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Quantification of CP4 EPSPS in Genetically Modified *Nicotiana Tabacum* Leaves by LC-MS/MS with ^{18}O-Labelling

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Abstract

The CP4 EPSPS gene is widely used in herbicide-tolerant crops/plants all over the world. In this study, a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated to quantify the amount of CP4 EPSPS expression in *Nicotiana tabacum* leaves. The quantification of protein was converted to measure the unique peptides of CP4 EPSPS protein. Two peptides unique to CP4 EPSPS were synthesized and labelled in $H_2{}^{18}O$ to give ^{18}O stable isotope labelled peptides served as internal standards. The validated method resulted in good specificity and linearity. The intra- and inter- day precisions and accuracy for all samples were satisfactory. The results demonstrated that the novel method was sensitive and selective to quantify CP4 EPSPS in the crude extract without time-consuming pre-separation or the purification procedures.

Key Words: CP4 EPSPS; absolute quantification; ^{18}O-labelling; MRM

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Introduction

5-Enolpyruvylshikimate-3-phosphate (EPSP) synthase (EC 2.5.1.19) is inhibited by the broad-spectrum herbicide glyphosate, which was found by Ahmed in 1969.[1] EPSP is the key enzyme catalyzing the penultimate step of the shikimate pathway toward the biosynthesis of aromatic amino acids.[2] Expression of CP4 EPSPS results in glyphosate-tolerant crops, enabling more effective weed control by allowing post-emergent herbicide application.[3] As a result, CP4 EPSPS gene is widely used in genetically modified crops/plants, such plants are marketed under the trade name Roundup Ready (RR) (Monsanto Co., St. Louis, MO). RR soybeans contain four 5-enol-pyruvyl-shikimate-3-phosphate synthase genes from *Agrobacterium sp.* CP4 (CP4 EPSPS).[4] Consequently, quantitative techniques that facilitate detection of the expreesion amount of CP4 EPSPS protein in genetically modified crops/plants are required.

The quantitative methodology of the transgenic proteins in crops/plants has become one of the most exciting topics of research in recent years. Either Real Time- or Reverse Transcription Polymerase Chain Reaction (RT-PCR) for quantification of CP4 EPSPS on DNA or RNA level were involved in conventional procedures.[5-8] The reliability of PCR methodologies depends on the integrity of the DNA, which can be degraded by heat or low pH. Immunological assays are alternative methods, such as western blotting and enzyme-linked immunosorbent assay (ELISA), which quantify genetically modified crops/plants on protein level.[9] However, getting a suitable antibody for each target genetically modified protein is challenging. What’s more,

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there are limits of applying these methods to highly processed crop products because of the effect on food processing. In addition, immunological methods might suffer from non-specific binding and cross contamination, which might reduce the accuracy of the quantitative method.

Recently, Mass Spectrometry with multiple-reaction monitoring (MRM) strategy was introduced into detection of target proteins and their modification from cell or tissue lysates on peptide level, which could provide a higher precision and sensitivity than quantification of proteins themselves directly.[10-12] For example, it was recently shown that stable isotope labelling strategies were applied for the quantification of CP4 EPSPS in genetically modified soya.[13] In that work, stable isotope-labelled peptide was used as an internal standard which was needed to be synthesized by the time-consuming *Fmoc strategy* using an expensive isotope-labelled amino acid. With the development of stable isotope labelling, ^{18}O-labelling has become increasingly popular and widely practiced because of its simplicity, low cost and good reproducibility.[14] Stable isotope labelled peptides are able to serve as internal standards in analytical methods after confirming the stability of labelling efficiency.

In the present study, a sensitive and precise method was developed and validated to quantify CP4 EPSPS in genetically modified *N. tabacum* leaves by high performance liquid chromatography coupled with electrospray ionization triple quadruple mass spectrometry (HPLC-ESI-Triple Quatrople MS) with ^{18}O-labelling. In this method, the ^{18}O-labelling technique was applied for the preparation of peptides which are unique to CP4 EPSPS as internal standards, and MRM mode in mass

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spectrometry was used for quantification of the unique peptides of CP4 EPSPS and the corresponding ^{18}O-labelled peptides in the complex mixture to promote the selectivity and specificity. *N. tabacum* was used as a model plant, which is widely used in agriculture field for genetically modified crop/plant study.[15] It not only offers a novel method for the accurate quantification of CP4 EPSPS in genetically modified *N. tabacum*, but also has an important reference value for quantification of the absolute amount of target genetically modified proteins in other crops/plants.

