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## Metabolic profiling by H NMR of ground beef irradiated at different irradiation doses

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### ABSTRACT

This work describes a metabolic profiling study of non-irradiated and irradiated beef (at 2.5, 4.5 and 8 kGy) using 1H NMR and chemometrics. The assignment of all major NMR signals of the aqueous/methanolic extracts was performed. A comprehensive multivariate data analysis proved the ability to distinguish between the irradiated and non-irradiated beef. Classification trees revealed that three metabolites (glycerol, lactic acid esters and tyra- mine or a *p*-substituted phenolic compound) are important biomarkers for classification of the irradiated and non-irradiated beef samples. Overall, the achieved metabolomic results show that the changes in the metabolic profile of meat provide a valuable insight to be used in detecting irradiated beef. The use of the NMR-based approach simplifies sample preparation and decrease the time required for analysis, compared to available official analytical procedures.

### Introduction

Irradiation is a food preservation technology by which ionising radi- ation is applied for various purposes including insect disinfestations, growth inhibition, control of parasites, and shelf-life extension. More- over, it is well-known that irradiation increases food safety by reduction of pathogenic bacteria, and its use is gradually increasing worldwide. The irradiation of food and agricultural products, as part of the larger ra- diation processing industry, is currently allowed by about 60 countries around the globe (Sommers & Fan, 2006). In the European Union, the Community positive list of foods and food ingredients that may be treated with ionising radiation, established by the Directives 1999/2/EC (EC, 1999a) and 1999/3/EC (EC, 1999b), in- cludes up to now the single category of “dried aromatic herbs, spices and vegetable seasoning”, although existing authorizations in certain Member States allow the irradiation of a number of foodstuffs. The treatment with ionising radiation of meat and meat products is not authorised in the European Union, except for chicken meat (The Netherlands), poultry (France and United Kingdom), and mechanically recovered chicken meat (France). However, each Member State has to consider the possible presence on the market of irradiated foods coming and eggs. However, they have the disadvantage of being quite time consuming and requiring the use of considerable amounts of organic solvents due to a long and complex sample preparation. Although

the analytical methods available for the detection of the ir- radiated foods are numerous, the European Commission promotes the development of new techniques and the setup of new protocols aimed to simplify or improve the already existing procedures (Boniglia, 2004; Califano, 2009) from other countries and must take all measures necessary to ensure that they comply with the regulations in force. In particular, every year the Member States have to inform the European Commission the information about the analytical method adopted and the results of con- trols carried out at the product marketing stage and aimed to evaluate the compliance with the provisions of the Directives. The controls have to be performed also on foods coming from third countries, consid- ering that in several extra-EU countries the use of food irradiation is much more widespread. The official control of the irradiated foods at the retail level has to be carried out by analytical methods validated according to the Commis- sion Decision 2002/657/EC (EC, 2002). A single analytical method to be used to control all the types of foods is not currently available. The European Committee of Standardisation (CEN) has validated ten methods of analysis specific for categories of foods. Four of them are screening methods and have been validated for herbs and spices, poul- try meat, food containing mineral debris and food containing DNA, re- spectively. The six other methods are reference methods and are based on the analysis of primary radiolytic products by thermolumines- cence or electron spin resonance spectroscopy, or on the analysis of sec- ondary radiolytic products from fatty acids, namely hydrocarbons and 2-alkylcyclobutanones (Marchioni, 2006). The latter ones are suitable for foods with a fat content higher than 1% treated at an irradiation dose higher than 0.5 kGy, and have been validated for pork, poultry. The application of nuclear magnetic resonance (NMR) spectroscopy to the analysis and quality control of foods has shown a great develop- ment in the last few years. The ability of high-resolution NMR to moni- tor in a non-invasive and reproducible way all abundant molecules present in a raw material or in a complex system is the major driver for NMR applications in food science. In this context the identification of each signal of the spectrum is unnecessary because all relevant infor- mation can be obtained by the application of chemometric or pattern recognition techniques which allow the use of the NMR spectrum as a fingerprint or metabolic profile of foods. Several examples of the NMR-based metabolomic characterisation of foods are available in the literature (Consonni & Cagliani, 2008; Jung et al., 2010; Rezzi et al., 2007). Recently, Villa, Castejón, Herraiz, and Herrera (2013) proposed $^{1}$H High Resolution magic angle spinning (HRMAS) NMR spectroscopy to differentiate between irradiated and non-irradiated cold-smoked Atlantic salmon (*Salmo salar*). $^{1}$H NMR lipid profiling was applied to differentiate irradiated and non-irradiated beef (Zanardi et al., 2013). However, to the best of our knowledge, studies on the appli- cation of the NMR-based metabolomics for the detection of irradiat- ed meats are not available in literature. Therefore, the present study aimed to investigate the metabolite profiling of beef subjected to irradiation treatment

and identify potential markers for detecting the irradiation in beef.

