SLOVENSKI STANDARD
SIST EN 13784:2002

01-junij-2002

Živila - DNA komet analiza za detekcijo obsevane hrane - Informativna metoda

Foodstuffs - DNA Comet Assay for the detection of irradiated foodstuffs - Screening method

Lebensmittel - DNA-Kometentest zum Nachweis von bestrahlten Lebensmitteln - Screeningverfahren

Produits alimentaires - Détection d'aliments ionisés en utilisant le test de comete d'ADN - Méthode par criblage

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

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English version

Foodstuffs - DNA Comet Assay for the detection of irradiated foodstuffs - Screening method

Produits alimentaires - Détection d'aliments ionisés en utilisant le test de comète d'ADN - Méthode par criblage

Lebensmittel - DNA-Kometentest zum Nachweis von bestrahlten Lebensmitteln - Screeningverfahren

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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 Management Centre has the same status as the official versions.

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

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Foreword

This European Standard has been prepared by Technical Committee CEN /TC 275 "Food analysis - Horizontal methods", 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of an identical text or by endorsement, at the latest by May 2002, and conflicting national standards shall be withdrawn at the latest by May 2002.

This European Standard was elaborated on the basis of a protocol developed following a concerted action supported by the Commission of European Union (XII C.). Experts and laboratories from E.U. and EFTA countries, contributed jointly to the development of this protocol.

WARNING: The use of this standard may involve hazardous materials, operations and equipment. This standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

Annex A is informative.

This standard includes a Bibliography.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard: Austria, Belgium, Czech Republic, Denmark, Finland, France, Germany, Greece, Iceland, Ireland, Italy, Luxembourg, Netherlands, Norway, Portugal, Spain, Sweden, Switzerland and the United Kingdom.

1 Scope

This European Standard specifies a screening method for foods which contain DNA. It is based on micro-gel electrophoresis of single cells or nuclei to detect DNA fragmentation presumptive to irradiation treatment [1] to [8]. The DNA Comet Assay is not radiation specific, therefore, it is recommended to confirm positive results using a standardized method to specifically prove an irradiation treatment of the respective food, e.g. EN 1784, EN 1785, EN 1786, EN 1787, EN 1788, EN 13708, and prEN 13751.

Interlaboratory studies have been successfully carried out with a number of food products, both of animal and plant origin such as various meats [9] to [11], seeds, dried fruits and spices [6], [12]. Other studies [13] to [32] demonstrate that the method is applicable to a large variety of foodstuffs, but also that limitations exist (see clause 8).

2 Normative references

This European Standard incorporates by dated or undated reference, provisions from other publications. These normative references are cited at the appropriate places in the text, and the publications are listed hereafter. For dated references, subsequent amendments to or revisions of any of these publications apply to this European Standard only when incorporated in it by amendment or revision. For undated references the latest edition of the publication referred to applies (including amendments).

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

3 Principle

DNA fragmentation can be caused by various chemical or physical treatments including ionizing radiation. When food containing DNA is treated by ionizing radiation, modification of these large molecules occurs including fragmentation either by single- or double-strand breaks. This fragmentation can be studied by microgel electrophoresis of single cells or nuclei. These are embedded in agarose on microscope slides, lysed for disruption of membranes using a detergent and electrophoresed at a set voltage. DNA fragments will stretch or migrate out of the cells forming a tail in the direction of the anode giving the damaged cells the appearance of a comet. This comet assay to measure DNA damage can be carried out under various conditions. Both alkaline and neutral protocols exist. In general, under alkaline conditions both DNA single- and double-strand breaks and alkali-labile sites are measured, whereas under neutral conditions only DNA double-strand breaks are observed. However, using neutral conditions [1] single-strand breaks also exert an influence on the comet appearance, due to relaxation of supercoiled DNA in the nucleus [7], [8]. Irradiated cells will show an increased extension of the DNA from the nucleus towards the anode thus considerably longer comets (more fragmentation) than unirradiated cells. Unirradiated cells will appear nearly circular or with only slight tails (see Figure A.1).

This European Standard describes the use of a simple agarose single-layer set-up employing neutral pH combined with a low voltage and short electrophoresis time.

4 Reagents

4.1 General

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and water of at least grade 1 according to EN ISO 3696.

4.2 Hydrochloric acid, substance concentration *c*(HCl) = 1 mol/l

4.3 Dimethylsulfoxide, DMSO[1)] (optional)

[1)] DMSO is a harmful substance and appropriate safety precautions should be taken.

4.4 Phosphate buffered saline (PBS), pH 7,4

Dissolve 8,0 g of sodium chloride, 0,2 g of potassium chloride, 2,94 g of disodium hydrogen phosphate dodecahydrate ($Na_2HPO_4 \cdot 12\ H_2O$) and 0,24 g of potassium dihydrogen phosphate (KH_2PO_4) in 900 ml water, adjust the pH to 7,4 with a few drops of hydrochloric acid (4.2) and adjust the volume with water to 1000 ml. The solution should be autoclaved or sterile-filtered.

