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Exploring the limits and losses in MALDI sample preparation of attomole amounts of peptide mixtures

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Abstract

We have used a non-contact piezo-electric liquid dispensing workstation to transfer 200 pL aliquots of both a sample solution containing 12 peptides, each at a concentration of 5 or 50 fmol/μL, and dilution buffer onto microcrystalline spots of α-cyano-4-hydroxycinnamic acid for thin-layer preparations on prestructured MALDI sample supports. This approach allowed us to systematically investigate the dependence of detection sensitivity on sample volume, spot size, concentration, as well as sample losses. We demonstrate that 1 amol of the peptide mixture was sufficient to detect all components if it was prepared in a volume of 0.2 nL across a spot of ca. 180 μm diameter. If the sample was diluted on the target to 200 nL and prepared on a spot of 400 μm diameter, 10 amol could be detected and if the sample volume was increased to 1 μL, 50 amol were required to detect all components. If the peptide mixture instead was diluted in a sample vial and 1 μL of that solution was prepared on a spot of 400 μm diameter, 50 amol of the peptide mixture could be detected as well and the signal-to-noise ratios of the analyte signals were comparable to those observed for the on-target dilution experiment.

Keywords: MALDI-TOF-MS; Peptide; Piezo-electric dispensing; Prestructured sample support; Detection sensitivity

1. Introduction

The objective of this study was to explore the limits of detection for MALDI-TOF-MS analysis of peptides with regards to sample volume, spot size, concentration, and analyte losses. For this purpose we chose the thin-layer sample preparation protocol [1] combined with prestructured sample supports (AnchorChips™, Bruker Daltonics, Germany) as sample preparation method [2–4]. This combination has become popular for a broad range of applications because it provides high-detection sensitivity and produces homogeneous samples of predefined size at predetermined locations on the sample support rendering automated analyses straightforward. This has, for example, enabled off-line coupling of nano-LC and MALDI-TOF-MS without the necessity to mix the LC effluent with MALDI matrix solution [5,6].

Non-contact picoliter dispensing techniques enable handling of tiny sample volumes in a controlled and reproducible way. For this study, several of the experiments were performed using a piezo-electric liquid dispensing workstation (sciFLEXARRAYER S5, Scienion AG, Germany) for transfer of 200 pL aliquots of sample solution as well as dilution buffer to the MALDI sample support. This instrument allows rapid aspiration of microliter volumes of biological sample solutions and their subsequent transfer in droplets of sub-nanoliter volumes onto various substrates including AnchorChips™. Accurate on-target dilution experiments over a broad volume range in short time are possible because, if necessary, more than two thousand droplets of either solution can be dispensed in 1 s.

In a previous study based on prestructured sample supports, the detection limit observed for a mixture of peptides was 50 amol based on a dilution series performed in vials [2]. To enable detection of 50 amol of peptide, the sample was concentrated to a spot of a diameter of 200 μm. The sample volume transferred to the sample support was kept constant (1 μL). The detection limit encountered was assigned to substantial sample loss by surface adsorption in the vials used for dilution as well as the pipette tip used for transfer to the sample support. This problem was avoided in the current study by transferring amol

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amounts of peptides in 200 pL instead of 1 μL and performing the dilution on the sample support.

2. Materials and methods

2.1. Chemicals

Trifloroacetic acid (TFA), tetrahydrofuran (THF), *n*-octylglucopyranoside (*n*-OGP), α-cyano-4-hydroxycinnamic acid (CHCA), and water used for HPLC solvents and MALDI matrix solutions were purchased from Fluka Chemie (Switzerland) and citric acid from Aldrich (Sigma–Aldrich, St. Louis, MO).

2.2. Peptides

The following peptides (peptides 1–12) were purchased from Bachem (Germany): human angiotensin I and II, substance P, fibrinopeptide A, Glu-1-fibrinopeptide A, bombesin, rennin substrate, ACTH clip 1–17, 18–39, 3–24 and 1–24, and somatostatin.

2.3. Materials

MTP AnchorChip 384/var MALDI sample support providing 4 arrays of 96 anchors of a diameter of 200, 400, 600, and 800 μm were purchased from Bruker Daltonics (Germany).

2.4. Solutions

2.4.1. Dilution buffer

One millimolar *n*-OGP dissolved in water/TFA, 99.9/0.1 (v/v).

2.4.2. Peptide mixture stock solution

A stock solution containing 5 pmol/μL of each of the 12 peptides in dilution buffer was prepared following the quantity specifications provided by the manufacturer.

2.5. MALDI sample preparation

Thin microcrystalline layers of α-cyano-4-hydroxycinnamic acid (CHCA) were prepared on AnchorChips 384/var as described [5] with the modification that instead of two peptides (calibrants), 50 mM citric acid was included in the matrix solution to reduce salt adduct formation.

