Metabolic profiling by 1H NMR of ground beef irradiated at different irradiation doses

Metabolic profiling by ^{1}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 ^{1}H NMR and chemometrics. The assignment of all major NMR signals of the aqueous/methanolic extracts was performed. A comprehensive multivariate data analysis proved able to distinguish between irradiated and non-irradiated beef. Classification Trees revealed that three metabolites (glycerol, lactic acid esters and tyramine or a *p*-substituted phenolic compound) are important biomarkers for classification of irradiated and non-irradiated beef samples. Overall, the achieved metabolomic results show that the changes in the metabolic profile of meat provide 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.

Keywords: beef, irradiation, NMR, metabolomics, Principal Component Analysis, Classification Trees

1. Introduction

Irradiation is a food preservation technology by which ionizing radiation is applied for various purposes including insect disinfestations, growth inhibition, control of parasites, and shelf-life extension. Moreover, it is well-known that irradiation increases food safety by reduction of pathogenic bacteria, and its use is gradually increasing worldwide. Irradiation of food and agricultural products, as part of the larger radiation processing industry, is currently allowed by about 60 countries around the globe (Sommers and Fan, 2006).

In the European Union, the Community positive list of foods and food ingredients that may be treated with ionizing radiation, established by the Directives 1999/2/EC (EC, 1999a) and 1999/3/EC (EC, 1999b), includes 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 ionizing radiation of meat and meat products is not authorized 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 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 controls 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, considering that in several extra-EU countries the use of food irradiation is much more widespread.

The official control of irradiated foods at the retail level has to be carried out by analytical methods validated according to the Commission 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

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86 categories of foods. Four of them are screening methods and have been validated for herbs and
87 spices, poultry meat, food containing mineral debris and food containing DNA, respectively. The
88 six other methods are reference methods and are based on the analysis of primary radiolytic
89 products by thermoluminescence or electron spin resonance spectroscopy, or on the analysis of
90 secondary radiolytic products from fatty acids, namely hydrocarbons and 2-alkylcyclobutanones
91 (Marchioni et al., 2006). The latter ones are suitable for foods with a fat content higher than 1%
92 treated at irradiation dose higher than 0.5 kGy, and have been validated for pork, poultry and eggs.
93 However, they have the disadvantage of being quite time consuming and requiring the use of
94 considerable amounts of organic solvents due to a long and complex sample preparation.
95 Although the analytical methods available for the detection of irradiated foods are
96 numerous, the European Commission promotes the development of new techniques and the set up
97 of new protocols aimed to simplify or improve the already existing procedures (Boniglia, 2004;
98 Califano, 2009).
99 The application of nuclear magnetic resonance (NMR) spectroscopy to the analysis and
100 quality control of foods has shown a great development in the last few years. The increase of new
101 applications and the attention to this technique by scientists, official control institutions, and food
102 industries can be attributed both to the high specificity and versatility of the NMR technique and to
103 the improvement of the NMR instrument performances and availability (Sacchi and Paolillo, 2007).
104 The ability of high-resolution NMR to monitor in a non-invasive and reproducible way all
105 abundant molecules present in a raw material or in a complex system is the major driver for NMR
106 applications in food science. In this context the identification of each signal of the spectrum is
107 unnecessary because all relevant information can be obtained by the application of chemometric or
108 pattern recognition techniques which allow to use the NMR spectrum as a fingerprint or metabolic
109 profile of foods. Several examples of NMR-based metabolomic characterization of foods are
110 available in the literature. Metabolomic studies using ^{1}H NMR spectroscopy coupled with
111 multivariate statistics were carried out for determining the quality of grapes and wines (Son et al.,

2009), differentiating tomato paste triple concentrated (Consonni et al., 2009), authentication, geographic origin and varietal traceability of virgin olive oil (Mannina et al., 2001; Rezzi et al., 2005). With regard to foods of animal origin, the exploitation of the NMR spectrum as a whole metabolic profile has been successfully applied for the geographical characterization of honey (Consonni and Cagliani, 2008), recognition of wild and farmed fish and discrimination of fish species (Aursand and Alexon, 2001; Rezzi et al., 2007), meat products authentication (Al-Jowder et al., 2001), and geographical origin discrimination of beef (Shintu et al., 2007; Jung et al., 2010).

A study on the application of ^{1}H NMR spectroscopy for the identification of irradiated meat based on the quantitative determination of fatty acids has been proposed by Stefanova et al. (2011). Recently, Villa et al. (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 application of the NMR-based metabolomics for the detection of irradiated 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.

2. Materials and methods

2.1. 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 sodium salt (TSP, 98% atom % D) were purchased from Sigma-Aldrich (Milan, Italy). Sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate were supplied by Merck (Darmstadt, Germany).

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2.2. 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 forequarter) 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 48h. Thirty samples were randomly chosen for comparison purposes (non-irradiated control samples), twenty aliquots were randomly allotted in each of two groups intended for treatment at irradiation dose 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 irradiation 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 ^{60}Co 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 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 0.2M (pH 7) sodium phosphate buffer, homogenized by a vortex homogenizer for 1 min and centrifuged twice at 2348 *g* for 20 s using a multispeed refrigerated centrifuge (PK 121R, Thermo Electron Corporation, Whaltham, MA). After homogenization, 210 µl of methanol-d_4 and 90 µl of 0.2M (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 15871 *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 centrifuged at 13000 rpm for 10 min. The supernatant was transferred into 5 mm NMR tubes.

