

Gas Chromatography-Mass Spectrometry (GCMS) of Amino Acids

Introduction

In this experiment you will learn about separation science using gas chromatography (GC) and mass spectrometry by measuring amino acids by GCMS. Separation of amino acids has always been a challenge because of the heterogeneous nature of this series of compounds due to the wide range of functional groups they possess. Mass spectrometry aids identification of which GC peak that elutes is which amino acid. Using mass spectrometry to positively identify the various amino acids, you should be able to make some generalizations as to how compounds separate by GC: which compounds elute first, which elute last, which functional groups cause retardation of elution, which pairs of compounds do not separate well, and which functional groups do not derivatize and chromatograph well.

This lab is devoted to biological molecules. The keys to biologically active molecules are the functional groups in the molecules. Functional groups define the class of molecules. For example, every amino acid has both a carboxyl and an amino group. However, functional groups also add polarity to the molecules and, often times, charge. The polar nature of these molecules aids their solubility in water, but greatly reduces their vapor pressure for analysis by GC. We must chemically modify amino acids to reduce polarity and increase volatility for measurement by GC. The types of modification chemistry you will do follows exactly what you learned in organic chemistry, only with some twists. You will complete organic reactions of biological molecules using nothing more than screw cap vials. In addition all reactions and manipulations will be done on a micro scale, using nanomole amounts of sample.

You will use mass spectrometry to identify the individual amino acids and to confirm the chemical modifications you have imposed with your “derivatization” chemistry. We will review some of the fragmentation rules used in mass spectrometry for organic compound analysis, and you will apply these rules to identify individual amino acids. Knowing what amino acids you have injected into the GCMS and knowing something about expected elution order will simplify the identification of the individual amino acids. You will also prepare and measure samples of *perdeuterated* amino acids to see the effect of adding deuterium on the various ions in the mass spectra of the various amino acids you are measuring.

What you will accomplish:

Lab period #1: Prepare derivatives of a mixture of amino acids and deuterated amino acids. You will also need to set up tables of information concerning what you expect the amino acid derivatives will yield in terms of modified amino acid structures and deuterated amino acids. You will need to make some predictions about what ions you will see in the different amino acid spectra.

Lab period #2: Perform EI-GCMS of the amino acid derivatives. You will optimize GC separation conditions for measuring the amino acids during this lab. You will collect all the spectra and GC-chromatogram information needed to identify the amino acids.

Equipment

- Varian Saturn 2100T ion trap gas chromatograph-mass spectrometer with a Varian 3900 GC and CP-8400 autosampler
- Micro-derivatization equipment

Experimental

Lab period #1: Preparation of samples (see below for details of what is in each solution):

1. Preset the heating block to 110° C.
2. Prepare 1-ml of a 1:1 mix of N-methyl-N-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) and acetonitrile.
3. Your instructor will have available (i) a limited mixture of amino acids in solution, (ii) a solution containing most amino acids, and (iii) a solution of deuterated amino acids. The concentration of leucine and most of the other amino acids will be known in each of these solutions.
 - a. Calculate the volume you will need to transfer ~200 nmol of leucine (which serves as an “average” amino acid because the different amino acids will be in different amounts depending upon the protein source of the amino acids) for each of the samples.
 - b. The amino acid solution will either be a standard mixture of amino acids or protein, such as bovine serum albumin, that was hydrolyzed to component amino acids by heating 6 M HCl for 12-24 h at ~100 °C. *If the sample is hydrolyzed protein, you will need to follow a special procedure to trap the amino acids away from the acid using a cation exchange resin column.*
4. Obtain 2-ml screw cap reaction vials from your instructor for preparing each sample in duplicate. Check to make sure there are no chips in the sealing lip of the glass of each vial. Prepare labels in duplicate as follows:
 - i. limited mixture AA
 - ii. AA sample
 - iii. ²H-AA
 - iv. AA + ²H-AA
5. Transfer using the hand-held disposable tip pipetter enough amino acids from each solution to contain ~200 nmol leucine to the correctly marked vials. The 4th solution is a combination of the unlabeled amino acid solution and the ²H-labeled amino acids.
6. **Special treatment for the BSA hydrolysate:**
 - a. Pipette the appropriate volume into 12x75 mm glass tubes.
 - b. Add 2-ml 1-N acetic acid & vortex.
 - c. Prepare the ion exchange columns:
 - i. Add 1-ml cation exchange resin (50W-X8) to each column.
 - ii. Rinse with ~4 ml distilled water.
 - d. Pour hydrolysate onto columns. Do NOT collect the acid eluate.
 - e. When all the fluid has passed through the column, rinse the column 3 times with distilled water. Let it drain.
 - f. Place reaction vials under columns. Add 2-ml 3-N ammonium hydroxide to each column; collect eluate.
7. *For all samples: Evaporate any liquid using a stream of N₂ gas in the N-Evap apparatus until the vials are dry (your instructor will show you how to use the N-Evap). You may wish to use a “squirt” of methylene chloride as an azeotrope agent after the vials are dry and then evaporate the methylene chloride off to be sure that all water is removed.*
8. Add 100 µl of 1:1 mix of MTBSTFA:acetonitrile to each vial. Cap the vials & vortex each one.
9. Place the vials in the heating block at 110° C for 30 minutes.
10. Remove the vials carefully, and let cool to room temperature.

