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Application of Response Surface Methodology (RSM) for the Optimization of Supercritical CO_2 Extraction of Elaeagnus Protein: A Comparison Study

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ABSTRACT: Elaeagnus contains a large amount of protein which can be used as a functional ingredient. In this study, Elaeagnus protein was extracted using supercritical carbon dioxide method as an effective method. Central composite design was utilized to identify the most effective operating conditions including supercritical temperature, supercritical pressure, time and solvent ratio of carbon dioxide and methanol in supercritical carbon dioxide extraction. According to the analysis of variance results, the optimum operating condition for the extraction of protein using supercritical CO_2 was as following:150.85 bar, 51.06°C, 59.80 min and 717.08 µL methanol, leading to extracting of 47.53 mg protein per 10 g the Elaeagnus dried sample. Among the operating conditions, supercritical pressure had the highest effect on amount of protein. Compared to conventional extraction method, the amount of extracted protein was 25% higher. Therefore, the results of this study suggested that the supercritical carbon dioxide extraction can be used as an effective method for protein extraction from plants.

Keywords: Elaeagnus angustifolia, Essential Amino Acids, Gel Electrophoresis, Supercritical CO_2 Extraction.

Introduction

Protein is one of the most important nutrients for human body that can be obtained from animal or plant sources. In recent years, much has been stressed on replacing plant proteins. Most fruits have the essential amino acids in their protein structure that are essential for human growth. Proteins are complex compounds that are composed of amino acid units. Proteins contain 20 amino acids or more in various tissues of the body. Of these, 10 amino acids must be present in the human diet because they are not made in the body. These essential amino acids include Leucine, Isoleucine, Lysine, Methionine, Phenylalanine, Threonine, Tryptophan, Valine, Histidine, and Arginine (Wen *et al.*, 2020; Kim *et al.*, 2020; Preece *et al.*, 2017). *Elaeagnus angustifolia* is a family of *Elaeagnaceae*. The Indigenous plant is native to North Asia and Europe. In Iran, it can be found in the provinces of Azerbaijan, Kurdistan, Chaharmahal and Bakhtiari, Hamedan, Isfahan, Tehran, and Khorasan. Elaeagnus is one of the fruits

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that contain high amount of protein and essential amino acids including Aspartic acid, Threonine, Serine, Phenylalanine, Threonine, Tryptophan, Valine, Histidine, Arginine, Methionine, Leucine, Lysine, Isoleucine, Alanine, Glycine, Proline and Glutamine., which can be a good substitute for animal protein and other sources (Hamidpour *et al.*, 2017). Different methods have been used to extract proteins from plant sources such as solvent extraction, ultrasound, microwave extraction, etc. Nowadays, the focus of recent studies has been on new methods of extraction with appropriate operating conditions. Supercritical carbon dioxide extraction as a novel method is a fast and effective approach to extract bioactive compounds from plant tissues with different advantages such as the non-toxic and non-flammable benefits, low price, easy accessibility, chemical stability, non-reaction with the sample and easy separation from the sample as well as the use of low heat (which has no thermal degradation on the protein structure), low extraction time and high selective power. In this type of extraction, the sample crosses along with the supercritical carbon dioxide in the extraction tank and then, the supercritical carbon dioxide is extracted in the separating tank and the extracted sample as well as extract are also separated by pressure change (Xiong *et al.*, 2020) Supercritical carbon dioxide method was used to extract different compounds such as, papaya fruit seed compounds such as fatty acids (Pedro *et al.*, 2016), avocado fruit oil (Corzzini *et al.*, 2017), ginger oil (Michele *et al.*, 2013), and allicin and oleoresin from garlic (Valle *et al.*, 2012).

To the best of our knowledge there is no study on supercritical carbon dioxide extraction of protein from dried Elaegnus using response surface methodology. Therefore, this study aims to optimize the protein extraction from the protein of dried fruits using supercritical carbon dioxide method and to identify the amino acid composition of the extracted protein. Experimental design with central composite design (CCD) was utilized to identify the most effective operating conditions in the extraction. Traditional protein extraction method with buffer was applied as a reference method for the comparison.

Materials and Methods

- *Materials*

The dried fruit used in this study was obtained from Chaharmahal and Bakhtiari province of Iran in autumn 2017. The fruits were rinsed, washed, milled, and then dried at 40°C for 72 h. Finally, dried powder of Elaeagnus was stored at 4°C until testing. All chemicals used in this study including acrylamide, chloric acid, glycine, glycerin, bromophenol blue, ammonium per sulfate, tetramethyl ethylenediamin, manol, glacial acetic acid, coomassie - brilliant blue R-250, coomassie-brilliant 250 and orthophosphoric acid (85%wt) were obtained from Merck Chemical Company, Germany.

- *Buffer Extraction*

The powdered fruit was crushed and then dried using some liquid nitrogen and 10 g of dried powder combined with 5 mL tris Hcl 0.2 M (pH = 8) in Falcone tubes and was then centrifuged for 20 min at 10000 *g*. The supernatant was finally removed using a sampler and transferred to a clean micro-tube until analysis.

