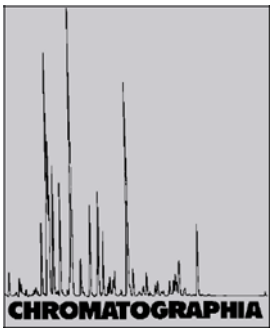
Effects of Experimental Parameters on the Signal Intensity of Capillary Electrophoresis Electrospray Ionization Mass Spectrometry in Protein Analysis



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Key Words

Capillary electrophoresis
Electrospray ionization – mass spectrometry
Interface conditions
Proteins

Summary

In recent years, the hyphenation of capillary electrophoresis (CE) and electrospray ionization mass spectrometry (ESI-MS) has been widely used for the analysis of biological molecules with high efficiency and high accuracy. However, the signal intensity of CE-ESI-MS is restricted by various parameters. This paper reports the effects on the CE-ESI-MS signal of the pH and the electrolyte concentration, the formic acid concentration of the sheath liquid, and the sheath liquid composition, using several proteins as samples. The study was performed systematically by experimental and theoretical analyses. The maximum signal intensity of three proteins (cytochrome c, insulin, bovine serum albumin) was attained with a pH 4.40 buffer containing 75 mM formic acid and 75 mM ammonium acetate. Investigation of the influence of the formic acid concentration in the sheath liquid (over the range of 0% – 1%) on the ESI-MS signal of bovine serum albumin showed that the feasible amount of the formic acid in sheath liquid could improve the signal intensity of the sample ions. However, considerable band broadening was observed that should be attributed mainly to column overloading, band spreading at the interface, and scanning data acquisition.

Introduction

In the past five years, the separation sciences have assumed an increasingly significant role in bioanalytical chemistry [1]. Since biological samples are usually complex, separation techniques are necessary in order to isolate the components of interest before their identification and characterization can be attempted. Of the various separation techniques, high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) are usually employed [2, 3].

CE is a modern analytical method that offers the advantages of high efficiency, short analysis time, and minimum consumption of both reagents and samples [4, 5]. It has therefore been widely used for the analysis of proteins for over three decades. Mass spectrometry (MS) provides high sensitivity and reliable detection of various samples, particularly in biochemical applications where the sample amount is very limited [6]. Moreover, it affords much information about the structure and identity of analytes. Accordingly, CE-MS has played an important role in the analysis of biological samples. However, the interface between CE and MS may affect the performance of such hyphenation. Among all the developed interfaces, electrospray ionization (ESI) interface permits the mass spectrometric analysis of large biomolecule with mass up to several hundreds thousand with significantly lower mass scan range. Thus it proves to be suitable for the coupling with HPLC, CE, and even electrochromatography [7–17].

The success of coupling CE separation systems to ESI-MS detection system depends on many factors, including the design and operating parameters of the electrospray interface [20], the composition and pH of the buffer solution [19], the chemical properties of the analyte [21], and the composition of the sheath liquid [18, 22–24]. However, up to now, few systematic studies have been reported on the effects of the electrolytes used in CE separations on the ESI-MS signal. In this paper the effects of pH and the electrolyte concentration in CE on the ESI-MS signal were studied experimentally and theoretically. Simultaneously, the influence of the formic acid concentration in the sheath liquid was investigated in order to better understand the most important factors that determine the performance of the coupled CE-ESI-MS system.

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Experimental

Chemicals and Instrumentation

Methanol (HPLC-reagent grade), ethanol (HPLC-reagent grade), formic acid (80%, analytical-reagent grade), ammonium acetate (analytical-reagent grade) were purchased from YuWang (Shandong, China). N,N,N,N-Tetra-methylethylenediamine (TEMED, 99%), acrolylic amide (>98%), 3-(trimethoxysilyl)propylmethacrylate (γ-MAPS, 98%), ammonium persulfate (ACS, >98%) were purchased from ACROS (New Jersey, USA). Cytochrome c, bacteriolysin, hemoglobin, insulin, bovine serum albumin were obtained from Sigma (St. Louis, Mo, USA). Recombinant human renal tissue Kallikrein were obtained from the Medical University of South Carolina (Charleston, USA). Insect cell supernatants were provided by Dr Qing Yang (Dalian Institute Chemical Physics, the Chinese Academy of Sciences, Dalian, China). High-purity water was prepared by a Milli-Q water purification system (Millipore, Milford, MA, USA). The ammonium acetate-formic acid electrolyte was prepared from the salt with subsequent adjustment of the pH with formic acid.

