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Evaluation of gamma rays influence on some biochemical and microbiological aspects in black truffles

Filomena Nazzaro [a,*], Florinda Fratianni [a], Gianluca Picariello [a], Raffaele Coppola [a,c], Anna Reale [c], Aldo Di Luccia [a,b]

[a] *Istituto di Scienze dell'Alimentazione-CNR, Via Roma, 52, 83100 Avellino, Italy*
[b] *Dipartimento PROGESA, Università degli Studi di Bari, Via G. Amendola, 165/A, 70126 Bari, Italy*
[c] *DiSTAAM, Università degli Studi del Molise, Via De Sanctis, 86100 Campobasso, Italy*

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Abstract

The effects of two gamma-ray doses (1.5 kGy and 2.0 kGy) on some biochemical aspects and on the microbiological profile of black truffles was monitored, immediately after treatment and after 30 days of storage at 4 °C. Electrophoretic and chromatographic analyses of proteins and peptides, just like monitoring of polyphenol content, peroxides formation and microbial profile, allowed for the first time a better understanding of the mechanisms responsible for biochemical alterations and bacterial pattern in black truffles during their storage. Treatment at 1.5 kGy appeared to better preserve the characteristics of the fresh product. In 2.0 kGy-samples, the protein profile was characterised by a 20 kDa-polypeptide, which could be considered as an useful marker of the irradiation treatment and of the storage time of the product. MALDI-TOF mass spectrometry analysis did not permit a correct identification from tryptic peptides in databases, although the nano-ES/MS/MS analyses performed on the 10 kDa tryptic digest peptides showed an amino acidic sequence entirely contained in a protein of filamentous fungus *Neurospora crassa*.

Keywords: Food irradiation; Food preservation; Gamma rays; Protein; Truffle

1. Introduction

The primary need of food has induced man to explore the most suitable technologies for its storage and transformation, both to improve its quality and reduce its scarcity during less favourable periods. Nowadays, the recent progress in food technology as well as a better knowledge of food physics and chemistry, biochemistry, microbiology and engineering, has allowed the introduction of more innovative food storage systems. Moreover, the widespread and increasing incidence of food-borne illness recently caused by pathogenic bacteria and parasites, and the consequent social and economic impact on the human population have also brought food safety to the forefront of public health concerns. In this aspect, irradiation is widely emerging as a recognised safe and effective method of food preservation, being used to extend the shelf life of raw and processed foods in many countries worldwide. Ionizing radiation is a method for preservation of foods that uses the high energy of gamma rays or accelerated electrons, thereby ionizing molecules (Andrews et al., 1998). At present, the international rules limit use of conventional additives; therefore, food industries should have many problems to limit the biological contamination, with a consequent loss of food and notable economic and social damages. The main benefit of irradiation is widely accepted for eliminating microorganisms, insects or parasites capable to lead to food spoilage and toxicity, replacing chemical fumigants, with-

* Corresponding author. Tel.: +39 0825299381; fax: +39 0825781585.
E-mail address: mena@isa.cnr.it (F. Nazzaro).

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out significantly affecting the physical state of the products (Delincee, Mancini-Filho, & Villavicencio, 1998). In spite of the several advantages giving rise from use of ionising radiation, food irradiation has not successfully been applied on a broad scale in Europe, even if, based on the available data on the wholesomeness and safety of irradiated foods, a Joint FAO/IAEA/WHO Expert Committee on irradiated foods recommended a widespread use of this technique and an unconditional acceptance of foods treated with doses up to 10 kGy (CAC, 1984; Thakur & Singh, 1995; WHO, 1994). Moreover, the interest of the consumer towards fresh food with a more extensive shelf life is stimulating experimentation with methodologies such as irradiation, also used for treatment and storage of fresh and highly perishable foods. One of these products is represented by truffles, the fruit body of an ectomycorrhizal Ascomycotina fungi of genus *Tuber* (Trappe, 1979). The best-known species are *Tuber magnatum* var Pico, or white truffle, and *Tuber aestivum* var Vittadini, or black truffle. One of the most important problems of its storage is the preservation of taste and aroma, essential parameters for its quality and the main reason why truffles are one of the most famous foods in the world. This problem in actual fact is of great consequence in Italy, where production has high potentialities, but low possibilities for its wider marketing, due to its limited shelf life as a fresh product. On the other hand, it is not capable to retain, for a long time, its sensory and biochemical peculiarities, being the most important characteristics for the consumer. As a consequence, several methodologies, such as use of gamma rays, are potentially attractive to improve its shelf life and safeguard its sensory and structural characteristics. However, if applied in an inappropriate mode, irradiation could trigger unwanted sensory and chemical changes, i.e. resulting in free radicals, whose reaction with other biomolecules, such as proteins, lipids and polyphenols, could give rise to detrimental and damaging effects. Several works are present in literature as regards genus *Tuber*. At present, most of them generally concern the investigation of truffle organic compounds, taxonomic and biomolecular analysis (Diaz, Ibanez, Senorans, & Reglero, 2003; Percudani, Trevisi, Zambonelli, & Ottonello, 1999). Furthermore, the two-dimensional profile and protein identification in different *Tuber* species have been obtained (Pierleoni et al., 2004). Eventually, the antimicrobial and antimutagenic effects of truffle extracts were also evaluated (Fratianni, Di Luccia, Coppola, & Nazzaro, 2005; Janakat, Al-Fakhiri, & Sallai, 2005). Therefore, no information appears about the main biochemical modifications taking place during the storage of this product. The aim of our work was to monitor the effect of gamma rays on some biochemical events as well microbial profile occurring in black truffles packed in modified atmosphere and treated with two doses of gamma rays, immediately after irradiation and after 30 days of storage at 4 °C. Hence, the natural and irradiated samples were compared for some specific aspects of degradation during the use of gamma rays and natural storage.

