

Fatty Acid determination of an unknown mixture by H¹ NMR and C¹³ NMR analysis

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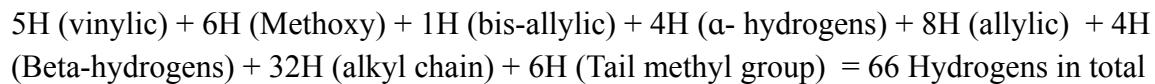
Objective: In this experiment we will analyze a solution of two fatty acid methyl esters by ¹H NMR and ¹³C NMR. We will determine the number of C=C bonds, the empirical formula for each molecule, and the molar ratios of the two molecules in the mixture.

Methods: (Rough parameters)

First 80 μL of an unknown fatty acid mixture was mixed with 720 μL of deuterated chloroform to make an 800 μL sample which was transferred into an NMR tube for measurement in a 400 mHz instrument NMR machine. For ¹H NMR, detection of the fatty acids in our unknown mixture, the acquisition time (**aq**) was set to 2.5 seconds. Spectral window is set from 0-14 ppm (**sw**) in ¹H NMR (0-220 ppm in ¹³C NMR). Dwell time (**dt**) is set to 25 seconds because it is usually determined by 10 times the maximum spin lattice relaxation time (T_1). We collected eight scans (**nt**), thus **nt**=8. The delay between pulses (**d1**) was set to 14 seconds. For our ¹³C NMR we used a 500 mHz instrument and an acquisition time (**aq**) of 2.17 seconds, spectral width (**sw**) of 0-220 ppm, delay between pulses (**d1**) of 40 seconds, and collected 1024 scans, thus **nt**=1024.

Calculations:

Part I. Relative Number of Hydrogens: To determine the relative number of hydrogens in the ¹H NMR Spectrum we used the set integration function in MNova to normalize integration where 0.198 is equivalent to one hydrogen. This was found by dividing the known methoxy peak (integration value of 1.19) by six; the number of hydrogens in that environment. Using this we calculated the presence of the following hydrogens:



The eight allylic hydrogens, 5 vinylic hydrogens, and the 1 bis-allylic hydrogen imply that there is a CH₂ group located between two double bonds and also that there is only one more double bond. This is because each double bond system would create 4 allylic hydrogens.

Part II. Relative Degrees of Unsaturation: (Estimate based on the single fatty acid Formula)

$$\text{DoU} = \frac{2\text{Carbons} + 2 - \text{Hydrogens}}{2} = \frac{2(36) + 2 - 66}{2} = 4$$

Because we know that we have two unsaturated fatty acids, we use this as a guideline. For a saturated fatty acid methyl ester, the molecular formula obeys the rule: $C_nH_{2n+2}O_2$

Because we know that we have two unsaturated fatty acids, we use this as a guideline and instead our molecular formula, accounting for degrees of unsaturation becomes: $C_{36}H_{66}O_4$ This does not obey the $C_nH_{2n+2}O_2$ rule.

Part III. Relative Number of Carbons: To determine the relative number of carbons in the ^{13}C NMR Spectrum we used the set integration function in MNova to normalize integration where 1.465 is equivalent to one carbon which was found by dividing the known tail methyl shift by 2 carbons seeing that both fatty acids have one.

2C (tail methyl group) + 2C (third to last carbon of the chain) + 2C (Beta carbon) + 0.5C (bis-allylic) + 4C (allylic carbons) + 12C (alkyl chain) + 2C (second to last) + 2C (alpha carbons) + 2C (Methoxy) + 5C (olefinic) + 2C (carbonyl) = 35.5 carbons

Part IV. Ratio of Lipid W to Lipid T

Int1 - maximum theoretical integration of olefinic bonds in a FAME with 1 C=C bond

Int2 - maximum theoretical integration of a FAME with two C=C bonds

Int1a - maximum theoretical integration of allylic protons in a FAME with 1 degrees of unsaturation

Int2a - maximum theoretical integration of allylic protons in a lipid with 2 degrees of unsaturation

$$\text{IntOlefinic (0.99)} = (\text{W} \times \text{Int1}) + (\text{T} \times \text{Int2});$$

$$\text{IntAllyl (1.61)} = (\text{W} \times \text{Int1a}) + (\text{T} \times \text{Int2a});$$

$$\text{IntBis (0.17)} = (\text{W} \times \text{Int1b}) + (\text{T} \times \text{Int2b});$$

$$\begin{pmatrix} 0.315 \\ 0.0883333 \end{pmatrix} = \begin{pmatrix} -\frac{1}{6} & \frac{1}{3} & -\frac{1}{3} \\ \frac{2}{9} & -\frac{1}{9} & \frac{5}{18} \end{pmatrix} \cdot \begin{pmatrix} 0.99 \\ 1.61 \\ 0.17 \end{pmatrix}$$

