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12**supercritical CO2 extraction of phenolic- phytochemicals from an epiphytic plant tuber (Myrmecodia pendans) Rebeca E. Sanjaya** a,b,1, **Yanuar Y. Tedjo** a,1, **Alfin Kurniawan** a,1, **Yi-Hsu Ju** b, **Aning Ayucitra** a, **Suryadi Ismadji**

a,* a

3**Department of Chemical Engineering, Widya Mandala Surabaya Catholic University, Kalijudan 37, Surabaya 60114, Indonesia** b **Department of Chemical Engineering, National Taiwan University of Science and Technology, 43 Sec. 4, Keelung Road, Taipei 106, Taiwan ARTICLE** INFO Article **history: Received** 3 **July** 2013 **Received in revised form** 1 March 2014 **Accepted 14**

March 2014 Available online Keywords: Ant-nest Supercritical extraction Antioxidant Solubility DPPH scavenging ABSTRACT Myrmecodia pendans (ant-nest) is an epiphytic plant with rich content of phytochemicals. In this work, the application of supercritical carbon dioxide (SC-CO2) for extracting several polyphenol and flavonoid compounds from ant-nest tuber include gallic acid, catechin, ferulic acid, caffeic acid, p-coumaric acid, quercetin, luteolin and kaempferol was investigated. Static-analytical extraction experiments were performed at pressures ranged between 9–22.5 MPa and temperatures of 313.15–343.15 K for 6–7 h. Solubility data of total polyphenols (gram gallic acid equivalent (GAE)/L CO2) and total flavonoids (gram quercetin equivalent (QE)/L CO2) were modeled using

1**Chrastil and Del Valle–Aguilera density-based** equations. Both equations **were**

able to represent actual solubilities satisfactorily in terms of correlation coefficient and parameter consistency. DPPH-scavenging test showed high antioxidant capacity of supercritical extract with an IC50 level of 3.62 0.12 mg/mL. It was observed a positive linear correlation between bioactive contents of antioxidant extract and the potency of scavenger activity against DPPH free radical. ß 2014 Elsevier Ltd. All rights reserved. Introduction Myrmecodia pendans (genus of Myrmecophytes),

8**also locally known by indigenous Papuans as ant-nest** is **native to Southeast Asia** and it **has** now widely distributed **to**

Australasian regions. Ant- nest

19**has proven to be rich in bioactive constituents such as flavonoids, tocopherols, tannins and a variety of essential minerals,**

which are an important part of our diet. This tropical plant is

8**highly valued as an alternative choice for cancer/tumor treatments and an efficacious herbal drug to prevent and cure**

various illnesses include hemorrhoid, ulcer, nosebleed, backache, skin rashes, allergy, gout, uric acid disorder, stroke, prostate, coronary heart, lung tuberculosis, rheumatism and diarrhea [1]. Many pharmacol- ogists and botanists strongly believe that the nutritional values as well as powerful in vitro anti-cancer and antioxidant properties of *

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http://dx.doi.org/10.1016/j. jcou.2014 **.03.**

001 2212-9820/ß 2014 Elsevier Ltd. All rights reserved. ant-nest are sourced from flavonoid compounds. The isolation technique of these compounds is therefore the primary interest to fully exploit this natural remedy for modern therapeutics and also for advances in the phytochemistry science. The isolation of flavonoid compounds from M. pendans employing various organic solvents has been conducted [2–4]. Soeksmanto et al. extracted ant-nest with n-butanol, ethyl acetate and water and the

5**anti-cancer activity of** organic extracts **was tested** for inhibiting **cancer cells derived from human cervix** (HeLa cells) **and canine breast** (MCM-B2 **cells)**

[2]. Hertiani et al. studied bioactivity of ant-nest extracts in vitro as an immunomodulatory agent against Balb/c mice lymphocytes proliferation and macro- phage phagocytosis [3]. Engida and colleagues had successfully identified five flavonoids of ant-nest extract by HPLC technique, which includes kaempferol, luteolin, rutin, quercetin and apigenin [4]. They also studied antioxidant potential (IC50) of the extract and

