

Separation of Palmitic and Palmitoleic Acid using Transition Metal Salts: Part 2

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ABSTRACT

Fatty acids (FAs) play a major role in many physiological functions and offer various health benefits. Separation and isolation of fatty acids thus is of great interest. A set of experiments were conducted to evaluate the effectiveness of select transition metal salts in separating a saturated FA from mixture of C16:0 and C16:1 FAs. In an initial study, ZnSO₄, CuSO₄ and AgCl were able to cause significant precipitation of C16:0 FA from a mixture of C16:0 and C16:1 FAs in methanol. A follow-up study was conducted to ascertain the effect of ZnSO₄ concentration on the ability to separate individual FAs from a mixture of saturated and unsaturated FAs in methanol. Results indicated that a 30 mg/ml ZnSO₄ solution could remove 58% of C16:0 FA from a mixture containing both the saturated and the unsaturated FA forms. IR spectra suggests formation of a complex between zinc ion and the carboxylate group of the C16:0 FA, which in turn may cause precipitation. Computational chemistry calculations indicate that specific complex formation with the saturated FA might be due to more favorable thermodynamics between zinc ion - C16:0 FA complexation (with more negative value of Gibbs free energy of the reaction) than that between the metal ion and the unsaturated FA.

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 contribute to many health effects. Palmitic acid has been proven to mediate hypothalamic insulin resistance [1]; Palmitoleic acid has been shown to boost insulin sensitivity thus improving hyperglycemia and hypertriglyceridemia [2]. Saturated FAs (SFA) are engaged in the synthesis of phospholipids and spingolipids, storage and production of energy, and lipid transport [3]. Poly-unsaturated fatty acids (PUFAs), especially the omega-3 fatty acids

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is reported to provide cardiovascular health benefits when integrated into the diet [4]. Many studies omega-3 fatty acids have shown that supplementation may render preventative and therapeutic effects on certain cancers including those of the prostate, colon, rectum, and breast, among others.[4] Therefore, isolation and separation of unsaturated fatty acids is of significant interest.

Various methods have been used for separation and isolation of saturated and unsaturated fatty acids: these include chromatography techniques such as gas chromatography[5-8], thin layer chromatography[9], and high pressure liquid chromatography[10, 11]; winterization; and urea complexation.[12, 13]¹

Silver ion chromatography is one of the most effective techniques used in fatty acid separation. This technique uses the property of olefinic compounds forming a complex with transition metals (especially silver ions), resulting in separation of fatty acids based on the degree of unsaturation. 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 [14]. This property to form charge-transfer type complexes with transition metal ions has been widely adapted in high-performance liquid chromatography based olefin separation [15].

The property of silver ion complexation with olefinic double bonds has also been used to extract fatty acid ester derivatives from various organic solvents [16]. To give an example, separation of ethyl esters of eicosapentaenoic acid and docosahexaenoic acid had been attempted via circulating liquid membranes using AgCl as a carrier [17]. Another example involves the use of supercritical extraction (SC) to extract polyunsaturated fatty acid ethyl esters (PUFA-Et) by aqueous AgCl[18].

Another method to separate fatty acids uses the property of particular metal ions' ability of form precipitates with certain FAs. An example includes the use of alkali metal salts in combination with alkali metal hydroxides to precipitate out free fatty acids in the form of insoluble fatty acid salts [19].

Certain metallic ions demonstrate the ability to form precipitates with specific types of fatty acids and this property has been utilized to isolate 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 [19]. In another example, saturated straight chain aliphatic monocarboxylates have been separated using the property of Cu, Fe, Zn, Pb and Mg to form complexes with the monocarboxylates[20-22].

A method widely used for large scale separation of fatty acid is urea complexation. In this method, a homogenous solution of urea and free fatty acids/saturated fatty acids is formed in alcoholic solvent at 65°C. This is followed by cooling and separation of the resultant urea complex to room temperature [13]. Studies reveal that in a mixture containing both saturated and unsaturated FA forms, by using urea complexation, the SFA concentration was decreased to almost zero from an initial concentration of 4.5 % while the PUFA concentration was increased from an initial concentration of 33.5 % to 53.87 % [23]. The process is considered uncomplicated, quick, cheap, robust and environmental friendly [12, 13].

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Winterization is another method adaptable for large scale separation of fatty acids. The 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 [24]. 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%[25].