Methods and Materials

Chemicals and Reagents

Urea, dithiothreitol (DTT), iodoacetamide (IAA), NH_4HCO_3, KH_2PO_4 and K_2HPO_4 were purchased from Sigma-Aldrich (Steinheim, Germany). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI, USA). HPLC-grade formic acid (FA) and acetonitrile (ACN) were purchased from Fisher Scientific (Edmonton, Canada). Water was obtained from a Millipore Milli-Q Plus purification system (Bedford, MA, USA). CP4 EPSPS was expressed and supplied by the Chinese Academy of Agricultural Sciences (CAAS, Beijing, China). Two synthesized peptides (IK, ITGLLEGEDVINTGK and LR, LAGGEDVADLR) with purities of 95.52 % and 98.76 %, assessed by MALDI-TOF MS and HPLC were obtained from Beijing SBS Genetech Co., Ltd (Beijing, China). $H_2{}^{18}O$ (purity > 97 %) was supplied by Cambridge Isotope Laboratories (Massachusetts, USA).

Sample Preparation

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N. tabacum (both genetically modified and non-genetically modified plants) were provided by CAAS. The *N. tabacum* leaf (300–500 mg) was first weighed and sliced into small pieces, which was then kept in a 2 mL EP tube. The extraction buffer (Tris-HCl, pH 7.6) was added, and the ratio of *N. tabacum* leaf to extraction buffer was fixed to 1 (mg) to 2 (μL). The leaf was fully ground by a grind rod in extraction buffer directly and then underwent ultrasonic extraction for 2 h. The homogenate was centrifuged at 17,000 × g for 30 min and the supernatant was collected. The protein concentration of the supernatant was determined by Bradford assay. Each sample was denatured and reduced by adding a solution (0.1 μL solution/ mg protein) containing 8 M urea and 10 mM DTT in 50 mM NH_4HCO_3 buffer (pH 8.3) at 37 °C for 4 h. Alkylation was performed in a 50 mM IAA (IAA:DTT = 5:1, *n/n*) at room temperature for 1 h in the dark. After alkylation, the sample was diluted using a 50 mM NH_4HCO_3 buffer to give a final urea concentration of 1 M. Tryptic digestion was then performed at a trypsin-to-protein ratio of 1:50 (*w/w*) for 20 h at 37 °C. The trypsin remaining in the sample was deactivated by boiling water bath for 10 min and the addition of 1% (v/v) FA.

Preparation of Internal Standards

Stock solutions of peptide IK and LR were mixed with 50 μL of 50 mM KH_2PO_4–K_2HPO_4 buffer (pH 4) and then lyophilized. The dry mixture was resuspended in 40 μL $H_2{}^{18}O$, and then 10 μL trypsin (0.1 μg/μL trypsin dissolved in $H_2{}^{18}O$) was added for the digestion of proteins. This solution was further incubated at 37 °C for 20 h. After the reaction finished, trypsin remaining in the solution was

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deactivated by boiling water bath for 10 min and the addition of 1 % FA.

HPLC-ESI-Triple Quatrople MS/MS analysis

Agilent 1290 series HPLC system was directly coupled to an Agilent 6460 Series Triple Quatrople MS. The separation was achieved on an analytical column (SB-C18, 1.8 μm, 150 mm × 2.1 mm) using a mobile phase that consisted of 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B) with the following gradient program: 5% B at 0–5.0 min; 5% B → 20% B at 5.1–7.0 min; 20% B → 30% B at 7.1–20.0 min; 30% B→5% B at 20.1–25.0 min; and 5% B at 25.1–30.0 min. The flow rate was 0.15 mL/min; the injection volume was 1 μL.

