Chapter 3

Standardization of calibration and quality control using Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry

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Clin Chim Acta 2006;366:249-56

Abstract

Background
Protein profiling by Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry is gaining importance as a diagnostic tool for a whole range of diseases. This report describes a quality control procedure, which acts prospectively by checking the calibration before starting profiling experiments.

Methods
A well-defined protocol for calibration of the Protein Biosystem IIc instrument was established, using a commercial quality control sample containing independent certified standards and by determination of acceptance criteria. Instrument calibration was performed externally every week with the standards provided by the manufacturer. Quality control was performed for the period of five months.

Results
According to the acceptance criteria defined in this study, data points should be in the established range of the process mean ± two standard deviations for the mass-to-charge ratio values, peak intensities, signal-to-noise ratios, and peak resolutions for insulin and apomyoglobin in the quality control sample. Moreover, it was demonstrated that the pipetting variability in the handling of the quality control sample significantly contributed to systematic errors and that spotting of a larger volume of quality control sample resulted in a better reproducibility.

Conclusions
Stringent quality control of the calibration part of Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry experiments prevents unreliable data acquisition from the very start.

Introduction

Proteomic pattern analysis by Surface-Enhanced Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (SELDI-TOF-MS) is one of the most promising new approaches for the discovery and identification of potential biomarkers for various diseases. The first clinical investigations using SELDI-TOF-MS for different types of cancer, e.g., ovarian[1-6], prostate[7-11], lung[12], and brain cancer or inflammatory diseases[13,14] revealed high diagnostic sensitivities and specificities. Notwithstanding using identical types of biological specimens and the same analytical platform[15], several groups identified different patterns for the same types of cancers. A recent study by Karsan et al.[16] provided evidence that both pre-analytical and analytical variation can affect profiled markers. They demonstrated that specimen collection and processing introduce significant biases in the spectral pattern. The differences between the pre-and post-analytical strategies used in various studies was also reviewed by Bons et al.[17]. The effect of pre- and post-analytical variables on protein profiling needs further and more systematic investigation. Therefore, a stringent standardized protocol is needed, not only for pre- and post-analytical aspects, but also for calibration and quality control (QC) performance.

In a recent study by Semmes et al.[18] the reproducibility of the SELDI-TOF-MS was examined. Instruments of six different laboratories were calibrated by use of an established set of protocols and the instrument output was standardized with respect to three prominent mass-to-charge ratio *(m/z)* "peaks" present in a pooled QC serum sample. They also evaluated the ability of the calibrated and standardized instrumentation to accurately differentiate between selected cases of prostate cancer and control by use of an algorithm developed from data derived from a single laboratory two years earlier. In that study was demonstrated that under strict operating procedures, they were able to achieve across-laboratory reproducibility for SELDI-TOF-MS analysis and showed that adequate calibration was one of the critical steps.

Recently Plebani et al.[19] indicated that only few studies have been made describing good quality control procedures that should be incorporated in proteomic experimental protocols. The aim of our study was to establish a well-defined protocol for calibration of the Protein Biosystem IIc (PBSIIc) instrument, to implement a QC sample with independent certified standards and to determine acceptance criteria for quality control. Because the QC samples were spotted on a normal phase (NP-20) ProteinChip array, which is a normal phase array, without washing or selective binding steps, only the Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) part of the PBSIIc instrument was checked. Stable instrument performance over time is a prerequisite before any proteomic experiments should be performed. The QC procedure described in our report acts prospectively by checking the calibration

every week in contrast to some other studies, where QC samples are included in the profiling studies and quality control thus acts retrospectively or where no quality control procedure is performed at all.

