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Development and validation of the Standard method EN ISO 19020 - microbiology of the food chain — Horizontal method for the immunoenzymatic detection of staphylococcal enterotoxins in foodstuffs

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ABSTRACT

In the frame of the CEN Mandate M/381 from the European Commission to CEN (European Committee for Standardization), a method for the detection of staphylococcal enterotoxins in foodstuffs has been developed, validated and standardized. An extraction procedure based on dialysis concentration followed by an immuno-enzymatic detection has been defined. In addition, performance criteria (minimum values of sensitivity, specificity and level of detection) to be achieved by the commercially available immuno-enzymatic kits that could be used to detect staphylococcal enterotoxins in food matrices, were developed. A 2-stage validation study was conducted: The first stage aimed at selecting the commercial kits to be included in the second stage, which consisted in an interlaboratory study, using eight matrices covering five food categories (ready-to-eat food, meat products, milk products, dessert and fish). Results showed that two detection kits included in the study met the pre-defined performance criteria. The implementation of dialysis concentration step increased significantly the sensitivity of the method. The method developed allowed to achieve the Benchmark Dose lower limit (BMD₁₀) estimated at 6.1 ng. In 2019, finally, the European Commission recognized this standard as the European Union reference method for the detection of staphylococcal enterotoxins in food.

1. Introduction

Over all of the food-borne outbreaks observed in the European Union (EU), the rate of staphylococcal food-poisoning outbreaks (SFPO) varied between 15 and 20% since 2011 (Anonymous, 2012, 2013, 2014, 2015, 2016a; Beloeil et al., 2020). In 2018, among the 950 outbreaks which occurred in EU countries, 15 EU countries declared 114 outbreaks (12%) caused by staphylococcal enterotoxins (SEs) (Beloeil et al., 2020).

SEs produced by coagulase-positive staphylococci (CPS), including mostly *Staphylococcus aureus*, have super-antigenic and emetic activities, leading to toxic shock syndrome and staphylococcal food poisoning. Thus, SEs are assumed to be a threat to public health, and notification of food poisoning outbreaks has been mandatory since 2005 (Anonymous, 2007).

Criteria for CPS enumeration and SEs detection have been set down in Commission Regulation (EC) No. 2073/2005 amended (Anonymous, 2007) on microbiological criteria for food. For milk and milk products,

SEs detection must be performed when the CPS count exceeds 10⁵ colony-forming units per gram (cfu/g).

Among the 27 SEs reported in literature (Argudín et al., 2010; Ono et al., 2015; Zhang et al., 2018), only five could be detected with commercially available immunoassay kits: SEA, SEB, SEC, SED and SEE (Nia et al., 2016a), or by using commercially available 6 antibodies. SEA is reported as the most frequent SE involved in SFPO (80%) (Ercoli et al., 2017; Pinchuk et al., 2010; Zeaki et al., 2015). SED and SEC are commonly detected during both outbreak investigations and routine control analysis (Balaban and Rasooly, 2000; Denayer et al., 2017; Ercoli et al., 2017; Guidi et al., 2018; Kadariya et al., 2014; Oliveira et al., 2010; Ostyn et al., 2010a; Schubert et al., 2017). However, enterotoxin type SEE was rarely confirmed in the frame of food-borne outbreak investigation (Ostyn et al., 2010a).

Immunological assays have become the method of choice for SEs detection due to the lack of other screening methods that could be easily implemented by routine laboratories. Chemical methods based on mass

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spectrometry are being developed for SEs detection and quantification, but they are neither finalized nor fully validated for routine use.

Today, several commercially available immunoassays are available for the detection of classical enterotoxins (SEA to SEE). Even if a reference method was developed by the EU Reference Laboratory for CPS (EURL) and was used by the EU network of National Reference Laboratories (NRL) for CPS (Hennekinne et al., 2007; Nia et al., 2016a; Ostyn et al., 2011; Ostyn et al., 2010b), there are no standardized methods available.

The development, validation and standardization of a method for SEs detection in foodstuffs was conducted in the frame of the Mandate M/381 from the European Commission to CEN (European Committee for Standardization) for the validation and standardization of 15 Standard methods in the field of food hygiene legislation (Anonymous, 2010). This work was conducted in Task Group 12 “Staphylococcal enterotoxins” of Working Group 6 “Microbiology of the food chain” of Technical Committee 275 “Food analysis, horizontal methods” (CEN/TC 275/WG 6/TAG 12).