The triple quatrople ionization mode was positive electospray and MRM scan type was selected. The nebulizer pressure 35 psi. Drying gas flow and temperature was 7 L/min 300 °C, respectively. Sheath gas flow and temperature was 11 L/min and 250 °C, respectively. The capillary voltage was -3.5 kV. Dwell time of each transition was 50 ms, the fragmentor and collision energy were optimized for each unique peptide. Cell accelerator voltage and Delta EMV were 7 V and 1 kV, respectively. The mass resolution was unit (0.6 *m/z*) for both Q1 and Q3.

Quantification of CP4 EPSPS in *N. tabacum* leaves by LC-MRM-MS

The proteins extracted from genetically- or non-genetically modified *N. tabacum* leaves were digested, added with 10 nM ^{18}O-labelled peptides as internal standards and quantified by LC-MRM-MS.

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Results and Discussions

Selection of unique peptides to CP4 EPSPS

The digested peptides from CP4 EPSPS were analyzed by LC-ESI-Ion Trap MS/MS, and then the MS/MS data was searched against a SwissProt database (updated on July 1st, 2011) using Mascot search engine (version 2.2) with the following parameters: taxonomy, *Viridiplantae*; fixed modification, carbamidomethyl; enzyme, trypsin; precursor tolerance, 2 Da; MS/MS tolerance, 0.8 Da; maximum number of missing cleavages, 0. CP4 EPSPS protein sequence was added into the Zea mays database manually. The CP4 EPSPS score was 979 and 20 candidate peptides were identified. To select the unique peptides to CP4 EPSPS, the matched peptides in Mascot need to be searched through BLAST to ensure no homology in *Viridiplantae*. In addition, candidate unique peptides for quantification should also abide by principles as previously discribed.[16] Brifely, selected peptides should be: 1) unique to CP4 EPSPS; 2) with high MS intensity and ionization efficiency; 3) without unstable amino acids and missed tryptic cleavage site; 4) could be synthesized easily. Finally, two peptides with the sequence of ITGLLEGEDVINTGK (IK) and LAGGEDVADLR (LR) were selected as unique to CP4 EPSPS and purchased as authentic materials. The chromatograms and Mass Spectra of two unique peptides are shown in Figure 1A-C. Figure 2 shows MS/MS spectra of peptide LR and IK, two y ions of each peptide with highest intensities would be selected to be target product ions in MRM detection.

Efficiency of Tryptic Digestion

In this work, the quantification of CP4 EPSPS protein was related to the

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quantification of the amount of its unique peptides, which ultimately reflect the amount of the target protein. The possible explaination to when CP4 EPSPS protein was completely digested. As a result, the different ratio of trypsin to protein and digestion time were optimized to obtain the highest digestion efficiency.

The relationship between trypsin amount and digestion efficiency was investigated. As shown in Figure 3A, there was no significant difference between different ratios of trypsin to protein ratio of 1:10, 1:25 and 1:50 (w/w). In order to save the amount of trypsin, the optimized trypsin to protein ratio was selected as 1:50 (w/w). In Figure 3B, the horizontal axis represents different digestion time and the longitudinal axis representing labelling efficiency. It can be seen that the digestion reached its saturation or a plateau at 16 h. Finally, 20 h digestion was chosen to digest the proteins extracted from the biological sample in order to ensure the fully digestion.

^{18}O-Labelling Efficiency and ^{18}O-^{16}O Back-exchange of Unique Peptides

^{18}O-labelling needs to be catalyzed by Serine enzymes (such as trypsin) in $H_2{}^{18}O$. Two ^{16}O atoms of the C-terminal Lysine or Arginine (-COOH) would be displaced by ^{18}O.[17] High labelling efficiency and no significant ^{18}O-^{16}O back-exchange of two unique peptides was the foundation of the accuracy and precision of the quantitative method.[18, 19] Two unique peptides were labelled in $H_2{}^{18}O$ at 37 °C with trypsin catalysis. The ^{18}O-labelled peptides were detected by HPLC-ESI-TOF MS to calculate the labelling efficiency. Labelling efficiency was calculated by the ratio between the peak area of ^{18}O-labelled peptides and the total area of ^{18}O-labelled peptides and

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^{16}O-labelled peptides.

