Grading of Emulsified Agarwood Oil Using Gel Electrophoresis Technique

Y. T. Boon, M. N. Naim, R. Zakaria, N. F. Abu Bakar, N. Ahmad, I. W. Lenggoro

Abstract—In this study, encapsulation of agarwood oil with non-ionic surfactant, Tween 80 was prepared at critical micelle concentration of 0.0167 % v/v to produce the most stable nano-emulsion in aqueous. The encapsulation has minimized the bioactive compounds degradation in various pH conditions thus prolong their shelf life and maintained its initial oil grade. The oil grading of the prepared samples were conducted using the gel electrophoresis instead of using common analytical industrial grading such as gas chromatography- mass spectrometry (GC- MS). The grading method was chosen due to their unique zeta potential value after the encapsulation process. This paper demonstrates the feasibility of applying the electrophoresis principles to separate the encapsulated agarwood oil or grading of the emulsified agarwood oil. The results indicated that the grading process are potential to be further investigate based on their droplet size and zeta potential value at various pH condition when the droplet were migrate through polyacrylamide gel.

Keywords—Electrophoretic mobility, essential oil, nanoemulsion, polyacrylamide gel electrophoresis, Tween 80, zeta potential.

I. INTRODUCTION

AGARWOOD is resin impregnated heartwood from *Aquilaria malaccensis,* which belongs to the family of genus *Aquilaria (Thymelaeaceae).* It is also known as gaharu, eaglewood, aloeswood, oud, jinkoh, and chenxiang and can be found in Southeast Asian countries such as Malaysia. The aromatic resin is formed as the tree sap gradually become harder and change it physical form from dark brown to black colour due to response to injury and fungal infection. Agarwood is recognized as one of the most valuable natural product trade internationally due to its endless uses, ranging from large sections of trunk to finished products such as incense and perfumes.

The quality of the agarwood oil which industrially extracted through hydrodistillation is depending on the degree of injury

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and its resin formation. High quality agarwood oil (grade A) may cost between USD 93- 465 per tola [1]. Many previous studies on grading agarwood oil quality have been carried out. Conventionally, grading of agarwood was based on its physical properties such as colour, aroma, density which mainly depend on individual perception and experience. Recently, several analytical methods such as gas chromatography- mass spectrometry (GC- MS), electronic nose, and nuclear magnetic resonance (NMR) were recognized to determine the chemical properties of agarwood oil [2]-[5]. However, some of the industrialist unable to afford the burden of the analytical cost due to their limited product for sampling purpose. Thus, gel electrophoresis is introduced as an alternative method to assess and grade the quality of the agarwood oil based on its electrical properties.

Gel electrophoresis is a powerful, yet, simple and cheaper tool used for separate nucleic acid and protein on the basis of their size and charges in an applied electric field [5]. Small and high charge molecule will travel faster through the gel than a large and low charge molecule. Sieving mechanism is used to describe the electrophoretic mobility of the molecules throughout the gel matrix [6]. The types of gel most commonly used are agarose and polyacrylamide gels. Agarose gel is used for separating large size molecules and has a relatively low resolving power; whereas, polyacrylamide gels have greater resolving power for small size molecule. Also, the average pore size is typically 200-500 nm for agarose, but 5-100 nm for polyacrylamide gel [7]. Therefore, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) was chosen for the emulsified agarwood oil droplets separation in this study.

Up to the author's knowledge, agarwood oil quality grading system based on its electrical properties has not been being reported elsewhere in the literature. The objective of this study was to separate or to grading the emulsified agarwood oil droplets with SDS PAGE based on their size and zeta potential value. Since pH is one of the most important factor that affects zeta potential value, thus relationship between pH and zeta potential was also studied. The size of emulsified oil droplets lying within nanometre range and response to specific zeta potential value and electrophoretic mobility when they are varies in different pH conditions, so that separation using SDS PAGE can be evaluated.

II. EXPERIMENTAL

A. Materials

Agarwood oil was purchased commercially from YSG Excellence Sdn. Bhd., Malacca, Malaysia; Tween 80

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(Polyoxyethylene (20) sorbitanmonooleate), acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), tris base, ammonium persulfate (APS), Tetramethylethylenediamine (TEMED), bromophenol blue, coomassie brilliant blue, acetic acid, and methanol were purchased from Sigma Aldrich (St. Louis, MO, USA).

