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Determination of irradiation markers in foods

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CHAPTER 1

Foreword

1. Foreword

This work addresses analytical methods to detect irradiated foodstuffs. These procedures concern both a scientific challenge and practical aspects behind food irradiation which is one of the most discussed food related issues of great importance to consumers and policymakers. The research activity herein carried out would also promote the awareness of the scientific community about the safety and security of the irradiated food supply through the development and application of sound analytical tools to detect treated commodities likely to be present at the marketing stage in all European countries, particularly in Italy. The overall goal is to ensure that all of the laws and regulations governing irradiated food marketing and production are endorsed and correctly implemented. Besides food industry and marketing requirements' fulfillment is to be considered the possibility to enhance consumers' confidence in properly processed food with approved radiation sources thus safeguarding consumer's freedom of choice between treated or non treated products. Therefore the analytical methods available for those national bodies involved in food surveillance activities are being considered as a definitive means to assure correct labeling. That it is true even in those cases where specific requirements are intentionally ignored by food processors to avoid adverse reaction of most uninformed consumers.

1.1 Food irradiation process

Food irradiation is a technological process in which several kind of food products are exposed to a controlled amount of radiant energy to achieve relevant benefits for both food industry and consumers. Nowadays the term "food irradiation" has generally come to describe the application of different ionising radiation sources such as electrons or energetic photons such as gamma rays and X-rays.

1.1.1 Food irradiation principles

The process can have a lot of beneficial applications such as to control the population of undesirable biological organisms in food or prevent the growth of both spoilage and pathogenic microorganisms. As a result of the interaction of radiation field with the exposed foods and biological agents associated with it, the process can control insects and parasites and reduce spoilage, as well as can inhibit ripening and sprouting. In order to achieve the desired effects in the process, the products are exposed to radiation fields in highly controlled and specific steps. First the food in bulk or packed form is moved by conveyer belt into a shielded room where is briefly exposed, depending on the strength of the

source, to a controlled radiation field. The food products are then moved around the source in a stop and go way to be irradiated in different positions when gamma rays are employed or in a continuous way if alternative non radioactive sources are used. Then all foods are removed from the radiation room by the conveyor system and, after the irradiation process is completed, the products are automatically taken out by conveyor and stored in the irradiated product area. Each stage is recorded automatically and the overall irradiation process is realized by means of a powerful and reliable computer system. Indeed the radiation devices and conveyor systems are always completely controlled by a computer in each stage of the irradiation treatment. Also in case of an abnormal situation, the irradiator turns off automatically providing audio and visual alarms. Three different irradiation technologies exist: *gamma rays sources*, electron beam accelerators and X-rays irradiators. The first system is routinely used to sterilize medical, dental and household products. The use of gamma rays from radioactive substance (Co-60 or Cs-137) permits the penetration of radiation through the food at depth of several centimeters. Both radionuclides do not emit any neutron particles avoiding induced radioactivity in food or around it. Irradiation is performed in a chamber equipped with massive concrete to shield gamma rays and it is therefore a safe practice. Irradiation source can also be made of an accelerator system with streams of *high energy electrons (maximum electron energy < 10 MeV)*, propelled out of an electron gun which can be switched on or off. No radioactivity is involved and the penetration power is only to a depth of about three centimetres; so the food to be treated must be of similar thickness otherwise two opposing beams can treat food that is twice as thick. The third technology is X-ray irradiation; the newest one that is still being developed. The *X-ray system (maximum photon energy < 5 MeV)* is a powerful version of the apparatus used in many hospitals and dental offices to take X-ray radiographies. The production of X-rays comes from the same electron accelerator systems when high energy electrons are directed at a thin plate target of a hard metal (high atomic number, Z- layer) generating a stream of X-rays coming out the other side. Like gamma rays, X-rays can pass through tick foods, also packed in pallet, and require a suitable shielding for safety. No radioactive materials are involved and X-ray machines will be the dominant technology in the next future. Provided the main function of irradiation is to preserve post harvest food and to improve the hygienic status and quality of commercial food commodities, the choice of source and irradiator configuration is dependent on several factors. The main elements to be considered are the following: the products to be treated, the operating expenses, the volume of commodities to be processed, the cost of the equipment and the sociopolitical environment in which the facility will operate. The dose of irradiation is usually expressed in Gy, a physical unit that measures the amount of energy absorbed by the food, a microbe or other

kind of substance being exposed to a radiation field (1 Gy = 1 Joule/kg). All kind of ionising radiations are sufficiently energetic to cause ejection of electrons from atoms or molecules, resulting in breakage of chemical bonds. Therefore the primary effects of interaction are ionization, dissociation and excitation. During the food irradiation process absorbed dose leads to chemical changes and free radicals are produced. Interacting with water these chemical species may induce secondary effects producing further free radicals which can diffuse far enough to reach and damage different biological compounds. So, radiolysis phenomenon is more important in foods with higher water content because of its influence on temperature, pH and dilution of solution by the presence or absence of oxygen. However adverse changes in food such as altered flavor, color and other properties along with rise in temperature minimally occur during the radiation treatment, thus also called as "cold process". All of chemical species generated during the process react with essential biomolecules, such as nucleic acids (DNA, RNA), membrane lipids, proteins and carbohydrates of bacteria, other pathogens and insects causing damage to them. As a result, most of organisms die or are unable to reproduce whereas food, with a very few exceptions related to losses of vitamins, is left virtually unchanged. Harmful bacteria, parasites and fungi are greatly reduced in number or eliminated thanks to the biological effect caused by the disruption of nucleic acid in the nuclei of cells, either through primary events (ionising) or through secondary free radical attack following water radiolysis. The sensitivity of a particular organism to the effects of ionising radiation is related to D-values, usually expressed as D_{10} , which is the decimal reduction value or the dose of radiation causing a 10-fold reduction in the numbers of a given microorganism. An important reason for the comparatively high sensitivity of DNA is its enormous molecular size. Indeed, parasites and insect pests, which have large amount of DNA, are rapidly killed by extremely low doses of irradiation with D-values of about 0.1 kGy or less. Bacteria are more resistant to radiation, because of smaller amount of DNA, with D-values ranging from 0.3 to 0.7 kGy, while bacterial spores exhibit D-values on the order of 2.8 kGy. The increasing D-values are correlated to the content of DNA and viruses, being the smallest pathogens that have nucleic acid, are therefore resistant to generally irradiation maximum doses approved for foods. Although they may have D-values higher than 10 kGy, recent studies suggest that, depending on food matrix, viruses can become sensitive to electron beam radiation at levels significantly lower than those produced with traditional Co-60 irradiation. In summary with relatively modest radiation doses (1-5 kGy) it is possible to kill the organisms responsible for foodborne illness and spoilage without affecting the nutritional and sensory qualities of foods. Furthermore, it's worth noting that heating, drying and cooking may cause higher nutritional losses compared to those caused by irradiation.

1.1.2 *Applications of food irradiation*

Benefits of food irradiation are strictly linked to the applied dose, the purposes to achieve, food matrix composition and its local environment (temperature, packaging and combination with other food processes) at the time of radiation treatment. Following the recommendations of the Joint FAO/IAEA/WHO Expert Committee the applications are organized according to the range of delivered dose, even if it should underline that such categorization is to some extent arbitrary. Application of food irradiation is generally divided into three categories. The first level regards low-dose applications for dose values in the range up to 1 kGy, which are concerned with inhibition of sprouting, delaying of maturation, parasite disinfection and insect disinfestations. The second range comprises medium dose applications in the range of 1-10 kGy, which are generally concerned with the control of food borne diseases and retardation of spoilage; the third category includes all those applications at doses higher than 10 kGy, which are associated with radiation sterilization of foods devoted to specific purposes such as space food. In Europe and in the USA, the corresponding regulatory agencies have already approved several petitions permitting the irradiation of a wide range of food including spices, red meats, poultry, fresh produce, eggs and others food categories of both animal and plant origin for the control of foodborne pathogens and shelf-life extension, as well as disinfestation applications for all foods. Further petitions to extend the irradiation to particular type of prepared food such as ready to eat foods are now under review or are still pending.

According to numerous studies conducted worldwide, irradiation within approved doses in the *low-dose* range has been shown to have two main applications: *sprouting inhibition* and insect disinfestation. Prolonged storage up to several months of various sprouting foods such as potatoes, garlicks and onions is desirable in international trade. Even if sprouting can be inhibited by refrigeration or other chemicals such as maleic hydrazide and isopropyl chlorocarbamate in a preharvest or postharvest steps respectively, irradiation can be a reasonable alternative. It has the advantages of being cheaper than physical method and as effective as application of chemical methods without leaving any residues. Because of health reasons many countries have already banned or are prohibiting the use of chemical sprout inhibitors. So many chemical agents are being replaced for irradiation measures. Sprouting of potatoes is effectively prevented at doses of 0.1 kGy while it is significantly delayed at doses as low as 30 Gy. Onions require a dose of 50-60 Gy while garlic bulbs require about 0.1 kGy. All of these industrial applications require a practical waiting period between harvest and irradiation of about two weeks because the treatment can diminish the ability of tubers to form a protective periderm against parasites causing spoilage.

Therefore, under accurate and established conditions, irradiation can be used as an alternative to chemical sprout inhibitors for tubers, bulbs and root crops. Controlling the germination of barley is also another useful application of considerable economic impact for the brewing industry. In fact very low doses of radiations retard root growth. In this way two positive effects are obtained: production of high quality malt and reduced losses of it resulting from too rapid sprouting. Low dose irradiation has also found useful application in stimulating and controlling seed germination to maximize the development of ascorbic acid and riboflavin in soybean sprouts. A promising and important application of food irradiation is for *insect disinfection*. So far food industry has achieved this result by using fumigants such as ethylene dibromide or ethylene oxide. However for health and environmental reasons many countries have banned or severely restricted the use of these chemicals while other fumigants are considered harmful to the environment and less effective than radiations. Therefore irradiation practice is now widely adopted as an effective means to decontaminate certain foods, especially in grain and grain products, where excellent control of insects is necessary. Many ongoing studies suggest that the necessary absorbed dose is in the range of 150 - 700 Gy where sterility is already achieved at doses in the lower end while higher levels have to be administered to kill adult insects. Generally low irradiation up to 1 kGy is effective against insects and is adopted as a pest control treatment on quarantined fruits and vegetables to prevent the importation of harmful pests, such as Mediterranean fruit fly. In fact insects are easily distributed by international trade and when such pests move from original environment to new one without their natural enemies they can flourish and become an unmanageable issue. Because of this potential consequence and to minimize the associated risk many countries have established and set up accurate quarantine procedures which require the use of fumigation or heat or cold treatment of fruit that is not ripe. However irradiation has the unique potential to be applied as alternative to classical measures for many type of fresh produce because it can be used on riper fruit and on fruit that cannot tolerate heat treatment. If one considers the increasingly decline of pesticide use in agricultural production it becomes clear the important role played by the numerous irradiation quarantine practices. Furthermore the softening and browning associated with the ripening of certain fruits and vegetables, such as bananas, mangoes and mushrooms can be also delayed with irradiation. In summary, at present no other method comes close to delivering the top fresh fruit quality that food irradiation does. Many and well established applications are in the range of *medium doses* (1-10 kGy). One of the principal uses of food irradiation is for *pathogen reduction*. Considerable attention has been paid by most governments to ensuring higher level of food safety. Due to our complex food supply and changing lifestyles greater chances of breakdowns are

occurring and as a result there is a need to employ more effective control measures. In that sense food irradiation has been shown to be extremely effective in controlling our exposure and decreasing associated risk to offending foodborne pathogens. The radiation technology has been used to reduce or eliminate various pathogens in beef, poultry, pork, lamb, fish and seafood and it has also been very successful in eliminating or greatly controls the heavy load of undesirable pathogens in dried vegetables, herbs and spices. Irradiation has also been employed to destroy pathogens from certain dairy products and from naturally fermented products such as Chinese soy sauce. Indeed, many studies have demonstrated that irradiation within approved dose levels has the ability to destroy at least 99.9 % of common foodborne organisms, including pathogens such as various species of *Salmonella*, *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*, which are associated with meat and poultry. It is also effective against *Vibrio* species associated with seafood and against parasites, such as *Toxoplasma gondii* very common in many animal species, and *Trichinella spiralis* particularly found in pork. Excellent control of all these organisms can be achieved with doses in the range 1-3 kGy but unfortunately, radiation treatment is not so effective against microbial toxins and toxins produced by molds. Depending on the product and its place in the distribution system, the methodology used to treat foods will vary and the minimum required dose for food safety purposes are essentially established by the desired degree of reduction in the pathogenic population and the D_{10} value of the organism of concern. On the other hand the maximum dose is established either by government regulations or by the tolerance of foods limited by the development of negative sensory characteristics. Another promising application of food irradiation is for killing the harmful microorganisms that cause spoilage or product deterioration resulting in a *shelf-life extension* of perishable foods. The useful shelf-life of many fruits and vegetables, meat, poultry, fish and seafood can be considerably prolonged at the same dose levels appropriate for control of foodborne pathogens. Moreover treatment in combination with refrigeration does not alter flavor or texture without any significant quality deterioration. Most food spoilage microorganisms are destroyed at doses well below 5 kGy and for instance, dose levels of 2, 5 kGy can extend the shelf life of meat products (chicken and pork) by as much as a few weeks, while the application of 5 kGy can prolong the shelf life of low fat fish to several weeks compared to a few days if not treated. In addition the shelf life of various cheese can be extended significantly by eliminating molds even at doses less than 0.5 kGy while promising application appear for strawberries, carrots, mushrooms, papayas and packaged leafy vegetables. A particular worldwide application of radiation deals with *spice irradiation* because fresh plants from which these products are derived are very often contaminated by microorganism living in the soil or present in

windblown dust or in bird droppings deposited on the food surfaces. Furthermore irradiation in the dose range of 5-10 kGy is considered the most effective and safe method for pathogens' control preserving the natural contents of essential oils, generally heat sensitive. Higher doses of radiations can also be used to greatly reduce or sterilize the non-pathogenic microorganism and bacterial spore load of dried spices, herbs and dry vegetable seasonings. Unlike EU countries, in the USA these products can be treated up to 30 kGy achieving a more easy spoilage prevention and microorganism control when spices are pre-treated. However irradiation does not sterilize food nor does make it shelf stable and irradiated foods must be properly handled: refrigerated and cooked prior to consumption.

The last category of food irradiation applications considers **high dose** food sterilization. Although some foods such as fresh fruits and vegetables deteriorate when exposed to high radiation doses (> 10 kGy) many others including meat, poultry and certain seafood still exhibit good quality, provided that certain precautions are taken. As a consequence it is possible to effectively sterilize these products with doses in the range of 25-45 kGy. Even if the cost of sterilized food increases related to higher delivered dose, these irradiated products are of great importance to hospitalized patients with suppressed immune systems or for special applications such as space foods for astronauts.

In conclusion, because of irradiation's effectiveness in controlling common foodborne pathogens and in treating packaged food, thereby minimizing the possibility either of recontamination or cross-contamination prior to consumer use, regulatory agencies should consider irradiation as an effective critical point in a HACCP system.

CHAPTER 2

Objectives

2. Objectives

Today irradiation is slowly gaining popularity as one of the successful techniques to preserve food with minimum change to the functional, nutritional, and sensory properties of food products. However the absence of common procedures and regulations can prevent the spread use of this preservation technology although its benefits are well established on sound scientific basis. The existence of different requirements and standards related to the application of food irradiation throughout the world is a major concern both in international trade by food processors and official checks by national authorities of a country.

2.1 Detection methods

Food irradiation involves radiation interactions with water and other biological molecules in a food system with the production of numerous radiolytic products, which generally act as oxidizing agents and can cause several changes in the molecular structure of organic matter. Radiations interact with foods as well as with all living organisms present in it. Indeed DNA interaction with high energy field (electrons or photons) results in two biological effects on microorganisms: population death, depending on the severity of radiation damage and the specific species' radiosensitivity, or reproduction prevention for living cells. On the other hand radiation processing, like other preserving techniques, results in physicochemical changes in all irradiated food. In principle any change to some food component can be used to detect the treatment. The nature and extent of these effects mainly depend on the kind of food products processed and the irradiation dose. At present the methods used for the detection of irradiated foods are based on physical, chemical, biological, and microbiological changes in food products during irradiation, although these changes are minimal. Extensive research has been undertaken which resulted in the development of a range of tests that can be used to reliably determine the irradiation status of a wide variety of foods. To date, the European Committee for Standardization (CEN) has issued 10 European standards for detection of irradiation process in various food commodities (EN 1784:1996, EN 1786:1996, EN 1787:2000, EN 1788:2001, EN 13708:2001, EN 13784:2001, EN 13783:2001, EN 1785:2003, EN 14569:2004, and EN 13751:2009). All of CEN methods have been adopted by Codex Alimentarius Commission (CAC). The application field of each standard is reported below.

EN 1784:1996 specifies a method for the identification of irradiated food containing fat by gas chromatography (GC). The method has been successfully applied in interlaboratory tests on raw chicken, pork, and beef as well as on

Camembert cheese, avocado, papaya, and mango. Detection of irradiated raw meat and Camembert cheese has been validated for doses of approximately 0.5 kGy and higher, whereas detection of irradiated fresh avocado, papaya, and mango has been validated for doses of approximately 0.3 kGy and higher.

EN 1786:1997 specifies a confirmatory method for the detection of meat and fish containing bone that have been treated with ionizing radiation, by analyzing the ESR spectrum of the bones prepared in both powder form and fragments. Interlaboratory studies have been successfully carried out only with beef bones, trout bones, and chicken bones. The main findings from those exercises are the following: in the case of meat bones, the results of this detection method are not significantly influenced by heating of the sample (e.g., boiling in water) and by storage times of up to 12 months.

EN 1787:2000 describes a method for the detection of food containing cellulose that has been treated with ionizing radiation, by analyzing the resulting ESR spectrum. Interlaboratory studies have been successfully carried out with limited foods: pistachio nutshells, paprika powder, and fresh strawberries. Detection of irradiated pistachio nuts has been validated for doses of 2 kGy and higher, whereas paprika powder has been validated for doses of 5 kGy and higher. Fresh strawberries have been validated for doses of 1.5 kGy and higher.

