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Electrophoretic mobility of Tween 80-encapsulated agarwood oil in aqueous

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Abstract

A study on the feasibility of using gel electrophoresis technique in grading the agarwood oil quality was investigated. Prior to electrophoresis, the emulsified agarwood oil droplets were screened by a diffuse layer of ions that have equal absolute charge to that of the droplets surface charge in aqueous phase. The condition was obtained by varying the concentrations of non-ionic surfactant; Tween 80 until the critical aggregation concentration (CAC) value of 0.0167% (v/v) was achieved. The prepared droplets suspended in the aqueous within nano-metre size and had ability to migrate through the agarose gel with its own specific electrophoretic mobility. However, due to the limitation of gel pore size, only large oil droplets (>200 nm) indicated visible bands. Overall, a novel work for grading the emulsified agarwood oil droplet with its own electrical properties was feasible.

Introduction

Gel electrophoresis is a well-established analytical technique that has been used to separate colloidal particles or macromolecule compounds such as protein and nucleic acid in the presence of an electrical field. The separation is mainly based on their electrical charge, size, shape, or a combination of charge and size (Crowe and Bradshaw, 2010). In the gel electrophoresis principles, molecules move through the matrix at different rates determined by their mass when the charges to mass ratio of the molecules are uniform. During gel electrophoresis, molecules with positive ionic charges will migrate toward the negative electrode, and vice versa with different velocities (Robyt, 1990). In species that have no net charge, the mobility is nil.

In general, gel electrophoresis is applied to determine the purity of protein or Deoxyribonucleic acid (DNA), the quaternary structure of protein, the isoelectric pH values, the nucleic acids sequencing and to estimate the molecular weight of Ribonucleic acid (RNA)(Peterson, 1971; Lehrach *et al*., 1977; Maxam and Gilbert, 1977; Vogelstein and Gillespie, 1979; Rabilloud *et al*., 1997; Powers *et al*., 2005). Separation in gel electrophoresis is based on physical characteristics rather than chemical properties (Adamson and Reynolds, 1997). However, the feasibility study of this technique in grading the quality of essential oil has not been demonstrated.

In this study, the grading of essential oil from agarwood is demonstrated using the agarose gel electrophoresis. Agarwood oil is the extracted product from the resinous heartwood of Aquilaria species in the Thymelaeaccae family (Qi, 1995). The formation of resin is often associated with the physiological reactions of these trees against wounding of the stem and infection by various fungi or insects (Donovan and Puri, 2004). Naturally inoculated resin has a high quality and purer fragrance due to the longer incubation time. As a result, Aquilaria trees are often being cut down indiscriminately in the search for those containing agarwood resins. The high value of the agarwood products is also encouraging the rising numbers of illegal harvest and trade in several countries. According to IUCN Red List Categories, the Aquilaria species have already declined to the point where they are considered threatened. In fact, Aquilaria Malaccensis has been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1995 (Barden *et al*., 2000).

The as-received agarwood oil in this work was distilled from the agarwood trees which growing wild.

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This kind of trees are preferable because it consist of high concentration components and the agarwood quality depends on the composition and concentration of the components in the oil (Ishihara *et al*., 1993; Tamuli *et al*., 2005; Naef, 2011; Pripdeevech *et al*., 2011; Subasinghe and Hettiarachchi, 2013). Unfortunately, very little techniques are known to be used for evaluating the quality of the extracted essential oil. In the global trading market, the agarwood oil is graded according to the basic specifications of resin content, colour and aroma. This grading technique has been conventionally accomplished by trained sensory panels, but it was limited by poor reproducibility, subjectivity, time consumption and large labour expense (Keller, 1999; Hidayat *et al*., 2010).

