Betalain Extraction from Beetroot Using Supercritical Carbon Dioxide and Microwave Pretreatment by Response Surface Method (RSM)

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ABSTRACT: *Extensive research has begun on the production of naturally occurring red colorants as permitted food additives. The Betalain in Beetroot (Beta vulgaris) cells can be used to prepare a natural red color additive for food products. Betalains are more resistant to acidic conditions and higher temperatures than other red pigments such as anthocyanins. However, the low stability of Betalain compared to synthetic colorants is considered a big challenge in its extraction, processing, and storage. Therefore, it is very important to use a suitable extraction method for it. In recent years, the use of supercritical carbon dioxide (scCO$_2$) for isolation and extraction of natural products instead of conventional methods such as extraction with organic solvents has attracted interest because the final product may become contaminated with the solvents. In the same vein, this research evaluated the effects of microwave power (100-450 W), carbon dioxide flow rate (1-3 mL/min), temperature (30-70℃), and pressure (15-40 MPa) on extraction efficiency, 2,2-diphenyl-1-picrylhydrazyl, and the quantities of phenolic compounds extracted from Beetroot. The results suggested that temperature, pressure, flow rate, and microwave power significantly influenced the amounts of phenolic compounds, extraction efficiency, and antioxidant properties of the compounds extracted from Beetroot. Increases in temperature, pressure, microwave power, and carbon dioxide flow rate in the (50-60℃), (20-30 MPa), (300-400 W), and (1-2 mL/min) ranges, respectively, increased the quantities of phenolic compounds, ability to suppress free radicals and extraction efficiency as the response. However, increases beyond the mentioned ranges caused decreases in the response variables. Optimal extraction of Betalain from Beetroot using supercritical carbon dioxide and microwave pretreatment was achieved at a temperature of 45 °C, pressure of 27.5 MPa, CO$_2$ flow rate of 2 mL/min, and microwave power of 300W.*

KEYWORDS: *Supercritical carbon dioxide; Beetroot; Betalain; Microwave; Antioxidant; RSM.*

INTRODUCTION

Recently, international organizations and research institutions have announced many restrictions on the use of synthetic food colorants, especially red food colorants. Hence, extensive research on producing natural red food colorants as a permissible additive has begun. Beetroot (*Beta vulgaris*), one of the most important sources of natural red food colorant, is a member of the spinach family [1]. It is grown in Asia and Europe and is a good source of fiber, sugar (glucose), Betalain (75-95%), B vitamins and vitamin C. This plant is reported to be rich in bioactive compounds including polyphenols, carotenoids, and flavonoids. Betalain refers to a class of water-soluble heterocyclic nitrogen pigments that are members of the phenolic and cyclic amine compounds present in Beetroot cell walls (vacuoles) that create a red-to-purple color. Compared to other red pigments such as anthocyanins, Betalains are more resistant to acidic pH and high temperatures and are therefore suited for neutral and low-acid foods, whereas it is impossible to use anthocyanin in these food materials [2]. In general, Betalains are categorized into two groups, namely Betacyanin (red-purple color with the maximum absorbance at 540 nm) and Betaxanthins (yellow-orange color with the maximum absorbance at 480 nm). Approximately, 130-450 mg of Betalain is extracted from every 100 g of fresh raw Beetroot. Beetroot extract can have up to 70% sugar and 0.5% Betanin pigments. The contents of Beetroot pigments can also be increased by fermentation and removal of ethanol [3]. At low concentrations, these pigments prevent lipid peroxidation due to the presence of hydroxyl groups that inhibit free radicals [4].

The stability of Betalains is affected by internal factors including activities of peroxidase and polyphenol oxidase and external factors such as temperature, oxygen, a_w, and pH. It is noteworthy that the low stability of these compounds is a big challenge during extraction, processing treatment, and storage of the Beetroot-derived bioactive compounds [2]. Therefore, using a suitable extraction method is very important since the extraction method and conditions influence its efficiency and the types of the resulting compounds. A common and widely used method for extracting bioactive compounds from plant sources is solid-liquid extraction using organic solvents. However, this method is not recommended for extracting heat-sensitive compounds because it needs large amounts of organic solvents and evaporation or concentration of the final extract, there is the possibility of thermal degradation of the compound of interest (in case the sample contacts the solvent for a long time), the extraction process is long, and some of the solvents remain in the final product [5]. New and green methods for extracting bioactive compounds have been developed because of these problems. Nowadays, gamma rays, microwaves, and ultrasound are used as extraction and pretreatment methods (to increase extraction efficiency and reduce extraction time).

Microwave pretreatment is one of the methods that can be used before the extraction process. Microwave heating is different from all other heat transfer methods including conduction, convection, and radiation due to its heat generation and transfer mechanisms. Unlike infrared waves, microwaves do not convey thermal energy but, when they strike the materials in the electromagnetic field, heat is produced in the materials. In general, many chemical solutions and most food materials contain water and various amounts of dissolved salts. When these salts dissolve, they are converted into two separate and oppositely charged particles. When ionic solutions are exposed to an alternating microwave field, the ions are forced to flow in one direction and immediately in the opposite direction. Charged particles hit their adjacent molecules and transfer kinetic energy to accelerate them and make them hit other molecules. The increased kinetic energy of the molecules heats the substance. Therefore, all parts of the food material uniformly absorb microwave energy and heat, which removes moisture from the food consistently. The absorption of microwaves by solids rapidly increases water temperature and quickly evaporates it to produce a high vapor pressure. This vapor pressure breaks the cell wall and makes its content more accessible [6, 7].

On the other hand, extraction with supercritical fluids is an old technique with slow commercial use due to the expenses of high-pressure equipment and the required technology. Supercritical Fluid Extraction (SFE) can be used to extract bioactive compounds from plants near room temperature, which prevents substance degradation and thermal denaturation [8]. In general, any substance at a pressure and temperature beyond its critical points is called a supercritical fluid. Using a compound as the supercritical fluid solvent is directly tied to its density. The viscosity and molecular permeability coefficient of

the molecules of supercritical fluids are close to those of gases, and they flow easily as gases do. Supercritical fluids also have very low surface tension so increasing the contact surface between the phases in the separation processes increases mass transfer intensity. Therefore, extraction using supercritical fluids is very fast, and there are fewer changes in the properties of materials compared to the use of organic solvents. The solubility of supercritical fluids is a function of temperature and pressure, and changing temperature and pressure will provide the necessary conditions for extraction with solvent and the separation of the extract from the solvent. Carbon dioxide is the most common solvent in SFE extraction since it is an inert and harmless (non-flammable, non-explosive), cheap, non-corrosive, odorless, and colorless substance, and leaves no residues in the product. Carbon dioxide has low surface tension and viscosity and high diffusivity, which make it a suitable solvent in supercritical extraction. It is non-toxic and is generally accepted as a harmless substance in the food industry [9, 10].

