

# *Biological Techniques*

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## 15.1 Biological Changes in Irradiated Foods

DNA is a large molecule particularly sensitive to ionizing radiation, which suffers several kinds of damage: fragmentation resulting from both single-strand and double-strand breaks, denaturation of the DNA helix, cross-linking (*e.g.*, production of thymine dimers, or between DNA and a protein) and base damage.<sup>1-3</sup> It causes primarily single strand breaks (SSBs) in genomic DNA, in addition to double strand breaks (DSBs) at ratios of SSB/DSB of 20/1 to 70/1, as well as some detectable membrane damage.<sup>4</sup> In foods, this DNA susceptibility is the cause of death of most if not all living contaminants, such as microorganisms, insects, or parasites,<sup>4</sup> and is also the cause of changes in the food's DNA itself, which can reflect on various morphological and physiological features.

DNA damage occurs predominantly by the indirect action of gamma rays, which interact with other atoms or molecules, particularly water, to produce reactive free radicals.<sup>5</sup> Cell death (defined for proliferating cells as the loss of reproductive capability) is predominantly induced by double-strand breaks in DNA, separated by not more than a few base pairs, which cannot generally be repaired by the cell.<sup>6</sup> Since irradiation with just 1 Gy introduces about 1000 DNA single-strand breaks and about 50 double-strand breaks per cell,<sup>7</sup>

the radiation doses of mostly several kGy employed in food irradiation will have an effect on DNA. Such DNA changes, and mostly its fragmentation, are excellent candidates to be used as biological markers for the detection of radiation treatments in foods.

One of the most evident effects of radiation treatment is a significant shift in the microbiota loads and profiles. This shift is based on the fact that microorganisms are, in general, inactivated by radiation treatments, so the final amount of viable cells in irradiated foods is significantly lower than that in non-irradiated foods.<sup>8</sup> Microbiota changes can thus be used as indicators of food irradiation treatment. Different microorganisms show different sensitivity to irradiation, as described in Chapter 10.

Owing to extensive DNA degradation, deep changes occur in the morphological and physiological characteristics of cells and tissues, mostly in plant meristems. Cell division is inhibited by irreparable defects in the cell cycle, seed germination is strongly delayed or hampered, and seedling morphology (root and shoot) is aberrant.<sup>9</sup> The enzymatic activity is also changed in physiologically active tissues.<sup>10</sup> While these effects are the central goal in irradiation treatment for sprouting inhibition in potatoes, onions, and garlic, or for ripening delaying in numerous fruits, they can also be used as irradiation markers.

## 15.2 Detection of Irradiated Foods by Biological Methods

The most commonly used biological methods for the detection of irradiated foods are the Direct Epifluorescent Filter Technique/Aerobic Plate Count (DEFT/APC), DNA comet assay, and Limulus Amebocyte Lysate (LAL) Test. These are currently standardized methods, but others have been tested for their ability to detect irradiated foods.

In theory, all types of food storage or processing, and not only irradiation, cause some kind of changes in the food product, be it in the DNA profile, cytological, or physiological features, or microbial loads and profiles. For that reason, methods of irradiation detection based on biological changes of test foods are usually presumptive and can be used only as screening methods. Being generally not radiation-specific, they can only give an indication of a possible treatment by ionizing radiation.

Both standardized and alternative methods currently in use or being tested for the detection of biological changes in irradiated foods are described below and summarized in Tables 15.1 and 15.2.

### 15.2.1 Measurement of DNA Changes

#### 15.2.1.1 Comet Assay

DNA strand breaks can be monitored by microgel electrophoresis of single cells or nuclei, a technique commonly called 'Comet Assay' (CA). In this

**Table 15.1** Standard biological methods validated to screen for irradiated foods.

Standard method	Principle of the method	Irradiation conditions	Validated foods
EN 13783:2001 Detection of irradiated food using direct epifluorescent filter technique/aerobic plate count (DEFT/APC) – screening method	Comparison of the viable number of cells obtained by APC with the total count obtained using DEFT	Gamma irradiation (5 and 10 kGy)	Herbs and spices (allspice, peppers, cardamom, ginger, thyme, marjoram, basil, oregano)
EN 13784:2001 DNA comet assay for the detection of irradiated foodstuffs – screening method	Quantification of DNA damage by micro-gel electrophoresis of single cells or nuclei	Gamma irradiation (0 to 5 kGy)	Various meat (chicken, pork, beef, veal, lamb, fish) and plant (seeds, dried fruits, spices) products
EN 14569:2004 Microbiological screening for irradiated food using LAL/GNB procedures	Identification of unusual microbiological profiles using the limulus amoebocyte lysate (LAL) test and the enumeration of total Gram-negative bacteria (GNB) in the test sample	Gamma irradiation (2.5 and 5 kGy)	Poultry meat (breast, legs, wings of fresh, chilled, or frozen carcasses, with or without skin)

**Table 15.2** Alternative biological methods tested for the detection of irradiated foods.

Method	Principle of the method	Irradiation conditions	Tested foods	Ref.
Real-time PCR	Quantification of DNA damage by PCR amplification of different sized amplicons	Gamma irradiation (0.25 to 9 kGy)	Rainbow trout	3
Mitochondrial DNA	Measurement of mitochondrial DNA breakage by agarose gel electrophoresis	Gamma irradiation (2 to 4 kGy)	Meat	62
Flow cytometry	Detection of changes in the DNA content	Gamma irradiation (0.06 to 0.09 kGy)	Onion bulbs	64
Shift in microbial load and profile	Detection of changes in microbial counts or profiles based on different microbial sensitivity to irradiation	Gamma irradiation (2.0 and 2.5 kGy)	Strawberries, raw poultry meat	25, 65
Bacterial spoilage profiles	Determination of the ability of bacteria to cause spoilage determined by measuring the generation of total volatile acids (TVAs) and total volatile basic nitrogen (TVBN)	Gamma irradiation (0 to 5 kGy)	Bombay duck, Indian mackerel, white pomfret, seer, shrimp, beef, chicken, mutton, pork, dried anchovies	81, 82
Germination and half-embryo tests	Quantification of physiological disorders caused to seeds, such as significant delay or full inhibition of seed germination and abnormal root and shoot growth	Gamma irradiation (0.025 to 10 kGy)	Wheat, maize, chickpea, lentils, black eye beans, watermelon, melon, citrus, onions, garlic, potatoes	29, 36, 49, 77, 85–94