2. Materials and methods

Fresh samples of black truffle (*T. aestivum* var. Vittadini) were purchased in Molise (Italy). After packaging in N_2 atmosphere, truffles were irradiated using a ^{60}Co gamma ray source to achieve the final doses of 1.5 and 2.0 kGy and stored until 30 days at 4 °C. The analyses were carried out through spectrophotometric, electrophoretic and chromatographic procedures to monitor the soluble N fraction. Furthermore, spectrophotometric methods were performed to evaluate the polyphenols and hydroperoxides content. The microbiological profile was evaluated through microbial counts. Truffles only packed and stored in the same conditions but not irradiated were used as control.

3. Protein extraction

Proteins were extracted in buffer (Tris/HCl 25 mM + Urea 3 M + β-mercaptoethanol 0.5%, pH 8.0) following the procedure of Nazzaro et al. (2004); the protein content was spectrophotometrically determined at 595 nm using the Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA) according to Bradford (1976). Bovine serum albumin was used as standard reference.

4. Electrophoretic analyses

4.1. Sodium dodecylsulfate gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). Protein samples (100 μg) were incubated at 95 °C for 10 min in sample buffer (60 mM Tris HCl, 2% SDS, 14.4 mM β-mercaptoethanol, 25% glycerol, 0.1% bromophenol blue, pH 6.8), resolved on a 12–15% polyacrylamide separation gel and revealed by staining with R-250 Coomassie Brilliant Blue (0.1%) in aqueous methanol (4:10 v/v) and diluted acetic acid (1:10 v/v). A protein mixture with a molecular weight ranging from 205 kDa to 6.5 kDa was used as standard.

4.2. Tricine SDS-PAGE

One hundred micrograms of each sample were incubated at 95 °C for 10 min in a denaturing mixture, and resolved onto an acrylamide/bis acrylamide 46.5:3% slab gel in Tris–Tricine buffer (Schagger & von Jagow, 1987). A mixture of polypeptides with a molecular weight ranging from 26.62 to 3.49 kDa was used as standard. After the electrophoretic run, the bands, previously fixed in 50% methanol/10% acetic acid solution, were revealed through G-250 Coomassie Brilliant Blue in 10% acetic acid for 2 h.

5. Chromatographic analyses

Chromatographic analyses were performed by RP-HPLC procedure, using a System Gold Beckman chromatograph with an UV detector. The protein profile was obtained with a Vydac Reverse Phase C-8 column (Nazzaro et al., 2004). After treatment of samples with ice cold-acetonitrile and centrifugation, the supernatant, containing the peptide fraction, was collected, dried and resuspended in H_2O + 0.01% TFA. The peptide profile was obtained through fractionation on Khromasyl C-18 at 214 nm with a gradient A (0.01%TFA in deionised water) towards B (acetonitrile-deionised water + 0.01% TFA).

6. Mass spectrometry analyses

6.1. In-gel digestion of excised protein spots

Manually excised spots from Coomassie stained gels, were de-stained, reduced and alkylated by carboxymethylation, and then in situ digested by trypsin (Sigma Chemical Co., St. Louis, MO), overnight at 37 °C, according to Shevchenko, Wilm, Vorm, and Mann (1996). Peptides resulting from digestion were extracted in 40 μl of ACN 5%: formic acid 1/1 (v/v) solution and concentrated by vacuum centrifugation for mass spectrometric analysis. All steps of the excision procedure, destaining, reduction/alkylation and peptide extraction, were carried out under a laminar flow hood to avoid contamination by keratins.

6.2. Peptide-mass mapping by MALDI-TOF MS

Mass spectrometry experiments were carried out on a PerSeptive Biosystems (Framingham, MA) Voyager DE-Pro instrument equipped with a N_2 laser (337 nm, 3 ns pulse width). An aliquot of peptide mixture (1 μl) from each digested spot was loaded on a stainless steel plate together with 1 μl of matrix (α-ciano-4-hydroxycinnamic acid) prepared by dissolving 10 mg in 1 ml of aqueous 50% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and the mixture was air dried. Typically, 250 laser pulses were acquired for each spectrum and the mass spectra were acquired in the reflector mode; the accelerating voltage was 20 kV. External mass calibration was performed with low-mass peptide standards.

6.3. Nano-electrospray tandem mass spectrometry (nano-ES/MS/MS)

Before electrospray mass spectrometry analysis, tryptic peptides were desalted by loading onto Zip-Tip (Millipore, Badford, USA) C-18 Reversed Phase pre-packed microcolumns, previously equilibrated with 0.1% TFA, washing with 0.1% TFA and eluting by aqueous 50% acetonitrile containing 0.1% TFA. The sample solutions (2 μl) were introduced into borosilicate needles (Protana Inc., Odense, Denmark) to run nano-ES/MS and MS/MS experiments. Nano-ES/MS and nano-ES/MS/MS analysis were performed using an hybrid quadruple-orthogonal acceleration time of flight Q-star Pulsar (PESciex, Toronto, Ontario, Canada) equipped with a nanospray source (Protana) operating in positive ion mode. Needle voltage was 800 V, and orifice voltage was set at 40 V to minimize cone fragmentation. Air at the pressure of 10 psi was used as "curtain gas". In nano-ES/MS/MS experiments N_2 was used as collision gas; Q0 and Q2 quadrupole voltages were respectively 58.0 V and 9.9 V.

