ESI-HIGH RESOLUTION TOF MS OF SMALL MOLECULES

1 Introduction

Electrospray ionization (ESI) is a frequently used technique for the analysis of polar species of various molecular weights.[1, 2] ESI coupled with a high-resolution time of flight mass spectrometer (HR-ESI-TOF-MS) allows for a relatively easy and accurate identification.[3] However, there are multiple factors, which may affect the accuracy and precision of the results obtained.

Among the major factors influencing the resulting mass spectrum are the analyte concentration, chemical nature and concentration of the electrolytes (frequently referred to as ionization agents) used, and the selection of solvents. Obviously, too low concentrations of the analyte may lead to the loss of a signal whereas too high concentrations may cause various spectral artifacts, e.g., dimerization of the analyte molecules in the ion source. The essential requirement on solvent and electrolyte is their volatility (to prevent their precipitation in the source) as well as nebulizing and ionization capability. The acquired mass spectrum may also be affected by the introduction of contaminants, which may be observed as interfering ions or may cause the suppression of ionization.[4] For this reason, only LCMS grade solvents (including water) and reagents should be used. Similarly, disposable glassware for sample dilutions is preferred.

The optimization of ionization and focusing voltages plays important role in quality of the resulting mass spectrum. The optimized voltages include the electrospray voltage providing ionization (i.e., capillary voltage), and voltage for further fragmentation [collision included dissociation (CID)] and transfer of the analyte from source to MS (fragmentor voltage). In order to perform the analysis with high mass accuracy, a mass calibration has to be performed frequently (typically daily). Addition of an internal reference standard, which may correct for the mass errors, is another way to improve mass accuracy. Although such reference may appear to be preferred for all of the measurements, it is important to note that reference compounds may also suppress the ionization of analytes or be suppressed themselves (e.g., have lower response, or do not appear in the mass spectrum at all).

In addition to measurements, data processing plays a key role in the presentation of data. The highest molecular weight ion observed in the mass spectrum does not have to be a protonated ion. Barring contamination, those ions may be various adducts, including the dimmers of the analyte or adducts of molecular ions/fragments and electrolytes/solvents.

2 Objectives

The aim of this laboratory assignment is to obtain practical experience in sample preparation and HR-ESI-TOF-MS analysis with respect to the main optimization parameters for small molecular weight species. The lab will be focus on optimization using direct infusion and one variable at time (OVAT) approach (training required for all users).

Within this lab assignment, a student will gain experience in 1) sample preparation for HR-ESI-TOF-MS analysis, 2) data acquisition and optimization on the ESI-TOF MS instrument, 3) data processing of data obtained 4) data reporting in the form of a Power Point and Excel files.

3 Preparation for the lab

Preparation for the lab includes following items described below in detail:

a) Take home prelab test

Prior to the lab, students are expected to complete the Home to Take Lab Test. The results of this test have to be provided at the beginning of the lab and will be discussed and reviewed with the instructor. **Unsatisfactory preparation/knowledge** will result in a cancellation of a lab appointment, loss of 5 pts, and the lab will be deferred until new time slot is available. *The test has to be handwritten; the study materials include this lab manual and TOF user guideline, which should be both read prior to the lab.*

b) Chemical structures of analytes and their calculated monoisotopic mass

Students must calculate a molecular mass of the assigned analytes as well as their protonated/deprotonated ions $[M+H]^+$ and $[M-H]^-$ (tip: do not forget to add or subtract the mass of electron; use the MS Excel calculator), and draw their structures in a chemical drawing software (e.g., Chem Draw or Chemsketch)

c) Calculations for sample dilutions

Students should also precalculate preparation of the tested solutions from 100 mM stock of electrolyte to 2.5 mM and 100 ppm stock of analyte to 10 ppm. So, they can be easily prepared during the lab.

