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Unravelling in vitro variables of major importance for the outcome of mass spectrometry-based serum proteomics☆

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Abstract

The use of mass spectrometry (MS) for analysing low-molecular weight proteins and peptides from biological fluids has a great, yet not fully realized, potential for biomarker discovery. To prune MS-data as much as possible for non-relevant non-biological variation the development of standardized protocols for handling and processing the samples before MS and adjusting data after MS to compensate for method-induced variability are warranted. This calls for knowledge about how different variables contribute to MS-based proteome analyses. In addition, identification of the peptides involved in pre-analytical variation will be helpful in evaluating the clinical significance of predictive models derived from MS data. Using human sera, extraction by weak cation-exchange magnetic beads, and analysis by MALDI-TOF MS we here evaluated pre-analytical variation and identify peptides involved in this. The influences of humidity, temperature, and time for preparation of sera on spectral changes were evaluated. Also, the reproducibility of the methods and the effect of a baseline correction procedure were examined. Low temperatures, short handling times, and a baseline correction procedure minimize the contribution of artifacts to sample variability as observed by MS. The complement split product C3f and fragments thereof appear to be sensitive indicators of sample handling induced modifications. Other peptides that are indicative of such variability are fibrin and kininogen fragments. Using strict experimental guidelines as well as standardized sample collection procedures it is possible to obtain reproducible peak intensities and positions in serum mass profiling using magnetic bead-based fractionation and MALDI-TOF MS.

Keywords: Proteomics; Mass spectrometry; Serum; In vitro variables; Proteins; Peptides; Variability; MALDI-TOF MS; Humidity; Temperature; Time; Baseline correction; Complement C3f; Magnetic bead-based fractionation; Reproducibility

1. Introduction

While potentially of great value for the discovery of novel markers of disease, mass spectrometry (MS) based proteomic investigation of biofluids has yet to fulfil its promise of providing new and better diagnostic targets in clinical practice. Instead, the validity of some of the early and quite optimistic reports [1–7] has been questioned [8–12]. The fact that not a single useful novel biomarker or an independently validated diagnostic mass pattern has yet resulted from MS-based proteome studies during the last 4–6 years attests to the difficulties of distilling true biological variation (e.g., disease-related) from pre-analytical and method (in vitro) variation.

Many biomarkers currently reported from MS studies of biofluids are peptides below 10,000 Da [1–3,13–17] and many appear to be fragments of a limited number of parent proteins (apolipoproteins, complement proteins, transthyretin, various enzyme inhibitors) irrespective of the condition studied [18], i.e., their appearance is related to sample handling or other pre-analytical parameters or to common processes in diseased individuals. In addition, in many cases the identities of putative diagnostic *m*/*z*-values are not reported and this hampers the evaluation of the relevance of the findings for diagnostic purposes. Also, reproducibility parameters such as the inter- and

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intra-assay variation of peak intensities are most often unknown even though it has been ambly documented that sample handling procedures have large effects on the analyses [19–21]. We here provide a systematic study of the influence of sample handling temperature, time, and humidity on the variability of the outcome of MS-based serum proteomics using derivatized magnetic beads for sample extraction and MALDI-TOF for analysis. We report on the relative contribution of time and temperature to the overall variability using statistical methods to evaluate the performance of replicate analyses, and offer a baseline correction approach that reduces the contribution of handling and analysis parameters to variability. Furthermore, we use tandem-MS to identify a number of peaks that appear to be strongly associated with non-biological variation. The study emphasizes the need to adhere to strictly standardized sample handling, processing, and analysis conditions to get results of relevance for diagnostic discoveries and diminish the risk of producing variables instead of discovering them.

2. Experimental

2.1. Chemicals

Weak cation-exchange magnetic beads were part of the purification kit MB–WCX and were purchased together with α-cyano-4-hydroxycinnamic acid (HCCA) from Bruker Daltonik GmbH (Leipzig, Germany).

Ethanol extra pure, HPLC grade water, acetone (Lichrosolv grade) was purchased from Merck (Darmstadt, Germany). The Complete™ protease inhibitor tablets were purchased from Roche Diagnostics GmbH (Mannheim, Germany).

2.2. Biological materials

For all experiments serum was prepared by drawing blood into standard blood collection tubes with no additions and let it clot at room temperature for 3–6 h. Serum was then isolated by centrifugation at 3000 × *g* for 6 min at room temperature. Finally, sera were aliquoted as described for each experiment and stored at −20 °C. Samples were stored at −20 °C for no more than 2 weeks before analysis. We have previously shown that storage for up to 14 days at either −20 °C or −80 °C has no impact on the mass spectra [21].