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2.3. NMR spectroscopy

^{1}H NMR spectra of aqueous/methanolic extracts from 80 beef samples (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 spectrometer (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 spectral 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 256 increments, with water presaturation during the relaxation delay of 1 s. 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. Buckets were chosen as large as to compensate the little chemical shifts fluctuation in each single spectrum. The defined pattern was used for the automatic integration of all the spectra and referred to TSP area.

2.4. Chemometric techniques

A matrix (80 x 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 chemometric techniques. In this study both unsupervised and supervised multivariate 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 classification and cluster individual samples solely on the basis of the variability/similarity expressed in their data; on the

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189 other hand, supervised learning methods require that the group information is known a priori and
190 use it to create a classification rule that may be applied to future samples.
191 Principal Components Analysis (PCA) is an unsupervised multivariate method whose aim is
192 to reduce the dimensionality of multivariate data while preserving most of the variance within it. In
193 this study, PCA was based on the correlation matrix (which is equivalent to using the covariance
194 matrix on mean centred and variance scaled variables). Data are visualized by plotting the PC
195 scores, i.e. projecting the individual samples on the plane formed by the first 2 principal
196 components, or the loading plot, which allows to identify the spectral regions with the greatest
197 influence on the possible clustering of the samples. However, PCA optimizes the directions of
198 largest variability (variance) and not the largest class separation ability, so it is not tailored to
199 optimise sample classification. A classification model was then adopted by means of Classification
200 Trees (CT). CT are a nonparametric supervised learning/discriminant analysis method proposed by
201 Breiman et al. (1984), which has been used also in food science applications (Zhang et al., 2005;
202 Debska & Guzowska-Swider, 2011; Cho & Kurup, 2011; Cirlini et al., 2011; Caligiani et al., 2014)
203 as it does not require a normal multivariate distribution of the data nor the equality of within-group
204 variances, two assumptions that frequently don't hold in such applications..
205 For a binary or categorical variable Y and n independent variables X_1,..., X_i,…,X_n, CT is a
206 tree-building method in which the data are split recursively into two groups on the basis of a
207 threshold value of one of the X_i's, with the final aim of predicting Y. The splitting is aimed to
208 optimise a measure of purity of the tree and is repeated until the tree has pure final branches, called
209 nodes (i.e. with samples belonging to one class only) unless some additional pre-set stopping rules
210 on the minimum number of elements in the parent or offspring nodes prevent further partitions.
211 There is also an option to prune the tree by a criterion which trades off tree purity with complexity,
212 with the aim of avoiding overfitting to the training data. A measure of classification accuracy can be
213 obtained by resubstitution (with the same statistical sample on which the rule has been derived) or
214 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:

$$I(t) = \sum_{i \neq j} p_i p_j = 1 - \sum_{j=1}^{J} p_j^2,$$

where I(t) is the impurity at node t, p_j is the proportion of training patterns at node t that belongs to class j (Cho & Kurup, 2011). An advantage of CT on other discriminant analysis methods like Partial Least Squares Discriminant Analysis (PLS-DA) is that each tree partition is based on one actual threshold value of the variable X_i, which makes the results more interpretable for the purpose of potential classification of new samples than one based on a linear combination of the variables. However as the number of samples in this application is relatively low, the prediction results should be generalised with caution.

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

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3. Results and discussion

The advantage of NMR spectroscopy is that all types of compounds give rise to signals simultaneously, so that the NMR spectrum represents a fingerprint of the sample under study. NMR is frequently applied to food samples that can be directly examined as liquids (Belton et al., 1996) but very simple extraction or sample preparation procedures may also be used (Schievano et al., 2008).

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Figure 1 reports a typical ^{1}H NMR spectrum of an aqueous/methanolic extract from non-irradiated and 8 kGy-irradiated beef sample showing the dominant resonances of main components.

