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ARTICLES

Detection of Irradiated Ingredients Included in Low Quantity in Non-irradiated Food Matrix. 1. Extraction and ESR Analysis of Bones from Mechanically Recovered Poultry Meat

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Protocol EN 1786 for the detection of irradiated food by electron spin resonance (ESR) spectroscopy was not conceived for the detection of irradiated bone-containing ingredients included in low concentration in non-irradiated food. An enzymatic hydrolysis method, realized at 55 °C, has been developed for the extraction of the bone fraction. When followed by a purification of the extracts by an aqueous solution of sodium polytungstate, this method made possible the detection of irradiated mechanically recovered poultry meat at very low inclusions (0.5%, wt/wt by ESR) in various meals (quenelles and precooked meals).

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

INTRODUCTION

During the production of mechanically recovered poultry meats (MRM), the poultry carcasses undergo, after a manual preparation of the neck, thighs, and wings, a step of high-pressure crushing, which generates contact between the interior of the carcass (prone to contamination by enterobacteria) and the flesh of the animal. After this mechanical step, the flesh is separated from the bone fragments by screening. This mechanical separation allows recover of the major part of the flesh of the animal, which would have been too long or too expensive to obtain by another process. A more or less large quantity of bone fragments remains in the produced MRM. The total quantity of bone residues must always remain <1% (*1*). The final product looks like very finely chopped meat. An irradiation process (5 kGy) of this product in a frozen state allows its use as an ingredient in culinary preparations [aerobic mesophilic count < 10^4 CFU g^{-1}, absence of *Salmonella* in 25 g (*2*)].

The MRM is used as a protein contributor in food and also for its technological qualities (emulsifying and binding). However, during the mechanical separation and freezing steps, some of the proteins are denatured, and the binding capacity of a MRM may then decrease by 70% compared to that of a normal meat (*1*). The quantity of MRM added to a food will thus always be higher than the quantity of a fresh meat used for the same objective. Moreover, the MRM is appreciated by the food-processing industries because of its low cost. In a practical way, the concentrations used by food industry are always ∼6−10% (wt/wt) and can even rise to 25% in some cases. The best dedicated European reference protocol for the detection of irradiated meats with radicals induced in bones [analysis by electron spin resonance (ESR) spectroscopy after manual recovery and drying)] (*3*, *4*) is not applicable when little bone fragments are included in small quantities in a lipidoproteic complex matrix (cheeses, quenelles, etc). It is actually essential to remove as efficiently as possible the food matrix before analysis of bone fragments by ESR spectrometry. Gray et al. (*5*) recommended, for the recovery of the bone fragments, an alkaline hydrolysis of the food matrix by alcoholic boiling KOH. When this treatment was used for the analysis of non-irradiated quenelles containing irradiated MRM, the detection was only possible for an inclusion rate >10% (wt/wt). This is probably due to the significant destruction of bone fragments by alcoholic boiling KOH and the presence of cellulose and pectin in the extracts (components of plants not hydrolyzed under the selected analytical conditions) contained originally in the pepper added at a level of 0.5% (wt/wt) in the analyzed quenelles (*6*). Improvement was made by these authors by replacing alcoholic

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boiling KOH by an aqueous solution of Alcalase intended to hydrolyze proteins at moderate temperature and to dissolve the fat content of the food with SDS. With these new soft hydrolysis conditions, the authors could lower the detection threshold to a minimal inclusion rate of 3% (wt/wt) of irradiated MRM, the lower limit being still imposed by the presence of nonhydrolyzable pepper.

The purpose of this work is to present a single protocol for the extraction and purification of bone fragments included in a food matrix, in a less drastic, more selective, and sensitive way than the already published protocols [comprising an enzymatic hydrolysis (Alcalase) of the proteins, a dissolution of the lipids by sodium dodecyl sulfate (SDS), and a purification of the extracts by decantation in an aqueous solution of sodium polytungstate] and to associate it with an analysis by ESR spectroscopy to carry out a high-sensitivity detection of irradiated MRM or fish products included in various culinary preparations.

