

Analysis of *Staphylococcus* enterotoxin B using differential isotopic tags and liquid chromatography quadrupole ion trap mass spectrometry

Khanh Dang Bao^a, Ann Letellier^b and Francis Beaudry^{a*}

ABSTRACT: *Staphylococcus aureus* produces enterotoxins, which are causative agents of foodborne intoxications. Enterotoxins are single-chain polypeptides and have a molecular weight of about 26–28 kDa. The consumption of food contaminated with *Staphylococcus aureus* enterotoxins results in the onset of acute gastroenteritis within 2–6 h. The objective of this study was the development of a new method for the quantification of Staphylococcal enterotoxin B (SEB) in food matrices. Tryptic peptide map was generated and nine proteolytic fragments were clearly identified (sequence coverage of 35%). Among these, three specific tryptic peptides were selected to be used as surrogate peptides and internal standards for quantitative analysis using an isotopic tagging strategy along with analysis by LC-MS/MS. The linearity of the measurement by LC-MS/MS was evaluated by combining mixtures of both isotopes at 0.1, 0.2, 0.5, 1.0 and 2.0 ¹H/²H molar ratios with a slope near to 1, values of *R*² above 0.98 and %CV obtained from six repeated measurement was below 8%. The precision and accuracy of the method were assessed using SEB spiked in chicken meat homogenate samples. SEB was fortified at 0.2, 1 and 2 pmol/g. The accuracy results indicated that the method can provide accuracy within a 84.9–91.1% range. Overall, the results presented in this manuscript show that proteomics-based methods can be effectively used to detect, confirm and quantify SEB in food matrices. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: mass spectrometry; stable isotope tags; quantitative proteomics; enterotoxins

Introduction

Staphylococcal food poisoning caused by enterotoxin-producing *Staphylococcus aureus* (*S. aureus*) is an important foodborne disease encountered worldwide (Argudín *et al.*, 2010; Arvidson and Tegmark, 2001; Sockett and Todd, 2000). It has been reported by regulatory agencies that most raw (fresh or frozen) poultry meat is contaminated with *S. aureus* (Waters *et al.*, 2011; Capita *et al.*, 2002). *S. aureus* is a Gram-positive bacterium producing enterotoxins, which are responsible for food-borne intoxications. *Staphylococcus* enterotoxins are a family of serologically defined, low-molecular-weight proteins (26–30 kDa) produced by some strains of *S. aureus*. Consumption of food contaminated with *S. aureus* enterotoxins results in the onset of acute gastroenteritis within 2–6 h (Seo and Bohach, 2007; Murray, 2005; Tranter, 1990). The most common symptoms associated with *S. aureus* food poisoning are nausea, vomiting, abdominal cramps and headache (Murray, 2005; Balaban and Rasooly, 2000). The symptoms normally resolve within 24 h, but *Staphylococcus* enterotoxins may cause toxic-shock-like syndromes and are frequently involved in allergic and autoimmune diseases (Argudín *et al.*, 2010; Ortega *et al.*, 2010; Le Loir *et al.*, 2003). Poor food handling is a very common source of contamination and, consequently, *S. aureus* can enter the food chain during the processing of animal products. It is challenging to prevent this type of food poisoning, especially since in most cases it is related to cultural practices, religion and lack of proper education. However, in most countries, regulatory agencies enforce food

safety surveillance programs along with a system of laboratories capable of analyzing pathogens and chemicals in food products (World Health Organization, 2002). In order to prevent food poisoning related to *S. aureus* enterotoxins, it is important to determine the level of contamination observed in retail meat or other food products susceptible to direct or indirect contamination.

Staphylococcus enterotoxin B (SEB) is a highly heat-resistant enteric toxin. SEB is responsible for over 50% of enterotoxin food poisonings and represents a particular problem for food requiring handling during processing, such as milk, cheese, canned meat, ham or cooked meals, because, even if the

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Abbreviations used: CID, collision-induced dissociation; ESI, electrospray ionization; MRM, multiple reaction monitoring; SEB, Staphylococcal enterotoxin B; TFA, trifluoroacetic acid.

bacteria has been sterilized, the biological activity of the toxin remains unchanged (Normanno *et al.*, 2007; Le Loir *et al.*, 2003). Moreover, SEB and other enterotoxins could be used as a biological warfare weapons (Pinchuk *et al.*, 2010; Ler *et al.*, 2006). Substantial researches have been conducted in the area of detection of enterotoxins in food resulting in the development of radioimmunoassay and enzyme-linked immunosorbent assay methods (Bennett, 2005; Candlish, 1991; Clark and Engvall, 1980). However, these methods are not used for the quantitative determination of enterotoxins but rather as a detection tool. The toxicity of enterotoxins is proportional to the quantity of the toxin ingested and, consequently, analytical methods capable not only of detection, but also of quantification of the toxins, are needed.

Latest liquid chromatography–mass spectrometry (LC-MS) technological developments along with the integration of new analytical strategies have significantly contributed to the acceleration of biomedical research (Halquist and Karnes, 2011; Kito and Ito, 2008; Brun *et al.*, 2007; Cravatt *et al.*, 2007; Mant *et al.*, 2007). Current trends highlight the emerging importance of LC-MS for the characterization, identification, confirmation and quantitation of proteins in complex biological or nonbiological matrices (Chaerkady and Pandey, 2008; Griffiths *et al.*, 2001). By coupling mass spectrometry with separation techniques such as high-performance liquid chromatography (HPLC), studies of biopolymer mixtures can be efficiently performed (Hoffmann and Stroobant, 2007; Wilson *et al.*, 2008). An atmospheric pressure ion source is required when an instrument that separates molecules in a liquid phase is coupled to a detector that subsequently identifies the ions by manipulation in the gas phase, as in the case of HPLC coupled to mass spectrometry (MS), to analyze complex mixtures of biomolecules. Electrospray ionization (ESI) is the most common atmospheric pressure ion source currently employed to couple HPLC to MS (Yamashita and Fenn, 1984; Aleksandrov *et al.*, 1984). Briefly, the liquid from HPLC is directed through the free end of a capillary typically set at 3–5 kV and the electric field transports the ions into the MS. Currently, ESI is one of the softest ionization techniques available and has the advantage of generating pseudo-molecular ions ($[M + nH]^{n+}$ or $[M - nH]^{n-}$). Moreover, little or no extra internal energy is provided to the ions and, therefore, little fragmentation occurs, allowing pseudomolecular ions to be studied (Bruins, 1987). However, one strong limitation when analyzing large proteins (>50 kDa) using ESI-MS is the formation of a wide distribution of multiply charged species expressed in positive mode as $[M + nH]^{n+}$ ions, resulting in reduced sensitivity for direct MS analysis (Lane, 2005; Dalluge, 2000). Recent strategies have been developed to overcome this important limitation. Protein sequence information can be obtained from several types of enzymatic digestion methods prior to liquid chromatographic separation and ESI-MS (D'Siva and Mine, 2010; Manz *et al.*, 2004). Enzymatic digestion involves reducing the target protein into smaller peptides (typically <4 kDa). This reaction yields a large number of single- and double-charged peptides in solution that may be separated by HPLC, prior to their molecular mass determination by ESI-MS, and creates a comprehensive peptide map specific to the molecular sequence of the original protein (Manz *et al.*, 2004). Peptide mapping is now a widely used tool for the identification and characterization of proteins (Xu and Ma, 2006). Peptide mapping is essentially a qualitative and comparative technique that permits protein sequencing using bioinformatic tools (Xu and Ma, 2006). Trypsin is the most widely

