USE OF TRANSITION METAL FORMS IN SEPARATION OF LIPID COMPONENTS

A Thesis

by

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ABSTRACT

Fatty acids (FAs) play an important role in physiological functions. Hence, separation and isolation of FAs is of great industrial importance. Studies presented here were conducted with the intention of developing scalable and cheaper means of separating different forms of lipid constituents. 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, select 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.

The third study involved elucidating the effect of Zn^{2+} concentration on the separation behavior of mixtures of saturated and unsaturated FAs (in methanol). Results showed that a concentration of 30 mg/ml of $ZnSO_4$ could decrease the concentration of C16:0 FAs in the solution by 58%. IR spectra of the precipitate obtained in the second study supported the fact that the precipitate obtained is due to a complex between zinc ion and the carboxylate group of the C16:0 FA. Computational chemistry calculations indicate

that the that specific complex formation with the saturated FA may be due to structural differences between C16:0 FA and C16:1 FA and their respective metal carboxylates.

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

1. INTRODUCTION AND LITERATURE REVIEW[*]

This section starts with an introduction on physiological and health effects of fatty acids and the need for effective separation techniques for isolating fatty acids. In addition to that, current separation techniques that are used for fatty acid isolation such as gas chromatography, liquid chromatography, winterization, urea complexation and liquid-liquid extraction are discussed. The need for exploring new separation techniques is also discussed.

1.1 Health Effects of Fatty Acids

Fatty acids (FA) play an important role physiological functions of the human body and are significant part of lipids which are one of the three major components of biological matter (other two being proteins and carbohydrates)(Sheehy & Sharma, 2010) . FAs contribute to around 30% of the total energy intake for humans. The adipose tissue stores FA in excess, especially when there's an increase of intake of fat in the diet, resulting in obesity (Tvrzicka, Kremmyda, Stankova, & Zak, 2011). Fatty acids are broadly categorized into saturated or unsaturated carboxylic acids. In the tissue of plants and animals, the most abundant FAs are those with chain lengths of 16 and 18 carbon atoms, while lengths longer than 22 and shorter than 14 occur in minor concentrations.

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Unsaturated ones make up approximately half of the FAs in animals and plants and contain 1-6 double bonds(Tvrzicka et al., 2011).Long chain (saturated) fatty acids (LCFA) such was lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0) and stearic acid (C18:0) have significant thrombogenic and atherogenic potential. These fatty acids represent 80-90 % of dietary saturated fatty acid (SFA) intake.

Consumption of saturated LCFA plays a role in increasing the levels of cholesterol, especially that of low density lipoprotein (LDL) cholesterol, which is in turn connected with coronary heart disease (CHD) (Astrup et al., 2011). Very long chain saturated fatty acids (VLCFA) (C20:0 – C30:0) contribute to metabolic diseases such as Zellweger syndrome, X-linked adrenoleucodystrophy, Refsum's disease and Menkes' disease (McPhee, Papadakis, & Rabow, 2010).Monounsaturated fatty acids (MUFAs) have been known to reduce key risk factors in metabolic syndrome (MetS) (Garg, 1998; Kris-Etherton, 1999; Ros, 2003). MUFAs help in promoting a healthy blood lipid profile, moderate insulin sensitivity and glycemic control and also play a role in mediate blood pressure (Gillingham, Harris-Janz, & Jones, 2011; Krauss et al., 2000).

N-3 polyunsatuarted fatty acids such as ecosapentaenoic acid (EPA), alpha-linoleic acid (ALA) and docosahexaenoic acid (DHA) are ligands of peroxisome proliferator-activated receptor (PPAR-α) thus have a significant number of pleiotropic effects on energy and lipid metabolism. They can decrease lipogenesis and VLDL secretion by activating (PPAR-α) along with suppression of sterol response element binding protein

(SREBP-1).(Burdge et al., 2009) n-3 PUFA also increase activity of lipoprotein lipase, facilitate reverse cholesterol transport and decrease concentrations of apo C-III(P. Calder & P. Yaqoob, 2009; P. C. Calder & P. Yaqoob, 2009). PUFA are involved in the carrier modulated transport of choline, glycine and taurine (Hyman & Spector, 1982; Yorek, Hyman, & Spector, 1983; Yorek, Strom, & Spector, 1984) and the function of delayed rectifier potassium channels (Poling, Karanian, Salem, & Vicini, 1995).

Palmitic acid has been shown to mediate hypothalamic insulin resistance (Benoit et al., 2009) while palmitoleic acid has been shown to increase insulin sensitivity thus improving hyperglycemia and hypertriglyceridemia (Yang, Miyahara, & Hatanaka, 2011). Saturated FAs have been linked to adverse health effects. On the other hand, unsaturated FA are considered to be more beneficial for human health(Tvrzicka et al., 2011).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.

1.2 Separation Techniques

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 (Morris, 1966). 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 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 (Morris, 1966). This property to form charge-transfer type complexes with transition metal ions has been widely adapted in high-performance liquid chromatography (HPLC) based olefin separation(Nikolova-Damyanova, 2009). Retention of lipids in Ag-HPLC (silver ion – HPLC) depends on (i) physiochemical properties of the mobile phase and the interaction of analytes with the stationary phase: like any other chromatographic system. (ii) the stability of complex formed between silver ions and olefinic double bond in the lipid molecule (Nikolova-Damyanova, 2009). Some of the retention patterns(Nikolova-Damyanova, 2009) seen in the Ag-HPLC system are as follows:

- Retention is proportional to the number of double bonds.
- The stability is inversely proportional to the number of substituents of the double bond.
- Trans isomers are weakly retained than cis isomers. The greater stability of the cis-isomer can be credited to either the release of strain when complex if formed or to the steric hindrance by the two alkyl moieties when they are in transposition to each other.
- Compounds with methylene interrupted double bonds are retained more strongly than compounds with conjugated double bonds, and the greatest stability is when

two methylene groups separate double bonds, perhaps because a chelate complex can be formed.

- Retention weakens with decreasing chain length.

The early developing stage of HPLC of fatty acids was reported as isocratic reversed-phase C18 HPLC of methyl esters using a refractive index detector or UV detector (Henke & Schubert, 1980; Pei, Henly, & Ramachandran, 1975; Scholfield, 1975). Till the present, the most commonly used stationary phase for separation of fatty acids by HPLC is the reversed-phase C18 column. Nevertheless, gradient elution which offers a more dynamic range than that of isocratic elution has become popular for separating different classes of fatty acids (Lin, McKeon, & Stafford, 1995; Marcato & Cecchin, 1996; Mehta, Oeser, & Carlson, 1998).

In order to gain detection sensitivity or separation efficiency, pre-column derivatizations such as esterification or introduction of appropriate chromophores are usually performed in the case of HPLC for fatty acids, direct separation of free fatty acids has also been shown in several reports(Lin et al., 1995; Marcato & Cecchin, 1996; Mehta et al., 1998). Capillary GC coupled with flame ionization detector (FID) is the most convenient analytical tool for the determination of fatty acids. The fatty acids are determined as their methyl ester derivatives. For routine fat analysis, fatty acid methyl esters (FAMEs) can be easily prepared via transesterification. Currently, it is generally accepted that flexible fused silica capillary columns coated with highly polar cyanosiloxane stationary phases such as SP-2560 , SP- 2380 , CP-Sil 88 and BPX-70 are effective even in the analysis of cis-trans isomers of FAs (Ratnayake, 2004). Column lengths of about 100m length provide best resolution even for geometrical and positional isomers of FAMEs. Helium is usually used as a carrier gas (Ratnayake, 2004).

A powerful alternative to GC-FID for fatty acid separation is gas-chromatography couple to mass spectrometry (GC-MS). GC-MS methods for separation of fatty acids usually suffer from long retention times (i.e. that is usually in excess of 30 minutes). A novel, fast GC-MS method based on a short, small diameter column characterized by a run time of 17.2 minute has been developed by Ecker et al. (Ecker, Scherer, Schmitz, & Liebisch, 2012), where FAMEs were separated by a highly polar BPX70 column (10 m length, 0.10 mm diameter, 0.20 um thickness, SGE) coated with 70% cyanopropyl polysilphenyl-siloxane using a GC-2010 coupled to a GCMS-QP2012 detector. A programmed temperature vaporizer (PVT) was used in the split mode 1:20 for 3 s, switched for 1.3 min to the split-less mode and split ratio if 1:100 until the end of the run. By the use of a PVT, there was a gain in sensitivity as well as superior peak shapes compared to conventional split/split less operation. In contrast to the frequently used WAX columns, the selected cyano column showed a high temperature stability allowing separation of very long chained FAs (up to C:28). A three-stepped temperature program had been used to achieve clear separation geometric as well as positional isomers.

Mass spectrometric analysis in the field desorption (FD) mode to characterize individual FAME from their molecular ion peaks was carried out by Chopra et al.(Chopra et al., 2011). They also carried out analysis by obtaining the ^{1}H-NMR spectra of FAMEs. The results obtained by ANOVA analysis showed that there was no significant difference between GC/GC-MS and ^{1}H-NMR techniques. FAMEs can also be analysed by using gas chromatography/electron ionization –mass spectrometry (GC/EI-MS) in the selected ion monitoring (SIM) mode(Thurnhofer & Vetter, 2005). A similar method was used to determine FAMEs in food samples by use of ethyl esters (FAEE) and trideuterium-labelled methyl esters (d_3-FAME) as internal standards(Thurnhofer & Vetter, 2006).

