Matrix assisted ionization mass spectrometry in targeted protein analysis – an initial evaluation

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Abstract

Rationale: Matrix assisted ionization (MAI) is a relatively new ionization technique for analysis by mass spectrometry (MS). The technique is simple and has been shown to be less influenced by matrix effects than e.g. electrospray (ESI) ionization. These features are of interest in targeted analysis of proteins from biological samples.

Methods: Targeted protein determination by MAI-MS was evaluated using a triple quadrupole mass analyzer equipped with a stripped nanoESI source in selected reaction monitoring (SRM) mode. The proteins were analyzed using the bottom-up approach with stable isotopic labeled peptides as internal standards (IS). The MAI matrix was 3-nitrobenzonitrile dissolved in acetonitrile. Aqueous sample and matrix solution were mixed in a 1:3 volume ratio. One

microlitre of the dried matrix/analyte sample was introduced into the mass spectrometer inlet where ionization commences.

Results: SRM settings established for ESI-SRM-MS of the peptides here investigated were applicable in MAI-SRM-MS for all evaluated peptides except one. Addition of IS provided efficient correction at most levels (relative standard deviation (RSD) ≤28% (except lowest digest level), $r^2 \geq 0.995$). This was also true for the more complex biological matrixes diluted urine (1:1; RSD=20% a synthetic peptide, NLLGLIEAK), and diluted digested serum (1:100; RSD=7% digested cytochrome C). Biological matrix influenced the signal intensity unless sufficiently diluted.

Conclusions: The results demonstrate that MAI-SRM-MS has promising potential in targeted protein determination by the bottom-up approach because of its simplicity, ease of use, and speed. However, more data is needed to confirm the results prior to application in a clinical setting.

Introduction

Ambient mass spectrometric techniques are gaining increased interest in analysis of macromolecules directly from their biological matrix requiring little or no sample clean-up.[1-3] These techniques have the potential to accelerate the current methodology for the analysis of biological matrices with regard to time consumption by eliminating many of the sample preparing steps required by conventional mass spectrometric methodologies. Techniques such as desorption electrospray ionization (DESI), and liquid extraction surface analysis (LESA) have already been applied in analysis of proteins directly from tissues and dried blood spots.[3-5] The techniques are mostly applied in analysis of intact proteins and often in combination with ion mobility separation.[5] In the last couple of years an additional direct ionization technique has

been introduced; the technique is called matrix assisted ionization (MAI) and can be applied both in vacuum and from ambient environments.[6-9] This ionization technique introduces the analyte co-crystallized with a matrix similar to matrix-assisted laser desorption/ionization (MALDI) except that it readily sublimes and does not require the use of a laser or high voltages. In contrast to MALDI, MAI produces multiply charged ions similar to electrospray ionization (ESI)[10] extending the mass range of the atmospheric pressure ionization mass spectrometers commonly used in laboratories and making the new ionization processes in principle readily accessible with minor source modifications.[11] MAI is also described to be more tolerant to salts than ESI,[6, 7, 12] this being an advantage when analyzing proteins from complex solution with minimal sample preparation. Mechanistic descriptions can be found elsewhere.[13-15]

The quantitative potential of MAI has previously been demonstrated for small molecule analytes showing linearity and sufficient reproducibility when using a labeled internal standard.[16] Furthermore, MAI has been applied to protein analyses, both intact proteins showing the typical charge state distributions and tryptic peptides in protein digests showing identification by MS/MS fragmentation, respectively.[7, 12, 17-20] However, to the author's knowledge no quantitative information is available for protein analyses.

Quantification of proteins in biological matrices is performed for several reasons e.g. in diagnosis and follow-up of treatment, drug discovery and doping analysis. Serum and plasma is the most prevalent matrices for analysis of protein biomarkers for diagnosis and treatment follow-up as well as for protein biopharmaceuticals in drug discovery, while both serum or plasma and urine is utilized as sample matrix in doping analysis. The relevant level is dependent of the analyte of interest and span from low pM (e.g. low abundance biomarkers such as for instance biomarkers for lung cancer,[21, 22] testicular and ovarian cancer[23, 24]) to medium to high µM (e.g. cardiovascular disease markers[25]). Quantification of proteins by MS is most often performed using the bottom-up approach after digestion of the proteins of interest into

peptides.[26-28] The tryptic peptide mixture is then analyzed and protein specific peptide(s), so-called proteotypic peptide(s), are used to indirectly quantify the protein. Triple quadrupole mass spectrometers are frequently used for targeted analysis of proteins by the bottom-up approach.[28, 29] To our knowledge, MAI has not been reported using a triple quadrupole mass analyzer.

Because MAI-MS has been shown to produce low background ions and less matrix effects compared to other ambient and direct ionization techniques, it is of interest to investigate MAI as an ionization technique for quantitative bottom-up protein analysis. In the current work the combination of MAI on a triple quadrupole mass spectrometer is evaluated.