B. Sample Preparation

The droplets of agarwood oil were dispersed in a continuous phase of surfactant solution according to [8], [9]. However, some modifications were done on the formulation. The emulsion formulations were prepared by using various surfactant concentration of 0.01, 0.0125, 0.0167, 0.025, 0.05, 0.1% (v/v) with a constant volume of agarwood oil. The emulsion was prepared by probe sonicator (Fisher Scientific Model 705 Sonic Dismembrator, Waltham, MA, USA) inside an ice bath for 4 minutes and 70 % amplitude ultrasonication.

The emulsion that produced at critical micelle concentration (CMC) was then subjected to pH variation in order to obtain large significant differences in zeta potential values so that separation based on charges can be performed through SDS PAGE.

C. Droplet Size and Zeta Potential

The stability of emulsion and pH- adjusted emulsion produced were evaluated in term of size and zeta potential [10]. Droplet size distribution and zeta potential were measured using built in dynamic light scattering technique and electrophoretic light scattering technique, respectively by zeta/ nano particle analyser (Nanoplus, Particulate Systems, USA) at ambient temperature. The zeta potential was not measurable directly but it was calculated using Smoluchowski equation which relate with the experimentally determined electrophoretic mobility.

D. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE)

The experimental setup of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) was performed using a protocol [11], but with a little modification, as shown in Fig. 1. Electrophoresis gel can be divided into stacking gel and separating gel. Resolving gel was prepared by mixing 0.1 mL of 10 % APS (ammonium persulfate) and 0.01 mL of N, N, N', N'-tetramethylethylenediamine (TEMED) for 10 mL of resolving gel solution and pour up to ¾ of short plate height. About 0.2 ml of isopropanol was added on top of the gel solution to make the surface even and to remove the bubbles from the top layer. The isopropanol was then removed after 30 minutes with Whatman filter paper and the polymerized gel surface was rinsed with distilled water. Whereas, stacking gel solution was prepared by mixing 0.15 mL of 10 % APS and 0.02 mL of TEMED for 10 mL of stacking gel solution prior pouring to fill the remaining volume of the glass plates. The comb was immediately inserted to avoid the entrapment of bubbles inside the gel. Then, the comb was removed from the glass plates and the glass plates was washed to remove the gel adhered on the outer surface of the glass plates after 30 minutes. The glass plate was then placed properly in the inner chamber and the chamber was tightened properly by clamping frame in order to avoid the leaking of running buffer from the inner chamber. Next, the chamber was filled with 1x running buffer. Finally, dyed samples were loaded into wells and electrophoresis was carried out at 50 mA, 150 V, for 70 minutes. The gel was then stained with staining solution for half an hour and destaining for an hour prior image scanning. The relative mobility of the bands appeared were then calculated based on:

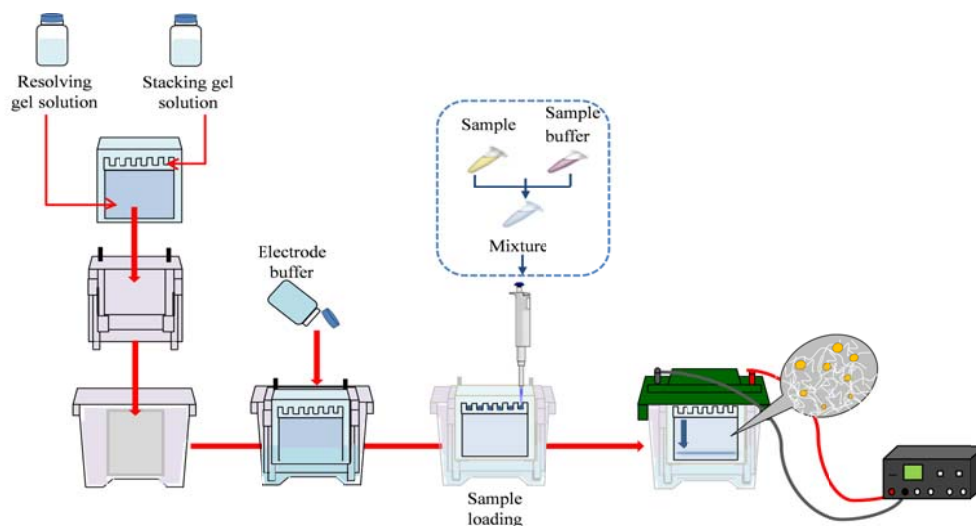$$\text{Relative mobility} = \frac{Migration\ distance\ of\ band}{Migration\ distance\ of\ dye} \qquad (1)$$