technique, DNA from single cells or nuclei are extracted from samples by cell lysis in appropriate buffers for 5 to 60 min (depending on the type of tissue), suspended in melted agarose, and casted on microscope slides. Following a rapid electrophoretic separation, the gel is stained with a fluorescent dye, observed through a microscope, and documented by photography or image analysis. The migration pattern of DNA indicates a possible irradiation treatment. In irradiated samples, the radiation-induced DNA fragments leak from the nuclei during electrophoresis, forming a tail in the direction of the anode. In non-irradiated samples, if not exposed to other DNA-fragmenting treatments, cells appear intact. Damaged and undamaged cells are thus easily differentiated. The size and shape of the tail, as well as the distribution of DNA within the comet, vary with the extent of DNA damage, which in turn correlates with the applied dose.<sup>11,12</sup>

The CA technique was initially developed by Östling and Johanson<sup>13</sup> to monitor DNA degradation in mammalian cells after radiation treatments, and was later adapted for the sensitive detection of irradiated foods by Cerda and colleagues.<sup>14</sup> Since then, CA has increasingly been studied and recognized as a valuable tool for the detection and quantification of irradiated foods of plant and animal origin, and has shown to be rapid, sensitive, inexpensive, and simple to perform.<sup>12,15</sup> The first tests developed on food matrices applied low stringent conditions similar to those of human cells. In the course of these experiments, it was observed that apparently intact cells with no comets also appeared in irradiated samples, potentially resulting from insufficient lysis of the membranes of the cells or nuclei. Consequently, the conditions were optimized: the concentration of the lysing agent sodium dodecyl sulphate (SDS) was increased from 0.1 to 2.5%, and tris-borate-ethylenediamine tetraacetic acid (TBE) buffer was employed. In addition, the electrophoretic conditions were adjusted to optimize discrimination and a potential of  $2 \text{ V cm}^{-1}$  for 2.0 min was applied.<sup>11,16</sup> Using these modifications, good results were obtained for chicken, both fresh and frozen, other poultry, *e.g.*, duck, quail, pheasant, and also for beef, pork, game, and fish such as salmon,<sup>17</sup> thus confirming the applicability of the method. As a consequence, the procedure was generally established as a routine protocol. A detailed description of this protocol has been given by Cerda and colleagues.<sup>15</sup> In addition to the described adjustments, the technique can be carried out under alkaline or neutral conditions, depending on the goal. 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.<sup>15</sup>

Following electrophoresis, comets can be analyzed by visual scoring, without the use of image analysis software, by visual classification of comets into categories based on the size and shape of the tail.<sup>2,4,12,15,18–21</sup> Although various differently shaped comets can be observed on the same electrophoresis slide, it is the lowest degree of DNA damage that will determine the classification of the sample.<sup>11</sup> Visual assessment of the radiation dose administered can be aided by a set of reference slides prepared from the

foods under investigation submitted to known doses of radiation and run along with the unknown samples to ensure identical conditions.<sup>15</sup> Alternatively, comets can be analyzed based on computer image analysis.<sup>22,23</sup> Image analysis systems for comet evaluation potentially strengthen the method by avoiding individual analyzer variation,<sup>22</sup> mostly for unexperienced laboratories and for very low irradiation doses (e.g., 0.1 kGy used to inhibit sprouting of potatoes, onions, and garlic).<sup>24</sup> Also, they allow fully quantitative discrimination between irradiated and non-irradiated samples, as well as they are able to set up standard dose-response curves, resulting in sufficiently accurate dose estimations.<sup>25</sup> Nevertheless, good correlation between visual scoring and image-based DNA damage measuring parameters (tail length, % of DNA in the tail, tail moment) has been reported.<sup>20</sup> In the cases where CA is applied as a screening technique to detect irradiated food, the use of an image analyzer may not be required.

Several kinds of foods such as whole fresh and frozen meats (chicken, turkey, pork, beef, duck, lamb, veal, pheasant, deer, among others), frozen hamburgers, fish (trout, salmon), figs, grams, pulses, cereals, nuts, dried fruits, fresh fruits (citrus, apples, watermelons, tomatoes, papaya, melon), and spices have already been subjected to analysis by this technique.<sup>2,4,12,18,23-40</sup>

Dry food stuffs (seeds) as well as moist foods (meat, fruits, and vegetables) were analyzed by Khawar and colleagues.<sup>12</sup> Also, Khan and colleagues<sup>2</sup> successfully detected radiation treatments in several types of whole pulses (green, red and yellow lentils, green and yellow peas, chickpeas, cowpeas) and grams (black, red, and white grams). Cetinkaya and colleagues<sup>23</sup> used it for quantification of applied low doses to various citrus. In this study, an applied dose as low as 0.1 kGy was detected, and the method was proposed as a potential quarantine control method for inspectors.

