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Improvement of extraction and stability of anthocyanins, the natural red pigment from roselle calyces using supercritical carbon dioxide extraction

Zuhaili Idham [a,b,*], Nicky Rahmana Putra [a], Ahmad Hazim Abdul Aziz [a], Ahmad Syahmi Zaini [b], Noor Azwani Mohd Rasidek [a], Norlisa Mili [b,c], Mohd Azizi Che Yunus [a,b,*]

[a] Centre of Lipids Engineering and Applied Research, Ibnu Sina Institute for Scientific and Industrial Research, Universiti Teknologi Malaysia, 81310, UTM, Johor Bahru, Malaysia
[b] School of Chemical and Energy Engineering, Faculty Engineering, Universiti Teknologi Malaysia, 81310, UTM, Johor Bahru, Malaysia
[c] Faculty of Engineering, Universiti Malaysia Sarawak (UNIMAS), Kota Samarahan, Malaysia

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ABSTRACT

In this work, anthocyanins, a natural red pigment, were isolated from roselle calyces using supercritical carbon dioxide (SC-CO_2). Three process conditions, namely pressure, temperature, and co-solvent ratio (ethanol-water), were investigated using a three-factorial design from response surface methodology (RSM). The method was used to model the extraction of anthocyanins, total phenolic content, total flavonoid content, and colour characteristics (i.e., lightness (L*), chroma (C*), and hue (h°)). The best conditions were 27MPa, 58 °C, and 8.86 % co-solvent ratio at maximal anthocyanin, phenolic, and flavonoid content, with minimal L* and C* values. The anthocyanin production was 1197 mg/100 g of dried roselle calyces. Next, the high relative value of cyanidin 3-sambubioside and phenolic compound in SC-CO_2 extract was analyzed using an ultra-high-performance liquid chromatogram (UHPLC). Anthocyanins stored at 4 °C, 25 °C, and 37 °C had average reaction rate (k) and half-life ($t_{1/2}$) values of 0.0032 and 216 days, 0.0098 and 70 days, and 0.024 and 28 days for SC-CO_2 and 0.0093 and 74 days, 0.0222 and 31 days, and 0.0444 and 15 days for solid-liquid extraction (SLE), respectively, in first-order degradation kinetics. These findings demonstrated that the studied conditions using SC-CO_2 provided lower degradation rates and a longer $t_{1/2}$ than the conventional SLE methods.

1. Introduction

Natural colourants have recently gained popularity due to the health benefits of the natural compounds used. The demand is due to several studies indicating that synthetic food colourants may have adverse effects such as high toxicity, allergic reactions, and carcinogenic potential [1]. Therefore, plant-derived anthocyanin pigments may serve as a natural substitute for synthetic red colourants. Additionally, these molecules have been linked to potential health benefits such as anti-inflammatory, antioxidant, and anti-diabetic properties [2–4]. Anthocyanins also provide excellent added value due to their dual nature as a colourant and a highly nutritious pigment.

Roselle (*Hibiscus sabdariffa*) calyces are an excellent source of anthocyanins [5]. Roselle is grown in tropical regions worldwide, including Malaysia, the Philippines, India, Mexico, and Senegal [6–8]. In Malaysia, roselle is cultivated commercially throughout the year, especially in Terengganu, Johor, Penang, Selangor, Perak, and Kedah [9]. Besides anthocyanins, roselle calyces contain many organic acids, phenolic, and glucoside compounds [3]. The recovery of anthocyanins from roselle dry calyces as a natural food colourant could expand the industry application for roselle crops.

Anthocyanins are produced in the cytoplasm and are stored in vacuoles. Several experiments have been conducted to determine the efficacy of various solvents for extracting and recovering anthocyanin compounds. Water-alcohol mixes have proven to be more efficient than pure solvent systems [10–12]. They are generally extracted using an acidified solvent extraction technique, which typically involves adding 1% acid (v/v), such as hydrochloric acid (HCl), acetic acid, or other acids to break cell walls and remove it from vacuoles [12–14]. Acids are needed to maintain a low pH environment where anthocyanins are in their more stable flavylium form [15]. This method, however, has several drawbacks, including stability issues, a long extraction time,

* Corresponding authors at: Centre of Lipids Engineering and Applied Research, Ibnu Sina Institute for Scientific and Industrial Research, Universiti Teknologi Malaysia, 81310, UTM, Johor Bahru, Malaysia.
E-mail addresses: zuhaili@cheme.utm.my (Z. Idham), r-azizi@utm.my (M.A.C. Yunus).

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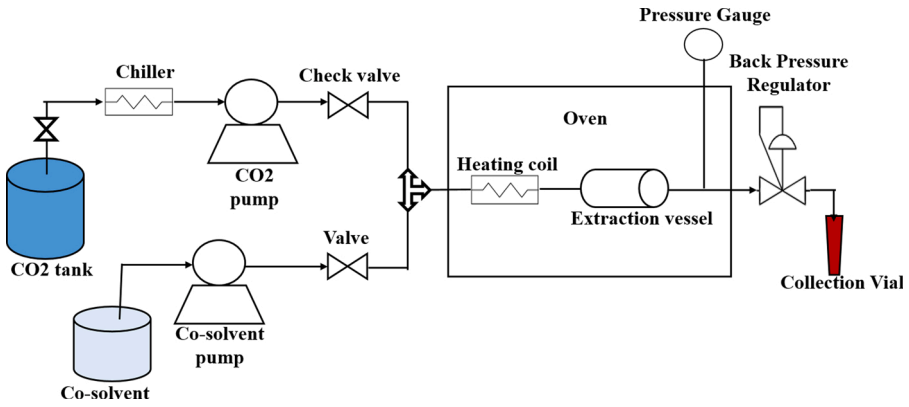

Fig. 1. SC-CO_2 schematic diagram at a CLEAR laboratory.

batch-to-batch variations, a large amount of solvent evaporation, and low selectivity with a high number of impurities [14,16–18]. Additionally, anthocyanins degrade quickly, especially under extraction and storage conditions due to high temperature and oxygen [16]. Therefore, developing innovative extraction methods with higher anthocyanin recovery yields and prolonged colour stability at storage temperatures is vital.

Using environmentally friendly technology such as supercritical carbon dioxide (SC-CO_2), anthocyanin recovery could be optimized while minimizing degradation during the process. The extraction process is carried out in a closed system with no oxygen and under mild conditions (i.e., low critical pressure of 7.38 MPa and temperature of 31.1 °C) [19]. In addition, SC-CO_2 extraction may disrupt intracellular electrolyte balance by modifying cell membranes and removing essential cell constituents and membranes [20]. The addition of ethanol and water (co-solvent) could increase the solvating power of carbon dioxide (CO_2) and enhance the solubility of polar compounds such as anthocyanins and phenolic compounds. Due to the low cost and possibility to be used directly in foods and pharmaceuticals, ethanol and water, with polarities of 5.2 and 9.0, respectively, have been widely used as co-solvents as both are considered green [21–23].

Meanwhile, several studies have reported that ethanol-water (EtOH/H_2O) mixtures function efficiently as a co-solvent in extracting anthocyanins and polyphenols with higher yields [24–26]. When water reacts with CO_2, carbonic acid is formed, which may lower the pH of the extraction system [22]. The acidic pH state may facilitate penetration into the roselle calyces plant matrix and remove anthocyanins within the vacuole. Lower pH conditions may also be beneficial for stabilizing the effect of anthocyanin molecules without organic acid.

We previously reported the feasibility of anthocyanins from roselle calyces employing SC-CO_2 extraction with EtOH/H_2O as a co-solvent to determine the effect of flow rate and particle size [27]. Temperature, pressure, and co-solvent types significantly impact the recovery of anthocyanins from plants [20,22,28]. When the source of the compound to be extracted varies, it is crucial to optimize the extraction conditions [29]. However, there is completely missing information on optimizing SC-CO_2 conditions for anthocyanins in roselle calyces. Therefore, this work examined the optimal extraction conditions of SC-CO_2 for anthocyanins from roselle calyces.