obtained comparable DPPH-scavenging performance with com- mercial ascorbic acid. The conventional extraction operation of flavonoid compounds using organic solvents, although simple, possesses limitations such as cost-prohibitive, unenvironmentally benign and non-selective, which requires post-treatment stages for purification of the product. The disposal of unrecyclable organic solvent may also pose a risk to health and the environment. On the other hand, the extraction of flavonoid compounds using supercritical fluids is an alternative viable for effective and greener extraction technology on laboratory- and pilot-scale. The working procedure is relatively simple and high purity extract can be easily obtained. The practical advantages of carbon dioxide as a supercritical fluid include cheap, inert, readily available in high purity, non-toxic, non-flammable and non-explosive have made it highly desired for isolating various heat-labile compounds from various plant and animal materials such as antioxidants [5], pigments [6], flavors [7], fragrances [8], lipids [9] and essential oils [10] at near-ambient temperature. Also, the recovery process of product is accomplished by simple pressure reduction and leaving no solvent residue thus does not affect the aroma or taste of the final product. Considering SC-CO2 extraction of polyphenolic compounds from plant materials, a nearly complete recovery can be achieved without adding a modifier (co-solvent). Gelmez et al. extracted phenolics and tocopherols from roasted wheat germ where 91% of phenolics and tocopherols in wheat germ oil were recovered using pure SC-CO2 at 442 bar and 313.15 K [11]. In the study conducted by Piantino et al. [12], four phenolic compounds in Baccharis dracunculifolia leaves were extracted by SC-CO2 without a co- solvent and they obtained higher yields of individual phenolics in the supercritical extract than that from conventional ethanolic extraction. It has been shown that some phenolic acids and their esters are readily soluble in SC-CO2 [13–15] while the addition of co-solvents would be required to increase solubilities of high molecular weight phenolics. Since there is no single study dealing with SC-CO2 utilization for extraction of phenolic-phytochemical compounds from ant-nest tuber, this study aims to evaluate the co- solvent free SC-CO2 extraction performance and determine total

21**contents of polyphenols and flavonoids in the** antioxidant **extracts.**

The effects of extraction parameters

7**(pressure and temperature) on the extraction yields and** compositions are **studied.**

Mathemat- ical modeling of solubility data of total polyphenol content and total flavonoid content

1**by Chrastil and Del Valle–Aguilera density- based models**

was conducted. The scavenging activity of antioxi- dant extract against stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical was assayed and the scavenging performance was compared with commercial gallic acid. Experimental Materials Dried ant-nest tuber was purchased

5**from a** local **medicine store in Wamena** city, **Papua, Indonesia.**

This plant material was collected by indigenous Papuans from forest areas of Central Mountains in Jayawijaya district (1388300–1398400 east longitude and 38450–48200 south latitude). The chemical compositions of ant- nest tuber were determined as per standard methods: moisture of 4.36% (vacuum oven-drying method, AOAC 934.01), crude protein of 2.75% (Kjeldahl method, AOAC 984.13), crude fat of 2.68% (acid hydrolysis method, AOAC 954.02), crude fiber of 4.84% (soxhlet method, AOAC 962.09), ash of 3.22% (ash oven method, AOAC 920.153) and total carbohydrates of 82.15% (by difference). Prior to extraction, dried ant-nest tuber was pulverized by a laboratory- scale grinder and sieved through 30/40 mesh screens. The product was then stored in a cold storage at 4–5 8C. Food grade liquid CO2 with a minimum purity of 99.9% was supplied by a local gas company. Folin-Ciocalteu's phenol reagent,

15**2,2-diphenyl-1-picryl- hydrazyl (DPPH), gallic acid** (99%), caffeic **acid** (HPLC grade, 98%), **p-** coumaric **acid**

(HPLC grade, 98%), ferulic acid (99%), ( )- epicatechin (HPLC grade, 90%), (+)-catechin hydrate (HPLC grade, 98%), rutin (HPLC grade, 94%), kaempferol (HPLC grade, 97%), apigenin (HPLC grade, 95%), luteolin (TLC grade, 98%) and quercetin (HPLC grade, 95%) were obtained from Sigma Aldrich Co., Singapore. Other chemicals such as ethanol, potassium acetate, anhydrous sodium carbonate, acetonitrile, aluminum chloride and acetic acid glacial were obtained from Merck, Germany. Distilled water was used throughout the experiments. Supercritical extraction apparatus Bench-scale supercritical extractor in this work was custom- manufactured, consisting of a 150 mL high-pressure double-ended extraction vessel, horizontal reciprocating pump with dual inlet ports

4**(Eldex AA-100-S-2), a pressure transducer (Druck PTX 611) connected to digital** pressure **indicator (Druck DPI 280), a** temperature-controlled **oven**

as the heating chamber (Memmert UNB500),

4**a vacuum pump (GAST DOA-P504-BN)** and **a calibrated wet gas flow meter (ZEAL DM3B).**

All tube fittings and micro- metering valves were made of

7**316SS-grade stainless steel (Swagelok, USA). The maximum working pressure and** working **temperature of** supercritical extraction unit **are** 40 MPa **and** 473.15 K, **respectively.**

Extraction procedures Soxhlet extraction experiments were conducted at atmospheric pressure and 343.15 K. Ten grams of dried powdered plant were wrapped with a filter paper and placed in the glass extraction thimble. Then, 250 mL 96% ethanol was transferred into a three- neck round bottom flask and heated by an electrothermal heating mantle (Barnstead EMA500/CEB). Both the flask and thimble were sealed tightly with

