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Automatic Identification of Proteins with a MALDI-Quadrupole Ion Trap Mass Spectrometer

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A matrix-assisted laser desorption/ionization (MALDI) ion trap mass spectrometer of new design is described. The instrument is based on a commercial Finnegan LCQ ion trap mass spectrometer to which we have added a MALDI ion source that incorporates a sample stage constructed from a compact disk and a new ion transmission interface. The ion interface contains a quadrupole ion guide installed between the skimmer and the octapoles of the original instrument configuration, allowing for operation in both MALDI and electrospray ionization modes. The instrument has femtomole sensitivity for peptides and is capable of collecting a large number of MALDI MS and MALDI MS/MS spectra within a short period of time. The MALDI source produces reproducible signals for 10^4–10^5 laser pulses, enabling us to collect MS/MS spectra from all the discernible singly charged ions detected in a MS peptide map. We describe the different modes of the instrument operation and algorithms for data processing as applied to challenging protein identification problems.

The growing interest in large-scale proteome research has created the need for improved methods for the rapid, automatic identification of proteins. A mass spectrometry-based method can satisfy this need if it (1) produces high-quality MS and MS/MS data; (2) has femtomole sensitivity; (3) is fast, i.e., collects spectra in seconds; (4) is capable of obtaining MS/MS spectra of every peptide in a complex proteolytic mixture; and (5) is robust. In this list, properties 1, 2, and 4 are necessary for solving the kinds of proteomic problems that are becoming standard in biological research; property 3 is needed if this analysis is to be high throughput; and property 5 is required for the technology to become widespread. In addition, if the technology is to become generally available to biochemists and biologist, it should not be excessively expensive.

To our knowledge, none of the superb instruments developed to date has yet achieved all of these properties simultaneously. For example, our prototype matrix-assisted laser desorption/ionization (MALDI)-QqTOF mass spectrometer,[1] while genuinely robust and capable of producing very high quality data, often requires more than 60 s to accumulate sufficient statistics to produce a usable MS or MS/MS spectrum. To obtain sufficient ion counts with the MALDI-QqTOF instrument, it also proves necessary to irradiate the sample with relatively high energy–density laser pulses—the practical result is that is often not possible to obtain more than 5−10 MS/MS spectra from a single sample before it is completely used up. In addition, the relatively high cost of the current commercial version of the instrument may limit its availability to biologists. Although the nanospray-QqTOF MS combination has been shown to produce data of impressive quality,[2] it may take up to 0.5 h to collect MS and MS/MS spectra from a single proteolytic digest and good results often depend on a highly skilled operator. An alternative strategy for the combined MS and MS/MS analysis of complex proteolytic peptide mixtures involves on-line LC/MS using data-dependent mass analysis.[3] One highly successful method utilizes microcapillary LC/electrospray ionization (ESI)-ion trap MS. This combination satisfies several of the properties mentioned above (including relatively low cost). However, as normally performed, it is not possible to obtain MS/MS spectra of all the eluted peptides—i.e., only the most intense peaks are usually measured. To address this problem, an innovative scheme has been developed wherein the HPLC flow can be interrupted in a data-dependent manner to allow sufficient lag for collection of the spectra from multiple peptides eluting at a given time.[4,5] Although effective in the developers' laboratories, this approach has not yet been widely adopted presumably because of the high degree of skill required. A similar approach has been used in the development of high-performance, high-sensitivity nano-HPLC micro-ESI Fourier transform ion cyclotron resonance mass spectrometry.[6] Despite its extraordinary capabilities, FTICR mass spectrometers are expensive and can be nontrivial to maintain. A final alternative for consideration is a new generation of MALDI-TOF-TOF instrument being developed at PerSeptive Biosystems.[7] Proper evaluation of this promising (but likely expensive) instrument awaits further published experimental data.

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Although numerous groups have coupled MALDI ion sources with ion traps,[8] these have generally not been commercialized and are thus not yet widely used. A few years ago, our laboratory also constructed a MALDI-ion trap mass spectrometer, based on a modified Finnigan ITMS electronics kit.[9] Although the software controlling this instrument was primitive and did not allow for data-dependent control of experiments, the instrument was found to be superb for solving biological problems.[10] Encouraged by these results, we have recently modified a commercial Finnigan LCQ instrument for use with an in-house constructed combined MALDI/ESI ion source.

To couple the MALDI ion source to the ion trap mass spectrometer, we used elements of a technology that implements high-pressure multipole ion guides.[11] Such multipole ion guides have previously been used to good advantage for gas dynamic interface coupling of ion sources to mass analyzers (see references in ref 12). Their ability to capture ions with large velocity distributions (as in MALDI-produced ions[13]) and large spatial distributions at the input of the ion guide and to collimate ions through a combination of collisional cooling and rf fields has been described.[12] Ion guides operating in this mode have been used to couple continuous ion sources (e.g., ESI) to different types of mass analyzers, yielding instruments with improved performance.[11,12,14,15] More recently, the pulsed MALDI ion source has also been efficiently coupled to the orthogonal injection TOF mass analyzer through a quadrupole ion guide.[16] Such coupling of the MALDI ion source to tandem QqTOF mass analyzers[1,17−19] and an FTICR mass spectrometer[20] has added substantially to the analytical capabilities of these instruments and opens the possibility of the interchangeable use of MALDI or ESI ion sources on the same QqTOF mass analyzer.[1,14]

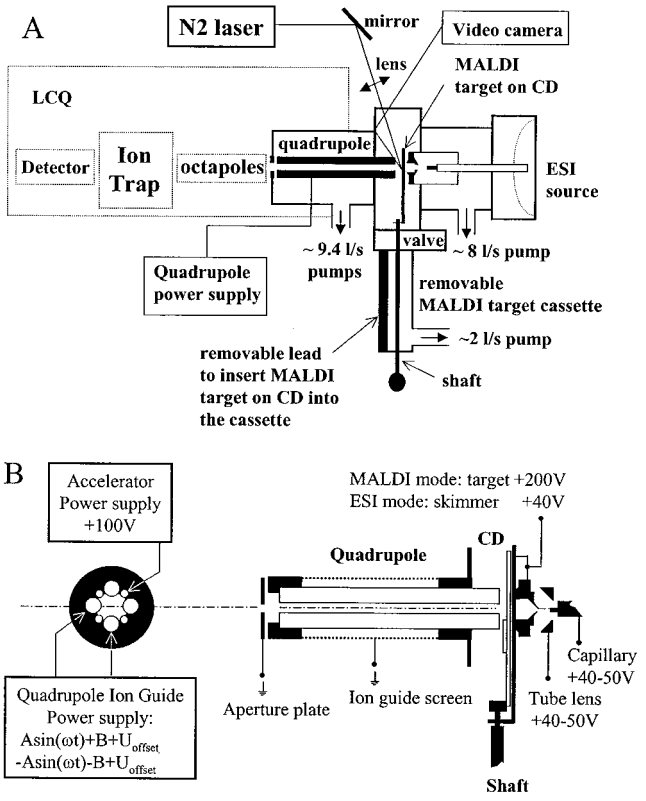

Figure 1. (A) Schematic diagram of the instrument showing modifications to the ion trap mass spectrometer. (B) Detailed diagram of the quadrupole ion guide construction indicating the voltages applied to the different components.

In the current development, we have added a MALDI ion source and a new interface to a commercial ion trap (LCQ Classic, Finnigan Thermoquest) that was originally designed by the company to operate exclusively in ESI mode. Here we took an approach similar to the one that we used to successfully couple a rapidly switchable MALDI/ESI source to our prototype Sciex Centaur QqTOF instrument.[1] However, in the present MALDI-ion trap design we have added some new features. These include a new MALDI target design that utilizes the polycarbonate surface of a compact disk (CD) and a quadrupole ion guide with improved properties (see Experimental Section). The modified instrument has femtomole sensitivity for peptides and MS/MS capabilities that make it capable of performing a large number of MS/MS experiments within a short period of time. Here we describe the design and performance of the instrument and discuss the different modes of its operation and algorithms for data processing.

EXPERIMENTAL SECTION

New Ion Interface. Figure 1A is a schematic diagram of the instrument showing the modifications that we have made. We have added a new vacuum chamber that admits both the MALDI target and the original ESI ion source (heated capillary−skimmer assembly). This new portion is shown with the solid lines in Figure 1A. The added vacuum chamber contains a quadrupole (~20 cm long, 0.635 cm rod diameter) installed between the octapoles and the skimmer of the original configuration. The quadrupole is separated from the octapoles by an aperture plate (aperture diameter ~0.3 cm). Figure 1B provides a detailed view of the

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quadrupole construction and the voltages applied to the different components. The additional quadrupole acts as a high-pressure ion guide.[11,12] The quadrupole is driven by an independent rf power supply, which consists of a 500-kHz crystal oscillator-controlled sine wave generator and a power amplifier (model 240L ENI, Rochester, NY). In addition to the rf voltage (typical value of the sine wave amplitude $A = +(300-500)$ V), we can apply dc potentials in order to operate the quadrupole ion guide as a mass filter ($B = \pm(0-50)$ V) and to provide the necessary quadrupole offset voltage ($U_{offset} = +(15-25)$ V). More details on the circuitry can be found elsewhere.[1,12]

The ion guide assembly also contains an accelerator, which provides an electrostatic force to drag the ions toward the exit of the ion guide. The accelerator consists of a set of four ~18-cm-long rods 0.32 cm in diameter inserted between the main rods of a quadrupole ion guide (see the assembly cross section in Figure 1B). The construction is assembled such that the accelerator rods are closer to the axis of the quadrupole ion guide at its entrance and further from the axis at the ion guide output. A constant voltage (typically +100 V) applied to all four rods of the accelerator creates a small electrical field along the axis of the quadrupole ion guide because of the changing proximity of these rods to the axis of the ion guide. The described construction is a simplified version of the linear accelerator LINAC II recently implemented in the QqTOF instrument.[21] Its presence in our construction improves the transport efficiency of ions, especially in the ESI mode of operation.