Experimental

Chemicals, proteins and peptides

Dithiothreitiol (DTT), iodoacetic acid (IAA), tosyl phenylalanyl chloromethyl ketone (TPCK) treated trypsin from bovine pancreas, formic acid (MS grade), ammonium bicarbonate (ABC) and 3-nitrobenzonitrile (3-NBN) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A). LC-MS grade acetonitrile was purchased from Merck (Darmstadt, Germany).

The model proteins bovine serum albumin (BSA), cytochrome C (Cyt C) from bovine heart, myoglobin from equine heart, carbonic anhydrase II (CAII) from bovine erythrocyte, catalase from bovine liver and bovine transferrin, and the synthetic peptide AYPTPLR and its corresponding stable isotopic labeled peptide (SIL-peptide) AYPTPL[R_$^{13}C_6$_$^{15}N_4$] were also obtained from Sigma-Aldrich. The synthetic peptide NLLGLIEAK and the remainder SIL-peptides (TGPNLHGLFG[R_$^{13}C_6$_$^{15}N_4$], LFTGHPETLE[K_$^{13}C_6$_$^{15}N_2$], QSPVDIDT[K_$^{13}C_6$_$^{15}N_2$], NFSDVHPEYGS[R_$^{13}C_6$_$^{15}N_4$],

DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$], ELPDPQESIQ[R_$^{13}C_6$_$^{15}N_4$] and NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$]) were purchased from Innovagen (Lund, Sweden).

Human serum from healthy donors were obtained from Oslo University Hospital (Oslo, Norway). Urine was obtained from a healthy voluntary. Grade 1 water was obtained using a Milli-Q Integral 5 water purification system (Merck Millipore, Billerica, MA, U.S.A.).

Preparation of stock solutions

Stock solutions (5 mM) of each protein were prepared in water, and stored in freezer prior to use (-32 °C). Stock solutions (1 mM of AYPTPLR and AYPTPL[R_$^{13}C_6$_$^{15}N_4$], 10 mM of NLLGLIEAK and NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$], 0.61 mg/mL of DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$] and 1 mg/mL of the remainder SIL peptides) were prepared in pure water except DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$] which was prepared in water:acetonitrile (80:20)). The peptide stock solutions were diluted to 100 µM with water prior to use as spiking solution (peptide level in samples were 0.5-25 µM).

In solution digestion

In solution protein digestion was performed using a 1:40 ratio (w/w) of trypsin to protein. The proteins were digested individually at a level of 50 µM after dilution of the stock solutions with freshly prepared 50 mM ABC. Digestion was performed at 37 °C (at 800 rpm) overnight using a Thermomixer (Eppendorf®, Hamburg, Germany). For the samples that were reduced and alkylated prior to digestion, a volume of 10 µL of 90 mM DTT (reduction at 60 °C at 800 rpm for 15 min) and 10 µL 450 mM IAA (alkylation at 25 °C in dark at 600 rpm for 15 min) were used when digesting 100 µL of a 50 µM protein solution. Multiple digestions to determine digestion efficiency were not performed.

Preparation of urine samples

The urine samples was prepared by mixing undiluted urine with a solution containing NLLGLLIEAK and NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$], both 50 µM, in water in a 1:1 ratio.

Preparation of digested serum samples

Serum digestion was performed on 200 µL of unspiked serum by addition of 50 µL of trypsin beads. Digestion was performed overnight at 37 °C at 1450 rpm). The trypsin beads were prepared as previously described.[30] After preparation of the serum digest the Cyt C containing diluted serum samples were prepared mixing undiluted serum digest, serum digest diluted five times with water and serum digest diluted fifty times with water with in-solution digest of Cyt C (50 µM) and Cyt C internal standard (TGPNLHGLFG[R_$^{13}C_6$_$^{15}N_4$]; 10 µM) in a ratio of 2:1:1. This resulted in 1:1, 1:10 and 1:100 dilution of serum all containing 25 µM of digest and 5 µM of internal standard each.

Preparation of sample and introduction to the mass spectrometer inlet

MAI was performed using 3-NBN as matrix. 3-NBN was dissolved in acetonitrile (5 mg/50 µL) and mixed with the aqueous sample (digest/peptide and corresponding SIL-peptide) in a 3:1 volume ratio. For introduction of the sample to the mass spectrometer, 1 µL of the matrix:analyte mixture solution was withdrawn using a Hamilton syringe (Sigma-Aldrich), subsequently ejected to the tip of the syringe and briefly let to dry to form the matrix:analyte crystals, similar to previous work.[16] The matrix/analyte sample was ionized in the following manner: the syringe tip was placed approximately 1 mm from the inlet of the heated capillary of the mass spectrometer. The sub-atmospheric gas flow associated with the vacuum of the mass spectrometer was sufficient to heat and flow entrap the matrix:analyte sample crystallized outside the syringe tip into the heated inlet tube of the mass spectrometer where ionization commences.

