Separation of Palmitic and Palmitoleic Acid Using Transition Metal Salts: Experimental vs Theoritical Analyses

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Authors' contributions

This work was carried out in collaboration between the two authors. The study was designed and conducted under author SF direction and management. Author SK conducted the studies, collected data, performed the statistical analysis, and wrote the first draft of the manuscript. Both authors read and approved the final manuscript.

Original Research Article

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, $ZnSO_4$, $CuSO_4$ 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 $ZnSO_4$ 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 $ZnSO_4$ solution could remove 58% of C16:0 FA from a mixture containing both the saturated and the unsaturated FA forms. IR spectra suggest formation of a complex between zinc ion and the carboxylate group of the C16:0 FA, which in turn may cause

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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.

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 with 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].

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 an 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 forming 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].

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, 100mg 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, $CuSO_4$, $ZnSO_4$, $MnCl_2$, $FeSO_4$, $FeCl_3$ and $CoCl_2$ (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 $AgNO_3$, $CuSO_4$ and $ZnSO_4$, 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).

For metal salts showing immediate precipitation, studies were conducted to measure the decrease in the amount of fatty acids in the solution. Once AgCl, $CuSO_4$ and $ZnSO_4$ 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 in triplicate 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. Fig. 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 an 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.
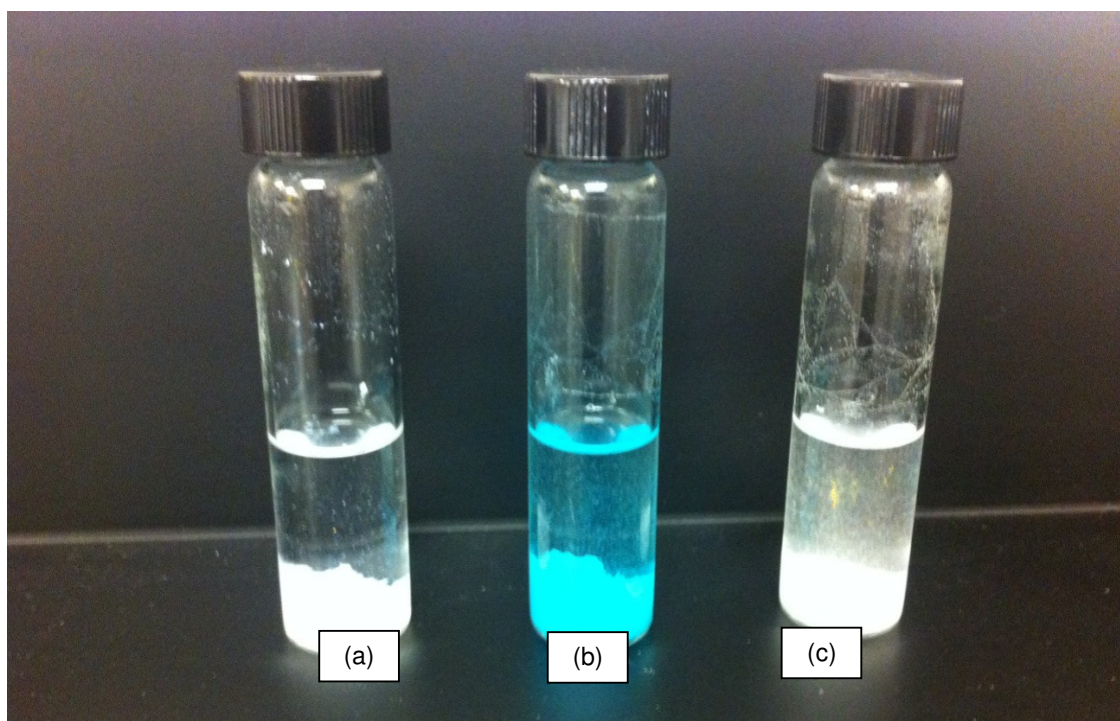
Photo 1. (Left to Right) a) AgCl b) CuSO$_4$ c) ZnSO$_4$ showing clear precipitation of saturated FA from a mixture containing both saturated and unsaturated FA
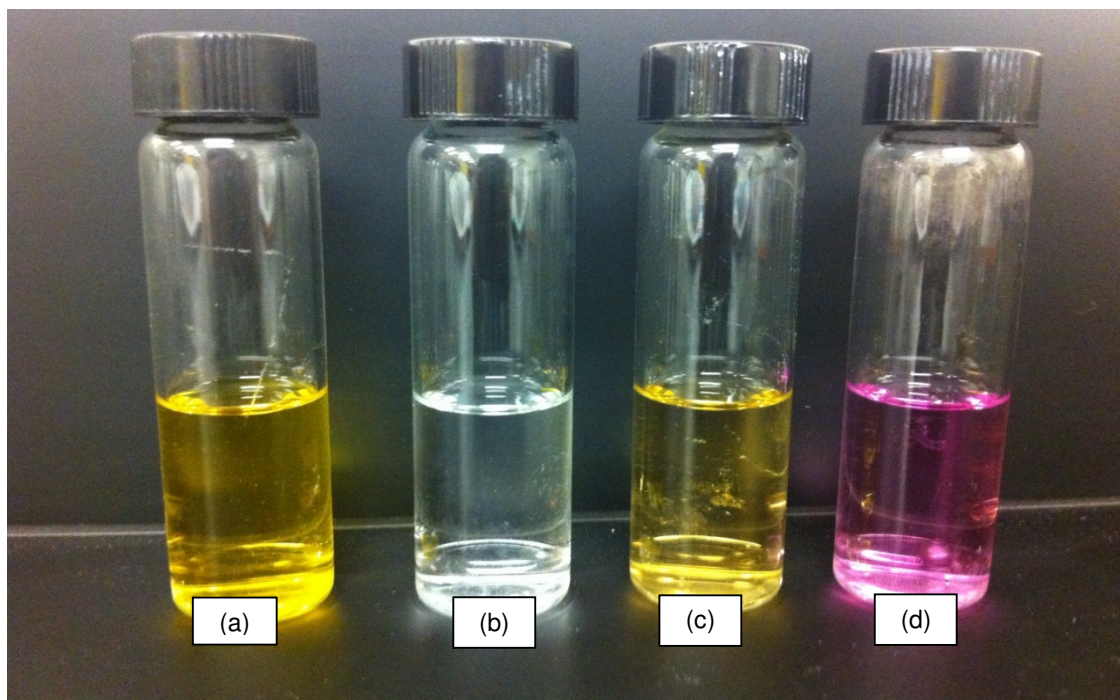

