Water Cluster Calibration Reduces Mass Error in Electrospray Ionization Mass Spectrometry of Proteins

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Herein we report a novel calibration routine for use in positive ion mode electrospray ionization mass spectrometry (ESI-MS). Monoisotopic masses were calculated for a series of water clusters and used as calibration reference files (available at http://www.hbcg.utmb.edu/xray). The water cluster series contains singly charged peaks every 18 Da, which allows calibration curves to be precisely defined over a broad mass-to-charge ratio range. Water clusters, induced by a combination of high flow rate and high cone voltage, were used to accurately calibrate a quadrupole mass spectrometer from 100 to 1900 m/z. Calibration curves thus generated have many more data points and greatly reduced standard deviations compared to those obtained from myoglobin, sodium iodide, cesium iodide, or poly(ethylene glycol) based calibration standards. This calibration routine reduces the error in protein mass measurements by a factor of 3, from ±0.01% to ±0.0035% at the 95% confidence limit. The implications of this increased mass accuracy and wider calibrated mass-to-charge ratio scale for the study of protein sequence, structure, and folding by ESI-MS are discussed. (J Am Soc Mass Spectrom 1997, 8, 1158–1164) © 1997 American Society for Mass Spectrometry

Since electrospray ionization mass spectrometry (ESI-MS) was developed by Fenn and coworkers [1, 2], myoglobin has been used as the primary calibration standard. It gives 17 peaks between 679 and 1885 in the mass-to-charge ratio (m/z) scale. When calibrated with myoglobin, the masses of proteins that produce multiply charged peaks in this region can be determined with an accuracy of ±0.01% [3–6]. Sodium iodide, cesium iodide, and poly(ethylene glycol) produce peaks in different regions of the mass-to-charge ratio scale and are also used as calibration standards yielding similar numbers of peaks and error [7, 8]. It is essential that the low mass region be calibrated prior to peptide analysis, including liquid chromatography–mass spectrometry (LC-MS) and fragmentation studies, since species with mass-to-charge ratio values below those that can be calibrated using myoglobin will be generated [9–13].

We have developed a new calibration method that utilizes a series of singly charged water clusters as the calibration standard. Water clusters are multimers of water molecules held together by extensive hydrogen bond networks [14, 15]. They are formed by condensation of water vapor in the supersonic beam as it passes from the atmospheric to low pressure region of the source through an orifice [16]. Singly protonated water clusters formed during electrospray ionization of acidic solutions have been used to calibrate the mass-to-charge ratio scale of a magnetic sector instrument up to near 1000 Da [17]. Others have considered using them to calibrate quadrupole mass spectrometers but failed to obtain significant cluster peaks above a mass-to-charge ratio of 1000 and abandoned this approach [18, 19]. A method is presented herein for generating water cluster series with mass-to-charge ratio values up to 2400. These series are ideal calibration standards as they do not suffer from the complications of multiple charging, standard decomposition, memory effects, or cation adduction common with the conventional reference compounds [7]. Using the monoisotopic masses for the water cluster series as calibration reference files, we have been able to use stringent calibration parameters and the maximum number of calibration points compatible with current software. This has resulted in precisely defined calibration curves that cover the com-

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monly used region of the mass-to-charge ratio scale and yields a threefold reduction in mass error.

Experimental

Materials

High-performance liquid chromatography (HPLC) grade water, bovine serum albumin, bovine β-lactoglobulin forms A and B, and horse heart myoglobin were all purchased from Sigma Chemical Company (St. Louis, MO). Trifluoroacetic acid (TFA) was purchased from Aldrich Chemical Company (Milwaukee, WI). Microfiltration centrifugation units (0.2 μm) were purchased from Gelman Sciences (Ann Arbor, MI).