Mass Spectrometer

The experiments were performed using a Thermo Scientific (Rockford, IL, U.S.A.) Quantum Access triple quadrupole equipped with a stripped Nanospray Flex^{TM} ESI source (Thermo Scientific) (Figure 1). The heated capillary was operated at 60 °C without ionizing voltage applied, similar to previous work.[7, 11] Data was collected by selected reaction monitoring using the transitions and collision energies described in Table 1. Argon was used for collision induced dissociation (CID) at a gas pressure of 1.5 mTorr (except for in analysis of NLLGLIEAK and NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$] where the CID gas pressure was 1.7 mTorr.

Results

Establishing MAI-SRM-MS conditions

Initial experiments were performed to establish MAI-SRM-MS conditions using the MAI matrix 3-NBN. We rationalized that if the analyte ions are formed promptly within the ion source region, the ESI-SRM-settings should be similar if not the same. To test our hypothesis, we evaluated if previously established ESI-SRM-MS-settings[22, 31, 32] could be directly applied to perform MAI-SRM-MS. Standard MAI conditions[11] were used: a matrix consisting of 3-NBN in acetonitrile, and peptides at 5 µM in water (volume ratio 3:1) without optimization using the syringe introduction.[16] The experiments were performed using synthetic peptide standards and SIL peptides (see Table 2). This was mainly to avoid the complicating factor of a digestion step.

Nine out of the ten peptides evaluated were detected, hence, the mass spectrometer settings established for ESI-SRM-MS are directly transferrable for the majority of the analytes for use with MAI-SRM-MS. The peptide that was not detected (DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$]) had the longest amino acid sequence (13 amino acids) and a high degree of hydrophobicity and, thus, was not soluble in pure water as the other peptides (DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$] was

dissolved in 80:20 water:acetonitrile). Optimization of both MAI and SRM conditions would be necessary for detection of this specific peptide.

Two peptides (AYPTPLR and NLLGLIEAK) were available as both non-labeled and $^{13}C/^{15}N$-labeled (SIL) peptide pairs. The MS/MS spectrum for the non-labeled and the labeled version were identical for both peptides. This is shown for one of the pairs (NLLGLIEAK and its SIL peptide) in Figure 2.

Initial quantitative data

Initial quantitative measurements were established using a synthetic peptide (NLLGLIEAK, signature peptide for ProGRP small-cell lung cancer marker) and its respective SIL peptide (NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$]) as IS. A synthetic peptide was used to avoid the possible confounding effect of digestion on the quantitative results.

A response curve was prepared using four levels of NLLGLIEAK (n=3) in the low µM-range (0.5-5 µM) while the SIL peptide was kept constant (2.5 µM). The samples were mixed with matrix in acetonitrile in a 1:3 volume ratio prior to introduction to the mass spectrometer inlet.

From this experiment, the internal standard correction was crucial for good quantitative data. Without IS correction the relative standard deviation (RSD) was between 18 and 43 % while IS correction resulted in RSD (%) ≤13 % for all four levels. IS correction also improved the correlation coefficient (R^2) from 0.9879 to 0.9992 as can be seen in Figure 3. The use of internal standards relates well with previous results using MAI for quantitation of drugs.[16]

The results from these studies indicate that sufficient quantitative quality for tryptic peptides can be obtained using MAI-SRM-MS. However, to be applicable in a real setting the data must be obtained for tryptic peptides obtained by protein digestion and not only using synthetic peptides.

MAI-SRM-MS of digested proteins

In-solution digestion was performed on a set of non-human proteins (Cyt C, myoglobin, CAII, catalase and transferrin). BSA was not included in the further study as its corresponding SIL peptide (DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$]) was not observed in the initial experiments (see Table 2). The specific digestion was performed without a preceding reduction and alkylation step.

After enzymatic digestion, the proteotypic peptide was observed for Cyt C, CAII and catalase, i.e. for three of the five digested proteins. Since the corresponding SIL-peptide previously had been detected for all five peptides (Table 2), the lack of detection of the proteotypic peptides was likely a result of insufficient digestion. One reason for a too low digestion output may be the lack of a reduction and alkylation step prior to digestion.[33] As a simple precautionary measure, initially this step was left out to avoid introduction of the DTT and IAA into the mass spectrometer during direct injection MAI-MS using the syringe method. However, to evaluate if inclusion of the reduction and alkylation step increased the digestion efficiency Cyt C and CAII was digested both with and without a preceding reduction and alkylation step and both digests were analyzed by MAI-MS. For these two proteins, the presence of reduction and alkylation agents, although these samples must have contained DTT and IAA and must have entered the mass spectrometer inlet, did not seem to influence the signal intensity of the proteotypic peptides in either direction.

Linearity and repeatability of digested proteins

Linearity and repeatability was evaluated for Cyt C using TGPNLHGLFGR as surrogate peptide and IS correction by the corresponding SIL peptide. An increased variability was seen compared to synthetic peptide analysis. The correlation coefficient was still satisfactory

(R^2=0.995, 5-25 µM, four levels), however, the repeatability even with IS correction was insufficient at the lowest level (see Table 3).

Application to realistic samples

Two different realistic settings were evaluated: Detection of synthetic peptide spiked to urine and detection of proteotypic Cyt C peptide from digested Cyt C in digested serum.