Interlaboratory studies have been successfully carried out with a number of food products, such as various meats, seeds, dried fruits, and spices,<sup>11,16</sup> yielding very high rates (>90%) of identification. In a collaborative study in Scandinavia on irradiated frozen chicken, all samples were correctly identified as having been irradiated or not.<sup>41</sup> In another test, five participants were able to differentiate between samples of trout, salmon, and chicken treated at various radiation doses (0, 1, 2, 3, and 5 kGy) with a probability of over 94%. An interlaboratory trial with nine participating laboratories, not all highly experienced in the technique, investigated cell suspensions made of irradiated and non-irradiated chicken bone marrow, chicken, and pork muscle, with radiation doses varying between 0 and 5 kGy. Of the total 148 results reported, 138 were correctly identified (93%).<sup>15</sup> A further collaborative trial was conducted with a variety of plant items, namely almonds, figs, lentils, linseed, rosé pepper, sesame seeds, soybeans, and sunflower seeds irradiated at doses of 0, 0.2, 1, and 5 kGy.<sup>42</sup> The results showed that CA can also be applied to plant tissues for the detection of irradiation treatment with high rates of identification. Experiments with other plant products (strawberries, beans)<sup>43</sup> also confirmed the applicability of the method even at low dose levels (0.5 kGy).

The DNA comet assay has been tested for the control of imported food to Sweden and a number of meat samples were found to indicate irradiation treatment. The suspected samples were also analyzed by gas chromatographic analysis of lipid-derived hydrocarbons, which confirmed the CA results.<sup>41</sup>

In 2001, the European Committee for Standardization (CEN) adopted the DNA Comet Assay in the European Standard EN 13784:2001,<sup>†</sup> and it became one of the currently ten approved standard methods for the detection of irradiated foods. The standard specifies this assay as a screening method for foods that contain DNA, namely meat, seeds, dried fruits, and spices. It has been adopted as a screening method to detect irradiated foods, but has not been officially considered for the determination of the applied dose.<sup>23</sup>

Despite all this, the technique is not free of drawbacks, and some limitations to its application must be considered. Foods that have been subjected to other treatments or processing that also induce DNA fragmentation (such as cooking, blanching, repeated freezing–thawing, or medium- to long-term storage) can display comets similar to those obtained from irradiated samples.<sup>3,4,11,15,21,22,27,31,44</sup> However, some studies have reported the successful application of the technique to frozen meats (chicken, beef hamburgers), even after long periods of storage of up to six months.<sup>31,40,45</sup> The results obtained for dry foodstuffs (seeds and nuts) are generally clearer than those for fresh foods (meat, vegetables, and fruits), most likely because DNA damage by other factors is eliminated in dry foods.<sup>12,19,29</sup> In fact, it is not advisable to use this assay in foods with rapid natural degradation such as seafood.<sup>31</sup> Accumulation of certain metals in animal organs also seems to induce DNA breakage analogous to that resulting from irradiation.<sup>46</sup>

Technical limitations also exist, as suitable DNA material is hard to obtain in some dry foods, especially nuts, seeds, and beans.<sup>30,32,39,47–49</sup> For example, suitable DNA material from Brazil cashew and pistachio nuts could not be extracted and, in the case of pine nuts, very few round intact cells were observed along with most comets, making the screening difficult.<sup>32</sup> Cells or nuclei are also difficult to extract from some fresh samples of seafood like squid and saithe.<sup>31</sup> The sensitivity to irradiation also differs among diverse types of tissues.<sup>19</sup> The preparation of cell suspensions must thus be optimized for each type of food material.<sup>39,47</sup>

Because of unspecific DNA degradation, this technique can result in high levels of false positives. Mangiacotti and colleagues<sup>44</sup> detected as high as 26% false positives in an official control by an accredited laboratory, whereas other methods such as photostimulated luminescence (PSL, also a screening method) yielded 11% false positives. CA false-positives were associated with freeze–thaw processes. In this study, it was stated that PSL is a more versatile screening technique for numerous food matrices, being more accurate, faster, and simpler than CA, and with lower consumable costs. In contrast,

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<sup>†</sup>Available at [http://ec.europa.eu/food/safety/biosafety/irradiation/legislation\\_en](http://ec.europa.eu/food/safety/biosafety/irradiation/legislation_en).

Merino and Cerda<sup>20</sup> found great consistency between CA and the hydrocarbon method. From over 15 analyzed samples, only one showed no agreement between the two methods.

Two main consequences rise from these limitations. On the one hand, given the high matrix effect observed, the method must be optimized and validated for each type of food.<sup>2,12</sup> On the other hand, as a result of the non-specificity of DNA damage detected by CA, it is mandatory that positive results are confirmed by other radiation-specific identification methods.

#### 15.2.1.2 Real-time PCR

Gamma irradiation induces random closely spaced lesions, including double-stranded DNA (dsDNA) breaks and about twice as many single strand DNA (ssDNA) breaks on opposing strands within about 10–20 base pairs (bp).<sup>50</sup> Successful amplification by polymerase chain reaction (PCR) normally depends on the intact nature of the targeted DNA sequence, and the degraded DNA may still be amplified only in cases where the average DNA strand is not shorter than the desired DNA sequence to be amplified. As a result of irradiation, genomic DNA is fragmented in such a way that efficient amplification by PCR is precluded, either by alteration in primer binding sites or by reduction of DNA into fragments smaller than the target.<sup>51–53</sup>

The quantification of DNA damage resulting from irradiation treatments is possible by real-time PCR analysis. In conventional PCR, the amplified DNA product, or amplicon, is detected in an end-point analysis, usually by gel electrophoresis. In real-time PCR, the accumulation of the amplification product is measured as the reaction progresses, in real time, with the product being quantified after each cycle. Real-time detection of PCR products is assisted by a fluorescent reporter molecule that yields increased fluorescence with the increasing amount of product DNA, and the changes in fluorescence over time are used to calculate the amount of amplicon being produced. Real-time PCR has several advantages over traditional PCR, the most important one being the ability to quantify initial DNA amounts present in the sample (initial number of copies of the target sequence), thus being also called quantitative PCR. Other advantages include enhanced speed and the absence of post-PCR steps such as gel electrophoresis, with consequent reduced bench time and increased throughput. DNA extracts of known cell concentrations are used to establish standard curves relating the log number of genomic targets (derived from the number of colony forming units (CFU) g<sup>-1</sup> of tissue) to the threshold cycle (*Ct* value) obtained by DNA amplification. The *Ct* values will determine the amount of template DNA; the lower the *Ct* value, the higher the amount of targeted nucleic acid.