240 The assignments of the metabolites observable in the spectra were carried out on the basis of the
241 analysis of 2D NMR (TOCSY) and information provided by other authors (Jung et al., 2010;
242 Brescia et al., 2002; Graham et al., 2010). A detailed analysis of the ^{1}H NMR spectra is reported in
243 Table 1, together with signal assignment.
244 The overall features of the ^{1}H NMR spectrum of non-irradiated and irradiated beef were
245 quite similar; however, some differences were observed in the abundance of some signals, therefore
246 multivariate statistical analyses were performed in order to gain an insight in such spectral
247 differences.
248 As a first step PCA was applied. The first three PCs explained 86.73% of cumulative
249 variance. The PCA model using projection onto three dimensions of PC1, PC2 and PC3 showed
250 some clustering according to the irradiation dose, indicating differences in metabolite composition
251 among the beef extracts. The PCA 3D score plot is shown in Figure 2. In particular, it can be seen
252 that the non-irradiated beef samples, with the lowest values on the PC1, clustered separately from
253 the samples irradiated at 4.5 and 8 kGy, and to a lower extent to those irradiated at 2.5 kGy.
254 To investigate the basis for the observed spectral clustering between the beef samples, the
255 PCA loadings were inspected (Figure 3). The loadings on PC1, which is the component that well
256 separates control samples from samples treated at higher irradiation doses (4.5 and 8 kGy), were
257 mainly positive, demonstrating that almost all metabolites in the ^{1}H NMR spectra are more
258 abundant in irradiated meat samples. Loadings on PC2 and PC3 (not reported) showed a more
259 complicated, harder to interpret distribution pattern, so it was preferred to proceed with a supervised
260 multivariate analysis by means of CT in order to focus the interpretation of classification on a
261 smaller set of variables. Thus, CT was carried out to identify the metabolites from ^{1}H NMR spectra
262 best discriminating between non-irradiated and irradiated beef. For CT the 112 integrated area of
263 the NMR signals of the beef samples were used and a binary tree based on Gini partitioning
264 criterion was constructed. Two different classification models were elaborated which formalise a
265 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-irradiated samples *vs.* all the irradiated samples grouped together, in order to highlight the variables able to discriminate treated meat. In the first model (Figure 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 trading off complexity and accuracy and yields a cross-validation misclassification 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 correlated 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 tentatively attributed to tyramine or to a *p*-substituted phenolic compound. In the case of the second model, comprising the three classes of irradiated 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 obtained after just two tree partitions. As shown in the graphs (Figure 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 substance). The other tree partitioning was determined by valine, that 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, although changes of these water soluble, low molecular weight compounds were observed during post mortem storage of beef (Jarboe and Mabrouk, 1974;

292 Lawrie and Ledward, 2006). Considerable variability was detected among aqueous extracts of beef
293 samples from different countries, suggesting that the metabolite levels and their relative
294 composition could be affected by breed, feeding regimen and production system. However, NMR-
295 based metabolomics of aqueous beef extracts was an efficient method to distinguish fingerprinting
296 difference between raw beef samples, and several metabolites including succinate and various
297 aminoacids (isoleucine, leucine, methionine, tyrosine and valine) can be possible biomarkers for
298 discriminating the geographical origin of beef, although the reasons for the differences in
299 metabolomic profiles as a function of geographical origin are not fully understood (Jung et al.,
300 2010).

301 In the present study ^{1}H NMR profiling of aqueous/methanolic extracts of beef samples
302 allowed to distinguish between irradiated and not irradiated meat. The irradiation-induced changes
303 in meat components occur via primary radiolysis effects, due to the direct absorption of energy, and
304 by secondary indirect effects. The high reactivity of the free radicals and excited molecular ions
305 produced by the radiolysis of water molecule form very reactive intermediates. These can undergo a
306 variety of reactions leading to stable chemical products, often referred to as radiolytic products. In
307 general, the extent of chemical reactions induced by irradiation in food components depends on
308 many variables; the most important are the irradiation treatment conditions like the absorbed dose,
309 facility type, presence or absence of oxygen and temperature. The composition of meat and its
310 physical state also influence the extent of the reactions induced by the treatment and the nature of
311 the formed products (Sommers & Fan, 2006). The effects of ionizing radiation on meat lipids
312 involve both oxidative and non-oxidative changes and are responsible of rancidity acceleration and
313 the formation of some hydrocarbons and 2-alkylcyclobutanones from the major fatty acids (Zanardi
314 et al., 2009; Stefanova et al., 2011; Zanardi et al., 2007). Also muscular proteins have been
315 extensively studied: radiation-induced major changes consist of dissociation, aggregation, cross-
316 linking and oxidation. Irradiation produced changes in the electrophoretic patterns of chicken
317 muscle proteins after irradiation in the range 6-20 kGy (Hassan, 1990). An increase of muscular

318 protein solubility and a decrease of shear force was observed with increasing irradiation dose in
319 *semitendinosus* beef muscle irradiated at 1, 3 and 5 kGy (Hong-Sun et al, 1999). Irradiation
320 increased significantly the content of sulfhydryls and the hydrophobicity of salt-soluble proteins of
321 ground pork irradiated at 0, 1.5, 3, 5 and 10 kGy (Koh, Lee & Whang, 2006). Radiation-induced
322 amino acid modifications have been well documented. Aromatic and sulphur containing amino
323 acids are most susceptible to irradiation. This is the case for the generation of three tyrosine isomers
324 (para-, meta- and ortho-tyrosine) after ionizing radiation of phenylalanine (Hein et al., 2000). The
325 compounds pointed out in the present study as reliable markers for distinguishing between
326 irradiated and non-irradiated beef are probably generated by the effects described above. It is
327 possible to make the hypothesis that glycerol can be released from glycerides that account more
328 than 50% of the intramuscular fat of beef . A significant oxidation effect of radiation is exerted on
329 the decomposition of fats with a release of free fatty acids from glycerol bond in a process similar to
330 rancidification (Dvorak et al., 1985). Regarding the other substances, no hypothesis can be
331 formulated or supported by literature. According to Jarboe & Mabrouk, 1974), lactic acid accounts
332 for 44.5% of the organic acid fraction of aqueous beef extract; tyrosine and phenylalanine, whose
333 decarboxylation can generate tyramine and *p*-substituted phenolic compounds, account for 5.44 and
334 6.04 mg/100 aqueous beef extract. The only consideration that can be made is that they tend to
335 increase in irradiated samples, following a general trend observed for almost all metabolites. It is
336 possible that the irradiation determinate a breakdown of cellular structures, making the metabolites
337 more extractable.
338
339 **4. Conclusions**
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341 This study represents a step forward in the metabolic profiling of irradiated beef. An
342 extensive assignment of ^{1}H NMR signals of beef aqueous/methanolic extracts was carried out to
343 interpret metabolic changes occurring as a consequence of the irradiation treatment. A