The synthetic peptide NLLGLIEAK and its SIL peptide were spiked to urine at 25 µM (final concentration in urine), and the sample was diluted 1:1 with water prior to addition of 3-NBN matrix. The MS/MS spectrum is similar as for the aqueous standard (Figure 4). An RSD of 20 % was obtained (n=4, with IS correction).

In this first experiment involving a biological sample (also referred to as (biological) matrix), a pure standard was used in the less complex although salt containing biological matrix urine. From the latter results, it was of interest to evaluate the applicability in a more complex biological matrix such as serum. As the signal intensity of the synthetic peptide NLLGLIEAK in urine (diluted 1:1 with water) was significantly lower than in pure aqueous sample, three different dilutions of digested serum were evaluated: 1:1, 1:10 and 1:100 dilution with water. For the 1:1 dilution, no signal was observed for neither Cyt C proteotypic peptide nor for the SIL peptide. For the 1:10 dilution, a signal was observed but it was significantly lower than for the pure aqueous digest, and a large variation in the signal intensity was present for both tryptic peptide and the SIL peptide. For the 1:100 dilution the signal was comparable (or even higher) than for the pure aqueous digest (Figure 5), and the RSD was improved (7 %, n=5 and 22 %, n=6, respectively).

Discussion

This initial evaluation of quantitative performance was carried out using a Quantum access triple quadrupole (Thermo Scientific). Although the sensitivity of this rather old instrument (2008) cannot match the newer triple quadrupole systems, it was considered sufficient for obtaining initial information of the quantitative performance of MAI in targeted analysis of proteins using the bottom-up approach. The levels of proteins applied in this work correspond to the levels of medium to high abundant proteins in human serum (high nM to low µM-level). Several relevant biomarkers are present at these levels in healthy humans (e.g apolipoproteins (cardiovascular disease biomarkers)[25] and ceruloplasmin (Wilson's disease)[34], and lower levels should be possible to reach by analyzing the samples using a newer mass spectrometer than the one used in the present study. Improvements in matrix:analyte transfer into the sub-atmospheric pressure of the mass spectrometer could potentially also assist in sensitivity gain.[35]

MS/MS transitions and collision energies originally optimized for ESI-SRM-MS were applied and the results demonstrate that these parameters seem to be feasible for most peptides, most likely due to the ESI like formation of multiple charged ions produced by MAI. Thus, this makes for simple conversions from already developed ESI methods into MAI-SRM-MS methods.

Two major concerns in using MAI for quantitative determination of proteins in complex biological matrices are relatively high variability in sample introduction in MAI-MS, and how to ensure sufficient digestion efficiency. The latter is crucial in order to obtain sufficiently low detection and quantification limits. The use of SIL peptide as IS was shown to be crucial for good quantitative data, although the correction was less efficient for the digested samples compared to the synthetic peptide samples. This was expected, as the internal standard did not correct for the digestion step. In addition, the sample matrix was more complex, containing other Cyt C peptides as well as trypsin autolysis peptides and the remainder of intact trypsin, which all may influence the analysis. From peptide in water to peptide in urine, the variability

increased but was comparable to what was seen for the digested Cyt C (see Table 3). This means that despite a relatively complex biological matrix it was possible to obtain quantitative results using internal standards. However, as the signal intensity of the target peptides in urine (diluted 1:1 with water) was significantly lower than for aqueous samples, the salts and other components of urine may have influenced the ionization efficiency, and further dilution might be necessary to circumvent this effect.

A similar effect was seen for digested Cyt C in serum; no signal was observed for the digested serum diluted 1:1 prior to MAI, while low abundant ions were observed for the digested serum diluted 1:10. However, further dilution (1:100) eliminated this negative effect: In other words when analyzing a spiked 1:100 dilution of the digested serum sample comparable or higher signal intensities of digested Cyt C and the internal standard relative to the aqueous samples were obtained (see Figure 5). In addition, better repeatability than for pure aqueous samples was seen indicating possibilities for development of methods with sufficient robustness for a clinical application.

The reason for the somewhat higher signals and improved repeatability for the more diluted biological matrices (1:100 dilution) compared to aqueous samples is unknown, but the most likely reason is that proteins present in serum dynamically coat the walls of the tubes and pipettes used resulting in less loss of target protein during sample preparation. Addition of small amounts of serum to aqueous protein standards has previously been applied with great success to minimize non-specific binding of the large peptide insulin by addition of 0.05 % plasma to aqueous standards.[36, 37] In addition higher signals and improved repeatability with more diluted biological matrices also relates well with previous results. [6] Higher dilution can be expected to reduce sample related contamination to the inlet of the mass spectrometer.

Cyt C is also present in human serum but the levels are in the pM-range[38] and would not be detectable using the current analytical approach.