About a decade ago, Armstrong et al. showed that room temperature ionic liquids (RTIL) possess the volatility, viscosity, solubility and polarity properties required for the use of stationary phases for capillary GC (Armstrong, He, & Liu, 1999) . When used as stationary phases, they showed dual nature retention selectivity, separating polar molecules as a polar stationary phase and non-polar molecules as a non-polar stationary phase(Delmonte, Fardin-Kia, & Rader, 2013). Comprehensive two-dimensional (2-D) gas chromatography can provide more detailed separation of FAMEs by combining selectivity of two different gas chromatographic separations (Adahchour, Beens, Vreuls, & Brinkman, 2006). The orthogonality between two separations is commonly achieved by employing GC columns of two different polarities, different elution temperatures for ^{1}D and ^{2}D or a combination of both. Non-polar capillary columns separate FAMEs primarily on their chain lengths and are often preferred for ^{1}D separation. Highly polar columns provide selectivity based on number, geometric configuration and position of double bonds preferred for ^{2}D separation. A study undertaken by Delmonte et al. , describes a novel approach to the GC x GC separation of FAMEs in which analytes undergo chemical reduction between ^{1}D and ^{2}D and ^{2}D separates the products of reaction.(Delmonte et al., 2013). Unsaturated FAMEs are reduced to their fully saturated forms by passing them through a capillary coated with Pd (reactor) in the presence of H_2 as the carried gas. This GC X GC separation is achieved by using the same highly polar (SLB-111) stationary column, and maintained at the same temperature. In thus method, the chemical structure of analytes is modified instead of applying different chromatographic conditions.

Winterization has been utilized to fraction triglycerides (present in edible oils) with different melting points. It involves the cooling of oils to allow the solid portion to crystallize, which is followed by filtration of the two phases (López-Martínez, Campra-Madrid, & Guil-Guerrero, 2004). The melting point of fatty acids varies with the types and degree of unsaturation and hence separation of mixtures of saturated and unsaturated fatty acids becomes feasible. At lower temperatures, long chain SFA which have higher melting points crystallize out leaving the PUFA in the solution (Shahidi & Wanasundara, 1998). There are two steps in the winterization process : (a) Crystallization during which selective nucleation and crystal growth take place under controlled cooling and slow agitation and (b) Filtration in which resultant slurry is filtered to separate solid and liquid portions(Gerpen, Knothe, & Krahl, 2005). For example, in the biodiesel industry, winterization has been used to decrease saturated fatty acid methyl ester (FAME) concentration from 86.9% to 73.4% while increasing unsaturated FAME concentration from 12% to ~20% (Doğan & Temur, 2013). In a study to concentration PUFA from fish oil, bleached oil was cooled progressively from 30 ^{0}C to 5 ^{0}C. Compared to bleached fish oil, a 9.2 % increase of unsaturated fatty acids and a 13.3% decrease of saturated fatty acids was obtained(Cunha, Crexi, & Pinto, 2009). PUFAs present in peanut oil were concentrated and at the same time SFA in the oil were reduced by a three-step winterization process where the temperature was reduced from 15 ^{0}C to 0 ^{0}C (Pérez, Casas, Fernández, Ramos, & Rodríguez, 2010). Hence it can be seen that winterization is effective in removing saturated fatty acids by crystallizing them at low temperatures and in the process, increasing the overall concentration of the unsaturated fatty acids present in the solution.

Another efficient method for large scale separation of fatty acids is urea complexation. This process involves formation of a homogeneous solution of urea and free fatty acid (FFA) /SFA at 65°C in an alcoholic solvent such as ethanol, followed by cooling and separation of the resultant urea complex to room temperature (Hayes, Bengtsson, Van Alstine, & Setterwall, 1998). 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 % (Fei, Salimon, & Said, 2010). The process is considered uncomplicated, quick, cheap and environmental friendly (Hayes et al., 1998; Wanasundara & Shahidi, 1999). Enrichment of omega-3-fatty acids from hoka oil has been attempted by using urea complexation(Tengku-Rozaina & Birch, 2013). In this particular study, by the use of urea complexation, the total SFA concentration was reduced to 1.6 % from an original concentration of 24 %, while the enrichments factor for various omega-3-fatty acids ranged from 2.83 to 3.88 (Tengku-Rozaina & Birch, 2013). The purity of alpha-linoleic acid (ALA) present in desilked silkworm pupae oil was increased to 34.8% from an initial value of 15 % by the use of urea complexation (Wang, Zhang, & Wu, 2013). Urea complexation was also successful in concentrating DHA and EPA from fish oil to 60.6% from initial concentrations of 24.2% and 36.4% respectively(Chen, Zhang, Mao, & Zu, 2012). Hence it can be seen that urea complexation is successful in concentrating PUFAs from various oils.

Apart from 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 (Teramoto, Matsuyama, Ohnishi, Uwagawa, & Nakai, 1994). For example, supercritical extraction (SC) has been used to extract polyunsaturated fatty acid ethyl esters (PUFA-Et) from aqueous silver nitrate (Suzuki, Tanaka, Kato, & Nagahama, 1994) while separation of ethyl esters of eicosapentaenoic acid and docosahexaenoic acid has been attempted via circulating liquid membranes using silver nitrate as a carrier (Teramoto, Matsuyama, & Nakai, 1996).

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 salts along with alkali metal hydroxides have been used to precipitate out free fatty acids via the formation of insoluble fatty acids salts (Awad & Gray, 2000). 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 (Daloz, Rapin, Steinmetz, & Michot, 1998; Hefter, North, & Tan, 1997; Rocca & Steinmetz, 2001).

1.2 Need for New Separation Methods

Chromatographic techniques are selective, but expensive (in terms of energy requirements) and difficult to scale-up. On the other hand, currently used large-scale separation techniques such as urea complexation and winterization, are costly due to required temperature variations (E.G. in urea complexation, temperature of solution is increased to 65°C and then decreased to 4-24°C (Mendes, da Silva, & Reis, 2007) whereas during winterization with temperature is decreased to -18°C(Mendes et al., 2007). They are also not quite selective. The essence of this study was to test methods that are scalable, energetically less costly, and selective for separation of FAs.

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

The effect on increasing concentration of one of the metal salts ($ZnSO_4$) on the precipitation of saturated (palmitic acid) and unsaturated (palmitoleic acid) in methanol is then evaluated. The next stage involves analyzing the IR spectra of the precipitate to characterize the nature of precipitate formed. The final stage utilizes computational chemistry tools to explain/verify experimental observations.

2. OBJECTIVES

The essence of this set of experiments is to evaluate the effectives of transition metal salts in selectively separating saturated fatty acids (or saturated FAMEs) from a mixture containing both the unsaturated and unsaturated form(s). The major specific objectives are as follows:

Specific objective 1: To determine whether transition metal salts are effective in separating the unsaturated FAME from a mixture of saturated and unsaturated forms.The variables for this study are:

1. Type of aqueous solution: Blank, $CuSO_4$, $AgNO_3$
2. Type of FAME in hexane: Saturated (palmitic) acid and unsaturated (palmitoleic) acid
3. Time of stirring: 0, 5, 15, 30 and 50 minutes.

Response measurement: Difference in decrease of unsaturated FAME and saturated FAME in the organic layer. Working hypothesis: Metal ions in the aqueous solution will form a complex with the olefinic double bond of unsaturated FAME and lead to a decrease in Palmitoleic FAME concentration in the organic layer.

Specific objective 2: To determine whether select transition metal salts show a selective binding behavior between saturated and unsaturated fatty acids.The variables in this study are:

1. Type of salts added in methanol: Blank, $CuSO_4$, $AgNO_3$, $ZnSO_4$
2. Type of fatty acid in methanol: Saturated (palmitic) acid and unsaturated palmitoleic) acid

Response measurement: Difference in the decrease of saturated fatty acid and unsaturated fatty acid concentrations in the methanol phase after the treatment.Working hypothesis: Select transition metal salts will bind differently to saturated and unsaturated fatty acids due to structural differences of the two fatty acid forms. From the findings of study 2, it could be identified: a) whether metal salts show selective precipitation with either the saturated or the unsaturated fatty acid form and b) if part (a) is true, the best performing metal salt will be selected for next study.

Specific objective 3: To determine the optimum concentration of the transition metal salt at which the fatty acid separation is maximum.The variables in this experiment are:

1. Concentration of the transition metal salt.
2. Type of fatty acid (saturated FA and unsaturated FA)

Response measurement: (a) Difference in the decrease of saturated fatty acid and unsaturated fatty acid concentrations in the methanol phase; (b) Decrease in concentration of C16:0 FA and C16:1 FA with varying concentrations of the selected transition metal salt. Working hypothesis: Increasing concentration of metal ion will lead to an increase in precipitation of either the saturated fatty acid or the unsaturated fatty acid.

Any significant results obtained from part 1, 2 and 3 will be attempted to be explained via theoretical calculations by the use of computational chemistry software - GuassView and Gaussian. Thermochemistry calculations performed will include free energy, enthalpy and Gibbs free energy values. These data will be used to ascertain why certain reaction(s) are preferable over the other and/or why a particular metal preferentially binds on a specific fatty acid form.

Note – A journal article(Sayali Kulkarni 2014) has been published which contains information on the first set (part 1 and 2) of experiments carried out

3. METHODS AND MATERIALS*

3.1 Evaluation of Effectives of Free Forms of Transition Metal Ions to form Complexes with Olefinic Double Bond in an Organic-Aqueous Two-Phase System

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/dm^3 in the solution (Solution A). A 2.5 ml aqueous solution of 4 mol/dm^3 of transition metal ion salt and was prepared by adding either 1.6 g of $AgNO_3$ or 2.5 g of $CuSO_4.5H_2O$ (Solution B).

The transition metal ion salts used in this study were either $AgNO_3$ or $CuSO_4$. 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.

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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 greater 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).

