

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

ABSTRACT

Fatty acids (FAs) play an important role in physiological functions. Hence, separation and isolation of FAs is of great industrial importance. With the intension of finding out effective means of separating different forms of lipid constituents, two studies were carried out. In the first study, aqueous solutions of metallic salt(s) were brought in contact with mixtures of saturated (C16:0) and unsaturated (C16:1) fatty acid methyl esters (FAMES). In the second study, FA forms were used. The goal here was to investigate whether metal ions in their solvent state form strong enough complexes with either the saturated or unsaturated forms of FAMES or FAs to selectively pull the ensembles out from a mixture. Results showed that although the FAME forms did not show a significant selectivity toward separation, saturated FA forms preferentially bound to select metal ions allowing separation of these from a mixture.

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 fatty acids (SFAs) involve in energy storage and production, lipid transport, and in the synthesis of phospholipids and spingolipids [1]. Poly-unsaturated fatty acids (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. One of the most effective techniques employed for fatty acid separation is silver ion chromatography [8, 9]. 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 [8]. An accepted model predicts formation of a 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].

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

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46 Certain metallic ions demonstrate the ability to form precipitates with specific types of fatty
47 acids and this property has been utilized to separate out one type of fatty acid from a
48 mixture. For example, alkali metal salts along with alkali metal hydroxides have been used to
49 precipitate out free fatty acids via the formation of insoluble fatty acids salts [13]. In a second
50 example, saturated straight chain aliphatic monocarboxylates have been separated using
51 the property of Cu, Fe, Zn, Pb and Mg forming complexes with the monocarboxylates [14,
52 15, 16].

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54 A method adaptable for large scale separation of fatty acids is winterization. This method
55 uses the difference of melting points of various fatty acids depending on the type and degree
56 of unsaturation. Long chain SFA crystallize out at lower temperatures leaving PUFA in liquid
57 form [17]. For example, in the biodiesel industry, winterization has been used to decrease
58 saturated fatty acid methyl ester (FAME) concentration from 86.9% to 73.4% while
59 increasing unsaturated FAME concentration from 12% to ~20%[18].

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61 Another efficient method for large scale separation of fatty acids is urea complexation. This
62 process involves formation of a homogeneous solution of urea and free fatty acid (FFA)
63 /SFA at 65°C in an alcoholic solvent such as ethanol, followed by cooling and separation of
64 the resultant urea complex to room temperature[19]. Optimization experiments indicate that
65 when urea complexation is carried out for a mixture of fatty acids, the SFA concentration can
66 be decreased to almost zero from an initial concentration of 4.5 % while the PUFA
67 concentration can be increased from an initial concentration of 33.5 % to 53.87 % [20]. The
68 process is considered uncomplicated, quick, cheap, robust, and environmental friendly [19,
69 21].

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71 Chromatographic techniques are selective, but expensive (in terms of energy requirements)
72 and difficult to scale-up. Currently used large-scale separation techniques such as urea
73 complexation and winterization, besides being costly due to required temperature variations
74 (E.G. in urea complexation, temperature of solution is increased to 65°C and then decreased
75 to 4-24°C[22] whereas during winterization, temperature is decreased to -18°C[22]) are not
76 quite selective. The essence of this study was to test methods that are scalable and
77 energetically less costly.

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79 In this experiment, two different mechanisms for separation of fatty acids or their ester
80 derivatives were tested. In the first study, the property of free forms of transition metal ions to
81 form a complex with unsaturated FAME was evaluated. In the second study, the property of
82 fatty acids to form precipitates with metallic salts was evaluated. In both cases, it was
83 checked whether that particular system was successful in separating a comparatively higher
84 fraction of an unsaturated form from a mixture containing both forms (i.e., saturated and
85 undaturated) or vice versa.

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2. EXPERIMENTAL DETAILS

2.1 Evaluation of effectiveness of free forms 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. To 2.5 ml of hexane, 32 mg of both FAMES were mixed, 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 and was prepared by adding either 1.6 g of silver nitrate (AgNO₃) or 2.5 g of copper sulphate (CuSO₄.5H₂O) (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 the saturated FAME and the 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 metal ions, the portion of unsaturated FAME which formed a complex with metal ions would migrate to the aqueous layer. After 0 (immediately after mixing), 5, 15, 30 and 50 minutes of stirring, the concentration of FAMES in the top (hexane) layer was measured using gas chromatography-flame ionization detector (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 the aforementioned procedure, the percentage decrease in concentration of FAME after 0, 5, 15, 30 and 50 minutes of stirring, 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 0, 5, 15, 30, and 50 minutes of stirring. The response was measured at 5 different stirring times in order to check whether the time of stirring had 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 , which is a statistical tool for design of experiment (DOE) methods, to determine the significance of the model (at $\alpha = 0.05$. α is the “significance level” or the probability of rejecting the null hypothesis when the null hypothesis is true).

