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PAPER

49

Specifications for identity and purity of certain food additives

Emulsifiers, enzyme preparations,
flavouring agents,
food colours,
thickening agents, miscellaneous food additives

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APPENDIX A

DETERMINATION OF ANTIBIOTIC ACTIVITY

Scope

This procedure is designed for the determination of antibiotic activity in enzyme preparations derived from microbial sources.

Principle

The assay is based on the measurement of inhibition of bacterial growth under specific circumstances.

Culture plates

Six organisms are tested: Staphylococcus aureus ATCC 6538; Escherichia coli ATCC 11229; Bacillus cereus ATCC 2; Bacillus circulans ATCC 4516; Streptococcus pyrogenes ATCC 12344; and Serratia marcescens ATCC 14041. Make a test plate of each organism by preparing a 1:10 dilution of a 24 hour Trypticase Soya Broth culture in Tryticase Agar (TSA) (for Streptococcus pyrogenes ATCC 12344 a 1: 20 dilution).

Pour 15 ml of plain TSA into a Petri dish and allow the medium to harden. Overlay with 10 ml of seeded TSA and allow to solidify. Place a paper disk (prepared according to disk preparation below) of the tested enzyme on each of the 6 inoculated plates.

Disk preparation

Make a 10% solution of the enzyme by adding 1 g of enzyme to 9 ml of sterile, distilled water.

Mix thoroughly with a Vortex mixer to obtain a homogeneous suspension. Autoclave suitably paper disks (for instance, S&S Analytical Filter Papers No. 740-E, 12.7 mm in diameter), then saturate them with the enzyme by application of 0.1 ml (about 3 drops) of a 10% solution of the enzyme to the disk surface. Prepare 6 disks (1 for each of the 6 organisms) for each enzyme: place one disk on the surface of the 6 inoculated agar plates.

Incubation

Keep the 6 plates in the refrigerator overnight to obtain proper diffusion. Incubate the plates at 37° for 24 hours. Examine the plates for any for any inhibition zones that may have been caused by the enzyme preparation.

Interpretation

A visually clear zone around a disk (total diameter: 16 mm) indicates the presence of antibacterial components in the enzyme preparation. If an enzyme preparation shows obvious antibacterial activity against 3 (or more) organisms it is concluded that antibiotic agents are present.

ASSAY OF ENDO-1,4-β-GLUCANASE ACTIVITY

Principle

Endo-1,4-β-glucanase in the sample hydrolyses the substrate, hydroxyethyl cellulose, and the reducing sugars thus produced are assayed spectrophotometrically using dinitrosalicylic acid.

Unit of activity

One endo-1,4-β-glucanase unit (ECU) is defined as the amount of enzyme producing one nmole of reducing sugars as glucose in one second (1 ECU = 1 nkat).

Field of application

The method is suitable for measurement of celluloses from *Trichoderma* origin. The method is not specific for endo-1,4-β-glucanase. Other hydroxyethyl cellulose degrading enzymes (e.g. β-glucosidase) may have an effect on the result.

Safety

DNS-reagent is harmful by inhalation, in contact with skin and eyes and if swallowed.

Assay conditions

Substrate	hydroxyethyl cellulose
pH	4.8
Incubation temperature	50 ºC ± 0.5 ºC
Incubation time	10 min

Equipment

Water bath	50 ºC
Water bath	100 ºC
Test tube mixer (vortex)	
Spectrophotometer	

Reagents

All solutions are prepared in deionized water, Milli-Q or equivalent.
1. Sodium Citrate Buffer (0.05 M, pH 4.8)
Dissolve 10.51 g of citric acid ($C_6H_8O_7 \cdot H_2O$, Merck 244) in about 800 ml of water. Adjust the pH to 4.8 with the 1.0 M NaOH (consumption about 90 ml). Make up to 1000 ml with water in a volumetric flask.

2. Substrate
Dissolve 1.00 g hydroxyethyl cellulose (mittelviskos, Fluka AG, 54290 or Aldrich 434965) in 100 ml of sodium citrate buffer. The powder is dissolved with magnetic stirring over at night. Store at + 4 ºC for a maximum of 2 weeks, may also freeze.

3. DNS reagent
Dissolve 50.0 g of 3,5-dinitrosalicylic acid (Sigma D-0550) in about 4 l of water. With continuous magnetic stirring, gradually add 80.0 g of NaOH and let it dissolve. Add 1500 g of Rochelle Salt (K-Na-tartrate, Merck 8087) in small portions with continuous stirring. The solution may be cautiously warmed to a maximum temperature of 45 ºC. Cool to room temperature and make up to 5000 ml with water in a volumetric flask. If the solution is not clear, filter through Whatman 1 filter paper. Storing in a dark bottle at room temperature.

Sample

The sample is diluted in 0.05 M sodium citrate buffer. A suitable dilution will yield an absorbance of 0.20 - 0.25 in the reaction.

Assay

Add 1.8 ml of substrate solution to each of two test tubes and equilibrate at 50 ºC for 5 min. Add 200 µl of diluted sample solution to one of the tubes and mix with a vortex mixer. After exactly 10 min incubation, add 3.0 ml of DNS reagent to both tubes and mix. Add 200 µl of sample solution to that tube (blank) which was incubated without sample. Place both tubes in a boiling water bath one at a time. After boiling for exactly 5 min, remove the tubes and cool to room temperature. Measure the sample absorbance against the blank at 540 nm. Read the activity from the standard curve and multiply the result by the dilution factor.

Standard

Prepare 0.1 M stock solution of glucose. Dissolve 1.802 g of glucose (Merck, 8337)[1.] in sodium citrate buffer and make the volume up to 100 ml. Make the following dilutions from the stock solution in sodium citrate buffer:

Dilution	Glucose concentration µmol/ml	Activity ECU/ml[2.]
1:25	4.0	6.67
1:15	6.67	11.11
1:10	10.00	16.67

The standard solutions can be frozen. Do triplicate assays of each standard dilution. Pipette to test tubes 1.8 ml of substrate, 200 µl of standard dilution and 3.0 ml of DNS reagent. Boil for exactly 5 min, cool and measure the absorbances against the reagent blank at 540 nm. The reagent blank is prepared by adding 200 µl of citrate buffer instead of the standard dilution.

1.) Store in a desiccator
2.) The corresponding endo-1,4-β-glucanase activity (nkat/ml) is obtained by multiplying the glucose concentration (µmol/ml) by 1000 and dividing by the time of the hydrolysis, 600 s

Reference

Bailey, M.J., Nevalainen, K.M.H., Enzyme Microb. Technol. 3, 1981, 153-157.

Updated version

The method have updated from version B-030-C, (5.7.1999, Marja Turunen)

- The assay of sodium citrate buffer has changed. The old assay has removed:" Prepare 0.05 M solutions of both citric acid ($C_6H_8O_7 \cdot H_2O$, 10.51 g/l) and sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$, 14.71 g/l) in water. Adjust the pH of the 0.05 M citrate solution to 4.8 with the 0.05 M citric acid solution (should require about 667 ml of citric acid solution per 1 l of sodium citrate solution)."
- The assay of substrate solution has changed. The old assay has removed: "Dissolve 1.00 g hydroxyethyl cellulose (mittelviskos, Fluka AG, 54290) in citrate buffer and make the volume up to 100 ml. The powder is dissolved with magnetic stirring for at least one hour, after which it must stand for a further 1 h to clarify. Store at + 4 ºC for a maximum of 2 weeks."
- To the Standard has added: "The standard solutions can be frozen".

DETERMINATION OF LIPASE ACTIVITY

Version history

Date	Describe shortly what was changed	Who made changes
26.9.2014	1st version, translated from Finnish method description "Lipaasiaktiivisuuden määritys"	Vilma Ikonen
11.12.2014	Revised	Outi Haapala
17.12.2014	Approved	Susanna Eerola

Principle

Method is based on titration of butyric acid, which is released when tributyrin is degraded. pH is kept constant at 7.0 during titration by adding 0.025 M NaOH. The best result is achieved when consumption of NaOH is in 10 minutes 1.0 – 3.0 ml (0.1 – 0.3 ml/min).

Unit of activity

1 ALU/g is an amount of enzyme which releases 1 µmol of butyric acid in 1 minute in specified conditions (30 °C, pH 7.0).

Field of application

Method is suitable for determination of lipase activity in lipase/esterase preparations and from mixed enzyme preparations, except for those that contain protease activity.

Safety and environmental aspects

No special remarks.

Assay conditions

Substrate	Tributyrin
pH	7.0
Temperature	30 °C ± 0.5 °C
Reaction time	10 min

Equipment

Titrator, e.g. Mettler T50M with 30 °C waterbath.

Reagents

Ion exchanged water (Milli-Q or equivalent) is used in preparation of all reagents and in all steps of analysis.

1. 0.025 M NaOH
   E.g. Titrisol Merck 9959 diluted in 2000 ml in volumetric flask. Further dilution 1:2 (e.g. 250 ml into 500 ml)

2. 0.01 M NaOH
   E.g. 10 ml of 1 M NaOH diluted in 1000 ml in volumetric flask.

3. Gum arabic emulsion
   3 g of gum arabic (Sigma G9752) is slowly added into approx. 30 ml of water on magnetic stirrer. Wait until dissolved.

   8.95 g of NaCl and 0.205 g of KH_2PO_4 are dissolved in approx. 80 ml of water. 270 ml of glycerol and dissolved gum arabic are added. pH is adjusted to 4.5 (1 M NaOH/1 M HCl). Solution is transferred into 500 ml volumetric flask and filled with water. Solution is stored in cold and can be used for one month.

4. 0.17 M tribytyrin substrate
   15 ml of tributyrine (Carl Roth 4813.1), 50 ml of gum arabic solution and 235 ml of water are combined in 2 liter plastic decanter. Substrate is emulsified with Ultra-Turrax mixer, 5 min, approx. 11000 rpm. Substrate is kept on magnetic stirrer during the analysis. Fresh substrate is prepared daily.

Sample preparation

Liquid samples:
Weigh 0.25 – 0.5 g accurately in 25 ml volumetric flask, fill up with water and mix carefully.

Dry samples:
Weigh 0.5 – 1.0 g accurately in 50 ml volumetric flask, fill up with water and mix carefully on magnetic stirrer for 20 – 30 min in room temperature.

Samples are diluted with water to reach 0.1 – 0.3 ml/min consumption of 0.025 M NaOH. Sample dilutions must be freshly prepared.

Assay

Calibrate pH-meter in titrator (Toimintaohje L011 Mettler T50M titraattorin käyttöohje). Pour 30 ml of substrate, which is on magnetic stirrer, into large ground glass test tubes. Each tube is in turn equilibrated at 30 °C for at least 5 min, after which the solution is poured into equilibrated titration vessel. After preliminary titration, 2 ml of room temperature enzyme solution is added. Titrator (Mettler T50M) has been programmed with titration protocol, which adjusts pH to 7.0 and calculates the NaOH consumption during 3 – 10 minutes. After the titration protocol is run, the titration vessel is rinsed with 0.01 M NaOH. Vessel is filled and the mixing program of the titrator is used for approx. 1 min.

Waste from titration contains butyric acid and it must be disposed in well ventilated room, e.g. in autoclave room.

Calculation

Formula for ALU calculation:

$$ALU/g = \frac{\Delta V*25*1000}{m*2}$$

where
ΔV = NaOH consumption as ml/min
25 = concentration of NaOH (µmol)
1000 = coefficient, which modifies the concentration into g/ml
m = concentration of sample dilution mg/ml

Example 1.
500 mg of enzyme has been weighed, diluted into 100 ml.
Titrator gives consumption 0.2067 ml/min.

$$ALU/g = \frac{0.2067\ ml/min*25*1000\ mg/g}{500/100\ (mg/ml)*2\ ml} = 516.75\ ALU/g$$

References

Determination of lipase (LU) –activity, 1/ABEG-FEA/001 Rev. 0
Toimintaohje L011 Mettler T50M titraattorin käyttöohje.

ASSAY OF ENDO-1,4-β-GLUCANASE ACTIVITY

Principle

Endo-1,4-β-glucanase in the sample hydrolyses the substrate, hydroxyethyl cellulose, and the reducing sugars thus produced are assayed spectrophotometrically using dinitrosalicylic acid.

Unit of activity

One endo-1,4-β-glucanase unit (ECU) is defined as the amount of enzyme producing one nmole of reducing sugars as glucose in one second (1 ECU = 1 nkat).

Field of application

The method is suitable for measurement of celluloses from *Trichoderma* origin. The method is not specific for endo-1,4-β-glucanase. Other hydroxyethyl cellulose degrading enzymes (e.g. β-glucosidase) may have an effect on the result.

Safety

DNS-reagent is harmful by inhalation, in contact with skin and eyes and if swallowed.

Assay conditions

Substrate	hydroxyethyl cellulose
pH	4.8
Incubation temperature	50 ºC ± 0.5 ºC
Incubation time	10 min

Equipment

Water bath	50 ºC
Water bath	100 ºC
Test tube mixer (vortex)	
Spectrophotometer	

Reagents

All solutions are prepared in deionized water, Milli-Q or equivalent.
1. Sodium Citrate Buffer (0.05 M, pH 4.8)
Dissolve 10.51 g of citric acid ($C_6H_8O_7 \cdot H_2O$, Merck 244) in about 800 ml of water. Adjust the pH to 4.8 with the 1.0 M NaOH (consumption about 90 ml). Make up to 1000 ml with water in a volumetric flask.

2. Substrate
Dissolve 1.00 g hydroxyethyl cellulose (mittelviskos, Fluka AG, 54290 or Aldrich 434965) in 100 ml of sodium citrate buffer. The powder is dissolved with magnetic stirring over at night. Store at + 4 ºC for a maximum of 2 weeks, may also freeze.

3. DNS reagent
Dissolve 50.0 g of 3,5-dinitrosalicylic acid (Sigma D-0550) in about 4 l of water. With continuous magnetic stirring, gradually add 80.0 g of NaOH and let it dissolve. Add 1500 g of Rochelle Salt (K-Na-tartrate, Merck 8087) in small portions with continuous stirring. The solution may be cautiously warmed to a maximum temperature of 45 ºC. Cool to room temperature and make up to 5000 ml with water in a volumetric flask. If the solution is not clear, filter through Whatman 1 filter paper. Storing in a dark bottle at room temperature.

Sample

The sample is diluted in 0.05 M sodium citrate buffer. A suitable dilution will yield an absorbance of 0.20 - 0.25 in the reaction.

Assay

Add 1.8 ml of substrate solution to each of two test tubes and equilibrate at 50 ºC for 5 min. Add 200 µl of diluted sample solution to one of the tubes and mix with a vortex mixer. After exactly 10 min incubation, add 3.0 ml of DNS reagent to both tubes and mix. Add 200 µl of sample solution to that tube (blank) which was incubated without sample. Place both tubes in a boiling water bath one at a time. After boiling for exactly 5 min, remove the tubes and cool to room temperature. Measure the sample absorbance against the blank at 540 nm. Read the activity from the standard curve and multiply the result by the dilution factor.

Standard

Prepare 0.1 M stock solution of glucose. Dissolve 1.802 g of glucose (Merck, 8337)[1.] in sodium citrate buffer and make the volume up to 100 ml. Make the following dilutions from the stock solution in sodium citrate buffer:

Dilution	Glucose concentration µmol/ml	Activity ECU/ml[2.]
1:25	4.0	6.67
1:15	6.67	11.11
1:10	10.00	16.67

The standard solutions can be frozen. Do triplicate assays of each standard dilution. Pipette to test tubes 1.8 ml of substrate, 200 µl of standard dilution and 3.0 ml of DNS reagent. Boil for exactly 5 min, cool and measure the absorbances against the reagent blank at 540 nm. The reagent blank is prepared by adding 200 µl of citrate buffer instead of the standard dilution.

1.) Store in a desiccator
2.) The corresponding endo-1,4-β-glucanase activity (nkat/ml) is obtained by multiplying the glucose concentration (µmol/ml) by 1000 and dividing by the time of the hydrolysis, 600 s

Reference

Bailey, M.J., Nevalainen, K.M.H., Enzyme Microb. Technol. 3, 1981, 153-157.

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- The assay of sodium citrate buffer has changed. The old assay has removed:" Prepare 0.05 M solutions of both citric acid ($C_6H_8O_7 \cdot H_2O$, 10.51 g/l) and sodium citrate ($C_6H_5O_7Na_3 \cdot 2H_2O$, 14.71 g/l) in water. Adjust the pH of the 0.05 M citrate solution to 4.8 with the 0.05 M citric acid solution (should require about 667 ml of citric acid solution per 1 l of sodium citrate solution)."
- The assay of substrate solution has changed. The old assay has removed: "Dissolve 1.00 g hydroxyethyl cellulose (mittelviskos, Fluka AG, 54290) in citrate buffer and make the volume up to 100 ml. The powder is dissolved with magnetic stirring for at least one hour, after which it must stand for a further 1 h to clarify. Store at + 4 ºC for a maximum of 2 weeks."
- To the Standard has added: "The standard solutions can be frozen".

ASSAY OF XYLANASE ACTIVITY

Principle

Xylanase in the sample hydrolyses the substrate, birch xylan, and the amount of released reducing carbohydrates is determined spectrophotometrically using dinitrosalicylic acid.

Unit of activity

One xylanase unit (BXU) is defined as the amount of enzyme that produces reducing carbohydrates having a reducing power corresponding to one nmol xylose from birch xylan in one second under the assay conditions (1 BXU = 1 nkat).

Field of application

The method is suitable for measurement of enzyme samples containing xylanase. The linearity of the assay should be checked before measurements are done.

Safety

DNS-reagent is harmful by inhalation, in contact with skin and eyes and if swallowed.

Assay conditions

Substrate	beech xylan
pH	5.3
Temperature	50 ºC ± 0.5 ºC
Incubation time	5 min

Equipment

Water bath	50 ºC
Water bath	100 ºC
Test tube mixer (vortex)	
Spectrophotometer	540 nm

or option
Liquid handling robot (Tecan Freedom EVO or equivalent)
Microplate reader (Tecan Infinite M200 or equivalent)

Reagents

All solutions are prepared in deionized water, Milli-Q or equivalent.

1. Sodium Citrate buffer (0.05 M, pH 5.3)
Dissolve 10.5 g of citric acid ($C_6H_8O_7 \cdot H_2O$, Merck 100244) in about 800 ml of water and adjust the pH with 1 M NaOH to 5.3 (the consumption should be about 110 ml). Make up to 1000 ml with water in a volumetric flask.

Note: Into the buffers which are used for premix samples add 20 g EDTA (disodium salt, Titriplex III, Merck 108418) before adjusting the pH and make up to 1000 ml in water.

2. Substrate - 1 % beech xylan.
Homogenize 1.0 g of xylan (from beechwood, Sigma X-4252) by adding the powder gradually in about 80 ml of citrate buffer heated to 60 ºC using a kitchen blender or magnetic stirrer. Heat to boiling point on a heating magnetic stirrer. Cool with continued stirring, cover and stir slowly overnight at room temperature. Make the volume up to 100 ml with the citrate buffer. If the solution is not clear centrifuge (e.g. Sorvall GSA, 8000 rpm, 20 min.) it to remove the precipitate. Store at + 4 ºC for a maximum of 1 week or freeze in aliquots at –20 ºC. Mix well after thawing.

3. DNS reagent
Dissolve 50.0 g of 3,5-dinitrosalicylic acid (Sigma D-0550) in about 4 l of water. With continuous magnetic stirring, gradually add 80.0 g of NaOH and let it dissolve. Add 1500 g of Rochelle Salt (K-Na-tartrate, Merck 108087) in small portions with continuous stirring. The solution may be cautiously warmed to a maximum temperature of 45 ºC. Cool to room temperature and make up to 5000 ml with water in a volumetric flask. If the solution is not clear, filter through Whatman No. 1 filter paper. Store in a dark bottle at room temperature.

Sample preparation

Liquid enzymes: Weigh approximately 0.25 g - 0.5 g of enzyme liquid accurately in 25 ml volumetric flask, fill the flask up to the mark by citrate buffer and mix properly.

Dry enzymes: Weigh approximately 0.5 g - 1 g enzyme accurately in 50 ml volumetric flask, fill the flask up to the mark by citrate buffer and stir on a magnetic plate for 20-30 minutes at room temperature. Sample size is recommended to be increased to 2.5 g (in 100 ml) if the sample is inhomogenous or high in bulk density (> 1kg/l). When needed centrifuge for 10 minutes at ca. 2800 x g.

Vitamin/mineral premixes: Weight approximately 5 g of enzyme-containing premix accurately in 100 ml beaker and add 40 ml citrate buffer containing 2% (w/v) EDTA. Stir samples for 30 minutes on a magnetic stirrer plate at room temperature and centrifuge for 10 minutes at ca. 2800 x g.

The sample is diluted in citrate buffer. A suitable dilution will yield an absorbance difference of 0.1 – 0.4 (*Trichoderma*) or 0.2 – 0.4 (*Bacillus, Aspergillus*). Using liquid handling robot the absorbance difference should be between 0.25 - 0.5.

Assay

Manual method:
Add 1.8 ml of substrate solution to each of two test tubes and equilibrate at 50 ºC for 5 minutes. Add 200 µl of diluted enzyme solution to one of the tubes and mix with a vortex mixer. Enzymeblanks don't need to incubate. After exactly 5 minutes, add 3.0 ml of DNS reagent to both tubes, mix and remove the tubes from the water bath. Add 200 µl of sample solution to the enzyme blank tubes. Place the tubes in a boiling water bath one at a time. After boiling for exactly 5 minutes remove the tubes and cool in cold water to room temperature. Measure the sample absorbance against the enzyme blank at 540 nm. Read the activity from the standard line and multiply by the dilution factor.

Liquid handling robot:
Work in accordance with the instructions of Tecan Freedom EVO

Standard

Prepare a 10 mM xylose stock solution. Dissolve 150 mg of xylose [1.](Merck 108689) in citrate buffer and make up to 100 ml with buffer in a volumetric flask. Stock solution can be frozen in small aliquots at - 20 ºC; after thawing the tubes must be carefully mixed. The stock solution is diluted in buffer as follows:

Dilution	Xylose µmol/ml	[2.]BXU/ml
1:1	10.0	33.3
1:2	5.0	16.7
1:3	3.33	11.0
1:5	2.0	6.7

[1.] Store in a desiccator.

[2.)] The xylanase activity (BXU/ml) is obtained by multiplying the xylose concentration (µmol/ml) by 1000 and dividing by the reaction time (300 s). Standard solutions can be frozen in small aliquots at -20 ºC. Do duplicate assays of each standard dilution in the same way as the enzyme blanks: pipette into test tubes 1.8 ml of substrate, add 3.0 ml of DNS and 200 µl of standard dilution. Prepare the reagent blank by adding 200 µl of citrate buffer instead of the standard dilution. Boil the tubes exactly 5 minutes, cool and measure the absorbances against the reagent blank at 540 nm. Construct a standard line for every series of assays.

Calculation

Subtract the blank absorbance from the sample absorbance. Read the activity (BXU/ml) from the standard curve. Multiply it by the dilution factor. The final calculated activity is represented in xylanase units per gram of original sample (BXU/g). Calculations (in BXU) are presented below

$$\text{BXU/g} = \frac{\text{BXU/ml x volume of extraction buffer (ml) x dilution factor}}{\text{weight of sample extracted (g)}}$$

where:

BXU/ml	*= activity of the unknown sample (from standard curve)*
Vol of extraction buffer	*= 25 ml/50 ml for enzymes and 40 ml for premixtures*
Dilution factor	*= total dilution factor*
Weight of sample extracted	*= appr. 0.25 g - 1.0 g for enzyme preparations and appr. 5 g for premixtures*

Reference

Bailey, M.J. and Poutanen, K. (1989). Production of xylanases by strains of Aspergillus. Appl. Microbiol. Biotechnol. 30:5-10

Additions/Changes

The method has updated from the version B-038-G (19.1.2012).

- Instructions of liquid handling robot
- substrate

ASSAY OF PROTEASE ACTIVITY

Version history

Date	Describe shortly what was changed	Who made changes
25.9.2014	Version 2.0. updated header	Sari Suominen
27.10.2014	Deleted all the references to kat/g –unit, updated calculation example, ε value checked	Vilma Ikonen, Markus Kaarnakoski
29.10.2014	Revised	Susanna Eerola
29.10.2014	Approved	Susanna Eerola
16.12.2014	2.1 Added Version number to header	Anni Honkanummi

Principle

Protease in a sample catalyzes hydrolysis of denatured hemoglobin substrate. Hemoglobin that is not hydrolyzed is precipitated with trichloroacetic acid and separated by filtration. The hydrolysis products of hemoglobin are measured by spectrophotometry.

Unit of activity

One unit of protease (UHb) is defined as enzyme activity, which in one minute under standard conditions (37 ºC, defined pH, 280 nm) catalyzes the release of trichloroacetic acid soluble hemoglobin compounds, equivalent to 1µmol tyrosin solution. Unit of activity is UHb/g. pH-value has to be report with results, e.g. 67.3 UHb6.8/g.

Field of application

The method is suitable for measurement of protease activity.

Safety and environmental aspects

Trichloroacetic acid (TCA) is strong toxic acid. The solutions corrode skin, mucous membranes and eyes. Vapors irritate airway. Wear protective glass and gloves. Strong reagent is preferably handled in a fume cupboard. TCA-waste is collected and taken to a hazardous waste treatment plant.

Equipment

Water bath 37 ºC± 0.5 ºC
Spectrophotometer

Reagents

All reagents are prepared in deionized water, Milli-Q or equivalent

1. 1.0 M Calcium chloride solution
Dissolve 147.0 g of $CaCl_2 \cdot 2\ H_2O$ (Merck 2382) in water and make up to 1000 ml.

2. 10 mM Calcium chloride solution
Dissolve 10 ml of 1.0 M calcium chloride solution in water and make up to 1000 ml.

3. Disodium hydrogen phosphate solution, 0.2 M
Dissolve 35.6 g of disodium hydrogen phosphate ($Na_2HPO_4 \cdot 2\ H_2O$, Merck 1.06580) in water and make up to 1000 ml. Stored in room temperature.

4. Citric acid, 0.1 M
Dissolve 21 g of citric acid monohydrate (Merck 244) in water and make up to 1000 ml. Stored in a refrigerator (+ 2-8 ºC).

5. Potassiumdihydrogenphosphate solution, 1 M
Dissolve 136 g of potassiumdihydrogenphosphate (KH_2PO_4, Merck 4873) in water and make up to 1000 ml. Stored in a refrigerator (+ 2-8 ºC).

6. Trichloroacetic acid, 0.3 M
Dissolve 49.02 g of trichloroacetic acid (Merck 807, stored in the desiccator) in water and make up to 1000 ml.

7. NaOH, 0.5 M

8. Tyrosine standard
Stock solution: dissolve 181.19 mg of L-tyrosine (Merck 8371) in 0.2 M hydrochloric acid and make up to 1000 ml with hydrochloric acid.
Dilution: dilute 30 ml of stock solution to 100 ml with 0.2 M hydrochloric acid. Tyrosine concentration of this dilution is 0.3 mmol/l.

9. Substrate solutions

Reagents	**pH 4.4**	**pH 6.8**	**pH 8.5**
Hemoglobin	4.60 g	4.60 g	4.60 g
Water	100 ml	100 ml	100 ml
Urea	88 g	88 g	88 g
0,5 M NaOH	32 ml	32 ml	32 ml
Na_2HPO_4, 0.2 M	17.64 ml	-	-
Citric acid, 0.1 M	22.36 ml	-	-

KH_2PO_4, 1 M	-	40 ml	30 ml
Hydrochloric acid 1M *	to pH adjustment		-
NaOH 1M	-	-	to pH adjustment
Water	filled up to the total weight of 280 g		

*Initial pH is adjusted with concentrated hydrochloric acid

Tare a 300 ml glass beaker and weigh hemoglobine (Bovine hemoglobin, MP Biomedicals, CAT NO. 100714), add water while stirring about 10 min. The temperature drops, when urea (Merck 1.08487) is added. The mixture must be warmed rapidly to not more than 25 ºC in a water bath (about 60 ºC) while stirring on magnetic plate. Add NaOH and stir on a magnetic plate for 30 min at room temperature (denaturation).
Add sodium phosphate, citric acid and potassium phosphate. Adjust the pH to right value with HCl or NaOH. The substrate solution is filled up to the total weight of 280g with water and stored in the refrigerator (+ 2-8 ºC) in dark bottle. The substrate solution is stable for four days.

Sample preparation

Liquid enzymes: Weigh accurately 0.25 g - 0.5 g of enzyme liquid in 25 ml volumetric flask, fill the flask up to the mark by 10 mM $CaCl_2$ solution and mix properly.

Dry enzymes: Weigh accurately 0.5 g - 1 g enzyme in 50 ml volumetric flask, fill the flask up to the mark by 10 mM $CaCl_2$ solution and stir on a magnetic plate for 15-20 minutes at room temperature.

The sample is diluted with 10 mM $CaCl_2$ solution. A suitable dilution will yield about 0.5-0.75 UHb/ml. An absorbance difference should be between 0.25-0.40. Enzyme dilution is stable not more than one hour.

Assay

Add 5 ml of substrate into 100 ml vessels and temperate for 10 min at 37 ºC. Add 1 ml of sample and mix. Stop the reaction exactly after 10 min by adding 10 ml of 0.3 M trichloroacetic acid. After being left to stand for minimum 30 min at room temperature, the composition is passed through a filter (e.g. Munktell V 5 100 pcs, 125 mm).

Enzyme blanks: Add 5 ml of substrate solution (at room temperature), 10 ml of 0.3 M trichloroacetic acid and 1 ml of sample into the 100 ml vessels. Enzyme blanks don't need to be incubated. After being left to stand for 30 min at room temperature, the enzyme blanks are treated as described above.

Samples and enzyme blanks are measured against water at 280 nm in UV-cuvettes.

Tyrosine standard

To determine the molar extinction coefficient the tyrosine standard is measured with each analysis. Absorbance of the diluted tyrosine solution is measured against 0.2 M hydrochloridic acid.

The measured absorbance is divided with the concentration and the cuvette diameter (10 mm). Absorbance should be 0.35-0.37. AutoLIMS uses for calculation the value of 0.360935.

