

1. Prior to the Lab

Before the lab, all students must complete the online safety training (students will not be allowed into the lab without its successful completion) and quantitation homework.

Further, students will be required to take a take-home test (the last section of this document) Students' answers to that test must be handwritten and students are expected to have a mastery of them (being able to present them without reading). The answers will be discussed with the instructor prior to the first lab at the time of meeting. The lack of preparation for this test will result in a delay in taking the lab to the next available time.

Students are expected to have their own laboratory notebook with page numbers (handwritten page numbers are acceptable). The safety glasses must be worn at all times in the lab (students should use their own; however, optionally they may use those available in the lab).

2. Lab Timeline

This timeline is tentative.

	Sign up for the lab (topic and time)
1 st session	1 st lab starting with home to take test and completion of the 1 st task (solutions prep)
2 nd session	GC-MS analysis of samples in the instructor's laboratory. Students will be responsible for recapping their samples and sequence preparation, they also need to ensure that they have record of the GC-MS method and sequence
3 rd session	Data processing and submission of a MS Excel spreadsheet to the Instructor via-Email for review (the student will not be allowed to start the second task without corrected data processing)
4 th session	Based on the results (correctly processed data in MS Excel) from the 1 st task, students will remediate the experimental error from the 1 st lab and will complete the second task
5 th session	GC-MS Analysis of samples in the instructor's laboratory. Students will be responsible for recapping their samples and sequence preparation, they also need to ensure that they have record of the GC-MS method and sequence
	Submission of the final report on Blackboard

3. Optional Topics

- Phthalates introduced to plastic (plastic toys) as plasticizers, regulated by EPA due to their toxicity.
- Caffeine, theobromine, theophylline, the active components in tea or coffee
- Fungicides (e.g., tebuconazole, propiconazole) used as preservatives in wooden products such as windows
- Essential oils (e.g., thymol, menthol, eucalyptol, carvone, carvacrol), in peppermint or savory.
- BTEX in gasoline, benzene, toluene, xylenes, ethylbenzene concentrations are sometimes used to aid in assessing the relative risk or extent of the contamination by gas stations
- Alkanes (C₆-C₁₄) in arson samples (or other matrices, e.g., fuels)
- Polycyclic aromatic hydrocarbons (PAHs), promutagenes found in charred meat products (or other matrices, e.g., fuels, air particles)

NOTE! Students can perform this assignment using their own analytes, but then they need to obtain an approval from the instructor.

4. Introduction

Gas chromatography coupled with mass spectrometry (GC-MS) is an analytical method used for identification and quantification of numerous volatile compounds. The qualitative GC-MS analysis is conducted by matching the retention time of given unknowns and mass spectra of known chemicals, typically electron ionization (EI) mass spectra with known standards or libraries. The quantitative analysis in the total ion current mode (TIC) can be performed as a traditional GC analysis based on the total peak area, however the correct approach to quantification is based on the peak area of a specific ion. In addition to the instrumentation, quantification is also dependent on the type of the calibration method used. Various calibration methods such as external standard calibration, internal standard calibration, and method of standard addition may be performed. Today the most common in gas chromatography is the internal standard calibration. The essential component of the work in analytical lab in industry and regulation agencies is following the rules of Good Laboratory Practice (GLP). Thus, the underlying component of the lab is placed on sample labeling, organization of experimental work and data recording.

The aim of this lab assignment is to evaluate GC-MS analyses using the internal standard calibration with selected compounds (corresponding to the 1st assignment) within two tasks. In the 1st task students will determine the instrumental limits of quantification and validate the method based on replicate determination of the targeted compound in an evaluation sample of a defined (known) composition. This will be followed by the second lab (upon successful completion of 1st lab) where students will determine the concentrations in an unknown sample.

