Clinical Chemistry 53:5
852–858 (2007)

Proteomics and
Protein Markers

Reproducibility in Protein Profiling by MALDI-TOF Mass Spectrometry

Jakob Albrethsen[1*]

Background: **Protein profiling with high-throughput sample preparation and MALDI-TOF MS analysis is a new potential tool for diagnosis of human diseases. However, analytical reproducibility is a significant challenge in MALDI protein profiling. This minireview summarizes studies of reproducibility of MALDI protein profiling and current approaches to improve its analytical performance.**

Methods: **The PubMed database was searched using combinations of the following search terms: *MALDI, SELDI, reproducibility, variation, precision, peak intensity, quantification, peptide, biomarkers,* and *proteomics*. Acceptance criteria were detailed reports on the reproducibility with MALDI protein profiling and studies describing efforts to improve the analytical performance with this technology.**

Results: **The reported intraexperiment CVs of the peak intensity vary highly between individual protein peaks, with the reported mean CV of the peak intensity varying among studies from 4% to 26%. There is additional interexperiment variation in peak intensity. Current approaches to improve the analytical performance of MALDI protein profiling include automated sample processing, extensive prefractionation strategies, immunocapture, prestructured target surfaces, standardized matrix (co)crystallization, improved MALDI-TOF MS instrument components, internal standard peptides, quality-control samples, replicate measurements, and algorithms for normalization and peak detection.**

Conclusions: **Further evaluation and optimization of MALDI-TOF MS is recommended before use in routine analysis.**

Protein profiling with high-throughput sample preparation and MALDI-TOF MS analysis is a new potential tool for the diagnosis of human diseases *(1)*. In MALDI protein profiling, biological fluids such as serum, urine, and tissue extracts are treated with a simple preparation step to capture proteins and remove lipids and salts. The protein solution is mixed with a so-called matrix solution that catalyzes the (co)crystallization of matrix molecules and proteins onto a target plate, and the target plate is then analyzed by MALDI-TOF MS. In MALDI-TOF MS the proteins are liberated in an ionized form from the target surface by firing a laser pulse at the crystallized proteins. The ionized proteins are accelerated through a vacuum tube by an electrical field and reach a detector. Smaller proteins are accelerated to higher velocities than heavier proteins, and the "time of flight" is proportional to m/z. The charge (z) of the ionized proteins is often 1, making the m/z value equal to the mass value. The spectral output produced by MALDI protein profiling consists of a number of protein peaks, which are described by an m/z value on the horizontal axis and by a peak-intensity value on the vertical axis, and is referred to as a "protein profile". Recently, MALDI protein profiling has been applied in proteomics biomarker research *(1)*; however, several aspects of MALDI protein profiling need further evaluation and optimization before clinical use *(2)*.

First, high-throughput MALDI protein profiling is presently confined to the detection of highly abundant proteins *(1)*. For example, with the MALDI protein-profiling platforms from Ciphergen Biosystems (the SELDI-TOF MS platform) and Bruker Daltonics, serum analyses primarily detect highly abundant plasma proteins and their fragments [e.g., *(3)*]. Sensitivity in clinical proteomics can be effectively improved with extensive prefractionation strategies, which still need critical evaluation before they can be used in high-throughput protein profiling. In one study, the use of reversed-phase HPLC and MALDI protein profiling to analyze plasma samples in a medium-throughput setup led to improved sensitivity *(4)*.

Second, MALDI protein profiling provides only a limited mass window. The matrix molecules produce highly intense signals in the low-mass range (~0–1000

[1] Glostrup Research Institute, Glostrup Hospital, Glostrup, Denmark
* Address correspondence to this author at: Glostrup Research Institute, Glostrup Hospital, DK 2600 Glostrup, Denmark. Fax number +45-43233929; e-mail jakalb01@glostrup-hosp.kbhamt.dk.

Received November 8, 2006; accepted March 1, 2007.
Previously published online at DOI: 10.1373/clinchem.2006.082644

Table 1. Reproducibility of peak intensity with MALDI protein profiling.

