ISSN print: 1224-5984
ISSN online: 2248-3942

Rom Biotechnol Lett. 2023; 28(2): 3959-3967
doi: 10.25083/rbl/28.2/3959.3967

Received for publication, October, 25, 2023
Accepted, November, 02, 2023

Original Article

The potential of the radiation technologies to improve the quality of dietary supplements

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Abstract

The use of dietary supplements has much increased over recent decades, principally due to the need to correct dietary deficiencies but also for their supportive role in certain medical treatments. While they are regulated as food, with certain specific regulations and requirements that distinguish them from conventional foods, the manufacturers can choose to adhere to higher standards, that allows them a future upgrade as pharmaceuticals or medical devices. This paper presents the results of exploratory studies for the introduction of gamma irradiation as a method of control of the microbial contamination for egg lyophilizates currently marketed as dietary supplements. Characterization studies were performed with the goal of upgrading the products for the pharmaceutical market. The irradiation treatment improved their microbiological quality by the exerted microbicidal effect and it can be used as long as the other properties (physico-chemical and/or therapeutic) are not affected.

Keywords

avian immunoglobulin, dietary supplements, pharmaceuticals, gamma irradiation, amino acids, microbiological contamination.

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Introduction

Dietary supplements are an important aspect of modern life. Their use has much increased over recent decades, because of their large availability, the need to try to reduce dietary deficiencies but also for their supportive role in medical treatment in certain situations [1].

Egg lyophilizates are used as dietary supplements due to their valuable nutritional profile. They are rich in several essential nutrients, making them a good source of vitamins, minerals, and protein. A particular case is when egg lyophilizates are enriched with immunoglobulins.

Egg yolk immunoglobulins (IgY) have been identified for more than a hundred years, having as their first biological effect the protection of chicks from infection. IgY represents the main antibody produced by plasma cells in the body of the domestic chicken (*Gallus domesticus*). It corresponds to the immunoglobulin IgG produced by mammals.

By inoculating chickens with different bacterial antigens, specific antibacterial IgY can be obtained, which are passively transferred from the hen's serum to the yolk. The process of obtaining IgY from egg yolk is relatively simple and inexpensive.

IgY is a relatively stable protein that can be stored at 2-4 °C. In lyophilized form, IgY activity remains constant for months at temperatures of -20 °C or even for a month at 37 °C. IgY, due to its multiple advantages, compared to IgG derived from mammals, can be used in several fields such as alternative therapies, prophylaxis and acute passive immunization. The effects of passive immunization by oral administration of specific IgY have been demonstrated in several clinical trials [2].

The therapeutic use of the egg lyophilizates with enriched IgY content need proper characterization of the biochemical compounds and, taking into account their intended use, the compliance with microbiological quality requirements.

For products of animal origin, ensuring batch to batch uniformity and microbiological quality is challenging. Lyophilization is known to reduce bioburden, but is not accepted as a sterilization method in the absence of precise quantification of a parameter related to bioburden reduction. Pharmaceutical GMP [3] guidelines call for the use, whenever possible, of a terminal sterilization method. Since thermal or gas (EO) sterilization are not appropriate for the egg lyophilizates, gamma irradiation was taken into consideration.

Radiation sterilization is widely accepted in the production of medical devices (almost 50% of sterile single-use medical devices are sterilized by radiation) and is gaining momentum in the pharmaceutical field. There are specific GMP guidelines for the use of radiation for sterilization of the medicinal products [4]. In case of dietary supplements there is no requirement for sterilization (oral use) but the competitive advantage obtained by introducing gamma irradiation is given by an increase in confidence in the microbial stability of the products for several years (increasing the period of validity/marketing). Furthermore, by proving the sterility of the egg lyophilizates the way is opened for their use as intermediate products in the aseptic manufacturing process.

The current study involves testing selected physical and chemical properties of egg lyophilizates before and after irradiation, as well as microbial characterization. Based on literature data concerning the behavior of egg products to irradiation [5, 6] the analytical methods taken into consideration are HPLC [7,8], for determining the amino acid profile and thermal analysis [9÷14], for determining the water content. The microbiological tests include determination of the total microbial load before and after irradiation and detection of specific microorganisms.