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344 comprehensive multivariate data analysis identified the metabolites involved which, in turn, make it
345 easier to understand how ionising radiation affects the meat composition. In particular,
346 Classification Trees proved to be a effective and interpretable tool for discrimination when dealing
347 with more than two different groups. Glycerol, lactic acid esters and tyramine or a *p*-substituted
348 phenolic compound proved to be reliable markers for distinguishing between irradiated and non-
349 irradiated beef. Overall, the achieved metabolomic results show that the changes in the metabolic
350 profile of meat represent valuable insight to be used in detecting irradiated beef. The use of the
351 NMR-based approach simplifies sample preparation and decrease the time required for analysis,
352 compared to available official analytical procedures, i.e. European Standard EN 1785 method
353 (Anonymous, 2001). Further investigations will be addressed to beef irradiated at doses lower than
354 2.5 kGy and to different meat species, with the caveat that the validation of this promising
355 technique for the purpose of classification of new samples will have to be based on a much larger
356 number of samples.
357
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363
364 **References**
365
366 Al-Jowder, O., Casuccelli, F., Defernez, M., Kemsley, E.K., Wilson, R.H., & Colquhoun, I.J.
367 (2001). High resolution NMR studies of meat composition and authenticity. In G.A. Webb,
368 B.S. Belton, A.M. Gil, & I. Delgadillo (Eds.) *Magnetic Resonance of Food Science: a View*
369 *to the Future* (pp. 232-238). Cambridge, United Kingdom: Royal Society of Chemistry.

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Anonymous (2001). Foodstuffs. Detection of irradiated food containing fat. Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones; EN 1785. Brussels, Belgium: European Committee for Standardization (revised 2003).

Aursand, M., & Alexon, D.E. (2001). Origin recognition of wild and farmed salmon (Norway and Scotland) using ^{13}C NMR spectroscopy in combination with pattern recognition techniques. In G.A. Webb, B.S. Belton, A.M. Gil, I. Delgadillo (Eds.) *Magnetic Resonance in Food Science: a View to the Future* (pp. 227-231). Cambridge, United Kingdom: Royal Society of Chemistry.

Belton, P.S., Delgadillo, I., Gil, A.M., Holmes, E., Nicholls, A., Nicholson, J.K., & Spraul, M. (1996). Use of high-field ^{1}H NMR spectroscopy for the analysis of liquid foods. *Journal of Agriculture and Food Chemistry*, *44*, 1483-1487.

Boniglia, C. (2004). Identificazione degli alimenti irradiati. In C. Boniglia, S. Onori, & O. Sapora, (Eds.) *Trattamento degli Alimenti con Radiazioni Ionizzanti - Report 04/21* (pp. 69-75). Roma, Italy: Istituto Superiore di Sanità.

Breiman, L., Friedman, J.H., Olshen, R.A., & Stone, C.J. (1984). *Classification and Regression Trees*, Monterey, CA: Wadsworth International Group.

Brescia, M.A., Caputi Jambrenghi, A., de martino, V., Sacco, D., Ginnico, F., Vonghia, G., & Sacco, A. (2002). High resolution nuclear magnetic resonance spectroscopy (NMR) studies on meat components: potentialities and prospects. *Italian Journal of Animal Science*, *1*, 151-158.

Califano, G. (2009). Ruolo ed iniziative del Ministero del Lavoro, della Salute e delle Politiche Sociali nel settore degli alimenti irradiati. *In Proceedings of Workshop Alimenti irradiati: aspetti scientifici, normativi e sanitari* (pp. 1-25). Foggia, Italy, 23th May 2009.

Caligiani, A., Coisson, J. D.l, Travaglia, F., Acquotti, D., Palla, G.. Palla, L., Arlorio, M. (2014). Application of 1H NMR for the characterisation and authentication of "Tonda Gentile Trilobata" hazelnuts from Piedmont (Italy). *Food Chemistry*, *148*, 77-85.

Cho, J.H., & Kurup, P.U. (2011). Decision tree approach for classification and dimensionality reduction of electronic nose data. *Sensors and Actuators B, 160*, 542-548.

Cirlini, M., Caligiani, A., Palla, L., Palla, G. (2011). HS-SPME/GC-MS and chemometrics for the classification of Balsamic Vinegars of Modena of different maturation and aging. *Food Chemistry*, *124(4)*, 1678-1683.