Tahmasb Hatami et al. (2019), obtained lycopene from processing tomato byproducts through SFE. This study evaluated the effects of temperature (40- 80°C), pressure (30-50 MPa), and skin-to-seed ratio on the extraction efficiency of lycopene. The results suggested that the highest extraction efficiency of lycopene (32.1 mg/kg of raw material) was obtained at 80°C, 50 MPa, and a skin-to-seed ratio of 70/30 [11]. *Ameer et al.* (2017), reported that factors such as chemical structure, pigment content, matrix, additives, enzymes, pH, heat treatment, water activity, oxygen, and light during storage affected the stability of Betalains. They stated that reducing heat during processing maintained Betalain coloring capacity [9]. *Jiao & Kermanshahi Pour* (2018), evaluated the extraction of anthocyanins from haskap berry pulp using supercritical carbon dioxide. Most anthocyanin compounds were captured from haskap berry pulp paste using supercritical carbon dioxide ($scCO_2$) and water as co-solvent. The pressure, temperature, and amount of water were the independent variables of extraction conditions. The highest Total Anthocyanins (TA) yield of 52.7% was attained at 45 MPa, 65 °C, 5.4 g water to 3.2 g berry pulp paste, 15 min static and 20 min dynamic time. Different combinations of ethanol as co-solvent and water did not significantly affect the TA yield. Compared with official extraction, the higher anthocyanin extraction efficiency (52.7% versus 38.3%)

with ameliorated antioxidant activity (89.8% versus 72.2%) was obtained by using $scCO_2$ and water as co-solvents [12]. *Fernando et al.* (2021), researched the recovery of Betalain and polyphenols from red beet waste. They used a mixture of water and ethanol as a solvent and ultrasound for extraction. The total of Betalain, polyphenol, antioxidant capacity, and stability were investigated over 4 weeks. Based on the results, Betalain was destroyed at high temperatures, but the antioxidant activity and polyphenols were less affected. These data showed that Beetroot waste can serve as a good source of Betalain for the industry [13]. The extraction of anthocyanins from grape pomace by using supercritical carbon dioxide was conducted by *Pazir et al.* (2020). Ethyl alcohol was used as a co-solvent. During the extraction, the temperature and pressure were retained constant (95°C and 100 bar). The total extraction time was 180 min. The total monomeric anthocyanin content (TMAC) and the total capacity of antioxidants (TAC) were analyzed by capturing specimens from the extract at the 30th, 60th, 90th, 120th, 150th, and 180th min. The TMAC of grape pomace is 1,932.1 mg/kg dry matter. The values of TMAC and TAC at each time were measured to be 579.2, 406.1, 123.7, 52.8, 38.5, and 16.4 mg/kg dry matter and 177.3, 171.1, 90.6, 44.7, 19.6, and 10.0 mg Trolox/100 g dry matter, respectively. As time increased, the extractable TMAC and TAC values gently decreased but the stockpiled total anthocyanin content and the capacity of antioxidants increased [14].

As mentioned earlier, the main goal of substituting traditional extraction methods with modern techniques is to reduce the extraction time and the amount of chemical solvent used for extraction, and improve extraction efficiency and the quality of the final extract. Considering the negative effects of heat on bioactive compounds and the other mentioned points, this study used microwave pretreatment to optimize the extraction conditions of the bioactive compound Betalain in Beetroot using the supercritical fluid method. However, no research, to the best of our knowledge, has been conducted on the extraction conditions, especially using microwave pretreatment and the supercritical fluid that was examined in the present study.

EXPERIMENTAL SECTION
Preparation of samples

The required Beetroot was purchased from the local market in the winter of 2019. The samples were transferred

to the lab, washed thoroughly, and kept in dark containers at refrigerator temperature before tests were conducted. To conduct the test, the Beetroots were peeled and diced into 1.5 cm^3 cubes and scattered in a dark, well-ventilated room until their moisture content decreased (to a maximum of 2%) and they dried to a constant weight. Before the extraction process, the Beetroot cubes were preheated in a microwave oven (Samsung ME3410W, South Korea) for 90 s at 100-450 W. In the following, extraction was performed the by supercritical fluid method and with an SCF6000 device. The module's load was 10 g, and the variables and their range for the extraction process included temperature (30-80 °C), pressure (15-40 MPa), and CO_2 flow rate (1-3 mL/min). To increase the polarity of the supercritical carbon dioxide, about 10 mL of ethanol and 5 mL of distilled water were injected into the extraction chamber as a modifier so that carbon dioxide could better dissolve the phenolic compounds. The response surface method was used to evaluate a wide range of the parameters affecting extraction including temperature, flow rate and pressure, and determine the optimal process conditions. In this study, the amounts of extracted materials and bioactive compounds, antioxidant activities, and Total Phenolic Content (TPC) were measured to evaluate the effect of various parameters on the extraction process.

Total phenolic content of the extract

The total phenolic content was measured using the Folin-Ciocalteu reagent. At first, 0.5 ml of the obtained extract was mixed with 3 mL of 85% ethanol. In the following, 300 µL of the Beetroot extract was combined with 1500 µL of the 10 % Folin-Ciocalteau. After 5 minutes, 1200 µL of 7 % sodium carbonate was added and then, the solution was shaken for 1.5 h at 120 rpm. Finally, the absorbance of the samples was measured using spectrophotometry at 765 nm. The total amount of phenolic compounds was expressed as (mg/g dry weight of the extract) using the line equation drawn for Gallic acid [15].