Another factor that most likely will improve the reproducibility and sensitivity even further is the introduction of the newly developed automated platform for MAI (Ionique from MS^{TM}, LLC, Newark, DE, U.S.A). Automated sample introduction will provide a more precise matrix:analyte sample introduction step to the inlet of the mass spectrometer, which in turn is anticipated to improve the reproducibility of the signal intensity.[39]

With respect to digestion and digestion efficiency in complex biological matrices, this remains a challenge. The most efficient approach is to reduce the sample complexity either by depletion of high abundant proteins or, even more efficient, by isolation of the protein or proteins of interest using generic or selective extraction methods (i.e. solid-phase extraction or immunoaffinity extraction). The different strategies available for this should hence be evaluated in combination with MAI.

Conclusions

Overall, this initial evaluation demonstrates that MAI-MS may have a potential in targeted determination of proteins directly from biological matrices. By diluting serum, it is possible to determine a digested protein with sufficient repeatability without any other sample pretreatment than protein digestion, however further optimization will be necessary before application in a clinical setting.

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Figure captions

Figure 1. Photograph of the commercial Nanosource Flex^{TM} ion source: (A) unmodified (B) after stripping providing direct access to the mass spectrometer inlet to perform MAI-MS. Briefly, 3-NBN matrix dissolved in acetonitrile and aqueous analyte solution are combined in a 1:3 volume ratio and 1 µL, preferably crystallized outside the syringe tip, is brought in close proximity of the mass spectrometer inlet where the sample is entrapped into the sub-atmospheric pressure where ionization commences.[13]

Figure 2. MS/MS spectrum of NLLGLIEAK and NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$] at 2.5 µM in water.

Figure 3. Linearity of NLLGLIEAK without (A) and with (B) internal standard correction.

Figure 4. MS/MS spectrum of NLLGLIEAK and NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$] at 25 µM in urine diluted 1:1 with water.

Figure 5. MS/MS spectrum of TGPNLHGLFGR from digested cytochrome C (25 µM) and TGPNLHGLFG[R_$^{13}C_6$_$^{15}N_4$] at 5 µM in digested diluted serum (A-C) and aqueous media (D). Digested serum was diluted 1:1 (A), 1:10 (B), and 1:100 (C) with water. MS/MS transitions: TGPNLHGLFGR: *m*/*z* 584.8 → *m*/*z* 505.9; 549.3; 686.4 and TGPNLHGLFG[R_$^{13}C_6$_$^{15}N_4$]: *m*/*z* 589.8 → *m*/*z* 510.9; 559.3; 696.4.

Table 1. Overview of transitions and collision energies for the tryptic peptides and SIL peptides used for MAI-SRM-MS, in analogy to ESI-SRM-MS. [22, 31-32]

Peptide	Precursor *m/z*	Product ions *m/z*	Collision energy (eV)
TGPNLHGLFGR	584.8	505.9; 549.3; 686.4	20
TGPNLHGLFG[R_$^{13}C_6$_$^{15}N_4$]	589.8	510.9; 559.3; 696.4	20
LFTGHPETLEK	636.3	716.4; 910.5; 1011.5	20
LFTGHPETLE[K_$^{13}C_6$_$^{15}N_2$]	640.3	724.4; 918.5; 1019.5	20
QSPVDIDTK	501.8	394.4; 591.3: 787.4	20
QSPVDIDT[K_$^{13}C_6$_$^{15}N_2$]	505.8	398.4; 599.3; 795.4	20
NFSDVHPEYGSR	704.3	708.4; 845.5; 944.	20
NFSDVHPEYGS[R_$^{13}C_6$_$^{15}N_4$]	709.3	718.4; 855.4; 954.5	20
DAFLGSFLYEYSR	784.4	334.3; 717.6; 1121.6	20
DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$]	789.4	727.3; 1131.5	20
ELPDPQESIQR	656.3	429.4; 535.4; 857.4	20
ELPDPQESIQ[R_$^{13}C_6$_$^{15}N_4$]	661.3	434.4; 540.4; 867.4	20
NLLGLIEAK	485.8	630.3; 743.2	15; 16
NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$]	489.9	638.4; 751.4	15; 16
AYPTPLR	409.3	583.4	20
AYPTPL[R_$^{13}C_6$_$^{15}N_4$]	414.3	593.4	20

Table 2. Overview of proteins and peptide standards used in this MAI-SRM-MS study.

