DEVELOPMENT OF A DETECTION METHOD OF SOME CLASSICAL STAPHYLOCOCCAL ENTEROTOXINS IN MEAT USING ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY AND TANDEM MASS SPECTROMETRY : A CONTRIBUTION

MARIE DAVIN

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ANNÉE ACADÉMIQUE 2012-2013

(CO)-PROMOTEUR(S): Georges Lognay, Micheline Vandenbol

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Résumé

Le but initial de ce travail était d'adapter une méthode de détection et de quantification des entérotoxines de staphylocoques développée dans le lait à une matrice différente mais aussi complexe °: la viande. Deux entérotoxines parmi les plus impliquées dans l'intoxication alimentaire staphylococcique étaient ciblées °: SEA et SEB. Après l'optimisation du protocole sur la viande, le second objectif était de caractériser ses performances par une validation et d'analyser des échantillons réels, dans lesquels les entérotoxines ont déjà été recherchées.

Le protocole s'articule autour de l'utilisation de filtres de porosités différentes afin d'extraire, de purifier et de concentrer les entérotoxines hors de la matrice. La détection et la quantification est réalisée par chromatographie liquide ultra performante couplée à la spectrométrie de masse en tandem (UPLC-MS/MS). L'optimisation du protocole a été entreprise en plusieurs étapes.

La première partie a été la sélection des peptides selon plusieurs critères spécifiques et à l'aide de bases de données. Après la sélection des peptides, les paramètres LC et MS ont été déterminés afin d'assurer l'identification et la quantification adéquates.

La deuxième partie est l'adaptation du protocole d'extraction du lait à la viande. Le principe général a été suivi, et plusieurs points ont été optimisés. Cette adaptation a été réalisée à l'aide de deux outils supplémentaires. Le premier est un outil immunologique basé sur le principe ELFA qui a été utilisé pour détecter la présence des entérotoxines de staphylocoques à plusieurs étapes de l'extraction, et le second est la méthode électrophorétique du SDS-PAGE, utilisée pour évaluer l'efficacité de la procédure de purification.

La troisième partie est l'étape de digestion qui fait la transition entre les deux parties précédentes. En effet, après l'extraction et la purification des entérotoxines, elles doivent être protéolysées en peptides, les analytes détectés par UPLC-MS/MS. L'efficacité de cette étape a été évaluée en comparant les rendements de digestion, calculés grâce aux facteurs de réponse des signaux obtenus.

Finalement, après avoir travaillé sur toutes les parties séparément, un test final a eu lieu en rassemblant les optimisations différentes afin d'estimer l'efficacité globale du protocole.

En raison de la grande complexité de la matrice et de retards causés par des problèmes techniques au niveau de l'appareil, le deuxième objectif (validation et essais sur des échantillons réels) n'a pas été atteint.

Plusieurs améliorations importantes ont été portées dans l'adaptation à la viande de la méthode.

Le protocole final est le suivant : Les entérotoxines sont extraites de la viande à l'aide d'un solvant aqueux composé de1,5 % de NaCl en solution dans un tampon acétate à pH 4. Le dichlorométhane est utilisé pour extraire les graisses des échantillons et la phase aqueuse est récupérée par centrifugation.

Une série de filtrations, (un filtre seringue en PTFE 0,1 µm comme premier filtre de purification ; un filtre de centrifugeuse 50 kDa MWCO PES comme deuxième filtre de purification et un filtre de centrifugeuse MWCO PES à 5 kDa comme filtre de concentration) est appliquée aux échantillons. Les toxines concentrées sont ensuite préparées pour la digestion à l'aide de dithiothréitol et d'iodoacetamide (respectivement un agent réducteur et un agent d'alkylation) et soumis à une protéolyse (digestion) en solution. La digestion est effectuée par une trypsine modifiée. La digestion se déroule dans un tampon de Trishydroxyméthylaminométhane (Tris) et de chlorure de calcium ($CaCl_2$).

Les peptides résultants de la digestion sont analysés par UPLC-MS/MS avec SPE en ligne. Des standards internes à marqueurs isotopiques sont utilisés pour la détection et la quantification.

Summary

The initial goal of this work was to adapt a detection and quantification method of Staphylococcal enterotoxins developed in milk to a different but also complex matrix°: meat. Two enterotoxins among the most implied in staphylococcal food poisoning were targeted°: SEA and SEB. After optimizing the protocol on meat, the second objective was to characterize its performances by a validation and to analyse real samples, already tested for Staphylococcal enterotoxins.

The protocol is articulated around the use of several size filters to extract, purify and concentrate the toxins out of the matrix. The detection and quantification takes place using Ultra Performance Liquid Chromatography coupled to Tandem Mass Spectrometry (UPLC-MS/MS). The optimisation of the protocol was undertaken in several parts.

The first part was the selection of peptides according to several specific criteria and databases. After the selection of the peptides, MS and LC parameters were determined in order to ensure proper identification.

The second part was the adaptation of the extraction protocol from milk to meat. The general principle was followed and several points were optimized. This adaptation was evaluated using two additional tools. The first is an immunological tool based on the ELFA principle used to detect the presence of Staphylococcal enterotoxins at several steps of the extraction, and the second is SDS-PAGE, an electrophoretic method, used to evaluate the efficiency of the purification steps.

The third part was the digestion step which makes the transition between the two previous parts. Indeed, after the extraction and purification of the enterotoxins, they have to be broken down into peptides, the analytes detected by UPLC-MS/MS. The efficiency of this step was assessed by comparing digestion yields, calculated with the peak areas and response ratios.

Finally, after working on all those parts separately, a final testing took place by bringing all the different optimizations together in order to estimate the efficiency of the global protocol.

Due to the high complexity of the matrix and to delays caused by successive technical issues of the device, the second objective (validation and testing of real samples) was not achieved.

Several important improvements have been brought in the adaptation of the method to meat.

The final protocol goes as follows. Enterotoxins are extracted from meat using an aqueous solvent composed with 1.5% NaCl in pH 4 acetate buffer solution. Dichloromethane is used as a fat extraction solvent and the aqueous phase is recovered by centrifugation.

A series of filtrations, implying a 0.1 µm PTFE syringe filter as first purification filter; a 50 kDa MWCO PES centrifuge filter as a second purification filter and a 5 kDa MWCO PES centrifuge filter as a concentration filter, is applied on the samples. The concentrated toxins are then prepared for digestion using dithiothreitol and iodoacetamid (respectively a reducing and alkylating agent) and submitted to in-solution proteolysis (digestion) by a modified trypsin. The digestion takes place in a buffer made of Trishydroxyméthylaminométhane (Tris) and calcium chloride ($CaCl_2$).

The peptides resulting from the digestion are analysed by online SPE-UPLC-MS/MS and isotopically marked internal standards are used for proper identification and quantification.

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Table 19. Estimation of the toxins recoveries obtained for each series of samples spiked with toxins at key steps of the meat protocol. The recoveries are sorted by selected transition.

List of abbreviations

AB	All blue Standard Molecular Proteins
ACN	Acetonitrile
ANSES	French Agency for Food, Environmental and Occupational Health & Safety
APC	Antigen Presenting Cells
AQUA	Absolute Quantification
C	Cysteine
CNS	Coagulase Negative Staphylococci
CPS	Coagulase Positive Staphylococci
DAD	Diode Array Detection
DB	Dual color Standard Molecular Proteins
DTT	Dithiothreitol
E	Glutamic acid
E_h	Redox Potential
ELFA	Enzyme-Linked Fluorescent Assay
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	ElectroSpray Ionization
ES+	Positive Electrospray Ionization
EU-RL	European Union - Reference Laboratory
FBDs	Foodborne Diseases
G	Glycine
HAc	Acetic Acid
HFO	Formic Acid
HPLC	High Performance Liquid Chromatography
IAA	Iodoacetamid
Ig	Immunoglobulin
IS	Internal Standard
K	Lysine
LC	Liquid Chromatography
M	Methionine
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption/ionization
MHC	Major Histocompatibility Complex
MRM	Multi-residual monitoring
mRNA	Messenger RNA (ribonucleic acid)
MRSA	Methicillin-Resistant *S. aureus*
MS	Mass Spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular Weight
MWCO	Molecular Weight Cut Off
N	Asparagine
PBS	Phosphate Buffer Saline (solution)
PCR	Polymerase Chain Reaction

PEG	Polyethylene glycol
PES	Polyethersulfone
PSAQ	Protein Standard Absolute Quantification
PT	Pyrogenic exotoxins
PTFE	Polytetrafluoroethylene
PVDF	Polyvinylidene Difluoride
Q	Glutamine
QconCAT	Quantification concatamer
QIT	Quadrupole Ion Trap
QqQ	Triple Quadripole
qTOF	Quadripole Time of Flight
R	Arginine
RPLA	Reverse Passive Latex Agglutination
RT-PCR	Reverse Transcriptase PCR
RT-qPCR	Real time PCR
SAgs	Superantigens
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Staphylococcal Enterotoxin
se	Gene coding for a staphylococcal enterotoxin
SEA, SEB,…	Staphylococcal Enterotoxin A, B,…
SE*l*	Staphylococcal Enterotoxins-*like* toxin
SETTECT	Staphylococcal Enterotoxin Detection
SFP(O)s	Staphylococcal Food Poisonning (Outbreaks)
SPE	Solid Phase Extraction
speA	Streptococcal pyrogenic exotoxin A
ssa	Streptococcal superantigen
T	Threonine
TCR	T-cell receptor
Tris	Trishydroxyméthylaminométhane
TSS(T)	Toxic Shock Syndrome (Toxin)
UPLC-MS/MS	Ultra Performance Liquid Chromatography coupled to Tandem Mass Spectrometry
W	Tryptophan

1 Introduction

Food poisoning caused by ingestion of *Staphylococcus aureus* enterotoxins is one of the most common foodborne diseases. *Staphylococcus aureus* is a well-studied, omnipresent bacterium which is not only found in the environment but is also part of the commensal mammalian flora. *S. aureus* produces enterotoxins which can cause gastro-enteritis, emesis or act as superantigen.

This work is a contribution to the development of a new method for the rapid detection and quantification of staphylococcal enterotoxins using online Solid Phase Extraction and Ultra Performance Liquid Chromatography coupled to Tandem Mass Spectrometry (SPE-UPLC-MS-MS).

S. aureus and its toxins have been thoroughly studied in the past decades and many descriptions of this micro-organism can be found in the literature. However a rapid review of its characteristics will help identify the specificities of this bacterium, its toxins and why they cause so much concern.

Afterwards, a review of different existing strategies that were developed in order to characterize staphylococcal food poisoning outbreaks will be described, along with the benefits that would be brought by the development of a new method involving UPLC-MS/MS.

Finally, the objectives of the present work will be presented, followed by the methodologies employed to try and achieve them, the results, and the conclusions and perspectives that can be drawn from it.

2 Staphylococcus aureus

Staphylococcus aureus belongs to the *Staphylococcus* genus which is part of the Staphylococcaceae family and accounts more than 50 species and subspecies. Species are classified in two groups: the coagulase positive Staphylococci (CPS), including *S. aureus*, *S. intermedius* and *S. delphini*, and coagulase negative Staphylococci (CNS), such as *S. epidermidis*, *S. haemolyticus* and *S. capitis*. Some species may also present either a coagulase positive or negative phenotype like *S. hyicus* (Gaebler Vasconcelos *et al*., 2010).

Biologically, *S. aureus* is described as a gram-positive, non-sporulated, catalase positive, facultative anaerobic, chemoorganotrophic and non-motile bacterium. Cells are spherical (cocci) and can be single, paired or form grape-like clusters (as *staphylo* means grape in greek). These organisms possess a respiratory and fermentative metabolism (Le Loir *et al.*, 2003).

The growth of *S. aureus* is influenced by environmental factors such as water activity (a_w), pH, redox potential, temperature… *S. aureus* is capable of growing in a temperature range from 7 to 48.5°C, with an optimum between 30 and 37°C. It is also very tolerant towards pH, as it can grow between pH 4.2 and 9.3 with an optimum between pH 7 and 7.5. Staphylococci are known for their resistance towards harsh environmental conditions, such as sodium chloride concentrations up to 10-15% (w/v), and have the ability to recover from non-physiological environments. This large tolerance exposed in Table1 makes *S. aureus* a ubiquitous organism that can be found in the air, dust, sewage, water, environmental surfaces but also animals and humans. Indeed *S. aureus* is part of the normal flora found on the skin and mucous membranes of mammals and birds (Hennekinne *et al.*, 2011).

Staphylococcus aureus, just as many microbial pathogens, have great capabilities when it comes to colonizing and infecting their hosts. These organisms, described as opportunistic, adhere very strongly to epithelial cells, colonize catheters or other devices and form biofilms. They easily reach the bloodstream and cause infections to high risk patients (intensive care unit patients, pre-term new-borns, cancer and transplanted patients…) (Gaebler Vasconcelos *et al*., 2010).

Table 1. Factors affecting growth and enterotoxin production by *Staphylococcus aureus* (Hennekine *et al*., 2011).

	Organism growth		**Staphylococcal enterotoxin production**	
Factor	**Optimum**	**Range**	**Optimum**	**Range**
Temperature (°C)	37	7-48	37-45	10-45
pH	6-7	4-10	7-8	4-9.6
Water activity (a_w)	0.98	0.83→0.99*	0.98	0.85→0.99**
NaCl (%) (w/v)	0	0-20	0	0-10
Redox potential (E_h)	> + 200 mV	< -200 mV to > +200mV	> + 200 mV	< -100 mV to > +200mV
Atmosphere	Aerobic	Anaerobic-aerobic	Aerobic (5-20% dissolved O_2)	Anaerobic-aerobic

*Aerobic (anaerobic 0.90 → 0.99)
**Aerobic (anaerobic 0.92 → 0.99)

2.1 Staphylococcal enterotoxins

2.1.1 Description and Classification

S. aureus is a pathogen capable of producing various toxins. Staphylococci in general and *S. aureus* in particular are capable of producing toxins named pyrogenic toxic superantigens, including the toxic-shock syndrome toxin (e.g. TSST-1) and staphylococcal enterotoxins (SEs). Many authors have reported the production of one or several enterotoxins by other *Staphylococcus* species such as S. *cohnii*, *S. xylosus*, *S. haemolyticus* and *S. epidermidis* (Ortega *et al*., 2010).

SEs are part of a large group of pyrogenic exotoxins (PT). This group includes SEs, two groups of Toxic Shock-Syndrome Toxins (TSSTs), exfoliatins A and B, and the streptococcal pyrogenic exotoxins. An interesting characteristic all those toxins share, besides their functional effects, is their common phylogenetic relationships, structure and sequence homology (see Balaban & Rasooly, 2000 for a review).

SEs are remarkably stable to factors that easily destroy the bacteria such as heat treatment, freezing, drying, low pH and most proteolytic enzymes (pepsin, trypsin, chymotrypsin, rennin, papain…), except for the TSST-1 toxin. An interesting property of those toxins is that while inactivation through heat varies according to SE type, SE concentration, pH and matrix, some inactivation can be reversed under alkaline pH. Furthermore, heating can in many cases cause a loss of serologic activity but not of biological activity. This means that those toxins are undetectable with antibodies because they lost their serological recognition but remain active (Hennekinne *et al.*, 2010).

2.1.2 Staphylococcal Enterotoxins and Staphylococcal Enterotoxin-like toxins

Staphylococcal Enterotoxins A and B (SEA and SEB) were the first described SEs. For a long time, only five SEs designated SEA to SEE were reported in the literature and because they all were discovered when some major food poisoning outbreaks occurred, all SEs were described as emetic substances, some being stronger than others (Ortega *et al.*, 2010).

Since then, different SEs have been described, bringing the actual number to 23. Many of the new toxins were only predicted by genotyping, from the study of the classic enterotoxins (SEA-SEE) genes sequences or *egc* locus (Pocsfalvi *et al*., 2008). As they were studied, it was discovered that their common property is named superantigenic effect. This effect will be described later on. Because of this, two groups can be identified among those superantigens (SAgs). The toxins named “SE”, standing for Staphylococcal Enterotoxins, possess an emetic property while the “SE*l*” toxins (Staphylococcal Enterotoxins-*like*) either do not induce emesis or have not yet been studied for this property. (Lina *et al.*, 2004).

Table 2. Major characteristics of staphylococcal enterotoxins (Le Loir *et al*., 2003).

SE type	ORF length (bp)	Precursor length (aa)	Mature SE length (aa)	Molecular mass (kDa)	pI
A	774	257	233	27,100	7.3
B	801	266	239	28,336	8.6
C1	801	266	239	27,531	8.6
C2	801	266	239	27,531	7.8
C3	801	266	239	27,563	8.1
C (bovine)	NA	NA	NA	27,618	7.6
C (sheep)	NA	NA	NA	27,517	7.6
C (goat)	NA	NA	NA	27,600	7.0
D	777	258	228	26,360	7.4
E	774	257	230	26,425	7.0
G	777	258	233	27,043	5.7
H	726	241	218	25,210	ND
I	729	242	218	24,928	ND
J	806	268	245	28,565	8.65
K	729	242	219	25,539	6.5
L	723	240	215	24,593	8.66
M	722	239	217	24,842	6.24
N*	720	258	227	26,067	6.97
O*	783	260	232	26,777	6.55

*Named SEK and SEL in Jarraud *et al*., 2001, renamed SEN and SEO, respectively, in a correction note published in *J. Immunol*. 166: 4260 (2001)
NA: not available
ND: not determined

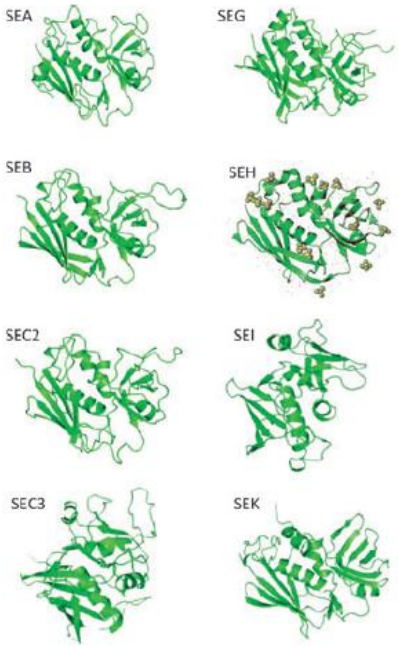

Figure 1. 3D structures of various staphylococcal enterotoxins (Hennekinne et al., 2011).

2.1.3 Nomenclature

So far, 23 serologically distinct staphylococcal SAgs have been described and include TSST-1, SEs A-E, G-J and the SE*l* K-R, U, U2 and V (Ortega *et al.*, 2010). Because of the distinctions that exist between staphylococcal superantigens as regards their emetic activity, the International Nomenclature Committee for Staphylococcal Superantigens introduced in 2004 a new nomenclature for the naming of all these rapidly discovered or predicted proteins.

Only staphylococcal superantigens causing emesis to primates after oral administration should be designated as staphylococcal enterotoxins. Other SAgs that either do not exhibit emetic properties or have not yet been tested should be designated as staphylococcal enterotoxin-*like* toxins (SE*l*) type X (Gaebler Vasconcelos *et al*., 2010). Letters from A to V simply identify toxins in the chronological order they were described, SE*l*V being the last discovered toxin. SEF is the only exception as it was later renamed TSST-1(Lina *et al.*, 2004)

2.1.4 Structure

SEs (and SE*l*s) are secreted proteins with a mature length of approximately 220–240 amino acids and low-molecular weights ranging from 24 to 30 kDa, and are soluble in water and saline solutions (Sospedra *et al.*, 2013). Their major characteristics are listed in Table 2. Their sequences are rich in lysine, aspartic acid, glutamic acid and tyrosine residues. Crystallographic studies show similar three-dimensional SEs structures. The common structural description of SEs is a "small N-terminal α-helix connected to a β-folded sheet known as domain B or oligosaccharide-binding fold (O/B). Such O/B fold is connected to a wall of β-folded sheets by a central diagonal α-helix forming domain A" (Gaebler Vasconcelos *et al*., 2010, pg.36). Several models are exposed in Figure 1.

Slight differences may be observed from one toxin to another, amongst which the cysteine fold is probably the most notable. As many SEs contain a cysteine loop it is believed that this structure is involved in their emetic activity. Interestingly, such fold has also been observed in streptococcal pyrogenic exotoxin A (*spe*A). This added to the observation that high sequence homologies exist between SEs, the streptococcal superantigen (*ssa*) and *spe*A supports the hypothesis of *Staphylococcus aureus* and *Streptococcus pyogenes* toxins to share an ancestral toxin gene or that horizontal gene transfer took place in the evolution of these species (Gaebler Vasconcelos *et al*., 2010).

Finally, the high similarities between SEs sequences, which are exposed in Table 3, allow a classification into five groups according to their homologies. A representation of this classification is schematised in Figure2. Note that only 15% of the amino acid residues are completely conserved throughout all SEs groups (Le Loir *et al.*, 2003; Ortega *et al*., 2010).

Table 3. Percentage of amino acid identity in different staphylococcal enterotoxins (Le Loir *et al*., 2003).

Toxin	SEA	SEB	SEC1	SED	SEE	SEG	SEH	SEI	SEJ	SEM	SEN	SEO
SEA	100	33	30	50	83	27	37	39	64	35	39	37
SEB		100	68	35	32	43	33	31	33	29	32	36
SEC1			100	31	29	41	27	26	30	26	29	33
SED				100	52	27	35	33	51	41	38	39
SEE					100	27	35	35	63	37	39	37
SEG						100	34	28	29	28	31	30
SHE							100	33	35	38	34	31
SEI								100	34	31	31	57
SEJ									100	38	42	33
SEM										100	28	31
SEN											100	42
SEO												100

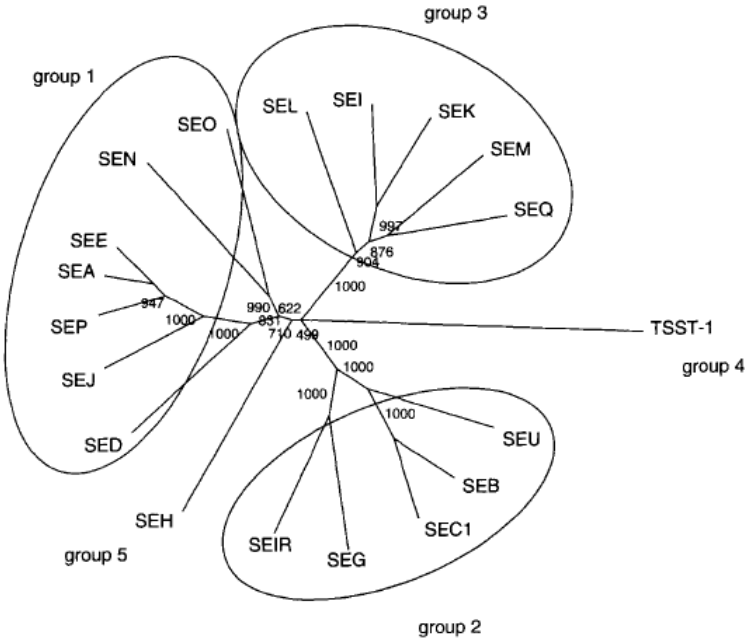

Figure 2. Dendrogram of staphylococcal SAgs (in Popoff's Comprehensive Sourcebook of Bacterial Protein Toxins, pg 832).

2.1.5 Genes

SAgs are accessory proteins meaning they are not necessary for growth and multiplication. Some of their corresponding genes are located on accessory movable genetic elements. A non-exhaustive list of SE genes and their supports is illustrated in Table 4 (Le Loir *et al*., 2003). Because enterotoxin genes are located on movable elements, there is an uneven distribution of SEs between *S. aureus* strains. About 77% of *S. aureus* strains are positive for one or several enterotoxin genes, and it has been observed that some genes tend to co-exist (i.e. *sei* and *seg* or *sej* and *sed*). Besides genes horizontal transfer takes place between strains, which constitutes an important part of their pathogenicity evolution (Ortega *et al*., 2010).

Table 4. Genetic support of some staphylococcal toxins (Hennekinne *et al*., 2010).

Toxin type	Genetic location
SEA	Prophage
SEB	Chromosome, plasmid, pathogenicity island
SEC $_{1\text{-}2\text{-}3}$	Plasmid
SED	Plasmid (pIB485)
SEE	Prophage
SEG	*Enterotoxin gene cluster* (*egc*), chromosome
SEH	Transposon
SEI	*egc*, chromosome
SE*l*J	Plasmid (pIB485)
SEK	Pathogenicity island
SE*l*L	Pathogenicity island
SE*l*M	*egc*, chromosome
SE*l*N	*egc*, chromosome
SE*l*O	*egc*, chromosome
SE*l*P	Prophage (Sa3n)
SE*l*Q	Pathogenicity island
SER	Plasmid (pIB485)
SES	Plasmid (pIB485)
SET	Plasmid (pIB485)
SE*l*U	*egc*, chromosome
SE*l*U_2	*egc*, chromosome
SE*l*V	*egc*, chromosome

2.1.6 Clinical Manifestations

S. aureus is considered a major public health issue because it can cause many infections that range from mild to severe or fatal, either on humans or animals. It is responsible for infecting superficial lesions (abscesses, wound infections…), causing systemic infections (septicaemia, endocarditis, and osteomyelitis) and toxin-mediated diseases like the Toxic Shock Syndrome, Kawasaki's Disease and staphylococcal food poisoning. Concern in nosocomial bacteraemia has recently increased the interest in *Staphylococcus* species, known for causing community- and hospital-acquired infections. There are multiple clinical manifestations because each strain produces a combination of toxins causing virulence and invasiveness (Pocsfalvi *et al*., 2008; Ortega *et al*., 2010). Besides, the increase of antibiotic resistance has led to the apparition of methicillin-resistant *S. aureus* (MRSA) strains considered by the American National Nosocomial Infections Surveillance System as being among the most common causes of healthcare-associated infections (Normanno *et al*., 2007; Kuehnert *et al*, 2010).

a) Food poisoning and SEs emetic effect

S. aureus is among the leading causes of food-borne diseases (FBDs) worldwide for two reasons. First *S. aureus* is often present in food contaminated by humans. As an estimated 30-50% of human population carries *S. aureus*, mainly in the nasopharynx or on the hands where the organisms can persist without causing any damage, simple coughing, sneezing or food handling combined with poor hygiene may cause contamination, especially when occurring after heat treatment. When it comes to raw foods, contamination from animal origins is more frequent (i.e. mastitis). The second reason is that *S. aureus* is capable of growing and producing toxins in a wide variety of foods (milk and milk-transformed products, meat, salads, cooked meals…) (Ortega *et al.*, 2010).

FBDs are defined by the World Health Organization as "diseases of infectious or toxic nature caused by or thought to be caused by the consumption of food or water". FBDs include food-borne infections, which are caused by pathogens that contaminate foods, and food-borne poisoning, caused by substances present in food (Le Loir *et al.*, 2003).

Staphylococcal food poisoning (SFP) belongs to food-borne poisoning as it is caused by the ingestion, through food, of preformed Staphylococcal Enterotoxins (Gaebler Vasconcelos *et al*., 2010). The exact implication of *S. aureus* in foodborne diseases (FBDs) is difficult to assess because available data is incomplete and thus not very representative (Ortega *et al*., 2010; Lecture Analyse de la Qualité, Pr. M. Sindic, ULg GxABT). Table 5 gives an insight of its implication in FBDs.

The most commonly observed symptoms of SFPs are abdominal cramps, nausea, vomiting and diarrhoea. Those symptoms can appear 1-4 hours after eating and usually disappear after 24-48 hours (Dupin H., 1992).

Historical association between SEs and food-borne poisoning is the reason why SEs were originally described as emetic substances. Strains isolated from foods involved in SFPs produce mainly SEA and to a lesser extent SEB, SEC and SED (Dupuis *et al*., 2008). In France, SEA is involved in 65% of SFPOs and SEB in 20% (Dupin H., 1992).

The infective dose required to induce SFP to humans remains uncertain. The first reason is that the infective dose depends on the patient's sensitivity (Le Loir *et al*., 2003). The second is that bioassays, consisting feeding a suspected food to a monkey, have shown that the amount triggering the food-poisoning symptoms is lower for humans than it is for monkeys. For instance, the 50% effective dose of SEA is 1 µg in humans and 5 µg in monkeys (Ikeda *et al*., 2005), but it has also been reported that the ingestion of doses as low as 20–100 ng of SEs are susceptible to cause food-poisoning (Rodriguez-Caturla *et al*., 2012). Finally, the knowledge on infective doses is limited by the sensitivity of the methods used to detect and quantify the enterotoxins. Therefore, it is important to lower the detection and quantification limits of the methods in order to establish properly this infective dose.

The physiopathology of SFPs and the emetic function of enterotoxins are very partially known and still the object of many hypothesis and researches (see Ortega *et al*., 2010; Gaebler Vasconcelos *et al*., 2010 for recent researches). A strong hypothesis is that SEs stimulate the emetic centre and the gut transit because they affect the vague nerve and the intestinal epithelium. More precisely, it is believed that the enterotoxins increase the permeability of the intestinal mucosal cells to chloride ions. This activation of the membrane pores leads to secretory diarrhoea.

At first, the cysteine loop, common to the first SEs, was suspected to be implied in the emesis mechanism but the fact that some SEs (like SEI and SEK) lack that specific structure while presenting the emetic property, even though it is significantly weaker than for other SEs, questions this hypothesis (Ortega *et al*., 2010).

Table 5. Causative agents of food-borne disease outbreaks recorded in France between 1999 and 2000. Frequencies of each type of agent are given in per cent (Le Loir *et al*., 2003).

Causative agents	Outbreaks (N=530)	Cases (N=6451)	Hospitalizations (N=872)	Death (N=7)
Salmonella sp. (Enteritidis, Typhimurium, Heidelberg, and other serotypes)	63.8	47.7	16.8	100
Staphylococcus aureus	16	25.6	17.1	0
Clostridium perfrigens	5.1	12.3	0.5	0
Bacillus cereus	2.8	3.7	10.0	0
Histamine	3.8	1.4	30.4	0
Other pathogens (*Campylobacter* sp., *Dinophysis*, *Clostridium botulinum*, *Shigella* sp., Calcivirus, HAV, *Vibrio* sp., *E. coli*, etc.)	8.5	9.2	7.6	0

b) Superantigenic effect

Historically, SEs were only described as emetic substances. It is only later, with the extensive study of their properties, that SEs have been described as SAgs.

Superantigens are defined as "microbial antigens with the common capacity to activate and induce uncontrolled mitosis on T-lymphocytes presenting any specific variable region" (Gaebler Vasconcelos *et al*., 2010, pg 34).

This term was suggested after a series of independent studies demonstrated that SEs and streptococcal pyrogenic exotoxins share two properties: (i) the ability to directly bind the class II Major Histocompatibility Complex (MHC) of the antigen presenting cells (APC) and (ii) the ability to bind to the T-cell receptors (TCR) β-chain in another way than the usual peptide recognition mechanism (Ortega *et al*., 2010). The mechanisms involved in the interactions between SAgs, TCR and APC have been characterized (Balaban & Rasooly, 2000).

In an usual immune response, as schematised on Figure 3a, the antigens are internalized and processed by APC then they are presented to TCR in the form of peptides bound to molecules of class-I and class-II MHC which themselves are proteins bound to the membranes of the APC. The binding between TCR and usual antigens requires the recognition of all five variable elements of TCR (Vβ, Dβ, Jβ, Vα and Jα). This recognition of the antigen is a primary step in the cellular immune response and makes the specificity of the immune response.

On the contrary, superantigens affect the immune system by binding directly with the TCR and the MHC of antigen-presenting cells, as exposed in Figure 3b. This binding only requires the recognition of specific Vβ chains of the TCR, which induces a non-specific polyclonal immune response as T-cells are activated at orders of magnitude higher than the antigen-specific activation. Indeed, SAgs can stimulate about 20% of all T-cells, against 0.01% for the conventional antigens.

This nonspecific and exaggerated activation results in a proliferation of T-cells and a massive secretion of interleukines, various cytokines and lymphokines. These compounds are the ones responsible for severe outcomes of superantigens. They act as capillary vasodilators, leading to fever, hypotension, systemic toxicity (shock), which can all cause death (Le Loir *et al.*, 2003).

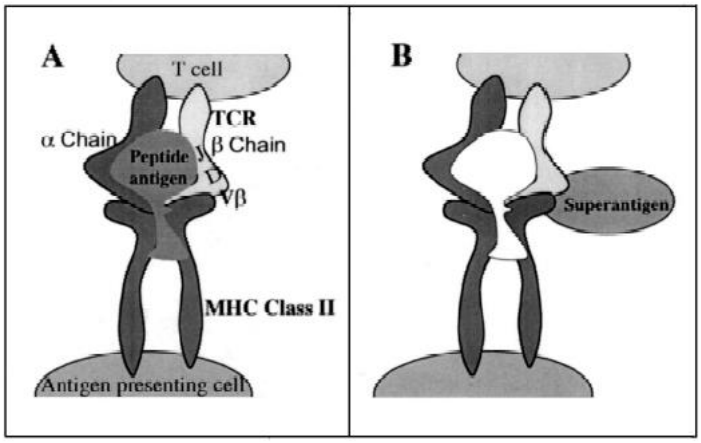

Figure 3. Model for the structure of the complex MHC class II and T-cell receptor. (A) Conventional antigen. (B) Superantigen. The model shows the processed antigen peptide presented by MHC class II which attracts specific T-cell bearing antigen specific T-cell receptor (TCR) variable chain. In contrast, superantigens bind directly to the outside of the MHC molecule and cross-link it to variable chain, which initiates non-specific activation of the cell (Balaban & Rasooly, 2000).

c) Emesis and superantigenicity: two functions

A precision about superantigens in general and staphylococcal toxins in particular is to be highlighted. SEs and SE*l*s do not present the same properties. All of them share superantigenic activity but only a few are emetic. A toxin is classified as enterotoxin only due to its ability to cause emesis when orally administrated to monkeys. All superantigens are thus not emetic (or one might say all superantigens are not enterotoxins). SAgs are actually a family of several groups of proteins, SEs and SE*l*s being part of them.

The interconnection of superantigenic and emetic activities is uncertain. The activities are located on different domains of the proteins but in most cases a correlation can be observed as a decreased superantigenicity often results in decreased emetic activity (Ortega *et al.*, 2010).

d) TSST-1

TSST-1 is secreted by some *S. aureus* strains. It is not emetic, probably because of its sensibility to proteolytic enzymes, but is capable of crossing mucosal barriers. This toxin is responsible for the Toxic Shock Syndrome (TSS) which affects the whole organic system and causes fever, hypotension, rash, vomiting, circulatory failure, organ failure… If not treated properly, a fatal shock may develop in less than 24h. This toxin's production is most known to happen to young women during their menstruation but also to post-op patients or in association with other infections (Ortega *et al*., 2010).

TSST-1 was first named SEF because it shows structural and functional similarities with other SEs. It was renamed after it was proved to show a different behaviour than other enterotoxins and to share less gene sequence homology with genes coding for SEs than these toxins usually do with one another (Gaebler Vasconcelos *et al*., 2010). To avoid any further confusion, the SEF appellation was chosen not to be attributed again to name an enterotoxin (Lina *et al.*, 2004).

For all the reasons previously mentioned, staphylococcal enterotoxins constitute a significant threat to Public Health. The American Center for Disease Control has even registered the staphylococcal enterotoxin B (SEB) as a potential warfare contaminant of food and water supplies (Brun *et al.*, 2007). A rapid overview of the existing strategies used to detect *S. aureus* and its toxins (or to confirm SFP) will show the need for the development of more efficient, reliable, fast and most important allowing quantification methods. It will show the interest of this work.

2.2 Investigating Staphylococcal Food Poisoning Outbreaks

As previously mentioned, SEs are responsible for a large part of FBDs. Because of this implication, most detection tools were developed in the purpose of confirming and identifying SFPOs and find applications in the field of alimentation. However, as will be developed later on, a tool allowing the accurate identification and quantification of staphylococcal toxins could find applications in other fields, such as health.

All existing tools developed to investigate SFPOs are based on the research of either the microorganism responsible for the outbreak, the gene coding for the toxin, the intermediate to the toxin production (the translated messenger RNA (mRNA)) or the toxin itself.

Their general principles are exposed along with advantages and drawbacks.

2.2.1 Bioassays

The first ways of detecting food contaminations were by biological methods. Bioassays are based on the capacity of a suspected food to induce food poisoning symptoms when fed to a monkey. This type of method is not only outdated because it faces serious ethical issues, but also because it does not provide any indication on the nature and severity of the contamination. Besides, as mentioned in the clinical manifestation, section 2.1.6, symptoms of SFP only appear with bioassays at higher doses than those involved in human food poisoning (Hennekine *et al*., 2010).

2.2.2 Molecular tools

Molecular tools usually involve Polymerase Chain Reaction (PCR), used for the detection of enterotoxin genes and the characterization of *S. aureus* strains involved in SFPOs, or Reverse Transcriptase PCR (RT-PCR), an intermediate to the detection of *se* genes and the actual detection of the SEs because it can prove gene expression by detecting mRNA sequences, responsible for the toxin's production (see reviews Hennekine *et al*., 2011 and Gaebler Vasconcelos *et al*., 2010 for details).

However, methods involving molecular tools cannot be used to confirm SFPOs (i.e. to link a SE to a food source because SFP can only be confirmed if the presence in food of one or several SEs has been demonstrated). Indeed, methods involving PCR can only detect the presence of genes encoding for SEs, which does not mean the enterotoxin has been secreted.

Duquenne (2010) developed an alternative method based on real time RT-PCR (RT-qPCR) to try and estimate the corresponding level of transcript toxins, but it still does not prove the SEs presence.

PCR and RT-PCR can however bring valuable information. For this reason the European Union - Reference Laboratory (EU-RL) for CPS decided in 2005 to use PCR procedure in an integrated approach to improve SFPO characterization (Hennekine *et al*., 2010).

Because molecular tools cannot be used to link a SE to a food source and because SFP can only be confirmed if the presence in food of one or several SEs has been demonstrated, alternative methods for detecting the toxins, the real causes of SFPs, have to be used.

2.2.3 Immunological assays

Immunological tools constitute the official method for detection of SEs in food. These assays rely on the recognition of an enterotoxin epitope by a specific antibody, either monoclonal or polyclonal (Dupuis *et al*., 2008). A wide range of commercial kits have been developed for the simultaneous detection of several enterotoxins (SEA to SEE). This development was made possible by the improvement of techniques that helped obtaining pure toxins and producing SEs antibodies (Gaebler Vasconcelos *et al*., 2010; Sospedra *et al*., 2013).

Commercial methods, referenced in Table 6, use various principles such as the enzyme-linked immunosorbent assay (ELISA), the enzyme-linked fluorescent assay (ELFA) or reverse passive latex agglutination (RPLA). ELISA and ELFA couple antibody-antigen recognition to an enzymatic reaction that liberates a colour compound. The visible compound is a signal measured by spectroscopy. In RPLA tests, the cross-linking of the latex particles by the specific antigen/antibody reaction results in a visible latex agglutination (Gaebler Vasconcelos *et al*., 2010; Sospedra *et al*., 2013; Thermoscientific).

The double antibody sandwich ELISA test is the most frequent because it is available both for screening and specific identification. Indeed commercial tests are divided in two categories. The tests that detect SEA to SEE as a whole and informs on the total SEs (screening), and the ones that can differentiate six or seven types of SEs (SEA, SEB, SEC_1, SEC_2, SEC_3, SED and/or SEE), or serotyping tests. However, none of those kits yet allows detection of SEG to SElV (Hennekinne et al, 2009).

Immunoassays all present the advantage of being simple, rapid and highly sensitive. Several drawbacks and limitations must however be mentioned.

- Manipulations frequently require time-consuming incubation periods.
- The development of a new specific antibody is expensive (~1 million dollar) and takes over a year.
- Not all toxins can yet be detected due to lack of available antibodies.
- High sequence and structural homologies exist between SEs and only a few specific antibodies are available. Therefore the techniques are less sensitive to small variations and not suited to the identification of SEs presenting antigenic similarities.
- Matrices like foods are complex and may lead to false positives when the antibody reacts with unrelated antigens or endogenous peroxides. For example, the Immunoglobulin G (IgG)-binding staphylococcal protein A is co-secreted in food with SE and is well-known for interfering with assays (Dupuis *et al*., 2008).
- If the enterotoxin epitope is damaged, for instance by heating, the enterotoxin is serologically but not biologically inactivated and the immunoassay leads to a false negative. Also, heat-treated enterotoxins may aggregate, reducing their reactivity with antibodies.
- The main drawback of methods based on specific monoclonal or polyclonal antibodies remains its high cost, preventing it from being used in routine tests. Therefore, immunoassays are reserved to serious epidemiology issues (Gaebler Vasconcelos *et al*., 2010; Dupuis *et al.*, 2008; Sospedra *et al*., 2013).

