



BSI Standards Publication

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

**National foreword**

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## 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.

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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of an identical text or by endorsement, at the latest by October 2014 and conflicting national standards shall be withdrawn at the latest by October 2014.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<sup>[1]</sup>, 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  $7,4 \pm 0,1$ : 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<sup>1)</sup>, (10x)<sup>2)</sup> 100 ml
- b) sodium bicarbonate solution<sup>1)</sup>, 7,5 % (m/V) 30 ml
- c) glutamine solution, 200 mmol/l (or Glutamax I®)<sup>1)</sup>, (100x)<sup>2)</sup> 10 ml
- d) non-essential amino acids solution<sup>1)</sup>, (100x)<sup>2)</sup> 10 ml
- e) foetal calf serum<sup>3)</sup> 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 Earle's salts<sup>1)</sup>, (10x)<sup>2)</sup> 100 ml
- b) sodium bicarbonate solution<sup>1)</sup>, 7,5 % (m/V) 30 ml
- c) glutamine solution, 200 mmol/l (or Glutamax I®)<sup>1)</sup>, (100x)<sup>2)</sup> 10 ml
- d) non-essential amino acids solution<sup>1)</sup>, (100x)<sup>2)</sup> 10 ml
- e) foetal calf serum<sup>3)</sup> 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)<sup>1)</sup> without Ca<sup>2+</sup> and Mg<sup>2+</sup>.

### 5.4 Cell dissociation reagent

Cells are detached using a trypsin/EDTA solution<sup>1)</sup> (0,25 % m/V trypsin, 1mM EDTA/Na<sub>4</sub>).

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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.