Materials and Methods

Calibration samples

Instrument calibration was performed externally with the All-in-1 peptide and All-in-1 protein standards (Ciphergen Biosystems, Inc., Fremont, CA, USA). Both standards as well as the sinapinic acid (SPA) solution as energy absorbing matrix (Ciphergen Biosystems) were prepared according to the recommendations of the manufacturer, with the exception that trifluoroacetic anhydride (TFAH) as a solvent component was used instead of trifluoroacetic acid (TFA). On a NP-20 array (Ciphergen Biosystems) with eight spots, 1 µl of All-in-1 peptide standard was applied to the spots A-D, while 1 µl of All-in-1 protein standard was applied to the spots E-H. After preparation, the same calibration array was used for all experiments during the whole period, using one spot each week. The spots, including within-spot positions were alternated weekly. All seven calibrants of the All-in-1 peptide standard, ranging in molecular weight from 1084 to 7034 Dalton (Da), were used to generate the peptide calibration equation. The four lowest calibrants of the All-in-1 protein standard, ranging in molecular weight from 7034 to 29,023 Da, and the three highest calibrants, ranging in molecular weight from 46,671 to 147,300 Da of the All-in-1 protein standard, were used to generate the protein-low calibration and protein-high calibration equations, respectively. In this study the protein-high calibration equation was not evaluated. The calibration array was stored in a dry and dark environment until further use.

Quality control sample

The QC sample consisted of the Proteomass MALDI-TOF-MS standards insulin and apomyoglobin (Sigma-Aldrich CO, St. Louis, MO, USA). According to the specifications the standards should produce in MALDI-TOF-MS analysis the $[M+H]^+$ ions at *m/z* 5734.51 and *m/z* 16,952.27 for insulin and apomyoglobin, respectively. Insulin and apomyoglobin were mixed together according to the manufacturer's specifications. One µl of QC sample was applied on a NP-20 array. SPA was prepared as described above. The array was dried and afterwards 1 µl SPA solution was applied. The spotting of SPA was repeated once. Each batch of the QC sample was divided into aliquots and stored at –20°C. A batch was used for one month, according to manufacturer's specifications and aliquots were spotted every week on a new spot of a NP-20 array.

Reproducibility

For the reproducibility test, spotting of 1 µl QC sample (QC_1) was performed as described above. From the same batch, additional QC samples were prepared by diluting the QC sample five times with 1% of TFAH solution. Five µl of this diluted QC sample (QC_5) were spotted on a NP-20 array, so the absolute amount of insulin and apomyoglobin on the spot was identical for QC_1 and QC_5 samples. The reproducibility of QC_1 and QC_5 was determined with the following two experiments. In one experiment (A), two NP-20 arrays were spotted with QC_1 samples and two other NP-20 arrays with QC_5 samples, to compare the reproducibility of both QC samples and to determine the inter-chip variability. In the other experiment (B) the QC_1 and QC_5 samples were spotted alternately over four NP-20 arrays to compare the reproducibility of both QC samples and to reduce the effect of inter-chip variability by dividing both QC samples over all NP-20 arrays. The reproducibility of QC_1 and QC_5 samples was determined by calculating the CV values for the *m/z* values, intensities, signal-to-noise (S/N) ratios, and peak resolutions of the insulin and apomyoglobin signal.

Instrumental settings and calibration

The calibration and QC arrays were analyzed on a PBSIIc instrument (Ciphergen Biosystems). The high mass setting for the peptide standard was set at 10 kDa, with an optimization range between 1 and 7.5 kDa. Focus mass and deflector were set at 4 kDa and 500 Da, respectively. Mass spectrometry profiles were generated by averaging 130 laser shots (laser intensity (LI) 165, detector sensitivity (SE) 6). For the protein standard low, the high mass setting was set at 50 kDa, with an optimization range between 7 and 30 kDa. The focus mass and deflector were set at 15 and 1 kDa, respectively. Mass spectrometry profiles were generated by averaging 130 laser shots (LI 215, SE 7). The high mass setting for the protein standard high was set at 200 kDa, with an optimization range between 25 and 150 kDa. Focus mass and deflector were set at 70 and 10 kDa, respectively. Mass spectrometry profiles were generated by averaging 130 laser shots (LI 220, SE 7). The calibration curves were generated with the 3-parameter calibration in the Biomarker ProteinChip Software 3.2.0 (Ciphergen Biosystems).
For the QC sample, the high mass setting was set at 20 kDa, with an optimization range between 5 and 20 kDa. Focus mass and deflector were set at 10 and 1 kDa, respectively. Mass spectrometry profiles were generated by averaging 130 laser shots (LI 160, SE 7). The mass accuracy of insulin and apomyoglobin were determined after calibrating with the most recent peptide and protein-low calibration equations, respectively. The mass accuracies were defined in this study as the quotient of the mass difference and the process mean of the *m/z* values.
For the reproducibility test, the high mass setting, optimization range, focus mass, and deflector settings were the same as described above. The mass spectrometry

profiles were also generated by averaging 130 laser shots and the optimal LI and SE for QC_1 and QC_5 samples were selected for both experiments. The spectra of the reproducibility test were normalized for total ion current, because they were generated during one experiment. However, the weekly generated QC spectra were not normalized.

