Title: GC Omega-3 Ethyl Esters Experiment

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Abstract:

Gas chromatography with a flame ionization detector with C23:0 as the internal standard was used in this experiment to determine the components that made up omega-3 ethyl ester dietary supplements. Using a data set provided, the GC performance parameters were calculated. N_{eff} (effective theoretical plates), H_{eff}(height equivalent to an effective plate), and FWHM (Full Width at Half Maximum) and the retention time for C16:0 were found. N_{eff} was 18830, H_{eff} was 1.593 mm, and FWHM was 0.02171 min. The retention time for C16:1 and C18:2 were 1.646 min and 2.356 min respectively. The resolution between C16:0 and C16:1 was 1.980 and between C16:0 and C18:2 was 27.155. Using these conditions found for the GC instrument, several samples of omega-3 ethyl ester dietary supplements were injected and peaks within the samples were found under a standard, GLC-68 FAEE by NuChek Prep. Using these peak values from the standard, % weight of EPA and DHA were calculated and compared to the label values which yielded a significant difference in % weight of both components. A linear relationship between the number of carbons and retention time was found.

Keywords: Effective theoretical plates, height equivalent to an effective plate, % weight, EPA, DHA, omega-3 dietary supplements, resolution, standard, linear relationship

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I. Introduction

We used gas chromatography (GC) to determine the composition of the fatty acid ethyl esters in omega-3 dietary supplements. For my pre-laboratory report, Filipowska et. al.[1] used GC to determine concentrations of aldehydes extracted from pale malts using different methods of sample preparation. I also read two additional peer-reviewed journal articles that were published in 2019 and they are summarized together with my pre-laboratory report journal article in Table 1. The first additional article I read was by Yang et. al.[2] where they used gas chromatography to determine the volatile components in jujube fruits under different types of cold storage periods. They concluded that aldehydes and ketones were the main components in early cold storage, but esters became the main components in the old cold storage. The second additional article I read was by Rutkowska et. al.[3] where they used gas chromatography with mass spectrometry to analyze pesticide residues. They concluded that a large percentage of the tested pesticides did not show effects of matrix compounds that were produced.

Table 1. My refereed journal articles.

Corresponding Author	**Date Submitted**	**Date Accepted**	**Samples**	**My reference number**
Weronika Filipowska	July 24, 2019	October 21, 2019	Methods of preparation of 2-methylpropanal, 2-methylbutanal, 3-methylbutanal, hexanal, furfural, methional, phenylacetaldehyde and trans-2-nonenal.	1
Lvzhu Yang	September 20, 2019	October 28, 2019	Jujube fruits composition under different cold storage periods	2
Ewa Rutkowska	July 8, 2019	November 25, 2019	Pesticide residue	3

From chapters 11 and 12 of the textbook,[4] gas chromatography is explained in depth. Gas chromatography is the analytical technique where an inert gas is passed through the column to separate compounds based on how fast each compound changes from the liquid to the gas phase, or their volatility. When a component of a sample is ready to be carried out by the mobile phase, a peak will be shown at the time when the component is separated, or leaves the sample. This is

the retention time of the compound that left, and polarity of the compound can also change this value. Polarity explains the interactions between the sample and the stationary phase with retention times. The more interactions, the higher the retention time. Each retention time is a fingerprint for each compound which means that no two compound will have the same retention time. This retention time is used to plot a chromatogram where the x-axis is the retention time usually in minutes, and the y-axis is the response of the compound in mV that is proportional to the concentration of the specific compound in the sample. From a chromatogram, if the sample had a higher retention time, it could mean that it had lower volatility and a high interaction with the stationary phase which is usually a silicon polymer.