The effect of urea concentration was involved in labelling quality, because urea could inhibit the ^{18}O-labelling activity of trypsin with changing protein structure. In this experiments, the final urea concentration less than 1 M was selected according to an optimized condition.[20] The pH value of the labelling buffer might be another factor of labelling quality. The relationship between buffer pH value (50 mM KH_2PO_4–K_2HPO_4 from pH 4 to 7) and the labelling efficiency was investigated. However, as shown in Figure 4, there was no significant difference among buffer with pH 4 to 7, and pH~4 was selected for further usage. The labelling reaction is a reversible chemical reaction. Double ^{18}O-labelled peptides with high labelling percentage were expected in this method. Due to the purity of $H_2{}^{18}O$ is 97%, the theoretical labelling percentage is: $97\%\times97\% = 94.09\%$. Finally, under the optimized condition, ^{18}O-labelling of two unique peptides was carried out by the optimized conditions, and labelling efficiency of IK and LR was 94.90% and ~100.00%, respectively.

To investigate the ^{18}O-^{16}O back-exchange of labelled peptides, the ^{18}O-labelled peptides were mixed with $H_2{}^{16}O$ and stored at different conditions (Table 1). It can be seen that the labelling efficiency had no significant decrease except stored at 4 °C for 10 days, which meant that the deactivation of trypsin was successful by boiling in water and FA. Based on the optimized conditions, two unique peptides have high efficiency in $H_2{}^{18}O$ and ^{18}O-labelled peptides have no significant back-exchange from ^{18}O to ^{16}O in $H_2{}^{16}O$, which indicated that ^{18}O-labelled unique peptides IK and LR had

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satisfied conditions to be internal standards.

Optimization of MS Parameters

MRM mode in mass spectrometry was applied to quantify the unique peptides to CP4 EPSPS in order to improve the selectivity and specificity. In MRM mode, the fragmentor and collision energy are the most important parameters to the sensitivity of the assay. The transmission efficiency of precursor ions depends on fragmentor voltage, and collision energy is related to collision induced dissociation. As a result, these two parameters of each transition needs to be optimized to obtain the best signal to noise ratio for each peptide. In our experiment, the two most intense product ions (singly charge y-ions) were selected for the unlabelled and labelled peptides. The fragmentor and collosion energy were optimized for each transition of each peptide (Table 2). It can be seen that ^{18}O-labelled peptides (double charge) have 2 Da and 4 Da (single charge) mass shift in Q1 and Q3 compared with the unlabelled peptides.

Investigation of Extraction Buffer

Nine different protein extraction buffers were tested to maximize the protein amount by each extraction. Different kinds of buffers and also different pH values of a certain kind of buffer were tested to extract crude protein mixture from pieces of tobacco leaves. The results of protein extraction efficiency were summarized in Table 3, and it could be seen that the best extraction buffer was Tris-HCl (pH 7.6). And then, the extracted crude protein mixture was digested then checked by LC-MS, the result showed the target product ions could be detected clearly. Thus, Tris-HCl (pH 7.6) would be the extraction buffer for the further real samples' extraction.

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Method Validation

Linearity of calibration curves, LOD and LOQ

Non-genetically modified *N. tabacum* leaves were used for blank matrix since it cannot express the genetically modified protein CP4 EPSPS. The stock solution of each unique peptide was serially diluted to obtain a series of concentration added in non-genetically modified *N. tabacum* leaves. The calibration curve was obtained by the peak area ratio against the concentration ratio of variable unlabelled peptide to fixed ^{18}O-labelled peptide as internal standard. Regression analysis resulted in equation of y = ax + b, where y represents the ratio of peak areas of synthetic peptides to those of corresponding ^{18}O-labelled peptides and x represents the concentration ratios of synthetic peptides to corresponding ^{18}O-labelled peptides added to the digested peptides from non-genetically modified *N. tabacum* leaves as blank matrix. The limit of detection (LOD) and limit of quantification (LOQ) for each peptide was obtained based on the signal to noise ratio (SNR) as 5 and 15, respectively. As shown in Table 4, calibration curves of two peptides were linear over the range of 5–500 and 5–1000 fmol with correlation coefficient (r^2) of 0.9992–0.9996. The LOD was down to 1 fmol and 2 fmol for IK and LR peptides, respectively. The LOQ was 3 fmol and 4 fmol for IK and LR peptides, respectively.

