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# Protein analysis by packed capillary liquid chromatography with electrospray ionization and time-of-flight mass spectrometry detection[1]

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**Abstract**

Packed capillary liquid chromatography (LC) has been interfaced on-line to electrospray (ES) ionization with time-of-flight (TOF) mass spectrometry (MS) detection for the separation and identification of standard proteins. Using this system, as little as 2.5 fmol of cytochrome c could be detected with a 0.1 mm I.D. column. The effect of column size on sensitivity has been investigated using various column diameters and a sensitivity increase of 428-fold has been observed between the 0.10- and 2.1 mm I.D. columns. Although the TOF-MS can ultimately record data at up to 100 mass spectra/s, the peak widths for these particular separations require only 1 spectra/s. Finally, the sensitivity obtained is a function of both the reduced diameter LC column and the high ion duty cycle of the TOF-MS. © 1997 Elsevier Science B.V.

*Keywords:* Mass spectrometry; Time-of-flight mass spectrometry; Proteins; Cytochromes

## 1. Introduction

The use of packed capillary or microcolumn LC separations has become increasingly popular due to the benefits of enhanced sensitivity and efficiency which are achievable with this technology. Because the characterization of proteins according to structure and function is one of the most critical challenges in modern biochemistry and because these samples are available in ever decreasing quantities, we have explored the use of capillary LC coupled with mass spectrometry for protein analysis.

Mass spectrometry (MS) detection is one of the most powerful tools available in modern chemical analysis due to its ability to provide, in most cases, the molecular weight of analytes. Unfortunately, interfacing MS with LC in an on-line arrangement has been notoriously difficult due the mutual incompatibilities of mobile-phase solvent systems and vacuum systems, as well as the limitations in ionizing fragile polar species without destroying them. Electrospray (ES) as introduced by Fenn and coworkers [1–3] has demonstrated an ability to overcome the classical difficulties associated with desolvation, ionization and transfer into vacuum required for the MS analysis of large, fragile polar species such as proteins. ES interfacing has been demonstrated with quadrupole [2–7], magnetic [8–12], Fourier-transform [13–16], ion-trap [17,18] and time-of-flight (TOF) machines [19–25]. None of these mass analyzers, except TOF, has the potential

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to realize a continuous high duty cycle or usage of ions. Other types of mass spectrometers are scanning and thus dispersion machines, and their ability to discriminate against ions of different mass/charge is based on the inherently wasteful process of individually selecting ions for stable trajectory through the analyzer by varying either electric or magnetic fields in time. This process results in the rejection of the majority of the ions sampled at any given time in favor of the particular $m/z$ ion being detected.

Because a TOF mass analyzer simply measures the flight times of an ion population which has received an initial pulse of energy and then traveled through an otherwise empty tube, nearly all ions of interest are expected to survive until they impinge on the detector. A major difficulty to overcome with this method is the sampling of a continuous ion beam from the ES source into essentially a discontinuous pulsing process. This problem can be solved through the method of orthogonal pulsing [24–28] from the ion source into the TOF analyzer chamber at a repetition rate high enough to extract a majority of the ion beam. Ultimately, the TOF analyzer described in this report can generate complete mass spectra at a rate of 8.2 kHz. These individual mass spectra are then summed or grouped together into appropriate time segments, as small or as large as is required by the time domain of the separation process, in order to generate final mass spectra.

Capillary LC offers increased sensitivity with detectors which are concentration dependent, and not mass dependent in nature. ES–MS seems to operate in a concentration-sensitive mode for liquid flow-rates ranging from approximately 100 nl/min up to 1 ml/min and thus gives the same signal strength regardless of flow-rate. This effect has been demonstrated and discussed by a number of groups [29–31]. The speculation for this behavior is the occurrence of droplet space-charge repulsion in the atmospheric region of the ES/MS source. As the liquid droplets carrying the sample analyte are formed and charged, they repel each other in space as they travel in the ES source towards the orifice which will transport the ions into vacuum. Because there is a limit or a maximum density of droplets due to like-charge repulsion, only a small portion of the droplets created will be sampled. As the liquid flow-rate increases, the droplet spacing near the vacuum orifice is conserved due to the repulsion, so the number of droplets contributing to the ion signal does not increase. In practice then, at high flow-rates, the liquid sample is split internally after it enters the source. Because of this behavior, optimum sensitivity can be achieved when separation methods which operate best at the low flow-rates are used.