EN 1788:2001 concerns a confirmatory method for the detection of irradiation treatment of food and/or food ingredients by which silicate minerals can be isolated and then analysed by TL technique. The method has been successfully tested in interlaboratory tests with herbs and spices as well as their mixtures, shellfish including shrimps and prawns, both fresh and dehydrated fruits, vegetables, and potatoes. Detection of irradiated shellfish has been validated in the range of 0.5–2.5 kGy, whereas it has been validated for doses of approximately 1 kGy for fresh fruits and vegetables.

EN 13708:2001 specifies a confirmatory method for the detection of foods containing crystalline sugars that have been treated with ionizing radiation, by analyzing the resulting ESR spectrum. Interlaboratory studies have been successfully carried out on dried figs, dried mangoes, dried papayas, and raisins. Detection of irradiated dried figs, dried mangoes, dried papayas, and raisins has been validated.

EN 13783:2001 specifies a microbiological screening method for the detection of irradiation treatment of herbs and spices, using the combined direct epifluorescent filter technique (DEFT) and aerobic plate count (APC). It is recommended to confirm positive results using a standardized method and it has been successfully tested in interlaboratory exercises with herbs and spices. Some spices such as cloves, cinnamon, garlic, and mustards may exhibit APC false-positive result due to the presence of components with antimicrobial activity.

EN 13784:2001 specifies a screening method for foods that contain DNA. The DNA comet assay is not radiation specific; therefore, it is recommended to confirm positive results using a standardized method. Interlaboratory tests have been successfully carried out with a number of food products, of both animal and plant origin, such as various meats, seeds, dried fruits, and spices.

EN 13751:2002 specifies a method for the detection of irradiated foods using photostimulated luminescence (PSL). It is necessary to confirm a positive screening result using calibrated PSL or another standardized or validated method. The method has been successfully tested in interlaboratory exercises using shellfish, herbs, spices, and seasonings.

EN 1785:2003 provides for a method to identify irradiated food containing fat by means of gas chromatography–mass spectrometry (GC/MS). The method has been successfully tested in interlaboratory trials on raw chicken, pork, liquid whole egg, salmon, and Camembert cheese, whereas it failed in the case of mangoes and papayas. The selected 2-ACBs analyzed in interlaboratory studies 2-dodecylcyclobutanone (DCB) and 2-tetradecylcyclobutanone (TCB) derived from palmitic acid and stearic acid, respectively.

EN 14569:2004 specifies a microbiological screening method comprising two procedures, to be carried out in parallel. It concerns the identification of an unusual microbiological profile in poultry meat. It is recommended that a positive result be confirmed using a standardized reference method for the detection of irradiated food. The method has been successfully tested by interlaboratory trials only to whole or parts of poultry (e.g., breast, legs, and wings of fresh, chilled, or frozen carcasses with or without skin).

2.1 Aims

The need for reliable and routine methods to determine whether or not food has been irradiated is a result of the progress made in commercialization of the food irradiation process. As a consequence consumer demand for clear labelling of the treated food is arisen due to greater international trade in irradiated foods and non harmonized regulations relating to the use of the technology in many countries. Because of the slight changes induced in foods by radiations, development of effective methods is a challenging scientific task. Although there is no a perfect and universal method to identify food products treated with ionising radiations it is important to have many sensitive analytical methods to detect irradiation processing independently if the food products have been labeled as such. Proper control of irradiation processing of food is very critical to facilitate trade of irradiated foods, safety and to enhance consumer confidence and freedom of choice. All regulatory authorities in all countries are thus

interested in having reliable methods to detect irradiated foods as well as estimation of dose. This research activity is aimed at improving current methods and developing new dosimetric protocol so that administrative control of food once it leaves the irradiation facility can be supplemented by an additional means of enforcement. Furthermore the availability of such analytical tools would help strengthen national regulations on irradiation of specific foods, and enhance consumer confidence in such regulations. Although extensive research has resulted in the development of a range of tests which can be used to properly determine the irradiation status of a wide variety of food, more analytical efforts are required. Reinforcing of consumer confidence in the overall process can be achieved through the availability of reliable identification methods which would be of assistance in establishing a system of legislative control, and help to enhance acceptance of irradiated foods.

The following objectives were identified for investigation in this work:

- a) Study the nature of radiation induced paramagnetic centres formed after exposure of food to commercially recommended doses of radiation.
- b) Extension of the ESR methodology to various types of food likely to be treated with radiations and present at the marketing stage.
- c) Implementation and standardization of luminescence techniques applied to two kinds of luminescence centres: polyminerals extracted from various foods and bioconstituents of foods.
- d) Comparison of TL characteristics induced in irradiated inorganic minerals extracted from shellfish using different isolation procedures.
- e) Study the properties of radiation induced radicals and their suitability as a dosimetric parameter to estimate absorbed dose level.
- f) Evaluation of reliable routine procedures in food tests comprising a screening method in conjunction with suitable confirmatory analysis for a wide range of food products.
- g) Application of versatile screening (PSL, DNA Comet Assay) and confirmatory methods (ESR, TL) in official checks covering many food categories.

CHAPTER 3

Irradiated bivalve mollusks: Use of EPR spectroscopy for identification and dosimetry

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3. Irradiated bivalve mollusks: Use of EPR spectroscopy for identification and dosimetry

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3.1 Abstract

High energy radiation treatment of foodstuff for microbial control and shelf-life extension is being used in many countries. However, for consumer protection and information, the European Union has adopted the Directives 1999/2/EC and 1999/3/EC to harmonize the rules concerning the treatment and trade of irradiated foods in EU countries. Among the validated methods to detect irradiated foods the EU directives also include Electron Paramagnetic Resonance (EPR/ESR) spectroscopy. We describe herein the use of EPR for identification of four species of bivalve mollusks, i.e. brown Venus shells (*Callista chione*), clams (*Tapes semidecussatus*), mussels (*Mytilus galloprovincialis*) and oysters (*Ostrea edulis*) irradiated with ⁶⁰Co γ -rays. EPR could definitely identify irradiated seashells due to the presence of long-lived free radicals, primarily CO₂⁻, CO₃³⁻, SO₂⁻ and SO₃⁻ radical anions. The presence of other organic free radicals, believed to originate from conchiolin, a scleroprotein present in the shells, was also ascertained. The use of one of these radicals as a marker for irradiation of brown Venus shells and clams can be envisaged. We also propose a dosimetric protocol for the reconstruction of the administered dose in irradiated oysters.

3.2 Introduction

Treating aliments of either animal or vegetable origin with high energy radiations is a rapid and environmentally friendly procedure to eliminate microbial growth, to disinfect pests, to prevent sprouting, and overall to increase the foodstuffs shelf life. The organoleptic properties of foods are not altered by irradiation, provided they have been administered an appropriate dose that may vary with the kind of food. At the same time, the nutrient value

and digestibility of proteins, fats and carbohydrates (macronutrients) are hardly affected, while among micronutrients some of the vitamins seem susceptible to irradiation (Diehl, 1995; Farkas, 2006). Despite these overall beneficial consequences, consumer organizations seem rather inclined to oppose food irradiation on a two-fold basis: an unjustified public concern on the use of a methodology that has been, and to some extent still is wrongly associated with nuclear power and radioactivity and, on a more rational ground, the paucity of reliable detection methods combined with the absence of a uniform codification at international level. A number of different techniques have been applied to the identification of irradiated foodstuffs, including both physical and chemical methods (Schreiber et al., 1993; Haire et al., 1997; Marchioni, 2006; Arvanitoyannis et al., 2009). As treatment of aliments with high energy radiations normally results in the formation of free radicals in either or both their organic or inorganic components, in the last three decades a substantial number of investigations have been addressed to the identification of all sorts of irradiated foodstuff through the use of EPR spectroscopy, the technique of choice for the detection and identification of paramagnetic species (Rahman et al., 1995; Raffi and Stocker, 1996; Desrosiers, 1996; Anderle et al., 1998; Delinceè, 2002). At the European level this resulted in the issuing of official protocols for the EPR detection of irradiated foodstuffs containing bone (EN, 1996), cellulose (EN, 2000) or crystalline sugar (EN, 2001). Several EPR studies have also been addressed to the identification of irradiated seafood, i.e. fishes, crustacea and mollusks. Early investigations showed that bones from irradiated fishes, e.g. brown trouts and sardines (Raffi et al., 1989), give rise to an EPR signal similar to that exhibited by irradiated meat bones and assigned to the orthorhombic CO_2^- radical trapped in the hydroxyapatite matrix. Salmon (Goodman et al., 1989) and mackerel (Abdel-Rehim et al., 1997) samples provided similar results. EPR spectroscopy has also proved successful in the identification of irradiated shrimps, the cuticula of which exhibits irradiation specific signals (Dodd et al., 1985; Desrosiers, 1989; Dodd et al., 1989; Goodman et al., 1989; Raffi and Agnel, 1990; Stewart et al., 1992; Stewart et al., 1994; Chung et al., 2002) that on the other hand varied with the examined species (Morehouse and Ku, 1992; Morehouse and Desrosiers, 1993) and were sometime undetectable for administered doses ≤ 1 kGy (Cutrubinis et al., 2007). The signal from the CO_2^- radical, superimposed to other minor signals and to a sextet due to naturally present Mn^{2+} ions, allows identification of irradiated crabs (Nam et al., 2000; Maghraby, 2007). Comparatively fewer EPR studies have been addressed to the identification of irradiated seashell mollusks, attention having been mainly focused on mussels (Desrosiers, 1989; Raffi et al., 1996; Sin et al., 2005), clams (Douifi et al., 1998; Strzelczak et al., 2001) and oysters (Raffi et al., 1996;

Strzelczak et al., 2001). While the above EPR studies have clear significance for identification purposes, we are not aware of reliable EPR protocols for the dose reconstruction of irradiated seashells.

Since the early 1970s the Italian legislation permits the treatment of some vegetables (i.e. garlic, onions and potatoes) with ionizing radiations (Italian Government Decree, 1973). Later on Italian rules on this matter have been harmonized with the European Community directives (Italian Government Decree, 2001) but irradiation, inside the country, of meat and seafood for human consumption remains prohibited. Seafood has a substantial role in the Mediterranean diet, and although Italy is a peninsula with a coastline extending for over 7500 km, a significant fraction of the consumed seafood is imported from other countries. Because of the international inhomogeneity of the guidelines concerning food irradiation, the Italian Ministry of Health has implemented a project aimed to define and/or update protocols for the identification of irradiated food. We report here on an identificative EPR study carried out within this framework on some seashell mollusks of large local consumption, namely clams (*Tapes semidecussatus*), brown Venus shells (*Callista chione*), mussels (*Mytilus galloprovincialis*) and oysters (*Ostrea edulis*). In addition, we outline a dose reconstruction EPR protocol believed to be acceptably reliable for irradiated oysters.

3.3 Materials and methods

3.3.1 Irradiation procedure

All the absorbed doses mentioned in this paper are absorbed dose to water. Irradiation of seashells with γ -rays was carried out at ISOF, Bologna, by means of a ^{60}Co Nordion Gammacell 220 having a dose rate of about 10 Gy/min. The temperature of the irradiation chamber was about 21-24°C. Whole mollusks were sealed in plastic bags and inserted into a plastic phantom with wall thickness of 0.4 g/cm², which is suitable for establishing electron equilibrium. Administered doses were in the range 0.15 to 7 kGy. The dose rate of the Gammacell for the reference geometry was determined with the alanine reference transfer dosimeters from Risø High Dose Reference (HRD) Laboratory. It should be pointed out that the dose rate distribution within the irradiation chamber of the Gammacell was not completely uniform, being larger towards the side walls and lower towards the top and bottom walls, with limiting variations of ± 10 -15%. This problem was minimised using only the central portion of the irradiation chamber, but it was impossible to avoid that individual samples experienced dose rate differences of $\pm 5\%$.

3.3.2 Materials

Seashells, distributed by UNI EN ISO 9001:2000 certified companies, were bought in a local market. Clams and brown Venus shells came from the Italian northern Adriatic sea, mussels from Atlantic Galicia coast in Spain, while oysters had been grown out of the Brittany coast in France. After irradiation the shells were opened, the animal was carefully cleaned off, the shells were first degreased with methanol then repeatedly washed with fresh water and eventually dried in a vacuum until their weight remained constant. The clean and dry shells were then ground in a knife-mill and the resulting fine powder (average grain diameter \approx 0.2 mm) was stored in glass bottles without any particular care.

3.3.3 EPR measurement

EPR spectra were recorded at room temperature using an upgraded Bruker ER200D/ESP300 EPR spectrometer operating in the X-band (9.3-9.7 GHz), equipped with an NMR Gaussmeter for the calibration of the magnetic field and with a Systron-Donner frequency counter for the determination of g-factors that were corrected with respect to that of the perylene radical cation in concentrated sulphuric acid ($g = 2.00258$) (Wertz and Bolton, 1972).

Irrespective of whether the EPR experiments were carried out for identification or for dosimetric purposes, all samples were prepared as follows: a pyrex tube (i.d. 4 mm) of known weight was filled up to an height of 15 mm with shell powder (this corresponded to ca. 200 mg of material) and the exact amount of the sample was determined by weight difference.

Identification EPR spectra – Spectra were the sum of 4 scans recorded over a field width of either 20 mT or 80 mT, with modulation amplitudes in the range 0.005 - 0.5 mT, a receiver gain of 1×10^4 and an attenuation of 20 dB (corresponding to a power of ca. 2.3 mW). No significant variations of the EPR spectral pattern were observed rotating the samples inside the EPR cavity.

Dosimetric EPR spectra – Single scan spectra of irradiated oysters carried out for dosimetric measurements were run on a field width of 20 mT, with a modulation amplitude of 0.2 mT and a receiver gain of 1×10^4 . The intensity A_{exp}^i of the signals was measured as the peak-to-peak amplitude of the spectral line with $g = 2.00349$ (see below). In a first step the saturation curve of this signal was determined for samples that had received the highest dose, i.e. 7 kGy, varying the attenuation from 35 dB (ca. 0.07 mW) to 0 dB (ca. 223 mW). As signs of signal saturation were evident for attenuations lower than 26 dB, an operative attenuation of 32 dB was chosen to avoid conditions where the amplitudes of the EPR signals might be unreliable. To correct sensitivity variations of the cavity for each individual sample, the spectrum of a synthetic ruby crystal

permanently located in a corner of the spectrometer resonant cavity was recorded without displacing the sample and its double integral was measured. The correction factor for each sample was determined as the ratio between the double integral of the ruby signal recorded with the empty cavity, I_{st} (taken as a standard) and that of the ruby signal recorded in the presence of the sample, I_{sa}^i , using exactly the same instrumental setup as to modulation amplitude, receiver gain, signal attenuation, scan width and number of points. As a simpler alternative, the double integrals can be replaced by the peak-to-peak amplitudes of the ruby lines, A_{st} and A_{st}^i recorded as indicated above. In our hands the two methods proved equivalent as they resulted in A_{norm} values differing by less than 0.05%. The signal intensity was monitored over a time span of more than 60 days, the reading interval increasing as time elapsed.

3.4 Results and Discussion

3.4.1 Identification studies

In general, the shells of unirradiated bivalvian mollusks are naturally EPR active. Their spectra normally show a single line with a g -factor value of ca. 2.0041, which is attributed to a free radical deriving from the organic residue present in the shell material, and a set of six lines due to Mn(II) ions, also present in the shell material, with a separation of ca. 9.3 mT, plus five equally spaced doublets in between each pair of contiguous Mn(II) lines due to forbidden transitions ($\Delta m_1 = \pm 1$). Although the four unirradiated matrices examined in the present investigation did not drastically deviate from the above general features, they did not exhibit the same EPR behaviour.

Brown Venus shells (Callista chione) – The examined samples of unirradiated brown Venus shells only exhibited a weak signal from a residual organic radical (single line with a g -factor value of 2.0045₀), and no Mn(II) derived signals (see Figure 1a). After irradiation a strong complex anisotropic signal appeared (see Figure 1b), similar to that previously observed for the shells of other irradiated mollusks, and resulting from the overlapping of the signals from the orthorhombic and isotropic CO_2^- and isotropic and SO_3^- radical anions ($g = 2.0020_0, 1.9974_3, 2.0007_5$ and 2,0031₀) (Ikeya, 1993; Raffi et al., 1996).

The spectral components have different intrinsic linewidth; this signal was strongly dependent on the instrumental setup, e.g. attenuation and modulation amplitude. In particular, a reduction of the modulation amplitude leads to a much more resolved signal (See Figure 1c) where several of the different components of the CO_2^- , and SO_3^- radical anions become detectable, possibly along with some components of CO_3^{3-} radical trianion.

In all instances this composite signal remained detectable over a period of several months, although its intensity decreased with time, and could therefore be safely used as a marker of irradiation. On the other hand, as already stated for other matrices (Strzelczak et al., 2001), the overlapping of components from different radicals and the variation of the spectral intensity may cause inaccurate dose estimates, especially when dealing with items for which the date of a possible treatment is not known.

For all the examined samples, irradiation also led to the appearance of weaker signals (see Figure 1c, green and red starred lines) in the wings and the centre of the spectral region, their detection being unprecedented to the best of our knowledge. While the inner signals (green starred lines) disappeared in a month or so, the outer signals (red starred lines) could be detected after more than one year from irradiation. We believe that these apparently isotropic signals originate from organic radicals embedded in the inorganic material of the shell.

The organic material most likely to be found in the ground shell of brown Venus shells is conchiolin, a water insoluble basic protein in which glycine, valine, lysine, leucine and arginine account for 87% of the aminoacid contents (Bowen and Tang, 1996).

