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Multiplex Detection of Seven Staphylococcal Enterotoxins Using Liquid Chromatography–Mass Spectrometry Combined with a Novel Capture Molecule

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Abstract: Food poisoning caused by Staphylococcal enterotoxins (SEs) is prevalent globally, making efficient detection of these toxins very important. Traditionally, liquid chromatography–mass spectrometry required immunosorbent enrichment by magnetic bead-coupled antibodies obtained by animal-specific immunization. However, this method is time-consuming and costly. In this study, two recombinant protein capture molecules were designed based on the principle of toxins binding to Major Histocompatibility Complex (MHCII) and T cell receptor (TCR) molecules. The two capture molecules are called MHCII and MHCII-D10. The design of the MHCII and TCR-D10 was achieved through searching for the binding site protein sequence of Staphylococcal enterotoxins in the relevant literature, and MHCII-D10 was to link MHCII sequence with TCR-D10 sequence using linker (G4S)3 linking peptide. These capture molecules were shown to effectively bind to seven types of toxins and to capture SEs in various matrices. The digestion time, ratio, and temperature were further optimized, reducing the overall digestion time to just 2 h. The specificity, linearity, sensitivity, precision (RSD%), and recovery of the two methods were verified by liquid chromatography–mass spectrometry. When the MHCII and MHCII-D10 captured the toxins, the limit of quantification (LOD) in the 1 × PBS, plasma, and milk matrices ranged from 1.5625 to 100 fmol/μL, with the recovery rate ranging from 18.4% to 96%. The design of these capture molecules eliminates the need for animal-specific immunization, simplifying the pre-detection process and avoiding ethical concerns. This development holds significant promise for clinical diagnosis and reference.

Keywords: capture molecules; liquid chromatography–mass spectrometry; staphylococcal enterotoxins detection; major histocompatibility complex; T cell receptor

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1. Introduction

Staphylococcus aureus (*S. aureus*) is a common bacteria associated with food poisoning [1]. Indeed, *S. aureus* poses a global food safety problem, as the bacteria can contaminate a range of foods from processed meats and dairy products to salads and cooked food [2]. It typically poisons through ingestion or inhalation, with common symptoms including fever, chills, headache, myalgia, coughing, and difficulty breathing; severe cases may exhibit vomiting, diarrhea, or even multi-organ and systemic damage, as well as immune dysfunction [3].

Staphylococcal enterotoxins (SEs) primarily cause this poisoning effect. These enterotoxins are a type of SEs, a group of monomeric polypeptide chains comprising 238–258 amino

acids, with a molecular weight ranging from 26 to 34 kDa [4]. There are approximately 27 distinct serological types of SEs that exhibit structural, functional, and sequential similarities [5]. The most prevalent serotypes associated with food poisoning, due to their superantigen activity, are SEA, SEB, SEC, SED, SEE, SEF, SEG, and SEH [6].

SEs are not only major causes of foodborne illness caused by bacterial toxins [7], but they also risk becoming potent biological warfare agents. With atomization and inhalation toxicity, ready availability, easy preparation, high toxicity, and a lack of medical countermeasures [8], SEs can serve as effective potential biological warfare agents. Therefore, establishing an efficient method for detecting SEs is crucial for clinical diagnosis, disease prevention, and anti-biowarfare applications. Various detection methods are currently employed, including biodetection, immunological molecular biology, biosensor technology, and liquid chromatography–mass spectrometry [5]. Of these, liquid chromatography coupled with mass spectrometry (LC-MS/MS) represents a newly developed analytical method recommended by the designated laboratory of the International Organization for the Prohibition of Chemical Weapons (IOPCW) and commonly employed by the network laboratories of the United Nations Secretary-General's Investigative Mechanism (UNSGM). LC-MS/MS offers several advantages over other techniques, such as a high sensitivity, low sample requirements, a rapid analysis, and a straightforward operation [9,10].

The detection of SEs using LC-MS/MS involves bottom–up methods. These methods rely on immune capture techniques for detecting the toxins in a complex sample, with specific antibodies playing an important role. Conventionally, specific antibodies, in this case polyclonal or monoclonal antibodies, are obtained through animal immunization, resulting in a cumbersome, time-consuming, and costly process. There is some literature on the mechanisms of the MHCII and TCR interactions with SEs and their application to the diagnosis and treatment of SE poisoning. There is also some literature on the use of T cell receptor structural domains for capturing the ELISA detection of SEs [11,12]. In this paper, we designed two capture molecules aiming to replace the traditional antibodies used in the LC-MS/MS detection of SEs, thereby making detection more efficient. These new capture molecules were designed to make use of the superantigen mechanism of SEs binding to MHCII class molecules [13–15]. Our molecules, MHCII and MHCII-D10, proved capable of simultaneously adsorbing seven serotypes of toxins (A, B, C, D, E, G, and H). Compared with the traditional antibodies, the new capture molecules simplified the detection process, eliminating the need for the time-consuming immunization of animals to obtain the antibodies. The capture molecules could be quickly obtained by protein expression and purification alone. Moreover, only one capture molecule was required to capture all seven toxins. Compared with the complicated procedure of obtaining the traditional antibodies, the capture molecules could be obtained quickly, and the detection time was shortened, which is crucial for clinical diagnosis and rapid detection.

As a second aim of our study, we explored the optimal digestive conditions for the LC-MS/MS detection of SEs. The LC-MS/MS detection of protein toxins typically involves the trypsin digestion process, which usually takes between 18 and 24 h [16]. This long digestion time and low yield of the target peptide are challenges for efficient detection. Researchers have attempted to address these issues by developing various digestion protocols, including organic solvent-assisted or surfactant-assisted methods [17–20], microwave-assisted or ultrasound-assisted protocols [21–25], and pressure-assisted methods [26,27]. However, the existing literature suggests that the auxiliary effect of adding organic reagents to *S. aureus* enterotoxin digestion is not optimal [18]. Therefore, this study focused on optimizing the digestion temperature, time, and protein-to-trypsin ratio of SEs. The optimized digestion method can improve the yield of peptides, shorten the digestion time, and improve the detection efficiency [28].

2. Materials and Methods

2.1. Safety Precautions

SEs are highly toxic, so the safety rules for handling toxic contamination and the use of personal protective equipment were strictly observed. The SE-contaminated consumables and solutions were inactivated overnight with 2 M of NaOH.

2.2. Chemicals and Reagents

Acetonitrile (HPLC grade) and formic acid (HPLC grade) (>98%) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), and iodoacetamide (IAA) were purchased from Sigma (Roedermark, Germany). Sequencing grade modified trypsin was sourced from Promega Corporation (Madison, WI, USA). Ultrapure water was obtained from Wahaha pure water (Hangzhou, China). Dynabeads® M-270 Epoxy magnetic beads were obtained from Thermo Fisher Scientific (USA). The capture molecule MHCII-D10 is a designed sequence, which was synthesized by the GenScript company and purified by the recombinant protein. Synthetic heavy peptides with labeled Lys [13C6; 15N2] (+8 Da) and Arg [13C6; 15N4] (+10 Da) were synthesized by the GenScript company at a high purity grade. Semi-skimmed milk was purchased from local stores. The animal immune polyclonal rabbit antibody was commissioned by Beijing Protein Innovation Company. Sheep plasma was purchased from Solebol. The anti-*Staphylococcus aureus* Enterotoxins A + B + TSST-1 antibody ab190337) were purchased from Abcam Company. Imidazole ($C_3H_4N_2$), disodium hydrogen phosphate (Na_2HPO_4), dihydrogen phosphate (NaH_2PO_4), sodium chloride (NaCl), and sodium hydroxide were obtained from the China National Medicines Corporation Ltd. Urea, reduced glutathione, oxidized glutathione, arginine, and ethylene diamine tetraacetic acid (EDTA) were obtained from the China National Medicines Corporation Ltd. Phosphate-buffered saline (1 × PBS) and 1 M Tris-HCl were obtained from Solarbio.