From page 859 of the textbook,[4] the Van Deemeter equation is used to explain the relationship between the chromatographic column efficiency and the height equivalent to a theoretical plate (HETP) with μ for a particular geometry and construction:

$$H = A + \frac{B}{\mu} + C\mu \qquad (1)$$

where A is for multiple paths, $\frac{B}{\mu}$ is due to longitudinal diffusion and C μ is due to equilibration over time. The next equation that was used was for the effective theoretical plates, N_{eff}:

$$N_{eff} = 5.545\frac{t_R}{FWHM} \qquad (2)$$

where t_R is the retention time of the compound, FWHM is the full width at half maximum value found by using some sort of graphing utility. The equation is for height equivalent to an effective plate, H_{eff}:

$$H_{eff} = \frac{L}{N_{eff}} \qquad (3)$$

where L is the length of the column, and N_{eff} is the effective theoretical plates. For the resolution of the machine, this equation was used.

$$R = \frac{t_2 - t_1}{.5(W_1 + W_2)} \qquad (4)$$

Where R is the resolution, t_1 and t_2 are the retention times of the respective compounds, and W_1 and W_2 are the FWHM of the respective compounds. For the percent error of omega-3 compounds, specifically EPA and DHA, an equation was given:

$$\frac{\%\ OM3\ Label - \%OM3\ GC}{\%\ OM3\ Label} * 100 \qquad (5)$$

Where % OM3 Label is either DHA or EPA from the product label and % OM3 GC is either DHA or EPA determined by the area %. The mean was also found and that was determined by this equation:

$$\overline{X} = \frac{\sum_1^n X_n}{n} \qquad (6)$$

with $\overline{X}$ is the mean (average), $\sum_1^n X_n$ is the sum of all the trials, and n is the number of samples conducted in the experiment. The next equation used was for standard deviation.

$$\sigma = \sqrt{\frac{\sum_{i=1}^{n}(\overline{X}-X_i)^2}{n-1}} \qquad (7)$$

Where σ is the standard deviation, $\overline{X}$ is the mean, $\sum_{i=1}^{n}(\overline{X}-X_i)^2$ is the sum of the mean minus the X_i, which is the trial of the specific term, i, squared, and n is the number of samples conducted in the experiment. Percent relative standard deviation, %RSD was also calculated where

$$\%\ \text{RSD} = \frac{\sigma}{\bar{x}} * 100 \qquad (8)$$

with σ being the standard deviation and $\bar{x}$ is the mean of the total trials conducted. The final equation that was used was for the capacity factor, k:

$$k = \frac{t_R - t_M}{t_M} \qquad (9)$$

where t_R is the retention time of the compound and t_M is the time for the unretained substance.

II. Experimental

Samples

Table 2 summarizes the omega-3 dietary samples that I chose to analyze and inject into the GC.

Table 2. Omega-3 dietary samples analyzed.

Sample ID	**Manufacturer**	**Label EPA (mg)**	**Label DHA (mg)**
C:23 Methyl Ester Standard	NuChek Prep	0	0
*OM1	Kenko Bio LT 363244 40-20	800	600
*OM2	OmegaGenics Mega 10	330	170
*OM3	Vitamin Shoppe 1290mg	735	165
OM4	Omera Lot 8516630055	400	300
OM5	Mother's Select Prenatal DHA 39422	50	80
OM6	Apotex POM3	465	375
GLC 68D EE	NuChek Prep	10% by weight	12% by weight
*Lovaza	Lovaza Lot 5ZP506	465	375

*Denotes the samples I injected

Materials and Supplies

HPLC grade Isooctane (CAS: 540-84-1), and n-hexane (CAS: 110-54-3) were purchased from Sigma-Aldrich (St. Louis, MO). Glass vials 4-mL were purchased from ThermoFisher (Waltham, MA) and 1-µL Hamilton Syringe (Model #86211) (Reno, NV) was used to inject the samples into the GC. The internal standard methyl Tricosanoate (C23:0) and GLC 68D ethyl esters were purchased from NuChek Prep (Elysian, MN). Several Hamilton syringes (1 µL, 5 mL). GC vials, 5-mL with septa, 5-mL vials without septa.

Instrumentation:

PerkinElmer Clarus 480 Gas Chromatograph (Waltham, MA) with flame ionization detector (FID), heated injector, and PerkinElmer Elite Wax capillary column (30 m x 0.25 mm x 0.25 µm film), controlled by Empower 3 software (Milford, MA).