Vacuum System. We have added three additional rotary pumps to the original vacuum system of the instrument. Two rotary pumps (Edwards 12, 4.7 L/s) connected in parallel maintain the pressure in the quadrupole ion guide region at 60−70 mTorr. This pressure, determined by the rate of gas flow through the capillary and the skimmer (which remain open all the time) and the resulting pumping speed of all pumps, was found to be optimal for both ESI and MALDI modes of the instrument operation. A small rotary pump (Edwards 5, ~2 L/s) is connected to the MALDI target cassette to pump out the vacuum lock prior to introduction of the MALDI target into the quadrupole ion guide region. The skimmer-heated capillary assembly of the original ESI source is evacuated to a pressure of ~1 Torr by a rotary pump (Pfeifer UNO 030B, ~8 L/s) that is an original component of the commercial instrument.

ESI Mode. When operating in the ESI mode, the MALDI target is retracted (Figure 1A) and ions produced in the ESI source are introduced into the instrument through the standard heated capillary and then through the original skimmer into the quadrupole ion guide. The distance separating the skimmer and the quadrupole ion guide is 5 mm, just sufficient to admit the CD MALDI target (see below). The offset voltages on the skimmer, the quadrupole, and the electrode that separates the ion guide from the original octapole are shown in Figure 1B. The remaining operating conditions are similar to those used before modification.

MALDI Mode. When operating in the MALDI mode, the MALDI target is inserted between the skimmer and the quadrupole ion guide (Figure 1A) and ions produced on the surface of the MALDI target are introduced into the quadrupole ion guide. All the MALDI-mode MS/MS spectra reported here were obtained from singly charged ions.

MALDI Target on a CD. MALDI samples are deposited on the surface of a CD, which serves as a MALDI target. We prepare such targets from standard blank CDs (74 min 650 mb, Silver/Blue, 1−12× certified CD-R, Cyanine Blue dye). First, we remove the metal layer that covers the CD on one side by making a small scratch in the metal layer and lift it off with sticky tape. We wash the freshly exposed layer of dye with methanol and then water and glue a paper label to this cleaned side. Labels are designed using the FreeHand8 or LabVIEW computer programs and printed with up to 1000 labeled positions patterned on circles or a spiral. Samples are deposited along the labeled positions on the opposite side, i.e., on the CD polycarbonate surface. The labels can be easily read because the CD is transparent.

MALDI CD Inlet System. We have added a sample inlet system, which allows rapid (1−2 min) introduction of the CD MALDI target through a vacuum lock. The CD with samples spotted on its surface samples is fixed with a screw to a metal CD support plate and introduced into the mass spectrometer. A MALDI target potential is applied to the plate when it makes a physical contact with the skimmer (see Figure 1B). The distance between the CD and the entrance of the ion guide is ~1 mm. Each sample on the CD is positioned at the entrance of the quadrupole ion guide by rotating and translating a shaft attached to the CD supporting plate. Rotation of the shaft is transmitted to the CD through a small rubber wheel at the end of the shaft.

Laser System. The 337 nm wavelength laser beam (VSL-337 nitrogen laser, Laser Science Inc., MA) operating at a repetition rate of 10−20 Hz is reflected by a mirror and introduced through a collimating lens ($f = 10$ cm) and then through a sapphire window to the surface of the CD at an angle of incidence of ~60°. The diameter of the laser spot on the sample surface is 0.3−0.5 mm. The power density of laser radiation in the spot is $(2-5) \times 10^7$ W/cm^2, which is somewhat less than the value that we usually use in our MALDI QqTOF system.[1]

Both the sample and the laser spot are monitored by a video camera. Desorbed ions are introduced directly into the quadrupole ion guide. The offset voltages were identical to those used for ESI mode (Figure 1B), except that in MALDI mode the potential is applied to the skimmer, which now makes contact with the CD supporting plate. We found empirically that a potential of +200 V is optimum for recording MALDI spectra.

Computer Programs. All of the modifications that we made to the ion trap mass spectrometer preserve the ability to control the instrument by the original Finnigan software designed to support spectral acquisition. However, these programs (LCQ Tune Plus and Xcalibur (version 1.2, Finnigan)) were written to support the LC-ESI mode of operation. To perform automatic experiments in the MALDI mode, we adapted some of the options provided in this commercial software and supplemented these with custom programs assembled using the LabVIEW graphical programming language (version 6, National Instruments, Austin, TX).

The methods that we used to conduct MALDI-ITMS protein identification experiments are illustrated in Figure 2. The figure depicts the major steps in the experiments and the computer programs that control the manipulation and the flow of data from

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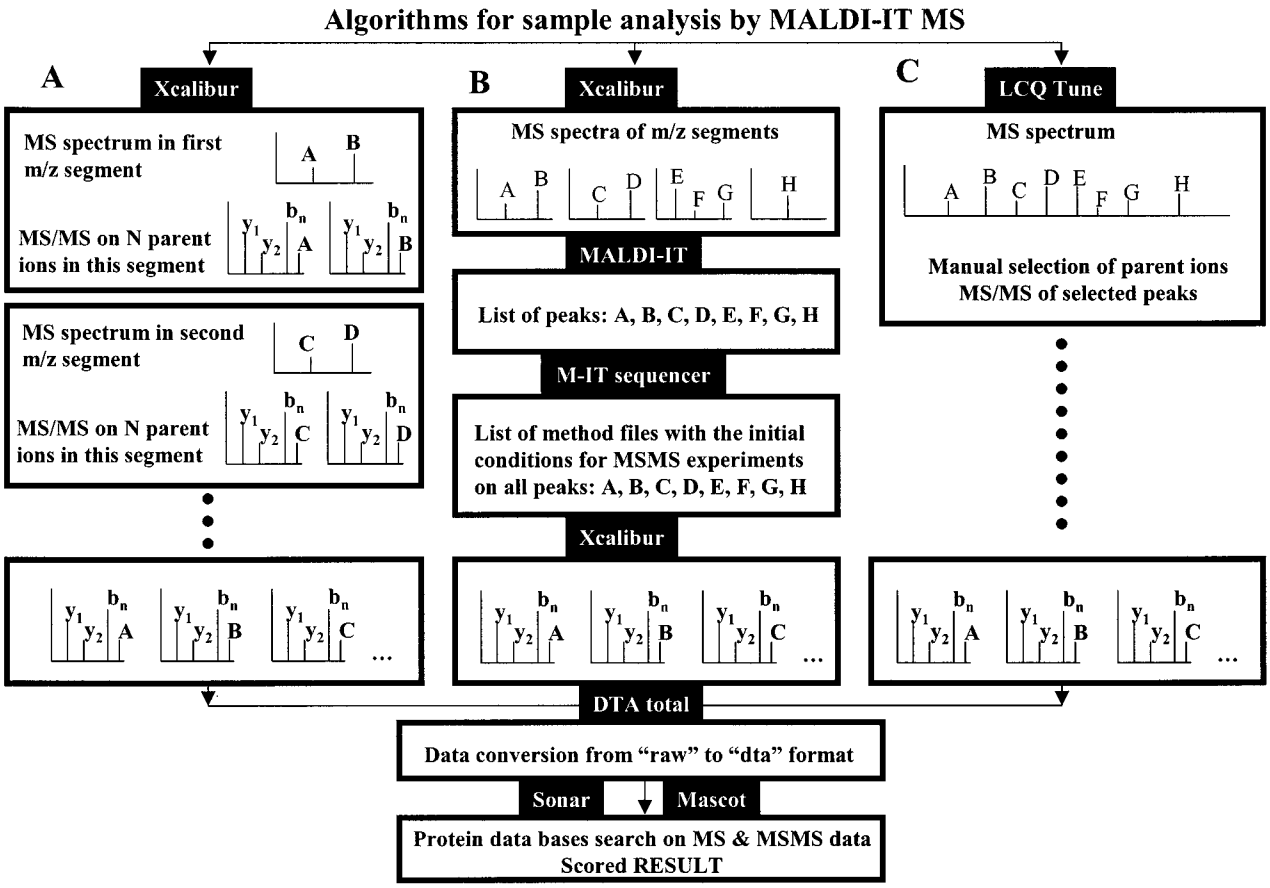

Figure 2. Algorithms for protein identification experiments performed in the MALDI-IT mass spectrometer. (A) Algorithm for automatic collection of MS/MS spectra of the *N* most abundant peaks found in a particular *m*/*z* segment (supported by the existing Xcalibur software that controls the Thermo-Finnigan ion trap mass spectrometer.) (B) Algorithm for automatic collection of MS/MS spectra on all detected peaks (supported by the existing Xcalibur software and several in-house written programs.) (C) Manual acquisition of spectra using the existing Thermo-Finnigan Xcalibur software.

one step to another. For example, Figure 2A illustrates the analysis of a sample using the commercial Finnigan Xcalibur program. One of the options of this program allows us to create a method file, which controls the collection of the MALDI MS spectrum in a short m/z segment (typically 200−400 m/z). The mass spectrum from the first segment is collected, whereupon the computer program automatically selects the N most intense peaks from this segment to perform automatic MALDI MS/MS measurements. After these MS/MS spectra are collected, the MS spectrum in the next short m/z segment is obtained and the whole procedure is repeated.

