Optimized Sample-Processing Time and Peptide Recovery for the Mass Spectrometric Analysis of Protein Digests

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Proteomics requires an optimized level of sample-processing, including a minimal sample-processing time and an optimal peptide recovery from protein digests, in order to maximize the percentage sequence coverage and to improve the accuracy of protein identification. The conventional methods of protein characterization from one-dimensional or two-dimensional gels include the destaining of an excised gel piece, followed by an overnight in-gel enzyme digestion. The aims of this study were to determine whether: (1) stained gels can be used without any destaining for trypsin digestion and mass spectrometry (MS); (2) tryptic peptides can be recovered from a matrix-assisted laser desorption/ionization (MALDI) target plate for a subsequent analysis with liquid chromatography (LC) coupled to an electrospray ionization (ESI) quadrupole ion trap MS; and (3) an overnight in-gel digestion is necessary for protein characterization with MS. These three strategies would significantly improve sample throughput. Cerebrospinal fluid (CSF) was the model biological fluid used to develop these methods. CSF was desalted by gel filtration, and CSF proteins were separated by two-dimensional gel electrophoresis (2DGE). Proteins were visualized with either silver, Coomassie, or Stains-All (counterstained with silver). None of the gels was destained. Protein spots were in-gel trypsin digested, the tryptic peptides were purified with ZipTip, and the peptides were analyzed with MALDI and ESI MS. Some of the samples that were spotted onto a wax-coated MALDI target plate were recovered and analyzed with ESI MS. All three types of stained gels were compatible with MALDI and ESI MS without any destaining. In-gel trypsin digestion can be performed in only 10–60 min for protein characterization with MS, the sample can be recovered from the MALDI target plate for use in ESI MS, and there was a 90% reduction in sample-processing time from overnight to ca. 3 h. (J Am Soc Mass Spectrom 2004, 15, 784–794) © 2004 American Society for Mass Spectrometry

The conventional method for protein characterization and identification from two-dimensional (2-D) gels involves the destaining of the excised gel pieces, followed by an overnight in-gel enzyme digestion and mass spectrometry (MS) [1–10]. Recently, 4 and 6 h in-gel trypsin digestions have been used for mass spectrometry (MS) analysis [7, 11].

Protein identification with matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) MS has been enhanced by employing methods such as ZipTip (Millipore Corp., Bedford, MA) purification [5, 12–16] and by the use of a hydrophobic sample support for sample purification and sample delivery into the MS. The use of sample supports has been developed to improve detection sensitivity and to remove salts. Some of the polymer materials that have been used for hydrophobic surfaces on MALDI target plates include polytetrafluoroethylene (Teflon; [4, 17–19]), polyurethane [20], paraffin wax film [21], and polyethylene and polypropylene [22]. For routine MS analysis, a sample is processed for either MALDI or ESI MS, or it is "split" for a MALDI and ESI MS analysis. There is no published report on the recovery of tryptic peptides from a MALDI target plate. The aims of this study were to determine the compatibility of in-gel trypsin digestion of stained protein spots with MS, to evaluate the time necessary for in-gel trypsin digestion for protein characterization with MS, and to recover tryptic peptides from a MALDI target plate for subsequent analysis with ESI.

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Experimental

CS Sample

A human CSF sample was obtained after appropriate institutional approvals by lumbar puncture (courtesy of Dr N. M. Oyesiku, Emory University, Atlanta), frozen immediately with liquid nitrogen, and shipped on dry ice to Memphis. It was thawed, a cocktail of protease inhibitors [10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), Complete mini (Roche, Mannheim, Germany)] was added, and the CSF was stored (−80 °C) until analyzed.

Desalting of Cerebrospinal Fluid

CSF samples were desalted by Bio-Spin chromatography, as described by the manufacturer (Bio-Rad Laboratories, Hercules, CA,). Bio-gel P-6 (30 g, Bio-Rad Laboratories) was washed with the standard cycle of washing. A column with a gel-bed volume of 3.5 mL was prepared by pouring a slurry of Bio-gel P-6 in buffer (150 mM sodium chloride, 17.5 mM sodium citrate, pH 7.0) into a 1 × 10 cm column. The buffer in the column was exchanged (3×) with deionized distilled water, and was centrifuged at 1000 × *g* for 1 min. Thawed CSF (1 mL) was applied to the Bio-Gel P-6 column, and was centrifuged (1000 × *g*, 4 min). An aliquot (10 μL) was removed from the desalted CSF for protein assay, and the remainder was stored (−20 °C).

Protein Assay

A Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL) was used to measure the CSF protein content. The protein content of the original and desalted CSF was measured by the Pierce-modified Bradford method [23], using bovine serum albumin as the protein standard. CSF (10 μL) was diluted (1:150, vol/vol) with water, and duplicate aliquots (0.7 mL) of diluted CSF, blank, and known concentrations of the standard, were measured. Coomassie Plus reagent (0.7 mL) was added to each tube, the contents were mixed, and the absorbance of the solution was measured at 595 nm. The standards were plotted, and the CSF protein concentration was obtained.

Two-Dimensional Gel Electrophoresis

An appropriate volume of rehydration buffer [7 M urea, 2 M thiourea, 2% CHAPS, 0.8% ampholyte pH 3–10 non-linear (NL), 0.02% bromophenol blue, and 60 mM dithiothreitol (DTT)] was added to desalted CSF (52–104 μL, contained 25–50 μg protein) to make a total volume of 350 μL. The mixture was agitated (1 h, room temperature) with a vortex mixer (Vortex-Genie mixer, Fisher Scientific, Pittsburgh, PA). The mixture (350 μL) was loaded onto an immobilized pH gradient (IPG) drystrip (pH 3–10, NL, 18-cm, Amersham Biosciences, Uppsala, Sweden). After rehydration (12 h), the proteins were focused with an isoelectric focusing (IEF) unit (IPGphor, Amersham Biosciences, San Francisco, CA), with a six-step program (100 V for 2 h, 500 V for 1 h, 1000 V for 1 h, 2000 V for 2 h, 3500 V for 8 h, and 8000 V for 7 h). A limiting current (50 μA per strip) was maintained. After IEF, the strips were stored (−80 °C) until they were analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The IPG strips were equilibrated (10 min) with 2% (wt/vol) DTT in 4.2 mL/strip of an equilibration solution [0.38 M Tris-base, pH 8.8, 6 mM urea, 2% (wt/vol) SDS and 20% (vol/vol) glycerol], and (10 min) with 2.5% (wt/vol) iodoacetamide in the equilibration solution. Each IPG strip was loaded onto a 12% acrylamide gel, which was sealed with 1% agarose in buffer (see below); and electrophoresed (200 V for 8 h) in a Protein-Plus Dodeca Cell (Bio-Rad Laboratories) in a buffer (25 mM Tris, 190 mM glycine and 0.1% SDS).