For temperature and time experiments fresh blood samples were obtained from an ongoing study with strictly standardized sampling procedures. Four different sera of 160 μl were obtained, and each sample was divided in 16 aliquots of 10 μl each that were stored and frozen. For the temperature and time experiment sera were thawed and kept at room temperature or on ice for 120, 60, 30, and 0 min before fractionation with WCX-beads using a robot (cf. below). Thus, the experiment generated four samples at four different time points at 0 and 22 °C, i.e., 32 spectra. This experiment was repeated twice over 2 consecutive days.

For the reproducibility experiments, serum from one volunteer was obtained at two time points separated by 1.5 year. The samples were divided into six aliquots of 50 μl and stored at −20 °C. All samples were subsequently analyzed in duplicate six times over five days. Each sample was divided in two each undergoing the same fractionation step and spotted out on the target plate twice.

2.3. Magnetic bead-based sample preparation for MALDI-TOF MS

Paramagnetic nonporous particles coupled with weak cation-exchange ligand (WCX) were used for preparing sera. The contents of the binding, washing, and elution solutions provided by Bruker Daltonics as part of the kits are proprietary. Briefly, 10 μl MB–WCX binding solution and 5 μl serum was transferred to a 0.2 ml thin-wall PCR-tube (ABgene, UK). A 10 μl homogenous magnetic particle solution was added, mixed and left for 5 min. The tubes were placed in a 2 × 8 well magnetic bead separator (MBS) (Bruker Daltonik, Germany) for 30 s for magnetic fixation of the MB–WCX particles. The supernatant was aspirated and the tubes were removed from the MBS device. Wash solution (100 μl) was added and carefully mixed with the magnetic beads. The tube was then replaced in the MBS device and moved back and forth sequentially between adjacent wells on each side of the magnetic bar in the MBS device. This facilitated washing of the magnetic particles as they are fixed to the tube wall, respectively move through the washing solution in succession. After fixation of the magnetic beads for 30 s in the MBS device the supernatant was aspirated. This washing procedure was repeated three times. After the final washing step bound molecules were eluted by incubation with 5 μl MB–WCX elution solution for 1 min before collecting the eluate using the MBS device. Finally, 5 μl MB–WCX stabilization solution was added to the eluate.

The eluate (1 μl) was then mixed with 10 μl matrix solution (0.3 g/l HCCA in ethanol:acetone 2:1) and 1 μl was spotted onto a 600 μm diameter spot size 384 AnchorChip™ target plate (Bruker Daltonik, Germany) and left to dry. For each run a new HCCA-matrix was prepared. Protein calibration standard (Protein Calibration standard 1, Bruker Daltonics) was dissolved in 125 μl 0.1% aq. TFA and 0.5 μl of the solution was applied to target spots in close proximity to the serum samples for external calibration.

For the time and temperature experiments an automated sampling handling robot (ClinProt robot, Bruker Daltonik, Germany) was used. The workflow conducted by the robot is similar to the workflow performed manually. Humidity and temperature were recorded using a hygrometer (Hygrometer testo 608-H1) from Testo GmbH & Co. Experiments took place in an air-conditioned, dehumidified, temperature-controlled room and variations in humidity were achieved by placing a beaker with heated water in the room.

2.4. MALDI-TOF MS

The AnchorChip™ target plate was placed in an UltraFlex® TOF/TOF mass spectrometer (Bruker Daltonik, Germany) controlled by Flexcontrol software v. 2.0 (Bruker Daltonik, Germany). The instrument is equipped with a 337-nm nitrogen

laser, delayed-extraction electronics, and a 2 GHz digitizer. The instrument was externally calibrated by standard procedures. All acquisitions were generated by an automated acquisition method included in the instrument software and based on averaging 150 randomized shots over 5 positions (30 shots/position). The acquisition laser power was set between 25 and 35%. Before each acquisition cycle the position was pretreated with 10 laser shots at 40% laser power to improve spectra quality [22]. Spectra were acquired in positive linear mode, in the mass range of 1000–10,000 Da using deflection of ions below 900 *m/z*-value. Pulsed ion extraction was set to 320 ns to assure appropriate time lag focusing.

The MS/MS method based on laser-induced dissociation (post-source decay) (Bruker LIFT^{TM}) was used for fragmentation and was in most cases done directly on spot. Parent mass ion was measured with the highest degree of precision and with precursor ion selector range set to ±1% of the parent mass. Parent mass ion was collected with 200 shots and for the MS/MS mode typically 800–1000 shots were needed.