2.3. Protein Sequence Information

We obtained the SE serotype sequences from Uniprot (http://www.uniprot.org). These serotypes were SEA, SEB, SEC, SED, SEE, SEH, and SEG. The full length of the seven enterotoxin sequences were 702 bp, 720 bp, 720 bp, 702 bp, 693 bp, 654 bp, and 702 bp, respectively. The protein sequences of the toxins were rearranged to remove the signal peptide. Two restriction sites of NcoI and XhoI, six His tags, and the restriction site R were added to the design. Then, the GenScript company added our designed recombinant protein sequence into the pET-28a(+) carrier plasmid to synthesize the glycerobacteria before carrying out the expression and transformation to produce the recombinant protein [29] The design of the recombinant capture molecules mainly relied on the antigenic peptide binding sites of the SEs and the MHCII and TCR molecules. Upon analysis, we determined that the binding part of the MHCII to the toxin was divided into the α1 chain domain. The α1 domain sequence 1–84 and the amino acid sequence of the MHCII were identified. MHCII-D10 capture molecules link MHCII and TCR-D10 antibodies via a (G4S)3 linker. The designed sequence was sent to the GenScript company, and our recombinant protein sequence was added to the pET-28a(+) carrier plasmid to synthesize glycerobacteria, which were then expressed and transformed into the recombinant protein. The sequence is shown in Figure S1.

2.4. Screening of Specific Marker Peptides for Staphylococcal aureus Enterotoxin

The amino acid sequences of the SEA (P0A0L1), SEB (P01552), SEC (P01553), SED (P20723), SEE (P12993), SEH (P0A0L9), and SEG (P0A0L8) were obtained from the UniProt database. The proteins were digested via an online theoretical digestion method, and the peptides were classified. DNAMAN 7.0 software was used to compare the sequences of the seven toxins and to screen the different peptides. As shown in Figure S2, the ACQUITY UPLC®I-Class-Xevo G2-XS Tof -MS/MS was used in a bottom–up approach to screen the

peptides with good signals, and the NCBI non-redundant protein database was used to search for specific peptides.

2.5. Induced Expression and Purification of the Target Protein

In total, 100 μL of the SE-engineered bacteria solution was added to two 5 mL of Luria–Bertani (LB) liquid mediums containing Kanamycin (Kana) and incubated at 180 rpm at 37 °C overnight. Then, 5 mL of the bacterial solution cultured overnight was added to 500 mL of the LB medium containing Kana, one of which was supplemented with Isopropyl β-D-Thiogalactoside (IPTG) inducer at a final concentration of 1 mM. The cultures were shaken at 180 rpm at 37 °C for 4 h until the OD_{600} reached between 0.6 and 0.8. Meanwhile, a blank control group was set up without the inducer.

The bacterial solutions of the induction group and the blank control group were then centrifuged at 8000 rpm for 20 min. After the centrifugation, the supernatant medium was discarded and the precipitation was re-suspended in 80 mL of 1 × PBS. After the suspension, the ultrasonic crushing precipitation was performed, followed by centrifugation at 8000 rpm for 20 min. After the centrifugation, the supernatant was separated from the precipitation, and the precipitation was suspended in 20 mL of 1 × PBS. The blank control group and the induced bacteria were tested with a 4–20% SDS-PAGE gel electrophoresis to verify whether the bacteria induced expression. For the gel electrophoresis, the voltage was set to 180 V, the current to 400 mA, and the run time to 40 min. The expressed strain was then expanded, and the target protein was purified with an affinity chromatography (HisTrap™HP column). After the centrifugation of the sonicated bacterial solution at 180 rpm for 20 min, the bacterial solution was filtered through a 0.45 mm flow-through membrane. The HisTrap™ HP column was first equilibrated with five times the column volume of the equilibration solution. The filtered sample was aspirated onto the column at a flow rate of 2 mL/min and purified according to the principle of a low concentration of imidazole for the binding and a high concentration elution. The equilibrium solution was a solution containing a low 19 nM concentration of imidazole ($C_3H_4N_2$), 39 μM of disodium hydrogen phosphate (Na_2HPO_4), 5.8 μM of sodium dihydrogen phosphate (NaH_2PO_4), and sodium chloride (NaCl), and the eluent was a high concentration equilibrium solution containing a high concentration of 500 μM of imidazole. The proteins were eluted in a gradient of 0–100% at a flow rate of 3 mL/min, and the eluted proteins were divided into 1–7 fractions. The proteins were collected as target proteins in 1–7 tubes. Then, the 4–20% SDS-PAGE gel electrophoresis was performed. The protein concentration was measured with a BCA by mixing the homogeneous proteins with a higher purity.

The recombinant MHCII and MHCII-D10 antibodies were expressed in inclusion bodies. Therefore, the culture medium was added and centrifuged at 8000 rpm for 20 min. After the separation, the precipitate was collected and the bacteria were suspended in 2.5 mL of 8 M urea–imidazole solution at 4 °C overnight. An ultrasonic disruption at 100% was performed by centrifugation at 8000 rpm for 20 min, and the supernatant was retained. The HisTrap™ HP column was first equilibrated with five times the column volume of the equilibration solution. The filtered sample was applied to the column and purified by the principle of a low concentration of imidazole for the binding and a high concentration elution. The equilibration solution was an imidazole-containing eluent, and the eluted proteins were collected as target proteins. After collecting the protein solution, the required volume of a commercial dialysis bag was removed and washed with distilled water. The revenge solution was composed of 1 L of purified water, 2 M of Urea, 0.002 M of reduced glutathione, 0.004 M of oxidized glutathione, 0.2 M of arginine, and 2 mM of ethylene diamine tetraacetic acid (EDTA). The protein solution was dialyzed overnight in 100 mM of Tris-HCl with fluid changes every 4 h. After 48 h, the protein solution was collected. Then, the 4–20% SDS-PAGE gel electrophoresis was performed. The results of the protein purification and gel electrophoresis are shown in Figure S3.

2.6. Optimization of Trypsin Digestion Conditions

The SEC was digested with modified trypsin. In total, 30 μL of SEC and 270 μL of 50 nM of amine bicarbonate (NH_4HCO_3) were added into EP tubes and mixed well. Then, 3 μL of 1 M dithiothreitol (DTT) was added into the mixture and incubated at 56 °C for 1 h. Then, 9 μL of 1 M iodoacetamide (IAA) was added into the mixture and incubated avoiding light for 45 min. The trypsin was added at a trypsin/SEC ratio of 1:100, 1:50, 1:25, and 1:5 and incubated at 37 °C. There was 50 μL of the sample that was taken at 4 h, 8 h, 12 h, 16 h, 24 h, and 28 h, and the digestion was terminated with 10% of formic acid (FA). After termination, the samples were centrifuged at 12,000× *g* for 10 min and analyzed with the LC-MS/MS.

In order to obtain the best digestion time, 50 μL of SEC was added into 300 μL of 50 nM NH_4HCO_3 and mixed well. Then, 3.5 μL of 1 M DTT was added and incubated at 56 °C for 1 h. In total, 10 μL of 1 M IAA was added into the mixture and incubated avoiding light for 45 min. The trypsin was added at a trypsin/SEC ratio of 1:50 and 1:5, and the mixture was incubated at different temperatures (50 °C and 60 °C) for 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, and 8 h. There was 50 μL of the sample that was taken and terminated with 10% formic acid. After termination, the samples were centrifuged at 12,000× *g* for 10 min and analyzed with the LC-MS/MS.