The spectrum consisting of the red starred lines has a g -factor of 2.0041_5 and can be rationalized as being due to a radical where the unpaired electron is coupled with two slightly magnetically nonequivalent hydrogen atoms ($a_{\text{H}} = 1.943$ and $a_{\text{H}} = 1.982$ mT), one nitrogen atom ($a_{\text{N}} = 0.110$ mT) and another hydrogen atom ($a_{\text{H}} = 0.110$ mT). These spectral parameters seem fairly consistent with a radical of the type $\text{R-CH}_2\text{-}\dot{\text{C}}(\text{NHR}')\text{-C(O)-R''}$ (Fischer and Paul, 1977; Neugebauer, 1987). Although at first sight the two outer multiplets might have been thought to reflect a weak interaction of the unpaired electron with a methyl group, their intensity ratio was less than the expected 1:3:3:1. Besides, none of the aminoacids present in the protein seems to have a structure that could justify the formation of a radical exhibiting a small coupling with a methyl group and large couplings with two magnetically nonequivalent hydrogen atoms. We therefore prefer to assign the red starred spectrum to a radical having the structure mentioned above, although it is impossible to identify the aminoacid from which this radical originates as this molecular fragment is present in many of the conchioline constituents (the lysine, leucine or arginine residues, and in the less abundant tyrosine, histidine, cysteine, phenylalanine, methionine, glutamic acid, serine and aspartic acid). The observation of this “isotropic” spectrum is unexpected for powder samples, and we tentatively explain it with the protein or its fragment bearing the radical centre being located inside a “closed porosity” (a sort of zeolite like cavity of large dimensions) of the shell inorganic material, which would also account for the remarkable persistence of the EPR signal. It appears worth stressing that, due to its distinctive features and its persistence,

this spectrum provides an additional marker of irradiation, especially valuable over long time interval (e.g. frozen mollusks).

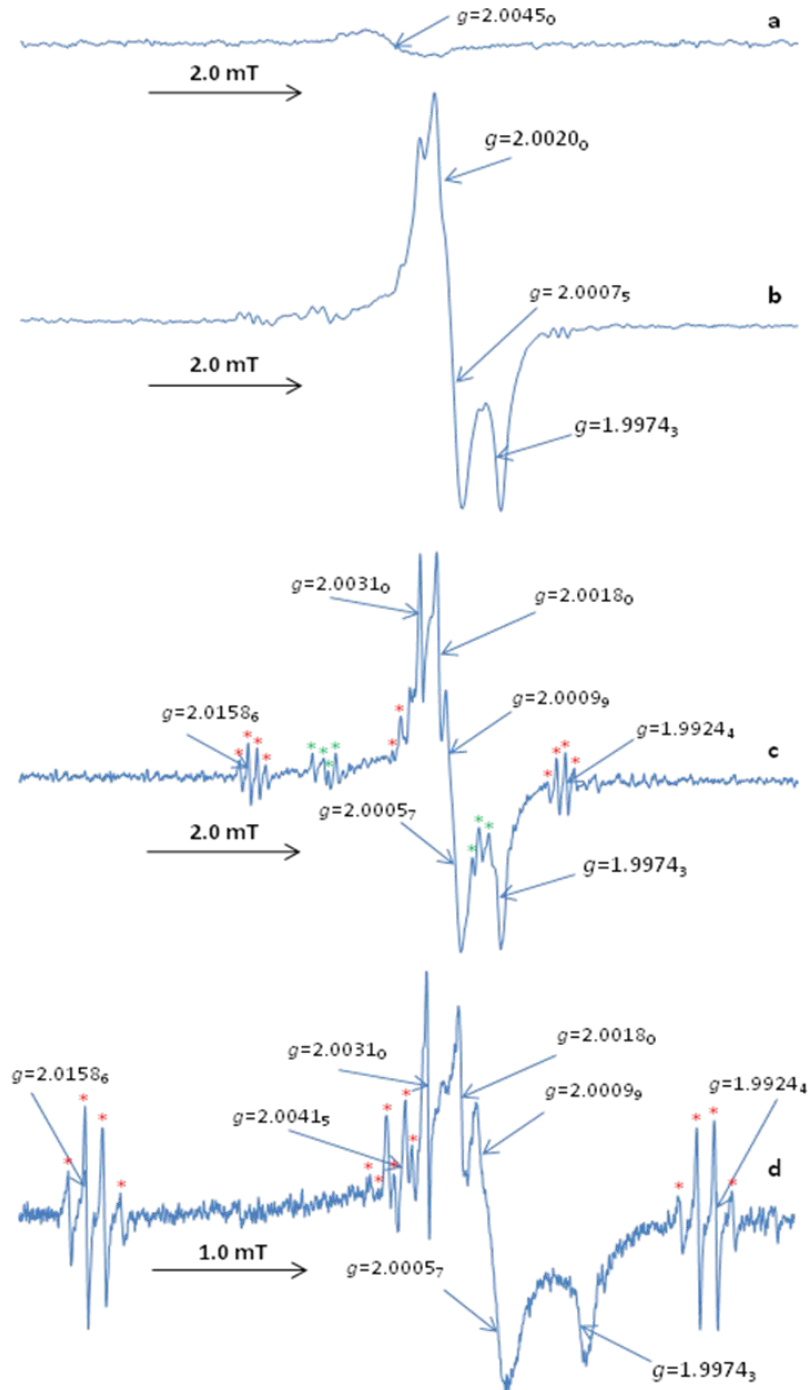


Figure 1 – EPR spectra exhibited at room temperature by brown Venus shells: **a)** unirradiated (0 kGy, sw 10 mT, ma 0.05 mT, rg 1×10^5); **b)** irradiated (1.5 kGy, sw 10 mT, ma 0.05 mT, rg 1×10^5); **c)** irradiated (1.5 kGy, sw 10 mT, ma 0.02 mT, rg 5×10^5); **d)** same as **c)** after six months (1.5 kGy, sw 5 mT, ma 0.02 mT, rg 1×10^6).

As for the radical responsible for the green starred lines its spectrum is characterized by a slightly higher g -factor (2.0049_5) and basically shows coupling of the unpaired electron with one hydrogen atom ($a_{\text{H}} = 1.970$ mT) and, possibly, with a nitrogen atom ($a_{\text{N}} \approx 0.140$ mT). While hydrogen abstraction from the methylenic group of a glycine (the most abundant conchiolin molar component) residue would lead to a radical with a single α -hydrogen the nitrogen splitting is smaller than expected for such a radical and we prefer to attribute this spectrum to a species deriving from a radiation induced fragmentation process of the protein.

Clams (Tapes semidecussatus) – In analogy with what found for the shells of other previously investigated mollusks (see above), EPR spectra of powdered shell of unirradiated clams featured a single line ($g = 0.20042_3$) due to an organic radical, and six barely detectable Mn(II) lines with an average separation of 9.355 mT (see Figure 2a), that become more evident at higher field modulation. After irradiation (1.5 kGy) a strong complex anisotropic signal appeared, similar to that observed for irradiated brown Venus shells, and due to the signals from the CO_2^- and SO_3^- radical anions. In particular, using a fairly low modulation amplitude (max 0.02 mT) components due to isotropic SO_3^- ($g = 2.0030_3$), isotropic ($g = 2.0005_2$) and orthorhombic CO_2^- ($g = 2.0017_4, g = 1.9973_8$) radical anions were detected (see Figure 2b). At higher amplification of the signal two groups of lines, hardly detectable in the spectra recorded at normal amplification (see Figure 2b), could be readily observed (red starred lines in Figure 2c) on the wings of the main spectrum. Their multiplicity and their g -factor values are the same already observed for the outer groups of lines in the spectra from irradiated brown Venus shells, and we therefore assign these lines to the same organic radical believed to originate from one of the aminoacids residues present in conchiolin. It is known that conchiolin protein is present in brown Venus shells in much a larger amount than in clams, which might explain the much weaker intensity of the related radical that we observed in the latter mollusks. On the other hand, because conchiolin is located in the periostracum (or mantle), the outer epithelium of the shell, it can be worn by the mechanical action of rocks, sand and/or water and therefore its amount is bound to significantly vary for each individual item of the same species. Despite this, we nevertheless observed signals from the conchiolin deriving radical of comparable intensities for all the samples of the same species, and we conclude that also for clams these signals are a reliable marker of irradiation.

Mussels (Mytilus galloprovincialis) – Unirradiated mussels naturally exhibited EPR spectra in line with what previously reported (Desrosiers, 1989; Raffi et al., 1996; Seletchi and Dului, 2007) showing the Mn(II) sextet (plus the ten lines

corresponding to the forbidden transitions) along with a fairly intense signal with $g = 2.0044_3$ due to an organic radical embedded in the inorganic material (see Figure 3a). The administration of a dose of 1.5 kGy led to a strong signal (see Figure 2b), apparently more intense than that observed with clams or brown Venus shells. The complexity of this signal became more evident when the spectra were recorded at low field modulation amplitude, components from axial and isotropic SO_3^- and orthorhombic CO_2^- radical anions being clearly detectable. Although the signal from irradiated mussels may appear similar to those exhibited by brown Venus shells and clams, it is characterized by a greater intensity of the signal at $g = 2.0052_9 \div 2.0056_0$ and attributable to the isotropic SO_2^- radical anion (see Figures 3b-d). A second noteworthy particularism of the EPR spectra of irradiated mussel samples is the vanishingly small intensity of the lines due to the conchiolin-derived radical, in fact hardly detectable even using a high amplification of the EPR signal. As shown in Figure 3d, the signal from orthorhombic CO_2^- decreases with time much more significantly than those from the SO_2^- and SO_3^- radical anions. Due to the drastic modification of the EPR signal consequent to irradiation and to the persistence of the SO_2^- and SO_3^- signals, the possibility of the safe identification of irradiated mussels is then confirmed both in the short and long term.

Oysters (Ostrea edulis) – The present results on the identification of irradiated oysters are in line with previous reports (Raffi et al., 1996; Douifi et al., 1998). Thus, samples of powdered shells of irradiated oysters led to the observation of EPR spectra only showing signals from Mn(II) ions, i.e. a sextet with average separation of 9.455 mT and the ten lines due to the forbidden transitions. None of the examined samples exhibited the single line signal with $g \approx 2.004$ due to organic radical(s) that was instead observed with the other three matrices (see Figure 4a). γ -irradiation (0.15 to 7.0 kGy) originated a strong signal that at low power and high modulation mainly consisted of lines from axial SO_3^- ($g = 2.0034_9$) and orthorhombic CO_2^- ($g = 1.9972_0$) (see Figure 4b). As the modulation amplitude was lowered, the signal became more complex due to partial resolution of components from orthorhombic CO_2^- and isotropic SO_3^- (see Figure 4c). The detection of the organic radical(s) from conchiolin that had been observed with irradiated brown Venus shells and clams proved instead impossible, even at high amplification of the signal. In the examined oysters, the very intense and persistent radiation-originated signals (see next section) allow an unambiguous identification of irradiated samples.

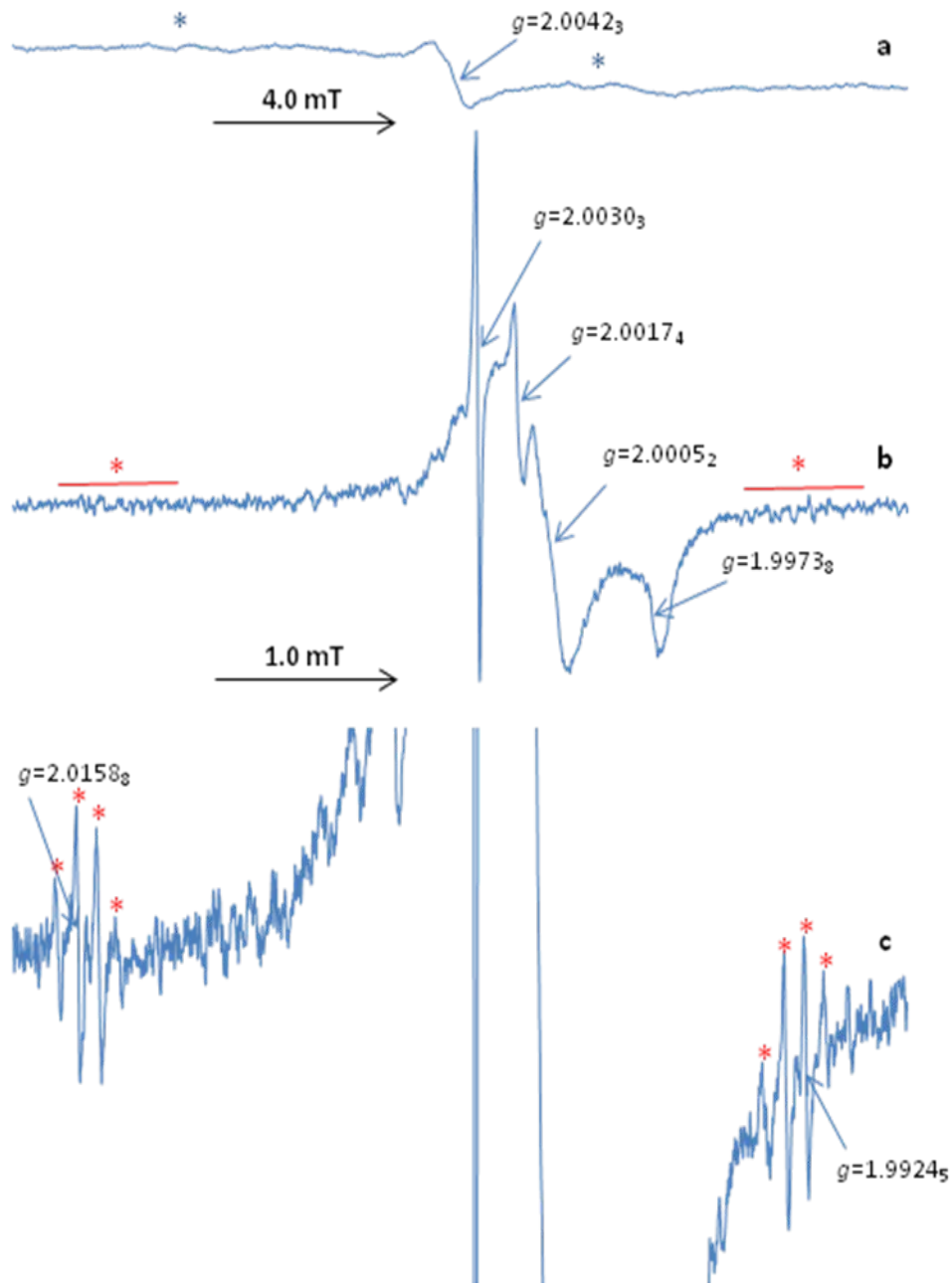


Figure 2 – EPR spectra exhibited at room temperature by clams: **a**) unirradiated (0 kGy, sw 20 mT, ma 0.05 mT, rg 1×10^4), blue asterisks indicate the third and fourth Mn(II) lines; **b**) irradiated (1.5 kGy, sw 5 mT, ma 0.02 mT, rg 1×10^5); **c**) after six months from irradiation (1.5 kGy, sw 5 mT, ma 0.02 mT, rg 5×10^6).

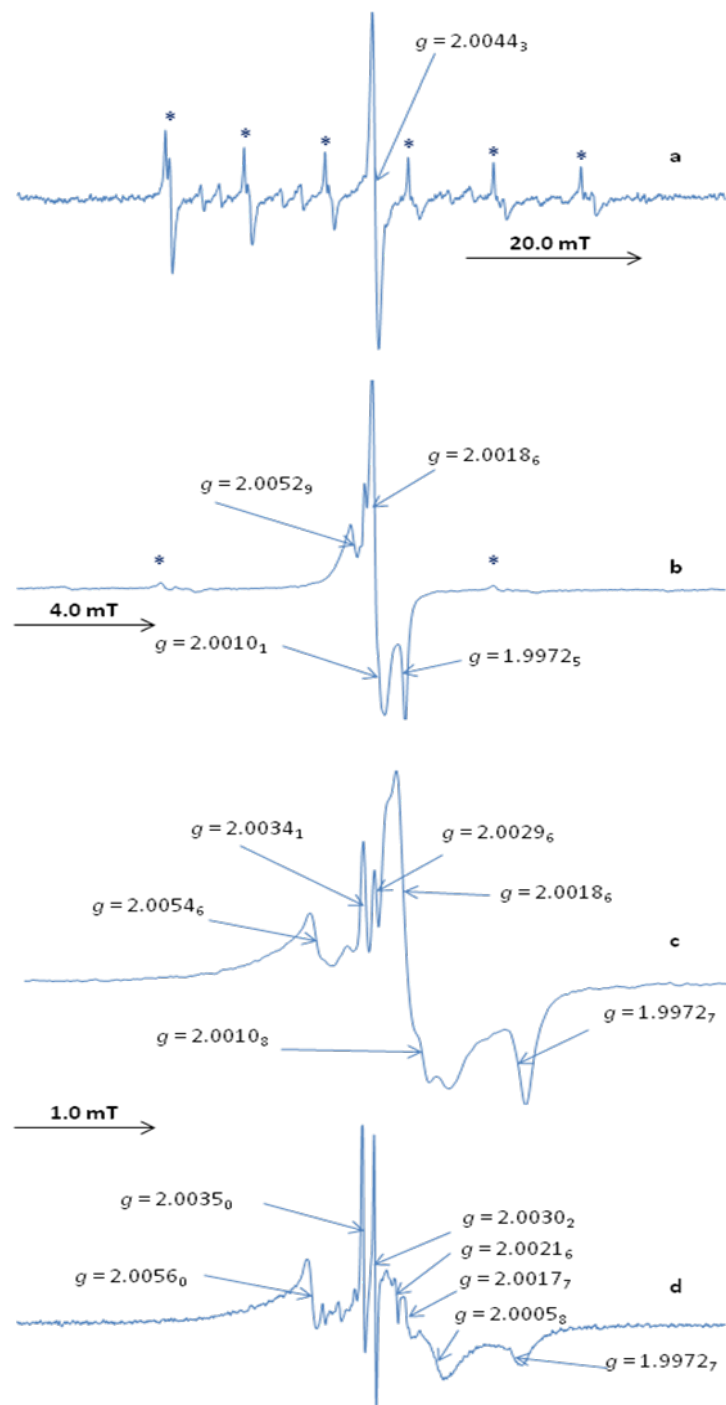


Figure 3 – EPR spectra exhibited at room temperature by mussels: **a)** unirradiated (0 kGy, sw 80 mT, ma 0.05 mT, rg 1×10^5), blue asterisks indicate Mn(II) lines; **b)** irradiated (1.5 kGy, sw 20 mT, ma 0.05 mT, rg 1×10^4); **c)** irradiated (1.5 kGy, sw 5 mT, ma 0.02 mT, rg 1×10^4); **d)** after six months from irradiation (1.5 kGy, sw 5 mT, ma 0.01 mT, rg 5×10^4).

