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Optimized Purification of Free Amino Acids from Molasses by Nanofiltration Membrane

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ABSTRACT: In this research, nanofiltration was utilized to purify free amino acids (AAs) from sugar and colloids of sugar cane molasses (SCM) and sugar beet molasses (SBM) based on their molecular weight. The impact of temperature (30-50˚C), pressure (2-7 bar), and pH (2-11) in optimizing the purification condition was evaluated using the response surface methodology. The SCM and SBM purification results revealed the same optimum conditions in both types of molasses: 47 ˚C temperature, 3 bar pressure, and 9.3-9.5 pH. In the optimum condition, the recovery values of AAs for SCM and SBM were 66% and 63% for aspartic acid, 68.5% and 66% for glutamic acid, 84% and 69% for alanine, and 82% and 69% for lysine, respectively. The total efficiency values of four AAs and flux were obtained as 75% and 70 $Lm^{-2}h^{-2}$ for SCM and 67% and 45 $Lm^{-2}h^{-2}$ for SBM, respectively. The results indicated the suitability of the nanofiltration system's purifying function for AAs from SCM and SBM with desirable selectivity and purification efficiency.

Keywords: Alanine, Aspartic acid, Dead-end filtration, Glutamic acid, Lysine.

Introduction

Molasses, the main byproduct of sugar cane (*Saccharum officinarum L.*) and sugar beet (*Beta vulgaris L. ssp. saccharata*) processing plants, is a dark, highly viscous syrup containing valuable compounds. The composition of molasses varies substantially depending on the non-sucrose compounds in the raw juice and the refining technology used. However, 75–85% of sugarcane molasses content is composed of total solids: 30–36% sucrose, 10–17% (fructose + glucose), 10–16% ash, and some smaller quantities of polysaccharides, oligosaccharides, organic acids, and several non-sugar organic compounds (approximately 6%). The sugar beet molasses has a similar composition but with higher sucrose content and a lower concentration of reducing sugars (Abdelmoez *et al.*, 2007; Abejón *et al.*, 2017; Alexander *et*

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al., 2009; Bernal *et al*., 2016; Betancur-Ancona *et al*., 2015; Table 1).

The global production of molasses amounts to around 50 million tons per year. It is evaluated that from about 100 tons of processed cane, 10 tons of sucrose and 4 tons of molasses are extracted. Molasses is considered a low-value product regarded as substrate environmental contaminant due to its high sugar content and organic compounds that cause environmental pollution over time. Furthermore, ethanol production is the primary use of molasses. Its wastewater is widely used in this process due to high biological oxygen demand (BOD) and chemical oxygen demand (COD). Thus, molasses can be used to extract valuable components, such as amino acids (Clarke, 2003).

Molasses are mainly used as soil fertilizer and cattle feed supplement in specialized yeast fermentation (production of citric acid, glutamate, and acetone), as flavoring agents in some foods, or as a feedstock for ethanol. Molasses production is highly correlated with sugar production, by extension sugarcane and sugar beets (Yadav & Solomon, 2006).

Table 1. The Composition of molasses (Clarke, 2003)

Main constituents	***Components***	***Normal range***
Water		17–25%
Sugars	Sucrose	30–40%
	Glucose (dextrose)	4–9%
	Fructose (levulose)	5–12%
	Other reducing substances (as invert)	1–4%
	Total reducing substances (as invert)	10–25%
Other carbohydrates	Gums, starch, pentosans, also traces of hexitols; myoinositol, d-mannitol, and uronic acids	2–5%
Ash	As carbonates[a]	7–15%
	Bases:	
	Potassium oxide (30–50%)	
	Calcium oxide (7–15%)	
	Magnesium oxide (2–14%)	
	Sodium oxide (0.3–9%)	
	Metal oxides (as ferric) (0.4–2.7%)	
	Acids:	
	Sulfur trioxide (7–27%)	
	Chloride (12–20%)	
	Phosphorus pentoxide (0.5–2.5%)	
	Silicates and insolubles (1–7%)	
Nitrogenous compounds	Crude protein (as N × 6.25)	2.5–4.5%
	True protein	0.5–1.5%
	Amino acids, principally aspartic and glutamic acids, including some pyrrolidine carboxylic acids	0.3–0.5%
	Unidentified nitrogenous compounds	1.5–3.0%
Nonnitrogenous	Aconitic acid (1–5%), citric, malic, oxalic, glycolic	1.5–6.0%
	Mesaconic, succinic, fumaric, tartaric	0.5–1.5%
Wax, sterols, and phosphatides		0.1–1.0%
Vitamins	Thiamin (B_1)	2–10 p.p.m.
	Riboflavin (B_2)	1–6 p.p.m.
	Pyridoxine (B_6)	1–10 p.p.m.
	Nicotinamide	1–25 p.p.m.
	Pantothenic acid	2–25 p.p.m.
	Folic acid	10–50 p.p.m.
	Biotin	0.1–2 p.p.m.

Valuable components in molasses such as amino acids (AAs) can be used for other segments as essential additives in food products, chemical raw materials, pharmaceutical products, and biotechnological processes (Bhagavan *et al*., 2011; Bowen and Mukhtar, 1996; Centenaro *et al*., 2014). The molecular weights of AAs range from 75 to more than 200 Da. However, the percentage of amino acids in molasses is low (0.3–0.5%) in comparison to other wastes such as waste of meat. The molasses is used not only as a pilot pattern for extracting amino acids for other industries (since sugar is the main component in molasses, amino acids can be easily separated from sugar based on the difference in molecular weight between amino acids and sugars), it also contains necessary amino acids, such as lysine, which cannot be synthesized in the body. Furthermore, glutamic acid and aspartic acids are the primary amino acids in molasses. These valuable amino acids can be found in other wastes in low amounts (Bhagavan et al., 2011).

Membrane filtration is a highly effective and economical process for separating valuable components from the waste suspended or dissolved in a liquid. Dead-end filtration is the most basic filtration system in which the whole feed flow is forced through the membrane. Nanofiltration (NF) is a new class of pressure-driven membrane processes with high selectivity in the purification of components in the molecular weight cut-off range of 300-1000 Da; thus, it is a valuable tool for purifying AAs (Centenaro *et al*., 2014; Chabeaud *et al*., 2009; Chakrabarty *et al.,* 2013; Clarke and edye, 1996; Coca *et al*., 2004; Córdovaa *et al*., 2017; Curtin, 2018; Eckera *et al.,* 2012; El-Geddawy *et al.,* 2012; Fabiani *et al.,* 2002; Ferry, 1936; Filipcev *et al.,* 2016; Garem *et al.,* 1996). The selectivity of NF membranes in the purification of AAs is defined based on the size and electrical charge of solutes during their transmission (Geanta *et al.,* 2013). The transmission of neutral AAs only depends on the size rejection effects of the membrane and can be modeled by employing the ferry pattern (Gómez-Díaz *et al.,* 2009), corrected by Zeman and Wales (Gotoh *et al*., 2004). However, the transmission related to charge interactions can be related to the Donnan theory. The Donnan exclusion is a non-sieving mechanism that refers to electrostatic interactions between charged compounds and the membrane related to the pH value of the solution (Goulas *et al*., 2002; Grib *et al.,* 2000; Guan *et al.,* 2014; Guo *et al.,* 2018; Gyura *et al.,* 2002; Hong and Bruening, 2006; Hubbard and Binder; 2016).