The effects of temperature, pressure and co-solvent ratio on the recovery of anthocyanins, polyphenols, and red colour characteristics from roselle calyces were explored. This study also determined the optimal parameters that allow the most efficient extraction yield using Response Surface Methodology (RSM). Subsequently, the storage stability of roselle anthocyanins was investigated as there is insufficient evidence on colour analysis and storage stability on the feasibility of the SC-CO_2 as a better alternative red pigment extraction method. The optimized result was then compared to a conventional solid-liquid extraction (SLE) process that used acidified EtOH/H_2O as a solvent to differentiate extract selectivity and storage stability for future commercial applications.

2. Materials and method

2.1. Chemicals and standard

CO_2 with 99.95 % purity was purchased from KRAS Instrument (Johor, Malaysia). Ethanol absolute 99.99 % (HaymanKimia) and acetic acid (VChem) were brought from VNK Supply and Services (Johor, Malaysia). For phytochemical characterization, all the chemicals used in this study were supplied from Sigma-Aldrich (Selangor, Malaysia). Those chemicals were potassium chloride, sodium acetate trihydrate, Folin–Ciocalteau, sodium carbonate, aluminium nitrate, potassium acetate, sodium chloride, and hydrochloric acid. Meanwhile, the standard reagent, gallic acid, and quercetin were obtained from Merck (Sigma-Aldrich (M) Sdn Bhd). These chemicals were used without further treatment or purification.

2.2. Preparation of roselle calyces

Roselle calyces var. UMKL-1 was obtained from a plant growing in the Terengganu from Ekomekar Resources Sdn Bhd. Roselle calyces were harvested and sun-dried at comparable stages of ripening (physiological maturity). Dried roselle calyces were ground in a commercial blender and sieved with a sieve shaker at particle sizes ranging from 255 to 355 μm. The sample was stored in a freezer at −20 °C in a tight-sealed storage plastic until further use.

2.3. SC-CO_2 extraction

The SC-CO_2 extraction was carried out at the Centre of Lipid Engineering and Applied Research (CLEAR), Universiti Teknologi Malaysia (UTM). Fig. 1 depicts the SC-CO_2 schematic diagram. The unit comprises a CO_2 tank, circulating water bath (WiseCircu), CO_2 pump (Lab Alliance, Supercritical 24), pressure gauge, extraction chamber with 5 ml extraction vessel, and back pressure regulator (BPR) (Jasco, Model BP-2080) with a restrictor valve. The co-solvent was pumped with a 10 ml Series II pump (Scientific Systems, Inc., USA). The extraction chamber was used to regulate the process temperature, which the BPR controlled. The restrictor valve was released when depressurization attained the required pressure. Meanwhile, to prevent CO_2 frost in the collecting valve, the restrictor valve temperature was set to 50 °C.

The 5 ml extractor was filled with approximately 1.5 g of dry roselle calyces (particle size $<$ 0.355 mm) mixed with glass beads to fill the remaining space and improve contact with SC-CO_2. A small amount of cotton wool was covered on both sides of the extractor to prevent any solid material residue from leaving the vessel. The pre-cooled liquefied CO_2 (chiller was set at 6 °C) was pumped into the system at a 4 ml/min

Table 1
Variables and levels value range in SC-CO_2 extraction condition.

Variable, factors (unit)	Levels		
	Minimum	Medium	Maximum
Temperature, X_1 (°C)	40	55	70
Pressure, X_2 (MPa)	10	20	30
Co-solvent ratio, X_3 (%)	5	7.5	10

total flow rate to achieve the desired pressure controlled by the BPR. Co-solvent (EtOH/H_2O, 75/25 v/v) was pumped at a 5–10 % ratio of the total flow rate. During the 120-minute continuous dynamic extraction, anthocyanin extract was collected at the collection unit every 15 min at room temperature and combined with the yield after depressurization of CO_2. The yield was collected in Falcon tubes and stored in the freezer at −20 °C until further analysis.

2.4. Experimental design

RSM was used to model and optimize anthocyanins, polyphenol and red colour extract from dry roselle calyces. All experimental yield was calculated on a dry plant weight basis. A three-factorial design was used to determine the recovery of anthocyanins via SC-CO_2 with EtOH/H_2O as a co-solvent, with a function of temperature, pressure, and co-solvent ratio. The three-factorial design is an experimental design used to gather as much information about a process as possible while not excluding any range of experimentation. Based on SC-CO_2 and anthocyanins property with equipment limitations, 40–70 °C [30], 10–30 MPa, and a ratio of co-solvent at 5–10 % [31] were selected. The experiments were designed after selecting independent variables and their ranges. Table 1 shows the coded values at three levels: maximum, medium, and minimum. Meanwhile, Table 2 shows that 29 runs were generated, with triplicates at the centre points. The experimental errors were calculated based on the replications, which is assumed to be constant in conjunction with the domains.

The given experimental data were subjected to an analysis of variance (ANOVA) from a Design-Expert software (Version 6, Stat-Ease, Inc., Minneapolis, USA). The coefficient of determination (R^2) was used to assess the quality of the fit of the polynomial model equation. Meanwhile, the values of adjusted R^2 (Adj R^2) were used to assess model adequacy. Each term in the equation is vital for estimating the goodness of fit in each scenario. The ANOVA was used to evaluate the effect and regression coefficients of each linear, quadratic, and interaction factor. The significance level was considered as a p-value of less than 0.05. Regression coefficients and regression models were used for statistical calculations and the generation of three-dimensional graphics.

2.5. Conventional SLE

Meanwhile, ground dried roselle calyces in a conventional SLE were combined with EtOH/H_2O (75:25 v/v) in 1:32 ratios with 1% acetic acid as reported by [18,32], with some modifications for the comparative method. After homogenizing the extract in a vortex (15 s), it was placed in a thermal bath (55 °C) with a constant stirring rate for two hours. After extract centrifugation, the solvent was evaporated by a vacuum evaporator at 35 °C. Subsequently, the SLE extract was kept at −18 °C in a dark area for further analysis.

2.6. Determination of total anthocyanins content (TAC)

The TAC of roselle calyces extract was determined using a pH differential method [33]. Two dilutions of the same sample were formed using potassium chloride solution (0.025 M) and sodium acetate trihydrate solution (0.4 M). Both were adjusted to pH 1.0 and 4.5 with

Table 2
Three factorial experimental design and responses results for SC-CO_2 of red roselle calyces extract.