- *Extraction using supercritical carbon dioxide*

As shown in Figure 1, the supercritical carbon dioxide is transferred into the liquid at specified dynamic times by

passing through the liquid refrigeration system by the pressure control pump until it reaches the desired pressure. Following that, it is pumped along with the aqueous solvent of methanol to the extraction column containing 10 g of the sample at a predetermined temperature. The desired solvents and the sample are then transferred to the separating chamber. At this stage, the pressure drops results in separation of the sample and solvent. Then the recovery pump return carbon dioxide to the process and on the other hand, the sample throw out of the collection section then collected in the syringe and transferred to the penicillin glass (Xiong *et al*,, 2020).

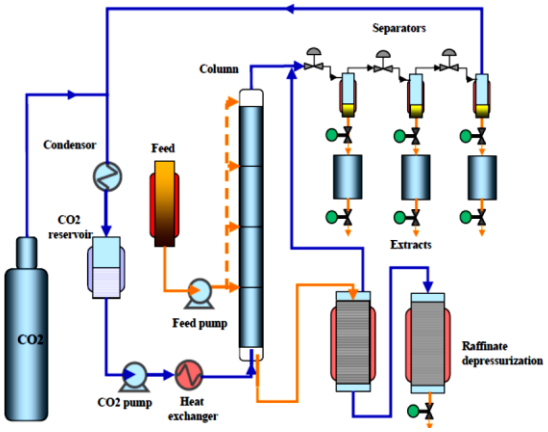

Fig. 1. Supercritical CO_2 extraction schematic

- Characterization

- High performance liquid chromatography (HPLC)

HPLC was employed to identify amino acids (Honarvar *et al*, 2019). Two derivatization steps were first implemented. In the first derivatization, the first solution (water, methanol and tri-methylamine with ratio of 2:2:1) was mixed with 10 ml of sample and then vortexed and finally dried using vacuum oven. In the second derivatization, the second solution (water, methanol, tri-methylamine and isothiocyanate with ratio 7:1:1:1) was mixed with 10 ml of sample and then vortexed. Finally, the solution was dried using a liquid nitrogen injection. The sample was mixed with solvent buffer ~~a~~ (sodium acetate, tri ethylamine, and acetone nitrile) and centrifuged to disperse the sample. Finally, the sample was injected using a Hamilton syringe.

- SDS-PAGE

In this method, as shown in Figure 2, the extracted sample is injected into the wells prepared within the gel between the glass blades, then the glass blades are inserted into the tank containing buffer electrolyte which is connected to the power of 100 V and 48 A for 90 min. Accordingly, based on different molecular weights, the protein bands sample are separated into gels.

Fig. 2. Sodium dodecyl sulfate electrophoresis (SDS-PAGE) schematic.

- Experimental design

In this research, Design Expert software

(version 11) was used to design experiments, analyze results and plot curves using response surface methodology (RSM), using central composite method at 5 levels with characteristic codes (+ α, +1, 0, -1 ,-α) . According to preliminary studies and experiments, four factors of temperature in Celsius (ºC), pressure in bar (bar), time in minutes (min) and volume of methanol were chosen as the independent variables and amount of protein extracted was considered as the response (R1). Based on the RSM design and considering 4 factors at five levels, 18 tests with α equal to 1 were proposed by the software (Biligic *et al*, 2019; Kosterzewa *et al*, 2020). The conditions and design of the proposed experiments are presented in Table 1.

Results and Discussion

- *Extraction efficiency*

In order to compare the extraction efficiency of SCF method with that of conventional method, two experiments with the same operating condition (i.e., temperature of 45ºC and time of 30 min) were carried out (Table 2). The amount of protein extracted via supercritical carbon dioxide extraction method was 44.6 mg/g while 35.6 mg/g by using conventional buffered extraction method showing higher efficiency of extraction using supercritical carbon dioxide method. Supercritical carbon dioxide has high diffusivity, low viscosity, and high density, which allows it to penetrate the dried sample more deeply and extract more protein than conventional method. Therefore, the results showed that the supercritical CO2 extraction method is a better method compared to the buffer method owing to its higher protein extraction efficiency.

Table 1. Design of the experiments by RSM method based on the actual values of the variables and the extracted protein as the response

Row	Temperature (℃)	Pressure (bar)	Time (min)	methanol content (µl)	Protein (m gr/gr)
1	50	210	60	900	40.95
2	45	165	30	600	40.10
3	[50]	[210]	[40]	900	42.30
4	45	165	50	600	47.53
5	50	210	20	900	42.50
6	40	210	40	900	36.75
7	50	300	40	900	41.04
8	[50]	[210]	[40]	1500	43.25
9	60	210	40	900	31.04
10	45	255	50	1200	31.83
11	[50]	[210]	[40]	900	42.30
12	55	165	50	1200	37.83
13	55	255	30	600	38.10
14	55	255	50	600	38.66
15	45	255	30	1200	44.60
16	50	120	40	900	41.04
17	[50]	[210]	[40]	300	38.75
18	55	165	30	1200	37.13

Table 2. Comparison between the SFC method and the conventional method

Method	Temperature (^{0}C)	Time (min)	Extraction (mg/g)
Conventional buffered extraction	45	30	35.6
Supercritical CO_2 extraction	45	30	44.6

In the present study, the supercritical carbon dioxide method was employed to extract the dried fruit protein. Variables of temperature, pressure, time, and volume of methanol-assisted solvent had different effects on the protein extraction under different operating conditions. Different operating conditions were optimized using RSM. The ability of model to justify the experimental data was studied using ANOVA at a confidence level of 95%. The results of the analysis of variance obtained by Design Expert software are presented in Table 3. The significance of the quadratic model was evaluated by two measures of Fisher's ratios (F test) and model accuracy test (LOF). In the proposed model for the extracted protein content, the experimental F value calculated for the model (832.97) is greater than its critical value (2.64) at 95% confidence level, which confirms the significance of the model. Also, the **P** value for the model is less than 0.05, which indicates that the model is suitable for predicting the experimental results.