## Materials and methods

### *Chemicals*

Methanol-$d_4$ (99.8 atom % D), deuterium oxide (99.9 atom% D), chloroform-d (99.8 atom% D), 3-(trimethylsilyl)propionic acid-$d_4$ sodi- um salt (TSP, 98% atom% D) were purchased from Sigma-Aldrich (Milan, Italy). Sodium dihydrogen phosphate monohydrate and disodium hy- drogen phosphate dihydrate were supplied by Merck (Darmstadt, Germany).

### *Irradiation and sample preparation*

Three batches of ground beef, each consisting of each of 10 different subsamples, for a total amount of about 3 kg prepared from the fore- quarter were purchased at a local supermarket. One hundred twenty portions of about 25 g each were vacuum packed and stored at −20 °C prior to irradiation for 48 h. Thirty samples were randomly cho- sen for comparison purposes (non-irradiated control samples), twenty aliquots were randomly allotted in each of the two groups intended for treatment at irradiation doses of 2.5 and 4.5 kGy, and ten aliquots for treatment at 8 kGy. 8 kGy irradiation dose is considerably higher than would ever be used in industrial practice; however, targeted irradi- ation doses were chosen for this study to include in the experimental design a high range of dose. The samples were arranged in polystyrene foam boxes able to keep their temperature in the range from −18 °C to −13 °C for all the treatment period. Irradiation was performed using a 60Co γ-irradiator (1.17–1.33 MeV) at the Gammatom S.r.l. facilities (Guanzate, Italy). Alanine dosimeters were positioned to the top and bottom surfaces of each box and the absorbed dose was within ± 5% of the targeted dose. After the irradiation treatment, the samples were stored for 5 days at 5 ± 1 °C prior to analysis. Beef samples were prepared for the $^1$H NMR analysis according to the procedure of Jung et al. (2010) with some minor modifications. In particular, from each sample, about 200 mg of beef was put into 1.5 ml Eppendorf tube with 350 µl of methanol-$d_4$ and 150 µl of 0.2 M (pH 7) sodium phosphate buffer, homogenised by a vortex homogeniser for 1 min and centrifuged twice at 2348 *g* for 20 s using a multispeed refrigerated centrifuge (PK 121R, Thermo Elec- tron Corporation, Waltham, MA). After homogenisation, 210 µl of methanol-$d_4$ and 90 µl of 0.2 M (pH 7) sodium phosphate buffer and 400 µl of chloroform-d were added to the tube. The mixture was vortexed vigorously for 1 min. The samples were allowed to separate for 15 min and centrifuged at 15,871 *g* for 10 min at 4 °C. The upper layer was transferred to a new Eppendorf tube and mixed with 70 µl 7 mM TSP dissolved in deuterium oxide. The mixture was then centri- fuged at 13,000 rpm for 10 min. The supernatant was

transferred into 5 mm NMR tubes.

*NMR spectroscopy*

H NMR spectra of aqueous/methanolic extracts from 80 beef sam- ples (30 non-irradiated and 20, 20 and 10 irradiated at 2.5, 4.5 and 8 kGy, respectively) were acquired with an INOVA 600 MHz spectrom- eter (Varian, Milan, Italy) operating at 599.736 MHz for $^{1}$H and equipped with a HCN probe. Spectra were acquired at 298 K, with 32 K complex points, using a 45° pulse length and 1 s of relaxation delay (d1). One hundred twenty-eight scans were acquired with a spec- tral width of 9595.8 Hz and an acquisition time of 1.707 s. TOCSY spectra were acquired at 298 K, with 2048 data points. Thirty-two scans were acquired for each of the 256 increments, with water presaturation during the relaxation delay of 1 s. The spectra were processed with a sinebell function in both dimensions. To analyse the profiles by pattern recognition, $^{1}$H NMR spectra were transferred to MestReNova software (release 6) and referenced to TSP (0 ppm). An integration pattern was defined choosing buckets manually on all the considered spectra in the overlapped form. The buckets were chosen to eliminate the spectral regions with no signals and as large as to compensate the little chemical shift fluctuation in each single spec- trum. The defined pattern was used for the automatic integration of all the spectra and referred to the TSP area.

