

JoVE: Science Education
Purification of Complex Organic Mixtures: Saponification
--Manuscript Draft--

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Earth Science Education Title: Purification of Complex Organic Mixtures:
Saponification

Overview:

The product of an organic solvent extraction, a total lipid extract (TLE), is often a complex mixture of hundreds, if not thousands, of different compounds. The researcher is often only interested in a handful of compounds or, if interested in many, may need to remove unwanted constituents that are “in the way” or co-eluting. For example, the concentrations of individual compounds in a sample are often determined on a gas chromatograph coupled to a flame-ionizing detector (GC-FID), because the relationship between FID response (in pA) and the amount of compound in a sample (*e.g.*, ng/ μ L) is both linear and sensitive. The GC portion of the instrument separates different compounds in a sample based on their boiling point, chemical structure, and affinity with a solid phase that can change according to application. The result is a chromatogram (**Figure 1**), showing the separation of different chemical constituents in time, as well as their relative concentration (calculated as the area under the curve). However, sometimes more than one compound elutes off the GC at a time (**Figure 1**). In this case, sample purification is required before compounds can be confidently quantified.

Principles:

FAMES (fatty acid methyl esters) are important constituents of terrestrial and marine microbes and are often used, in conjunction with genetic techniques, to describe ecosystem microbial diversity in modern and ancient systems. However, the presence of FAMES in a sample is not always helpful. Due to their similar size and chemistry, FAMES called alkenonates commonly co-elute with long-chain alkyl ketones, called alkenones (Figure 2). The distribution of alkenones in a sample relates information on sea surface temperature at the time and/or the place the sample was taken, and so their accurate and precise characterization is important.

Saponification is a common purification technique used to convert FAMES into fatty acids, thus changing their chemical characteristics enough to remove them from co-elution with alkenones (Figure 3). Saponification is a form of hydrolysis. In hydrolysis, water is used to split molecules. Saponification is a hydrolysis ~~that is~~ accelerated in the presence of a base, such as potassium hydroxide (KOH). KOH dissolves into K^+ and OH^- in water. The hydroxide anion (OH^- , negatively charged ion) adds to the slightly, positively charged tertiary carbon atom at the heart of the FAME (Figure 3, top). However, this chemical configuration is unstable, however (carbon has just bonded to too many other atoms) and an alkoxide (ROH^-) is expelled. However, But the H of the conjugate base this expulsion

Commented [JJ1]: The principles of the separation will be touch upon in the analysis videos, so this manuscript needs to focus on saponification and FAMES. Provide more detail on the saponification process, and why FAMES inhibit the analysis of the alkenones.

forms quickly moves to the alkoxide to form the alcohol methanol and fatty acid potassium salt (**Figure 3, middle**). At this point, the offending FAME (alkenoate) has been converted to a chemical that will no longer co-elute with it. However, if one wishes to also analyze FAME chemistry, they can be reclaimed by the addition of acid (HCl) to the solution, until pH reaches ~2. At this pH, the fatty acid potassium salt is split to form a carboxylic acid and ionic salt (KCl; **Figure 3, bottom**). Saponification is often used to remove fatty acid methyl esters (FAMES) from samples. FAMES are organic compounds containing a carbon atom that is double bonded to an oxygen atom and singly bonded to a long alkyl group, as well as another oxygen followed by a methyl group (**Figure 2**). FAMES commonly coelute with long chain alkyl ketones, called alkenones. The distribution of alkenones in a sample relates information on sea surface temperature at the time and/or the place the sample was taken.

Procedure:

1. Setup and Preparation of Materials

1.1. Obtain a total lipid extract (TLE) using a solvent extraction method (Sonication, Soxhlet, or Accelerated Solvent Extraction (ASE)).

1.2. Prepare a solution of 2 N KOH in 5% H₂O in methanol.

1.2.1. KOH and methanol can be purchased from chemical retailers. These chemicals should be pure and free of hydrocarbons.

1.2.1.1. The molar mass of KOH is ~56 amu yielding 1 mol of OH⁻ for each mol of KOH. Thus, to achieve a 2 N concentration, dissolve 112 g of KOH in 0.95 L of pure water and 0.05 L of methanol.

1.2.1.2. Note that the dissolution of KOH in water is exothermic and creates a large amount of heat. Take care to add the KOH pellets to the water slowly to avoid a violent reaction.

1.2.2. Dissolve 112 g of KOH pellets into 0.95 L of pure water on an automatic stir plate.

1.2.3. Add 0.05 L of methanol once all of the KOH is dissolved to aid in the dissolution of the organic biomarkers into the aqueous solution.