11. Store the samples in a ZIP-lock bag in the freezer.
12. When ready to analyze the samples, make sure samples come to room temperature while still in the bag to prevent condensation inside the vials.

Lab period 2: EI-GCMS

1. With the mass spec TA or with the Chem 201 TA, orient yourself to the Varian Saturn GCMS:
 - a. Note the major components
 - b. Note operation of the GC
 - c. Note the vacuum of the system
 - d. Note the GC column installed
2. With your TA, set the mass spec in parameters for EI mode and a scan range from 50-550 Da.
3. Start with a general temperature program for the GC:
 - a. 1 ml/min He,
 - b. Injector: 250 °C,
 - c. GC temperature program: initial: 80 °C, hold for 2 min, then program the GC: 8 °C/min to 250 °C; hold for 15 min.
4. Inject 1 µl of one of the “limited mix AA” samples.
 - a. Did you get all the peaks you expected and were they resolved?
 - b. Check the spectrum of each peak and confirm each peak’s identity.
 - c. Were the earliest and last eluting amino acids the ones you expected?
5. Repeat, injecting one of the regular AA derivatives.
 - a. Confirm the first and last peaks for their identity.
 - b. Did you get the earliest expected eluting amino acid?
 - c. Were all of the peaks resolved?
6. Change the temperature program to increase the initial temperature for quicker elution of the 1st AA to reduce the time. Repeat the injection of the TBDMS AA sample. If needed, readjust the initial temperature and repeat the injection again.
7. Reduce the temperature ramp from 8 °C/min to 4 °C/min. Repeat injection of the TBDMS AA sample.
 - a. Print a good “mass chromatogram” of the total ion current (TIC) trace versus time of each of the samples.
 - b. Integrate, process, and plot the spectrum of each of various peaks one of the AA sample injections.
8. Chose the best GC program and inject one of the ²H-AA samples.
 - a. Prepare a mass chromatogram of the ²H-AA sample.
 - b. Integrate each peak and plot each peak’s spectrum.
 - c. Identify the ²H-amino acids in the run.
9. You may also measure the ²H-AA + AA sample if there is enough time.

Solutions of amino acids (AA) available:

Bovine serum albumin (BSA) protein already hydrolyzed to component amino acids (AA)

vial #1A: 12.9 mg BSA in 4 ml 6M HCl

Uniformly (U) ²H-labeled algal amino acid mixture (CIL #DLM-2082, 98%+ ²H)

49.02 mg total AA in 10.0382 g H₂O

Algal amino acid mix U-¹³C:

50.18 mg total AA in 10.1024 g H₂O
Estimated leucine content: 10.6 g Leu/100 g AA

Four AA mixture #1 (Ala, Leu, Lys, Phe):

Ala - 7.82 mg
Lys - 13.38 mg
Leu - 10.44 mg
Phe - 13.82 mg
in 19.93 g H₂O

Four AA mixture #2 (Ala, Arg, Glu, Leu):

Ala - 14.81 mg
Arg - 14.62 mg
Glu - 15.92 mg
Leu - 14.91 mg
in 10.03 g H₂O

Approximate amino acid composition (weight basis) of the samples:

Amino Acid	BSA	CIL ² H-AA mix
Ala	4.7%	7%
Arg	4.0%	7%
Asn	5.5%	
Asp	5.5%	10%
Cys	5.7%	
Glu	9.2%	10%
Gln	4.5%	
Gly	2.7%	6%
His	2.2%	2%
Ile	4.7%	4%
Leu	9.2%	10%
Lys	8.5%	14%
Met	1.5%	1%
Phe	7.2%	4%
Pro	4.7%	7%
Ser	5.8%	4%
Thr	5.7%	5%
Trp	0.0%	
Tyr	2.8%	4%
Val	6.0%	5%

Questions

1. What is the effect of changing the initial temperature or the rate of temperature programming upon the separation of the various amino acids?
2. Which amino acids eluted first, which amino acids eluted late, and why?
3. Which amino acids did *not* give you a peak or spectrum? Why do you think that was?

