

# Certified reference materials for testing of the presence/absence of *Staphylococcus aureus* enterotoxin A (SEA) in cheese: IRMM-359a-c

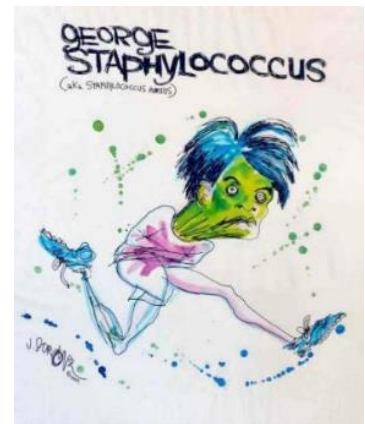
Reinhard Zeleny, EC-JRC  
EURACHEM/CITAC online workshop, 19.09.2024



1

## Setting the scene

- Staphylococcal enterotoxins (SEs) are causative agents in food-poisoning outbreaks (FPOs)
- 6 ng SEA in a 100 g food serving size is estimated as BMDL<sub>10</sub> (lower benchmark dose to induce effects in 10 % of the exposed population)
- SEs are proteins (ca. 22 – 28 kDa mass); high stability to changing temperature and pH, resistivity to proteolytic digestion
- More than 30 SEs are known by now, only 5 can be detected with commercially available detection kits
- SEA is the serotype most frequently involved in FPOs



2



2

## Setting the scene

- Commission Regulation No. 1441/2007 (microbiological criteria for foodstuffs): cheeses, milk powders and whey powders shall be tested using the European Screening method (ESM) of the EURL CPS (Coagulase-Positive Staphylococci): “Detection of Staphylococcal enterotoxins types SEA-SEE in milk and milk products”
- In 2019, the updated ESM (“all types of food matrices”) was converted into ISO 19020 (“Microbiology of the food chain – horizontal method for the immunoenzymatic detection of staphylococcal enterotoxins in foodstuffs”)
- Prescribed sample intake: 25 g of food; 5 replicate analyses shall be performed. For compliance with legislation, the result has to be “not detected” in all 5 cases.

3



3

## Setting the scene

### General method outline

#### Sample preparation

- Extraction of the samples with warm water
- pH adjustment, centrifugation
- dialysis concentration

#### Analysis

Employment of one of the two commercially detection assays

VIDAS SET2: enzyme-linked fluorescent assay

Ridascreen SET Total: enzyme-linked immunosorbent assay

Each assay can detect SEA/SEB/SEC/SED/SEE or combinations thereof.  
The assays do not distinguish among the toxins (screening).

4



4

## Setting the scene

Each assay has a threshold/cut-off, determined in extensive validation studies of the assay providers.

A result below the threshold/cut-off is interpreted as “not detected”.

A result at or above the threshold/cut-off is interpreted as “detected”.

### Choice of matrix

Tomme de Savoie  
(raw milk cheese from France)

### Choice of SEA concentrations

close to method LOD  
+ strong positive  
+ blank material



5



5

## Processing

Sets with 3 materials were prepared:

IRMM-359a, blank material

IRMM-359b, SEA-containing material (ca. 0.1 ng SEA/g cheese)

IRMM-359c, SEA-containing material (ca. 0.25 ng SEA/g cheese)

Major processing steps:

Removal of rind – cutting cheese into cubes

IRMM-359a: grinding – freeze-drying – mixing

IRMM-359b/c: addition of water – grinding to a slurry – spiking with SEA solution  
mixing – freeze-drying – mixing – combining IRMM-359a with this  
powder to obtain desired SEA concentrations – mixing

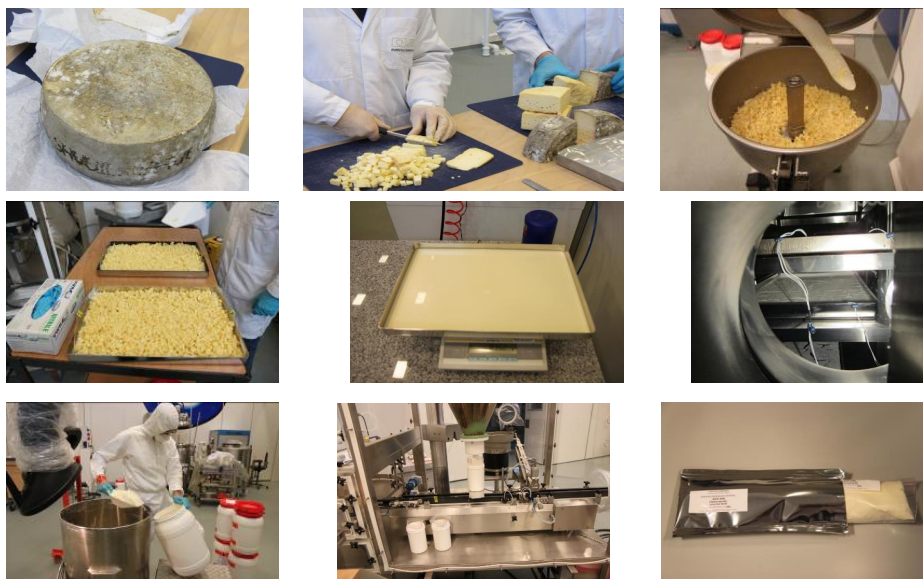
All materials: filling under inert gas atmosphere into sachets

