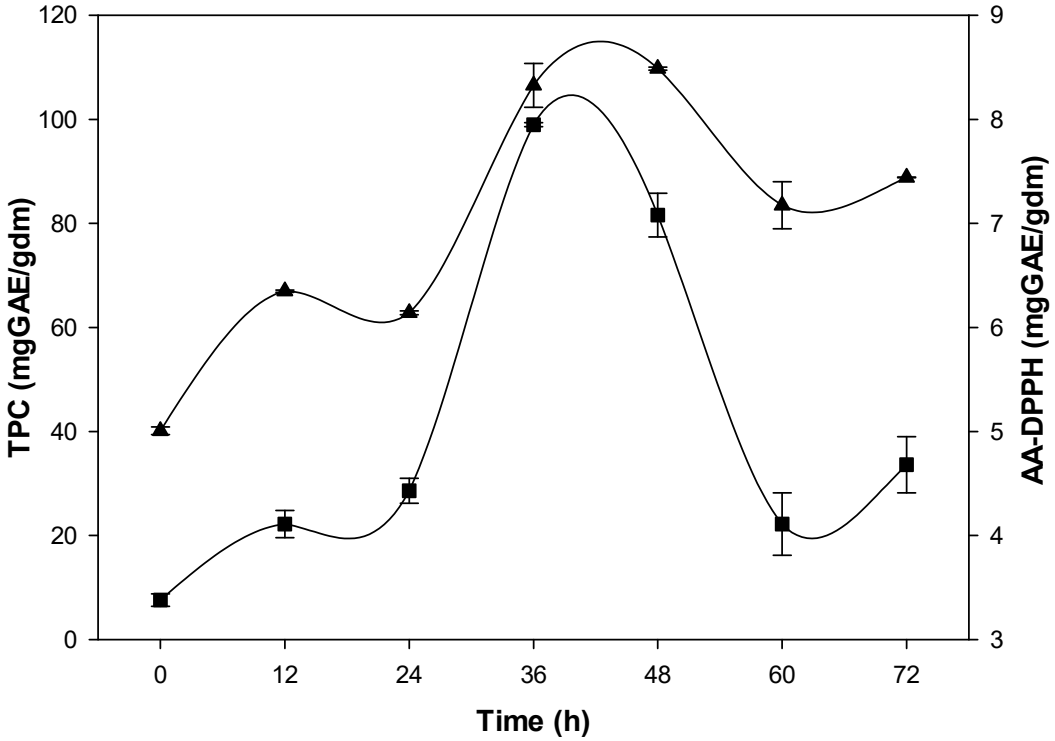

Figure 1. Kinetics of total polyphenols compounds (▲) and antioxidant activity (■) from fermented pomegranate peels.

3.3. Significant factors for TPC recovery by SSF

To identify the variables with significant effect on the TPC release by *A. niger* GH1, seven variables (temperature, pH, moisture, $NaNO_3$, KH_2PO_4, $MgSO_4$ and KCl) were evaluated by a Box-Hunter-Hunter design (BHH) (Tab. 1). 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, Pareto Chart was plotted (Fig 2). The factors whose values exceeded the dotted line (moisture, $MgSO_4$, KCl and temperature) have a significant effect (α=0.05) on TPC production. The $MgSO_4$ and temperature have a positive as their level value increases, the response value also increases. In contrast, moisture and KCl have a negative effect on the response variable, thus at any increase in those variables, the TPC extraction decreases therefore, lower moisture and KCl levels should be used to increase the TPC release. The $NaNO_3$, KH_2PO_4 and pH variables had no significant impact (α=0.05) on the TPC extraction by SSF, thus were not considered in further studies.