Materials and methods

Five types of samples (lyophilized powder or liquid extract) from products named as "Imunyze Wellness" (Wellness), "Imunyze Complete" (Complete), "Imunyze Defence" (Defence), "Imunyze Health" (Health) and "Imunyze Healthcare" (oral spray, 5 mL bottle) (Healthcare), all manufactured by Health Laboratory SRL, were physically, chemically and microbiologically characterized, before and after irradiation.

Protein hydrolysis

In order to identify the amino acid profile (AA), a digestion of the samples was carried out in order to hydrolyze the proteins from the freeze-dried samples, to break the peptide bonds and increase the concentration of free amino acids. The Ultrawave Single Reaction Chamber Microwave Digestion System equipment from Milestone (Italy) was used to hydrolyze the samples as follows: 50 mg of each sample were subjected to acid hydrolysis in the vapor phase assisted by microwaves - 6.5 mL hydrochloric acid 6 N , at a temperature of 160°C, for 40 min, at a power of 1000W and an initial pressure of 40 bars in an inert nitrogen atmosphere (N_2, purity 5.0 or 99.999%). After digestion, the samples were diluted to 15 mL, from which approximately 2 mL were taken in Eppendorf tubes and centrifuged at 9000 RPM. Then 0.1 mL was taken from the supernatant and diluted to 2 mL with deionized water in glass vials for HPLC sample injection. In the HPLC injection method, a volume of approximately 1 µL was taken, which was subsequently subjected to a pre-column derivatization method, using o-phthalaldehyde for the derivatiza-

tion of primary amino acids and fluorenylmethoxycarbonyl chloride for the secondary ones.

Chromatographic system

The experiments were carried out using liquid chromatography - HPLC (High-performance liquid chromatography), using an Agilent (USA), type 1260 INFINITY II system, with the following configuration: degasser (G1379A); quaternary pump (G7111B); sample thermostat (G7129A), column thermostat (G7116A); UV-Vis detector (DAD) (G7115A). The chromatographic separation was performed on an AdvancedBio AAA column, having 4.6 x 100 mm and particle size of 2.7 µm, thermostated at 40°C. The mobile phase consists of a mixture of aqueous solution of 10 mM Na_2HPO_4 and 10 mM $Na_2B_4O_7$ brought to pH 8 (solvent 1) and methanol/acetonitrile/water (solvent 2). Gradient elution was applied, using the following profile: at 0 min - 2% solvent 2, for 0.5 min, from min 0 to 14 - linear increase to 55% solvent 2, followed by another linear increase until min 14.2, 100% solvent 2, from min 14.2 to 16.5 - jump back to 2% solvent 2, with a re-equilibration period of 2 minutes. The used flow was 1.5 mL/min, and the UV-Vis spectra were simultaneously recorded in the range 190-400 nm, with a frequency of 0.03 min and a resolution of 2 nm. Four wavelengths were monitored simultaneously to generate the chromatograms (210, 241, 260 and 330 nm).

Water content tests by thermal analysis (TG)

In the determinations made by thermal analysis, a TG/DSC simultaneous thermal analysis equipment type STA 409 PC Luxx, produced by Netzsch Geratebau GmbH, with the following configuration was used:

- purged and thermostated Sartorius microbalance, maximum mass of 18000 mg, mass resolution of 0.002 mg;
- TG/DSC (sample crucible and reference crucible) sample holder with type S thermocouple (Pt/Rh, ± 3K); - for DSC, the comparative heat flow is measured between the crucible (cell), the test sample and the reference crucible, by applying a constant heating rate; in practice, the calorimetric technique with differential thermal flux (heat-flux DSC), described above, is used;
- alumina furnace: temperature range 20 - 1500°C, heating speed 0.1 - 50 K/min, temperature control by thermocouple type S;
- dynamic inert atmosphere (N_2), gas with a minimum purity of 99.999%, flow rate 100ml/min: microbalance purge 50 ml/min, furnace purge 50 ml/min from the level of the thermal radiation shield; - aluminum DSC crucibles with perforated lid (25 µL);

Mass of the test specimen: The test specimen consisted of 10 mg of lyophilized powder transferred into a 25 µL aluminum crucible, with a perforated lid, manually closed and weighed on an analytical balance (5 decimals per gram).