used proteolytic enzyme for protein cleavage because of its high specificity and ability to digest insoluble or adsorbed protein. Trypsin cleaves peptide bonds at the carboxylate-terminal side of lysine (K) and arginine (R) residues (Liu *et al.*, 2007; Manz *et al.*, 2004). Although detailed structural information can be obtained with this method, data analysis and interpretation are relatively tedious. However, several computer algorithms have now been developed to employ sequences of segments of the analyte protein and compare them with databases of known proteins, for the purpose of protein identification (Liu *et al.*, 2007; Xu and Ma, 2006). A major advantage of this approach is that only the sequences of a few fragments and the protein's molecular mass are necessary to unambiguously identify a protein (Callahan *et al.*, 2006). Tandem MS and collision-induced dissociation (CID) provide a comprehensive spectrum allowing structural information to be derived (Callahan *et al.*, 2006). More recently, new protein quantification strategies were developed based on tryptic peptides. Specifically, non-isobaric amine labeling reagents were developed to perform relative and absolute quantitation experiments of targeted proteins and peptides by LC-MS/MS using multiple reaction monitoring (MRM) (D'Siva and Mine, 2010; Callahan *et al.*, 2006). To facilitate protein quantification by MRM, internal standards are required and can be easily created through chemical labeling. In the reference internal standard approach, synthetic tryptic peptide standards can be used to react with a heavy labeling reagent (2H , ^{13}C , ^{15}N and/or ^{18}O). Because of the internal standard, the ratios for all MRM transitions of each peptide can be obtained and concentration determined (D'Siva and Mine, 2010).

Quantitative LC-MS/MS analyses of SEB using signature peptides in food matrices have been investigated using a label-free approach and unrelated internal standards (Callahan *et al.*, 2006). However, very few quantitative methods are available in the literature, particularly methods based on mass spectrometry. Stable isotope labeling in combination with mass spectrometry has emerged as a central method to identify, detect and quantify proteins within complex matrices (Elbert *et al.*, 2008; Bantscheff *et al.*, 2007). The objective of this study is to demonstrate that a proteomic-based strategy can effectively be used to detect and quantitate the SEB in food matrices within accepted criteria for bioassays (Callahan *et al.*, 2006).

Experimental

Chemicals and reagents

Acetic anhydride 99.5% (Ac_2O , $^2H_6-Ac_2O$), ammonium bicarbonate (NH_4HCO_3) and trypsin (proteomic grade) were obtained from Sigma Aldrich Inc. (Saint Louis, MO, USA). SEB was obtained from Toxin Technology Inc. (Sarasota, FL, USA). Synthetic tryptic peptides were synthesized and characterized by CanPeptide Inc (Pointe-Claire, QC, Canada). Acetonitrile was purchased from Fisher Scientific (NJ, USA) and trifluoroacetic acid (TFA), formic acid and ammonium hydroxide (NH_4OH) 28.0–30.0% were purchased from J.T. Baker (Phillipsburg, NJ, USA).

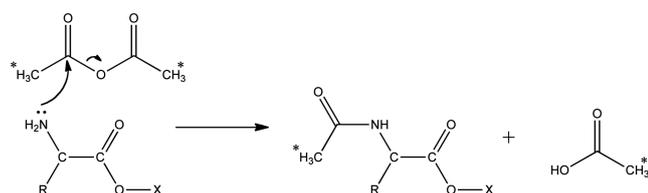
Stock solution

Staphylococcal enterotoxin B protein (100 μg) was dissolved in 2 mL of 0.1% (v/v) TFA–water solution (50 $\mu g/mL$). Further dilution (1:10) in 100 mM ammonium bicarbonate (pH 8.5) was necessary to perform the trypsin digestion and generate tryptic peptides. Synthetic tryptic peptides were dissolved in a 0.1% (v/v) TFA–water solution (100 $\mu g/mL$). The peptide stock solutions (100 $\mu g/mL$) were diluted in a 0.2 M

ammonium bicarbonate buffer (pH 8.5) at a concentration of 200 pmol/mL to prepare the nonisobaric tagged standards and internal standard. This approach is referred as the reference internal standard method in quantitative proteomics (DeSouza *et al.*, 2008).

Synthesis of the internal standards

Selected tryptic peptides were specifically used as internal standards. Ac₂O reacts principally with the N-terminal primary amine as illustrated in Figure 1, but also with lysine primary amine. Briefly, the selected tryptic peptides were diluted in a 0.2 M ammonium bicarbonate buffer (pH 7.5). Two hundred microliters of standard peptides solution were mixed with 10 μL of Ac₂O (Standards) or ²H₆-Ac₂O (Internal standards) (>10,000 molar excess) in a microcentrifuge vial (Che and Fricker, 2002). Ten microliters of NH₄OH were added and the reaction was stopped after 30 min by further diluting the peptide with 0.25% TFA solution to obtain a final concentration of 2 pmol/mL. The standards and the internal standard mixtures were tested by LC-MS/MS and <1% of the original peptides was observed. The



* ¹H or ²H

²H is deuterium

Figure 1. Generic reaction of tryptic peptide N-terminal modifications with acetic anhydride (Ac₂O) and introduction of differential isotopic tag.

linearity measurement of the LC-MS/MS response was evaluated by combining the two mixtures in 0.1, 0.2, 0.5, 1.0 and 2.0 ¹H/²H molar ratios.

Instrumentation

The HPLC system contained a Thermo Surveyor autosampler and a Thermo Surveyor MS pump (San Jose, CA, USA). The quadrupole ion trap (QIT) system used was a Thermo LCQ Advantage (San Jose, CA, USA). Data were acquired and analyzed with Xcalibur 1.4 (San Jose, CA, USA), and regression analyses were performed with PRISM (version 5.0 d) GraphPad software (La Jolla, CA, USA) using the nonlinear curve fitting module with an estimation of the goodness of fit. The calibration lines were constructed from the peak-area ratios of the acetylated peptides and the corresponding ²H₆-acetylated peptides internal standard.

