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Targeted Analyte Detection by Standard Addition Improves Detection Limits in MALDI Mass Spectrometry

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Abstract

Matrix-assisted laser desorption/ionization has proven an effective tool for fast and accurate determination of many molecules. However, the detector sensitivity and chemical noise compromise the detection of many invaluable low-abundance molecules from biological and clinical samples. To challenge this limitation, we developed a targeted analyte detection (TAD) technique. In TAD, the target analyte is selectively elevated by spiking a known amount of that analyte into the sample, thereby raising its concentration above the noise level, where we take advantage of the improved sensitivity to detect the presence of the endogenous analyte in the sample. We assessed TAD on three peptides in simple and complex background solutions with various exogenous analyte concentrations in two MALDI matrices. TAD successfully improved the limit of detection (LOD) of target analytes when the target peptides were added to the sample in a concentration close to optimum concentration. The optimum exogenous concentration was estimated through a quantitative method to be approximately equal to the original LOD for each target. Also, we showed that TAD could achieve LOD improvements on an average of 3-fold in a simple and 2-fold in a complex sample. TAD provides a straightforward assay to improve the LOD of generic target analytes without the need for costly hardware modifications.

Keywords

MALDI; mass spectrometry; limit of detection; sensitivity; targeted detection; serum biomarker; carrier effect; method of standard additions

INTRODUCTION

Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) serves as a major technique for fast and accurate analysis of a number of molecules from complex mixtures such as cells, tissues and serum samples¹. Although MALDI-TOF MS has been successfully applied for detection, identification and validation of many peptides and molecules, it has proven ineffective for analyzing low abundance molecules from complex mixtures. Considering the extremely wide range of protein concentrations in plasma (i.e. from albumin at 10¹⁰ pg/mL to interleukins at 10 pg/mL²), the lower-abundant proteins or peptides are dominated by the abundant serum contents and fail to be detected in a mixture. In addition, background and chemical noise-coming from desorbed matrix clusters-interfere with the MS signal and further compromise the sensitivity and detectability for low-abundance analytes³. Despite the technological advances in MALDI-TOF instrumentation, the suboptimal transmission efficiency of the mass analyzer and detection

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efficiency of the detector also result in some loss of analyte, which is another factor that reduces the detection limit and sensitivity of MALDI-TOF MS to low-abundant analytes. On the other hand, the concentration of potential disease biomarkers lies in the lower range of concentrations in serum, particularly at the early stages of the disease where screening is crucial⁴. Therefore, the sensitivity and detection limit of MALDI-TOF MS must be improved in order to be effective for biomarker discovery research.

Sample preparation, matrix development or optimization, sample target plates, spotting technology and mass spectrometry hardware are possible places where the sensitivity of MALDI-MS can be improved. Sample treatment with solvents helps remove the adducts and salts, which consequently enhances the analyte desorption/ionization and sensitivity⁵. Additionally, sample derivatization procedures play a significant role in enhancing ionization efficiency, thus improving the sensitivity of MALDI-MS to small molecules, proteins and peptides, oligosaccharides and synthetic polymers⁶. Although these techniques have proven effective in improving the sensitivity, they should be tailored to the specific analyte of interest^{7,8,9}. In comparison, matrix optimization provides a benefit for more generic groups of analytes. Cohen et al showed that the quality of the mass spectra of peptides and proteins could be improved by optimizing the matrix preparation conditions¹⁰. Many novel matrices have been developed for various MALDI-MS applications^{11,12,13}. The binding affinity of the analytes to the surface of target plate greatly influences the efficiency of analyte ionization, and on-plate sample purification and target capture can enrich the analyte of interest, thus improve both the sensitivity and specificity of MALDI-MS to certain analytes^{14,15,16,17}. Besides the sample plate, studies suggest that the sample deposition and the spot size significantly influence the sensitivity of MALDI, where nanoliter spots - with a diameter greater than that of the laser beam - produce signal with greater intensity¹⁸. Hardware modifications to optimize efficiency of the analyzer and the sensitivity of the detector, mainly led by the mass spectrometry manufacturing companies, also play a crucial role in improving this technology. Despite their effectiveness, hardware modifications require total or partial replacement of the instrument; hence, they represent a costly option.