3.2 Evaluate Formation of Precipitates of Metal Salts with Fatty Acids

In the second study, the ability of fatty acids to form precipitates with metallic salts was investigated. The metal salts tested include $AgNO_3$, $CuSO_4$, $ZnSO_4$, $MnCl_2$, $FeSO_4$, FeCl3 and $CoCl_2$ (all metals salts were hydrated except for $AgNO_3$). 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 (~ 0.17×10^{-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 $ZnSO_4$ showed formation of a precipitate (figure 1) while for others it was absent (figure 2). Transition metal salts showing distinct precipitation were selected for more in-depth quantitative studies.

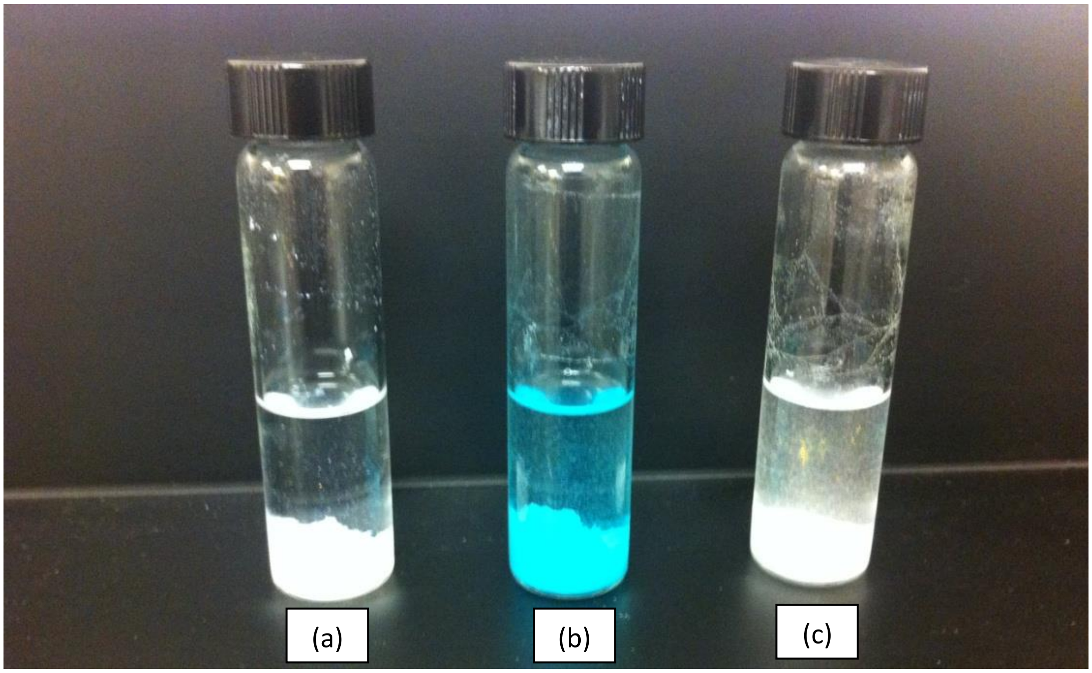

Figure 1:. (Left to Right) a) $AgNO_3$ b) $CuSO_4$ c) $ZnSO_4$ showing clear precipitation of saturated FA from a mixture containing both saturated and unsaturated FA

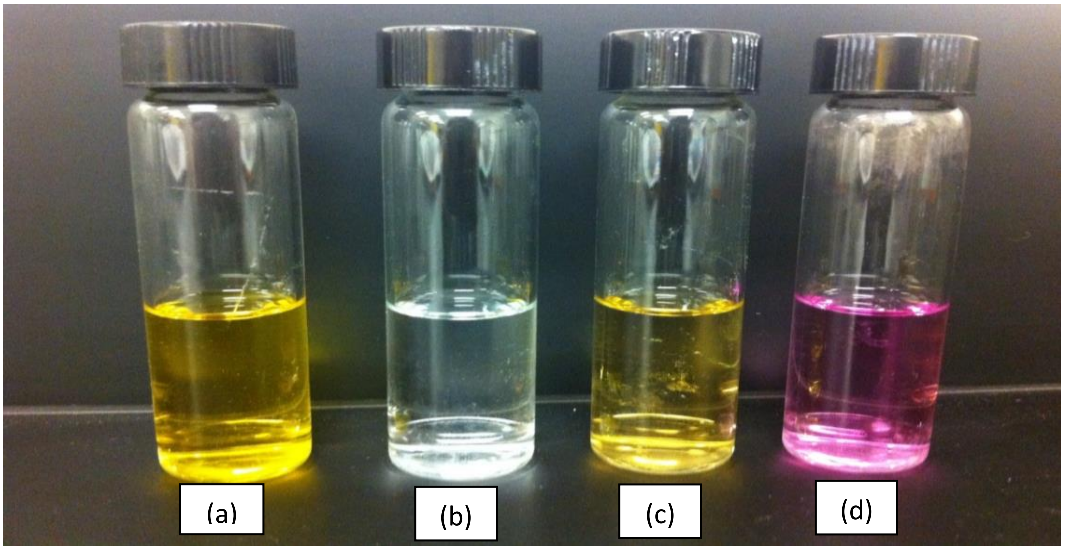

Figure 2: (Left to Right) a) $FeSO_4$ b) $MnCl_2$ c) $FeCl_3$ d) $CoCl_2$ did not show any precipitation from a mixture containing both saturated and unsaturated FA

For blank runs, FAs 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^{o}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 (~ 0.17 x 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 µm 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 (~ 0.17 x 10^{-3} moles) of $CuSO_4$:5H_2O 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 (~ 0.17 x 10^{-3} moles) of $ZnSO_4.7H_2O$ 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 α = 0.05 (α is the “significance level” or the probability of rejecting the null hypothesis when the null hypothesis is true).

$$\% \, Reduction\ of\ C16{:}0\ FAME (section\ 3.1\ )\ or\ FA (section\ 3.2)$$
$$= \left(\frac{Initial\ concentration\ of\ C16{:}0\ in\ solution - Concentration\ of\ C16{:}0\ after\ precipitation\ \ in\ supernatant}{Initial\ concentration\ of\ C16{:}0\ in\ solution}\right) 100$$

.....Eq. 1

$$\% \, Reduction\ of\ C16{:}1\ FAME (section\ 3.1)\ or\ FA (section\ 3.2)$$
$$= \left(\frac{Initial\ concentration\ of\ C16{:}1\ in\ solution - Concentration\ of\ C16{:}1\ after\ precipitation\ in\ supernatant}{Initial\ concentration\ of\ C16{:}1\ in\ solution}\right) 100$$

…..Eq. 2

$$Difference\ in\ reduction\ (Response\ of\ experimental\ model)\ for\ Section\ 3.1\ = \{(Eq.2) - (Eq.1)\}$$

…..Eq. 3

$$Difference\ in\ reduction\ (Response\ of\ experimental\ model)\ for\ Section\ 3.2 = \{(Eq.1) - (Eq.2)\}$$

…..Eq. 4

3.3 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. The response factors for C15:0 , C16:0 and C16:1 were obtained to be 0.9325, 0.8904 and 0.8973 respectively

$$RF_{C16:0/C16:1/C15:0} = \frac{(Peak\ Area\ of\ C13:0)\ X\ (Concentration\ of\ C16:0/C16:1/C15:0)}{(Peak\ Area\ of\ C16:0/C16:1/C15:0) X\ (Concentration\ of\ C13:0)}$$

…..Eq. 5

C13:0/C19:0 FAME was used as the internal standard and was added to every experimental sample. Fig. 3 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.

$$Concentration\ of\ C15:0/C16:0/C16:1\ FAME = \frac{(Peak\ Area\ of\ C15:0/C16:0/C16:1\ FAME) X\ (RF) X\ (Concentration\ of\ C13:0/C19:0\ FAME)}{(Peak\ Area\ of\ C13:0/C19:0\ FAME)}$$

…..Eq. 6

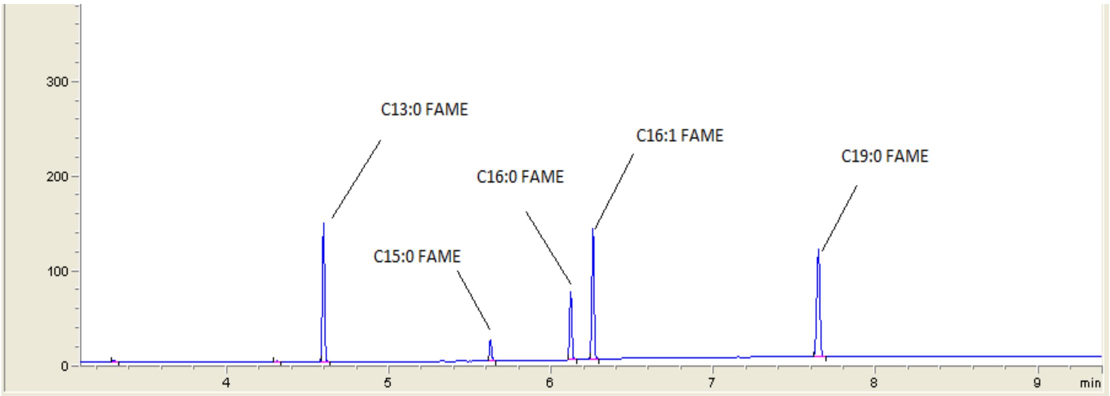

Figure 3 : Chromatogram showing distinct FAME peaks in a typical sample

3.4 Optimization of $ZNSO_4$ Concentration

Preliminary studies indicated that $ZnSO_4$ preferentially precipitates palmitic acid from a mixture containing both palmitic and palmitoleic acids. Consequently, the present study was aimed at elucidating whether an increase in concentration of $ZnSO_4$ leads to increase in the formation of precipitates and if so, to what extent.

