CHARACTERISATION OF OPTIMISED SUPERCRITICAL CARBON DIOXIDE CHIA SEED OIL USING TOCOPHEROL QUANTIFICATION, FATTY ACID PROFILE, OXIDATION KINETIC AND DIGESTION STUDY

By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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Particle size affects the yield of seed oil extraction other than supercritical carbon dioxide (SC-CO_2) parameters. Chia seed oil (CSO) extracted by supercritical fluid extraction (SFE) is vulnerable to oxidation which contains more than 80% polyunsaturated fatty acids (PUFA). However, limited study has been conducted on examining the effect of different particle sizes of chia seed in extracting a high yield of oil using SC-CO_2. Therefore, the objectives of this study are: i) To determine the optimum SC-CO_2 extraction parameters and particle size of the ground sample to extract the highest yield of CSO. ii) To compare the optimised CSO extracted by SC-CO_2 (SC-CO_2-CSO) characteristics with Soxhlet (SOX-CSO) in terms of oxidation levels, tocopherols, PUFA, and oxidative stability. iii) To evaluate the oxidation stability of SC-CO_2-CSO during storage with SOX-CSO. iv) To identify the bioaccessibility of tocopherols and volatile oxidation compounds of SC-CO_2-CSO (fresh and stored) using *in vitro* stomach and small intestine. The optimisation parameters include different particle sizes of chia seeds based on grinding times (10-

30 s) and SFE at different temperatures (40-80 °C) and pressure (220-340 bar) to maximise the CSO yield. The optimum yield (30.7%) of CSO based on Central Composite Design is similar to the predicted value (31.1%) at the pressure (335 bar), temperature (40 °C), and grinding time of chia seed (20 s). The oxidative stability shows both CSO (SC-CO_2-CSO: 0.88 h and SC-CO_2-SOX: 1.49 h) are less protected against oxidation due to the high amount of PUFA (85%) in CSO. For objective 3, the oxidation level, degradation of tocopherols, and antioxidant activity of SC-CO_2-CSO and SOX-CSO stored at different temperatures (25-60 °C) were evaluated kinetically based on the reaction rate constant (*k*) and activation energy (*Ea*). The *k* for oxidation level (PV:0.047-0.468 mEq O_2/kg oil day^{-1}), degradation of antioxidant activity (0.000-0.552 % day^{-1}) and α- (0.031-0.233 mg/kg oil day^{-1}) and γ-tocopherols (0.003-0.03 mg/kg oil day^{-1}) of SC-CO_2-CSO and SOX-CSO increased significantly ($p<0.05$) as storage temperatures increased from 25-60 °C. Lower *Ea* for the degradation rates of tocopherols (0.012-0.032) than antioxidant activity (147.429-149.26) and oxidation levels (52.779-54.756) in SC-CO_2-CSO and SOX-CSO were obtained due to the tocopherol acted as a hydrogen donor to prevent oxidation during storage. As storage temperature increases, the shelf life of SC-CO_2-CSO and SOX-CSO is decreased significantly ($p<0.05$) from 2.35 to 0.23 months. Finally, fresh (PV: 0.6 mEq O_2/kg oil) and stored (PV: 26.7 mEq O_2/kg oil) SC-CO_2-CSO were submitted to the *in vitro* stomach and small intestine digestion models to determine the bioaccessibility of tocopherols and fatty acids, antioxidant activity and volatile oxidation compounds. Stored SC-CO_2-CSO has significantly ($p<0.05$) higher concentration of aldehydes (2.58-12.29 Bp x 10^6), lower tocopherols (37.87-73.54%), α-linolenic acid (36.53-89.97%) and antioxidant activity (6.6-12.6%) are prone to oxidation than fresh SC-CO_2-CSO using *in vitro* stomach and small intestine models due to occurrence of

oxidation process. Therefore, fresh SC-CO_2-CSO has higher bioaccessibility of PUFAs and tocopherols, antioxidant activity, and lower oxidation than stored SC-CO_2-CSO, which can be diversified as vegetable-based oil supplement and functional food ingredient.