2.5. Data handling

Spectra were converted into the ASCII file format using Flexanalysis v. 2.0 software (Bruker Daltonik, Germany). Analyses of the MS–MS spectra were performed using Biotools v. 2.2. Protein database searches were performed by Mascot (http://www.matrixscience.com).

The Unscrambler v. 8.05 program (Camo, Norway) was used for linear analysis of the data, i.e., principal components analysis (PCA). The objective of PCA is to reduce the dimensionality of data sets to allow visual analysis of the data and thus to discover important data trends. PCA finds the linear transformation (combinations of rotation and scaling) of the original variables that gives the maximum amount of variation in the data, and this axis is the first principal component, PC1. The axis orthogonally on PC1 that captures the maximum amount of variation left in the data is the second principal component PC2. Each data set will have a projection on the PC axes and the loadings are the correlations between the variables and the PC. Thus, PCA allows the visualization of sample groupings and the determination of which variables contribute most to differences between samples and if individual variables are correlated (i.e., contribute in the same way).

As model building in Unscrambler takes every point in the spectra into account, we reduced our data with a factor of four, leaving each spectrum with about 8363 (*m/z*, intensity) coordinates. All spectra were normalized by a mean normalization algorithm.

In an effort to apply a measure of reproducibility the coefficient of variation (CV) was calculated as described by Noo et al. [19] with some modifications. The data was pretreated as mentioned earlier and the average (μ_i) and the local noise (s_i) were estimated in each data point with an analysis of variance (ANOVA) model. Unless otherwise mentioned the ANOVA only assumed a difference between blood samples from different individuals, $\gamma = \mu + \beta + \varepsilon$, where β is the effect of different blood samples and ε is the residual. The CV is calculated as $\mathrm{CV} = \sum_i(s_i\mu_i)/\sum_i(\mu_i^2)$ equivalent to the slope of a linear regression through origin in a scatter plot of s versus μ [19].

3. Results and discussion

3.1. Time and temperature

From earlier studies it is known that blood samples left for many hours at room temperature are subject to large changes in the MS-profiles [21] and that this has an impact on the model building. In the present study we wanted to investigate the putative effects of time and temperature on a shorter time scale. To determine the effect of thawing time and temperature, these were varied systematically in an experiment with a replicate run the next day. Here we report these effects (biology, time, temperature, and day-to-day variation) and quantitate the contribution of each compared to the total variation. The results show that a large part of the total variation comes from leaving serum samples at room temperature for more than 30 min. It is therefore, advisable in all proteomics studies immediately to aliquot sera for storage at least at −20 °C when they are received.

In Table 1, the overall CV (0.30) of peak intensities is estimated as the average of the CV of the four individual sample sets (to exclude the variation caused by the biological differences between the samples) using noise estimates from an ANOVA. Thus, this CV reflects the variability induced by in vitro factors. The CVs are high, i.e., reflecting a relatively poor reproducibility. However, upon separation of the data sets into high and low temperature sets, the CVs show that reproducibility is much better at the lower temperature (Table 1). Splitting up the data with respect to time also shows that the CV is lowered as the time is shortened, especially at 22 °C meaning that the samples deteriorate over time and faster at higher temperature. Thus, the relative contribution of biological variation is increased at lower temperature. A low sample handling temperature and short handling time therefore, are to be recommended to obtain decent reproducibility.

In order to quantify the effects mentioned above a principal component analysis was performed on the dataset and the scores from the first seven components were used sequentially in a univariate ANOVA model with the same assumptions as above. The sum of squares (SSQ) relating to each effect was added and the relative proportion of each SSQ summation was calculated (Fig. 1). When comparing both within and between these plots it must be pointed out that the influences of the degrees of free-

Table 1
Coefficient of variation (CV) values calculated for the time/temperature experiment

CV	Accumulated CV	0 °C	22 °C
Day 1/day 2	0.30	0.16	0.32
Time 0 min	0.14	0.148	0.124
Time 30 min	0.20	0.151	0.233
Time 60 min	0.30	0.157	0.281
Time 120 min	0.39	0.159	0.319

CV values are based on the time-accumulated sample data.