Protein extraction from chicken meat products

The extraction method used was based on a published procedure available from the Food Directorate (Health Canada) (2008). Briefly, 2.5 g of raw chicken was mixed with 2.5 mL of distilled water. The mixture was blended at high speed for 3 min to obtain a homogeneous suspension. The resulting suspension was fortified with SEB at three distinct concentrations (0.2, 1 and 2 pmol/g). The pH of the suspension was then adjusted to 4 with HCl. The samples were mixed and centrifuged at 3000 g for 15 min. The supernatants were transferred into new centrifuge tubes and 5% (v/v) of a 90% TCA solution was added to precipitate the proteins. The sample was mixed and centrifuged at 3000 g for 30 min. The protein pellets were then suspended in 250 μL of 100 mM ammonium bicarbonate (pH 8.5) and 1 μg of proteomic-grade trypsin was added. The incubation time was 24 h at 60 °C, as previously suggested (Callahan *et al.*, 2006). The sample was then processed through a 0.5 mL, 10 kDa MWCO spin filter at 12,000 g for 60 min. The sample

Table 1. The mass transitions for quantitation in MRM mode

| SEB tryptic peptide | Precursor ion | Precursor ion charge state (z) | Product ion | Fragment ion | Collision energy (%) |
|---|---------------|--------------------------------|-------------|--|----------------------|
| (¹ H ₃ -Ac)- <u>L</u> GNVDNVR | 992 | 1+ | 974 | b ₈ | 37 |
| | | | 818 | b ₇ | |
| (² H ₃ -Ac)- <u>L</u> GNVDNVR | 995 | 1+ | 977 | b ₈ | 37 |
| | | | 821 | b ₇ | |
| (¹ H ₃ -Ac) ₂ - <u>I</u> EVYLTTK | 1051 | 1+ | 1014 | b ₈ ^{+H} ₂ ^O | 37 |
| | | | 844 | b ₇ ^{+H} ₂ ^O | |
| (² H ₃ -Ac) ₂ - <u>I</u> EVYLTTK | 1057 | 1+ | 1020 | b ₈ ^{+H} ₂ ^O | 37 |
| | | | 847 | b ₇ ^{+H} ₂ ^O | |
| (¹ H ₃ -Ac) ₂ - <u>F</u> TGLMENMK | 1155 | 1+ | 966 | b ₈ | 37 |
| | | | 721 | b ₆ | |
| (² H ₃ -Ac) ₂ - <u>F</u> TGLMENMK | 1161 | 1+ | 969 | b ₈ | 37 |
| | | | 724 | b ₆ | |

Table 2. Summary of peptides obtained following the digestion of SEB with trypsin

| Number | m/z Observed | Sequence | Charge state (z) | Retention time (min) |
|--------|--------------|--------------------|------------------|----------------------|
| 1 | 475.9 | LGNYDNVR | 2 | 3.8 |
| 2 | 794.0 | VLYDDNHVSAINVK | 2 | 9.7 |
| 3 | 966.2 | IEVYLTTK | 1 | 10.1 |
| 4 | 1191.2 | YLMMYNDNK | 1 | 9.8 |
| 5 | 535.9 | FTGLMENMK | 2 | 11.3 |
| 6 | 655.0 | VTAQELDYLTR | 2 | 12.0 |
| 7 | 919.7 | LYEFNNSPYETGYIK | 2 | 12.5 |
| 8 | 640.0 | NLLSFDVQTNK | 2 | 13.0 |
| 9 | 1146.4 | FIENENSWYDMMPAPGDK | 2 | 16.3 |

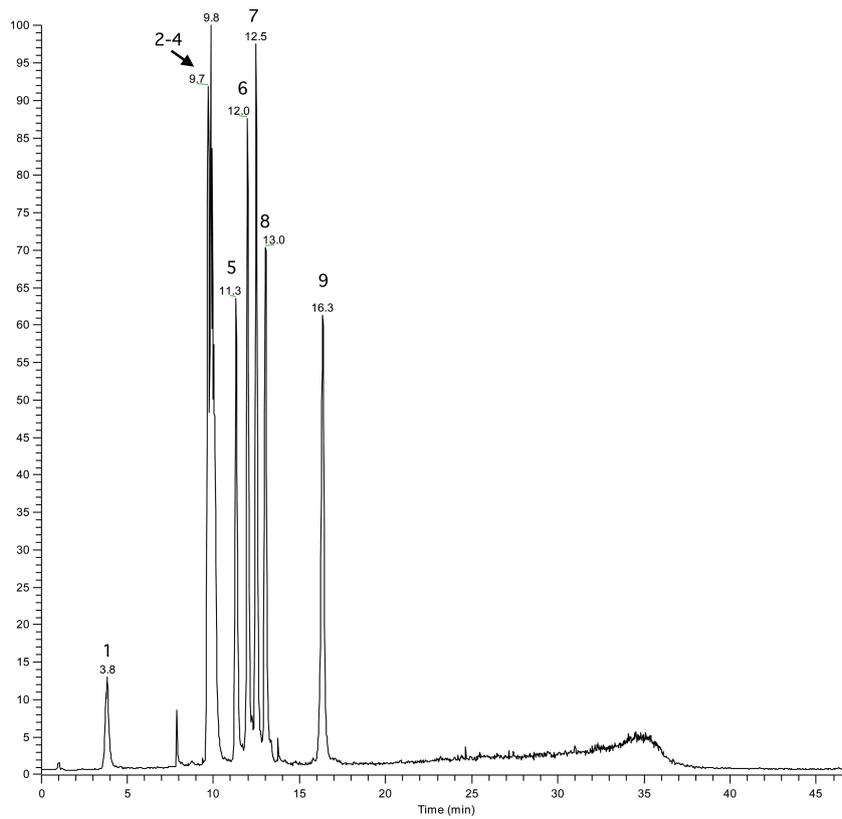


Figure 2. Full-scan LC-MS chromatogram of SEB tryptic peptides.