4 Lab assignment

The measurements will be performed in Abbott Hall room 110 under supervision of an instructor or a TOF MS operator. Each student/pair of students will be responsible for the data acquisition of at least two samples. The data processing must be accomplished **on an individual basis** in room 343 using MassHunter Qualitative software 10. Although students may discuss (and this is encouraged) their data, the processing files cannot be duplicated even if students worked on the same analytes. *Submission of the same or nearly the same files will be considered to be a plagiarism and students will be dismissed from the lab with zero score and no further access to the MS lab.*

5 Work in lab

5.1 Sample preparation

Each student will receive one analyte (students may propose to use their own analyte, but this must be approved by the instructor). The analyte (purchased from Sigma-Aldrich, St. Louis, MO, USA) will be provided by the instructor in the concentration of 100 µg/mL (w/v) dissolved in 100% methanol, acetonitrile, THF, or water. For diluting the analytes the solvents used will either be 50% (v/v) ACN or 50% MeOH in water. The electrolytes will be 100 mM acetic acid or ammonium acetate. All solvents and electrolytes were purchase from Sigma-Aldrich and are of LC-MS purity. The solvents and electrolytes labeled a color coordinated system that will be reviewed by the instructor during the lab.

For the optimization experiment, students will prepare 1 mL of the following solutions:

4 solutions of BLANKS consisting of

- 2.5 mM acetic acid in 50 % ACN
- 2.5 mM acetic acid in 50% MeOH
- 2.5 mM ammonium acetate in 50 % ACN

- 2.5 mM ammonium acetate in 50% MeOH
  4 solutions of analytes, consisting of
- 1 µg/mL analyte with 2.5 mM acetic acid in 50 % ACN
- 1 µg/mL analyte with 2.5 mM acetic acid in 50% MeOH
- 1 µg/mL analyte with 2.5 mM ammonium acetate in 50 % ACN
- 1 µg/mL analyte with 2.5 mM ammonium acetate in 50% MeOH

All blanks should be run before analytes. Next analytes should be run sequentially, meaning that if analyte A is run first, all of its different electrolyte/solvent systems should be run back to back. Optimizing an analyte should take place on a singular day; trying to optimize on separate days will result in bad results as the instrument response change daily due to its high resolution. For this assignment, each student in the group should have 4 samples, excluding the blanks (these can be shared).

The solvents/electrolytes labeled in blue are for solution making and solvents labeled in yellow are for washing/cleaning steps (this labeling and correct use of solvents is essential to prevent contamination).

Students will record in "walking user notebook" samples they prepared, the labels must be in a following format YYMM_543IN representing year, month, course and their initials.

Laboratory tasks

Student(s) will then evaluate ESI conditions for two analytes in following steps

- Analysis of blanks
- Analysis of samples
  a) First task will be to select the mode (positive or negative) of ionization (note the selection must be justified in the final report)

Then, in selected polarity mode students will evaluate ionization in two different electrolytes and solvent systems.

5.2 Measurements

The mass spectrometric analyses will be performed using a high-resolution time of flight G1969A MS equipped with ESI (Agilent, Santa Clara, CA, USA). Initial demonstration will be performed either by the instructor or the TOF operator. The student will observe the direct infusion and data acquisition. Data processing will be covered after the data has been collected.

Initial setup for work in a direct infusion mode

Direct infusion involves analysis by direct injection

1) Software Agilent MassHunter Workstation is open.
2) The system is **calibrated** (check with the operator).
3) **Check and record vacuum** (top left panel) and compare to the previous values in the log sheets. If you find discrepancies inform the operator. The high vacuum should be ~4 x 10^{-7} Torr.
4) Open Analyst and create working directory: Go to Tools/Create project and name it in the following format: **YY-MMDD-IN** where IN stands for the initials of the user.
5) Go to Software Agilent Mass Hunter Workstation, load method C:/PE Sciex Data/Projects/calibration Methods/ and in "Name" window select Infusion_POS.m or

Infusion_NEG.m. **Save the method in your project method directory before any further work.**

6) Check that TOF is ON (green), if not contact the operator.
7) **Record required information** (initial and throughout the work) in the log sheet and walking user notebook (as specified in record section).

Blank Analysis

1) First measure all blanks (solvent and solvent + electrolyte) at 15 psi, 3500 V/150 V) with drying gas set to 4 L/min and nebulizer gas pressure 15 psi at 300 °C, in both positive and negative mode.
2) Before running sample check MS spectra to make sure there is not cross contamination of ion of interest, look at the abundance of ions present. The mass spectra in second panel should be both changed to profile (right click/TOF spectrum/profile. In MassHunter one mass spectrum pane can be used in a full range and the other can be zoomed to see targeted ions.
3) On the syringe pump set syringe volume and flow rate to 5 µl/min (0.3 mL/h), the syringe pump is typically preset for a 1-mL syringe (*additional setting must be changed for work with different volume syringes*).
4) Set the project name, file name and sample name in the sample tab (see above).
5) When the TIC signal is stable, acquire data by hitting "START" (RED VIAL) on the very top of the Workstation window.