$$\text{RelativeW} = 0.31499;$$

$$\text{RelativeT} = 0.08833;$$

$$\text{NormalizedTotalAbundance} = \text{RelativeW} + \text{RelativeT}$$

$$\text{RoughPercentTotalW} = (0.31499/0.403333) \times 100$$

$$\text{RoughPercentTotalW} = (0.08833/0.403333) \times 100$$

$$\text{ApproxRatioW} = 21.9008/20 = 1.09504$$

$$\text{ApproxRatioW} = 78.0992/20 = 3.90496$$

Results

Hydrogen Type	Name	Integration	Multiplicity	Chemical Shift (ppm)	# of Hydrogens
Olefinic Carbon (C=CH)	•	0.99		5.3	5
Methoxy (3HC-O)	•	1.19	1	3.64	6
Bis-allylic (CH-CH ₂ -CH=)	Δ	0.17	3	2.75	1
Alpha Carbon (3HC-O-CO-CH ₂)	•	0.83	3	2.28	4
Allylic (CH ₂)	•	1.61	6	2	8
Beta Carbon (3HC-O-CO-CH ₂ -CH ₂)	•	0.85	5	1.6	4
Alkyl Chain (CH ₂)	•	6.34	4	1.28	32
Methyl Tail (CH ₃)	•	1.19	6	0.86	6

Figure 1.1 Integrations, multiplicities, chemical shift, and number of hydrogens as determined from our collected data for the H NMR are tabulated above.

Empirical Formula:

Based on the number of carbons and hydrogens calculated from our H NMR and C NMR spectra of our mixture the following empirical formulas were found to fit the data.

Palmitoleic acid methyl ester: C₁₇H₃₂O₂

Linoleic Acid methyl ester: C₁₉H₃₄O₂

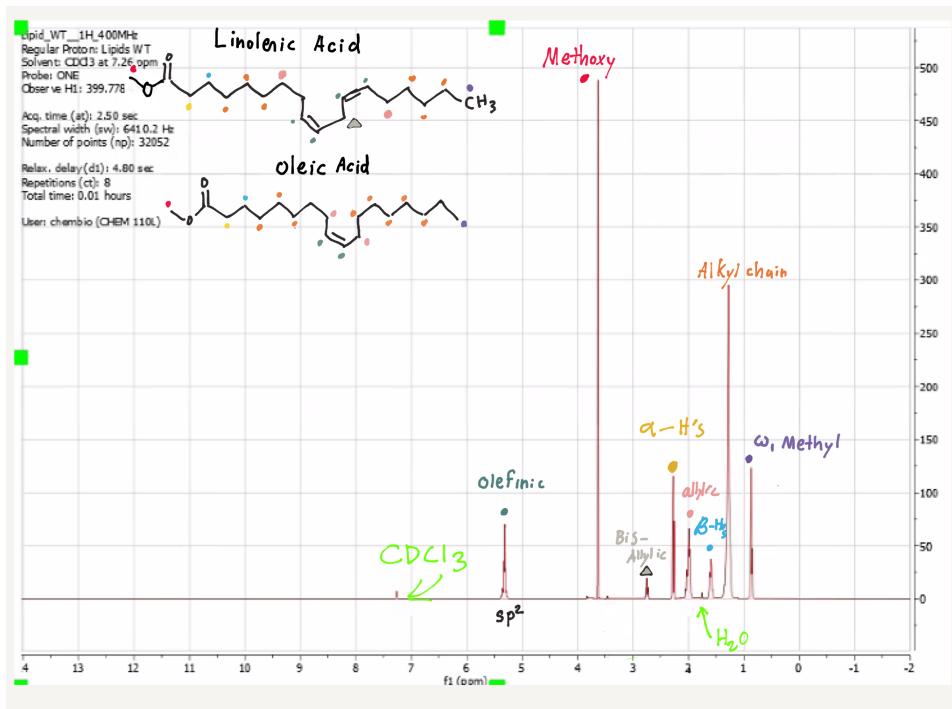


Figure 1.2 Above represents the ¹H NMR annotated with plausible structures of the FAMEs, as well as labelled peaks for various hydrogens found in the structures.