For information, a list of applications and studies based on immuno-assays and using some of the kits referenced in Table 6 is available in a review written by Sospedra *et al*. (2013).

So far, the official method for the detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices (milk and milk products and other food matrices) is based on the use of the VIDAS SET2 and the RIDASCREEN SET TOTAL commercial kits, as mentioned by the "European Screening Method of the European Union – Reference Laboratory for Coagulase Positive Staphylococci, including *Staphylococcus aureus* (EU-RL for CPS)", in the Version of September 5th, 2010 (ANSES).

Table 6. Commercial kits based on immunoassays for detection of staphylococcal enterotoxins.

SE detected	Name	Principle	Limit of detection (LOD)*	Manufacturer	Matrix	Time for analysis**
SEA-SEE	VIDAS SET2 [3]	ELFA	0.25 ng/ml	bioMérieux	Food samples	1h30
SEA-SEE	TRANSIA PLATE SE[4]	ELISA sandwich	0.25 to 1 ng/g	BioControl Systems	Water, milk, dairy products	1h
SEA-SEE	Ridascreen SET Total [5]	ELISA double sandwich	0.25 ng/ml	R-Biopharm	Fluid and solid foods Bacterial supernatants	3h
SEA-SEE	3M™TECRA™ Staph enterotoxin [6]	ELISA sandwich	1 ng/ml	3M	Food samples	4h
SEA-SED	Oxoid SET-RPLA [7]	RPLA	0.5 ng/ml	Thermo Fisher Scientific	Food samples Culture filtrates	24h

*Limits of detection indicated in kits manual

**Time of extraction is not included in time for analysis

Note: A commercial kit named TSST-RPLA has also been developed for the specific detection of TSST (Thermo scientific).

[3]Biomérieux
[4]Biocontrol
[5]R-Biopharm
[6]3M
[7]Thermo scientific

2.2.4 Detection, identification and quantification of SEs

The methods previously exposed are not suitable for preventing outbreaks or to properly identify them. Indeed, they are too laborious, too long or too expensive to be applied in preventive analysis and it is usually after several FBD cases have been reported that research is done to identify the responsible foodstuff and the associated pathogen or toxin. Besides, none of those methods allow unambiguous identification, let along quantification, as molecular tools are inefficient at proving the existence of the toxins in foods and immunoassays are not specific enough, not suitable for quantification and more importantly limited in the range of toxins they can identify.

Recently, researchers have started to explore proteomics approaches in an attempt to develop fast and specific methods to detect and quantify SEs. The main matrixes investigated are foods, as SEs are mainly investigated for food-poisoning.

The development of mass spectrometers and more specifically of two soft ionization methods has revolutionized the analysis of biomolecules. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) allowed the development of new analysis strategies (Hennekine *et al*., 2010).

Mass spectrometry (MS) is a very sensitive technique that provides specific and rapid results. New detection and quantification strategies were thus developed around MS for protein and peptide mixtures analysis. Besides, MS-based methods allow multiplex analyses (Brun *et al.*, 2009), which is a real advantage for the confirmation and characterization of SFPOs, as several SEs may be involved.

Several detection and quantification strategies have been explored, all articulated around the use of mass spectrometry. There are several variations but also common points to the recently developed methods. In food analysis, extraction and purification steps are critical for SEs detection and quantification and must be carefully optimized. Those parts of the protocols tend to differ, depending on the food, for instance. On the other hand, many of the developed strategies lead to the proteolysis (or digestion) of the extracted enterotoxins into specific peptides, even though some strategies also aim the detection of whole proteins. Enzymatic digestion is about reducing the target protein into smaller peptides, yielding to a large number of peptides in solution. Those peptides can be separated by Liquid Chromatography (LC). Afterwards, their molecular mass is determined by ElectroSpray Ionization Mass Spectrometry (ESI-MS) or Matrix-Assisted Laser Desorption/Ionization Time Of Flight Spectrometry (MALDI-TOF).The most widely used proteolytic enzyme for protein cleavage is trypsin because of its high specificity and ability to digest insoluble or adsorbed protein (Bao *et al*., 2011). Finally, because foods often result into strong matrix effects, several quantification strategies using internal calibration, with or without isotope-labelled internal standards were developed.

The following section will be dedicated to the three proteins quantification strategies that use isotope-labelled internal standards. Next, a rapid overview of protocols using some of these quantification strategies along with the extraction, purification, concentration, and eventual LC and Mass spectrometry MS tools employed for SEs detection and quantification will be exposed.

a) Quantification methods – Internal Standards

Nowadays, mass spectrometry permits the simultaneous characterization of several proteins in a very specific and rapid way. The actual challenge lays in the accurate quantification of these proteins.

The internal standard calibration strategy is a very useful quantification tool for analysing samples with strong matrix effects, like foods. More specifically isotope dilution strategies, which are part of the large family of internal calibration, have been developed to provide correct identification and absolute quantification. Three kinds of quantification strategies using this isotope dilution principle are exposed in the literature°: Protein Standard Absolute Quantification (PSAQ), using intact labelled proteins, Absolute quantification (AQUA) peptides, using chemically synthesised labelled peptides or Quantification concatamer (QconCAT), using concatamers of tryptic labelled peptides in an artificial protein. All their principles are schematised in Figure 4 (Brun *et al.*, 2009).

AQUA peptides

The AQUA peptide strategy uses chemically synthesized isotope-labelled peptides. Those peptides are spiked in known quantities into the samples either before the digestion step or just before MS analysis.

The AQUA method is fast and easy to use because a large range of AQUA peptides are commercially available and affordable compared to other isotope standards.

However, several drawbacks must be mentioned.

First, this strategy but does not take any of the sample preparation step into account. If those standards were injected at early stages of the protocol, all extraction, purification and concentration steps could significantly decrease the AQUA peptides recovery, and consequently the quantification accuracy. Besides, as those standards are peptides, they are not submitted to digestion and the efficiency of this step is not taken into account either. So if this approach is chosen, special attention must be taken in evaluating the digestion yield and the recovery of the pre-fractionation steps.

Second, the peptides must be chosen carefully with regards to their sequences because some peptides are more stable than others and some chemical synthesis limits exist. For example, peptides shorter than 15 amino acids are preferred and reactive residues such as tryptophane, methionine, cysteine… or some sequence patterns (N-terminal glutamine, aspartate-glycine…) should be avoided (Polyquant). Also their sequences can affect their conservation as peptides can have a tendency to adhere to certain surfaces, resulting in quantification underestimations. Storage at -80°C and careful over-time monitoring of the quality and concentration are recommended (Brun *et al.*, 2009).

Finally, in case of multiplex analysis, the experiment cost can rapidly increase because very pure AQUA peptides are needed. This is the reason why often, only one carefully selected highly specific of the target protein AQUA peptide is used for quantification.

Studies where AQUA peptides have been successfully employed are listed in Table 7.

Protein Standard Absolute Quantification (PSAQ)

The ideal internal standard for the quantification of a protein is its corresponding full-length isotope-labelled *in vitro*-synthesized protein.

Here again, this quantification tool presents advantages and drawbacks.

First, those ideal standards can be spiked into the samples at very early stages of the analytical process, as shown on the scheme Figure 4 even if the sample is to undergo intensive pre-treatment, which is often required considering the complexity of food matrices (Brun *et al.*, 2009; Adrait *et al.*, 2011). Because PSAQ standards display the same characteristics and behaviour as the target proteins they will account for possible protein losses and avoid differences in digestion between internal standard and target protein (Dupuis *et al.*, 2008).

Second, using a full-length marked protein instead of a peptide allows a larger coverage of the protein sequence, increasing specificity and robustness.

A third characteristic of PSAQ standards is that being artificially synthesised, those proteins do not carry the post-translational modifications of the targets. The generation of such proteins is possible but challenging, time consuming and expensive (Polyquant). It is therefore only feasible for a very small number of proteins. Intact protein standards, lacking post-translational modifications, have however been used successfully in absolute quantification experiments. Some are referenced in Table 7.

Quantification concatamer (QconCAT)

QconCAT concatamers are chimerical proteins made of different marked peptides whose sequences originate from different proteins. QconCAT proteins constitute an interesting intermediate to both previously exposed quantification tools (AQUA peptides and PSAQ proteins).

QconCAT concatamers were specifically developed for multiplex absolute quantification of proteins as up to 100 peptides can be included in a structure. Because they are proteins, those concatamers can be spiked into the samples just before the protein digestion step, as exposed in Figure 4. During digestion the isotope-labelled peptides are released by the digestion and serve in MS analysis as standards for quantification (Brun *et al.*, 2009).

But because they are artificial proteins without a three-dimensional structure, they differ in their biological characteristics and behaviour and are digested at higher rates than folded proteins. Therefore, QconCAT proteins cannot be spiked at early stages of the protocol and their different sensitivity to digestion must be estimated, and maybe compensated by surrounding each proteotypic peptide of the concatamer with its native flanking sequences (Kito *et al.*, 2007).

The main advantage of the QconCAT methodology really is that it facilitates multiplex protein quantification. With regards to the cost of those standards, it is probably more economical to use AQUA peptides if only a few proteins are to be quantified. But if a high number of proteins are targeted, using QconCATs is the economical solution (Polyquant).

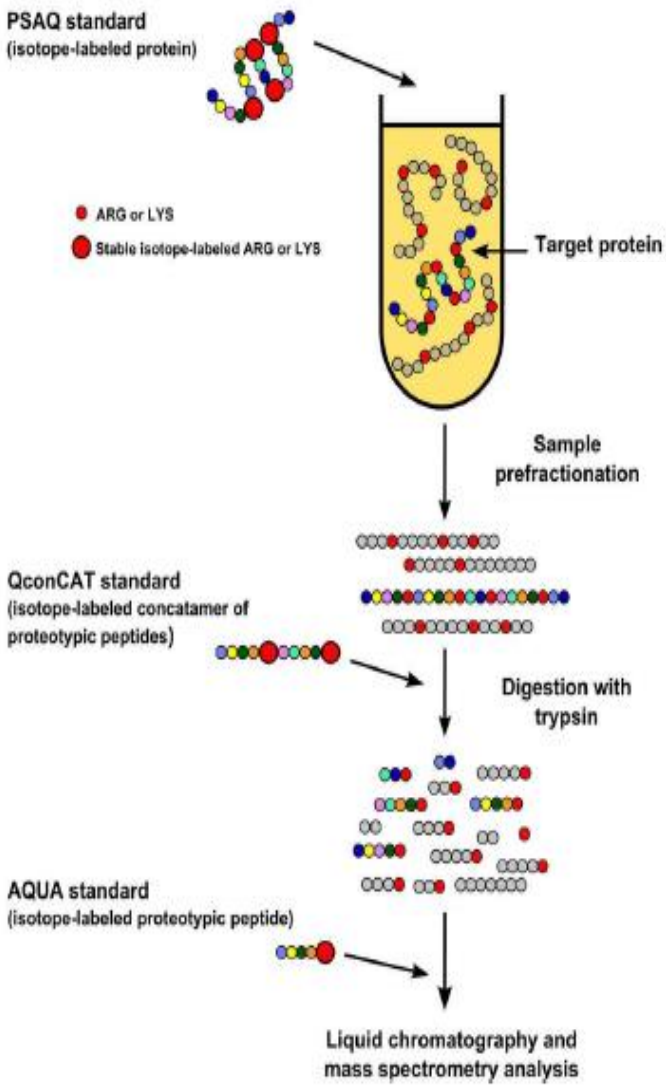

Figure 4. Isotope dilution strategies for MS-based absolute quantification of proteins. Three types of internal standards are available°:
(1) The PSAQ protein standard ("Protein Standard Absolute Quantification") is an isotope-labelled version of the target protein which is directly added into the sample;
(2) The QconCAT ("Quantification concatamer") standard is a chimerical protein containing one/several isotope-labelled proteotypic peptide(s) of the targeted protein. This concatamer is added before the digestion step so that the standard peptide(s) is/are released in the samples;
(3) The AQUA peptides ("Absolute Quantification") are synthetic isotope-labelled copies of the target proteotypic peptides. They are generally added to the samples before LC-MS analysis (Brun *et al*., 2009).

b) Protocols based on MS

This section is dedicated to a rapid overview of protocols that were developed to detect and quantify SEs and end up in the use of a Mass Spectrometry technology.

The methods exposed hereunder either target whole proteins or specific protein fragments (peptides). Proteolysis can either be achieved in solution or in-gel. Several extraction and purification strategies exist, using immunocapture, precipitation, filtration, solid phase extraction (SPE) or sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Finally, quantification is achieved by internal or external calibration.

Dupuis (2008) combined dialysis against polyethylene glycol (PEG) for the concentration of the enterotoxins, immunocapture and SDS-PAGE for their purification, a proteolytic step on SDS-PAGE gel to produce specific peptides and PSAQ strategy, for quantification of SEA in cheese and coco-pearls (a Chinese dessert). Peptides specific for SEA and its internal standard were analysed in nanoLC-MS using a quadripole time of flight (qTOF) mass spectrometer.

Adrait (2011) combined immunoaffinity and SDS-PAGE purification, in-gel digestion and nanoLC coupled to a hybrid quadrupole/linear ion trap mass spectrometer analysis. PSAQ internal standards were used.

Brun (2007) compared the three quantification strategies (AQUA, QconCAT and PSAQ) on urine and water samples to detect SEA and TSST-1. Digestion was performed in solution for water samples and on-gel for urine samples, after the toxins were concentrated on resin and purified by SDS-PAGE. Peptides were analysed using nanoLC-MS (qTOF) and nanoLC coupled to tandem mass spectrometry (MS/MS).

Sospedra (2011) performed the detection of SEA in milk using matrix proteins precipitation, centrifugation and SDS-PAGE to purify the toxin. In-gel digestion was performed and the analysis took place using Matrix-assisted laser desorption/ionization coupled to a Time of Flight analyser (MALDI-TOF).

An inconvenient of immunoaffinity, besides its cost, is that the target proteins range is limited to available antibodies for the searched toxins. So SE for which specific antibodies are not yet available are more difficult to purify, and consequently to analyse.

When it comes to gel-based methods like SDS-PAGE purification and in-gel digestion, some drawbacks can be mentioned. For example, those protocols can be time-consuming, require careful handling and include many fastidious steps such as gel-excision, peptides extraction from the gel…

Some methods use in-solution digestion, which is by itself a long step but is faster because of the absence of SDS-PAGE purification.

Bao (2011) developed a method to detect SEB in solid food°: meat. The toxins were purified using centrifugation and precipitation steps. In this protocol, the toxins were precipitated along with matrix protein. Digestion was performed in solution and the resulting peptides were purified by ultrafiltration. Peptides were analysed by LC and a quadripole ion trap MS (QIT).

Callahan (2006) used a different purification and concentration strategy, based on ultrafiltration to detect SEB in juice. Digestion in solution was employed and the peptides were analysed by LC-MS using either a qTOF or a triple quadripole (QqQ). In this case, a different quantification strategy, using Leu-enkephalin as an internal standard, was applied.

Also, some protocols detecting whole proteins were developed. Sospedra (2012) developed a protocol targeting SEA and/or SEB in juice and milk. As the whole proteins were targeted, external standard calibration was used for quantification. SEs were purified from juice by filtration and from milk by caseins precipitation and fat extraction. Analysis of the proteins was performed using LC-ESI/MS using a triple quadripole (QqQ).

It is interesting to note that SE detection has been successfully applied in culture supernatant using High Performance Liquid Chromatography coupled to Diode Array Detection (HPLC-DAD). SEB was successfully detected and quantified as a whole protein using external standard calibration.

A synthesis of the methods described here above along with their performances is available in Table 7.

The conclusion of this short overview is that many pre-fractionation, proteolysis and analysis tools are available for the detection and quantification of SEs in food. Many methods have been developed or are still being optimized. In any case, liquid chromatography coupled to mass spectrometry is a technique that is more and more explored as it can provide fast, sensitive and specific results.

Table 7. Overview of methods for Staphylococcal enterotoxins (SEs) detection and quantification.

Reference	Matrix	Staphylococcal enterotoxins	Methodology	Quantification	LOD
Callahan *et al*., 2006.	Apple juice; Water soluble food matrixes.	SEB	Ultrafiltration; Tryptic digestion; LC-ESI-MS or LC-ESI-MS/MS (MRM mode) : Micromass QTOF Micro quadrupole time-of-flight Or Micromass Quattro Premier QqQ	IS (leu-enkaphalin)	5 ppb
Brun *et al.*, 2007.	Water; Urine	SEA TSST-1	Resin extraction; SDS purification; In-gel digestion or In-solution tryptic digestion; nanoLC-MS or nanoLC-MS/MS	AQUA QconCAT PSAQ	3.8 pM in water (TSST-1) 0.4 nM in urine (SEA)
Dupuis *et al.*, 2008.	Cheese; Coco pearls (Chinese desert).	SEA	Dialysis against PEG; Immunoaffinity enrichment; SDS-PAGE purification; In-gel tryptic digestion; nanoLC-MS/ QTOF	PSAQ	1.47 ng/g (coco pearls) 2.5ng/g (cheese)
Sospedra *et al*., 2011.	Milk	SEA	Sample clean-up; SDS-PAGE separation; In-gel tryptic digestion; MALDI-TOF MS	Peptide calibration standard	N.A.
Bao *et al*., 2011.	Chicken meat	SEB	Toxin precipitation; In-solution tryptic digestion; Peptide purification; LC-ESI-MS/MS (MRM mode)	AQUA	0.2 pmol/g
Adrait *et al*., 2012.	Serum	SEA	Immunocapture extraction; SDS-PAGE fractionation; In-gel tryptic digestion; Peptide ultrafiltration; nanoLC-MS/MS (SRM Mode)	PSAQ	LOD: 352 pg/ml LOQ: 1057 pg/ml
Sospedra, Soler *et al*., 2012.	Apple juice ; Orange juice ; Milk.	SEA SEB	Sample clean-up; LC-ESI/MS (SCAN and SIR modes) →Whole protein detected	External calibration	LOD°: 0.025µg/ml (SEA) 0.01µg/ml (SEB) LOQ°: 0.05µg/ml (both)
Sospedra, Marìn *et al*., 2012.	Culture filtrates	SEB	HPLC-DAD →Whole protein detected	External calibration	LOD: 0.5 µg/ml LOQ: 1 µg/ml

2.3 Objectives of the thesis

2.3.1 SETTECT Project

The present work is part of the SETTECT (Staphylococcal EnteroToxin DeTECTion) project aiming at the detection and quantification of Staphylococcal Enterotoxins in various matrixes (foods, culture supernatant…) using UPLC-MS/MS. The objective is to develop a multi residual detection and quantification method that would allow the simultaneous detection of several SEs in a single, fast analysis. As reported in the literature, there are 23 known SEs so far and such analysis would find many applications in food poisoning prevention as well as clinical set-up.

2.3.2 SETTECT Strategy

The general SETTECT strategy is illustrated in Figure 5. The specific detection and quantification of each toxin is achieved through the analysis of peptides (toxin fragments) unique to a toxin. The peptides are obtained by extraction, purification and concentration of the enterotoxins out of the matrix and submitting them to a proteotypic digestion. Trypsin, the chosen protease, cuts the toxins into specific peptides. The goal is to select two specific peptides per toxin to ensure proper identification and quantification. The selection of those peptides (the analytes) is first based on a theoretical research that will be explained farther down. This theoretical search results into a list of potential peptides that are to be tested and among which two will be selected for each toxin. The theoretical peptides are the basis for the method development. First those peptides are used in an isotopically marked form for the determination of the analytes MS/MS transitions. Second, the marked peptides serve for the identification and quantification of the analytes. The analytes and the marked peptides present the same amino acid sequence and differ only by their weight. When both the analyte and the marked peptide are analysed by UPLC-MS/MS and since the LC separation is based on hydrophobicity for this method, one analyte and its internal standard co-elute. Weight difference allows the segregation of the marked peptide and the analyte, or endogenous peptide, in the mass spectrometer, resulting in different signals. Not only do marked peptides allow proper identification, they are also used as internal standards and allow quantification, following the AQUA principle described in section 2.2.4. Consequently, the marked peptides will be named IS, standing for Internal Standards.

The similar part of the SETTECT project for all protocols that are or will be developed is the digestion of the toxins and the detection and quantification using IS. However, several extraction protocols must be developed and optimized depending on the matrix, and peptides must be selected for each toxin.

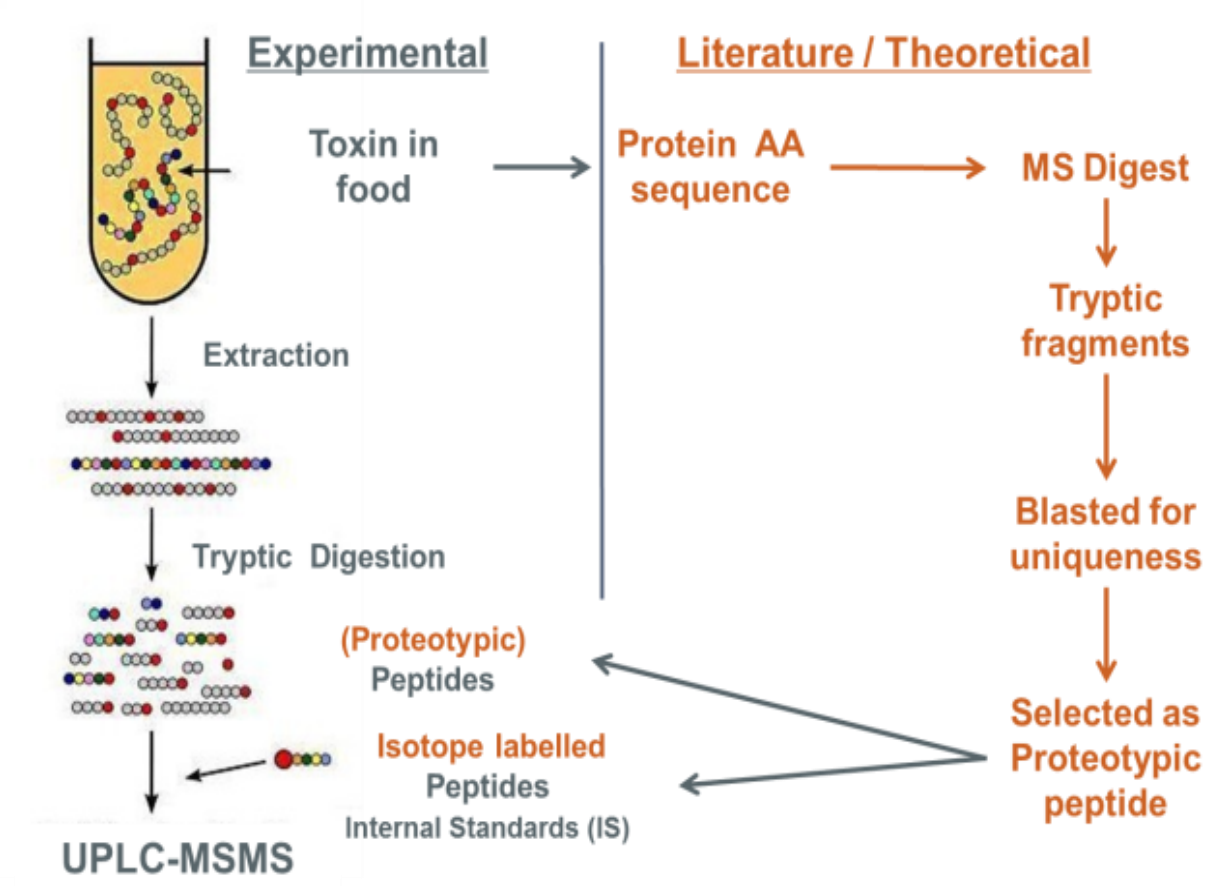

Figure 5. SETTECT (Staphylococcal EnteroToxin DeTECTion) detection and quantification strategy.

2.3.3 Objectives of the thesis

The initial goal of this work was to adapt an already developed detection and quantification method of Staphylococcal enterotoxins developed in milk to a different but also complex matrix°: meat. Besides, only two enterotoxins among the most implied in staphylococcal food poisoning were targeted°: SEA and SEB.

After optimizing the protocol on meat, a validation plan was to be established and applied to characterize the performance of the adapted protocol. As part of that validation process, real food samples, either with already detected SEs or coming straight from the market, would have been processed with the new protocol.

Due to the high complexity of the matrix and to delays caused by successive technical issues of the device, the objectives were not all reached. Several important improvements have however been brought in the adaptation of the method to meat and will be exposed in the present work. The optimisation of the protocol was undertaken in several parts.

The first part was the selection of peptides according to several specific criteria and databases. After the selection of the peptides, MS and LC parameters were determined in order to ensure proper identification.

The second part was the adaptation of the extraction protocol from milk to meat. The general principle was followed and several points were optimized. This adaptation was evaluated using two additional tools. The first is an immunological tool based on the ELFA principle used to detect the presence of Staphylococcal enterotoxins at several steps of the extraction, and the second is SDS-PAGE, an electrophoretic method, used to evaluate the efficiency of the purification steps.

The third part was the digestion step which makes the transition between the two previous parts. Indeed, after the extraction and purification of the enterotoxins, they have to be broken down into peptides, the analytes that are detected by the UPLC-MS/MS. The efficiency of this step was assessed by comparing estimated digestion yields, calculated with peak areas and response ratios.

Finally, after working on all those steps separately, a final testing took place by bringing all the different optimizations together in order to estimate the efficiency of the global protocol. For this part, several series of samples, spiked with the enterotoxins at different steps of the protocol, were run through the whole process and analysed by UPLC-MS/MS. This experiment was useful for determining which steps of the protocol caused toxins losses and needed further improvements.

2.3.4 Milk protocol

The principle of the protocol developed in milk, which will be referred to as “milk protocol”, is articulated around the use of several size filters to extract, purify and concentrate the SEs out of the matrix. The general milk protocol goes as follows. First toxins are spiked in the matrix. Milk caseinates are precipitated by acidification and milk fat is eliminated by an organic solvent. After a centrifugation step, the supernatant is cleaned up by microfiltration and ultrafiltration and the toxins are concentrated by ultrafiltration as well. Once concentrated, the enterotoxins are submitted to digestion. A digestion preparation step unfolds the toxins to ease the protease access to its cleavage sites. This preparation involves dithiothreitol (DTT) to reduce disulphide bonds and iodoacetamide (IAA), an alkylation agent, to avoid the formation of intra-molecular or inter-molecular disulphide bonds.

The digestion step involves a surfactant named Rapigest, a modified trypsin as a protease and a digestion buffer used to adjust the pH in the optimal trypsin working range (pH 7-9). Rapigest helps to solubilize toxins, making them more susceptible to tryptic digestion, without inhibiting the enzyme activity. The protease used for the digestion is a modified trypsin. The cleavage site of this serine protease is at the carboxyl side of the amino acids lysine (K) or arginine (R), except when either is followed by proline. The digestion buffer used in the milk protocol is ammonium bicarbonate 50 mM. The IS are added to the samples before digestion. Once digestion is over, the trypsin is quenched by formic acid and the samples are centrifuged before UPLC-MS/MS analysis.

3 Material and methods

3.1 Materials

Material

Plastic tubes°:

15 ml High-Clarity Polypropylene Conical Tube, 17 x 120 mmm style (BD Falcon)

1.5 ml plastic tubes with Safe-Lock (Eppendorf)

Centrifuges°: Biofuge pico (Hearaeus Instruments); Centrifuge 5810 R (Eppendorf)

Pipettes°:1-10 µl (Labsystems); 2-20 µl, 10-100 µl, 50-200 µl (Eppendorf reference); 5-50 µl; 100-1000 µl; 1-5 ml (Socorex)

Multipipette°: Multipipette plus, 10 ml (Eppendorf)

Gloves°: Peha-Soft nitrile (FINO)

Classical laboratory glassware

Vortex°: IKA vortex Genius 3

Ultrasonic bath°: 3510 Branson

pHmeter°: BECKMAN Φ32 pHmeter

Minicentrifuge°: Mini Star silverline (VWR)

Scale°: Sartorius analytic and its printer

Blender °: Le Mini Plus Automatic (Magimix)

Plastic syringes°: 5 ml single-use syringes with Luer Lock Tip (TERUMO)

Syringe filters°:

0.2µm PVDF (Polyvinylidine Difluoride), 25 mm, GD/X^{TM} (Whatman)

0.1µm PTFE (Polytetrafluoroethylene), 25 mm, SIMPLEPURE (Membrane solutions)

Centrifuge filters :

50,000; 30,000; 10,000; 5,000 MWCO PES (Polyethersulfone)

Vivaspin 6 **(**Sartorius Stedim biotech)

Vials°: 1.2 ml Ultra Recovery Clear Vial with Blue 9 mm Bonded Preslit PTFE/silicone cap (GRACE)

Solvents

MilliQ water obtained from a Gradient A10 Millipore device.

Formic acid (HFo), 98-100%°: Merck

Acetic acid (HAc), 100%°: Merck

Dichloromethane (CH_2Cl_2)°: HiPerSolv (BDH)

n-hexane°: UniSolv ® (Merck)

Acetonitrile (ACN)°: HPLC-S (Biosolve chemicals)

0.1% formic acid°: 1 ml of HFO, 100%, is diluted in 999 ml of MilliQ water.

0.1% formic acid (mobile phase B) °: 1 ml of HFO, 100%, is diluted in 999 ml of MilliQ water. The bottle containing the solution is placed for 15 minutes in a degasser.

Acetonitrile with 0.1%formic acid (mobile phase A) °: 1 ml of HFO, 100%, is diluted in 999 ml of ACN. The bottle containing the solution is placed for 15 minutes in a degasser.

50 % acetonitrile, 0.1 % formic acid°: 100 µl of HFo, 100%, are diluted in 49.5 ml of ACN and 49.5 ml of MilliQ water.

Toxins

50 ng/µl working solution of SEA°: 1 mg of SEA (Sigma) was dissolved in 20 ml of MilliQ water and stored at -20°C. To minimize the number of freeze/thawing cycles aliquots of 1 ml were made.

50 ng/µl working solution of SEB°: 5 mg of SEB (Sigma) were dissolved in 100 ml of MilliQ water and stored at -20°C. To minimize the number of freeze/thawing cycles aliquots of 1 ml were made.

Internal standards (IS)

All internal standards were purchased at Thermo Scientific.

100 pmol/µl individual stock solution of internal standard°:

The ordered peptides were delivered in a dissolved form (in 50% Acetonitrile, 0.1 % trifluoroacetic acid).

Internal Standard stock solutions were brought to 100 pmol/µl by dissolving an aliquot of the IS in 50 % acetonitrile, 0.1 % formic acid. Details are available in Annex 1**Erreur ! ource du renvoi introuvable.**. To minimize the number of freeze/thawing cycles aliquots of 500 µl were made. The solutions were stored at -20°C.

10 pmol/µl individual working solution of internal standard°:

100 µl of 100 pmol/µl individual stock solution of internal standard and 900 µl of 50 % acetonitrile, 0.1 % formic acid were mixed together. The solutions were stored at -20°C.

1 pmol/µl working solution of mixed internal standards (IS SEA_1 to SEA_5 and SEB_1 to SEB_6)°:

50 µl of each internal standard individual stock solution (100 pmol/µl) and 4450 µl of 0.1% formic acid were mixed together. The mix was stored at -20°C.

10 pmol/µl working solution of mixed internal standards (for IS SEA_2; SEA_4; SEA_5; SEB_1; SEB_2; SEB_4; SEB_5; and SEB_6) °:

200 µl of each internal standard individual stock solution (100 pmol/µl) and 400 µl of 0.1% formic acid were mixed together. The mix was stored at -20°C.

SDS-PAGE

Cold MilliQ water°: stored overnight in the fridge.

Electrophoresis buffer°: 250 ml of buffer 10 x Tris/Glycine/SDS buffer (Biorad 161-0732, 5l) brought to 2500 ml with Milli-Q water and stored overnight in the fridge.

Tris = trishydroxyméthylaminométhane

Sample buffer°: 54 mg of dithiothreitol or DTT (Sigma Aldrich 43817) are diluted in 1 ml of Laemli sample buffer (Biorad 161-0737) and stored in the fridge.

Filling buffer°: 100 µl of electrophoresis buffer and 100 µl of sample buffer are mixed together in an eppendorf tube.

Molecular weight markers°:

Precision plus protein all blue standard or AB (Biorad 161-0373)

Precision plus protein dual color standard or DB (Biorad 161-0374)

Ready-to-use 50 µl aliquots stored at -20°C

Biosafe coomassie blue°: Biorad 161-0786

Ready-to-use gels°: Criterion TGX 4-20 % 26 wells (Biorad 567-1095)

Heating block°: Thermomixer Compact Eppendorf

Electrophoresis tank°: Criterion blotter with plate electrodes (Biorad 170-4071) and its specific material

Generator°: PowerPac HC power supply 220-240V (Biorad 164-5052)

Eppendorf tubes °: with safe-lock caps 1,5 et 0,5 ml (Eppendorf)

Surgical blade°: Martin

Micropipette°: Biohit proline 1-10 µl and adapted tips

Rocking platform°: Ultrarocker (Biorad 166-0719)

Scanner°: Hewlett Packard

VIDAS SET2

miniVIDAS (bioMérieux) and its related material

Extraction-Digestion

Meat°: pork meat (pork sirloin chops) was purchased at a local store and chopped very thinly using a food processor.

Acetic acid, 0.2 M°: 11.4 ml of pure acetic acid (Merck) were diluted in 988.6 ml of MilliQ water.

Sodium acetate, 0.2 M°: 13.6 g of $CH_3COONa*3H_2O$ were dissolved in 500 ml of MilliQ water.

Acetate buffer, 0.2 M, pH~4°: 180 ml of Sodium acetate, 0.2 M, is mixed with 820 ml of Acetic acid, 0.2 M.

Phosphate Buffer Saline solution 2X concentrated (PBSX2) °: 1.6 g NaCl (VWR), 0.04 g KCl (Merck), 0.28 g $Na_2HPO_4*2H_2O$ (Merck) and 0.04 g KH_2PO_4 (Merck) were dissolved in 100 ml of MilliQ water.

Phosphate Buffer Saline solution (PBS)°: 0.8 g NaCl (VWR), 0.02 g KCl (Merck), 0.14 g $Na_2HPO_4*2H_2O$ (Merck) and 0.02 g KH_2PO_4 (Merck) were dissolved in 100 ml of MilliQ water.

PBS-Tween 20 (0.1 %)°: 0.8 g NaCl (VWR), 0.02 g KCl (Merck), 0.14 g $Na_2HPO_4*2H_2O$ (Merck) and 0.02 g KH_2PO_4 (Merck) were dissolved in 99 ml MilliQ water and 1 ml 10 % Tween 20 (Bio-Rad).

Phosphate Buffer Saline solution in acetate buffer, 0.1 M (pH~4)°: 50 ml of PBSX2 were diluted in 50 ml of Acetate buffer, 0.2 M, pH~4.

Sodium chloride, 1.8%°: 1.8 g of NaCl (VWR) was dissolved in 98.2 g of MilliQ water.

Sodium chloride, 6%°: 6 g of NaCl (VWR) were dissolved in 94 g of MilliQ water.

Sodium chloride, 3%°: 50 ml of sodium chloride, 6%, were diluted in 50 ml of MilliQ water.

Sodium chloride, 0.9%, in acetate buffer, 0.1 M (pH~4)°: 50 ml of sodium chloride, 1.8%, were diluted in 50 ml of Acetate buffer, 0.2 M, pH~4.

Sodium chloride, 1.5%, in acetate buffer, 0.1 M (pH~4)°: 50 ml of sodium chloride, 3%, were diluted in 50 ml of Acetate buffer, 0.2 M, pH~4.

Sodium chloride, 3%, in acetate buffer, 0.1 M (pH~4)°: 50 ml of sodium chloride, 6%, were diluted in 50 ml of Acetate buffer, 0.2 M, pH~4.

Ammonium bicarbonate (NH_4HCO_3), 50 mM°: 0.4 g NH_4HCO_3 (Sigma) was dissolved in 100 ml MilliQ water.

Tris HCl- $CaCl_2$, (500 mM; 100 mM) : 6.06 g Tris (Sigma) and 1.46 g $CaCl_2$ (Vel) were dissolved in 100 ml MilliQ water.

Tris (100 mM) : 1.22 g Tris (Sigma) was dissolved in 100 ml MilliQ water.

NaOH (1M) °: 4 g of NaOH (brand) were dissolved in 100 ml MilliQ water.

Rapigest 0.01%°: 1 mg of rapigest powder (Waters) was dissolved in 1 ml of 50 mM ammonium bicarbonate.

Dithiothreitol (DTT in water) 40 mM°: 0.0121 g of DTT (Sigma) was dissolved in 2ml MilliQ water.

Iodoacetamid (IAA in water) 50 mM°: 0.0184 g of IAA (Sigma) was dissolved in 2ml MilliQ water.

Trypsin 100ng/µl working solution°: 20 µg of lyophilized Sequencing grade modified Trypsin (Promega) were reconstituted in 200 µl of Resuspension buffer (= 50mM acetic acid).

UPLC-MS/MS

UPLC °: Ultra performance liquid chromatographic system (Waters Acquity)

MS/MS°: Xevo TQ-MS mass spectrometer (Waters)

High pressure switching valves°: Vici

Additional loading pump°: to make on-line SPE possible (Waters)

Column switching software°: Micromass 4.1

Mass spectrometry software°: MassLynx (Waters)

Data processing°: TargetLynx (Waters)

Solid phase extraction column °: Oasis HLB 2.1 mm × 20 mm, 15 µm column (Waters)

Analytical column°: Acquity BEH C18 2.1 × 100 mm, 1.7 µm column (Waters)

3.2 Methods

3.2.1 Identification by UPLC-MS/MS

a) Objectives

The objectives of this section are related to the development of the UPLC-MS/MS method. The first part is the selection of unique peptides or analytes that will be detected by the device, according to several specific criteria and databases. The second part is the determination of MS and LC parameters in order to ensure proper identification and detection. This part of the process is framed in orange in the general protocol for SEs detection in meat exposed in Figure 6.

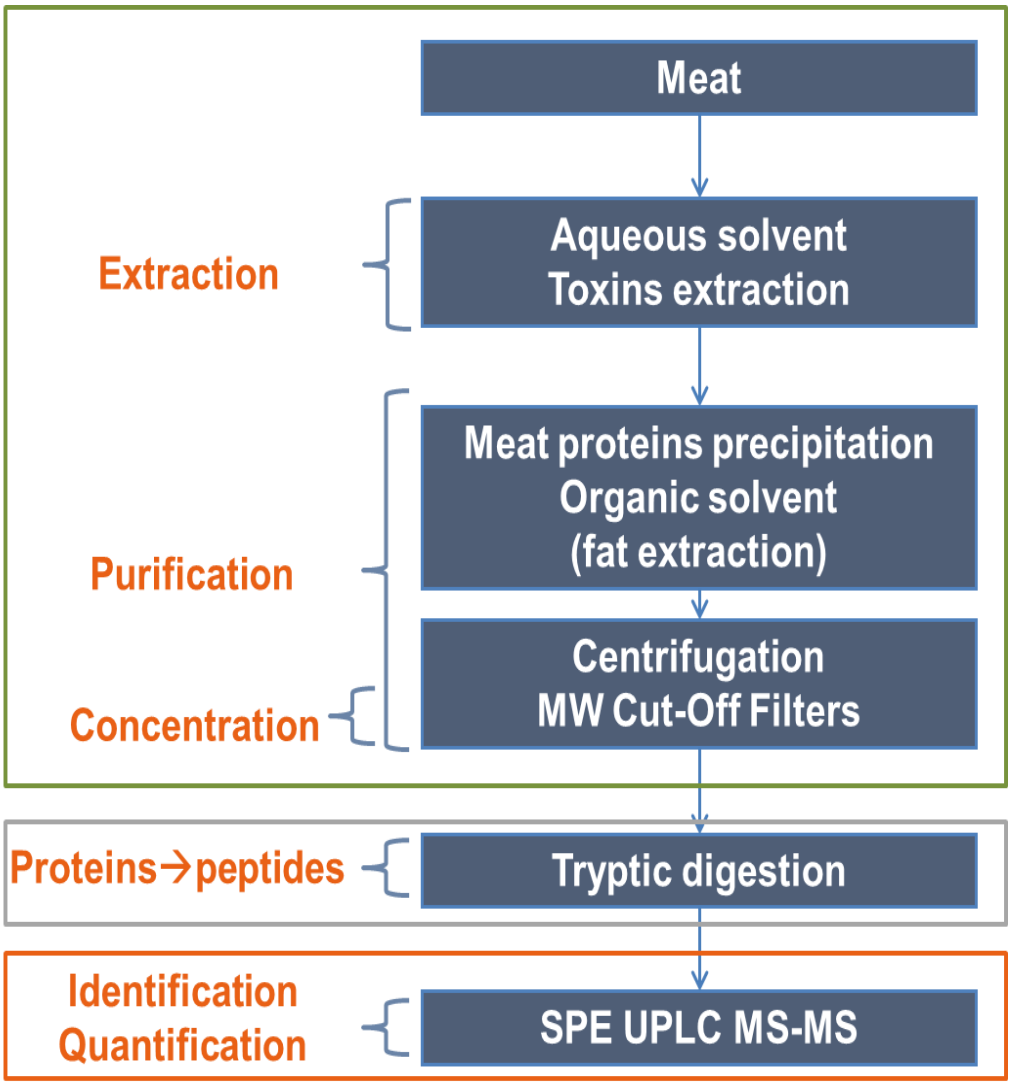

Figure 6. General protocol for Staphylococcal Enterotoxins detection in meat.
Green°: extraction, purification and concentration optimization;
Grey°: Digestion optimization; Orange°: UPLC-MS/MS optimization.

b) Pre-selection of peptides

The purpose of this part is to select unique peptides for each staphylococcal enterotoxin. Because the goal of the SETTECT project is to develop a multi-residual method and to adapt it on several matrixes, peptides were pre-selected for several SEs (SEA, SEB, SEC, SED, SEE, SEG, SEH, SEI, SEM, SEN, SEO and TSST-1).