Data analysis

Data analysis was performed with in house developed software (ShewhartPlots), which was based on the Shewhart control chart principle[20]. Two-dimensional Youden plots were made by drawing insulin (x-axis) and apomyoglobin (y-axis) in one plot for the *m/z* values, intensities, S/N ratios, and peak resolutions of insulin and apomyoglobin in the QC sample. The fulfilment of the following Westgard rules was checked: 1_{3s}, 2_{2s}, 4_{1s}, 8_x, 10_x, and 12_x[21].
Statistical analysis to compare CV values was performed with the two-tailed F-test.

Results

Acceptance criteria

The process mean and standard deviations for the *m/z* values, intensities, S/N ratios, and peak resolutions of insulin and apomyoglobin in the QC-sample are shown in Figure 4.1. A process mean will change when a new data point is added in comparison to a fixed mean, where the mean is always constant. A few data points occasionally exceeded the process mean ± 2SD range, which was noticed using the Westgard 1_{2s} rule as the warning signal. After inspection it was clear that none of the measurements violated the rules 1_{3s}, 2_{2s}, 4_{1s}, 8_x, 10_x, and 12_x and that therefore the 1_{2s} observations were related to random errors.
The two dimensional Youden plots, in which the data points of insulin and apomyoglobin were combined in one plot, also demonstrated that most points were within the process mean ± 2SD range and none of the points were outside the process mean ± 3SD range. Figure 3.2 illustrates two examples of two-dimensional Youden plots, one of the *m/z* values (Figure 3.2A) and the other of the intensities of insulin and apomyoglobin (Figure 3.2B). The advantage of two-dimensional Youden plots is that when the QC sample contains two proteins, random errors can be distinguished from systematic errors. In all cases evaluated this way, random errors were noticed, except for the intensities of insulin and apomyoglobin, where a systematic error was noticed (Figure 3.2B). As with the 1_{2s} Westgard rule, the observation that the data points in the Youden plots are within the mean ± 3SD, but outside the mean ± 2SD range, can be used as a warning signal.