5. Instrumentation

The experiments will be performed on a HP5790 Series II GC-MS system equipped with a split/splitless injector and a mass spectrometer (HP 5975C) with an electron ionization source, quadrupole analyzer and HP 7873 autosampler. The analyses will be performed on ca. 50 m long DB-5 (5%phenyldimethylpolysiloxane) capillary column with a 0.25 mm internal diameter and 0.25 μm film thickness. *Note: in the experimental section of your report you will need to provide specific operation conditions (e.g., flowrate, temperatures, etc.) and the instrument used for the analysis. The conditions reported are essential to ensure that another chemist can repeat your work if needed.*

6. Materials

Each student is responsible to have his/her own laboratory notebook with page numbers (handwritten page numbers are acceptable). The safety glasses must be worn at all times in the lab (students should use their own; however, optionally they may use those available in the lab).

Students will be provided with gloves. If solutions are spilled on the gloves, students need to replace them.

For task 1 each student will receive two target compounds (corresponding to the 1st assignment) and an internal standard diluted solution. For task 2, an unknown sample will be provided. In the lab, solvents of GC or HPLC quality including dichloromethane, *n*-hexane and methanol will be available for sample dilution. At the beginning of each lab period, each student will also receive a check-out box, which must be returned to the instructor at the end of the lab period. This box will include volumetric flasks, two 10-mL vials, autosampler vials (2 mL), several syringes (10, 250 μL and 1 mL) for solution preparation, readymade internal standard solution, and Pasteur pipettes.

Throughout the labs, all the flasks and vials must be labeled with the student's initials and the page number of the notebook on which the sample is described. Omitting labeling of any samples will result in a loss of points. At the end of this lab assignment, each student will be responsible for returning the cleaned syringes (toolbox) to the instructor. All disposable materials must be discarded, and glassware washed with water and

ethanol. The samples and solvents should be disposed in the properly labeled waste containers separating chlorinated and non-chlorinated waste.

7. Instructions

Students have to sign up based on the timeline provided above to ensure the instrument availability.

In Section 7, several “How to” videos are provided with detailed descriptions showing sample and standard preparation and data processing.

The lab assignment consists of the following tasks:

TASK 1

- 1) Home to take test
- 2) Preparation of stock solutions, calibration standards and evaluation samples.
- 3) Analysis of calibration solutions and samples using GC-MS with MS conducted with either the supervising graduate student or instructor (ensure that you schedule an appointment in advance).
- 4) Data processing and submission of MS excel sheet:
 - a. Identification of compounds based on retention times and mass spectra.
 - b. Generation of calibration data including main calibration parameters, graphical presentation of calibration curves using the internal standard method, and determination of LOQs.
 - c. Validation of the method based on the quantified content of analytes in the evaluation samples and its comparison with the theoretically prepared concentration using the t-test (i.e., accuracy). Evaluation of repeatability of the GC analysis and also that of sample preparation (i.e., precision).
 - d. Submission of the MS Excel to the instructor

TASK II

- 5) Preparation of unknown samples and their analysis with the calibration solutions. If the calibrations from the 1st lab are correct and solutions are not evaporated, they can be reused for this analysis (otherwise they need to be remade).
- 6) Data processing and determination of concentrations in unknowns.
- 7) Preparation of the report in the form of a paper following J. Chromatography instructions at <http://www.elsevier.com/journals/journal-of-chromatography-a/0021-9673/guide-for-authors>. The exception are references which must follow the ACS style format. The paper should cover validation of the method based on the accuracy of determination of the analytes in the evaluation sample and repeatability of the determination and application of the method to the unknown samples. The details are described in Section 8.

7.1. Preparation of Solutions

Sample preparation for this project consists of the preparation of:

- stock solutions of the target analytes and internal standard (IS) (two analytes by each student)
- an initial calibration mixture (a) and its serial dilution (one set prepared jointly by two students)
- five evaluation samples (i.e., defined mixtures of two targeted analytes) prepared by each student
- triplicate unknown sample prepared by each student (task 2)

Sample labeling: Each sample and solution must be labeled with the initials of the student preparing the sample, page number in the notebook, and number (or letter) referring to the location on the particular page. Omitting the labeling of the solutions and/or its recording the labels in the notebook will lead to the loss of points!!

