8

chapter

Gas Chromatography

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8.1 INTRODUCTION

8.1.1 Background

Gas chromatography (GC) has many applications in the analysis of food products. GC has been used for the determination of fatty acids, triglycerides, cholesterol, gases, water, alcohols, pesticides, flavor compounds, and many more. While GC has been used for other food components such as sugars, oligosaccharides, amino acids, peptides, and vitamins, these substances are more suited to analysis by high performance liquid chromatography. GC is ideally suited to the analysis of volatile substances that are thermally stable. Substances such as pesticides and flavor compounds that meet these criteria can be isolated from a food and directly injected into the GC. For compounds that are thermally unstable, too low in volatility, or yield poor chromatographic separation due to polarity, a derivatization step must be done before GC analysis. The two parts of the experiment described here include the analysis of alcohols that requires no derivatization step and the analysis of fatty acids which requires derivatization. The experiments specify the use of capillary columns, but the first experiment includes conditions for a packed column.

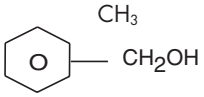
8.1.2 Reading Assignment

Ellefson, W.C. 2017. Fat analysis. Ch. 17, in *Food Analysis*, 5th ed. S.S. Nielsen (ed.), Springer, New York.

Pike O.A., and O'Keefe, S.F. 2017. Fat characterization. Ch. 23 in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

Qian, M.C., Peterson, D.G., and Reineccius, G.A. 2017. Gas chromatography. Ch. 14, in *Food Analysis*, 5th ed. S.S. Nielsen (Ed.), Springer, New York.

8.1 table Alcohol structure and boiling point

Alcohol	*Structure*	*b.p.(°C)*
Methanol	CH_3OH	64.5
Ethanol	$CH_3\text{-}CH_2OH$	78.3
n-Propanol	$CH_3\text{-}CH_2\text{-}CH_2OH$	97
Isobutyl alcohol (2-methyl-1-propanol)	$CH_3\text{-}CH(CH_3)\text{-}CH_2OH$	108
Isoamyl alcohol (3-methyl-1-butanol)	$CH_3\text{-}CH(CH_3)\text{-}CH_2CH_2OH$	
Active amyl alcohol (2-methyl- 1-butanol)	$CH_3\text{-}CH_2\text{-}CH(CH_3)\text{-}CH_2OH$	128
Benzyl alcohol		205

8.2 DETERMINATION OF METHANOL AND HIGHER ALCOHOLS IN WINE BY GAS CHROMATOGRAPHY

8.2.1 Introduction

The quantification of higher alcohols, also known as fusel oils, in wine and distilled spirits is important because of the potential flavor impact of these compounds. These higher alcohols include n-propyl alcohol, isobutyl alcohol, and isoamyl alcohol. Some countries have regulations that specify the maximum and/or minimum amounts of total higher alcohols in certain alcoholic beverages. Table wine typically contains only low levels of higher alcohols, but dessert wines contain higher levels, especially if the wine is fortified with brandy.

Methanol is produced enzymatically during the production of wine. Pectin methyl esterase hydrolyzes the methyl ester of α-1,4-D-galacturonopyranose. The action of this enzyme, which is naturally present in grapes and may also be added during vinification, is necessary for proper clarification of the wine. White wines produced in the United States contain less methanol (4–107 mg/L) when compared with red and rosé wines (48–227 mg/L). Methanol has a lower boiling point than the higher alcohols (Table 8.1), so it is more readily volatilized and elutes earlier from a gas chromatography (GC) column.

Methanol and higher alcohols in distilled liquors are readily quantitated by gas chromatography, using an internal standard such as benzyl alcohol, 3-pentanol, or n-butyl alcohol. The method outlined below is similar to AOAC Methods 968.09 and 972.10 [Alcohols (Higher) and Ethyl Acetate in Distilled Liquors].

8.2.2 Objective

Determine the content of methanol, n-propyl alcohol, and isobutyl alcohol in wine by gas chromatography, using benzyl alcohol as the internal standard.

8.2.3 Principle of Method

Gas chromatography uses high temperatures to volatilize compounds that are separated as they pass through the stationary phase of a column and are detected for quantitation.

8.2.4 Chemicals

	CAS no.	*Hazards*
Benzyl alcohol	100-51-6	Harmful
Ethanol	64-17-5	Highly flammable
Isobutyl alcohol	78-83-1	Irritant
Methanol	67-56-1	Extremely flammable
n-Propyl alcohol	71-23-8	Irritant, highly flammable

8.2.5 Reagents

(**It is recommended that these solutions be prepared by the laboratory assistant before class.)

