BS EN 16418:2014

BSI Standards Publication

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

bsi.

...making excellence a habit.™

National foreword

This British Standard is the UK implementation of EN 16418:2014.

The UK participation in its preparation was entrusted to Technical Committee PAI/11, Methods of test for paper, board and pulps.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

ISBN 978 0 580 78119 3

ICS 85.060

Compliance with a British Standard cannot confer immunity from legal obligations.

This British Standard was published under the authority of the Standards Policy and Strategy Committee on 30 April 2014.

Amendments issued since publication

Date	Text affected

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN 16418

April 2014

ICS 85.060

English Version

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

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)

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

This European Standard was approved by CEN on 8 February 2014.

CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration. Up-to-date lists and bibliographical references concerning such national standards may be obtained on application to the CEN-CENELEC Management Centre or to any CEN member.

This European Standard exists in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and United Kingdom.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Contents

Page

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.

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

According to the CEN-CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, Former Yugoslav Republic of Macedonia, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland, Turkey and the United Kingdom.

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

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.

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

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.

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.

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:

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

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

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.

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

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

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.

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);

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).

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).

8.2 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

— 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**.

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.

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).

Whatman 3MM®[5)] sheets or equivalent should be used.

9.3.9 Liquid scintillation counter or microplate scintillation counter for tritium measurements.

9.3.10 Scintillation flasks.

10 Preparation of specimens

10.1 General

10.1.1 The sample preparation procedures are designed to mimic contact with wet foods by applying hot and cold water extraction.

10.1.2 In cases where the selected extraction temperature is higher than (20 ± 2) °C, the water extract (3.2) is to be chilled to room temperature before the cytotoxicology assessment.

10.1.3 The paper and board materials to be tested shall be clean and without any external contamination. They may be tested after different storage times and conditions (temperature, humidity, light, etc.) but it shall be noted that these factors may affect the results and shall be known when interpreting the results of the assays. The samples shall be provided enclosed in non-transparent inert cover material (i. e. unlacquered aluminium foil) stored at ambient temperature and extracted within a week after reception.

10.2 Paper and board intended for wet contact

The paper and board extracts shall be prepared according to EN 645 and EN 647, with the exception that reference water (3.1) shall be used. The water extracts can be stored frozen (- 20 ˚C) for a limited time (up to three months). The extracts should be thawed slowly at ambient temperature. Ultrasound can be used to disperse any precipitations. Before testing, the extracts are sterilized by filtration using filters defined in 9.3.3.

11 Cytotoxicity assessment

11.1 Principle

HepG2 cells are incubated in a culture medium prepared with water extract from paper and board intended for wet food contact with reference water (3.1), negative control (3.3) and positive control water (3.4). After a (19 ± 2) h incubation period, the rate of incorporation of tritiated uridine in cell RNA is measured. The rate of cell RNA synthesis remains linear for at least 30 min after an initial lag phase of about 2 min to 3 min, which is the time-lapse required for the uridine to penetrate into the cell.

RNA synthesis rate is determined by calculating the slope of the regression straight line plotted using experimental values obtained for each kinetics run. This rate is then compared to the rate obtained for the reference sample, which is arbitrarily set at 100 %. The result is expressed as percentage RNA synthesis compared to the reference sample, where 100 % represents zero cytotoxicity. The value obtained with the positive control should be < 60 % of the reference sample value.

11.2 General

11.2.1 Using the cell line (6.1) and the routine culture medium (5.2.2), prepare a sufficient quantity of cells to carry out the test.

---

5) 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.

11.2.2 If the cells to be grown are taken from stock cell culture, eliminate any cryoprotectant (5.5) that may be present. Before the cells can be used, they shall be sub-cultured at least three times, but no more than ten times, as specified in 6.3.

11.2.3 Annex B shows the test work flow of the test procedure.

11.2.4 All operations up to measurement of the rate of RNA synthesis shall be carried out in asepsis, preferably in laminar air flow facilities.

