Characterisation of the staphylococcal enterotoxin research method in a dairy product

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The staphylococcal enterotoxin research method is a quantitative method, involving a sample preparation and an immunoenzymatic detection. In order to estimate its performance characteristics, a method characterisation is carried out with a single dairy product and for staphylococcal enterotoxin A. Statistical analysis shows that the method is linear over a contamination interval between 0 – 1 ng SEA per gram of cheese. The limit of detection is about 0.01 ng SEA per gram of cheese and the limit of quantification is about 0.04 ng SEA per gram of cheese. The intra laboratory repeatability and intermediate precision are estimated on two levels of contamination. The repeatability coefficient of variation is included between 3.1 % and 6.2 %, the intermediate precision coefficient of variation is included between 4.7 % and 11.0 %.

Introduction

Staphylococcal food poisoning is the second bacterial foodborne illness in France after *Salmonella* and before *Clostridium perfringens* [1-3]. Enterotoxins are proteins, whose molecular weight is between 26000 and 29000. Foods involved are usually rich in proteins, such as dairy products, meat products and egg mixtures. The staphylococcal enterotoxin A (SEA) is responsible for 75 % of staphylococcal food poisonings. Nowadays, staphylococcal enterotoxin research is concentrated on dairy products, contaminated by *S. aureus* at a level more than 10^4 cfu.g [4]. Products contaminated by staphylococcal enterotoxins are withdrawn from the market.

There is no reference method and no accreditation program on the staphylococcal enterotoxin topic. Due to the lack of a validated method, the AFSSA method is considered as the "reference method" by AFNOR (Association Française de Normalisation) and DGAL (Direction Générale de l'Alimentation) and by other laboratories.

The staphylococcal enterotoxin research method consists of two steps, sample preparation and immunoenzymatic detection. In order to characterize and to determine the method performance criteria, we have carried out a statistical intra laboratory evaluation.

A full method evaluation includes method characterisation and comparison with a reference method. Due to lack of reference method in staphylococcal enterotoxin research, we have only studied the method characterisation. Three criteria considered as compulsory (linearity, sensitivity and intra laboratory repeatability) have been estimated in addition to the intra laboratory intermediate precision, the limit of detection and the limit of quantification.

General guidelines of this method evaluation are described in the *Guide d'évaluation et de validation d'une méthode d'analyse* [5].

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Material and methods

Material

Distilled water.

PBS (Phosphate Buffered Saline): sodium phosphate 10 mM (Merck), sodium chloride 0.145 M (Merck), pH = 7.4.

PBS Tween: PBS with 0.1 % Tween 20.

Sodium hydroxide 5N or 1N (Merck).

Hydrochloric acid 5N or 1N (Merck).

Polyethylene glycol 20000 (Merck).

Dialysis tubings: Spectra/Por® molecular porous regenerated cellulose membranes, MWCO 6000-8000 (Poly-Labo).

Staphylococcal enterotoxin A (SEA) produced and purified at AFSSA Paris and used for artificial contamination of samples: solution at 96 ng SEA per ml PBS with 2 mg BSA (Bovine Serum Albumin) per ml and 0.02 % sodium azide.

Experimental sample: soft cheese from supermarket.

Methods

Sample preparation

12.5 g of cheese are mixed with 25 ml of distilled water at $\theta \leq 40$ °C, using Ultra Turrax. The slurry is either artificially contaminated by SEA solution or not. The slurry is adjusted to pH ≤ 4 with HCl then centrifuged at 12500 g, at 4 °C for 15 min. The pH of the aqueous supernatant is adjusted to 7.3 ± 0.3 with NaOH, centrifuged and filtered on glass wool. The filtered supernatant is concentrated by dialysis against PEG solution (15 g of PEG plus 50 ml of distilled water), overnight, at 4 °C.

The concentrated extract is recovered and adjusted to 2.5 ml with PBS.

Detection of SEA

Detection of SEA in extracts is carried out by indirect double sandwich EIA (Enzyme ImmunoAssay) [6]. This detection method uses specific monoclonal antibodies as coating antibodies and polyspecific polyclonal antibodies as probing antibodies. The presence of enterotoxins is revealed by using goat anti-rabbit immunoglobulins coupled to horseradish peroxydase and determined by a colorimetric measure. In the same step, to quantify the SEA amount in samples, SEA solution at an increasing final concentration from 0.125 ng ml^{-1} to 1 ng ml^{-1} is incubated separately in order to obtain a SEA standard curve: DO = f [SEA(ng.ml^{-1})].

Results are determined by measuring absorbance at 405 nm using a microtiter plate reader. The amount of SEA (Y ng.ml^{-1}) in extracts is calculated from the SEA standard curve.

