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An Improved Method for Synthesis of *N*-stearoyl and *N*-palmitoylethanolamine

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Abstract Certain *N*-acylethanolamines interact with cannabinoid receptors and have anorexic and neuroprotective effects. Traditional methods for the synthesis of *N*-acylethanolamines use fatty acid chlorides, fatty acid methyl esters, free fatty acids and triacylglycerols as acyl donors to react with ethanolamine. In the present study, we investigated the feasibility of using fatty acid vinyl esters as the acyl donor to synthesize *N*-stearoyl and *N*-palmitoylethanolamine. Theoretically, the use of fatty acid vinyl esters should lead to an irreversible reaction because the volatile acetaldehyde by-product is easily removed. Four reaction conditions, i.e. catalyst concentration, substrate ratio, temperature, and time were evaluated. The reaction performed at mild temperatures and with an excess amount of ethanolamine which acted as both reactant and solvent resulted in the formation of high purity *N*-stearoyl and *N*-palmitoylethanolamine. When 20 mmol ethanolamine was reacted with 1 mmol vinyl stearate at 80 °C for 1 h with 1% sodium methoxide as catalyst, *N*-stearoylethanolamine with 96% purity was obtained after the removal of excess ethanolamine without further purification, while *N*-palmitoylethanolamine with 98% purity was obtained by reacting with the same substrate ratio at 60 °C for 1.5 h with 3% catalyst. Complete conversion of vinyl stearate and palmitate was achieved.

Keywords Acyl donor · Amidation · Fatty acid vinyl esters · *N*-Acylethanolamines · Optimization · Synthesis

Introduction

N-Acylethanolamines from fatty acids are an important class of alkanolamides that function as nonionic surfactants and have a wide range of applications in the lubricants, surfactants and detergents, cosmetics, and other industries [1–3]. Certain *N*-acylethanolamines are lipid mediators in animals and plants [4–6]. It has been reported that *N*-acylethanolamines of different chain length and structure exhibit a variety of biological activities. *N*-Palmitoylethanolamine has anti-inflammatory activity, and attenuates pain sensation [5, 7]. They have also been shown to reduce allergic reaction, inhibit mast cell degranulation [8] and exert neuroprotective effects in rats and mice [9]. These actions were accompanied by changes in nitric oxide production [10] and the expression of pro-inflammatory proteins [11]. *N*-Oleoylethanolamine exerts anorexigenic effects by binding to the nuclear receptor in the periphery tissues, leading to body fat loss [12, 13]. *N*-Stearoylethanolamine shows pro-apoptotic and anorexic effects [14]. They could affect cell signaling and elicit biological effects potentially through targets other than cannabinoid receptors, such as exerting anorexic effects in mice via down-regulation of a liver enzyme expression [6], and having anti-inflammatory activity by a passive IgE-induced cutaneous anaphylaxis [15]. Therefore, these are an interesting class of compounds for animal and human applications.

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N-Acylethanolamines from fatty acids theoretically can be formed from a fatty acyl donor and ethanolamine. Four basic methods have been reported for synthesizing *N*-acylethanolamines according to the differences in the acyl donor. In general, they are synthesized by reacting fatty acid chlorides [16, 17], fatty acid methyl esters [18, 19] and triacylglycerols [20] with alkanolamine, or by a direct reaction between free fatty acids and alkanolamine in the presence of catalysts at low temperatures or without any catalyst at high temperatures [1]. Yield and purity improvement was usually achieved by removal of water, methanol, glycerol or hydrochloric acid. However, only 60–90% *N*-acylethanolamines were produced from free fatty acids, fatty acid methyl esters and triacylglycerols at temperatures above 100 °C, usually at 180 °C for 6–12 h [21]. It has been reported that about 99% *N*-acylethanolamine was obtained if two moles of fatty acid and one mole of ethanolamine were first reacted at 180 °C to give the *N*,*O*-bis-acylethanolamine, which was then transesterified with another mole of ethanolamine to form the *N*-acylethanolamine [19]. Nevertheless, the reaction conducted at high temperature resulted in the formation of products with dark color which influences the product's quality. The addition of deodorizers and antioxidants has been suggested to improve the quality of the final products [21]. The main problems for synthesis of *N*-acylethanolamines are the low conversion of reactants and the esterification reaction occurring in the reaction, which result in low yield of *N*-acylethanolamines.

Purity of commercial alkanolamides for surfactant purposes was about 80% [22], but with the increasing knowledge and interest in *N*-acylethanolamines as lipid mediators in animals, plants, or humans, high purity *N*-acylethanolamines are needed to validate their biological functions in cellular and animal systems. Currently, this type of study is limited to small animal experiments due to the lack of access to these compounds in a desired purity and quantity. For example, the prices of *N*-stearoyl and *N*-palmitoylethanolamines from the Sigma Chemical Company are $82/5 mg and $60.8/10 mg. Thus, it is necessary to establish a simple, effective and economical synthesis method to support studies examining the biological and nutritional properties of *N*-acylethanolamines in large animal and human subjects.

