AM19002 Building Capacity in Irradiation

Literature review – Panorama of detection methods available or under development for testing and confirming exposure to phytosanitary irradiation

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Contents

EXECUTIVE SUMMARY

This report summarizes the results of a literature review carried out to assess the availability of state-of- the-art detection methods for their ability to detect and to validate irradiation treatment of fruits and vegetables at doses used for phytosanitary purposes. The information gathered has revealed that none of the considered methods, whether standardized, developed or under development, fulfills the requirements to either confirm the exposure to ionizing radiation or to validate that the correct phytosanitary dose has been used.

Currently, a set of well-established procedures are conducted within the irradiation facility to measure the dose delivered throughout the product to a high degree of accuracy. These procedures, backed by accreditation of the facility by regulatory authorities, are used as part of a certification system that is recognized by the International Plant Protection Commission to assure importing countries that any pest risk has been managed. The availability of a post-irradiation detection method that meets the criteria outlined below could act as an extra insurance for border control officials in the importing country.

Post-irradiation detection of phytosanitary irradiation treatment requires the application of analytical techniques. Ideally, such techniques must be accurate, applicable to a wide range of commodities and pests, and sensitive enough to allow the detection of irradiation at low doses (less than 1 kGy). The measured test parameter should be able to clearly identify if the commodity has been irradiated at any point during its storage life, without requiring a non-irradiated sample for comparison. Moreover, it would be optimal if the test could also fulfil practical criteria, in line with the expertise and means necessary for its implementation.

None of the standardized methods, nor the experimental or emerging ones described in the literature, can fully meet these criteria. Indeed, each of the techniques/methods reviewed has limitations in line with one or more of the criteria listed below:

- **Technical criterion**: Method Performance (accuracy, sensitivity threshold detection), specificity of the marker to irradiation, stability of the marker throughout the lifetime of the products and the extent of application domain (fruit and vegetables or pests)
- **Practical criteria**: ease of implementation, level of expertise required, cost and speed
- **Market accessibility**: maturity of the method (validated, tested in several laboratories, ready for collaborative testing), equipment/techniques availability.

The literature review has highlighted the advantages and the limits of the reviewed methods, and on this basis, their classification into three groups. The first group includes the methods considered inappropriate mainly because of the inconsistency of the results and the lack of sensitivity, as well as the inadequacy of the implementation conditions for use in quality control. The second group includes methods that are potentially sensitive but cannot be used as confirmatory methods, but rather as screening methods. The third group includes mature methods, or methods with a promising concept, both of which require development and optimization work. This classification was done for each of the application areas, i.e., commodity or pest.

This literature review was conducted as part of project AM19002 - Building Capacity in Irradiation, the objectives of this project are to:

- Build a body of knowledge concerning phytosanitary irradiation for the Australian horticulture sector, government and our international trading partners
- Fill gaps in our knowledge regarding the effective use of phytosanitary irradiation
- Identify future research and development activities that will increase the use and acceptance of phytosanitary irradiation domestically and internationally.

The next step taken by the project will be to discuss and enrich the results of this literature review with international experts in order to prepare a roadmap forward that seeks to answer the questions raised.

1. INTRODUCTION

Irradiation has several advantages over traditional phytosanitary treatments such as fumigation and other chemical and physical (heat/cold/modified atmosphere) treatments (Follett 2009, Follett et al. 2011; Hallman 2011). Numerous countries use irradiation to disinfest fruit and vegetables from a multitude of regulated pests (Hallman 2011; Hallman *et al.* 2011). Assessment of whether exported or imported fruit has been irradiated to a dose that meets an agreed treatment for pest management includes accurate dose qualification measurements within the irradiation facility. However, regulated pests may be found alive, but sterile, during inspections prior to export or on import. The availability of a reliable test to retrospectively confirm radiation exposure can increase market confidence in a situation where live pests are detected.

The purpose of this desk top review is to identify detection methods and to evaluate their ability to detect irradiation treatment of fruits and vegetables at doses used for phytosanitary purposes. To this end, current knowledge of analytical methods, techniques and indicators developed, internationally standardized or being developed in the framework of other research projects are assessed.

This document reports on:

- the methodology used to gather the information available in the scientific literature
- the critical review of the current standards with regards to their applicability to detect fruits and vegetables irradiated at low doses (lower than 1.0 kGy)
- the critical review of the methods being tested for the detection of phytosanitary irradiation treatment
- a classification attempt of the reviewed methods according to their relevance to detect irradiation phytosanitary treatment.

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2. METHODOLOGY

The methodology of the bibliographic research was to define key words and then to launch queries on different search engines (Sci Finder, Google Scholar and PubMed).

The keywords searched were as follows:

(1) "food irradiation"

(2) "detection method" "irradiated food"

(3) "food irradiation" "phytosanitary treatment"

(4) "detection method" "irradiated food" "phytosanitary treatment"

(5) "irradiation" "phytosanitary" "treatment"

(6) "detection" "method" "irradiation" "phytosanitary" "treatment"

- 2 283, 186 000 and 6 060 references were found containing keywords (1) on Sci Finder, Google Scholar and PubMed respectively.
- 518, 29 700 and 565 references were found containing keywords (2) on Sci Finder, Google Scholar and PubMed respectively.
- 12, 2 870 and 28 references were found containing keywords (3) on Sci Finder, Google Scholar and PubMed respectively.
- 3 050 and 1 references were found containing keywords (4) on Google Scholar and PubMed respectively. No references were found containing all the concepts on Sci Finder.
- 452 references were found containing keywords (5) on Google Scholar.
- 197 references were found containing keywords (6) on Google Scholar.

Among all the references found by the search engines, we selected the most relevant, i.e., those that contained the most searched keywords, and whose content was related to our study subject. We have also kept track of the relevant references cited in the selected texts.

In order to classify and provide a hierarchy to our findings, we considered the following information: application domain of the technique (product and/or pest), technique used, specificity of the technique (is this specific to irradiation?), specificity of the markers, performance (detection threshold), maturity of the method (validation completed, in progress or experimental method), ease of implementation, other advantages and drawbacks of the methods.

3. DESCRIPTION OF THE MAIN RESULTS ACHIEVED

The analysis was first carried out on standardized detection methods and their applicability to fruits and vegetables irradiated at low doses. A second analysis (see figure 1) was carried out to identify experimental and emerging methods. It is worthy to note that the selected publications deal with detection methods applied to insects and/or products, but none dealing with detection methods applied to the packaging was found.

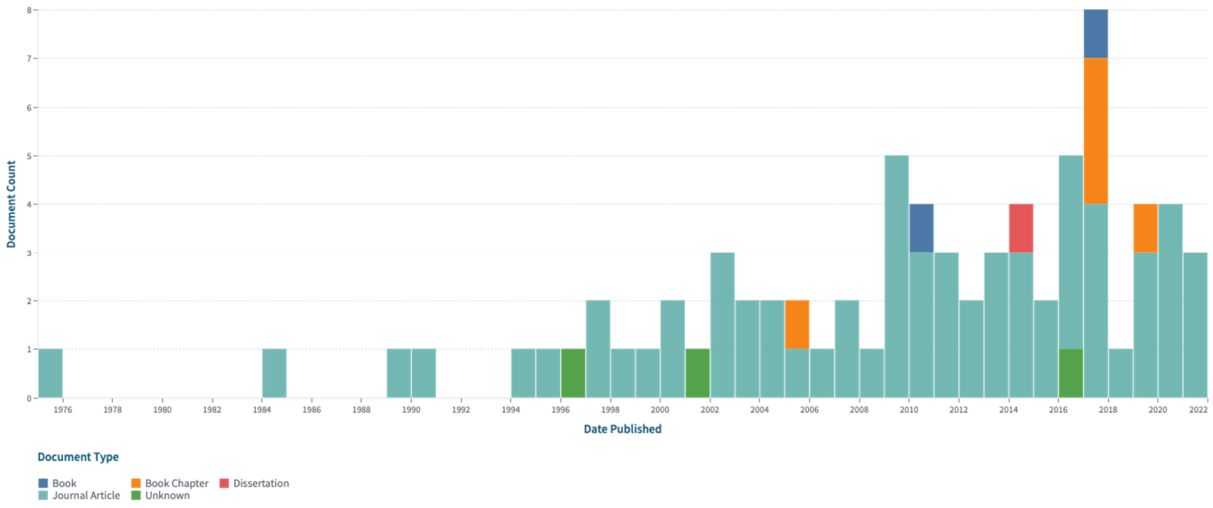

Figure 1. Aerial Curated Data Statistics: Yearly publications (sum = 77)

Among all the publications detected by the search engines, less than a hundred were found to be relevant. For scientific questions as precise as the detection of phytosanitary treatment by irradiation, we observed that less than 5 publications of interest came out per year, even though the average number of scientific works seems to grow from decade to decade,. The publications selected and presented in this report are therefore rich in information and we tried to exploit them to the fullest.

The word cloud presented in the figure 2 allows us to visualize the themes most often mentioned in these publications. Thus, we can immediately notice the prevalence of the keyword “DNA”, which seems to be a focus point of the search for irradiation traces. The term "food irradiation" appears 24 times against 35 times for "irradiation", hinting that the research concentrates on the irradiated product, and less on the pests.

