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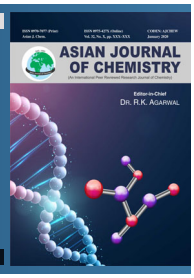

Separation of Fatty Acid Ethanolamides using High Performance Liquid Chromatography

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This study aimed to separate fatty acid ethanolamides (FAE) through an enzymatic reaction of ethanolamine and the *Terminalia catappa* (Local name: Ketapang) seed oil by using reverse-phase high-performance liquid chromatography (RP-HPLC). A 4.6 mm × 150 mm, 5 µm SunFire C18 column was used. Separation optimization was performed by analyzing different variables, which includes mobile phase composition (acetonitrile/water), flow rate and sample concentration. The separation results showed that the retention time for all components was 4.5; 2.4; 12.9; and 6.3 min for fatty acid ethanolamides: oleoylethanolamide, linoleoylethanolamide, stearyl diethanolamide and palmitoylethanolamide, respectively. Inter-peak resolution of >1.5 indicated that each component was accurately separated.

Keywords: *Terminalia catappa* seed oil, Ethanolamine, Fatty acid ethanolamides, HPLC.

INTRODUCTION

Fatty acid ethanolamides (FAEs) are the active surfactant ingredients having two active ends, non-polar and polar sides comprising amide bonds. Surfactants play a role of reducing voltage between water and oil caused by the amide bonds in emulsion systems. These bonds are chemically stable in alkaline media [1]. It behaves as thickeners and solvents and component adhesive, wetter, clumping, emulsifying, foaming and penetrating materials for aerosol materials and certain drugs. Moreover, it is applied to different industrial fields that employ multiphase systems [2].

The requirement and usefulness of FAEs in studies and industries are not in agreement with the sufficient availability of materials. Studies have been conducted for FAEs synthesis from various basic materials by using enzymatic and chemical catalysts [3-6]. However, the resulting FAE obtained are a mixture of long and short fatty acid chains depending on the used base materials. Commercially available pure ethanolamide fatty acids are expensive and rare; thus, studies are required to use the appropriate separation techniques having inexpensive basic materials. Fatty acid ethanolamide (FAE) has been synthesized enzymatically or chemically by using enzymes as industrial biocatalysts that offers numerous advantages over chemistry [7]. Vegetable-oil-based FAE was synthesized using ketapang (*Terminalia cattapa*) comprising C16, C18 and C20 fatty acid chains with enzyme and ethanolamine catalysts [8]. This led to the synthesis of FAEs in the form of a mixture, which was mandatory to acquire pure compounds.

Fatty acid ethanolamide (FAE) comprises amide fatty acids having long chain hydrocarbons that are similar to the carbon amount in compounds having several double bonds C (18:1), C (18:2), and C (18:3) and thus causes a small variation in the polarity between components. Additionally, FAEs are the least reactive derivatives of hydrocarbons with low volatility, which makes their separation using liquid or column gas chromatography (GC) difficult. Because of the stable nature of FAEs, a separation method with high performance column, such as HPLC, is required. Thus, this study aimed to investigate the influential variables responsible for separation to form pure ethanolamide fatty acid products.

EXPERIMENTAL

The ingredients used were hexane, chloroform, methanol, ethanolamine, lipozyme@RM IM (immobilized *Rhizomucor miehei* lipase) Novo Nordisk, standard ethanolamide fatty acid and Ketapang seed oil. FT-IR spectrophotometer from Perkin Elmer Model Frontier™ and C-18 HPLC (Shimadzu LC-10 ATVP) reverse phase were used.

Synthesis and purification of fatty acid ethanolamide (FAE): FAE was enzymatically synthesized through a reaction of the ketapang seed oil and ethanolamine under the optimal conditions acquired from studies [8]. Ketapang seed oil (10 g) was treated with 125 mL of ethanolamine, which was first dissolved in distilled water at pH 7 through the addition of 1 M HCl. The reaction was conducted using lipozyme@RM IM catalyst and 100 mL of the 7.5% (w/w) hexane solvent. Moreover, in a horizontal water bath shaker at 150 rpm, the mixture was incubated for 2 h at 40 ºC. The product of synthesis was purified using various stages, including enzyme separation in the bottom and top layers of the mixture through funnel filtration. The organic or top layer containing FAEs was cooled at -4 ºC for 5 h and was filtered through vacuum filtration. The filtered products were placed in a desiccator for drying and weighed using analytical scales.

Characterization of fatty acid ethanolamide (FAE): The initial identification of the amides produced through thin layer chromatography (TLC) was employed to acquire bottled TLC sheets, This was further established into a chloroform: methanol (90:10, v/v) solvent system that was characterised by identifying functional groups through Fourier-transform infrared (FT-IR).