In order to obtain the optimal digestion temperature, 5 μL of SEC was added to 45 μL each of 50 nM NH_4HCO_3 and mixed well. Then, 0.5 μL of 1 M DTT was added and incubated at 56 °C for 1 h. Then, 1.5 μL of 1 M IAA was added into the mixture and incubated avoiding light for 45 min. The trypsin was added at a trypsin/SEC ratio of 1:50 and 1:5, and the mixture was incubated at different temperatures (50 °C, 60 °C, 65 °C, and 70 °C) for 2 h. After termination with 10% FA, the samples were centrifuged at 12,000× *g* for 10 min and analyzed with the LC-MS/MS.

2.7. The Ability of Recombinant Antibodies to Bind to Toxins

The binding ability of the capture molecules to the toxins was verified by molecular interaction experiments. The capture molecule MHCII was labeled with biotin and then immobilized onto an Streptavidin (SA) sensor at a concentration of 1 μg/mL. Different concentrations of the toxin were then tested to explore and determine the binding capacity of the MHCII capture molecule to the toxin, with concentrations set at 3200 nM, 1600 nM, 800 nM, 400 nM, 200 nM, and 0 nM. Similarly, the recombinant antibody MHCII-D10 was used at a curing concentration of 1 μg/mL, and its binding concentration to the toxin was tested at 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, and 0 nM. The concentrations of polyclonal antibodies obtained through animal immunization (pAb), namely pAb-SEA and pAb-SEB, were determined, and the binding concentrations were set at 200 nM, 100 nM, 50 nM, 25 nM, 12.5 nM, and 0 nM. The binding concentrations of the pAb-SEC, pAb-SED, pAb-SEE, pAb-SEH, and pAb-SEG to the toxins were tested at 3200 nM, 1600 nM, 800 nM, 400 nM, 200 nM, and 0 nM. The Octet molecular interaction platform was used for analysis, and the binding ability of the toxin to the capture molecules was determined through data processing.

2.8. SEs Extraction from Complex Matrices

Different concentrations of SEs were added to the 1 × PBS milk and plasma samples at concentration gradients of 100 fmol/μL, 50 fmol/μL, 25 fmol/μL, 12.5 fmol/μL, 6.25 fmol/μL, 3.125 fmol/μL, 1.5625 fmol/μL, and 0.7825 fmol/μL. Each sample was enriched by adding 0.2 mg of magnetic beads before incubation with SEs at room temperature for 1 h.

2.9. Multiplex Immunocapture of SEs

The capture molecules were coupled to the Dynabeads® Capture Molecules Coupling Kit magnetic beads (Thermo Fisher Scientific, USA) at a ratio of 100 µL (10 mg/mL) of beads to 25 µg of capture molecules and incubated for 1 h in complex matrices. We used mass spectrometry to quantify the toxin in the complex matrix after the magnetic bead adsorption, and the results showed that 1 mg of antibody could bind approximately 10 µg of toxin. The capture molecules conjugated to the magnetic beads were incubated with different concentrations of the toxin in the complex matrix. After incubation, the magnetic beads were adsorbed using a magnetic rack. The complex matrix adsorbed by the magnetic beads was removed and washed once with 1 × PBS and twice with 50 mM of NH_4HCO_3. Then, 50 µL of 50 mM NH_4HCO_3. DTT (0.5 µL and 10 mM) was added to the beads, and they were subsequently heated for 1 h at 56 °C to induce the toxin reduction. Next, 1.5 µL of IAA (30 mM) was added, and the mixture was incubated for 45 min in the dark at room temperature. The trypsin was added at a trypsin/SEs ratio of 1:5, and the SEs were digested at 60 °C for 2 h. The digestion was stopped with 2.5 µL of 10% FA, and 5 µL of the labeled peptide mixture was added to the samples. Finally, 10 µL was injected into the UPLC-MS/MS system.

2.10. Liquid Chromatography–Mass Spectrometry Analysis

The UPLC-MS/MS experiment was performed using the ACQUITY UPLC®I-Class-Xevo TQ-S. The peptide was isolated with a C18 reversed-phase chromatography (ACQUITY UPLC@BEH C18 300A 2.1×*50 mm 1.7 µm). The column temperature was 40 °C, and the mobile phases were 0.1% formic acid water (A) and 0.1% formic acid ACN (B). After an initial equilibration step with 95% of water, a gradient from 95% to 10% of the aqueous phase was applied over 10 min at a flow rate of 0.3 mL/min. The ion source used for the mass spectrometry was UniSpray+. The data acquisition was performed in positive ion mode using multi-reaction monitoring (MRM). The capillary voltage and ion source temperature were set to 3.5 kV and 500 °C, respectively. The precursor ions were selected from both unlabeled peptides and internal labeled peptides, and the daughter ions were optimized by adjusting the cone voltage and collision energy.

2.11. Method Evaluation

The performance of the method was evaluated across the milk, 1 × PBS, and sheep plasma matrices. The specificity, linearity, sensitivity, immunocapture recovery, and precision of the proposed method were evaluated.

2.11.1. Specificity

The capture molecules conjugated to the magnetic beads were added to blank matrices (milk, plasma, and 1 × PBS) and the matrices containing Botulinum toxin and Ricin (milk, plasma, and 1 × PBS) before incubation at room temperature. The specificity was verified by mass spectrometry after digestion.

2.11.2. Linearity Range and Sensitivity

An 8-point standard curve was generated for the milk and plasma between 0.7825 fmol/µL and 100 fmol/µL. The internal standard peptide was added after digestion, and the results were analyzed with the UPLC-MS/MS. The calibration curves were generated by linear regression analysis. The lower limit of qualitative (LOD) was defined as three times the signal-to-noise ratio (S/N). The lower limit of quantification (LOQ) was defined as S/N > 10.

2.11.3. Immunocapture Recovery and Precision (RSD)

To evaluate the immunocapture recovery of the SEs from different matrices, we compared the content of the total SES in the eluent with that in the eluate during the immunocapture at three levels (QCL, QCM, and QCH) in triplicate. Three concentration

samples were set: 100 fmol/μL, 25 fmol/μL, and 3.125 fmol/μL or 6.25 fmol/μL were added into the samples of PBS, milk, plasma, and other complex substrates, and 0.2 mg of magnetic beads coupled with the MHCII and MHCII- D10 antibodies were added. After incubation at room temperature for 1 h, the digestion was performed. After digestion, the sample concentration was detected on the UPLC-MS/MS. The relative deviation of peptide (RSD%) was measured.

3. Results and Discussion

3.1. Sequence Characterization of Toxin Standards and Selection of Proteotypic Peptides

The SE toxin protein sequences were collected for the theoretical digestion analysis. The Peptide Cutter tool was used to predict the theoretically digested peptides (https://web.expasy.org/peptide_cutter/, accessed on 26 March 2024), and the DNAMAN 7.0 software was used to compare the toxin protein sequences of seven serotypes and to identify different peptide sequences, as shown in Table S1. The ACQUITY UPLC®I-Class-Xevo G2-XS Tof-MS/MS uses a bottom–up approach to match the peptide profiles after trypsin digestion. The peptides with stronger signals were selected by screening. The specific protein sequences were determined by a BLAST search of the National Center for Biotechnology Information (NCBI) non-redundant database. The peptide profile matching was performed using the ACQUITY UPLC®I-Class-Xevo G2-XS Tof-MS/MS after the trypsin digestion. The matching coverage areas were as follows: SEA 74%, SEB 93%, SEC 93%, SED 83%, SEE 99%, SEG 99%, and SEH 96%, as shown in Table S2.

3.2. Optimization of Digestion Conditions

The results showed that the highest peptide yield of peptides was achieved at a ratio of 1:5 at 37 °C. In addition, there was no decrease in the digestion efficiency even after 28 h, indicating that the digestion was continuing. We then increased the temperature and shortened the time to explore the shortest digestion time to obtain the maximum peptide yield. The result is shown in Figure 1a.