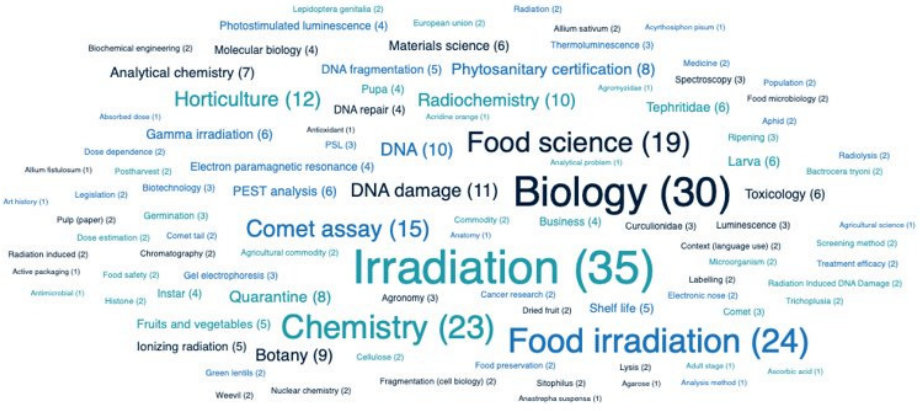

Figure 2. Aerial Curated Data Statistics: Top Fields of Study Word Cloud

Another interesting visualization of the data is the mapping of the most active countries with citations (figure 3). This is helpful to understand which regions of the world are concerned by phytosanitary problems and use irradiation to remedy them. Australia is often quoted, with Pakistan, India, South Korea, and China in Asia; the USA, Mexico and Brazil for the Americas; Italy is one of the most active countries in Western Europe, while Turkey and Egypt are active in the Middle East.

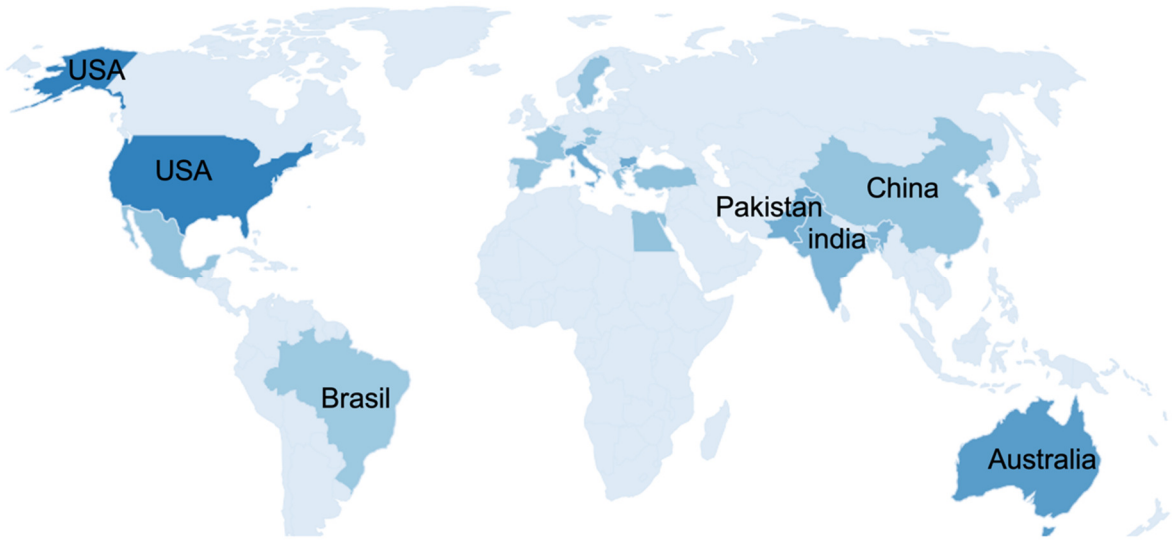

Figure 3. Aerial Curated Data Statistics: Most Actives Countries with Citations (the intensity of the blue is positively correlated to the number of citations)

3.1 Review of the current standards with regards to their applicability to detect fruit and vegetables irradiated at low doses

There has been considerable research since the late 1980s in developing and validating a series of reliable detection methods that can be used to distinguish irradiated from non-irradiated foods. No fewer than fifteen analytical methods for the detection of irradiated food were developed, of which ten were standardized by the European Committee for Standardization (CEN) (Marchioni, 2006).

Detection methods are focused on changes in chemical composition, physical or biological changes that occur specifically during food irradiation. A single analytical method to be used to control all types of foods is not currently available, but useful detection methods are developed for specific foods. However, in some cases the distinction between irradiated and non-irradiated foods is still an unsolved analytical problem.

Six of the EU standardized methods are reference methods and are based on the analysis of primary radiolytic products by thermoluminescence validated for food containing silicate minerals (EN 1788 2001); by electron spin resonance (ESR) spectroscopy validated for food containing cellulose (EN 1787 2001), bones (EN 1786 1996), and sugars (EN 13708 2001); and on the analysis of secondary radiolytic products from fatty acids, namely, hydrocarbons (EN 1784 1996) and 2-ACBs (EN 1785 2003).

Four others are less specific than the reference methods, namely: EN 13751 (2009) using photo stimulated luminescence; EN 13783 (2001) based on the direct epifluorescence filter technique/aerobic plate count, EN 14569 (2004) related to the determination of Limulus amoebocyte lysate/Gram-negative bacteria and EN 13784 (2001) implementing DNA Comet test or single gel microelectrophoresis. These methods are used as screening methods to establish a suspicion of irradiation treatment.

The EU methods were adopted by the Codex Alimentarius Commission as General Methods and referred to in the Codex General Standard for Irradiated Foods (Parlato *et al,* 2014). The European Standardized detection methods are reported in table 1 grouped in physical, chemical, and biological methods.

Table 1. European Standardized detection methods

Method classification	Method description	
Physical	EN 1786: 1996	Detection of irradiated food containing bone - Method by ESR spectroscopy
	EN 1787: 2000	Foodstuffs - Detection of irradiated food containing cellulose by ESR spectroscopy
	EN 13708: 2002	Detection of irradiated food containing sugar by ESR spectroscopy
	EN 1788: 2001	Thermoluminescence detection of irradiated food from which silicate materials can be isolated
	EN 13751: 2009	Detection of irradiated food using photo-stimulated luminescence
Chemical	EN 1785: 2003	Detection of irradiated food containing fat - Gas chromatographic analysis of 2-alkylcyclobutanones"
	EN 1784: 2003	Detection of irradiated food containing fat - Gas chromatography of hydrocarbons
Biological	EN 13783: 2002	Detection of irradiated food using Direct EpiFluorescent Filter Technique/Aerobic Plate Count (DEFT/APC)
	EN 13784: 2002	DNA comet assay for the detection of irradiated foodstuffs – Screening method
	EN 14569: 2004	Microbiological screening for irradiated food using LAL/GNB procedures

Several literature reviews reporting on the detection of irradiated food generally were already published (Hasselmann and Marchioni 1991; Delincée 1991; McMurray et al, 1996; Marchioni, 2006; Chauhan et al, 2008). It is established that several methods are available to detect irradiation treatments of most foods treated at doses above approximately 1 kGy. In the following sections of this report, the literature review will be limited to detection methods that were implemented on fruits and vegetables (F&V) irradiated at low doses (1 kGy or less).

3.1.1 European Standardized irradiation detection methods- physical methods

a) Electron Paramagnetic Resonance Spectroscopy (ESR) methods are the base for European standards EN 1787: 2001 Foodstuffs - Detection of irradiated food containing cellulose by ESR spectroscopy and EN 13708: 2002 Foodstuffs - Detection of irradiated food containing sugar by ESR spectroscopy. These detection methods involve detection of free radicals containing unpaired electrons, which are paramagnetic in an applied external magnetic field. The following paragraphs describe the two methods in more details highlighting their advantages and limitations as well as feedback on their use to detect irradiated F&V at low doses.

- **EN 1787: 2001 Foodstuffs - Detection of irradiated food containing cellulose by ESR spectroscopy**. Radiation treatment produces specific radicals that can be mostly detected in solid and dry parts of the foodstuff. The intensity of the signal obtained increases with the concentration of the paramagnetic compounds and thus with the applied dose. A single central signal (C), with approximately gs = 2.004 is observed in the ESR spectra of all foodstuffs containing cellulose, including unirradiated samples. In the case of irradiated samples, the intensity of this signal is usually much greater than that of non-irradiated samples and, a pair of lines appears at the left (at lower magnetic field) and right (at higher magnetic field) of the central signal. This pair of lines is due to cellulose radicals formed by the ionizing radiation. The spacing of this radiation-induced signal pair is 6.05 mT ± 0.05 mT and is symptomatic of radiation treatment having taken place (Figure 4).

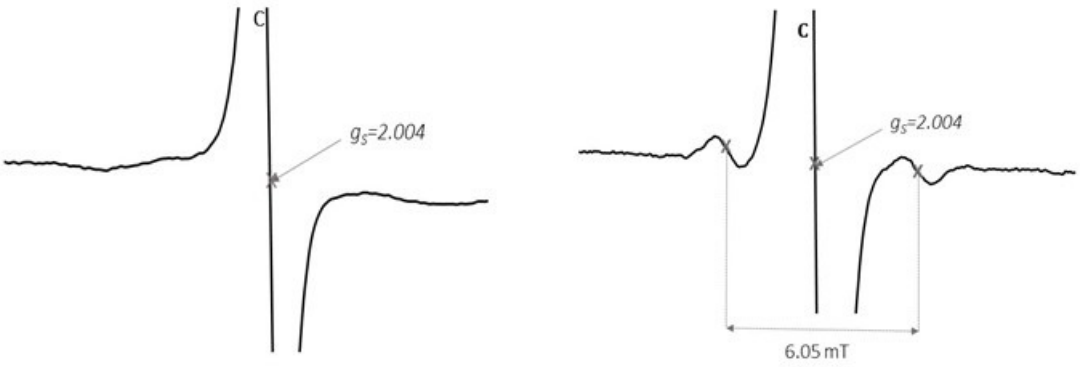

Figure 4. ESR spectrum of seeds from (a) unirradiated and (b) irradiated strawberries

ESR technique is specific and combines simplicity with rapid measurement. The availability of desktop ESR spectrometers reduces the cost of equipment, but this is still substantial. CEN standard EN 1787, relies on the formation of cellulose radicals upon irradiation if the content of crystalline cellulose in the food is adequate and moisture content is low enough, typical `cellulosic' radicals can be detected by ESR. A limitation of the ESR cellulose method is that positive identification of the cellulose radicals is evidence of irradiation, but absence of the signal does not constitute evidence that the sample is not irradiated. Another limitation of the technique is that the lifetime of the radicals is more stable in solid, dry food or foodstuffs with lower water content (Stefanova *et al*, 2010).

