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PHYSICOCHEMICAL CHARACTERISATION OF EMULSIFIED AGARWOOD OIL USING GEL ELECTROPHORESIS

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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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PHYSICOCHEMICAL CHARACTERISATION OF EMULSIFIED AGARWOOD OIL USING GEL ELECTROPHORESIS

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Chairman : Mohd. Nazli Naim, PhD
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Quality of the agarwood oil is very subjective and the market price of the oil mainly depends on the quality itself. GC-MS is one of the expensive analytical techniques that is used to determine the quality of the agarwood oil based on the major chemical compound detected. There is no simple and cheaper method established to determine the quality of the agarwood oil based on their physicochemical and electrical properties. Therefore, this dissertation describes three interdependent studies from the beginning in which sample preparation till sample quality were characterised using gel electrophoresis. The first study examined the role of non-ionic surfactant and its critical aggregation concentration (CAC) (0.0167% v/v) on the emulsification process of agarwood oil. The physicochemical properties of emulsified agarwood oil were studied with focus on their droplet size, surface charge, and chemical constituents. Each emulsified droplet consisted of a specific zeta potential value that represented its specific bioactive compounds that were trapped inside the droplets and responded to a specific molecular mass and electrophoretic mobility. Thus, gel electrophoresis technique was performed in the second study to separate the emulsion produced. Due to the nano size (80–100 nm) of emulsified droplet produced at CAC condition, SDS-polyacrylamide gel electrophoresis technique, which is an in-situ, reliable, and sustainable technique, was successfully performed to separate the emulsified oils based on their mobility through the gel in terms of molecular size. The third study used the mechanism and technique studied previously to determine the quality of two inoculated oil emulsions. GC-MS result reported that the high concentration of major compounds present in naturally inoculated agarwood oil led to a large molecular weight. To obtain a vivid

visible band in the gel electrophoresis, the optimisation of the gel formulation was conducted to control the necessary pore size with the optimised formulation at 21% resolving gel with 48% acrylamide concentration and 3.33% cross-linker. Electrophoretic mobility of droplet through gel matrix was the main factor in evaluating the quality of emulsified oils in terms of their molecular weight. The high quality oil with large molecular weight (211 Da) migrated slower than the low quality oil with small molecular weight (195 Da) when both samples were subjected to gel electrophoresis.

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

PENCIRIAN FIZIKOKIMIA MINYAK GAHARU TEREMULSI MENGGUNAKAN PRINSIP DAN TEKNIK ELEKTROFORESIS GEL

Oleh

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Kualiti minyak gaharu sangat subjektif dan harga pasaran minyak bergantung terutamanya pada kualiti minyak itu sendiri. GC-MS adalah salah satu teknik beranalisis yang mahal yang digunakan untuk menentukan kualiti minyak gaharu berdasarkan sebatian kimia utama yang dikesan. Tiada kaedah yang lebih mudah dan murah untuk menentukan kualiti minyak gaharu berdasarkan sifat fizikokimia dan elektrik minyak tersebut. Oleh itu, disertasi ini menerangkan tiga kajian berkaitan penyediaan sampel dan pencirian kualiti sampel menggunakan elektroforesis gel. Kajian pertama mengkaji peranan surfaktan bukan ionik dan kepekatan agregat kritikal (CAC) (0.0167% v/v) terhadap proses pengemulsian minyak gaharu. Ciri-ciri fizikokimia emulsi minyak gaharu dikaji berdasarkan factor-faktor saiz titisan, caj permukaan, dan unsur-unsur kimia. Setiap titisan emulsi mempunyai nilai zeta tentu masing-masing dan mewakili sebatian bioaktif tertentu yang terperangkap di dalam titisan tersebut dan mempunyai jisim molekul dan mobiliti elektroforetik tertentu. Oleh itu, teknik elektroforesis gel dilakukan dalam kajian kedua untuk memisahkan emulsi yang dihasilkan. Oleh kerana saiz nano (80–100 nm) titisan emulsi yang dihasilkan pada keadaan CAC, teknik elektroforesis gel menggunakan SDS-poliakrilamida yang merupakan teknik in-situ, boleh dipercayai, dan mampan, berjaya dilakukan untuk memisahkan minyak teremulsi berdasarkan saiz. Kajian ketiga menggunakan mekanisme dan teknik yang dikaji sebelum ini untuk menentukan kualiti dua emulsi minyak yang dihasilkan. Hasil GC-MS melaporkan bahawa kepekatan tinggi sebatian utama yang terdapat dalam minyak gaharu terinokulasi secara semula jadi menyumbang kepada berat molekul yang besar. Untuk memperoleh jalur yang jelas dalam elektroforesis

