Université de Montréal

DETECTION AND QUANTIFICATION OF *STAPHYLOCOCCUS AUREUS* ENTEROTOXIN B IN FOOD PRODUCT USING ISOTOPIC DILUTION TECHNIQUES AND MASS SPECTROMETRY

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Mémoire présenté à la Faculté de médecine vétérinaire
en vue de l'obtention du grade de
Maître ès Sciences (M.Sc.)
en sciences vétérinaires
option pharmacologie

Mai, 2012

RÉSUMÉ

L'entérotoxine B staphylococcique (SEB) est une toxine entérique hautement résistante à la chaleur et est responsable de plus de 50 % des cas d'intoxication d'origine alimentaire par une entérotoxine. L'objectif principal de ce projet de maîtrise est de développer et valider une méthode basée sur des nouvelles stratégies analytiques permettant la détection et la quantification de SEB dans les matrices alimentaires. Une carte de peptides tryptiques a été produite et 3 peptides tryptiques spécifiques ont été sélectionnés pour servir de peptides témoins à partir des 9 fragments protéolytiques identifiés (couverture de 35 % de la séquence). L'anhydride acétique et la forme deutérée furent utilisés afin de synthétiser des peptides standards marqués avec un isotope léger et lourd. La combinaison de mélanges des deux isotopes à des concentrations molaires différentes fut utilisée afin d'établir la linéarité et les résultats ont démontré que les mesures faites par dilution isotopique combinée au CL-SM/SM respectaient les critères généralement reconnus d'épreuves biologiques avec des valeurs de pente près de 1, des valeurs de R^2 supérieure à 0,98 et des coefficients de variation (CV%) inférieurs à 8 %. La précision et l'exactitude de la méthode ont été évaluées à l'aide d'échantillons d'homogénat de viande de poulet dans lesquels SEB a été introduite. SEB a été enrichie à 0,2, 1 et 2 pmol/g. Les résultats analytiques révèlent que la méthode procure une plage d'exactitude de 84,9 à 91,1 %. Dans l'ensemble, les résultats présentés dans ce mémoire démontrent que les méthodes protéomiques peuvent être utilisées efficacement pour détecter et quantifier SEB dans les matrices alimentaires.

Mots clés : spectrométrie de masse; marquage isotopique; protéomique quantitative; entérotoxines

ABSTRACT

Staphylococcal enterotoxin B is a highly heat-resistant enteric toxin and it is responsible for over 50% of enterotoxin food poisoning. It represents a particular challenge during food processing since, even if the bacteria have been destroyed, the biological activity of the toxin remains unchanged. The objective of this study was to develop and validate a new method based on a novel proteomic strategy to detect and quantify SEB in food matrices. Tryptic peptide map was generated and 3 specific tryptic peptides were selected and used as surrogate peptides from 9 identified proteolytic fragments (sequence coverage of 35%). Peptides were label with light and heavy form of acetic anhydride to create an isobaric tag that will allow quantification. The linearity was tested using mixtures of different molar ratios and the results showed that measurements by LC-MS/MS were within generally accepted criteria for bioassays with slope values near to 1, values of R^2 above 0.98 and less than 8% coefficient of variation (%CV). The precision and accuracy of the method were assessed using chicken meat homogenate samples spiked with SEB at 0.2, 1 and 2 pmol/g. The results indicated that the method can provide accuracy within 84.9 – 91.1% range. Overall, the results presented in this thesis show that proteomics-based methods can be effectively used to detect, confirm and quantify SEB in food matrices.

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

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LIST OF ABBREVIATIONS

agr	Accessory genome regulator
APC	Antigen-Presenting cell
CA-MRSA	Community-associated methicillin resistant *S. aureus*
CID	Collision-induced dissociation
CPL-SM/SM	Chromatographie en phase liquide- Spectrométrie de masse/Spectrométrie de masse
CPS	Coagulase Positive Staphylococci
Da	Dalton
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ETs	Exfoliative toxins
FAO	Food and Agricultural Organization
FBD	Foodborne disease
FTICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
HPLC	High performance liquid chromatography
LC	Liquid chromatography
LC-MRM-MS	Liquid chromatography-Multiple reaction monitoring-Mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantitation
MALDI	Matrix-assisted laser desorption/ionization

MHC class II	Major histocompatibility complex class II
MODS	Multiple organ dysfunction syndrome
MRM	Multiple Reaction Monitoring
MRSA	Methicillin Resistant *Staphylococcus aureus*
MS	Mass spectrometry
m/z	Mass to charge ratio
ng	nanogram
PCR	Polymerase Chain Reaction
pmol	picomole
PVL	Panton Valentine Leukocidin
RIA	Radioimmunoassay
RPLA	Reversed passive latex agglutination assay
SAgs	Superantigens
SaPIs	*Staphylococcus aureus* pathogenicity islands
S. aureus	*Staphylococcus aureus*
SCC	Staphylococcal cassette chromosome
SE	Staphylococcal enterotoxin
se	Enterotoxin genes
SEB	Staphylococcal enterotoxin B
SE*l*	Staphylococcal enterotoxin like proteins
SFP	Staphylococcal food poisoning
SIS	Synthetic internal standard
SSSS	Staphylococcal scalded skin syndrome
RT-PCR	Reverse transcription polymerase chain reaction

TCR	T cell Receptor
TOF	Time of flight
TSS	Toxic Shock Syndrome
VFA	Vietnam Food Administration
VISA	Vancomycin-intermediate *S. aureus*
VRSA	Vancomycin resistant *S. aureus*
WHO	World Health Organization
%CV	Coefficient of variation (precision)
%NOM	Percent nominal (accuracy)

ACKNOWLEDGMENTS

First of all, I would like to express my special thanks to my director Dr Francis Beaudry for his important guidance, encouragement, patience and invaluable advices which made this thesis a reality. I do not know what I would have done without you. Thank you for all.

I would also like to give my sincere appreciation to my Co-director Dr Ann Letellier for providing supports and valuable comments for my study and qualified research works.

My acknowledgement also goes to Dr Sylvain Quessy, the program director for organizing this project which not only gives me a chance of working this thesis but also give me to better understand the cultures and people of Canada.

I want to thanks all the staff of FAPQDCP-CIDA project especially Mrs Hélène Bergeron, my second mother who helped me a lot. Your care, supports have made an unforgettable moment and made my stay so nice during the past two years.

I would like to thanks to Floriane Pailleux, Mohamed Rhouma, my best friends for all the wonderful times spent both inside and outside the laboratory. Appreciation also goes to Vietnamese students in Canada for providing help and encouragements during the entire period of completing thesis.

I would like to send my deepest thanks to my beloved family, my parents, without them I would not be the person I am today.

Finally, I owe special gratitude to my wife, whom without her full love and supports, I would never achieved fruitful results. I wish to dedicate my result to my beloved family members.

Chapter 1. INTRODUCTION

In recent years, foodborne diseases (FBD) have been a widespread and growing public health problem and a financial burden for the public and the food industry (Sockett and Todd, 2000). More than 250 known diseases can be transmitted through food and bacteria are the most common reasons of FBD outbreaks (Le Loir *et al.,* 2003). Among these, *Staphylococcus aureus* (*S. aureus)* is a one of the major pathogenic bacteria which causes gastroenteritis resulting from the consumption of staphylococcal enterotoxins (SEs) produced in contaminated food (Hennekinne *et al.,* 2009; Chiang *et al.,* 2008). Consumption of food contaminated with SEs of *S. aureus* results in the onset of acute gastroenteritis within 2-6h (Seo and Bohach, 2007; Murray, 2005). The symptoms associated with staphylococcal food poisoning are characterized by nausea, vomiting, abdominal cramps and headache (Hennekinne *et al.,* 2010; Pinchuk *et al.,* 2010; Balaban and Rasooly, 2000). The illness usually resolves within 24h. Improper food handling is the most common reason of contamination and consequently, *S. aureus* enters the food chain during preparation and handling (Pinchuk *et al.,* 2010). Additionally, SEs are also responsible for toxic shock syndromes and other conditions frequently involved in allergic and autoimmune diseases (Vasconcelos and Cunha, 2010; Balaban and Rasooly, 2000). In order to better recognize food poisoning related to SEs, the identification and quantification of SEs in food is important and therefore analytical assays need to be specific, sensitive, accurate and precise.

Staphylococcal enterotoxin B (SEB) is an exotoxin produce by *S. aureus* (Arvidson and Tegmark, 2001). SEB is a single polypeptide chain containing a total of 239 amino acid residues with one disulfide bond (Spero *et al.,* 1973). The molecular weight of SEB is 28,336 Da (Hennekinne *et al.,* 2010). It belongs to a family of microbial proteins called ‘‘pyrogenic toxin superantigens’’ (Chiang *et al.,* 2008*)*. SEB is a highly heat resistant

enteric toxin (Pinchuk *et al.,* 2010; Balaban and Rasooly, 2000). Although there are more than 20 different SEs only a few of them have been clearly understood and SEB is one of the most common toxins associated with food poisoning (Pinchuk *et al.,* 2010). Moreover, SEB is also considered as agents of biological warfare (Ahanotu *et al.,* 2006). During the 1960s, the USA deployed an offensive biological warfare program and SEB was one of the agents studied. SEB was an attractive agent due to low quantities requires to trigger acute poisoning (Ulrich *et al.,* 1997). Extensive researches have been conducted in the area of detection of enterotoxins in food resulting in the development of radioimmunoassay and enzyme linked immunosorbernt assay methods (ELISA) (Bennett, 2005; Candlish, 1991). However, these methods are not used for the quantitative determination of enterotoxins but rather as a detection tool. Consequently, the development of a rapid, sensitive, selective, accurate and precise method for the direct detection and quantification of enterotoxins in food is needed (Vasconcelos and Cunha, 2010).

The purpose of this research project is to demonstrate that proven targeted quantitative proteomic strategies can be applied for the quantification of *S. aureus* enterotoxin B in food products. The method will use the principles of an isotope dilution technique using surrogate tryptic peptides to quantify SEB by liquid chromatography tandem mass spectrometry. Specific tryptic peptides of SEB will be labeled with a non-isobaric amine labeling reagent to create internal standards used to develop a robust and reproducible Liquid Chromatography-Multiple Reaction Monitoring-Mass Spectrometry (LC-MRM-MS) assay. The project includes SEB tryptic mapping and molecular characterization of selected tryptic peptide based on MS/MS analysis. Moreover, method linearity, precision and accuracy will be assessed and compared with generally accepted criteria.

Chapter 2. LITERATURE REVIEW

2.1. Microbiology of staphylococci

2.1.1. Historical background

The genus *Staphylococcus* belongs to the bacterial family of *Staphylococcaceae*; they are gram-positive and catalase-positive bacteria, nonsporulating, nonmotile and forming grape-like clusters when observed under the microscope (Vasconcelos and Cunha, 2010; Bennett and Monday, 2003; Le Loir *et al.,* 2003). The organism was first described in 1881 by Alexander Ogston in purulent infections (Bergdoll and Wong, 2006). After microscopic analysis, Ogston discovered grape-like clusters of round, golden cells (Ogston, 1881). In 1884, Rosenbach described staphylococci according to the colony types: the pigmented type of the cocci which produced yellow colonies, called *Staphylococcus aureus* (*S. aureus)* and the other produced non-pigmented or white colonies, named *Staphylococcus albus* (Cowan *et al.,* 1954). The latter species is now known as *Staphylococcus epidermidis* forming relatively small white colonies (Bergdoll and Wong, 2006).

2.1.2. Taxonomy

Coagulase has been used to distinguish between different types of *Staphylococcus* isolates and allowed the classification of 50 species and subspecies (Hennekinne *et al.,* 2010, Vasconcelos and Cunha, 2010). Coagulase producing staphylococci strains are divided into two groups: coagulase-positive staphylococci (CPS) (Table 1) and coagulase-negative staphylococci with more than different 30 species (Cunha, 2009). However, only CPS are clearly involved in food poisoning incidents. Among CPS

group, *S. aureus* sp. *aureus* is the main causative agent described in staphylococcal food poisoning (Hennekinne *et al.,* 2010; Cunha*,* 2009; Murray *et al.,* 2005).

Table 1. *Staphylococcus* Genus*:* coagulase-positive species (Hennekinne *et al.,* 2010)

Species	**Main sources**
S. aureus sp. *aureus*	Humans, animals
S. aureus sp. *anaerobius*	Sheep
S. intermedius	Dog, horse, mink, pigeon
S. hyicus	Pig, chicken
S. delphini	Dolphin
S. schleiferi sp. *coagulans*	Dog (external ear)
S. lustrate	Otter

2.1.3. Biochemical and metabolic characteristics

Staphylococci are non-motile, facultative anaerobes and they can grow by aerobic respiration (facultative anaerobes) or by fermentation producing lactic acid (Bennett and Monday, 2003). The ability to grow in high saline concentrations is a special characteristic of the organism and most of them are able to grow in media with 10% NaCl. The organism is able to grow at a wide temperature ranging from of 7°C to 48.5°C with an optimum of 30°C to 37°C, a pH ranging from 4.2 to 9.3 (with an optimum of 7 to 7.5) (Le Loir *et al.,* 2003; Stewart *et al.,* 2002). *S. aureus* has positive

reactions when testing for coagulase, heat stable nuclease, alkaline phosphatase and mannitol fermentation (Todar, 2009).

2.2. Epidemiology

Staphylococci are ubiquitous bacteria and they are common inhabitants of the nasal passage, skin and others anatomical sites on human and other warm blooded mammals such as on mucous membranes of the upper respiratory tract, lower urogenital tract and gastrointestinal tract (Tolan *et al*., 2010; Bergdoll and Wong, 2006). Approximately 20% of healthy individuals are carriers the organism, 60% of individuals who carry the organism intermittently and 20% are non-carriers (VandenBergh *et al.,* 1999; Kluytmans *et al.,* 1997). The common *S. aureus* infections are superficial infections like styes. Initially, these infections start locally and then are spread into bloodstream and may result in life threatening condition like bacteremia, endocarditis, meningitis and pneumonia (Lee, 1998; Lowy, 1998). The clinical manifestations of some staphylococcal diseases are such as impetigo, staphylococcal scalded skin syndrome (SSSS), toxic shock syndrome (TSS) and staphylococcal food poisoning. *S. aureus* is also notorious for its resistance to antibiotics such as penicillin resistance, methicillin resistant *S. aureus* (MRSA), vancomycin resistant *S. aureus* (VRSA) and vancomycin-intermediate *S. aureus* (VISA).

2.3. Virulence factors of staphylococci

2.3.1. Overview of staphylococcal virulence factors

Virulence factors are often involved in direct interactions with the host tissues or in concealing the bacterial surface from the host's defense mechanisms (Wu *et al.,* 2008). Staphylococcal virulence factors can be divided into several groups based on the mechanism of virulence and the function: (i) Surface proteins that promote colonization of host tissues; (ii) immune-avoidance (Protein A, coagulase, capsule, leukocidin, biofilm formation ability); (iii) invasion that promote bacterial spread in tissues (leukocidin, kinase, hyaluronidase, staphylokinase, ADNase, fatty acid modifying enzyme); (iv) biochemical properties that enhance their survival in phagocytes (carotenoids, catalase production); (v) damage to cell membranes (hemolysins, leukocidin); (vi) damage to host tissues (SEs, Toxic shock syndrome toxin, Exfoliative toxins) and (vii) antimicrobial resistance factors (Todar, 2009; Wu *et al.,* 2008; Haghkhah, 2003, Nilsson *et al.,* 1999).

2.3.2. Staphylococcal toxins

S. aureus is notorious not only for its ability to develop antibiotic resistance quickly but also for the wide variety of virulence factors which contribute to its ability to invade and colonize tissues. *S. aureus* can produce several molecules associated to virulence factors including cell surface-associated proteins, capsular polysaccharides, exoenzymes and exotoxins (Nilsson *et al.,* 1999). *S. aureus* can produce five different membrane damaging toxins and four hemolysins (alpha-, beta-, gamma-, and delta hemolysin) (Nilsson *et al.,* 1999).

2.3.2.1. Hemolysins

Alpha toxin (α haemolysin) is encoded in the bacterial chromosome and plays an important role in pathogenesis (Haghkhah, 2003; O'Callaghan *et al.,* 1997). *S. aureus* ability to adhere to plasma and extracellular matrix proteins is a significant factor in the pathogenesis of infections (Harris *et al.,* 2002). Several specific adhesins are expressed on the surface of *S. aureus*, which interact with a number of host proteins, such as fibronectin, fibrinogen, collagen, vitronectin and laminin (Foster and McDevitt, 1994). *S. aureus* is able to penetrate host cell by producing a number of membrane damaging toxins. Specific integration in the hydrophobic regions of the host cell membrane can lead to pore formations. Alpha toxin can produce cytolysis, due to an osmotic imbalance that has caused excess water to move into the cell (Krull *et al.,* 1996; Harshman *et al.,* 1989).

Beta (β) toxin is an important cause of the reduction of the macrophage activity induced by most strains of *S. aureus*. The toxin, Mg^{2+}-dependent sphingomyelinase C degrades sphingomylin in the outer phospholipid layer of the erythrocytes (Nilsson *et al.,* 1999). The toxin lyses a variety of cells such as erythrocytes, leukocytes, macrophages and fibroblasts, known to scavenge nutrients (Huseby *et al.,* 2007). Beta toxin is responsible for tissue destruction and abscess formation characteristic of staphylococcal disease (Murray *et al.,* 2005; Haghkhah, 2003).

The gamma (γ) toxin locus occurs in 99% of *S. aureus* (Frinck-Barbancon, 1991). The γ toxin locus expresses three proteins, two class S components (HlgA and HlgC) and one

class F component (HlgB) (Nilsson *et al.,* 1999). The gamma toxin is able to lyses human erythrocytes as well as human lymphoblastic cells (Haghkhah, 2003). The gamma toxin is believed to be responsible for the pathogenesis of Toxic shock syndrome (TSS) together with TSS toxin 1 (TSST-1) (Nilsson *et al.,* 1999).

Delta (δ) toxin is surface active protein and can readily insert itself into hydrophobic membrane structures and form ion channels (Schmitz *et al.,* 1997; Colacicco *et al.,* 1977). Delta toxin is responsible for various pathological effects during an infection. Differentiation between delta toxins from other hemolysins is done by determining heat stability and the pattern of its activity on erythrocytes of various species (Bohach *et al.,* 1997). The toxin is able to lyse erythrocytes as well as mammalian cells by formation of pores in the membrane (Haghkhah, 2003) and has different affinities for different cells such as neutrophils, monocytes and erythrocytes (Alouf and Freer, 1999).

2.3.2.2. Leukocidin

Panton-Valentine Leukocidin (PVL) is a staphylococcal exotoxin belonging to the pore forming toxin family that induces lysis of some immune system cells such as polymorphonuclear neutrophils, monocytes and macrophages (Genestier *et al.,* 2005). Recently, PVL have been strongly associated with human primary necrotizing infection including community-associated methicillin resistant *S. aureus* (CA-MRSA) and the often lethal necrotizing pneumonia (Labandeira-Rey *et al.,* 2007; Gillet *et al.,* 2002). PVL plays a significant role in leukocyte destruction and tissue necrosis (Genestier *et al.,* 2005; Lina *et al.,* 1999). Pore formation of PVL requires the assembly of two components of the toxin, LukS-PV and LukF-PV which alter phospholipid metabolism

and cause disruption of normal cellular activities (Haghkhah, 2003). The toxin damages membranes of host defense cells and erythrocytes by the synergistic action of those components (Lina *et al.,* 1999).