Protein Staining

Three different staining methods (silver, Coomassie blue, and Stains-All counterstained with silver) were used to visualize the proteins. (1) The 2DGE gels were stained with silver nitrate, employing a slight modification of Amersham Biosciences' procedure [24]. The gels were fixed (1 h) in an aqueous solution of 40% ethanol:10% acetic acid (vol/vol) for 1 h with gentle shaking on a Maxi Rotator (Lab-Line Instruments, Inc., Melrose Park, IL). They were incubated (30 min) in 100 mL/gel of an aqueous sensitizing solution (30% ethanol, 0.2% sodium thiosulfate, 6.8% sodium acetate), and were washed with three changes of water (5 min each). Thereafter, the gels were incubated (20 min) in 0.25% (wt/vol) silver nitrate solution. They were washed twice with water (1 min each), and were incubated in 100 mL/gel of a developing solution [2.5% (wt/vol) sodium carbonate, 0.015% (vol/vol) formaldehyde]. The developer was changed whenever it turned brown or when a smokey precipitate appeared. After the desired staining intensity was achieved (usually 4 min), the development was stopped by replacing the solution with 100 mL/gel of 5% (vol/vol) acetic acid, and the solution was gently agitated (10 min). The gels were washed with water 3x (5 min each) and were stored. (2) For Coomassie blue, the gels were fixed in an aqueous solution as in (1), and were incubated (1 h) with GelCode® Blue stain reagent (Pierce, Rockford, IL). They were rinsed with water until the background was completely colorless. (3) For Stains-All, the gels were fixed in 25% isopropanol, stained with Stains-All, and counterstained with silver nitrate, according to the method of Goldberg and Warner [25]. Briefly, gels were fixed in 25% (vol/vol) isopropanol (150 mL) on a shaker (50 min). The isopropanol was replaced with Stains-All solution ([150 mL], 30 mM Tris, 7.5% formamide [vol/vol], 25% isopropanol [vol/vol], adjusted to pH 8.8 with HCl, followed by addition of 0.025% Stains-All [wt/vol]). Because Stains-

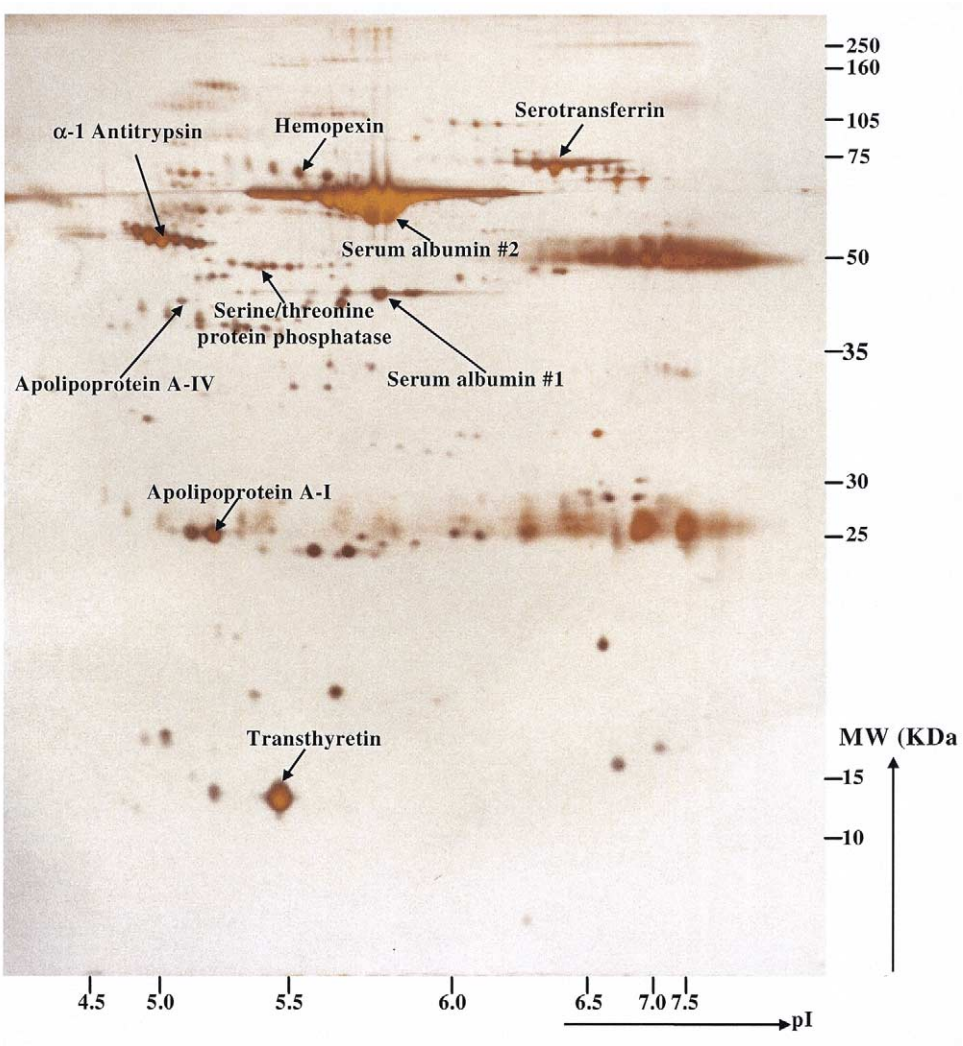

Figure 1. 2-D map of CSF proteins. CSF proteins were separated on a pH 3–10 non-linear IPG strip, followed by 12% SDS-PAGE. The gels were stained with silver nitrate. The nine protein spots that were selected for MS analysis are marked.

All is photosensitive, the gels were incubated (2 h, room temperature) in light-tight containers (i.e., covered with aluminum foil) on a shaker. The gels were destained with 25% isopropanol until the background became clear (approximately 1 h). They were rinsed (3×) with water, and were silver-stained as described above.

Image Analysis

The stained 2-D gels were scanned at 300-dpi resolution with an Epson Expression 800 scanner (Epson, Singapore) and Photoshop software (version 6.2.1, Adobe Systems Inc., San Jose, CA). The gel images were saved as .tiff files, and were analyzed with PDQuest 2-D gel analysis software (version 6.2, Bio-Rad Laboratories).