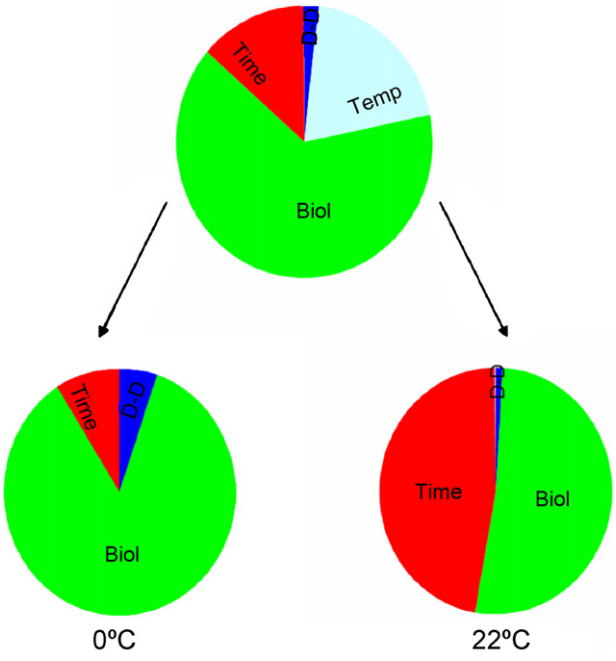

Fig. 1. Graphical view of the contribution of handling parameters (time, temperature, biology, and day-to-day variation (D–D)) to total variation in the analysis of four different samples. Samples were left for 0, 30, 60 or 120 min, at either 0 or 22 °C and were measured twice. PCA scores were used in a univariate factorial design enabling estimation of primary effects (time, temperature, biology, and day-to-day variation). The upper figure comprises all data and is split into variation at 0 and 22 °C in the lower figures. The relative contribution of biological variation (green) is increased at lower temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dom of each effect as well as the possible change in total variance have been ignored in this analysis. However some comparisons can be made. Thawing time and biological samples can be compared as they contain equal degrees of freedom. As depicted in (Fig. 1), the effect of time at high temperature is actually reaching the same magnitude as the effect of biological variance. Inspection of the mass spectra reveals that the main contribution to variability originates from intensity changes of the low mass range. Especially *m/z*-values of 2022 and 1865 strikingly contribute to variability. Sequencing by MS–MS identifies (data not shown) these peaks as complement C3f and C3f fragments (see also below). This is in good agreement with Villanueva et al. [18]. Further support comes from additional, independent experiments that involved 8 different sera processed after incubation at room temperature for 0, 15, 30 and 60 min. The results (data not shown) indicated that seven peaks correlate highly with increasing incubation time. The C3f and C3f-fragments (*m/z*-values of 2022, 1865, 1778) were prominent among these peaks. Thus, C3f and C3f subfragments are sensitive indicators of sample handling-induced variability. A number of other fragment peaks also contribute significantly to the overall variability and have been identified by MS–MS (Table 2). This fragment population is indicative of the complex enzymatic activities present in sera after clotting. While plasma may prove to be a more stable biofluid than serum, we have found in preliminary experiments that the number of peaks using the magnetic bead sample preparation procedure on plasma samples was lower than the number found in serum. The reasons for this remain to be elucidated and in the present study we only focus on serum.

3.2. Serum mass profile reproducibility

The reproducibility of mass spectra derived from subfractions of sera obtained by ion exchange beads was evaluated by repeating analyses under identical conditions with the temperature being 22 °C ± 0.5 °C and the humidity 35% ± 1%. We tested the reproducibility by analyses on five different days with a weekend between days 2 and 3. Additionally, two analyses separated by 3 h were performed on day 1. Thus, each sample (two sera from the same individual taken 1.5 years apart) was analyzed six times. PCA on all samples (Fig. 2A and B) describes the variation observed in the dataset. The samples were located in well-separated clusters (Fig. 2A). The loading plot (Fig. 2B) reveals that practically all variation (both PC1 and PC2) is due to the two larger peaks near 2000 Da marked with a circle (Fig. 2B). A two-component model sufficiently describes 97% of the variation with 87% within PC1, the first principal component. PC1 explains the differences between old and the new samples (1.5 years apart) and is illustrated with the blue trace in the loading plot (Fig. 2B). The red trace illustrates the *m/z*-contributors to the second principal component (PC2). PC2 explains the difference between days 1–2 and days 3–5 (cf. Fig. 2A). The same two peaks as above appear in the loading plot illustrates PC2 (red spectrum, Fig. 2B). The peaks have opposite signs corresponding to the two groups with either pos-