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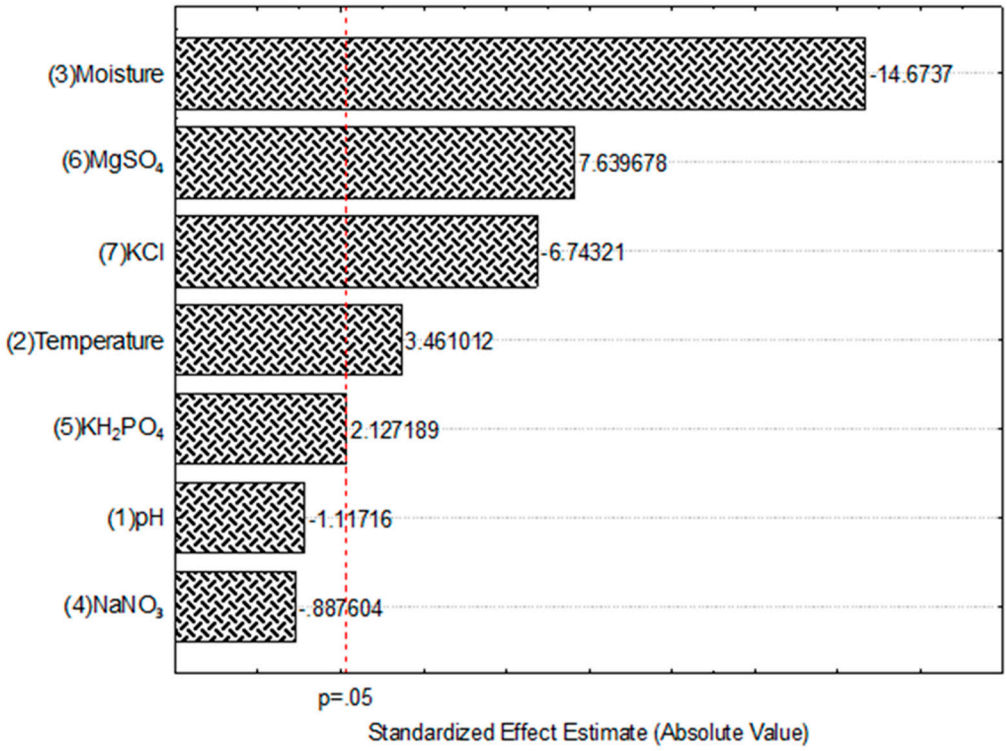

Figure 2. Pareto chart: significative independent variables on TPC recovery by *A. niger* GH1.

3.4. Optimization of the culture conditions for release of TPC

An experimental design was set to evaluate the influence of temperature, moisture, $MgSO_4$ levels on the maximal TPC release from pomegranate by-products by SSF. Tab. 2 shows the values and coded levels of the variables studied in the CCD as well as the TPC release. According to the CCD, maximum TPC release was achieved in treatments 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 interaction between factors were obtained from the CCD analysis. Linear term of temperature was significant in the process at a level of α <0.05. For quadratic terms, only moisture was significant. The interaction between moisture and $MgSO_4$ as well as the interaction between temperature and $MgSO_4$ were also significant ($\alpha \leq 0.05$). The quadratic effect of temperature and $MgSO_4$, linear effect of $MgSO_4$, as well as interaction between moisture and temperature were not significant ($\alpha \leq 0.05$). To determine the influence of the resulted significant variables and find the ideal process condition for the TPC release by SSF from pomegranate by-products, contour plots were constructed (Fig. 3). The tendency to obtain higher TPC release was observed at central-low moisture (Fig.3a, 3b), central-high temperature (Fig. 3a) and high $MgSO_4$ values (Fig. 3b,3c), respectively. To optimize the selected factors (moisture, temperature and $MgSO_4$) over the TPC release, a second order polynomial model (Eq. 1), experimental data and regression coefficients were obtained by multiple lineal regressions (Eq. 2) as follow:

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

Under ideal condition, the simplified model (Eq. 2) predicted a maximal yield of TPCmax= 234.85 mgGAE/gdm (R2= 0.95). In order to validate the model, another experiment design was set (in triplicate) at the following values: temperature of 42 °C, moisture 50 %, pH 5, $NaNO_3$ 3.83 g/L, KH_2PO_4 1.52 g/L, $MgSO_4$ 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 the predicted but within of the significance model error.