Consonni, R., Cagliani, L.R., Stocchero, M., & Porretta, S. (2009). Triple concentrated tomato paste: discrimination between Italian and Chinese products. *Journal of Agricultural and Food Chemistry*, *57*, 4506-4513.

Consonni, R., & Cagliani, L.R. (2008). Geographical characterization of polyfloral and acacia honeys by nuclear magnetic resonance and chemometrics. *Journal of Agricultural and Food Chemistry*, *56*, 6873-6880.

Debska, B., & Guzowska-Swider, B. (2011) Decision trees in selection of featured determined food quality. *Analytica Chimica Acta, 705*, 261-271.

Dvorak, J, Smid, K., & Hrusovsky, J. (1985) The effect of ionizing radiation on structural changes in the energy nutrients in animal feed. *Veterinarni Medicina, 30*, 629-636.

EC (2002). Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. *Official Journal of European Communities*, *L 221*, 8-36.

EC (1999a). Directive 1999/2 of the European Parliament and the Council of February 22, 1999, on the approximation of the laws of the Member States concerning foods and food ingredients treated with ionizing radiation. *Official Journal of European Communities*, *L 066*, 16-23.

EC (1999b). Directive 1999/3 of the European Parliament and the Council of February 22, 1999, on the establishment of a Community list of foods and food ingredients treated with ionizing radiation. *Official Journal of European Communities*, *L 066*, 24-25.

Graham, S. F., Kennedy, T., Chevallier, O., Gordon, A., Farmer, L., Elliott, C., & Moss, B. (2010) The application of NMR to study changes in polar metabolite concentrations in beef *longissimus dorsi* stored for different periods post mortem. *Metabolomics*, *6*, 395–404.

Hassan, I.M. (1990). Electrophoretic analysis of proteins from chicken after irradiation and during cold storage. *Food Chemistry*, *35*, 263-276.

Hein, W.G., Simat, T.J., & Steinhart, H. (2000). Detection of irradiated food - Determination of non-protein bound o-tyrosine as a marker for the detection of irradiated shrimps. *European Food Research and Technology*, *210*, 299-304.

Hong-Sun, Y., Ju-Woon, L., Hyun-Ja, L., Jong-Goon, K., Kyoung-Pyo, K., & Myung-Woo, B. (1999). Effect of gamma irradiation on the protein solubility, purge loss and shear force of beef. *Korean Journal of Food Science and Technology*, *31*, 665-671.

Imafidon, G.I., & Spanier, A.M. (1994). Unraveling the secret of meat flavour. *Trends in Food Science and Technology*, *5*, 315-321.

Jarboe, J.K., & Mabrouk, A.F. (1974). Free amino acids, sugars, and organic acids in aqueous beef extract. *Journal of Agricultural and Food Chemistry*, *22*, 787-791.

Jung, Y., Lee, J., Kwon, J., Lee, K.S., Ryu, D.H., & Hwang, G.S. (2010). Discrimination of the geographical origin of beef by ^{1}H NMR-based metabolomics. *Journal of Agricultural and Food Chemistry*, *58*, 10458-10466.

Koh, K-H., Lee, S. P., & Whang, K. (2006) Effects of electron beam irradiation on functional and other associated properties of pork myofibrillar salt-soluble proteins. *Journal of Food Science and Nutrition*, 11, 73-77.

Lawrie, R.L., & Ledward, D.A. (2006) *Lawrie's Meat Science* (seventh edition). Cambridge, United Kingdom: Woodhead Publishing Ltd.

Mannina, L., Patumi, M., Proietti, N., Bassi, D., & Segre, A.L. (2001). Geographical characterization of Italian extra virgin oils using high-field 1H NMR spectroscopy. *Journal of Agricultural and Food Chemistry*, *49*, 2687-2696.

Marchioni, E. (2006). Detection of irradiated foods. In C.H. Sommers and X. Fan (Eds.) *Food Irradiation Research and Technology* (pp. 85-103). Oxford, United Kingdom: Blackwell Publishing Ltd.

Pereira-Lima, C.I., Ordoñez, J.A., Garcia de Fernando, G.D., & Gambero, M.I. (2000). Influence of heat treatment on carnosine, anserine and free amino acid composition on beef broth and its role in flavour development. *European Food Research and Technology*, *210*, 165-172.

Rezzi, S., Giani, I., Héberger, K, Axelson, D.E., Moretti, V.M., Reniero, F., & Guillou, C. (2007). Classification of gilthead sea bream (Sparus aurata) from 1H NMR lipid profiling combined with principal component and linear discriminant analysis. *Journal of Agricultural and Food Chemistry*, *55*, 9963-9968.

Rezzi, S., Axelson, D.E., Héberger, K, Reniero, F., Mariani, C., & Guillou, C. (2005). Classification of olive oils using high throughput flow ^{1}H NMR fingerprinting with principal component analysis, linear discriminant analysis and probabilistic neural networks. *Analytica Chimica Acta*, *52*, 13-24.

Sacchi, R., & Paolillo, L. (2007). NMR for food quality and traceability. In L.M.L. Nollet, F. Toldrá, Y.H. Hui (Eds.) *Advances in Food Diagnostics* (pp. 101-117). Oxford, United Kingdom: Blackwell Publishing Ltd.