Capillary Preparation

Fused-silica capillaries were treated by the following procedure. First, the capillary column was treated in order with 1.0 M NaOH, 1.0 M HCl, and deionized water for 30 min, the residual water being removed by nitrogen. Second, the capillary was filled with a methanol solution containing 50% (*w/w*) 3-(trimethoxysilyl)propylmethacrylate and allowed to react at room temperature for 12 h. Then the capillary was washed with several capillary volumes of methanol, followed by a 15 min wash with deionized water. Third, the capillary was filled with an aqueous solution containing 3% (*w/w*) acrolylic amide, 0.1% (*w/w*) ammonium persulfate and 0.1% (*w/w*) TEMED and left at room temperature for 5–6 h for reaction. Subsequently the capillary was rinsed with deionized water for 30 min. Before use, the capillary was rinsed with the running buffer for 20 min.

CE Operation and the Hyphenation with ESI-MS

All experiments were performed on a Beckman Coulter P/ACE CE™ MDQ capillary electrophoresis system (Beckman, Fullerton, CA, USA). A fused-silica capillary (80 cm, 75 μm I.D., 375 μm O.D., Beckman) coated with acrolylic amide was used. Samples were injected hydrodynamically for 13 s at 7.0 kPa. Between the runs, the capillary was rinsed with the running buffer for 2.0 min at 175 kPa.

ESI-MS was performed on a Finnigan LCQ^{DUO} ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). The mass spectrometer was equipped with an electrospray ion source and was calibrated by direct infusion of a solution of methyl reserpate 3,4,5-trimethoxybezoic acid ester (10 pg μL^{-1}, Finnigan) in methanol-water (50:50, *v/v*) containing 1% acetic acid. A spray voltage of 4.0 kV was employed. The temperature of the heated transfer capillary was set to 200 °C. The sheath-liquid flow comprising 0.25% (*v/v*) formic acid in methanol-water (50:50, *v/v*) was delivered at a flow rate of 4 μL min^{-1} via a syringe pump (Mode 22, Harvard apparatus, South Natick, MA, USA). Mass electropherograms and mass spectra were recorded on an LCQ™ DUO workstation with Core data system software, version 1.2 (Finnigan).

Results and Discussion

Theory of ESI-MS

In ESI, the number of ions that escape from droplets is related to the charge on the droplets, which can be derived from the spray current measurement. Kebarle et al. [25, 26] have proposed equations for a binary electrolyte system, [+]

$$I_A = fp\frac{k_A[A^+]}{K_A[A^+] + k_B[B^+]}I_{spray} \quad (1)$$

$$I_B = fp\frac{k_A[B^+]}{K_A[A^+] + k_B[B^+]}I_{spray} \quad (2)$$

where I_A is the A^+ ion signal at the MS detector; I_B is the B^+ ion signal at the MS detector; f represents fraction of charges on the droplets that are converted to gas-phase ions; p is the fraction of gas-phase ions transported into the mass analyzer; k_A and k_B are sensitivity coefficients for A^+ and B^+; I_{spray} is the total droplet current (spray current); $[A^+]$ and $[B^+]$ are the concentration of A^+ and B^+.

Eq. (1) can be extended for a muilt-electrolyte system by extending the denominator with the appropriate number of $K_X[X^+]$ terms.

For an increasing concentration of electrolytes in solution, the spray current increases weakly with conductivity [25, 27],

$$I_{spray} \propto (conductivity)^n \quad (3)$$

where $n < 1$.

Formic acid-ammonium acetate buffer should be considered as a ternary-electrolyte system. Accordingly, Eq. (1) and Eq. (2) should be modified as follows.

see below Eqs. (4)–(6).