Keywords: Chia seed oil, supercritical carbon dioxide, tocopherols, oxidation, digestion

SDG: GOAL 9: Industry, innovation and infrastructure

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN MINYAK BIJI CHIA KARBON DIOKSIDA SUPERKRITIKAL DIOPTIMUMKAN DENGAN MENGGUNAKAN KUANTIFIKASI TOKOFEROL, PROFIL ASID LEMAK, KINETIK PENGOKSIDAAN DAN KAJIAN PENCERNAAN

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Saiz partikel mempengaruhi hasil pengekstrakan minyak biji selain daripada parameter karbon dioksida superkritikal (SC-CO_2). Minyak biji chia (CSO) yang diekstrak oleh SC-CO_2 terdedah kepada pengoksidaan yang mengandungi lebih daripada 80% asid lemak poli tak tepu (PUFA). Walau bagaimanapun, kajian terhad telah dijalankan untuk mengkaji kesan saiz partikel biji chia yang berbeza untuk mengekstrak hasil minyak yang tinggi dengan menggunakan SC-CO_2. Oleh itu, objektif kajian ini adalah: i) Untuk menentukan optimum parameter pengekstrakan SC-CO_2 dan saiz partikel sampel yang telah dikisar untuk mengekstrak hasil CSO yang paling tinggi. ii) Untuk membandingkan pencirian CSO yang telah dioptimumkan dari pengekstrakan SC-CO2 (SC-CO_2-CSO) dengan Soxhlet (SOX-CSO) dari segi tahap pengoksidaan, tokoferol, PUFA, dan kestabilan oksidatif. iii) Untuk menilai kestabilan pengoksidaan SC-CO_2-CSO semasa penyimpanan dengan SOX-CSO. iv) Untuk mengenal pasti bioakses tokoferol dan sebatian pengoksidaan meruap SC-CO_2-CSO (segar dan disimpan) menggunakan perut dan usus kecil secara

in vitro. Parameter pengoptimuman termasuk saiz partikel biji chia yang berbeza berdasarkan masa pengisaran (10-30 s) dan SFE pada suhu berbeza (40-80 °C) dan tekanan (220-340 bar) untuk memaksimumkan hasil CSO. Hasil optimum (30.7%) CSO berdasarkan Reka Bentuk Komposit Pusat adalah serupa dengan nilai ramalan (31.1%) pada tekanan (335 bar), suhu (40 °C), dan masa pengisaran biji chia (20 s). Kestabilan oksidatif menunjukkan kedua-dua CSO (SC-CO_2-CSO: 0.88 h dan SC-CO2-SOX: 1.49 h) kurang dilindungi daripada pengoksidaan disebabkan oleh jumlah PUFA yang tinggi (85%) dalam CSO. Untuk objektif 3, tahap pengoksidaan, degradasi tokoferol, dan aktiviti antioksidan SC-CO_2-CSO dan SOX-CSO yang disimpan pada suhu berbeza (25-60 °C) dinilai secara kinetik berdasarkan pemalar kadar tindak balas (*k*) dan tenaga pengaktifan (*Ea*). *k* untuk tahap pengoksidaan (PV:0.047-0.468 mEq O_2/kg minyak $hari^{-1}$), kemerosotan aktiviti antioksidan (0.000-0.552 % $hari^{-1}$) dan α- (0.031-0.233 mg/kg minyak $hari^{-1}$) dan γ-tokoferol (0.003-0.03 mg/kg minyak $hari^{-1}$) SC-CO^2-CSO dan SOX-CSO meningkat dengan ketara ($p<0.05$) apabila suhu penyimpanan meningkat daripada 25-60 °C. *Ea* yang lebih rendah untuk kadar degradasi tokoferol (0.012-0.032) daripada aktiviti antioksidan (147.429-149.26) dan tahap pengoksidaan (52.779-54.756) dalam SC-CO_2-CSO dan SOX-CSO diperolehi kerana tokoferol bertindak sebagai penderma hidrogen untuk mengelakkan pengoksidaan semasa penyimpanan. Apabila suhu penyimpanan meningkat, jangka hayat SC-CO_2-CSO dan SOX-CSO berkurangan dengan ketara ($p<0.05$) daripada 2.35 kepada 0.23 bulan. Akhirnya, segar (PV: 0.6 mEq O_2/kg minyak) dan disimpan (PV: 26.7 mEq O_2/kg minyak) SC-CO_2-CSO dimasukkan kepada model pencernaan perut dan usus kecil secara *in vitro* untuk menentukan bioakses tokoferol dan asid lemak, aktiviti antioksidan dan sebatian pengoksidaan yang meruap. SC-CO_2-CSO yang disimpan dan telah didedahkan kepada pengoksidaan mempunyai kepekatan

