ESI-HIGH RESOLUTION TOF MS OF POLYMERS

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 (TOF) mass spectrometer allows for 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 chemical nature and concentration of the electrolytes (in lab slang referred to as ionization agents), and the selection of solvents. 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 solvents and electrolytes is their volatility (to prevent their precipitation in the source) as well as ensure 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 cause ionization suppression.[4] For this reason, only LCMS-grade solvents (including water) and reagents should be used. Similarly, disposable glassware for sample dilutions is preferred. However, the compatibility of solvents with materials used MUST be verified. *For example, tetrahydrofuran can be used only with stainless steel tubing and connections.*

Optimizing ionization and focusing voltages plays an important role in the resulting mass spectrum quality. The optimized voltages include the electrospray voltage providing ionization (i.e., capillary voltage), 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). Adding an internal reference standard may also correct the mass errors. Although such reference may appear to be preferred, it is essential to note that reference compounds may suppress the ionization of analytes or be suppressed themselves (e.g., have a lower response or do not appear in the mass spectrum at all).

The analysis of small molecules typically focuses on the highest molecular weight ion observed in the mass spectrum. These ions may be protonated molecular ions (pseudomolecular ions in older terminology), or various adducts, including the dimmers of the analyte, fragments and/or ions from electrolytes/solvents.

The advantage of ESI is the formation of multiply charged species, thus allowing to measure species that in size would be below the range of mass analyzer (e.g., compound of mass 15,000 amu with three charges will be observed as ~5000 *m/z*). The charge (*z*) may be estimated using the isotopic pattern from protonated ions $[M+zH]^{z+}$ (or other adducts), where the *m/z* ratio may be expressed as in the Equation 1.

$$\frac{m}{z} = \frac{M_i + z(H - e^-)}{z} \qquad \text{Eq. 1}$$

Where *m/z* is the observed value in the mass spectrum, M_i is monoisotopic molecular weight of the analyte, H is the mass of hydrogen and e^- is the mass of electron.

It is well known that species of a single charge have isotopes separated by one amu. By contrast, doubly charged species will have isotopic peaks separated by 0.5 amu, etc. Thus the charge of two adjacent isotopic peaks, m_1/z and m_2/z can be determined using Equation 2.

$$\frac{1}{z} = \frac{m_1}{z} - \frac{m_2}{z} = \Delta\frac{m}{z} \qquad \text{Eq. 2}$$

For example for two peaks of *m/z* 981.2361 and 980.9897, the Δ *m/z* is 0.2464 and thus z= 1/0.2464 = 4.05

Synthetic polymers are typically polydisperse that contain polymer molecules with chains of unequal length, so the molecular weight is not a single value. As a result, the polymers exist as a distribution of molecular weights. Typically, the molecular weight of a polymer is therefore described by three parameters: a number average molecular weight (M_n) defined in Equation 3, weight average molecular weight (M_w) shown in Equation 4, and z-average molecular weight (M_z) shown in Equation 5.

$$M_n = \frac{\sum N_i M_i}{\sum N_i} \qquad \text{Eq. 3}$$

$$M_w = \frac{\sum N_i M_i^2}{\sum N_i M_i} \qquad \text{Eq. 4}$$

$$M_z = \frac{\sum N_i M_i^3}{\sum N_i M_i^2} \qquad \text{Eq. 5}$$

Where M_i is the mass of one molecule with a specific chain length, and N_i is the number of these molecules. The equations are typically used in size exclusion chromatography but have also been used in mass spectrometric determinations, where N_i is replaced by *the* absolute abundance of the deconvoluted species of a given molecular weight (M_i). The deconvolution of mass spectra refers to the conversion process of acquired mass spectrum from peaks with multiple charges (i.e., X axis is denoted with *m/z* variable) to spectrum, where variable on X-masses is mass (i.e., based on recalculating using Equations 1, 2).

2 Objectives

This laboratory assignment aims to obtain practical experience in sample preparation and ESI-HRMS analysis with respect to the main optimization parameters for large molecular weight species. The lab will focus on optimization using direct infusion.