11.3 Cell seeding

Cells from a 90 % confluent flask (9.2.1) can be used to initiate cultures for a toxicity test. Follow the steps b) to g) of the routine cell culturing protocol as described in 6.2 and continue with the following steps:

a) From the single cell suspension obtained (if several flasks were used, all the cells have to be pooled in a single flask), count cells using a hemacytometer to determine the cell concentration. Use a 1:1 mixture of cell suspension and 0,4 % (*w/V*) trypan blue solution. Check that cell viability is ≥ 95 %.

b) Adjust the cell concentration to 5 x 10^5 cells/ml using routine culture medium (5.2.2).

c) Using a multichannel pipette, dispense 100 µl of routine culture medium (5.2.2) only into the row H (Annex A) of a 96-wells tissue culture microplate (no cell blanks).

d) Agitate gently the cell suspension and place it in a sterile reservoir. Ensure a uniform cell suspension by filling and removing contents back several times with the multichannel pipette.

e) Dispense 100 µl of the cell suspension (5 x 10^4 cells/well) into the 84 remaining wells of the plate.

f) Cover the plate and allow the cells to grow for 28 h in the tissue incubator (9.2.6) to form a half–confluent monolayer. This incubation period allows for cell recovery and adherence and for exponential growth.

11.4 Preparation of samples

11.4.1 The test sample shall be prepared by adding one volume of concentrated culture medium (5.2.3) to 5,45 volumes of water extract (3.2) prepared according to 10.2.

11.4.2 The reference sample shall be prepared by adding one volume of concentrated culture medium (5.2.3) to 5,45 volumes of reference water (3.1) sterilized by filtration on filter defined in 9.3.3.

11.4.3 The negative control sample shall be prepared by adding one volume of the concentrated culture medium (5.2.3) to 5,45 volumes of negative control water (3.3) sterilized by filtration on filter defined in 9.3.3.

11.4.4 The positive control sample shall be prepared by adding one volume of the concentrated culture medium (5.2.3) to 5,45 volumes of positive control water (3.4) sterilized by filtration on filter defined in 9.3.3

11.5 Cell culture treatment

a) After incubating, check the correct growth of the cells under an inverted microscope.

b) Remove carefully the culture medium from the microplate by inverting over a catch basin and blotting on a stack of autoclaved paper towels.

c) Add 100 µl of the samples (11.4) following Annex A distribution of the columns (C): C1 to C3 with negative control sample, C4 to C6 with reference sample, C7 to C9 with test sample, C10 to C12 with positive control sample.

d) Place the plate for a period of (9 ± 2) h in incubator (9.2.6).

11.6 Preparation of the chromatography sheet

The chromatography sheet is to be prepared before the uridine incorporation kinetics analysis described in 11.7.

a) Trim the chromatography paper (9.3.8) to a suitable format for the chromatography chamber. For example, chromatography paper sheets (9.3.8) are cut.

b) Position the paper sheets from top to bottom.

c) At a sufficient distance from the top of the sheet, rectangles are drawn on each piece of paper with a soft pencil.

d) Another sheet of paper is prepared in the same way. Rectangles are drawn on this sheet.

11.7 Kinetics of uridine incorporation in the cell RNA

a) Examine the cell cultures under an inverted microscope. Note the general appearance of the cultures and score severe alterations such as growth inhibition, vacuolisation, rounding, detachment and lysis by a + to ++++ rating system.

b) Examine also the no cell wells (row H of the plate) which allows detection of microbial contamination or precipitate in well samples.

c) Place a sufficient volume of uridine solution (5.10) in a sterile reservoir.

d) Place a sufficient volume of SDS solution (5.7) in a second reservoir.

e) Remove carefully the exposure medium by inverting the plate over a catch basin and blotting on a stack of paper towels.

f) Incubate the plate at (37 ± 1) °C in an incubator (9.3.1).