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

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

$$\begin{aligned} & \text{Difference in reduction (Response of experimental model) for Study 1} = \{(\text{Eq. 2}) - (\text{Eq. 1})\} \quad \dots (\text{Eq. 3}) \\ & \text{Difference in reduction (Response of experimental model) for Study 2} = \{(\text{Eq. 1}) - (\text{Eq. 2})\} \quad \dots (\text{Eq. 4}) \end{aligned}$$

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, ferrous sulphate, ferric chloride, cobalt chloride and manganese chloride (all metals salts were hydrated except for silver nitrate). Hexadecanoic acid (C16:0) was obtained from Sigma Aldrich while 9-cis-hexadecanoic (C16:1) acid was purchased from Nu-Chek Prep. 100 mg each of C16:1 and C16:0 were thoroughly mixed in 10 ml of methanol. Preliminary studies included testing of a visible formation of precipitate after the addition of aforementioned metallic salts ($\sim 0.17 \times 10^{-3}$ moles) to the fatty acid solution in methanol. Once individual metallic salts were added to the FA solution, they were left undisturbed for 24 hours to observe any form of precipitation. Of the tested transition metal salts, silver nitrate, copper sulphate and zinc sulphate showed formation of a precipitate (photo 1) while for others it was absent (photo 2). Transition metal salts showing distinct precipitation were selected for more in-depth quantitative studies.

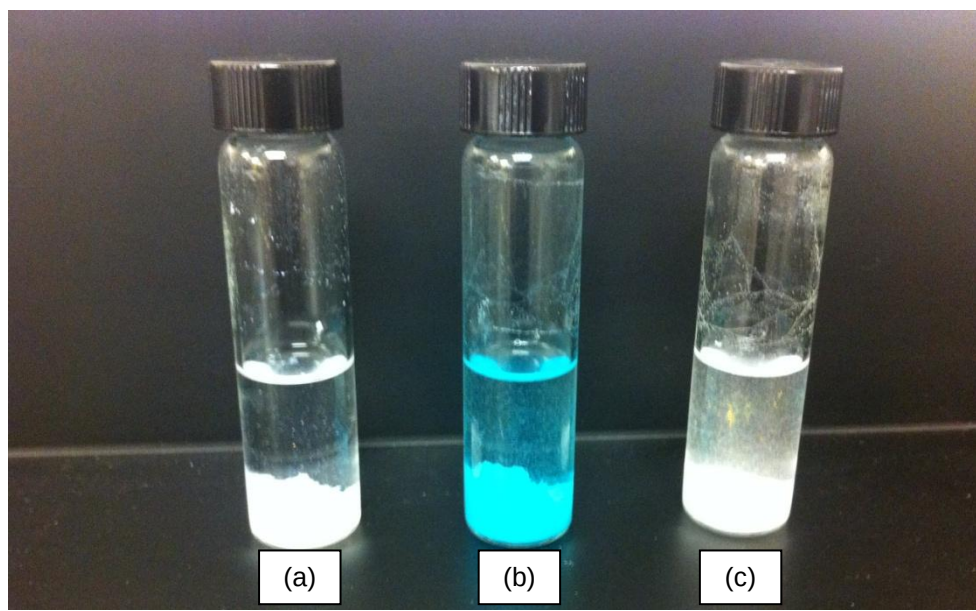


Photo 1: (Left to Right) a) Silver Nitrate b) Copper Sulphate c) Zinc Sulphate showing clear precipitation of saturated FA from a mixture containing both saturated and unsaturated FA.