General

All experiments were performed using a Fisons VG Platform single quadrupole mass spectrometer with electrospray ionization source. Data were acquired and processed on a Digital Equipment Company 560 XL PC using MassLynx software version 2.10. A Rainin HPLC system controlled with Dynamax software version 4.1 running on an Apple MacIntosh SE computer was connected to an electrospray probe via a six-way Rheodyne valve in series with a Rheodyne injection port. A model 55-1111 Harvard Apparatus syringe pump was connected to the six-way Rheodyne valve to provide flow rates below 20 μL/min. Throughout this series of experiments, the instrument was set to positive ion mode, the source temperature was maintained at 60 °C, the capillary voltage was 3.50 kV, the HV lens voltage was 0.81 kV, and the ion energy was 1.0 V. The electrospray process was assisted by gas nebulization using nitrogen at 12 L/h. Nitrogen at 250 L/h was also used as a drying gas. High and low resolution settings were kept at 15, which corresponds to a peak width at half-height of slightly less than 1.5 Da for singly charged solvent peaks.

Calibration Method

Prior to calibration, the instrument was tuned on the water cluster series by setting the HPLC system to pump solvent A [water with 0.05% trifluoroacetic acid (TFA)] at a flow rate of 100 μL/min, setting the cone voltage to 200 V, and mechanically adjusting the probe orientation within the electrospray source to optimize the intensity of the 1405.83 Da peak. Modest source temperature, high solvent flow rate, low drying gas flow rate, and high cone voltage all favor water cluster formation. The exact values for each of these parameters may vary with different instrument/ion source combinations. Two solvent calibration reference files were generated from the data in Table 1. The water cluster static calibration file contains 32 data points starting with the 109.07 Da peak and taking every third point to 1892.12 Da (109.07, 163.10, 217.14, etc.); this file is limited to 32 data points by the MassLynx software. The water cluster scanning calibration file contains 100 data points starting with the 109.07 Da peak and ending with the 1892.12 Da peak and is also limited by the MassLynx software. All peak intensities were set to 100% in the calibration files.

Static calibration, which accurately sets the quadrupole mass analyzer on specific masses for tuning or single ion response (SIR), was performed in multiple channel acquisition (MCA) mode by loading the water cluster static calibration file and using the default calibration parameters with the exception that the calibrated region was from 100 to 1900 in the mass-to-charge ratio scale and a run duration of 10 min was used. The maximum run duration was used to ensure good peak shape for the low intensity calibration peaks near 1900 m/z. The following mass measure parameters were used during the calibration: a 0.1% first order polynomial background subtraction, two times 1.50 Da peak width at half-height Savitzky Golay smoothing routine, and peak top centering using a minimum peak width at half-height of four channels.

Scanning calibration, which ensures that peaks acquired in a scanning acquisition are mass measured accurately at a given scan speed, and scan speed compensation, which compensates for lag time in the system when the instrument is scanning at speeds other than that used for the scanning calibration, were performed in MCA mode by loading the water cluster scanning calibration file and using the same calibration and mass measure parameters as were used for the static calibration. One scan was acquired every 50 s during the scanning calibration. During scan speed compensation calibration, scan speed ranged from one scan every 5 s to one scan every 50 s. In each case the mass measure parameters used for static calibration were also used.

When calibrating the instrument using 10 pmol/μL myoglobin as the calibration standard, all of the above source parameters were used except that a cone voltage of 38 V was used to maximize signal. Data were acquired from 650 m/z to 1550 m/z and fit with a third-order polynomial; many fewer points were generated by the standard.

Protein Mass Measurements

Lyophilized proteins were dissolved in water with 0.05% TFA at a concentration of 10 pmol/μL. The solutions were filtered using 0.2 μm centrifugation units and injected into the mass spectrometer at a rate of 5 μL/min. The instrument was tuned on the myoglobin 1060 peak and a cone voltage of 27–38 V was used to optimize the various protein signals. Ten 6 s scans were summed in MCA mode for each sample and then smoothed twice using a 1.50 Da peak width in the Savitzky Golay method. Peak top centering of the smoothed data was then done using a minimum peak width at half-height of four channels. Analyte masses

Table 1. Monoisotopic masses of the water cluster series ($H_3O^+ + X\ H_2O$) in Da