Effects of pH Value of Electrolyte on ESI-MS Signal

The effects of the electrolyte pH on the sample ions signal intensity have been investigated and the results are shown in Figure 1. It can be seen that the sample ion signal intensities of cytochrome c, insulin and bovine serum albumin increase with the increase in the electrolyte pH from 4.0 to 4.4, and decreases following the increase of the electrolyte pH from 4.4 to 4.8. This phenomenon can be explained by Eq. (4). When the electrolyte pH increases from 4.0 to 4.4, the sample ions have been fully ionized and there is residual $[H^+]$ in the system. In this pH range, $[H^+]$ is the main effective factor on the sample ions signal intensity. Accordingly,

$$I_S = fp\frac{k_S[S^+]}{K_S[S^+] + k_H[H^+] + k_{NH_4}[NH_4^+]}I_{spray} \quad (4)$$

$$I_H = fp\frac{k_H[H^+]}{K_S[S^+] + k_H[H^+] + k_{NH_4}[NH_4^+]}I_{spray} \quad (5)$$

$$I_{NH_4} = fp\frac{k_{NH_4}[NH_4^+]}{K_S[S^+] + k_H[H^+] + k_{NH_4}[NH_4^+]}I_{spray} \quad (6)$$

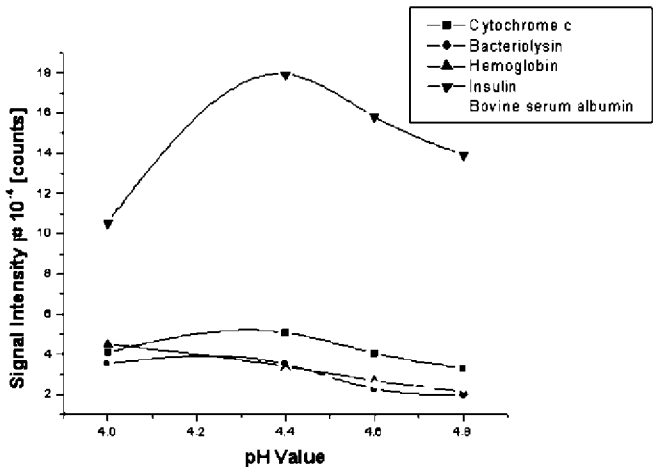

Figure 1. Influence of the buffer pH on the ESI-MS signal. CE conditions: separation voltage 16 kV; capillary, polyacrylamide coated, 80 cm × 75 μm I.D.; temperature, 25 °C; electrolyte, formic acid-ammonium acetate. MS conditions: sheath-gas, 6 unit min^{-1}; spray voltage, 4 kV; sheath-liquid, 0.5% formic acid in methanol-water (50:50, *v/v*), 4 μL min^{-1}; scan range, 400 2000. Sample, cytochrome c, bacteriolysin, hemoglobin, insulin, bovine serum albumin, 100 ~ 200 μg mL^{-1} each.

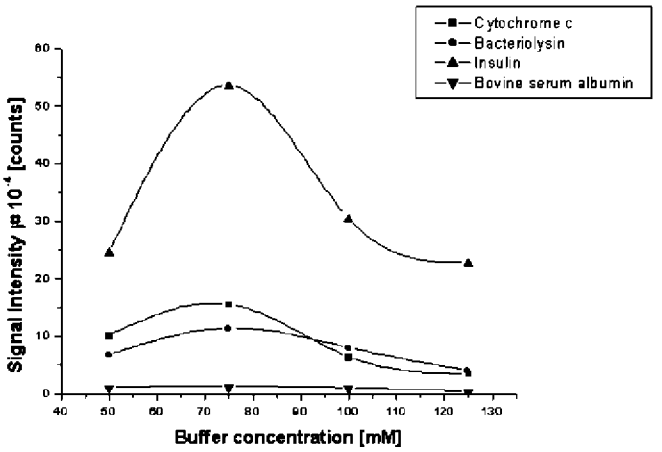

Figure 2. Influence of the buffer concentration on the ESI-MS signal. Electrolyte, 50 125 mM formic acid-ammonium acetate; sheath-liquid, 0.25% formic acid in methanol-water (50:50, *v/v*), 3.4 μL min^{-1}. Sample, cytochrome c, bacteriolysin, insulin, bovine serum albumin, 100 ~ 200 μg mL^{-1} each. Other conditions as in Figure 1.