Currently used large-scale separation techniques such as urea complexation and winterization, besides being 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 [26] whereas during winterization, temperature is decreased to -18°C[26]), are not quite selective. Chromatographic techniques are selective, but expensive (in terms of energy requirements) and difficult to scale-up. The essence of this study was to test methods that are scalable and energetically less costly.

During preliminary studies, the ability of different metal salts to selectively precipitate saturated FAs from a mixture containing both saturated and unsaturated FAs dissolved in methanol were evaluated [27]. Once a suitable salt was identified (in this case zinc sulphate), the effect of increasing ion concentration on selective precipitation of saturated (palmitic acid) from a mixture containing palmitic and palmitoleic acid in methanol was evaluated. **Spectroscopic** and computational studies were conducted to verify the composition and mechanistic aspects of formation of precipitates respectively.

2. EXPERIMENTAL DETAILS

2.1 Preliminary Studies

Preliminary studies were aimed at elucidating interaction of various transition metal salts with saturated and unsaturated fatty acids. For this purpose, 100 mg each of palmitic acid (saturated) and palmitoleic acid (unsaturated) were mixed in methanol. Approximately 0.17×10^{-3} moles of metal salts such as AgCl, CuSO₄, ZnSO₄, MnCl₂, FeSO₄, FeCl₃ and CoCl₂ (all salts were hydrated except for AgCl) were added individually to the solution of palmitic acid and palmitoleic acid in methanol. **Experiments were carried out at room temperature.** In the case of AgNO₃, CuSO₄ and ZnSO₄, immediate precipitate formation was observed as seen in photo 1, while in case of the other salts, no precipitation was observed (as seen in photo 2).

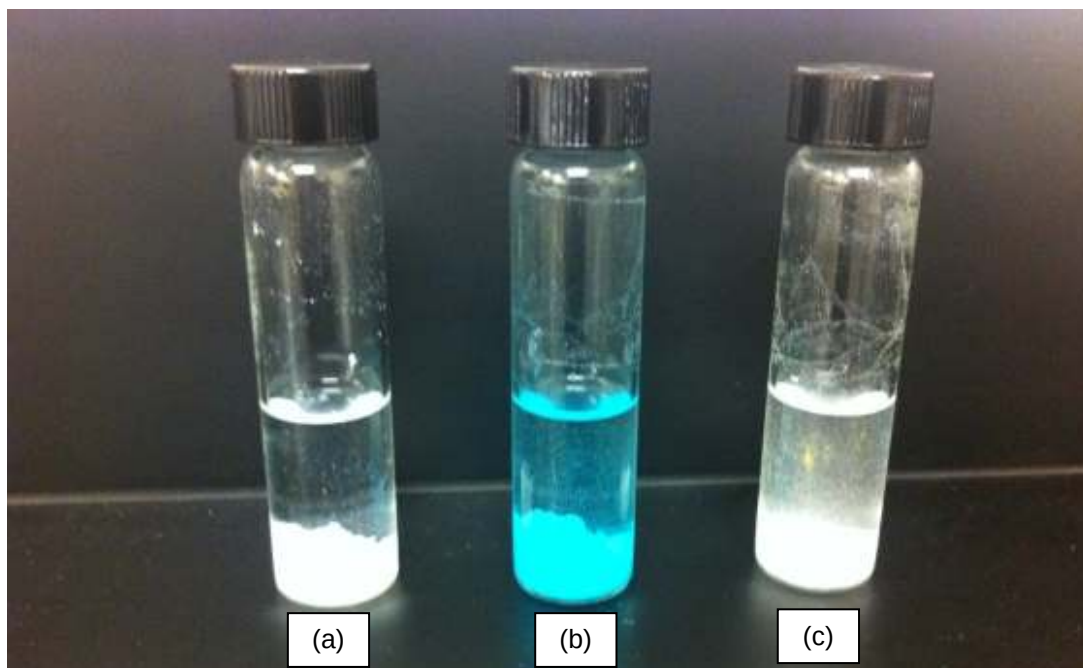


Photo 1: (Left to Right) a) AgCl b) CuSO₄ c) ZnSO₄ showing clear precipitation of saturated FA from a mixture containing both saturated and unsaturated FA