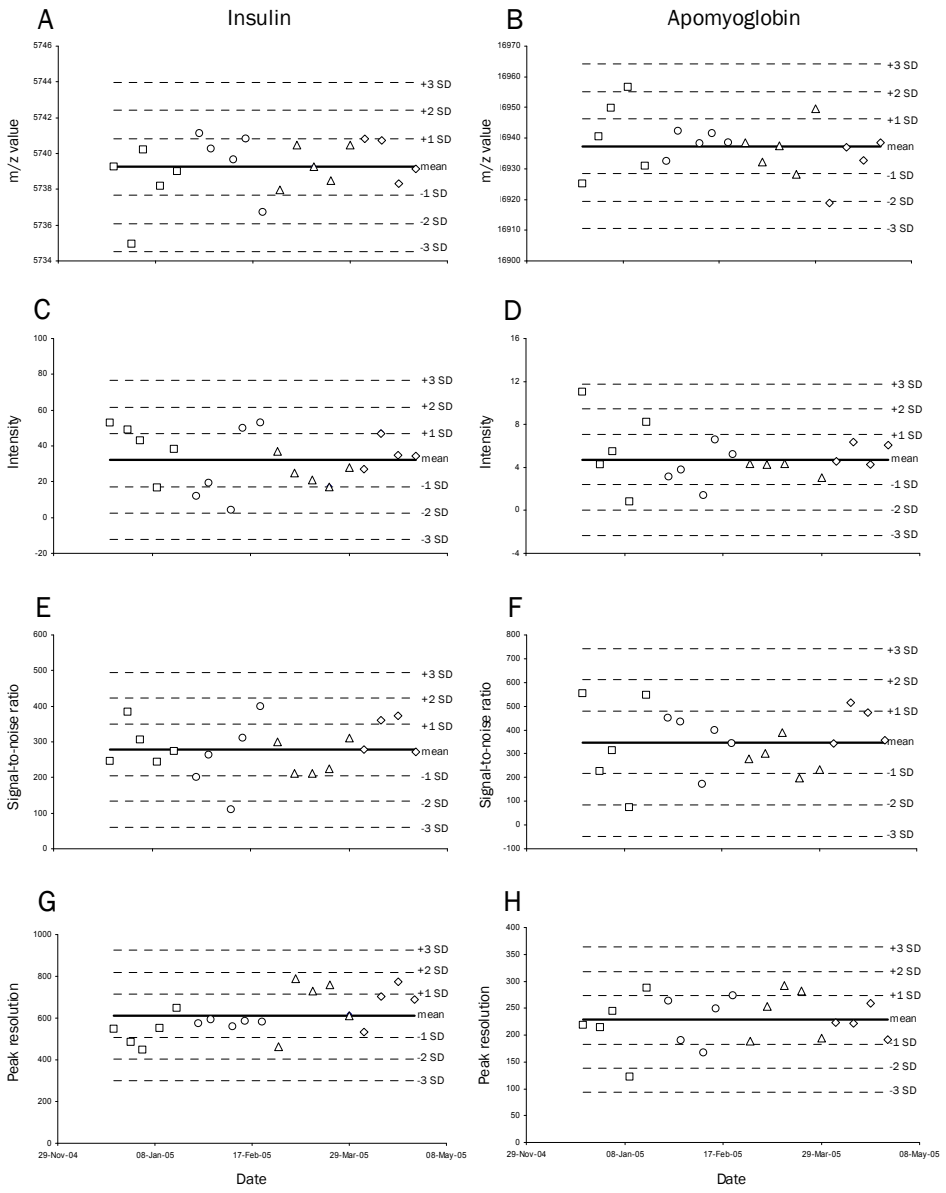

Figure 3.1 The *m/z* values (A, B), intensities (C, D), S/N ratios (E, F), and peak resolutions (G, H) of insulin and apomyoglobin, respectively in the QC sample.
The process mean and standard deviations (+ 1, 2 and 3 × SD, -1, 2 and 3 × SD) are indicated in the figures. Four different batches of QC sample were used; batch 1 (□), batch 2 (○), batch 3 (△), batch 4 (◇).

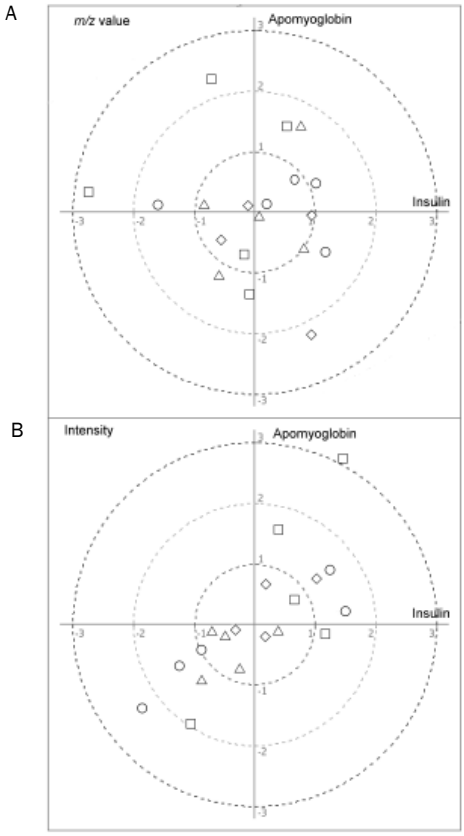

Figure 3.2 Two-dimensional Youden plots of the *m/z* values (A), and intensities (B) of insulin and apomyoglobin in the QC sample.
The measurements of the insulin and apomyoglobin signals are indicated on the x- and y-axis, respectively. The standard deviations (+ 1, 2 and 3 × SD, -1, 2 and 3 × SD) are indicated on the x- and y-axis. Four different batches of QC sample were used; batch 1 (□), batch 2 (○), batch 3 (△), batch 4 (◇).