MATERIALS AND METHODS

Food Samples. The salmon fillets (irradiated at 5 kGy) and MRM (irradiated at 5 kGy) were provided by French food companies. The MRM was delivered by the supplier after being irradiated in an industrial plant. The salmon samples were irradiated in the laboratory.

Industrial poultry quenelles with inclusions of irradiated MRM (0, 0.5, 2, 4, 6, and 8%, wt/wt) and pepper (1%, wt /wt) were manufactured in the food factory, starting from the industrial base of the natural quenelles, before poaching (20 min, internal quenelle temperature = 80 °C) according to the usual recipe (containing tallow, flour, eggs, salt, water, and milk proteins). More complex cooked dishes [fish paupiettes (14%, wt/wt irradiated salmon, 38%, wt/wt non-irradiated salmon, and 17%, wt/wt whiting; poultry quenelles (5%, wt/wt irradiated MRM) cooked for 20 min at 100 °C (internal temperature > 65 °C); and fish quiches (12% non-irradiated salmon and 7%, wt/wt irradiated salmon) cooked for 10 min at 220 °C] were carried out by the Gathering and Tourism College of Strasbourg (Illkirch, France). Sliced samples of MRM (3 mm thickness, 100 g) were conditioned in aluminum foil before cooking (100, 150, and 200 °C, 60 min). All of the foods were preserved at −20 °C until they were analyzed.

Chemicals. Sodium monohydrogen phosphate, potassium dihydrogen phosphate, 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 was 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. Cooking of foods prepared at the Gathering and Tourism College of Strasbourg was carried out in a drying oven Memmert UM 600 (Schwabach, Germany). 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 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. 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 get a good homogeneous dose distribution within the thickness of the food (±10%) (*7*).

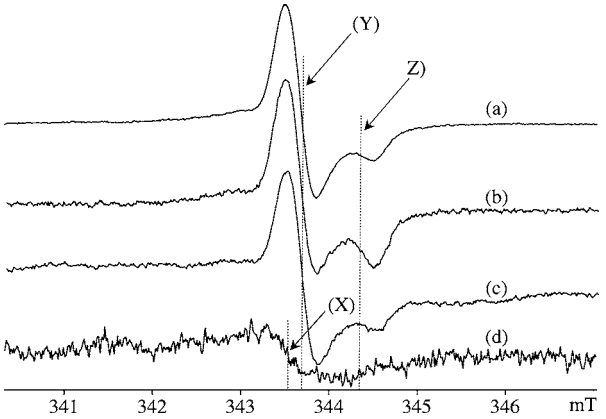

Figure 1. ESR spectra of chicken bones: (**a**) 3 kGy irradiated tight-bone; (**b**) bone fragments extracted from 5 kGy irradiated MRM, (**c**) fishbone fragments extracted from 5 kGy irradiated salmon; (**d**) non-irradiated tight-bone. Sample preparation was performed only with Alcalase hydrolysis.

Analytical Protocol. 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 vigorous magnetic agitation during 1 h at 55 ± 5 °C. During the hydrolysis, the pH of the suspension was controlled through pH testing paper every 15 min and was adjusted using NaOH pellets. After hydrolysis, the suspension was subjected to a 20 min ultrasonic treatment and then sieved in portions through a 100 μm nylon mesh (Polylabo, Strasbourg, France) into a large beaker, leaving the bone fragments on the nylon surface. After filtering, the bone fragments were washed with a strong jet of water.

The bone fragments recovered on the nylon mesh were placed in a 300 mL test tube and rinsed at least three times per addition of 200 mL of water for each washing step. The bone fragments were allowed to settle for 5−10 min, and most of the water was withdrawn by suction. The bone fragments were transferred together with the remaining water in a 50 mL conical centrifuge tube. After suction of the water, a volume of sodium polytungstate aqueous solution (density of 2 g mL^{-1}), equivalent to the one of the deposit, was added. After short and vigorous agitation, the tube was briefly centrifuged (2 min, 1000 *g*). The bone fragments settled, whereas the organic impurities floated. After suction of the sodium polytungstate solution, the bone fragments were rinsed twice by 40 mL of water for each rinsing step. A few milliliters of acetone was added to rinse the sample and then removed. The fragments were finally placed for 10 min in the laboratory vacuum oven (40 °C, 14 kPa).