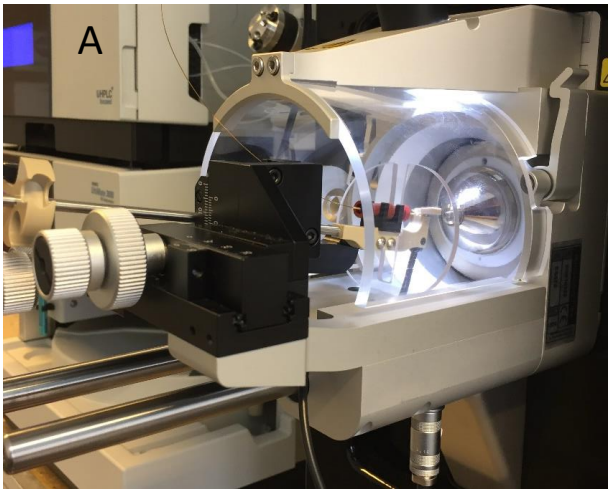
Proteins[a]	**Molecular mass (kDa)**	**Proteotypic peptide**	**SIL peptide**	**Synthetic peptide observed**	**SIL peptide observed**
Cytochrome C (Cyt C)	11.7	TGPNLHGLFGR	TGPNLHGLFG[R_$^{13}C_6$_$^{15}N_4$]	NA	+
Myoglobin	17.1	LFTGHPETLEK	LFTGHPETLE[K_$^{13}C_6$_$^{15}N_2$]	NA	+
Carbonic anhydrase II (CAII)	29.1	QSPVDIDTK	QSPVDIDT[K_$^{13}C_6$_$^{15}N_2$]	NA	+
Catalase	59.9	NFSDVHPEYGSR	NFSDVHPEYGS[R_$^{13}C_6$_$^{15}N_4$]	NA	+
Serum albumin (BSA)	66.4	DAFLGSFLYEYSR	DAFLGSFLYEYS[R_$^{13}C_6$_$^{15}N_4$]	NA	-
Transferrin	77.8	ELPDPQESIQR	ELPDPQESIQ[R_$^{13}C_6$_$^{15}N_4$]	NA	+
Progastrin releasing peptide (ProGRP)	13.7[b]	NLLGLIEAK	NLLGLIEA[K_$^{13}C_6$_$^{15}N_2$]	+	+
Chorionic gonadotropin alpha subunit (hCGα)	14.0	AYPTPLR	AYPTPL[R_$^{13}C_6$_$^{15}N_4$]	+	+

[a]Origin see experimental [b]Molecular mass of ProGRP isoform 1; NA: Not available as synthetic peptide

Table 3. Repeatability of Cyt C digest at different levels both without and with internal standard correction (n=8)

Concentration (µM)	RSD (%) w/o IS correction	RSD (%) w/IS correction
5	87	55
10	85	28
15[a]	67	19
25[b]	56	22