## 2. Experimental

### 2.1. Apparatus

The electrospray ionization source was from Analytica of Branford (Branford, CT, USA) and is schematically depicted with the TOF system in Fig. 1. This system was similar to its original form [32] except for the use of a micro flow-rate pneumatic nebulization probe which has been previously discussed [31]. Large windows on three sides of the atmospheric region of the source allowed for the visualization of the electrospray process itself and greatly aided in the optimization of instrumental conditions necessary to obtain good results. The voltages in the source were set as follows: the cylinder electrode ($V_{cyl}$) at −2500 V, the endplate

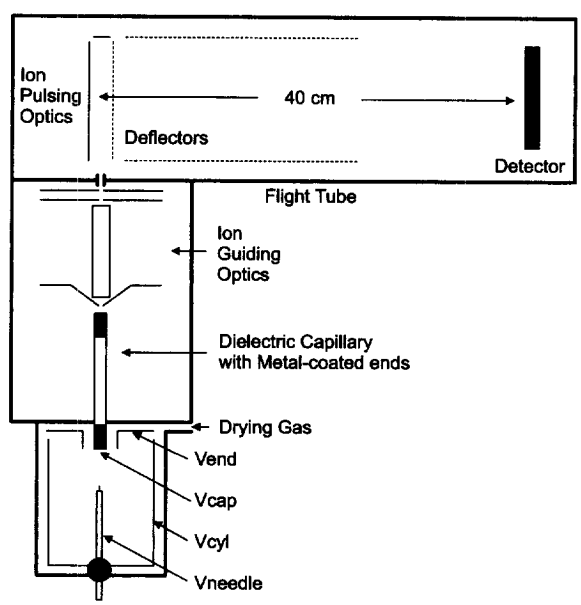


Fig. 1. Schematic of the ES–TOF-MS system.

electrode ($V_{end}$) at −3970 V, and the capillary entrance ($V_{cap}$) at −4480 V.

From the exit of the ion guiding lenses, the ion beam is focused through a 2-mm aperture into the first stage of the Wiley/McLaren [33] accelerator. The distance from the center of the extraction volume to the detector surface was 40 cm and the drift energy in the field free flight tube was 3000 eV for a singly charged ion. Electrostatic steering deflectors were used to compensate for the effect of the initial orthogonal velocity component. A mass resolution of $R$(fwhm)=1000 was determined for leucine enkephalin at $m/z$ 556 using a Tektronix Model TDS 540 digital oscilloscope (Wilsonville, OR, USA), operated at 500 Mhz. For rapid and continuous analog to digital conversion and signal averaging, the output signal from the electrically floating ion detector was capacitively coupled into a Model 9825 integrating-transient-recorder (ITR) data acquisition device (200 Mhz) from Precision Instruments (Knoxville, TN, USA).

The LC system used was a 1090 series II L from Hewlett-Packard (Palo Alto, CA, USA). The mobile phase was taken directly from the high-pressure output pump for the 1.0- and 2.1-mm I.D. columns. For the 0.10-mm I.D. column, a splitting tee was constructed using a 5-ft section of 0.0025″ I.D. peek tubing, and the pump was operated at a flow-rate of 2.5 ml/min. Injection was accomplished with a Valco CI4W (Houston, TX, USA) internal loop injection valve with a 60-nl rotor. The packed capillary LC column was fabricated from 100-μm I.D. fused silica from Polymicro Technologies (Phoenix, AZ, USA) and was 15 cm in length. The reversed-phase packing material used was Capcell Pak C18, 5-μm particle size from Shiseido Co. (Tokyo, Japan). The microbore or 15 cm×1 mm I.D. column as well as the 15 cm×2.1 mm I.D. column were purchased from Vydac (Hesperia, CA, USA) and were packed with 5-μm $C_{18}$ particles.

### 2.2. Materials

Buffers were prepared from distilled water obtained from a Barnstead NANOpure II (Boston, MA, USA) system. Acetonitrile (ACN) and trifluoroacetic acid (TFA) were obtained from Mallinckrodt (Paris, KY, USA). All solvents were filtered through nylon 66 membranes from Anspec (Ann Arbor, MI, USA). Protein samples were purchased from Sigma (St. Louis, MO, USA).