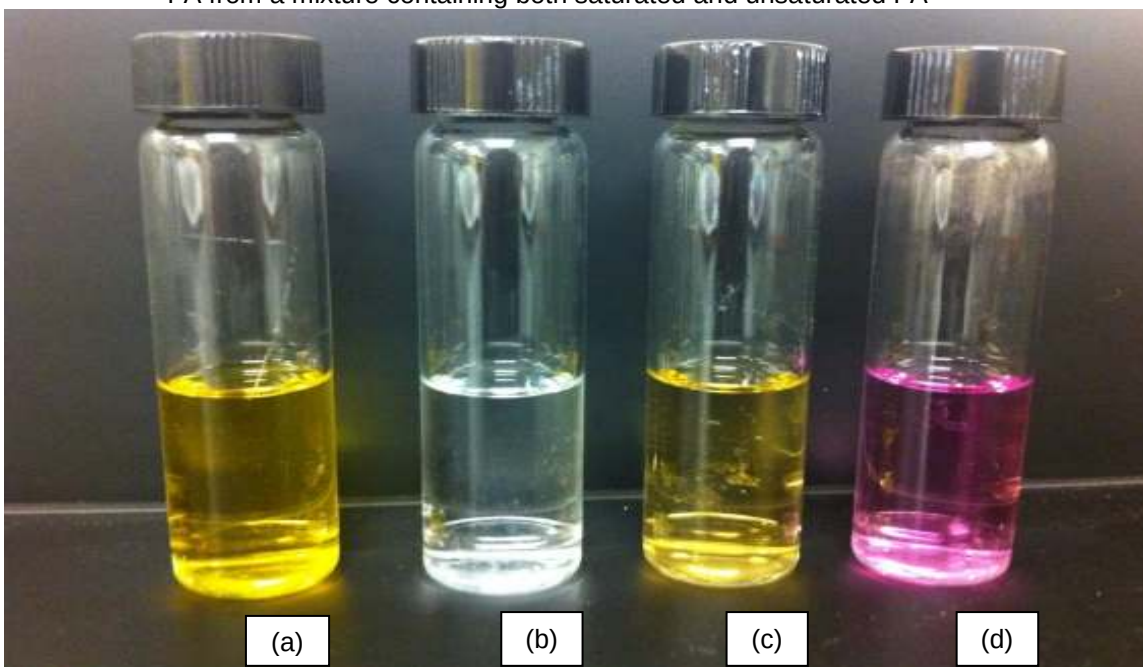


Photo 2: (Left to Right) a) Ferrous Sulphate b) Manganese Chloride c) Ferric Chloride d) Cobalt Chloride did not show any precipitation from a mixture containing both saturated and unsaturated FA

For metal salts showing immediate precipitation, studies were conducted to measure the decrease in the amount of fatty acids in the solution. Once AgCl, CuSO₄ and ZnSO₄ were added individually to the solution of FAs in methanol, the precipitate was allowed to settle for 24 hours, after which, the precipitate was filtered out and the supernatant was analyzed for

the fatty acids concentration. FAs were first transesterified to FAMES using 4% solution of H_2SO_4 in methanol. The concentration of FAMES was estimated using a GC-FID. The procedure was repeated thrice from every metal salt. As the internal standard, a blank solution 100 mg of C16:0 and 100 mg of C16:1 FA in methanol was prepared - without addition of any metal salts. Figure 1 represents the results. The response of the study is measured as the difference is decrease of concentration of palmitoleic acid from the decrease in palmitic acid (decrease in concentration C16:0 – decrease in concentration of C16:1).

2.2 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. The working hypothesis for this experiment is that increasing the concentration of metal ion will lead to an increase in precipitation of either the saturated fatty acid, since more zinc ions will be available to interact with the carboxylate group of the saturated fatty acids molecules.

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°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.

2.3 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°C 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 $500\text{--}4000\text{ cm}^{-1}$. Pure samples of palmitic acid and palmitoleic acid were also analyzed using FTIR-ATR to compare their spectra to that of the precipitate.

2.4 Computational Chemistry Calculations

In order to explain the experimental results, theoretical calculations were performed. Computational chemistry software, GaussView 5 and Gaussian 09 [28] 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: this means that the fatty acid molecules were ionized in solution, giving the carboxylate group of the FA a net negative charge. The metal ions (in methanol) possess a net positive charge. It was also observed from earlier studies[27] 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, following computational chemistry calculations were undertaken:

- 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[28];
- 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; and
- 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.