Precision, Accuracy and Recovery

Quality control (QC) peptides were used to validate the precision and accuracy of the quantitative method. Three different QC concentration (5 fmol/μL for LQC, 10

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fmol/μL for MQC and 50 fmol/μL for HQC) were prepared by addition of the unlabelled unique peptide (IK and LR) solution to blank matrix. The intra- and inter-day precisions were evaluated by the relative standard deviation (RSD) of six replicate preparations on three different validation days at 3 different concentration levels for each QC peptide. The accuracy was assessed by the ratio of calculated concentration to actual concentration for each QC peptide, spiked into the blank matrix.

As shown in Table 5, the precision and accuracy (both intra-day and inter-day) of 5, 10 and 50 fmol/μL samples for peptide IK and LR were calculated, respectively. These results demonstrated that the quantitative method was accurate and precise, which achieves the standards for biological sample analysis. The investigation of recovery was performed by addition of known amount of IK and LR peptides to non-genetically mdified *N. tabacum* leaf samples. The resulting mixtures were assayed and the recoveries obtained in average were summarized in Table 6.

Quantification of CP4 EPSPS in genetically modified *N. tabacum* leaves

Twenty genetically modified *N. tabacum* plants and 10 non-genetically modified N. tabacum plants were given from CAAS. The amount of CP4 EPSPS extracted from genetically modified *N. tabacum* leaves was measured using LC-MS/MS and ^{18}O-labelled internal standard peptides. Due to peptide IK and LR are unique to CP4 EPSPS. As a result, The moles of peptides and protein CP4 EPSPS were the same. Based on our method, the concentration of peptides would be know from the calibration curve. And then, the amount of protein can be calculate according to the following formulae:

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$$m_{protein} = \frac{C_{peptide} \times V_{peptide} \times M_{protein}}{M_{peptide}} = \frac{C_{peptide} \times 47585.5}{M_{peptide\ IK\ or\ LR}}$$

The injection volume: $V_{protein} = 1\ \mu L$

The molecular weight of peptide IK: $M_{peptide\ IK} = 1558.8$

The molecular weight of peptide LR: $M_{peptide\ LR} = 1115.6$

The CP4 EPSPS in leaves from different genetically modified *N. tabacum* plants was detected and the concentration was 1.6–4.9 pg/mg fresh *N. tabacum* leaf. However, CP4 EPSPS cannot be detected in non-genetically modified *N. tabacum* plants.

Conclusions

In this work, a liquid chromatography multiple reaction monitoring tandem mass spectrometry method was developed and validated to quantify the CP4 EPSPS in genetically modified *N. tabacum* leaves. The novel method offered a high level of sensitivity, accuracy and precision. The discovery of unique peptides enabled quantification of these peptides instead of quantifying the protein itself. ^{18}O-labelling coupled with MRM strategy changed this originally relative quantitative method to absolute quantification. This method might be also applied to detection, identification and quantification of CP4 EPSPS in other genetically modified plants/crops.

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Dr. Yulin Deng is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

We acknowledge that no conflicts exist for all authors.

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Figure Legends

Figure 1: The chromatograms and Mass Spectra of two unique peptides: (A) The chromatograms of peptide IK and LR; (B) product ions of peptide LR; (C) product ions of peptide IK.

Figure 2: HPLC-ESI Ion Trap MS/MS analysis of the peptide mixture extracted and digested from *N. tabacum* leaf: A: MS/MS spectrum of peptide (ITGLLEGEDVINTGK) product ions (*m/z* = 932.4 and 1061.5) unique to CP4 EPSPS; B: MS/MS spectrum of peptide (LAGGEDVADLR) product ions (*m/z* = 931.5 and 1002.3) unique to CP4 EPSPS.