*Chemometric techniques*

A matrix (80 × 112) having rows representing the acquired beef samples (cases) and columns corresponding to the integrated area of the NMR signals (variables) was the basis for the application of chemo- metric techniques. In this study both unsupervised and supervised mul- tivariate methods were applied to determine whether the metabolic fingerprint of beef samples allowed identification of metabolic markers for the detection of the irradiation treatment in meat. On the one hand, unsupervised methods do not require prior information for classifica- tion and cluster individual samples solely on the basis of the variabili- ty/similarity expressed in their data; on the other hand, supervised learning methods require that the group information is known a priori and use it to create a classification rule that may be applied to future samples. By principal components analysis (PCA) data are visualised by plot- ting the PC scores, i.e. projecting the individual samples on the plane formed by the first 2 principal components, or the loading plot, which allows the identification of the spectral regions with the greatest influ- ence on the possible clustering of the samples. However, the PCA opti- mises the directions of largest variability (variance) and not the largest class separation ability, so it is not tailored to optimise sample classification. A classification model was then adopted by means of classification trees (CT). CT are a non-parametric supervised learning/ discriminant analysis method proposed by Breiman,

Friedman, Olshen, and Stone (1984), which has been used also in food science applications(Caligiani ,et al., 2014; Cho & Kurup, 2011; Cirlini, Caligiani, Palla, & Palla, 2011; Debska & Guzowska-Swider, 2011; Zhang, Xu, Daeyaert, Lewi, & Massart, 2005) as it does not require a normal multivariate distribution of the data nor the equality of within-group variances, two assumptions that frequently don't hold in such applications. For a binary or categorical variable Y and n independent variables $X_1$, $X_i$,…,$X_n$, CT is a tree-building method in which the data are split re- cursively into two groups on the basis of a threshold value of one of the $X_i$'s, with the final aim of predicting Y. The splitting is aimed to optimise a measure of purity of the tree and is repeated until the tree has pure final branches, called nodes (i.e. with samples belonging to one class only) unless some additional pre-set stopping rules on the minimum number of elements in the parent or offspring nodes prevent further partitions. There is also an option to prune the tree by a criterion which trades off tree purity with complexity, with the aim of avoiding overfitting to the training data. A measure of classification accuracy can be obtained by resubstitution (with the same statistical sample on which the rule has been derived) or by cross-validation (leaving out a fraction of the sample to attain an unbiased estimate). Since the interest of this study was mainly to identify the variables most influential in the classification, the criteria to grow the tree were set as follows: prior probability equal to frequency (as we built trees with two categories of fairly balanced size), misclassification by 10-fold cross-validation (leave out 10% of the sample), no stopping rule and as a measure of impurity the Gini index which is defined as: but very simple extraction or sample preparation procedures may also be used (Schievano, Pasini, Cozzi, & Mammi, 2008). The overall features of the $^{1}H$ NMR spectrum of non-irradiated and irradiated beef were quite similar; however, some differences were ob- served in the abundance of some signals, therefore multivariate statisti- cal analyses were performed in order to gain an insight in such spectral differences. As a first step PCA was applied. The first three PCs explained 86.73% of cumulative variance. The PCA model using projection onto three dimensions of PC1, PC2 and PC3 showed some clustering according to the irradiation dose, indicating differences in metabolite composition among the beef extracts. The PCA 3D score plot is shown in Fig. 2. In par- ticular, it can be seen that the non-irradiated beef samples, with the lowest values on the PC1, clustered separately from the samples irradi- ated at 4.5 and 8 kGy, and to a lower extent to those irradiated at 2.5 kGy. To investigate the basis for the observed spectral clustering between the beef samples, the PCA loadings were inspected (Fig. 3). The loading on PC1, which is the component that well separates control samples from samples treated at higher irradiation doses (4.5 and 8 kGy), were mainly positive, demonstrating that almost all metabolites in the $^{1}H$ where $I(t)$ is the impurity at node $t$, $p_j$ is the proportion of training pat- terns at node $t$ that belongs to class $j$ (Cho & Kurup, 2011). However, as the number of samples in this application is relatively low, the predic- tion results should be generalised with caution.