Temperature program: heating rate of 10 K/min from room temperature (RT) to 590°C, followed by a 10 min isothermal segment.

The temperature (°C) and enthalpy (J/g) calibration curves used in the test were drawn through 3 experimental points using high-purity metals (In, Bi and Zn) as reference materials, in the same experimental configuration and the same operating parameters as in the method described above for the test specimen. The processing of the experimental data and the generation of the calibration curves used in the analysis was carried out according to the specifications of the instrument manufacturer.

The sampling was carried out by Health Laboratory Ltd., according to its internal procedures. The sampling of test specimens was carried out with disposable plastic spatulas, transferring a lyophilized product powder into 25 µL aluminum crucibles, with a perforated lid, reproducibly closed with a manual press. For each sample, two samples- the reference (as received from the Ltd company) and the irradiated (decontaminated) sample were tested.

Conditioning method of the tested specimen: before and after irradiation, these were kept in a refrigerator at 8 °C. During the irradiation, the samples stayed at the temperature of the irradiation chamber, which is located around 30 °C. The samples removed from the refrigerator were reweighed on the analytical balance and transferred directly to the TG/DSC sample holder at room temperature, after which the oven was closed and the temperature program started as soon as possible, in order to minimize the unrecorded time of unbound water.

Microbiological characterization

The microbiological characterization of lyophilized samples included determination of total aerobic microbial count, total fungi and detection of some pathogens (*Escherichia coli, Salmonella* sp., *Staphylococcus aureus*), before and after irradiation of the samples.

Culture media and diluents: Tryptic Soy Agar (TSA) for Total Aerobic Microbial Count, Sabouraud Chloramphenicol Agar (SABcfa) for fungi (Yeasts and Mold), Buffered sodium chloride-peptone with 0,1% (w/v) Tween 80 (APS 0,1 % Tw) as diluent.

Reference test strains used for suitability of the microbial contamination test method: *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027, *Candida albicans* ATCC 10231, *Aspergillus niger* ATCC 16404.

The detection of *Escherichia coli*, Presence/g, *Salmonella* sp., Presence / 10 g, *Staphylococcus aureus*, Presence/g were done by culturing on enrichment non-selective culture media (Tryptic soya broth, TSB) and subculturing on selective media (MacConkey broth, Mckb, MacConkey agar – Mcka, Rappaport-Vassiliadis (RV) enrichment broth, Xylose Lysine Deoxycholate Agar, Mannitol salt agar).

The determination of microbial contamination was carried out in accordance with the requirements of the reference document European Pharmacopoeia, 10th edition, chapter 2.6.12 Microbiological examination of non-sterile products, respectively chapter 2.6.13 Microbiological examination of non-sterile products. Test for specified microorganisms [15–17].

Gamma irradiation

Gamma irradiation of the test samples was performed at the Gamma Chamber GC-5000 Research Irradiator of the IRASM Technological Irradiation Center from NIPNE. The target dose was 3 kGy. The dose was chosen to achieve the product quality control requirements for industrial scale treatment with ionizing radiation, based on preliminary tests (organoleptic and biochemical) performed by Health Laboratory Ltd. (not reported here).

Results and discussion

Amino acids profiling

For the quantification of amino acids from these samples, standard solutions of amino acids were prepared in 0.1 N hydrochloric acid at five different concentrations and kept at a temperature of 4°C. To check linearity and reproducibility, concentrations between 210 and 0.6 ppm were prepared by repeated dilutions on the day of analysis.

In Figure 1, an example of linearity domain is represented, having lysine in concentrations ranging from 0.1827 to $0.1827*10^{-2}$ mg/mL. Furthermore, in all the 17 analyzed amino acids, the correlation coefficient (R^2) has values higher than 0.997.

