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Detection of Irradiated Ingredients Included in Low Quantity in Non-irradiated Food Matrix. 2. ESR Analysis of Mechanically Recovered Poultry Meat and TL Analysis of Spices

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Protocols EN 1786 and EN 1788 for the detection of irradiated food by electron spin resonance spectroscopy (ESR) and thermoluminescence (TL) were not conceived for the detection of irradiated ingredients included in low concentration in nonirradiated food. An enzymatic hydrolysis method, realized at 55 °C, has been developed for the extraction of silicate minerals and bone fragments. When followed by a purification of the extracts by an aqueous solution of sodium polytungstate, this method made it possible to detect very low inclusions of irradiated spices (0.05%, wt/wt by TL) included in various meals (cheeses and precooked meals). Even for food containing together two ingredients (spices and mechanically recovered meat), it was possible to detect and identify them simultaneously.

KEYWORDS: Food irradiation; detection of irradiated foods; ingredient; spices; mechanically recovered meat; thermoluminescence; ESR

INTRODUCTION

European Directive L66/16 (*1*) forces the food industry of all member states of the European Union to indicate on the label if one of the food ingredients was irradiated, whatever the inclusion rate might be. The electron spin resonance (ESR) detection of irradiated foods, when sold as items, and thus the control of their labeling do not present any major analytical problem because 10 standardized protocols were published by CEN. The situation is not the same when the detection of an irradiated ingredient included in low amounts in a non-irradiated food is to be considered.

Spices, aromatic herbs, and mechanically recovered poultry meat (MRM) are currently the foods most frequently subjected to irradiation. These foods are primarily used as ingredients by the food-processing industry. The best dedicated reference protocol (*2*) for the detection of irradiated herbs and spices [analysis of excited electrons trapped in silicates by thermoluminescence (TL) after ultrasound treatment of the samples and decantation of silicates in an aqueous solution of sodium polytungstate] and meats (analysis of induced radicals in bones by ESR spectroscopy after manual recovery and drying) (*3*) is irrelevant when silicates and small bone fragments are included in low amounts in a lipidoproteic complex matrix (cheeses, quenelles, etc). It is actually essential to remove the food matrix as much as possible before analysis of silicates by TL and of bone fragments by ESR spectrometry. Regarding the detection of irradiated bone fragments, Gray and Stevenson recommended an alkaline hydrolysis of food matrix by alcoholic boiling KOH (*4*) and Stevenson et al. an enzymatic hydrolysis by Alcalase (*5*). More recently, Marchioni et al. (*6*) proposed to perform the purification step with an aqueous solution of sodium polytungstate to get higher purity bone fragments and better ESR spectra. These authors achieved the detection of very low amounts (0.5%, wt/wt) of irradiated MRM included in industrial foods. However, the method was proposed for the detection of only irradiated MRM or fish bones in food and not for the detection of irradiated spices. Regarding the detection of irradiated spices (*2*), Carmichael et al. (*7*) proposed an acidic hydrolysis of the shellfish matrix to extract the silicate minerals. It might be possible to use this hydrolysis method for the TL detection of irradiated spices included in non-irradiated food. However, this protocol, proposed for the detection of only irradiated crustaceans and shellfishes, was never proved to be efficient for such a use. Furthermore, the acid hydrolysis (6 M HCl, 110 °C, 2 h) would certainly alter the integrity of the bone fragments, and this method cannot thus be used for the ESR detection of irradiated MRM or fishes in food.

Some previous works (*5*, *8*) have demonstrated the powerful hydrolytic capacities of Alcalase, which is an industrial protein enzyme reaching its maximum effectiveness under soft condi-

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tions (55 °C, pH 8). By using this protein it is now possible to release minerals adhering to the food matrix without altering the bone fragments.

The aim of this work is to present a single protocol for the extraction of silicates and bone fragments included in a food matrix [including an enzymatic hydrolysis (Alcalase) of the proteins, a dissolution of the lipids (sodium dodecyl sulfate; SDS), and a purification of the extracts by decantation using aqueous solution of sodium polytungstate] and to associate it with an analysis by ESR spectroscopy (bone fragments) or by TL (silicates) to carry out the detection of irradiated ingredients (MRM, fishes, and spices) included in various culinary preparations.