A second approach (Figure 2B) allows us to obtain MALDI MS/MS spectra of all peaks detected in the MALDI MS spectrum. After the entire MS tryptic map of a sample is obtained by collection of the spectra in small segments, the custom program MALDI-IT processes the data. This program labels ion peaks in the spectra using a peak selection algorithm. It can also select either a given number of the most intense ion peaks in the spectrum or any number of peaks with a signal-to-noise ratio above a given value. Thus, it produces a list of m/z-values of the selected peaks and stores this list in text format for generating the method files for the Xcalibur software. These method files are automatically generated by M-IT Sequencer, a custom-designed LabVIEW program that uses Macro Magic (Iolo Technologies, LCC, Pasadena, CA) and the peak lists to control the "instrument setup" program of the original Xcalibur software package. Besides a list of parent masses, the method files contain information about the m/z selection window, excitation energy, q value, tune parameter files, and m/z range of the fragment spectrum to be observed. Without any user interaction, M-IT Sequencer efficiently assembles the method files into ready-to-go sequences that are subsequently executed by the preexisting Xcalibur software.

Alternatively, in some special cases, manual collection and manipulation of the MS and MS/MS spectra may still be useful and can be performed with the commercial LCQ Tune program (Figure 5C).

The resulting MALDI MS/MS spectra collected from a sample by any of the discussed means are processed with the LabVIEW program "DTA total". This program functions as a graphical interface for the command line-operated "lcqdta" program written by John Yates. It converts the original MS/MS spectra into text files in dta format and concatenates all the MS/MS data obtained from a given sample. The resulting dta files are readily readable by several protein identification engines. In the present work, we used the search engines Sonar (Proteometrics, LLC, New York) and Mascot (Matrix Science Ltd., London), both of which use unintepreted MS/MS data for identifying proteins.

Ion Trap Instrumental Settings. All spectra were obtained at the following settings of the instrument (using standard Finnigan notation): ion injection time 500 ms ("maximum inject

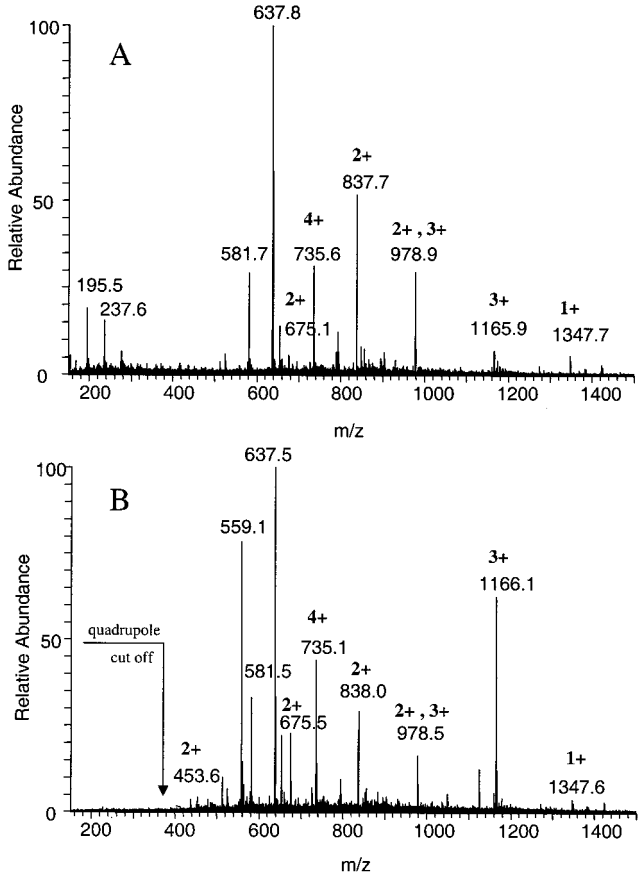

Figure 3. Electrospray spectra of a mixture of six peptides at equimolar concentration (100 fmol/μL) in water/MeOH/acetic acid (35/60/5 v/v) sprayed at a rate 1 μL/min obtained (A) with the original ion interface and (B) after installing the new ESI/MALDI interface. The observed multiply charged ions of bradykinin fragment 2−9 (monoisotopic mass 903.5 Da), at *m*/*z* 453.6 (2+), substance P (1346.73 Da) at *m*/*z* 675 (2+) and *m*/*z* 1347 (1+), neurotensin (1671.9 Da) at *m*/*z* 837.7 (2+), amyloid β-protein fragment 12−28 (1954.0 Da) at *m*/*z* 978.9 (2+), ACTH fragment 1−24 (2931.6 Da) at *m*/*z* 735.6 (4+) and 978.9 (3+), and insulin chain B, oxidized, from bovine insulin (3493.6 Da) at *m*/*z* 1166 (3+) are indicated in the figure. Acquisition time was 2 s for each spectrum.

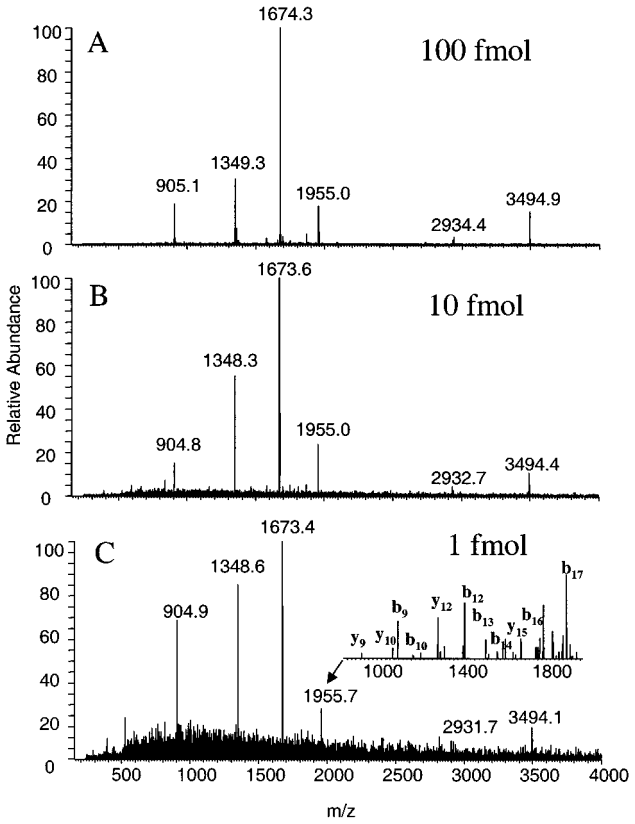

Figure 4. MALDI mass spectra of the same six peptide mixture described in the legend to Figure 3. The amount of sample applied to the CD target is indicated. Acquisition time was 2 s for each spectrum. The inset in panel C shows the MS/MS spectrum of 1 fmol of amyloid β-protein fragment 12−28 peptide with the sequence VHHQKLVFFAEDVGSNK and molecular mass 1954.0 Da. The MS/MS spectrum acquisition time was 2 s.

time"), automatic gain control (AGC) on, and maximum number of ions allowed to fill the trap 5×10^9 ("full MS target"). In addition, MS/MS spectra were obtained with the following settings: *m*/*z* window 3−4 ("isolation width"), excitation energy 23−25% ("Normalized collision energy"), *q* of excitation 0.25 ("activation *q*"), and excitation time 300 ms ("activation time"). We used 6 microscans for the collection of MS spectra and 12 microscans for the MS/MS spectra.

Sample Preparation. (1) Peptide Mixture. A stock mixture of bradykinin fragment 2−9, (monoisotopic mass 903.5 Da), Substance P (1346.73 Da), neurotensin (1671.9 Da), amyloid β-protein fragment 12−28 (1954.0 Da), ACTH fragment 1−24 (2931.6 Da), and insulin chain B, oxidized, from bovine insulin (3493.6 Da) was prepared at a concentration 200 fmol/μL per component in water/methanol/acetic acid (35/60/5 v/v/v).

(2) Yeast Protein Complexes. The immunopurified YJL041w: protein-A fusion protein in complex with its associated yeast proteins was kindly provided by Drs. Julia Kipper and Michael P. Rout (Rockefeller University, New York). Approximately 80% of the material (∼2 pmol) was buffered in 50 mM ammonium bicarbonate and further purified by ultrafiltration using a Microcon 3 device (Millipore) in a refrigerated centrifuge (Eppendorf 5417R) at 4 °C. The remaining 20% of the protein material was separated by SDS−PAGE and visualized by silver staining. On the ultrafiltration membrane, the proteins were reduced (10 mM tris(2-carboxyethyl)phosphine (Pierce), 20 min at 70 °C), alkylated (50 mM iodoacetamide, 30 min at room temperature), and enzymatically digested (sequencing grade modified trypsin (Roche), 4 h at 37 °C). Cleanups by ultrafiltration were performed between the steps. A 10% aliquot of the resulting solution of digest peptides was removed for reversed-phase purification and MALDI-IT MS analysis. The complexity of the peptide mixture was reduced by a two-step elution using respectively 20 and 60% organic solvents (methanol/acetonitrile 2:1 v/v) with 0.1% TFA. The resulting two samples were deposited on the CD target using DHB as matrix (see below).