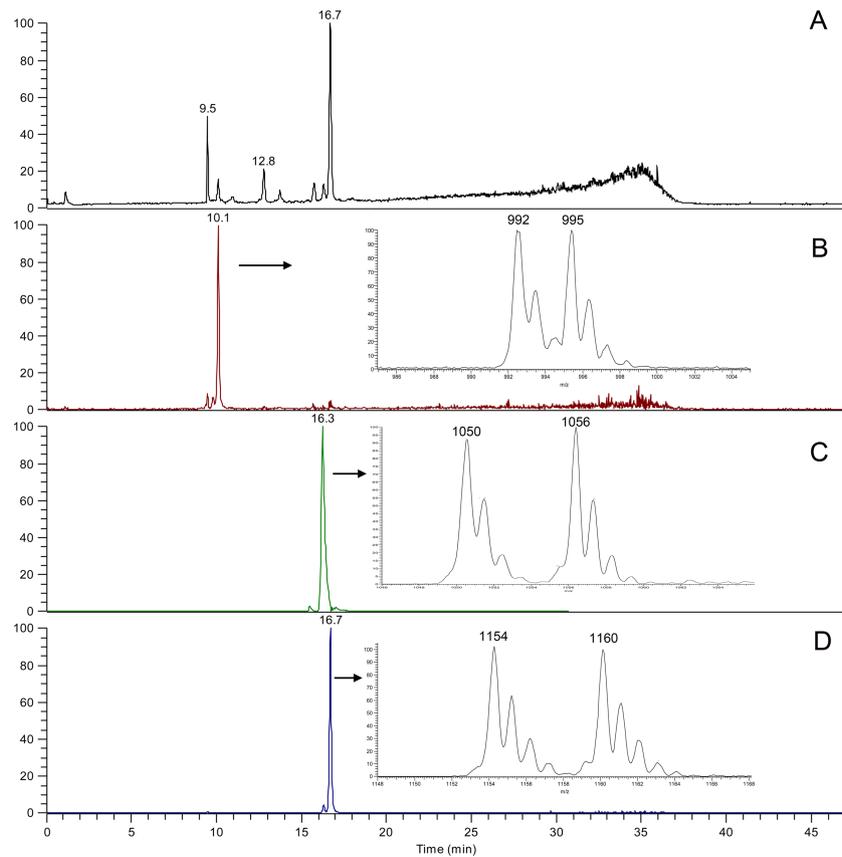


Figure 3. Representative chromatograph of acetylated SEB tryptic peptides mixed at a molar 1:1 ($^1\text{H}/^2\text{H}$).

was evaporated by vacuum evaporation and reconstituted in 200 μL of 0.2 M ammonium bicarbonate buffer (pH 7.5). Ten microliters of Ac_2O were added to the sample and vortex vigorously followed by the addition of NH_4OH . After 30 min of reaction, the sample was evaporated by vacuum evaporation and reconstituted in 25 μL of 0.25% TFA solution containing 100 pmol/mL of the $^2\text{H}_6$ -acetylated peptides internal standard generating a nominal $^1\text{H}/^2\text{H}$ (H/D) ratio of 0.2, 1 and 2.

LC-MS/MS methods

The chromatography was achieved using a gradient mobile phase along with a microbore column Thermo Biobasic C_8 100 \times 1 mm with a particle size of 5 μm . The initial mobile phase conditions consisted of acetonitrile and water (both fortified with 0.4% of formic acid) at a ratio of 5:95, respectively. From 0 to 1 min, the ratio was maintained at 5:95. From 1 to 31 min a linear gradient was applied up to a ratio of 60:40 and maintained for 2 min. The mobile phase composition ratio was returned to the initial conditions and the column was allowed to re-equilibrate for

14 minutes for a total run time of 47 min. The flow-rate was fixed at 75 $\mu\text{L}/\text{min}$. All acetylated peptides eluted between 10 to 17 min. Two microliters of sample were injected using full loop mode. The mass spectrometer was coupled with the HPLC system using a pneumatic assisted electrospray ion source. The sheath gas was set to 5 units and the ESI electrode was set to 4000 V in positive mode. The capillary temperature was set at 300 $^\circ\text{C}$ and the capillary voltage to 34 V. The mass spectrometer was operated for quantitative analysis in MRM mode and the mass transition and collision energy are presented in Table 1.

Results and discussion

SEB tryptic peptide mapping by LC-MS

Peptide mapping is essentially a qualitative and comparative technique that permits protein sequencing using bioinformatic tools. It is an essential step to adequately identify specific tryptic