Experimental design	Sample preparation	MALDI-TOF MS instrument	Normalization, Software	Peak detection	Mass range, M_r	Peak count, n	CV of PI/PA[a,b]	Ref.
12 Serum samples, measured 4 times	ProteinChips (automated)	PBS II (Ciphergen)	ProteinChip 3.0	SNR >2 in ≥1 of 4 replicates	2.5–10.0	~110	PI: 26% (20%–33%)	*(13)*
1 Serum sample, measured 8 times	ProteinChips (manual)	PBS II (Ciphergen)	Total ion current, ProteinChip 3.0	SNR >3 in all 8 replicates	2.0–30.0	36	PI: 16% (9.8%–36%)	*(5)*
1 Serum sample, measured 9 times	Magnetic beads, ultracentrifugation, Gold array	PBS II (Ciphergen)	Total ion current, BRB Array Tools 3.1	Iterative algorithm *(38)*	1.0–5.0	194	PI: 17%	*(17)*
1 Plasma sample, measured 3 times	Magnetic beads, AnchorChip	OmniFLEX (Bruker Daltonics)	Total ion current, ClinProt	Randomly selected	1.0–10.0	10	PA: 18%	*(22)*
2 Plasma samples, measured 12 times	Magnetic beads, AnchorChip	Ultraflex (Bruker Daltonics)	Normalization of each peak, ClinProt		1.0–6.0	3	PI: <4% (2.3%–5.0%)	*(24)*
1 Serum sample, measured 12 times	Magnetic beads, ultracentrifugation, AnchorChip	Ultraflex (Bruker Daltonics)	Total ion current, BRB Array Tools 3.1	Iterative algorithm *(38)*	1.0–5.0	276	PI: 11%	*(17)*
1 Serum sample, measured 8 times	Miniaturized solid-phase extraction	Voyager-DE STR (Applied Biosystems)	Normalization to base peak, Proteometrics	Objectively selected	1.5–10.0	16	PI: 3.5%–40%	*(25)*
1 Serum sample, measured 3 times	Carrier-bound peptide isolation	prOTOF 2000 (Perkin Elmer)	Outlier rejection, BAMF		1.0–10.0		PI: 5%–10%	*(31)*

[a] PI, peak intensity; PA, peak area.

[b] CV data are presented as the mean and/or the range.

M_r) that obscure some of the signals of small peptides, and larger proteins have a lower detection limit, primarily because they fly slower than smaller proteins and detector response is related to ion velocity *(1)*. For example, the majority of protein peaks detected in SELDI-TOF MS analyses of serum samples are in the M_r range of 2000–15 000, some peaks are observed in the M_r range of 15 000–30 000, and only a few peaks >30 000 M_r are observed *(5)*. In a protein profiling experiment of snake venom, MALDI-TOF MS with cryodetection showed increased sensitivity for high mass ions, as compared to MALDI-TOF MS with standard ion detectors (such as microchannel plates) *(6)*.

The 3rd challenge in MALDI protein profiling is establishing the reproducibility of peak intensity. In biomarker research with MALDI protein profiling, the aim is to identify peak intensities (or peak areas) that are different between case and control samples, and the reproducibility of peak intensities is of highest importance. However, poor reproducibility has been considered one of the major problems in protein profiling with MALDI-TOF MS. The matrix (co)crystallization and desorption/ionization steps in MALDI-TOF MS have been derived empirically, and the processes are poorly understood. Different matrix molecules crystallize in different shapes and dimensions, proteins tend to accumulate at the droplet periphery, and the composition of the matrix solution and the rate of crystal growth influence the spectral output *(7, 8)*. These phenomena produce shot-to-shot variation, which is related to sampling different parts of the target surface and progressive sample ablation with repeated sampling. The desorption/ionization step in MALDI-TOF MS is a complex process involving optical and mechanical phenomena, as well as thermodynamic and physicochemical processes of phase transition and ionization, which are not well understood *(9)*. Studies have demonstrated ion-suppression effects in MALDI-TOF MS. Ion suppression occurs when an ion suppresses the peak signal of other ions in the sample, and peptides with greater hydrophobicity show the greatest suppression effects *(8, 10)*. The presence of basic residues may favor ionization in MALDI-TOF MS analysis *(8, 11)*. One study found that highly acidic compounds produced weak signals in MALDI-TOF MS analyses, but when such compounds were mixed with a basic peptide to form a noncovalent complex, the signals improved *(12)*. In summary, peak intensity in MALDI protein profiling has significant analytical variation and is poorly understood. Peak intensity is related to the concentration of the individual protein, to its primary structure, and to the complexity of the sample.