The absorbance reader is a MR 5000 model (Dynatech).

Method characterisation

Linearity

Experiments are carried out with 3 replicates (n = 3) of the 6 contamination levels (k = 6) – 0, 0.2, 0.4, 0.6, 0.8 and 1 ng SEA per gram of cheese (X). Samples are contaminated by SEA solution before the extraction procedure.

Our purpose is to characterize the linearity of the entire method, that is to say sample preparation and immunoenzymatic detection.

Experiments on pure solutions are not carried out because this would only characterize the detection method.

From the 18 (N = kn = 18) data obtained, the following statistical parameters are calculated: the slope (b), the standard deviation of the slope (Sb), the intercept (a) and the standard deviation of the intercept (Sa), and the correlation coefficient (r).

To determine the linearity of the method, the following statistical tests are studied:

- Comparison of the intercept with zero by the Student test (t-test) (α-error = 5 %; df (degrees of freedom) = N-2)

$$t = \frac{|a|}{Sa} \quad (1)$$

  If $t < t$ (α; N-2), the intercept is not significantly different from zero.

- Homogeneity of intra group variances (S^2_j) determination by the Cochran test (C-test) (α-error = 5 %; k; n)

$$C = \frac{S^2_{max}}{\sum_{j=1}^{k} S^2_j} \quad (2)$$

  If $C < C(\alpha; k; n)$, intra group variances are homogeneous.

- Slope significance test by the Fisher test (F-test): Guidelines are given in table I.

  If $F_1 > F$ (α; 1; N-2), the linear regression slope is significative.

- Linear regression significance test by the Fisher test: Guidelines are given in table II.

  If $F_2 < F$ (α; k-2; N-k), the linear regression model is valid.

Intra laboratory repeatability and intermediate precision (study of recovery level's quality)

According to the *Guide d'évaluation et de validation d'une méthode d'analyse* [5], experiments must be carried out with 3 contamination levels. But, within the context of a preliminary study, our purpose is to estimate the intra laboratory

Table I. Slope significance test.

Variations	*Degrees of freedom (df)*	*Sum of squares*	*Variances*	*F calculated*
Total variance	*N*-1	$\sum T^2 = \sum_{j=1}^{k} \sum_{i=1}^{nj} \left(Y_{ij} - \overline{\overline{Y}}\right)^2$		
Regression variance	1	$\sum I^2 = b^2 \sum_{j=1}^{k} n_j \left(\overline{X}_j - \overline{\overline{X}}\right)^2$	$S^2_I = \sum I^2$	$F_1 = \frac{S^2_I}{S^2_R}$
Residual variance	*N*-2	$\sum R^2 = \sum T^2 - \sum I^2$	$S^2_R = \frac{\sum R^2}{N-2}$	

Table II. Linear regression significance test.

Variations	*df*	*Sum of squares*	*Variances*	*F calculated*
Experimental variation	*N*-k	$\sum E^2 = \sum_{j=1}^{k} \sum_{i=1}^{nj} \left(Y_{ij} - \overline{Y}_j\right)^2$	$S^2_E = \frac{\sum E^2}{N-k}$	
Regression variation	*k*-2	$\sum L^2 = \sum R^2 - \sum E^2$	$S^2_L = \frac{\sum L^2}{k-2}$	$F_2 = \frac{S^2_L}{S^2_E}$

repeatability and intermediate precision. Experiments are only carried out with 2 contamination levels.

To determine the quality criteria of the method, experiments are carried out with 6 replicates (n = 6) of 2 contamination levels (0.3 and 0.7 ng SEA per gram of cheese) for 3 days (k = 3). Samples are contaminated by SEA solution before the extraction procedure.

For the intermediate precision study, the only variation parameter is the day of the sample preparation and SEA detection. The response is SEA recovery level. The repeatability (r), the repeatability standard deviation (S_r), the repeatability coefficient of variation (CV_r), the intermediate precision (R), the intermediate precision standard deviation (S_R) and the intermediate precision coefficient of variation (CV_R) are estimated from guidelines given by NF ISO 5725-2 Norm (December 1994) [7].

To characterize the quality of the method, the following statistical tests are carried out:

- Homogeneity of intra group variances determination by the Cochran test (α-error = 5 % and 1 %; k; n).

  Guidelines are described in the paragraph "*Linearity*" (equation (2)).

- Determination of isolated and aberrant data by the Grubbs test.

  Statistical data are estimated from averages $\overline{Y}_j$ of each contamination level. The G-test allows to determine isolated or aberrant values among averages.