6



6

## Processing



7

7

## Homogeneity and stability

The direct read-outs of the VIDAS assay (quantity/sort-of-quantity) were used to evaluate the H and STS and LTS studies.

This was seen more appropriate than a simple “not detected” result for IRMM-359s and “detected” for IRMM-359b and IRMM-359c.

Uncertainties were calculated but not used in the end, as a lower limit of confidence was given instead of a one-sided uncertainty

8

8

## Homogeneity

Only done for IRMM-359b and c

The absence of SEA in the cheese was confirmed by VIDAS SET2 measurements before processing.

10 units, random stratified sampling scheme, 3 replicate analysis per unit  
VIDAS SET2

All 60 results were correct (SE detected)

Also, all results in the characterisation study were correct, serving as an additional homogeneity check/assessment.

No outliers (Grubbs test, 99 % confidence level)

No trends (regression analysis, 95 % confidence level)

9

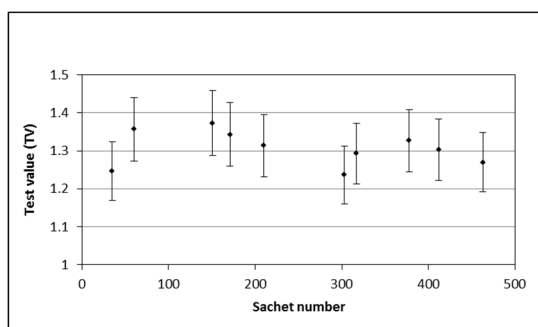


9

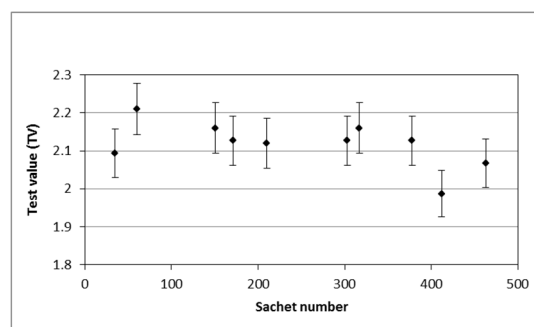
## Homogeneity summary

	IRMM-359b	IRMM-359c
RSD [%]	6.145	3.753
$s_{wb}$ [%]	6.186	3.078
$s_{bb}$ [%]	n.c. <sup>1)</sup>	2.224
$u_{bb}^*$ [%]	2.008	0.999

<sup>1)</sup> n.c.: cannot be calculated as  $MS_{between} < MS_{within}$



IRMM-359b



IRMM-359c



10

10

## Short-term stability

Only done for IRMM-359b and c

VIDAS SET2

Test temperatures +18 °C and +60 °C, reference temperature: -70 °C

Isochronous study: 0/1/2/4 weeks

No outliers (Grubbs test, 99 % confidence level)

No trends (regression analysis, 95 % confidence level)

Uncertainties calculated, but not used in the end

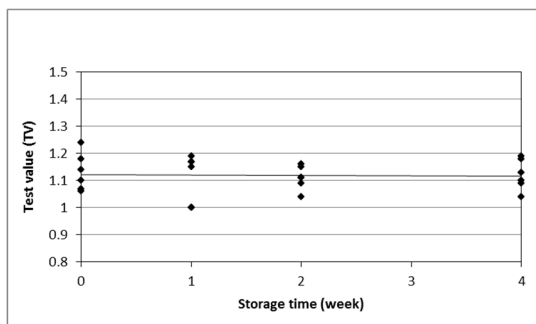
11



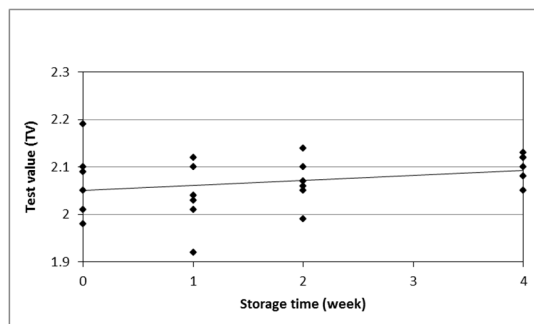
11

## Short-term stability - summary

	IRMM-359b		IRMM-359c	
	18 °C	60 °C	18 °C	60 °C
$u_{sts}$ [%/week]	0.775	1.475	0.434	0.399