The ESR spectra were recorded at room temperature while placing 5−10 mg of dry and clean bone fragments in a quartz tube (Suprasil, 6 mm i.d.) placed at the center of the resonator. Data acquisition was carried out according to the EN 1786 protocol (*3*). The ESR signal strengths were measured as the differences of signal heights between the two major first peaks 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 repetition (4 degrees of freedom for the independent Student *t* test), with the assumption that all measured parameters were normally distributed. For each comparison the obtained *t* values are presented.

RESULTS

Extraction of Bone Fragments without Purification. The bone fragments extracted from the studied MRM by means of the enzymatic suspension showed an intense asymmetrical ESR signal [$g = 2.003$ and 1.997 (proportional to the ratio of the microwave frequency and the magnetic field imposed to the sample), **Figure 1**, spectrum b, signals Y and Z], which was superimposed on the symmetrical signal ($g = 2.004$) present in

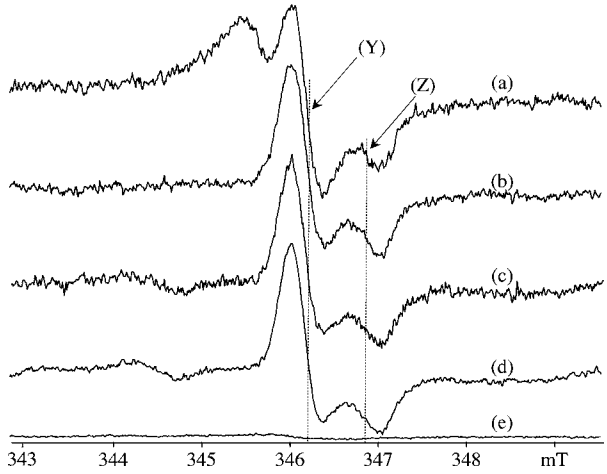

Figure 2. ESR spectra of bone fragments extracted from chicken quenelles containing irradiated MRM: 2%, wt/wt MRM inclusion without (**a**) and with (**b**) purification by aqueous solution of polytungstate of samples after the Alcalase hydrolysis; 0.5%, wt/wt of MRM inclusion [spectra acquired by one sweep (**c**) and five sweeps (**d**)] obtained by sample preparation with Alcalase hydrolysis and purification by aqueous solution of polytungstate; for reference (**e**) non-irradiated bone.

the non-irradiated bones (**Figure 1**, spectrum d, signal X). This asymmetrical signal, which was until now detected only in irradiated bones (*3*), was not significantly different ($t = 0.1$) from the one observed with whole irradiated bones analyzed following the EN 1786 procedure (**Figure 1**, spectrum a). This indicates that the bone fragments extracted from the MRM can be directly analyzed by ESR spectroscopy and that the enzymatic digestion cleanup was efficient for the elimination of proteins and lipids from this food matrix. On the other hand, when poultry quenelles were analyzed, the extracted bone fragments were contaminated by important quantities of nonhydrolyzable impurities (flour and spices used as ingredients in the industrial quenelles). These impurities led to an important interfering singlet signal in the ESR spectrum, making difficult the unambiguous identification of the irradiated bone spectra in the case of low inclusion (<4%, wt/wt) of MRM (**Figure 2**, spectrum a). These results were in agreement with those reported by Stevenson et al. (*6*), who could not detect inclusions of irradiated MRM lower than 10% (wt/wt) in poultry quenelles containing together 0.5% (wt/wt) of pepper. A purification step of the hydrolysates was therefore essential.

