

University of Groningen

Mass spectrometry-based methods for protein biomarker quantification

Klont, Frank

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version

Publisher's PDF, also known as Version of record

Publication date:

2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Klont, F. (2019). *Mass spectrometry-based methods for protein biomarker quantification: On the road to clinical implementation*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen.

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

CHAPTER II

Assuring consistent performance of a novel IGF1 MALDIImmunoassay by monitoring measurement quality indicators

Frank Klont^a, Nick H.T. ten Hacken^b, Péter Horvatovich^a,
Stephan J.L. Bakker^c, Rainer Bischoff^a

^aDepartment of Analytical Biochemistry, Groningen Research Institute of Pharmacy, University of Groningen, The Netherlands; ^bDepartment of Pulmonary Diseases, University Medical Center Groningen, University of Groningen, The Netherlands; ^cDepartment of Nephrology, University Medical Center Groningen, University of Groningen, The Netherlands

Analytical Chemistry 2017; 89(11): 6188-6195

ABSTRACT

Analytical methods based on mass spectrometry (MS) have been successfully applied in biomarker discovery studies, while the role of MS in translating biomarker candidates to clinical diagnostics is less pronounced. MALDIImmunoassays – methods that combine immunoaffinity enrichment with MALDI-TOF mass spectrometric detection – are attractive analytical approaches for large-scale sample analysis by virtue of their ease of operation and high-throughput capabilities. Despite this fact, MALDIImmunoassays are not widely used in clinical diagnostics, which is mainly due to the limited availability of internal standards that can adequately correct for variability in sample preparation and the MALDI process itself. Here we present a novel MALDIImmunoassay for quantification of insulin-like growth factor I (IGFI) in human plasma. Reliable IGFI quantification in the range of 10-1,000 ng/mL using 20 μ L of plasma was achieved by employing ^{15}N -IGFI as internal standard. The method was validated according to FDA guidelines fulfilling all relevant criteria, and was subsequently tested on > 1,000 samples from a cohort of renal transplant recipients to assess its performance in a clinical setting. Based on this study, we identified readouts to monitor the quality of the measurements. Our work shows that MALDI-TOF mass spectrometry is suitable for quantitative biomarker analysis provided that an appropriate internal standard is used and that readouts are monitored to assess the quality of the measurements.

2.1. INTRODUCTION

The number of newly discovered biomarker candidates has increased dramatically in recent years following the rise of modern omics approaches. However, only few of these biomarkers have made their way into clinical practice.¹ This discrepancy reflects the gap between biomarker discovery and validation, and stresses the need for breaking the bottleneck(s) of the biomarker development pipeline.²⁻⁴ To address this need, many efforts are currently being deployed to translate biomarker research into clinical practice.^{1,2,5}

In the past decade, MS has found wider acceptance in biomarker validation studies.^{4,5} In particular, the combination of immunoaffinity enrichment and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry is gaining momentum. This approach, which we denote by the generic term ‘MALDImmunoassay’, holds considerable promise for biomarker validation studies because of its ease of use as well as its automation and multiplexing capabilities.⁶ In fact, a substantial number of these approaches have been described in the past years, including various MSIATM (*i.e.* on-target elution of intact proteins/peptides which are enriched using antibody-coated microcolumns)⁷⁻²¹, SISCAPA[®]-MALDI (*i.e.* spotting of proteotypic peptides which are enriched using antibody-conjugated magnetic beads)^{22,23}, and iMALDI methods (*i.e.* spotting of antibody-conjugated magnetic beads containing enriched proteotypic peptides)²⁴⁻²⁸ as well as other approaches without distinct denominations.²⁹⁻³⁴

In light of the potential application of MALDImmunoassays in clinical diagnostics, it is important to note that MALDI-TOF MS has already made its entrance into routine clinical practice. Bruker’s Biotyper[®] and bioMérieux’s Vitek[®] are two approved analytical platforms that have transformed species determination in medical microbiology.³⁵ Although clinical application of MALDI-TOF MS has been successful for microbial species determination, its application for biomarker quantitation has not yet reached its full potential, and challenges for MALDImmunoassays are still numerous and substantial. In particular, a cornerstone of high quality quantitative assays is good internal standardization.³⁴ As MALDImmunoassays employ antibodies which may be sources of variation, an internal standard must be able to compensate for variability during the immunoaffinity enrichment step.³⁶ Furthermore, inasmuch as MALDI-TOF detection is known for its nonlinear relationship between signal intensity and analyte concentration, internal standards (preferably stable-isotope-labeled, SIL) must also compensate for detection variability.³⁷ Indeed, most MALDImmunoassays employ internal standards, although some of these standards exhibit substantial structural and chemical differences compared to the authentic analyte. Therefore, some methods may benefit from improving the internal standardization which may even advance their maturation into clinical diagnostics.

An example of a clinically relevant biomarker that has been targeted by MALDIImmunoassays is insulin-like growth factor 1 (IGF1).^{9,18} IGF1, a 7.65 kDa polypeptide hormone, is the main mediator of growth hormone (GH)-stimulated cell and tissue growth. In laboratory medicine, IGF1 is routinely measured to diagnose GH deficiency as well as to test for an excess of GH leading to abnormal growth in children (*e.g.* gigantism) or as the result of a pituitary tumor (*e.g.* acromegaly).¹⁸ Furthermore, IGF1 is an important measure to detect abuse of GH and IGF1 in sport, and numerous IGF1 measurements are annually conducted in the field of doping analysis.^{38,39}

The most recently published IGF1 MALDIImmunoassay is a high-throughput assay based on the MSIATM principle.¹⁸ This method employs specific antibody-coated microcolumns that are compatible with selected liquid handling platforms, and is thereby capable of measuring > 1,000 samples per day. The method employs the doping agent LONG^R3IGF1 as internal standard, which is an IGF1 analogue with increased potency due to a lower binding affinity to circulating IGF binding proteins (IGFBPs) compared to IGF1.³⁸ This feature, however, likely affects the appropriateness of LONG^R3IGF1 as internal standard for IGF1, since it implies that this analogue may not correct adequately for the extraction of IGF1 from IGFBP-containing matrices, such as serum and plasma. Furthermore, the two additional methionine residues in the N-terminal extension of this protein may lead to formation of different oxidation products compared to IGF1 during the analytical procedures.³⁸ Thus, chemical differences between IGF1 and LONG^R3IGF1 may cause variation in the signals for both compounds.

In this work, we present a MALDIImmunoassay for quantification of IGF1 in human plasma which uses a fully ¹⁵N-labeled recombinant version of IGF1 as internal standard. The method was validated according to FDA guidelines⁴⁰, and its performance was subsequently tested in a clinical setting using > 1,000 samples from a cohort of renal transplant recipients. Based on this large-scale study, we identified indicators of measurement quality which may aid in making MALDI-TOF MS a reliable bioanalytical assay platform.

2.2. EXPERIMENTAL SECTION

2.2.1. Materials

Recombinant human IGF1 (Cat. No. CYT-216), ¹⁵N-IGF1 (Cat. No. CYT-128), and IGFBP3 (Cat. No. CYT-300) were purchased from ProSpec (Ness-Ziona, Israel). Polyclonal anti-IGF1 antibody (Cat. No. PA0362) was obtained from Cell Sciences (Newburyport, MA, U.S.A.). PierceTM Protein A/G magnetic beads (Cat. No. 88802/3) were acquired from Fisher Scientific (Landsmeer, The Netherlands), and these were separated using a Promega MagnaBot[®] 96 separation device. Acetonitrile (ACN; LC-MS grade) was purchased from

Biosolve (Valkenswaard, The Netherlands), sinapinic acid (Cat. No. M002) was from LaserBio Labs (Sophia-Antipolis, France), and polystyrene U-bottom microtiter plates (Cat. No. 650-101) were obtained from Greiner Bio-One (Alphen aan den Rijn, The Netherlands). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

2.2.2. Plasma samples

For method development and preparation of QC-samples, a bulk quantity of human plasma from Seralab (West Sussex, U.K.) was used. This plasma was either used directly as QC-medium sample, diluted four times with rat plasma (obtained from Seralab) to prepare the QC-low sample, or fortified with recombinant IGF1 to obtain the QC-high sample. Spike recovery experiments were carried out using six different sources of human plasma (all from Seralab). For method testing, 1,038 plasma samples were analyzed from a cohort of renal transplant recipients (plus screened donors and healthy controls) that is being studied at the University Medical Center Groningen (UMCG).⁴¹ For this study, ethical approval has been granted by the UMCG's review board (METc 2008/186), and the study adheres to the Declaration of Helsinki. All samples were stored at -80 °C.

2.2.3. Calibrants and internal standard

Lyophilized IGF1 was reconstituted in 2% ovalbumin (in 100 mM PBS, pH 7.2) to obtain a 200 µg/mL solution. This solution was diluted to 10 µg/mL with rat plasma or 2% ovalbumin to obtain a stock solution for calibration or sample fortification purposes, respectively. Using the stock solution in rat plasma, calibration samples were prepared in blank rat plasma at 10, 20, 50, 100, 200, 500, and 1,000 ng/mL. For the internal standard (IS), lyophilized ¹⁵N-IGF1 was reconstituted in 10 mM ammonium bicarbonate to obtain a 500 µg/mL solution. After checking the compound's (isotopic) purity by MALDI-TOF MS, the stock was diluted sequentially in 2% ovalbumin to obtain a 400 ng/mL IS working solution.