Al foil to prevent light interference. A seven- bulb condenser filled with flowing water at 20–25 8C was used as a cooling media to condense solvent vapor and a thermometer for sensing temperature. The extraction process was carried out for 4– 5 h until clear liquid was obtained in the glass capillary column. Afterward, the system was cooled and the extract was removed from the flask. The product was stored in an amber bottle under cold condition prior to analysis. Supercritical CO2 extraction experiments were performed in a static-analytical mode because it is simpler and allowed to conduct extraction with less fluid than with dynamic extraction. Briefly, a known amount of ant-nest powder (10 g) was introduced into high-pressure equilibration vessel packed with clean glass beads. At both ends of the vessel, sintered stainless steel filters (50 mm) were installed to prevent any carryover of the solid particles. Prior to extraction, the vessel was evacuated using a vacuum pump. Then, the vessel

1**was heated from room temperature to** operating **temperatures (313.** 15 K, **323.** 15 K, **333.** 15 K **and 343.**

15 K). Once the operating temperature was reached, liquid CO2 was charged to the system by a reciprocating pump at

6**constant flow rate of** 10 **mL/min**

and compressed to desired pressures in the range of 9–22.5 MPa. The extraction process was performed for 6–7 h to attain equilibrium. Determination of equilibrium time was based on the condition when the amounts of extracted analytes in SC-CO2 no longer change with time. During this period, temperature was controlled with a precision of 1 K and pressure was monitored electronically by a digital transducer system to establish isobar and isothermal conditions. After a given extraction period was accomplished, the outlet micrometering valve was opened to depressurize saturated CO2-rich phase while pressure was maintained constant inside the vessel. A precipitated fraction was collected in a trapping flask containing some volume of 96% ethanol. To prevent freezing and possible solute precipitation during depressurization, the discharge channel was gently heated with an electrical heating mantle. The expanded gas was escaped through

4**a vent needle mounted on the**

trapping flask and 28 R.E. Sanjaya et al. /

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1**total volume of** analytes **-free ambient gas was** measured by **a calibrated wet gas flow meter at a known pressure and temperature (0.** 05 L). **The**

collected samples were stored in an amber bottle at 4 8C for further use. Tubings and valves throughout the extraction apparatus were cleaned with ethanol at the end of each run to ensure good quantification. The equilibrium loading

14**(solubility) at a given pressure and temperature was determined by quantifying the mass of** analytes collected in **the trapping solvent for a given volume of CO2.** Extraction yield **was**

determined gravimetrically and expressed as percentage of weight of the extract collected to the original sample. Determination of total polyphenol content (TPC) Total polyphenol content was determined by Folin-Ciocalteu spectrophotometric assay [16]. Briefly, 1 mL aliquot (ethanolic solution of extract) was mixed with 5 mL 1:10 dilution of Folin- Ciocalteu's phenol reagent and vortexed for 5 min. Then, 4 mL saturated Na2CO3 solution (75 g/L)

25**was added** and **the mixture was incubated in dark for 2 h at room temperature.** Blank **was**

concomitantly prepared, containing

11**0.5 mL ethanol, 2.5 mL 10% (v/v) Folin-Ciocalteu's phenol reagent and 2.5 mL 7.5% Na2CO3 solution** by **the same procedure. Dilutions** were **made when needed. The absorbance**

was read at 764.6 nm on a Shimadzu 1700 UV/vis spectrophotometer. The standard solutions of gallic acid at five different concentrations (0.4–2 mM) were used to prepare the calibration curve. Three replications were performed for each run. The results were expressed as milligram gallic acid equivalent (mg GAE) per gram dry plant. Here, the justification for expressing a mixture of polyphenols as a single number (GAE) is because gallic acid is the significant phenolic unit in most macromolecular complexes; also this hydroxybenzoic acid is inexpensive, water soluble, readily dried, recrystallized easily from water and stable in dry form [16]. Determination of total flavonoid content (TFC) Total flavonoid content was assayed by modified aluminum chloride colorimetric assay [17]. The procedure was described

3**as follows: 1** mL **of** aliquot **was** separately **mixed with 3 mL** 95% **ethanol, 0.2 mL** 10% **aluminum chloride, 0.2 mL 1 M potassium acetate and 5.6 mL distilled water**

in a series of test tubes. The mixtures were allowed to stand for 30 min at room temperature prior to spectrophotometric determination. The absorbance was read at 415.2 nm. Blank was prepared in similar way without adding aluminum chloride (the volume of 10% AlCl3 solution was replaced by the same volume of distilled water). The

1**calibration curve was prepared** from **standard solutions of** quercetin at varying **concentrations**

of

20**12.5 mg/mL, 25 mg/mL, 50 mg/mL, 75 mg/mL and 100 mg/mL**

in ethanol. The results

21**were expressed as** milligram **quercetin equivalent** (mg **QE)/g dry plant.**

Here, quercetin was selected as a standard for calibration and to represent total flavonoids as a single number (QE) because the complex formed between quercetin and aluminum chloride gave a more sensitive absorbance reading at 415 nm among other flavonoid standards such as rutin, apigenin, morin or luteolin [17]. Additionally, quercetin belongs to flavonols which are widely found in plant materials. Determination of total antioxidant capacity Total antioxidant capacity was estimated by DPPH scavenging activity using the modified method of Blois [18]. A known amount of aliquot (0.1 mL)

