Original Article

Technical Evaluation of MALDI-TOF Mass Spectrometry for Quantitative Proteomic Profiling

Matrix Formulation and Application

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Abstract

Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) has been recently used to identify disease markers by directly profiling and quantifying the peptide/proteins in biological samples under different physiological or experimental conditions. The information of reproducibility of such quantitative profiling method has not been available. It is important to evaluate and reduce error from technical variation. In this study, an unbiased signal acquisition strategy was used to evaluate the effects of three sample-matrix spotting methods and two matrix chemicals, α-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid, on the reproducibility of the peptide/protein signal intensities. The sandwich spotting method using 0.1% nitrocellulose coating film and CHCA gave the best quantitative results for the standard peptides and proteins with mass <66.5 kDa. The normalized signal intensities of the standard peptides and proteins were directly proportional to their concentrations with intra-assay (within-day) coefficient of variations (CVs) ranging from 6.5% to 17%. When analyzing serum peptides <6000 m/z, the interassay (between-days) CVs of all the evaluated peptide peaks were <15%. These data indicate that with the right MS analysis conditions, MALDI-TOF MS appears to be a feasible tool for directly profiling and quantifying the peptide/proteins in biological samples.

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Key Words: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; quantitative proteomics; nitrocellulose; reproducibility; variation.

Introduction

Quantitative proteomics is concerned with the identification of biological and physiological changes in protein expression patterns between biological samples under different physiological, pathological, or experimental conditions. Two-dimensional polyacrylamide gel electrophoresis (2D PAGE) is the most commonly used technology to separate individual proteins, followed by image analysis to obtain the quantity of individual protein spots. Subsequently, the protein identity of a spot of interest can be obtained by peptide mass fingerprinting with the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) *(1)*.

Recently, there has been increasing interest in using gel-free approaches, such as surface-enhanced laser desorption/ionization (SELDI)-TOF ProteinChip technology *(2–4)* and direct application of MALDI-TOF MS *(5–8)*, to profiling and quantification of individual peptides/proteins in biological samples. MALDI-TOF MS was applied to direct profiling and imaging of peptides and proteins from mammalian cells and tissue sections *(5,6)*. Liquid-phase isoelectric focusing electrophoresis has been used to reduce signal suppression effects in the MALDI-TOF MS analysis of serum proteins *(7)*. This approach was used to identify potential lung cancer markers by comparing the serum peptide/protein profiles between lung cancer patients and individuals without cancer *(8)*.

MALDI-TOF MS has been being regarded as a semiquantitative technology, and the quantitative aspects have not been well examined. It has been shown that when a mixture of very pure peptides/proteins is analyzed, the relative ion yields of different peptide and protein molecules can vary significantly from preparation to preparation *(9)*. As quantitative proteomics identifies disease markers by measuring relative differences between biological samples under different physiological or experimental conditions, it is important to evaluate the signal linearity and error caused by technical variation and to increase the precision.

In this study, using an unbiased automatic mass spectrum acquisition protocol, we examined how different sample-matrix spotting methods and matrix chemicals affect the reproducibility of peptide/protein ion signals when MALDI-TOF MS was used for direct quantitative measurement of mixtures of peptides and proteins. Finally, using serum as an example, we attempted to apply our optimized methodology in analyzing the peptide profile of a complex peptide-protein mixture and examined the interassay variation.

Materials and Methods

Nitrocellulose Preparation

A 2% (w/v, 20 g/L) nitrocellulose (NC) solution was prepared by dissolving Hybond C NC membrane (Amersham Biosciences, Piscataway, NJ) in pure acetone and subsequently diluting it with same volume of isopropanol to form the 1% NC stock solution. Different concentrations of NC solution (0.01%, 0.05%, 0.1%) were then prepared by diluting with 1:1 (v/v) acetone-isopropanol solution.