Calculation

Formula for calculating the molar extinction coefficient:

$$(1)\quad \varepsilon(\text{l/mmol/mm}) = \frac{E_{280}}{c(\text{mmol/l}) \cdot d(\text{mm})}$$

c = 0,3 mmol/l

Formula for calculating the activity:

$$(2)\quad \text{UHb}(\mu\text{mol/min}) = \frac{\Delta E_{280} \cdot V(\text{l}) \cdot 1000}{\varepsilon(\text{l/mmol/mm}) \cdot d(\text{mm}) \cdot v(\text{l}) \cdot t(\text{min})}$$

Final formula for calculating the activity concentration UHb/g:

$$(3)\ \text{UHb/g} = \frac{\Delta E_{280} \cdot V(\text{l}) \cdot 1000}{\varepsilon(\text{l/mmol/mm}) \cdot d(\text{mm}) \cdot v(\text{l}) \cdot t(\text{min}) \cdot c_s(\text{g/l})}$$

The established parameters can be combined to form a constant:

$$(4)\ K = \frac{V}{d \cdot v \cdot t} = \frac{0{,}016\text{l}}{10\text{mm} \cdot 0{,}001\text{l} \cdot 10\ \text{min}}$$

$$\Delta E_{280}$$

$$(5)\ UHb/g = \frac{}{\varepsilon \cdot c_s} \cdot K \cdot 1000$$

The average E_{280} value in 2005 for tyrosine solution was 0.360935. The ε value calculated from that was 0.120337 l/mmol/mm. These values are used as constants in the calculations. (The value has been checked in October 2014 with the reference to the absorbance data from 2012-2013 and found out to be valid.)

Example

0,755 g of sample was weighed into 50 ml bottle and filled to mark. The sample was further diluted in a ratio 1:20. The concentration c_s of this solution was 0.755 g/l. The measured absorbance difference was 0.371.

ε = 0,120337 l/mmol/mm
v = 0.001 l
V = 0.016 l
t = 10 min
d = 10 mm
K = 0.16 mm/min
1000 = conversion factor from mmol to µmol

$$UHb/g = \frac{0.371}{0{,}120337\ (mmol/mm) \cdot 0.755(g/l)} \cdot 160(l/mm/min) = 653\ UHb/g$$

Symbols

ε (l/mmol/mm)	molar extinction coefficient
E_{280}	extinction at 280 nm
ΔE_{280}	difference in extinction at 280 nm
V (l)	volume of sample
v (l)	volume of sample
t (min, s)	time
d (mm)	layer thickness
c_s (g/l)	concentration in the composition

Reference

Determination of proteinase activity (UHb), 1/REG-FEA/009, Rev 6

Water quality — Application of inductively coupled plasma mass spectrometry (ICP-MS) —

Part 2:
Determination of 62 elements

WARNING — Persons using this part of ISO 17294 should be familiar with normal laboratory practice. This part of ISO 17294 does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices and to ensure compliance with any national regulatory conditions.

IMPORTANT — It is absolutely essential that tests, conducted in accordance with this part of ISO 17294, be carried out by suitably qualified staff.

1 Scope

This part of ISO 17294 specifies a method for the determination of the elements aluminium, antimony, arsenic, barium, beryllium, bismuth, boron, cadmium, caesium, calcium, cerium, chromium, cobalt, copper, dysprosium, erbium, europium, gadolinium, gallium, germanium, gold, hafnium, holmium, indium, iridium, lanthanum, lead, lithium, lutetium, magnesium, manganese, molybdenum, neodymium, nickel, palladium, phosphorus, platinum, potassium, praseodymium, rubidium, rhenium, rhodium, ruthenium, samarium, scandium, selenium, silver, sodium, strontium, terbium, tellurium, thorium, thallium, thulium, tin, tungsten, uranium, vanadium, yttrium, ytterbium, zinc, and zirconium in water [for example drinking water, surface water, groundwater, wastewater and eluates (9.2)].

Taking into account the specific and additionally occurring interferences, these elements can also be determined in digests of water, sludges and sediments (for example digests of water as specified in ISO 15587-1 or ISO 15587-2).

The working range depends on the matrix and the interferences encountered. In drinking water and relatively unpolluted waters, the limit of application is between 0,1 µg/l and 1,0 µg/l for most elements (see Table 1).

The detection limits of most elements are affected by blank contamination and depend predominantly on the laboratory air-handling facilities available.

The lower limit of application is higher in cases where the determination is likely to suffer from interferences (see Clause 5) or in case of memory effects (see 8.2 of ISO 17294-1).

Table 1 — Limits of application for unpolluted water

Element	Isotope often used	Limit of application[a] µg/l
Ag	^{107}Ag	1
	^{109}Ag	1
Al	^{27}Al	5
As	^{75}As	1
Au	^{197}Au	0,5
B	^{10}B	10
	^{11}B	10
Ba	^{137}Ba	3
	^{138}Ba	0,5
Be	^{9}Be	0,5
Bi	^{209}Bi	0,5
Ca	^{43}Ca	100
	^{44}Ca	50
	^{40}Ca	10
Cd	^{111}Cd	0,1
	^{114}Cd	0,5
Ce	^{140}Ce	0,1
Co	^{59}Co	0,2
Cr	^{52}Cr	1
	^{53}Cr	5
Cs	^{133}Cs	0,1
Cu	^{63}Cu	1
	^{65}Cu	2
Dy	^{163}Dy	0,1
Er	^{166}Er	0,1
Eu	^{151}Eu	0,1
	^{153}Eu	0,1
Ga	^{69}Ga	0,3
	^{71}Ga	0,3
Gd	^{157}Gd	0,1
	^{158}Gd	0,1
Ge	^{74}Ge	0,3
Hf	^{178}Hf	0,1
Ho	^{165}Ho	0,1
In	^{115}In	0,1
Ir	^{193}Ir	0,1
K	^{39}K	50
La	^{139}La	0,1
Li	^{6}Li	10
	^{7}Li	1
Lu	^{175}Lu	0,1
Mg	^{24}Mg	1
	^{25}Mg	10
Mn	^{55}Mn	3
Mo	^{95}Mo	0,5
	^{98}Mo	0,3
Na	^{23}Na	10
Nd	^{146}Nd	0,1
Ni	^{58}Ni	1
	^{60}Ni	3
P	^{60}P	5,0
Pb	^{206}Pb[b]	0,2
	^{207}Pb[b]	0,2
	^{208}Pb[b]	0,1
Pd	^{108}Pd	0,5
Pr	^{141}Pr	0,1
Pt	^{195}Pt	0,5
Rb	^{85}Rb	0,1
Re	^{185}Re	0,1
	^{187}Re	0,1
Rh	^{103}Rh	0,1
Ru	^{101}Ru	0,2
	^{102}Ru	0,1
Sb	^{121}Sb	0,2
	^{123}Sb	0,2
Sc	^{45}Sc	5
Se	^{77}Se	10
	^{78}Se	10
	^{82}Se	10
Sm	^{147}Sm	0,1
Sn	^{118}Sn	1
	^{120}Sn	1
Sr	^{86}Sr	0,5
	^{88}Sr	0,3
Tb	^{159}Tb	0,1
Te	^{126}Te	2
Th	^{232}Th	0,1
Tl	^{203}Tl	0,2
	^{205}Tl	0,1
Tm	^{169}Tm	0,1
U	^{238}U	0,1
V	^{51}V	1
W	^{182}W	0,3
	^{184}W	0,3
Y	^{89}Y	0,1
Yb	^{172}Yb	0,2
	^{174}Yb	0,2
Zn	^{64}Zn	1
	^{66}Zn	2
	^{68}Zn	3
Zr	^{90}Zr	0,2

[a] Depending on the instrumentation significantly lower limits can be achieved.

[b] In order to avoid mistakes due to the different isotope ratios in the environment, the signal intensities of ^{206}Pb, ^{207}Pb and ^{208}Pb shall be added.

AE 114

Nr. 161
1998

POHJOISMAINEN
ELINTARVIKKEIDEN
METODIIKKAKOMITEA

NORDIC COMMITTEE ON FOOD ANALYSIS

No 161
1998

METALLIT. MÄÄRITTÄMINEN ELINTARVIKKEISTA ATOMIABSORPTIOSPEKTROME TRISESTI MIKROAALTOUUNISSA TAPAHTUVAN MÄRKÄPOLTON JÄLKEEN

Tämä NMKL-menetelmä on validoitu kollaboratiivisessa tutkimuksessa AOAC International Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis -ohjeiden mukaan.

1. TARKOITUS JA SOVELTAMISALA

Tämä menetelmä kuvaa metallien lyijy, kadmium, sinkki, kupari ja rauta kvantitatiivisen määrittämisen erityyppisistä elintarvikkeista lukuunottamatta öljyjä, rasvoja ja erittäin rasvaisia elintarvikkeita. Menetelmässä käytetään atomiabsorptiospektrometria (AAS) mikroaaltouunissa tapahtuvan paineenalaisen hajoituksen jälkeen. Menetelmä on testattu vain kuivilla materiaaleilla, mutta sitä voidaan tietyissä olosuhteissa käyttää näytteille, jotka sisältävät vettä.

2. PERIAATE

Näyte märkäpoltetaan käyttäen typpihappoa ja vetyperoksidia suljetussa astiassa, jota kuumennetaan mikroaalloilla. Näyteliuos laimennetaan vedellä ja metallien pitoisuudet määritetään AAS:lla käyttäen liekki- tai grafiittiuunitekniikkaa.

3. REAGENSSIT

Reagenssien on oltava vähintään pro analysi -laatua, mieluummin suprapur-laatua, tai vastaavaa.

3.1 Vesi, tislattu tai ionivaihdettu (Millipore tai vastaava laatu).

3.2 Typpihappo, väkevä. (65% w/w).

3.2.1 Typpihappo, 0,1 mol/l: Laimenna vedellä 7 ml väkevää typpihappoa 1000 ml:ksi.

METALS. DETERMINATION BY ATOMIC ABSORPTION SPECTRO-PHOTOMETRY AFTER WET DIGESTION IN A MICROWAVE OVEN

This NMKL method has been validated in a collaborative study according to the AOAC International Guidelines for Collaborative Study Procedures to Validate Characteristics of a Method of Analysis

1. SCOPE AND FIELD OF APPLICATION

This method describes quantitative determination of the metals: lead, cadmium, zinc, copper and iron in various types of foods, with the exception of oils, fats and extremely fatty products. The method employs atomic absorption spectrophotometry (AAS) after microwave oven digestion under pressure. The method has been tested on dry materials only, but may under certain conditions be used for samples containing water.

2. PRINCIPLE

The sample is wet digested with nitric acid and hydrogen peroxide in a sealed container heated by microwaves. The sample solution is diluted with water and the concentrations of the metals are determined by flame or graphite furnace AAS.

3. REAGENTS

Reagents should be of at least analytical grade, preferably of suprapur quality, or equivalent.

3.1 Water, redistilled or deionised (Millipore or equivalent quality).

3.2 Nitric acid, Concentrated. (65% w/w).

3.2.1 Nitric acid, 0.1 mol/l: Dilute 7 ml of conc. nitric acid with water to 1000 ml.

In-house method validation for

lipase activity

December 2014

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1. Introduction

Lipase (ALU) assay for enzyme preparations was validated in-house at Roal Oy's Quality Control Laboratory. The method is based on titration of butyric acid which is released on degradation of tributyrin. The method is suitable for measurement of lipase activity from lipase/esterase preparations or mixed enzyme preparations, excluding those that contain protease activity. The samples used in this study are presented in table 1. Sample referred to as "Control" is a lipase preparation used as control sample in routine analytical procedures.

Table 1. Samples used in the validation process.

Sample name	LIMS ID	Batch	Sample type
RLOP	2014-451	140225915	Semifinal enzyme preparation
Veron Hyperbake-ST	2014-1533	140630249	Enzyme preparation
Control	2014-7	201401358	Enzyme preparation

2. Accuracy

Accuracy was determined with repeatability and intermediate precision (reproducibility) measurements (table 2).
Also bias was measured between Roal Oy QC Laboratory and AB Enzymes laboratory in Darmstadt, Germany. The results have been recorded but are not relevant in this context, since this validation was done only to show accuracy with-in the Roal QC Laboratory.

Table 2. Summarized results of repeatability and reproducibility of RLOP, Veron Hypebake-ST and Control.

	RLOP	Veron Hyperbake-ST	Control
Repeatability (U1)	5738	526	289
Reproducibility (U2)	15520	1298	408
Combined standard uncertainty (U_c)	16547	1400	500
Expanded uncertainty (U)	33094	2801	1000
Measured lipase activity (ALU/g)	126214	23301	11306

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%U	26	12	9

The method uncertainties for RLOP, Veron Hyperbake-ST and Control were 26 %, 12 % and 9 %, respectively.

3. Precision

a) Repeatability

Repeatability was evaluated by analyzing lipase activity of the samples with 15 individual weighings (table 3.)

Table 3. Results of the repeatability measurements.

Sample	n	Lipase activity (ALU/g)	% RSD
RLOP	15	124825 ± 5738	4,6
Veron Hyperbake-ST	15	24280 ± 526	2,2
Control	15	11314 ± 289	2,6

Repeatability for RLOP, Veron Hyperbake-ST and Control were 4,6 %, 2,2 % and 2,6 %, respectively.

b) Intermediate precision

RLOP, Veron Hyperbake-ST and Control were analyzed from 15 weighings by 3 different technicians of Roal Oy Quality Control Laboratory giving altogether 45 observations per sample type. This set-up gave variation between days and between technicians.

Table 4. Results of the intermediate precision measurements.

Sample	Technician	Day	Mean lipase activity (ALU/g)	% RSD
RLOP	1	1	109227 ± 4600	4,2
	2	2	124825 ± 5738	4,6
	3	3	144590 ± 5473	3,8
		1 & 2 & 3	126214 ± 15520	12,3

Veron Hyperbake-ST	1	1	24280 ± 526	2,2
	2	2	23895 ± 710	3,0
	3	3	21728 ± 676	3,1
		1 & 2 & 3	22301 ± 1298	5,6
Control	1	1	11314 ± 289	2,6
	2	2	11589 ± 285	2,5
	3	3	11016 ± 426	3,9
		1 & 2 & 3	11306 ± 408	3,6

Intermediate precision for RLOP, Veron Hyperbake-ST and Control were 12,3 %, 5,6 % and 3,6 %, respectively.

4. Range

According to method B079 (version 1.0) the samples must be diluted so that the consumption of 0,025 M NaOH is between 0,1-0,3 ml/min. Different dilutions of Control were tested and the results are shown in figure 1.
The range was shown to be correct, since the results were quite constant within this range. If the NaOH consumption decreases below 0,1 ml/min or is above 0,3 ml/min there is a notable decline in the lipase activity results.

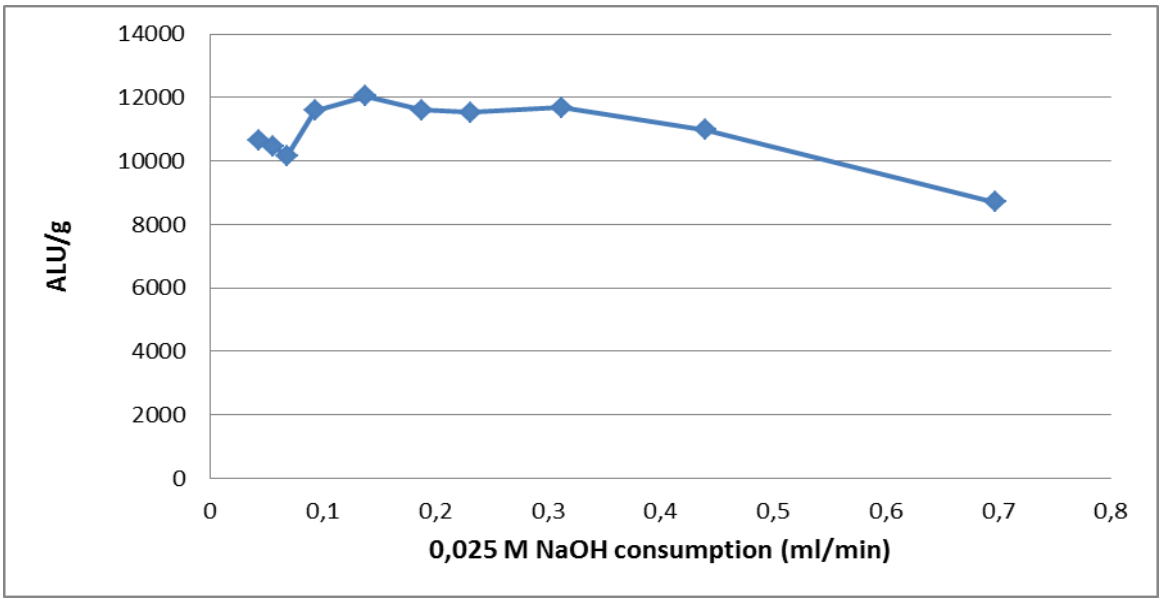

Figure 1. Range for lipase (ALU/g) assay.

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5. Conclusions

The method was validated with three dry enzyme preparations with different activity levels. Method uncertainties for the two products with lower lipase activity levels were approx. 10 %, ie. for Veron Hyperbake-ST and Control. The concentrated dry semifinal product RLOP had notably higher uncertainty (26 %). Reason for this might be the higher dilution factor needed to reach the NaOH consumption of 0,1 – 0,3 ml/min.
When semifinal concentrate RLOP is planned to be used in final product manufacturing, several measurement repetitions are needed to assure that the correct amount of semifinal is dosed into the product mix.

6. Method descriptions

Lipase (ALU) assay description for enzyme preparations is presented in Enclosure 1.

7. References

Determination of lipase (LU) -activity, 1/ABEG-FEA/001 Rev. 0
Operating procedure L011 for Mettler T50M titrator, version 1.0

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ENUMERATION OF COLIFORMS BY A COLONY COUNT TECHNIQUE

Principle

The number of viable coliforms in liquid or dry samples is determined by plating a specified quantity of a sample or sample dilution on Crystal Violet - Neutral Red – Bile – Lactose - Agar (VRBL) dishes. Dishes are incubated at 37°C for 24h under aerobic conditions and the number of coliform colonies is counted.

A typical coliform colony is purple red in color with a diameter › 0,5 mm. Occasionally a reddish precipitate of bile salts is formed around the colonies.

Field of application

Method is applicable for enumeration of viable coliforms from liquid and dried enzyme semifinal- and final products.

Equipment

Autoclave	
Water bath	45 °C ± 0.5°C
Incubator	37 °C ± 1 °C
Vortex	
Sterile Petri dishes:	Ø 80 mm to 100 mm
Sterile dilution bottles	100 ml
Sterile dilution tubes	10 ml
Sterile glass flasks	250 ml
Sterile pipettes	1 ml and 10 ml
Colony counter	

Reagents

All solutions are prepared in deionized water, Milli-Q or equivalent.

1. Crystal Violet - Neutral Red – Bile – Lactose - Agar (VRBL)

Weigh 41.5 g of VRBL Agar (e.g. Difco 0012) and dissolve into 1000 ml of water. Boil to ensure complete dilution of medium components. Obs! Maximum boiling time is 2 minutes. Divide to 100 ml aliquots to sterile 250 ml glass flasks. Do not sterilize in autoclave. VRBL-agar is melted in a microwave oven or water bath and tempered to 45°C before use.

2. Dilution fluid (0.9 % w/v NaCl)

Dilute 9.0 g NaCl (e.g. Merck 6404) into 1000 ml of water. Divide 90 ml aliquots to appropriate glass flasks. Sterilize in autoclave for 15 min at 121 °C.

Samples

1. Dry samples

Aseptically weigh 10.0 g of sample to 90 ml of dilution fluid. Vortex until the mixture is homogenous (= 10^{-1} dilution). Prepare dilution series from the 10^{-1} dilution by pipetting 1 ml of 10^{-1} dilution to 9 ml of dilution fluid (= 10^{-2} dilution) and 1 ml of 10^{-1} dilution to 99 ml of dilution fluid (= 10^{-3} dilution). Vortex all the samples carefully. The dilution series can be continued further by following the dilution principle described above.

2. Liquid samples

Aseptically pipet 10.0 ml of sample to 90 ml of dilution fluid. Vortex until the mixture is homogenous (= 10^{-1} dilution). Prepare dilution series from the 10^{-1} dilution by pipetting 1 ml of 10^{-1} dilution to 9 ml of dilution fluid (= 10^{-2} dilution) and 1 ml of 10^{-1} dilution to 99 ml of dilution fluid (= 10^{-3} dilution). Vortex all the samples carefully. The dilution series can be continued further by following the dilution principle described above.

Procedure

1.0 ml of all samples including the original sample mix or undiluted enzyme sample and all the required dilutions are pipetted aseptically on empty sterile Petri dishes as duplicates. 15 ml of tempered 45°C VRBL-agar is poured to each of the plates. Samples are mixed to VRBL-agar by carefully swaying the plates. Sterility control plates are also prepared by pouring 15 ml of tempered 45°C VRBL-agar only to two sterile Petri dishes. Sterility controls are prepared from every new VRBL-agar batch used. The procedure from preparing the first sample dilution to pouring the VRBL-agar should not take more than 15 minutes. Dishes are placed on an even surface and the agar is let to solidify. Once the agar is solid ~4 ml of tempered 45°C VRBL-agar is poured on top of the first layer of agar on all dishes. Agar is again let to solidify on an even surface. Dishes are moved to 37°C ± 1 incubator and incubated upside down for 24h.

Results

Number of typical coliform colonies (see the "principle" section) is counted from dishes that contain no more than 150 colonies. If the dishes contain more than 150 colonies the appearance of colonies might be non-typical. Colonies are counted from both of the duplicate dishes from two dilutions.

The number of coliforms /ml or /g is calculated accordingly:

$$N = \frac{\Sigma C}{[n_1 + (0{,}1 \times n_2)]\, d}$$

ΣC = combined number of colonies (a´2 dishes from 2 dilutions)
n_1 = number of dishes from the first dilution
n_2 = number of dishes from the second dilution
d = dilution factor of the first dilution

Results are reported with the accuracy of two significant digits/ml or /g.

Example:

First dilution 10^{-2}; colonies on dishes 83 + 97 = 180
Second dilution 10^{-3}; colonies on dishes 13 + 8 = 21

$$N = \frac{180 + 21}{[2 + (0{,}1 \times 2)] \times 10^{-2}} = \frac{201}{0{,}022} = 9\,136 = 9{,}1 \times 10^3 \text{ cfu/ml or /g}$$

cfu = colony forming unit

If no typical colonies are detected in any of the plates the result is reported as ‹ d^{-1} cfu/ml (liquid product) or ‹ d^{-1} cfu/g (dry product) (d = dilution factor from the smallest dilution).

References

International Standard ISO 4832, 2nd edition 1991-03-01. Microbiology - General Guidance for the Enumeration of Coliforms – Colony count technique.

Reviewed by Päivi Paukku

21.8.2013 Päivi Paukku

Approved by Eino Väisänen

21.8.2013 Eino Väisänen

ESCHERICHIA COLI -BAKTEERIN TOTEAMINEN JA PITOISUUDEN MÄÄRITTÄMINEN MPN-TEKNIIKALLA

Kvalitatiivinen määritys

Punnitse 25 g näytettä laktoosipeptonilihaliemeen ja inkuboi 44,0±0,5^{0}C.ssa 2 vuorokautta.

Mikäli inkuboinnin jälkeen liemen väri on muuttunut keltaiseksi/kellertäväksi, suorita positiivisen tuloksen varmistus.

Siirrosta rikasteesta silmukallinen viiteen Fluorocult-LMX-putkeen ja inkuboi 37±1^{0}C:ssa 1 vuorokausi.

Tuloksen ilmoittaminen
Näyte on *Escherichia coli* todettu/25 g, mikäli yksikin LMX-putkista antaa positiivisen *E.coli* –reaktion ja positiivisen testituloksen Kovac´s reagenssilla suoritettavassa indolitestissä.

Kvantitatiivinen määritys

Annostele 10:een koeputkeen kuhunkin 5 ml 3 x väkevöityä laktoosipeptonilihalientä.
Punnitse 25 g entsyyminäytettä + 100g steriiliä vettä.

Sekoita hyvin ja annostele 10:een koeputkeen, kuhunkin putkeen 10 ml.
Mikäli näytettä jää sekoituksesta huolimatta pullon pohjalle, annostele loppu tasaisesti putkiin.

Inkuboi seosta 44,0±0,5^{0}C 2 vrk.

Laske kuinka monta putkista on positiivista (keltainen/kellertävä) ja siirrosta niistä 100 µl LMX-liemiputkiin.

Inkuboi LMX-putkia normaalisti ja tarkista inkuboinnin jälkeen värinmuutos/fluoresenssi/indoli.

Tuloksen ilmoittaminen
Ilmoita niiden putkien määrä, jotka olivat alkuperäisessä viljelmässä positiivisia ja jotka LMX:ssä osoittivat E.colia. Mikrobiologi laskee tuloksen tilastollisesta MPN-taulukosta

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STANDARDI

SFS 4089

Vesi- ja ympäristöhallitus

Vahvistettu
1988-04-18

VEDEN KOLIFORMISTEN BAKTEERIEN LUKUMÄÄRÄN MÄÄRITYS PUTKIMENETELMÄLLÄ

Bestämning av antalet coliforma bakterier i vatten med rörmetoden
Water quality. Detection and enumeration of coliform bacteria by enrichment in a liquid medium

Sisällys

1 JOHDANTO

Juomaveden kautta leviävät taudit ovat ennenkaikkea erilaisia suolistosairauksia, joissa tartunnanaiheuttajat (bakteerit, virukset, alkueläimet) esiintyvät eläinten ja ihmisen ulosteissa. Vedessä tautia-aiheuttavat (patogeeniset) mikrobit esiintyvät yleensä pieninä pitoisuuksina verrattuna luonnollisiin suolistobakteereihin (1). Menetelmiä erilaisten tautia aiheuttavien mikrobien osoitukseen vedestä on olemassa, mutta ne ovat usein työläitä ja kalliita. Kaikille taudinaiheuttajille ei ole olemassa luotettavia osoitusmenetelmiä. Jos vesistä haluttaisiin suoraan osoittaa taudinaiheuttajat, se olisi tehtävä jokaiselle taudinaiheuttajalle eri menetelmällä. Sen vuoksi veden mikrobiologisessa rutiinivalvonnassa etsitään erilaisia indikaattoribakteereita, joita on eläinten ja ihmisen ulosteissa.

Ulosteiden aiheuttaman saastutuksen parhaana indikaattorina pidetään **Escherichia coli** lajia. **E. colin** esiintyminen katsotaan ulostesaastutuksen osoitukseksi, kun taas muut koliformiset bakteerit voivat olla ulosteista peräisin, mutta niitä voi myös muutoin esiintyä maassa ja vedessä. Näistä muista lajeista vain harvat, lämpökestoiset koliformiset bakteerit, kykenevät kasvamaan 44,5 °C lämpötilassa. **E. coli** kuuluu tähän joukkoon, mutta sen erottamiseen muista tarvitaan lisäksi vähintään indolitesti.

2 SOVELTAMISALA

Tässä standardissa kuvataan putkimenetelmät (SFS 4447) koliformisten bakteerien kokonaismäärän ja lämpökestoisten koliformisten bakteerien määrittämiseksi. Lisäksi esitetään menetelmät **E. colin** alustavaksi tunnistukseksi. Näiden indikaattoribakteerien alustava määritys ja varmistus tehdään määrätyssä järjestyksessä. Analyysin laajuus riippuu vesinäytteestä ja tarvittavasta tiedosta.

Putkimenetelmiä voidaan käyttää tutkittaessa erilaisia vesiä, kuten juomavettä, raakavettä, uimavettä, vastaanottavan vesistön vettä ja jätevettä. Ne soveltuvat myös käytettäväksi puhdistamolietteen, sedimentin ja maaperän tutkimiseen edellyttäen, että bakteerit on saatu tasaisesti jakautumaan näytteeseen.

On huomattava, että putkimenetelmä on epätarkka (SFS 4447). Yleisemmin ovat käytössä kalvosuodatusmenetelmät SFS 3950, koliformisten bakteerien kokonaismäärälle SFS 3016 ja lämpökestoisille koliformisille bakteereille SFS 4088. Jos näyte on samea tai sisältää kalvolle pidättyviä ja bakteerien kasvua estäviä aineita, käytetään putkimenetelmää. Suurempi osa esim. desinfektiossa vaurioituneista bakteereista voidaan saada esiin putkimenetelmällä kuin kalvosuodatusmenetelmällä etenkin, jos kalvosuodatusmenetelmässä käytetään etsityn organismin maksimikasvulämpötilaa lähellä olevaa lämpötilaa ilman esi-inkubointia alemmassa lämpötilassa.

3 VIITTAUKSET

SFS 3016 Veden koliformisten bakteerien kokonaismäärän määritys kalvosuodatusmenetelmällä.

SFS 3950 Kalvosuodatusmenetelmä veden mikrobiologisessa tutkimuksessa.

SFS 3951 Vesinäytteenotto mikrobiologista tutkimusta varten.

Kyriiri Oy 750.88-04

SFS 4088 Veden lämpökestoisten (fekaalisten) koliformisten bakteerien lukumäärän määritys kalvosuodatusmenetelmällä.

SFS 4447 Putkimenetelmä veden mikrobiologisessa tutkimuksessa.

4 MÄÄRITELMÄT

KOLIFORMISET BAKTEERIT tarkoittaa tässä standardissa fakultatiivisesti anaerobisia, oksidaasi-negatiivisia, gram-negatiivisia, itiöitä muodostamattomia sauvabakteereita, jotka fermentoivat laktoosia muodostaen happoa ja kaasua 48 tunnin kuluessa lämpötilassa 35 °C tai vaihtoehtoisesti 37 °C.

LÄMPÖKESTOISET KOLIFORMISET BAKTEERIT tarkoittaa koliformisia bakteereita, jotka lisäksi tuottavat sokerista kaasua 24 tunnissa lämpötilassa 44,5 °C.

E. COLI (ALUSTAVA) tarkoittaa lämpökestoista koliformista bakteeria, joka lisäksi muodostaa indolia tryptofaanista.

5 PERIAATE

Koliformisten bakteerien osoitus ja lukumäärän määritys tietyssä vesitilavuudessa edellyttää rikastusviljelyä ja tarkistustutkimusta. Lämpökestoisuuden osoitus ja **E. colin** alustava osoitus edellyttävät vielä lisätestejä. Lämpökestoisen koliformisten ja alustavan **E. colin** osoitus voidaan tehdä ilman koliformien tarkistustutkimusta.

5.1 Rikastusviljely

Näyte inkuboidaan bromikresolipurppura-laktoosi -liemessä lämpötilassa 35 °C tai 37 °C 44 ± 4 h. Koliformiset bakteerit kasvavat tässä alustassa tuottaen laktoosista happoa ja kaasua. Happo aiheuttaa indikaattorin värin muuttumisen violetista keltaiseksi. Kaasun muodostuminen havaitaan koeputken sisään ylösalaisin sijoitetusta kaasunkeräysputkesta (durhamputkesta).