aldehid yang lebih tinggi (2.58-12.29 Bp x 10^6), tokoferol (37.87-73.54%), asid α-linolenik (36.53-89.97%) dan aktiviti antioksidan (6.6 -12.6%) yang rendah secara ketara ($p<0.05$) daripada SC-CO_2-CSO segar dengan menggunakan model perut dan usus kecil secara *in vitro* kerana berlakunya proses pengoksidaan. Oleh itu, SC-CO_2-CSO segar mempunyai bioakses PUFA dan tokoferol yang lebih tinggi, aktiviti antioksidan, dan pengoksidaan yang lebih rendah daripada SC-CO_2-CSO yang disimpan, yang boleh dipelbagaikan sebagai tambahan minyak berasaskan sayuran dan bahan makanan berfungsi.

Kata Kunci: Minyak biji chia, karbon dioksida superkritikal, tokoferol, pengoksidaan, pencernaan

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DPPH	2,2-diphenyl-1-picrylhydrazyl
ALA	α-linolenic acid
BCBA	β-carotene bleaching assay
p-AV	*p*-anisidine value
ANOVA	analysis of variance
E_a	activation energy
Bp	base peak
CO_2	carbon dioxide
°C	degree celcius
CCD	Central Composite Design
CCP	commercial cold-pressed
DSC	differential scanning calorimeter
DHA	docosahexaenoic acid
DPA	docosapentaenoic acid
EPA	eicosapentaenoic acid
ΔH	enthalpy
EFSA	European Food Safety Authority
FESEM	field emission scanning electron microscopy
GRAS	generally recognised as safe
g	gram
ha	hectares
HPLC	High-Performance Liquid Chromatography
h	hour
IP	induction period
IT	induction time

J/g	joule per gram
K	kelvin
kcal	kilocalorie
kJ/mol K	kilojoule per mole kelvin
kcal/mol	kilocalorie per mole
kg	kilogram
kHz	kilohertz
LA	linoleic acid
LPG	liquefied petroleum gas
L	litre
m/z	mass for charge number of ions
T_{max}	maximum temperature
m	metre
metric ton	metric tonne
µg	micro gram
µL	micro Litre
µm	micro metre
mg	milligram
mm	millimetre
mmol/L	millimoles per litre
mL	milli Litre
mmHg	millimetres of mercury
mEq O_2	milliequivalents of oxygen
min	minute
MUFA	monounsaturated fatty acids
N	normality
T_{off}	offset temperature

T_{on}	onset temperature
PV	peroxide value
PUFA	polyunsaturated fatty acid
k	reaction rate constant
RSM	Response Surface Methodology
rpm	revolutions per minute
SFA	saturated fatty acid
s	second
m^2/g	specific surface area
SPME-GC/MS	Solid Phase Microextraction Gas Chromatography/ Mass Spectrometry
SOX	Soxhlet
SC-CO_2	supercritical carbon dioxide
SFE	supercritical fluid extraction
TAG	triglyceride
TOTOX	total oxidation
UV-Vis	ultraviolet-visible