Table 2
Table of identified peaks involved in post-sampling changes in serum

Sequence name	Parent mass	Score (threshold 64)
Complement C3f: aa 2–16 (SKITHRIHWESASLL)	1777.9	68.00
Complement C3f: aa 1–16 (SSKITHRIHWESASLL)	1864.9	93.70
Complement C3f: aa 1–17 (SSKITHRIHWESASLLR)	2021.7	99.40
Fibrin alpha C term fragment: aa 81–105 (SSSYSKQFTSSTSYNRGDSTFESKS)	2767.4	72.50
Fibrin alpha C term fragment: aa 81–106 (SSSYSKQFTSSTSYNRGDSTFESKSY)	2931.5	79.00
Fibrinopeptide A: aa 1–12 (EGDFLAEGGGVR)	1206.5	84.30
Fibrinopeptide A: aa 3–16 (SGEGDFLA EGGGVR)	1350.7	110.00
Fibrinopeptide A: aa 2–16 (DSGEGDFLAEGGGVR)	1465.5	73.10
Fibrinopeptide A (modifications: Ser-3 phosphorylated): aa 1–16 (AD**S**GEGDFLAEGGGVR)	1616.9	130.00
Kininogen: aa 439–456 (HNLGHGHKHERDQGHGHQ)	2080.9	69.70

Score values greater than 64 indicate a false positive rate of less than 5% for the identification by Mascot. Bold S indicates a phosphorylated site found on serine.

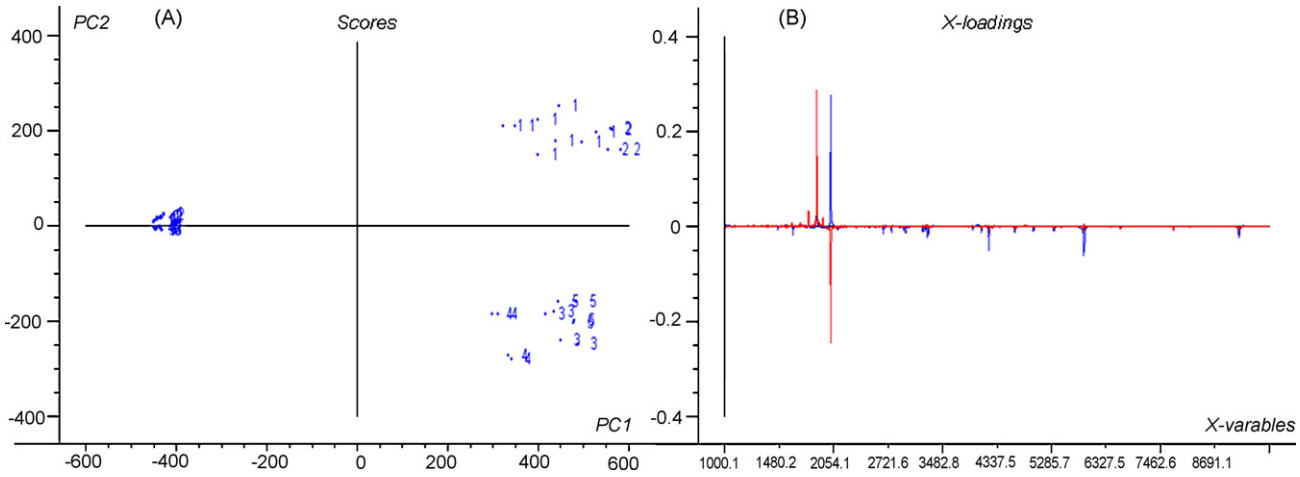

Fig. 2. Reproducibility of serum mass profiles illustrated by principal component analysis (PCA). Three distinctive groups can be observed in the score plot (A). The left group represents sample one (sample drawing point 1) whereas the two groups to the right represent sample two (sample drawing point 2) see text. The loading plot (B) describes the variation seen in (A) and is depicted as two spectra (blue/red) each corresponding to the two first principal components PC1 (blue trace) and PC2 (red trace). Eighty seven percent of the variation is within PC1 illustrating the difference between the two samples. Ten percent of the total variation in the dataset is depicted in PC2 and describes the variation between days 1–2 and days 3–5 (red spectrum in the loadings plot). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

itive or negative score values in the direction of PC2 (y-axis). Both peaks were identified by MS–MS that showed that both originated from the complement split product C3f with peak 1865 lacking the C-terminal arginine (156 Da) (Fig. 3). The relative amount (intensity) of each peak suggests that there is a correlation between the two fragments, i.e., that the larger fragment decreases when the smaller increases. Subsequently also an $m/z = 1778$ fragment corresponding to C3f with both the C- and N-terminal residue removed appears (cf. Table 2).