Detection limits and stability are influenced by the crystalline cellulose content and the moisture content of the samples. If dose levels are lower than 1.0 kGy, as is the case for the irradiation of some fruit and vegetables, difficulties may arise in detecting the cellulosic radical. Detection of irradiated fresh strawberries has been validated for doses of 1.5 kGy and above. Whereas detection of irradiated berries has been assessed for doses of 0.5 kGy and above. Detection is typically limited to about the first 3 weeks after treatment. Stability of cellulose radicals in berries depends on storage conditions and can be shorter than the shelf-life of the products (EN 1787:2001).

The continuous improvement of ESR techniques would allow gain in detection sensitivity. In addition, a number of studies proposed sample pre-treatments (freeze-drying, alcoholic extraction, and nitric acid extraction) to overcome the major drawback of this technique, namely the instability of the relatively weak radiation-specific signals (zanardi, 2017). Jesus *et al,* (2000) show that detection sensitivity can be improved for the soft tissue of fruit by alcoholic extraction to remove water and other constituents that can affect the specific ESR signal. By this method, irradiation can be detected at doses as low as 0.1 kGy; a fair estimation of the applied dose can be obtained by the additive dose method (Jesus *et al,* 1999). The method was also successfully tested on fruit juice [Aleksieva *et al,* 2014), and fresh vegetables [Kwak *et al,* 2014).

- **EN 13708: 2002 Foodstuffs - Detection of irradiated food containing sugar by ESR spectroscopy**.

Irradiated foodstuff containing crystalline sugar show typical multicomponent ESR spectra reflecting the presence of radiation-induced radicals in the sample. Dried fruits often contain sugar particles in crystalline form, and therefore the appearance of a typical multicomponent ESR spectrum indicates radiation treatment (Figure 5). Due to different mono- and disaccharides and due to the changes in saccharide composition, various ESR spectrum types can occur.

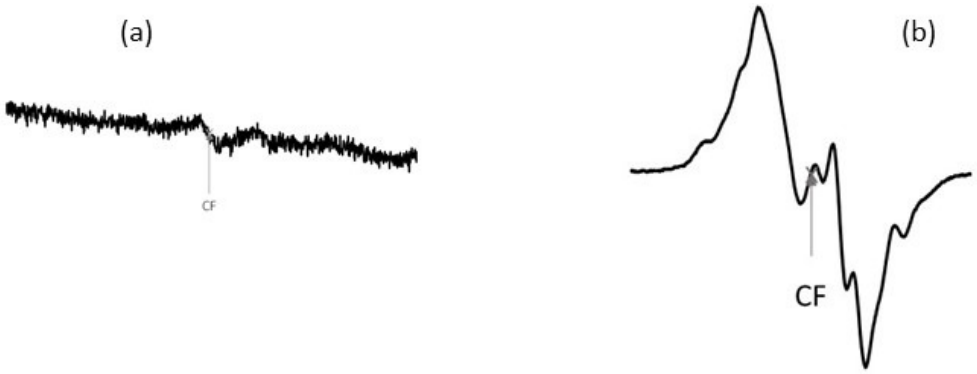

Figure 5 —Typical ESR spectrum of unirradiated (a) and irradiated (b) dried mangoes

EN 13708 allows unequivocal identification of irradiated samples (e.g mangos, papayas, figs, raisins). Multicomponent ESR spectra prove irradiation, but the absence of the specific spectrum does not constitute evidence that the sample is not irradiated. If no sugar crystals are present in the sample, irradiation will not produce specific ESR signals. Detection of irradiated dried figs, dried mangoes, dried papayas, and raisins has been validated by inter-laboratory tests (EN 17308:2002). The limit of detection as reported in this standard method mainly depends on the crystallinity of the sugar in the sample. Detection of irradiation treatment is not significantly influenced by storage of at least several months.

b) Luminescence methods are based on the stimulation, either thermally or optically, of minerals that have been previously exposed to ionizing radiation. During irradiation, minerals electrons absorb a part of the energy of irradiation and are placed in an energy state known as excited. Instead of immediately emitting a photon to stabilize at the fundamental state, the electrons are maintained during a long-term in a metastable state of intermediate energy.

The de-energizing of these electrons can be stimulated by subjecting the sample to stimulation either thermally or optically. This de-energizing causes a return to the fundamental state characterized by a release of energy in the form of luminous photons. Thus, the light emission is measured thanks to 2 techniques: Thermoluminescence (TL) and Photo Stimulated Luminescence (PSL).

- **Thermoluminescence (TL)** is the base for European standard EN 1788:2001. The silicate minerals isolated from the sample, in a sufficient amount, are thermally stimulated and electron–hole pairs induced by the radiation and trapped in the minerals, are released. This results in a recombination and in an emission of light that is measured as a function of temperature. The signal is compared with the re-irradiated minerals at 1.0 kGy approx. (standardization curve) and if the ratio is higher than 0.1 the sample is considered irradiated (Figure 6). A prerequisite of the calculation of the TL glow ratio is that the area of Glow 2 evaluated over the defined temperature interval is 10 times higher than the Minimum Detectable integrated TL intensity Level (MDL).

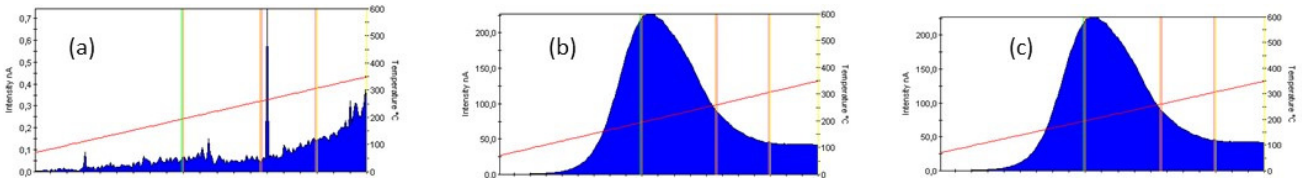

Figure 6. TL Glow curves 1 of unirradiated product (a), of irradiated product (b) and TL Glow curve 2, standardization curve (c)

In principle, TL methods can be applied to detect irradiation of any food from which silicate minerals can be isolated such as herbs, spices, bulbs, tubers, vegetables, cereals, shellfish, and fruits containing silicate minerals. Detection limits and stability of the method depend on the quantities and types of minerals recovered from individual samples. Detection of irradiated fresh and dehydrated fruits and vegetables has been validated for doses of about 1.0 kGy for fresh fruits and vegetables (EN 1788/2001).

TL method is laborious, limited by the quantity of extracted minerals (Arvanitoyannis, 2010; Stefanova, 2010). Suitable TL readers are quite costly, and in addition access to a radiation source is needed for normalization of the result. On the other hand, the TL method mostly enables an unequivocal classification of irradiated and unirradiated samples. For products irradiated at low doses such as fresh fruit and vegetables, the normalizing dose must be adjusted.

- **Photo stimulated luminescence (PSL)** is the base for European standard EN 13751:2009. This technique is analogous to thermoluminescence, but in this case, optical radiation (pulsed infrared light) is used to excite the sample and to stimulate the recombination of electrons with holes. This results in the emission of light, which is the measured signal. Contrary to TL, no mineral extraction is needed in PL measurement. Indeed, whole samples or a mixture of organic and inorganic materials can be analyzed.

The PSL method may in principle be applied to detect irradiation of any foods, which contain mineral debris, especially silicate mineral and bioinorganic material such as calcite, which originates from shells or exoskeletons, or hydroxyapatite from bones or teeth. The PL sensitivity depends on the quantities and types of minerals present in the sample.

PSL method obviates the need to isolate minerals. The measurement is carried out in a few minutes. The sensitivity of current reference methods might be insufficient for low-dose irradiation treatments (less than 1.0 kGy) of food.

The review of the literature gives feedback on the use or test of EU standardized irradiation methods e.g Electron Spin Resonance (ESR), Thermoluminescence (TL), Photo Stimulated Luminescence (PSL) to detect irradiation of fruit and vegetables at low doses (Amilcar *et al,* 2012). The results of the studies described in the literature have been somewhat mixed. A non-exhaustive overview of these studies is summarized below.

ESR, TL and PSL were tested to identify irradiated Korean chestnuts. The samples were irradiated with 0.5 kGy. TL technique was adequate to distinguish irradiated from non-irradiated samples, while PSL signal was too low to distinguish irradiated from non-irradiated samples. With ESR spectroscopy, no radiation induced cellulose radicals were detected leading to the observation that this low dose of radiation induces small changes that are not easily detectable by the available techniques (Chung *et al,* 2004).

ESR, TL and PSL standards were also tested on European chestnuts irradiated at different doses ranging between 0.1–1.0 kGy. With the TL technique it was possible to correctly identify the irradiated samples even at a low dose of 0.15 kGy. The PSL signal was only just above the negative threshold for all doses, except for the lower dose of 0.15 kGy. With ESR spectroscopic methods, no radio-induced signal was observed for chestnut shell or pulp (Mangiacotti *et al,* 2009). This confirms that EN Standards based on ESR technique are not useful for the correct identification of this food product irradiated at doses lower than 1.0 kGy. Only the PSL and TL techniques could be useful for detecting irradiated fresh chestnuts.

ESR, TL and PSL standards were performed successfully to detect various fruits (oranges, grapefruits, mandarins, lemons, limes, and pineapples) irradiated with 1.0 kGy (Yunhee, 2015). However, ESR signal stability over the time life of the product was not assessed.