## 3. Results and discussion

### 3.1. Effect of column diameter on ES–MS signal

In order to demonstrate the sensitivity enhancement available from the packed capillary column, a 500-fmol sample of horse heart myoglobin was injected onto three LC columns having internal diameters of 2.1, 1.0 and 0.10 mm. Each column was operated at its optimum flow-rate, being 200, 50 and 0.40 μl/min, respectively. These experiments were performed in triplicate, and the resulting total ion chromatograms (TIC)s from the ES–TOF-MS instrument are combined into a single composite TIC, as shown in Fig. 2. For these and all remaining experiments the TOF-MS acquired data from 500 to 2000 $m/z$ at a rate of 1 spectra/s, the sum of 8192 individual mass spectra. A linear gradient of 30–90% ACN in water with 0.1% TFA over 30 min was used in each case. The results show that signal in the TIC can be greatly increased through the use of smaller-bore LC columns and is in agreement with theory which states that the concentration sensitivity of LC columns of varying diameters is equal to the inverse of the square of the two column radii:

$$\frac{\text{sensitivity column 2}}{\text{sensitivity column 1}} = \frac{(\text{column 1 radius})^2}{(\text{column 2 radius})^2}$$

assuming the length, efficiency, porosity and particle size to be identical in each column. By measuring the signal-to-noise ($S/N$) ratio of the peaks in Fig. 2, an increase of 428-fold in sensitivity is calculated upon decreasing the column I.D. from 2.1 to 0.10 mm. From the column diameters and the relation above, a theoretical value of 441 is expected. This is a reasonable result since, in the work described here, the assumption that the remaining physical properties of the columns and packing material are identical is only partially valid. Also, the assumption that the signal response of the ES–MS system is equal across the flow-rate range used is only partially valid.

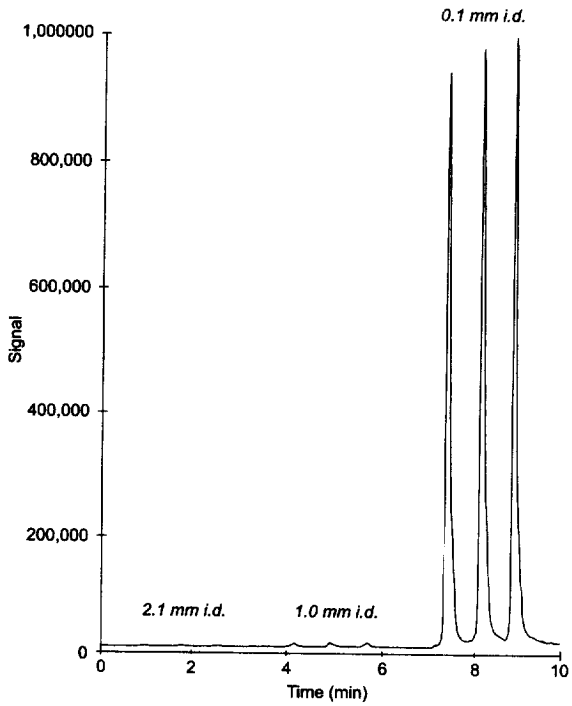


Fig. 2. Comparison of TIC signal from the ES–TOF-MS machine using LC columns of different diameters. Flow-rate: 2.1-mm I.D. column, 200 μl/min; 1.0-mm I.D. column, 50 μl/min; and 0.10-mm I.D. column, 0.400 μl/min. Gradient: 30–90% ACN, 0.1% TFA, in 30 min; MS integration rate, 1 spectra/s; mass range, 500–2000 *m/z*.

