

METHOD OF DETECTION AND QUANTIFICATION OF STAPHYLOCOCCAL ENTEROTOXIN TYPE "A" IN ALL TYPES OF FOOD MATRICES BY THE ELISA METHOD AFTER EXTRACTION AND DIALYSIS CONCENTRATION

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1. PURPOSE AND SCOPE

From all types of food matrices, proceed to the extraction, concentration by dialysis, then quantification of staphylococcal enterotoxins type A (SEA) by double sandwich ELISA. This method was developed as part of the UMT TERESA Action 3 in charge of development and transfer of methods enabling rapid extraction and detection of the various toxins produced by *Staphylococcus aureus* and evaluation of toxin production conditions in products.

Prior to implementation of the SEA quantitative ELISA detection, it is recommended to perform a combined detection of enterotoxins (SEA to SEE) using the European Screening Method of the EURL for CPS including *Staphylococcus aureus* applicable to the detection of SEs in all types of food matrices.

2. REFERENCES

- Commission Regulation (EC) No. 1441/2007 of 5 December 2007 amending Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, Official Journal of the European Union L 332, 7/12/2007, pp 12-29.
- European Screening Method of the EURL for CPS including *Staphylococcus aureus* applicable to the detection of SEs in all types of food matrices including milk and milk products.
- A. Gateaux, S. Herbin and JA. Hennekinne. «Développement de tests ELISA quantitatifs des entérotoxines staphylococciques de types SEA, SEB, SEC, SED, SEE et SEH. Travaux UMT TERESA Action 3. Rapports d'avancement octobre 2008 – juillet 2009 / juillet 2010».
- Pace, CN, Vajdos, F, Fee, L, Grimsley, G, Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. Protein Sci. 4(11):2411-2423.

3. ABBREVIATIONS

A.U.: Absorbance Unit

BSA: Bovine Serum Albumin

DC: Dialysis-Concentration

ELISA: Enzyme Linked ImmunoSorbent Assay

IC: Internal control

LoD: Limit of Detection

LoQ: Limit of Quantification

MSDS: Material Safety data Sheet

SE: Staphylococcal enterotoxin

UMT TERESA Action 3: Joint Technological Unit – Transfer for Risk Approval in Food Safety – For quality control of food with respect to the risk associated with staphylococcal enterotoxins. Priority study of the contamination of primary production and its derived products, milk, cheese and milk powders.

4. PRINCIPLE

- Extraction of staphylococcal enterotoxins;
- Concentration of extract by dialysis;
- Treatment of the extract with rabbit immunoglobulins (IgG);
- Treatment using raw meat kit if needed
- Detection of staphylococcal enterotoxins type SEA by double sandwich ELISA method.

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5. REAGENTS AND PRODUCTS

- 5.1 Rabbit immunoglobulins G [1 mg/mL] distributed by Bethyl Laboratories Inc., Montgomery, USA (reference P120-101)
- 5.2 Transia Additive Raw meat extraction (only in case of raw/cooked meat and seafood products) distributed by BioControl Systems Inc., Bellevue, USA (reference AK 0220).
- 5.3 Anti-SEA capture antibodies: reference SLAI101 (Toxin Technology Inc., Curtiss Ave, Sarasota, FL, USA)
- 5.4 Secondary anti-SEA antibodies: reference LAI101 (Toxin Technology Inc., Curtiss Ave, Sarasota, FL, USA)
- 5.5 Staphylococcal enterotoxin type SEA: reference AT101 (Toxin Technology Inc., Curtiss Ave, Sarasota, FL, USA)
- 5.6 In-house positive control (IC+): extraction and dialysis-concentration of a milk product matrix spiked with a known value, or uncontaminated and supplemented by SEA toxin after treatment with IgG
- 5.7 In-house negative control (IC-): extraction and dialysis-concentration of a milk product matrix uncontaminated by SE
- 5.8 Goat anti-rabbit IgG labelled with peroxidase (GAR-POX) (KPL, reference 074-1516)
- 5.9 ABTS [2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonate)] detection solution (e.g.: ABTS Microwell Peroxide Substrate System, KPL, ref: 50-62-01)
- 5.10 PBS, PBS-Tween, PMT, PGT and PBS/BSA/Azide solutions

Note: For Sections 5.3 to 5.4, refer to Annex II. For Section 5.5, refer to Annex III. For Sections 5.6 and 5.7 refer to Annex IV. For Section 5.10, refer to Annex I.