2.2.4. Immunoaffinity enrichment

Three microliters of magnetic beads were washed thrice with 100 µL Wash Buffer (0.1 % Tween-20 in 100 mM PBS, pH 7.2), and incubated (1 hour; 750 RPM) in 100 µL Wash Buffer containing 0.5 µg antibody. Next, unbound antibody was removed following three washing steps with 100 µL Wash Buffer. During incubation of the beads with the antibody, 20 µL of sample was combined with 10 µL IS working solution, and the sample was incubated (5 min; 900 RPM) to allow complexing of the IS with the IGF1s. Subsequently, 50 µL of Dissociation Buffer (0.3% SDS in Wash Buffer) was added, and the sample was incubated (30 min; 900 RPM) to enable dissociation of IGF1/IGF1BP-complexes. After diluting the dissociated

sample with 50 μL of Wash Buffer, this mixture was added to the antibody-conjugated beads for immunoaffinity enrichment of IGF1 (1 hour; 750 RPM). Subsequently, the beads were washed thrice with 100 μL Wash Buffer and once with 100 μL Milli-Q water, prior to elution of IGF1 from the beads (10 min; 900 RPM) with 20 μL Elution Solution (0.45% TFA + 33% ACN in H_2O). Finally, 5 μL of eluate was mixed 1:1 with a saturated solution of sinapinic acid in Elution Solution, and 1 μL of this mixture was spotted in quadruplicate onto a polished steel MALDI target plate. The immunopurification workflow was automated with an Agilent Bravo liquid handling platform equipped with a 96-channel LT pipetting head.

2.2.5. MALDI-TOF MS

Linear positive MALDI-TOF spectra were recorded between 4,000 and 10,000 Da with a Bruker ultrafleXtreme mass spectrometer operated under Bruker flexControl software (version 3.4). Acquisition was performed under the following conditions: 50 ns delayed extraction; signal deflection up to m/z 4,000; 2 kHz Smartbeam-II™ UV laser (Nd:YAG; $\lambda = 355$ nm) operating with the “4_large” parameter set; 5 GS/s digitizer sampling rate; and ion source 1, 2, and lens voltages of 25.00, 23.30, and 5.75 kV, respectively. For every sample, 2,500 shots were acquired in 100 shot steps following a ‘hexagon’ measuring raster, although only spectra of sufficient resolution (≥ 500 , after “Centroid” peak detection (peak width = 5 m/z) using “TopHat” baseline subtraction) were averaged for each mass spectrum.

2.2.6. Data processing

MALDI spectra were smoothed (SavitzkyGolay filter; width = 5 m/z ; cycles = 1), baseline subtracted (Median; flatness = 0.1; median level = 0.5), and peaks were detected and integrated (Centroid algorithm; peak width = 5 m/z) using Bruker flexAnalysis software (version 3.4). Peak intensity values for the IGF1 and ^{15}N -IGF1 peaks as well as for their oxidation peaks were retrieved from obtained mass lists, and processed further using customized Microsoft Excel (version 2010 & 2013) spreadsheets.

2.2.7. Method validation

The method was validated based on FDA guidelines on bioanalytical method validation.⁴⁰ The following criteria were addressed: selectivity (*e.g.* spike recovery and IGFBP3 challenge test), accuracy & precision, calibration curve, and stability (*e.g.* 24h benchtop, 3x freeze-thaw, and 7 days MALDI sample stability). With respect to the selectivity tests, samples were spiked with IGF1 (25, 100 and 500 ng/mL) or IGFBP3 (2,500 ng/mL; protein was reconstituted & diluted in 2% ovalbumin), and incubated for 30 min prior to analysis with the MALDIImmunoassay. This incubation step was included to allow complexing of IGF1 with IGFBP3 and other IGF

binding proteins. Furthermore, the method was compared with the IDS-iSYS IGF1 assay using a cohort consisting of 20 ‘normal’ samples and 20 samples from patients with growth hormone deficiency or excess.⁴²

2.3. RESULTS AND DISCUSSION

2.3.1. Characterization of mass spectra

Figure 1-A shows a linear positive MALDI-TOF MS spectrum representative of the clinical samples that were measured. The intense peaks at m/z 7,650 and 7,743 represent IGF1 and ¹⁵N-IGF1, respectively. Both peaks are accompanied by their sinapinic acid adduct peaks (+ 206 mass units), as well as by a peak around m/z 8,350, which was previously observed and denoted as a possible IGF1 variant.¹⁸ Figure 1-A also features a zoom-in of the spectrum between 7.6 and 7.8 kDa clearly displaying the oxidation peaks of both IGF1 and ¹⁵N-IGF1, which likely arise as the result of oxidation of the methionine residue at position 59. The percent abundance of these oxidation peaks (relative to the cumulative intensity of the oxidized and non-oxidized peaks) was monitored and on average, oxidation peak abundances for IGF1 and ¹⁵N-IGF1 were around 15% for the clinical samples. In order to assess analytical accuracy, the constancy of the ratio between these abundances was monitored and ensured for all samples (see ‘Quality assessment of MALDI measurements’ section below).

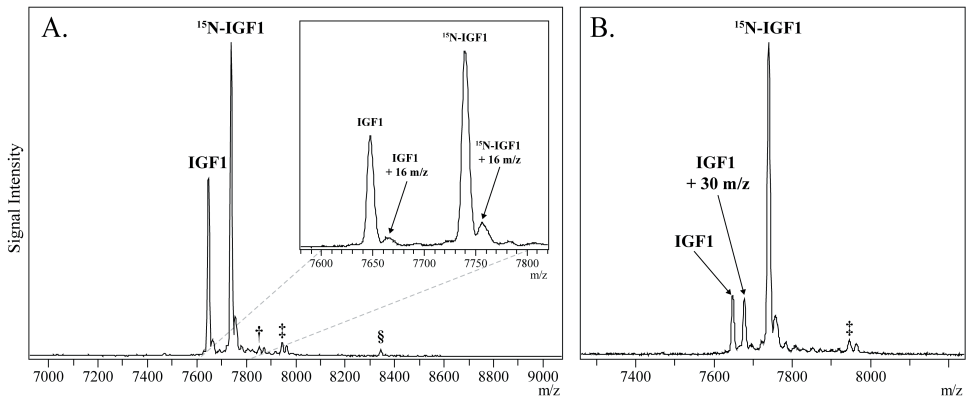


Figure 1. (A) MALDIImmunoassay spectrum of IGF1 in plasma from an individual expressing wild-type IGF1 and (B) from an individual expressing wild-type IGF1 and an IGF1 variant giving rise to a 30 m/z mass increase which likely arises from an alanine-to-threonine substitution at position 67 or 70. Besides peaks originating from IGF1 and ¹⁵N-IGF1, MALDI spectra also displayed peaks representing sinapinic acid adducts of IGF1 (†) and ¹⁵N-IGF1 (‡) as well as an unknown peak that was previously¹⁸ denoted as a possible IGF1 variant (§). In addition, Figure 1-A features a zoom-in of the spectrum between 7.6 and 7.8 kDa displaying oxidation peaks of IGF1 and ¹⁵N-IGF1.

Figure 1-B displays a spectrum that contains an additional IGF1 signal at m/z 7,680, which was observed in one out of more than 1,000 clinical samples. This IGF1 variant has been observed previously and could originate from a nonsynonymous single nucleotide polymorphism (SNP) giving rise to an alanine-to-threonine substitution at position 67 (rs17884626) or 70 (rs151098426).^{18,43} In samples from patients carrying these SNPs, a large discrepancy can be expected between IGF1 levels based on wild-type IGF1 as obtained with the MALDIImmunoassay and those that are obtained with conventional immunoassays or even with available LC-MS methods targeting proteotypic IGF1 peptides that do not cover the regions relevant for detection of these SNPs. Intensities of the peaks at m/z 7,650 and 7,680 may be summed up to give the total concentration of these IGF1 proteoforms, however it is currently unknown whether the potencies of these variants are the same as the potency of wild-type IGF1.

2.3.2. Selection of internal standard and calibration matrix

For quantitative MALDI-TOF MS (and quantitative MS methods in general), calibration is ideally performed with authentic analyte in authentic matrix, and by using a stable-isotope-labeled (SIL) version of the authentic analyte as internal standard (IS).^{44,45} Given that IGF1-free human plasma was not available, we studied the applicability of several surrogate matrices, including bovine serum albumin in PBS and plasma from other species. Corresponding experiments indicated that a high degree of similarity between the authentic and surrogate matrix was needed, notably to compensate for technical variation during the IGF1/IGFBP-complex dissociation step and for the influence of SDS during the subsequent immunocapture of IGF1. Ultimately, rat plasma was selected as surrogate matrix since it enables reliable IGF1 quantitation (as demonstrated during method validation; see below), and because it does not interfere with measuring human IGF1 or the internal standard (as depicted in Figure S-1). In addition, rat plasma does not give rise to signals that interfere with known endogenous IGF1 variants (*e.g.* des(1-3)IGF1, IGF1 A67T, and IGF1 A70T) or synthetic IGF1 analogues that may be used as doping agents (*e.g.* R³IGF1 and LONG^RIGF1).