To the best of the authors' knowledge, the literature has not yet reported electrophoresis application on oil droplets for grading of agarwood oil. This study therefore proposed to produce uniform charge droplets prepared from the as-received agarwood oil. The uniform charge droplets can be achieved when the agarwood oil droplets are emulsified with non-ionic surfactant within the CAC value. The CAC is defined as the concentration of surfactants above which aggregates are formed (MC Clements, 2014). The successful emulsified droplets are electro sterically stable and exist within the size of nano-metre range. The optimal surfactant concentration also tends to lower the interfacial tension between the oil and the surfactant solution to an ultralow level which provide a stable suspension. Since only non-ionic surfactant was used to emulsify the oil droplets, it is expected that the charged droplets have their individual zeta potential value that represent the specific bioactive compounds that trapped in the droplet. Thus, the droplet characteristics at the CAC such as droplet size and zeta potential value were measured and investigated. Here we assume that each droplet responds to its own specific charge and mobility so that the separation using the gel electrophoresis method can be performed.

Materials and Methods

Materials

Agarwood oil was purchased from YSG Excellence Sdn. Bhd., Malacca, Malaysia; Tween 80 (Polyoxyethylene (20) sorbitanmonooleate), agarose powder, TAE (Tris-acetate-EDTA) buffer, Bromophenol blue, Coomassie Brilliant Blue, acetic acid were purchased from SYSTERM, Classic Chemicals Sdn. Bhd., Malaysia.

Sample preparation

The emulsion was prepared with minimal light exposure in order to prevent the degradation of compounds. The emulsion was prepared using the surfactant solution as the continuous phase and agarwood oil as the dispersed phase. Several concentrations of surfactant solutions were prepared by adding different surfactants volume to distilled water. The aqueous phases were placed in a glass beaker and mixed at ambient temperature using a magnetic stirrer for 30 minutes. The oil phase was added to the beaker slowly. The resulting mixture was then sonicated (Model 705 Sonic Dismembrator, Fisher Scientific) for 4 minutes at an amplitude of 70% with 15 seconds duty cycle. The sonication was performed in an ice water bath to prevent the mixture from overheating. The Tween 80 surfactant concentration was further investigated to determine the critical aggregation concentration (CAC) of the emulsion.

Gas chromatography –mass spectrometry (GC-MS) analysis

As received naturally-inoculated agarwood oil was sent for GC-MS analysis, which was performed by GC-MS electron impact ionization (EI) method on GC-17A gas chromatograph (Shimadzu) coupled to a GC-MS QP 5050A mass spectrometer (Shimadzu). The capillary column used was a ZB-FFAP (30 m × 0.25 mm I.D x 0.25 μm film thickness). The oven temperature was initially held at 50ºC for 1 minute, then increased at a rate of 20ºC/min to a temperature of 100ºC and finally, increased to 240ºC with a rate of 5ºC/min for 25 min. The injector temperature was 250 ºC. Helium gas was pressure-controlled to give linear gas velocity of 44 cm/s at 50ºC. Electron Ionization mass spectra were collected at 70 eV ionization voltages over the range of m/z 35-450. The interface temperature was maintained at 255ºC. Identification of volatile components was performed by comparison of the mass spectra of individual components with the reference mass spectra in the NIST Mass Spectral Library.

Interfacial tension (IFT)

In order to identify the critical aggregation concentration (CAC), the interfacial tension (IFT) measurements between the surfactant solution and the agarwood oil were studied. The IFT measurement was performed using a Spinning Drop Video Tensiometer (SVT 15, Dataphysics) at 25˚C. A small drop of agarwood oil (less dense fluid) and the surfactant solution (denser fluid) were placed in a rotating glass tube. The interfacial tension was

determined through the radius of elongated droplet and centrifugal force. Each sample was rotated at a speed greater than 3000 rpm to cut off the gravity. The IFT value was measured automatically after the oil droplet reached the equilibrium position in the aqueous phase.