A pre-selection of peptides was based on a theoretical research and aimed at selecting peptides that were susceptible of giving strong and unique signals with the method. A second selection happened once the internal standards transitions were selected and their separation by liquid chromatography was optimized.

The final selection took place in the experiments related to the meat protocol, where internal standards and their corresponding endogenous peptides were analysed. The goal was to select two peptides for each toxin, every peptide being identified by two transitions.

First, every enterotoxin's amino acid sequence was obtained from the Uniprot database. An illustration of the database and the list of all SEs amino acid sequences are available in Annexes 2 and 3.

As in the protocol the extracted toxins will be digested by trypsin, the second step was to obtain fragments resulting from a theoretical tryptic digestion. Those fragments were simulated using the MS-Digest tool. The obtained fragments are expected to present a lysine and/or an arginine residue to its C-terminal residue. The search parameters were set as follow°:

- No missed cleavage
- Minimal peptide length: 5

The reason for imposing a minimal length is that if the peptides are too small, future MS-MS fragmentation will be problematic because too small molecules give less fragment possibilities.

An illustration of the tool and the complete list of all theoretical tryptic fragments obtained for each SE are available in Annexes 4 and 5.

The third step was to select peptides according to several criteria°:

- All peptides longer than 25 amino acids were dismissed because of the mass to charge ratio (m/z) upper limit of the triple quadripole.
- Peptides with glutamic acid (E) or glutamine (Q) residues present at an end were also avoided because they present a risk of cyclization of the peptide;
- Peptides with methionine (M) or cysteine (C) residues present anywhere in the peptide sequence were eliminated because they may form disulphide bonds within the peptide;
- Peptides with tryptophan (W) residues present anywhere within the sequence were withdrawn from the list because they present a risk of oxidation, which would cause a shift in the m/z ratio (the same remark applies for M and C).

- Finally, peptides with glycine (G) or asparagine (N) residues present next to each other present a risk of adduct formation and were also removed from the selection.

After selecting the peptides according to practical criteria, the fourth step was to ensure their uniqueness. Indeed, if the analysis confirms the presence of a peptide in the sample extract, the peptide must only originate from a staphylococcal enterotoxin. It is important to keep in mind that the uniqueness of the peptide is limited to the actual range of known proteins as the peptide sequence is compared to every known and studied protein so far. It is very possible for an unknown or unstudied protein to present a similar sequence. The peptides sequences were thus confronted to the Blast Proteins database. Any sequence found to appear in a protein other than the corresponding SE was ruled out. More specifically, amongst the Blast P results presenting 100% identity with one theoretical peptide sequence, all the results presenting a query cover of 100% had to come from *Staphylococcus aureus*. Otherwise, the peptide is not considered specific to the toxin. The database is illustrated in Annex 6, as well as the list of peptides unique to each SE, in Annex 7.

Because the LC separation is based on the peptides average hydrophilicity, the fifth step was to examine the corresponding parameter, calculated using the Bachem peptide calculator, illustrated in Annex 8. If the average hydrophilicity value for one peptide was very different from the values of the other peptides, the peptide was eliminated to avoid long separation times.

In the meantime, peptides were also examined for their stability, using the manufacturer's Peptide Analysing Tool, also illustrated in Annex 8. Unstable peptides were eliminated. The final list of theoretically selected peptides is exposed in Table 13 (see the results section 4.1.1.)

Once selected, the theoretically selected peptides were ordered at Thermo Scientific where they were synthesized with isotope markers. More specifically, the C-terminal residue of all peptides contains ^{13}C and ^{15}N. The internal standards present thus a higher molecular weight than the endogenous peptides (analytes).

c) LC-MS/MS parameters

Instrument description

A standard Waters Acquity ultra performance liquid chromatographic system (UPLC, Waters Corp., USA) was coupled with a Xevo TQ-S mass spectrometer (Waters Corp., USA). To this standard configuration, one additional loading pump (Waters Corp., USA) and two high pressure switching valves (Vici, Switzerland) were added to make on-line SPE possible. All actions of this column switching system were coordinated in the Micromass 4.1 software. An Oasis HLB 2.1 mm × 20 mm, 15 µm column (Waters Corp., USA) was used as a trapping column in combination with an Acquity BEH C18 2.1 × 100 mm, 1.7 µm column (Waters Corp., USA) as analytical column (heated at 40 °C).

Peptides transitions

The pre-selected peptides marked with isotopes were infused in the Mass Spectrometer to search specific transitions. 10 pmol/µl individual working solutions of internal standard were infused in ES^+ mode (positive electrospray ionization) and with a 0.5 ml/min infusion flow.

The first step was to select the parent ion for each internal standard. Theoretical masses of parent ions, obtained with a fragment ion calculator, furnished guidelines for the research of these transitions. An illustration of this tool is available in Annex 10.

To search parent ions, infusion was done using the MS mode, the MS SCAN function and with only the desolvation gas turned on.

Signals around the theoretical parent ions ratios (m/z) were compared and the most intense was selected. The experimental m/z value for the parent ion was determined with precision.

The second step was to select daughter ions. Here again, theoretical masses of daughter ions, obtained with the fragment ion calculator, furnished guidelines.

To search daughter ions, infusion was done using the MS/MS mode, the Daughter SCAN function and with both the desolvation gas and the collision gas turned on.

Acquisitions were run for each peptide. Acquisition scans furnish all daughter ions coming from the fragmentation of a specific parent ion (the selected parent ion) when using a determined collision energy.

Several full scans were run for several collision energies and the daughter ions were examined and compared to theoretical ions furnished by the calculator. A compromise between advanced fragmentation of the parent ion and strong intensities of specific daughters was made.

The objective was to select four transitions for each peptide before selecting the two best.

If less than four daughters were available for the parent ion, acquisitions were run on a different parent ion (i.e the one presenting the second strongest intensity).

All experimental m/z values were determined for every transition and marked down.

Once the transitions were determined for the internal standards, the transitions for their corresponding analytes (or endogenous peptides) were calculated.

For every transition, the parent m/z value is corrected according to the parent ion charge (z value) and the extra weight added by the isotope amino acid on the internal standard.

For example, if a peptide is marked on the K residue, the extra weight is 10 Da (8 for R residue and 7 for the T residue).

If the parent ion m/z value is 698.7 and its charge is 2, then the weight difference between the IS and the endogenous parent ion will be of 10/2=5 Da for a K residue, bringing the endogenous parent ion m/z value to 693.7.

If the daughter ion is a B-ion (meaning the ion is a fragment of the internal standard that does not wear the isotope marker), the endogenous daughter ion m/z value is equivalent.

Table 8. Mobile phase gradients and flows applied on the solid phase extraction (SPE) and analytical (LC) columns.

	SPE			LC	
Time (min)	Flow rate (ml/min)	%ACN	%H2O	%ACN	%H2O
0	1	0	100	0	100
2	1	0	100	0	100
2.5	0.2	0	100	5	95
7	0.2	100	0	50	50
7.5	1	100	0	100	0
8.5	1	0	100	100	0
9	1	0	100	0	100
11	1	0	100	0	100

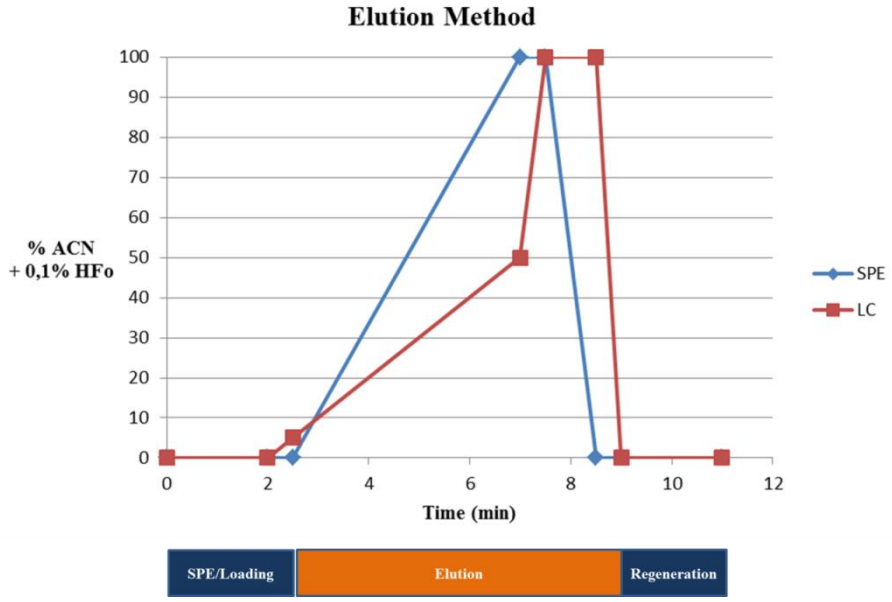

Figure 7. Mobile phase gradients applied on the SPE and LC columns.
The analytical steps are represented under the time axis.

If the daughter ion is a Y-ion (meaning the ion is a fragment of the IS wearing the isotope marker), the endogenous daughter ion m/z value must be corrected. If the marked residue is K and the internal standard daughter ion is charged once (z=1) with a m/z value of 983.0, then the endogenous daughter ion m/z value is 983.0 – (10/1) = 973.0.

All measured and calculated transitions are exposed in Table 14.

LC parameters

The applied LC-gradients and flow rates are referenced in Table 8 and illustrated in Figure 7. Solvent A consisted of 0.1% formic acid in acetonitrile, while solvent B consisted of 0.1% formic acid in water. The LC flow rate was 0.2 ml/min.

During injection, 10 µl were injected on the trapping column and then backflush eluted to the analytical column. Total run time (including SPE step and regeneration time of the column) was 11 min.

MS/MS parameters

The analytes were measured in positive electrospray ionization (ESI^+) mode. The monitored transitions and related parameters are exposed inT able 14. Capillary voltage was set to 3.0 kV, desolvation temperature was 550 °C. Desolvation and cone gas flow were set respectively to 1100 and 80 l/h.

Retention times and Multiple Reaction Monitoring (MRM) functions

In order to ensure proper LC separation and MS/MS detection, a pure mix of the internal standards (1 pmol/µl working solution of mixed internal standards°: SEA_1 to SEA_5 and SEB_1 to SEB_6) was injected. As the objective is to determine the retention times of each internal standard (and its matching endogenous peptide) in order to separate them in MRM (Multiple Reaction Monitoring) functions, no windows were imposed to the device. After injection, retention times and proper separation were examined and internal standards were divided into MRM functions or “windows” in order to rise the dwell time on each transition and to increase precision. The results are exposed in Table 15.

3.2.2 Extraction, purification and concentration

a) Objectives

The objectives of this section are to extract the toxins from the matrix, to eliminate impurities and to concentrate the toxins in a small volume (about 150µl). This major part of the process is framed in green in the general protocol for SEs detection in meat exposed in Figure 6.

Adapted from the milk protocol, the general meat protocol goes as follows. First the toxins are extracted in an aqueous phase (in milk, they are already in solution, so this step had to be added to the meat protocol). Then, matrix proteins and fat, both either from milk or from meat are removed from the aqueous phase using an organic solvent and precipitation. The toxins are then purified and concentrated using several filters before moving on to digestion.

This main part of the protocol was optimized using VIDAS SET2, SDS-PAGE and visual examination.

In order to facilitate the understanding and the reading of the present work, all optimizations are exposed following the logical order of the protocol and not the chronological order of the experiments. Phosphate buffer saline (PBS) solution was used as a reference extraction buffer because it is mentioned in the official method for "Detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices" (ANSES). But as phosphates are known for causing interferences in Mass Spectrometry analysis (because there are not volatile and therefore are not eliminated during desolvation), alternative aqueous phases were tested. Besides, the milk protocol uses acetic acid to lower the pH precipitate milk proteins so for the optimization of the meat protocol, the reference precipitation mode was pH lowering with 100% acetic acid and using a pHmeter. Because it is quite tedious to lower the pH of each sample by hand, alternative ways of precipitation were also tested.

The first exposed optimization tests on extraction, purification and concentration were made in order to choose a combination of aqueous toxins extraction solvent, meat proteins precipitation mode and organic solvent. Therefore, those parameters were tested and optimized using visual examination and SDS-PAGE analysis. As the general protocol uses filters and as meat is a complicated matrix, the objective was to find a combination that would allow going through filters without being blocked.

After a first combination was found, a VIDAS SET2 test was used to assess if the toxins were properly extracted from the matrix.

The second optimization tests on extraction, purification and concentration that are exposed were aiming at the choice of purification and concentration filters. Those parameters were tested using observations, SDS-PAGE analysis and VIDAS testing.

b) Extraction and first steps of purification°: choice of aqueous solvent, precipitation mode, extraction efficiency and organic solvent

As toxins are soluble in water, this solvent was chosen to extract them from the matrix. However, two major constituents of the matrix interfere with proper toxins recovery and have to be eliminated in the purification steps°: meat proteins and meat fat.

Precipitation by acidification and solvent extraction were the two strategies respectively explored to purify the toxins from the meat proteins and the meat fat in the extract.

Several schemes were tested. In every scheme, an extraction solution was added to the samples, the toxins were extracted, meat proteins were precipitated and an organic solvent was used to remove the fat from the samples. Finally the different phases (aqueous, solid and organic) were separated by centrifugation.

This procedure constitutes the extraction step and the first part of the toxins purification. Three parameters were optimized in this step°: the nature of the extraction solvent, the meat proteins precipitation mode and the organic solvent. Note that two extraction steps take place in the process°: one by adding the extraction solvent, to recover the toxins, and one by adding the organic solvent, to eliminate the fat.

Seven types of aqueous phases were tested, using observations and SDS-PAGE. Those phases are each coupled to a precipitation mode. Either pH adjustment by hand or with a buffer°:

- water brought to pH 4 using glacial acetic acid;
- an acetate buffer at pH 4;
- PBS brought to pH 4 using glacial acetic acid;
- PBS in a pH 4 acetate buffer;
- 0.9% NaCl in a pH 4 acetate buffer;
- 1.5% NaCl in a pH 4 acetate buffer;
- 3% NaCl in a pH 4 acetate buffer.

Two types of organic phases were tested, using also observations°: n-hexane and dichloromethane (CH_2Cl_2).

Figure 8. Scheme of the manipulations applied to each series of samples tested in the experiment "Choice of an aqueous solvent for toxins extraction and of a precipitation mode (part 1/2)".

Choice of an aqueous solvent and of a precipitation mode (part 1/2)

The optimization of the toxins extraction by an aqueous solvent took place using the selected organic solvent (dichloromethane, see section 4.2.1., choice of an organic solvent for fat extraction).

Seven series of samples were realized in triplicate, each named after one of the tested aqueous solvents. The manipulations applied to each series are exposed in Figure 8.

One gram of meat was weighed in each falcon tube. All were spiked with 20 µl of 50 ng/µl working solution of SEA and 20 µl of 50 ng/µl working solution of SEB.

5 ml of each aqueous solvent to test were added to three different tubes and the mix was passed on the vortex for about 15 seconds.

At this point, pH had to be adjusted for some samples. For the aqueous solvents already buffered (0.9% NaCl in a pH 4 acetate buffer; 1.5% NaCl in a pH 4 acetate buffer; 3% NaCl in a pH 4 acetate buffer; PBS in a pH 4 acetate buffer and pH 4 acetate buffer), no pH adjustment was necessary. All samples extracted either with water or simple PBS were brought to pH 4 adding glacial acetic acid with constant stirring and measuring the pH.

After proper homogenization of all the samples, first visual examinations were made and 100 µl aliquots were withdrawn from each sample and stored in small eppendorf tubes, in the fridge, for further SDS-PAGE analysis.

2 ml of dichloromethane were then added, each tube was passed on the vortex for about 15 seconds then phases were separated by centrifugation (3200 X g; 20 min).

At this point, other examinations were made and only aqueous phases that had properly separated from the other phases were removed and stored in fresh falcon tubes for further experimentation (see following section, Choice of an aqueous solvent and of a precipitation mode (part 2/2)). Also, 100 µl aliquots were withdrawn from each sample and stored in eppendorf tubes, in the fridge, for SDS-PAGE analysis.

Finally, to evaluate the protein contents of the aliquots, the efficiencies of the different saline solutions, and the effect of centrifugation, a SDS-PAGE gel was run according to the procedure describe in section 3.2.4, VIDAS SET2.

It is important to note that due to the limited material; especially the little number of gels available for the experiment, only some selected aliquots were submitted to SDS page.

Table 9 lists all aliquots from the present experiment along with their identification. Since visual examination helped drawing some important conclusions, not all groups of aliquots were analysed by SDS-PAGE. The groups of aliquots that were examined by SDS-PAGE are highlighted in Table 9. The reasons why only those samples were analysed are exposed in section 4.2.1.a).a)

Due to lack of gels, only one aliquot of groups 0.9 % -↓; 1.5 % -↓; and 3 % -↓ and two aliquots of groups 0.9 % - centri; 1.5 % - centri; and 3 % - centri were analysed. The aliquots selection was random.

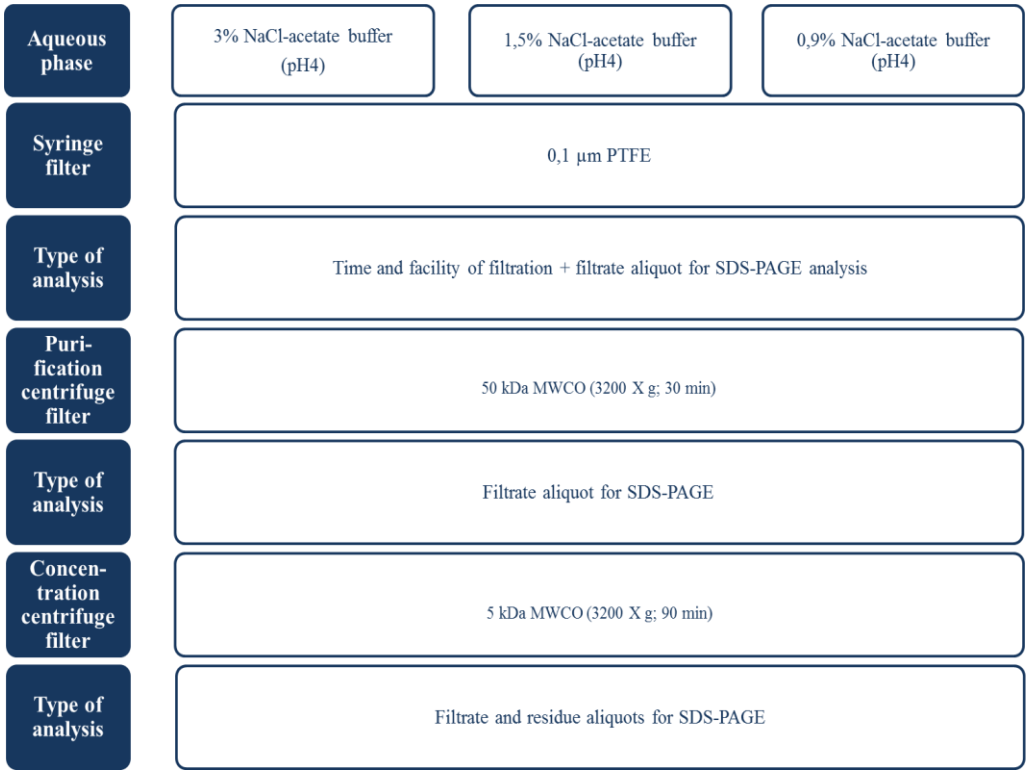

Figure 9. Scheme of the manipulations applied to each series of samples tested in the experiment "Choice of an aqueous solvent for toxins extraction and of a precipitation mode (part 2/2)".

Table 9. Identification of the aliquots withdrawn during the experiment "Choice of an aqueous solvent for toxins extraction and of a precipitation mode (part 1/2)".
The aliquots highlighted in orange were submitted to SDS-PAGE analysis.

Step	Aliquots names (n=3)	Standing for…
Toxins extraction + meat proteins precipitation	H_2O to pH 4-↓	Extracted in H_2O, precipitated by adjusting at pH 4
	PBS to pH 4-↓	Extracted in PBS, precipitated by adjusting at pH 4
	Acetate-↓	Extracted in acetate buffer, precipitated because pH fixed at 4
	PBS-↓	Extracted in PBS - acetate, precipitated because pH fixed at 4
	0.9 % -↓	Extracted in 0.9% NaCl - acetate, precipitated because pH fixed at 4
	1.5 %-↓	Extracted in 1.5% NaCl - acetate, precipitated because pH fixed at 4
	3 % -↓	Extracted in 3% NaCl - acetate, precipitated because pH fixed at 4
Fat extraction + centrifugation	PBS to pH4-centri	Extract from PBS adjusted at pH 4 + CH_2Cl_2 + centrifugation
	PBS-centri	Extract from PBS - acetate + CH_2Cl_2 + centrifugation
	0.9 %-centri	Extract from 0.9 % NaCl - acetate + CH_2Cl_2 + centrifugation
	1.5 % -centri	Extract from 1.5 % NaCl - acetate + CH_2Cl_2 + centrifugation
	3 % -centri	Extract from 3 % NaCl - acetate + CH_2Cl_2 + centrifugation

Choice of an aqueous solvent and of a precipitation mode (part 2/2)

This experimentation uses some of the aqueous phases that were removed and stored in the "Choice of an aqueous solvent for toxins extraction and of a precipitation mode (part 1/2)" experiment, exposed just above. More specifically, it uses the extracts in aqueous phases that contain sodium chloride in acetate buffer, after the fat was removed and they were centrifuged. Details are referred in Figure 8. The following manipulations, applied to those samples, are exposed in Figure 9.

The 0.9%; 1.5% and 3% NaCl solutions in acetate buffer (pH~4) supernatants were filtered using 0.1 µm PTFE syringe filters. Observations were made on the facility and the time taken to filter the extracts through those syringe filters. 100 µl aliquots were saved in eppendorf tubes for SDS-PAGE analysis.

Afterwards, the 0.1 µm filtrates were all centrifuged through a 50 kDa Molecular Weight Cut Off (MWCO) filter (3200 X g; ~30 min). Ultrafiltration was considered successful if the respective volumes of the residues remaining in the filters were below 100 µl. (Any filter that contained residue larger than 100 µl after 60 min of centrifugation would be considered to be blocked and was unsuited for further analysis). 50 kDa filtrates aliquots were saved for SDS-PAGE analysis.

Finally, the 50 kDa filtrates were centrifuged through a 5 kDa MWCO filter (3200 X g; 90 min). Aliquots of the 5 kDa residues and 5 kDa filtrates were saved as well. Those syringe and centrifuge filters were used because they were determined to be the right sizes to let pass or retain the toxins (see section 4.2.2).

Table 10 lists all aliquots related to this experiment along with their identification.

Here again, due to lack of available SDS-PAGE gels, only two aliquots of each group were analysed. The aliquots selection was random as well.

Table 10. Identification of the aliquots withdrawn during the experiment "Choice of an aqueous solvent for toxins extraction and of a precipitation mode (part 2/2)".

Step	Aliquots names (n=3)	Standing for…
0.1 µm syringe filtration	0.9 % - 0.1 µm	Supernatant from 0.9% NaCl – acetate + 0.1 µm PTFE syringe filter →syringe filtrate
	1.5 % - 0.1 µm	Supernatant from 1.5% NaCl - acetate + 0.1 µm PTFE syringe filter →syringe filtrate
	3 % - 0.1 µm	Supernatant from 3% NaCl - acetate + 0.1 µm PTFE syringe filter →syringe filtrate
50 kDa MWCO centrifuge filtration	0.9 % → 50 kDa	Syringe filtrate from 0.9 % NaCl - acetate + 50 kDa centrifuge filter →50 kDa filtrate
	1.5 % → 50 kDa	Syringe filtrate from 1.5 % NaCl - acetate + 50 kDa centrifuge filter →50 kDa filtrate
	3 % → 50 kDa	Syringe filtrate from 3 % NaCl - acetate + 50 kDa centrifuge filter →50 kDa filtrate
5 kDa MWCO centrifuge filtration	0.9 % → 5 kDa (filtrate)	50 kDa filtrate from 0.9 % NaCl - acetate + 5 kDa centrifuge filter →5 kDa filtrate
	1.5 % → 5 kDa (filtrate)	50 kDa filtrate from 1.5 % NaCl - acetate + 5 kDa centrifuge filter →5 kDa filtrate
	3 % → 5 kDa (filtrate)	50 kDa filtrate from 3 % NaCl - acetate + 5 kDa centrifuge filter →5 kDa filtrate
	0.9% → 5 kDa (residue)	50 kDa filtrate from 0.9 % NaCl - acetate + 5 kDa centrifuge filter →5 kDa residue
	1.5 % → 5 kDa (residue)	50 kDa filtrate from 1.5 % NaCl - acetate + 5 kDa centrifuge filter →5 kDa residue
	3 % → 5 kDa (residue)	50 kDa filtrate from 3 % NaCl - acetate + 5 kDa centrifuge filter →5 kDa residue

Extraction efficiency

Once an extraction protocol was selected (see results in 4.2.1), it was important to determine whether the aqueous solvent is successful at extracting the toxins from the matrix. This was assessed using a VIDAS SET2 test.

To determine whether all toxins are extracted by the selected aqueous solvent-precipitation mode combination, 1 µg of SEA and 1 µg of SEB were added to1 gram of meat, in a falcon tube so the initial contamination was of 1 ppm for each toxin. 5 ml of 1.5% NaCl, in pH 4 acetate buffer (0.1 M) were added to the tube and the mixture was passed on the vortex for about 15 seconds. Then 2 ml of dichloromethane were added and the mix was vortexed again for 15 extra seconds. The different phases were separated by centrifugation (20 minutes; 3200Xg).

The upper aqueous phase was withdrawn and a 500 µl aliquot saved for further testing. This aliquot is named "Extraction 1".

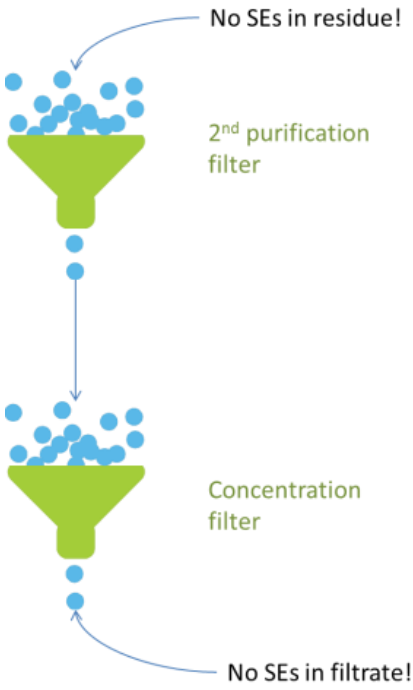

Figure 10. Theoretical purification and concentration of the toxins when using ultrafiltration centrifuge filters.

The organic phase (CH_2Cl_2) was removed from the falcon with a pipette and 5 ml of fresh aqueous extraction solvent (1.5% NaCl in pH4 acetate buffer) were added to the residual meat. Vortex was used again (15 seconds) and the two phases (meat and aqueous phase) were separated by centrifugation (20 minutes; 3200Xg). A second 500 µl aliquot was saved for testing and named "Extraction 2".

Both aliquots were then analysed following the VIDAS SET2 method (exposed in section 3.2.4, VIDAS SET2.

Choice of an organic solvent for fat extraction

The optimization of the fat extraction by an organic solvent took place using PBS as a reference aqueous solvent. The reference precipitation mode was used.

Two series of samples were realized in triplicate, bringing it to a total of six samples. The first was the "hexane series", and the second the "dichloromethane series". One gram of meat was weighed in each 15 ml falcon tube. 5 ml of PBS solution were added to each tube and the mix was passed on the vortex for about 15 seconds. All samples were brought to pH 4 adding glacial acetic acid with constant stirring and measuring the pH. 2 ml of n-hexane were added to three tubes and 2 ml of dichloromethane to the three others. Each tube was passed on the vortex for about 15 seconds then phases were separated by centrifugation (3200 X g; 20 min). Examination of fat removal and phase separations helped choosing one organic solvent.

c) Second part of purification and concentration°: choice of filters

The next part of the process involves microfiltration (syringe filters) and ultrafiltration (centrifuge filters). Some filters are part of the purification process and some allow the concentration of the toxins.

The first filter is a syringe filter and a purification step. It is followed by two Molecular Weight Cut Off filters (MWCO) that are used with a centrifuge.

Two pore sizes of syringe filters were tested as well as several membranes°: 0.2µm (Polyvinylidene Difluoride PVDF membrane) and 0.1µm (Polytetrafluoroethylene PTFE membrane) respectively were the two tested filters.

The next filter is also a purification filter and is the final step for toxins purification. The two tested sizes were 50 and 30 kDa MWCO PES (Polyethersulfone) filters. Finally, a last filter is used for concentrating the toxins before the digestion step. Two sizes of concentration filters were tested°: 10 kDa and 5 kDa MWCO PES filters. Figure 10 shows the contribution of the centrifuge filters to the toxins clean-up and concentration.

Microfiltration with a syringe filter

The choice of a syringe filter took place using PBS as a reference aqueous solvent. The reference precipitation mode was used.

An extraction protocol was applied on two samples and aliquots were withdrawn at two steps for SDS-PAGE analysis. One sample is named "0.2 µm testing" and the other is named "0.1 µm testing". The respective manipulations applied to those samples are exposed in Figure 11.

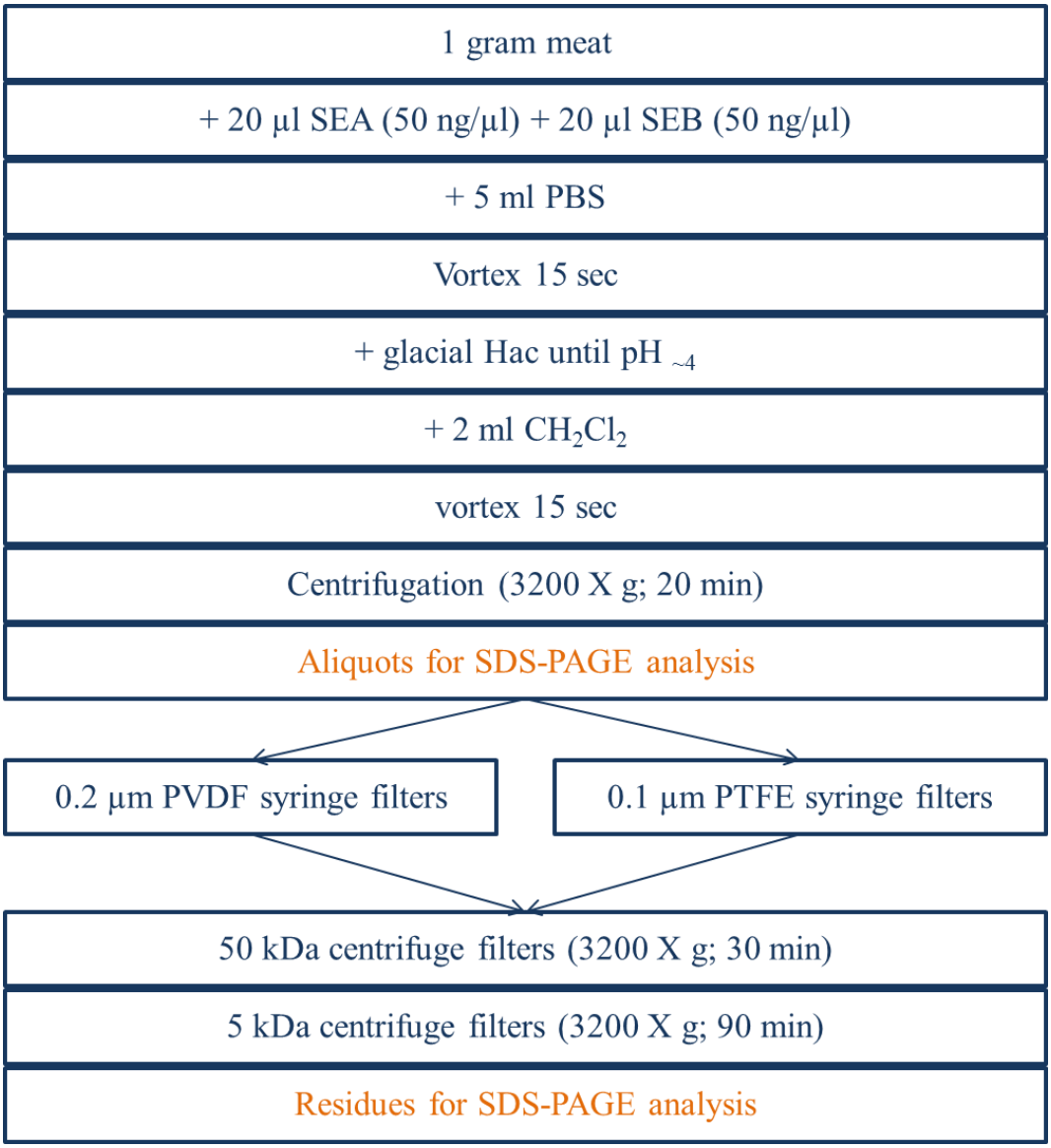

Figure 11. Scheme of the manipulations applied to each sample tested in the experiment "Microfiltration with a syringe filter" aiming at the choice of a syringe filter.

One gram of meat was weighed in each falcon tube. Both were spiked with 20 µl of 50 ng/µl working solution of SEA and 20 µl of 50 ng/µl working solution of SEB. 5 ml of PBS solution were added to each tube and the mix was passed on the vortex for about 15 seconds. Samples were brought to pH 4 adding glacial acetic acid with constant stirring and measuring the pH, 2 ml of dichloromethane were added and each tube was passed on the vortex for about 15 seconds. Finally, phases were separated by centrifugation (3200 X g; 20 min).

At this point, 100 µl aliquots were withdrawn from each sample and stored in eppendorf tubes, in the fridge, for SDS-PAGE analysis.

The aqueous phase from the "0.2 µm testing" sample was filtered through 0.2 µm PVDF syringe filters and the aqueous phase from the "0.1 µm testing" sample was filtered through 0.1 µm PTFE syringe filters.

All filtrates were next centrifuged through 50 kDa MWCO centrifuge filters (3200 X g; 30 min). Observations on the residual volume of the retentate were made, as the criteria for successful ultrafiltration was set to less than 100µl of residual retentate volume. The 50 kDa filtrates were passed through 5 kDa MWCO filters also by centrifugation (3200 X g; 90 min). The 5 kDa residues were withdrawn from the filters and saved in eppendorf tubes for SDS-PAGE analysis.

The efficiency of the 0.2 µm PVDF and the 0.1 µm PTFE syringe filters was evaluated running SDS-PAGE gels with the aliquots according to the procedure described in section 3.2.4, VIDAS SET2. Two different gels were run, as the syringe filters were tested at different times. A well of each gel was also filled with pure solutions of enterotoxins SEA and SEB (20 µl of each 50 ng/µl working solution were mixed in an eppendorf tube and prepared according to the SDS-PAGE protocol). Table 11 lists all aliquots from the present experiment along with their identification.

Table 11. Identification of the aliquots withdrawn during the experiment "Microfiltration with a syringe filter" aiming at the choice of syringe filter.

Step	Aliquots names	Standing for…
Extraction → centrifugation	"S → P" (A; B)	Sample + toxins + PBS + 100% HAc + CH_2Cl_2 + centrifugation (samples A and B)
Filtrations	"0.2; 50; 5" (A)	Supernatant + 0.2 µm PVDF + 50 kDa + 5 kDa → residue (sample A)
	"0.1; 50; 5" (B)	Supernatant + 0.1 µm PTFE + 50 kDa + 5 kDa → residue (sample B)

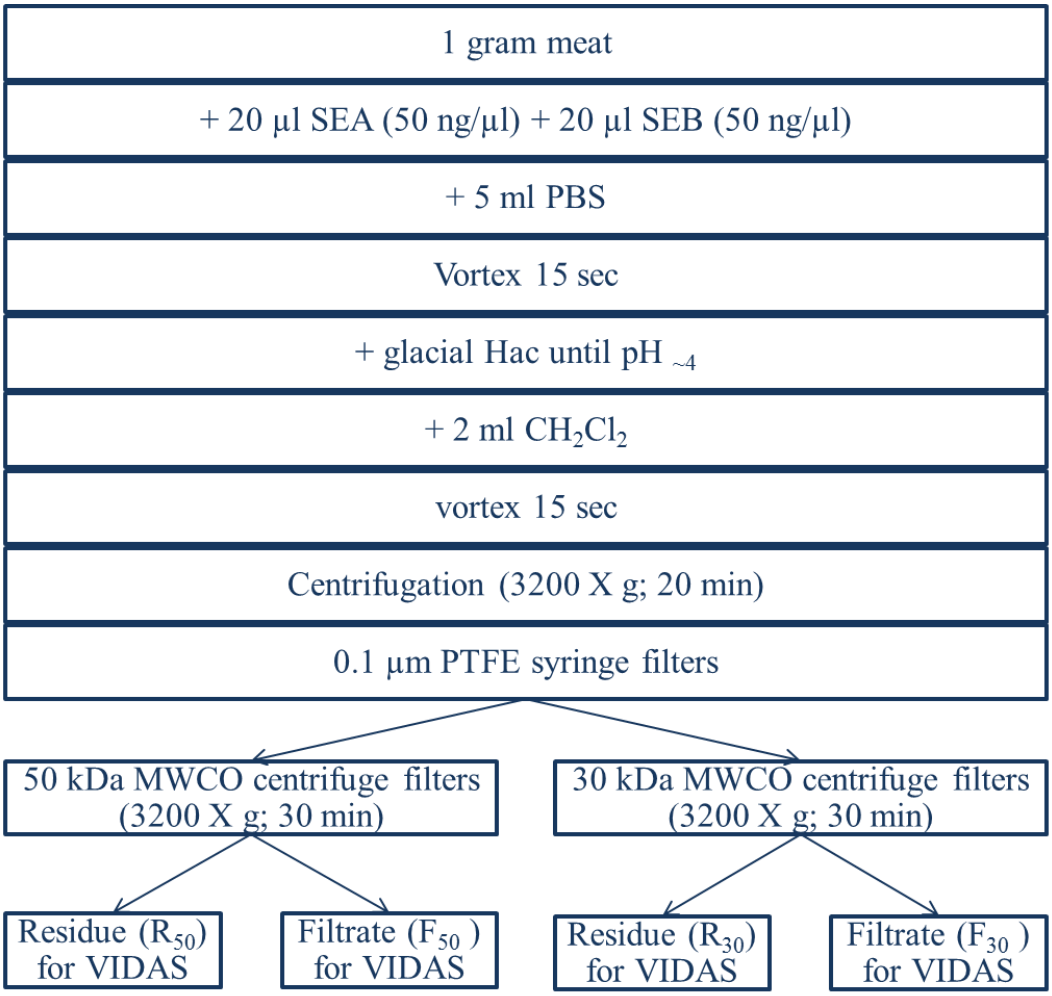

Figure 12. Scheme of the manipulations applied to each sample tested in the experiment "Ultrafiltration with centrifuge filters" aiming at the choice of the purification centrifuge filters.