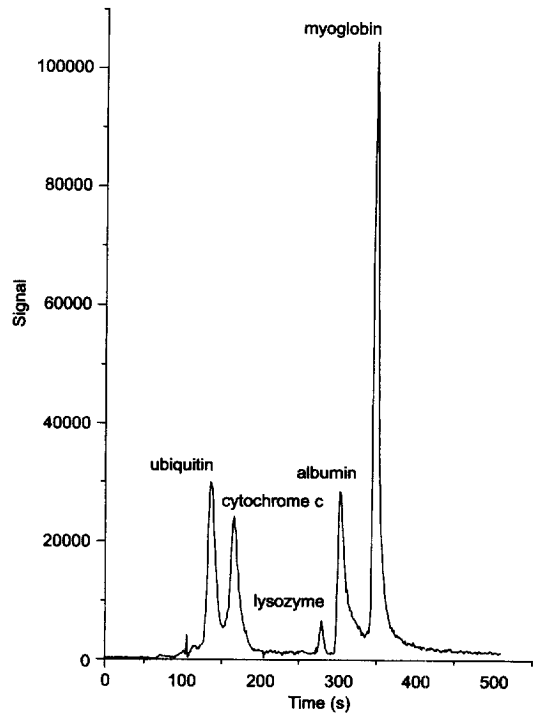


Fig. 3. TIC from the separation of a standard mixture of proteins (25 fmol each) on a 0.10-mm I.D. LC column. Flow-rate, 400 nl/min. Remaining conditions same as Fig. 2.

### *3.2. Separation of proteins using a 0.10-mm I.D. column*

To demonstrate the performance of packed capillary LC coupled with ES–TOF-MS, a mixture of standard proteins containing 25 fmol per component was separated using the 0.1-mm I.D. column. The volumetric flow-rate delivered ultimately to the column was 400 nl/min, and it is useful to note that the electrospray gas nebulizer functioned adequately at this low flow-rate. Fig. 3 shows the TIC acquired for this separation. The peaks appearing in the TIC range in width from 5 to 12 s, and so an integration time of 1 spectra/s was used with the TOF-MS. Although the mass spectra can be recorded at rates of up to 100/s using the TOF-MS, the chromatographic width of these peaks do not justify such a rapid rate of collection. It is important to remember that the TOF is generating mass spectra at a rate of over 8 kHz, regardless of the 'integration time' chosen. This integration time is an arbitrary figure and represents only the time over which these continual mass spectra are summed to create a final mass spectrum. However, it is also immediately obvious that shorter integration times will result in lower signal since ions will be summed for shorter time periods. Fig. 4 shows the averaged mass spectra obtained by summing the individual mass spectra recorded underneath each LC peak. Even with these relatively small quantities, the TIC shows impressive signal strength, as do the mass spectra in Fig. 4. The myoglobin data can be easily deconvoluted [34] to give the protein molecular mass as shown in Fig. 5.

### *3.3. Sensitivity*

The sensitivity and linear dynamic range of this system for the analysis of a single protein was explored. Again, the 0.1-mm I.D. column was used for maximum sensitivity and the TOF-MS integra-

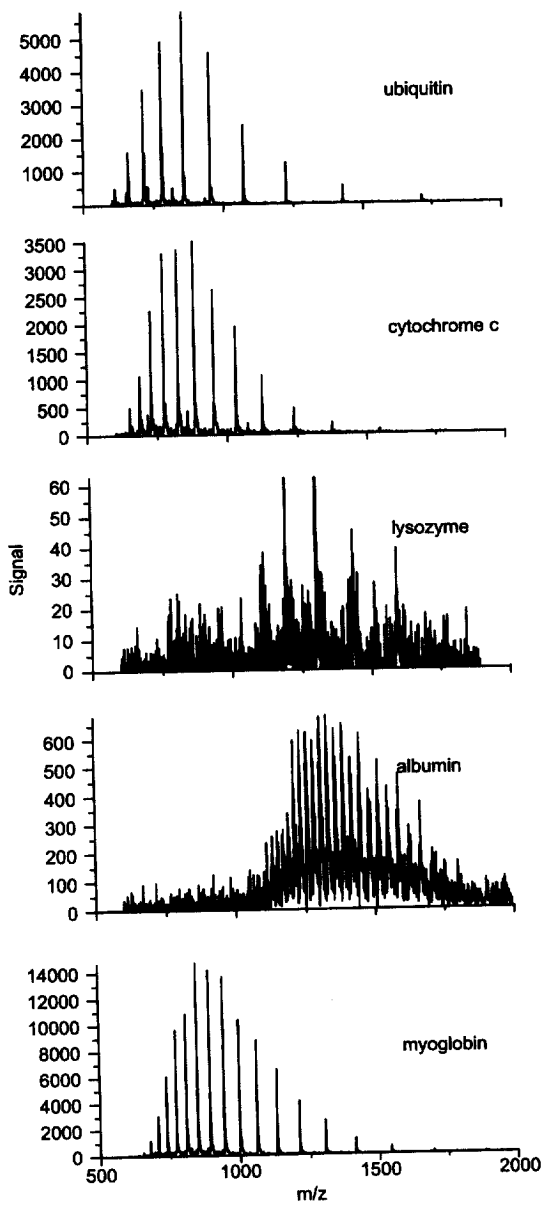


Fig. 4. Mass spectra of proteins, averaged from underneath each peak in Fig. 3.