In the case of viable cells exposed to irradiation, the maximum correlation between the viability (CFU) and *Ct* values is critically dependent on several factors.<sup>52</sup> One such factor is the irradiation dose, which determines the mean length of ruptured DNA strands. This allows the technique to be used for quantitative determination of the irradiation dose. For this, a standard



curve correlating the viability with the irradiation dose needs to be created. A second critical factor is the number of genomic targets available for amplification. A single genomic target per cell will yield a closer correlation, while multi-copy sequences will introduce biases to this correlation. A third critical factor is the size of the amplicon to be detected. The larger the amplicon, the closer the correlation.

Only few studies have tested the use of real-time PCR for the detection of irradiation treatment in food products<sup>3,52–55</sup> using different approaches. One approach relies on the acknowledgment that every unprocessed food product is associated with a given microbial load (usually bacteria). It is then possible to evaluate food irradiation *via* the quantification of microbial DNA present in the test product. For this, the highly conserved 16S rRNA gene can be used as a universal bacterial DNA sequence that will identify the presence of any bacteria contaminating the product. The 16S rRNA gene is present as multiple copies in the genome of most bacterial species but absent in animal, plant, viral, or fungal genomes.<sup>56</sup> The same can be applied to fungal genomes using the corresponding pan-fungal 18S rRNA gene. The presence of multiple copies of this target in the genome increases the assay sensitivity, but also introduces a bias in the correlation between the viability and *Ct*, as demonstrated by Trampuz and colleagues.<sup>56</sup> Alternatively, primers to highly conserved species-specific DNA target regions from bacteria closely associated with specific food materials can be used. *Vibrio vulnificus* has been successfully tested in clam tissue homogenates<sup>52–54</sup> and the virulence gene *hilD* from *Salmonella enterica* serovar Typhimurium in chicken breast.<sup>55</sup>

In this technique, DNA from food products is extracted and amplified with at least two primer pairs that target notably different-sized DNA sequences. One primer pair will target a long-sized sequence, which will be amplifiable only if non-degraded template DNA is present. The other one will target a small-sized sequence, which is present in both degraded and non-degraded DNA, hence indicating the approximate initial number of target cells subjected to irradiation. Lee and Levin<sup>52</sup> exposed a viable cell suspension (with a density of  $1.0 \times 10^6$  CFU mL<sup>-1</sup>) of *Vibrio vulnificus*, a pathogen usually associated with fishery products, to 0, 1, 3, and 5 kGy, and applied real-time PCR using species-specific primer pairs to obtain amplicons sized 1000, 700, and 70 bp. With a gamma radiation dose of 1 kGy or above, amplification of the 1000 bp sequence failed, showing the suitability of this sequence for the rapid detection of the irradiation destruction of *V. vulnificus*. The additional use of the primer pair for amplification of small sized amplicons (70 bp) was used as a control. Trampuz and colleagues<sup>56</sup> failed to establish a clear correlation between *Ct* and irradiation using a 528 bp target sequence in cell suspensions of *Staphylococcus aureus* and *Escherichia coli*. In a subsequent study by Lee and Levin<sup>54</sup> with *V. vulnificus* cells suspended in clam-tissue homogenate, a detection limit of  $10^3$  to  $10^5$  CFU g<sup>-1</sup> of clam tissue was reported. The detection of the destruction of less than  $10^3$  CFU g<sup>-1</sup> of tissue will depend primarily on the detection sensitivity of the real-time PCR assay

system. These are, however, conclusions from tissue homogenates and not from original food matrices.

Ethidium bromide monoazide (EMA) has allowed real-time PCR detection of viable bacterial pathogens in numerous food products.<sup>57</sup> EMA penetrates only membrane-damaged cells and cross-links double-stranded DNA, preventing its amplification and detection. The increased ability of EMA to further reduce the detectable number of target sequences *via* PCR with DNA from cells exposed to increased doses of radiation can be considered to reflect the accompanying increase in membrane damage, which allows EMA to penetrate the cells. Under such conditions, the inability to detect extensively degraded DNA *via* PCR can be taken as evidence of cell death. The effect of irradiation on *V. vulnificus* was examined by EMA real-time PCR for the first time by Lee and Levin.<sup>53</sup> This study was able to discriminate irradiation-destroyed cells from viable cells by real-time PCR in cell suspensions subjected to irradiation doses of 0.15 to 1 kGy. EMA inhibits the DNA fluorescence mediated by ethidium bromide<sup>58</sup> and it also reduces the real-time PCR fluorescence signal;<sup>59</sup> therefore, quantitative studies must be based on the standard curve generated with DNA derived from EMA-treated cells.

