Improved Mass Accuracy in Matrix-Assisted Laser Desorption / Ionization Time-of-Flight Mass Spectrometry of Peptides

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One problem of matrix-assisted laser desorption ionization coupled to time-of-flight mass spectrometry is the moderate mass accuracy that typically can be obtained in routine applications. Here we report improved mass accuracy for peptides, even when low amounts and complex peptide mixtures are used. A new procedure for preparing matrix surfaces is used, and there is no need to mix the matrix with the sample or to add internal standards. Examples are shown with a mass accuracy better than 50 ppm in a peptide mixture. Peptide mapping as well as sequencing by creating "ragged ends" or "ladder sequencing" should benefit especially from the improved mass accuracy. *(J Am Soc Mass Spectrom 1994, 5, 955–958)*

Matrix-assisted laser desorption/ionization (MALDI), introduced in 1988 by Hillenkamp and Karas [1], has proven to be an excellent technique for the study of large biological molecules. It is a fast, simple, and sensitive method, and has already become a widely used tool in protein chemistry [2].

Hitherto, one main drawback of MALDI time-of-flight (TOF) mass spectrometry has been that mass calibration is difficult. Mass accuracies of up to 0.01% have been reported in the analysis of peptides and proteins of molecular weight up to 20 ku [3]. These measurements required the addition of internal calibrants to the sample. This procedure increases analysis time and—more seriously—the extra components introduced into the sample can interfere with the measurement of the analyte. Even with internal calibration, we and others [4–7] have found it difficult in many cases to achieve 0.01% mass accuracy, especially when analyzing complex peptide mixtures and low amounts of sample. The time of flight did not appear to be always completely proportional to the square root of the mass-to-charge ratio, which is a prerequisite for empirical mass calibration of time-of-flight spectra.

In a recent paper [8] we described a new sample preparation procedure, which decouples matrix and sample handling. Matrix solution is applied to the probe tip of the mass spectrometer in a solvent that evaporates very fast and thus leads to the formation of a thin layer of very small crystals of matrix. A small volume of analyte solution is then placed on top of the matrix surface and the solvent is allowed to evaporate slowly. Significantly better sensitivity (subfemtomole), washability, surface homogeneity, and mass resolution were obtained with this procedure compared to the conventional preparation procedure, where sample and matrix are mixed in solution before drying on a probe tip.

Here we show that the new sample preparation technique, together with the use of a reflector, also leads to a much better proportionality between time of flight and the square root of the mass-to-charge ratio. Thus, very simple and highly accurate mass calibration has become possible, and we routinely obtain better than 0.01% mass accuracy without the addition of peptide standards for internal calibration. With internal calibration, MALDI promises to become even more accurate. Results of MALDI TOF of peptide mixtures are demonstrated where very high mass accuracy was obtained without adding an internal standard, but by using the time of flight and mass of the protonated matrix dimer instead.

Experimental

Microcrystalline matrix surfaces were made on the probe tips of the mass spectrometer following the sample preparation procedure described in detail in ref 8 and outlined below.

The matrix material (α-cyano-4-hydroxy-cinnamic acid) was dissolved in acetone that contained 1–2%

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pure water or 0.1% aqueous trifluoroacetic acid. The concentration of matrix ranged between the point of saturation (approximately 40 g/L) and one-third of that concentration. Approximately 0.5 μL of matrix solution was deposited at the center of the probe tip. The transfer was done quickly to avoid evaporation of matrix solution in the pipette tip. The solution spread rapidly by itself, which allowed evaporation of the acetone within a second or two. Because of the extremely rapid crystallization of matrix material, an even and homogeneous surface of very small crystals was formed.

Analyte solution (volumes in the range 0.3–1 μL) was then deposited onto these matrix surfaces and the solvent was allowed to dry at ambient temperature. The analyte solution can be chosen freely within the constraint that it must not completely redissolve the matrix surface. For example, aqueous solutions with up to 30% acetonitrile can normally be tolerated.

Samples were washed by placing a 5–10-μL volume of water or dilute organic acid on the matrix surface after the analyte solution had dried completely. The liquid was left on the sample for 2–10 s and was then shaken off or blown off by pressurized air. The procedure was repeated once or twice. The washing procedure was found to be critical to the success of the procedure with most samples other than diluted standards.

The mass spectrometer used was a Bruker REFLEX time-of-flight instrument (Bruker-Franzen, Bremen, Germany) equipped with the LaserOne data acquisition system [9]. Pressures were below 10^{-6} mbar in the source region and below 10^{-7} mbar in the analyzer region. About 100 shots usually were averaged per spectrum. The number of shots did not have much influence on mass accuracy once a sufficient signal-to-noise ratio had been achieved.