The members of CEN/TC 275/WG 6/TAG 12 agreed that SEB should not be used in these studies as this toxin has not been involved in SFPOs and that shipment of food containing SEB could be tricky due to its classification as a potential bioweapon. However, in the frame of the European EQUATOX project, an interlaboratory test was performed on SEB detection using different immuno-enzymatic detection assays and other methods such as mass spectrometry. Results obtained by Vidas® SET2, Ridascree® SET Total and Tecra® VIA kits on dairy matrices spiked by SEB are in agreement with those obtained in this study (Nia et al., 2016b).

The aim of this study was to perform an in-depth characterization and validation of the method standardized by CEN and ISO (International Organization for Standardization) for the detection of SEs in foods. The study included a first stage, consisting of intra-laboratory studies to test all of the available commercial immuno-enzymatic kits and to select the kits to be included in the second stage. The latter stage consisted of interlaboratory validation studies, on eight matrices covering five food categories: ready-to-eat food, meat products, milk products, dessert and fish. These studies were conducted from 2012 to 2014.

2. Materials and methods

2.1. Design of the studies

Prior to the inter-laboratory studies (ILS), intralaboratory studies were conducted by different laboratories to test the commercially available kits for the detection of SEs in food. Seven detection kits were identified and tested on five food categories (dairy products, meat products, fish products, dessert, and buffet meals) represented by 10 matrices: raw milk cheese (cow), ewe's raw milk cheese, fermented sausage, roasted chicken, prawns, smoked fish, tiramisu, chocolate or coffee éclair, mixed salad and mixed salad with rice. Each matrix was spiked separately with two or three toxins among the five common toxins (SEA to SEE) at different concentrations (0.10, 0.25 and 0.50 ng/g). Blank samples of each matrix were also tested to evaluate the specificity of each detection kit.

During this intra-laboratory step, among the seven detection kits tested, four kits gave deviations (false positives or false negatives). Thus only three detection kits, Vidas® SET2 (bioMérieux), Ridascree® SET Total (R-Biopharm) and Tecra® VIA (3M) were selected and tested during the interlaboratory validation studies.

Two interlaboratory validation studies were organized to cover the five food categories requested to standardize a method applicable to all food:

- in 2013, a first study on milk and meat-based products;

- in 2014, a second study on ready-to-eat food, fish and dessert products.

2.2. Methods submitted to interlaboratory studies

Samples were dispatched frozen and then were completely thawed at $3 \pm 2^\circ\text{C}$ or $5 \pm 3^\circ\text{C}$ before starting the analysis.

The method implemented was the so-called European Screening Method (ESM), which was the official method for the EU NRLs network (Nia et al., 2016a). Briefly, 25 g of sample was mixed in 40 mL of distilled water at $38 \pm 2^\circ\text{C}$, using rotary homogenizer, blender or peristaltic homogenizer (e.g. an Ultra-Turrax® IKA homogenizer). This step is particularly important in case of high fat content products. Then, samples were shaken at room temperature (18°C to 27°C) for 30 to 60 min to allow toxin diffusion.

The pH of the slurry was adjusted between 3.5 and 4.0 with appropriate hydrochloric acid solutions to precipitate caseins (in the case of dairy products) and centrifuged at a minimum of 3130 g for 15 min under refrigeration (approximately 4°C). The aqueous supernatant was sampled and adjusted to $\text{pH } 7.5 \pm 0.1$ with sodium hydroxide solutions and centrifuged as above. During this step:

- the necessary volume (depending on the kit used) of the neutralized aqueous phase was sampled and analyzed with the kits.
- The rest of the supernatant was filtered through glass wool and concentrated on 50 cm to 60 cm of a dialysis membrane with a molecular weight cut-off (MWCO) of 6000–8000 Da (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) against 30% (w/w) polyethylene glycol 20,000 (Merck, Darmstadt, Germany) overnight at 4°C . The concentrated protein extract was recovered and adjusted to a final weight of 5.0 to 5.5 g using phosphate buffered saline (PBS: 145 mmol/L NaCl + 10 mmol/L Na_2HPO_4 , $\text{pH } 7.3 \pm 0.2$) or osmosed water.

This dialysis concentration step was mandatory for milk and dairy products. For other matrices, participants implemented, for the same sample, the extraction protocol with and without dialysis concentration against PEG solution.

SEs detection was performed on both extracts obtained and the supernatants, using the three studied qualitative commercial assays, Vidas® SET2 (bioMérieux) and/or Ridascree® SET Total (R-Biopharm) and/or Tecra VIA® (3M) (see Fig. 1).