Figure 3: Investigation of digestion efficiency by the different trypsin to CP4 EPSPS protein ratio (A) and digestion time (B).

Figure 4. Investigation of digestion efficiency by the different pH value of the labelling buffer.

Tables

Table 1: ^{18}O-^{16}O Back-exchange of Two Unique Peptides.

Table 2: The Optimized Fragmentor and Collision Energy for Each Transition.

Table 3. The Investigation of Extraction Buffers.

Table 4. Calibration Curves of the Two Unique Peptides with Four Transitions.

Table 5. Precision and Accuracy by HPLC-ESI-MRM Analysis (n = 6).

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Tables

Table 1. ^{18}O-^{16}O Back-exchange of Two Unique Peptides

storage condition with $H_2{}^{16}O$	labelling efficiency (%)	
	IK	LR
original labelling efficiency	94.90	~ 100.00
- 80 °C for 7 days	95.18	~ 100.00
- 80 °C for 10 days	95.07	~ 100.00
4 °C for 7 days	94.47	~ 100.00
4 °C for 10 days	88.77	98.68

Table 2. The Optimized Fragmentor and Collision Energy for Each Transition

peptide	charge	Q1	Q3	fragmentor (V)	collision energy (eV)
IK	+2	780.4	932.4 (y9+)	145	28
			1061.5 (y10+)	145	26
IK-labelled[a]	+2	782.4	936.4 (y9+)	145	28
			1065.5 (y10+)	145	26
LR	+2	558.5	931.5 (y9+)	145	20
			1002.3 (y10+)	145	18
LR-labelled[a]	+2	560.5	935.5 (y9+)	145	20
			1006.3 (y10+)	145	18

[a] The two ^{16}O atoms in the carboxyl of lysine (K) or arginine (R) were substituted by two ^{18}O atoms.

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Table 3. The Investigation of Extraction Buffers

Entry	Extraction buffer	Extraction efficiency (%)[a]
1	50 mM Tris-HCl, pH 7.6	0.937
2	50 mM Tris-HCl, pH 6.8	0.856
3	50 mM NH_4HCO_3	0.820
4	100 mM PBS, pH 7.0	0.681
5	100 mM PBS, pH 6.0	0.651
6	100 mM PBS, pH 5.0	0.634
7	100 mM KH_2PO_4	0.576
8	H_2O	0.583
9	Na_2CO_3 + NaCl + H_2O	0.612

[a] Extraction efficiency was calculated by the ratio of extracted crude proteins to fresh *N. tabacum* leaf, w/w.

Table 4. Calibration Curves of the Two Unique Peptides with Four Transitions

Peptide	Transition	Equation	linear range (fmol)	r^2	LOD[a] (fmol)	LOQ[b] (fmol)
IK	780.4/1061.5	y = 1.0297x + 46.288	5-500	0.9994	1	3
	780.4/932.4	y = 2.7373x - 0.7665	5-500	0.9996	2	5
LR	558.5/931.5	y = 0.9054x + 4.7032	5-1000	0.9995	2	4
	558.5/1002.3	y = 0.7691x + 6.3659	5-1000	0.9992	2	4

[a] LOD: limit of detection, SNR = 5;

[b] LOQ: limit of quantitation, SNR = 15.

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Table 5. Precision and Accuracy by HPLC-ESI-MRM Analysis (n = 6)

Peptide	Transition	Conc. of QC sample (fmol/μL)	Precision (RSD %)		Accuracy (RE %)	
			intra-day	inter-day	intra-day	inter-day
IK	780.4 / 1061.5	5	1.33	10.21	2.87	12.11
		10	0.35	10.26	-2.39	-12.47
		50	0.21	10.75	-2.12	-4.38
	780.4 / 932.4	5	1.18	11.43	1.05	6.41
		10	0.42	5.79	-1.94	2.22
		50	1.84	9.03	2.41	4.55
LR	558.5 / 931.5	5	2.17	11.69	4.57	-7.91
		10	0.31	8.95	-1.69	-2.37
		50	1.16	9.76	5.66	1.70
	558.5 / 1002.3	5	1.59	11.33	-2.62	-8.14
		10	0.41	7.38	-8.80	-4.32
		50	0.30	6.96	4.36	3.65