Extraction and Purification of Extracts. After purification (with sodium polytungstate) of extracts obtained with the Alcalase suspension, the bone fragments were cleaner and the ESR signals (**Figure 2b**) were identical to those obtained with whole irradiated bones prepared following the EN 1786 procedure. The mass of bones obtained from a 100 g test sample of poultry quenelles followed a linear progression according to the MRM inclusion rate. The regression slope was 2.9 ± 0.2 mg of bones per percent of MRM inclusion for 100 g of quenelles (correlation coefficient = 0.98, **Table 1**). The extracts were clean and could be analyzed by ESR spectroscopy. Even for the lowest studied inclusion rate (0.5%, wt/wt, **Figure 2**, spectrum c), the asymmetrical characteristic ESR signal was detected and its amplitude remained 11 times higher than the background noise. By increasing the number of sweeps (from one to five sweeps), the signal (**Figure 2**, spectrum d) was cleaner and the signal-to-noise ratio became equal to 17, allowing an unambiguous detection of irradiated MRM. The statistical analysis performed on the mean ESR intensities proved that all food samples containing irradiated MRM showed

Table 1. Mass of Bones Extracted from Quenelles Containing Different MRM Inclusions[a]

inclusion (%)	mean[b] (mg)	inclusion (%)	mean[b] (mg)
0	0	4	7.6 ± 4.0
0.5	2.2 ± 0.3	6	18 ± 2.0
2	5.0 ± 1.5	8	22 ± 2.0

[a] Sample preparation with Alcalase hydrolysis and purification by aqueous solution of polytungstate. [b] ± standard deviation ($n = 3$).

Table 2. ESR Signal Intensities Obtained from Fragments Extracted from 5 kGy Irradiated MRM and Cooked at Various Temperatures (Room Temperature, 55, 100, 150, and 200 °C, 60 min)[a]

temperature (°C)	normalized ESR signal[b] (AU mg^{-1} of bones)	sensitivity[b] (AU g^{-1} of food)
room	1014 ± 50	0.88 ± 0.05
55	637 ± 140	1.47 ± 0.32
100	440 ± 80	1.18 ± 0.22
150	427 ± 70	1.28 ± 0.21
200	491 ± 120	0.98 ± 0.24

[a] Sample preparation with Alcalase hydrolysis and purification by aqueous solution of polytungstate. [b] ± standard deviation ($n = 3$).

bone fragments with ESR asymmetrical signals significantly different ($t = 4.0$, 3.6, 5.3. 5.8, and 13.9 for inclusion rates of 0.5, 2, 4, 6, and 8%, respectively) from the one obtained with non-irradiated bones.

The heating (100, 150, and 200 °C, 60 min) of the MRM prior extraction showed a decrease of the normalized ESR signal (ESR signal strength normalized to the mass of bones present in the ESR tube). This happened essentially below 100 °C (30% decrease). Above this temperature, the normalized ESR signal remained stable whatever the temperature studied (**Table 2**).

DISCUSSION

Measurements of ESR signals induced in animal bones is not really new, having been used for accidental post-irradiation dosimetry (*8*−*10*) and for dating bones and shells (*11*−*13*). Applications for the detection of irradiated foods containing bones, scales, teeth, or shells have been widely reported (*14*−*17*). Extraction of bone fragments from tertiary products was reported by Stevenson et al. (*6*), but the analysis failed when the inclusion rate was below 3% because of coextracted impurities (**Figure 2**, spectrum a). It was then impossible to detect irradiated MRM in food matrices more complex than burgers.

Following the proposed protocol of extraction and purification, the extraction rate of bones from industrial quenelles (2.7 ± 0.2 mg of bones per percent of MRM inclusion for 100 g of quenelles) was consistent with that obtained when the pure MRM samples were analyzed (2.5 ± 0.4 mg of bones g^{-1} of MRM, correlation coefficient = 0.97). The consistency between the values obtained with the MRM and that obtained with the quenelles indicated that the hydrolysis and purification steps remained very effective even in the case of a complex food matrix that underwent technological treatments such as cooking or freezing. The low variability of the mass of bones extracted from the quenelles was undoubtedly linked to the industrial manufacturing process, which ensures a good distribution of the MRM within the quenelles. Stevenson et al. (*6*) observed much more important fluctuation (200%) in the case of quenelles prepared in the laboratory.