Drawing the curve of changes in the values predicted by the model in terms of actual values (Figure 3), a high correlation coefficient (99.97%) was obtained, which indicates that the model is satisfactory.

Table 3. Analysis of variance for evaluating the proposed model by RSM

Source	sum of squares	Degrees of freedom	average of squares	P-value
Model	284.74	14	20.34	$< 0.0001^{**}$
The linear effect of temperature (A)	87.75	1	87.75	$< 0.0001^{**}$
Linear pressure effect (B)	20.17	1	20.17	$< 0.0001^{**}$
Linear effect of time (C)	0.07550	1	0.07550	0.115^{*}
Linear effect of solvent volume (The interaction of temperature and pressure D)	14.11	1	14.11	0.0002^{*}
(A×B)	30.25	1	30.25	$< 0.0001^{**}$
The interaction of temperature and time (A×C)	5.44	1	5.44	$< 0.0007^{**}$
The interaction of temperature and volume of solvent (A×D)	5.52	1	5.52	$< 0.0006^{**}$
Interaction of pressure and time) B×C(	51.71	1	51.71	$< 0.0001^{**}$
Interaction of pressure and volume of solvent (B×D)	0.0529	1	0.05290	0.2374^{ns}
Interaction time and volume solvent(C×D)	50.30	1	50.30	$< 0.0001^{**}$
Temperature squared effect (A^2)	74.84	1	74.84	$< 0.0001^{**}$
Squeeze pressure effect (B^2)	1.62	1	1.62	$< 0.0039^{*}$
The squeeze of time (C^2)	0.3183	1	0.3183	$< 0.0365^{*}$
Squalor effect of solvent volume (D^2)	1.72	1	1.72	$< 0.0035^{*}$
Residual	0.0733	3	0.0244	
Lack-of-fit	0.0733	2	0.0366	0.1772^{ns}
Pure error	0.0000	1	0.0000	
Explanation factor (R^2)	99.97			
Adjusted correction factor (R^2_{adj})	99.85			
Predicted coefficient (R^2_{Pre})	98.62			
Necessary precision	115.6290			

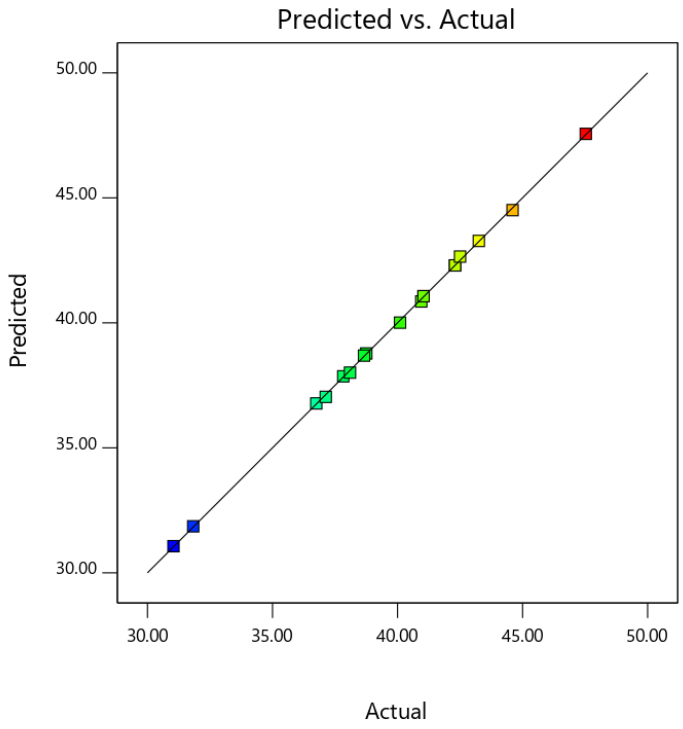

Fig. 3. Comparison of experimental results calculated by the proposed model

The significance of direct effects and interaction of variables in the prediction model were considered based on the *p*-value statistical parameter values. *P* values less than 0.05 for an expression indicate that the expression effectively affects the response of the system. To obtain a simple equation, non-significant terms with *p* values greater than 0.05 were omitted from the final model. Thus, the pressure and temperature factors had the highest and the most significant effect, respectively, and the time and volume of the injectable solvent to the sample input system had the lowest effect on the protein extraction efficiency. Accordingly, the final prediction model (reduced model) in terms of actual values of variables is presented by following equation:

$$R1=+37.78 - 5.62*A + 5.50*B + 0.75*C + 4.60*D + 5.50*AB + 1.65*AC + 2.35*AD - 10.17*BC -10.03*CD - 2.09*A^2 - 1.23*B^2 -0.54*C^2 - 1.27*D^2 \quad \text{[Eq. 1]}$$