Table 6. The mean recoveries of peptide IK and LR (n = 3)

Peptide	Transition	Conc. of QC sample (fmol/μL)	Recovery (%, mean ± SD)
IK	780.4 / 1061.5	5	93.7 ± 4.7
		10	92.8 ± 2.8
		50	94.1 ± 2.0
	780.4 / 932.4	5	93.2 ± 3.3
		10	96.3 ± 3.2
		50	92.5 ± 2.8
LR	558.5 / 931.5	5	91.3 ± 5.1
		10	93.7 ± 1.9
		50	92.8 ± 2.3
	558.5 / 1002.3	5	89.9 ± 6.9
		10	94.2 ± 2.6
		50	92.5 ± 3.5

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Fig. 1

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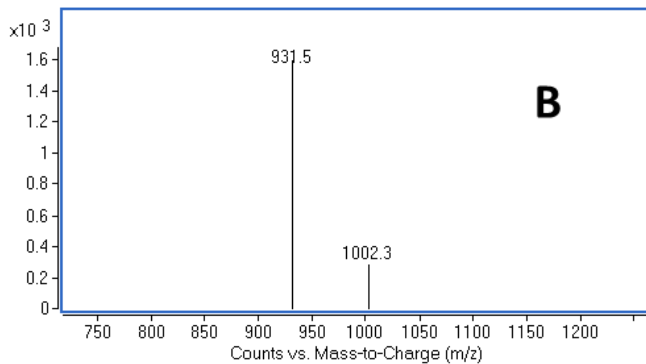
peptide LR

peptide IK

×10^6

Counts vs. Acquistion Time (min)