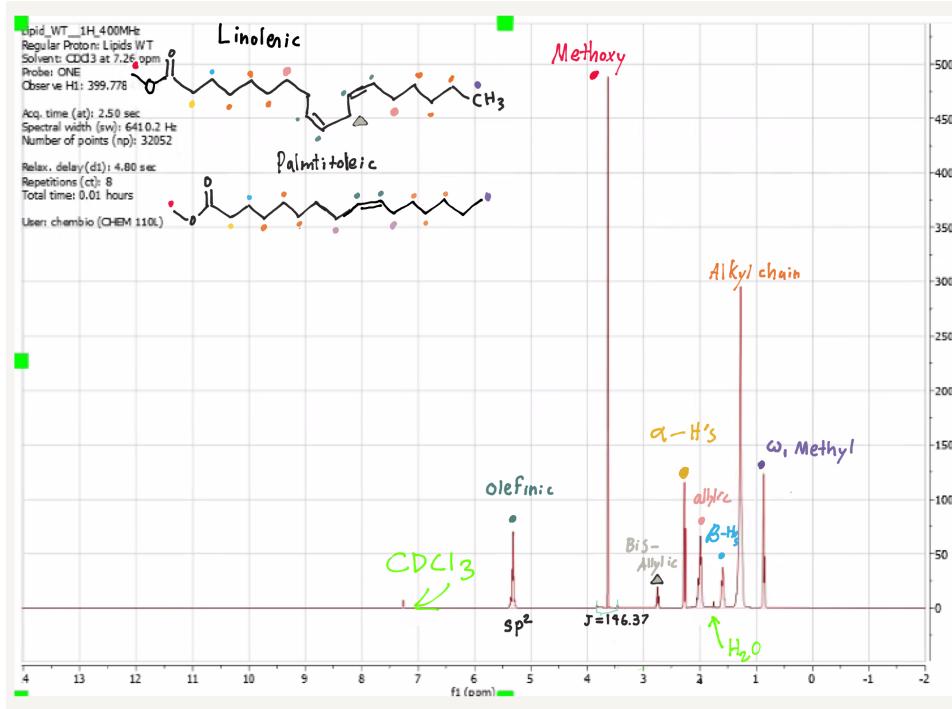


Figure 1.3 Above represents the ¹H NMR annotated with plausible structures of the FAMEs (linoleic acid and palmitoleic acid methyl ester), as well as labelled peaks for various hydrogens found in the structures. The Bis-Allylic hydrogen identified herein corresponds to the spectrum of the linoleic acid methyl ester.

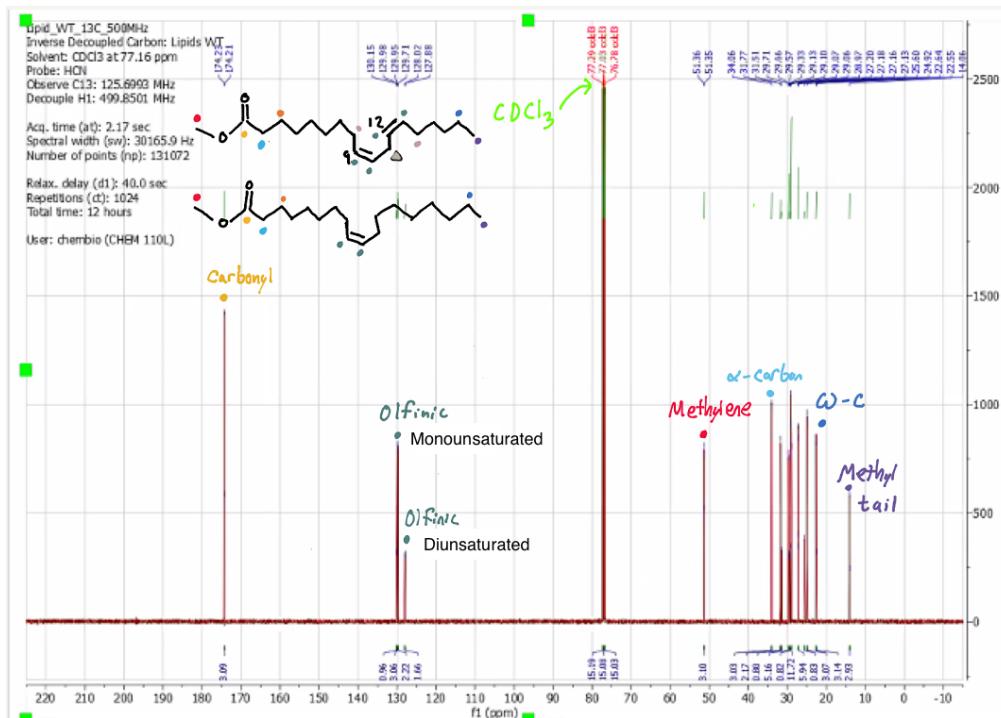


Figure 2.1 Above represents the ^{13}C NMR annotated with plausible structures of the FAMEs (linoleic acid and oleic acid methyl ester), as well as labelled peaks for various carbons found in the structures.

