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Microbiology of the food chain — Horizontal method for the immunoenzymatic detection of staphylococcal enterotoxins in foodstuffs

Microbiologie de la chaîne alimentaire — Méthode horizontale de détection des entérotoxines staphylococciques par test immuno-enzymatique dans les aliments

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by the European Committee for Standardization (CEN) Technical Committee CEN/TC 275, *Food Analysis — Horizontal methods*, in collaboration with ISO Technical Committee TC 34, *Food products*, Subcommittee SC 9, *Microbiology*, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Introduction

Staphylococcal enterotoxins (SEs) are proteins that can be produced in foods, by certain strains of the coagulase positive staphylococci (CPS), mainly *Staphylococcus aureus*. These SEs are heat and acid stable toxins that cause nausea, vomiting, abdominal pain and diarrhoea when ingested. Due to their stability SEs might still be present even when coagulase positive staphylococci cannot be detected. SEs consist of a family of more than 20 structurally-related globular monomeric proteins with molecular weights of 19 kDa to 30 kDa.[1] These proteins are relatively stable under changing environmental conditions, such as heat treatment, freezing and change in pH; moreover, they are resistant to proteolytic digestion. Typically, and depending on the sensitivity of affected individuals, nanogram (ng) amounts of enterotoxin can cause intoxication with the symptoms described above. Due to the influence of SEs on human health, the European Union has adopted legislation in order to increase consumer protection by defining microbiological criteria for foodstuffs, such as CPS enumeration and detection of SEs.[2]

Several methods have been developed for the detection and/or quantification of SEs. Some of these methods are based on enzyme immunoassay (EIA). Other methods are based on the chemical analysis using liquid chromatography with tandem mass spectrometry (LC-MS/MS) for the detection and quantification of SEs. As these latter methods are currently under development, EIA methods have been chosen as the starting point for standardization of a detection method for SEs.

The aim is to detect SEs using commercially available test kits. This document describes the protocol for the extraction of SEs from food samples. Moreover, criteria for the performance of the kits have been evaluated on five types of food matrices before use based on the criteria given in this document.

Response rates of different staphylococcal food poisoning outbreaks were modelled as a function of ingested doses.[3] For this purpose, data from the literature as well as data from the European Union Reference Laboratory for CPS were used.

The United States Environmental Protection Agency (US EPA) benchmark dose methodology was applied to this data set and helped to establish the benchmark dose (BMD).[4] The BMD is defined as the dose of a hazard (staphylococcal enterotoxin) likely to trigger health symptoms in a given percentage of the exposed population. The BMD lower limit (BMDL) is the lower 95 % (or 90 %) confidence interval of the BMD. This value was used to set up the acceptable value for the limit of detection 50 (LOD_{50}) of the various commercially available SE detection kits.

Microbiology of the food chain — Horizontal method for the immunoenzymatic detection of staphylococcal enterotoxins in foodstuffs

1 Scope

This document specifies a screening method for the detection of staphylococcal enterotoxins SEA, SEB, SECs, SED and SEE in foodstuffs. It consists of two main steps: a) extraction followed by a concentration based on dialysis principle; and b) an immunoenzymatic detection using commercially available detection kits.

This document is applicable to the screening of staphylococcal enterotoxins SEA to SEE in products intended for human consumption.

Other staphylococcal enterotoxins such as types SEG, SEH, SEI, SER, SES and SET can also cause illness. Due to the lack of commercially available detection kits, this document is applicable only to types SEA to SEE, but may apply to other types of toxins, subject to validation of the method.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

ISO 7218, *Microbiology of food and animal feeding stuffs — General requirements and guidance for microbiological examinations*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

3.1
staphylococcal enterotoxin A, B, C, D, E
SEA, SEB, SEC, SED, SEE
exoprotein SEA, SEB, SEC, SED and SEE produced by enterotoxigenic strains of coagulase positive staphylococci, mainly *Staphylococcus aureus* with a molecular weight ranging from 19 kDa to 30 kDa

3.2
specificity
SP
number of samples found to be negative divided by the total number of blank samples tested