REPRODUCIBILITY IN MALDI PROTEIN PROFILING

With the SELDI-TOF MS platform from Ciphergen Biosystems, proteins are captured on target surfaces (ProteinChips) that are coated with chromatographic resins and analyzed by linear MALDI-TOF MS with the PBS II or Protein Reader Series 4000 instruments from Ciphergen Biosystems (now marketed by Bio-Rad Laboratories). Studies have concluded that peak intensities with SELDI-TOF MS are highly sensitive to experimental details *(13–15)*. In addition, the performance of the SELDI-TOF MS instrument may change over time because of varying laser intensity and detector sensitivity *(16)*. In one study, a SELDI-TOF MS procedure that had been optimized through the use of automated sample loading and a standardized drying time for the ProteinChips before matrix application improved the reproducibility of peak intensities *(13)*. To evaluate intraexperiment variation, the investigators measured 12 serum samples 4 times in 1 experiment. On average, 110 protein peaks with signal-to-noise ratio (SNR)[2] >2 were detected in the M_r range of 2500–10 000 in a minimum of 1 of the 4 measurements of the 12 samples; the mean intraassay CV was 26% (range, 20%–33%; Table 1). Long-term variation was assessed with 30 serum samples that were analyzed in 2 experiments 7 months apart. Approximately 50 protein peaks with SNR >2 were detected in duplicate measurements of the 30 samples; the mean CV was 33%. With a higher threshold (SNR >5), ~25 protein peaks were detected in duplicate measurements of the 30 samples, and the mean CV was 26%. In another evaluation study, 1 serum sample was analyzed 8 times by SELDI-TOF MS in 1 manual experiment. Only protein peaks detected with SNR >3 in all 8 measurements were counted. The mean intraexperiment CV of the peak intensity was 16% (range, 9.8%–36%) for 36 protein peaks in the M_r range of 2000–30 000 *(5)* (Table 1). Next, these investigators measured 1 serum sample on 4 different days. Only 16 protein peaks with an SNR >3 were detected in all 4 measurements, and the mean CV was 18% (range, 5.9%–34%). Another study *(17)* combined prefractionation and SELDI-TOF MS analysis with an inert target surface (Gold array; Ciphergen Biosystems). First, 9 aliquots of 1 serum sample were desalted with C8 magnetic beads and then subjected to ultracentrifugation through membranes with a 50 000-M_r cutoff. Next, the filtrate was analyzed by conventional MALDI-TOF MS analysis with the SELDI-TOF MS instrument. The mean CV of peak intensity was 17% for 194 protein peaks in the M_r range of 1000–5000 *(17)* (Table 1).

Attempts have been made to advance SELDI-TOF MS into clinical validation. In a SELDI-TOF MS study of prostate cancer, randomly selected samples were rerun after a year and correctly classified by a decision-tree classification algorithm *(18)*. In another SELDI-TOF MS study, however, the sensitivity and specificity of a biomarker pattern for renal cell carcinoma were initially >80%, but the sensitivity dropped to 41% when the same procedure was used 10 months later with a new set of patient samples *(19)*. In a SELDI-TOF MS study of serum samples from patients with ovarian cancer, the spectra of patients and controls from 3 separate experiments were

[2] Nonstandard abbreviation: SNR, signal-to-noise ratio.

compared. The investigators found that the sets of features that distinguished patient samples from controls in one experiment could not be generalized to other experiments *(20)*. In a study of interplatform variation, 6 SELDI-TOF MS PBS II instruments with automated sample-handling robots were synchronized and evaluated *(16)*. The investigators used the 3 most prominent protein peaks in a standard serum sample to standardize spectral output between platforms and found it necessary to monitor and adjust each instrument continuously. These alterations included adjusting laser intensity, detector voltage, and sensitivity; having a Ciphergen engineer check the PBS II instruments; and replacing instrument components. When the criteria were met, the investigators assessed across-site variation in peak intensity by analyzing 96 replicates of the standard sample at each of the 6 instrument sites. The interlaboratory CVs of the peak intensity were 15%, 17%, and 36% for the 3 selected peaks, which were comparable to the intralaboratory CVs for the same 3 peaks at each of the 6 sites. The SELDI-TOF MS platform was also evaluated in a multicenter analysis of 4 serum and plasma reference samples from the Human Proteome Organization. The samples were analyzed in replicates in 8 laboratories with 8 different SELDI-TOF MS instruments *(21)*; however, only 5 of the 8 submissions were acceptable for a reproducibility analysis. Each of the 5 laboratories analyzed 3 or 4 samples in different numbers of replicates (minimum 2) to produce a total of 42 spectra. Sixty protein peaks were assigned in the M_r range of 1500–20 000, and the correlation coefficient was >0.7 for 37 of the 42 spectra. The mean intralaboratory CV of the peak intensity in the 5 laboratories varied from 15% to 43%.