3. RESULTS AND DISCUSSION

3.1 Preliminary Studies

Figure 1 depicts the differences of the decrease of concentration of palmitoleic acid from the decrease in palmitic Acid (decrease in concentration C16:0 – decrease in concentration of C16:1).

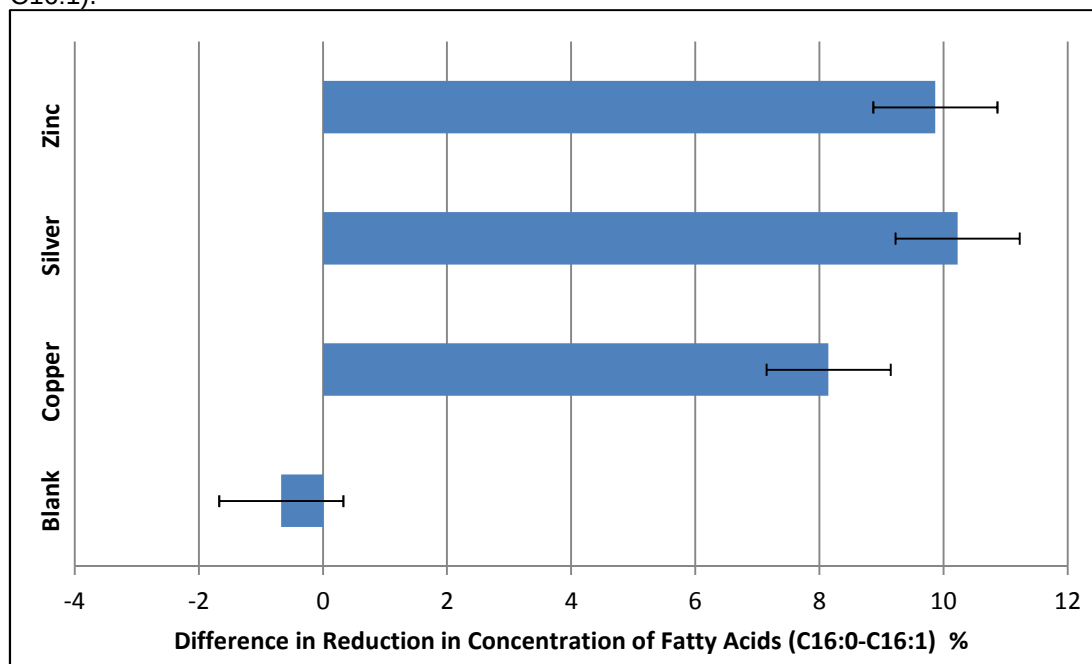


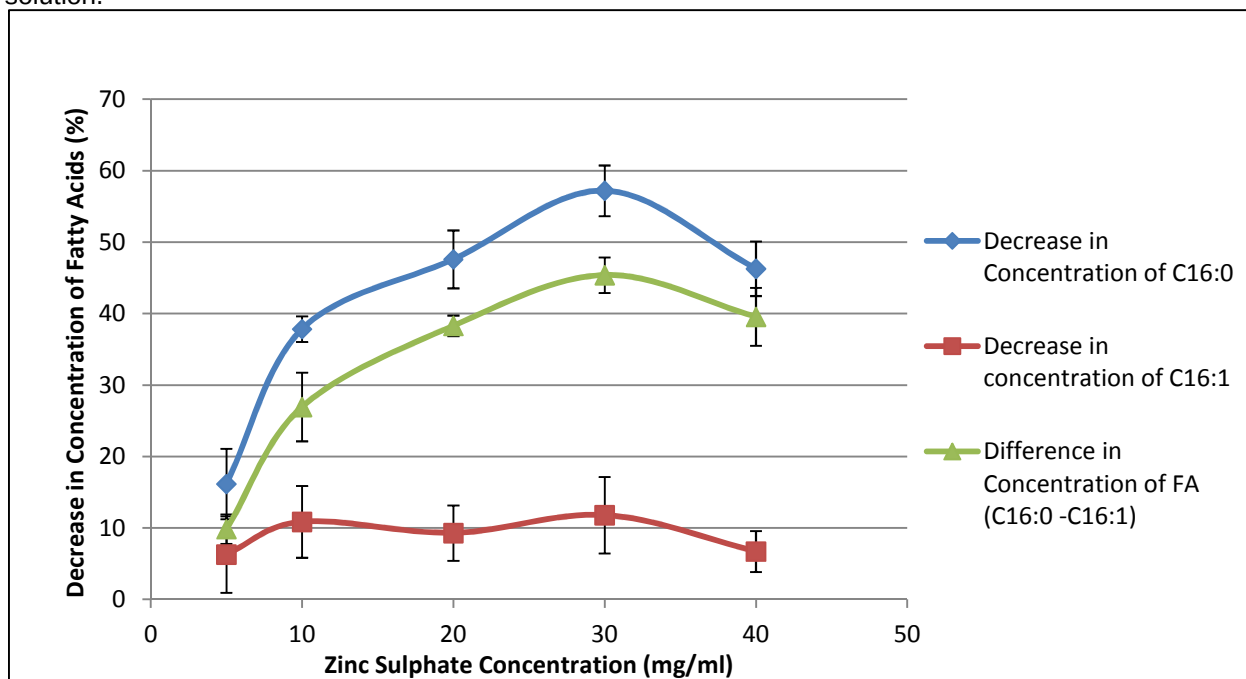
Figure 1: Results of preliminary studies showing that metal salts selectively precipitate the saturated fatty acid (C16:0) more than the unsaturated fatty acid (C16:1)

