

Original Research Article

Separation of Palmitic and Palmitoleic Acid and their FAMES using Transition Metal Salts

ABSTRACT

Fatty acids play an important role in physiological functions. Hence, separation and isolation of fatty acids (FAs) are of great importance in lipid industry. In this experiment, two different mechanisms have been tested for their effectiveness in separating one from a mixture of either palmitic acid and palmitoleic acid or their methyl esters. In the first study, a mixture of C16:0 and C16:1 Fatty Acid Methyl Esters (FAMES) in hexane was put in contact with an aqueous solution of silver nitrate and copper sulphate to check the strength of complex formation of the metal ions with the olefinic double bond of the unsaturated compound. The second study consisted of checking the difference in formation of a precipitate of palmitic acid and palmitoleic acid with transition metal ions arising due to the structural differences of both acids, i.e., testing if the bend in the structure of the cis-C16:1 fatty acid makes its carboxyl group less readily available to the metal ions for precipitation. Analysis of variance for both parts of the study showed that the difference in amount of precipitation of fatty acids was significant ($P < 0.001$) while the property of complex formation of the metal with the olefinic double bond wasn't significant ($P > 0.1$) in the two-phase, organic-aqueous system used.

Keywords: *Palmitic acid, Palmitoleic acid, C16:0 FAME, C16:1 FAME, copper sulphate, silver nitrate, zinc sulphate, precipitation, complex formation*

1. INTRODUCTION

Fatty acids (FAs) play an important role in various physiological functions and have a range of health effects. For example, saturated FAs (SFA) involve in energy storage and production, lipid transport, and in the synthesis of phospholipids and spingolipids[1]. Poly-unsaturated FAs (PUFAs) like docosohexanoic acid are involved in the carrier modulated transport of choline, glycine and taurine[2-4] and the function of delayed rectifier potassium channels [5]. Palmitic acid has been shown to mediate hypothalamic insulin resistance[6] while palmitoleic acid has been shown to increase insulin sensitivity thus improving hyperglycemia and hypertriglyceridemia [7]. Hence there's a widespread industrial interest in the development of separation techniques and isolation of fatty acids – especially those that are beneficial to human health.

Various methods have been used for the separation of saturated and unsaturated fatty acids. These include: chromatographic techniques such as thin layer chromatography, high pressure liquid chromatography, and gas chromatography and other physical techniques such as winterization and urea complexation. One of the most effective techniques employed for fatty acid separation is silver ion chromatography. This technique is based on the property of olefinic compounds forming a complex with transition metals (especially silver ions) that helps in eventual separation of the complex from the bulk. An accepted model

predicts formation of σ -type bond between occupied 2p orbital of an olefinic double bond and the free 5s and 5p orbitals of the silver ion. Also there is a π – acceptor backbond between occupied 4d orbital of silver ion and the free antibonding 2p π^* orbital of the olefinic bond [8]. This property to form charge-transfer type complexes with transition metal ions has been widely adapted in high-performance liquid chromatography based olefin separation[9].

Apart from in chromatographic separations, the property of silver ion complexation with olefinic double bonds has been used to extract fatty acid ester derivatives from various organic solvents [10]. For example, supercritical extraction (SC) has been used to extract polyunsaturated fatty acid ethyl esters (PUFA-Et) from aqueous silver nitrate [11] while separation of ethyl esters of eicosapentaenoic acid and docosahexaenoic acid had been attempted via circulating liquid membranes using silver nitrate as a carrier [12].

Certain metallic ions demonstrate the ability to form precipitates with specific types of fatty acids and this property has been utilized to separate out one type of fatty acid from a mixture. For example, alkali metal hydroxides paired with precipitation by alkaline earth metal salt has been used to separate free fatty acids in the form of insoluble fatty acid salts. [13]. In a second example, saturated straight chain aliphatic monocarboxylates have been separated using the property of Cu, Fe, Zn, Pb and Mg forming complexes with the monocarboxylates[14,15,16].

Another method adaptable for large scale separation of fatty acids is winterization. This method uses the difference of melting points of various fatty acids depending on the type and degree of unsaturation. Long chain SFAs crystallize out at lower temperatures leaving PUFAs in liquid form [17]. For example, in biodiesel industry, winterization has been used to decrease saturated FAMES from 86.9% to 73.4% while increasing unsaturated FAMES from 12% to ~20%[18].