2.6. Non-contact liquid handling

All non-contact liquid handling was performed with a sciFLEXARRAYER S5 workstation (Scienion AG, Germany) provided, and configured by the manufacturer. Two PDC50 piezo-dispensing capillaries were installed in Positions 1 and 3, the first for transfer of sample solution and the second for transfer of dilution buffer. The sample solution was contained in a 0.6 mL Eppendorf tube from which aliquots were aspirated. The dilution buffer was used as system liquid for both dispensing capillaries. Fig. 1 shows a stroboscope image of 200 pL droplets of the peptide mixture diluted in dilution buffer to a concentration of 50 fmol/μL. The dispensing parameters (voltage amplitude and pulse width) were tuned by the manufacturer such that both dispensing capillaries generated droplets of 200 pL volume with a maximum error of 10 pL, at a frequency of 1000 Hz. The droplet volume was confirmed before each experiment by determining the weight of a total of 50,000 droplets (10 μL) collected in a 0.2 mL Eppendorf tube. The concentration of the sample solution that fills the channel of the dispensing nozzle (50-μm orifice) increases over time due to solvent evaporation (Fig. 1). This problem is well known, especially in the non-contact production of DNA and protein microarrays, for which the sciFLEXARRAYER S5 was developed. It is overcome by dispensing a series of droplets to a nearby waste position directly before the sample is transferred to the target positions. In this study, always 500 droplets of the sample solution were dispensed to a neighboring CHCA spot not used for analysis before the specified aliquot was transferred to the target position.

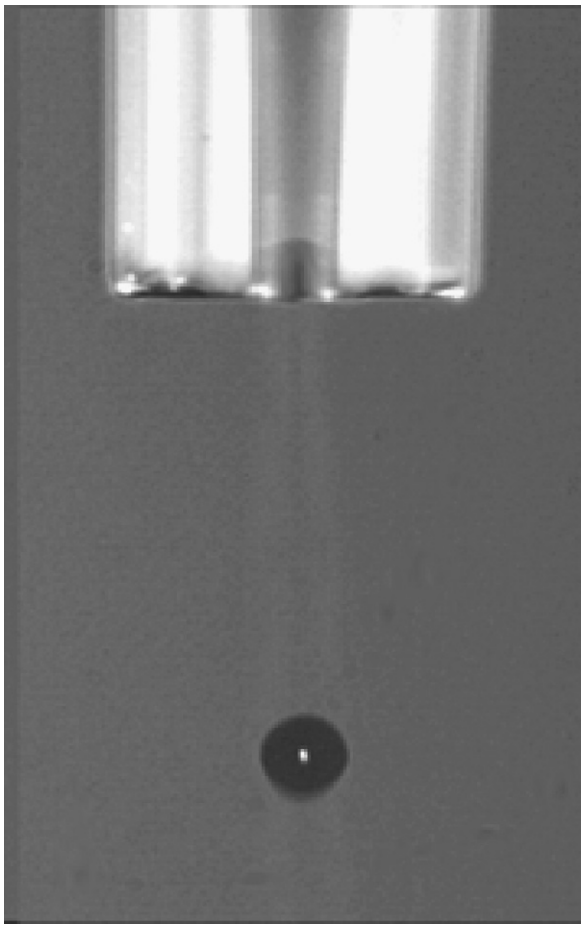

Fig. 1. Non-contact liquid handling. A stroboscope image of 200-pL droplets of sample solution dispensed with a frequency of 500 Hz.

2.7. MALDI-TOF-MS

All mass spectra were acquired on a Bruker Ultraflex II MALDI TOF mass spectrometer equipped with the

smartbeam^{TM} laser technology [7] (Bruker Daltonics, Germany). Positively charged ions of *m/z* 900–3300 were analyzed in manual and automatic mode. Five-hundred single-shot spectra were accumulated from 10 different positions on a sample in subsets of 50 using the smartbeam laser focus setting minimum, which focuses the laser beam on the target to a circular spot of 10–15 μm diameter. Automatic detection of the peptide monoisotopic signals was performed using the algorithm SNAP, implemented in the FlexAnalysis software (Bruker Daltonics, Germany). The spectra were calibrated internally using the monoisotopic mass of the singly charged molecular ions of peptide 1 (*m/z* 1296.6853) and peptide 9 (*m/z* 2465.1989) as reference. The spectra shown in Figs. 2–6 were not baseline-corrected and no smoothing algorithm was applied.