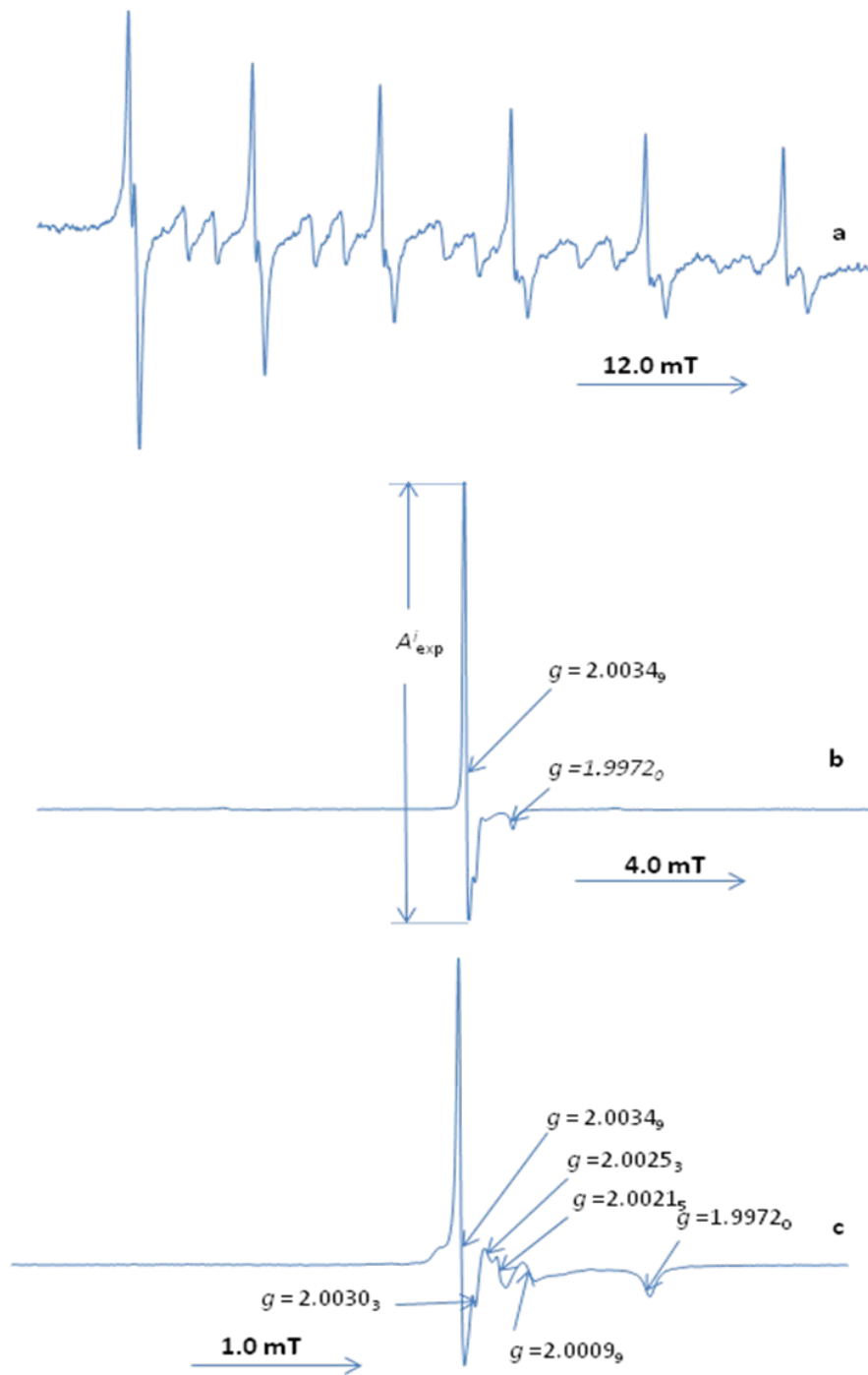


Figure 4 – EPR spectra exhibited at room temperature by Oysters: **a)** unirradiated (0 kGy, sw 60 mT, ma 0.05 mT, rg 1×10^5); **b)** irradiated (1.5 kGy, sw 20 mT, ma 0.05 mT, rg 1×10^4); **c)** irradiated (1.5 kGy, sw 5 mT, ma 0.005 mT, rg 1×10^4).

3.4.2 Dose reconstruction

Although the EPR signals exhibited by all the investigated mollusks were fairly persistent, their intensity slowly decreased with time for brown Venus shells, clams and mussels, thus making unreliable a possible dose reconstruction for samples of unknown history, that is when the date of their possible irradiation is not available. In the case of the examined oysters the peak-to-peak intensity of the EPR signals of treated samples recorded at low power (32 dB) and a modulation amplitude of 0.05 mT remained instead virtually constant for more than 60 days, i.e. a time interval largely longer than their shelf-life (see Table 1), and we therefore set up an EPR dose reconstruction protocol for these mollusks.

The interpolating function – The average values of the normalized peak to peak amplitude, A_{exp}^i , (see Figure 4b) of the EPR lined with $g = 2.0034$, measured after 24 hours and 60 days from irradiation at different doses are collected in Table 1. In all cases, as evidenced in Figure 5, the EPR signal amplitude increased with the administered dose, although not exactly linearly.

Table 1 - Peak-to-peak average amplitude of the EPR line with $g = 2.0034$, measured for irradiated *Ostrea edulis* after 24 hours (initial) and 60 days (final) from treatment. Values are normalized to 100 mg of substance and corrected for the spectrometer sensitivity.

Administered dose/kGy	Initial pp amplitude/a.u.	Final pp amplitude/a.u.
0.15	$1.24 \times 10^3 \pm 2.62 \times 10^2$	$1.32 \times 10^3 \pm 2.65 \times 10^2$
0.50	$4.17 \times 10^3 \pm 9.21 \times 10^2$	$3.93 \times 10^3 \pm 7.51 \times 10^2$
1.00	$8.45 \times 10^3 \pm 7.72 \times 10^2$	$8.15 \times 10^3 \pm 7.27 \times 10^2$
3.00	$2.06 \times 10^4 \pm 2.54 \times 10^3$	$2.13 \times 10^4 \pm 1.52 \times 10^3$
7.00	$3.66 \times 10^4 \pm 5.79 \times 10^3$	$3.398 \times 10^4 \pm 6.66 \times 10^3$

A satisfactory interpolation of the experimental data is necessary for a reliable dose reconstruction. Slightly different functions are required for the best interpolation of the initial and final amplitude values, which represents a problem for unknown samples where there is no indication as to the time elapsed between their possible irradiation and the recording of the EPR spectra.

Because the decrease of the spectral amplitude with time fell within the variability of the spectral amplitudes of different samples with the same irradiation history, we focused our attention on the average values of all the available measurements for each sample for every dose. A linear as well as a quadratic fitting of these averaged data were attempted and Eqs. (1) and (2), where y stands for the spectral amplitude A_{exp}^i and x for the dose value D^i , represent the functions providing the best fit of the experimental data for irradiated oysters.

$$y = 4899.6x + 2550 \quad (R^2 = 0.9724) \quad \text{Eq. (1)}$$

$$y = -493.81x^2 + 8489.4x + 59.447 \quad (R^2 = 0.9999) \quad \text{Eq. (2)}$$

As it is clearly shown in Figure 5, the parabolic function interpolates the experimental data much better than the linear one, as indicated by the respective coefficients of determination R^2 . Indeed, because of the R^2 value of Eq. (2) no attempt were made to fit the data with a higher order polynomial or an exponential function.

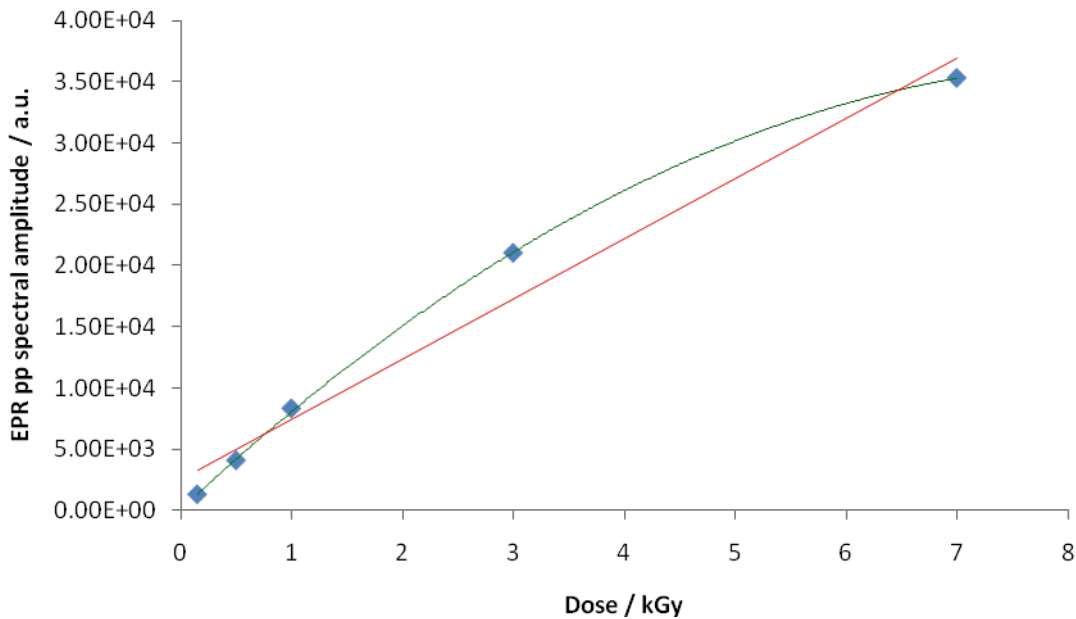


Figure 5 – Linear (red) and quadratic (green) fitting of the EPR signal amplitude vs. the administered dose for all the samples of *Ostrea edulis* independently from the time elapsed from irradiation (24 h or 60 days). Symbols indicate average values.

Evaluation of unknown samples – Blind experiments were carried out on 34 samples of *Ostrea edulis* for which the irradiation history was not known. Table 2 collects the doses administered to each sample as evaluated through the use of Eq.s (1) and (2), along with their percent deviation from the doses actually administered to the samples. All the unirradiated oysters were correctly identified as such. As for the irradiated samples, the large majority of the dose values reconstructed using Eq. (2) differed by less than $\pm 25\%$ from the actually administered ones, this value being lower than the uncertainty considered reasonable in EPR dose estimation (Sin et al., 2005). It is worth stressing that only 6 estimated values out of 34 differ from the administered dose by more than 25%. Three exceedingly low reconstructed values were observed for low doses (in between 0.15 and 0.5 kGy), and the worst performance was observed for the highest administered dose, three estimated values out of six falling outside an acceptable range. It should however be noted that 7 kGy is a dose much higher than those possibly administered to commercialized oysters. As it could be anticipated on the basis of the R^2 values, the performance of Eq. (1) was much poorer and for 50% of the irradiated samples it led to dose values unacceptably deviating from the actually administered doses. All in all, dose reconstruction provided by Eq. (2) seems fairly good and suggests the possibility of a reliable dose estimation through the use of EPR spectroscopy.

Table 2 - Administered and reconstructed irradiation doses for oysters. Numbers in bold indicate deviation greater than $\pm 25\%$.

Administered dose /kGy	Reconstructed dose (Eq.1)/kGy	Deviation/%	Reconstructed dose (Eq.2) /kGy	Deviation/%
0.0	0.0	0.0	0.0	0.0
0.0	0.0	0.0	0.0	0.0
0.0	0.0	0.0	0.0	0.0
0.0	0.0	0.0	0.0	0.0
0.0	0.0	0.0	0.0	0.0
0.15	0.038	-74.3	0.093	-32.97
0.15	0.062	-58.9	0.153	+2.26
0.15	0.060	-61.4	0.144	-4.13
0.15	0.052	-65.6	0.156	+4.00
0.15	0.071	-52.5	0.127	-15.30
0.15	0.062	-68.3	0.170	+19.07
0.50	0.392	-21.6	0.536	+7.25
0.50	0.406	-18.8	0.546	+9.01
0.50	0.089	-82.2	0.356	-28.78
0.50	0.318	-36.4	0.491	-1.77
0.50	0.394	-21.2	0.537	+7.50
0.50	0.116	-76.8	0.368	-26.31
1.00	0.926	-7.3	0.872	-12.80
1.00	1.349	+34.9	1.148	+14.87
1.00	1.110	+11.0	0.991	- 0.87
1.00	1.260	+26.0	1.094	+9.46
1.00	1.149	+14.9	1.017	+1.66
1.00	1.087	+8.78	0.976	- 2.33
1.50	1.765	+17.7	1.431	- 4.57
1.50	2.031	+35.4	1.617	+7.84
1.50	2.132	+42.2	1.690	+12.70
3.00	3.940	+31.6	3.149	+4.96
3.00	3.766	+25.5	2.984	-0.53
3.00	4.194	+39.8	3.337	+12.65
3.00	3.378	+12.6	2.652	-11.60
3.00	3.378	+12.6	2.652	-11.60
3.00	3.520	+17.3	2.772	-7.60
7.00	4.786	-31.6	3.974	-43.22
7.00	4.643	-33.7	3.823	-45.37
7.00	6.704	-4.2	7.072	+1.03
7.00	5.317	-24.0	4.584	-34.51
7.00	7.419	+6.0	7.716	+10.23
7.00	7.827	+11.8	8.113	+11.59

3.4.3 Dose estimating protocol

Based on the above results, we suggest the following protocol for the reconstruction of the radiation dose administered to oysters. In this context, it should be emphasized that because the EPR dose quantification is based on the use of an internal standard, the nature of which is related to the actual instrumental set-up available to each individual operating unit, the functions described by Eq. (1) and (2) do not have a general validity. Thus, a preliminary key step is the construction of the necessary calibration curves. This in turn requires the availability of an appropriate radiation source.

Irradiation of the samples – Irradiate oysters administering different radiation doses in the appropriate range. The use of at least four different doses for decade of kGy is suggested.

Preparation of the samples – Separate the edible part of the irradiated mollusks from the shells. Degrease the latter with methanol, wash them thoroughly with fresh water and eventually dry them until their weight remains constant. Grind the clean and dry shells to a fine powder, put some powder in an EPR tube of known weight and determine by difference the weight (W_{sa}^i) in milligrams of the introduced material.

Calibration curve – Record the signal of the standard with the empty cavity and determine its double integral (I_{st}). Record the spectrum of the sample(s) irradiated with known doses and determine the peak-to-peak amplitude (A_{exp}^i) of the line with $g = 2.0034$. Without displacing the sample(s) record the signal of the standard and determine its double integral (I_{sa}^i). Normalize to 100 mg the spectral amplitude values corrected for the different cavity sensitivity through the formula

$$A_{norm}^i = A_{exp}^i \times 100 \times I_{st} / (W_{sa}^i \times I_{sa}^i).$$

Plot the resulting A_{norm}^i values vs the corresponding dose (D^i) and determine the function $A = f(D)$ that best interpolates the experimental values.

Dose reconstruction – Prepare the sample of unknown irradiation history as indicated above, determine its A_{norm}^i value and estimate the administered dose using the calibration function $A = f(D)$.

3.5 Conclusions

The results reported here confirm the applicability of EPR spectroscopy in the identification of four types of irradiated mollusks, i.e. brown Venus shells, clams, mussels and oysters. For the first three species only a dose of 1.5 kGy was tested, that is the one commercially more used. For oysters doses in the range 150 Gy to 7.0 kGy were administered and irradiated mollusks could be identified also at the lowest dose.

While identification of the irradiated species is mostly based on the detection of the EPR signal from the SO_3^- and CO_2^- radical anions, in the case of brown Venus shells and, to a lesser extent, of clams the isotropic EPR signal from an organic radical believed to derive from one of the aminoacids present in the scleroprotein conchiolin was found to be an as yet unreported very persistent and unequivocal irradiation marker.

In the case of oysters a protocol was defined that allows reconstruction of the administered dose within a reliability of $\pm 25\%$ in the above dose interval. The reproducibility of the signal from the internal standard is critical to the reliability and proposed protocol, and we suggest that whenever possible a “fixed and undisplaceable” internal standard is used (e.g. an appropriately cut synthetic ruby crystal located in a corner of the resonant cavity of the EPR spectrometer).

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CHAPTER 4

Identifying irradiated oysters by luminescence techniques (TL & PSL)

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4. Identifying irradiated oysters by luminescence techniques (TL & PSL)

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4.1 Abstract

Recent growing interest by the food industry in irradiation technology and the need to facilitate trade in irradiated foods have led regulatory authorities to develop reliable and suitable methods to distinguish between irradiated and non-irradiated foodstuffs and, consequently, to check compliance with labeling requirements. The aim of this study is to correctly identify non-irradiated and irradiated oysters in the range 0.1 – 2 kGy, with different procedures that use two techniques (TL and PSL) and to investigate signal stability over the course of their commercial shelf life. Furthermore, all of the alternative procedures were compared with several practical and analytical parameters. We report here on a practical and reliable means being used as official tools to enforce correct food labeling regulations and to enhance routine analysis.

4.2 Introduction

Oysters are considered one of the most delicious and refined foods, but epidemiological evidence shows that many outbreaks of foodborne illness in the world today are due to the intentional consumption of raw seafood, such as oysters. Indeed, tainted oysters are one of the main entries in the FDA (Food and Drug Administration) Top Ten Riskiest Foods Report in 2009 (Klein, Tian, Witmer and Smith, 2009). They are filter feeders and selectively accumulate human enteric viruses (e.g. noroviruses and hepatitis A virus) (Gillespie, Adak, O'Brien, Brett and Bolton, 2001; Lees, 2000) and indigenous marine bacterial species (e.g. *Vibrio spp.*) (Potasman, Paz and Odeh, 2002; Rippey, 1994; Richards, 1988). Consumers usually eat oysters raw and whole or following very mild heat treatment (Gram & Huss, 2000; Lees, 2000), as all of the currently available processing techniques (steaming, pressurizing and freezing) can affect the taste and reduce their post-processing shelf-life, making them unacceptable for many consumers. (Murchie et al., 2005).

