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# Gel Protein Capillary Extraction Apparatus. Electronic Protein Transfer

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**A gel protein capillary extraction apparatus is developed and demonstrated for its rapid and effective transfer of SDS−protein complexes from polyacrylamide gel to a fused-silica capillary. The small dimensions of capillary columns permit the application of high voltages for achieving rapid and effective transfer of gel proteins. Furthermore, the fused-silica capillaries are internally coated with polyacrylamide for the elimination of electroosmotic pumping and protein adsorption onto the capillary wall. The extracted proteins are present in a highly concentrated solution plug as the result of field amplification and sample stacking during the extraction process. Three model proteins, including cytochrome *c* (14 kDa), ovalbumin (45 kDa), and *β*-galactosidase (116 kDa), are visualized using coomassie blue staining and electrophoretically extracted from the gels with protein loading as low as 50 ng. The SDS−cytochrome *c* complexes extracted from a 50-ng protein loading are concentrated in a 30-nL solution plug inside the capillary with an estimated concentration of 0.1 mg/mL or $10^{-5}$ M. The capillary format allows the straightforward integration of a miniaturized trypsin−membrane reactor for on-line proteolytic digestion and ESI-MS analysis for protein/peptide identification.**

Proteomics is an emerging area of research in the postgenomic era involving the global analysis of gene expression via a combination of bioanalytical techniques for resolving, identifying, quantitating, and characterizing proteins.[1] Due to the ability to separate thousands of proteins while offering the additional advantage of "differential display", two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) has been the method of choice for the comprehensive analysis of protein composition in a given cell type.[2]

Mass spectrometry (MS) employing matrix-assisted laser desorption/ionization (MALDI)[3,4] and electrospray ionization (ESI)[5] has evolved to become an essential tool for protein identification and sequencing.[6] Prior to MS analysis, individual protein spots are typically excised from the 2-D gel, washed, and in-gel digested with an excess of trypsin. Repeated washing, drying, and swelling of gel pieces are needed between each step of chemical or enzymatic reaction. Peptides are then extracted using aqueous/organic mixtures at acidic or basic conditions and prepared for peptide mass mapping or sequencing using MALDI or ESI-MS.[7,8]

However, all of these procedures are time-consuming tasks prone to sample loss and analyte dilution. For example, Aebersold and co-workers[9] recently showed that the current techniques of combining 2-D PAGE with MS identified only the most abundant proteins even though more than 1500 spots were visualized by silver staining in a narrow pH range (4.9−5.7). Thus, questions remain concerning the ability to characterize all of the elements of a proteome using 2-D PAGE and MS.[9,10] Doubts are raised about the overall effectiveness of large efforts currently devoted to implementing this proteomics strategy.

In this study, we have developed a gel protein capillary extraction apparatus which potentially provides the critically needed link between 2-D PAGE and MS. Our results demonstrate rapid and efficient extraction of negatively charged sodium dodecyl sulfate (SDS)−protein complexes from polyacrylamide gel into a coated capillary. The small dimensions of capillary columns (high surface-to-volume ratio) provide efficient dissipation of Joule heat, which allows the application of relatively high voltages and transforms conventional protein electroblotting into a high-speed and effective protein extraction platform.

## EXPERIMENTAL SECTION

**Materials and Reagents.** Fused-silica capillaries with the dimensions of 50-*μ*m i.d. and 192-*μ*m o.d. (Polymicro Technologies, Phoenix, AZ) were coated internally with linear polyacrylamide for the elimination of electroosmotic flow and protein adsorption onto the capillary wall.[11] Model proteins (horse heart cytochrome *c*, chicken egg albumin, *Escherichia coli β*-galactosidase), mercaptoethanol, and methanol were acquired from Sigma (St. Louis, MO). Bromophenol blue, coomassie blue R, Tris(hydroxymethyl)aminomethane (Tris), glycine, and SDS were