As mentioned above, SIL versions of analytes are the preferred internal standards for MALDIImmunoassays. Such standards allow accurate compensation for variability in both sample preparation and MS detection; however, SIL-analogues are not readily available for every protein. In cases when such analogues are not available, alternative internal standards (*e.g.* close structural analogues) may be appropriate, though justification of their applicability must be supported by full method validation according to internationally recognized guidelines (*e.g.* EMA, FDA and/or CLSI guidelines).⁴⁶

Differences in analytical behavior between analytes and alternative internal standards should ideally be absent, though it is not inconceivable that differences become apparent, which we experienced when using LONG³IGF1 as internal standard for IGF1.¹⁸ We found that LONG³IGF1 is not an ideal internal standard for IGF1, since an equimolar mixture of both compounds yielded an over 5-fold higher intensity for IGF1 compared to LONG³IGF1. More importantly, some MALDI-TOF spectra revealed three oxidation peaks for LONG³IGF1 compared to only one for IGF1 (see Figure S-2). Most probably, the two additional methionine residues of the LONG³-peptide were oxidized and gave rise to these peaks. On the contrary, ionization efficiency and oxidation behavior of ¹⁵N-IGF1 were highly similar to IGF1 (see Figure 1), and therefore we employed ¹⁵N-IGF1 as internal standard to accurately compensate for variability during the entire analytical procedure.

2.3.3. Assay characteristics

Results from the method validation experiments are included in Tables S1-S10 (Supporting Information), while Table 1 displays a concise summary of the validation data. The calibration curve (1/x weighting) consisted of 7 non-zero standards with values ranging from 10 ng/mL (LLOQ: CV & bias \pm 20%) to 1,000 ng/mL. Signal intensities based on peak height and peak area were both evaluated during method validation, yet peak height was ultimately selected for calculation of the IGF1 levels as it gave more accurate results, which has also been reported previously.^{34,47,48}

Evaluation of accuracy and precision as well as all stability assessments demonstrated biases and CVs within \pm 15%. Notably, observed CVs were lowest for the midrange QC-samples, which has also been observed by others.^{22,34,49,50} For corresponding IGF1 levels, the analyte and internal standard were present on the MALDI spot in near equimolar amounts, which appears to be favorable for the internal standard's effectiveness in correcting for variation arising from the MALDI-TOF process. This effect was further demonstrated by calculating 4-spot CVs for each sample and by relating these to the corresponding (4-spot) IGF1/¹⁵N-IGF1-ratios (Figure S-3 displays graphical representations of these relationships for four selected analytical runs carried out for clinical sample analysis). Observed variation was typically lowest for IGF1/¹⁵N-IGF1-ratios around 1 and increased with both higher and lower ratios. These observations illustrate the generally limited span of calibration ranges for MALDI-TOF MS-based quantitative methods. Furthermore, these results also emphasize the need to match the amount of spiked internal standard to the median of expected concentrations, or to the level that is most important for clinical decision-making.

Table 1. Summary of validation data.

		QC-low		QC-medium		QC-high	
		CV	bias ^a	CV	bias ^a	CV	bias ^a
Accuracy & precision (3 runs in 6-fold)	run 1	5%	1%	5%	8%	13%	4%
	run 2	6%	-4%	4%	-6%	15%	-10%
	run 3	10%	2%	4%	-2%	15%	6%
Bench-top stability (24h, in 3-fold)		14%	-9%	-	-	1%	9%
Freeze-thaw stability -20 °C (3 cycles, in 3-fold)		6%	-13%	-	-	4%	12%
MALDI-sample stability (7 days, in 6-fold)	day 0	5%	1%	5%	8%	13%	4%
	day 7	3%	10%	4%	10%	4%	12%
		20 ng/mL calibrant		QC-low		QC-medium	
		CV	bias	CV	bias ^a	CV	bias ^a
IGFBP3 challenge test (in 5-fold)		5%	-7%	4%	-6%	1%	-2%
		+ 25 ng/mL		+ 100 ng/mL		+ 500 ng/mL	
		CV	bias	CV	bias	CV	bias
Spike recovery (6 different plasma samples)		9%	4%	7%	11%	12%	4%

^a The average value of measured concentrations during the precision and accuracy experiments was used as nominal concentration.

It is of particular relevance for quantitative IGF1 assays to ensure that IGF1 is properly liberated from its binding proteins (*e.g.* IGFBP3) and to demonstrate that these binding proteins do not interfere with the assay. For this assay, disruption of IGF1/IGFBP-complexes was realized by treating samples with SDS, similar to the approaches of previously published IGF1 methods.^{9,18,38,51} The effectiveness of this step was demonstrated by means of an IGFBP3 challenge test, in which calibration and QC samples were spiked with an excess of IGFBP3, as well as through spike recovery experiments using six different sources of human plasma. After the samples were spiked with IGFBP3 or IGF1, they were incubated for 30 minutes to allow IGF1/IGFBP-complex formation. Subsequently, samples were analyzed with the MALDImmunoassay to assess accuracy and precision. Results of these experiments showed that SDS treatment does not introduce a significant bias or imprecision into the assay ($\pm 15\%$), and thereby demonstrate (to our understanding for the first time) the effectiveness of an SDS-based strategy for IGF1/IGFBP-complex dissociation.

The MALDImmunoassay was compared with the IDS-iSYS IGF1 immunoassay using a set of 40 clinical samples⁴² (corresponding scatter and Bland-Altman plots are shown in Figure 2). The negative intercept of the regression line in Figure 2-A and the positive relative differences in Figure 2-B indicate that there is a bias between the measurements with the IDS-iSYS IGF1 immunoassay giving higher values than the MALDImmunoassay. This bias may be explained

by the different assay principles of both methods. With the MALDImmunoassay, IGF1 levels are calculated solely based on the response of IGF1 with a mass of 7,649 Da, while the IDS-iSYS IGF1 immunoassay may also respond to other IGF1 proteoforms, such as des(1-3)IGF1, proteolytic fragments and potential post translational modifications of IGF1 that escape the MALDImmunoassay.

Moreover, Figure 2 indicates that there are two regions with different biases, one for lower IGF1 concentrations (below ± 150 ng/mL) and one for higher IGF1 concentrations (above ± 150 ng/mL). For the lower concentrations, there is a relative difference between the assays of approximately 60% which decreases to about 20% for the higher concentrations. Lower values for the MALDImmunoassay may be due to pre-analytical variables leading to a reduced availability of wild-type IGF1 (*e.g.* proteolytic degradation, methionine oxidation) or may be caused by incomplete IGF1 extraction from specific plasma samples. Higher levels for the IDS-iSYS IGF1 immunoassay may be the result of cross-reactivity of the antibodies, which cannot be checked since the readout is indirect. In order to elucidate the reason(s) for the observed bias, further research is needed.

As for the abovementioned pre-analytical variables, we must acknowledge that potential degradation products may be 'missed' by the MALDImmunoassay. Yet, this characteristic could either be an advantage or a disadvantage of this assay depending on which samples and clinical questions are being studied. The MALDImmunoassay has the distinct advantage over IGF1 immunoassays that the levels obtained are based on defined chemical information and thereby relate to one IGF1 proteoform with a given potency, whereas methods that respond to multiple IGF1 proteoforms with different potencies yield IGF1 levels that cannot be directly related to potency. In particular, des(1-3)IGF1 and LONG^RIGF1 are known to be more potent than wild-type IGF1, which is presumably caused by altered binding affinities towards IGF1 binding proteins as a result of N-terminal structural differences.^{38,52} The MALDImmunoassay discriminates wild-type IGF1 from these variants, and thereby allows separate detection of these variants in the same experiment. When including calibrants and proper internal standards for these compounds, the resulting assay may even be used to quantify specific variants, which could be of interest, for example, in the field of doping analysis. Ultimately, one method is not necessarily better than the other, and the choice of the method for specific applications should depend on the available samples as well as the relevant clinical questions.

