Fundamentals of Biomolecule Analysis by Electrospray Ionization Mass Spectrometry

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An Instrumental Analysis Laboratory Experiment

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Mass Spectrometry (MS) has become one of the premier modern analytical tools for the analysis of molecules of biological interest. The development of electrospray ionization *(1)* (ESI), a soft ionization technique that allows transfer of fragile biomolecules directly from solution into the gas phase, merited a shared Nobel Prize in Chemistry awarded in 2002 to John Fenn *(2).* ESI revolutionized the breadth of MS applications in bioanalytical chemistry. Most notably, the marriage of ESI–MS and high-performance liquid chromatography (HPLC) created a very powerful separation technique called liquid chromatography–mass spectrometry (LC–MS). Despite the high cost of MS instrumentation, LC–MS has become one of the most commonly used analytical techniques. Numerous MS analyzers have been coupled to LC, or used as a stand-alone technique for analysis of biomolecules. Quadrupole ion traps represent one of the most common types of mass spectrometers combining relatively low cost, decent resolution, and multiple-stage tandem MS capabilities.

More and more chemistry departments now feature shared MS equipment. The use of mass spectrometers has not been limited to research. Several MS experiments have been described for analytical *(3),* instrumental analysis *(4),* biochemical *(5),* general *(6),* and organic *(7)* undergraduate laboratories. Several articles in this *Journal* are devoted to ESI *(2, 8, 9),* but none of them has an experimental component. The only experimental example of ESI is an LC–MS experiment *(3)* that focuses on separation and quantitation and uses ESI–MS as an LC detector. Since ion traps are becoming increasingly popular, we decided to design an instrumental analysis laboratory experiment that would introduce our students to the ESI technique, major parameters of the ion trap mass spectrometers (mass range, resolving power), as well as some caveats in interpretation of mass spectra of biomolecules.

The problem in analysis of biological molecules is that the molecular mass (M) could be substantially larger than the high mass-to-charge limit of quadrupole ion traps (often as low as 2000 m/z). This problem is overcome in ESI by multiple protonation that converts a biomolecule M into multiply charged ions with the formula $(M + nH)^{n+}$ (in positive-ion mode). Mass spectra containing a series of *charge states* (ions with different number of charges on them) can be mathematically *deconvoluted* to deduce the M of the neutral analyte. This is one of the focuses of this experiment.

Many elements have more than one isotope *(10).* For instance, the natural abundance ratio of bromine isotopes ^{79}Br:^{81}Br is about 1:1, while for chlorine isotopes ^{35}Cl:^{37}Cl it is 3:1. This leads to very distinct mass spectra of chlorine and bromine-containing species *(6)* and any major instrumental analysis textbook will have such examples. The elements in most biomolecules, namely, C, H, O, and N, do not have multiple isotopes of high abundance. For instance, ^{13}C natural abundance is only 1.1%. However, isotopic contribution is purely additive and biomolecules containing dozens, or hundreds of carbon atoms will have very complex isotopic patterns. This was realized and explained in the scientific literature *(11)* years before the development of ESI and recently at the educational level *(12).* In this experiment, students will be able to observe an isotopic pattern of a model peptide and compare it with a theoretical prediction based on the natural abundance of C, N, H, and O isotopes.

To observe isotopic patterns of a biomolecule, the mass spectrometer must possess sufficient *resolving power*. The resolving power of a typical quadrupole ion trap is sufficient to observe isotopic peaks of molecules with M < 2000. For a biomolecule of a higher M, the isotopic peaks will collapse into one broad peak that allows students to see the average mass only.

This experiment is suitable for chemistry or biochemistry majors enrolled in an instrumental analysis (or analytical chemistry) laboratory course. The experiment takes about 1.5–2 hours for a four-student group.

Experiment

Overview

The students first acquire an ESI mass spectrum of a model peptide, bradykinin. They observe and identify the charge states +1, +2, and +3. By acquiring a narrow mass-to-charge range around the charge state +1 ("zoom scan" mode), students obtain the isotopic pattern of the bradykinin $(M + H)^+$ ion. The students then calculate a theoretical isotopic pattern by using Table 1 and compare it to the experimental result.