With the MALDI protein-profiling platform from Bruker Daltonics, magnetic beads and prestructured sample supports (AnchorChip technology; Bruker Daltonics) are used to prepare biological fluids before analysis with the Ultraflex/OmniFLEX MALDI-TOF MS instruments (Bruker Daltonics) *(22, 23)*. With this approach, investigators achieved a mean intraexperiment CV of the peak area of 18% (range, 11%–25%) for 3 measurements of 10 protein peaks in human plasma in the M_r range of 1000–10 000 *(22)* (Table 1). For assessment of interexperiment variation, 1 sample was measured on 3 different days; the mean CV was 26% (range, 4%–43%) for 10 protein peaks in the M_r range of 1000–10 000. In another study with the Bruker Daltonics platform *(24)*, investigators analyzed 2 plasma samples 12 times and obtained a mean CV of the peak intensity of <4% (range, 2.3%–5.0%) for 3 protein peaks in the M_r range of 1000–6000 (Table 1). The mean interassay CV for 8 replicates was <7% (range, 4.6%–8.2%) for these 3 peaks. Finally, in another study with the Bruker Daltonics platform, 15 aliquots of 1 serum sample were first prepared for analysis by desalting with magnetic beads followed by ultracentrifugation. The mean CV of the peak intensity was 11% for 276 protein peaks in the M_r range of 1000–5000 *(17)* (Table 1), and a comparison of randomly selected peaks of low, medium, and high intensity in this M_r range revealed that the CV of the peak intensity did not correlate with the mean signal intensity.

Other MALDI protein-profiling platforms combining high-throughput sample preparation with MALDI-TOF MS are being developed. For example, one study used solid-phase extraction and MALDI-TOF MS *(25)*. In this study, 1 serum sample was prepared 8 times with C8 material and eluted directly onto the MALDI target. The intraexperiment CV varied from 3.5% to 40% for 16 protein peaks in the M_r range of 1500–10 000 (Table 1).

High-throughput MALDI platforms have thus far shown acceptable analytical performance in the quantification of single peptides. One study showed high linear correlation ($r > 0.9$) between the concentration of serum amyloid A measured by ELISA and concentrations of serum amyloid A variants measured by SELDI-TOF MS in crude serum (serum amyloid A concentration range, mg/L) *(26)*. SELDI-TOF MS also detected myoglobin added to diluted serum (10 mL/L), and the myoglobin concentration showed a good correlation ($r^2 > 0.9$) with the SELDI-TOF MS signal; the dynamic concentration range was 1.00–2000 nmol/L *(27)*. In another study, off-line coupling of reversed-phase HPLC and MALDI protein profiling for improved sensitivity was used in a setup of medium-high throughput to analyze 13 peptides added to plasma samples. The investigators found a good linear correlation ($r > 0.9$) in the concentration range of 50–800 pmol/L *(4)*. Immunocapture of single candidate biomarkers followed by antigen quantification by MALDI-TOF MS is a promising tool: Immunocapture increases sensitivity and bypasses the problem of cross-reactivity, which may affect the performance of conventional immunoassays. For example, a SELDI immunoassay developed for serum prostate-specific membrane antigen showed a high linear correlation (r^2 >0.9) between the concentration of this antigen (in μg/L) in serum and the peak intensity *(28)*.

APPROACHES TO IMPROVE THE ANALYTICAL PERFORMANCE OF MALDI PROTEIN PROFILING

Further experiments must be performed to identify and control the sources of analytical variation with high-throughput MALDI protein profiling *(2, 20)*. Differences in reagents and handling, and changes in room temperature, pressure, and humidity may influence the (co)crystallization step and cause day-to-day variation *(29, 30)*. Matrix solutions usually contain the volatile solvent acetonitrile and solutions may not be left in open containers for a prolonged time as evaporation of acetonitrile may affect the matrix crystallization *(29)*.

The performance of the MALDI-TOF MS instrument may change over time because of variation in laser intensity and detector sensitivity *(16)*, and improved MALDI-TOF MS instrument components may reduce the analytical variation. The SELDI-TOF MS instrument is

designed to produce a reproducible protein profile over a relatively wide mass range at the price of lower mass accuracy and lower resolution of the spectral output. With the PBS II SELDI-TOF MS instrument, the mean interlaboratory CV of the m/z value is <0.1% *(16)*, whereas the latest SELDI model (the Protein Reader Series 4000) has improved resolution *(27)*. In a study with a high-resolution MALDI orthogonal TOF MS instrument (PerkinElmer), a single external calibrant was used to achieve a mass accuracy of <10 ppm (<0.001%) in a high-throughput screening setup. In a triplicate measurement of 1 serum sample, the CV of the peak intensity was 5%–10% for peaks in the M_r range of 1000–10 000 *(31)* (Table 1).