  Inferior datum

$$G_i = \frac{\left(\overline{\overline{Y}} - \overline{Y_{j\min}}\right)}{S} \quad (3)$$

  Superior datum

$$G_S = \frac{\left(\overline{Y_{j\max}} - \overline{\overline{Y}}\right)}{S} \quad (4)$$

  With

$$\overline{\overline{Y}} = \frac{\sum_{j=1}^{k} \overline{Y}_j}{k} \quad (5)$$

$$S = \sqrt{\frac{1}{k-1} \sum_{j=1}^{k} \left(\overline{Y}_j - \overline{\overline{Y}}\right)^2} \quad (6)$$

  If $G < G$ (α-error = 5 %; k), there is no isolated value. If $G < G$ (α-error = 1 %; k), there is no aberrant value.

- Determination of repeatability and intermediate precision criteria: guidelines are given in table III.

Limits of detection and quantification (method of blank matrix)

Experiments are carried out with 20 uncontaminated cheese samples which are extracted. For each blank extract, the absorbance (OD) at 405 nm is measured.

Table III. Determination of repeatability and intermediate precision parameters.

Number of groups	*k*	
Number of replicates per group	*n*	
Total number of experiments	*N*	*kn*
Sum of averages	$\sum_{j=1}^{k} \overline{Y_j}$	
Squares of averages' sum	$\sum_{j=1}^{k} \overline{Y_j^2}$	
Sum of variances	$\sum_{j=1}^{k} S_j^2$	
Repeatability variance	S_r^2	$\frac{\sum_{j=1}^{k} S_j^2}{k}$
Inter group variance	S_g^2	$\frac{\left(k\sum_{j=1}^{k}\overline{Y_j^2}\right)-\left(\sum_{j=1}^{k}\overline{Y_j}\right)^2}{k(k-1)}-\frac{S_r^2}{n}$
Intermediate precision variance	S_R^2	$S_r^2 + S_g^2$
General average	$\overline{\overline{Y}}$	$\frac{\sum_{j=1}^{k}\overline{Y_j}}{k}$
Repeatability	*r*	2.83Sr
Repeatability coefficient of variation	CVr	$\frac{S_r}{\overline{\overline{Y}}}*100$
Intermediate precision	R	2.83SR
Intermediate precision coefficient of variation	CVR	$\frac{S_R}{\overline{\overline{Y}}}*100$

The average OD, noted $\overline{OD}$, and the standard deviation S_{OD} are calculated from these 20 measures.

Limits of detection and quantification are estimated from these two data.

- Limit of detection (LOD)

$$LOD = \overline{OD} + 3S_{OD} \quad (7)$$

- Limit of quantification (LOQ)

$$LOQ = \overline{OD} + 10S_{OD} \quad (8)$$

Limits are estimated in the unity $ng.ml^{-1}$ from a SEA standard curve.

Determination of statistical data

Statistical data are generated using Excel software.

Results and discussion

Linearity

Figure 1 gives a visual plot of the experimental data and regression line.

From these data, the linear regression parameters are calculated and the t-test is carried out. The results are shown in table IV.

The intercept is not significantly different from zero with α-error = 5 %. The dairy extract does not interfere with SEA detection. The method sensitivity is determined from the linear regression slope.

- Homogeneity of intra group variances determination

Table V gives statistical results about homogeneity of variances.

Variances S^2_j are homogeneous with α-error = 5 %.

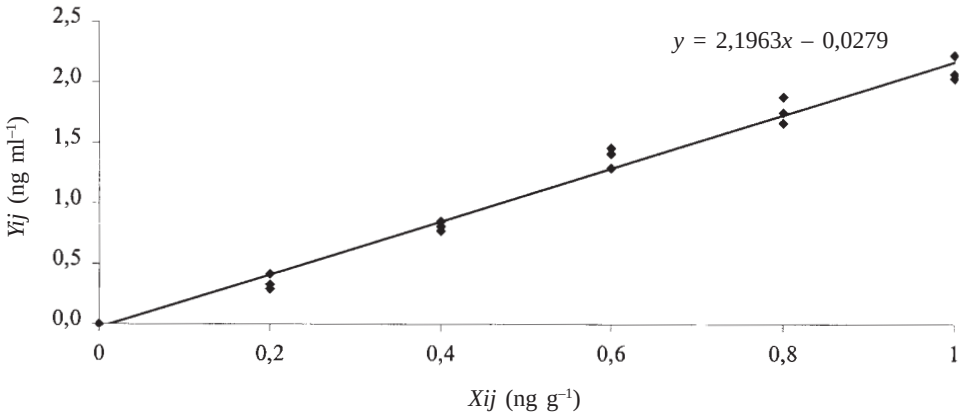

Figure 1. Experimental data and regression line.

Table IV. Linear regression parameters and results of the t-test.