CHAPTER 1

INTRODUCTION

Chia (*Salvia hispanica* L.) is a herbaceous plant with flowers and edible seeds under the family of Labiate, cultivated annually and originally from Mexico and Guatemala (Ixtaina et al., 2011a). Mexico, Bolivia and Paraguay are the world's primary chia seed producers (approximately 30,000 metric tons annually) (Statistica, 2022a; Hrnčič et al., 2020; Grancieri et al., 2019). In 2017, the global sales of chia seeds generated about 66,000 metric tons, but rising demand (100,000 metric tons) is predicted for chia seeds until 2027 (Wunsch, 2020). Recently, the consumption of chia seeds has greatly increased due to their high polyunsaturated fatty acids (PUFA) and dietary fibre (Biswas et al. 2023). Furthermore, chia seeds contain a high content of oils (33%), carbohydrates (40%), proteins (25%), vitamins (A, E, C, and B3), minerals (magnesium, phosphorus, calcium and potassium), and phenolic compounds (Hrnčič et al., 2020). Oil from chia seed has the largest concentration of PUFA, mainly α-linolenic (65–68%) and linoleic acids (17–23%) compared to other vegetable oils (Motyka et al., 2022). The demand for chia seed oil (CSO) is increasing, specifically in America, Europe and Asia-Pacific (Future Market Insights et al., 2017a).

Besides PUFA, CSO has a potential source of γ-tocopherol and phytosterols as natural antioxidants (Dąbrowski et al., 2018a; Dąbrowski et al., 2018b; Shen et al., 2018). The therapeutic properties of CSO have been demonstrated to prevent obesity, diabetes and several cardiovascular diseases (Fonte-Faria et al., 2019; Gazem et al., 2016; Marineli et al., 2015a; Marineli et al., 2015b; Sierra et al., 2015). Increasing consumption of PUFA from fish oil contributes to the overutilisation of certain fish

species like cod, sardine, shark and tuna, while consumers like vegetarians exclude PUFA-rich oil supplements from fish or seafood products (Lane et al., 2021). Therefore, CSO can be applied on food products to improve the content of PUFA and antioxidant properties.

CSO can be obtained by conventional extraction methods (mechanical pressing and Soxhlet) (Hrnčič et al., 2020; Fernandes et al., 2019). However, several experimental results indicated that the mechanical pressing (parameters: temperature, restriction dye and screw speed) obtained the lowest yield (20-25%) of CSO compared to other extraction methods, which are laborious and have a high tendency to undergo oxidation (Fernandes et al., 2019; Ixtaina et al., 2011b). Furthermore, various products (vegetable oils, fats, flavours, fragrances or other bioactive ingredients) extracted by the Soxhlet procedure using hexane consist of residues and toxic solvents dangerous to human health and the environment and unacceptable for the food industry (Cravotto et al., 2022). Alternatively, supercritical fluid extraction technology provides a practical and clean procedure without toxic solvents to obtain the oil with the desired compound of interest from a plant sources (plant seeds and vegetables) (Masturah and Masniza, 2019). Supercritical fluid extraction (SFE) is a safe, green and sustainable technology to obtain oil from plant seed that has attracted the attention of large-scale industries to replace conventional methods (Sánchez-Camargo et al., 2019). Supercritical carbon dioxide (SC-CO_2) is broadly applied because it has low critical extraction parameters (temperature of 32 °C and pressure of 74 bar), economical, not toxic, non-flammable, and non-polar solvent which suitable for extracting oils and lipophilic compounds from plant seeds (cumin, sunflower, watermelon and pepper) (Ahangari et al., 2021). The application of response surface methodology to optimise

the SFE conditions (pressure, temperature, time and SC-CO_2 flow rate) and particle size of the ground sample has contributed to the wide use of this green technology in extracting oil from plant seeds (sesame, annatto, watermelon, *Portulaca oleracea* and quinoa) (Ahangari et al., 2021; Sodeifian et al., 2018; Benito-Román et al., 2018).