gel, pengoptimuman formulasi gel telah dijalankan bagi mengawal saiz liang yang diperlukan dengan rumusan yang optimum pada 21% gel pemisahan dengan kepekatan akrilamida sebanyak 48% dan 3.33% bahan pemautsilang. Mobiliti elektroforetik titisan melalui matriks gel merupakan faktor utama dalam menilai kualiti minyak emulsi dari segi berat molekul. Minyak yang berkualiti tinggi dengan berat molekul yang besar (211 Da) bergerak dengan lebih perlahan daripada minyak berkualiti rendah dengan berat molekul yang kecil (195 Da) apabila kedua-dua sampel dianalisis menggunakan elektroforesis gel.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS AND NOMENCLATURES

Abbreviations

o/w	Oil-in-water
w/o	Water-in-oil
HLB	Hydrophilic-Lipophilic Balance
IFT	Interfacial tension
CMC	Critical micelle concentration
CAC	Critical aggregation concentration
TEM	Transmission electron microscopy
GC-MS	Gas chromatography-mass spectrometry
FTIR	Fourier transform infrared spectroscopy
PAGE	Polyacrylamide gel electrophoresis
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
NMR	Nuclear magnetic resonance
DL	Double layer
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
DNA	Deoxyribonucleic acid
APS	Ammonium persulfate
TEMED	Tetramethylethylenediamine
HCl	Hydrochloric acid
NaOH	Sodium hydroxide
ANOVA	Analysis of variance

Nomenclatures

Symbol	Description	Unit
F_E	Electric force	N
Q	Charge	C
E	Electric field	N C^{-1}
F_f	Frictional force	N
f	Frictional coefficient	dimensionless
R	Particle's radius	m
R_f	Relative mobility	dimensionless
M_W	Molecular weight	$gmol^{-1}$
M_a	Average molecular weight	g mol^{-1} / Da
%T	Total concentration of the acrylamide monomer	%
%C	Proportion of the cross-linker bis-crylamide	%

Greek letter

Symbol	Description	Unit
v	Velocity of the particle	ms^{-1}
η	Viscosity	Pas
ε_o	Permittivity of free space	$C^2N^{-1}m^{-2}$
Δρ	Densities difference	kgm^{-3}
μ_e	Electrophoretic mobility	cm^2/Vs
ζ	Zeta potential	mV
ε_r	Dielectric constant of the dispersion	dimensionless
σ	Centrifugal force	$kgms^{-2}$
ω	Interfacial tension	mNm^{-1}
λ	Wavelength	nm