Hexadecanoic acid (C16:0) was obtained from Sigma Aldrich while 9-cis-hexadecanoic (C16:1) acid was purchased from Nu-Chek Prep. A 100 mg of each acid was added to 10 ml of methanol and the solution was mixed using a vortex mixer until the FAs were completely dissolved and a clear solution was obtained. To this solution, 50 mg of $ZnSO_4$ was added. Formation of an immediate precipitate was observed. The precipitate was allowed to form and settle for 24 hours. The precipitate was filtered and the supernatant was then transesterified to convert the remaining fatty acids in the solution to FAMEs. For the transesterification process, the FA solution in methanol was mixed with 4% H_2SO_4 solution in methanol and heated at 120 ^{0}C for 1 hour. The concentration of FAMEs was estimated using a GC-FID. The same procedure was repeated for different $ZnSO_4$ concentrations of 5, 10, 20, 30 and 40 mg/ml. Experiments at each concentration were run in triplicates.

3.5 FTIR-ATR Analysis of Precipitate

The precipitate obtained after adding $ZnSO_4$ to a mixture of C16:0 FA and C16:1 FA was filtered and dried at 70^0C to evaporate methanol present in the sample. Then the precipitate was analyzed in a FTIR-ATR (ATR-Attenuated Total Reflectance mode) spectrometer to obtain the spectra between 500-4000 cm^{-1}. Pure samples of palmitic acid and palmitoleic acid were also analyzed using FTIR-ATR to compare their spectra to that of the precipitate.

3.6 Computational Chemistry Calculations

In order to explain the results that were obtained in the earlier experiments, theoretical chemistry calculations were undertaken. Computational chemistry software, GuassView 5 and Gaussian 09 (M. J. Frisch, M. Ehara, & K. Raghavachari, 2009) were used to build, optimize and find the energies of molecules and potential structures formed.

In the procedure it is mentioned that both fatty acids were completely mixed in methanol - which means that the fatty acid molecules were ionized in the solution, giving the carboxylate group of the FA a net negative charge. In the case of the metal salts in solution (in methanol) the metal ions possess a net positive charge(s). It was also observed from earlier studies that certain metal salts showed selective binding preference with the saturated fatty acid forms. Accordingly, in order ascertain why certain metals showed the tendency to form precipitates selectively with the saturated FA, computational chemistry calculations were undertaken as follows:

a. Structures of palmitic acid, palmitoleic acid, a few of the metal ions used in the experiment, and their corresponding "metal carboxylates" were optimized using the method "Hartree-Fock" with basis set of "321-G" available in Gaussian 09.

b. Frequency calculations of the optimized structures were undertaken using the same method and basis set, from which the sum of electronic and thermal free energies (Standard Gibbs Free Energies) of ions and molecules were calculated.

c. Assuming that positively charged metal ions formed a metal carboxylate by reacting with the negatively charged fatty acid ion in methanol, the Gibbs free energies of reactions of fatty acid ions with metals ions resulting in the formation of metal carboxylate was calculated.

4. RESULTS AND DISCUSSION[*]

4.1 Evaluation of Effectives of Transition Metal Ions to form Complexes with Olefinic Double Bond in an Organic-Aqueous Two-Phase System

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 4 - 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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[*] Part of this chapter is reprinted with the permission from "Separation of Palmitic and Palmitoleic Acid and their FAMEs using Transition Metal Salts" by Sayali Kulkarni and Sandun Fernando,2014. *International Research Journal of Pure and Applied Chemistry,* 4, 551-561,Copyright [2014] by ScienceDomain International

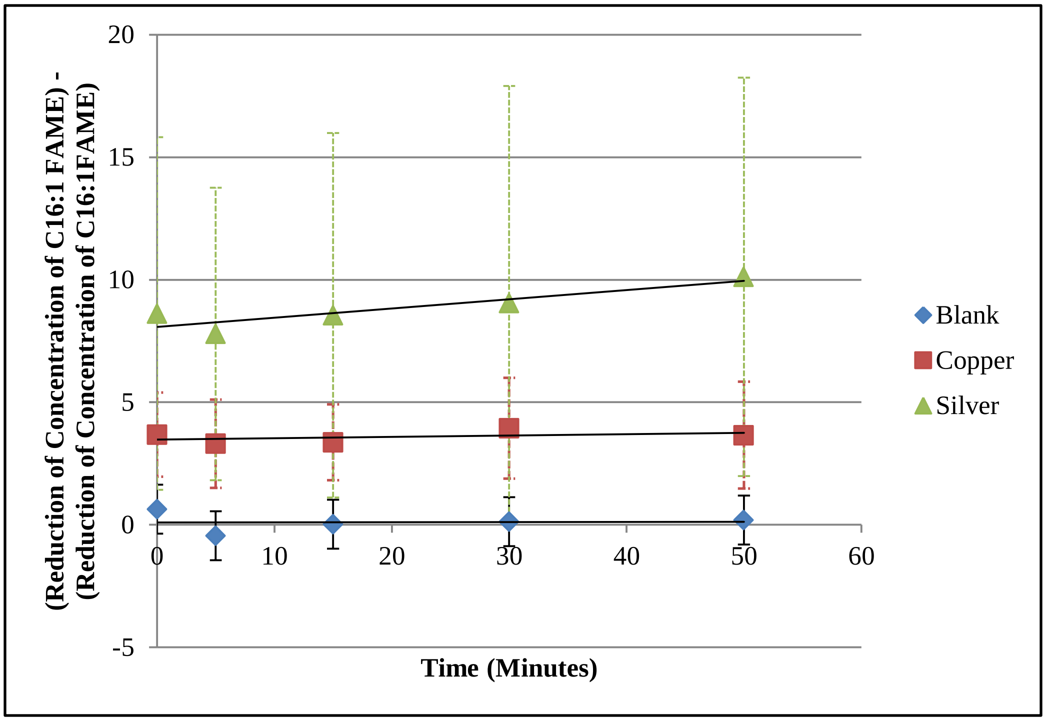

Figure 4: Means of the replicates plotted against the time along with error margins with one standard deviation

Table 1: Statistical output for study 4.1

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

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) are 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.

4.2 Formation of Precipitates of Metal Salts with Fatty Acids

For this second set of experiments, we chose fatty acids instead of fatty acid methyl esters. Figure 5 graphically represents the results. We can distinctly see the effectiveness of metal 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.

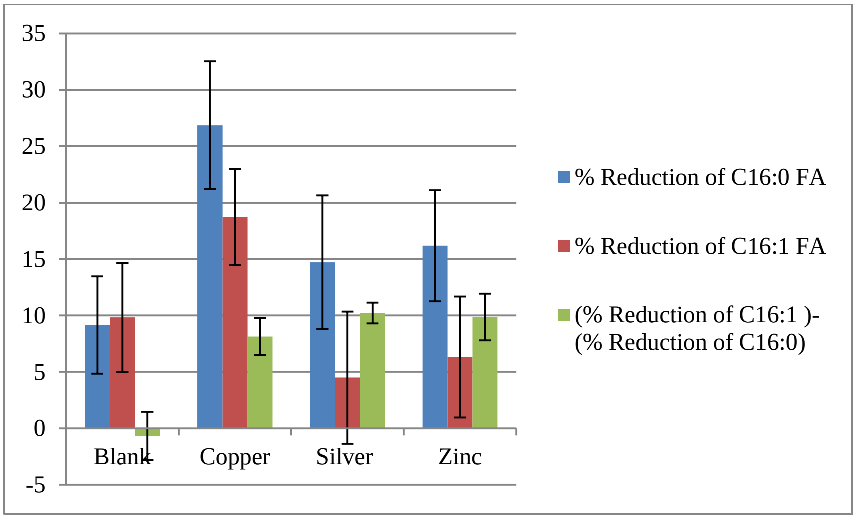

Figure 5: Plot of the means of replicates of different metal ions with that of the blank solution. Error margins are drawn with one standard deviation

Significant responses ($P < 0.001$) were obtained for samples where $CuSO_4$, $AgNO_3$ and $ZnSO_4$ were added to the FAs mixture. ANOVA output from Design Expert is given in table 2, indicating the significance of the model. Figure 6 and 7 represent the normal and the box-cox plot for this experimental model respectively. It can be surmised from these results that the normality assumption is satisfied. All residuals' plots seem to be compliant. Box-cox plot recommends no transformation of data.