In contrast to thermally processed foods, irradiation is a cold treatment which serves especially to keep bacterial *Vibrio* species in check, to reduce microbiological contamination and also to increase the shelf-life of raw seafood. Some studies have found that at low doses (1-3 kGy), contamination in *Vibrio spp* and *Salmonella spp* is reduced considerably and have also provided evidence for oyster survival with no change in their odor, flavor or appearance (Jakabi et al., 2002). According to the List of Member States' authorizations of food and food ingredients which may be treated with ionizing radiation (Directives 1999/2/EC) mollusks, including oysters, can be irradiated in a range of 0.5-1 kGy only in authorized countries (UK, Belgium and Czech Republic). Therefore, these irradiated foodstuffs are likely to be present in our markets and found on our tables. Since foods like oysters are not consumed regularly, there is no effective intervention strategy available for preventing outbreaks of foodborne illness, but recent growing interest on the part of the food industry in irradiation technology and the need to facilitate trade in irradiated foods have led the regulatory authorities to develop reliable and suitable methods to distinguish between irradiated and non-irradiated foodstuffs and, consequently, to check compliance with labeling requirements (Bhaskar, Chawla and Arun Sharma, 2009).

Photoluminescence (PSL) and Thermoluminescence (TL) are two of the physical techniques (for screening and confirmatory analysis, respectively) able to detect many irradiated foodstuffs such as herbs, spices, vegetables, eggs, fruit, etc. (Mangiacotti et al., 2009; Leth, Hansen & Boisen, 2006) by using constituent biocarbonate minerals (calcite, aragonite etc.) or contaminating silicate minerals (quartz, feldspar etc.). In particular, both techniques are also used to identify irradiated shellfish (Bhatti et al., 2008; Sanderson, Carmichael and Fisk, 2003; Pinnioja and Lindberg, 1998), even though very few specific works have been carried out on oyster samples (Ziegelman, Bögl and Schreiber, 1999; Sanderson, Carmichael, Spencer and Naylor, 1996). The aim of this study is to identify non-irradiated and irradiated oysters at increasing dose levels, with different procedures, using two techniques (TL and PSL) that analyze luminescence signals both from contaminating silicate minerals, especially feldspar and quartz, and from constituent biocarbonate minerals, in particular calcite and aragonite, originating from white mother-of-pearl. Furthermore, the present work intends to investigate signal stability over the course of the commercial shelf life and to compare all of alternative procedures with respect to several qualitative validation parameters. It also offers practical and reliable means being used as official tools enforcing correct food labeling regulations as required by European Food legislation (Directives 1999/2/EC and 1999/3/EC). Finally, it could be also considered as an attempt to enhance consumer confidence in food processing technology involving irradiation.

4.3 Materials and methods

4.3.1 Sampling and sample irradiation

A total number of 110 samples of oysters (*Ostrea edulis*), farmed in the North Sea and bought from local retail, were collected and divided into three groups, as reported in Table 1: the first, made up of 10 samples, to determine native signal (non irradiated), the second group, made up of 50 samples, irradiated at 5 different dose levels (0.1 – 0.5 – 1 – 1.5 – 2 kGy) and the last, made up of 50 samples, to study signals over time (fading). Each group was processed using five different analytical procedures, named A, B, C, D and E (A and B, related to PSL and C, D and E related to TL). All samples were stored in subdued light conditions at a refrigeration temperature of 4 °C, except for those samples involved in the fading investigation, stored after irradiation at a freezing temperature of -18 °C. Irradiation operations were carried out using non-nuclear irradiator that utilizes low energy X-ray beams, thus avoiding complex regulation issues and improving operator safety conditions. Oyster samples were irradiated at room temperature at different dose levels with a low-energy X-Ray machine (RS 2400 Radsources Inc.) operating at 150 kV and 45 mA with a dose rate of $15 \text{ Gy min}^{-1} \pm 10 \%$ measured with a calibrated ion chamber (Radcal Inc.) Each sample was wrapped in a plastic bag and inserted into a carbon-fiber reinforced resin canister with a diameter of 7.62 cm located in a carousel rotating around the X-ray tube, assuring a uniform dose delivery within 20% tested by Gafchromic film HD-810. All of the absorbed doses mentioned in this investigation are dose to water.

4.3.2 Experimental PSL procedures

A rapid method for detecting irradiated foods is based on photostimulated luminescence (PSL) that uses light, rather than heat, to stimulate electromagnetic emission from irradiated bioorganic materials such as calcite or mineral debris, typically silicates. Exposure to IR stimulation sources releases trapped charge carriers that stored energy during the irradiation process. The PSL method thus features the unique possibility to analyze inorganic systems either extracted or in the presence of organic matter. An irradiated food screening system (SURRC; Scottish Universities Research and Reactor Centre, UK) comprising a sample chamber, stimulation source, pulsed stimulation and synchronized photon counting systems was sufficiently sensitive to allow direct measurements of powder shell and whole pulp (Schreiber, 1996).

Table 1 - Oyster sampling plan

Analytical Procedures	1 st group	2 nd group					3 rd group
	Non Irradiated	0.1 kGy	0.5 kGy	1 kGy	1.5 kGy	2 kGy	Fading Signal After ~ 90 days
A Contaminating minerals from pulpes in PSL	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples	10 samples (two for every dose level)
B Constituent minerals of shells in PSL	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples	10 samples (two for every dose level)
C Contaminating minerals from intestine in TL	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples	10 samples (two for every dose level)
D Contaminating minerals from shells in TL	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples	10 samples (two for every dose level)
E Constituent minerals of shells in TL	2 samples	2 samples	2 samples	2 samples	2 samples	2 samples	10 samples (two for every dose level)
Total samples	10 samples	50 samples					50 samples

Sample preparation for procedure A was very simple and easy including mechanical removal of the pulp from the shell using a scalpel, while a rough separation of the white inner part of the shell, ground to a powder form by a knife miller and sieved in the range 0.5-1 mm was sufficient for procedure B. Sample preparation, handling and PSL measurement were all conducted under safelight conditions to minimize optical bleaching of PSL. Each sample was dispensed into disposable Petri dishes (50 mm diameter) and the instrument provided for quantitative screening measurements in 60 seconds. The instrumental setup procedure included checks on dark count, irradiated and unirradiated standard materials (paprika) and empty test, also establishing measurement parameters (cycle time, threshold and data recording conditions). The accumulated counts corresponding, for shellfish, to the lower threshold (T1) of 1000 counts/60 s were classified as “negative samples”, whereas those higher than the upper threshold (T2) of 4000 counts/60 s were classified as “positive samples”. Signal levels between T1 and T2 thresholds were classified as intermediate results for which further investigation was needed. Empty tests were repeated periodically every 10 negative results or after a positive one. This action was required to guarantee a quality assurance program and to prevent false positive results following strong light emission output. To ensure that the chamber was free of contamination, in all cases in which a contamination is suspected such as cross contamination after sensitive irradiated sample, it was

necessary to clean the chamber using an air duster until a negative empty test was achieved. The two PSL analytical procedures, previously classified A and B, analyzed signals from contaminating silicate minerals in whole oyster pulp (procedure A) and signals from ground constituent bicarbonate minerals of oyster shell (procedure B), respectively. They were successfully performed, with differences and limitations which will be discussed in the next paragraph.

After initial PSL measurement (to establish the status of the sample), all samples were processed by an optional second measurement: calibrated PSL (according to European standard EN 13751:2009), consisting in irradiating samples with a known dose level of 1 kGy and then re-measuring for a second PSL reading. Usually, calibrated PSL measurements are recommended to estimate sample PSL sensitivity, in case of ambiguous results of an irradiation treatment or for shellfish with low mineral contents. To be sure that the surface of the samples was the same as in procedure B, minerals were fixed deposited on a Petri dish by a layer of a silicon gel spray, assuring that the mineral amount is similar in weight.

4.3.3 Experimental TL procedures

Among applied and standardized physical methods to detect irradiated food, thermoluminescence is certainly a reliable and accurate confirmatory luminescence technique based on the principle that in solid dielectric materials (e.g. quartz, feldspar, calcite, aragonite etc.) energy is stored during irradiation as trapped charge carriers. This excess energy, in contrast to the PSL method, is released as luminescence emission by thermal stimulation of isolated silicate minerals, heated under controlled conditions which give rise to a measurable glow curve. Light emission at a certain temperature depends on the depth of the trap and on the type of minerals (Reuven Chen & Stephen W.S. McKeever, 1997). Furthermore, the stability of the excited electron increases either with the depth of the trap or with the temperature at which light is released. For the TL investigations, oyster samples were analyzed by three different procedures (C, D and E). Procedure C verified and extended the application of standard protocol (EN 1788, 2001) to irradiated and unirradiated oyster samples. This procedure involved extracting the contaminating silicate minerals from the intestine of each sample, using wet sieving to pre-concentrate minerals, and density separation in sodium polytungstate solution to ensure that isolated silicate minerals are as free of organic constituents as possible so as to avoid obscuring phenomena or spurious TL signals. These minerals, after hydrochloric acid treatment to remove carbonate, using an acetone suspension, were deposited onto a 0.10 mm Ø and 0.20 mm thick stainless steel disc and dried at 50°C overnight before measurement. Procedure D is alternative, but very similar to procedure C, where the minerals, because of their origin, were extracted from oyster shells without

the sodium polytungstate density separation step. By contrast, procedure E involves constituent biocarbonate minerals located in the inner part (mother-of-pearl) of oyster shells. These shell parts were ground, sieved in the range 0.5-1 mm, deposited (~ 30 mg) on a thick stainless steel disc and then dried at $50\text{ }^{\circ}\text{C}$ overnight before measurement. The following operations were common to all procedures. TL glow curves were recorded immediately after preparation (1st Glow curve: G1) and following a standard calibrating radiation dose of 1 kGy delivered with X-ray equipment (2nd Glow curve: G2). Sample preparation, handling and TL measurement were all conducted under safelight conditions to minimize optical bleaching of TL. TL measurements were carried out by a Risø TL/OSL reader, model DA-20, equipped with a sealed surface β -source ($^{90}\text{Sr}/^{90}\text{Y}$) with a nominal activity of 1.48 GBq and a photomultiplier (PMT) for light detection. The curves were recorded with a heating rate of $6\text{ }^{\circ}\text{C}/\text{s}$ from room temperature to 400°C , in nitrogen atmosphere. Table 2 shows operational instrumental settings and validation parameters. The evaluation of samples needs to calculate the TL ratio of the first and second integral glow intensity. If the TL ratio is more than 0.1 then the samples were classified as “irradiated”, otherwise the samples were considered “non irradiated”. Quality assurance steps included full process blank measurements for every sample batch and the positive verification that all glassware and sample discs were free from luminescence materials. The process blank levels were also used to define minimum detectable levels (MDL) for the analysis, evaluated as the mean value plus 3 standard deviations using long term data. Temperature intervals to calculate the TL glow integral were defined by evaluating the glow curve of lithium fluoride (LiF, TLD-100) pellets, a well characterized phosphorous material, that were irradiated with a dose level of about 0.5 Gy with a sealed β -source of $^{90}\text{Sr}/^{90}\text{Y}$ included in the TL/OSL Risø Reader. Figure 1 shows the TL glow curve of an irradiated LiF where the positions of the peaks V (PV) and VI of the LiF glow curve on the temperature axis are measured and the temperature difference IS between the two values (using the mean values of at least 10 measurements) is calculated. The temperature interval I, extending from (PV-IS), is recommended for evaluation (EN 1788, 2001).

Table 2 - Operating setting methods and validation parameters

Starting temperature	Room temperature ($\sim 22\text{ }^{\circ}\text{C}$)
Final temperature	400 $^{\circ}\text{C}$
Heating rate	6 $^{\circ}\text{C}/\text{sec}$
Nitrogen flow	3 ml/s
Irradiation source	X-Ray
Normalization dose	1 kGy
Temperature range	201 – 268 $^{\circ}\text{C}$
MDL	750
TL limit for G2 (10 time MDL)	7500
Data point	330

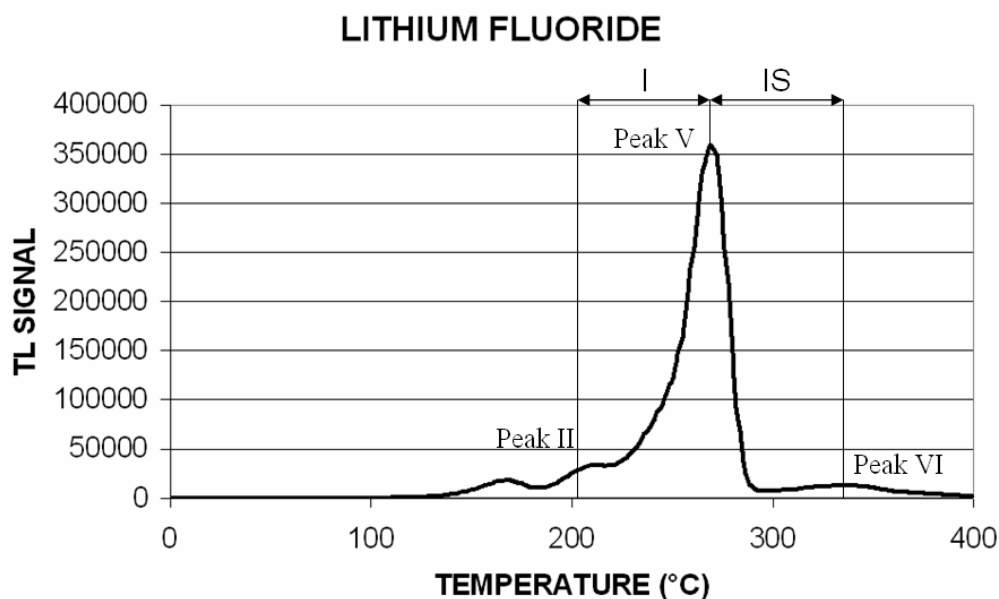


Figure 1 – TL glow curve of an irradiated LiF pellet

4.4 Results and discussion

4.4.1 PSL results

Table 3 reports the PSL results obtained with procedures A and B related to initial PSL, calibrated PSL, sample sensitivities and fading behaviour (after 60 days). All samples analyzed with procedure A were identified correctly, both non

irradiated ones and those irradiated at different dose levels, except samples irradiated at the lowest dose level (0.1 kGy), probably due to the presence of a small amount of contaminating minerals in oyster pulps. Procedure B was also able to correctly distinguish non irradiated samples (< T1) from treated samples at increasing dose levels (> T2). To determine PSL sensitivity and to confirm screening results, in cases of doubt, according to European Standard (EN 13751:2009), all samples were re-irradiated at a defined radiation dose of 1 kGy (dose comparable to the expected treatment dose). After irradiation, all further handling took place under subdued lighting whenever possible. Calibrated measurements were conducted after storage overnight either at ambient temperature for procedure B (powder of shells) or at chilled storage for procedure A (pulp). According to EN 13751:2009, negative calibrated results (< T1) are indicative of insufficient PSL sensitivity and can be common in shellfish analysis, leading to unclassified cases which require another standardised method.

On the other hand, positive calibrated results (> T2) within the same order of magnitude as the screening results are indicative of irradiation. Finally, in cases where calibrated PSL gives signals which are much greater than their negative or intermediate screening results, samples are likely to be unirradiated. Following the above evaluation scheme, our data confirmed that the application of the calibrated PSL method is reliable for all samples in the range 0.1 – 2 kGy.

Table 3 - PSL results: Initial, second measurement, index sensitivity and fading of oyster samples

DOSE LEVELS (kGy)	PROCEDURE A (Contaminating minerals from pulp)				PROCEDURE B (Constituent minerals of shells)			
	Initial PSL (counts/60s)	Calibrated PSL (counts/60s)	* Index Sensitivity	PSL Fading (60 days)	Initial PSL (counts/60s)	Calibrated PSL (counts/60s)	* Index Sensitivity	PSL Fading (60 days)
0	207	10643	52	432	466	1794023	3850	650
0.1	693	4715	6.8	6214	89792	170622	1.9	46536
0.5	11365	34430	3.0	70774	906039	2078954	2.3	104432
1	4363	7323	1.7	166618	2533599	3632424	1.4	170938
1.5	18399	24287	1.3	67689	1502226	1987314	1.3	358897
2	9449	9624	1.0	164574	1093154	1485491	1.4	443296

*Calibrated PSL/Initial PSL

The preliminary hypothesis about samples analysed by procedure A at 0.1 kGy was also supported by the calibrated PSL results characterized by a small gain with respect to the screening results. Indeed, the calibrated PSL signal, very close to the upper threshold (4000 cps/60s), confirmed the presence of a few silicates.

To further investigate our findings, an index of sensitivity was calculated as the ratio of calibrated over initial PSL signals. Non irradiated samples processed by procedure A presented an average sensitivity index of 52, while all irradiated samples had a sensitivity index in the range 1.0 - 6.8. Procedure B shows that calibrated PSL signals of non irradiated samples were about 4000-fold greater than the initial measurements, while irradiated samples had a sensitivity index in the range 1.3 – 2.3.

Comparison of initial and calibrated mean PSL results obtained with the two procedures established a higher sensitivity index for non irradiated samples in the latter procedure (i.e. B). Indeed, experimental data clearly indicated that a different sensitivity was present and particularly for non irradiated samples it was possible to state that calibrated PSL signal from constituent minerals increased much more than the corresponding calibrated signal obtained by analysing contaminants and minerals from oyster pulps. It is worth noting that this behaviour is likely due to major changes in amount and mineral composition. Furthermore, signal fading studies evidenced that samples, stored in subdued lighting, were correctly classified even after approx. 60 days, double the commercial life of irradiated oysters.