(3) In-Gel Protein Digestion. Protein in-gel digestion and extraction from gel bands were performed essentially as described in ref 1. The only modification to the described in-gel digestion protocol was made at the peptide extraction step, where we terminated the digestion by adding 25−40 μL of 7% formic acid and then added 100−300 nL (dry volume) of Poros 20 or Poros 50 (PerSeptive Biosystems, MA) reversed-phase (RP) beads directly to the solution containing the gel pieces. The peptides

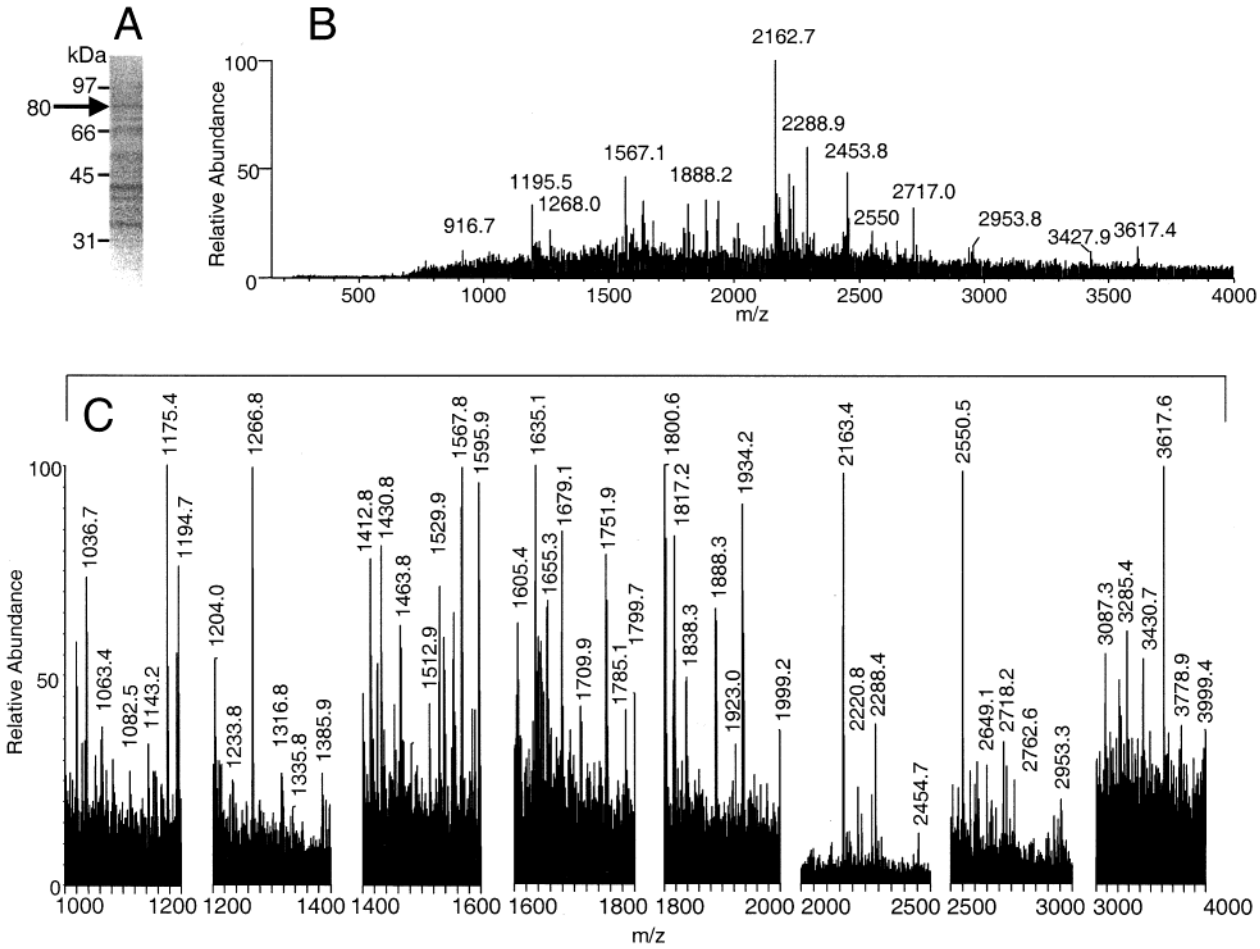

Figure 5. (A) Zn-stained 1D SDS−PAGE gel of separated human proteins. (B) MALDI mass spectrum of tryptic peptides extracted from the 80-kDa gel band. (C) MALDI mass spectrum of the same sample sequentially obtained in automatic mode in eight *m*/*z* segments. Because of space limitations, we label only 49 out of the total of 91 peaks that were subjected to MS/MS analysis.

were extracted in the presence of the RP beads for 3−12 h while constantly shaking. The solution containing the RP beads was aspirated into either an Eppendorf Geloader tip pinched at its tip or a ZipTip (which was used essentially as a frit). The column formed at the pipet tip was washed with 5−10 μL of water/0.1%TFA and the peptides were directly eluted to the MALDI probe with a DHB matrix solution saturated in 60%MeOH/5%acetic acid. Addition of RP beads to the extraction solution was found to improve the recovery of peptides.

RESULTS AND DISCUSSION

Performance in ESI Mode. We compared the performance of the instrument operating in ESI mode prior to and after installation of the quadrupole ion guide using a mixture of six peptides at equimolar concentration (100 fmol/μL) in MeOH/H_2O/acetic acid (60/35/5 v/v/v), electrosprayed at a rate 1 μL/min (Figure 3). Each spectrum was acquired in ~2 s. All the components of the six-peptide mixture were observed in the spectra. The signal intensity and the appearance of the spectra were similar in the new versus old configurations, indicating that the modification of the ion transport interface did not result in significant changes in performance of the ESI mode of operation.

Improvements in the detected signal due to the "accelerator" in the quadrupole were especially noticeable for low-concentration samples. For example, when we applied ~100 V to the accelerator, we were able to reproducibly obtain ESI signals from 10 fmol/μL of the six-peptide mixture in the same ~2-s acquisition period.

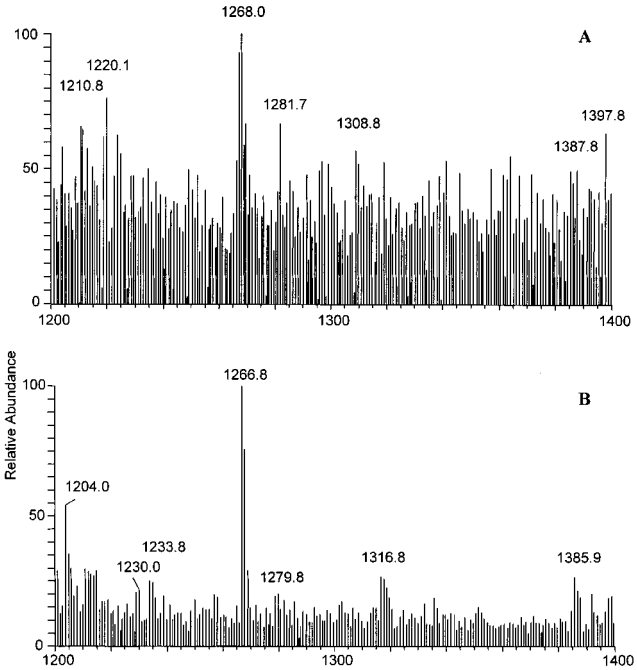

Figure 6. Comparison of a portion (between *m*/*z* 1200 and 1400) of (A) the full-scan MALDI mass spectrum (from Figure 5B) with (B) the corresponding segmented spectrum (from Figure 5C).