2.3. Test materials of the ILS

EFSA data on SFPOs in Europe were investigated to determine the main food matrices involved and contamination levels. On this basis, eight matrices covering five food categories (ready to eat food, meat products, milk products, dessert cream, and fish) were used for the inter-laboratory studies. SEA, SEC, SED, and SEE, identified in the introduction section as implicated in several food poisoning outbreaks in Europe, were selected for sample contamination.

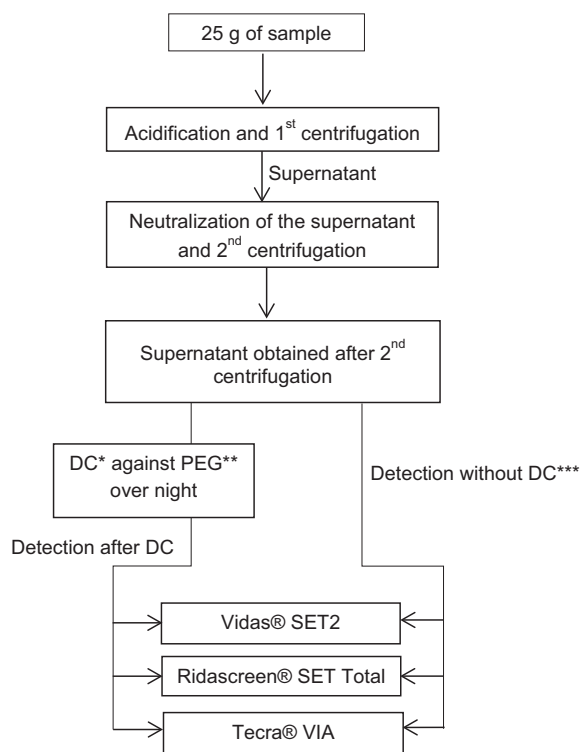
2.3.1. Toxins

Highly purified freeze-dried SEs were purchased from Toxin Technology, Sarasota, FL, USA (batch no. 120794 A for SEA, no 113094C2 for SEC2, and no 70595E for SEE) and were rehydrated according to the manufacturer's instructions to obtain stock solutions. Briefly, 1 mL of osmosed water was added to 1 mg of SE powder to obtain a theoretical concentration equal to 1 mg/mL.

2.3.2. Samples selection

Test items were naturally or artificially contaminated by the desired SE type to achieve the targeted concentration.

Cow's raw milk cheese (“Comté”), meat (roasted chicken), fish (mackerel), ready-to-eat (RTE) food (pie) and dessert cream (“crème



*dialysis-concentration, ** polyethylene glycol ***not performed for cheese since dialysis-concentration is mandatory,

Fig. 1. Experimental design for staphylococcal enterotoxins detection in food matrices during the inter-laboratory studies.

brûlée”) were purchased from a retail outlet. Twenty-five grams test portions were tested for SEs before sample contamination.

In addition, SED naturally contaminated cow's raw milk cheeses (“Bleu de Gex” and “Tomme au Marc”) from our sample collection were tested. These cheese samples were obtained from producers in the frame of regular sampling (own check). SED was quantified using an in-house specific ELISA method (Denayer et al., 2017).

2.3.3. Preparation of blank and contaminated samples

Uncontaminated blank samples were grinded, homogenized and dispatched into flasks in order to obtain 25 ± 0.1 g test portions.

Sample contamination was performed as follows. After mixing the matrix sample, 25 g test portions were taken and spiked individually by 500 μ L of SE solution in PBS-BSA-Azide (BSA 2 mg/mL, sodium azide 0.02%) in each flask to achieve the target concentration (Table 1). Note that for cheese with rind, about 10% of rind and 90% of core were sampled and mixed.

In order to prevent any cross contamination, each food/contamination level combination was prepared and contaminated separately. After their preparation, all samples were stored at -18 °C until they were tested for homogeneity and shipped to participating laboratories in the interlaboratory studies.

2.3.4. Identification of the test samples

The organizer guaranteed full confidentiality, in terms of identity of the participants in the studies. In accordance with its internal Quality Manual, a random encrypted coding for all test samples was used, including homogeneity and stability-dedicated samples. Samples were labelled after dispatch into flasks.

The samples were randomly coded independently of the laboratories' codification to avoid any collusion between laboratories. Parcel preparation and shipment was also randomly performed.