Droplet size and zeta potential

The diameter of emulsified agarwood oil droplet was determined by dynamic light scattering using zeta/ nano particle analyser (Nanoplus, Particulate Systems, USA) at 25°C which measured the scattered light of particles that were in Brownian motion. Zeta potential value was used to indicate the stability of the produced oil droplets. Electrophoretic light scattering was used to determine the velocity of the particles in the suspension. Since the particles' velocity was proportional to the amount of the charge of the particles, zeta potential was determined from the electrophoretic mobility of the essential oil droplets using the Smoluchowski equation. The zeta potential of each sample was calculated from the average of five time measurements. The refractive index and the viscosity were set at 1.33 and 1.0 cps respectively, in order to mimic the values of pure water (Meyer *et al*., 2006; Shah and Amiji, 2006; Vyas *et al*., 2008; Affandi *et al*., 2011).

Agarose gel electrophoresis

Agarose powder (electrophoresis grade) was mixed with electrophoresis TAE buffer (Tris-acetate-EDTA) to the desired concentrations, then heated in a microwave oven until the powder was completely dissolved. The gel concentration was varied from 1-4% in order to obtain the appropriate pore size for separating the emulsified oil. The mixture was then cooled to 60˚C and poured into the casting tray with a comb for gel solidification. The comb was removed and a row of wells were made once the gel solidified. The samples were then mixed with a dye before being injected into the well by a disposable micropipette. Then, electrophoresis was carried out at 50 mA, 150 V, for 1 hour. Finally, the gel was stained for half an hour and destained for 1 hour prior to image scanning.

Table 1 Chemical composition of volatile oils of agarwood

Chemical compounds	Chemical formula	As received		(Tajuddin *et al*., 2013)	
		RT (min)	Intensity (%)	RT (min)	Intensity (%)
4-Methyl-3-penten-2-one	$C_6H_{10}O$	4.20	0.13	-	-
2-Pentanone, 4-hydroxy-4-methyl	$C_6H_{12}O_2$	5.12	44.31	-	-
α- Gurjunene	$C_{15}H_{24}$	8.33	13.09	-	-
β -Gurjunene	$C_{15}H_{24}$	8.78	8.47	-	-
Benzaldehyde	C_7H_6O	-	-	7.17	1.93
β -Caryophyllene	$C_{15}H_{24}$	10.53	1.05	23.50	0.12
γ-Gurjunene	$C_{15}H_{24}$	10.90	1.00	31.50	1.96
Alloaromadendrene	$C_{15}H_{24}$	11.32	0.16	29.08	0.12
α-Muurolene	$C_{15}H_{24}$	11.99	0.10	30.67	0.97
δ-Cadinene	$C_{15}H_{24}$	12.21	0.08	27.50	0.14
Benzylacetone	$C_{10}H_{12}O$	13.39	0.12	16.92	5.83
Ledene oxide-(II)	$C_{15}H_{24}O$	14.55	33.00	35.00	2.17
Caryophyllene oxide	$C_{15}H_{24}O$	15.47	0.30	-	-
Isoaromadendrene epoxide	$C_{15}H_{24}O$	16.20	0.37	34.17	0.68
α -Elemol	$C_{15}H_{26}O$	16.59	0.71	31.75	2.35
γ-eudesmol	$C_{15}H_{26}O$	18.64	6.60	32.42	2.68
β-Eudesmol	$C_{15}H_{26}O$	19.24	6.78	-	-
α -Bulnesene	$C_{15}H_{24}$	-	-	31.17	2.47
(-)-Spathulenol	$C_{15}H_{24}O$	17.27	0.60	-	-
β-Selinene	$C_{15}H_{24}$	-	-	26.33	0.14
aromadendrene	$C_{15}H_{24}$	-	-	30.58	0.12
α-Humulene	$C_{15}H_{24}$	-	-	25.58	0.19
Agarospirol	$C_{15}H_{26}O$	-	-	32.42	2.08
(-)-Aristolene	$C_{15}H_{24}$	-	-	31.33	0.49
α -Guaiene	$C_{15}H_{24}$	-	-	24.25	0.19