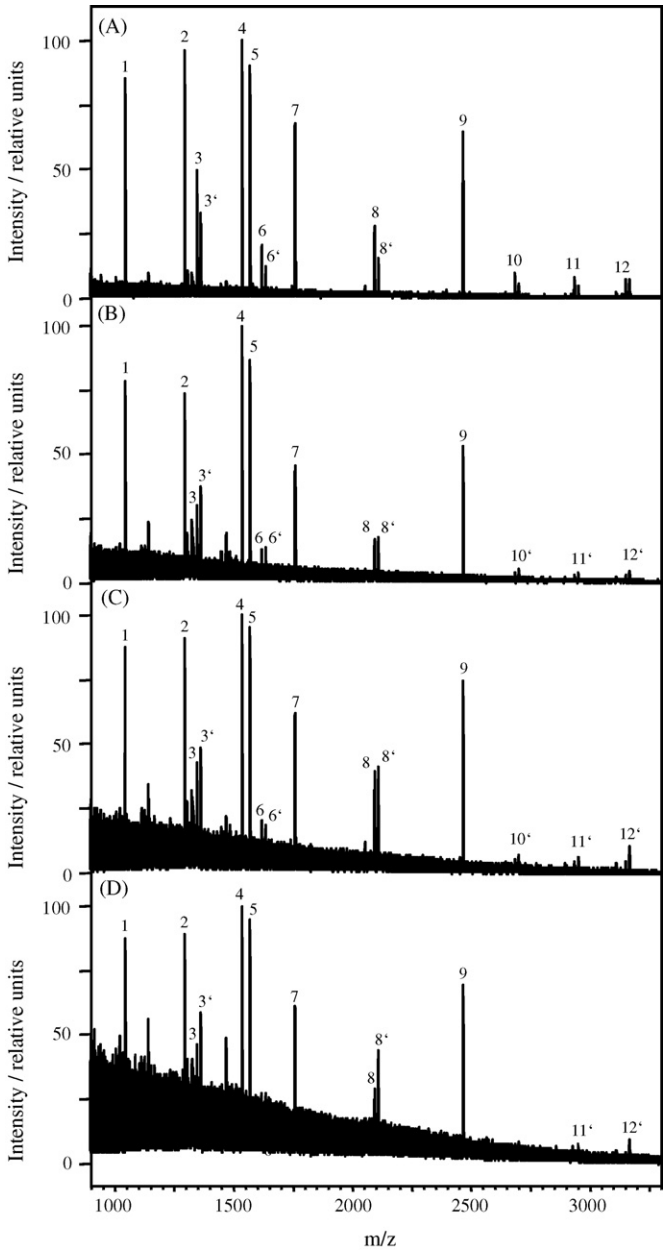

Fig. 2. Dependence of detection sensitivity on sample spot size. (A–D) Mass spectra of 10 amol of the peptide mixture prepared in a volume of 20 nL on 200, 400, 600 and 800 μm CHCA spots. 1–12, peptides 1–12; (′) singly oxidized peptide.

3. Results and discussion

3.1. Experimental conditions

The thin-layer sample preparation method combined with the AnchorChip^{TM} technology was chosen as the sample preparation technique in these studies because it combines high-detection sensitivity for peptides with high-sample homogeneity, and the size of the sample spot is predetermined by the surface design of the AnchorChip^{TM}. With high-sample homogeneity is inferred that across the entire sample spot, including regions close to the rim as well as the center of the sample, the signal response upon laser irradiation is comparable and the so-called 'sweet spot' phenomenon, which is often observed when samples are prepared by the dried-droplet preparation technique, is not observed. High-sample homogeneity renders systematic comparative experiments straightforward. The AnchorChip^{TM} technology is characterized by a specific design of the surface of the sample support: this is coated with a strongly water repellent, Teflon-like composite material except for an array of circular interruptions measuring 200, 400, 600, or 800 μm in diameter, respectively, at which the underlying stainless-steel surface acts as hydrophilic sample anchors. Available formats include among others the MTP 384 format used in this study. An important analytical parameter is the size of the anchor spot, which determines the horizontal expansion of the thin layer of matrix crystals prepared on the anchor before the sample is added. This study included all four available anchors sizes. These anchors coated with a thin layer of crystalline CHCA prepared according to our protocol, are referred to in the following as 200-, 400-, 600-, or 800-μm CHCA spots.

3.2. Detection sensitivity dependence on MALDI sample dimensions

Because the area of an 800-μm CHCA spot is four times larger than that of a 400 μm and 16 times larger than that of a 200-μm CHCA spot, the loss of detection sensitivity when using an 800-μm instead of a 200-μm CHCA spot should be considerable. An important fact that has to be taken into consideration when interpreting the details of the results is that the contact angle of the dilution buffer (0.1% TFA/1 mM *n*-OGP) on the hydrophobic coating of an AnchorChip^{TM} is approximately 100° with the consequence that the diameter of the footprint of a 20-nL sample drop on that surface falls around 400 μm (the diameter of a sphere with a volume of 40 nL is 420 μm). Consequently, the area covered by the droplet can be four times larger than a 200-μm CHCA spot, which provides ample surface area for peptide loss by hydrophobic interactions. To estimate whether a certain sample volume is likely to spread over the hydrophobic barrier surrounding the different CHCA spots employed in this study, we used the calculated half-volumes of a sphere with a diameter of 200, 400, 600, and 800 μm as a reference, which is 2.1, 16.8, 56.7, and 134 nL, respectively.