2.3.2.3. Exfoliative toxins A and B

Staphylococcal scalded skin syndrome (SSSS) is a blistering skin disorder caused by *S. aureus* strains which produce exfoliative toxins (ETs) (Mockenhaupt *et al*., 2005). The illness usually begins abruptly with a fever and redness of the skin, often near the mouth and spreading over the entire body in the course of a few days. When the skin is lightly rubbed, the top layer of skin may be begins to peel off the epidermal layer wrinkles. Among 4 different serotypes of ETs (named ETA, ETB, ETC and ETD), ETA and ETB are the major causes of SSSS (Prévost *et al.,* 2003). ETD was associated with epidermal blister (Hanakawa *et al.,* 2002). ETA is heat resistant protein and retains its exfoliative activity after being heated whereas ETB is heat labile. ETA is encoded on a chromosome whereas the gene encoding ETB is located on a large plasmid. Similar to the other virulence factors of *S. aureus,* the regulation of the ETs is under control of the *agr* locus (Novick, 2003). Both ETA and ETB have significant amino acid identity (share 40% identical to each other) (Amagai *et al.,* 2000). ETs have proteolytic activity manifested only under specific, still undermined, conditions (Bukowski *et al.,* 2010). Their proteolytic activity seems directly responsible for skin exfoliation while mitogenic activity, despite being physiologically relevant or observed under particular experimental conditions, was probably not directly associated with the primary manifestations of SSSS (Bukowski *et al.,* 2010).

2.3.2.4. Toxic shock syndrome toxin-1

Toxic shock syndrome (TSS) is an acute systemic disease caused by TSS toxin 1 (TSST-1) which is secreted by *S.aureus* (Vasconcelos and Cunha, 2010). It is characterized by a rapid fever, arterial hypotension, diffuse cutaneous rash, myalgias, vomiting, diarrhea, multiple organ failure (multiple organ dysfunction syndrome – MODS) and desquamation of hands and feet (Chesney, 1989). A fatal shock may be developed 24 h after the onset of symptom if it is not treated promptly. The disease was associated with young women in their menstrual period (Bergdoll *et al.,* 1981). More than 45% of TSS cases are associated with menstruation and most cases caused by TSST-1. In non menstrual TSS, 50% is due to TSST-1 and another is also attributed to staphylococcal enterotoxin B and C (Vasconcelos and Cunha, 2010; Bohach *et al.,* 1990). Non menstrual TSS may occur in any individuals with other infections such as post-surgery TSS, influenza associated TSS skin infections, erythematous syndrome and TSS associated with the use of diaphragms such as contraceptive methods (McCormick *et al.,* 2001).

2.3.3. Staphylococcal enterotoxins

2.3.3.1. Structure and Properties

Staphylococcal enterotoxins are proteins produced by certain of *Staphylococcus* strains. SEs belongs to a family of various types of heat stable enterotoxins that are a leading cause of gastroenteritis resulting from consumption of contaminated food (Balaban and Rasooly, 2000). In addition, SEs are powerful superantigens that stimulate non-specific T-cell proliferation. SEs share close phylogenic relationship with similar structures and

activities. Twenty two different types of enterotoxins have been described including staphylococcal enterotoxins (SEs: SEA to SEE, SEG to SEI, SER to SET) which are characterized by emetic activity and staphylococcal enterotoxin–like (SE*l*) proteins, which are not emetic in a primate model (SE*l*L and SE*l*Q) or have yet to be tested (SE*l*J, SE*l*K, SE*l*M to SE*l*P, SE*l*U, SE*l*U2 and SE*l*V) (Argudín *et al.*, 2010; Vasconcelos and Cunha, 2010).

Based on amino acid sequence comparisons, they can be divided into five major groups (Table 2). SEH has been placed within Groups 1 or 5 (Vasconcelos and Cunha, 2010; Thomas *et al,* 2007; Uchiyama *et al.,* 2006). The percentage of amino acid in the primary sequence was used for classification and comparison purposes. SEA, SED and SEE share 70-90% of homology in the amino acid sequence, while only 40-60% with SEB, SEC and TSST-1 (Pinchuk *et al.,* 2010; Balaban and Rasooly, 2000).

Table 2. Grouping of SEs and SE*l*s based on amino acid sequence comparisons *.

Group	**SEs and SE*l*s**
Group 1	SEA, SED, SEE, (SEH), SE*l*J, SE*l*N, SE*l*O, SE*l*P, SES
Group 2	SEB, SEC, SEG, SER, SE*l*U, SE*l*U2
Group 3	SEI, SE*l*K, SE*l*L, SE*l*M, SE*l*Q, SE*l*V
Group 4	SET
(Group 5)	(SEH)

*Modified from Larkin *et al.,* 2009.

These toxins are globular single chain proteins with molecular weights ranging from 22-29kDa (Table 3) (Argudín *et al.,* 2010) and their mature length is approximately 220-240 amino acids (Pinchuk *et al.,* 2010). The three dimensional structure of several SEs and TSST-1 have been determined by crystallography. They are compact ellipsoidal proteins with two major unequal domains with a β strand and a few α helices, separated by a shallow cavity. The larger of the two domains contains both the amino and carboxyl termini (Pinchuk *et al.,* 2010; Balaban and Rasooly, 2000). The two domains are highly conserved among all SEs.

Table 3. General properties of SEs and SE*l*s (Hennekinne *et al.,* 2010)

Toxin type	Molecular weight (Da)	Genetic basis of SE	Superantigenic Action	Emetic action
SEA	27,100	Prophage	+	+
SEB	28,336	Chromosome, plasmid, pathogenicity island	+	+
SEC_{1-2-3}	27,500	Plasmid	+	+
SED	26,360	Plasmid (pIB485)	+	+
SEE	26,425	Prophage	+	+
SEG	27,043	*enterotoxin gene cluster (egc)*, chromosome	+	+
SEH	25,210	Transposon	+	+
SEI	24,928	*egc*, chromosome	+	+
SE*l*J	28,565	Plasmid (pIB485)	+	nk
SEK	25,539	Pathogenicity island	+	nk
SE*l*L	24,593	Pathogenicity island	+	-
SE*l*M	24,842	*egc*, chromosome	+	nk
SE*l*N	26,067	*egc*, chromosome	+	nk
SE*l*O	26,777	*egc*, chromosome	+	nk
SE*l*P	26,608	Prophage (Sa3n)	+	nk
SE*l*Q	25,076	Pathogenicity island	+	-
SER	27,049	Plasmid (pIB485)	+	+
SES	26,217	Plasmid (pIB485)	+	+
SET	26,614	Plasmid (pIB485)	+	+
SE*l*U	27,192	*egc*, chromosome	+	nk
$SEIU_2$	26,672	*egc*, chromosome	+	nk
SE*l*V	24,997	*egc*, chromosome	+	nk

* +: positive reaction; -: negative reaction; nk: not known

SEB is a single polypeptide chain containing total 239 amino acid residues with one disulfide and no free group (Spero *et al.,* 1973). The molecular weight of SEB is 28,336 Da.

Figure 1. 3D structure of SEB (Papageorgiou *et al.,* 1998)

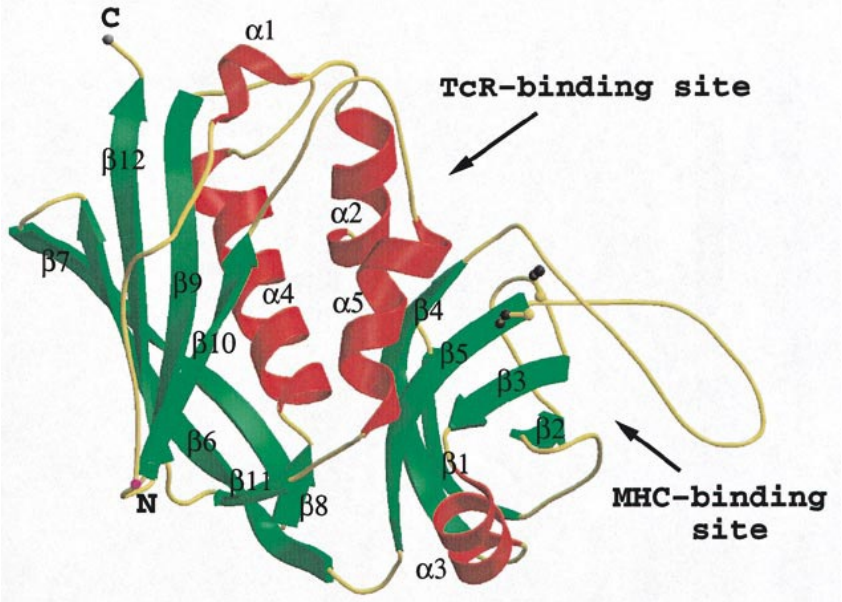


Figure 2. Staphylococcal enterotoxin B sequence (Nema *et al.,* 2007)

ESQPDPKPDE	LHKASKFTGL	MENMKVLYDD	NHVSAINVKS	IDQFLYFDLI
YSIKDTKLGN	YDNVRVEFKN	KDLADKYKDK	YVDVFGANYY	YQCYFSKKTN
DINSHQTDKR	KTCMYGGVTE	HNGNHLDKYR	SITVRVFEDG	KNLLSFDVQT
NKKKVTAQEL	DYLTRHYLVK	NKKLYEFNNS	PYETGYIKFI	ESENSFWYDM
MPAPGDKFDQ	SKYLMMYNDN	KMVDSKDVKI	EVYLTTKKK	

One of the most important properties of SEs is thermal stability. Generally, heat treatments commonly used in food processing are not effective for complete inactivation of enterotoxins. SEs are also partially resistant to proteolytic enzymes (e.g. pepsin, trypsin, rennin and papain) retaining some activities in the digestive tract after ingestion (Balaban and Rasooly, 2000).

They are pyrogenic and share some other important functions in the ability to induce emesis and gastroenteritis as well as superantigenicity (Pinchuk *et al.,* 2010). SEs belong to the broad family of pyrogenic toxin superantigens (Sags). Unlike conventional antigens, Sags do not need to be processed by antigen-presenting cell (APC) before being presented to T cells. Balaban and Rassoly (2000) suggested that the enterotoxin activity may facilitate transcytosis, enabling the toxin to enter the bloodstream, that allow the interaction with T cells and leading to superantigenic activity. Superantigens bind directly to class II MHC complex (Major histocompatibility complex class II) on the surface of APC. Interaction typically occurs to the variable region of TCR β chain (Vβ). Thus, a large number of T cells are stimulated and proinflammatory cytokines are released in large amounts causing systematic toxicity and suppression of the adaptive immune response (Pinchuk *et al.,* 2010; Vasconcelos and Cunha, 2010; Balaban and Rasooly, 2000). Although the superantigenic activity of SEs is well understood, the mechanisms leading to the emetic activity are not clearly define (Balaban and Rasooly, 2000). The biological strength of the Sags is determined by its affinity for the TCR. Sags with the highest affinity for the TCR elicit the strongest response.

2.3.3.2. Enterotoxin gene location

Genes encoding SEs have different genetic supports. All *se* and *sel* genes are located on accessory genetic elements, including plasmids, prophages, *S. aureus* pathogenicity islands (SaPIs), genomic island *v*Sa, or next to the staphylococcal cassette chromosome (SCC) elements (Argudín *et al.*, 2010). Most of these are mobile genetic elements and their spread among *S. aureus* isolates can modify their ability to cause disease and contribute to the evolution of this important pathogen. For instance, *sea* gene is carried

by a family of temperate phages (Coleman *et al*., 1989). SEB is encoded by *seb* gene and is chromosomally located in some clinical isolates (Shafer and Iandolo, 1978). Some of the SE genes are controlled by the accessory gene regulator (*arg*) which is the main regulatory system controlling the gene expression of virulence factors in *S. aureus* (Kornblum *et al*., 1990).

2.3.3.3. Environmental factors that affect staphylococcal enterotoxin production

Staphylococcal food poisoning (SFP) is often associated with growth in protein rich food such as meat and dairy products. These products are highly complex matrices. Many studies have been carried out in laboratory media and in diverse foodstuff to investigate the conditions for producing SEs of *S. aureus.* Some amino acids are essential elements: valine is necessary for growth, cystein and arginine are necessary for both growth and SE production strains of *S. aureus* producing specifically SEA, SEB or SEC (Le Loir *et al.,* 2003). The same factors that affect growth of the organism in general also affect the production of enterotoxin (Bergdoll and Wong, 2006). Moreover, growth ability of *S. aureus* is influenced by a variety of microorganisms and *S. aureus* is quite sensitive to microbial competition. Lactic organisms may inhibit the production of proteases and enterotoxins associated to *S. aureus* (Haines and Harmon, 1973).

2.4. Food poisoning an overview

2.4.1. Food safety activities

2.4.1.1. Food safety activities in the world

Based on report of World Health Organization (WHO), up to one third of the population in developed countries acquire foodborne illnesses each year (WHO, 2006). A great proportion of these cases can be attributed not only by food contamination of food but also by contaminated drinking water (WHO, 2007; Mead *et al.,* 1999). Many incidents of food poisoning were not reported because symptoms are mild and can be resolved quickly. In addition, the evaluation of FBD incidences is difficult to monitor in many countries due mainly to poor monitoring and health systems (Le Loir *et al.,* 2003). To date, more than 250 known FBDs were identified (Le Loir *et al.,* 2003). Food poisoning can be due to known or unknown causes. The known causes of food poisoning are infectious agents and toxic agents. Mainly, the infectious agents are bacteria, viruses, prions, parasites and the toxic agents are toxins as well as inorganic and organic chemicals. They can be found and detected in food with appropriate analytical methods (Le Loir *et al.,* 2003; Mead *et al*., 1999). Food poisoning caused by infectious agents can be classified into two groups: (i) foodborne infections and (ii) foodborne intoxications. Foodborne infections occur when pathogenic bacteria present in food are consumed. Among these, bacteria have accounted for more than 70% of deaths related to foodborne diseases (Hughes *et al.,* 2007; Lynch *et al.,* 2006; Le Loir *et al.,* 2003; Mead *et al.,* 1999). The symptoms vary widely depending on the etiological agent (Le Loir *et al.,* 2003). They include abdominal pain, vomiting, diarrhea, and headache (Kass and Riemann, 2006). More serious cases can result in life-threatening neurologic, hepatic, and renal syndromes leading to permanent disability or death particularly in susceptible

groups such as the elderly, people with diminished immunity or infants and young children (Kennedy *et al.,* 2004; Le Loir *et al.,* 2003).

The WHO identified five factors associated with these illnesses: (i) improper cooking procedures, (ii) incorrect temperature during storage, (iii) lack of hygiene and sanitation by food handler, (iv) cross-contamination between raw and fresh ready-to-eat foods and (v) acquiring food from unsafe source (WHO, 2007). Among these factors mentioned above, four of five factors are related directly to food handler behaviours (acquiring foods from unsafe sources is the exception) (Chapman *et al.,* 2010). According to the CDC, the majority of food poisoning occurrence is related to improper food handling (97%). Among the food poisoning cases, 79% of cases are associated with food prepared in commercial or institutional establishment and 21% of cases are associated with preparation of food at home (Gamarra *et al.,* 2009) and meals prepared outside of the home have been implicated in up to 70% of traced outbreaks (Klein and DeWaal, 2008; Lee and Middleton, 2003). Cross-contamination is often a cause of food poisoning that is overlooked. It occurs when harmful bacteria are spread between food and contaminated surfaces or equipment. Thus food can become contaminated at any stage of food chain and contamination can occur at anytime from farm to folk.

2.4.1.2. Food safety activities in Vietnam

Vietnam has been facing enormous challenges in improving its food safety and safety regulations. A fundamental problem is directly related to the lack of trained resources, including management, leading inevitably to poor implementation of a surveillance system. Moreover, the current legal framework is inadequate and ambiguous (ASIA

Invest Program 2006-2007; Van, 2007). With a population of more than 86 million and 75% of the population living in rural areas (General Statistic Office of Vietnam, data in 2009), the country is facing a lot of challenges related to food safety. Based on report of Vietnam Food Administration (VFA, 2011), the incidence of food poisoning have slightly decreased from 2006 to 2010 (on average 7.8/100,000 person-year) with 944 cases of FBDs which resulted in 32,259 victims and 259 people died (Table 4). Almost all food poisoning outbreaks were associated with preparation of food at home followed by collective kitchen and street vended food (Table 5) (VFA, 2011). In fact, the true incidence of diarrheal disease (includes food borne and waterborne etiologies) could be significantly higher than the official figures due to poor implementation food surveillance program, lack of trained resources, lack of appropriately laboratory equipments as well as the poor organization and inaccessibility of the health system (Khan, 2009; Van, 2007; Kim, 2002).

Table 4. Reported foodborne disease in Vietnam from 2006-2010 (VFA, 2011)

Year	Outbreaks	Number of cases	Deaths
2006	165	7,135	57
2007	247	7,329	55
2008	205	7,828	61
2009	152	4,303	35
2010	175	5,664	51
Total	**944**	**32,259**	**259**

Table 5. Distribution of food poisoning outbreaks in Vietnam 2009-2010

Categories	Results of surveillance			
	2007	**2008**	**2009**	**2010**
Home	120 (48.6)	112 (54.6)	79 (52.0)	106 (60.6)
Restaurant	4 (1.6)	1 (0.5)	2 (1.3)	4 (2.3)
Kindergarten	3 (1.2)	4 (2.0)	1 (0.7)	0 (0.0)
Collective kitchen	51 (20.6)	32 (15.6)	30 (19.7)	23 (13.1)
Hotel	3 (1.2)	1 (0.5)	2 (1.3)	1 (0.6)
Parties (wedding parties)	33 (13.4)	34 (16.6)	22 (14.5)	16 (9.1)
Street food	11 (4.5)	11 (5.4)	6 (3.9)	10 (5.7)
School	10 (4.0)	4 (2.0)	3 (2.0)	4 (2.3)
Others	12 (4.9)	6 (2.9)	7 (4.6)	11 (6.3)
Total	**247**	**205**	**152**	**175**

- In parenthesis: percentage of cases (based on statistics of VFA, 2011)

Currently, the Vietnamese government is making great efforts to improve the food safety and management system in order to reduce risks (Kim, 2002). The Food Law has been approved and promulgated by the Vietnamese Congress in 2010. Moreover, Vietnam has applied international standards for food hygiene and safety in the entire production chain following the warning of WHO and the United Nations Food and Agricultural Organisation (FAO). Food safety must be controlled from “farm to fork”, meaning from the growing, harvesting to processing, distribution and consumption phases (FAO, 2002).

