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Electrophoretic mobility of nano-emulsified cinnamon oil in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) system

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Abstract

A novel application of sodium dodecyl sulphate-polyacrylamide gel electrophoresis was evaluated to differentiate the quality of two commercial cinnamon bark oil samples extracted by steam distillation and cold pressed. Prior to the electrophoresis, cinnamon oils were emulsified with non-ionic surfactant, tween 80 at 2% *v/v* of critical aggregation concentration. The droplets prepared were within 30 to 70 nm size and zeta potential values of -4 to -12 mV consisted of certain amount of bioactive compounds that responded to specific molecular mass and electrophoretic mobility so that separation using SDS-PAGE can be performed. The resolution bands at 21% gel with 48% acrylamide concentration and 3.33% of cross-linker demonstrated that high quality (steam distilled) cinnamon oil migrated slower through gel due to its large molecular weight components when compared to the low quality (cold pressed) cinnamon oil. The difference in the relative mobility, 0.68 and 0.75 of emulsified steam distilled and cold pressed cinnamon bark oils, respectively, was attributed by their entrapped components impinging upon their electrophoretic mobility.

1. Introduction

Cinnamon is one of the oldest herbal plants, genus of tree *Cinnamomum* has been widely used as a spice, a flavouring agent in food and beverage industries and for various application in medicines (Wijesekera, 1978; Lee and Balick, 2005; Wong *et al*., 2014). Cinnamon gets its distinctive smell and aroma from volatile oil that can be extracted from its bark, leaves and roots. The traditional extraction technology such as water and steam distillation, effleurage, cohobation, maceration is of great significance in pertaining to and improving the quality of essential oil (Handa *et al*., 2008). Among these methods, steam distillation was recognized as the simplest and most economical method to extract and isolate the volatile oil (Farag *et al*., 1989; Suhaj, 2006). The method requires no solvent, safer and cheaper than other methods. However, due to the advanced technology demands, more techniques were invented such as cold press, supercritical fluid extraction, solid phase micro-extraction are used to extract the volatile oil without alteration of their heat-sensitive components (Handa *et al*., 2008; Kamaliroosta *et al*., 2012). The efficiency of extraction methods mainly depends on understanding the nature of the plant matrix, the chemistry of bioactive compounds and critical input parameters (Azmir *et al*., 2013). Due to various numbers of extraction methods, the qualities of the extracted oil varies at the downstream processes and difficult to be evaluated.

Extracted cinnamon oil contains numbers of chemical compounds with different chemical structures that affect its physical properties. These compounds possess specific functional groups (i.e aldehyde and phenolic group) that exhibit different properties such as antimicrobial, antioxidant, stabilizing and flavouring (Kamaliroosta *et al*., 2012). Therefore, the quality of the cinnamon oil also depends on the physical properties of the samples as well as the number of major compounds detected during the analytical analysis. For example, extracted cinnamon leaf oil consists of more eugenol, which smells like clove oil with hints of citrus, while cinnamon bark oil has high levels of cinnamaldehyde, which smells spicier and sweeter. Since cinnamaldehyde gives cinnamon its trademark scent, hence bark oil has the stronger cinnamon aroma than leaf oil. Moreover, cinnamon bark oil is usually more expensive than cinnamon leaf oil as the oils in the bark are difficult to be extracted, and it takes greater quantity to produce similar amount of oil.

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Many studies have been carried out to determine the quality of cinnamon oil by analysing the chemical components of the extracted oil using Gas Chromatography-Mass Spectrometry (GC-MS) (Jayaprakasha *et al*., 2002; Trajano *et al*., 2010; Wong *et al*., 2014; Abdelwahab *et al.*, 2017). However, high equipment maintenance cost, well-trained personnel, high operating pressure and large quantities of expensive organic solvents are required when using the chromatographic technique. Thus, gel electrophoresis is introduced in the study as a non-destructive, convenient and low operational cost method to assess the quality of the extracted cinnamon oil. Prior to electrophoresis, the extracted oil was first emulsified. Emulsified extracted cinnamon oil is preferable instead of the pure cinnamon oil due to the immiscibility of the essential oil and water that allows electrophoretic mobility of the suspended compounds during electrophoresis. Moreover, oil-in-water emulsification processes also allow the delivery of active compounds in aqueous solution and enhance the stability of chemically unstable compounds from degradation (Chime *et al*., 2014).