Instrument operating parameters are summarized in Table 3.

Table 3. Instrument operating parameters.

Parameter	**Value**
Injector temperature	250.0°C
Detector temperature	250.0°C
Carrier gas flow rate	1.0ml/min
Injected Volume	0.8µL
Temperature program	150°C start, 220°C end
Split flow	100:1

Table 4 summarizes the GC performance parameters that were calculated during week 1.

Table 4. GC Performance Parameters From Week 1.

Parameter	**Compound**	**Value**
L (column length)	Elite-Wax	30 m or 30,000 mm
N_{eff}	C16:0	18830
H_{eff}	C16:0	1.593 mm
FWHM (W)	C16:0	0.02171 min

Parameter	Compound	Value
Retention Time	C16:0	1.600 min
Retention Time	C16:1	1.646 min
Retention Time	C18:2	2.356 min
R (resolution)	C16:0 and C16:1	1.980
R (resolution)	C16:0 and C18:2	27.155

Analytical Procedure:

There were two weeks to this experiment, but only week two was performed in the laboratory. Week 1 required us to understand the OriginPro software in analyzing chromatograms that were provided to us. Using the data, a chromatogram can be plotted that yields N_{eff}, H_{eff}, retention times, and the resolution of the machine. Week 2 was in the laboratory where the PerkinElmer Clarus 480 Gas Chromatograph was used. This week taught us how to inject samples and read the chromatogram that was plotting in the Empower 3 software included on the computer connected to the machine. To prepare an injection sample, the clean syringe was washed once with the injection sample, then the experimental sample was collected and ready to inject into the GC. To inject a sample, two hands are used where the dominant hand is to hold the syringe as well as the plunger while the non-dominant hand is used to guide the needle into the hole. After the needle is fully in the hole, the sample is injected quickly, the syringe removed quickly, and the run button on the machine is pressed. If the sample was injected too slowly, sample could be going through the column and a chromatogram with incorrect retention times are given. After injecting sample, the syringe was rinsed three times with n-hexane and placed back into the syringe box. The retention time of the internal standard was shown on the chromatogram, but could usually be distinguished from the other samples.

III. Results and Discussion

Table 5 summarizes the retention times of the ethyl ester compounds in GLC 68D standard.

Table 5. Retention times of the ethyl ester compounds in GLC 68D standard.

CHAIN	Compound	% BY WT.	Retention time (min)
C14:0	ETHYL MYRISTATE	6	2.383
C14:1	ETHYL MYRISTOLEATE	1	2.555
C16:0	ETHYL PALMITATE	16	3.496
C16:1	ETHYL PALMITOLEATE	5	3.630
C18:0	ETHYL STEARATE	8	4.969
C18:1	ETHYL OLEATE	13	5.052
C18:1	ETHYL VACCENATE	4	5.455
C18:2	ETHYL LINOLEATE	2	5.567
C18:3	ETHYL LINOLENATE	2	6.004
C20:0	ETHYL ARACHIDATE	1	6.621
C20:1	ETHYL 11-EICOSENOATE	9	6.710
C20:2	ETHYL 11,14-EICOSADIENOATE	1	7.041
C20:4	ETHYL ARACHIDONATE	3	7.302
C20:3	ETHYL 11-14-17 EICOSATRIENOATE	1	7.438
C20:5	ETHYL EICOSAPENTAENOATE (EPA)	10	7.735
C22:0	ETHYL BEHENATE	1	8.031
C22:1	ETHYL ERUCATE	3	8.119
C22:6	ETHYL DOCOSAHEXAENOATE (DHA)	12	9.296
C24:0	ETHYL LIGNOCERATE	1	9.501
C24:1	ETHYL NERVONATE	1	9.646
C23:0	Methyl Tricosanoate (Internal standard)	----------	8.428

I used Table 4 above to identify the three most abundant fatty acid ethyl esters in the three omega-3 dietary supplements that I chose to analyze. They are summarized below in Table 6.

Table 6. Three most abundant fatty acid ethyl esters in my chosen omega-3 dietary supplements.