1.3. Prepare a solution of 6 N hydrochloric acid (HCl) in H₂O.

Commented [JJ2]: Do you do anything to counteract KOH's hygroscopic tendencies?

Commented [JS3R2]: KOH pellets are kept in a plastic jug with our other basic reagents. No special care.

Commented [JJ4]: What is the purpose for the MeOH? Does it help keep the biomolecules in solution?

Commented [JS5R4]: Yep. I've said that now.

1.3.1. HCl can be purchased from chemical retailers. This chemical should be pure and free of hydrocarbons.

1.3.2. HCl generally comes concentrated as 13 N. Thus, a 1:1 mixture of HCl and pure water produces a 6.5 N mixture of HCl, which is close enough to 6 N for the purposes of this experiment.

1.3.2.1. Be sure to add the HCl to the water and not the other way around, as adding water to concentrated HCl is exothermic and generates heat. This may cause HCl to splash.

1.3.3. Gently and slowly pour 100 mL HCl into a beaker containing 100 mL pure water, swirling the mixture between additions of HCl.

1.4. Prepare a solution of 5% NaCl (table salt) in H₂O.

1.4.1. NaCl can be purchased from chemical retailers. This chemical should be pure and free of hydrocarbons.

1.4.2. Calculate and weigh the mass of salt needed to make ~1 L of 5% solution ([w/w:m:m](#)). 1 L of water weighs ~1 kg or 1,000 g. Thus, 50 g of NaCl dissolved into 950 mL gives a 5% solution (50 g + 950 g = 1,000 g; 50 g / 1,000 g = 0.05 or 5%).

1.4.3. Gently add 50 g of NaCl to pure water on an automatic stir plate and wait for it to dissolve.

1.5. Obtain the following materials: 2 clean and combusted (550 °C for 6 h) 40 mL borosilicate glass vials with PTFE-lined cap; a warming oven or heating blocks; combusted (550 °C for 6 h) borosilicate glass pipettes and bulbs; pH tape (acidic range); Hexane (hexane can be purchased from chemical retailers. This chemical should be Optima Grade or equivalent).

2. Methods

2.1. Start with the dried TLE (containing esters) in one of the 40 mL borosilicate glass vials.

2.2. Add approximately 10 mL of 2 N KOH to the TLE and cap.

2.3. Heat in the oven or on a heating block to 60 °C for 2.5 h to cleave the ester bond (Figure 22).

2.4. Remove the sample from heat and allow it to cool to room temperature.

2.5. Add approximately 10 mL of the 5% NaCl solution to the TLE ~~to flood the solution with Na⁺ to interact with the O⁻ produced, and ensure the reaction does not reverse~~ (Figure 23). This helps keep the interface between the aqueous and organic phase (to be added) from foaming. Cap and shake briefly.

2.6. Add 6 N HCl to the salted TLE dropwise until pH 2 is reached to protonate the O⁻ and form the final product, a stable carboxylic acid (Figure 23; use pH paper to test). If the TLE was colored, there may be a shift in color coinciding with pH 2.

2.7. Add approximately 20 mL of hexane to the acidified solution, which is now ester-free. Cap and shake vigorously for 5 s to extract organic compounds from the water.

2.8. Allow to set until aqueous and organic phases completely separate. Salts, ions, water, and unreacted HCl remain in the aqueous phase. Organic compounds are now in the hexane.

2.9. Remove approximately 75% of the supernatant hexane using a pipette and dispense into the other 40 mL borosilicate glass vial.

2.10. Repeat 2.7 – 2.9 twice, adding approximately 10 mL of hexane each time.

2.11. Discard leftover aqueous mixture in a proper waste container.

2.12. Label the vial containing hexane and ester-free organics “TLE – saponified.”

Representative Results:

This purification produces a TLE free of esters that may be co-eluting with the alkenones ~~that were~~ set out to be analyzed. However, the purification produced carboxylic acids, which cannot be injected onto instruments commonly used to analyze samples for alkenone concentrations ~~because of their low volatility~~. For example, the boiling point of hexane, a 6 carbon hydrocarbon is 68 °C, but the boiling point of that of its acid, (hexanoic acid) is 205 °C. Most GC amenable biomarkers have from 20 to 35 carbon atoms (boiling point generally increases with an increasing number of atoms), and most GC temperature programs stop around 300 °C. Carboxylic acids injected into a GC quickly accumulate and ruin inlets, inlet liners, and the front end of columns. To remove these

Commented [JJ6]: Go into more detail on how the Na inhibits the reverse reaction.