Measurement of antioxidant activity of the extract

DPPH assay is used to evaluate the compounds that inhibit free radicals or donate hydrogen. This test measures the antioxidant activity of water-soluble phenolic compounds. In this regard, 500 µL of the extract was centrifuged together with 500 µL of distilled water for 5 min at 10,000 rpm. Then, 75 µL of the resulting solution was transferred into test tubes, and finally, 2925 µL of a DPPH methanolic solution (2.4 mg per 100 mL) was added, and after a few seconds of vortex mixing, the absorbance of the samples was read at 515 nm using a spectrophotometer. Absorbance was read again after 30 min in a dark room at room temperature, and finally, the antioxidant activity of the phenolic compounds was measured using Equation (1([15].

$$\text{Inhibition (\%)} = \frac{(A_0 - A_{30}) \times 100}{A_0} \tag{1}$$

Where, A_0 = sample absorbance at time A_0 and A_{30} = sample absorbance 30 min later.

Measurement of extracted bioactive materials

To measure the efficiency of Beetroot extraction, the extract was placed in an oven at 25 °C for 16 h, and extraction efficiency was measured using Eq. (2) after the extract was placed in a desiccator to cool and reach a constant weight [16].

$$\text{Extraction ef eficiency (\%)} = \frac{A \times 100}{B} \tag{2}$$

Where, A = weight of the dried compounds, and B = the initial weight of the compounds.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

The GC/MS (Shimadzu, Japan with spectrometer) was conducted on a Varian 3400 gas chromatograph equipped with a DB5 capillary column (60 m, 0.25 mm ID, 0.25 µm film thickness). In this regard, absorption spectra were identified in the 200-700 nm. For this purpose, at first, the extract was passed through filters with diameters of 0.15 to 0.3 micrometers. Next, the extract (pH=5.5-6.5) was injected into the chromatography column and the visible spectrum of the output material was determined by using spectrophotometry.

Statistical analysis

The Response Surface Method (RSM) designs the test matrix based on the number of variables and the maximum and minimum range for each independent variable. In this way, the variable levels in each test as well as the number of tests are determined. In the design of experiments, the arrangement is such that reliable statistical results can be

Table 1: Values of independent variables (actual and coded).

Independent variable	Symbols	Levels		
		-1	0	1
Temperature	x_1	30	50	70
Pressure	x_2	15	25.5	40
Flow rate	x_3	1	2	3
Microwave power	x_4	100	275	450

Table 2: Regression coefficients of the proposed models for prediction of the phenolic, DPPH and extraction yield of the composite samples containing Temperature, Pressure, Flow rate and Microwave power.

Regression coefficients	Phenolic compounds	F-value	p-value	DPPH	F-value	p-value	Extraction efficiency	F-value	p-value
β_0	534.24	20.84**	0.0098	58.9	11.41**	0.0026	70.96	22.07**	0.0072
β_1	7.44	18.28**	0.0060	0.316	5.01*	0.0115	1.23	21.53**	0.0047
β_2	15.41	20.40**	0.0051	1.19	10.42**	0.0016	1.40	14.80**	0.0038
β_3	13.98	16.14**	0.0099	2.28	12.60**	0.0044	0.6829	12.18**	0.0067
β_4	63.77	20.54**	0.0031	10.12	20.23**	0.0052	6.61	31.49**	0.0015
β_{12}	-8.06	32.24**	0.0062	0.732	8.039**	0.0084	0.5447	10.08**	0.0077
β_{13}	20.89	10.61**	0.0021	1.5	7.163**	0.0021	2.12	5.18*	0.0029
β_{14}	-19.63	4.42*	0.0049	-5.51	4.21*	0.0048	-2.29	5.37*	0.0025
β_{23}	5.23	10.32**	0.0078	0.6598	15.03**	0.0087	1.27	18.48**	0.0049
β_{24}	8.78	4.13*	0.0072	3.68	5.01*	0.0032	1.32	5.46*	0.0050
β_{34}	-2.53	5.02*	0.0088	2.12	6.30*	0.0077	0.170	4.00*	0.0092
β_{11}	-59.63	4.90*	0.0001	-8.87	8.16**	0.0015	-5.59	5.49*	0.0078
β_{22}	-24.8	0.92ns	0.3502	0.335	0.003ns	0.9549	0.4455	0.021ns	0.0088
β_{33}	3.19	2.01ns	0.9001	3.72	0.434ns	0.5183	3.59	1.46ns	0.0024
β_{44}	-1.96	0.06ns	0.9388	3.2	0.319ns	0.5791	1.22	0.133ns	0.0071
Lack of fit	-	0.087ns	0.6521	-	0.125ns	0.5412	-	0.215ns	0.5123
R^2	0.9788	-	-	0.9858	-	-	0.9733	-	-
adj-R^2	0.8879	-	-	0.9122	-	-	0.8932	-	-

**Significant difference at $\alpha = 1\%$ probability level; ns Not significant difference; *Significant difference at $\alpha = 5\%$ probability level

obtained even if the test is not repeated. Also, this method can evaluate the interaction of parameters with each other. The model used for RSM is often a second-degree model. In the RSM, a model is defined for each dependent variable which states the main and interaction effects of the factors on each variable. In Equation 3, Y is the predicted response, β_0 represents the constant coefficient, $\beta_1, \beta_2, \beta_3,$ and β_4 the linear effects, $\beta_{11}, \beta_{22}, \beta_{33},$ and β_{44} the square effects, and $\beta_{12}, \beta_{13}, \beta_{14}, \beta_{23}, \beta_{24},$ and β_{34} the interaction effects.

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 x_4 + \quad (3)$$
$$\beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{44} x_4^2 + \beta_{12} x_1 x_2 +$$
$$\beta_{13} x_1 x_3 + \beta_{14} x_1 x_4 + \beta_{23} x_2 x_3 + \beta_{24} x_2 x_4 + \beta_{34} x_3 x_4$$

This study used the central composite design with four independent variables (temperature, pressure, flow rate, and microwave power) each at three levels, three blocks, and six replications at the central point (for calculating process repeatability) to evaluate the effect of Betalain extraction conditions and optimize the process. Dependent (response) variables, phenol percentage, DPPH, and extraction efficiency were also considered. The optimal operational conditions for extracting Betanin from Beetroot were obtained using the numerical optimization technique.

