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DSC AND EPR STUDY OF THE EFFECTS OF GAMMA RAYS UPON CARNOSINE AND L-ALANYL-GLYCINE

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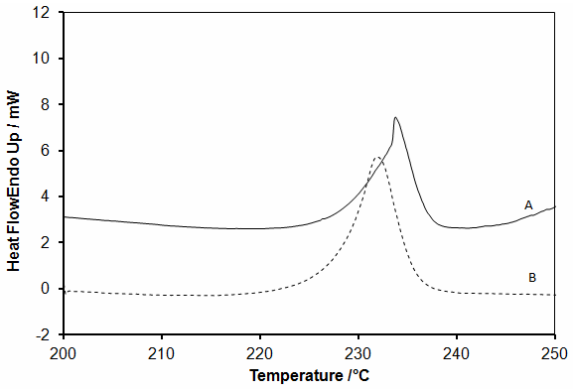
The differential scanning calorimetric (DSC) technique has been used in order to investigate the thermal behavior of non-irradiated and irradiated carnosine (β-alanyl-L-histidine) and L-alanyl-glycine. The phase transition points (melting) and decomposition processes occurring by heating were evidenced by DSC thermograms in the temperature range between ambient to 250 ^{0}C. The corresponding enthalpies were calculated. The effects of gamma rays upon thermal parameters and their variation as a function of dose were emphasized.
The radiolysis of carnosine (β-alanyl-L-histidine) and L-alanyl-glycine, under γ-rays, in solid polycrystalline state was performed at room temperature. The Electron Paramagnetic Resonance (EPR) spectra recorded after radiolysis allowed the identification of three paramagnetic centers. A possible mechanism of formation of the three radical species is suggested.



INTRODUCTION

The mechanism of ionizing radiation interaction with the substances that present biological activity is similar to the mechanism of interaction with the substances without biological activity, but what distinguishes the two actions are the consequences to which they lead. It is well established that ionizing radiations react with the substance both directly and indirectly. [1]

The direct action leads to atoms and molecules ionization and excitation. The action of radiations on weak bonds organic combinations, such as biological media, has as a consequence the breaking of these bonds.

Due to the high water content (~85%) of biological environments, ionizing radiations also produce indirect effects by splitting the water molecules into radicals. In this context it should be noted that further reactions involving these radicals and those molecules depend on a number of factors such as: their chemical structure, spatial arrangement of the atoms, the presence or absence of the oxygen etc. It must be specified that irradiation of biological activity compounds produces chemical modifications with consequences that are not present in the case of other substances irradiation. These modifications can cause finally alteration of the functions that living cells accomplish in tissue occurring the so-called radiations biological effect.

Irradiation of solid state biomolecules with ionizing radiations provides extremely interesting and varied information on the effect of high energy radiation, such as: direct chemical transformations

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produced by radiations, information regarding chemical auto radioprotection mechanism of living matter, explanation of different radiosensibilities of molecules correlated with their structure, and so on.

Biological molecules, such as proteins and DNA, are very sensitive to the ionizing radiation and this problem has a fundamental importance in biology and medicine (*e.g.* mechanism of mutagenesis and radioprotection).[2] Radiation damage manifests in the disturbance of a long range crystalline or supramolecular order[3-6] and in chemical modification of studied systems through free radical formation[7-9] or mass loss.[10-12] For a better understanding of the effect of ionizing radiation on biological materials it is useful to investigate the influence of ionizing radiation on simple biological molecules such as amino acids.

Amino acids are structural units that make up proteins and are the simplest organic molecules with biological importance, therefore serving as model systems to study degradation by irradiation of peptides and proteins. In solid state amino acids present zwitterion structure with a protonated amino group and a deprotonated carboxyl group.

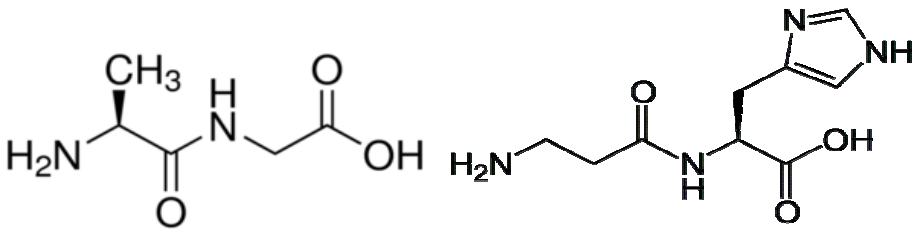
The first transformation process which is supposed to occur under the action of radiation in molecules is decarboxylation preceded by a proton transfer from amino group to carboxylic group (zwitterion → neutral molecule transition).[2]

Under the action of ionizing radiations, in amino acids molecules occur numerous transformations accompanied with break bonds and formation of a new ones, in this case deamination processes with NH_3 removal and formation of corresponding keto acids, decarboxylation with CO and CO_2 removal, with opening cycles (proline), with HCN formation etc. In the case of peptides, besides similar processes with those occuring in amino acids, it is also produced the breaking of peptidic bonds between the constituents amino acids.[13]

In literature there are numerous articles since 1960 by the pioneering works of Gordy, Iwasaki, Miyagawa and Morton[14-18] on amino acids and peptides irradiation in which the method of study was especially electronic spin resonance (RPE).[19-23] Recent studies were also conducted on behavior of the amino acids under irradiation.[2,13,23-27] Most of these studies have used electron spin resonance (RPE) to highlight the radicals formed by irradiation and the effects on structure and properties of studied amino acids. Differential scanning calorimetry (DSC) became an useful method for the study of the denaturation of non-irradiated and gamma irradiated amino acids[24,28-33] permitting the detection of the differences between decomposition of non-irradiated and gamma irradiated compounds.

In our previous contributions we used differential scanning calorimetry to study the influence of gamma radiations on some amino acids, namely the isomers of aspartic acid[34], monohydrated asparagines[35] and monohydrated and anhydrous asparagines.[36] The variation with irradiation dose of the melting/decomposition temperatures and of the enthalpies corresponding to the recorded thermal effects was emphasized and these effects were correlated with transformations caused by radiation.

