SLOVENSKI STANDARD
SIST EN 16418:2014

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Papir, karton in lepenka - Ugotavljanje citotoksičnosti vodnih ekstraktov z uporabo metabolno kompetentnih hepatom celic linije (HepG2)

Paper and board - Determination of the cytotoxicity of aqueous extracts using a metabolically competent hepatoma cell line (HepG2)

Papier und Pappe - Bestimmung der Zytotoxizität von wässrigen Extrakten unter Vewendung einer metabolisch kompetenten Hepatom-Zelllinie (HepG2)

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Papier et carton - Détermination de l'effet cytotoxique d'extraits aqueux en utilisant une lignée cellulaire d'hépatome possédant des enzymes du métabolisme (cellules HepG2)

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85.060 Papir, karton in lepenka Paper and board

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Paper and board - Determination of the cytotoxicity of aqueous extracts using a metabolically competent hepatoma cell line (HepG2)

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Foreword

This document (EN 16418:2014) 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of an identical text or by endorsement, at the latest by October 2014 and conflicting national standards shall be withdrawn at the latest by October 2014.

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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 intended to come into contact with foodstuffs using specifically the HepG2 cell line.

Compared to the EN 15845[1], HepG2 cells are more representative of a human oral exposure to xenobiotics, due to the presence in the cells of phase I, II and III enzymes of the metabolism.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. 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
tap water which 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

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Note 1 to entry: Alternatively, any other purification regime, which produces HPLC-quality water (resistance > 18,0 MΩ/cm, total organic carbon < 3 ppb, no microorganism) or waters of grade 1 or 2 according to EN ISO 3696, can be used.

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3.2
water extract
reference water that has been exposed to contact with paper or board

Note 1 to entry: See EN 645 or EN 647.

3.3
negative control water
reference water that has been treated according to the same conditions as the water extract but without being in contact to paper or board

3.4
positive control waters
1) fresh 2,5 mg/l solution of potassium dichromate (CAS 7778-50-9) prepared in reference water and sterilized by filtration through 0,22 µm filter and kept at room temperature in the dark

and

2) a stock 2 mg/ml solution of benzo[|a]pyrene (CAS 50-32-8) prepared in dimethylsulfoxide (DMSO) and kept at 4 °C in the dark. Dilute this stock solution 1 000 times in reference water before use

3.5
reference sample
culture medium prepared with reference water as specified in 3.1

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3.6
test sample
culture medium prepared with water extract as specified in 3.2

3.7
negative control sample
culture medium prepared with negative control water (3.3) alone or with 0,1 % of DMSO (if benzo[a]pyrene is used as positive control)

3.8
positive control sample
culture medium prepared with positive control water as specified in 3.4

3.9
test material
a specified quantity of paper or board, that is randomly sampled from a batch

4 Principle

The test method specified in this document is intended to evaluate the cytotoxic effect of water extracts from materials for wet foods intended for human consumption. The test evaluates the impact of the water extract on the rate of RNA (Ribonucleic Acid) synthesis by measuring the incorporation of a radioactive marker (tritiated uridine) in human cells (HepG2).

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

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The extracts then undergo cytotoxicity assessment, and the results obtained are compared to the results of a non-cytotoxic control purified water (for which the rate of RNA synthesis is considered optimal and arbitrarily set at 100 %). Potassium dichromate and/or benzo[a]pyrene, prepared from stock solutions described in 3.4 and diluted as described in 11.4.4, are used as positive controls.

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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. Complete medium with additives and serum should be stored at 4 °C for no longer than two weeks.

5.2.2 Medium for routine culture

Composition:

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a) minimum Essential Medium Eagle with Earle's salts[1], (10x)[2] 100 ml

b) sodium bicarbonate solution[1], 7,5 % (*m/V*) 30 ml

c) glutamine solution, 200 mmol/l (or Glutamax I®)[1], (100x)[2] 10 ml

d) non-essential amino acids solution[1], (100x)[2] 10 ml

e) foetal calf serum[3] 100 ml

f) reference water 800 ml

5.2.3 Concentrated culture medium for testing samples

A 5,45-fold concentrated culture medium is prepared by successively mixing:

a) minimum Essential Medium Eagle with Earl's salts[1], (10x)[2] 100 ml

b) sodium bicarbonate solution[1], 7,5 % (*m/V*) 30 ml

c) glutamine solution, 200 mmol/l (or Glutamax I®)[1], (100x)[2] 10 ml

d) non-essential amino acids solution[1], (100x)[2] 10 ml

e) foetal calf serum[3] 5 ml

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The serum concentration of reconstituted treatment medium is reduced to 0,5 % since serum proteins can mask the toxicity of the water extract.