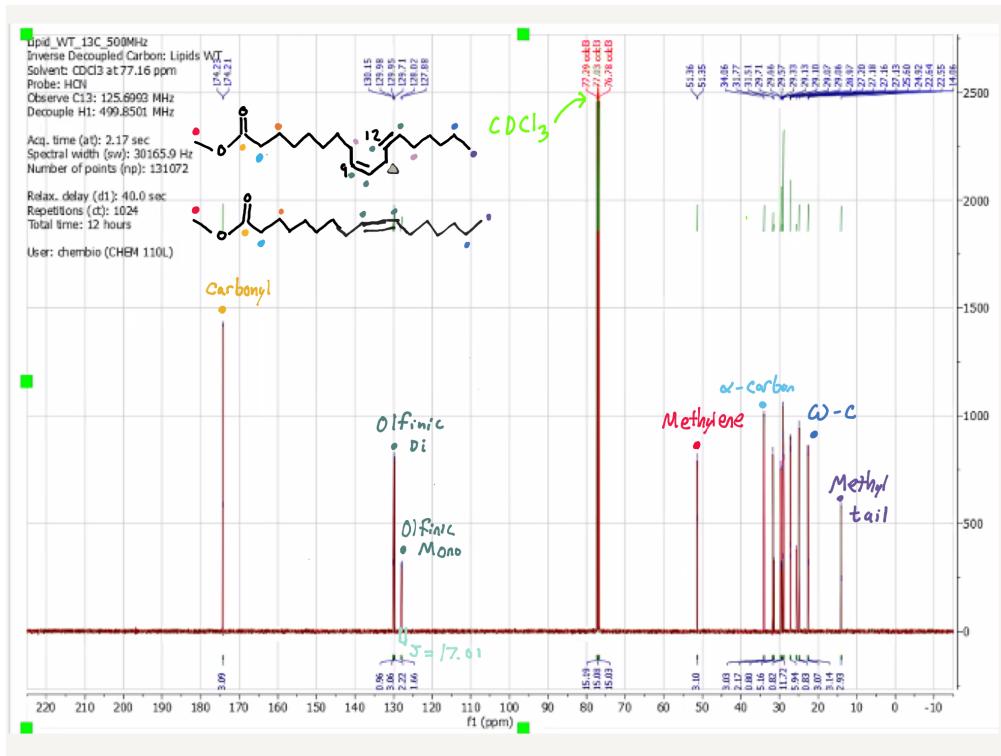
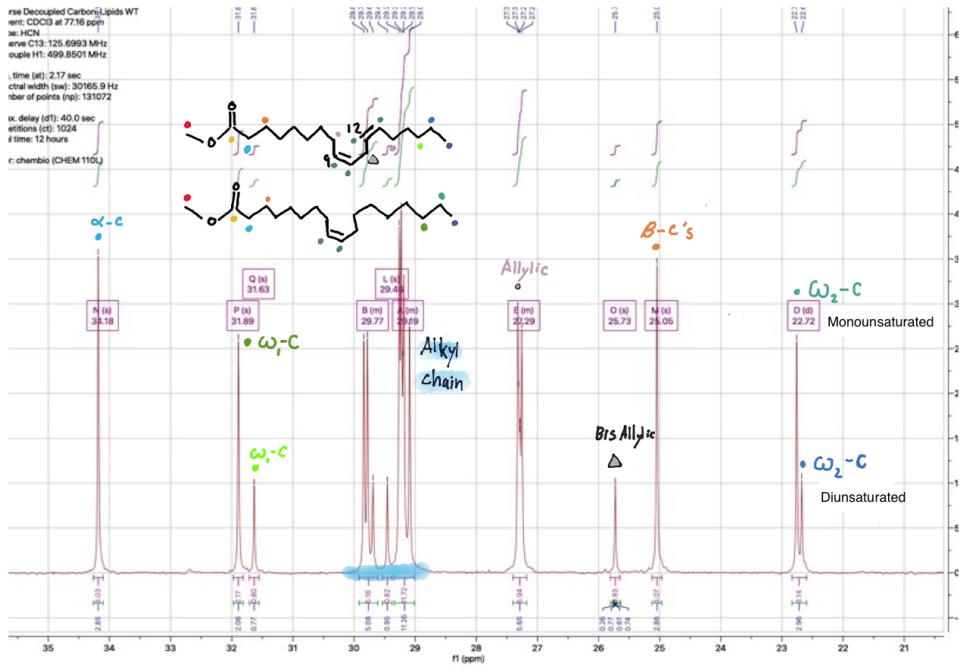


Figure 2.2 Above represents the ^{13}C NMR annotated with plausible structures of the FAMEs (linoleic acid and palmitoleic acid methyl ester), as well as labelled peaks for various carbons found in the structures.