In sum, the importance of factors affecting the amount of protein extracted can be summarized as follows:
Time> solvent volume> pressure> temperature

- ***Effect of Supercritical condition on extraction***

Using the model proposed to describe the method studied by RSM, two-dimensional graphs of the response surface were drawn to investigate the influence of linear factors on the process. The single-factor response surface curves in Figure 4 (a-d) and the two-factor interaction effect curves are presented in Figure 5 (a -f). As shown in Figure 4a, an increase in temperature (from 40 to 60°C) resulted in a significant decrease in the amount of protein extracted. This decrease can be

probably due to the negative effect of temperature on the protein structure as well as the heat-induced protein denaturation. The protein extraction efficiency ranged from 31.04 to 47.43 mg/g. Figure 4b shows the effect of pressure on the amount of protein which was extracted. As shown, the extraction efficiency increased significantly with increasing the pressure level from 120 to 300 bars. The density of supercritical carbon dioxide increases with increasing pressure at a specified operating temperature leading to higher solvating capacity in supercritical carbon dioxide (Kosterzewa *et al*, 2020). Consequently, more dissolution of protein and therefore more mass transfer of the protein from the sample tissue takes place with increasing the pressure (Sahena F *et al*, 2009). Figure 4c shows the effect of extraction time on the amount of extracted protein. As investigated, more protein was extracted over the time as a result of more opportunity for dissolution of protein in solvent and further higher diffusing out of protein from the dried sample. Figure 4d also shows the effect of solvent volume on the protein content. The results showed that the extraction efficiency was increased significantly with increasing of the solvent volume from 300 to 1500 µL. The reason can be attributed to the good interaction between the protein and the methanol as a result of polarity of both the protein and methanol which increased mass transfer from the sample to solvent and ultimately increasing protein extraction (Sahena *et al.*, 2009).

Figure 5a shows the effect of interaction of temperature and extraction pressure on the protein extraction efficiency. It is well known that the increase in temperature reduces the yield due to the negative effect of temperature on the protein structure and protein loss due to the denaturation. In this study the highest protein yield was obtained by lower temperature extraction and higher pressure. Figure 5b shows the effect of interaction of temperature and extraction time on the rate of return. It is clear from the observations that the temperature factor had a negative effect on the extraction due to the loss of protein texture as previously mentioned and the highest protein extraction was obtained at lower temperatures. Figure 5c shows the effect of interaction of temperature and solvent volume on the protein extraction efficiency. As shown, the extraction yield was increased significantly by increasing of solvent level and decreased with increasing the extraction temperature. The highest protein extraction efficiency was obtained at the low temperatures and the highest solvent content which can be due to the polarity of the methanol solvent resulting in in a good interaction between the protein and the solvent. Figure 5d shows the effect of interaction of time and pressure on the rate of return. As the pressure and time levels increased, the extraction efficiency was also increased, which is more than the impact of time. This positive effect of pressure can be due to the increased mass transfer from the striated tissue that results in a more protein extraction. The highest protein extraction efficiency was obtained at the lowest and highest pressure and time. Extraction at high pressure and over a longer period of time reduced the extortion efficiency. The reason is that over longer periods of time, protein builds up negative pressure, which in turn destroys the building chains and finally denatures the protein structure which can decrease its extraction efficiency. Figure 5e shows the effect of interaction between pressure and volume of the solvent on the protein extraction yield. As noted in the analysis of variance

table, the effect of interaction between pressure and solvent volume on the extraction yield was not significant and the linear effects were significant by increasing the yield and solvent volume at higher extraction pressure, respectively. Figure 5f shows the effect of interaction between time and volume of the solvent on the extraction yield. The effect of time and volume of solvent alone was positive on the extraction efficiency and also the effect of solvent was greater than time, however, with higher solvent extraction and longer extraction times, the yield decreased and the highest extraction efficiency was obtained by using higher solvent volume at shorter times.