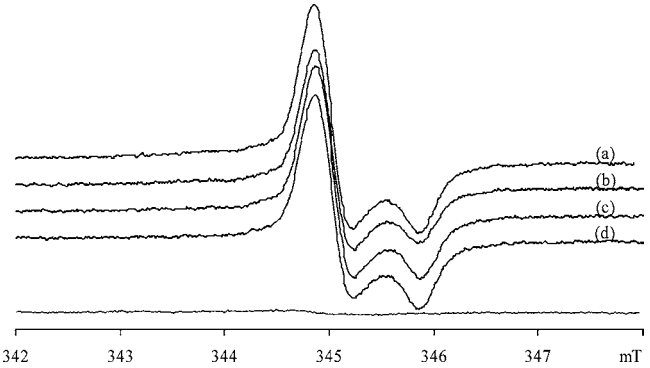

Figure 3. ESR spectra of bone fragments extracted from MRM samples cooked for 60 min at 200 °C (**a**), 150 °C (**b**), and 100 °C (**c**) and uncooked (**d**). Sample preparation was performed with Alcalase hydrolysis and purification by aqueous solution of polytungstate. For reference, spectrum (**e**) is non-irradiated bone.

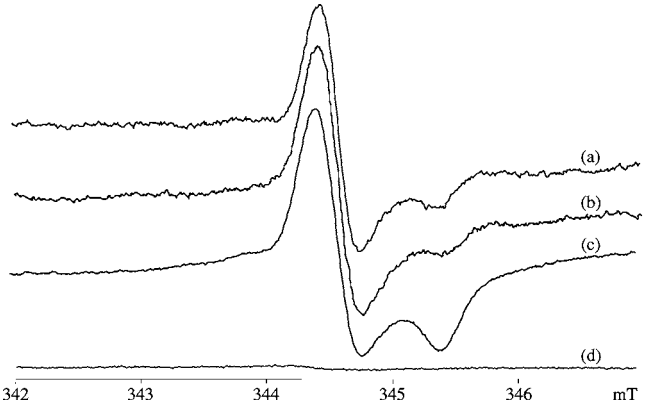

Figure 4. ESR spectra of bone fragments extracted from chicken quenelle with curry sauce (**a**), salmon quiches (**b**), and salmon paupiettes (**c**). Sample preparation was performed with Alcalase hydrolysis and purification by aqueous solution of polytungstate. For reference, spectrum (**d**) is non-irradiated bone.

The bone fragments extracted from the MRM essentially consist of cartilages originating from the ribs, the sternum, and the neck of the poultry [the long bones shall be excluded from the raw materials used for the manufacture of MRM (*1*) to minimize possible contamination by marrow]. According to Gray et al. (*14*), these bones of lower crystallinity degree provide ESR signals of lower amplitude than those of the femur or tibia. That is why the quantity of analyzed bone fragments extracted from MRM (thus, that which is obtained after hydrolysis) must be higher than that of tight bone, to obtain detectable ESR signals. The enzymatic suspension should then be used at the temperature at which it reaches its maximum effectiveness (55 °C). To evaluate the effect of the temperature increase on the ESR signal strength, 100 g samples of MRM were hydrolyzed at 55 °C and ambient temperature. In the latter case, due to the weak activity of the Alcalase, 12 h of extraction was necessary (but not even sufficient to perform complete extraction) to hydrolyze the whole of the MRM organic fraction. The quantity of bones extracted in this case, although largely sufficient to record an ESR spectrum, was much lower than the one obtained after a 55 °C hydrolysis (respectively, 55 and 275 mg). On the other hand, the normalized ESR signal was 50% higher (**Table 2**) when the extraction was performed at room temperature. When considering the normalized ESR signal strength and the effectiveness of the extraction step, one can define the sensitivity of the method [expressed in AU g^{-1} of food, i.e., normalized ESR signal multiplied by yield of extraction (mass of extracted bone per gram of test sample)]. This sensitivity was higher when extraction was performed at 55 °C, certainly because the decrease of the ESR signal intensities (due to heating) was largely compensated by the improvement of the bones extraction yield (**Table 2**).