6. EQUIPMENT AND MATERIALS

Standard laboratory equipment, including:

- 6.1 Analytical balance
- 6.2 Vortex
- 6.3 Microplates shaker-incubator
- 6.4 Thermostatic chamber ($5^{\circ}\text{C} \pm 3^{\circ}\text{C}$; $\leq -18^{\circ}\text{C}$)
- 6.5 Microplate reader with wavelength range from 405 nm to 620 or 630 nm
- 6.6 Micropipettes
- 6.7 Laboratory glassware or polypropylenware
- 6.8 Rotary tube shaker
- 6.9 96-flat-bottom well microtitration plates with high binding capability (Nunc 467320 MediSorp™ plates for ELISA)
- 6.10 Microplate adhesives

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7. PROCEDURE

7.1. SE EXTRACTION AND CONCENTRATION BY DIALYSIS AND EXTRACT RECOVERY

For extraction of staphylococcal enterotoxins type SEA by dialysis-concentration and extract recovery, refer to method Anses Maisons-Alfort CAT-BAC 06 or to its European equivalent (European Screening Method of the EURL for CPS including *Staphylococcus aureus*) for the detection of staphylococcal enterotoxins types SEA to SEE in all types of food matrices.

7.2. PROCEDURE FOR DETECTION AND QUANTIFICATION BY ELISA METHOD

7.2.1. Implementation of the method

Before any implementation of the method, it is absolutely necessary to characterize the batches of toxins and antibodies that will be used.

Indeed:

For the toxins, the real concentration does not always correspond to the value announced by the purchaser. It is recommended to check and to assign a value to the purchased stock solution using:

- Mass spectrometry method (e.g.: Amino Acid Analysis method).
- Measure the absorbance of the solution using the spectrophotometer at a wavelength of $\lambda = 280$ nm (Annex III)

For the antibodies, the concentration can change from one batch to another.

Therefore, the Food safety laboratory of Maisons-Alfort has tested different batches of toxins (according to the procedure described in Annex V) and has adjusted the concentration of the different batches of antibodies to use.

The batch of toxin in the following table has been tested and is therefore ready to use after reconstitution of the powder toxins according to the manufacturer procedure.

Type of toxin	Batch number	Concentration defined by AAA method
SEA	120794A	1.10 mg/mL

The batch numbers of the antibodies and their concentrations are reported in Annex V.

7.2.2. Preparation of microtitration plates

Stock solutions of anti-SEA antibodies are prepared according to annex II.

- Coat the wells of a plate (6.9) with 50 μ L of a solution of capture antibody (5.3) specific to the enterotoxin SEA to implement detection (See Annex V).
- Incubate the coated plate with the adhesive (6.10) for 12 to 48 hours at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$.
- Wash the plate twice with phosphate buffered saline (PBS) solution (5.10). The plate can then be stored in PBS for up to 48 hours at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$, if necessary.

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- Saturate the plate wells with 200 µL of PMT (5.10) and incubate for 45 minutes at 37°C while shaking in a shaker-incubator (shaking at 900 rpm for Labsystems iEMS (6.3)).
- Wash the plate twice with PBS-Tween (5.10) solution. At this stage, the plate can be stored in PBS for up to 72 hours at 5°C ± 3°C, if necessary.

7.2.3. Treatment of the extract and in-house controls by rabbit immunoglobulins (5.1)

Before beginning the detection method it is necessary to treat the concentrated extracts and the positive and negative in-house controls* by adding rabbit immunoglobulins G (5.1).

- Take 1 mL of the concentrated extract to be tested and treat it by adding 2 µL of rabbit serum (immunoglobulins G);
- Homogenise (6.2);
- Incubate for one hour at room temperature while shaking (6.8).