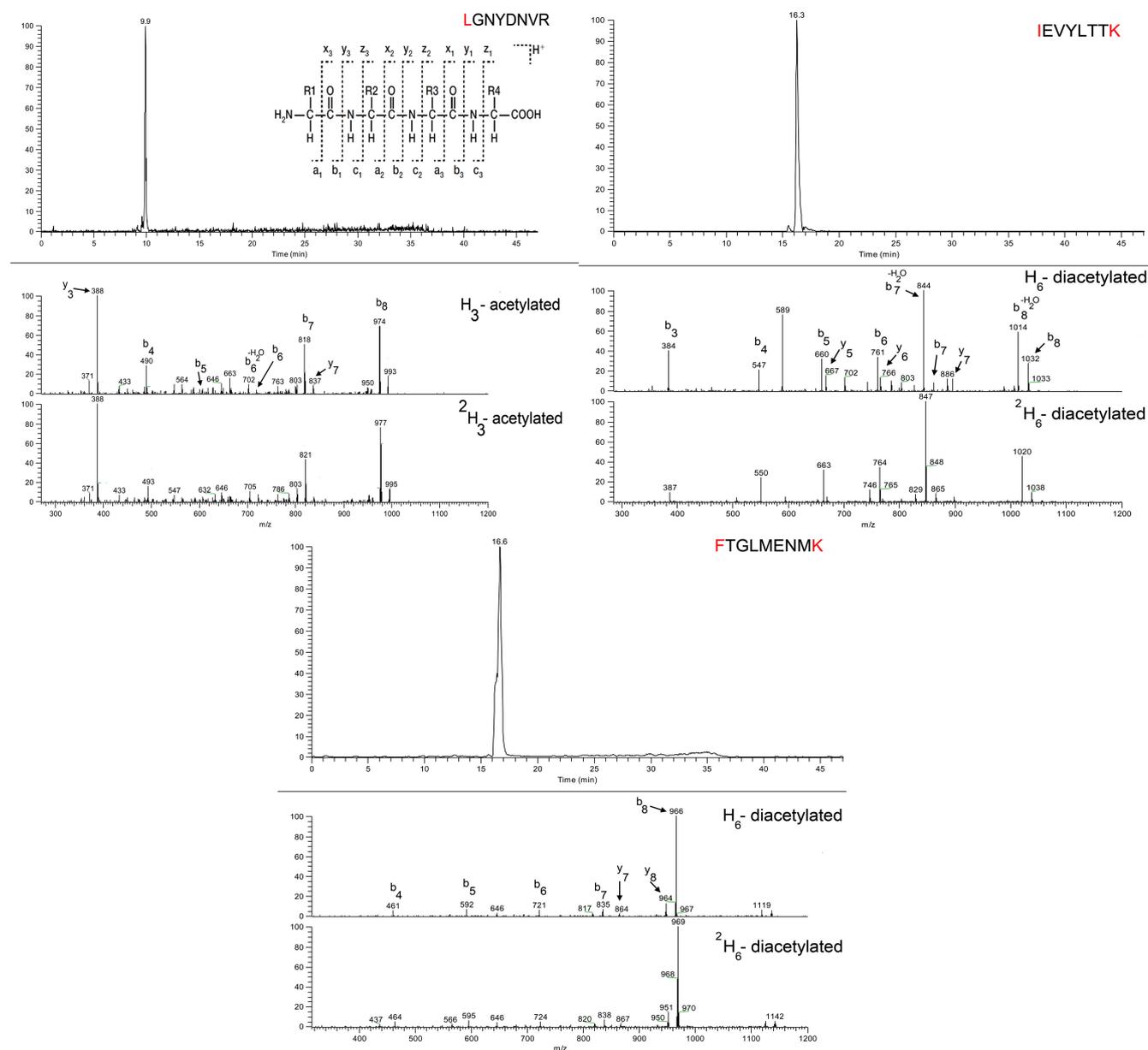


Figure 4. Product ion spectra of targeted Ac_2O -derivatized tryptic peptides.

peptides that will be used for quantification and to build MRM methods. Tryptic digest samples were analyzed by LC-ESI/MS and the observed ions (m/z) were surveyed against an SEB-predicted peptide list generated with mMass (version 3.11, ICT; Strohm *et al.*, 2010). Following the analysis of the peptide mixtures, nine proteolytic fragments were identified with a total sequence coverage of 35% determined using MASCOT (Matrix Science, London, UK) based on an NCBI GI-108515206 sequence (Nema *et al.*, 2007). Table 2 summarizes the molecular weight and amino acid sequence of each SEB tryptic peptides observed, their retention times, the charge states and m/z ratios derive from the full-scan LC-MS experiment (Figure 2). Other tryptic fragments predicted *in silico* were not observed with sufficient certainty, principally owing to the relatively low ion abundance. Moreover, certain fragments were not observed since they were sheltered by an unreduced cysteine bound. Sequence coverage could be improved if reduction with dithiothreitol and alkylation

with iodoacetamide was used prior to the trypsin digestion. However, we believe there was no analytical benefit to perform this additional step since the objective was to generate at least three specific tryptic peptides for quantitative analysis.

Selection of peptide for analytical measurements and tandem MS analysis

Tryptic digestion of SEB generates multiple peptide fragments, many of which could be used for quantification, but some consideration is needed. Long peptides may lead to a wider charge state envelope characteristic of electrospray ionization and consequently hinder our ability to detect and quantify the peptides at low concentrations. Additionally, the reaction with acetic anhydride (Ac_2O and $^2\text{H}_6\text{-Ac}_2\text{O}$) with long peptides may lead to several products since the reagent will react with the

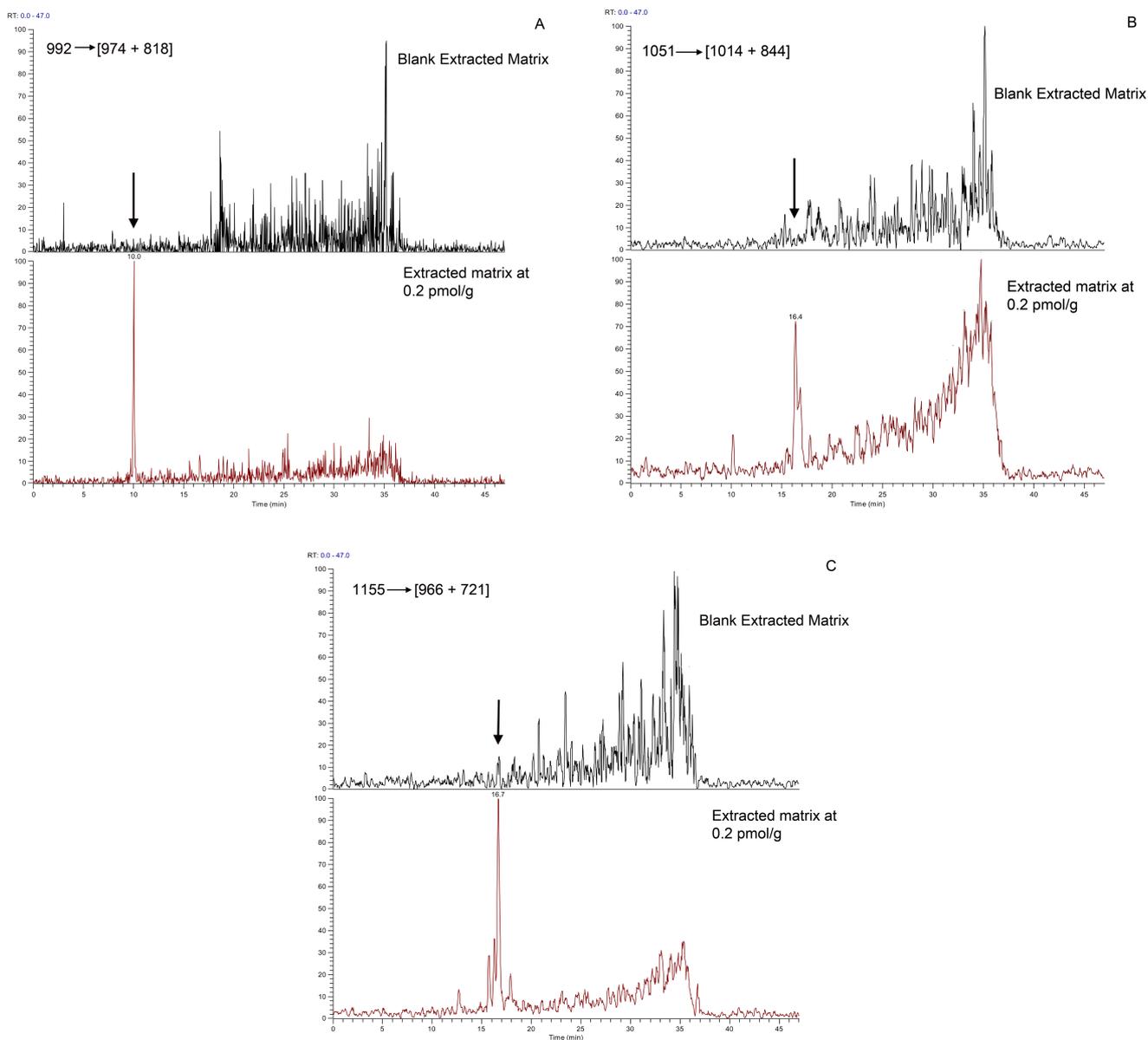


Figure 5. Representative blank and LOQ chromatogram for (A) LGNYDNVR, (B) IEVYLTK and (C) FTGLMENMK.