MATERIALS AND METHODS

Food Samples. The salmon fillets (irradiated at 5 kGy), the spice mix containing garlic, pepper, chive, and parsley (non-irradiated or irradiated at 5 kGy), individual spices of parsley, pepper, and curry (irradiated at 10 kGy), and the MRM (irradiated at 5 kGy) were provided by French food companies. The spice mix and the MRM were delivered by the supplier after being irradiated in an industrial plant. All other samples were irradiated in the laboratory.

Inclusions of irradiated spice mix (0.5 and 1%, wt/wt) in cheese were carried out in the cheese dairy. Lower inclusion rates (0.05 and 0.1%, wt/wt) and higher inclusion rates (3, 5, and 10%, wt/wt) were carried out in the laboratory using the cheesemaking base provided by the dairy. Cooked dishes [poultry quenelles (5%, wt/wt irradiated MRM, 0.6%, wt/wt irradiated curry) cooked for 20 min at 65 °C and fish quiches (7 and 12%, wt/wt, respectively, of irradiated and non-irradiated salmon, 3 and 0.2%, wt/wt, respectively, of irradiated parsley and pepper) cooked for 10 min at 220 °C] were carried out by the Gathering and Tourism College of Strasbourg (Illkirch, France). All of the foods were preserved at −20 °C until they were analyzed.

Chemicals. Sodium monohydrogen phosphate, potassium dihydrogen phosphate, aqueous solution of ammonia (25% v/v), carboxymethyl cellulose, and SDS were provided by Merck (Darmstadt, Germany). Sodium polytungstate was an Interchim product (Montluçon, France). Sodium hydroxide pellets were provided by BDH (Poole, U.K.). Acetone and hydrochloric acid (37% v/v) were provided by Carlo Erba (Rodano, Italy). The proteolytic enzyme (Alcalase 2.5 L, DX) was a food quality industrial protease provided by Novo Nordisk (Bagsvaerd, Denmark). The ultrapure water was produced with Milli-Q PLUS equipment from Millipore (Saint Quentin, France) equipped with a 0.45 μm filter.

Materials. The ultrasonic treatments (42 kHz, 120 W) were carried out in a Deltasonic (Meaux, France) ultrasonic bath able to contain two beakers of 1000 mL. The centrifuge (MR1822, Jouan, Saint Nazaire, France) was equipped with a swing-out rotor for 10−15 mL conical bottom tubes. The Bruker ESR spectrometer, type ECS 106 (Wissembourg, France), was equipped with a TMH ECS 4108/9105 cylindrical resonator. The Harshaw-Bicron thermoluminometer, type QS 3500 (Solon, OH), was equipped with stainless steel (quality 18/10) measurement disks (diameter = 8 mm, thickness = 1 mm). The measurement TL cell was purged with N45 quality nitrogen provided by Air Liquide (Paris La Défense, France). The vacuum oven was a Heraeus product (RVT 360 type, Henau, Germany).

Irradiation Treatments. A Van de Graaff electron beam accelerator, 2.2 MeV, 75 μA (Vivirad High Voltage, Handschuheim, France), was used to carry out the radiation treatments of the food samples and disks containing silicates for TL measurements. The dose rate was of ∼1 kGy s^{-1}. The dosimetry was carried out using radiachromic FWT 60.00 optical dosimeters (Far West Technology, Goleta, CA) calibrated against alanine dosimeters (Laboratoire National Henri Becquerel, Gif sur Yvette, France), the French national reference for high absorbed doses. A 100 μm copper scattering foil was put over each sample to obtain a good homogeneous dose distribution within the thickness of the food (±10%) (*9*).

Analytical Protocol. To avoid any contamination by dust, all of the glassware was rinsed several times by a strong jet of water, the various containers were covered during analysis by aluminum foil, and the working solutions were filtered through filter paper.