Recently, several researchers have attempted to purify AAs by NF membranes (Geanta *et al.,* 2013; Hong and Bruening, 2006; Ingole *et al.,* 2014; Katz, 1986; Khan *et al.,* 2006; Kovacs and Samhaber, 2008; Kovacs and Samhaber, 2009). Tsuru *et al.* tested polymeric NF membranes to purify single AAs and peptides at their isoelectric point (pI) (Ingole *et al.,* 2014). Garem et al. fractionated various AAs and peptides through a charged organic-inorganic NF membrane (Katz, 1986). They also used multi-layer polyelectrolyte NF membranes to separate four neutral AAs from their mixture (Khan *et al.,* 2006). In another work, the separation of AAs and peptides was investigated using an inorganic NF membrane (Geanta *et al.,* 2013). The role of the membrane-solute charge interaction was evaluated. The influence of sorption on the elimination of AAs with thin-film composite NF membranes was studied by Shim *et al.* (2008). Moreover, Grib *et al.* (2000), studied the separation of AAs with

γ-alumina NF membranes. Timmer *et al.* (1998) reported the effect of various experimental parameters, such as pressure, salt concentration, and pH, on the experimental retention coefficient of NF membranes.

The present work was undertaken to fill the existing research gap involving industrial wastes such as sugar cane molasses (SCM) and sugar beet molasses (SBM). An attempt was also made to develop an operating condition to clarify the effect of variable factors such as temperature, pressure, and pH on identifying optimum conditions to separate AAs using NF membranes. Thus, the current work aimed to optimize the purification of AAs extracted by supercritical fluid extraction (SFE) from SCM and SBM using a commercial NF membrane, given that the AA-selectivity of the SFE process was not high, and an additional membrane process was needed to achieve more pure products. We aimed to evaluate the effects of three operating variables of temperature, pressure, and pH using the response surface methodology (RSM). The aim was to achieve a desirable membrane efficiency based on optimum conditions for the purification of AAs with NF membranes and investigate the yield value of purified AAs. Preparing an optimum operating condition is an effective and practical solution to developing and up-scaling existing methods for purifying sources with small molecular weight, such as AAs and peptides, from industrial wastes and reducing the environmental pollution.

Materials and Methods

- *Chemicals, raw materials and standard substances*

SCM and SBM with 18-25 wt% water content were provided by Hegmatan Co., Ltd. (Hamedan, Iran) and Developed Sugar Cane Co., Ltd. (Ahvaz, Iran), respectively. Hydrochloride acid, sodium hydroxide, sodium borate, HPLC-grade acetonitrile, and methanol were procured from Merck (Darmstadt, Germany). Moreover, FMOC-Cl (9-fluorenylmethyl-chloroformate), ADAM (1-amino-adamantane hydrochloride), and amino acid standards including Aspartic acid (Asp), Glutamic acid (Glu), Lysine (Lys), and Alanine (Ala), were purchased from Sigma (Milano, Italy). Some chemical properties of the AAs are summarized in Table 2. All standard solutions were kept at 4 ^{o}C and protected from light.

Table 2. Chemical property of AAs (Katz, 1968) and properties of NF membrane

AAs properties	Aspartic acid	Glutamic acid	Lysine	Alanine	NF-1
pK_{ai} at 25 ˚C	1.88 (COOH) 9.60 (NH3) 3.65 (R group)	2.19 (COOH) 9.67 (NH3) 4.25 (R group)	2.18 (COOH) 8.95 (NH3) 10.53 (R group)	2.34 (COOH) 9.69 (NH3)	
p*I* at 25 ˚C	2.77	3.22	9.74	6.00	
Molecular weight (Dalton)	133	147	146	89	
Parameters of membrane					
Active area, m^2					3.5×10^{-4}
Active layer material					Polyamide
Transmembrane pressure (MPa)					0.35-1.6
Test pressure (MPa)					1.03
Molecular weight cut off, Dalton					150-300
Requirement feed pH scope					2-11
Operational temperature (max.), ˚C					90
Water flux ($Lm^{-2}h^{-1}$)					110

- Molasses pretreatments

Firstly, AAs were extracted from molasses (SCM and SBM) using carbon dioxide supercritical fluid extraction (SFE). A Separex (Champigneulles, France) system in the SFE model was utilized in all experiments. Extractions were performed using a 100 mL volume stainless steel extraction vessel. An adjustable separator (240 mL) from Separex Co. (Champigneulles, France) was used to collect the extracted AAs. The method's suitability was evaluated by Varaee et al. for preparing and separating AAs (Lameloise and Lewandowski, 1994). The AAs were extracted from SCM and SBM under 316 and 184 bar pressure, at 50 ˚C and 43˚C, and in periods of 76 min and 76 min, respectively.

- Membrane and experimental set up

The NF membrane implemented in the present study was NF-1 (Spero Co., USA) as flat sheet samples with a polyamide active layer. The properties of the used membrane are shown in Table 2. The membrane was cut into a round shape with a diameter of 5.0 cm put into the setup to filtrate distilled water for 30 min to ensure the sealing and degassing pore of the membrane.

NF experiments were applied in a batch mode. The NF apparatus, shown in Figure 1, was equipped with a feed solution cell. Also, a magnetic ring was placed straight above a membrane support disk. Two clamps were placed on the top and at the bottom of the cell to seal and fix it.

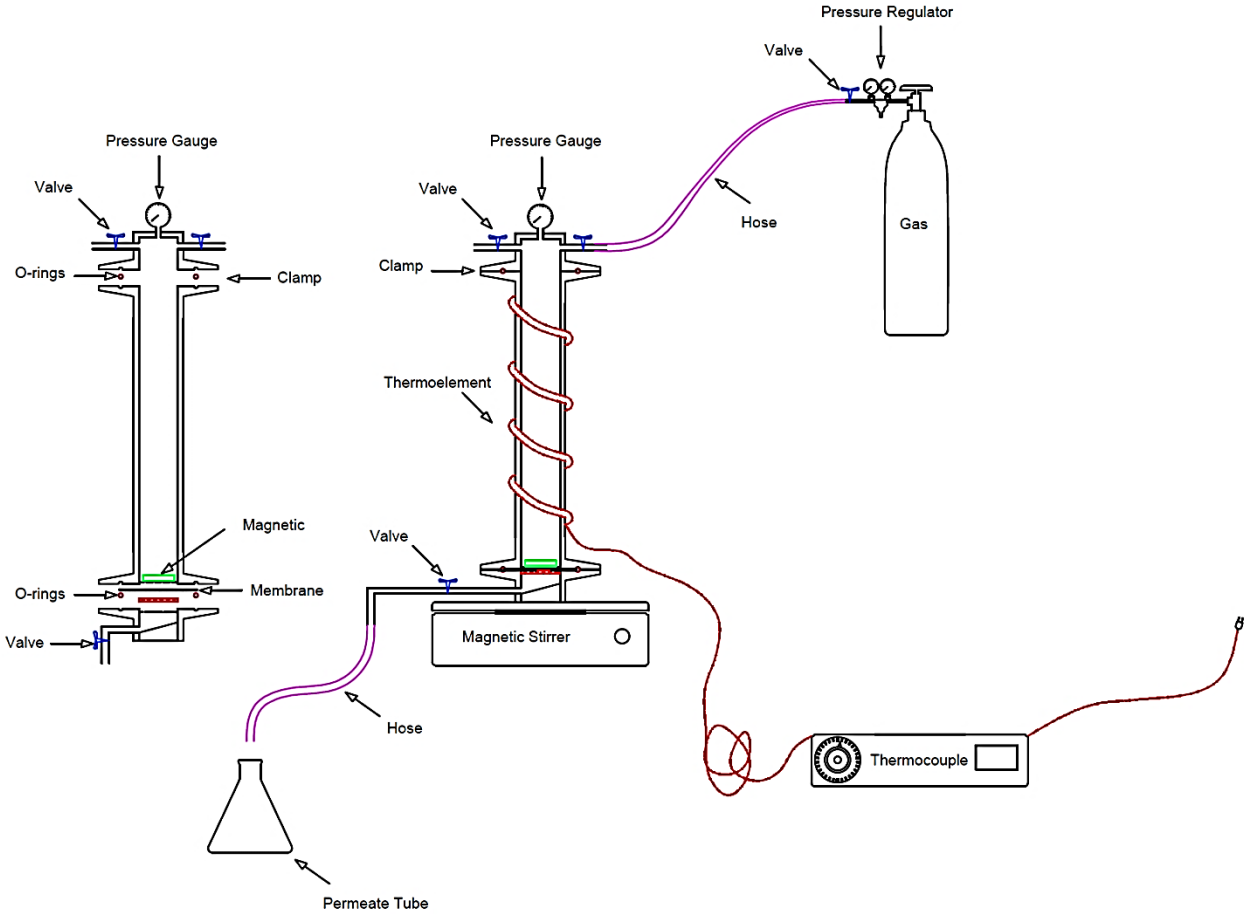


Fig. 1. Figure of NF apparatus.

