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RESEARCH ARTICLE

Effects of pressure variation and dynamic extraction time in supercritical fluid extraction (SFE) with co-solvent on bioactive compounds from *Orthosiphon stamineus*

Masniza Mohamed-Mahmood [a, b], Wan Ramli Wan Daud [a], Masturah Markom [a,*], Che Nurul Ain Nadirah Che Mansor [c], Jalifah Latif [c]

[a] *Jabatan Kejuruteraan Kimia dan Proses, Fakulti Kejuruteraan dan Alam Bina, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia*
[b] *Universiti Kuala Lumpur, Malaysian Institute of Chemical and Bioengineering Technology, Lot 1988 Vendor City Taboh Naning, 78000 Alor Gajah, Malacca*
[c] *Pusat Pengajian Sains Kimia, Fakulti Sains dan Teknologi, Universiti Kebangsaan Malaysia (UKM), 43600 Bangi, Selangor, Malaysia*

* *Corresponding author: masturahmarkom@ukm.edu.my*

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Abstract

Supercritical Fluid Extraction (SFE) is an advance method used in extracting bioactive compounds from plants. However, carbon dioxide used as an extraction solvent in SFE has limitation in extracting polar compounds. By adding a small amount of co-solvent (polar solvent) to extract wide range (polar to non-polar) of bioactive compounds, thereby affecting the extract yields. Pressure and dynamic time play important roles in this technique. In this study, the effects of pressure variation and continuous dynamic extraction time on the bioactive compounds from *Orthosiphon stamineus*, which were rosmarinic acid, 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, sinensetin, and eupatorin using ethanol (co-solvent) modified CO_2 SFE were discussed. The pressure variations studied were included 150, 225, and 300 bar with continuous dynamic extraction. The co-solvent used was 10% (v/v) of absolute ethanol with a flow rate at 4 mL/min. The quantification of bioactive compounds was done using HPLC. The variation of pressure and dynamic time significantly affected the total component yields of targeted bioactive compounds obtained after the extraction process, as it was increased along with increasing of extraction pressure and time. The total yields of bioactive compounds at different pressures were 4.08% (150 bar), 4.18% (225 bar) and 4.51% (300 bar). The application of SFE with co-solvent and the relationship with the yields of targeted bioactive compounds obtained from of *O.stamineus* were given.

Keywords: *Orthosiphon stamineus*, supercritical fluid extraction, co-solvent

INTRODUCTION

Orthosiphon staminues is a herbal in the family of *Lamiacea*, which is originated from South-East Asia regions (Abdullah *et al.*, 2011). This herbal plant has medicinal properties such as anti-inflammatory, anti-oxidant, anti-bacterial (Hsu *et al.*, 2010), and anti-cancer potential against prostate malignancy (Fouad *et al.*, 2014). The bioactive compounds reported for this plant are rosmarinic acid, sinensetin, eupatorin, 3-hydroxyl-5,6,7,4'-tetramethoxyflavone, caffeic acid, cirrchoric acid, diterpenes, orthosiphols, monoterpenes, triterpenes, saponins, hexoses, and other organic acids (Olah *et al.*, 2003; Akowuah *et al.*, 2004; Tezuka *et al.*, 2000).

Various extraction methods can be used to extract the targeted bioactive compounds from herbal plants such as solvent extraction and pressurised liquid extraction. These methods have some disadvantages such as the long extraction time, the use of large amount of chemical solvents (e.g methanol, acetone, ethanol and chloroform) and the presence of residual solvents in the final product. The solvents used in these extraction methods can strongly affect the yield of the component because of their uneven distribution and chemical properties of the plant matrix and also due to the presence of different chemical characteristics of the compounds and their polarities (ability to be extracted and soluble in a particular solvent) (Sultana *et al.*, 2009; Jakopic *et al.*, 2009). One of the best alternatives for plant extraction with the capability to extract numerous constituents in plants is supercritical fluid extraction (SFE). The selection of carbon dioxide in SFE is mainly due to its critical properties, less toxicity, inert, cheap, and capability to dissolve non-polar to moderately polar compounds.

Carbon dioxide is non-polar and it has limitation in extracting very polar compound and normally, by adding a small amount of co-solvents (polar solvents), the characteristics of supercritical CO_2 can be modified to extract a wide range of bioactive compounds (non-polar to polar) from herbal plant. The use of polar solvents is crucial for the recovery of polyphenols from plant. Other than that, the ratio of sample to solvent is important in order to obtain a high yield of extract (Wong *et al.*, 2013). Ethanol, methanol, acetone and ethyl acetate are frequently employed as co-solvents in SFE (Sultana *et al.*, 2009).