RESULTS AND DISCUSSION

Table 2 shows the analysis of the estimated regression coefficients in the second-order polynomial model

Table 3: Tested and predicted values of all response variables.

						Tested values			Predicted values	
	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2	Response 3	Response 1	Response 2	Response 3
	Temperature	Pressure	Flow rate	Microwave power	DPPH	Phenolic compounds	Extraction Yield	DPPH	Phenolic compounds	Extraction Yield
Run	°C	MPa	mL/min	W	%	µg/mL GAE	%	%	µg/mL GAE	%
1	45	40	1.8	450	78	423	81	83	452	85
2	50	40	3	250	73	534	79	71	540	75
3	30	40	3	450	75	554	79	80	570	77
4	55	35	1.5	400	76	557	82	79	570	85
5	30	30	1.5	360	75	551	78	71	549	76
6	60	25.5	3	450	81	358	87	79	362	91
7	30	15	3	450	55	403	65	58	410	60
8	70	15.5	1	180	50	400	60	48	385	64
9	45	15	1	450	51	507	76	57	525	80
10	70	25	1.5	180	42	403	62	45	390	69
11	45	27.5	2	300	70	504	76	68	510	78
12	50	15	3.5	300	56	490	70	58	475	75
13	45	15	1	250	54	477	70	57	468	72
14	70	40.5	2.6	180	56	487	70	53	495	69
15	70	25	3	400	55	477	96	59	475	95
16	70	15	1	450	54	444	70	57	440	75
17	40	20.5	2	180	42	400	60	48	385	61
18	30	40	1	180	41	405	60	39	400	56
19	70	35	2	180	40	393	60	42	385	68
20	50	40	2.3	180	40	390	61	41	400	64

for response variables (phenol percentage, DPPH, and extraction efficiency). Also, the results of tested and predicted values of all response variables are shown in Table 3.

Gas Chromatography-Mass Spectrometry (GC-MS)

The main betacyanin of red beet is betanin, which constitutes 75-95% of all beet pigments. Therefore, to measure the concentration and efficiency of betacyanin pigments, it was examined in terms of betanin (Betalain) at 538 nm wavelengths. Also, the amount of betaxanthin pigment in terms of violaxanthin, which had an absorption wavelength of 480 nm, is investigated. It should be noted that the total content of Betalain was calculated as the sum of betacyanin and betaxanthin. According to Fig. 1, the maximum absorption peak for betacyanin, betaxanthins
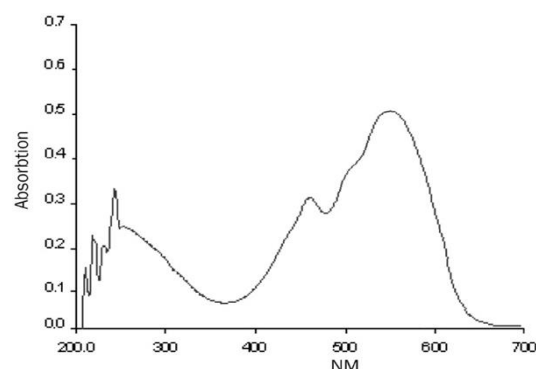

Fig. 1: Chromatogram of beetroot extract compounds.

and betanin appeared at 537, 478 and 538, respectively. According to the obtained, a high amount of the extract contains betanin.

Total Phenolic Content (TPC)

According to ANOVA results (Table 2), the effects of temperature, pressure, flow rate, and microwave power on TPC were significant (*p≤0.05*). Fig. (2. a) shows the interaction effects of temperature and pressure on the TPC. The results suggest that increasing these two parameters (to 60 °C and 30 MPa) increased the TPC, but further increases in pressure (up to 40 MPa) and temperature (up to 70 °C) significantly reduced it (*p≤0.05*). According to Fig. (2. f), the TPC at 1 mL/min and 300 W (minimum temperature and pressure were considered) was 544.307 µg/mL GAE, now increasing the temperature to 60℃ improved the TPC to 565.588 µg/mL GAE, but further temperature increases decreased it to 546.213 µg/mL GAE (*p≤0.05*). Also, increasing pressure to 30 MPa improved the TPC from 544.307 µg/mL GAE to 577.515 µg/mL GAE, but the TPC decreased under higher pressure (*p≤0.05*). Fig. (2. b) shows that increasing temperature and flow rate increased the TPC. Likewise, at 27.5 MPa and 300 W, the TPC was 548.491 µg/mL GAE, but increasing the flow rate to 3 mL/min improved it to 553.748 µg/mL GAE (*p≤0.05*). In addition, increasing the temperature to 45 °C increased the TPC from 548.91 to 563.392 µg/mL GAE (*p≤0.05*). On the other hand, Fig. (2. c) shows that increasing temperature and microwaving power (the pretreatment) increased the TPC, whereas higher or lower than 300 W and higher or lower than 45 °C significantly reduced it (*p≤0.05*). At 1 mL/min and 27.5 MPa (minimum flow rate and pressure were considered), increasing the temperature to 40-50 °C improved the TPC, but the TPC decreased substantially at higher temperatures. In the same conditions, the TPC increased significantly with a relatively fixed slope from 544.307 to 577.515 µg/mL GAE by increasing microwave power (*p≤0.05*). However, the TPC decreased to 365.822 µg/mL GAE when the power level was gradually raised to 450 W. Also Fig. (2. c) shows that at 45 °C and 300 W, the TPC reached 579.044 µg/mL GAE, but further gradual increases in pressure reduced it to 574 µg/mL GAE (*p≤0.05*). It is noteworthy that, increasing the flow rate from 1 mL/min to 2 mL/min decreased the TPC from 579.044 to 566.08 µg/mL GAE, and increasing it to 3 mL/min lowered it to 558.583 µg/mL GAE (*p≤0.05*). Eventually, the TPC at the optimal central point (2 mL/min, 27.5 MPa, 45 °C, and 300 W) was determined at 605µg/mL GAE. The regression equation (Eq.4) indicated that the total variation in phenolic extraction was 78.23%.

$$\text{Phenolic } \left(\mu\frac{g}{mL}\text{ GAE}\right) = 534.24 + 7.44x_1 + \quad (4)$$
$$15.41x_2 + 13.98x_3 + 63.77x_4 - 59.63x_1^2 - 24.8x_2^2 + 3.19x_3^2 - 1.96x_4^2 - 8.06x_1x_2 + 20.89x_1x_3 - 19.63x_1x_4 + 5.23x_2x_3 + 8.78x_2x_4 - 2.53x_3x_4$$