15**was mixed with** 0 **.5 mL of 0.** 5 **mM DPPH** solution **in ethanol**

and 0.4 mL of 0.1 M Tris–CIH buffer (pH 7.4). The mixture was aged

10**for 30 min in** a **dark** chamber. **The absorbance of the mixture was measured at 517.** 3 **nm.** The following equation **was** applied to determine **the**

deterioration rate of stable free radical DPPH species at a characteristic wavelength in presence of the sample: Scavenging percentageð%Þ ¼ 1 As A0 100 (1) where As and A0 are the absorbance of extract and DPPH solution as control, respectively. Gallic acid solutions of different concentra- tions ranging from 0.1 mg/mL to 10 mg/mL were employed as positive control. Quantification of polyphenol compounds High performance liquid chromatography (HPLC) is one of versatile instrumental techniques in analytical chemistry and biochemistry with the purposes of separating, identifying and quantifying individual components contained in a

5**sample that can be dissolved in HPLC compatible liquid**

[19]. The chromatographic instrument consists of a quaternary low pressure gradient pump (Jasco PU-2089 Plus), a dual/multi-wavelength intelligent UV/vis detector (Jasco UV-2077 Plus)

7and a LC-NetII/ADC hardware interface system for data acquisition. The analysis was performed following a

method described by Weiss et al. [20] with some modifications. All samples were filtered through a 0.22 mm PVDF syringe filter prior to injection. The chromatogram was acquired in a SGE Enduro C18-5 mm-120 Å reversed-phase HPLC column (250 mm 4.6 mm ID). The samples

5were eluted with a linear gradient elution of mobile phases A and B. Solvent A was consisted of

3% (v/v) acetic acid in distilled water and solvent B was consisted of a mixture of acetonitrile and 3% acetic acid in water (97:3, v/v). Acetic acid was added to increase the peak resolution. Flow rate was set at 1.0 mL/min and 30 8C. The gradient elution was programmed as follows: isocratic at 10% phase B (10 min), linear increase of phase B from 10% to 20% (5 min), isocratic at 20% phase B (10 min), 20% B–32% B (13 min), 32%

13B–46% B (12 min), 46% B– 55% B (5 min), 55% B–100% B (5 min), 100% B isocratic (8 min), 100% B– 10% B (2 min) and 10% B isocratic (5 min).

10Total run time was 75 min. The injection volume was 20 mL. Polyphenol compounds were separately monitored at 280 nm

(hydroxyben- zoic acids), 320 nm (hydroxycinnamic acids) and 360 nm (flavones and flavonols) for maximizing detection sensitivity. Identification of peaks was conducted by matching retention time and UV/vis spectra with those previously obtained from the injection of authentic standards of known identity and purity. Calibration curves were used to quantify the concentrations of each compound. Data were recorded and processed using a Jasco ChromNav software system (Version 1.18.04, build 6) available within the instrument. Statistical analysis All measurements were reported as mean standard deviation (SD) for three replications. One-way

6analysis of variance (ANOVA) using Minitab 16 software was conducted to

examine statistical significance and post hoc comparisons between means were obtained by means of Duncan's multiple range tests. Significant difference between groups was statistically considered at 95% confidence level (p-values <5%). Results and discussion Densities of SC-CO2 at various pressures and temperatures The liquid-like solvent power and gas-like diffusion ability are two main attracting features of supercritical fluids which allow such fluids to have an exceptional extraction capability. The cubic equation of state proposed by Peng and Robinson [21] was applied to compute density of SC-CO2 in dependence on pressure and temperature with the aid of ChemCAD software (Version 6.4, ChemstationsTM). Details about

calculation procedure can be found elsewhere [22]. The values of Tc, Pc and v for CO2 are 304.25 K, 7.39 MPa and 0.225, respectively. It is well-reported that the density of SC-CO2 increased with increasing pressures at a given temperature. At higher pressure, the available volume occupied by the molecules becomes lesser due to compression action, causing the intermolecular spaces to be closer and consequently greater density of the fluid. The density of SC-CO2 ranged between 549.98 g/L and 879.26 g/L at pressures of 9–22.5 MPa and 313.15 K. As comparison, the density of liquid ethanol at ambient pressure and temperature is 789.0 g/L. Therefore, higher densities of SC-CO2 at nearly ambient tempera- ture (313.15 K) were noted in the range of pressures from 1.65 MPa to 2.25 MPa. An opposite behavior was observed between density and temperature in which higher temperature causes the fluid density to decrease. This phenomenon may be ascribed

1**to greater molar volume at** higher temperature. **Since**

molar volume and density are

1**inversely proportional hence the** higher the **molar volume,** the **lower**

is the density and vice versa. Chemical compositions and extraction yields of antioxidant extracts Fig. 1A–E shows the chromatography patterns of antioxidant extracts obtained from soxhlet extraction and SC-CO2 extraction. The antioxidant extract obtained from soxhlet extraction (SE extract) contains polyphenols of five subclasses include hydro- xybenzoic acids, hydroxycinnamic acids, flavan-3-ols, flavones and flavonols with the following retention time: gallic acid (4.41 min), (+)-catechin (17.83 min), caffeic acid (21.18 min), p-coumaric acid (23.55 min), ferulic acid (24.48 min), rutin (62.78 min), quercetin (64.63 min), luteolin (66.97 min) and kaempferol (69.91 min). The identified flavonoids (i.e., rutin, quercetin, luteolin and kaempferol) are in good conformance with previous results [4]. We found that apigenin was undetectable, likely due to trace concentration of this compound. On the other hand, the chromatograms of four supercritical extracts (denoted as