Table 1

Design of the inter-laboratory validation studies on SE detection in food matrices.

ILVT year	Food matrices	Toxin type	Spiking levels	Level attributed for the ILPT	Replicates/participant	
2013	Cheese “Comté”	/	/	Blank	4	
	Cheese Bleu de Gex ^a	SED ^a	0.20 ng/g	Level 1	8	
	Cheese “Tomme au Marc”	SED ^a	0.90 ng/g	Level 2	4	
	Meat sample	/	/	Blank	4	
	Meat sample	SEA	0.10 ng/g	Level 1	8	
	Meat sample	SEA	0.50 ng/g	Level 2	4	
	2014	Ready To Eat Food	/	/	Blank	1
		Ready To Eat Food	SEA	0.06 ng/g	Level 1	3
		Ready To Eat Food	SEA	0.11 ng/g	Level 2	1
		Ready To Eat Food	SEA	0.22 ng/g	Level 3	1
Fish		/	/	Blank	1	
Fish		SEC	0.15 ng/g	Level 1	3	
Fish		SEC	0.25 ng/g	Level 2	1	
Dessert cream		/	/	Blank	1	
Dessert cream		SEE	0.15 ng/g	Level 1	3	
Dessert cream		SEE	0.25 ng/g	Level 2	1	
Dessert cream	SEE	0.40 ng/g	Level 3	1		

^a Naturally contaminated.

2.4. Homogeneity and stability of the test samples

Samples dedicated to the inter-laboratory study must be sufficiently homogeneous and stable over the period of analysis by participating laboratories in order to avoid deviation that could be due to the quality of samples. Homogeneity and stability studies must be performed on the same batch of samples used for the ILS. The homogeneity study was performed before sample dispatch. Stability of enterotoxins in samples was verified after receiving all participants' data.

2.4.1. Homogeneity study

According to the Standard EN ISO 13528 (ISO, 2015), the homogeneity study was performed on 20 flasks randomly sampled from each matrix/contamination level combination. Each sample was analyzed once on the same day according to European Screening Method using both Vidas® SET2 and Ridascreen® SET Total kits. For blank samples, 100% of the obtained intermediate results (Test Value (TV) for Vidas SET2 and absorbance unit (AU) for Ridascreen SET Total) had to be below the positive threshold for the detection assay. For contaminated samples, 100% of the obtained intermediate results had to be above or equal to the positive threshold for the detection kits and their relative standard deviation (RSD) had to be less than or equal to 15% (Nia et al., 2016a). It should be noted that this RSD was calculated using the mean and the standard deviation of the 20 AU/TV values.

2.4.2. Stability study

Six samples of each matrix/level combination were randomly selected and analyzed according to ESM (Nia et al., 2016a). For the blank level, 100% of the obtained intermediate results (AU/TV) had to be below the positive threshold. For spiked levels, 100% of the obtained

intermediate results had to be above or equal to the positive threshold of the detection kits.

2.5. Data processing

Vidas SET2, Ridascreen SET Total and Tecra VIA are qualitative SE assays that are used as primary screening tools. These detection assays can detect the presence or absence of five SEs (SEA to SEE) but they are not able to identify the SE type.

Using the method for the immunoenzymatic SE detection in food-stuffs being validated and standardized, interlaboratory participants reported their final results as “SE not detected” if their raw data were below the positive threshold, and as “SE detected” if their raw data were above or equal to the positive threshold.

However, to assess results of the homogeneity and stability studies, additional quantitative intermediate results (see Section 2.4) were evaluated to better control the quality of the samples used over the ILS period.

2.5.1. Assessment of participants' data

Participants' results were excluded from calculations in the following cases:

- at reception, if temperature was higher than 10 °C (samples could not be analyzed);
- if the test portions were not stored as specified;
- if the protocol defined by CEN/TC 275/WG 6/TAG 12 was not followed as specified;
- If participating laboratories failed to perform analysis during the fixed period;
- if several results for an individual sample were sent to ILS organizer.

Results obtained by participants were interpreted as “SE not detected” or “SE detected” and compared with the expected results defined by ILS organizer.

The accuracy of the qualitative results was assessed according to the two following criteria:

Specificity: ability to obtain a negative response for a sample known by the ILS organizer to not contain any SE

$$\text{Specificity} = \frac{N^-}{N_{\text{expected}}^-} \times 100 \quad (3)$$

where N^- is the number of negative responses and N_{expected}^- is the number of samples expected to be negative.