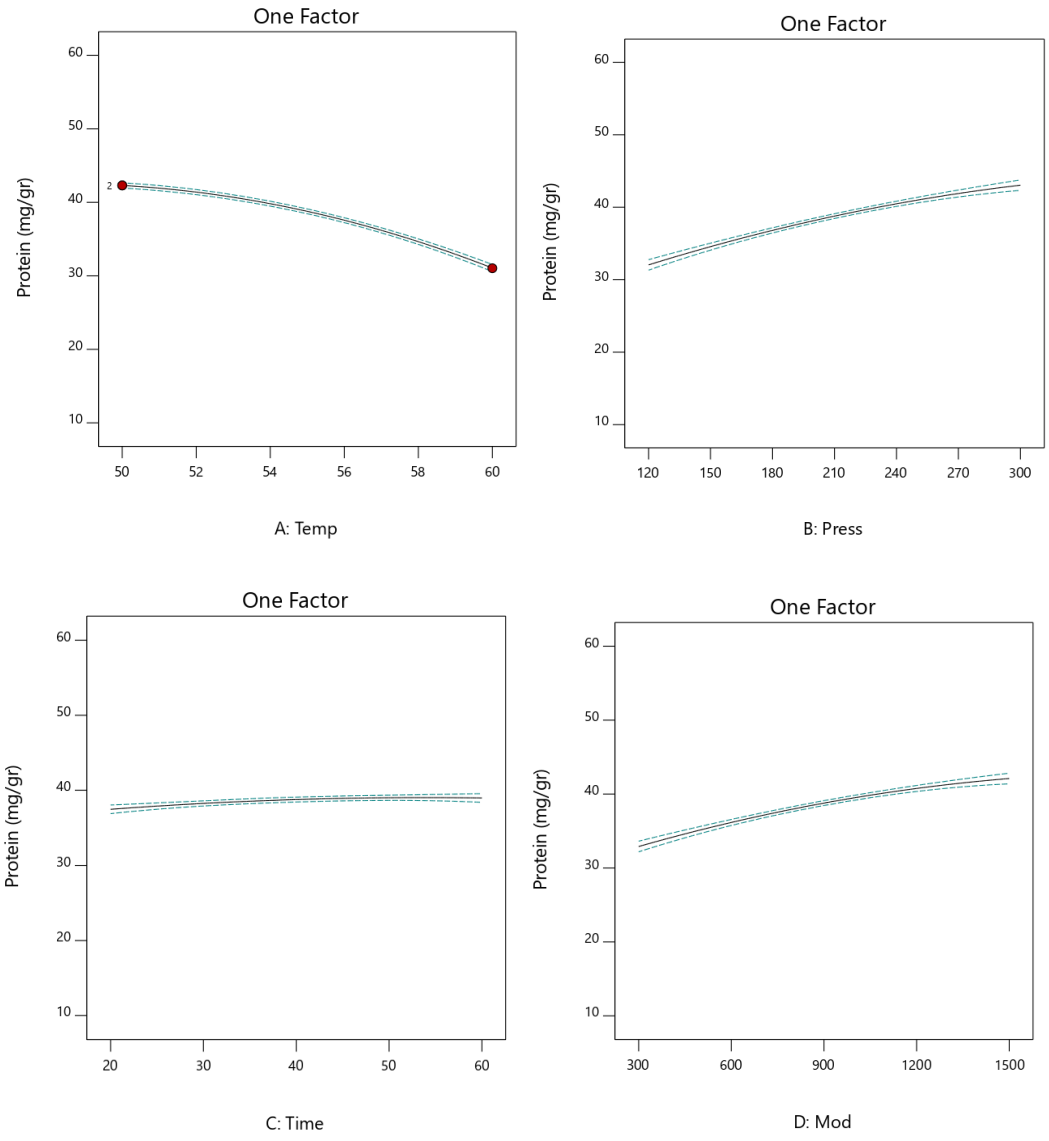

Fig. 4. Effect of different parameters on the amount of protein extracted by supercritical fluid from *Elaeagnus* fruit, A) Temperature, B) Pressure, C) Time, D) Solvent volume