Another efficient method for large scale separation of fatty acids is urea complexation. This process involves formation of a homogeneous solution of urea and FFAs/SFAs at 65°C in an alcoholic solvent such as ethanol, followed by cooling and separation of the resultant urea complex to room temperature[19]. Optimization experiments indicate that when urea complexation is carried out for a mixture of fatty acids, the SFA concentration can be decreased to almost zero from an initial concentration of 4.5% while the PUFA concentration can be increased from an initial concentration of 33.5 % to 53.87%[20]The process is considered uncomplicated, quick, cheap, robust, and environmental friendly [19, 21].

In this experiment, two different mechanisms of separation of fatty acids or its ester derivatives were tested. First, the property of transition metal ions to form a complex with the olefinic double bond of the unsaturated was evaluated. In the second experimental model, the property of fatty acids to form precipitates with metallic salts was studied. In both cases, it was checked whether the system was successful in separating a comparatively higher fraction of an unsaturated FA than an saturated FA or vice versa. The essence of this study was to test methods which can be successful in separating saturated and unsaturated fatty acids (or its ester derivatives) and which could be easily scalable for bulk mixtures of fatty acids. Hence the use of any chromatographic techniques was avoided. Also, targeting energy and cost savings, experiments were carried out at room temperature and pressure.

2. EXPERIMENTAL DETAILS

2.1 Evaluation of effectiveness of transition metal ions to form complexes with olefinic double bond in an organic-aqueous two-phase system (Study 1)

For this study, a procedure similar to that carried out by Teramoto et al.(1994) was adopted. The property of metal ions to form a complex with the olefinic double bond of the unsaturated fatty acid methyl esters (FAME) was tested for a liquid-liquid system. The unsaturated fatty acid, cis-C16:1 FAME and a saturated fatty acid, C16:0 FAME were purchased from Sigma Aldrich. Both FAMES were mixed in 2.5 ml of hexane so that the concentration of each FAME was 0.05 mol/dm³ in the solution (Solution A). A 2.5 ml aqueous solution of 4 mol/dm³ of transition metal ion salt was prepared (Solution B).

The transition metal ion salts used in this study were either silver nitrate or copper sulphate. Solution A and B were put together in a reaction vial. Two distinct layers were observed – the heavier aqueous layer containing metal salts and an upper hexane layer consisting of saturated FAME and unsaturated FAME. Both layers were mixed together by the use of a magnetic stirrer at 800 rpm. This gave an opportunity for the metal ions in the aqueous layer to interact with FAMES in the organic layer. So if there were a complex formation between the olefinic bond of the unsaturated FA and the silver/copper ions, the portion of unsaturated FAME which formed a complex with metal ions would migrate to the aqueous layer. At the time intervals of 0 (immediately after mixing), 5, 15, 30 and 50 min, the concentration of FAMES in the top (hexane) layer was measured using GC-FID(Agilent Technologies 6850). The same procedure was also carried out using a blank aqueous layer i.e, water, with no metal ions.

Using above procedure, the percentage decrease in concentration of FAMES at the five time intervals was calculated (Eq.1, Eq.2 and Eq.3). This decrease can be attributed either to: (i) complex formation of unsaturated FAME with metal ions and hence the transfer of FAMES into the aqueous layer or (ii) transfer of both FAMES to the aqueous solution due to mixing. The response was measured as the percentage difference in decrease of concentration of C16:1 FAME and C16:0 FAME from the hexane layer. The more the difference, the more effective the aqueous solution is in selectively separating the unsaturated FAME from the mixture containing both saturated and unsaturated FAMES. The response was measured at 5 time intervals: 0, 5, 15, 30, and 50 minutes of stirring. The response was measured at different time intervals in order to check whether the time of stirring has any effect on the experimental model. Each experiment was carried out with three replicates. For each time response, the analysis of variance was calculated using Design Expert to determine the significance of the model (at $\alpha = 0.05$).