Second, students acquire a spectrum of the unknown. Deconvolution of the multiply charged peaks results in the molecular weight of the unknown, which allows students to identify melittin from the list of possible unknowns (Table 2). The experimental isotopic pattern of bradykinin is used to estimate the resolution of the mass spectrometer. Acquiring a zoom scan of a charge state of melittin does not result in resolving isotopes. Only average masses are observed from that experiment.

Sample Solutions

The peptide solutions are prepared by the instructor before the experiments; if time permits the students can prepare the peptide solutions. Peptide (bradykinin and melittin) solutions should be about 10–100 μM in 49% water, 49% acetonitrile (or methanol), and 2% glacial acetic acid (v:v:v).

Table 1. Exact Masses and Natural Abundances of Some Isotopes

Element	Symbol	Exact Mass /Da	Abundance (%)	M + 1 Factor[a]	M + 2 Factor[a]
Hydrogen	^{1}H	1.0078	99.99		
	^{2}H	2.0141	0.01	0.01 x N_H	
Carbon	^{12}C	12.0000	98.90		
	^{13}C	13.0034	1.10	1.1 x N_C	0.006 x N_C^2
Nitrogen	^{14}N	14.0031	99.63		
	^{15}N	15.0001	0.37	0.37 x N_N	
Oxygen	^{16}O	15.9949	99.76		
	^{17}O	16.9991	0.04	0.04 x N_O	
	^{18}O	17.9992	0.20		0.2 x N_O
Phosphorus	^{31}P	30.9738	100		
Sulfur	^{32}S	31.9721	95.02		
	^{33}S	32.9715	0.76	0.8 x N_S	
	^{34}S	33.9679	4.22		4.4 x N_S

[a]Contribution of the isotope to the first and second isotopic peaks, respectively.

Instrumentation

Experiments were done on a ThermoFinnigan LCQ Advantage ion trap mass spectrometer equipped with an ESI source. In past years, identical results were obtained on a Bruker Esquire 3000 ion trap mass spectrometer. In principle, a wide array of other mass analyzers coupled to an electrospray ion source should be suitable for this experiment (e.g., single quadrupole, triple quadrupole, time-of-flight). Standard ESI conditions recommended by the manufacturer for the analysis of peptides were used. Solutions of peptides are infused into the ESI source of the instrument by a syringe pump at a rate of about 5 μL/min.

Table 2. List of Potential Peptide or Protein Unknowns and Their Masses

Unknown	Monoisotopic Mass[a]/ Da	Average Mass[b]/ Da
Angiotensin II	1045.53	1046.2
Substance P	1346.74	1347.7
Neurotensin	1689.93	1691.0
Melittin	2844.76	2846.5
Glucagon	3480.62	3482.8
Insulin	5729.61	5733.6
Ubiquitin	8559.63	8564.8

[a]Monoisotopic mass is the mass of a molecule calculated using the mass of the most abundant isotope for each element from Table 1.

[b]Average mass is the mass of a molecule calculated from a given empirical formula using the relative atomic weight (which is an average of the isotopes and is found in the periodic table) for each element.

Hazards

Bradykinin and melittin are not hazardous at the concentrations used in the experiment. Skin or eye contact of the peptide solutions should be avoided owing to the presence of acetonitrile (Caution: Avoid breathing vapors. May cause skin irritation) or methanol (Caution: Poisoning may occur from ingestion, inhalation, or percutaneous absorption); instructors or students making the solutions and loading them into a syringe should wear gloves. If concentrated acetic acid (Caution: Ingestion may cause severe corrosion of mouth and G.I. tract) is used in solvent preparation, it should be done in a fume hood to avoid exposure to vapors. Safety goggles should be worn at all times.