X = 0–33	X = 34–67	X = 68–101	X = 102–135	X = 136–169
19.0184	631.3788	1243.7392	1856.0996	2468.4600
37.0290	649.3894	1261.7498	1874.1102	2486.4706
55.0396	667.4000	1279.7604	1892.1208	2504.4812
73.0502	685.4106	1297.7710	1910.1314	2522.4918
91.0608	703.4212	1315.7816	1928.1420	2540.5024
109.0714	721.4318	1333.7922	1946.1526	2558.5130
127.0820	739.4424	1351.8028	1964.1632	2576.5236
145.0926	757.4530	1369.8134	1982.1738	2594.5342
163.1032	775.4636	1387.8240	2000.1844	2612.5448
181.1138	793.4742	1405.8346	2018.1950	2630.5554
199.1244	811.4848	1423.8452	2036.2056	2648.5660
217.1350	829.4954	1441.8558	2054.2162	2666.5766
235.1456	847.5060	1459.8664	2072.2268	2684.5872
253.1562	865.5166	1477.8770	2090.2374	2702.5978
271.1668	883.5272	1495.8876	2108.2480	2720.6084
289.1774	901.5378	1513.8982	2126.2586	2738.6190
307.1880	919.5484	1531.9088	2144.2692	2756.6296
325.1986	937.5590	1549.9194	2162.2798	2774.6402
343.2092	955.5696	1567.9300	2180.2904	2792.6508
361.2198	973.5802	1585.9406	2198.3010	2810.6614
379.2304	991.5908	1603.9512	2216.3116	2828.6720
397.2410	1009.6014	1621.9618	2234.3222	2846.6826
415.2516	1027.6120	1639.9724	2252.3328	2864.6932
433.2622	1045.6226	1657.9830	2270.3434	2882.7038
451.2728	1063.6332	1675.9936	2288.3540	2900.7144
469.2834	1081.6438	1694.0042	2306.3646	2918.7250
487.2940	1099.6544	1712.0148	2324.3752	2936.7356
505.3046	1117.6650	1730.0254	2342.3858	2954.7462
523.3152	1135.6756	1748.0360	2360.3964	2972.7568
541.3258	1153.6862	1766.0466	2378.4070	2990.7674
559.3364	1171.6968	1784.0572	2396.4176	3008.7780
577.3470	1189.7074	1802.0678	2414.4282	3026.7886
595.3576	1207.7180	1820.0784	2432.4388	3044.7992
613.3682	1225.7286	1838.0890	2450.4494	3062.8098

were determined from the peak top centered data using all peaks above 5% of the base peak.

Results

A water cluster series spectrum from 100 to 1900 m/z is shown in Figure 1. It is extremely regular and completely free of doubly charged peaks, degradation products, and cationic adducts that can nearly overlap with certain reference peaks and thereby complicate the spectra of other calibration standards. Minor peaks on either side of a given water cluster peak are from the acetonitrile + $X\ H_2O$ and 2 acetonitrile + $X\ H_2O$ cluster series. These peaks are the result of a small amount of acetonitrile bleeding from the HPLC system that provides the high flow rate needed to produce water clusters. They are predictable and can be used as further calibration points, eliminated by using a syringe pump to deliver pure water/TFA solution, or ignored. Since the offset of the acetonitrile + $X\ H_2O$ series from the pure water cluster peaks is constant, they do not overlap. The monoisotopic masses for the water cluster series used as calibration reference files are shown in Table 1. Static and scanning calibrations were performed separately due to differing software limitations on the number of usable data points (see Experimental). The high number of data points allowed the calibration curves to be precisely defined and were best fit with a fifth-order polynomial, the highest order available.

Calibration curves generated from this approach are shown in Figure 2. 10 min acquisitions were used to ensure that the low intensity peaks at high mass-to-charge ratios were adequately defined. Initially 3 min acquisitions had been used but these resulted in increased scatter about the calibration curve in the high mass-to-charge ratio region. This scatter was reduced to the level of that in the low mass-to-charge ratio region with 10 min acquisitions. With repeat calibration, standard deviations for static calibrations varied from 0.020 to 0.029 and showed a distinct pattern in the residuals below 1000 m/z. This indicates that further improvement may be obtained by fitting with a higher-order polynomial or a combination of polynomial and Fourier series. Standard deviations for scanning calibrations varied from 0.035 to 0.040 using optimized calibration parameters.