In the present study, we investigated an alternative method for the preparation of high purity *N*-stearoyl and *N*-palmitoylethanolamines. Vinyl stearate was used as the acyl donor to study the reaction conditions and the effects of catalyst type, solvent and temperature on the purity of *N*-stearoylethanolamine. Finally, a solvent-free system and sodium methoxide catalyst were chosen to synthesize *N*-stearoyl and *N*-palmitoylethanolamines and a large excess ethanolamine was used as both the reactant and solvent for the synthesis.

Materials and Methods

Materials

Most chemicals including *N*-acylethanolamines standards were purchased from the Sigma Aldrich Chemical Company (St. Louis, MO, USA). Vinyl stearate and palmitate were purchased from Tokyo Chemical Industry (Tokyo, Japan). Ethanolamine (>99%) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). *Candida antarctica* lipase (Novozym 435) was provided by Novozymes America (Blair, NE, USA). This is an immobilized lipase and has a declared activity of 10,000 PLU (propyl laurate unit)/g.

Synthesis of *N*-Stearoylethanolamine Using Vinyl Stearate Based on Procedures Already Reported for Other Acyl Donors

Amidation of Ethanolamine with Vinyl Stearate at 105 °C in a Solvent-Free System

The procedure of Farris [18] was followed in which a fatty acid methyl ester was used as the acyl donor. Ethanolamine (1.1 mmol) was placed in a 10-mL round bottom flask and then vinyl stearate (1 mmol) was added followed by 0.5% sodium methoxide (w/w, relative to total reactants). The mixture was agitated at 105 °C. Samples were withdrawn after 1 and 12 h of reaction.

Amidation of Ethanolamine with Vinyl Stearate at 45 °C in Hexane

This experiment was carried out based on the procedure of using a free fatty acid as acyl donor [23]. Ethanolamine (1 mmol) in hexane (5 mL) was mixed with vinyl stearate (1 mmol) in a 10-mL round bottom flask in the presence of Novozym 435 lipase at 10, 20, 30, 40 and 65% (relative to total reactants) level. The reaction was conducted with agitation at 45 °C for 5 and 20 h before hexane was removed under reduced pressure. The enzyme was filtered out so it could be reused if needed. The experiment was also conducted under similar conditions except that sodium methoxide was used at 1, 2 and 3% levels, and the reaction was at ambient temperature (25 °C) for 14 h.

Amidation of Ethanolamine with Vinyl Stearate at 50 °C in a Solvent-Free System

The procedure of Kolancilar [24], in which laurel oil was used as the acyl donor, was followed. Vinyl stearate (1 mmol), ethanolamine (10 mmol), and sodium methoxide (2%, relative to total reactants) were placed in a 10-mL round bottom flask and the mixture was agitated at 50 °C

for 2 and 3 h. The reaction mixture was then mixed with 5 mL distilled water at 6 °C for 1 h and the process was repeated three times to remove excess ethanolamine.

After all the reaction conditions as described above were examined, a set of conditions was identified to optimize the synthesis of *N*-stearoyl and *N*-palmitoylethanolamines.

Optimization for Amidation Reaction at Mild Temperature in a Solvent-Free System

The design for the optimization experiments is outlined in Tables 1, 2 and Fig. 1. The yields of *N*-stearoyl and *N*-palmitoylethanolamines were studied as a function of one of the four following variables while the other three variables were held constant: sodium methoxide concentration, molar ratio of ethanolamine to fatty acid vinyl ester, reaction temperature and time. For catalyst concentration optimization, ethanolamine (20 mmol) and vinyl stearate or palmitate (1 mmol) were mixed at 60 °C for 1 h with different concentrations of sodium methoxide (0.5–4% for the synthesis of *N*-stearoylethanolamine, 1–4% for *N*-palmitoylethanolamine). For optimizing the reactant ratio, different quantities of ethanolamine (5–25 mmol) were reacted with 1 mmol fatty acid vinyl ester at 60 °C for 1 h. Sodium methoxide at 1% was used to optimize the molar ratio of ethanolamine to vinyl stearate, while 3% sodium methoxide was used to optimize the molar ratio of ethanolamine to vinyl palmitate since these catalyst concentrations were confirmed to be the optimum values for the two reactions. After 20:1 molar ratio was confirmed as the optimal ratio for the synthesis, ethanolamine (20 mmol) and vinyl stearate or palmitate (1 mmol) were mixed at different temperatures (40–80 °C) for 1 h with 1 or 3% sodium methoxide to optimize the reaction temperature. Finally, ethanolamine (20 mmol) and vinyl stearate

Table 1 Experimental design for optimization of amidation between vinyl stearate and ethanolamine

Level	X_1 (%, relative to total substrates)	X_2 (molar ratio)	X_3 (°C)	X_4 (h)
1	0.5	5:1	40	0.5
2	1.0	10:1	50	1.0
3	2.0	15:1	60	2.0
4	3.0	20:1	70	3.0
5	4.0	25:1	80	4.0