Gel electrophoresis is mainly used to separate macromolecules such as deoxyribonucleic acid, ribonucleic acid, or proteins based on molecular size, charge and molecular weight by an electrical field through gel medium. It is widely used to determine the molecular weight, charges of nucleic acid or protein, the subunit structures of proteins, and the purity of protein (Wrolstad *et al*., 2005; Rath *et al*., 2013). When an electric field is applied, the suspended molecules migrate towards the opposite charge electrode allowing the molecules to migrate at different velocities, which mainly depends on the size and charge of the molecules. Using the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the gel acts as a sieving medium, and thus retards the passage of molecules.

For comparison purposes, two different essential oils qualities obtained from the bark of cinnamon were characterized by GC-MS in order to differentiate the quality of the samples. The samples were commercial steam distilled and cold pressed oils respectively. Since the separation principles are obtained from the molecular sieving property of the gel or the gel pore size, different electrophoretic mobility can be observed from the samples that consist of distinguished extracted compounds properties or quality.

2. Materials and methods

2.1 Materials

Two cinnamon bark oils with purity 95-99% extracted using steam distillation and cold press and methods were purchased commercially from Best Formula Industries Sdn. Bhd., Malaysia and Fairy Essential Gallery Sdn. Bhd., Malaysia respectively. Tween 80 (Polyoxyethylene (20) sorbitan monooleate), acrylamide, bisacrylamide, sodium dodecyl sulphate (SDS), tris base, ammonium persulphate (APS), Tetramethylethylenediamine (TEMED), bromophenol blue (loading dye), Coomassie brilliant blue (staining dye), acetic acid, isopropanol and methanol were purchased from Sigma-Aldrich Co. LLC., Saint Louis, MO, USA.

2.2 Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Steam distilled and cold pressed cinnamon oils were sent for GC-MS analysis in order to identify the quality of the oil (Jayaprakasha *et al*., 2002; Trajano *et al*., 2010; Wong *et al*., 2014). The GC-MS analysis was performed by GC-7890B gas chromatograph coupled to a 5975C inert mass selective detector (MSD) with Triple Axis Detector (TAD) (Agilent Technologies, Palo Alto, CA). The capillary column used was an HP-1MS (30 m x 0.25 mm i.d. x 0.25 µm film thickness). The oven temperature was initially held at 50°C for 1 min, and then increased to a final temperature of 230°C at a rate of 3°C/min, which was then held for 4 mins. The injector and interface temperature was held at 230°C. Helium gas was pressure controlled with a flow rate of 1 mL/min at 50ºC. Electron ionization mass spectra were collected at 70 eV ionization voltage over the range of m/z 5-241.5. Identification of volatile components was performed by comparing the mass spectra of individual components with the reference mass spectra in the NIST Mass Spectral Library. The average molecular weights of the two cinnamon oil samples were calculated directly from compound peak area percentage and molecular weight analysed through GC-MS (Lee, 2012).

2.3 Sample preparation

The cinnamon oil emulsions were prepared according to Komaiko *et al*. (2015) with a little modification on the formulation. The surfactant solution was prepared by using surfactant concentration of 1-5% *v/v* 0.2 mL cinnamon oils were then dispersed in the continuous phase of surfactant solution to produce emulsions. Energy input was supplied by sonicator (Model 705 Sonic Dismembrator, Fisher Scientific, USA) at 700 Watts and 20 kHz with 70% amplitude, 1 min process time, 15 s of pulse on and pulse off time. The heat generated during the emulsification process was eliminated by placing the sample container inside an ice bath.

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2.4 Droplet size and zeta potential

The droplet size and zeta potential values are the representative parameters used to predict the emulsion stability (Achouri *et al*., 2012). The measurement of droplet size in term of diameter was carried out at 25°C with zeta potential and nanoparticle analyzer (NanoPlus-3, Particulate System, USA). The measurement was based on the droplets' Brownian motion in the bulk; with the principle of slower motion for larger droplets.