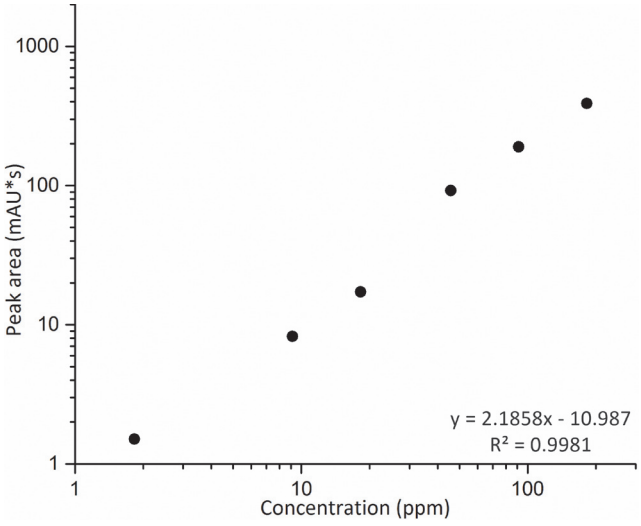


Figure 1. Lysine's calibration curve.

Amino acids from freeze-dried samples

In order to optimize the sample mass for achieving a proper digestion and thus protein hydrolysis, one of the four solid matrices was chosen, "Imunyze Health", five different masses were taken into consideration (5, 10, 50, 100 and 200 mg), subjected to the digestion process and analyzed. For the masses of 5 mg and 10 mg, the amino acids could not be quantified (below LoQ – limit of quantification), however, in Figure 2 it can be seen that each identified amino acid (for the samples of 50, 100 and 200 mg) - represented as a percentage of the total mixture - the fact that the digestion process is stable, apart from the amino acids serine, threonine and proline, in which case more tests are needed in a subsequent method validation. Thus, the mass of 50 mg was selected as the ideal mass to be taken into analysis due to the fact that it allows a good identification and quantification of the analytes. When it comes to the digestion method, the consumption of reagents is reduced 7 times in comparison with the ones found in the literature, while the number of simultaneously processed samples is the maximum for this class of digesters (use of 15 mL ampoules with 15-positions rack).

The amino acid profiles from the four types of non-irradiated lyophilized samples are represented comparatively in Figure 3(a), by their chromatograms at a wavelength of 330 nm The presence of the same 16 essential and non-essential amino acids can be observed, where certain peaks are coming from the solvent, although with small variations in intensities and in the peak area. It should be mentioned that certain minor variations in concentration may also come from

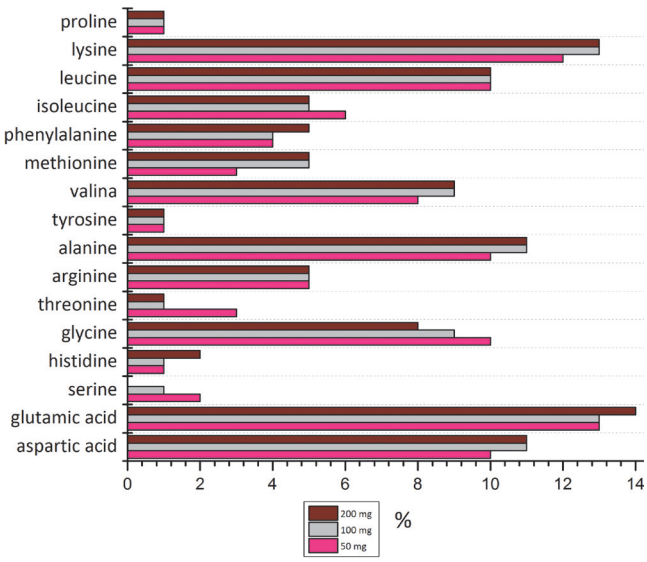
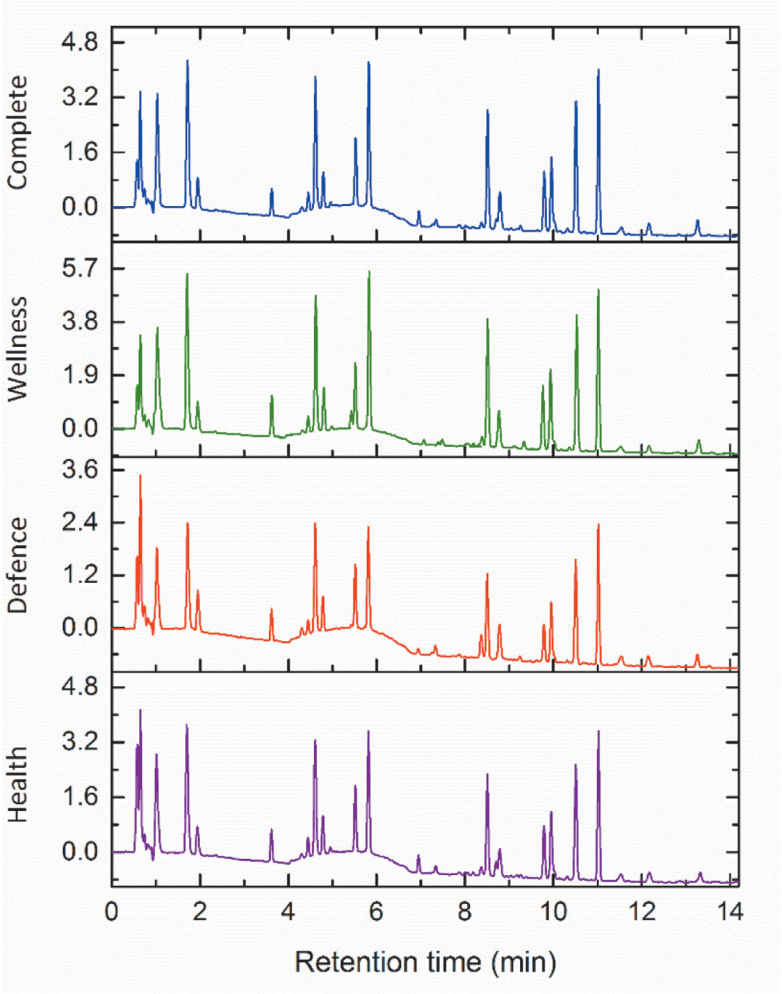