The particle sizes (200-500 μm) of different ground plant seeds (roselle, raspberry, quinoa and grape affected the extraction oil yield using SC-CO_2 (Peng et al., 2020; Pavlić et al., 2020; Benito-Román et al., 2018; Jokić et al., 2016). The small particle size of the ground roselle seed (300 μm) contributed to higher oil extraction yield (Peng et al. 2020). The grinding process by grinder increased the surface area with reduced particle size by minimising their diffusion path to penetrate the sample, thus it increased the oil recovery from the SC-CO_2 extraction (Sodeifian et al. 2018). In contrast, the small particle size of ground quinoa seed (125-250 μm) causes a compact form of the sample which resulted in insufficient contact during SC-CO_2, thus decreasing the efficiency of oil extraction (Benito-Román et al., 2018). Therefore, the appropriate particle size of ground chia seed should be determined to obtain the maximum oil yield for SC-CO_2 extraction. However, optimisation of different grinding times to determine the various particle sizes of ground chia seed (independent variables) for extracting high yield of oil using SC-CO_2 is not well reported. Only a study by Ixtaina et al. (2010) indicated the effect of various SFE variables, including temperatures (from 40 to 80 °C), pressures (from 250 to 450 bar), and duration (from 60 to 240 min) to predict the best parameters for the highest yield of CSO based on the optimisation study. However, these authors did not report the effect of particle sizes of the ground chia seed, oil extraction using optimised parameters of SC-CO_2. They also did not study on the characterisation (tocopherols, antioxidant activity,

oxidation kinetic and oxidative stability) and bioaccessibility of the optimised CSO. Limited publication is available on optimising various grinding times to evaluate the particle size ranges of the ground sample, extraction pressure and time used as independent variables in the SFE procedure to extract the optimum yield of CSO. Therefore, the SFE process optimisation is implemented to extract the highest yield of CSO with important lipophilic compounds like PUFAs and tocopherols.

The quality of vegetable oils (refined canola, corn, soybean, sunflower and nut) should be tested extensively by investigating the parameters of different extraction methods, nutritional quality and oxidative stability (Castelo-Branco et al., 2016). Tocopherol and fatty acid profile of different plant oils and animal fats (22 types) are important parameters for determining their oxidative stability based on the linear correlation analysis (Redondo-Cuevas et al., 2018). Fernandes et al. (2019) and Dąbrowski et al. (2016) observed that the different extraction methods (conventional and SC-CO_2) have chemical characteristics (peroxide value, induction period and saponification value) of CSO. Only a study reported on the highest yield of CSO obtained by optimisation of operating conditions (temperature: 80 °C, pressure: 450 bar and time: 4 h) of the SFE, which had similar PUFA (consists of α-linolenic and linoleic acids) and rheological properties with the oil extracted by Soxhlet method (Ixtaina et al., 2011a). Therefore, complete characterisation related to the physicochemical, profile of fatty acid and tocopherols, antioxidant activity, oxidative stability and thermal profiles of optimised CSO obtained by SC-CO_2 compared to Soxhlet (using hexane) and commercial CSO (obtained by cold pressing) is important.

Regardless of extraction procedures (conventional and alternative); CSO with high polyunsaturated fatty acids (more than 80%) is vulnerable to lipid oxidation based on the induction hour (0.14-3.45 h) measured by the Rancimat test (Shen et al., 2018; Dąbrowski et al., 2018a; Dąbrowski et al., 2018b; Dąbrowski et al., 2016). Even though the Rancimat method is carried out to measure the oxidative stability of several vegetable oils (avocado and blends of chia seed and sesame oils) for a short period (approximately several hours), the oxidation procedure operated more than 100 °C contributed to the excessive oxygen exposure and overproduction of lipid oxidation products indicating the inconsistent to determine the oxidative stability of vegetable oils (Rodríguez et al., 2020; Aktar and Adal, 2019; Guitto et al., 2014). Therefore, Şahin et al. (2020) suggested that the peroxide, p-anisidine and TOTOX values are employed to measure the vegetable oil's primary and secondary oxidation products at different storage temperatures (25 to 60 °C) to determine the rancidity level at each stage of the lipid oxidation. However, an elevated temperature of more than 25 °C is the primary factor that accelerates the vegetable oils' lipid oxidation by decomposing the hydroperoxides to alkoxy radicals and finally converting to aldehydes and ketones during storage which produces food spoilage and harmful oxidation products related to human cardiovascular diseases (Bodoira et al., 2017; Kim and Min, 2008; Marquez-Ruiz et al., 2008; Choe and Min, 2006). For example, increasing storage temperatures (from 20 to 80 °C) elevated the oxidation and degraded the tocopherol concentrations and polyunsaturated fatty acid structures in perilla and corn oils (Wang et al., 2010; Choe and Min, 2006). Concerning CSO, previous studies reported exclusively on the improvement of their oxidative stability by adding antioxidants (synthetic or natural) or blending with other vegetable oils (sunflower, walnut, almond, and sesame) to prevent lipid oxidation during storage at room and accelerated temperatures (20, 40,