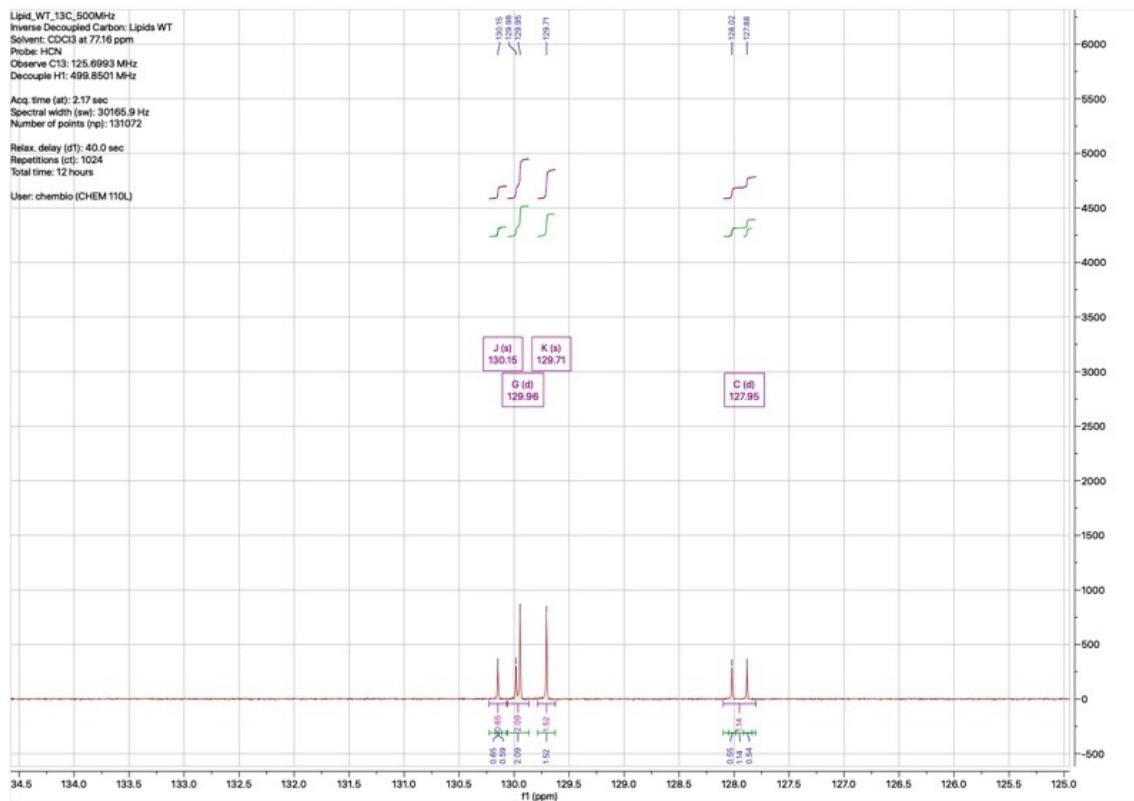


Figure 2.4 Depicted above are the olefinic carbon peaks in the 127.95-130.15 ppm region. Which display coupling constants of (from left to right) 17.31, 30.21, and 20.19. If an additional peak was present here calculation of E/Z isomer ratio may be calculated.

Carbon Type	Color	Integration	Multiplicity	Chemical Shift (ppm)	# of Carbons
Methyl Tail (Ω)	●	2.93	2	14.17	2
Ω -2 (second to last carbon of the chain) - Linoleic acid -	●	0.82	1	22.55	0.5
Ω -2 (second to last carbon of the chain) -Monounsaturated FAME -	●	2.11	1	22.64	1.5
Beta Carbon	●	3.03	1	25.05	2
Bis Allylic Carbon	△	0.835	1	25.73	0.5
Allylic Carbon	●	5.95	4	27.29	4
Alkyl Chain Carbons	■	5.17, 0.82, 11.72	Region of multiple singlets	29.19-29.77	12
Ω -1 (third to last carbon of the chain) - Methyl linoleate -	●	0.80	1	31.63	0.55
Ω -1 (third to last carbon of the chain) -Monounsaturated FAME -	●	2.17	1	31.89	1.35
Alpha Carbon	●	3.03	1	34.18	2
Methylene Carbon	●	3.10	1	51.36	2
Olefinic Carbon	●	0.952, 3.06, 2.23, 1.67	Region consists of singlets	127.95-130.15	5
Carbonyl Carbon	●	3.09	2	174.23	2

Figure 2.5 Above represents the ^1H NMR annotated with plausible structures of the fatty acid methyl esters, as well as labelled peaks for various hydrogens found in the structures.