X_1, sodium methoxide, reactions conducted by reacting 20 mmol ethanolamine with 1 mmol vinyl stearate at 60 °C for 1 h; X_2, molar ratio of ethanolamine to vinyl stearate, reactions conducted at 60 °C for 1 h with 1% sodium methoxide; X_3, temperature, reactions conducted by reacting 20 mmol ethanolamine with 1 mmol vinyl stearate for 1 h with 1% sodium methoxide; X_4, time, reactions conducted by reacting with 20 mmol ethanolamine with 1 mmol vinyl stearate at 80 °C with 1% sodium methoxide

Table 2 Experimental design for optimization of amidation between vinyl palmitate and ethanolamine

Level	X_1 (% by substrate)	X_2 (molar ratio)	X_3 (°C)	X_4 (h)
1	1.0	5:1	40	0.5
2	2.0	10:1	50	1.0
3	2.5	15:1	60	1.5
4	3.0	20:1	70	2.0
5	3.5	25:1	80	2.5
6	4.0			3.0

X_1, sodium methoxide, reactions conducted by reacting 20 mmol ethanolamine with 1 mmol vinyl palmitate at 60 °C for 1 h; X_2, molar ratio of ethanolamine to vinyl palmitate, reactions conducted at 60 °C for 1 h with 3% sodium methoxide; X_3, temperature, reactions conducted by reacting 20 mmol ethanolamine with 1 mmol vinyl stearate for 1 h with 3% sodium methoxide; X_4, time, reactions conducted by reacting with 20 mmol ethanolamine with 1 mmol vinyl stearate at 60 °C with 1% sodium methoxide

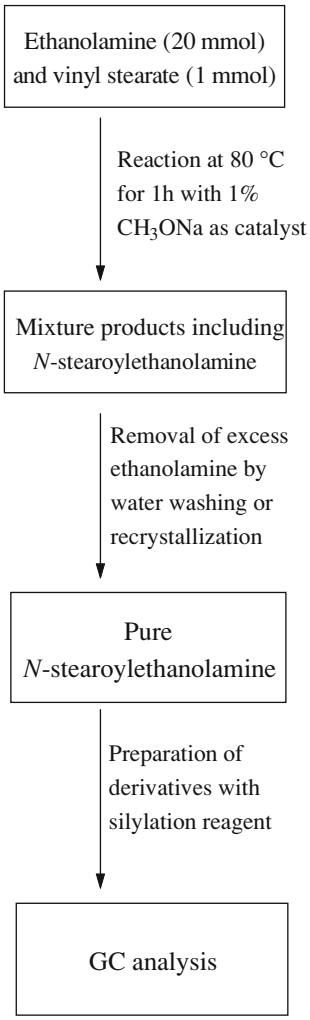

Fig. 1 A general flow chart for the synthesis of *N*-stearoylethanolamine

(1 mmol) were mixed at 80 °C with 1% sodium methoxide to optimize the reaction time since 80 °C was chosen as the optimum. The same ratio of reactants was mixed at 60 °C with 3% sodium methoxide to optimize the reaction time for the *N*-palmitoylethanolamine synthesis. For all the products, excess ethanolamine was removed from the product by addition of distilled water (5 mL) and crystallization of the amide at 6 °C for 1 h. The *N*-acylethanolamines were washed three more times with water. All optimization experiments were conducted in duplicate.

Procedure for Preparing of *N*-Stearoyl and *N*-Palmitoylethanolamines Derivatives for GC Analysis

The anhydrous reaction mixture containing amide (about 5 mg) was placed in a 2-mL glass vial and treated with pyridine (0.5 mL), hexamethyldisilazane (0.15 mL) and trimethylchlorosilane (0.05 mL). The mixture was shaken for 15–30 s and then allowed to stand for 1 h or stored in a freezer (0 °C) overnight during which time the upper layer became clear [25]. *N*-Stearoyl and *N*-palmitoylethanolamines standards were used as external standards and the peaks were identified according to the GC retention time. The purity of the *N*-acylethanolamines was calculated based on their peak areas relative to the total peak area of a particular sample.

Samples were identified and quantified by an HP 5890 Series II capillary GC (Hewlett-Packard, PA) equipped with a flame ionization detector (FID) using a 30 m × 0.25 mm × 0.25 μm (length × ID × film thickness) fused silica bonded phase capillary column SP-1 (Supelco, Bellefonte, PA). The carrier gas (helium) flow rate was 32.3 mL/min, and the split ratio was seven. The oven temperature was programmed from 140 to 300 °C at a rate of 10 °C/min, and then held at 300 °C for 5 min. Injector and detector temperatures were set at 300 °C.

TLC Chromatography of *N*-Stearoyl and *N*-Palmitoylethanolamine Products

A developing solvent of A (toluene:ethyl acetate:ether:acetic acid = 80:10:10:0.2, v/v) and B (100% methanol) in 80:15 ratio (v/v) were used to separate the products on a 20 × 20 cm silica plate (250-μm thickness), with the standards applied in separate lanes to further validate the purity of the water-washed final lipid products.

NMR Analysis for Structure Confirmation

^{1}H-NMR qualitative analysis of *N*-stearoyl and *N*-palmitoylethanolamine products was done by using a Varian MR-400 Spectrometer (Foster City, CA, USA) with $CDCl_3$ as solvent and TMS as the internal standard (chemical shift of 0 ppm).