Schievano E., Pasini G., Cozzi G., & Mammi, S. (2008). Identification of the production chain of Asiago d'allevo cheese by nuclear magnetic resonance spectroscopy and principal component analysis. *Journal of Agricultural and Food Chemistry*, *56*, 7208-7214.

500 Shintu, L., Caldarelli S., & Franke, B.M. (2007). Pre-selection of potential molecular markers for
501 the geographic origin of dried beef by HR-MAS NMR spectroscopy. *Meat Science*, *76*, 700-
502 707.
503
504 Sommers, C.H., & Fan, X. (2006). *Food Irradiation Research and Technology*. Oxford, United
505 Kingdom: Blackwell Publishing Ltd.
506
507 Son, H.S., Hwang, G.S., Kim, K.M., Ahn, H.J., Park, W.M., Van Den Berg, F., Hong, Y.S., & Lee,
508 C.H. (2009). Metabolomic studies on geographical grapes and their wine using ^{1}H NMR
509 analysis coupled with multivariate statistics. *Journal of Agricultural and Food Chemistry*,
510 *57*, 1481-1490.
511
512 Stefanova, R., Toshkov, S., Vasilev, N.V., Vassilev, N.G., & Marekov, I.N. (2011) Effect of
513 gamma-ray irradiation on the fatty acid profile of irradiated beef meat. *Food Chemistry*, *127*,
514 461-466.
515
516 Villa, P., Castejón D., Herraiz, M., & Herrera, A. (2013) 1H-HRMAS NMR study of cold smoked
517 Atlantic salmon (Salmo salar) treated with E-beam. *Magnetic Resonance in Chemistry*, *51*,
518 350-357.
519
520 Zanardi, E., Battaglia, A., Ghidini, S., Conter, M., Badiani, A., Ianieri, A. (2007) Evaluation of 2-
521 alkylcyclobutanones in irradiated cured pork products during vacuum-packed storage.
522 *Journal of Agricultural and Food Chemistry*, 55, 4264-4270.
523
524 Zanardi, E., Battaglia, A., Ghidini S., Conter M., Badiani A., Ianieri A. (2009) Lipid oxidation of
525 irradiated pork products. *LWT-Food Science and Technology*, *42*, 1301-1307.

Zanardi, E., Caligiani, A., Padovani, E., Mariani, M., Ghidini, S., Palla, G., & Ianieri, A. (2013) Detection of irradiated beef by nuclear magnetic resonance lipid profiling combined with chemometric techniques. *Meat Science*, *93*, 171-177.

Zhang, M.H., Xu, Q.S., Daeyaert, F., Lewi, P.J., & Massart, D.L. (2005). Application of boosting to classification problems in chemometrics. *Analytica Chimica Acta, 544*, 167-176.

535 **FIGURE CAPTIONS**

536

537 **Fig 1.** Representative ^{1}H NMR spectra of polar extract from beef irradiated at 8 kGy (a) and non-
538 irradiated beef (b).

539

540 **Fig 2.** PCA 3D score plot from ^{1}H NMR spectra of polar extract from beef irradiated at different
541 irradiation doses (0; 2,5; 4,5 and 8 kGy).

542

543 **Fig 3.** Loadings for the first PC of the analysed beef samples. X-axis reports the ppm scale of the ^{1}H
544 NMR spectra.

545

546 **Fig 4.** Classification tree (Gini criterion, prior probabilities equal to frequencies, 10-fold cross-
547 validation) of irradiated and non-irradiated beef samples.

548

549 **Fig 5.** Classification tree (Gini criterion, prior probabilities equal to frequencies, 10-fold cross-
550 validation) of beef samples irradiated at three different doses (2.5, 4.5 and 8 kGy).

551
552

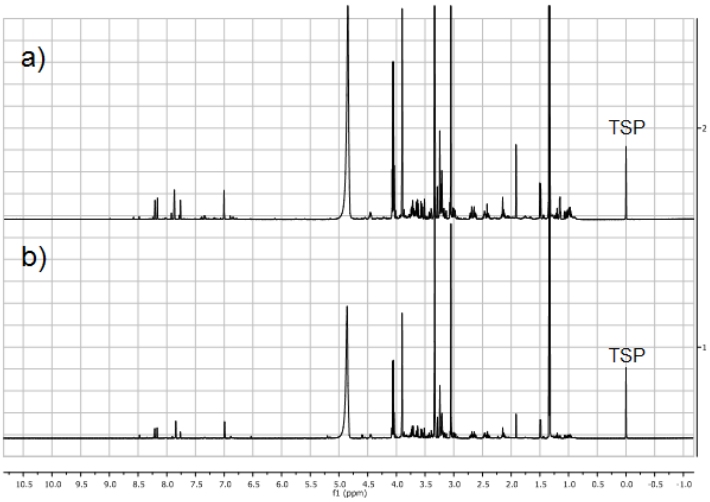
553 **Table 1**. Summary of the signals evidenced in 600 MHz ^{1}H NMR spectra of beef hydroalcoholic
554 extracts. Solvent D_2O. Chemical shifts are reported with respect to TSP (δ=0.000 ppm)
555