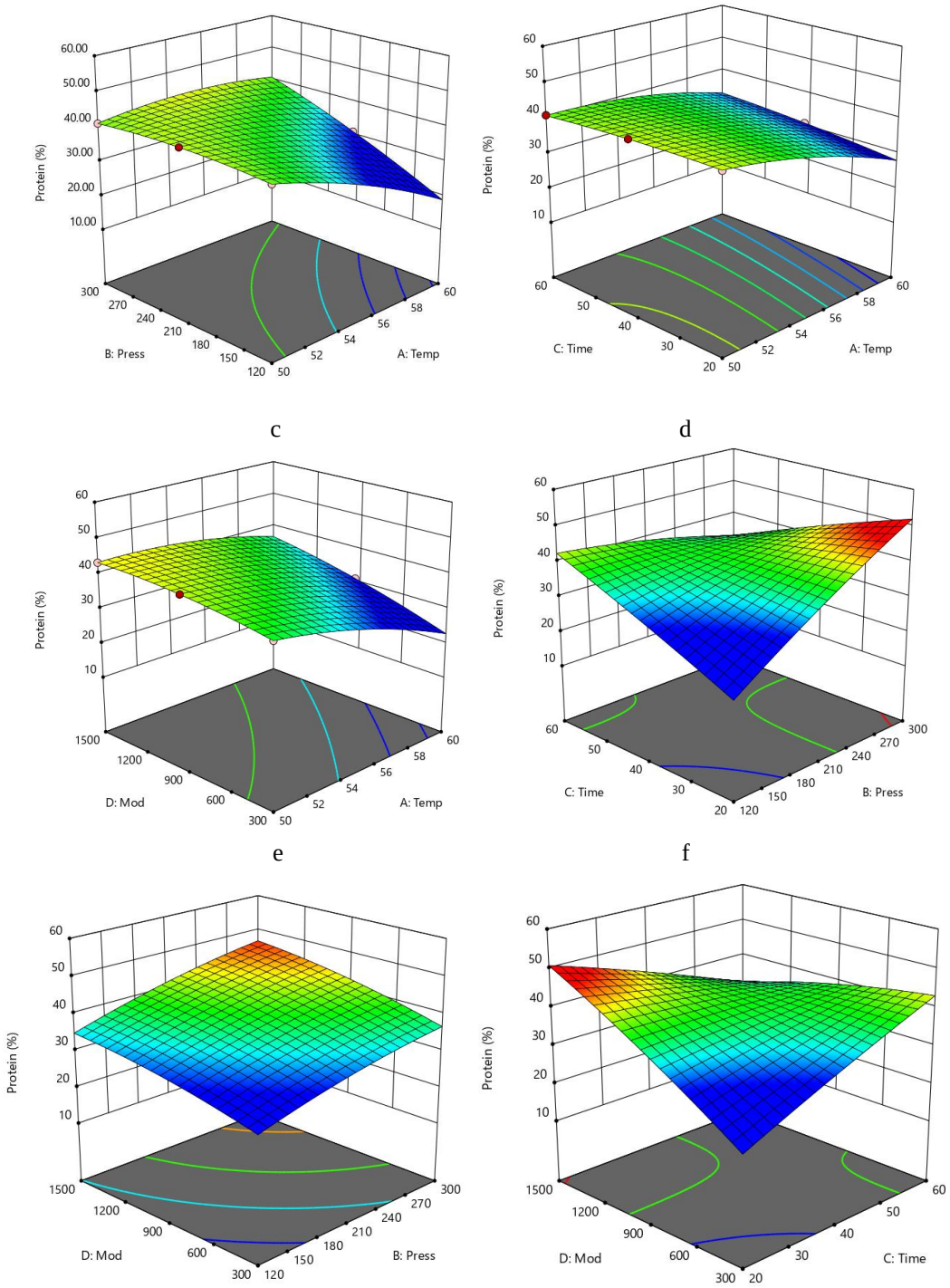

Fig. 5. The interaction of different parameters on the rate of extraction efficiency of protein by supercritical fluid from *Elaeagnus* fruit, a) temperature and pressure, b) temperature and time, c) temperature and volume of solvent, d) pressure and time, e) pressure and volume of solvent, f) between time and volume of solvent ple)

- ***SDS-PAGE analysis***

SDS-PAGE method was used to isolate the extracted proteins. The extracts were load into pre-prepared gels and then, after one and a half hours of loading, the protein bands appeared as shown in Figure 6. The

results showed that the proteins extracted via supercritical carbon dioxide method (Figure 6a) were mostly with molecular weights of 55-70 KDa and 32 to 34 KDa were extracted. To compare the proteins extracted by the supercritical carbon dioxide method with the buffer extraction method, with extraction buffer into SDS-PAGE gel extracts were also injected (Figure 6b). They appeared in different molecular weights ranging from 25-100 KDa according to the shape of the protein bands.

- ***HPLC analysis***

Figure 7 is shown HPLC chromatograms of extracted Elaeagnus amino acids using different methods. As can be seen from Figure 7a, HPLC chromatogram of the sample extracted from buffer extraction shows the smaller number of amino acids peaks with the low peak areas, indicating the low number of amino acids extracted from this method. In contrast, the high number of peaks with high peak areas are revealed from the sample extracted using supercritical carbon dioxide (Figure 7b) indicates that most amino acids are identified. Figure 7c also shows the amino acid profile of dried powders injected directly with the derivative as a control sample. The number of peaks and the area below the peaks are not satisfactory.

Figure 8 show the percentage of extracted amino acids from super critical carbon dioxide, buffer and the dried sample. As can be seen, most of the essential amino acids in human body have been identified with different percentages by HPLC. The most amino acids extracted using supercritical carbon dioxide were 35.15% alanine amino acid and 11.70% aspartate and glutamate (Figure 8a). The analysis of the data showed that the conditions of supercritical carbon dioxide extraction have significant effects on amino acids. With increasing temperature, most of the amino acids are removed because of their high sensitivity to temperature. Pressure has a positive effect