Eq. (4) can be further modified as follows:

$$I_S = fp\frac{k_S[S^+]}{K_S[S^+] + k_H[H^+]} I_{spray} \qquad (7)$$

When the electrolyte pH increases from 4.0 to 4.4, I_{spray} increases and $k_H[H^+]$ decreases. Accordingly I_S increases. When the electrolyte pH increases from 4.4 to 4.8, the sample ions have not been fully ionized, and $[NH_4^+]$ exerts the main effect on the sample ion signal intensity. Consequently, Eq. (4) can be further modified as follows:

$$I_S = fp\frac{k_S[S^+]}{K_S[S^+] + k_{NH_4}[NH_4^+]} I_{spray} \qquad (8)$$

In this pH range, I_{spray} increases and $k_{NH_4}[NH_4^+]$ increases as well. According to Eq. (3), because $n < 1$, I_S is reduced with the increase in the $[NH_4^+]$ concentration. For bacteriolysin and hemoglobin, the sample ions signal intensity reduces in the investigated pH range, which could also be explained by Eq. (8).

Effect of the Electrolyte Concentration on the ESI-MS Signal

The effect of the electrolyte concentration on the ESI-MS signal has been investigated, with results shown in Figure 2. The maximum sample signal intensity was attained with 75 mM formic acid-ammonium acetate, pH 4.4. Comparing 50 mM with 75 mM formic acid-ammonium acetate, the sample signal intensity decreased.

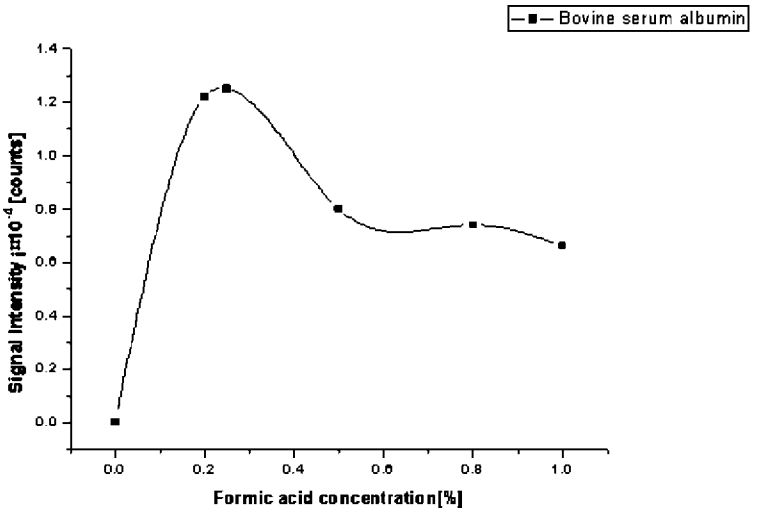

Figure 3. Influence of the formic acid in sheath liquid on the ESI-MS signal. CE conditions: separation voltage 16 kV; capillary, polyacrylamide coated, 80 cm × 75 μm I.D.; temperature, 25 °C; electrolyte, formic acid-ammonium acetate. MS condition: sheath-gas, 6 unit min^{-1}; spray voltage, 4 kV; sheath-liquid, 0.25% formic acid in methanol-water (50:50, *v/v*), 3.4 μL min^{-1}; scan range, 400 2000; sample, Bovine serum albumin, 100 μg mL^{-1}.

One possible explanation for this behavior is that a low concentration can increase the zeta potential of the capillary surface and enhance the adsorption of proteins to the capillary surface, which leads to band broadening and the shortening of the retention time. Consequently the sample ions signal intensity decreases. For 125 mM formic acid-ammonium acetate, the sample ions signal intensity decreases also. This may be caused by the high NH_4^+concentration which competes with the sample ions in the conversion process from solution to gas phase ions.