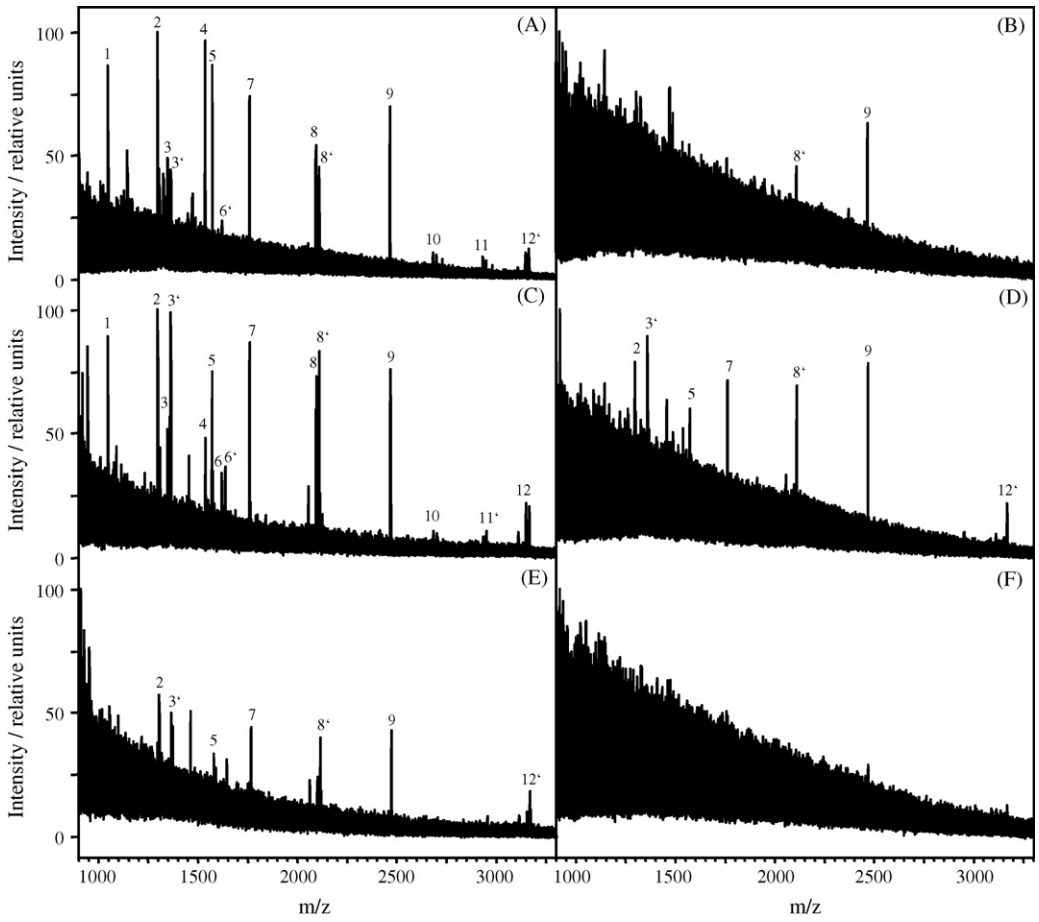

Fig. 3. Dependence of detection sensitivity on sample volume. (A and B) Mass spectra of 10 amol of the peptide mixture prepared in 100 nL on a 400 and 800 μm CHCA spots. 1–12 peptides, 1–12; (′) singly oxidized peptide. (C and D) Mass spectra of 50 amol of the peptide mixture prepared in a volume of 1 μL on a 400 and 800 μm CHCA spots. (E and F) The same sample amount prepared in 2 μL on a 400 and 800 μm CHCA spots. 1–12, peptides 1–12; (′) singly oxidized peptide.

The contact angle of dilution buffer on the hydrophobic surface of an AnchorChip™ was estimated by observing under a light microscope from the side a 2-μL droplet placed close to one of the long edges. A more accurate determination was considered of little value because it does not necessarily reflect the hydrophobicity of the surface surrounding the anchors, which can be subject to local variations due to imperfections in the production process, contamination with particles from the room air or left from previous sample preparations, and aging effects. An important consequence of this is that the ratio of the area of the CHCA spot to the total surface wetted by the sample is subject to considerable variation and concomitantly potential sample losses due to hydrophobic interactions with the surface surrounding the CHCA spot. In our experiments, always including at least five replicates, variations of up to 25% of the signal-to-noise ratios for the same peptide were indeed observed.

In a first experiment we investigated the dependence of detection sensitivity on CHCA spot area. A 1.5-μL aliquot of the peptide mixture stock solution was diluted in dilution buffer in a 1.5 mL Eppendorf tube to a final concentration of 5 fmol/μL for each of the 12 peptides. Fifteen minutes after dilution, 5 μL of that solution were aspirated into the piezo-dispensing capillary installed in Position 1 and used for the following experiments: 90 droplets of the dilution buffer, each of a volume of 0.2 nL were dispensed with a frequency of 1000 Hz onto a CHCA spot, directly followed by addition of 10 droplets of the peptide solution, each containing 1 amol of the peptide mixture. This corresponds to 10 amol of each peptide diluted in 20 nL to a final concentration of 500 amol/μL. This experiment was programmed to be repeated 20 times using five 200-, 400-, 600-, and 800-μm CHCA spots. After solvent evaporation the samples were washed and analyzed.

Fig. 2 shows four of the recorded mass spectra obtained from a 10-amol peptide mixture prepared on 200-, 400-, 600-, and 800-μm CHCA spots. Comparing these confirms the expected trend, i.e., the signal-to-noise-ratio and the absolute signal intensities for each peptide declines significantly as the CHCA spot size increases. For instance, in the mass spectrum recorded from the 200-μm CHCA spot, 10 amol yielded a signal-to-noise ratio exceeding 100 for peptides 1, 2, 4, 5, and 7 whereas for the 800-μm CHCA spot these values dropped below 10 for peptides 1, 2, 4, 5 and 7. Comparing the data for the 400- and 200-μm CHCA spot does not indicate that in the latter case the majority of the peptides were lost by interactions with the hydrophobic surface surrounding the spot.

The trend visible in Fig. 2 has important practical implications. For instance, 10 amol of the analyte mixture prepared on 200 μm CHCA spots resulted in peptide signal intensities that

