SLOVENSKI STANDARD
SIST EN 15845:2010

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Papir, karton in lepenka - Ugotavljanje citotoksičnosti vodnih ekstraktov

Paper and board - Determination of the cytotoxicity of aqueous extracts

Papier und Pappe - Bestimmung der Zytotoxizität in wässrigen Extrakten

Papier et carton - Détermination de la cytotoxicité des extraits aqueux

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ICS:

67.250	Materiali in predmeti v stiku z živili	Materials and articles in contact with foodstuffs
85.060	Papir, karton in lepenka	Paper and board

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Paper and board - Determination of the cytotoxicity of aqueous extracts

Papier et carton - Détermination de la cytotoxicité des extraits aqueux

Papier und Pappe - Bestimmung der Zytotoxizität von wässrigen Extrakten

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EUROPEAN COMMITTEE FOR STANDARDIZATION
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Foreword

This document (EN 15845:2010) has been prepared by Technical Committee CEN/TC 172 “Pulp, paper and board”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by July 2010, and conflicting national standards shall be withdrawn at the latest by July 2010.

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1 Scope

This European Standard specifies a test method for the laboratory assessment of the potential cytotoxic effect of paper and board materials. This test method is intended to assess wet contact with food simulant.

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 645, *Paper and board intended to come into contact with foodstuffs — Preparation of a cold water extract*

EN 647, *Paper and board intended to come into contact with foodstuffs — Preparation of a hot water extract*

3 Terms and definitions

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

3.1
reference water
purified water or pyrodistilled water used as the reference in the cytotoxicity assay

3.2
purified water
produced starting with tap water, which then undergoes the following treatment sequence: pre-filtration, reverse osmosis, filtering through activated carbon powder (adsorption) then through cartridges of mixed-bed ion exchange microresins (demineralisation), ultrafiltration (molecular weight cut-off at 10 kDa), and UV photo-oxidation

NOTE Alternatively, any other purification regime, which produces HPLC-quality water (resistance > 18,0 MΩ/cm, Total organic carbon < 3 ppb, no micro-organisms) or waters of grade 1 or 2 (see EN ISO 3696), can be used.

3.3
pyrodistilled water
water prepared as described in Annex A, that is used to maintain the cell line, and which can also be used for cleaning laboratory glassware

3.4
water extract
reference water that has been exposed to contact with paper or board

NOTE See EN 645 or EN 647 with necessary modifications.

3.5
control water
reference water that has been treated according to the same conditions as the water extract but without being exposed to contact with paper or board

3.6
positive control
potassium dichromate (CAS 7778-50-9) 5 mM solution freshly made in reference water

3.7
sample
culture medium prepared with water extract as specified in 3.4

3.8
test material
one or more components, or a specified quantity of paper or board, that is randomly sampled from a batch

4 Principle

The test protocol specified in this document is intended to evaluate the cytotoxic effect of substances migrating from food contact paper and board into wet foods intended for human consumption. The food simulant used is water as described in Clauses 7 and 10. The test evaluates the impact of the water extract on the rate of RNA (Ribonucleic Acid) synthesis by measuring the incorporation of a radioactive tracer (tritiated uridine) in human cells (HeLa S3).

The food contact paper and board samples to be tested are exposed to the extraction water as described in Clause 10.

The extracts then undergo cytotoxicity assessment, and the results obtained are compared to the results for a non-cytotoxic control (a purified water for which the rate of RNA synthesis is considered optimal and is therefore arbitrarily set at 100 %). Potassium dichromate, as described in 11.4.3, is used as a positive control.

5 Reagents

5.1 Liquid scintillant for tritium counts on dry filters

5.2 Culture media

The pH of all culture media used shall be 7,4 ± 0,1. pH to be adjusted using a sterile NaOH (or HCl) solution.

5.2.1 Culture media – quality and storage

All culture media, foetal serum and solutions used for cell culture shall be sterile and of sufficiently high quality to guarantee optimal cell growth (see 12.2).

They shall be stored in compliance with manufacturer's instructions, where given.

5.2.2 Medium for maintaining HeLa S3 cells in monolayer culture

Composition:

a)	minimum Essential Medium Eagle[1)] (10x)[2)]	100 ml
b)	sodium bicarbonate solution[1)], 7,5 % (*m*/*V*)	30 ml
c)	glutamine solution, 200 mM (or Glutamax I®)[1)] (100x)[2)]	10 ml
d)	non-essential amino acids solution[1)] (100x)[2)]	10 ml
e)	foetal calf serum[3)]	50 ml

1) Commercially available, the composition is based on the used of Earle's salts in the minimum essential medium. Glutamax I® is an example of a suitable product available commercially. This information is given for the convenience of this European Standard and does not constitute an endorsement by CEN of this product.