Results and Discussion

Gas chromatography –mass spectrometry (GC-MS) analysis

The quality of agarwood oil depends highly on the composition of the compound (Ishihara *et al*. 1993b). Many researchers have studied the chemical constituents in agarwood oil and most of them have stated that sesquiterpenes components and chromone derivatives such as agarospirol, α-agarofuran, agarospirol, 10-epi-eudesmol, kusunol and jinkoheremol were the major constituents in agarwood oil (Tamuli *et al*., 2005; Nor Azah *et al*., 2008; Pripdeevech *et al*., 2011).

The chemical compounds found in commercial agarwood oil and their intensities are summarized in Table 1. More than fifteen compounds were identified in this study, with the major constituents of the agarwood oil being 2-pentanone, 4-hydroxy-4-methyl, α-gurjunene, β-gurjunene, β-eudesmol, 10-epi-γ-eudesmol, and α-eudesmol with percentage of area 44.31 %, 13.09 %, 8.47 %, 6.78 %, 6.60 % and 2.71 %, respectively. Other compounds including benzylacetone, ledene oxide-(II), alloaromadendrene, elemol, and γ-eudesmol also existed with a lower percentage of area. Some of the obtained compounds in this study are in agreement with those in the work by Tajuddin *et al*. (2013). In the percentage of area of chemical compounds reported, however, there were some similarities as well as variations. This might be due to the different sources of agarwood oil and systematic errors occurring during the analysis.

Effect of surfactant concentration on the interfacial tension, size and zeta potential of agarwood oil droplets

Two immiscible liquids, such as oil and water, will separate into two layers, with the less dense liquid on the top of the denser one. A surfactant which is soluble in the continuous phase adsorbs at the oil/water interface and an emulsion is produced when the mechanical shear is applied to the system. When the surfactant is applied to the system, the large oil droplets break up into small droplets and the adsorption of surfactant at the interface inhibits the droplets from coalescence. Interfacial tension (IFT) is defined as the free energy required for creating a unit of surface area at the boundary of two immiscible phases (Sarapardeh *et al*., 2014). A system with high interfacial tension exhibits significant resistance to one immiscible liquid dispersing in another in the form of droplets. The efficiency in interfacial tension reduction of oil/ water system greatly depends on the adsorption of surfactant at the oil/ water interface. A non-ionic surfactant offers many advantages over an ionic surfactant including flexible formulation, wider compatibility, higher stability and the most importantly in this study it contributes a very minimal influence to the produced oil droplets' charge. Thus, the zeta potential value in the system was derived originally from the individual oil droplet charge since non-ionic surfactants do not indicates any zeta potential value in this work. Tween 80 with a critical aggregation concentration (CAC) of 0.0016% (v/v) in water is selected as the surfactant in this study. Tween 80 is preferable over other types of non-ionic surfactants because of its high hydrophilic and lipophilic balance (HLB) value of about 15 which is of particular interest for solubilising volatile components (le Maire *et al*., 2000; Choi *et al*., 2010).

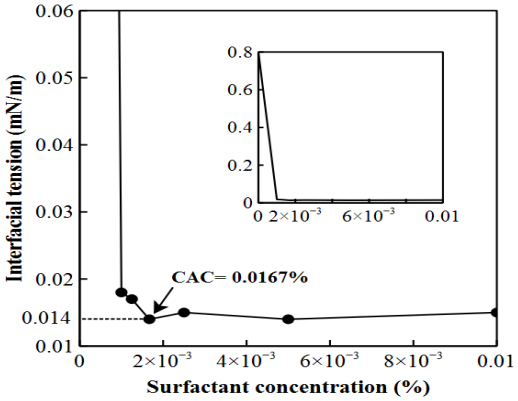

Figure 1. Interfacial tension as a function of surfactant concentration of Tween 80