Zeta potential designates the magnitude of the repulsion or attraction between droplets in the emulsion system. The emulsions were injected into the sample flow cell and their zeta potential values were determined by measuring the electrophoretic mobility of the dispersed droplets in a charged field via a built-in laser Doppler micro-electrophoresis technique. The refractive index and viscosity were kept at 1.33 and 1.0 cps respectively in order to mimic the values of pure water. (Meyer *et al*., 2006; Vyas *et al*., 2008; Affandi *et al*., 2011).

2.5 Sodium dodecyl sulphate- polyacrylamide gel electrophoresis (SDS-PAGE)

Since SDS-PAGE has higher resolving power for small fragments, thus it was selected in this study for grading the emulsified cinnamon oil. The experimental setup was performed by the method proposed by Boon *et al.* (2016) using Owl dual-gel vertical electrophoresis system (P8DS, Thermo Fisher Scientific, USA). Electrophoresis gel can be divided into stacking gel and resolving gel. The resolving gel pore sizes, controlled by acrylamide concentration percentage (%T) and cross-linker percentage (%C) were varied between 40-55%T and 2.6-5%C, respectively in order to obtain the best resolution. The gel percentage was varied in 16% and 21% to improve the resolution. The formula for the preparation of acrylamide/ bisacrylamide stock solution was summarised in Supplementary Table 1 while the formula for the preparation of 16% and 21% resolving gel and 4% stacking gel solution was summarised in Supplementary Table 2. 10 mL of the prepared resolving gel solution was poured up to 75% of short plate height. About 0.2 mL of isopropanol was then 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 min with Whatman filter paper (#1) and the polymerized gel surface was rinsed with distilled water. The prepared stacking gel solution was then poured to fill the remaining volume of the glass plates. The comb was immediately inserted to avoid the entrapment of bubbles inside the gel. The comb was then removed from the glass plates. The glass plates were then washed to remove the adhered gel on the outer surface of the glass plates as the gel solidified. The gel cassette was then placed properly in the electrode chamber and the chamber was tightened by clamping the frame in order to avoid the leaking of running buffer from the inner chamber. The chamber was then placed into a tank and the tank was filled with electrode buffer. Finally, the sample, which was the mixture of emulsion and loading buffer, were loaded into the wells. The electrophoresis was carried out initially at 150 mA, 150 V, once the sample migrated down to resolving gel, the current and voltage were increased up to 300 mA and 300 V, respectively, for about 1 hour. The gel was then stained with staining solution for an hour and destaining for an additional hour prior to image scanning. The relative mobility, R_f of each visible band was calculated and tabulated. It is the distance migrated by a band divided by the distance migrated by the bromophenol blue dye front (Equation 1).

$$R_f = \frac{Migration\ distance\ of\ the\ band}{Migration\ disance\ of\ the\ dye\ front} \quad (1)$$

Table 1. Summary properties of both types cinnamon oil emulsion (mean ± standard deviation, n = 3)

Properties	Steam distilled cinnamon oil emulsion	Cold pressed cinnamon oil emulsion
Droplet Size (nm)	63.17 ± 2.32	36.97 ± 0.64
Molecular weight (g/mol)	188.56	171.31
Zeta potential (mV)	-9.76 ± 0.19	-10.50 ± 0.09
Relative mobility	0.71 ± 0.03	0.76 ± 0.03

2.6 Statistical analysis

All data were expressed as mean ± standard deviation. All experiments were carried out in triplicate. Any differences in values were determined by one-way analysis of variance (ANOVA) ($p<0.05$) using Minitab 16.