In this study, we developed a generic technique for improving the sensitivity and detection limit of MALDI-MS. This method, named targeted analyte detection (TAD), selectively enhances the detection of analytes of interest. In TAD, a small known amount of analyte of interest is spiked into the sample, thereby elevating the concentration to levels above the noise, where the interference of the noise is relatively reduced and the sensitivity is increased. The added analyte acts as a *carrier* to suppress the matrix effect (introduced by interferences with other compounds in the sample) and enhances ion abundance of analyte of interest. The measured signal is thus contributed by both the endogenous and exogenous (spiked-in) analytes. Therefore, TAD uses the added standard to reveal the endogenous target analyte that was otherwise buried in the noise. In this study, we assess the feasibility of TAD in improving the detection limit of MALDI-MS. Additionally, we provide a systematic method for optimizing the spiking amount needed to achieve the maximum improvement in the limit of detection (LOD). The main advantage of TAD is that it is not limited to certain types of analytes, provided that the analyte of interest is available or can be synthesized for spiking into the unknown sample. Furthermore, this approach takes advantage of the generic sigmoidal shape of the calibration curve, which is very reproducible in a wide range of analytical instruments. Therefore, this method might be capable of improving the sensitivity in a wide range of instruments regardless of the detection technologies, including but not limited to mass spectrometers.

EXPERIMENTAL SECTION

Materials and reagents

All peptides were purchased from Anaspec (Fremont, CA, USA). Single-use alpha-cyano-4-hydroxy-cinnamic acid (CHCA) and 2,5-dihydroxy benzoic acid (DHB) MALDI matrices were from Thermo Scientific (Waltham, MA, USA) and were prepared freshly with 50% acetonitrile and 0.1% trifluoric acid. Bovine serum and all other reagents and chemicals were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated.

In our experiment three test peptides of different molecular weights were chosen, including Angiotensin I ($m_a = 1296.685$), Angiotensin II ($m_a = 1046.542$) and β -Amyloid (1-15) ($m_a = 1826.785$) and their heavy-isotope labeled counterparts, Angiotensin I ($m_a = 1309.798$), Angiotensin II ($m_a = 1053.603$) and β -Amyloid (1-15) ($m_a = 1836.871$).

Simple Background Experiment

A 100 fmol/ μ L solution of target heavy-isotope labeled peptide in deionized water was prepared as the diluent. Heavy-isotope labeled peptides serve as internal standards for better quantification. Control TAD solution with concentration of 0, and three additional TAD solutions with final concentrations of 4, 20 or 100 fmol/ μ L were prepared by dissolving the target peptide into its diluent. To determine the LOD of the target peptides, the target peptide was sequentially diluted by a factor of two in the diluent to cover the range of 0 to 2000 fmol/ μ L. For each experiment, the sample containing targeted peptide was mixed at a 1:1 ratio with the TAD solution containing 0, 4, 20 or 100 fmol/ μ L exogenous target peptide, thus elevating the target peptide concentration by 0, 2, 10 and 50 fmol/ μ L, respectively. The spiking concentration and exogenous concentration both refers to the target peptide concentration elevation in the TAD solution, and will be used interchangeably throughout this paper.

Samples, including serial dilutions of target peptides mixed with four sets of TAD solution, were spotted on a MALDI plate, and coated with either recrystallized CHCA or DHB matrix dissolved in 50% acetonitrile and 0.1% trifluoric acid. The samples were analyzed by Applied Biosystems 4800 MALDI TOF/TOF analyzer in the positive reflector mode. Twenty sub-spectra (100 shots/sub-spectrum) were averaged to yield the mass spectrum for each sample. Four replicates were conducted to study the functionality of TAD in the simple background experiment.

For each spot, ten spectra were acquired and saved into a .T2D file. The .T2D files were then converted into .txt text files using ProteomeCommons.org IO Framework Peak List Convertor. Finally, the text files were opened in Matlab (Mathworks, Natick, MA USA). For each peptide monoisotopic peak, the area under the curve was calculated and normalized to the area under the curve of the monoisotopic peak of their heavy-isotope labeled counterpart. This quantity was used as the normalized signal intensity for further analysis of the target analyte detection sensitivity using TAD solutions.