Preparation of Matrix Chemical Solution and Standard Peptide/Protein Mixture

An α-cyano-4-hydroxycinnamic acid (CHCA; Sigma, St. Louis, MO) matrix solution was prepared by dissolving 1 mg of CHCA in 250 μL of 50% acetonitrile (ACN) containing 0.3% (v/v) trifluoroacetic acid (TFA). The saturated solution was then diluted four times with 40% ACN containing 0.1% TFA. A sinapinic acid (SA; Fluka, St. Louis, MO) matrix solution

Table 1
Peptide and Protein Standards
Used in the Present Study

Peptide/protein standard	Theoretical average mass, MH$^+$	Concentration (pmol/μL)
Des-Arg-bradykinin	905.05	1.00
Angiotensin I	1297.51	3.30
Glu-fibrinopeptide B	1571.61	1.30
ACTH (clip 1–17)	2094.46	2.00
ACTH (clip 18–39)	2466.72	1.50
ACTH (clip 7–38)	3660.19	3.00
Insulin (bovine)	5734.59	4.50
Thioredoxin (*E. coli*)	11674.48	5.50
Apomyoglobin (horse)	16952.56	8.00
Bovine serum albumin	66431.00	2.40
IgG (mouse)	148500.00	6.00

The concentrations that were used in the intra-assay error experiments are listed. One microliter of the standard mixture was subjected to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis. ACTH, adrenocorticotropic hormone.

(1 g/L) was prepared by dissolving SA in 30% ACN and 0.2% TFA. Peptide and protein standards were prepared from the Sequazyme peptide mass standard kit (Applied Biosystems, Foster City, CA). Components of the standard mixture are listed in Table 1.

Sample–Matrix Spotting Methods

The application of sample and matrix chemical on a 100-shallow-well gold MALDI sample stage (Applied Biosystems) was performed on open bench at 24°C and approx 70% humidity. Three types of sample-matrix spotting methods were used, as summarized below:

Direct Mixing Method

One microliter of sample was mixed with 1 μL of matrix chemical. Two microliters of the sample-matrix mixture was then deposited onto a shallow well (3.14 mm^2) and air-dried.

Overlaying Method

One microliter of sample was directly spotted onto a shallow well and air-dried. Then 1 μL of matrix chemical was overlaid on the sample afterward and air-dried.

NC Sandwich Method

One microliter of NC solution (0.01%, 0.05%, 0.1%, 0.25%, 0.5%, or 1.0%) was first spotted onto a shallow well with a positive displacement pipette and air-dried. Then 1 μL of matrix chemical was spotted and air-dried, followed by addition of 1 μL of sample and air-drying. Finally, another 1 μL of matrix chemical was spotted over the sample and air-dried.

Unbiased Automatic MALDI-TOF MS Mass Spectrum Acquisition

All MALDI-TOF MS mass spectra were acquired on a Voyager DE-PRO Biospectrometry Workstation (Applied Biosystems) in positive linear mode using a nitrogen laser (337 nm). The acceleration voltage was 25 kV. The low- (600–10,000 Dalton) and high- (10–200 kDa) molecular-weight proteins were detected by different settings to achieve highest resolutions. The laser power, grid voltage, and guide wire voltage were optimized for different sample-matrix spotting methods, different matrix chemicals, and different mass ranges.

To overcome the problems of variations of MS signals from different sample-matrix cocrystals, we collected each sample mass spectra automatically from 104 different positions on the sample-matrix spot in a spiral distribution pattern. For each position, MS data were collected from 35 laser shots. The final mass spectrum of a sample was the average of the data (total of 3640 laser shots) from the 104 different positions. This automatic MS

acquisition method aimed to avoid biased selection of a "good" sample-matrix cocrystal for mass spectrum collection, which might not reflect accurately the peptide/protein content of a sample.

Normalization of Peptide/Protein Signal Intensity

The raw spectra were processed with the Voyager Data explorer software (version 4.0.0, Applied Biosystems). The whole isotope envelope was used to calculate the signal intensity of a peptide/protein. After baseline subtraction, the signal intensity of each peptide/protein was normalized by presenting either as a percentage of total peak area, or as a relative peak intensity with the use of the following equations.