We tested the digestion at 50 °C and 60 °C, and the ratios of trypsin to toxin of 1:50 and 1:5. The digestion durations of 0.5 h, 1 h, 1.5 h, 2 h, 4 h, 6 h, and 8 h were tested. After digestion, the peak areas of five peptides were determined with the mass spectrometry. The results indicated that 2 h after digestion, the digestion effect reached its peak at 60 °C, with the highest peptide yield observed at this time. Subsequently, the efficiency of the digestion began to decline. Thus, the shortest digestion time of 2 h resulted in the highest peptide yield. The result is shown in Figure 1b.

Finally, the optimal digestion temperature was determined. By controlling the digestion time, different temperatures were set as 50 °C, 60 °C, 65 °C, and 70 °C, and the digestion ratio of trypsin to protein was set as 1:50 and 1:5. The termination reaction was compared after the digestion for 2 h, and the peak area was determined with the mass spectrometry peptide segment to find out the highest peptide yield at the optimal temperature. The results showed that the optimal digestion temperature was 60 °C at 2 h. The result is shown in Figure 1c.

The recently published literature did not specifically reflect the digestion time and conditions of SEs by modified trypsin [5,6], and only one article explored the kinetic properties of the sequencing grade modified trypsin and the temperature at which the peptide yield was improved [28]. We systematically explored the optimal digestion efficiency and peptide yield from three aspects: the digestion temperature, digestion time, and digestion ratio. The results were significant.

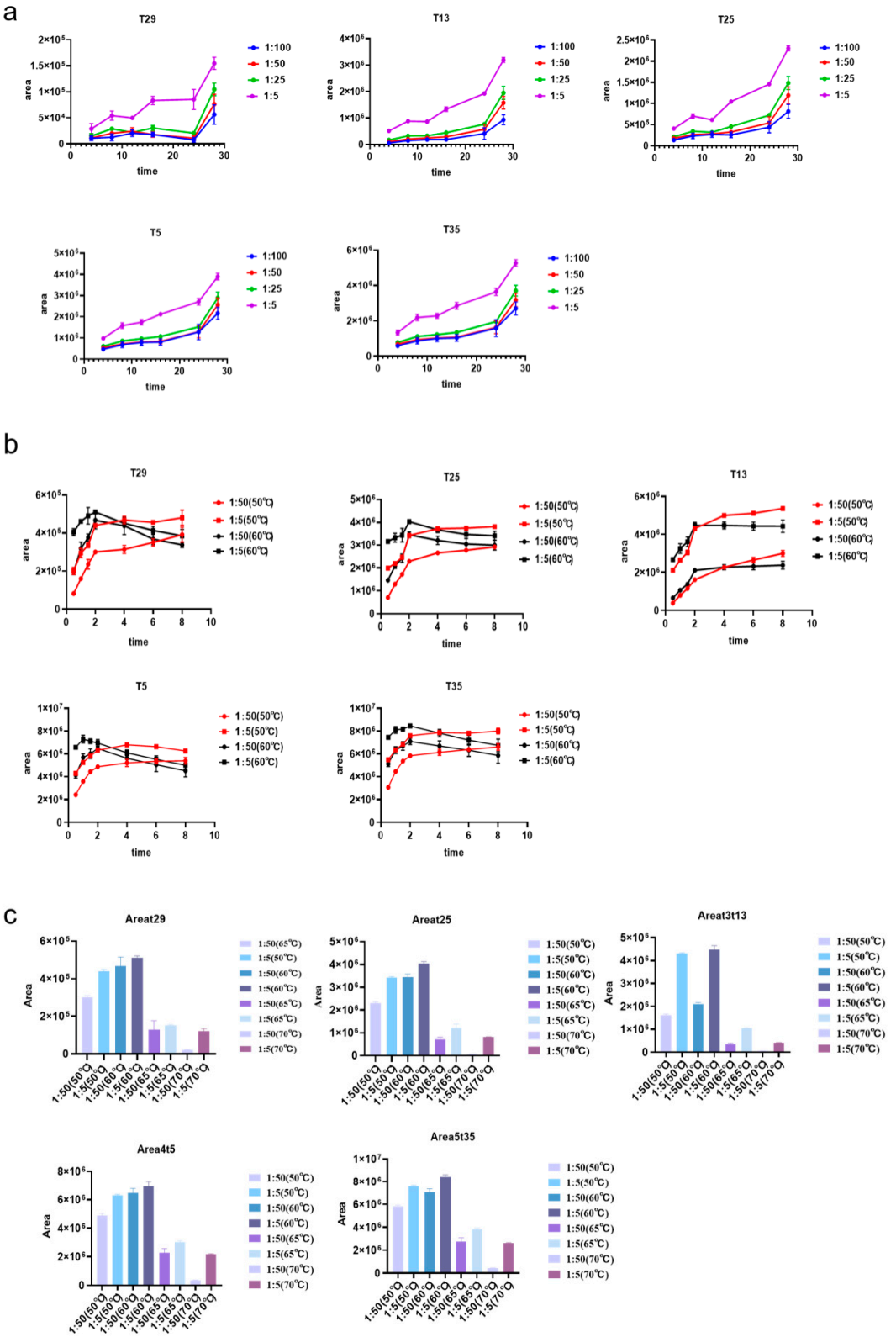

Figure 1. (**a**) The optimal ratio of trypsin to protein was studied at 37 °C by selecting digestion ratios of 1:100, 1:50, 1:25, and 1:5. (**b**) The ratio of the protein and the digestion was set at 1:5 and 1:50, and the digestion temperature was set at 50 °C and 60 °C. Seven time points were selected to explore the optimal digestion time. (**c**) The ratio of protein to trypsin was 1:5 and 1:50. Four digestion temperatures were selected as 50 °C, 60 °C, 65 °C, and 70 °C, to explore the optimal digestion temperature.

3.3. The Ability of Capture Molecules to Bind Toxins

The affinity of the two new capture molecules to various enterotoxins was tested using the Octet molecular interaction platform. Data processing was conducted to obtain the binding ability of the toxins and capture molecules. The Data Analysis 12.0 software was used for the data analysis; the baseline concentration was removed; and the fitting curve analysis was performed according to the dissociation signal data. The value of the correlation affinity constant KD, which equals the dissociation rate constant/binding rate constant (KD = kdis/kon), was obtained, where Kdis reflects the stability of the complex and the percentage of the dissociated complex per second. The faster the dissociation of Kon represents the rate of complex formation (AB). KD represents the size of the binding capacity of the interaction. The results showed that the lowest concentration of the MHCII binding to the seven toxins was 200 nM, with a range of equilibrium dissociation constant (KD) values from 10^{-8} M to 10^{-12} M. Specifically, the KD of the SED was in the range of 10^{-12} M. The lowest binding concentration of the MHCII-D10 with the seven toxins was 12.5 nM. The KD range was 10^{-9}–10^{-11} M, of which the KD of the SED was most optimal at 10^{-11} M. The minimum binding concentration of the specific antibody pAb, obtained by the immunization of animals, to the toxins was 200 nM, and the range KD was 10^{-8}–10^{-12} M. The results showed that two capture molecules, MHCII and MHCII-D10, had the same affinity for toxins as the pAb. The results are presented in Table 1.

Table 1. Protein binding to capture molecules.