4.4.2 TL results

All remaining TL procedures (C, D and E) identified correctly either samples non irradiated with a TL ratio < 0.1 or irradiated at increasing irradiation doses in the range 0.1 – 2 kGy. TL ratio related to the lowest level (0.1 kGy) is very close to the discriminating value (0.1) and in all cases it was important to extract the maximum amount of mineral possible. Table 4 shows TL results along with the validation and practical parameters considered in this work: TL ratio, regression expressions, coefficient of determination, signal fading, preparation time, difficulty and cost.

Table 4 - Validation and practical parameters of oyster samples analyzed by three different TL procedures (C, D and E).

TL RESULTS, VALIDATION AND PRACTICAL PARAMETERS												
TL PROCEDURE	TL RATIO (G1/G2)						REGRESSION EXPRESSIONS	COEFFICIENTS (R ²)	SIGNAL FADING (60 days)	PREPARATION TIME	DIFFICULTY	COST
	Non irradiated	0.1 kGy	0.5 kGy	1 kGy	1.5 kGy	2 kGy						
C	0.0022	0.11	0.30	0.51	0.48	0.56	$y = 0.266x + 0.094$	0.84	> 0.1	~6 h	+++	+++
D	0.0019	0.12	0.45	0.60	0.62	0.73	$y = 0.341x + 0.126$	0.85	> 0.1	~7 h	++	++
E	0.0100	0.15	0.45	0.99	1.43	1.31	$y = 0.726x + 0.104$	0.92	> 0.1	~1 h	+	+

Comparing the three procedures, it was evident that the TL ratio increased on increasing the administered dose, markedly for samples analyzed by procedure E, but a saturation effect was found for all procedures at high dose levels, in particular for samples analyzed with procedure C. Furthermore, these trends were confirmed by the coefficient of determination (R²) for the linear regression curves of the TL ratio versus dose levels. Moreover, procedure E exhibited the greatest slope (0.726) of the procedures as a consequence of the different type of minerals. Indeed, procedures C and D, extracting the same kind of minerals, show close values for some statistical parameters (R² and slope). Comparing the different extraction sites, outer shell and pulp, for procedures C and D respectively, it was possible to stress that contaminating minerals from shells were the most sensitive ones. In terms of time spent for the sample preparation, procedure E resulted the most convenient, enabling the whole operation to be run in less than 1 h against times of 6–7 h for other procedures. Also considering other practical operating parameters, procedure E involved less operator difficulty, consisting in simple mechanical treatment against complex chemical steps in order to concentrate and purify minerals. Another economic aspect that should be considered is the cost savings for reagents and consumables that characterize procedure E, which also improves operator safety. Figure 2 reports examples of first glow curves from oyster samples analyzed by the three procedures (C, D and E). The first TL glow curves of non-irradiated samples present no peaks in the integration range (201-268°C) but only in the

high temperature region (ca. $> 300^{\circ}\text{C}$) where it was possible to find geological signals. In particular, the first glow curves were independent of increasing irradiation dose levels. All glow curves show similar shapes and peak value shifts in the range $234\text{--}276^{\circ}\text{C}$ for each exposure and treatment dose. This trend in oyster samples accounts for the different quantity, quality and composition of minerals, mainly due to their origin and site of extraction. Signal stability was also analyzed over 60 days for all dose levels considered, and fading studies confirmed the applicability of each procedure providing a TL ratio that was always greater than 0.1.

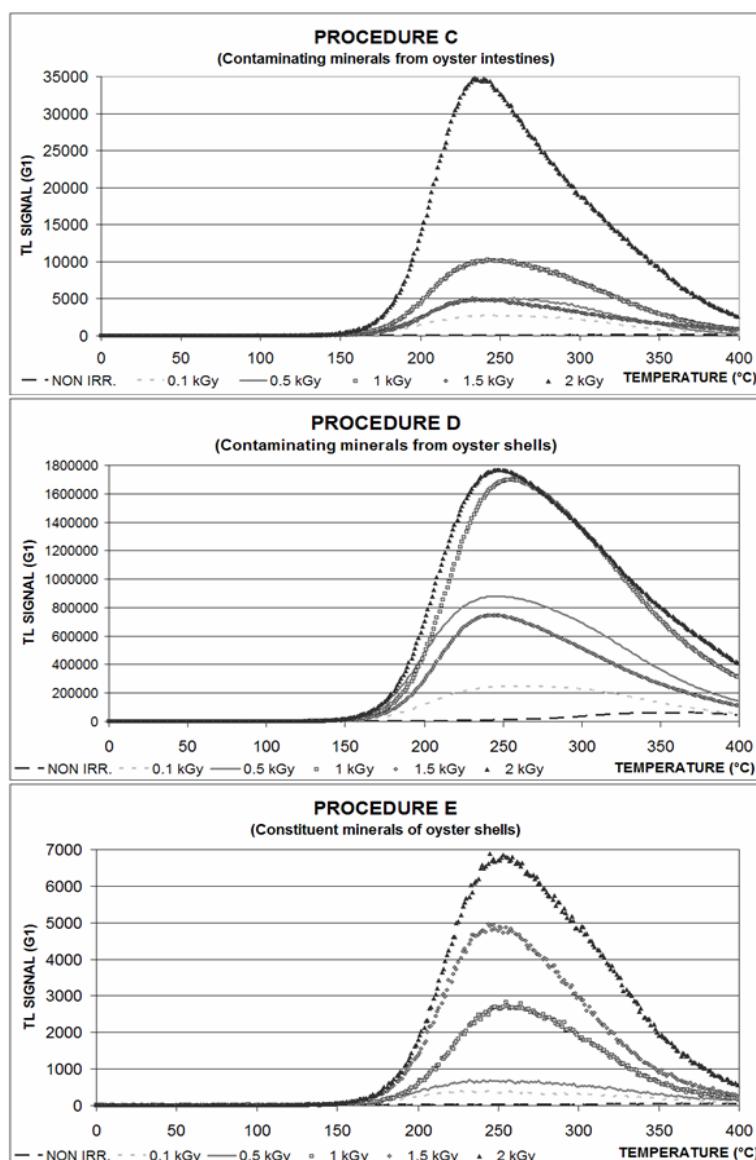


Figure 2 – Glow curve 1 showing examples of non irradiated and irradiated oysters at different dose levels analyzed by procedures C, D and E.

4.5 Conclusions

This work extends the application of standardized PSL and TL methods, identifying a seafood that is suitable for such treatment, i.e. oysters. Both alternative and simple screening and confirmatory methods were investigated in order to detect irradiated and non irradiated oysters. Both PSL screening methods tested in this study were able to be used for rapid and efficient routine analysis. In doubtful cases, it was useful to confirm the results using a calibrated PSL signal to estimate sensitivity samples, especially when samples were irradiated at very low dose levels (e.g. 0.1 kGy) or native signals of minerals were very high (e.g. constituent minerals). Irradiated samples showed only a small increase in PSL intensity after radiation exposure, whereas unirradiated samples usually showed a substantial increase of two or three orders of magnitude. After analyzing all experimental calibrated PSL data, it was possible to affirm that procedure B was better than procedure A, both because the constituent minerals were more sensitive than the contaminant minerals and because the constituent minerals are naturally abundant. It was verified that oysters can be analysed using both procedures and in cases of doubt or discordant results, if a radiation source is available, then it was useful to carry out a calibrated PSL experiment. Although reliable results were found applying both procedures A and B, we advice to adopt procedure B thanks to its higher constituent mineral sensitivity and the greater amounts of material at our disposal. Indeed, it should be considered that positive PSL results need to be further analysed by performing a confirmatory method. Thus, according to international standards and considering that sample preparation of PSL procedure B is in common with TL procedure E, the following sequential routine protocol can be applied: screening PSL (procedure B) and confirmatory TL (procedure E). Three alternative TL analyses were also carried out and all procedures (C, D and E) confirmed the application of the technique to the identification both of non irradiated and of irradiated oyster samples. Among all procedures studied, procedure E was the best one comparing the three TL approaches in terms of a set of practical and validation parameters (TL signal sensitivity, preparation operation, simplicity and cost). Procedure E also resulted in shorter routine analysis times and in a safer protocol for operator health issues. Signal stability was also assessed over time (60 days) for PSL and TL analysis, assuring that potential treatment with ionization radiations could be evaluated for the whole shelf-life of oyster samples (ca. 20-30 days). Furthermore, the chosen experimental dose range (0.1 - 2 kGy) covers current commercial application doses (0.5 – 1 kGy) intended to extend shelf-life and reducing the number of pathogenic microorganisms. In conclusion, this work establishes a sound basis to enhance consumer's confidence in official checks and presents a simple and sequential routine

analysis protocol, including PSL screening and confirmatory TL, to be implemented as a fast and reliable tool to correctly identify irradiated oysters at marketing stage.

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CHAPTER 5

Official checks by an accredited laboratory on irradiated foods at an Italian market

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5. Official checks by an accredited laboratory on irradiated foods at an Italian market

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5.1 Abstract

Food irradiation can be used to increase the microbiological safety and to extend the shelf life of foods. European legislation states that any food or food ingredients must be labeled and every year each Member State, particularly Italy, has to carry out checks at marketing stage. This work reports on the results of analytical controls on 451 foodstuff samples over the period 2006-2011 performed by an Italian accredited laboratory using 4 different screening and confirmatory techniques: PSL, DNA Comet Assay, TL and ESR.

A total of 18 samples were found non compliant: 6 frog legs, 3 clams, 3 cuttlefish, 1 octopus and 1 shrimps from Vietnam; 3 squids, 1 white pepper and 1 chilli tofu from China. Non compliances are due to both incorrect labeling and irradiation in not approved facilities in extra European/third countries. Check results also showed that among screening methods PSL is the most accurate, simple and practical standard to analyze most of samples (spices, herbs, supplements, mollusks, crustaceans and vegetables) with a low false positive classification (11%) whereas DNA Comet assay revealed the highest percentage of false positive cases (26 %). ESR is the suitable confirmatory method to detect dried fruits and foodstuffs (meat and fish product) containing bone, while TL is the best confirmatory method to detect herbs, spices and supplements, cephalopods, mollusks and crustaceans, besides fresh fruits and vegetables.

In conclusion, by comparison with European data, this study suggests more checks on meat products (frog and poultry meats), fish products (cephalopods, mussels and crustacean) spices and supplements especially at import stage from countries where non approved irradiation facilities are operating (e.g. Vietnam and China).

5.2 Introduction

Ionising radiations (gamma rays, X-rays and electron beams) are used in the food industry for various purposes, such as reducing contamination by putrefactive and especially by pathogenic spoilage micro-organisms, inactivating any parasites, inhibiting germination in some vegetables and slowing the senescence of fruits and vegetables. The major advantage of ionising radiation on foods is a considerable increase in shelf-life, while leaving the organoleptic and nutritional quality of the starting product practically intact. Despite being a technology with undisputed potential, food irradiation is at the centre of controversy, owing mostly to distrust on the part of most consumers who, being uninformed, intimidated and influenced by the terminology, are scared of risks to their health (Ehlermann, 2009; Junqueira-Gonçalves et al., 2009). The numerous studies conducted so far have confirmed that this technology has no harmful effects on foods, apart from a slight loss of some vitamins, comparable to that obtained with other more widespread storage technologies, such as heat treatment. Various organisations such as EFSA (European Food Safety Agency), IAEA (International Atomic Energy Agency) and FAO (Food Agriculture Organization) have expressed a favourable opinion on food irradiation stating that, subject to certain criteria, there are no dangers associated with the consumption of these foods (Statement of EFSA, 2011; WHO, 1999; WHO, 1994; WHO, 1980).

The European regulatory framework, which is much more restrictive than the international one, includes two Community Directives, i.e. 1999/2/EC and 1999/3/EC (Directive 1999/2/EC; Directive 1999/3/EC), which Italy has implemented by Government Decree No. 94 dated 30/01/01 (Italian Government Decree, 2001). These directives give guidelines and obligations on the use of this technology, including a positive list of foods that can be treated at Community level, the national authorisations with an indication of the doses for each product, the maximum dose of 10 kGy, the rules for authorising irradiation facilities, mandatory labelling of irradiated foodstuffs with the wording “irradiated” or “treated with ionising radiation” and in particular mandatory controls by each Member State both on radiation facilities and on the foods during the marketing stage. To this end, the European Committee for Standardisation (CEN) has developed, validated, and published several identification methods based on food characteristics and radiation-induced changes. This set, divided into screening and confirmatory methods, has proved sufficient for analysing most food types present on the market. In order to comply with regulatory requirements and ensure consumer choice, here in Italy, the laboratory at the Istituto Zooprofilattico Sperimentale della Puglia e della Basilicata, has taken steps to validate and accredit two screening methods: the

first one is based on physical technique (Photostimulated Luminescence - PSL) which involves optical stimulation (generally infra-red radiation) of the silicate minerals present in the food as contaminants, causing the emission of photons which are then detected by a photomultiplier tube (EN 13751, 2009); the second method is based on biological technique (DNA Comet Assay) that consists of an electrophoretic separation on agarose gel of individual cells extracted from animal or plant tissue, followed by an analysis of the image under an epifluorescence microscope (EN 13784, 2001). Along with screening methods the laboratory has also implemented four physical confirmatory methods. Three standards are based on Electron Spin Resonance (ESR) spectroscopy that is suitable to reveal the presence of free radicals, i.e. molecules characterised by a non-zero magnetic moment, generated by the presence of one or more unpaired electrons. The physical phenomenon on which ESR spectroscopy is based is that of resonant absorption in a magnetic field. ESR methods for identifying irradiated foods involve determining stable radio-induced radical species in matrices containing bones, cellulose or crystalline sugar (EN 1786, 1996; EN1787, 2000; EN 13708, 2001). The remaining confirmatory standard is based on Thermoluminescence (TL) that uses heat to stimulate the silicate minerals present as contaminants and to induce the emission of photons detected later by a photomultiplier tube (EN 1788, 2001). This paper presents the results of official controls on food matrices of both animal and vegetable origin in the period 2006-2011, thus providing an updated overview on the status of imported irradiated foodstuffs from emerging markets, as well as information on the distribution of irradiated food on the Italian market. Analysis of the results will also provide useful information for the following purposes: to analyse the effectiveness of official methods and highlight the strengths and weaknesses of each technique; to focus future controls in an appropriate manner, to promote the international irradiated foods market, and to strengthen consumer confidence.

5.3 Materials and methods

5.3.1 Sampling

Since the first phase of the controls consists of sampling, to ensure proper representation and consistency of each sample, official sampling offices around the country were involved (local offices of the Italian Ministry of Health and Italian National Health Service facilities). The operating instructions for the sampling were provided by our laboratory and apply to quantity – in order to ensure implementation of the screening and confirmatory analyses – as well as to storage and transport. For samples to be analysed by DNA Comet Assay, it is

essential for a constant temperature to be maintained, while for analyses with luminescence techniques (PSL and TL), samples need to be protected from sources of light and/or heat. Sampling initially focused on the different types of foods that may be irradiated in Member States and marketed there, and was then extended to imported products. A total of 451 samples, collected from 2006 to 2011, were selected on the basis of validated and accredited techniques, and divided into six food categories: meat products (MP), seafood products (SP), herbs, spices and seasonings (HSS), fruit (F), vegetables (V) and other (O). Samples in the MP category consisted mainly of unboned poultry (such as chicken, turkey, duck etc.), frogs' legs, beef, pork, horse, sheep, goat, etc. Samples in the SP category consisted mainly of shellfish (clams, mussels, tellins, etc.), crustaceans (shrimps, prawns, etc.), cephalopods (octopus, squid and cuttlefish) and unboned fish (salmon, red mullet, anchovy, turbot, etc.). The HSS category consisted mainly of oregano, black pepper, chilli pepper, mixed herbs etc., whereas category V consisted of garlic, potatoes, onions, mushrooms, wheat etc. Category F consisted primarily of dried fruit (walnuts, peanuts, almonds, pistachios and hazelnuts) and some fresh fruit (strawberries, pineapples, loquats, papayas etc.). Finally, category O included samples of various kinds: three food supplements, a sample of tofu with chilli and a sample of brown sugar. The sampling plan is shown in Table 1 with the total number of samples divided up per year and per food category.

Table 1 - Number of samples per year per food category.

FOOD CATEGORY	2006	2007	2008	2009	2010	2011	TOTAL	%
MEAT PRODUCTS (MP)	2	12	8	8	17	49	96	21.3%
SEAFOOD PRODUCTS (SP)					6	169	175	38.8%
VEGETABLES (V)			6	9	19	44	78	17.3%
FRUIT (F)				1	13	23	37	8.2%
HERBS, SPICES AND SEASONING (HSS)			4	3	19	33	59	13.1%
OTHER (O)					2	4	6	1.3%
TOTAL	2	12	18	21	76	322	451	100%

5.3.2 Experimental

The screening and confirmatory standards used to perform the checks are reported in Table 2 with the corresponding fields of application. Each method was previously optimised and extended to the most common matrices on the

domestic market likely to be treated with ionising radiation. The equipment used in the laboratory includes an SURRC pulsed photo stimulated luminescence system, a Risø TL/OSL DA-20 thermoluminescence reader, an Optica B-350 epifluorescence microscope, a Bruker EMX-113 electron spin resonance spectrometer and a Rad Source RS 2400 X-ray irradiator. The glassware, reagents, solvents and disposable materials were those commonly used in laboratories. All analytical procedures were performed by qualified personnel and include rigorous quality assurance programs meeting the requirements of the international standard (ISO/IEC 17025, 2005), such as metrological confirmation, internal quality control and participation in proficiency tests. In most cases, the protocol developed by the laboratory involves a preliminary screening analysis, followed by confirmatory analysis only in the case of doubtful or positive results in the first phase. Samples in the MP category were analysed according to a protocol that involves screening with the DNA Comet Assay, and ESR for the confirmatory analysis, although this protocol has only been applied since 2010, when the DNA Comet Assay method was first validated and accredited.

Table 2 - Standard used for official controls and their field of application.