Run	Factors			Responses						
	X_1 Temperature (°C)	X_2, Pressure (MPa)	X_3, Co-solvent Ratio (%)	TAC (mg Cya-3-glu/100g dry calyces)	TPC (mg GAE/100g dry calyces)	TFC (mg QUE/100g dry calyces)	Lightness (L*)	Chroma (C*)	Hue (h°)	RGB colour
1	40	10	5	431.05 ± 1.21	71.70 ± 0.56	672.49 ± 0.69	39.50 ± 0.01	78.79	34.89	
2	40	20	5	427.58 ± 0.92	68.45 ± 0.89	584.27 ± 0.97	39.61 ± 0.05	77.01	39.13	
3	40	30	5	465.12 ± 2.13	91.53 ± 0.75	726.49 ± 0.23	36.37 ± 0.03	74.52	42.05	
4	55	10	5	467.12 ± 0.82	69.70 ± 0.96	628.93 ± 0.74	39.68 ± 0.02	80.35	40.64	
5	55	20	5	344.53 ± 0.53	78.10 ± 1.11	611.82 ± 0.29	36.82 ± 0.02	77.43	40.99	
6	55	30	5	671.87 ± 0.98	102.22 ± 0.69	656.49 ± 0.47	36.50 ± 0.01	83.04	43.29	
7	70	10	5	433.41 ± 0.87	95.30 ± 0.33	553.82 ± 0.67	36.58 ± 0.03	85.24	43.50	
8	70	20	5	215.22 ± 0.97	52.85 ± 0.58	517.16 ± 0.43	47.64 ± 0.04	68.26	29.24	
9	70	30	5	363.10 ± 0.86	88.22 ± 0.49	666.93 ± 0.51	28.22 ± 0.03	69.52	41.29	
10	40	10	7.5	350.83 ± 0.59	91.70 ± 0.54	788.04 ± 0.67	34.56 ± 0.03	81.25	43.01	
11	40	20	7.5	878.49 ± 1.35	120.27 ± 0.25	721.16 ± 0.36	28.35 ± 0.01	73.67	41.03	
12	40	30	7.5	868.70 ± 0.88	110.45 ± 0.74	788.04 ± 0.43	30.86 ± 0.01	76.73	42.32	
13	55	10	7.5	741.21 ± 0.23	96.90 ± 0.14	656.93 ± 0.65	32.69 ± 0.02	76.15	42.65	
14	55	20	7.5	1156.72 ± 0.49	123.53 ± 0.26	838.04 ± 0.84	22.40 ± 0.03	62.75	37.95	
15	55	20	7.5	1134.01 ± 0.35	111.02 ± 0.77	822.27 ±0 .32	26.73 ± 0.04	70.50	40.34	
16	55	20	7.5	1494.62 ± 0.77	138.50 ± 0.39	876.49 ± 0.58	19.15 ± 0.03	57.29	35.18	
17	55	30	7.5	963.86 ± 1.16	115.36 ± 0.51	809.61 ± 0.63	29.44 ± 0.05	76.45	41.22	
18	70	10	7.5	870.35 ± 0.30	104.62 ± 0.64	707.62 ± 0.28	32.21 ± 0.02	80.41	42.63	
19	70	20	7.5	1082.04 ± 1.47	111.13 ± 0.86	776.27 ± 0.87	27.04 ± 0.04	69.61	40.78	
20	70	30	7.5	928.90 ± 0.95	118.67 ± 0.67	703.61 ± 0.57	21.24 ± 0.03	60.57	37.18	
21	40	10	10	612.87 ± 0.24	77.87 ± 0.75	644.93 ± 0.62	36.46 ± 0.02	82.01	42.98	
22	40	20	10	927.66 ± 0.61	123.30 ± 0.87	681.82 ± 0.74	30.96 ± 0.02	79.86	41.57	
23	40	30	10	1045.62 ± 0.38	113.13 ± 0.35	799.23 ± 0.26	21.87 ± 0.01	61.20	37.93	
24	55	10	10	666.17 ± 1.22	70.56 ± 0.28	586.71 ± 0.98	38.14 ± 0.01	85.19	43.53	
25	55	20	10	1049.18 ± 1.69	127.65 ± 0.85	770.49 ± 0.21	24.57 ± 0.02	67.37	38.90	
26	55	30	10	973.12 ± 1.32	120.62 ± 0.35	716.27 ± 0.57	24.17 ± 0.04	66.33	38.80	
27	70	10	10	599.60 ± 0.97	80.79 ± 0.42	699.38 ± 0.73	33.58 ± 0.03	78.96	42.93	
28	70	20	10	1056.04 ± 1.25	128.33 ± 0.68	726.27 ± 0.59	25.92 ± 0.01	70.35	39.32	
29	70	30	10	1098.61 ± 0.28	125.02 ± 0.71	731.16 ± 0.60	28.54 ± 0.01	73.22	41.28	

TAC = Total anthocyanins content, cya 3-glu = cyanidin 3-glucoside.
TPC = Total phenolic content; GAE = Gallic acid.
TFC = Total flavonoid content, QUE = Quercetin.

hydrochloric acid, respectively. The absorbance was measured with an ultraviolet-visible (UV–vis) spectrophotometer (Jasco, Japan) at wavelengths of 520 and 700 nm and calculated based on Eq. (1).

Absorbance, A: (A520 - A700) pH1.0 – (A520 - A700) pH4.5 (1)

The TAC was calculated as mg cyanidin-3-glucoside (cya 3-glu)/100 g of dry roselle calyces as in Eq. (2);

Total anthocyanins content, TAC (mg/L): A x MW x DF x 1000/Ɛ x L (2)

whereby A is absorbance, MW is the molecular weight of cyanidin 3-glucoside (449.2 g/mol), DF is a dilution factor, $\mathcal{E}$ *is* an extinction coefficient of cyanidin 3-glucoside (26,900 L/cm/mol), and L is the cell path length (1 cm).

2.7. Determination of total phenolic content (TPC)

The TPC in roselle calyces extract was determined using the Folin-Ciocalteu method [34]. In this study, gallic acid, a type of phenolic acid, was used as a standard. Briefly, 0.1 ml of the sample was transferred into a test tube. The mixture was then added with 1.0 ml of Folin-Ciocalteu reagent and left for 3 min. Next, a test tube was filled with 300 µl of sodium carbonate solution and left at room temperature for 90 min. A UV–vis spectrophotometer was then used to measure mixture absorbance at 765 nm. The TPC results were calculated based on a gallic acid standard curve and expressed as mg of gallic acid equivalents (GAE)/100 g of dry roselle calyces.

2.8. Determination of total flavonoid content (TFC)

The TFC of the roselle calyces extract was analyzed according to the published method [35] with slight modifications. Approximately, 1 ml of the roselle calyces extract diluted in ethanol (concentration of 1 mg/mL) was mixed with 8.6 ml of 80 % ethanol solution, 0.2 ml of 10 % aluminium nitrate, and 0.2 ml of 1 M potassium acetate. Afterwards, the mixture was left at room temperature for 40 min. The absorbance of each sample mixture was set to 415 nm using a UV–vis spectrophotometer. TFC values in roselle extract were calculated using a quercetin standard curve and expressed as quercetin equivalent (mg QE/g 100 dry roselle calyces).

2.9. Analysis of colour properties

The CIELAB or CIE L*, a*, b* colour space diagram was used to determine the colour of the anthocyanins in roselle calyces extract using a UV–vis Spectrophotometer (Jasco, Japan) with a D65 light source and 10 ° observation angle. The experimental responses were Lightness (L*), Chroma (C*), and hue (h°) calculated from a* and b* values using Eqs. 3 and 4. The colour parameters were measured as a mean of three replicates.

$$C^* = \sqrt{a^{*2} + b^{*2}} \quad (3)$$

$$h^\circ = \tan^{-1} b^*/a^* \quad (4)$$

These values were later converted to red, green, and blue colour values (RGB), using the OpenRGB (Logicol) software.

Table 3
ANOVA for the predicted quadratic polynomial models for properties of red pigment roselle extract.