In-Gel Trypsin Digestion

The in-gel digestion methods were a modification of Gharahdaghi et al. [1]. Each selected spot was excised (with a cut pipette tip) from a wet, stained 2-D gel, and the gel pieces were placed into a siliconized polypropylene microcentrifuge tube (0.6 mL). Some of the gel pieces were destained with a freshly prepared destaining solution (20 μL) that contained 30 mM potassium ferricyanide and 100 mM sodium thiosulfate. The gel pieces destained in <2 min. The destaining solution was removed, and the gel pieces were washed with 50 μL water (5–7 times) until the yellow color disappeared. The gel pieces were washed (2×) with 50 μL of 50% acetonitrile (ACN):50% 200 mM ammonium bicarbonate for 5 min, dehydrated with 50 uL of 100% ACN until the gel turned opaque white, and dried in a vacuum centrifuge for 30 min (Eppendorf Vacufuge; Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany). Some of the gel pieces were not destained, but were washed (2×) with 50 μL of 50% ACN:50% 200 mM ammonium bicarbonate (5 min), dehydrated (50 μL 100% ACN) until the gel turned opaque white, and dried in a vacuum centrifuge (30 min). The gel pieces were rehydrated in 5–10 μL of 50 mM ammonium

Table 1. Sequence coverage vs digestion time of destained gel plugs

(A) MALDI		
Duration of trypsin digestion	Sequence coverage (%)	No. of peptides matched
10 min	85 ± 0	12 ± 1
30 min	86 ± 1	11 ± 1
1 h	100 ± 0	14 ± 0
2 h	85 ± 6	10 ± 1
4 h	88 ± 4	11 ± 1
Overnight	80 ± 11	8 ± 2
(B) ESI		
Duration of trypsin digestion	Sequence coverage (%)	No. of peptides matched
1 h	20 ± 3	14 ± 3
2 h	21 ± 1	15 ± 1
4 h	21 ± 1	14 ± 1
Overnight	21 ± 4	16 ± 2

TR (A) or serum albumin (B) spots were destained, in-gel digested with trypsin at various time intervals, and the digest was analyzed with MS. Those data are mean ± s.d., n = 2–4.

bicarbonate (37 °C, 4 min), an equivalent volume (5–10 μL) of trypsin (Promega, Madison, WI) solution (8.35 ng/μL in 50 mM ammonium bicarbonate) was added, and digestion was performed (10 min, 30 min, 1 h, 2 h, 4 h, and overnight). After digestion, the peptides were extracted (3×) with 50 μL of 60% ACN in 5% TFA (vol/vol) by sonication (10 min). The supernatants were pooled, and were dried in a vacuum centrifuge.

MALDI-TOF MS Analysis

Within a day prior to MALDI MS analysis, the dried tryptic digest samples were reconstituted (10 μL, 0.1% TFA), and were purified with a ZipTip$_{C18}$ (Millipore, Billerica, MA) [15, 26], using the procedure recommended by the manufacturer. Because hydrophobic surfaces have been shown to enhance protein identification [10, 11], a wax hydrophobic surface was used as the standard. A solution (50 mg/mL) of paraffin wax (Surgipath Medical Industries, Richmond, IL) dissolved in chloroform was smoothed onto a stainless steel MALDI target plate (PerSeptive Biosystems, Inc., Framingham, MA) with a Caliber™ cotton-tipped applicator (Allegiance Healthcare Corporation, McGaw Park, IL) to provide a hydrophobic surface. The plate was allowed to dry (5 min, room temperature). The purified peptides were eluted from the ZipTip onto a wax-coated stainless steel MALDI target plate with 2 μL of α-cyano-4-hydroxycinnamic acid (Fluka Chemie GmbH, Steinheim, Germany) solution (2 mg/mL in 50% acetonitrile:0.1% TFA (vol/vol). The solution was allowed to dry (room temperature), and a spot (diameter = 1.25 mm) was produced. The tryptic peptides were analyzed with a Voyager-DE

Table 2. Stained and destained gel spots

MALDI	Sequence coverage (%)	No. of peptides matched
Stained gel piece	69	6
Destained gel piece	69	5
ESI		
Stained gel piece	88	10
Destained gel piece	78	9

TR spots were obtained from replicates of silver-stained gels, and were in-gel trypsin digested overnight (n = 2).

RP MALDI-TOF mass spectrometer (PerSeptive Biosystems/Applied Biosystems, Inc., Framingham, MA). Mass spectra were recorded in the positive-ion, delayed-extraction (DE) mode. All spectra were acquired with 20 kV accelerating voltage, 57% grid voltage, 1.08 mirror voltage ratio, 0.07% guide wire, 150 ns delayed extraction time, and 300 Da low-mass gate. All spectra were internally mass-calibrated with the protonated molecule ions, $(M + H)^+$, of trypsin autodigestion peptides (m/z 515.33, 842.51, and 2211.10) and matrix peaks (m/z 379.11 and 568.14).

Comparing MALDI Target Plate Surfaces

The MS characterization of a model protein digest, transthyretin (TR), on a wax-coated stainless steel MALDI target plate was compared to other MALDI target plates: the AnchorChip (Bruker Daltonics, Bremen, Germany, adapted to the Voyager) and a plain stainless plate. A sample pool was made from the TR protein spot excised from four similarly prepared silver-stained 2-D gels, with 50 μg CSF protein loaded per gel. The four spots were pooled, and were in-gel trypsin digested. Peptides were extracted (3×) with 150 μL (rather than 50 μL) of an aqueous solution of 60% ACN:5% TFA (vol/vol), the extract was dried, reconstituted in 50 μL of 0.1% TFA, and purified with a ZipTip, 10 μL at a time. The peptides were eluted from the

Table 3. Sequence coverage vs. digestion time

Protein	1 h incubation	Overnight incubation
Transthyretin	11 ± 0/82 ± 2	9 ± 0/75 ± 0
Apolipoprotein A-I	13 ± 1/52 ± 3	8 ± 2/29 ± 3
Apolipoprotein A-IV	19 ± 0/46 ± 2	19 ± 2/46 ± 5
Serum albumin (1)	17 ± 1/34 ± 2	16 ± 1/34 ± 0
Serine/Threonine protein phosphatase	6 ± 0/12 ± 0	3 ± 1/7 ± 2
α-1 Antitrypsin	11 ± 3/42 ± 7	14 ± 3/42 ± 6
Hemopexin	12 ± 1/31 ± 2	9 ± 1/26 ± 2
Serum albumin (2)	34 ± 1/59 ± 3	33 ± 0/57 ± 2
Serotransferrin	35 ± 1/56 ± 3	31 ± 2/46 ± 4

No. peptides/% protein coverage.
The protein containing spots were excised from silver-stained 2-D gels, the stained gel plugs were in-gel trypsin digested, and the tryptic peptides were processed for MALDI-TOF MS analysis as described in the Experimental section (Mean ± s.d., n = 3–5).