5.2 Tarkistustutkimus

Kaikista positiivisista ja lisäksi sellaisen rikastusviljelyn laimennoksen kasvua osoittavista putkista, joissa yksikin putki osoittaa hapon muodostusta siirrostetaan näytettä briljanttivihreä-sappi-laktoosi -liemeen. Juomavesinäytteitä tutkittaessa olisi hyvä tarkistaa kaikki kasvua osoittavat putket. Kaasun muodostus briljanttivihreä-sappi-laktoosi -liemessä lämpötilassa 35 °C tai 37 °C 44 ± 4 tunnissa on positiivinen tulos.

5.3 Lämpökestoisten koliformisten bakteerien osoitus

Siirros steriileihin lauryyli-tryptoosi-mannitoli-tryptofaani-liemiputkiin tehdään rikastusviljelyn bromikresolipurppura-laktoosi -liemiviljelmistä mieluiten heti rikastusviljelyn jälkeen (tai vasta tarkistustutkimuksen jälkeen, jolloin tutkittaviksi valitaan vain sekä rikastusviljelyssä että tarkistustutkimuksessa positiivisen tuloksen antaneet viljelmät). Putkia inkuboidaan vesihauteessa lämpötilassa 44,5 °C 22 ± 2 h. Kaasun muodostus tulkitaan positiiviseksi tulokseksi.

5.4 E. colin alustava osoitus

Alustavana **E. coli** bakteerina pidetään rikastus (5.1) ja/tai tarkistustutkimuksessa (5.2) positiivisen tuloksen antavaa kantaa, joka on lisäksi lämpökestoinen (5.3) ja joka muodostaa tryptofaanista indolia lämpötilassa 44,5 °C 24 tunnissa.

6 LAITTEET JA VÄLINEET

Laimennusveden ja tarvikkeiden sterilointiohjeet on annettu standardissa SFS 3950. Tavanomainen bakteriologisen laboratorion välineistö ja

6.1 Inkubaattori, asetettu lämpötilaan 35 ± 1 °C tai 37 ± 1 °C.

6.2 Inkubaattori, asetettu lämpötilaan 44,5 ± 0,5 °C. Vesihaude ja haluttaessa lisäksi inkubointikaappi.

6.3 Autoklaavi, asetettu lämpötilaan 121 ± 1 °C ja 110 ± 1 °C.

6.4 Koeputket, esim. 160 ... 180 mm x 16 ... 21 mm borosilikaattilasia.

Pullot esim. 200 ... 300 ml borosilikaattilasia.

6.5 Kaasunkeräysputket, esim. durhamputket 36 mm x x 7 mm koeputkiin ja esim. 80 mm x 10 mm durhamputket pulloihin.

7 KASVUALUSTAT JA KEMIKAALIT

Veden tulee olla tislattua tai vastaavaa puhtausastetta.

Tulosten toistettavuuden vuoksi on suositeltavaa käyttää joko hyviksi tunnettuja vedettömiä kasvualusta-aineita ja pro analysis -kemikaaleja tai luotettavan valmistajan kasvualustajauheita tai -rakeita valmistajan ohjeiden mukaan.

pH säädetään tarvittaessa joko 1,0 mol/l natriumhydroksidiliuoksella (40 g NaOH litrassa vettä) tai 1,0 mol/l suolahapolla (82,5 ml väkevää HCl litrassa vettä).

7.1 Laimennusvesi

Valmistus ja käyttö, kuten standardissa SFS 4447 on esitetty.

7.2 Kasvualustat

7.2.1 Bromikresolipurppuralaktoosiliemi (väkevöimätön) (2)

Koostumus:

lihauute	3,0 g
peptoni	10,0 g
NaCl	3,0 g
laktoosi	5,0 g
bromikresolipurppuraliuos	
1 g/100 ml 94 % etanolia	1 ml
tislattu vesi	1000 ml

Liuota lihauute, peptoni ja NaCl veteen ja tarkista, että liuoksen pH on 7,4. Lisää laktoosi liuosta kuumentaen ja lisää sen jälkeen bromikresolipurppuraliuos. Tarkista, että valmiin alustan pH on 7,4.

Annostele alusta putkiin (esim. 10 ml) tai pulloihin (esim. 100 ml väkevyydeltään kaksinkertaista kasvualustaa), joihin on asetettu kaasunkeräysputki ylösalaisin.

Sulje putket ja pullot. Steriloi ne autoklaavissa väljästi sijoitettuna 15 minuuttia lämpötilassa 110 °C. Jos sterilointi ei vaikuta käytännössä riittävältä näin, voidaan alusta ilman laktoosilisäystä steriloida ensin 15 minuuttia lämpötilassa 121 °C ja laktoosilisäyksen ja annostelun jälkeen uudelleen 15 minuuttia lämpötilassa 110 °C.

Huom. Jos tutkitaan millilitraa suurempia näyte-eriä, on syytä valmistaa väkevyydeltään kaksinkertainen kasvualusta, joka annostellaan tilavuudeltaan tutkittavien näyte-erien kokoisiin eriin. Myös muita kasvualustan väkevyys-näytetilavuus -suhteita voidaan käyttää.

7.2.2 Briljanttivihreä-sappiliemi (2)

Koostumus:

peptoni	10 g
laktoosi	10 g
briljanttivihreäliuos 0,1 g/100 ml vettä	13 ml
naudansappea	20 g
tislattu vesi	1000 ml

Peptoni ja laktoosi liuotetaan 500 millilitraan vettä. Naudansappi liuotetaan 200 millilitraan vettä ja saadun liuoksen pH säädetään arvoon 7,4 ... 7,5. Molemmat liuokset sekoitetaan keskenään, siihen lisätään 75 millilitraa vettä ja pH säädetään arvoon 7,4. Briljanttivihreäliuos lisätään ja liuoksen määrä täytetään 1000 millilitraksi tislatulla vedellä.

Annostele 10 millilitran erät kasvualustaa koeputkiin, joissa on kaasunkeräysputket ylösalaisin. Sulje putket ja steriloi ne autoklavoimalla niitä väljästi asetettuna 15 minuuttia lämpötilassa 110 °C.

7.2.3 Lauryyli-tryptoosi-mannitoli-tryptofaaniliemi (3)

Koostumus:

tryptoosi	20 g
mannitoli	5 g
NaCl	5 g
dikaliumvetyfosfaatti, K_2HPO_4	2,75 g
kaliumdivetyfosfaatti, KH_2PO_4	2,75 g
natriumlauryylisulfaatti ($C_{12}H_{25}SO_4Na$)	0,1 g
L-tryptofaani	0,2 g
tislattu vesi	1000 ml

Ainekset, lukuunottamatta natriumlauryylisulfaattia, liuotetaan kuumentamalla. Sitten lisätään natriumlauryylisulfaatti, joka muutoin vaahtoaisi. pH-arvo säädetään alueelle 6,8 ± 0,2. Kasvualusta annostellaan 10 ml eriksi koeputkiin, joihin on sijoitettu kaasunkeräysputket ylösalaisin. Tulpitetut putket steriloidaan autoklaavissa lämpötilassa 115 °C 10 minuuttia.

7.3 Indolireagenssi (4)

Koostumus:

p-dimetyyliaminobentsaldehydi [1)]	5,0 g
amyylialkoholi [1)]	75 ml
väkevä HCl [1)]	25 ml

Reagenssi on keltainen. Kemikaalit eivät kestä hyvin pitkää säilytystä. Kaikki p-dimetyyliaminobentsaldehydi-valmisteet eivät ole tyydyttäviä.

8 NÄYTE

Näyte otetaan ja kuljetetaan, kuten standardissa SFS 3951 mikrobiologisesta näytteenotosta esitetään.

9 SUORITUS

9.1 Näytteen käsittely

Näyte käsitellään ja laimennetaan standardin SFS 4447 mukaisesti. Esimerkkejä sopivista tutkittavista tilavuuksista on esitetty taulukossa 1.

Taulukko 1 Esimerkkejä sopiviksi tutkittaviksi näytetilavuuksiksi koliformisten bakteerien määrityksessä putkimenetelmällä.

	Putkien tai pullojen lukumäärä x näyte-erän tilavuus
Juoma- ja talousvesi vesilaitoksella	5 x 100 ml, 5 x 10 ml, (5 x 1 ml)
Juoma- ja talousvesi vesilaitoksella rutiinitutkimuksessa ja vesijohtovesi	1 x 50 ml ja 5 x 10 ml tai 10 x 10 ml
Yksityistalouksien vesi ja kotieläinten juomavesi	1 x 50 ml, 5 x 10 ml, 5 x 1 ml 5 x 0,1 ml (tai 10 x 10 ml)
Uimarantavesi	(5 x 10 ml), 5 x 1 ml, 5 x 0,1 ml, 5 x 0,01 ml
Raakavesi	5 x 10 ml, 5 x 1 ml, 5 x 0,1 ml, 5 x 0,01 ml
Jätevesi	5 x 1 ml, 5 x 0,1 ml, 5 x 0,01 ml jne.

[1)] Katso Opastavia tietoja, turvallisuusohjeet.

9.2 Rikastusviljely

Siirrä bromikresolipurppura-laktoosi-liemiputket lämpötilaan 35 ± 1 °C tai 37 ± 1 °C. Hylkää putket, jotka osoittavat kaasunmuodostusta tai kasvua ennen siirrostusta. Millilitraa suuremmat näyte-erät tulee lisätä näyte-erän kokoiseen tilavuuteen väkevyydeltään kaksinkertaista kasvualustaa. Kvantitatiivisen tuloksen saamiseksi käytetään MPN-menetelmää (standardi SFS 4447).

Kaikki putket, joissa havaitaan hapon ja kaasun muodostusta inkuboinnin jälkeen lämpötilassa 35 ± 1 °C tai 37 ± 1 °C, tulkitaan positiivisiksi. Kaasunmuodostus saadaan joskus esiin vasta kevyesti putkea napauttamalla, jolloin kuplat nousevat ylös. Kaasunmuodostus tulkitaan positiiviseksi, kun kaasu täyttää vähintään kaasun keräysputken kupolin. Hapon muodostus havaitaan liuoksen muuttumisena keltaiseksi. Tuloksen voi alustavasti tarkistaa jo 24 tunnin kuluttua, mutta lopullinen tulos rikastusviljelystä saadaan vasta 44 ± 4 tunnin kuluttua. Kunkin laimennustason kaikkien putkien reaktiot merkitään muistiin.

9.3 Tarkistusviljely

Tarkista jokainen positiivinen rikastustulos ja joitakin kasvua osoittavia negatiivisia putkia sellaisesta laimennoksesta, jossa jossakin putkessa on positiivinen tulos. Tarkistusviljely tehdään briljanttivihreäsappi -liemessä (7.2.2).

Tarkistus briljanttivihreä-sappi -liemessä

Rikastusviljelyputkista (9.2) siirrostetaan steriilillä pipetillä 2 tippaa tai 100 µl viljelmää vastaavaan briljanttivihreä-sappi -liemiputkeen. Putkia inkuboidaan lämpötilassa 35 ± 1 °C tai 37 ± 1 °C. Kaasunmuodostus 44 ± 4 tunnin kuluessa on positiivinen tulos. Alustavasti putket voi tarkastaa jo 22 ± 2 tunnin kuluttua. Kunkin laimennustason kaikkien putkien reaktiot merkitään muistiin.

9.4 Lämpökestoiset koliformiset bakteerit

Samoin kuin tarkistusviljelyssä (9.3) lämpökestoisten koliformisten bakteerien ja alustavan **E. colin** määrityksessä lähdetään rikastusviljelyn putkista tai pulloista ja testattavat viljelmät valitaan saman periaatteen mukaan (9.3).

Rikastusviljelyputkista (9.3) siirrostetaan 2 tippaa tai 100 µl steriilillä pipetillä steriileihin lauryyli-tryptoosi-mannitoli-tryptofaaniliemiputkiin (7.2.4). Siirrostetut putket asetetaan lämpötilaan 44,5 ± 0,5 °C vesihauteeseen, josta ne voidaan haluttaessa kahden tunnin kulutua siirtää inkubointikaappiin samaan lämpötilaan. 22 ± 2 tunnin kulutua luetaan positiiviseksi tulokseksi kaasun muodostus. Tulos merkitään muistiin siten, että se voidaan yhdistää vastaavan viljelmän rikastus- ja tarkistusviljelyiden tulosten kanssa.

9.5 E. colin alustava osoitus

Alustavassa **E. colin** osoituksessa indolin muodostus tutkitaan samanaikaisesti lämpökestoisten koliformisten bakteerien määrityksen kanssa lisäämällä indolireagenssia lauryyli-tryptoosi-mannitoli-tryptofaaniliemiputkiin. Alustavasti **E. coliksi** katsotaan lämpökestoiset indolipositiiviset koliformiset bakteerit.

9.5.1 Indolikoe

Haluttaessa indolitesti voidaan tehdä vain niille kannoille, jotka ovat olleet positiivisia muissa testeissä.

Heti, kun on luettu kaasun muodostus lauryyli-tryptoosi-mannitoli-tryptofaaniliemiputkista, kuhunkin putkeen lisätään 0,2 ... 0,3 ml indolireagenssia ja tulos luetaan heti. Jos se on negatiivinen, tulos tarkistetaan 10 min kuluttua. Positiivinen tulos on tummanpunainen väri ylemmässä amyylialkoholikerroksessa. Jos reagenssin väri ei muutu tulos on negatiivinen.

Testin toimivuus voidaan varmistaa indoli-positiivisen ja -negatiivisen tunnetun kannan avulla.

Indolitestin tulos merkitään muistiin siten, että se voidaan yhdistää vastaavan viljelmän rikastus-, tarkistus- ja lämpökestoisuusviljelyiden tulosten kanssa.

10 TULOSTEN LASKEMINEN

MPN-menetelmän tulosten laskeminen on esitetty standardissa SFS 4447. Rikastusviljelyn tulos ei ole riittävän luotettava koliformisten bakteerien lukumäärän mitta, vaan tuloksena ilmoitetaan tarkistusviljelyn (9.2) tulos. Lämpökestoisten koliformisten bakteerien lukumäärä lasketaan ottamalla huomioon vain ne viljelmät, jotka antavat positiivisen tuloksen lämpökestoisuudessa (9.3). Alustavan **E. colin** lukumäärä lasketaan ottamalla huomioon ne koliformiset bakteerit, jotka ovat lisäksi lämpökestoisia ja muodostavat indolia tryptofaanista (ks. Opastavia tietoja).

11 TULOSTEN ILMOITTAMINEN

Tulosta ilmoitettaessa on viitattava tähän standardiin. Kaikki tiedot näytteen täydellistä tunnistusta varten on ilmoitettava. Tulokset ilmoitetaan erikseen koliformisille bakteereille, lämpökestoisille koliformisille bakteereille ja alustavalle **E. colille**.

SFS 4089 Suomen Standardisoimislautakunta 1988-04-18

OPASTAVIA TIETOJA

Tämän standardin mukaan lämpökestoiset koliformiset bakteerit tuottavat kaasua lämpötilassa 44,5 °C, kun taas standardissa SFS 4088 lämpökestoiseksi koliformiseksi bakteeriksi luokitellaan tässä lämpötilassa kasvavat ja happoa muodostavat pesäkkeet. Osa indolia lämpötilassa 44,5 °C muodostavista bakteeriviljelmistä, jotka eivät muodosta kaasua lämpötilassa 44,5 °C vuorokaudessa saattavat kuitenkin olla **E. coli** -kantoja (5).

Turvallisuusohjeet

p-dimetylaminobentsaldehydi, amyylialkoholi ja väkevä suolahappo ovat sosiaali- ja terveysministeriön päätöksen (409/78) mukaan terveydelle vaarallisia aineita.

p-dimetylaminobentsaldehydin joutumista iholle on vältettävä. Sitä käsitellään mieluiten vetokaapissa suojakäsineitä käyttäen.

Amyylialkoholi ärsyttää limakalvoja ja unettaa suurina määrinä. Sitä tulee käsitellä vetokaapissa.

Väkevä suolahappo on ärsyttävä ja syövyttävä. Sitä on käsiteltävä vetokaapissa käyttäen tarpeen mukaan suojakäsineitä, hengitys- ja silmäsuojaimia.

Lähdekirjallisuutta

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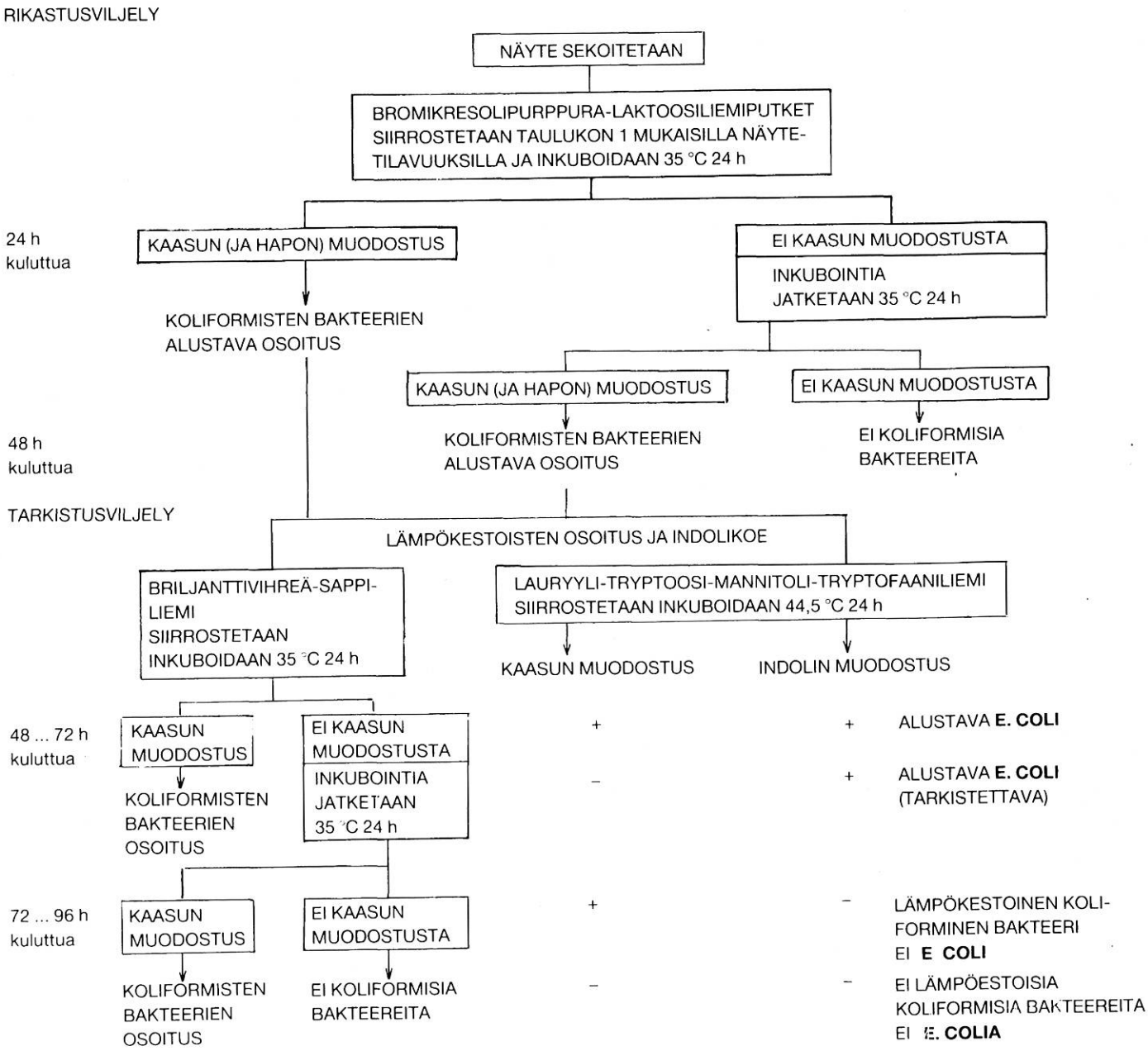

Kuvio 1 Koliformisten bakteerien, lämpökestoisten koliformisten bakteerien ja **E. colin** (alustava) tutkiminen

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POHJOISMAINEN ELINTARVIKKEIDEN METODIIKKAKOMITEA

NORDIC COMMITTEE ON FOOD ANALYSIS

No 71 5. ed., 1999

SALMONELLA. OSOITTAMINEN ELINTARVIKKEISTA.

SALMONELLA. DETECTION IN FOODS.

Menetelmä on validoitu kollaboratiivisessa testauksessa.

This NMKL method has been validated in a collaborative study.

1. TARKOITUS JA KÄYTTÖALUE

Menetelmä on tarkoitettu salmonellojen osoittamiseen elintarvikkeista. Menetelmä soveltuu kaikenlaisille elintarvikkeille.

2. MÄÄRITELMÄ

Salmonellasuku kuuluu *Enterobacteriaceae*-heimoon. Sukuun kuuluu noin 2300 serotyyppiä.

Salmonellabakteeri on fakultatiivisesti anaerobinen gram-negatiivinen sauva. Se liikkuu peritrikkisten flagellojen avulla, mutta liikkumattomia mutantteja voi esiintyä. Lisäksi eräs serotyyppi (S. Gallinarum) on aina liikkumaton. Salmonellabakteeri muodostaa happoa glukoosista ja mannitolista, mutta ei sakkaroosista eikä laktoosista. Se ei muodosta indolia eikä hajota ureaa. Useimmat muodostavat rikkivetyä ja dekarboksyloivat ornitiinia ja lysiiniä.

Salmonellasuku jaetaan somaattisten (O) ja flagella-(H) antigeenien perustella serotyyppeihin Kauffman-White'n kaavan mukaan. Serotyypit Enteritidis, Typhi, Paratyphi ja Typhimurium faagityypitetään.

3. KIRJALLISUUSVIITTEET

3.1 NMKL menetelmä nro 91, 2. painos, 1988: Elintarvikkeiden esikäsittely mikrobiologista tutkimusta varten.

3.2 NMKL raportti nro 5, 2. painos, 1994: Laadunvarmistusohjeita mikrobiologisille laboratorioille.

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1. SCOPE AND FIELD OF APPLICATION

This method describes the detection of *Salmonella* in foods. The method is applicable to all kinds of foodstuff.

2. DEFINITION

The genus *Salmonella* comprises about 2300 serotypes and belongs to the family *Enterobacteriaceae.*

Salmonellae are facultatively anaerobic Gram negative rods. They are motile with peritrichous flagella, but unmotile mutants may occur, and one serotype (Gallinarum) is always non-motile. *Salmonella* strains produce acid from glucose and mannitol but not from saccharose and lactose. They do not produce indole and do not decompose urea. Most types produce hydrogen sulphide and decarboxylate ornithine and lysine.

The genus *Salmonella* is divided into serotypes after the Kauffmann-White scheme according to its somatic (0) and flagella (H) antigens. Some serotypes (Enteritidis, Typhi, Paratyphi and Typhimurium) are phage typed.

3. REFERENCES

3.1 NMKL method No. 91, 2nd ed., 1988: Pre-treatment of foods for microbiological examination.

3.2 NMKL Report No. 5, 2nd ed., 1994: Quality Assurance Guidelines for Microbiological Laboratories.

3.3 Peterz, M., Wiberg, C. and Norberg, P. (1989): The effect of the incubation temperature and magnesium chloride concentration on growth of *Salmonella* in homemade and in commercially

available dehydrated Rappaport-Vassiliadis broths. J. Appl. Bacteriol. 66, 523-528.

3.4 Maijala, R., Johansson, T. and Hirn, J. (1992): Growth of *Salmonella* and competing flora in five commercial Rappaport-Vassiliadis (RV)-media. Int. J. Food Microbiol. 17, 1-8.

4. PERIAATE

Salmonellatutkimus on kvalitatiivinen ja vastaus ilmoitetaan: Salmonella todettu/ei todettu tutkitussa näytemäärässä.

Salmonellatutkimukseen kuuluu neljä vaihetta. Nämä vaiheet ovat välttämättömiä, koska salmonella esiintyy yleensä pieninä pitoisuuksina ja solut saattavat olla vaurioituneita. Lisäksi näyte useinkin sisältää runsaasti muita *Enterobacteriaceae*-heimon bakteereita.

4.1 Esirikastus. Tietty määrä näytettä esirikastetaan ei-selektiivisessä elatusaineessa (puskuroidussa peptonivedessä) 37°C:ssa noin 18 tuntia.

4.2 Rikastus. Tietty määrä esirikastetta siirrostetaan selektiiviseen rikastusliemeen (Rappaport-Vassiliadis soijapeptoniliemi) ja inkuboidaan 42°C:ssa noin 24 tuntia.

4.3 Viljely kiinteälle selektiiviselle elatusaineelle. Selektiivistä rikastetta viljellään hajotusviljeltynä kahdelle kiinteälle selektiiviselle agarelatusaineelle (Ksyloosi-lysiini-desoksikolaatti-agar ja lisäksi toinen valinnainen elatusaine), jotka inkuboidaan 37°C:ssa noin 24 tuntia.

4.4 Varmistus. Tyypillisistä ja/tai epäillyistä salmonellapesäkkeistä tehdään puhdasviljelmät sopivalle kiinteälle elatusaineelle ja varmistetaan biokemiallisesti ja serologisesti.

5. ELATUSAINEET

Ensisijaisesti suositellaan kaupallisten kuivaelatusaineiden käyttöä. Ne valmistetaan valmistajan ohjeiden mukaisesti. Ellei toisin ole mainittu, niiden koostumus voi hieman poiketa tässä menetelmässä esitetyistä elatusaineista. Käytettyjen kemikaalien tulisi olla "mikrobiologista laatua" tai sitäkin puhtaampia. Tislattua vettä tai vähintään vastaavan laatuista vettä tulisi käyttää liuosten ja elatusaineiden valmistamiseen.

Käyttövalmiit elatusaineet ja liuokset on säilytettävä,

available dehydrated Rappaport-Vassiliadis broths. J. Appl. Bacteriol. 66, 523-528.

3.4 Maijala, R., Johansson, T. and Hirn, J. (1992): Growth of *Salmonella* and competing flora in five commercial Rappaport-Vassiliadis (RV)-media. Int. J. Food Microbiol. 17, 1-8.

4. PRINCIPLE

This method is a qualitative method only, and the result is reported as: *Salmonella* detected/not detected in the amount of sample taken.

Four separate steps are required to detect *Salmonella* most efficiently. These steps are necessary because *Salmonella* often occur in low numbers, sometimes sublethally injured, and often in the presence of much greater numbers of other bacteria of the *Enterobacteriaceae* family.

4.1 Pre-enrichment. A certain amount of sample is pre-enriched in a non-selective medium (buffered peptone water) at 37°C for about 18 hours.

4.2 Enrichment. A certain amount of pre-enriched sample is transferred to a selective enrichment broth (Rappaport-Vassiliadis soy peptone broth) and is incubated at 42°C for about 24 hours.

4.3 Plating out. An aliquot from the selective enrichment broth is inoculated to two selective agar plates (Xylose lysine desoxycholate agar and a further agar plate free of choice), which is incubated at 37°C for about 24 hours.

4.4 Confirmation. Presumptive salmonellae are subcultured on a suitable plate and are biochemically and serologically verified.

5. CULTURE MEDIA

Dehydrated commercially available culture media are primarily recommended. Such media are prepared according to the manufacturer's instructions. If not otherwise stated, the composition of the media may deviate somewhat from those described below. The chemicals used should be of the quality "for microbiology" or purer. Distilled water or purified water of at least the same quality should be used for the preparation of solutions and media.
Prepared media and solutions should, if not otherwise stated, be stored in the dark at ca. 4°C (4±2°C) for not

ellei toisin ole mainittu, pimeässä ja kylmässä noin 4°C:ssa (4±2°C) enintään kuukauden ajan. Elatusainemaljojen kuivumisen estämiseksi agarkerroksen on oltava riittävän paksu (noin 4 mm). Mikäli maljojen pinnalla on kosteutta, maljat tulee kuivattaa ennen käyttöä (kannet poistettuna agarpinta alaspäin) 37°C:ssa 30 minuuttia tai kunnes agarin pinta on kuiva.

5.1 Puskuroitu peptonivesi

Peptoni	10,0 g
Natriumkloridi	5,0 g
Dinatriumvetyfosfaatti (Na_2HPO_4)	3,6 g
Kaliumdivetyfosfaatti (KH_2PO_4)	1,5 g
Tislattu vesi	1000 ml

Liuota ainesosat veteen keittämällä. Jaa liuos sopiviksi annoksiksi (normaalisti 225 ml) pulloihin. Autoklavoi 121°C:ssa 15 minuuttia. Steriloinnin jälkeen tulee käyttövalmiin elatusaineen pH:n olla 7,0±0,2 mitattuna 25°C:ssa.

5.2 Rasvaton maitojauhe -liemi

Rasvaton maitojauhe (esim. "Skim milk powder" tai "Bacto skim milk")	100 g
Briljanttivihreä	0,02 g
Tislattu vesi	1000 ml

Liuota ainesosat veteen, mahdollisesti lämmittämällä. Autoklavoi 121°C:ssa enintään 15 minuuttia, koska muutoin maitosokeri voi karamellisoitua. Steriloinnin jälkeen tulee käyttövalmiin elatusaineen pH:n olla 6,8±0,2 mitattuna 25°C:ssa.

5.3 Rappaport-Vassiliadis soijapeptoni(RVS)-liemi

5.3.1 Peruselatusaine

Soijapeptoni	5,0 g
Natriumkloridi	8,0 g
Kaliumdivetyfosfaatti (KH_2PO_4)	1,4 g
Dikaliumvetyfosfaatti (K_2HPO_4)	0,2 g
Tislattu vesi	1000 ml

Lämmitä noin 80°C:een, niin että kaikki ainesosat liukenevat. Valmista tämä liuos samana päivänä kuin täydellinen RVS-elatusaine.

5.3.2 Magnesiumkloridiliuos

Magnesiumkloridi ($MgCl_2 \cdot 6\ H_2O$)	400 g
Tislattu vesi	1000 ml

Liuota magnesiumkloridi veteen. Tämä suola on hyvin hygroskooppista. Sen vuoksi koko avatun

longer than one month. Poured plates should be thick enough – ca. 4 mm - to avoid drying out. Immediately prior to use, it should be checked that the surface of the plate is dry. If necessary, the plates are dried (with lids removed and with the agar surface downward) at 37°C for 30 min, or until the agar surface is dry.