Table 2: ANOVA output for experimental model for evaluation of formation of precipitates of metal salts with fatty acids

Response	1	Difference in Reduction in Concentration of Fatty Acids (C16:0-C16:1)				
ANOVA for selected factorial model						
Analysis of variance table [Classical sum of squares - Type II]						
	Sum of		Mean	F	p-value	
Source	Squares	df	Square	Value	Prob > F	
Model	236.1494	3	78.71645	25.39143	0.000193	significant
A-Aqueous Layer	236.1494	3	78.71645	25.39143	0.000193	
Pure Error	24.80095	8	3.100119			
Cor Total	260.9503	11				

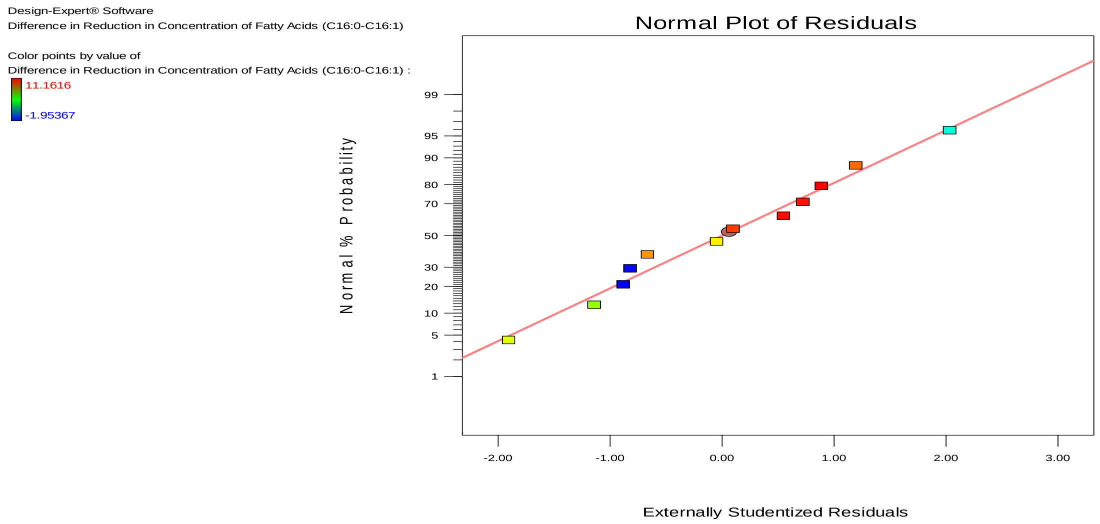

Figure 6: Normal plot for residuals for experimental model for formation of precipitates of metal salts with fatty acids

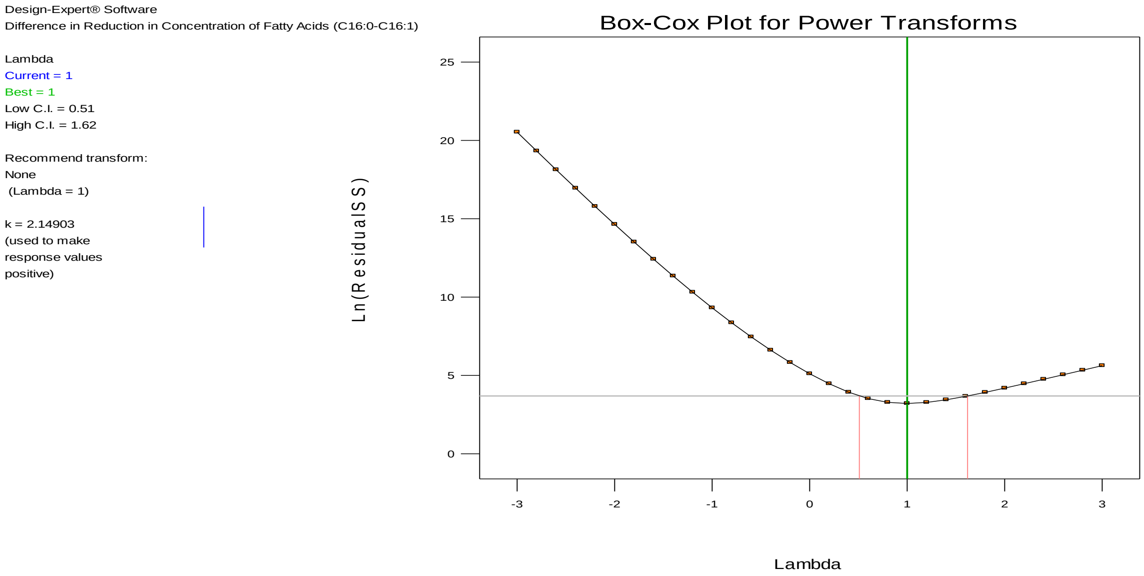

Figure 7: Box-cox plot for residuals for experimental model for formation of precipitates of metal salts with fatty acids

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.

We can see that $AgNO_3$, $CuSO_4$ and $ZnSO_4$ 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.

4.3 Optimization of $ZnSO_4$ Concentration

This study was geared toward ascertaining whether the $ZnSO_4$ concentration has any impact on the amount of FAs that can be selectively separated. Figure 8 depicts how increased concentrations of $ZnSO_4$ lead to enhancement of the selective precipitation of the saturated FA (in comparison to unsaturated FA). Precipitation of C16:0 FA goes on increasing to up to 58% while that of C16:1 FA remains at around 10% irrespective on the concentration of $ZnSO_4$ added. This phenomenon leads to greater difference between the saturated FA and unsaturated FA that is being precipitated.

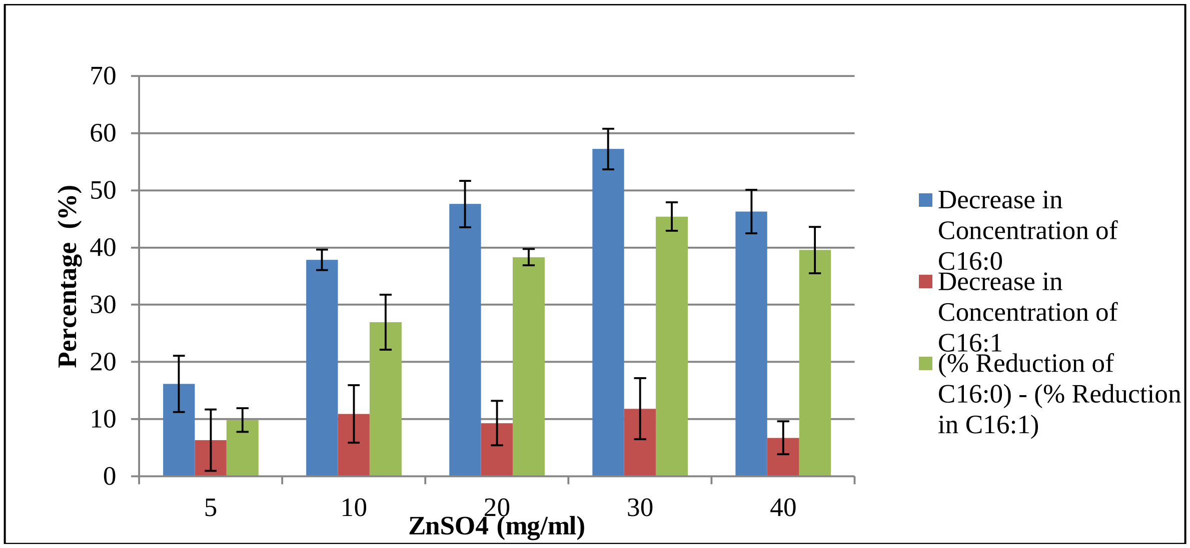

Figure 8: Fatty acid precipitation with increasing concentration of $ZnSO_4$

Analysis of variance (ANOVA) was conducted using Design-Expert (version 9.0.3.1) with concentration of $ZnSO_4$ as a factor with 5, 10, 20, 30 and 40 mg/ml as levels. The ANOVA resulted in a p-value of 0.0002 - which is significant. The ANOVA table generated from Design Expert for this model is given in table 3. The normality plot and box-cox plot for this experimental model are represented by figure 9 and 10 respectively. It can be surmised from these results that the normality assumption is satisfied. All residuals' plots seem to be compliant. Box-cox plot recommends no transformation of data.

Table 3: ANOVA for concentration of $ZnSO_4$ as a factor with 5, 10, 20, 30 and 40 mg/ml as levels

Response 1 Difference in decrease of concentration (C16:0 -C16:1)
Transform: Power Lambda: 1.28 Constant: 0
ANOVA for Response Surface Quadratic model
Analysis of variance table [Partial sum of squares - Type III]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	23675.69	2	11837.84	94.09809	4.64E-08	significant
A-Concentration of Zinc Sulphate	15962.04	1	15962.04	126.881	9.76E-08	
A^2	6888.139	1	6888.139	54.75328	8.28E-06	
Residual	1509.639	12	125.8032			
Lack of Fit	324.1888	2	162.0944	1.367366	0.298573	not significant
Pure Error	1185.45	10	118.545			
Cor Total	25185.33	14				

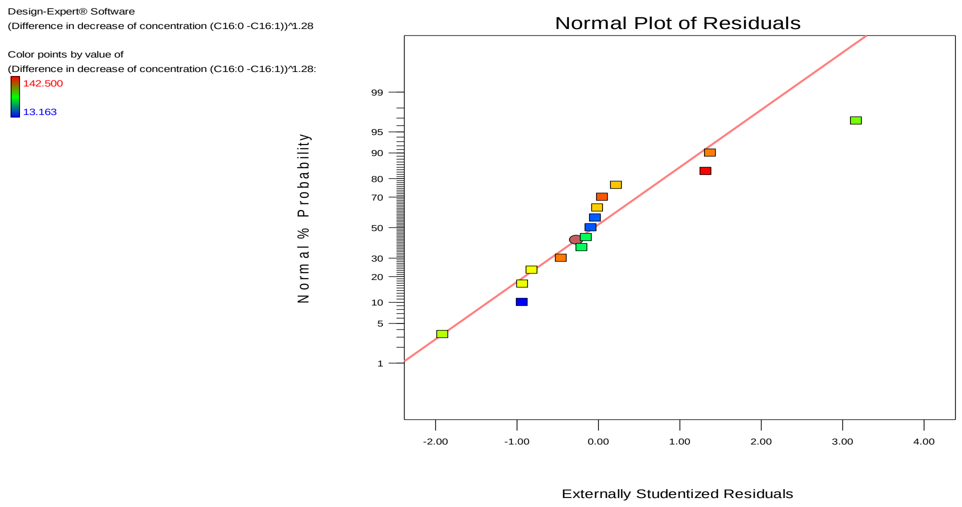

Figure 9: Normality plot for experimental model with concentration of $ZnSO_4$ as a factor with 5, 10, 20, 30 and 40 mg/ml as it levels