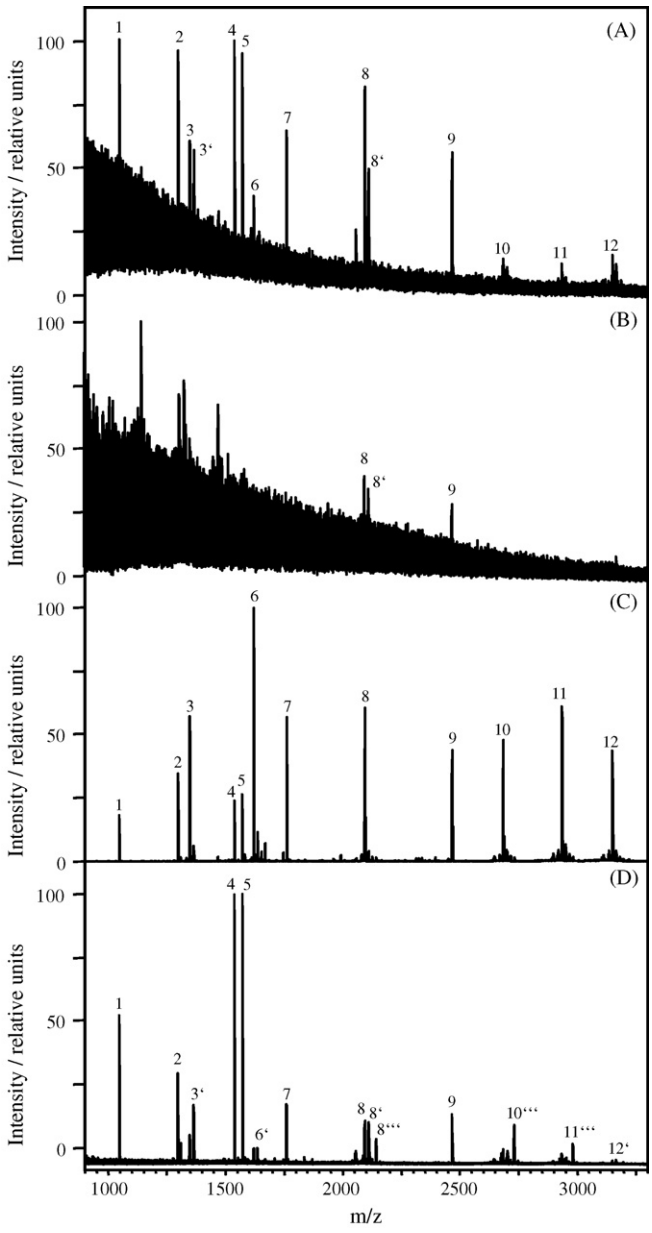

Fig. 4. Detection limits. Mass spectra of 1 amol of the peptide mixture prepared in a volume of (A) 0.2 nL on a CHCA spot of a diameter of ca. 180 μm and (B) 2 nL on a 400-μm CHCA spot. (C and D) Mass spectra of 50 fmol and 10 amol of the peptide mixture prepared in a volume of 1 μL and 0.2 nL on a 400-μm CHCA spot and a CHCA spot of a diameter of ca. 180 μm, respectively. 1–12, peptides 1–12; (′) singly oxidized peptide; (′′′) triply oxidized peptide.

would have classified all 12 peptides as suitable candidates for further characterization by MS/MS if required. In contrast, if prepared on an 800-μm CHCA spot, only peptide 9 would have come near that criterion. On the other hand, it has been shown that 600-μm CHCA spots provide sufficient sample material (surface area) to perform up to 15 MS/MS experiments each including more than two thousand single-shot-spectra [5]. In contrast to this, on a 200-μm CHCA spot one MS/MS experiment can easily consume most of the sample. The dilemma is that in the latter case for the analysis of one peptide by MS/MS most of the 110 amol of the other 11 peptides are consumed as

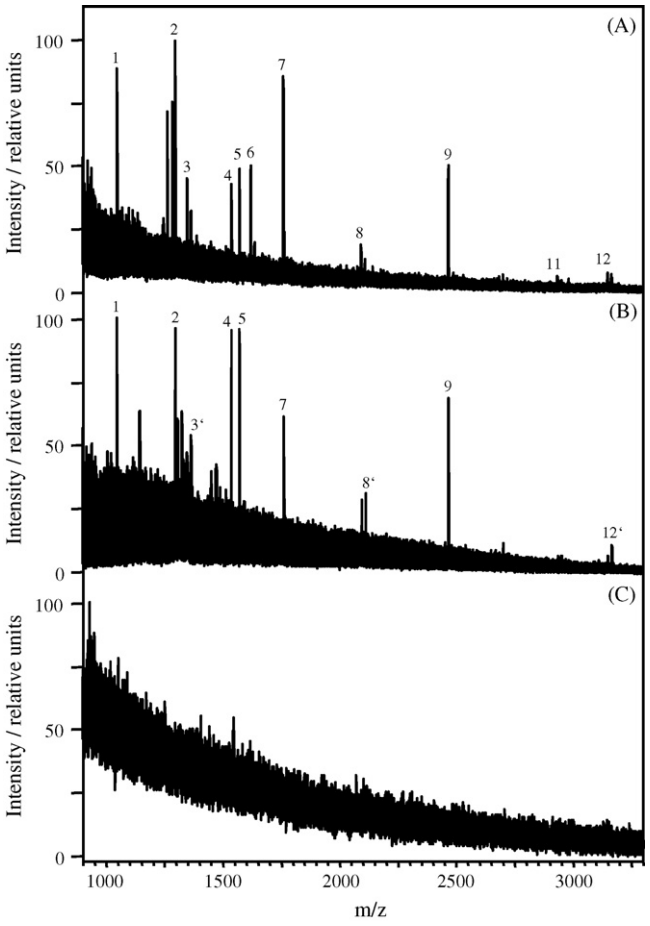

Fig. 5. Time-dependence of peptide loss by surface adsorption in plastic vials. Mass spectra of 1-μL aliquots of the peptide mixture diluted in a 2-mL Eppendorf tube to a concentration of 50 amol/μL. (A) Prepared directly after dilution, (B) after 2 h, and (C) after 24 h. 1–12, peptides 1–12; (′) singly oxidized peptide.

well, whereas on the 800-μm spot significantly more analyte molecules are required to enable the analysis of all 12 peptides by MS/MS. A second, well-known trend that affects the sample consumption is the dependence of the laser irradiance threshold above which peptide signals are observed on the peptide amounts available for MALDI across the irradiated sample spot. Close to the detection limit the consumption of the sample spot per laser shot is significantly higher in both, MS and MS/MS experiments.