Assays were also carried out to discriminate **irradiated pests** from untreated ones based on electron spin resonance (ESR) signal derived from treated insects. Adults of the confused flour beetle, Tribolium confusion were irradiated with 0.75 and 1.0 kGy, whereas adults of the rice weevil, S. oryzae, were irradiated with 0.25, 0.5, 0.75, and 1.0 kGy. Two weeks after irradiation, insects were killed by a temperature of 65°C, slowly dried, and used for ESR spectroscopic studies by measuring peak heights of ESR signals. Peak heights obtained from the confused flour beetle were variable and seem not to be affected by irradiation with a dose of 1.0 kGy or lower. In the rice weevil, a reduction of ESR peak heights as the

dose of radiation increased up to 1 kGy was noted. However, this reduction of peak heights is too small to be used as a criterion for detection of irradiated rice weevils (Ignatowitz, 1994).

3.1.2 European Standardized irradiation detection methods - Biological methods

Direct Epifluorescent Filter Technique/Aerobic Plate Count (DEFT/APC) - EN 13783: 2002). The characteristics of microbial population of irradiated foods have been used for developing detection methods for irradiated foods. **DEFT/APC (EN 13783-2002)** was approved and validated as screening microbiological method for identification of irradiated foods. This method is based on a comparison of the APC with the count obtained using DEFT. The APC gives the number of viable microorganisms in the sample after a possible irradiation and DEFT count indicates the total number of microorganisms, including non-viable cells, present in the sample.

DEFT/APC technique is easy to implement but not efficient. It becomes limited if the initial contamination before irradiation is very low $<10^3$ CFU/g or the radiation dose is very low. Besides, some spices such as cloves, cinnamon, garlic, and mustards contain inhibitory components with antimicrobial activity that can lead to a decrease in APC (false positive result). In addition, similar differences between DEFT and APC counts may be induced by other food treatments that cause the death of microorganisms, such as fumigants and heat, so positive results should be confirmed (Chauhan *et al,* 2008). The applicability of this technique in detecting minimally processed vegetables (MPV) (lettuce, chard, watercress, escarole, chicory, spinach, and cabbage) irradiated with 0.5 and 1.0 kGy was evaluated by Araujo and *al*., 2009. The authors show, that 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. Research carried out on cereal grains and beans found a log DEFT/APC ratio between 2.0 and 3.0 for doses of 0.5 kGy or more (Oh *et al,* 2002).

DNA Comet Assay -EN 13784, 2002. Ionizing radiation causes changes both in the DNA of living cells and in the food molecules. The Single-Cell Gel Electrophoresis (SCGE) or comet assay can detect DNA strand breaks and alkali labile sites by measuring the migration of DNA from immobilized nuclear DNA. **The DNA Comet Assay EN 13784, 2002** is one of ten approved standard detection methods in many food items such as meat, fish, grains, and fruits.

This method is based on the radiation induced damage of the DNA molecules of the food that causes chain breakage, double-strand breaks, single- strand breaks and base damage. This approach was proposed by Ostling (1988) and was later adapted to the sensitive detection of irradiated foods by Cerda *et al,* (1993 and 1997). It consists of an extraction of the cells by a simple shaking in a buffer solution. The cellular suspension, mixed with low-melt agarose, is coated on a microscope slide, and subjected to short (2 min) electrophoresis. The cells whose DNA have a high molecular weight (unaltered DNA) will migrate shortly into the gel. The cells extracted from irradiated food have shorter DNA (because of strand breaks) and will migrate over longer distances (Figure 7). Electrophoretic pattern of such cells appears as comets whose heads are represented by the cell and the tails consist of the low molecular-weight DNA. The higher the absorbed dose, the longer the tail of the comet. The DNA Comet Assay is working as a screening test and may detect irradiation of any food containing DNA: both animal foods and plant foods.

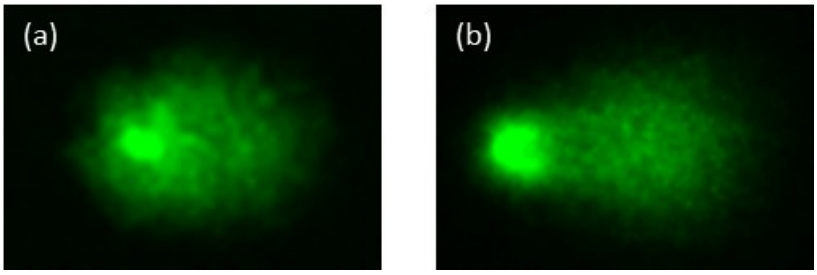

Figure 7. DNA comet assay from non-irradiated (a) and irradiated (b) product

DNA method (Comet Assay) is considered rapid, sensitive and simple to perform and inexpensive. However, this technique is not radiation-specific and thus limited to food products that have not been submitted to other processes, like cooking or freezing that cause similar damages (Stefanova *et al,* 2010). Therefore, any positive result obtained with this screening method must be confirmed by using a reference method (Zanardi *et al,* 2017). Indeed, it has been extensively reported that physical agents, e.g., gamma radiations and X-rays, and a variety of chemical compounds can damage DNA in living cells (Lee & Steinert, 2003). This technique can result in high rates of false positives, Mangiacotti *et al,* (2013) detected as high as 26% false positives in an official control by an accredited laboratory. Thus, suspicious samples should be confirmed by another validated method.

Technical limitations also exist, as suitable DNA material is hard to obtain in some dry foods, especially nuts, seeds, and beans (Khan *et al,* 2002; Khan *et al,* 2005; Khan *et al,* 2008; Delincée *et al,* 2003).

When it comes to the implementation of this method on food pests further studies are needed to evaluate it in specific pests under conditions closer to practical irradiation doses, and in consideration of the duration of post-irradiation transportation. Indeed, stored-product insects differ markedly in their tolerance to gamma radiations (Hasan & Khan 1998). Tolerance varies considerably even within a single genus. It is also known that a particular stage of an insect species life cycle may determine its sensitivity to ionizing radiations, as some stages may be capable of continuing development after sub lethal exposure (Arthur *et al,* 2015); Bakri *et al,*2005). Thus, the results may differ from one type of insect to another and according to the phase of development of the insect. Besides, to date, there is no threshold or maximum value for the different parameters measured including percentage DNA in tail, tail length, tail moment, olive-tail moment, and percentage DNA damage which makes it possible to make a statement whether a product has been irradiated or not, hence the need for a control sample to be able to conclude.

The literature provides several examples of the application of **DNA method (''Comet Assay'', EN 13784, 2002)** to irradiated fruits and vegetables (Chung *et al,* 2004; Horak *et al,* 2009; Khan *et al,* 2011; Khawar *et al,* 2011; Cetinkaya, 2016) as well as to food pests (Todoriki *et al,* 2006; Hasan *et al,* 2008; Kameya *et al,* 2012).

Assays implementing DNA method tested to identify irradiated with 0.5 kGy Korean chestnuts, C. Bungena, shown that no difference was observed between irradiated and non-irradiated samples (Chung *et al,* 2004). This method was also tested to detect radiation treatment of apples and to evaluate the possibility to estimate the irradiation dose. To this end, seeds obtained from the apples were irradiated at 0.1, 0.2 and 0.3 kGy (Horak *et al,* 2009). The authors reported that with the incremental doses, an increase in the tail length was seen due to the DNA fragments migration out of the cell. The irradiation samples showed much longer comets and could be clearly identified. The differences in tail length, evaluated to see the dose effect between the control and the three incremental doses, were significant (p=0.01).

Comet assay was also tested under neutral conditions to evaluate the radiation sensitivities of stored product insect, C. sikkimensis. This latter was exposed to electrons at different acceleration voltages of 300, 750, 1000, and 1500 kV at doses of 1.0 and 4.0 kGy. Percent DNA damage varied significantly between the irradiated and non-irradiated larvae of C. sikkimensis. Damage increased as the acceleration voltage of electron beam increased at both doses, 1.0 and 4.0 kGy, which clearly indicates that electron beam tolerance of larvae was dose-dependent and has certain upper limits (Todoriki *et al,* 2006). The authors concluded that comet assay would be a potentially useful tool for detecting DNA damage in insects in control strategies of the pest management.

DNA comet assay was also implemented to evaluate the effects of gamma irradiation dose of 0.28 kGy (as quarantine dose) on the DNA of the adult stage of R. dominica and wheat grains. Abotaleb *et al,* (2020) reported that this method

has allowed detecting DNA damage in the R. dominica cells, while in the case of wheat treated with the same dose (0.28 kGy), there has been no significant increase in all DNA damage parameters (percentage of DNA tail, tail length, tail moment) in comparison with non-irradiated (control) wheat samples.

DNA damage in larvae, pupae, and adults of S. Zeamais after exposure to gamma radiation was investigated by Hasan *et al,* (2008). Mature larvae (22-days-old), pupae (1-day-old) and adults (1-day-old) of maize weevil were irradiated with doses of 0.5 and 1.0 kGy. Exposure of developmental stages of S. Zeamais to gamma radiation caused a significant increase in DNA damage. DNA damage increased as radiation dose increased for all the developmental stages of S. Zeamais, which indicates that radiation damage of all the stages is dose-dependent and has certain upper limits. The authors observed comparatively-less DNA damage in the adults and larvae than the pupae of maize weevil.

Comet assay has been conducted under alkaline conditions to observe DNA variation and to investigate its use for identifying the irradiation treatment history of pests using the cigarette beetle, Lasioderma serricorne, as a test pest. L. serricorne adults collected from stock cultures within 2 days of emergence were irradiated with gamma rays at 1.0 kGy. According to Kameya *et al,* (2012), broken DNA strands appeared to be repaired as the post-irradiation period lengthened. However, even 7 days after irradiation, clear differences were observed between the irradiated and control samples indicating the potential of this method, to identify the irradiation history of insect pests.

3.2 Methods being tested for the detection of phytosanitary irradiation treatment.

Several methods and techniques are being evaluated for their potential as diagnostic tools for detecting irradiated fruits and vegetables and parasites. These include non-standardized methods dating from the 1990s or earlier (e.g., total protein profiles, midgut structure, phenoloxidase activity, and melanization reaction during microbial loading, germination, and half-embryo) and newer, emerging methods implementing advanced analytical strategies (based on histone H2A immune detection, or using flow cytometry, PCR, and DNA analysis techniques).