For obvious health reasons, MRM-containing foods must necessarily be subjected to a cooking (*1*) quickly after their manufacture. This step may reduce the ESR signal intensity, as already reported by Gray et al. (*18*), who noticed a reduction of 23% of the intensity of ESR signal after cooking chicken carcasses for 80 min at 190 °C (internal breast temperature = 85 °C). In this work, the samples heated at different temperatures between 100 and 200 °C for cooking purposes (**Table 2**) presented ESR signal intensities that remained quite stable. The shape (asymmetrical signal) of the ESR spectra remained also identical whatever the heating temperature was (**Figure 3**), allowing an easy and unambiguous detection of irradiated bones. As the normalized ESR signal strength remained constant for a cooking temperature ranging from 100 to 200 °C, it can be assumed that the analyzed bone fragments contained two energy levels of radicals, the last being either already recombined at a temperature below 100 °C or still stable up to 200 °C, the maximal temperature investigated in this work. The sensitivity of the proposed detection method showed a maximum at 55 °C, which was the temperature of hydrolysis and was not significantly reduced (comparison of sample cooked at 55, 100, 150, and 200 °C to the room temperature sample, obtained t values are, respectively, 5.5, 16.1, 7.9, and 61.4) by a cooking up to 200 °C (60 min) (**Table 2**). The protocol can therefore be used for the analysis of cooked culinary preparations.

Due to the good sensitivity and selectivity of the suggested analysis method, it seemed possible to detect other irradiated ingredients, such as fish fillets containing 10 times less bone (0.3 mg of fish bones g^{-1} of fish) than MRM, as well as complex food containing a great number of nonhydrolyzable ingredients such as plants. As the crystallinity degree of the fish bone is generally much lower than that of meat bones, one may assume that the ESR signals are of lower intensity [8 times weaker (*19*)] and less stable during storage or heating (*18*, *20*−*23*). Stewart et al. (*21*) already observed a rapid decrease of the ESR signal in the carcasses of scampi (27 and 8% of the signal after 4 days of storage, respectively, at 5 and −20 °C). On the other hand, Gray et al. (*18*) showed that the ESR signals detected in the poultry bones are not prone to such variations during storage, as long as it is carried out at temperatures of <5 °C, which is generally required for food conservation. In the case of chicken bones it thus seems that under good storage temperature conditions (<5 °C), the storage period of food does not influence the intensity of the recorded ESR signals and thus the detection limit of the irradiated MRM included in culinary preparations. Despite these difficulties, the characteristic asymmetric ESR signal has been clearly obtained in the extracted fish bones from irradiated salmon samples (**Figure 1**, spectrum c). The method also was used to detect the presence of irradiated MRM and salmon in various culinary preparations (quiches, fish paupiettes, and poultry quenelles with curry sauce). Fish inclusions were in fact made up of three varieties of fish (fresh whiting, fresh salmon, and irradiated salmon). The irradiated salmon thus was only a component of the fish ingredient. Whatever the type of the analyzed bone, it was always possible to recover the typical asymmetric ESR signal in the different studied foods even when the irradiated ingredient was a component of the fish ingredient (**Figure 4**). These results proved without any ambiguity the use of irradiated fish in the studied dishes.

The present protocol showed an excellent sensitivity, which makes possible the detection of irradiated MRM or fish present in low amounts in non-irradiated cooked meals with a single extraction, whether they were subjected to various processes such as cooking, freezing, or conservation. This method completes the official protocols for the detection of irradiated food published by CEN and represents significant progress for the control of irradiated food as recommended in the European Directive L66/16 (*24*); it also represents a significant argument in favor of acceptance by the consumer of irradiated foods or of foods containing irradiated MRM or fish.

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