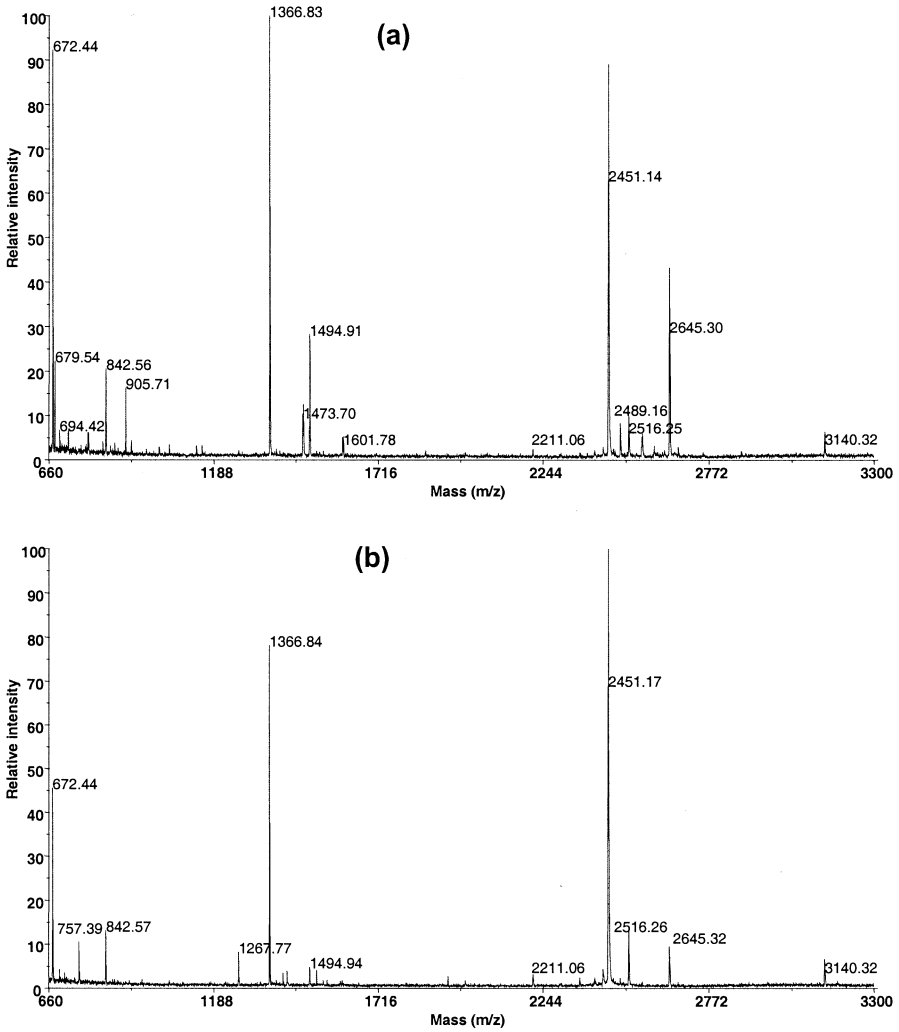

Figure 2. Comparison of MALDI spectra obtained after 1 h versus overnight digestion of transthyretin. The TR spot was excised from silver-stained 2-D gels (25 μg CSF protein/gel). The destained TR spot was in-gel trypsin digested for 1 h (**a**) or overnight (**b**), and was analyzed on a wax-coated stainless MALDI target plate.

ZipTip with 4 μL of 5% ACN:0.1% TFA (vol/vol) followed by 2× 4 μL of 50% ACN:0.1% TFA, and the pooled eluate was concentrated to ca. 30 μL (that eluate was designated the "TR stock"). The TR stock was diluted (1/2 to 1/50) with 5% ACN/0.1% TFA, and each dilution was mixed 1:1 (vol/vol) with a CHCA solution, and spotted onto a MALDI target plate. For wax-coated stainless steel and anchor chip MALDI target plates, 1 part TR dilution was mixed with 1 part of 4 mg CHCA/mL in 50% acetonitrile:0.1% TFA (vol/vol). Therefore, the concentration of CHCA was 2 mg/mL in the spotted sample on the AnchorChip and wax-coated stainless steel MALDI target plates. For a stainless steel MALDI target plate, 8 mg CHCA/mL was used with a CHCA concentration of 4 mg/mL in the spotted sample. Each CHCA concentration was optimized for each particular MALDI target plate. Two microliters of each solution was spotted. The diameters of the dried spots were 1.25, 2, and 3 mm for wax-coated, anchor chip, and stainless MALDI target plates, respectively.

Protein Database Search

The matching of the experimental tryptic peptide mass with the in silico-derived tryptic peptide masses from the database was performed with Peptident proteomic tools in the ExPASy Molecular Biology Server (http://www.expasy.ch/). The SWISS-PROT database was searched within a mass tolerance of ±100 ppm for human proteins; one missed cleavage was allowed. The alkylation of a cysteine residue and the oxidation of methionine were considered as modifications. Proteins were evaluated by considering the number of matched tryptic peptides, the percentage coverage of the entire protein sequence, the experimental M_r, and the experimental *pI* of the 2DGE-separated protein.

Table 4. Sequence coverage vs. stain type

MALDI	Sequence coverage (%)	No. of peptides matched
Silver	75	8
Stains-all + Silver	70	7
Coomassie blue	75	9
ESI	**Sequence coverage (%)**	**No. of peptides matched**
Silver	65	8
Stains-all + Silver	63	7
Coomassie blue	45	6

TR spots were from gels stained with silver, Coomassie blue, or stains-all counterstained with silver nitrate. The 1 h trypsin digests were analyzed with either MALDI, using a wax-coated stainless steel MALDI target plate, or ESI MS (n = 2).