6.4. Protein database searching

The measured tryptic peptide molecular masses were used as inputs to search the NCBI and Swiss-Prot databases. Searches by peptide mass fingerprinting were carried out by using the PROTEIN PROSPECTOR/MS FIT software developed at the University of California, San Francisco (http://prospector.ucsf.edu) and were restricted to mammalian species proteins. The allowed protein molecular mass range was chosen by referring to electrophoretic MW standards from 10,000 to 200,000 Da. Isoelectric points were allowed to range from 3.0 to 10.0, and oxidation of Met was included as a possible side reaction. Up to two missed tryptic cleavages were considered, and a conservative mass accuracy of +0.4 Da was used for all tryptic-mass searches. Searches by homology, based on sequences deduced by ES/MS/MS, were carried out on the National Center for Biotechnology Information BLAST server using the Genbank database.

7. Polyphenols and hydroperoxides determination

Ten grams of truffle were mixed with 30 ml of methanol and immediately homogenized for 30 s. The mixture was centrifuged at 4 °C for 10 min at 1500 × *g*, and the supernatant was collected and dried. Total phenolic concentration was performed by spectrophotometric analysis, using the Folin-Ciocalteu colorimetric method (Singleton & Rossi, 1965). The absorbance was spectrophotometrically measured at 760 nm. The phenolic concentration was calculated as nM quercetin equivalent from the calibration curve of quercetin standard solutions (covering the concentration range between 0.01 and 2.0 mM). For each measurement, three replicates of five truffles (untreated and irradiated with different dose rate of gamma rays) were tested. Hydroperoxides were spectrophotometrically determined (Griffiths, Leverentz, Silkowski, Gill, & Sanchez-Serrano, 2000) at 560 nm based on oxidation of Fe(II) to Fe(III) under acidic conditions, followed by complexation of Fe(III) by Xylenol Orange (FOX2 assay). FOX2 reagent contained Xylenol Orange (Sigma, UK) and ammonium ferrous sulphate in 250 mM H_2SO_4 (1 mM and 2.5 mM final concentrations, respectively). One volume of reagent was added to 9 volumes of methanol containing 4.4 mM BHT. Triphenylphosphine (TPP) was added (final concentration 2.5 mM) to one series of samples to reduce the

hydroperoxides to their corresponding unreactive hydroxide derivatives and was used to authenticate the signal generated in the samples minus TPP following the addition of FOX2 reagents. After former 30 min incubation in the dark at room temperature, samples were then allowed to stand at room temperature for further 30 min following the addition of the working FOX2 reagent. The values were related to a standard curve made with a 10^{-6} M H_2O_2 solution. For each measurement, three replicates of five truffles (untreated and irradiated with different dose rate of gamma rays) were tested. The results were submitted to an analysis of variance followed by Student's *t* test. The degree of confidence was fixed at 95% for all of the analyses (Snedecor & Cochran, 1978).

8. Microbial profile

Microbial counts were determined after the irradiation treatment and packaging in N_2 atmosphere, at the beginning and at the end of storage (Reale, Sorrentino, Maiuro, & Coppola, 2005). Each sample (10 g) was aseptically homogenised in 90 ml sterile quarter-strength Ringer's solution in a stomacher Lab Blender (Seward Medical, London, UK) for 30 s. Serial dilutions were made and plated for microbial enumeration. Each microbial group was investigated using standard procedure on specific growth media (Table 1). As control, the same analyses were performed on untreated truffle.

9. Results and discussion

9.1. Chromatographic analyses

9.1.1. Protein analysis

The most interesting chromatographic protein profiles were divided into three portions: in the first, all peaks eluted between 0 and 10 min were included, the second portion contained the peaks eluted between 10 and 16 min, and, in the last one, all peaks eluted between 16 and 28 min were taken in. The areas of these peaks, expressed as absolute areas (mm^2), were used to design histograms, as showed in Fig. 1. The first area gave high values for all samples. Significant differences were found between the beginning and the end of storage, all samples having superior values after 30 days in comparison with time 0;

Table 1
Growth media and conditions used for the different microbial groups evaluated in untreated and irradiated truffles

Microbial group	Growth medium	Incubation (°C)	Time
Lactobacilli	MRS	28	48–78 h
Lactococci	M17	28	4 d
Micrococcaceae	MSA	28	36–48 h
Clostridia	RCM	28	72 h
Mesophilic bacteria	PCA	28	48 h
Enteobacteriaceae	VRBGA	37	6 h
Coliform	VRBLA 37 °C	37	36 h
Faecal Coliforms	VRBLA 44 °C	44	36 h
Enterococci	SB	37	36 h