Percentage of total peak area

$$= \frac{\text{Peak area of the interested peak}}{\text{Sum of areas of all peaks}} \times 100\%$$

Relative peak intensity

$$= \frac{\text{Peak area of the interested peak}}{\text{Peak area of an internal reference peptide}} \times 100\%$$

Measurement of Signal Variations and Linearity

The peptide/protein standards and their quantities (at low picomole levels) used in the evaluation of intra-assay error are listed in Table 1. For each combination of sample-matrix spotting method and matrix chemical, 10 identical sample-matrix spots were prepared and subjected to MS analysis independently for the examination of intra-assay (within-day) coefficient of variation (CV) for each standard peptide/protein. For determination of interassay (between-days) CV, a control sample was analyzed in duplicate, and the MS analysis was performed on five different days for a total of five times. For assessing the linearity of the peptide/protein signals, the amount of a peptide/protein of interest was varied while fixing the concentration of the internal reference peptide.

Quantitative Profiling of Serum Peptides

One microliter of peptide standards at low picomole levels (Table 1) were spiked into 20 μL serum as an internal reference. The serum was diluted and acidified with 60 μL of 1% TFA solution. The mixture was then subjected to ultrafiltration with a 30-kDa-cutoff Microcon centrifugal filter device (Millipore, Billerica, MA) according to the manufacturer's instructions. Two microliters of the filtrate was analyzed with the use of the NC sandwich method and CHCA matrix. The signals within the range of 900–6000 m/z were collected with the use of the unbiased automated acquisition method. When examining the interassay variation, the control serum sample was processed and applied onto the source plate on the day of measurement.

Results

Effect of Sample-Matrix Spotting Methods and Matrix Chemicals on Reproducibility of Peptide/Protein Signals

Because it is well established that CHCA gives the highest sensitivity and resolution in the detection of peptides, we only used CHCA as the matrix chemical when quantitatively profiling proteins <10 kDa (i.e., peptides) in the peptide/protein standard mixture. The results are summarized in Table 2. With the use of the unbiased automatic MS acquisition protocol and the total peak area normalization method, the direct mixing sample-matrix spotting method (i.e., the conventional dried-drop method) resulted in intra-assay CV values from 10.5 to 27.5%. Among all the tested sample-matrix spotting

Table 2
Summary of Coefficient of Variations (CV, %) of Different Sample-Matrix Spotting Methods in Detecting Low-Molecular Weight Proteins (Peptides)

Protein standard	CV (%) of percentage of total peak area					CV (%) of relative peak intensity (angiotensin as internal reference)				
	Direct mixing	Sandwich				Direct mixing	Sandwich			
		Overlaying	0.1% NC	0.05% NC	0.01% NC		Overlaying	0.1% NC	0.05% NC	0.01% NC
Des-arg-bradykinin (905.05 Dalton)	22.1	12.5	12.5	12.0	11.1	24.5	25.4	6.4	9.2	10.1
Angiotensin (1297.5 Dalton)	27.5	23.1	13.5	11.8	6.1	NA	NA	NA	NA	NA
Glu-fibrinopeptide B (1571.6 Dalton)	21.8	15.0	12.2	18.9	17.6	25.4	16.3	10.9	12.1	14.9
ACTH (clip 1–17) (2094.5 Dalton)	23.0	9.2	10.5	6.3	13.5	28.2	15.3	6.5	7.3	14.3
ACTH (clip 18–39) (2466.7 Dalton)	16.4	5.9	10.7	11.0	22.6	21.9	23.3	13.1	21.3	28.6
ACTH (clip 7–38) (3660.2 Dalton)	21.2	23.2	11.1	12.7	17.5	25.9	37.9	16.2	24.3	18.8
Insulin (5734.6 Dalton)	10.6	32.2	14.3	25.6	22.4	34.1	49.7	16.9	36.9	25.4

The protein peak intensities were normalized with either the total peak area or the peak area of the internal reference. α-cyano-4-hydroxycinnamic acid was used as the matrix chemical. NA, Not applicable; ACTH, adrenocorticotropic hormone; NC, nitrocellulose.

methods, the sandwich method with the use of 0.1% NC provided the best reproducibility of the peptide ion signals. The peak intensity could be normalized with either the total peak area or with the peak area of an internal reference peptide. These two normalization procedures resulted in intra-assay CVs in the range of 6.5 to 17%.