Omega-3 Supplement	**Compound 1**	**Retention Time**	**Compound 2**	**Retention Time**	**Compound 3**	**Retention Time**
Kenko Bio LT 363244 40-20	ETHYL EICOSAPENTAENOATE (EPA)	7.586 min	ETHYL DOCOSAHEXAENOATE (DHA)	9.193 min	ETHYL ARACHIDONATE	7.126 min
OmegaGenics Mega10	ETHYL EICOSAPENTAENOATE (EPA)	7.614 min	ETHYL DOCOSAHEXAENOATE (DHA)	9.217 min	ETHYL PALMITOLEATE	3.618 min
Vitamin Shoppe 1290mg	ETHYL EICOSAPENTAENOATE (EPA)	7.630 min	ETHYL DOCOSAHEXAENOATE (DHA)	9.272 min	ETHYL ARACHIDONATE	7.186 min

Table 7 summarizes the amount (% by Wt.) of EPA and DHA in my chosen omega-3 dietary supplements and in Lovaza. These values are determined by normalizing the area % by re-normalizing by only including all components detected expect that of the internal standard.

Table 7. % by weight EPA and DHA.

Omega-3 Supplement	**EPA wt %**	**Label wt%**	**% Error**	**DHA wt %**	**Label wt%**	**% Error**
Kenko Bio LT 363244 40-20	48.46%	57.14%	15.19%	30.85%	42.86%	28.02%
OmegaGenics Mega10	33.01%	66.00%	49.98%	20.77%	34.00%	38.91%
Vitamin Shoppe 1290mg	54.09%	81.67%	33.77%	15.04%	18.33%	17.95%
Lovaza	37.59%	55.36%	32.10%	22.56%	44.64%	49.46%

Determine % error by using Equation 5 from the introduction:

$$\frac{\%\ OM3\ Label - \%OM3\ GC}{\%\ OM3\ Label} * 100 \qquad (5)$$

Figure 1 shows the impurity profile of the omega-3 dietary supplement that I would recommend taking.