In case of raw/cooked meat or seafood products, concentrated extracts have to be treated using the Transia raw meat extraction kit (5.2):

Defreeze one aliquot of solution 1 and 2 included in the kit:

- Add 20 µL of the solution 1 to 1 mL of extract
- Homogenise.
- Incubate for 10 min at room temperature (18-25 °C), under agitation (6.8).
- Add 20 µL of the solution 2.
- Homogenise.
- Incubate for 10 min at room temperature (18-25 °C), under agitation (6.8).

* For preparation of the in-house controls, refer to Annex IV.

7.2.4. Preparation of an enterotoxins standards range

Stock solution of SEA is prepared according to Annex III. (See Section 9.1 on precautions for use of toxins).

This stock solution has then to be diluted in order to obtain the defined concentration: 0.55 µg/ml.

Therefore, dilute 4 µL of the stock solution of SEA in 2 mL of PBS-Gelatine-Tween (PGT) (5.10). From this solution, make serial ½ dilutions in PGT (four tubes: 1 mL of spiked solution + 1 mL of PGT) to obtain the concentrations from 0.0688 to 1.1 ng/mL .

7.2.5. IC deposits, extracts to be assayed, and series of standards

- Deposit 200 µL of PGT (5.10) in the wells used as reagent blanks and in the first wells of the serie of standards corresponding to the blanks in the serie.
- Deposit 200 µL per well of the serial dilutions of SEA standards (see Annex V). Repeat the procedure on another column of the plate.
- Deposit in duplicate (2 wells per dilution) the IC+ (5.6), the IC- (5.7) and the treated extracts to be assayed, i.e. for each well:
 - o concentrated extract: 200 µL of treated extract;
 - o extract diluted to one-half: 100 µL of treated extract + 100 µL of PGT;
 - o extract diluted to one-quarter: 50 µL of treated extract + 150 µL of PGT;
 - o or extracts at any other appropriate dilution.
- Incubate the plate for 45 minutes at 37°C while shaking in a shaker-incubator (shaking at 900 rpm for Labsystems iEMS (6.3)).

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Example of plate diagram for detection of SEA toxin in six extracts (Ext 1 to Ext 6):

	1	2	3	4	5	6	7	8	9	10	11	12
A	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks
B	Reagent blanks	Reagent blank range	Reagent blank range	IC+ pure	IC- pure	Ext1 pure	Ext2 pure	Ext3 pure	Ext4 pure	Ext5 pure	Ext6 pure	Reagent blanks
C	Reagent blanks	R 0.0688	R 0.0688	IC+ pure	IC- pure	Ext1 pure	Ext2 pure	Ext3 pure	Ext4 pure	Ext5 pure	Ext 6 pure	Reagent blanks
D	Reagent blanks	A 0.1375	A 0.1375	IC+ 1/2	IC- 1/2	Ext1 1/2	Ext2 1/2	Ext3 1/2	Ext4 1/2	Ext5 1/2	Ext6 1/2	Reagent blanks
E	Reagent blanks	N 0.275	N 0.275	IC+ 1/2	IC- 1/2	Ext1 1/2	Ext2 1/2	Ext3 1/2	Ext4 1/2	Ext5 1/2	Ext6 1/2	Reagent blanks
F	Reagent blanks	G 0.55	G 0.55	IC+ 1/4	IC- 1/4	Ext1 1/4	Ext2 1/4	Ext4 1/4	Ext4 1/4	Ext5 1/4	Ext6 1/4	Reagent blanks
G	Reagent blanks	E 1.1	E 1.1	IC+ 1/4	IC- 1/4	Ext1 1/4	Ext2 1/4	Ext 4 1/4	Ext 4 1/4	Ext5 1/4	Ext6 1/4	Reagent blanks
H	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks	Reagent blanks