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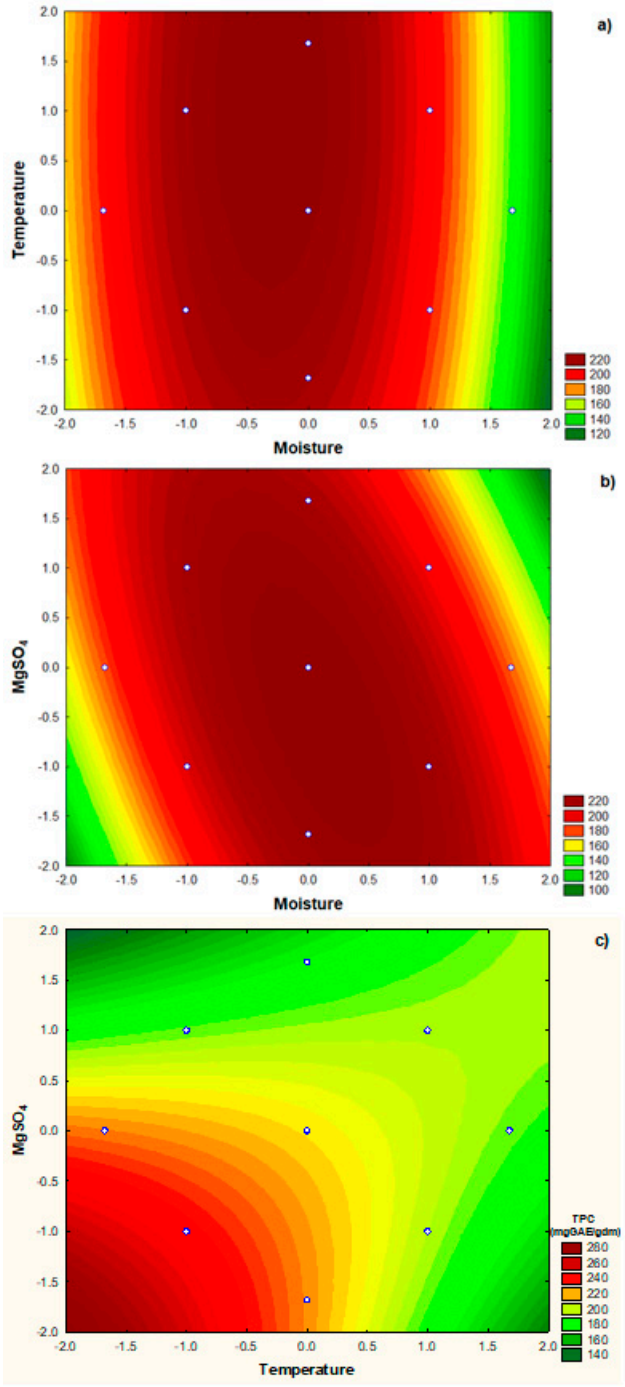

Figure 3. Combined effect of a) Moisture and temperature; b) Moisture and $MgSO_4$. and c) Temperature and MgSO4 on TPC recovery by *A. niger* GH1.

3.5. Identification of phenolic

TPC extracts obtained at optimal SSF culture conditions were characterized by HPLC-MS. A total of eight phenolic compounds were identified, mass spectrum (Fig. 4) matched as the 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-

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(hexoside or corilagin) respectively. Finally, compound 8 corresponds to 2,3,7,8-tetrahydrxy-chromen [5,4,3-cde] chromene-5, 10-dione or ellagic acid (m/z 300.9).

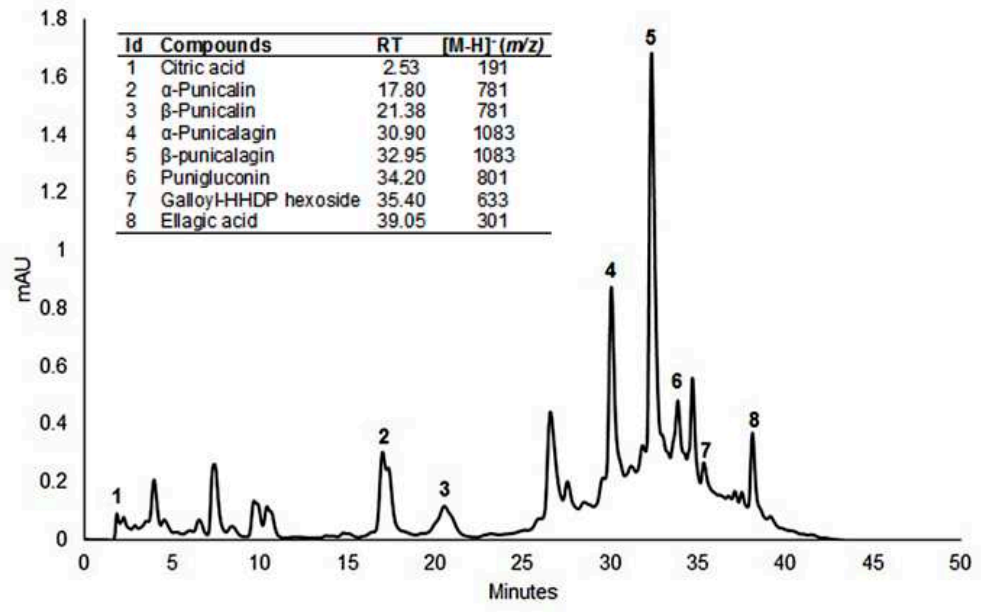

Figure 4. HPLC-MS chromatogram of TPC extracted from pomegranate peels by *A. niger* GH1.