3. Results and discussion

3.1 GC-MS

Cinnamon extracts were analyzed using GC-MS for compound identification, which indirectly reflected the quality of the oil. About fifty compounds were found in the steam distilled cinnamon bark oil. The identified oil consisted of cinnamaldehyde (8.18%), caryophyllene (67.26%), coumarin (0.85%), benzyl benzoate (0.51%), p -cymene (1.59%), δ-limonene (0.45%), linalool (4.93%), eugenol (3.52%) and 2-propanamine (6.28%) and other small percentage impurities. Whereas, forty eight compounds were identified in the cold pressed cinnamon bark oil. The major compounds were cinnamaldehyde, caryophyllene, coumarin, benzyl benzoate, p-cymene, linalool, eugenol, diethyl phthalate, δ-limonene and 2,4-diethyl-6-methyl-1,3,5-trioxan, with intensity percentage

of 0.20%, 0.86%, 0.05%, 0.31%, 0.18%, 0.26%, 10.94%, 16.54%, 0.05% and 66.48%, respectively as well as other small percentage impurities. From the obtained results, steam distilled cinnamon oil contained higher amounts of cinnamaldehyde and caryophyllene than cold-pressed cinnamon oil, these compounds were identified as major compounds that are responsible for the delicate flavor. The spicy taste and fragrance of cinnamon are due to the absorption of oxygen by cinnamaldehyde (Singh *et al*., 2007). The quantitation of essential oils components reflects the quality as well as their market price (Kamaliroosta *et al*., 2012; Porel *et al*., 2014; Wong *et al*., 2014). Ravindran *et al*. (2004) reported that high-quality cinnamon oil has a high amount of cinnamaldehyde; while Caiger (2016) reported that the oil is priced according to its cinnamaldehyde content, with current price in the range of US dollar 150-200 per kg. The results indirectly designated that steam distillation produced high-quality cinnamon oil as compared to cold pressed due to its higher content of cinnamaldehyde. Cinnamaldehyde possesses a wide range of pharmacological functions. Studies have found that this compounds had antimicrobial, anti-inflammatory, antioxidant, and antidiabetic properties (Zhu *et al.,* 2011; Roth-Walter *et al.*, 2014; Kawatra and Rajagopalan, 2015). Several studies have reported the effectiveness of cinnamon in treating HIV infected cells, cardiovascular disease and Parkinson's disease (Fink *et al*., 2009; Khasnavis and Pahan, 2012; Rao and Gan, 2014).

3.2 Surfactant concentration effect on droplet size and zeta potential of extracted cinnamon oil

Figures 1 and 2 show the droplet size and zeta potential for cold pressed and steam distilled cinnamon oil emulsions, respectively. The results showed that non-ionic surfactant, tween 80 was capable to stabilize the emulsion by size reduction significantly ($p<0.05$). Tween 80 was used as a surfactant in this study as it has a high hydrophilic-lipophilic balance (HLB-15) and favourable for oil in water emulsion (Ghosh *et al*., 2013). Tween 80 is a non-ionic surfactant that consists of a hydrophobic tail and a hydrophilic head. The hydrophobic tail adsorbs on the oil droplet surface while its hydrophilic head faces outwards into the aqueous medium. The condition creates a stabilized layer and prevents the aggregation amongst the emulsified droplet. If the droplet collisions were faster than the surfactant molecules absorption rate, the droplet coalescence occurred due to the hydrophobic attraction between droplets (Tamjidi *et al*., 2013).

Both results also showed that the droplet size decreased significantly with increasing surfactant concentration ($p<0.05$). After a minimal value was reached which indicated as the critical aggregation concentration (CAC); about 2% *v/v* of surfactant concentration, the droplet size increased which indicated that droplet interface was saturated with surfactant monomers. Among the surfactant concentrations, maximum interfacial tension reduction occurred at this point, the smallest droplet size of 63 nm and 37 nm was achieved for steam distilled, and cold pressed cinnamon oil emulsions, respectively. Thus, 2% *v/v* was determined as critical aggregation concentration (CAC) in this study. Interfacial tension (IFT) is defined as energy required for creating a unit surface area at the boundary of two