Coefficients of variation were also calculated on this dataset. The overall CV was 0.27, which may be compared to the value of 0.16–0.20 from the study by de Noo et al. [19]. Looking closer at the CV values by calculating them separately for sections of the spectra (Fig. 4), it is evident that the lower region of the MS-spectrum is responsible for most of the variation (CV in this region is 0.37). The peaks responsible for the higher CV-value are mainly C3f and fragments generated thereof. The rest of the spectrum appears to be more reproducible with CV-values

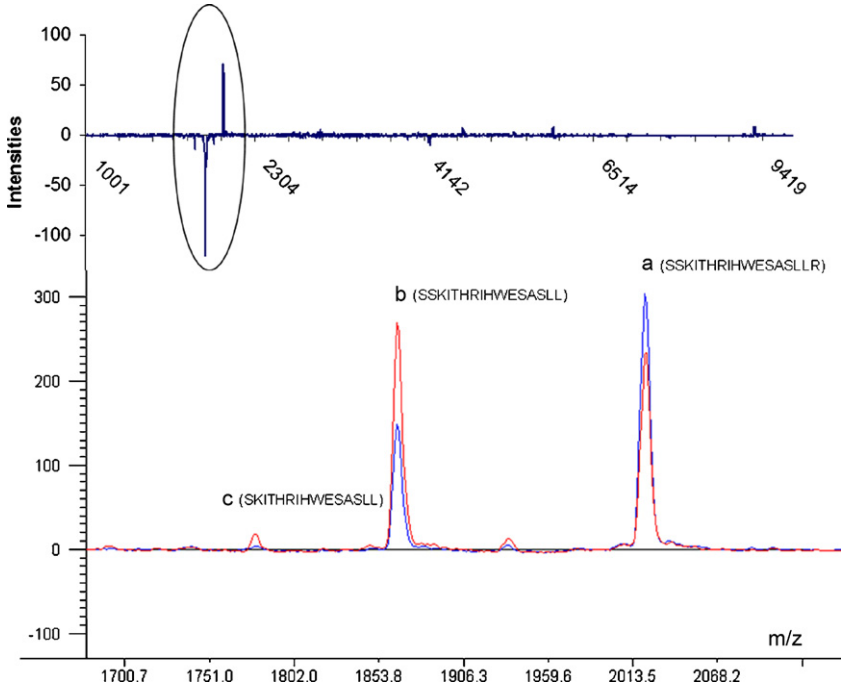

Fig. 3. Differences seen between samples from days 1, 2 (red) vs. days 3–5 (blue). (A) (upper trace) is an example of the subtracted mass spectrum of a short time sample (1 day) from a longer time sample (5 days). The circle in (A) indicates the most variable peaks. (B) is an expansion of the m/z 1700–2090 range with an overlay of the two spectra (1 day (blue) and 5 days (red)). The peaks marked b and c are subfragments of the complement C3f split product (peak a). Amino acid sequences are indicated (cf. also Table 2). The only difference between peaks a and b is the removal of the C-terminal amino acid arginine from a. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

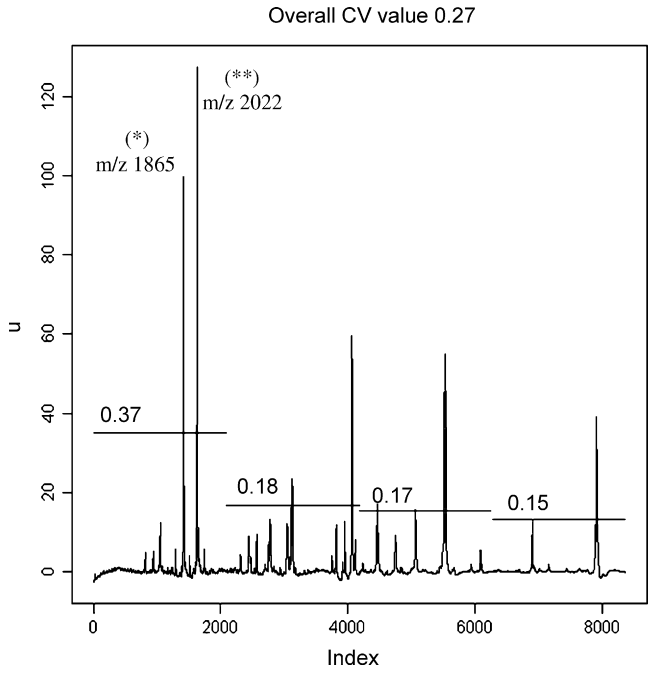

Fig. 4. Inter-assay variation coefficient (CV). Six repetitions of two different samples over 5 days. High variation is especially pronounced in the area of the two large peaks at 1865 (*) and 2022 (**). Please note that the *x*-axis is not depicted as *m*/*z* values but is an index axis, and that the *y*-axis is depicted as CV-values (*u*).

in the range of 0.15–0.18 (Fig. 4). Fragments of C3 have occasionally been reported in the literature as biomarker candidates [12]. However, our results indicate that these markers should be used with caution as diagnostic peaks as they seem to be heavily associated with processing variability.