18**SFE-1, SFE-2, SFE-3 and SFE-4)**

showed fewer peak signals with lower intensities. The extraction yields of each phenolic compound are graphically presented in Fig. 2. It can be seen that soxhlet extraction is capable to leach more polyphenol and flavonoid compounds from the plant matrix. The extraction yield was 122.36 mg crude extract/g dry plant. In the soxhlet extraction, the condensed solvent vapor penetrates internal structure of the solid and separates out solutes that have similar polarity to that of liquid solvent. Meanwhile, the dissolution phenomenon in SC-CO2 extraction not solely depends on the solute solubility in supercritical solvent, but also on several factors such as effective solute diffusivity, external mass transfer and solute–matrix interaction. In the native state, SC-CO2 behaves as a lipophilic solvent whose extraction performance toward phenolic antioxidants is not impressive. Thus, it is not surprisingly to obtain antioxidant extracts with lower bioactive content from SC-CO2 extraction. Total content of polyphenols and flavonoids in SE extract are 28.07 1.28 mg GAE/g dry plant and 16.44 0.58 mg QE/g dry plant, respectively. These concentrations are higher than those of SFE extracts, even for SFE-4 obtained at 22.5 MPa and 343.15 K. However, the purity of phenolic antioxidants in SFE extracts was higher than in SE extract, indicating that SC-CO2 was a

more selective solvent in comparison to liquid ethanol. The purity of phenolic antioxidants ranged between 34.3 wt% and 48.7 wt% for SFE extracts compared to 18.3 wt% of SE extract. The concentrations of gallic acid, caffeic acid, p-coumaric acid, quercetin and kaempferol in SFE extracts were increased by Fig. 1. HPLC chromatograms of antioxidant extracts obtained from soxhlet extraction and SC-CO2 extraction. The identified compounds are designated by numbers as follows:

24**gallic acid (1); (+)-catechin (2); caffeic acid (3); p-** coumaric **acid (4);** ferulic acid **(5);** rutin **(6);** quercetin **(7);** luteolin **(8)** and kaempferol **(9).**

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Fig. 2. Extraction yields of several phenolic compounds (* quercetin; gallic acid; p-coumaric acid; rutin; luteolin; kaempferol; ferulic acid; (+)-catechin; and caffeic acid) obtained from soxhlet extraction and SC-CO2 extraction at a pressure of 22.5 MPa and various temperatures (SFE-1/313.15 K; SFE-2/323.15 K; SFE-3/333.15 K; and SFE-4/343.15 K). increasing extraction temperature. Interestingly, two or more phenolic compounds such as (+)-catechin, ferulic acid and luteolin were detected in SFE-2, SFE-3 and SFE-4 chromatograms, which might be attributed to improved extraction performance of SC-CO2 toward more polar and high molecular weight compounds as a result of synergistic action between pressure and temperature. In this work, the use of co-solvent to improve extractability of phenolic antioxidants is not considered because it would compli- cate further downstream processing and also several co-solvents (e.g., methanol, propylene glycol, chloroform or ethyl acetate) may not be environmentally acceptable. Another explanation is that several phenolic acids include gallic acid, ferulic acid, kaempferol and p-coumaric acid is readily soluble in SC-CO2 according to previous studies [13–15]. Analysis of TPC and TFC of antioxidant extracts Fig. 3 shows the error-bar plots of TPC and TFC of supercritical extracts at various conditions. The uncertainty of the measurement ranged between 4.84% and 9.12%. As can be seen, both TPC and TFC increased by increasing pressure for constant temperature, which might be ascribed to enhanced density and solvent capacity of SC- CO2. Total flavonoid content in the supercritical extracts ranged between 0.061 0.006 and 0.392 0.035 g QE/L CO2 while total phenolic content ranged between 0.368 0.021 and 2.182 0.180 g GAE/L CO2. The extraction yields of polyphenols and flavonoids also rose from 2.78 0.21 to 16.41 1.08 mg GAE/g dry plant and from 0.48 0.03 to 2.96 0.15 mg QE/g dry plant with increasing extraction pressure and temperature. Considering the retrograde solubility behavior existed in the solid-supercritical fluid systems, the effect of temperature may be of importance toward the extraction yield. Generally speaking, the increase of temperature lowers the extraction yield due to reduced density of the fluid. As shown in Fig. 2A and B, an increase in temperature from 313.15 K to 343.15 K at 9.5 MPa and 10.5 MPa does not give a clear influence on the solubilities of polyphenols and flavonoids in SC-CO2. The increasing solubilities of solutes below crossover point were mainly attributed to dominate effects of solvent density. Johnston et al. defined crossover pressure