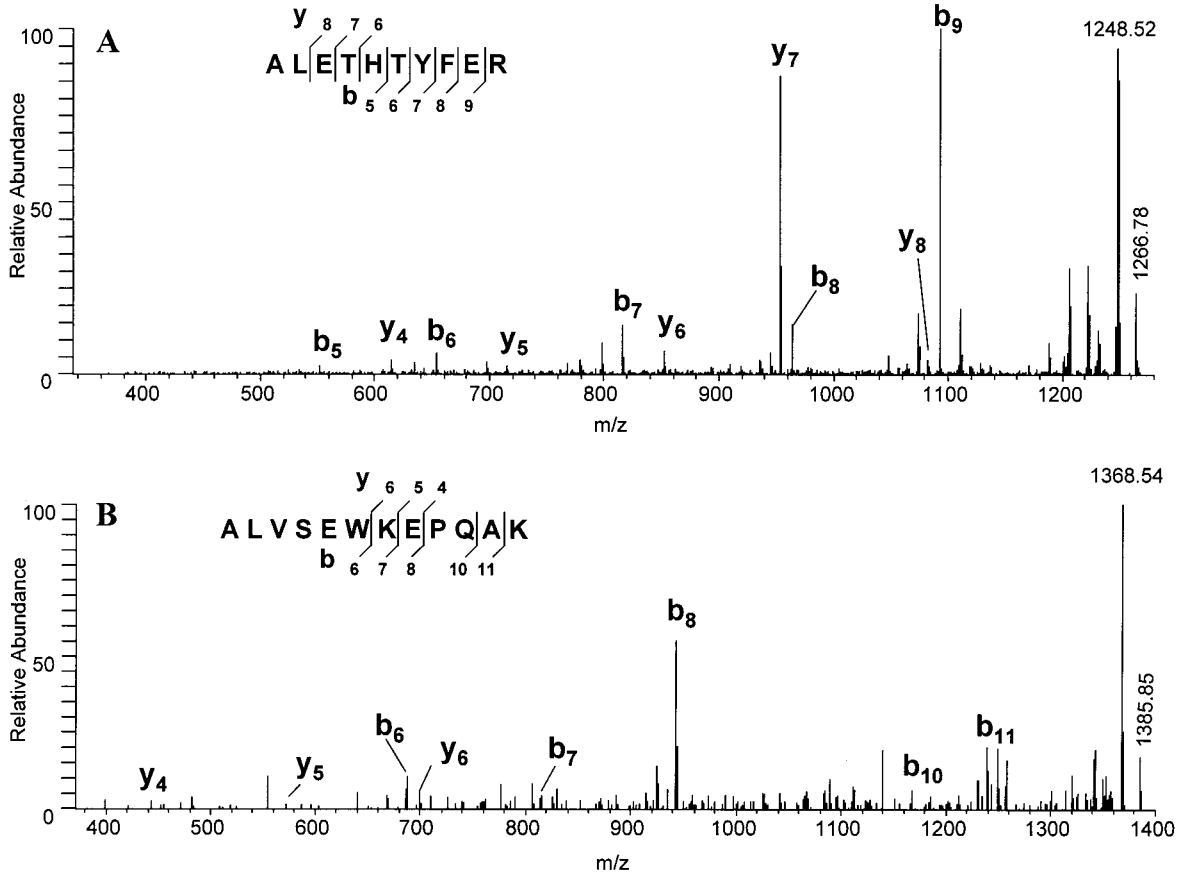

Figure 7. Example of 2 from a total of 91 MS/MS spectra of the tryptic peptides extracted from the 80-kDa gel band. The parent *m*/*z* values were (A) 1266.8 and (B) 1385.9. The sequence of the identified peptides and their fragments are indicated.

The amplitude of the rf voltage on the quadrupoles determines a low-*m*/*z* cutoff for the transmission of ions through the guide. Although the cutoff in the spectrum shown in Figure 3B occurs at *m*/*z* ~400, it can be varied in the *m*/*z* range 300−1500 by appropriate adjustment of the rf voltage amplitude. This cutoff is of considerable utility for rejecting the often-present low-*m*/*z* background. The high-pressure quadrupole guide can also be operated as a mass filter[12] by supplying small positive and negative dc voltages to opposite pairs of quadrupole rods. A transmission window of 50 *m*/*z* units was readily achieved without incurring losses in the intensity of the selected peak (data not shown). The implications of preselection of ions injected into the ion trap for sensitivity improvement are currently being investigated.

Performance in MALDI Mode. Figure 4 shows MALDI spectra of the same six-peptide mixture used to obtain the ESI spectra. The MALDI spectra reproducibly exhibited singly charged ion signals from all six components of the peptide mixture even when only 1 fmol of each peptide was applied to the CD. All spectra were acquired in ~2 s.

We have found that MALDI signals from samples desorbed from the polycarbonate surface of a CD have higher intensities and better signal-to-noise ratios than those obtained from a metal substrate. For reasons that we do not fully understand, the influence of the target potential (+200 V on the metallic back plate supporting the CD) on the overall MALDI signal from the polycarbonate surface is less dramatic than that observed from a metal surface.

The inset in the bottom panel of Figure 4 shows the MS/MS spectrum of the peptide at *m*/*z* 1955.7 selected from the MALDI spectrum of the 1-fmol mixture. This fragmentation spectrum was also acquired in ~2 s. The majority of the peaks in the spectrum can be rationalized as b- or y-type fragments. This high-speed acquisition of both MS and MS/MS data from small amounts of sample opens up unique opportunities for the design of the experiments on relatively complex peptide mixtures (see below).

Use of a CD as the MALDI Target. Advantages of the present CD target design include the following: (1) We have observed a low signal background from the polycarbonate surface. (This CD surface was found to produce the lowest background of the 20 different materials that we have tested[1].) (2) CDs are thin (1.2 mm) and have very plane surfaces so that they can be interposed between the skimmer and the quadrupole without significantly increasing the skimmer quadrupole spacing (thus maintaining the high transport efficiency of ESI-produced ions). (3) The CD surface has hydrophobicity/hydrophilicity properties that ensure that the matrix/sample solutions do not spread out unduly on the surface and at the same time permits the matrix crystals to adhere strongly to the surface. (4) The CD has a large accessible surface area, making it straightforward to spot >1000 samples on its surface. (5) The ability to use a variety of inexpensive methods for accurately and rapidly positioning any given sample in the path of the desorbing/ionizing laser beam is important for high-throughput applications. In summary, CDs provide a clean, convenient, compact, and very inexpensive means of sample interrogation and storage.

Applications of MALDI-IT for Protein Identification. The high sensitivity and speed of the MALDI MS and MS/MS analyses enable us to perform a great many protein identification experi-

Table 1. Top Three Protein Candidates[f] Found in the NCBInr Database (2001/04/02, Taxonomy: *Mamalia*) by the Search Engine Sonar Based on the Interpretation of 91 MALDI-IT MS/MS Spectra Obtained from Tryptic Digestion of the Species in a Single ~80-kDa Gel Band

Expect[a]	Result[b]			
7.9×10^{-5}	**72.3 kDa glucose-regulated protein [Homo sapiens]**			
	A/T[c]	**a:b:y[d]**	**m/z**	**Peptide[e]**
	24/50	2:19:4	1817.2	199 IINEPTAAAIAYGLDKR 215
	22/49	8:7:17	1430.8	103 TWNDPSVQQDIK 114
	25/50	4:13:9	1888.3	166 VTHAVVTVPAYFNDAQR 182
	20/50	1:14:10	1605.4	125 TKPYIQVDIGGGQTK 139
	24/45	1:11:20	1934.2	476 DNHLLGTFDLTGIPPAPR 493
	14/50	1:5:8	1512.8	326 AKFEELNMDLFR 337
	20/50	2:13:7	1975.2	603 IEWLESHQDADIEDFK 618
	13/50	2:3:8	1228.8	51 VEIIANDQGNR 61
	15/50	1:4:12	1211.0	378 EFFNGKEPSR 387
	14/50	1:7:8	1999.2	494 GVPQIEVTFEIDVNGILR 511
	7/50	2:4:1	1317.6	523 NKITITNDQNR 533
1.2×10^{-2}	**76.6 kDa, cytochrome P450 reductase [Homo sapiens]**			
	A/T	**a:b:y**	**m/z**	**Peptide**
	18/50	2:13:5	1203.9	279 NPFLAAVTTNR 289
	17/46	2:12:8	1423.8	267 SYENQKPPFDAK 278
	12/50	2:8:3	1537.9	298 HLMHLELDISDSK 310
	11/50	1:1:11	2453.5	199 IFELGLGDDDGNLEEDFITWR 219
	10/49	2:6:5	2117.6	78 NIIVFYGSQTGTAEEFANR 96
3.9×10^{-2}	**126.9 kDa, DDB1_HUMAN DNA DAMAGE BINDING PROTEIN 1 (DAMAGE-SPECIFIC DNA BINDING PROTEIN 1)**			
	A/T	**a:b:y**	**m/z**	**Peptide**
	15/50	0:10:9	1644.0	515 ALYYLQIHPQELR 527
	17/50	4:7:7	1385.9	485 ALVSEWKEPQAK 496
	9/49	3:0:10	1052.6	271 YLLGDMEGR 279
	11/50	4:8:3	1194.6	159 LEELHVIDVK 168
	10/49	4:5:4	1635.1	288 EEQMDGTVTLKDLR 301
	9/50	6:2:2	1203.9	61 IAVMELFRPK 70

[a] Search parameters: 1 m/z was the error for parent ions, 1 m/z for daughter ions. No modifications were expected. See explanations for the expectation values and other parameters at the web site http://canada.proteometrics.com/prowl/sonar.html. [b] No redundant protein candidates have been included in the list of the results. [c] Shows the ratio of the number of matched fragments to the total number of fragments supplied for the search. Currently the number of fragments is restricted to less or equal to the 50 most abundant fragments. [d] Shows the number of fragments that were interpreted as a-, b-, or y-type of fragments according to the Roeppstorf nomenclature. [e] Shows a matched peptide sequence and its position in the protein. Bars between amino acid codes show a spectrogram of relative intensities of the fragment peaks. [f] Also identified but not shown, were bovine trypsin and IgG heavy chain (see text).

Table 2. Details of the Analysis of 2 out of 91 MALDI-IT MS/MS Spectra Supplied to the Search Engine Sonar To Determine Proteins in a Single ~80-kDa Gel Band

MALDI-IT MS/MS of 1266.78 m/z.