Ultrafiltration with centrifuge filters

The purpose of centrifuge filters is either to let the enterotoxins through and to retain meat proteins and other particles (purification) or to retain them and let smaller meat proteins, ions and extraction solvents through (concentration). For both, two different filter sizes were tested°: 50 and 30 kDa MWCO for the purification filter and 10 and 5 kDa MWCO for the concentration filter.

The initial choice was set on the 30 kDa, which is the filter that presents a MWCO that is the closest to the toxins molecular weight (~27 kDa) that should let them through, and on the 10 kDa MWCO filter, because it is the filter with the closest MWCO to the toxins molecular weight that should retain them.

However, the manufacturer's recommendations on filtration stipulate that the chosen filter's MWCO has to be smaller than the third of the proteins-to-retain molecular weight. Therefore, 50 kDa and 5 kDa MWCO filters were respectively tested for purification and concentration.

The filters efficiencies were assessed using PBS as a reference aqueous solvent and the reference precipitation mode was used.

A complete extraction was run and aliquots were withdrawn at several points of the tested centrifuge filters for further VIDAS SET2 testing.

The experiment took place in two steps. First, the purification centrifuge filters were tested, then the concentration centrifuge filters.

Two samples were prepared. The first is named "50 kDa testing" and the second "30 kDa testing". The respective manipulations applied to those samples are exposed in Figure 12.

One gram of meat was weighed in each falcon tube. 5 ml of PBS solution were added to each tube and the mix was passed on the vortex for about 15 seconds. Samples were brought to pH 4 adding glacial acetic acid with constant stirring and measuring the pH, 2 ml of dichloromethane were added and each tube was passed on the vortex for about 15 seconds. Phases were then separated by centrifugation (3200 X g; 20 min), and the aqueous phases were all filtered through 0.1 µm PTFE syringe filters.

The syringe filtrate from the "50 kDa testing" sample was next put into 50 kDa MWCO centrifuge filters and the syringe filtrate from the "30 kDa testing" sample was put into 30 kDa MWCO centrifuge filters. All filters were centrifuged (3200 X g; 30 min).

The retentates and the filtrates of both purification centrifuge filters (50 and 30 kDa MWCO) were tested for SEs. Therefore, the retentates were withdrawn from the filters and submitted to a VIDAS SET2 test, according to the VIDAS procedure, section 3.2.4, VIDAS SET2. The samples are named "R_{50}" and "R_{30}". They are purification residues or "residues P".

500 µl aliquots of each filtrate were submitted to a VIDAS test as well. Those samples are named "F_{50}" and "F_{30}". They are purification filtrates or "filtrates P".

After the purification centrifuge filter was selected (50 kDa MWCO, see section 4.2.2, Ultrafiltration with centrifuge filters), two samples were prepared. The first is named "10 kDa testing" and the second "5 kDa testing". The respective manipulations applied to those samples are exposed in Figure 13.

One gram of meat was weighed in each falcon tub. 5 ml of PBS solution were added to each tube and the mix was passed on the vortex for about 15 seconds. Samples were brought to pH 4 adding glacial acetic acid with constant stirring and measuring the pH, 2 ml of dichloromethane were added and each tube was passed on the vortex for about 15 seconds. Phases were then separated by centrifugation (3200 X g; 20 min), the aqueous phases were both filtered through 0.1 µm PTFE syringe filters and syringe filtrates were centrifuged through the selected 50 kDa MWCO centrifuge filters and centrifuged (3200 X g; 30 min).

The 50 kDa centrifuge filtrate from the "10 kDa testing" sample was next put into a 10 kDa MWCO centrifuge filter and the 50 kDa centrifuge filtrate from the "5 kDa testing" sample was put into a 5 kDa MWCO centrifuge filter. The filters were centrifuged (3200 X g; 90 min).

The retentates and the filtrates of both concentration centrifuge filters (10 and 5 kDa MWCO) were tested for SEs. Therefore, the retentates were withdrawn from the filters and submitted to a VIDAS SET2 test, according to the VIDAS procedure, section 3.2.4, VIDAS SET2. The samples are named "R_{10}" and "R_5". They are concentration residues or "residues C".

500 µl aliquots of each filtrate were submitted to a VIDAS test as well. Those samples are named "F_{10}" and "F_5". They are concentration filtrates or "filtrates C".

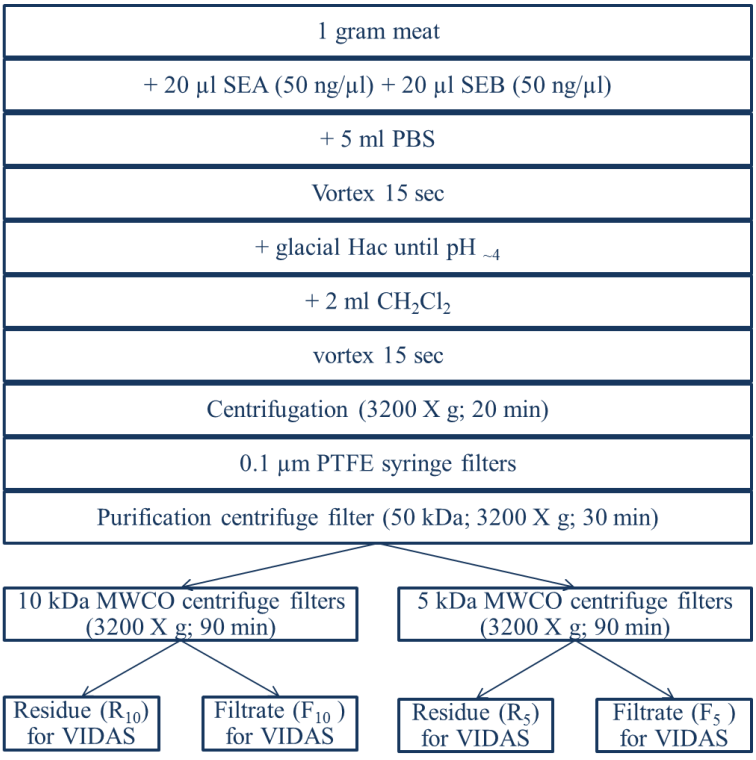

Figure 13. Scheme of the manipulations applied to each sample tested in the experiment "Ultrafiltration with centrifuge filters" aiming at the choice of the concentration centrifuge filters.

3.2.3 Digestion

a) Objectives

The objectives of this section are to digest the extracted, purified and concentrated toxins into peptides. The peptides are the analytes that will be detected and quantified by UPLC-MS/MS. The digestion is divided in two major parts°: the preparation of the toxins for digestion and the digestion itself. This major part of the process is framed in grey in the general protocol for SEs detection in meat exposed in Figure 6.

b) Protocol

For this section, the toxins digestion method of the milk protocol has almost exclusively been applied to the toxins digestion in the meat protocol.

The digestion conditions were optimised on the level of standards. The matrix was not considered since the objective here is to optimize the proteolysis of toxins into peptides. The efficiency of this step was assessed by comparing the chromatograms and response ratios of the several series of samples.

A single parameter was optimized when transferring the digestion step from the milk to the meat protocol°: the nature of the digestion buffer, used to adjust the pH in the adequate trypsin pH range. This parameter needed optimization because of a major difference between the milk and the meat protocols°: the toxins aqueous extraction solvent is also a buffer (1.5% NaCl in acetate buffer, see section 4.2.1). Indeed in the meat protocol, an aqueous solvent prepared with acetate buffer is used and is still present with the concentrated toxins at the end of the filtration steps. Because the toxins are contained in a pH 4 buffer and need to be put in a 7-9 pH range, two additional, stronger digestion buffers were tested.

In order to evaluate if interferences were caused by the presence of the toxins extraction buffer, this solution was added to half the series of samples.

The tested digestion buffers were°:

- Ammonium bicarbonate (50 mM) buffer at pH 8.5 (digestion buffer used in the milk protocol);
- Tris-$CaCl_2$ (500 mM; 100 mM) buffer at pH 9.5;
- Tris (100 mM) buffer, pH 9.5.

The two additional digestion buffers containing Tris have a pH that is higher than the searched pH range because, as the solution containing the toxins is a strong buffer, it is expected to lower the final pH of the digestion area.

Six series of toxins mix were prepared for digestion, in triplicate. Three series contained aqueous extraction solvent along with one of the three digestion buffers to test, and three only contain one of the pure digestion buffers.

Each series of tubes was prepared as follows and the corresponding manipulations are exposed in Figure 14.

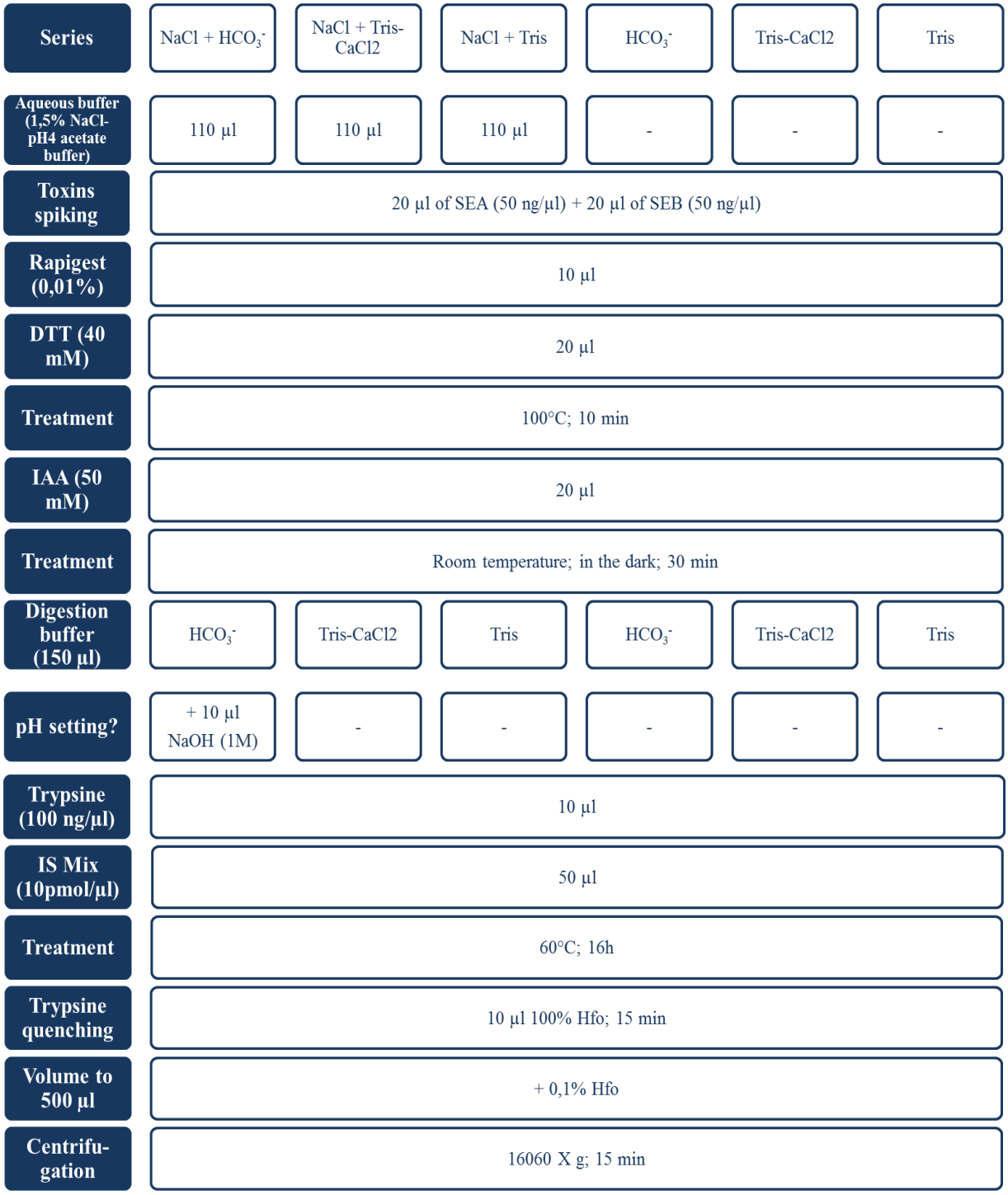

Figure 14. Scheme of the manipulations applied to each sample of the experiment "digestion" aiming at the choice of the digestion buffer.

110 µl of NaCl were added in all eppendorf tubes of the « NaCl + HCO_3^- » ; « NaCl + Tris-$CaCl_2$ » ; and « NaCl + Tris »series;

110 µl of digestion solution were added in all eppendorf tubes of the « HCO_3^- » ; « Tris-$CaCl_2$ » ; and « Tris » series;

20 µl of SEA (50 ng/ul) and 20 µl of SEB (50 ng/ul) were spiked in each tube.

Were successively added 10 µl of Rapigest (0.01%) and 20 µl of 40mM DTT ($[DTT]_{final}$: 4 mM) to each tube. They were then put 10 minutes at 100 °C on the thermoblock, removed and condensation was brought down the tubes with the minicentrifuge.

20 µl of 50mM IAA ($[IAA]_{final}$: 5 mM) were added and the tubes were left 30 min in the dark, at room temperature. 150 µl of HCO_3^-, Tris-$CaCl_2$, and Tris buffers were added to their corresponding series and all the samples pH were measured to ensure they were in the proper range (7-9). If necessary, the pH was adjusted using NaOH 1M.

50 µl of Internal Standards Mix at 10 pmol/ µl, containing SEA_2, SEA_4, SEA_5, SEB_1, SEB_2, SEB_4, SEB_5 and SEB_6, were added ($[IS]_{final}$: 1 pmol/µl) as well as 10 µl of 100ng/µl of Trypsin. The tubes were vortexed and placed for 16 hours at 60 °C on the thermoblock. After the digestion, 10 µl of pure formic acid were added and the tubes were left at room temperature for 15 minutes. The samples volumes were adjusted to 500 µl with 0.1% formic acid, the tubes were centrifuged (16000Xg, 15 min) and the supernatants were transferred to UPLC vials for analysis, according to parameters exposed in section 3.2.1, LC-MS/MS parameters. The monitored MRM transitions and the compound dependent parameters can be found in table 15 (section 4.1.2).

Two types of results may be drawn from the resulting chromatograms. First, some internal standards (and their corresponding endogenous peptides) can be eliminated from the selection if the obtained signals are not satisfying.

Second, the digestion yields are estimated and examined. It is important to keep in mind that those digestion yields are only an estimation. The first reason being that the Internal Standards used in the study (Thermo Fisher) are of ~70% purity. This is an estimated value for lower-price standards used in the process of method optimization. Higher purity standards are commonly used for established functioning methods. Second, the analytical method is not validated and the linearity range is unknown.

The examination of the digestion yields (one for each endogenous peptide), in the different tested combinations, will show if the nature of the buffer influences the digestion efficiency, if so which one works best, and if the presence of the toxins extraction buffer constitutes an interference. The digestion yield is the ratio between the amount of digested toxin and the amount of added toxins (equation 1).

Equation 1°:

$$digestion\ yield\ (\%) = \frac{Toxins_{\ digested}}{Toxins_{\ spiked}} * 100$$

Where°:

$Toxins_{digested}$ is the amount of toxins digested that is recalculated with the chromatograms peak areas [g].

$Toxins_{spiked}$ is the known amount of toxins submitted to digestion [g].

The recalculation of the digested toxins goes as follows.

Theoretically, the digestion of one mole of toxins results into one mole of each searched endogenous peptide.

The amount of resulting endogenous peptide is calculated by the use of the added Internal Standard (IS) which is the corresponding peptide with an isotope marker (equation 2).

Equation 2°:

$$Conc._{endo.pept.} = \frac{Area_{endo.pept.}}{Area_{IS}} * Conc._{IS} = Response * Conc._{IS}$$

Where°:

$Conc._{endo.pept.}$ is the concentration of the searched endogenous peptide [mol/l];

$Conc._{IS.}$ is the concentration of the corresponding Internal Standard [mol/l];

$Area._{endo.pept}$ is the peak area of the searched endogenous peptide [area units];

$Area._{IS}$ is the peak area of the corresponding Internal Standard [area units];

Response is the ratio of the respective peak areas [/].

Since the final volume containing the endogenous peptide is known, the amount of endogenous peptides (mol) can be calculated. The amount of endogenous peptides is equal to the amount of toxins (mol). The molecular weights of the toxins are known and give the reverse calculated amount of toxins (g). Finally, the digestion yield is calculated.

3.2.4 Additional tools

a) VIDAS® SET2

The VIDAS® SET2 is a commercial kit used for the detection of Staphylococcal enterotoxins. It is described by the European Union – Reference Laboratory for CPS as official method for staphylococcal enterotoxins testing. As this test only gives an indication on the presence or absence of toxins in a sample (positive or negative sample), no exact quantification is possible. Besides, this detection kit evaluates the presence of five SEs (SEA, SEB, SEC, SED and SEE), without quantifying them. However it proved a very useful tool as it was used to assess the presence of the toxins in different phases of the protocol, therefore providing indication on the efficiency of the extraction phase.

The official immunologic detection method was used as an accessory tool for the optimization of three steps of the protocol : the toxins extraction, their purification and their concentration.

The samples do not need any additional preparation. 500 µl aliquots of the samples are injected in the appropriate well of a VIDAS SET2 kit (one per sample) and placed in the miniVIDAS device according to the manufacturer's instructions. In this well, the searched toxin (antigen) is captured by an antibody fixed in the well. One ready-to-use syringe containing all the necessary reagents is added to the device (also one per sample). The analysis is fully automated and the results are available within 80 minutes. The intensity of the fluorescence is interpreted by the system which converts it into a positive or negative answer for the presence of SEs (SEA-SEE).

b) SDS-PAGE

The purpose of the SDS-PAGE procedure is to evaluate the protein composition of a sample. This type of electrophoresis allows separation of proteins according to their molecular weight. This tool was used as an indicator of the purification brought by every step of the protocol. Aliquots withdrawn at several steps of the optimization were separated on a gel by the following protocol.

Protocol

Gloves are required during the whole manipulation to avoid contaminations and the colouring of fingerprints during the staining step.

The staining by Coomassie blue was used as it is more suited for highly concentrated proteins.

Samples and markers preparation

Aliquots containing the molecular weight markers are thawed at room temperature and homogenized with a vortex.

A 10 µl aliquot of the sample is mixed to 10 µl of sample buffer in an eppendorf tube. The tubes are carefully locked and put five minutes on the heating block previously set on 100 °C. After the heating, the tubes are left to cool down at room temperature and passed on a vortex.

Gel preparation

The electrophoresis tank is filled with electrophoresis buffer up to the mark. The gel is removed from its packaging and placed in the tank according to manufacturer's instructions. The plastic box that contained the gel is washed and filled with cold MilliQ water as it will be used later.

Every well of the gel is filled by 10 µl of either the prepared samples or filling buffer (as wells are never left empty) using a micropipette. A new tip is used for every well. Gels were loaded by filling one well out of two with treated samples and the other with filling buffer.

Electrophoresis separation

When all the wells are filled, the tank is closed and connected with electric wires to the power generator. The separation parameters, programed on the generator, are 150 volts, 3.0 amperes and 90 minutes.

Gel coloration

When the run is over, the generator is turned off, the tank disconnected and the gel removed. The case is cracked open and the gel's edges are carefully cut out with a surgical blade.

The gel is placed in the plastic container filled with cold MilliQ water and washed three times during five minutes with agitation (on the rocking platform). Fresh cold MilliQ water is used for every wash. Afterwards, the water is removed and replaced by Coomassie solution. The gel is incubated under agitation for about 60 minutes. When the gel is stained, it is rinsed 30 minutes in cold MilliQ water, still under agitation and stored in fresh cold MilliQ water in the closed plastic container, in the fridge, until it is scanned.

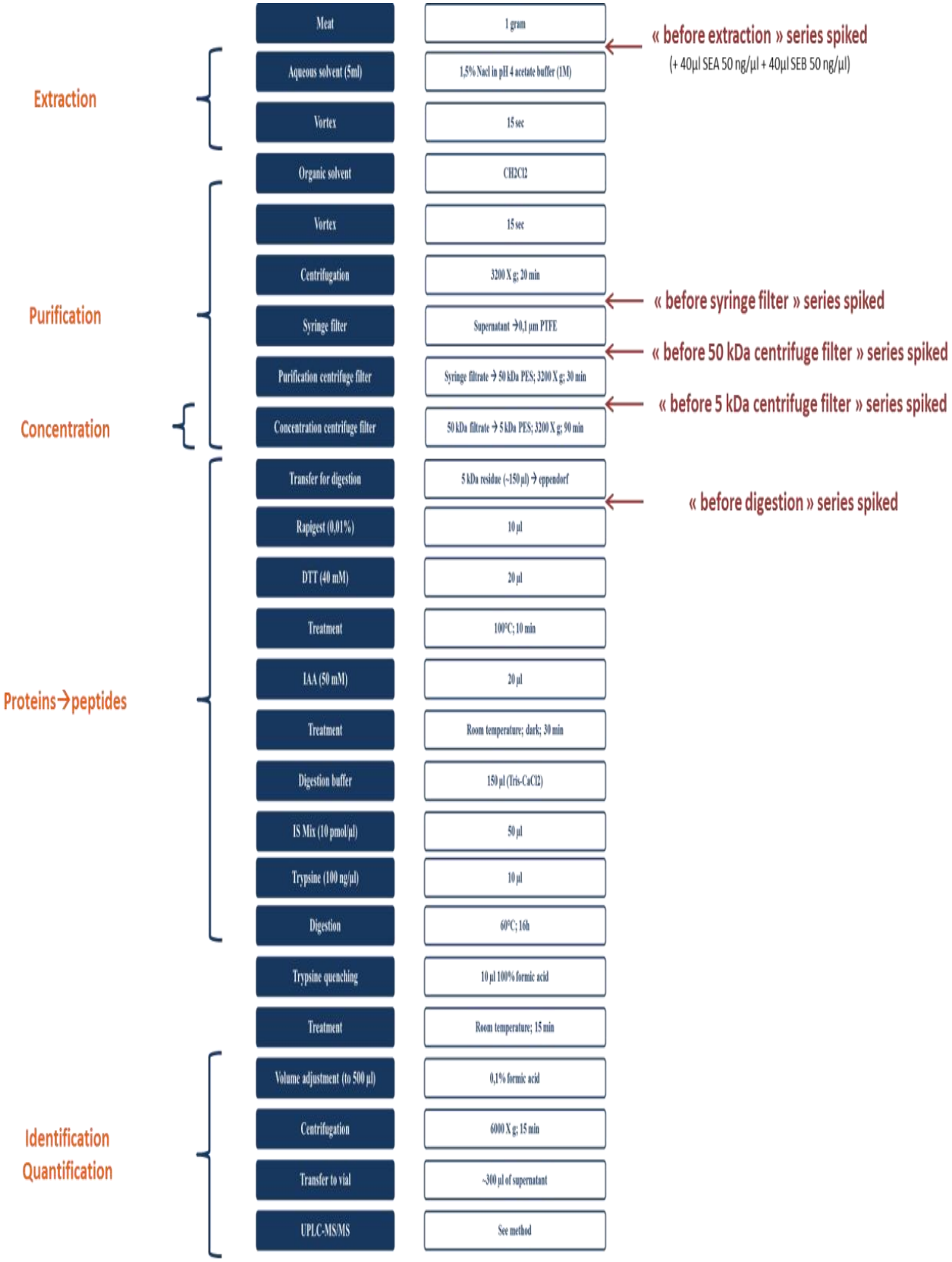

Figure 15. Scheme of the manipulations applied to the series of the experiment "full method evaluation" aiming at the estimation of toxins losses in the meat protocol.

3.2.5 Full method evaluation

The objective of the final part of this work was to integrate all the optimizations brought to the meat protocol in order to evaluate the efficiency of the global process. This general testing pointed out the steps still in need for further improvements.

For this experiment, all individually optimized parts of the protocol were brought together. Five series of samples were tested, as exposed in Figure 15 and spiked with the enterotoxins at several key points of the process. The series names and their meanings are grouped in Table 12. As the objective of the experiment is to examine eventual toxin losses, the spiking level was very high. Therefore, the initial contamination was set on 2000 ppb.

Rapidly, one gram of meat was weighed in each falcon tube. 5 ml of aqueous extraction solvent (1.5% NaCl in pH4 acetate buffer) were added to the tubes and the mix was passed on the vortex for about 15 seconds. 2 ml of dichloromethane were then added and each tube was passed on the vortex for 15 seconds then phases were separated by centrifugation (3200 X g; 20 min). All supernatants were filtered using 0.1 µm (PTFE) syringe filters, the 0.1 µm filtrates were then all centrifuged through a 50 kDa MWCO filter (3200 X g; 30 min) and the 50 kDa filtrates were centrifuged through a 5 kDa MWCO filter (3200 X g; 90 min).

The 5 kDa residues are transferred to eppendorf tubes, 10 µl of 0.01% rapigest are added together with 20 µl of 40 mM DTT ($[DTT]_{final}$: 4 mM) to the transferred residue and samples are heated at 100°C for 10 minutes. They are then cooled down to room temperature and 25 µl of 50 mM IAA ($[IAA]_{final}$: 5 mM) are added. The samples are placed in the dark for 30 minutes, 150µl of Tris-$CaCl_2$ digestion buffer is added, followed by 50 µl of 10 pmol/µl IS mix (($[IS]_{final}$: 1 pmol/µl) and 10 µl of 100 ng/µl Trypsin. The samples are placed in a heating block (Thermomixer Compact Eppendorf) at 60°C, overnight (~16h).

After incubation 10 µl of pure formic acid are added and after 15 minutes the samples are brought to a final 500 µl volume with 0.1% formic acid and centrifuged (16060 X g; 15 min). The final supernatant is transferred to UPLC vials for analysis, according to parameters exposed in section 3.2.1, LC-MS/MS parameters. The monitored MRM transitions and the compound dependent parameters can be found in table 15 (section 4.1.2).

The resulting chromatograms were processed and the toxins recoveries were evaluated for every sample. The same remark as in the digestion yield calculation applies with regards to the recoveries calculations. What is here named recoveries is only a rough estimation of the toxins passing through the protocol steps.

The toxins recovery is the ratio between the amount of extracted and digested toxin and the amount of added toxins (equation 3).

Equation 3°:

$$toxins\ recovery\ (\%) = \frac{Toxins_{\ extracted\ and\ digested}}{Toxins_{\ spiked}} * 100$$

Where°:

$Toxins_{extracted\ and\ digested}$ is the amount of toxins extracted and digested that is recalculated with the chromatograms peak areas [g].

$Toxins_{spiked}$ is the known amount of toxins spiked at one step of the protocol [g].

The amount of extracted and digested toxins was recalculated using the same reasoning as exposed in 3.2.3.

As this calculation was done for each peptide transition and at each crucial step of the protocol, it was possible, by comparing the toxins recoveries at every step, to estimate which step causes losses.

Table 121. Series tested in the experiment "full method evaluation" and their identification.

Series name	Spiking step
"before digestion" (n=3)	Before extraction (realized in triplicate)
"before syringe filter" (n=3)	Before syringe filter (realized in triplicate)
"before 50 kDa centrifuge filter" (n=3)	Before purification centrifuge filter (realized in triplicate)
"before 5 kDa centrifuge filter" (n=3)	Before concentration centrifuge filter (realized in triplicate)
"before digestion" (n=3)	Before digestion (realized in triplicate)

Table 14. List of the peptides related to Staphylococcal Enterotoxins SEA and SEB. The table lists for each peptide, its name, sequence, the different transitions followed by the MS/MS for the endogenous peptide and for the matching internal standard, and some MS/MS parameters (cone voltages and collision energies). The peptides highlighted in red were eliminated after injection on column of a pure mix.

Internal Standard	Sequence	Internal Standard Transition		Parent charge	Daughter charge	Endogenous peptide Transition		Cone Voltage (V)	Collision Energy (eV)
		Parent (m/z)	Daughter (m/z)			Parent (m/z)	Daughter (m/z)		
SEA_1	VPINLWLDGK	582,20	212,20	2	1	578,20	204,20	30	20
		582,20	532,60	2	2	578,20	528,60	30	20
		582,20	626,60	2	1	578,20	618,60	30	20
		582,20	966,80	2	1	578,20	958,80	30	20
SEA_2	YNLYNSDVFDGK	722,30	278,20	2	B-ion	718,30	278,20	30	27
		722,30	391,30	2	B-ion	718,30	391,30	30	27
		722,30	474,40	2	1	718,30	466,40	30	27
		722,30	212,20	2	1	718,30	204,20	30	27
SEA_3	NVTVQELDLQAR	698,70	214,20	2	B-ion	693,70	214,20	35	27
		698,70	983,00	2	1	693,70	973,00	35	27
		698,70	612,50	2	1	693,70	602,50	35	27
		698,70	1081,60	2	1	693,70	1071,60	35	27
SEA_4	SELQGTALGNLK	619,70	330,10	2	B-ion	615,70	330,10	30	22
		619,70	217,15	2	B-ion	615,70	217,15	30	22
SEA_5	GFFTDHSWYNDLLVDFDSK	772,80	619,50	3	1	770,13	611,50	30	20
		772,80	686,40	3	B-ion	770,13	686,40	30	20
		772,80	718,60	3	1	770,13	710,60	30	20
		772,80	831,70	3	1	770,13	823,70	30	20
SEB_1	VLYDDNHVSAINVK	798,20	185,20	2	2	794,20	181,20	30	31
		798,20	213,30	2	B-ion	794,20	213,30	30	31
		798,20	692,30	2	2	794,20	688,30	30	31
		532,60	185,20	3	2	529,93	181,20	30	16
SEB_2	VTAQELDYLTR	660,00	677,60	2	1	655,00	667,60	30	20
		660,00	790,70	2	1	655,00	780,70	30	20
		660,00	919,80	2	1	655,00	909,80	30	25
		660,00	562,50	2	1	655,00	552,50	30	30
SEB_3	TNDINSHQTDK	641,00	724,00	2	1	637,00	716,00	30	30
		641,00	837,50	2	1	637,00	829,50	30	30
		641,00	950,50	2	1	637,00	942,50	30	30
		641,00	1066,00	2	1	637,00	1058,00	30	30
SEB_4	SIDQFLYFDLIYSIK	625,30	518,50	3	1	622,63	510,50	30	15
		625,30	859,80	3	1	622,63	851,80	30	15
		625,30	1006,90	3	1	622,63	998,90	30	15
		625,30	631,60	3	1	622,63	623,60	30	15
SEB_5	NLLSFDVQTNK	644,20	1059,85	2	1	640,20	1051,85	30	20
		644,20	946,80	2	1	640,20	938,80	30	20
		644,20	341,35	2	B-ion	640,20	341,35	30	20
		644,20	228,20	2	B-ion	640,20	228,20	30	20
SEB_6	LGNYDNVR	481,00	185,20	2	1	476,00	175,20	30	25
		481,00	398,40	2	1	476,00	388,40	30	21
		481,00	847,70	2	1	476,00	837,70	30	21

4 Results and discussions

4.1 Identification by UPLC-MS/MS

4.1.1 Pre-selection of peptides

Table 13 shows the pre-selection of unique and stable peptides related to enterotoxins SEA and SEB. The complete list of preselected peptides for each toxin implied in the SETTECT project is illustrated in Annex 9.

Table 13. Pre selection of unique and stable peptides related to each Staphylococcal Enterotoxin SEA and SEB.

Name	Abbreviation	Sequence
Staphylococcal Enterotoxin A	SEA_1	VPINLWLDGK
	SEA_2	YNLYNSDVFDGK
	SEA_3	NVTVQELDLQAR
	SEA_4	SELQGTALGNLK
	SEA_5	GFFTDHSWYNDLLVDFDSK
Staphylococcal Enterotoxin B	SEB_1	VLYDDNHVSAINVK
	SEB_2	VTAQELDYLTR
	SEB_3	TNDINSHQTDK
	SEB_4	SIDQFLYFDLIYSIK
	SEB_5	NLLSFDVQTNK
	SEB_6	LGNYDNVR

4.1.2 LC-MS/MS parameters

a) Peptides transitions

Table 14 shows all selected transitions for each infused internal standard and the corresponding endogenous peptides transitions that were calculated according to the principles explained in section 0 pre-selection of peptides. The cone voltages used for the parent ion selection and the collision energy employed for its fragmentation are also exposed. Calculation details are available in Annex 11.

b) LC parameters

The injection on column of the 1 pmol/µl mix of all SEA and SEB internal standards with the gradients exposed in section 3.2.1, LC-MS/MS parameters aimed the determination of retention times and the repartition of the peptides in several MRM functions. It also allowed a second peptides selection.

Indeed, SEA_1, SEA_3 and SEB_3 were not detected by MS/MS after UPLC separation. They were eliminated of the list of selected peptides that could be used for the identification of SEA and SEB in matrix since due to the complexity of the matrix they may be even more difficult to detect.

Note°: Because of device technical issues and to make sure the transitions were always accurate, the transitions were checked several times, especially after reparations were made on the device. The m/z ratio values were therefore often readjusted as shifting was observed, for example after one of the quadripoles was replaced. The transitions exposed in Table 14 are the final transitions of internal standards (and corresponding endogenous peptides) that were used for the experiments implying the UPLC-MS/MS.

Figure 16 illustrates the separation of the internal standards by liquid chromatography. Every peak is labelled with the internal standard name. As the endogenous peptides will present the same retention times as their matching IS, they are marked down as well. The resolution between SEB_2 and SEA_2, (circled in blue) is the least obtained but still may be used, once the transitions are separated in MRM functions as listed in Table 15, the peaks present satisfying intensities and symmetries. Those peaks are available in Annex 12.

Table 15 shows the repartition of all transitions (from IS and endogenous peptides) and the MS Method parameters (cone voltages, collision energies, dwell times). The complete details of the UPLC-MS/MS analysis are in Annex 13 (MS Method report, Inlet Method report and MS Tune Detector Parameters).

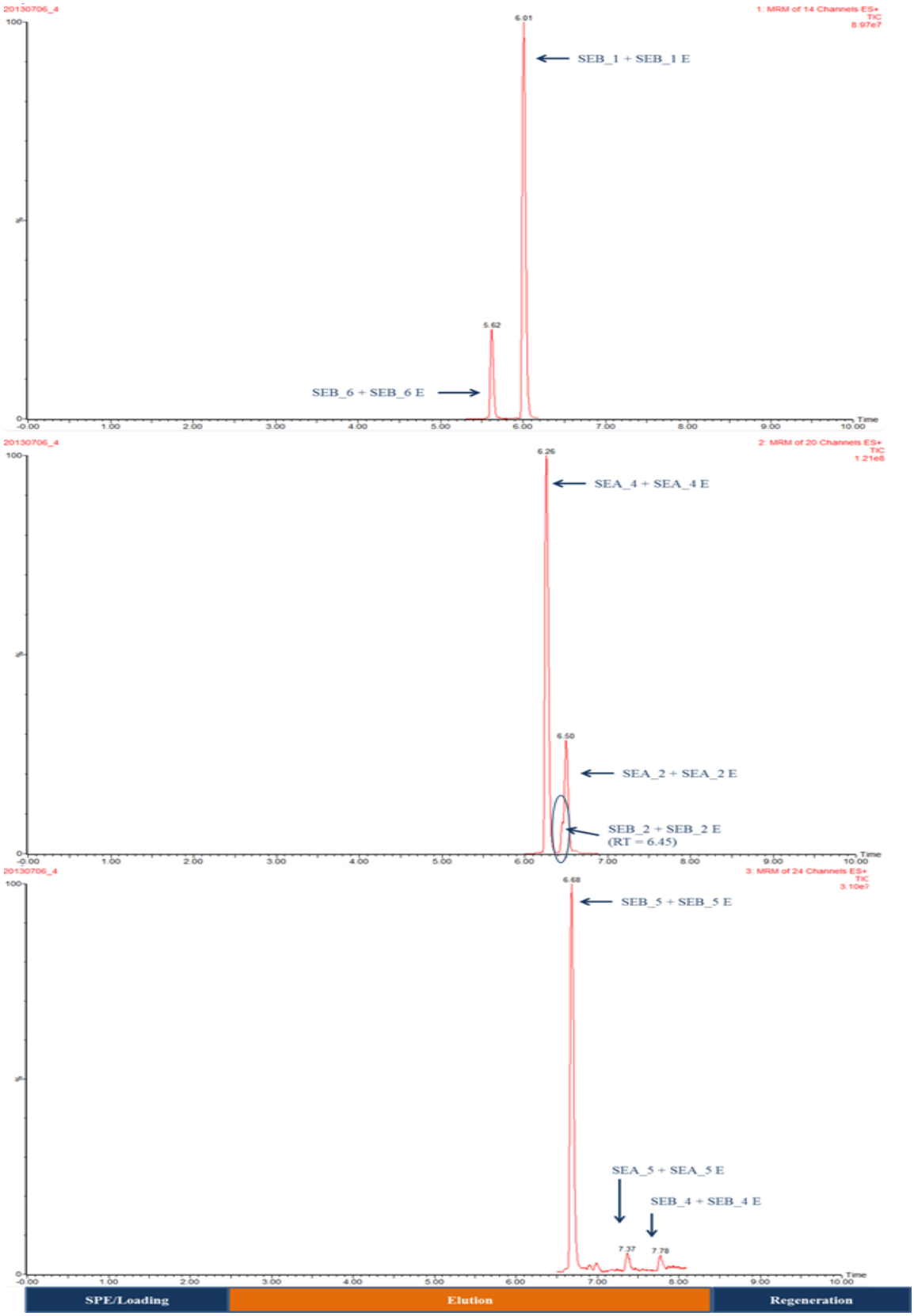

Figure 16. Chromatograms of the internal standards injected in 0.1% formic acid. Each signal is labelled by the internal standard and corresponding endogenous peptide name. The analytical steps are represented under the time axis.

Table 15. Repartition of the followed transitions into three MRM functions. For each peptide transition, start and end times of the functions are exposed, along with their retention and dwell times, cone voltages and collision energies.