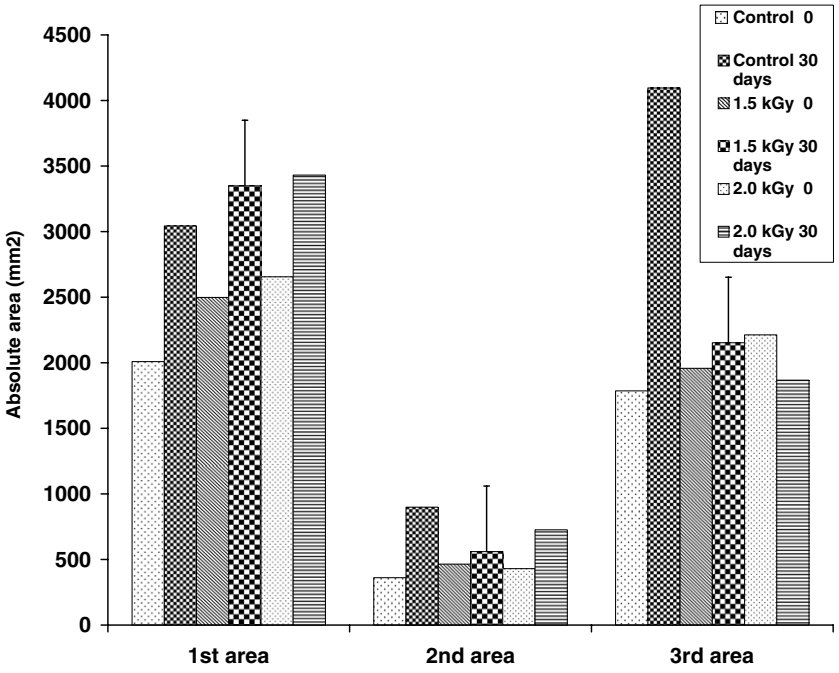

Fig. 1. Histogram of chromatographic protein profiles, from fresh and irradiated truffle (1.5 or 2.0 kGy), at zero and after 30 day of storage. The analyses were performed by RP-HPLC procedure. The representation is divided in three portions, based on elution time of peaks: portion 1 (elution within 10 m), portion 2 (elution between 10 and 16 m) and portion 3 (elution between 16 and 28 m). In the *y*-axis, the values of the groups of peaks taken into consideration are expressed as absolute areas (mm^2). For details see Section 2.

in the irradiated truffles, they were always higher than in the control. Interestingly, since time 0, in the 2.0 kGy irradiated truffles, all peaks gave the highest values. So, in addition to the normal biochemical process, taking place during the time of storage considered, it appeared that a further modification could be induced by treatment with gamma rays. However, at the same time, it could be also assumed that a low dose of gamma rays could, in a certain manner, slow the protein degradation, normally occurring in the untreated truffle. In fact, by the analysis of all the chromatographic groups, the most evident degradation phenomena were observed in the control, while they were not so evident in the treated samples, especially in the 1.5 kGy-irradiated one. In the third group, the 2.0 kGy-treated sample showed decreasing values after 30 days of storage. Probably, this was due to a greater degradation of protein with consequent release of peptides: this event was not observed in the samples irradiated with 1.5 kGy, which, after the same period of storage, presented just about constant values.

9.1.2. Peptide analysis

Histograms, created on the basis of the most significant peaks, showed interesting results (Fig. 2); in the first group, control showed greater values at the end of the storage if compared to time zero, an event certainly due to the natural product degradation. In truffle irradiated with 1.5 kGy, this group gave a slightly higher area; the event was probably due to the effect of gamma rays, as therefore it was more evident in the 2.0 kGy sample; on the contrary, after 30 days, 1.5 kGy truffle presented similar values to those shown by fresh control. This effect was also revealed for the other groups. Moreover, the 2.0 kGy truffle exhibited values always higher than in the other two samples, this signifying a certain protein degradation that should favour the development of a greater quantity of polypeptides fragments.

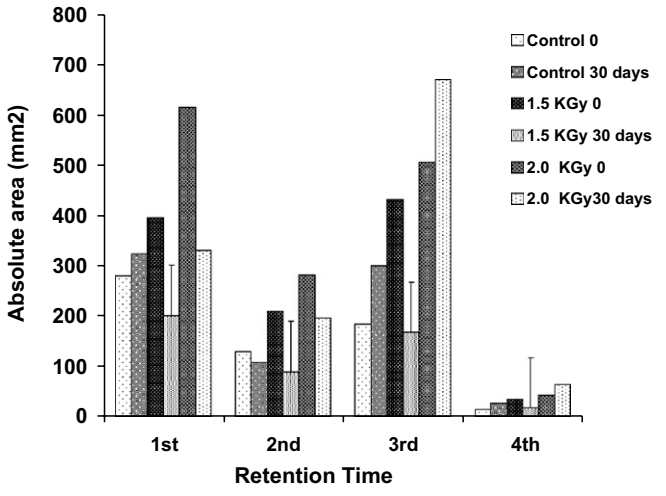

Fig. 2. Histogram of chromatographic peptide profiles, from fresh and irradiated truffle (1.5 or 2.0 kGy), at zero and after 30 day of storage. The analyses were performed by RP-HPLC procedure. In the *y*-axis are represented the values of the most interesting peaks considered, expressed as absolute areas (mm^2). For details see Section 2.

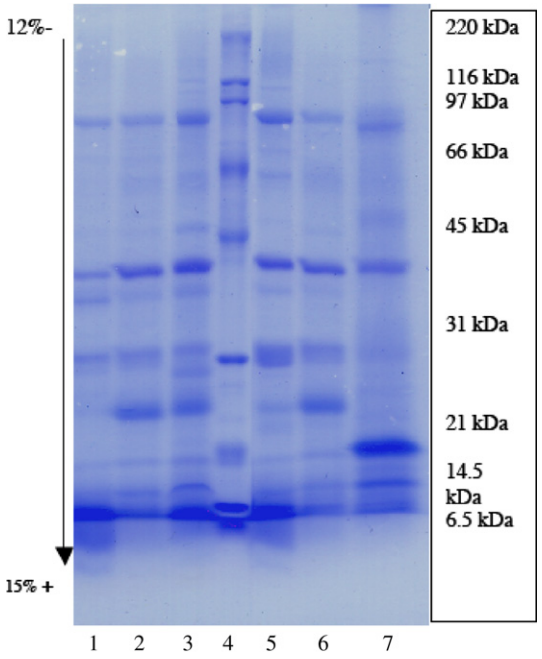

Fig. 3. SDS-PAGE patterns of truffle protein extracts from fresh and irradiated truffle (1.5 or 2.0 kGy) at beginning and at the 30th day of storage. SDS-PAGE was performed according to the method of Laemli. Lane 1: fresh truffle; Lanes 2 and 3: truffle irradiated with 1.5 and 2.0 KGy respectively, time zero; Lane 4: molecular weight standards; Lane 5: 30-days stored untreated truffle; Lanes 6 and 7: 30-days stored truffles. On the right are indicated the molecular weights of standards used in the experiment.