<i>Protons</i>	<i>One C=C Bond</i>	<i>Two C=C bonds</i>
<i>Olefinic</i>	2	4
<i>Allylic</i>	4	4
<i>Bis-Allylic</i>	0	2

Figure 1.4 Above is pictured the maximal theoretical integrations of each proton signal of a given type. [2]

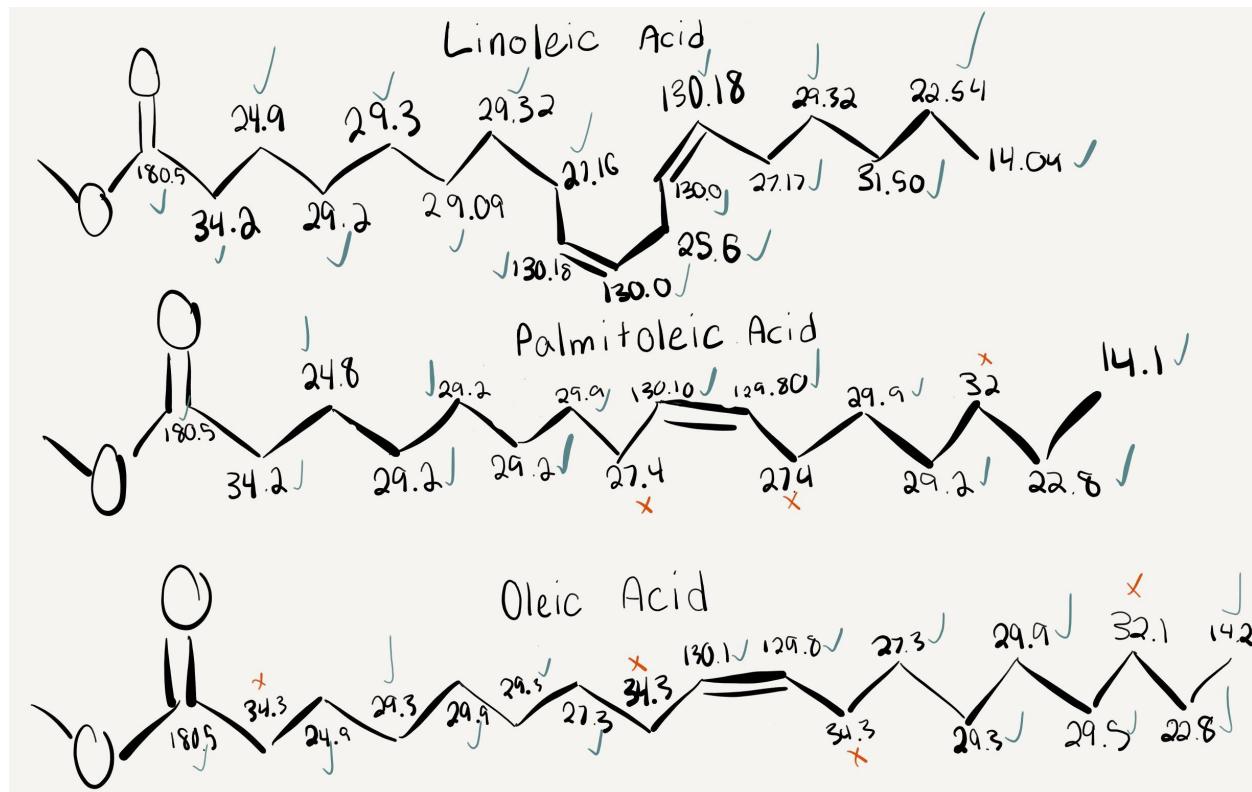


Figure 2.6 The figure above shows the expected chemical shifts corresponding to each carbon according to SpectraBase[7]. The ✓ denotes the existence of a peak in our spectra within 0.2 ppm of the literature values. The ✗ indicates that no peak was found within 0.2 ppm of the expected value in our experimental spectra.

Discussion

To determine the structure of the two unknown fatty acid methyl esters (FAME's), a sample peak assignment for characteristic regions of the fatty acid chain in both ^1H & ^{13}C NMR was achieved. Peak integrations in the ^1H NMR allowed determination of the relative number of hydrogens between both fatty acids in the mixture. Proton chemical shifts were determined from literature values^[3] accounting for shielding and deshielding effects of relevant chemical environments. One characteristic peak which guided our structural determination is the bis-allylic proton peak (2.75ppm). This peak informed us that at least one FAME possessed two or more double bonds to shift the corresponding protons downfield of typical alkyl chain protons, that the FAME with these two double bonds was in lower abundance (Integration =0.17), and that there are only two adjacent hydrogens to cause splitting at this position as the peak is a triplet ($M=3$). All of these attributes are present in Linoleic acid; in addition the bis-allylic carbons in cis dienes are known to appear more upfield (25-27ppm) which is consistent with our peak at 25.73ppm.

