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Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —

Part 3:
Detection and MPN technique for low numbers

*Microbiologie des aliments — Méthode horizontale pour le dénombrement des staphylocoques à coagulase positive (*Staphylococcus aureus *et autres espèces) —*

Partie 3: Recherche et méthode NPP pour les faibles nombres

Reference number
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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 6888-3 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 9, *Microbiology*.

ISO 6888 consists of the following parts, under the general title *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus *and other species)*:

— *Part 1: Technique using Baird-Parker agar medium*

— *Part 2: Technique using rabbit plasma fibrinogen agar medium*

— *Part 3: Detection and MPN technique for low numbers*

This corrected version of ISO 6888-3:2003 incorporates the following correction:

Subclause 9.1.1

The second paragraph has been amended to resolve any ambiguity.

Introduction

Because of the large variety of food and feed products, this horizontal method may not be appropriate in every detail for certain products. In this case, different methods, which are specific to these products, may be used if absolutely necessary for justified technical reasons. Nevertheless, every attempt should be made to apply this horizontal method as far as possible.

When this part of ISO 6888 is next reviewed, account will be taken of all information then available regarding the extent to which this horizontal method has been followed and the reasons for deviations from this method in the case of particular products.

The harmonization of test methods cannot be immediate and, for certain groups of products, International Standards and/or national standards may already exist that do not comply with this horizontal method. It is hoped that when such standards are reviewed they will be changed to comply with this part of ISO 6888 so that eventually the only remaining departures from this horizontal method will be those necessary for well-established technical reasons.

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) —

Part 3:
Detection and MPN technique for low numbers

1 Scope

This part of ISO 6888 specifies a horizontal method for the enumeration and detection of coagulase-positive staphylococci, using the most probable number (MPN) technique. It is applicable to

— products intended for human consumption and the feeding of animals, and

— environmental samples in the area of food production and food handling.

This method is recommended for products where staphylococci are expected to be stressed and in low numbers as, for example, in dried products. Coagulase-positive staphylococci will primarily be *Staphylococcus aureus* but *Staphylococcus intermedius* and some strains of *Staphylococcus hyicus* also produce coagulase.

2 Normative references

The following referenced documents are indispensable for the application 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 6887 (all parts), *Microbiology of food and animal feeding stuffs — Preparation of test samples, initial suspension and decimal dilutions for microbiological examination*

ISO 8261, *Milk and milk products — General guidance for the preparation of test samples, initial suspensions and decimal dilutions for microbiological examination*

ISO 6888-1:1999, *Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus *and other species) — Part 1: Technique using Baird-Parker agar medium*

ISO 6888-2:1999, *Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus *and other species) — Part 2: Technique using rabbit plasma fibrinogen agar medium*

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

ISO/TS 11133-1, *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 1: General guidelines on quality assurance for the preparation of culture media in the laboratory*

ISO/TS 11133-2:—[1)], *Microbiology of food and animal feeding stuffs — Guidelines on preparation and production of culture media — Part 2: Practical guidelines on performance testing culture media*

3 Terms and definitions

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

3.1
coagulase-positive staphylococci
bacteria which form typical and/or atypical colonies on the surface of a selective culture medium and which show a positive coagulase reaction or a specific rabbit plasma reaction on rabbit plasma fibrinogen agar

NOTE For the purpose of this part of ISO 6888, the confirmation of coagulase-positive staphylococci is based on a strongly positive coagulase reaction, but it is recognized that some strains of coagulase-positive staphylococci give weakly positive coagulase reactions. These latter strains can be confused with other bacteria but they can be distinguished from such other bacteria by the use of additional tests such as the production of thermonuclease (for details, see IDF 83).

3.2
enumeration of the coagulase-positive staphylococci
determination of the number of coagulase-positive staphylococci found per millilitre or per gram of sample when the test is carried out according to the method specified in this part of ISO 6888

4 Principle

4.1 Detection method

4.1.1 A selective culture medium is inoculated with a specified quantity of the test sample if the initial product is liquid, or a specified quantity of an initial suspension in the case of other products.

4.1.2 The tubes are incubated at 37 °C, anaerobically, for 24 h and 48 h. The presence of presumptive coagulase-positive staphylococci is indicated by the reduction of potassium tellurite.

NOTE In this part of ISO 6888, anaerobiosis is obtained by pouring a plug of agar or paraffin into each tube, but an alternative procedure is to incubate the tubes in a jar or an incubator under anaerobic conditions.

4.1.3 The surface of solid selective Baird-Parker medium is inoculated from presumptive positive tubes (4.1.2) after 24 h, and all the remaining tubes after 48 h.