Commented [JS7R6]: Upon further research, the salt is actually added to help the analyst. It keeps the interface between the aqueous and organic phase (to be added) from foaming. This helps the analyst pipette away the supernatant hexane.

Commented [JJ8]: As a way to give this video some sense of conclusion, can you run the TLE pre- and post-saponification and provide the chromatograms, to demonstrate the reaction took place?

Commented [JS9R8]: I can definitely do this. It may take a few weeks to get this to you...

Commented [JJ10]: Why? Is it still a co-eluting issue, or something else?

acids. The sample first needs to undergo another purification technique: separation via column chromatography.

Applications:

As mentioned previously above, saponification is commonly used in organic geochemistry labs to remove fatty acid methyl esters (FAMES) of alkenones, called alkenoates, which co-elute with alkenones on gas chromatographs (Figure 1). Saponification is also used to “free” fatty acids “bound” to sediment or macromolecules. The degradation and preservation of organic matter and biomarkers in sediments involves the removal of functional groups (N, O, and S) and the eventual polymerization of individual biomarkers into macromolecules and/or the adsorption of biomarkers and macromolecules onto mineral surfaces. Not all biomarkers in a setting become bound, and the ratio of bound-to-free biomarkers may change by setting and sediment age for reasons still not fully explained. Saponification, sometimes at temperatures higher than those discussed here (>200 °C), is used to free these bound biomarkers in the effort to describe them, their source, and the mechanisms responsible for their bound nature.

Legend:

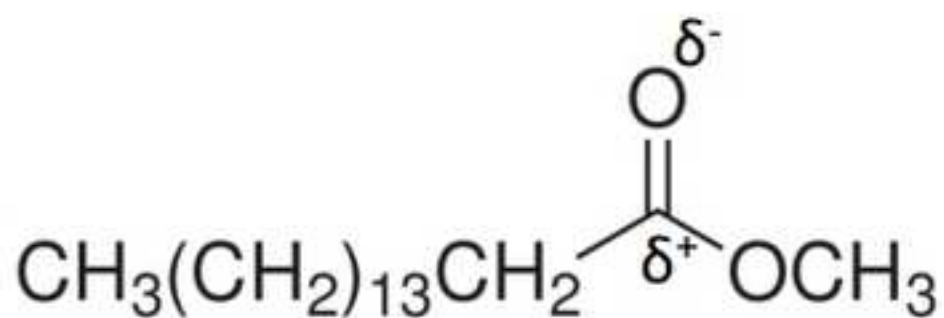
Figure 1: A chromatogram showing the separation of different chemical constituents over time and their relative concentration (area under the curve). Co-eluting and separated peaks are shown.

Figure 2: The chemical structures of an alkenone with 37 carbon atoms and two double bonds (top) and its associated alkenoate FAME (bottom).

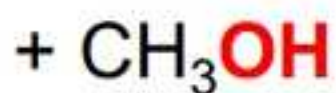
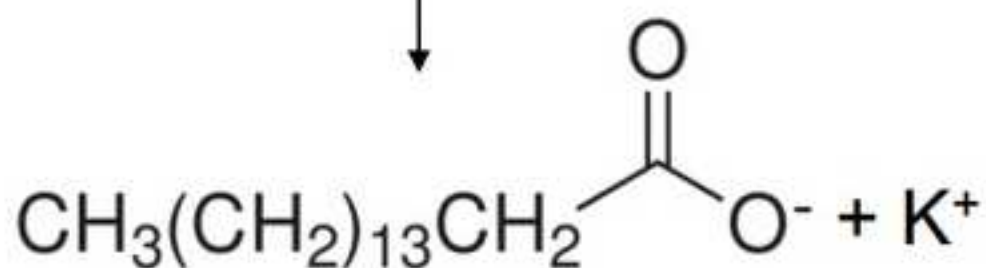
Figure 3: A schematic of the saponification of palmitic acid using potassium hydroxide (KOH) to increase the rate of hydrolysis (<http://www.mpbio.com/>).

Commented [JJ11]: The bulk of this section applies to the entire 8-video collection. Provide saponification-specific applications. What are some other uses of saponification in organic geochemistry? What other complex samples rely on moving the alkenoate peak?