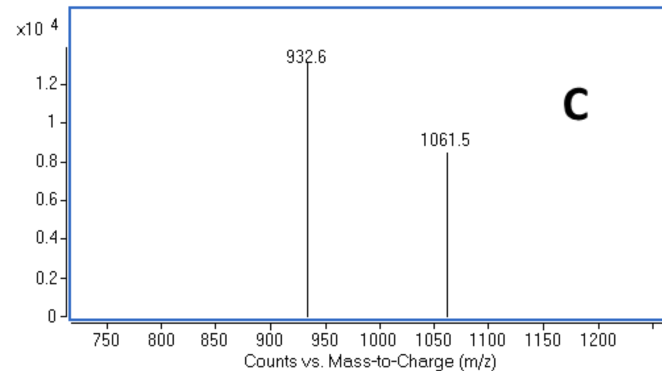


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Fig. 2

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Fig. 3

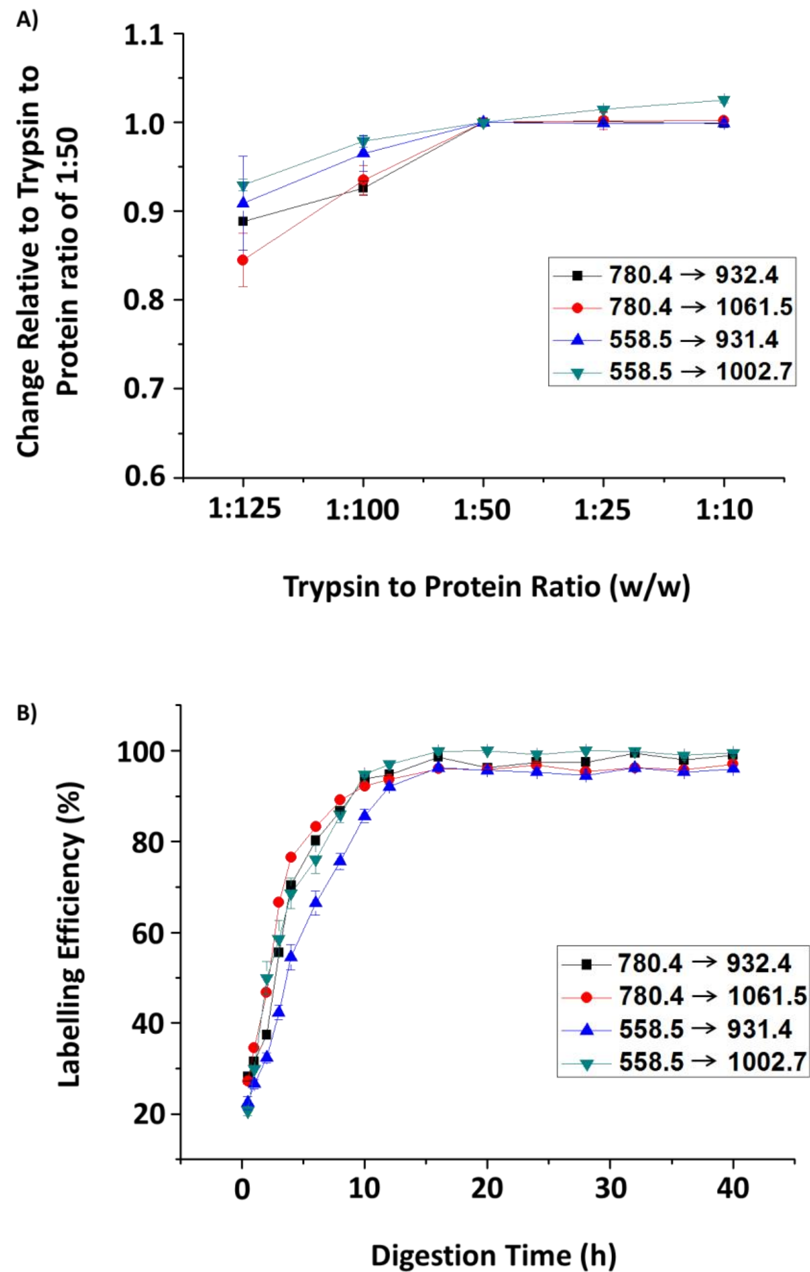

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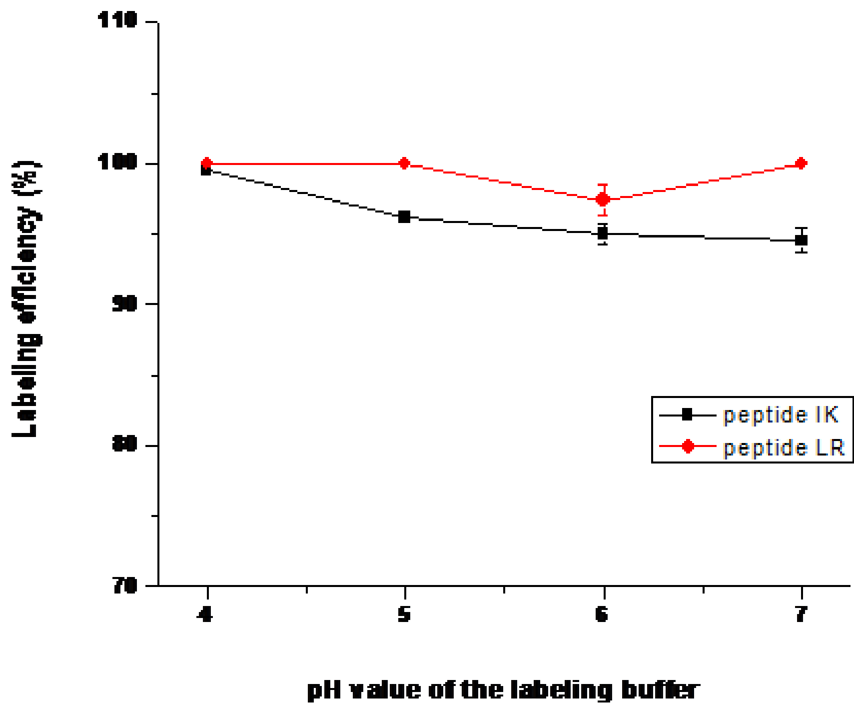
Fig. 4



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