Effect of flow rate, particle size and modifier ratio on the supercritical fluid extraction of anthocyanins from *Hibiscus sabdariffa* (L).

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\textbf{Abstract.} This work aims to evaluate the effects of different particle size, flow rate and modifier ratio on the extraction yield and anthocyanins content of *Hibiscus sabdariffa* L (Roselle) by using supercritical carbon dioxide (SC-CO\textsubscript{2}) method. SC-CO\textsubscript{2} extraction was carried out at constant pressure and temperature, 10MPa and 70°C respectively with 75\% (v/v) ethanol as a modifier. The result shows that 120 minutes was the efficient extraction time at a determination of flow rate. The trend and results for the extraction yield and anthocyanins were varied in all parameters studied. The finding shows that a low flow rate (4 ml/min), smaller particle size (200-355 µm) and high percentage ratio of modifier (10\%) could obtain more anthocyanins content. Thus, the manipulation of several parameters in the SC-CO\textsubscript{2} extraction process could enhance the selectivity of anthocyanins extraction from the total extract.

\textbf{1. Introduction}

Recently, several researchers are focussing on finding new plant sources of anthocyanins that can be found in more stable form \cite{1–3}. Anthocyanins can be found in a more stable form. Most of the anthocyanins potential sources are normally in plant materials with high availability or in the manufacturing industry by-products. Roselle, or scientifically known as *Hibiscus sabdariffa*, is widely and easily grown in Malaysia and tropical areas. Dried roselle is one of the potential sources of anthocyanins, however, the application to date is only served as tea or infusion drink \cite{4}. The benefits of roselle include (i) source of vitamins, (ii) minerals, (iii) bioactive compounds (e.g. organic acids, phytosterols, and polyphenols), and (iv) antioxidant properties.

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The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, delphinidin-3-sambubioside, cyanidin-3-sambubioside; cyanidin-3-glucoside, other flavonoids like gossypetin, hibiscetin, and their respective glycosides; protocatechuic acid, eugenol, and sterols like – sitoesterol and ergosterol [5].

Supercritical fluid extraction (SFE) offers great advantages for the recovery of anthocyanins from a plant due to inert atmospheres, the absence of light, low temperature and a short time of an extraction process. There is an increasing number of studies that applied SFE for extraction of anthocyanins from plants such as haskap berry pulp [6], jucara [7], chokeberry [8], Crocus sativus petals [9] and purple corn cob [10]. Moreover, the extraction using supercritical fluids aims to maximize the recovery and quality of the extracted material since it is more selective than conventional extraction methods, thus, easily extract anthocyanins from roselle.

A major used solvent in the SFE is carbon dioxide (CO\textsubscript{2}). CO\textsubscript{2} is cheap, non-flammable and less toxic compared to other solvents. Extraction technique using supercritical carbon dioxide (SC-CO\textsubscript{2}) is certified as Generally Recognised as Safe (GRAS) by the American Food and Drug Administration, which permits the addition of the extracted components into any food without undesirable effects for health [11]. However, SC-CO\textsubscript{2} has a limitation to extract anthocyanins as they are the water soluble pigment. Therefore, the choice of mixing ethanol with water as modifiers will perhaps change the non-polar nature of CO\textsubscript{2} to improve its affinity towards anthocyanins implementing SC-CO\textsubscript{2} extraction technique [12].

In order to improve the efficiency of SC-CO\textsubscript{2} extraction, several factors must be considered. These include the process and operational factors such as the pressure, temperature, flow rate, type and ratio of modifiers, extraction time, the particle size, as well as method of fluid feeding [13]. Therefore, in this study, the effects of several parameters, such as flow rate, particle size and modifier ratio on the yield and anthocyanins content of roselle extract were observed.

2. Methodology

2.1. Sample preparation

Dry roselle calyces’ variety UMKL-1 were purchased from Ekomekar Resources Sdn Bhd in their farm located at Ladang Setiu, Terengganu. The samples were grounded and sieved into powder form before stored in an airtight container. The samples were then kept in the freezer at -20°C for further extraction process.

2.2. Supercritical carbon dioxide extraction

The SC-CO\textsubscript{2} extraction process was carried out using the instrument at the Centre of Lipid Engineering and Applied Research (CLEAR), Universiti Teknologi Malaysia (UTM). Approximately 1.5 g of dry roselle powder was weighed and placed inside the 3-litre SFE extraction vessel. For the start-up process, the circulating water bath was set to at least 6°C and the oven was set at 70°C. During the experiment, CO\textsubscript{2} was cooled and chilled to enable the gas to be pumped. 75% of ethanol was used as a modifier. Initially, the extraction process was done dynamically for a total extraction time of 150 min and the yields were collected at every 15-min interval time for determination of extraction time. The remaining experiments were then followed by the best extraction time. Next, the extracts were centrifuged using centrifugal vacuum evaporator to remove the modifier at 40°C. The extract was then stored in a freezer (-20°C) until further analysis. The yield of extracted roselle was calculated by using equation (1).

\[
\text{Percentage of extracted yield (\%) = } \left[ W_a - W_b \right] \times 100
\]

where:

\( W_a \) is the weight of dried vial with roselle extract (g) and

\( W_b \) is the weight of empty vial (g).
2.3. Total Anthocyanins Content
The total anthocyanin content (TAC) of the extracts was conducted by using a differential pH method followed by AOAC Official Method 2005.02 [14]. The TAC is based on spectrophotometric readings as a function of pH (to observe the changes in anthocyanins structures). Potassium chloride (pH 1.0, 0.025 M) and sodium acetate trihydrate (pH 4.5, 0.4 M) were prepared as buffer solutions. A concentrated hydrochloric acid was used to adjust the sample pH. Samples were then diluted in the buffer solutions. After leaving for 30 minutes in the dark, a spectrophotometer at wavelengths of 510 and 700 nm was used to identify the absorbance of each sample. The final calculation of TAC is shown in equation (2).

\[
TAC \left(\frac{mg}{L}\right) = \frac{(A \times MW \times DF \times 1000)}{(ε \times l)}
\]  

(2)

where:
A: Absorbance = (A520 − A700), pH 1.0 − (A520− A700), pH 4.5;
MW: the molecular weight, calculated as cyanidin-3-glucoside (449.2 g/mol);
DF: the dilution factor;
l: the cuvette radius, 1 cm;
ε: the molar absorptivity, calculated as cyanidin-3-glucoside (26, 900 L/cm.mol).

2.4. Effect of solvent flow rate and determination of extraction time
The effect of flow rate and determination of extraction time were carried out experimentally at 70°C, 10 MPa and 10% modifier ratio. The solvent flow rate was studied at 4 mL/min, 5 mL/min and 6 mL/min. The yield and amount of TAC versus extraction time were recorded and plotted every 15 minutes. From the extraction curves plotted, the equilibrium of the diffusion region was selected as the best extraction time for further experiment.

2.5. Effect of particle size
A Vibrator Sieve Shaker was used to categorizes the particle size distribution. The ground dry roselle was divided into three different range of sizes; viz. 200-355 μm (dp1), 355-500 μm (dp2) and 500-710μm (dp3). In order to study the effect of particle size of dried ground roselle using SC-CO₂ extraction, the experiments were carried out at 70°C, 10 MPa, 10% modifier ratio and 4 ml/min of flow rate.

2.6. Effect of modifier percentage
75% ethanol is a modifier that used to the SC-CO₂ to enhance its ability to extract more polar compounds in the sample. The effect of the modifier amount was observed experimentally at 70°C, 10 MPa, and 4 ml/min of flow rate. Three percentage of modifiers ratio (i.e. 5%, 7.5% and 10%) were compared.

3. Result and Discussion
3.1. Effect of solvent flow rate and determination of extraction time
The extraction rate of the easily accessible solute can be affected by the solvent flow rate in the SC-CO₂ extraction process. Figure 1 and 2 show the effect of three different flow rates on the extraction yield and total anthocyanins content. Both figures show that increased the solvent flow rates would support extraction during the initial process stages. It is reported that in the initial stage of extraction, the solute amount in the extracted stream is rapidly increased [15]. This is because, at this stage, the extraction of surface-attached solute occurred. Meanwhile, the external mass transfer resistance will reduce by increasing the flow rate as it enhances the initial rate of extraction. Nevertheless, at a later stage of the process, the extraction was manipulated mostly by solute diffusion from the cell matrix.
Simultaneously, by increasing the flow rate, the rate of diffusion could not be controlled which resulted in an almost similar rate of extraction [16].

The highest extraction yield was achieved at the high solvent flow rate, i.e. 6 ml/min. The surge in the flow rate led to a decline in mass transfer resistance until the existing solvent is saturated. At an equilibrium state, the maximum yield is then attained. As flow rate increase, residence time will decrease, which resulting in the system shift from equilibrium. This might cause the solvent to leave the extractor unsaturated despite the high mass transfer rate [17,18]. This finding was comparable with a study by Kumoro and Hasan et al. [17]. They reported that the solvent flow rate was increased from \(7.95 \times 10^{-6}\) kg/s to \(3.18 \times 10^{-5}\) kg/s as the extraction yield increased when applying SC-CO\(_2\) extraction of andrographolide.

However, the higher solvent flow rates can have negative, marginal, moderate or poor positive effects on the extraction process, depending on the situation [19]. As can be seen in Figure 2, a low flow rate (4 ml/min) gave the highest total of anthocyanins content. The low flow rate could allow the solvent to pass through the overall surface of the sample matrix even longer and valuable compounds such as anthocyanins could be extracted. A study by Radzali et al. [16] revealed that the maximum amount of astaxanthin could be extracted at a low extraction flow rate. This is because there is enough residence time that allows more effective contact between solvent and solute. In contrast, when the flow rate increased, a contacting time becomes shorter, thus lead to a less viscous or pale orange astaxanthin extract due to incomplete extraction.

The extraction time could be a useful parameter for achieving a complete SC-CO\(_2\) extraction. The efficiency of the SC-CO\(_2\) process could be improved by increasing the contact time of the SC-CO\(_2\) with the sample material [20]. The infiltration of the solvent and the diffusion of the targeted compounds in the particles is quite low due to the sample physical structure. Since the amount of extraction yield recovered in the slow extraction period is insignificant, therefore extraction time is restricted to the fast extraction [20].