Sample Recovery from MALDI Target Plates

After MALDI MS, 4 μL 0.1% TFA was applied onto the dried spot of tryptic peptides on the MALDI target plate; that volume was pipetted up and down twice, and was transferred to a 0.6 mL siliconized polypropylene microcentrifuge tube. That process was repeated twice with 4 μL 0.1% TFA; the samples were pooled, and were purified with the ZipTip as described earlier. The pooled eluate (16 μL) was concentrated to ca. 4 μL in a vacuum centrifuge (ca. 6 min), and the concentrate was stored (4 °C) until analyzed with ESI MS within 24 h, or it was stored at −20 °C for longer times. Tryptic peptides have been recovered after mass fingerprinting, were used for a sulfonation reaction, and were re-analyzed by MALDI post-source decay MS [27].

LC MS Sample and Analysis

In-gel tryptic peptides were purified with a ZipTip as described above. The tryptic peptides were analyzed with a Finnigan LCQ Deca ion-trap mass spectrometer (ThermoFinnigan Corporation, San Jose, CA) equipped with a nanoESI interface and an in-house packed microcapillary column (PicoFrit; New Objectives, Woburn, MA). The microcapillary column (75 μm i.d. × 360 μm o.d.) was packed with 10.5 cm of 5 μm MagicC18 AQ particles (Michrom BioResources, Inc., Auburn, CA). The tryptic peptide solution (4 μL) was loaded onto the column, and the column eluate was sprayed directly into the ESI source. The mobile phase A was ACN/water (2:98 vol/vol) 0.1% formic acid, and mobile phase B was ACN/water (90:10 vol/vol) 0.1% formic acid. A linear gradient program, from 0 to 80% of B over a period of 15 min (flow-rate 5 μL/min) was used. The LCQ Deca was operated in a data-dependent scan mode in which a MS scan produced three MS/MS scans. The MS/MS spectra were analyzed with the SEQUEST (Revision 2.0) computer program (ThermoFinnigan Corporation), which correlated the experimental data with the theoretical spectra that are generated from known protein sequences.

Table 5. Comparison of nine stained and destained protein spots

Protein	Stained gel	Destained gel
Transthyretin	11 ± 0/82 ± 0	9 ± 1/73 ± 8
Apolipoprotein A-I	13 ± 1/52 ± 3	9 ± 4/50 ± 8
Apolipoprotein A-IV	19 ± 0/46 ± 2	10 ± 3/28 ± 7
Serum albumin (1)	17 ± 1/34 ± 2	17 ± 1/35 ± 3
Serine/Threonine protein phosphatase	6 ± 0/12 ± 0	5 ± 1/11 ± 1
α-1 Antitrypsin	11 ± 3/42 ± 7	11 ± 1/34 ± 5
Hemopexin	12 ± 1/31 ± 2	10 ± 1/27 ± 2
Serum albumin (2)	34 ± 1/59 ± 3	28 ± 1/53 ± 2
Serotransferrin	35 ± 1/56 ± 3	22 ± 5/39 ± 7

No. of peptides matched/% protein sequence coverage.
The stained and destained protein spots from silver-stained 2-D gels were in-gel trypsin digested 1 h, and the peptides were analyzed with MALDI-TOF MS on a wax-coated stainless steel target plate (Mean ± s.d., n = 3–5).

Results and Discussion

The nine protein spots that were excised from stained 2-D gels for this study are shown in Figure 1.

Evaluation of Time Adequate for In-Gel Trypsin Digestion of Protein

The in-gel trypsin digestion of TR and serum albumin spots from replicate silver-stained gels at different times (10 min to overnight) showed no difference in the percentage of protein sequence coverage and the number of peptides (Table 1). For the TR, the highest sequence coverage was obtained at 1 h. Because TR and serum albumin were characterized for each trypsin digestion time, it was not necessary to carry out an in-gel digestion for more than 2 h.

A Comparison of Stained and Destained Gel Plugs Prior to Trypsin Digestion

TR spots from replicates of silver-stained gels were in-gel trypsin digested (overnight), and the tryptic peptides were analyzed with MS (MALDI, ESI). The data showed no significant difference in the sequence coverage between the stained and destained gel pieces (Table 2). Replicate experiments produced similar results.

Nine different spots were excised from silver-stained 2-D gels, in-gel trypsin digested for 1 h or overnight (18 h) and were analyzed with MALDI. There was no difference in the percentage of protein sequence coverage for apolipoprotein A-IV, serum albumin, α-1 antitrypsin, and serotransferrin (Table 3). However, there was a decrease in the number of tryptic peptides and protein sequence coverage for TR, apolipoprotein A-I, serine/threonine protein phosphatase, and hemopexin