2.4.2. Staphylococcal food poisoning

SFP is caused by the consumption of SEs produced in contaminated foods. SFP are also the second most common cause of reported foodborne illnesses (Argudín *et al.,* 2010; Hennekinne *et al.,* 2010; Pinchuk *et al.,* 2010; Le Loir *et al.,* 2003; Balaban and Rasooly, 2000). *S. aureus* is an ubiquitous bacteria and is found in variety of domestic animals as well as humans and transfer to food through two main sources: human carriage and dairy animals in cases of mastitis (Hennekinne *et al.,* 2010; Pinchuk *et al.,* 2010). The amount of toxin ingested from contaminated food needed to cause disease is less than 1.0 μg, comparable to 10^6 CFU/g (Pinchuk *et al.,* 2010; Bergdoll and Wong, 2006). Onset of the illness can occur rapidly (2 to 6h) with symptoms such as nausea followed by vomiting and diarrhea, abdominal cramps, dizziness and shivering (Seo and Bohach, 2007; Balaban and Rasooly, 2000). In more severe cases, headache, muscle cramping, and transient changes in blood pressure and pulse may occur. The disease resolves within 24-48h without specific treatment (Bennett, 2005; Murray, 2005; Dinges *et al.,* 2000). Occasionally, it can be severe enough to require hospitalization, particularly when infants, elderly or debilitated people are concerned (Murray, 2005). Death is rare (Balaban and Rasooly, 2000; Mead *et al.,* 1999).

Amongst the SEs family, only a few have been the focus of specific studies (Pinchuk *et al*., 2010; Vasconcelos and Cunha, 2010). SEA is the most common toxin in staphylococcus- related food poisoning (80%) with relatively mild symptoms while SEB is a toxin associated with severe symptoms. SEB has very low toxic and lethal doses and was studied for potential use as an inhaled bio-weapon (10%) (Ahanotu *et al.,* 2006; Ler *et al.,* 2006; Casman, 1965). There are other identified SEs. SED is one of the most

common staphylococcal toxin associated to food poisoning with relatively low toxic dose but it is associated to mild symptoms (Pinchuk *et al.,* 2010). SEE was identified in rare cases, while SEF was presumed to be implicated with the toxic shock syndrome (Vasconcelos and Cunha, 2010). SEG, SEH and SEI were not studied in depth, however SEH was identified as one of the cause for a massive outbreak associated with the reconstituted milk consumption in Osaka (Japan) in 2000 (Ikeda *et al.,* 2005).

Foods that require hand preparation and kept at slightly elevated temperatures after preparation are frequently involved in staphylococcal food poisoning due to the insufficient pasteurization/decontamination of raw material or its contamination during preparation and handling by food handlers. Foods that are incriminated in SFP include meat, poultry products, eggs products, canned meat, salads, cooked meals (especially pasta based products), sandwich fillings and other dairy products. Milk and milk products are also related to staphylococcal food poisoning. Although, bacteria can be killed by heating, the SEs are heat resistant, they will not degrade extensively and consequently contaminated food products will remain toxic even after cooking (Evenson *et al.,* 1988).

2.5. Analytical method for the detection of *S. aureus* related toxins

2.5.1. Bioassays

Detection and identification of SEs in food were initially performed using biological methods. Surgalla *et al* (1953) successfully identify the enterotoxins. Biological assays are based on the capacity of an extract of the suspected food to induce symptoms such as vomiting, gastrointestinal symptoms in animals or superantigenic action in cell cultures.

Initially, animal studies were conducted in order to find out a link between the enterotoxicity of foods and different organisms isolated from foods. SEs have been identified based on their emetic activity in monkey feeding test and kitten-intraperitoneal test and more recently, house musk shrews *Suncus murinus* are used as animal models (Ono *et al.,* 2008). Monkey feeding tests were not sufficiently sensitive to detect the amount of toxin in food during outbreaks and the procedure of injecting cats or kittens was rapidly considered non-specific (Casman and Bennett, 1965). Therefore, the biological assays utilizing animals have been replaced by immunoassays, and molecular biological methods (Normanno *et al.,* 2006; Martin *et al.* 2004; Nakano *et al.,* 2004).

2.5.2. Molecular tools

Polymerase chain reaction (PCR) is a noteworthy technique used in molecular biology. This method usually detects genes encoding enterotoxins in strains of *S. aureus* isolated from contaminated foods (Vasconcelos and Cunha, 2010). However, these molecular methods have two major limitations: (i) staphylococcal strains must be isolated from food and (ii) the results inform on the presence or absence of genes encoding SEs but do not provide any information on the concentration of the toxins in food. This method therefore cannot be the sole method to detect SEs in food (Hennekinne *et al.,* 2010). Other related techniques such as reverse transcription polymerase chain reaction (RT-PCR) detect and quantify mRNA. The method includes the reverse transcription of RNA strand into its DNA complement. The resulting cDNA is amplified using PCR (Vasconcelos and Cunha, 2010). This method offers more specificity but still does not allow direct detection and/or quantification of the toxins (Hennekinne *et al.,* 2010). Recently, several PCR-based methods have been used for staphylococcal enterotoxin

genotyping but these methods are time-consuming and laborious because many separate reactions are required to identify subsets of different enterotoxin genes (*se*). The advantage of this method is its sensitivity for the detection of enterotoxin genes. In contrast, results of these methods may show false positive due to the presence of different copy numbers of the genes resulted (including new and unexpected toxin genes) in varying signal intensities in the array (Vasconcelos and Cunha, 2010).

2.5.3. Immunological tools

Several immunological methods have been used for the detection of staphylococcal enterotoxins in food: (i) immunodiffusion assays (ii) radioimmunoassays (RIA) and (iii) enzyme-linked immunosorbent assays (ELISA) (Thompson *et al.,* 1986).

2.5.3.1. Gel diffusion

There are two kinds of gel diffusion: (i) single gel diffusion tube assay and (ii) double gel diffusion tube assay. A single gel diffusion assay was described for the detection of SEA and SEB by Hall and colleagues (Hall *et al.,* 1965). In single and double gel diffusion tube assay, when enterotoxin antigen reacts with its corresponding antibody, a visible precipitate may occur, called precipitin reaction. Melted agar containing antiserum is poured into test tubes and is overlaid with a solution containing enterotoxins. The enterotoxin diffuses downward into the antiserum agar layer and forms a precipitin band at the interface in the test tube. The diameter of the precipitin ring is plotted against the concentration of enterotoxin resulting in a straight line. The double gel diffusion tube assays are modified from the single gel system. A layer of

plain agar separates the antibody containing agar layer and the enterotoxin solution. The limit of detection of double gel diffusion is approximately 1µg/ml. The tube diffusion assays has been supplanted by the micro slide and plate assays. In 1969, Casman *et al* (1969) developed the micro slide gel double diffusion assays and now it is used as the current standard for evaluation new methods. In micro slide gel double diffusion assay, small wells are cut in agar coated micro slides, antiserum added to the center well and enterotoxin placed in the wells surrounding the antiserum well.

2.5.3.2. Radioimmunoassay

Radioimmunoassay (RIA) is a highly sensitive technique used for measurement of primary antigen and antibody interactions and for the determination of the amount of substances present in samples. First discovered by Rosalyn Yalow and Solomon Berson, RIA was used to measure blood volume, iodine metabolism and hormones like insulin (Yalow and Berson, 1960). Since its development, RIA has been considered a revolution in bioanalysis because of its rapidity, precision, sensitivity and simplicity. The principle of this method is based on the reaction of the antigen with specific antibody resulting in a competitive binding assay using an antigen as a ligand and an antibody as the binding protein (‘‘carrier’’). To perform a radioimmunoassay, a known quantity of an antigen is made radioactive (i.e. labeled with gamma-radioactive isotopes of iodine attached to tyrosine). Radiolabeled antigens are then mixed with a known amount of the antibody for that antigen, and both will bind to one another (Smith and Bencivengo, 1985). The unknown concentration of the antigenic substance in a sample is obtained by comparing its inhibitory effect on the binding of radiolabeled antigen to a specific amount of

specific antibody (Smith and Bencivengo, 1985). A standard curve is established and the amount of antigen in the unknown samples can be calculated based samples with increasing concentrations of unlabeled ligand. The analysis of food samples by RIA require minimal preparation allowing fast and sensitive detection of staphylococcal enterotoxins from foods with sensitivity near 1 ng/g (Thompson *et al.,* 1986; Smith and Bencivengo, 1985). Several methods were developed, validated and used for the detection of SEA, SEB, SEC: (i) solid-phase RIA with polystyrene tubes; (ii) RIA with bromoacetyl-cellulose as an immunoadsorbent; (iii) the double-antibody technique with anti-rabbit gamma globulin as co-precipitant and RIA with cells containing protein A as coprecipitant (Miller *et al.,* 1978). The assay involved labeling of the enterotoxins with radioactive ^{125}I or ^{131}I-chloramine-T, lactoperoxidase and gaseous iodine (Miller *et al.,* 1978). Although, RIA has been widely used in research and routine analysis, there are many limitations. Limitations include the lack of specificity and linearity leading to accuracy problems. Moreover, the handling and disposal of radioactive waste are a concern and represent an additional cost (Smith and Bencivengo, 1985).

2.5.3.3. Enzyme linked immunosorbent assay

ELISA can also be referred as enzyme immunoassay but does not have the same limitation compared to RIA regarding the handling and disposal of radioactive chemicals and wastes (Freed *et al.,* 1982; Kauffman, 1980). ELISA is an immunoassay technique involving an enzymatic reaction to detect the presence of a specific antigen-antibody reaction (Candlish, 1991; Clark and Engvall, 1980). The enzyme converts a colorless substrate to a colored product that allows the detection of antigen-antibody binding. An ELISA can be used to detect either the presence of antigens or antibodies in a sample,

depending on how the test is designed. In 1971, Engvall and Perlmann published their first paper on ELISA and demonstrated quantitative measurement of IgG in rabbit serum with alkaline phosphatase as the reporter label (Engvall and Perlmann, 1971). Saunders and Bartlett (1977) described a double antibody solid-phase enzyme immune assay for detection of SEA from foods. Presently, many ELISA methods are available for the detection of staphylococcal enterotoxins in food products. Among ELISA techniques, there are three types which are frequently for the detection of SEs: (i) the single sandwich ELISA; (ii) the double sandwich ELISA; and (iii) competitive methods. In the single sandwich ELISA technique, a solid phase is coated with antibody and enterotoxin is added and allowed to react. This technique could be carry out in microtiter plate tubes or spheres, or polystyrene tubes. The assay uses peroxidase or alkaline phosphatase antibody conjugates (Smith and Bencivengo, 1985; Saunders and Bartlett, 1977). In the double sandwich method, the enzyme is coupled to the specific antibody. A solid phase anti-enterotoxin complex with enterotoxin reacted with a second anti enterotoxin produced in an animal species different from that of the first. An anti-IgG-enzyme conjugate is used in the assay (Smith and Bencivengo, 1985; Saunders and Bartlett, 1977). With the competitive method, the enzyme is conjugated with the toxin molecule (Kauffman, 1980). There are many advantages including speed, simplicity and sensitivity. However, there are also many limitations. ELISA methods are prone to false positive and false negative results due to the cross reactivity of the antibodies with antigens. ELISA methods provide information on the presence of an analyte but no information on its chemical properties (i.e. chemical structure) and consequently, the specificity of the technique can be questioned. The method accuracy strongly depends on the specificity and ELISA methods are rather used as a detection tool that should be

used in combination with more sophisticated analytical techniques for confirmation and accurate quantification.

2.5.3.4. Reversed passive latex agglutination assay

For rapid detection of enterotoxin, a reversed passive latex agglutination assay was developed (Shingaki *et al.,* 1981; Oda *et al.,* 1979). This method allows the detection of soluble antigens such as enterotoxins. The antibody is coated to particles such as latex beads and reacts with the soluble antigen which is visibly agglutinated in the presence of the corresponding enterotoxin. The latex particles are sensitized with rabbit globulins and these latex particles agglutinate in the presence of staphylococcal enterotoxins. This is now commercially available in SET-RPLA kit from Basingstoke (Hampshire, UK). The kit is more convenient, simple and rapid to use and is more sensitive than the immune diffusion assay with a limit of detection of 1 ng/g (Bankes and Rose, 1989). Fujikawa and Igarashi (1988) modified the RPLA which using high density latex particles for the detection of SEA, SEB, SEC, SED and SEE. The assay can be performed using the SET-PPLA commercial kit (Denka Seiken Co.Ltd, Tokyo Japan). The uses of this method provides some advantages including a simpler procedure and a shorter incubation time (reduce from 20-24h to 4h). Moreover, there is no need for expensive equipment but similar limitations are seen compared to traditional ELISA assays. Therefore, SET-RPLA kits could be used for the rapid detection of toxins in variety of foods (Park and Szabo, 1986) but will require more sophisticated analytical techniques to adequately identify and quantify the toxins.

2.5.4. Mass-spectrometry based methods

The limitations outlined in previous sections, more specifically the fact that all methods described above do not provide any information on the toxin chemical properties, the development of new analytical alternatives to detect, identify and quantify SEs in food matrices are needed. Moreover, the lack of available antibodies against the newly describes SEs has lead bioanalytical scientist to develop direct detection methods base on specific physicochemical properties. Mass spectrometry (MS) has become an indispensable technique for the identification, characterization and quantification of proteins. The method provides unparalleled specificity, rapidity and reliable analytical results (Hennekinne *et al*., 2010; Chaerkady and Pandey, 2008; Griffiths *et al*., 2001). The development and implementation of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) has allowed structural characterization of biomolecules especially proteins and peptides (Griffiths *et al*., 2001). However, single MS cannot be used for all proteins and all purposes (Hennekinne *et al*., 2010). Therefore, the MS method requires the development of a series of techniques and liquid chromatography coupled to tandem MS is applied for the sensitive identification of complex protein mixtures (Chaerkady and Pandey, 2008). Recent studies illustrated that LC-MS proteomic strategies can be applied to develop more selective, accurate and precise assays for the characterization and quantification of SEs (Brun *et al.,* 2007; Callahan *et al*., 2006; Bernardo *et al*., 2002). Isotopically labeled internal standard is one of the popular strategies in which the relative concentration of proteins can be measured by isotopic dilution. Various labeling methods have been developed and the mass tags can be introduced into peptides by chemical or metabolic labeling techniques (Kito and Ito, 2008). Accordingly, known amounts of isotope labeled synthetic peptide standards

are combined with the analyte and then two peptide ions of isotope pairs can be simultaneously analysed by LC-MS/MS. The mass difference between the labeled and unlabeled peptide can be distinguished and the absolute amount of the analyte is calculated based on ratio of peak intensity between isotope pair ions (Kito and Ito, 2008).

2.6. Analytical strategies

2.6.1. Proteomic methods used in mass spectrometry

Proteomic is a promising tool for studying global gene expression profiles at the protein levels (Yan and Chen, 2005). In general, proteomics involve the profiling of protein component, identifying their modifications and measurement of protein abundance through the use of purification and characterization techniques (Kito and Ito, 2008). There are many proteomic methods that play an important role for understanding the alterations of biological systems especially protein structures, activities and interactions (Cravatt *et al*., 2007). Among proteomic techniques, mass spectrometry has become the most powerful tool to generate information on the structure and mass of the peptide due to its high sensitivity and specificity. Furthermore, MS-based analyses can provide accurate and precise concentrations (Kito and Ito, 2008; Brun *et al*., 2007; Cravatt *et al.,* 2007). Currently, there are several widely used methods to generate global quantitative protein profiles including top down and bottom up approaches but most of these methods include stable isotope labeling for quantitation (Yan and Chen, 2005).

2.6.2. Liquid chromatography- Mass spectrometry

2.6.2.1. General overview

Since the first introduction of chromatography in the early 20th century, chromatography has become the preferred technique in most bioanalytical laboratories. Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction (IUPAC, 1993). Chromatographic methods include two categories depending on the nature of the mobile phase such as gas chromatography (GC) and liquid chromatography (LC). A mobile phase is described as ‘‘a fluid which percolates through or along the stationary bed in a definite direction’’. It may be a liquid, a gas or a supercritical fluid, while the stationary phase may be a solid, a gel or a liquid. If a liquid, it may be distributed on a solid, which may or may not contribute to the separation process (IUPAC, 1993).

GC is suitable for gaseous or volatile substances that are heat-stable (Manz *et al.,* 2004) and is not adequate for peptide and protein analysis. Unlike GC, liquid chromatography (LC) is more versatile and can be applied to safely separate a very wide range of organic compounds from small molecules, such as drugs and metabolites up to larger molecules such as peptides and proteins (Manz *et al.,* 2004). The majority of LC separations can be classified as normal and reversed phase chromatography. In normal phase chromatography, the stationary phase consists of hydrophilic material for instance silica particles and the mobile phase is a hydrophobic organic solvent such as hexane. In reversed phase chromatography, the stationary phase is hydrophobic and the mobile phase is a mixture of polar solvents such as water and acetonitrile. Chromatography can

separate components from a complex mixture by differential adsorption between a stationary phase and a mobile phase. The separation is based on the chemical properties of the analytes, the stationary phase and the mobile phase which requires optimization to obtain adequate selectivity. Traditional LC methods use ultraviolet-visible, fluorescence, electrochemical and refractive index detectors, but more recently LC was couple to single or multistage MS to significantly enhance the selectivity and sensitivity as well as to obtain structural information on targeted analytes.

Nowadays, MS is a widely used in a number of fields such as chemistry, biochemistry (Siuzdak, 1994), pharmacology (Fenselau, 1992), microbiology (Easterling *et al.,* 1998) and the proteomics (Pandey and Mann, 2000). The development of MS is a direct consequence of the improvement of soft ionization techniques like electrospray (ESI) and matrix-assisted laser desorption ionization (MALDI) (Karas and Hillenkamp, 1988) which allowed the direct analysis of polar, thermally labile biomolecules without fragmentation (Lane, 2005; Griffiths *et al.,* 2001). MS accurately measures the mass to charge (m/z) ratios of ionizable compounds. Generally, ESI-MS analysis requires the samples to be injected using a chromatographic system to avoid problems associated with ionization suppression when analytes are introduced into the ion source (Griffiths *et al.,* 2001; Jonsson, 2001).

2.6.2.2. Electrospray ionisation

ESI was first described by Malcolm Dole when transferred large molecules to the gas phase in the late 1960s (Dole *et al.,* 1968). In 1984, ESI was used for the first time to create gas phase ions for MS analysis (Yamashita and Fenn, 1984). This achievement

was recognized in 2002. The Nobel Prize in Chemistry 2002 was awarded to John B. Fenn specifically for his contribution into the development of ESI-MS. Electrospray ionization is the most common atmospheric pressure ion source currently employed to couple LC to MS (Jonsson, 2001; Yamashita and Fenn, 1984). The ion source is necessary to evaporate the liquid, produce ions and generate an electric field to transport the ions into the orifice of the MS. ESI-MS was introduced by Yamashita and Fenn in 1984 (Yamashita and Fenn, 1984) and has made a significant commercial impact since 1990 (Balogh, 1997). In an ESI source, the liquid from the HPLC is directed through the free end of an electrode (capillary) set at 3 to 5 kV. In the case of pure ESI, the high electric field at the tip of the capillary pulls the liquid emanating from the electrode into a fine jet that breaks up, typically a millimeter from the tip of the electrode, into a fine spray of electrified droplets. The fine droplets in the spray evaporate in about one millisecond to liberate charged molecules from the droplets as ions, which the electric field of the electrode tip then transports toward the entrance of the MS. Currently, the ESI process is one of the softest ionization techniques available and has the strong advantage of generating molecular ions ($[M+nH]^{n+}$ or $[M-nH]^{n-}$). Several other techniques were derived for this general concept such as ionspray (Bruins *et al.*, 1987), microspray (Covey, 1995) and nanospray (Wilm and Mann, 1996). The latter techniques are mainly used for proteomic analysis and the ionspray or pneumatic assisted ESI has a higher nebulization capacity and can accommodate significantly higher flow rates. Ionspray is the most often used version of ESI currently in bioanalysis. ESI is considered one of the mildest desorption techniques available since little or no extra internal energy is imparted to the ions and, therefore, little fragmentation occurs (Bruins *et al*, 1987). Furthermore, one of the most widely accepted features of ESI-MS is that ions observed in a given mass spectrum are preformed in solution. This implies that unless the species

are charged in solution, they will not be seen in the ESI mass spectrum. However, one strong limitation when analyzing large proteins (>50kDa) using ESI-MS is the formation of a wide distribution of multiple charged species expressed in positive mode as $[M+nH]^{n+}$ ions resulting in reduced sensitivity for direct MS analysis (Lane, 2005; Dalluge, 2000).