- Ethanol, 16% (vol/vol) with deionized distilled (dd) water, 500 mL**
- Ethanol, 50% (vol/vol) with dd water, 3200 mL**
- Ethanol, 95% (vol/vol) with dd water, 100 mL**
- Stock solutions**
  Prepared with known amounts of ethanol and fusel alcohols or methanol:
  1. 10.0 g of methanol and 50% (vol/vol) ethanol to 1000 mL
  2. 5.0 g of n-propyl alcohol and 50% (vol/vol) ethanol to 1000 mL
  3. 5.0 g of isobutyl alcohol and 50% (vol/vol) ethanol to 1000 mL
  4. 5.0 g of benzyl alcohol in 95% (vol/vol) ethanol to 100 mL
- Working Standard Solutions**
  Prepared from stock solutions, to contain different amounts of each of the fusel alcohols; aliquots of these are used to get standard curves. Prepare four working standards by combining:
  1. 0.5 mL of stock solutions 1, 2, and 3 with 4.5 mL of 50% (vol/vol) ethanol plus 16% (vol/vol) ethanol to 100 mL
  2. 1.0 mL of stock solutions 1, 2, and 3 with 3 mL of 50% (vol/vol) ethanol plus 16% (vol/vol) ethanol to 100 mL
  3. 1.5 mL of stock solutions 1, 2, and 3 with 1.5 mL of 50% (vol/vol) ethanol plus 16% (vol/vol) ethanol to 100 mL
  4. 2.0 mL of stock solutions 1, 2, and 3 with 16% (vol/vol) ethanol to 100 mL

(*Note*: The final concentration of ethanol in each of these working standard solutions is 18% (vol/vol) ethanol.)

8.2.6 Hazards, Precautions, and Waste Disposal

The alcohols are fire hazards; avoid open flames, breathing vapors, and contact with skin. Otherwise, adhere to normal laboratory safety procedures. Wear safety glasses at all times. Aqueous waste can go down the drain with a water flush.

8.2.7 Supplies

(Used by students)

- Mechanical pipettor, 1000 µL, with tips
- Round bottom flask, 500 mL
- Syringe (for GC)
- 6 Volumetric flasks, 100 mL
- 4 Volumetric flasks, 1000 mL

8.2.8 Equipment

- Analytical balance
- Distillation unit (heating element to fit 500-mL round bottom flask; cold water condenser)
- Gas chromatography unit:

Column	DB-Wax (30 m, 0.32 nm ID, 0.5-µm film thickness) (Agilent Technologies, Santa Clara, CA) or equivalent (capillary column) or 80/120 Carbopack BAW/5% Carbowax 20 M, 6 ft × 1/4 in OD × 2 mm ID glass column (packed column)
Injector temperature	200 °C
Column temperature	70–170 °C at 5 °C/min
Carrier gas	He at 2 mL/min (N_2 at 20 mL/min for packed column)
Detector	Flame ionization
Attenuation	8 (for all runs)

ID inner diameter, *OD* outer diameter, *BAW* base and acid washed

8.2.9 Procedure

(Instructions are given for single standard and sample analysis, but injections can be replicated.)

8.2.9.1 *Sample Preparation*

1. Fill a 100-mL volumetric flask to volume with the wine sample to be analyzed.
2. Pour the wine into a 500-mL round bottom flask and rinse the volumetric flask several times with dd water to complete the transfer. Add additional water if necessary to bring the volume of sample plus dd water to ca. 150 mL.
3. Distil the sample and recover the distillate in a clean 100-mL volumetric flask. Continue the distillation until the 100-mL volumetric is filled to the mark.
4. Add 1.0 mL of the stock benzyl alcohol solution to 100 mL of each working standard solution and wine sample to be analyzed.

8.2.9.2 *Analysis of Sample and Working Standard Solutions*

1. Inject 1 µL of each sample and working standard solution in separate runs on the GC column (split ratio 1:20) (For packed column, inject 5.0 µL.)
2. Obtain chromatograms and data from integration of peaks.

8.2.10 Data and Calculations

1. Calculate the concentration (mg/L) of methanol, n-propyl alcohol, and isobutyl alcohol in each of the four Working Standard Solutions (see sample calculation below).

Alcohol concentration (mg/l):

Working standard	*Methanol*	*N-Propyl alcohol*	*Isobutyl alcohol*
1			
2			
3			
4			

Example calculations:

Working Standard Solution #1 contains methanol + n-propyl alcohol + isobutyl alcohol, all in ethanol.