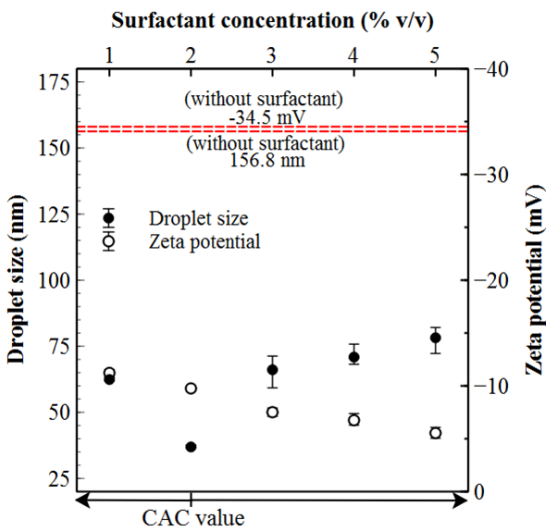

Figure 1. Droplet size and zeta potential for cold pressed oil emulsion as a function of surfactant concentration. ABCD Significant difference ($p<0.05$), one sample over the other(s) if their droplet sizes carry different superscript; abcde Significant difference ($p<0.05$), one sample over the other(s) if their zeta potential values carry different superscript. (n = 3, error bars show standard deviation).

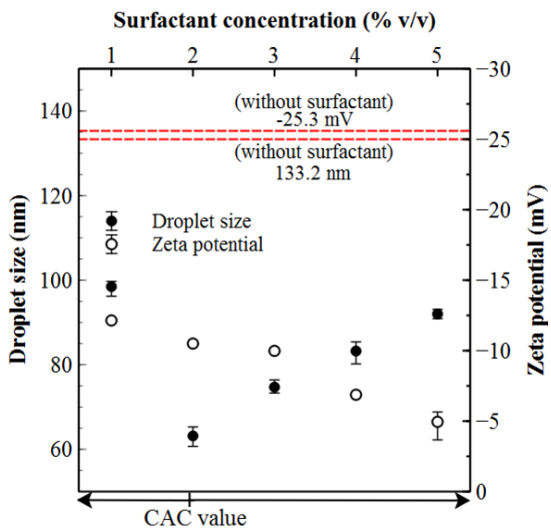

Figure 2. Droplet size and zeta potential for steam distilled oil emulsion as a function of surfactant concentration. ABCDEF Significant difference ($p<0.05$), one sample over the other(s) if their droplet sizes carry different superscript; abcde Significant difference ($p<0.05$), one sample over the other(s) if their zeta potential values carry different superscript. (n = 3, error bars show standard deviation).

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immiscible liquids (Sarapardeh *et al*., 2014). A system with high interfacial tension resisted one immiscible liquid dispersing in another in the form of droplets. Tween 80 which is soluble in the water phase was therefore added to stabilise the unstable system by adsorption at the oil/water interfaces. They oriented themselves by facing hydrophilic group toward water phase and hydrophobic group toward oil phase. This oriented film reduced the interfacial tension and consequently caused the thermodynamical instability of the system resulting from the increase in the interfacial area between the two-phase (Rosen and Kunjappu, 2012).

In this work, larger droplet size was observed for steam distilled cinnamon oil emulsion compared to cold pressed cinnamon oil. This might due to the low viscosity of the cold pressed cinnamon oil, and thus, better size reduction was observed for cold pressed cinnamon oil when the same amount of mechanical energy was applied. Additional surfactant concentration gave rise to the droplet size due to the changes in the spherical micelle structure (Langevin, 1992; Guo *et al*., 2001). Furthermore, the emulsion with a smaller average droplet size was more stable than with larger size (Chung *et al*., 2001).

Figures 1 and 2 also present that the zeta potential values decreased with the increasing surfactant concentration. This was due to the shift of the shear plane further from the droplet surface with increasing surfactant monomers. The range of zeta potential magnitude (-4 to -12 mV) indicated the destabilization of emulsions. In general, systems with zeta potential > ±30 mV are considered pharmaceutically stable (Hanaor *et al*., 2012). Despite the decrease observed in the magnitudes of the zeta potential, the emulsions may be stable, as there were non-ionic steric interactions between the droplets. The reduction in zeta potential value had confirmed that the importance of CAC value in representing the uniform barrier that screening the droplet surface with a particular amount of charge.