From figure 1 it can be seen that copper, silver and zinc ions are effective in selectively precipitating out the saturated fatty acid from the solution. Considering cost and health benefits, it was decided to carry out further experiments with zinc [29] (sulphate).

3.2 Optimization of ZnSO₄ Concentration

This study was geared toward ascertaining whether the ZnSO₄ concentration has any impact on the amount of FAs that can be selectively separated. Figure 2 depicts how increased concentrations of ZnSO₄ lead to enhancement of the selective precipitation of the saturated FA (in comparison to unsaturated FA). The Y-axis in figure 2 is a measure of the difference

218 in reduction of concentration of the unsaturated FA from that of the saturated FA. A positive
 219 difference indicates that the amount of C16:0 FA outweighs the amount of C16:1 in the
 220 solution.



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 223 **Figure 2:** Fatty acid precipitation with increasing concentration of ZnSO_4
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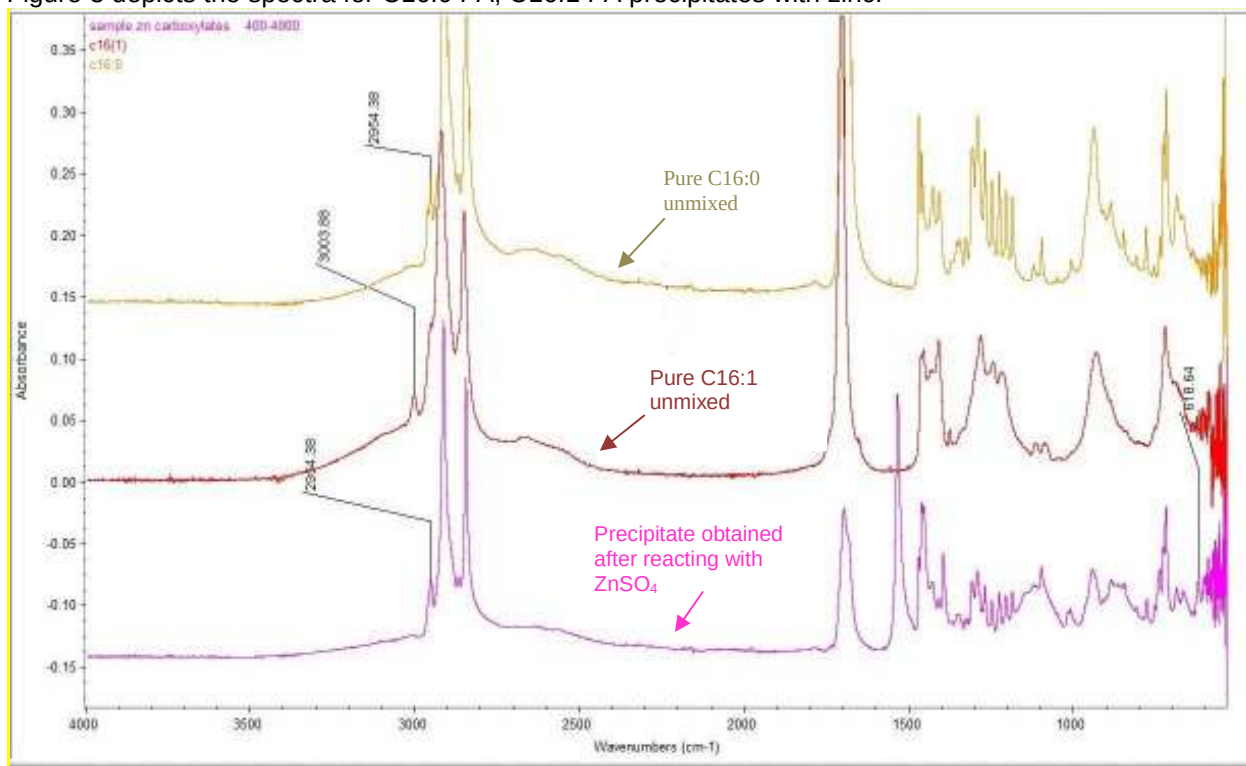
225 An Analysis of variance (ANOVA) was conducted using Design-Expert (version 9.0.3.1) with
 226 concentration of ZnSO_4 as factors with 5, 10, 20, 30 and 40 mg/ml as levels. The ANOVA
 227 resulted in a p-value of 0.0002 - which is significant. This means that varying concentration
 228 of ZnSO_4 significantly affects the concentration difference between C16:1 FA and C16:0.
 229 From figure 2, it can be confirmed that increasing the ZnSO_4 concentration up to about 30
 230 mg/ml leads to an increase in the relative amount of C16:0 FA precipitated. However,
 231 beyond 30 mg/ml ZnSO_4 concentration, the separatory effect seems to taper off. It can be
 232 clearly seen that the decrease in the concentration of C16:1 FA remains more or less
 233 constant at 10% whereas the reduction in concentration of C16:0 FA increases to a
 234 maximum of around 58%.
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236 Another experimental model was analyzed where there were two factors: (a) Amount of
 237 ZnSO_4 added and (b) Type of fatty acid (which in turn has two levels: (i) C16:0 FA and (ii)