More recently, Sakalar and Mol<sup>3</sup> tested a different approach. Real-time PCR was applied as an irradiation detection technique directly in food tissue. The effects of gamma irradiation on the DNA were tested on fish (*Oncorhynchus mykiss*) by real-time PCR. Fish was exposed to gamma radiation doses in the range of 0.25–9 kGy. Primers were designed for regions with different lengths of both nuclear (18S rRNA gene) and mitochondrial (12 rRNA gene) DNA, and each primer was used to amplify the DNA from the irradiated samples. Irradiation was found to result in extensive reduction of the molecular size of DNA. Nuclear DNA was found to be more sensitive to the irradiation technique than mitochondrial DNA. One of the reasons could be the redundancy in the number of repetitions of the 18S rRNA gene.<sup>60</sup> In addition, nuclear DNA is longer than mitochondrial DNA.<sup>61</sup> The number of mitochondria and contained DNA vary from species to species, tissue to tissue, and cell to cell. The authors also found a significant correlation between DNA detection (amplicons) and the radiation dose applied, even after three months of storage. In this study, irradiated fish meat quantified by real-time PCR was confirmed by the CA method. As a consequence, a molecular methodology to analyze irradiated fish meat qualitatively and also for the estimation of administered doses was developed.

In a study by Trampuz and colleagues,<sup>56</sup> irradiation of DNA in viable bacterial cells, subsequently subjected to extraction, had less effect on amplifiable DNA than did irradiation of already extracted DNA, even at high radiation doses. In addition, standardized DNA extraction methods must be validated for each type of food matrix, since different methods and different matrices result in different amounts of extracted DNA,<sup>55</sup> as well as different DNA quality. Effects on the PCR amplification, such as contaminated DNA, matrix effects, quantity and quality of extracted DNA, physical and enzymatic degradation of DNA during storage, and improved understanding of the

dose–effect relationships, especially at low doses, require further investigation. Contrary to other methods such as CA, not enough studies have been developed to ascertain the validity of real-time PCR as an irradiation detection method in food. Even though the few existing studies foresee success, its sensitivity, precision, and specificity must be clearly defined by interlaboratory tests before real-time PCR can be validated.

### 15.2.1.3 *Measurement of Mitochondrial DNA Changes*

Generally, strong enzymatic degradation of genomic DNA occurring in fresh produce like meat and fish hinders the identification of DNA fragmentation specifically caused by irradiation. For instance, Sakalar and Mol<sup>3</sup> recently applied direct agarose electrophoresis to genomic DNA extracted from irradiated and non-irradiated fish meat. DNA derived from fish exposed to an irradiation range of 0 to 9 kGy exhibited a notable decrease in molecular weight and increased visible degradation with the increasing irradiation dose. However, no studies on irradiation specificity were applied, and enzymatic degradation could have also occurred.

Mitochondrial DNA (mtDNA) is thought to be protected from enzymatic reactions due to the presence of mitochondrial walls, but it is not protected from radiation. Based on this assumption, mtDNA breakage can be assumed as a radiation-specific change.<sup>25</sup> In foods of animal origin, mtDNA has low molecular weight (approximately 16 base pairs) and is normally in super-coiled forms, which after irradiation (2 to 4 kGy) relax into circular and then linear DNA.<sup>62</sup> These three forms can be separated by agarose gel electrophoresis and be used as irradiation detectors. In non-irradiated food, super-coiled mtDNA remains perfectly stable, even during storage of 25 days at 4 °C as well as during abrupt temperature changes (freezing at –20 °C and thawing at 20 °C). For plant products, the more complex and heavier DNA (200 to 250 Kb) makes the analysis more difficult.<sup>63</sup>

Although this method has been considered useful in meat analysis,<sup>62</sup> the process of mtDNA extraction is rather complex, which reduces its practical application. In addition, not enough studies have demonstrated its validity.

### 15.2.1.4 *Flow Cytometry*

Flow cytometry (FCM) has been rarely tested as a detection method for radiation-induced changes in DNA. Selvan and Thomas<sup>64</sup> used FCM to monitor changes in the DNA content of irradiated onion bulbs using a fluorescent dye (the fluorochrome 4,6-diamidino-2-phenylindole), which binds specifically to double strand regions. Since the amount of nucleic acids in the meristem tissues (inner buds) is higher than that in the storage parenchyma of onion bulbs, the irradiation effect on nucleic acids should be discernible in meristem tissue cells.<sup>64</sup> Nuclei from onions irradiated at low gamma doses (0.06 to 0.09 kGy) exhibited a broader DNA distribution profile, appearing as a high coefficient of variation ( $cv = 4.78\%$ ) of the  $G_0/G_1$

peak compared to non-irradiated samples ( $cv = 2.39\%$ ). The DNA index (DI) of the diploid cells in control onions was 1, against the 0.74 value of irradiated samples, indicative of the presence of  $G_0/G_1$  cells with abnormal DNA content in the meristem tissue cells of irradiated onions. These differences were detected even after 150 days storage at ambient conditions. These results indicate the potential of the FCM technique for the differentiation of irradiated and non-irradiated bulbs.

## 15.2.2 Measurement of Microbiological Changes

### 15.2.2.1 Shift in Microbial Load and Profile

Different microorganisms have different sensitivity to irradiation, Gram-negative bacteria (GNB) being much more sensitive than Gram-positive bacteria and yeasts. For this reason, selective destruction of the first ones is expected in food irradiation. Studies have been carried out on fruits, vegetable products, and raw poultry meat. With raw poultry meat, a characteristic microbiological profile is generally seen with significant numbers of Gram negative bacteria, predominantly of the genus *Pseudomonas*. In contrast, the microflora of raw chicken after irradiation at a dose of 2.5 kGy mostly consists of Gram-positive bacteria and yeasts.<sup>25</sup> For strawberries, the initial microflora mostly of *Pseudomonas* was completely removed after irradiation at 2 kGy.<sup>65</sup> Nevertheless, this method has considerable disadvantages as it is very dependent on the initial microbial load, which varies regionally and with agronomic practices (*e.g.*, traditional cultivation *versus* greenhouse cultivation). Thus, data obtained for a particular food under specific conditions may not be valid for another food, or even the same food obtained under different conditions.