Capture Molecules	Protein	KD (M)	Kon (1/Ms)	Kdis (1/s)	R^2	X^2
MHCII	SEA	3.47×10^{-8}	5.60×10^{4}	1.94×10^{-3}	0.9853	0.2332
MHCII	SEB	3.34×10^{-9}	3.39×10^{5}	1.13×10^{-3}	0.9646	1.5755
MHCII	SEC	9.47×10^{-9}	2.53×10^{5}	2.40×10^{-3}	0.9951	1.0342
MHCII	SED	1.0×10^{-12}	3.40×10^{4}	1.0×10^{-7}	0.993	3.4625
MHCII	SEE	1.43×10^{-8}	1.47×10^{5}	2.10×10^{-3}	0.9694	0.7162
MHCII	SEG	6.48×10^{-9}	9.98×10^{4}	6.47×10^{-4}	0.9773	0.7921
MHCII	SEH	4.45×10^{-8}	1.49×10^{4}	6.64×10^{-4}	0.9821	2.1512
MHCII-D10	SEA	3.89×10^{-9}	2.27×10^{5}	8.82×10^{-4}	0.9971	0.0083
MHCII-D10	SEB	2.62×10^{-9}	4.84×10^{5}	1.27×10^{-3}	0.9909	0.1127
MHCII-D10	SEC	3.38×10^{-9}	3.98×10^{5}	1.35×10^{-3}	0.9914	0.0527
MHCII-D10	SED	1.91×10^{-11}	5.00×10^{4}	9.55×10^{-7}	0.9326	3.8586
MHCII-D10	SEE	4.99×10^{-9}	3.18×10^{5}	1.59×10^{-3}	0.9947	0.045
MHCII-D10	SEG	4.42×10^{-9}	1.54×10^{5}	6.80×10^{-4}	0.9979	0.0347
MHCII-D10	SEH	9.19×10^{-9}	1.79×10^{5}	1.65×10^{-3}	0.9849	0.0224
pAb-SEA	SEA	4.84×10^{-9}	1.39×10^{5}	6.72×10^{-4}	0.9984	0.0202
pAb-SEB	SEB	1.76×10^{-9}	2.94×10^{5}	5.18×10^{-4}	0.9949	0.0991
pAb-SEC	SEC	1.36×10^{-8}	8.27×10^{4}	1.12×10^{-3}	0.9600	0.4866
pAb-SED	SED	1.0×10^{-12}	1.56×10^{4}	1.0×10^{-7}	0.9957	2.9424
pAb-SEE	SEE	1.54×10^{-8}	1.17×10^{5}	1.80×10^{-3}	0.9483	0.2682
pAb-SEG	SEG	1.41×10^{-8}	3.13×10^{3}	4.40×10^{-5}	0.9673	2.454
pAb-SEH	SEH	7.00×10^{-8}	1.01×10^{4}	7.05×10^{-4}	0.9986	0.1528

3.4. UPLC-MS/MS (MRM) Method Development

A sensitive UPLC-MS/MS (MRM) method was developed using the MassLynxV4.2 software (Waters) to refine the MRM parameters of SE-specific peptides such as precursor ion, product ion, cone voltage, and collision energy. Table 2 shows the optimization parameters of the precursor ions and product ions of each serotype-labeled peptide and its corresponding isotope-labeled synthetic peptide. For the optimization of the collision energy, Skyline (64 bit) software was used. Using the software algorithms, the product ions with a high response signal and the optimal collision energy were selected.

Table 2. Optimized MRM parameters of specific peptide segment.

Peptide	Amino Acid Sequences	Precursor Ion (*m*/*z*)	Product Ions (*m*/*z*)	Cone (V)	Collision Energy (V)
SEA3	QNTVPLETVK	565.00 (2+)	686.30, 476.10, 380.20	35	15, 25, 20
SEA4	NVTVQELDLQAR	693.70 (2+)	487.30, 396.10, 214.10	35	35, 20, 20
SEA3R*	QNTVPLETV	569.00 (2+)	560.00, 694.90, 894.60	35	20, 15, 20
SEB2	VLYDDNHVSAINVK	529.90 (3+)	744.60, 687.90, 213.20	35	15, 15, 15
SEB4	LGNYDNVR	476.10 (2+)	837.40, 503.25, 86.10	35	15, 15, 25
SEB6	VTAQELDYLTR	654.90 (2+)	909.50, 780.50, 276.20	35	20, 20, 20
SEB6R*	VTAQELDYLT	660.10 (2+)	919.50, 562.40, 201.20	35	20, 30, 20
SEC1	VLYDDHYVSATK	471.20 (3+)	656.60, 600.00, 461.00	35	15, 10, 15
SEC2	TELLNEGLAK	544.30 (2+)	744.42, 631.34, 344.18	35	19, 19, 19
SEC3R*	FLAHDLIYNISD	519.80 (3+)	583.30, 469.60, 356.61	35	15, 15, 20
SED4	NVDVYPIR	488.60 (2+)	762.50, 647.50, 548.30	35	15, 20, 15
SED5	LYNNDTLGGK	548.30 (2+)	818.40, 249.20, 136.10	35	18, 18, 30
SED5R*	LYNNDTLGG	552.10 (2+)	989.50, 826.40, 249.20	35	20, 15, 20
SEE1	NALSNLR	394.40 (2+)	602.30, 489.30, 299.20	35	10, 10, 10
SEE4	QTTVPIDK	451.40 (2+)	472.30, 326.30, 262.10	35	15, 20, 20
SEE4R*	QTTVPID	455.40 (2+)	680.40, 480.50, 270.30	35	15, 15, 20
SEH1	SDEISGEK	432.90 (2+)	662.30, 533.30, 420.40	35	15, 15, 15
SEH3	FATADLAQK	483.00 (2+)	746.40, 473.90, 364.90	35	15, 10, 15
SEH5R*	NVTLQELDI	591.40 (2+)	867.40, 625.40, 214.40	35	20, 20, 15
SEG2	TELENTELANNYK	769.87 (2+)	609.30, 538.26, 344.18	35	27, 27, 27
SEG4	NMVTIQELDYK	677.34 (2+)	1108.59, 345.16, 310.18	35	20, 20, 20
SEG6	FLNIYGDNK	542.80 (2+)	824.50, 596.40, 233.40	35	15, 15, 15
SEG6R*	FLNIYGDN	546.60 (2+)	831.40, 604.30, 233.30	35	15, 15, 15

R* Lys [13C6; 15N2] (+8 Da) and Arg [13C6; 15N4] labeled arginine.

3.5. Method Validation

The performance of the method was evaluated across the milk, 1 × PBS, and sheep plasma matrices. The specificity, linearity, sensitivity, immunocapture recovery, and precision of the proposed method were evaluated.

3.5.1. Specificity

The specificity was evaluated by analyzing analytical matrix blank samples (1 × PBS, milk, and plasma) and samples spiked with Botulinum toxin and Ricin in 1 × PBS, milk, and plasma. In each case, the background of each labeled peptide showed no interference peaks.

3.5.2. Linearity Range and Sensitivity

The SEs were added to 1 × PBS, milk, and plasma to prepare a sample of seven standard calibrations, ranging from 1.5625 fmol/μL to 100 fmol/μL. Each calibrator sample was captured on MHCII molecule beads, digested with trypsin, added with internal standard peptides, and analyzed with the UPLC-MS/MS (MRM). Similarly, seven serotypes of SEs were added to 1 × PBS, milk, and plasma to produce eight standard calibration samples in the range of 0.78125–100 fmol/μL. Each calibration agent sample was captured on MHCII-D10 molecule beads, digested with trypsin, added with internal standard peptide, and analyzed with the UPLC-MS/MS (MRM). Using MHCII as the capture molecule, the qualitative and quantitative detection limit, detection ranges, and linearity of different serotypes of toxins in different substrates are shown in Tables 3–5.

Table 3. Standard curve, linear range, limit of detection, and limit of quantification of different SES isoforms in 1× PBS. (MHCII as the capture molecule).