Complex Background Experiment

In this part of the experiment, a 30 ng/ μ L tryptic digested bovine serum solution was mixed with the target peptides. This complex solution, the concentration of which is roughly three orders of magnitude higher than the LOD of the target peptides, mimics a practical biological or clinical sample. Thus, this experiment creates a benchmark for assessing the feasibility of TAD in realistic complex mixtures. Three heavy-isotope labeled peptides, Angiotensin I, Angiotensin II and β -Amyloid (1-15) were mixed into the above serum solution to formulate the diluent with a final heavy-isotope labeled peptide concentration of

200 fmol/ μ L. Then, three target peptides were dissolved into the diluent and serially diluted by a factor of two to cover the range of 0 to 1000 fmol/ μ L. Control peptide solution with concentrations of 0, and five additional TAD solutions with a final concentration of 12.5, 25, 50, 100 and 200 fmol/ μ L were mixed with the original peptide dilution at a 1:1 ratio, to boost the peptide concentrations by 0, 6.25, 12.5, 25, 50 and 100 fmol/ μ L, respectively. Then the samples were spotted on the MALDI plate and analyzed as described in the *Simple Background Experiment*. Triplicate experiments were conducted to study the functionality of TAD in the complex background experiment.

RESULTS AND DISCUSSION

Estimation of LOD

A code was developed to estimate the predicted LOD. The mean and standard deviation of the measurements for the control group without TAD solution was used as the input to the code. For each target analyte, the original LOD was calculated based on the commonly used definition of LOD as shown in eq 1:

$$Signal(LOD_{orig}) = Signal(Background) + 3SD, \quad (1)$$

where LOD_{orig} is the limit of detection in the absence of any exogenous target analyte and $Signal(LOD_{orig})$ is the total signal at this concentration. $Signal(Background)$ represents the background signal mean and SD denotes the background signal standard deviation (Figure 1).

For a given exogenous concentration of the target peptide in TAD solution, the LOD was estimated by shifting the reference point of the background to the given exogenous spiking peptide concentration (C) used in TAD solution. The signal and standard deviation at any concentration were estimated by interpolating the signal and standard deviation of the calibration curve without exogenous peptide in TAD solution, respectively. Denote the limit of detection at the spiking concentration C fmol/ μ L of the target analyte in the TAD solution, as LOD_C and LOD_C should then satisfy eq 2:

$$Signal(C + LOD_C) = Signal(C) + 3SD_C. \quad (2)$$

It is noted that for the control set with no spiking target peptide in the TAD solution, C is set to zero (Figure 1 and eq 1).

Ideally, the measured signal for each analyte is proportional to the amount of that analyte in the sample. However, the detection accuracy is compromised by factors such as background, detection efficiency, sample preparation and signal detection variations, and the limit of detection in mass spectrometry. Presence of background results in a nonzero signal even at zero concentration of the analyte. Suboptimal detection efficiency compromises the output signal. Analyte concentration variations introduced by analyte-matrix cocrystallization, desorption/ionization, analyzer and detector add noise to the measurements, thus limiting the threshold as well as the confidence of low abundance analyte detection. Therefore, the practical calibration curve of MALDI-MS, which is the measured mass spectral signal versus a given analyte concentration, differs from the ideal curve in crucial aspects (Figure 1). The sigmoidal shape of the practical calibration curve arises from these differences, whereas the signal intensity is linearly proportional to the analyte concentration in the ideal curve. LOD corresponds to the concentration of an analyte that produces a signal at least as large as the background mean plus three times the background standard deviation. Thus the

LOD can be estimated by the standard deviation of the background as well as the sensitivity, based on eq 3 (Figure 2):

$$LOD=3SD/Sensitivity, \quad (3)$$

where sensitivity is defined as the slope of the calibration curve. Eq 3 indicates that LOD is directly proportional to the standard deviation and inversely proportional to the sensitivity; therefore, lower noise level and higher sensitivity improve the LOD. This provides the opportunity that one can improve the LOD by improving the reproducibility or sensitivity.