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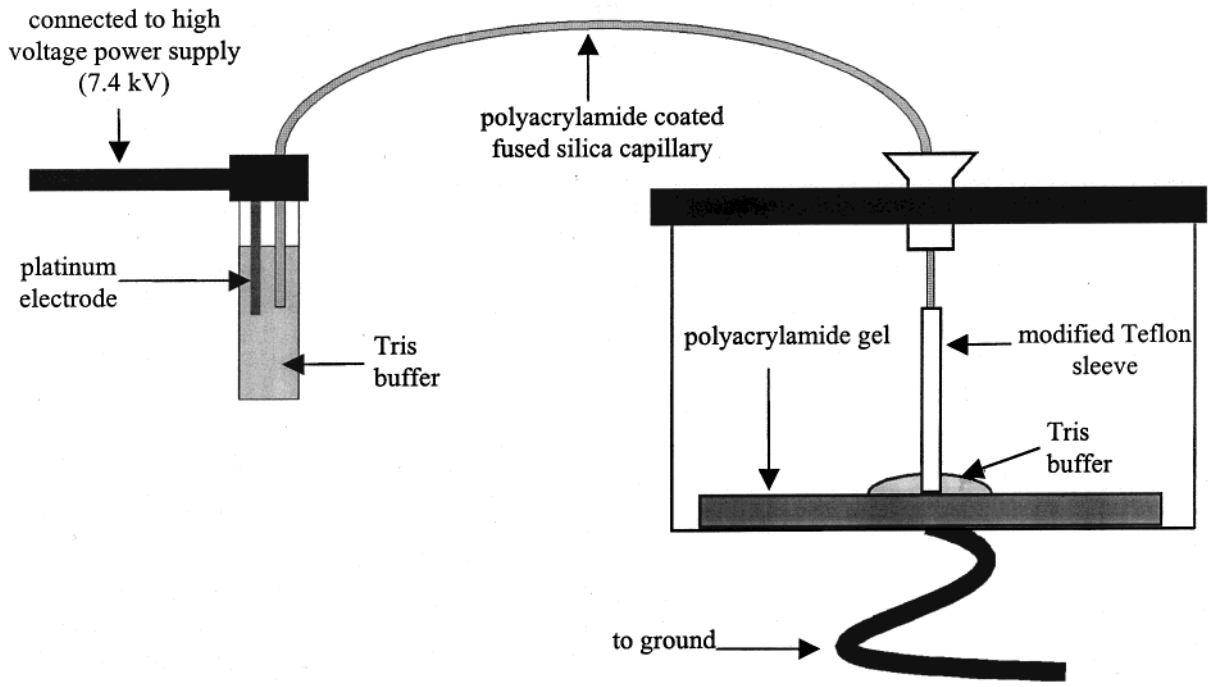


Figure 1. Schematic of gel protein capillary extraction apparatus.

purchased from Bio-Rad (Hercules, CA). All solutions were prepared using water purified by a Nanopure II system (Dubuque, IA) and further filtered with a 0.22-$\mu$m membrane (Costar, Cambridge, MA).

**SDS−PAGE and Modified Coomassie Blue Staining.** Samples of standard proteins at various concentrations were denatured for 10 min at 97 °C in a solution containing 10 mM Tris (pH 6.8), 1% mercaptoethanol, 2% SDS, 1% glycerol, and 0.05% bromophenol blue. Electrophoretic separations of standard proteins were performed by employing 4−20% polyacrylamide gradient gels (Bio-Rad) in the electrophoresis buffer of 25 mM Tris (pH 8.3), 200 mM glycine, and 0.1% SDS. An electric potential of 200 V was applied until the bromophenol blue eluted off the end of the gel.

Upon completion of electrophoresis, the gel was transferred to a plastic dish and stained with 0.1% coomassie blue R in 30% methanol. Destaining was performed using 30% methanol. The gel was rinsed with water to remove excess methanol and then reequilibrated in a 10% SDS solution. SDS solution was removed with subsequent water rinses, and the gel was incubated with 20 mM Tris at pH 6.8 prior to protein extraction.

**Gel Protein Extraction.** A gel protein capillary extraction apparatus (see Figure 1) was developed in our laboratory for rapidly and effectively transferring protein analytes from polyacrylamide gel to fused-silica capillary. The fused-silica capillaries (50-$\mu$m i.d./192-$\mu$m o.d.) internally coated with polyacrylamide were filled with electrophoresis buffer of 20 mM Tris at pH 6.8. A modified Teflon sleeve allowed the extraction end of the capillary to be in contact with the gel. A 200-$\mu$L sample of 20 mM Tris buffer was placed at the gel−capillary interface to establish the electrical contact, and the gel was connected to a common ground through a platinum wire.