4. What do you think you might need to do to get spectra of the “missing” amino acids?
5. Did you record molecular (M^+) ions for any amino acids?
6. What were the characteristic fragmentation patterns for the EI TBDMS amino acids?
7. Were the spectra from the ^2H -amino acids helpful in identifying amino acids and the origin of various fragments?
8. Did each amino acid contain the number of ^2H 's per amino acid expected?

Points to include in your lab report

Introduction

- Discuss the challenge of measuring individual members of a homologous series of compounds and how a chromatographic technique can affect separation.
- Discuss how the mass spectrometer provides additional specific information as to compound identification.
- Discuss the problems with measuring amino acids in terms of heterogeneity of functional groups and polarity that keeps them from passing through a GC.

Experimental

- Describe the derivatization reaction that occurs.
- Describe the instrument & instrumental conditions used.

Results

- Include the results of changing GC program conditions on the elution of the amino acids.
- Present figures for the TIC trace for each of the sets of amino acids. Be sure to annotate each peak with what you think the identity of the peak is.
- You can include complete spectra for all peaks in the APPENDIX. However, include as figures key spectra highlighting the major ions and fragmentation pattern of the TBDMS amino acids.
- Prepare a table listing all the amino acids measured for each derivative. The 1st column will be a list of the individual amino acids. The 2nd column would be for the M^+ ion (may not exist), and following columns would be major fragments. List under each amino acid whether each ion appears and approximately how intense ion that ion is in the spectrum.
- Present results for the ^2H -amino acids and highlight the differences between the unlabeled and the ^2H -amino acids.

Discussion

- Outline the mechanism by which the derivatization reaction occurs. Discuss why you did not get GCMS peaks corresponding to some of the amino acids. Suggest why you did not get peaks and what you might be able to do to get peaks for the missing amino acids.
- Compare your results to the literature for amino acid elution order from the GC. Discuss differences.
- Discuss why some amino acids did not provide a predicted spectrum.
- Discuss whether the use of the ^2H -labeled amino acids was helpful in assigning amino acid identities and fragments.
- Was the expected number of ^2H present in each amino acid spectrum of the ^2H -labeled amino acids? Were any unlabeled hydrogen present or positions in the amino acids that did not have a ^2H label?

Appendix

- Put complete sets of mass spectra with the key peaks annotated for each in the APPENDIX.

Handouts - none

References

- D.A. Skoog, F.J. Holler & S.R. Crouch, *Principles of Instrumental Analysis*, 6th ed, 2007:
 - Chap 11: Atomic mass spectrometry
 - Chap 20: Molecular mass spectrometry
 - Chap 26: An introduction to chromatographic separations
 - Chap 27: Gas chromatography
- J.T. Watson, *Introduction to Mass Spectrometry*, 3rd ed, 1997:
 - Chaps 1 & 2
 - Chap 7: Electron ionization
 - Chap 8: Chemical ionization
 - Chap 16: Gas chromatography-mass spectrometry
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 - Part I: Introduction
 - Part II, Chap 5: Amino acids and peptides
- J.B. Lambert, H.F. Shurvell, D.A. Lightner, & R.G. Cooks, *Organic Structural Spectroscopy*, 1998
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Silyl amino acid derivatization and GC articles (PDF files available on the CHEM 201 website):

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- RJ Early, JR Thompson, GW Sedgwick, JM Kelly & RJ Christopherson: Capillary gas chromatographic analysis of amino acids in blood and protein hydrolysates as *tert.*-butyldimethylsilyl derivatives. *J. Chromatogr.* 416: 15-23, 1987.
- C-J Goh, KG Craven, JR Lepock & EB Dumbroff: Analysis of all protein amino acids as their *tert.*-butyldimethylsilyl derivatives by gas-liquid chromatography. *Anal. Biochem.* 163: 175-181, 1987