Results and Discussion

Selection of Reaction Parameters According to the Reported Conditions

The reaction conditions which were used for the synthesis of *N*-acylethanolamines in previous studies that used other acyl donors were tested in this study, and the purity of *N*-acylethanolamines in the reaction mixture was chosen as a parameter to study the reaction conditions. Since *N*-stearoylethanolamine has a high melting point (about 95 °C), amidation reaction between vinyl stearate and ethanolamine should be conducted at >95 °C in a solvent-free system or at lower temperature in the presence of solvent and catalyst, such as a lipase or sodium methoxide.

When the amidation reaction was carried out at 105 °C in the solvent-free system, 67.9 and 73.0% *N*-stearoylethanolamine was obtained at 1 and 12 h, respectively. The reaction after 12 h did not result in a dramatic increase in *N*-stearoylethanolamine content compared to that at 1 h. The reaction at 105 °C resulted in the formation of products with a dark color, which influences product quality. Therefore, the reaction in a solvent system at a lower temperature was then tested.

Results for the synthesis of *N*-stearoylethanolamine in the solvent system are presented in Table 3. Although the previous researchers used these reaction conditions to synthesize *N*-acylethanolamine, the desired product was not formed in this study under low concentrations of lipase when vinyl stearate was used as the acyl donor. Instead, a

Table 3 Synthesis of *N*-stearoylethanolamine in a solvent system with lipase as catalyst

	Time of reaction (h)									
	5					20				
Lipase (% relative to total substrate)	10	20	30	40	65	10	20	30	40	65
Vinyl stearate (%)	85.4	0	0	0	0	73.6	0	0	0	0
O-stearoylethanolamine (%)	7.2	100	100	100	17.4	5.3	100	100	100	26.5
N-stearoylethanolamine (%)	0	0	0	0	72.3	10.9	0	0	0	61.6

side reaction product, *O*-stearoylethanolamine was produced in high concentration. When the enzyme addition was increased to 65%, there was 72.3% *N*-stearoylethanolamine produced after 5 h. Further addition of enzyme is not feasible for the experiment. When the enzyme was substituted with sodium methoxide, no *N*-stearoylethanolamine was produced after 14 h with the catalyst concentration at 1, 2 and 3% (w/w) level (based on total reactants). Therefore, a reaction using excess ethanolamine as solvent to dissolve *N*-stearoylethanolamine was tested in the next experiment.

When excess ethanolamine (10×) was used to act as both reactant and the solvent to dissolve the *N*-stearoylethanolamine product, 92.9% *N*-stearoylethanolamine was produced with 2% sodium methoxide at 50 °C after 2 h. This experiment proved that the *N*-stearoylethanolamine can be synthesized by using excess ethanolamine as a solvent rather than using hexane probably because hexane diluted the catalyst and the reactants.

From all above preliminary experiments using the unique vinyl stearate as the acyl donor and various conditions as reported in the literature, we identified the reaction system and variables to examine in our single factor optimization experiments.

Optimization of Reaction Conditions for the Synthesis of *N*-Stearoyl and *N*-Palmitoylethanolamine with Sodium Methoxide Catalyst

The effects of sodium methoxide concentration, molar ratio of ethanolamine to fatty acid vinyl ester, temperature, and time on the amidation reaction were investigated. The results are shown in Figs. 2, 3, 4, and 5. Data are expressed as means ± SD.

For sodium methoxide concentration optimization (Fig. 2), the content of *N*-stearoylethanolamine formed with 1% catalyst was high but it tended to decrease slightly when the sodium methoxide was increased from 2–4%. An explanation for the changes is that the increase in the catalyst concentration may result in the formation of *O*-stearoylethanolamine produced by the esterification reaction (Fig. 6). Spontaneous acyl migration of *O*-stearoylethanolamine will happen, and thus the reaction typically proceeds towards amidation [26]. In contrast, 3% sodium methoxide was optimal for the synthesis of *N*-palmitoylethanolamine. In general, the amidation reaction is dominant.

Responses of *N*-stearoyl and *N*-palmitoylethanolamine to the molar ratio of ethanolamine to vinyl ester were similar. The content of *N*-acylethanolamines increased gradually with the increase in molar ratio from 5:1 to 20:1 (Fig. 3). Reactions with 5:1 and 10:1 ratio were incomplete because it was difficult to completely dissolve the final