Figure 1 shows the IFT as a function of Tween 80 surfactant concentration. The IFT value of agarwood oil/water system was found to be 0.8 mNm^{-1} which was noticeably higher than in any surfactant-added systems. Subsequently, when the surfactant concentration on oil/water was increased, the free energy of the system decreased. The interfacial tension at the oil/water interface, which arose because of the imbalance of attractive forces between molecules at the surface of both immiscible liquid, was found to be largely decreased by the surfactant adsorption (Mark, 2007). Once the interface of the oil droplet was completely covered by the surfactant molecules, the critical aggregation concentration (CAC) was achieved. Beyond this point, aggregates were formed in the bulk solution, the interfacial tension reached its ultralow condition and no further decrease was detected. In this study, the CAC value was found at 0.0167% (v/v) and at this condition, the IFT value was reduced to 0.014 mNm^{-1}. After reaching the CAC condition, the interfacial tension was noticed to be almost constant, as shown in the Figure 1. The study on the optimal surfactant concentration is very important to ensure an optimum amount

of surfactants that necessary to produce a stable emulsion system (Tadros *et al*., 2004). Furthermore, the optimum surfactant condition also reduce the IFT value and assist the suspension process by providing a electrosteric film that surround the droplets (Vispute *et al*., 2013).

The interfacial tension (IFT) is significantly reduced with the presence of surfactant monomers by orientating the hydrophobic group of surfactant towards the agarwood oil and the hydrophilic group of the surfactant towards water molecules (McClements, 1999). Since the strength of these interactions are now much stronger than the initial interaction between the agarwood oil and the water molecules, the tension across the interface is significantly reduced (Goffin, 2008). Ariyaprakai (2007) reported that the mechanism of the filled micelle formation upon CMC are divided into five steps: micelles diffusion to the oil/water interface, micelle adsorption at the interface and lowering its IFT, filled micelle formation as the oil incorporates into micelles, filled micelle desorption from the interface, and diffusion into the water continuum. The optimal surfactant concentration of 0.0167% (v/v) is referred as the CAC value and will be used in subsequent sections of this work.

Droplets size distribution is the most important characteristic of emulsion. The defined size of emulsion varies in the literature (Solans *et al*., 2005; Mason *et al*., 2006). However, a well-accepted typical droplets size for nanoemulsion falls in the range of 20- 200 nm (Aboofazeli, 2010; Sadtler *et al*., 2010; Thakur *et al*., 2012; Jaiswal *et al*., 2014). As shown in Figure 2, the size of oil droplets decreased with increasing surfactant concentration up to the CAC value, with a concomitant reduction in the IFT value, in which the smallest droplet size of 89.64 nm was obtained at 0.0167% (v/v) of Tween 80. Larger droplets size was observed below the CAC value which was probably due to insufficient surfactant coverage at the oil/water interfaces. A further increase in the surfactant concentration above the CAC value resulted with the increasing droplet size. It is likely due to the formation of interbilayer-cross linked multilamellar vesicles (ICMVs) and lyotropic liquid crystals with the excessive surfactant monomers. Increase of surfactant concentration causes a transition of typical spherical micellar to a more elongated or rod-like micelles (Borzenkov and Hevus, 2014) . Further increase in the concentration may cause the orientation and closer packing of the elongated micelles into hexagonal phase (Florence and Attwood, 2015).

The zeta potential of the droplets produced at different Tween 80 concentration is also shown in Figure 2. The zeta potential values lie between -39 to -45.8 mV. All condition indicates with the zeta potential values greater than -30 mV. Thus, the emulsion system with a high degree of physical stability was obtained in this study. The emulsified agarwood oil without Tween 80 showed a negative value of –45.80 mV, indicating that the oil droplet interface was negatively charged in the aqueous. The zeta potential value for oil droplets before and after 0.0167% (v/v) of surfactant addition was -45.8 mV and -40.9 mV, respectively. The reduction might occurred due to the adsorption layer of the surfactant monomers that shift the shear plane at which the zeta potential value was measured to a further distance from the particle surface, thus, resulting in the lower zeta potential value (Ridaoui *et al*., 2006). Since only slightly difference in zeta potential (-5 mV) was observed in the system, we assumed that the zeta potential values were independent from Tween 80 influence, as predicted in the previous discussion.