7.1.1. Stock Solutions

For details on the experimental and organization of data in MS Excel see videos (Section 10).

The students will prepare stock solutions of target analytes typically in a volume of 5-10 mL in volumetric flasks with final concentrations of 1-3 mg/mL, typically in dichloromethane (for more polar compounds, methanol may be used). Two stocks will be prepared by each student.

The internal standard (IS) should be a compound of similar chemical and physical properties as are your target analytes and may NOT be naturally occurring in the samples. However, for the purpose of this lab, we will use a single IS, *o*-terphenyl or, for the caffeine lab, 4-chloroacetophenone diluted to ca. 5 mg/mL.

All of the stock solutions need to be prepared in volumetric flasks by weighing approximate amounts of the target analyte into the volumetric flask (e.g., ~5 mg for 5 mL flask) and adding the solvent up to the meniscus of the volumetric flask. The exact concentration should be determined after the sample preparation and a pertinent calculation needs to be shown in MS Excel. Each stock solution needs to be labeled and recorded in the lab notebook as described above.

7.1.2. Calibration Solutions

Note that most of solutions are prepared in dichloromethane, which has a low boiling point (37 °C) and thus evaporates very fast even at ambient temperature. The exception is the caffeine lab, which employs methanol. Make sure that you close your solutions between the sample preparation steps to ensure that the concentrations of your stock solutions, standards, internal standard, and samples are not changing over time.

For the internal standard (IS) calibration method, the standards should be prepared by a serial dilution, then the IS is added to each standard solution (using the same volume/mass ratios). The same amount of IS should also be added to each sample (in the same ratio).

First prepare from stock solutions 1 mL of mixture (A) consisting of 200 µg/mL (ppm) of each target analyte, these will be prepared jointly by two students thus consisting of four analytes. For example, if you have stock solutions of target analytes in concentrations 3 and 5 mg/mL in stock solutions, you need to start with 60 and 40 µL (round the volumes so you can measure them) of each analyte respectively. Then add the other two analytes and add DCM, so the final volume is 1 mL. Using this preparation, the final concentrations of these analytes in mixture A will be 180 and 200 µg/mL.

Prepare five additional calibration solutions of 5x fold serial dilutions (e.g., use 200 µL of mixture A + 800 µL of a solvent to prepare solution B, then 200 µL of mixture B + 800 µL of the same solvent to prepare solution C and so on). Thus you will obtain 6 calibration standards of 800 µL. that is **you have to remove 200 µL from the lowest concentration standard**. To each of these calibration standards (A, B, C, D, E, F), add 10 µL of IS (or other consistent volume if applicable).

7.1.3. Evaluation Samples

- The evaluation samples should be prepared as mixtures **by each student individually starting with a preparation of a new mixture, containing two targeted analytes (but not IS)**. Note that this sample should have a different label in your lab notebook.
- From this mixture, prepare five evaluation samples by spiking the known volume of the mixture to the known volume of the solvent, thus resulting in 800 µL of the solution. The final concentration of each analyte should be ~20 ppm (you need to know the exact concentration).
- Add 10 µL of IS (or the same volume as added to the standard).

7.1.4. Unknown Samples (Task 2)

- Solution of ~4.0 mL of unknown sample will be given to each student. Each student will need to take three 800 μ L aliquots and add 10 μ L of IS (or the same volume as added to the standard) to each thus preparing three replicated for the analysis.

7.2. GC-MS Method

Work in this section is to be performed with an instructor of the course or a graduate student in charge of the instrument. In the GC-MS logbook, record that this is a Chem 443 session, your name, date, the pressure in the He gas cylinder. The MS performance should be verified based on the last autotune report, to determine if there was a leak (level of nitrogen should be below 5%). If the GC-MS software is running (if not, turn it on by clicking the instrument ion "MS Top 1"), upload the "testmix" method. The testmix solutions are analyzed and evaluated by the instructor/graduate student, prior to the samples. The testmix analyses are used to confirm the instrument performance by the instructor/graduate student. Select the method for the analysis NY-443-1.m for most of the analytes for BTEX NY-443-5.m.