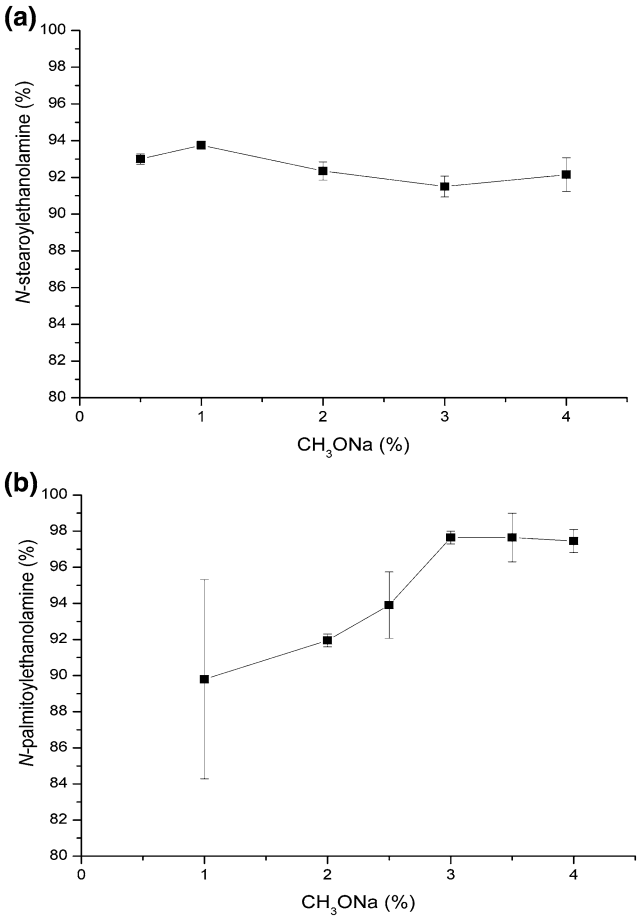

Fig. 2 Effect of sodium methoxide concentration on the content of *N*-stearoyl and *N*-palmitoylethanolamine in the final amidation product

products at 60 °C. The reason for the slight reduction in the content of *N*-acylethanolamine at a molar ratio above 20:1 is unknown. The 20:1 molar ratio was selected as optimal reaction condition for further reaction optimization.

For selecting the best temperature, the reaction temperature was varied from 40 to 80 °C. Results from Fig. 4a show that the increase in temperature was beneficial to the reaction. This is probably because of the increase in the rate of both amidation reaction and acyl migration, which resulted in more ester being converted to amide. The reaction at 80 °C resulted in the formation of 95.4% *N*-stearoylethanolamine. The color of the product became dark if the temperature was increased further. Therefore, 80 °C was selected as the optimal temperature for the synthesis of *N*-stearoylethanolamine. However, the response of *N*-palmitoylethanolamine to temperature was different from that of the *N*-stearoylethanolamine, in that *N*-palmitoylethanolamine yield did not change as sharply with the changes in temperature. The content of *N*-palmitoylethanolamine seemed to have reached a maximum at 60 °C (Fig. 4b). The different optimal temperatures for

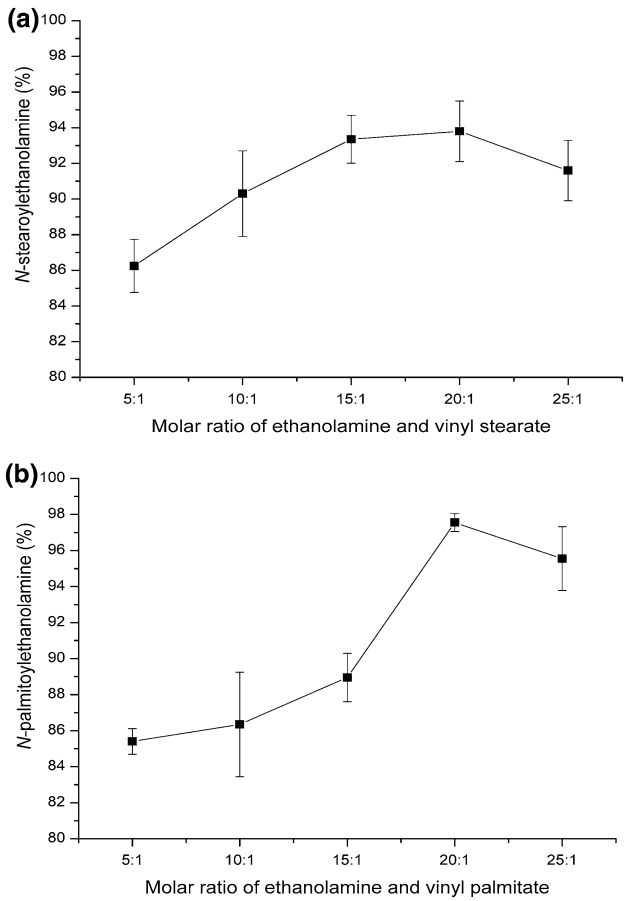

Fig. 3 Effect of molar ratio of ethanolamine to vinyl ester on the content of *N*-stearoyl and *N*-palmitoylethanolamine in the final amidation product