A consequence of the limitations discussed above is that the MS detection sensitivity is connected to the MS/MS analysis capacity required. When using the AnchorChip^{TM} technology, four options are available for this trade off. The best choice in each particular case depends on the expected sample complexity and thus the number of required MS/MS spectra, which is usually not known a priori in proteomic peptide mixture analyses.

3.3. Detection sensitivity dependence on sample volume

Above considerations are important when planning bottom-up proteome identification projects based on off-line nano-LC–MALDI-TOF-MS/MS. In such experiments fractions of the LC effluent typically in the range of 50–200 nL are collected on the MALDI target. For many applications, however, the avail-

able sample volumes are larger and one is left with the choice to either prepare a sample of 1–2 μL directly on the MALDI target or implement a miniaturized sample enrichment step prior to MALDI sample preparation to decrease the sample volume. Our findings above motivated us to test whether the detection sensitivity observed in the first experiments, where the samples were diluted to a final volume of 20 nL on the target, would change if the samples instead were diluted five times more. It was clear that in this case the sample droplet would spread considerably even over the borders of a 600-μm CHCA spot and could also exceed the dimensions of an 800-μm CHCA spot. What we wanted to know was whether such conditions affect the detection sensitivity, for instance because more peptides escape from the analysis by binding to the hydrophobic coating of the AnchorChip™.

For this purpose the protocol used for the previous experiment was modified in some respects. Ten droplets of the sample solution were mixed on the target with 490 droplets dilution buffer yielding a total volume of 100 nL, and to ensure proper mixing, 390 of the 490 droplets were added directly before, and the remaining 100 droplets directly after addition of the sample solution (10 droplets). The complete protocol including three dispensing steps and two moves was finished in less than 1 s.

Comparing the results with those discussed before revealed that extending the sample volume from 20 to 100 nL while keeping the total analyte amount constant degraded the detection sensitivity significantly. This is documented in Fig. 3A and B showing mass spectra recorded from a 400- and an 800-μm CHCA spot, respectively. Comparing these with the corresponding spectra shown in Fig. 2 illustrates the trend. In both cases (400 and 800-μm CHCA spot) the loss in signal-to-noise is clearly visible.

This observation encouraged us to extend the dilution of the sample on the target by another order of magnitude and more. A first experiment showed that at this level (10 amol peptide in 1 μL instead of 100 nL dilution volume) we had crossed the detection limit in all respects: not a single peak could be assigned to any of the 12 peptides. This changed first when we raised the peptide amounts to 50 amol (50 droplets). Now we could assign again all 12 peptides to peaks when the sample was prepared on a 400-μm CHCA spot (Fig. 3C), and 7 when it was prepared on an 800-μm CHCA spot (Fig. 3D). When we extended the dilution volume from 1 to 2 μL, only 7 peptides could be detected from 400 μm CHCA spots (Fig. 3E) and no peptide anymore from 800 μm CHCA spots (Fig. 3F).

Compared to where we started (10 amol in 20 nL) and where we stopped our dilution experiments (50 amol in 2 μL), the loss in detection sensitivity we observed is certainly not negligible, and to detect 10 amol of a peptide in a sample of 2 μL volume requires preconcentration by one order of magnitude.

3.4. Limit of detection

Drawing on the observations above, to explore the limit of detection it is thus necessary to minimize both sample volume and spot size. For this purpose we performed experiments where we reduced the sample volume in several steps from 20 to 0.2 nL and the total amount of each peptide from 10 to 1 amol. An inevitable consequence of this is that the sample volume finally becomes too small to wet the entire CHCA spot. This was certainly the case when we transferred only one droplet (0.2 nL) of the peptide solution containing 1 amol of each peptide onto a 400-μm CHCA spot. Scanning across that spot vertically and horizontally in 10 μm steps with MALDI-TOF-MS and monitoring the occurrence of peptide signals revealed a spot of 180 ± 20 μm across which peptide signals were detected. Fig. 4A shows a mass spectrum that was acquired from this spot, documenting that all 12 peptides could be detected. If the sample droplet, however, was diluted with nine droplets dilution buffer to a volume of 2 nL, the detection failed except for two very weak signals that could be assigned to peptides 8 and 9 (Fig. 4B). This result confirmed the observed trend regarding the dependency of the detection sensitivity on sample volume and illustrates what is required to detect peptides in the sub-amol range. Considering that the laser spot size on the target used in these experiments was 10–15 μm and that typically 50 single-shot spectra could be recorded from one position before no more peptide signal was registered, it is also clear that the ultimate limit for this approach is not too far away.