Results and Discussion

Bradykinin

An ESI–MS of bradykinin ($C_{50}H_{73}N_{15}O_{11}$) is given in Figure 1A. One can clearly identify the three charge states: $(M + H)^+$ at m/z 1060.6, $(M + 2H)^{2+}$ at m/z 531.4, and $(M + 3H)^{3+}$ at m/z 354.9. Zooming in the +1 charge state gives a spectrum shown in Figure 1B. The isotopic pattern of this peak is well resolved. The first peak of the pattern corresponds to the *monoisotopic mass*. To calculate a monoisotopic mass of a compound one cannot use the periodic table since it gives atomic weights averaged for naturally occurring isotopes. Instead, atomic weights of individual isotopes should be used. Masses and abundances of isotopes of the most relevant biologically elements are listed in Table 1.

Using the masses from this table, the monoisotopic mass of bradykinin is calculated to be 1059.57. The +1 charge state has an experimental mass of 1060.6, which is almost exactly 1 au higher than the predicted monoisotopic mass since this charge state is formed by attaching a proton, (H^+, 1.007 au) to the bradykinin molecule.

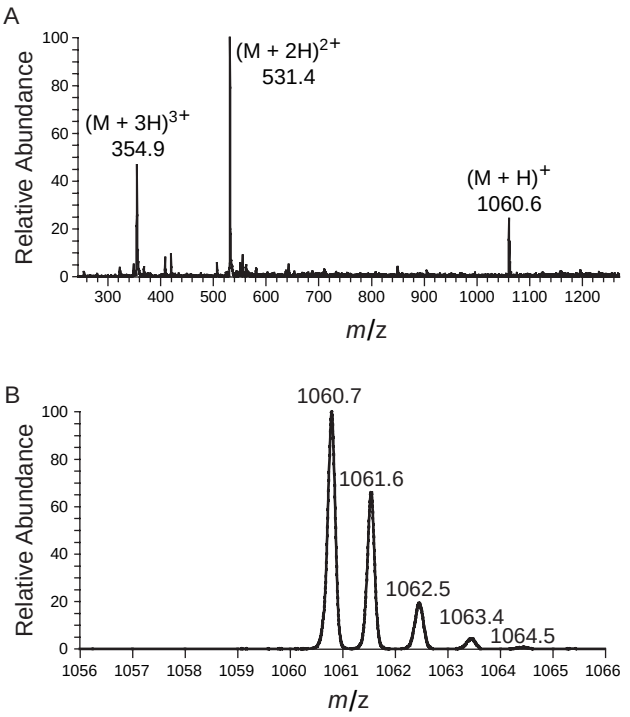

Figure. 1. ESI–MS of bradykinin: (A) full scan and (B) zoom scan of $(M + H)^+$ peak.

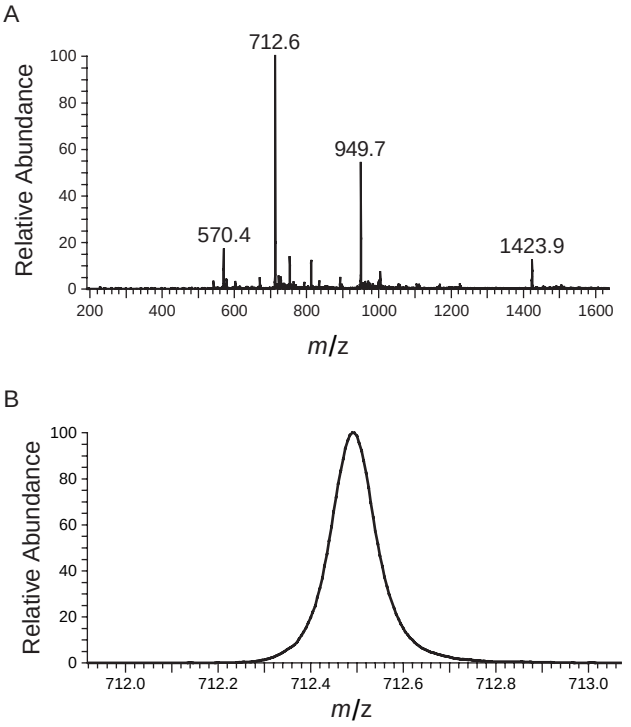

Figure. 2. ESI–MS of unknown: (A) full scan and (B) zoom scan of $(M + 4H)^{4+}$ peak.