[a]n=10; [b]n=6; w/o: without; w: with



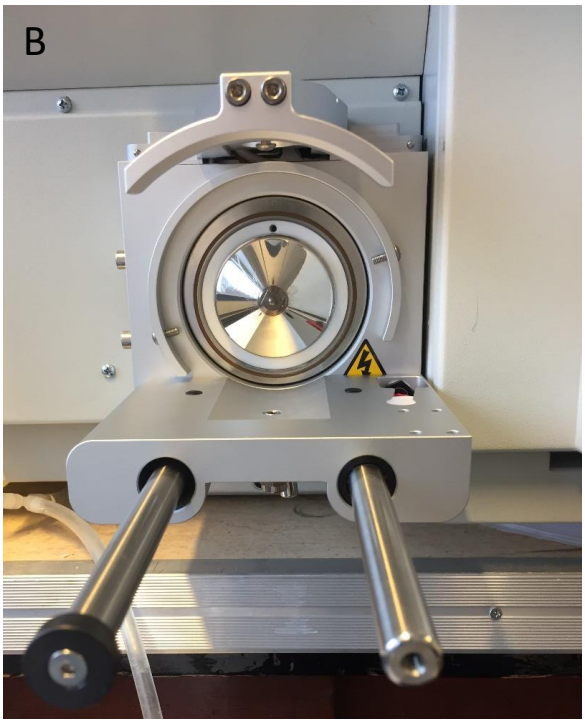


Figure 1

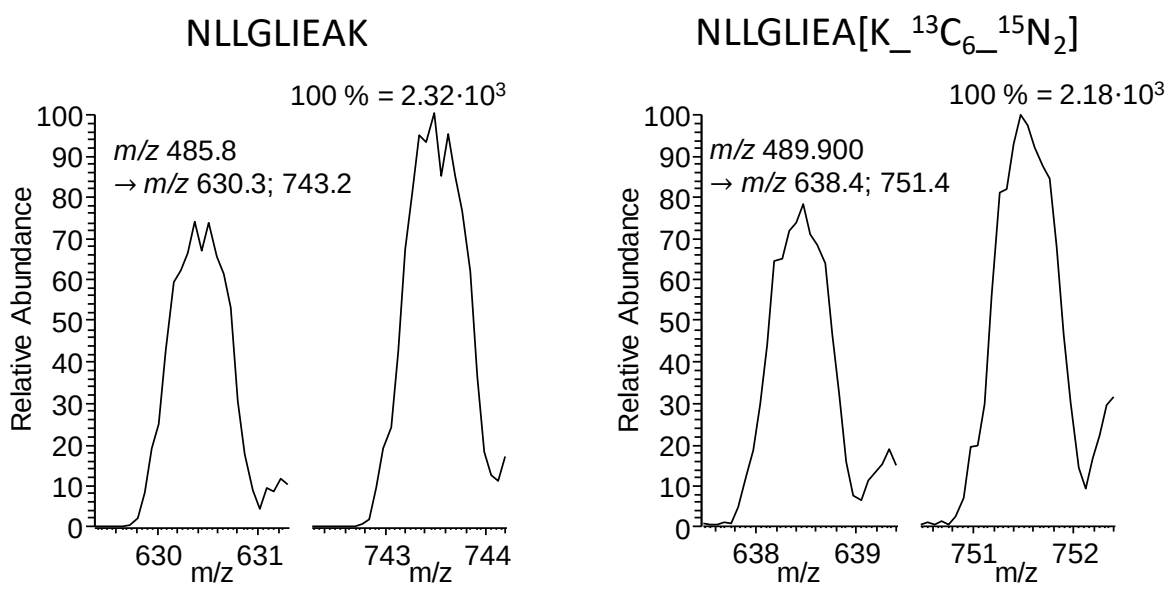

NLLGLIEAK
100 % = 2.32·10^3
m/z 485.8
→ m/z 630.3; 743.2
Relative Abundance
m/z
NLLGLIEA[K_13C6_15N2]
100 % = 2.18·10^3
m/z 489.900
→ m/z 638.4; 751.4
Relative Abundance
m/z

Figure 2

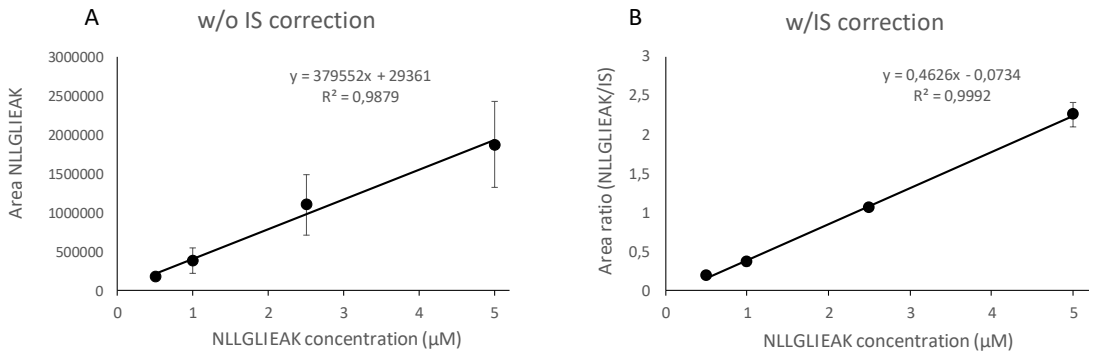

A
w/o IS correction
y = 379552x + 29361
R² = 0,9879
Area NLLGLIEAK
NLLGLIEAK concentration (µM)
B
w/IS correction
y = 0,4626x - 0,0734
R² = 0,9992
Area ratio (NLLGLIEAK/IS)
NLLGLIEAK concentration (µM)

Figure 3

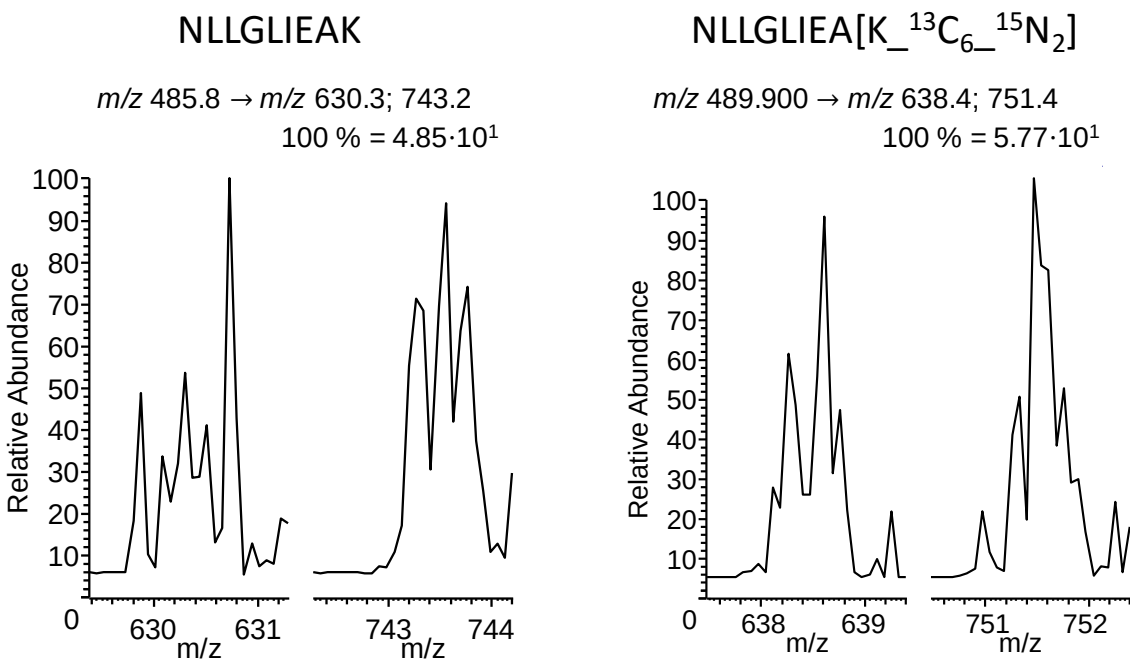

NLLGLIEAK
m/z 485.8 → m/z 630.3; 743.2
100 % = 4.85·10¹
Relative Abundance
m/z
NLLGLIEA[K_¹³C₆_¹⁵N₂]
m/z 489.900 → m/z 638.4; 751.4
100 % = 5.77·10¹
Relative Abundance
m/z