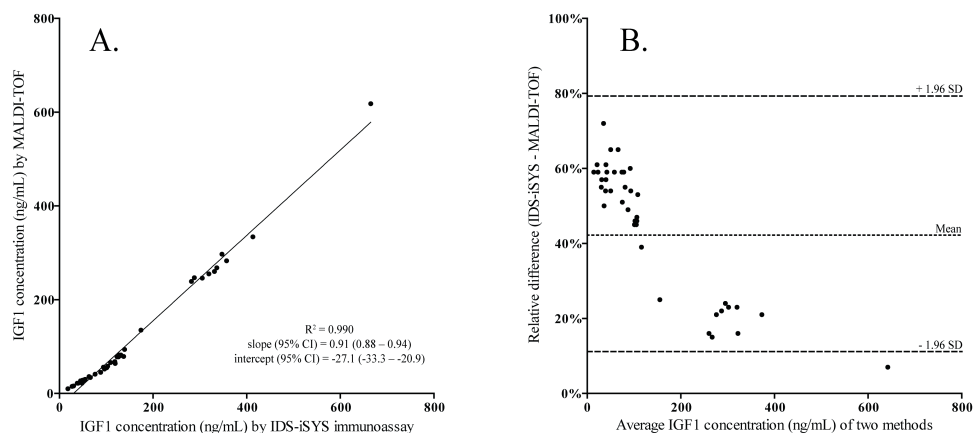


Figure 2. Comparison between the IGF1 MALDIImmunoassay and the IDS-iSYS IGF1 immunoassay using (A) linear regression and (B) the Bland-Altman plot.

2.3.4. Quality assessment of MALDI measurements

To study the performance of the MALDIImmunoassay more extensively, the method was applied to over 1,000 clinical samples. Ninety-six samples were processed per analytical run (*i.e.* 81 clinical samples, 8 calibrants, 1 blank, and duplicate QC-L, QC-M & QC-H samples). After a few runs, we observed that more time was needed per sample to reach the required number of acceptable spectra (with sufficient resolution). Peaks that fulfilled the preset acquisition specifications could not be found easily, and total MALDI measurement time increased significantly as a consequence. Ultimately, we found that this prolongation of analysis time was due to accumulation of matrix deposits in the MALDI source, and that this prolongation could be reversed by cleaning the source. Cleaning, however, necessitates venting of the instrument, so it goes hand in hand with considerable instrument downtime. Thus, maintaining good analytical quality comes at the price of reducing the method's (weekly) throughput.

To assess whether matrix deposits in the source affect data quality, we searched for readouts that allowed monitoring of data quality. In this regard, we observed that in parallel with the increasing analysis time the relative abundances of oxidation peaks also increased (Figure 3-A: run 1, 4 & 8). These abundances decreased again after cleaning of the source (Figure 3-A: run 12), thereby confirming that accumulating deposits in the source led to increased IGF1 oxidation during MALDI-TOF analysis, which is most likely due to prolonged exposure of the samples to UV irradiation. Subsequently, we calculated the ratio between the relative oxidation peaks of IGF1 and ^{15}N -IGF1, since methionine oxidation is not necessarily problematic if the internal standard can correct for this phenomenon. Figure 3-B shows these ratios for some of

the analytical runs, and indicates that corresponding distributions are slightly different for the displayed runs. The impact of these differences on the reported IGF1 levels is, however, limited which becomes apparent when comparing ‘regularly calculated’ IGF1 levels with IGF1 levels that are calculated using the sum of peak intensities from both non-oxidized and oxidized IGF1 (see Figure S-4). The differences between the obtained concentrations are well within $\pm 15\%$ with the exception of two samples for run 8 (see Figure S-5), and indicate that data was not substantially affected by matrix deposits in the source. Nonetheless, these figures highlight the significance of an appropriate cleaning interval for the MALDI source and also emphasize the need for using ^{15}N -IGF1 as internal standard. Eventually, we believe that monitoring oxidation peaks would be of interest for IGF1 (and potentially also for other methionine-containing proteins) as it enables to follow changing conditions in the MALDI source thus allowing to establish criteria for regular cleaning.

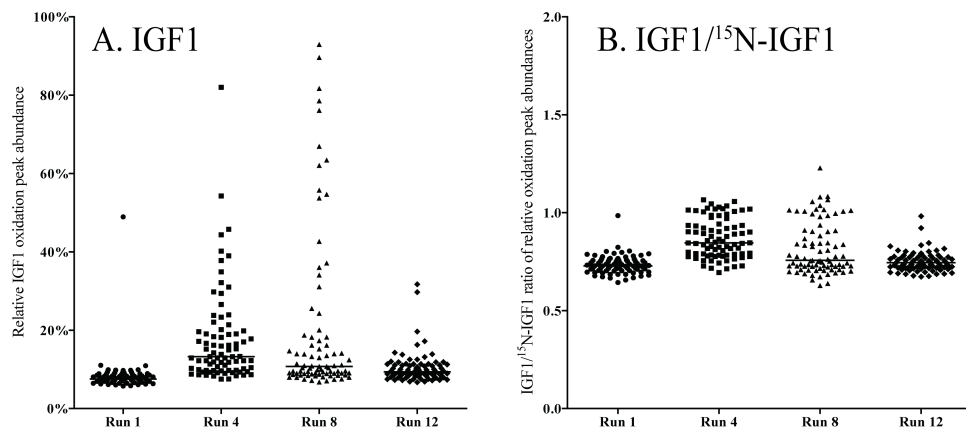


Figure 3. (A) Bee swarm plots of the relative abundance of the IGF1 oxidation peak and (B) the ratio of the IGF1 and ^{15}N -IGF1 relative abundances as observed in 4 (of the 13) analytical runs carried out for clinical sample analysis. With respect to the selected runs, the MALDI source was cleaned after run 8, thus run 1, 4 and 8 are shown to illustrate the effect of an increasing level of matrix deposits in the source, and run 12 is shown to illustrate the effect of cleaning the source. In order to calculate the relative abundances, the peak intensity of the oxidized analyte was divided by the sum of the peak intensities from the ‘native’ and the oxidized analyte. To calculate the ratio, the relative abundance of the IGF1 oxidation peak was divided by the relative abundance of the ^{15}N -IGF1 oxidation peak.

Besides evaluating oxidation peak abundances, we also monitored the variation between the results obtained for the different MALDI spots belonging to the same sample. Following the calculation of 4-spot CV values for every sample, a straightforward measure for monitoring MALDI measurement quality was obtained, which is not dependent on an analyte’s chemical composition (e.g. whether it contains one or more methionine residues). Figure 4 shows

observed 4-spot CV values plotted against the corresponding IGF1/¹⁵N-IGF1-ratios for the samples of a run that was performed under optimal analytical conditions (run 1) and for samples that were obtained with a ‘dirty’ source (run 8). This graph is rather revealing in several ways. Firstly, the patterns of both data series show that variation is typically lowest when IGF1 and the IS are present in equimolar amounts. This finding is in line with our previous observation that the precision for the midrange QC-samples was better than that of the QC-low and QC-high samples (see above). Secondly, 4-spot variation is clearly larger when the source contains matrix deposits and thus is in need of cleaning. We adopted a 4-spot CV cutoff value of 10% to ensure acceptable measurement quality. All samples with 4-spot CVs exceeding this value were re-analyzed with a clean source which resulted in CVs well below 10%. Admittedly, monitoring 4-spot variation necessitates using multiple spots per sample which affects the method’s throughput. Nevertheless, we recommend to monitor this quality indicator to ensure accurate data acquisition and to follow accumulation of matrix deposits in the source (additional data that support this recommendation are shown in Figure S-7 and the Tables S-11 and S-12).

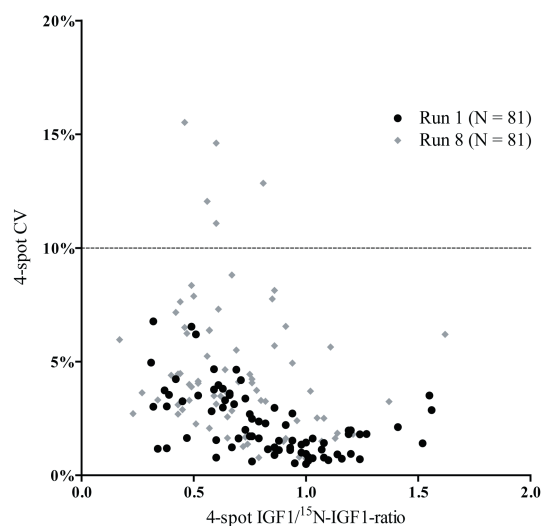


Figure 4. Scatter plot of observed 4-spot coefficients of variation plotted against the relative IGF1 quantities for run 1 (black dots, clean source) and run 8 (grey diamonds, source containing excessive matrix deposits). Individual plots for run 1, 4, 8, and 12 are shown in the Supporting Information in Figure S-6.

2.4. CONCLUSIONS

We describe a MALDImmunoassay for quantification of IGF1 in human plasma which complies with current international guidelines on quantitative bioanalysis. The assay shows good correlation with the IDS-iSYS IGF1 immunoassay. However, a positive bias was observed for the IDS-iSYS immunoassay as compared to the MALDImmunoassay, and the exact reasons for this bias are still unknown.

MALDImmunoassays combine immunoaffinity enrichment with MALDI-TOF MS detection, and both these methodological features are known sources of analytical variability. Consequently, the most critical feature of a reliable quantitative assay is the application of an appropriate internal standard which is capable of correcting for these sources of analytical variability. A stable-isotope-labeled (SIL) version of the full-length analyte is preferred for MALDImmunoassays, and therefore ^{15}N -IGF1 was used as internal standard in our IGF1 MALDImmunoassay. Another critical step for an IGF1 assay is proper liberation of IGF1 from its binding proteins which could interfere with the detection of IGF1. We demonstrate in an IGFBP3 challenge experiment as well as in spike recovery experiments that the SDS-based dissociation step is effectively leading to dissociation of the IGF1/IGFBP-complexes.