4. Discussion

Culture media is a mixture of nutrients that, in adequate concentrations and under optimal physical conditions, allow the growth and metabolic processes of the desirer microorganisms. Obtained results (Tab. 3) are similar to those reported by Bhol *et al.* [2016] in fat (2.37 ± 0.15 %) and protein (8.03 ± 0.21). However, differences were observed in ash (0.67 ± 0.02 %), fibre (4.80 ± 0.10 %) and carbohydrates (46.21 ± 0.11 %) content, while C/N value (65) was lower than the reported by Ben-Ali *et al.* (2017). 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 to biomass or to secondary metabolites production. Then, the C/N ratio must be established according to the product of main interest [Lopez-Flores et al., 2016]. 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 toxic and affect fungal growth and enzymes production then, enzymatic breakdown of the cell wall of the substrate and subsequent polyphenols release is reduced [Rajarathnam *et al.* 1989]. 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 materials to be used as a substrate-support (S-S) in SSF. WAI is related to hydroxyl groups present on substrate fibre, which allows additional water-interaction throughout hydrogen bonding [Martins *et al.* 2017], 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 high WAI, since the moisture content of these materials can be modified to required values either for the microorganism growth or for a bioprocess convenience. Pomegranate by-products presented a WAI of 4.38 g/g; similar WAI values were reported for creosote bush leaves [Orzua, *et al.* 2009], candelilla stalks [Ascacio-Valdés *et al.* 2010; Buenrostro-Figueroa *et al.* 2014] and grape by-products (Martínez-Ávila *et al.* 2012), all of them reported as good S-S for SSF. The CHP is the amount of water linked to the support macromolecules and represents the water that cannot be used for the microbe for their metabolic processes. High CHP value represents a high amount of water bounded to the material, which can select the type of strain able to grow over the substrate, then materials with low CHP are preferred in SSF [Martins *et al.* 2017]. 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 % (Buenrostro-Figueroa *et al.* 2014; Martínez-Ávila *et al.* 2012; Orzua *et al.* 2009]. Based on tHe physicochemical characterization, pomegranate by-products are suitable to be used as S-S for SSF. Physicochemical characterization suggested that pomegranate by-products possessed the required characteristics for its potential use as substrate-support for SSF.

Kinetics of metabolite production provide a quick-look of the microbial growth, suitability of the substrate and culture conditions, maximal production time and the process yield. Lower TPC extraction values when using conventional methods or commercial enzymes for have been reported (Coetzee *et al.* 2012). In contrast, in SSF, different enzymes such 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 the result of the breakdown of the links between polyphenols moieties and other macromolecules, then the amount of TPC release and AA is increased in a short time process [Ascacio-Valdés *et al.* 2014; Santos da Silveira *et al.* 2019].

Positive effect of $MgSO_4$ on the TPC release is explained on the fact that magnesium is related to the growth of hyphae in *A. niger*, the increase in sporulation rate ensures an efficient enzyme synthesis, increasing the nutrients availability and in consequence, the microbial biomass proliferation (Jamal *et al.* 2011). Sepúlveda *et al.* [2012] 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 enzymes production rate, thus impacting the TPC release. Most studies related to growth and enzyme production for *A. niger* GH1 are performed at 30 °C [De la Cruz *et al.* 2014; Lopez-Trujillo *et al.* 2017]. In this study, temperature had a positive effect (Fig. 2), showing good TPC release from 30 °C. Different microbial species have different needs of specific moisture content to support their growth and metabolites production, in this study, moisture had a negative effect on the TPC release. Therefore, fermentation processes require a close control of water content as it affects the adequate 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 [Beniwal *et al.* 2013].

The addition of KCl exhibited a negative effect on the TPC release (Tab. 1 and Fig. 2). Potassium ions may trigger the conformational transition when binding to a distant protein enzyme site promoting suitable conformational changes in the active site [Vašák and Schnabl, 2016]. However, experimental results (Tab. 1, Fig. 2) show, that the concentration of KCl used was high (3.04 g/L) that the possible positive effect of the K+ was reversed causing a decrease in the enzymatic activity for the TPC release, then KCl was set at its lower level (1.52 g/L). Then, the variables of $MgSO_4$, temperature and moisture were further considered in the CCD to optimize the TPC release from pomegranate by-products by SSF.