Record all operation conditions: including details about the instrument, temperatures of the injector and transfer line, the temperature program, the pressure on the injector (B) and flow rate, the split/splitless program, the volume of your sample injected (should be 1 μ L) and MS conditions such as mass range for the total ion scan (TIC).

7.3. Setup of the GC-MS Sequence & Analysis

Work in this section is to be performed with the course instructor or a graduate student in the charge of the instrument. To analyze samples using an autosampler, you need to prepare a sequence (i.e., a table listing all standards and samples to be analyzed). Click "Sequence/Sample log table" to insert the samples, which you will be analyzing. The sequence should be analyzed in the order specified below. You need to specify the sample name, file name (your filename should always start with your initials), name of the method, and vial position on the carousel.

- 1) Blank (just DCM or solvent),
- 2) Testmix (run 3x)
- 3) Calibration standards from the lowest to highest concentration
- 4) Evaluation samples for task 1 or unknown samples for task 2. To be able to evaluate the GC injection and sample preparation repeatability, analyze one of the samples three times and all other samples only a single time. Unknown samples should be analyzed only once (i.e., 3 for each student)
- 5) Following the samples, reanalyze both calibration standard sets and testmix (if multiple groups are running samples, the testmix should be run only at the end).

Save "Sequence/save," name your sequence by YY-MMDD.

Before running the sequence, make sure that the vials with wash solvents on the autosampler are filled with methylene chloride. Recap samples as soon as possible to ensure that you can use the same samples for the analysis of unknowns (otherwise you will have to prepare them again).

To run the sequence, go to "Sequence/run"; in the data file directory specify C:\HPCHEM\1\DATA\CH443\, click off "Overwrite existing datafiles," and hit "Run data sequence." The autosampler should start injecting. Make sure that the sample is injected and after the first run look at the analysis.

7.4. Evaluation of Results

For data processing, use the commands shown in the pertinent videos (Section 7) or Section 7.5 for the use of MS ChemStation software.

TASK 1

7.4.1. Identification of Analytes and Interpretation of Mass Spectra

The first task of your project is to identify the compounds you have analyzed. For this, you need to determine the retention times and report mass spectra of each analyte and IS. Prepare Figure 1 including the chromatogram (a) and the mass spectra (b, c) of your analytes, **discuss the main ions** observed and their abundance from the perspective of mass spectra interpretation. You may need to obtain “clean” mass spectra by subtracting the background.

7.4.2. Quantitative Data Analyses

To generate the calibration curves for TIC GC-MS analyses, you need to obtain the peak area of each peak. Higher accuracy and sensitivity are achieved when processing specific m/z ions instead of the total area. Thus, select the m/z of the ions you intend to quantify for each analyte (the first part of your report should support your selection), and extract them. You can integrate your peaks manually or automatically. For automatic integration you may need to adjust the integration parameters – mainly the threshold in *Chromatogram/MS Signal Integration Parameters*. For manual integration, you need to switch to the manual integration mode in *Tools/Options*. The integration results (peak areas) are obtained in *Chromatogram/Integration report*. Copy the retention data and peak areas into the MS Excel spreadsheet for further data processing. In MS Excel, obtain the slope and intercept, standard deviation of the linear curve (s_y) and limit of quantification (instrumental) for each analyte. Report these data in a tabulated form. The calibration curves should be obtained for GC-MS analyses in TIC mode using the areas and concentrations for 2 calibrations (*do not average the data points*). The data processing in MS Excel should be done using the LINEST function and demonstrating calibration charts, see pertinent videos on LINEST, charts and LODs.