g) At time zero, using a 12-channels pipette, add 30 µl (0,3 µCi/well) of uridine solution to the wells of row B. The introduction of the uridine into the subsequent wells of rows C, D, E, F and G is performed successively at equal time ranges using the same micropipette tips.

h) At time-point 5 min, using a 12-channels pipette, stop the reaction by addition of 30 µl of SDS solution to the wells of row B. The introduction of the SDS into the subsequent wells of rows is performed successively at time-point 10 min for the row C, 15 min for the row D, 20 min for the row E, 25 min for the row F and 30 min for the row G.

i) Independently of the operations performed for the kinetics analysis, add 30 µl of SDS solution to the three wells of row A corresponding to reference water (point zero of the kinetic run) and to six wells of row H (no cell blanks). Then add 30 µl of the uridine solution.

j) Agitate the plate (9.3.7).

11.8 Measurements of the RNA synthesis

11.8.1 option A

a) Using a 12-multichannel pipette, transfer each well of rows B to G into the corresponding wells of a new 96-well microplate equipped with GF/C glass filters wetted with 100 µl of cold 20 % (*w/V*) TCA (5.8).

b) Then transfer the three wells of row A corresponding to reference water.

c) Add again 100 µl of cold 20 % (w/*V*) TCA (5.8) into the new microplate and let 10 min in contact (precipitation of nucleic acids).

d) Eliminate the acid-soluble fraction (non incorporated uridine) by vacuum filtration (9.3.4) and wash filters by 2 x 300 µl of cold ethanol (5.9).

e) Dry filters (place microplate 1 h to 2 h in an oven at < 50 °C).

f) Dispense 30 µl of scintillation liquid solution into each well.

g) Measure the activity of the uridine incorporated in RNA cell by microplate scintillation counting (9.3.9), the results being expressed in cpm (counts per min).

h) Spot the six wells of row H (no cell blanks) on the chromatography sheet prepared according to 11.6 d) and leave to dry. Cut and place each spotted rectangle in a scintillation flask (the spot side being outside) and add the volume of scintillation mixture required. Measure by liquid scintillation counting. Calculate the mean value in order to obtain the total initial radioactivity introduced into each well, expressed in cpm (counts per minutes).

11.8.2 option B

a) Using a pipette, spot each content well of rows B to G and the three wells corresponding to the reference sample of row A on the chromatography sheet prepared according to 11.6 c).

b) When the spots are dry, eliminate the acid-soluble fraction (non incorporated uridine) by putting the chromatography sheet into the descending chromatography tank.

c) The descending chromatography is realised in 5 % (*w/V*) TCA (5.8) with a migration time of approximately 1,5 h to 2 h.

d) After migration, cut away the spot area (free from any trace of medium), rinse it in ethanol (5.9) for 10 min, and then leave it to dry.

e) Cut each spotted rectangle and place it into a scintillation flask (the spot side being outside). Add the volume of scintillation mixture required. Measure the activity of the uridine incorporated in RNA cell by liquid scintillation counting (9.3.9), the results being expressed in cpm (counts per min).

f) Spot the six wells of row H (no cell blanks) on the chromatography sheet rectangles prepared according to 11.6 d) and leave to dry. Place each spotted rectangle in a scintillation flask (the spot side being outside) and add the volume of scintillation mixture required. Measure by liquid scintillation counting. Then calculate the mean value in order to obtain the total initial radioactivity introduced into each well, expressed in cpm (counts per min).

12 Expression of the results

12.1 Graphic representation of the results

12.1.1 Since the uridine is not incorporated instantaneously, do not include the time point corresponding to time zero of the kinetic run in the regression line plotted. It should nevertheless be recorded, since excessive variation generally indicates that the uridine is substandard.

12.1.2 Each kinetic run includes six experimental time-points (ranging from time 5 min to 30 min).

12.1.3 For each experimental time-point, average the three cpm values measured and plot the mean cpm values of uridine incorporated as a function of time (expressed in min).