4.1.4 The plates are inoculated at 37 °C for 24 h and 48 h. The presence of presumptive coagulase-positive staphylococci is indicated by the reduction of potassium tellurite and an egg yolk reaction.

4.1.5 Typical and/or atypical colonies are confirmed by a coagulase reaction.

4.1.6 Alternatively, the surface of rabbit plasma fibrinogen agar may be inoculated and, after appropriate incubation, the presence of coagulase-positive staphylococci is indicated by colonies showing a specific rabbit plasma fibrinogen reaction.

4.1.7 The results are given as the “presence” or “absence” of coagulase-positive staphylococci in x g or x ml of product.

4.2 Enumeration method

4.2.1 Serial dilutions of product are inoculated into liquid selective culture medium.

1) To be published.

4.2.2 The tubes are incubated at 37 °C, anaerobically, for 24 h and 48 h. The presence of presumptive coagulase-positive staphylococci is indicated by the reduction of potassium tellurite.

NOTE In this part of ISO 6888, anaerobiosis is obtained by pouring a plug of agar or paraffin into each tube, but an alternative procedure is to incubate the tubes in a jar or an incubator under anaerobic conditions.

4.2.3 The surface of solid selective Baird-Parker medium is inoculated from presumptive positive tubes (4.2.2) after 24 h, and all the remaining tubes after 48 h.

4.2.4 The plates are incubated at 37 °C for 24 h and 48 h. The presence of presumptive coagulase-positive staphylococci is indicated by the reduction of potassium tellurite and an egg yolk reaction.

4.2.5 Typical and/or atypical colonies are confirmed by a coagulase reaction.

4.2.6 Alternatively, the surface of rabbit plasma fibrinogen agar may be inoculated and, after appropriate incubation, the presence of coagulase-positive staphylococci is indicated by colonies showing a specific rabbit plasma fibrinogen reaction.

4.2.7 The most probable number of coagulase-positive staphylococci per gram or per millilitre of sample is calculated by reference to most probable number tables for confirmed dilutions (4.2.5 or 4.2.6).

5 Diluents and culture media

For current laboratory practice, see ISO 7218.

The chemical products used for the preparation of the culture media and reagents shall be of recognized analytical quality.

5.1 Diluents

Refer to the relevant part of ISO 6887, or to ISO 8261, or the specific standard dealing with the product to be examined.

5.2 Modified Giolitti and Cantoni broth

5.2.1 Base medium

5.2.1.1 Composition

	Double-strength medium	Single-strength medium
Enzymatic digest of casein	20,0 g	10,0 g
Meat extract	10,0 g	5,0 g
Yeast extract	10,0 g	5,0 g
Lithium chloride	10,0 g	5,0 g
Mannitol	40,0 g	20,0 g
Sodium chloride	10,0 g	5,0 g
Glycine	2,4 g	1,2 g
Sodium pyruvate	6,0 g	3,0 g
Polyoxyethylene sorbitan mono-oleate (Tween 80)	2,0 g	1,0 g
Water	1 000 ml	1 000 ml

5.2.1.2 Preparation

Dissolve the ingredients in the water, by heating and mixing if necessary, to obtain complete dissolution. Cool to room temperature and adjust the pH, if necessary, so that after sterilization the final pH is 6,9 ± 0,2.

Dispense the medium in appropriate quantities into tubes of suitable dimensions (e.g. 16 mm × 160 mm in the case of single-strength medium, and 20 mm × 200 mm in the case of double-strength medium).

Sterilize for 15 min in an autoclave set at 121 °C.

5.2.2 Potassium tellurite solution

5.2.2.1 Composition

Potassium tellurite [2] (K_2TeO_3)	1,0 g
Water	100 ml

5.2.2.2 Preparation

Dissolve the potassium tellurite in the water with minimal heating.

The powder should be readily soluble. If a white insoluble material is present in the water, discard the potassium tellurite.

Sterilize by filtration using 0,22 µm pore size membranes.

The solution may be stored for a maximum of one month at 3 °C ± 2 °C.

Discard the solution if a white precipitate forms.

5.2.3 Complete medium

Shortly before use, heat the base medium (5.2.1) for 15 min at 100 °C to expel air.

Cool to 44 °C to 47 °C and aseptically add the potassium tellurite solution (5.2.2) using 0,1 ml per tube for single-strength medium and 0,2 ml per tube for double-strength medium.

5.2.4 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-1. Table 1 shows the performance testing criteria of modified Giolitti and Cantoni broth.