5.1 Buffered peptone water

Peptone	10.0 g
Sodium chloride	5.0 g
Disodium hydrogen phosphate (Na_2HPO_4)	3.6 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.5 g
Distilled water	1000 ml

Dissolve the components in the water by boiling. Distribute the solution in suitable portions (normally 225 ml) to flasks. Autoclave at 121°C for 15 min. Adjust the pH so that after sterilization it is 7.0±0.2 at 25°C.

5.2 Skim milk broth

Skim milk powder (e.g. "Skim milk powder" or "Bacto skim milk")	100 g
Brilliant green	0.02 g
Distilled water	1000 ml

Dissolve the ingredients in the water, eventually by heating. Autoclave at 121°C for a maximum of 15 min, otherwise there is the risk of caramelization. Adjust the pH so that after sterilization it is 6.8±0.2 at 25°C.

5.3 Rappaport-Vassiliadis soy peptone (RVS) broth

5.3.1 Base

Soy peptone	5.0 g
Sodium chloride	8.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	1.4 g
Dipotassium hydrogen phosphate (K_2HPO_4)	0.2 g
Distilled water	1000 ml

Heat to about 80°C to dissolve all ingredients. Prepare this solution on the same day as the complete RVS medium is prepared.

5.3.2 Magnesium chloride solution

Magnesium chloride ($MgCl_2 \cdot 6\ H_2O$)	400 g
Distilled water	1000 ml

Dissolve the salt in the water. Because this salt is very hygroscopic, it is advisable to dissolve the entire

pakkauksen sisältö tulisi liuottaa tislattuun veteen. Magnesiumkloridiliuos voidaan säilyttää ilman sterilointia tummassa kierrekorkillisessa pullossa, huoneenlämmössä 2 vuotta.

5.3.3 Malakiittivihreäliuos

Malakiittivihreäoksalaatti	0,4 g
Tislattu vesi	100 ml

Liuota suola veteen. Liuos voidaan säilyttää steriloimattomana tummassa kierrekorkillisessa pullossa, huoneenlämmössä 8 kuukautta.

5.3.4 Täydellinen elatusaine

Peruselatusaine (5.3.1)	1000 ml
Magnesiumkloridiliuos (5.3.2)	100 ml
Malakiittivihreäliuos (5.3.3)	10 ml

Sekoita liuokset huolellisesti yllämainitussa järjestyksessä. Jaa liuos 10 ml annoksiin kierrekorkillisiin putkiin. Autoklavoi 115°C:ssa 15 minuuttia. Steriloinnin jälkeen tulee käyttövalmiin elatusaineen pH:n olla 5,2±0,2 mitattuna 25°C:ssa. Käyttövalmis elatusaine voidaan säilyttää kierrekorkillisissa putkissa noin 4°C:ssa 4 kuukautta.

Käyttövalmiin RVS-liemen $MgCl_2$ –pitoisuuden (sisältäen 6 H_2O) tulee olla 29 g/l. Liemen toimivuuden kannalta on tärkeää, että pitoisuus on oikea. Siksi kaupallisia kuivaelatusaineita käytettäessä on pitoisuus aina tarkastettava.

5.4 Ksyloosi-lysiini-desoksikolaatti (XLD)-agar

Hiivauute	3,0 g
Natriumkloridi	5,0 g
Ksyloosi	3,75 g
Laktoosi	7,5 g
Sakkaroosi	7,5 g
L-lysiini-HCL	5,0 g
Natriumtiosulfaatti	6,8 g
Rauta(III)ammoniumsitraatti	0,8 g
Fenolipuna	0,08 g
Natriumdesoksikolaatti	1,0 g
Agar	15,0 g
Tislattu vesi	1000 ml

Liuota ainesosat veteen. Kuumenna koko ajan sekoittaen, kunnes elatusaine alkaa kiehua. Vältä ylikuumentamista. Vältä suurten tilavuuksien valmistamista, koska tämä pidentää kuumennusaikaa. Siirrä liuos välittömästi vesihauteeseen, joka on temperoitu noin 50°C:een. Jatka sekoittamista,

contents of a newly opened container in distilled water. The magnesium chloride solution can be stored unsterilized, in a dark bottle with screw cap, at room temperature for 2 years.

5.3.3 Malachite green solution

Malachite green oxalate	0.4 g
Distilled water	100 ml

Dissolve the salt in the water. The solution can be stored unsterilized, in a dark bottle with screw cap, at room temperature for 8 months.

5.3.4 Complete medium

Base (5.3. 1)	1000 ml
Magnesium chloride solution (5.3.2)	100 ml
Malachite green solution (5.3.3)	10 ml

Mix the solutions well in the order specified above. Distribute the solution in portions of 10 ml to tubes with screw caps. Autoclave at 115°C for 15 min. Adjust the pH so that after sterilization it is 5.2±0.2 at 25°C. The ready-to-use medium can be stored, in tubes with screw caps, at about 4°C for 4 months.

As the amount of $MgCl_2 \cdot 6\ H_2O$ is very important for the RVS medium to function satisfactorily, it is necessary, when using commercially available dehydrated media, to ascertain that the amount is about 29 g/l in the ready-to-use medium.

5.4 Xylose lysine desoxycholate (XLD) agar

Yeast extract	3.0 g
Sodium chloride	5.0 g
Xylose	3.75 g
Lactose	7.5 g
Sucrose	7.5 g
L-Lysine hydrogen chloride	5.0 g
Sodium thiosulphate	6.8 g
Iron(III)ammonium citrate	0.8 g
Phenol red	0.08 g
Sodium desoxycholate	1.0 g
Agar	15.0 g
Distilled water	1000 ml

Dissolve the components in the water. Heat under constant stirring until the medium starts to boil. Avoid over-heating. Avoid preparing too large volumes of medium, as this requires prolonged heating. Immediately transfer the solution to a water bath tempered to about 50°C, continue stirring until the

kunnes elatusaineen lämpötila on noin 50°C. Steriloinnin jälkeen tulee käyttövalmiin elatusaineen pH:n olla 7,4±0,2 mitattuna 25°C:ssa. Valmiita agarmaljoja voidaan säilyttää pimeässä muovipussiin pakattuna enintään 14 päivää noin 4°C:ssa.

Elatusaineen selektiivisyyden lisäämiseksi siihen voidaan lisätä natriumnovobiosiinia. Kun elatusaine on jäähdytetty noin 50°C:een, lisätään 10 ml 0,15 % steriilisuodatettua natriumnovobiosiinin vesiliuosta.

6. LAITTEISTO

6.1 Lämpökaappi 37,0±1,0°C

6.2 Vesihaude (veden kierrätyksellä) 42,0±0,2°C, vaihtoehtoisesti lämpökaappi 41,5±0,5°C. Lämpökaappia voidaan käyttää, mikäli alla olevat ehdot täyttyvät.

Vesihaudetta käytetään rikastusliemen (RVS) inkubointiin. Tarkka inkubointilämpötila on tärkeä tekijä rikastuksen optimoimiseksi. Optimaalinen rikastuslämpötila on 42,0°C. Vesihauteen lämpötilanvaihtelu on ajan ja tilavuuden suhteen minimaalinen verrattuna lämpökaappiin. Tällä hetkellä saatavissa olevien parhaimpien lämpökaappien (joissa on inkubaattori-ilman kierrätys puhaltimella) lämpötilanvaihtelu ajan ja tilavuuden suhteen on noin 1°C. Jotta vesihauteen sijasta voitaisiin käyttää (puhaltimella varustettua) lämpökaappia, tulee varmistautua mittauksin siitä, että lämpötila ajallisesti ja koko tilavuudelta pysyy 41,5±0,5°C:ssa. Tämä on erityisen tärkeää, mikäli lämpökaapin koko tilavuutta käytetään monien rikastusliemien inkubointiin.

7. NÄYTTEENOTTO

Näytteet otetaan tavanomaisen mikrobiologisen käytännön mukaisesti (3.1).

Otettaessa näytteitä salmonellatutkimuksiin on tärkeätä tietää mahdollinen saastutuskohta. Esim. tutkittaessa pakastettua lihaa, näyte on otettava lihan pinnasta mahdollisimman laajalta alueelta. Tutkittaessa jauheita tai hiutalemaisia tuotteita, on näytteen määrä sen sijaan ratkaiseva. Tällöin näytteeksi on suositeltavaa ottaa tavallista suurempia näytemääriä, esim. 100 g. Mikäli mahdollista, otetaan näyte useasta pakkauksesta. Näytteet tutkitaan joko erikseen tai yhteisnäytteenä.

Tutkittaessa raakaa siipikarjan lihaa menetelmän herkkyys kasvaa, jos esirikastetaan koko lintu. Vaihtoehtoisesti voidaan näytteeksi ottaa vähintään

medium has reached about 50°C. Adjust the pH so that after heating it is 7.4±0.2 at 25°C. Poured agar plates can be stored for a maximum of 14 days, stored in plastic bags in the dark, at about 4°C.

Sodium novobiocin can be added in order to increase the selectivity of the medium. Add, after cooling of the medium to about 50°C, 10 ml of a 0.15% filter-sterilized aqueous solution of sodium novobiocin.

6. APPARATUS

6.1 Incubator for incubation at 37.0±1.0°C

6.2 Water-bath (with circulation), controllable at 42.0±0.2°C. An incubator can be used, on condition that the requirements below are fulfilled.

The water-bath is used for incubation of the enrichment broth. An exact incubation temperature is a very important factor for optimal enrichment with this substrate. The optimal enrichment temperature is 42.0°C. A water-bath has a minimal variation of temperature in time and space compared to an incubator. The best incubators today (equipped with a fan for re-circulation of the air in the incubator) have a variation of temperature in time and space of about 1°C. Therefore, before you use an incubator (equipped with a fan) instead of a water-bath, you should measure the temperature in time and in the whole space of the incubator, to control that the temperature is maintained within 41.5±0.5°C. This is especially important when the whole space of the incubator is used for incubation of many enrichment broths.

7. SAMPLING

Take samples according to established bacteriological practice (3.1).

When sampling for *Salmonella* examinations it is important to have some knowledge about the probable sites of the *Salmonella* contamination. For example when sampling frozen meat, the sample must be taken from an area as large as possible, because possible contamination will be on the surface. When sampling dry powder or cereals, on the other hand, the quantity of the sample is crucial. It is desirable to take larger samples of this type of products, e.g. 100 g. If many packages are available, several packages should be sampled. These samples are either examined separately or are well mixed and examined as a composite sample. When sampling whole raw poultry the detection frequency increases if the whole bird is pre-enriched.

25 g kaulanahkaa. Mikäli kaulanahkaa ei ole, otetaan näytteeksi nahkaa muista linnun osista.

8. SUORITUS

8.1 Näytteen esikäsittely

Näyte sekoitetaan hyvin. Näytteeksi otetaan vähintään 25g (määrän tulee olla tarkka). Tarvittaessa näyte homogenoidaan.

8.2 Esirikastus

8.2.1 Tavanomainen esirikastus

Periaatteena on lisätä 1 osa näytettä 9 osaan puskuroitua peptonivettä (5.1). Näyte (25g) punnitaan pulloon, jossa on 225 ml puskuroitua peptonivettä.

8.2.2 Esirikastus – erityistapaukset

Eräiden elintarvikkeiden esirikastamiseen tulee käyttää tavanomaisesta poikkeavia rikasteliemiä tai muuta laimennossuhdetta kuin 1:10. Syynä voi olla se, että elintarvikkeessa on luonnostaan salmonellalle toksisia aineita tai että elintarvikkeen koostumus on erityislaatuinen.

Raaka siipikarja: Siirrä koko lintu suureen vahvaan muovipussiin. Lisää 1 l puskuroitua peptonivettä (5.1). Ravista voimakkaasti vähintään 30 sekuntia niin, että peptonivesi huuhtelee koko linnun. Inkuboi koko nestemäärä ohjeen 8.2.3 mukaisesti.

Kuivamaitotuotteet: Punnitse 25 g näytettä 225 ml:aan puskuroitua peptonivettä (5.1). Älä sekoita, vaan anna pullon seistä huoneenlämmössä 60±10 minuuttia. Sekoita sen jälkeen niin, että näyte liukenee kokonaan, ja inkuboi kohdan 8.2.3 mukaisesti.

Yrtit, mausteet ja elintarvikkeet, joissa on vahvasti turpoavia aineita: Normaalin 1:10 laimennussuhteen sijasta voidaan käyttää suhdetta 1:100, jotta vältetään näytteen estävien aineiden vaikutukset tai näytteen epätäydellinen homogenoituminen.

Kaseiini, juusto, voi ja vastaavat tuotteet: Jotta nämä tuotteet saataisiin täydellisesti homogenisoiduksi, esilämmitetään puskuroitu peptonivesi (5.1) ennen käyttöä noin 40°C:ksi.

Kaakaota sisältävät tuotteet: Sekoita 25 g näytettä 225 ml:aan kuorittu maito -lientä (5.2). Suklaan homogenoimiseksi liemi on esilämmitettävä noin 40°C:een.

Alternatively, 25 g of neck skin may be taken - or other skin if the neck is not available.

8. PROCEDURE

8.1 Sample pre-treatment

The sample is mixed well and a sample of at least 25 g (exact amount) is taken. If needed the sample is homogenized.

8.2 Pre-enrichment

8.2.1 Normal pre-enrichment

The principle is to take 1 part of sample and 9 parts of buffered peptone water (5.1). If 25 g has been taken, transfer the sample to a flask containing 225 ml buffered peptone water.

8.2.2 Pre-enrichment – certain foods

Some foods require special pre-enrichment broths. The reason may be either that the food contains naturally occurring substances that are toxic to *Salmonella*, or may be connected with other aspects of the composition of the food. These negative effects can be suppressed by using a special pre-enrichment broth or other dilution ratio than 1:10.

Raw poultry: Place the whole bird in a large strong plastic bag. Add 1 L buffered peptone water (5.1). Shake vigorously for at least 30 sec so that the peptone water rinses the whole bird. Incubate the entire broth as described below (8.2.3).

Dried milk products: Add 25 g sample to 225 ml pre-enrichment broth (5.1). Without mixing, allow the flask to stand undisturbed at room temperature for 60±10 min. Thereafter, mix the content until it is completely dissolved and incubate as described below (8.2.3).

Herbs, spices and foodstuffs containing heavily swelling agents: A dilution factor of 1:100 could be used instead of the normal 1:10 to eliminate the effects of inhibitory substances in the sample or incomplete homogenization of the sample.

Casein, cheese, butter and similar products: For complete homogenization of these products, pre-enrichment broth (5.1) could be pre-warmed to about 40°C prior to use.

Products containing cocoa: Mix a 25 g sample with 225 ml skim milk broth (5.2). For the homogenization of chocolate, the broth must be pre-warmed to about 40°C.

Kookos ja sen kaltaiset tuotteet, joiden rasvapitoisuus on hyvin korkea: Sekoita 25 g näytettä 225 ml:aan puskuroitua peptonivettä (5.1). Lisää sen jälkeen 2-3 pisaraa Triton X-100.

Coconut and similar products with a very high fat content: Mix 25 g of sample with 225 ml buffered peptone water (5.1). Then add 2–3 drops of Triton X-100.

8.2.3 Esirikasteliemien inkubointi

Esirikasteliemet inkuboidaan 37,0±1,0°C:ssa 18±2 tuntia. Mikäli annettua inkubointiaikaa ei voida noudattaa käytännön syistä, inkubointiaikaa voidaan pidentää 21±3 tuntiin.

8.2.3 Incubation of pre-enrichment broths

Incubate all samples at 37.0±1.0°C for 18±2 hours. If the stated incubation time can not be used for practical reasons the time could be extended to 21±3 hours.

8.3 Rikastus

8.3.1 Esirikaste sekoitetaan. Siitä siirretään 0,1 ml 10 ml:aan RVS-lientä (5.3), joka on esilämmitetty inkubointilämpötilaan.

8.3 Enrichment

8.3.1 Mix the pre-enrichment broth prior to removal and transfer of 0.1 ml pre-enrichment broth to 10 ml Rappaport-Vassiliadis soy peptone broth (5.3), which has been pre-warmed to the incubation temperature.

8.3.2 Inkuboi vesihauteessa 42,0±0,2°C:ssa (tai inkubaattorissa 41,5±0,5°C:ssa, katso 6.2) 24±3 tuntia. Vaihtoehtoisesti inkubointiaikaa voidaan pidentää 48±4 tuntiin.

8.3.2 Incubate in a water-bath at 42.0±0.2°C (or incubator at 41.5±0.5°C, see 6.2) for 24±3 hours. As an alternative, the incubation time can be extended to 48±4 hours.

8.4 Viljely kiinteälle selektiiviselle elatusaineelle

8.4.1 Ota silmukalla materiaalia rikasteesta ja viljele hajotusviljelmäksi XLD-agarmaljan (5.4) pinnalle. Toista viljely valinnaiselle selektiiviselle elatusaineelle. Maljan koko pinta on käytettävä siten, että saadaan erillisiä pesäkkeitä.

Esimerkkejä valinnaisista kiinteitä selektiivisiä elatusaineita ovat briljanttivihreä-fenolipuna-agar, Rambach-agar ja mannitoli-lysiini-kristallivioletti-briljanttivihreä(MLCB)-agar. MLCB-agarin etuna on, että laktoosi- ja sakkaroosipositiiviset pesäkkeet voidaan todeta helposti.

8.4 Spreading on agar plates

8.4.1 Using a loop take material from the enrichment broth and inoculate on the surface of an XLD agar plate (5.4). Repeat the procedure on another optional and selective plate. Use the whole surface of the plate in order to get well-isolated colonies. Begin with a main streak from the edge of the plate and downwards about one third. Continue with the same loop and make a secondary streak – starting at the beginning of the main streak and diagonally towards this – from edge to edge over the whole surface of the plate.

Example of optional plates is brilliant green phenol red, Rambach- and mannitol lysine crystal violet brilliant green (MLCB) agar. One advantage of the MLCB agar plate is that lactose- or saccharose positive colonies can easily be detected.

8.4.2 Inkuboi maljat ylösalaisin käännettyinä 37,0±1,0°C:ssa 24±3 tuntia.

8.4.2 Incubate the plates in inverted position at 37.0±1.0°C for 24±3 hours.

8.5 Lukeminen

XLD-agar: Tyypillinen salmonellapesäke on mustakeskustainen ja sen reunassa on hieman läpinäkyvä, punertava vyöhyke. Melkein aina (erityisesti kun salmonellakasvu on massiivisen runsas) näkyy elatusaineessa pesäkkeiden ympärillä vaihtelevan suuruinen vaaleanpunainen-punainen vyöhyke.

8.5 Reading

XLD agar plate: A typical *Salmonella* colony has a slightly transparent zone of reddish colour due to the indicator change in the medium, and has a black centre. Almost always (especially in the case of massive *Salmonella* growth) a larger or smaller pink-red zone is seen in the medium surrounding the colonies.

8.6 Varmistus

Tyypilliset ja/tai epäiltävät pesäkkeet varmistetaan biokemiallisesti ja serologisesti.

Maljoilta poimitaan vähintään viisi tyypillistä tai epäiltävää pesäkettä, jotka viljellään puhtaaksi sopivalle ei-selektiiviselle agarelatusaineelle siten, että saadaan erillisiä pesäkkeitä. Maljat inkuboidaan 37,0±1,0°C:ssa, kunnes saadaan varmistusta varten riittävästi kasvustoa.

8.6.1 Biokemiallinen varmistus
Biokemiallisessa varmistuksessa käytetään tavanomaisia salmonellan biokemialliseen identifiointiin tarkoitettuja testejä. Myös kaupallisia testisarjoja voidaan käyttää, jos ne valmistajan ilmoituksen mukaan on tarkoitettu salmonellojen identifiointiin. Sopivia testejä ovat Triplesugar-iron(TSI)-agar, mannitoli, urea, ornitiinidekarboksylaasi ja lysiinidekarboksylaasi.

8.6.2 Serologinen varmistus
Serologinen varmistus tehdään fysiologisella keittosuolaliuoksella (auto-agglutinoivien kantojen eliminoimiseksi), salmonellapolyvalentilla O-antiseerumilla sekä salmonellapolyvalentilla H-antiseerumilla.

Täydellistä serologista tyypitystä varten kanta lähetetään hyväksyttyyn salmonella-referenssilaboratorioon.

8.6 Confirmation

Presumptive colonies should be verified biochemically and serologically.

Pick from agar plates used at least five colonies believed to be typical or presumptive, and inoculate on a suitable non-selective plate, so that well isolated colonies are developed. Incubate the plates at 37.0±1.0°C until a good growth for the confirmation is developed.

8.6.1 Biochemical confirmation. In the biochemical verification an established test for the biochemical identification of *Salmonella* should be used. So-called mini-diagnostica may also be used, if they, according to the manufacturer, are designed for *Salmonella* identification. Suitable tests may be Triple sugar iron (TSI) agar, mannitol, urea, ornithin decarboxylase and lysine decarboxylase.

8.6.2 Serological confirmation. Serological confirmation should be performed against physiological saline solution (to eliminate auto-agglutinating strains), *Salmonella* polyvalent O-antiserum and *Salmonella* polyvalent H-antiserum.

For complete serological typing, send the strain to a recognized reference laboratory for *Salmonella*.

9. TULOSTEN ILMOITTAMINEN

Tutkimustulos ilmoitetaan: Salmonella todettu / ei todettu 25 g:ssa näytettä tai tutkitussa näytemäärässä.

Vastausta täydennetään serotyypillä heti kun tulos saadaan.

9. REPORTING OF RESULTS

According to the result, state: *Salmonella* detected or not detected in 25 g sample (or the amount analyzed).

As soon as the serotyping is complete, the result can be supplemented with serotype.

10. KOLLABORATIIVISEN TESTAUKSEN TULOKSET JA JOHTOPÄÄTÖS

Menetelmän kollaboratiivisessa testauksessa käytettiin ISO 6579:1993-menetelmää referenssimenetelmänä. Näillä kahdella menetelmällä saatuja tuloksia arvioitiin tilastollisesti ja verrattiin McNemar-testillä, joka antoi χ^2-arvon 1,23. Mikäli testin antama χ^2-arvo on suurempi kuin 3,84, eroavat menetelmien antamat tulokset tilastollisesti merkitsevästi 5% tasolla. ISO- ja NMKL-menetelmien antamat tulokset eivät eronneet tilastollisesti merkitsevästi.

10. RESULTS OF THE COLLABORATIVE STUDY AND CONCLUSION

In the collaborative study of this NMKL method ISO 6579:1993 was used as the reference method. The results obtained using the two methods were statistically evaluated and compared by means of a McNemar test, which gave a χ^2 value of 1.23. In this test a χ^2 value of above 3.84 indicates a statistically significant difference between the two methods at the 5% level. Therefore the observed difference in the performance of the ISO and the NMKL methods was not statistically significant.

11. MENETELMÄN REFERENTTI

NMKL-menetelmän 71:1991 on uusinut Christer Wiberg, (Livsmedelsverket, Box 622, SE-751 26 UPPSALA, SVERIGE).

11. REFEREE OF THE METHOD

This revised NMKL method has been elaborated by Christer Wiberg, National Food Administration, Box 622, SE-751 26 UPPSALA, SWEDEN.

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SALMONELLA -SUVUN BAKTEERIEN MÄÄRITYS RIKASTUSMENETELMÄLLÄ

Yleistä

Salmonella-sukuun kuuluu kaksi lajia *Salmonella Enterica* ja *Salmonella Bongori. Salmonella Enterica* jakautuu kuuteen alalajiin ja nämä edelleen serotyypeiksi, joita tällä hetkellä tunnetaan yli 2300. Osa salmonellatyypeistä on patogeenisia ihmisille ja monille eläinlajeille.

Salmonella aiheuttaa useita erilaisia tauteja nopeasti paranevista **ripuleista vaikeisiin septisiin infektioihin**. Tartunta saadaan useimmiten ruuasta tai juomasta, ja tartunta on lähes aina peräisin eläinkunnan tuotteesta tai iduista. harvinaisempaa on, että tartunnan lähteenä on oireeton salmonellan uloste-erittäjä.

Tyypillisimmillään oireet ovat **pahoinvointi, 1-2 vrk kestävä kuume ja alle viikon ripuli. Vaikeat taudinkuvat ovat tavallisia lapsilla ja vanhuksilla sekä erilaisia perustauteja sairastavilla.**

Salmonella pystyy lisääntymään elintarvikkeissa lämpötila-alueella 5-47^{0}C ja pH-alueella 4-9.**Salmonella kestää pakastamista, mutta tuhoutuu kuumennettaessa yli 65^{0}C.** Salmonella ei lisäänny kuivissa elintarvikkeissa, mutta säilyy niissä hengissä.

Näytetyypit

Elintarvikkeet, rehut, ympäristönäytteet (tähän menetelmään on kirjattu ohjeet myös salmonellavalvontaohjelmaan liittyvien näytteiden tutkimiseksi). Jos tutkittavan elintarvikkeen käsittelystä on olemassa standardi, joka poikkeaa tässä kuvatusta, käytetään elintarvikkeen omaa standardia, kuitenkin niin, että poikkeamat tässä kuvatusta menetelmästä ovat mahdollisimman vähäiset.

Määritelmä

Salmonellat ovat *Enterobacteriaceae* -heimoon kuuluvia fakultatiivisesti anaerobeja, gramnegatiivisia sauvoja. Salmonellat liikkuvat tavallisesti peritrikkisin flagelloin, mutta liikkumattomia kantoja tavataan. *S. Gallinarun* ja *S. pullorum* ovat aina liikkumattomia. Salmonella muodostaa happoa glukoosista ja mannitolista, mutta ei sakkaroosista eikä yleensä laktoosista. Diagnostiikassa on kuitenkin erittäin tärkeä muistaa, että on myös laktoosipositiivisia salmonellakantoja. Salmonella on urea- ja indolinegatiivinen. Rikkivedyn muodostuskyky vaihtelee. Salmonella -suku jaetaan antigeenisten ominaisuuksien perusteella serotyyppeihin (O, H, Vi, Kauffman-White).

Periaate

Salmonellan osoittaminen on nelivaiheinen

1. Esirikastus

Punnittu määrä näytettä esirikastetaan esi-selektiivisessä liemessä (puskuroitu peptonivesi) 37±1^{0}C:ssa 16-20 tuntia. Rehunäytteitä pitää esirikastaa vähintään 24 tuntia (-30 tuntia).

2. Rikastus

Tunnettu määrä esirikastettua näytettä siirrostetaan selektiiviseen rikastusliemeen (RV, seleniittikystiini) ja inkuboidaan 41,5±1^{0}C:ssa 18-24 tuntia/RV tai 37±1^{0}C:ssa 48±4 tuntia/seleniittikystiini.

3. Rikastusliemen viljely selektiivisille maljoille

Silmukallinen selektiivisestä rikastusviljelmästä siirrostetaan XLD- ja Rambach- maljoille, joita inkuboidaan 37±1^{0}C:ssa 24±3 tuntia.

4. Varmistus

Salmonellaksi epäillyt pesäkkeet XLD:llä ja/tai Rambachilla varmistetaan biokemiallisin ja serologisin testein. Lopullinen varmistus teetetään alihankintana Evirassa (tarvittaessa KTLssä).

Elatusaineet, reagenssit ja välineet
Katso tarkemmin elatusainevalmistuksen agarkansio kunkin elatusaineen kohdalta.

Esirikastus:	puskuroitu peptonivesi
	maitojauheelle 1% briljanttivihreäliuos ja steriili puhdistettu vesi
	kaakaota sisältäville tuotteille kuorittu maito-ravintoliemi-brilj.vihreä
	(jos näin sovitaan tutkimuksen tilaajan kanssa)
Rikastus:	Rappaport-Vassiliadis-rikastusliemi (RV)
Agarit:	Ksyloosi-lysiini-desoksikolaatti-agar (XLD)
	Rambach
	TSI
	Urea
Antiseerumi:	Omnivalent

API 20 E tunnistusjärjestelmä

Inkubointikaapit:	41,5$\pm$1^{0}C
	37$\pm$1^{0}C

Suoritus

Näytteiden tutkiminen aloitetaan viimeistään saapumista seuraavana päivänä. Jos niitä ei pystytä heti tutkimaan, ne säilytetään jääkaapissa tutkimuksen aloittamiseen asti. Yli 3 vrk matkalla viipyneet näytteet eivät enää ole tutkimuskelpoisia. Tästä ilmoitetaan viipymättä tutkimuksen tilaajalle.

Otettaessa viljelynäytettä salmonellan varalta tehtäviä tutkimuksia varten **on tärkeää ennalta tietää salmonellasaastutuksen todennäköiset kohteet.** Esimerkiksi jäädytetystä lihasta näyte on otettava mahdollisimman laajalta alueelta, koska mahdollinen saastutus voi olla pinnalla. Otettaessa viljelynäytettä kuivista jauhoista tai muroista on näytteen määrä ratkaiseva. Tämän tyyppisistä näytteistä on suotavaa ottaa suurempia näytteitä, esim. 100 g. Jos saatavilla on runsaasti pakkauksia, on syytä ottaa näyte useasta pakkauksesta. Nämä näytteet tutkitaan joko erikseen tai sekoitetaan hyvin ja tutkitaan kokoomanäytteenä. Happamien tuotteiden esirikasteen pH ei saa laskea alle 4,5.

Otettaessa viljelynäytettä kokonaisista raaoista siipikarjaruhoista tai nahkaa sisältävistä ruhonosista kasvaa määritysherkkyys, jos esirikastukseen otetaan koko ruho tai sen osa. Vaihtoehtoisesti voidaan näytteeksi ottaa pinta-alaltaan vähintään 25 cm^2:n pala niskanahkaa - tai muuta nahkaa ellei niskanahkaa ole saatavilla. Näytteenottotavasta sovitaan mikrobiologin kanssa.

Yleisohje (huom. erikseen mainitut näytetyypit kts. jäljempänä).

<u>Esirikastus</u>

Punnitse **elintarviketta** 10g/25 g näytettä 90 g/225 g:aan puskuroitua peptonivettä pulloon.

Salmonellavalvontaohjelman mukaisia näytteitä koskee näytteenkäsittelyohje ”Salmonellavalvontaan kuuluvien näytteiden käsittely ja koostaminen”, Eviran ohje 6002-1/6, 31.6.2012

Jos näytemäärä poikkeaa edellä mainituista, valitaan puskuroidun peptoniveden tilavuus siten, että näytemäärän suhde rikasteen tilavuuteen on 1:9 (eli näyte laimenee 1:10, yksi osa näytettä + 9 osaa rikastetta). **Kirjaa rikastettu näytemäärä työraportille.**

Inkubointi: Näyte sekoitetaan puskuroituun peptoniveteen ravistelemalla pulloa kevyesti. Rikastetta inkuboidaan 37±1^{0}C:ssa 16-20 tuntia. Rehunäytteitä pitää esirikastaa vähintään 24 tuntia (-30 tuntia). **Esirikaste voidaan siirtää inkuboinnin jälkeen jääkaappiin enintään kahdeksi vuorokaudeksi, mikäli sitä ei voidaan välittömästi inkuboinnin jälkeen jatkoviljellä (esim. viikonloppu). Tällöin esirikastetta on inkuboitava 37±1^{0}C:ssa 2 tuntia ennen jatkoviljelyä.**

Rikastus

Lämmitä 10 ml Rappaport-Vassiliadis- rikastuslientä (RV) 37±1^{0}C:een. Mikäli puskuroitu peptonivesirikaste on ollut viikonlopun takia jääkaappilämpötilassa, inkuboi sitä 37±1^{0}C:ssa 2 tuntia ennen siirrostamista.