A CZE 1000R high-voltage power supply (Spellman High-Voltage Electronics, Plainview, NY) was employed for providing a positive electric voltage of 7.4 kV at the outlet reservoir through a platinum electrode. Thus, an electric field strength of 200 V/cm was generated over a 37-cm total capillary length during the protein extraction period. At the end of extraction period, the voltage was turned off and the extraction end of the capillary was quickly transferred from the gel to a solution reservoir filled with the electrophoresis buffer and connected to a common ground. The voltage was reapplied for inducing electrophoretic migration of SDS−protein complexes inside the capillary. A Linear UVIS 200 detector (Linear Instruments, Reno, NV) was placed near the outlet reservoir (7 cm to the end of the capillary) for monitoring the extracted proteins using UV absorbance at 214 nm.

**SDS−Cytochrome *c* Calibration Curve.** To measure the concentration of extracted cytochrome *c* in the capillary, cytochrome *c* samples of various concentrations were reduced and denatured as per gel electrophoresis sample conditions described above. The samples were then passed through a PD-10 size exclusion column (Amersham Pharmacia Biotech., Uppsala, Sweden) to remove excess SDS. Capillary zone electrophoresis (CZE) of cytochrome *c* samples was performed in a 37-cm coated capillary (50-$\mu$m i.d./192-$\mu$m o.d.) filled with an electrophoresis buffer of 20 mM Tris at pH 6.8. An electric potential of 7.4 kV was applied at the outlet reservoir during electrokinetic injection for 15 s and during separation. CZE separations of cytochrome *c* samples with known concentrations were performed and measured by UV absorbance at 214 nm. Measured peak heights were used to construct a calibration curve in an effort to estimate the concentrations of extracted cytochrome *c*−SDS complexes from polyacrylamide gels.

**ESI-MS Analysis of Extracted Cytochrome *c*.** The capillary containing cytochrome *c*−SDS complexes extracted from polyacrylamide gel was connected to a microdialysis junction prior to a Perkin-Elmer Sciex (Foster City, CA) API 150EX single quadrupole mass spectrometer. The other end of the capillary was

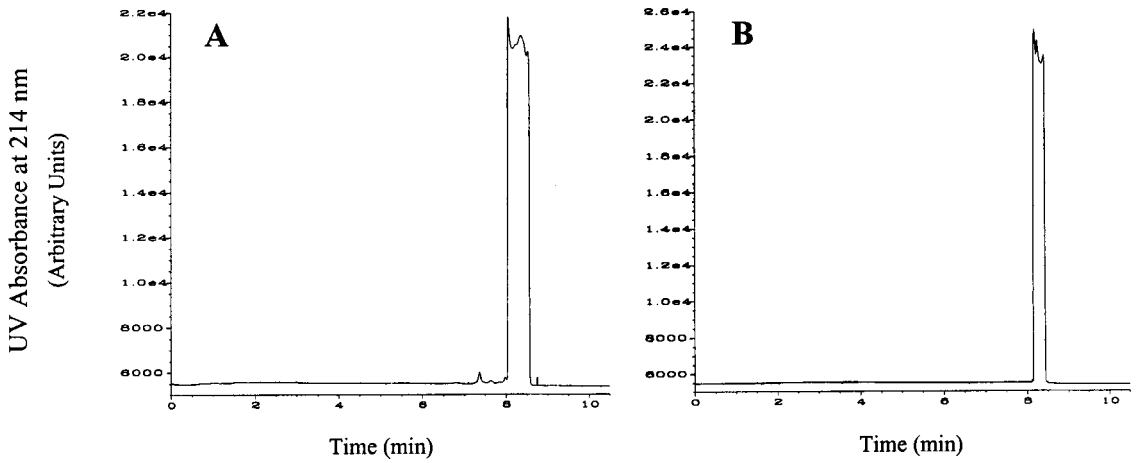


Figure 2. Electropherograms of (A) extracted SDS−cytochrome *c* complexes from a protein loading of 25 $\mu$g and (B) CZE separation of cytochrome *c* complexes with a concentration of 4 mg/mL. Conditions: capillary, polyacrylamide coating, 37 cm × 50 $\mu$m i.d. × 192 $\mu$m o.d., 30-cm effective separation length; electrokinetic injection, 7.4 kV for 15 s; electrophoresis buffer, 20 mM Tris at pH 6.8; electric field strength for separation, 200 V/cm; detection, UV absorbance at 214 nm.