The aim of this article is to extend these studies to more complex biomolecules, namely two peptides: L-alanyl-glycine and carnosine (ß-alanyl-L-histidine) respectively. Differential scanning calorimetry technique is used to track the thermal behavior in the temperature range between ambient and the melting/decomposition point of the non-irradiated and irradiated forms of the two peptides L-alanyl-glycine and carnosine (ß-alanyl-L-histidine). The structural formulas of the two peptides are:



L-alanyl-glycine ß-alanyl-L-histidine (carnosine)

Scheme 1 – The chemical structure of the studied dipeptides.

L-Carnosine (2-[(3-aminopropanoyl) amino]-3-(1*H*-imidazol-5-yl) propanoic acid) is a naturally occurring dipeptide of considerable biological and pharmacological importance. It is composed exclusively of covalently bonded alanine and histidine residues, and is found in the brain, heart, skin, muscle and gastrointestinal tissues.[37] Carnosine has a radioprotective action of tissues. This action is related to its affinity for free radicals, mainly for hydroxyl, and its ability to interact with the products of lipid peroxidation, preventing cell membrane damage. Carnosine acts as a great hydroxyl radical scavenger, protecting biological structures and playing an important role in the anti-oxidative process.

L-Alanyl-glycine is a dipeptide found in human urine. It is a breakdown product from endogenous and exogenous proteins.[38]

EXPERIMENTAL

1. Materials

Commercially available polycrystalline powders of carnosine (Fluka, purity ≥99.0%) and L-alanyl-glycine (Fluka, purity>99%) were used.

2. Methods

Irradiation

A source with an activity of $3 \cdot 10^{13}$Bq and a dose rate of $1.05 \cdot 10^{2}$Gy h^{-1} was used for γ-irradiation of carnosine and L-alanyl-glycine at room temperature. The exposure doses were between 0.3 and 1.47 kGy. The studied compounds were white fine crystallite powders prior to irradiation.

Differential scanning calorimetry

A Perkin Elmer power compensated DSC (model 8500) was used for the measurement of the enthalpies of the processes occurring during heating (fusion and decomposition). The calorimeter was calibrated with indium ($\Delta_{fus}H = 28.46$ J g^{-1}). The areas of the peaks corresponding to the standard and studied substances were used to calibrate the instrument and calculate the thermal effects of the studied peptides. Aluminium sealed pans for samples which decompose or sublime were used.

The DSC experiments were done at a heating rate 4^0C/min and by using nitrogen with purity > 99.996% as carrier. The heat flow curves were processed with Pyris Software for Windows, calculating both the thermal effects and the purity.

Electron Paramagnetic Resonance

The EPR spectra were recorded with an EPR spectrometer ART 5 (IFIN-Magurele) operating in the X band of 100 kHz modulation Mn^{2+} ion, while CaO matrix was used as a standard for determining "g" factor.

Thermogravimetry

The weight loss was measured using a TGA SETSYS EVOLUTION 17 instrument. The measurements were performed between ambient and 300^0C at a heating rate of 4^0C/min under argon flow using alumina crucibles.

RESULTS AND DISCUSSION

Dipeptides, compounds made up of two α-amino acids, are the smallest unit of protein chains. For this reason, dipeptides allow to study the reciprocal influence of different α-amino acids.

Like most amino acids, these dipeptides having an acidic and an alkaline group one neutralize each other intra molecular performing a zwitterion structure.

The electrostatic interaction between positively charged amino groups and negatively charged carboxyl group are always accompanied by N-H…..O hydrogen bonds and are such as to maximize the hydrogen bonding possibility. The EPR spectra of the two dipeptides irradiated at room temperature in polycrystalline solid state were recorded (Figs. 1 and 2). After L Ala-Gly and β Ala-LHis irradiation no changes were observed in their organoleptic properties.

In order to explain the EPR spectrum structure, in Scheme 2 is presented the radiolysis mechanism of L Ala-Gly.

It is fully established that the primary process in the solid state radiolysis of amino acids involves the expulsion of an electron from an oxygen atom of the deprotonated carboxyl group.[13]

Direct ionization of L Ala-Gly molecule, leads to the L Ala-Gly COO^- radical-cation formation, called primary oxidated species. L Ala-Gly COO^- radical- cation having excess positive charge, being unstable, spontaneously decomposes in two ways. One way of decomposition is CO_2 expulsion accompanied by R_1 radical formation, a stable species, identified in the EPR spectrum (Fig. 1).

The decarboxylation process was also proved in the literature by highlighting CO_2 using gas chromatography method of irradiated amino acid.[23]

L Ala-Gly COO^- radical having excess positive charge spontaneously deprotonates at the amino group and forms the unstable L Ala-Gly COO^- radical.

The expelled proton from NH_3^+ group is the one which builds the shortest hydrogen bond with a neighbor carboxyl group. The deprotonation process is produced because the protons of the NH_3^+ amino group build three hydrogen bonds

with neighbor carboxyl groups. The resulted radical after deprotonation being unstable, decomposes through decarboxylation and forms the R_2 radical, similarly with R_1.

In conclusion, the oxidation mechanism transforms the unstable radical $\text{L Ala-Gly}(\text{COO})^{\bullet+}$ through two decomposition processes in the R_1 and R_2 species stable at room temperature and identified in the EPR spectrum from Fig. 1.

The electron eliminated during the primary radiolytic process, gradually loses its kinetic energy and, after thermalization, it is captured by another aminoacid molecule with formation of the $\text{L Ala-Gly}(\text{COO})^{\bullet-}$ radical-anion. In order to compensate the negative charge excess, an oxygen atom of the carboxyl group captures a proton.

The transferred proton comes from the NH_3^+ group of a neighbor molecule, close to the radical which performed the shortest hydrogen bond. The radicalic entity $\text{L Ala-Gly}(\text{COOH})^{\bullet-}$ being unstable at room temperature, spontaneously deaminates and forms the paramagnetic species $\text{L Ala-Gly}(CH_3\text{-}\dot{C}H)R_3$, stable at room temperature and having a signal in the EPR spectrum.