Figure 4

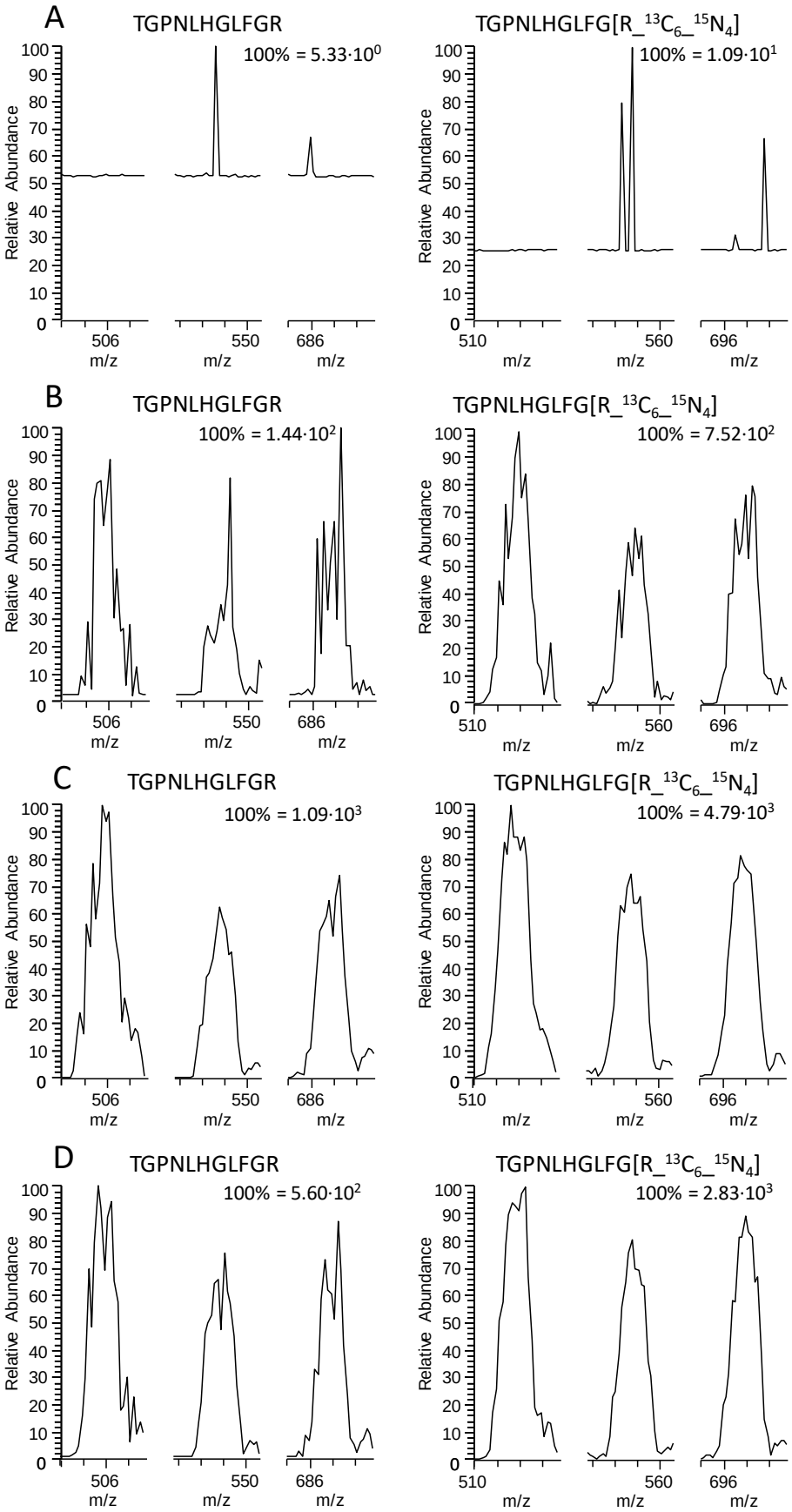

A
TGPNLHGLFGR
100% = 5.33·10^0
TGPNLHGLFG[R_13C6_15N4]
100% = 1.09·10^1
B
TGPNLHGLFGR
100% = 1.44·10^2
TGPNLHGLFG[R_13C6_15N4]
100% = 7.52·10^2
C
TGPNLHGLFGR
100% = 1.09·10^3
TGPNLHGLFG[R_13C6_15N4]
100% = 4.79·10^3
D
TGPNLHGLFGR
100% = 5.60·10^2
TGPNLHGLFG[R_13C6_15N4]
100% = 2.83·10^3
Relative Abundance
m/z
506
550
686
510
560
696

Figure 5