3.2.1 Available (but non standardized) methods tested

Protein profiles. Lescano *et al,* (1994) assessed the ability of electrophoretic protein profiles to detect irradiated larvae and pupae with 0.075 kGy of Bactrocera tryoni. They showed that protein profiles for control and irradiated larvae were similar. Thus, the authors concluded that this technique is not suitable to discriminate between irradiated and non-irradiated larvae of B. tryoni. However, Yulo-Nazarea *et al,* (1991) showed the absence of a specific protein band, when fruit fly larvae were irradiated at a dose of 0.1 kGy, which is an integral part of the bands of proteins that appear only at the pupal and adult stages. They concluded that the absence of Gs protein in the SDS-PAGE gel pattern of pupae could be used as a marker for irradiated fruit flies.

Midgut structure. A consistent reduction in the size of the supra esophageal ganglion of B. tryoni when irradiated at 0.75 kGy was observed by Lescano *et al,* (1994). These results converge with those of Rahmann *et al,* (1990). The latter find a reduction in the size of the ganglion in larvae treated with doses as low as 0.5-1.0 kGy and a significant correlation between the size of the supraoesophageal ganglion of the Ceratitis capitata larvae and the irradiation dose applied. Lescano *et al,* (1994) as well as Rahmann *et al,* (1990), suggested that this could be used to detect larvae irradiated at quarantine dose rates within fruit at commercial scales. This technique has been tested sporadically on a very limited number of insects. The last published work dates from 1994.

Phenoloxidase activity and melanization process. The effects of gamma radiation on the melanization process were addressed on Plodia interpunctella Hubner (Larvae of the Indian meal moth), Ephestia kuehniella Zell (Mediterranean flour moth), and Trogoderma grananum Ev. (Khapra beetle) irradiated at doses of 0.1, 0.3, 0.5 and 0.7 kGy. Index of melanization was calculated and the activity of phenoloxidase enzyme in irradiated and control larvae was determined using the 2-methyl-DOPA spot tests. It was shown that melanization decreased with increasing dose and with the elapsed time after the treatment. Activity of phenoloxidase enzyme was highly variable for both control and irradiated larvae of the Mediterranean flour meal moth and the khapra beetle. Therefore, they found that a test based on phenoloxidase activity or on the melanization reaction are unreliable to be applied in quarantine procedure (Ignatowicz and Dorota,

1998). While another study demonstrated inhibited melanization and reduced phenoloxidase activity in irradiated insects (Nation *et al,* 1995).

Shift in microbial load was implemented on fruit and vegetable products to detect irradiation treatment. Tamminga *et al,* (1975) shown that, the initial microflora of strawberries mostly of Pseudomonas, was completely removed after irradiation at 2.0 kGy. 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.

Germination and Half-embryo Tests. Germination test, used to differentiate irradiated commodities from non-irradiated ones, relies on the fact that irradiated seeds germinate at significantly slower rates than control seeds. However, this test, even though simple and inexpensive, is limited to vegetable seeds and too slow for routine analysis takes several days to get results (e.g 6 to 14 days for grapefruit seeds) (Chauhan *et al,* 2009). Kawamura *et al,* (1989) developed an improved germination test known as "**half embryo test**" for detection of irradiated grapefruit and other fruits. In this test, the embryo was used for germination instead of seeds thereby accelerating the germination process. They reported that at a dose of 0.15 kGy radiation treatment could be detected within 2 to 4 days, which is still an important time period even though it is shorter than the duration required for the germination test.

3.2.2 Emerging methods

Immune detection of H2A histone has been proposed by Siddiqui *et al,* (2013) to be used for identifying the irradiation status of live insects found in exported or imported consignments of fruit and vegetables. This method is well-established for biological dosimetry of irradiation exposure. It is based on immune detection of phosphorylated H2A histone variants. Phosphorylation of the C-terminal of the core histone protein H2AX (termed γH2AX when phosphorylated) is an early known response to DNA double-strand breaks in living organisms that can be due to exposure to ionizing radiation. Two types of γH2AX foci have been found in cells: firstly, transient γH2AX foci that are associated with rapid DNA double-strand repair and dephosphorylation of γH2AX to H2AX, usually within minutes to hours. The second type of γH2AX foci are residual and tend to persist for days to months (Siddiqui *et al,* 2020). The study on the Queensland fruit fly (Bactrocera tryoni) carried out by Siddiqui et al demonstrated that irradiation exposure leads to a persistent γH2AvB response (a fruit fly variant of γH2AX). The authors showed that the higher the dose of irradiation, the higher the amount of γH2AvB that is produced in the flies. This was tested over irradiation doses from 0 kGy (not irradiated) to 0.4 kGy. γH2AvB was detectable using ELISA test for a significant time period after irradiation treatment (up to 17 days). These results could foresee the possibility of using γH2AvB as a biomarker of prior irradiation exposure of fruit flies. However, the authors identified some potential limitation such as γH2AvB kinetics which can differ among species and suggested to assess the kinetics of persistent γH2AvB responses in diverse fruit flies of quarantine concern.

Another limitation was highlighted by Lei *et al,* (2020) linked to the assessment of γH2AvB, by antibodies that are designed to target short C-terminal peptides of phosphorylated H2A variants. They assumed that due to species specificity, it is difficult for one or a few antibodies to detect phosphorylated H2A in all insects with high sensitivity. Conversely, cross-reactivity occasionally can cause failures in distinction of irradiated and control tissues.

Flow cytometry has been used extensively as a clinical diagnostic tool, in pharmaceutical development, and in basic medical research. In recent years, it has increasingly been applied in the food and dietary supplement industries but has been rarely tested as a detection method for radiation-induced changes in DNA. In 1995, Selvan *et al,* tested the use of this technique to monitor changes in the DNA content of irradiated onion bulbs at 60 Gy, using a fluorescent dye, which binds specifically to double strand regions. The DNA content in the nucleus (from onion meristem) proportional to the fluorescence signal, was expressed as arbitrary C values in which 2C and 4C values representing the DNA content of the diploid and tetraploid chromosome complements, respectively. The DNA indices (DI) for 2C and 4C populations in the irradiated samples were calculated using the formulae of Hruban *et al,* (1990). The authors reported that DNA indices of nuclei of control onion meristem was 1 (range 0-98-1.02) whereas a highly significant lowering of the DI (range 0-60-0.90)

was noted in irradiated meristem nuclei. Similarly, highly significant difference in the coefficient of variation (CV) were recorded consistently between control and irradiated samples. The authors concluded that both CV and DI determination by Flow cytometry analysis can be used for detection of irradiated onions and may complement existing methods based on chemical changes in nucleic acids for the detection of irradiated foods.

More recently, Lei *et al,* (2020), investigated the feasibility of using flow cytometry detection of holometabolous insects irradiated at 0.2 and 0.4 kGy. The authors observed that irradiated Cowpea bruchid (Callosobruchus maculatus), corn earworm (Helicoverpa zea) and fruit fly (Drosophila melanogaster) displayed significantly higher proportions of polyploid cells than their respective non-irradiated controls. The authors reported that the eBeam-triggered DNA endoreplicative response was strong in larvae but became weaker as insects continued to develop into pupae and was even further diminished in adults. From this fact, they considered that the flow cytometry method has the potential to be a diagnostic tool to determine whether juvenile insects particularly those that underwent complete metamorphosis, have received irradiation as a quarantine treatment.

The advantages of flow cytometry are that it is rapid and requires a relatively small number of samples. However, it is premature to state that this technique is a reliable diagnostic test for detecting irradiated insects or for discriminating between irradiated and non-irradiated fruit and vegatbles. Confirmation is needed on a wide range of commodities and insects (representative of quarantine insects). Likewise, robustness of this technique as well as the failure rate must be evaluated.

Real-time PCR is based on fluorescence measurements. Fluorescence is used to measure the accumulation of products of polymerase chain after each cycle. Measurement of fluorescence allows for direct quantification of target DNA present in the sample. PCR amplification curves (fluorescence measured as a function of the number of PCR cycles) allows Ct (Cycle threshold) values determination. These Ct values correspond to the number of amplification cycles necessary for the fluorescence emitted by a PCR product (i.e., target/amplicon) reaches the point where it will be greater than the background noise. Eugster *et al,* (2017), used a variation of real-time PCR, LATE-PCR (Linear-after-exponential polymerase chain reaction) to distinguish irradiated and non-irradiated garlic, dried figs, sweet paprika powder, papaya, and dried mango sliced. This technique differs from conventional real-time PCR by preferential amplification of one of the two strands of DNA. Using this real-time PCR method, authors have shown a distinction between irradiated and non-irradiated garlic with doses of 0.125, 0.25, 0.5, 1.0 and 2.0 kGy. They have shown a decrease of the Ct values in the irradiated garlic sample since DNA is damaged (double strand breaks for example). However, from 0.5 kGy the number of Ct values is the same regardless of the dose applied (around 30 Ct for garlic samples). This technique is highly specific since it targets a particular DNA sequence and requires DNA extraction. If this extraction is not optimal for the product treated, the amount of DNA extracted may not be sufficient for PCR amplification.

In addition, inhibitors can be found in various matrices (especially in spices), and these substances can interfere with PCRs by interacting directly with DNA and blocking the activity of the polymerase or other PCR mixture components (e.g., MgCl2), thereby preventing target amplification.

NGS (Next Generation Sequencing) technologies. Metagenomics is the study of genetic material recovered directly from environmental samples, such as on the animal gut, ocean, soil, air, etc. This method has been already used to describe the diversity of the microbiome of insects such as Bactrocera dorsalis in various conditions (Andongma *et al,* 2015; Gujjar *et al,* 2017; Liu *et al,* 2016; Khaeso *et al,* 2018). To date, the most classic approach is to extract bacterial DNA from the desired environment and then, after a step of amplification, to sequence the gene encoding 16S rRNA. This gene is a universal marker of diversity, since it is present in all bacteria species, but is variable enough to distinguish bacterial species. Using this method, Stathopoulou *et al,* (2019) have shown that the microbial profiles are different between samples irradiated at 50 Gy and non-irradiated samples. In addition, the bacterial profile of larvae appeared to be different compared to that of adult B. dorsalis flies. The subsequent application of irradiation at the pupal stage led to the development of different microbiota between treated and untreated samples, affecting diversity and operational taxonomic unit composition.