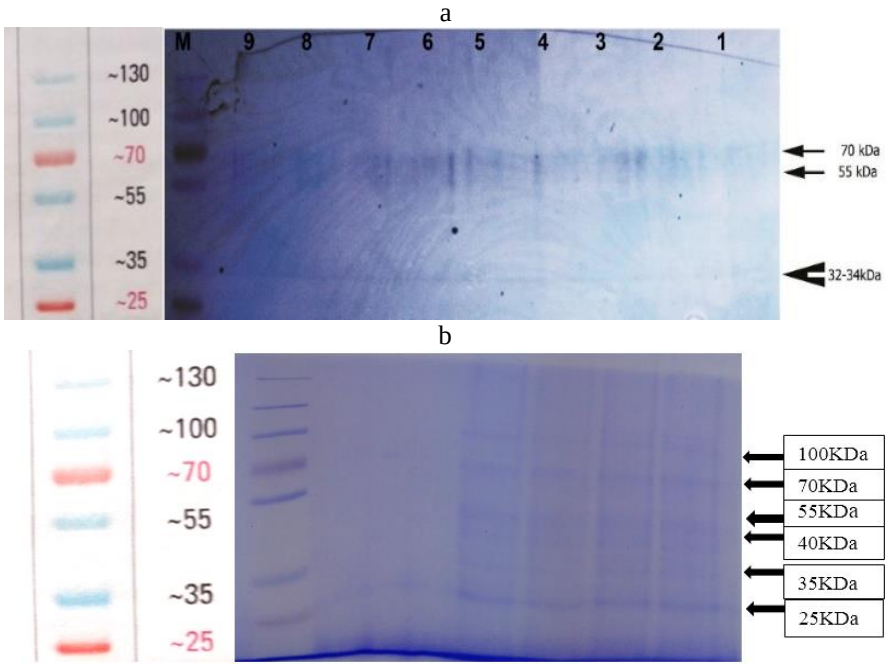

Fig. 6. Protein bands appearance, a) supercritical carbon dioxide extraction, b) buffer extraction (as a control sample)

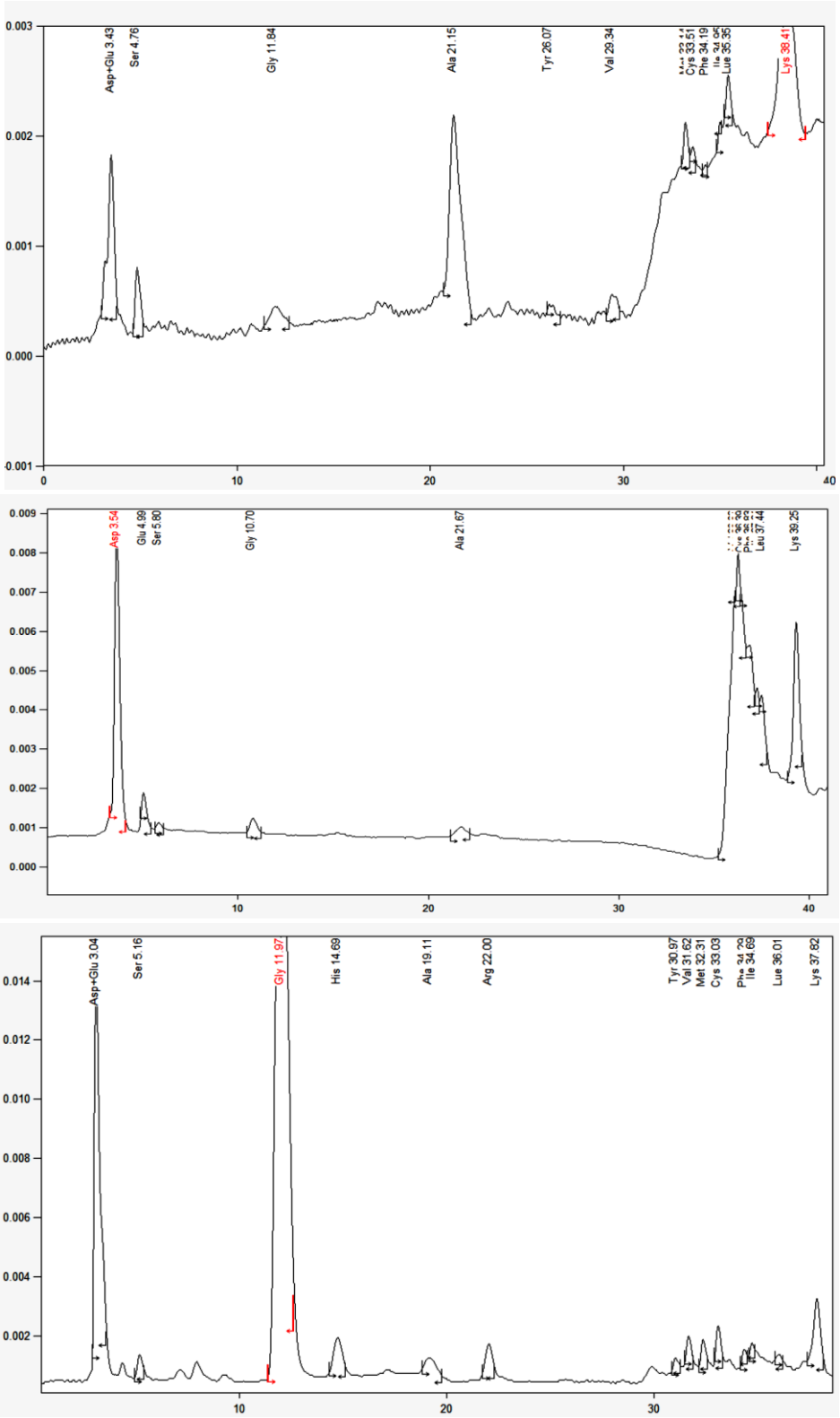

Fig. 7. Chromatogram graphs of a) Buffer extraction sample, b) Supercritical carbon dioxide extraction sample, c) Dried samples of *Elaeagnus* powder as a control sample

on this process. Extraction time has a negative effect on amino acid identification. Methanol solvent has a positive effect on the appearance of amino acids due to polarity. It should be noted that the amino acid tryptophan has been removed due to its high sensitivity at the acid hydrolysis stage. In buffer extraction, the highest percentage was related only to the glycine amino acid (66.1%) (Figure 8b). Other percentages of amino acids were not satisfactory. And according to the Figure 8c, Aspartat and Lysine are the most amino acids extracted from the dried sample.