Separation of FAE components using HPLC: Each unit comprises Waters 2489 UV/Visible detector, SunFire C18 5 µm (4.6 mm × 150 mm), 1525 Binary HPLC pump and fixed flow method using a gradient sensitivity system and wavelength of 4,000 AUFS and 213 nm, respectively. After HPLC commencement, the pump was started and then the motion phase was allowed to flow for 30 min till the base line stopped producing noise. It shows that the system stabilized.

Preparation of sample solutions: The synthesized FAEs obtained as a fine white solid were dissolved in 0.05 g of acetonitrile and diluted to 10 mL. The sample solution was filtered into a vial tube by using a nitrate filter of 0.45 µm cellulose.

Determination of flow rate, motion phase and the optimum concentration: The solution of sample with 5000 ppm concentration was injected into HPLC with motion phase of 95:5 acetonitrile/water and flow rate variation of 1.5, 1.0 and 0.5 mL/min. Subsequently, a 5000 ppm solution was injected into HPLC with a variation of the ratio of the motion phase of 95:5, 90:10 and 85:15 and flow rate of 0.5 mL/min. For concentration differences, the 1000, 3000 and 5000 ppm samples were injected into the HPLC system by using the motion phase of 9010 acetonitrile/water and flow rate of 1 mL/min. Additionally, for each component of the compound, the area and retention time were observed with different variations.

Components were characterized under the optimum conditions acquired in previous stages. Moreover, an identification test was performed on FAE components by comparing the retention times of oleoylethanolamide, palmitoylethanolamide, linoleoylethanolamide and stearyl diethanolamide standards at 213 nm wavelength.

RESULTS AND DISCUSSION

The reaction of Ketapang seed oil (in *n*-hexane) with ethanolamine produces a solution having two phases, which were immiscible. The upper and lower layers are the oil containing *n*-hexane and water phase containing ethanolamine, respectively. Moreover, the two layers were formed in the solution because of the differences in the polarity of *n*-hexane and water. After the reaction is conducted using the lipozyme@RM catalyst, the enzyme tends to remain between water and *n*-hexane phases. The reaction was easily stopped by separating the enzyme from reaction products. Currently, immobilized enzymes are preferably used as catalysts because reactions proceed efficiently in terms of time, cost and materials [9]. The reaction products are expected to comprise ethanolamide fatty acids, *i.e.* oleoyl-ethanolamide, palmitoylethanolamide, linoleoyl-ethanolamide and stearyl diethanolamide [10].

At this stage of synthesis, 75.44% yield conversion is obtained after the refining process. This finding is slightly lower than that of Sari *et al.* [11]. Sari *et al.* [11] synthesized FAE from palm oil by using chemical catalysts. However, the obtained results of this study are greater than those of FAE synthesis performed using *Calophyllum inophyllum* oil with enzymatic catalysts [12].

Characterization: Initial identification of FAE using TLC with chloroform-methanol (90:10 v/v) eluent provided a retention factor (R_f) value, which was identical for the samples and standard FAEs. This finding indicated that FAEs were successfully synthesized because it has the same properties according to the similarity of R_f values which were used to identify a compound. Considering that identification values are similar, the compound comprises similar characteristics and *vice-versa*. Based on FTIR (Fig. 1), similarities were observed between synthesized and standard spectral FAE. In the region of 3450-3012 cm^{-1}, absorption appears, indicating O–H vibrations with a coincident N–H cluster. In the absorption region of 1690-1650 cm^{-1}, amide ester group appears with the successful synthesis of FAE from the ketapang seed oil. The wavenumber shifts towards the 1745-1710 cm^{-1} range is due to the presence of the ester group in the oil. Moreover, successful synthesis is supported by the appearance of highest absorption peak near 1296 cm^{-1}, which corresponds to the C–N vibration. Similar results were also obtained by Adewuyi [2] while synthesizing diethanolamide from the seed oil of *Baphia nitida*.