4.5 Coating agarose solution, 0,5% agarose in distilled water

Dissolve 50 mg agarose in 10 ml water by boiling or microwaving (no flakes, clear solution). Keep the solution in a water bath at 45 °C for precoating the microscope slides.

4.6 Casting gel solution, 0,8% agarose in PBS

Dissolve 80 mg of low melting temperature agarose, in 10 ml of PBS (4.4) by boiling or microwaving. Keep the solution in a water bath at 45 °C, ready to be mixed with the cell suspension and to cast the gel on the slides.

4.7 EDTA stock solution *c*(EDTA) = 0,5 mol/l

Add 93,05 g of ethylenediaminetetraacetic acid, disodium salt dihydrate to 300 ml of distilled water, mix well, and adjust the pH to 8,0 with 40 % sodium hydroxide solution. Dilute to 500 ml with distilled water, and autoclave.

4.8 TBE stock solution

Dissolve 54 g Tris(hydroxymethyl)aminomethane (Tris base) and 27,5 g of boric acid in 20 ml of EDTA stock solution (4.7), dilute to 1000 ml with distilled water (TBE). This TBE stock solution can be stored in glass bottles at room temperature. Discard any batches that develop a precipitate.

4.9 Electrophoresis buffer

Dilute one volume part of the TBE stock solution (4.8) with nine volume parts of water. If necessary, adjust the pH to 8,4.

4.10 Lysis buffer

Dissolve 25 g of sodium dodecylsulfate (SDS) in electrophoresis buffer (4.9) and adjust the volume to 1000 ml.

4.11 Staining solutions

4.11.1 Acridine orange stock solution[2)]

Dissolve 100 mg of acridine orange in 100 ml of water. Keep in the dark at approximately 4 °C to 6 °C.

4.11.2 Acridine orange staining solution[2)]

Dilute 0,5 ml of acridine orange stock solution (4.11.1) to 100 ml with PBS (4.4). This solution may be stored at 4 °C to 6 °C for up to one week.

4.11.3 Propidium iodide stock solution[2)]

Dissolve 100 mg of propidium iodide in 100 ml of water. Keep in the dark at approximately 4 °C to 6 °C.

4.11.4 Propidium iodide staining solution[2)]

Dilute 1 ml to 5 ml of propidium iodide stock solution (4.11.3) to 100 ml with PBS (4.4).

[2)] Acridine orange, ethidium bromide and propidium iodide are harmful substances and appropriate safety precautions should be taken.

4.11.5 Ethidium bromide stock solution[2])

Dissolve 100 mg of ethidium bromide in 100 ml of water. Keep in the dark at approximately 4 °C to 6 °C.

4.11.6 Ethidium bromide staining solution[2])

Dilute 2 ml of ethidium bromide stock solution (4.11.5) to 100 ml with water.

4.11.7 Silver staining

4.11.7.1 General

The following silver staining solutions and procedure [33] has been employed in the interlaboratory trials [6]. Other procedures [34] as well as commercial silver staining kits for nucleic acids may be used, provided they have been found satisfactory.

4.11.7.2 Fixing solution A

To 150 g of trichloroacetic acid add 50 g of zinc sulfate and 50 g of glycerol and dilute to 1000 ml with water.

4.11.7.3 Staining solution B

Dissolve 12,5 g of sodium carbonate in water and adjust the volume to 250 ml.

4.11.7.4 Staining solution C

Dissolve 100 mg of ammonium nitrat, 100 mg of silver nitrate and 500 mg of tungstosilic acid in water, add 250 µl of formaldehyde (min. 37%) and dilute to 500 ml with water.

4.11.7.5 Staining solution D

Immediately before use, add 68 ml of staining solution C (4.11.7.4) to 32 ml of a vigorously stirred staining solution B (4.11.7.3).

4.11.7.6 Stopping solution E

Dilute 10 ml glacial acetic acid to 1000 ml with water.

5 Apparatus

Usual laboratory apparatus and, in particular, the following:

5.1 DNA horizontal submarine electrophoresis chamber,

5.2 Power supply, e.g. 0 V to 100 V, 0 mA to 400 mA

5.3 Stopwatch

5.4 Balance

5.5 Water bath

5.6 Hot plate magnetic stirrer

5.7 Microwave oven

5.8 Sieve cloth, 100 µm, 200 µm and 500 µm pore size, e.g. of nylon

5.9 Microscope slides

(76 mm x 26 mm) with one frosted end.

5.10 Cover slips

(24 mm x 60 mm).