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5.3 Solution for rinsing cell lawns

Phosphate buffered saline (PBS)[1] without Ca^{2+} and Mg^{2+}.

5.4 Cell dissociation reagent

Cells are detached using a trypsin/EDTA solution[1] (0,25 % *m*/*V* trypsin, 1mM EDTA/Na_4).

[1] Commercially available. Glutamax I® is an example of a suitable product available commercially and based on Earle's salt with a minimum of the medium necessary. 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.

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5.5 Trypan blue, at 0,4 % (*w/V*)[1)]

5.6 Dimethyl sulfoxide (DMSO), analytical-grade

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

5.8 Trichloroacetic acid (TCA), for analysis

5.9 Ethanol, 95 % to 96 % (*v*/*V*)

5.10 [5,6-^{3}H] uridine

Use uridine (35 Ci/mmol to 50 ; 1 mCi/ml or 1,29 TBq/mmol to 1,85, 37MBq/ml): a sterile and non-cytotoxic aqueous solution. On the day of kinetic measurement of uridine incorporation, the required volume of uridine is taken under sterile conditions and put into a sterile tube to prepare a uridine solution at 10 µCi/ml in culture medium (5.2.3) prepared with reference water (3.1).

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 the rate of RNA synthesis meets the conditions set out in 13.3.

6 Cell line

6.1 Generating the cell strain

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The cell line used is HepG2, a human hepatoma cell line.

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This line shall be generated from recognised sources, such as the HB-8065 line of the American Type Culture Collection (ATCC) or European Collection of Cell Cultures (ECACC) No. 85011430.

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6.2 Maintaining the cell strain

Cells shall be cultured without antibiotics, and checks of absence of mycoplasma shall be run at frequent intervals:

a) seed the HepG2 cells in the culture medium (5.2.2) in a flask (9.2.1) and incubate at (37 ± 1) °C until a 90 % confluent lawn of growth is formed;

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

c) cover the cell lawn with 2 ml of the dissociation solution (5.4) and swirl around gently to cover the entire bottom of the flask. Leave on for 1 min and remove excess trypsin/EDTA solution;

d) incubate the flask at (37 ± 1) °C for 2 min to 5 min. Observe the cells detaching under the inverted microscope periodically tapping on the side of the flask to assist in detachment;

e) once the cells are detached, add 10 ml of the culture medium (5.2.2) to stop the reaction. Pipette the medium up and down over the bottom of the flask ensuring that all the cells are detached and resuspend in the medium;

f) transfer the cell suspension to a sterilized polypropylene tube;

g) homogenise cell suspension with a syringe and needle (9.2.3);

h) redistribute the cell suspension obtained into the required number of flasks, and add the appropriate volume of culture medium (5.2.2);

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i) place the flasks in an incubator at (37 ± 1) °C. When the cultures are approximately 90 % confluent, the cells can be subcultured or harvested for use in an experiment.

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 (5.6) (10 % *v/V*, final concentration). The cells should be propagated in monolayers through the minimum of three passages after thawing, before they can be used for testing.

7 Food simulants used for testing

7.1 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 liquids for laboratory glassware

8.1.1 Laboratory detergent: RBS 25® [4] at 5 % (*v/V*) or Aquet® at 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).

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8.1.3 Rinsing water

8.1.3.1 Reference water (3.1)

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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).

8.2 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 negative control water (3.3).

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;

[4] RBS and Aquet are examples of suitable products available commercially. This information is given for the convenience of the user of this European Standard and does not constitute an endorsement by CEN of these products

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

NOTE This procedure can be automated.

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 Tissue culture **flasks** 7500 mm^2.

9.2.2 Flat-bottomed **96-well** tissue culture **plates**.

9.2.3 Sterile disposable **polypropylene syringes** and **sterile long-needle syringes** (21G).

9.2.4 Sterile disposable **tubes** and **pipettes**.

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9.2.5 Work area with laminar air flow.

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

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9.2.7 Inverted microscope.

9.2.8 Routinely-used laboratory cell culture equipment.

9.3 Equipment used for cytotoxicity testing

9.3.1 Incubator for microplate working at a temperature of (37 ± 1) °C.

9.3.2 Oven at 50 °C.

9.3.3 Sterile syringe filters 0,22 µm, non-cytotoxic.

9.3.4 System for microplate **vacuum filtration** (11.8, option A).

9.3.5 Shaker for microplate.

9.3.6 8 and 12-channel pipettes and sterile tips.

9.3.7 96 deep wells microplates equipped with GF/C filters (11.8, option A).

9.3.8 Chromatography paper in pure cellulose (thickness: 340 µm, water capillary rise rate: 130 mm/30 min) (11.8.2, option B).