In the case of pressure variation in SFE, it has been demonstrated that a pressure increase enhances the plant extraction yield for certain group of extracts but it varies to other group of compounds (Al-Asheh

et al., 2012). It has been demonstrated that in the extraction of Lavender (*Lavandula* spp.), pressure and dynamic time of the extraction showed a significant linear effect on the extracts yield, while temperature did not show any improvement on extract yield unless the involvement of interaction with pressure.

In supercritical CO_2 extraction, the static and dynamic extraction times can vary significantly from a case to another due to sample composition and solute concentration. For example, 5–10 min of static extraction time applied during an extraction did not show any enhanced yield of fatty acids and sterols in plant tissues (Klink *et al.*, 1994). A similar trend for methanol modified SFE of michellamines A and B was observed where 6–60 min static time employed also did not result in any improved yield (Ashraf-Khorassani & Taylor, 1997). It could be an indication that the static extraction time would not be necessary to vary for such extraction.

The aim of this work was to determine the pressure and dynamic time that necessary for the extraction of bioactive compounds from *O.stamineus* using supercritical carbon dioxide with the addition of absolute ethanol as co-solvent. The yield and dynamic time were determined at different extraction pressures, related to the quantity of the bioactive compounds extracted, which were rosmarinic acid, sinensetin, 3'-hydroxy5,6,7,4'-tetramethoxyflavone, and eupatorin. To the best of our knowledge, the effects of pressure variation and dynamic extraction on supercritical fluid with co-solvent of bioactive compounds from *O.stamineus* have not been reported till to date.

In this study, the flow of compressed carbon dioxide (4 mL/min) was continuously passed through the sample with the addition of 10% (v/v) of co-solvent (±97% purity grade absolute ethanol; >190 proof) for 160 min to increase the dissolving power and enhance the extraction efficiency. The main goal was the determination of components yield in dynamic extraction mode by varying the process pressures at 150, 225, and 300 bar.

EXPERIMENTAL

Materials

Approximately 2 kg of *O.stamineus* was purchased from Herbagus, Penang. The moisture content of the leaves was ±12.8 % (dry basis) and it was determined by using Sartorious Moisture Analyzer. The samples were ground and sieved to obtain a sufficient particle size of 0.5 µm with high surface area. The samples were kept at -10°C in bottles covered with aluminium foil to avoid degradation of the material.

Supercritical fluid extraction

The SFE unit used was a lab-scale system consisted of carbon dioxide and co-solvent pumps (Lab Alliance, USA), an automated back pressure regulator (model JASCO BP 2080 Plus), and air circulating oven (Memmert), as shown in Fig. 1. The extraction was performed in a dynamic mode, which allowed the continuous supply of fresh CO_2 with co-solvent (liquid) to the sample. The liquid was pumped to the heating zone (oven) to achieve its supercritical conditions. It was later diffused into the solid matrix/sample in the extraction vessel to dissolve the compounds to be extracted. The flow rate, co-solvent content (±97% purity grade absolute ethanol; >190 proof), and temperature were fixed at 4 mL/min, 10% (v/v), and 60 °C, respectively. The dissolved compounds were swept from the extraction vessel and settled out into collecting vials at lower pressure. Three different pressures were tested (150, 225, and 300 bar) and each fraction was collected at 20 min interval during the extraction process. The extracts were dried at 50 °C and subsequently kept at -20 °C. The quantification of bioactive compounds in the extract was done using high performance liquid chromatography (HPLC).

High performance liquid chromatography (HPLC)

Analyses were performed using high performance liquid chromatography (HPLC) model 2998 (Waters Corporation, USA) equipped with an autosampler and a photodiode array detector. The column used was a reverse phase C18, Chromolith (i.d. 100 × 4.6 × 5 mm). An acetonitrile/water/triflouro acetic acid mobile phase system was used for the chromatographic separation.