Methanol in Stock Solution #1:

$$\frac{10\,\text{g methanol}}{1000\,\text{mL}} = \frac{1\text{g}}{100\,\text{mL}} = \frac{0.01\text{g}}{\text{mL}}$$

Working Standard Solution #1 contains 0.5 mL of Stock Solution #1.

=0.5 mL of 0.01 g methanol/mL
=0.005 g methanol = 5 mg methanol

That 5 mg methanol is contained in 100 mL volume.

=5 mg/100 mL = 50 mg/1000 mL
= 50 mg methanol/L

Repeat procedure for each alcohol in each working standard solution.

2. Calculate the peak height or peak area ratios for methanol, n-propyl alcohol, and isobutyl alcohol, compared to the internal standard, for each of the Working Standard Solutions and the wine sample. To identify which is the methanol, n-propyl alcohol, and isobutyl peak, see the example chromatogram that follows. Note that data from automatic integration of the peaks can be used for these calculations. Report the ratios in a table as shown below. Show an example calculation of concentration for each type of alcohol.

Peak hseight ratios for alcohol peaks at various concentrations of methanol, n-propyl alcohol, and isobutyl alcohol, with benzyl alcohol as internal standard:

	Peak height ratio[a]		
	Methanol	*n-Propyl alcohol*	*Isobutyl alcohol*
Alcohol Conc. (mg/l)	*Benzyl alcohol*	*Benzyl alcohol*	*Benzyl alcohol*
25			
50			
75			
100			
150			
200			
Wine sample			

[a]Give individual values and the ratio

3. Construct standard curves for methanol, n-propyl alcohol, and isobutyl alcohol using the peak height ratios. All lines can be shown on one graph. Determine the equations for the lines.
4. Calculate the peak ratios for methanol, n-propyl alcohol, and isobutyl alcohol in the wine sample and their concentrations in mg/L.

8.2.11 Questions

1. Explain how this experiment would have differed in standard solutions used, measurements taken, and standard curves used if you had used external standards rather than an internal standard.
2. What are the advantages of using an internal standard rather than external standards for this application, and what were the appropriate criteria to use in selecting the internal standard?

8.3 PREPARATION OF FATTY ACID METHYL ESTERS (FAMEs) AND DETERMINATION OF FATTY ACID PROFILE OF OILS BY GAS CHROMATOGRAPHY

8.3.1 Introduction

Information about fatty acid profile on food is important for nutrition labeling, which involves the measurement of not only total fat but also saturated, unsaturated, and monounsaturated fat. Gas chromatography is an ideal instrument to determine (qualitatively and quantitatively) fatty acid profile or fatty acid composition of a food product. This usually involves extracting the lipids and analyzing them using capillary gas chromatography. Before such analysis, triacylglycerols and phospholipids are saponified, and the fatty acids liberated are esterified to form fatty acid methyl esters (FAMEs) so that the volatility is increased.

Two methods of sample preparation for FAME determination will be used in this experiment: (1) sodium methoxide method and (2) boron trifluoride (BF_3) method. In the sodium methoxide method, sodium methoxide is used as a catalyst to interesterify fatty acid. This method is applicable to saturated and unsaturated fatty acids containing from 4 to 24 carbon atoms. In the BF_3 method, lipids are saponified, and fatty acids are liberated and esterified in the presence of a BF_3 catalyst for further analysis. This method is applicable to common animal and vegetable oils and fats and fatty acids. Lipids that cannot be saponified are not derivatized and, if present in large amount, may interfere with subsequent analysis. This method is not suitable for preparation of methyl esters of fatty acids containing large amounts of epoxy, hydroperoxy, aldehyde, ketone, cyclopropyl, and cyclopentyl groups and conjugated polyunsaturated and acetylenic compounds because of partial or complete destruction of these groups.

It should be noted that AOAC Method 969.33 is used in this laboratory exercise, rather than AOAC Methods 996.06, which is the method for nutrition labeling, with a focus on *trans* fats. Compared to AOAC Method 969.33, Method 996.06 used a longer and more expensive capillary column, requires a longer analysis time per sample, and involves more complicated calculations.

8.3.2 Objective

Utilize two methods to prepare methyl esters from fatty acids in food oils and then determine the fatty acid profile and their concentration in the oils by gas chromatography.