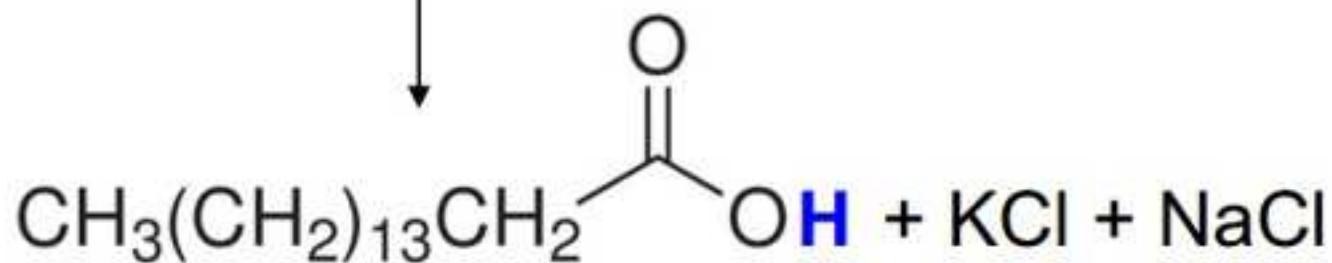
Commented [JR12]: The first image was created by the author, but Figure 2 and 3 will need to be recreated (I had trouble finding rights information on the website provided, so better safe than sorry).



(K⁺OH⁻ and heat)



(NaCl, HCl)



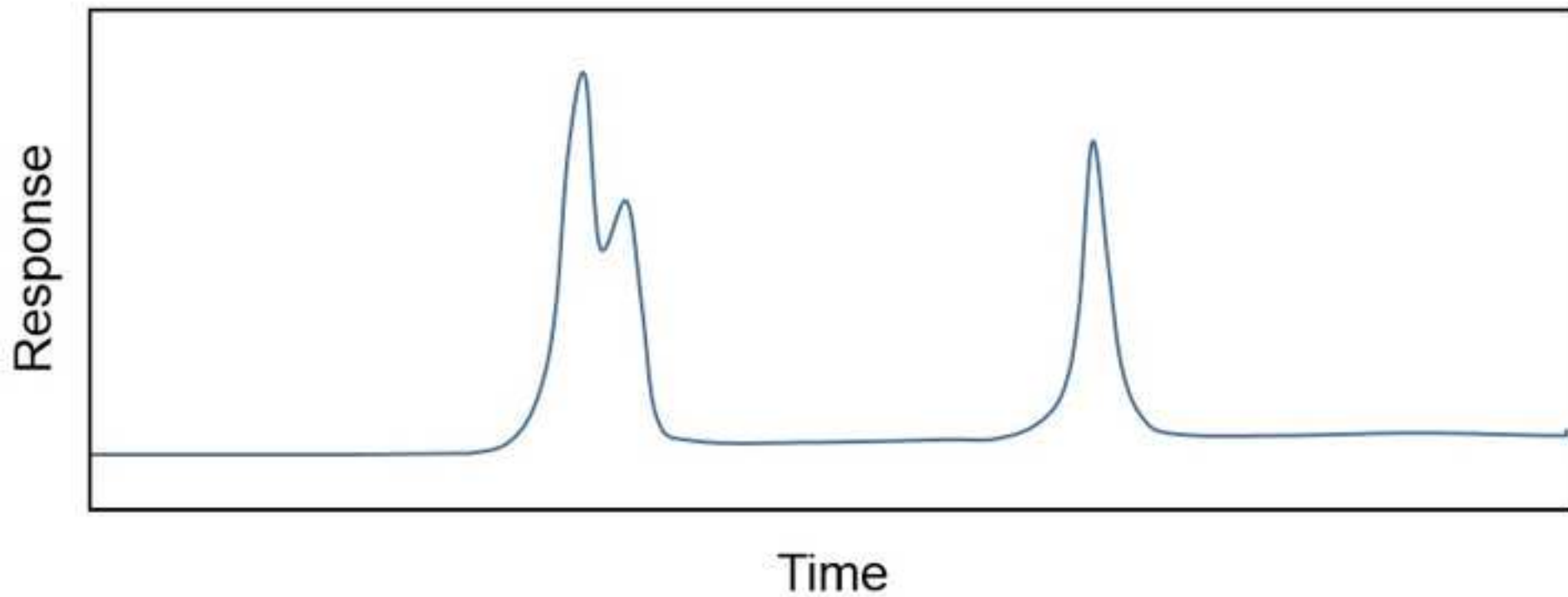


Photo or Graphic File

[Click here to download Photo or Graphic File: Jeff Salacup_Saponification_Figure 2.jpg](#)