3.3
sensitivity
SE
number of samples found to be positive divided by the total number of samples tested at a given level of contamination

3.4
limit of detection 50
LOD_{50}
concentration (ng SE/g) for which the probability of detection is 50 %

3.5
benchmark dose
BMD
dose of a hazard (e.g. staphylococcal enterotoxin) likely to trigger health symptoms in a given percentage of the exposed population

4 Principle

This document specifies a method for the detection of staphylococcal enterotoxins (SEA to SEE) in all foodstuffs, consisting of two main steps: a) extraction followed by a concentration based on dialysis principle; and b) an immunoenzymatic detection using commercially available detection kits.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

5.1 Distilled or **demineralized water** or **water of equivalent quality** according to ISO 3696.

5.2 Hydrochloric acid (e.g. concentrations 5N, 1N or other dilutions).

5.3 Sodium hydroxide (e.g. concentrations 5N, 1N or other dilutions).

5.4 PBS (phosphate buffered saline), pH 7,3 ± 0,2 [$NaCl/Na_2HPO_4$: 145 mM/10 mM].

5.5 PEG, molecular weight 20 000 g/mol (PolyEthylene Glycol) solution.

Prepare a concentrated PEG solution: weigh 30 g of PEG powder, and add 70 ml of water (5.1).

5.6 Electrode cleaning solution (e.g. ethanol 70 %).

5.7 Immunoenzymatic detection kit dedicated to SEs. Any kit shall comply with the performance criteria in 8.7.

6 Apparatus

Usual microbiological laboratory equipment (in accordance with ISO 7218) and, in particular, the following.

6.1 Blender.

6.2 Balance.

6.3 Homogenization equipment, e.g. rotary homogenizer, blender or peristaltic homogenizer.

It is highly recommended to use a rotary homogenizer, in particular for all types of food difficult to mix in order to obtain a homogeneous sample. If a peristaltic homogenizer is used, only use bags without filter.

6.4 Shaker at room temperature, e.g. orbital shaker, magnetic stirrer, etc.

6.5 pH-meter and electrode, e.g. combination electrode.

6.6 Centrifuge, capable of operating at 3 130*g* minimum; if possible, capable of being refrigerated.

6.7 Dialysis membrane, molecular weight cut off (MWCO) of 6 000 Da to 8 000 Da.

6.8 Closures for dialysis membrane.

6.9 Filtering material, e.g. funnel and cotton-wool, glass-wool, etc.

6.10 Shallow tray.

6.11 Refrigerator (3 °C ± 2 °C or 5 °C ± 3 °C) and **freezer** (≤ −18 °C).

6.12 Laboratory ware in glass or polypropylene to avoid the adsorption of toxins (funnel, beaker, vial, centrifuge tube, etc.).

6.13 Equipment suitable for the detection kit used, see 5.7.

6.14 Water bath (38 °C ± 2 °C).

7 Sampling

Sampling is not part of the method specified in this document.

8 Procedure

8.1 Preparation of test portion

In the case of cheese with rind, take about 10 % of rind and 90 % of core.

As enterotoxins can be heterogeneously distributed in the sample, if possible, mix and homogenize the whole sample or a representative part of it with a blender (6.1). Use 25 g of the homogenized sample as the test portion.

In the case of a suspected staphylococcal food poisoning outbreak (SFPO), the test sample size may be less than 25 g. Perform the analysis as described below and adapt the steps 8.3.1 to 8.5.2 accordingly. The ratio of the weight of the test portion and concentrated extract (8.5.2) should be approximately five [e.g. 25 g test portion for 5,0 g to 5,5 g (maximum 5,8 g for the sticky extracts) of concentrated extract, 12,5 g test portion for 2,5 g to 2,8 g (maximum 2,9 g for the sticky extracts) of concentrated extract].

8.2 Storage of the test sample

It is recommended to store the samples at 3 °C ± 2 °C or 5 °C ± 3 °C (6.11) before analysis.