The spectra were calibrated according to the usual time-of-flight equation $T = C_1 * (m/z)^{1/2} + C_0$, where T is the time of flight and C_1 and C_0 are constants. To avoid the use of internal standards, we first determined a value for C_0 from the statistical average of a large number of measurements of known substances. The value obtained ($C_0 = -7$ ns) is instrument specific and has not been changed for months. Having fixed C_0 we then use the time of flight and mass of the protonated matrix dimer (379.0929-u monoisotopic MW) to calibrate each spectrum. The value can be set as a "preference" in the LaserOne data system. After acquiring a spectrum the data system performs the calibration on the protonated dimer molecule automatically.

Results and Discussion

Figure 1 shows part of the reflector mass spectrum of a peptide mixture obtained from a tryptic digest of human α-chain hemoglobin. All peaks are isotope resolved and seven tryptic peptides have been identified in this part of the spectrum. (There are 14 tryptic limit peptides in the hemoglobin sequence, and the full mass range of this spectrum covers 67% of the sequence.) From the amount of starting material, we estimate that less than 14 fmol of each peptide has been applied to the target. The spectrum was mass calibrated according to the time-of-flight equation as mentioned above by using a predetermined value for C_0 and the time of flight and mass of the protonated matrix dimer. As Table 1 shows, the average value of the relative mass accuracy was 20 ppm calculated over all seven peaks.

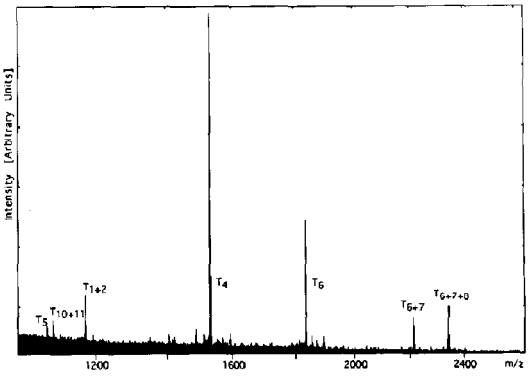

Figure 1. Reflector mass spectrum of a peptide mixture obtained from a tryptic digest of human α-chain hemoglobin. The identified tryptic peptide peaks are marked by the letter T above or next to each peak.

Figure 2 shows the reflector mass spectrum of a "ragged end" peptide mixture obtained from a combined carboxypeptidase Y and P digest of substance P. No more than a total of 50 fmol of peptide was applied to the target out of 1 pmol of substance P used in the digestion. Six peaks have been identified from the digested substance P and allow the sequence determination of five amino acid residues. Again the spectrum was calibrated by using a predetermined value for C_0 and the time of flight and mass of the protonated matrix dimer. The six peptide masses could be obtained with an average relative accuracy of 12 ppm as shown in Tables 2 and 3. Additionally, the measured mass differences between neighboring peaks were

Table 1. Measured and calculated masses for some tryptic peptides from human α-chain hemoglobin (data from Figure 1)

Peak	Peptide Sequence	Meas. Mass[a] (u)	Calc. Mass[a] (u)	Error (ppm)
T5	32–40	1070.57	1070.55	19
T10+11	91–99	1086.58	1086.62	37
T1+2	1–11	1170.70	1170.66	34
T4	17–31	1528.73	1528.73	0
T6	41–56	1832.87	1832.88	5
T6+7	41–60	2212.13	2212.08	23
T6+7+8	41–61	2340.12	2340.17	21

[a] Monoisotopic masses (proton mass has been subtracted from measured values).

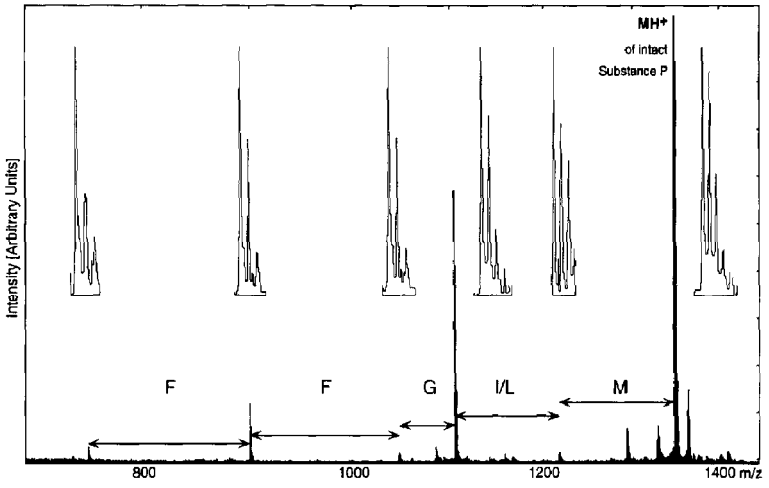

Figure 2. Reflector mass spectrum of a peptide mixture obtained from a combined carboxypeptidase Y and P digest of substance P (seq.: RPKPQQFFGLM amide C-terminal, monoisotopic MR 1347.736). Identified peaks are marked by their corresponding amino acid symbols and normalized zooms are inserted above or next to each peak. The average mass resolution was around 2400 (full width at half-maximum). Five amino acid residues were sequenced from substance P and have been marked on the spectrum.

compared to their corresponding calculated amino acid residue masses, and for all residues the discrepancy was found to be less than 0.03 u. Such high mass accuracy opens up the possibility to distinguish mass spectrometrically between one lysine (monoisotopic residue MR 128.0950) and glutamine (monoisotopic residue MR 128.0586) residue. However, in other analyses we found that the discrepancy can be as high as 0.07 u. Further developments will show whether or not these two residues can be distinguished reliably in this mass range. In any case, the accuracy presented here is about a factor of 5–10 higher than that achieved in proposals to sequence by ragged ends [6, 7]. With realistic samples, that is, samples with residual contamination, there will often be background peaks. The correct series can be read off much more confidently if mass accuracy is high.