2.6.2.3. Mass analyzers

After ionization, ionized species are then introduced into a mass analyzer, which separates ions according to their mass to charge (m/z) ratio. There are four basic kinds of mass analyzers currently being used in proteomics research: the ion trap, time of flight (TOF) analyzer, a quadrupole mass analyzer and Fourier transform ion cyclotron resonance (FTICR) analyzers (Lane, 2005; Manz *et al.,* 2004). All four differ considerably in sensitivity, mass resolution, mass accuracy, mass range and capability. However, they can either be used as stand-alone or in some cases put together in tandem to strengthen their advantages (Guerrera and Kleiner, 2005; Lane, 2005). The ion trap uses three-dimensional electric fields to trap ions in a small volume: the ring electrode, the entrance end cap electrode and the exit end cap electrode (Lane, 2005; Mann *et al*., 2001). Hence, the ion trap is also known as the quadrupole ion trap. Ions are subjected to additional electric fields by a radio frequency applied to the ring electrode only. Ions of all m/z values enter the trap at the same time. The ions are held inside once again by changing the electrode voltage and take on an oscillating frequency that related to their m/z value. The amplitude on the ring electrode increases leading to the frequencies of the ion oscillations also increase. When the resonant frequency of an ion reaches the end cap frequency, the ion will become excited into an oscillating motion that is so large.

Consequently, the ion becomes destabilized and depending on voltages applied and their individual m/z ratio, the ion is ejected from the trap along the axis of the end cap by a gradual change in the potentials (Lane, 2005). The coupling of LC with ESI-MS together can be used to determine the molecular structure of an analyte. A particular m/z is selectively isolated from all the other ions in the trap. Fragmentation of this isolated precursor ion can then be induced by collision-induced dissociation (CID) experiments. This stream of ions is ejected selectively onto the detector of instrument to produce the mass spectrum.

2.6.2.4. Detector

A detector collects the signals, transfers information to a computer for calculating the abundances of each ion present. There are several types of detector that are used for ion detection such as: a Faraday cup, a secondary electron multiplier, a scintillation counter or a multichannel plate (Manz *et al.,* 2004). The most common detector is an electron multiplier specifically used to detect the presence of ion signals emerging from the mass analyzer of a mass spectrometer. The task of the electron multiplier is to detect every ion of the selected mass passed by the mass filter. The basic physical process that allows an electron multiplier to operate is called secondary electron emission. When a charged particle strikes a surface it causes secondary electrons to be released from atoms in the surface layer and electrons generate a current that can be recorded.

2.6.3. Peptide separation

Protein identification in biological samples is a substantial task in proteomic. In bottom up proteomic approaches, proteins are digested by enzymes (e.g. trypsin) in order to obtain a collection of proteolytic fragments (e.g. tryptic peptides). Trypsin is the most commonly used enzyme to digest proteins into proteolytic fragments due to its high specificity and ability to digest insoluble or adsorbed proteins. Trypsin cleaves proteins at the carboxyl side of lysine (K) and arginine (R) residues (Liu *et al.,* 2007; Manz *et al.,* 2004). The digestion of target proteins (> 20kDa) into smaller peptides (typically < 4 kDa) is a necessary preparation step to reduce the complexity of the chromatographic condition and MS results (D'Siva and Mine, 2010). This reaction yields a large number of single- and double-charged peptides in solution that maybe separated by LC. Generally, LC is the most common method for the separation of peptides, especially a combination of ion-exchange with reverse phase (Issaq *et al*., 2005). Peptide mixtures are separated to decrease the negative influence of contaminants, thus improving the sensitivity and accuracy by preventing competitive ionization (D'siva and Mine, 2010).

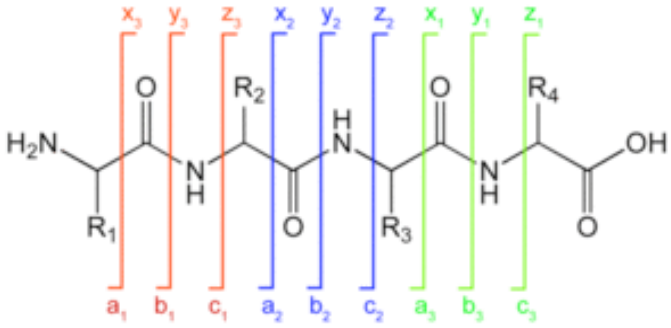
2.6.4. Peptide identification

Peptide mass fingerprinting is now a widely used tool for the identification and characterization of protein (Xu and Ma, 2006; Jonsson, 2001). Peptides of interest are selected for further fragmentation to produce tandem MS spectra using CID tandem MS. Tandem MS and CID provide a comprehensive spectrum allowing structural information to be derived (Callahan *et al.,* 2006). Peptide fragment ions are indicated by a, b, or c if the charge is retained on the N terminal and by x, y or z if the charge is maintained on

the C terminus (van den Broek *et al.,* 2008; Manz *et al*., 2004). Generally using low energy collision cell, the fragment pattern consists of a ladder of peaks of the y ion and a ladder of peaks of b ion. Consequently, the peptide mapping can be derived by the mass differences of adjacent peaks in each of the two ladders (Xu and Ma, 2006). A major advantage of this approach is that only sequences of few fragments and the protein's molecular weight are necessary to unambiguously identify a protein (Callahan *et al*., 2006).

Methods for identifying peptides from tandem spectra can be classified into two basic approaches. In the first, *de novo* sequencing methods, this class of algorithms requires high quality spectra with nearly complete ladders of *b/y* ions. Consequently, the peptide sequence can be derived by the mass differences of adjacent peaks in each of two ladders. Information about protein origin and an estimate of its molecular weight are required in order to improve the chances of a correct match. The second method is database search. There are many types of software programs that have been developed for tandem mass spectrometry peptide identification which can be classified into four classes: (i) database searching, (ii) *de novo* peptide sequencing, (iii) peptide sequence tagging and (iv) consensus of multiple search engines (Xu and Ma, 2006). With tandem mass spectrum, an experimental spectrum will be compared with protein sequence database to find the best matching peptide; *de novo* sequencing computes a peptide directly from the spectrum; sequence tagging combines the two approaches by first conducting *de novo* sequencing to obtain a partial sequence (sequence tag) (Xu and Ma, 2006).

Figure 3. Nomenclature for the product ions generated in the fragmentation of peptide molecules by tandem mass spectrometry (van den Broek *et al*., 2008).



- The fragment ions containing the carboxyl terminus: x_1,, y_1, z_1

- The fragment ions containing the amino terminus: a_1, b_1, c_1

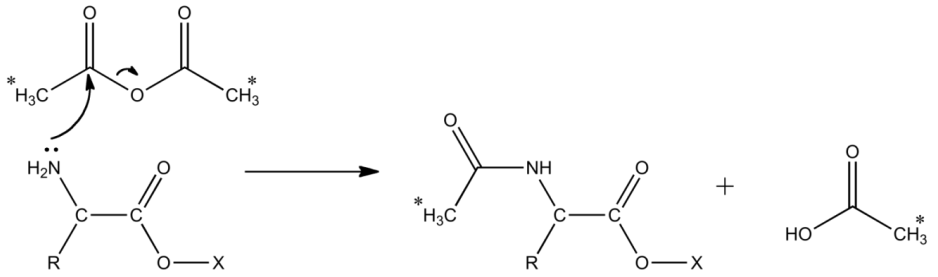
2.6.5. Peptide quantification

For quantification purposes, specific peptides are used as surrogates for the protein of interest (Halquist and Karnes, 2011). Internal standards are used for quantification of peptides with mass spectrometry as the addition of known concentrations to the biological sample will provide accurate concentrations (Kirkpatrick *et al*., 2005). An internal standard is often a surrogate of the molecule containing one or more heavy stable isotopes (D'siva and Mine, 2010). 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 reactions 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. MRM allows researchers to select peptides of interest while

all other peptides are filtered out. Peptides are then detected by MS and the exact concentration can be determined.

In the synthetic internal standard (SIS) approach, synthetic tryptic peptide standards can be used to react with a heavy labeling reagent (^{2}H, ^{13}C, ^{15}N and/or ^{18}O). With the use of the internal standard, the ratios for all MRM transitions of each peptide can be obtained and concentration determined (D'siva and Mine, 2010). These isotopic labels are incorporated into the peptides after their extraction. The relative intensity of MS signals of the heavy and light forms of the labeled peptides reveals the relative amount of the substance (D'siva and Mine, 2010; Wu *et al.,* 2006; Che and Fricker, 2002). For example, peptides can be labeled with either the heavy ($^{2}H_6$) and the light ($^{1}H_6$) form of acetic anhydride. Acetyl group is transferred from acetic hydride to the amino group of the peptide following the reaction (Figure 4).

Figure 4. Generic reaction of tryptic peptide N-terminal modifications with acetic anhydride (Ac_2O) and introduction of differential isotopic tag.



* ^{1}H or ^{2}H
^{2}H is deuterium

HYPOTHESIS

1. Proteomic strategies can be applied to analyze Staphylococcal enterotoxin B in food matrices

2. Isotopic labeling will provide adequate mean for quantification of Staphylococcal enterotoxin B

OBJECTIVES

1. Identify and characterize specific Staphylococcal enterotoxin B tryptic peptides

2. Optimize and adapt the labeling strategy

3. Develop a precise and accurate LC-MS/MS method base on isotopic dilution to quantify SEB in food matrices

Chapter 3. Analysis of *Staphylococcus* Enterotoxin B using Differential Isotopic Tags and Liquid Chromatography Quadrupole Ion Trap Mass Spectrometry

PUBLISHED ARTICLE

Bao,K.D., Letellier,A., Beaudry,F., Biomedical Chromatography, 2012;26:1049–1057

Analysis of *Staphylococcus* Enterotoxin B using Differential Isotopic Tags and Liquid Chromatography Quadrupole Ion Trap Mass Spectrometry

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Keywords: Mass Spectrometry, Stable isotope tags, Quantitative proteomics, Enterotoxins

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 $^{1}H/^{2}H$ molar ratios with a slope near to 1, value of R^2 above 0.98 and %CV obtained from six repeated measurement was below 8%. The precision and accuracy of the method was 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.

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, 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 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 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 radioimmunoassays and enzyme-linked immunosorbent assays 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. 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 to 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 *et al.,* 1987). However, one strong limitation when analyzing large proteins (>50kDa) 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 to 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) provides 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 (^{2}H, ^{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 synthetized and characterized by CanPeptide Inc (Pointe-Claire, QC, CA). 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 (100µg) was dissolved in 2mL of 0.1% (v/v) TFA–water solution (50 µg/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 µg/mL). The peptide stock solutions (100 µg/mL) were diluted in a 0.2 M ammonium bicarbonate buffer (pH 8.5) at a concentration of 200 pmol/mL to prepare the non-isobaric 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. Ac_2O 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 Ac_2O (Standards) or $^2H_6$-Ac_2O (Internal standards) (> 10,000 molar excess) in a microcentrifuge vial (Che and Fricker, 2002). Ten microliters of NH_4OH 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 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 5.0d) 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 $^{2}H_{6}$-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 mix 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 centrifuge at 3000 g for 30 min. The protein pellets were then suspended in 250 µL of 100 mM ammonium bicarbonate (pH 8.5) and 1µg of proteomic-grade trypsin was added. The incubation time was 24h 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, 000g for

60 min. The sample was evaporated by vacuum evaporation and reconstituted in 200µL of 0.2M 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 $^2H_6$-acetylated peptides internal standard generating a nominal $^1H/^2H$ (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 × 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 µL/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°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 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; Strohalm *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) base on 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 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 $^2H_6$-Ac_2O) with long peptides may lead to several products since the reagent will react with the 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 Figure 1. Product ion spectra (MS/MS) of selected acetylated peptides were collected and typical b and y positive charge ion fragments were observed. Figure 4 shows the CID spectra of acetylated **L**GNYDNVR at m/z 992 (H_6-Ac_2O) and 995 ($^2H_6$-Ac_2O) 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- H_2O), 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

IEVYLTT**K** at m/z 1051 (H_6-Ac_2O) and 1057 ($^2H_6$-Ac_2O) 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 – H_2O), 895/898 (y_7), 862/865 (b_7), 844/847 (b_7 - H_2O), 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 **F**TGLMENM**K** at m/z 1155 (H_6-Ac_2O) and 1161 ($^2H_6$-Ac_2O) 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 show 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.

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-Ac_2O or $^2H_6$-Ac_2O 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 Figure 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 kit such as mTRAQ, iTRAQ or TMT. However, this paper suggests an alternative using an acetylation strategy with acetic anhydride ($Ac_2O/^2H_6$-Ac_2O), 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.

Acknowledgment

Khanh Dang Bao is a recipient of a Canadian International Development Agency (CIDA) trainee scholarships and the project is partially funded by the CIDA (Grant No. A032157-001). Francis Beaudry's proteomic research is funded by the National Sciences and Engineering Research Council of Canada (NSERC; Grant No. 386637). The authors would like to thank Ms Karine Giguère for the level 2 confinement coaching and assistance in dealing with pathogenic substances.

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 rapids identification in foods by enzyme-linked immunosorbent assay-based methodology. *Journal of Food Protection* 2005; **68**: 1264-1270.

Bruins AP, Covey TR and Henion JD. Ion spray interface for combined liquid chromatography/atmospheric pressure ionization mass spectrometry. *Analytical Chemistry* 1987; **59**: 2642-2646.

Brun V, Depuis A, Adrait A, Marcellin M, Thomas D, Court M, Vandenesch F and Garin J. Isotope-labled 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 matrixes 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; 167-179.

Cravatt BF, Simon GM and Yates JR. The biological impact 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's). Detection of Staphylococcal enterotoxins in food products using the Vidas® Staph Enterotoxin Ii (Set2), an ELFA (enzyme linked fluorescent assay) technique, method MFLP-65. 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 ES 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. 2003. *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, Dimitri L, 2004. 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 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 enterotoxingenic 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, Beswick EJ and Reyes VE. Staphylococcal enterotoxins. *Toxins* 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. *Analalytical 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.

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)-**L**GNYDNVR	992	1+	974	b_8	37
			818	b_7	
($^{2}H_3$-Ac)-**L**GNYDNVR	995	1+	977	b_8	37
			821	b_7	
($^{1}H_3$-Ac)$_2$-**I**EVYLTT**K**	1051	1+	1014	$b_8^{-H_2O}$	37
			844	$b_7^{-H_2O}$	
($^{2}H_3$-Ac)$_2$-**I**EVYLTT**K**	1057	1+	1020	$b_8^{-H_2O}$	37
			847	$b_7^{-H_2O}$	
($^{1}H_3$-Ac)$_2$-**F**TGLMENM**K**	1155	1+	966	b_8	37
			721	b_6	
($^{2}H_3$-Ac)$_2$-**F**TGLMENM**K**	1161	1+	969	b_8	37
			724	b_6	

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

No	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	FIENENSFWYDMMPAPGDK	2	16.3

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: **L**GNYDNVR (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: **I**EVYLTT**K** (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: **F**TGLMENM**K** (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%
Combined Results				
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%

Figure legends

Figure 1. Generic Reaction of Tryptic Peptide N-terminal Modifications with Acetic Anhydride (Ac_2O) and Introduction of Differential Isotopic Tag

Figure 2. Full Scan LC-MS Chromatogram of SEB Tryptic Peptides

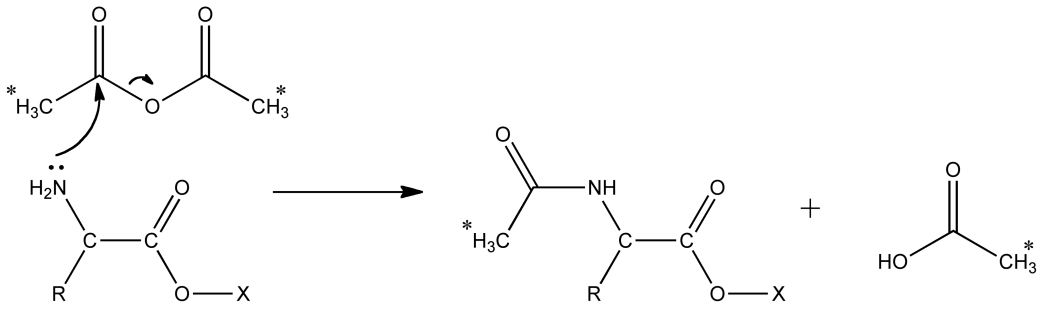
Figure 3. Representative chromatograph of Acetylated SEB Tryptic Peptides Mixed at a molar 1/1 ($^1H/^2H$).