Sekoita puskuroitu peptonivesirikaste ja siirrosta siitä 0,1 ml 10 ml:aan RV-lientä.

Säilytä esirikasteet jääkaapissa, kunnes tutkimustulos yhteisnäytteestä on valmis. Jos toteat salmonellan yhteisnäytteestä, tutki jokainen yhteisnäytteen osanäyte erikseen salmonellan varalta.

Inkubointi
RV-lientä inkuboidaan 41,5±1^{0}C:ssa 24±3 tuntia.

Viljely agarmaljoille
Sekoita rikastusliemi. Siirrosta silmukalla (10 µl) rikastusliemestä XLD-maljalle ja –Rambach -maljalle hajotusmenetelmällä, jolloin saadaan selvästi erillisiä pesäkkeitä.

Maljojen inkubointi
Inkuboi XLD- ja Rambach maljoja 37±1^{0}C:ssa 24±3 tuntia.

Maljojen tarkastelu
XLD: tyypillinen salmonellapesäke on punertava, läpinäkyvähkö ja sillä on musta keskusta. Lähes aina (erityisesti voimakkaan salmonellakasvun yhteydessä) on pesäkettä ympäröivässä elatusaineessa laajempi tai kapeampi vaaleanpunainen/punainen vyöhyke.

Rambach maljalla tyypillinen salmonellapesäke on voimakkaan vaaleanpunainen, fuksianpunainen, tasaisen pyöreä tai leviävä pesäke kasvuston ympärillä voi olla alustassa vaaleanpunainen saostuma. Huomioi myös epätyypilliset pesäkkeet: väritön/persikanvärinen (esim. epätyypillinen S. parathypi) tai rakeinen epätasaisesti leviävä kasvusto (esim. epätyypillinen S. senftenberg).

Pesäkkeiden varmistaminen

Epäillyt pesäkkeet varmistetaan biokemiallisin ja serologisin menetelmin.

Valitse kummaltakin maljalta 2-10 (mikäli mahdollista) tyypillistä tai epäiltyä pesäkettä jatkotutkimuksiin. **Mikäli pesäke on selvästi erillään muista pesäkkeistä, siirrosta samalla pesäkkeestä ensin urea-putki, sitten TSI-putki ja lopuksi selektiivinen malja jäljempänä olevan ohjeen mukaan.**

Mikäli maljalla **ei ole erillisiä pesäkkeitä, viljele selektiiviselle agarille** jatkoviljelmä siten, että saadaan erillisiä pesäkkeitä varmistustestien suorittamista varten. Inkuboi 37±1^{0}C:ssa 24±3 tuntia.

Tarkista viljelmien puhtaus ja siirrosta siitä pistoviljelmänä TSI- ja pintaviljelynä ureaputkeen Mikäli ne eivät ole puhtaita, valitse kultakin taas tyypillinen tai epäilty pesäke ja viljele sitä edelleen selektiiviselle agarille ja siirrosta samalla TSI- ja urea-putkiin.

Käytä tarvittaessa biokemiallisten testien tulosten tulkinnan varmistamiseksi **positiivista ja tarvittaessa negatiivista testikantaa.** Kirjaa testikantojen antamien reaktioiden tulokset työraporttiin.

TSI- ja ureaputkien inkubointi:
37±1^{0}C 24±3 tuntia.

TSI-putken tarkastelu:
Salmonella käyttää glukoosia, muttei laktoosia ja sakkaroosia sekä muodostaa yleensä rautasulfidia, jolloin tyypillinen salmonella-reaktio TSI-putkessa on:
pinta punainen (emäksinen)- pohja keltainen (hapan) - keskiosa musta (rauta-(II)-sulfidi)
Voimakas rautasulfidin muodostus voi peittää alleen pohjan keltaisen värin (putki punamusta).
Rikkivetynegatiiviset salmonellat eivät muodosta rautasulfidia, ja aiheuttavat pelkän punakeltaisen reaktion.
Laktoosipositiiviset salmonellat muuttavat vinopinnan keltaiseksi. Varmista epäselvät tapaukset API20 E:llä.

Urea-putken tarkastelu:
Salmonella ei hydrolysoi ureaa, joten urea-putken väri säilyy keltaisena.

Mikäli XLD/Rambach tyypillinen pesäke antaa salmonellalle tyypilliset reaktiot TSI/Urea:lla, testataan kanta API20E:llä.

Serologinen varmistus
Tee selektiivisellä maljalla olevasta puhdasviljelmän pesäkkeestä objektilasilla agglutinaatiotesti omnivalentilla antiseerumilla seuraavasti:
1. Tiputa tummalla taustalla olevalle puhtaalle objektilasille tippa antiseerumia
2. Siirrä tutkittavaa bakteerimassaa tasaiseksi suspensioksi seerumipisaraan. Bakteerimassan voi ensin liettää pisaraan fysiologista (0,9%) suolaliuosta seerumipisaran vieressä ja kun suspensio on tasainen, sekoita sitä antiseerumiin.
3. Kallista objektilasia edestakaisin.
4. Tummaa taustaa vasten tarkasteltuna salmonella-solut liimautuvat vaaleiksi kokkareiksi tai hiutaleiksi, negatiivisessa tapauksessa samennus on tasainen.
5. Tee myös kontrollinäyte niin, että antiseerumin tilalla käytät fysiologista keittosuolaliuosta (autoagglutinaation toteaminen).
6. Käytä testissä kontrollina positiivista salmonellakantaa.
7. Kirjaa tulokset työraportille.

Biokemiallisin testein (TSI- ja urea, API 20E ja serologisesti) varmistettu bakteerikanta lähetetään EVIRA:an (Kuopio/Henry Kuronen) lopullisesti varmistettavaksi, erityistapaukissa myös KTL:ään.
Lähetä kanta (tavallisesti 5 viljelmää/kanta) maljoilla asianmukaisesti pakattuna EVIRA:an käyttäen EVIRAN:n lähetettä. Lähetä myös ne kannat, jotka autoagglutinoivat. Kirjaa lähetteeseen tutkimuspyynnöksi **”Salmonella-serotyypitys”.**

Jos kyseessä on omavalvontanäyte, on sekin mainittava lähetteessä, koska muuten tulos menee tiedoksi ulkopuolisille tahoille.

Salmonellavalvontaohjelman mukaisissa näytteissä lähetetään aina myös karjojen uusintatutkimuksissa todetut salmonellakannat. Lähetteeseen kirjataan myös omistajan kunta ja lääni.

Lähetä puhdas kanta **suoraan EVIRAN:n salmonellalaboratorioon (nimi pakkauksen päälle)**, niin tulos saadaan nopeammin.

Toimita työraportti mikrobiologille mahdollisen välivastauksen kirjoittamista varten.

Tuloksen ilmoittaminen
Tulos ilmoitetaan seuraavasti:

Negatiivinen tulos:	Salmonella	Ei todettu./10 g tai 25 g
Positiivinen tulos:	Salmonella	Todettu /10 g tai 25 g

Huuhtelunäytteiden tulos ilmoitetaan määritysrajan mukaan.
Mikäli näytemäärä on poikkeava, myös **tutkittu näytemäärä** ilmoitetaan ja esim. **pinta-ala**, jos se on saatavilla.
Lisäksi ilmoitetaan näytteiden tutkimisesta yhteisnäytteenä.

Virhelähteet
Joskus *Proteus* -kannat voivat aiheuttaa TSI-putkessa samanlaisen reaktion kuin salmonellat. Proteukset ovat kuitenkin ureaasipositiivisia (ureaputki muuttuu punaiseksi).
Jotkut *Citrobacter-*, *Proteus* ja *Enterobacter* -lajit voivat agglutinaatiotestissä aiheuttaa ristireaktion, joka ilmenee heikkona kokkaroitumisena.

Määritysmenetelmän laadunvarmistus
Kyseessä on kansainvälinen standardimenetelmän muunnos. Laboratorio osallistuu menetelmällä laboratorioiden välisiin vertailututkimuksiin. Mikäli vertailututkimusta ei jonain vuonna järjestetä, laboratorio osoittaa tulostensa oikeellisuuden siirrostetun näytteen kvalitatiivisella (herkkyys) tutkimuksella. Elatusaineiden toimivuus tarkistetaan toimintaohjeiden mukaisesti.

Viitemenetelmä
NMKL 71:1999

ENUMERATION OF MICRO-ORGANISMS BY A COLONY COUNT TECHNIQUE

Principle

The number of viable micro-organisms in liquid or dry samples is determined by plating a specified quantity of sample or sample dilution on Plate Count Agar (PCA) dish. Dishes are incubated at 30°C for 72h under aerobic conditions and the number of microbial colonies is counted.

Field of application

Method is applicable for enumeration of viable bacteria, yeasts, and molds from liquid and dried enzyme semifinal- and final products.

Equipment

Autoclave	
Water bath	45 °C ± 0.5°C
Incubator	30 °C ± 1 °C
Vortex	
Sterile Petri dishes:	Ø 80 mm to 100 mm
Sterile dilution bottles	100 ml
Sterile dilution tubes	10 ml
Sterile pipettes	1 ml and 10 ml
Glass flasks	250 ml
Colony counter	

Reagents

All solutions are prepared in deionized water, Milli-Q or equivalent.

1. Plate Count Agar (PCA)

Weigh 23.5 g of Plate Count Agar (e.g. Difco 0479) and dissolve into 1000 ml of water. Boil to ensure complete dilution of medium components. Divide 200 ml aliquots to 250 ml glass flasks. Sterilize in autoclave for 15 min at 121 °C. Sterile PCA agar is melted in a microwave oven or water bath and tempered to 45°C before use.

2. Dilution fluid (0.9 % w/v NaCl)

Dilute 9.0 g NaCl (e.g. Merck 6404) into 1000 ml of water. Divide 90 ml aliquots to appropriate glass flasks. Sterilize in autoclave for 15 min at 121 °C.

Samples

1. Dry samples

Aseptically weigh 10.0 g of sample to 90 ml of dilution fluid. Vortex until the mixture is homogenous (= 10^{-1} dilution). Prepare dilution series from the 10^{-1} dilution by pipetting 1 ml of 10^{-1} dilution to 9 ml of dilution fluid (= 10^{-2} dilution) and 1 ml of 10^{-1} dilution to 99 ml of dilution fluid (= 10^{-3} dilution). Vortex all the samples carefully. The dilution series can be continued further by following the dilution principle described above.

2. Liquid samples

Aseptically pipet 10.0 ml of sample to 90 ml of dilution fluid. Vortex until the mixture is homogenous (= 10^{-1} dilution). Prepare dilution series from the 10^{-1} dilution by pipetting 1 ml of 10^{-1} dilution to 9 ml of dilution fluid (= 10^{-2} dilution) and 1 ml of 10^{-1} dilution to 99 ml of dilution fluid (= 10^{-3} dilution). Vortex all the samples carefully. The dilution series can be continued further by following the dilution principle described above.

Procedure

1.0 ml of all samples including the original sample mix or undiluted enzyme sample and all the required dilutions are pipetted aseptically on empty Petri dishes as duplicates. 15-20 ml of tempered 45°C PCA-agar is poured to each of the plates. Samples are mixed to PCA-agar by carefully swaying the plates. The procedure from preparing the first sample dilution to pouring the PCA-agar should not take more than 15 minutes. Dishes are placed on an even surface and the agar is let to solidify. Dishes are moved to a 30°C ± 1 °C incubator and incubated upside down for 72h.

Results

Number of microbial colonies is counted from dishes that contain less than 300 colonies. Colonies are counted from both of the duplicate dishes from two dilutions.

The number of micro-organisms/ml or /g is calculated accordingly:

$$N = \frac{\Sigma C}{[n_1 + (0{,}1 \times n_2)]\, d}$$

Reviewed by Päivi Paukku

21.8.2013 Päivi Paukku

Approved by Eino Väisänen

21.8.2013 Eino Väisänen

ΣC = combined number of colonies (a´2 dishes from 2 dilutions)
n_1 = number of dishes from the first dilution
n_2 = number of dishes from the second dilution
d = dilution factor of the first dilution

The number of colonies from the second dilution should be at least 15.
Results are reported with the accuracy of two significant digits/ml or /g.

Example:

First dilution 10^{-2}; colonies on dishes 168 + 215 = 383
Second dilution 10^{-3}; colonies on dishes 14 + 25 = 39

$$N = \frac{383 + 39}{[2 + (0{,}1 \times 2)] \times 10^{-2}} = \frac{422}{0{,}022} = 19\,182 = 1{,}9 \times 10^4 \text{ cfu/ml or /g}$$

cfu = colony forming unit

If no colonies are detected in any of the plates result is reported as ‹ d^{-1} cfu/ml (liquid product) or ‹ d^{-1} cfu/g (dry product) (d = dilution factor of the smallest dilution).

References

International Standard ISO 4833, 2nd edition 1991-03-01. Microbiology - General Guidance for the enumeration of micro-organisms – Colony count technique at 30 °C.

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Determination of T-2 and HT-2 Toxins in Cereals Including Oats after Immunoaffinity Cleanup by Liquid Chromatography and Fluorescence Detection

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A reliable method for the determination of T-2 toxin and HT-2 toxin in different cereals, including oats, as well as in cereal products was developed. After extraction with methanol/water (90/10, v/v) and dilution with a 4% NaCl solution, the toxins were purified with immunoaffinity columns, derivatized with 1-anthroylnitrile, separated by HPLC, and determined using fluorescence detection. Due to the unspecific derivatization reagents, validation parameters were matrix dependent: in the range 10−200 μg/kg, recovery rates of 74−120% with relative standard deviations between 0.5 and 20.3% were obtained. On average, the limit of quantitation was shown to be 8 μg/kg for each toxin. For naturally contaminated samples, comparable results were obtained when analysis was performed according to this method without derivatization as well as according to a method based on a SPE cleanup utilizing tandem mass spectrometric detection in both cases. Using aqueous acetonitrile as extractant resulted in incorrectly high toxin concentrations due to water absorption of dry samples and toxin accumulation in the organic phase in the subsequent phase separation of the extractant. Furthermore, when comparing the commercially available immunoaffinity columns for T-2 and HT-2 toxins, significant differences regarding capacity and cleanup performance were observed.

KEYWORDS: T-2 toxin; HPLC-FLD; analysis; cereals; immunoaffinity column; derivatization; matrix effects; HPLC-MS/MS

INTRODUCTION

Mycotoxins are toxic secondary metabolites produced by different genera of fungi that grow on agricultural commodities in the field and during storage (*1*). Trichothecene mycotoxins are a family of tetracyclic sesquiterpenoids divided into four groups, referred to as types A, B, C, and D, whose production in cereals has been reported for several *Fusarium* species, for example *F. sporotrichioides*, *F. poae*, and *F. acuminatum*. The mycotoxins T-2 toxin (T-2) and HT-2 toxin (HT-2) are predominantly found in oats, but other cereals may also contain these toxins (*2, 3*). The toxic effects of T-2 and its metabolite HT-2 in animals and cell cultures are inhibition of protein synthesis and mitochondrial function, immunosuppression, and general cytotoxicity (*3*). Because of a lack of data regarding exposure of consumers, maximum levels for T-2 and HT-2 have been discussed within the European Commission for about 5 years and are expected to be established in 2008 (*4*). T-2 and HT-2 toxins are important representatives of type-A trichothecenes owing to an isovaleryl group at the C-8 position (**Figure 1**). Thus, compared to type-B trichothecenes (e.g., deoxynivalenol), which are characterized by a carbonyl function at this position, T-2 and HT-2 are not detectable via UV or fluorescence devices (*5*).

Today, different methods for the determination of T-2 and HT-2 are used. In general, analysis via gas-chromatography (GC) with an electron capture detector (ECD), GC with mass spectrometric detection (MS), and high-performance liquid-chromatography (HPLC) with tandem mass spectrometry (MS/MS) are the most frequently applied techniques (*6*). When using methods based on GC, several problems have to be coped with. Matrix compounds and analytes adsorb to active sites in the injector as well as to the first part of the column, which leads to higher toxin responses in the presence of the matrix. Drifting responses of the detected trichothecenes and carry over or memory effects from previous samples were also observed (*7, 8*). Furthermore, an elaborate derivatization in the course of sample workup is necessary for GC analysis, in order to increase volatility and sensitivity of the analytes (*9*).

In recent years, probably because of a simplified sample preparation without derivatization, HPLC-MS/MS methods are

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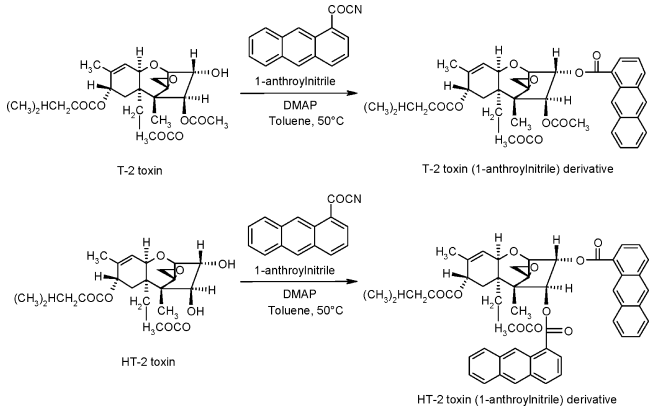

Figure 1. Scheme of the derivatization reaction of T-2 and HT-2 toxins with 1-anthroylnitrile in the presence of dimethylaminopyridine, according to Visconti et al. (*19*).

used more frequently (*10–12*). A critical point when using this detection method is the ionization of the analytes. Kloetzel et al. (*13*) described interfering effects of matrix compounds during the ionization of trichothecenes, which have to be overcome.

However, for laboratories without mass spectrometric equipment, no alternative to GC-ECD methods for T-2 and HT-2 analysis in cereals, especially in oats, exists. The structure of the T-2 and HT-2 molecules (i.e., the lack of any chromophore or fluorescent structure) makes it difficult to quantify these toxins. Derivatization procedures for T-2 and HT-2 using coumarin-3-carbonyl chloride, thus making flourescence detection possible, are reported by different research groups (*14–17*). Although the sensitivity of this method seems to be satisfying, its main disadvantage lies in a time-consuming synthesis of the reagent in the laboratory. In 2003, Pascale et al. (*18*) described the derivatization of T-2 with 1-anthroylnitrile in the presence of dimethylaminopyridine. This reaction was utilized for the precolumn derivatization of T-2 and the subsequent HPLC-fluorescence detection. The method was applied to extracts of wheat, corn, barley, oats, rice, and sorghum after immunoaffinity cleanup. Based on Pascale's method (*18*), 2 years later Visconti et al. (*19*) published an improved method, which was suitable for HT-2 quantitation as well. Reliable results were obtained applying the method to wheat, corn, and barley, while oats and oat containing products could not be analyzed due to interfering compounds eluting at the retention time of HT-2 (*19*). Therefore, the aim of this work was to develop a reliable HPLC-FLD method for the analysis of T-2 and HT-2 in cereals, especially in oats, using immunoaffinity cleanup and precolumn derivatization with 1-anthroylnitrile. In order to determine even the lowest toxin concentrations, the limit of detection (LOD) should be as low as possible but in any case well below 100 μg/kg for each toxin. Furthermore, different items (e.g., comparison of extraction solvents (methanol/water, acetonitrile/water)) including trials regarding recovery rates and different IAC brands should be investigated.

MATERIALS AND METHODS

Chemicals and Materials. Methanol (MeOH, hyper grade), acetonitrile (MeCN, gradient grade), sodium chloride (NaCl, p.a.), ammonia (25%, p.a.), and ammonium acetate (p.a.) were purchased from Merck (Darmstadt, Germany). For all experiments, ultrapure water (H_2O) provided by a Millipore Milli-Q-System (Billerica, MA) was used. Toluene (chromasolv Plus, HPLC grade, ≥ 99.9%), 4-dimethylaminopyridine, T-2 toxin (T-2), HT-2 toxin (HT-2), and silanized amber vials (4 mL) with green melamine resin screw-caps with PTFE-liner were purchased from Sigma (Taufkirchen, Germany). 1-Anthroylnitrile was purchased from Wako (Neuss, Germany). Cellulose filters MN 619 1/4 were obtained from Machery-Nagel (Dueren, Germany), glass microfiber filters (GF/A) from Whatman (Maidstone, U.K.). Immunoaffinity columns EASI EXTRACT T-2 and HT-2 (IAC 1) were from R-Biopharm Rhone Ltd. (Darmstadt, Germany), and immunoaffinity columns T-2 test HPLC (IAC 2) were purchased from VICAM (Watertown, MA). The SPE-columns Bond Elut Mycotoxin (BEM, 1000 mg) were obtained from Varian (Darmstadt, Germany). An oat quality control test material T-2234 was purchased from Central Science Laboratory (Sand Hutton, U.K.). For all experiments, immunoaffinity columns EASI EXTRACT T-2 and HT-2 (IAC 1) were used, unless stated otherwise.

Standard Stock Solutions and Derivatization Reagent. T-2 and HT-2 toxin stock solutions were prepared by dissolving the solid commercial toxins in acetonitrile, resulting in concentrations of 50 μg/mL for each toxin. Dimethylaminopyridine and 1-anthroylnitrile stock solutions were prepared in toluene at concentrations of 3.25 and 3.00 mg/mL, respectively. Dimethylaminopyridine and 1-anthroylnitrile working solutions (0.325 and 0.300 mg/mL, respectively) were prepared by 1:10 dilution of stock solutions with toluene.

APPARATUS

HPLC-FLD. HPLC analysis was performed using an Agilent 1100 series system consisting of a binary pump, degasser, column oven, autosampler, fluorescence detector, and Chemstation-Software (Agilent Technologies, Waldbronn, Germany). The separation of T-2 and HT-2 toxins was performed using a 150 mm × 4.6 mm i.d., 5 μm, Luna Phenyl-Hexyl column with a 4 mm × 3.0 mm i.d. guard column (Phenomenex, Aschaffenburg, Germany). The column temperature was set at 40 °C, and the injection volume was 100 μL. A binary gradient at a flow rate of 1 mL/min was performed as follows: 70% acetonitrile and 30% water as starting composition was kept constant for 5 min. Within 10 min, the acetonitrile content was increased to 85% and kept constant for 10 min. Within 2 min, the acetonitrile content was raised to 100% and kept constant for 5 min. To equilibrate the system, the initial composition was held for 8 min before injecting the next sample. The excitation and emission wavelengths of the fluorescence detector were set at 381 and 470 nm, respectively.

HPLC-MS/MS. The MS/MS-quantitation of T-2 and HT-2 was performed according to Kloetzel et al. (*13*) using an in-house validated multimethod, which includes the trichothecenes T-2 tetraol, nivalenol, deoxynivalenol, fusarenon X, neosolaniol, 15-acetyldeoxynivalenol, monoacetoxyscirpenol, 3-acetyldeoxynivalenol, T-2 triol, diacetoxyscirpenol, HT-2 toxin, T-2 toxin, and zearalenone.

HPLC Parameters. HPLC analysis was performed using an Agilent 1100 series system consisting of a binary pump, degasser, column oven, and autosampler (Agilent Technologies, Waldbronn, Germany). The separation of T-2 and HT-2 toxins was performed using a 250 mm × 2 mm i.d., 4 μm, Synergi Fusion RP 80A column with a 4 mm × 2.0 mm i.d. guard column (Phenomenex, Aschaffenburg, Germany). The column temperature was set at 25 °C, and the injection volume was 10 μL. Solvent A was 1.84 mM ammonia in water and solvent B was 0.92 mM ammonia and 0.13 mM ammonium acetate in acetonitrile. A binary gradient at a flow rate of 0.2 mL/min was performed as follows: within 25 min, changing solvent B from 20 to 70%. Then, the content of solvent B was lowered within 1 min to 20%, which was held for 10 min.

MS/MS Parameters. MS/MS was performed on an API 3000 triple quadrupole mass spectrometer equipped with a TurboIonSpray electrospray ionization (ESI) source (Applied Biosystems, Darmstadt, Germany) heated at 400 °C in the positive ionization mode. The ion spray voltage was set at 4200 V. As the nebulizer and auxiliary gas, zero grade air (8 arbitrary units and 8 L/min,

respectively) was used. Nitrogen served as the curtain gas (8 arbitrary units) and as the collision gas in quadrupole 2 (2.5×10^{-5} Torr corresponding to 4 arbitrary units). Quantitation was performed using multiple reaction monitoring (MRM) with a dwell time of 150 ms. The following transition reactions of T-2 and HT-2 with the respective declustering potential (DP), collision energy (CE), and cell exit potential (CXP) in brackets were recorded using the first mass transition for quantitation. T-2: *m/z* 484.4−245.4 (DP 26 V, CE 19 V, CXP 16 V), *m/z* 484.4−215.4 (DP 26 V, CE 25 V, CXP 14 V), *m/z* 484.4−185.4 (DP 26 V, CE 31 V, CXP 12 V). HT-2: *m/z* 442.2−215.0 (DP 26 V, CE 19 V, CXP 14 V), *m/z* 442.2−233.2 (DP 26 V, CE 17 V, CXP 16 V), *m/z* 442.2−197.2 (DP 26 V, CE 21 V, CXP 14 V). Data aquisition was carried out using Analyst 1.4.2 software (Applied Biosystems, Darmstadt, Germany).

SAMPLE PREPARATION

HPLC-FLD. *IAC Cleanup after Extraction with Methanol/Water.* By using an Ultra Turrax blender, 25 g of finely ground sample mixed with 2.5 g of NaCl were extracted with 100 mL of methanol/water (90/10; v/v) for 3 min. To ensure a complete extraction of the toxins, the samples were then shaken for 30 min at a speed of 230 rpm. The mixture was filtered through a MN 619 1/4 filter. A portion of the filtrate (7.5 mL) was diluted with 30 mL of 4% NaCl. To precipitate all proteins, the mixture was left to settle for 5 min, following 3 min of stirring and then again left to settle for 5 min. After filtration through a GF/A filter, 25 mL of the filtrate was passed through an immunoaffinity column at a flow rate of 1 drop/s. The IAC was washed with 20 mL of water and dried by rapidly passing air through it. The toxins were eluted in screw-cap amber vials with 1 mL of methanol. In order to ensure the complete release of toxins, the backflush technique (i.e., reversing the direction of the flow by generating a low pressure with a syringe) with the mentioned solvent was applied 3 times. Afterward, the IAC was rinsed with 1 mL of methanol. The combined eluates were evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen.

IAC Cleanup after Extraction with Acetonitrile/Water. By using an Ultra Turrax blender, 25 g of finely ground sample mixed with 2.5 g of NaCl were extracted with 100 mL of methanol/water (90/10; v/v) for 3 min. To ensure a complete extraction of the toxins, the samples were then shaken for 30 min at a speed of 230 rpm. The mixture was filtered through a MN 619 1/4 filter. A portion of the filtrate (6.4 mL) was evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen. The residue was reconstituted in 8.0 mL of acetonitrile/water (80/20; v/v). Part of the reconstituted mixture (7.5 mL) was diluted with 30 mL of 4% NaCl. The following steps were identical to those of the extraction with methanol.

Derivatization Procedure. The dried residues were dissolved in 100 μL of dimethylaminopyridine and 100 μL of 1-anthroylnitrile working solutions by vortexing for at least 1 min. The derivatization procedure was performed by heating for 20 min at 50 °C, followed by cooling for 15 min in an ice bath. The solvent was evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen. The residue was redissolved in 1 mL of acetonitrile/water (70/30; v/v). A portion of this extract (100 μL) was injected into the HPLC-FLD.

HPLC-MS-MS. *IAC Cleanup.* In addition to the cleanup for fluorescence detection, another aliquot of each extract was treated identically for mass spectrometric detection. After evaporation of the IAC eluate in a heated aluminum block at 50 °C using a gentle stream of nitrogen, the residue was reconstituted in 0.5 mL of acetonitrile/water (20/80; v/v). The solution was mixed, and a 10 μL aliquot was used for HPLC-MS/MS analysis.

Bond Elut Mycotoxin (BEM) Cleanup. According to Kloetzel et al. (*20*), the samples were cleaned-up via solid phase extraction (SPE) using Bond Elut Mycotoxin columns. The finely ground sample (25 g) was extracted with 100 mL of a mixture of acetonitrile/water (80/20; v/v) by blending at a high speed for 3 min using an Ultra Turrax blender. The extract was filtered through a MN 619 1/4 filter. A portion of the filtrate (4 mL) was passed through a BEM column. An aliquot of 2 mL of the eluate was evaporated to dryness in a heated aluminum block at 50 °C using a gentle stream of nitrogen. The residue was reconstituted in 0.5 mL of acetonitrile/water (20/80; v/v). The solution was mixed, and a 10 μL aliquot was used for HPLC-MS/MS analysis.

Validation of the HPLC-FLD Method with Methanol/Water Extraction. For external calibration, standard solutions containing both T-2 toxin and HT-2 toxin were prepared at concentrations of 5 μg/mL, 100 ng/mL, and 10 ng/mL of each toxin by diluting the stock solutions in acetonitrile. Thirteen different concentration levels were prepared. Therefore, aliquots (corresponding to 1−420 ng and 0.8−336 μg/kg of each toxin, respectively) were placed in screw-cap amber vials, evaporated in a heated aluminum block at 50 °C using a gentle stream of nitrogen, and derivatized as described above. The resulting peak areas of the toxins were plotted against the concentrations. Calibration curves were calculated by linear regression. Because of the lack of blank matrices, recovery experiments were performed in triplicate (three independent extractions) using naturally contaminated oats, infant food, muesli, corn grits, and breakfast cereals. Corresponding toxin amounts were added to aliquots of the raw extract before cleanup via IAC, resulting in levels of 10, 50, 100, and 200 μg/kg of each toxin. The precision of the derivatization procedure was determined by triplicate derivatization of evaporated standard solutions at concentrations of 1, 10, 100, and 250 ng of each toxin. The precision of the complete method was determined by a 10-fold workup (10 independent extractions) of naturally contaminated oat and infant food samples. A commercially available Food Analysis Performance Assessment Scheme (FAPAS) oat quality control test material (T-2234) was worked up in duplicate (two independent extractions) in order to check the trueness of the validated method. The workup of this quality control test material included recovery experiments, which were also performed in duplicate by spiking 100 μg/kg of each toxin to aliquots of the raw extracts. All mentioned experiments were performed according to the method using extraction with methanol/water, IAC cleanup, and determination via HPLC-FLD after precolumn derivatization with 1-anthroylnitrile.