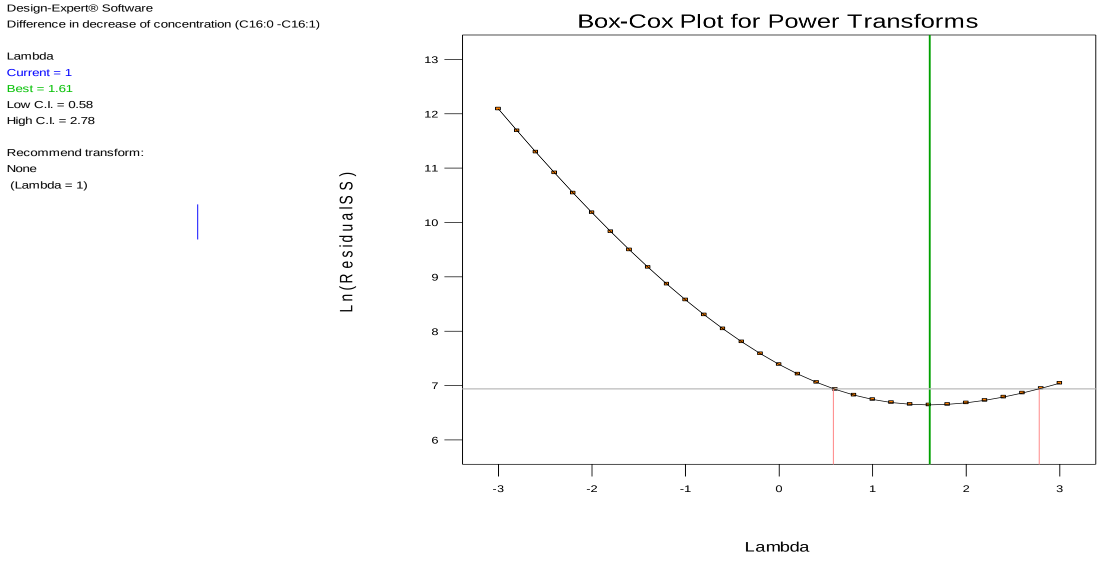

Figure 10: Box-cox plot for experimental model with concentration of $ZnSO_4$ as a factor with 5, 10, 20, 30 and 40 mg/ml as it levels

This means that varying concentration of $ZnSO_4$ significantly affects the concentration difference between C16:1 FA and C16:0. From figure 6, it can be confirmed that increasing the ZnSO4 concentration up to about 30 mg/ml leads to an increase in the relative amount of C16:0 FA precipitated. However, beyond 30 mg/ml $ZnSO_4$ concentration, the separatory effect seems to taper off. It can be clearly that the decrease in the concentration of C16:1 FA remains more or less constant at 10% whereas the reduction in concentration of C16:0 FA increases to a maximum of around 58%. Another experimental model was analyzed where there were two factors : (a) amount of $ZnSO_4$ added and (b) type of fatty acid, which in turn has two levels : (i) C16:0 FA and (ii) C16:1 FA. The response of this model was measured as the decrease in concentration of fatty acid after the addition of $ZnSO_4$. The ANOVA for this experimental model resulted in a p-value of < 0.0001. This means that the model is significant. The data for this experimental model is also represented in figure 6. ANOVA output from Design Expert is given in table 4, indicating the significance of the model. Figure 11 and 12 represent the normal and the box-cox plot for this experimental model respectively. It can be surmised from these data that the normality assumption is satisfied. All residuals' plots seem to be compliant. Box-cox plot recommends no transformation of data.

Table 4: ANOVA for experimental model with two factors : (a) amount of $ZnSO_4$ added and (b) type of fatty acid , which in turn has two levels : (i) C16:0 FA and (ii) C16:1

Response	1	Decrease in Concentration of Fatty Acid				
Transform:	Square Root	Constant:	0.5			
ANOVA for Response Surface Quadratic model						
Analysis of variance table [Partial sum of squares - Type III]						
Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	105.0473071	4	26.26183	54.04254	6.05E-12	significant
A-Concentration of Zinc Sulphate	6.512524821	1	6.512525	13.40171	0.001177	
B-Fatty Acid	87.82187164	1	87.82187	180.723	6.06E-13	
AB	5.416283532	1	5.416284	11.14582	0.002641	
A^2	8.574940575	1	8.574941	17.64582	0.000295	
Residual	12.14868301	25	0.485947			
Lack of Fit	3.642584948	5	0.728517	1.712929	0.177653	not significant
Pure Error	8.506098064	20	0.425305			
Cor Total	117.1959901	29				

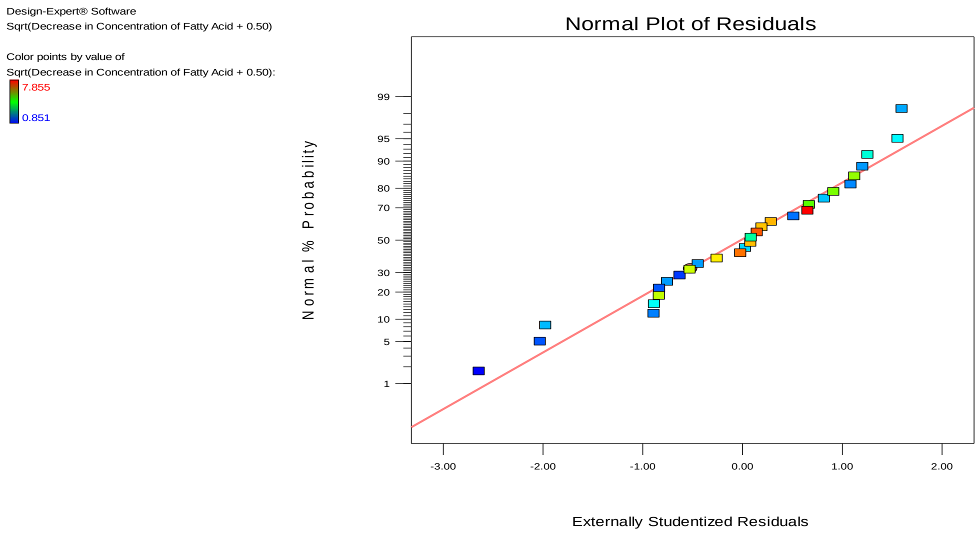

Figure 11: Normality plot for experimental model with two factors : (a) amount of $ZnSO_4$ added and (b) type of fatty acid, which in turn has two levels : (i) C16:0 FA and (ii) C16:1

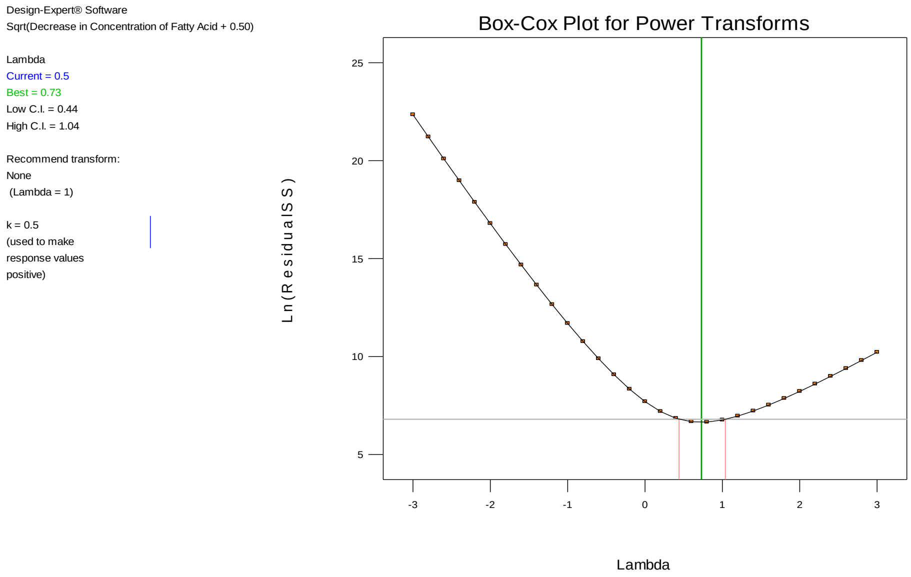

Figure 12: Box-cox plot for experimental model with two factors : (a) amount of $ZnSO_4$ added and (b) type of fatty acid has two levels (i) C16:0 FA and (ii) C16:1

Hence it can be surmised that the amount of $ZnSO_4$ added to the solution has a significant impact on (i) the type and (ii) the amount of FA precipitated.

4.4 FTIR-ATR Analysis of Precipitate

Figure 13 depicts the spectra for C16:0 FA, C16:1 FA precipitates with zinc.