The PCA and CT were performed by PASW Statistics (release 18.0.0, IBM SPSS, Armonk, NY).

**Results and discussion.**

The advantage of NMR spectroscopy is that all types of compounds give rise to signals simultaneously, so that the NMR spectrum repre- sents a fingerprint of the sample under study. NMR is frequently applied to food samples that can be directly examined as liquids (Belton et al., NMR spectra are more abundant in the irradiated meat samples. Load- ings on PC2 and PC3 (not reported) showed a more complicated, harder to interpret distribution pattern, so it was preferred to proceed with a supervised multivariate analysis by means of CT in order to focus the interpretation of classification on a smaller set of variables. Thus, CT was carried out to identify the metabolites from $^{1}$H NMR spectra best discriminating between non-irradiated and irradiated beef. For CT the 112 integrated areas of the NMR signals of the beef samples were used and a binary tree based on Gini partitioning criterion was constructed. Two different classification models were elaborated which formalise a two-stage procedure with a hierarchy of priorities, i.e. detecting first presence of any irradiation and then the dose of irradiation: in the first, two classes were considered, non-irradiate samples vs. all the irradiated samples grouped together, in order to highlight the variables able to discriminate treated meat. In the first model (Fig. 4), the group of non-irradiated samples can be separated from the treated group mainly by the level of glycerol. The (pruned) tree with only the split due to glycerol represents the optimal tree trad- ing off complexity and accuracy and yields a cross-validation misclassi- fication rate (leaving out 10% of the samples) of 11.3% (s.e. = 3.5). Other variables contributing to a further separation of the two groups were NMR signals centred at 1.41 ppm and 6.79 ppm. The signal at 1.41 ppm was a doublet with a coupling constant of 6.88 Hz. TOCSY cor- related with a signal at 4.1 ppm. This spectroscopic pattern was very similar to that of lactic acid signal centred at 1.334 ppm (methyl group, see Table 1), so the signal centred at 1.41 ppm was attributed to a lactic acid derivative, probably an ester. The signal at 6.79 ppm was a doublet with a coupling constant of 8.46 Hz and it presented a TOCSY correlation at 7.126 ppm. For these characteristics it could be chemically related to tyrosine signal at 6.844 ppm, so it could be tenta- tively attributed to tyramine or to a *p*-substituted phenolic compound. In the case of the second model, comprising the three classes of irradiat- ed samples only, the misclassification rate estimated by cross- validation, leaving out 10% of the samples, was 14% (s.e. = 4.9) (decreasing to 4% by resubstitution, s.e. = 2.8). In this case an almost perfect separation of the three different treated groups could be obtain- ed after just two tree partitions. As shown in the graphs (Fig. 5), the first tree partitioning was due to a substance giving a NMR signal at 2.23 ppm (singlet unknown) able to separate two main groups, one containing all (except one) 2.5 kGy treated beef samples, the other 4.5 and 8 kGy treated beef