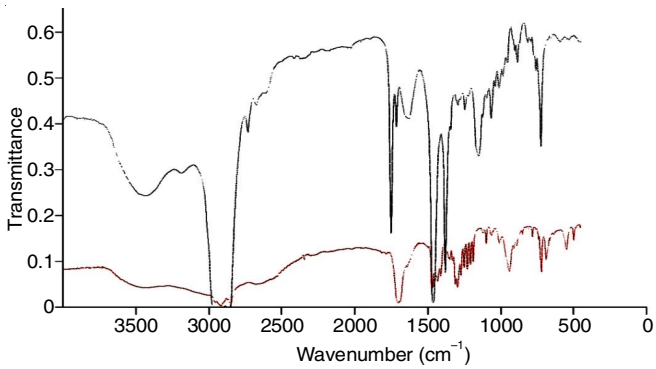

Fig. 1. FTIR spectrum of FAE standard and product

Separation of FAE components using HPLC: HPLC selection for separating alkanolamide fatty acids synthesized using ethanolamine and the ketapang seed oil is based on high

efficiency and selectivity than can be used to identify and determine the small quantities of ingredients quantitatively. Various advantages related with separation, including low cost, rapid analysis, ability to change the composition of utilized motion phases [13] and possibility of analyzing unstable samples are present. Additionally, under three conditions, its optimal parameters are determined: (i) eluent composition (acetonitrile: water), (ii) optimum flow rate and (iii) concentration of the sample. This optimization is conducted to acquire the optimal method for separating alkanolamide fatty acids.

The HPLC system comprises UV detection system at 213 nm wavelength and a C18 inverted phase column. Additionally, this wavelength was selected because amide compounds have the highest absorbance at 213 nm. The effluent employed has a wavelength of < 213 nm such as water (λ_{max} 167 nm) and acetonitrile (λ_{max} 190 nm); consequently, the effluent was not detected during separation.

Determination of optimum flow rate: The optimal flow rate was determined using the Sunfire C-18 ODS 2 column with an eluent acetonitrile-water composition and injection volume of 95:5 (v/v) and 20 µL, respectively. The flow rate variation was 0.5, 1.0 and 1.5 mL/min. Moreover, FAEs (0.05 in 10 mL of acetonitrile) were separated in the vial tube with 20 µL injected into an instrument by using a 0.45 µm micro filter. The samples flowed in columns for 60 min. Chromatograms and four main components were observed (Fig. 2). This was indicated by the presence of four highest points that were separated at various retention times after sample injection to obtain the highest point of analyte by using the detector. In the qualitative test, the retention time indicated the existence of a compound in the mixture, which exhibited the time values of each fatty acid ethanolamide (FAE) [14] (Table-1).

TABLE-1
RETENTION TIME OF FAE AT VARIATION OF FLOW RATES

Flow rate (mL/min)	Retention time of FAEs (min)			
	a	b	c	d
0.5	5.828	7.632	10.176	11.768
1.0	3.614	4.732	5.556	33.042
1.5	2.747	3.441	4.108	18.174

The retention time value at the flow rate of 1.5 mL/min is smaller than that of 1.0 and 0.5 mL/min (Table-1), leading to faster retention time and analyte influenced by column length (L) and flow rate (µ) [15]. When the column is long or the flow rate is slow, t_R increases; thus, to determine the optimum flow rate, consideration of separation power (resolution) is necessary. In the sequential method, the separation degree between these two results is quantitatively defined using resolution (R_S), the ratio of the distance of the results at average width and maximum height. Table-2 presents the resolution values of each flow rate. The optimum resolution is observed at highest FAEs with a flow rate of 1 mL/min according to the highest resolution value. According to Dolan [16], the recommended resolution value is > 1.5 with a flow rate of 1 mL/min.

TABLE-2
PEAK RESOLUTION AT VARIOUS FLOW RATES

Flow rate (mL/min)	Peak of FAE	Resolution
0.5	a and b (Rs_1)	1.1180
	b and c (Rs_2)	0.6592
	c and d (Rs_3)	0.7960
1.0	a and b (Rs_1)	1.7950
	b and c (Rs_2)	1.7020
	c and d (Rs_3)	12.2160
1.5	a and b (Rs_1)	0.9250
	b and c (Rs_2)	0.8893
	c and d (Rs_3)	9.7373

Determination of optimum mobile phases: The mobile phase plays a crucial role in analyte separation because mobile phase migration is regulated through the interaction between mobile and stationary phases. Analytic migration is caused by a competition of the mobile phase, a mixture of two or more solvents having different strengths, with the analyte phase. This study employed the mobile phase and inverse columns of acetonitrile-water and C-18 ODS 2, respectively. Fatty acid ethanolamide (FAE) comprise polar amine and hydroxyl groups and consequently the acetonitrile-water solvent composition is highly appropriate for separation [17]. The composition of acetonitrile-water eluent varies with a ratio of 90:10 (v/v); 95:5 (v/v) and 85:15 (v/v). Table-3 presents the FAE retention time at various mobile/eluent phases, while Table-4 shows the peak resolution at different mobile phases/eluents.