Peptide	Matrices	Calibration Curve	R^2	LOD (fmol/μL)	LOQ (fmol/μL)	Range (fmol/μL)
SEA4	PBS	Y = 0.005120X − 0.000189	0.997	1.5625	3.125	3.125–100
SEB2	PBS	Y = 0.061606X − 0.049921	0.999	1.5625	3.125	3.125–100
SEC2	PBS	Y = 0.000118X − 0.000156	0.981	1.5625	3.125	3.125–100
SED4	PBS	Y = 0.170828X − 0.403331	0.951	1.5625	3.125	3.125–100
SEE4	PBS	Y = 0.002636X + 0.001088	0.992	1.5625	3.125	3.125–100
SEH3	PBS	Y = 0.009839X − 0.002605	0.990	3.125	6.25	6.25–100
SEG6	PBS	Y = 0.155547X + 0.019418	0.990	3.125	6.25	6.25–100

Table 4. Standard curve, linear range, limit of detection, and limit of quantification of different SES isoforms in plasma. (MHCII as the capture molecule).

Peptide	Matrices	Calibration Curve	R^2	LOD (fmol/μL)	LOQ (fmol/μL)	Range (fmol/μL)
SEA4	Plasma	Y = 0.001202X − 0.000736	0.990	3.125	6.25	6.25–100
SEB2	Plasma	Y = 0.020269X − 0.011634	0.995	1.5625	3.125	3.125–100
SEC2	Plasma	Y = 0.004754X − 0.001350	0.990	3.125	6.25	6.25–100
SED4	Plasma	Y = 0.050879X − 0.069610	0.944	1.5625	3.125	3.125–100
SEE4	Plasma	Y = 0.001211X + 0.002182	0.997	3.125	6.25	6.25–100
SEH3	Plasma	Y = 0.004225X − 0.004713	0.992	6.25	12.5	12.5–100
SEG4	Plasma	Y = 0.157611X − 0.149189	0.987	1.5625	3.125	3.125–100

Table 5. Standard curve, linear range, limit of detection, and limit of quantification of different SES isoforms in milk. (MHCII as the capture molecule).

Peptide	Matrices	Calibration Curve	R^2	LOD (fmol/μL)	LOQ (fmol/μ)	Range (fmol/μL)
SEA4	Milk	Y = 0.001729X − 0.000568	0.998	3.125	6.25	6.25–100
SEB2	Milk	Y = 0.012888X + 0.004582	0.998	1.5625	3.125	3.125–100
SEC2	Milk	Y = 0.003255X − 0.003341	0.965	1.5625	3.125	3.125–100
SED4	Milk	Y = 0.019634X + 0.015828	0.956	1.5625	3.125	3.125–100
SEE4	Milk	Y = 0.002358X − 0.001658	0.993	1.5625	3.125	3.125–100
SEH3	Milk	Y = 0.15241X − 0.280831	0.980	3.125	6.25	6.25–100
SEG6	Milk	Y = 0.001537X − 0.004049	0.956	3.125	6.25	6.25–100

The result shows that one of the specific peptides of the different serotypes was quantified, and the calibration curves were generated by linear regression (Figure S4). Using MHCII as the molecule, the SEA, SEB, SEC, SED, and SEE showed good linearity in the range of 3.125–100 fmol/μL in the PBS substrate, with a coefficient of determination $R^2 > 0.951$. However, the SEE and SEH showed good linearity in the range of 6.25–100 fmol/μL, with $R^2 > 0.990$. In the plasma matrix, the SEB, SED, and SEG showed good linearity in the range of 3.125–100 fmol/μL, with $R^2 > 0.944$. However, the SEA, SEC and SEE showed good linearity in the range of 6.25–100 fmol/μL, with $R^2 > 0.990$. The SEH showed good linearity in the range of 12.5–100 fmol/μL, and $R^2 > 0.992$. In the milk samples, the SEB, SEC, SED, and SEE showed good linearity in the range of 3.125–100 fmol/μL, and $R^2 > 0.956$. The SEA, SEH, and SEG showed good linearity in the range of 6.25–100 fmol/μL, with $R^2 > 0.956$.

Using MHCII-D10 as the capture molecule, the qualitative and quantitative detection lines, detection ranges, and linearity of different serotypes of toxins in different substrates are shown in Tables 6–8. In the PBS matrix samples, the SEA, SEB, SEC, SED, SEE, and SEG showed good linearity in the range of 1.5625–100 fmol/μL, with $R^2 > 0.953$. However,

the SEH showed good linearity in the range of 12.5–100 fmol/μL, with $R^2 > 0.964$. In the plasma matrix, the SEB and SED showed good linearity in the range of 1.5625–100 fmol/μL, with $R^2 > 0.935$. The SEG showed good linearity in the range of 3.125–100 fmol/μL, with $R^2 > 0.971$. The SEA, SEC, and SEE showed good linearity in the range of 6.25–100 fmol/μL, with $R^2 > 0.960$. The SEH showed good linearity in the range of 12.5–100 fmol/μL, with $R^2 > 0.997$. In the milk matrix samples, the SED showed a good linearity in the range of 3.125–100 fmol/μL, with $R^2 > 0.986$. The SEA, SEB, SEC, SEE, and SEG had good linearity in the range of 6.25–100 fmol/μL with $R^2 > 0.943$. The SEH had good linearity in the range of 12.5–100 fmol/μL, with $R^2 > 0.967$.

Table 6. Standard curve, linear range, limit of detection, and limit of quantification of different SES isoforms in 1 × PBS. (MHCII-D10 as the capture molecule).

Peptide	Matrices	Calibration Curve	R^2	LOD (fmol/μL)	LOQ (fmol/μL)	Range (fmol/μL)
SEA4	PBS	Y = 0.041789X + 0.003444	0.974	0.78125	1.5625	1.5625–100
SEB2	PBS	Y = 0.038587X + 0.005922	0.998	0.78125	1.5625	1.5625–100
SEC2	PBS	Y = 0.000209X + 0.000238	0.977	0.78125	1.5625	1.5625–100
SED4	PBS	Y = 0.1172X − 0.10204	0.993	0.78125	1.5625	1.5625–100
SEE4	PBS	Y = 0.003741X − 0.000546	0.999	0.78125	1.5625	1.5625–100
SEH3	PBS	Y = 0.033934X + 0.026944	0.964	6.25	12.5	12.5–100
SEG6	PBS	Y = 0.24238X + 0.008165	0.953	0.78125	1.5625	1.5625–100

Table 7. Standard curve, linear range, limit of detection, and limit of quantification of different SES isoforms in plasma. (MHCII-D10 as the capture molecule).

Peptide	Matrices	Calibration Curve	R^2	LOD (fmol/μL)	LOQ (fmol/μL)	Range (fmol/μL)
SEA4	Plasma	Y = 0.003135X + 0.002139	0.960	3.125	6.25	6.25–100
SEB2	Plasma	Y = 0.001340X + 0.000483	0.958	0.7825	1.5625	1.5625–100
SEC2	Plasma	Y = 0.003198X + 0.000597	0.994	3.125	6.25	6.25–100
SED4	Plasma	Y = 0.055135X + 0.004234	0.935	0.7825	1.5625	1.5625–100
SEE4	Plasma	Y = 0.002209X + 0.001913	0.980	3.125	6.25	6.25–100
SEH3	Plasma	Y = 0.120729X − 0.003764	0.997	6.25	12.5	12.5–100
SEG6	Plasma	Y = 0.083461X − 0.040817	0.971	1.5625	3.125	3.125–100

Table 8. Standard curve, linear range, limit of detection, and limit of quantification of different SES isoforms in milk. (MHCII-D10 as the capture molecule).