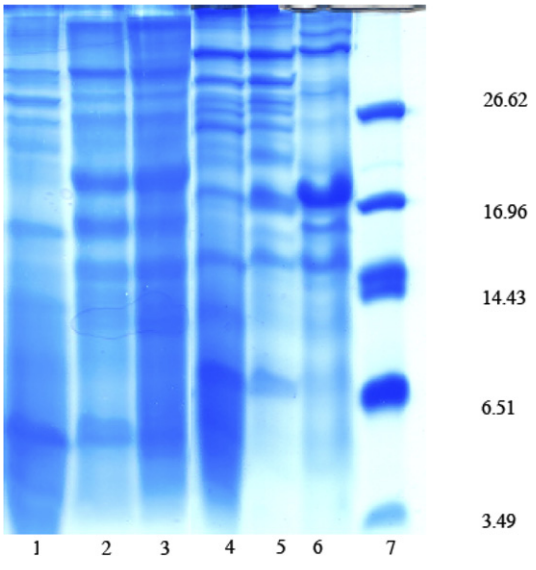

Fig. 4. Tricine SDS-PAGE patterns of truffle protein extracts from fresh and stored truffles, with and without irradiation. The analysis was carried out according to Schagger and von Jagow. Lane 1: fresh truffle; Lanes 2 and 3: irradiated truffle (1.5 and 2.0 kGy, respectively), time zero; Lane 4: 30-days untreated truffle; Lanes 5 and 6: 30 days stored truffles irradiated with 1.5 and 2.0, respectively; Lane 7: molecular weight standards.

10. Electrophoretic analyses

The electrophoretic analysis, performed by SDS-PAGE, showed a similarity between protein pattern in the control and in the 1.5 kGy irradiated sample, after 30 days of truffle storage at 4 °C (Fig. 3). Molecular weight of bands (M_r) ranged from 97 kDa to 14.5 kDa; in particular, proteins with an estimated M_r of 97 kDa, 43 kDa, 40 kDa and 37.5 kDa were revealed. In the 2.0 kGy truffle, the disappearance of 40 kDa and 37 kDa bands and the appearance of a highly intensive band having an estimated M_r of about 20 kDa were observed, less evident in the other two samples. Apparently, the intensity of irradiation involved a more rapid protein hydrolysis, inducing the formation of lower M_r compounds. Similar results were observed in other studies (La Maire et al., 1990; Schuessler & Schilling,