Application of the MALDImmunoassay to a clinical study comprising more than 1,000 clinical samples indicated that contamination of the MALDI source led to various degrees of oxidation of Met⁵⁹. This variation in IGF1 oxidation was corrected for by the ^{15}N -IGF1 internal standard emphasizing the need for a SIL internal standard. Furthermore, variation in IGF1 oxidation as well as the inter-spot variation were useful indicators of MALDI-TOF performance. Therefore, we recommend to monitor these quality indicators in order to assure consistent performance of the assay.

In conclusion, our work reports a validated MALDImmunoassay for quantification of IGF1 in human plasma and addresses some of the challenges of MALDImmunoassays that must be met in order to advance implementation of this technology into routine clinical diagnostics.

2.5. REFERENCES

1. Poste G. Bring on the biomarkers. *Nature*. 2011;469(7329):156-157.
2. Butler D. Translational research: Crossing the valley of death. *Nature*. 2008;453(7197):840-842.
3. Drucker E, Krapfenbauer K. Pitfalls and limitations in translation from biomarker discovery to clinical utility in predictive and personalised medicine. *EPMA J*. 2013;4(1):7-5085-4-7.
4. Parker CE, Borchers CH. Mass spectrometry based biomarker discovery, verification, and validation--quality assurance and control of protein biomarker assays. *Mol Oncol*. 2014;8(4):840-858.
5. Mitchell P. Proteomics retrenches. *Nat Biotechnol*. 2010;28(7):665-670.
6. Nelson RW, Borges CR. Mass spectrometric immunoassay revisited. *J Am Soc Mass Spectrom*. 2011;22(6):960-968.
7. Niederkofler EE, Tubbs KA, Gruber K, et al. Determination of beta-2 microglobulin levels in plasma using a high-throughput mass spectrometric immunoassay system. *Anal Chem*. 2001;73(14):3294-3299.
8. Tubbs KA, Nedelkov D, Nelson RW. Detection and quantification of beta-2-microglobulin using mass spectrometric immunoassay. *Anal Biochem*. 2001;289(1):26-35.
9. Nelson RW, Nedelkov D, Tubbs KA, Kiernan UA. Quantitative mass spectrometric immunoassay of insulin like growth factor 1. *J Proteome Res*. 2004;3(4):851-855.
10. Kiernan UA, Addobati R, Nedelkov D, Nelson RW. Quantitative multiplexed C-reactive protein mass spectrometric immunoassay. *J Proteome Res*. 2006;5(7):1682-1687.
11. Kiernan UA, Nedelkov D, Nelson RW. Multiplexed mass spectrometric immunoassay in biomarker research: A novel approach to the determination of a myocardial infarct. *J Proteome Res*. 2006;5(11):2928-2934.
12. Niederkofler EE, Kiernan UA, O'Rear J, et al. Detection of endogenous B-type natriuretic peptide at very low concentrations in patients with heart failure. *Circ Heart Fail*. 2008;1(4):258-264.
13. Trenchevska O, Kamcheva E, Nedelkov D. Mass spectrometric immunoassay for quantitative determination of protein biomarker isoforms. *J Proteome Res*. 2010;9(11):5969-5973.
14. Kiernan UA, Phillips DA, Trenchevska O, Nedelkov D. Quantitative mass spectrometry evaluation of human retinol binding protein 4 and related variants. *PLoS One*. 2011;6(3):e17282.
15. Oran PE, Jarvis JW, Borges CR, Sherma ND, Nelson RW. Mass spectrometric immunoassay of intact insulin and related variants for population proteomics studies. *Proteomics Clin Appl*. 2011;5(7-8):454-459.
16. Trenchevska O, Kamcheva E, Nedelkov D. Mass spectrometric immunoassay for quantitative determination of transthyretin and its variants. *Proteomics*. 2011;11(18):3633-3641.
17. Trenchevska O, Nedelkov D. Targeted quantitative mass spectrometric immunoassay for human protein variants. *Proteome Sci*. 2011;9(1):19-5956-9-19.
18. Oran PE, Trenchevska O, Nedelkov D, et al. Parallel workflow for high-throughput (>1,000 samples/day) quantitative analysis of human insulin-like growth factor 1 using mass spectrometric immunoassay. *PLoS One*. 2014;9(3):e92801.
19. Sherma ND, Borges CR, Trenchevska O, et al. Mass spectrometric immunoassay for the qualitative and quantitative analysis of the cytokine macrophage migration inhibitory factor (MIF). *Proteome Sci*. 2014;12(1):52-014-0052-3. eCollection 2014.
20. Trenchevska O, Schaab MR, Nelson RW, Nedelkov D. Development of multiplex mass spectrometric immunoassay for detection and quantification of apolipoproteins C-I, C-II, C-III and their proteoforms. *Methods*. 2015;81:86-92.

21. Trenchevska O, Sherma ND, Oran PE, Reaven PD, Nelson RW, Nedelkov D. Quantitative mass spectrometric immunoassay for the chemokine RANTES and its variants. *J Proteomics*. 2015;116:15-23.
22. Razavi M, Johnson LD, Lum JJ, Kruppa G, Anderson NL, Pearson TW. Quantification of a proteotypic peptide from protein C inhibitor by liquid chromatography-free SISCAPA-MALDI mass spectrometry: Application to identification of recurrence of prostate cancer. *Clin Chem*. 2013;59(10):1514-1522.
23. van den Broek I, Nouta J, Razavi M, et al. Quantification of serum apolipoproteins A-I and B-100 in clinical samples using an automated SISCAPA-MALDI-TOF-MS workflow. *Methods*. 2015;81:74-85.
24. Jiang J, Parker CE, Hoadley KA, Perou CM, Boysen G, Borchers CH. Development of an immuno tandem mass spectrometry (iMALDI) assay for EGFR diagnosis. *Proteomics Clin Appl*. 2007;1(12):1651-1659.
25. Reid JD, Holmes DT, Mason DR, Shah B, Borchers CH. Towards the development of an immuno MALDI (iMALDI) mass spectrometry assay for the diagnosis of hypertension. *J Am Soc Mass Spectrom*. 2010;21(10):1680-1686.
26. Mason DR, Reid JD, Camenzind AG, Holmes DT, Borchers CH. Duplexed iMALDI for the detection of angiotensin I and angiotensin II. *Methods*. 2012;56(2):213-222.
27. Camenzind AG, van der Gugten JG, Popp R, Holmes DT, Borchers CH. Development and evaluation of an immuno-MALDI (iMALDI) assay for angiotensin I and the diagnosis of secondary hypertension. *Clin Proteomics*. 2013;10(1):20-0275-10-20.
28. Popp R, Malmstrom D, Chambers AG, et al. An automated assay for the clinical measurement of plasma renin activity by immuno-MALDI (iMALDI). *Biochim Biophys Acta*. 2015;1854(6):547-558.
29. Gobom J, Kraeuter KO, Persson R, Steen H, Roepstorff P, Ekman R. Detection and quantification of neurotensin in human brain tissue by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Anal Chem*. 2000;72(14):3320-3326.
30. Boyer AE, Quinn CP, Woolfitt AR, et al. Detection and quantification of anthrax lethal factor in serum by mass spectrometry. *Anal Chem*. 2007;79(22):8463-8470.
31. Gelfanova V, Higgs RE, Dean RA, et al. Quantitative analysis of amyloid-beta peptides in cerebrospinal fluid using immunoprecipitation and MALDI-tof mass spectrometry. *Brief Funct Genomic Proteomic*. 2007;6(2):149-158.
32. Kuklenyik Z, Boyer AE, Lins R, et al. Comparison of MALDI-TOF-MS and HPLC-ESI-MS/MS for endopeptidase activity-based quantification of anthrax lethal factor in serum. *Anal Chem*. 2011;83(5):1760-1765.
33. Kaneko N, Yamamoto R, Sato TA, Tanaka K. Identification and quantification of amyloid beta-related peptides in human plasma using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Proc Jpn Acad Ser B Phys Biol Sci*. 2014;90(3):104-117.
34. Meyer K, Ueland PM. Targeted quantification of C-reactive protein and cystatin c and its variants by immuno-MALDI-MS. *Anal Chem*. 2014;86(12):5807-5814.
35. Faron ML, Buchan BW, Hyke J, et al. Multicenter evaluation of the bruker MALDI biotyper CA system for the identification of clinical aerobic gram-negative bacterial isolates. *PLoS One*. 2015;10(11):e0141350.
36. Baker M. Reproducibility crisis: Blame it on the antibodies. *Nature*. 2015;521(7552):274-276.
37. Szajli E, Feher T, Medzihradzky KF. Investigating the quantitative nature of MALDI-TOF MS. *Mol Cell Proteomics*. 2008;7(12):2410-2418.
38. Bredehoft M, Schanzer W, Thevis M. Quantification of human insulin-like growth factor-1 and qualitative detection of its analogues in plasma using liquid chromatography/electrospray ionisation tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2008;22(4):477-485.