FURTHER EXPERIMENTS

Comparison of Different Cleanup (IAC, SPE) and Detection Systems (FLD, MS/MS). The same extracts of the samples used for validation experiments were cleaned-up in triplicate for HPLC-MS/MS detection without derivatization with 1-anthroylnitrile. Additionally, the same samples were cleaned-up in triplicate using Bond Elut Mycotoxin columns and detected via HPLC-MS/MS.

Comparison of Extraction Solvents. In addition to the validation experiments, the oat, muesli, and breakfast cereals samples as well as the FAPAS oat quality control test material

(T-2234) were cleaned-up according to the acetonitrile extraction procedure for HPLC-FLD, instead of using methanol, in triplicate and in duplicate, respectively.

Spiking Experiments (Direct, Extract) Using Blank and Naturally Contaminated Oat Samples and HPLC-FLD Detection. T-2 and HT-2 blank oat samples were directly spiked with about 150 μg/kg of each toxin and worked up in triplicate (three independent extractions) according to the method with methanol/water extraction, as well as according to the method with acetonitrile/water extraction. Additionally, to compare the spiking procedures, the sample was identically analyzed in triplicate (three independent extractions) but with the difference that the raw extracts, rather than the sample itself, were spiked at a level of 150 μg/kg of each toxin. Furthermore, three naturally contaminated samples were treated in the same way (direct and extract spiking, three independent extractions) with a spiking level of about 100 μg/kg of each toxin.

Comparison of IAC. Standard solutions of methanol/water (18/82; v/v) containing absolute toxin amounts of 10, 50, and 500 ng of each toxin were passed through IAC of both brands in triplicate each and prepared for fluorescence detection according to the validated method. Performance experiments were conducted in triplicate using a naturally contaminated oat sample. Therefore, corresponding toxin amounts at levels of 10, 50, 100, and 200 μg/kg of each toxin were spiked to aliquots of the raw extract before the cleanup via IAC of both brands, and the extracts were then prepared for fluorescence detection according to the validated method.

Statistical data for all experiments mentioned above was calculated using the software Valoo 2.1 (Analytik-Software, Leer, Germany).

RESULTS AND DISCUSSION

Preliminary Work. In general, methods based on derivatization of the analytes bring about challenges that have to be overcome. The derivatization process using 1-anthroylnitrile and dimethylaminopyridine is based on an esterification of hydroxyl groups (**Figure 1**). Because of this unspecific derivatization mechanism, not only the toxins T-2 and HT-2 but also many compounds originating from matrix as well as from solvents or from impurities of the reagents are potential reaction partners for 1-anthroylnitrile. In the first experiments where standard solutions were derivatized according to Visconti's method (*19*), chromatograms showing interfering peaks at the retention time of the toxins were obtained, thus making a reliable determination impossible. Experiments with different brands of toluene (all labeled "for residue analysis") as solvent for dimethylaminopyridine and 1-anthroylnitrile reagents resulted mainly in chromatograms of unsatisfying quality. Only one of the four tested brands of toluene resulted in chromatograms that were free of interfering signals at the toxins' retention time. In practice, even the chromatogram of a reagent blank (containing only dimethylaminopyridine and 1-anthroylnitrile) using this toluene led in general to a significantly higher base fluorescence as well as to a multitude of signals within the whole chromatographic process (**Figure 2**A). A chromatogram as shown in **Figure 2**B was obtained by the derivatization of standard solution. As Visconti et al. (*19*) observed, the clean-up of oat samples resulted in chromatograms with interfering matrix signals at the retention time of HT-2, making a reliable quantification for this toxin impossible. Hence, the method could not be validated for oats matrix. Recently new commercially available IAC for T-2 and HT-2 determination in cereals were launched together with a protocol based on the slightly modified method of Visconti et

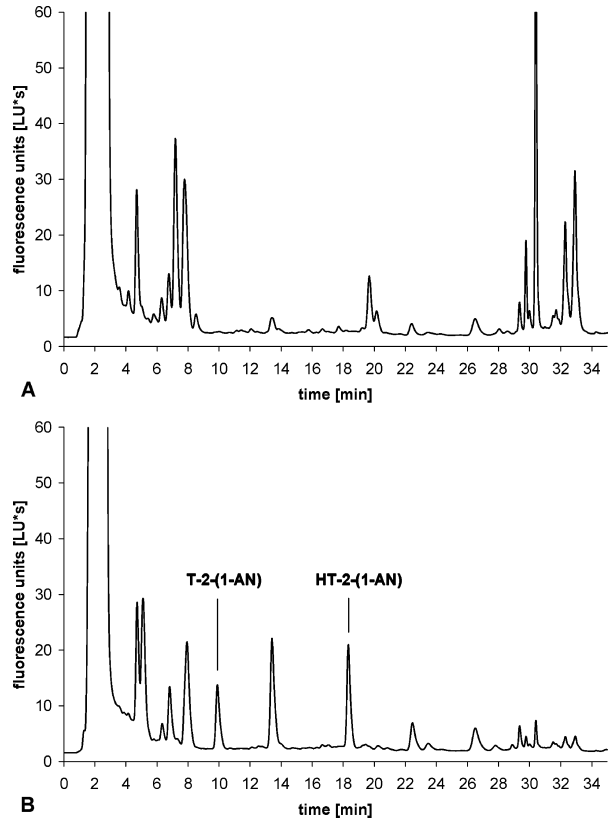

Figure 2. HPLC-FLD chromatograms of (**A**) a reagent blank (containing only 1-anthroylnitrile and dimethylaminopyridine) and (**B**) a standard solution of T-2 and HT-2 toxins (10 ng each) derivatized with 1-anthroylnitrile and dimethylaminopyridine.

al. (*19*). Application of this modified protocol to oat samples unfortunately led to no improvement in terms of purity of the chromatograms (**Figure 3**A). According to the protocols based on Visconti et al. (*19*), dilution of the extracts had to be performed using water, which resulted for most of the samples in turbid solutions. To precipitate oat-specific compounds (e.g. proteins) within this dilution, tests with different salts (e.g., sodium chloride) were performed. Interpretable chromatograms could be obtained by diluting the raw extract with a 4% sodium chloride solution instead of using water. To ensure the precipitation of proteins, the mixture was left to settle for 5 min after addition of 4% sodium chloride solution, followed by stirring for 3 min, and again left to settle for 5 min. Nearly all kind of matrices, including oats, were cleaned-up in this manner, giving reliable results. **Figure 3**B presents a chromatogram of an oat sample after clean-up including this step. Only with these improvements, the determination of toxin concentrations, which correspond to those obtained with our internal reference method (BEM-clean-up and quantitation via HPLC-MS/MS) (*20*), were acquired (**Figure 4**). It is evident that all hydroxyl group containing molecules compete with the toxins for the derivatization reagents. Easily accessible hydroxyl groups were derivatized better and faster than sterically hindered ones. Especially for HT-2, it was observed that the derivatization step was more critical due to the presence of a second hydroxyl group (**Figure 1**).

Pascale et al. (*18*) used 50 μL of each derivatization solution for the determination of only T-2 toxin in cereals including oats. The same volumes containing similar concentrations of reaction agents were used by Visconti et al. (*19*) for the derivatization of T-2 and HT-2 in cereals, with the exception of oats. Taking the high amount of oat-specific potential reaction partners for

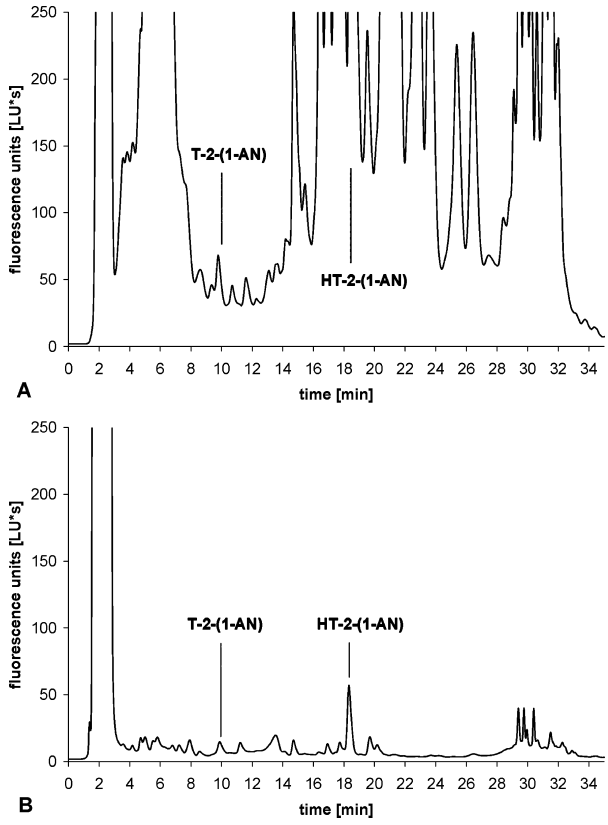

Figure 3. HPLC-FLD chromatogram of a naturally contaminated oat sample: (**A**) Cleanup according to the protocols of IAC-manufacturers—the dilution of the extract was performed using water; (**B**) Clean-up according to the validated method—the dilution of the extract was performed using 4% NaCl solution. The concentrations of T-2 and HT-2 toxins were 6.2 and 24.9 μg/kg, respectively.

1-anthroylnitrile into consideration, we tested volumes of 50 and 100 μL of each reagent for derivatization of the toxins extracted from different matrices (oats, infant food, breakfast cereals). Furthermore, the reaction time of 15 and 20 min was tested. Within the investigated matrices, there was no significant higher toxin response observable using higher volumes of derivatization reagents and/or more reaction time, but 3-fold trials showed lower standard deviations. In order to ensure a complete derivatization of toxins extracted from all considerable matrices, volumes of 100 μL of each reagent and a reaction time of 20 min were used for all experiments.

Validation of an HPLC-FLD Method for the Determination of T-2 and HT-2 Toxins after Methanol/Water Extraction, IAC Cleanup, and Precolumn Derivatization. Linearity for standard solutions with concentrations between 1 and 420 ng/mL (corresponding to 0.8−336 μg/kg) was confirmed for both toxins via a plot of the residuals. Correlation coefficients (R^2) ranged from 0.995 to 0.9999 and from 0.9980 to 0.9994 for T-2 and HT-2, respectively. To demonstrate the method's scope of application, a broad range of naturally contaminated matrices (oats, infant food, muesli, corn grits, and breakfast cereals) was cleaned-up according to the method stated above. The native contents of T-2 and HT-2 in these samples are given in **Table 1**. Statistics showed that the relative standard deviation (RSD_r, generated under repeatability = intralaboratory conditions) increased with decreasing quantities of the measured toxin content. This was mainly observable for the corn grits sample. However, even for this very lowly contaminated sample, the limits of RSD_r set by the EU to be ≤40% were fulfilled for T-2 (contamination level 50−250 μg/kg) and HT-2 (contamination level 100−200 μg/kg) (*21*). Due to a lack of oat- and oat-containing blank material for T-2 and HT-2, recovery trials were carried out with the same naturally contaminated materials mentioned above. Within this method, direct and extract spiking were verified to give comparable results, as described below. Therefore, the spiking solution was added to aliquots of the raw extracts. In the spiking range from 10 to 200 μg/kg of each toxin, reliable results were obtained (**Table 2**). The European Commission has set recovery limits for T-2 and HT-2: thus, recoveries should be in the range 60−130% at contamination levels of 50−250 μg/kg for T-2 and at 100−200 μg/kg for HT-2 (*21*). As a result, recovery rates of the validated method were in alignment with EU requirements. The limit of detection and the limit of quantitation (LOD, LOQ) were determined using standard solutions. They were based on a signal-to-noise ratio of 3/1 and 9/1, respectively. Limits were found to be theoretically clearly lower than 1 and 3 ng/mL (corresponding to 0,8 and 2,4 μg/kg), respectively. In practice, nontarget signals resulting from the derivatization process appeared close to the signals of T-2 and HT-2 in the chromatograms, even without matrix (**Figure 2**B). The composition of the matrix is mainly responsible for the abundance of signals. As per experience with this method, an average LOQ of 10 ng/mL (equivalent to 8 μg/kg) for each toxin is applicable in matrix samples. Depending on the matrix type, the LOD and LOQ may vary, resulting in slightly lower or higher values. However, based on the assumption of a future maximum limit of about 100−150 μg/kg (sum of T-2 and HT-2),the LOD and LOQ, even with slight variations from matrix to matrix, are acceptable. The RSD_r of the 3-fold derivatization of toxin standards was acceptable for all tested concentrations and both toxins (data not shown). With a value of ≤5%, the RSD_r for HT-2 was higher than for T-2 (≤2%), which correlates with the necessity to derivatize two hydroxyl groups in the HT-2 molecule instead of only one hydroxyl group as in the T-2 molecule. The precision of the complete method was performed 10-fold for oat and infant food samples. The mean contents ± SD (RSD_r) of the oat sample were 66.0 ± 3.4 μg/kg (5.2%) for T-2 and 187.5 ± 10.8 μg/kg (5.8%) for HT-2. The results for T-2 and HT-2 in the infant food sample were 5.0 ± 0.7 μg/kg (14.0%) and 40.3 ± 3.6 μg/kg (8.9%), respectively. In addition to recovery experiments, the trueness of the method was approved using a commercially available Food Analysis Performance Assessment Scheme (FAPAS) oat quality control test material (T-2234). This test material was a real food matrix with T-2 and HT-2 contents that have been sufficiently well-established from the results of laboratories participating in a proficiency test. The values have been derived as a consensus of a number of laboratories using a variety of methods. The mean values (corrected for recovery) for T-2 and HT-2 were 83.3 ± 18.3 and 113.4 ± 24.9 μg/kg, respectively, summarized from 41 (T-2) and 35 (HT-2) participating laboratories. The satisfactory range, which indicates the range between which results would have been awarded a satisfactory z-score in the proficiency test, was 46.6−120.0 μg/kg for T-2 and 63.5 - 163.3 μg/kg for HT-2, respectively. This broad range reflects the current situation regarding analysis of T-2 and HT-2 toxins. The values resulting from the newly developed method range slightly below but still close to the mean values of all participating laboratories (**Table 3**). Considering the performance data of the method, it is applicable for the determination of T-2 and HT-2 in all kind of cereals including oats. Thus, it provides an alternative to all GC as well as HPLC-MS/MS methods for the analysis of samples regarding compliance with future EU maximum limits.

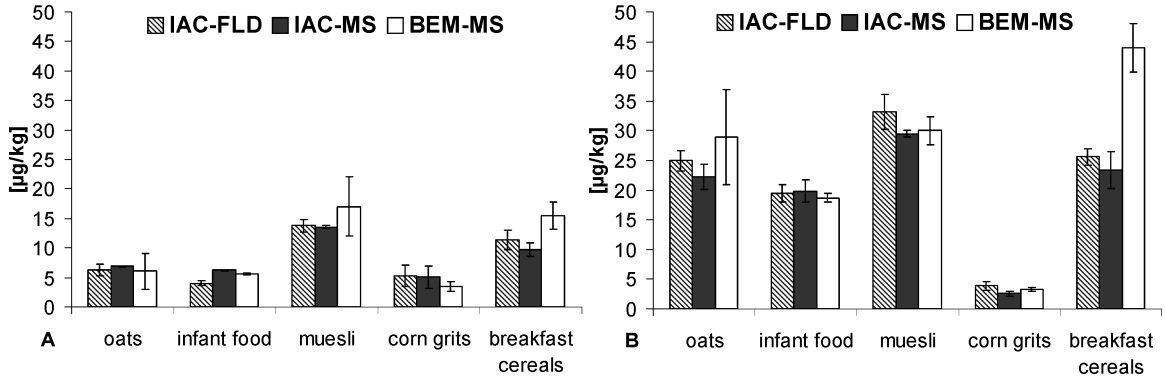

Figure 4. T-2 (**A**) and HT-2 (**B**) concentrations ± SD ($n = 3$) in different samples depending on cleanup and detection system. IAC-FLD and IAC-MS values are based on the very same extracts yielded after methanol/water extraction followed by FLD and MS/MS detection, respectively. BEM-MS values are based on acetonitrile/water extraction followed by MS/MS detection.

Comparison of Different Cleanup (IAC, SPE) and Detection Systems (FLD, MS/MS). In order to investigate the quality of derivatization via 1-anthroylnitrile, the extracts of the samples used for validation were worked up simultaneously for HPLC-MS/MS detection. The first and second bars in **Figure 4**A and **B** show the T-2 and HT-2 values determined after identical IAC cleanup, differing only in the type of detection: the first bar represents the fluorescence detection while the MS/MS detection is represented by the second bar. Within the variation of values, there is no difference between fluorescence and MS/MS detection, which confirms the reliability and quality of the precolumn derivatization used for the FL-detection. The third bar shows the values of an in-house validated method including a SPE cleanup using Bond Elut Mycotoxin columns with subsequent HPLC-MS/MS-detection based on Kloetzel et al. (*20*). While the T-2 values quantified with the three mentioned methods matched for all matrices, the HT-2 value of the breakfast cereals cleaned-up according to the BEM-protocol represented a trend to higher values. Beside the different cleanup principles (immunoaffinity chromatography versus solid phase extraction), the used extraction solvents (methanol/water versus acetonitrile/water) were probably the reason for this trend. Different research groups are convinced that, in general, a mixture of acetonitrile/water is a better alternative for T-2 and HT-2 extraction than methanol/water. The main problem concerning cleanup via immunoaffinity columns is the denaturation of the antibodies in the presence of even low concentrations of acetonitrile (3−5%) in comparison to methanol (15−20%) (*18, 19*).

Comparison of Extraction Solvents. In order to compare the obtained results using methanol/water extraction with those resulting from a method using acetonitrile/water extraction, an additional step had to be included in the latter. An aliquot of the raw extract had to be evaporated to dryness and reconstituted in methanol/water (90/10; v/v). The subsequent cleanup was then performed as described for the original method. Significantly higher results for both toxins were obtained for oats and especially for breakfast cereals after acetonitrile/water extraction but not for the investigated muesli sample. As presented in **Table 3**, the FAPAS oat quality control test material (T-2234) extracted with acetonitrile/water also resulted in higher toxin values in comparison to those determined via methanol/water extraction. This points out that, at least in some matrices, a more effective extraction is obtained using acetonitrile/water. As reported by Stroka et al. (*22*), the use of aqueous acetonitrile to extract aflatoxin B1 from dry samples like spices, infant formula, or animal feed can result in incorrect, higher toxin values. This is due to the water absorption of the investigated samples, resulting in phase separation with accumulation of the toxins in the organic phase. The author recommended the use of aqueous methanol for extraction because, for this solvent, the absorption effect was not observed (*22*). Taking this fact into consideration, the higher results after extraction with acetonitrile/water are doubtful and have to be verified by further investigations. The chromatograms resulting from the acetonitrile/water extraction were slightly better than those from methanol/water extraction regarding peak shape and resolution. This was probably due to less interfering compounds extracted by acetonitrile/water but also strongly depending on the matrix type.

Spiking Experiments (Direct, Extract) with Blank and Naturally Contaminated Oat Samples. To verify whether the higher results of the acetonitrile/water extraction were caused by the mentioned effect, we performed spiking trials with the only available oat blank sample, which was brought from New Zealand. Recovery rates for both toxins and extractants of about 96−105% were determined after spiking of the extracts. Interestingly, when the sample was spiked directly, we obtained significantly higher recovery rates for T-2 and HT-2 (118−130%) after acetonitrile extraction but not after methanol extraction. In the latter, recovery rates of about 100% were determined.

In order to test if the effect is also observable in naturally contaminated samples oats, muesli and breakfast cereals were worked up in the same way. For all three samples, the identical pattern was obtained—the recovery rates for both toxins determined after direct spiking and acetonitrile/water extraction were about 130% (data not shown). Stroka et al. (*22*) proposed salting out effects in matrices containing high amounts of water-soluble constituents as well as water absorbing effects being responsible for these higher values. In the case that this effect is the only reason for higher values obtained after acetonitrile/

Table 1. T-2 and HT-2 Concentrations ± SD ($n = 3$) in Different Samples after Methanol/Water Extraction, IAC Cleanup, and Determination via HPLC-FLD[a]

matrix	T-2 toxin		HT-2 toxin	
	content ± SD (µg/kg)	RSD_r (%)	content ± SD (µg/kg)	RSD_r (%)
oats	6.2 ± 1.0	16.2	24.9 ± 1.7	6.8
infant food	4.0 ± 0.4	10.0	19.4 ± 1.5	7.7
muesli	13.8 ± 1.0	7.2	33.1 ± 2.9	8.8
corn grits	5.3 ± 1.8	34.0	3.9 ± 0.7	17.9
breakfast cereals	11.3 ± 1.7	15.0	25.6 ± 1.4	5.5

[a] The values are not corrected for recovery (recovery rates are given in **Table 2**).

Table 2. T-2 and HT-2 Recovery Rates ± SD ($n = 3$) in Different Samples after Methanol/Water Extraction, IAC Cleanup, and Determination via HPLC-FLD[a]

		T-2 toxin		HT-2 toxin	
matrix	spiking level of each toxin (μg/kg)	recovery ± SD (%)	RSD_r (%)	recovery ± SD (%)	RSD_r (%)
oats	10	86.3 ± 11.4	13.2	99.2 ± 8.6	8.7
	50	86.9 ± 5.5	6.3	90.7 ± 4.0	4.4
	100	86.3 ± 2.3	2.3	93.9 ± 2.2	2.3
	200	89.7 ± 0.7	0.8	95.3 ± 0.5	0.5
infant food	10	90.4 ± 10.8	11.9	119.6 ± 17.3	14.5
	50	95.7 ± 4.2	4.4	107.3 ± 7.2	6.7
	100	95.4 ± 0.2	0.2	109.6 ± 2.7	2.5
	200	97.0 ± 2.8	2.9	112.4 ± 6.4	5.7
muesli	10	88.9 ± 12.6	14.2	101.2 ± 16.4	16.2
	50	96.3 ± 0.9	0.9	103.5 ± 0.8	0.8
	100	97.8 ± 0.7	0.7	109.2 ± 1.8	1.6
	200	97.3 ± 2.1	2.2	105.0 ± 3.4	3.2
corn grits	10	80.9 ± 9.1	1.1	98.8 ± 5.5	5.6
	50	86.6 ± 4.6	5.3	102.5 ± 2.8	2.7
	100	87.9 ± 3.4	3.9	100.2 ± 2.6	2.6
	200	90.8 ± 4.2	4.6	101.5 ± 4.8	4.7
breakfast cereals	10	74.3 ± 15.1	20.3	102.1 ± 2.3	2.3
	50	81.0 ± 8.8	10.9	95.5 ± 5.4	5.7
	100	89.0 ± 2.2	2.5	97.5 ± 1.6	1.6
	200	87.2 ± 3.0	3.4	93.6 ± 5.0	5.3

[a] Recovery rates were determined by spiking of the extract.

water extraction, the T-2 and HT-2 values corrected for recovery (determined for each single sample) should be identical for both extractants. However, taking recoveries into account, still higher results were observed. For two of the three tested matrices, the significance was confirmed (two-sided t-test, $p = 0.05$). This concluded that especially for HT-2 the efficiency of the acetonitrile/water extraction was better than those of the methanol/water extractant. According to Stroka et al. (*22*), the addition of salt can have a strong influence on the results. This fact was tested by comparison of toxin values and recovery rates after extraction with and without sodium chloride using a naturally contaminated oat sample (data not shown). The findings of Stroka et al. (*22*) could not be confirmed as there was no significant influence of sodium chloride observable. It is supposed that the composition of the matrix has a more distinct influence regarding the mentioned effect.

Actually, since recovery rates are strongly matrix dependent and can vary significantly within the same group of food (e.g., oats), a spiking of each sample to be analyzed would be necessary. Irrespectively, because of the absorbing effects, toxin values resulting from extraction with acetonitrile/water have to be corrected mandatorily for recovery rates (resulting from direct spiking of the same sample) in order to obtain reliable results. Otherwise, too high concentrations of T-2 and HT-2 will be determined. In practice, this procedure is unsuitable as there would be for example an increased need for standard substances, sample matrix, and time. As a precondition for validation of an appropriate method based on an extraction with acetonitrile/water, the absorbing effects have to be coped with.

Comparison of IAC. At the moment, immunoaffinity columns for cleanup of T-2 and HT-2 toxins are commercially available from two manufacturers. In order to compare these columns for using in combination with the validated method, the capacity as well as the cleanup performance in naturally contaminated and spiked samples were tested. Pascale et al. (*18*) determined the maximum capacity of the utilized IAC 2 only for T-2 toxin to be 1.6 μg, while Visconti et al. (*19*) determined this parameter for the sum of T-2 and HT-2 to be 1.4 μg. Within the IAC-capacity experiments, it can be concluded that IAC 1 gave about 90−100% recovery for both toxins at all tested concentrations. IAC 2 worked well for 10 and 50 ng of each toxin, but for 500 ng, the HT-2 recovery was only about 50% in comparison to 100% for T-2. The toxin contents in a naturally contaminated oat sample were 6.2 ± 1.0 μg/kg for T-2 and 24.9 ± 1.7 μg/kg for HT-2 after cleanup with IAC 1. Utilizing

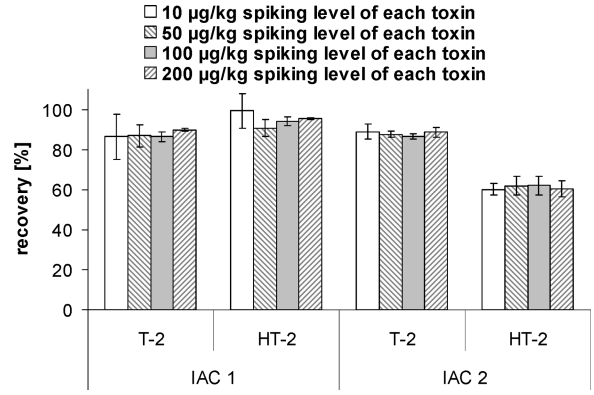

Figure 5. T-2 and HT-2 recovery rates ± SD ($n = 3$) depending on spiking level and used IAC determined in a spiked oat sample via HPLC-FLD.

Table 3. T-2 and HT-2 Concentrations ± SD ($n = 2$) in FAPAS Oat Quality Control Test Material (T-2234) after Different Extraction Procedures, IAC Cleanup, and Determination via HPLC-FLD[a]

	T-2 toxin			HT-2 toxin		
method	content ± SD (μg/kg)	z-score	recovery ± SD (%)	content ± SD (μg/kg)	z-score	recovery ± SD (%)
MeOH/H_2O extraction	72.4 ± 1.4	−0.6	96.5 ± 2.0	99.6 ± 3.9	−0.6	102.8 ±1.2
MeCN/H_2O extraction	83.7 ± 3.4	0.0	99.9 ± 0.7	114.5 ± 3.0	0.0	113.3 ± 4.1

[a] The values are corrected for recovery; recovery rates were determined by spiking of about 100 μg/kg of each toxin to the extract. The calculation of the z-score is based on the FAPAS proficiency test T-2234.

IAC 2 lower results for both toxins (4.9 ± 0.4 μg/kg for T-2 and 19.7 ± 1.0 μg/kg for HT-2) were obtained.

Recovery rates for the same sample spiked at four levels (10, 50, 100, and 200 μg/kg) are presented in **Figure 5**. This data revealed the high quality grade of both IAC regarding the determination of T-2 toxin. With IAC 1 a simultaneous determination of HT-2 is possible in the same quality, but using IAC 2 for the determination of HT-2 in naturally contaminated and spiked samples, the performance was unsatisfying. According to the results from the capacity experiments, the recovery rate at least for the lowest spiking level (10 μg/kg = 8 ng absolute) is expected to be about 100% when using IAC 2. However, all obtained recovery rates of HT-2 remained constant at about 60% independent of the spiking levels. It is assumed that, beside the lower capacity concerning HT-2, the antibodies of IAC 2 are obviously affected by matrix compounds. A satisfying determination of HT-2 according to the validated method is therefore only achievable using IAC 1.

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Short method descriptions for regulatory purposes:

JJ0EW/JJ0F0 Aflatoxins:
Extraction with methanol / water 80/20 (v/v) + NaCl
Dilute with Tween solution
Apply on Immunoaffinity column
Wash Column
Elution with Methanol
Dilute with water
Measurement: HPLC-FLD with cobra cell post column derivatisation

Fumonisins JJ0BG/ JJ088
Weight in ~10 g of a grinded, homogenized sample
Double-extraction with acetonitril/water/methanol (25/50/25, v/v/v) via shaking, centrifugation, dilution, measurement with LC-MS/MS.

Fusarium toxins JJ0FE/J5012/A7193
Weight in: ~ 5 g of a grinded, homogenized sample.
Addition of internal standard (ZAN). Extraction with 20 ml Acetonitril / water (80/20, v/v) by shaking (30 min).
Centrifugation, dilution, addition of C13 labelled standard, measurement with LC-MS/MS

JJ0G5/JJ0FH Ochratoxin A:
weighing: 10 g
extraction: 50 m MeOH, 50 ml 5 % NaHCO3 solution, shake 30 min, filtration, dilution
clean-up with immuno-affinity column
detection: HPLC-FLD with postcolumn derivatisation

Sterigmatocystin JJW2Z
Weight in ~10 g of a grinded, homogenized sample
Extraction with acetonitril/water (85/15, v/v/v), solvent exchange, measurement with LC-MS/MS

Crude Fat in food, SBR modified LP06X
Principle of determination: gravimetric. The sample is hydrolyzed with 7.7 M hydrochloric acid at 75 Celsius degrees in order to release the bounded fat from test material before extraction. After hydrolysis some alcohol is added to the sample and the fat is extracted using a mixture of diethyl ether/petroleum ether (1:1). The solvents are evaporated to dryness and the residue (fat) weighed.

Raisio, 3rd October 2016

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NORDISK METODIKKOMITÉ FOR LEVNEDSMIDLER

NORDIC COMMITTEE ON FOOD ANALYSIS

No. 173
2nd Ed.
2005

ASKE, gravimetrisk bestemmelse i levnedsmidler.

ASH, gravimetric determination in foods.