The NF membrane was installed in the filtration apparatus and further washed by filtering 20 mL of distilled water through the membrane. Then, 10 mL of a feed solution of SFE-extracted SCM and SBM was added to the cell. The SFE-extracted SCM and SBM samples were prepared by mixing 1 mL of SCM or SBM and 9 mL of methanol. A pH meter (ADWA-12, Romania) was used to adjust the pH, and the temperature of the solutions was controlled with a thermocouple (TMC 101, Pooyesh, Iran) within the range of 30-50˚C. In addition, the cell was insulated with fiberglass to prevent heat loss. The filtration membrane operation was carried out by stirring the magnetic ring at about 30 rpm. A pressure of between 2 and 7 bar was applied to the apparatus by an N2 cylinder (50 l). The pH of the solutions was adjusted between 2 and 11 according to a value with HCL (1M) and NaOH (1M). For each run, first, 1 mL of permeate was discarded. Then, 7 mL of permeate was collected in test tubes for high-performance liquid chromatography (HPLC) analysis.

- ***Derivatization procedure***

AAs were derivatized (FMOC-AA) at room temperature by employing a precolumn procedure. In these circumstances, 300 µL of filtered AAs were extracted from the molasses, and 600 µL of standard AAs was added to 200 mM of borate buffer (pH 10.0). Then, 600 µL of 15 mM FMOC-CL (in acetonitrile) was added to the extracted/filtered molasses for derivatization. The reaction was stopped after 5 min by adding 600 µL of 300 mM ADAM (water-acetonitrile, 1:1, v/v). The final reaction step lasted for 1 min to constitute the FMOC-ADAM complex. Next, the sample was filtered through a 0.45 µm polytetrafluorethylene and analyzed by HPLC-UV in the wavelength of 263 nm. The total time to perform the derivatization process was 6 min (Liu *et al*., 2013).

- ***HPLC analysis***

The filtered AAs separated from the molasses were analyzed by high-performance liquid chromatography. The HPLC system containing a Spectra-Physics (San Jose, CA) was equipped with an 8700 XR ternary pump, a 20-µL Rheodyne (Cotati, CA) injection loop, an SP8792 column heater, an 8440 XR UV-Vis detector set at 263 nm, and a 4290 integrator linked via Labent to a computer. The chromatographic data were analyzed using ChromanaCH (ver. 3.6.4). For separation, a 250-×4.6 mm column packed with 5-µm particle size C_{18} (Sugelabor, Madrid, Spain) was applied at 25 °C. A mixture of sodium acetate 50Mm (pH 4.2) as eluent A and acetonitrile (60:40) as eluent B was employed at a flow rate of 1.0 mL min^{-1} as the mobile phase. All the chromatographic measurements were performed in the linear range (Liu *et al.*, 2013).

- ***Response surface methodology***

Based on the orthogonal central composite design (OCCD), RSM was applied to independently evaluate the role of the temperature, pressure, and pH variables on the separation of AAs from other components in SCM and SBM. Primarily, it led to approximating the principle effects individually. Then, a fitted second-order model was implemented to determine optimum conditions. Also, the OCCD permitted the optimization of the filtration process.

The first-order two-level design with center runs was used to estimate second-order terms since the second-order response surface model applies to system optimization. The total number of

experiments was equal to twenty.

The three independent variables included temperature (X_1) from 30 to 50 ˚C, pressure (X_2) from 2 to 7 bar, and pH (X_3) from 2 to 11 with five levels selected for each variable: -α, -1, 0, +1 and +α. The axial points were arranged at +α and -α from the center of the experimental area, which was calculated equal to ±1.5. The codes, ranges, and levels of the independent variables operated in the RSM design are classified in Table 3. For both SCM and SBM, the empirical design was performed with twenty experiential runs at six central points (Table 4). All the experiments were carried out randomly to minimize the effect of extraneous variables. A central point (0, 0, 0) was considered for all the experiments. In addition, all the experiments were implemented with three replications, and the central point was considered six times.

The experimental data were adjusted in Eq. (1) as a second-order polynomial equation comprising of the linear and interaction effects of each variable to suppose the Y variable (Martin-Orue *et al.*, 1998):

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j Z_j + \sum_{j=1}^{k} \beta_{jj} Z_j^2 + \sum \sum_{1<j}^{k} \beta_{ij} Z_i Z_j \quad (1)$$

Table 3. Independent factors, their symbols and levels for the OCCD used for SCM and SBM

Factor	Symbol	Levels				
		-α	-1	0	+1	+α
Temperature (˚C)	X_1	30	33.50	40	47	50
Pressure (bar)	X_2	2	3	4.50	6	7
pH	X_3	2	3.50	6.50	9.50	11

Table 4. Experimental values of the total peak area obtained for SCM and SGM

No.	X_1	X_2	X_3	Total peak area	
				SCM	SGM
1	40	4.50	6.50	767	1512.71
2	47	3.00	9.50	1988	1997.45
3	30	4.50	6.50	1212	576
4	40	4.50	6.50	544	1324.2
5	50	4.50	6.50	1657	1324
6	40	4.50	6.50	822.97	1456.78
7	40	4.50	6.50	786	1650.38
8	47	6	9.50	884.40	1770.78
9	40	2	6.50	1354	1465.21
10	40	7	6.50	1002	1753.35
11	33.50	6	9.50	697.90	1440.10
12	40	4.50	11	607	1879.21
13	40	4.50	6.50	544	1483.61
14	47	6	3.50	810.96	1456.3
15	33.50	6	3.50	1295.88	1878.22
16	47	3	3.50	1123	2000
17	40	4.50	6.50	945	1752.71
18	33.50	3	9.50	796.61	324
19	33.50	3	3.50	617.19	1278.34
20	40	4.50	2	324	1988.09

where Y is the response or output (yield as the total peak area), k is the number of patterns, i and j are the index numbers for patterns, and β_0, β_i, β_{ii}, and β_{ij} are the offset, linear, quadratic, and interaction terms, respectively. Moreover, Z_i and Z_j are the independent variables (temperature, pressure, and pH). Response surfaces were illustrated by the fitted model. Design-Expert (8.0.3) (Stat-Ease, Minneapolis, MN, USA) was used to design experiments, analyze data, and obtain the response surface plots.