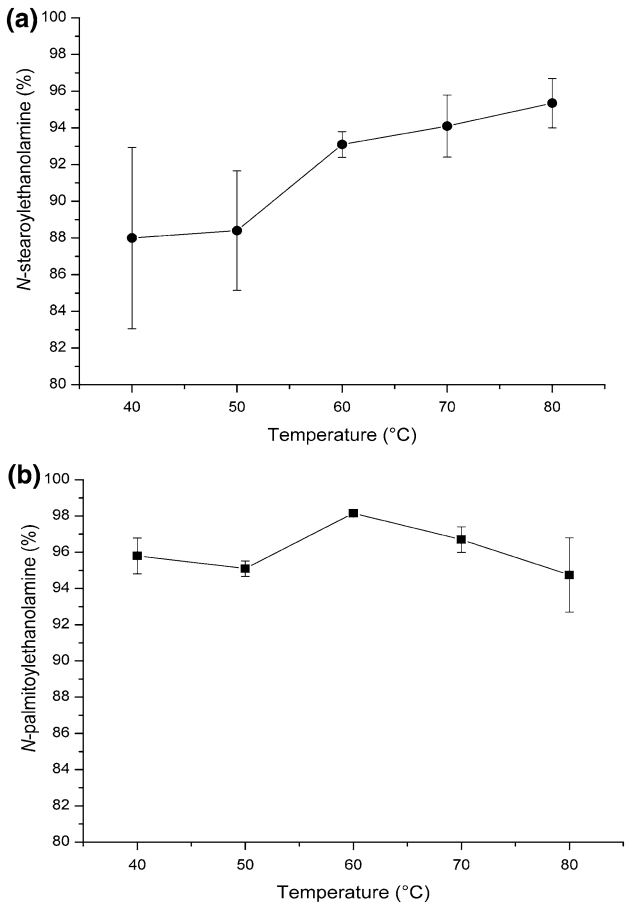

Fig. 4 Effect of reaction temperature on the content of *N*-stearoyl and *N*-palmitoylethanolamine in the final amidation product

these two *N*-acylethanolamines may be due to the difference in the sodium methoxide concentration used.

The content of *N*-stearoylethanolamine after 1 h of the reaction was significantly higher than other time points (Fig. 5a). This may be due to the side reactions if the reaction time was prolonged. In contrast, the reaction time did not seem to be a significant factor for the synthesis of *N*-palmitoylethanolamine. Therefore, 1 and 1.5 h were considered as the optimal times for the synthesis of *N*-stearoyl and *N*-palmitoylethanolamine.

After all the factors were examined, 1% sodium methoxide, 20:1 molar ratio of ethanolamine to vinyl stearate, 80 °C and 1 h were chosen for the synthesis of *N*-stearoylethanolamine, compared to 3%, 20:1, 60 °C and 1.5 h, respectively, for the synthesis of *N*-palmitoylethanolamine. The purity of *N*-stearoyl and *N*-palmitoylethanolamine was 95.7 ± 0.6 and 98.0 ± 0.5%, respectively, under these optimal conditions.

For a scaled-up synthesis, ethanolamine (600 mmol) was reacted with vinyl stearate or palmitate (30 mmol) with agitation under the optimal reaction conditions identified above. There was 95.3% *N*-stearoylethanolamine and 96.5% *N*-palmitoylethanolamine in the final product after washing with water.

Thin layer chromatography purity confirmation of the *N*-acylethanolamine was done to show that there were no other lipids that may not have been eluted from the GC column. The Rf values of *N*-stearoyl and *N*-palmitoylethanolamine were about 0.49–0.48, and the Rf values of the corresponding standards were all about 0.48. There was no other major lipid impurity except some very faint bands of residual fatty acyl vinyl esters or *O*-acylethanolamine.

Structural confirmation of *N*-acylethanolamine was done by using ^{1}H NMR. Nuclear magnetic resonance analysis gave the expected amide peak (δ 5.9, 1H, –CH_2CH_2 CON**H**–) and we did not see any NMR peak of –COO $\mathbf{CH_2}CH_2NH_2$ (δ 4.2–4.4, 2H) and –$COOCH_2CH_2N\mathbf{H_2}$ (δ 1.1–1.5, 2H).

Traditionally, synthesis of *N*-acylethanolamines has been performed with free fatty acids, fatty acid methyl esters, fatty acid chlorides and triacylglycerols. To the best of our knowledge, this was the first time a fatty acid vinyl