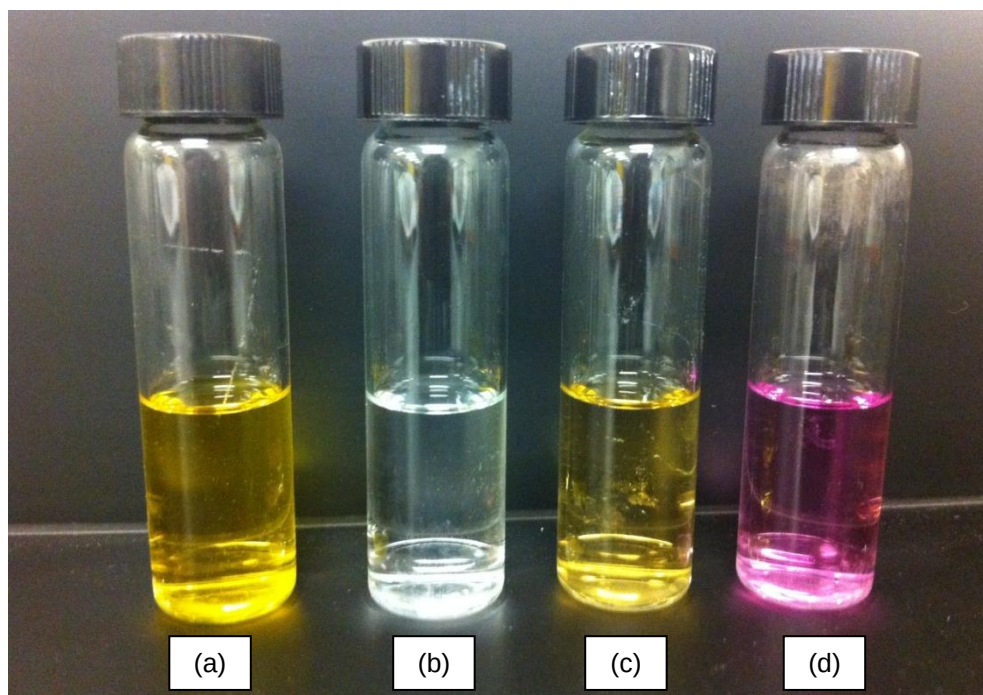


Photo 2: (Left to Right) a) Manganese Chloride b) Ferrous Sulphate c) Ferric Chloride d) Cobalt Chloride did not show any precipitation from a mixture containing both saturated and unsaturated FA.

For blank runs, fatty acids that were mixed in methanol were trans-esterified using an acidic medium of 4% H_2SO_4 in methanol. A known amount of C15:0 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 $0.2 \mu\text{m}$ polytetrafluoroethylene (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. For quantification studies, the procedure carried out for silver ions was adopted for copper ions. In order to evaluate 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. Quantification was done similar to the procedure adopted for silver ions above.

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. Significance of the model was compared at $\alpha = 0.05$ (α is the “significance level” or the probability of rejecting the null hypothesis when the null hypothesis is true).

2.3 GC-FID Analysis

For estimating the amount of C16:0, C16:1 and C15:0 FAME in experimental samples, the response factor (RF) for every FAME was calculated with respect to C13:0/ C19:0 FAME (using Equation 5). To calculate the response factors, known amounts of C13:0, C19:0, C16:0, C16:1, and C15:0 FAME were mixed in hexane and analyzed via GC-FID.

$$RF_{C16:0/C16:1/C15:0} = \frac{(\text{Peak Area of C13:0}) \times (\text{Concentration of C16:0/C16:1/C15:0})}{(\text{Peak Area of C16:0/C16:1/C15:0}) \times (\text{Concentration of C13:0})} \quad \dots \text{Eq. 5}$$

C13:0/C19:0 FAME was used as the internal standard and was added to every experimental sample. **Figure 1** is an example of the chromatogram obtained after analysis. Distinct peaks pertinent to C13:0, C15:0, C16:0, C16:1 and C19:0 FAMES are observable in Figure 3. The amounts of C15:0, C16:0 and C16:1 FAME were calculated using equation 6.

$$\text{Concentration of C15:0/C16:0/C16:1 FAME} = \frac{(\text{Peak Area of C15:0/C16:0/C16:1 FAME}) \times (RF) \times (\text{Concentration of C13:0/C19:0 FAME})}{(\text{Peak Area of C13:0/C19:0 FAME})} \quad \dots \text{Eq. 6}$$