7.4.3. Validation of the Method Using the Evaluation Sample

To validate the method, calculate the analyte concentration based on your calibration curve in your evaluation samples and compare it to the true value (i.e., the concentration you prepared). Determine the accuracy of their preparation using the t-test at 95% confidence interval. Evaluate the repeatability of GC analysis by comparison of the standard deviations and relative standard deviation of concentrations of three replicate runs of one sample. The repeatability of the sample preparation method should be based on the analyses of five different samples (do not include the same injection, just one of them). Support your results with tabulated data.

Prior to starting on Task 2, you need to submit the MS Excel spreadsheet to the instructor (at least a day before the lab) with all calculations to receive an approval to continue in the experimental work and if needed to determine remediation steps.

TASK 2

7.4.4. Characterization of Unknown Sample

Based on the analyses performed as part of task 2, the student will need to identify all components in the sample, determine concentrations of the found analytes and discuss possible implications regarding the concentrations of other compounds identified if observed.

7.5. GC-MS Data Processing using MSD ChemStation

The data can be processed in the computer lab on Windows based computers. Copy your analyses' files from the GC-MS computer and to the computer in the computer lab.

To open files in HP ChemStation software, click on the desktop the icon "MS data analysis 1." To view the data file, go to "File\load data file", find directory to which you have saved the data, and open the file. The commands for work with the data are listed below. Before proceeding with the integration, ensure that you use the Chemstation Integrator.

Commands to review the GC-MS files

Copy chromatogram of Mass spectrum to Word = click Tools/Copy Window and select the appropriate number (2 for chromatogram and (1) for Mass spectrum.

Chemstation Integrator = "Chromatogram/Select Integrator"

Extract ions from single sample = "Chromatogram/extract ion chromatogram" type in m/z of interest

Extract ion from multiple samples (data files) = "Tools/Options/Overlay Chromatograms" "and overlay either TIC or selected ion chromatograms

Integrate = "Chromatogram/Integrate or autointegrate"

Integrate manually = "options/manual integration", then you can integrate by using the right key on the mouse (click and drag)

Integration parameters = "Chromatogram/Integrate Parameters"

Integrate smaller peaks = "Chromatogram/Integration Parameters", change the threshold to lower number ENTER "Chromatogram/Integrate"

Integration report = "Chromatogram/Integration results"

Magnify = Left click, drag and view

Mass spectra of the peak = Right double click (if it does not work, go to Tools/options and make sure that the manual integration is off)

Select library = "Spectrum/select library" type in "?" select NIST98.L (or 2005)

Search spectra in the library = Right double click on the spectra

Subtract spectra = right double click on the peak, right double click on the background "Spectrum/subtract"

View extracted ions overlaid (on/off) = "Chromatogram/Display ion chromatograms in merged format"

Zoom = left drag over the area to be zoomed

Zoom out = left double click

8. Report

You will be evaluated on the basis of (1) your hand-written take-home test (5 pt) (2) ability to deal with the assigned problems in the lab and based on the accuracy and precision of your work (25 points); (3) your report (in electronic form) (50 points), consisting of the written and spreadsheet sections (30 and 20 points, respectively) with the total of 80 points.

The ability to deal with the assigned problems including the accuracy and precision of your data will be based on the responsible conduct in the lab, use of lab notebook, and final precision and accuracy of the data. For full score the data must be accurate thus being within the expected value on 95% confidence level and precision of the data must be 5% (RSD) for the least tailing compound (some compounds may have a lower precision).

The report should include a title page with the date, the experiments that were performed, and name of the person performing experiments. The report should be written in a manner similar to the articles published in peer reviewed journals including formatting (*see, for example, J. Chromatography guidelines for authors and/or some article*). Thus it should include:

- 1) **Title and Abstract** providing quantitative summary of the project performed.
- 2) **Introduction section** (primarily based on the 1st assignment paper)
- 3) **Experimental** section describing materials, operation condition of the analytical system, approach to the data processing (check the journal article for an example).