Concentration Dependent Sensitivity and SD

Sensitivity and standard deviation (SD) of an analyte greatly depend on the concentration of the target analyte in the sample. To illustrate the dependence, we calculated the sensitivity and SD versus analyte concentration using Angiotensin II as an example and the results are shown in (Figure 3). The calibration curve of Angiotensin II was generated by analyzing sequential dilutions of this peptide with the mass spectrometer. The sensitivity was calculated from the calibration curve by dividing the signal difference by the concentration difference for two adjacent data points at each concentration. The standard deviation was computed from ten normalized mass spectral signals and is denoted as SD. Due to the sigmoidal shape of the calibration curve (Figure 1 and Figure 3A), the sensitivity, i.e. the slope of this curve stays stable at its maximum value over the linear range of the assay, and decreases as the concentration falls below the LOD or above the upper linear range (Figure 1 and Figure 3B). The standard deviations are usually modest at lower analyte concentration and increase with analyte concentration (Figure 3C). Moreover, the coefficient of variation (CV) defined by the standard deviation divided by the signal mean also associated with the concentration of the target analyte and drops rapidly as the concentration of the analyte increases (Figure 3D). As a result, there are certain concentrations of analyte with a lower SD to sensitivity ratio than this ratio at the background. These concentrations generally lie near the LOD of the target analyte, where the sensitivity increases dramatically (Figure 1, Figure 3A and 3B), but SD still remains relatively low (Figure 3C). Considering that the LOD depends on the ratio of SD to sensitivity, the LOD can then be improved by shifting the reference point of concentration to this range of a higher slope on the calibration curve by spiking additional analytes into the sample.

Determining LODs of Target Analytes in Simple Mixture

To determine whether we could lower the LOD with the target analytes spiked in TAD solution, we analyzed the target peptides in different dilutions in control solution (which did not have any spiking target peptides added) and in various TAD solutions (which had a different amount of target peptides spiked in the solution). The mass spectral peaks of Angiotensin I is depicted in Figure 4 for the control and the TAD solution group. For this peptide, the measured LOD_{orig} was 64.5 fmol/ μ L. Therefore, the mass spectral peak of Angiotensin I ($m_a=1296.685$), averaged over the ten measurements, is not distinguishable from the background at concentration of 31.25 fmol/ μ L (Figure 4A). By spiking the target analyte with a concentration $C = 50$ fmol/ μ L, the spectral signal is boosted and the LOD reduces to 22.5 fmol/ μ L. Consequently, as shown in Figure 4B, the averaged mass spectral peak of Angiotensin I at the endogenous concentration of 31.25 fmol/ μ L and exogenous concentration of 50 fmol/ μ L ($X = 31.25$ and $C = 50$ fmol/ μ L, i.e. 81.25 fmol/ μ L) is significantly higher than the background at exogenous concentration of 50 fmol/ μ L.

To determine whether this LOD improvement method and the dependence of the LOD improvement on the spiking target analyte concentration could be applicable to other analytes, three analytes, Angiotensin I, Angiotensin II, and β -Amyloid (1-15), with three

different concentrations of target analytes spiked in TAD solution were tested. Nine experimental conditions (three exogenous concentrations for each of the three targeted peptides) were studied in four replicate measurements with independent sample preparation. The experiment was performed in both CHCA and DHB as the MALDI matrix. On average, in CHCA matrix, TAD successfully improved the LODs in all nine experimental conditions (Figure 5A). TAD successfully improved the LODs in eight of nine experimental conditions when using DHB as matrix (Figure 5B). Improvement factor is defined by LOD_{orig} divided by LOD_C , where LOD_{orig} is the LOD of the control group with no exogenous peptides spiked to the solution and LOD_C represents the LOD that was achieved by boosting the concentration by spiking the analyte of a concentration C fmol/ μL .

Additionally, we estimated the LOD improvement expected at each exogenous concentration using the MALDI-MS calibration curve for each peptide. The experimental LOD_{orig} was calculated using eq 1 and the predicted LOD_C was estimated using eq 2 where the LOD_{orig} and LOD_C are graphically depicted in Figure 1. For all three peptides, the predicted improvement factor is very close to 1 at the lower end of the target analyte spiking concentrations, but it has local maxima at the midranges and decreased at higher spiking concentration. For example, the predicted LOD_C for Angiotensin II using CHCA and DHB matrices in the simple background experiment is plotted as a function of spiking concentration C divided by LOD_{orig} (Figure 6). Maximum predicted LOD improvement was achieved when the spiking peptide concentration is close to LOD_{orig} . For all three peptides, there is a local maximum on the estimated LOD curve (corresponding to an optimal LOD improvement) at a spiking concentration of the target analyte around the control LOD_{orig} . The optimal spiking concentration and the corresponding estimated LOD improvement factor for the three peptides are listed in Table 1. This quantitative method suggests that maximum improvement of the detection limit for low abundance analytes depends not only on the analyte but also on the MALDI matrix, which affect the signal to noise ratio of each analyte. Maximal improvement factor for the analyte of interest can be reached by spiking the target analyte at a concentration close to LOD_{orig} into the unknown sample for the analysis of target analytes.