This non-targeted technique has the advantage of being able to access a precise description of the bacterial species present in the sample, without *a priori*. It also makes it possible to compare bacterial communities from different samples. However, this technique requires (1) DNA extraction from samples and (2) understanding and mastering the bioinformatics challenges of analyzing sequencing data.

Others approaches. Non-targeted approaches combined with chemiometric analysis have been proposed to detect irradiation treatment (Zanardi, 2017). The principle of these approaches is to compare the profiles/fingerprints of irradiated and non-irradiated products and to identify correlations between constituents of these profiles (corresponding to the presence or absence of metabolites) and the irradiation treatment. The choice of the instrumental method is of prime importance for the establishment of such correlations since it determines the quality and the quantity of the generated data. In addition to the instrumental aspects, the other key elements of the metabolomic approaches are the processing and the statistical analysis of the data. This is based on multivariate approaches using unsupervised, supervised, and applied methods. More generally, the exploitation of analytical data should provide a description of the chemical complexity of the analyzed products and an identification of correlations between the presence/absence of certain molecules and the irradiation treatment. The analysis of the data should also contribute to highlight markers of irradiation. These markers must then be identified to consider more targeted monitoring strategies.

Techniques such as NMR, NIR and MIR or GC-MS have been implemented to identify changes due to irradiation (Zanardi *et al,* 2017; Yunhee *et al,* 2019). The ability of NMR and MIR to detect changes in the composition profile of products irradiated at low doses is questionable due to insufficient sensitivity compared to MS. Furthermore, the use of these approaches raises questions about the validity of the correlations or markers identified. Indeed, the quality of the data and the validity of the model or marker depends on the representativeness of the defined experimental domain and the accounting of the parameters of variability due, among other, to the species, origin, technical courses, degree of maturity, etc. This poses a non-negligible problem of sampling or availability of data base which are often owner of the manufacturers of the equipment.

3.3 Classification of the reviewed methods according to their relevance to detect irradiation phytosanitary treatment

For confirming the correctness of phytosanitary irradiation treatment, analytical techniques are needed. Ideally, such techniques must be accurate, applicable to a wide range of commodities and pests, and sensitive enough to allow the detection of irradiation at low doses (less than 1 kGy). The measured test parameter should be able to clearly identify if the commodity has been irradiated at any point during its storage life, without requiring a non-irradiated sample for comparison from the particular batch tested. Moreover, it would be optimal if the test could also fulfil practical criteria, in line with the expertise and means necessary for its implementation.

None of the standardized methods, nor the experimental or emerging ones described in the literature, can fully meet these criteria. Indeed, each of the techniques/methods reviewed has limitations in line with one or more of the criteria listed below (see table 2):

- **Technical criterion**: Method Performance (accuracy, sensitivity threshold detection), specificity of the marker to irradiation, stability of the marker throughout the lifetime of the products and the extent of application domain (fruit and vegetables or pests)
- **Practical criteria**: ease of implementation, level of expertise required, cost and speed
- **Market accessibility**: maturity of the method (validated, tested in several laboratories, ready for collaborative testing), equipment/techniques availability.

Based on the available information from literature, we have attempted to classify the methods reviewed into 3 groups:

- The first group includes methods that are considered inappropriate mainly due to the inconsistency of the results and lack of sensitivity, as well as inadequacy of the implementation conditions for quality control use.
- The second group includes methods that are potentially sensitive but cannot be used as confirming methods but rather as screening methods.
- The third group includes mature methods or methods with a promising concept, both requiring development and optimization work

This classification has been carried out for each of the fields of application, i.e., the product or the pest (figures 8 and 9). It is worth to note that all the methods mentioned are qualitative methods. They do not allow an evaluation of the applied radiation dose.

Table 2. Methods tested for the detection of phytosanitary irradiation treatment: advantages and limitations

Group	Method/ Technique	Technical criteria	Practical criteria	Market accessibility	Remarks	References
Physical methods	ESR	Specific to irradiation. Detection may be difficult for dose levels < 1.0 kGy. Concerns about stability of the signals	Simple. Rapid. Expensive equipment.	Wide availability. Mature and normed	Discernable minimum dose depends on product cellulose content the continuous improvement of ESR techniques and sample preparation would allow to gain in detection sensitivity	[1], [13], [29], [31] [32], [41], [47], [59], [64], [66]
	(TL)	Specific to irradiation. May be limited by the quantity of extracted minerals	Costly. Time - consuming,	Wide availability. Mature and normed	Requires minerals extraction and radiation source	[7], [13], [47], [59], [64]
	PSL	Specific to irradiation but low sensitivity	Quick	Wide availability	Signal decreases over time (optical blanching). Not suitable for samples with low mineral contents	[13], [47] [64].
Biological methods	(DEFT/APC)	Nonspecific to irradiation Not efficient. Limited if the initial contamination before irradiation is low (<10^3 CFU/g).	Quick Simple Costless	Wide availability	Needs knowledge of microflora High Risk of false positives	[5], [12], [51].
	DNA methods (‘‘Comet Assay’’)	Nonspecific to irradiation Sensitive. Sensitivity may vary between pest species	Rapid Simple Inexpensive	Wide availability	High Risk of false positives.	[52], [9], [10], [59], [65], [42], [48], [36], [37], [15], [39], [25], [13], [27], [28], [40], [11], [62], [33], [3]
Other methods	Protein profiles	Nonspecific to irradiation	Simple Inexpensive	Wide availability	Inconsistent results between publications	[44], [63]

	Midgut structure	Nonspecific to irradiation, applicable to pests only	Inexpensive	Available	Few published works.	[44], [54]
	Phenoloxidase activity and melanization reaction	Nonspecific to irradiation. Unreliable	Inexpensive	Available	Inconsistent results between publications	[30], [50]
	Shift in microbial load	Nonspecific, need control sample	Simple Inexpensive	Available	Very dependent on the initial microbial load, which varies regionally and with agronomic practices	[61]
	Germination and Half-embryo Tests	Nonspecific to irradiation	Simple Inexpensive Time - consuming	Available	It takes several days to get results	[12], [34]
	Immune detection of H2A histone	Specific to irradiation Sensitivity may vary between pest species.	Simple rapid	Well-established for biological dosimetry Premature for irradiation detection	Further development and validation work needed	[56], [57], [43],
	Flow cytometry	Nonspecific to irradiation	Rapid, Equipment expensive	Premature for irradiation detection	Few publications	[55] [43]
	PCR and NGS	Highly specific	Needs a DNA step extraction	Premature	Inhibitors (interfering with PCR) can be found in various matrices (specially in spices) NGS Needs expertise and knowledge in bioinformatics	[17], [4], [20], [45], [35], [58]

- **Technical criterion**: Method Performance (accuracy, sensitivity threshold detection), specificity of the marker to irradiation, stability of the marker throughout the lifetime of the products and the extent of application domain (fruit and vegetables or pests)
- **Practical criteria**: ease of implementation, level of expertise required, cost and speed
- **Market accessibility**: maturity of the method (validated, tested in several laboratories, ready for collaborative testing), equipment/techniques availability.

3.3.1 Classification of the detection methods targeting the commodities

Group 1. Unreliable Methods

- PSL and DEFT/APC are not efficient. DEFT/APC also becomes limited if the initial microbial contamination before irradiation is low.
- The measured test parameters in the shift in microbial load method are unable to clearly identify whether commodity has been irradiated since results are dependent on the initial contamination
- Germination and half- embryo tests tend to be inaccurate and time consuming

Group 2. Promising as screening methods

- The Comet assay method has been widely tested at doses below 1.0 kGy and has led to conclusive results (but not always). This method has 2 important limitations: it is not radiation specific, and it requires a control (non-irradiated) sample for comparison. Nevertheless, it may have useful roles as rapid assay methods.
- Real-time PCR technique have been used to distinguish irradiated from non-irradiated products in one publication (Eugster, 2017). This discrimination is based on a lower CTs value in the case of irradiated product than in the case of non-irradiated product. However, it has not been demonstrated at what Ct value the product is considered irradiated. This suggests that this method requires a comparison with a control sample. Besides, further work is needed to confirm that the analytical response of this method is radiation specific although the publication states that the mechanisms of DNA degradation by heating and irradiation are different. Once these questions are answered, this method can be used as screening method, probably universally applicable and the analysis can be performed in a relatively short time.

Group 3. Mature methods but further optimization needed

- TL and ESR techniques are mature and widely applied. TL has a high sensitivity and reliability, but the difficulty lies in the possibility to find enough silicate minerals. As for ESR, the results of the studies addressed in the literature are mixed: the sensitivity of the method is not always sufficient at low doses. Therefore, developments are needed to improve its detection threshold and the stability of the EPR signal before concluding on its use as a confirmatory method for phytosanitary irradiation treatment.

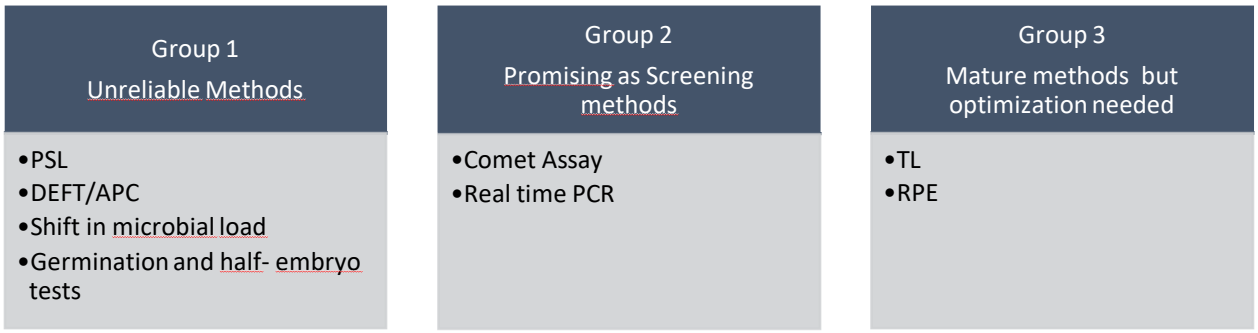

Figure 8. Classification of the methods based on their ability to detect F&V irradiated at low doses for phytosanitary purpose

3.3.2 Classification of the detection methods targeting pests

Group 1. Unreliable Methods

- Protein profiles and ESR are not sensitive enough, and results are often inconsistent
- The study of midgut structure changes is time-consuming and tend to be inaccurate.