5.11 Staining jars

5.12 Microscope

In the case of DNA silver staining a standard transmission microscope can be used, but using fluorescent staining, a microscope with epifluorescence illumination is needed, with a filter set of e.g. 460 nm to 485 nm (blue excitation) for acridine orange or a filter set of 515 nm to 560 nm (green excitation) combined with a barrier filter at 590 nm for propidium iodide or ethidium bromide.

The microscope should allow a magnification of 100x to 400x.

6 Procedure

6.1 Preparation of single cell suspensions

6.1.1 General

For a suitable evaluation of electrophoresed slides, the distribution of cells in the agarose gel should be even and not overlapping each other. If too few cells are present, the amount of tissue can be increased, and vice versa. The cell suspensions may be stored on ice but no longer than 10 min. By addition of DMSO to a final level of 5 % to 10 % as a freeze protectant, the cell suspensions can be stored for extended periods at - 18 °C.

6.1.2 Animal tissues

6.1.2.1 Bone marrow

Split the bone (e.g. chicken leg) and transfer about 50 mg of bone marrow to a test tube with 3 ml of ice-cold PBS. Suspend the cells using a glass rod. Filter the cell suspension through sieve cloth with a pore size of 100 µm. Keep the filtrate on ice. Use the supernatant for further analysis.

6.1.2.2 Muscle tissue

Scrape off the tissue or cut it (without visible fat) in very thin slices with a scalpel and transfer about 1 g to a small beaker with 5 ml of ice-cold PBS. Cool the beaker in a larger one with crushed ice and stir for 5 min at about 500 min^{-1}. Filter the suspension sequentially through 500 µm and 200 µm sieve cloth. Leave to settle on ice for about 5 min. Use the supernatant as cell extract.

6.1.3 Plant tissues

6.1.3.1 Seeds, nuts and spices

Crush about 0,25 g of the samples with a mortar and pestle (if present remove outer shell before grinding, sometimes immersion in water facilitates the removal) and transfer to a small beaker with 3 ml of ice-cold PBS. Cool the beaker in a larger one with crushed ice and stir for 5 min at about 500 min^{-1}. Filter the suspension sequentially through 200 µm and 100 µm sieve cloth. Leave to settle on ice for 15 min to 60 min. A longer time gives a suspension with less contaminants, but also with less cells/cell nuclei. Use the supernatant for further analysis.

6.1.3.2 Strawberries

Isolate the achenes of strawberries just by picking or by blending the strawberries in a large amount of water, allowing the heavier achenes to settle. Weigh about 0,25 g of achenes and proceed as for seeds, nuts and spices (6.1.3.1).

6.1.3.3 Potatoes

Cut the meristem of potato in very thin slices with a scalpel and transfer about 4 g to a small beaker with 5 ml of ice-cold PBS, and proceed as for seeds, nuts and spices (6.1.3.1).

6.1.3.4 Onions

Cut the meristem of onions in very thin slices with a scalpel and transfer about 2 g to a small beaker with 4 ml of ice-cold PBS, and proceed as for seeds, nuts and spices (6.1.3.1).

6.2 Pre-coating slides

To improve adhesion of the agarose gel to the slide, the latter is pre-coated with a thin agarose layer. Before coating, the slides are freed from fat by immersion overnight in methanol, and are allowed to air dry. Precoat the cleaned dustfree slide by spreading one drop (approximately 50 µl) of the warm coating agarose solution (4.5) with a second slide across the first slide and allow air dry for about 30 min. Precoating can also be done by dipping and cleaning one side with paper tissue. Coated slides can be stored dustfree for several weeks.

6.3 Casting the gels

Mix 100 µl of cell suspension with about 1 ml of warm casting gel solution (4.6). Transfer 100 µl of this mixture on a precoated slide and spread it roughly by the pipette tip. Cover immediately with a cover slip in such a way that the gel is spread evenly and avoid air bubbles. Place the slide on ice for 5 min to solidify the agarose gel. Move the cover slip aside with the tip of a scalpel, and gently slide the slip off the agarose. The gel shall be even without bubbles. Several slides can be prepared in parallel using the same gel solution.

6.4 Lysis of the cells

DNA fragments migrate out of the cells during electrophoresis under the condition that the cell membranes are permeable. Therefore, the lysis of the cells is a necessary prerequisite for the application of the comet assay. Immerse the casted slides completely in lysis buffer in a staining jar for at least 5 min for animal cells and at least 15 min for plant cells at room temperature. Do not touch the agarose layer. (To check for complete lysis, cells may be stained and observed with a microscope: lysed cells will show diffusion of DNA out of the cells.)

6.5 Conditioning

Immerse the slides after lysis in electrophoresis buffer (4.9) for 5 min.

6.6 Electrophoresis

Place the slides in the horizontal electrophoresis chamber side by side, avoiding spaces and with the frosted end facing the cathode. Fill the tank with fresh electrophoresis buffer (4.9) to a level approximately 2 mm to 4 mm above the slides (do not displace the slides). Conduct electrophoresis at room temperature at a potential of 2 V/cm