- 4) **Results and Discussion** including measured and calculated data showing results evaluated in section 4.4. Use MS Excel for data processing. Make sure that all data are properly labeled and referenced. Discuss the parameters affecting the results. The results should consist of four sections:
- Identification of the target analytes based on retention time and mass spectra and discussion of main ions observed and their abundance. Add one figure including be mass spectra of your analytes.
 - Calibration method data for GC-MS TIC as required by J. Chromatography including also LOQs (see a sample article as an example). This should include discussion and comparison of the obtained LOQs to those reported in the literature.
 - Evaluation of your method based on the analysis of the Evaluation samples. This section should include the GC method and sample preparation repeatability based on the mean, standard deviation, and relative standard deviation values.
 - Characterization of the unknown sample. Identify the major components and support the identification by mass spectra. Quantify the target analytes, discuss possible implications regarding concentrations of other compounds identified.
- 5) **Acknowledgement**
- 6) **References in ACS format**

9. Lab check out

Each student is responsible for cleaning all glassware, i.e., syringes, volumetric flasks, spatulas by washing with a series of solvents and water and returned to the instructor. Disposable glass items should be discarded to the waste glass container. Students will not receive grades unless the lab checkout is completed.

10. Instructional Videos - Tutorials

10.1. Solution Preparation

- Preparing stock solutions I: from solid samples <https://youtu.be/1x3psJldwE>
File name: Lab stock preparation from solid chemical.mp4
- Preparing stock solutions II: from liquid samples <http://youtu.be/YY67EnW8evl>
File name: Lab stock preparation from liquid chemical.mp4
- Stock solution: How to Organizing MS Excel Spreadsheets https://youtu.be/r_S9IPtnf_s
File name: Stock_solution_spreadsheet_2015-03-08.mp4
- Performing serial dilution for IS calibration <https://youtu.be/SefSLzMJL8>
File name: Lab serial dilution.mp4

10.2. Viewing Data in MSD ChemStation

- Transferring GC-MS data to another computer <https://youtu.be/Odmnqjd1hpl>
File name: Data_Storing_Transfer.mp4
- Chromatograms and mass spectra: <http://youtu.be/yZ6mkfQUgZg>
File name: Chemstation_View Data.mp4

- Basic Integration: <http://youtu.be/PoR7YhT3v4Q>

File name: ChemStation_Simple Integration in MSD Chemstation.mp4

10.3. ChemStation for Advanced Users

- Creating Quant method <https://youtu.be/ish26zecnZE>

File name: ChemStation_QMethod_Setup__Quantification method.mp4

- Creating database for peak areas in ChemStation (Custom reports) <https://youtu.be/IXGXGRJ6bMw>

10.4. MS Excel data processing

- Serial dilution in MS Excel <https://youtu.be/zQcYctc9K6U>

File name: MS Excel_Serial_dilution_2015-03-09.mp4

- Internal standard calibration in Excel Part 1 <http://youtu.be/bDgXHxO906g>

File Name: MS Excel_Internal_standard_calibration_in_Excel_2015-03-08.mp4

- Internal standard calibration in Excel Part 2: Charts <http://youtu.be/6W5IHZ5E-kY>

File Name: MS Excel_Internal_standard_calibration_in_Excel_Part2_charts_2015-03-08

- LOD and LOQs with LINEST in MS Excel <http://youtu.be/ap-EwhseyI>

File name: MS Excel_LOD_and_LOQs_with_LINEST_2015-03-09.mp4

11. Take-home Test

The answers must be provided **in a handwritten form** at the beginning of the lab, however the student should have the knowledge the content without looking at notes. Lack of preparation will result in cancelation of the lab

1. What is the purpose of this assignment?
2. What are the main tasks to be completed?
3. Explain the approach to internal standard calibration and its purpose.
4. What compound should be used for the internal standard quantification method?
5. What GC-MS temperatures and other instrumental operation values should you record in the report?
6. What is repeatability and how is it evaluated?
7. What types of repeatability are evaluated in this study?
8. How do you prepare the evaluation sample in this lab (provide 3 step approach)?
9. What are the LOD and LOQ and how do you determine them?