27**as the point where the slope of plot of solubility versus temperature changes sign** [23]. The **crossover**

phenomenon was obviously observed at 12–22.5 MPa. In this pressure range, the role of temperature seems to be more influential than pressure in increasing solubilities of solutes. Beyond the crossover pressure, the solubilities of both polyphenols and flavonoids in SC-CO2 Fig. 3. Solubilities of total polyphenols (A) and total flavonoids (B) in SC-CO2 at various pressures and temperatures (error bars represent standard deviation), values not sharing same letters are

26**significantly different ($p < 0.05$) based on one- way analysis of variance and Duncan's**

multiple range tests. doubled for a thirty degrees-increase in temperature at constant pressure. Increasing temperature would enhance volatility of solutes thus more solutes were readily dissolved in the supercritical solvent. The significance of solute vapor pressure has been investigated by Hojjati et al. [24] and Foster et al. [25]. Hojjati et al. reported the increasing solubilities of five statin drugs in SC-CO2 with temperature increase beyond the crossover point. Similarly, Foster et al. found that crossover pressure is a phenomenological observation in the solid-supercritical fluid systems and may also be of fundamental significance

17**to indicate reliability and consistency of** actual **solubility data.**

Density-based modeling of solubility data Modeling solubility data of a solute in SC-CO2 is highly important for scale-up,

1**design and optimization of supercritical processes. For**

this purpose,

4**Chrastil and Del Valle–Aguilera** density-based **models were** employed **to correlate solubilities of**

various phenolic antioxidants in SC-CO2.

18**Here, SFE-1, SFE-2, SFE-3 and SFE-4**

extracts were applied as the models to be analyzed. Because the concentrations of phenolic antioxidants in the supercritical extracts were determined at equilibrium condition for a given quantity of CO2, the values could be regarded as solubility data. The correlation of solubility data by empirical or semi-empirical density-based models is preferred than cubic equations of state due to it is simpler and does not require any thermophysical data that normally cannot be experimentally determined. Moreover, most of density-based models give high level of consistency between predicted and actual solubilities over a wide range of

1**pressures and temperatures** [26–29]. **Chrastil was the first who** developed a **density-based model**

from the conception of chemical association laws and/or entropies of the components for correlating

23**solubility of solids and liquids in** dense **gases**

[30]. He suggested that solute and solvent molecules are interacted each other

17**to form a** solvato **complex, which is in equilibrium with supercritical solvent,**

according to the following reaction: A þ kB $ ðABÞk (2) From reaction above,

16**one molecule of solute A associates with k molecules of solvent B to form a** solvato **complex** of **(ABk)** at **equilibrium. The** values **of constant**

k are not an integer and often defined

1**as an average association number for a given solute- supercritical solvent** pair. This **model**

expressed a linear relation- ship between logarithmic solubility of

1**solute in** pure **supercritical solvent** and **that of solvent density and temperature**

as follows: c ¼ rkexp a T þb (3) where c is

1**the solubility of solute (kg/m3),** r is **solvent density (kg/ m3),** T is **temperature (K),**

6**a is a function of enthalpy of solvation** (DHsolv) **and enthalpy of vaporization** (DHvap) **and b is a function of average association number that depends on molecular weight of solute and solvent. The** values **of** adjustable constants **a,**

b and k are

1**specific for each solute-solvent pair and** independent **on** tempera- ture **and pressure.**

1**The second semi-empirical density-based** model **used was Del Valle and Aguilera. They**

proposed a modification to Chrastil

1**model to compensate for variation of vaporizing** enthalpy **with temperature**

[31]. The

1**model has a mathematical** expression **as follows: c** ¼ rkexp þ **b**

þ T2 a c T (4) where c/T2 is an adjustable term added to account for

1**variation of solute's vaporizing heat with temperature.**

This four-parameter model has proven to adequately predict solubility data under 100 g/L within 293–353 K and 150–880 atm [31]. The

9**agreement between the model** predictions **and experimental** solubility **data is** evaluated based on **the**

1**absolute average relative deviation (AARD) as**

an objective function: AARDð%Þ ¼ 100 XN yicalc yiexp N (5) i¼1 yiexp

28**where N is the number of experimental data,** yicalc **and** yeixp **are** the calculated **and** actual **solubility values, respectively. The**

fitness of

1**Chrastil and Del Valle–Aguilera models** toward **solubility data** is depicted **in**

Fig. 4. In this figure, the symbols represent solubility data and wire-mesh plots indicate simulation results. Solubility of polyphenols and flavonoids was represented as based on analysis. Nonlinear regression fitting using SigmaPlot software (Version 12.3, Systat Software Inc.) was conducted to determine parameter values of the model and the results are shown in Table 1. The initial values of fitted parameters were determined with the following constraints: a < 0, b > 0, c > 0 and k > 0. Visually, both Chrastil and Del Valle–Aguilera models can represent actual solubilities of phenolics and flavonoids in SC-CO2 very well. The value of average association number is always positive because it describes the number of CO2 molecules in solute-solvent complexes. The fitted values of k for Chrastil model Fig. 4. 3D-fitting of