$$\begin{aligned} & \% \text{ Reduction of C16:0 FAME(study 1) or FA(study 2)} \\ & = \left(\frac{\text{Initial concentration of C16:0 in solution} - \text{Concentration of C16:0 after precipitation in supernatant}}{\text{Initial concentration of C16:0 in solution}} \right) 100 \quad \text{Eq. 1} \end{aligned}$$

$$\begin{aligned} & \% \text{ Reduction of C16:1 FAME(study 1) or FA(study 2)} \\ & = \left(\frac{\text{Initial concentration of C16:1 in solution} - \text{Concentration of C16:1 after precipitation in supernatant}}{\text{Initial concentration of C16:1 in solution}} \right) 100 \quad \text{Eq. 2} \end{aligned}$$

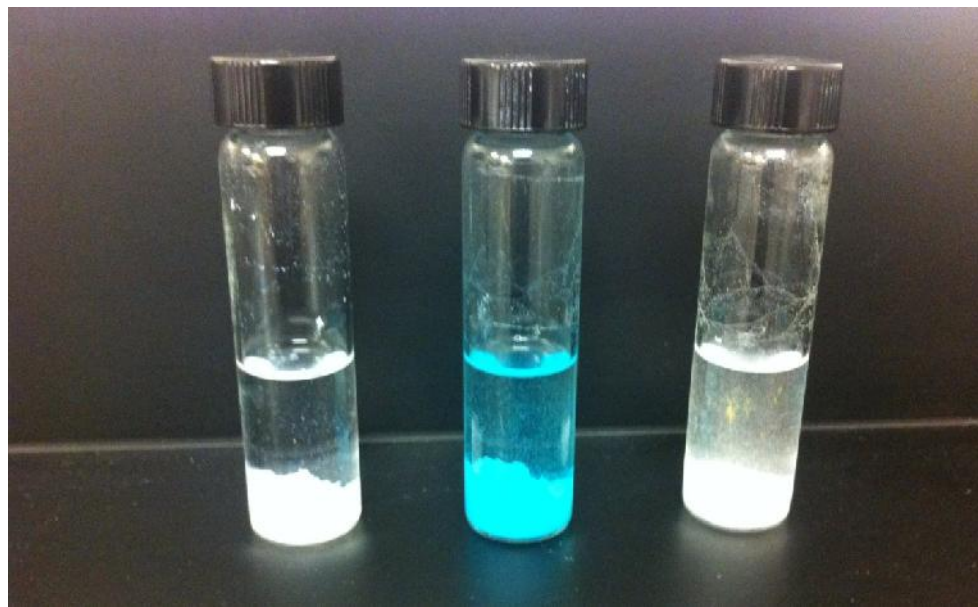
$$\begin{aligned} & \text{Difference in reduction (Response of experimental model) for Study 1} \\ & = \{(\text{Eq. 2}) - (\text{Eq. 1})\} \quad \text{Eq. 3} \end{aligned}$$

$$\begin{aligned} & \text{Difference in reduction (Response of experimental model) for Study 2} \\ & = \{(\text{Eq. 1}) - (\text{Eq. 2})\} \quad \text{Eq. 4} \end{aligned}$$

2.2 Evaluate formation of precipitates of metal salts with fatty acids (Study 2)

In the second study, the ability of fatty acids to form precipitates with metallic salts was investigated. The metal salts tested include copper sulphate, silver nitrate, zinc sulphate,

133 ferrous sulphate, ferric chloride, cobalt chloride and manganese chloride. Hexadecanoic acid
134 (C16:0) was obtained from Sigma Aldrich while 9-cis-hexadecanoic (C16:1) acid was
135 purchased from Nu-Chek Prep. A 100 mg of both fatty acids were thoroughly mixed in 10 ml
136 of methanol. Preliminary studies included testing of a visible formation of precipitate after the
137 addition of aforementioned metallic salts ($\sim 0.17 \times 10^{-3}$ moles) to the fatty acid solution in
138 methanol. Once individual metallic salts were added to the FA solution, they were left
139 undisturbed for 24 hours to observe any form of precipitation. Of the tested transition metal
140 salts, silver nitrate, copper sulphate and zinc sulphate showed formation of a precipitate
141 (figure 1) while for others it was absent (figure 2). Transition metal salts showing distinct
142 precipitation were selected for more in-depth quantitative studies.
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145
146 **Figure 1: (Left to Right) a) Silver Nitrate b) Copper Sulphate c) Zinc Sulphate showing**
147 **clear precipitation of saturated FAs from a mixture containing both saturated and**
148 **unsaturated FAs.**
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Figure 2: (Left to Right) a) Manganese Chloride b) Ferrous Sulphate c) Ferric Chloride d) Cobalt Chloride did not show any precipitation from a mixture containing both saturated and unsaturated FAs.