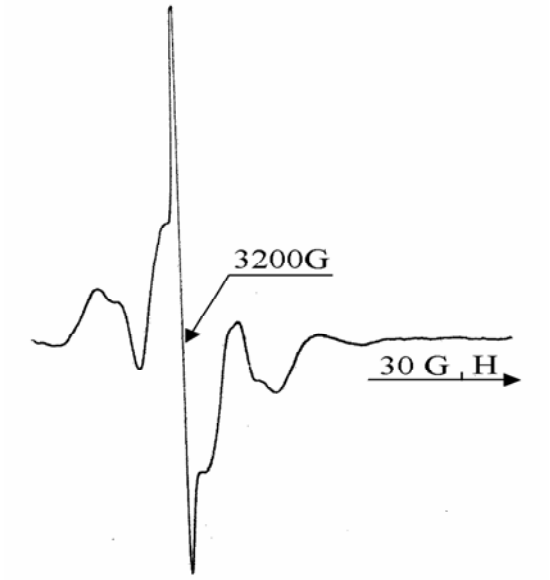


Fig. 1 – The EPR spectrum of L Ala-Gly irradiated with a 2 kGy dose.

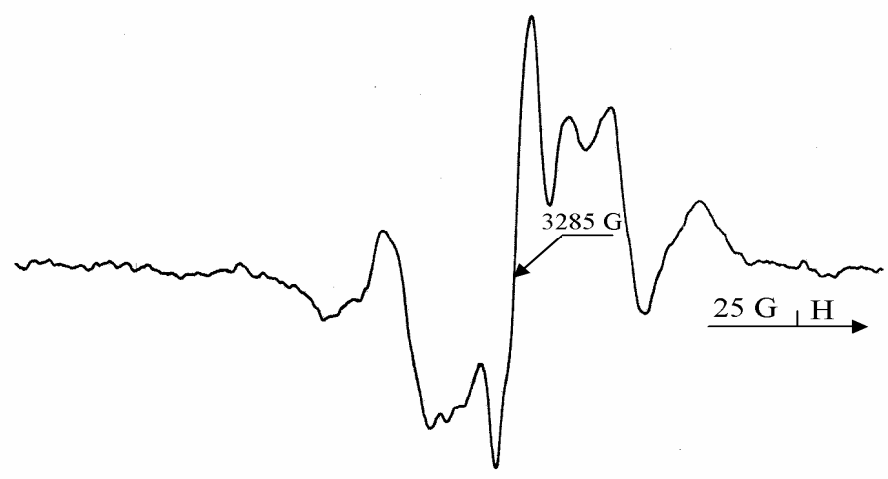
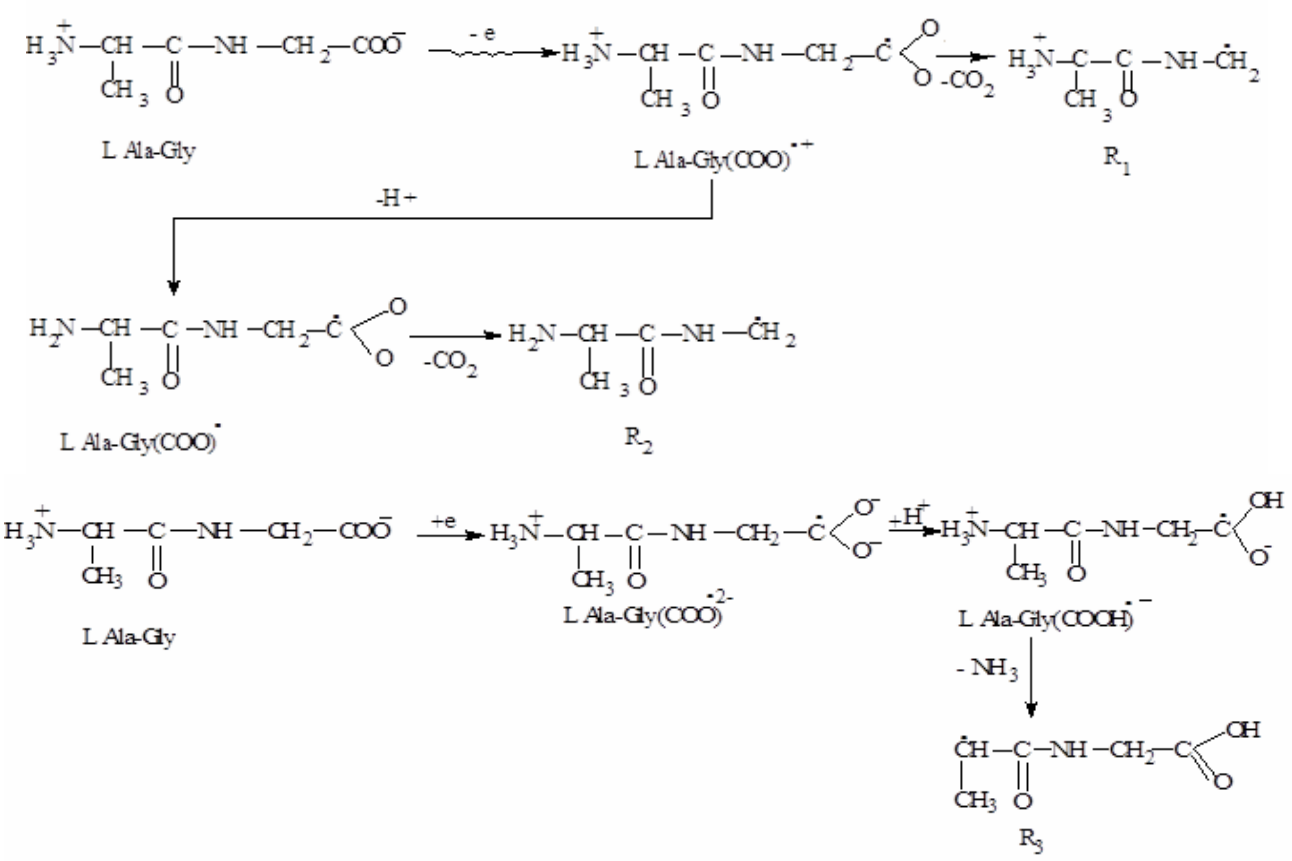


Fig. 2 – The EPR spectrum of β Ala-LHis irradiated with a 2 kGy dose.



Scheme 2 – The mechanism of the L Ala-Gly radiolysis process.

It should be noted that the process of NH_3 elimination involves neutralization of the electric charges, a positive one of the amino group and a negative one of the carboxyl group.

In the case of R_1 and R_2 radicals resulted from decarboxylation, the odd electron interacts with two α equivalent protons and produce the triplet hyperfine structure recorded by the central signal in the EPR spectrum (Fig. 1).