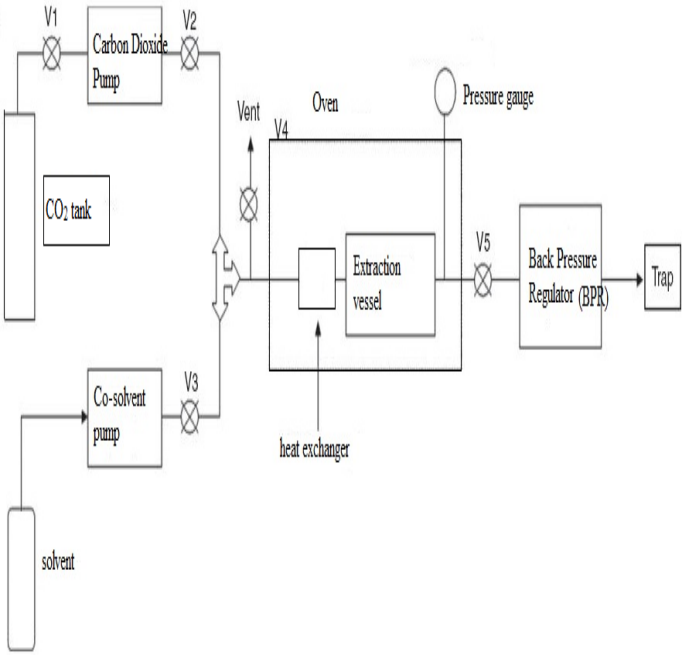

Fig. 1 Schematic diagram for SFE system.

RESULTS AND DISCUSSION

Effect of pressure variation on rosmarinic acid, 3'-hydroxy-5,6,7,4'- tetramethoxyflavone, sinensetin and eupatorin

The obtained results suggested that an increase in the pressure from 150 to 300 bar at constant temperature enhanced the total amount of bioactive compounds extracted. As described in Fig. 2, pressure variation showed significant effect (p<0.05) on bioactive compounds contents (EUP, TMF and RA) but not on TMF (p>0.05). However, the total yields of all targeted bioactive compounds were increased as the pressure was increased. The highest recovery of all targeted bioactive compounds was obtained at the highest pressure (300 bar) with a total components yield of 4.51% followed by 4.18% (225 bar) and 4.08% (150 bar) as can be seen in Fig. 3. The possible reason for this trend is due to the increase of density which can enhance the solvent power and thus, increasing the extraction rate. Fig. 4 shows the HPLC profile of bioactive components in the extract.

Increasing the dynamic time of extraction from 20 to 160 min would increase the targeted bioactive compounds yields. Fig. 5 shows the total component yield for every 20 min of dynamic extraction. Beyond the pressure variation, RA, EUP, TMF and SEN were significantly increased (p<0.05) along with increasing of dynamic time. Generally, positive linear relationships were obtained between targeted bioactive compounds (EUP, RA, TMF and SEN) and dynamic extraction time, as shown in Fig. 6.

Pressure and time play important roles to increase extract yield. In *O.stamineus* extraction, the pressure variation is crucial in extracting its bioactive components. Similar finding was observed in SFE in order to obtain tocopherols and carotenoids as the yield of oil extracted from enriched rapeseed oil which mostly influenced by pressure, followed by temperature and extraction time (Uquiche *et al.*, 2012). Nevertheless in the case of lavender SFE extraction, the three operative parameters, namely as pressure, temperature, and co-solvent did not have any impacts on the chemical composition of the extracts (Lu *et al.*, 2012).

Supercritical carbon dioxide extraction for European available vanilla sugars at 18.9 MPa and 45°C for 10 min extraction time under dynamic conditions has been reported for the recovery of vanillin and ethyl vanillin. The result indicated good average recovery of 98–104% (concentration range of 10–60 mg) with simple and rapid sample preparation which made the technique was reliable for chemical analysis (Auklam & Muller, 1993).

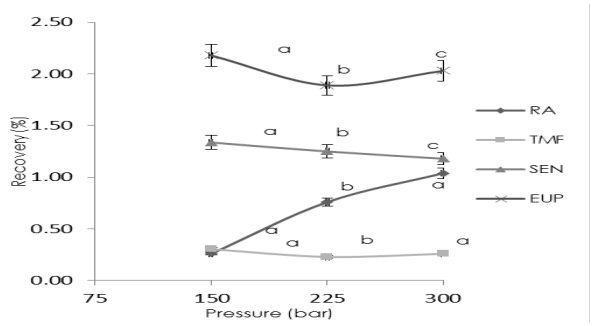

Fig. 2 Effect of pressure (bar) variation on RA, TMF, SEN, EUP from *O. stamineus* (n= 2)*. RA: rosmarinic acid, TMF: 3'-hydroxy-5,6,7,4'-tetramethoxyflavone, SEN: sinensetin, EUP: eupatorin Values marked by different lower case letters (a-c) are significantly different (p<0.05). *Replication of extractions. Note: Error bars represent the standard deviation.