Assigned sequence[a]: 1 ALETHTYFER 10
Full sequence[b]: IgM heavy chain constant region

Parent Properties

m/z[c]	z	mass (m)	assigned[d] (a)	m - a
1266.78	1	1265.77	1265.61	0.16

Ion assignments

m/z (m)	m/z (a)	m - a	z	Ion
653.20	653.33	-0.13	1	b6 T/Y
798.40	798.38	0.02	1	b7 Y/F (-18)
816.30	816.39	-0.09	1	b7 Y/F
935.50	935.44	0.06	1	y7 E/T (-18)
935.50	935.45	0.05	1	a8 F/E
945.20	945.45	-0.25	1	b8 F/E (-18)
953.40	953.45	-0.05	1	y7 E/T
963.30	963.46	-0.16	1	b8 F/E
1074.40	1074.49	-0.09	1	b9 E/R (-18)
1082.40	1082.49	-0.09	1	y8 L/E
1092.40	1092.50	-0.10	1	b9 E/R

MALDI-IT MS/MS of 1386.85 m/z.

Assigned sequence:1 ALVSEWKEPQAK 12
Full sequence: UV-Damaged DNA-Binding Protein 1 (UV-DDB1)

Parent Properties

m/z	z	mass (m)	assigned (a)	m - a
1386.85	1	1385.84	1384.74	0.10

Ion assignments

m/z (m)	m/z (a)	m - a	z	Ion
554.10	554.29	-0.19	1	y5 K/E (-18)
641.20	641.32	-0.12	1	a6 W/K (-17)
668.40	668.34	0.06	1	b6 W/K (-18)
686.10	686.35	0.25	1	b6 W/K
700.30	700.40	-0.10	1	y6 W/K
815.00	814.45	0.55	1	b7 K/E
886.20	886.48	-0.28	1	y7 E/W
925.40	925.48	-0.08	1	b8 E/P (-18)
926.30	926.47	-0.17	1	b8 E/P (-17)
943.30	943.49	-0.19	1	b8 E/P
997.50	997.51	-0.01	1	y8 S/E (-18)
1085.40	1085.54	-0.13	1	y9 V/S (-17)
1168.40	1168.60	-0.20	1	b10 Q/A
1239.40	1239.63	-0.23	1	b11 A/K

[a] Bars between one-letter codes for amino acids show a spectrogram of relative intensities of the fragment ions. [b] No redundant protein matches are shown. [c] Observed m/z. [d] Calculated mass.

ments within a relatively short period of time. Thus, we have used the instrument to identify >1000 proteins over a period of a few months. Here, we show a few selected examples, where we illustrate the performance of the MALDI-IT mass spectrometer and point out its major characteristics.

Identification of the Proteins in an 80-kDa Gel Band Illustrates the Mass Accuracy of the Instrument. Figure 5A shows a Zn-stained 1D SDS−PAGE gel of human proteins that were co-immunoprecipitated from a HeLa nuclear cell extract using stably expressed UV-DDB1 (ultraviolet DNA damage binding protein 1) tagged with the Flag peptide. Although we did not observe the expected Flag-tagged UV-DDB1 band at 130 kDa in this particular preparation, we did observe a set of lower molecular weight bands that we sought to analyze. Thus, for example, the 80-kDa band in Figure 5A was excised from the gel and digested with trypsin, and the tryptic peptides were extracted and deposited on the CD MALDI target. The resulting mass spectrum (in the range of 200−4000 m/z) is shown in Figure 5B. The spectrum was collected in 8 s (average of four spectra, three microscans each) with the instrument settings allowing a maximum number of ions (5×10^9 in the Finnigan nomenclature) to enter the ion trap. However, at this setting, the large number of ions admitted by the ion trap caused not only the well-described shift in the measured m/z of the peptides[22] but also obscured the observation of the less intense ion peaks. To overcome this problem (while still trapping a relatively large number of ions), we collected the same spectrum in a series of eight segments using the automatic mode of spectral acquisition provided by the

(22) (a) Cox, K. A.; Cleven, C. D.; Cooks, R. G. *Int. J. Mass Spectrom. Ion Processes* **1995**, *144*, 47−65. (b) Schwartz, J. C.; Jardine I. *Methods Enzymol.* **1996**, *270*, 552−586.

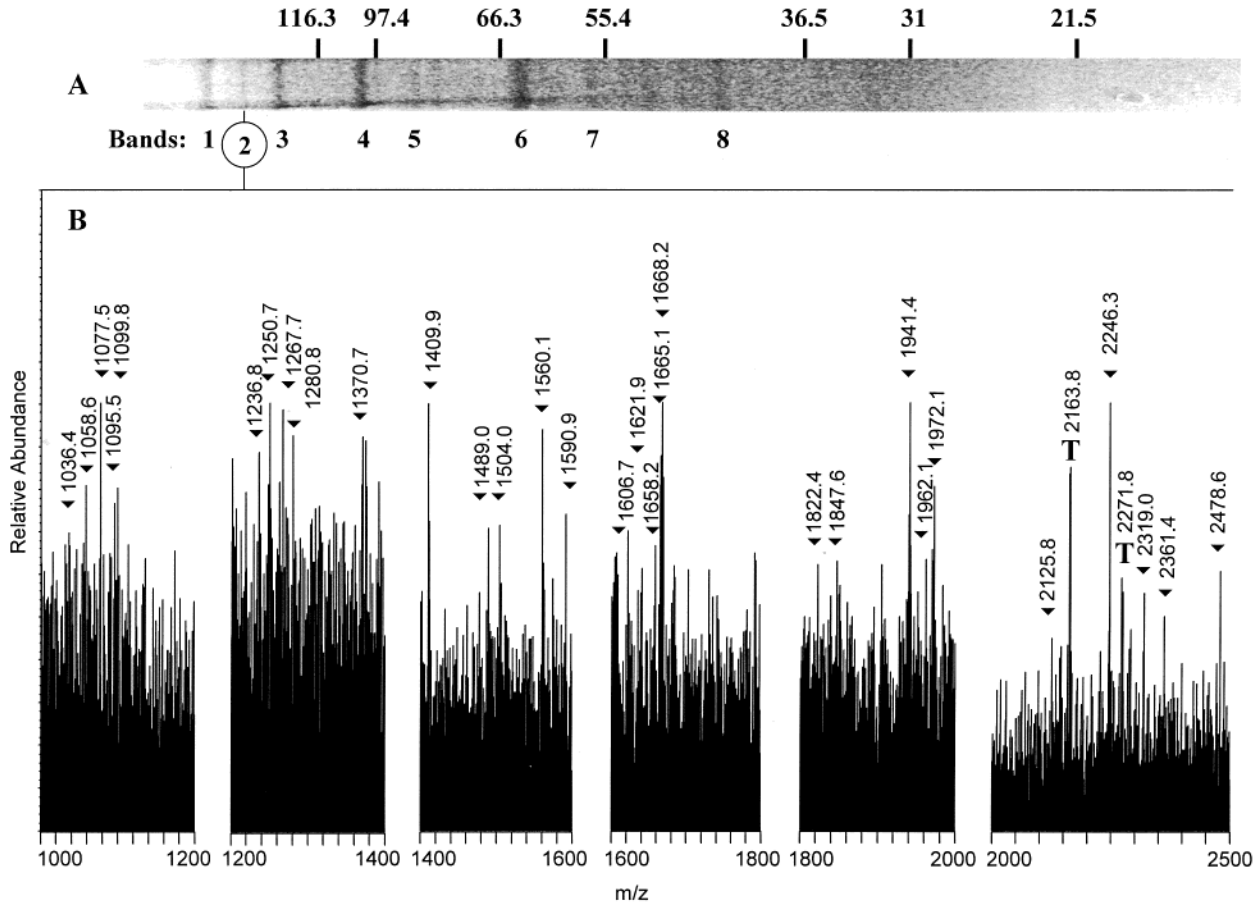

Figure 8. (A) Zn-stained 1D SDS−PAGE gel of a subset of proteins isolated from a rat nuclear pore complex preparation. (B) MALDI mass spectra of tryptic peptides extracted from the indicated 150-kDa gel band. The MALDI mass spectrum was obtained in automatic mode in six separate *m*/*z* segments.

Xcalibur software. The spectra of these segments, each obtained in 8 s (i.e., 12 microscans) are shown in Figure 5C. As expected, the ion peaks in each segment become more apparent and the shift in the observed *m*/*z* values less pronounced. Figure 6 shows a comparison of the *m*/*z* 1200−1400 portion of the full-scan spectrum from Figure 5B with the corresponding segment from Figure 5C. Although both spectra were obtained in 8 s, the segment scan spectrum (Figure 6B) exhibits better a signal-to-noise ratio than the same portion of the full-scan spectrum (Figure 6A). In addition, the *m*/*z* values of the ion peaks measured in the segment-scan spectrum were more accurate (as deduced from the calculated peptide masses of the identified species, see below).

The same Xcalibur routine used for collecting the MS spectra was also configured to collect MS/MS spectra of the *X* most abundant ion peaks from each segment, using a data collection algorithm (Figure 2A) similar to that used in the HPLC "stop-flow" experiment.[4,5] However, a difference between the MALDI and normal LC-ESI experiments is the long persistence of the MALDI ion signal—i.e., a given sample yields MALDI signals for 10^4−10^5 laser pulses. This persistence allows us to collect MS/MS spectra from all the intense ions in the sample—in this example, 91 MS/MS spectra with a total acquisition time of 10 min.