connected to a Harvard Apparatus 22 (Holliston, MA) syringe pump. The solution containing extracted cytochrome *c* was delivered into ESI-MS through the microdialysis junction at a flow rate of 0.1 $\mu$L/min. The microdialysis junction, which provided the necessary electrical connection for inducing the electrospray process, was constructed as described previously.[12−14] An Eppendorf pipet tip housing the junction contained a solution of 50% methanol, 49% water, and 1% acetic acid (v/v/v) at pH 2.6. The solution offered postacidification and increased organic solvent content via the polysulfone dialysis tubing for enhancing the protonation and the ionization efficiency of proteins.

## RESULTS AND DISCUSSION

**Electrophoretic Extraction of Nonstained SDS−Cytochrome *c* Complexes.** The cytochrome *c* spots on the gels can be directly visualized without coomassie blue staining at high protein loadings of 2.5−50 $\mu$g. Thus, the capability of the gel protein capillary extraction apparatus for protein electronic transfer was first investigated by electrophoretically extracting nonstained SDS−cytochrome *c* complexes from a 4−20% polyacrylamide gradient gel. The electropherogram of extracted cytochrome *c* at a protein loading of 25 $\mu$g (see Figure 2A) was compared with CZE separation of SDS−cytochrome *c* complexes with a concentration of 4 mg/mL (see Figure 2B). The migration time of extracted cytochrome *c* complexes to reach the UV detector was ~8.1 min and was slightly shorter than that obtained from CZE.

The peak width at base was ~0.5 min for a 2-min protein extraction. This peak width corresponded to a solution plug of ~30 nL inside a 50-$\mu$m i.d./192-$\mu$m o.d. capillary. Furthermore, the electric current, measured by the current-monitoring method,[15] continuously decreased from 9 to 6 $\mu$A during the protein extraction period. This continuous current drop indicated the depletion of limited electrolytes near the end of the capillary during protein extraction.

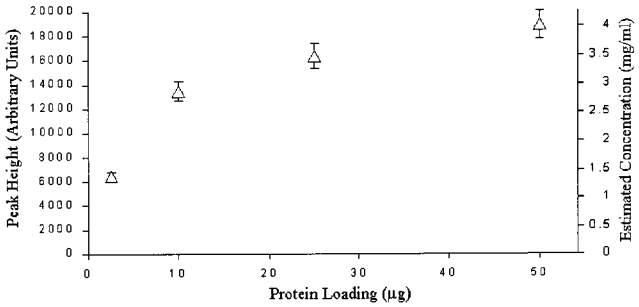


Figure 3. Dependence of peak heights and estimated concentrations of extracted cytochrome *c* complexes upon protein mass loadings.

The extracted protein analytes were therefore present in the low-conductivity zone containing depleted electrolytes and were subjected to sample stacking/concentration as the result of field amplification.[16−18] After the 2-min protein extraction, the high-voltage power supply was turned off and the end of the capillary facing the gel was lifted and placed in a reservoir containing the electrophoresis buffer. Once the positive electric voltage was reapplied, the current was restored to 9 $\mu$A and the negatively charged SDS−protein complexes electrophoretically migrated toward the UV detector near the anodic end.

Based on the calibration curve constructed from CZE separations of SDS−cytochrome *c* complexes with known concentrations, the concentration of extracted SDS−cytochrome *c* complexes from a protein loading of 25 $\mu$g was estimated to be ~3.5 mg/mL in a solution plug of ~30 nL. The peak heights and the estimated concentrations of extracted cytochrome *c* complexes during the 2-min extraction period are summarized in Figure 3 for various protein loadings. Both the peak height and the concentration increased with increasing protein loading. Furthermore, the peak height typically increased with increasing extraction time while the peak width remained approximately constant. For example, the peak height of extracted cytochrome *c* from a