Figure 2. The composition of the samples having 50, 100 and 200 mg from "Imunyze Health" represented as percentage.



(a)

Amino acid	Retention time (min)	Mw (Da)	Structure
aspartic acid	0.99	133.103	
glutamic acid	1.61	147.129	
serine	3.56	105.093	
histidine	4.40	155.155	
glycine	4.55	75.067	
threonine	4.73	119.119	
arginine	5.49	174.201	
alanine	5.76	89.093	
tyrosine	6.90	181.189	
valine	8.43	117.146	
methionine	8.63	149.211	
phenylalanine	9.70	165.189	
isoleucine	9.86	131.173	
leucine	10.42	131.173	
lysine	10.95	146.188	
proline	12.13	115.131	

(b)

Figure 3. Comparative chromatograms for the four types of lyophilized samples (a), the studied amino acids and their retention times (b).

the inhomogeneity of the samples. The identification of the analytes was carried out on the basis of standard solutions of amino acids, retention time and in certain cases, on the basis of the UV-Vis spectrum, which can be quite similar for most amino acids. The structure of the analyzed or identified compounds, their molecular masses, as well as their retention time is presented in Figure 3(b). On the other hand, 1 g was also sampled from the IH-s spray and analyzed, but there were no quantifiable amino acids.

Figure 4 shows of the four types of freeze-dried, non-irradiated samples, represented as a percentage of the total amino acid content. Their variability can be noticed in each sample, observing discrete variations (probably falling within the standard deviation of the method) between matrices for the same analyte. AA ratios are similar to those reported in literature [18].

Regarding the differences before and after irradiation, in Table 1 the most important percentage decreases in concentration are marked in green in the case of histidine, arginine, tyrosine, methionine and phenylalanine, while the significant increases in the concentrations of glycine, alanine, valine and lysine are marked in orange. The assumption that these