Taking into consideration the number of observations of the performance of the QC sample during five months, it was concluded that an adequate numbers of data points were collected to define acceptance criteria for the performance of the PBSIIc instrument. It was decided that data points should be in the range of the process mean ± 2SD and accordingly that the acceptance criteria for *m/z* values, intensities, S/N ratios and peak resolutions for insulin and apomyoglobin in the QC sample can be defined (Table 3.1).

Table 3.1 Defined acceptance criteria (process mean ± 2SD) for *m/z* values, intensities, S/N ratios and peak resolutions for insulin and apomyoglobin in the QC sample.

	m/z value	Intensity	S/N	Peak resolution
Insulin	5736.1 - 5742.4	2.5 - 61.7	133.3 - 422.6	404.7 – 820.3
Apomyoglobin	16,919.5 - 16,955.2	0.03 - 9.4	83.9 - 611.2	138.2 – 318.3

The manufacturer has also defined specifications for the mass accuracy, which are 0.1% for masses with molecular weights ranging from 1 to 10 kDa and 0.2% for masses with molecular weights ranging from 10 to 300 kDa for the PBSIIc instrument. The mass accuracies of the insulin and apomyoglobin of the QC sample were exactly in accordance with the manufacturer's specifications.

Reproducibility

The reproducibility of QC_1 and QC_5 samples was compared in two experiments. In one experiment (A), where two NP-20 arrays were spotted with QC_1 samples and two other NP-20 arrays with QC_5 samples, the pooled CV values were significantly better for QC_1 samples for both insulin and apomyoglobin. However, for almost all parameters it was obvious for the QC_5 samples that array two resulted in inferior CV values for insulin and apomyoglobin compared to array one (Table 3.2A). Different factors could have been responsible for the inferior CV values: the amount of sample spotted, the crystallization, and the chip quality.
In the other experiment (B) the QC_1 and QC_5 samples were spotted alternately over four NP-20 arrays, the pooled CV values were significantly better for QC_5 samples compared to QC_1 samples (Table 3.2B). In general the reproducibility of QC_5 samples was superior compared to the reproducibility of QC_1 samples. This was also true for the individual arrays.

Table 3.2 CV values (%) of insulin (INS) and apomyoglobin (APO) in the QC_1 and QC_5 samples.

A	Mass accuracy	Array 1 (n=8)	Array 2 (n=8)	Pooled (n=16)		
	QC1 INS	0.04	0.04	0.04		
	QC5 INS	0.03	0.02	0.03		
	QC1 APO	0.04	0.05	0.04		
	QC5 APO	0.04	0.12	0.10		
	Intensity	Array 1 (n=8)	Array 2 (n=8)	Pooled (n=16)		
	QC1 INS	30	21	25		
	QC5 INS	13	30	32		
	QC1 APO	19	18	18		
	QC5 APO	8	47	42		
	S/N	Array 1 (n=8)	Array 2 (n=8)	Pooled (n=16)		
	QC1 INS	28	24	26		
	QC5 INS	18	37	37		
	QC1 APO	74	62	67		
	QC5 APO	31	100	66		
	Peak resolution	Array 1 (n=8)	Array 2 (n=8)	Pooled (n=16)		
	QC1 INS	8	10	9		
	QC5 INS	11	9	10		
	QC1 APO	15	13	14		
	QC5 APO	9	53	36		
B	Mass accuracy	Array 1 (n=4)	Array 2 (n=4)	Array 3 (n=4)	Array 4 (n=4)	Pooled (n=16)
	QC1 INS	0.02	0.03	0.03	0.03	0.02
	QC5 INS	0.05	0.05	0.06	0.05	0.05
	QC1 APO	0.02	0.03	0.03	0.03	0.03
	QC5 APO	0.05	0.06	0.04	0.06	0.05
	Intensity	Array 1 (n=4)	Array 2 (n=4)	Array 3 (n=4)	Array 4 (n=4)	Pooled (n=16)
	QC1 INS	28	30	47	35	32
	QC5 INS	25	21	26	22	27
	QC1 APO	14	3	3	8	8
	QC5 APO	5	9	5	1	5
	S/N	Array 1 (n=4)	Array 2 (n=4)	Array 3 (n=4)	Array 4 (n=4)	Pooled (n=16)
	QC1 INS	31	35	52	39	36
	QC5 INS	18	21	33	30	29
	QC1 APO	11	17	10	14	13
	QC5 APO	22	19	18	5	17
	Peak resolution	Array 1 (n=4)	Array 2 (n=4)	Array 3 (n=4)	Array 4 (n=4)	Pooled (n=16)
	QC1 INS	18	13	16	16	15
	QC5 INS	8	10	14	11	12
	QC1 APO	15	8	12	2	11
	QC5 APO	5	10	11	2	7