39. Cox HD, Lopes F, Woldemariam GA, et al. Interlaboratory agreement of insulin-like growth factor 1 concentrations measured by mass spectrometry. *Clin Chem*. 2014;60(3):541-548.
40. Food and Drug Administration (FDA). *Guidance for industry: Bioanalytical method validation*. Washington, DC, U.S.A.: U.S. Department of Health and Human Services; 2001.
41. van den Berg E, Engberink MF, Brink EJ, et al. Dietary acid load and metabolic acidosis in renal transplant recipients. *Clin J Am Soc Nephrol*. 2012;7(11):1811-1818.
42. Grimminger P, Frystyk J, Blankenstein O, et al. Inter-laboratory comparison of IGF-I concentrations measured by an automated immunoassay: Results from a multicentre study across europe. *Experimental and Clinical Endocrinology & Diabetes*. 2015;123:LB_11.
43. Hines J, Milosevic D, Ketha H, et al. Detection of IGF-1 protein variants by use of LC-MS with high-resolution accurate mass in routine clinical analysis. *Clin Chem*. 2015;61(7):990-991.
44. Nicola AJ, Gusev AI, Proctor A, Jackson EK, Hercules DM. Application of the fast-evaporation sample preparation method for improving quantification of angiotensin II by matrix-assisted laser desorption/ionization. *Rapid communications in mass spectrometry : RCM* [- 12]. 1995;9(12):1164-1171.
45. van de Merbel NC. Quantitative determination of endogenous compounds in biological samples using chromatographic techniques. *TrAC Trends in Analytical Chemistry*. 2008;27(10):924-933.
46. Bronsema KJ, Bischoff R, van de Merbel NC. High-sensitivity LC-MS/MS quantification of peptides and proteins in complex biological samples: The impact of enzymatic digestion and internal standard selection on method performance. *Anal Chem*. 2013;85(20):9528-9535.
47. Helmke SM, Yen CY, Cios KJ, et al. Simultaneous quantification of human cardiac alpha- and beta-myosin heavy chain proteins by MALDI-TOF mass spectrometry. *Anal Chem*. 2004;76(6):1683-1689.
48. Chang PC, Reddy PM, Ho YP. Quantification of genetically modified soya using strong anion exchange chromatography and time-of-flight mass spectrometry. *Anal Bioanal Chem*. 2014;406(22):5339-5346.
49. Anderson DS, Kirchner M, Kellogg M, et al. Design and validation of a high-throughput matrix-assisted laser desorption ionization time-of-flight mass spectrometry method for quantification of hepcidin in human plasma. *Anal Chem*. 2011;83(21):8357-8362.
50. Anderson NL, Razavi M, Pearson TW, Kruppa G, Paape R, Suckau D. Precision of heavy-light peptide ratios measured by maldi-tof mass spectrometry. *J Proteome Res*. 2012;11(3):1868-1878.
51. Niederkofler EE, Phillips DA, Krastins B, et al. Targeted selected reaction monitoring mass spectrometric immunoassay for insulin-like growth factor 1. *PLoS One*. 2013;8(11):e81125.
52. Thomas A, Kohler M, Schanzer W, Delahaut P, Thevis M. Determination of IGF-1 and IGF-2, their degradation products and synthetic analogues in urine by LC-MS/MS. *Analyst*. 2011;136(5):1003-1012.

2.6. SUPPORTING INFORMATION

The Tables S-1 to S-10 can be found in the online version of the Supporting Information which is available on the ACS Publications website at DOI: 10.1021/acs.analchem.7b01125.

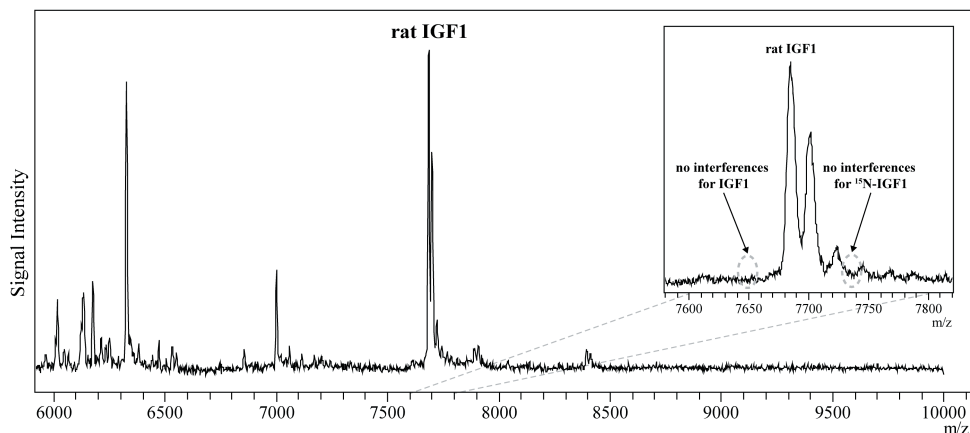


Figure S-1. MALDI mass spectrum from non-spiked rat plasma (used as calibration matrix) showing that rat plasma does not give rise to peaks that interfere with the peaks originating from human IGF1 (used for preparation of the calibration curves) and ^{15}N -labeled human IGF1 (used as internal standard), or relevant IGF1 variants.

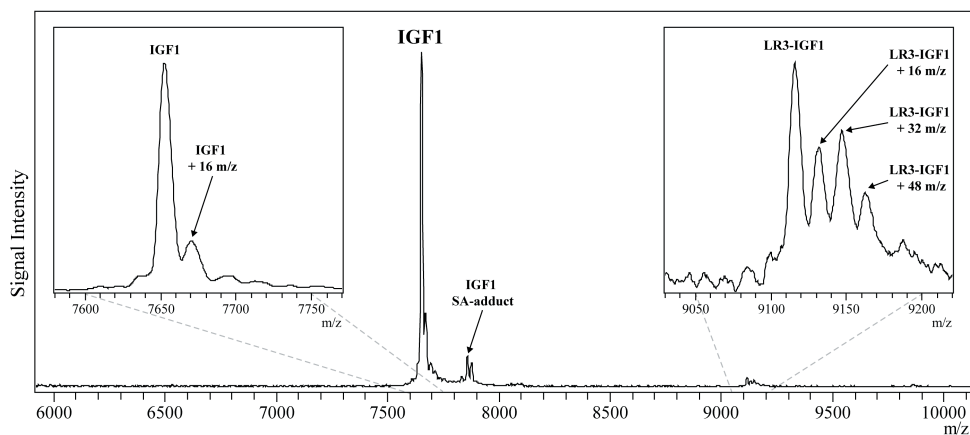


Figure S-2. MALDI mass spectrum showing differences in the number of (putative) oxidation peaks for IGF1 and LONG^{R} IGF1 (LR3-IGF1).