After water cluster calibration of the region shown in Figure 2, positive ion electrospray spectra of horse heart

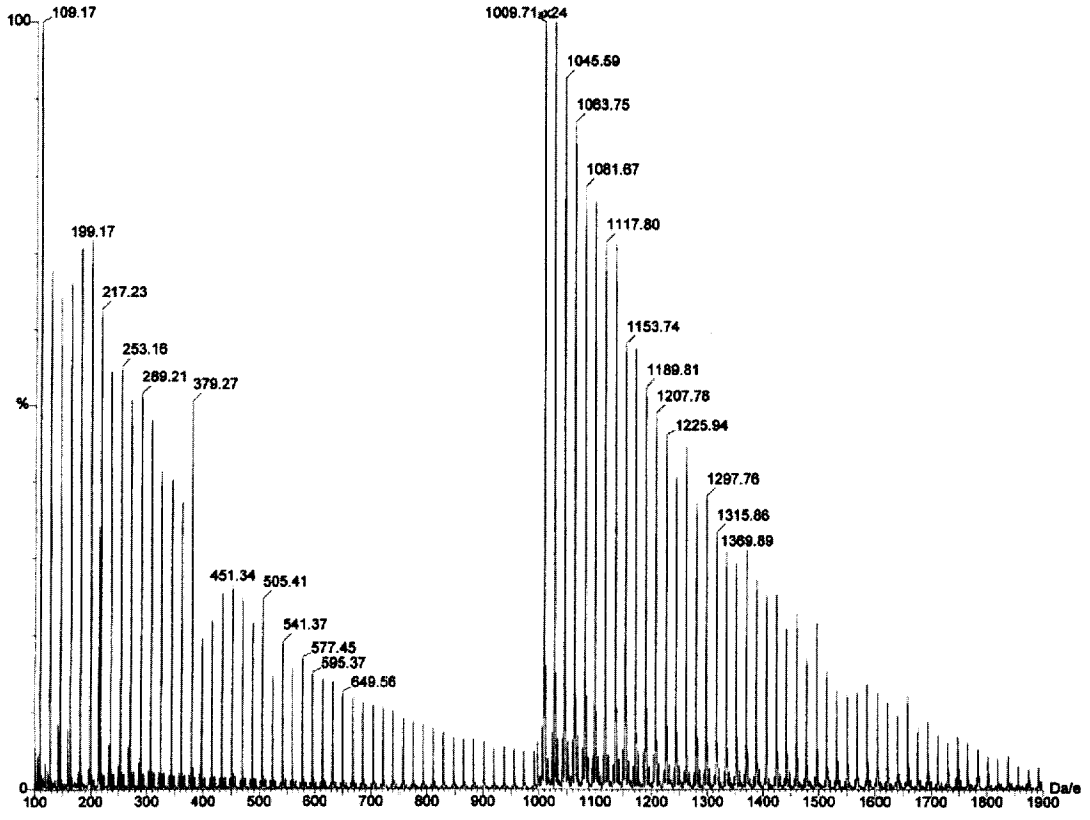

Figure 1. Positive ion electrospray ionization mass spectrum of a water cluster series. The spectrum in the result of the summation of ten 6 s scans acquired in MCA mode. Peaks beyond 1000 in the mass-to-charge ratio scale have been magnified ×24 for clarity.

myoglobin M_c = 16,951.50) [20], bovine beta-lactoglobin forms A (M_c = 18,363.19) [21–24] and B (M_c = 18,277.10) [21], and bovine serum albumin (M_c = 66,430.30) [21, 25], were acquired between 600 and 1800 m/z (see Figure 3 and Experimental). Table 2 shows the results of repeat mass measurements from data collected on two separate days using independent calibrations. The standard deviation in percent error is 0.0017, giving an accuracy of ±0.0035% at the 95% confidence limit. This represents a threefold improvement in accuracy over protein masses measured using standard myoglobin calibration and is accompanied by a concomitant decrease in the standard deviation of the calculated mass for a given protein from a given spectrum.