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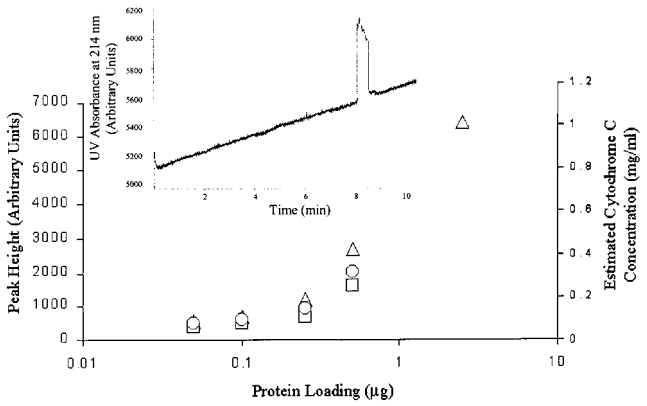


Figure 4. Dependence of peak heights and estimated concentrations of extracted SDS−protein complexes (Δ, cytochrome *c*; □, ovalbumin; ○, β-galactosidase) upon protein mass loadings. Inset: electropherogram of extracted SDS−cytochrome *c* complexes from a 50-ng protein loading. The peak height of 455 arbitrary absorbance units above the background corresponds to a concentration of 0.1 mg/mL.

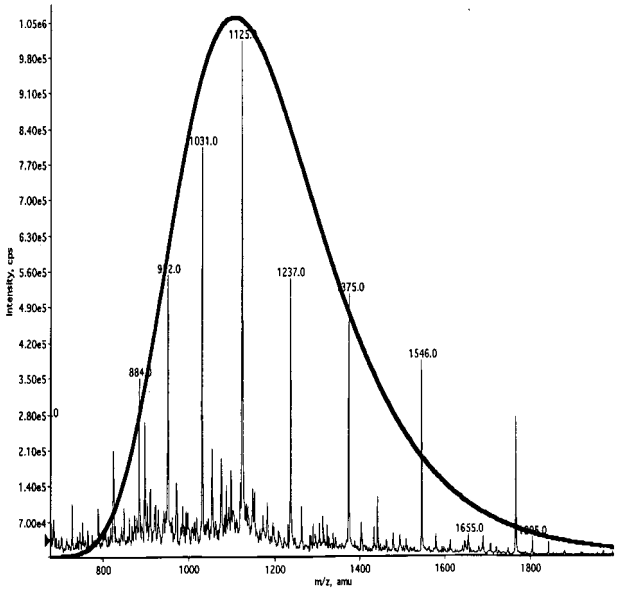


Figure 5. Positive ESI mass spectrum of extracted SDS−cytochrome *c* complexes from a 250-ng protein loading.

protein loading of 50 μg increased more than 5-fold by extending the extraction time from 2 to 5 min (data not shown).

**Electrophoretic Extraction of Coomassie Blue-Stained SDS−Protein Complexes.** Modified coomassie blue staining and destaining procedures were developed and employed to enhance the visualization of cytochrome *c* spots on gels at low protein loadings. Our current protein loading limitation on polyacrylamide gels for capillary extraction studies lies around 50−100 ng/spot and is in good agreement with those (0.1−0.2 μg/spot) reported in the literature using coomassie blue staining.[19]

At a protein loading of 2.5 μg, both the migration time and the peak profile of stained SDS−cytochrome *c* complexes were identical to those of nonstained complexes extracted from the gels. It should be noted that the coomassie blue staining allows the visualization of gel proteins at low mass loadings but exhibits negligible influence on protein mobility and UV absorbance. These observations are further supported by the practice in the Bradford protein assay,[20] where the assay involves the application of coomassie blue for measuring total protein concentration using visible absorbance at 595 nm.

The results summarized in Figure 4 further demonstrated the ability to rapidly transfer the SDS−cytochrome *c* complexes over a wide range of protein loadings, from 2.5 μg to 50 ng. Peak height of extracted SDS−cytochrome *c* complexes obtained from a 2-min extraction period decreased with decreasing protein loading on polyacrylamide gel. However, the extent of decrease in peak height was reduced at low protein loadings as the size of a protein spot shrunk with decreased protein loading. The concentration of extracted SDS−cytochrome *c* complexes from a 50-ng protein loading was estimated to be ~0.1 mg/mL on the basis of calibration curve established from CZE separation of protein complexes with known concentrations.