- ***Data processing***

For the NF process, the yield values (Y) for AAs separated in the optimum filtration condition through the dead-end NF were estimated in Eq. (2) as the fraction of the original concentration of AAs remaining in the feed:

$$Y = \frac{Cp}{Cf} \times 100 \quad (2)$$

where C_f and C_p are the concentrations of AAs in the feed (mg/kg) and permeate (mg/kg), respectively (Mee *et al.*, 1979; Muir *et al.*, 2005; Munir, 2006). The retention factor of AAs in the optimum filtration condition, which considered the amount of AAs, remained in the retentate phase. The retention depended on the concentration obtained from the sample analysis and was computed from Eq. (3).

$$R\% = \frac{(Cf - Cp)}{Cf} \times 100 \quad (3)$$

The permeate flux (J_p) was calculated as Eq. (4) ($Lm^{-2}h^{-1}$):

$$J_p = \frac{Vp}{t.A_m} \quad (4)$$

where V_p is the permeate volume, t is the time needed for the permeate volume to be collected, and A_m is the effective membrane area (Mee *et al.*, 1979; Muir *et al.*, 2005; Munir, 2006).

Results and Discussion

In the present study, the NF technique separated AAs existing in an SFE extract obtained from SCM and SBM. After SFE, a mixture containing AAs and sugar was obtained, as mentioned before. SCM and SBM are mixtures of sugar (53 and 64% w/w) and non-sugar materials, i.e. AAs (19 and 10% w/w), water (16.5 and 20% w/w) and ash (11.5 and 8% w/w), respectively (Rearik and Mckey, 1996).

NF membrane with the molecular weight cut-off range of 150-300 Daltons was selected because the molecular weight of AAs is in the range of 89-147 Daltons. Therefore, after filtration, all sugar (sucrose, fructose, glucose, and raffinose with molecular weights of 342, 180, 180, and 504 Daltons, respectively) and colloid components (pectin and starch with molecular weights of 190 and 734 Daltons, respectively) remained in retentate, and most AAs were collected and separated in permeate. Therefore, the main aim of NF was to purify AAs from other components of SCM and SBM, especially sugar and colloids, based on the molecular weight of these components. Some important variables can affect the efficiency of the NF process: temperature, pressure, and pH. Optimizing such parameters was examined using RSM. The results were discussed in the following sections.

- ***HPLC analysis of AAs from SFE extracts for SCM and SBM***

SCM and SBM contain comparatively eighteen AAs (Rodríguez *et al.*, 2013; Saidi *et al.*, 2013; Saric *et al.*, 2016). However, Asp, Glu, Ala, and Lys were only selected for this study. Asp and Glu were chosen based on prevailing SCM and SBM (Saidi *et al.*, 2013) and as both are the most abundant neurotransmitters in the central nervous system (Sato *et al.*, 2004; Sharma, Chellam, 2005). Although Ala

and Lys are inconsiderable AAs in SCM and SBM (Saidi *et al.,* 2013), Ala collaborates in metabolizing glucose for energy and has a notable task in transferring nitrogen from tissues to the liver, inducing the balance of glucose and nitrogen in the body. Lys is an essential AA and is the first limiting AA for mammalians (Shim *et al.,* 2000; Tanaka *et al.,* 2017). Samples consisting of 20 μL of the SFE extract for SCM and SBM were derived using the FMOC method and analyzed using HPLC as control samples to estimate the AAs mentioned above. AAs in the samples were detected by comparing the relative retention times of the AAs extracts with the standards. The HPLC results of the SFE extracts for SCM and SBM are presented in Table 5. A mixture containing AAs and sugar was obtained after SFE extraction. Consequently, NF was carried out to separate AAs from sugar and colloids in SCM and SBM.

- ***Statistical analysis***

The values obtained for the total peak area of the analyzed AAs for each experiment of the OCCD design are summarized in Table 4. Responses (R_{SCM} and R_{SBM}) showed the total peak area. The results of analysis of variance (ANOVA) for SCM and SBM are listed in Tables 6 and 7, respectively. ANOVA was accomplished to verify the property of the response surface model and examine whether the effect of the process parameters and their interactions was statistically significant on the response (α=0.05).

At first, the regressions of the models indicated that the NF process was significant. Then, ANOVA was performed with an F-test (lack of fit) for verification. The "lack of fit" was not significant ($p>0.05$) for both SCM and SBM molasses. The F-values of SCM and SBM were 21.74 and 24.28, respectively, and both were significant. F-ratio is a tool to determine which parameter significantly affects the purification of AAs from sugar by the membrane. A larger F-ratio has a more significant effect on the separation. The F-value in the ANOVA test determines p-values that present significant factors ($p<0.05$). As shown in Tables 6 and 7, only the significant parameters were considered to constitute the model. The pH did not significantly affect SCM, whereas the quadric pressure factor did not have any significant effect on SBM. The pH did not have any significant effect on SCM since components with a tampon or buffer potentials, such as colloids, gums, starch (29-38%), fructose, and glucose (9-18%) in SCM, are more than those in SBM (colloids, gums, starch 19-23% and fructose and glucose 1-3%). Tampon or buffer can regulate and balance pH at a nearly constant value (Thuy *et al.,* 2014; Timmer *et al.,* 1998).

The response equation was adapted to the experimental data, comprising the R^2-value of was 0.9514, 0.9562 for SCM and SBM, respectively. Moreover, the adjusted R^2 value was 0.9076 and 0.9169 for SCM and SBM, respectively, certainly within acceptable limits of $R^2 \geq 0.9$. The total peak area in the both cases was chosen as the optimization criterion.

The mathematical model for the experimental design was expressed as Eqs. (5) and (6) for SCM and SBM, respectively.

$$R_{SCM} = 7.728\times10^{3} - 4.447\times10^{2}X_1 + 5.633\times10^{2}X_2 - 23.19\,X_1X_2 + 8.765\,X_1X_3 - 40.54\,X_2X_3 + 6.464x_1^2 + 62.39x_2^2 - 15.93\,x_3^2 \quad (5)$$

$$R_{SBM} = -9.7\times10^{3} + 5.487\times10^{2}\,X_1 + 9.408\times10^{2}X_2 - 8.420\times10^{2}\,X_3 - 28.88\,X_1X_2 + 11.01\,X_1X_3 + 21.52\,X_2X_3 - 5.609\,x_1^2 + 20.87\,x_3^2 \quad (6)$$

Table 5. Concentrations of AAs from SFE extracted (C_f) and permeate separation NF (C_P), retention and yield values at the optimum conditions (%) from SCM and SBM

	Asp	Glu	Ala	Lys	Mean value
SFE extract (mg/kg)(C_f)					
SCM	150	162	133	98	135.75
SBM	159	152	169	121	150.25
Permeate NF (mg/kg)(C_p)					
SCM	99	111	112	80	100.5
SBM	100	100	117	84	100.25
Retention (%)					
SCM	34	31.5	16	18	24.87
SBM	37	34	31	31	33.25
Yield Values (%)					
SCM	66	68.5	84	82	75.13
SBM	63	66	69	69	67.75

Table 6. ANOVA for SCM

Source	Sum of square	df[a]	Mean square	*F*-Ratio	*p*- Value	Effect
Model	2.946E+006	9	3.273E+005	21.74	<0.0001	significant
X_1-Tempreture	3.411E+005	1	3.411E+005	22.66	<0.0008	
X_2-Pressure	1.489E+005	1	1.489E+005	9.89	0.0104	
X_3-pH	71559.66	1	71559.66	4.75	0.0542	Not significant
X_1X_2	4.978E+005	1	4.978E+005	33.07	0.0002	
X_2X_3	3.077E+005	1	3.077E+005	20.44	0.0011	
X_1X_3	2.302E+005	1	2.302E+005	15.29	0.0029	
X_1^2	8.359E+005	1	8.359E+005	55.53	<0.0001	
X_2^2	3.042E+005	1	3.042E+005	20.21	0.0012	
X_3^2	2.079E+005	1	2.079E+005	13.81	0.0040	
Residual	1.505E+005	10	15052.56			
Lack of fit	22100.93	5	4420.19	0.17	0.9620	Not significant
Pure Error	1.284E+005	5	25684.93			
Cor Total	3.096E+006	19				
R^2				0.9514		
R^2 (adj)				0.9076		

[a] degrees of freedom.