Sensitivity: ability to obtain a positive response for a sample known by the ILS organizer to contain SE:

$$\text{Sensitivity} = \frac{N^+}{N_{\text{expected}}^+} \times 100 \quad (4)$$

where N^+ is the number of positive responses and N_{expected}^+ is the number of samples expected to be positive.

2.5.2. LOD₅₀

The level of detection at 50% (LOD₅₀) was calculated according to the statistical approach described in Standard EN ISO 16140-2 (Anon-ymous, 2016b) by using the program developed according to this standard by Dr. Cordula Wilrich and Prof Peter Wilrich, available at: <http://www.wiwiss.fu-berlin.de/fachbereich/vwl/iso/ehemalige/wilrich> (Wilrich and Wilrich, 2009).

2.5.3. Performance criteria

The whole methods, including the commercially available detection kits, were evaluated according to the performance criteria established by CEN/TC 275/WG 6/TAG 12: 90% for both sensitivity and specificity.

As to determine whether LOD₅₀ values were fit-for-purpose, a link

was established between the technical performance of the method, in terms of LOD₅₀, and its use for food safety control. To set this link, it was necessary to assess the minimal dose of ingested SEs which would trigger symptoms in exposed consumers. This dose is generally derived by modelling from dose-response studies. The dose which gives an effect in 10% of the exposed population is usually taken as a reference value (Guillier et al., 2016). For enterotoxin SEA, the BMD dose was estimated at 0.06 ng/g.

3. Results and discussion

3.1. Stability and homogeneity of the test materials

Materials were prepared as outlined in the materials and methods section, and were checked for suitable homogeneity before dispatch to the ILS participating laboratories. Homogeneity data for all studied levels are presented in Table 2. The produced samples were accepted for use in the ILS based on achieving satisfactory homogeneity and stability according to Sections 2.4.1 and 2.4.2.

The homogeneity study was performed on 20 samples in one week before sample dispatch. Table 2 shows that SEs were detected in all spiked samples whatever the level of contamination was. For blank levels, SEs were not detected in 100% of the tested samples, regardless of the assay used. Therefore, these samples were considered to be homogeneous for a qualitative assessment.

For the Vidas SET2 kit, mean values ranged from 0.52 to 2.80 Test Value (TV). For the Ridascreen SET Total kit, mean values ranged from 0.66 to 4.00 AU. On the other hand, the Relative Standard Deviations (RSD) calculated for each food type/contamination level combination, from 20 individual samples, were less than 15% (2.80% to 14.80%). Thereby, these samples were considered to be homogeneous.

The stability study was performed after receiving all participants' data in order to cover the entire ILS period. SEs were not detected in 100% of the blank samples (data not shown), and SEs were detected in 100% of the contaminated samples, regardless of the assay used.

Table 2
Homogeneity data obtained for matrices used for ILS.

Food matrices	Toxin type	Spiking levels	Vidas SET 2 assay		Ridascreen SET Total assay	
			n = 20	RSD %	n = 20	RSD %
Cheese “Comté”	Blank	Blank	nd ^a	/	nd	/
Cheese “Bleu de Gex”	SED	Level 1	1.06	12.5%	0.66	13.3%
Cheese “Tomme au Marc”	SED	Level 2	np ^b	/	np	/
Dessert cream	Blank	Blank	nd	/	nd	/
Dessert cream	SEE	Level 1	0.76	10.7%	0.73	14.8%
Dessert cream	SEE	Level 2	1.13	7.5%	1.23	8.6%
Dessert cream	SEE	Level 3	1.56	5.3%	2.06	9.5%
Fish	Blank	Blank	nd	/	nd	/
Fish	SEC	Level 1	0.52	9.2%	1.42	12.2%
Fish	SEC	Level 2	1.01	8.0%	2.74	9.9%
Ready To Eat Food	Blank	Blank	nd	/	nd	/
Ready To Eat Food	SEA	Level 1	0.69	7.3%	0.88	7.0%
Ready To Eat Food	SEA	Level 2	1.25	6.7%	1.78	6.5%
Ready To Eat Food	SEA	Level 3	1.88	2.7%	3.02	5.5%
Meat sample	Blank	Blank	nd	/	nd	/
Meat sample	SEA	Level 1	2.41	4.20%	2.25	12.90%
Meat sample	SEA	Level 2	2.80	2.80%	4.00 ^c	/

^a Nd: not detected.