R_3 radicalic species resulting from deamination shows a hyperfine coupling with an α proton and three β equivalent protons. The two large lateral components of Fig. 1 spectrum having a splitting tendency belong to this radicalic entity.

In conclusion, the spectrum from Fig. 1 recorded at L Ala-Gly irradiation in polycrystalline solid state positioned at 3200 G and a total width ΔH_{pp}=57 G is due to the overlapping spectra of the radicals resulted from decarboxylation and deamination. The formation of the three R_1, R_2 and R_3 radicals presented in the previous mechanism is in agreement with the results obtained by Makino and Riesz[39] using 2-methyl-2-nitrosopropane (MNP) trapping method. In the study of Makino and Riesz[39], the solid sample of L Ala –Gly was dissolved after irradiation in aqueous solution with MNP. L Ala-Gly radicals were captured and converted into nitroxide stable radicals. Those radicals were separated by liquid chromatography and then identified in the EPR spectra.

A complex spectrum having a broad triplet centered at 3285 G and a width of 103 G (Fig. 2) was recorded in the case of carnosine solid state irradiation.

Carnosine radiolysis mechanism is similar to L Ala-Gly one, and the radical formation occur simultaneously in two ways: oxidation and reduction. The similarity of the two oxidation and reduction proceses between solid state irradiated L Ala-Gly and carnosine is potentially due to their related molecular structure.

The primary oxidized species is the radical centered on the carboxyl group and, being unstable, decarboxylates and forms the stable $(\text{-NH - }\dot{\text{C}}\text{H - CH}_2\text{ –})$ radical.

The radiolysis process includes the formation of the radical-anion, the reduced species, complementary to the oxidized species. This radicalic entity being unstable, spontaneously

deaminates and forms the ($\dot{C}H_2$-) radicalic species, stable at room temperature. Due to the overlapping spectra of the two radicals, Fig. 2 spectrum is complex. The intense central triplet belongs to the radical produced through deamination due to the odd electron interaction with two equivalent protons. In the radical formed through decarboxylation, the even electron interacts with an α proton, the nitrogen atom and two β protons, their spectral lines overlap.

In conclusion, on solid state irradiation of the two dipeptides, the specific amino acids reactions occur. Accumulation with the absorbed dose of the radicals formed on L Ala-Gly irradiation is shown in Fig. 3, representing the intensity of the central line (arbitrary units) as a function of irradiation dose.

One may be noticed from Fig. 3 that the signal intensity increases with the irradiation dose initially linear, then the increase becomes slower, the curve reaches a maximum and then the radical concentration starts to decrease at doses higher than 0.8 kGy, proving that the radiolytic process involves not only the formation, but also the decay of the radicals under the action of gamma radiation. The predominant effect of gamma rays upon the sample is radicals destruction and not that of formation, and it is the prevalent process at small doses.

It is known that the radiolytic process is not selective, meaning that the radicals turns into non paramagnetic species under the action of radiation.

DSC thermograms of non-irradiated and irradiated L Ala-Gly and carnosine (Figs. 4 and 5) are attributed to melting accompanied by decomposition.

The effect of gamma radiation upon L Ala-Gly and carnosine is highlighted in the DSC curves of the irradiated samples both by a shift of the melting/decomposition temperatures towards lower values and by a decrease of the temperature interval of occurrence of the corresponding processes.

It can be seen from DSC thermograms that the shape of endothermic curves of irradiated samples of the two dipeptides is similar to that of non-irradiated one. The temperature values of the thermal effects obtained from DSC curves for non-irradiated and irradiated L-alanyl-glycine and carnosine with different doses are shown in Tables 1 and 2.

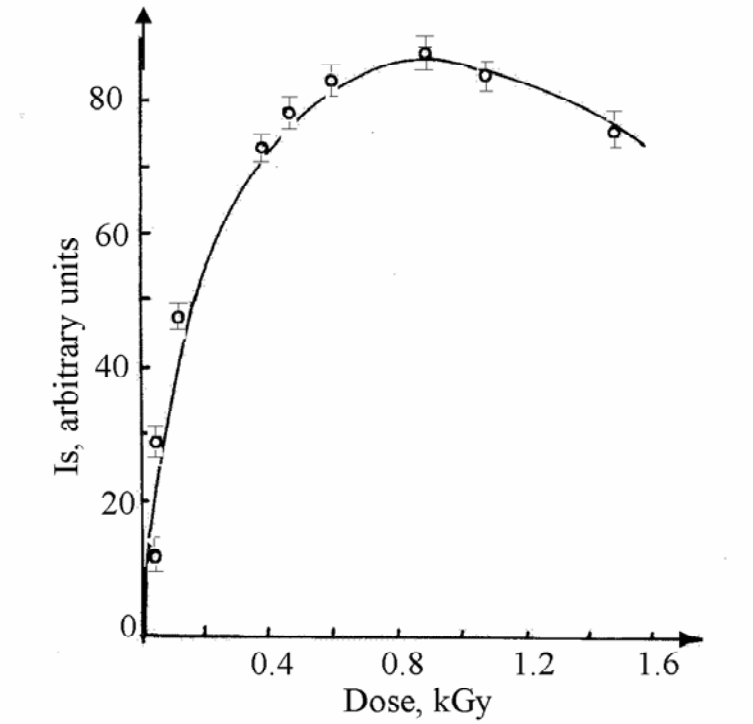


Fig. 3 – Variation of the intensity of the EPR signal (arbitrary units) as a function of irradiation dose.