MRM Function n°	Start time (min)	Retention time (min)	End time (min)	Compound	Transition		Dwell (s)	Cone Voltage (V)	Collision energy (eV)
					Parent (m/z)	Daughter (m/z)			
1	5,3	6,2	5,62	SEB_6 E	476,00	175,20	0,006	30	25
				SEB_6 E	476,00	388,40			21
				SEB_6 E	476,00	837,70			21
				SEB_6	481,00	185,20			25
				SEB_6	481,00	398,40			21
				SEB_6	481,00	847,70			21
			6,01	SEB_1 E	529,90	181,20			16
				SEB_1	532,60	185,20			16
				SEB_1 E	794,20	181,20			31
				SEB_1 E	794,20	213,30			31
				SEB_1 E	794,20	688,30			31
				SEB_1	794,20	185,20			31
				SEB_1	794,20	213,30			31
				SEB_1	794,20	692,30			31
2	6	6,9	6,26	SEA_4 E	615,70	217,15	0,004	30	22
				SEA_4 E	615,70	330,10			22
				SEA_4	619,70	217,15			22
				SEA_4	619,70	330,10			22
			6,45	SEB_2 E	655,00	552,50			30
				SEB_2 E	655,00	667,60			20
				SEB_2 E	655,00	780,70			20
				SEB_2 E	655,00	909,80			25
				SEB_2	660,00	562,50			30
				SEB_2	660,00	677,60			20
				SEB_2	660,00	790,70			20
				SEB_2	660,00	919,80			25
			6,50	SEA_2 E	718,30	204,20			27
				SEA_2 E	718,30	278,20			27
				SEA_2 E	718,30	391,30			27
				SEA_2 E	718,30	466,10			27
				SEA_2	722,30	212,20			27
				SEA_2	722,30	278,80			27
				SEA_2	722,30	391,30			27
				SEA_2	722,30	474,40			27
3	6,5	8,1	7,78	SEB_4 E	622,63	510,50	0,004	30	15
				SEB_4 E	622,63	623,60			15
				SEB_4 E	622,63	851,80			15
				SEB_4 E	622,63	998,90			15
				SEB_4	625,30	518,50			15
				SEB_4	625,30	631,60			15
				SEB_4	625,30	859,80			15
				SEB_4	625,30	1006,90			15
			6,68	SEB_5 E	640,20	228,20			20
				SEB_5 E	640,20	341,35			20
				SEB_5 E	640,20	938,80			20
				SEB_5 E	640,20	1051,85			20
				SEB_5	644,20	228,20			20
				SEB_5	644,20	341,35			20
				SEB_5	644,20	946,80			20
				SEB_5	644,20	1059,85			20
			7,37	SEA_5 E	770,20	611,50			20
				SEA_5 E	770,20	686,40			20
				SEA_5 E	770,20	710,60			20
				SEA_5 E	770,20	823,70			20
				SEA_5	772,80	619,50			20
				SEA_5	772,80	686,40			20
				SEA_5	772,80	718,60			20
				SEA_5	772,80	831,70			20

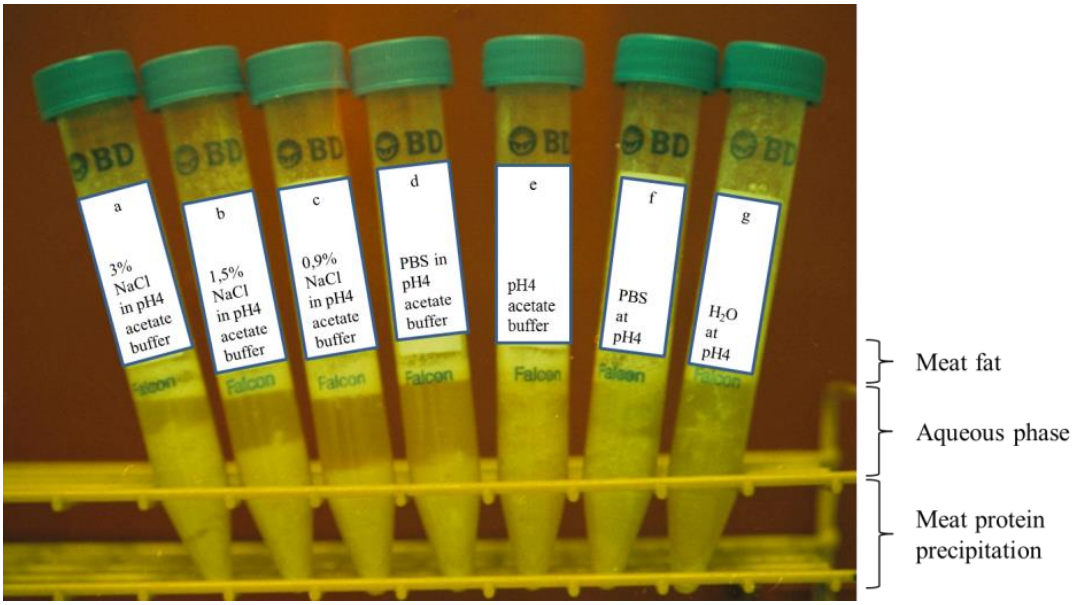

Figure 17. Visual examinations in the testing of extraction solvent. This picture presents the states of the samples after the addition of the aqueous extraction solvent, eventual pH adjustments and vortexing.

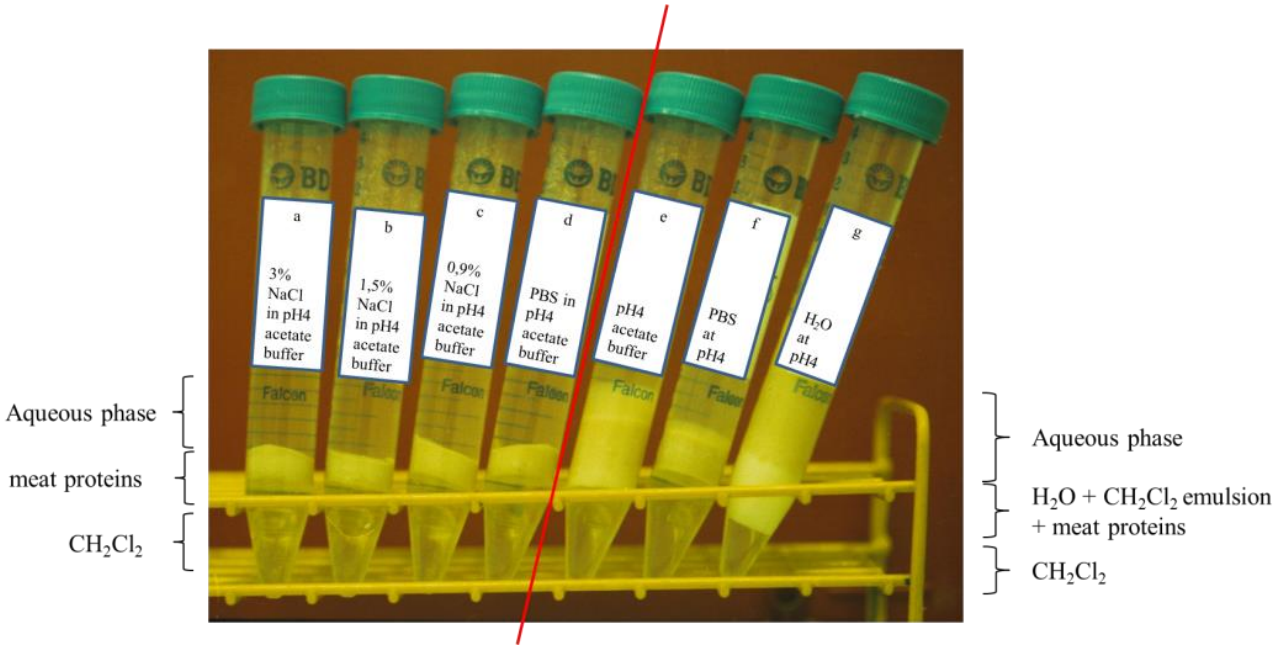

Figure 18. Visual examinations in the testing of phase separation. This picture presents the states of the samples after extraction with an aqueous solvent, precipitation, addition of the organic extraction solvent, vortexing, and centrifugation.

4.2 Extraction, purification and concentration

4.2.1 Extraction and first steps of purification°: choice of aqueous solvent, precipitation mode, extraction efficiency, and organic solvent

a) Choice of an aqueous solvent and of a precipitation mode (part 1/2)

To choose the aqueous phase, several aqueous solvents were tested and the results are visually presented in Figures 17 and 18.

First, the lowering of the pH to 4 helps precipitating some of the proteins as they are denatured by those acidic conditions. The layer of precipitated meat proteins is more pronounced in samples a, b, c and d (Figure 17), when a buffer was used. Sample e was also precipitated with a buffer but the meat protein layer is less distinct than in samples a, b, c and d, pointing on to less efficient precipitation. Samples f and g were acidified with pure acetic acid, and they too present a less important meat protein precipitation.

This resulted in selection of acidification in a buffer to precipitate meat proteins. Moreover, this acidification method is easier than the lowering with pure acetic acid followed by pHmeter, and is time saving.

Second, samples a, b, c, d and f, which contain a saline solution, either PBS or NaCl, were easier to centrifuge after the organic solvent (dichloromethane) addition and present a better phase separation, as illustrated in Figure 18. When using salt-free solutions (samples e and g), a thick emulsion remains between water and dichloromethane. The presence of salt in the solution probably helps destabilizing the emulsion and therefore facilitates phase separation, and subsequently the retrieval of the upper aqueous phase containing the toxins. This may even be observed in sample e where the emulsion is less dense than in sample g, probably due to the presence of sodium acetate in sample e which brings about slightly better separation.

Third, visual comparisons on the effect of the different saline solutions were made. The simple addition of the different extraction solvents prepared with sodium chloride showed a precipitation of the meat proteins that is proportional to the NaCl concentration, as exposed in Figure 17. The higher the salt concentration, the more meat proteins precipitate. Also, the 1.5% and 3% NaCl supernatants (samples a and b) are clearer and less turbid than the 0.9% NaCl supernatant (sample c). Because of the emulsion destabilization and more important protein precipitation in presence of salt, one of the NaCl saline solutions was chosen.

Since the PBS solution (sample d) showed a similar effect as the 0.9% NaCl solution it was ruled out right away from the initial selection to avoid the presence of phosphates in the mass spectrometer. Additionally NaCl solutions are easier to prepare than the PBS solution.

Finally, in order to select the best sodium chloride solution, some of the aliquots named°: 0.9 %-↓ (1, 2, 3); 1.5 %-↓ (1, 2, 3); 3 %-↓ (1, 2, 3); 0.9 % - centri (1, 2, 3); 1.5 % - centri (1 ,2, 3); and 3 % - centri (1, 2, 3) were separated by SDS-PAGE to examine the aqueous phases protein contents. Since other aqueous phases (H_2O and PBS brought to pH4, acetate buffer and PBS in acetate buffer) have already been eliminated, only those aliquots were examined by SDS-PAGE. Due to the limited availability of the materials randomly two out of three aliquots were separated by SDS-PAGE.

Figure 19 shows the protein contents of each series of samples (0.9% NaCl, 1.5% NaCl, and 3% NaCl) in the aqueous phases after toxins extraction and meat proteins precipitation but before centrifugation. The presence of the matrix proteins is clearly observed. Figure 20, on the left part of the gel, shows the samples after centrifugation. The SDS-PAGE shows a reduction in protein content during centrifugation opposite to increase of sodium chloride concentration. Solutions at 1.5% and 3% NaCl probably help precipitating meat proteins much better than the 0.9% NaCl solution.

The conclusions of the first part of this experiment are that meat proteins precipitation seems more efficient when using a buffer, that a saline solution helps to recover the aqueous phase after centrifugation, probably by destabilizing the emulsion formed between water and dichloromethane, that a saline solution helps precipitating proteins, and that it is a 1.5% or 3% NaCl solution that is to be used to eliminate matrix proteins. Further observations from the experiments described below helped to determine which of these two concentrations was best.

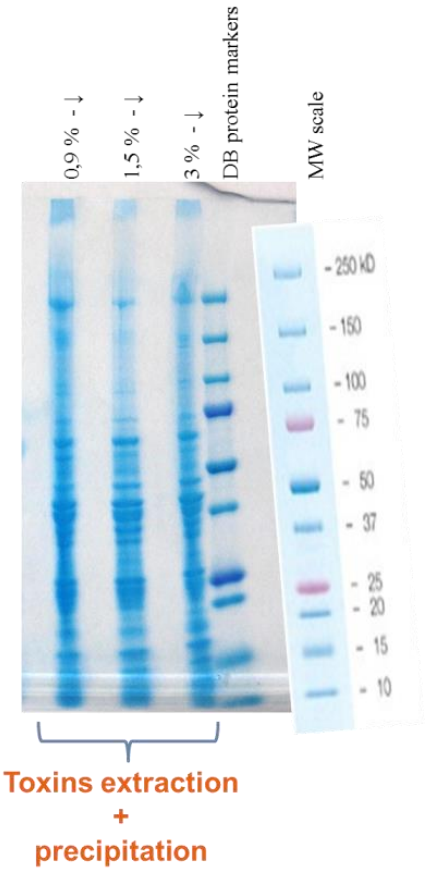

Figure 19. Protein contents of the samples after toxins extraction and meat precipitation by solutions of 0.9%, 1.5% and 3% NaCl in pH4 acetate buffer, obtained by SDS-PAGE.

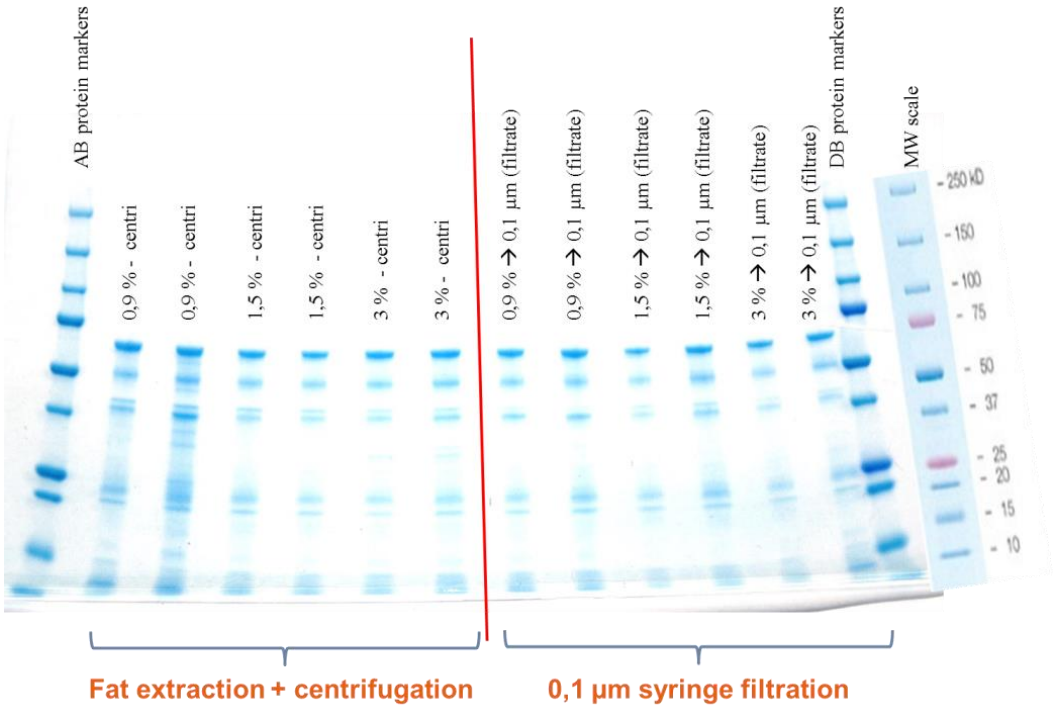

Figure 20. Left°: Protein contents of the samples after toxins extraction and meat precipitation by solutions of 0.9%, 1.5% and 3% NaCl in pH4 acetate buffer, fat extraction by CH_2Cl_2 and centrifugation, obtained by SDS-PAGE. Right°: Protein contents of the samples after toxins extraction and meat precipitation by solutions of 0.9%, 1.5% and 3% NaCl in pH4 acetate buffer, fat extraction by CH_2Cl_2, centrifugation and 0.1 µm syringe filtration, obtained by SDS-PAGE.

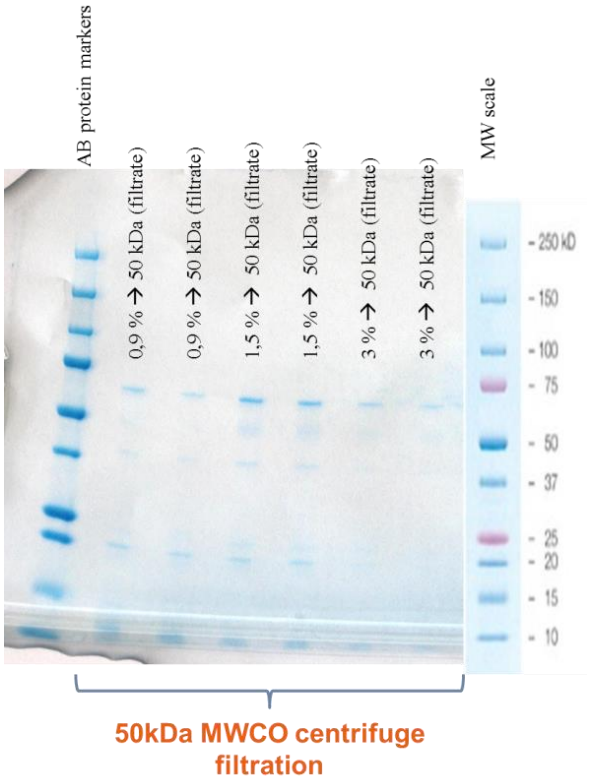

Figure 21. Protein contents of the samples after toxins extraction and meat precipitation by solutions of 0.9%, 1.5% and 3% NaCl in pH4 acetate buffer, fat extraction by CH_2Cl_2, centrifugation, 0.1 µm syringe filtration, and 50 kDa MWCO centrifuge filtration, obtained by SDS-PAGE.

b) Choice of an aqueous solvent and of a precipitation mode (part 2/2)

Other observations were made from the successive filtrations of the 0.9%, 1.5% and 3% NaCl - acetate supernatants.

First, the facility and the time taken to filter the extracts through the 0.1 µm syringe filters were examined. Samples extracted with 0.9% NaCl were more difficult and took more time to pass through the syringe filters than the ones extracted with 1.5 % and 3% NaCl. This observation is in accordance with the above-mentioned analysis of the SDS-PAGE (Figure 20, on the left part of the gel). As the samples extracted by 0.9% NaCl solutions contain more proteins after centrifugation than the "1.5%" and "3%" samples, they are more difficult to pass through the syringe filter. This observation is another reason why the 0.9% NaCl aqueous solvent was ruled out. The "1.5%" and "3%" supernatants presented similar behaviour when passing the syringe filters, even though the 3% samples were a little faster. It was thus tempting to select the 3% NaCl solution as the final aqueous extraction solvent, as it seems to precipitate more meat proteins, as explained in section 4.2.1, and because samples are easier to filtrate with the syringe filters.

Second, the respective volumes of the residues remaining in the ultra-filters of 50 kDa size were examined. The residues remaining in the 50 kDa filters used for samples precipitated with 0.9% NaCl and 1.5% NaCl were lower than 100 µl in contrast to 300 µl left after micro filtration of the samples precipitated with 3% NaCl. A possible explanation is that 50 kDa MWCO filters were blocked by the content of the samples extracted by the 3% NaCl aqueous phase but not by the content of the samples extracted by the "0.9%" and "1.5%" samples. When imposing a hypertonic environment to meat cells, an osmotic shock happens and they "dry out", collapse and release their cytoplasmic content in their environment. The higher the salt concentration, the more cells content is released in the solution. Some of the proteins are invisible at first sight but could cause the filters to block. This blocking is a major disadvantage for the recovery of the toxins as they may be lost in the residue instead of moving on to the next filter within the filtrate. Therefore, at this point, the 1.5% NaCl aqueous solvent was considered as the best option for the precipitation.

This was further confirmed by examination of SDS-PAGE results of the aliquots withdrawn at every filtration step for the different series of samples. The SDS-PAGE results were used for selection of the salt concentration to be used in the final protocol.

Figure 20 shows the evolution of the samples protein contents when they are cleaned-up with the 0.1µm syringe filter.

The "0.9%" samples are really cleaner after the 0.1 µm syringe filtration step, so even if this filtration step took longer and was more difficult for those samples, it was probably useful. On the other hand, the "1.5%" and "3%" samples do not present a clear amelioration after passing through the syringe filters.

Figure 21 shows the samples protein contents after they are cleaned-up with the 50 kDa MWCO centrifuge filter. When comparing with the previous filtration step showed in Figure 20, on the right part of the gel, all samples present an important general clean-up.

All 50 kDa filtrates still contain proteins of about 70 kDa, which is more than the filter MWCO. This could easily be explained by the manufacturer's recommendations which stipulate that proteins will be retained only by a filter that has a MWCO inferior to the third of their weight.

The "1.5%" samples present similar but also more concentrated protein contents than the "0.9%" samples. This is probably caused by the higher salt concentration which caused a more important release of the matrix cells contents. On the other hand, the "3%" samples are far less concentrated than the "1.5%" and the "0.9%" samples. If a higher salt concentration releases more matrix proteins, those samples should also be more concentrated in matrix proteins. The first explanation could be that the "3%" samples global clean-up worked very well and that the proteins that should be observed in these samples were eliminated in previous clean-up steps, like the precipitation step for instance. However, the "1.5%" and "3%" aliquots from the previous step (after 0.1 µm syringe filtration) show similar contents and concentrations. Besides, another important fact is to be taken under consideration°: the 50 kDa filters blocked when the "3%" samples were passed through. Therefore, it is probably the blocking of the filters that prevented the matrix proteins to pass. Only the liquid content was let through, resulting in an apparent excellent clean-up. Let us remind that the blocking of the purification centrifuge filter is highly undesired as it might cause an important toxins loss.

A question remains°: why did the "3%" samples block the 50 kDa MWCO filters and not the "1.5%" samples when, as mentioned just above, the aliquots from the previous step show similar contents and concentrations?

This might be due to the coomassie staining sensitivity. If a protein concentration is too low, the coloration doesn't work. So no coloration does not mean "no protein", it simply means "not concentrated enough proteins". Therefore, it is very possible that large but low abundant proteins, visible in Figure 19 aliquots from the "Toxins extraction + precipitation" step but invisible on Figure 20 "0.1 µm syringe filtration" aliquots blocked the filters.

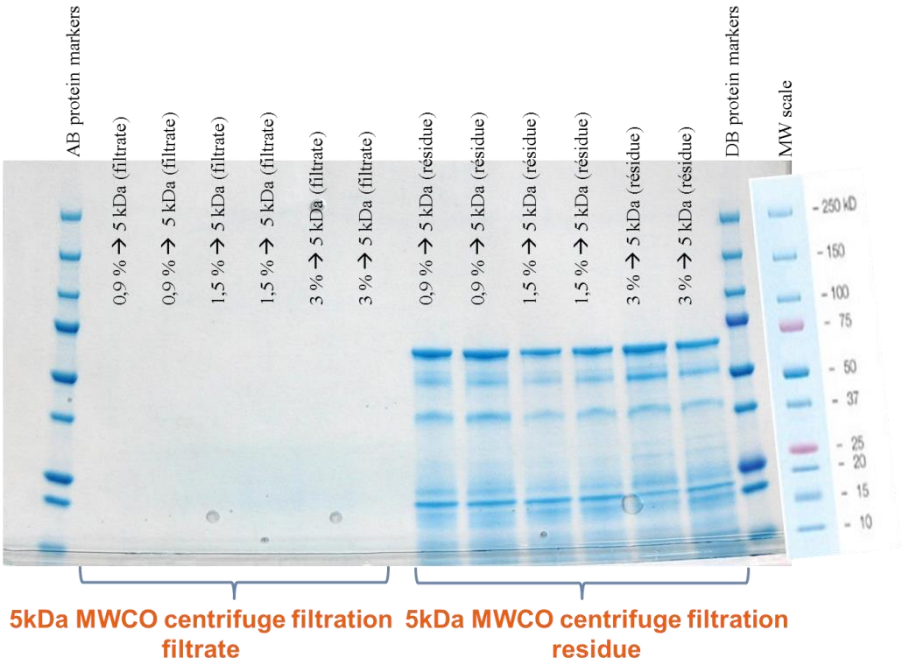

Figure 21. Protein contents of the samples after toxins extraction and meat precipitation by solutions of 0.9%, 1.5% and 3% NaCl in pH4 acetate buffer, fat extraction by CH_2Cl_2,centrifugation, 0.1 µm syringe filtration, 50 kDa and 5 kDa MWCO centrifuge filtration, obtained by SDS-PAGE.
Left°: 5 kDa MWCO filters filtrates. Right°: 5 kDa MWCO filters residues.

Finally, Figure 22 shows the 5 kDa MWCO samples residues and filtrates. There are no or very little proteins left in the 5 kDa filtrates and the residues contents are quite similar, ranging from 10 to 75 kDa. The fact that the 5 kDa MWCO samples filtrates all show inexistent protein content, could either mean there is no protein left in the final filtrates, or their concentration is beneath the Coomassie staining LOD.

The presence of proteins ranging from 10 to 75 kDa in the residues shows that proteins the size of the target toxins are retained in the residue. The "1.5%" samples are less concentrated which is the final argument in favour of the 1.5% NaCl aqueous solvent. Indeed, the 5 kDa residue is the final toxins concentration and will be submitted to digestion. It is thus important that the samples present the lowest possible proteins content. First because those contaminations will create interferences in the final UPLC-MS/MS analysis and second because those proteins will also be submitted to tryptic digestion and present a risk of lowering the digestion efficiency, either because the matrix proteins could enter in competition for trypsin or because they could hinder the trypsin access to the toxins.

The conclusion to this general experiment aiming at the choice of an aqueous solvent is that the 1.5% NaCl solution in acetate buffer (pH 4) is a good compromise between proteins precipitation, easiness of filtration, toxins recovery, and the sample final contamination in matrix proteins. This general extraction, purification and concentration protocol however still needs to be improved as a better matrix proteins removal will both improve the digestion yield and lower the matrix effect, and therefore raise the general sensitivity of the method.

c) Extraction efficiency

The VIDAS SET2 test was performed on two samples. The first (“Extraction1”) is the aqueous phase resulting from the phase separation by centrifugation. The second (“Extraction2”)comes from a second extraction of the meat remaining in order to check whether the toxins are properly extracted in the first extraction step in spite of the precipitation, fat removal and centrifugation steps. Indeed, lowering the pH constitutes a risk for precipitating the toxins as well as the meat proteins. However, since SEs are stable between pH 3.0 and 9.0, it should not be a problem.

The VIDAS tests results, available in Annex 12, are positive on both samples. This means that the toxins are not completely recovered into the aqueous phase when performing the extraction. This could be caused by several parameters that require confirmation. First, the pH lowering might cause toxins precipitation. Second, the extraction might be incomplete, requiring a second extraction step. Finally, the use of a milder extraction device, as a rotative agitator, should be considered, as the vortex may not allow a proper recuperation of the toxins because they don’t have enough time to diffuse in the extraction solution.

The spiked amount of toxins (1000 ng of each enterotoxin) is not representative of a real contamination. However, the VIDAS SET2 LOD is of 0.25ng/ml and since the second extraction is positive for enterotoxins, it means there is still at least 0.25*5=1.25 ng of toxins that were not extracted with the first 5 ml of aqueous solvent, inducing considerable loss on the scale of real samples which will probably present lower contamination.

d) Choice of an organic solvent for fat extraction

Dichloromethane was chosen over n-hexane for the extraction of fat. Figure 23 first shows that fat extraction is better with dichloromethane than with hexane as some fat remains in the sample cleaned-up with n-hexane. In order to extract as much fat with hexane as with CH_2Cl_2, several steps would be needed, which would mean more manipulations and more risks of toxins losses. Second, dichloromethane is of higher density than hexane and sinks to the bottom of the tube during centrifugation, making it easier to remove the aqueous phase which ends up on top of the organic phase and the matrix remaining’s.

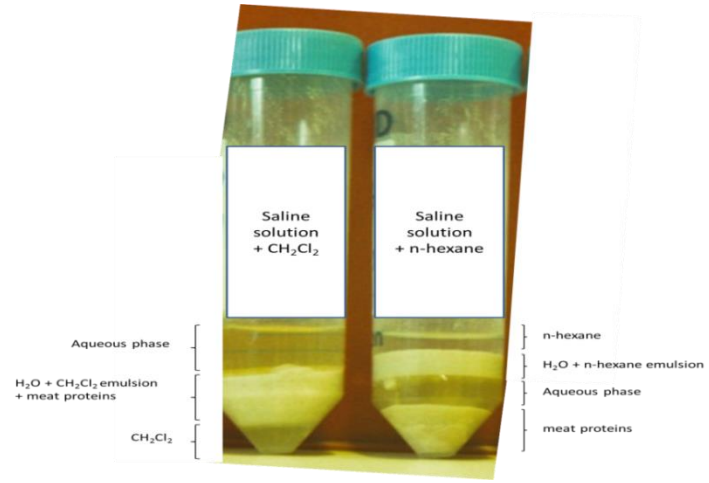

Figure 23. Visual examination of fat removal from the tested samples by two organic solvents (dichloromethane on the left and n-hexane on the right).

4.2.2 Second part of purification and concentration°: choice of filters

The method involves two types of filtrations°: microfiltration followed by ultrafiltration.

Two types of syringe filters of different porosity and composition were tested.

The first filter is a 0.2 µm pore size filter with a PVDF membrane. It was tested first because it was available in the regular laboratory material and second because it is described by its manufacturer (Whatman) as particularly suited for food analysis, as it eliminated contaminations down to 0.7 µm and requires less hand pressure, and still provides fast flow rates. Besides, according to other manufacturers (Sterlitech), the fact that the membrane is hydrophilic will cause less proteins binding than nitrocellulose membranes, for instance.

The second filter that was tested is a 0.1 µm pore size filter with a PTFE membrane (membrane solutions is the manufacturer). The membrane is of different composition because PVDF membranes are not available in other sizes. Because of its hydrophobic nature, PTFE requires pre-wetting with alcohol for filtering aqueous solutions, to establish flow with reasonable pressure differentials. However, PTFE membranes are described as chemically and biologically inert and thicker meaning they are more resistant to concentrated acids and bases, and are easier to handle (Sterlitech).

a) Microfiltration with a syringe filter

The microfiltration was evaluated by examination of the protein contents of the aliquots taken before the microfiltration and after the ultrafiltration.

As listed in Table 16 two microfiltration paths were tested but the ultrafiltration is common.

Table 16. Experimental set-up of the micro-filter selection.

Filtration	**“0.2; 50;5”**	**“0.1; 50; 5”**
Microfilter (size µm)	0.2	0.1
Ultra-purification (size kDa)	50	50
Untra-concentration (size kDa)	5	5

The aliquots “S→P”, on Figure 24, represent the protein contents of samples extracted with the reference method (PBS solution and pH lowering by hand, with acetic acid), extracted with CH_2Cl_2 and centrifuged (3200 X g; 20 min).

The aliquot “0.2; 50; 5” on Figure 24Figure, on the left gel, appears protein-free. This might imply the good sample clean-up after successive filtrations. However, the “S→P” aliquots clearly show that the samples contain proteins with sizes ranging roughly from 10 to 100 kDa. In a perfect filtration, anything bigger than 50 kDa would be retained in the 50 kDa filter residue (and therefore eliminated) and anything between 5 and 50 kDa would be concentrated in the 5 kDa filter residue. Nothing bigger than 50 kDa appears in the “0.2; 50; 5” aliquot, which is fine, but nothing between 5 and 50 kDa is visible either. This absence of proteins could be caused by a blocking of the 50 kDa filter during centrifugation. All proteins would have been retained in the residue and only water and very small components of the extract would have passed through. Therefore, there would be no protein left to concentrate in the 5 kDa residue. This is a major inconvenient as the target SEs present an average 27 kDa MW. This means they are also blocked in the 50 kDa residue instead of passing to the next filter where they should be concentrated.

On the other hand, the “0.1; 50; 5” aliquot, on Figure 24, on the right gel, shows no protein content that is higher than 50 kDa but presents proteins ranging from 10 to about 40 kDa, as expected.

During centrifugation, the biggest proteins sink first to the bottom of the filter and therefore may block the filter. Using a 0.1µm syringe filter probably allows the removal of bigger proteins than with a 0.2 µm filter and therefore avoids the 50 kDa filter blocking.

It is thus the 0.1 µm PTFE syringe filter that was selected for this first filtration step.

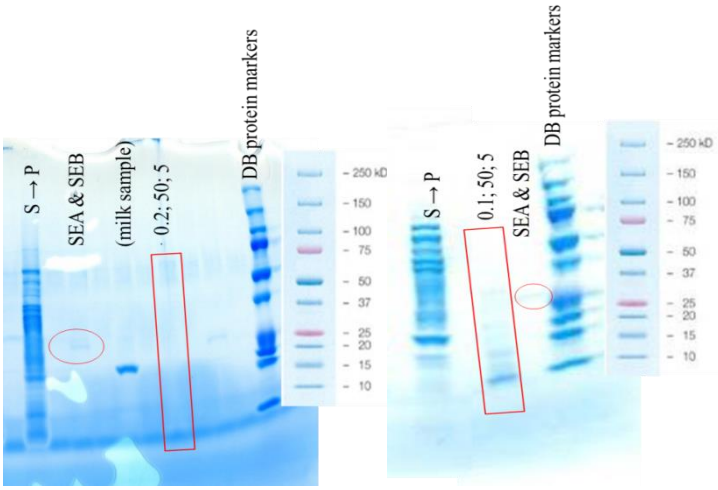

Figure 24. Protein contents of the samples after toxins extraction by PBS solution, meat precipitation by lowering the pH with pure acetic acid, fat extraction by CH_2Cl_2,centrifugation, syringe filtration, 50 kDa and 5 kDa MWCO centrifuge filtration, obtained by SDS-PAGE.
Left°: Testing of the 0.2 µm PVDF filter. Right°: Testing of the 0.1 µm PTFE filter.
“S→P” are protein contents of samples extracted with the reference method (PBS solution and pH lowering by hand, with acetic acid), extracted with CH_2Cl_2 and centrifuged (3200 X g; 20 min)
“0.2; 50;5” and “0.1; 50; 5” are the samples after the filtration steps.

b) *Ultrafiltration with centrifuge filters*

Two sizes of purification (50 and 30 kDa) and two sizes of concentration (10 and 5 kDa) centrifuge filters were tested because of the manufacturer's recommendations exposed in section 0 second part of purification: choice of filters. All of them were of PES composition because centrifuge filters were only available with a PES membrane (Sartorius).

The best combination would be the "30 kDa-10 kDa" MWCO filters, because they would permit the elimination of more matrix proteins in the purification centrifuge filter's residue and in the concentration centrifuge filter filtrate.

The specificity of the ultrafiltration was tested by VIDAS SET2. Theoretically the purification filter would let the toxins through, resulting in "negative" result with VIDAS SET2 for the residues of the purification filters (residues P) and the filtrates of the concentration filters (filtrates C), and "positive" for the filtrates of the purification filters (filtrates P) and the residues of the concentration filters (residues C), as illustrated in Figure 25.

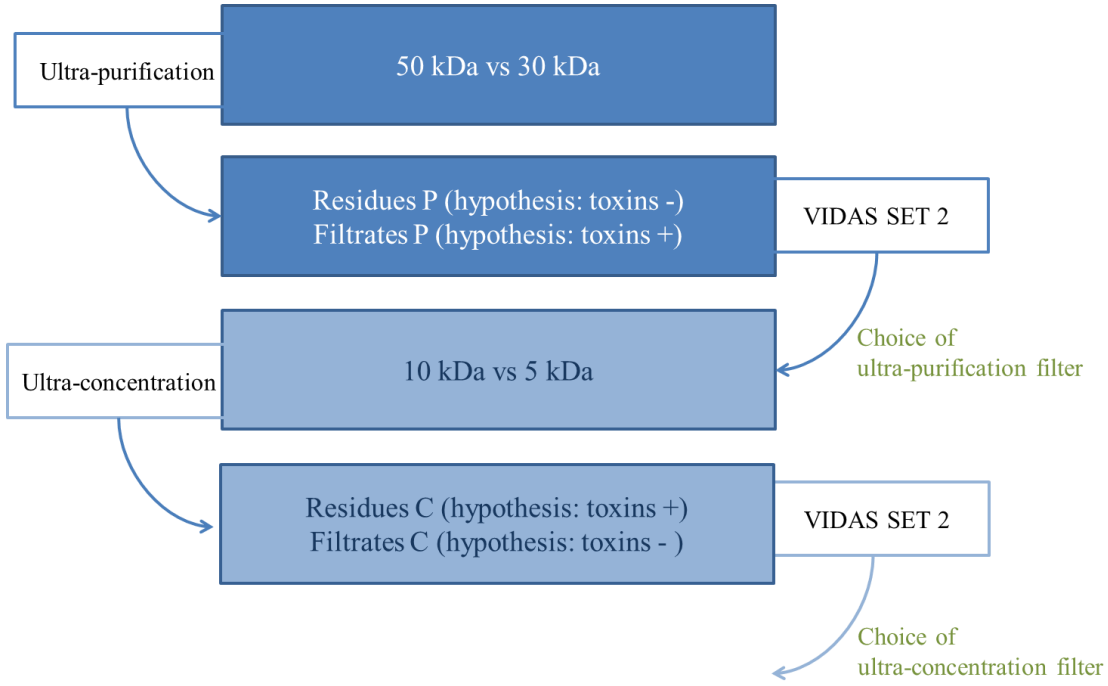

Figure 25. Testing the specificity of ultra-filters by VIDAS SET2.

The results exposed in Table 17 show that not all size filters are suited for those steps of the protocol. Some SEs are retained in the residue of the 30 kDa MWCO filter which makes it unsuitable as a purification filter because it would cause a significant loss of the target toxins at this point. On the other hand, the 50 kDa MWCO filter absence of the toxins by VIDAS SET2 making it suitable for further use in the study. However, as the 50 kDa filter presents a higher molecular weight cut-off than the 30 kDa filter, smaller impurities are not retained and pass through with the toxins.

The concentration filters also gave different results, in Table 17. The 10 kDa MWCO filter does not fully retain the toxins as some are found in the "F_{10}" filtrate C. The 5 kDa MWCO filter blocks the toxins in its residue and does not let any toxin through. Therefore, the 5 kDa filter was selected for final protocol. However, the 5 kDa filter also retains small impurities that are concentrated with the toxins, as shown by the analysis of SDS-PAGE gels (Figure 22 in section 4.2.1).

The combination of 50 kDa for ultra-purification and 5 kDa for ultra-concentration MWCO centrifuge filters was chosen for the final protocol of detection of toxins in meat.

Table 17. VIDAS results on the testing of the different purification and concentration filters.

Type of filter	Residue	Filtrate
Purification filter (50 kDa MWCO)	-	+
Purification filter (30 kDa MWCO)	+	+
Concentration filter (10 kDa MWCO)	+	+
Concentration filter (5 kDa MWCO)	+	-

The conclusion of the experiments enclosed in the "extraction, purification and concentration" section are that the adopted parameters were the 1.5% NaCl in pH 4 acetate buffer solution as the toxins extraction aqueous solvent, the integration of a buffer in the aqueous solvent as a precipitation mode; dichloromethane as a fat extraction solvent; a 0.1 µm PTFE syringe filter as the first purification filter; a 50 kDa MWCO PES centrifuge filter as a second purification filter and a 5 kDa MWCO PES centrifuge filter as a concentration filter.

4.3 Digestion

Three digestion buffers were tested for the digestion step°: 50 mM ammonium bicarbonate, Tris-$CaCl_2$ (500 mM; 100 mM), and Tris (100 mM).

Only the samples containing the toxins extraction buffer and HCO_3^- had to be adjusted between 7.5 and 9 with 10 µl NaOH 1M. When other samples pH were measured, all were in the 7-9 pH range required for tryptic digestion. This proves that the ammonium bicarbonate 50 mM is not strong enough to adjust the pH. This pH setting constitutes an unnecessary manipulation that could potentially cause toxins loss. This reason constitutes a first argument against the 50 mM bicarbonate buffer.

The first observations of the MS/MS peaks concerned the intensities and signal shapes of the internal standards. Peaks had to be as intense and steady as possible. As the objective is to keep one or two peptides per toxin, with two transitions per peptide, the final selection was made among the peptides that were selected for SEA and SEB detection.

SEB_1, SEB_2, SEB_5, and SEA_4 transitions (for the isotopes or IS) present good peak symmetry and very high intensities. All corresponding transitions are conserved.

Two of the three transitions of the peptide named SEB_6 (one of enterotoxin SEB's selected peptides) present unsatisfying peaks. Therefore, this peptide was eliminated.

All four transitions for SEA_2 and SEB_4 are satisfying, and kept.

The resulting peaks of all SEA_5 transitions were of less quality but were kept as an alternative confirmatory peptide for SEA.

Second, estimated digestion yields were calculated according to the path explained in section b). The estimated digestion yields obtained for each transition are exposed in Table 18 where they are sorted by series. An illustration of all the steps calculation is available in Annex 15. In this table, the estimated digestion yields are rounded in order to facilitate the reading and because a big precision is not necessary to draw conclusions. The exact values can be consulted in Annex 16.

Several conclusions are drawn from those results.

None of SEA_2 E; SEA_4 E and SEA_5 E transitions gave satisfying signals. As a result, the corresponding digestion yields are close to zero. The corresponding results are highlighted in red in Table 18.

This observation is not surprising for SEA_5 E, as the corresponding IS SEA_5 was already giving uncertain signals and kept only as a possible back-up. There are three possible explanations to the fact that none of the peptides selected for toxin SEA show good signals. First, none of the tested digestion buffers works. However, good signals are obtained for some transitions related to toxin SEB. Second, toxin SEA is more resistant to digestion or is not enough unfolded before the digestion step starts, which would mean that the trypsin cannot access some cleavage sites. Indeed Callahan (2006) performed digestion of SEB without any preparation step while Sospedra (2011) used DTT and IAA to prepare the toxins to digestion.