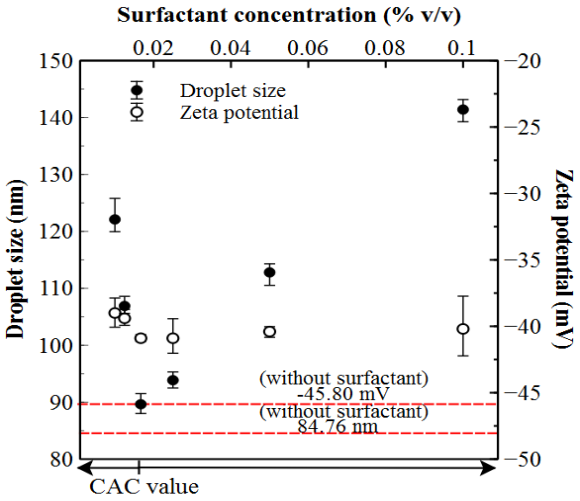

Figure 2. Droplet size and zeta potential as a function of Tween 80 concentration.

From Figure 2, the independence of oil droplet from Tween 80 were continuously noticed when no significant difference in the zeta potential values were observed with the increment of Tween 80 concentration. This study also found that at all Tween 80 concentration, the average zeta potential values were lies within -40.1 mV. Therefore, the separation or grading of the agarwood oil using the gel electrophoresis were feasible because the oil droplets charges were depending on the components that trapped in the droplet.

When a non-ionic surfactant adsorbed at the interface of oil droplets in aqueous, surprisingly, the surface charge rather than being neutralised because of the non-ionic characteristics, caused a slight reduction in the zeta potential. Steric repulsion force

not only occurred due to the addition of the non-ionic surfactant, but an additional of electrostatic stabilization mechanism might be present whereby in this case the zeta potentials (-45.8 mV) were contributes by the oil droplets itself when no Tween 80 surfactant was added. Thus, it further stabilizing the droplet dispersion.

Agarose gel electrophoresis

Gel pore size is the most important parameter for electrophoretic separation, and can be controlled by the gel concentration (Manz *et al*., 2004). High gel concentration resulted in smaller gel pores. The droplets size produced with the surfactant concentrations of 0.01-0.1% (v/v) and 1-2.2% (v/v) were shown in Figures 3a and 3b, respectively. At surfactant concentration of 0.01-0.1% (v/v), it was found that oil droplets were in the range of 80-150 nm. The droplets size was too small and unable to be trapped by the gel pores which indicated in Figure 4a. As a result, no band were noticed in Figure 4a even the gel concentration varies (1, 2, and 4%). Interestingly, when the Tween 80 concentrations were increased within 1-2.2% (v/v), the droplets size increased from 200-1000 nm and the droplets could be visibly separated using 1 and 2% of agarose gel concentration. However, at the 4% of gel concentration, the visible bands were unable to be separated due to small gel pores that prevented the droplets migration (Figure 4b).