Photo 2. (Left to Right) a) Ferrous Sulphate b) Manganese Chloride c) Ferric Chloride d) Cobalt Chloride did not show any precipitation from a mixture containing both saturated and unsaturated FA

Hexadecanoic acid (C16:0) was obtained from Sigma Aldrich while 9-cis-hexadecanoic (C16:1) acid was purchased from Nu-Chek Prep. A 100mg 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, 50mg 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 120ºC 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 40mg/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 70ºC 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 500-4000cm^{-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, Guass View 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

Fig. 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).

From Fig. 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 $ZnSO_4$ 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. Fig. 2 depicts how increased concentrations of $ZnSO_4$ lead to enhancement of the selective precipitation of the saturated FA (in comparison to unsaturated FA). The Y-axis in Fig. 2 is a measure of the difference in reduction of concentration of the unsaturated FA from that of the saturated FA. A positive difference indicates that the amount of C16:0 FA outweighs the amount of C16:1 in the solution.
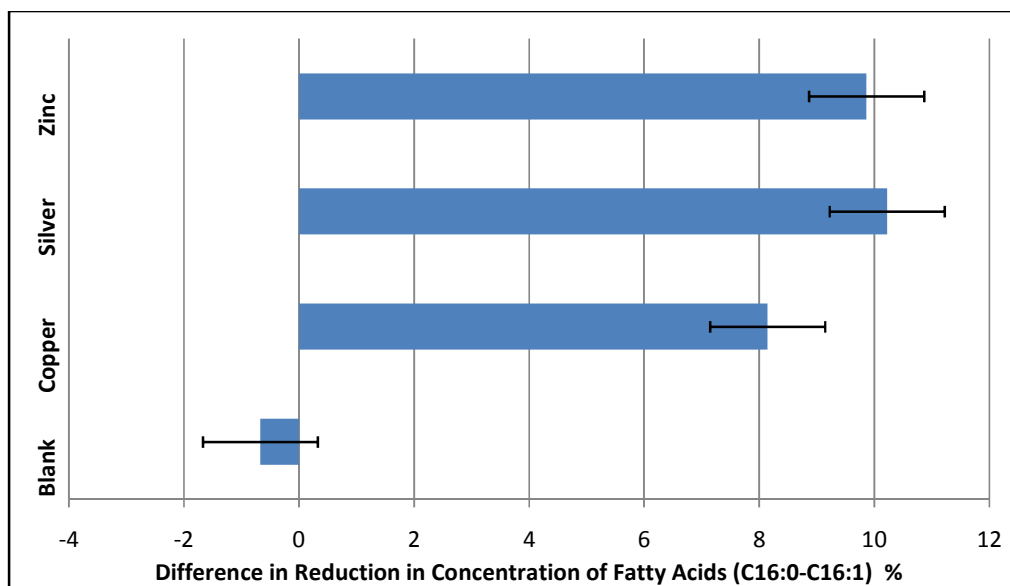
Fig. 1. Results of preliminary studies showing that metal salts selectively precipitate the saturated fatty acid (C16:0) more than the unsaturated fatty acid (C16:1)