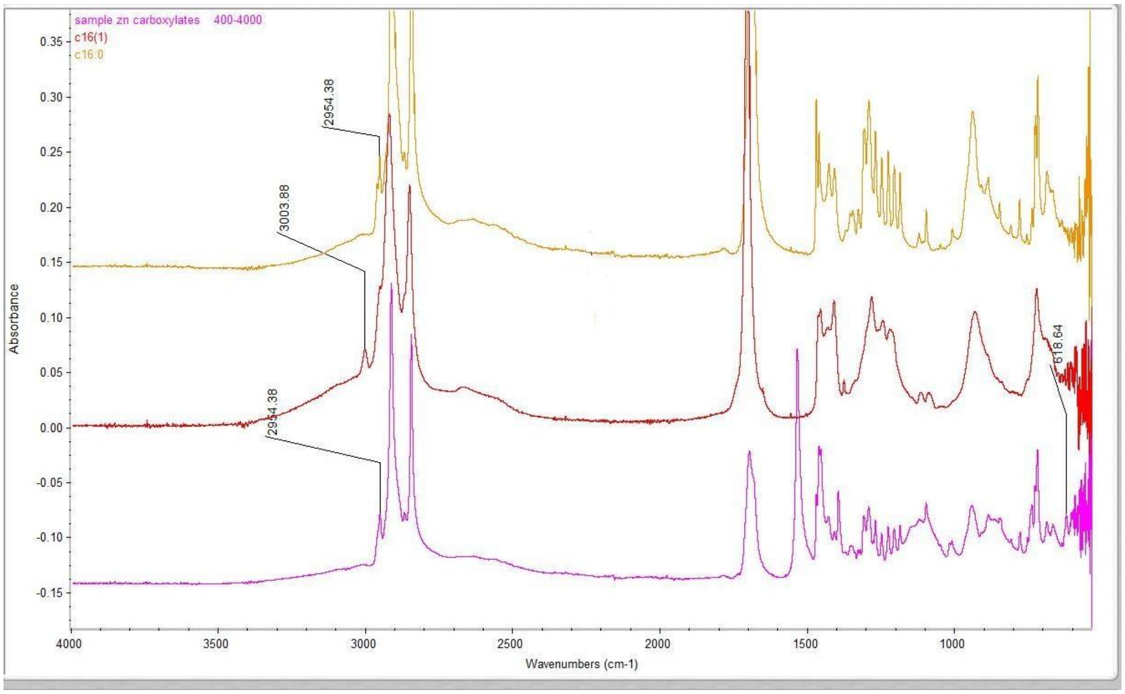

Figure 13: Spectra of C16:0 FA, C16:1 FA and zinc carboxylate precipitate

A stretch band above 3000 cm^{-1} indicates the presence of a alkene or “=C-H” bond(Volland, 1999), which can also be verified by looking at the differences in the spectra of C16:0 FA and C16:1 FA in figure 13- where there’s a distinct stretch band at 3003 cm^{-1}. The spectra for the zinc precipitate did not contain a stretch band above 3000

cm^{-1}, indicating the absence of a carbon-carbon double bond in the precipitate. One distinct stretch band to be noted in the case of the precipitate is the one just above 1500 cm^{-1} -- which is indicative of the asymmetric carboxylate vibration in the IR spectra [$\nu_{as}(COO-)$] (Zeleňák, Vargová, & Györyová, 2007). It also confirms the presence of a zinc forming complex with the carboxylate group of the fatty acid (Zeleňák et al., 2007) and this case, a complex with the C16:0 FA.

4.5 Computational Chemistry Calculations

The Gibbs free energies of reactions (generated from computational chemistry calculations) are given in Table 5-8. Table 9 gives information on the solubility of metal salts in methanol. The last column of each table gives the difference between the Gibbs free energy of the reaction of the metal ion with the with palmitoleic acid and with palmitic acid. If this value is negative, then it can be hypothesized that the reaction of the metal ion with palmitic acid is more favorable than that with palmitoleic acid.

According to Table 5, negative Gibbs free energies for in column 6 indicate that Zn^{2+} has a tendency to complex with both C16:0 FA ion and C16:1 FA spontaneously. A more negative value with palmitic acid would indicate a preference for forming a complex with the metal ion when palmitic acid is mixed with palmitoleic acid. But in this case, the difference of Gibbs free energies of both reactions is just 3.25 KJ/mol which is negligible. Hence, phenomenon of selective precipitation of saturated FA cannot be explained through theoretical calculations alone.

Table 5: Computational calculations of Zn^{2+}– fatty acid chemistry

	Fatty acid ion (-1) (Ha)	Zn^{2+} (Ha)	Total Energy of Rectants (Ha)	Zinc Carboxylate (Ha)	Gibbs Free Energy (Ha)	Difference of Gibbs Free Energies (C16:0 - C16:1) (KJ/mol)
Palmitic acid	-807.778275	-1768.29	-3383.84747	-3384.88857	-1.041103	-3.257799999
Palmitoleic acid	-767.825167	-1768.29	-3303.94125	-3304.9811	-1.03985	

In the case for Cu^{2+}, as can be seen from table 6, Gibbs free energies for both reactions are negative - indicating that Cu^{2+} also has the potential to spontaneously form complexes with both forms of FAs. A more negative value with palmitic acid would indicate a preference for forming a complex with the metal ion when palmitic acid is mixed with palmitoleic acid. But in this case, the difference of Gibbs free energies of both reactions is just 2.76 KJ/mol which is negligible. Hence, phenomenon of selective precipitation of saturated FA by addition of Cu^{2+} cannot be explained through theoretical calculations alone.

. Table 6: Computational calculations of Cu^{2+}– fatty acid chemistry

	Fatty acid ion (-1) (Ha)	Cu^{2+} (Ha)	Total Energy of Reactants (Ha)	Copper Carboxylate (Ha)	Gibbs Free Energy (Ha)	Difference of Gibbs Free Energies (C16:0 - C16:1) (KJ/mol)
Palmitic acid	-807.778275	-1630.13	-3245.68509	-3246.72107	-1.035988	-2.761200001
Palmitoleic acid	-767.825167	-1630.13	-3165.77887	-3166.8138	-1.034926	

Fe^{2+} also behaved consistent to above cases: As can be seen from table 7, Gibbs free energies of Fe^{2+} complexing with both forms of FAs are negative indicating thermodynamic favorability. However, in this case, the reaction with palmitoleic acid is more favored. Nevertheless, experimentally, no precipitate was observed either with palmitic acid nor palmitoleic acid. This can be attributed to the fact that the solubility of ferrous sulphate is negligible in alcohols (Oldberg & Long, 1891). For both reactions to proceed, the availability of Fe^{2+} ions is essential.

Table 7: Computational calculations of Fe^{2+}– fatty acid chemistry

	Fatty acid ion (-1) (Ha)	Fe^{2+} (Ha)	Total Energy of Reactants (Ha)	Ferrous Carboxylate (Ha)	Gibbs Free Energy (Ha)	Difference of Gibbs Free Energies (C16:0 - C16:1) (KJ/mol)
Palmitic acid	-807.778275	-1255.39	-2870.94686	-2871.91519	-0.968329	67.8834
Palmitoleic acid	-767.825167	-1255.39	-2791.04064	-2792.03508	-0.994438	

To validate the results, a calculation was run with select metal ions that did not form any precipitates experimentally. In the case of Co^{2+}, it can be seen from table 8 that Gibbs free energies for formation of complexes with both forms of FAs are positive and thus non-spontaneous (even though cobalt chloride is soluble in methanol indicating readily available Co^{2+} ions ("Cobaltous Chloride, Hexahydrate :Safety Data Sheet,")

Table 8: Computational calculations of Co^{2+}– fatty acid chemistry

	Fatty acid ion (-1) (Ha)	Co^{2+} (Ha)	Total Energy of Reactants (Ha)	Cobalt Carboxylate (Ha)	Gibbs Free Energy (Ha)	Difference of Gibbs Free Energies (C16:0 - C16:1) (KJ/mol)
Palmitic acid	-807.778275	-1373.78	-2989.33515	-2871.76541	117.569746	78.1196
Palmitoleic acid	-767.825167	-1373.78	-2909.42894	-2791.88924	117.5397	

Table 9: Solubility of metal ions in methanol

$ZnSO_4$(heptahydrate)	Soluble ("DOSE/Dictionary of substances and their effects,")
$CuSO_4$ (pentahydrate)	Soluble (Haynes, 2011)
$FeSO_4$ (heptahydrate)	Insoluble / Sparingly Soluble (Oldberg & Long, 1891)
$CoCl_2$ (hexahydrate)	Soluble ("Cobaltous Chloride, Hexahydrate :Safety Data Sheet,")

Theoretical calculations of Gibbs free energies for reactions of various metal ions were inconclusive. So, it can be said that, energetically, there isn’t much difference in how reaction of metal ions with either a saturated FA or unsaturated FA will proceed. Although, experimental results have shown that saturated FA forms a precipitate with certain metal salts in the solution. Hence, structural differences of palmitic acid and palmitoleic acid as well as those between the complex formation of metal and fatty acids have to be taken into account. As seen in figure 14 and 15, because of the presence of a double bond, there’s a bend in the carbon backbone of palmitoleic acid, while palmitic acid is straight-chained. This difference in spatial arrangements of both the molecules makes the carbolylic group of the straight-chained saturated FA more easily available for metal ions to form a complex with.

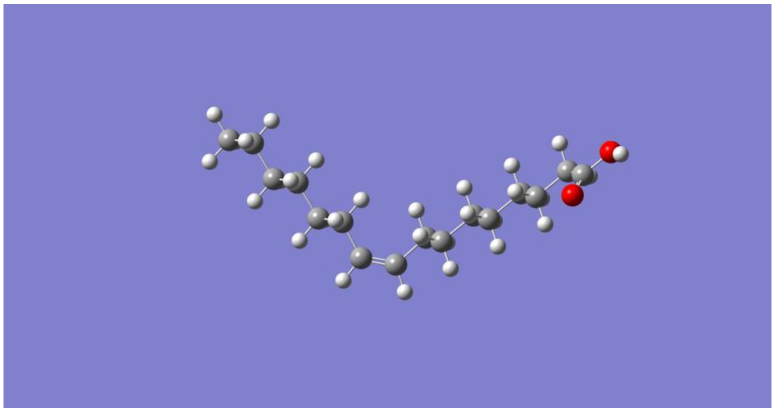

Figure 14: Optimized structure of palmitoleic acid

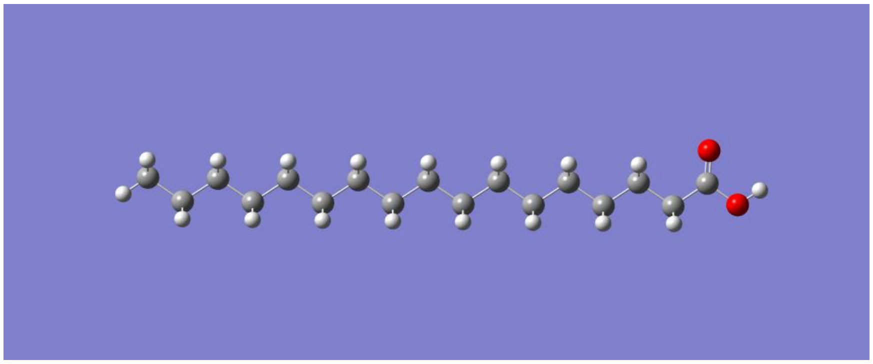

Figure 15: Optimized structure of palmitic acid

Optimized structures of zinc pamitate and zinc palmitoleate are given in figure 16 and 17. The energy for an optimized molecule of zinc palmitate is -3385.811 Hatrees while that of zinc palmitoleate is -3305.797 Hatrees. Hence the complex between zinc and C16:0 is much more energetically stable that that with C16:1 FA. In figure 12 and 13, it is visible that the structure of zinc palmitate is linear when compared to that of zinc palmitoleate. Hence zinc palmitoleate molecules can stack themselves in a closely packed arrangement, promoting in precipitation and crystallization of the compound. These structural and energetic differences between the molecules of zinc pamitoleate and zinc palmitate could be the reason why zinc ions (and other metal ions) can easily form precipitates with palmitic acid than with palmitoleic acid.