Fig. 1 Experimental setup for SDS PAGE

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III. RESULTS AND DISCUSSION

A. Effect of Surfactant Concentration on Size Distribution and Stability of Emulsion
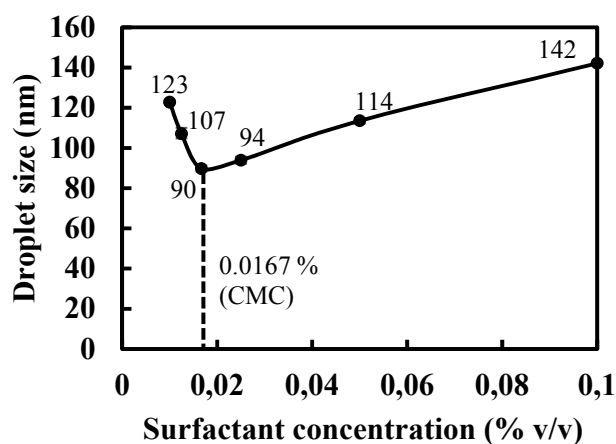

Fig. 2 Surfactant concentration effect on emulsified droplets size

The size of emulsified oil droplets is controlled by the interaction between the droplet breakup and droplet coalescence [12], [13]. Droplet break up is controlled by the type and amount of energy applied to the droplets whereas droplet coalescence is controlled by the ability of surfactant adsorb at the droplet interface, which depend on surfactant surface activity and concentration. In the present study, surfactant Tween 80 was chosen and its concentration effect on droplet size was shown in Fig. 3. Droplet surface coverage by surfactant can used to describe the relationship between droplet size and surfactant concentration. Initially increasing in surfactant concentration resulted in a large decrease in droplets size as the surfactant adsorbed at the oil/ water interface and instantaneously oriented themselves between the two phases. Next, the surfactant facilitated reduction of interfacial tension, and formed a protective film around the droplet to prevent droplets from coalescence [14], [15]. Large droplet size was observed at low surfactant concentration which owing to insufficient coverage of surfactant at the interface of newly formed droplets.

When there was an excess addition of surfactant, at 0.0167% v/v, smallest droplets size about 88 nm was obtained. This was owing to the sufficient concentration of surfactant that rapidly adsorbed at the interface of newly formed droplets, facilitates a maximum reduction in the interfacial tension [16] and provided strongest repulsive forces to prevent droplets from coalescence. Further increase in surfactant concentration, above 0.0167% v/v, led to increasing of droplets coalescence. This might due to changes of sphere micelle to rod shape, lamellar or mesophase owing to excessive surfactant in the system. In this system, Tween 80 concentration of 0.0167% was close to the limit of the reduction of droplet size, thus, 0.0167% of Tween 80 was recognized as critical micelle concentration (CMC) along this study.

Fig. 3 showed the effect of surfactant concentration on zeta potential value of emulsion produced. Zeta potential is one of the factors determining the physical stability of emulsions. The higher the zeta potential value, the higher is the electrostatic repulsion between the droplets and the higher is the physical stability. In the present study, zeta potential values ranged from -20 to -40 mV for emulsion produced with Tween 80, a non-ionic surfactant were obtained, but in principle, droplets stabilized by non-ionic surfactants should have no charge. Negative charge values were obtained may be explained by the presence of composition of agarwood oil, pH, electrolyte or other ionic impurities remaining from their production or generated during storage [17].