### 15.2.2.2 Direct Epifluorescent Filter Technique Combined with Aerobic Plate Count (DEFT/APC)

This method is based on the combined use of the total cell count by the direct epifluorescent filter technique (DEFT) and the viable cell count by the conventional aerobic plate count (APC) method. The APC indicates the number of microorganisms present in the sample at the time of analysis capable of growth under the culture conditions used. The DEFT count is the total number of microorganisms, both viable and non-viable, that have ever been present in the sample.<sup>66</sup> For non-irradiated samples, DEFT counts are in line with those obtained by APC. If the APC value is found to be considerably smaller than that obtained by DEFT, it indicates that the sample may have been irradiated.

DEFT is a method originally developed for the rapid enumeration of microorganisms in raw milk samples,<sup>67</sup> and it has been used for the detection on several foodstuffs, such as spices, beans, poultry, meat, and minimally processed vegetables.<sup>66,68–75</sup> In this method, a specified volume of

the sample is passed through a membrane filter to concentrate the microorganisms on the filter. The microorganisms are then stained with the fluorochrome acridine orange. After staining, the membrane is rinsed and mounted on a microscope slide. The microorganisms in the filter result in orange and orange-yellow fluorescence when submitted to illumination with blue light at 450–490 nm, and are easily counted using an epifluorescence microscope to give the DEFT count. The complete procedure can take as little as 30 min.<sup>76</sup>

APC is determined from another portion of the same test sample. It results from the standardized method universally used for counting viable cells from food samples, where samples are serially diluted and plated in nutrient agar (usually Plate Count Agar, PCA).

Oh and colleagues<sup>75</sup> applied doses up to 10 kGy to spices. The log DEFT/APC ratios of non-irradiated and irradiated samples with 1.0 kGy were 1.14 and 2.38, respectively, with the log DEFT/APC ratio increasing with the dose. In general, spices may contain initial microbial levels of  $10^5$ – $10^8$  before application of any hygiene treatment. If the foodstuffs are irradiated, the level of viable microorganisms generally decreases to below  $10^4$ . Samples of minimally processed lettuce, chard, watercress, escarole, chicory, spinach, and cabbage were tested immediately after irradiation.<sup>76</sup> All the studied vegetables showed similar DEFT counts despite the irradiation treatment; however, the APC showed a negative correlation with the radiation dose. Even at the lowest radiation dose tested, 0.5 kGy, the viable count (log APC) was reduced by approximately two log units, while the DEFT count remained at the same level.<sup>76</sup> Research carried out on cereal grains and beans<sup>73,74</sup> found a log DEFT/APC ratio between 2.0 and 3.0 for doses of 0.5 kGy or more. Wirtanen and colleagues<sup>69</sup> applied the DEFT/APC method to assess the possible irradiation treatment of samples of frozen poultry meat and, using a ratio level of 2.0 as the threshold, successfully identified poultry meat that had been irradiated at doses of 3, 5, and 7 kGy.

As a result of the abovementioned studies, a log DEFT/APC ratio of 2.0 has been suggested as a threshold criterion for sample irradiation at doses of 0.5 kGy or higher. Nonetheless, this method has limitations when there are too few microbes in the sample ( $\text{APC} < 10^3 \text{ CFU g}^{-1}$ ) as the log DEFT/APC ratio can vary with the degree of initial contamination<sup>69,76</sup> and, for that reason, the suggested log DEFT/APC ratio should not be an absolute criterion. In addition, similar differences between DEFT and APC values can be induced by other food treatments leading to the death of microorganisms, such as heat, preservatives, or storage. Some spices such as cloves, cinnamon, garlic, and mustards contain inhibitory components with an antimicrobial activity that may lead to decreasing APCs (false positives), and because of this the threshold for screening irradiation in herbs and spices may be increased. Wirtanen and colleagues<sup>69</sup> reported some differences in the application of the method for spices and poultry meat, because of the characteristic high fat and protein content of meat interfering with the filtration process. For the analysis of meat products, the authors also argued

that the conditions of the sample material are of utmost importance. When using this method, poultry meat or carcasses should be irradiated in a deep frozen state (below  $-20^{\circ}\text{C}$ ) or should be frozen immediately after irradiation. Furthermore, they find it mandatory that samples should be kept frozen from the end of production until analysis. Despite the deep frozen state, microbial levels of samples may be somewhat higher after a storage period of a few months. The resulting higher loads of living microbes give rise to smaller differences between the DEFT and APC assessments and lower apparent levels of irradiation.<sup>69</sup> An advantage of the microbial method is that it provides additional information on the hygienic quality of the food.<sup>77</sup>

The DEFT/APC method is specified in EN 13783:2001 as a screening method for the detection of irradiation treatment of herbs and spices, where a threshold criterion for irradiation of 3 to 4 is recommended. The method has been successfully tested in interlaboratory tests with herbs and spices,<sup>66</sup> but positive results must be confirmed using a standardized method to specifically prove irradiation of the suspected food.

#### 15.2.2.3 *Reduced Viable Gram-negative Bacteria: Limulus Amoebocyte Lysate Test Combined with Gram-negative Bacteria Count (LAL/GNB)*

A microbiological method comprising the Limulus Amoebocyte Lysate (LAL) test in conjunction with a Gram-negative bacterial (GNB) plate count has been proposed by Scotter and colleagues<sup>78–80</sup> as a screening method for the presumptive detection of radiation treatments. When large numbers of GNB are present in a sample, a high LAL titer will be obtained, and *vice versa*. However, when a high LAL titer is detected in the absence of the corresponding high GNB load, it is indicative of high numbers of dead cells. In an irradiated food matrix, it is assumed that GNB are easily inactivated, while the bacterial endotoxin present on their surface as lipopolysaccharides (the LPS layer) are not destroyed by the treatment. The number of viable GNB present at the moment of analysis is determined by the GNB plate count test, while the concentration of bacterial endotoxin (which reveals the total number of GNB in the product before treatment) is set by the LAL counterpart.<sup>25</sup> If the difference between the GNB count and LAL titer is high, it is assumed that the sample was treated by a method of preservation, possibly by irradiation. Scotter and colleagues<sup>80</sup> applied this test to both irradiated and non-irradiated samples of chicken pieces, and found a lower GNB count in samples irradiated at 2.5 kGy, while no toxin differences were observed between the two sets of samples.