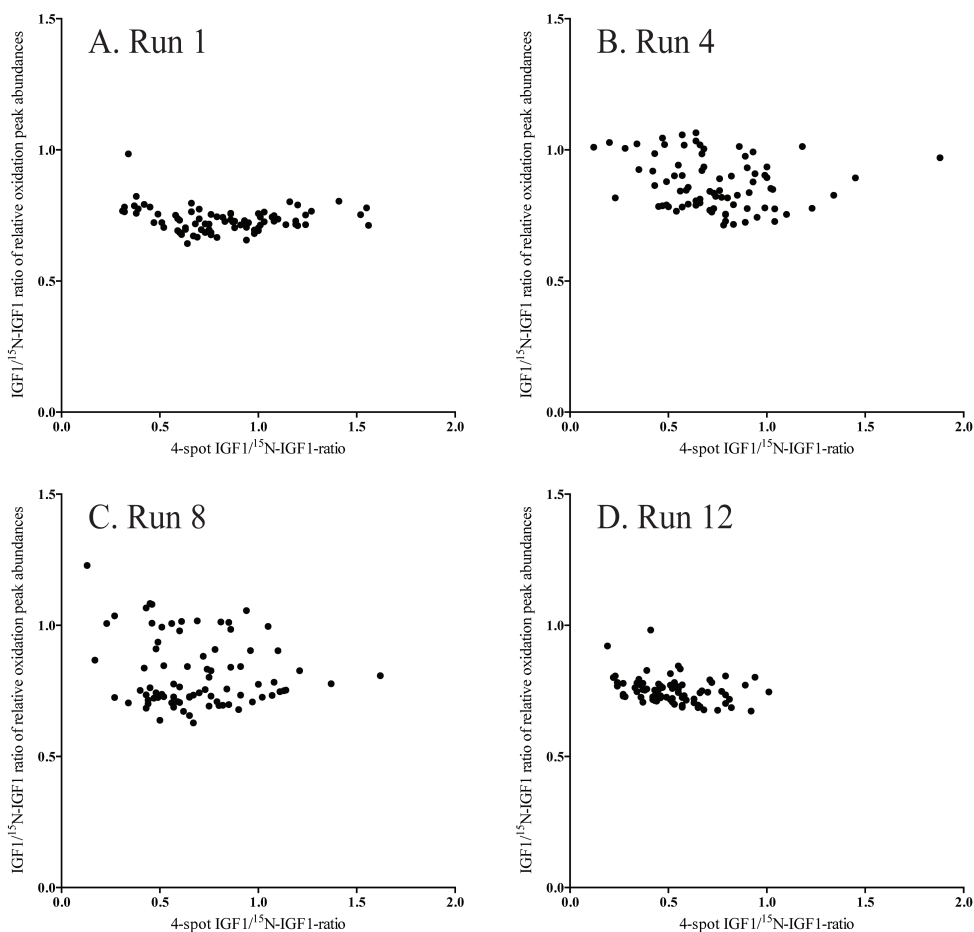


Figure S-3. Scatter plot of observed IGF1/¹⁵N-IGF1 ratios of relative oxidation peak abundances plotted against the relative IGF1 quantities for 4 (of the 13) analytical runs carried out for clinical sample analysis. With respect to the selected runs, the MALDI source was cleaned after run 8, thus run 1, 4 and 8 are shown to illustrate the effect of accumulating matrix deposits in the source, and run 12 is shown to illustrate the effect of cleaning the source. In order to calculate the relative abundances, the peak intensity of the oxidized analyte was divided by the sum of the peak intensities from the native and the oxidized analyte. To calculate the ratio, the relative abundance of the IGF1 oxidation peak was divided by the relative abundance of the ¹⁵N-IGF1 oxidation peak.

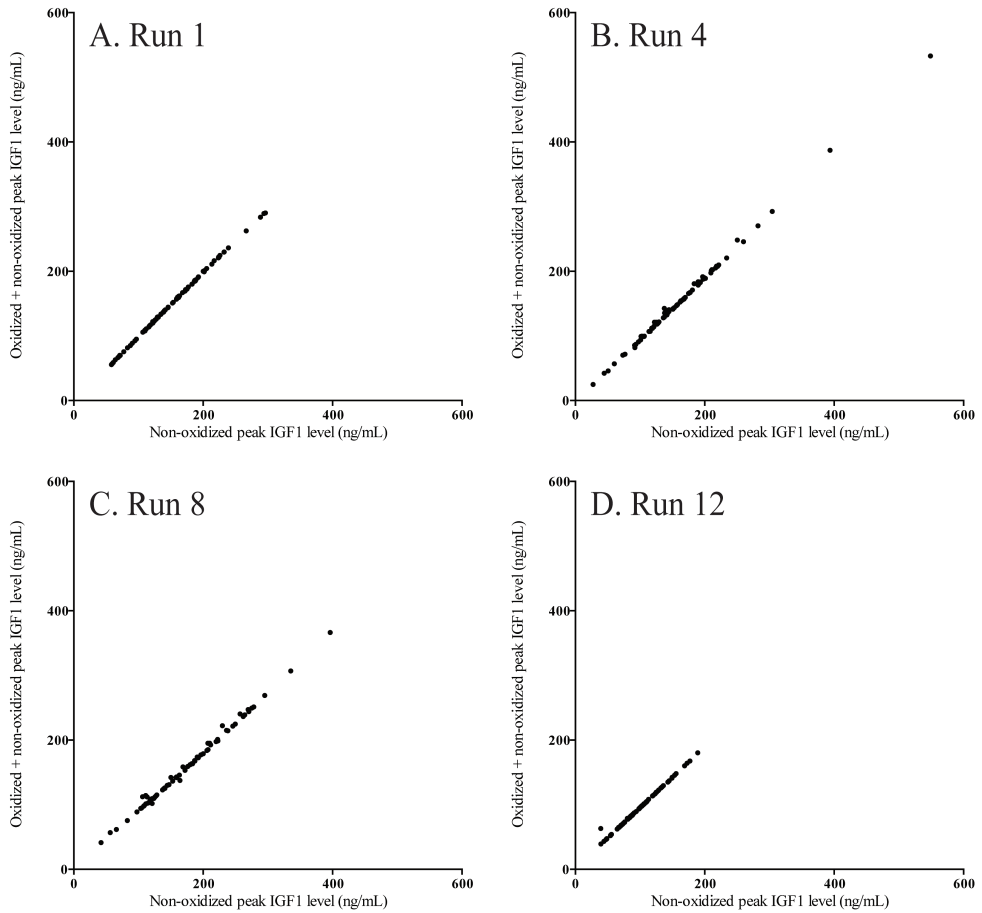


Figure S-4. Scatter plot of IGF1 levels based on the sum of non-oxidized and oxidized IGF1 plotted against IGF1 levels calculated using solely non-oxidized IGF1 for 4 (of the 13) analytical runs carried out for clinical sample analysis. With respect to the selected runs, the MALDI source was cleaned after run 8, thus run 1, 4 and 8 are shown to illustrate the effect of accumulating matrix deposits in the source, and run 12 is shown to illustrate the effect of cleaning the source.

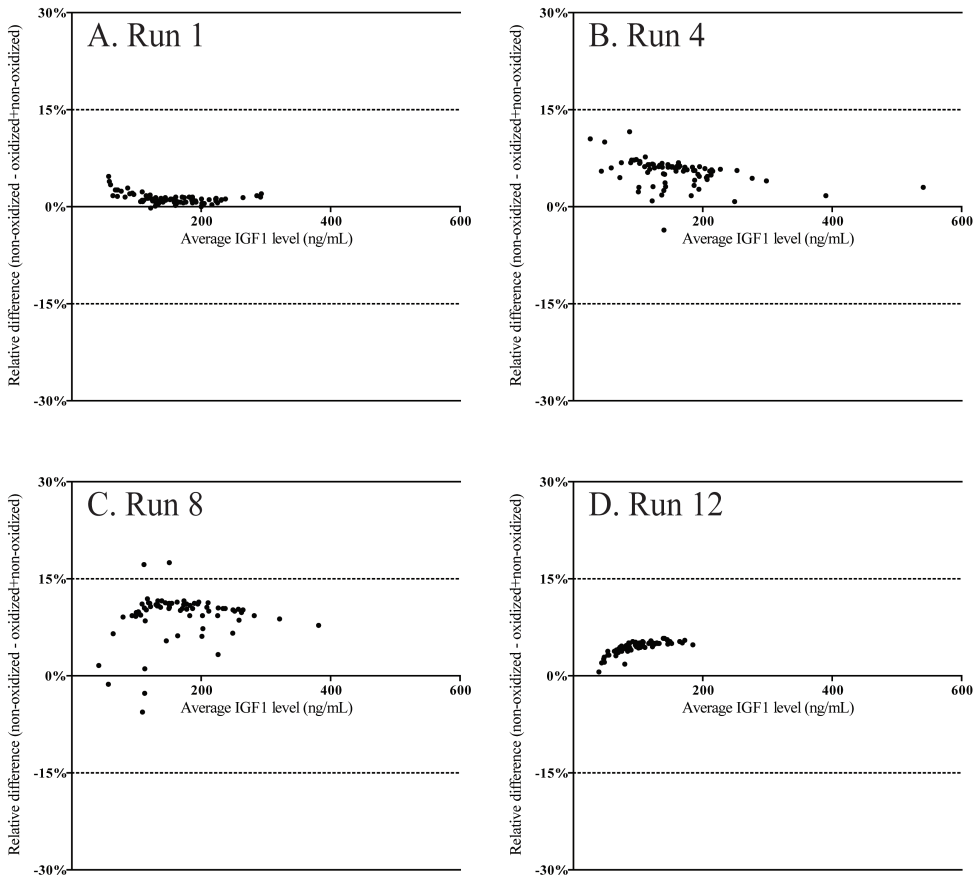


Figure S-5. Bland-Altman plots displaying the relative differences between IGF1 levels calculated using solely non-oxidized IGF1 and levels based on the sum of non-oxidized and oxidized IGF1 plotted against the average of both IGF1 levels for 4 (of the 13) analytical runs carried out for clinical sample analysis. With respect to the selected runs, the MALDI source was cleaned after run 8, thus run 1, 4 and 8 are shown to illustrate the effect of accumulating matrix deposits in the source, and run 12 is shown to illustrate the effect of cleaning the source.