The mass accuracies in the results above are not single events, but have been reproduced in hundreds of analyses of other peptide mixtures and carbohydrates. In rare cases, the one-point calibration procedure that was used here would yield only a 200-ppm mass accuracy. A reason for these outliers may be that some of those sample surfaces were not washed thoroughly enough and that interfering substances remained on the target. Although these few occurrences were not especially limiting, it was sometimes preferred to use internal calibration standards. Preliminary results show that an accuracy of 50 ppm is routinely obtained for peptides under 2000 u in reflector TOF spectra; that is, masses are expected to be correct within 0.1 u in this mass range.

By using identical instrumentation but the conventional sample preparation (i.e., matrix–analyte coprecipitation) we previously determined the limits of mass accuracy when no internal standard was added [10]. Five synthetic peptide samples in the mass range from 750 to 2500 u were analyzed on five consecutive days. All factors that influence the measured mass accuracy, such as irradiance, type of matrix, and voltage settings, were held as constant as possible under routine conditions. The result was that the mass could be determined within a mass unit as the average of several measurements, but individual measurements on different days could differ by more than 0.5 u in some cases. When analyzing peptide mixtures in low femtomole amounts, results were not certain within a mass unit. The procedure described improves mass accuracy by about 1 order of magnitude and does not impose

Table 2. The measured and calculated masses of identified peaks in Figure 2 are shown along with the relative mass accuracy (data from Figure 2)

Amino Acids	Meas. Mass[a] (u)	Calc. Mass[a] (u)	Error (ppm)
1–11	1346.735	1346.728	5
1–10	1216.651	1216.672	17
1–9	1103.577	1103.588	10
1–8	1046.569	1046.566	3
1–7	899.484	899.498	16
1–6	752.444	752.429	20

[a] Monoisotropic masses (proton mass has been subtracted from measured values).

Table 3. Measured and calculated masses of the identified amino acids from substance P along with the absolute mass accuracy (data from Figure 2)

Residue	Meas. Difference[a] (u)	Cal. Residue Mass[a] (u)	Meas – Calc (u)
M	130.084	130.056	0.028
L/I	113.074	113.084	−0.010
G	57.008	57.021	−0.013
F	147.085	147.068	0.017
F	147.040	147.068	−0.028

[a] Monoisotopic masses

practical constraints on the irradiance and voltage setting.

The reasons for the improved mass accuracy observed here are not clear yet. However, our experiments support the following reasoning: The surfaces produced by our new sample preparation method are very flat, probably within about a micrometer, and the matrix surface is composed of a layer rather than a collection of relatively large crystals with much space in between [8]. Furthermore, because of the decoupling of the matrix surface preparation and sample loading, the analyte molecules are probably embedded in the outmost layers of the crystals. Our experiments are consistent with the idea that much less gaseous material is produced in the desorption event. Thus the ions are extracted from the well defined potential of the surface rather than the ill defined starting point from within a cloud of desorbed matrix ions (in terms of electrical potential, time delay and point in space). This conjecture is supported by the linearity of the mass scale, which indicates common starting conditions for all ions, by lack of internal energy of the ions as manifest in the virtual absence of fragmentation, and by the much larger independence of both resolution and ion-flight times from the level of irradiance when compared to standard preparations [8]. Because the desorption event does not impart time delays, the reflector can compensate for remaining energy deficits. The level of performance achieved is close to the limits of the system components, such as time width of the laser pulse, finite digitizer rate, vacuum pressure, and reflector quality. Therefore, we speculate that the desorption event is no longer the limiting factor, but that the limit on achievable performance is now set by the mass spectrometer.

Proteins are normally analyzed in the linear mode where there is no energy compensation for their ions or for the matrix dimer ions used in the calibration procedure. We have not yet systematically studied the effect of our procedure on protein mass accuracy. Nevertheless, improvements in mass accuracy have been obtained, and chicken lysozyme could be measured with an accuracy of 0.02% in the linear mode without the addition of standards.

Prospects

The increase in mass accuracy is closely tied to the new procedure for preparing matrix surfaces. We are currently performing a detailed study to obtain statistical figures for mass accuracy and precision. The computer routine for automatic calibration on the peak from the matrix dimer will be tested. In addition, we will try to examine some mechanistic aspects to clarify why the time-of-flight equation holds more closely with the new matrix surfaces.

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