In comparison with a 50-ng protein loading, a 30-nL extraction plug containing 0.1 mg/mL SDS−cytochrome *c* complexes corresponded to a 6% total protein recovery during the 2-min extraction period. However, the extraction efficiency around the end of the capillary in touch with the gel would be significantly higher and close to quantitative protein transfer due to the small ratio of capillary cross section (50-μm i.d./192-μm o.d.) to the area of a 50-ng protein spot (0.1 cm × 1 cm). The small capillary dimensions potentially allow high-resolution analysis and identification of overlapping protein spots using MS.

The results summarized in Figure 4 also demonstrated the ability to extract higher molecular weight proteins such as ovalbumin and β-galactosidase with molecular mass around 45 and 116 kDa, respectively. The peak heights of extracted ovalbumin and β-galactosidase from a 4−20% polyacrylamide gradient gel were about 60−80% of those measured from cytochrome *c* at various protein loadings. Due to the use of UV absorbance at 214 nm, the weight concentrations of extracted ovalbumin and β-galactosidase would also be about 60−80% of those measured for cytochrome *c*.

The capillary containing extracted SDS−cytochrome *c* complexes from a 250-ng protein loading was connected to a microdialysis junction. The solution inside the capillary was delivered using a syringe pump at a flow rate of 0.1 μL/min. By applying a 4-kV electric potential at the microdialysis junction, a 25-s solution plug containing cytochrome *c* was identified using ESI-MS. The mass spectrum (see Figure 5) taken from the average of scans within the elution of extracted cytochrome *c* clearly indicated the presence of cytochrome *c* envelope.

Our high-voltage electrophoretic protein extraction is 1−2 orders of magnitude faster than the conventional electroelution and membrane electroblotting processes, which take 3−18 h to complete.[21−24] A key to our rapid and effective protein transfer

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includes the use of small-dimension capillaries (50-$\mu$m i.d./192-$\mu$m o.d.) in physical contact with the gel, which provides a large potential drop and high electric field strength at the capillary−gel interface for protein extraction. For example, Clarke and coworkers[25] reported the electroelution of proteins from SDS−PAGE to a membrane preconcentration cartridge within 2 h. The Teflon tubing, which encases a polymeric preconcentration membrane, exhibits an inner diameter of at least 400 $\mu$m.[25,26] Similarly, it took Timperman and Aebersold 4 h to electrophoretically elute the digested peptides from polyacrylamide gel to a cation exchange cartridge through a 1500-$\mu$m-i.d. channel.[27]

Another key factor contributing to the effectiveness of our electronic protein transfer technique involves the use of the large, negative electrophoretic mobilities of SDS−protein complexes in the electrophoresis buffer of 20 mM Tris at pH 6.8. In contrast, acidic electrolytes, including solutions of 20 mM ammonium acetate/1% acetic acid (pH 4.5) and 0.05% trifluoroacetic acid/10% acetonitrile, were utilized by Clarke[25] and Timperman[27] for positively charging the analytes in their studies.

## CONCLUSION

Our approach, a gel protein capillary extraction apparatus, allows rapid and effective transfer of SDS−protein complexes directly from polyacrylamide gel to fused-silica capillary by exploiting electrophoretic extraction. The technique harnesses electrophoretic forces to mobilize negatively charged SDS−protein complexes from the gel and concentrate them in the capillary tip. The small dimensions of fused-silica capillaries not only permit the application of high electric voltages during electrophoretic extraction for rapid and quantitative protein transfer but also offer the promise of providing the high-resolution analysis of overlapping protein spots on polyacrylamide gels. Furthermore, the presence of sample stacking and the absence of electroosmotic pumping in the coated capillary contribute to significant increase in protein concentration inside a narrow solution plug during protein extraction.

For a 50-ng cytochrome *c* loading, the extracted protein mass (3 ng or 0.3 pmol) and concentration (0.1 mg/mL or $1 \times 10^{-5}$ M) should allow us to fully exploit the mass detection sensitivity achievable with conventional mass spectrometers from the low-femtomole to the subfemtomole level[28,29] and enable much more efficient identification of protein spots from 2-D PAGE. Smith reported a sensitivity of better than 10 amol using Fourier transform ion cyclotron resonance MS.[10] The capillary setup in our gel protein extraction apparatus further provides straightforward integration with on-line proteolytic digestion using a miniaturized trypsin−membrane reactor[30] and subsequent ESI-MS analysis.

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