To determine the number of hydrogens in our structure we normalized the integration of a strong, well characterized methoxy proton peak (3.6ppm) which represents the relative abundance of the six protons (3 from each FAME) in our ^1H NMR. One sixth of this relative integration value was normalized to one hydrogen allowing us to obtain a relative number of 66 hydrogens. The multiplicity of the hydrogen peaks allowed us to further analyze the number of hydrogens adjacent to a specific peak. The ^{13}C NMR was used to determine the relative number of carbons; this could also have been estimated using the relative number of hydrogens. The ^{13}C NMR was also used to analyze the chemical shifts between our three suspected fatty acid methyl esters (Figure 2.6). Here we see that all expected peaks for linoleic acid methyl ester were present in our spectrum. Further evidence for the presence of Linoleic acid methyl ester is shown in the characteristic peak at the bis-allylic carbon peak (25.73 ppm). We also see that not all expected peaks are present for oleic acid methyl ester and palmitoleic acid methyl ester. Specifically, the peaks at 27.4 and 32.0 for palmitoleic acid methyl ester, and the peaks at 32.0 and 34.3 for oleic acid methyl ester. Taking all of the above into account it seems very probable that linoleic acid methyl ester was one of the FAME's in our mixture. Deciding between the two monounsaturated FAME's was more difficult because they were both missing peaks. That being said, we believe it is more likely that the palmitoleic acid methyl ester is present because the estimated number of hydrogens and carbons are equivalent to the number of carbons and hydrogens in a mixture of palmitoleic acid methyl ester and linoleic acid methyl ester. This is not the case for the oleic acid methyl ester.

To determine the molar ratio of each fatty acid we used integrations of characteristic hydrogen peaks for each chemical shift environment. We identified olefinic protons (5.3–5.4ppm), allylic protons (2.0–2.1ppm), and bis-allylic protons (2.7–2.8ppm) in our fatty acid mixture. We then determined maximal theoretical integrations for each hydrogen of a given type

based on the degrees of unsaturation for the olefinic (2,4), allylic (4,4), and bis-allylic protons (0,2) assuming the presence of both one and two C=C double bonds. Different combinations of one and two intrachain unsaturations were used because eight allylic hydrogens were observed in the ¹H NMR. The observed integration of each hydrogen from respective chemical environments is then set equal to the product of that component's (W/T) relative abundance and the theoretical integration for a peak of the given type.^[4] Using three different versions of this system of equations we determined the relative abundance of each lipid in the mixture by multiplying experimental integrations by the inverse of the expected integration matrix. This yields the relative abundance of lipids W and T, which are then totaled, ratios are taken, and provides the percent relative amount of each respective lipid. Thus, the determined ratio of mono-unsaturated to di-unsaturated fatty acid methyl ester is 4:1.

While a qualitative approach to NMR analysis can yield basic structural information for the compound, quantitative NMR analysis allows one to determine the amounts of the desired compound(s) in solution. For quantitative ¹H NMR analysis to give precise outcomes, the signal to noise ratio must be at least 250:1.^[4] It is easier to experimentally achieve a high S:N ratio with ¹H NMR, due to the increased gyromagnetic ratio for the proton nucleus, which allows for a decreased number of scans (8) compared to ¹³C NMR (>1000). To obtain a qualitative ¹³C NMR within time constraints one may decrease the degrees of deviation from the random magnetic field and only allow the nuclei to do a 30° flip, rather than a quantitative scan which allows a full 90° flip against or with the orientation of the applied magnetic field. Relative quantitation was used to determine the approximate molar ratio of the two unknown lipids. A fundamental concept for quantitative NMR analysis is the equation that relates the signal intensity and the number of nuclei that are contributing to the signal:

$$I_x = K_s \times N.$$
^[4]

Parameters such as pulse delay and repetition time can affect the intensities of the signals. As seen in the calculations section, we were able to determine the relative number of hydrogens and carbons present in the lipid mixture by normalizing the NMR spectra, ultimately giving us 66 relative hydrogens and 35.5 carbons. Using the relative numbers of hydrogens and carbons to find degrees of unsaturation allowed us to propose possible structures for the two unknown lipids.

Sensitivity of NMR depends on how many spins are in the upper or lower state, which is represented by the gyromagnetic ratio. ¹H has a higher gyromagnetic ratio than ¹³C , which makes ¹H 64 times more sensitive than ¹³C. The abundance of ¹³C is ~1.1%, whereas the natural abundance of ¹H is ~99.9%.^[5] Furthermore, the relaxation time of hydrogen is much faster in ¹H NMR than the carbons of ¹³C NMR. The advantage of using ¹³C NMR analysis is that it can determine the presence or lack of signals for different types of carbons that are commonly found in fatty acids. For example, olefinic carbons will show up around 127-131 ppm.^[6] Our ¹³C NMR spectra has several peaks in this region, around 127.95-130.15 ppm. Based on these data it was determined that our lipid mixture consists of linolenic, and palmitoleic FAME's. Our reason for choosing Palmitoleic over linoleic is due to the relative number of carbons calculated for our

mixture, and the presence of olefinic carbons can explain the ^1H NMR peak we see at around 5.3 ppm, which represents the hydrogen of the sp₂ carbon.