Source	d.f	TAC (mg Cya 3-glu)/100g		TPC (mg GAE/100g)		TFC (mg QUE/100g)		Lightness (L*)		Chroma (C*)		Hue (h°)	
		Coefficient	Prob > F	Coefficient	Prob > F	Coefficient	Prob > F	Coefficient	Prob > F	Coefficient	Prob > F	Coefficient	Prob > F
Model	9	1099.01	< 0.0001	119.11	0.0012	786.33	0.0029	26.17	0.0023	68.27	0.0327	ns	0.2709
Linear													
T	1	35.52 ns	0.3951 ns	2.03 ns	0.5633 ns	−18.00 ns	0.2235 ns	−0.98 ns	0.3780 ns	−1.61 ns	0.2769 ns	−0.38 ns	0.6005 ns
P	1	122.57	0.0073	12.56	0.0017	36.60	0.0192	−3.68	0.0030	−4.82	0.0033	−0.63 ns	0.3804 ns
CR	1	233.88	< 0.0001	13.84	0.0007	40.98	0.0099	−4.26	0.0009	−1.65 ns	0.2647 ns	0.68 ns	0.3478 ns
Quadratic													
T*T	1	−126.31 ns	0.0755 ns	−3.25 ns	0.5731 ns	−17.46	0.4672 ns	1.31 ns	0.4703 ns	0.47 ns	0.8427 ns	−0.42 ns	0.7243 ns
P*P	1	−143.59	0.0458	−8.08 ns	0.1705 ns	−9.46	0.6921 ns	1.49 ns	0.4115 ns	5.27	0.0379	2.76	0.0280
CR*CR	1	−260.86	0.0010	−17.92	0.0052	−103.13	0.0003	5.58	0.0054	3.39 ns	0.1675 ns	−0.55 ns	0.6416 ns
Interaction													
T*P	1	−41.45 ns	0.4174 ns	−1.89 ns	0.6603 ns	−5.60 ns	0.7528 ns	−0.25 ns	0.8547 ns	−0.97 ns	0.5853 ns	−0.89 ns	0.3135 ns
T*CR	1	40.01 ns	0.4335 ns	1.26 ns	0.7683 ns	23.03 ns	0.2043 ns	0.15 ns	0.9115 ns	0.56 ns	0.7519 ns	0.26 ns	0.7688 ns
P*CR	1	89.18 ns	0.0905 ns	7.02 ns	0.1127 ns	10.06 ns	0.5726 ns	−1.58 ns	0.2483 ns	−2.34 ns	0.1981 ns	−1.59 ns	0.0820 ns
Lack of fit	17		0.7309		3706.60		70035.00		371.06		678.50		0.5005
CV		22.51			14.16		8.27		14.63		7.81		7.44
R^2		0.7996			0.7118		0.6721		0.6892		0.5613		0.3925
Adj R^2		0.7046			0.5965		0.5628		0.5420		0.3535		0.1047

T = Temperature.
P = Pressure.
CR = Co-solvent ratio.
ns = no significance at level P < 0.05.
CV = Coefficient of variations.
TAC = Total anthocyanins content, cya 3-glu = cyanidin 3-glucoside.
TPC = Total phenolic content; GAE = Gallic acid.
TFC = Total flavonoid content, QUE = Quercetin.

2.10. Identification of major anthocyanins, phenolic, and flavonoid compounds

This study used an ultra-high-performance liquid chromatogram (UHPLC) to analyze the optimized SC-CO_2 and SLE extracts. Using a Waters Acquity ultra-performance liquid chromatography (UPLC) (Milford, USA) consisting of a binary pump, a vacuum degasser, an auto-sampler, and a column oven, a chromatographic analysis was conducted with HSS T3 column (100 mm x 2.1 mm x 1.8 m) at 40 °C. A linear binary gradient of water (0.1 % formic acid) and acetonitrile were used as mobile phases A and B, respectively. During the process, the composition in mobile phase B was changed based on these conditions: 0 m in. 1% B; 0.5 m in. 1% B; 16.00 m in. 35 % B; 18.00 m in. 100 % B; and 20.00 m in. 1% B [36]. The injection volume and flow rates were 1 µl and 0.6 mL/min, respectively.

A mass spectrometry analysis was performed on a Water Vion IMS quadrupole time-of-flight mass spectrometry QToF, equipped with a Lock Spray ion source. Under the following specific conditions, the ion source was operated in negative electrospray ionisation (ESI) mode; capillary voltage = 1.50 kV; reference capillary voltage = 3.00 kV; source temperature = 120 °C; desolvation gas temperature = 550 °C; desolvation gas flow = 800 L/h, and cone gas flow = 50 L/h. Nitrogen (99.5 %) was applied as a desolvation and cone gas, while argon (99.999 %) was used as a collision-induced-dissociation (CID) gas. A high-definition MSE (HDMSE) mode data were acquired in the range *m/z* 50–1500 at 0.1 s/scan. Therefore, two independent scans with different collision energies (CE) were alternatively obtained during the run: a low-energy (LE) scan at a fixed CE of 4 eV and a high-energy (HE) scan where the CE was ramped from 10 to 40 eV.

2.11. Storage stability of anthocyanins-rich extract

The storage stability of the extracts was evaluated using anthocyanin concentrations (22.5 mg/L) in buffer solutions (pH 3), containing 0.025 M potassium chloride solution under optimal SC-CO_2 conditions and via SLE processes [18]. The solution was adjusted using 10 % sodium hydroxide or concentrated hydrochloric acid to obtain pH 3. For the stability tests, 2 ml of each sample was put into amber microtubes and stored at 4 °C, 25 °C, and 37 °C for 49 days. The TAC was calculated using samples taken at day 0, 3, 6, 9, 17, 30, and 49, while TPC, TFC, and colour properties (L*, C*, h°) were analyzed only at days 0 and 49.

Since the anthocyanin degradation in aqueous solutions follows first-order kinetics, therefore, the first-order reaction rate constants (k), $t_{1/2}$, change in the k at 10 °C (Q_{10}), and energy of activation (Ea) were determined using Eqs. (5)–(7):

$$\ln C_t = C_0 - k_t;\ t_{1/2} = -\ln 0.5 \times k^{-1} \quad (5)$$

$$Q_{10} = (k_2/k_1)^{10/(T2-T1)} \quad (6)$$

$$\ln k = \ln A - Ea/R\ (1/T) \quad (7)$$

where C_t is the TAC at time t, C_0 is the TAC at time zero; k is the reaction constant in days^{-1}; t is the time in days; A is the Arrhenius pre-exponential factor; E_a is the activation energy (kJ/mol); R is the gas constant (8.314 J/mol-K), and T is the temperature in Kelvin.

2.12. Data analysis

All analysis was carried out in triplicate and expressed as mean ± standard deviation (SD). Kinetic data were analyzed using linear regression analysis with Microsoft Excel 2010 software (Microsoft Corporation). The significant difference between means to compare the optimization data were determined by paired *t*-test in Statistica 8.0 (StatSoft Inc, USA). A p-value of 0.05 was used to verify the significance of the tests.

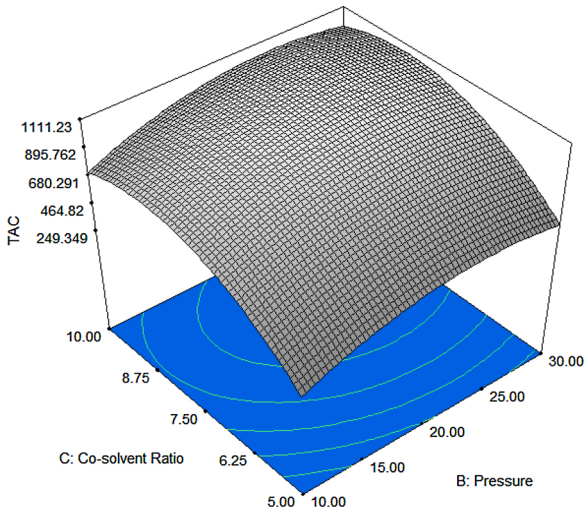

Fig. 2. Response surface of the total anthocyanins content (mg cya 3-glu/100 g dry roselle calyces) as a function of co-solvent ratio and pressure at a constant temperature (55 °C).

3. Results and discussion

A dynamic extraction procedure using SC-CO_2 and EtOH/H_2O as co-solvents was developed to obtain a natural red pigment from roselle calyces. RSM was employed to maximize anthocyanin recovery as a red pigment. The results from the extraction conditions of TAC, TPC, TFC, and colour quality (L*, C*, h°) were used for optimization (Table 2). The ANOVA for all significant response models is shown in Table 3. These parameters were calculated under optimum conditions. Subsequently, the optimum predicted value was compared to the experimental result to validate the model.

3.1. Effect of process condition on anthocyanins

The findings in Table 2 suggested that the linear coefficients of co-solvent ratio and pressure had a significant effect ($p < 0.05$) on the anthocyanins yield (TAC). On the other hand, the temperature did not affect within the specified range (40–70 °C). Additionally, quadratic terms of extraction pressure had a significant effect ($p < 0.05$) on TAC, but no interaction occurred between any factors that significantly affected the TAC value. Nonetheless, the R^2 of the predicted models was satisfactory (0.7996). This finding indicated that at least 80 % of the variation of the response variables could be precisely described by the regression models relating the independent variables and responses. The generated models significantly illustrated the actual relationships among the reaction parameters and sufficiently explained the data variation.