Denne NMKL-metode er valideret i to kollaborative afprøvninger.

This NMKL method is validated in two collaborative studies.

1. FORMÅL OG ANVENDELSESOMRÅDE.

Metodens formål er at bestemme askeindholdet i levnedsmidler til brug ved energiberegning. Med mindre modifikationer for specielle levnedsmidler kan metoden anvendes til alle levnedsmidler jf. bilag 1. Denne metode er kollaborativt valideret for askeindhold fra 0,07 til 8,0 g/100g.

1. SCOPE AND FIELD OF APPLICATION.

The method is intended for the determination of ash content in foods when calculating their energy content. With minor modifications for certain types of foods, as mentioned in the text, the method is applicable to all foods, see appendix 1. This method is collaboratively studied for ash content between 0.07 to 8.0 g /100 g.

2. DEFINITION.

Ved askeindholdet forstås den uorganiske rest, som er tilbage efter, at vand og alle organiske bestanddele er fjernet ved ophedning til 550 °C**.**

2. DEFINITION.

Ash content is defined as the inorganic residue, present when water and all organic matter have been removed by heating to 550 °C**.**

3. PRINCIP.

Prøven foraskes i muffelovn ved 550 ºC til konstant vægt, om nødvendig efter forudgående tørring.

3. PRINCIPLE.

The sample is ashed to constant weight in a muffle furnace at 550 °C. If necessary, samples are dried prior to ashing.

4. APPARATUR.

4.1 Muffelovn, elektrisk opvarmet og termostatstyret til foraskning ved 550 ± 25 °C. For bestemmelse af aske i mælk og mælkeprodukter skal temperaturen være 525 ± 25 °C. Mulighed for energiregulering (programmerbar ovn), så opvarmningshastigheden kan afpasses efter prøvernes art, vil være en fordel for visse typer levnedsmidler.

4.2 Infra-rød lamper, 250 watt, anbragt i stativ, så de enkelte lamper uafhængig af hinanden kan

4. APPARATUS.

4.1 Muffle furnace, controlled to ± 25 °C for ashing at 550 °C. For ash determination in milk and milk products the temperature should be 525 ± 25 °C. It is an advantage with some types of food if the furnace is equipped with an energy regulator (programmable furnace), to allow adjustment of the rate of heating.

4.2 Infra-red lamps, 250 watts, mounted on stands, allowing the lamps to be independently raised and

[1] This NMKL method replaces NMKL method No 7, No 23, No 108, No 128 and the previous version of No 173.

hæves og sænkes i forhold til de på et bord anbragte digler. Det vil være en fordel at komplettere infrarødlampen med en varmeplade med trinvis opvarmning.

lowered in relation to crucibles on a table. A supplementary hot plate with graduated heating is an advantage.

Andre former for udstyr til fortørring kan også anvendes f.eks. elektrisk varmebad, der passer til diglerne.

Other heating equipment for drying may also be used, e.g. electrical mantles suitable for the crucible.

4.3 Porcelæns-, kvarts-, nikkel-, eller platindigler lav form ca. 30, 50 og 100 mL.

4.3 Crucibles of porcelain, quartz, nickel or platinum shallow design ca 30, 50, and 100 mL.

4.4 Ekssikkator. Anvend tørret silikagel som tørremiddel.

4.4 Desiccator. Use dried silica gel as drying agent.

4.5 Analysevægt (gram med mindst 4 decimaler).

4.5 Analytical balance (in gram with at least 4 decimals).

5. FREMGANGSMÅDE.

5. PROCEDURE.

5.1 Opvarm de rengjorte digler i ovnen i mindst 30 min. ved 550 °C (eller 525 °C for mælk og melkeprodukter) og afkøl i ekssikkator til stuetemperatur. Vej diglen hurtigt med 0,1 mg's nøjagtighed. Homogeniser prøven, indvej en prøvemængde svarende til ca. 5 g tørstof, dog højst 10-15 g vådvægt.

5.1 Heat the cleaned crucible for at least 30 min in an oven at 550 °C (or 525 °C for milk and milk products). Cool to room temperature in a desiccator. Quickly weigh the crucible to the nearest 0.1 mg. Homogenize the food and weigh a sample corresponding to ca. 5 g dry matter, but not more than 10-15 g wet weight.

5.2 Hvis muffelovn skal anvendes, anbring først diglen med prøve under infrarødlampen (4.2), hvis højde indstilles, så fordampningen sker med rimelig hastighed, uden at diglernes indhold medrives. Fortsæt tørringen, indtil prøven er tør, og derefter til prøven viser begyndende brunfarvning.

5.2 If a muffle furnace is used, first put the crucible with the sample under an infra-red lamp (4.2) the height of which is adjusted allowing evaporation to take place at a reasonable rate without flash burning of the sample. Continue heating, until the sample is dry, and then shows initial browning.

Ved analyse af tørre prøver, udfør fortørringen direkte i ovnen. Indsæt diglen i ovnen ved 200 °C med temperaturen indstillet på 550 °C og lad temperaturen stige gradvis.

When analysing dry samples, place the crucible directly in the muffle furnace at 200 °C, set the temperature to 550 °C and allow the temperature to rise gradually.

For sukkerrige produkter som marmelade og honning, udfør fortørringen forsigtigt og fortsæt, indtil prøverne er sorte og helt tørre, således at der ikke sker opblæring ved den efterfølgende foraskning. Fedtholdige prøver behandles tilsvarende, så det smeltede fedt ikke sprøjter ud af diglen. (Forbehandling i øvrigt jf. bilag 1).

Materials rich in sugar (e.g. marmalade and honey) should be dried very carefully. Avoiding air bubbles in the subsequent ashing, continue to heat until the sample is black and completely dry. Treat samples with a high fat content in the same manner to avoid fat splashing out of the crucible. (Pretreatment see Annex 1).

5.3 Indsæt diglerne i muffelovn ved stuetemperatur og opvarm til 550 (525) ± 25 °C (4.1) og forask i 16 til 20 timer til gråhvidt udseende af asken.

5.3 Place the crucibles in the muffle furnace at room temperature and warm to 550 (525) ± 25 °C (4.1) for 16 to 20 hours until the ash has a grey-white appearance.

5.4 Hvis programmerbar ovn anvendes til foraskningen, indsæt diglerne i ovnen ved rumtemperatur, og indstil energireguleringen til en opvarmningshastighed, således at opblæring undgås.

5.4 As an alternative to separate drying and ashing, place the crucible in a programmable oven at room temperature, and adjust the heating to a rate that will not cause air bubbles in the samples. Continue the

Fortsæt med foraskning som under 5.3.

5.4.1 Ved langt de fleste prøver kan følgende program anbefales:

Start: rumtemperatur
Opvarm til 250 °C over 1 time
Lad stå på 250 °C i 2 timer
Opvarm til 550 °C over 3 timer
Lad stå på 550 °C i 18 timer

5.5 Afkøl diglen i ekssikkator og vej med en nøjagtighed på 0,1 mg.

Visse typer aske, især fra sukkerholdige levnedsmidler, kan være meget lette og voluminøse. Diglerne må da tages meget forsigtigt ud af ovnen og ekssikkator. Bedst er det i så fald at fugte asken forsigtigt med vand, inddampe til tørhed med infrarødlampe og derpå foraske ½-1 time i ovn.

For visse levnedsmidler f.eks.levnedsmidler med højt phosphatindhold som tørmælkspulver, buddingpulver og lignende, kan asken opnå konstant vægt, selvom fuldstændig foraskning ikke er opnået, idet der nede i asken er uforbrændte partikler. Opblød i så fald asken, så tilstedeværende partikler frigøres. Herefter gentages tørring og foraskning, kontroller at der ikke findes kulpartikler tilbage.

Man bør altid være opmærksom på askens farve, - hvis den ikke er gråhvid bør askeresten fugtes med vand og opvarmning gentages.

5.6 Anbring atter diglen i ovnen ved 550 °C (525 °C) i 4 timer, afkøl og vej. Hvis vægttabet ved denne vejning i forhold til vejning efter første foraskning overstiger 1% af askens vægt, gentages opvarmningen i ovnen, indtil to på hinanden følgende vejninger ikke overstiger 1% af askens vægt.

6. BEREGNING AF ASKEINDHOLD.

Beregn askeindholdet som:

g aske /100 g = (a-c)*100/(b-c)

hvor
a = endelig vægt (g) af digel med aske
b = vægt (g) af digel med prøve
c = vægt (g) af forglødet, tom digel

6.1 Beregning af SiO_2-fri "renaske":
g "renaske"/100 g = ((a-d)-c) * 100/(b-c)
hvor:
a = endelig vægt (g) af digel med aske
b = vægt (g) af digel med prøve.
c = vægt (g) af forglødet, tom digel
d = vægt (g) af den glødede, saltsyrebehandlede askeremanens fra punkt 5.2.

ashing as under 5.3.

5.4.1 The following program is used for the majority of samples:

Start: Room temperature
Heat to 250 °C during 1 hour
Stabilize at 250 °C for 2 hours
Heat to 550 °C during 3 hours
Stabilize at 550 °C for 18 hours

5.5 Cool the crucible in a desiccator and weigh to the nearest 0.1 mg.

The ash from foods with high sugar content may be very light and voluminous. Take care when removing the crucible from the oven and desiccator. The ash may be carefully wetted with water, the water evaporated under an infra-red lamp and the ashing continued for 0.5-1 hour.

Foods with high phosphate content, such as milk powder, may yield ash of constant weight even if complete ashing has not been achieved. This is due to the presence of incompletely incinerated particles in the ash, in which case, dissolve the ash in water to liberate the incompletely incinerated particles. Repeat the drying and ashing procedures. After ashing is completed, check the ash for residual carbon particles

The colour of the ash should always be noted - if the ash is not grey-white, the residue should be wetted with water and heated again.

5.6 Place the crucible in the oven for 4 more hours at 550 °C (525 °C). Cool and weigh. If the weight loss at this weighing is greater than 1% of the ash weight after the first ashing, heat repeatedly in the oven until two subsequent weighings do not differ by more than 1%.

6. CALCULATION OF ASH CONTENT.

Calculate the ash content from:

g ash /100 g = (a-c)*100/(b-c)

where
a = final weight (g) of crucible and ash
b = weight (g) of crucible and original sample
c = weight (g) of empty incinerated crucible

6.1 Calculating of SiO_2-free "ash":
g "ash"/100 g = ((a-d)-c) * 100/(b-c)
hvor:
a = final weight(g) of crucible and ash
b = weight (g) of crucible and original sample.
c = weight (g) of empty incinerated crucible
d = weight (g) of incinerated, acidtreated residue of ash from. 5.2.

7. METODENS PRÆCISION.

Denne metode er kollaborativt afprøvet i 1987 og i 1999. Begge afprøvninger blev udført af 14 laboratorier på 7 levnedsmidler. Askeindholdet varierede fra 0,07 – 8,0 g/100g. Metoden, der blev afprøvet i 1987, foreskrev strengere temperaturkontrol på muffelovnen, 550 ± 5 °C i stedet for 550 ± 25 °C. Resultaterne af afprøvningerne er anført i bilag 2.

Den relative standardafvigelse på repeterbarheden (RSD_r) varierede fra 0,21% for askeindhold på 8,0 g/100 g til 8,5 % for askeindhold på 0,07 g/100 g. Den relative standardafvigelse på reproducerbarheden (RSD_R) varierede fra 1,3% for askeindhold på 8,0 g/100 g til 11,9% for askeindhold på 0,07 g/100 g.

Afprøvningene var vellykket for de 14 levnedsmidlerne med undtagelse af æblemos.

8. BEMÆRKNINGER.

Calciumcarbonat, tilstedeværende i prøven eller dannet under foraskningen, indgår helt i askeindholdet.

9. REFERENT.

Cand. pharm. Arne Højgård Jensen, Fødevaredirektoratet, Fødevareregion Århus, Danmark har udarbejdet og arrangeret afprøvning af denne metode.

7. PRECISION OF THE METHOD.

This method was collaboratively studied in 1987 and 1999. In both studies, 14 laboratories participated analysing 7 foodstuffs. The method studied in 1987 required stricter temperature control on the muffle furnace, 550 ± 5 °C, rather than 550 ± 25 °C. The results from the collaborative study are given in appendix 2.

The relative standard deviation on repeatability (RSD_r) varied from 0.21% for an ash content of 8.0 g/100 g to 8.5% for an ash content of 0.07 g/100 g. The relative standard deviation of reproducibility (RSD_R) varied from 1.3% for ash content of 8.0 g/100 g to 11.9% for an ash content of 0.07 g/100 g.

The results of collaborative studies were acceptable for all the 14 foodstuffs except apple purée.

8. REMARKS.

Any calcium carbonate, present either in the sample or formed during the ashing, will be included in the final result.

9. REFEREE.

This method was elaborated and collaboratively tested by Cand. pharm. Arne Højgård Jensen, Danish Veterinary and Food Administration, Regional Veterinary and Food Control Authority, Århus, Denmark.

Askemetode: Forbehandling af visse levnedsmidler.

Kød.
En repræsentativ prøvemængde udtages f. ex. 200 - 500 g, der hakkes fint i kødhakker. Ca 10 g udtages og tørres under IR-lampe.

Mel, brød, korn.
Prøver af mel, brød, korn, som indeholder stensplinter eller –støv, sand eller lignende, underkastes efter foraskning og slutvejning følgende behandling, der korrigerer for hovedparten af indholdet af SiO_2: Hvis der forekommer sand i prøven koges med 15 mL 3 M saltsyre et øjeblik. Asken filtreres gennem askefrit filtrerpapir, idet skålen skylles 3 gange med vand. Prøve og filtrerpapir anbringes i muffelovn i l time ved 550 °C (eller 525 °C). Afkøl i ekssikkator og vej som angivet ovenfor. Det korrigerede indhold af "renaske" beregnes som angivet i 6.

Mælk.
Det sikres, at mælken er homogen, ved omrøring (20 °C). Hvis der er klumper af fedt opvarmes til 38 °C og mixes. Ca 10 g prøve inddampes til tørhed under IR-lampe.

Smør.
Det sikres at prøven er homogen ellers opvarmes til ca. 35 °C i vandbad, hvorved prøven kan blandes. (Smørret må ikke smelte). Prøven gøres vandfri ved forsigtig opvarmning under IR-lampe (skumdannelse ophører). Efter afkøling ekstraheres fedtet fra prøven ved gentagen vaskning med 15-20 mL petroleumsether og dekantering. Rest af petroleumsether fjernes ved forsigtig opvarmning under IR-lampe, hvorefter prøven forkulles ved yderligere opvarmning under lampe.

Is.
Isen gøres flydende ved opvarmning til 45 °C og mixes. 5 g prøve inddampes til tørhed under IR-lampe (evt. kogende vandbad).

Ash method: Pre-treatment of certain types of food.

Meat.
A representative sample is taken, e.g. 200 - 500 g, and finely chopped in a mincing machine
A sample of approx. 10 g is evaporated to dryness under an infra-red lamp.

Flour, bread, grain.
After ashing and weighing, samples of flour, bread, grain containing stone splints, dust, sand or something of the sort are corrected for most of the SiO_2 as follows: Boil for a very short time the crude ash in the crucible with 15 mL of 3M hydrochloric acid. Transfer the contents of the crusible onto ash-free filter paper and wash 3 times with distilled water. Return the filter paper to the crucible and place it in the furnace at 550 °C (or 525 °C) for 1 hour. Allow to cool in a desiccator and weigh as above. The corrected content of the pure ash is estimated as described in 6.

Milk.
The homogeneity of the milk is tested by stirring at 20 ^{o}C. In the event of lumps of fat, the milk is heated to 38 °C and mixed. Evaporate about 10 g sample to dryness under an IR lamp.

Butter.
The homogeneity of the butter sample is tested, and heated to approx. 35 ^{o}C in a water-bath, at which temperature, the sample can be mixed (the butter should not melt). Water is removed from the sample by carefully heating under an infra-red lamp (bubbles cease to form). After cooling the sample, fat is extracted by repeated washing in 15-20 mL petroleum ether, followed by decantation. The residual petroleum ether is removed by carefully heating under an infra-red lamp, and the sample is ashed by further heating under the lamp.

Ice-cream.
The ice-cream is melted by warming to 45 ^{o}C and then mixed. A sample of 5 g is evaporated to dryness under an infra-red lamp (alternatively boiling water-bath).

Table 1: The collaborative study conducted in 1986, arranged by Birthe Mortensen at the Danish Meat Research Institute.

Foodstuffs	Maize starch	Marmalade	Mayonnaise	Rolled oats	Feta cheese	Sausage	Milk powder
No of labs minus outliers	12	13	13	14	14	12	13
No of outliers	2	0	1	0	0	2	1
Mean, (g/100g)	0.068	0.27	0.59	1.8	6.1	7.2	8.0
Repeatability std., s_r, (g/100g)	0.0058	0.010	0.029	0.023	0.078	0.072	0.017
Relative std. repeatability RSD_r (%)	8.5	4.7	4.9	1.3	1.3	1.0	0.21
Repeatability value $r = (2,8*s_r)$	0.0162	0.0364	0.0812	0.0644	0.218	0.202	0.0476
Reproducibility std. S_R, (g/100g)	0.0081	0.029	0.042	0.040	0.230	0.075	0.100
Relative std. SD_R (%)	11.9	10.6	7.1	2.2	3.8	1.0	1.3
Reproducibility value, $R= 2.8*S_R$	0.0227	0.0812	0.118	0.112	0.644	0.21	0.28
Predicted RSD_R	5.99	4.86	4.33	3.66	3.05	2.97	2.92
HorRat value	2.0	2.2	1.7	0.60	1.2	0.35	0.43

Table 2: The collaborative study conducted in November 1999 arranged by Cand. pharm. Arne Højgård Jensen at Danish Veterinary and Food Administration, Regional Veterinary and Food Control Authority, Århus.

Foodstuffs	Salami	Tuna	Semi skimmed milk	Fromage Frais	Green beans	Apple purée	Wheat bran	Rye bread
No labs minus outliers	14	13	14	14	14	14	13	14
No of outliers	0	1	0	0	0	0	1	0
Mean, (g/100g)	5.25	0.87	0.72	0.9	0.45	0.22	4.64	1.97
Repeatability std., s_r, (g/100g)	0.052	0.023	0.013	0.014	0.02	0.056	0.079	0.02
Relative std. repeatability RSD_r (%)	0.99	2.64	1.81	1.56	4.44	25.45	1.70	1.02
Repeatability value $r = (2,8*s_r)$	0.1456	0.0644	0.0364	0.0392	0.056	0.1568	0.2212	0.056
Reproducibility std., S_R, (g/100g)	0.071	0.03	0.021	0.021	0.03	0.052	0.072	0.04
Relative std., RSD_R (%)	1.4	3.4	2.9	2.3	6.7	23.6	1.6	2.0
Reproducibility value, $R= 2.8*S_R$	0.1988	0.084	0.0588	0.0588	0.084	0.1456	0.2016	0.112
Predicted RSD_R	3.12	4.08	4.20	4.06	4.51	5.02	3.17	3.61
HorRat value	0.43	0.84	0.69	0.57	1.48	4.71	0.49	0.56

UDC 637.5	NORDISK METODIKKOMMITTÉ FÖR LIVSMEDEL NORDIC COMMITTEE ON FOOD ANALYSIS	No 23 3e uppl. 3rd ed. 1991

VATTEN OCH ASKA. GRAVIMETRISK BESTÄMNING I KÖTT OCH KÖTTVAROR

1. ÄNDAMÅL OCH ANVÄNDNINGSOMRÅDE

Denna metod beskriver gravimetrisk bestämning av vatten och aska i kött och köttprodukter. Metoden kan användas för alla typer av kött och charkprodukter, dock endast för vått material.

2. DEFINITION

Med vatten avses viktförlusten som uppstår vid torkning av kött och köttvaror enligt metodbeskrivningen. Aska definieras som den oorganiska rest som kvarstår, efter det att vatten och organiska beståndsdelar avlägsnats genom upphettning.

3. PRINCIP

Vid vattenbestämning torkas provet vid 102-105°C i 16-18 timmar. Inaskningen utförs vid 550°C.

4. APPARATUR

4.1 Köttkvarn med 2 eller 3 mm hålskiva.

4.2 Hushålls- eller storhushållsmixer, 1500-3000 v/min.

4.3 Skålar i metall, porslin eller glas (t ex aluminium, nickel, rostfritt stål, Duran-glas), 60-80 mm diameter, höjd ca 25 mm.

4.4 Värmeskåp, 102-105°C.

MOISTURE AND ASH. GRAVIMETRIC DETERMINATION IN MEAT AND MEAT PRODUCTS

1.SCOPE AND FIELD OF APPLICATION

This method describes the gravimetric determination of moisture and ash in meat and meat products. The method is applicable to all types of meat and meat products, but only to wet materials.

2.DEFINITION

By moisture is meant the loss in mass obtained after drying meat and meat products as described in this method. Ash is defined as the inorganic residue obtained after removal of moisture and organic matter by heat treatment.

3.PRINCIPLE

Determination of moisture involves drying of the sample at 102-105°C for 16-18 hours. The incineration is performed at 550°C.

4. APPARATUS

4.1 Mechanical meat chopper with plate openings of 2 or 3 mm.

4.2 Food processor, 1500-3000 r/min.

4.3 Dishes of metal, porcelain or glass (e.g. aluminium, nickel, stainless steel, Duran glass), 60-80 mm diameter, height about 25 mm.

4.4 Drying oven, 102-105°C.

4.5 Porslins- eller kvartsdeglar, 30 mm diameter.

4.6 Muffelugn, 550±5°C. Om tid/temperaturregulator saknas, behövs också en elektrisk värmeplatta.

4.7 Exsickator, innehållande blågel eller ett annat effektivt torkmedel.

5. PROVTAGNING OCH PROVBEREDNING

5.1 Uttagning av laboratorieprov

Uttag ett representativt prov på minst 200 g.

Förpacka provet i plastpåse, burk med tättslutande lock eller annan tättslutande behållare. Förvara prover vid högst +5°C i väntan på vidare behandling, högst -18°C om provet skall lagras längre. Provmaterial som inte förvaras i frysen skall analyseras inom tre dagar.

5.2 Provberedning

Ta ut provet kvantitativt ur förpackningen. Ta med köttsaft, fett eller annat som avskilts från köttvaran i förpackningen.

Tina fryst material i kylskåp. Mal provet direkt efter uttag ur kylskåpet.

Skär provet i småbitar. Mal det sedan två gånger i köttkvarn (4.1) genom 2 eller 3 mm hålskiva. Mal feta prover såsom späck enbart genom 3 mm hålskiva för att förhindra att fett avsättes i kvarnen. Blanda materialet omsorgsfullt efter såväl första som andra malningen. Alternativt kan provet finfördelas och homogeniseras i en mixer (4.2). Prover med mycket lös konsistens kan blandas med en gaffel. Provets homogenitet efter provberedningen kan kontrolleras genom att hälla en sked kolpulver i kvarnen eller mixern innan finfördelningen av ett kontrollprov påbörjas och därefter kontrollera färgen i materialet.

6. UTFÖRANDE

6.1 Vattenhaltsbestämning

Bred ut ca 6 g prov i ett jämntjockt lager över bottenytan av en torr och vägd skål (4.3). Väg med 4 decimalers noggrannhet. Utför dubbelprov.

Torka provet i ett värmeskåp (4.4) vid 102-105°C i 16-18

4.5 Porcelain or quartz crucibles, 30 mm diameter.

4.6 Muffle furnace, 550±5°C. If the furnace has no time/temperature regulator, an electric hot-plate is also needed.

4.7 Desiccator, containing silica gel, blue-indicating, or other efficient desiccant.

5.SAMPLING AND SAMPLE PREPARATION

5.1 Sampling

Use a representative sample of at least 200 g.

Put the sample into a plastic bag, an airtight container or similar airtight wrapping. Store the sample at max. +5°C until further treatment or at max. -18°C for periods longer than three days. If the sample is not frozen, analyses must be performed within three days.

5.2 Sample preparation

Remove the sample quantitatively from the bag. Include gravy, jelly, fat or anything else which has separated from the product in the package.

Thaw frozen material in a refrigerator. Chop the sample immediately after removing from the refrigerator.

Cut the sample into small pieces. Pass the pieces twice through a chopper (4.1). In the case of very fatty samples, use 3 mm plate openings to prevent the sample from greasing in the chopper. Mix thoroughly after each grinding. Alternatively, the sample can be homogenized in a food processor (4.2). Samples with a very loose consistency can be mashed and mixed well with a fork. The homogeneity of the minced sample can be confirmed by adding a spoonful of charcoal to the chopper or food processor before starting homogenization of a control sample and afterwards checking the colour of the material.

6. PROCEDURE

6.1 Determination of moisture content

Spread out about 6 g of the sample evenly over the bottom of a dry and weighed dish (4.3). Weigh to the nearest 0.0001 g. Carry out two determinations from each sample.

Dry the sample in the drying oven (4.4) at 102-105°C for 16-18 hours. Allow the sample to cool to room

timmar. Låt provet svalna till rumstemperatur i exsickator (4.7) och väg med 4 decimalers noggrannhet.

6.2 Askhaltsbestämning

Väg in 1,5-2,0 g prov med 4 decimalers noggrannhet i en glödgad och vägd degel (4.5). Utför dubbelprov.

Om laboratoriet inte har tillgång till en muffelugn med tid/temperaturregulator (4.6), inaska provet på en elektrisk värmeplatta så att temperaturen gradvis stiger till 550°C i loppet av 5-6 timmar. Slutföraska därefter i muffelugn vid 550°C tills askan blivit grå-vit.

Om laboratoriet disponerar över en muffelugn med tid/temperaturregulator, utför den ovan beskrivna inaskningsproceduren i ugnen.

Låt degeln svalna till rumstemperatur i exsickator (4.7) och väg med 4 decimalers noggrannhet.

7. BERÄKNING

Beräkna vatten- och askhalten i provet ur följande formler:

g vatten/100 g = ((a-b) x 100)/a

g aska/100 g = (c x 100)/a

a = invägd mängd prov i g
b = massan av det torkade provet i g
c = massan av askan i g

8. METODENS TILLFÖRLITLIGHET

Metoden har blivit kollaborativt avprövad i 14 laboratorier. Provmaterialet utgjordes av 16 dolda paralellprover av kött och charkvaror. Vattenhalten i materialen varierade mellan 44,1 och 74,8 g/100 g och askhalten mellan 1,0 och 5,4 g/100 g.

Såväl hos vatten- som hos askhaltsbestämningen var repeterbarheten (r) och reproducerbarheten (R) ej speciellt haltberoende, vilket framgår av nedanstående ekvationer, som inkluderar samtliga prover:

Vatten

r = 0,593 + 0,0017 x vattenhalten
R = 0,797 + 0,00471 x vattenhalten

temperature in a desiccator (4.7) and weigh to the nearest 0.0001 g.

6.2 Determination of ash content

To the nearest 0.0001 g, weigh duplicate samples of 1.5-2.0 g into an incinerated and weighed crucible (4.5).

If a muffle furnace with a time/temperature regulator is not available, preash the sample on an electric hot-plate so that the temperature gradually, in the course of 5-6 hours, increases to 550°C. Then ash in the furnace at 550°C until the ash has a gray-white appearance.

If a muffle furnace with time/temperature regulator is available, perform the incineration procedure described above in the furnace.

Allow the crucible to cool to room temperature in a desiccator (4.7) and weigh to the nearest 0.0001 g.

7. CALCULATION

Calculate the moisture and ash contents of the sample, using the formulae:

g moisture/100 g = ((a-b) x 100)/a

g ash/100 g = (c x 100)/a

a = mass of the sample (g)
b = mass of the dried sample (g)
c = mass of the ash (g)

8. RELIABILITY OF THE METHOD

The method has been collaboratively studied in 14 laboratories. The study included 16 samples of meat and meat products presented as blind duplicates. The materials ranged in moisture content from 44.1 to 74.8 g/100 g and in ash content from 1.0 to 5.4 g/100 g.

The repeatability (r) and reproducibility (R) of the moisture and ash methods were not markedly dependent on the concentration of the analytes in the sample, as can be seen from the equations below, calculated for all the samples:

Moisture

r = 0.593 + 0.0017 x moisture content
R = 0.797 + 0.00471 x moisture content

Aska

r = 0,0990 + 0,00933 x askhalten
R = 0,138 + 0,0046 x askhalten

Repeterbarheten låg i medeltal på 0,7 g/100 g hos vattenresultaten och 0,12 g/100 g hos askresultaten. Upprepningsanalyser utförda av en och samma laborant gav en motsvarande repeterbarhet på 0,4 resp. 0,08 g/100 g.

Ash

r = 0.0990 + 0.00933 x ash content
R = 0.138 + 0.0046 x ash content

The average repeatability for all the samples was 0.7 g/100 g for the moisture results and 0.12 g/100 g for the ash results. Repeated analyses of all the samples by the same analyst gave a corresponding repeatability of 0.4 and 0.08 g/100 g, respectively.

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NORDISK METODIKKOMITÉ FOR NÆRINGSMIDLER

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Nitrogen. Bestemmelse i levnedsmidler og foderstoffer efter Kjeldahl.

Nitrogen. Determination in foods and feeds according to Kjeldahl.

Denne NMKL metode er ikke valideret i en kollaborativ afprøvning.

This NMKL method is not validated in a collaborative study.

1. ANVENDELSESOMRÅDE.

Der beskrives en referencemetode til kvantitativ bestemmelse af nitrogen i levnedsmidler og foderstoffer. Referencemetoden omfatter anvendelse af blok-destruktion og eventuelt (semi)automatisk destillation og titrering, men også den konventionelle Kjeldahlmetode.
Hovedvægten er lagt på blok-destruktion og delvis automatisering, da dette efterhånden er den mest benyttede fremgangsmåde i Norden.
Metoden afviger på nogle punkter fra standarderne IDF 20, ISO 937 og ISO 8968. Kjeldahlmetoden er så robust, at det må antages, at alle disse metoder giver overensstemmende resultater.

1. FIELD OF APPLICATION.

This method describes a reference method for quantitative determination of nitrogen in foods and feeds. The reference method includes block digestion and (semi) automatic distillation and titration, as well as the conventional Kjeldahl method.
The main emphasis of the method is placed on block digestion and semi automation, as this is gradually the most applied technique in the Nordic countries.
On some points, the method deviates from the following standards: IDF 20, ISO 937 and ISO 8968. The Kjeldahl method is so robust, that it has to be assumed that all these methods give consistent results.

2. DEFINITION.

Nitrogenindholdet er den mængde, der svarer til det ammoniak, der er udviklet og bestemt ved den nedenstående metode.
Nitrogenindholdet udtrykkes som masseprocentindhold (w/w).