Peptide	Matrices	Calibration Curve	R^2	LOD (fmol/μL)	LOQ (fmol/μL)	Range (fmol/μL)
SEA4	Milk	Y = 0.002878X + 0.005775	0.973	3.125	6.25	6.25–100
SEB2	Milk	Y = 0.008302X − 0.001616	0.986	3.125	6.25	6.25–100
SEC2	Milk	Y = 0.008098X − 0.000650	0.984	3.125	6.25	6.25–100
SED4	Milk	Y = 0.006779X − 0.005443	0.986	1.5625	3.125	3.125–100
SEE4	Milk	Y = 0.000726X − 0.000004	0.963	3.125	6.25	6.25–100
SEH1	Milk	Y = 0.223869X + 0.588802	0.967	6.25	12.5	12.5–100
SEG6	Milk	Y = 0.027874X − 0.000883	0.943	3.125	6.25	6.25–100

3.5.3. Immunocapture Recovery and Precision (RSD)

The ample recovery was measured with three replicates at three levels (QCL, QCM, and QCH). The results showed that when the MHCII was used for the capture molecule, the immune capture recovery rates were 19.2–70.4% in the PBS matrix, 18.4–80% in the plasma matrix, and 19.2–96% in the milk matrix. The results are given in Tables 9–11. In the PBS substrate, the recovery rates of the SEC immune capture at three levels (QCL, QCM,

and QCH) ranged from 30.4% to 70.4%. In the plasma matrix, the recovery rates of the SED at three levels (QCL, QCM, and QCH) ranged from 24% to 80%. The recovery rates of the SEH were 36.7–57.8%. In the milk matrix, the immune capture recovery of the SEA, SEB, SEC, SED, and SEE at three levels (QCL, QCM, and QCH) ranged from 32.4% to 96%. The results are shown in Tables 9–11.

Table 9. Immunocapture extraction recovery and precision (RSD%) of SEs in 1 × PBS (n = 3). (MHCII as the capture molecule).

Peptide	Matrices	Added (fmol/µL)	Found (fmol/µL)	Recovery (%)	RSD%
SEA4	PBS	3.125	2.1 ± 0.04	67.2%	2.7%
		25	6.3 ± 0.8	25.2%	17.1%
		100	41 ± 3	41%	8.1%
SEB2	PBS	3.125	0.6 ± 0.04	19.2%	9.2%
		25	10 ± 0.05	40%	0.7%
		100	30 ± 0.9	30%	3.9%
SEC2	PBS	3.125	2.2 ± 0.01	70.4%	0.7%
		25	7.6 ± 0.6	30.4%	10.3%
		100	43.7 ± 0.05	43.7%	0.2%
SED4	PBS	3.125	0.7 ± 0.01	22.4%	2.2%
		25	6.8 ± 0.04	27.2%	0.7%
		100	63 ± 0.5	63%	0.9%
SEE4	PBS	3.125	0.9 ± 0.1	28.8%	17%
		25	5.7 ± 0.3	22.8%	7.4%
		100	30 ± 0.7	30%	2.9%
SEH3	PBS	6.25	2.9 ± 0.4	46.4%	15.2%
		25	11.5 ± 0.9	46%	9.3%
		100	55.1 ± 0.05	55.1%	0.1%
SEG6	PBS	6.25	2.4 ± 0.01	38.4%	0.7%
		25	7.6 ± 0.4	30.4%	6.3%
		100	44.8 ± 3.2	44.8%	8.8%

Table 10. Immunocapture extraction recovery and precision (RSD%) of SEs in plasma (n = 3). (MHCII as the capture molecule).

Peptide	Matrices	Added (fmol/µL)	Found (fmol/µL)	Recovery (%)	RSD%
SEA4	Plasma	6.25	2.1 ± 0.01	33.6%	0.8%
		25	6.7 ± 0.1	26.8%	2%
		100	45.9 ± 5.0	45.9%	13.6%
SEB2	Plasma	3.125	0.9 ± 0.09	28.8%	12.4%
		25	7.0 ± 0.8	28%	14.5%
		100	32.3 ± 3.4	32.3%	12.9%
SEC2	Plasma	6.25	1.6 ± 0.1	25.6%	9.7%
		25	7.9 ± 0.2	31.6%	2.7%
		100	30.8 ± 1.6	30.8%	6.4%
SED4	Plasma	3.125	0.8 ± 0.01	25.6%	2.2%
		25	6.0 ± 0.1	24%	2.8%
		100	80 ± 0.7	80%	1.1%
SEE4	Plasma	6.25	2.6 ± 0.05	41.6%	2.6%
		25	14.7 ± 1.4	58.8%	11.4%
		100	30 ± 1.1	30%	4.6%
SEH3	Plasma	12.5	5 ± 0.1	40%	2.6%
		25	9.1 ± 0.02	36.4%	0.4%
		100	57.8 ± 0.1	57.8%	0.2%
SEG4	Plasma	6.25	1.4 ± 0.01	22.4%	1.1%
		25	4.6 ± 0.01	18.4%	0.4%
		100	27.4 ± 1.2	27.4%	5.4%

Table 11. Immunocapture extraction recovery and precision (RSD%) of SEs in milk (n = 3). (MHCII as the capture molecule).

Peptide	Matrices	Added (fmol/μL)	Found (fmol/μL)	Recovery (%)	RSD%
SEA4	Milk	6.25	3.6 ± 0.1	57.6%	4.9%
		25	9.2 ± 0.2	36.8%	3.2%
		100	42.4 ± 4.8	42.4%	13.9%
SEB2	Milk	3.125	1.6 ± 0.1	52.1%	12.2%
		25	11.6 ± 1.7	46.5%	17.4%
		100	57.9 ± 6.9	57.9%	14.6%
SEC2	Milk	3.125	1.7 ± 0.02	54.4%	1.9%
		25	8.1 ± 0.5	32.4%	7.5%
		100	61.5 ± 0.08	61.5%	0.1%
SED4	Milk	3.125	3.0 ± 0.02	96%	2%
		25	12.8 ± 0.03	51.2%	3%
		100	89.1 ± 1.7	89.1%	2.4%
SEE4	Milk	3.125	2.3 ± 0.2	73.6%	12%
		25	20 ± 2.2	80%	13.2%
		100	41 ± 2.6	41%	7.8%
SEH3	Milk	6.25	1.6 ± 0.1	25.6%	9.4%
		25	12 ± 1.6	48%	16.4%
		100	55.2 ± 0.09	55.2%	0.2%
SEG6	Milk	6.25	1.2 ± 0.01	19.2%	1.4%
		25	11.2 ± 0.8	44.8%	9.3%
		100	37.1 ± 6.0	37.1%	19.8%

When MHCII-D10 was used as the capture molecule, the immune capture recovery rates were 19.2–75.1% in the PBS matrix, 19.2–85.1% in the plasma matrix, and 22.4–59.2% in the milk matrix. In the plasma matrix, the immune capture recovery of the SED was 51.2–85.1% in the three plasma matrices (QCL, QCM, and QCH). The recovery rates of the SEC immune capture were 30.2–52.7%. In the milk matrix, the immune capture recoveries of the SEA at the three levels (QCL, QCM, and QCH) were 34.4–56%, while those of the SEC were 52.3–58% and those of the SEH were 43.6–59.2%. The results are shown in Tables 12–14.

Table 12. Immunocapture extraction recovery and precision (RSD%) of SEs in 1 × PBS (n = 3). (MHCII-D10 as the capture molecule).