	Estimate	*Theoretical (α = 5 %)*	*Unity*
Sensitivity b	2.1963		
Sb	0.0861		
Intercept a	–0.0279		ng.ml^{-1}
Sa	0.0154		ng.ml^{-1}
Correlation coefficient *r*	0.9935		
t-test	1.8065	t (5 %; 16)	
	ns	2.12	

ns: non significant

Table V. Homogeneity of intra groups variances test.

	Estimate	*Theoretical (α = 5 %)*
C-test	0.3333	C (5 %; 6;3)
	ns	0.616

ns: non significant

- Slope significance test

  Statistical results are given in table VI and show a significant slope, which means a linear dependence between the added SEA contamination (X) and the SEA detected amount (Y) with α-error = 5 %.

- Linear regression significance test

  Statistical results are given in table VII and show a significant linear regression, which means that the linear model is suitable with α-error = 5 %.

Intra laboratory repeatability and intermediate precision (study of recovery level's quality)

- Homogeneity of intra groups variances determination

  Table VIII gives statistical results.

  Variances S^2_j are homogeneous with α-error = 5 % and α-error = 1 %.

- Determination of isolated and aberrant values

  Statistical results are shown in table IX and are calculated from averages $\overline{Y_j}$ of each contamination level.

  For each contamination level, the G-test shows no isolated value and no aberrant value.

Table VI. Slope significance test.

Variations	*df*	*Sum of squares*	*Variances*	*Estimated F*	*Theoretical F (5 %;1; 16)*
Total variance	17	10.2637			
Regression variance	1	10.1300	10.1300	1211.5624	4.49
Residual variance	16	0.1338	0.0084	s	

s: significant

Table VII. Linear regression significance test.

Variations	*df*	*Sum of squares*	*Variances*	*Estimated F*	*Theoretical F (5 %; 4; 12)*
Experimental variation	12	0.0703	0.0059	ns	
Regression variation	4	0.0635	0.0159	2.7092	3.26

ns: non significant

Table VIII. Homogeneity test of intra groups variances.

	Level 1 = 0.3 ng g^{-1}			*Level 2 = 0.7 ng g^{-1}*		
	Group 1	*Group 2*	*Group 3*	*Group 1*	*Group 2*	*Group 3*
n	6	6	6	6	6	6
S_j^2	16.3261	6.6627	5.3313	4.2113	1.6348	0.5535
$\overline{Y_j}$ (%)	44.84	50.09	54.22	45.87	48.97	45.92
C		0.5765			0.6581	
C (5%;3;6)			0.707			
C (1%;3;6)			0.793			
C-test		ns			ns	

ns : non significant

Table IX. Isolated and aberrant data determination.

Level	*Inferior*	*Superior*	
1 (0,3 ng g^{-1})	1.0374	0.9578	Estimated
2 (0,7 ng g^{-1})	0.5901	1.1546	
Isolated values (α = 5 %)	1.155	1.155	Theoretical
Aberrant values (α = 1 %)	1.155	1.155	
G-test	ns	ns	

ns: non significant

- Intra laboratory repeatability and intermediate precision

  Repeatability and intermediate precision parameters are estimated by applying mathematical formulas given in table III.

  Table X shows statistical results.

Limit of detection and limit of quantification

Table XI gives statistical results. Limits ($ng.ml^{-1}$) are calculated from a SEA standard curve. The concentrations in the unity $ng.g^{-1}$ (ng SEA per gram of cheese) are determined from the extract volume (2.5 ml), the sample mass (12.5 g) and corrected by the average recovery level, estimated by the quality study.

Conclusion and prospects

Results allow us to estimate the method performance characteristics.

The method characterisation is carried out with a single dairy matrix. The purpose of the next study is the evaluation of the method in several food matrixes.

Table XI. Limit of detection and limit of quantification ($ng.ml^{-1}$ and $ng.g^{-1}$)

		DO	*[SEA] $ng.ml^{-1}$*	*[SEA] $ng.g^{-1}$*
LOD	$m_b + 3S_b$	0.1831	0.03	0.01
LOQ	$m_b + 10S_b$	0.2809	0.1	0.04

In the same way, it would be interesting:

– to study method repeatability and intermediate precision through a larger scale of concentrations;

– to evaluate the research method for other staphylococcal enterotoxins.

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Table X. Intra laboratory repeatability and intermediate precision.

Level	*Recovery level SEA (%)*	*S_r (%)*	*r (%)*	*CV_r (%)*	*S_R (%)*	*R (%)*	*CV_R (%)*
0.3 ng g^{-1}	49,71	3,1	8,7	6,2	5,5	15,5	11,0
0.7 ng g^{-1}	46,92	1,5	4,1	3,1	2,2	6,3	4,7