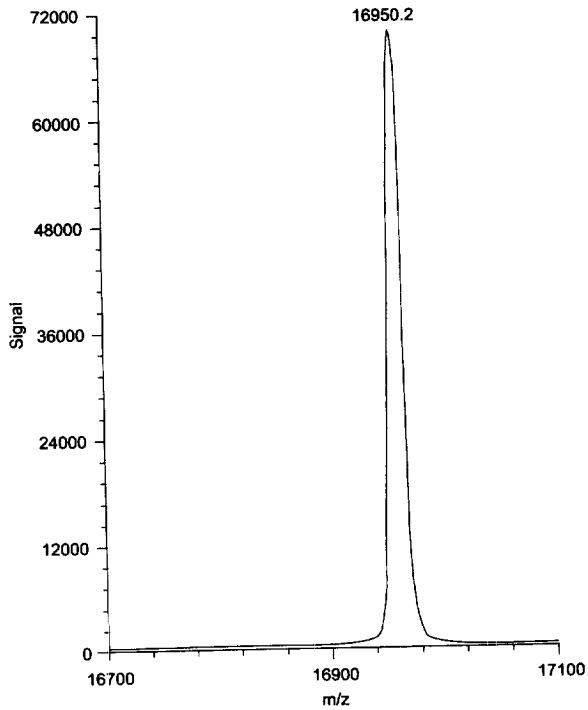


Fig. 5. Protein molecular mass obtained from the deconvolution of mass spectrum for myoglobin in Fig. 4.

tion time was 1 spectra/s. Fig. 6 shows a plot of the observed TIC peak height against the amount of cytochrome c injected. The smallest sample quantity which could be reliably detected was 2.5 fmol. The TIC for this injection is shown in Fig. 7, and an average of mass spectra acquired underneath the peak is shown in Fig. 8. Even with the small amount of material present, the multiply-charged spectra of cytochrome c is discernible. At sample levels much greater than 1000 fmol, peak shape is compromised due to the loadability limit of the packed capillary column. Thus, linearity of instrument response with the overall system is approximately 3–3.5 orders of magnitude.

The increased sensitivity observed in these experiments is due to the combined effects of a reduced LC column diameter and the use of a TOF mass spectrometer. In previous work [30] using a 1.0-mm I.D. column and quadrupole MS, a sensitivity limit of 4 pmol for the same protein was determined. From the reduction in column diameter alone, an increase in sensitivity of 100-fold, and thus a detection limit of 40 fmol is expected. The difference between this expected limit and the observed limit of 2.5 fmol may then be attributed largely to the MS. Since the TOF is summing the signal from all ions continuously as they elute from the LC column outlet and not 'scanning' the $m/z$ range by methodically

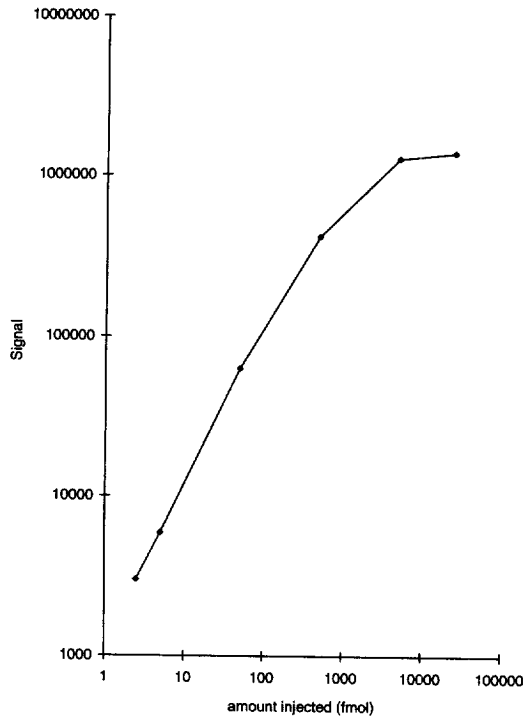


Fig. 6. TIC signal vs. sample amount for cytochrome c injections. Remaining conditions same as Fig. 3.