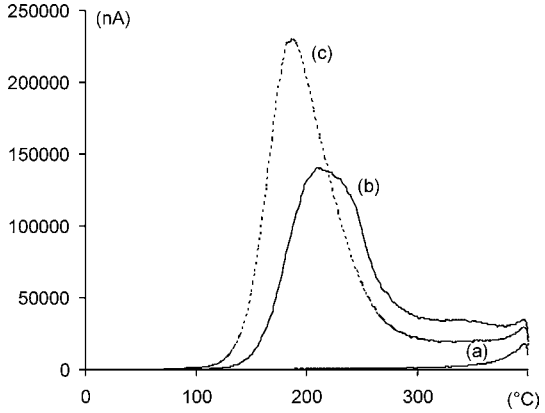

Figure 1. Glow 1 curves obtained with minerals extracted from 3 g of non-irradiated (**a**) and 5 kGy irradiated (**b**) spice mix, and glow 2 curve for re-irradiated minerals (**c**) extracted from spice mix.

Two hundred grams of food sample was coarsely cut, crushed with a knife homogenizer, and placed in a beaker containing a mixture of 100 mL of Alcalase, 6 g of SDS, and 400 mL of 0.2 M phosphate buffer (KH_2PO_4, 0.92 g L^{-1}; $Na_2HPO_4 \cdot 2H_2O$, 35.6 g L^{-1}), pH 8.2. This suspension was subjected to a vigorous magnetic agitation during 1 h at 55 ± 5 °C. During the hydrolysis, the pH of the suspension was controlled every 20 min through pH testing paper and was adjusted using NaOH pellets. After hydrolysis, the suspension was subjected to a 10 min ultrasonic treatment and then sieved in portions through a 100 μm nylon mesh (Polylabo, Strasbourg, France) into a large beaker, rinsing the minerals through and leaving the bone fragments in with a strong jet of water. The minerals recovered in the beaker were purified and analyzed according to protocol EN 1788 (*2*). The bone fragments recovered on the nylon mesh were analyzed following the protocol proposed by Marchioni et al. (*6*), and the ESR recording was performed according to the EN 1786 European standard (*3*). The ESR signal strengths were measured as the differences of signal heights between the major two first peaks present in the ESR spectrum (peak-to-peak height). Statistical evaluations were performed with the Student *t* test (with a confidence level of 95%) with three measurement repetitions (4 degrees of freedom for the independent Student *t* test), with the assumption that all measured parameters were normally distributed and that the ESR signal is dependent on the amount of radical present in the sample. For each comparison the obtained *t* values are presented.

RESULTS

Spice Mix. The absence of complex food matrix allowed the simple application of protocol EN 1788 for the analysis of this sample. Non-irradiated spice mix did not show any TL signal at 200−220 °C (**Figure 1a**). The TL signal resulting from the analysis of extracted minerals from irradiated sample (glow 1) was quite important (**Figure 1b**) despite the low value of the test sample (3 g). The glow 2 curve (**Figure 1c**), obtained after 1 kGy re-irradiation of the minerals having been measured for the glow 1 curve, presented an area >10 times the minimal detectable level (MDL, area of the glow 1 curve plus 3 times its standard deviation when the full analytical process without food sample is performed), indicating that enough silicates were extracted from the sample and that glassware and reagents were free of mineral particle contamination. The glow 2 area (**Figure 1c**) was also higher than the one obtained for the glow 1 curve (**Figure 1b**), producing a glow ratio (ratio between the areas of glow 1 and glow 2) of 0.8 ± 0.2. The analyzed minerals presented thus all of the characteristics of irradiated silicates (important TL signal around 220 °C and glow ratio close to 1). This spice mix would be a good model for the detection of irradiated ingredients.

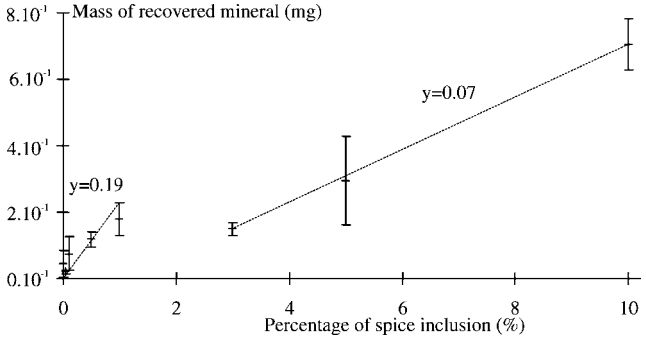

Figure 2. Mass of silicates (in mg) extracted from cheese samples (100 g) containing different inclusions rate (in %, wt/wt) of 5 kGy irradiated spice mix.