The LAL/GNB method is specified in EN 14569:2004 as a microbiological screening method through the identification of unusual microbiological profiles and is applicable to poultry meat (*e.g.*, breast, legs, and wings of fresh, chilled, or frozen carcasses with or without skin). This screening method has been successfully tested in interlaboratory trials;<sup>79,80</sup> however,



since high levels of bacterial inactivation can arise from several reasons, it is recommended that a positive result is confirmed using a standardized reference method for the detection of irradiated food.

#### 15.2.2.4 Bacterial Spoilage Profiles

Some decades ago, several researchers proposed that bacterial spoilage profiles could potentially be used as a tool to identify irradiated flesh foods, namely seafood and meat.<sup>81–83</sup> This is based on the premise that irradiated foods are less susceptible to bacterial spoilage than non-irradiated ones. In this method, irradiated and non-irradiated (control) foods are inoculated with known amounts of one or a mix of bacterial species (e.g., *Aeromonas hydrophila*, *Salmonella* Typhimurium, *Bacillus megaterium*, and *Pseudomonas marinoglutinosa*) and incubated for some hours to allow bacterial growth.<sup>81,82</sup> The ability of bacteria to cause spoilage is determined by measuring the generation of total volatile acids (TVAs) and total volatile basic nitrogen (TVBN). While bacteria maintain the ability to grow in both treated and non-treated food matrices, their metabolism will generate different spoilage profiles.

The effects of low gamma irradiation doses (0 to 5 kGy) on fish products (Bombay duck, Indian mackerel, white pomfret, seer, and shrimp) on the spoilage potential of several bacteria (*Aeromonas hydrophila*, *Salmonella* Typhimurium, *Bacillus megaterium*, and *Pseudomonas marinoglutinosa*) and mixed flora were examined by Alur and colleagues<sup>81</sup> in terms of their ability to proliferate in radurized fish and to produce TVAs and TVBN. The researchers concluded that bacteria proliferated well in both non-irradiated and irradiated fish, but the formation of TVAs and TVBN was significantly lower in the latter (30 to 50% those of the non-irradiated controls). Later on, Alur and colleagues<sup>82</sup> applied a similar method to meat products. Beef, chicken, mutton, and pork were exposed to gamma-radiation doses up to 5 kGy and then inoculated with *Aeromonas hydrophila* after 7 days and 15 days of storage at 3 °C and –11 °C. After 18 h of incubation at 30 °C or 6–7 h at 37 °C, the TVA and TVBN values of irradiated samples were found to be 40–50% lower than those found in non-irradiated samples.

In a different study, samples of non-irradiated and irradiated (5 kGy) dried anchovies (*Engraulis encrasicolus*) were transported from Korea to India.<sup>83</sup> The non-irradiated anchovies showed mold growth and increased total bacterial counts by three log cycles over the initial load, after four months of storage at 25 °C. However, 5 kGy irradiated samples exhibited 10<sup>2</sup> bacterial cells per gram even after six months of storage. The differences in the levels of TVBN correlated to irradiated and non-irradiated samples.

This method seems to correlate well with irradiated food, but these tests were applied more than two decades ago and, to our knowledge, no reports exist on more recent applications. Updated tests using current state-of-the-art techniques such as gas chromatography (either linked to mass

spectrometry or not) or reflectance spectroscopy are needed to confirm its use as an irradiation screening method.

### 15.2.3 Measurement of Histological and Morphological Changes: Germination and Half-embryo Tests

It is now fully accepted that ionizing radiation introduces metabolic disorders in the seeds and irreversibly affects the viability of the germ or embryo, probably due to effects caused by the free radicals generated by irradiation.<sup>84</sup> The consequences to these disorders are a significant delay or even full inhibition of seed germination, as well as an abnormal root and shoot growth. Based on these changes, a germination test was proposed for the differentiation of irradiated and non-irradiated vegetable commodities. In this test, seeds are generally soaked for a number of hours in distilled water and then placed on a distilled water-moistened absorbent cotton layer and cultured at around 28 °C in a plant growth chamber. Germination percentages, as well as root and shoot growth (in length), are measured periodically for one to two weeks, depending on the type of seed. The parameter 50% inhibition dose rate (IDR50) can be used as a measure of the radiosensitivity. IDR50 is the amount of radiation that reduces the root length to 50% that of non-irradiated seeds.<sup>85</sup> Germination tests have been successfully used for the detection of irradiated cereal grains and legumes.<sup>85–89</sup> This simple and cheap test was shown to be able to discriminate between all the irradiated and non-irradiated tested seeds, and does not require trained technicians or expensive equipment; however, it is time-consuming, as at least 4–6 days are needed for seed germination.