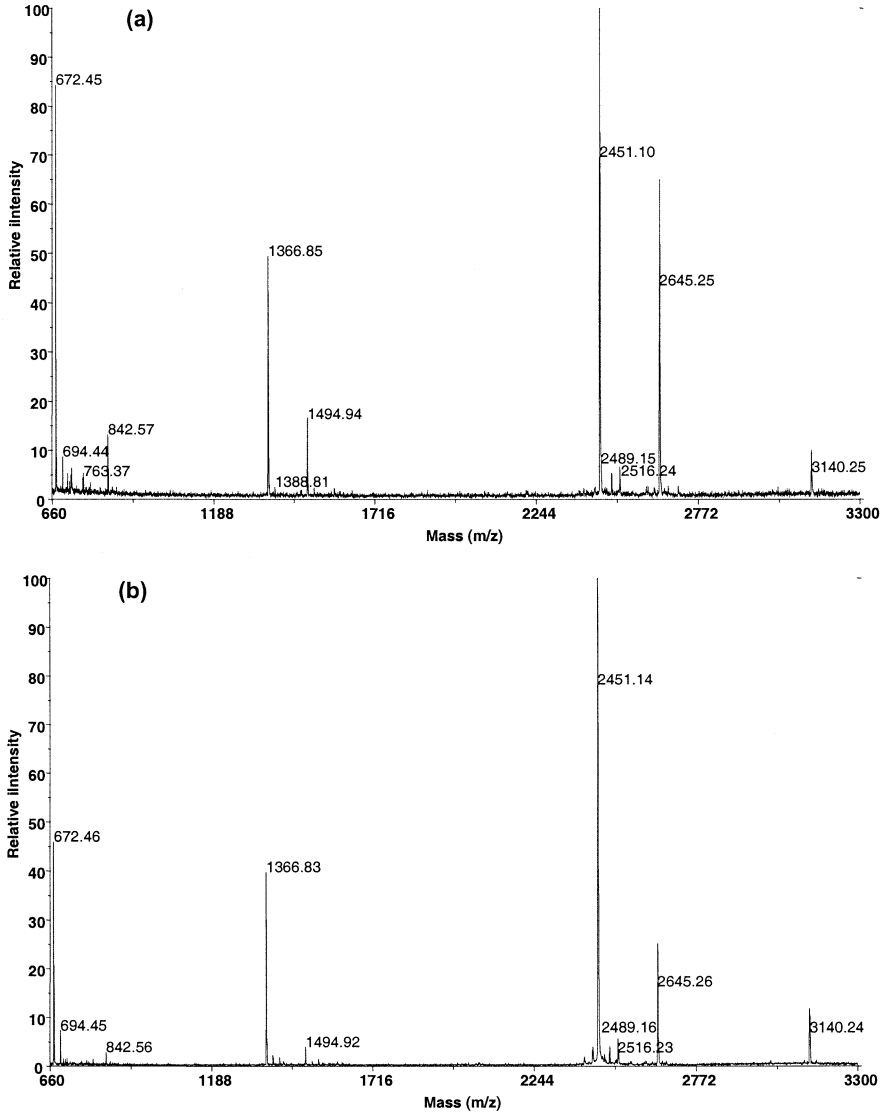

Figure 3. MALDI spectra obtained from stained (**a**) and destained (**b**) TR spot.

after 18 h digestion compared to 1 h. The MALDI spectra for the 1 h and overnight in-gel digestion of TR samples are shown in Figure 2. The relative intensity of the peaks at *m/z* 2645.3, 1494.91, and 905.71 were higher in the 1 h, compared to the overnight, in-gel digestion sample. The relative intensities of the different peptide signals may be due the further digestion of those peptides by trypsin during the prolonged (overnight) incubation, giving rise to smaller peptide fragments. The sequences of the tryptic peptides obtained after a database search show that the peptide at *m/z* 2645.3 has two missed cleavages, and may undergo further digestion by trypsin to give rise to ions at *m/z* 2516.25, 2489, or 2360. Also, *m/z* 1494.91, which has one missed cleavage, can undergo further digestion with prolonged incubation with trypsin to give an ion at *m/z* 1366.83. The decrease in the protein coverage was commonly observed with those spots that contained smaller amounts of protein (based on size and intensity of stained spot).

The compatibility of the three differently stained 2-D gels with MALDI-TOF and LC MS was evaluated. The TR spot excised from gels stained with silver, Coomassie blue, or Stains-All counterstained with silver nitrate were identified with MALDI-TOF and ESI MS (Table 4). Replicate experiments produced similar results.

The characterization of the above destained nine different protein spots was compared between stained and destained spots. The excised spots were in-gel digested, and were analyzed with MALDI-TOF MS. There was no difference between the destained and the stained gel data in terms of the number of peptides and the percentage of protein sequence coverage for serum albumin no. 1, serine/threonine protein phosphatase, α-1 antitrypsin, and hemopexin (Table 5). The MALDI spectra for the tryptic peptide mixture from TR and

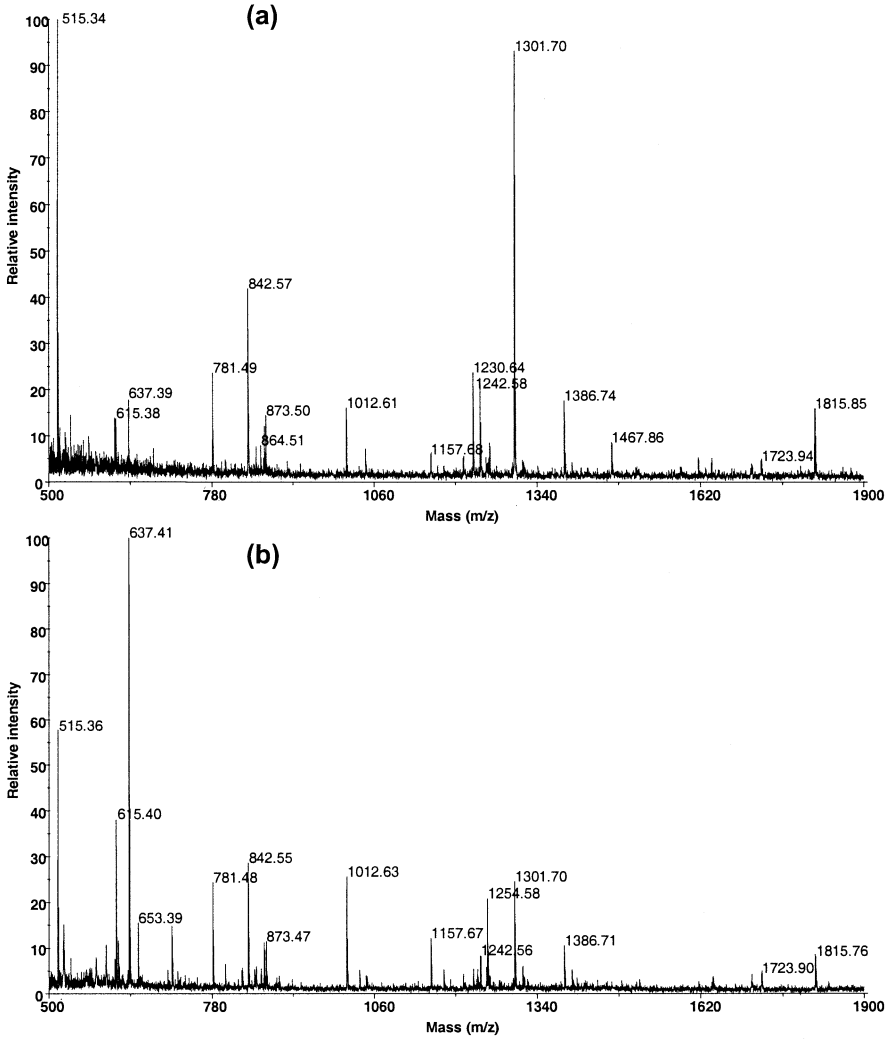

Figure 4. MALDI spectra obtained from stained (**a**) and destained (**b**) apolipoprotein A-I spot.

apolipoprotein A-I are shown in Figures 3 and 4, respectively. The dramatic decrease observed in the relative intensities of the different peptide signals for the destained gel plugs may be due to the loss of protein during the process of destaining. Destaining involved an incubation of gel plugs with a destain solution (30 mM potassium ferricyanide and 100 mM sodium thiosulfate) and several washings with water, and may have led to a leaching-out of the gel plugs water-soluble (hydrophilic) proteins, resulting in some loss in the amount of those proteins. In addition, proteins that have the tendency to refold may be soluble. Therefore, initiating an in-gel trypsin digestion with a smaller amount of protein may result in lower relative intensities of some of the peptide signals.