Sample Analysis

To measure your samples, follow the steps in procedure for the blank: set syringe volume and flow rate, project, file and sample names.

1) The typical sample should be prepared at concentration of 1 ppm; 2.5mM electrolyte.
2) The initial TOF conditions are described under "operation tabs" in the previous section, the good starting point is drying gas at 12 L/min, nebulizer 15 psi, capillary voltage of 3500 V and fragments of 150 V.
3) View the spectra of samples, use profile to find your analytes. The common adduct ions to look for are $[M+H]^+$ (M+1) and $[M+Na]^+$ (M+23). Use *mass calculator.xls* to calculate the exact *m/z* values.
4) Maximize the response of adduct ions of interest by varying capillary and fragmentor voltages and record the response values in the walking user logbook along with the tested conditions.
5) Acquire data, when satisfactory mass spectra (upon testing positive/negative mode, capillary and fragmentor voltages) are obtained in the MassHunter.
6) In the top window hit "START" (RED VIAL) to start data acquisition.
7) After acquisition is completed, open the data file in Analyst (refer to the work guideline for Analyst software) and verify whether you obtained high mass accuracy <10 ppm error for your samples.
8) If data are good, proceed to the next sample, if higher mass accuracy error > 15 ppm is obtained, consult with the operator to recalibrate.

5.3 Data Processing, Interpretation & Reporting

The processing will be performed using MassHunter Qualitative software version 10 in Abbott Hall, computer lab, room 343. The guideline on the use of the software is provided on the website including the instructions *"Dataprocessing MassHunter.docx," and links to videos"* After the lab completion, students with the operator will transfer a copy of their data onto the flash drive or Onedrive to transfer data to the computer in the lab (room 343).

The two reports (intermediate and final) will be submitted within PowerPoint and Excel files. The intermediate report needs to show sections I and II for the analyte and one electrolyte as described below. The final report should be comprehensive.

- The lab assignment should be reported in the form of Power Point presentation with calculation supported with Excel.
- The presentation, Excel files and all graphics must have explicit headings and labeling addressing required components report component.
- The supporting explanatory text with conclusions should be provided a bulleted text.
- Each mass spectrum must include operating conditions (concentrations of analyte, electrolyte solvents, flow rate, ESI and fragmentor voltage) specified in heading or footnote.
- The specific sections of the report should include:
  - I. **Title page** (1 slide) showing structure of the target analyte and their molecular formula
  - II. **Identification of major ions/fragments** (1-2 slides). The identification should be based on the evaluation of mass spectra ions, with respect to mass accuracy for the representative electrolytic system. If mass spectra can be acquired in both positive and negative modes, the example of mass spectra should be provided for each mode for one of the electrolyte. If only mode can acquired, the presentation should include rationale for this observation. The identification should be demonstrated within figures consisting of mass spectra shown **a full and magnified** (pertinent portion of the mass spectrum), with labeled ions (adducts, fragments) found at different voltages. The identification should show whether the observed species are for example $[M+H^+]$ or $[M+Na^+]$ etc. or specific fragments obtained by a loss of neutrals (e.g., $[M-Cl^+]$). The tentative identification provided (if possible) should be confirmed by calculating the mass accuracy error (preferably in tabulated form) and isotopic ratios. Furthermore, the identification should be supported by the determination of a number of charges using isotopic panel.
  - III. **Qualitative impact of different ionization conditions** (1-2 slides) should be shown by comparison of the mass spectra obtained for different electrolytes and or voltages. This can be, for example, done by comparing mass spectra for each electrolyte and/or comparing the effect of different ionization voltages and other operation conditions, showing impact on the abundance if specific ions or mass spectra profile.
  - IV. **Evaluation of ESI ionization conditions** (1 slide) considering the quantitative impact of the electrolyte and fragmentor voltages and nebulizing conditions. The evaluation of ESI ionization results should be based on peak heights or areas recorded in the laboratory notebook and reported as a table or a figure (chart) in the presentation. Students need to justify, which ions were used (i.e., protonated or adducts) highlighting the optimal conditions with regard to electrolyte use, solvent system and voltages tested.