8.3.3 Chemicals

	CAS no.	*Hazards*
Boron trifluoride (BF_3)	7637-07-2	Toxic, highly flammable
Hexane	110-54-3	Harmful, highly flammable, dangerous for the environment
Methanol	67-56-1	Extremely flammable
Sodium chloride (NaCl)	7647-14-5	Irritant
Sodium hydroxide (NaOH)	1310-73-2	Corrosive
Sodium sulfate (Na_2SO_4)	7757-82-6	Harmful
Sodium methoxide	124-41-4	Toxic, highly flammable

8.3.4 Reagents and Samples

- Boron trifluoride (BF_3) – in methanol, 12–14% solution
- Hexane (GC grade. If fatty acids contain 20 C atoms or more, heptane is recommended.)
- Methanolic sodium hydroxide 0.5 N (Dissolve 2 g of NaOH in 100 mL of methanol.)
- Oils: pure olive oil, safflower oil, salmon oil
- Reference standard [Gas–liquid chromatography (GLC)-60 Reference Standard FAME 25 mg is dissolved in 10 mL hexane, (Table 8.2) (Nu-Chek Prep, Inc. Elysian, MN)]
- Sodium methoxide, 0.5 *M* solution in methanol (Aldrich)
- Sodium chloride, saturated
- Sodium sulfate, anhydrous granular

8.3.5 Hazards, Precautions, and Waste Disposal

Do all work with the boron trifluoride in the hood; avoid contact with the skin, eyes, and respiratory tract. Wash all glassware in contact with boron trifluoride immediately after use. Otherwise, adhere to normal laboratory safety procedures. Wear safety glasses at all times. Boron trifluoride, hexane, and sodium methoxide must be disposed of as hazardous wastes. Other wastes likely may be put down the drain using a water rinse, but follow good laboratory practices outlined by environmental health and safety protocols at your institution.

8.3.6 Supplies

(Used by students)

- Boiling flask, 100 mL, with water-cooled condenser for saponification and esterification

8.2 table FAME GLC-60 reference standard

No.	*Chain*	*Item*	*Weight %*
1	C4:0	Methyl butyrate	4.0
2	C6:0	Methyl caproate	2.0
3	C8:0	Methyl caprylate	1.0
4	C10:0	Methyl caprate	3.0
5	C12:0	Methyl laurate	4.0
6	C14:0	Methyl myristate	10.0
7	C14:1	Methyl myristoleate	2.0
8	C16:0	Methyl palmitate	25.0
9	C16:1	Methyl palmitoleate	5.0
10	C18:0	Methyl stearate	10.0
11	C18:1	Methyl oleate	25.0
12	C18:2	Methyl linoleate	3.0
13	C18:3	Methyl linolenate	4.0
14	C20:0	Methyl arachidate	2.0

- Pasteur pipette
- Syringe
- Vials or sample bottle with tight-seal cap

8.3.7 Equipment

- Analytical balance
- Centrifuge
- Vortex mixer
- Gas chromatography unit (with running conditions):

Instrument	Gas chromatograph (Agilent 6890 or similar)
Detector	Flame ionization detector
Capillary column	DB-Wax or equivalent
Length	30 m
Internal diameter (ID)	0.32 mm
Df	1.0 µm
Carrier gas	He
Makeup gas	Nitrogen
Sample injection	1 µl
Split ratio	1:20
Flow rate	2 mL/min (measured at room temperature)
Injector temperature	250 °C
Detector temperature	250 °C
Temperature program	
Initial oven temperature	100 °C
Initial time	2 min
Rate	5 °C/min
Final temperature	230 °C
Final time	10 min

8.3.8 Procedure

(Instructions are given for single sample preparation and injection, but injections of samples and standards can be replicated.)

8.3.8.1 *Preparation of Methyl Esters*

Method A. Preparation of Methyl Esters by Boron Trifluoride (Adapted from AOAC Method 969.33)

Notes Methyl ester should be analyzed as soon possible or sealed in an ampule and stored in a freezer. You might also add equivalent 0.005% 2, 6-di-*tert*-butyl-4-methylphenol (BHT). Sample size needs to be known to determine the size of the flask and the amount of reagents, according to Table 8.3.

1. Add 500 mg sample (see Table 8.3) to 100-mL boiling flask. Add 8 mL methanolic NaOH solution and boiling chip.

8.3 table Determination of flask size and amount of reagent from approximate sample size

Sample (mg)	*Flask (mL)*	*0.5 N NaOH (mL)*	*BF_3 reagent (mL)*
100–250	50	4	5
250–500	50	6	7
500–750	100	8	9
750–1000	100	10	12

2. Attach condenser and reflux until fat globules disappear (about 5–10 min).
3. Add 9 mL BF_3 solution through condenser and continue boiling for 2 min.
4. Add 5 mL hexane through condenser and boil for 1 more min.
5. Remove the boiling flask and add ca. 15 mL saturated NaCl solution.
6. Stopper flask and shake vigorously for 15 s while solution is still tepid.
7. Add additional saturated NaCl solution to float hexane solution into neck of flask.
8. Transfer 1 mL upper hexane solution into a small bottle and add anhydrous Na_2SO_4 to remove H_2O.