The F-value of Lack of Fit in this model indicated that the Lack of Fit is insignificant compared to the pure error. These values are consistent with the mathematical model. The repeatability (coefficient of variation) of the SC-CO_2 extraction method was 22.51 % (acceptable) when three design trials were included (centre points). CV < 10 is considered a very good CV number, 10–20 is good, 20–30 is acceptable, and CV > 30 is not acceptable. As a result, 22.51 % is acceptable, implying that the experiments were conducted quite consistently. Eq. (8) depicts the relationship between process variables and anthocyanin content by excluding all insignificant regression coefficients.

$$Y_1 = +\ 1099.01 + 122.57 * X_2 + 233.88 * X_3 - 143.59 * X_2 2 - 260.86 * X_3 2 \quad (8)$$

Based on Eq. (8), Y_1 is the TAC (mg cya 3-glu/100 g dry materials) in roselle calyces, X_2 is the extraction pressure, and X_3 is the co-solvent

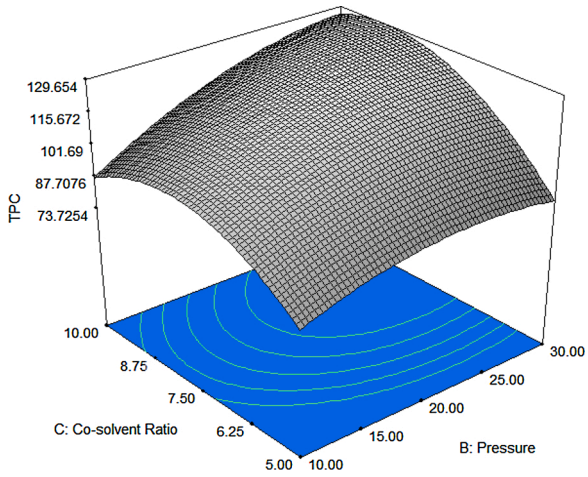

Fig. 3. Response surface of the total phenolic content (mg gallic acid/100 g dry roselle calyces) as a function of co-solvent ratio and pressure at a constant temperature (55 °C).

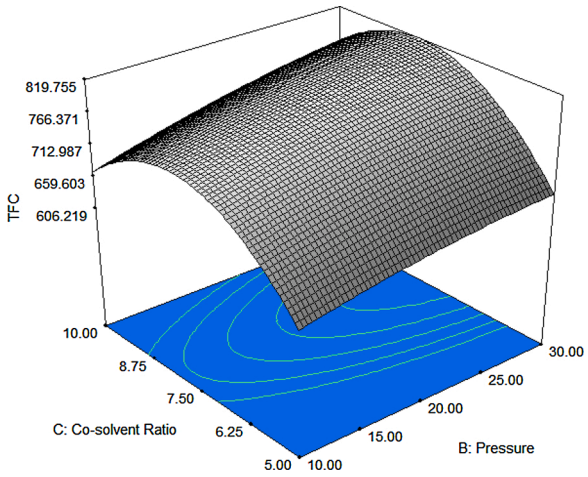

Fig. 4. Response surface of the total flavonoid content (mg quercetin/100 g dry roselle calyces) as a function of co-solvent ratio and pressure at a constant temperature (55 °C).

ratio. The equation was based on the data of regression coefficients presented in Table 3.

The quadratic model regression equations were examined using a three-dimensional graphical response to determine the influence of pressure and co-solvent, which were the most significant factors ($p < 0.050$). Fig. 2 illustrates the effect of pressure and co-solvent on TAC at 55 °C.

Fig. 2 shows that as the co-solvent ratio increased, TAC increased significantly ($p < 0.05$) until it gradually decreased around the 10 % co-solvent ratio. The role of co-solvent is proved by the molecules solubility and the ability to form hydrogen bonds when the EtOH/H_2O solution is added to the SC-CO_2, which allows for more thorough anthocyanin release from the matrix [37]. As a result, the co-solvent volume was maintained once the co-solvent ratio increased to a sufficient concentration to allow the entire TAC release.

Based on Fig. 2, increasing the pressure had increased the TAC value. The beneficial effect of pressure could be attributed to increased CO_2 density and diffusivity at higher pressures. When pressure increases, the fluid density fluctuates, rupturing the pressure effect and intensifying the interaction between the fluid and matrix. The chemical compounds in the plant components are rapidly released into the extraction solvents, increasing the solute and TAC solubility [20].

Meanwhile, as the pressure was elevated from 20 to 30 MPa, the TAC value decreased. This trend could be explained by the fact that the properties of supercritical CO_2 change at higher pressures, increasing mass transfer resistance and limiting its diffusion into sample matrices [38], hence inhibiting solubility. This phenomenon could also be explained by the extracted analytes' polarity and their interaction with the co-solvent ratios [20,39]. The results followed the same pattern as those reported on TAC from *crocus sativus* petals, with yield increasing gradually as the pressure increased from 9 to 15 MPa. However, as the pressure was increased to 20 MPa bar, the yield started to decline [20].

Contrarily, several studies have reported that temperature significantly affects anthocyanins [20,40,41]. The variation in extraction temperature is mainly due to plant matrices since the susceptibility of anthocyanins from diverse plants varies. Meanwhile, similar findings demonstrated that temperature has no influence on the recovery of phyllanthin when the dynamic extraction procedure was used at a range of 40–80 °C [42]. Nonetheless, the pressure increased when the amount of co-solvent increased, allowing the fluid's density to rise.

3.2. Effect of process condition on phenolic and flavonoid content

Table 2 shows the total phenolic and flavonoid contents of roselle calyces extracts obtained by SC-CO_2. The TPC was expressed as gallic acid, while TFC was expressed as quercetin. The effect of co-solvent ratio and pressure on phenolic and flavonoid compound extraction was highly significant ($p < 0.05$). TPC had a considerable regression model with a good R^2 of 0.7118, while TFC had a slightly lower yet acceptable ($R^2 = 0.6721$).

Moreover, the lack of fit test did not result in a significant F-value for both responses, indicating that the models accurately predicted TPC and TFC. Eqs. (9) and (10) show the relationship between process variables for TPC and TFC extractions, respectively, by omitting all significant regression coefficients.

$$Y_2 = +\,119.11 + 12.56 * X_2 + 13.84 * X_3 - 17.92 * X_3^2 \quad (9)$$

$$Y_3 = +\,786.33 + 36.60 * X_2 + 40.98 * X_3 - 9.46 * X_3^2 \quad (10)$$

From the equations, Y_2 is the TPC (mg gallic acid equivalent/100 g dry materials), Y_3 is the TFC (mg quercetin equivalent/100 g dry materials), X_2 is the extraction pressure, and X_3 is the co-solvent ratio. The equations were based on the data of regression coefficients presented in Table 3.

The relationship between total phenols and flavonoids of roselle calyces extract, and their significance factors are depicted in Figs. 3 and 4. Both figures clearly show that a 7.5 % maximum yield of phenolic and flavanoid was obtained at a medium co-solvent ratio. The pattern demonstrated that the co-solvent volume is considered minimum when using SC-CO_2 to extract valuable highly polar compounds. Higher pressures (> 20 MPa) may also cause the solvent's polarity to change slightly, reducing the amount of co-solvent assisted (Figs. 3 and 4). The TPC and TFC decreased when the extraction pressure exceeded a certain threshold, consistent with previous research on phenolic and flavonoid compounds. The studies discovered that the fluid viscosity (at higher pressure) increased and supercritical fluid diffusivity, on the other hand, decreased [20,21].