Phenolic compounds and the other phytochemicals present in fruits and vegetables have antioxidant properties, are bioactive compounds, can neutralize free radicals, and play a very important role in preventing various diseases [9]. It is noteworthy that this extraction method requires drying of the prepared samples for the extraction process because the water in them turns into ice during extraction and creates a physical barrier to the supercritical fluid flow. In addition, water may compete with carbon dioxide for dissolving the compounds and affect the rate of mass transfer [17]. According to

the results, increasing the temperature to about 45 °C improved the TPC but further temperature increases reduced it. The results showed that increasing the temperature increased the vapor pressure in the sample, and improved mass transfer and the rate of diffusion thereby increasing the TPC. However, temperatures higher than 45℃ broke down the structure of phenolic compounds due to enzymatic or thermal degradation [18]. Moreover, the increase in the quantity of extracted phenolic compounds by raising the extraction temperature may be due to the effect of temperature in softening and fracturing cell walls and plant tissue that makes it easier for these compounds to leave plant cells and tissues [19]. The sensitivity of phenolic compounds to heat and double bond breakage by heat reduce these compounds at high temperatures [20]. Research by *Xu et al*. (2012), on extracting polyphenolic compounds from tea showed that increasing extraction time at low temperatures increased the TPC, but the TPC decreased at high temperatures due to the sensitivity of phenolic compounds to heat. In addition, increasing the pressure to 30 MPa increased the extraction of phenolic compounds but harmed extraction at higher pressure levels. The reason for the increase in phenolic compounds at higher pressure levels is the increase in carbon dioxide density which improves its solubility. Meanwhile, excessive increases in pressure reduce fluid permeation ability in the sample matrix because pressure increases also increase viscosity thereby reducing extraction of phenolic compounds [22]. Interestingly, increasing the temperature up to a certain point at low
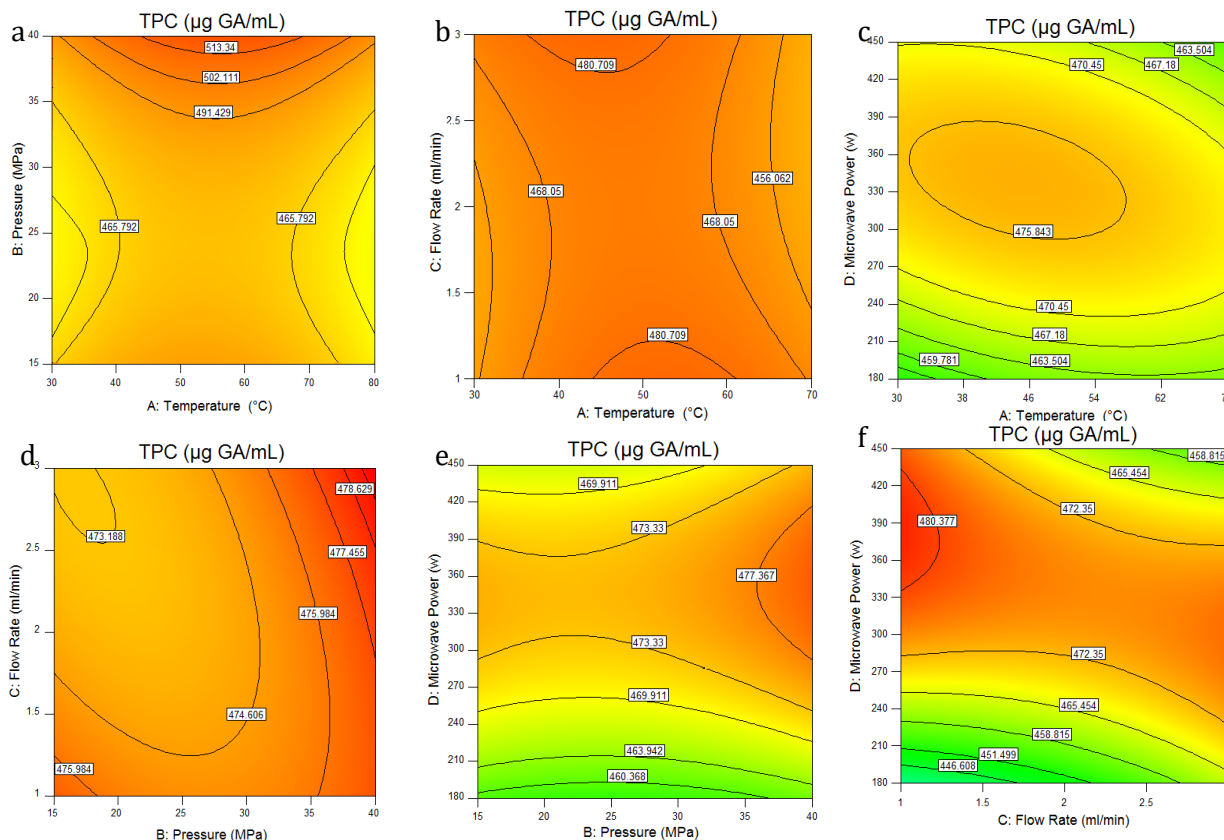
Fig. 2: Interaction effects of temperature and pressure (a), temperature and flow rate (b), temperature and microwave power (c), pressure and flow rate (d), pressure and microwave power (e), flow rate and microwave power (f) in total phenolic content.

pressures somewhat increased phenolic compounds but beyond that decreased it. However, the increasing temperature at high pressures reduced the TPC due to the prolonged exposure of phenolic compounds to high temperatures as high pressures reduce mass transfer rates. Such conditions also increase impurities, which negatively affect the TPC [23]. In addition, the interaction effects of temperature and flow rate on phenolic compounds were positive so increases in these two variables improved the TPC. As mentioned earlier, higher temperatures increased mass transfer and diffusion rates. At the same time, increasing the flow rate up to 2 mL/min put the fluid in constant contact with the sample and ultimately improved the extraction of phenolic compounds. Higher and lower flow rates than 2mL/min reduced extraction efficiency due to the inability of the solvent to separate and extract phenolic compounds. The results suggest that increasing temperature and microwaving power (300 W) increased the extraction efficiency of phenolic compounds. Microwaves increased

the temperature and fractured cell walls rapidly thereby enhancing the release of the contents of cells during extraction and reducing extraction time. However, at much higher or lower microwave power than 300 W and temperatures below or above 50 °C, the amounts of extracted polyphenolic compounds decreased significantly. It appears that the use of high-power microwaves breaks cell walls in addition to degrading heat-sensitive compounds such as polyphenols and flavonoids. Moreover, microwave power lower than 300 W is not able to break plant cell walls, which reduces the extraction of phenolic compounds in supercritical fluids. The results showed that applying microwave pretreatment significantly increased the quantities of extracted phenolic compounds [4, 7, 13, 14, 16].