Determining LODs of Target Analytes in Complex Mixture

To determine whether the LOD improvement observed with a solution of a single analyte using the TAD method could apply to target analytes in a complex mixture, the above three target analytes were analyzed in the mixture of serum peptides. Five spiking concentrations of each target peptide were considered and the triplicate experiments were performed for each case. Of the fifteen experimental conditions (five exogenous concentrations for each of the three target peptides), the average LOD of triplicate experiments was improved in ten targeted peptide-exogenous peptide pairs compared to the corresponding control (Figure 7). In general, highest spiking concentrations of target peptides result in low improvement factors, and in some cases fail to achieve any improvements. The estimated LOD curve for Angiotensin II in complex mixture shows similar pattern to the simple background experiment (Figure 8), with a local maximum at exogenous concentration of LOD_{orig} . The optimal exogenous concentrations and the achieved improvement factors for complex background experiment are shown in Table 2. The optimal predicted exogenous concentrations are estimated to be close to $2 \text{ LOD}_{\text{orig}}$, which is slightly higher than that of the simple background experiment. Also, the predicted maximum LOD improvement factor in the complex background experiment is lower than that of the simple background experiment for the same three examined peptides.

TAD takes advantage of the carrier effect of standard additions to reveal the signal that is buried in noise due to complexity of the sample¹⁹. The carrier effect is a repeatedly reported phenomenon²⁰²¹, which to the best of our knowledge has not previously been used in

quantitative mass spectrometry as a technique for improving the detection. TAD provides a 3-fold LOD improvement in simple background, and a 2-fold LOD improvement in complex background experiments. This enhancement achieved through TAD might be modest compared to signal enrichment techniques such as chromatography, fractionation, or affinity enrichment; however, TAD can be applied in combination with these techniques to further improve the detection limit by 2- to 3-fold. Also, further improvement of detection limit using TAD technique can be achieved by highly controlled conditions with high reproducibility. Therefore, we think that functionality of TAD might improve in a more controlled and reproducible experimental setting such as automated clinical assays.

CONCLUSION

A novel technique, targeted analyte detection (TAD), was developed for improving the detection limit of MALDI-MS. This technique takes advantage of the carrier effect of the added standard analytes, which occurs due to the generic sigmoidal shape of the calibration curve. The functionality of TAD depends on the relative enhancement of sensitivity over the increase of the standard deviation at the analysis of target analytes with spiking in exogenous concentration. At certain ranges of exogenous concentration, the increment in the sensitivity overcomes the standard deviation, resulting in an improved LOD. Theoretically, exogenous concentrations approximately at 1 LOD_{orig} would generate the optimum LOD improvement. TAD is a cost-effective LOD improvement method, which is not limited to a certain group of analytes, or detection methods or instruments. It can be applied to enhance the detection of any analyte with different detection methods, provided that the analyte of interest can be extracted or is available in synthetic form.

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Figure 1.

Presence of background, limited dynamic range, limit of detection, limited detection efficiency and variations cause the deviation between the realistic calibration curve from the ideal curve where the signal is proportional to the analyte concentration. Shifting the reference point to the linear dynamic range of the calibration curve will enhance the sensitivity and improve the LOD by spiking in certain exogenous concentrations of target analyte. The original calibration curve of the analyte of interest is used for this estimation of the predicted LOD_C . The reference point for calculation of LOD_C is shifted to the given exogenous concentration.

Figure 2.

Correlation between the LOD, sensitivity and the standard deviation is depicted. The slope of the dotted line is by definition the average sensitivity over the concentration range from 0 to LOD. On the other hand, this slope equals three times the standard deviation divided by LOD. Therefore, LOD is proportional to the SD divided by the sensitivity.

Figure 3.

Sensitivity and standard deviation of the measurements change with the analyte concentration. A) Calibration curve for Angiotensin II generated using Applied Biosystems 4800 MALDI-TOF/TOF analyzer is depicted. B) Sensitivity sharply rises as the concentration increases to supra-LOD levels. C) The variation in standard deviation at low concentrations is modest compared to increments in the sensitivity. D) Coefficient of variation (CV) rapidly decreases from 40% at the background to ~5% for higher end of the curve. The LOD is marked by the vertical dashed line.