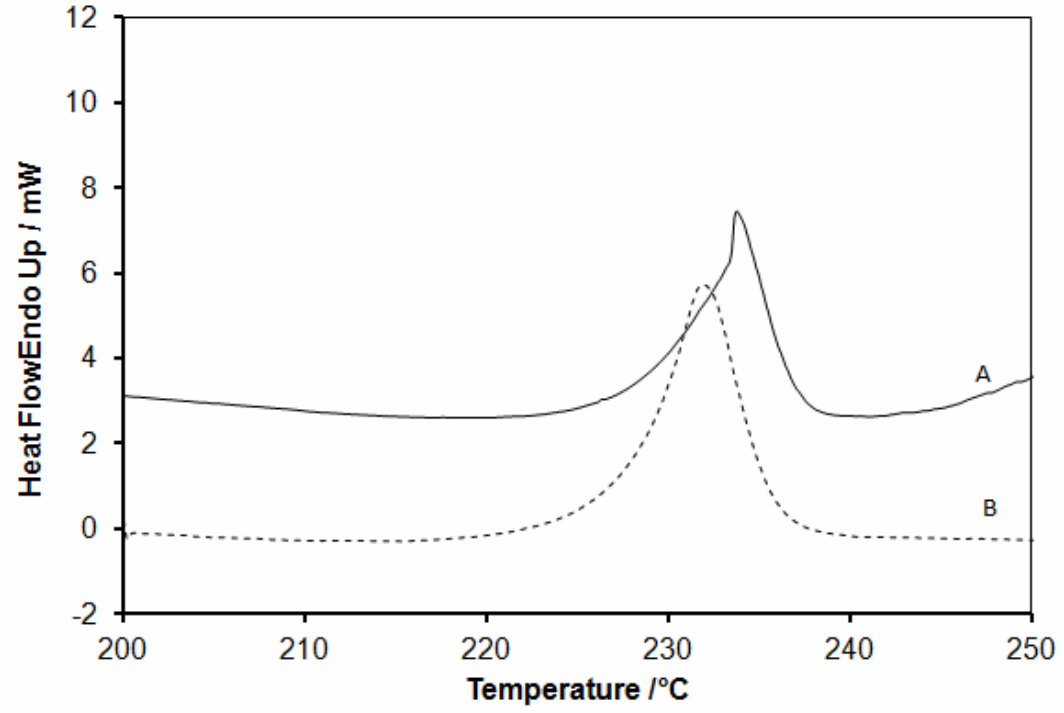


Fig. 4 – DSC thermograms of L-alanyl-glycine (L Ala-Gly) (A)- non-irradiated and (B)-3h- irradiated (0.315 kGy).

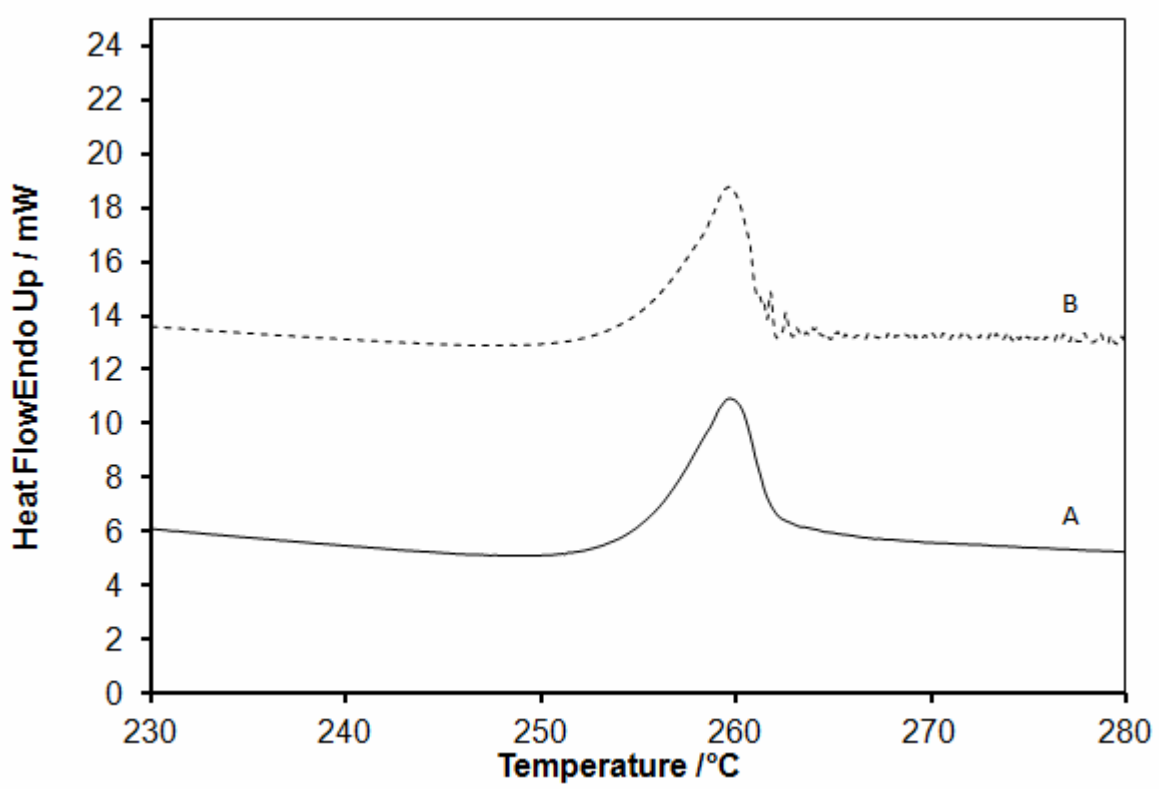


Fig. 5 – DSC thermograms of carnosine (A)- non-irradiated and (B)-3h- irradiated (0.315 kGy).

Table 1

DSC parameters for L-alanyl-glycine non-irradiated and irradiated with different doses

Dose, kGy	*0*	*0.31*	*0.73*	*1.47*
t_{onset}, ^0C	230.2±0.2	227.4±0.1	227.2±0.11	229.0±0.21
t_{max}, ^0C	232.5±0.3	232.09±0.22	231.4±0.2	232.3±0.27
ΔH_{dec}, $kJmol^{-1}$	90.8±0.9	79.6±0.8	79.2±0.8	79.1±0.8

Table 2

DSC parameters for carnosine non-irradiated and irradiated with different doses

Dose, kGy	*0*	*0.31*	*0.73*	*1.47*
t_{onset}, ^0C	256.2±0.15	256.1±0.11	255.5±0.1	254.7±0.12
t_{max}, ^0C	259.2±0.21	260.1±0.3	260.1±0.3	260.1±0.3
ΔH_{dec}, $kJmol^{-1}$	68.0±0.6	50.3±0.5	50.5±0.5	50.8±0.5

The thermal effects observed for the irradiated samples are smaller than those of the non-irradiated one. They decrease rapidly at small doses reaching an almost constant value for moderate and large doses. Such a behavior was also observed for several amino acids.[34] The decrease of the onset temperatures of the endothermic effect of the irradiated samples is also significant at lower doses. This behavior was also found in the case of other organic compounds such as antibiotics and steroids.[40]

The dipeptide carnosine (N-β-alanyl-L-histidine) when pyrolyzed alone produced acrylic acid and acrylamide. There are two possible pathways of formation of acrylamide from carnosine, one through hydrolysis of the peptide bond and release of β-alanine and its subsequent deamination, the second through release of 3-aminopropanamide and its deamination.[41-42]

Plotting the variation of the melting-decomposition enthalpy versus irradiation dose (Fig. 6) is ascertained that the decrease of the enthalpy (ΔH) is significant at low doses (0.3 kGy) and at doses higher than 0.3 kGy, the decrease reaches a plateau.