Percentage of DPPH inhibition

According to Table 2, the effects of temperature, pressure, flow rate, and microwave power on DPPH were significant ($p \leq 0.05$). Based on the Fig. (3. a) shows the

interaction effects of temperature and pressure on the inhibition of the free radical DPPH, its inhibition reached 67.97% at a flow rate of 1mL/min and microwave power of 300W (minimum flow rate and microwave power). Increasing the temperature from 30 to 50°C significantly increased DPPH inhibition from 67.97 to 77.16% ($p \leq 0.05$). However, when the temperature was raised to 70 °C, DPPH inhibition declined to 69.84%. Fig. (3. b) shows the interaction effects of temperature and flow rate on DPPH inhibition. At 27.5 MPa and 300 W (minimum pressure and microwave power), DPPH inhibition at 30 ℃ was 72.43%, and raising the temperature to about 45°C increased it to 78.87% ($p \leq 0.05$). Under the same conditions, when the temperature was raised to 70 °C, DPPH inhibition decreased to 71.12%. Also, changing the flow rate from 1 to 3 mL/min increased DPPH inhibition from 72.43 to 74.05%. Moreover, Fig. (3. c) shows that at 27.5 MPa and 1 mL/min (minimum pressure and flow rate), the measured DPPH inhibition was 60.70%. However, in the same conditions, increasing the temperature up to 55 °C improved DPPH inhibition to 72.27% but, when the temperature was raised to 70°C, DPPH inhibition declined to 68.99%. Fig. (3. e) demonstrates the interaction effects of pressure and microwave power on DPPH inhibition. At 45 °C and 1 mL/min (minimum temperature and flow rate), DPPH inhibition was 73.85%, but increasing the power to 450 W increased DPPH inhibition to 86.74% ($p \leq 0.05$). However, increasing the power levels to higher than 450 W reduced DPPH inhibition. Moreover, increasing the pressure from 15 to 27 MPa improved free radical inhibition from 73.85% to 78.90%, but further pressure increases reduced DPPH inhibition to 70.50% ($p \leq 0.05$). Fig. (3. f) shows the interaction effects of microwave power and flow rate. At 45 °C and 27.5 MPa (minimum temperature and pressure), DPPH inhibition was 75.34%, and increasing the flow rate to 3 mL/min increased it to 75.61%. Also, increasing the microwave power to 300 W improved inhibition to 89%, and increasing it to 450 W increased inhibition to 90.47%, but DPPH inhibition declined at higher microwave power. In total, the antioxidant activity level at the optimal center point (2 mL/min, 300 W, 45 °C, and 27.5 MPa) and based on the regression equation (Eq.5) was 89%.

$$
\begin{aligned}
Antioxidant\ activity\ (\%) = {} & 58.9 + 0.316x_1 + \quad (5) \\
& 1.19x_2 + 2.28x_3 + 10.12x_4 - 8.87x_1^2 + 0.335x_2^2 + \\
& 3.72x_3^2 + 3.2x_4^2 + 0.732x_1\,x_2 + 1.5x_1\,x_3 - \\
& 5.51x_1\,x_4 + 0.6598x_2\,x_3 + 3.68x_2\,x_4 + 2.12x_3\,x_4
\end{aligned}
$$