Based on above results, the optimized process conditions defined satisfactorily the TPC release by SSF at 36-h process. Robledo *et al.* (2008) reported TPC recovery of 6.3 and 4.6 mg/gdm from pomegranate peels with *A. niger* GH1 and *A. niger* PSH respectively. Ascacio-Valdés *et al.* [2014] reported TPC production of 42.02 mg/g for the fungal biodegradation of punicalin previously recovered and purified from pomegranate peels used as carbon source. The TPC released from pomegranate in the present work is 24 – 80 % higher from values previous reported [Robledo *et al.* 2008; Ascacio *et al.* 2014]. In addition, optimal SSF conditions provided an increase of 5.81-fold 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) at 36-h process. The increase in AA is attributed to the amount and type of the released phenolic compounds. The obtained results show the suitability of SSF to obtain TPC with AA from by-products over of the chemical synthesis or by the use of commercial enzymes.

Identification of phenolic compounds (Fig. 4) starts with a compound signal (compound 1) at m/z 191 and 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 peels [Kalaycıoğlu and Erim

2017; Pande and Akoh 2009]. 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), both molecules are considered intermediate compounds during ellagitannins biodegradation [Aguilera-Carbó *et al.* 2008; Ascacio-Valdés *et al.* 2014]. 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 [Amyrgialaki *et al.* 2014; Fischer *et al.* 2011]. Punicalagin is considered a key precursor in pomegranate ellagitannins degradation and it is determinant molecule for the induction of fungal *ellagitannase* production by SSF [Ascacio-Valdés *et al.* 2016]. 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 [Gómez-Caravacan *et al.* 2013] and seeds [Ambigaipalan *et al.*, 2016]. Finally, compound 8 corresponds to 2,3,7,8-tetrahydrxy-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.* [2016] suggested the complete biodegradation pathway of ellagitannins by SSF of ellagitannins previously extracted from pomegranate by-products by *A. niger* GH1. The same authors reported that punicalin, gallagic and ellagic acids were obtained from punicalagin, identifying the intermediate molecules and immediate precursor of ellagic acid. In this study, gallic acid was not detected at the final process time (36 h). Fischer *et al.* [2011] 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) nor punicalagin. Li *et al.* [2015] reported the gallic acid, punicalagin- α, punicalagin-β, catechin, chlorogenic acid, epicatechin, rutin, and ellagic acid as the eight characteristics chemical fingerprint of polyphenols extracted from pomegranate peel, but they don't find punicalin (α and β), citric acid punigluconin nor galloyl HHDP hexoside. According to Gumienna *et al.* [2016], differences among the formed bioactive compounds are explained by reactions of polymerization, condensation, oxidation, hydrolysis, enzyme activity and molecules 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 [Ascacio-Valdés *et al.* 2011] and when obtained from by-products are considered as high-added value product [Holic *et al.* 2018]. Then, bearing in mind that all pomegranate peels were treated under the described process, it could be obtained up to 248 kg of TPC per ton dm of pomegranate peels, and considering that in Mexico (2021) 5937.28 tons dm of pomegranate peels, then 1,472,445.44 kg of valuable TPC may be obtained from dry pomegranate peels. Considering the commercial price of the ellagic acid and punicalagin is 94 USD/50 mg and 494.70 USD/10 mg respectively (Sigma-Aldrich®), the SSF extraction process may result quite profitable for industrial interest. The improved biotechnological extraction process has a foremost impact on the recovery of high-value molecules from pomegranate peels, providing higher TPC and AA values in a short-time process. The recovered molecules are of a great interest in the food, pharmacy and cosmetic industries, and at the same time a diversification in the use of agroindustry by-products is obtained thus approaching the highly desired circular economy model.

Authors contribution: Buenrostro-Figueroa, José Juan: Conceived the study, acquisition of the pomegranate by-products, performed lab research, analyze and discussed data, wrote and reviewed the manuscript. Nevárez-Moorillón Guadalupe Virginia: Analyze and discussed data and reviewed the manuscript. Chávez-González, Mónica Lizeth: Performed substrate characterization and part of the fermentation research, analyze and discussed data and reviewed the manuscript. Sepúlveda, Leonardo: Performed part of fermentation research, performed graphical abstract and reviewed the manuscript. Ascacio-Valdés, Juan Alberto: Conceived and performed molecules identification, analyze and discussed data and reviewed the manuscript. Aguilar, Cristóbal Noé: Conceived the study, participate in manuscript integration and reviewed the manuscript. Pedroza-Islas, Ruth: Analyze and discussed statistic data and reviewed the manuscript. Huerta-Ochoa, Sergio: Conceived the study, analyze, discussed and integrate data, wrote and reviewed the manuscript. Prado-Barragán, Lilia Arely:

Conceived the study, supervised the experimental work, analyze, discussed and integrate data, wrote and reviewed the manuscript.

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