3.3. Humidity

One of the least understood and examined steps in getting from sample to mass spectra is the crystallization of the sample with matrix on the target plate. Besides the chemical composition and concentration of matrix and solvent there are at least two other factors that may be controlled in the crystallization step, viz. temperature and humidity. However, the effects of temperature and humidity on matrix crystallization as visualized by mass spectral changes appear not previously to have been studied systematically. The possibility that the humidity level could be an issue was noted on very cold and dry days where the humidity level went down as far as 15%. Under these conditions it proved impossible to obtain suitable mass spectra of fractionated serum samples spotted on target with HCCA-matrix, i.e., no peaks appeared. With increase in humidity this problem disappeared. It was also observed that the morphology of the crystals changed with the ambient humidity level (data not shown). To investigate humidity effects, two sera were fractionated twice using weak cation-exchanged magnetic beads and the same eluate was used for target preparation under different humidity levels. The effect on crystallization of 20%, 33%, and 46% relative humidity was examined and the same eluate as well as matrix preparation was used for all three evaluations. As seen in Fig. 5, there are four spots for each humidity setting because each sample was fractionated twice and each eluate was placed on the target in duplicate. The experiment was repeated with another serum sample, which gave similar results (data not shown). Analysis of the results show that PC1 describes 73% of the variability and hence the differences in mass profile caused by crystallization. PC2 only accounts for 12% of the variation in the dataset. After PC2 no more information can be obtained. The changes in the spectra cannot be ascribed to specific peaks but are more general changes in the serum mass profiles and this was found for both samples. Even though the differences in these experiments are not detrimental to the generation of mass spectra they do contribute considerably to the overall variability and hence add complexity to the challenge of biomarker discovery by model building. Also it is clear from this part of the study that the fractionation step and spotting on target (if the same eluent and matrix is used) have very little impact on sample variability. Normally the contribution from these steps is below 1% (data not shown).

3.4. Baseline correction

Baseline drift may be corrected by fitting an algorithm that describes the curvature of the baseline and subtract that from the originating baseline thereby getting a zero baseline. This

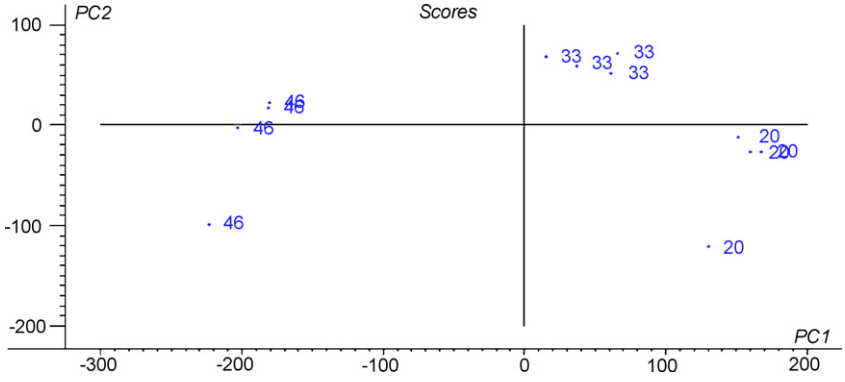

Fig. 5. Effect of humidity during sample crystallization on MS analysis variability. PCA scores showing three groups each corresponding to the three different humidity levels. Each sample was fractionated twice and the eluate was mixed with HCCA-matrix and spotted twice on target at a humidity level at 20%, 46%, and 33%, respectively.