Influence of the Concentration of Formic Acid as Sheath Liquid on the ESI-MS Signal

In our experiments, the sheath liquid interface has been adopted for the hyphenation of CE and MS. The influence of the concentration of formic acid in the sheath liquid on the ESI-MS signal of bovine serum albumin in the range of 0%–1% was studied in order to better understand the factors that determine the performance of the coupled CE-ESI-MS system. From Figure 3, it can be seen that the signal intensity increased first with more formic

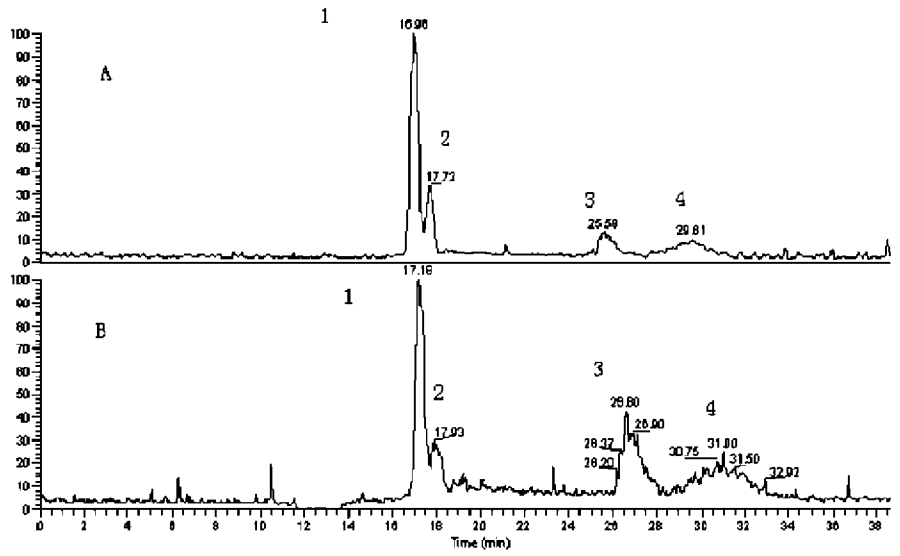

Figure 4. Influence of the sheath liquid composition on the migration times of the proteins. **A)** 0.25% formic acid in ethanol-water (50:50, *v/v*), 3.4 μL min^{-1}; **B)** 0.25% formic acid in methanol-water (50:50, *v/v*), 3.4 μL min^{-1}; 1, cytochrome c; 2, bacteriolysin; 3, insulin; 4, bovine serum albumin; Other conditions as Figure 3.

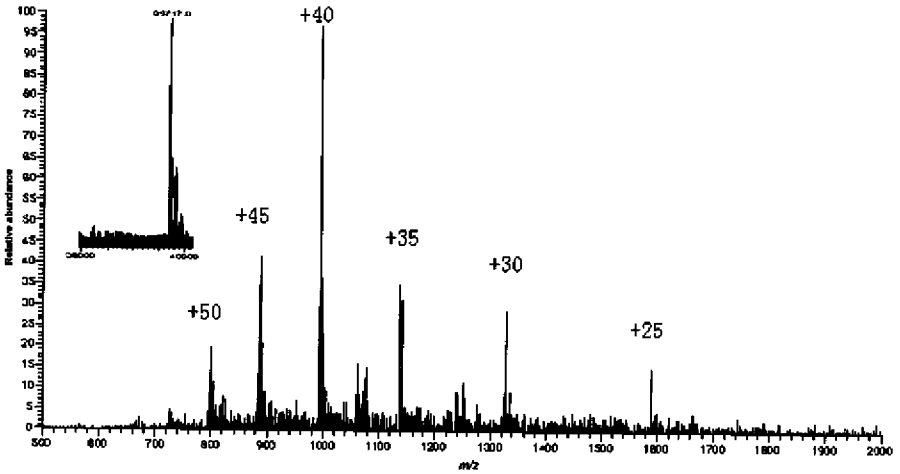

Figure 5. Full scan mass spectra of recombinant human renal tissue Kallikrein. CE conditions: separation voltage 25 kV; capillary, polyacrylamide coated, 80 cm × 75 μm I.D.; temperature, 25 °C; electrolyte, formic acid-ammonium acetate, pH 3.0; MS condition: sheath-gas, 10 unit min^{-1}; spray voltage, 5 kV; sheath-liquid, methanol-water (50:50, *v/v*), 6 μL min^{-1}; Scan range: 400 2000.

acid in the buffer. However, the trend reversed when its concentration is over 0.25%. The increase in the signal intensity with increasing formic acid concentration from 0.0% to 0.25% should be attributed to the more efficient protonation of the proteins at higher hydronium ion concentration, whereas the decrease in signal intensity at formic acid concentrations above 0.25% may be rationalized by the competition of analyte ions and hydronium ions in the conversion process from solution to gas phase ions.