The other peaks in Figure 1B at m/z 1061.6, 1062.5, 1063.4, and 1064.5 are called *isotopic peaks*. Since the contribution of the isotopes is purely additive, one can make a theoretical prediction of the isotopic pattern. A simplified approach to such a prediction is provided in Table 1. Summing all $M + 1$ factors will predict the abundance of the first isotopic peak as $[(1.1 \times 50) + (0.01 \times 74) + (0.37 \times 15) + (0.04 \times 11)] = 61.7\%$, very close to 64% observed in Figure 1B. Similarly, for the second isotopic peaks we will use the M + 2 factors: $[(0.006 \times 50^2) + (0.2 \times 11)] = 17.2\%$, again close to the experimental 19%. As the number of carbons and other elements in a biomolecule increases, manual calculations of isotopic patterns become cumbersome. A number of isotopic pattern calculators available on the Internet provide fast and accurate way to predict abundances of isotopic peaks, even for large proteins *(13)*.

The *resolving power* of a mass spectrometer is its ability to distinguish between ions that differ just slightly in mass-to-charge ratios. Resolving power is loosely related to *resolution* (R). One of the resolution definitions is $R = M/\Delta M$, where M is the m/z of the peak and ΔM is full width of the peak at half maximum (FWHM) *(14)*. Unit resolution, resolution sufficient to resolve isotopic peaks, can be estimated as $R_{unit} = M/0.5$ *(12)*. Thus, for the +1 charge state of bradykinin, the resolution is $R \geq 2120$.

Unknown

An ESI–MS of an unknown protein or peptide is shown in Figure 2A. Several charge states are observed at m/z 570.4, 712.6, 949.7, and 1423.9. The purpose of this experiment is to identify the unknown by *deconvoluting* its spectrum. Since students do not know the charges associated with each peak, an algebraic solution is necessary. If we pick two adjacent charge states n and $n + 1$ of a biomolecule with molecular mass M, their m/z values, M_n and M_{n+1}, are given by (rounding the mass of H to 1):

$$M_n = \frac{M + n}{n} \tag{1}$$

$$M_{n+1} = \frac{M + n + 1}{n + 1} \tag{2}$$

Solving this system for n and M gives:

$$n = \frac{M_{n+1} - 1}{M_n - M_{n+1}} \tag{3}$$

$$M = n(M_n - 1) \tag{4}$$

Thus, both the charge states of the two peaks and the molecular weight of the unknown are obtained. For instance, taking the pair of adjacent peaks at m/z 570.2 and 712.6 gives $n = 4$ and $M = 2846.4$. Thus, the unknown is identified as melittin from Table 2. A spreadsheet program for performing deconvolutions of ESI spectra and averaging the resulting molecular weight is available in the Supplemental Material.[W]

Zooming in at the charge states +5, +4, or +3 of melittin will show that the isotopic pattern is *not* resolved. For ex-

ample, the +4 charge state should have 0.25 m/z separation between isotopic peaks since the x axis in mass spectra is mass/charge. However, only a single broad peak can be seen in Figure 2B. Thus, the resolution of the ion trap mass spectrometer must be below "unit" for melittin, or $R < M/0.5 = 5692$, at least for the analysis of higher charge states. Because the isotopic peaks are not resolved, the students observe *average masses*. They can be obtained by using the average atomic weights of the elements from the periodic table. Indeed, for melittin ($C_{131}H_{229}N_{39}O_{31}$) the average M is be calculated to be 2846.5 Da, which is within 0.1 Da of the experimental value.

[W]Supplemental Material

Detailed instructor notes, instructions for students, including postlab questions, and a program for the charge state deconvolution are available in this issue of *JCE Online*.

Acknowledgments

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