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

Figure 5. Representative blank and LOQ chromatogram for (A) **L**GNYDNVR, (B) **I**EVYLTT**K** and (C) **F**TGLMENM**K**

Figure 6. LC-MS/MS Quantitative Analysis of Selected SEB Tryptic Peptides Labeled with $^1H_6$-Ac_2O or $^2H_6$-Ac_2O using a Reference Internal Standard Strategy

Figure 1



* ^{1}H or ^{2}H

^{2}H is deuterium

Figure 2

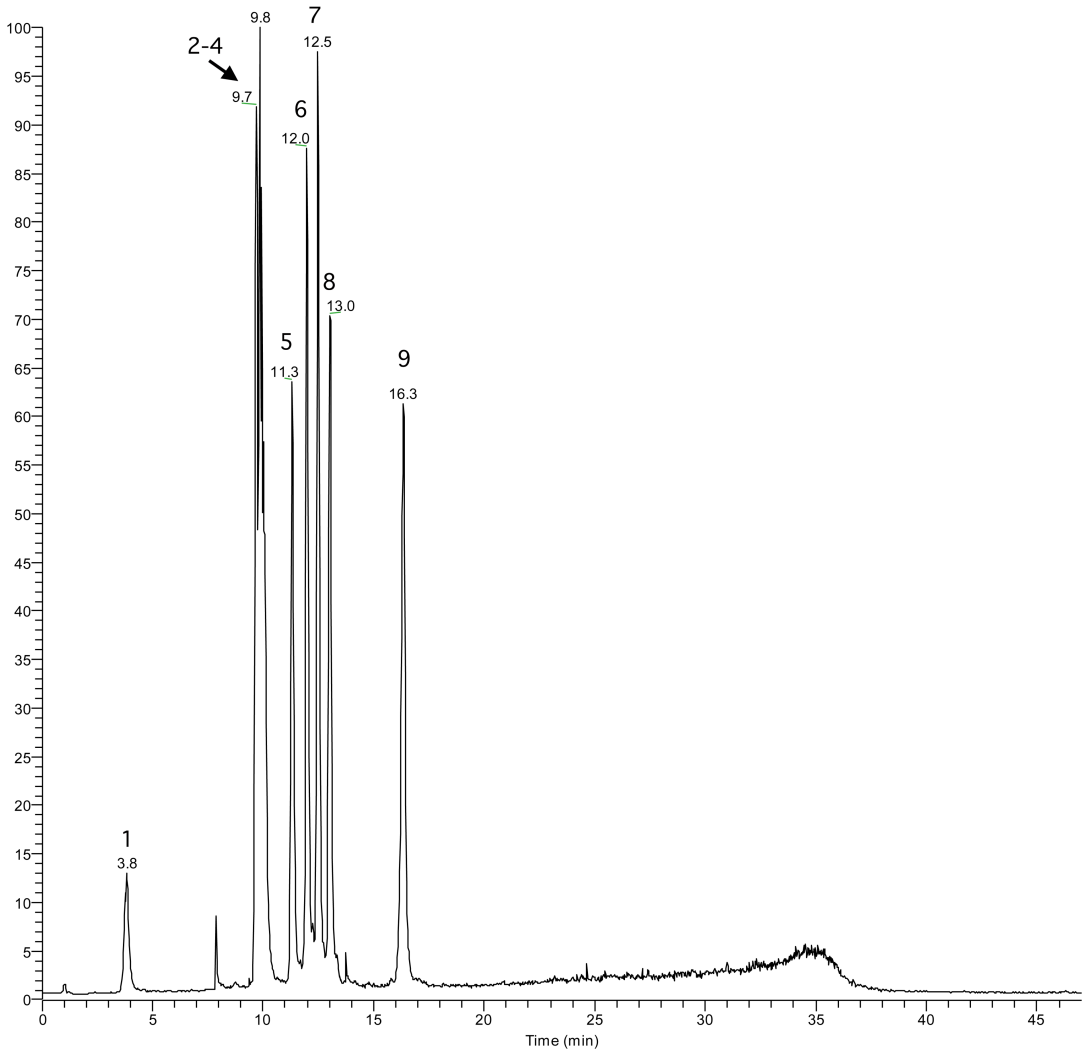


Figure 3

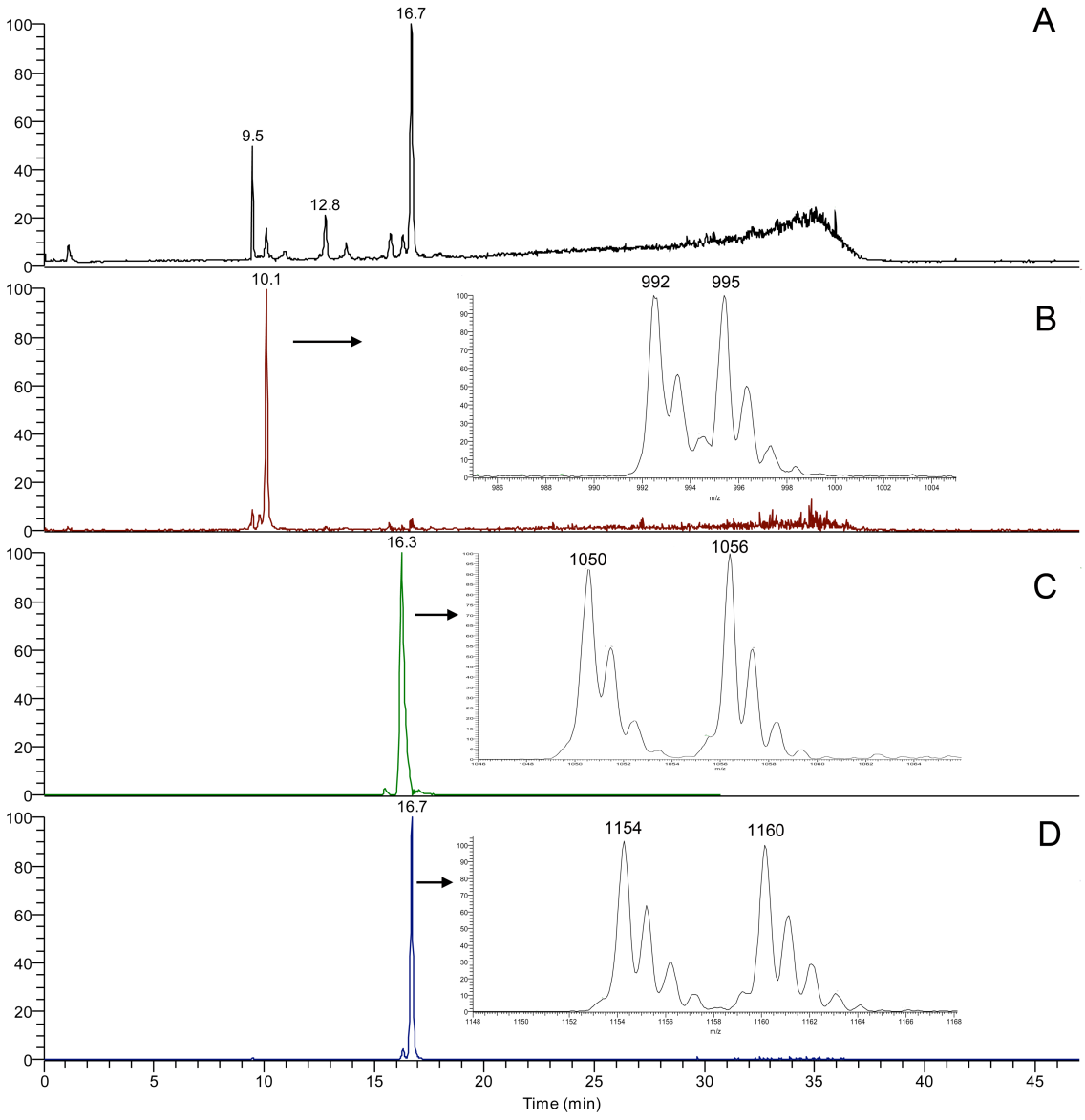

A
16.7
9.5
12.8
B
10.1
992
995
C
16.3
1050
1056
D
16.7
1154
1160
Time (min)

Figure 4

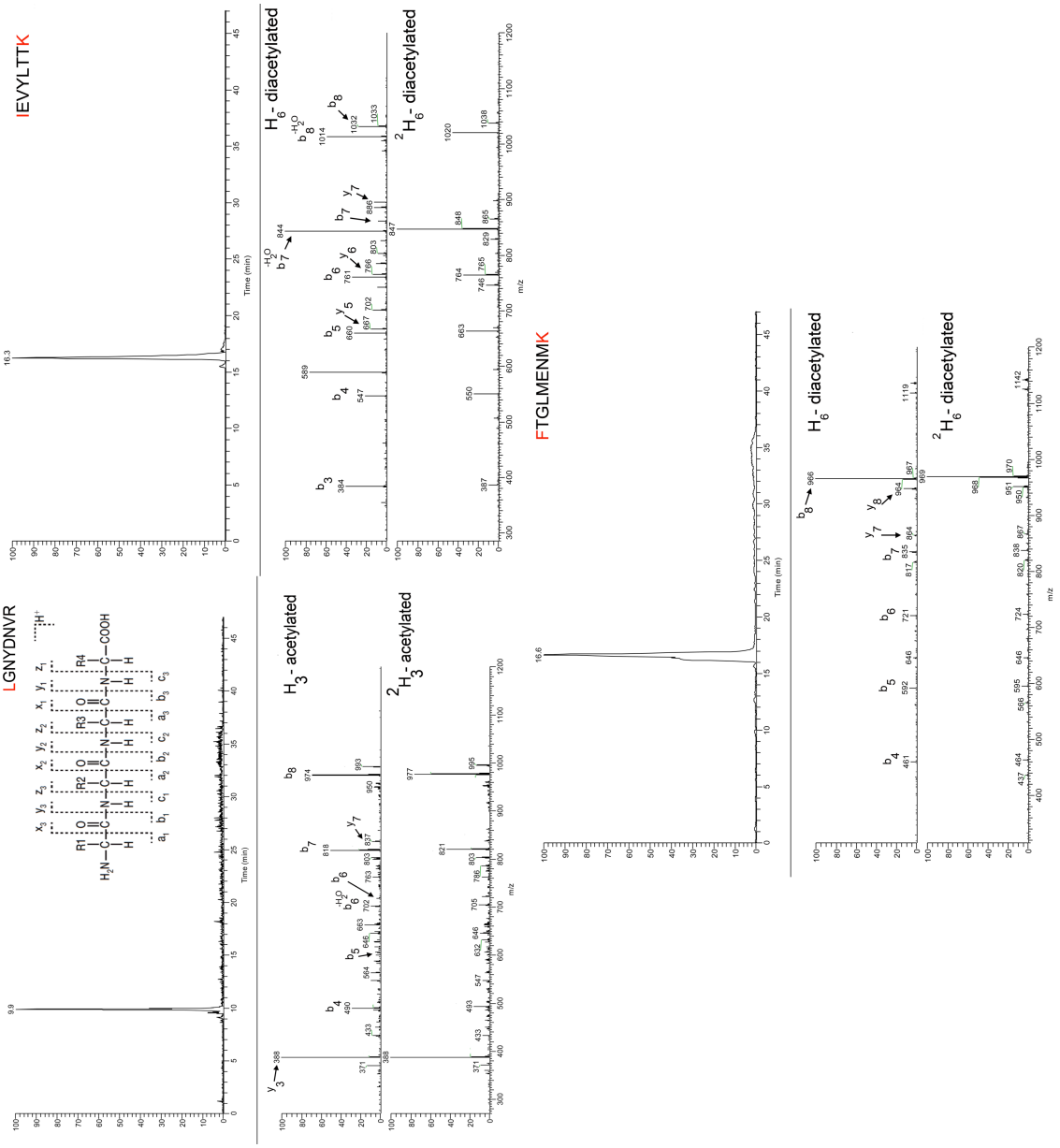

IEVYLTTK
LGNYDNVR
FTGLMENMK
H6- diacetylated
2H6- diacetylated
H3- acetylated
2H3- acetylated
Time (min)
m/z

Figure 5 (A)

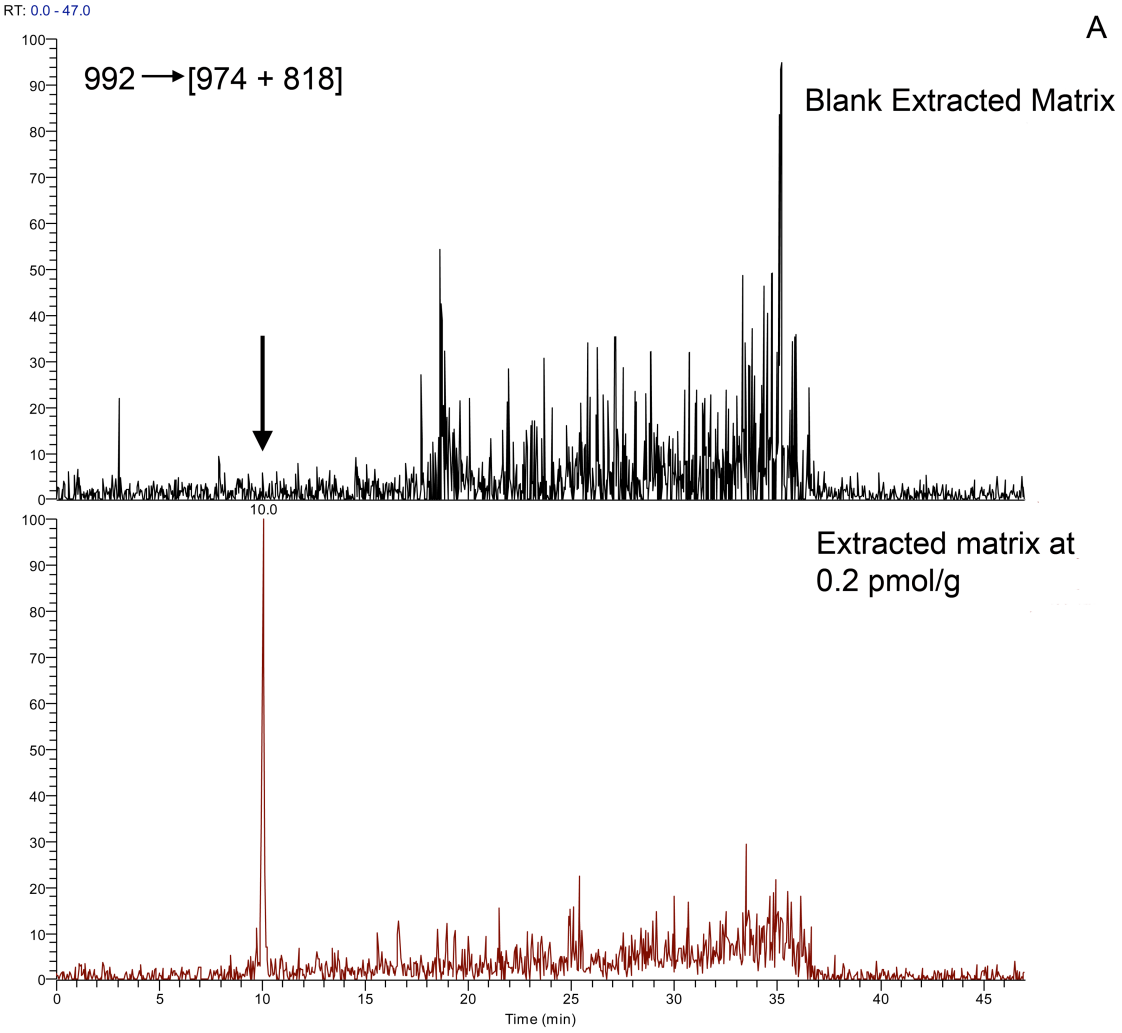

RT: 0.0 - 47.0
A
992 → [974 + 818]
Blank Extracted Matrix
10.0
Extracted matrix at
0.2 pmol/g
Time (min)

Figure 5 (B)

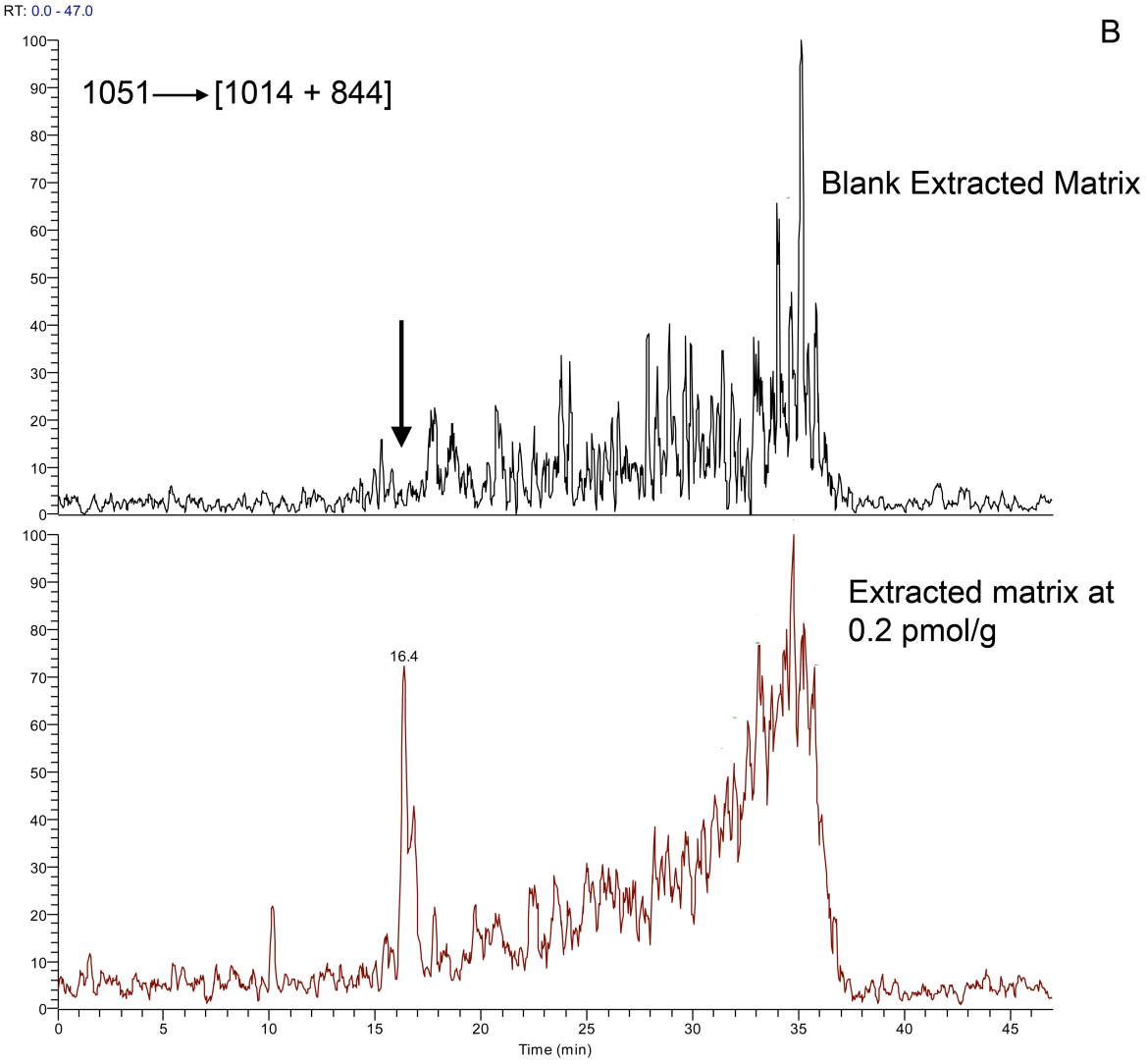

RT: 0.0 - 47.0
B
1051 → [1014 + 844]
Blank Extracted Matrix
Extracted matrix at
0.2 pmol/g
16.4
Time (min)

Figure 5 (C)