It is important to note that no 2D NMR experiments for our sample of WT lipids were used in our structural analysis. However, there are 2D NMR experiments that would have provided unique information. A 2D COSY NMR experiment would have provided information on the coupling of hydrogens or carbons to each other. Because coupling was determined well from the multiplicity of the peaks, COSY would have been of little utility. HMBC is a 2D heteronuclear experiment that would explicitly relate carbons and hydrogens that are more than one bond apart. Though likely convoluted with our two unknown fatty acids, with high resolution, this experiment could have enabled us to determine the exact structure of the two fatty acids. HSQC is another 2D heteronuclear experiment that would have helped to determine the exact structures because it would tell us which hydrogens are directly coupled to which carbons. Despite these alternative 2D experiments, it seems realistic to state that we did not need the additional experiments in order to come to a reasonable conclusion.

Potential sources of error include but are not limited to the theoretical limitation of resolution on acquisition time as it cannot exceed 150 milliseconds, and the small decoupling error caused by the coupling of protons to ^{13}C in the ^1H NMR scans. The acquisition time is limited by the transverse spin lattice relaxation time of our analyte (T_2) as we do not want to end signal acquisition before at least 90% if the signal has decayed into noise. Additionally the acquisition time is related to the product of the time between data sampling (dwell time), and the dwell time is usually set at the inverse of two times the spectral window. These dependencies create a minimal threshold for the acquisition time of a given analyte. We did not perform any heteronuclear decoupling because there is very little present in our spectra. One example of this ^{13}C and proton coupling is seen around the methoxy peak in the H NMR spectra at 3.45 and 3.82 ppm. The integration value of these peaks are less than 0.01 and thus represent less than 1% of the methoxy hydrogen peak (integration of 1.19). Despite this seeming small, it is still a cause of error in our spectra. Removing this heteronuclear decoupling would make peaks in crowded regions easier to interpret by removing small overlapping peaks. It would also increase the accuracy of the integration values in these crowded areas.

Conclusion

In this experiment, we analyzed ^1H NMR and ^{13}C NMR spectra to determine that the two most likely lipids in our mixture were palmitoleic acid methyl ester ($\text{C}_{17}\text{H}_{32}\text{O}_2$) and linoleic acid methyl ester ($\text{C}_{19}\text{H}_{34}\text{O}_2$). The spectral information indicating palmitoleic acid methyl ester is similar to literature spectra of oleic acid methyl ester, however relative carbon and hydrogen numbers are indicative of the mono-unsaturated fatty acid being palmitoleic. Determining the degrees of unsaturation, number of hydrogens and carbons, and the identity of each NMR peak allowed us to propose these two structures as our unknown lipids. Using quantitative NMR

analysis and linear algebra, it was found that the molar ratio of palmitoleic acid methyl ester to linoleic acid methyl ester was 4:1.

References

Additional question source

[1] Teesalu, T.; Sugahara, K, N.; Ruoslahti, E. “Tumor-penetrating peptides” *Front. Oncol.* 3:216 (2013).

Matrix algebra approach

[2] Knothe, G., & Kenar, J. A. (n.d.). Determination of the fatty acid profile by ^1H -NMR spectroscopy. *Eur. J. Lipid Sci. Technol.* **106** 88–96, doi:10.1002/ejlt.200300880 (2004)

H & C NMR peaks

[3] Coy, A. Characterizing Fatty Acids with advanced multinuclear NMR methods
<https://magritek.com/2018/04/06/characterizing-fatty-acids-with-advanced-multinuclear-nmr-methods/> (2018)

Relationship between signal intensity and number of nuclei

[4] Bharti, S.K.; Roy,R. “Quantitative ^1H NMR Spectroscopy” *TrAC Trends in Analytical Chemistry*. 35:5-26 (2012).

Carbon and hydrogen isotope abundance

[5] Arbogast, L.W.; Brinson, R.G.; Marino, J.P. “Chapter One - Application of Natural Isotopic Abundance ^1H - ^{13}C - and ^1H - ^{15}N -Correlated Two-Dimensional NMR for Evaluation of the Structure of Protein Therapeutics” *Methods in Enzymology*. 566:3-34 (2016).

Olefinic carbon chemical shift

[6] Gunstone, F.D.; Shukla,V.K.S. “NMR of Lipids” *Annual Reports on NMR Spectroscopy*. 31:219-237 (1995).

Reference chemical shifts for ^{13}C NMR

[7] Free Spectral Database. (n.d.). Retrieved December 03, 2020, from <https://spectrabase.com>

Appendix:

^1H NMR _____ 1.0
Figures

¹³C NMR _____ 2.0

Figures

FAME _____ Fatty Acid Methyl
Ester

OAME _____ Oleic Acid Methyl
ester

LAME _____ Linolenic acid methyl
ester

PAME _____ Palmitoleic acid methyl
ester