N-terminal amino acid but also with lysine residue present within the sequence. Another consideration is that we wanted to select tryptic peptides that are present at different locations of the SEB structure, and obtain a sequence coverage of approximately 10%. The acetic anhydride reaction was tested with tryptic peptides and the best results (data not shown) were obtained with peptides 1, 3 and 5 (Table 2) covering a total of 10.54% of SEB sequence. The selected tryptic peptides were acetylated and peptides 3 and 5 existed in two acetylation states (i.e. with one and two groups), but the main product (>90%) was with two groups since peptides 3 and 5 contain a lysine group at the C-terminus. Figure 3 shows that the number of acetyl groups incorporated was apparent from the mass difference between the two peaks, as illustrated by the difference of 3 mass units per acetyl group illustrated in Fig. 1. Product ion spectra (MS/MS) of

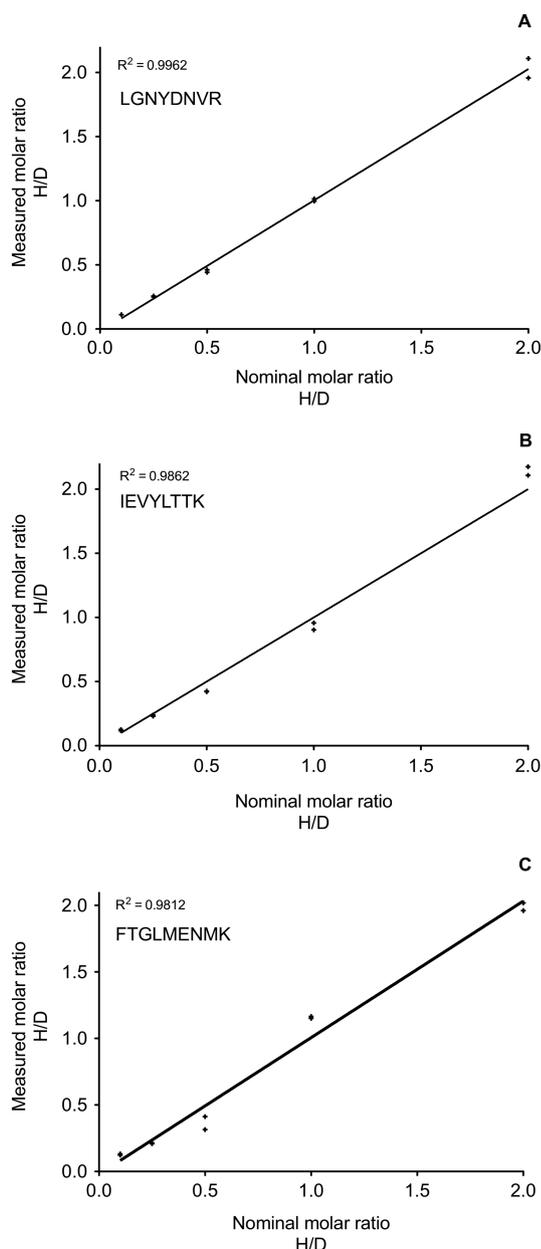


Figure 6. LC-MS/MS quantitative analysis of selected SEB tryptic peptides labeled with $^1\text{H}_6\text{-Ac}_2\text{O}$ or $^2\text{H}_6\text{-Ac}_2\text{O}$ using a reference internal standard strategy.

selected acetylated peptides were collected and typical b and y positive charge ion fragments were observed. Figure 4 shows that the CID spectra of acetylated LGNYDNVR at m/z 992 ($\text{H}_6\text{-Ac}_2\text{O}$) and 995 ($^2\text{H}_6\text{-Ac}_2\text{O}$) are compatible with the expected products based on the amino acid sequence. The spectra revealed the presence of the characteristic b and y fragments at m/z 974/977 (b_8), 837 (y_7), 818/821 (b_7), 719/722 (b_6), 702/705 ($b_6 - \text{H}_2\text{O}$), 605/608 (b_5), 490/493 (b_4) and 388 (y_3) for the acetylated tryptic peptide observed at 9.9 min. The CID spectra of acetylated IEVYLTTK at m/z 1051 ($\text{H}_6\text{-Ac}_2\text{O}$) and 1057 ($^2\text{H}_6\text{-Ac}_2\text{O}$) were compatible with the expected products based on the amino acid sequence. The spectrum also revealed the presence of characteristic b and y fragments at m/z 1032/1038 (b_8), 1014/1020 ($b_8 - \text{H}_2\text{O}$), 895/898 (y_7), 862/865 (b_7), 844/847 ($b_7 - \text{H}_2\text{O}$), 766/769 (y_6), 761/764 (b_6), 667/670 (y_5), 660/663 (b_5), 547/550 (b_4) and 384/387 (b_3) for the acetylated tryptic peptide observed at 16.3 min. Finally, CID spectra of acetylated FTGLMENMK at m/z 1155 ($\text{H}_6\text{-Ac}_2\text{O}$) and 1161 ($^2\text{H}_6\text{-Ac}_2\text{O}$) were compatible with the expected products based on the amino acid sequence. As illustrated for the other peptides, characteristic b and y fragments prevailed at m/z 966/969 (b_8), 964/967 (y_8), 864/867 (y_7), 835/838 (b_7), 721/724 (b_6), 592/595 (b_5) and 461/464 (b_4) for the acetylated tryptic peptide observed at 16.6 min. The observed low-energy CID spectra were compatible with the expected acetylated peptides. Furthermore, b ions predominated and this is particularly important for the selection of suitable MRM transitions to accomplish protein quantitation based on reference internal standard combined with amine-modifying isotopic tags labeling strategies. Additionally, specificity of the assay is important and the selected tryptic peptides were surveyed using MASCOT and NCBI databases against all *Staphylococcus enterotoxins* to verify whether potential interference exists. Moreover, similar surveys were performed with recorded mammalian proteins and no proteins showed the presence of these three tryptic peptides. As illustrated in Figure 5, extracted blank samples did not show any significant interferences at the mass transition and retention time for each tryptic peptide compare to LOQ.