For profiling proteins in the range of 10–150 kDa, the effects of the three sample-matrix spotting methods and two matrix chemicals were examined. The results are summarized in Table 3. When only thioredoxin (11.67 kDa), apomyoglobin (16.85 kDa), and bovine serum albumin (BSA; 66.43 kDa) were considered, the sandwich method with the use of 0.1% NC and CHCA matrix was still a reliable method, resulting in the low CV values for various proteins. When the signal intensity was normalized with an internal reference, the CV for of measurement of BSA was only 8.5%.

When SA was used as the matrix chemical, all of the proteins within the detectable range, including mouse IgG, were ionized and detected. However, in general, the results were less reproducible (relatively higher CVs) when compared with those obtained with the use of CHCA. Because the sandwich method (0.1% NC and CHCA) gave the best quantitative results for profiling peptides/proteins of mass <66,5 kDa, this approach was used and referred to as NC-MALDI-TOF MS in the following experiments.

Assay Linearity of the Nitrocellulose-MALDI TOF MS for Peptides <6000 Dalton

The linearity of the NC-MALDI-TOF MS method in quantitative profiling of a mixture of peptides between 900 and 6000 Dalton was examined. Adrenocorticotropic hormone (ACTH; clip 18–39) or glu-fibrinopeptide B was used as the internal reference peptide, and was added to the samples at a fixed concentration. Figure 1 shows typical mass spectra of the

quantitative analysis of the peptides when using glu-fibrinopeptide B as the internal reference. Using glu-fibrinopeptide B (1571.61 m/z) as the internal reference peptide, the relative peak intensities of ACTH clip 1–17 (2094.46 m/z), ACTH clip 18–39 (2466.72 m/z), ACTH clip 7–38 (3660.19), and insulin (5734.59 m/z) increased with their concentrations (Fig. 2A–D). Using ACTH clip 18–39 as the internal reference peptide, similar correlations were observed when measuring the intensities of des-arg-bradykinin (905.1 m/z) and glu-fibrinopeptide B (Fig. 2E,F). Linear equation was used in the curve fitting. All R^2 values were ≥0.97. The results demonstrate that the relative peak intensity is directly proportional to the peptide concentration regardless of the mass of a peptide.

Accuracy of Mass Measurement

The accuracy and precision of the NC-MALDI-TOF MS method in obtaining the peptide mass values were examined. The results are summarized in Table 4. For the assessed mass range between 900 and 6000 Dalton, percentage errors for all of the tested standard peptides were <0.05%. The CVs were all <0.03%. These results suggest that introduction of a NC layer does not significantly affect the mass accuracy of MALDI-TOF MS.

Application in Quantitative Profiling of Serum Peptides

Serum samples from four individuals were used to examine whether the NC-MALDI-TOF MS method could be used in the quantitative profiling of peptides in complex biological samples. In the initial study, peptide standards were first added to the serum as internal control, and the mixture was diluted fourfold and acidified with 1% TFA. No detectable signal in the peptide range was observed. To reduce the complexity, we ultrafiltrated the fourfold diluted and acidified samples with a 30-kDa-cutoff filter to remove all of the large molecular

Table 3
Summary of Coefficient of Variations (CV, %) of Different Sample-Matrix Spotting Methods in Detecting
High-Molecular-Weight Proteins

Matrix	Protein standard	CV (%) of percentage of total peak area					CV (%) of relative peak intensity (apomyoglobin as internal reference)				
		Direct mixing	Sandwich				Direct mixing	Sandwich			
			Overlaying	0.1% NC	0.05% NC	0.01% NC		Overlaying	0.1% NC	0.05% NC	0.01% NC
CHCA	Thioredoxin (11.7 kDa)	7.3	15.6	9.0	37.6	65.3	9.4	8.6	9.3	41.2	78.7
	Apomyoglobin (17.0 kDa)	5.1	11.7	11.7	3.6	11.8	NA	NA	NA	NA	NA
	Albumin (66.4 kDa)	11.9	32.5	18.6	11.8	22.6	15.0	37.7	8.5	14.2	36.3
	IgG (148.5 kDa)	ND	10.2	ND	ND	ND	ND	14.3	ND	ND	ND
SA	Thioredoxin (11.7 kDa)	27.6	11.4	13.4	9.1	7.4	27.1	10.6	14.2	10.4	13.1
	Apomyoglobin (17.0 kDa)	8.3	3.6	7.9	9.7	14.7	NA	NA	NA	NA	NA
	Albumin (66.4 kDa)	5.3	20.5	22.5	22.7	42.3	8.7	22.3	26.3	25.9	16.0
	IgG (148.5 kDa)	ND	33.5	56.7	61.9	42.3	ND	35.5	57.7	64.5	45.7