Table 7. ANOVA for SBM

Source	Sum of square	df[a]	Mean square	*F*-Ratio	*p*- Value	Effect
Model	3.441E+006	9	3.823E+005	24.28	<0.0001	significant
X_1-Tempreture	9.379E+005	1	9.379E+005	59.57	<0.0001	
X_2-Pressure	1.516E+005	1	1.516E+005	9.63	0.0112	
X_3-pH	1.228E+005	1	1.228E+005	7.80	0.0190	
X_1X_2	7.727E+005	1	7.727E+005	49.08	<0.0001	
X_2X_3	86786.95	1	86786.95	5.51	0.0408	
X_1X_3	3.631E+005	1	3.631E+005	23.06	0.0007	
X_1^2	6.295E+005	1	6.295E+005	39.98	<0.0001	
X_2^2	19331.32	1	19331.32	1.23	0.2938	Not significant
X_3^2	3.571E+005	1	3.571E+005	22.68	0.0008	
Residual	1.575E+005	10	15745.60			
Lack of fit	43197.37	5	8639.47	0.38	0.8453	Not significant
Pure Error	1.143E+005	5	22851.73			
Cor Total	3.598E+006	19				
R^2				0.9562		
R^2 (adj)				0.9169		

[a] degrees of freedom.

where X_1, X_2, and X_3 are temperature, pressure, and pH. The response surface plots explained the design and OCCD data. Figure 2 depicts the goodness-of-fit of the empirical model for SCM (a) and SBM (b). The horizontal axis exhibits the predicted peak area where peak areas are obtained, and the vertical axis displays an experimental peak area obtained during examinations. Fig. 2a and Fig. 2b indicate the short distance between the predicted peak area and the experimental peak area.

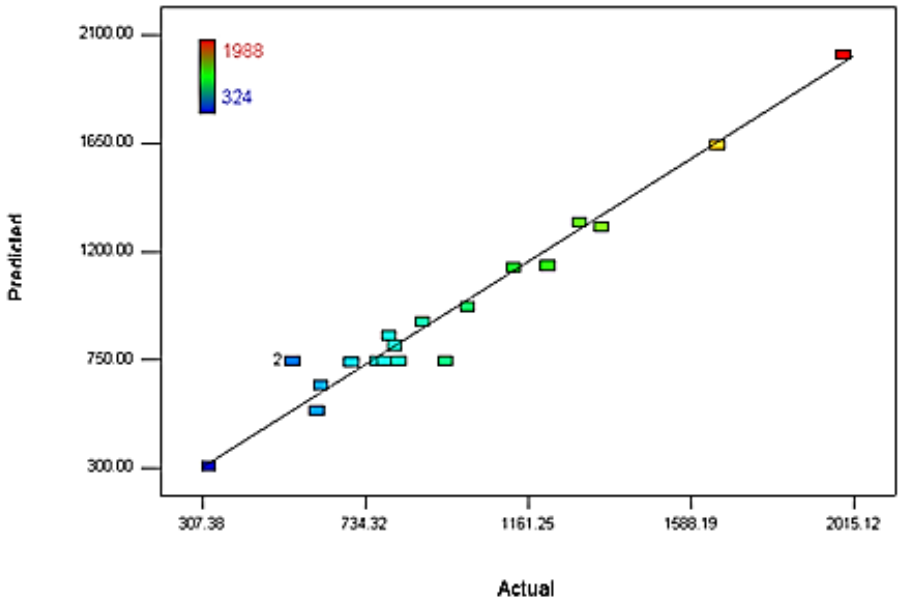


Fig 2a

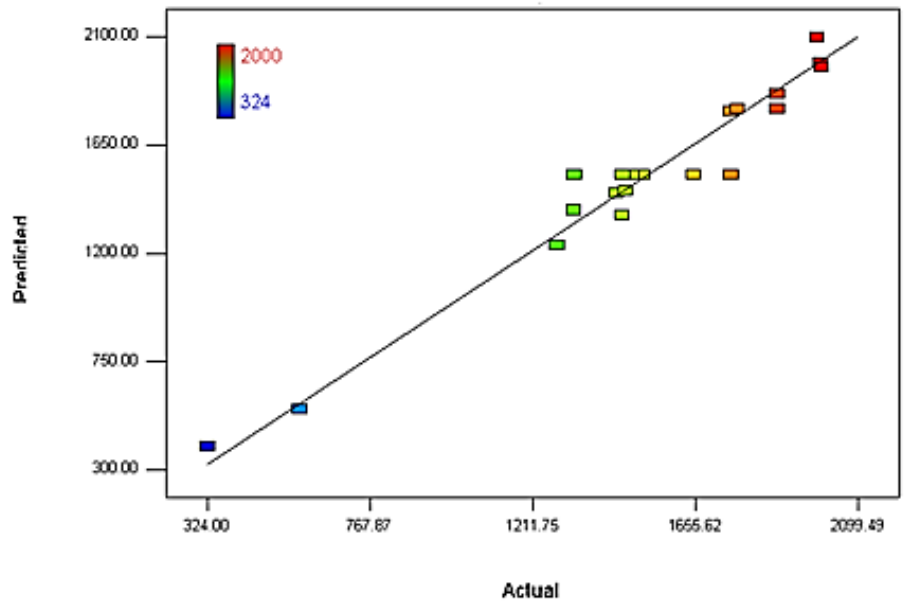


Fig 2b

Fig. 2. Goodness-of-fit of the empirical model with predicted for SCM (a) and SBM (b).

- ***Evaluation of factors that influence of NF system***

Wide ranges of temperature, pressure, and pH were selected because AAs are sensitive to temperature and pressure variation and might be denaturized (Katz, 1968; Khan *et al.,* 2006; Tsuru *et al.,* 1994; Valli *et al.,* 2012; Varaee *et al.,* 2019). In addition, pH (or the pI value) can be utilized to investigate AAs' global basic or acidic character. As stated above, Asp, Glu, Ala, and Lys had acidic, neutral, and basic pI values, respectively (Valli *et al.,* 2012; Varaee *et al.,* 2019). Figs. 3a-f and 4a-f show the relationship between the perceptive and response variables in a three-dimensional representation of the response surface and two-dimensional contour plots. The desirability function for detecting optimum conditions was measured based on the maximization of total peak areas for the four responses of AAs. Figures 3a-b and 4a-b represent the interaction between pressure and temperature in SCM and SBM, respectively. As shown in Fig. 3a-b and Fig. 4a-b, the temperature was 47˚C for SCM purification, the same as for SBM separation. However, raising the purification temperature in the SBM samples from 43 to 47 ˚C improved the purification efficiency significantly. The high temperature had a great effect on the purification of AAs from both molasses since the increased temperature in the liquid fluid led to decreased viscosity and increased the purification rate of AAs (Vyas and Ray, 2015).

Moreover, excessive temperature caused the denaturation and decomposition of AAs and revealed changes in the efficiency of the NF membrane permeability of AAs solutes. This result agrees with other previous research (Tsuru *et al.,* 1994; Valli *et al.,* 2012; Varaee *et al.,* 2019; Wang *et al.,* 2017). Moreover, lower pressure (3 bar) significantly impacted the purification of AAs from both molasses since components had a greater permeability at lower pressure, and AAs were not degraded. Likewise, the results are in line with other published results (Khan *et al.,* 2006; Varaee *et al.,* 2019; Wang *et al.,* 2009).