2. DEFINITION.

The nitrogen content corresponds to the amount of ammonia developed and determined as described in this method.
The nitrogen content is expressed as mass percentage content (w/w).

3. REFERENCER.

3.1 ISO 937-1978 Meat and meat products - Determination of nitrogen content (Reference method).

3.2 ISO 8968-1:2001 | IDF 20-1:2001 Milk - Determination of nitrogen content – Part 1: Kjeldahl method.

3.3 ISO 8968-2:2001 | IDF 20-2:2001 Milk -

3. REFERENCES.

3.1 ISO 937-1978 Meat and meat products - Determination of nitrogen content (Reference method).

3.2 ISO 8968-1:2001 | IDF 20-1:2001 Milk - Determination of nitrogen content – Part 1: Kjeldahl method.

3.3 ISO 8968-2:2001 | IDF 20-2:2001 Milk -

Determination of nitrogen content – Part 2: Block digestion method (Macro method).

3.4 Handbook for Kjeldahl Digestion. Tecator AB 1996.

4. PRINCIP.

Prøven destrueres ved opvarmning med koncentreret svovlsyre og kaliumsulfat, med kobbersulfat som katalysator. Herved omdannes organisk nitrogen til ammoniumioner. Efter køling tilsættes overskud af natriumhydroxid, og det dannede ammoniak destilleres over i en borsyreopløsning med indikator. Ammoniak og dermed nitrogen bestemmes ved titrering med en saltsyreopløsning. Denne fremgangsmåde er almindelig kendt som Kjeldahlmetoden.
Nitrogen i form af nitrit/nitrat vil ikke eller kun delvist blive bestemt ved denne metode. Heterocyklisk bundet nitrogen vil blive bestemt i varierende grad.

I denne metode anvendes meget ætsende og reaktive kemikalier ved høje temperaturer. Sikkerhedsforanstaltningerne skal omfatte grundig instruktion af laboratoriepersonalet, brug af sikkerhedsbriller, handsker og kittel.

5. REAGENSER.

Alle reagenser skal være af analysekvalitet, bortset fra natriumhydroxid (5.5). Det anvendte vand skal være destilleret eller af samme kvalitet som destilleret vand. Som kontrol af reagensernes kvalitet foretages blindprøver som omtalt i 8.8 og 8.9.

5.1 **Koncentreret svovlsyre (H_2SO_4),** mindst 98% (m/m), massefylde 1,84 g/ml.

5.2 **Kaliumsulfat (K_2SO_4).**

5.3 **Kobbersulfat ($CuSO_4$, $5H_2O$) .**

5.4 **Katalysatorblanding.**
I stedet for 5.2 og 5.3 kan anvendes specielle tabletter indeholdende fx 3,5 g kaliumsulfat og 0,4 g kobbersulfat ($CuSO_4$, $5H_2O$).
Katalysatorer baseret på kviksølv (Hg) eller selen (Se) bør ikke anvendes af miljømæssige årsager.

Determination of nitrogen content – Part 2: Block digestion method (Macro method).

3.4 Handbook for Kjeldahl Digestion. Tecator AB 1996.

4. PRINCIPLE.

The sample is digested by heating it with concentrated sulphuric acid and potassium sulphate, using copper sulphate as catalyst. Hereby, organic nitrogen is converted into ammonium ions. After cooling, an excess of sodium hydroxide is added, and the formed ammonia is distilled into a boric acid solution including indicator. The amount of ammonia, and thereby the nitrogen content, is determined by titration with a hydrochloric acid solution. This procedure is known as the Kjeldahl method.
Nitrogen in the form of nitrite/nitrate will not or only partly be determined by this method. Heterocyclic bound nitrogen would only be determined to a varying degree.

Highly corrosive and reactive chemicals at high temperatures are used in this method. Precautions have to include careful instruction of the analysts, use of safety glasses, gloves and coat.

5. REAGENTS.

All reagents should be of analytical grade, except for the sodium hydroxide (5.5). The water used in the analysis should be distilled, or of the same quality as distilled water. In order to check the quality of the reagents, blind sampling is performed as described in 8.8 and 8.9.

5.1 **Concentrated sulphuric acid (H_2SO_4),** at least 98% (w/w), density 1.84 g/ml.

5.2 **Potassium sulphate (K_2SO_4).**

5.3 **Copper sulphate ($CuSO_4$, $5H_2O$) .**

5.4 **Catalyst mixture.**
Instead of 5.2 and 5.3, special tablets containing e.g. 3.5 g potassium sulphate and 0.4 g copper sulphate ($CuSO_4$, $5H_2O$) can be used.
Catalysts based on mercury (Hg) or selenium (Se) should not be used due to the detrimental environmental effects.

5.5 **Natriumhydroxid (NaOH)**, 33 – 50 % (w/w) teknisk kvalitet (lavt nitrogenindhold).
Ved brug af automatisk destillationsudstyr anvendes den af fabrikanten angivne koncentration af NaOH.

5.6 **Saltsyre (HCl),** 0,1 ± 0,0005 M.
Koncentrationen bestemmes med 4 decimalers nøjagtighed.

5.7 **Natriumhydroxidopløsning (NaOH)**, 1 M.
Opløs 20,0 g NaOH i 500 ml vand i en 500 ml målekolbe.

5.8 **Methylrødtopløsning**.
100 mg methylrødt opløses i 96% ethanol (5.11) i 50 ml målekolbe.

5.9 **Bromkresolgrøntopløsning.**
500 mg bromkresolgrønt opløses i 96% ethanol (5.11) i 250 ml målekolbe.

5.10 **Borsyreopløsning med indikator.**
Til 1 L af en 4 % borsyreopløsning (fx ved at opløse 40 g borsyre (H_3BO_3) i 1000 ml vand) sættes 0,5 ml methylrødtopløsning (5.8) og 2,5 ml bromkresolgrønopløsning (5.9). Desuden tilsættes 5 ml (0,5 %) af en 1 M natriumhydroxidopløsning (5.7). Bland forsigtigt.
Ved brug af automatiske titratorer skal producentens anvisninger for fremstilling af borsyre- og indikatoropløsning nøje overholdes.

5.11 **Ethanol,** 96%.

5.12 **Ammoniumsulfatstandard.**
Ammoniumsulfat ($(NH_4)_2SO_4$, mindst 99,5%, svarende til 21,094 % N) tørres straks før brug ved 102-105 °C i mindst 2 timer. Derpå køles i ekssikkator. Til en Kjeldahl kolbe (6.7) eller et destruktionsrør (6.2) overføres 0,1500 g ammoniumsulfat (afvejes på analysevægt). Derpå tilsættes 75 ml vand.

5.13 **Nitrogenstandard, vanskeligt nedbrydelig.**
Afhængig af prøverne anvendes forskellige standarder, fx en af de følgende:

5.13.a **Lysin. L-lysin, monohydrochlorid,** $NH_2(CH_2)_4CH(NH_2)COOH$; HCl (15,34% N).

5.13.b **Tryptophan,** $C_8H_6NCH_2CHNH_2COOH$ (13,73 % N).

5.13.c **Acetanilid,** $C_6H_5NHCOCH_3$ (10,36 % N)

5.5 **Sodium hydroxide (NaOH)**, 33 – 50% (w/w) technique quality (low nitrogen content).
When utilizing automatic distillation equipment, the NaOH concentration stated by the manufacturer should be used.

5.6 **Hydrochloric acid (HCl),** 0.1 ± 0.0005 M.
The concentration is determined with an accuracy of 4 decimals.

5.7 **Sodium hydroxide solution (NaOH)**, 1 M.
Dissolve 20.0 g of NaOH in 500 ml water in a 500 ml volumetric flask.

5.8 **Methyl red solution.**
Dissolve 100 mg of methyl red in 96% ethanol (5.11) in a 50 ml volumetric flask.

5.9 **Bromocresol green solution.**
Dissolve 500 mg of bromocresol in 96% ethanol (5.11) in a 250 ml volumetric flask.

5.10 **Boric acid solution with indicator.**
Make 1 L of 4% boric acid solution by dissolving 40 g of boric acid (H_3BO_3) into 1000 ml of water. Add 0.5 ml of methyl red solution (5.8), 2.5 ml of bromocresol green solution (5.9), and 5 ml (0.5%) of the 1 M sodium hydroxide solution (5.7). Mix carefully.
When using automatic titrators, the manufacturer's procedures for making boric and indicator solutions should be followed carefully.

5.11 **Ethanol,** 96%.

5.12 **Ammonium sulphate standard.**
Ammonium sulphate ($(NH_4)_2SO_4$, at least 99.5%, corresponding to 21.094% N) is dried before use at 102-105 °C for at least 2 hours, and thereafter cooled in a desiccator. Transfer 0.1500 g of ammonium sulphate (weighed on an analytical balance) to a Kjeldahl flask (6.7) or a digestion block tube (6.2). Add 75 ml of water.

5.13 **Nitrogen standard, slowly digestible.**
Depending on the samples, different standards are used, e.g. one of the following:

5.13.a **Lysine. L-lysine, mono hydrochloride,** $NH_2(CH_2)_4CH(NH_2)COOH$; HCl (15.34% N).

5.13.b **Tryptophane,** $C_8H_6NCH_2CHNH_2COOH$ (13.73% N).

5.13.c **Acetanilide,** $C_6H_5NHCOCH_3$ (10.36% N).

5.14 **Hydrogenperoxid**, H_2O_2, ca. 30 %.

5.14 **Hydrogen peroxide**, H_2O_2, approx. 30%.

6. APPARATUR.

Almindeligt laboratorieapparatur og -udstyr, specielt det følgende:
Ved den almindelige, traditionelle Kjeldahlanalyse anvendes det apparatur, der er mærket med (A), mens der ved blok-destruktion anvendes det apparatur, der er mærket med (B).

6.1 **Stativ** med plads til fx 20 destruktionsrør (B).

6.2 **Destruktionsrør/destillationsrør** á 250 ml (B).

6.3 **Analysevægt** (A og B).

6.4 **Destruktionsblok** med temperaturjustering og termometer (B).

6.5 **Destillationsenhed:** manuel, semi-automatisk eller automatisk (B).
Dette apparat kan indeholde en (semi-)automatisk titreringsenhed.

6.6 **Ekssikkator (A og B).**

6.7 **Kjeldahl kolbe** med kapacitet på 500 ml eller 800 ml (A).

6.8 **Destruktionsapparat til Kjeldahl kolbe (A).**
Apparatet skal holde Kjeldahl kolben (6.7) på skrå, ca. 45°, og opvarmningssystemet må kun opvarme kolben, hvor der er væske indeni. Hvis der ikke er et specielt udsugningssystem, skal kolben lukkes med en glaskugle med en kort stilk.

6.9 **Destillationsapparat med dråbefanger og køler (A og B).**
Til destillationen bør anvendes den Kjeldahl kolbe (6.7) eller destruktionsrør (6.2), hvori prøven blev destrueret.

6.10 **Vejepapir, nitrogenfrit (A og B).**
Vejepapir anvendes ikke til alle typer af prøver, fx ikke til flydende prøver.

8. FREMGANGSMÅDE.

Punkterne 8.1, 8.2, 8.3 og 8.7 er fælles for de to metoder. Punkterne A.8.5 og A.8.6 er specielle for den almindelige, traditionelle Kjeldahlmetode.

6. EQUIPMENT.

Ordinary laboratory apparatus and equipment, particularly:
Equipment indicated with (A) is intended for the traditional Kjeldahl analysis, while equipment indicated with (B) should be used for block digestion.

6.1 **Rack** for e.g. 20 digestion block tubes (B).

6.2 **Digestion block tubes /distillation tubes,** 250ml (B).

6.3 **Analytical balance** (A and B).

6.4 **Digestion block** with temperature adjustment and thermometer (B).

6.5 **Distillation unit,** manual, semi automatic or automatic (B).
This apparatus can contain a (semi) automatic titration unit.

6.6 **Desiccator (A and B).**

6.7 **Kjeldahl flask** with a capacity of 500 ml or 800 ml (A).

6.8 **Digestion apparatus for Kjeldahl flask (A).**
The apparatus shall keep the Kjeldahl flask (6.7) aslant, at an angle of about 45°. The heating system must only heat the flask where it contains liquid. If there are no special means of withdrawing the fumes formed, the flask should be closed with a glass ball with a short stem.

6.9 **Distillation equipment with drip trap and cooler (A and B).**
For distillation use the Kjeldahl flask (6.7) or the digestion block tube (6.2) in which the sample was digested.

6.10 **Weighing paper, free of nitrogen (A and B).**
Weighing paper is not used for all types of samples, for instance not for liquids.

8. PROCEDURE.

Para 8.1, 8.2, 8.3 and 8.7 are common for the two methods. Para A.8.5 and A.8.6 are specific for the ordinary, traditional Kjeldahl method. Para B.8.5 and

Punkterne B.8.5 og B.8.6 er specielle for anvendelse af blok-destruktion.

8.1 **Afvejning af prøve.**
På analysevægt (6.3) afvejes prøven med en nøjagtighed på 0,1 mg. Eventuelt bruges vejepapir. Flydende prøver kan pipetteres direkte ned i Kjeldahl kolben eller destruktionsrøret. Ofte afvejes ca. 1 g prøve, men prøvemængden afhænger af nitrogenindholdet. I den afvejede prøve bør der være 15-35 mg nitrogen.

8.2 **Forberedelse.**
I Kjeldahl kolben (6.7) anbringes kogesten, glaskugler eller lignende, og derpå ca. 15 g kaliumsulfat (5.2), 0,5 g kobbersulfat (5.3) og den afvejede prøve (se A.8.1) samt eventuelt vejepapir. Det samme gøres ved anvendelse af destruktionsrør (6.2), men det er ikke altid nødvendigt at anvende kogesten, glaskugler eller lignende. Mængden af kobbersulfat kan variere. I nogle tilfælde bruges over 1 g, i andre tilfælde ca. 200 mg. I stedet for kalium- og kobbersulfat kan anvendes en passende mængde af den under 5.4 omtalte katalysatorblanding.

8.3 **Svovlsyretilsætning.**
I Kjeldahl kolben (6.7) eller destruktionsrøret (6.2) hældes 15 ml koncentreret svovlsyre (5.1), så eventuelle rester af prøve m.m. skylles ned. Ryst kolben/røret forsigtigt. Er der afvejet mere end 1 g prøve, og specielt hvis prøven har et højt fedtindhold, kan det være nødvendigt at anvende mere end 15 ml svovlsyre for at sikre det store støkiometriske overskud, der er nødvendigt for oxidationen.
Nedenstående tabel (Tecator 1996) giver en indikation af anvendt svovlsyre:

Prøvetype	Svovlsyreforbrug, ml/g
Fedt	9.7
Protein	4.9
Kulhydrat	4.0

8.4 **Hydrogenperoxidtilsætning (eventuel).**
Hydrogenperoxid fungerer som et effektivt skumdæmpende middel. Brugen er ikke ukritisk, da H_2O_2 ændrer systemets oxidationspotentiale. Analyseresultatet kan påvirkes i såvel positiv retning som følge af mere effektiv oxidation til NH_4^+ som i negativ som følge af dannelse af N_2 som undviger. H_2O_2 bør derfor kun anvendes efter parallelbestemmelser på den aktuelle matrix. Til nedbrydningsrøret/-kolben sættes forsigtigt 5 ml

B.8.6 are specific for the use of block digestion.

8.1 **Weighing of sample.**
Weigh the sample on an analytical balance (6.3) with an accuracy of 0.1 mg. Use weighing paper if necessary. Liquids can be pipetted directly into the Kjeldahl flask or the digestion block tube. Usually, about 1 g of the sample is weighed out. However, this depends on the nitrogen content. The sample should contain 15-35 mg of nitrogen.

8.2 **Preparations.**
Add bumping preventatives, granulated silicon carbide, glass balls or similar, to the Kjeldahl flask (6.7), and add 15 g of sodium sulphate (5.2), 0.5 g of copper sulphate (5.3) and the weighed sample (see A.8.1) together with the weighing paper, if relevant. Prepare the digestion block tubes (6.2) in the same manner. It is, however, not always necessary to use bumping preventatives in these tubes. The amount of copper sulphate may vary. In some cases, more than 1 g is used, whereas in other cases 200 mg is sufficient. A suitable amount of the catalyst mixture mentioned in 5.4 can be used instead of sodium and copper sulphate.

8.3 **Addition of sulphuric acid.**
Pour 15 ml concentrated sulphuric acid (5.1) into the Kjeldahl flask (6.7) or the digestion block tube (6.2) in such a way that possible sample remnants etc. are washed down. Shake the flask/tube carefully. If the amount of sample is more than 1 g, and especially if the sample has a high fat content, it may be necessary to add more than 15 ml of sulphuric acid in order to ensure the significant stochiometric excess necessary for the oxidation.
The following table (Tecator 1996) gives an indication of the amount of sulphuric acid used.

Matrix	Sulphuric acid consumption, ml/g
Fat	9.7
Protein	4.9
Carbohydrate	4.0

8.4 **Hydrogen peroxide (if required).**
Hydrogen peroxide is an effective anti foaming agent. The use of it is not uncritical, as H_2O_2 changes the oxidation potential of the system. It may influence the analysis result both in a positive direction, due to the more efficient oxidation to NH_4^+, as well as in a negative direction due to the formation of escaping N_2. H_2O_2 may therefore only be used after parallel determinations of the relevant matrix. Add 5 ml of hydrogen peroxide (5.14) carefully to the

hydrogenperoxid (5.14). Blandingen skal henstå 15 minutter inden opvarmningen startes.

A.8.5 **Destruktion.**
Kjeldahl kolben (6.7) placeres i destruktionsapparatet (6.8), hvorpå den opvarmes forsigtigt, til skumdannelse er ophørt. Derpå koges kraftigt, idet kolben drejes med mellemrum, indtil væsken er helt klar med en blågrøn farve. Der fortsættes med jævn kogning af væsken yderligere mindst 1 time. Den samlede destruktionstid afhænger af produkttype m.m., men er ofte ca. 2 timer. Kjeldahl kolben stilles ved stuetemperatur til afkøling i ca. 15-25 min. Når væsken er kølet til ca. 30 °C, tilsættes eventuelt kogesten eller lignende og derpå forsigtigt ca. 300 ml vand, idet vandet bruges til at vaske indersiden af kolbens hals. Der tilsættes mere vand til Kjeldahl kolber på 800 ml. Kolbens indhold blandes.

A.8.6 **Destillation.**
Til kolbens indhold sættes hurtigt men forsigtigt overskud af NaOH (5.5), ca. 70 ml. Umiddelbart efter forbindes Kjeldahl kolben med destillationsapparatet (6.9). Forlaget, evt. en konisk kolbe, skal indeholde en passende mængde, fx 50 ml borsyreopløsning med indikator (5.10). Kjeldahl kolben rystes og opvarmes derpå forsigtigt til kogning. Der skal destilleres mindst 150 ml over i forlaget. Derpå renses røret fra destillationsapparatet med lidt vand, der opsamles i forlaget.
Fortsæt med 8.7.

B.8.5 **Destruktion.**
Destruktionsblokken (6.4) opvarmes til 420 °C. Stativet (6.1) med destruktionsrør placeres i blokken (6.4), udsugningsshatten sættes over stativet, og udsugningen startes. Kraftig udsugning i den første periode, op til 10 min. Af hensyn til skumdannelse kan det for nogle prøvetyper være nødvendigt at starte med en temperatur i destruktionsblokken på ca. 200 °C. Temperaturen hæves gradvis til 420 °C. Skumdannelsen må ikke stige til op over 4-5 cm under udsugningen i hvert rør. Ved kraftig skumdannelse øges temperaturen ikke, før skumdannelse er ophørt. Den samlede destruktionstid skal være mindst 75 minutter, nogle tilfælde mere end 2 timer. Efter destruktionen tages stativet op af varmeblokken og får lov at afkøles ved stuetemperatur. Udsugningshatten skal forblive over stativet. Efter 15-25 minutters køling fjernes udsugningshatten, og der tilsættes ca. 75 ml vand til hvert rør, hvorpå der blandes ved omrystning. Væsken skal være grønblå og klar. Hvis der dannes krystaller, rystes røret til krystallerne er forsvundet.

tube/flask. The mixture should be left standing for 15 minutes before it is heated.

A.8.5 **Digestion.**
Place the Kjeldahl flask (6.7) in the digestion apparatus (6.8), and heat it gently until foaming has stopped. While swirling the flask at given intervals, boil the liquid at full strength until it is completely clear and has a blue-green colour. The liquid should then boil evenly for at least another hour. The total digestion time depends on product type etc., but is often about two hours. The Kjeldahl flask is then left to cool in room temperature for 15-25 minutes. When the liquid has cooled down to approx. 30 °C, add (if necessary) bumping preventatives, and subsequently 300 ml of water which is used to rinse the inside of the neck of the flask. Add more water to 800 ml Kjeldahl flasks. Mix the contents of the flask.

A.8.6 **Distillation.**
Quickly but gently, add an excess of NaOH (5.5), approx 70 ml. Immediately connect the Kjeldahl flask to the distillation equipment (6.9). The receiver, or a conical flask, should contain a suitable amount, e.g. 50 ml, of boric acid solution with indicator (5.10). Gently shake the Kjeldahl flask, and bring its contents carefully to the boil. At least 150 ml should be distilled over to the receiver. Thereafter, rinse the distillation equipment with a little water, which is then collected in the receiver. Proceed to 8.7.

B.8.5 **Digestion.**
Heat the digestion block (6.4) to 420 °C. Place the rack (6.1) with the digestion block tubes in the block (6.4), position the suction hood over the rack, and start the suction. Use strong suction at first, for up to 10 minutes. Because of foaming, some matrices may require a start temperature in the digestion block of approx. 200 °C. The temperature is then gradually increased to 420 °C. The foam must not rise higher than 4-5 cm below the suction hood in each tube. If too much foam forms, the temperature is not increased until the foaming has stopped. The total digestion time must be at least 75 minutes, and may take as long as 2 hours.
After the digestion, remove the rack from the block, and allow to cool down at room temperature. The suction hood should remain over the rack. After cooling down for 15-25 minutes, remove the suction hood, add approx. 75 ml of water to each tube, and mix by shaking. The liquid should be clear and blue-green. Should crystals form, continue to shake the tubes until the crystals have disappeared. Careful heating may be

Det kan være nødvendigt med en forsigtig opvarmning. I nogle tilfælde, især hvis prøven indeholder meget fedt, kan der tilsættes lidt H_2O_2 til prøven for at stoppe skumdannelse (se 8.4).

B.8.6 **Destillation.**
Destruktionsrørene tilsluttes destillationsenheden, hvor der i forlaget er en passende mængde, fx 50 ml, borsyreopløsning med indikator (5.10). Der tilsættes forsigtigt overskud af natriumhydroxid (5.5), ca. 70 ml, afhængig af koncentrationen af natriumhydroxid, afvejet prøvemængde (8.1), tilsat mængde svovlsyre (5.1) m.m. Der destilleres mindst 150 ml over i forlaget. Hvis der anvendes en automatisk eller semi-automatisk destillations- og titreringsenhed, vises eller udskrives den anvendte mænge 0,1 M HCl, jf. 8.7. I nogle automatiske enheder indtastes afvejet prøvemængde, saltsyrens molaritet m.m., hvorefter der sker en visning eller udskrivning af prøvens nitrogen- eller proteinindhold. Ved anvendelse af en sådan enhed fortsættes med 8.8.

8.7 **Titrering.**
Borsyreopløsningen (5.10) i forlaget titreres med 0,1 M HCl (5.6) til det første spor af lyserødt. Aflæs den forbrugte mængde af 0,1 M HCl med en nøjagtighed på ca. 0,1 ml. Denne mængde (ml) betegnes V_a.

8.8 **Blindprøver.**
Til kontrol af reagenser m.m. foretages en blindprøve, hvor der startes i 8.1, idet der dog ikke tilsættes prøve. Hvor der ikke anvendes vejepapir, tilsættes ofte 5 ml vand med ca. 0,85 g sucrose. I øvrigt fortsættes til og med 8.7. Den i 8.7 (eventuelt i B.8.6) forbrugte mængde 0,1 M HCl betegnes V_b. Der foretages blindprøve og bestemmelse af V_b ved ændringer som fx overgang til nyt vejepapir, ny katalysatorblanding, ny svovlsyre etc.

8.9 **Kontrol af destillationen.**
Der bør hyppigt foretages kontrol af destillationen ved at foretage punkt 8.6 (A.8.6 eller B.8.6) og 8.7.
Blindværdi for destillationen fås ved at destillere ca. 75 ml vand, og derpå titrere. Dette bør foretages ved skift af 0,1 M HCl (5.6) eller af borsyreopløsning (5.10). Der bør hyppigt foretages destillation og titrering af en ammoniumsulfatstandard (5.12). Denne standard indeholder 21,094% N, og genfindingen af nitrogen bør være mellem 99% og 100%. Ved brug af en kombineret destillations- og

required. In some cases, especially if the sample has a high content of fat, it may be necessary to add a small amount of H_2O_2 to the sample to stop the foaming (see 8.4).

B.8.6 **Distillation.**
Connect the digestion block tubes to the distillation unit, in which the receiver contains a suitable amount, e.g. 50 ml, of boric acid solution with indicator (5.10). Carefully add an excess of sodium hydroxide (5.5), approx 70 ml depending on the concentration of the sodium hydroxide, the weighed sample (8.1), the added amount of sulphuric acid (5.1) etc. At least 150 ml should be distilled over to the receiver. When using an automatic or semi automatic distillation or titration unit, the used amount of 0.1 M HCl will be displayed or printed, see 8.7. In some automatic units, the sample's nitrogen or protein content is displayed or printed after entering the weight of the sample, the molarity of the hydrochloric acid etc. If you are using such a unit, proceed to 8.8.

8.7 **Titration.**
Titrate the boric acid solution (5.10) with 0.1 M HCl (5.6) until the first trace of pink. Read the used amount of 0.1 M HCl with an accuracy of approx. 0.1 ml. This amount (ml) is called V_a.

8.8 **Blind samples.**
Blind samples are used to check the quality of the reagents etc., by performing the procedure from 8.1 without actually adding the sample. In cases where weighing paper is not used, 5 ml of water with approx. 0.85 g of sucrose is often added. Apart from that, all the steps of the method are followed up to 8.7. The used amount of 0.1 M HCl determined in 8.7 (or B.8.6), is called V_b. Blind sampling and the determination of V_b should be performed upon changes like taking unto use new weighing paper, a new catalyst mixture, a new hydrochloric acid, etc.

8.9 **Checking the distillation.**
The distillation should be checked regularly by performing 8.6 (A.8.6 or B.8.6) and 8.7. Blank values for the distillation are obtained by distilling approx. 75 ml of water, and subsequently titrating it. This should be performed when taking into use new 0.1 M HCl (5.6) or boric acid solutions (5.10). An ammonium sulphate standard (5.12) should be distilled and titrated regularly. This standard contains 21.094% N, and the recovery of nitrogen should be between 99% and 100%. When using a combined distillation and

titreringsenhed bør der destilleres prøver af både vand og ammoniumsulfat hver morgen. Analyse af prøver bør ikke påbegyndes, før kontrolresultaterne er accepteret.

8.10 **Genfinding.**
Genfinding er kontrol af analysemetoden og kan foretages på forskellige måder. Generelt er, at der analyseres som ved almindelige prøver, fra 8.1 til 8.7. Der anvendes en 'standard' med et kendt indhold af nitrogen, fx 5.13 a, 5.13.b eller 5.13.c. I alle tilfælde skal genfindingen af N være 99,0 %-100,5 %. Der kan også anvendes en "husstandard" med et kendt indhold af nitrogen. Sådanne standarder kan tillige anvendes til at fastlægge destruktionstid, tilsat mængde af svovlsyre, anvendt mængde borsyreopløsning m.m.

9. RESULTATANGIVELSE.

9.1 **Beregning af nitrogenindholdet.**
Nitrogenindholdet i % (w/w) beregnes af:

% nitrogen = 1,401 (V_a-V_b) M_{HCl} / w

V_a er den i 8.7 forbrugte mængde saltsyre (5.6); V_b er blindværdien, bestemt i 8.8.
M_{HCl} er molariteten af denne saltsyre (5.6)
w er den i 8.1 afvejede mængde prøve (i gram).

Formlen ovenfor er en forkortelse af :
% nitrogen = 100% · 14,007·(V_a-V_b)·M_{HCl} /(1000·w)
hvor 14,007 er molvægten for nitrogen.

9.2 **Omregning fra nitrogen til protein.**
For de fleste levnedsmidler, inkl. kød, fisk, fjerkræ m.m multipliceres indholdet af nitrogen med faktoren 6,25 for at få prøvens indhold af protein. For mejeriprodukter multipliceres med faktoren 6,38. For korn og kornprodukter, samt andre vegetabilier bruges andre omregningsfaktorer.

10. REFERENT.

Leif Bøgh-Sørensen, Fødevaredirektoratet, Søborg, Danmark har revideret denne NMKL metode.

titration unit, samples of both water and ammonium sulphate should be distilled every morning. Sample analysis should not commence until the control results have been accepted.

8.10 **Recovery.**
Recovery is a means of checking the analysis method, and can be performed in many different ways. Generally, the analysis is performed as a normal analysis from 8.1 to 8.7, using a standard with a known nitrogen content, e.g. 5.13 a, 5.13 b or 5.13 c. In all cases, the recovery of nitrogen should be 99.0% - 100.5%. An "in-house" standard with known nitrogen content may also be used. Such standards may also be used to determine digestion time, added amount of sulphuric acid, used amount of boric acid solution etc.

9. EXPRESSION OF THE RESULTS.

9.1 **Calculating the nitrogen content.**
The nitrogen content in % (w/w) is calculated based on the following:

% nitrogen = 1.401 (V_a-V_b) M_{HCl} / w

V_a is the amount of hydrochloric acid (5.6) used in 8.7, and V_b is the blank value determined in 8.8.
M_{HCl} is the molarity of this hydrochloric acid (5.6)
w is the weighed amount of sample from 8.1 (in grams).

The above mentioned formula is an abbreviation of:
% nitrogen = 100% · 14.007· (V_a-V_b) · M_{HCl} /(1000·w)
where 14.007 is the molar weight of nitrogen.

9.2 **Conversion from nitrogen to protein.**
For most foods, including meat, fish, poultry etc., the nitrogen content is multiplied with the factor 6.25 to obtain the sample's protein content. The factor 6.38 is used for dairy products. Other conversion factors are used for grain and grain products.

10. REFEREE.

Leif Bøgh-Sørensen, Danish Veterinary and Food Administration, Denmark has revised this NMKL method.