This compares to an accuracy of ±0.011% on our instrument using standard myoglobin calibration [6]. When myoglobin at 10 pmol/μL is used as the calibrant and all parameters except cone voltage (myoglobin fragments at cone voltage = 200 V), calibrated region, and polynomial fitting are kept the same (see Experimental), an error of ±0.0041% at the 95% confidence limit (n = 31) is obtained (see Table 3).

Discussion

Water clusters are superior calibration compounds. Fresh standards do not need to be made before each calibration as the reference compound does not degrade with time. The calibration peaks can also be removed instantly from the spectrum by lowering the cone voltage and increasing the drying gas flow rate. This is in contrast to other calibration compounds that can linger in the system. Because the solution being sprayed is essentially 100% reference compound, very low intensity peaks around 2400 m/z can be used as calibration points; there is virtually no background signal. The $H_2{}^{16}O$:[$HD^{16}O$ + $H_2{}^{17}O$] ratio is 99.73:0.07; thus, even at high mass the isotopic satellite peaks contribute little to the spectrum. This allows nearly the entire working range of a quadrupole mass spectrometer to be calibrated with one calibration standard.

The lens voltage, capillary voltage, resolution settings, and ion energy remain constant while the cone voltage, solvent flow rate, source temperature, or drying gas flow rate may change from calibration to acquisition mode, depending on the application. In practice, altering these latter values does not invalidate the calibration. Scan speed may also be changed within the limits of the scan speed compensation calibration, from 35.667 to 356.67 Da/s above, without introducing error into the measurement. The total time required for water cluster calibration is either 35 min without verification or 65 min with verification (verification is not routinely needed) after generation of, and tuning on, the water cluster series. The time required for the latter

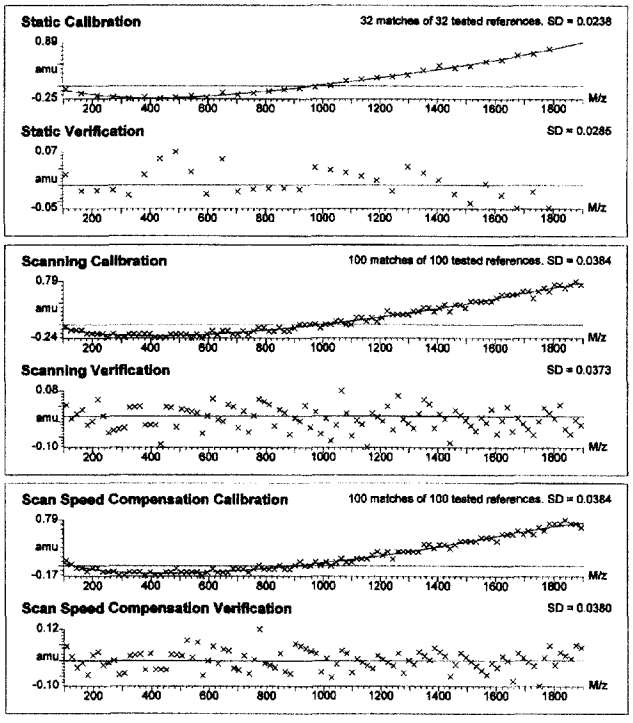

Figure 2. Calibration curves generated from a water cluster series spectrum fit to reference peaks from Table 1 (see Experimental) with a fifth-order polynomial. Verification plots are obtained from completely independent scans and provide a measure of the precision of the calibration routine.