Table 1. Mass of Extracted Minerals from Various Ingredients

food	mass of extracted minerals (mg/100 g of food)	extracted material
MRM	250	bones
salmon	30	fish bones
cheese mix	4.0	silicate minerals
parsley	0.16	silicate minerals
black peppercorns	1.82	silicate minerals
white pepper	0.25	silicate minerals
paprika	2.90	silicate minerals
powdered black pepper	96.0	silicate minerals
nutmeg	41.7	silicate minerals
curry	49.9	silicate minerals
cayenne pepper	148	silicate minerals

Extraction of Minerals without Purification. The silicates extracted from the cheese samples contained significant amounts of organic impurities. The last were certainly due to the high amount of cheese samples that had to be analyzed to recover 0.1 mg of silicates, the minimal required quantity for the TL analysis. Whatever the percentage of inclusion was, the organic impurities adhering to the minerals prevented the direct analysis by TL as suggested by protocol EN 1788 (*1*). An additional purification step was therefore necessary.

Extraction and Purification of Extracts. The mass of extracted silica minerals from a cheese sample increased with the percentage of spice inclusion. However, this progression was not linear (**Figure 2**). When a test sample of 200 g of cheese with a spice inclusion of >1% (wt/wt) was analyzed, a gel was formed (due to the garlic component of the spice mixture) and slowed the minerals deposition, reducing considerably the recovered silicates quantity to a value of 0.07 mg of silicate per percent of inclusion for 100 g of cheese (**Figure 2**, cor = 0.97), which was not far from the recovery of minerals (0.04 mg of silicate per gram of spices, cor = 0.97; **Table 1**) from the pure spice mix. The analysis of cheese samples containing <1% of spices (in this case the test sample has to be increased to 1 kg to compensate for the low percentage of inclusion) did not give way to the formation of the gel, allowing then a higher recovery rate of 0.19 mg of silicate per percent of inclusion for 100 g of cheese (cor = 0.64), which was nearly 3 times higher than the value obtained with spice inclusions >1% (**Figure 2**).

Thermoluminescence Glow Curves. Whatever the inclusion rate was, the glow ratios (mean value of 0.6 ± 0.1) were below 1 (except for the 0.1% inclusion) but always above 0.4 (**Table 2**). Furthermore, these values were not significantly different from each other (for statistical analysis, see **Table 2**). Even in the case of cheese containing the lowest spice inclusion (0.05%, wt/wt), the glow curves (**Figure 3b**,**c**) presented a peak

Table 2. Thermoluminescence Glow Ratios of Silicate Minerals Extracted from Cheese Samples Containing Irradiated Spice Mix at Different Inclusion Rates[a]

inclusion (%) (wt/wt)	glow ratio	inclusion (%) (wt/wt)	glow ratio
0.05	0.7 ± 0.1	3	0.4 ± 0.2
0.1	*1.3 ± 0.8*	5	0.5 ± 0.2
0.5	0.5 ± 0.1	10	0.6 ± 0.1
1	0.6 ± 0.1		

[a] All values are not significantly different from the value of other inclusion rates (*t* compared against the 0.05% inclusion rate are 1.3, 2.4, 1.2, 2.3, 1.5, and 1.2 for the sample of inclusion rate with 0.1, 0.5, 1, 3, 5, and 10%). Standard deviation is given as ± value ($n = 3$).

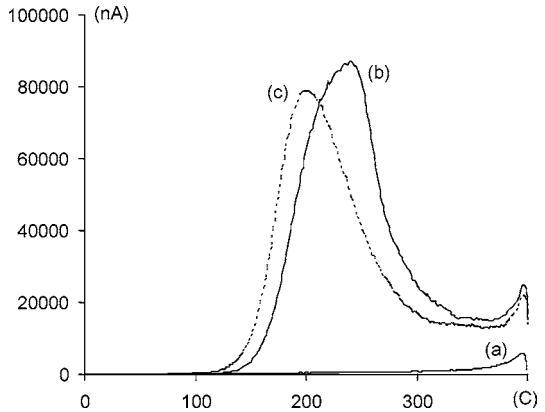

Figure 3. Thermoluminescence glow curves 1 (**b**) and 2 (**c**) of silicate minerals extracted from cheese samples containing 0.05%, wt/wt of 5 kGy irradiated spice mix. For reference, (**a**) glow 1 curve of silicate minerals extracted from non-irradiated spice mix is given.

around 200−220 °C and the glow 1 curve (**Figure 3b**) had an intensity of >10 times the full process blank level plus 3 times its standard deviation (i.e., MDL = 0.28), stating clearly that the recorded signals were significant and that no contamination of silicates from glassware or reagents occurred.