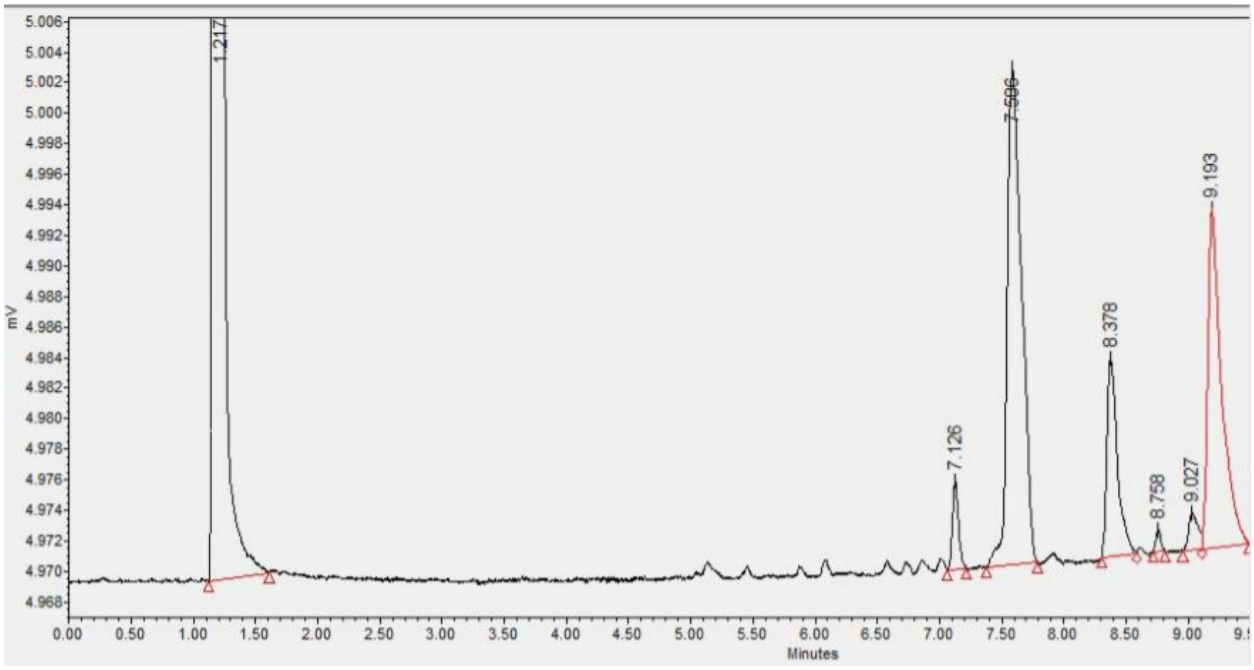

Figure 1. Chromatogram of my recommended omega-3 dietary supplement, Kenko Bio.

The statistics from the internal standard injections comparison between myself and my laboratory partner are summarized in Table 8.

Table 8. Statistics of internal standard injections.

Sample ID with student	**Internal Standard Retention Time**
Kenko Bio (Alan Yu)	8.378 min
OmegaGenics Mega 10 (Alan Yu)	8.378 min
Vitamin Shoppe (Alan Yu)	8.411 min
Mean (Alan Yu)	8.389 min
Standard Deviation (Alan Yu)	0.0191
%RSD (Alan Yu)	0.2277%

Sample ID with student	Internal Standard Retention Time
Omera (Haroon Chaudhry)	8.391 min
Mother's Select Prenatal DHA (Haroon Chaudhry)	8.370 min
Apotex POM3 (Haroon Chaudhry)	8.443 min
Mean (Haroon Chaudhry)	8.401 min
Standard Deviation (Haroon Chaudhry)	0.0376
%RSD (Haroon Chaudhry)	0.4476%

The %RSD of the internal standard of the four samples that I injected was 0.2277% and that for my laboratory partner was 0.4476%. Since my %RSD for the internal standard of the four samples I injected was less than my laboratory partner's it can be concluded that I had the better injection technique.

Based on the injection volume of 0.8µL, the density of isooctane for Bioglan with sample mass 0.02576g and isooctane mass of 2.12562g, 6.71×10^{-6} g of the sample was injected into the column. Using the same values as before, but a split value of 100:1 yields 6.7×10^{-8} g of sample was injected into the column.

Figure 2 shows the relationship between the retention time and C number (number of carbons in molecule) for saturated FAEE and for mono-unsaturated FAEE on one graph using the GLC 68D FAEE chromatogram standard.