In the varying composition of the acetonitrile eluent water of 90:10 v/v, 85:15 v/v, and 95:5 v/v, FAEs present four well-separated results (Fig. 3). In acetonitrile:water 85:15 v/v composition, the retention time of FAEs is smaller than that in aceto-

TABLE-3
RETENTION TIME OF FAE AT VARIOUS OF PHASA MOBILE/ELUENT

Eluent acetonitrile: water (v/v)	Retention time of FAE (min)			
	a	b	c	d
85:15	1.622	4.964	7.414	18.941
90:10	7.373	9.810	12.024	42.335
95:5	3.614	4.732	5.556	33.042

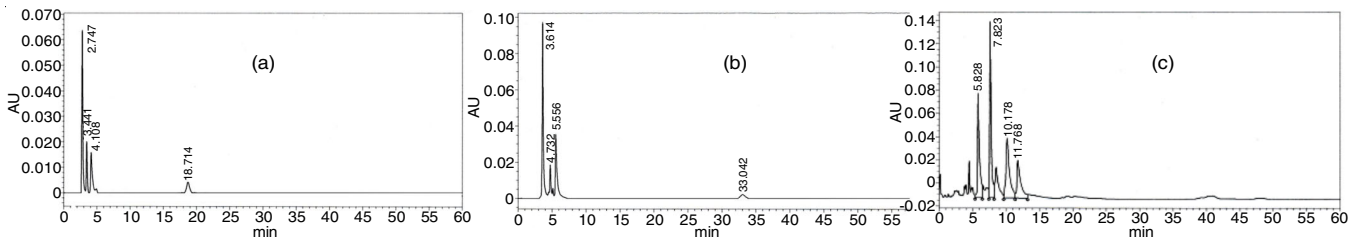

Fig. 2. Chromatogram of fatty acid ethanolamide (FAE) at various flow rate (a) 1.5 mL/min; (b) 1 mL/min; (c) 0.5 mL/min), eluent acetonitrile:water (95:5)

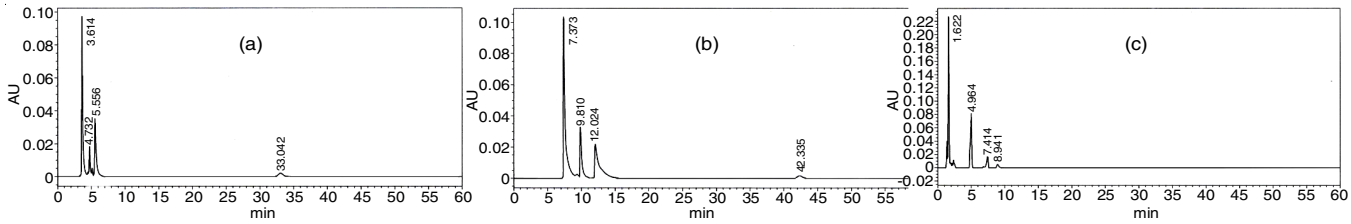

Fig. 3. Chromatograms of fatty acid ethanolamide (FAE) at various mobile phase/eluen, flow rate: 1.0 mL/min (a) acetonitrile:water (95:5), (b) acetonitrile:water (90:10), (c) acetonitrile:water (85:15)

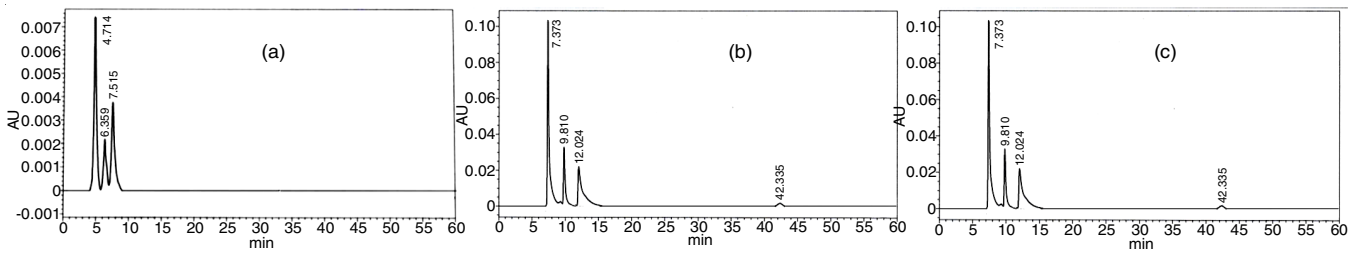

Fig. 4. Chromatograms of fatty acid ethanolamide (FAE) at various concentration (a) 1000 ppm, (b) 3000 ppm (c) 5000 ppm

TABLE-4
PEAK RESOLUTION AT VARIOUS MOBILE PHASE/ELUENT

Flow rate (mL/min)	Peak of FAE	Resolution
85:5	a and b (Rs_1)	4.4560
	b and c (Rs_2)	2.4500
	c and d (Rs_3)	1.2216
90:10	a and b (Rs_1)	3.2493
	b and c (Rs_2)	2.9520
	c and d (Rs_3)	15.1555
95:5	a and b (Rs_1)	1.1180
	b and c (Rs_2)	0.6592
	c and d (Rs_3)	12.216

nitrile:water 90:10 v/v and 95:15 v/v composition because of the polarity nature in all eluent compositions. The retention time of analytes decreases with an increase in the polar solvent content and water is more polar than acetonitrile.