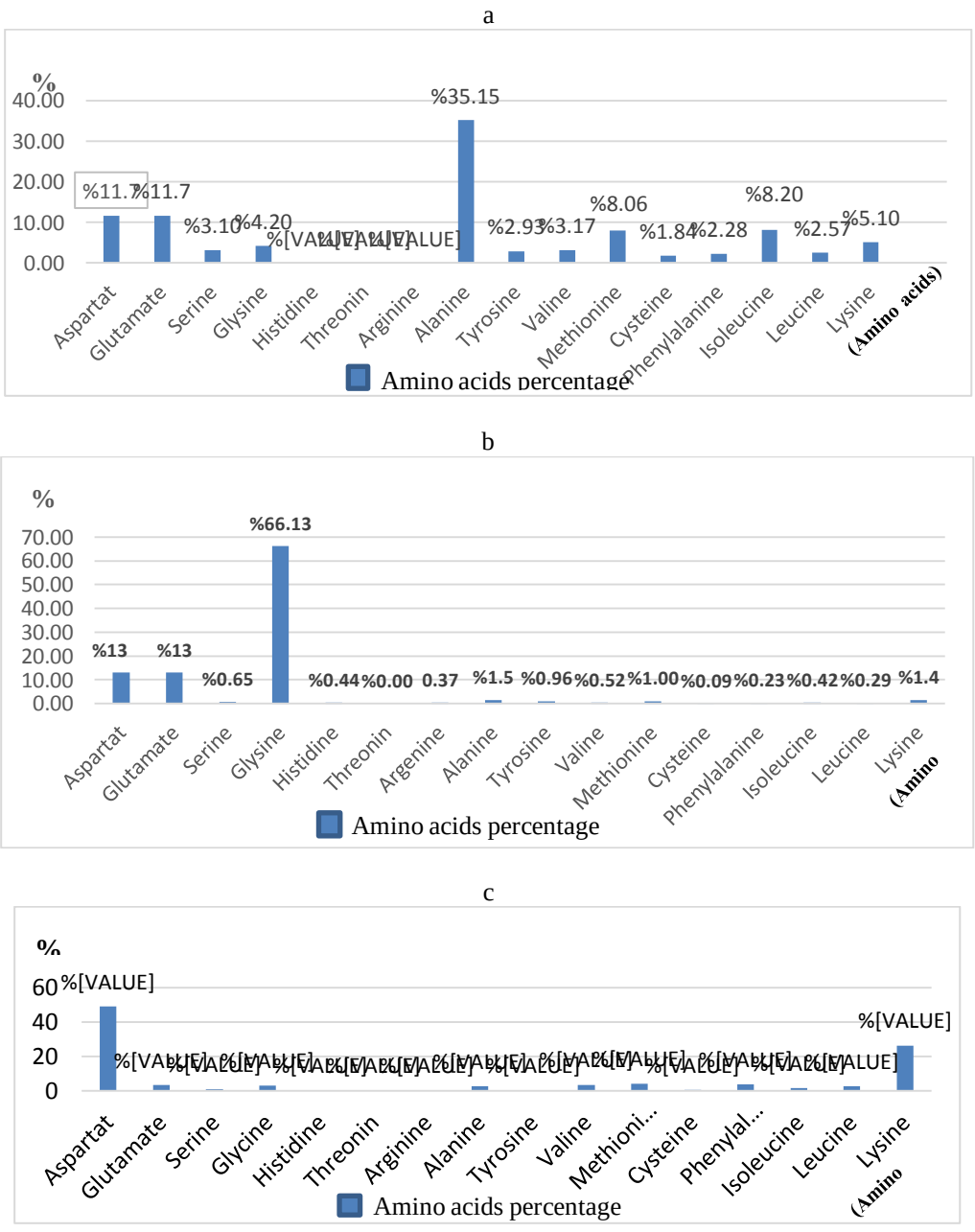

Fig. 8. Percentage of amino acids identified from samples extracted with a) supercritical CO_2, b) buffer, c) dry sample

Conclusion

In this research, the super critical CO_2 extraction method was used for the first time to extract a plant protein and as a suitable method for extracting amino acids. This method has some advantages such as separating the solvent more easily by adjusting the pressure, using the carbon dioxide solvent as a non-toxic, pure, cheap or a critical temperature correcting solvent. As the solubility behavior of the fluid is closer to the liquid phase and the fluid diffusivity in the solid is similar to the gas phase, it results in a significant increase in the rate of extraction and phase separation in comparison to the usual extraction. Since the supercritical fluid surface tension is zero, penetration of the fluid to the bottom of the solid material holes is easy and causes higher extraction than the conventional method. It also dramatically increases the extraction efficiency due to the increase in pressure which is resulted by the solubility of the fluid. The results of this study showed that the pressure factor was the most effective factor in protein extraction. The second factor with a high impact on protein extraction was temperature factor, that has a negative effect on the protein extraction when temperature increases. Subsequent methanol solvent factors had a positive effect on the protein extraction.

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