Increased anthocyanins in conjunction with increased phenolic and flavonoid content may promote anthocyanin stability by creating non-covalent molecular complexes known as co-pigmentation [43,44]. Co-pigmentation is referred to the ability of anthocyanins to develop supramolecular connections with other pigments. As an outcome, increased TPC and TFC recovery would stabilize the anthocyanins during storage.

3.3. Effect of process condition on colour properties

The red colour properties of anthocyanin-rich roselle extract were

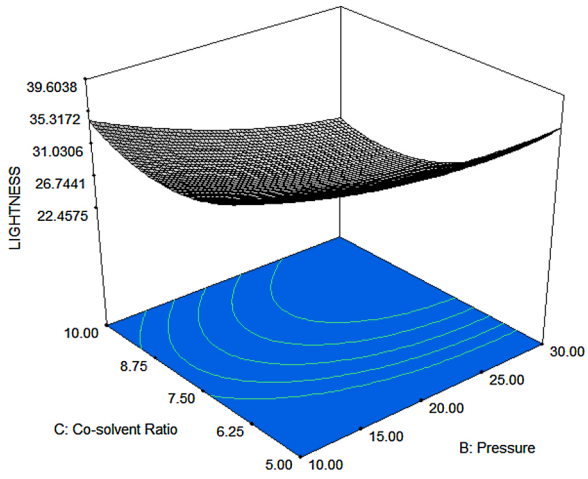

Fig. 5. Response surface of the L* of red roselle calyces extract as a function of co-solvent ratio and pressure at a constant temperature (55 °C).

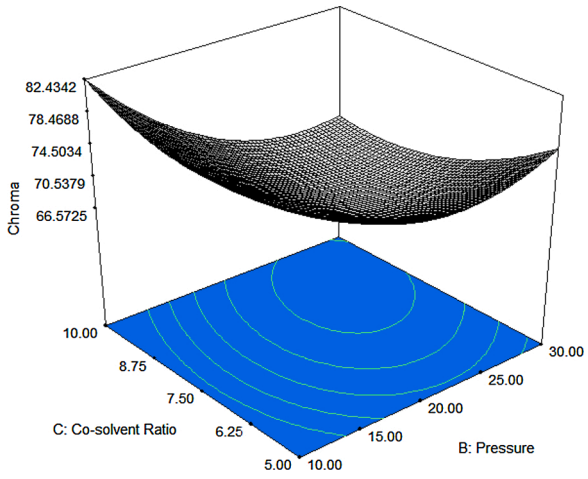

Fig. 6. Response surface of the C* value of red roselle calyces extract as a function of co-9solvent ratio and pressure at a constant temperature (55 °C).

represented by L*, C*, and h°, with L* values representing the perceived brightness or darkness. A value of 0 denotes black, while 100 represents white. C* refers to the colour intensity or saturation. A red-coloured sample with various dilution strengths ranging from pink to red will have the same h°, but the C* values will be higher. C* will increase to a maximum regarding pigment concentration but decreases as the colour darken (perplexing phenomenon). Consequently, a pink and dark red colour can have the same C* value.

In this study, when the extract was reddish-brown (dark red), it indicated that anthocyanin compounds and other polyphenols were more likely to be extracted. Compared to other literature on colour parameter quality, the desired values within the experimental ranges at the extraction process are minimum L* and C* [40,45]. Table 3 displays the colour visual using the RGB calculation. In the predicted model, the h° value was insignificant within the selected ranges in all variables, implying that all experiments produced red results. Hence, the h° was excluded from the effect of the process condition discussion.

3.3.1. L*

Based on Table 2, the values for extracts' L* colour parameter ranged from 19.15 to 47.64. This finding demonstrated that the darker colour of the extract was the greatest, which is consistent with the findings obtained by [1]. From the experimental results and ANOVA coefficient, the following second-order polynomial equation was generated to predict L* response to various process conditions by neglecting the insignificance variables. From Eq. 11, Y_4 is the L* value, X_2 is the extraction pressure, and X_3 is the co-solvent ratio. The equation was based on the data of regression coefficients presented in Table 3.

$$Y_4 = +\ 26.17 - 3\ 0.68 * X_2 - 4.26 * X_3 + 5.58 * X_3 2 \quad (11)$$

Co-solvent and pressure showed the most significant impact on the L* value of the extract ($p < 0.05$). Temperature and parameter interaction statistically had no significant effect on the L* colour parameter of extracts ($p > 0.05$). The R^2 value was relatively low but acceptable ($R^2 = 0.6892$). The model's validity was further supported by a non-significant value of $p > 0.05$ for lack of fit, indicating that a linear model with no interaction is statistically significant for the response. Based on Fig. 5, increasing pressure at a constant temperature of 55 °C reduced the L* value and darkened the extracts. Findings in Fig. 5 and RSM analysis showed that the lowest L* colour parameter of extracts, 19.15, was obtained at 55 °C, 20 MPa, and a co-solvent ratio of 7.5 %.

3.3.2. C*

Table 2 shows that the values obtained for the C* of an extract's red colour parameter were 57.29−85.24. The constant coefficient of the model ($p \leq 0.05$), the linear term of pressure ($p \leq 0.05$), and the quadratic term of pressure ($p \leq 0.05$) had a statistically significant effect on the dependent variable of extracts' L* colour parameter. The effects of other parameters presented in this table were insignificant on this dependent variable ($p > 0.05$). In this response, the R^2 of the predicted models was 0.5613, and the F-value for model lack of fit was 0.6794 ($p > 0.05$). Thus, the finding demonstrated that these values continue to provide an excellent fit to the mathematical model. The final predicting for the C* response, taking only the significant terms into account, is presented in Eq. (12) as follows:

$$Y_5 = +\ 68.27 - 4.82 * X_2 + 5.27 * X_2{}^2 \quad (12)$$

where Y_5 is the C* value, and X_2 is the extraction pressure. The equation was based on the data of regression coefficients presented in Table 3. As shown in Fig. 6, the C* value was lower when extracted at higher pressures of 20–30 MPa. The C* lowest colour parameter was obtained at 20 MPa, 55 °C, with 7.5 % co-solvent. The darker colour of the extract could be attributed to an increase in TAC content of the extracts concurrent with TPC and TFC. Moreover, the C* value did not affect either the temperature or the co-solvent.

Table 4
Comparison of experimental and predicted values of TAC, TPC, TFC, and colour properties using optimal levels of pressure, temperature, and co-solvent ratio.

Characteristics	Goal	Extraction methods		
		$SC-CO_2$		SLE
		Predicted values	Experimental values	Experimental values
TAC (mg cya-3-glu /100 g)	Maximum	1191.88^{a}	$1197 \pm 1.44^{a,A}$	748.11 ± 0.77^{B}
TPC (mg GAE /100 g)	Maximum	128.09^{a}	$128.16 \pm 0.53^{a,A}$	60.79 ± 0.58^{B}
TFC (mg QUE /100 g)	Maximum	797.05^{a}	$731.55 \pm 0.69^{a,A}$	546.71 ± 0.36^{B}
Lightness (L*)	Minimum	22.48^{a}	$22.91 \pm 0.02^{a,A}$	41.63 ± 0.03^{B}
Chroma (C*)	Minimum	66.02^{a}	$64.32^{a,A}$	73.67^{B}
Hue (h°)	ns	ns	37.88^{A}	35.432^{A}

Results are expressed by the mean (±) standard deviation of the analysis. $SC-CO_2$ = supercritical carbon dioxide; SLE = solid-liquid extraction; TAC = Total anthocyanins content, cya 3-glu = cyanidin 3- glucoside; TPC = Total phenolic content; GAE = Gallic acid, TFC = Total flavonoid content, QUE = Quercetin. The similar small letters between predicted and experimental values of $SC-CO_2$ are not significant at $p < 0.05$. The similar large letters between experimental values of $SC-CO_2$ and SLE methods are not significant at $p < 0.05$.

Table 5
Qualitative analysis of the primary compound in roselle calyces' extracts obtained by SC-CO_2 and SLE.