For blank runs, aforementioned solutions were trans-esterified using an acidic medium of 4% H_2SO_4 in methanol -- without the addition of any metallic salts. A known amount of C:15 FA was added as the internal standard during transesterification in order to calculate the transesterification efficiency. The transesterification process was carried out for 1 hour at 120°C . Then, water and hexane were added to the solution which was followed by centrifugation of at 1000 rpm for 6 minutes. FAME concentration was then measured using a GC-FID.

To check the effect of silver ions on the mixture of saturated FA and unsaturated FA in methanol, about 30 mg ($\sim 0.17 \times 10^{-3}$ moles) of AgNO_3 was added to the solution and mixed thoroughly. Formation of a white precipitate was visible. The precipitate was allowed to form and settle for 24 hours after which the supernatant was filtered out using a $0.2 \mu\text{m}$ PTFE filter to ensure that a clear solution is obtained for the next step. This sample was then transesterified following the same procedure as mentioned above. In order to evaluate the effect of copper ions, about 44 mg ($\sim 0.17 \times 10^{-3}$ moles) of copper sulphate pentahydrate was added to the C16:0/C16:1 solution in methanol and mixed thoroughly. Formation of a blue precipitate was observable. Same procedure as that for silver ions was adopted for quantification studies. In order to check the effect of Zinc ions, about 49 mg ($\sim 0.17 \times 10^{-3}$ moles) of zinc sulphate heptahydrate was added to the C16:0/C16:1 solution in methanol and mixed thoroughly. Formation of a white precipitate was observable. Same procedure as that for silver and copper ions was adopted for quantification studies.

The response was measured as the difference in percentage reduction of concentration of C16:0 FA from that of C16:1 FA and was calculated using Eq.1, Eq.2 and Eq. 4. Each experiment was carried out with three replicates. For each time response, the analysis of

variance was calculated using Design Expert to determine the significance of the model (at $\alpha = 0.05$).

3. RESULTS AND DISCUSSION

3.1 Evaluation of effectiveness of transition metal ions to form complexes with olefinic double bond in an organic-aqueous two-phase system (Study 1)

The first experiment concentrated on evaluating whether the type of metal played a significant role in distinctive separation of the two FAMEs. A closer look at the figure 3 - which is a plot of the mean (average of the three replicates) of the response at every time interval along with error bars with standard deviation - it can be seen that there's a specific pattern in the differences in the decrease of fatty acid concentrations, with the silver ion being the most effective in separating out the unsaturated fatty acid than the saturated one, followed by copper while blank solution is the least effective for the desired separation. A model is considered insignificant if the p-value for it is more than 0.05. We can see from table 1 that the model is not significant at any of the time response/interval. The reason for the model being insignificant at all time-responses can be explained by the large error bars -- which are results of non-uniformity of data points for replicates.