Table 3. Summary of precision and accuracy data for SEB determination in chicken meat

| Concentration (pmol/g) | Mean concentration (pmol/g) | SD | % NOM | %CV |
|---------------------------------------|-----------------------------|---------|--------|-------|
| Peptide: <u>LGNYDNVR</u> ($n = 6$) | | | | |
| 0.2 | 0.179 | 0.00324 | 89.4% | 1.8% |
| 1 | 0.952 | 0.02912 | 95.2% | 3.1% |
| 2 | 1.982 | 0.01618 | 99.1% | 0.8% |
| Peptide: <u>IEVYLTTK</u> ($n = 6$) | | | | |
| 0.2 | 0.166 | 0.01342 | 83.2% | 8.1% |
| 1 | 1.017 | 0.02806 | 101.7% | 2.8% |
| 2 | 2.055 | 0.09211 | 102.8% | 4.5% |
| Peptide: <u>FTGLMENMK</u> ($n = 6$) | | | | |
| 0.2 | 0.164 | 0.03482 | 82.0% | 21.2% |
| 1 | 0.694 | 0.02631 | 69.4% | 3.8% |
| 2 | 1.428 | 0.20242 | 71.4% | 14.2% |
| <i>Combined results</i> | | | | |
| 0.2 | 0.170 | 0.01819 | 84.9% | 10.7% |
| 1 | 0.888 | 0.15428 | 88.8% | 17.4% |
| 2 | 1.822 | 0.32240 | 91.1% | 17.7% |

Quantitation of selected tryptic peptides using differential isotopic tags

The LC-MS/MS method linearity was assessed by acetylating a mixture of three synthetic SEB tryptic peptides using H_6 -Ac₂O or 2H_6 -Ac₂O and combining the two mixtures in 0.1, 0.2, 0.5, 1.0 and 2.0 $^1H/^2H$ molar ratios. Figure 6 shows that the peak area ratios of specific MRM transitions related to each isotopic pair of the targeted tryptic peptides were consistent with the mixing ratio of the two labeling pools, yielding a linear dynamic range with a slope near to 1 and a value of R^2 above 0.98, which denotes that this quantification strategy is accurate. Moreover, the %CV obtained from six repeated measurements was below 8% for 0.1, 0.2, 0.5, 1.0 and 2.0 $^1H/^2H$ molar ratios, suggesting that the method is precise. These results are within generally accepted criteria for bioassays.

Quantitative determination of SEB in chicken meat

Since SEB is one of the major food poisoning agents, it was necessary to evaluate the efficiency of the present analytical method for detecting and quantifying the toxin in food matrices. Samples were prepared by spiking chicken meat homogenates with SEB at 0.2, 1 and 2 pmol/g to evaluate the recovery, precision and accuracy of the proposed method. The analyses were performed in MRM mode (refer to Fig. 4 for supporting information from MS/MS spectra of each acetylated tryptic peptide). The precision and accuracy of the method were evaluated for each tryptic peptide monitored and further statistical analyses were performed, including all the results in order to assess the overall precision and accuracy of the method. Statistical results are presented in Table 3.

In the current study, the determination of the accuracy (%NOM) is important since very few methods are available to estimate the concentration of SEB in food matrices. The method is based on three selected SEB tryptic peptides and the accuracy ranges from 69 to 103%; however, when all the data are combined together, the accuracy results are improved, which indicates that the method can provide accuracy within an 85–115% range. Moreover, the recovery is an integral part of the estimation of the accuracy and, considering the complexity of the matrix and the preparation procedure, the results obtained were excellent. Generally for bioassay, the precision around the mean value should not exceed 20% of the CV. The data provided in Table 3 show that most precision values were well below that limit, with only one exception. Considering the lack of quantitative or semi-quantitative methods, the results show that the general approach suggested in this manuscript can be used for the rapid detection, confirmation and quantification of SEB in meat matrices.

Conclusion

The results presented in this manuscript show that proteomics-based methods can be effectively used to detect, confirm and quantitate SEB in food matrices. More specifically, amine-modifying labeling reagents are an interesting strategy to achieve protein quantitation in complex food matrices using differential isotopic tags, reference internal standards and LC-MS/MS analysis. Interestingly, these approaches are perceived to be costly, especially when using commercial kits such as MTRAQ, ITRAQ or TMT. However, this paper suggests an alternative using an acetylation

strategy with acetic anhydride ($Ac_2O/^2H_6$ -Ac₂O), which is affordable and reliable, but more importantly, provides adequate figures of merit for identification and quantification of SEB in food matrices. In addition, regulatory agencies enforce the conditions under which laboratories can manipulate these toxins, making routine analysis more difficult. The method proposed in this manuscript does not directly require SEB to be manipulated during routine analysis and therefore represent a significant advantage.

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References

- Aleksandrov ML, Gall LN, Krasnov NV, Nikolaev VI, Pavlenko VA and Shkurov VA. *Doklady* Extraction of ions from solutions at atmospheric pressure – a method for mass spectrometric analysis of bioorganic substances. *Doklady Akademii nauk SSSR (Physical Chemistry)* 1984; **277**: 379.
- Argudín MÁ, Mendoza MC and Rodicio MR. Food poisoning and *Staphylococcus aureus* enterotoxins. *Toxins* 2010; **2**: 1751–1773.
- Arvidson S and Tegmark. Regulation of virulence determinants in *Staphylococcus aureus*. *International Journal of Medical Microbiology: IJMM* 2001; **291**: 159–170.
- Balaban N and Rasooly A. review: Staphylococcal enterotoxins. *International Journal of Food Microbiology* 2000; **61**: 1–10.
- Bantscheff M, Schirle M, Sweetman G, Rick J and Kuster B. Quantitative mass spectrometry in proteomics: a critical review. *Analytical and Bioanalytical Chemistry* 2007; **389**: 1017–1031.
- Bennett RW. Staphylococcal enterotoxin and its rapid identification in foods by enzyme-linked immunosorbent assay-based methodology. *Journal of Food Protection* 2005; **68**: 1264–1270.
- Bruins AP. Ion spray interface for combined liquid chromatography/atmospheric pressure ionization mass spectrometry. *Analytical Chemistry* 1987; **59**: 2642–2646.
- Brun V, Dupuis A, Adrait A, Marcellin M, Thomas D, Court M, Vandenesch F and Garin J. Isotope-labeled protein standards: toward absolute quantitative proteomics. *Molecular and Cell Proteomics* 2007; **6**: 2139–2149.
- Callahan JH, Shefcheck KJ, Williams TL and Musser SM. Detection, confirmation, and quantification of Staphylococcal enterotoxin B in food matrices using liquid chromatography–mass spectrometry. *Analytical Chemistry* 2006; **78**: 1789–1800.
- Candlish AAG. Immunological methods in food microbiology. *Food Microbiology* 1991; **8**: 1–14.
- Capita R, Alonso-Calleja C, García-Fernández MC and Moreno B. Characterization of *Staphylococcus aureus* isolated from poultry meat in Spain. *Poultry Science* 2002; **81**: 414–421.
- Chaerkady R and Pandey A. Applications of proteomics to lab diagnosis. *Annual Review of Pathology: Mechanism of Disease* 2008; **3**: 485–498.
- Che F and Fricker L. Quantitation of neuropeptides in Cpe(fat)/Cpe(fat) mice using differential isotopic tags and mass spectrometry. *Analytical Chemistry* 2002; **74**: 3190–3198.
- Clark BR and Engvall E. Enzyme linked immunosorbent assay (ELISA): theoretical and practical aspects. In *Enzyme Immunoassay*, Maggio ET (ed.). CRC Press: Boca Raton, FL, 1980; 167–180.
- Cravatt BF, Simon GM and Yates JR. The biological of mass-spectrometry-based proteomics. *Nature* 2007; **450**: 991–1000.
- Dalluge JJ. Mass spectrometry for direct determination of proteins in cells: applications in biotechnology and microbiology. *Fresenius' Journal of Analytical Chemistry* 2000; **366**: 701–711.
- DeSouza LV, Taylor AM, Li W, Minkoff MS, Romaschin AD, Colgan TJ and Siu KW. Multiple reaction monitoring of mTRAQ-labeled peptides enables absolute quantification of endogenous levels of a potential