Figure 3 shows the visual appearance of cinnamon oil emulsion as a function of surfactant concentration. The turbidity decreased with increasing surfactant concentration which contributed by the reduction of droplet size. The reduction in turbidity of the emulsion, is a key indicator for evaluating emulsion stability. The emulsion produced at 2% *v/v* showed the most optical transparent appearance, as compared to others surfactant concentration, indirectly showed that the most stable emulsion was produced at 2% *v/v*. In this study, the produced emulsion were in the size range of 30 - 100 nm, which indicated that the droplets were on nanometer scale or known as nanoemulsion (Aboofazeli, 2010; Thakur *et al*., 2012 Jaiswal *et al*., 2014).

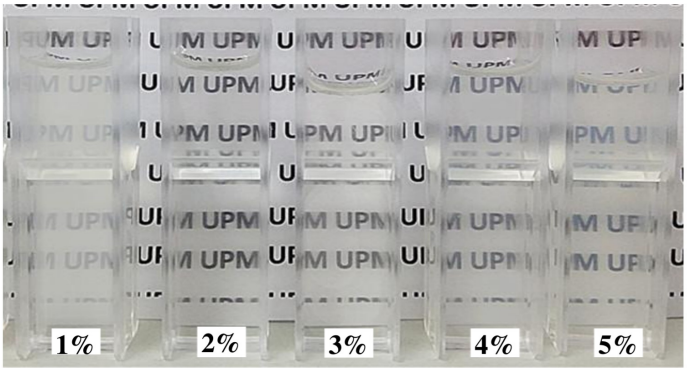

Figure 3. Visual appearance of emulsions produced with different surfactant concentration. The smallest emulsified droplet for steam distilled and cold pressed cinnamon oils of 63 nm and 37 nm respectively at 2% v/v surfactant concentration that showed the optical transparent appearance

3.3 Effect of acrylamide concentration (% T) and ratio of acrylamide to bisacrylamide (% C)

In the present work, the resolving gel percentages were cast in 16% and 21%, with various acrylamide and bisacrylamide concentrations, as shown in Figure 4. To the best of our knowledge, the smaller the molecular weight of the molecules, the higher the percentage of gel matrix should be used in order to obtain the best resolution. Therefore, 21% of gel was chosen in this study as the droplet size range of emulsified cinnamon oil was found below 100 nm. Furthermore, no distinct band was observed for 16% of gel owing to its large pore size. At the same time, the gel pore size decreased when resolving gel was increased to 21%, thereby retarding the small and fine emulsified droplets when the droplet migrates through the gel. Manz *et al*. (2004) reported that large molecules were stopped after only a short migrating distance, whereas smaller molecules could migrate further until they encountered a pore size too small for them to pass through.

Among the concentrations, 48%T and 3.33%C with gel percentage of 21% gave the best resolution with distinct bands due to its suitable pores size. Decreasing of gel pore size above 48%T limited the migration of the droplets, thus, no distinct band was observed for 50-55% T even the ratios of acrylamide to bisacrylamide were varied from 2.6-5%C. In contrast, 40%T with any %C with slightly larger pore size unable to entrap the droplets effectively. The results rather than the ideal condition (48%T, 3.33%C, and 21% gel), showed multi defects on a resolution such as smear and streaks. The polyacrylamide gel pore size can be controlled by adjusting the total acrylamide concentration (%T) and the ratio of acrylamide to bisacrylamide (%C) (Hong *et al*., 2016). Generally, the pore size decreases in a nearly

Ratio of acrylamide to bisacrylamide (% C)	Gel percentage (%)	Percentage of total acrylamide (% T)				
		40%	48%	50%	53%	55%
2.6	16					
	21					
3.33	16					
	21		Lane 1 Lane 2 Lane 3 Lane 4			
5	16					
	21					

Figure 4. SDS-PAGE resolution image for cinnamon oil emulsions produced with various acrylamide and bisacrylamide percentages in 16% and 21% gel (Lane 1 and 2: cold pressed and Lane 3 and 4: steam distilled). Ideal condition was identified as 48%T, 3.33%C, and 21% gel (marked with square dotted line)