7.2.6. Double sandwich for SEA

- 7.2.6.1 Dump the liquid out of the wells. Tap the plate onto a clean paper to remove all liquid from the well with PBS-Tween (5.11). Repeat this procedure 4 times.
- 7.2.6.2 Deposit 50 µL per well of secondary antibody solution (5.4) specific to SEA (See Annex IV).
- 7.2.6.3 Incubate the plate for 45 minutes at 37°C while shaking in a shaker-incubator (shaking at 900 rpm for Labsystems iEMS (6.3)).
- 7.2.6.4 Wash the plate as described in 7.2.6.1.
- 7.2.6.5 Deposit 50 µL per well of GAR-POX solution (5.8) diluted in PGT (5.10).
- 7.2.6.6 Incubate as described in 7.2.6.3

7.2.7. Detection and reading

- 7.2.7.1 Dump the liquid out of the wells. Tap the plate onto a clean paper to remove all liquid from the well with PBS (5.10). Repeat this procedure 5 times.
- 7.2.7.2 Deposit 100 µL of ABTS detection solution (5.9) (solution A + solution B, volume to volume) (See Section 9.2 on precautions for use).
- 7.2.7.3 Incubate the plate at 37°C while shaking in a shaker-incubator (shaking at 900 rpm for Labsystems iEMS (6.3)).
- 7.2.7.4 Read the plate using the microplate reader (6.5), in dual wavelengths (405/620 or 630 nm: λ of absorbance of ABTS/ λ of absorbance of the microtitration plate).

Measure the absorbance regularly until the highest point in the range reaches an absorbance of approximately 1.0.

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8. CALCULATIONS AND REPORTING OF RESULTS

8.1. PLOTTING THE CALIBRATION CURVE: ABSORBANCE = F ([SEA])

The calibration curve is constructed from the raw absorbance values (AU) obtained for the range of standards tested in duplicate, including reagent blanks in the range (See the example plate diagram in columns B2 and B3).

- Calculate the mean of the two absorbance values at each point of the calibration range and reagent blanks in the standard range;
- From the values obtained, perform a linear regression **on at least four values**. Set an intercept value for the linear regression corresponding to the mean of the two reagent blanks in the SEA standards range.

The formula for the curve obtained is: $y = ax + b$

where: y : mean of the two absorbance values (in A.U.)
 a : slope of the curve (in mL/ng)
 b : intercept (in AU)
 x : concentration of SEA (in ng/mL)

Thus, the enterotoxin concentration is expressed as: $x = \frac{(y-b)}{a}$

The correlation coefficient (R^2) of this curve must be **at least 0.95**. **If this condition is not met, the test is invalid and must be repeated.**

Note: if one of the two absorbance values is considered as aberrant, only one value can be used for performing the linear regression (remove the duplicate value considered to be aberrant).

Record the values **a** (slope of the curve) and **b** (intercept) obtained from the equation for the curve, for each standard range.

8.2. CALCULATING THE LOD AND LOQ

The LoD and LoQ are calculated from 20 raw absorbance values of reagent blanks: predefine the blanks used in the calculation, including the reagent blanks of the range series.

- Calculate the mean (M_{abs}), the standard deviation (S_{abs}) and the coefficient of variation (CV^*) of the 20 absorbance values of the reagent blanks chosen.

$$*CV (\%) = S_{abs} / M_{abs} \times 100$$

The CV must be less than or equal to 15% to obtain a compliant test. Otherwise, it is possible to remove up to three aberrant reagent blank values. **If the condition is still not met, the test is invalid and must be repeated.**

- Calculate the LoD and LoQ values:

$$LoD (\text{in AU}) = M_{abs} + 3 \times S_{abs} \times 0.15$$

$$LoQ (\text{in AU}) = M_{abs} + 10 \times S_{abs} \times 0.15$$

For conversion to ng/mL, the values a and b noted above are required:

$$LoD (\text{ng/ml}) = \frac{(LoD (AU) - b)}{a} \quad LoQ (\text{ng/ml}) = \frac{(LoQ (AU) - b)}{a}$$

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8.3. ANALYSIS OF RESULTS

8.3.1. Interpretation of results

The interpretation of results depends on the absorbance values obtained at each concentration of the extracts deposited: y_{pure} , $y_{1/2}$, $y_{1/4}$ (y representing the mean of the two absorbance values obtained for each concentration).