If analysis is not performed within 24 h, it is possible to freeze the samples. In this case, completely thaw the samples at 3 °C ± 2 °C or 5 °C ± 3 °C before starting the analysis.

To avoid loss of toxins, it is highly recommended not to freeze and thaw the samples repeatedly before analysis.

8.3 Extraction

8.3.1 Add approximately 40 ml of water (5.1) at 38 °C ± 2 °C to the 25 g test portion, except in the case of liquid products. For liquid products, proceed directly as described in 8.3.2. In the case of SFPO, if the test portion is smaller than 25 g, reduce the amount of water (5.1) with the equal ratio.

Homogenize the mixture using a rotary homogenizer or a blender (6.3). This step is particularly important in the case of high fat content products. It is recommended to use a rotary homogenizer for all types of food samples that are difficult to mix in order to obtain a homogeneous sample.

8.3.2 Recover the entire sample and rinse the system (stem of the rotary homogenizer, the stomacher bag or the bowl of the blender) with a minimal volume of water (5.1).

NOTE The greater the volume of liquid used the longer the length of dialysis membrane required.

8.3.3 Allow the toxins to diffuse by shaking the sample (6.4) at room temperature (18 °C to 27 °C) for 30 min to 60 min.

8.3.4 Acidify the mixture with appropriate hydrochloric acid solutions (5.2) in order to obtain a pH between 3,5 and 4,0 measured with a pH meter (6.5).

8.3.5 Centrifuge the entire mixture at 3 130*g* minimum for 15 min under refrigeration temperature (approximately 4 °C) or at room temperature (18 °C to 27 °C) (6.6).

In the case of fatty samples, a centrifugation at refrigeration temperature (approximately 4 °C) is recommended to eliminate the fat particles before the dialysis.

8.3.6 Recover the supernatant in a beaker (6.12). If the supernatant is opaque, repeat centrifugation as described in 8.3.5. After centrifugation pH shall be between 3,0 and 4,5.

If the pH > 4,5, proceed as described in 8.3.4.

If the pH < 3,0, the 3D structure of SEs might be damaged. Take another 25 g test portion and proceed as described in 8.3.1.

8.3.7 Neutralize the mixture with the appropriate sodium hydroxide solutions (5.3) in order to obtain a pH between 7,4 and 7,6.

If pH > 9,0, the 3D structure of SEs might be damaged. Take another 25 g test portion and proceed as described in 8.3.1.

8.3.8 Centrifuge according to 8.3.5.

8.3.9 Recover the entire neutralised aqueous phase for the concentration step.

To recover the maximum amount of toxins, at the end of the acidification and neutralization steps, rinse the electrode and beaker with some drops of water (5.1).

In the case of high fat content samples, the electrode can be cleaned using ethanol 70 % (5.6) to dissolve fat particles after the analysis is complete.

8.3.10 Alternative extraction procedure (optional).

This alternative procedure may only be used in limited circumstances, such as a suspected food poisoning event, and may not be used for milk and milk products. This alternative procedure differs from the described procedure by omitting the dialysis concentration step.

— Take the necessary volume (depending on the kit used) of the neutralized aqueous phase obtained in step 8.3.9 and proceed to the detection step 8.7. Store the remaining neutralized aqueous phase at 3 °C ± 2 °C or 5 °C ± 3 °C.

— If a SEs-negative result is obtained, implement the concentration step (8.4) of the remaining neutralized aqueous phase the same day and repeat the detection using the concentrated extract.

If this procedure is not strictly followed, a new test portion should be analysed.

8.4 Concentration of the extract (mandatory for milk and dairy products)

8.4.1 For each sample, use the PEG solution prepared according to 5.5.

8.4.2 Cut a piece of dialysis membrane (6.7) with sufficient length to contain the entire extract.

8.4.3 Soak the membrane in water (5.1) for rehydration, following the manufacturer's instructions (e.g. at least for 30 min at room temperature).

Before use, rinse the membrane (outside and inner parts) with water (5.1).

