EN 13784 - English version

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

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 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.

3 Limitations

The DNA Comet Assay may, in principle, be applied to detect irradiation of any food containing DNA. The comet assay has already been applied successfully to a number of foodstuffs, both animal foods, e.g. chicken, duck, quail, pheasant, pork, boar, beef, veal, lamb, deer, fish (trout, salmon), and plant foods, e.g. almonds, figs, lentils, soybeans, carioca and macaçar beans, strawberries, grapefruit, linseed, sesame seeds, sunflower seeds, rosé pepper.

It is emphasized that the comet assay is working as a screening test, and the results need to be confirmed by another technique specific for irradiation, since DNA fragmentation may be obtained by other means (see for example mustard seeds as described below in paragraph 8).

Each new type of foodstuff shall be tested before unknown samples are analyzed: lysis solution and treatment time, as well as electrophoresis time and field strength can be changed in order to obtain an adequate migration of DNA in the gel.

Since at present, knowledge of DNA comet patterns for various foodstuffs is still limited, it would be of advantage to use products from different sources to gain experience with the variability of comet patterns. Also the effect of irradiation and storage parameters should be further studied. In addition, the preparation of cell suspensions is an important step since this is a necessary prerequisite for applying the comet assay. For some foodstuffs, e.g. papayas, tamarinds [32], a lot of crude cell debris interferes with the pattern of comets, and some additional steps in the preparation of the cell suspension to arrive at a suitable background are necessary. For some other products, it might be difficult to obtain appropriate cells, e.g. for some nuts, some spices, some fish [31] and [32]. For heated foods, no cells will be available due to extensive heat damage. Thus, no appropriate cells can usually be found for blanched shrimps or for cooked (microwaved) chicken [6].

In the case of meat, it should be recognized that depending on storage conditions (temperature and time between slaughter and freezing) of the fresh meat, a natural (enzymatic) degradation of DNA takes place, producing DNA fragmentation [33]. The comets in this case are of varying shapes. The presence of many cells showing advanced DNA degradation might be only a sign of poor storage conditions. Also, repeated freeze-thawing of meat produces extensive DNA damage (fragmentation). The patterns are similar to those obtained from natural DNA degradation and can usually be distinguished from irradiated samples which give a homogenous pattern [6]. Only if all the cells show advanced DNA degradation one can not determine whether the sample was irradiated or not. This is a clear limitation of the comet assay.

Another limitation may be insufficient lysis of the cells to make membranes permeable, this permeability enabling the DNA fragments to migrate out of the nuclei. In earlier experiments [4], [5], [15], [17], [19], [20] insufficient lysis was experienced using an SDS concentration of only 0,1%. By increasing the SDS concentration to 2,5% this problem was overcome at least for animal cells [6]. For some plant cells the duration of lysis time had to be increased, e.g. up to 30 min to 60 min for soybeans or grapefruits [6], [26], [30]. For some other plant products, e.g. mushroom spores from *Agaricus bisporus*, lysis of the cell wall was not achieved [6], [19], and therefore, the comet assay could not be applied. Probably, insufficient lysis is also the reason for intact cells being observed in irradiated samples, such as dried gram, sliced almonds [28], red kidney beans and tamarinds [32]. Stronger lysis solutions or longer duration of incubation might alleviate this problem.

For some food items cell preparations could be obtained but difficulties arose to differentiate between unirradiated and irradiated samples, e.g. for some nuts, some seeds or some spices [31]. Difficulties were also observed for food items to be irradiated with low radiation doses, such as onions and potatoes [6] and [32]. Possibly, the use of sophisticated image analysis may help with identification, but just by visual inspection the judgement whether the sample was irradiated or not was doubtful.

A special case was observed for mustard seeds, which showed a comet pattern corresponding to an irradiated sample with clear comets and no intact cells. This sample was tested by thermoluminescence (EN 1788), but shown to be unirradiated. Also the germination capacity of the mustard seeds indicated no irradiation treatment [6]. Similar problems were encountered with a millet sample. Although only comets could be observed in the sample, other detection methods like thermoluminescence measurements (EN 1788) or hydrocarbon formation from the lipid part of the food (EN 1784) identified the sample as unirradiated [34]. Probably the state of the seeds, whether in dormancy or not, may play a role.

4 Validation

The procedure as described in this European Standard is based on interlaboratory studies with animal (chicken, pork) [9] to [11] and plant foods [6], [12] as well as on studies with a number of food items [13] to [32].

In an interlaboratory test organized on behalf of the Swedish National Food Administration, nine participating laboratories analyzed three kinds of coded cell suspensions made of irradiated and unirradiated chicken bone marrow, chicken and pork muscle tissue. The radiation doses varied between 0 kGy, 1 kGy, 2,5 kGy, 3 kGy, and 5 kGy. From a total of 162 samples dispatched, valid results were reported from 148 samples. Of these, 138 were correctly identified. From 106 irradiated samples, 99 were correctly detected, while 39 out of 42 unirradiated were correctly classified, see Table 1 [11].

Table 1 — Interlaboratory data for chicken and pork

Product	Total No. of samples	No. of samples (valid results)[a]	No. of correct identifications	No. of false negatives[b]	No. of false positives[c]
Chicken bone marrow	54	54	52	1	1
Chicken muscle	54	46	42	3	1
Pork muscle	54	48	44	3	1
All	162	148	138	7	3

[a] no results reported for lacking samples.

[b] false negatives are irradiated samples identified as unirradiated.

[c] false positives are unirradiated samples identified as irradiated.

It should be recognized that some laboratories did not have much experience with the comet assay at the time of this collaborative trial. Although each laboratory received a set of reference samples made from chicken bone marrow irradiated with 0 kGy, 1 kGy, 3 kGy or 5 kGy and labelled with the given doses, difficulties with this new method were experienced in some laboratories. However, six laboratories successfully identified all the samples correctly.

A further collaborative trial [6] was conducted with a variety of plant items, namely almonds, figs, lentils, linseed, rosé pepper, sesame seeds, soybeans and sunflower seeds. The coded samples were either unirradiated or irradiated with doses of 0,2 kGy, 1 kGy or 5 kGy. In addition to the 20 coded samples, the participants received a reference set of 12 samples with known radiation dose. Four laboratories participated in this intercomparison. Of the total 78 answers received, 74 were correct (95%). The results are shown in Table 2.

Table 2 — Interlaboratory data for plant cells tested (for 10 months of storage after irradiation)

Sample	Total No. of samples	No. of samples (valid results)[a]	No. of correct identification	No. of false negatives[b]	No. of false positives[c]
Unirradiated	32	31	29	0	2
Irradiated	48	47	45	2	0
All	80	78	74	2	2

[a] One laboratory did not provide results with soybeans, since it had problems with the lysing conditions.

[b] False negatives are irradiated samples identified as unirradiated.

[c] False positives are unirradiated samples identified as irradiated.