The effects of the pH-temperature interaction are shown in Figs—3c-d and 4c-d for SCM and SBM, respectively. Although pH alone does not significantly affect SCM, its interaction with temperature and pressure significantly influences the separation of AAs. Figures 3c-d and 4c-d demonstrate that the basic pH (between 9.3 and 9.5) had an essential role in separating AAs from both molasses, as most AAs were detected to be labile at the acidic pH and more persistent at a highly basic pH (Valli *et al.,* 2012; Wang *et al.,* 1995). The result is in line with other reproduced results (Katz, 1968). Furthermore, the experiments were performed at temperatures in the range of 30-50˚C, and the appropriate temperature was calculated at 47 ˚C for both molasses. This result is in agreement with other published results (Tsuru *et al.,* 1994; Valli *et al.,* 2012; Varaee *et al.,* 2019; Vyas and Ray, 2015; Wang *et al.,* 2017).

The influence of the pressure-pH interaction is presented in Figs. (3e-f) and (4e-f) for SCM and SBM, respectively. Pressure and pH had a significant impact on the purification function simultaneously (Wang *et al.,* 1997). The tests were performed at diverse pressure in the range of 2-7 bar. The results indicated that the purification of AAs from both molasses was enhanced at lower pressure (3 bar). The result conforms to other published results (Khan *et al.,* 2006; Valli *et al.,* 2012; Wang *et al.,* 2002). However, the experiments were carried out at pH values between 2 and 11. The purification

performance for both molasses increased considerably in the basic pH (9.3-9.5). The results revealed that the acidic pH led to the degradation of AAs (Valli *et al.*, 2012; Wang *et al.*, 1995). It can be concluded that the best conditions, including temperature, pressure, and pH for the purification of AAs using NF, were the same for both SCM and SBM. It is evident that SCM and SBM had nearly similar compositions; (Thuy *et al.*, 2014; Timmer *et al.*, 1998) thus, they needed the same purification condition.

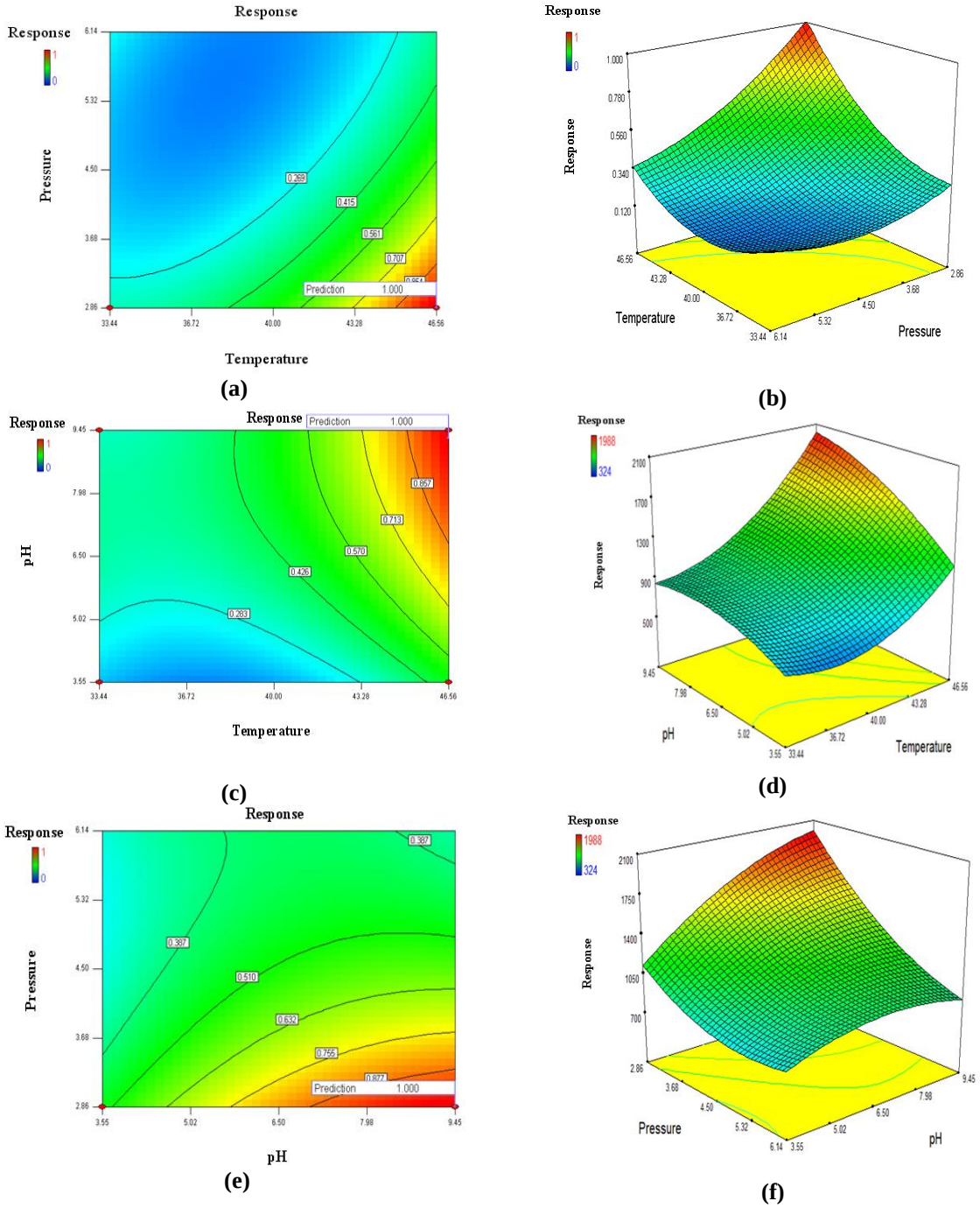


Fig. 3. Response surfaces and contour plots for: (a, b) Pressure (bar) vs. Temperature (˚C) in pH of 9.3; (c, d) pH vs. Temperature (˚C) at 3 bar; (e, f) Pressure (bar) vs. pH at 47 ˚C for SCM.