STANDARD	TECHNIQUE	METHOD	FIELD OF APPLICATION
EN 13784:2001	DNA COMET ASSAY	SCREENING	MEATS, SEEDS, DRIED FRUITS, SPICES
EN 13751:2009	PSL	SCREENING	HERBS, SPICES, MOLLUSCS AND CRUSTACEANS
EN 1788:2001	TL	CONFIRMATORY	HERBS, SPICES, SHRIMPS, POTATOES, FRUITS AND VEGETABLES
EN 1786:1996	ESR	CONFIRMATORY	FOOD CONTAINING BONE
EN 1787:2000	ESR	CONFIRMATORY	FOOD CONTAINING CELLULOSE (PISTACHIOS, PAPRIKA, STRAWBERRIES)
EN 13708:2001	ESR	CONFIRMATORY	FOOD CONTAINING CRYSTALLINE SUGAR (DRIED FIGS, MANGO, PAPAYA AND RAISINS)

Most of the samples in the SP and HSS categories were analysed according to a protocol involving PSL for the screening and TL for the confirmatory analysis, while in just a few cases the confirmatory analysis was performed with ESR. Matrices lacking the requirements for screening analysis (e.g. fruit, octopus, squid etc.) were analysed directly with the respective confirmatory techniques. The methods used are described below.

5.3.2.1 Screening techniques

a) *Photostimulated Luminescence (PSL)*

Sample preparation using PSL is very simple in the case of herbs, spices, seasonings or some dried matrices, it involves completely covering the base of a Petri dish with the sample. In the case of molluscs, by contrast, 3 to 6 edible parts are placed in the Petri dish (depending on shell size), while for crustacea a minimum of 6 intestines are required. The sample is always analysed in duplicate. The results were interpreted by comparing the values obtained by counting the sample for 60 seconds expressed as cts/60 s, with two threshold values which differ depending on the type of matrix.

For herbs, spices and seasonings, the threshold values are as follows: T1=700 cts/60 s and T2= 5000 cts/60 s, while for molluscs and crustaceans: T1=1000 cts/60 s and T2=4000 cts/60 s. All samples with PSL count values below T1 were classified as “negative”, those with counts between T1 and T2 were classified as “doubtful”, while all samples with counts above T2 were regarded as “positive”. Where results were discordant, the procedure involved preparing and analysing four additional plates, to ensure that the final result for the sample would be found in at least four of the six replicates. Any positive or doubtful samples were analysed with a suitable confirmatory technique. The applicability of the method to matrices not yet validated by the European standard (EN 13751, 2009) has also been demonstrated recently by the Authors (Marchesani, Mangiacotti & Chiaravalle, 2012) as well as by other studies (Sanderson et al., 1996 a, b).

b) *DNA Comet Assay*

Preparation of the sample involves obtaining a monocellular suspension transferred onto coverslips pretreated with a thin layer of agarose, followed by cell lysis using a detergent to remove the membranes. After brief washing in TBE (Tris-hydroxymethylaminomethane) buffer, the electrophoretic run was performed at 2 V/cm for two minutes at room temperature to migrate DNA fragments from the nucleus. The migrated DNA is analysed and quantified using a solution of acridine orange dye, measuring the intensity of fluorescence under a microscope. In the presence of intact DNA, extracted from untreated tissues, an integral fluorescent nucleus is observed; where, by contrast, the DNA is damaged following exposure to ionising radiation, the DNA fragments migrate to the anode, which takes on the shape of a comet, made up of a head that is not always visible and a more or less extensive tail, depending on the degree of DNA damage.

5.3.2.2 Confirmatory techniques

c) Thermoluminescence (TL)

Sample preparation by thermoluminescence is very time-consuming and complex, and consists of extracting the silicate minerals present in the sample as contaminants in various stages: initial gravimetric separation is followed by density separation using sodium polytungstate and subsequent washing with hydrochloric acid, sodium hydroxide, water and acetone in order to obtain mineral silicates free of organic matter. The minerals are separated, deposited on specific clean stainless disks, and placed in an oven overnight at 50°C. Instrumental analysis provides an initial reading of the extracted minerals to obtain the first glow curve (G1), irradiation at a default dose of 1 kGy or 250 Gy (for potato, garlic and onion samples) and a re-reading of the disk to record the second glow curve (G2). Subsequently the glow ratio (G1/G2) is calculated, i.e. the ratio between the integral of the two glow curves in a default temperature range common to G1 and G2. The interpretation of the result is qualitative and consists in comparing the glow ratio with the discrimination limit of 0.1 and verifying the presence of a peak in the region of interest as reported in the international standard (EN 1788:2001). All samples with a glow ratio of less than 0.1 are classified as “non-irradiated”, while samples with a glow ratio of above 0.1 are regarded as “irradiated”. Extension of this method to matrices not yet validated by the European standard has also recently been carried out by the Authors (Marchesani, Mangiacotti & Chiaravalle, 2012; Mangiacotti et al., 2009) as well as in studies performed by other authors (Correcher & Garcia-Guinea, 2011; Bhatti et al. 2008; Khan, Bhatti & Delincée, 2002).

d) Electron Spin Resonance (ESR)

Sample preparation for matrices containing bone consists in the elimination of the organic part in order to obtain bone pieces in the form of fragment or powder, while for matrices containing cellulose or crystalline sugars, the sample is used as is or cut into very small pieces. In all three methods, the samples are dried in an oven for about 3 hours at a controlled temperature of 40 +/- 5°C, after which an amount of about 50-100 mg is transferred into suitable quartz tubes for instrumental analysis. The ESR spectrum for irradiated matrices containing bones presents an orthorhombic symmetrical signal for CO₂⁻ superimposed on a symmetrical signal due to organic impurities, that in the case of non-irradiated foods is the only visible signal. For matrices containing cellulose, the spectrum consists of a broad singlet line with unresolved hyperfine splitting upon which an additional narrow signal is superimposed, due to semiquinone radicals produced by the oxidation of polyphenolics in plants, whereas treated samples show the formation of a typical cellulose paramagnetic

species with two satellite lines at a distance of about 3 G from the central signal. For matrices containing sugars, the spectrum typically exhibits a single-line symmetrical signal for non-irradiated matrices, while irradiated samples show a complex multi-component spectrum with a total amplitude of about 90 G, due essentially to the presence of radicals produced by the irradiation of crystalline mono- and disaccharides.

5.4 Results and discussion

5.4.1 Results

The distribution of the samples analysed in the period 2006-2011 is presented in Table 1, which shows that most of the samples were analysed in 2011 (71.4%), owing both to the increase in the number of techniques accredited by the laboratory, and to the increased participation in control activities by local official authorities. The percentage of samples from 2010 amounted to 16.8%, with the rest being analysed between 2006 and 2009. Most of the samples belonged to the SP category, followed by MP and HSS. The results showed that a total of 18 samples out of 451 were non-compliant (irradiated and improperly labelled). The majority of the non-compliant samples were in the SP (10 samples) and MP (6 samples) categories. In 2010, two non-compliant samples were found out of a total of 76: 1 sample of frogs' legs from Vietnam and 1 sample of tofu with chilli from China. In 2011, however, when 322 samples were analysed, a total of 16 non-compliant samples were found, including 5 samples of frogs' legs, 3 samples of clams, 2 samples of cuttlefish, 1 sample of octopus and 1 sample of prawn from Vietnam, as well as 3 samples of squid and 1 sample of white pepper from China.

Figure 1 shows, in summary, the distribution of irradiated and unirradiated samples, divided by category and expressed as percentages. The techniques used to analyse non-compliant samples were as follows: the samples of frogs' legs were analysed only in ESR, the cuttlefish, octopus and squid were analysed only by TL, whereas the clams, white pepper and shrimp were first screened by PSL, and then confirmed by TL. Finally, the tofu with chilli sample was screened positive by PSL (analysed whole), whereas confirmatory analysis was performed by ESR on only one of its components, i.e. chilli. According to the law, any sample that has one or more irradiated ingredients is considered irradiated, so the sample of tofu with chilli was classified as non-compliant. Figure 2 summarises information on the non-compliant samples.

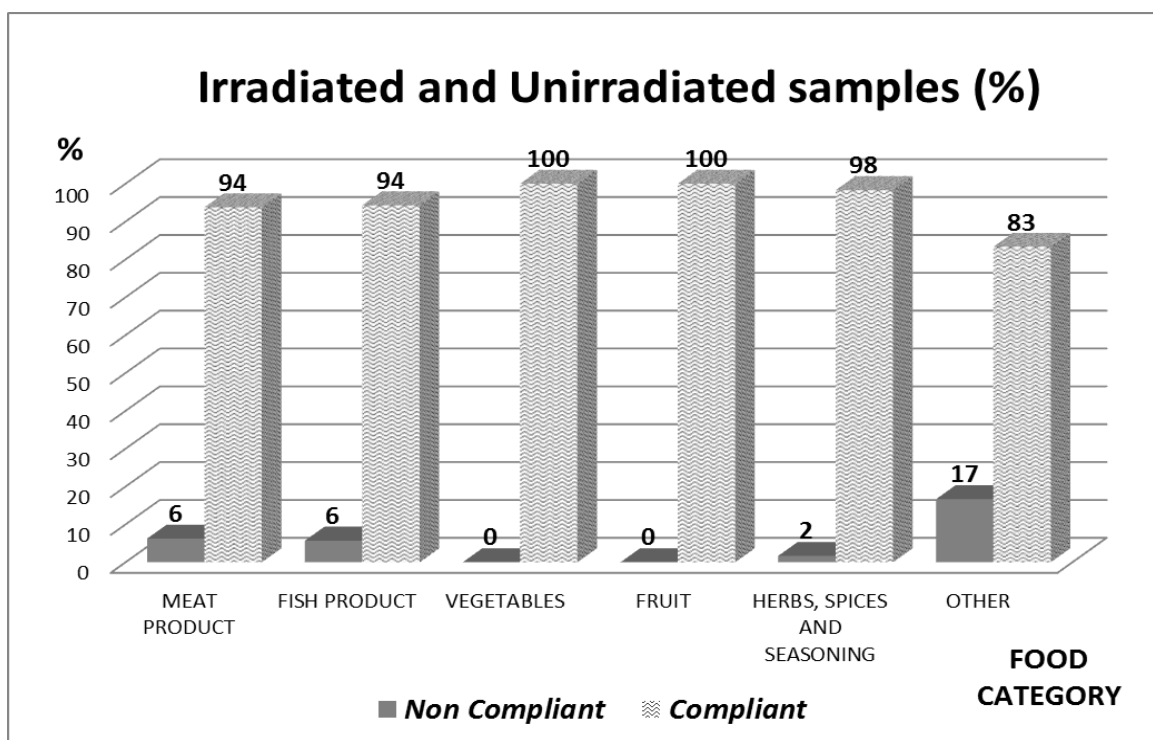


Figure 1 - Percentages of irradiated and unirradiated samples per food category.

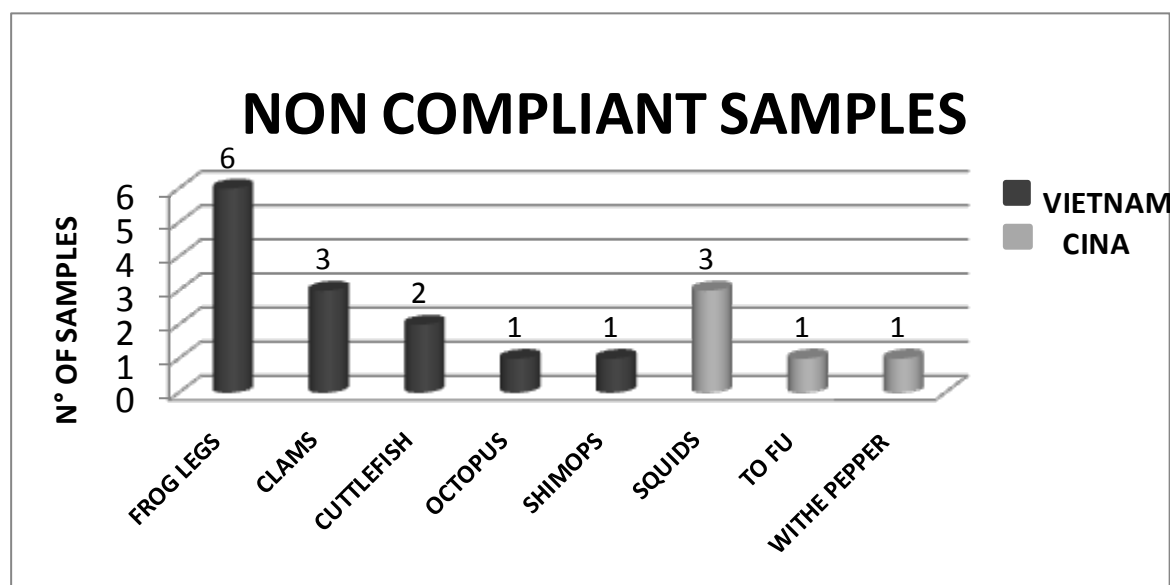


Figure 2 - Number, type and origin of non-compliant samples.

Table 3 shows the number of tests carried out from 2006 to 2011, divided by year and by analytical technique. ESR was the first technique to have been validated and accredited and was therefore available throughout the period 2006-

2011. PSL and TL, meanwhile, were used from 2008 to 2011, while the DNA Comet Assay was only performed from 2010 onwards.

Table 3 - Number of analyses per technique 2006-2011.

ANALYTICAL TECHNIQUE	2006	2007	2008	2009	2010	2011	TOTAL
PSL			10	10	37	165	222
DNA COMET ASSAY						23	23
TL				3	4	98	105
ESR	2	12	8	8	36	70	136
TOTAL	2	12	18	21	77	356	486

Regarding the choice of technique, 45.6% of total analyses were screened with PSL, with another 4.7% by DNA Comet Assay. The remaining 27.9% and 21.6% of the analyses were carried out using the confirmatory techniques, ESR and TL, respectively. This table shows that the total number of analyses performed is greater than that of the samples, given that some of them had screened positive or doubtful and so were reanalysed with the most appropriate confirmatory technique. With regard to the field of application of the various techniques, the biological method was used to analyse 23 samples of meat. PSL, however, enabled us to analyse the widest variety of samples (39%), i.e. all samples in categories HSS and V, molluscs and crustaceans in category SP and various dietary supplements. Unboned fish, dried fruit, strawberries, brown sugar, some spices and vegetables (wheat) and most of the samples in category MP were analysed in ESR, while TL was used to analyse fresh fruit, cephalopods and all positive or doubtful samples from PSL analysis (e.g. molluscs, spices and vegetables). One of the qualitative parameters for assessing the accuracy of a screening method is the percentage of false positives. In this regard, of the 245 samples analysed with the screening techniques, 36 were doubtful or positive. Six in particular, from category MP, were doubtful using the DNA Comet Assay and were later confirmed to be false positives using ESR, while of the remaining 30 samples screening positive or intermediate with PSL, six were confirmed as non-compliant and 24 identified as false positives (by TL or ESR). Thus, the DNA Comet Assay had a false positive rate of 26% (6/23), while for PSL the percentage fell to 10.8% (24/222). Figure 3 shows the annual trends of the percentage use for each analytical technique. PSL showed a slight decrease over the years, dropping from 55% in 2008 to 46% in 2011, while ESR, which for the first two years was 100%, in the following years showed a marked downward trend from 44.5% in 2008 down to about 20% in 2011. TL shows an upward

trend, starting from 14%, declining to 5% in 2010, but then increasing significantly in 2011 (28%). This is due to the fact that sampling in the final year focused mainly on seafood products (cephalopods, molluscs and crustaceans), a category in which most of the non-compliance has been found.

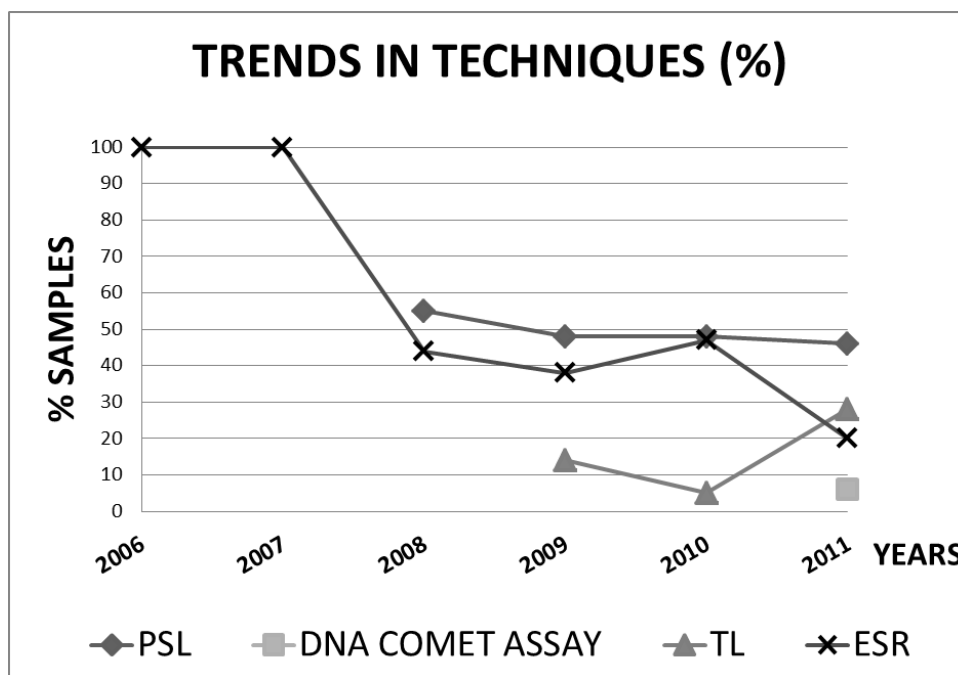


Figure 3 - Trends in techniques used 2006-2011 (shown as percentages).

5.4.2 Discussion

Recent studies (Kume et al., 2009) have reported that the amount of food irradiation is decreasing in the EU and growing significantly in Asia. In fact, the non-compliance found in this study concerned only food from non-European countries, Vietnam and China, which have no treatment facilities accredited at European level. All samples of Italian origin analysed in this work, mostly foods authorised at national level (e.g. garlic, onion and potatoes) were compliant (non-irradiated), which confirms that irradiation technology is not used in Italy.