1**Chrastil (A–** B) **and Del Valle–Aguilera** (C–D) **models**

on the experimental solubilities of polyphenols (g GAE/L CO2) and flavonoids (g QE/L CO2). 32 R.E. Sanjaya et al. /

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Table 1 The

4**fitted parameters of Chrastil and Del Valle–Aguilera models for**

solubility correlations of total polyphenols and total flavonoids in SC-CO2. Mathematical models Parameters Polyphenols Flavonoids Chrastil a b k AARD (%) 4621.05 1.46 1.93 5.96 5089.02 1.74 1.84 1.22 Del Valle–Aguilera a b c k AARD (%) 3149.41 0.77 2.43 105 1.93 5.84 18,543.43 22.09 2.22 106 1.83 0.98 are 1.93 for polyphenols/SC-CO2 and 1.84 for flavonoids/SC-CO2,

1**which is comparable to that of Del Valle–Aguilera model.**

The values of adjustable constant a are 4621.05 for polyphenols and 5089.02 for flavonoids from Chrastil fitting. As aforesaid, the adjustable constant a is the function of total reaction heat, which equals to the

23**sum of** vaporization **heat of** solute **and heat of solvation.** For Chrastil model, **the**

constant a is expressed as DHsoln/ R while

1**for Del Valle and Aguilera is**

DHsoln/R – 2d/T.

1**Total reaction heat for the** dissolution of polyphenols **and**

flavonoids in SC-CO2 was 38.42 kJ/mol and 42.31 kJ/mol for Chrastil model and 44.23 kJ/mol and 51.07 kJ/mol

1**for Del Valle and Aguilera model,**

respectively. The negative sign indicates the exothermicity of the associating process between

1**solute and supercritical solvent,** in which **the** magnitude **of**

solvation heat is much greater than heat of vaporization. By judging the AARD values (%), both

4**Chrastil and Del Valle and Aguilera** model give **satisfactory agreement for**

solubility correlations at studied pressures and temperatures. The adjustable constant q in both models can be calculated using following expression: q ¼ b þ klnMB lnðMA þ kMBÞ (6) In Eq. (6), MA and MB refer to the molecular weight of solute and carbon dioxide, respectively. The values of MA are taken as the molecular weight of gallic acid (170.12 g/mol) to represent polyphenols and quercetin (302.23 g/mol) to represent flavonoids. The calculated values of constant q for polyphenols/SC-CO2 and flavonoids/SC-CO2 systems are 5.45 and 5.73 (Chrastil model) and 3.22 and 26.08 (Del Valle–Aguilera model). DPPH free radical scavenging potential of antioxidant extracts The in vitro antioxidant potential of antioxidant extracts was tested for scavenging stable DPPH free radical. Five antioxidant extracts namely SE, SFE-1, SFE-2, SFE-3 and SFE-4 were assayed. The dosage of antioxidant extracts used in the experimental tests was 10 mg/mL. Fig. 5I illustrates the DPPH free radical scavenging percentage of each sample (data were given as mean SD for three replicate assays). The DPPH scavenging percentages of antioxidant extracts ranged between 37% and 76%. For the same dosage, SFE-4 extract showed the highest scavenging percentage of ca. 76.17 0.27% while SE was the lowest (36.64 0.15%). The DPPH scavenging capacity ranks from highest to lowest as follows: SFE- 4 > SFE-3 > SFE-2 > SFE-1 > SE, which is in good agreement with the purity of antioxidant extracts. Therefore, it can be concluded that SC- CO2 extraction is a more viable option than conventional soxhlet extraction to yield extracts containing high concentrations of antioxidative compounds for effective inhibition against free radicals. Fig. 5. Scavenging percentage of various antioxidant extracts, different letters indicate significant differences between means (I), DPPH-scavenging activity between SFE-4 extract and gallic acid (inset is the potency values (P), determined as log (IC50)) (II), and 3D-linear correlation between scavenging potency and bioactive contents (* TPC and TFC) of SFE extracts (III). Table 2 The half maximal inhibitory concentrations of various plant-derived antioxidant extracts obtained by static SC-CO2 extraction without a co-solvent. Plants Extraction parameters IC50 (mg/mL) References P (MPa) T (K) CO2 flow rate (g/min) N. sativa P. nigrum T. articulata A. satureioides Clove buds Ant-nest 35 323.15 30 313.15 9 313.15 12 313.15 <10 313.15 22.5 343.15 15 2590 10 0.04 103.28 5 243.0 10 8.33 20.40 10.33