δ ^{1}H (ppm)	Multiplicity[a]	Assignment	J (Hz)	TOCSY
0.962	t	Isoleucine	7.21	
0.992	d	Leucine	6.28?	
1.013	d	Valine	7.01	
1.031	d	Isoleucine	7.02	
1.066	d	Valine	7.04	
1.149	d	2,3-butanediol	6.17	3.577
1.198	t	Ethanol	7.08	3.648
1.334	d	Lactic acid	6.88	4.056
1.365	d	Threonine	7.23	
1.411	d	Ester of lactic acid	6.87	
1.492	d	Alanine	7.22	
1.664	m	Leucine		
1.755	m	Arginine + leucine		3.655
1.915	s	Acetate		
2.134	m	Glutamate + glutamine		
2.146	s	Methionine		
2.229	s	Unknown		
2.28	m	Valine		
2.417	s	Succinate		
2.433	m	Carnitine		3.41;4.55
2.458	m	Glutamine + methionine		
2.617	t	Anserine	6.19	3.218
2.644	s	Carnosine	6.10	3.218
2.685	t	Carnosine	6.78	3.218
2.712	t	Anserine	6.87	
2.801	m	Aspartate		2.70
2.978	d	Anserine	8.88	3.185;4.44
3.004	d	Carnosine	8.94	3.185;4.44
3.050	s	Creatine		3.912
3.065	s	Creatinine		4.005
3.195	s	Choline		4.45;2.99
3.208	s	Carnitine		3.41;4.55
3.267		Choline derivate		3.476;4.04
3.285	s	Betaine		3.869
3.41	m	Carnitine		2.407
3.513	s	Glycine		
3.597	m	Glycerol	5.42;11.52,17.85	3.72
3.721	m	Glycerol + Methionine		
3.900	s	Creatine		
4.01	s	Creatinine		
4.391	dd	Adenosina	5.39, 8.47	4.70, 4.23
4.057	q	Lactate	6.89	
4.45	m	Carnitine		3.41;2.43
4.593	d	Beta-glucopyranose	7.90	
4.599	d	Monosaccharide	7.94	
5.198	d	Alpha-glucopyranose	3.74	
6.048	d	Inosine	5.88	-
6.115	d	Sugar of nucleotide	5.24	-
6.534	s	Fumarate		
6.789	d	Tyramine	8.46	7.126
6.844	d	Tyrosine	8.41	7.176
6.891	s	Anserine		7.777
6.998	s	Carnosine		7.857
7.174	d	Tyrosine	8.38	
7.335	m	Phenylalanine		

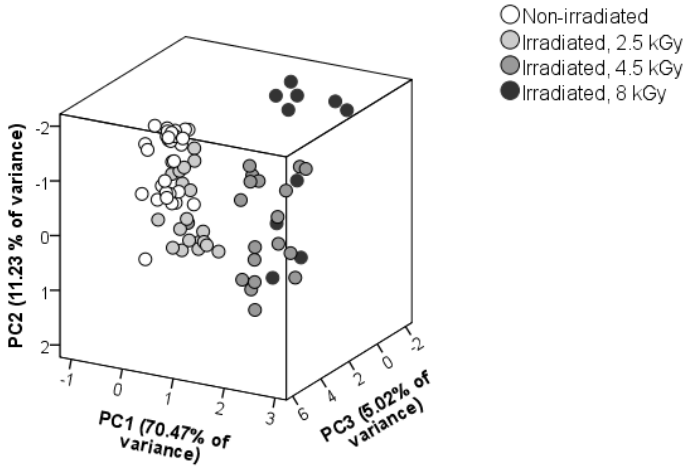
7.399	m	Phenylalanine		
7.51	m	Nicotinic acid		8.28;8.58;8.98
7.777	bs	Anserine		7.04;6.888
7.857	bs	Carnosine		
8.016	d	Uridine	7.68	4.441
8.165	s	Hypoxanthine		
8.208	s	Hypoxanthine		
8.287	td	Nicotinic acid	1.93;8.18	7.510;8.587
8.347	s	Inosine		9.05
8.587	dd	Nicotinic acid		
8.985	d	Nicotinic acid	1.8	7.51

[a] s, singlet; bs, broad singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quadruplet; m, multiplet.

Figure 1


a)
TSP
b)
TSP
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
f1 (ppm)