Group 2. Promising as screening methods

- For the comet assay method, the same advantages and limitations discussed above for products remain valid when this technique is applied to pests.
- NGS technique is only used in one study (Stathopoulou et al., 2019) to discriminate between irradiated and non-irradiated pests based on their microbial profile. However, the environmental conditions could influence the diversity of microbiota, making it difficult to have a clear unequivocal answer and requiring a control sample. Moreover, the exploitation of the results uses statistical tools, which suggests a certain level of expertise. For these reasons, the relevance of this technique as a control method of phytosanitary irradiation treatment (even as screening method) is questionable.

Group 3. Methods with a promising concept

- Immune detection of H2A Histones and Flow cytometry are both emerging but promising methods. Their advantages and limitations have been discussed above (see section 3.2.2). These new approaches for detecting irradiated pests raise several questions and require additional development and validation work before their suitability for the intended purpose can be definitively assessed.

Group 1 Unreliable Methods	Group 2 Promising as Screening methods	Group 3 Methods with a promising concept
•Protein profils •Midgut structure changes •ESR	•NGS •Comet assay	•Immune detection of H2A Histones •Flow cytometry

Figure 9. Classification of the methods based on their ability to detect irradiated pests at low doses

4. CONCLUSION

Phytosanitary treatments are often required to disinfest host commodities of pests. Irradiation has proven effectiveness against most insect and mite pests at low dose levels (less than 1.0 kGy). At these doses, death of pests is generally not immediate. An advantage of irradiation is that the product is available for immediate dispatch, but this can increase the possibility of live insects being found, becoming an issue especially in countries where a zero-pest requirement is mandatory. The finding of a live insect can cause an unnecessary remedial treatment to be applied which will be detrimental to quality. The effectiveness of the irradiation process is based on an accredited process of facility and product qualification (including accurate dose measurement), process control, product tracking, record keeping and documentation, a process trusted for over 60 years in other radiation applications. Although international authorities and national legislations do not require a post-irradiation detection method for trade in irradiated fruits, a valid detection method could provide extra confidence for inspectors and the market.

An ideal method for detection should be specific for irradiation and not influenced by other processes, accurate and reproducible, have a detection limit below the minimum dose likely to be applied for phytosanitary purpose, applicable to a range of pests and commodities, quick, easy, and cost effective to perform, and capable of providing an estimate of irradiation dose.

In this context, a literature review was carried out to assess state-of-the-art detection methods and for their ability to confirm exposure to phytosanitary irradiation. The analysis of the information gathered revealed that none of the methods considered, whether standardized, developed or under development, fulfilled all these requirements.

The exploitation of the collected information also served to highlight the advantages and the limits of the considered methods. On this basis, a classification was proposed. This was carried out by considering both the marker intrinsic to the product and intrinsic to the insect. In both cases, the methods were classified into 3 groups according to whether they are considered as unreliable (group 1), promising as screening methods (group 2), emerging methods with a promising concept (group 3- case of the methods targeting pest) or mature methods with further optimization needed (group 3- case of the methods targeting the product).

The promising methods to detect irradiated pests that are classified in group 3 use advanced techniques but raise several questions. Thus, further development work is required before being able to confirm their potential to detect, in an unequivocal way, phytosanitary irradiation treatment. Some of these issues are listed below:

- Are these tests universal, applicable to all insect or at least reliable for the detection of the most important irradiated quarantine pests?
- Are these tests reliable whatever the development stage of the pest considered?
- Are these techniques able to distinguish irradiation dose level (within the range of irradiation doses usually used for phytosanitary purpose)?
- What is the sensitivity of these techniques at very low doses since many insects have approved doses in the range of 0.07 to0.15 kGy?
- How often are the tests inconclusive or wrong?

Similarly, the methods targeting commodities and using mature techniques in group 3 also need further optimization and validation work. This is required to answer questions raised concerning the feasibility of improving their detection threshold that must be able to detect very low doses.

The findings of this literature review will be discussed and enriched with experts through a series of individual and/or collective interviews to prepare a roadmap that will overcome these barriers and seek to answer the questions raised.

REFERENCES

1. Aleksieva, K.I., Dimov, K.G., Yordanov, N.D. (2014) Identification of gamma-irradiated fruit juices by EPR spectroscopy, Radiat. Phys. Chem 103 (1) (2014) 27–30.

2. Amilcar, L. A., Márcio, C., Albino, B., Quintana, B., Botelho M. L., Ferreira I. C.F.R. (2012) Effects of gamma radiation on the biological, physico-chemical, nutritional and antioxidant parameters of chestnuts – A review- Food and Chemical Toxicology 50 (2012) 3234–3242

3. Abotaleb, A. O., Zinhoum, R. A , Gabarty, A. (2020) Evaluation of irradiation quarantine dose – induced DNA damage in Rhyzopertha dominica and its effect on wheat grains using comet assay. International Journal of Tropical Insect Science, 2020

4. Andongma ,A.A., Wan L, Dong, Y-C., Li P, Desneux N. (2015) Pyrosequencing reveals a shift in symbiotic bacteria populations across life stages of Bactrocera dorsalis. Scientific Reports 5: 9470.

5. Araujo, M.M., Duarte R.C., Silva, P.V., Marchioni, E., Villavicencio, A.L.C.H. (2009) Application of the microbiological method DEFT/APC to detect minimally processed vegetables treated with gamma radiation Radiation Physics and Chemistry 2009 (78) 691–693

6. Arthur, V., Machi, A., and Mastrangelo T., (2015). Ionizing Radiations in Entomology, in Evolution of Ionizing Radiation Research, chap 9, IntechOpen

7. Arvanitoyannis, I.S., (2010). Irradiation of Food Commodities: Techniques, Applications, Detection, Legislation, Safety and Consumer Opinion. Academic Press, Elsevier Publisher, USA.

8. Bakri, A., Mehta, K., Lance, D.R., (2005). Sterilizing insects with ionizing radiation. Sterile insect technique: principles and practice in area-wide integrated pest management. In: Dyck VA, Hendrichs J, Robinson AS (Eds.). Springer; 2005. pp. 233–269

9. Cerda, H., Hofsten, B. and Johanson, K. J., (1993). Recent Advances on Detection of Irradiated Food, ed. M. L. Conardi, J. L. Belliardo and J. J. Raffi, Commission of the European Communities, Luxembourg, 1993, EUR 14315, pp. 401–405.

10. Cerda, H., Delincée, H., Haine, H. and Rupp, H., (1997). The DNA `comet assay' as a rapid screening technique to control irradiated food Mutation Research/ Fundamental and Molecular Mechanisms of Mutagenesis, 1997, 375, 167-181

11. Cetinkaya, N., Ercin, D., Zvatan, S. Ö and Erel, Y., (2016). Quantification of applied dose in irradiated citrus fruits by DNA Comet Assay together with image analysis Food Chem., 2016, 192, 370.

12. Chauhan, S. K., Kumar, R., Nadanasabapathy, S., and Bawa, A.S. (2009). Detection Methods for Irradiated Foods. COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY—2009 (8), 4-14

13. Chung, H.W., Delincée, H., Han, S.B., Hong, J.H., Kim, H.Y., Kim, M.C., Kwon, J.H., (2004). Trials to identify irradiated chestnut (Castanea bungena) with different analytical techniques. Radiat. Phys. Chem. 2004, 71, 179–182.

14. Delincée H. (1991). Analytical detection methods for irradiated foods—a review of the current literature, IAEA Tec Doc 587.Vienna (Austria): IAEA. 172

15. Delincée H., Khan A. A. and Cerda H., (2003). Some limitations of the comet assay to detect the treatment of seeds with ionising radiation. Eur. Food Res. Technol., 2003, 216 (4), 343-346

16. Delincée H., (1998). Detection of food treated with ionizing radiation Trends in Food Science & Technology 1998, (9) 73-82

17. Eugster, A., Murmann P., Känzig A., and Breitenmoser A., (2017). A specific but nevertheless simple real-time PCR method for the detection of irradiated food shown detailed at theexample of garlic (Allium sativum) Eur Food Res Technol., 2017

18. Follett, P. A. (2009). Generic radiation quarantine treatments: The next steps. Journal of Economic Entomology. 2009, 102 (4): 1399–1406.

19. Follett, P. A., T. W. Phillips, J. W. Armstrong, and J. H. Moy. (2011). Generic phytosanitary radiation treatment for Tephritid fruit flies provides quarantine security for Bactrocera latifrons (Diptera: Tephritidae). Journal of Economic Entomology.2011, 104(5): 1509–1513.

20. Gujjar NR, Govindan S, Verghese A, Subramaniam S & More R (2017) Diversity of the cultivable gut bacterial communities associated with the fruit flies Bactrocera dorsalis and Bactrocera cucurbitae(Diptera: Tephritidae). Phytoparasitica. 2017, 45: 453–460

21. Gichner, T., Ptácek, O. D., Stavreva, A., Wagner, E. D., Plewa, M. J. (2000). Comparison of DNA repair using the comet assay in tobacco seedlings after exposure to alkylating agents or ionizing radiation. Mutation Research 470 (2000) 1–9

22. Hallman, G. J. (2011). Phytosanitary applications of irradiation. Comprehensive Review in Food Science and Food Safety 2: 143–151.