Kawamura and colleagues<sup>90</sup> developed an improved germination test known as the 'half-embryo test' for the rapid detection of irradiated grapefruit and other fruits. In this test, seeds are removed from the fruit and half-embryos, consisting of one cotyledon and embryo axis, are dissected from the surrounding tissue. Non-irradiated half-embryos thus germinate faster than intact or partially dissected (outer seed coat removed) seeds. In a follow-up study,<sup>91</sup> the half-embryo test was optimized to reduce the incubation period needed for germination. The duration of the half-embryo test used for identification of gamma-irradiated grapefruit was shortened by increasing the germination temperature to 35 °C, and maximum shooting percentages were reached within three days. At a dose of 0.15 kGy, radiation treatment could be detected within 2 to 4 days. Application of the phyto-hormone gibberellin further allowed the reduction of the incubation time to two days. Half-embryos extracted from irradiated orange and lemon gave similar results to those of grapefruit. This half-embryo test was thus proposed as an identification method for irradiated citrus, where radiation assessment could be made after 3 to 4 days using shooting percentages greater than 50%. Shoot elongation was also quicker, occurring within six days. In this test, irradiated half-embryos showed markedly reduced root growth, and



shoot elongation was almost totally retarded. Differences between irradiated and non-irradiated half-embryos were not affected when the variety, harvest date, and fruit storage conditions varied. Chaudhuri<sup>85</sup> also reported a similar standardized germination and seedling test for the identification of irradiated lentil seeds. Based on the germination efficiency and root/shoot lengths, gamma irradiated pulse seeds could be easily identified at the critical dose range of 0.1–0.5 kGy, even in seeds stored for 12 months after irradiation.

A collaborative study used the half-embryo test for the detection of irradiated citrus fruit.<sup>92</sup> Seeds were removed from fruits and incubated at 35 °C for several days. Shooting of less than 50% of the seeds after 4 or 7 days of incubation was taken as indicative of irradiation. Samples irradiated at 0.2 and 0.5 kGy were easily identified. Khawar and colleagues<sup>93</sup> tested the applicability of the germination test to distinguish non-irradiated and irradiated samples of wheat, maize, chickpea, and black eye beans. Samples were gamma-irradiated to absorbed doses up to 10 kGy. In all the irradiated samples, root and shoot lengths decreased with the increasing radiation absorbed doses, and germination was fully inhibited in all seeds irradiated at absorbed doses higher than 2 kGy. Barros and colleagues,<sup>29</sup> however, applied the germination test to wheat seeds irradiated with doses up to 2 kGy, and found a high coefficient of variation, indicating low accuracy experiments. In addition, in a study by Marín-Huachaca and colleagues,<sup>36</sup> melon seeds were irradiated with doses of 0.5 and 0.75 kGy and, on the first day after incubation, both irradiated and non-irradiated samples reached 100% germination. In watermelon, on the second day of incubation, all irradiated half-embryos up to 0.75 kGy germinated, whereas the germination percentage of the samples irradiated at 1.0 kGy was 92%. Clear differences between irradiated and non-irradiated samples were observed only in root growth from the second and third days after incubation for melon and watermelon, respectively. The roots of irradiated samples were markedly reduced and very limited secondary root elongation was observed. In this study, root elongation inhibition showed to be a better differentiating parameter than germination. In a half-embryo test applied to citrus seeds, Marín-Huachaca and colleagues<sup>34</sup> reported that shoot elongation and root growth were markedly inhibited at 0.5 kGy doses, particularly for oranges and lemons, but no dose-dependent estimation could be established, since samples irradiated at doses at 0.5 kGy or higher showed similar levels of germination retardation.

One of the major advantages of the germination test over physical and chemical methods, and even over most of the other biological methods, is that it is capable of detecting irradiation doses as low as 0.025 kGy, such as those used on onions, garlic, and potatoes for sprouting control during storage.<sup>94</sup> Selvan and Thomas<sup>94</sup> evaluated the rooting characteristics and rate of root elongation in onions and shallots irradiated with up to 0.15 kGy, and also compared the morphology of the roots in onions that had been subjected to pre-harvest spraying with maleic hydrazide for sprout

inhibition. They found a highly significant difference in root number and root elongation between the control and irradiated bulbs, with root length measurement being a better method for discriminating between them. Their results also indicated that maleic hydrazide-treated onions showed root growth similar to that of non-irradiated onions, hence showing the possibility to discriminate irradiated onions from chemically treated ones. Cutrubinis and colleagues<sup>49</sup> tested the germination test on irradiated garlic. The results showed that the germination test was reliable as a detection method even for samples treated with 0.025 kGy, but only during the dormancy period.

Sprout inhibition of potatoes by irradiation is irreversible and may serve as proof of irradiation, but the method is too slow for routine analysis, even if growth hormones are used to accelerate sprouting.<sup>77</sup>

### 15.3 Conclusions

It is well established that gamma irradiation causes biological changes in foods and their ingredients. The major cellular target of ionizing radiation is DNA, as it is reported that 1 Gy may introduce up to 1000 DNA breaks. This degradation is easily detected by different methodologies, but it is mainly used for the qualitative screening of irradiation, and only in a few cases for radiation dose estimation.

In food products, irradiation will affect the DNA of the food itself, as well as the DNA of other living organisms present on the food surface or mixed with it. Current methodologies are able to screen for DNA changes in either one of these two targets, and since different microorganisms have different sensitivity to irradiation, changes in the surviving microbiota can also be used for irradiation screening. The most commonly used biological methods for the detection of irradiated foods are Direct Epifluorescent Filter Technique/Aerobic Plate Count (DEFT/APC), DNA comet assay, and *Limulus* Amebocyte Lysate (LAL) test, which have been established as European Norms.

However, DNA damage by irradiation is not specific, and many other food-processing operations give rise to the same effects. In addition, validation of normalized biological methods is still limited to specific types of foods, and application to a broader range of matrices still lacks validation. For this reason, biological methods are being used just for screening, and need subsequent confirmation by standard chemical or physical methods.

As DNA knowledge and technology evolves, it is envisioned that DNA-based methods (namely real-time PCR and flow cytometry), although not yet fully explored, will be developed and/or further tested and validated as potential highly specific quantitative methods of irradiation detection for various matrices and processing conditions, without the need for further confirmation. Validation of quantitative biological methods is also needed to determine compliance with irradiation authorized doses.

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