12.1.4 Based on the six experimental time-points, or on at least five if one of the time-points yields an outlier, plot the linear regression line for each kinetic run. Determine the equation for this linear regression $y = at + b$ as well as its coefficient of correlation R.

12.2 Calculation of percentage RNA synthesis and the validity of the test

12.2.1 General

The test may only be deemed valid if it meets the conditions according to 12.2.1 to 12.2.5.

The reference sample and the control samples (positive and negative) shall be incubated at the same time as the test sample, and with cells from the same batch.

12.2.2 Reference sample

The mean linear regression line as defined in 12.1.4 shall meet the following conditions:

1) $b < 0$;

2) $R \geq 0{,}980$;

3) $Pt = \frac{slope\ of\ the\ straight\ line\ of\ the\ reference\ sample}{total\ initial\ radioactivity\ introduced\ into\ each\ well} \times 100$ shall be ≥ 0,70

NOTE Slope of the straight line expressed in cpm/min standardised to the total initial radioactivity introduced in a well (11.7 g). $Pt < 0{,}70$ indicates that the cells are not fully viable, which distorts the results.

If any of the above mentioned conditions is not met, the test has to be repeated.

Assign a 100 % value to the slope value (RNA synthesis rate in cpm/min).

12.2.3 Negative control sample

The mean linear regression line as defined in 12.1.4 shall meet the following conditions:

1) $b < 0$;

2) $R \geq 0{,}980$.

If any of the above mentioned conditions is not met, the test has to be repeated.

Compare the slope value obtained for the test sample with the slope value obtained for the reference sample, expressing the result as a percentage of RNA synthesis rate in relation to the reference sample.

12.2.4 Positive control sample

The mean linear regression line as defined in 12.1.4 shall meet the following conditions:

1) $b < 0$;

2) $R \geq 0{,}980$.

If any of the above mentioned conditions is not met, the test has to be repeated.

Compare the slope value obtained for the positive control sample with the slope value obtained for the reference sample, expressing the result as a percentage of RNA synthesis rate in relation to the reference sample.

12.2.5 Test sample

The mean linear regression line as defined in 12.1.4 shall meet the following conditions:

1) $b < 0$;

2) $R \geq 0{,}980$.

If any of the above mentioned conditions is not met, the test has to be repeated.

Compare the slope value obtained for the sample with the slope value obtained for the reference sample, expressing the result as a percentage of RNA synthesis rate in relation to the reference sample.

13 Interpretation of the results

13.1 Results for the reference sample

If the percentage of RNA synthesis rate obtained for the negative control sample is not in the same order of magnitude as for the reference sample (the tolerance ± 10 %), then the test has to be repeated. This measurement in fact acts as a control of the level of cleanliness of the laboratory glassware used: any clear deviation between these two values would invalidate the test results.

13.2 Results of the positive control sample

The RNA-synthesis rate of the positive control sample should be less than 60 % of that of the reference sample.

13.3 Results for the test sample

If the percentage of RNA synthesis rate obtained for the test sample by comparison to this obtained for the reference sample is less than 60 %, test sample has a cytotoxic potential.

If necessary in order to obtain a precise value for a toxic concentration, a dose response analysis using different dilutions can be performed for further evaluation.

14 Precision

From an interlaboratory test by three different laboratories with two different samples the test method described above gave repeatability (r) and reproducibility (R) results as shown in Table 1. The repeatability (r) has been calculated as the coefficient of variation (CV) from the total seven tests performed (two of the laboratories provided replicates, the third a single experiment). The reproducibility (R) is given as the coefficient of variation (CV) between the means from the three laboratories.