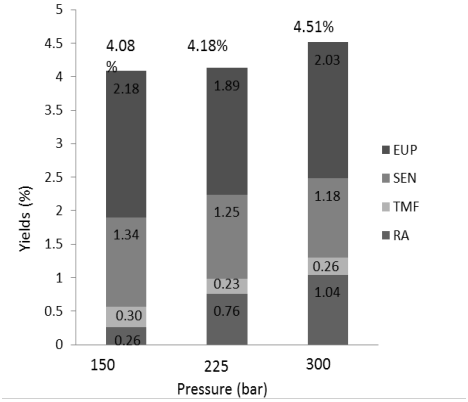

Fig. 3 Yields of bioactive compounds at different pressures. RA: rosmarinic acid, TMF: 3'-hydroxy-5,6,7,4'- tetramethoxyflavone, SEN: sinensetin, EUP: eupatorin.

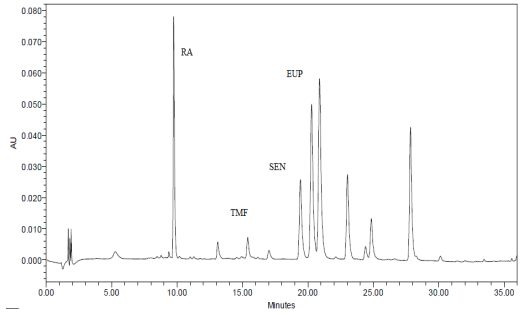

Fig. 4 HPLC profile for bioactive compounds in *O. stamineus*. RA: rosmarinic acid, TMF: 3'-hydroxy-5,6,7,4'- tetramethoxyflavone, SEN: sinensetin, EUP: eupatorin.

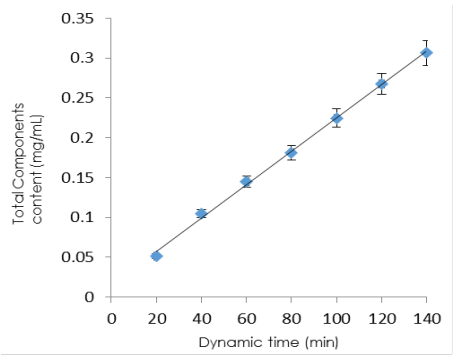

Fig. 5 Total components content (mg/mL) obtained after 140 min of dynamic extraction at 300 bar. Error bars represent standard deviation.

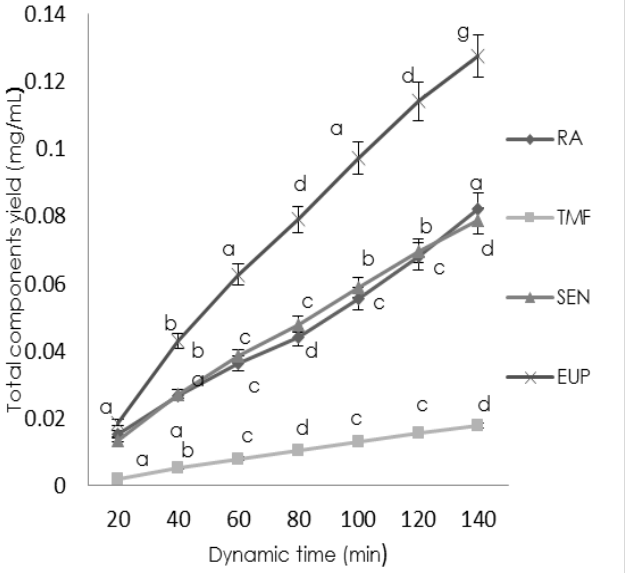

Fig. 6 Effect of extraction dynamic time (min) on RA, TMF, SEN, EUP from *O. stamineus* (n= 2)* at 300 bar. Values marked by different lower case letters (a-g) are significantly different (p<0.05). *Replication of extractions. Note: Error bars represent the standard deviation.

Table 1 Total components yield (mg/mL) of targeted bioactive compounds (RA, TMF, SEN and EUP) obtained after 140 min of dynamic extraction. SFE conditions (P= 225 bar, flow rate=4 mL/min and T=60 °C).