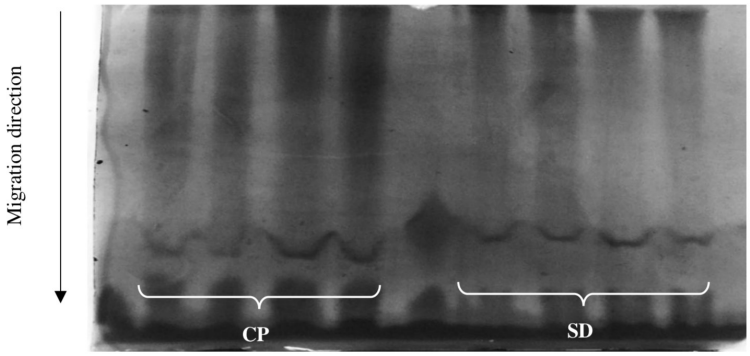

Figure 5. Higher resolution of cinnamon oil emulsion at optimized 21% gel percentage, with 48%T and 3.33%C. Four replicate samples were run simultaneously for reproducibility purpose. Lane 1-4 were CP samples; Lane 6-9 were SD samples (CP referred to cold pressed cinnamon oil emulsion; SD referred to steam distilled cinnamon oil emulsion).

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linear relationship with increasing %T. Yet, the relationship between %C and gel pore size is more complex. Minimum pore size occurs at 5%C (ratio acrylamide to bisacrylamide 19:1); decreasing %C results in a more open pore structure as there are less cross-linked molecules. In contrast, increasing %C beyond 5%, the gel pore size increases due to the non-homogeneous bundling of strands in the gel.

3.4 Molecular weight and relative mobility of two different cinnamon oil quality

In Figure 5, the migration patterns of both samples indicated that steam distilled cinnamon emulsion oil migrated slower than cold-pressed cinnamon oil emulsion. This phenomenon can be explained by referring to the larger average molecular weight of the steam distilled cinnamon oil emulsion. Steam distilled cinnamon oil emulsion is defined as high-quality emulsion earlier consisted of major compounds with high molecular weights, thus the average molecular weight for steam distilled cinnamon oil emulsion (188.6 g/mol) was higher than the cold pressed cinnamon oil emulsion (171.3 g/mol).

The steam distilled cinnamon oil emulsion with larger droplet size/molecular weight but slightly lower charge, in turn, migrated more slowly than cold-pressed cinnamon oil emulsion. This might due to the SDS-encapsulated droplets covered with uniform charges, providing all droplets with the same charge per unit weight. As a result, the rate of an SDS-encapsulated droplet migrated through gel depends primarily on its molecular weight (Helms, 2008). In addition, the frictional force of a droplet during movement through gel mainly depends on the size and shape of the droplet. Thus, a small droplet faces only a small frictional force while a large droplet faces a larger frictional force (Mordacq and Ellington, 1994). This study confirmed the feasibility of using gel electrophoresis principles and techniques in grading the emulsified essential oil based on their relative mobility through the gel in terms of their molecular weight. The finding is expected to provide a non-destructive *in-situ* analysis for not only cinnamon oil quality determination, but also for other essential oils. These findings will be of interest as the technique provides new insight into how electrophoresis can be applied in emulsion science.

4. Conclusion

This work was successful in differentiating two different oil qualities based SDS-PAGE technique and principles. Using the GC analysis, high-grade quality oil was determined from steam distilled oil extracts which consist of higher amounts of cinnamaldehydes compared to the cold pressed one. By transforming the oil extracts into nano-emulsified oil, a simple comparison method was developed using the SDS-PAGE system. From a series of scientific analytical methods, it was found that high-quality oil contributed to low relative mobility value, large particles size and low zeta potential value. The conditions caused the oil samples to be differentiated from each other when subjected to SDS-PAGE system. The information is expected to provide an *in-situ* analysis to the manufacturer/post-harvest process in terms of the quality of extracted oil without the necessity to frequently send the samples to laboratory for detailed analysis.

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