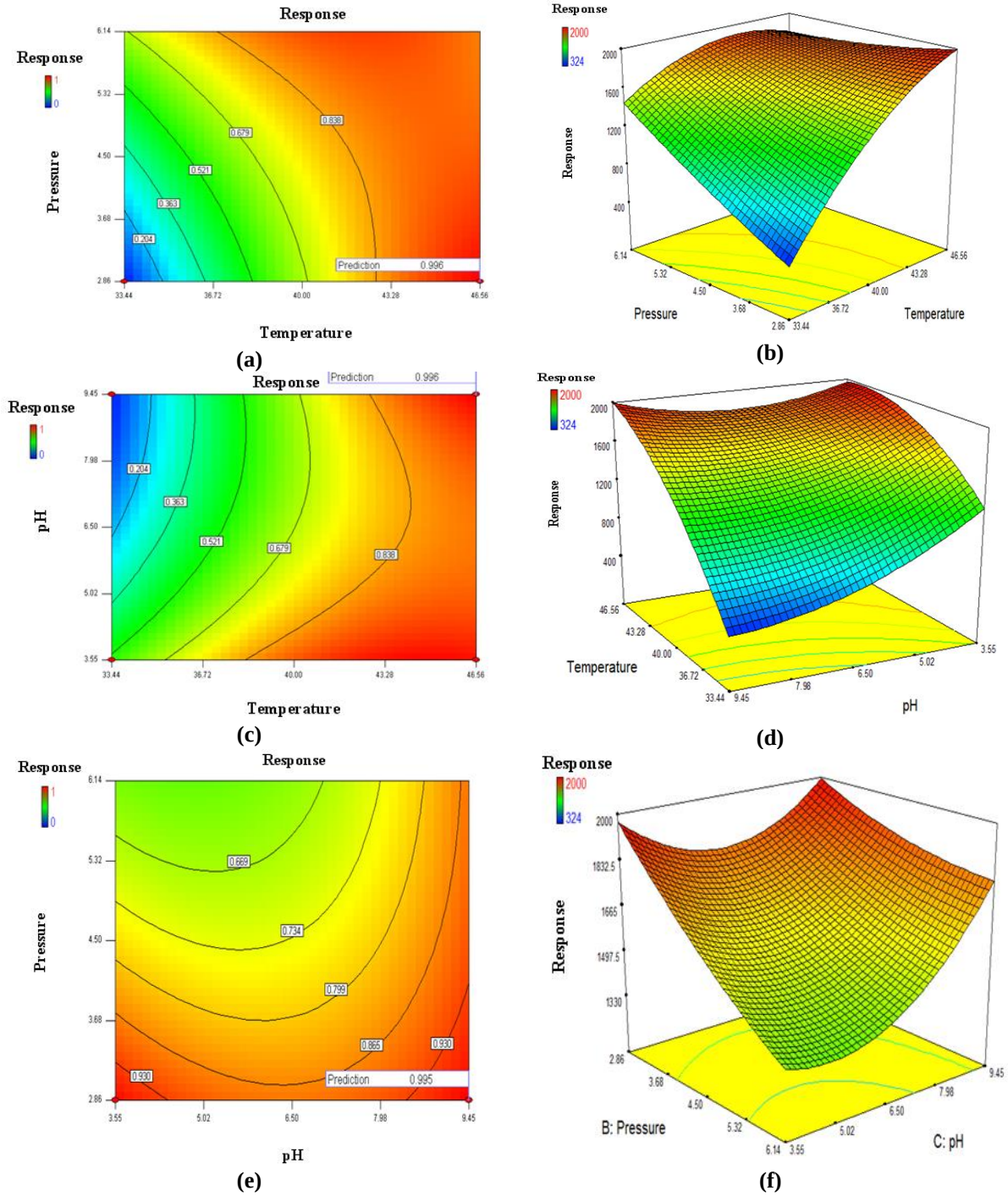


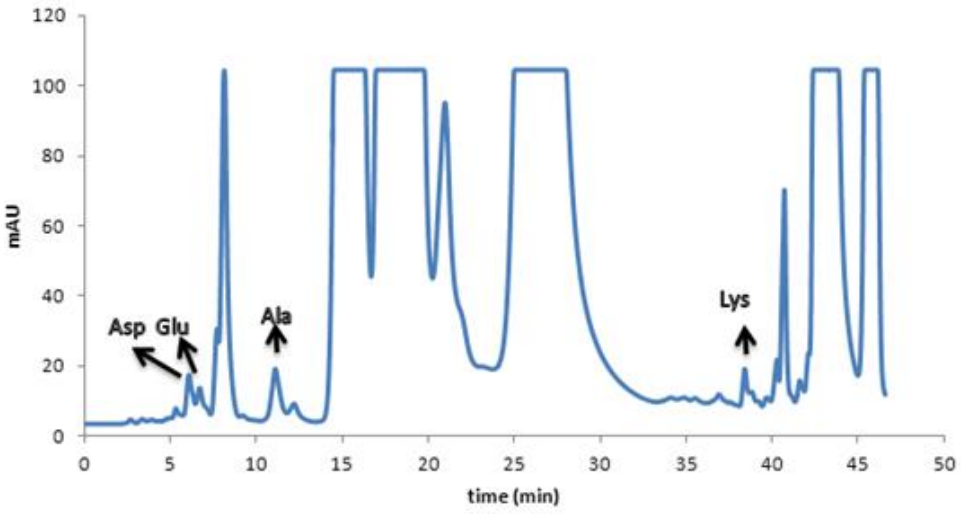
Fig. 4. Response surfaces and contour plots for: (a, b) Pressure (bar) vs. Temperature (˚C) in pH of 9.5; (c, d) pH vs. Temperature (˚C) at 3 bar; (e, f) Pressure (bar) vs. pH at 47 ˚C for SBM.

- ***Determination of optimal NF separation***
- ***In this research, the optimum*** condition for NF purification of SCM within the selected parameter values was obtained at 47˚C temperature, 3 bar pressure, and 9.3 pH, with the greatest total peak area of 1994. Thuy *et al.* (2014) evaluated the optimization of NF to obtain fish protein isolates from byproducts at the pressure of 11 bar and temperature of 45 ˚C. Furthermore, the optimum condition for SBM was obtained at the temperature of

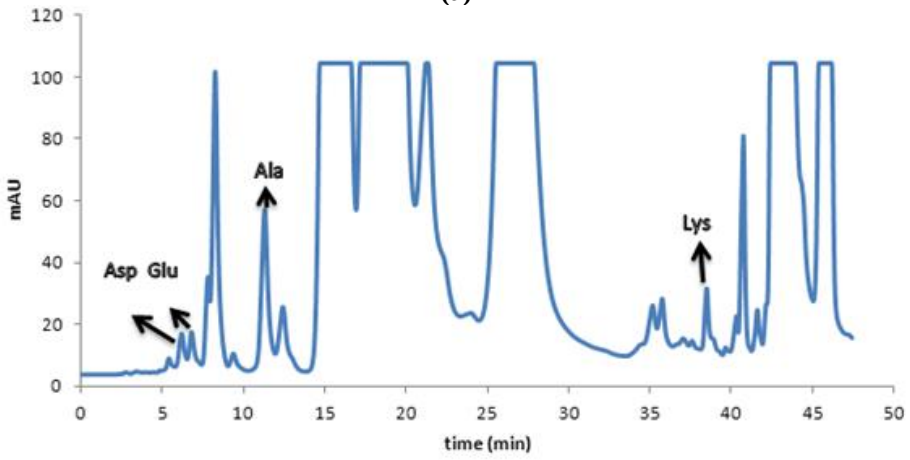
47˚C, 3 bar pressure, and 9.5 pH, with the greatest total peak area of 1993.

It should be noted that both molasses needed similar situations for purification of AAs by NF since they had approximately similar compounds and nature (Thuy *et al.*, 2014; Timmer *et al.*, 1998). Gyura *et al.* (2002) investigated the separation of non-sucrose compounds from the SB syrup using ultra and NF polymer membrane. Their results moderately agree with our results concerning the temperature and pressure effects.

The HPLC chromatograms and configuration of the optimum SFE and SFE-NF purification for SCM and SBM are presented in Figs. 5a-b and Figs. 6a-b, respectively. By comparing the chromatograms of SFE and SFE-NF purification for SCM and SBM, it was indicated that the SFE-NF peak was sharper and more evident than the SFE peak. The support experiment was implemented at optimum working conditions.



(a)



(b)

Fig. 5. The HPLC-UV chromatograms of optimized the SFE separation at 316 bar, 76 min and 50 ˚C for SCM (a) and at 147 bar, 76 min and 43 ˚C for SBM (b).