The differences found in the decreasing of the temperatures and melting-decomposition enthalpies on the dipeptides irradiated samples are due to the physical and chemical effects produced by ionizing radiation. Physical effects consist in producing the crystalline lattice defects. It is known that the high gamma radiation energy produces the breaking of the covalent bonds and disturb the interactions that stabilize the amino acid crystalline lattice.

The significant changes of the values of the melting-decomposition enthalpy relative to the initial value, as a result of irradiation (Fig. 6) could be explained by the cleavage of the peptide bond, as well as by changes in the crystal structure and the implication of decomposition products. The decomposition as a result of radiolysis affects the physico-chemical properties of the compound.[43]

To obtain further information about the influence of radiations upon the thermal behavior of L Ala-Gly and carnosine, the weight losses of the irradiated and non-irradiated samples were measured using thermogravimetry method. The weight losses of the two dipeptides are shown in Fig. 7 (L-alanyl-glycine) and Fig. 8 (carnosine) as a function of the irradiation dose.

From the radiolysis mechanism of L Ala-Gly shown in Scheme 2 is found that some of the amino acid molecules are converted into other chemical products that remain trapped in the crystalline lattice, except for the gaseous ones (NH_3, CO_2) which are thermally eliminated through diffusion.

The effect of temperature (t onset) and melting-decomposition enthalpy (ΔH) decreasing is significant at low doses (<0.7 kGy) only. The explanation of this behavior is due to the fact that

at doses higher than 0.7 kGy the radiolysis products concentration increases. Under these conditions a part of the irradiation dose is absorbed by the radiolitically formed products which produce a decrease of the radiation effect upon parent molecules, having a protective effect. This is the explanation why the irradiation was not performed at doses higher than 2 kGy.

From the thermal analysis of dipeptides having α-alanine using TG-DSC measurements,[44] Rodante *et al.*, established that the main process of the thermal decomposition consists from CO_2 and NH_3 removal, similarly with the radiolytic one. In the case of L Ala-Gly, the peak at 247^{0}C is assigned to the loss of methyl-amine (CH_3-NH_2) from α-alanine.

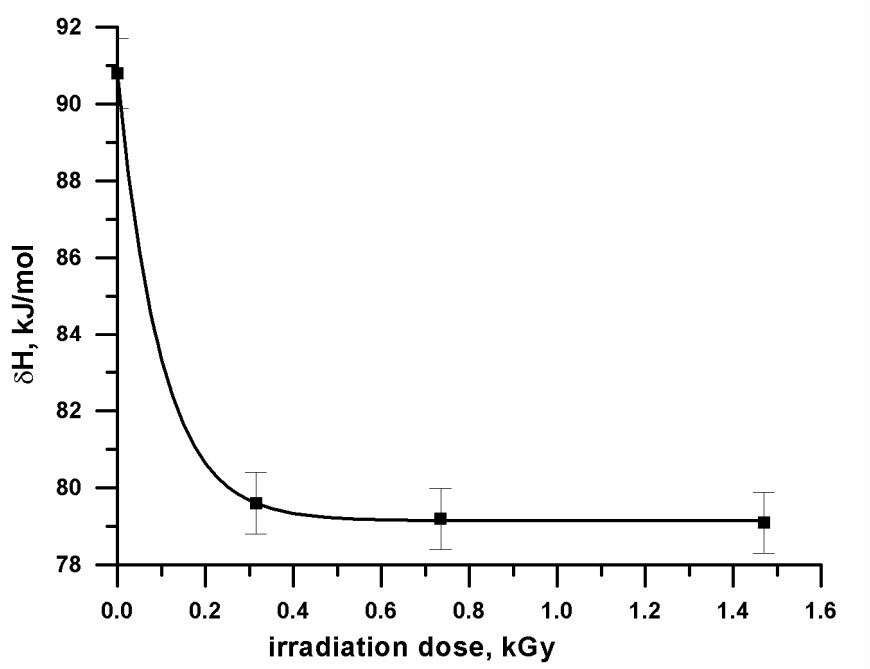


Fig. 6 – Decrease of L Ala-Gly melting-decomposition enthalpy versus irradiation dose (kGy).

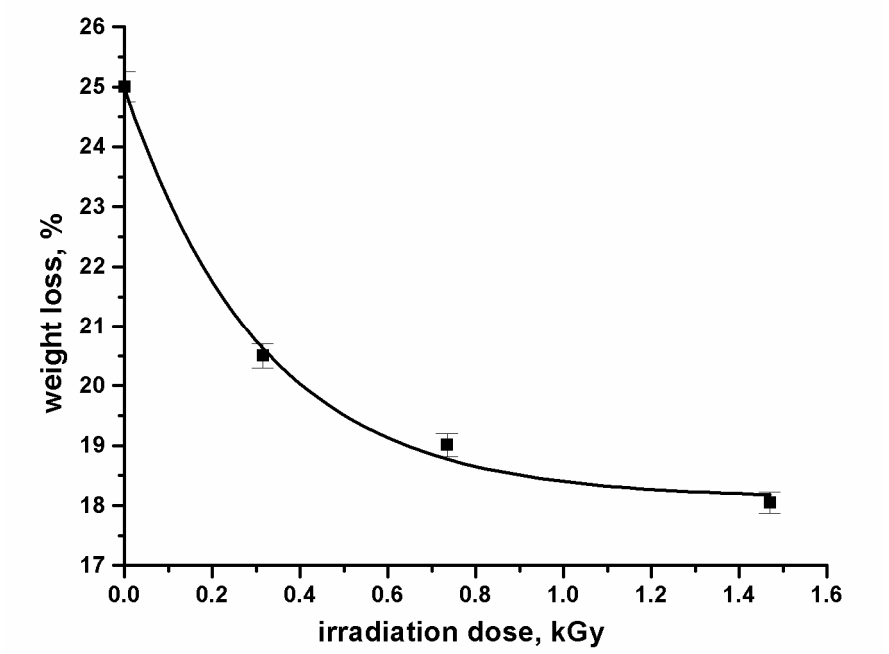


Fig. 7 – Dependence of the weight loss of L-alanyl-glycine on heating to 240^{0}C, on irradiation dose (kGy).