Determination of optimum concentrations: In this method, the effect of concentration or injection volume is important and has to be considered for investigating peak area of each compound and the 'overloading' effect. The optimum sample concentration was determined by injecting various concentrations of 1000, 3000 and 5000 ppm with 213 nm wavelength, an eluent composition of acetonitrile-water of 90:10 (v/v) and a flow rate of 1 mL/min. The samples were subjected to flow in the column for 60 min at 1000 ppm concentration. The peak area of FAE at 100 ppm is smaller than that at 3000 and 5000 ppm because the concentrations of all fatty acids in the samples were directly proportional to chromatogram areas. The area is determined using the detector sensitivity factor and data processing equipment accuracy.

At 1000 ppm concentration, the fourth compound was not detected (Fig. 4a). At 3000 and 5000 ppm concentrations, all four compounds were accurately detected (Fig. 4b-c). When the separation between peaks was considered, the chromatogram at the 5000 ppm concentration has no over loading effect and a better resolution.

Separation of FAE using optimum HPLC conditions: The alkanolamide samples were synthesized using the Ketapang seed oil, ethanolamine and triglycerides. It was further separated through HPLC under obtained optimum conditions (Table-5). Alkanolamide fatty acids synthesized from ethanolamine and triglycerides in the ketapang seed oil comprise four unknown compounds (Fig. 5). To determine these compounds, the highest point of chromatogram was identified through the comparison of the retention time (R_t) value of compounds with the standard retention time.

TABLE-5
OPTIMUM CONDITION OF HPLC

Stationery phase	SGE C-18 ODS 2 (250 mm, ID 4 mm, Frit 4/m μm)
Mobile phase	Acetonitrile:water (90:10 v/v)
Flow rate	1.0 mL/min
Volume injection	20 mL
Detector	UV 213 nm

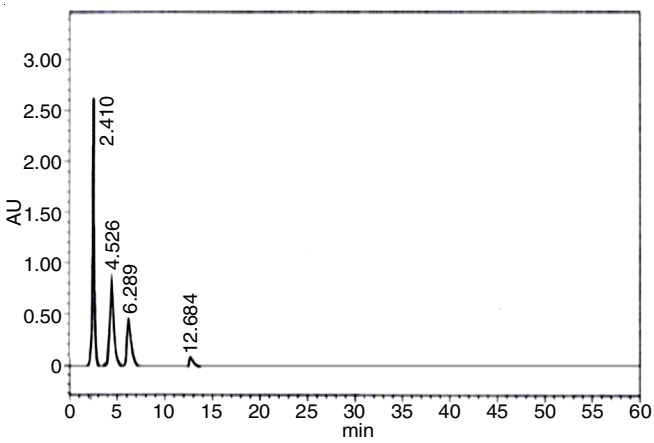

Fig. 5. Chromatogram of FAEs at optimum condition

Initially, linoleoylethanolamide compounds were eluted and then oleoylethanolamide was eluted because the polarities of hydrocarbons having a double bond are higher than those having single bonds. Because of its double bonds, the reactivity of alkenes is higher than that of alkanes and mainly, alkene

reactions occur at double bonds, affecting the polarity level. The polarity order is as follows: alkane < alkene < alkyne. Linoleoylethanolamides are the amide compounds having hydrocarbons with double bonds and oleylethanolamide has one. The stationary phase of non-polar octadecylsilan (C18 or ODS) binds considerably strongly to fatty acids, including palmitoylethanolamide and stearyl diethanolamide. Similarly, a mixture of acetoneitrile-water, such as oleylethanolamide and linoleoylethanolamide, is sufficiently polar in the mobile phase.

Conclusion

Fatty acid ethanolamides (FAEs) were successfully separated using HPLC under the optimum conditions of 90:10 motion phase composition, 1.5-mL flow rate and 5000 ppm concentration. According to the retention time, the successfully separated compounds include oleoylethanolamide, palmitoylethanolamide, linoleoylethanolamide and stearyl diethanolamide.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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