Table 1 — Performance testing criteria of modified Giolitti and Cantoni broth

Function	Incubation	Control strains	Reference media	Method of control	Criteria
Productivity	37 °C for 48 h	*Staphylococcus aureus* ATCC 6538 P or *Staphylococcus aureus* ATCC 25923 plus a competitive strain (*E. coli* ATCC 8732 or 25922), or same strain registered in other collections		semi-quantitative	> 10 colonies on selective medium
Selectivity	37 °C for 48 h	*Escherichia coli* ATCC 25922 or 8739 or same strain registered in other collections	TSA	semi-quantitative	no growth on non-selective medium

2) It is recommended to ensure beforehand that the potassium tellurite available is suitable for this test (5.3.2.2).

5.3 Agar solution (20 g/l)

5.3.1 Composition

Agar	15 to 20 g[3)]
Water	1 000 ml

5.3.2 Preparation

Suspend the agar in the water by boiling, and autoclave at 121 °C for 15 min.

Cool to 44 °C to 47 °C before use. Pour into tubes of suitable capacity. Store in accordance with ISO 7218.

5.4 Baird-Parker agar medium

5.4.1 Composition and preparation

See ISO 6888-1:1999, 5.3.

5.4.2 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:—, Table B.1.

5.5 Rabbit plasma fibrinogen agar medium (see references [6] and [7])

5.5.1 Composition and preparation

See ISO 6888-2:1999, 5.3.

5.5.2 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:—, Table B.1.

5.6 Brain-heart infusion broth

5.6.1 Composition and preparation

See ISO 6888-1:1999, 5.4.

5.6.2 Performance testing for the quality assurance of the culture medium

For the definition of selectivity and productivity, refer to ISO/TS 11133-1. For the performance criteria, refer to ISO/TS 11133-2:—, Table B.4.

5.7 Rabbit plasma

Refer to ISO 6888-1:1999, 5.5.

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3) Depending on the gel strength of the agar.

6 Apparatus

NOTE Disposable apparatus is an acceptable alternative to reusable glassware if it has similar specifications.

Usual microbiological laboratory apparatus (ISO 7218) and, in particular, the following.

6.1 Incubator, capable of operating at 37 °C ± 1 ºC.

6.2 Drying cabinet or **oven**, ventilated by convection, capable of being maintained at between 37 °C and 55 °C. A laminar airflow cabinet may also be used.

6.3 Petri dishes, sterile.

6.4 Loop, of platinum-iridium, nickel-chromium or plastic, of approximately 3 mm diameter, or 10 µl **sterile disposable loops**, and **stab inoculation wires** of the same material.

6.5 Test tubes, of suitable dimensions (e.g. 16 mm × 160 mm, 20 mm × 200 mm and 10 mm × 75 mm).

6.6 Graduated pipettes, of 1 ml nominal capacity, graduated in 0,1 ml divisions, and with an outflow opening of appropriate diameter.

6.7 Water baths, capable of operating between 44 °C and 47 ºC and at 37 °C.

6.8 Spatula, to cut agar.

6.9 Anaerobic jar.

7 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this part of ISO 6888. If there is no specific International Standard dealing with sampling of the product concerned, it is recommended that the parties concerned come to an agreement on this subject.

8 Preparation of test sample

Prepare the test samples in accordance with the appropriate part of ISO 6887, or ISO 8261, or the specific International Standard appropriate to the product concerned. If there is no specific International Standard available, it is recommended that the parties concerned come to an agreement on this subject.

9 Procedure

9.1 Detection method

9.1.1 Test portion and initial suspension

See the appropriate part of ISO 6887 depending of the product concerned, or ISO 8261.

Add 1 ml of the initial suspension to 9 ml of single-strength modified Giolitti and Cantoni broth (5.2) (i.e. 0,1 g or 0,1 ml of the sample) or 10 ml of the initial suspension to 10 ml of double-strength modified Giolitti and Cantoni broth (i.e. 1 g or 1 ml of the sample). For larger volumes of test portions, prepare the initial suspension by adding x ml or x g of test portion to $9x$ ml of the diluent (see ISO 6887 or ISO 8261). Then add the entire initial suspension to $90x$ ml of single-strength modified Giolitti and Cantoni broth, previously deaerated and with potassium tellurite added. There shall be a minimum air volume in the tube or flask

(e.g. add 5 ml or 5 g of the sample to 45 ml of the diluent, and add this entire initial suspension to 450 ml of single-strength modified Giolitti and Cantoni broth). Carefully pour a plug of agar (5.3) or paraffin, cooled to between 44 °C and 47 °C, onto the top of the medium and allow it to solidify to form a seal.