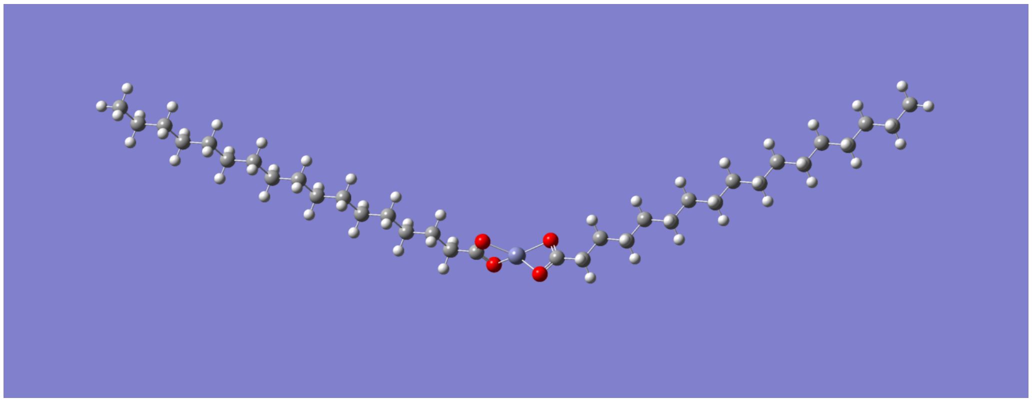

Figure 16: Optimized structure of zinc palmitate

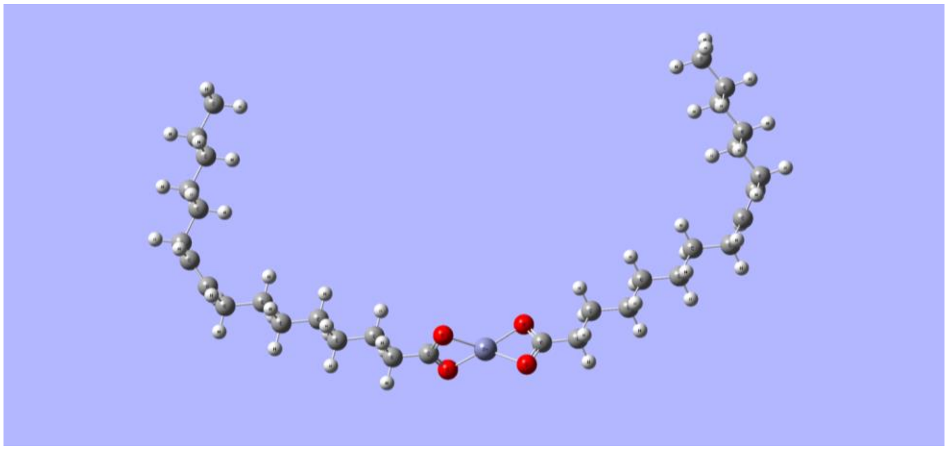

Figure 17: Optimized structure of zinc palmitoleate

5. CONCLUSIONS[*]

Conclusions for specific objectives listed in chapter 2 are as follows:

Conclusion for specific objective 1: 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.

Conclusion for specific objective 2: Transition metal salts such as $AgNO_3$, $CuSO_4$ and $ZnSO_4$ were able to effectively separate the unsaturated FA form from the mixture of saturated and unsaturated FA. Transition metal salts such as $AgNO_3$, $ZnSO_4$ and $CuSO_4$ form complexes preferentially with palmitic acid precipitating it out from a mixture containing both saturated palmitic (C16:0) and unsaturated palmitoleic (C16:1) acid forms in methanol.

Conclusion for specific objective 3: A concentration of 30 mg/ml of $ZnSO_4$ can precipitate about 58 % of C16:0 FA, leaving about 90% of C16:1 FA still in the solution.

Conclusion for specific objective 4: Theoretical calculations of the differences in Gibbs free energies of the saturated FA and unsaturated FA with metal ions were inconclusive.

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The free energy values, though showing a more favorable tendency for the saturated fatty acid to form complexation with the select metal ions, do not indicate conclusive evidence due to the relatively small numerical differences. However, differences in the optimized structure of palmitic acid and palmitoleic acid and their respective carboxylates with metals indicate that complex formation and precipitation is enhanced in the case of the saturated FA due to its more stable straight-chained structure that in turn favors stacking.

All experiments were carried out at room temperature and atmospheric pressure; therefore, the ability of such metal ions to form complexes with select forms of fatty acids needs to be explored further for the potential use for energy efficient and less costly large scale separation of fatty acids. Since these experiments were carried out at room temperature and pressure, this technique is worth further exploration for low-cost separation of high-value fatty acids such as DHA, EPA, and ALA. Future studies also should be aimed at removal of metal ions from the solution and further purification of fatty acids.

Computational chemistry tools such as Gaussian made it possible to some extent to explain the selective precipitation of metal ions with saturated FA. Future computational studies should involve the study of interaction of solvent molecules with metal salts as well as that with fatty acids so that computational tools can be used to make reasonable predictions before experimentation in the laboratory.

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APPENDIX

Table 10: Data for results for study where interaction of metal ions in aqueous solution with organic solution of C16:0 FAME and C16:1 FAME was checked

Type of Aqueous Solution ↓	Difference in Decrease of Concentration (C16:1-C16:0) at 0 minutes (%)	Difference in Decrease of Concentration (C16:1-C16:0) at 5 minutes (%)	Difference in Decrease of Concentration (C16:1-C16:0) at 15 minutes (%)	Difference in Decrease of Concentration (C16:1-C16:0) at 30 minutes (%)	Difference in Decrease of Concentration (C16:1-C16:0) at 50 minutes (%)
Blank	4.272915999	2.66670097	2.313154178	2.549300979	2.677097792
Blank	-2.205506427	-2.30354	-2.083383148	-1.964142171	-1.967350129
Blank	-0.166879734	-1.704576501	-0.166137639	-0.204185884	-0.130924539
Silver	4.439483293	3.265841523	3.704114892	3.113549098	5.673063881
Silver	16.92450961	14.55603627	17.11406347	19.22501415	19.50036326
Silver	4.493506393	5.547146496	4.832551337	4.803240451	5.176993529
Copper	2.645212149	2.685654003	2.733591194	3.018724783	2.747488003
Copper	2.739419771	1.90406698	2.235742799	2.508458623	2.080063951
Copper	5.657228085	5.344065858	5.129080968	6.292722576	6.142151747

Table 11: Data for interaction metal ions with a solution of C16:0 FA and C16:1 FA in methanol

Type of Aqueous Solution ↓	C16:0 FA Reduction in Concentration (%)	C16:1 FA Reduction in Concentration (%)	Difference in Reduction of Concentration (C16:0 FA - C16:1 FA) %
Blank	9.197795796	7.389985464	1.807810332
Blank	13.43742672	15.39109371	-1.953666991
Blank	4.817985773	6.690132309	-1.872146536
Copper	33.40369512	23.58206862	9.821626497
Copper	23.41892433	16.88187857	6.537045758
Copper	23.7690661	15.69493718	8.074128914
Silver	21.54745096	11.16895186	10.3784991
Silver	11.37048703	2.135585922	9.234901107
Silver	11.22795022	0.173993838	11.05395638
Zinc	11.38661284	0.224986146	11.1616267
Zinc	15.94358188	8.453321004	7.490260881
Zinc	21.21465733	10.27484304	10.93981429

Table 12: Data for varying concentration of $ZnSO_4$ and its effect on a mixture of C16:0 FA and C16:1 FA in methanol

$ZnSO_4.7H_2O$ (mg/ml)	Reduction in concentration of C16:0 FA (%)	Reduction in concentration of C16:0 FA (%)	Difference in reduction of Concentration (C16:0-C16:1) (%)
5	11.38661	0.224986	11.16163
5	15.94358	8.453321	7.490261
5	21.21466	10.27484	10.93981
10	39.60462	15.31672	24.28789
10	36.02163	11.92388	24.09776
10	37.95178	5.431238	32.52055
20	42.97652	5.329255	37.64726
20	49.50966	9.53268	39.97698
20	50.39416	13.05956	37.33459
30	61.19902	17.9022	43.29683
30	54.40789	9.643262	44.76462
30	56.05664	7.898705	48.15793
40	42.23549197	7.045284951	35.19020702
40	46.89498509	3.71679037	43.17819472
40	49.78859984	9.424123288	40.36447655