samples (with higher level of the unknown sub- stance). The other tree partitioning was determined by valine, which was able to perfectly separate the groups of 4.5 kGy treated samples from the group of 8 kGy treated samples. Free amino acids, peptides, amines, sugars, sugar amines, sugar phosphates, and organic acids account for 0.55% of bovine muscle, al- though changes of these water soluble, low molecular weight com- pounds were observed during post mortem storage of beef (Jarboe & Mabrouk, 1974; Lawrie & Ledward, 2006). Considerable variability was detected among aqueous extracts of beef samples from different countries, suggesting that the metabolite levels and their relative com- position could be affected by breed, feeding regimen and production system. However, the NMR-based metabolomics of aqueous beef ex- tracts was an efficient method to distinguish fingerprinting difference between raw beef samples, and several metabolites including succinate and various amino acids (isoleucine, leucine, methionine, tyrosine and valine) can be possible biomarkers for discriminating the geographical origin of beef, although the reasons for the differences in metabolomic profiles as a function of geographical origin are not fully understood (Jung et al., 2010). In the present study we showed that $^{1}$H NMR profiling of aqueous/ methanolic extracts of beef samples allowed us to distinguish between irradiated and non-irradiated meat. The irradiation-induced changes in the meat components occur via primary radiolysis effects, due to the direct absorption of energy, and by secondary indirect effects. The high reactivity of the free radicals and excited molecular ions produced by the radiolysis of water molecule form very reactive intermediates. These can undergo a variety of reactions leading to stable chemical products, often referred to as radiolytic products (Sommers & Fan, 2006). In general, the extent of chemical reactions induced by irradia- tion in food components depends on many variables; the most impor- tant are the irradiation treatment conditions like the absorbed dose, facility type, and presence or absence of oxygen and temperature. The composition of meat and its physical state also influence the extent of the reactions induced by the treatment and the nature of the formed products (Sommers & Fan, 2006). The effects of ionising radiation on meat lipids involve both oxidative and non-oxidative changes and are responsible of rancidity acceleration and the formation of some hydrocarbons and 2-alkylcyclobutanones from the major fatty acids (Zanardi et al., 2009 Stefanova, Toshkov, Vasilev, Vassilev, & Marekov, 2011; Zanardi et al., 2007). Also muscular pro- teins have been extensively studied: radiation-induced major chang- es consist of dissociation, aggregation, cross-linking and oxidation. Irradiation produced changes in the electrophoretic patterns of chicken muscle proteins after irradiation in the range of 6–20 kGy (Hassan, 1990). An increase of muscular protein solubility and a de- crease of shear force was observed with increasing irradiation dose in *semitendinosus* beef muscle irradiated at 1, 3 and 5 kGy (Hong- Sun et al., 1999). The irradiation increased significantly the content of sulfydryls and the hydrophobicity of salt-soluble proteins of ground pork irradiated at 0, 1.5, 3, 5 and 10 kGy (Koh, Lee, & Whang, 2006). Radiation-induced amino acid modifications have been

well documented. Aromatic and sulphur containing amino acids are most susceptible to irradiation. This is the case for the gen- eration of three tyrosine isomers (para-, meta- and ortho-tyrosine) after ionising radiation of phenylalanine (Hein, Simat, & Steinhart, 2000). The compounds pointed out in the present study as reliable markers for distinguishing between irradiated and non-irradiated beef are probably generated by the effects described above. It is pos- sible to make the hypothesis that glycerol was released from glycer- ides that constitute more than 50% of the intramuscular fat of beef (Marchioni, 2006). A significant oxidation effect of radiation is exerted on the decomposition of fats with a release of free fatty acids from glycerol in a process similar to rancidification (Dvorak, Smid, & Hrusovsky, 1985). Regarding the other substances, no hy- pothesis can be formulated or supported by literature. According to Jarboe and Mabrouk (1974), lactic acid accounts for 44.5% of the organic acid fraction of aqueous beef extract; tyrosine and phenylala- nine, whose decarboxylation can generate tyramine and *p*-substituted phenolic compounds, account for 5.44 and 6.04 mg/100 aqueous beef extract. The only consideration that can be made is that they tend to in- crease in irradiated samples, following a general trend observed for al- most all metabolites. It is probable that metabolites are more extractable from muscular tissue submitted to irradiation treatment.

## Conclusions

This study represents a step forward in the metabolic profiling of irradiated beef. An extensive assignment of $^{1}H$ NMR signals of beef aqueous/methanolic extracts was carried out to interpret metabolic changes occurring as a consequence of the irradiation treatment. A comprehensive multivariate data analysis identified the metabolites in- volved which, in turn, make it easier to understand how ionising radia- tion affects the meat composition. In particular, Classification Trees proved to be an effective and interpretable tool for discrimination when dealing with more than two different groups. Glycerol, lactic acid esters and tyramine or a *p*-substituted phenolic compound proved to be reliable markers for distinguishing between irradiated and non- irradiated beef. Overall, the achieved metabolomic results show that the changes in the metabolic profile of meat represent a valuable insight to be used in detecting irradiated beef. The use of the NMR-based approach simplifies sample preparation and decrease the time required for analysis, compared to available official analytical procedures, i.e. European Standard EN 1785 method. Further investigations will be ad- dressed to beef irradiated at doses lower than 2.5 kGy and to different meat species, with the caveat that the validation of this promising tech- nique for the purpose of classification of new samples will have to be based on a much larger number of samples.

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