 238 C16:1). The response of this model was measured as the decrease in concentration of fatty
 239 acid after the addition of zinc sulphate. The ANOVA for this experimental model resulted in a
 240 p-value of < 0.0001. This means that the model is significant. The data for this experimental
 241 model is also represented in figure 2. Hence it can be surmised that (i) amount of ZnSO_4
 242 added to the solution has a significant effect on the amount of precipitation and (ii) the
 243 saturated fatty acid selectively form a complex with zinc sulphate.
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3.3 FTIR-ATR Analysis of Precipitate

Figure 3 depicts the spectra for C16:0 FA, C16:1 FA precipitates with zinc.



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Figure 3: Spectra of C16:0 FA, C16:1 FA and Zinc Carboxylate precipitate

259 A stretch band above 3000 cm^{-1} indicates the presence of an alkene or “=C-H” bond [30],
260 which can also be verified by observation of the differences in the spectra of C16:0 FA and
261 C16:1 FA in figure 4 -- where there's a distinct stretch band at 3003 cm^{-1} . The spectra for
262 the zinc precipitate did not contain a stretch band above 3000 cm^{-1} -- indicating the absence
263 of a carbon-carbon double bond in the precipitate. One distinct stretch band to be noted in
264 the case of the precipitate is the one just above 1500 cm^{-1} -- which is indicative of the
265 asymmetric carboxylate vibration in the IR spectra [$\nu_{\text{as}}(\text{COO}^-)$] [31]. It also confirms the
266 presence of a zinc forming complex with the carboxylate group of the fatty acid [31] -- and
267 this case, a complex with the C16:0 FA.

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3.4 Computational Chemistry Calculations

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The Gibbs free energies of reactions (generated from computational chemistry calculations) are given in Table 1-4. Table 5 depicts 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 that with palmitic acid. If this value is negative, then it could be conjectured that the reaction of the metal ion with palmitic acid is more favorable than that with palmitoleic acid.