^b Np: not performed by Vidas® SET2 and Ridascreen® SET Total, this homogeneity was performed by Tecra® VIA and RSD was 12.5%, ^craw data over the maximal values of the reader.

Therefore, these samples were considered to be stable for a qualitative assessment.

The six stability raw data obtained by Ridascreen® SET Total and Vidas® SET2 were compared to the mean of values obtained during the homogeneity test (Eqs. (1) and (2)). Figs. 2 and 3 were obtained by dividing the value of each replicate tested for stability by the mean of homogeneity values (in %).

$$\frac{AU_{n \text{ stability}}}{AU_{\text{assigned}}} \times 100 \text{ for Ridascreen SET Total assay} \quad (1)$$

where $AU_{n \text{ stability}}$ is the test value of the n^{th} replicate and AU_{assigned} is the mean value obtained in the homogeneity study, which was thus considered as the assigned value (Table 2).

$$\frac{TV_{n \text{ stability}}}{TV_{\text{assigned}}} \times 100 \text{ for Vidas SET2 assay} \quad (2)$$

where $TV_{n \text{ stability}}$ is the test value of the n^{th} replicate and TV_{assigned} is the mean value obtained in the homogeneity study, which was thus considered as the assigned value (Table 2).

For the Ridascreen® SET Total assay (see Fig. 2), most values were included in the interval of $\pm 25\%$, except for RTE food at level 1, and two replicate values of dessert cream at level 1, which were included in the interval of $\pm 40\%$.

For the Vidas® SET2 assay (see Fig. 3), regardless of the food matrix, all replicate values were included in the interval of $\pm 25\%$, except for one replicate of fish at level 1 (56%, ILS 2014) and one replicate of dessert cream at level 1 (69%, ILS 2014).

This quantitative assessment globally confirmed the qualitative results and the satisfactory stability of samples over the ILS analysis period.

3.2. General results of the interlaboratory study and performance characteristics of the method

All the results from the participating laboratories were analyzed and some data were excluded when the instructions for the extraction or detection steps were not followed (pH of acidification and neutralization, final mass of extract, value of positive and negative controls). The excluded data represent less than 7% of all data returned by participants.

The remaining data were used to determine the performance characteristics of the method, that are specificity, sensitivity and LOD_{50} .

3.2.1. Specificity

A total of 11 negative samples were analyzed by each participant. The percentage of false positive results, corresponding to the lack of specificity of the method with and without performing dialysis concentration step, is shown in Table 3. Although a few false positive results were obtained with Tecra VIA for RTE food, chicken, and fish, and with Vidas SET2 for cheese, the specificity calculated for all matrices together remains higher than 90%. One-hundred percent specificity was obtained in 12 over 15 cases studied with dialysis concentration (all kits combined), and in 11 over 12 cases studied without dialysis concentration step (all kits combined).

3.2.2. Sensitivity

One objective of this ILS was to determine the influence of dialysis concentration step on the sensitivity of the method (see Table 4).

Using Vidas® SET2 detection assay,

- with dialysis concentration step, 100% of sensitivity was obtained for all contamination levels (all matrices combined);
- without dialysis concentration step, 100% of sensitivity was obtained for four level/matrix couples, 99% was obtained once in the case of meat sample spiked at level 1, and 1% to 87% of sensitivity was found in 5 matrix/level combinations.

Using Ridascreen® SET total kit,

- with dialysis concentration step, 100% of sensitivity was obtained for 7 level/matrix couples. Between 90% and 99% of sensitivity was obtained for four combinations, and 87% of sensitivity was obtained for one combination (cheese contaminated at level 1);
- without dialysis concentration step, 0% to 67% of sensitivity was obtained in most cases (8 couples), and 100% of sensitivity was obtained in only two combinations.

Using Tecra® VIA kit,

- with dialysis concentration step, 100% of sensitivity was obtained in only three matrix/level combinations. Between 83% and 97% of sensitivity was obtained in four combinations, and 33% to 57% of sensitivity was obtained in 5 combinations;
- without dialysis concentration step, 100% of sensitivity was obtained in only one case meat level 2 (chicken). All other combinations showed poor sensitivity, between 0% and 30%.