CV values of *m/z* values, intensities, S/N ratios, and peak resolutions were determined in two experiments. Two NP-20 arrays were spotted with QC_1 samples and two NP-20 arrays with QC_5 samples (A). In the other experiment (B), the arrays were alternately spotted with QC_1 and QC_5 samples divided over four NP-20 arrays.

Discussion

Any new technology, particularly one being presented as a potential clinically used diagnostic tool, requires stringent quality control to evaluate analytical performance over time. Instrument performance, however, must be compared not only during one experiment, but also over the course of time[22]. In this study a standard protocol for calibration of the MALDI-TOF-MS part of the PBSIIc instrument was defined and acceptance criteria for the independent certified QC samples were established. This is also possible for other instrument types. By checking the calibration every week, the QC procedure acts prospectively, while in some studies the quality control acts retrospectively by including the QC samples in the profiling experiments and in some studies there is no quality control procedure described at all. In contrast to the acceptance criteria reported by Semmes et al.[18] we also defined an upper limit and not only a threshold value for the *m/z* values, intensities, S/N ratios and peak resolutions. Actions need to be taken not only when the data of the QC samples are below the acceptance criteria, but also if the upper limit of the acceptance criteria are exceeded, because of processing or instrumental errors. During our prospective QC procedure, the QC samples were only spotted on a NP-20 array, to evaluate the MALDI-TOF-MS performance. However, during the profiling studies, the QC samples can also be spotted on other surfaces, whatsoever is appropriate to verify the quality of surface-enhanced arrays.

The composition of the QC samples was based in this case on the way of calibration, which was divided into a peptide, a protein-low and a protein-high calibration equation. Insulin and apomyoglobin were chosen to validate the peptide and protein-low calibration equations, respectively. The molecular weights of biomarkers investigated in proteomic patterns analysis by SELDI-TOF-MS are in general smaller than 20 kDa and therefore justify the current composition of the QC sample. However, with respect to the molecular range of interest, different certified proteins with different molecular weights can be chosen to validate the MALDI-TOF-MS part of the instrument. If for example the protein-high calibration becomes relevant, the composition of the QC sample should be extended with a QC component in the respective molecular weight range or an additional QC sample should be implemented. Using the right calibration equation to determine the *m/z* values is important. In our study, the peptide calibration was used to determine the *m/z* value of insulin and the protein low calibration was used to determine the *m/z* value of apomyoglobin. However, the *m/z* values of insulin clearly increased after calibrating with the protein-low calibration and the *m/z* values of apomyoglobin clearly decreased after calibrating with the peptide standard calibration. Choosing the wrong or an inadequate calibration equation can lead to a significant shift of the *m/z* values for the peak maximum. In the report by Semmes et al.[18] was also

described that by using an inadequate calibration, the *m/z* values shifted outside the acceptable range

Obviously, stringent quality control of the calibration part of the MALDI-TOF-MS experiments prevents unreliable data acquisition from the very start, because when the data of the QC sample exceeds the acceptance criteria, actions needs to be undertaken before starting new protein profiling experiments. The criteria can be exceeded because of different factors, like errors during preparations and handling of the calibration or QC sample as well as instrumental problems. In order to illustrate some of the errors we will discuss some experiences and problems we met. In the first example, an incorrect *m/z* value was accidentally assigned to a calibrant in the software for generating the calibration equation, which resulted in a shift of the calibration equation. The mass accuracy of the standard was outside the defined acceptance criteria and specifications. By checking the calibration spectra and subsequently selecting the correct *m/z* value, the mass accuracy changed and proved to be in accordance with the defined acceptance criteria. In the second case an instrumental problem with the spot alignment was detected. By adding QC samples every week, a decrease in signal from spot A to H was detected. After adjustment of the spot alignment, the signal was similar for spots A to H. In the third case differences between batches were seen. In the figures with the process mean and SDs and in the two dimensional Youden plots was demonstrated that the intensities of the insulin and apomyoglobin signal in the third QC batch were lower compared to the other batches. Probably the handling of that batch preparation had been less adequate than usual. All these examples demonstrate how important it is to use a well-defined protocol for calibration and to determine acceptance criteria.