IRMM-359b; +18 °C



IRMM-359c; +60 °C

12



12

## Long-term stability

Only done for IRMM-359b and c

VIDAS SET2

Test temperatures +4 °C and -20 °C, reference temperature: -70 °C

Isochronous study: 0/4/8/12 months

IRMM-359b: 1 statistical outlier, kept

IRMM-359c: 1 technical outlier, rejected

IRMM-359b: significant trend at +4 °C; no trend at -20 °C

IRMM-359c: no trends detected

Uncertainties calculated, but not used in the end

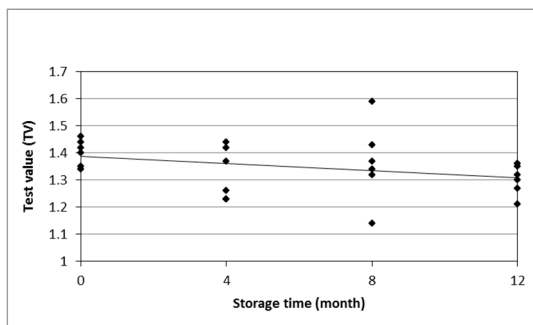
13



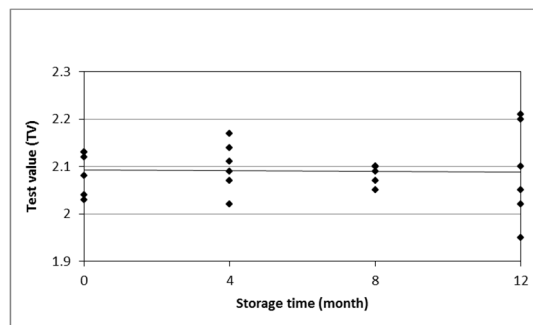
13

## Long-term stability – summary\*

	IRMM-359b		IRMM-359c	
	4 °C	-20 °C	4 °C	-20 °C
$u_{lis}$ [%/year]	6.234	3.945	1.545	1.697



IRMM-359b; -20 °C



IRMM-359c; +4 °C

14

\* A reduced two year study (0/24 months) confirmed the 1-year results



14

## Characterisation

3 analyses days, 3 independent analyses per day  
 Reconstitution: 15.1 g cheese powder + 9.9 g water  
 Method: ESM with either VIDAS SET2 or Ridascreen SET Total assay

8 data sets obtained using ESM with VIDAS SET2

7 data sets obtained using ESM with Ridascreen SET Total

Some data excluded for technical reasons (e.g. reconstitution masses not respected, pH adjustments outside the prescribed range, final mass of concentrated sample outside the prescribed range).

### Final results for statistical evaluation:

IRMM-359a, 112 valid results, all reported as “SEs not detected”

IRMM-359b, 122 valid results, all reported as “SEs detected”

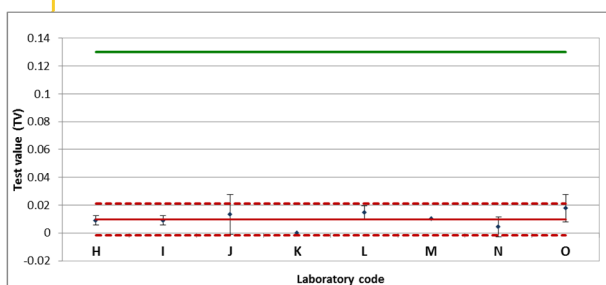
IRMM-359c, 125 valid results, all reported as “SEs detected”

15

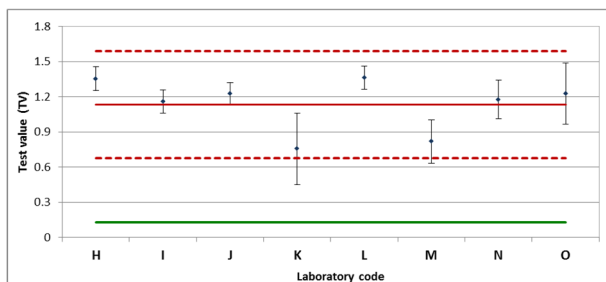


15

## Characterisation



IRMM-359a; ESM/VIDAS SET2



IRMM-359b; ESM/VIDAS SET2

16



16



## Value assignment: certified values uncertainties

Diagnostic specificity<sup>1)</sup> (IRMM-359a): 
$$\text{SPEC \%} = \frac{\text{TN}}{\text{TN} + \text{FP}} * 100$$