The antioxidant properties of Beetroot extract depend on Betalains and phenolic compound contents, but phenolic compounds are better inhibitors of free radicals than Betalains. Nevertheless, Betalains are also particularly capable of reducing metal ions [24]. According to the results, the temperature had a significant effect on the extraction of antioxidant compounds, and the highest antioxidant level was measured at 40-50 °C. However, further temperature increases noticeably decreased antioxidant compounds due to their sensitivity and degradation resulting in higher free radical levels. Impurities also increased at high temperatures, which could negatively affect antioxidant activity. Researchers have reported that levels of phenolic compounds significantly affect the antioxidant activity and free radical inhibition due to their high ability to reduce free radicals and donate hydrogen to them [25]. The results show that antioxidant levels were low at low pressures, increased when pressure was raised to 30 MPa, but declined significantly with further pressure increases ($p \leq 0.05$). Increasing pressure is effective in the degrading extracellular matrix by exerting greater force on the tissues. Also, the decrease in antioxidant compounds with pressure increases can be attributed to their structural degradation. Therefore, free radical inhibition decreases at high pressures due to the reduction in antioxidant compounds [24]. According to the results, the highest antioxidant levels were observed at a flow rate of 2 mL/min, but excessive increases in flow rate reduced antioxidant compounds. This was due to the continuous and desirable contact between the fresh fluid and the sample. Higher and lower flow rates reduced free radical inhibition due to the insufficient efficiency of the solvent in separating and extracting phenolic compounds. The results in Table 2 indicate that different microwave power levels had the most significant effect on free radical inhibition ($p \leq 0.05$). The reason for this is that microwaves increase temperature and thereby break the covalent bonds between phenolic compounds and other substances and also cell walls thus improving the release of phenolic compounds during the extraction process [7, 12, 16, 18]. In other words, water evaporates when exposed to high
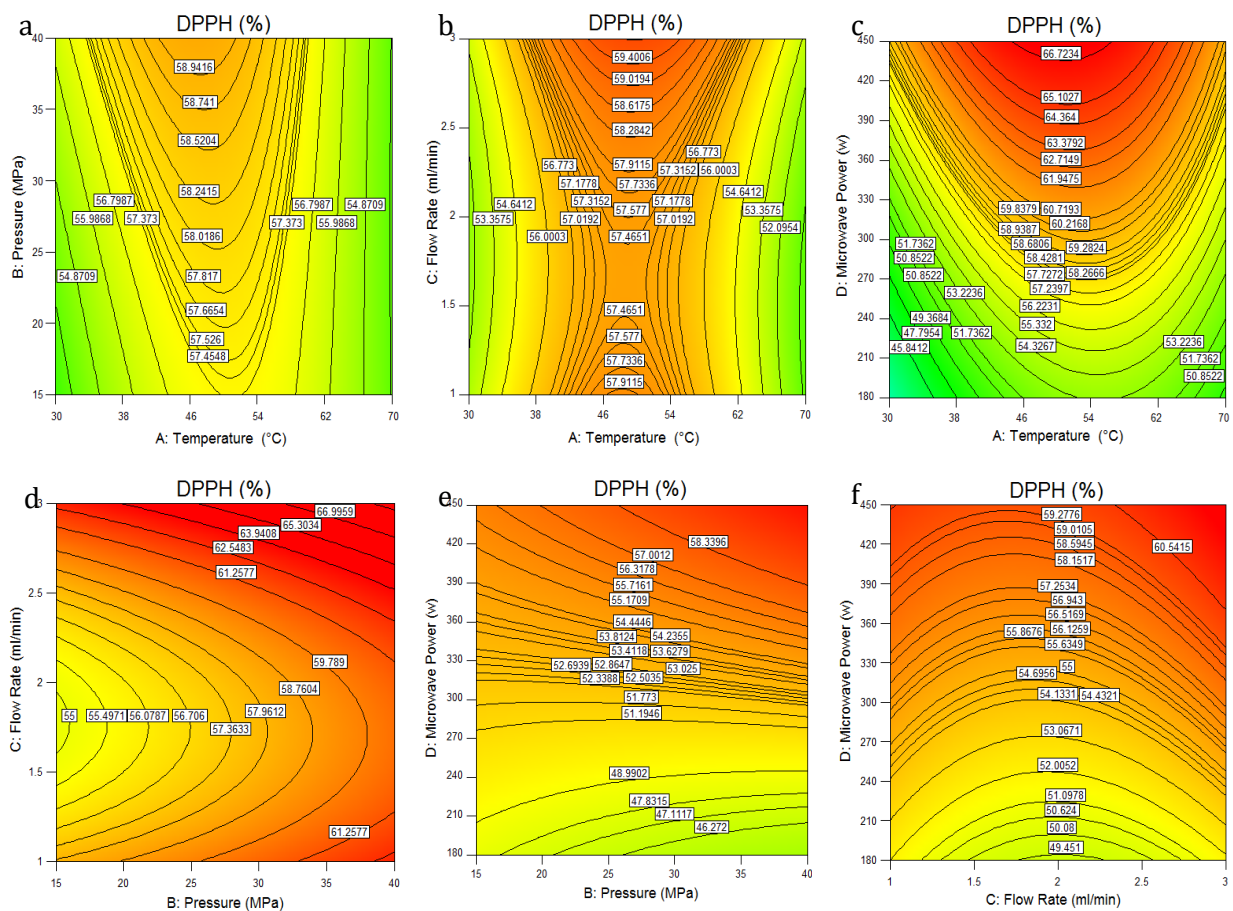
Fig. 3: Interaction effects of temperature and pressure (a), temperature and flow rate (b), temperature and microwave power (c), pressure and flow rate (d), pressure and microwave power (e), flow rate and microwave power (f) in DPPH.

thermal tension, local pressure, and microwave heat and, due to plant cell turgor, great pressure is exerted on cell walls that finally breaks them thereby facilitating the release of the active compounds from the degraded cells into the surrounding solvent thus improving extraction efficiency. The high temperatures created by microwaves can hydrolyze all bonds formed by cellulose, the main constituent of cell walls, within a few minutes and convert it into soluble components. The high temperatures created in cell walls during extraction with microwaves increase cellulose dehydration and reduce its mechanical strength, which allows the solvent easy access to the compounds inside cells. At the same time, power levels of more than 400 W significantly reduced antioxidant compounds. Higher power levels increase the temperature and decompose heat-sensitive antioxidant compounds like polyphenols thus significantly reducing antioxidant capacity [26]. *Khajenoori* and *Haghighi* (2014),

stated that increases in the number of hydroxyl groups in the structure of flavonoids increase their thermal degradation. At the same time, the presence of sugars and methoxyl groups in their structure protects them against thermal degradation [27].