Table 18. Estimated digestion yields calculated for each followed transition. The yields are sorted by tested digestion buffer, in presence of the aqueous extraction solvent or not.
Red values are the digestion yields obtained for all SEA related transitions.
Green values are the best obtained values for the final transitions selection, in the presence of aqueous buffer.

Peptides Transitions	Estimated digestion yields (%)					
	Pure buffer			Buffer + extraction solvent		
	HCO3-	Tris-CaCl2	Tris	NaCl/HCO3-	NaCl/Tris-CaCl2	NaCl/Tris
SEB_2 E T1	131 ± 33	181 ± 36	139 ± 38	8 ± 2	79 ± 16	35 ± 3
SEB_2 E T2	132 ± 33	183 ± 41	147 ±42	9 ± 4	79 ± 17	32 ± 5
SEB_2 E T3	139 ± 34	188 ± 41	148 ± 41	8 ± 2	82 ± 17	36 ± 4
SEB_2 E T4	135 ± 30	181 ± 33	149 ± 41	9 ± 2	84 ± 17	35 ± 5
SEA_2 E T1	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEA_2 E T2	0 ± 0	1 ± 1	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEA_2 E T3	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEA_2 E T4	2 ± 1	2 ± 2	2 ± 1	0 ± 0	0 ± 0	0 ± 0
SEB_5 E T1	14 ± 7	79 ± 24	5 ± 1	0 ± 0	41 ± 15	2 ± 1
SEB_5 E T2	13 ± 8	83 ± 23	5 ± 2	0 ± 0	43 ± 12	3 ± 0
SEB_5 E T3	17 ± 9	116 ± 29	7 ± 3	1 ± 0	49 ± 13	4 ± 1
SEB_5 E T4	17 ± 9	116 ± 29	7 ± 3	1 ± 0	48 ± 12	4 ± 1
SEB_1 E T1	74 ± 15	61 ± 21	86 ± 34	10 ± 3	47 ± 14	20 ± 4
SEB_1 E T2	80 ± 15	94 ± 22	90 ± 26	11 ± 3	50 ± 5	25 ± 3
SEB_1 E T3	0 ± 0	0 ± 0	1 ± 0	0 ± 0	0 ± 0	0 ± 0
SEB_1 E T4	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEA_4 E T1	3 ± 1	4 ± 1	4 ± 1	3 ± 0	3 ± 0	3 ± 1
SEA_4 E T2	2 ± 0	2 ± 1	2 ± 0	1 ± 0	1 ± 0	1 ± 0
SEA_5 E T1	1 ± 1	3 ± 4	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEA_5 E T2	0 ± 0	2 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEA_5 E T3	3 ± 6	3 ± 1	5 ± 5	2 ± 1	1 ± 1	1 ± 1
SEA_5 E T4	1 ± 2	2 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEB_4 E T1	78 ± 29	528 ± 40	39 ± 20	0 ± 0	163 ± 102	7 ± 6
SEB_4 E T2	137 ± 58	599 ± 80	66 ± 14	2 ± 3	318 ± 62	6 ± 2
SEB_4 E T3	0 ± 0	704 ± 164	0 ± 0	0 ± 0	0 ± 0	0 ± 0
SEB_4 E T4	132 ± 65	485 ± 75	71 ± 10	2 ± 3	258 ± 56	7 ± 2

E= endogenous peptide

T+ n°= transition n°…

A more efficient digestion preparation may be necessary and could be achieved by using more concentrated DTT and/or IAA solutions, for instance. Third, the amount of trypsin added to the samples is not sufficient. A protease to protein ratio of 1:100 to 1:20 (w/w) is recommended by the fabricant. Since 1000 ng of each toxin is submitted to digestion and 10 µl of 100ng/µl of trypsin are added, the working ratio is 1:2 (w/w) which should be enough.

When it comes to SEB transitions, two of SEB_1 E transitions (T3 and T4, also highlighted in red in Table 18) don't give any signal, in any condition.

However, SEB_2 E, SEB_5 E, SEB_4 E and two of SEB_1 E transitions provide some signals. Because of those observations, the following conclusions were drawn from the single analysis of SEB_2 E (T1 to T4), SEB_5 E (T1 to T4), SEB_4 E (T1 to T4) and SEB_1 E (T1 and T2).

First, the comparison of the digestion yields for the different series shows that the digestion yields are always smaller when the digestion media contains aqueous extraction solvent (1.5% NaCl in acetate buffer, pH4). This could indicate that some of the aqueous solvent components cause interferences to the digestion.

Besides, the series that contains Tris-$CaCl_2$, either with or without extraction aqueous solvent, leads to the highest digestion yields. The only exception is for SEB_1E T1 and T2. Without extraction aqueous solvent, the highest yields are obtained in the Tris buffer for those transitions. But since in the global protocol the digestion is to take place in the presence of the aqueous buffer and that SEB_1E T1 and T2 signals are also higher in Tris-$CaCl_2$ when the aqueous solvent is present, it is the digestion buffer Tris-$CaCl_2$ that is considered optimal.

Finally, SEB_4 E transitions are also removed from the peptides selection, this for two reasons. First, the standard deviation values of all transitions in the "NaCl + Tris-$CaCl_2$" series are lower than 20%, except for SEB_4 E T1, T2 and T4, where they are higher than 50 %, and second transition SEB_4 E T3 does not give any signal. The conclusions of this digestion experiment are thus that no signal could be observed for the transitions related to toxin SEA, and therefore a better digestion preparation should be tested; that transitions SEB_2 E (T1 to T4); SEB_5 E (T1 to T4) and SEB_1 E (T1 and T2) give the best signals and constitute the final selection for the detection of toxin SEB°; and finally that the Tris-$CaCl_2$ buffer is optimal for toxin SEB digestion, as it provides the highest digestion yields in the presence of 1.5% NaCl in pH4 acetate buffer aqueous extraction buffer, with standard deviations lower than 20%. The yields matching those selections are highlighted in green in Table 18.

4.4 Full method evaluation

Two types of results can be commented out of this experiment. First, the results consisting in the toxins recovery calculation for each series of samples, according to each followed transition, and their comparison. The path explained in section 0 was followed and the toxins recoveries obtained for each spiking step are exposed in Table 19 where they are sorted by transition (SEB_2 E T1 to T4, SEB_5 E T1 to T4 and SEB_1 E T1 and T2). An illustration of all the steps calculation is available in Annex 17.

Figure 26 shows the same data in graphs (one par transition). Those graphs are the comparison of the estimated recoveries and actually represent the toxins losses throughout the protocol.

Second, the comparison of the estimated digestion yields with or without a meat matrix effect. Indeed, as the samples for the series named “before digestion” were spiked with toxins after the meat was submitted to the whole process, the recovery values can be assimilated to a digestion yield similar to the ones calculated in the digestion experiment. The only difference between those yields is the matrix presence. Therefore the digestion yields comparison, exposed in Figure 26 can bring useful information too. The corresponding rough data is available in Annex 18.

Ideally, recoveries would all be at 100%, meaning 100% of the toxins spiked in meat would have been extracted, purified and concentrated by the filters and digested by the trypsin.

The main information shown by all the graphs is that there is an important toxins loss in the 50 kDa centrifuge filter, and therefore an important need for improvement in the meat protocol.

Indeed on each graph the recoveries can be divided in two groups. The samples named “before extraction”, “before syringe filter” and “before 50 kDa centrifuge filter” constitute the “pre 50 kDa filtration” group. The samples named “before 5 kDa centrifuge filter” and “before digestion” constitute the “post 50 kDa filtration” group. Each group is circled in red on graphs from Figure 26, except for transition SEB_1 E T1, where no group can be pointed out because all recoveries are close and low.

In the “pre 50 kDa filtration” group, recoveries are low but show close values. In the “post 50 kDa filtration”, recoveries are also close but show higher values, suggesting an important toxins loss in the 50 kDa filter.

A toxins loss in the steps previous to the 50 kDa filter cannot be determined for sure, as it is hidden by the loss in the 50 kDa filter. This major toxins loss must be solved before pointing out previous losses.

Another important remark concerns the amount of toxin spiked in this experiment. Meat samples were spiked at a very high level (2000 ppb) to make sure that some signals would result from the experiment. Even at such level, important losses (up to 40 % according to some transitions) are pointed out in the protocol. This means that if a much smaller amount of toxins, more representative of real contaminations, were to be extracted from a real sample, no signal would result from the method and it would result in a false negative. It is thus very important to bring optimizations to the extraction, purification and concentration protocol in order to lower the losses, and consequently the LOD of the general method.

This important loss seems contradictory with the "choice of centrifuge filters" experiment which showed that no toxins were present in the 50 kDa MWCO residue. This could be explained by the fact that the centrifuge filters membrane is of PES composition, known for binding proteins in a non-specific way. The VIDAS SET2 test only concluded there was no enterotoxin remaining in the 50 kDa filter residue (as far as the VIDAS SET2 LOD is concerned). But it doesn't mean the enterotoxins were not lost on the membrane, instead of being blocked on top of it. Further testing is necessary to determine whether the toxins bind with the membrane, for example by backwashing the filter.

Finally, the comparison of the digestion yields with or without the matrix is illustrated in Figure 27. The blue dots and error bars show the digestion yields from the digestion experiment and are the yields without matrix effect. The red dots and error bars show the toxins recoveries or digestion yields from the full method evaluation experiment. Those are the digestion yields with matrix effect. According to every followed transition, the digestion yields in the presence of matrix are lower. This could be caused, as explained previously, by the matrix proteins presence which interferes with the toxins digestion, either because they are in competition with the toxins for the trypsin, or because they lower the trypsin access to the toxins. Not only does this prove a matrix effect, but it once again shows the need for a more efficient sample clean-up.

Table 19. Estimation of the toxins recoveries obtained for each series of samples spiked with toxins at key steps of the meat protocol. The recoveries are sorted by selected transition.

Spiking step	SEB_2 E T1		SEB_2 E T2		SEB_2 E T3		SEB_2 E T4		SEB_5 E T1		SEB_5 E T2		SEB_5 E T3		SEB_5 E T4		SEB_1 E T1		SEB_1 E T2	
	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)
before extraction	11.7	2.0	13.5	1.9	10.9	2.0	11.4	2.2	2.5	0.7	4.0	0.9	13.4	1.4	5.0	0.6	4.9	2.0	5.3	0.8
before syringe filter	13.4	3.9	16.1	2.5	12.7	3.8	15.1	3.4	3.9	0.3	5.3	1.0	13.6	0.9	6.4	2.3	3.0	0.4	15.4	0.7
before 50 kDa filter	11.0	2.1	12.8	1.2	10.7	1.2	11.4	0.8	3.6	1.9	4.7	0.8	14.1	0.8	5.0	0.6	4.0	1.8	10.2	0.9
before 5 kDa filter	55.4	2.3	61.8	3.1	63.6	1.5	61.7	1.3	26.1	1.5	25.6	0.0	40.1	5.7	33.3	0.8	6.6	1.4	36.7	8.3
before digestion	54.7	11.2	63.6	4.8	58.9	3.9	65.4	1.8	26.1	3.8	29.6	2.4	41.2	2.5	35.7	2.6	5.6	0.4	39.4	1.3

Erreur ! Source du renvoi introuvable.

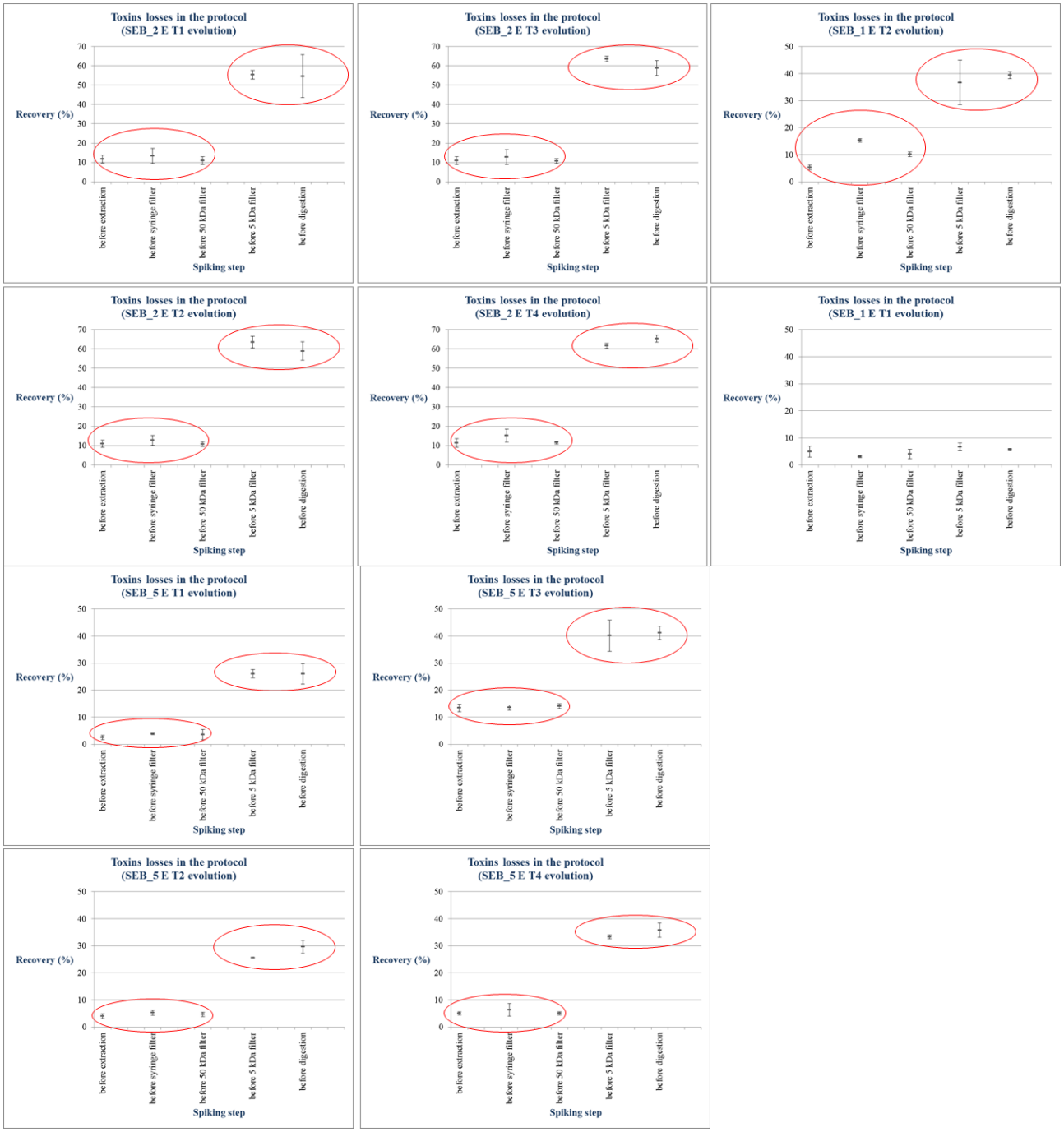

Figure 26. Estimation of the toxins losses throughout the meat protocol. Each graph represents the toxins recoveries for each series of samples (spiked at a key point of the meat protocol), according to one of the peptides followed transitions.

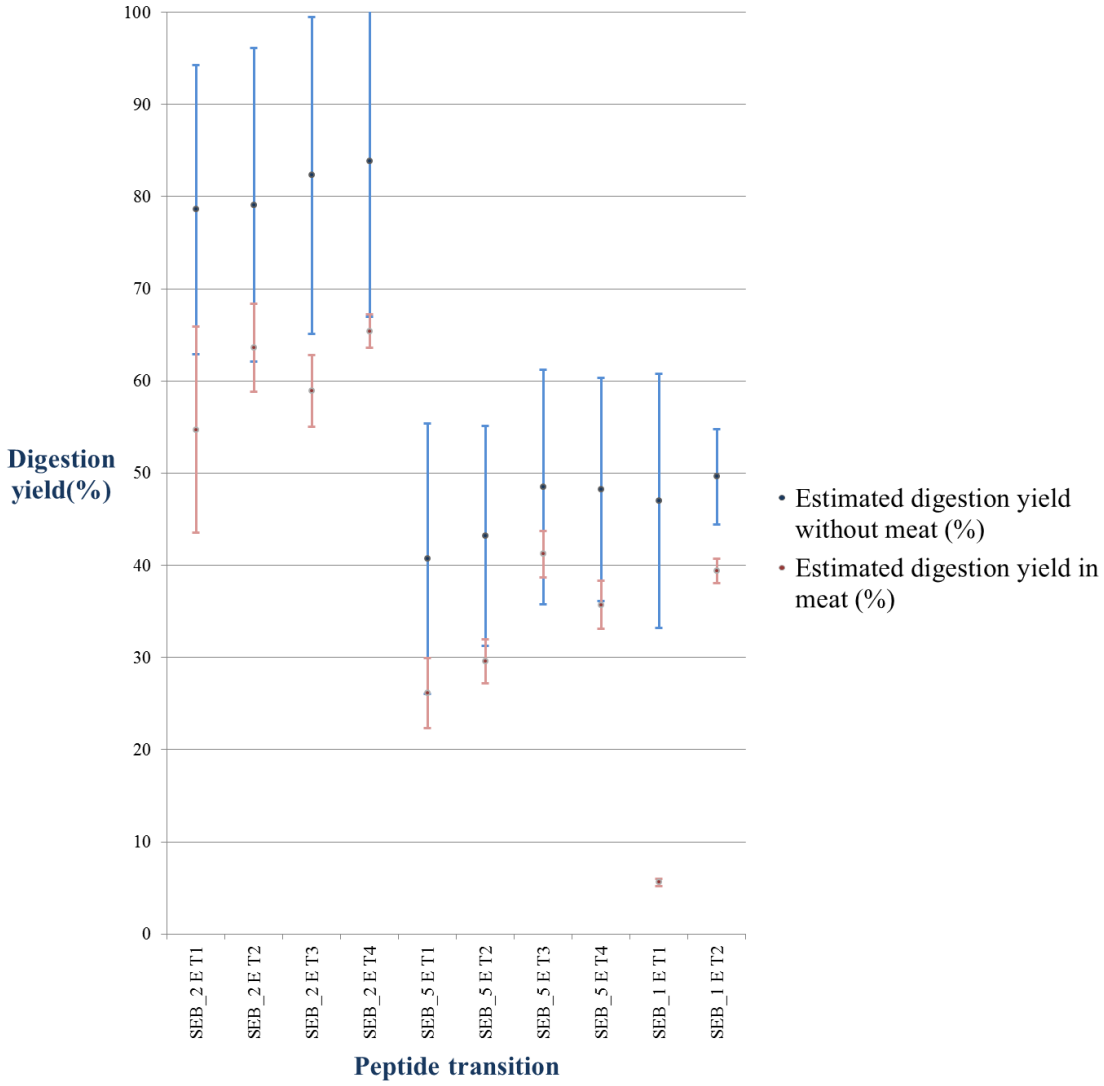

Figure 27. Comparison of the estimated toxins digestion yields for digestion in presence or absence of meat.

5 Conclusions and perspectives

The results may be discussed from three aspects. First, general conclusions about the achieved work and leads for optimizing the protocol are discussed. Second, the objectives of the work that were not achieved are presented along with the alternatives for their achievement. Finally, a few words about possible extrapolations of the general method will end the discussion.

5.1 Conclusions on the achieved work

The objectives of the work were to adapt and optimize a detection and quantification method of Staphylococcal Enterotoxins from milk to meat, to validate the method and to test real samples.

Meat is a complex matrix which requires thorough purification to allow the detection of the toxins. Not only do matrix interferences lower the detection limit of the UPLC-MS/MS method, but it also lowers the general efficiency of the method because matrix proteins could lower the enterotoxins digestion efficiency.

The adaptation of the method faced several challenges but several optimizations were developed. First, the protocol had to be adapted from a liquid to a solid matrix, meaning an aqueous toxin extraction solvent had to be chosen. Second, the samples charged with matrix fat needed clean-up, so an organic solvent (dichloromethane) was selected for fat removal. Third, the proper phase separation by centrifugation remained challenging too as an emulsion was forming, so the aqueous solvent was optimized (1.5% NaCl) in order to break the emulsion and improve the toxins recovery. Fourth, the samples charged with matrix proteins also needed clean-up. Therefore a purification strategy based on the use of proteins precipitation by acidification (pH 4 acetate buffer) and several filters (0.1µm PTFE micro-filter, 50 kDa and 5 kDa MWCO PES ultra-filters) was elaborated to eliminate as many matrix proteins as possible. The proteins precipitation was also improved by the salt presence in solution. Finally, the digestion buffer was optimized (Tris-$CaCl_2$) to adjust the pH to the range that suits the chosen protease (trypsin works in between pH 7 and 9).

The general protocol could be improved, as demonstrated by the experiment integrating all the optimized steps. Indeed, the “full method evaluation” (see section 4.4) shows that some toxins are lost in the process.

Apparently, the 50 kDa MWCO ultra-filter causes loss, but the previous steps (toxins extraction, matrix proteins precipitation, micro-filtration) could also be improved.

Several strategies could be explored to improve the recovery.

Besides the fact that toxins loss may result from the numerous steps of the protocol, the chosen material might also have an influence. The selection of filters (and their preparation) could therefore be revisited.

First, the centrifuge filters have a PES membrane. This membrane is described as presenting low protein binding characteristics (Whatman, Sterlitech). But some binding may occur anyway. The testing of the newly available Hydrosart® membrane which is described as highly hydrophilic, and therefore non-protein binding could be investigated. Note that nowadays those membranes only exist in 2, 5, 10 and 30 kDa MWCO sizes (Sartorius).

Second, passivation of the centrifuge filters could be investigated as a way of lowering non-specific binding. If the filters were pre-treated with a BSA (Bovine Serum Albumin) solution, the albumin proteins would first bind the membrane and consequently could diminish the toxins loss. This preparation would however increase the complexity, the number of steps and the length of the global protocol as it would need to be done a day before the filters are used.

Third, the syringe filter (from the micro-filtration step) might also cause loss due to their PTFE composition. PVDF membranes are described (Sterlitech) as binding less protein than PTFE membranes and would be more suited for this step but those membranes are not available in pore size under 0.2 µm, which already proved inefficient (see section a)).

Considering the lack of available filters that would serve this method (no 50 kDa MWCO Hydrosart® membrane and no 0.1 µm PVDF syringe filters), an alternative purification and concentration strategy could be investigated, solid phase extraction. For example, Brun (2007) already used a similar strategy to purify Staphylococcal enterotoxins in urine samples, using a resin made of hydroxylated silica particles.

Set aside the toxins loss in the filters, general purification could be improved beforehand. For instance, the extraction efficiency experiment (see section c)) showed that some toxins remain in the meat after phase separation by centrifugation. A several-steps extraction involving smaller volumes might be considered.

Inspiration could be sought in the official method for “Detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices” (ANSES).

This method, based on immunoassay detection, also gives recommendations in the samples preparation. The treatment of meat by the official method would be as follows. 25g of mixed sample and 40 ml of warm distilled water are homogenised, for example by blender, and kept 30 min at room temperature, under agitation, so the toxins diffuse to the water. The sample is then acidified down to pH 3.5-4.0 using HCl and centrifuged The matrix proteins precipitate because they are less acid stable than the toxins (which precipitate only under pH 3.0). The supernatant is then neutralized and centrifuged again. The concentration step takes place overnight by dialysis against PEG. Toxins are recovered in water and submitted to the immuno-enzymatic VIDAS SET2 test.

The official method and the meat protocol optimized in this work use some common principles. The following discussion is a comparison of the two extraction protocols as an attempt to find new ways to optimize the method.

The toxins extraction is quite similar between the two protocols, except for the physical part. Indeed the newly developed protocol uses a vortex and the official method uses agitation. The 15 seconds vortex step is much faster than the 30 minutes agitation one but it would be interesting to try and compare both methods as far as the toxins recovery is concerned.

The sample clean-up of both methods present a common acidification step but the official one uses a neutralization step which could maybe be tested in the current protocol as a way to eliminate more matrix proteins.

On the other hand, there is no mention of fat elimination, meaning the supernatant is probably difficult to recover properly in the official method. This is a big difference between the two protocols as the matrix fat interferes with proper toxins recovery. The fat extraction step of this protocol could be considered a bonus compared to the official method.

Finally, the concentration step of the official method is quite long as it takes place overnight whereas it is the digestion step that is time consuming in the present protocol. So both protocols are quite equivalent regarding their length.

This first discussion could be concluded by saying that many testing involving new filters, several-step extraction, toxins extraction by agitation and solid phase extraction could be undertaken in order to improve the sample clean-up and enterotoxins recovery.

5.2 Future work

Once optimized, the meat protocol was to be validated following the official guidelines furnished by legislation EC/657/2002. According to ISO 17025 for which the Institute of Public Health (WIV-ISP) has accreditation, the validation plan must be prepared. Internal guidelines were followed and the plan can be consulted in Annex 1919.

The validation is defined as "the confirmation by examination and the provision of effective evidence that the particular requirements of a specific intended use are fulfilled" (EC/657/2002). The parameters to be calculated are°:

The linearity of the method is the range of concentrations for which the response will be proportional to the concentration of the substance to measure. The Mandel's Fitting test (Mandel J., 1964) is used by the WIV-ISP as an acceptation criteria for linearity.

The matrix effect is the influence of the matrix on the response given by the device. It is obtained by comparing the response for a matrix sample spiked with a standard compared to the response obtained for the pure standard, in solution.

The recovery is the percentage of the true concentration of a substance recovered during the analytical procedure.

The repeatability represents the variation between three repetitions, on the same concentration level, on the same day, by the same operator. It is calculated as the standard deviation of the three repetitions, for each point and must satisfy certain criteria exposed in legislation EC/657/2002. The criteria depend on the concentration levels.

The intermediate precision represents the variation between three repetitions, on the same concentration level, between three days of work, by the same operator. It is calculated as the standard deviation of the nine repetitions, for each point and must satisfy certain criteria exposed in legislation EC/657/2002. The criteria depend on the concentration levels.

The Limit of Detection (LOD) is the smallest concentration of a substance that can be detected. It is defined as the substance concentration for which the signal to noise ratio (S/N) is greater than or equal to 3.

The Limit of Quantification (LOQ) is the smallest concentration of a substance that can be quantified with a certain precision. It is defined as 3.66 X the LOD. In other words it is the substance concentration for which the signal to noise ratio (S/N) greater than or equal to 10.

Eventually, the ruggedness of the method could have been checked for instance by lowering the enterotoxins digestion time to two hours. Real samples resulting from confirmed SFPOs, would also have been tested. Those parts of the work were not achieved, first due to delays caused by successive breakdowns of the UPLC-MS/MS device, and second because the meat protocol is still in need for further improvements.

5.3 Perspectives

The development of a fully optimized, validated, multi-residual detection and quantification method based on UPLC-MS/MS could open many perspectives in the analytical world.

As exposed in the introduction, there are three main methodologies based on the isotope dilution principle that could be used for quantification. The AQUA principle was used in this work because it is the cheapest but it does not account for recoveries or digestion yields. However, once peptides are selected for the target toxins, the use of a QconCAT concatamer could be considered so that the digestion yield could be evaluated. In any case, one very attractive advantage of the methodology using *à la carte* synthesised peptides is that once validated on a complex matrix such as meat, the protocol could be enlarged to other Staphylococcal Enterotoxins (SEC-SEE, SEH, TSST-1, *egc* locus SEs) and permit analysis of previously undetected SEs.

The general strategy could be extrapolated to the detection and quantification of other bacterial toxins originating for example from *Clostridium* sp., *Shigella* sp, *Vibrio* sp… which are also widely involved in food poisoning outbreaks (Le Loir *et al.*, 2003).

Such method could also find applications in more extended health fields such as respiratory diseases or nutrition.

There is evidence (Pastacaldi *et al.*, 2011; Bachert *et al.*, 2008) that *Staphylococcus aureus* is involved in persistent severe airway diseases such as chronic rhinosinusitis with nasal polyps, allergic respiratory diseases, asthma… *S. aureus* invades the airway mucosa (Corriveau *et al.*, 2009) and may locally release classical and *egc*-locus enterotoxins, which have superantigenic activities. As exposed in the introduction (section 2.1.6) SEs activate T- and B-cells and amplify the airway inflammation. The development of an appropriate tool to detect enterotoxins within the mucosal tissues or fluids could kick off the understanding of the involvement of SEs in severe airway diseases**.** Later on, this technique could be used to diagnose SE related asthma and to clarify the impact of specific SEs on asthma. The new knowledge may lead to novel treatment approaches.

Nutrition could also be improved by the methods developed in the present work. The use of different size filters has allowed the separation of meat proteins according to their weight. If this methodology were integrated in a foodomics approach (Capozzi F. & Bordoni A., 2013) it could bring valuable information to human nutrition science and possibly help improve food quality.

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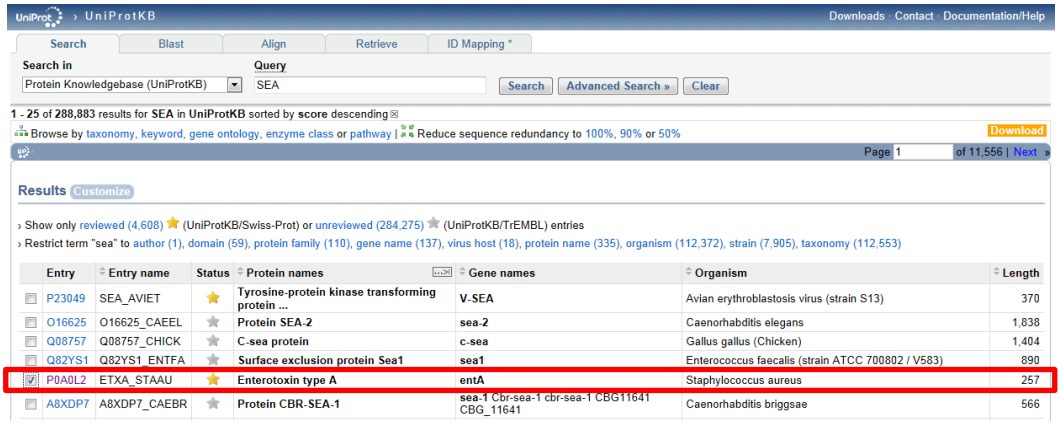
Annexes

1. Preparation of Internal Standard individual stock solution (100 pmol/µl).

IS named SEA_1, SEA_3, and SEB_3 were available at the desired concentration as they had been prepared for the milk protocol.

Internal Standard	Delivered concentration (pmol/µl)	Stock concentration (pmol/µl)	Final volume (µl)	Dissolved volume (µl)
SEA_2	3810	100	5000	131
SEA_4	4850	100	5000	103
SEA_5	3020	100	5000	165
SEB_1	3920	100	5000	128
SEB_2	3410	100	5000	147
SEB_4	3340	100	5000	150
SEB_5	5830	100	5000	86
SEB_6	2860	100	5000	175
SEG_1	3310	100	5000	151
SEG_2	4690	100	5000	107
SEG_3	2450	100	5000	204
SEI_1	2890	100	5000	173
SEI_2	4800	100	5000	104
SEI_3	6470	100	5000	77
SEI_4	2950	100	5000	170
SEI_5	4450	100	5000	112
SEM_1	2550	100	5000	196
SEM_2	5970	100	5000	84
SEM_3	2230	100	5000	224
SEN_1	4810	100	5000	104
SEN_2	6720	100	5000	74
SEN_3	3480	100	5000	143
SEN_4	4080	100	5000	123
SEO_1	5450	100	5000	92
SEO_2	5160	100	5000	97
SEO_3	3470	100	5000	144
SEO_4	6350	100	5000	79
SEO_5	5160	100	5000	97
TSST-1_1	2000	100	5000	250
TSST-1_2	2880	100	5000	174
TSST-1_3	2330	100	5000	215
TSST-1_4	3570	100	5000	140

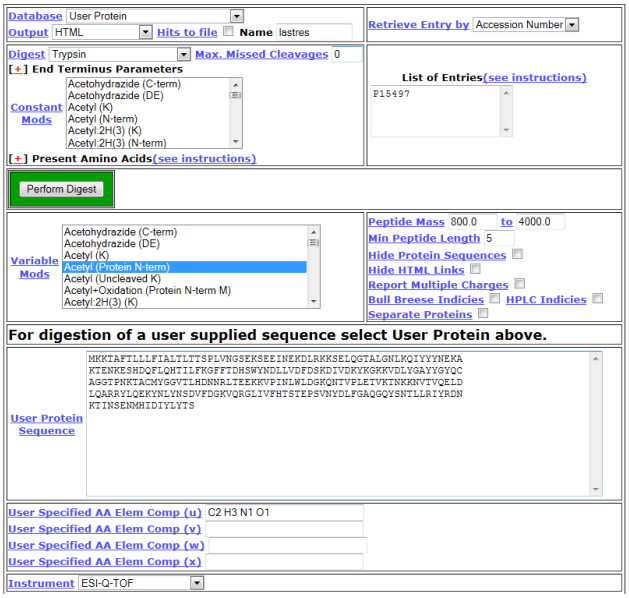
2. Search for protein sequences using the Uniprot database (www.uniprot.org)



3. SEs amino acid sequences

SE	Amino acid sequence
SEA	MKKTAFTLLL FIALTLTTSP LVNGSEKSEE INEKDLRKKS ELQGTALGNL KQIYYYNEKA
	KTENKESHDQ FLQHTILFKG FFTDHSWYND LLVDFDSKDI VDKYKGKKVD LYGAYYGYQC
	AGGTPNKTAC MYGGVTLHDN NRLTEEKKVP INLWLDGKQN TVPLETVKTN KKNVTVQELD
	LQARRYLQEK YNLYNSDVFD GKVQRGLIVF HTSTEPSVNY DLFGAQGQYS NTLLRIYRDN
	KTINSENMHI DIYLYTS
SEB	MYKRLFISHV ILIFALILVI STPNVLAESQ PDPKPDELHK SSKFTGLMEN MKVLYDDNHV
	SAINVKSIDQ FLYFDLIYSI KDTKLGNYDN VRVEFKNKDL ADKYKDKYVD VFGANYYYQC
	YFSKKTNDIN SHQTDKRKTC MYGGVTEHNG NQLDKYRSIT VRVFEDGKNL LSFDVQTNKK
	KVTAQELDYL TRHYLVKNKK LYEFNNSPYE TGYIKFIENE NSFWYDMMPA PGDKFDQSKY
	LMMYNDNKMV DSKDVKIEVY LTTKKK
SEC-1	MNKSRFISCV ILIFALILVL FTPNVLAESQ PDPTPDELHK ASKFTGLMEN MKVLYDDHYV
	SATKVKSVDK FLAHDLIYNI SDKKLKNYDK VKTELLNEGL AKKYKDEVVD VYGSNYYVNC
	YFSSKDNVGK VTGGKTCMYG GITKHEGNHF DNGNLQNVLI RVYENKRNTI SFEVQTDKKS
	VTAQELDIKA RNFLINKKNL YEFNSSPYET GYIKFIENNG NTFWYDMMPA PGDKFDQSKY
	LMMYNDNKTV DSKSVKIEVH LTTKNG
SEC-2	MNKSRFISCV ILIFALILVL FTPNVLAESQ PDPTPDELHK SSEFTGTMGN MKVLYDDHYV
	SATKVMSVDK FLAHDLIYNI SDKKLKNYDK VKTELLNEDL AKKYKDEVVD VYGSNYYVNC
	YFSSKDNVGK VTGGKTCMYG GITKHEGNHF DNGNLQNVLI RVYENKRNTI SFEVQTDKKS
	VTAQELDIKA RNFLINKKNL YEFNSSPYET GYIKFIENNG NTFWYDMMPA PGDKFDQSKY
	LMMYNDNKTV DSKSVKIEVH LTTKNG
SEC-3	MYKRLFISRV ILIFALILVI STPNVLAESQ PDPMPDDLHK SSEFTGTMGN MKVLYDDHYV
	SATKVKSVDK FLAHDLIYNI SDKKLKNYDK VKTELLNEDL AKKYKDEVVD VYGSNYYVNC
	YFSSKDNVGK VTGGKTCMYG GITKHEGNHF DNGNLQNVLV RVYENKRNTI SFEVQTDKKS
	VTAQELDIKA RNFLINKKNL YEFNSSPYET GYIKFIENNG NTFWYDMMPA PGDKFDQSKY
	LMMYNDNKTV DSKSVKIEVH LTTKNG
SED	MKKFNILIAL LFFTSLVISP LNVKANENID SVKEKELHKK SELSSTALNN MKHSYADKNP
	IIGENKSTGD QFLENTLLYK KFFTDLINFE DLLINFNSKE MAQHFKSKNV DVYPIRYSIN
	CYGGEIDRTA CTYGGVTPHE GNKLKERKKI PINLWINGVQ KEVSLDKVQT DKKNVTVQEL
	DAQARRYLQK DLKLYNNDTL GGKIQRGKIE FDSSDGSKVS YDLFDVKGDF PEKQLRIYSD
	NKTLSTEHLH IDIYLYEK
SEE	MKKTAFILLL FIALTLTTSP LVNGSEKSEE INEKDLRKKS ELQRNALSNL RQIYYYNEKA
	ITENKESDDQ FLENTLLFKG FFTGHPWYND LLVDLGSKDA TNKYKGKKVD LYGAYYGYQC
	AGGTPNKTAC MYGGVTLHDN NRLTEEKKVP INLWIDGKQT TVPIDKVKTS KKEVTVQELD
	LQARHYLHGK FGLYNSDSFG GKVQRGLIVF HSSEGSTVSY DLFDAQGQYP DTLLRIYRDN
	KTINSENLHI DLYLYTT
SEG	MKKLSTVIII LILEIVFHNM NYVNAQPDPK LDELNKVSDY KNNKGTMGNV MNLYTSPPVE
	GRGVINSRQF LSHDLIFPIE YKSYNEVKTE LENTELANNY KDKKVDIFGV PYFYTCIIPK
	SEPDINQNFG GCCMYGGLTF NSSENERDKL ITVQVTIDNR QSLGFTITTN KNMVTIQELD
	YKARHWLTKE KKLYEFDGSA FESGYIKFTE KNNTSFWFDL FPKKELVPFV PYKFLNIYGD
	NKVVDSKSIK MEVFLNTH
SEH	MINKIKILFS FLALLLSFTS YAKAEDLHDK SELTDLALAN AYGQYNHPFI KENIKSDEIS
	GEKDLIFRNQ GDSGNDLRVK FATADLAQKF KNKNVDIYGA SFYYKCEKIS ENISECLYGG
	TTLNSEKLAQ ERVIGANVWV DGIQKETELI RTNKKNVTLQ ELDIKIRKIL SDKYKIYYKD
	SEISKGLIEF DMKTPRDYSF DIYDLKGEND YEIDKIYEDN KTLKSDDISH IDVNLYTKKK
	V
SEI	MKKFKYSFIL VFILLFNIKD LTYAQGDIGV GNLRNFYTKH DYIDLKGVTD KNLPIANQLE
	FSTGTNDLIS ESNNWDEISK FKGKKLDIFG IDYNGPCKSK YMYGGATLSG QYLNSARKIP
	INLWVNGKHK TISTDKIATN KKLVTAQEID VKLRRYLQEE YNIYGHNNTG KGKEYGYKSK
	FYSGFNNGKV LFHLNNEKSF SYDLFYTGDG LPVSFLKIYE DNKIIESEKF HLDVEISYVD
	SN
SEM	MKRILIIVVL LFCYSQNHIA TADVGVLNLR NYYGSYPIED HQSINPENNH LSHQLVFSMD
	NSTVTAEFKN VDDVKEFKNH AVDVYGLSYS GYCLKNKYIY GGVTLAGDYL EKSRRIPINL
	WVNGEHQTIS TDKVSTNKKL VTAQEIDTKL RRYLQEEYNI YGFNDTNKGR NYGNKSKFSS
	GFNAGKILFH LNDGSSFSYD LFDTGTGQAE SFLKIYNDNK TVETEKFHLD VEISYKDES
SEN	MKNIKKLMRL FYIAAIIITL LCLINNNYVN AEVDKKDLKK KSDLDSSKLF NLTSYYTDIT
	WQLDESNKIS TDQLLNNTII LKNIDISVLK TSSLKVEFNS SDLANQFKGK NIDIYGLYFG
	NKCVGLTEEK TSCLYGGVTI HDGNQLDEEK VIGVNVFKDG VQQEGFVIKT KKAKVTVQEL
	DTKVRFKLEN LYKIYNKDTG NIQKGCIFFH SHNHQDQSFY YDLYNVKGSV GAEFFQFYSD
	NRTVSSSNYH IDVFLYKD
SEO	MIKNSKVMLN VLLLILNLIA ICSVNNAYAN EEDPKIESLC KKSSVDPIAL HNINDDYINN
	RFTTVKSIVS TTEKFLDFDL LFKSINWLDG ISAEFKDLKV EFSSSAISKE FLGKTVDIYG
	VYYKAHCHGE HQVDTACTYG GVTPHENNKL SEPKNIGVAV YKDNVNVNTF IVTTDKKKVT
	AQELDIKVRT KLNNAYKLYD RMTSDVQKGY IKFHSHSEHK ESFYYDLFYI KGNLPDQYLQ
	IYNDNKTIDS SDYHIDVYLF T
TSST-1	MNKKLLMNFF IVSPLLLATT ATDFTPVPLS SNQIIKTAKA STNDNIKDLL DWYSSGSDTF
	TNSEVLDNSL GSMRIKNTDG SISLIIFPSP YYSPAFTKGE KVDLNTKRTK KSQHTSEGTY
	IHFQISGVTN TEKLPTPIEL PLKVKVHGKD SPLKYGPKFD KKQLAISTLD FEIRHQLTQI
	HGLYRSSDKT GGYWKITMND GSTYQSDLSK KFEYNTEKPP INIDEIKTIE AEIN