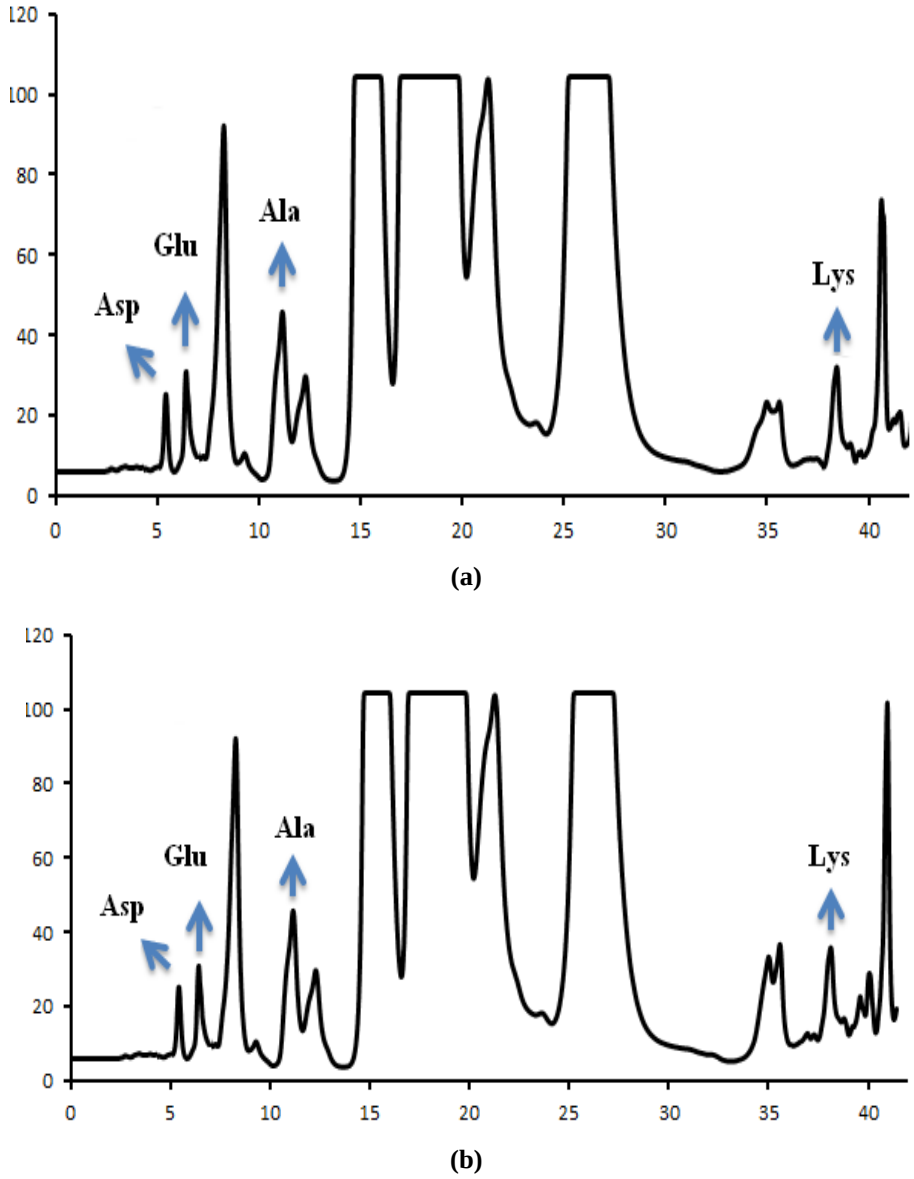


Fig. 6. The HPLC-UV chromatograms of optimized the SFE-NF separation at 47˚C, 3 bar and pH of 9.3 for SCM (a) and at 47 ˚C, 3 bar and pH of 9.5 for SBM (b).

- ***Evaluation of separation efficiency***

The concentration, yield value, and retention of AAs for SCM and SBM are displayed in Table 5. As mentioned before, the yield value and retention were calculated according to Eqs. (2) and (3), respectively.

According to the results of the yield value, the recovery and concentration of AAs in SCM were higher than those in SBM, meaning that the yield value of purification for the four AAs was 75% and 67% in SCM and SBM, respectively. The SCM had a lower Brix (75), leading to a reduction in its viscosity. However, SBM had a higher Brix (82), causing an increase in its viscosity. Therefore, some AAs might have adhered to other components in the viscous solution, hence their reduced purification (Wilkes *et al.*, 1971; Wu *et al.*, 2017).

The reasons for differences in the amount of AAs purified for SCM and SBM are explained as follows: as mentioned before, pH was not individually significant for SCM samples because of having more tampon compounds compared to SBM samples. However, pH interactions with other parameters, including temperature and pressure, were significant and substantially affected the purification performance of AAs in SCM. Meanwhile, the effect of pH, both individually and the combined effects of the other two variables, were regarded as significant on the purification efficiency of AAs in SBM. Furthermore, the molecular weight can influence purified AAs in SCM and SBM. The lower purification potential of Asp and Glu in SCM and SBM was with respect to their high molecular weight (133, 147 Dalton as Table 2); moreover, their pI was acidic while the observed optimum pH was basic (9.3, 9.5). These features might affect the decreased purification of Asp and Glu in SCM and SBM. However, Ala and Lys had neutral and basic pI. Moreover, Ala had a lower molecular weight (86), causing their purification to be more efficient (Geanta *et al.,* 2013; Gómez-Díaz *et al.,* 2009; Valli *et al.,* 2012; Varaee *et al.,* 2019; Wu *et al.,* 2014; York *et al.,* 2017). The reason is that the molecular weight of other components such as sugar and colloids was higher; consequently, these components were kept in the retentate, and most AAs were collected and purified in permeate (Weiss *et al.,* 2018).

Due to the reasons mentioned above, the purification of AAs was higher for SCM than for SBM. The results demonstrated that the NF function displayed properly purified AAs from sugar in both molasses. The results are close to those published elsewhere. The NF recovery of sericin from silk processing waste performed by Wu and Zhang (2014) showed a recovery of about 75%.

The retention factor demonstrates the number of components that remained in the retention phase. This study intended to purify AAs from other components. It can be observed that the retention of AAs from both molasses was expectedly negligible. AAs had low molecular weight and mostly passed through a nano-filter. However, some of them might have remained in the retention phase due to adherence of AAs to other components; the pH of purification might be effective for the purification of AAs. Based on Table 5, the concentration of AAs in the retention phase was 24.87 and 33.25 for SCM and SBM, respectively. The higher retention for SB compared to SCM was related to its higher Brix (82) and higher viscosity. Thus, the purification of AAs from other components was complicated (Wilkes and Jenning, 1971; El-Geddawy *et al.,* 2012). The obtained results are almost similar to results published elsewhere.

The permeate flux (J_p) was obtained using Eq. (4). In the optimum condition, the permeate volume and time were 9 mL and 23 min for SCM and 8 mL and 30 min for SB, respectively. Furthermore, the effective membrane area was 3.5×10^{-4} m^2. Thus, the permeate flux in the optimum condition was obtained 70 and 45 $Lm^{-2}h^{-1}$ for SCM and SBM, respectively. Although both molasses had an approximately similar optimum condition, the declined flux for SBM was due to its Brix. As a result, the SBM solution could be filtered through membranes over a more extended period than the SCM solution, which was fluent and had a lower Brix (Katz, 1968; Eckera *et al.,* 2012). Thus, the permeate flux for SBM was less than that for SCM.

Conclusion

In this study, NF purification was performed for the first time, with significant results concerning the purification of AAs from other components such as sugar and colloids for SCM and SBM. Furthermore, the yield value of the separated AAs was evaluated. The optimum conditions for purifying AAs with the highest total peak area were determined. Although both molasses had an approximately similar optimum purification condition, the recovery and yield of AAs were higher in SCM than in SBM due to the lower Brix and viscosity of SCM. In addition, the flux of SBM was less than SCM as SBM had a greater Brix that increased its viscosity. The optimum operating condition reported here corresponded to a laboratory scale-free purification of AAs from two types of molasses. In conclusion, the mentioned optimized method with NF membranes can be proposed as an efficient techno-economic method for purifying constituents with small molecular weight, such as AAs and peptides, from industrial wastes to eliminate environmental pollution and improve industrial profitability.

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