8.4.4 Lock one end of the membrane with a closure (6.8).

8.4.5 Fill the prepared membrane with all of the neutralized aqueous phase (8.3.9) using a funnel and a small piece of filtering material (6.9) to filter out suspended particles. Lock the other end of the membrane with a second closure (6.8).

8.4.6 Lay down the filled dialysis membrane in a shallow tray (6.10) filled with the PEG solution (5.5).

8.4.7 Allow the extracts to concentrate, overnight at 3 °C ± 2 °C or 5 °C ± 3 °C (6.11). If the extract is not concentrated enough (i.e. more than 5 ml left in the dialysis membrane), lay it down in the PEG solution for more time (up to 3 days) or add some PEG powder over the membrane.

8.5 Recovery of the concentrated extract

8.5.1 Take the dialysis membrane out of the PEG solution and rinse the outer-parts of the membrane with water (5.1) to remove all traces of PEG solution.

8.5.2 Open one end of the membrane and recover the concentrated extract by rinsing thoroughly the inner-part of the dialysis membrane using

— PBS (5.4) in the case of milk and dairy products, or

— water (5.1) in the case of other matrices.

Rinse thoroughly the inner-parts of the dialysis membrane to obtain a final concentrated extract mass ranging from 5,0 g to 5,5 g (maximum 5,8 g for the sticky extracts).

Carefully transfer the concentrated extract into a glass or polypropylene vial (6.12).

During this critical step, to recover the maximum amount of enterotoxins it is recommended

— to rub the inner-parts of the dialysis membrane (one part against another inner-part) in order to remove and to recover the maximum of SEs, and

— to maximize the quantity of SEs recovered, carry out the recovery of the extract by repeatedly adding small quantities of PBS (5.4) or water (5.1) into the membrane (e.g. add 1 ml or 2 ml), rubbing the membrane as described above and adding the recovered extract into the vial. Repeat these steps until a final mass of 5,0 g to 5,5 (5,8) g per 25 g test portion is obtained.

In the case of a SFPO, the mass of the sample analysed may be lower than 25 g (8.2). The final mass of the concentrated extract (8.5.2) will be adjusted to obtain a final ratio of 1 to 5 between the concentrated extract mass and the test portion mass.

8.6 Storage and steps before detection

If the concentrated extract (8.5.2) will be analysed within 48 h, store it at 3 °C ± 2 °C or 5 °C ± 3 °C (6.11). If the detection cannot be performed within 48 h, store the extract at ≤ −18 °C (6.11) unless otherwise stated by the manufacturer of the detection kit used.

In the case of frozen extract, completely thaw and homogenize it using a vortex before performing the detection step.

If foaming appears, make sure to pipet in the liquid phase.

8.7 Detection

Select a detection kit that fulfils the performance criteria (sensitivity, specificity, LOD_{50}) for the entire procedure, defined in this document (see 8.8).

Carefully follow the manufacturer's instructions for the detection step of the kit used.

8.8 Performance criteria

Performance criteria including specificity (SP, 3.2), sensitivity (SE, 3.3), limit of detection 50 % (LOD_{50}, 3.4) have been defined for the entire procedure, including extraction and detection. The calculation for LOD_{50} was performed using a dedicated programme available from the ISO website.

The performance criteria that the commercial kits shall achieve are defined as follows:

— SP and SE should be higher than 90 %.

— LOD_{50} should be less than 0,06 ng SEs/g. This value is based on the estimated BMD for SEA of 6,1 ng and the assumed ingestion of 100 g of food.

As consolidated data were only available for SEA, the staphylococcal enterotoxin most frequently involved in SFPO, it was decided to use this value for the other toxin types SEB to SEE.[4]

Values obtained by different detection kits and food matrices with and without dialysis are presented in Clause 12. Laboratories shall refer to the data obtained to perform the selection of the detection kit which fulfils the criteria mentioned above.

The data are summarized in Annexes A and B for the interlaboratory studies organized in 2013 and 2014, respectively. The values derived from the interlaboratory studies may not be applicable to food types other than those given in Annexes A and B.

9 Quality control

It is recommended to check the entire procedure, with reference materials. An example of a suitable reference material is given in Reference [5].