6 Lab Check Out

At the end of this lab assignment, each student will be responsible for returning the cleaned syringes and any materials to the instructor. All disposable materials must be discarded, and glassware must be washed with water and ethanol. Each student needs to demonstrate proper use of the laboratory notebook to the instructor.

7 Grading

The grading will be based on

(1) Prelab test (5 pt) – see section 9

(2) Intermediate report submitted as Excel and PowerPoint files (10 pt)

(3) Final Report (60 points), consisting of the PowerPoint presentation and supported by Excel file sheet. The final score will be based on the results and data processing. Consideration will be given to proofreading and language expressions, clarity of the presentation, graphical presentation of Tables and/or Figures with clear labeling and captions and appropriate references to them within the text.

(4) Lab checkout and demonstration of effective use of the lab notebook (5 pt)

8 References

1. Oss, M.; Kruve, A.; Herodes, K.; Leito, I., Electrospray ionization efficiency scale of organic compound. *Anal. Chem.* **2010,** 82, (7), 2865-2872.
2. Cech, N. B.; Enke, C. G., Practical implications of some recent studies in electrospray ionization fundamentals. *Mass Spectrom. Rev.* **2001,** 20, (6), 362-387.
3. Jiejun Wu, H. M., Exact mass measurement on an electrospray ionization time-of-flight mass spectrometer: error distribution and selective averaging. *J. Mass Spectrom.* **2003,** 38, (10), 1043-1053.
4. Trufelli, H.; Palma, P.; Famiglini, G.; Cappiello, A., An overview of matrix effects in liquid chromatography-mass spectrometry. *Mass Spectrom. Rev.* **2011,** 30, (3), 491-509.

9 Test Prior to the ESI-MS-TOF Lab

The answers must be provided prior to the lab; however, the student should have the knowledge of the content without looking at notes. Lack of preparation will result in the cancelation of the lab and its delay to a further date. The uploaded answers must be handwritten.

1) Sketch and explain the principles of electrospray ionization (ESI).
2) a) Why is it important to use an electrolyte with ESI? What electrolytes are commonly used?
   b) What are the required properties and concentrations of the electrolyte? Why do you limit the concentration of ammonium acetate?
3) Draw a diagram and explain the working principle of the TOF-MS.
4) Explain what *high resolution* and *accuracy* mean in terms of MS, provide formulas, compare the variables, and explain their importance.
5) Imagine you are given a stock solution of your analyte at a concentration of 100 ppm (w/v) and electrolyte solution at 100 mM, each dissolved in 50 % ACN in water. Show

how you would prepare a solution (1 mL) with 1 ppm analyte and 2.5 mM electrolyte in 50% ACN in water. Show your work.

6) a) What is the capillary voltage? What is the working range of the capillary voltages?
b) Explain how and why varying the capillary voltage affects the analyte response.

7) a) What is the fragmentor voltage? What is the working range of the fragmentor voltage?
b) Explain how and why varying the fragmentor voltage affects the analyte response.

b) Is it generally best to have the fragmentor voltage low to achieve larger peak areas of the molecular ion? Why?

8) What types ions/fragments do you typically expect in the mass spectra when using ESI?

9) a) What is an ammonium adduct? What is the m/z of it?
b) What is the added benefit of observing it in your mass spectra? What are possible negative effects of observing it?

10) Would you observe the ammonium adduct while analyzing in negative mode? Why or why not?

11) a) What is the sodium adduct? What is the *m/z* of it?
b) What is the source for sodium adduct? (Hint: You didn't add it to your sample)

12) How do you calculate the mass error? Show your calculation in determining the mass error of an ion whose theoretical mass is 245. 678340 *m/z* and measured mass is 245.679435 *m/z*.

13) What are isotopic ratios? How can they be important in analyzing an unknown compound?

14) Typically, the molecular weight of a polymers is described by a number average molecular weight (M_n) and weight average molecular weight (M_w) provide their definitions and explain why are they used.

15) Describe what tasks you have to pursue within the lab assignment (training).