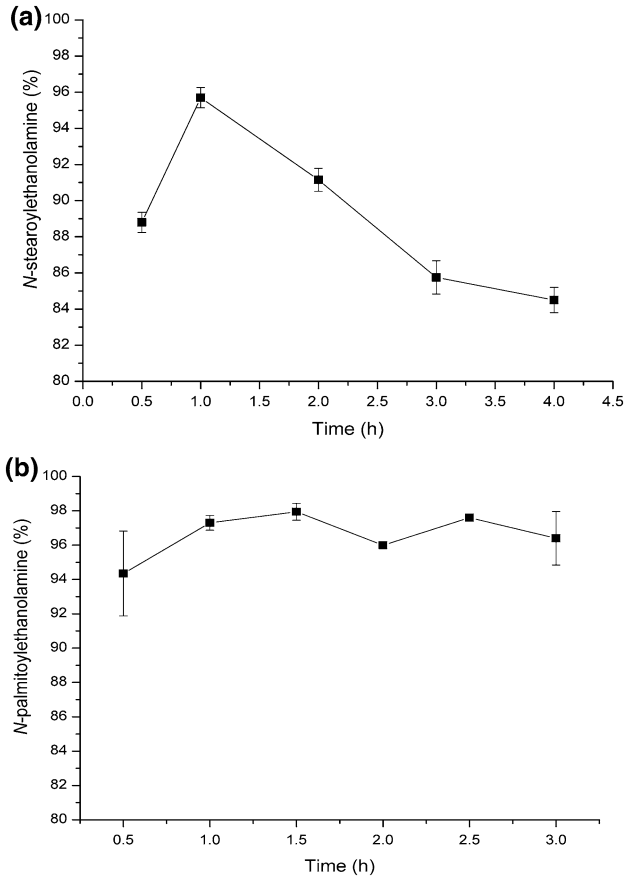

Fig. 5 Effect of reaction time on the content of *N*-stearoyl and *N*-palmitoylethanolamine in the final amidation product

ester has been used as the acyl donor to synthesize *N*-acylethanolamines. The possible reaction routes for acylation of ethanolamine with vinyl stearate are presented in Fig. 6. Three reactions might have occurred in the system. The amidation reaction was dominant and resulted in the formation of *N*-acylethanolamines and ethenol which tautomerized to a non-nucleophilic acetaldehyde immediately [27, 28]. Acetaldehyde (boiling point of 20.8 °C) could be evaporated at ambient temperature. The main side reaction product was *O*-acylethanolamine, resulting from an esterification reaction between ethanolamine and the fatty acid vinyl ester. However, spontaneous acyl migration would happen if a large amount of *O*-acylethanolamine were to be produced [21]. Since the acyl migration step was too fast to be monitored, the reaction appeared to proceed via direct amidation when the conditions were suitable [21, 26]. In our system, the formation of *N*,*O*-bis-acylethanolamine was not favored due to the large excess of ethanolamine and it was not seen. Therefore, the impurities in the final product might have included ethanolamine (if not completely washed away by water), fatty acid vinyl ester and *O*-acylethanolamine. Under the optimal reaction conditions, almost all the fatty acid vinyl ester was converted as verified by GC, while ethanolamine was removed by washing with water. Thus, the main impurity was *O*-acylethanolamine, as shown in the GC chromatogram (Fig. 7b). During the process of product purification, the non-polar acyl vinyl esters, *N*-acylethanolamines and *O*-acylethanolamine could not be removed by washing and thus no loss of these compounds could occur. Therefore, the purity or percentage of *N*-acylethanolamines in the final lipid extract as determined by GC could be used as an indicator to monitor the amidation reaction, as we have done in this report.

For GC product characterization, the peak at 11.1–11.2 min was attributed to the silylation reagent based on retention times determined by control injections. In addition, the area of *N*-acylethanolamines increased proportionally to the amount of sample used, whereas the area of the peak at 11.1–11.2 min remained almost constant even after the sample size was increased from 3 to 12 mg. This observation was also reported by another researcher [29]. Peaks that had common retention times as the silylating reagent peak were not included for the purity calculation.

As reported in the literature, products with a *N*-acylethanolamine content ranging from 60 to 90% were obtained when free fatty acids [1, 23], fatty acid methyl