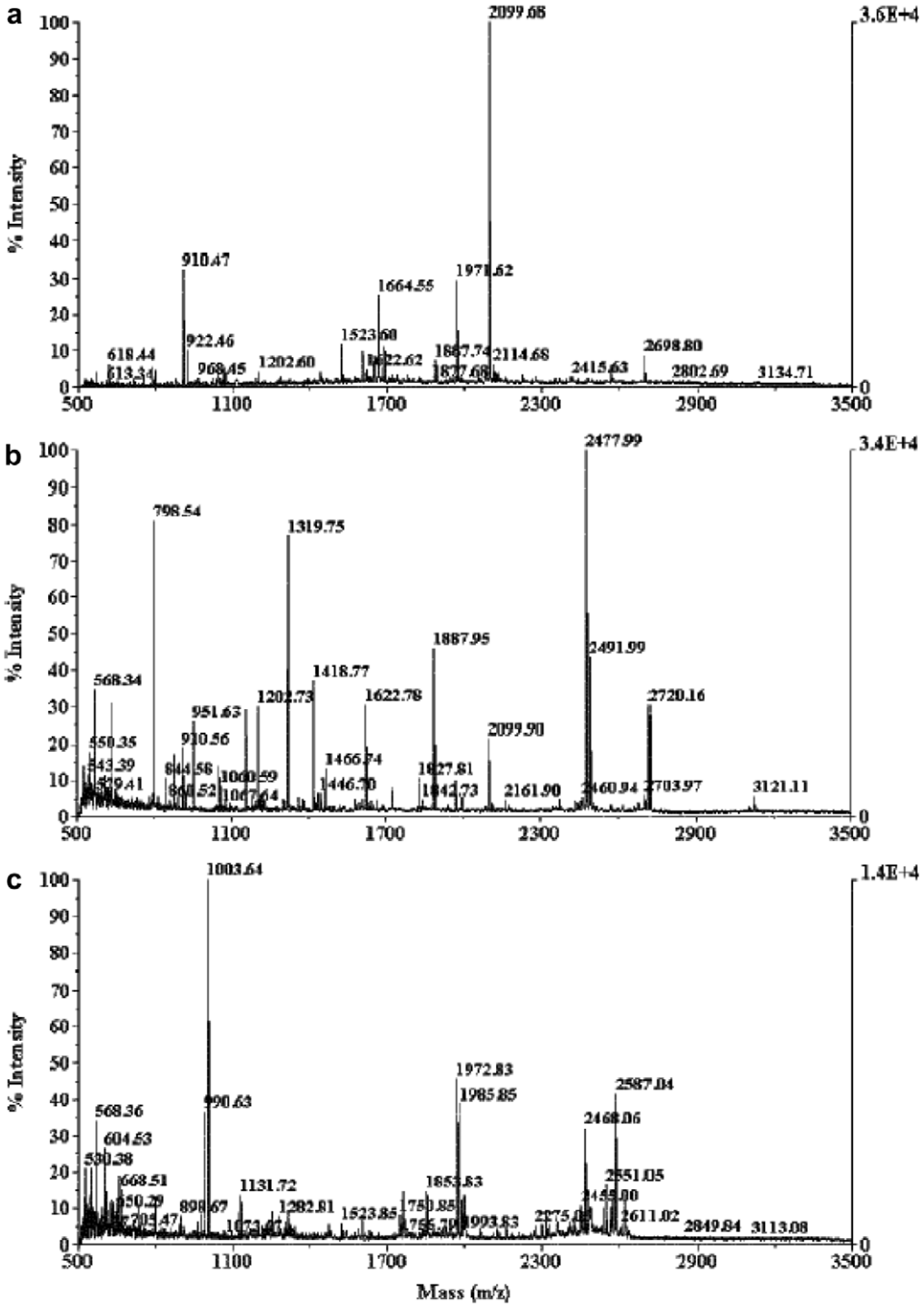

Fig. 5. Mass spectrometry mapping of protein fragments separated by SDS-PAGE electrophoresis. MALDI-TOF spectra of in gel tryptic digests from (a) spot n.1, corresponding to 10 kDa, (b) spot n.2, corresponding to 20 kDa and (c) spot n.3, corresponding to 14 kDa.

1984). This appearance was more evident from the electrophoretic peptide analysis (Fig. 4). In the 2.0 kGy sample, essentially at the beginning but also at the end of the storage, a loss of polypeptides with an estimated M_r of 26, 16 and 6.51 kDa and a concurrent, remarkable, increased intensity of an estimated 20 kDa band was monitored; this last band could be used as signal stress in truffle (senescence or irradiation treatment), taking into consideration that it was absent in the untreated sample but present in the irradiated truffles already at time 0. Therefore, after 30 days of storage, the band was present both in the control (natural degradation) and in the 1.5 kGy samples; but it was most of all evident in samples treated with a final dose of 2.0 kGy.

11. Mass spectrometry analyses

Mass-spectrometry experiments were carried out in order to identify the proteins separated by electrophoresis. Spots of 10–14–20 kDa from 1D-gels were excised, digested overnight with trypsin, and the tryptic peptide mixtures analysed by MALDI/TOF. The peptide mass fingerprinting was limited to search for homologous proteins that might show conserved tryptic peptides in databases of fungi or other similar species, but no entries were found, maybe because of a lack of information regarding the truffle genome. This was just an attempt, in fact it is well known that MALDI MS approach can be successfully used with fully-sequenced genomes or when enough ESTs are available. The peptides digested from the 10 kDa spot analysed by nano-electrospray mass spectrometry and the most abundant peptides were subjected to tandem mass spectrometry analysis with the aim of deducing amino acidic sequences ranging from 5 to 10 residues and searching protein and genomic databases to find homology with already known proteins. The three peptide maps showed effectively non-superimposable signals; this should suggest their different origin, or rather, they could arise from completely different proteins or from far portions of the same protein, but they can not be longer or smaller sequences of the same protein fragment (Fig. 5). In Fig. 6, the nano-ES/MS/MS spectrum of the selected ion $m/z =$ 676.50 is shown. It was possible to deduce the following amino acidic sequence: VYGLPK. Even longer sequences were obtained from fragmentation of larger peptides (data not shown). The above reported sequence is entirely contained in the protein XP_324353 from the filamentous fungus *Neurospora crassa*, but no other peptide molecular masses matched. The peptide sequences obtained from different peptides from spot n.1 were submitted to the homology search, but no certain identification was possible. This result eliminates every possible hypothesis on the microbial nature of the approximate 20 kDa polypeptide. From these data it could be thought that the effective product degradation could involve mostly endogenous reactions, in particular concerning the proteins, well-known easily degradable structures. Like lipids, proteins could be injured from gamma rays, mainly due to oxygen radicals generated by the radiolysis of water. Generally, radiation causes irreversible changes at the molecular level by breakage of covalent bonds of the polypeptide chains. The chemical changes activated in proteins by irradiation can be represented by both non-random and random fragmentation. In addition, there have been also reports on protein aggregation and cross-linking by irradiation. Hydroxyl radicals and super oxide anion radicals generated by radiation can also modify the primary structures of proteins, which results in distortions of secondary and tertiary structure (Davies,

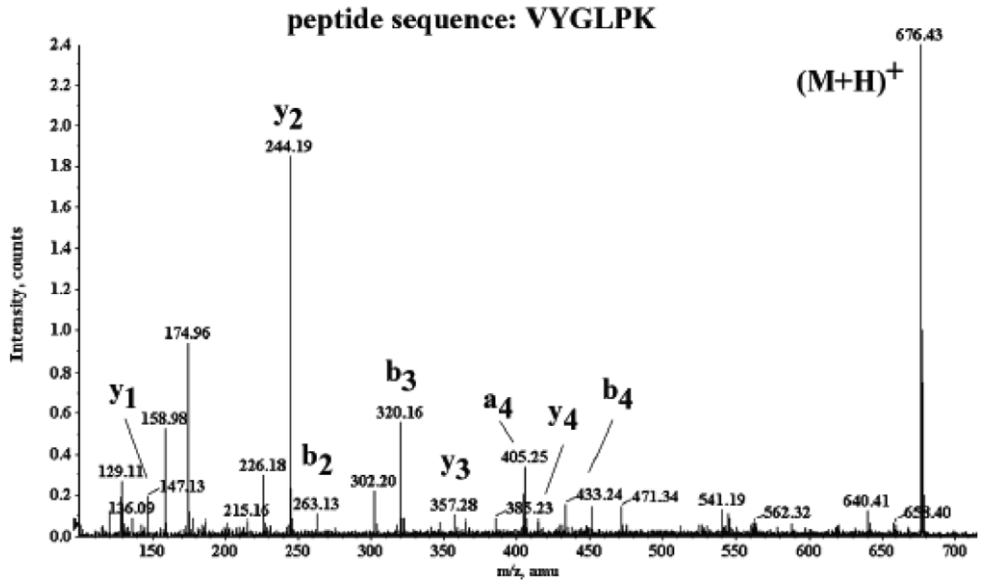

Fig. 6. Reconstruction of amino acidic sequence of the mono-charged selected ion with M+H+ = 676.43 by nano-ES/MS/MS. Deduced sequence was used to search protein and gene database by homology.