9.1.2 Enrichment

Incubate (see 6.1) the initial suspension (9.1.1) for 24 h ± 2 h at 37 °C. If blackening or black precipitate is detected, subculture as indicated in 9.1.3. If no blackening has developed, incubate for a further 24 h ± 2 h and subculture as indicated in 9.1.3 (whether or not blackening or a black precipitate has developed).

9.1.3 Subculture of tubes

Aseptically remove the plug of agar or paraffin by using a sterile spatula (6.8) to cut the agar plug (see Note in 4.1.2) into quarters lengthwise. If necessary, insert the spatula around the plug to release it from the glass wall of the tube. Agitate the tube to cause the pieces of plug to fall to the bottom of the tube and to ensure an even suspension of the culture.

With a sterile loop (6.4), spread a loopful of each selected broth onto the surface of separate plates of Baird-Parker agar (5.4) or plates of rabbit plasma fibrinogen agar (5.5) to obtain isolated colonies.

Invert the prepared dishes and place them in the incubator (6.1) set at 37 °C for 24 h ± 2 h and 48 h ± 2 h.

9.2 Enumeration method

9.2.1 Test portion and initial suspension

See the appropriate part of ISO 6887 depending of the product concerned, or ISO 8261.

9.2.2 Inoculation

Take three tubes of double-strength medium, deaerated and with potassium tellurite added (5.2.3). Transfer to each of these tubes 10 ml of the test sample for liquid products or 10 ml of the primary dilution (i.e. 1 g of sample) for other products.

Take three tubes of single-strength medium, deaerated and with potassium tellurite added (5.2.3). Transfer to each of these tubes 1 ml of the test sample for liquid products or 1 ml of the primary dilution (i.e. 0,1 g of sample) for other products.

For each of the subsequent dilutions (i.e. 10^{-1}, 10^{-2} and 10^{-3} for liquid products, or 10^{-2}, 10^{-3} or 10^{-4} for other products), proceed as specified above, using a fresh sterile pipette for each dilution.

Prepare a sufficient number of dilutions to ensure that the final dilution is sufficient to yield three negative results.

Carefully mix the inoculum and medium, in each case avoiding the introduction of air.

Carefully pour a plug of agar (5.3), cooled to between 44 °C to 47 ºC, onto the top of the medium in each inoculated tube and allow it to solidify to form a seal.

9.2.3 Incubation

Incubate (see 6.1) the inoculated tubes of double-strength medium and single-strength medium (9.2.2) at 37 °C for 24 h ± 2 h. Subculture any tubes showing any blackening or black precipitate as indicated in 9.5.

Incubate the remainder of the inoculated tubes for a further 24 h ± 2 h and subculture all tubes (i.e. those that do or do not develop a black precipitate after 48 h ± 2 h), as indicated in 9.1.3.

9.2.4 Subculture

See 9.1.3.

9.3 Selection of plates and interpretation

9.3.1 Baird-Parker agar medium

9.3.1.1 Selection of colonies

After 24 h incubation of the dishes (9.1.3 or 9.2.4), mark on the bottom of the plates the positions of any typical colonies present.

NOTE 1 Typical colonies are black or grey, shining and convex (1 mm to 1,5 mm in diameter after incubation for 24 h and 1,5 mm to 2,5 mm in diameter after incubation for 48 h) and surrounded by a clear zone which may be partially opaque. After incubation for at least 24 h, an opalescent ring, immediately in contact with the colonies, may appear in this clear zone.

NOTE 2 Atypical colonies have the same size as typical colonies and may present one of the following morphologies:

a) shining black colonies with or without a narrow white edge; the clear zone is absent or barely visible and the opalescent ring is absent or hardly visible;

b) grey colonies free of clear zones.

Atypical colonies are formed mainly by strains of coagulase-positive staphylococci contaminating, for example dairy products, shrimps and giblets. They are less often formed by strains of coagulase-positive staphylococci contaminating other products.

NOTE 3 Other colonies are all the remaining colonies possibly present on the plates that do not show the typical or atypical appearance described in Notes 1 and 2 and are considered as background flora.

Re-incubate (see 6.1) all plates at 37 °C for a further 24 h $\pm$ 2 h and mark any new typical colonies. Also mark any atypical colonies present.

9.3.1.2 Confirmation

From the surface of each selected colony (9.3.1.1), remove an inoculum with a sterile wire and transfer it to a tube or bottle of brain-heart infusion broth (5.6).

Incubate (see 6.1) for 24 h $\pm$ 2 h at 37 °C.