Mix of Two Ingredients. Complex samples may contain spices and MRM together. Silicate minerals could then be contaminated by a fine powder of bone passing through the nylon sieve. A treatment of the mineral extract with an acidic solution (4 M HCl, 2 mL), followed by the density purification with sodium polytungstate (after neutralization with ammonia), allowed the removal of the bone powder from the fraction containing silicate minerals. It was then possible, starting from 200 g of quenelles containing 8% (wt/wt) of irradiated MRM together with 1% (wt/wt) of irradiated black pepper, to recover 52 ± 19 mg of bone fragments presenting the characteristic asymmetric ESR signal and 0.8 ± 0.2 mg of silicate minerals presenting the characteristic TL signal at 220 °C with a glow ratio ranging between 0.4 and 1.5. It was thus possible, with the proposed protocol, to detect, differentiate, and identify simultaneously two irradiated ingredients present in low amount in a non-irradiated food (**Figures 4** and **5**).

The quantities of the mineral (**Table 1**) varied, of course, with the ingredient (1000 times less silicates in parsley than in cayenne pepper and 10 times less bone fragments in salmon than in the MRM) used for the preparation of the quenelle. Even in the case of food containing only salmon and parsley as irradiated ingredient (fish quiches), it was possible to obtain TL and ESR signals from the extracts (**Figure 4**). These results proved without any ambiguity the eventual use of irradiated spices, MRM, or fish in the studied dishes.

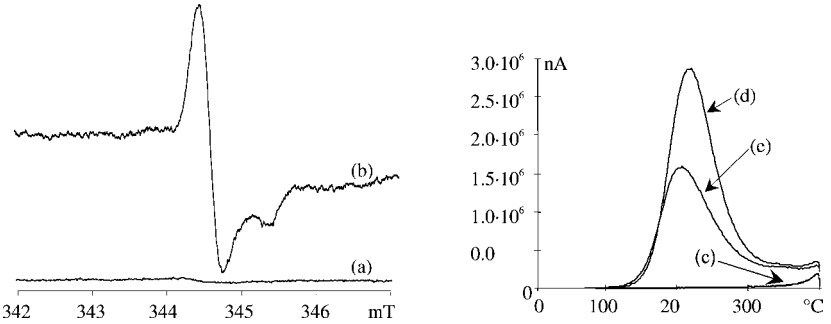

Figure 4. ESR spectrum of bone fragments (**b**) and TL glow curves [glow 1 (**d**), glow 2 (**e**)] of silicate minerals extracted from fish quiches containing 7% (wt/wt) of 5 kGy irradiated salmon, 12% of non-irradiated salmon, and 0.2 and 3% (wt/wt) of 10 kGy irradiated pepper and parsley, respectively. For reference, (**a**) ESR spectra and (**c**) glow 1 curve of silicate minerals extracted from non-irradiated MRM and spice mix, respectively, are shown.

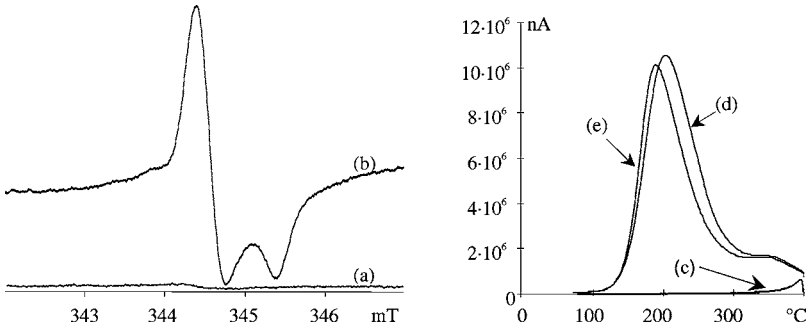

Figure 5. ESR spectrum of bone fragments (**b**) and TL glow curves [glow 1 (**d**), glow 2 (**e**)] of silicate minerals extracted from poultry quenelles with curry sauce containing 5% (wt/wt) of 5 kGy irradiated MRM and 0.6% (wt/wt) of 10 kGy irradiated curry. For reference, (**a**) ESR spectra and (**c**) glow 1 curve of silicate minerals extracted from non-irradiated MRM and spice mix, respectively, are shown.