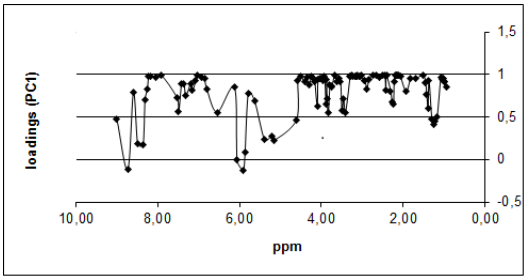
560 **Figure 2**
561



562 **Figure 3**

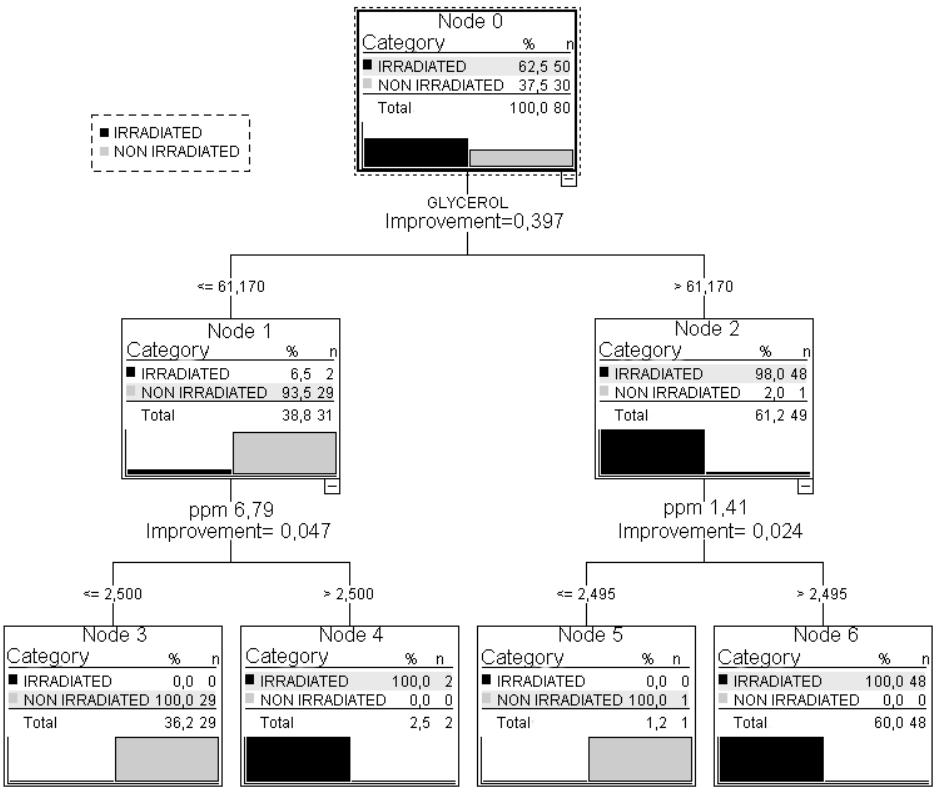
563

564



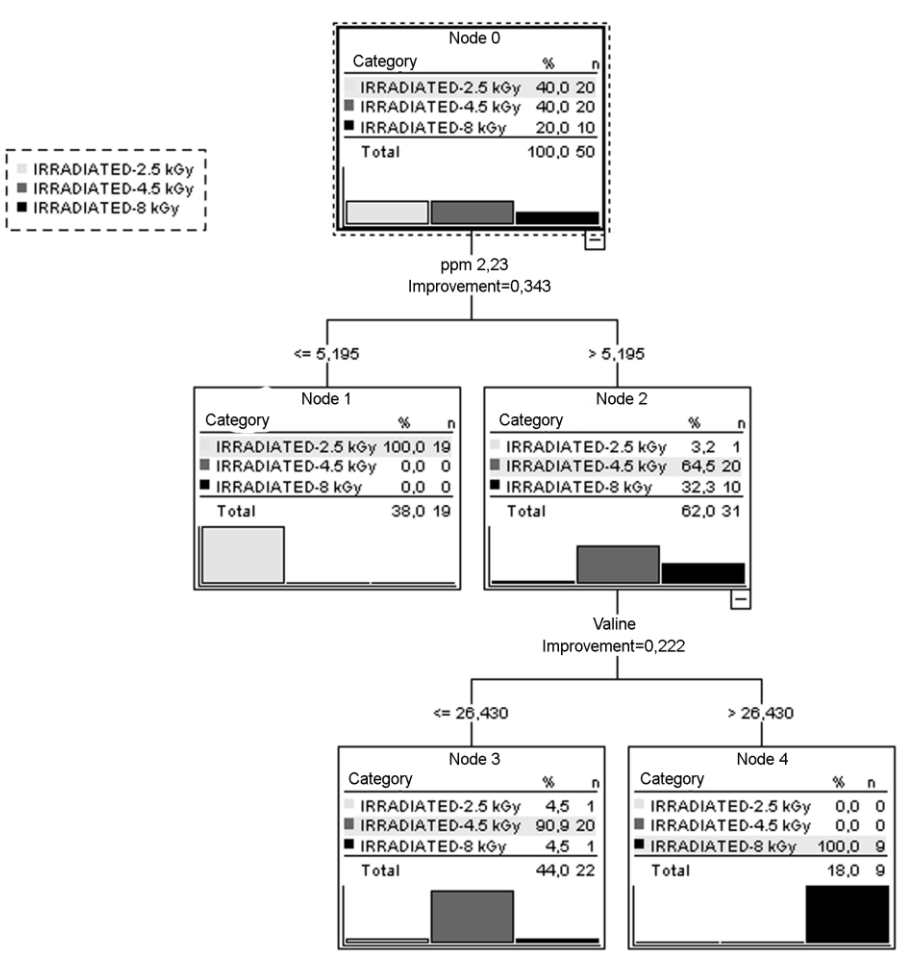
565

566 **Figure 4**



567
568
569

570 **Figure 5**



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