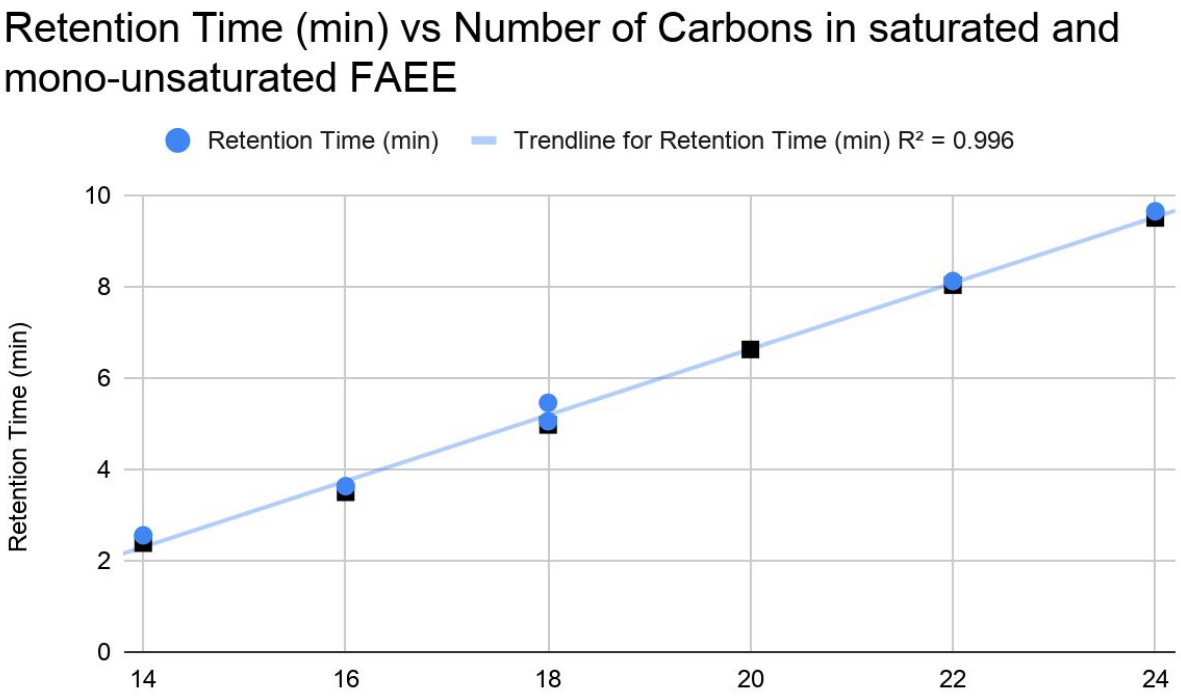

Figure 2. Correlation between C number for saturated and mono-unsaturated FAEE. The black squares are the saturated compounds and the blue circles are the mono-unsaturated compounds.

Based on the graph, the correlation between retention time and the number of carbons in the FAEE is linear. The graph can be used to determine a general range of what the retention time for saturated and mono-unsaturated compounds under the same conditions these were taken under. A factor that could affect what the retention time could be structure of the compound (linear or has different conformations).

Use t_m= 0.25 minutes for the unretained substance, determine k (capacity factor) for the two largest intensity peaks in your recommended dietary supplement excluding the internal standard. Use equation 9 from the introduction:

$$k = \frac{t_r - t_m}{t_m} \qquad (9)$$

Table 9 summarizes the capacity factor k for the two largest intensity peaks in my recommended dietary supplement, Kenko Bio.

Table 9. Capacity factor k for Kenko Bio.

Compound	**Retention Time**	**k**
EPA	7.586 min	29.344
DHA	9.193 min	35.772

IV. Conclusions

Samples of omega-3 ethyl esters in dietary supplements were injected swiftly into the gas chromatography instrument for the best chromatograms. Using the chromatogram, retention times were matched based on a rough estimation on some peaks that were not counted as peaks in the Empower 3 program. After estimating the retention times of the peaks, rough integrations were used based on the peaks of the heights surrounding the peaks not counted. Based on this, Figure 2 visualizes the linear trend of saturated and mono-unsaturated FAEE. This trend can be used to explain the retention times of the omega-3 ethyl esters where the larger the number of carbons and higher unsaturation value should yield a higher retention time under the same conditions.

From the three samples that I chose to inject into the instrument, Kenko Bio, OmegaGenics, and Vitamin Shoppe all had peaks in the right general location for the compounds analyzed from Table 4, but the values were shifted to the left by 0.100 min to 0.200 min. For OmegaGenics and Vitamin Shoppe, there was a large peak around the 7.150 min region which lies between ethyl 11,13-eicosadienoate and ethyl arachidonate. Since the assumption was that the peaks were shifted to the left, both were assumed to be ethyl arachidonate.

Using these three along with the standard sample, Lovaza, the weight % EPA and DHA were found. Kenko Bio had 48.46% EPA and 30.85% DHA with % error of 15.15% and 28.02% respectively. OmegaGenics had 33.01% EPA and 20.77% DHA with % error of 66.00% and 38.91% respectively. Vitamin Shoppe had 54.09% EPA and 15.04% DHA with % error of 33.77% and 17.95%. Lovaza had 37.59% EPA and 22.56% DHA with % error of 32.10% 49.46%. From these values of percent errors, Kenko Bio was the best fit because of the 15.15% and 28.02% error of EPA and DHA respectively.

Using peak areas to determine the concentrations of the % weights in the samples should be theoretically correct considering that both the % peak area and % weights are ratios that are solved to become the concentrations of the sample that was injected.

V. References

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