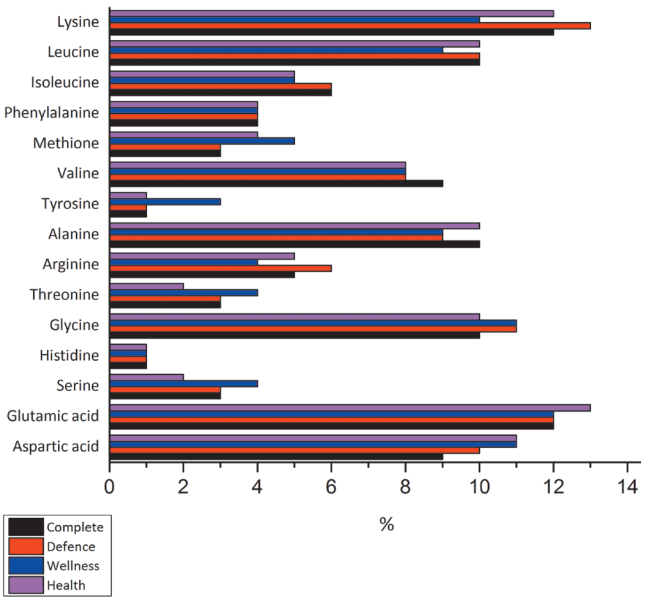


Figure 4. The percentage of amino acids in the freeze-dried samples: Complete, Defence, Wellness and Health.

Table 1. The difference between the amino acids profiles before and after irradiation.

	Complete	Complete*	Defence	Defence*	Wellness	Wellness*	Health	Health*
aspartic acid	8%	9%	8%	10%	9%	11%	9%	11%
glutamic acid	12%	12%	11%	12%	13%	12%	12%	13%
serine	3%	3%	4%	3%	3%	4%	4%	2%
histidine	6%	1%	7%	1%	5%	1%	6%	1%
glycine	4%	10%	4%	11%	4%	11%	4%	10%
threonine	4%	3%	5%	3%	4%	4%	4%	2%
arginine	8%	5%	8%	6%	7%	4%	8%	5%
alanine	6%	10%	5%	9%	6%	9%	6%	10%
tyrosine	4%	1%	6%	1%	4%	3%	5%	1%
valine	7%	9%	6%	8%	7%	8%	7%	8%
methionine	5%	3%	5%	3%	5%	5%	4%	4%
phenylalanine	7%	4%	6%	4%	7%	4%	7%	4%
isoleucine	7%	6%	7%	6%	7%	5%	7%	5%
leucine	9%	10%	9%	10%	9%	9%	9%	10%
lysine	10%	12%	10%	13%	9%	10%	10%	12%
Total	100%	100%	100%	100%	100%	100%	100%	100%

* Irradiated samples

variations in the content of amino acids after irradiation are due to the transamination reaction with amino transfer is not very viable because lysine - one of the amino acids that does not participate in this type of biochemical reaction - also undergoes concentration variations. Regarding the metabolism of amino acids, transamination reactions are reversible, their reversibility being dependent on the concentration of reactants and reaction products, in which an amino group is removed from an amino acid and transferred to keto-acid acceptors, such as pyruvic acid, from eggs, in order to generate a version of amino acids derived from the keto-acid and a keto-acid generated from the original amino acid.

The variation in the content of amino acids in the irradiated lyophilized samples could be determined by the cleavage of the backbone, thus being transformed into other amino acids, where the newly formed radical is able either to recombine, to be digested into smaller fragments, otherwise it was not separated and identified using this chromatographic method. Thus, in the case of histidine, the imidazole side chain in the structure could have suffered a breakage induced by gamma irradiation, leaving only the α-amino group and the carboxylic group, thus transforming into glycine, therefore explaining the increase in the concentration of the latter and the decrease of histidine's concentration.