2) 10x or 100x imply tenfold or hundredfold concentrated media or solutions.

3) Heat inactivated (56 °C for 40 min) before use.

f) reference water 800 ml

5.2.3 Concentrated culture medium for cytotoxicology testing

A 5-fold concentrated culture medium is prepared by successively mixing:

a) minimum Essential Medium Eagle[4] (10x)[5] 100 ml

b) sodium bicarbonate solution, 7,5% (*m*/*V*) 30 ml

c) glutamine solution, 200 mmol (or Glutamax I®)[4] (100x)[5] 10 ml

d) non-essential amino acids solution[4] (100x)[5] 10 ml

e) foetal calf serum[6] 50 ml

5.3 Solution for rinsing cell lawns

Composition:

a) Dulbecco's PBS (Phosphate buffered saline) solution, (10x)[5] 100 ml

b) reference water added up to 1 000 ml

5.4 Cell dissociation reagent

Cells are detached using a solution of Versene[7] 1/5 000.

5.5 Analytical-grade dimethyl sulfoxide or glycerol

5.6 Sodium dodecyl sulphate (SDS) for analysis, at 3 % (*m*/*V*)

5.7 Trichloroacetic acid (TCA) for analysis, at 5 % (*m*/*V*)

5.8 Ethanol, 95 % to 98 % (*v*/*v*)

5.9 [5,6-^{3}H] uridine (35Ci/mmol to 50 Ci/mmol; 1 m Ci/ml): a sterile and non-cytotoxic aqueous solution.

NOTE The products and materials referred to in the present document are considered non-cytotoxic if they do not trigger a cytotoxic response, i.e. if the linear regression line generated by measuring rate of RNA synthesis meets the conditions set out in 12.2.

6 Cell line

6.1 Generating the cell strain

The cell line used is HeLa S3, a human cell line.

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4) Commercially available, the composition is based on the used of Earle's salts in the minimum essential medium. Glutamax I® is an example of a suitable product available commercially. This information is given for the convenience of this European Standard and does not constitute an endorsement by CEN of this product.

5) 10x or 100x imply tenfold or hundredfold concentrated media or solutions.

6) Heat inactivated (56 °C for 40 min) before use.

7) Or equivalent amount of aqueous solution of the tetrasodium salt of ethylenediaminetetraacetic acid (NA_4EDTA).

This line shall be generated from recognized sources, such as the CCL2.2 HeLa S3 line of the American Type Culture Collection.

6.2 Maintaining the cell strain

Cells should be cultured without antibiotics, and checks should be run at frequent intervals to screen for mycoplasma:

a) seed the HeLa S3 cells in the culture medium (5.2.2) in a flask as defined in 9.2.1 (75 cm^3 or 150 cm^3) and incubate at (37 ± 1) °C, 5 % CO_2 and 95 % humidity until a confluent lawn of growth is formed;

b) remove the culture medium, and rinse the cell lawn at least twice with around 10 ml of the rinse medium (5.3);

c) cover the cell lawn in a film of the Versene solution or equivalent (5.4). Leave the flask at (37 ± 1) °C for a few minutes. Detach the cells by gently shaking:

   1) add a few millilitres of the culture medium (5.2.2);

   2) disperse the cells by repeat pipetting;

d) redistribute the cell suspension obtained into the required number of flasks, and add the appropriate volume of culture medium.

6.3 Storing the cell strain

If a stock of the cell line culture is stored, then it shall be stored in liquid nitrogen, with the cells preserved in the culture medium with added dimethyl sulfoxide (10 % *v/v*, final concentration) or glycerol (10 % *v/v*, final concentration), as per 5.5. The cells should be propagated through the minimum of three passages after the storage, before they can be used for testing.

7 Food simulants used for testing

7.1 Test water: Reference water (3.1) that will be used directly for contact with paper and board intended for wet foodstuffs.

8 Cleaning laboratory glassware

8.1 Cleaning fluids for laboratory glassware

8.1.1 Laboratory detergent: RBS 25®[8] 5 % (*v/v*) or Aquet®[8], 1 % (*v/v*) or any equivalent alkaline detergent prepared in reference water (3.1).

8.1.2 Nitric acid: in solution, at 5 % (*v/v*), prepared by diluting 65 % to 70 % analysis-grade nitric acid in the reference water (3.1).

8.1.3 Rinsing water

8.1.3.1 Reference water (3.1).

8.1.3.2 Water prepared by mixing 3,30 g of analytical-grade $CaCl_2 \times 2H_2O$ in 20 l of reference water (3.1).

8) RBS 25 and Aquet are examples of suitable products available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of these products.