- If $y_{\text{pure}} < LoD$: SE are not detected;
- If a dilution effect is observed between each concentration, i.e., $y_{\text{pure}} > y_{1/2} > y_{1/4}$, and if:
 - $LoD \leq y < LoQ$: SE are detected but not quantifiable;
 - $y \geq LoQ$: SE are detected and quantifiable (see below).
- If no dilution effect is observed and if $y_{\text{pure}} \geq LoD$: the results cannot be interpreted.

8.3.2. Determination of the concentration of detected and quantified SEA

SEA quantification is calculated from absorbance values obtained for each concentration deposited (y) and greater than the LoQ . Use only for calculations the absorbance values that fall within the range of the linear regression selected.

The amount of toxin in this sample is then calculated as follows:

$$[toxin](ng/ml) = \frac{(y - b) \times df}{a}$$

To obtain the equivalent of this concentration in ng/g:

$$[toxin](ng/g) = \frac{[toxin](ng/ml) \times V}{t.s..}$$

- where:
- df : dilution factor
 - a : slope of the curve
 - b : intercept
 - V : final volume of extract (in mL, normally 5 mL)
 - $t.s.$: test sample (in g, normally 25 g)

The whole **test will be validated** if it appears that the negative control (IC-) was not detected and the positive control (IC+) was detected, quantifiable and at the level of supplementation values. **If these conditions are not met, the test is invalid and must be repeated.**

9. DECONTAMINATION/DISPOSAL OF MATERIAL, STORAGE CONDITIONS, AND DISPOSAL OF SAMPLES

9.1. HANDLING PRECAUTIONS WITH STAPHYLOCOCCAL ENTEROTOXINS

Staphylococcal enterotoxins are hazardous to humans by oral absorption and by inhalation. Thus, all necessary precautions for dealing with hazardous materials must be taken when handling these toxins, particularly the wearing of gloves: refer to the material safety data sheets [MSDS].

9.2. HANDLING PRECAUTIONS WITH REAGENTS [E.G.: ABTS]

Refer to the Material Safety data Sheet (MSDS) of the reagents used.

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Annex I: Preparation of solutions

- **Phosphate Buffered Saline (PBS)** – Store up to one month at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$

E.g.: For 10 litres of PBS:

10 mM disodium hydrogen phosphate dodecahydrate: 35.8 g Na_2HPO_4 , 12 H_2O

145 mM sodium chloride: 90 g NaCl *q.s.p.* 10 litres with H_2O RO

Mix and adjust to $\text{pH } 7.3 \pm 0.2$ with HCl and/or NaOH.

- **PBS, Tween 20 (PBS Tween)** – Store up to one month at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$

E.g.: For 1 litre of PBS Tween:

PBS: 1 litre of PBS

Tween 20 0.1%: 1 mL of Tween 20

Mix.

- **PBS, Gelatine, Tween 20 (PGT)** – Prepare immediately before use

E.g.: For 200 mL of PGT (quantity for four plates):

Gelatine 0.2%: 0.4 g of gelatine

Tween 20 0.1%: 200 μL of Tween 20 *q.s.p.* 200 mL with PBS

Melt the gelatine in a water bath in a small amount of PBS and make up to 200 mL with cold PBS, and only then add the Tween 20; mix.

- **PBS, Milk, Tween 20 (PMT)** – Prepare immediately before use

E.g.: For 100 mL of PMT (quantity for four plates):

Powdered milk 1%: 1 g powdered milk

Tween 20 0.1%: 100 μL of Tween 20 *q.s.p.* 100 mL with PBS

Dissolve the powdered milk in a small amount of PBS, then make up to 100 mL, add the Tween 20 and mix.

- **PBS/BSA/Azide (SEs storage solution)** – Store up to three months at $5^{\circ}\text{C} \pm 3^{\circ}\text{C}$

E.g.: For 100 mL of PBS/BSA/Azide:

BSA 2 mg/mL: 200 mg of BSA

Sodium azide 0.02%: 0.02 g of NaN_3 *q.s.p.* 100 mL with PBS

Mix. For this preparation, **follow the precautions** for handling NaN_3 (refer to the MSDS).