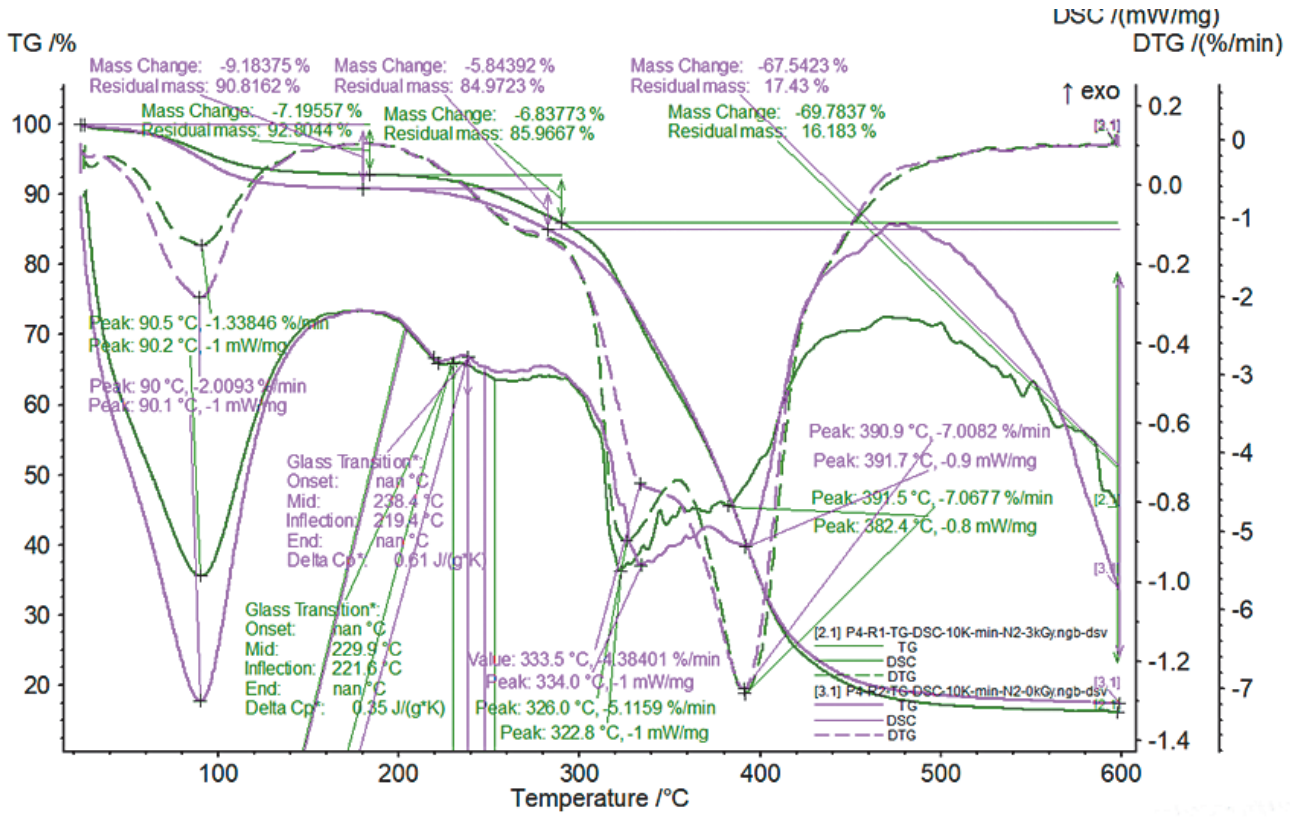


Figure 5. TG/DSC curves in nitrogen, 10K/min, for IMUNYZE HEALTH (P4), batch no.: 1/2023.

Table 2. Water loss and glass transition (ovalbumin) by thermogravimetry (TG).

Sample	First step (% mass)	Water loss		Kinetics (%/min)	Kinetics (mW/mg)	Ovalbumin Glass	
		Temp (DTG)	Temp (DSC)			Temp (MID DSC)	Delta Cp (DSC) J/(g*K)
P1(0kGy)	12	89	91	2.3	1.4	232	1.0
P1*(3kGy)	13	81	87	2.5	1.6	231	0.8
stdev	2	3	1	0.5	0.1	1	0.1
P2(0kGy)	6	87	90	1.3	1.0	234	1.1
P2*(3kGy)	9	81	84	1.9	1.4	231	0.9
stdev	1	10	6	0.1	0.1	1	0.1
P3(0kGy)	10	87	90	2.1	1.3	230	0.8
P3*(3kGy)	10	89	91	2.2	1.3	231	0.6
stdev	1	7	6	0.2	0.1	1	0.2
P4(0kGy)	9	87	89	2.0	1.0	229	0.4
P4*(3kGy)	8	83	86	1.6	1.0	229	0.4
stdev	1	11	8	0.2	0.1	1	0.1

* Irradiated samples

Similarly, the guanidino group from arginine could also suffer a breakage of the covalent bond generated by ionizing radiation, leading to an increase in the concentration of alanine, while the increase in the concentration of valine can be determined either by breaking the phenolic functionality from tyrosine, of the S-methyl thioether side chain from methionine or the phenyl group from phenylalanine.