and 65 °C) (Jung et al., 2021; Bordón et al., 2019; Bodoira et al., 2017; Guitto et al., 2014; Ixtaina et al., 2012). Therefore, the impact of storage temperatures on the antioxidant activity, tocopherol composition, and PUFAs of the CSO is also crucial to study their quality changes during lipid oxidation. According to Aktar and Adal (2019) and Conte et al. (2020), the nutritional composition of avocado and extra virgin olive oils deteriorated due to thermal exposure during storage (25 to 60 °C); therefore, it can be expressed kinetically by the Arrhenius equation to determine the reaction rate constant and activation energy value (E_a) of the changes in oxidation level and bioactive compounds of vegetable oils for each storage temperature examined (Calligaris et al., 2019). Furthermore, the shelf life of CSO can be predicted based on the lipid oxidation markers of virgin olive and perilla oils which are high in unsaturated fatty acids according to the acceptable limit for oxidation products (primary and secondary) during storage at 25-75 °C (Conte et al., 2020; Shim and Lee, 2011). There is limited information on the impact of storage temperatures on the oxidation levels, antioxidant activity, tocopherols, and fatty acids of optimised CSO extracted by SFE together with shelf-life prediction of CSO based on the oxidation parameters and the Arrhenius equation.

Vegetable oils (soybean, flaxseed and sunflower oils) high in unsaturated fatty acids are further oxidised, and changes in nutritional composition (based on the bioaccessibility study) under *in vitro* gastrointestinal digestion model were observed (Nieva-Echevarría et al., 2020a). The simulated gastrointestinal digestion system consists of stomach and small intestine to predict the presence of main volatile oxidation compounds (aldehydes), bioaccessibility levels of lipid-soluble compounds (tocopherols and fatty acids) and antioxidant activity of several vegetable oils rich in

unsaturated fatty acids (grape seed, soybean, palm, rapeseed, and linseed oils) and fish oil (Fruehwirth et al., 2020; Nieva-Echevarría et al., 2020a; Gomes et al., 2019; Martin-Rubio et al., 2019a; Ye et al., 2019; Ye et al., 2018). Only a study conducted on on the digestion behaviours (oil release rate and amount of free fatty acids) of CSO (with or without encapsulation) using simulated mouth, stomach, and small intestine models (Timilsena et al. 2017b). However, the authors did not report the bioaccessibility of bioactive compounds, antioxidant activity, and lipid oxidation of CSO during the gastrointestinal digestion process. Therefore, the digestion of CSO in the gastrointestinal at different stages (stomach and small intestine) is not well discussed, especially on the bioaccessibility of tocopherols and polyunsaturated fatty acids, antioxidant activity, and lipid oxidation.

Therefore, the objectives of this study are:

i. To determine the highest yield of CSO extracted by the SFE based on optimisation parameters (pressure and temperature) and particle sizes of ground chia seed by Central Composite Design.

ii. To characterise the optimised CSO extracted using SFE and Soxhlet and cold press based on oxidation levels, fatty acids and tocopherols profiles, oxidative stability, antioxidant activity and thermal profiles (melting and crystallisation).

iii. To evaluate the effects kinetic changes on oxidation levels, DPPH free radical scavenging activity, tocopherols, and fatty acid composition of the CSOs extracted by Soxhlet and optimised SC-CO_2 under different storage temperatures (25, 40, and 60 °C).

iv. To identify the bioaccessibility of tocopherols, fatty acids, antioxidant activity, and volatile oxidation compounds of fresh and stored CSOs using *in vitro* stomach and small intestine digestion models.

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