With the Youden plots random errors can be distinguished from systematic errors. Random errors can for instance be caused by residual potentials on the deflection plates after the deflection pulse, which affect the flight times for ions arriving at the deflection plates after the pulse. Plate planarity imperfections, which alter the distance between the sample and the first extraction element of the ion source, can result in mass errors with external calibration. So the ion flight times are dependent on the sample plate position[23]. Systematic errors can be caused by pipetting errors, crystallization process of the energy absorbing matrix, and chip variability. The systematic error for the intensities (Figure 2B) must be related to the pipetting variability in the handling of the QC sample. The intensities, S/N ratios and peak resolutions were lower for some data points for both insulin and apomyoglobin (Figure 3.1 and 3.2). Probably variations in the amount of QC sample applied resulted in simultaneous variations in insulin and apomyoglobin signals.

The results of the reproducibility experiments also gave insight in the possible sources of systematic errors. At first sight, 1 µl QC sample was spotted, but because the CV values were not excellent, we tried to improve the reproducibility by spotting 5 µl diluted QC sample. In the first experiment (A), the QC_5 experiment

resulted in a large inter-chip variability, while the inter-chip variability of the QC_1 samples was acceptable. Because differences in quality of chips can occur, it is important to divide the samples alternately or at random over all arrays especially in studies based on comparison of more groups. Chip variability may be controlled by using arrays from the same batch, and chemicals used during a single experiment should also be from the same batch[22]. In the other experiment (B) was shown that the pooled CV values and the CV values per array were superior by spotting of QC_5 samples compared to QC_1 samples when the QC samples were spotted alternately. This demonstrates that although all pipettes used were calibrated regularly, pipetting of a larger volume, but with the identical absolute amount spotted of insulin and apomyoglobin, gave a better reproducibility of the QC sample. Therefore, in the future, only 5 µl QC sample will be spotted.

The CV values in our study are comparable with the CV values reported by Semmes et al. [18] for SELDI-TOF-MS serum profiling. Potential sources of variability that arise during SELDI-TOF-MS profiling include spot-to-spot variation of chip surfaces, laser detector variability over time, pipetting variability[24], and the crystallization process of the energy absorbing matrix[25,26]. Colantonio et al.[27] also described that the pre-analytical variation and error can be reduced by using single lot reagents, chemicals and SELDI chips and proper specimen handling and processing should be utilized to reduce pre-analytical errors. It is important to use replicates especially when profiling experiments were performed with SELDI-TOF-MS. In the report by Aivado et al.[28] was demonstrated that the use of 2-4 replicates significantly increases the reliability of protein profiles. White et al.[22] also reported that replicates of spotted samples are highly recommended. In this way, the effect of spotting errors is reduced. Variability can also be reduced by using an automated robot during sample transfer and processing. Because an error introduced during processing can be difficult or impossible to trace once the experiment is completed, it is best to rigorously control the experimental procedure to minimize the introduction of variation in the first place[22]. Unfortunately, the same machine parameters will not continue to generate identical spectra over time. In SELDI-TOF-MS, several parts of the instrument, such as the laser and detector, have a limited life span[22].

According to the manufacturer's protocols, the All-in-1 peptide and All-in-1 protein mixtures are stable for three months after reconstitution and storage in aliquots at –20°C or lower. A new aliquot should be used before every new calibration, however in this study was demonstrated that after using the same calibration array for five months by alternating the spots and within-spot positions, there were still adequate signals of the calibrants. This indicates that the calibration array can be used for a longer time period when the array is stored under appropriate conditions.

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