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Annex II: Preparation of anti-SEA antibody solution

- Purpose and principle

Prepare solutions of anti-SEA antibody from lyophilisate (refer to the manufacturer's instructions).

The quality of the antibody may vary from batch to batch. It is necessary to test and perhaps optimise the concentration to be used in order to obtain quantification results that are similar to those obtained with previously used and tested batches. Please refer to instructions for use dispatched with this method to perform reagent controls.

- Reagents

PBS buffer

- Preparation of stock solutions of antibodies

Rehydrate the lyophilisate from 1 mg of capture antibody (i.e., SLAI 101) in 2 mL of PBS (C = 500 µg/mL).

Rehydrate the lyophilisate from 1 mg of secondary antibody (i.e., LAI 101) in 1 mL of PBS (C = 1 mg/mL).

Pipette up and down using a micropipette to fully resuspend and collect all the lyophilisate at the bottom and sides of the vial.

Wait a few minutes for the powder to dissolve, then stir the mixture.

- Preparation of aliquots of antibody solution

Once the concentration to be used has been determined: prepare aliquots of stock solution in a sufficient volume to perform no more than two operations, in order to avoid repeated freezing/thawing that is damaging to the antibodies.

- Storage

Store the anti staphylococcal enterotoxin antibodies lyophilisates at 5°C ± 3°C and the solutions obtained after rehydration at a temperature less than or equal to -18°C.

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Annex III: Preparation of SE standards solutions

- Purpose and principle

Prepare SEA solution from lyophilisate (refer to the manufacturer's instructions).
The quality of the toxin may vary from batch to batch; test and optionally optimise the concentrations to be used in order to obtain quantification results similar to those obtained with previously used and tested batches.
Please refer to instructions for use dispatched with this method to perform reagent controls.

- Equipment and reagents

- Spectrophotometer (e.g.: Lightwave);
- Quartz microcuvettes;
- Eppendorf tubes (0.65 mL);
- Vortex;
- Freezer ($\leq 18^{\circ}\text{C}$);
- Osmosed water or of equivalent purity;
- Lyophilised SEA (Toxin Technology Inc., Curtiss Ave, Sarasota, FL, USA);
- PBS buffer;
- PBS/BSA/Azide buffer.

- Preparation of the SEA stock solution

For this preparation, rigorously implement the precautionary measures described in the method (Section 9.1).
Rehydrate the SEA lyophilisate (1 mg) with reversed osmosis water (1 mL). Pipette up and down several times in order to resuspend and collect all the lyophilisate at the bottom and on the sides of the vial.
Wait a few minutes for the powder to dissolve and then stir the mixture.

- Calculation of the concentration of SEA stock solution

This calculation and the testing that follow are to be performed only if the batch have never been used.

- Transfer 500 μL of rehydrated lyophilisate into a quartz microcuvette;
- Measure the absorbance of the solution using the spectrophotometer at a wavelength of $\lambda = 280 \text{ nm}$.

The enterotoxin stock solution concentration is calculated with the BEER-LAMBERT equation:

$$C = \frac{A \times d}{\epsilon' \times l}$$

Where :

- A =absorbance (A.U.)
- ϵ' = extinction coefficient ($\text{L}\cdot\text{g}^{-1}\cdot\text{cm}^{-1}$)
- l = length of the container (1 cm)
- C = concentration ($\text{g}\cdot\text{L}^{-1}$)
- d = dilution factor

Extinction coefficient (Pace *et al.*, 1995) for SEA: ϵ' (280 nm) = $1.40 \text{ L}\cdot\text{g}^{-1}\cdot\text{cm}^{-1}$

Comments:

SEA solutions at concentrations less than 100 $\mu\text{g}/\text{mL}$ (dilution of stock solution, for example) must be made in a protein carrier to prevent their degradation during freezing (e.g.: containing Bovine Serum Albumin (BSA)). The dilutions are typically made in a solution of PBS, BSA 2 mg/mL, sodium azide 0.02% (See Annex I). Caution: the absorbance measurements taken at that point no longer reflect the toxin concentration.