8.2 Cleaning procedure for laboratory glassware

Cleanliness of the laboratory glassware is a very important factor, since it affects the quality of the results. Glassware cleanliness is therefore checked by measuring the rate of RNA synthesis with control water (3.5).

The cleaning procedure consists of the following steps:

— soaking in the laboratory detergent (8.1.1) for at least 12 h;

— washing and copiously rinsing using the rinsing water (8.1.3.2);

— soaking in analytical grade nitric acid (8.1.2) for about 2 h;

— copiously rinsing using the rinsing water (8.1.3.1);

— air-drying in a dust-free area away from toxic vapour;

— sterilisation by autoclaving (at 120 °C for 30 min) of laboratory glassware intended for cell culturing.

NOTE This procedure can be automated.

Any traces of organic materials left on the incubation tubes (9.2.2) after the last tube rinsing cycle should be eliminated by sterilising in an oven at 550 °C for 2 h.

8.3 Alternative cleaning procedure

— Standard washing programme for laboratory glassware is applied, but the utensils intended for cell culture work are washed separated from other glassware;

— the glassware is subsequently dried at 100 °C for 2 h;

— sterilisation by autoclaving (at 120 °C for 30 min) of laboratory glassware intended for cell culturing;

— all cell culture glassware is subsequently heat sterilised in an oven at 550 °C for 2 h.

NOTE This alternative procedure can be applied, if it can be demonstrated that the cytotoxicity assay and its results as described in Clauses 11 and 12 are not affected by the treatment

9 Equipment

9.1 Equipment for the migration test

9.1.1 Equipment or clean room able to maintain the temperature required for the test within a tolerance of ± 2 °C.

9.1.2 Borosilicate wide-necked (about 40 mm) **glass flasks** into which test material (paper and board) can be introduced, and which are fitted with a stopper in a material that does not affect the migration testing (borosilicate glass).

9.2 Cell culture equipment

9.2.1 Flasks for cell culture.

9.2.2 Round-bottom, sterile, borosilicate glass **cell culture tubes**, with a preferably polypropylene or otherwise a bakelite stopper with a PTFE-seal, and fitted with magnetic stir bars (9.3.11), for cell incubation with the water extract.

Polypropylene tubes can be used as a workaround solution.

NOTE 16 ml culture tubes should be used for a 5 ml culture volume.

9.2.3 Round-bottom, sterile, disposable polypropylene **culture tubes**, with stoppers, for kinetics runs.

NOTE 5 ml culture tubes should be used.

9.2.4 50 ml sterile, disposable, polypropylene **centrifuge tubes**.

9.2.5 **Sterile** disposable **pipettes**.

9.2.6 **Work area with laminar air flow.**

9.2.7 **Incubator** able to maintain a temperature of (37 ± 1) °C, 95 % humidity and 5 % CO_2 atmosphere.

9.2.8 **Inverted microscope.**

9.2.9 Routinely-used laboratory cell culture equipment.

9.3 Equipment used for cytotoxicity testing

9.3.1 **Centrifuge** able to centrifuge at up to 1 000 g.

9.3.2 **Water bath** working at a temperature of (37,0 ± 0,5) °C.

9.3.3 **Multipoint magnetic stirrer.**

9.3.4 **0,22 µm filters adaptable on syringes**, either in cellulose acetate or else nylon, shall be non-cytotoxic, and in particular, they shall be free of surfactants.

9.3.5 Sterile disposable polypropylene **syringes** for the media filtration, and long-needle syringes for cell homogenisation before sampling for kinetics analysis (or any other appropriate apparatus).

9.3.6 **Micropipettes** with sterilised cones.

9.3.7 **Chamber for descending chromatography.**

9.3.8 **Chromatography paper**: in pure cellulose (thickness: 0,34 mm; capillary rise rate: 130 mm/30 min [water]).

NOTE Whatman 3MM®9) sheets or equivalent should be used

9.3.9 **Vortex stirrer.**

9.3.10 **Liquid scintillation counter** for tritium measurements.

9.3.11 Sterile, **magnetic stir bars** adapted to fit the incubation tubes.

NOTE Cylindrical stir bars (10 mm x 5 mm) should be used for 16-ml tubes.

9.3.12 Borosilicate **glassware** set aside exclusively for the cytotoxicity test.

Any scratched glassware shall not be used.

9.3.13 **Scintillation flasks.**

9.3.14 **Infrared radiation heater** (optional)

9) Whatman 3MM is an example of a suitable product available commercially. This information is given for the convenience of users of this European Standard and does not constitute an endorsement by CEN of this product.