CHAPTER 1

INTRODUCTION

1.1 Background of study

Aquilaria malaccensis, one of the species in the genus *Aquilaria*, produces resin-impregnated heartwood that is fragrant and highly prized in international trading (Soehartono & Newton, 2000; Chakrabarty *et al*., 1994). Formation of resin is due to the physiological reactions of the tree against pathogenic fungal infections from *Aspergillus* sp., *Fusarium bulbiferum*, and *Penicillium* sp. (Dias *et al*., 2015; Mohamed *et al*., 2010). There are many names associated with the resinous wood, including agarwood, aloeswood, eaglewood, gaharu, chenxiang, jinkoh, oud, kalamabak, and so on, depending on the tree-growing region. Agarwood is divided into several grades in the market, based on their fragrance strength and longevity, resin content, wood density, and colour (Husni *et al*., 2013; Barden *et al*., 2000). The high-grade woods are used in incense mixtures, while the low-grade woods are extracted to produce agarwood oil used in perfumery (Gibson, 1977). However, the high-grade agarwood which grow naturally is hardly available. Furthermore, the resin which is produced naturally by fungal infection happens slowly and very infrequently. As a result, the price of agarwood has tremendously increased in the past few decades and range from a few US dollars per kilo for the lowest quality to over thirty thousand US dollars for the highest quality agarwood and its oil (Akter *et al*., 2013).

Agarwood oil, which is produced from the resin by a common extraction process such as hydro-distillation, is widely used in aromatherapy as an anxiety treatment or for stress and depression release. Based on the literature, agarwood oil is often graded by trained panels according to the individual perception on basic specifications such as composition, colour, and aroma. However, this method is limited in terms of subjectivity, time consumption, large labour expenses, and poor reproducibility (Ismail *et al*., 2014). Currently, many studies have been carried out to classify and analyse the quality of agarwood oil based on its chemical composition (Pripdeevech *et al*., 2011; Mailina *et al*., 2009; Ishihara *et al*., 1993b). Several analytical methods such as electronic nose, gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) are widely used for analysing and identifying the active compounds (Hidayat *et al*., 2010; Brage *et al*., 2007; Pourmortazavi & Hajimirsadeghi, 2007; Mohd. Rosli, 2006). However, the equipment and maintenance costs for each technique are particularly high. Moreover, a highly trained person is required to operate those instruments. The techniques also contribute to samples' destruction. High operating pressure, large organic solvents quantities, high cost standard compounds, and long column preparation time will be required to achieve efficient separation when using chromatography technique (Lord & Bralley, 2008; Lioy, 1991).

In the Southeast Asia region, such as Malaysia, the major chemical compounds of *Aquilaria malaccensis* oil consist of 4-phenyl-2-butanone, α-bulnesene, α-guaiene, agarospirol, ledene oxide-(II), elemol, and γ-eudesmol (Tajuddin *et al*., 2013). Unfortunately, the chemical components that are responsible for the unique aromatic characteristics of agarwood oil are lipophilic and very sensitive to the environmental factors such as oxygen, temperature, pH, and light, which can shorten their shelf life as well as reduce the quality of the agarwood oil. Due to the high sensitivity of the compounds to the environment, plus the stock depletion in the wild forest environment, a shortage of supply occurs, resulting in annually increasing price of agarwood oil (Lim & Noorainie, 2010). As a solution to the increasing price, active ingredients of the agarwood oil are diluted in a solvent, in which an aqueous solution is more preferable compared to an organic solvent. Oil-in-water emulsification processes allow the delivery of active compounds in aqueous solution and enhance the stability of chemically unstable compounds (Chime *et al*., 2014). However, the emulsified oil produced may be stable for a few hours to a number of years only, depending on the emulsification process parameter. In order to produce a stable emulsion without coalescence, encapsulation of the emulsified oil with a surfactant or emulsifier is necessary (Mason *et al*., 2006). The surfactant provides a thin layer at the oil/water interface that enhances droplet protection and provides extra stability in the colloidal system due to electrostatic or steric repulsion (Vaclavik & Christian, 2014). During the preparation of emulsified oil, the addition of a surfactant at critical aggregation concentration (CAC) also assists droplet break-up by reducing the interfacial tension (IFT) value to the maximum and preventing the immediate re-coalescence of newly-formed droplets by rapid adsorption and stabilisation of the newly formed interface of the oil droplet, which facilitates droplet deformation (Mason *et al*., 2006; Briceno *et al*., 2001). Furthermore, encapsulation of the bioactive compounds in aqueous media is a viable and efficient approach to increase the physical stability of the bioactive compounds, protecting them against environmental factors such as oxygen and light, facilitating colloidal-based delivery system, improving bioavailability, and increasing the shelf life of the oil. Other than the advantages stated earlier, the emulsified oil droplet in aqueous media also possesses a unique value in terms of electrical properties. The electrical properties are measured in terms of zeta potential value which is defined as electric potential at location of slipping plane in the electrical double layer and commonly used to measure the stability of emulsion against creaming and coalescence.