Compound Name	t_R (min)	m/z	% relative area	
			SC-CO_2	SLE
Anthocyanins				
Cyanidin 3-sambubioside	5.64	579.1363	11.20	2.52
Malvidin 3,5-diglucoside	9.58	653.1727	0.12	0.24
Pelargonidin 3,5-diglucoside	10.35	629.1305	0.20	0.16
Delphinidin 3,5-diglucoside	12.74	661.1173	0.01	0.003
Cyanidin-3-rutinoside	8.92	629.1310	0.03	0.02
Phenolic acids				
4-O-Caffeoylquinic acid	3.69	353.0881	10.97	9.89
Chlorogenic acid	5.15	353.0880	2.72	1.74
Shikimic acid	5.15	173.0455	0.70	0.58
cis-Caffeic acid	3.69	179.0348	1.39	1.13
Quinic acid	3.53	191.0559	5.18	3.71
Garcinia acid (hibiscus acid)	0.47	189.0041	0.60	0.19
Ethylchlorogenate	9.03	381.1197	3.44	4.59
Gallic acid	5.22	169.0142	0.08	0.07
Flavonoids				
Rutin	8.37	609.1471	4.74	3.84
Kaempferol	13.73	285.0405	0.23	0.53
Quercetin	11.83	301.0354	1.77	2.99
Quercetin 3-sambubioside	5.11	595.1308	2.78	2.04
Myricetin	9.8	317.0305	1.14	2.17

SC-CO_2: supercritical carbon dioxide (optimum value), SLE: solid-liquid extraction. The results are given as total area percentage, representing the relative abundance of one specific peak related to the sum of the areas of all peaks in the chromatogram for each extraction method [45]. *m/z* values are related to negative ionization [45].

3.4. Verification of the SFE and comparison with a conventional method

As previously described in this study, optimal extraction parameters correlate with the observed effects and the statistical analysis of each extraction parameter using the multivariate tool. Indeed, a co-solvent ratio of 8.86 % was the optimal condition for maximum TAC, TPC, and TFC recovery and is the most satisfactory red colour characteristics. Furthermore, when paired with a temperature of 58 °C, a pressure of 27 MPa proved to be the most effective for extracting TAC and other independent variables. As a result, the mathematical function determined and confirmed the multiple optimum extraction parameters of 27 MPa, 58 °C, and an 8.86 % co-solvent ratio. TAC was predicted to be 1192 mg cya 3-glu/100 g of dry calyces under these conditions.

In this study, TPC and TFC maximum concentrations were predicted to be 128 mg gallic acid/100 g and 797 mg quercetin/100 g, respectively. The experiment was conducted under optimal conditions predicted by the function. The results were quite similar to the expected values generated by the model, indicating its feasibility to optimize the extraction parameters for all SC-CO_2 responses at $p < 0.05$ (Table 4). The experimental extraction yield of TAC (1197 ± 1.44 mg cya 3-glu/100 g dry materials) and other responses was considerably higher in SC-CO_2 at the optimal condition than the yield achieved with SLE with TAC (748.11 ± 0.77 mg cya 3-glu/100 g dry materials), as indicated in Table 4.

A similar study reported that SC-CO_2 extract contained more TAC and TPC than other extraction methods such as pressurized liquid extraction and leaching [45]. Meanwhile, our findings showed a higher TAC recovery than other studies using the conventional acidified solvent method on roselle extract [14,46,47].

Besides TAC, other TPC and TFC also show better recovery with SC-CO_2 with EtOH/H_2O as co-solvent. This similar pattern could be due to the peculiar dissolving property of SC-CO_2, which intensified mass transfer [48,49]. Generally, co-solvent water and ethanol could exist in five states, including supercritical CO_2, carbonic acid, and dissociated products (H+, HCO3-, and CO32-) [15,50]. These various forms may have a function in the anthocyanin extraction process.

Initially, as the fastest solvent entering the dynamic extraction system, SC-CO_2 will dissolve the phospholipid layer of cell membranes by combining the high diffusivity of gas with the solvent strength of non-polar and lipophilic liquids. Water penetration into the cellular matrix will be increased, as will anthocyanin efflux from cell vacuoles outside the cell. Subsequently, when water enters the extraction system, the pH drops due to the formation of in situ carbonic acids. Carbonic acid has a beneficial effect on the extraction and stabilization of anthocyanin molecules. The explosive impact of rapid CO_2 depressurization destroys cell vacuoles and increases the availability of anthocyanins and other phenolic compounds, thereby increasing extraction and recovery [51]. As a result, SC-CO_2 outperforms the acidified solvent in optimizing the TAC and other polyphenols recovery. Storage stability analysis may provide additional support for SC-CO_2 as a potential process for producing natural red colourant from roselle calyces.

Table 6
Degradation kinetic parameters of roselle extract at 4 °C, 25 °C, and 37 °C for seven weeks' storage period.

Parameter	Storage condition (°C)	Anthocyanins	
		SC-CO_2 Optimum	SLE
Rate (k, d^{-1})	4	0.0032 ± 0.0005	0.0093 ± 0.0013
	25	0.0098 ± 0.0002	0.0222 ± 0.0008
	37	0.024 ± 0.0001	0.0444 ± 0.0004
Half-life ($t_{1/2}$, d)	4	216.61 ± 2.86	74.53 ± 3.25
	25	70.73 ± 0.25	31.22 ± 0.18
	37	28.89 ± 1.58	15.61 ± 0.38
Q_{10}	4−25	1.70 ± 0.05	1.51 ± 0.04
	25−37	2.11 ± 0.04	1.78 ± 0.07
Activation energy, Ea (kJ/mol)		42.67 ± 1.25	33.11 ± 2.34
Regression coefficient, R^2		0.998	0.9662

Results are expressed by the mean (±) standard deviation of the analysis. SC−CO_2 = supercritical carbon dioxide; SLE = solid-liquid extraction.

3.5. Identification of anthocyanins, phenolic acids, and flavonoids

Table 5 shows five types of anthocyanins found in optimized SC-CO_2 and SLE extract. The most significant anthocyanin in both extracts was cyanidin-3 sambubioside, with SC-CO_2 having a higher % relative than SLE. Other researchers have found a similar observation using roselle calyces extracts [52–54]. Other anthocyanins compounds were also detected, such as Malvidin 3,5-diglucoside, Pelargonidin 3,5-diglucoside, Delphinidin 3,5-diglucoside, and Cyanidin-3-rutinoside. This identification value has confirmed the high purity of anthocyanins in SC-CO_2 extract.

Meanwhile, eight phenolic acids were identified in the anthocyanin-rich extract. The compounds were identified using the software library's retention time (UNIFI Software (Waters). Except for Ethylchlorogenate, all identified phenolic acid compounds (4-O-Caffeoylquinic acid, Chlorogenic acid, Shikimic acid, cis-Caffeic acid, Quinic acid, Hibiscus acid, and Gallic acid) were found to be more abundant in SC-CO_2 than in SLE extract. Meanwhile, for flavonoids, the SC-CO_2 shows a higher relative value for the two identified compounds (rutin and quercetin 3-sambubioside), except the other dominant flavonoids, kaempferol, quercetin, and myricitin. The same flavonoid compound detected in solvent extraction of roselle calyces is supported by a different study reported here [18].

Natural co-pigmentation is when plant compounds such as phenolic acid and flavonoids interact and stabilize anthocyanins [55]. Based on identification and separation by % relative area, we can conclude that SC-CO_2 has better co-pigment compounds from hydroxycinnamic acid groups such as quinic, chlorogenic, 4-O-caffeoylquinic, and caffeic [43,

Table 7
Changes in polyphenol and colour parameters of roselle extract at 4 °C, 25 °C, and 37 °C during initial and after seven weeks' storage period.