Diagnostic sensitivity<sup>2)</sup> (IRMM-359b and c): 
$$\text{SENS \%} = \frac{\text{TP}}{\text{TP} + \text{FN}} * 100$$

Uncertainty expressed as lower limit of confidence <sup>2,3)</sup>

95 % lower limit of confidence<sup>\*,3)</sup>: 
$$\text{LL \%} = \left[ 1 - \left( \frac{1}{2n} * \chi^2_{(2k+2)} \right) \right] * 100$$

<sup>1)</sup> ISO 33406, Annex B2;

<sup>2,3)</sup> ISO 33406, clause 5.3.2.5.; Matrella 1963, Experimental Statistics, Handbook 91, NIST

	Certified value	95 % lower limit of confidence
<b>IRMM-359a</b>	SPEC, 100 %	97.3 %
<b>IRMM-359b</b>	SENS, 100 %	97.5 %
<b>IRMM-359c</b>	SENS, 100 %	97.6 %

17



17

## Value assignment – additional material information

<b>ESM/VIDAS SET2</b>		
	Test value	
	Mean value <sup>1)</sup>	Interval <sup>2)</sup>
SEA in IRMM-359a	0.01	0.00 – 0.05
SEA in IRMM-359b	1.14	0.47 – 1.53
SEA in IRMM-359c	1.97	1.10 – 2.42
<b>ESM/Ridascreen SET Total</b>		
	Absorbance units	
	Mean value <sup>1)</sup>	Interval <sup>2)</sup>
SEA in IRMM-359a	0.08	0.01 – 0.19
SEA in IRMM-359b	0.61	0.28 – 1.11
SEA in IRMM-359c	1.36	0.45 – 2.31

<sup>1)</sup> Mean of lab mean values

<sup>2)</sup> Lowest and highest individual value from all labs

18



18

## Metrological traceability

The ESM has to be strictly followed.  
Therefore, the measured properties are operationally defined.

The identity of SEA was assessed by SDS-PAGE (molecular mass deduced from gel) and confirmatory ELISA (SEA-specific).

Metrological traceability of the obtained results is based on the traceability of all relevant input factors. Instruments in individual laboratories were verified and calibrated with tools ensuring traceability to the International System of Units (SI).

Consistency of results in the interlaboratory comparison demonstrates that all relevant input factors were covered. As the assigned values are combinations of agreeing results individually traceable to the SI, the assigned values themselves are traceable to the SI as well.

19



19

## Intended use of the CRMs

**Method validation**

**Method performance control**

### Same/very similar approach used for these CRMs:

- BCR-599, ewes' and goats' curd
  - The certified value is the lower limit of confidence,
  - The property is called "probability of correct identifications"
- SB-0/0.1/0.5/2 (dried soy beans powder containing Roundup Ready™ soya)
  - The properties are called "sensitivity, specificity and efficiency of the 35S and NOS PCR methods"

20



20

## References

- Commission Regulation No. 1441/2007 – microbiological criteria for foodstuffs
- ISO 19020: Microbiology of the food chain – Horizontal method for the immunoenzymatic detection of Staphylococcal enterotoxins in foodstuffs
- ISO 16140 Microbiology of food and animal feeding stuffs – Protocol for the validation of alternative methods
- M.G. Natrella (1963) Handbook 91, Experimental Statistics, NIST
- R. Zeleny, J. Charoud-Got, H. Emteborg, H. Schimmel, Y. Nia, I. Mutel, A. Ostyn, S. Herbin, J.-A. Hennekinne (2015) Development of a reference material for *Staphylococcus aureus* enterotoxin A in cheese: feasibility study, processing, homogeneity and stability assessment. Food Chem. 168:241-246
- R. Zeleny, Y. Nia, H. Schimmel, I. Mutel, J.-A. Hennekinne, H. Emteborg, J. Charoud-Got, F. Auvray (2016) Certified reference materials for testing of the presence/absence of *Staphylococcus aureus* enterotoxin A (SEA) in cheese. Anal. Bioanal. Chem. 408:5457-5465

21



21

## Acknowledgements

Processing team of the Reference Materials Unit at JRC-Geel (formerly IRMM)

EURL CPS

Participating laboratories

22



22

Thank you for your attention!