Water content tests by thermal analysis (TG)

In order to define suitable processing, storage conditions, shelf life and use of a product, it is required to determine the product's water content and the mechanism of water–solid interactions. Therefore, in our study there were performed thermal analysis, the TG/DSC curves in nitrogen, 10K/min, for IMUNYZE HEALTH (P4, batch no.: 1/2023) being presented in Figure (5) and the results for all the tested samples summarized in Table 2.

The composition in unbound (absorbed) and bound water was determined to be between 6 - 13% for the analyzed samples - Step 1 TGA - maximum speed DTG/DSC between 81 and 91°C. It does not change significantly before and after irradiation, taking into account the variability of the samples. Also, the maximum peak position does not change significantly.

Beside the water loss peak, on the DSC thermograms can be observed the decomposition peak and a glass transition which was attributed to Ovalbumin. From the DTG and DSC peaks it appears that the treatment by gamma irradiation at 3 kGy does not significantly affect the structure of the protein, identified as Ovalbumin, having a glass transition temperature of approximately 230-231°C.

Table 3. Microbiological test results for non-irradiated and irradiated samples.

No	Samples	Non irradiated CFU / g		Irradiated CFU / g	
		TAMC	TYMC	TAMC	TYMC
1.	Health	2.4 x 104	35	10	< 10
2.	Complete	85	102	< 10	< 10
3.	Wellness	< 10	< 10	< 10	< 10
4.	Defence	1.7 x 104	60	20	< 10

TAMC *– Total aerobic microbial count*

TYMC *– Total yeasts and mould count*

Microbiological characterization

The results of the microbiological tests are summarized in table 3.

After analyzing the results obtained for the irradiated products, it is observed that the microbiological parameters are within the maximum limits allowed for pharmaceutical products (for example, the microbial load ID and IH decreased by 2 $\log_{10}$, to a total number of aerobic microorganisms TAMC < 10^2 CFU / g). For the other samples, the bioburden after irradiation was below the detection limit. The pathogenic microorganisms were not detected either before or after irradiation. In this context, the treatment with ionizing radiation improves the microbiological quality of products.

Conclusions

An HPLC-DAD chromatographic method was developed for the qualitative and quantitative analysis of the amino acid profile in protein powders/spray, based on lyophilized egg, as well as for evaluating the variation of their concentrations following the treatment using ionizing radiation. The method is fast, sensitive and selective; however, only the amino acids from the studied reference materials were reported. For more advanced fundamental research related to the changes in the molecular structure of the amino acids from the investigated products, it would be necessary to develop an LC-MS technique.

The digestion process was stable, the investigated samples show the same amino acid profile, with slightly differ-

ent distributions depending on the matrix. Certain analytes such as histidine, glycine, arginine, alanine, tyrosine, valine, methionine, phenylalanine and lysine, show significant differences (>1%) after gamma irradiation, both increasing and decreasing the concentration. However, to validate that these changes are produced by the treatment with ionizing radiation, more in-depth radiation qualification studies must be carried out, with an extended range of target doses and a statistically significant number of replicates included in the analysis. To optimize the qualitative analysis, but especially the quantitative one, additional experiments are needed, as well as the validation of the method.

For the matrix tested by simultaneous TG-DSC thermal analysis, no significant differences could be identified in both the TGA and DSC signals between the irradiated (target dose of 3 kGy) and non-irradiated samples.

The treatment with gamma radiation at low doses (≤ 3 kGy) improves the microbiological quality of these products through the microbicidal effect exerted and can be used as long as the other properties (physico-chemical and/or therapeutic) are not affected.

The sterility can be achieved by the increase of the irradiation dose, while the properties of interest for the intended use can be maintained by using other physical means (for example: high dose rate, irradiation in modified - non-oxidative atmosphere, or at ultra-low temperatures – dry ice).

Acknowledgments

This work was supported by the project POC-G, ID: P_40_276 (SMIS 107514) “GAMMA-PLUS” - Enhancing competitiveness through innovation and improving manufacturing processes with gamma irradiation technology - subsidiary project no 61/2023 funded by European Regional Development Fund (FEDR), Minister of European Funds in Romania (MFE) and co-funded by Health Laboratory Ltd. Core and IOSIN Programs are supporting IRASM gamma irradiators and laboratories of NIPNE.

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