Peptide	Matrices	Added (fmol/μL)	Found (fmol/μL)	Recovery (%)	RSD%
SEA4	PBS	1.5625	0.4 ± 0.05	25.6%	17.6%
		25	7.8 ± 0.9	31.2%	14.6%
		100	66.4 ± 10.2	66.4%	18.8%
SEB2	PBS	1.5625	0.6 ± 0.09	38.4%	18.7%
		25	9.4 ± 1.3	37.6%	18.1%
		100	47.7 ± 1.8	47.7%	4.6%
SEC2	PBS	1.5625	0.5 ± 0.01	32.3%	3.3%
		25	8.2 ± 1.3	32.8%	19.5%
		100	49.6 ± 2.1	49.6%	5.2%
SED4	PBS	1.5625	0.4 ± 0.01	25.6%	4.7%
		25	11.1 ± 1.2	44%	13.6%
		100	44.7 ± 6	44.7%	16.4%
SEE4	PBS	1.5625	0.4 ± 0.06	25.6%	18.4%
		25	4.8 ± 0.5	19.2%	13.8%
		100	28.2 ± 1.1	28.2%	4.6%
SEH3	PBS	12.5	2.5 ± 0.3	20%	14.5%
		25	7 ±0.2	28%	4.1%
		100	75.1 ± 5.1	75.1%	8.4%
SEG6	PBS	1.5625	0.7 ± 0.04	44.8%	6.9%
		25	7.6 ± 0.3	30.4%	5.2%
		100	58.8 ± 2.4	58.8%	5.02%

Table 13. Immunocapture extraction recovery and precision (RSD%) of SEs in plasma (n = 3). (MHCII-D10 as the capture molecule).

Peptide	Matrices	Added (fmol/μL)	Found (fmol/μL)	Recovery (%)	RSD%
SEA4	Plasma	6.25	2.4 ± 0.3	38.4%	0.7%
		25	8.2 ± 0.7	32.8%	10.1%
		100	30.7 ± 0.6	30.7%	2.4%
SEB2	Plasma	1.5625	0.6 ± 0.09	38.4%	18.2%
		25	9.4 ± 1.2	37.6%	15.8%
		100	24.8 ± 1.2	24.8%	6.1%
SEC2	Plasma	6.25	2.8 ± 0.2	44.8%	8.4%
		25	7.5 ± 1	30%	17.1%
		100	52.7 ± 0.1	52.7%	0.3%
SED4	Plasma	1.5625	0.8 ± 0.1	51.2%	19.7%
		25	11.5 ± 0.08	46%	0.9%
		100	85.1 ± 0.1	85.1%	0.1%
SEE4	Plasma	6.25	1.2 ± 0.1	19.2%	5.5%
		25	6.4 ± 0.2	25.6%	2.9%
		100	23.2 ± 0.9	23.2%	4.9%
SEH3	Plasma	12.5	2.7 ± 0.04	21.6%	2.1%
		25	5.2 ± 0.2	20.8%	4.7%
		100	24.1 ± 0.9	24.1%	4.4%
SEG4	Plasma	3.125	2 ± 0.2	64%	11.2%
		25	9.9 ± 0.6	39.6%	7.3%
		100	32.4 ± 1.1	32.4%	4.1%

Table 14. Immunocapture extraction recovery and precision (RSD%) of SEs in milk (n = 3). (MHCII-D10 as the capture molecule).

Peptide	Matrices	Added (fmol/μL)	Found (fmol/μL)	Recovery (%)	RSD%
SEA4	Milk	6.25	3.5 ± 0.3	56%	9.3%
		25	8.6 ± 0.6	34.4%	8.8%
		100	51.6 ± 1.9	51.6%	4.46%
SEB2	Milk	6.25	2.1 ± 0.3	33.6%	15.1%
		25	10.9 ± 1.3	43.6%	14.8%
		100	30 ± 0.1	30.0%	0.6%
SEC2	Milk	6.25	3.6 ± 0.5	57.6%	15.8%
		25	14.8 ± 0.9	58%	7.6%
		100	52.3 ± 0.7	52.3%	1.57%
SED4	Milk	3.125	0.9 ± 0.04	28.8%	5.4%
		25	6.4 ± 0.3	25.6%	4.8%
		100	29.8 ± 0.1	29.8%	0.4%
SEE4	Milk	6.25	3.2 ± 0.2	51.2%	8.5%
		25	10.2 ± 0.7	40.8%	8.1%
		100	27.7 ± 0.9	27.7%	3.9%
SEH1	Milk	12.5	7.4 ± 0.7	59.2%	12.1%
		25	10.9 ± 0.2	43.6%	2.1%
		100	49.1 ± 2	49.1%	4.94%
SEG6	Milk	6.25	1.4 ± 0.09	22.4%	8.2%
		25	6.9 ± 0.3	27.6%	4.6%
		100	23.3 ± 0.7	23.3%	3.9%

From the above results, we can see that in different complex matrix samples, it can be known that the two designed capture molecules have the ability to capture seven toxins.

Liquid chromatography–mass spectrometry using immunosorbent enrichment technology to detect SEs in complex matrices requires antibodies. There are 27 SEs that are enriched for antibodies. Antibodies must be prepared for each toxin, but there is no univer-

sal enrichment method. In addition, antibody selection is an issue when testing unknown samples. The capture molecules we have developed can capture at least seven toxins but have the potential to bind all SEs. The capture molecules are versatile and suitable for screening unknown samples [13–15].

4. Discussion

In this study, a new method for the detection of various SEs in complex substrates was established by capture molecules combined with liquid chromatography–mass spectrometry. This study consists of three parts. The first part was the design and acquisition of novel capture molecules and their affinity with SEs. The affinity of the capture molecules was compared with traditional animal-specific immune polyclonal antibodies and SEs. The results showed that the KD values of the new capture molecules binding to the toxin were in the range of 10^{-8}–10^{-12} M, indicating that the binding strength of the capture molecules and the toxin was the same as that of the traditional animal-specific immune polyclonal antibodies. The second part was the detection of toxins in complex substrates with liquid chromatography and mass spectrometry. Detection is usually performed using bottom–up methods, so the pretreatment of the sample is required. In the process of the sample pretreatment, trypsin digestion usually takes a long time and has a low efficiency. Most of the literature studies usually use physical methods, such as microwave-assisted or ultrasonic-assisted methods [22,23], chemical methods, or organic solvent-assisted methods to improve the enzyme digestion efficiency [19], due to the lack of research on modified trypsin digestion of SEs. This paper mainly explored the digestion of Staphylococcus aureus enterotoxin by a modified trypsin enzyme by controlling the temperature, time, and digestion ratio. The optimal conditions for digestion were with a trypsin/SEs ratio of 1:5 and an optimal digestion temperature of 60 °C. A reduction in the digestion time and an improvement of the digestion efficiency followed.

The third part was to verify the specificity, recovery accuracy, and linearity of SEs in complex substrates with liquid chromatography and mass spectrometry to prove whether the novel capture molecules can replace the traditional animal-specific immune antibodies. The results showed that, although the linearity and detection limit of the new trapping molecules were not better than that of the traditional animal-specific immune antibodies, their universality and recovery rate mean that they are potential replacements for the traditional animal-specific immune antibodies.

5. Conclusions

The two capture molecules developed in this paper are different from animal immune antibodies and can be obtained by the purification of recombinant proteins. This new approach not only yields many antibodies but also saves cost and time. Additionally, it eliminates the ethical issues with the use of animals. The two capture antibodies have a good binding effect on the seven serotypes of toxins, though the detection limits of the two capture antibodies appeared higher than that of the specific monoclonal or polyclonal antibodies obtained by animal immunization reported in the literature [6].

Furthermore, this study systematically explored the optimal digestion conditions of modified trypsin for SEs. By controlling the ratio, temperature, and time of the trypsin and toxin digestion, the optimum digestion conditions were identified. This step represents the most time-consuming aspect of the sample processing. Therefore, the endeavor to shorten the digestion time, streamline the processing of the complex matrix samples, and improve the detection efficiency are of great significance for clinical diagnosis and other real-world applications of the detection of SEs.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/separations11050136/s1. Figure S1: sequence diagrams of the capture molecules MHCII and MHCII-D10; Figure S2: sequence alignment map of the SE proteins; Figure S3: Toxin and capture molecules gel electrophoresis; Figure S4: standard curves of the seven serotypes of toxins in three different substrates of the MHCII and MHCII-D10 capture molecule:

1 × PBS, milk, and plasma were used; Table S1: specific peptide sequence; Table S2: peptide profile match rate of the protein sequence; and Table S3: target protein information.

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