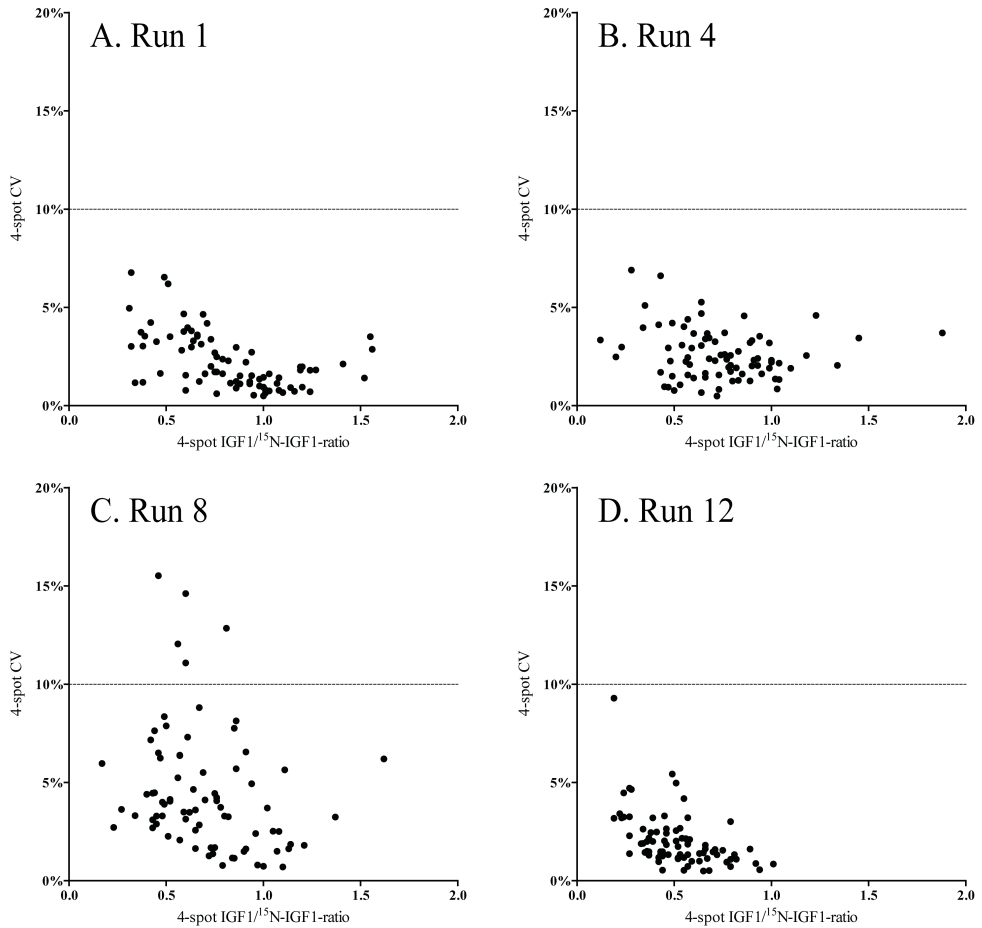


Figure S-6. Scatter plot of observed 4-spot coefficients of variation plotted against the relative IGF1 quantities for 4 (of the 13) analytical runs carried out for clinical sample analysis. With respect to the selected runs, the MALDI source was cleaned after run 8, thus the runs 1, 4 and 8 are shown to illustrate the effect of accumulating matrix deposits in the source, and run 12 is shown to illustrate the effect of cleaning the source.

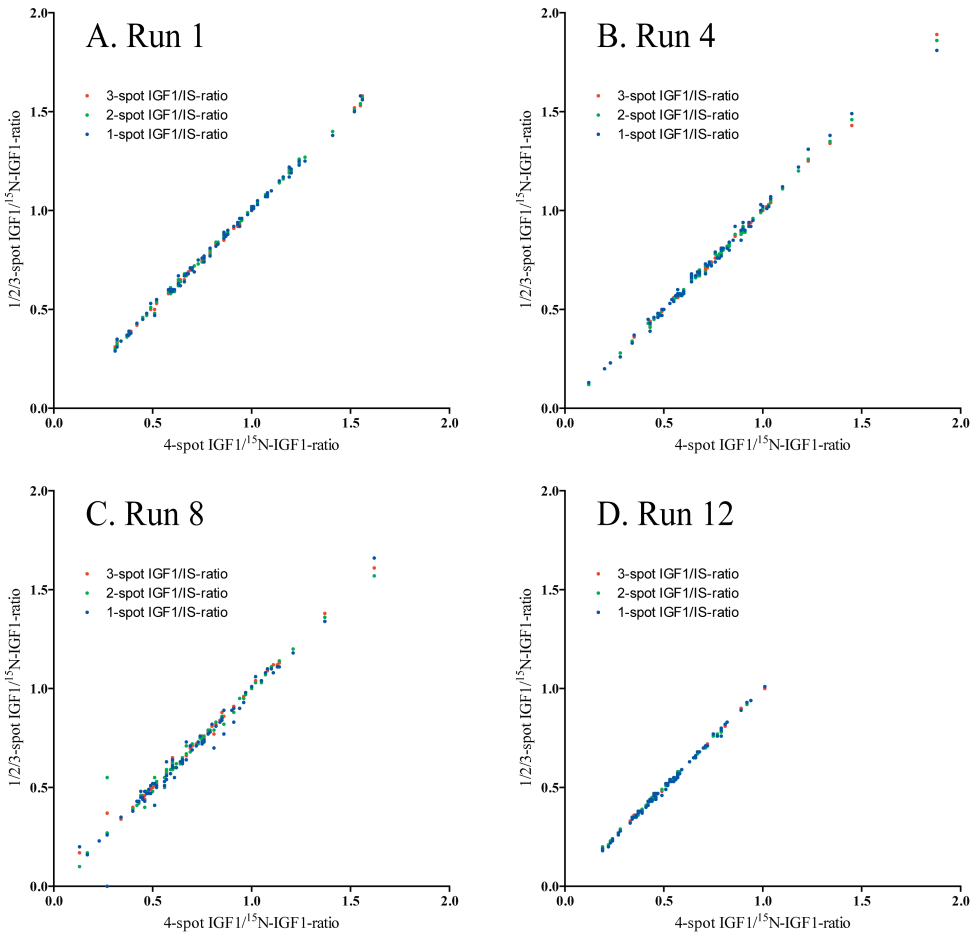


Figure S-7. Scatter plots for the comparison of IGF1 levels that are calculated using 1, 2 or 3 spots per sample versus IGF1 levels that are calculated using 4 spots per sample, as observed for 4 (of the 13) analytical runs carried out for clinical sample analysis. With respect to the selected runs, the MALDI source was cleaned after run 8, thus the runs 1, 4 and 8 are shown to illustrate the effect of accumulating matrix deposits in the source, and run 12 is shown to illustrate the effect of cleaning the source. Linear regression data belonging to the data series in the graphs above are presented in Table S-11. Furthermore, Table S-12 provides an overview of the relative differences between IGF1 levels that are calculated based on 4-spot IGF1/ ^{15}N -IGF1-ratios and levels that are calculated based on fewer spots per sample.

Table S-11. Linear regression data belonging to the comparisons of Figure S-7.

Run	Linear regression	3- vs. 4-spot IGF1/IS-ratio	2- vs. 4-spot IGF1/IS-ratio	1- vs. 4-spot IGF1/IS-ratio
1	slope (95% CI)	0.9999 (0.9943 to 1.0060)	0.9992 (0.9912 to 1.0070)	0.9974 (0.9854 to 1.0090)
	intercept (95% CI)	0.0015 (-0.0034 to 0.0065)	0.0040 (-0.0031 to 0.0110)	0.0057 (-0.0048 to 0.0161)
	R ²	0.9994	0.9987	0.9972
4	slope (95% CI)	0.9966 (0.9918 to 1.0010)	0.9991 (0.9916 to 1.0070)	1.0030 (0.9885 to 1.0180)
	intercept (95% CI)	0.0042 (0.0001 to 0.0082)	0.0029 (-0.0033 to 0.0092)	0.0026 (-0.0096 to 0.0149)
	R ²	0.9995	0.9989	0.9958
8	slope (95% CI)	0.9875 (0.9744 to 1.0010)	0.9700 (0.9408 to 0.9992)	1.0050 (0.9696 to 1.0390)
	intercept (95% CI)	0.0114 (0.0016 to 0.0211)	0.0211 (-0.0006 to 0.0428)	-0.0144 (-0.0403 to 0.0116)
	R ²	0.9965	0.9823	0.9765
12	slope (95% CI)	1.0000 (0.9938 to 1.0060)	1.0030 (0.9951 to 1.0110)	1.0090 (0.9966 to 1.0210)
	intercept (95% CI)	0.0004 (-0.0030 to 0.0037)	-0.0016 (-0.0058 to 0.0027)	-0.0071 (-0.0138 to -0.0004)
	R ²	0.9992	0.9988	0.9971

Table S-12. Relative differences between IGF1 levels calculated using 1, 2 or 3 spots per sample and IGF1 levels that are calculated using 4 spots per sample. The table shows the number of samples per run (=81 samples) clustered based on the magnitude of the differences between the calculated levels.

Run	Difference	3- vs. 4-spot IGF1/IS-ratio	2- vs. 4-spot IGF1/IS-ratio	1- vs. 4-spot IGF1/IS-ratio
1	<5.0%	81	79	75
	5.0-15.0%	0	2	6
	>15.0%	0	0	0
4	<5.0%	81	80	72
	5.0-15.0%	0	1	9
	>15.0%	0	0	0
8	<5.0%	77	74	64
	5.0-15.0%	2	5	14
	>15.0%	2	2	3
12	<5.0%	80	80	77
	5.0-15.0%	1	1	4
	>15.0%	0	0	0