Extraction efficiency

Based on Table 2, the effects of temperature, pressure, flow rate, and microwave power on the extraction efficiency of phenolic compounds were significant ($p \leq 0.05$). Fig. (4. a) shows the interaction effects of temperature and pressure on extraction efficiency. Increasing the temperature to 50 °C and the pressure to 30 MPa significantly increased the extraction percentage, but higher temperatures and pressures decreased it ($p \leq 0.05$). The regression equation (Eq. (6)) demonstrated that the total variation in extraction efficiency was 83.41%.
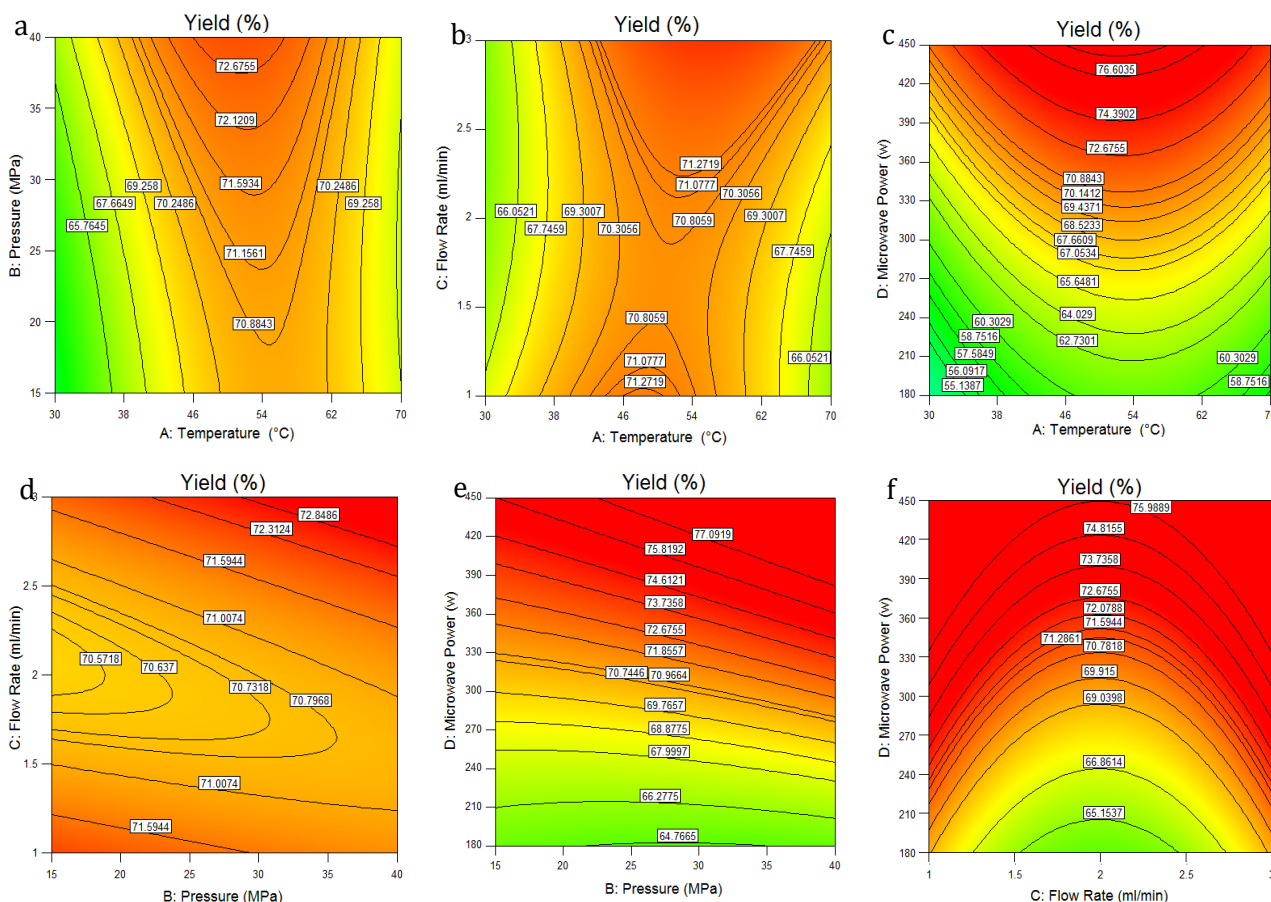
Fig. 4: Interaction effects of temperature and pressure (a), temperature and flow rate (b), temperature and microwave power (c), pressure and flow rate (d), pressure and microwave power (e), flow rate and microwave power (f) in extraction yield.

Extraction efficiency (%) = $70.96 + 1.23x_1 +$ (6)
$1.40x_2 + 0.6829x_3 + 6.61x_4 - 5.59x_1^2 + 0.4455x_2^2 + 3.59x_3^2 + 1.22x_4^2 + 0.5447x_1x_2 + 2.12x_1x_3 - 2.29x_1x_4 + 1.27x_2x_3 + 1.32x_2x_4 + 0.170x_3x_4$

As mentioned earlier, Betalains content decreases at higher temperatures due to its degradation at high temperatures. The main stage in Betalains degradation by heat is bond cleavage at C_{11} to form cyclo-Dopa 5-O-glucoside and balsamic acid [28, 29, 30]. Increasing the temperature to a certain point at low pressure somewhat increased extraction efficiency but beyond that point decreased it because the density of the supercritical fluid and the solubility of compounds decreased with temperature increases. At high pressures, however, increasing temperatures to a certain point somewhat increased extraction efficiency, but beyond that point reduced it due to the reduction in mass transfer rate [23, 29]. According to Fig. (4. b), increasing temperature and

flow rate improved extraction efficiency ($p{\leq}0.05$). This was caused by the increase in vapor pressure and the intermolecular interactions in the fluid that ultimately increased extraction efficiency. The supercritical fluid has a very low surface tension, which increases the contact surface between the phases in the separation and extraction process and increases the mass transfer rate. Moreover, increases in flow rate also improved extraction efficiency because the fresh fluid came into contact with the sample and there was a greater exchange between the sample matrix and the fluid. Fig. (4. c) shows that higher temperatures and microwave power increased extraction efficiency. It is noteworthy that temperature increases reduce carbon dioxide density, which decreases the solubility of the compounds and hence extraction efficiency. However, temperature increases also increase the vapor pressure of the solute and intensify intermolecular interactions of carbon dioxide with other

compounds thereby improving extraction efficiency. Meanwhile, higher vapor pressure has the dominant effect of reducing density and increasing the extraction efficiency of the compounds. Notably, accelerating the process and producing less heat by microwaves better preserve the compounds and improve extraction efficiency. However, high microwave power may reduce extraction efficiency due to the degradation of heat-sensitive compounds [31]. *Zhang et al.* (2013), used *Radix astragali* roots and microwave power of 200-1000 W to extract flavonoids. Their results showed that increasing temperature beyond 110 °C reduced extraction efficiency due to the instability of flavonoids. Fig. (4. e) shows that increases in pressure and microwave power improved extraction efficiency. Note that microwave pretreatment at high pressure had a greater and more significant effect on the quantities of the extracted compounds [32]. As mentioned above, compound extraction depends on the solvent's density and permeability. Pressure increases increased solvent density and permeability thereby improving extraction efficiency [22]. In addition, the use of microwaves increased extraction efficiency due to cellular degradation.

CONCLUSIONS

This study investigated the effects of pressure, temperature, flow rate, and microwave power (as pretreatment) on the extraction efficiency of Betalain from Beetroot using supercritical carbon dioxide extraction. The results suggest that microwave pretreatment significantly increases total phenolic compounds, antioxidant capacity, and efficiency of Betalain extraction from Beetroot. Given the results, Betalain was extracted from Beetroot using supercritical carbon dioxide and microwave pretreatment at 45 °C, 27.5 MPa, 2 mL/min, and 300 W. The results show that microwaves and supercritical fluids can be used as an effective extraction method that preserves heat-sensitive phytochemicals.

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