DISCUSSION

The major component of the spice mix was garlic, the minerals of which adhered on the surface of the bulb and were eliminated during hulling. The minerals present in the spice mix came then mainly from pepper (the content of which in the mixture was only ∼1%), chive, and parsley. Moreover, the presence of garlic in strong amount gave way to the formation of a gel during purification (rinsing and separation by density). The viscosity of this gel slowed the minerals pellet settlement and reduced considerably the extracted silicates mass obtained from food containing an important quantity of spice mix. That is why, when spices contaminated by higher quantities of minerals (**Table 2**) and containing less garlic are analyzed, the detection limit of the proposed method will be lower.

The glow curves from minerals extracted from non-irradiated spices according to protocol EN 1788 did not present any TL signal around 220 °C (**Figure 1a**). Only the natural radioactivity could be detected by the signals measured above 400 °C (used to carry out archaeological dating). The minerals extracted from irradiated foods presented also an intense TL signal, but around 220 °C. The differences of temperatures observed between the positions of the maxima of the glow curves 1 and 2 (**Figure 1b**,**c**) were due to the time difference elapsed between irradiation and analysis. Longer time differences resulted in more important moves of the peak position toward high temperatures because the low energy trapped electrons (i.e., signals recorded at the low temperatures) would be released (Sanderson, private communication). It is realistic to assume that irradiation is commonly performed several months before analysis and that glow 2 curve, for normalization purpose, is generally measured only one night after the 1 kGy re-irradiation.

Protocol EN 1788 forces a one night heating step of the extracts at 50 °C to release all low energy trapped electrons of the sample. The TL signals were then more stable, and the glow curves did not present TL signals below 100 °C. Sample heating during food hydrolysis (55 °C, 1 h) should then not affect the intensity of the TL signals determined, according to protocol EN 1788. In the same way, cooking steps should not affect significantly the intensity of the TL signals, whereas the temperature during cooking rarely exceeded 100 °C inside the sample.

The extracted silicates from non-irradiated foods containing irradiated spices were not only provided by the irradiated ingredient but could also come from other (non-irradiated) components of food. The recovered silicates were then often a mixture of irradiated and non-irradiated minerals. The intensity of glow 2 curve could thus be increased by the contribution of silicates extracted from the non-irradiated part of the food, but irradiated for normalization purpose (glow 2). The surface ratios of the two glow curves were thus very often <1. In our experiments the mean glow ratio was $\sim 0.6 \pm 0.1$.

This analytical protocol was used to control several types of industrially prepared cheeses, taken from the European market, and different precooked meals (poultry quenelles with curry sauce, fish quiches) containing inclusions of various irradiated spices (pepper, nutmeg, and parsley) with concentrations below 1%, MRM, and fishes (**Figures 4** and **5**). The technological treatments realized during the food preparation (cooking, freezing, etc.) never affected the reliability of the analysis, and the results obtained were always appropriate (positive identification was accepted when the glow 1 curve presented a TL peak at 220 °C with intensity 10 times higher than the MDL and glow ratios >0.4).

The presented protocol showed excellent sensitivity and selectivity, allowing—by single extraction—the detection of irradiated spices, MRM, or fish present in low amounts in non-irradiated cooked meals, whether they were subjected to various processes such as cooking, freezing, or conservation. Moreover,

it showed also how to establish that MRM or spice ingredients used in tertiary food products are not irradiated (absence of the specific ESR or TL signals). This method completes the official protocols for the detection of irradiated food published by CEN, is a considerable progress for the control of irradiated food as recommended by European directive L66/16 (*1*), and presents a significant argument in favor of acceptance by the consumer of irradiated foods or foods containing irradiated ingredients.

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