20.64 0.15 0.02 3620 120 [32] [33] [34] [35] [36] This study The IC50 values (i.e., the inhibition percentage corresponding to the half reduction of absorbance of DPPH free radical) were determined graphically by plotting concentrations versus DPPH scavenging percentages (Fig. 5I). The results are also presented in terms of potency (log IC50) as an inset figure. By regressing plot of C versus percent scavenging, the IC50 value of SFE-4 extract was found to be 3.62 0.12 mg/mL (r2 = 0.94). This value is about twofold higher compared to that of gallic acid with an IC50 level of 1.73 0.08 mg/mL (r2 = 0.91). The potency of scavenger activity corresponds to the reduction of absorbance of DPPH free radical by 50.12% for SFE-4 extract and 50.22% for gallic acid. For comparison study, the half maximal inhibitory concentrations (IC50) of various plant-derived antioxidant extracts are summarized in Table 2. It is also important to interpret the relevancy between the potency of scavenger activity and bioactive contents of the extract. For this purpose, linear regression method using SigmaPlot software with Pearson's correlation analysis was employed. Fig. 5I and II shows the 3D-view of regressed plot of TPC (x-axis, mg GAE/mL) and TFC (y-axis, mg QE/mL) of SFE extracts versus the scavenging potential. A positive linear correlation (r2 of 0.97) is obtained, suggesting the linear increase of DPPH scavenging potential with increasing TPC and TFC of antioxidant extracts. The following mathematical equation describes a linear relation between the potency of DPPH free radical scavenging and bioactive contents of SFE extracts: P ¼ 0:55 þ 0:0327 ðm þ nÞ (7) where P is the potency value, m and n are total polyphenol content (mg GAE/mL) and total flavonoid content (mg QE/mL) of the extract, respectively. Conclusions An eco-friendly extraction process of active phenolic-phyto- chemicals from ant-nest tuber by SC-CO2 has been demonstrated in this work. The extraction yields and solubilities of phenolic- phytochemicals in SC-CO2 are strongly affected by the changes of pressure and temperature. The solubilities of phenolic antioxidants were increased significantly with increasing pressure and temper- ature beyond the crossover point (P 12 MPa). The maximum yields of flavonoids and polyphenols are 2.96 0.15 mg QE/g dry plant and 16.41 1.08 mg GAE/g dry plant, respectively. The solubility data are well-correlated

1**by Chrastil and Del Valle–Aguilera density-based models**

over studied pressures and temperatures. Free radical scavenging test proved the inhibitory potency of SFE extract obtained at 22.5 MPa and 343.15 K against DPPH free radical with an IC50 concentration of 3.62 0.12 mg/mL. Acknowledgement This work was partially funded by Directorate General of Higher Education through Student Creativity Program (PKM-P) 2013 to Rebeca E. Sanjaya and Yanuar Y. Tedjo. References [1] M.N. Hamsar, H.H. Mizaton, J. Pharm. Res. 5 (2012) 3063–3066. [2] A. Soeksmanto, M.A. Subroto, H. Wijaya, P. Simanjuntak, Pak. J. Biol. Sci. 13 (2010) 148–151. [3] T. Hertiani, E. Sasmito, Sumardi, M. Ulfah, OnLine J. Biol. Sci. 10 (2010) 136–141. [4] A.M. Engida, N.S. Kasim, Y.A. Tsigie, S. Ismadji, L.H. Huynh, Y.H. Ju, Ind. Crop. Prod. 41 (2013) 392–396. [5] A.M. Farias-Campomanes, M.A. Rostagno, M.A.A. Meireles, J. Supercrit. Fluids 77 (2013) 70–78. [6] E. Vagi, B. Simandi, H.G. Daood, A. Deak, J. Sawinsky, J. Agric. Food Chem. 50 (2002) 2297–2301. [7] N. Assmann, S. Kaiser, P. Rudolf Von Rohr, J. Supercrit. Fluids 67 (2012) 149–154. [8] M. Poiana, V. Sicari, B. Mincione, Flavour Frag. J. 13 (1998) 125–130. [9] A. Nisha, K. Udaya-Sankar, G. Venkateswaran, Food Chem. 133 (2012) 220–226. [10] L.T. Danh, N.D.A. Triet, L.T.N. Han, J. Zhao, R. Mammucari, N. Foster, J. Supercrit. Fluids 70 (2012) 27–34. [11] N. Gelmez, N.S. Kincal, M.E. Yener, J. Supercrit. Fluids 48 (2009) 217–224. [12] C.R. Piantino, F.W.B. Aquino, L.A. Follegatti-Romero, F.A. Cabral, J. Supercrit. Fluids 47 (2008) 209–214. [13] R. Murga, M.T. Sanz, S. Beltran, J.L. Cabezas, J. Supercrit. Fluids 23 (2002) 113–121. [14] R. Murga, M.T. Sanz, S. Beltran, J.L. Cabezas, J. Supercrit. Fluids 27 (2003) 239–245. [15] E.S. Choi, M.J. Noh, K.P. Yoo, J. Chem. Eng. Data 43 (1998) 6–8. [16] V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventos, Methods Enzymol. 299 (1999) 152–178. [17] C.C. Chang, M.H. Yang, H.M. Wen, J.C. Chern, J. Food Drug Anal. 10 (2002) 178–182. [18] M.S. Blois, Nature

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