Within this lab assignment, a trainee will gain experience in 1) a sample preparation for ESI-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 pre-lab 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, and the lab will be deferred until a new time slot is available. *The test must be handwritten; the study materials include this lab manual and TOF user guideline, which should be read before the lab.*

b) Schemes of chemical structures (repeating units)

The structures of tested analytes. The polymers studied will be typically water-soluble polymers, including polyethylene glycols (PPG), polypropylene glycols (PPG), polymethylmethacrylates (PMMA). With the instructor's permission, students may inquire about other polymers or bring their own samples.

Students must calculate a monoisotopic mass of the monomeric unit of the assigned analytes, the MS Excel calculator may be used, and draw their structures in a chemical structure drawing software (e.g., Chem Draw or Chemsketch).

c) Calculations for sample dilutions

Students should also precalculate preparation of the tested solutions

Stocks of Analytes: 1,000-10,000 ppm (w/v) stocks in acetonitrile (ACN), methanol (MeOH) or if necessary in tetrahydrofuran (THF).

Electrolytes: formic acid, ammonium acetate, and sodium iodide all of LC-MS grade 100 mM.

Preparation of samples for analysis of 100 µg/mL of analyte and 2.5 mM electrolytes (final concentrations), typically in in 50% ACN in water in the final volume of 1 mL.

The blanks will be prepared the same way but without an analyte.

4 Lab assignment and expectations

The measurements will be performed in Abbott Hall room 110 under the 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 version 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 plagiarism and students will be dismissed from the lab with zero score and no further access to the MS lab.*

Within the lab, students are expected to optimize ESI conditions and evaluate the obtained mass spectra. The ESI optimizations will be performed for one polymer sample using one variable at time (OVAT) screening analysis. For the OVAT, students will manually vary three electrolytes (in a lab slang terminology called ionization agents), capillary and fragmentor voltages for each analyte, and determine the applicability of the positive and negative ESI mode.

5 Work in lab

5.1 Sample preparation

Each student (student group) will receive 1 analyte stocks (200 µL of each) as well as stocks of electrolytes from the instructor or the lab operator.

As mentioned in the previous section, students will dilute the stocks to obtain samples in a volume of 1-mL in 50% acetonitrile (ACN):

a) 3 blank solutions (i.e., samples without the analyte) each with a different electrolyte (2.5 mM final concentration): formic acid, ammonium acetate, and sodium iodide.
b) 3 samples for the analyte (100 µg/mL final concentration) in 3 different electrolytes (2.5 mM final concentration).

The solvents/electrolytes labeled in blue are for solution making, and the 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.

5.2 Laboratory tasks

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

1) Analysis of blanks
2) Analysis of analytes samples (one at a time)
    - The first task will be to select the mode (positive or negative) of ionization (note the selection must be justified in the final paper)
    - Then students will evaluate mass spectra obtained with ESI ionization in three different electrolytes in selected polarity mode.

5.3 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). The initial demonstration will be performed either by the instructor or the TOF operator**. The students will observe the direct infusion of PEG, data acquisition**. All these processes are described in detail below.

Instructions for nitial 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 the 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 a 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 analyzing the sample, check MS spectra to make sure there is no cross contamination of ion of interest, look at the abundance of ions present. The mass spectra in the 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

Check on reporting requirements in Section 5.4 to ensure correct data are acquired.

The typical polymer sample should be prepared in concentration 100 µg/mL with 2.5mM electrolyte.

1) The initial TOF conditions are described under "operation tabs" in the previous section, the good starting point is drying gas at 4 L/min, nebulizer 15 psi, capillary voltage of 3500 V and fragments of 150 V.
2) View the spectra of samples, use profile mass spectrum to find ions, which belong to the polymer pattern, which typically resembles Gaussian distribution.
    a) Look at the spectrum and make sure you see polymer distribution, determine whether positive or negative mode is more suitable for further analysis.
        - If the distribution is observed, go to step (b)
        - If you do not observe the polymer or if TIC is not consistent:

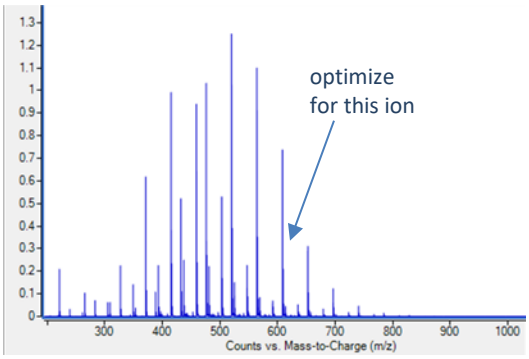
        Make sure the sample is well nebulized: drying gas may be increased to 7 L/min, the nebulizer may be increased to 25 psi, and temperature may be lowered to 250 °C.
    b) Find a repeating unit

        First estimates can be done within the MassHunter software caliper tool  . In Excel, open the mass calculator and calculate the estimated mass of the repeating unit based on number of C, H, O and see if you can find the repeat in the mass spectrum.

        c) Try to determine the charge from the isotopic pattern (refer to the question in your take-home-test) by scaling the mass spectrum to a specific cluster. Repeat for

several clusters within different chain lengths. The charge can also be viewed through Masshunter software using by adjusting "Spectrum display options"  .

3) Maximize the response of main ion in one of the clusters in 75% polymer Gaussian distribution toward a higher m/z end (use the left MS window). Keep in the right MS window full spectrum to ensure that the polymer distribution is not affected.



Optimized the following variables:

a) Nebulizing conditions i.e., N_2 pressure and flow (see 2a for ranges), when you change settings, make sure the actual pressure is stabilized. While optimizing make sure you see polymer distribution besides the main ion. (keep other parameters constant, i.e., capillary and fragmentor potentials at 3500 and 150 V)
b) Capillary voltage 2500–5500 V in positive mode, in negative mode (if considered) up to 5000 V.
c) Fragmentor voltage 100–250 V.

4) Acquire data, when the highest abundance (area) for a selected ion is achieved without losing polymer distribution and the TIC signal is stable (upon optimizing nebulizer, capillary, and fragmentor voltages) are obtained in the MassHunter. Other conditions for future comparisons can be recorded as well.

5.4 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 flashdrive 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 one analyte as described below. The final report should be comprehensive.

- The PowerPoint slides and Excel files must have explicit headings and labeling addressing the 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 the heading.
- The specific sections of the intermediate report:
  I. **Title page** (1 slide) showing the structure of the target analyte and its molecular formula.
  II. **The demonstration and evaluation of the polymer mass spectra** (3 slides) acquired for three electrolytes with a brief discussion on the differences and similarities. For each condition (one slide per electrolyte), four mass spectra should be shown (initial, blank

and subtracted, deconvoluted) in panels a,b,c. A magnified portion of subtracted mass spectra (in panel d) for one of the electrolytes should also be provided, supporting the determination of a number of charges. Furthermore, students also need to demonstrate identification of repeating units (also in blue box instructions) using an Excel template and discuss obtained results in the text along with mass accuracy error. Finally, isotopic distribution should be considered.

III. **Discussion of a role of ESI conditions** (1-2 slides) considering the impact of the electrolyte and fragmentor voltages and nebulizing conditions. 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. **The determination of polymer molecular weights,** including a number average molecular weight (M_n), weight average molecular weight (M_w), z-average molecular weight (M_z) as well as a polydispersity index PI (ratio of M_w/M_n) for each acquired condition within MS excel file. The determination of these values should be carried out based on the instructions and performed in Excel template provided. The mass spectra before and after deconvolution (noted in the Excel template) should be included in the MS excel results along with the data. The evaluation of the values obtained should be presented in the text along with a small table summarizing molecular weight values (M_w, M_n, M_z and PI) obtained for three electrolytes compared with each other or if available to those specified by other methods (e.g., SEC).

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. Wesdemiotis, C., Williams-Pavlantos, K.N., Keating, A.R., McGee, A.S., Bochenek, C. Mass spectrometry of polymers: A tutorial review. *Mass Spectrom. Rev*. **2023**; 1- 50.

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 the 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).