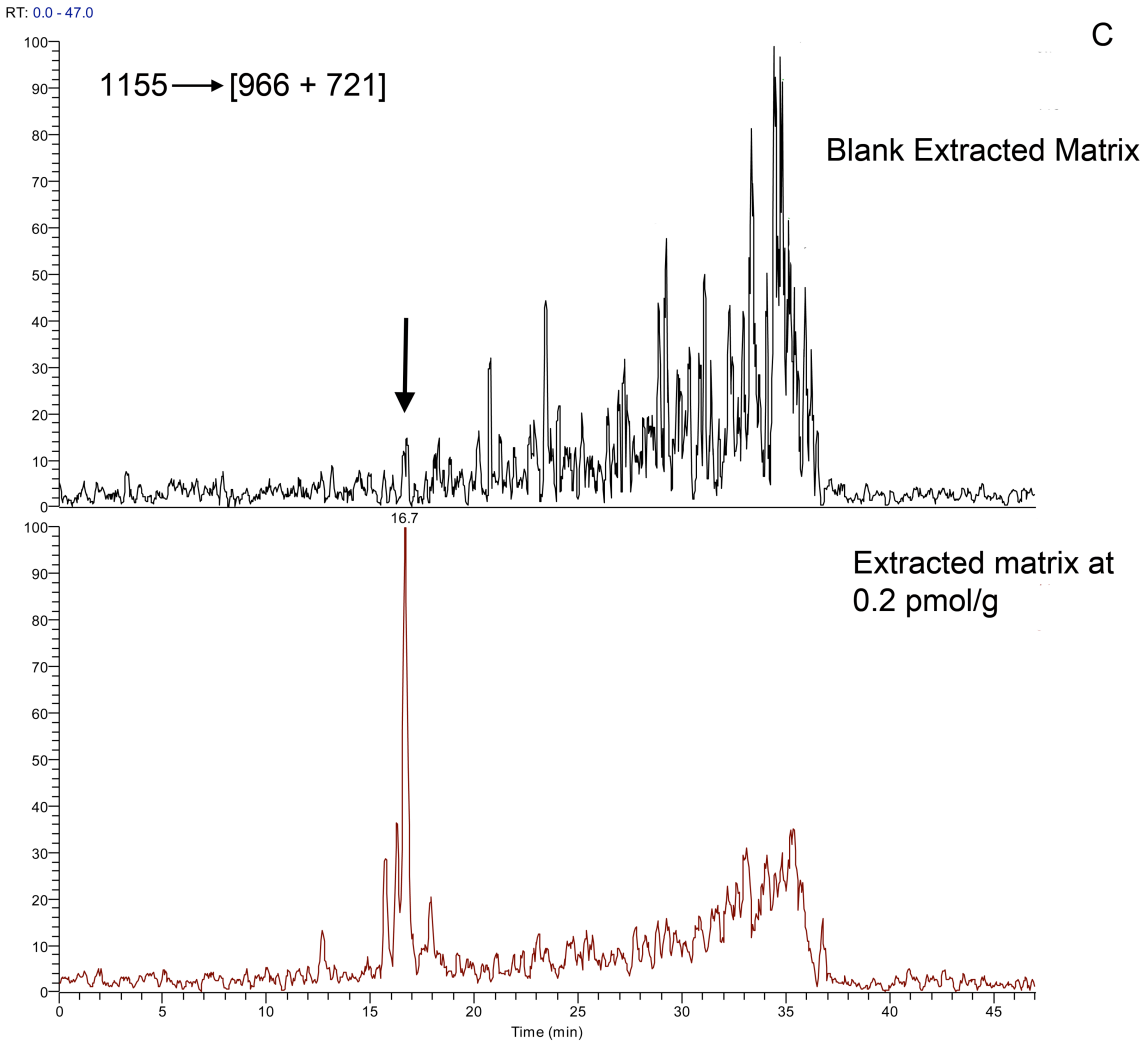
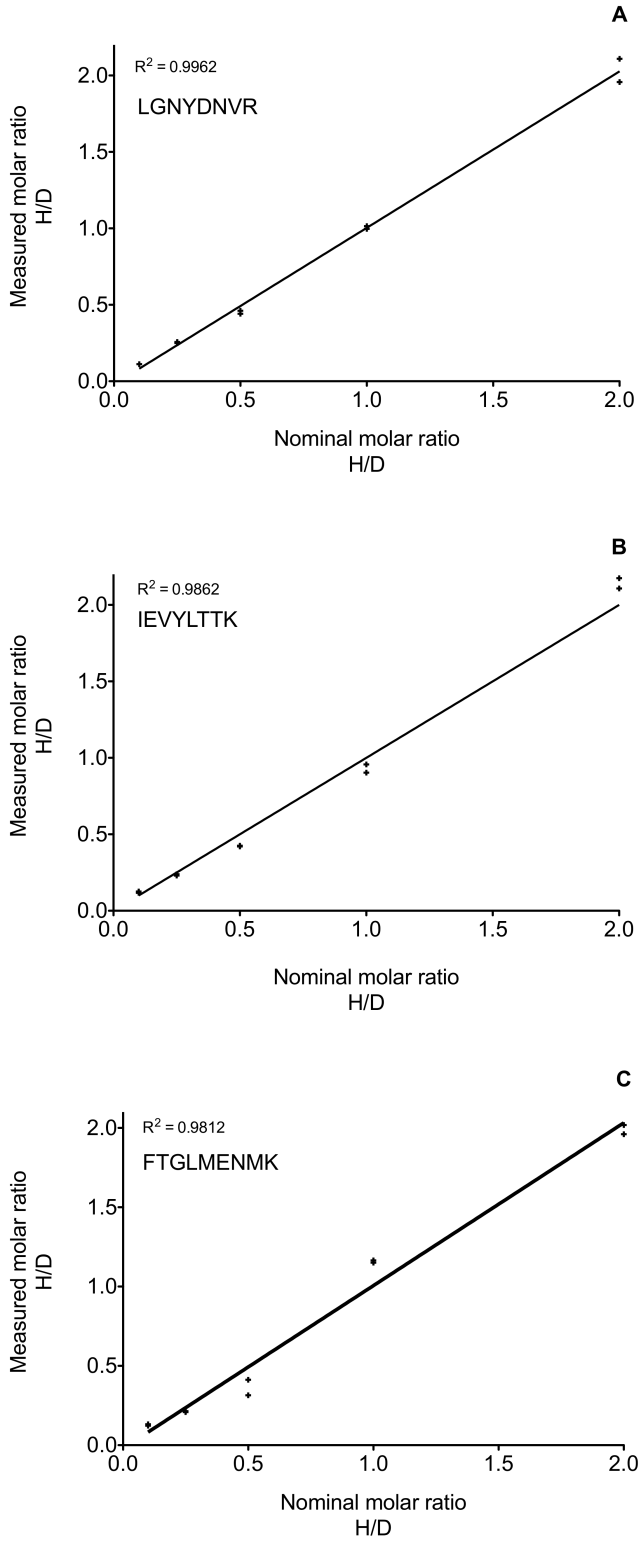

RT: 0.0 - 47.0
C
1155 → [966 + 721]
Blank Extracted Matrix
16.7
Extracted matrix at
0.2 pmol/g
Time (min)

Figure 6


A
R2 = 0.9962
LGNYDNVR
Measured molar ratio H/D
Nominal molar ratio H/D
0.0
0.5
1.0
1.5
2.0
B
R2 = 0.9862
IEVYLTTK
Measured molar ratio H/D
Nominal molar ratio H/D
C
R2 = 0.9812
FTGLMENMK
Measured molar ratio H/D
Nominal molar ratio H/D

Chapter 4. GENERAL DISCUSSION

Contamination by toxigenic *S. aureus* in food matrices is an important problem in both developing countries (e.g. China, Vietnam) and developed countries (e.g. Canada, France, USA, Japan). Staphylococcal food poisoning is a common foodborne disease caused by enterotoxin-producing *S. aureus*. The contamination is mainly associated with improper handling of cooked or processed foods (Pinchuk *et al.,* 2010). Furthermore, inadequate storage can allow *S. aureus* growth and toxins production, resulting in food poisoning. Based on report of The European Food Safety Authority, in 2008, 9.8% of notified food poisoning outbreaks were related to bacterial toxins. Among bacterial toxin, SEs were responsible for 5.5% of all notified outbreaks, but this percentage is certainly underestimated due to poor analytical performances of the reference methods to detect and quantify SEs in food (Hennekinne *et al.,* 2010). Classical SEs can be routinely detected by immunoassay-based methods such as EIA, ELISA, immunodiffusion, RIA and RPLA and a number of commercial kits are currently available (e.g. SET-RPLA and SET-EIA). However, the unavailability of immunoassay kit for new SEs or SAgs can lead to an incomplete diagnosis in the analysis of food extracts from SFP outbreaks (Hennekinne *et al.,* 2010; Vasconcelos and Cunha, 2010). Moreover, false positive or false negative results may occur when other molecules specifically or non-specifically reacts with the antibody binding site (Callahan *et al.,* 2006). The most common disadvantage involved in EIA kits designed for detecting SEs, is the high frequency of false positive results which can range up to 85% as a result of cross-reaction with unrelated antigens (Park *et al.,* 1992). Immunological assays rely on the reaction between antibody and antigen but the reaction between peroxidases in foods and the colorogenic used in the assay may give misleading results (Vernozy-Rozand *et al.,* 2004). SEs detection on culture supernatant by classic immune diffusion, agglutination and ELISA assays is lengthy and does not always selectively detect toxins

at low concentration. In addition, immunological assays detect all forms of the peptide that react with antibody and do not provide any information on the precise molecular entity. Another drawback of the immunological assay is that it requires advanced knowledge of the epitope and generation of the appropriate antiserum. The sensitivity and specificity of these methods always depends on obtaining detectable amount of toxins and may vary significantly with reagent purity (Cremonesi *et al.,* 2005). The limit of detection of RIA is less than 1 ng/g of food (Janin *et al.,* 1983; Miller *et al.,* 1978). RPLA method is able to detect concentration of SEs only above 1 ng/g of foods. Sensitivity levels of ELISA were reported 0.25-0.1ng of SEB per g (Kijek *et al*., 2000); LOD of time resolved fluorometry is 4 to 20 pg/g (Nedelkov and Nelson, 2003). Recently, experimental tests were developed for some SEs (SEG, SEH and SEI), but they are not commercialized due to difficulties in purification and preparation of specific antibodies (Cremonesi *et al.,* 2005). Due to the lack of specificity and sensitivity of the assays, the SEs detection in food matrices by immunological methods as a routine analysis is unreliable.

In order to investigate the trace of the strains isolated from SFP outbreaks, molecular methods involving PCR analysis were used for the detection of superantigenic toxin genes. PCR is considered as a reliable tool for detecting genes with high sensitivity and accuracy (Vasconcelos and Cunha, 2010). PCR can detect not only live but also damaged and dead micro-organisms in food subjected to thermal processing (Cremonesi *et al.,* 2005). Various staphylococcal strains carry more than one *se* gene therefore the presence of new and unexpected genes may lead to false-positive or false-negative results (Vasconcelos and Cunha, 2010). Moreover, PCR can only demonstrate the presence or absence of genes encoding SEs in contaminated food without indicating

whether enterotoxins are produced (Hennekinne *et al.,* 2010; Vasconcelos and Cunha, 2010). Recently, several PCR-based methods were used for SEs typing but these methods are time-consuming and laborious because many separate reactions are required to identify subsets of different *se*. This method allows for characterization of *S. aureus* strains involved in food poisoning but it is not applicable for the SEs detection and confirmation of *S. aureus* in SFP outbreaks because those methods do not indicate whether those strains were able to produce detectable or poisonous levels of toxin in food.

More recently, some authors reported results using proteomics approaches for detection and absolute quantification of SE in foods (Hennekinne *et al.,* 2010). The development of new analytical strategies based on LC-MS/MS has emerged as a new perspective to properly characterize and investigate SFP. Kientz *et al* (1997) has reported that they were able to detect SEB at levels down to 3 pmol/g by on-line (micro) liquid chromatography-electrospray mass spectrometry. They demonstrated that MS is useful tool for the analysis of protein toxins and specifically they were able to generate SEB tryptic peptides and obtain coherent MS and MS/MS data (Kientz *et al.,* 1997). In 2002, Kawano *et al* (2002) carried out rapid isolation, quantification and identification of SEs by LC-ESI/MS determined by its N-terminal amino acid sequences of separated peaks. Based on Kawano's method, a wide variety of SEs were characterized by LC-MS (Kawano *et al.,* 2002). Bernardo *et al.* (2002) developed a MALDI-TOF method for detection of SEs and demonstrated that this approach was adequate for the detection of SEs in culture supernatants. Callahan *et al.* (2006) detected and quantified SEB in apple juice using LC-ESI/MS. The method was able to provide a limit of detection of 80 ng of SEB. Callahan *et al.* (2006) showed that, the presence of SEB can be confirmed at

concentration as low as 5 ng/g and the method is applicable for the detection of SEB in other water miscible food matrixes (Callahan *et al.,* 2006). More recently, Brun *et al.* (2007) used isotope-labeled protein standard to perform absolute quantification of SEA and TSST-1 in spiked water or urine samples but not in food matrices. These previous studies have shown that MS is a sensitive technique and it provides specific, rapid and reliable analytical quantification (Callahan *et al.,* 2006). However, almost all the current methods described in the literature are laborious, expensive and time consuming (Che and Fricker, 2002).

In our study, we developed a new mass spectrometry based method to detect, identify and quantify SEB. A reliable method for the analysis of SEB in food matrices is a corner-stone in the detection and quantification of the enterotoxigenic strains. Stable isotope labeling in combination with mass spectrometry is an alternative method to typical analytical techniques and commonly used in proteomic. The method for the quantification of SEB described in our manuscript has several advantages over previous methods (e.g. RIA, ELISA) including improved specificity essentially because the mass analyzer separate molecule based on the specific molecular structure of the analytes. Additionally, only the sequences of a few fragments (tryptic peptides) and the protein's molecular mass are necessary to clearly identify a protein. The method proposed does not directly require SEB to be manipulated during routine analysis since it used specific tryptic peptides to perform the detection and quantification. The manipulation SEB in laboratory represents an added safety challenge and regulatory agencies limit the availability of pure reference standard making traditional approach difficult to perform during routine analysis. The analytical method described has been developed to overcome those limitations. Protein sequence information can be obtained from several

types of enzymatic digestion methods. Tandem MS and collision-induced dissociation (CID) provide a comprehensive spectrum allowing structural information to be derived. Another advantage of labeling the amines is that, for many peptides, acetylation of the N-terminus and the Lys side chains reduces the charge state of the parent peptide, thus facilitating the interpretation of tandem MS data. Additionally, there are many software programs for interpretation of MS data taking into account this specific modification. Furthermore, the MS- based methods are extremely selective and the sensitivity and particularly in MRM. The utilization of MRM mode reduces background noises and enhances the specificity of measurement. Absolute quantification was achieved by the addition of a known quantity of stable isotope labeled standard peptides. The precision and accuracy of the method were evaluated and showed that this method is accurate and precise. The variation between the nominal and observed ratios of standard peptide was below 8%. The accuracy results indicated that the method provided accuracy within 84.9-91.1% range. The quantification using differential isotopic tags provided very good sensitivity, linearity and dynamic range. The selectivity of MS permits the identification of peptides that are specific to one particular enterotoxin.

Despite all the methodological and technical advantages mentioned above, our method still has some shortcomings. The principal limitation of this technique is the possible variable yield of the derivatization reaction observed during inter-laboratory studies, thus limiting the method transferability. Moreover, the robustness and repeatability of the method depend on the quality of the reagents used for derivatization and the level of training provided to technicians. Quantitative LC-MRM assays used for SEs analysis must use a standardization method to reduce technical and instrument variation. Stable isotope-labeled peptides, created chemically *de novo* have certainly many advantages

since resulting internal standards would preserved very high similarities with the physico-chemical properties (except for the mass) of the targeted peptides. It is widely recognized that using stable isotope-labeled internal standard will offer the best guarantee for high specificity, reproducibility and precision of the method, since it diminishes problems with calibration and sample preparation matrix effects associated with the analysis of complex biological samples (Aman *et al.,* 2006). Different amino acids residues are available incorporating labeled atoms such as ^{13}C, ^{15}N or ^{2}H (or d for deuterium). Conceptually, since stable isotope-labeled internal standard are nearly identical in structure and assuming they co-elute with the analyte, the degree of ionization suppression or enhancement caused by the co-eluting matrix components should be compensated by the internal standard (Elliot *et al.,* 2009; Aman *et al.,* 2006). Therefore, while the absolute response might be affected, the analyte to IS peak area ratio should be unaltered and consequently, the figure of merits should improved using stable isotope-labeled internal standards. There are other considerations when using stable isotope-labeled internal standard. Isotopic clusters of light and heavy peptides, especially for multiply charged species need to be assessed adequately. Bioinformatic tools are available to quickly make simulations that would help to properly determine the adequate number of label atoms needed to be incorporated to avoid isotopic pollution (Ong and Mann, 2005; Cappadona *et al*., 2011). Ideally, singly charged precursor ion isotope cluster should be separated by at least 3 Da but doubly and triply charged species are commonly more abundant in ESI-MS for peptides with more than 10 amino acids (Julka and Regnier, 2004). The analysis of doubly or multiply charged species would benefit of larger precursor ion isotope cluster mass differences especially when using unit mass resolution mass spectrometers (Cox and Mann, 2011).

Isotopic tagging reactions are not easily performed since food matrices contains a wide variety of proteins, lipids and other molecular species that can generates non-specific reactions and affect the yield resulting into the distortion of the quantification. Another limitation of our method is that the number of acetyl groups incorporated must provide sufficient mass difference between H-and D-labeled peptides to clearly separate them. Isotopic clusters of light and heavy peptides, especially for multiply charged species need to be assessed adequately to avoid the problem of isotopic pollution. The number of acetyl groups incorporated was apparent from the mass difference between the two peaks (3 mass units per acetyl group). The reaction products obtained from endogenous toxins and structurally identical standards were nearly identical, which is an important detail to apply the analytical strategy proposed in our manuscript.

The results obtained in this study show that proteomics-based methods can be effectively applied for the detection, confirmation and quantitation of SEB in food matrices as well as the diagnosis of SFP outbreaks. Investigation of SFP is generally based on the symptoms and on the presence of the SEs in both food remnants and diarrhea patients (Hennekinne *et al.,* 2010). In some food poisoning cases, it is not possible to characterize a food poisoning outbreak by enumerating the cell in food remnant because in heat treated food matrices, *S. aureus* may be destroyed while the SEs are still present. The amount of enterotoxins needed to cause intoxication is very small and hence sensitive and specific detection is essential. The symptoms intensities depend on the amount of toxins ingested and the susceptibility of each individual. The toxic dose reported for SEB is 200 ng/kg producing symptoms such as vomiting (Mossel *et al.,* 1995). Thus, an adult would need to consume approximately 10-20 μg of SE to suffer from symptoms. However, during SFP outbreaks, the total intake of SEs causing

food poisoning appears to be lower. For instance, an outbreak of food poisoning disease caused by small amounts of staphylococcal enterotoxins A and H in Japan was documented and the total intake of SEA was estimated about 20-100 ng (Ikeda *et al.,* 2005). Ostyn *et al*. (2010) investigated SFP outbreaks caused by SEE and reported that the total intake of SEE was 90 ng. However, the precision and accuracy of the analytical method used were questionable. Therefore, to apply a new method for investigation of SFP outbreak, we need a selective and sensitive method that can detect SEs at very low concentration. In most clinical laboratories, detection methods used rely on microbiological culture, biochemical tests on the isolated bacteria, selective culture medium for coagulase detection, hemolysis and the laborious test for the thermonuclease (Cremonesi *et al.,* 2005). The LC-MRM assay we proposed is suitable for diagnosis and for epidemiological investigation with high accuracy, precision with a limit of detection of 0.2 pmol/g whereas other approaches for the analysis of SEB are not as selective despite being sensitive. Although, stable isotope labeled peptides are quite expensive, they provide significant improvement in method precision. Other commercial kits can be used (e.g. iTRAQ, mTRAQ, TMT) but they are very expensive and not developed for routine analysis. MS-based methods can be developed and validated to analyze all SEs involved in SFP outbreaks. The sensitivity and selectivity of described method show that proteomics based methods can be used for detection, confirmation and quantification of SEB in meat and can be used as a template model for the analysis of other enterotoxins in food matrices for the surveillance program. As mentioned above, the principal advantage of the method is using synthetic peptides as internal standard, the method analytical error was significantly reduce and allow absolute quantification with adequate figures of merits. 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. In summary, the generic method described in the present study can be used to detect, confirm and quantify SEB in food matrices. The method can also be used as models for other SEs and other more toxic proteins. MS based method can be used as a tool for diagnosis and epidemiological investigation for all SEs involves in staphylococcal food poisoning outbreaks. In addition, regulatory agencies enforce food safety surveillance programs along with a system of laboratories can now performed the SEs analysis rapidly using the latest analytical technologies.