3.5. Loss of analyte due to oxidation

The mass spectrum shown in Fig. 4A, obtained from 1-amol peptide mixture, demonstrates in fact the detection of sub-amol amounts of peptides 3, 8, and 10–12 because a significant fraction of these peptides has been oxidized during the sample preparation. For instance, nonoxidized and singly oxidized peptide 3 were detected with nearly the same signal-to-noise ratio suggesting that for one or the other species maximum 0.5 amol were available for detection. An important consequence of this observation is that the presence of methionine and tryptophane residues in peptides limits their detection sensitivity in MALDI-TOF-MS. It has been shown that their oxidation is primarily caused by traces of ozone molecules present in the room air, that this reaction is independent of which matrix compound is used for the sample preparation, and that the reaction stops when the samples are dry [8]. Important parameters for the degree to which these peptides are oxidized, are the concentration of ozone in the air surrounding the samples, the concentration of the peptides in the sample droplet and the reaction time. For the experiments performed in this study, this means that at the same concentration of ozone the level of oxidation should correlate with the volume of the sample and correlate inversely with the sample concentration. This trend was indeed observed for samples that were prepared at the same time. If, however, prepared at different times, e.g., in the morning, during the day and in the evening, that correlation was weaker or non-existing. This observation is in good agreement with the finding of Cohen [8] that the seasonal and daytime dependence of the outdoor ozone formation through the ventilation system affects the indoor concentration levels, on which the oxidation of methionine and tryptophane residues depends.

In all the experiments described and discussed so far, both the sample solutions and the dilution buffer contained 1 mM of the detergent *n*-OGP, which in addition to reducing peptide loss

by surface adsorption phenomena [9] was reported to decrease the level of oxidation of methionine and tryptophane residues in the MALDI sample preparation of peptides [3]. Considering the substantial levels we observed in above experiments, we were curious to see whether in the absence of *n*-OGP they were even higher, which should be the case if *n*-OGP inhibits the oxidation of peptides to a significant degree. First we dispensed 10,000 droplets of a solution containing 50 fmol/μL of the peptide mixture in dilution buffer onto a 400-μm CHCA spot corresponding to a total sample volume of 1 μL containing 50 fmol of each peptide. This was done to confirm that the peptides were not already oxidized to a substantial degree in that solution. Analysis of that sample yielded the spectrum shown in Fig. 4C proving that only a small fraction of peptides 3 and 6 (one methionine residue) as well as peptides 8, and 10–12 (one methionine and one tryptophane residue) were oxidized under these conditions. In the following experiment we excluded *n*-OGP from the sample solution and dispensed only one sample droplet onto a 400-μm CHCA spot corresponding to 10 amol of each peptide transferred in a volume of 0.2 nL. The analysis of this sample provided the spectrum shown in Fig. 4D documenting a high level of oxidation for all peptides containing a methionine (+O) and/or tryptophane residue (+2O). In none of the experiments performed in the presence of *n*-OGP, not even when instead of 10 only 1 amol of each peptide was prepared on a 400-μm CHCA spot (Fig. 4A), was a triply oxidized peptide an abundant species. In the last experiment, in contrast, triply oxidized peptides were detected at high levels and for peptides 10 and 11 represented the most abundant molecular ion. This observation clearly confirms the inhibitory effect of *n*-OGP on the oxidation of methionine and tryptophane residues. The fact that in that experiment the total sample volume was only 0.2 nL and that the evaporation of so little solvent takes only a second or two can be considered as evidence that the oxidation of methionine and tryptophane residues we observed is not a slow reaction. Escaping from it by shortening the time the sample is wet, e.g., by raising the temperature of the sample support, appears not to be a very attractive strategy. An obviously better approach would be to perform the sample preparation in an inert gas atmosphere.

3.6. Loss of analyte due to surface adsorption

Unexpectedly steep loss of signal in MALDI-MS, observed when a sample is diluted, is often speculated to be caused by analyte adsorption to the inner surfaces of sample vials and pipette tips. The piezo-electric liquid dispensing instrument used in this study is a good tool to test this hypothesis by enabling the preparation of very small peptide amounts from relatively concentrated solutions in which analyte losses caused by surface adsorption are not expected to reduce the analyte concentration significantly. The spectrum in Fig. 3C was prepared in such a way: 50 amol of the peptide mixture were dispensed onto the sample anchor from a 5 fmol/μL solution, and thereafter diluted further by addition of 1 μL solvent. To assess potential analyte losses caused by surface adsorption, a sample with the same end concentration was prepared by consecutive dilutions in the following way: a 4-μL aliquot of the peptide stock solution (5 pmol/μL) was diluted with 1996 μL dilution buffer in a 2-mL Eppendorf tube to a final concentration of 10 fmol/μL and a 10-μL aliquot of this solution was further diluted with 1990 μL dilution buffer in a second 2 mL tube to a final concentration of 50 amol/μL. After 1 min vortexing, 1-μL aliquots of that solution were prepared on 400 and 800 μm CHCA spots and analyzed. Fig. 5 shows a spectrum acquired from a 400-μm CHCA spot. On 400 μm CHCA spots 10 or more peptides could be detected whereas on 800 μm CHCA spots this was limited to 4 or 5. Comparing this spectrum with the one shown in Fig. 3C does not indicate that a significant amount of the peptides were lost in the two Eppendorf tubes or the three pipette tips used for the off-target dilution experiment. In fact, the signal-to-noise ratios for peptides 5, 7 and 9–11 are comparable, for peptides 3, 8 and 12 it is better in the spectrum obtained from the on-target dilution experiment, and for peptides 1, 2, 4, and 6 it is better in the spectrum that was obtained from the off-target dilution experiment. The differences in the signal-to-noise ratios for peptide 3 correlate with the different levels of oxidation observed in the two spectra. By comparing spectra of several samples from both experiments, we came to the conclusion that the detection sensitivity in both experiments was similar, which means it was mainly limited by the sample preparation on the sample support and that losses due to surface adsorption did not contribute significantly. On the other hand the better results obtained using the 400-μm spots clearly shows that even for large sample volumes, a smaller CHCA spot enhances detection sensitivity.