- cancer marker in cancerous and normal endometrial tissues. *Journal of Proteome Research* 2008; **7**: 3525–3534
- D'Siva I and Mine Y. Peptidomics for bioactive peptide analysis. In *Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals*, 1st edn. Blackwell and Institute of Food Technologists: Chichester, 2010; 307–324.
- Elbert LD, Mawuenyega KG, Scott EA, Wildsmith KR and Bateman RJ. Stable isotope labeling tandem mass spectrometry (SILT): integration with peptide identification and extension to data-dependent scans. *Journal of Proteome Research* 2008; **7**: 4546–4556.
- Food Directorate (Health Canada). Detection of Staphylococcal enterotoxins in food products using the Vidas® Staph Enterotoxin li (Set2), an ELFA (enzyme linked fluorescent assay) technique, method MFLP-65, 2008. Available from: http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index_e.html (accessed 30 August 2011).
- Griffiths WJ, Jonsson AP, Liu S, Rai DK and Wang Y. Electrospray and tandem mass spectrometry in biochemistry. *Biochemistry Journal* 2001; **355**: 545–561.
- Halquist MS and Karnes HT. Quantitative liquid chromatography tandem mass spectrometry analysis of macromolecules using signature peptides in biological fluids. *Biomedical Chromatography* 2011; **25**: 47–58.
- Hoffmann E and Stroobant V. Analysis of biomolecules. In *Mass Spectrometry, Principles and Applications*. Wiley: Chichester, 2007; 305–324.
- Kito K and Ito T. Mass spectrometry-based approaches toward absolute quantitative proteomics. *Current Genomic* 2008; **9**: 263–274.
- Lane CS. Mass spectrometry-based proteomics in the life sciences. *Cellular and Molecular Life Sciences* 2005; **62**: 848–869.
- Le Loir Y, Baron F and Gautier M. *Staphylococcus aureus* and food poisoning. *Genetics and Molecular Research* 2003; **2**: 63–76.
- Ler SG, Lee FK and Gopalakrishnakone P. Trends in detection of warfare agents. Detection methods for ricin, staphylococcal enterotoxin B and T-2 toxin. *Journal of Chromatography. A* 2006; **1133**: 1–12.
- Liu J, Bell WA, Bergeron JJM, Yanofsky CM, Carrillo B, Beaudrie CEH and Kearney RE. Methods for peptide identification by spectral comparison. *Proteome Science* 2007; **5**: 3.
- Mant CT, Chen Y, Yan Z, Popa TV, Kovacs JM, Mills JB, Tripet BP and Hodges RS. Part I: characterization. In *Peptide Characterization and Application Protocols*, Fields GB (ed.). Humana Press: Totawa, NJ, 2007.
- Manz A, Nicole P and Dimitri L. Mass spectrometry. In *Bioanalytical Chemistry*. Imperial College Press: London, 2004; 85–107.
- Murray RJ. Recognition and management of *Staphylococcus aureus* toxin-mediated disease. *Internal Medicine Journal* 2005; **2**: S106–S119.
- Nema V, Agrawal R, Kamboj DV, Goel AK and Singh L. Isolation and characterization of heat resistant enterotoxigenic *Staphylococcus aureus* from a food poisoning outbreak in Indian subcontinent. *International Journal of Food Microbiology* 2007; **117**(1): 25–39.
- Normanno G, La Salandra G, Dambrosio A, Quaglia NC, Corrente M, Parisi A, Santagada G, Firinu A, Crisette E and Celano GV. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. *International Journal of Food Microbiology* 2007; **115**: 290–296.
- Ortega E, Abriouel H, Lucas R and Gálvez A. Multiple roles of *Staphylococcus aureus* enterotoxins: pathogenicity, superantigenic activity, and correlation to antibiotic resistance. *Toxins* 2010; **2**: 2117–2131.
- Pinchuk IV, Beswich EJ and Reyes VE. Staphylococcal enterotoxins. *Toxin* 2010; **2**: 2177–2197.
- Seo KS and Bohach GA. *Staphylococcus aureus*. In *Food Microbiology: Fundamentals and Frontiers*, Doyle MP and Beuchat LR (eds). ASM Press: Washington, DC, 2007; 493–518.
- Sockett PN and Todd ECD. The economic cost of foodborne disease. In *The Microbiological Safety and Quality of Food*. Aspen: Gaithersburg, MD, 2000; 1563–1588.
- Strohalm M, Kavan D, Novak P, Volny M and Havlicek V. mMass 3: a cross-platform software environment for precise analysis of mass spectrometric data. *Analytical Chemistry* 2010; **82**: 4648–4651.
- Tranter HS. Foodborne staphylococcal illness. *Lancet* 1990; **336**: 1044–1046.
- Waters AE, Contente-Cuomo T, Buchhagen J, Liu CM, Watson L, Pearce K, Foster JT, Bowers JM, Driebe EM, Engelthaler DM, Keim PS and Price LB. Multidrug-resistant *Staphylococcus aureus* in US meat and poultry. *Clinical Infectious Diseases* 2011; **52**: 1227–1230.
- Wilson R, Belluoccio D and Bateman JF. Proteomic analysis of cartilage proteins. *Methods* 2008; **45**: 22–31.
- World Health Organization. *WHO Global Strategy for Food Safety: Safer Food for Better Health*. WHO: Geneva, 2002
- Xu C and Ma B. Software for computational peptide identification from MS–MS data. *Drug Discovery Today* 2006; **11**(13/14): 595–600.
- Yamashita M and Fenn JB. Another variation on the free-jet theme. *Journal of Physical Chemistry* 1984; **88**: 4451–4459.