23. Hallman, G. J., K. Guo, and Liu T.X. (2011). Phytosanitary irradiation of Liriomyza Trifolii (Diptera: Agromyzidae). Journal of Economic Entomology 104(6): 1851–1855.

24. Hasan, M.,Todoroki, S.,Miyanoshita, A.,Imamura,T., (2008). Detection of gamma radiation-induced DNA damage in maize weevil, Sitophilus zeamais Motschulsky (Coleoptera:Curculionidae) assessed using the comet assay. Int. J.Radiat.Biol.84, 815–820.

25. Hasselmann, C., Marchioni, E., (1991). Physicochemical methods for the detection of food irradiation. In: S Thorne, ed. Food Irradiation. Amsterdam (The Netherlands): Elsevier Appl Sci 129–168.

26. Horak C., DiGiorgio M., Kairiyama, E., (2009). Identification of irradiated apples for phytosanitary purposes. Radiation Physics and Chemistry 78 (2009) 707–709

27. Hruban R H, Andrew G H, Traganos F, Reuter V, Lieberman P H, Melamed M R 1990 Follicular neoplasms of the thyroid in men older than 50 years of a g e A DNA flow cytometric study. Am J Clin Path0194 527-532.

28. Ignatowicz S, (1994). Detection methods for irradiated pests of stored products. Proceedings of the 7th International Working Conference on Stored-product Protectum - Volume 2, pp 1076-1082

29. Ignatowicz S, Lupa DA (1998). Melanization process in irradiated larvae of moths and beetles, pests of stored products. In: Proceedings of the 7th international working conference on stored product protection (2), 1065–1075

30. Jesus, E.F.O., Rossi, A.M., Lopes, R.T. (2000). Identification and dose determination using ESR measurements in the flesh of irradiated vegetable products. Applied Radiation and Isotopes 52 (2000) 1375-1383

31. Jesus, E.F.O., Rossi, A.M., Lopes, R.T. (1999) An ESR study on identification of gamma-irradiated kiwi, papaya and tomato using fruit pulp, Int. J. Food Sci. Tech. 34 (2) (1999) 173–178

32. Kameya, H., Miyanoshita, A., Imamura, T., Todoriki, S. (2012). Assessment of gamma ray-induced DNA damage in Lasioderma serricorne using the comet assay. Radiation Physics and Chemistry 81 (2012) 316–321

33. Kawamura, Y., Uchiyama, S., Saito.Y., (1989). A Half-Embryo Test for Identification of Gamma-Irradiated Grapefruit. J. OF food science, 1989 (54) 2, 379-382

34. Khaeso, K., Andongma, A.A., Akami, M., Souliyanonh B., Zhu J. (2018) Assessing the effects of gut bacteria manipulation on the development of the oriental fruit fly, Bactrocera dorsalis (Diptera; Tephritidae). Symbiosis 74: 97–105.

35. Khan A., Khan H. M. and Delincée H., (2002). Detection of radiation treatment of beans using DNA comet assay Radiat. Phys. Chem., 2002, 63, 407-410

36. Khan A., Khan H. M. and Delincée H., (2005). Comet Assay—a rapid screening method for detection of irradiated cereals and tree nuts- Food Control, 2005, 16, 141-146

37. Khan, H. M., Khan A. A. and Khan S., (2011), Application of DNA comet assay for detection of radiation treatment of grams and pulses. J Food Sci Technol (2011) 48 (6):718–723

38. Khan, A. A. and Khan, H. M., (2008) DNA comet assay: A simple screening technique for identification of some irradiated foods J. Radioanal. Nucl. Chem., 2008, 275, 337-342

39. Khawar, A, Bhatti, I. A., Khan, Q., Asi, M. R. and Ali, T., (2011). Evaluation of irradiation in foods using DNA Comet assay J. Food Sci. Technol., 2011, 48, 106.

40. Kwak J.-Y., Akram, K., Ahn, J.-J., Kwon, J.-H. (2014). ESR-based investigation of radiation-induced free radicals in fresh vegetables after different drying treatments, Int. J. Food Prop. 17 (6) (2014) 1185–1198

41. Lee, R.F. and Steiner, T. S. (2003). Use of the single cell gel electrophoresis/comet assay for detecting DNA damage in aquatic (marine and freshwater) animals. Mutation Research/Reviews in Mutation Research Volume 544, Issue 1, September 2003, Pages 43-64

42. Lei, J., Chen, I. W., Wrigh G., Pillai, S., Salzman K.Z., (2020). Electron beam irradiation induces DNA endoreplication in holometabolous juvenile insects: a rapid flow cytometry-based diagnosis. Journal of Pest Science, 2020

43. Lescano H. G., Congdon l B. C., and Heather N. W. (1994). Comparison of Two Potential Methods to detect Bactrocera tryoni (Diptera: Tephritidae) Gamma-Irradiated for quarantine purposes. Journal of Economic Entomology, Volume 87, Issue 5, 1994, 1256–1261,

44. Liu, L., Martinez-Sanudo, I., Mazzon, L., Prabhakar, C.S., Girolami V. (2016). Bacterial communities associated with invasive populations of Bactrocera dorsalis (Diptera: Tephritidae) in China. Bulletin of Entomological Research 106: 718–728.

45. McMurray CH, Stewart EM, Gray R, Pearce J. (1996). Detection Methods for Irradiated Foods—Current Status. Cambridge (United Kingdom): The Royal Society of Chemistry.

46. Mangiacotti, M., Chiaravalle, A.E., Marchesani, G., De Sio, A., Boniglia, C., Bortolin, E., Onori, S., 2009. Detection of irradiated chestnuts: preliminary study using three analytical techniques. Radiat. Phys. Chem. 78, 695–698

47. Mangiacotti M., Marchesani G., Floridi F., Siragusa G. and. Chiaravalle A. E, Food Control, 2013, 33, 307.

48. Marchioni, E. (2006). Detection of irradiated foods, in Food Irradiation: Research and Technology Edited by Christopher H. Sommers, Xuetong Fan Blackwell Publishing and the Institute of Food Technologists 2006

49. Nation, JL., Smittle, BJ., Milne, K., (1995) Radiation-induced changes in melanization and phenoloxidase in Caribbean fruit-fly larvae (Diptera, Tephritidae) as the basis for a simple test of irradiation. Ann Entomol Soc Am 88:201–205

50. Oh K. N., Lee, S. Y. and Yang, J. S., (2002). Detection of gamma irradiated grains by using DEFT/APC method Food Sci. Technol., 2002, 34(3), 380-384.

51. Ostling, O. and Johanson, K. J., (1984) Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells Biochem. Biophys. Res. Commun., 1984,123, 291-298.

52. Parlato, A., Giacomarra, M., Galati A., and Crescimanno, M., (2014). ISO 14470:2011 and EU legislative background on food irradiation technology: The Italian attitude. Trends in Food Science & Technology Volume 38, Issue 1, July 2014, Pages 60-74

53. Rahman, R., Rigney, C. & Bush·Petersen, E., (1990). Irradiation as a quarantine treatment against Ceratitis capitata (Diptera: Tephritidae): anatomical and cytogenetic changes in mature larvae after gamma irradiation. J. Econ. Entomol. 83: 1449-1454.

54. Selvan, E. and Thomas, P.,(1995). Application of Flow Cytometric DNA Measurements in the Detection of Irradiated Onions J. Sci. Food Agric., 1995, 67, 293-297

55. Siddiqui, M. S., E. Filomeni, M. Francois, S. R. Collins, T. Cooper, R. V. Glatz et al. 2013. Exposure of insect cells to ionising radiation in vivo induces persistent phosphorylation of a H2AX homologue (H2AvB). Mutagenesis 28(5): 531–541.

56. Siddiqui, M.s., Taylor, P., and Crisp, P., (2020). Gamma-H2AX A Promising Biomarker for Fruit Fly Phytosanitary Irradiation Exposure in area world- wide management of fruit fly pests CRC PressTaylor & Francis Group,2020, 169-172

57. Stathopoulou P., Asimakis E.D., Khan M., Caceres C., Bourtzis K., Tsiamis G., (2019). Irradiation effect on the structure of bacterial communities associated with the oriental fruit fly, Bactrocera dorsalis, Entomologia Experimentalis et Applica 167:209-219, 2019.

58. Stefanova, R., Vasilev, N., Spassov, V., Stefan.L., (2010). Irradiation of food, current legislation framework, and detection of irradiated foods. Food Anal. Meth. 3, 225–252.

59. Stewart, E.M., (2001). Detection methods for irradiated foods. In: Molins, R. (Ed.), Food Irradiation – Principles and Applications. Wiley Interscience, New York, pp. 347–386.

60. Tamminga, S. K., Beumer, R. R., Kooij, J. G. and van Kampelmacher, E. H. (1975). Microbiological possibilities to demonstrate that strawberries have been irradiated Eur. J. of Appl. Microbiology.and biotechnology, 1975, 1, 79-93

61. Todoriki, S., Hasan, M., Miyanoshita, A., Imamura, T., Hayashi, T.,(2006). Assessment of electron beam-induced DNA damage in larvae of chestnut weevil, Curculio sikkimensis (Heller) (Coleoptera: Curculionidae) using comet assay. Rad. Phys. Chem.75, 292–296.

62. YULO-NAZAREA, M.T., COBAR, M.L.C., MANOTO, E.C., BILOG, G., (1991). Polyacrylamide gelelectrophoresis as a detection method for irradiated orientalfruit fly, The Nucleus, 29 (1991) 47-52.

63. Yunhee, J., Hyun-Kyu K., Hae-Jun P., .Joong-Ho K., (2015). Irradiated fruits can be identified by detecting radiation-induced markers with luminescence and ESR analyses for different trading fruits. Appl Biol Chem (2016) 59(1):59–65

64. Zanardi, E., Caligiani, A., Novelli E., (2017). New Insights to Detect Irradiated Food: an Overview. Food Anal. Methods, 2017Start typing here.