With regard to the techniques used, the results obtained confirm that PSL is a versatile technique, suitable for screening numerous food matrices, with a small percentage of false positives (about 11%) compared to the DNA Comet Assay (about 26%). Moreover, comparison between the two screening techniques shows that the benefits of PSL, as confirmed by our research, lie not only in its greater accuracy, but also in its speed and simplicity both when preparing samples, and when reading instruments, as well as incurring significantly lower laboratory consumables costs. Of the confirmatory techniques, however, ESR is the best for analysing meat and fish containing bones and for dried fruit, with a signal that remains stable over time, while for samples containing cellulose, such

as spices, our laboratory confirmed the finding in the literature (Raffi et al., 2000), that the ESR signal tends to decay over time, so we preferred TL for official controls. The TL technique has the major disadvantage of being expensive and time-consuming, and sample preparation requires great dexterity and precision. Even in TL, the signal is subject to fading, so all matrices were analysed within seven days of sampling. Most of the false-positive samples in PSL were shrimps, prawns and clams, because contributions from the geological signal can lead to a misclassification. The false positive results from the DNA Comet Assay, meanwhile, are closely correlated with how the samples were transported and stored; indeed, we found that thawing and refreezing a sample can destroy the cell wall of animal cells and lead to leakage of DNA, with the consequent formation of comets in the analysis phase, a result that has also been found by other authors (Duarte et al., 2009). The results of the official controls described in this paper were then compared with official data from the European controls, as per the annual reports (European Commission 2001-2010) and with data reported in the literature by other authors (Chen et al., 2012). The European data used for the comparisons range from 2007 to 2010, as they are the more recent data and very homogeneous. Over this period, between 6637 and 9263 tonnes of food are estimated to have been irradiated, with the most commonly irradiated foodstuffs being frogs' legs (37%), poultry (20%) and aromatic herbs and spices (20%). The number of controls performed ranged from 6220 and 6463; the percentage of non-compliant samples varied between 2 and 4%, while the doubtful samples amounted to between 0.7 and 1.5%. Most of the non-compliance was due to mislabelling and to the irradiation of products from non-authorized categories, whereas doubtful results were often due to a lack of confirmatory identification, after positive tests, and/or difficulty in determining which ingredient in compound foodstuffs had been irradiated. The number of non-compliances found, during 2010-2011 alone, was in the range 2.6 - 4.9%, almost comparable with the European figures (2-4%), where in the category of seafood products 34 samples were found to be non-compliant out of 1632, a rate of 2.1% which when compared to the rate of 5.7% found in this study (10 non-compliant samples out of 175) suggests that in future it would be appropriate to increase the number of controls, especially on imported products, considering the low amount of seafood products irradiated in Europe in recent years (about 2.5%). The remaining non-compliant samples found in this work, however, fall into the category of foods most subject to irradiation in the EU (frogs' legs and spices). A total of 7 out of 29 samples of frogs' legs in the European study (24.1%) were non-compliant, compared with 6 out of 16 samples in this study (37.5%). Such high percentages of non-compliance for such a small number of samples, given the large quantities of frogs' legs irradiated in Europe (37% of total irradiated foods), suggest continuing and intensifying the controls on this

type of product during both the marketing and import phases. Finally, a comparison of the HSS category showed that in this study 59 samples were collected, one of which was non-compliant (1.7%). At European level, however, 17343 samples were collected, of which 129 were non-compliant (0.7%); therefore, given that the amount of irradiated herbs and spices in Europe is very high (20%), more focused controls are needed for samples in this category. The same reasoning applies for poultry sampling, as even though about 20% of products irradiated in Europe are poultry, in the EU study only 1/545 samples (0.18%) was non-compliant, while all of the poultry samples in this study (25) were compliant. Finally, comparison of the data in this study with those reported by Chen et al. (2012) shows that China irradiates many types of food matrix, both of plant origin, with high rates of non-compliance, and of animal origin, on which controls need to be strengthened, as demonstrated by the results set out in this paper.

5.5 Conclusions

The samples from official controls used for this work were chosen in order to provide a wide variety of matrices, that would in turn provide an overview of irradiated foods in Italy and more detailed knowledge on the prevalence of irradiation in the food sector. The number of non-compliant samples, 18 out of a total of 451, although in line with average European figures, is probably underestimated because they included so many vegetable matrices (e.g. garlic, onions and potato), authorised in Italy but not often irradiated either on the domestic market or elsewhere. Seafood, however, is the most irradiated category, with frogs' legs and cephalopods having the highest percentages of non-compliance. Regarding the source of non-compliant samples, 13 out of 18 came from Vietnam, while the remaining five were from China, given that these two countries have numerous irradiation facilities, none of which have so far been accredited by the competent authorities in the European Union.

These controls also enabled us to test and apply methods for identifying a wide range of food products treated with ionising radiation, confirming the usefulness and highlighting the limits of each technique. The results obtained, the skills acquired and the experience gained through this work will help plan future official control programmes, in order to meet specific regulatory requirements in force regarding foods and food ingredients treated with ionising radiation. On the basis of the non-compliances reported in this paper, and a comparison with the data made available by the European Union, it can be concluded that future official controls should focus especially on food at the import stage. In conclusion, in order to ensure free movement of goods, as well as consumer

right to choose, official controls should continue to be carried out and further suitable methods should be developed in order to identify irradiated foods, given the increasing commercial use of irradiation technology.

5.6 References

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CHAPTER 6

General Discussion

6. General discussion

In this chapter are discussed the overall results obtained in the investigations undertaken through the research work.

6.1 Electron Spin Resonance methods

The outcomes described in the previous papers prove the applicability of ESR spectroscopy in the detection of various foodstuffs of animal or plant origin. The methods are based on the detection of stable paramagnetic centres formed in several food after radiation processing. The presence of asymmetric signals is assigned to radiation induced bioconstituent radicals either in the mineral component of bones or in mollusks' shell inorganic part, whereas symmetric paramagnetic signal is related to the identification of cellulose radicals or mollusks' shell organic protein. Although during storage or subsequent manufacturing process electron spin resonance signals can be transformed due to the complexity of involved radical reactions with the matrix components of food, all of the developed methods are specific and sensitive enough to assure a reliable identification of irradiated foods and are recommended to perform routine control on marketed food items. The results reported confirm the applicability of ESR spectroscopy in the identification of four types of irradiated mollusks (brown Venus shells, clams, mussels and oysters) not validated in the European standards. For the first three species only a dose of 1.5 kGy was tested, that is the one commercially more used. For oysters, doses in the range 150 Gy–7.0 kGy were administered and irradiated mollusks could be identified also at the lowest dose. While identification of the irradiated species is mostly based on the detection of the ESR signal from the SO_3^- and CO_2^- radical anions, in the case of brown Venus shells and, to a lesser extent, of clams the isotropic ESR signal from an organic radical, believed to derive from one of the aminoacids present in the scleroprotein conchiolin, was found to be an as yet unreported very persistent and unequivocal irradiation marker. In the case of oysters a protocol was defined that allows reconstruction of the administered dose within a good reliability (ca. 25 %) in the studied dose interval. The cavity sensitivity is critical to the reproducibility of the signal from the samples and thus the use of an internal standard is needed to assure the accuracy of the proposed quantitative protocol.

In conclusion, the investigation undertaken demonstrated the feasibility of using ESR spectroscopy as a practical tool to identify or estimate dose levels of irradiated mollusks. The ESR technique has proven to be suitable as a

quantitative methods after having taken into account and studied the possible influencing parameters on the yield of radiation induced radicals.

Furthermore analytical methods exhibited many positive features: specific response to ionising radiation treatment, until now not found in similar control samples; detection of different signals able to discriminate between irradiated and not treated foods; ability to choose irradiation marker of both organic or inorganic nature depending on the sensitivity required or detection purposes (identification or dose estimation); determination of fading behavior over typical commercial shelf-life; simple and rapid sample preparation steps required; application on limited sample size with amount of foods ranging from 100 to 200 mg; reproducibility of the procedures based on sufficiently stable markers to enhance detection through the expected shelf-lives even at doses lower than the minimum level applied in common commercial practices; capability of rapid and repeated inexpensive measurements; dose estimation of the actual dose of treatment received by the product at the irradiation facility.

Although the analytical technique has been successfully applied on common and wide consumed foods such as meats, seafood, and fruits there is evidence that current ESR based standard methods (EN 1786, EN 1787 and EN 13708) have to be more studied and improved to be effectively used in routine control programs or surveillance plans by food control bodies involved in official checks on correct labeling of irradiated foods. However the developed detection methods confirmed the efficacy of ESR methodology in identifying commercial foods if suitable studies are previously carried out in order to evaluate the signal intensity and spectrum pattern change over the time elapsed from irradiation, also establishing the minimum detectable dose level for each food categories. Indeed the instability of the induced peaks along with low absorbed doses may greatly limit the period over which detection can be performed. Therefore further research is needed to extend the field of application of current standards and to study more accurately the factors influencing signal intensities and consequently the quantification estimations of administered dose in treated foods.

Finally, as a definite test of any analytical method and especially in reconstruction dose protocols, it would be desirable to assess their validity in a collaboration blind trial from various laboratories using analytical equipments with different performances and sensitivities.

6.2 Luminescence based methods

Among physical methods standardized at European level and approved as Codex Standards by Codex Alimentarius Commission of FAO-WHO there are

those analytical procedures based on luminescence phenomena: photo-stimulated luminescence (PSL) as a screening method and thermoluminescence (TL) as a powerful confirmatory method. Both screening and confirmatory methods are based on the detection of radio-induced luminescence centres present in foods either as constituents or as contaminating minerals. Regardless the stimulus applied, heating or optical radiations, the luminescence techniques have been proved of extensive applicability being suitable for the detection of different and various food categories of both plant and animal origin.

A comprehensive study on irradiated oysters using both PSL and calibrated PSL in conjunction with three different TL analytical procedures has shown luminescence performances of each approach. The results confirm the great importance to carry on experimental procedures to set up more reliable and rapid methods on every food category where a possibility to choice among more radiation markers, bioinorganic compounds and polyminerals (from outer shell or intestines), exists.

6.3 Application of standards

In view of the continuously increasing interest in identifying irradiated foods, there is a strong activity in developing rapid, reliable, more efficient and validated detection methods. To underline the importance of validation and to extend the field of application of current standards the results of analytical controls on a large number of foodstuff samples over a period 2006 - 2011, long enough to evaluate the performances of mostly used methods, were analysed. The determinations were carried out using 4 different screening and confirmatory techniques: PSL, DNA Comet Assay, TL and ESR. For each method have been established the limit of application, including the list of food categories and the lowest doses at which the food matrix can be correctly identified. Check results also showed that among screening methods PSL is the most accurate, simple and practical standard to analyse most of samples (spices, herbs, supplements, mollusks, crustaceans and vegetables) with a low false positive classification, whereas DNA Comet Assay revealed the highest percentage of false positive cases. ESR is the suitable confirmatory method to detect dried fruits and foodstuffs (meat and fish product) containing bone, while TL is the best confirmatory method to detect herbs, spices and supplements, cephalopods, mollusks and crustaceans, besides fresh fruits and vegetables. This study, based on the extent of the non compliance and by comparison with European data, suggests more checks on meat products (frog and poultry meats), fish products (cephalopods, mussels and crustacean) spices and supplements especially at import stage from countries where non approved

irradiation facilities are operating (e.g. Vietnam and China). It is also evident the importance of the experimental evaluation as a practical criterion for the selection of most suitable procedures depending on the food matrix to be analyzed and its storage status. One important finding of the comparative studies is that ESR, TL and PSL are the most reliable, rapid, and promising methods. Only for limited kind of samples such as foods containing likely irradiated ingredients, proper combination of two or more methods have been applied for testing the irradiation status. In conclusion, one should consider all detection methods as dynamic analytical tools because in practice there is a strong need for introducing modifications to improve their effectiveness and avoid potential interferences.

6.4 Future of food irradiation detection methods

To date, more than 50 countries have approved over 100 products to be irradiated. The USA, South Africa, Thailand, emerging economies such as Brazil, China, Mexico and among European countries The Netherlands, are leaders in adopting the technology. Currently regulations on food irradiation in the European Union are not fully harmonized. Although Directive 1999/3/EC establishes a positive list of foods which may be irradiated and traded freely between Member States, so far only one food category – dried aromatic herbs, spices and vegetable seasonings – has been approved. Some countries, such as Belgium, France, The Netherlands, Poland, and the UK allow other foods to be irradiated, whereas other countries, such as Denmark, Germany and Luxembourg remain opposed.

In Italy in addition to the only food category authorized in all Europe, other three categories of foods (potatoes, onions and garlicks) are cleared for irradiation at specified doses, but only in 2006 a limited amount of foodstuffs (spices and dried herbs) was irradiated.

Even if food irradiation is slow to gain support within many parts of Europe, including Italy, in the USA and several other countries this technology is gaining popularity with increasing consumer acceptance. Indeed many consumers, initially hostile to irradiation, after a proper understanding of the process become generally more in favour. It is evident that there is an important role for respected professional bodies and regulatory agencies to inform consumers of the advantages and limitations of the technology so that they can make informed decisions on purchasing and eating irradiated foods. This view has been endorsed by international bodies such as the World Health Organisation, the Food and Agricultural Organisation and Codex Alimentarius. Therefore is increasingly critical to all stakeholders (food industry, policymakers and

consumers) to continue in developing detection methods able to meet the requirements of changing regulations across the world ensuring that consumers are fully informed whether foods or ingredients have been irradiated and eventually what dose levels they absorbed. Thus the research activities finalized to develop quantitative methods and improving already existing qualitative methods will be enhanced in the near future. Those methods will establish a means to further promote the international trade for informed consumers able to make reasoned choice in favor of irradiate foods. Then the symbol "radura" in conjunction with the phrase "treated with ionizing radiation" or other explicative statements, will not serve anymore as a warning but likely as a value-added element, synonymous of safer and higher quality food.

CHAPTER 7

Summary

7. Summary

Today's food industry is faced with several important challenges, including food product deterioration and the constant increase of diseases related to the presence of pathogenic microorganisms in food products. Thus, adequate and effective food preservation strategies are even more important. Food irradiation is a technological process that can improve the microbiological quality of foodstuffs and extend the period in which it can be safely consumed. The radiation treatment, carried out under conditions of Good Manufacturing Practice, is considered as an effective, widely applicable food processing method judged to be safe on extensive available evidence. This technology can reduce the risk of food poisoning, control food spoilage and extend the shelf-life of foods without detriment to health and with minimal effect on nutritional or sensory quality. Due to its numerous positive effects, including those of a commercial nature, food irradiation has assumed a highly important role in the field of food preservation, and increasingly large numbers of foodstuffs are subjected to this treatment each year. For some time now, countries equipped with adequate food irradiation facilities have used this technology at well defined doses for the preservation of various foodstuffs. Because of the divergent opinions expressed by many consumers' organizations, the European Union has issued two directives (1999/2/EC and 1999/3/EC), which have been implemented in Italy by Legislative Decree No. 94 of 30 January 2001. Those directives aims at harmonizing the rules concerning the treatment and trade of irradiated foods in EU countries. With the open market, each country is obliged to accommodate the presence in its internal market of irradiated food commodities treated in other EU states or in extra-European countries. To further safeguard the consumer, the EU legislation provides for official annual checks at the product marketing stage, with the purpose of identifying improperly labeled or unauthorized products. Thus far, only limited food categories has been studied and subjected to interlaboratory validation by analytical detection methods for irradiated food identification. To meet the specific requirements of the laws and to increase acceptance of this type of food preservation technology, we have extended the field of application of both screening (PSL) and confirmatory (ESR, TL) physical methods to check compliance with labeling of irradiated foodstuffs. Therefore for consumer protection and information, following the invitation from the European Commission to improve and develop more reliable analytical standards, research work was focused on new applications of these physical methods. Relevant contributions have been made to the extension of the current field of application, with the development of promising analytical procedure able to estimate the actual dose administered to treated foods. The first goal was

achieved investigating, even at low doses (0.1 kGy), the luminescence yield of oysters, considered a great delicacy in many parts of the world, and validating its identification with two physical techniques: PSL as screening method and TL as a confirmatory one. Besides oysters other seafood, including bivalve mollusks, i.e. brown Venus shells, clams, and mussels, all of which are widely consumed and likely to be treated with irradiation were studied with Electron Spin Resonance (ESR) spectroscopy.

It is well known that irradiation by ionising radiation leads to the formation of many radical species which, if stable, could be detected in calcified tissue such as mollusks' shell. Identification of four irradiated species of bivalve mollusks, i.e. brown Venus shells (*Callista chione*), clams (*Tapes semidecussatus*), mussels (*Mytilus galloprovincialis*) and oysters (*Ostrea edulis*) was performed. ESR could definitely identify irradiated seashells due to the presence of long-lived free radicals, primarily CO_2^- , CO_3^{3-} , SO_2^- and SO_3^- radical anions. The presence of other organic free radicals, believed to originate from conchiolin, a scleroprotein present in the shells, was also ascertained. The use of one of these radicals as a marker for irradiation of brown Venus shells and clams can be envisaged. In addition to detection procedures a reliable dosimetric protocol for the reconstruction of the administered dose in irradiated oysters was proposed. Finally the results of a study on official checks by an accredited laboratory aimed at both evaluating the performances of detection methods and the presence of irradiated food on the Italian market, are discussed. Non-compliances found are due to both incorrect labelling and irradiation in non approved facilities in extra European countries. In summary, two physical methods, electron spin resonance (ESR) spectroscopy and thermoluminescence (TL) were studied most extensively and applied on a wide range of foods with successful results, whereas limitations of current standards were also assessed. The development and application of analytical methods for correct identification of irradiated samples from non-irradiated samples, along with protocols for dose evaluation, have become important for several purposes: upholding regulatory controls, checking compliance against labeling requirements, facilitating international trade, and reinforcing consumer confidence. Therefore the research on new detection methods represents a key area and more studies in this field should be encouraged.

CHAPTER 8

Acknowledgements

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