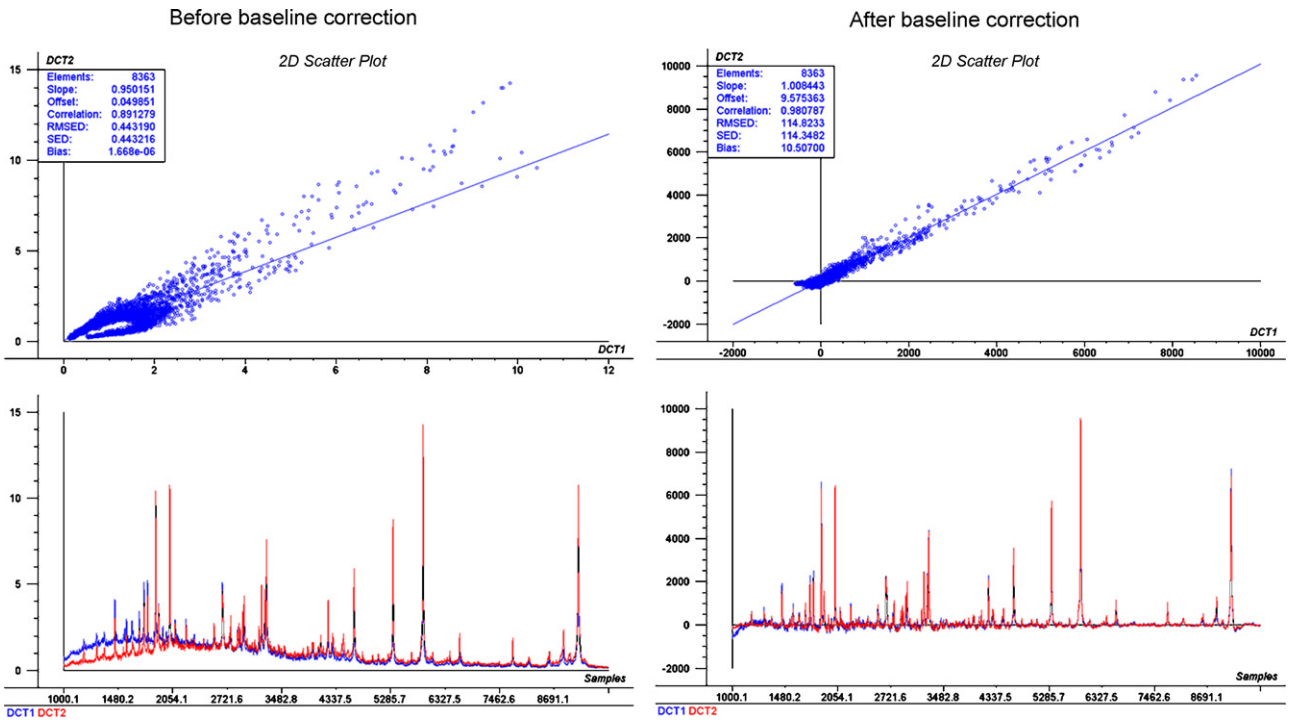

Fig. 6. Target plate-induced variability. Same sample spotted on two different MALDI targets (AnchorChip^{TM} 600 μm). Lower left panel shows the two analyses of the same sample on two different target plates superimposed. Lower right panel shows these data after baseline correction. In the upper panel the data are represented before (left) and after (right) baseline correction in scatter plots of peak intensities from each experiment plotted on *x*- and *y*-axes, respectively.

can be done by applying the algorithm to the total spectrum or dividing the spectrum in segments and applying the algorithm to each segment. We used the segmented method with the median algorithm set to 0.8. This feature is available in the commercial software from Bruker Daltonics (Flexanalysis 2.4^{TM}).

A MALDI target plate can accommodate up to 384 samples and may be reused. We found that washing the target or using a new target discernibly affects the crystallization and hence the MS-spectra (Fig. 6). Such target-induced variability may be reduced using baseline correction. After baseline correction the spectra are almost identical. The correlation coefficient (R) before baseline correction is 0.89 and 0.98 after baseline correction, see Fig. 6. But the more the target is used the more difficult it is to correct the baseline. It is probably warranted to include, e.g. a serum pool in each run as a control for overall reproducibility performance.

4. Conclusions

Serum sample polypeptide profiling using ion-exchange-based pre-fractionation and MALDI-TOF MS is very sensitive to different types of pre-analytical and analytical variables. However, we have established that robust serum mass profiling is possible using strict sample handling rules.

When thawing serum samples for pre-fractionation with cation-exchange chromatography it is beneficial to keep the samples on ice for as short a period of time as possible. One should avoid leaving the serum samples for thawing at room temperature for more than 30 min. We found and identified several peaks in the low mass range that was influenced by different experimental variables especially connected with sample handling. Notably, C3f complement inactivation split product and fragments thereof appear to be sensitive markers for sample handling and processing-induced variables.

We have also shown that crystallization of samples with HCCA-matrix is subject to variability when the ambient humidity changes. Finally, we propose a reproducibility measure that will be helpful when comparing different studies in the field of clinical proteomics.

Our results suggest that optimal in vitro conditions for MS-based proteomics include carefully optimized and controlled sample handling time, temperature, and humidity as well as baseline correction measures to compensate for variability, e.g. due to target plate factors. In contrast, the variability associated with the fractionation procedure itself and with repeated spotting was found to be minimal in comparison with the other factors mentioned above. These findings will help in designing experiments that reduce the non-informative non-biological data noise and thus ensure better chances for discriminating true biological and disease-associated peak patterns. In addition, our study has identified a number of molecules that appear to be excellent markers of sample handling variability and thus should be regarded with caution if reported of diagnostic significance.

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