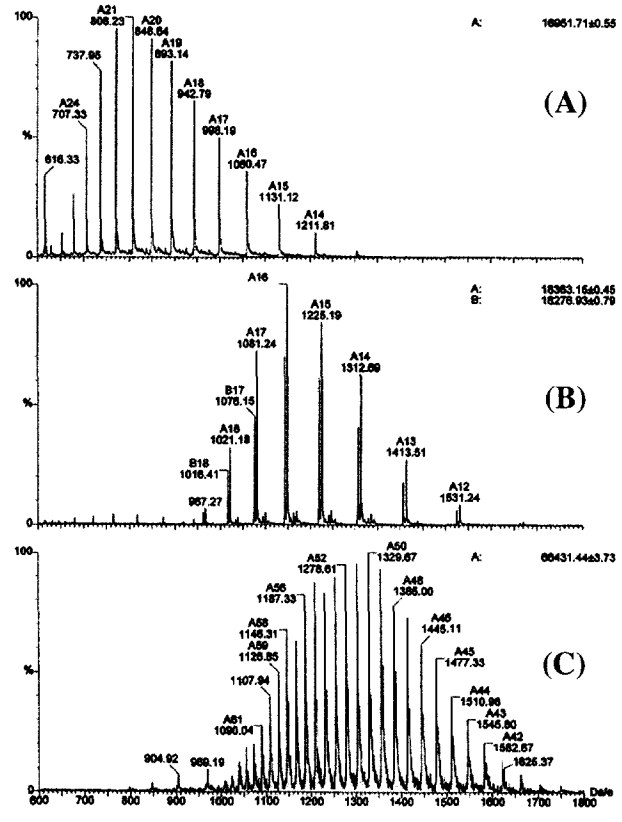

Figure 3. Positive ion electrospray ionization mass spectra of **(A)** horse heart myoglobin, **(B)** bovine β lactoglobulin forms A and B, and **(C)** bovine serum albumin acquired after water cluster calibration of the quadrupole mass analyzer.

varies greatly in our laboratory with the experience of the user.

We have demonstrated the calibration of the region from 100 to 1900 m/z fit with a single calibration curve. This region is optimal for LC-MS separation of complex mixtures of biological origin. Intact proteins from such mixtures may occupy very different regions of the mass-to-charge ratio scale (see Figure 3) and peptides may yield peaks well below the region able to be calibrated with myoglobin. The lower region of the mass-to-charge ratio scale contains the most intense water cluster peaks and is easily calibrated along with the high mass-to-charge ratio region using our method. Calibration must be performed daily in order to obtain such accurate measurements as the VG quadrupole mass analyzer is only stable to within ±0.01% over 3 days, under normal conditions. Calibration drift is a function of instrumentation, not the calibration routine, and we have observed an increased error in measurements made 18 h post calibration. Increased error also occurs with dilute samples, when a low number of scans is acquired, and for complex spectra in which analyte peak shape is distorted by overlap with contaminant [26].

This water cluster calibration method should be valid for any instrument/source combination in which the source is capable of producing water clusters over the desired region and the operating system can accept the calibration files and operating parameters presented herein. As mentioned in the Experimental section, the exact combination of solvent flow, drying gas flow, and source temperature required to generate optimal cluster series may vary from system to system. Indeed, depending upon the initial positioning of the probe within the source, differing drying gas flow rates may be required to yield optimum cluster formation on the same instrument (from 50 to 250 L/min on our Fisons VG single quadrupole). The drying gas flows in the same direction as the solvent flow in the Fisons source and thus also serves in part to focus the spray. This presumably accounts for much of the variability in the aforementioned parameter.

Generation of an appreciable level of water clusters may be very difficult, with sources using a countercurrent drying gas flow. Users with sources incapable of generating sufficient water clusters may still benefit from application of the calibration parameters, developed for optimal water cluster calibration, to standard myoglobin calibrant solutions. This technique offers a superior calibration within the standard mass-to-charge ratio range (see Table 3) but lacks the major advantage of a wide calibrated mass-to-charge ratio range obtained from water cluster calibration.