Two examples of these 91 MS/MS spectra are shown in Figure 7. The parent ions for these MS/MS spectra were selected from the *m*/*z* 1200−1400 segment (Figure 5C). Both peptides were identified as arising from the top protein candidates identified from the NCBInr database when all 91 MS/MS spectra were supplied to the search engine Sonar (Table 1). Details of the analysis of these two peaks are provided in Table 2, showing the attained accuracy in the assignment of parent masses and *m*/*z* values for the observed fragments. All but one of the assigned peaks fall within 0.3 Da of the predicted values, an accuracy comparable to that achieved in the ESI mode. In addition to trypsin and IgG heavy chain (arising from leakage of the protein from the IgG column used to pull out the flag-tagged proteins), the Sonar search engine confidently identified three human proteins in the 80-kDa band (Table 1). The top candidate is glucose-regulated protein, an abundant member of the heat shock 70-kDa family of proteins that is frequently pulled out in this type of the experiment. The biological meaning of the presence of the second candidate in the band, cytochrome P450 reductase, remains unclear, although it may play a role in DNA repair.[23] The third candidate is the UV-DDB1 protein, which is present in the 80-kDa band presumably as a degradation product of the tagged protein (MW ∼130 000).

Analysis of the Proteins in a Weak 150-kDa Gel Band Illustrates the Sensitivity and Speed of the Instrument. Highly purified nuclear pores from rat liver nuclei[24] were separated using

(23) Kruyt, F. A.; Hoshino, T.; Liu, J. M.; Joseph, P.; Jaiswal, A. K.; Youssoufian, H. *Blood* **1998**, *92*, 3050−3056.

(24) Fontoura, B. M.; Blobel, G.; Matunis, M. J. *J. Cell Biol.* **1999**, *144*, 1097−1112.

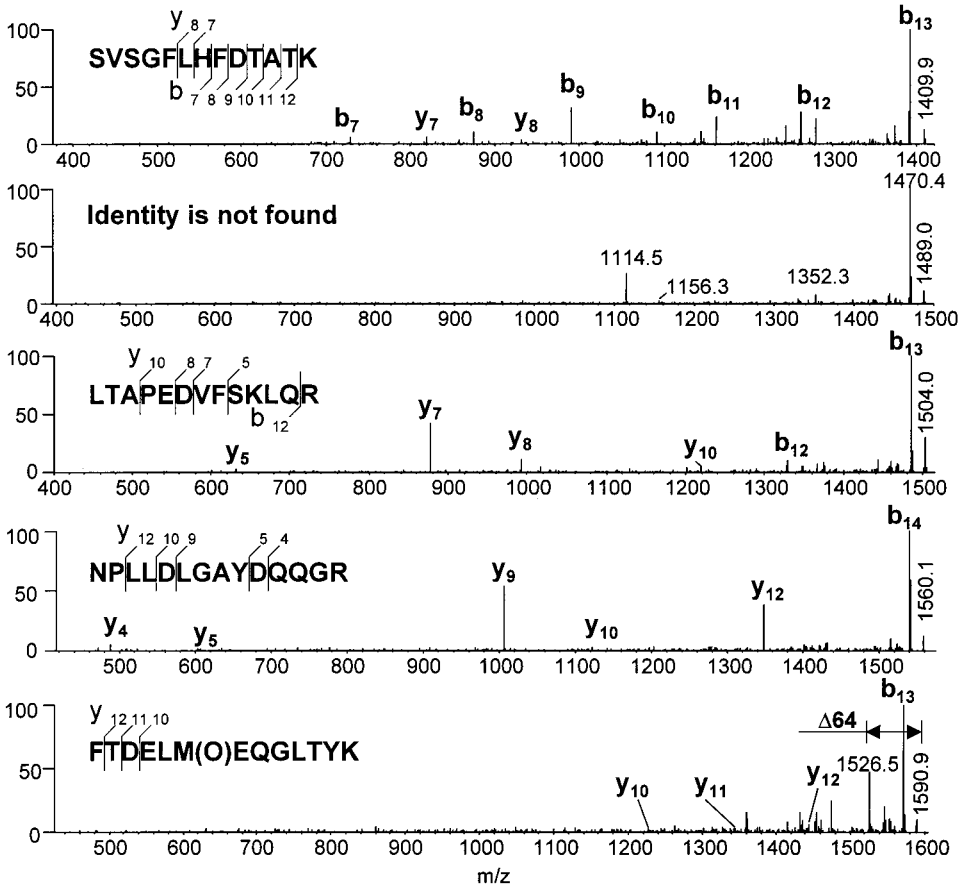

Figure 9. MS/MS spectra of the five most abundant tryptic peptides from the 150-kDa gel band that were detected in the *m*/*z* segment 1400−1600. The sequences of the identified peptides and their fragments are indicated.

C4 reversed-phase HPLC and the eluting fractions subjected to 1D SDS−PAGE. Figure 8A provides an example of one of these gel separations. In an attempt to make a complete inventory of the nuclear pore proteins from rat, it proved necessary to identify proteins from more than 350 such gel bands.

To come up with an appropriate strategy to complete this project, it was necessary to take into account two important factors. The first is that the existing databases of rat proteins, ESTs, and genomic sequence are largely incomplete. Thus, our MS approach had to provide the maximum possible peptide coverage in order to optimize our chance of finding homologous proteins in the human and mouse databases. A second critical factor is the time needed to complete the analysis of 350 gel bands. We considered using the LCQ-ESI ion trap combination, but rejected this choice as being too time-consuming—i.e., 30 min per LC/MS run, resulting in 175 h of run time. Extensive experience tells us that we should allow an additional factor of 2 for LC troubleshooting, source cleaning, etc., that is inevitably required in such experiments—leading to a total analysis time of 350 h (i.e., 35 days at 10 h/day). This long analysis time prompted us to test the performance of our new MALDI-IT instrument for this challenging task.

An Xcalibur-based data collection routine was set up to collect the requisite MS and MS/MS spectra from a given band in 3.5 min. These MS data were automatically collected in 6 segments (Figure 8B) along with MS/MS spectra of the 5 most abundant peaks from each segment (i.e., a total of 30 MS/MS spectra/band). All known trypsin and contaminant peaks were excluded from the MS/MS experiment. This strategy provided reasonably high quality data within a short analysis time. For example, Figure 8B shows the six segments of the MS spectrum of tryptic peptides extracted from the weak Zn-stained 150-kDa gel band shown in Figure 8A. All the peaks selected by the Xcalibur routine for MS/MS analysis are labeled in this spectrum. Figure 9 shows the five MS/MS spectra of the parent ions selected from one of the segments (1400−1600 *m*/*z*). Despite the low signal-to-noise ratio (2−3) in the segment, the quality of the MS/MS spectra (each obtained in 5 s) was sufficiently high to identify four out of these five tryptic peptides.

Table 3 shows the top three protein candidates found by the Sonar search engine in the NCBInr database after analysis of the 30 MS/MS spectra. The first candidate is a 227.9-kDa protein (gi/1504030), which is homologous to a *Caenorhabditis elegans* protein encoded in cosmid K12D12, which in turn is highly homologous to the yeast nuclear pore protein nup192.[25] The second and the third candidates are previously annotated nuclear pore proteins, nup155 from mouse and nuclear pore membrane glycoprotein gp210 from both rat and mouse. Thus, within less than 4 min (3.5-min spectrum acquisition + 0.3-min database search with the Sonar search engine), we identified three component proteins within a single very weak gel band. It took a total of ∼30 min to identify the proteins in all eight excised gel bands labeled in the

(25) Kosova, B.; Pante, N.; Rollenhagen, C.; Hurt, E. *J. Biol. Chem.* **1999** *274*, 22646−22651.

Table 3. Top Three Protein Candidates Found in the NCBInr Database (2001/04/02, Taxonomy: *Mamalia*) by the Search Engine Sonar Based on the Interpretation of 30 MALDI-IT MS/MS Spectra Obtained from Tryptic Digestion of the Species in a Single ~150-kDa Gel Band

4.5×10^{-3}	**227.9 kDa, similar to a *C.elegans* protein encoded in cosmid K12D12 (Z49069) [Homo sapiens]**			
	A/T	**a:b:y**	**m/z**	**Peptide**
	28/50	10:13:7	1409.9	1166 SVSGFLHFDTATK 1178
	14/50	4:2:8	1099.8	893 KADNVVNIAR 902
	17/50	0:4:17	1504.0	1453 LTAPEDVFSKLQR 1465
	15/50	2:9:6	1668.2	47 HKPDFISLFKNPPK 60
	8/50	0:3:7	1250.7	573 DLPSADSVQYR 583
	13/50	1:12:6	1665.1	1166 SVSGFLHFDTATKVR 1180
	10/50	4:7:2	1504.0	71 ASTEGVAIQGQQGTR 85
	17/50	5:8:7	1665.1	846 AVQHCLALLNLTLQK 860
1.0×10^{-2}	**155.0 kDa, nucleoporin 155 [Mus musculus]**			
	A/T	**a:b:y**	**m/z**	**Peptide**
	23/50	6:17:6	1370.7	1153 IQLQIQETLQR 1163
	8/12	0:1:7	1972.1	952 HGEPEEDVVGLQTFQER 968
0.36	**204.0 kDa, Rat Integral Membrane Glycoprotein GP210 Precursor**			
	A/T	**a:b:y**	**m/z**	**Peptide**
	21/50	2:11:12	1658.2	1871 KASPPSGLWSPAYASH 1886
	16/50	4:6:12	1560.1	784 NPLLDLGAYDQQGR 797
	9/50	2:6:1	1621.9	516 AHDVQNPLHFGEMK 529
0.36	**204.0 kDa, Nuclear pore membrane glycoprotein 210 [Mus musculus]**			
	A/T	**a:b:y**	**m/z**	**Peptide**
	21/50	2:11:12	1658.2	1871 KASPPSGLWSPAYASH 1886
	16/50	4:6:12	1560.1	784 NPLLDLGAYDQQGR 797
	11/50	2:5:6	1409.9	1548 EIVVGTPQKIVAR 1560
	8/50	2:4:3	1962.1	799 FDNFSSLSIQWESFPR 814

Figure 8A, in which we found four known nuclear pore proteins, a novel human protein that we identified as a previously unknown nuclear pore protein, a karyopherin (i.e., nuclear transport protein), and an abundant enzyme, which most likely was a contaminant. The full dataset will be published elsewhere.