Chapter 5. CONCLUSIONS

The linearity of the measurement by LC-MS/MS was evaluated with a slope near to 1 and R^2 above 0.98; the % CV from six repeated measurement was below 8%. The precision and accuracy of the method was evaluated using SEB spike in chicken meat homogenate sample at three distinct concentrations (SEB was fortified at 0.2, 1 and 2 pmol/g) and accuracy results obtained show that the method can provide accuracy within 84.9-91.1% range with a LOQ 0.2 pmol/g of tissue. The use of labeled peptides with $^2H_6$-acetic anhydride for MS absolute quantification of proteins provides adequate figures of merit for identification and quantification of SEB in food matrices. 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 to effectively minimize analytical errors and noise. The method provides excellent sensitivity, selectivity and robustness. All results are within generally accepted criteria for bioassay which denote that quantification strategy are accurate and can be applied to the rapid detection, confirmation and quantification of SEB in meat matrices. The method can be used as models for other SEs and other more toxic proteins. Proteomic-based methods are viable alternative to immunological and molecular methodologies. MS based methods can be used as a tool for diagnosis and epidemiological investigation for all SEs involves in staphylococcal food poisoning outbreaks.

In conclusion, we suggest an alternative isotopic tags using an acetylation strategy with acetic anhydride (Ac_2O / $^2H_6$-Ac_2O) that is affordable, reliable but more importantly, that provide adequate figures of merits for the quantification of SEB in food matrices. Additionally, regulatory agencies reinforced the conditions laboratory can manipulates 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.

BIBLIOGRAPHICAL REFERENCES

Ahanotu, E., Alvelo-Ceron, D., Ravita, T., Gaunt, E. Staphylococcal Enterotoxin B as a Biological Weapon: Recognition, Management, and Surveillance of Staphylococcal Enterotoxin. Applied Biosafety 2006; **11**(3): 120-126.

Alouf, J.E., Freer, J.H. The comprehensive source book of bacterial protein toxins. 2nd edition 1999. Academic Press. PP. 443-456.

Amagai, M., Matsuyoshi, N., Wang, Z.H., Andl, C., Stanley, J.R. Toxin in bullous impetigo and staphylococcal scalded-skin syndrome targets desmoglein 1. Nature Medicine 2000; **6**: 1275-1277.

Aman, C.S., Pastor, A., Cighetti, G., de la Guardia, M. Development of a multianalyte method for the determination of anabolic hormones in bovine urine by isotope-dilution GC-MS/MS. Analytical Bioanalytical Chemistry 2006; **386**: 1869-1879.

Argudín, M.Á., Mendoza, M.C, Rodicio, M.R. Food Poisoning and *Staphylococcus aureus* Enterotoxins. Toxins 2010; **2**(7): 1751-1773.

Arvidson, S., Tegmark, K. Regulation of virulence determinants in *Staphylococcus aureus*. International Journal of Medical Microbiology 2001; **291**(2): 159-170.

ASIA Invest Programme 2006-2007. The Real Situation of Food Production, Processing

Technologies and Trading and some Issues on Food Safety in Vietnam. Available at: http://techfood.progetti.informest.it/regions/survey/vietnam_en.pdf

Avrameas, S. Coupling of enzymes to proteins with glutaraldehyde. Use of the conjugates for the detection of antigens and antibodies. Immunochemistry 1969; **6**(1): 43-52.

Balaban, N., Rasooly, A. Review: Staphylococcal enterotoxins. International Journal of Food Microbiology 2000; **61**: 1-10.

Balogh, M.P. Commercialization of LC-MS 1987-1997: A successful decade in review. LCGC International, 1997; **10**(11):728-737.

Bankes, P., Rose, S.A. Rapid detection of staphylococcal enterotoxins in foods with a modification of the reversed passive latex agglutination assay. Journal of Applied Bacteriology 1989; **67**: 395-399.

Bennett, R.W. Staphylococcal enterotoxin and its rapid identification in foods by enzyme-linked immunosorbent assay-based methodology. Journal of Food Protection 2005; **68**: 1264-1270.

Bennett, R.W., Monday, S.R. Chapter 4. *Staphylococcus aureus*. In: International Handbook of foodborne pathogens. Edited by Miliotis M.D. and Bier J.W. Marcel Dekker Inc. 2003; PP. 41-59.

Bergdoll, M.S., Wong, A.C.L. Staphylococcal intoxications. In: Foodborne infections and intoxications. Edited by Hans Riemann P. and Dean O. Cliver, third edition. Elsevier Inc. 2006; PP. 523-552.

Bergdoll, M.S., Reiser, R.F., Crass, B.A., Robbins, R.N., Davis, J.P. A new staphylococcal enterotoxin, enterotoxin F, asscociated with Toxic Shock Syndrome *Staphylococcus aureus* isolates. Lancet 1981; **317**: 1017-1021.

Bernardo, K., Pakulat, N., Macht, M., Krut, O., Seifert, H., Fleer, S., Hunger, F., Kronke, M. Identification and discrimination of *Staphylococcus aureus* strains using matrix assisted laser desorption/ionization-time of flight mass spectrometry. Proteomics 2002; **2**(6): 747-753.

Bohach, G.A., Dinges, M.M., Mitchell, D.T., Ohlendorf, D.H., Schlievert, P.M. Exotoxins. In: Crossley KB, Archer GL eds. The staphylococci in human disease. New York: Churchill Livingstone 1997; PP. 83-111.

Bohach, G.A., Fast, D.J, Nelson, R.D., Schlievert, P.M. Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses. Critical Review in Microbiology 1990; **17**(4): 251-272.

Bruins, A.P., Covey, T.R., Henion J.D. Ion spray interface for combined liquid chromatography/atmospheric pressure ionization mass spectrometry. Analytical Chemistry 1987; **59**(22): 2642-2646.

Brun, V., Dupuis, A., Adrait, A., Marcellin, M., Thomas, D., Court, M., Vandenesch, F., Garin, J. Isotope-labeled protein standards: toward absolute quantitative proteomics. Molecular and Cellular Proteomics 2007; **6**: 2139-2149.

Bukowski, M., Wladyka, B., Dubin, G. Exfoliative toxins of *Staphylococcus aureus*. Toxins 2010; **2** :1148-1165.

Callahan, J.H., Shefcheck, K.J., Williams, T.L., Musser, S.M. Detection, Confirmation, and Quantification of Staphylococcal Enterotoxin B in Food Matrixes Using Liquid Chromatography-Mass Spectrometry. Analytical Chemistry 2006, **78**: 1789-1800.

Candlish, A.A.G. Immunological methods in food microbiology. Food Microbiology 1991; **8**: 1-14.

Cappadona, S., Munoz, J., Spee, W.P.E., Low, T.Y., Mohammed, S., Van Breukelen, B., Heck, A.J.R. Deconvolution of overlapping isotopic clusters improves quantification of stable isotope-labeled peptides. Journal of Proteomics 2011; **74**: 2204-2209.

Casman, E.P., Bennett, R.W., Dorsey, A.E., Stone, J.E. The micro-slide gel double diffusion test for the detection and assay of staphylococcal enterotoxins. Health Laboratory Science 1969; **6**(4): 185-198.

Casman, E.P. Staphylococcal enterotoxin. Annals of the New York Academy of Sciences 1965; **128**: 124-131.

Casman, E.P., Bennett, R.W. Detection of staphylococcal enterotoxin in Food. Applied Microbiology 1965; **13**(2): 181-189.

Chaerkady, R., Pandey, A., Applications of Proteomics to Lab Diagnosis. Annual Review Pathology: Mechanisms of Disease 2008; **3**: 485-498.

Chapman, B., Eversley, T., Fillion, K., MacLaurin, T., Powell, D. Assessment of Food Safety Practices of Food Service Food Handlers (Risk Assessment Data): Testing a Communication Intervention (Evaluation of Tools). Journal of Food Protection 2010; **73**(6): 1101-1107.

Che, F., Fricker, L.D. Quantitation of neuropeptides in Cpe^{fat}/Cpe^{fat} mice using differential isotopic tags and mass spectrometry. Analytical Chemistry 2002; **74**(13): 3190-3198.

Chesney, P.J. Clinical aspects and spectrum of illness of toxic shock syndrome. Overview. Review of Infectious Diseases 1989; **11**: S1-7.

Chiang, Y.C., Liao, W.W., Fan, C.M., Pai, W.Y., Chiou, C.S., Tsen, H.Y. PCR detection of Staphylococcal enterotoxins (SEs) N, O, P, Q, R, U, and survey of SE types in *Staphylococcus aureus* isolates from food-poisoning cases in Taiwan. International Journal of Food Microbiology 2008; **121**: 66-73.

Clark, B.R., Engvall, E. Enzyme linked immunosorbent assay (ELISA): theoretical and practical aspects. In Enzyme Immunoassay, Maggio ET (ed), CRC Press Inc. Boca Raton, FL 1980; PP. 167-179.

Colacicco, G., Basu, M.K., Buckelew, A.R.Jr., Bernheimer, A.W. Surface properties of membrane systems. Transport of staphylococcal delta-toxin from aqueous to membrane phase. Biochim Biophys Acta 1977; **465**(2): 378-390.

Coleman, D.C., Sullivan, D.J., Russel, R.J., Arbuthnott, J.P., Carey, B.F., Pomeroy, H.M. *Staphylococcus aureus* bacteriophages mediating the simultaneous lysogenic Conversion of β-Lysin, Staphylokinase and Enterotoxin A: Molecular Mechanism of Triple Conversion. Journal of General Microbiology 1989; **135**: 1679-1697.

Covey, T. Analytical characteristics of the electrospray ionization process. In Biochemical and biotechnological applications of electrospray ionization mass spectrometry; Snyder, P., Ed; ACS Symposium Series 619; American Chemical Society: Washington, DC, 1995; Chapter2. PP 21-59.

Cowan, S.T., Shaw, C., Williams, R.E.O. Type strain for *Staphylococcus aureus* Rosenbach. Journal of General Microbiology 1954; **10**: 174-176.

Cox, J., Mann, M. Quantitative, high-resolution proteomics for data-driven systems biology. Annual Review of Biochemistry 2011; **80**: 273-299.

Cravatt, B.F., Simon, G.M., Yates, J.R. The biological impact of mass-spectrometry-based proteomics. Nature 2007; **450**: 991-1000.

Cremonesi, P., Luzzana, M., Brasca, M., Morandi, S., Lodi, R., Vimercati, C., Agnellini, D., Caramenti, G., Moroni, P., Castiglioni, B. Development of a multiplex PCR assay for the identification of *Staphylococcus aureus* enterotoxigenic strains isolated from milk and dairy products. Molecular and Cellular Probes 2005; **19**(5): 299-305.

Cunha, M.L.R.S. Pathogenesis of *Staphylococcus aureus* and Bovine Mastitis. International Journal of Medical and Biological Frontiers 2009; **15**: 1031-1042.

Dalluge, J.J. Mass spectrometry for direct determination of proteins in cells: applications in biotechnology and microbiology. Fresenius' Journal of Analytical Chemistry 2000; **366**: 701-711.

Dinges, M.M., Orwin, P.M., Schlievert, P.M. Exotoxins of *Staphylococcus aureus*. Clinical Microbiology Reviews 2000; **13**(1): 16-34.

Dole, M., Mach, L.L., Hines, R.L., Mobley, R.C., Ferguson, L.D., Alice, M.B. Molecular beams of macroions. Journal of Chemical Physics 1968; **49**: 2240-2249.

D'siva, I., Mine, Y. Chapter 21. Peptidomics for Bioactive Peptide Analysis. In: Bioactive Proteins and Peptides as Functional Foods and Nutraceuticals. 1st Edition Blackwell and Institute of Food Technologists. Chichester, 2010; PP. 307-324.

Easterling, M.L., Colangelo, C.M., Scott, R.A., Amster, I.J. Monitoring protein expression in whole bacterial cells with MALDI time-of-flight mass spectrometry. Analytical Chemistry 1998; **70**(13): 2704-2709.

Elliot, M.H., Smith, D.S., Parker, C.E., Borchers, C. Current trends in quantitative proteomics. Journal of Mass Spectrometry 2009; **44**: 1637-1660.

Engvall, E., Perlmann, P. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 1971; **8**(9): 871-874.

Evenson, M.L., Hinds, M.W., Bernstein, R.S., Bergdoll, M.S. Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. International Journal of Food Microbiology 1988; **7**(4): 311-316.

FAO (Food and Agricultural Organisation). Pan-European Conference on Food Safety and Quality 2002. Available at: http://www.who.int/foodsafety/publications/fs_management/pec/en/index.html

Fenselau, C. Tandem mass spectrometry: the competitive edge for pharmacology. Annual Review of Pharmacology and Toxicology 1992; **32**: 555-578.

Foster, TJ., McDevitt D. Surfac-associated proteins of *Staphylococcus aureus* : their possible role in virulence. FEMS Microbiol Lett 1994; **118**:199-206.

Freed, R.C., Evenson, M.L., Reiser, R.F., Bergdoll, M.S. Enzyme-linked immunosorbent assay for detection of Staphylococcal enterotoxins in Foods. Applied and Environmental Microbiology 1982; **44**(6): 1349-1355.

Frinck-Barbancon, V., Prévost, G., Piémont, Y. Improved purification of leukocidin from *Staphylococcus aureus* and toxin distribution among hospital strains. Research in Microbiology 1991; **142**: 75-85.

Fujikawa, H., Igarashi, H. Rapid latex agglutination test for detection of staphylococcal enterotoxins A to E that uses high-density latex particles. Applied and Environmental Microbiology 1988; **54**: 2345-2348.

Gamarra, R.M., Manuel, D.M., Piper, M.H., Nachimuthu, S., Balasundaram, P. Food Poisoning 2009. Available at: http://emedicine.medscape.com/article/175569.

Genestier, A.L., Michallet, M.C., Prévost, G., Bellot, G., Chalabreysse, L., Peyrol, S., Thivolet, F., Etienne, J., Lina, G., Vallette, F.M., Vandenesch, F., Genestier, L. *Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. Journal of Clinical Investigation 2005; **115**(11): 3117-3127.

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J.C., Lina, G., Bes, M., Vandenesch, F., Piémont, Y., Brousse, N., Floret, D., Etienne, J. Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine Leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients. Lancet 2002; **359**: 753-759.

Griffiths, W.J., Jonsson, A.P., Liu, S., Rai, D.K., Wang, Y. Electrospray and tandem mass spectrometry in biochemistry. Biochemistry Journal 2001; **355**: 545-561.

Guerrera, I.C., Kleiner, O. Application of Mass Spectrometry in Proteomics. Bioscience Reports 2005; **25**: 71-93.

Haghkhah, M. Study of virulence factors of *Staphylococcus aureus.* Degree of doctor of Philosophy 2003. University of Glasgow, Scotland, UK.

Haines, W.C., Harmon, L.G. Effect of Selected Lactic Acid Bacteria on Growth of *Staphylococcus aureus* and Production of Enterotoxin. Applied Microbiology 1973; **25**: 436-441.

Hall, H.E., Angelotti, R., Lewis, K.H. Detection of the staphylococcal enterotoxins in food. Health Laboratory Science 1965; **2**: 179-191.

Halquist, M.S., Karnes, H.T. Quantitative liquid chromatography tandem mass spectrometry analysis of macromolecules using signature peptides in biological fluids. Biomedical Chromatography 2011; **25**: 47-58.

Hanakawa, Y., Schechter, N.M., Lin, C., Garza, L., Li, H., Yamaguchi, T., Fudaba, Y., Nishifuji, K., Sugai, M., Amagai, M., Stanley, J.R. Molecular mechanisms of blister formation in bullous impetigo and staphylococcal scalded skin syndrome. Journal of Clinical Investigation 2002; **110**: 53-60.

Harris, L.G., Foster, S.J., Richards., R.G. An introduction to *Staphylococcus aureus*, and techniques for identifying and quantifying *S. aureus* adhensins in relation to adhesion to biomaterials: Review. European Cells and Materials 2002; **4**:39-60.

Harshman, S., Boquet, P., Duflot, E., Alouf, J.E., Montecucco, C., Papini, E. Staphylococcal alpha-toxin: a study of membrane penetration and pore formation. Journal of Biological Chemistry 1989; **264**: 14978-14984.

Hennekinne, J.A., Ostyn, A., Guillier, F., Herbin, S., Prufer, A.L., Dragacci, S. How should staphylococcal food poisoning outbreaks be characterized? Toxins 2010; **2**: 2106-2116.

Hennekinne, J.A., Brun, V., De Buyser, M.L., Dupuis, A., Ostyn, A., Dragacci, S. Innovative application of Mass Spectrometry for the Characterization of Staphylococcal Enterotoxins Involved in Food Poisoning Outbreaks. Applied and environmental microbiology 2009; **75**(3): 882-884.

Hoffmann, E., Stroobant, V. Analysis of biomolecules. In: Mass Spectrometry Principles and Applications. 3rd Edition. John Wiley & Sons Ltd. PP. 305-324.

Hughes, C., Gillespie, I.A., O'Brien, S.J. Foodborne transmission of infectious intestinal disease in England and Wales, 1992-2003. Food control 2007; **18**: 766-772.

Huseby, M., Shi, K., Brown, C.K., Digre, J., Mengistu, F., Seo, K.S., Bohach, G.A., Schlievert, P.M., Ohlendorf, D.H., Earhart, C.A. Structure and biological activities of

beta toxin from *Staphylococcus aureus*. Journal of Bacteriology 2007; **189**(23): 8719-8726.

Ikeda, T., Tamate, N., Yamaguchi, K., Makino, S. Mass outbreak of food poisoning disease caused by small amounts of staphylococcal enterotoxins A and H. Applied and Environmental Microbiology 2005; **71**: 2793-2795.

Issaq, H.J., Chan, K.C., Janini, G.M., Conrads, T.P, Veenstra, T.D. Multidimensional separation of peptides for effective proteomic analysis. Journal of Chromatography B 2005; **817**(1): 35-47.

IUPAC (International Union of Pure and Applied Chemistry). Recommendations on Nomenclature for Chromatography. Pure and Applied Chemistry 1993; **65**: 819-872.

Janin, F., De Buyser, M.L., Lapeyre, C., Feinberg, M. Radio immunological quantitative determination of *Staphylococcus aureus* enterotoxin A in various foods. Sciences des Aliments 1983; **3**: 397-412.

Jonsson, A.P. Mass spectrometry for protein and peptide characterisation. Cellular Molecular Life Sciences 2001; **58**: 868-884.

Julka, S., Regnier, F.E. Quantification in proteomics through stable isotope coding: a review. Journal of Proteome Research 2004; **3**: 350-363.

Kass, F.H, Riemann, H.P. Epidemiology of foodborne diseases. In: Infections and Intoxications. 3rd edition edited by Hans P. Reimann and Dean O. Cliver. 2006; PP. 3-24.

Karas, M., Hillenkamp, F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Analytical Chemistry 1988; **60**(20): 2299-2301.

Kauffman, P.E. Enzyme immunoassay for staphylococcal enterotoxin A. Journal Association Official Analytical Chemists 1980; **63**(5): 1138-1143.