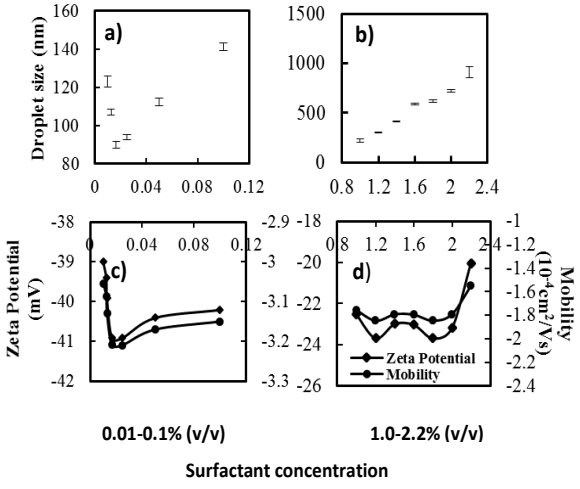

Figure 3. Droplets size with surfactant concentrations of (a) 0.01-0.1% (v/v) and (b) 1-2.2% (v/v). The droplet also indicated various zeta potential values with surfactant concentrations of (c) 0.01-0.1% (v/v) and (d) 1-2.2% (v/v).

Figures 3c and 3d showed the zeta potential value and the electrophoretic mobility of the oil droplets produced with the surfactant concentrations from 0.01-2.2% (v/v), respectively. The zeta potential and the electrophoretic mobility value of fine droplets (80-150 nm) oil droplets were higher than in the case of large droplets (160-950 nm). Although the fine oil droplets produced at surfactant concentrations 0.01-0.1% (V/V) consist of high zeta potential and electrophoretic mobility value, no visible band was observed from the work even the gel pores were reduced by increasing the gel concentration from1 to 4% (Figure 4a).

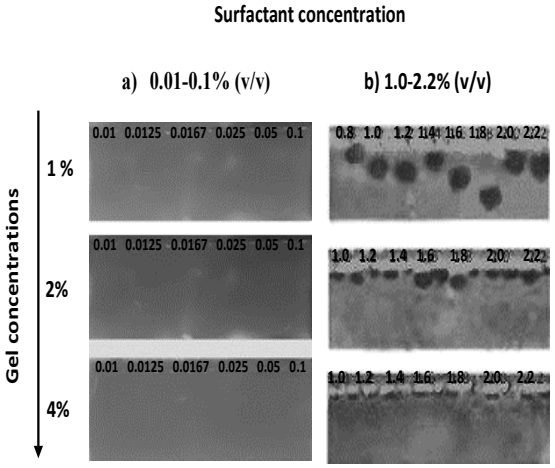

Figure 4. The agarose gel electrophoresis image for emulsion produced with surfactant concentration of (a) 0.01-0.1% and (b) 1.0-2.2% at different gel concentration. Visible bands in the gel were noticed when the droplets size increased from 200-1000 nm or equivalent to 1-2.2% (v/v) surfactant concentration. The patterns of the visible band were identical to droplets zeta potential value, not droplets size.

A visible bands separation was observed for the large range droplet with surfactant concentration of 1-2.2% (v/v) using the gel concentration of 1-2% (Figure 4b). However, the separation of the visible bands or mobility of the large range droplets were limited by 4% gel concentration, suggesting that gel pore size retarded the migration of the large droplets. Referring to the visible band, we concluded that the droplet separation patterns tended to follow zeta potential and electrophoretic mobility values which indicated that the grading of an essential oil quality can be performed using the gel electrophoresis because each oil grades are differs by the components that trapped within the droplet. Here we hypothesised that the zeta potential values rather than size of the particular oil droplets were the key factor separating the droplets via the gel electrophoresis.

Conclusion

This study demonstrated the separation of the emulsified agarwood oil droplets using agarose gel electrophoresis. Critical aggregation concentration (CAC) for the agarwood oil droplet was obtained at 0.0167% (v/v) of Tween 80 concentration. The value

was crucial in this study since it represented equal diffuse ions with same absolute charges to that of the emulsified droplets in aqueous. However, due to the agarose gel pore size limitation, only the large size emulsified oil droplets which are not produced within CAC value were visible to be separated. Thus, a further investigation on electrophoresis of the emulsified agarwood oil droplet charge characterization using smaller gel pores size than agarose gel i.e. polyacrylamide gel will be performed in the next following work to improve the current findings.

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