Table 1 — Repeatability and reproducibility found in an interlaboratory test

Sample	Repeatability (r) CV (%)	Reproducibility (R) CV (%)
Paper/board (non food)	13,9	13,1
Paper/board (food contact grade)	16,1	15,7

15 Test report

The test report shall include the following information:

a) reference to this European Standard, i. e. EN 16418;

b) date and place of testing;

c) complete identification of the sample tested;

d) result, expressed as percentage of RNA synthesis;

e) any deviation from the specified procedure, or other circumstances that may have affected the results.

Annex A
(informative)

96-well plates configuration

Table A.1 — 96-well plates configuration

	1	2	3	4	5	6	7	8	9	10	11	12
A	bc	bc	bc	bc	bc	bc	bc	bc	bc	bc	bc	bc
B 5 min	NC	NC	NC	R	R	R	T	T	T	PC	PC	PC
C 10 min	NC	NC	NC	R	R	R	T	T	T	PC	PC	PC
D 15 min	NC	NC	NC	R	R	R	T	T	T	PC	PC	PC
E 20 min	NC	NC	NC	R	R	R	T	T	T	PC	PC	PC
F 25 min	NC	NC	NC	R	R	R	T	T	T	PC	PC	PC
G 30 min	NC	NC	NC	R	R	R	T	T	T	PC	PC	PC
H	b	b	b	b	b	b	b	b	b	b	b	b

Key:
NC = Negative control sample
R = Reference sample
T = Test sample
PC = Positive control sample
bc = blanks with cells
b = blanks, no cells

Annex B
(informative)

RNA Synthesis rate inhibition cytotoxicity test work flow

Table B.1 — RNA Synthesis rate inhibition cytotoxicity test work flow

ASSAY STEPS	PROCEDURE	
Preparation of water extract and negative control water	- According to EN 645 or EN 647 with reference water as food simulant	
Cell growth prior to incubation	- Seed 96-well plates: 5.10^4 cells/100µl of culture medium/well - Incubate (37 °C/5 % CO_2/28 h)	
Cell treatment	- Remove culture medium - Treat with test, reference, negative and positive control samples (precise 100 µl/well) - Incubate (37 °C/5 % CO_2/20 h)	
Uridine Uptake Kinetic Assay	- Microscopic evaluation of morphological alterations; - Remove treatment medium; - Incubate (37 °C); - Add 30 µl tritiated uridine solution (0.3 µCi/well); - Stop uridine incorporation by addition of 30 µl SDS (3 %, *w*/V) after the desired uptake time (5, 10, 15, 20, 25 and 30 min); - Shake plate for 5 min.	
Measurement of RNA synthesis	***Option A*** - Transfer samples on a 96-well plates equipped with GF/C glass filters wetted with 100 µl of TCA (20 %); - Add 100 µl TCA/well to precipitate nucleic acids for 10 min; - Filter using a vacuum manifold system to eliminate non incorporated uridine; - Wash wells twice with cold ethanol; - Dry 96-well plate filters (oven 50 °C, 1 h to 2 h) - Add scintillation liquid; - Count directly radioactivity in cpm on microplate reader; - Determine the total introduced radioactivity into well in parallel.	***Option B*** - Spot samples on a chromatography paper sheet; - Use TCA for the descending chromatography to precipitate nucleic acids and to eliminate non incorporated uridine (1,5 h to 2 h); - Cut the spot areas of the sheet and immerse them in ethanol (10 min); - Dry the paper strips; - Introduce each spot area in a scintillation vial; - Add scintillation liquid; - Count directly radioactivity in cpm in a liquid scintillation counter; - Determine the total introduced radioactivity into well in parallel.
Data analysis	- The rate of RNA synthesis is determined by the mean slope of the regression straight line obtained from the experimental values. It is then expressed in relation to that obtained with the reference and converted to a percentage value.	