Kawano, Y., Ito, Y., Yamakawa, Y., Yamashino, T., Horii, T., Hasegawa, T., Ohta, M. Rapid isolation and identification of staphylococcal exoproteins by reverse phase capillary high performance liquid chromatography-electrospray ionization mass spectrometry. FEMS Microbiology Letters 2002; **189**: 103-108.

Kennedy, M., Villar, R., Vugia, D.J., Rabarsky-Ehr, T., Farley, M.M., Pass, M., Smith, K., Smith, P., Cieslak, P.R., Imhoff, B., Griffin, P.M., Emerging infections program FoodNet working group. Hospitalizations and deaths due to *Salmonella* infections, FoodNet, 1996-1999. Clinical infectious Diseases 2004; **38**: S142-148.

Khan, N.C. Ensuring Food safety in Vietnam: Challenges and prospects. In: Proceedings of Scientific Research and Studies on Food Safety in 2009. PP. 11-22.

Kientz, C.E., Hulst, A.G., Wils, E.R.J. Determination of staphylococcal enterotoxin B by on-line (micro) liquid chromatography-electrospray mass spectrometry. Journal of Chromatography A 1997; **757**:51-64.

Kijek, T.M., Rossi, C.A., Moss, D., Parker, R.W., Henchal, E.A. Rapid and sensitive immunomagnetic-electrochemiluminescen detection of staphylococcal enterotoxin B. Journal of Immunological Methods 2000; **236**(1-2): 9-17.

Kim, P.T. Food safety activities in Vietnam. FAO/WHO Global Forum of Food Safety Regulators 2002. Available at: http://www.fao.org/docrep/meeting/004/ab532e.htm.

Kirkpatrick, D.S., Gerber, S.A., Gygi, S.P. The absolute quantification strategy: A general procedure for the quantification of proteins and post-translational modifications. Methods 2005; **35**(3): 265-273.

Kito, K., Ito, T. Mass spectrometry-Based approaches toward absolute quantitative proteomics. Current Genomic 2008; **9**(4): 263-274.

Klein, S., DeWaal, C.S. Dirty dining. Center for Science in the Public Interest 2008. Available at: cspinet.org/new/pdf/ddreport.pdf.

Kluytmans, J., van Belkum, A., Verburgh, H. Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms and associated risks. Clinical Microbiology Reviews 1997; **10**(3): 505-520.

Kornblum, J., Kreiswirth, B.N., Projan, S.N., Ross, H., Novick, R.P. *agr*: a polycistronic locus regulating exoprotein synthesis in *Staphylococcus aureus*. In: Molecular Biology of the Staphylococci (Novick, R.P. and Skurry, R., eds.). 1990. VCH, New York, USA, PP. 373-402.

Krull, M., Dold, C., Hippenstiel, S., Rosseau, S., Lohmeyer, J., Suttorp, N. *Escherichia coli* hemolysin and *Staphylococcus aureus* alpha-toxin potently induce neutrophil adhesion to cultured human endothelial cells. Journal of Immunology 1996; **157**: 4133-4140.

Labandeira-Rey, M., Couzon, F., Boisset, S., Brown, E.L., Bes, M., Benito, Y., Barbu, E.M., Vazquez, V., Hook, M., Etienne, J., Vandenesch, F., Bowden, G.M. *Staphylococcus aureus* Panton-Valentine leukocidin causes necrotizing pneumonia. Science 2007; **315**(5815): 1130-1133.

Lane, C.S. Mass spectrometry-based proteomics in the life sciences. Cellular and Molecular Life Sciences 2005; **62**(7-8): 848-869.

Larkin, E.A., Carman, R.J., Krakauer, T., Stiles, B.G. *Staphylococcus aureus*: the toxic presence of a pathogen extraordinaire. Current Medicinal Chemistry 2009; **16**(30): 4003-4019.

Le Loir, Y., Baron, F., Gautier, M. *Staphylococcus aureus* and food poisoning. Genetic and Molecular Research 2003; **2**(1): 63-76.

Lee, J.C. An experimental vaccine that targets staphylococcal virulence. Trends in Microbiology 1998; **6**(12): 461-463.

Lee, M.B., Middleton, D. Enteric illness in Ontario, Canada, from 1997 to 2001. Journal of Food Protection 2003; **66**: 953-961.

Ler, S.G., Lee, F.K., 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.

Lina, G., Piémont, Y., Godail-Gamot, F., Bes, M., Peter, M.O., Gauduchon, V., Vandenesch, F., Etienne, J. Involvement of Panton-Valentine leukocidin-producing *Staphylococcus aureus* in primary skin infections and pneumonia. Clinical Infectious Diseases 1999; **29**(5): 1128-1132.

Liu, J., Bell, A.W., Bergeron, J.J.M., Yanofsky, C.M., Carrillo, B., Beaudrie, C.E.H., Kearney, R.E. Methods for peptide identification by spectral comparison. Proteome Science 2007, **5**:3 doi:10.1186/1477-5956-5-3.

Lowy, F.D. *Staphylococcus aureus* infections. New England Journal of Medicine 1998; **339**: 520-532.

Lynch, M., Painter, J., Woodruff, R., Branden, C. Surveillance for foodborne-disease outbreaks- United States, 1998-2002. Morbidity and Mortality Weekly Report 2006; **55**: 1-34.

Mann, M., Hendrickson, R.C., Pandey, A. Analysis of Proteins and Proteomes by Mass Spectrometry. Annual Review of Biochemistry 2001; **70**: 437-473.

Mant, C.T., Chen, Y., Yan, Z., Popa, T.V., Kovacs, J.M., Mills, J.B., Tripet, B.P., Hodges, R.S. Part I: Characterization. In Peptide Characterization and Application Protocols. Edited by Fields GB. Humana Press: Totawa, NJ, 2007; PP. 33-55.

Manz, A., Nicole, P., Dimitri, L. Mass spectrometry. In Bioanalytical Chemistry. Imperial College Press: London. 2004; PP. 85-107.

Martin, M.C., Fueyo, J.M., Gonzalez-Hevia, M.A., Mendoza, M.C. Genetic procedures for identification of enterotoxigenic strains of *Staphylococcus aureus* from three food poisoning outbreaks. International Journal of Food Microbiology 2004; **94**(3): 279-286.

McCormick, J.K., Yarwood, J.M., Schlievert, P.M. Toxic shock syndrome and bacterial superantigens: an update. Annual Review of Microbiology 2001; **55**: 77-104.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M., Tauxe, R.V. Food-related illness and death in the United States. Emerging Infectious Diseases 1999; **5**(5): 607-625.

Miller, B.A., Reiser, R.F., Bergdoll, M.S. Detection of staphylococcal enterotoxins A, B, C, D, and E in foods by radioimmunoassay, using staphylococcal cells containing protein A as immunoadsorbent. Applied and Environmental Microbiology 1978; **36**(3): 421-426.

Mockenhaupt, M., Idzko, M., Grosber, M., Schopf, E., Norgauer, J. Epidemiology of staphylococcal scalded skin syndrome in Germany. Journal of Investigative Dermatology 2005; **124**: 700-703.

Mossel, D.A.A., Corry, J.E.L., Struijk, C.B., Baird, R.M. Essentials of the microbiology of foods: A textbook for advanced studies 1995; Wiley & Sons, Chichester. PP. 146-150.

Murray, P.R., Rosenthal, K.S., Pfalter, M.A. *Staphylococcus* and related organisms. In: Medical Microbiology, 5th edition, 2005. Elsevier Mosby. Edinburgh, UK. PP. 221-236.

Murray, R.J. Recognition and management of *Staphylococcus aureus* toxin-mediated disease. Internal Medicine Journal 2005, **2**: S106-S119.

Nakano, S., Kobayashi, T., Funabiki, K., Matsumura, A., Nagao, Y., Yamada, T. PCR detection of *Bacillus* and *Staphylococcus* in various foods. Journal of Food Protection 2004; **67**: 1271-1277.

Nedelkov, D., Nelson, R.W. Detection of staphylococcal enterotoxin B via biomolecular interaction analysis mass spectrometry. Applied and Environmental Microbiology 2003; **69**: 5212-5215.

Nema, V., Agrawal, R., Kamboj, D.V., Goel, A.K., Singh, L. Isolation and characterization of heat resistant enterotoxigenic *Staphylococcus aureus* from a food

poisoning outbreak in Indian subcontinent. International Journal Food Microbiology 2007; **117**(1): 29-35.

Nilsson, I.M., Hartford, O., Foster, T., Tarkowski, A. Alpha-toxin and gamma-toxin jointly promote *Staphylococcus aureus* virulence in murine septic arthritis. Infection and Immunity 1999; **67**(3): 1045-1049.

Normanno, G., La Salandra, G., Dambrosio, A., Quaglia, N.C., Corrente, M., Parisi, A., Santagada, G., Firinu, A., Crisette, E., Celano, G.V. Occurrence, characterization and antimicrobial resistance of enterotoxigenic *Staphylococcus aureus* isolated from meat and dairy products. International Journal of Food Microbiology 2007; **115**: 290-296.

Normanno, G., Dambrosio, A., Quaglia, N.C., Lorusso, V., Merico, A., Germinario, G.L, Abenante, Narcisi, D., Firinu, A., Celano, G.V. Detection of Staphylococcal enterotoxin A from Ricotta cheese by cell culture. Journal of Food Quality 2006; **29**: 374-382.

Novick, R.P. Pathogenicity factors and their regulation. In: Gram Positive Pathogens (Fischetti, V.A., Novick, R.P., Feretti, J.J., Portnoy, D.A. and Rood, J.I., eds.) 2003. ASM Press, Washington, D.C, USA. PP. 392-407.

O'Callaghan, R.J., Callegan, M.C., Moreau, J.M., Green, L.C, Foster, T.J., Hartford, O.M., Engel, L.S., Hill, J.M. Specific roles of Alpha-Toxin and Beta-Toxin during *Staphylococcus aureus* corneal infection. Infection and Immunity 1997; **65**(5): 1571-1578.

Oda, T., Ohkubo, T., Nagai, M., Nishimoto, Y., Ohmaru, K. Detection of staphylococcal enterotoxins in foods by reversed passive agglutination test. Annu. Rep. Fakuoka City Hyg. Lab. 1979; **4**: 33-37.

Ogston, A. Report upon micro-organisms in surgical diseases. The Bristish Medical Journal 1881; **1**(1054): 369-375.

Ong, S.E., Mann, M. Mass spectrometry-based proteomics turns quantitative. Nature Chemical Biology 2005; **1**: 252-262.

Ono, H.K., Omoe, K., Imanishi, K., Iwakabe, Y., Hu, D.L., Kato, H., Saito, N., Nakane, A., Uchiyama, T., Shinagawa, K. Identification and characterization of two novel staphylococcal enterotoxins, types S and T. Infection and Immunity 2008; **76**: 4999-5005.

Ostyn, A., De Buyser, M.L., Guillier, F., Groult, K., Félix, B., Salah, S., Delmas, G., Hennekinne, J.A. First evidence of a food poisoning due to staphylococcal enterotoxin type E, France, 2009. Eurosurveillance 2010; **15**(13): 19528.

Pandey, A., Mann, M. Proteomics to study genes and genomes. Nature 2000; **405**(6788): 837-846.

Papageorgiou, A.C., Tranter, H.S., Acharya, K.R. Crystal structure of microbial superantigen staphylococcal enterotoxin B at 1.5 Å resolution: Implications for

superantigen recognition by MHC class II molecules and T-cell receptors. Journal of Molecular Biology 1998; **277**: 61-79.

Park, C.E., Akhtar, M., Rayman, M.K. Nonspecific reactions of a commercial enzyme-linked immunosorbent assay kit (TECRA) for detection of staphylococcal enterotoxins in foods. Applied and Environmental Microbiology 1992; **58**(8): 2509-2512.

Park, C.E., Szabo, R. Evaluation of the reversed passive latex agglutination (RPLA) test kits for detection of staphylococcal enterotoxins A, B, C and D in foods. Canadian Journal of Microbiology 1986; **32**: 723-727.

Pinchuk, I.V., Beswick, E.J., Reyes, V.E. Staphylococcal enterotoxins. Toxins 2010; **2**(8): 2177-2197.

Prévost, G., Couppié, P., Monteil, H. Staphylococcal epidermolysins. Current Opinion in Infectious Diseases 2003; **16**(2): 71-76.

Saunders, G.C., Bartlett, M.L. Double-antibody solid-phase enzyme immunoassay for the detection of staphylococcal enterotoxin A. Applied and Environmental Microbiology 1977; **34**(5): 518-522.

Schmitz, FJ., Veldkamp, K.E., Van Kessel, K.P., Verhoef, J., Van Strijp, J.A. Delta-toxin from *Staphylococcus aureus* as a costimulator of human neutrophil oxidative burst. Journal of Infectious Diseases 1997; **176**(6): 1531-1537.

Seo, K.S., Bohach, G.A. *Staphylococcus aureus*. In: Food Microbiology: Fundamentals and Frontiers. Doyle MP and Beuchat LR (eds). ASM Press, Washington, DC 2007, PP. 493-518.

Shafer, W.M., Iandolo, J.J. Chromosomal locus for staphylococcal enterotoxin B. Infection and Immunity 1978; **20**(1): 273-278.

Shingaki, S., Igarashi, H., Fujikawa, H., Ushioda, H., Terayama, T., Saki, S. Study on reversed passive latex agglutination for the detection of staphylococcal enterotoxins A-C. Annual Report of the Tokyo Metropolitan Research Laboratory of public Health 1981; **32**: 128-131.

Siuzdak, G. The emergence of mass spectrometry in biochemical research. Proceedings of the National Academy of Sciences of the USA 1994; **91**: 11290-11297.

Smith, J.L., Bencivengo, M.M. A review of methods for detection of Staphylococcal enterotoxins and evaluation applied to foods. Journal of Food Safety 1985; **7**: 83-100.

Sockett, P.N., Todd, E.C.D. The economic cost of foodborne disease. In: The Microbiological Safety and Quality of Food. Aspen: Gaithersburg, MD, 2000; PP. 1563-1588.

Spero, L., Warren, J.R., Metzger, J.F. Effect of single peptide bond scission by trypsin on the structure and activity staphylococcal enterotoxin B. Journal of Biological Chemistry 1973; **248**: 7289-7294.

Stewart, C.M., Cole, M.B., Legan, J.D., Slade, L., Vandeven, M.H., Schaffner, D.W. *Staphylococcus aureus* growth boundaries: moving towards mechanistic predictive models based on solute-specific effects. Applied and Environmental Microbiology 2002; **68**(4): 1864-1871.

Surgalla, M.J., Bergdoll, M.S., Dack, G.M. Some observations on the assay of staphylococcal enterotoxin by the monkey-feeding test. Journal of Laboratory and Clinical Medicine 1953; **41**(5): 782-788.

Thomas D., Chou, S., Dauwalder, O., Lina, G. Diversity in *Staphylococcus aureus* enterotoxins. Chemical Immunology and Allergy 2007; **93**: 24-41.

Thompson, N.E., Razdan, M., Kuntsmann, G., Aschenbach, J.M., Evenson, M.L., Bergdoll, M.S. Detection of staphylococcal enterotoxins by enzyme-linked immunosorbent assays and radioimmunoassays: comparison of monoclonal and polyclonal antibody systems. Applied and Environmental Microbiology 1986; **51**(5): 885-890.

Todar, K. *Staphylococcus aureus* and Staphylococcal disease. Todar's online textbook of bacteriology 2009. Available at: http://www.textbookofbacteriology.net/staph.html.

Tolan, R.W.J., Baorto, E.P., Baorto D. *Staphylococcus Aureus* infection 2010. Available at: http://emedicine.medscape.com/article/971358-overview.

Uchiyama, T., Imanishi, K., Miyoshi-Akiyama, T., Kato, H. Staphylococcal superantigens and the diseases they cause. In: The Comprehensive Source book of Bacterial Protein Toxins, 3rd ed.; Alouf, J.E., Popoff, M.R., Eds.; Academic Press: Burlington, VT, USA, 2006; PP. 830-843.

Ulrich, R.G., Sidell, S., Taylor, T.J., Wilhelmsen, C.L., Franz, D.R. Staphylococcal enterotoxin B and related pyrogenic toxins. In the textbook of military medicine, warfare, weaponry, and the casuality. Medical aspects of chemical and biological warfare. Falls Church, VA: Office of the Surgeon General, Department of the Army. 1997. Available at: www.bordeninstitute.army.mil/cwbw/Ch31.pdf.

Van, HT. Detection of enteric bacteria in raw food samples from Vietnam and evaluation of antibiotic resistance. Doctor of philosophy 2007, RMIT university, Melbourne Victoria Australia.

VandenBergh, M.F., Yzerman, E.P., van Belkum, A., Boelens, H.A., Sijmons, M., Verburgh, H.A. Follow up of *Staphylococcus aureus* nasal carriage after 8 years: redefining the persistent carrier state. Journal of Clinical Microbiology 1999; **37**(10): 3133-3140.

van den Broek, I., Sparidans, R.W., Schellens J.H.M., Beijnen J.H. Quantitative bioanalysis of peptides by liquid chromatography coupled to (tandem) mass spectrometry. Journal of Chromatography B 2008; **872**: 1-22.

Vasconcelos, N.G., Cunha M.L.R.S. Staphylococcal enterotoxins: Molecular aspects and detection methods. Journal of Public Health and Epidemiology 2010; **2**(3): 29-42.

Vernozy-Rozand, C., Mazuy-Cruchaudet, C., Bavai, C., Richard, Y. Comparison of three immunological methods for detecting staphylococcal enterotoxins from food. Letters in Applied Microbiology 2004; **39**: 490-494.

VFA (Vietnam Food Administration). Conference on summed up the national target program on food safety. Period 2006-2010. 2011. PP. 95-108.

WHO (World Health Organization). Food safety and foodborne illness. 2007; Fact sheet no. 237. Available at: www.who.int/mediacentre/ factsheets/fs237/en/.

WHO (World Health Organization). Five keys to safer food manual. 2006. Available at: www.who.int/entity/foodsafety/publications/consumer/manual_keys.pdf.

Wilm, M and Mann, M. Analytical properties of the Nanoelectrospray ion source. Analytical Chemistry 1996; **68:**1-8.

Wu, H., Wang, A.H., Jennings, M.P., Discovery of virulence factors of pathogenic bacteria. Current Opinion in Chemical Biology 2008; **12**: 1-9.

Wu, W.W., Wang, G., Baek, S.J., Shen, R.F. Comparative study of three proteomic quantitative methods. DIGE, cICAT, and iTRAQ, using 2D gel or LC – MALDI TOF/TO. Journal of Proteome Research 2006; **5**(3): 651-658.

Xu, C., Ma, B. Software for computational peptide identification from MS–MS data. Drug Discovery Today 2006, **11**(13-14): 595-600.

Yalow, R.S., Berson, S.A. Immunoassay of endogenous plasma insulin in man. Journal of clinical investigation 1960; **39**(7): 1157-1175.

Yamashita, M., Fenn, J.B. Electrospray ion source: another variation on the free-jet theme. Journal of Physical Chemistry 1984; **88**: 4451-4459.

Yan, W., Chen, S.S. Mass spectrometry-based quantitative proteomic profiling. Briefings in functional genomics and proteomics 2005; **4** (1):1-12.