The CMC 0.0167% v/v gave highest zeta potential of -39.6 mV among the others surfactant concentration. This might be caused by the strongest repulsive force provided by sufficient coverage of surfactant barrier around the oil droplets, providing the most stable emulsion. However, there was no significant change for zeta potential value of emulsified oil with less or excess addition of surfactant which might be due to the non-ionic characteristic of surfactant used.
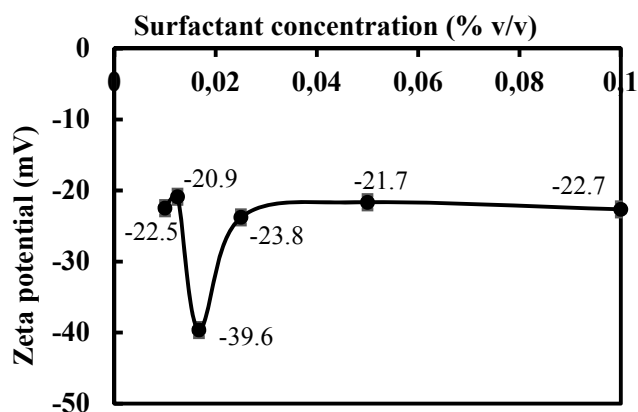

Fig. 3 Zeta potential values of emulsified agarwood oil as a function of surfactant concentration

B. Effect of pH on Size Distribution and Stability of Emulsion Produced at CMC

The oil droplets which comprise of bioactive compounds tend to undergo physical and chemical changes when they are subjected to an extreme pH condition. However, when the most stable emulsion which produced at CMC with pH 6.5 was subjected to various pH conditions starting from pH 3 to pH 10, the droplet size of emulsified oil has no significant changes as shown in Fig. 4. This might be due to the sufficient protection provided by the surfactant barrier toward pH condition as the droplets were fully encapsulated by surfactant monomers.

The zeta potential of the particles is used as a measure of particle charge and electrostatic repulsion, and is one of the fundamental parameters that affects stability. In aqueous media, the pH is one of the most important factors that affects zeta potential value. The relationship between the effects of pH on the zeta potential of emulsion produced at CMC was shown in Fig. 5. It can be seen that small changes in pH has caused significant changes in the zeta potential values of the emulsified oil in which the zeta potential values decreased at low pH and increased at high pH. This may be owing to the adsorption of H^+ and OH^- ions, which are the zeta potential- determining ions

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at the oil/ water interface. In addition, the effect of pH on the zeta potential of the emulsified oil may be explained by considering that the surfaces of the oil droplets might contain pH- dependent ionizable functional groups which can undergo dissociation and protonation [18]. Highest zeta potential at simultaneously low electrolyte concentration led to formation of the smallest particle size. Consequently, the emulsified oil produced initially at CMC value (approximately pH 6.5) has high zeta potential value of -39.6 mV in correlate with smallest droplet size. However, the zeta potential value of the emulsified oil tended to decrease with decreasing pH value.

At acidic pH condition (pH 3- 4), the emulsified oil showed destabilisation due to its low zeta potential values that less than 30 mV which is the marker value of stabilisation. This might be explained as the negative charge density was gradually reduced by the adsorption of H^+ ions at the oil/ water interface, which resulting in reduction of zeta potential value as well as the electrostatic repulsion force between droplets. As pH increased to pH 6-7, a significant increase in zeta potential value was observed indicating that the formation of stable emulsion. The highest zeta potential value of -75.6 mV was achieved at pH 8, which might owing to strongest electrostatic repulsion force acting between droplets. Also, OH^- ions concentration have greatly increased with increasing pH and their adsorption at the fully surfactant covered oil/ water interface significantly increased the negative charge density of oil droplets and thus, increased the zeta potential value. As pH value further increase up to pH 10, an increase in OH- ions concentration in the system reduced the emulsion zeta potential to -60.5 mV by compressing the electrical double layer [19].
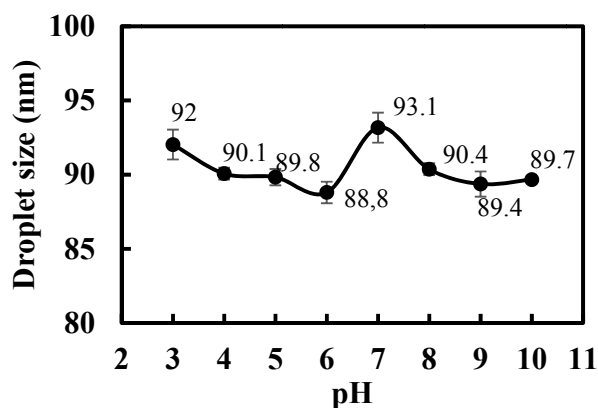