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In addition to measuring absorbance value, it is necessary to test the toxin concentration by concurrently performing a series of the new and old batch on the same plate. To do this, make pre-dilutions of the stock solution (5.5 µg/mL for SEA prepared in a volume of PBS/BSA/Azide solution of between 100 and 1000 µL), to facilitate the preparation of the diluted stock solution (0.55 µg/mL for SEA prepared in a volume of 100 µL of PBS/BSA/Azide solution).

- Preparation of aliquots of SEA solution

Once the concentration to be used has been determined: prepare aliquots of diluted SEA stock solution to obtain the standard ranges and supplement the positive internal control, and a sufficient volume for performing no more than two operations, to avoid repeated freezing/thawing that is damaging to the enterotoxins.

- Storage

Store the SEA lyophilisate at 5°C ± 3°C and the standard solutions (stock or diluted) obtained after rehydration at a temperature less than or equal to -18°C.

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Annex IV: Preparation of positive and negative in-house controls

- Purpose and principles

Obtain positive and negative in-house controls that can be used to validate the tests performed to detect and quantify SEA.

- Equipment and reagents

Refer to Sections 5 and 6 of this method.

- Preparation, storage and use of in-house controls

Perform extraction by dialysis concentration from uncontaminated milk product matrix.
After recovering the extract, aliquot in fractions of 1 mL and store at -18°C.

Note: During the first preparation of the matrix uncontaminated with SEs, verify that the sample is actually negative for each type of toxin using this method.

During implementation of the quantitative ELISA test:

- Thaw one aliquot for each control;
- Treat with rabbit IgG, in the same manner as for extracts to be tested;
- For the positive control, supplement the treated extract by adding SEA in order to obtain 0.275 ng/mL;
- Deposit the positive and negative controls on the plate according to this method.

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Annex V: Procedure for the detection/quantification of SEA by quantitative ELISA (example)

COATING (Capture antibody) Concentrations for SEA	Preparation of antibodies for one plate from stock solutions (See Annex II)	Dilute in PBS Distribute 50 µL/well
SLAI101 0.5 µg/mL (batch 120700AI) 0.25 µg/mL (batch 70609AI)	5 µL/5 mL (batch 120700AI) 2.5 µL/5 mL (batch 70609AI)	Incubation 12 to 48 h at 5 ± 3°C or 2 hours at 37°C

Wash twice with PBS (The plate can be stored in PBS up to 48 hours at 5°C ± 3°C)

SATURATION	200 µL/well of PMT Incubation 45 min at 37°C
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Wash twice with PBS Tween (The plate can be stored in PBS up to 72 hours at 5°C ± 3°C)

Toxin for standard range, internal positive and negative controls		
Concentration of stock solution diluted aliquots	Dilute 4 µL of SEA stock solution diluted in 2 mL of PGT. Then, perform serial dilution at ½ in PGT. Concentration to deposit on the wells:	Deposit 200 µL/well Incubation 45min at 37°C
SEA: 0.55 µg/mL	SEA : de 0.06875 à 1.10 ng/mL	

Wash four times with PBS Tween

SECONDARY SEA ANTIBODY Concentration for use	Preparation of Antibody for one plate from stock solution (See Annex II)	Dilute in PGT Distribute 50 µL/well
LAI101 1 µg/mL (batches 42408AI and 41408AI)	5 µL in 5 mL	Incubation 45 min at 37°C

Wash four times with PBS Tween

CONJUGATE GAR-POX (Goat Anti Rabbit IgG Peroxidase) Batches 060506 and 080184	Dilute to 1:15,000 in PGT the stock solution at 1 mg/mL (e.g.: 4 µL (of the stock solution ½-diluted) in 30 mL for four plates) Distribute 50 µL/well Incubation 45 min at 37°C
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Wash five times with PBS

ABTS SUBSTRATE	Mix equal volumes solution A + solution B (e.g.: 6 mL + 6 mL for one plate)	100 µL/well Incubation at 37°C
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READING (when the absorbance high point of the standard range ≈ 1.0 AU)	λ = 405 nm (reference filter 630 nm)
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