Gel electrophoresis is a powerful, yet simple, convenient, and inexpensive technique used for separating charged macromolecules such as nucleic acids and proteins through the gel matrix according to their sizes and charges when an electric current is applied (Wrolstad *et al.,* 2005). A small and high-charge molecule will migrate faster through the gel matrix than a large and low-charge molecule. The electrophoretic mobility of the molecules throughout the gel matrix is described by sieving mechanism (Stellwagen, 1985). Two main types of gels commonly used are agarose and polyacrylamide (PA) gels. Agarose gel is used for separating large-size molecules and has a relatively low resolving power, whereas polyacrylamide gel has a greater resolving power for small-size molecules. Likewise, the average pore size is typically 200–500 nm for agarose gels, and 5–100 nm for polyacrylamide gels (Viovy, 2000). However, currently, there is no study on the assessment and evaluation of the quality of the agarwood oil based on its physicochemical and electrical properties by using gel electrophoresis. Therefore, based on the preliminary approach, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was chosen to assess the quality of emulsified agarwood oil droplets produced at CAC by using SDS-PAGE based on their relative mobility either in terms of their size, molecular weight, and/or zeta potential value.

1.2 Problem statements

Agarwood species has been classified as endangered species due to the increasing demand of the agarwood and its extracted valuable essential oil (Lim & Noorainie, 2010). It has been used in cultural and religious activities and for medicinal purposes for hundreds of years in the Middle East, China, and Japan. Currently, agarwood oil is also used as ingredients in perfume, essence, and others products such as soap and shampoo, whereby these products are marketed at prices about ten times more expensive than the common brands of toiletries products. With advancing technology, it is expected that in the future, more new products derived from agarwood oil will be sold in the market. However, serious depletion of the wild resource in the global market leads to a reduced supply of agarwood oil. In this regard, a sustainable effort of emulsification was applied in the handling of the extracted agarwood oil. Emulsification allows delivery of active materials in green solvent, water, which is an inexpensive and innocuous solvent. Emulsification not only helps to reduce the size of bulk oil down to nano size, the process also maintains the optimum concentration of the oil's active ingredient and allows the dilution of the expensive functional materials to be effectively suspended in water, and thus the appropriate dosage of active ingredient can be controlled. However, the emulsion produced is inherently unstable due to the immiscible properties of oil and water, which separate into two layers very quickly after the mixing process. Thus, a surfactant was added to encapsulate agarwood oil within the colloidal system (Wrolstad *et al*., 2005).

The next problem is creating an in-situ, inexpensive, and sustainable technique to evaluate and assess the quality of essential oil extracted from agarwood. There are many grades of agarwood oil, and the highest quality of the agarwood oil in terms of major chemical compound is extremely expensive. As a matter of fact, the quoted prices for pure agarwood oil, which is one of the most valuable natural products in the world, in average, is 236 dollar per tola (*Agarwood and Oud Oil Quarterly Market Price Report - Sep 2015*). Conventionally, the grading method of agarwood is based on its physical properties such as aroma, colour, and density, which is very subjective, time consuming, associated with expensive labour cost, and lacks repeatability (Ali *et al*., 2012; Hidayat *et al*., 2010; Hung *et al*., 2014). Currently, many studies have been carried out to analyse the quality of agarwood oil based on its chemical compositions (Ishihara *et al*., 1993b; Mailina *et al*., 2009; Pripdeevech *et al*., 2011). Several analytical methods such as electronic nose, gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR) are widely used to identify the active compounds (Brage *et al*., 2007; Hidayat *et al*., 2010; Mohd Rosli, 2006; Pourmortazavi & Hajimirsadeghi, 2007). However, the equipment and maintenance costs for each technique are particularly high and a highly trained person is required to operate those instruments. Furthermore, quality assessing or evaluating system based on physicochemical and electrical properties of agarwood oil has not yet been extensively studied.