Fig. 4 Measurement of droplet size as a function of pH for emulsion produced at CMC

A. SDS-PAGE Electrophoresis

Electrophoresis refers to the movement of a molecule through a matrix of gel under an electric field. The separation of molecules within a gel was determined by the relative size of the pores formed within the gel. While, the pore size of the gel can be controlled by choosing appropriate concentration of acrylamide and cross- linking agent. Higher percentage gels with smaller pores are used to separate smaller molecules and vice versa. In the present study, single-percentage gel of 15 % was used to separate emulsified droplets with size below 100

nm. Fig. 6 represented the SDS PAGE of emulsion produced at CMC with pH varies from 3-10 at 15% of gel concentration.
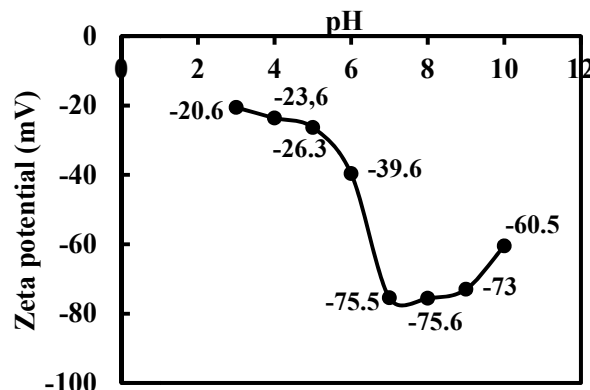

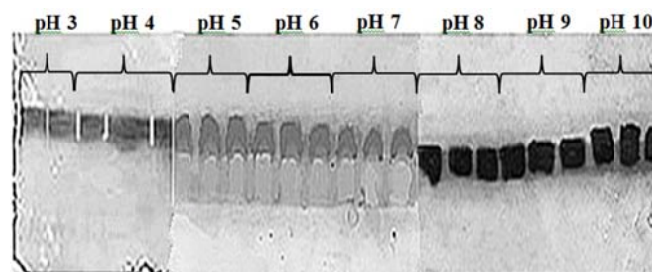Fig. 5 Measurement of zeta potential as a function of pH for emulsion produced at CMC

Fig. 6 Visible bands of emulsion produced at CMC 0.0167 % v/v at various pH conditions

Since bioactive compounds of agarwood oil with close molecular weight, for example, γ-eudesmol, α-Elemol, β-Caryophyllene, alloaromadendrene, α-Gurjunene with molecular weight 222.4 g/mol, 222.4 g/mol, 222.4 g/mol, 204.4 g/mol and 204.4 g/mol, respectively were encapsulated within a droplet, thus a broad and single band was observed for each sample lane. As mentioned earlier, there was not much different in the size distribution of the emulsified oil droplets produced at CMC with pH varies from pH 3-10, thus separation was mainly based on the significant difference values of zeta potential at various pH condition. Also, by referring to the band mobility pattern, the emulsified droplets tended to follow the zeta potential trend during pH variation. Thus, a hypothesis was made in which the zeta potential was the domain factor in separating emulsified droplets instead of molecular weight using SDS PAGE.

IV. CONCLUSION

The paper studied the feasibility of applying SDS PAGE in separation of the emulsified agarwood oil. Characteristics of emulsions produced were evaluated in term of size and zeta potential. Emulsion produced at CMC provided the most stable condition with smallest z-average diameter ~90 nm, and highest zeta potential value of -39.6 mV. Emulsion produced below CMC or above CMC value contributed to large oil droplet and low zeta potential. Variation of pH has a significant

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effect on zeta potential of emulsion, owing to H^+ and OH^- ions adsorption at the oil/water interface. Visible bands were observed for emulsified droplets produced at various pH condition within size range of 85 to 95 nm and zeta potential range of -20 to -80 mV due to suitable pore size of 15 % of SDS PAGE gel. Zeta potential value was the dominant factor for emulsified agarwood oil separation as compared to droplet size.

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