Table 2
Hydroperoxide concentration (nM/g of truffle) in untreated and irradiated (1.5 kGy and 2.0 kGy) truffles at the beginning of experimentation and stored for 30 days

Hydroperoxides (nM/g truffle)			
Time (days)	0.0 kGy	1.5 kGy	2.0 kGy
0	53.5^{A}	54.4^{A}	61.3^{A}
30	$263.7^{a,B,C}$	$238.8^{a,B}$	$315.0^{b,C}$

Hydroperoxides were spectrophotometrically determined at 560 nm based on oxidation of Fe(II) to Fe(III) under acidic conditions, followed by complexation of Fe(III) by Xylenol Orange (FOX2 assay). The data are the mean of five independent experiments. The results were submitted to an analysis of variance followed by the Student's test. The degree of confidence was fixed at 95% for all the analyses.
$^{a,b}P < 0.01$.
$^{A,B,C}P < 0.001$.

1987; Davies & Delsignore, 1987; Davies, Lin, & Delsignore, 1987; Kempner, 2001; Lacroix et al., 1992; Moon & Bin Song, 2000; Puchala & Schlesser, 1993; Taub, Robbins, Simic, Walker, & Wierbick, 1979). By this point, we could hypothesize that use of a final dose of 1.5 kGy might be considered acceptable for the treatment of truffles; in fact, although a slight degradation of proteins was present at the beginning of storage, a good preservation of protein profile was observed after 30 days of storage, therefore an excessive protein degradation was demonstrated in samples treated with a final dose of 2.0 kGy, at the end of storage.

Our hypothesis was also supported by the spectrophotometric results. In fact, as shown in Table 2, after 30 days of storage, the peroxides content was significantly higher ($p < 0.001$) with respect to samples at the beginning of the experiment. In particular, after 30 days of storage, control and 1.5 kGy-treated samples did not show significant differences whereas the difference between the control and 2.0 kGy sample was less significant ($p < 0.01$) than between the two irradiated samples ($p < 0.001$). Therefore, when a dose of 2.0 kGy was applied, a superior damage was also evident, revealing the higher content in peroxides (6 times), as also shown in other food models, in which the auto-oxidative process induced by ionizing radiation was quite accelerated (ICGFI, 1992).

12. Microbial profile

Previous studies were performed on different *Tuber borchii* microbial communities, describing the presence of Proteobacteria, aerobic spore-forming bacteria and Actinomycetes (Barbieri et al., 2005; Bedini et al., 1999; Citterio et al., 1995). In our experiment, the microbial profile present in fresh samples of *T. aestivum* was investigated (Fig. 7). It was mainly represented by Lactobacilli, Lactococci, Micrococcaceae, Clostridia, mesophilic bacteria and Enterobacteriaceae; therefore Enterococci and faecal coliforms were less present. In addition, the effect of the different intensities of gamma rays on truffle microflora was investigated. As shown in Fig. 7, the treatment with 1.5 and 2.0 kGy affected

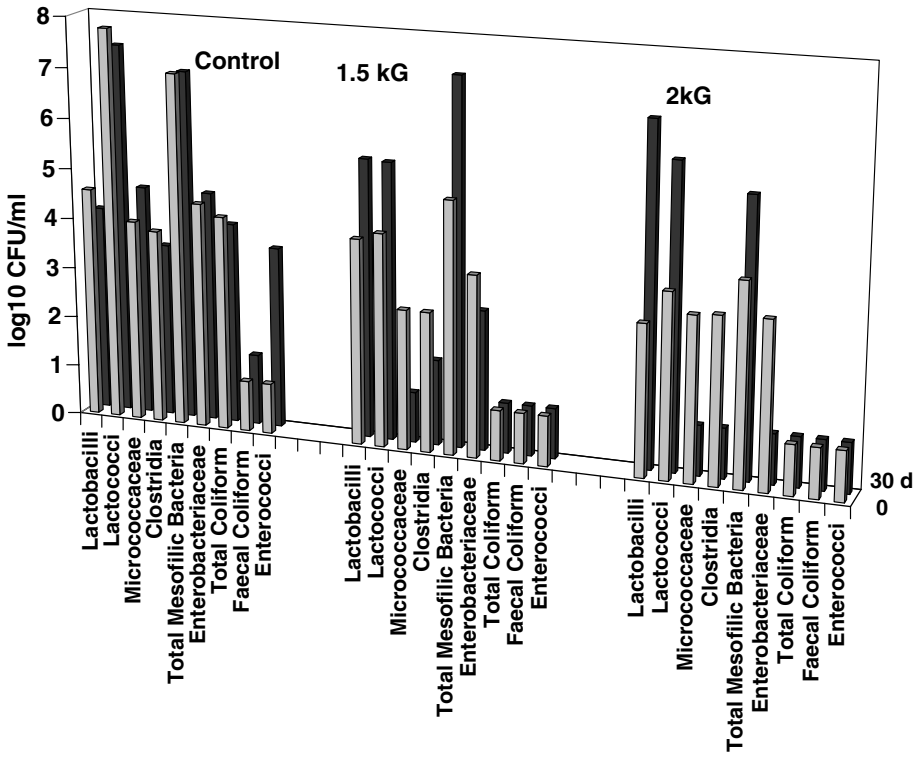

Fig. 7. Graphical representation of microbial count (log10 CFU/g) in untreated (control) and irradiated (1.5 kGy or 2.0 kGy) truffles, at zero and after 30 d of storage at 4 °C.