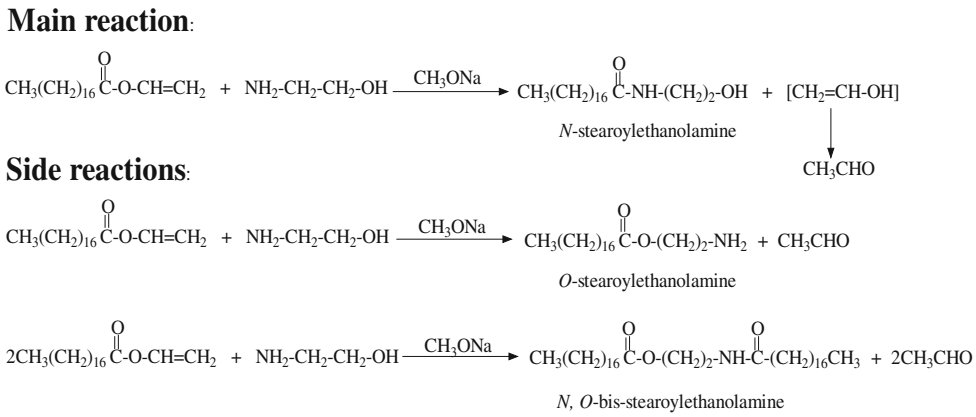

Fig. 6 Possible reactions between ethanolamine and vinyl stearate

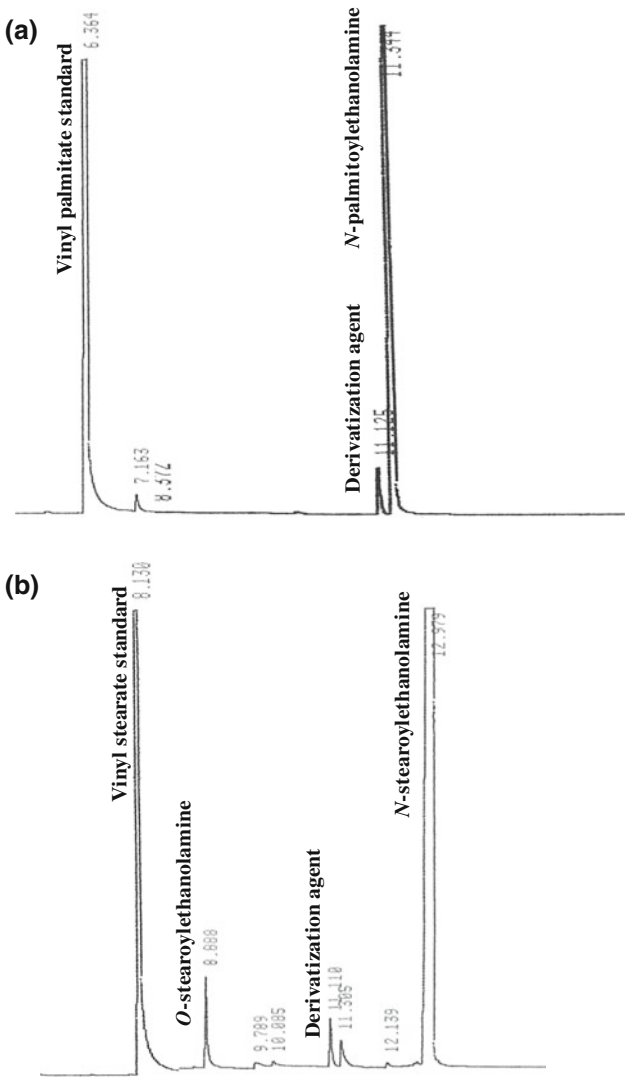

Fig. 7 GC chromatograms of *N*-palmitoyl (**a**) and *N*-stearoylethanolamine (**b**). Peaks of fatty acid vinyl ester standards and peaks from reaction products are superimposed for retention time comparison

esters [18] and triacylglycerols [22] were used as the acyl donor. A high temperature and long reaction time (100 °C for 6–12 h) in the absence of a catalyst resulted in undesirable product quality. In a recent study, much milder reaction conditions were used for the synthesis. Plastina et al. [23] used a free fatty acid to synthesize *N*-acylethanolamines at 40 °C for 6–15 h in hexane with lipase (Novozym 435). However, a high concentration of lipase was shown to be essential for the reaction, which made the reaction uneconomical. Their study did not show product purity, but did report a 80% yield after preparative LC purification. By comparison, the purity of *N*-stearoyl and *N*-palmitoylethanolamines in the present study was approximately 96 and 98% after the removal of excess ethanolamine. We also showed that 100% fatty acid vinyl ester was converted when the reaction was performed at the optimal conditions because none of these reactants were detected by GC.

The unique property of our reaction is its irreversibility. The amidation reaction between the free fatty acids, fatty acid methyl esters or triacylglycerols and ethanolamine is reversible. Yield of *N*-acylethanolamines might be increased by removal of water, methanol or glycerol under reduced pressure. However, this method was not desirable since the ethanolamine would also be evaporated due to its relatively low boiling point (170 °C) [21]. When a fatty acid vinyl ester is used as the acyl donor, the amidation reaction is irreversible because of the tautomerization of the by-product to a non-nucleophilic acetaldehyde. Acetaldehyde has a boiling point of 20.8 °C, so it can be evaporated easily without the loss of ethanolamine. Thus, this irreversibility promoted the amidation reaction. In fact, fatty acid vinyl esters were previously applied to the synthesis of symmetrical diacylglycerol and triacylglycerol [27, 30], but this was the first time they have been used for *N*-acylethanolamine synthesis.

The amount of ethanolamine influenced the solubility of products, and it perhaps acted as a catalyst [3, 24, 31]. Using ethanolamine as a solvent was economical because this material is readily available and affordable ($50.4 per liter from the Sigma Chemical Company).

The objective of optimization for this amidation reaction was to find a set of reaction conditions to reduce the esterification and promote the amidation reaction. The series of reactions we conducted was single-factor reactions. We knew that there might be interactions among these factors or variables, and a multi-factor response surface type of experimental design might be used to generate some mathematical equations or prediction models. However, a recently published paper [32] discouraged us from doing so. This work gave basic reaction information for possible future modeling and optimization trials on a large scale.

Conclusion

We evaluated four reaction conditions which had been frequently tested in earlier studies for the synthesis of *N*-acylethanolamines. We showed that high temperatures were not desirable when vinyl stearate was used as the acyl donor as this might lead to the formation of unwanted color. No reaction occurred when the reaction was conducted at 45 °C in hexane with sodium methoxide or a low concentration of lipase. The use of excess ethanolamine as the solvent proved suitable for the synthesis of *N*-acylethanolamines. The novelty of this study was that a new route for the synthesis of *N*-stearoyl and *N*-palmitoylethanolamines was established, and the reaction was fast and the

conditions were mild. This reaction resulted in the formation of *N*-acylethanolamines with high purity (> 95%) after simply washing with water. These products could be used in biological activity evaluations in the future.

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