The protein peak intensities were normalized with either the total peak area or the peak area of the internal reference. NA, not applicable; ND, not detectable; CHCA, α-cyano-4-hydroxycinnamic acid; SA, sinapinic acid; NC, nitrocellulose.
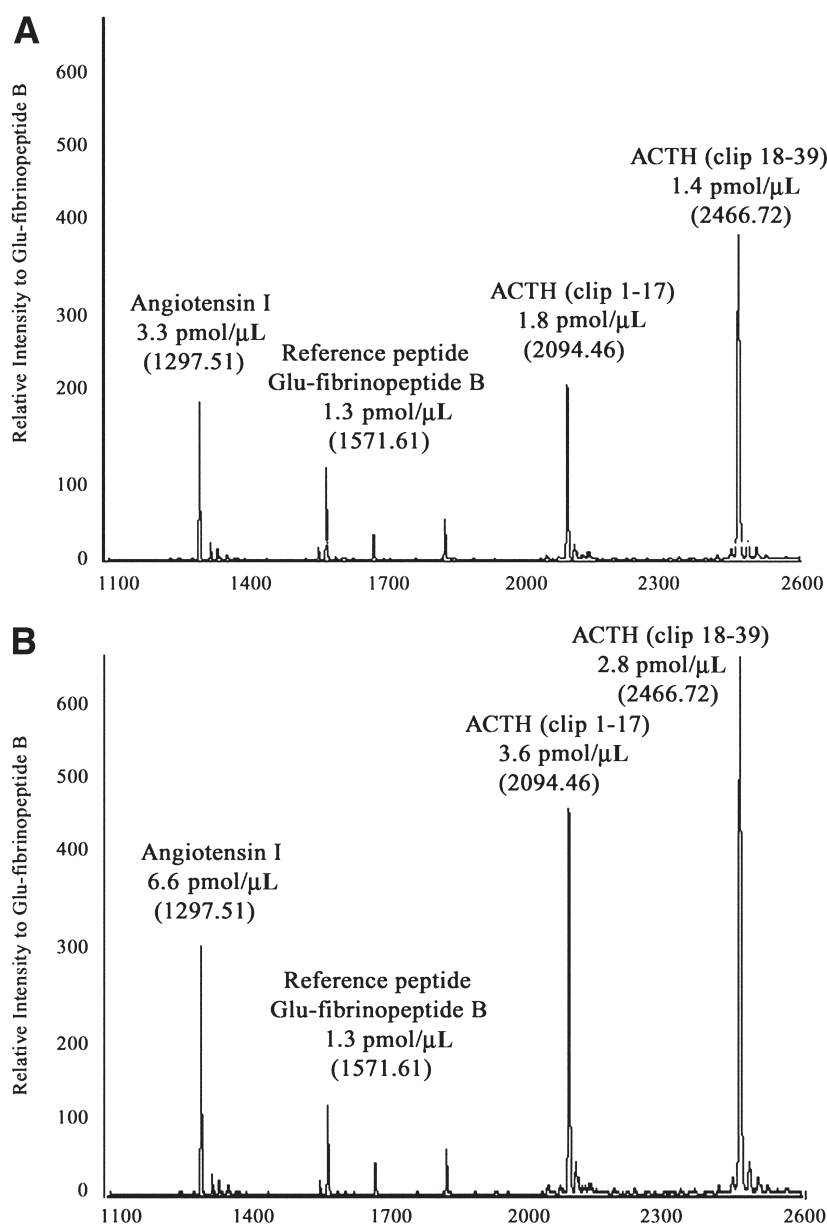
Fig. 1. Typical mass spectra of the quantitative analysis of the peptides when using glu-fibrinopeptide B as the internal reference. When the concentration of the internal reference peptide glu-fibrinopeptide B was kept constant, the relative intensities of angiotensin I, adrenocorticotropic hormone (ACTH; clip 1–17), and ACTH (clip 18–39) increased with their concentrations.