4. **Search for theoretical triptych fragments using the MS-Digest tool (http://prospector.ucsf.edu/prospector/cgi-bin/msform.cgi?form=msdigest)**



5. Tryptic fragments obtained for all SEs with the MS Digest tool

SEA	SEB	SEC	SED
(K) SEEINEK(D)	(K) LGNYDNVR(V)	(K) IEVHLTTK(N)	(K) NPIIGENK(S)
(K)QIYYYNEK(A)	(K) IEVYLTTK(K)	(K) NLYEFNSSPYETGYIK(F)	(K) EMAQHFK(S)
(K)QNTVPLETVK(T)	(K) FTGLMENMK(V)	(K) TCMYGGITK(H)	(K)EMAQHFK(S)
(K) QIYYYNEK(A)	(K)FTGLMENMK(V)	(K) HEGNHFDNGNLQNVLVR(V)	(R) YLQKDLK(L)
(K) QNTVPLETVK(T)	(K)FTGLMENMK(V)	(K)TCMYGGITK(H)	(K) NVDVYPIR(Y)
(K) VPINLWLDGK(Q)	(K) YLMMYNDNK(M)	(K) SVTAQELDIK(A)	(K) ANENIDSVK(E)
(K) SELQGTALGNLK(Q)	(K)YLMMYNDNK(M)	(R) VILIFALILVISTPNVLAESQPDPMPDDLHK(S)	(K) IEFDSSDGSK(V)
(K) NVTVQELDLQAR(R)	(K)YLMMYNDNK(M)	(R)VILIFALILVISTPNVLAESQPDPMPDDLHK(S)	(K) VSYDLFDVK(G)
(K) YNLYNSDVFDGK(V)	(K) TNDINSHQTDK(R)	(K) TELLNEDLAK(K)	(K) GDFPEKQLR(I)
(K) TACMYGGVTLHDNNR(L)	(K) NLLSFDVQTNK(K)	(K) FIENNGNTFWYDMMPAPGDK(F)	(K) LYNNDTLGGK(I)
(K)TACMYGGVTLHDNNR(L)	(K) VTAQELDYLTR(H)	(K) DEVVDVYGSNYYVNCYFSSK(D)	(K) EMAQHFKSK(N)
(K) ESHDQFLQHTILFK(G)	(K) VLYDDNHVSAINVK(S)	(K)FIENNGNTFWYDMMPAPGDK(F)	(K)QLRIYSDNK(T)
(K) TINSENMHIDIYLYTS(-)	(K) LYEFNNSPYETGYIK(F)	(K)FIENNGNTFWYDMMPAPGDK(F)	(K)EMAQHFKSK(N)
(K)TINSENMHIDIYLYTS(-)	(K) SIDQFLYFDLIYSIK(D)	(K) YLMMYNDNK(T)	(K) QLRIYSDNK(T)
(K) VDLYGAYYGYQCAGGTPNK(T)	(K) TCMYGGVTEHNGNQLDK(Y)	(K)YLMMYNDNK(T)	(K) SKNVDVYPIR(Y)
(K) GFFTDHSWYNDLLVDFDSK(D)	(K)TCMYGGVTEHNGNQLDK(Y)	(K)YLMMYNDNK(T)	(K) ANENIDSVKEK(E)
(K) TAFTLLLFIALTLTTSPLVNGSEK(S)	(K) YVDVFGANVYYQCYFSK(K)	(R) NTISFEVQTDK(K)	(K) EVSLDKVQTDK(K)
(R) GLIVFHTSTEPSVNYDLFGAQGQYSNTLLR(I)	(K) FIENENSFWYDMMPAPGDK(F)	(K) SSEFTGTMGNMK(Y)	(R) GKIEFDSSDGSK(V)
	(K)FIENENSFWYDMMPAPGDK(F)	(K)SSEFTGTMGNMK(Y)	(K) SELSSTALNNMK(H)
	(K)FIENENSFWYDMMPAPGDK	(K)SSEFTGTMGNMK(Y)	(K)SELSSTALNNMK(H)
		(K) YLYDDHYVSATK(V)	(K) NVTVQELDAQAR(R)
		(K) FLAHDLIYNISDK(K)	(R) YSINCYGGEIDR(T)
		(R) VILIFALILVISTPNVLAESQPDPMPDDLHK(S)	(K) IPINLWINGVQK(E)
		(R)VILIFALILVISTPNVLAESQPDPMPDDLHK(S)	(K) KSELSSTALNNMK(H)
		(K) NLYEFNSSPYETGYIK(F)	(K)KSELSSTALNNMK(H)
		(K) HEGNHFDNGNLQNVLVR(V)	(K) DLKLYNNDTLGGK(I)
		(K) FIENNGNTFWYDMMPAPGDK(F)	(K) KNVTVQELDAQAR(R)
		(K) DEVVDVYGSNYYVNCYFSSK(D)	(K) LYNNDTLGGKIQR(G)
		(K)FIENNGNTFWYDMMPAPGDK(F)	(K) NVTVQELDAQARR(Y)
		(K)FIENNGNTFWYDMMPAPGDK(F)	(K) KIPINLWINGVQK(E)
		(R) VILIFALILVISTPNVLAESQPDPMPDDLHK(S)	(R) TACTYGGVTPHEGNK(L)
		(R)VILIFALILVISTPNVLAESQPDPMPDDLHK(S)	(K) HSYADKNPIIGENK(S)
			(K) STGDQFLENTLLYK(K)
			(K) STGDQFLENTLLYKK(F)
			(K) VSYDLFDVKGDFPEK(Q)
			(R) TACTYGGVTPHEGNKLK(E)
			(K) TLSTEHLHIDIYLYEK(-)
			(K) SELSSTALNNMKHSYADK(N)
			(K)SELSSTALNNMKHSYADK(N)
			(K) IPINLWINGVQKEVSLDK(V)
			(K) IEFDSSDGSKVSYDLFDVK(G)
			(K) FFTDLINFEDLLINFNSK(E)
			(K) KFFTDLINFEDLLINFNSK(E)
			(K) NVDVYPIRYSINCYGGEIDR(T)
			(K) FNILIALLFFTSLVISPLNVK(A)
			(K) KFNILIALLFFTSLVISPLNVK(A)
			(K) NPIIGENKSTGDQFLENTLLYK(K)
			(R) IYSDNKTLSTEHLHIDIYLYEK(-)
			(R) YSINCYGGEIDRTACTYGGVTPHEGNK(L)
			(K) FFTDLINFEDLLINFNSKEMAQHFK(S)
			(K)FFTDLINFEDLLINFNSKEMAQHFK(S)
			(K) FNILIALLFFTSLVISPLNVKANENIDSVK(E)

SEE	SEG	SEH	SEI
(K) TACMYGGVTLHDNNR(L)	(K)MEVFLNTH(-)	(K) SDDISHIDVNLYTK(K)	(K)HDYIDLK(G)
(K)TACMYGGVTLHDNNR(L)	(K)MEVFLNTH(-)	(K) AEDLHDK(S)	(K)FYSGFNNGK(V)
(K) SEEINEK(D)	(K)FLNIYGDNK(V)	(K) SDEISGEK(D)	(K)VLFHLNNEK(S)
(K) ESDDQFLENTLLFK(G)	(K)ELVPFVPYK(F)	(K) GLIEFDMK(T)	(K)LVTAQEIDVK(L)
(K)QTTVPIDK(V)	(R)QSLGFTITTNK(N)	(K) FATADLAQK(F)	(K)IPINLWVNGK(H)
(K) QTTVPIDK(V)	(R)QSLGFTITTNK(N)	(K) ILFSFLALLLSFTSYAK(A)	(K)LDIFGIDYNGPCK(S)
(K) TINSENLHIDLYLYTT(-)	(K)LITVQVTIDNR(Q)	(K)GLIEFDMK(T)	(K)FHLDVEISYVDSN(-)
(K) VDLYGAYYGYQCAGGTPNK(T)	(K)NMVTIQELDYK(A)	(K) ISENISECLYGGTTLNSEK(L)	(K)DLTYAQGDIGVGNLR(N)
(K) GFFTGHPWYNDLLVDLGSK(D)	(K)NMVTIQELDYK(A)	(R) NQGDSGNDLR(V)	(K)YSFILVFILLFNIK(D)
(R)QIYYYNEK(A)	(K)NNTSFWFDLFPK(K)	(K) GENDYEIDK(I)	(K)YMYGGATLSGQYLNSAR(K)
(R) GLIVFHSSEGSTVSYDLFDAQGQYPDTLLR(I)	(K)TELENTELANNYK(D)	(K) NVTLQELDIK(I)	(K)YMYGGATLSGQYLNSAR(K)
(R) QIYYYNEK(A)	(K)LYEFDGSAFESGYIK(F)	(K) SELTDLALANAYGQYNHPFIK(E)	(R)YLQEEYNIYGHNNTGK(G)
(K) VPINLWIDGK(Q)	(R)QFLSHDLIFPIEYK(S)	(R) DYSFDIYDLK(G)	(K)SFSYDLFYTGDGLPVSFLK(I)
(K) TAFILLLFIALTLTTSPLVNGSEK(S)	(R)QFLSHDLIFPIEYK(S)	(R) VIGANVWVDGIQK(E)	(K)NLPIANQLEFSTGTNDLISESNNWDEISK(F)
(K) FGLYNSDSFGGK(V)	(K)VDIFGVPYFYTCIIPK(S)	(K) NVDIYGASFYYK(C)	
(K) EVTVQELDLQAR(H)		(K) SDDISHIDVNLYTK(K)	
(K) TACMYGGVTLHDNNR(L)		(K) ILFSFLALLLSFTSYAK(A)	
(R) GLIVFHSSEGSTVSYDLFDAQGQYPDTLLR(I)		(K) ISENISECLYGGTTLNSEK(L)	
(K)TACMYGGVTLHDNNR(L)		(K) SELTDLALANAYGQYNHPFIK(E)	
(K) ESDDQFLENTLLFK(G)			
(K) TINSENLHIDLYLYTT(-)			
(K) VDLYGAYYGYQCAGGTPNK(T)			
(K) GFFTGHPWYNDLLVDLGSK(D)			
(K) TAFILLLFIALTLTTSPLVNGSEK(S)			
(R) GLIVFHSSEGSTVSYDLFDAQGQYPDTLLR(I)			

SEM	SEN	SEO	TSST-1
(K)FSSGFNAGK(I)	(K)VTVQELDTK(V)	(R)MTSDVQK(G)	(K) ASTNDNIK(D)
(K)YIYGGVTLAGDYLEK(S)	(K)DGVQQEGFVIK(T)	(R)MTSDVQK(G)	(K) LPTPIELPLK(V)
(K)NHAVDVYGLSYSGYCLK(N)	(K)VEFNSSDLANQFK(G)	(K)NIGVAVYK(D)	(R) HQLTQIHGLYR(S)
(R)YLQEEYNIYGFNDTNK(G)	(K)ISTDQLLNNTIILK(N)	(K)SIVSTTEK(F)	(K)QLAISTLDFEIR(H)
(R)IPINLWVNGEHQTISTDK(V)	(K)GSVGAEFFQFYSDNR(T)	(K)FHSHSEHK(E)	(K) QLAISTLDFEIR(H)
(R)ILIIVVLLFCYSQNHIATADVGVLNLR(N)	(K)TSCLYGGVTIHDGNQLDEEK(V)	(K)VTAQELDIK(V)	(K) ITMNDGSTYQSDLSK(K)
(K)ILFHLNDGSSFSYDLFDTGTGQAESFLK(I)	(K)LFNLTSYYTDITWQLDESNK(I)	(K)VEFSSSAISK(E)	(K)ITMNDGSTYQSDLSK(K)
	(K)GCIFFHSHNHQDQSFYYDLYNVK(G)	(K)FLDFDLLFK(S)	(K) FEYNTEKPPINIDEIK(T)
		(K)TVDIYGVYYK(A)	(K) NTDGSISLIIFPSPYYSPAFTK(G)
		(K)SINWLDGISAEFK(D)	(K) SQHTSEGTYIHFQISGVTNTEK(L)
		(K)ESFYYDLFYIK(G)	(K) DLLDWYSSGSDTFTNSEVLDNSLGSMR(I)
		(K)DNVNVNTFIVTTDK(K)	(K)DLLDWYSSGSDTFTNSEVLDNSLGSMR(I)
		(K)TIDSSDYHIDVYLFT(-)	(K) LLMNFFIVSPLLLATTATDFTPVPLSSNQIIK(T)
		(K)GNLPDQYLQIYNDNK(T)	(K)LLMNFFIVSPLLLATTATDFTPVPLSSNQIIK(T)
		(K)SSVDPIALHNINDDYINNR(F)	
		(K)AHCHGEHQVDTACTYGGVTPHENNK(L)	
		(K)VMLNVLLLILNLIAICSVNNAYANEEDPK(I)	
		(K)VMLNVLLLILNLIAICSVNNAYANEEDPK(I)	

6. **Verification of uniqueness of selected theoretical peptides using the Blast Proteins database (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins).**

Sequences producing significant alignments:

Select: All None Selected:25

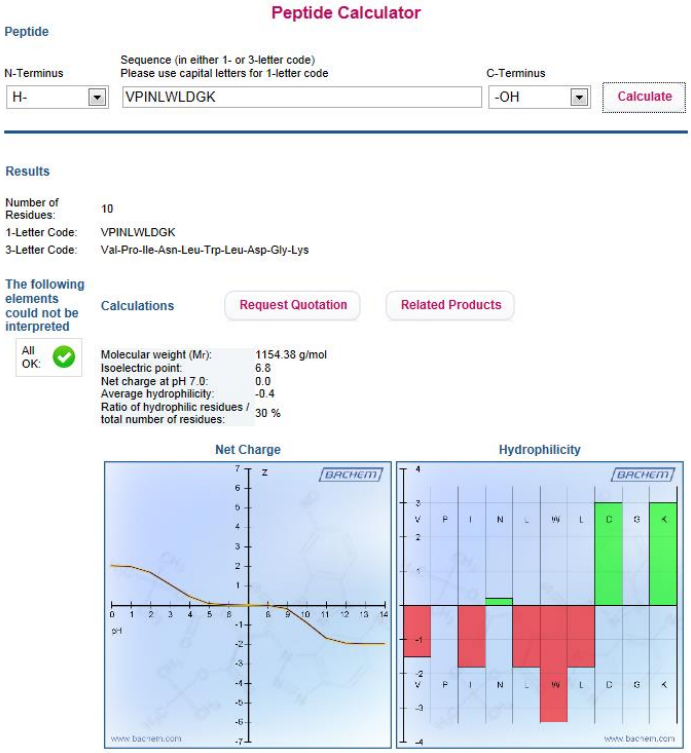
Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Max ident	Accession
enterotoxin [Staphylococcus aureus]	38.4	38.4	100%	0.013	100%	ABS50252.1
enterotoxin type B [Staphylococcus aureus] >gb|EHT21120.1| enterotoxin type B [Staphylococcus aureus subsp. aureus CIG1114]	38.4	38.4	100%	0.015	100%	WP_001813987.1
enterotoxin B [Staphylococcus aureus subsp. aureus]	38.4	38.4	100%	0.016	100%	AAR99634.1
enterotoxin [Staphylococcus aureus] >gb|EFB50676.1| enterotoxin [Staphylococcus aureus subsp. aureus D139]	38.4	38.4	100%	0.017	100%	WP_001798366.1
enterotoxin seb variant [Staphylococcus aureus]	38.4	38.4	100%	0.017	100%	AAP37186.1
Chain D, Complex Of The Human Mhc Class Ii Glycoprotein Hla-Dr1 And The Bacterial Superantigen Seb >pdb|1SEB|H Chain H, Complex Of	38.4	38.4	100%	0.017	100%	1SEB_D
Chain A, Staphylococcal Enterotoxin B	38.4	38.4	100%	0.018	100%	3SEB_A
Chain D, X-Ray Crystal Structure Of Hla-Dr4 Complexed With A Peptide From Human Collagen Ii >pdb|1SBB|B Chain B, T-Cell Receptor Beta	38.4	38.4	100%	0.018	100%	2SEB_D
Chain A, Structural Basis For The Altered T-Cell Receptor Binding Specificity In A Superantigenic Staphylococcus Aureus Enterotoxin-B Mutant	38.4	38.4	100%	0.018	100%	1GOZ_A
Chain A, Staphylococcal Enterotoxin B Mutant N23yk97sk98s >pdb|3GP7|B Chain B, Staphylococcal Enterotoxin B Mutant N23yk97sk98s	38.4	38.4	100%	0.018	100%	3GP7_A
enterotoxin B precursor, partial [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	ABJ97619.1
enterotoxin B [Staphylococcus aureus] >gb|ABF93357.1| enterotoxin B [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	ABF93356.1
enterotoxin B [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	AAW21709.1
staphylococcal enterotoxin B precursor (SEB) [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	CAJ43561.1
enterotoxin B precursor, partial [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	ABJ97620.1
enterotoxin B precursor, partial [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	ABJ97621.1
enterotoxin type B [uncultured Staphylococcus sp.]	38.4	38.4	100%	0.018	100%	AGH13407.1
enterotoxin type B [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	BAM66955.1
enterotoxin type B [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	BAM66936.1
optimized enterotoxin B [synthetic construct]	38.4	38.4	100%	0.018	100%	AAS87602.1
enterotoxin B [Staphylococcus aureus subsp. aureus COL] >ref|YP_005297270.1| Superantigen enterotoxin SEB [Staphylococcus aureus subs	38.4	38.4	100%	0.018	100%	YP_185778.1
hypothetical protein [Staphylococcus aureus] >gb|EHS28977.1| enterotoxin type B [Staphylococcus aureus subsp. aureus IS-122]	38.4	38.4	100%	0.018	100%	WP_000278087.1
hypothetical protein [Staphylococcus aureus] >gb|EFB48235.1| superantigen enterotoxin SEB [Staphylococcus aureus subsp. aureus C427] >(	38.4	38.4	100%	0.018	100%	WP_000278086.1
hypothetical protein [Staphylococcus aureus] >dbj|BAH30700.1| enterotoxin B [Staphylococcus aureus] >dbj|BAH30702.1| enterotoxin B [Staph	38.4	38.4	100%	0.018	100%	WP_000282760.1
enterotoxin B precursor [Staphylococcus aureus]	38.4	38.4	100%	0.018	100%	ABJ97622.1
exotoxin A [Streptococcus pyogenes]	29.1	29.1	72%	20	100%	ABX44734.1
Chain A, Structural Features Of A Zinc-Binding Site In The Superantigen Streptococcal Pyrogenic Exotoxin A (Spea1): Implications For Mhc Cl	29.1	29.1	72%	20	100%	1HA5_A
Chain A, Streptococcal Pyrogenic Exotoxin A1 >pdb|1B1Z|B Chain B, Streptococcal Pyrogenic Exotoxin A1 >pdb|1B1Z|C Chain C, Streptococ	29.1	29.1	72%	20	100%	1B1Z_A
type A exotoxin [Streptococcus pyogenes]	29.1	29.1	72%	20	100%	CAA43752.1
Chain A, Crystal Structure Of A Dimeric Form Of Streptococcal Pyrogenic Exotoxin A (spea1) >pdb|1UUP|B Chain B, Crystal Structure Of A Di	29.1	29.1	72%	20	100%	1UUP_A
Chain A, Structure Of Streptococcal Pyrogenic Exotoxin A >pdb|1FNU|B Chain B, Structure Of Streptococcal Pyrogenic Exotoxin A >pdb|1FNU	29.1	29.1	72%	20	100%	1FNU_A
Chain B, Tcr Beta Chain Complexed With Streptococcal Superantigen Spea >pdb|1L0X|D Chain D, Tcr Beta Chain Complexed With Streptoco	29.1	29.1	72%	20	100%	1L0X_B
exotoxin type A [Streptococcus pyogenes] >gb|AAL06068.1| pyrogenic exotoxin A [Streptococcus dysgalactiae subsp. equisimilis]	29.1	29.1	72%	20	100%	AAD21315.1
exotoxin A [Streptococcus pyogenes]	29.1	29.1	72%	20	100%	AAD11624.1
exotoxin type A precursor (allele 3) - Streptococcus pyogenes phage (strain MGAS158 isolate Nebraska and others) (fragment) >emb|CAA437	29.1	29.1	72%	20	100%	S18783
exotoxin type A precursor (allele 2) - Streptococcus pyogenes phage (strain MGAS250 isolate California and others) (fragment) >emb|CAA437	29.1	29.1	72%	20	100%	S18786
type A exotoxin [Streptococcus pyogenes]	29.1	29.1	72%	20	100%	CAA43771.1
type A exotoxin [Streptococcus pyogenes] >emb|CAA43754.1| type A exotoxin [Streptococcus pyogenes] >emb|CAA43757.1| type A exotoxin [	29.1	29.1	72%	20	100%	CAA43758.1
enterotoxin [Streptococcus pyogenes phage 5005.1] >ref|YP_006933065.1| exotoxin type A [Streptococcus pyogenes A20] >ref|YP_00758757	29.1	29.1	72%	21	100%	YP_282359.1
exotoxin type A precursor - phage associated [Streptococcus pyogenes MGAS315] >ref|NP_795658.1| exotoxin type A precursor [Streptococci	29.1	29.1	72%	21	100%	NP_665105.1
exotoxin type A [Streptococcus pyogenes MGAS8232] >ref|WP_009880239.1| hypothetical protein [Streptococcus pyogenes] >sp|P62560.1|SF	29.1	29.1	72%	21	100%	NP_606642.1
SpeA variant [Streptococcus pyogenes MGAS10394] >ref|WP_011184439.1| SpeA variant [Streptococcus pyogenes] >gb|AAT86876.1| SpeA \	29.1	29.1	72%	21	100%	YP_060059.1
hypothetical protein [Leucobacter chromiiresistens]	29.1	29.1	72%	21	100%	WP_010156334.1
hypothetical protein CAPTEDRAFT_226653 [Capitella teleta]	29.1	29.1	72%	22	100%	ELU14775.1
phage integrase/site-specific recombinase [Haloarcula amylolytica] >gb|EMA18094.1| phage integrase/site-specific recombinase [Haloarcula ai	27.8	46.2	81%	53	100%	WP_008311643.1
Predicted ATP-dependent endonuclease of the OLD family [Cronobacter dublinensis] >emb|CCJ82895.1| Predicted ATP-dependent endonucle	34.1	34.1	100%	0.48	91%	WP_007730207.1
Predicted ATP-dependent endonuclease of the OLD family [Cronobacter condimenti] >emb|CCJ71013.1| Predicted ATP-dependent endonucle	34.1	34.1	100%	0.48	91%	WP_007664600.1

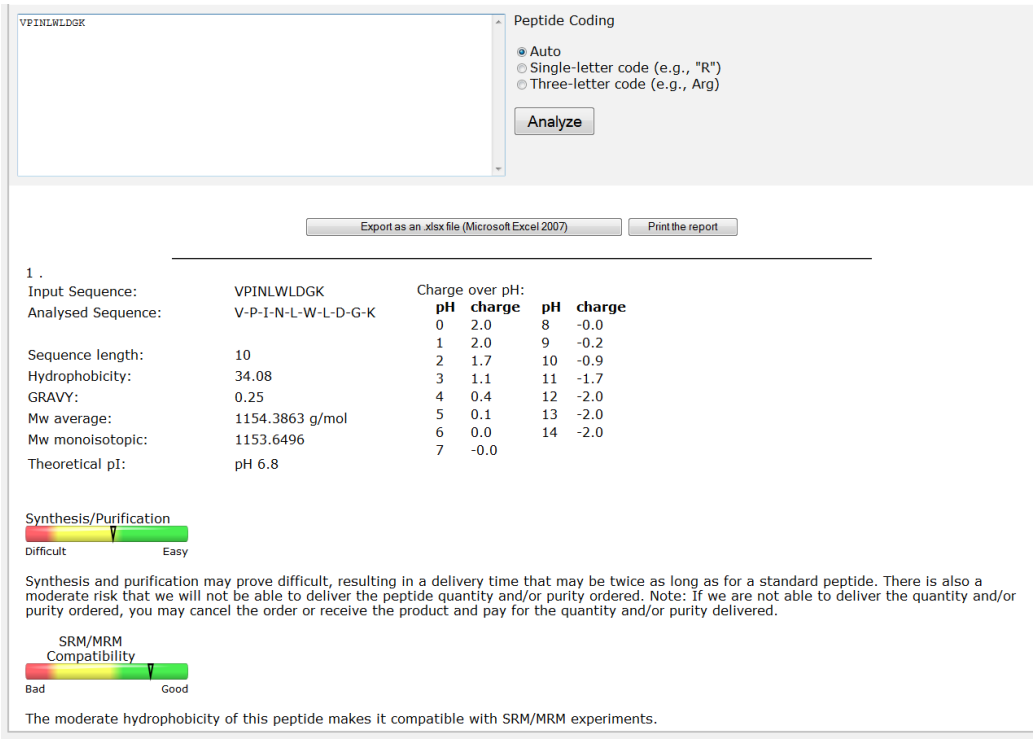
7. Unique selected peptides for each enterotoxin.

SEs	Unique proteotypic peptides
SEA	VPINLWLDGK
	GFFTDHSWYNDLLVDFDSK
	YNLYNSDVFDGK
	SELQGTALGNLK
	NVTVQELDLQAR
SEB	SIDQFLYFDLIYSIK
	NLLSFDVQTNK
	VLYDDNHVSAINVK
	VTAQELDYLTR
	LGNYDNVR
	TNDINSHQTDK
SEC	FLAHDLIYNISDK
	YLYDDHYVSATK
	HEGNHFDNGNLQNVLVR
SED	TLSTEHLHIDIYLYEK
	FNILIALLFFTSLVISPLNVK
	LYNNDTLGGK
	ANENIDSVK
SEE	TINSENLHIDLYLYTT
	GFFTGHPWYNDLLVDLGSK
	FGLYNSDSFGGK
SEG	LITVQVTIDNR
	TELENTELANNYK
	LYEFDGSAFESGYIK
SEH	ILFSFLALLLSFTSYAK
	NVDIYGASFYYK
	SELTDLALANAYGQYNHPFIK
	FATADLAQK
	SDDISHIDVNLYTK
	NVTLQELDIK
	DYSFDIYDLK
	NQGDSGNDLR
	GENDYEIDK
	SDEISGEK
SEI	YSFILVFILLFNIK
	SFSYDLFYTGDGLPVSFLK
	FYSGFNNGK
	VLFHLNNEK
	FHLDVEISYVDSN
	YLQEEYNIYGHNNTGK
	LVTAQEIDVK
SEM	YIYGGVTLAGDYLEK
	FSSGFNAGK
	YLQEEYNIYGFNDTNK
	ILFHLNDGSSFSYDLFDTGTGQAESFLK
SEN	VTVQELDTK
	DGVQQEGFVIK
	VEFNSSDLANQFK
	ISTDQLLNNTIILK
SEO	NIGVAVYK
	TVDIYGVYYK
	FLDFDLLFK
	TIDSSDYHIDVYLFT
	DNVNVNTFIVTTDK
	GNLPDQYLQIYNDNK
	VEFSSSAISK
	SIVSTTEK
	FHSHSEHK
TSST-1	HQLTQIHGLYR
	NTDGSISLIIFPSPYYSPAFTK
	LPTPIELPLK
	QLAISTLDFEIR
	SQHTSEGTYIHFQISGVTNTEK
	ASTNDNIK
	FEYNTEKPPINIDEIK

8. **Peptides hydrophilicity and stability using the Bachem peptide calculator (http://www.bachem.com/service-support/peptide-calculator/) and the Peptide Analysing tool (http://test.thermohybaid.de/cgi-bin/analysis.app).**



Peptides average hydrophilicity calculation

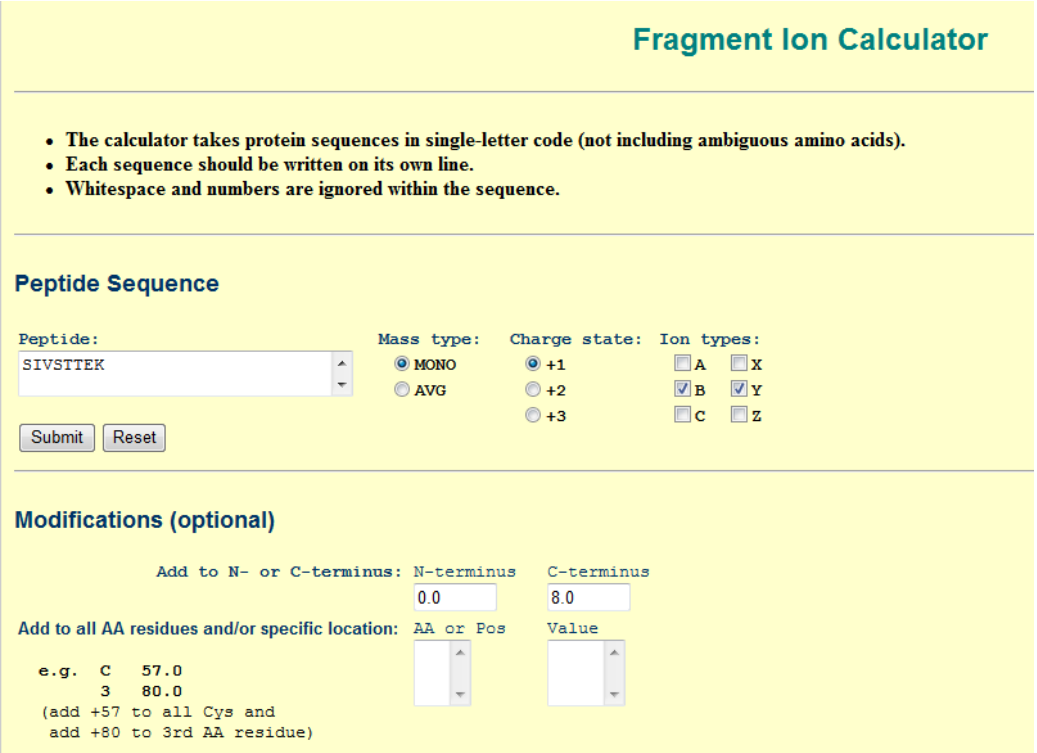


Peptide stability calculation

9. Complete list of pre-selected peptides related to the SETTECT project

Name	Abbreviation	Sequence
Staphylococcal Enterotoxin A	SEA_1	VPINLWLDGK
	SEA_2	YNLYNSDVFDGK
	SEA_3	NVTVQELDLQAR
	SEA_4	SELQGTALGNLK
	SEA_5	GFFTDHSWYNDLLVDFDSK
Staphylococcal Enterotoxin B	SEB_1	VLYDDNHVSAINVK
	SEB_2	VTAQELDYLTR
	SEB_3	TNDINSHQTDK
	SEB_4	SIDQFLYFDLIYSIK
	SEB_5	NLLSFDVQTNK
	SEB_6	LGNYDNVR
Staphylococcal Enterotoxin C	SEC_1	FLAHDLIYNISDK
	SEC_2	YLYDDHYVSATK
	SEC_3	HEGNHFDNGNLQNVLVR
Staphylococcal Enterotoxin D	SED_1	FNILIALLFFTSLVISPLNVK
	SED_2	LYNNDTLGGK
	SED_3	ANENIDSVK
Staphylococcal Enterotoxin E	SEE_1	TINSENLHIDLYLYTT
	SEE_2	GFFTGHPWYNDLLVDLGSK
	SEE_3	FGLYNSDSFGGK
Staphylococcal Enterotoxin G	SEG_1	LITVQVTIDNR
	SEG_2	TELENTELANNYK
	SEG_3	LYEFDGSAFESGYIK
Staphylococcal Enterotoxin H	SEH_1	NVDIYGASFYYK
	SEH_2	FATADLAQK
	SEH_3	DYSFDIYDLK
Staphylococcal Enterotoxin I	SEI_1	SFSYDLFYTGDGLPVSFLK
	SEI_2	FYSGFNNGK
	SEI_3	VLFHLNNEK
	SEI_4	YLQEEYNIYGHNNTGK
	SEI_5	LVTAQEIDVK
Staphylococcal Enterotoxin M	SEM_1	YIYGGVTLAGDYLEK
	SEM_2	FSSGFNAGK
	SEM_3	YLQEEYNIYGFNDTNK
Staphylococcal Enterotoxin N	SEN_1	VTVQELDTK
	SEN_2	DGVQQEGFVIK
	SEN_3	VEFNSSDLANQFK
	SEN_4	ISTDQLLNNTIILK
Staphylococcal Enterotoxin O	SEO_1	NIGVAVYK
	SEO_2	TIDSSDYHIDVYLFT
	SEO_3	GNLPDQYLQIYNDNK
	SEO_4	VEFSSSAISK
	SEO_5	SIVSTTEK
Toxic Shock Syndrome Toxin 1	TSST-1_1	HQLTQIHGLYR
	TSST-1_2	LPTPIELPLK
	TSST-1_3	SQHTSEGTYIHFQISGVTNTEK
	TSST-1_4	FEYNTEKPPINIDEIK

10. Fragment Ion Calculator (http://db.systemsbiology.net/proteomicsToolkit/FragIonServlet.html)



Theoretical ions masses and charges for a peptide according to its sequence. As the C-terminal residue is isotopically marked, the peptide weight is adjusted (+8 for lysine, +10 for arginine and +7 for threonine). All three possible charged states are searched (+1, +2 and +3)

Theoretical masses of parent ions

Mass/Charge Table

	Mass	
	Mono	**Avg**
(M)	1317.67217	1318.45397
$(M+H)^{+}$	1318.68000	1319.46191
$(M+2H)^{2+}$	659.84393	660.23494
$(M+3H)^{3+}$	440.23191	440.49262
$(M+4H)^{4+}$	330.42590	330.62147

* C-terminus modification: 10.0

Theoretical masses of daughter ions

Sequence: **VTAQELDYLTR**, pI: **4.37019**

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+1)
V	1	100.07628	1318.68000	11
T	2	201.12396	1219.61158	10
A	3	272.16108	1118.56391	9
Q	4	400.21965	1047.52679	8
E	5	529.26225	919.46821	7
L	6	642.34631	790.42562	6
D	7	757.37325	677.34156	5
Y	8	920.43658	562.31462	4
L	9	1033.52064	399.25129	3
T	10	1134.56832	286.16722	2
R	11	1300.66943	185.11955	1

Fragment Ion Table, monoisotopic masses

Seq	#	B	Y	# (+2)
V	1	50.54208	659.84393	11
T	2	101.06592	610.30973	10
A	3	136.58447	559.78589	9
Q	4	200.61376	524.26733	8
E	5	265.13506	460.23804	7
L	6	321.67709	395.71675	6
D	7	379.19056	339.17471	5
Y	8	460.72223	281.66124	4
L	9	517.26426	200.12958	3
T	10	567.78810	143.58755	2
R	11	650.83865	93.06371	1

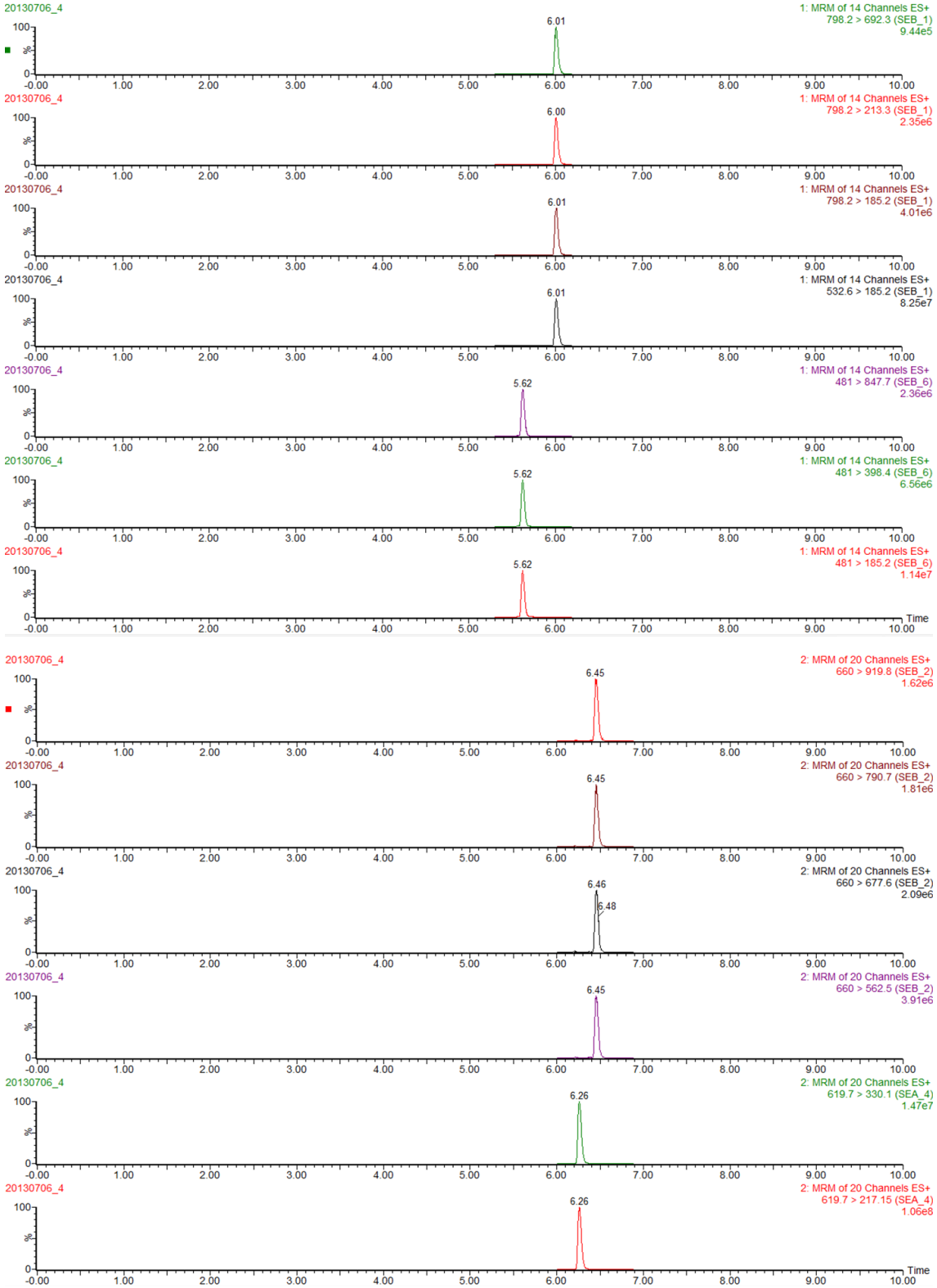
Fragment Ion Table, monoisotopic masses

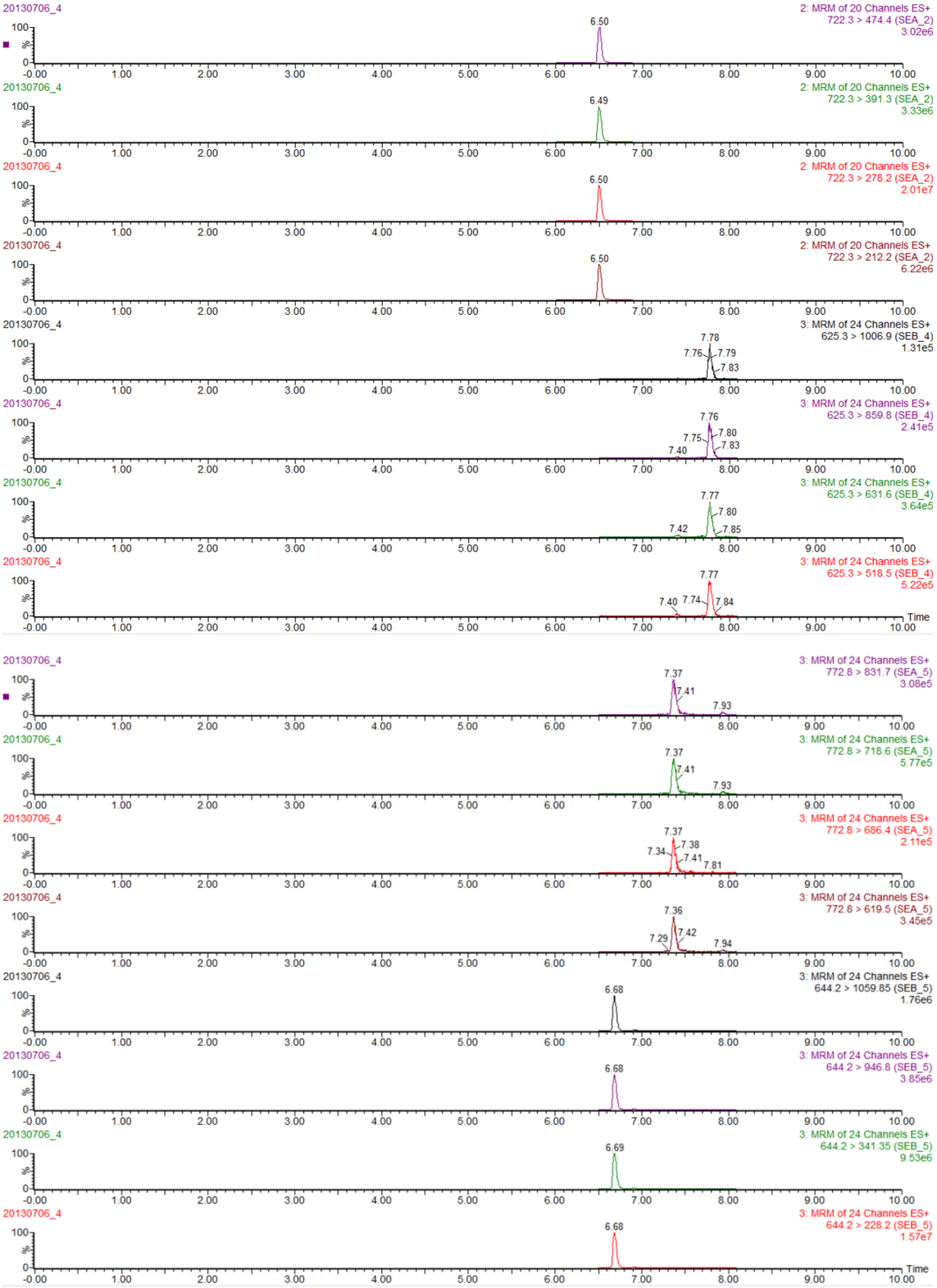
Seq	#	B	Y	# (+3)
V	1	34.03067	440.23191	11
T	2	67.71323	407.20911	10
A	3	91.39227	373.52655	9
Q	4	134.07846	349.84751	8
E	5	177.09266	307.16132	7
L	6	214.78735	264.14712	6
D	7	253.12966	226.45243	5
Y	8	307.48411	188.11012	4
L	9	345.17879	133.75568	3
T	10	378.86135	96.06099	2
R	11	434.22839	62.37843	1