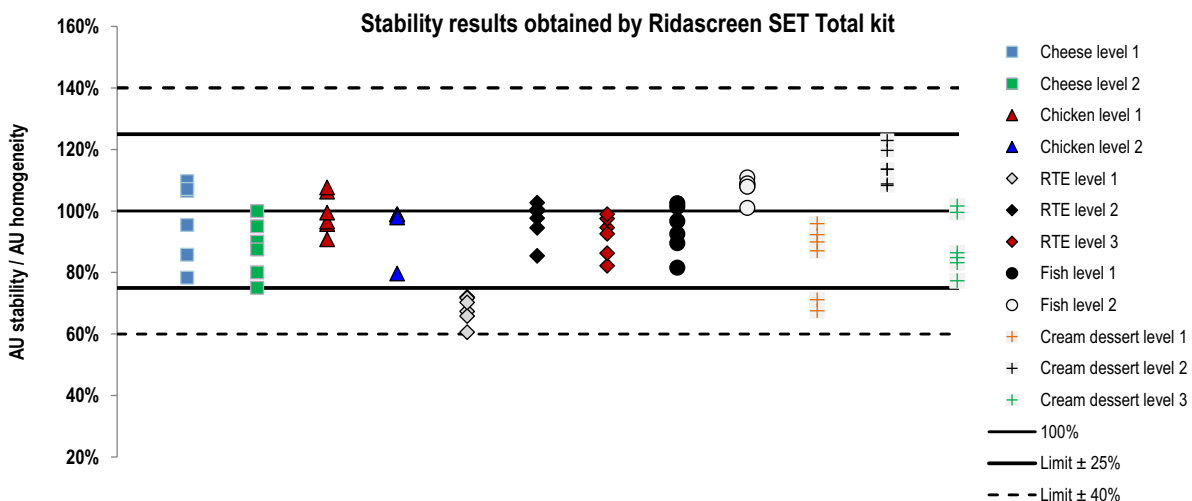


Fig. 2. Results of the stability study by Ridascreen® SET Total. Comparison of data obtained after ILS period (six replicates) to the assigned value obtained during the homogeneity study ($n = 20$).

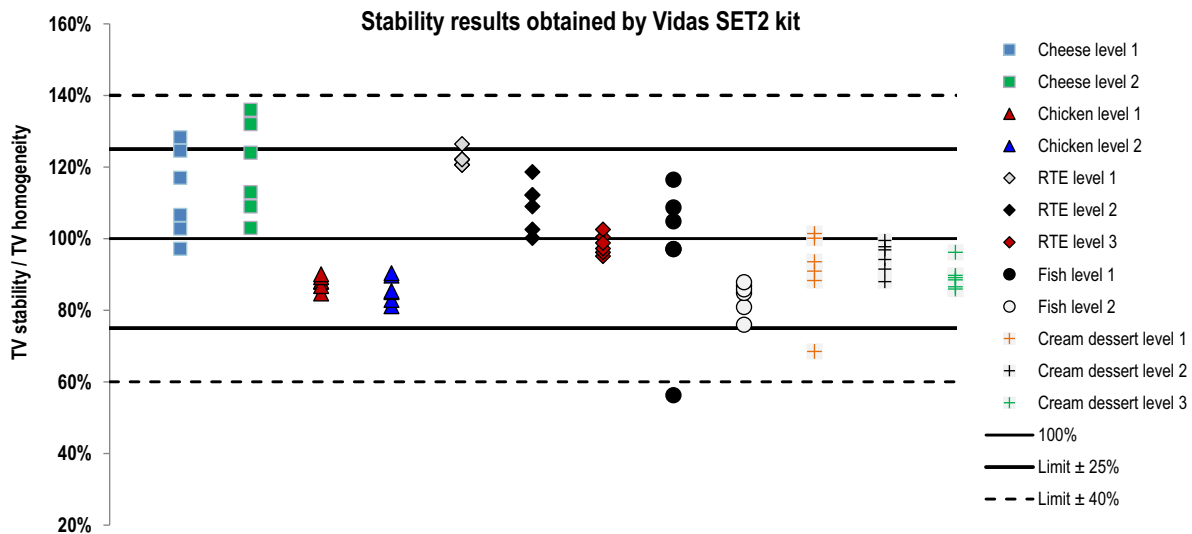


Fig. 3. Results of the stability study by Vidas® SET2. Comparison of data obtained after ILS period (six replicates) to the assigned value obtained during the homogeneity study (n = 20).

Table 3
Specificity (%) values obtained with the three detection kits used for the five food categories.