According to Table 1, 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. However, the more negative value with palmitic acid indicates a preference for forming a complex with the metal ion when palmitic acid is mixed with palmitoleic acid. This result is consistent with the observations made experimentally.

Table 1: Computational calculations of Zn^{2+} – fatty acid chemistry

	Fatty acid ion (-1) (Ha)	Zn^{2+} (Ha)	Total Energy of Reactants (Ha)	Zinc Carboxylate (Ha)	Gibbs Free Energy (Ha)	Difference of Gibbs Free Energies (C16:0 - C16:1) (KJ/mol)
Palmitic acid	-807.778	-1768.290	-3383.847	-3384.888	-1.04110	-3.257
Palmitoleic acid	-767.825	-1768.290	-3303.941	-3304.981	-1.0398	

In the case for Cu^{2+} , as can be seen from table 2, 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. However, Cu^{2+} complexation with palmitic acid is favored in the presence of both forms of FAs since the Gibbs free energy with palmitic acid is more negative. This is again consistent with the results obtained experimentally.

Table 2: 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	-807.778	-1630.130	-3245.685	-3246.721	-1.0360	-2.761
Palmitoleic acid	-767.825	-1630.130	-3165.779	-3166.813	-1.0350	

Fe^{2+} also behaved consistent to the above cases: As can be seen from table 3, 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 or palmitoleic acid. This can be attributed to the fact that the solubility of ferrous sulphate is negligible in alcohols [32]. For both reactions to proceed, the availability of Fe^{2+} ions is essential.

Table 3: 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	-807.778	-1255.390	-2870.947	-2871.915	-0.968	67.883

Palmitoleic acid	-767.825	-1255.390	-2791.041	-2792.035	-0.994	
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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 4 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 [33]).

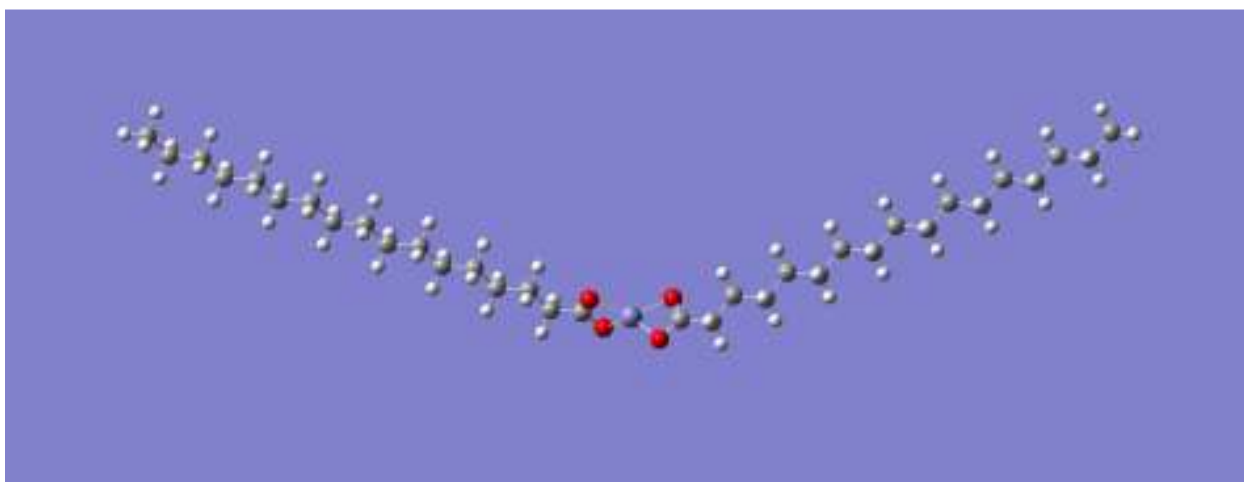
Table 4: Computational calculations of Co^{2+} – fatty acid chemistry

	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	-807.778	-1373.780	-2989.335	-2871.765	117.569	78.119
Palmitoleic acid	-767.825	-1373.780	-2909.429	-2791.889	117.539	