An Analysis of variance (ANOVA) was conducted using Design-Expert (version 9.0.3.1) with concentration of $ZnSO_4$ as factors with 5, 10, 20, 30 and 40mg/ml as levels. The ANOVA resulted in a p-value of 0.0002-which is significant. This means that varying concentration of $ZnSO_4$ significantly affects the concentration difference between C16:1 FA and C16:0. From Fig. 2, it can be confirmed that increasing the $ZnSO_4$ concentration up to about 30 mg/ml leads to an increase in the relative amount of C16:0 FA precipitated. However, beyond 30mg/ml $ZnSO_4$ concentration, the separatory effect seems to taper off. It can be clearly seen 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). The response of this model was measured as the decrease in concentration of fatty acid after the addition of zinc sulphate. 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 Fig. 2. Hence it can be surmised that (i) amount of $ZnSO_4$ added to the solution has a significant effect on the amount of precipitation and (ii) the saturated fatty acid selectively form a complex with zinc sulphate.

3.3 FTIR-ATR Analysis of Precipitate

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

A stretch band above 3000cm^{-1} indicates the presence of a alkene or "=C-H" bond [30], which can also be verified by observation of the differences in the spectra of C16:0 FA and C16:1 FA in Fig. 4 -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 [v_{as}(COO–)] [31]. It also confirms the presence of a zinc forming complex with the carboxylate group of the fatty acid [31]-and this case, a complex with the C16:0 FA.
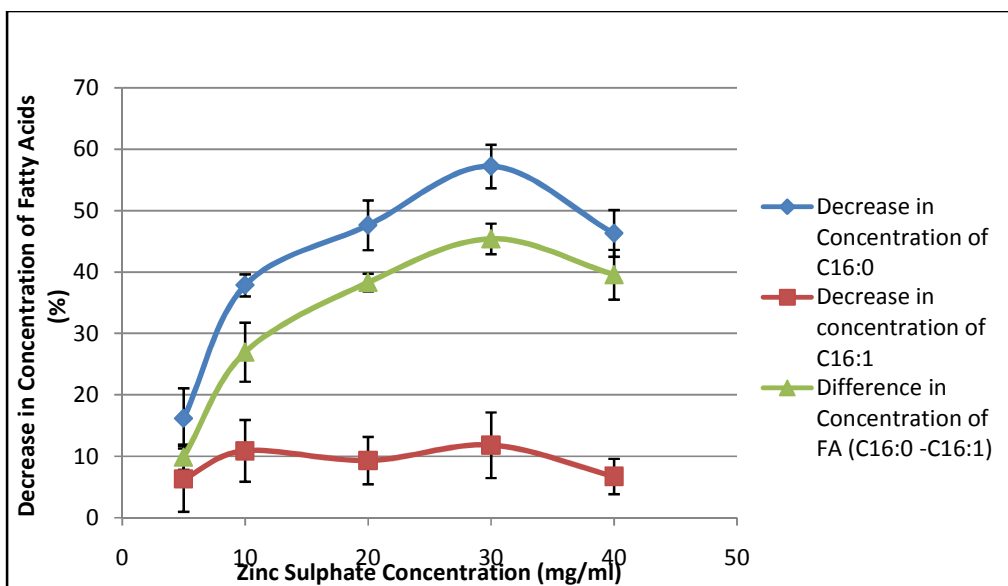
Fig. 2. Fatty acid precipitation with increasing concentration of ZnSO₄

3.4 Computational Chemistry Calculations

The Gibbs free energies of reactions (generated from computational chemistry calculations) are given in Tables 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.

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.

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.