SFE	Total components yield (mg/mL)
SFE with ethanol (10% (v/v) of co-solvent content)	0.286
SFE without ethanol *control	0.243

Table 1 shows the total components yield for all targeted bioactive compounds for 140 min of dynamic extraction. A comparison was made between SFE with ethanol and without ethanol (control). The addition of ethanol as co-solvent helped to increase 18% of the extracted amount of polar and non-polar compounds especially eupatorin, 3-hydroxyl-5,6,7,4'-tetramethoxyflavone and sinensetin.

Using ethanol as co-solvent for SFE also showed the highest yield of essential oil containing compounds with therapeutic activities and several substances of industrial interest (Almeida *et al.*, 2012). In addition, other researchers also reported that ethanol used as a co-solvent could enhance the yield of onion oil (Dron *et al.*, 1997). It was believed that the change in polarity of the supercritical solvent mixture caused a significant increase in the extraction performance of components of coffee when ethanol was added (Banchero *et al.*, 2013).

In this study, a lower content of rosmarinic acid as the main constituent was extracted (0.23–0.26%) compared to solid phase extraction (10–27%) using a more polar solvent, as reported by other researchers (Lau *et al.*, 2014). This could be explained by the polarity nature of rosmarinic acid (very polar). As shown in Fig. 7**,** rosmarinic acid (a) is a very polar compound with many hydroxyl groups in its structure compared to other compounds such as 3-hydroxyl-5,6,7,4'-tetramethoxyflavone (b), sinensetin (c), and eupatorin (d), which are considered as non-polar and semi-polar. Even though ethanol contains hydroxyl group that can create hydrogen bonding with polar solute, the use of water-ethanol combination is recommended because water has shorter chain with higher polarity (Pin *et al.*, 2010) compared to absolute ethanol which makes it more suitable to extract wide range of non-polar to polar compounds from *O.stamineus.*

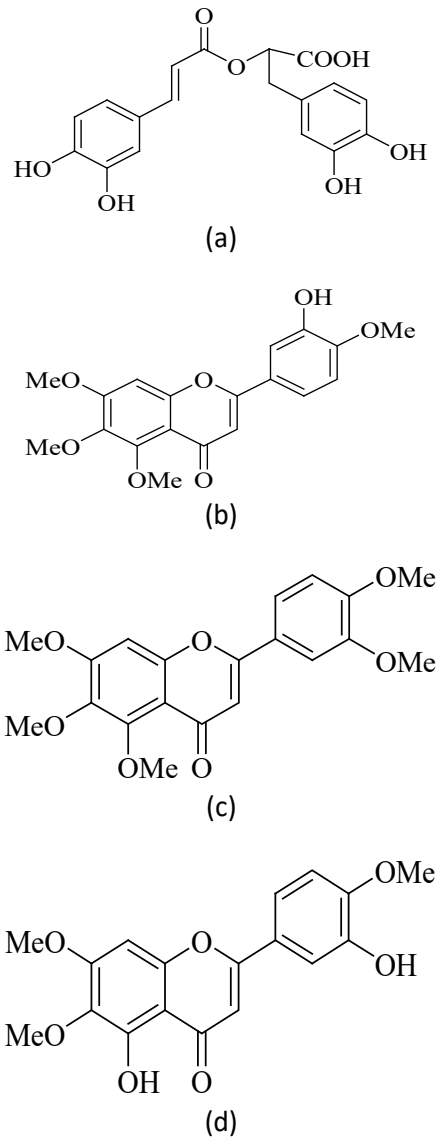

Fig. 7 Chemical structures of targeted bioactive compounds in *O.stamineus:* (a) rosmarinic acid, (b) 3'-hydroxy-5,6,7,4'-tetramethoxy-flavone, (c) sinensetin and (d) eupatorin.

CONCLUSION

The effects of pressure and dynamic extraction time in SFE on the bioactive compounds of *O.stamineus* were investigated. The total yield of targeted bioactive compounds was increased along with the increase in extraction pressure and dynamic time. It was also found that absolute ethanol was an effective co-solvent in the extraction of rosmarinic acid, sinensetin, eupatorin and 3-hydroxyl-5,6,7,4'-tetramethoxyflavone using supercritical CO_2. For future work, SFE-CO_2 with co-solvent of herb extraction with different polarity compounds can be implemented and thus, replacing the common SFE using carbon dioxide which extracts only non-polar compounds.

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