Influence of the Sheath Liquid Composition on CE-ESI-MS System

The influence of two sheath liquids, methanol-water (50:50, *v/v*) containing 0.25% formic acid, and the ethanol-water (50:50, *v/v*) containing 0.25% formic acid, on the migration times of four proteins (cytochrome c, bacteriolysin, insulin, bovine serum albumin) have been investigated, with the results shown in Figure 4. It can be seen that the migration time of the proteins are different with various kinds of sheath liquid. In addition, the currents are also different from each other. Due to the potential gradient across the CE capillary, anions from both the sheath flow and background electrolyte migrate towards the anode. When the anions differ in the two solutions, a moving ionic boundary is formed inside the capillary. Since the pH may be different within this moving boundary, the electroosmotic flow rates, the effective charge on the analytes and their migration rates will change once they enter the boundary, which will affect the migration of analytes.

CE-ESI-MS Analysis of Human Renal Tissue Kallikrein in the Insect Cell Supernatant

Insect cell supernatant was chosen to test the applicability of CE-ESI-MS to the separation and detection of recombinant human renal tissue Kallikrein in real sample. Figure 5 shows the full scan mass spectra of recombinant human renal tissue Kallikrein. Figure 6 shows the separation and detection of recombinant human renal tissue Kallikrein in an insect cell supernatant by using CE-ESI-MS with selected ion monitoring. According to Figure 5, the *m/z* values of selected ions used to acquire this electropherogram were: 794.3, 882.6, 992.9, 1134.8, 1323.9, 1588.7. The migration time at 25 kV separation voltage in a 80 cm long coated capillary were 17.23 min for human renal tissue Kallikrein. Comparing with SDS-PAGE [28, 29] and ELISA [29], this method can easily prove the presence of the recombinant human renal tissue Kallikrein in the insect cell supernatant.

Conclusion

Effects of the pH and electrolyte concentration, the formic acid concentration and the composition of sheath liquid on the CE-ESI-MS system have been investigated. Our results show that these parameters not only affect the CE separation, but also influence the MS detection system. Therefore, good performance of the CE-ESI-MS system for proteins could be obtained with these parameters optimized.

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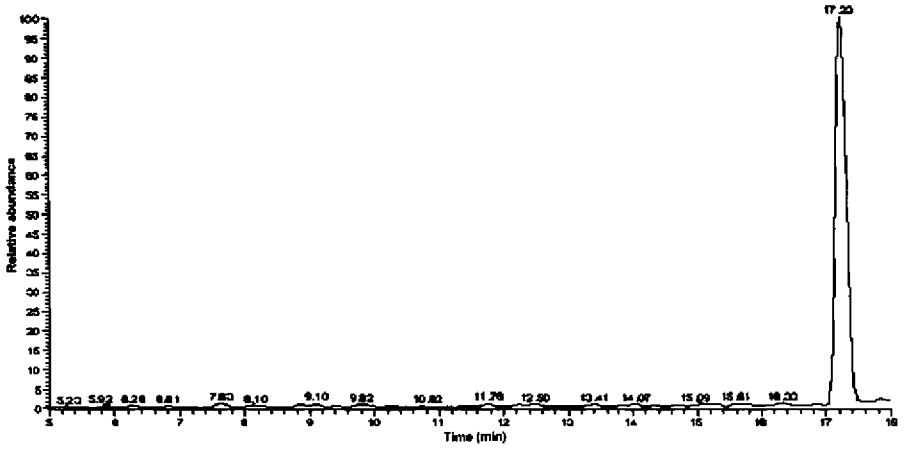

Figure 6. Analysis of recombinant human renal tissue Kallikrein in the insect cell supernatant with selected ion monitoring. CE conditions: separation voltage 25 kV; capillary, polyacrylamide coated, 80 cm × 75 μm I.D.; temperature, 25 °C; electrolyte, formic acid-ammonium acetate, pH 3.0; MS condition: sheath-gas, 10 unit min^{-1}; spray voltage, 5 kV; sheath-liquid, methanol-water (50:50, *v/v*), 6 μL min^{-1}; SIM *m/z* values: 794.3, 882.6, 992.9, 1134.8, 1323.9, 1588.7; Sample: insect cell supernatant.

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