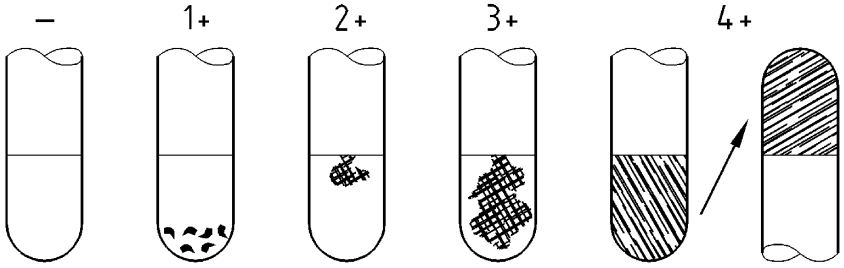
Aseptically, add 0,1 ml of each culture to 0,3 ml of the rabbit plasma (5.7) (unless other amounts are specified by the manufacturer) in sterile tubes of suitable dimensions (e.g. 10 mm $\times$ 75 mm) and incubate at 37 °C.

By tilting the tube, examine for clotting of the plasma after 4 h to 6 h of incubation and, if the test is negative, re-examine after 24 h $\pm$ 2 h of incubation, or examine at the incubation times specified by the manufacturer.

Consider the coagulase test to be positive if the cultures yield at least 3+ coagulase reactions according to the scoring guidance in Figure 1. Reactions from 1+ to 2+ are considered as intermediate.

As a negative control, for each batch of plasma, add 0,1 ml of sterile brain-heart infusion broth (5.6) to the recommended quantity of rabbit plasma (5.7) and incubate without inoculation. For the test to be valid, the control plasma shall show no signs of clotting.

Record as positive each tube from which at least one colony is confirmed as coagulase positive.



– negative: no evidence of fibrin formation
1+ positive: small unorganized clots
2+ positive: small organized clot
3+ positive: large organized clot
4+ positive: entire content of tube coagulates and is not displaced when tube is inverted

Figure 1 — Scoring of coagulase test reactions

9.3.2 Rabbit plasma fibrinogen agar medium

After incubation for 24 h ± 2 h, and for an additional 24 h if necessary, coagulase-positive staphylococci form black or grey (or even white) small colonies surrounded by a halo of precipitation indicating a coagulase activity. *Proteus* colonies may show, at the beginning of incubation, an appearance similar to coagulase-positive staphylococci colonies. However, after 24 h or 48 h of incubation, they appear as a spreading colony more or less brownish in colour that therefore allows distinction from staphylococci.

The test is positive when the presence of at least 1 colony indicating coagulase activity is seen.

NOTE As the rabbit plasma fibrinogen agar is based on a coagulase reaction, it is not necessary to confirm this activity.

10 Expression of results

10.1 Detection method

In accordance with the interpretation of the results, report the presence or absence of coagulase-positive staphylococci in the test portion, specifying the mass in grams, or the volume in millilitres, of the test sample.

10.2 Enumeration method

10.2.1 Selection of dilutions

NOTE The initial suspension and the test sample, if liquid, are considered as dilutions.

For each dilution of liquid selective culture medium inoculated (9.1.2 and 9.2.2), record the number of tubes in which the presence of coagulase-positive staphylococci has been confirmed with the Baird-Parker agar test (9.3.1) or the presence of colonies with positive coagulase reactions on the rabbit fibrinogen agar medium (9.3.2). Designate these as positive tubes.

Select three consecutive dilutions in accordance with ISO 7218:1996, 9.4, and determine the MPN index (see ISO 7218).

10.2.2 Calculations

See ISO 7218:1996, 9.4.

11 Precision

It is well known that wide variations in results may occur with the MPN technique. The specified confidence limits are based entirely on a random distribution of results. Other sources of variation, which may sometimes be of even greater importance, do not enter into the MPN estimate. To allow for such effects, use the categories in Table B.2 of ISO 7218:1996. These summarize the possible tube combinations according to the probability of their occurrence.

NOTE An international collaborative study (see reference [3]) on dried milk samples showed that, using a MPN technique similar to the one described in this part of ISO 6888, the difference between two single test results was less than 1,25 times the arithmetic mean of the two results, in 75 % of cases. The highest difference observed between two single test results was 1,94 times the arithmetic mean of the two results.

12 Test report

The test report shall specify:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used (detection or enumeration; media used), with reference to this part of ISO 6888;

d) all operating details not specified in this part of ISO 6888, or regarded as optional, together with details of any incidents which may have influenced the results;

e) the results obtained, indicating clearly the method of expression used.

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