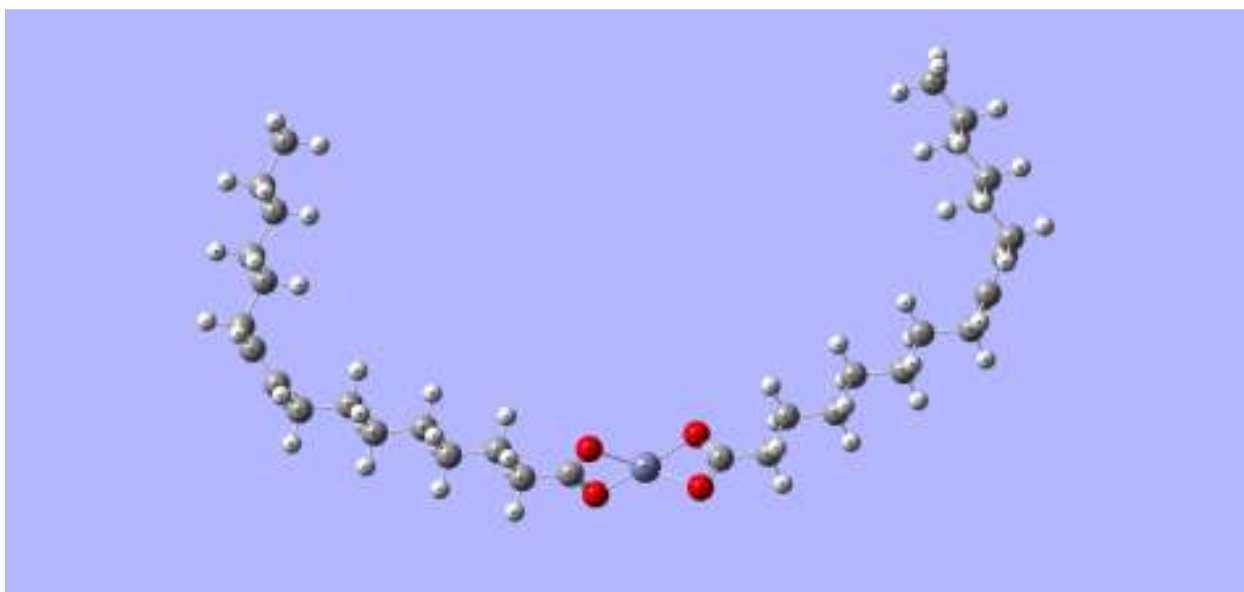
Table 5: Solubility of metal ions in methanol

ZnSO_4 (heptahydrate)	Soluble [34]
CuSO_4 (pentahydrate)	Soluble [35]
FeSO_4 (heptahydrate)	Insoluble / Sparingly Soluble [32]
CoCl_2 (hexahydrate)	Soluble [33]

Optimized structures of zinc pamitate and zinc palmitoleate are given in figure 4 and 5. 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 than 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 the linear palmitic acid than with the skewed palmitoleic acid.



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Figure 4: Optimized structure of zinc-palmitate

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Figure 5: Optimized structure of zinc palmitoleate

330 Differences in the optimized structure of palmitic acid and palmitoleic acid and their
331 respective carboxylates with metals indicate that complex formation and precipitation is
332 enhanced in the case of the saturated FA due to its more stable straight-chained structure
333 that in turn favors stacking.

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4. CONCLUSIONS

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339 Transition metal salts such as AgCl , ZnSO_4 and CuSO_4 form complexes preferentially with
340 palmitic acid helping precipitating it out from a mixture containing both saturated palmitic
341 (C16:0) and unsaturated palmitoleic (C16:1) acid forms in methanol. A concentration of 30
342 mg/ml of ZnSO_4 can precipitate about 58 % of C16:0 FA, leaving about 90% of C16:1 FA still
343 in the solution. Theoretical calculations indicate that the saturated FA preferentially forms
344 complexes with these metal ion species (i.e., the Gibbs free energy of the saturated FA-ion
345 complexation is more negative than that of the unsaturated FA-ion complexation reaction). All
346 experiments were carried out at room temperature and atmospheric pressure; therefore, the
347 ability of such metal ions to form complexes with select forms of fatty acids needs to be
348 explored further for the potential use in energy efficient and less costly large scale fatty acid
349 separations. Computational chemistry tools helped to gain insights on the selective
350 precipitation of metal ions with saturated FA to some extent. Future studies should involve
351 combined computational and experimental interrogations to reveal the interaction of solvent
352 molecules with other fatty acids and metal salts so that more accurate predictions could be
353 made.

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