Not only does affinity capture of molecules directly on the MALDI target surface permit a fast and simple analysis, the structure of the target surface may also reduce the variation in peak intensity. Prestructured target surfaces are used to support the drying and (co)crystallization step with the MALDI protein-profiling platforms from Ciphergen Biosystems (ProteinChips) and Bruker Daltonics (AnchorChip technology) *(23, 27)*. One study has shown that the use of a silicone/graphite coating on the MALDI target increases peak intensities and generates a more homogeneous crystal layer *(32)*. Nitrocellulose may also improve the sensitivity and reproducibility of MALDI-TOF MS by causing a more homogeneous crystallization of the matrix *(33)*. In MALDI protein profiling with the material-enhanced laser desorption/ionization (MELDI) approach, samples are prepared with various carrier materials followed by MALDI-TOF MS analysis *(34)*. Disposable MALDI targets pre-spotted with matrix have been developed by Bruker Daltonics (pre-spotted AnchorChip) and Ciphergen Biosystems, Inc. (the "SEND" array) for improved spectral quality. In addition, target structures for matrix-free laser desorption/ionization are being developed *(35)*.

The use of reference materials may reduce the variation in peak intensity in MALDI protein profiling. Traditionally in semiquantitative MALDI-TOF MS, internal calibrant peptides of defined concentrations and with physicochemical properties comparable to the analyte of interest are added to the sample, and relative ion intensities are compared *(9)*. For example, in a study of tissue homogenates, a single reference peptide was included to allow correction for crystallization variation in MALDI sample preparation *(36)*. In another study, calibration curves with synthesized peptides were used for absolute quantification of tryptic peptides with MALDI-TOF MS *(37)*. Including quality-control samples in high-throughput analyses in combination with iterative algorithms may improve the robustness of MALDI protein profiling over time *(38)*. Algorithms are being developed for baseline subtraction, normalization, and peak detection in MALDI protein profiling [see, for example, *(39)*], which may compensate for some of the analytical variation. Finally, 2 to 4 replicate measurements of each sample may increase the reliability of MALDI protein profiling *(13)*.

Discussion

The semiquantitative nature of the peak intensity and the high-throughput capability of MALDI-TOF MS permit its use in preclinical explorative research of biomarkers. Different MALDI protein-profiling strategies are now being developed for improved analytical performance. In particular, MALDI protein profiling has been combined with advanced bioinformatics to identify proteomic biomarker patterns for human diseases and improved reproducibility of the spectral output is critical for avoiding false discovery with this "black box" approach *(20)*.

It is difficult to generalize about the level of reproducibility required for diagnostic tests. The CVs for established markers used in clinical diagnostics have been argued to be typically in the range of 1.5%–10%, including interlaboratory variation *(40)*. As summarized here, large differences exist between MALDI protein-profiling studies in the reported mean CVs of the peak intensity (<4%–26%), but comparisons of these studies are difficult because of differences in the number of replicate measurements, mass range, and the number of peaks (Table 1). Significant interexperiment variation has been reported with the MALDI protein-profiling platforms from Ciphergen Biosystems and Bruker Daltonics, and day-to-day variation is a well-known problem in biomarker research with MALDI protein profiling. Such findings suggest that the negative influences of the matrix molecules and instrument components in MALDI-TOF MS have not yet been sufficiently reduced to allow the use of this technology for routine analysis.

In addition to reproducibility (or precision), important considerations for any clinical assay are the detection limit, linearity, and dynamic range. Studies of MALDI-TOF MS have shown acceptable linearity and dynamic range in the quantification of single proteins, and the detection limit can be improved with extensive prefractionation steps or immunocapture. However, the method's analytical performance in the simultaneous quantification of numerous proteins requires critical examination. It is emphasized that intraexperiment reproducibility of peak intensity in MALDI protein profiling varies dramatically between individual protein peaks (2%–40%, Table 1), and evaluation studies based on a few selected mass peaks may bias the imprecision estimate downward.

Current approaches to improve the analytical performance of MALDI protein profiling include automated sample processing, extensive prefractionation strategies, immunocapture, prestructured target surfaces, standardized matrix (co)crystallization, improved MALDI-TOF MS instrument components, the use of internal calibrant peptides, quality-control samples, replicate measurements, and algorithms for normalization and peak detection.

In conclusion, MALDI protein profiling holds the potential to bridge the present gap between laboratory research and clinical validation in proteomics biomarker research, but further evaluation and optimization of MALDI protein profiling is recommended before it can be used in routine analysis.

Grant/funding support: This work was supported by the Department of Paediatrics, Glostrup University Hospital, Glostrup, Denmark.
Financial disclosures: None declared.

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