The increased accuracy obtained, over a more limited mass-to-charge ratio range, using myoglobin as the

Table 2. Protein analysis after water cluster calibration

Protein	Calculated mass (Da)	Measured mass (Da)	Error (Da)	Percent error
HHMB[a]	16,951.50	16,951.33	−0.17	−0.0010
		16,951.52	0.02	0.00012
		16,951.92	0.42	0.0025
		16,951.63	0.13	0.00077
		16,951.71	0.21	0.0012
		16,951.63	0.13	0.00077
		16,951.90	0.40	0.0024
		16,952.05	0.55	0.0032
		16,952.05	0.55	0.0032
		16,951.85	0.35	0.0021
		16,951.18	−0.32	−0.0019
		16,951.08	−0.42	−0.0025
		16,951.43	−0.07	−0.0004
		16,952.13	0.63	0.0037
		16,951.70	0.20	0.0012
		16,951.63	0.13	0.00077
		16,951.79	0.29	0.0017
Bβ-LGA[b]	18,363.19	18,363.15	−0.04	−0.00022
		18,362.89	−0.30	−0.0016
		18,363.24	0.05	0.00027
Bβ-LGB[b]	18,277.10	18,276.93	−0.17	−0.00093
		18,276.87	−0.23	−0.0013
		18,277.19	0.09	0.00049
BSA[c]	66,430.30	66,430.36	0.06	0.000090
		66,431.44	1.14	0.0017
		66,431.44	1.14	0.0017
		66,431.31	0.01	0.000015

[a]Horse heart myoglobin.
[b]Bovine β-lactoglobulin forms A and B.
[c]Bovine serum albumin.

Table 3. Protein analysis after enhanced myoglobin calibration

Protein	Calculated mass (Da)	Measured mass (Da)	Error (Da)	Percent error
HHMB	16,951.50	16,952.04	0.54	0.0031
		16,951.86	0.36	0.0021
		16,951.80	0.30	0.0018
		16,951.91	0.41	0.0024
		16,951.85	0.35	0.0021
		16,951.91	0.41	0.0024
		16,951.44	−0.06	−0.00035
		16,951.53	0.03	0.00018
		16,951.80	0.30	0.0018
		16,951.46	−0.04	−0.00024
		16,951.60	0.10	0.00059
		16,951.66	0.16	0.00094
		16,951.96	0.46	0.0027
		16,951.71	0.21	0.0012
		16,951.58	0.08	0.00047
		16,951.80	0.30	0.0018
		16,951.90	0.40	0.0024
		16,951.83	0.33	0.0019
		16,951.90	0.40	0.0024
		16,951.88	0.38	0.0022
		16,951.79	0.29	0.0017
		16,951.65	0.15	0.00088
		16,951.83	0.33	0.0019
		16,951.88	0.38	0.0022
		16,952.29	0.79	0.0047
		16,951.86	0.36	0.0021
		16,951.78	0.28	0.0017
		16,951.52	0.02	0.0017
		16,951.99	0.49	0.0029

calibrant and the parameters developed for water cluster calibration, is likely the result of the excellent peak shape that this method affords. High myoglobin concentration, combined with 10 min acquisitions for each calibration type, and slight oversmoothing generate highly reproducible peak top assignments. This process does, however, result in a systematic error in the measurement (only two of 31 measurements were low: −0.04 and −0.06 Da). Thus, accuracy may be further improved, for proteins that produce all of their peaks in this region, by applying a correction factor to the calculated mass.

The central issue in every mass spectral technique is the accuracy with which an analyte mass can be determined. The threefold reduction in mass error afforded by this method has implications for nearly every application of ESI-MS to protein science. One advantage is that the maximum mass that can be unambiguously assigned to within ±0.50 Da is increased from 5000 to 15,000 Da. This means that point mutants that generate single Da differences (Asp to Asn, Glu to Gln) can now be confirmed for a number of biologically active proteins (i.e., cytochrome *c* [27], ubiquitin [28], and lysozyme [29]) without resorting to peptide mapping or FT-MS. Similarly, the upper limit for unambiguous assignment of intramolecular disulfide bond formation, which results in a 2 Da decrease in protein mass, is increased from 10,000 to 30,000 Da. The increased mass accuracy will also minimize the ambiguity in sequence assignment of chemical cleavage products using the EPD [(EDTA-2 aminoethyl)2-pyridyl disulfide]-Fe reagent we have developed for protein conformation and folding studies [30–35].

Acknowledgments

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