11. Endogenous transitions calculation

Internal Standard	Sequence	Internal Standard Transition		Extra weight on IS	Parent charge	Daughter charge	Δ= (mass IS daughter)-(mass endog daughter) = Extra weight on IS/parent charge	Δ= (mass IS parent)-(mass endog parent) = Extra weight on IS/daughter charge	Endogenous peptide Transition		Cone Voltage (V)	Collision Energy (eV)
		Parent (m/z)	Daughter (m/z)						Parent (m/z)	Daughter (m/z)		
SEA_1	VPINLWLDGK	582,20	212,20	8	2	1	4	8	578,20	204,20	30	20
		582,20	532,60	8	2	2	4	4	578,20	528,60	30	20
		582,20	626,60	8	2	1	4	8	578,20	618,60	30	20
		582,20	966,80	8	2	1	4	8	578,20	958,80	30	20
SEA_2	YNLYNSDVFDGK	722,30	278,20	8	2	B-ion	4	B-ion	718,30	278,20	30	27
		722,30	391,30	8	2	B-ion	4	B-ion	718,30	391,30	30	27
		722,30	474,40	8	2	1	4	8	718,30	466,40	30	27
		722,30	212,20	8	2	1	4	8	718,30	204,20	30	27
SEA_3	NVTVQELDLQAR	698,70	214,20	10	2	B-ion	5	B-ion	693,70	214,20	35	27
		698,70	983,00	10	2	1	5	10	693,70	973,00	35	27
		698,70	612,50	10	2	1	5	10	693,70	602,50	35	27
		698,70	1081,60	10	2	1	5	10	693,70	1071,60	35	27
SEA_4	SELQGTALGNLK	619,70	330,10	8	2	B-ion	4	B-ion	615,70	330,10	30	22
		619,70	217,15	8	2	B-ion	4	B-ion	615,70	217,15	30	22
SEA_5	GFFTDHSWYNDLLVDFDSK	772,80	619,50	8	3	1	2,67	8	770,13	611,50	30	20
		772,80	686,40	8	3	B-ion	2,67	B-ion	770,13	686,40	30	20
		772,80	718,60	8	3	1	2,67	8	770,13	710,60	30	20
		772,80	831,70	8	3	1	2,67	8	770,13	823,70	30	20
SEB_1	VLYDDNHVSAINVK	798,20	185,20	8	2	2	4	4	794,20	181,20	30	31
		798,20	213,30	8	2	B-ion	4	B-ion	794,20	213,30	30	31
		798,20	692,30	8	2	2	4	4	794,20	688,30	30	31
		532,60	185,20	8	3	2	2,67	4	529,93	181,20	30	16
SEB_2	VTAQELDYLTR	660,00	677,60	10	2	1	5	10	655,00	667,60	30	20
		660,00	790,70	10	2	1	5	10	655,00	780,70	30	20
		660,00	919,80	10	2	1	5	10	655,00	909,80	30	25
		660,00	562,50	10	2	1	5	10	655,00	552,50	30	30
SEB_3	TNDINSHQTDK	641,00	724,00	8	2	1	4	8	637,00	716,00	30	30
		641,00	837,50	8	2	1	4	8	637,00	829,50	30	30
		641,00	950,50	8	2	1	4	8	637,00	942,50	30	30
		641,00	1066,00	8	2	1	4	8	637,00	1058,00	30	30
SEB_4	SIDQFLYFDLIYSIK	625,30	518,50	8	3	1	2,67	8	622,63	510,50	30	15
		625,30	859,80	8	3	1	2,67	8	622,63	851,80	30	15
		625,30	1006,90	8	3	1	2,67	8	622,63	998,90	30	15
		625,30	631,60	8	3	1	2,67	8	622,63	623,60	30	15
SEB_5	NLLSFDVQTNK	644,20	1059,85	8	2	1	4	8	640,20	1051,85	30	20
		644,20	946,80	8	2	1	4	8	640,20	938,80	30	20
		644,20	341,35	8	2	B-ion	4	B-ion	640,20	341,35	30	20
		644,20	228,20	8	2	B-ion	4	B-ion	640,20	228,20	30	20
SEB_6	LGNYDNVR	481,00	185,20	10	2	1	5	10	476,00	175,20	30	25
		481,00	398,40	10	2	1	5	10	476,00	388,40	30	21
		481,00	847,70	10	2	1	5	10	476,00	837,70	30	21

12. Pure internal standards peaks (injection of pure mix of IS at 1 pmol/µl)




20130706_4
2: MRM of 20 Channels ES+
722.3 > 474.4 (SEA_2)
3.02e6
6.50
20130706_4
2: MRM of 20 Channels ES+
722.3 > 391.3 (SEA_2)
3.33e6
6.49
20130706_4
2: MRM of 20 Channels ES+
722.3 > 278.2 (SEA_2)
2.01e7
6.50
20130706_4
2: MRM of 20 Channels ES+
722.3 > 212.2 (SEA_2)
6.22e6
6.50
20130706_4
3: MRM of 24 Channels ES+
625.3 > 1006.9 (SEB_4)
1.31e5
7.78
7.76
7.79
7.83
20130706_4
3: MRM of 24 Channels ES+
625.3 > 859.8 (SEB_4)
2.41e5
7.76
7.75
7.80
7.83
7.40
20130706_4
3: MRM of 24 Channels ES+
625.3 > 631.6 (SEB_4)
3.64e5
7.77
7.80
7.42
7.85
20130706_4
3: MRM of 24 Channels ES+
625.3 > 518.5 (SEB_4)
5.22e5
7.77
7.40
7.74
7.84
Time
20130706_4
3: MRM of 24 Channels ES+
772.8 > 831.7 (SEA_5)
3.08e5
7.37
7.41
7.93
20130706_4
3: MRM of 24 Channels ES+
772.8 > 718.6 (SEA_5)
5.77e5
7.37
7.41
7.93
20130706_4
3: MRM of 24 Channels ES+
772.8 > 686.4 (SEA_5)
2.11e5
7.37
7.38
7.34
7.41
7.81
20130706_4
3: MRM of 24 Channels ES+
772.8 > 619.5 (SEA_5)
3.45e5
7.36
7.29
7.42
7.94
20130706_4
3: MRM of 24 Channels ES+
644.2 > 1059.85 (SEB_5)
1.76e6
6.68
20130706_4
3: MRM of 24 Channels ES+
644.2 > 946.8 (SEB_5)
3.85e6
6.68
20130706_4
3: MRM of 24 Channels ES+
644.2 > 341.35 (SEB_5)
9.53e6
6.69
20130706_4
3: MRM of 24 Channels ES+
644.2 > 228.2 (SEB_5)
1.57e7
6.68
Time

13. UPLC-MS/MS technical reports

MS Method Report

MS Method Report - MassLynx 4.1 SCN 843 Page 1 of 2

File: c:\masslynx_project\201304-settect.pro\acqudb\20130706 settect sea1-5 seb1-6 endog.exp

Printed: Thursday, July 18, 2013 13:21:41 Romance Daylight Time

Creation Time	Wed 10 Jul 2013 09:45:05
Instrument Identifier	XEVO-TQS#WAA080
Version Number	1.0
Duration (min)	2.8
Calibration Filename	C:\MassLynx\IntelliStart\Results\Unit Mass Resolution\Calibratio
n_20130607_3.cal	
Initial States	
Stop flow	No Change
Switch 2	No Change
Switch 3	No Change
Switch 4	No Change
Infusion	No Change
Flow state	LC
Flow rate	5 µl/min
Reservoir	No Action
Refill	No Action
API Probe Delay Temp	20 °C
Run events	Yes
Number Of Functions	3

Function 1 : MRM of 14 mass pairs, Time 5.30 to 6.20, ES+

Type	MRM
Ion Mode	ES+
Inter Channel Delay (sec)	-1.000
InterScan Time (sec)	-1.000
Span (Da)	0.0
Start Time (min)	5.3
End Time (min)	6.2

Ch	Prnt(Da)	Dau(Da)	Dwell(s)	Cone(V)	Coll(eV)	Delay(s)	Compound
1	476.00	175.20	0.006	30.00	25.00	-1.000	SEB_6 E
2	476.00	388.40	0.006	30.00	21.00	-1.000	SEB_6 E
3	476.00	837.70	0.006	30.00	21.00	-1.000	SEB_6 E
4	481.00	185.20	0.006	30.00	25.00	-1.000	SEB_6
5	481.00	398.40	0.006	30.00	21.00	-1.000	SEB_6
6	481.00	847.70	0.006	30.00	21.00	-1.000	SEB_6
7	529.90	181.20	0.006	30.00	16.00	-1.000	SEB_1 E
8	532.60	185.20	0.006	30.00	16.00	-1.000	SEB_1
9	794.20	181.20	0.006	30.00	31.00	-1.000	SEB_1 E
10	794.20	213.30	0.006	30.00	31.00	-1.000	SEB_1 E
11	794.20	688.30	0.006	30.00	31.00	-1.000	SEB_1 E
12	798.20	185.20	0.006	30.00	31.00	-1.000	SEB_1
13	798.20	213.30	0.006	30.00	31.00	-1.000	SEB_1
14	798.20	692.30	0.006	30.00	31.00	-1.000	SEB_1

Function 2 : MRM of 20 mass pairs, Time 6.00 to 6.90, ES+

Type	MRM
Ion Mode	ES+
Inter Channel Delay (sec)	-1.000
InterScan Time (sec)	-1.000
Span (Da)	0.0
Start Time (min)	6.0

End Time (min) 6.9

Ch	Prnt(Da)	Dau(Da)	Dwell(s)	Cone(V)	Coll(eV)	Delay(s)	Compound
1	615.70	217.15	0.004	30.00	22.00	-1.000	SEA_4 E
2	615.70	330.10	0.004	30.00	22.00	-1.000	SEA_4 E
3	619.70	217.15	0.004	30.00	22.00	-1.000	SEA_4
4	619.70	330.10	0.004	30.00	22.00	-1.000	SEA_4
5	655.00	552.50	0.004	30.00	30.00	-1.000	SEB_2 E
6	655.00	667.60	0.004	30.00	20.00	-1.000	SEB_2 E
7	655.00	780.70	0.004	30.00	20.00	-1.000	SEB_2 E
8	655.00	909.80	0.004	30.00	25.00	-1.000	SEB_2 E
9	660.00	562.50	0.004	30.00	30.00	-1.000	SEB_2
10	660.00	677.60	0.004	35.00	20.00	-1.000	SEB_2
11	660.00	790.70	0.004	30.00	20.00	-1.000	SEB_2
12	660.00	919.80	0.004	30.00	25.00	-1.000	SEB_2
13	718.30	204.20	0.004	30.00	27.00	-1.000	SEA_2 E
14	718.30	278.20	0.004	30.00	27.00	-1.000	SEA_2 E
15	718.30	391.30	0.004	30.00	27.00	-1.000	SEA_2 E
16	718.30	466.10	0.004	30.00	27.00	-1.000	SEA_2 E
17	722.30	212.20	0.004	30.00	27.00	-1.000	SEA_2
18	722.30	278.20	0.004	30.00	27.00	-1.000	SEA_2
19	722.30	391.30	0.004	30.00	27.00	-1.000	SEA_2
20	722.30	474.40	0.004	30.00	27.00	-1.000	SEA_2

Function 3 : MRM of 24 mass pairs, Time 6.50 to 8.10, ES+

Type	MRM
Ion Mode	ES+
Inter Channel Delay (sec)	-1.000
InterScan Time (sec)	-1.000
Span (Da)	0.0
Start Time (min)	6.5
End Time (min)	8.1

Ch	Prnt(Da)	Dau(Da)	Dwell(s)	Cone(V)	Coll(eV)	Delay(s)	Compound
1	622.63	510.50	0.004	30.00	15.00	-1.000	SEB_4 E
2	622.63	623.60	0.004	30.00	15.00	-1.000	SEB_4 E
3	622.63	851.80	0.004	30.00	15.00	-1.000	SEB_4 E
4	622.63	998.90	0.004	30.00	15.00	-1.000	SEB_4 E
5	625.30	518.50	0.004	30.00	15.00	-1.000	SEB_4
6	625.30	631.60	0.004	30.00	15.00	-1.000	SEB_4
7	625.30	859.80	0.004	30.00	15.00	-1.000	SEB_4
8	625.30	1006.90	0.004	30.00	15.00	-1.000	SEB_4
9	640.20	228.20	0.004	30.00	20.00	-1.000	SEB_5 E
10	640.20	341.35	0.004	30.00	20.00	-1.000	SEB_5 E
11	640.20	938.80	0.004	30.00	20.00	-1.000	SEB_5 E
12	640.20	1051.85	0.004	30.00	20.00	-1.000	SEB_5 E
13	644.20	228.20	0.004	30.00	20.00	-1.000	SEB_5
14	644.20	341.35	0.004	30.00	20.00	-1.000	SEB_5
15	644.20	946.80	0.004	30.00	20.00	-1.000	SEB_5
16	644.20	1059.85	0.004	30.00	20.00	-1.000	SEB_5
17	770.20	611.50	0.004	30.00	20.00	-1.000	SEA_5 E
18	770.20	686.40	0.004	30.00	20.00	-1.000	SEA_5 E
19	770.20	710.60	0.004	30.00	20.00	-1.000	SEA_5 E
20	770.20	823.70	0.004	30.00	20.00	-1.000	SEA_5 E
21	772.80	619.50	0.004	30.00	20.00	-1.000	SEA_5
22	772.80	686.40	0.004	30.00	20.00	-1.000	SEA_5
23	772.80	718.60	0.004	30.00	20.00	-1.000	SEA_5
24	772.80	831.70	0.004	30.00	20.00	-1.000	SEA_5

Inltet Method report

Method File: C:\MassLynx_Project\201304-SETTECT.PRO\Acqudb\20120123_SETTECT_Xevo2
Last Modified: Monday, April 22, 2013 16:23:24 Romance Daylight Time

Printed: Thursday, July 18, 2013 13:22:07 Romance Daylight Time

Waters Gl Pump Method

1 ----------
loading pump
Solvent A Name: Acetonitrile + 0.1% AF
Solvent B Name: Water + 0.1% AF
Solvent C Name:
Solvent D Name:
Low Pressure Limit: 0 psi
High Pressure Limit: 15000 psi
Seal Wash Period: 5.00 min
[Gradient Table]
Time(min) Flow Rate(mL/min) %A %B %C %D Curve
1. Initial 1.000 0.0 100.0 0.0 0.0 Initial
2. 2.00 1.000 0.0 100.0 0.0 0.0 6
3. 2.50 0.200 0.0 100.0 0.0 0.0 6
4. 7.00 0.200 100.0 0.0 0.0 0.0 6
5. 7.50 1.000 100.0 0.0 0.0 0.0 6
6. 8.50 1.000 0.0 100.0 0.0 0.0 1
7. 11.00 1.000 0.0 100.0 0.0 0.0 1
Comment:
Flow Ramp Rate: 0.45 min
D Solvent Selection (if supported): No Change
System Pressure Data Channel: No
Flow Rate Data Channel: No
%A Data Channel: No
%B Data Channel: No
%C Data Channel: No
%D Data Channel: No
Primary Data Channel: No
Accumulator Data Channel: No
Degasser Data Channel: No
Gradient Start: At Injection
Gradient Start Volume: 0 uL
Gradient Start Time: 0.00 min
Participate in pre-analysis: No
2 ----------
elution pump
Run Time: 11.00 min
Comment:
Solvent Selection A: A1
Solvent Selection B: B1
Low Pressure Limit: 0 psi
High Pressure Limit: 15000 psi
Solvent Name A: Water
Solvent Name B: Acetonitrile
Switch 1: No Change
Switch 2: No Change
Switch 3: No Change
Seal Wash: 5.0 min
Chart Out 1: System Pressure
Chart Out 2: %B
System Pressure Data Channel: No
Flow Rate Data Channel: No
%A Data Channel: No
%B Data Channel: No
Primary A Pressure Data Channel: No
Accumulator A Pressure Data Channel: No
Primary B Pressure Data Channel: No
Accumulator B Pressure Data Channel: No
Degasser Pressure Data Channel: No
[Gradient Table]
Time(min) Flow Rate %A %B Curve

Method File: C:\MassLynx_Project\201304-SETTECT.PRO\Acqudb\20120123_SETTECT_Xevo2
Last Modified: Monday, April 22, 2013 16:23:24 Romance Daylight Time

Printed: Thursday, July 18, 2013 13:22:07 Romance Daylight Time

1. Initial 0.200 0.0 100.0 Initial
2. 2.00 0.200 0.0 100.0 6
3. 7.00 0.200 50.0 50.0 6
4. 7.50 0.200 100.0 0.0 6
5. 8.50 0.200 100.0 0.0 6
6. 9.00 0.200 0.0 100.0 6
7. 11.00 0.200 0.0 100.0 1

Run Events: Yes
[Event Table]
Run Time(min) Event Action Parameter
1. 2.00 Switch 2 Off 0.00
2. 2.00 Switch 3 On 0.00
3. 7.50 Switch 2 On 0.00
4. 7.50 Switch 3 Off 0.00

Gradient Start (Relative to Injection): 0 uL
Participate in pre-analysis: No

Waters Acquity Autosampler Method

Run Time: 10.00 min
Comment:
Load Ahead: Disabled
Injection Mode: Full Loop
LoopOffline: Disable
Weak Wash Solvent Name: Water/Acetonitrile
Weak Wash Volume: 600 uL
Strong Wash Solvent Name: Acetonitrile
Strong Wash Volume: 600 uL
Target Column Temperature: 40.0 C
Column Temperature Alarm Band: 5.0 C
Target Sample Temperature: 10.0 C
Sample Temperature Alarm Band: 5 C
Full Loop Overfill Factor: Automatic
Syringe Draw Rate: Automatic
Needle Placement: Automatic
Pre-Aspirate Air Gap: Automatic
Post-Aspirate Air Gap: Automatic
Column Temperature Data Channel: No
Ambient Temperature Data Channel: No
Sample Temperature Data Channel: No
Sample Organizer Temperature Data Channel: No
Sample Pressure Data Channel: No
Switch 1: No Change
Switch 2: No Change
Switch 3: No Change
Switch 4: No Change
Chart Out: Sample Pressure
Sample Temp Alarm: Enabled
Column Temp Alarm: Enabled
Run Events: Yes
Needle Overfill Flush: Automatic
NoInjection: false
End Of Report

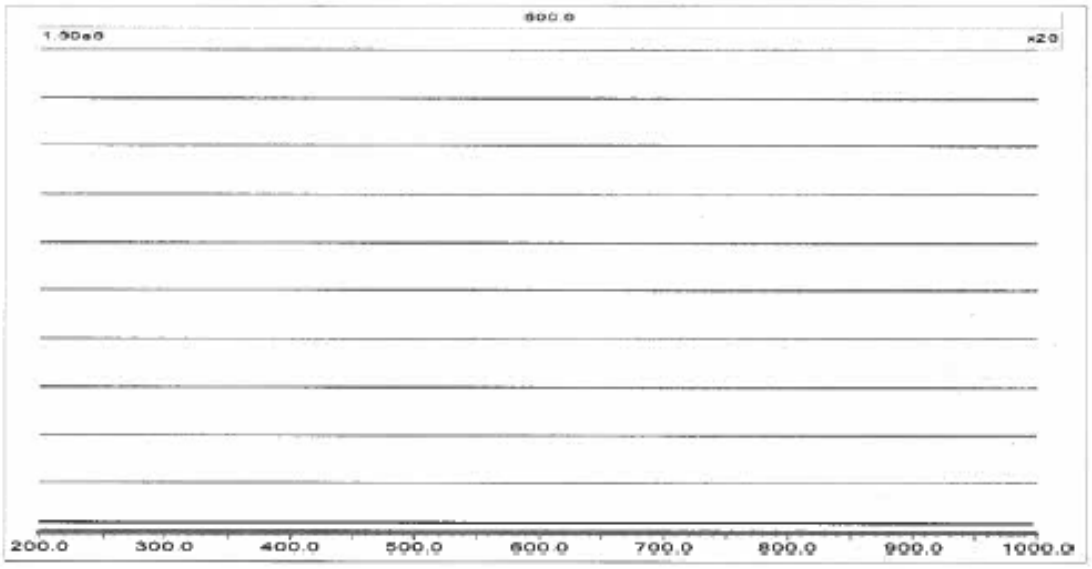
MS Detector Tune Parameters

Waters Xevo TQ-S MS Detector Tune Parameters - MassLynx 4.1 SCN 843 Page 1 of 2

File: C:\MassLynx_Project\201304-SETTECT.PRO\ACQUDB\20130424 SETTECT_Xevo2.ipr

Instrument: XEVO-TQS#WAA080

Printed: Thursday, July 18, 2013 13:23:43 Romance Daylight Time



Type	**Start Mass**	**End Mass**	**Set Mass**
MS2 Scan	200.00	1000.00	

Source (ES+)	**Settings**	**Readbacks**
Capillary (kV)	3.00	0.00
Cone (V)	50.00	-1.84
Source Offset (V)	60.0	
Source Temperature (°C)	150	150
Desolvation Temperature (°C)	550	57
Cone Gas Flow (L/Hr)	OFF	
Desolvation Gas Flow (L/Hr)	OFF	
Collision Gas Flow (mL/Min)	0.17	0.00
Nebuliser Gas Flow (Bar)	7.00	0.00

Analyser	**Settings**	**Readbacks**
LM 1 Resolution	2.8	
HM 1 Resolution	15.0	
Ion Energy 1	0.5	
MS Mode Collision Energy	4.00	
MSMS Mode Collision Energy	28.00	
MS Mode Entrance	1.00	
MS Mode Exit	1.00	
Gas On MS Mode Entrance	1.00	
Gas On MS Mode Exit	1.00	
Gas On MSMS Mode Entrance	1.00	
Gas On MSMS Mode Exit	1.00	
Gas Off MS Mode Entrance	30.00	
Gas Off MS Mode Exit	30.00	
Gas Off MSMS Mode Entrance	30.00	
Gas Off MSMS Mode Exit	30.00	
ScanWave MS Mode Entrance	1.00	
ScanWave MS Mode Exit	1.00	
ScanWave MSMS Mode Entrance	1.00	
ScanWave MSMS Mode Exit	1.00	

File: C:\MassLynx_Project\201304-SETTECT.PRO\ACQUDB\20130424 SETTECT_Xevo2.ipr

Instrument: XEVO-TQS#WAA080

Printed: Thursday, July 18, 2013 13:23:43 Romance Daylight Time

LM 2 Resolution	2.7
HM 2 Resolution	15.0
Ion Energy 2	0.6
Gain	1.00
Multiplier	0.24
Active Reservoir	Wash

Pressure Gauges

Collision Cell Pressure (mbar)	7.638852e-005

Instrument Configuration

Automatic Mode

MS Inter-scan delay (secs)	0.003	
Polarity/Mode switch Inter-scan delay (secs)		0.020
Enhanced Inter-scan delay (secs)		0.020

Inter-channel delay - See Tables

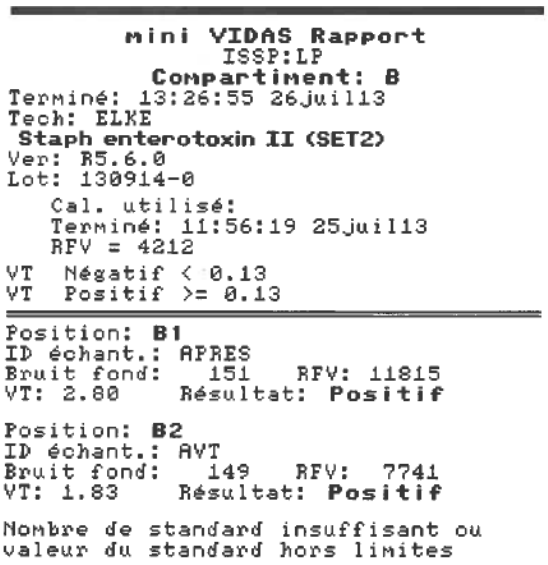
MS 1 Delay Table:

R	delay
<=-1800.000	0.006
<=-1500.000	0.005
<=-1000.000	0.004
<=1000.000	0.003
<=1500.000	0.004
<=1800.000	0.005
> 1800.000	0.006

RF 2 Delay Table:

R	delay
<=-150.000	0.006
<= -75.000	0.005
<= -10.000	0.004
<= 10.000	0.003
<= 20.000	0.004
<= 40.000	0.005
<= 70.000	0.006
<= 100.000	0.007
<= 150.000	0.008
<= 200.000	0.009
<= 250.000	0.010
<= 275.000	0.011
> 275.000	0.012

14. VIDAS SET2 report on the extraction efficiency



mini VIDAS Rapport
ISSP:LP
Compartiment: B
Terminé: 13:26:55 26juil13
Tech: ELKE
Staph enterotoxin II (SET2)
Ver: R5.6.0
Lot: 130914-0
Cal. utilisé:
Terminé: 11:56:19 25juil13
RFV = 4212
VT Négatif < 0.13
VT Positif >= 0.13

Position: B1
ID échant.: APRES
Bruit fond: 151 RFV: 11815
VT: 2.80 Résultat: Positif

Position: B2
ID échant.: AVT
Bruit fond: 149 RFV: 7741
VT: 1.83 Résultat: Positif

Nombre de standard insuffisant ou
valeur du standard hors limites

15. Illustration of the digestion yields calculation

Peptide transition	Sample n°	NaCl/Tris-CaCl2						
		Response (Area endo/Area IS)	Endo pept		Toxins		Statistics	
		[-]	concentration (mol/l)	amount in vial (mol)	amount in vial (g)	digestion yield (%)	digestion yield average (%)	digestion yield standard deviation (%)
SEA_4 E T1	1	3.06E-03	2.14E-09	1.07E-12	2.90E-08	2.9	3.0	0.1
	2	3.23E-03	2.26E-09	1.13E-12	3.06E-08	3.1		
	3	3.16E-03	2.21E-09	1.10E-12	2.99E-08	3.0		
SEA_4 E T2	1	1.14E-03	7.99E-10	4.00E-13	1.08E-08	1.1	1.2	0.1
	2	1.27E-03	8.87E-10	4.44E-13	1.20E-08	1.2		
	3	1.27E-03	8.92E-10	4.46E-13	1.21E-08	1.2		
SEA_5 E T1	1	2.82E-04	1.98E-10	9.88E-14	2.68E-09	0.3	0.2	0.1
	2	2.39E-04	1.67E-10	8.36E-14	2.26E-09	0.2		
	3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0		
SEA_5 E T2	1	4.66E-04	3.26E-10	1.63E-13	4.41E-09	0.4	0.1	0.3
	2	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0		
	3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0		
SEA_5 E T3	1	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0	1.3	1.5
	2	9.44E-04	6.61E-10	3.31E-13	8.95E-09	0.9		
	3	3.08E-03	2.16E-09	1.08E-12	2.92E-08	2.9		
SEA_5 E T4	1	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0	0.0	0.0
	2	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0		
	3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0		
SEB_4 E T1	1	1.13E-01	7.88E-08	3.94E-11	1.12E-06	111.6	163.4	101.9
	2	9.85E-02	6.90E-08	3.45E-11	9.77E-07	97.7		
	3	2.83E-01	1.98E-07	9.91E-11	2.81E-06	280.8		
SEB_4 E T2	1	2.62E-01	1.84E-07	9.18E-11	2.60E-06	260.1	318.3	61.5
	2	3.15E-01	2.21E-07	1.10E-10	3.12E-06	312.3		
	3	3.86E-01	2.70E-07	1.35E-10	3.83E-06	382.7		
SEB_4 E T3	1	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0	0.0	0.0
	2	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0		
	3	0.00E+00	0.00E+00	0.00E+00	0.00E+00	0.0		
SEB_4 E T4	1	2.26E-01	1.58E-07	7.91E-11	2.24E-06	224.0	257.5	55.6
	2	2.29E-01	1.60E-07	8.01E-11	2.27E-06	226.8		
	3	3.25E-01	2.27E-07	1.14E-10	3.22E-06	321.7		

IS supposed concentration (mol/l)	IS Average purity (%)	IS average concentration (mol/l)
1.00E-06	70%	7.00E-07

Final volume (l)	SEA (g/mol)	SEB (g/mol)	Amount of toxins spiked (g)
5.00E-04	27091	28327	1.00E-06

16. Summary of the estimated digestion yields (unrounded values)

Peptides transitions	Pure digestion buffer						Digestion buffer + aqueous solvent					
	HCO3-		Tris-CaCl2		Tris		NaCl/HCO3-		NaCl/Tris-CaCl2		NaCl/Tris	
	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)	Average (%)	Standard deviation (%)
SEB_2 E T1	131.4	32.8	180.8	36.4	139.0	37.6	8.1	1.8	78.6	15.7	34.8	3.1
SEB_2 E T2	131.6	32.9	183.1	40.8	147.0	41.6	8.7	3.7	79.1	17.0	32.1	4.7
SEB_2 E T3	139.4	34.1	187.7	40.5	148.2	41.4	8.0	1.9	82.3	17.2	36.2	3.7
SEB_2 E T4	135.2	29.8	180.8	32.9	148.5	41.1	8.6	1.6	83.8	16.8	35.0	5.4
SEA_2 E T1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SEA_2 E T2	0.0	0.0	0.9	1.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SEA_2 E T3	0.1	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0
SEA_2 E T4	1.9	1.3	2.1	2.0	1.7	1.0	0.0	0.1	0.4	0.2	0.0	0.0
SEB_5 E T1	14.1	7.4	78.6	24.1	4.9	1.5	0.5	0.4	40.7	14.7	2.3	0.5
SEB_5 E T2	12.9	7.7	83.3	23.1	5.1	2.2	0.4	0.3	43.2	11.9	2.8	0.5
SEB_5 E T3	17.2	8.8	115.6	29.4	7.1	2.9	1.3	0.3	48.5	12.7	4.4	1.3
SEB_5 E T4	16.7	8.7	115.8	28.8	7.2	2.7	1.1	0.0	48.2	12.1	4.1	0.8
SEB_1 E T1	74.4	15.5	61.0	20.6	85.6	34.2	10.0	3.2	47.0	13.8	20.5	4.1
SEB_1 E T2	79.8	14.7	93.8	21.8	89.9	26.3	11.2	3.4	49.6	5.2	25.2	3.3
SEB_1 E T3	0.4	0.1	0.4	0.1	0.6	0.2	0.0	0.0	0.4	0.1	0.1	0.1
SEB_1 E T4	0.2	0.0	0.0	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SEA_4 E T1	3.5	0.6	4.0	0.9	3.7	0.8	3.1	0.1	3.0	0.1	2.9	0.5
SEA_4 E T2	1.7	0.4	2.4	0.9	1.8	0.5	1.0	0.0	1.2	0.1	1.1	0.1
SEA_5 E T1	0.7	0.6	3.3	3.6	0.0	0.0	0.0	0.0	0.2	0.1	0.1	0.2
SEA_5 E T2	0.1	0.2	1.8	1.7	0.4	0.4	0.0	0.0	0.1	0.3	0.0	0.0
SEA_5 E T3	3.5	6.0	3.4	1.4	5.3	4.6	2.0	0.6	1.3	1.5	0.7	0.9
SEA_5 E T4	1.3	2.0	2.0	1.6	0.4	0.4	0.0	0.0	0.0	0.0	0.0	0.0
SEB_4 E T1	77.8	28.9	528.0	39.5	39.0	19.7	0.0	0.0	163.4	101.9	7.3	6.3
SEB_4 E T2	137.2	58.2	599.2	80.0	65.5	13.6	2.0	3.5	318.3	61.5	6.0	2.2
SEB_4 E T3	0.0	0.0	704.1	163.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
SEB_4 E T4	131.7	65.0	485.1	75.0	71.4	10.1	2.2	3.0	257.5	55.6	6.9	2.2

17. Illustration of the recoveries calculation

SEB_2 E T1										
		Response	Endo pept		Toxins		Concentration	Recovery	Stat	
Spiking step	Sample n°	[-]	concentration (mol/l)	amount in vial (mol)	amount in vial (g)	amount in vial (ng)	ppb (ng/g)	(%)	Average recovery (%)	Standard deviation of recovery (%)
before extraction	1	2.46E-02	1.72E-08	8.61E-12	2.44E-07	2.44E+02	244	12.2	11.7	2.0
	2	1.91E-02	1.34E-08	6.69E-12	1.89E-07	1.89E+02	189	9.5		
	3	2.70E-02	1.89E-08	9.46E-12	2.68E-07	2.68E+02	268	13.4		
before syringe filter	1	2.11E-02	1.47E-08	7.37E-12	2.09E-07	2.09E+02	209	10.4	13.4	3.9
	2	3.59E-02	2.51E-08	1.26E-11	3.56E-07	3.56E+02	356	17.8		
	3	2.43E-02	1.70E-08	8.49E-12	2.41E-07	2.41E+02	241	12.0		
before 50 kDa filter	1	2.70E-02	1.89E-08	9.43E-12	2.67E-07	2.67E+02	267	13.4	11.0	2.1
	2	1.98E-02	1.39E-08	6.94E-12	1.97E-07	1.97E+02	197	9.8		
	3	1.96E-02	1.37E-08	6.85E-12	1.94E-07	1.94E+02	194	9.7		
before 5 kDa filter	1	1.15E-01	8.04E-08	4.02E-11	1.14E-06	1.14E+03	1138	56.9	55.4	2.3
	2	1.14E-01	7.98E-08	3.99E-11	1.13E-06	1.13E+03	1130	56.5		
	3	1.06E-01	7.45E-08	3.73E-11	1.06E-06	1.06E+03	1056	52.8		
before digestion	1	1.36E-01	9.52E-08	4.76E-11	1.35E-06	1.35E+03	1348	67.4	54.7	11.2
	2	1.02E-01	7.13E-08	3.56E-11	1.01E-06	1.01E+03	1009	50.5		
	3	9.32E-02	6.52E-08	3.26E-11	9.24E-07	9.24E+02	924	46.2		

IS supposed concentration (mol/l)	IS Average purity (%)	IS average concentration (mol/l)
1.00E-06	70%	7.00E-07

Final volume (l)	SEA (g/mol)	SEB (g/mol)	Amount of toxins spiked (g)	Meat weight (g)
5.00E-04	27091	28327	1.00E-06	1

18. Comparison of digestion yields in presence or absence of meat (rough data)

Peptide transition	Estimated digestion yield without matrix (%)	Estimated digestion yield in matrix (%)
SEB_2 E T1	78.6 ± 15.7	54.7 ± 11.2
SEB_2 E T2	79.1 ± 17.0	63.6 ± 4.8
SEB_2 E T3	82.3 ± 17.2	58.9 ± 3.9
SEB_2 E T4	83.8 ± 16.8	65.4 ± 1.8
SEB_5 E T1	40.7 ± 14.7	26.1 ± 3.8
SEB_5 E T2	43.2 ± 11.9	29.6 ± 2.4
SEB_5 E T3	48.5 ± 12.7	41.2 ± 2.5
SEB_5 E T4	48.2 ± 12.1	35.7 ± 2.6
SEB_1 E T1	47.0 ± 13.8	5.6 ± 0.4
SEB_1 E T2	49.6 ± 5.2	39.4 ± 1.3

19. Validation plan

Linearity + Recoveries

Two series of samples have to be prepared and injected twice.

First range°: 0 – 125 ng – 250 ng – 500 ng – 750 ng – 1000 ng per g, but lower if possible!!

N°1: spiking of toxins in the matrix (1g meat) at several concentrations, before the extraction.

N°2: spiking of toxins without the matrix at several concentrations, before the extraction.

Mandel's fitting test°: x= concentrations; y= ratio ($^{area_{endogene}}/_{area_{isotope}}$); one file for each transition of each peptide and selection of the best transitions and best range.

Recovery°: ratio between recalculated amount and amount spiked in series n°1.

($^{amount_{measured}}/_{amount_{spiked}}$)*100

Matrix effect

Two series of samples have to be prepared.

N°3: spiking of toxins in matrix at the intermediate concentration of the selected linearity range, in triplicate.

N°4: spiking of toxins in water at the intermediate concentration of the selected linearity range, in triplicate. On those samples°: application of the same protocol as for matrix, from beginning but without meat (NaCl+toxins+CH_2Cl_2+centri+filters+Tris-buffer+trypsin+IS)

Repeatability

3X3 samples are prepared and cover the linearity range. Then RSD are calculated for each level.

N°5, (6, 7)°: spiking of toxins in the matrix (1g meat) at the **first, (second, third)** level of concentration of the selected range, before the extraction.

Intermediate precision

Repetition of series N°5-6-7 on 2 extra days and calculate RSD for ratios, on each concentration level, through the 9 repetitions

LOD

Spike toxins with concentration under linearity range, inject, and diminish the level until $^{S}/_{N}$ = 3. Once the concentration is found, make 10 repetitions of the corresponding level

LOQ

LOQ ~ LOD X 3.66 ~ $^{S}/_{N}$ = 10