Method B Preparation of Methyl Esters by Sodium Methoxide Method

1. Using a Pasteur pipette to transfer, weigh 100 mg (±5 mg) of sample oil to the nearest 0.1 mg into a vial or small bottle with a tight-sealing cap.
2. Add 5 mL of hexane to the vial and vortex briefly to dissolve lipid.
3. Add 250 µl of sodium methoxide reagent, cap the vial tightly, and vortex for 1 min, pausing every 10 s to allow the vortex to collapse.
4. Add 5 mL of saturated NaCl solution to the vial, cap the vial, and shake vigorously for 15 s. Let stand for 10 min.
5. Remove the hexane layer and transfer to a vial containing a small amount of Na_2SO_4. Do not transfer any interfacial precipitate (if present) or any aqueous phase.
6. Allow the hexane phase containing the methyl esters to be in contact with Na_2SO_4 for at least 15 min prior to analysis.
7. Transfer the hexane phase to a vial or small bottle for subsequent GC analysis. (Hexane solution can be stored in the freezer.)

8.3.8.2 *Injection of Standards and Samples into GC*

1. Rinse the syringe three times with hexane and three times with the reference standard mixture (25 mg of 20A GLC Reference Standard FAME dissolved in 10 mL hexane). Inject 1 µl of standard solution, remove syringe from injection port, and then press start button. Rinse syringe again three times with solvent. Use the chromatogram obtained as described below.
2. Rinse the syringe three times with hexane and three times with the sample solution prepared by Method A. Inject 1 µl of sample solution, remove syringe from injection port, and then press start button. Rinse syringe again three times with solvent. Use the chromatogram obtained as described below.
3. Repeat Step 3 for sample solution prepared by Method B.

8.3.9 Data and Calculations

1. Report retention times and relative peak areas for the peaks in the chromatogram from the FAME reference standard mixture. Use this information to identify the 14 peaks in the chromatogram.

Peak	*Retention time*	*Peak area*	*Identity of peak*
1			
2			
3			
4			
5			
6			
7			
8			
9			
10			
11			
12			
13			
14			

2. Using the retention times for peaks in the chromatogram from the FAME reference standard mixture, and your knowledge of the profile of the oil, identify the peaks in the chromatograms for each type of oil analyzed, [Cite your source(s) of information on the fatty acid profile of each oil.] Report results for samples from both methods of derivatization.

Results from chromatograms using *boron trifluoride method* to prepare methyl esters:

	Safflower oil		*Pure olive oil*		*Salmon oil*	
Peak	*Retention time*	*Identity*	*Retention time*	*Identity*	*Retention time*	*Identity*
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						

Results from chromatograms using *sodium methoxide method* to prepare methyl esters:

	Safflower oil		*Pure olive oil*		*Salmon oil*	
Peak	*Retention time*	*Identity*	*Retention time*	*Identity*	*Retention time*	*Identity*
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						

3. For the one oil analyzed by your group, prepare a table (with appropriate units) comparing your experimentally determined fatty acid profile to that found in your cited literature source.

	Quantity determined		
	Quantity in literature	*Boron trifluoride method*	*Sodium methoxide method*
C4:0			
C6:0			
C8:0			
C10:0			

	Quantity determined		
	Quantity in literature	*Boron trifluoride method*	*Sodium methoxide method*
C12:0			
C14:0			
C14:1			
C16:0			
C16:1			
C18:0			
C18:1			
C18:2			
C18:3			
C20:0			

Type of oil tested:

8.3.10 Questions

1. Comment on the similarities and differences in the fatty acid profiles in question #3 of Data and Calculations, comparing experimental data to literature reports. From the results, compare and decide which method of esterification to obtain FAMEs was better for your sample.
2. The approach taken in this lab provides a fatty acid profile for the oils analyzed. This is sufficient for most analytical questions regarding fatty acids. However, determining the fatty acid profile is not quite the same thing as quantifying the fatty acids in the oil. (Imagine that you wanted to use the results of your GC analysis to calculate the amount of mono- and polyunsaturated fatty acids as grams per a specified serving size of the oil). To make this procedure sufficiently quantitative for a purpose like that just described, an internal standard must be used.
   (a) Why is the fatty acid profiling method used in this lab inadequate to quantify the fatty acids?
   (b) What are the characteristics required of a suitable internal standard for FAME quantification by GC and how does this overcome the problem(s) identified in (a)?
   (c) Would the internal standard be added to the reference standard mixture and the sample or only to one of these?
   (d) When would the internal standard be added?

RESOURCE MATERIALS

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