weight proteins before MS analysis. After removing the proteins >30 kDa, peptide peaks were observed in the mass spectra of the serum samples (Fig. 3). One of the serum samples was used to examine the interassay errors. The interassay errors of 18 representative peptide peaks (sample A) were examined and are summarized in Table 5. All of the interassay CVs were between 3.5 and 15%.
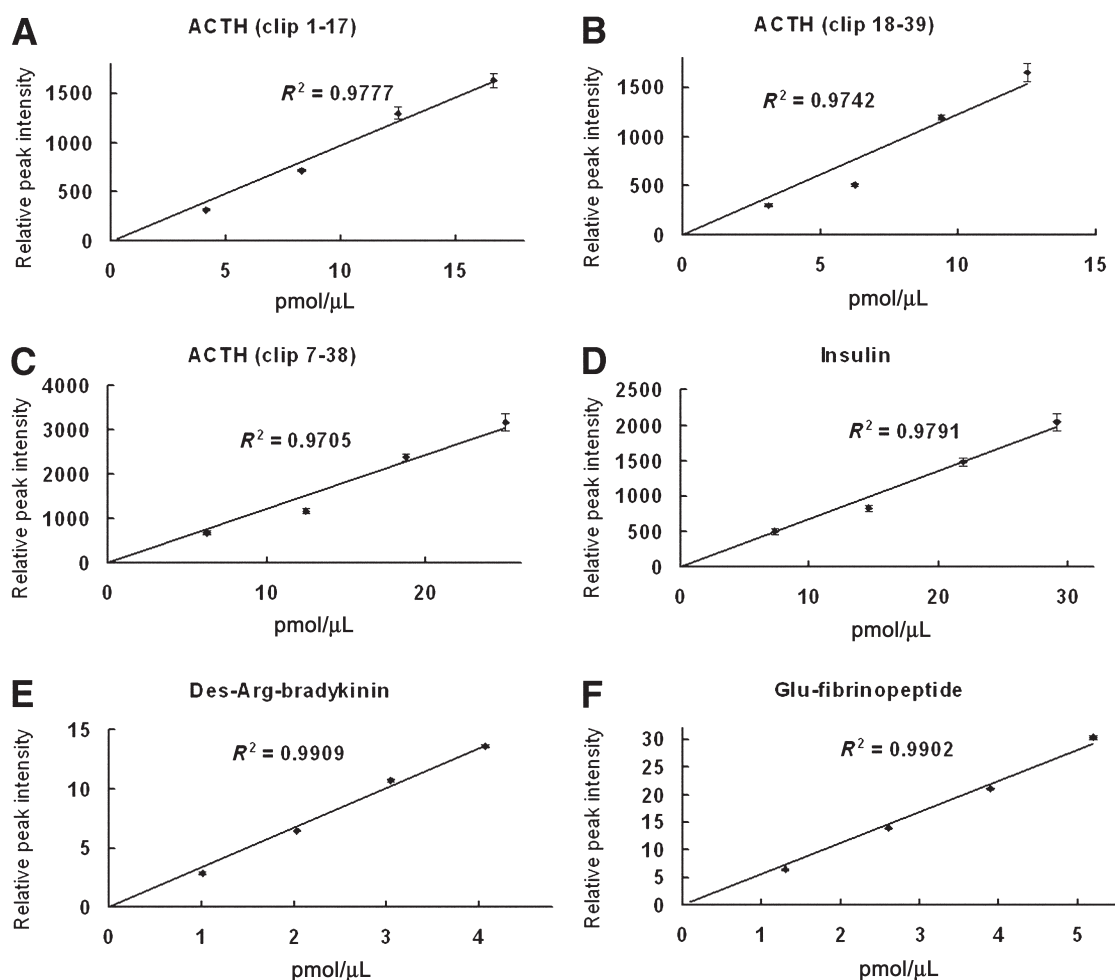
Fig. 2. The relative intensities of the tested peptides were plotted against their concentrations. Assayed peptides: **(A)** adrenocorticotropic hormone (ACTH; clip 1–17; 2094.46 m/z); **(B)** ACTH (clip 18–39; 2466.72 m/z); **(C)** ACTH (clip 7–38; 3660.19); **(D)** insulin (5734.59 m/z); **(E)** des-arg-bradykinin (905.05 m/z); and (F) glu-fibrinopeptide B (1571.61). Internal reference peptide: **(A–D)** glu-fibrinopeptide B; **(E,F)** ACTH (clip 18–39). Linear equation was used in the curve fitting. The error bars represent the standard errors of the mean relative intensities.