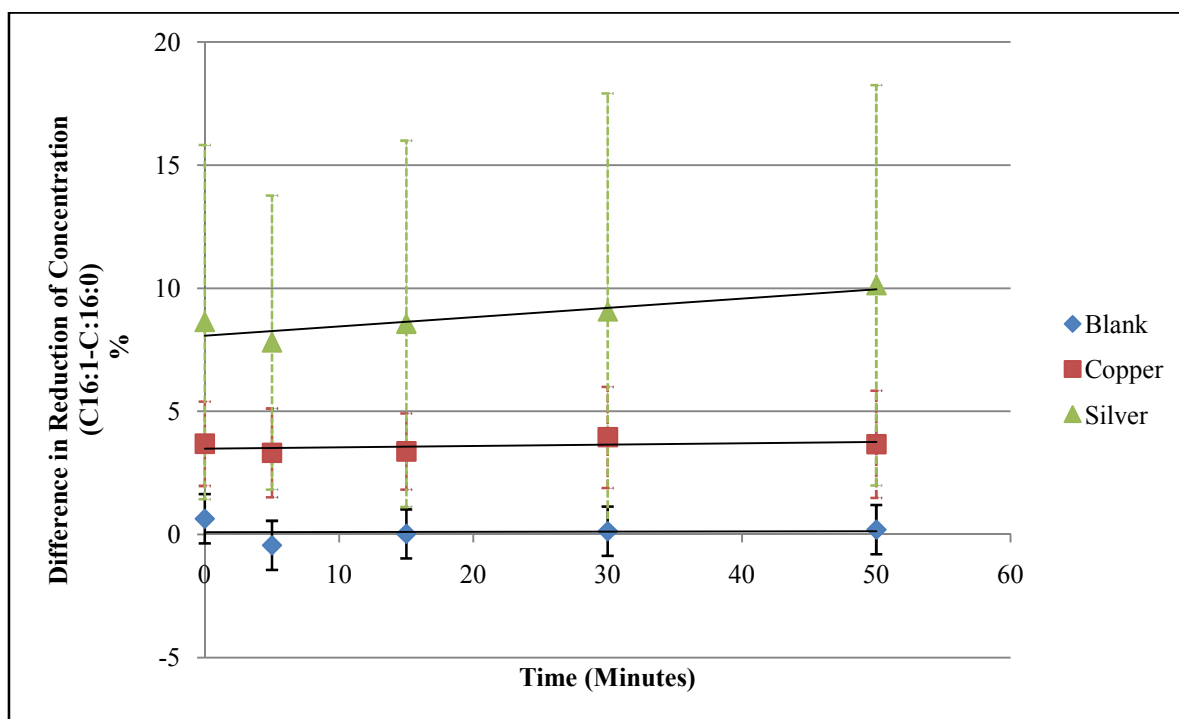


Figure 3: Means of the replicates plotted against the time along with error bars with one standard deviation. We can clearly see a trend where the metal ions are more selective to the unsaturated FAME

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Table 1: Statistical output for Study 1

Time Interval (minutes)	0	5	15	30	45
P-Values	0.19	0.11	0.15	0.21	0.13

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215 Statistically speaking, the type of aqueous solution used doesn't significantly affect the
 216 response (which is to selectively differentiate between the unsaturated and saturated
 217 FAMES) nor does the time of stirring. While the transition metal ions do seem to show a
 218 trend in extracting more of the unsaturated FAME than the saturated FAME, there seems to
 219 be variation in the readings which likely has an effect on the significance of the model. One
 220 of the reasons of this variation may be due to the fact that the complexes formed between
 221 the FAME and the metal ion being not strong enough, i.e., the FAMES not staying in the
 222 aqueous layer long enough to cause separation. Consequently, the second study was
 223 geared toward amplifying another property present in fatty acid derivatives that may cause
 224 crystal formation in the presence of metallic salts.

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226 **3.2 Evaluate formation of precipitate of metal salts with fatty acids (Study 2)**

227 For this second set of experiments, we chose fatty acids instead of fatty acid methyl esters.
 228 Figure 4 graphically represents the results. We can distinctly see the effectiveness of
 229 addition of metal ions in separating out C16:0 FA more than C16:1 FA. While the difference
 230 in case of blank solution is negligible, that of metal salts is around 8-10%. Which means that
 231 the reduction in the concentration of the saturated FA is far more than that of the
 232 unsaturated FA.

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234 Significant responses ($P < 0.001$) were obtained for samples where CuSO_4 , AgNO_3 and
 235 ZnSO_4 were added to a mixture of FAs. The slight reduction in concentrations in blank
 236 solutions where no precipitate was seen can be attributed to two factors - i) not all fatty
 237 acids getting transesterified at the same rate and/or ii) Not all FAMES get transferred from
 238 methanol to the hexane layer. Reduction in concentration with metal ion salts can be
 239 attributed to the precipitate formation in addition to the above two factors. It is possible that
 240 because of the presence of a kink in the structure of the cis-unsaturated FA, the carboxyl
 241 group at the end of its structure won't be as easily available to the Ag^+ or Cu^{2+} or Zn^{2+} ions to
 242 form a precipitate. Hence a positive value of response would indicate that there's more
 243 reduction in C16:0 FA than C16:1 FA; and higher positive value of response the better the
 244 system is in selectively precipitating out the saturated FA more than the cis-unsaturated FA.