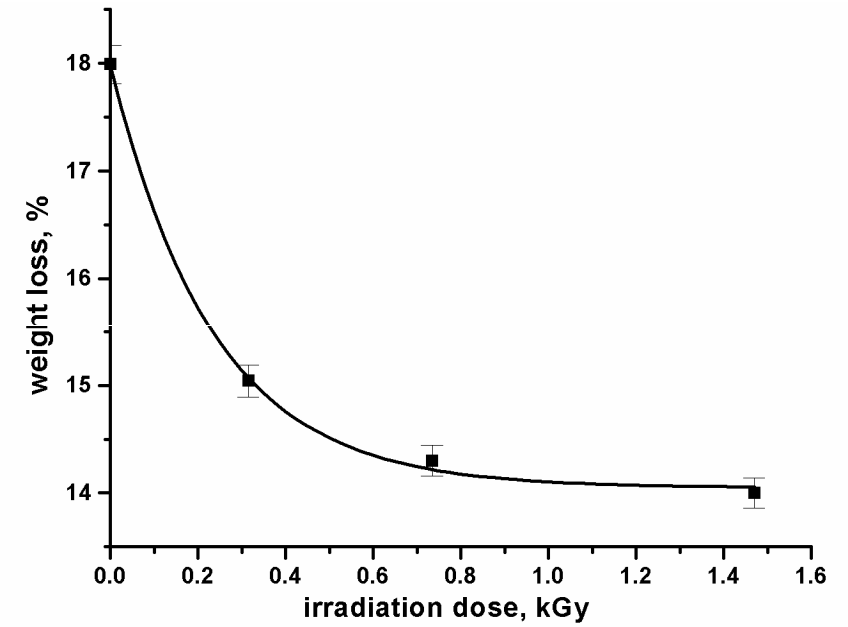


Fig. 8 – Dependence of the weight loss of carnosine on heating to 270^0C, on irradiation dose (kGy).

From Figs. 7 and 8 is observed that the decrease of the irradiated samples weight is lower than that of the non-irradiated ones. At higher doses the curves tend to a plateau, a similar behavior as in the case of enthalpy variation. The significant decrease of the weight loss of the two dipeptides takes place at lower irradiation doses only. The 0.7 kGy irradiated sample of L Ala-Gly present a mass decrease of 19% and the non-irradiated one 25%. In the case of irradiated carnosine the decrease is 14.5% and 18% for the non-irradiated sample. The lower weight loss of the irradiated samples is due to the radiations effect that decomposed some of the molecules through decarboxylation and deamination, similar to the thermal effect. The presented experimental results proved that the two dipeptides have a high radiolytical sensitivity and that is the reason for which the study was performed at low doses.

Determination of purity by DSC

The PerkinElmer Pyris DSC purity analysis software permits the assessment of purities for samples which undergo simultaneous degradation during melting. The purity of a sample for which only the molecular weight and sample weight are known can be ascertained by a simple mathematical treatment of the data from a DSC scan.

The thermal method is generally effective for determining the total amount of impurities, even if their species are unknown. The melting point of a pure substance is estimated from the fusion process and compared with that of a sample to find the degree of depression of the melting point. This method has an excellent feature not found in other methods. It cannot determine the content of each of the individual impurities, but can accurately determine the total amount of impurities, including the unknown species.[45] In Fig. 9 is plotted the purity using Pyris software for L-alanyl-glycine 3h-irradiated (0.31 kGy).

In the T vs $1/F_s$ curve, the corrected data points are shown as black circles and the line drawn through them shows how the data fit the Van't Hoff relationship (ec. 1). The black boxes represent the uncorrected values calculated for $1/F_s$ (fraction melted) at given temperatures (Fig.10).

$$1/Fs = [\Delta H/R] \bullet [To - Ts] / To^2 \bullet [1/X_2] \quad (1)$$

T_s – sample temperature and the melting temperature, K
T_0 – the melting temperature of the pure substance
ΔH – heat of melting of the pure material, J/g
X_2 – mole fraction of impurity in the sample
R – constant (8,314 J/mole·K)
F_s – fraction of sample melting at temperature T_s

$$F_s \quad \frac{A_s}{A_t}$$

A_s – area of the melting endotherm up to temperature T_s
A_t – total area of melting endotherm