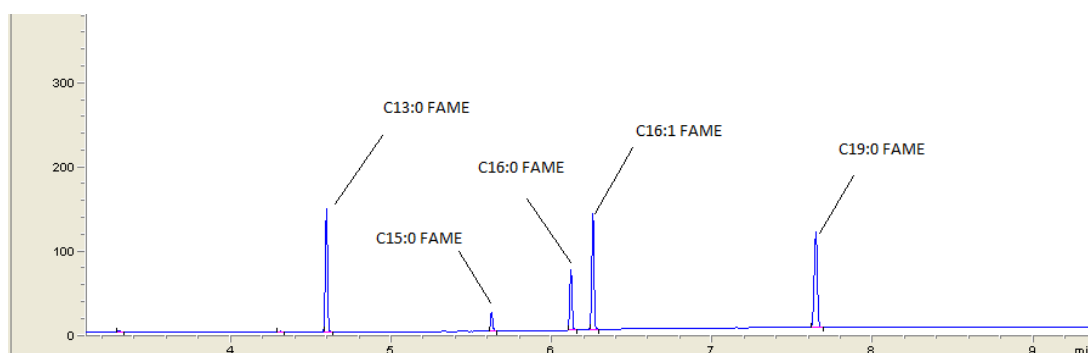


Figure 1: Chromatogram showing distinct FAME peaks in a typical sample

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 2** - which is a plot of the mean (average of the three replicates) of the response 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 margins -- which are results of non-uniformity of data points between replicates.

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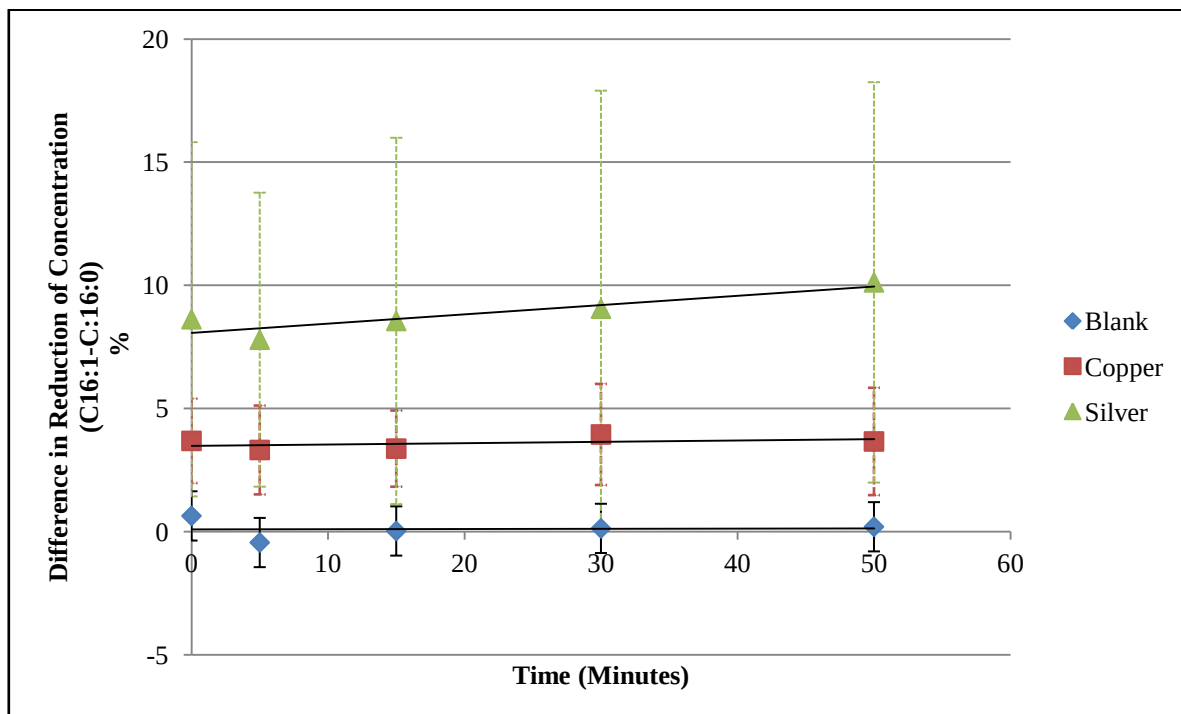


Figure 2: Means of the replicates plotted against the time along with error margins with one standard deviation.

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

253 **3.2 Formation of precipitates of metal salts with fatty acids (Study 2)**

254 For this second set of experiments, we chose fatty acids instead of fatty acid methyl esters.
255 **Figure 3** graphically represents the results. We can distinctly see the effectiveness of metal
256 ions in separating out C16:0 FA more than C16:1 FA. While the difference is negligible in the

case of blank solutions, that of the metal salts is around 8 – 10% - which means that the reduction in the concentration of the saturated FA is far more than that of the unsaturated FA.