To study the influence of the delay time between off-target dilution and on-target sample preparation, we prepared additional aliquots after 15 min and 1, 2, and 24 h. We observed that after 15 min the results were comparable to those obtained from the samples prepared 1 min after dilution, but after 2 h a significant loss was noticed (Fig. 5B), and after 24 h the detection failed (Fig. 5C). In conclusion, if our peptide mixture was diluted quickly, signals could be detected at sample concentrations as low as 50 amol/μL, but if the diluted sample was left for 2 h in the sample vial, significant analyte losses were observed. The kinetics of this reaction is likely to depend on the sequence and size of the peptide and general conclusions cannot be drawn from the small set of peptides in our experiments.

In a previous study based on prestructured sample supports combined with the dried-droplet sample preparation protocol using the MALDI matrix 2,5-dihydroxybenzoic acid, 100 amol of peptides 1, 2, 8, and 12 could only be detected if sample dilution and following transfer onto the MALDI target were performed quickly. This observation is not in conflict with the results discussed above because our dilution series included the detergent *n*-OGP, which was not included in the previous study. In those experiments the sample was diluted with matrix solution in the absence of any detergent. When using the thin-layer sample preparation technique, diluting the sample with matrix solution is not possible. Instead, we repeated the dilution experiment described above but without including *n*-OGP. The result was that losses were observed already 15 min after the sample had been diluted to 50 amol/μL (data not shown).

Finally, we made an attempt to investigate whether a significant fraction of the analyte molecules is lost during the on-target washing procedure. This step is an important part of the thin-layer sample preparation technique, especially when *n*-OGP or contaminants are present in the samples. If omitted in the experiments of this study, the results were significantly worse and in many cases no peptides could be detected whereas they could when the samples were washed. This was, for instant, the case when the samples were diluted on target to a volume of 1 or 2 μL. In one set of experiments 200 droplets (200 amol) of the peptide mixture were dispensed onto 400 and 800 μm CHCA spots and, after the samples were dry, a 1-μL aliquot of 0.1% TFA was manually deposited on each sample spot. After 10 s most of the remaining washing solution was aspirated into a new tip and transferred to a new 200- or 400-μm CHCA spot. Analysis of these spots before and after washing did not reveal any significant loss. In most cases no peptides could be detected and in the others a maximum of 4 from 12 was detected with a signal-to-noise ratio of 3–5 (data not shown).

3.7. Limitations of this study

Several limitations restrict extrapolation of the results of our experiments, especially the fact that only 12 water-soluble peptides and only one sample preparation technique and one matrix compound were included in this study. We consider it likely that the experiment where a single droplet containing 1 amol of each peptide was prepared on a CHCA spot would have benefited from restricting the sample spot diameter from 180 to 100 or 50 μm by using a 100- or 50-μm anchor. Proving this was not possible because anchors of such small sizes are currently not available.

When we diluted 10 amol of the peptide mixture on the target to 100 nL we observed a loss in detection sensitivity, and when we diluted the sample to 1 μL the signal was lost. With 50 instead of 10 amol, peptides could be detected but the results were worse than for a 10-amol aliquot diluted to 20 nL. This raises two important questions we cannot answer. Why is the detection sensitivity reduced when the same amount of sample is prepared on the same anchor size but in a larger volume? If this is caused by analyte loss, where are the peptides lost? Answering these questions requires more experimental data and probably additional analytical techniques, for instance localization of peptides by the use of radioactive isotopes.

One potential reason for loss of peptides in the on-target dilution experiments is reverse–phase interactions with the hydrophobic coating of the AnchorChips^{TM} either across the entire liquid–solid contact area or mostly at the liquid–solid–gas phase intersection because the peptides are enriched at the surface of the sample droplet exposed to the air as a consequence of their structure and solvent evaporation. A second possible reason that could explain the results when the sample was diluted to 1 or 2 μL, is the accumulation of *n*-OGP to a level that it negatively affects the sample preparation, e.g., by competition with the peptides for binding sites on the surface of the CHCA spot. A third possibility is partial recrystallization of the matrix CHCA, which is little but not a 100% insoluble in water below pH 2.

4. Conclusions

The influence of several parameters on the MALDI-TOF-MS detection sensitivity of peptides prepared by the thin-layer preparation technique was studied. These included the sample spot size, volume, concentration, as well as analyte oxidation and surface adsorption. All experiments showed that the smaller the CHCA spot size, the higher the detection sensitivity, even if the sample volume is as low as 20 nL. Another clear trend was that the smaller the sample volume, the higher the detection sensitivity. We could show that 1 amol of the peptide mixture was sufficient to detect all components if the sample was prepared in a volume of 0.2 nL across a spot of ca. 180 μm diameter. This was not possible anymore when the sample was diluted to 2 nL and spread out over a spot of 400 μm. The conclusion is that for MALDI-TOF-MS of peptides a detection sensitivity below 1 amol requires tiny sample volumes prepared on a spot whose dimensions come close to the laser spot size on the target. Another important observation in this context was that oxidation of methionine and tryptophane residues can drastically decrease the detection sensitivity for peptides that contain these amino acids, especially if they contain more than one of them. To achieve sub-amol detection sensitivity this reaction must be inhibited.

Our results suggest that loss of peptides in dilute samples by adsorption to the walls of sample vials and pipette tips is not necessarily a quick process, but can over hours be severe. For the peptide mixture and dilution buffer used in this study, the conclusion is that the pipette tips are of little concern, whereas sample storage is.

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