Comparison of Three Different MALDI Target Surfaces

Three different MALDI target plate surfaces (AnchorChip, wax-coated stainless steel, and stainless MALDI plates) were compared. There was no difference in the number of peptides and the percentage of sequence coverage for the three different target plates (Table 6). The AnchorChip provides no advantage over the wax-coated stainless steel MALDI target plate in the identification of proteins, provided that the correct concentration of CHCA solution is used. The wax-coated

Table 6. Three different MALDI target plate surfaces

Transthyretin dilution	AnchorChip plate	Wax-coated stainless steel plate	Stainless steel plate
Stock	10 ± 0/75 ± 0	10 ± 0/75 ± 0	10 ± 0/75 ± 0
1/2	10 ± 0/73 ± 2	10 ± 0/73 ± 2	9 ± 0/72 ± 2
1/5	4 ± 0/34 ± 0	6 ± 1/61 ± 4	4 ± 0/34 ± 0
1/10	3 ± 0/16 ± 0	4 ± 0/28 ± 6	3 ± 0/16 ± 0
1/50	1 ± 0/5 ± 0	1 ± 0/5 ± 0	0

No. peptides/% protein coverage.
The same TR gel spot pool was diluted and analyzed on three different MALDI target plate surfaces. Mean ± s.d., $n = 3$.

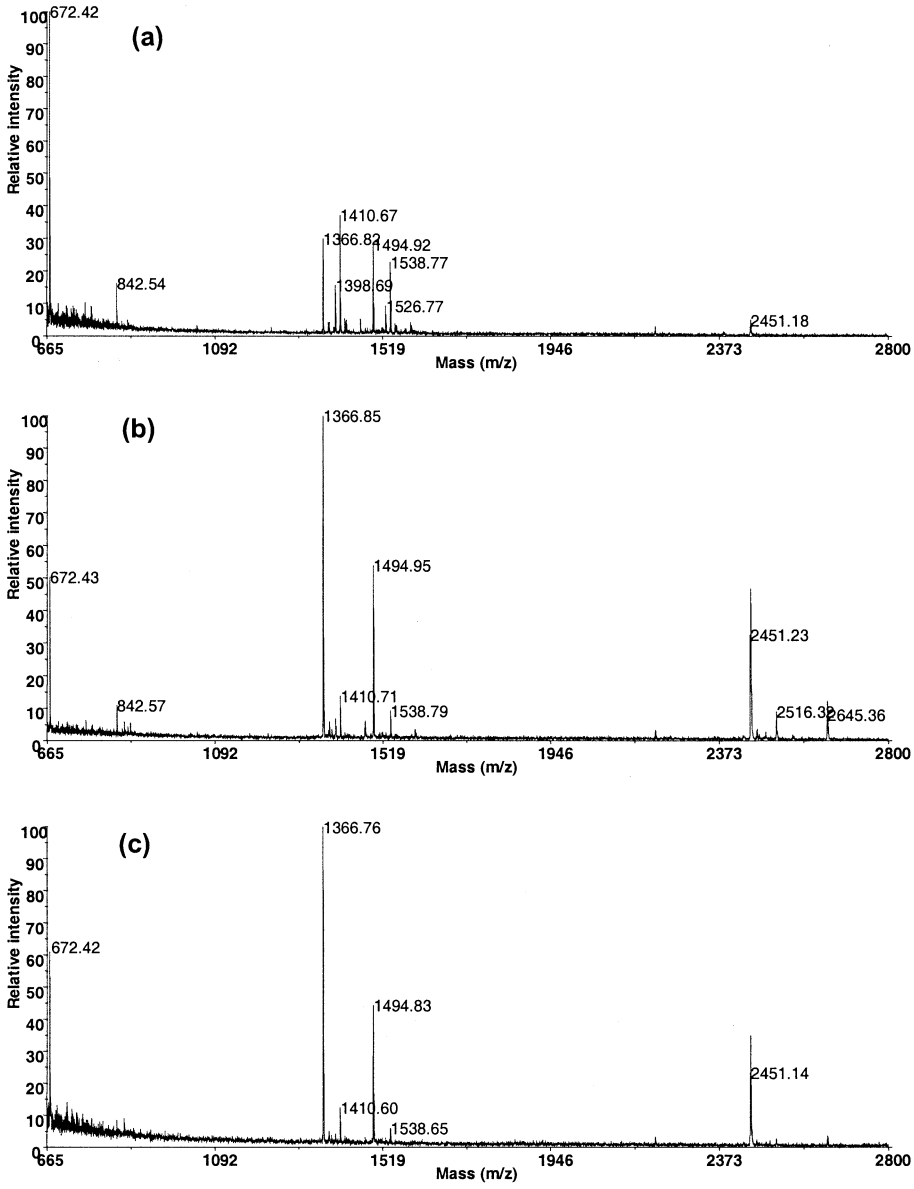

Figure 5. Comparsion of a tryptic digest on three different MALDI target plate surfaces. A pool of TR protein spots (4 silver-stained 2-D gels) was in-gel trypsin digested for 1 h. The extracted tryptic peptides were ZipTip-purified, and were concentrated (to 30 μL; referred to as stock). The stock TR was diluted 1/5, and was analyzed on either an AnchorChip (**a**), wax-coated stainless steel (**b**), or stainless steel (**c**) MALDI target plate. Note that the trypsin autodigestion peak (*m/z* 842.51) is absent on the stainless steel target plate.