Annex C
(informative)

Validation of the two methods (option A and B)

Table C.1 — Validation of the two methods (option A and B)

	Original method option A	Automated method option B
2-AAF	(122 ± 20) µM	(115 ± 20) µM
B[a]P	(3,4 ± 0,4) µM	(5,2 ± 0,37) µM
4NQO	(1,9 ± 0,3) µM	(1,95 ± 0,21) µM
MMS	(0,9 ± 0,07) mM	(0,42 ± 0,01) mM
DMN	(110 ± 24,1) mM	(159 ± 7,8) mM
1-NP	(14,3 ± 1,12) µM	(10,7 ± 1,7) µM
CP	(6,5 ± 1,6) mM	(5,5 ± 1,5) mM
2,4 DNP	(156 ± 41,3) µM	(175 ± 46,5) µM
PYR	No cytotoxicity	No cytotoxicity
Values of three independent experiments ± Standard deviation		

IC50 results in the uridine uptake assay (original (option A) and automated (option B) method)

Both methods (option A and B) were compared with nine compounds belonging to different classes of chemicals. These results were published in Toxicology (2002)[2].

Bibliography

[1] EN 15845, *Paper and board - Determination of the cytotoxicity of aqueous extracts*

[2] EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*

[3] VALENTIN-SEVERIN I. et al. Uridine uptake inhibition assay: an automated micromethod for the screening of cytotoxicity. *Toxicology*. 2002, **171** pp. 207–213

This page deliberately left blank

British Standards Institution (BSI)

BSI is the national body responsible for preparing British Standards and other standards-related publications, information and services.

BSI is incorporated by Royal Charter. British Standards and other standardization products are published by BSI Standards Limited.

About us

We bring together business, industry, government, consumers, innovators and others to shape their combined experience and expertise into standards -based solutions.

The knowledge embodied in our standards has been carefully assembled in a dependable format and refined through our open consultation process. Organizations of all sizes and across all sectors choose standards to help them achieve their goals.

Information on standards

We can provide you with the knowledge that your organization needs to succeed. Find out more about British Standards by visiting our website at bsigroup.com/standards or contacting our Customer Services team or Knowledge Centre.

Buying standards

You can buy and download PDF versions of BSI publications, including British and adopted European and international standards, through our website at bsigroup.com/shop, where hard copies can also be purchased.

If you need international and foreign standards from other Standards Development Organizations, hard copies can be ordered from our Customer Services team.

Subscriptions

Our range of subscription services are designed to make using standards easier for you. For further information on our subscription products go to bsigroup.com/subscriptions.

With **British Standards Online (BSOL)** you'll have instant access to over 55,000 British and adopted European and international standards from your desktop. It's available 24/7 and is refreshed daily so you'll always be up to date.

You can keep in touch with standards developments and receive substantial discounts on the purchase price of standards, both in single copy and subscription format, by becoming a **BSI Subscribing Member**.

PLUS is an updating service exclusive to BSI Subscribing Members. You will automatically receive the latest hard copy of your standards when they're revised or replaced.

To find out more about becoming a BSI Subscribing Member and the benefits of membership, please visit bsigroup.com/shop.

With a **Multi-User Network Licence (MUNL)** you are able to host standards publications on your intranet. Licences can cover as few or as many users as you wish. With updates supplied as soon as they're available, you can be sure your documentation is current. For further information, email bsmusales@bsigroup.com.

BSI Group Headquarters

389 Chiswick High Road London W4 4AL UK

Revisions

Our British Standards and other publications are updated by amendment or revision.

We continually improve the quality of our products and services to benefit your business. If you find an inaccuracy or ambiguity within a British Standard or other BSI publication please inform the Knowledge Centre.

Copyright

Useful Contacts:

Customer Services
Tel: +44 845 086 9001
Email (orders): orders@bsigroup.com
Email (enquiries): cservices@bsigroup.com

Subscriptions
Tel: +44 845 086 9001
Email: subscriptions@bsigroup.com

Knowledge Centre
Tel: +44 20 8996 7004
Email: knowledgecentre@bsigroup.com

Copyright & Licensing
Tel: +44 20 8996 7070
Email: copyright@bsigroup.com

bsi.

...making excellence a habit.™