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]).
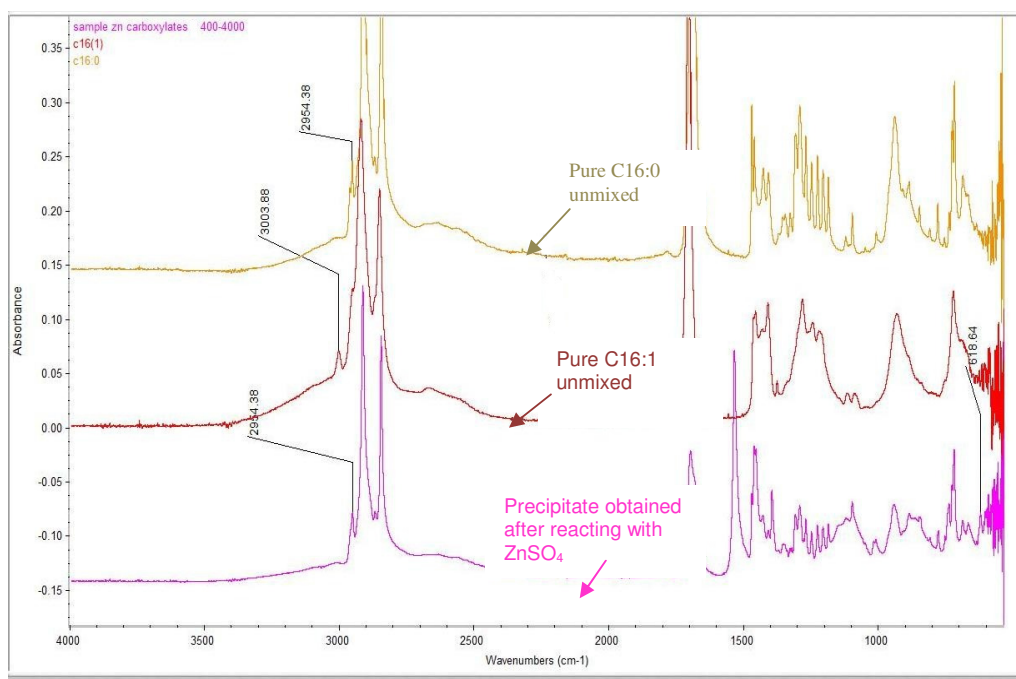
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Fig. 3. Spectra of C16:0 FA, C16:1 FA and Zinc Carboylate precipitate

Table 1. 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	-807.778	-1768.290	-3383.847	-3384.888	-1.04110	-3.257
Palmitoleic acid	-767.825	-1768.290	-3303.941	-3304.981	-1.0398	

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	

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	

**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 Figs. 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 that that with C16:1 FA. In Figs. 4 and 5, 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.

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.
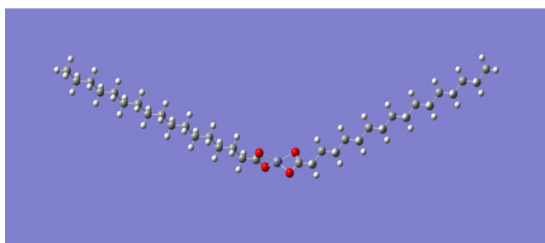
Fig. 4. Optimized structure of zinc-palmitate
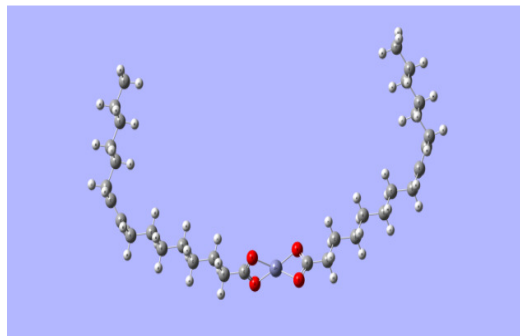
Fig. 5. Optimized structure of zinc palmitoleate

4. CONCLUSION

Transition metal salts such as AgCl, ZnSO$_4$ and CuSO$_4$ form complexes preferentially with palmitic acid helping precipitating the saturated fatty acid out from a mixture containing both saturated palmitic (C16:0) and unsaturated palmitoleic (C16:1) acid forms in methanol. A concentration of 30mg/ml of ZnSO$_4$ can precipitate about 58% of C16:0 FA and only 10% of C16:1 from the solution leaving an unsaturated FA-rich solution behind. Theoretical calculations indicate that the saturated FA preferentially forms complexes with these metal ion species (i.e., the Gibbs free energy of the saturated FA-ion complexation is more negative than that of the unsaturated FA-ion complexation reaction). 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 in energy efficient and less costly large scale fatty acid separations. Computational chemistry tools helped to gain insights on the selective precipitation of metal ions with saturated FA to some extent. Future studies should involve combined computational and experimental interrogations to reveal the interaction of solvent molecules with other fatty acids and metal salts so that more accurate predictions could be made.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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