MALDI target plate provided an increased detection sensitivity, especially at low peptide concentrations (1/5 and 1/10 TR stock dilutions). At the 1/5 and 1/10 TR dilutions, the number of peptides and the percentage of protein sequence coverage were the highest with the wax-coated stainless steel plate compared to the AnchorChip and stainless steel MALDI target plates. At the 1/50 TR dilution, only one peptide was identified for the AnchorChip and wax-coated stainless steel MALDI target plates, and none for the stainless steel MALDI target plate. The MALDI spectra for the 1/5-diluted TR tryptic peptides spotted onto the three different MALDI target plates showed two peptide peaks (*m/z* 2517.32 and 2645.36) that were present in only the wax-coated stainless steel MALDI target plate (Figure 5). The calculated hydrophobicity, using a scale for the relative hydrophobicity of amino acids [28], for the tryptic peptides at *m/z* 2517.32 and 2645.36 are −4 and −13.9, respectively. The absence of those two tryptic peptides in the AnchorChip and stainless steel plates may not be due to the hydrophobicity, because other tryptic peptides at *m/z* 1366.82 and 1410.67 are −4. A trypsin autodigestion peak (*m/z* 842.51) was present in the AnchorChip and wax-coated stainless steel

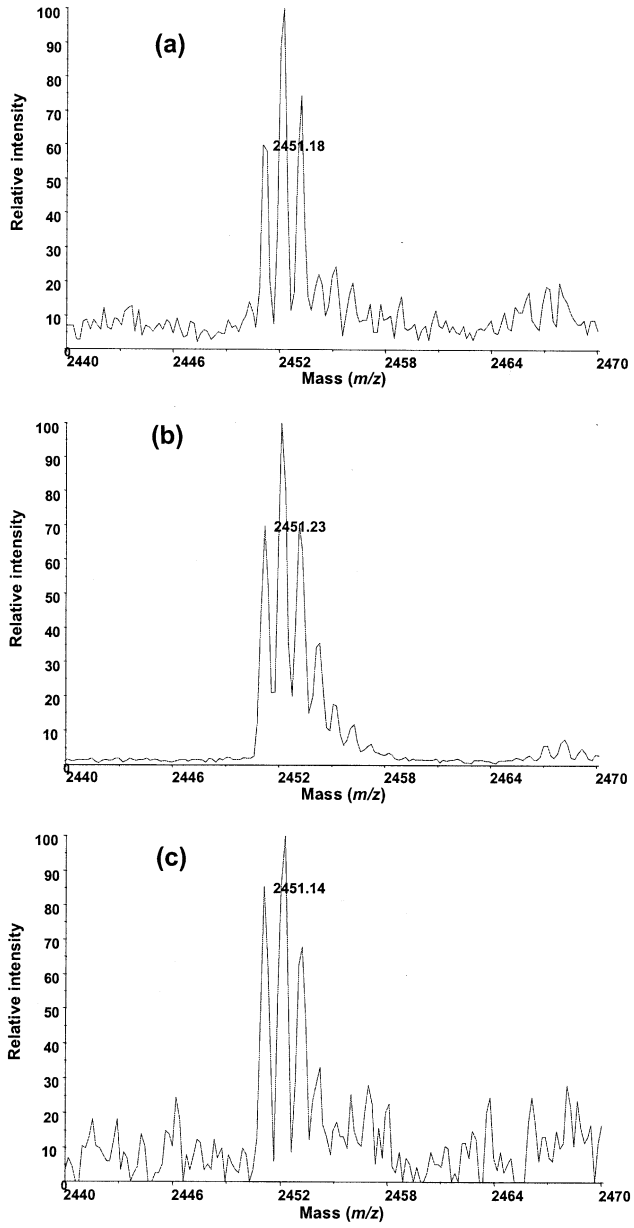

Figure 6. Quality of MALDI spectra with different MALDI target plates. The spectra of a selected TR tryptic peptide (*m/z* 2451.21) obtained from the MALDI-TOF MS analysis of 1/5-diluted TR tryptic peptides spotted onto either an AnchorChip (**a**), wax-coated stainless steel (**b**), or stainless steel (**c**) MALDI target plate.

plates, but not the stainless steel MALDI target plate. The signal/noise ratio of the TR peptide peak *m/z* 2451.21 was best with the wax-coated stainless steel MALDI target plate (Figure 6). One of the more striking features of the waxed plate was the increase in the signal/noise ratio. The highest percentage of protein-sequence coverage obtained with the wax-coated stainless steel plate compared to the AnchorChip and stainless steel MALDI target plates could be due to the fact that the wax-coated stainless steel MALDI target plate concentrates the spotted sample ca. 2× more than the other conventional target plates (AnchorChip and stainless steel MALDI target plates). Two microliters of sample eluted from the ZipTip and spotted onto the AnchorChip, wax-coated stainless steel, or stainless steel MALDI target plate gave 2, 1.25, and 3 mm dried spot size, respectively.

Table 7. Recovery of tryptic peptides from three different MALDI target plates

MALDI	AnchorChip plate	Wax-coated stainless steel plate	Stainless steel plate
Transthyretin	10 ± 0/75 ± 0	10 ± 0/75 ± 0	10 ± 0/75 ± 0
Serum albumin	19 ± 0/34 ± 1	22 ± 0/36 ± 0	16 ± 2/27 ± 1
Apolipoprotein A-I	11 ± 1/37 ± 2	12 ± 1/38 ± 3	13 ± 0/40 ± 0

ESI	AnchorChip plate	Wax-coated stainless steel plate	Stainless steel plate
Transthyretin	7 ± 1/69 ± 7	8 ± 1/72 ± 9	5 ± 1/60 ± 7
Serum albumin	15 ± 1/23 ± 3	14 ± 1/21 ± 1	12 ± 1/18 ± 1
Apolipoprotein A-I	10 ± 2/38 ± 6	13 ± 2/48 ± 10	9 ± 2/33 ± 4

No. peptides/% protein coverage.
The tryptic peptide samples were analyzed with MALDI MS, were recovered from the MALDI target plate, and were analyzed with ESI MS. Mean ± s.d., $n = 3$.

Tryptic Peptide Recovery from MALDI Target

The recovery of tryptic peptides from the three different types of MALDI target plates (AnchorChip, wax-coated stainless steel, stainless steel) was evaluated. Three protein spots (TR, serum albumin, and apolipoprotein A-I) from silver-stained 2-D gels were in-gel trypsin digested (1 h). The tryptic peptides were analyzed with MALDI-TOF MS, using AnchorChip, wax-coated stainless steel, or stainless steel MALDI target plates. Thereafter, the tryptic peptides were recovered and analyzed with ESI MS (Table 7); the proteins were characterized. This combined strategy could readily be employed for unknown and unidentified samples from MALDI-TOF MS because the sample can be analyzed subsequently with ESI MS to obtain amino acid sequence data.

Conclusion

A wax-coated stainless steel MALDI target plate improved the quality of the MALDI spectra, and is also less costly compared with an AnchorChip target plate. It is not necessary to destain 2-D gel pieces prior to an in-gel enzyme digestion, and tryptic peptides can be recovered from a MALDI target plate for LC MS/MS to obtain amino acid sequence data. Improved protein identification sensitivity and a reduction in sample-processing time are of particular benefit to the characterization of low-abundance proteins, and for a cost-effective, high-throughput system.

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