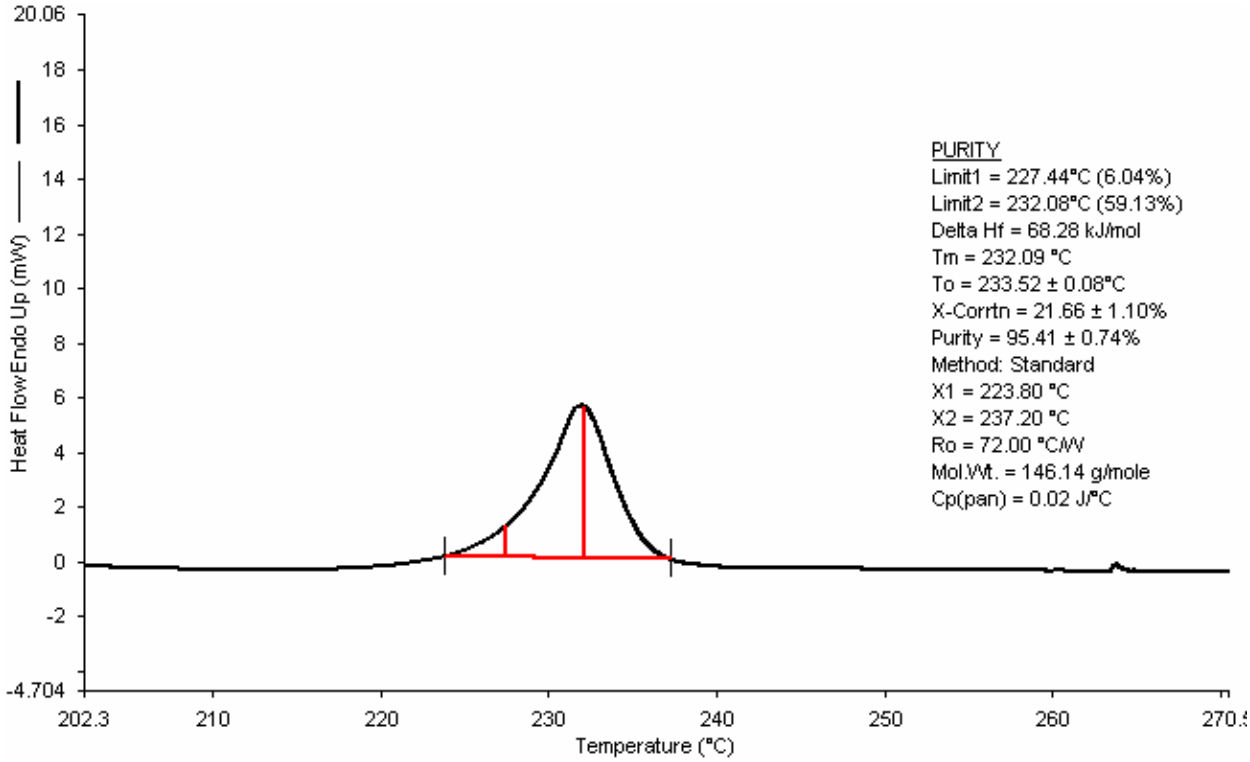


Fig. 9 – Determination of purity using Pyris software for L-alanyl-glycine 3h-irradiated (0.31 kGy).

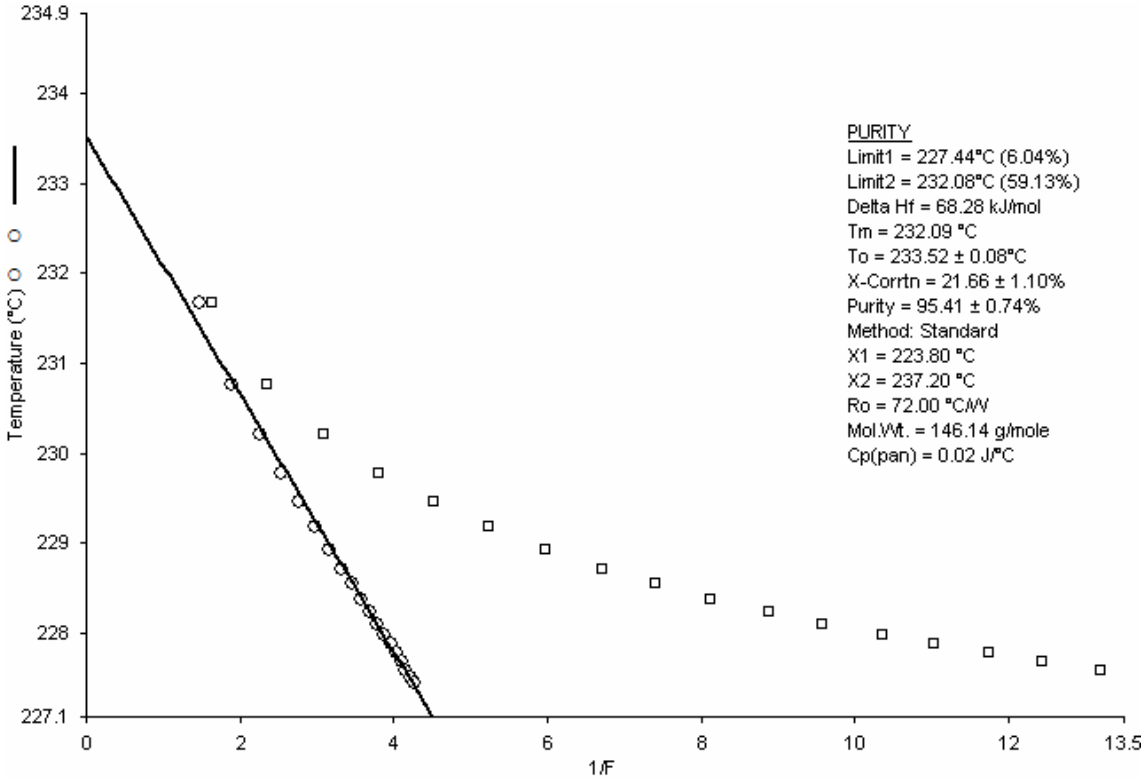


Fig. 10 – Van't Hoff plot for 3h-irradiated (0.31 kGy) L-alanyl-glycine.

Data of Table 3 indicate the decreasing of purity with increasing irradiation dose.

The presence in the irradiated samples of radiolytic products of the dipeptides, even in a small concentration, has the same influence as the presence of impurities: damages the crystalline structure of the dipeptides, and produces the decrease of the presented experimental parameters.

Table 3

Variation of the purity, % versus irradiation dose for L-alanyl-glycine and carnosine

Compound	*Dose*, *kGy*	Purity, %
L-alanyl-glycine	0	100.49 ± 0.59
	0.31	95.41 ± 0.74
	0.73	95.03 ± 0.83
L-carnosine	0	98.64 ± 0.54
	0.31	93.43 ± 1.96
	0.73	86.95 ±4.03

CONCLUSIONS

The EPR spectra of the L Ala-Gly and carnosine samples irradiated at room temperature are the result of the overlapping of two radicalic entities presented in the radiolysis mechanism. The radicals identified at room temperature result by the decarboxylation and deamination of the dipeptides, specific processes of the amino acids radiolysis.

The thermal effects of the irradiated dipeptides samples are different from the non-irradiated ones. This finding proves that the existence of the radiolysis products affect the thermal decomposition process. It is noted the decrease of the dipeptides melting-decomposition enthalpy at low irradiation doses.

The weight loss due to the thermal decomposition is 4-6% lower for the irradiated samples than the non-irradiated ones and occurs at low doses too.

The weight loss decrease of the dipeptides is due to the CO_2 and NH_3 removal, a similar process to the effect of radiations. From EPR and DSC study it has been found that the radiation effect upon the two dipeptides is significant at low irradiation doses only (~ 1 kGy). This experimental finding is derived from two factors: first it is known that the two dipeptides having an important biological role become sensitive to the action of ionising radiations at low doses; the second factor having general character is the formation of the radiolysis products lowering the effects of radiations at high doses.

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