1.3 Objectives of study

The general objective of this study is to assess the quality of agarwood oils using gel electrophoresis via emulsification mechanism based on their relative mobility through gel matrix while the specific objectives of this study are:

1. To investigate the ultrasonic parameters and surfactant critical aggregate concentration (CAC) to obtain stable emulsion in terms of interfacial tension, droplet size, and zeta potential, as well as under effect of storage and pH;
2. To characterize the physical properties of the emulsified agarwood oil in terms of droplet size, molecular weight, and/or charge using gel electrophoresis separation technique;
3. To assess the relative mobility as the quality indicator of emulsified agarwood oil.

1.4 Scope of study

The scope of this study is essentially to compare and assess the quality of commercial agarwood oil (as-received) with different resin inoculation techniques (naturally or artificially) through gel electrophoresis based on their relative mobility either in terms of their droplet size, molecular weight, and/or zeta potential. Prior to electrophoresis separation, several parameters such as ultrasonication power and time, and surfactant concentration were manipulated to produce the most stable emulsion. Fine and monodispersed oil droplets are preferred in most cases because a homogeneous emulsion can be produced easily and each emulsified droplet is able to entrap an equal amount of bioactive compounds. The effects of pH and stability during sample preparation were also controlled and investigated. The critical aggregation concentration (CAC) was determined at which the emulsified droplets were screened uniformly with diffused counter-ions in aqueous media, so that each commercial oil sample has its own specific zeta potential value based on the oil chemical constituents or oil quality. A particular concern was given to the optimisation of gel pore size by controlling their acrylamide and bis-acrylamide percentage. The visible vivid bands that appeared were then used for the oil quality comparison. As a result, the droplet that responded to a specific molecular mass and electrophoretic mobility can be separated using SDS-PAGE.

1.5 Rationale and significant

Due to the problem statements stated above, especially the analytical cost of the GC-MS technique, the concern of finding an in-situ, inexpensive, and sustainable green technique that can be used to evaluate the quality of agarwood oils based on their physicochemical and electrical properties as well as major compounds are the crucial parts of this study. Prior to the separation, emulsification mechanism for sample preparation in terms of size, molecular weight, and/ or charge will be investigated. The discovery of such technique or any research leading to that discovery would concrete all steps towards solving the problems stated above. It is believed that the technique can be more widely used for the separation of other oils, and provides a new insight into how electrophoresis can be applied in emulsion science.

1.6 Hypothesis

Here, the hypothesis proposed is that the addition of surfactant Tween 80 not only stabilises the emulsified oil, but also creates a thin layer that protects the oil droplets against various pH conditions. It is also hypothesised that SDS coats the emulsified droplets with a uniform negative charge, and the intrinsic charges of emulsified droplets become negligible when compared to the negative charges contributed by SDS. Thus, SDS-coated droplets possess a uniform charge density, which is the same net charge per unit weight. Therefore, there will be no differential migration based on charge and the separation mobility is hypothesised to be based on the molecular weight of the droplets. The zeta potential values in the system were also hypothesised to be derived originally from the individual oil droplet charge and were independent from non-ionic surfactant (Tween 80) effects. It is speculated that zeta potential values represented the specific bioactive compounds trapped in the droplets, and responded to a specific molecular mass and electrophoretic mobility so that separation using SDS-PAGE can be performed. High-quality emulsified oil with greater major chemical compounds that contribute to the aromatic characteristics is hypothesised to have larger molecular weight. Thus, high-quality emulsified oil migrates slower through the gel medium when an electric field is applied.

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