Extraction method	Storage condition (°C)	TPC (mg GAE/100g)		TFC (mg QUE/100g)		Lightness (L*)		Chroma (C*)		Hue (h°)		Colour	
		Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final	Initial	Final
SC-CO_2	4	128.16 ± 0.53	126.14 ± 0.28	731.55 ± 0.69	460.27 ± 0.25	22.91 ± 0.02	32.85 ± 0.01	64.32	85.34	37.88	41.39		
	25	128.16 ± 0.53	53.62 ± 0.87	731.55 ± 0.69	196.49 ± 0.85	22.91 ± 0.02	48.15 ± 0.03	64.32	67.3	37.88	36		
	37	128.16 ± 0.53	40.42 ± 0.63	731.55 ± 0.69	83.38 ± 0.24	22.91 ± 0.02	54.82 ± 0.04	64.32	52.52	37.88	38.42		
SLE	4	60.79 ± 0.58	52.94 ± 0.41	546.71 ± 0.36	215.82 ± 0.43	41.63 ± 0.03	55.06 ± 0.03	73.67	26.54	35.43	20.54		
	25	60.79 ± 0.58	21.22 ± 0.82	546.71 ± 0.36	27.82 ± 0.73	41.63 ± 0.03	48.97 ± 0.01	73.67	23.75	35.43	21.47		
	37	60.79 ± 0.58	15.57 ± 0.25	546.71 ± 0.36	8.71 ± 0.35	41.63 ± 0.03	91.84 ± 0.02	73.67	-1.11	35.43	268.35		

Results expressed by the mean (±) standard deviation of the analysis. SC-CO_2 = supercritical carbon dioxide; SLE = solid-liquid extraction; TPC = Total phenolic content; GAE = Gallic acid, TFC = Total flavonoid content, QUE = Quercetin.

56]. Rutin also has been identified as one of the most effective co-pigments. Due to its comprehensive pi (π) systems conjugated in their cyclic structure, the increased rutin content in the SC-CO_2 extract would be of additional utility in boosting the stability of anthocyanins extract [43].

3.6. Degradation kinetic and storage stability of roselle extract

Food shelf-life prediction is based on environmental conditions such as temperature, humidity, microbes, and reaction kinetics [57]. Of all the factors mentioned, the temperature is the most critical that influencing the storage kinetics. The stability of the anthocyanin-rich extract obtained by SC-CO_2 was assessed and compared to the quality of the SLE extract. Based on the current regression coefficient, anthocyanin extract degradation from SC-CO_2 and SLE followed first-order reaction kinetics, consistent with previous research [58–61].

Table 6 displays the anthocyanin degradation kinetic parameter at various storage temperatures. The degradation rate values (k) for TAC at 4, 25, and 37 °C were 0.0032 – 0.024 d^{-1} for SC-CO_2 extract and 0.0093 – 0.044 d^{-1} for SLE extract. The SC-CO_2 extract had a two-fold increase in $t_{1/2}$ (216−29 days) than the SLE extract (74−15 days). As the storage temperature increases, the degradation rate constant increases, resulting in greater anthocyanin preservation at lower temperatures. In extraction, the temperature is well-known in affecting the stability of anthocyanins. It has been previously reported that the loss of total anthocyanin in roselle beverages, with k values, increased with temperature [62]. It is worth mentioning that degradation can occur in two ways. Firstly, a hydrolytic opening of the heterocyclic ring produces colourless chalcone, and secondly, the hydrolysis of the 3-glucoside that produces unstable aglycone [45,63].

The Ea value represents the amount of energy required by the degradation reaction. The higher the value, the more stable the anthocyanin is since it needs more energy to degrade [46,64]. Ea value was obtained from linearised Arrhenius equation by plotting ln k against 1/T. In this study, the calculated Ea values were 42.67 kJ/mol for SC-CO_2 extract and 33.11 kJ/mol for SLE extract. Only a single study discovered Ea of 40.61 kJ/mol for the degradation of total anthocyanins in the SC-CO_2. The study used black bean extract, which was stored at nearly the same temperatures (4, 25, and 32 °C) [45]. However, their findings show that SC-CO_2 had a much lower Ea value than the conventional leaching method.

The temperature dependence is also observed by Q_{10}, which is calculated from Eq. (6). Q_{10}denotes the various reaction rate constants at 10 °C. In our study, a higher Q_{10} value was obtained for anthocyanins employing SC-CO_2 extract. The Q_{10} values were 1.70 ± 0.05 and 2.11 ± 0.04 for storage temperatures of 4–25 °C and 25–37 °C, respectively. The extracted anthocyanins by SC-CO_2 exhibited a more temperature-dependent reaction, whereas slightly lower Q_{10} values were detected when using SLE.

Table 7 shows the TPC and TFC values and the colour parameters L*, C*, and h° for extracts at the beginning and end of storage. The current findings showed that all responses at both extracts decreased significantly during storage, especially at higher temperatures. On the other hand, TPC was more stable, with high retention values at the end of storage (98 % at 4 °C, 41 % at 25 °C, and 31 % at 37 °C). The same pattern was observed on SLE extract, although the retention rate was much lower (87 % at 4 °C, 35 % at 25 °C, and 25 % at 37 °C). This observation was consistent with previous research using Litchi pericarp [65]. Despite a significant decrease in anthocyanin content at 4 and 27 °C, they discovered that the TPC of Litchi pericarp was not significantly reduced at storage temperature. Table 7 also shows that after 49 days of storage, the SC−CO_2 could retain the red colour of the roselle extract. On the other hand, SLE extract lost the red colour after 49 days at 37 °C storage.

Colour stabilization phenomena could be caused by various reactions, including co-pigments of anthocyanins with other compounds. Co-pigmentation is regarded as one of the primary mechanisms of plant colour stabilization [66]. The pigments and other colourless compounds, such as phenolic acids and flavonoids, form molecular or complex associations, resulting in colour intensity changes. The identification of a noble co-pigment from a phenolic compound in roselle extract and the high value of TPC at the end of storage of SC-CO_2 extract suggested a possible relationship between phenolic acids and anthocyanin colour stability. The stacking of anthocyanin and co-pigments in SC-CO_2 extract via π-π interaction may contribute to anthocyanin structural stability [67]. The higher stability of anthocyanins using SC-CO_2 over SLE in various storage temperature conditions proves that the former extraction has a distinct advantage concerning anthocyanin extraction or storage.

4. Conclusion

Our findings demonstrated that co-solvent and pressure were the most significant factors in extracting TAC, TPC, TFC, and L*. The pressure was the only factor that significantly affected C* value. The optimum SC-CO_2 operating conditions were 27 MPa, 58 °C, and 8.86 % co-solvent ratio with maximum TAC, TPC, TFC, and minimal L* and C* values. The predicted values were reliable to the experimental work. The anthocyanins extraction yield was 2-fold higher than the conventional SLE, resulting in an anthocyanin-rich extract with a deep red colour.

Furthermore, cyanidin 3-sambubioside was identified as a primary anthocyanin compound in roselle extract. The high relative area percentage of cyanidin 3-sambubioside confirms the high concentration of anthocyanins in SC-CO_2 extract. The main phenolic acid was 4-O-caffeoylquinic acid, while the primary flavonoid compound was rutin. Both are known as good co-pigments that could influence the colour stability of roselle extract. This study proved that higher stability of anthocyanins was achieved using SC-CO_2. This technique is also better

at protecting the anthocyanins and maintaining colour stability in refrigerated, room, or ambient temperatures, which are more practical in industry.

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CRediT authorship contribution statement

Zuhaili Idham: Conceptualization, Writing - original draft, Investigation, Methodology. **Nicky Rahmana Putra:** Methodology, Writing - review & editing. **Ahmad Hazim Abdul Aziz:** Methodology, Writing - review & editing. **Ahmad Syahmi Zaini:** Validation, Writing - review & editing. **Noor Azwani Mohd Rasidek:** Methodology, Writing - review & editing. **Norlisa Mili:** Validation, Writing - review & editing. **Mohd Azizi Che Yunus:** Supervision, Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors report no declarations of interest.

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