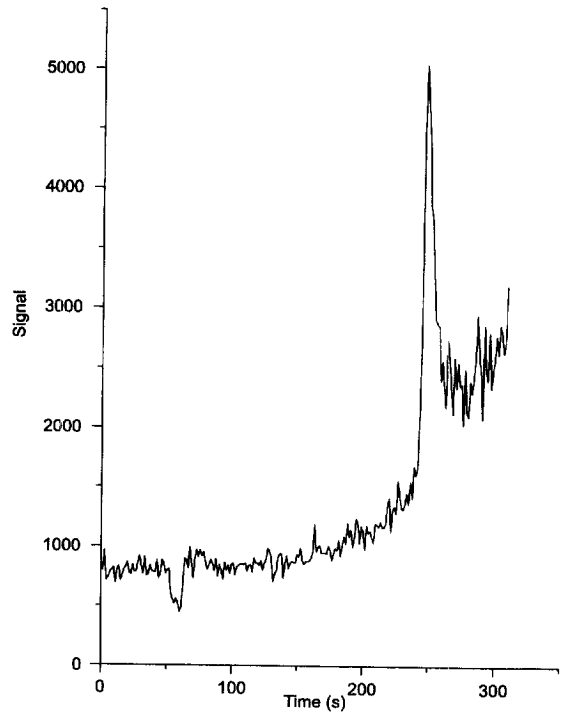


Fig. 7. TIC from the injection of 2.5 fmol of cytochrome c on a 0.10-mm I.D. LC column. Remaining conditions same as Fig. 3.

stabilizing the trajectory of selected ions, the duty cycle for ions achievable with the TOF mass analyzer is considerably higher than that of the quadrupole. Dispersive types of mass spectrometers such as quadrupoles have low duty cycles and are in fact discarding the majority of useful ions at any given time, thus spending relatively little time analyzing the ions of interest.

## 4. Conclusions

The interfacing of capillary LC and electrospray ionization with time-of-flight mass spectrometry detection can provide a very sensitive analysis method for the separation and identification of proteins. Capillary LC offers greatly enhanced sensitivity due to the reduction of solute dilution in a decreased mobile-phase volume. This effect is especially useful for electrospray ionization which seems to function as a concentration-sensitive detector throughout a large span of the liquid flow-rate range. By reducing the scale of the analytical separation, and thus its flow-rate, substantial increases in sensitivity can be obtained. In truth, the ES–MS sensitivity enhancement is the result of increasing the concentration of the ions present in each droplet which is sampled by the MS.

The sensitivity can be further enhanced through the use of a TOF mass analyzer which inherently samples the ions created in the ES source more efficiently than other machines. Ultimately, the combination of these techniques can result in the ability to separate, detect and identify very low fmol amounts of proteins.

## Acknowledgements

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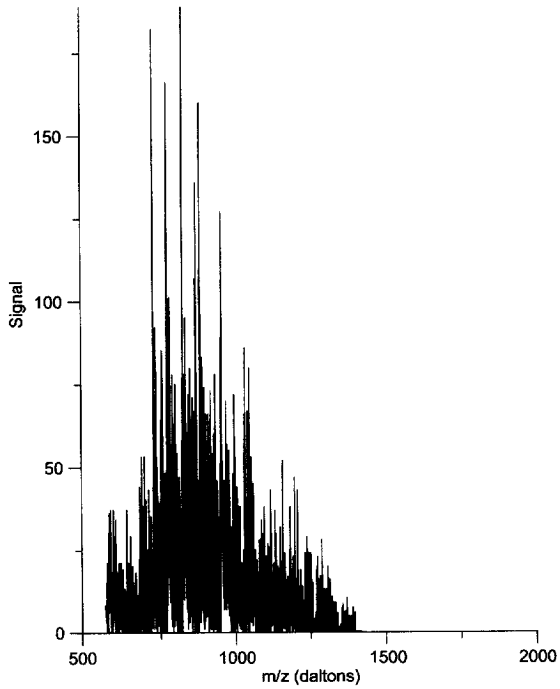


Fig. 8. Mass spectrum from the average of data underneath the cytochrome c peak in Fig. 7.

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