As a result, both Figures 3 and 4 show the same trend whereby the extraction yield and TAC had increased significantly for 60 minutes. This is called a constant extraction rate (CER) period. Further increased and tends to be saturated up until 120 minutes is called a falling extraction rate (FER) period. As time increases, the extraction yield reached its maximum and become equilibrium from 120 minutes to 150 minutes whereby the diffusion (DIFF) happened. According to Taylor [21], extraction takes place rapidly during the CER region and is dependent on the solubility of the solute in SC-CO\(_2\) fluids. The solubilized solute is easily removed until the condition of the near equilibrium controls the extraction of the solute into the fluid.

Meanwhile, during the FER region, the solute-matrix interaction was disrupted, thus causing a slower extraction rate. At the same time, there is also a transition in this region to a diffusion-controlled process. Hence, the causes of the phenomenon of the DIFF region is either by the limited access to supercritical carbon dioxide fluids to the target solute or by the limited mobility of the solute within the matrix. The sample nature of complex matrix could also influence the extraction rate in this region. In the DIFF regions (equilibrium state), complete extraction demonstrated that sample solute extracted has stopped to increase with a prolonged extraction time. Therefore, an extraction time of 120 minutes (2 hours) is needed to reach the equilibrium state to get the highest yield and anthocyanins from roselle calyces.
3.2. Effect of particle size

Mass transfer kinetics and the access of CO₂ with a modifier into soluble components are affected by particle size. Three different particle sizes were represented with dp1, dp2 and dp3 as shown in Figure 3 and 4 to observe their effect on the extraction yield and total anthocyanins content, respectively. In Figure 3, the trends show that the highest percentage of extraction yield was achieved by using dp2 (355-500 µm), followed by dp3 (500-700 µm) and dp1 (200-355 µm). This might be due to the existence of other compounds in the extract.

Meanwhile, for the extraction of bioactive content, the highest total anthocyanins content of 4.95E+04 mg/L was obtained by using the lowest particle size (dp1, 200-355 µm). As the sample particle size increased (dp1>dp2>dp3), the total content of anthocyanins decreased. The smaller
particle size enhances the extraction of a bioactive compound due to the higher surface area to volume ratio, that in turn, enhanced the contact between solvent molecules and plant material during the extraction process. When the size of the solute particle increases, the path between the inside of the particle and surface particle also increases, consequently lowering the extraction of the solute. In fact, if the particle size is small, the effect would be a relatively large surface area, thus increasing the area of contact between the solvent. Size reduction (deep inside the plant matrix) also helps the soluble substrates to be more easily accessible. In addition, the diffusion direction of the compound within the particle was reduced, hence, facilitate the extraction to be easier and faster [22].

Figure 3. Extraction yield (%) versus extraction time (min) of roselle SC-CO$_2$ extract at three different particle sizes

Figure 4. Total anthocyanins content (mg/L) versus extraction time (min) of roselle SC-CO$_2$ extract at three different particle sizes.
3.3. Effect of modifier ratio

In order to choose the type of modifier, several factors such as toxicity, solvent cost, and the final use of the extract must be considered. In pharmaceutical and food-grade products, the use of ethanol is acceptable. Meanwhile, water is clean, cheaper, tasteless, and less restrictive in terms of residual solvent due to its process [19]. Therefore, the combination of water and ethanol could improve the polarity of SC-CO$_2$ to extract anthocyanins (i.e. a highly polar compound). However, the use of a modifier and their amount should be controlled so that the amount of organic solvents in the process could be reduced and processing time could be saved in order to extract the concentrated sample. Hence, the effect of the three-modifier ratio was examined in this study (Figures 5 and 6) at the low-density condition of SC-CO$_2$ (70°C and 10MPa). The low extraction yield (52.96%) was achieved when using a higher modifier ratio (10%). Meanwhile, when using a high percentage of modifier ratio (10%), a high concentration of anthocyanins (3.84E+04) was obtained. This result indicates that the modifier amount should be sufficiently high to extract the anthocyanins from the sample at the low-density condition.

![Figure 5](image1.png) ![Figure 6](image2.png)

**Figure 5.** Effects of different modifier ratio (%) on the extraction yield (%).

**Figure 6.** Effects of different modifier ratio (%) on total anthocyanins content (mg/L).

4. Conclusion

The effect of particle size, flow rate and a modifier ratio could give different findings of overall extraction yield and total anthocyanins content. Therefore, it is compulsory to determine the characteristics of the desired compound in order to obtain the highest anthocyanins concentration using the SC-CO$_2$ extraction process as possible. The best extraction time of anthocyanins extraction used in this study was observed at 120 minutes. Prolong extraction time is not economically efficient due to the high amount of solvents, energy and cost consumption. To conclude, a low flow rate of 4 ml/min, smaller particle size (200-355 µm) and a high percentage of modifier ratio (10%) gave the highest anthocyanins content in the roselle calyces using SC-CO$_2$ extraction.

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