Automatic Identification of the Components of a Protein Complex without Prior Electrophoretic Separation. The present instrument can identify proteins from relatively complex protein mixtures without prior separation. Here, we illustrate this capability by identification of the six major protein components of a *Saccharomyces cerevisiae* protein complex. We have previously identified the six components, after genomically tagging one of them (YJL041w) with protein A,[26] immunoprecipitating the complex with IgG, separating the components by SDS−PAGE, cutting out and tryptically digesting the individual bands, and identifying the proteins by MALDI-QqTOF MS and MS/MS analysis. These previously identified proteins are shown annotated in the silver-stained Figure 10C gel (using ~ 400 fmol of the immunoprecipitated complex).

Figure 10A shows the MALDI-IIT MS tryptic map obtained from ~200 fmol of the same complex without prior electrophoretic separation. To identify as many proteins as possible in the sample, we used the algorithm depicted in Figure 2B. Thus, an MS tryptic map of the sample was obtained in several segments (each 400 m/z in width) and MS/MS spectra were obtained from all the discernible peaks as shown in Figure 10B. The total number of MS/MS spectra measured from this sample was 130.

All six expected proteins were identified by both the Mascot and Sonar search routines. This ability to obtain MS/MS spectra on all ion peaks that are discerned in the MS spectrum enables the identification of proteins in relatively complex mixtures. In a second experiment (data not shown), we identified 11 out of 12 proteins in an artificial protein mixture of known composition without prior protein separation.

We believe that the algorithm used for the identification of proteins in this experiment (Figure 2B) is better than algorithms borrowed from an HPLC-LCQ experiment and described in the previous sections (Figure 2A). However, because it is not integrated into the computer software controlling the instrument, it takes considerable time to set up the methods, increasing the total analysis time to 15 min for 130 MS/MS spectra. Future algorithms in which the current software is integrated into the instrument control software should reduce the analysis time for such experiments.

CONCLUSIONS

We have described a novel MALDI-IT mass spectrometer that can be used to rapidly obtain informative MS and MS/MS spectra from femtomole amounts of peptides. The instrument can collect MS/MS spectra from all the discernible singly charged ions in a MS peptide map. The current speed of spectral acquisition is ~8 s for MS and ~5 s for MS/MS spectra although we have shown that we can acquire usable spectra in as little as 2 s. We believe that the acquisition speed can be further decreased through the use of a high repetition rate UV laser (~ kHz), which will increase the number of ions entering the ion trap. Under these conditions, we plan to minimize space charge effects (in MS/MS measurements) through the use of the quadrupole ion guide to preselect ions with a particular m/z. We also plan to design software that optimizes the time spent on any given MS/MS analysis, depending on the intensity of the fragmentation signals.

We have observed that the quality of the MS/MS spectra obtained from singly charged ions is sufficiently high to identify proteins in relatively complex protein mixtures. Analysis of large numbers of MS/MS spectra confirms our earlier observation of selective fragmentation of singly charged tryptic peptides.[10] Thus, for example, selective fragmentation occurs on the C-terminal side of aspartic and glutamic residues as well as on the N-terminal side of proline residues, yielding abundant y- and/or b-type fragment ions. We plan to incorporate this information about systematic selective fragmentation into a search engine to further improve the identification procedure.

The present instrument is robust and needs minimal operation skills. Biologist and students from other laboratories in our institute, who do not have prior experience with mass spectrometry, can effectively run the instrument after a 15-min instruction period. Because an identification experiment consists of a series of repetitive applications of the existing automatic Xcalibur software routine to collect spectra from each sample, the only intervention that is currently required of the operator during

(26) Aitchison, J. D.; Blobel, G.; Rout, M. P. *J. Cell Biol.* **1995**, *131*, 1133−1148.

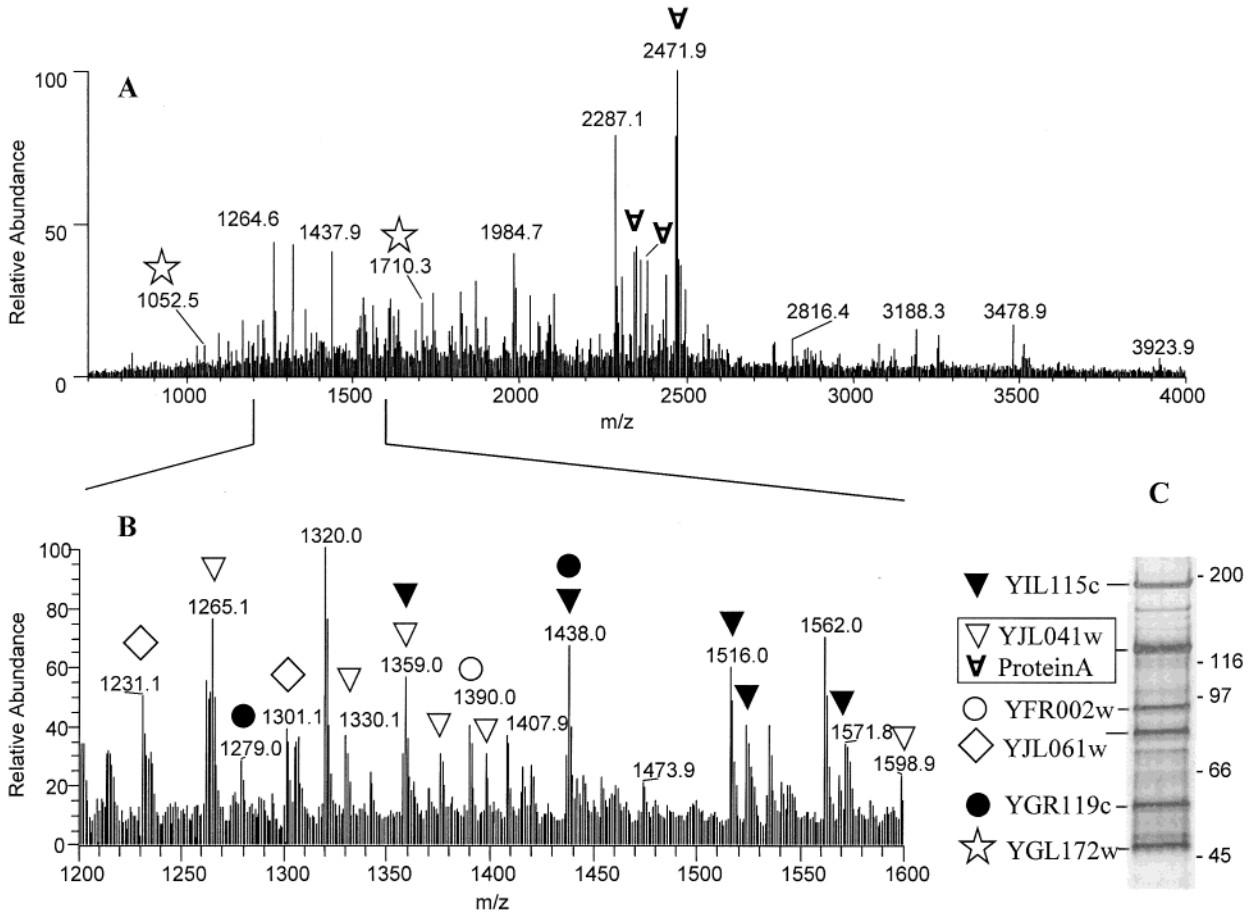

Figure 10. Identification of proteins from an unfractionated protein complex by MALDI-ion trap MS/MS analysis. (A) MALDI MS tryptic map of a ~200-fmol mixture of proteins from a *S. cerevisiae*-derived complex obtained without electrophoretic separation of the component proteins. (B) MS map of the segment between *m*/*z* 1200 and 1400. (C) Silver-stained 1D SDS−PAGE of ~ 400 fmol of the same protein mixture with identities of the major proteins determined by a separate experiment on each individual gel band. Six proteins that give rise to the six major bands were directly identified from the unfractionated mixture through analysis of some 130 MS/MS spectra.

spectra acquisition is positioning of a new sample and the occasional repositioning of the laser spot on the sample. These two operations can be readily automated and we plan to put an automatic sample positioning system and laser scanning system into operation soon.

The MALDI ion trap needs little maintenance. We clean the ion guide rods that become covered with dust from desorbed matrix after 1−2 months of extensive use, even though it is not obvious that this buildup causes degradation in the performance of the instrument. We periodically check sensitivity by collecting MALDI spectra from 100, 10, and 1 fmol of a peptide mixture (Figure 4). The present instrument also supports the ESI mode of operation. However, if it is desired to dedicate its use solely to MALDI experiments the requirements of the pumping system can be considerably reduced.

In summary, we have constructed an instrument that satisfies the requirements discussed in the introduction for high-throughput protein identification.

ACKNOWLEDGMENT

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