Figure 4.

The mass spectral peak of Angiotensin I ($m_a=1296.685$) is depicted at the reference point of LOD measurement (dotted line) and endogenous concentration $X = 31.25$ fmol/ μ L (solid line) for A) control group, where $C = 0$ and $LOD_{orig} = 64.5$ fmol/ μ L and B) TAD experiment where $C = 50$ fmol/ μ L and $LOD_C = 22.5$ fmol/ μ L. A) In the control group, the solid line is hardly differentiated from the background. B) However, improving the LOD in the TAD experiment leads to significant distinction of the signal from the background at concentration $X = 31.25$ fmol/ μ L. The peak intensities were normalized to the heavy isotope-peptide.

Figure 5.

Experimental improvement factors for three peptides in simple background are averaged over four replicates. A) When using CHCA, the LOD is improved in all of the 9 experimental pairs yielding improvement factors greater than 1. B) The LOD is improved in 8 of the 9 experimental pairs when DHB is used as the MALDI matrix. The dotted line shows the threshold of LOD improvement. The error-bars show the standard error of the mean.

Figure 6.

An example of the predicted improvement factor for Angiotensin II in the simple background experiment using A) CHCA and B) DHB is shown. The highest improvement factor is achieved at exogenous concentrations close to the LOD_{orig} of the target peptide for both CHCA ($0.67 LOD_{orig}$) and DHB ($1.46 LOD_{orig}$) matrices. Due to the increase in the standard deviation of the measurements, the improvement factor decreases at higher exogenous concentrations.

Figure 7.

Experimental LOD improvement factors for three peptides in complex background using CHCA as the matrix are averaged over triplicate experiments. In 10 out of the 15 experiments, the LOD is improved. The improvement factors depend on the concentration of the exogenous analyte spiked into the sample as well as the analyte of interest. The error-bars depict the standard error of the mean.

Figure 8.

An example of predicted improvement factor for Angiotensin II in the complex background experiment is shown. The highest theoretical improvement factor is achieved at exogenous concentrations close to the LOD_{orig} of that peptide, similar to the simple background experiment. The curve is bell-shaped yielding an improvement factor close to 1 at lower concentrations, and decreasing at higher concentrations.

Table 1

The optimal exogenous concentrations and improvement factors for simple background experiment averaged over four replicates. The optimal exogenous concentration is close to LOD_{orig} . On average, the optimal exogenous concentration is $1.26 LOD_{orig}$ over all three peptides in CHCA matrix, which is lower than the optimal exogenous concentration of $1.58 LOD_{orig}$ for DHB matrix. Additionally, CHCA yields a higher predicted optimal improvement factor compared to DHB matrix.

Peptide	Matrix	Local maximum exogenous concentrations (C/LOD_{orig})	Local maximum improvement factors (LOD_{orig}/LOD_C)
Angiotensin I	CHCA	0.72 ± 0.09	3.80 ± 0.68
	DHB	1.75 ± 0.62	2.58 ± 0.92
Angiotensin II	CHCA	0.85 ± 0.21	3.93 ± 1.41
	DHB	1.83 ± 0.18	1.63 ± 0.22
β -Amyloid (1-15)	CHCA	2.21 ± 0.82	1.82 ± 0.31
	DHB	1.17 ± 0.30	4.04 ± 1.28
All three peptides	CHCA	1.26 ± 0.47	3.18 ± 0.68
	DHB	1.58 ± 0.21	2.75 ± 0.70

Table 2

The optimal exogenous concentrations and improvement factors for complex background experiment using CHCA matrix averaged over triplicate independent experiments. On average, the optimal exogenous concentration required for predicted maximum LOD improvement factor is close to 2 LOD_{orig} .

Peptide	Local maximum exogenous concentrations ($C/\text{LOD}_{\text{orig}}$)	Local maximum improvement factors ($\text{LOD}_{\text{orig}}/\text{LOD}_C$)
Angiotensin I	2.57 ± 0.31	3.05 ± 0.81
Angiotensin II	0.71 ± 0.31	1.85 ± 0.85
β -Amyloid (1-15)	3.15 ± 2.59	1.59 ± 0.48
All three peptides	2.14 ± 0.74	2.16 ± 0.45