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

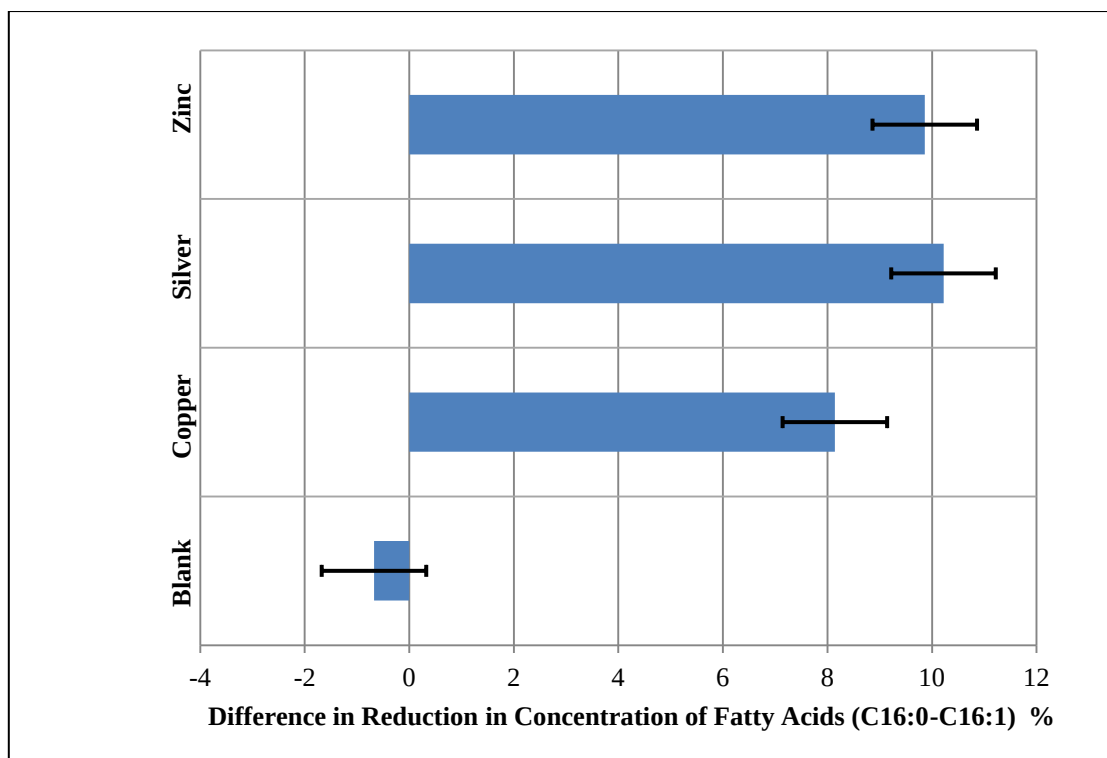


Figure 3: Plot of the means of replicates of different metal ions with that of the blank solution. Error margins are drawn with one standard deviation.

We can see that silver nitrate, copper sulphate and zinc sulphate interact with the saturated form of the FA and forming crystallites while preventing formation of the same with the unsaturated version. The precipitates are most likely carboxylates (of the saturated fatty acid and the metal ion) now crystallized with similar molecules. The unsaturated FAs, though still metal carboxylates, did not form the crystallites likely due to the absence of a condition that promotes intermolecular attractive forces. We conjecture that the preclusion of these

interactions is as a result of the bend in the structure of the cis-unsaturated fatty acid that prevents molecules coming closer to generate such interactions.

The results look promising and further studies are needed to be carried out for exploring the ability of different metal ions to selectively precipitate a straight chain saturated fatty acid(s) from a mixture of fatty acids. If proven effective, the applicability to extend this method toward separating high value omega-3-fatty acids such as DHA and EPA whose structure isn't linear from a mixture of FAs needs to be explored.

4. CONCLUSION

Free forms of metal ions (in solvent medium), when mixed with a mixture containing saturated and unsaturated forms of a FAME, were not quite effective in separating the unsaturated form from the other. However, when the FA forms were used, transition metal salts such as silver nitrate, copper sulphate and zinc sulphate were able to effectively separate the unsaturated form from the mixture of the two. Since these experiments were carried out at room temperature and pressure, this technique is worth further exploration for low-cost separation of component fatty acids.

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