Matrices	Detection kits	+DC ^a	-DC ^b
Ready To Eat Food (SEA)	Vidas SET2	100	100
	Ridascreen SET Total	100	100
	Tecra Staph VIA	90	100
Fish (SEC)	Vidas SET2	100	100
	Ridascreen SET Total	100	100
	Tecra Staph VIA	100	80
Dessert (SEE)	Vidas SET2	100	100
	Ridascreen SET Total	100	100
	Tecra Staph VIA	100	100
Cheeses (SED)	Vidas SET2	98	Not performed
	Ridascreen SET Total	100	
	Tecra Staph VIA	100	
Meat sample (SEA)	Vidas SET2	100	100
	Ridascreen SET Total	100	100
	Tecra Staph VIA	93	100

^a With dialysis concentration.
^b Without dialysis concentration.

In conclusion, whatever the detection kit tested, the sensitivity was clearly improved when the DC step was implemented.

3.2.3. LOD₅₀

LOD₅₀ values have been calculated and reported in Table 5.

Table 4
Sensitivity (%) values obtained by the three kits used for the five food categories.

Kit	Level	RTE (SEA)		Fish (SEC)		Dessert (SEE)		Cheese (SED)		Meat (SEA)	
		+DC ^a	-DC ^b	+DC	-DC	+DC	-DC	+DC	-DC	+DC	-DC
Vidas SET2	L1	100	87	100	3	100	1	100	* ^c	100	99
	L2	100	100	100	68	100	86	100	*	100	100
	L3	100	100	*	*	100	100	*	*	*	*
Ridascreen SET Total	L1	98	10	98	10	94	0	87	*	100	37
	L2	100	52	100	44	100	26	98	*	100	100
	L3	100	100	*	*	100	67	*	*	*	*
Tecra Staph VIA	L1	33	10	97	23	20	0	83	*	95	10
	L2	57	17	100	30	37	10	97	*	100	100
	L3	100	30	*	*	50	10	*	*	*	*

^a With dialysis concentration.
^b Without dialysis concentration.
^c Not performed.

In the literature, response rates of 36 SFPO involving SEA alone were modelled as a function of ingested doses. The lower 95%-confidence interval of the Benchmark Dose lower limit (BMD₁₀) was estimated at 6.1 ng. With assumption of a 100 g serving size, LOD₅₀ for qualitative methods should be lower than 0.06 ng/g for SEA. In Table 5, LOD₅₀ obtained using both Vidas® SET2 and Ridascreen® SET Total kits, after dialysis-concentration step, were satisfactory. However, absence of DC step increased LOD₅₀ for these two kits up to BMD₁₀. This highlights the relevance of DC step that allows concentrating 5 times SEs present in food matrices. Finally, for the third tested kit (Tecra Staph VIA), LOD₅₀ seemed to be satisfactory only for toxin SEC in fish using DC step. This observation showed that Tecra Staph VIA sensitivity did not achieve BMD₁₀ even when implementing DC step.

4. Conclusion

The aim of this study was to develop and validate a horizontal method for the immuno-enzymatic detection of staphylococcal enterotoxins in foodstuffs. For the first time, a representative panel of food safety laboratories (54 over the whole of the studies) participated in intra-laboratory and interlaboratory validation studies of the methods selected. Performance criteria (sensitivity, specificity and LOD₅₀) were established for the method. The validated method has been standardized and the resulting Standard EN ISO 19020 has been published in June 2017, together with its performance criteria. Dialysis concentration step has been identified as relevant and allowing improvement in term of

Table 5LOD₅₀ (ng/g) values obtained by the three kits tested on the five food categories.

LOD ₅₀ ng/g	RTE food (SEA)		Fish (SEC)		Dessert (SEE)		Cheese (SED)		Meat (SEA)	
	+DC ^a	-DC ^b	+DC	-DC	+DC	-DC	+DC	-DC	+DC	-DC
Vidas SET 2	0.007	0.019	0.017	0.552	0.017	0.375	*c		0.007	0.015
Ridascreen SET Total	0.010	0.184	0.027	0.815	0.036	1.444	0.076		0.010	0.120
Tecra Staph VIA	0.082	0.406	0.031	0.35	0.408	2.673	0.092		0.082	0.211

^a With dialysis concentration.^b Without dialysis concentration.^c Not calculated with naturally contaminated samples, level above LOD50.

sensitivity of the method.

This first Standard method on SE detection in foodstuffs is used by food suppliers and scientific community for the development and the validation of future commercial assays, and has been recently included as reference method in the EC Regulation 2073/2005 on microbiological criteria for food, amended by EC Regulation 2019/229 (Anonymous, 2019).

Declaration of competing interest

The authors declare no conflict of interest.

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