the decrease of Enterococci, Enterobacteriaceae, and total and faecal coliforms. Variations related to doses were found as to total mesophilic bacteria and Lactococci, which showed a linear decrease related to the intensity of gamma rays applied to the sample. In all probability, treatment with gamma rays generated a damaging effect against some microbial populations; this was evident especially for total coliform bacteria, exhibiting a dramatic decrease of about 3 logarithmic units in comparison to the fresh product. After 30 days, an increase of Lactobacilli, Lactococci and mesophiles and a concurrent loss of Micrococcaceae and Clostridia were evident, these last showing a decrease of 2 logarithmic units. These data demonstrated how gamma rays, although not determining a whole inhibition of all microbial flora present on the sample, caused an evident decrease of unwanted and partially of pathogen bacteria, preserving also the sensory peculiarities of truffles.

13. Polyphenol content

The analysis of total polyphenol content (Table 3), carried out at the beginning and at the end of the storage considered in our experiments, supported the trend observed from the monitoring of peroxides. In fact, the results confirmed that, with a dose of 1.5 kGy, the polyphenol content was constant after 30 days of storage, so it should produce neither considerable biochemical modifications nor, in particular, compounds of degradation, which are notably evident both in the control (5 times higher) and, slowly lower, in the sample irradiated with 2.0 kGy (3.5 higher). At time 0, significant differences among control and irradiated samples were not found. After 30 days, differences ($p < 0.001$) were significant for control and sample treated with 2.0 kGy, whereas it was less significant for 1.5 kGy treated sample ($p < 0.01$). Very significant differences among control and irradiated samples were found ($p < 0.001$). The presence of polyphenols, as known in literature (Booker & Miller, 1998; Benoit, D'Aprano, & Lacroix, 2000) may be expected to vary in vegetable organisms both as reaction against biotic or abiotic stress and during the course of senescence. Among stress factors influencing the polyphenol content, phenylalanine ammonia-lyase (PAL) and polyphenol oxidase or tyrosinase (PPO) activities are the most known. PAL is generally involved in polyphenol synthesis, while PPO catalyzes the oxidation of polyphenols to quinones, which condense to form brown melanin pigments (Long & Alben, 1969; Stussi & Rast, 1981). It was demonstrated in *Agaricus bisporus* mushrooms that γ-irradiation affected some biochemical parameters, such as PAL activity, total phenols content, and PPO activity (Benoit et al., 2000; Beaulieu, Beliveau, D'Aprano, & Lacroix, 1999). Therefore, it has also to be considered that, under stress conditions, mushrooms produced tyrosinase inhibitors, such as dimethyl sulfide (DMS), to slow down the enzymatic browning event (Perez-Gilabert & Garcia-Carmona, 2001). Saggese, Luongo, Fiume, Coppola, and Di Luccia (2005), studying the influence of γ-irradiation on black truffle flavours, found that a dose of 2.0 kGy induced a higher content of DMS at 20 days, which decreased up to 60 days, whereas a dose of 1.5 kGy increased DMS content through the storage. In our experiments, injuries caused to truffles by irradiation should trigger biochemical mechanisms, that should affect polyphenol synthesis by increasing PAL activity and the increase of polyphenol content should indicate the action of PPO; this last activity, probably, could stimulate the production of inhibitors, such as DMS. We observed that the untreated truffle met a normal senescence with a higher microbial count, that induced polyphenol synthesis as cellular defence. After treatment with 1.5 kGy, the lowest polyphenol content was recorded, due to the microbial reduction (Fig. 7), as well as to a low dosage of γ-irradiation and probably to the constant increase of enzyme inhibitors. On the contrary, whereas a dose of 2.0 kGy reduced microbial contamination (Fig. 7) it was less capable, in comparison to treatment with 1.5 kGy, to effectively control the biochemical mechanisms above thought. In conclusion we could assume that the treatment with 1.5 kGy did not induce truffle to biosynthesise these compounds against stress; moreover it also could slow down the natural senescence process in truffle. The preliminary biochemical data and microbial results obtained in this work, as well as scientific references, could constitute a comforting starting basis for using gamma rays to increase the shelf life of stored truffle, and indicate that the extension of this technique to increase the shelf life and safety of "highly perishable" and fresh food products should be well calibrated in the dosage, to avoid injuries to their biochemical, structural and technological qualities.

Table 3
Polyphenol content (nM quercetin equivalent) in untreated and irradiated truffle (1.5 kGy and 2.0 kGy) at the beginning of experimentation and at the 30th day of storage

Polyphenol content (nM quercetin equivalent)			
Time (days)	0.0 kGy	1.5 kGy	2.0 kGy
0	424.5^{A}	$472.3^{a,A}$	$4\ 37.4^{A}$
30	$2145.0^{a,B}$	$621.0^{b,D}$	1515.1^{C}

Total phenolic concentration was performed by spectrophotometric analysis, using the Folin-Ciocalteu colorimetric method. The data are the mean of five independent experiments. The results were submitted to an analysis of variance followed by the Student's test. The degree of confidence was fixed at 95% for all the analyses.
^{a,b} $P < 0.01$.
^{A,B,C,D} $P < 0.001$.

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