Discussion

Understanding the precision and linearity is crucial to all quantitative studies, including quantitative proteomic experiments. In experiments aiming to identify significant differences between two sample groups, the statistical power depends on the CVs of the quantitative measurements. For assays with higher CVs, a larger sample size is needed to achieve the same degree of statistical power

(10). In this study, we have demonstrated that different sample-matrix spotting methods and matrix chemicals do affect the reproducibility of the intensity of peptide/protein ion signals. The quantitative capability of MALDI-TOF MS can be easily improved with the use of a thin film of NC coating for sample-matrix cocrystallization. The normalized signal intensities of the peptides/proteins were directly proportional to their concentrations with

Table 4
Summary of the Accuracy and Precision of Measurement of the Masses for the Peptide
Standards by the NC MALDI-TOF MS Method

Peptide	Theoretical average mass, MH⁺	Mean of masses from five independent assays, MH⁺	Percentage error	CV (%)
Des-arg-bradykinin	905.05	904.68	0.041	0.025
Angiotensin I	1297.51	1297.18	0.025	0.024
Glu-fibrinopeptide B	1571.61	1571.35	0.017	0.026
ACTH (clip 1–17)	2094.46	2094.32	0.007	0.025
ACTH (clip 18–39)	2466.72	2466.68	0.002	0.025
ACTH (clip 7–38)	3660.19	3660.26	−0.002	0.025
Insulin	5734.59	5734.95	−0.006	0.025

The masses shown were obtained from five independent assays. NC, nitrocellulose; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis; CV, coefficient of variations; ACTH, adrenocorticotropic hormone.
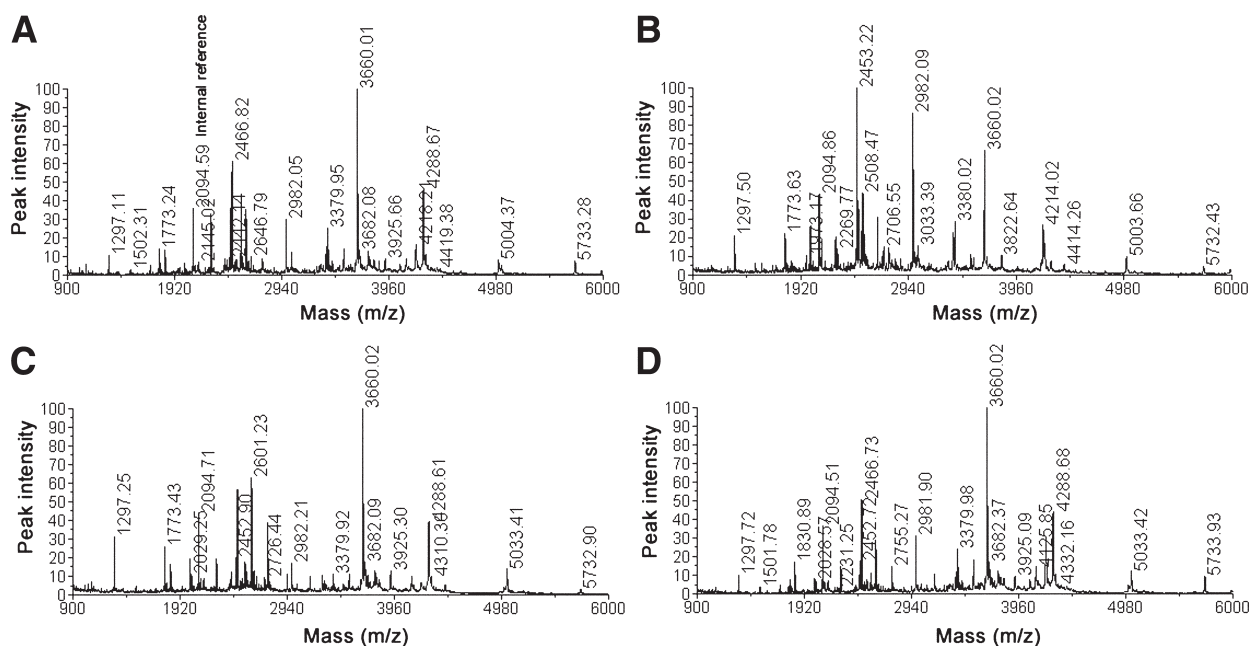