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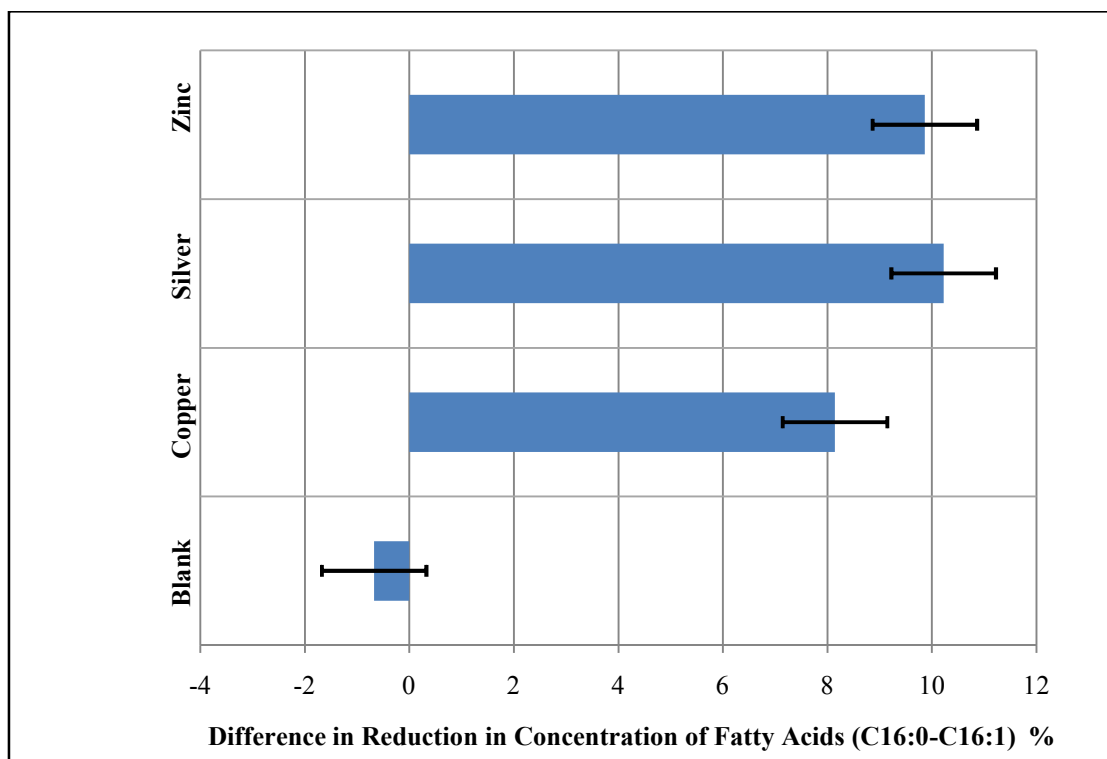


Figure 4: Plot of the means of replicates of different metal ions with that of the blank solution. Error bars are drawn with one standard deviation. We can clearly see the effectiveness of all metal salts in distinctly separating the two fatty acids.

We can see that the addition of silver nitrate, copper sulphate and zinc sulphate into the system is successful for separating out one type fatty acid more than the other. This could be attributed to the bend in the structure of the cis-unsaturated fatty acid. The results look promising and further studies are needed to be carried out for exploring the efficiency of different metal ions in selectively precipitating a straight chain saturated fatty acid than cis-unsaturated fatty acids. Also, high value omega-3-fatty acids such as DHA and EPA whose structure isn't linear owing to several bends due to the double bonds present can be introduced into this system to check their precipitation with various metal salts.

4. CONCLUSION

The organic-aqueous system used was not significantly effective in separating a singular ester from a mixture of saturated and unsaturated FAME in the organic/aqueous bi-phasic system used. However, it was observed that saturated FA forms were able to be separated from a mixture containing both forms (saturated and unsaturated FAs) in methanol. It is likely that the saturated FAs formed precipitated selectively with Ag^+ , Cu^{++} , Zn^{++} . Since these experiments were carried out at room temperature and pressure, this technique is worth further exploration for low-cost and scalable separation of component fatty acids.

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