Fig. 3. Representative mass spectra of serum samples from four individuals with the peptide range of 900–6000 m/z. The peptide standard adrenocorticotropic hormone 1−17 clip (2094.46 m/z) was used as the internal reference. The interassay errors for sample A are listed in Table 5.

intra-assay and interassay CVs <17%. The reproducibility of the NC-MALDI-TOF MS method is comparable to technical variations (20–30% CV) of the 2D PAGE technique *(11)*

and those (CVs <15%) of SELDI ProteinChip technology *(12)*.

NC was first used by Preston et al. in sample preparation for MALDI-TOF MS *(13)*.

Table 5
Interassay Errors of Quantitative Measurements
of 18 Representative Peptides Identified in
Serum Sample A

Average mass(m/z)	Coefficient of variation (CV, %)	
	Percentage of total peak area	Relative peak intensity
1206.65	3.5	2.9
1260.57	5.2	6.2
1350.59	10.0	11.6
1466.47	2.6	2.8
1617.60	3.7	5.1
1830.87	4.6	5.7
1897.02	12.7	11.4
2029.36	9.5	10.4
2112.35	4.9	4.6
2210.17	14.3	13.3
2489.07	4.7	6.6
2553.24	5.9	4.6
2933.10	7.5	6.0
3274.74	14.6	14.7
3530.76	5.5	9.9
3680.02	4.4	5.9
3704.09	11.4	13.1
5004.78	7.5	8.9

The signal intensities were normalized with the total peak area or with the peak area of the internal reference, adrenocorticotropic hormone (clip 1–17).

They observed that addition of NC increased the yield of peptide ions and improved the sample-to-sample reproducibility and the precision of peptide quantification. Their observations are consistent with our results. In the conventional sample-matrix spotting methods, it is well known that the sizes of sample-matrix cocrystals are highly variable. Optical microscopy results suggest that NC can modify the crystallization of sample-matrix solution to allow more even coverage over the sample stage surface *(13)*. It is possible that a thin NC coating film provides "a crystallization network" or "crystallization cores" for the formation of protein-matrix cocrystals. A higher concentration of NC solution creates a denser network and provides more crystallization cores for the formation of a layer of homogeneous small sample-matrix cocrystals, resulting in more homogeneous relative peptide ion signals. NC concentrations of 0.01–3% were tested in initial experiments. We found that low NC concentrations (0.01 and 0.05%) were ineffective in the formation of a layer of homogenous NC film (data not shown). When the NC concentration was too high (>1%), the signal intensities of the peptide/protein peaks fall significantly (data not shown). It is possible that peptides/proteins are adsorbed to the NC fiber by physicochemical forces such as hydrophobic interactions, hydrogen bonding, and electrostatic interactions when too much NC is coated on the MALDI sample stage.

It is important to emphasize that in this study we have only evaluated the effect of matrix formulation and application on the reproducibility of MALDI-TOF MS in quantitative proteomic profiling. In future studies, other matrix factors, such as drying conditions, should be examined.

In conclusion, with the right MS analysis conditions, MALDI-TOF MS appears to be a feasible tool for directly profiling and quantifying the peptides/proteins in biological samples with intra- and interassay CVs <17%.

Acknowledgments

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