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Protection of Human Albumin against UV-C Irradiation by Natural Antioxidants

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Abstract: The use of UV-C light as a means to inactivate pathogens in biological media containing blood components or plasma derivatives has recently been rediscovered. Although highly effective, these treatments may have deleterious effects on plasma proteins such as albumin, fibrinogen and immunoglobulins. Here we investigate the use of three natural antioxidants, gallic acid, nicotinic acid and ascorbic acid, as potential stabilizers of human albumin (HA) during irradiation by UV at 254 nm. The effects of UV-C on HA were monitored by measuring the absorbance changes in the protein spectrum at 320 nm. Experiments carried out at irradiance levels of 3.5–15 W m^{-2} and application times of up to 40 min showed that albumin undergoes rapid aggregation and that this process is dose-dependent. When added at 0.2 mM to the protein solution, all of the antioxidants inhibited HA aggregation, with ascorbic acid being the most effective. To quantitatively describe the observed effects, a fluence-based model was developed. The model predicted a quadratic dependence of the spectral changes of HA on the radiation dose and was validated by experiments performed under different exposure conditions.

Keywords: Albumin, Antioxidants, UV-C, Sterilization, Viral Inactivation

Introduction

Over the last years, the risk of transmission of viruses or other pathogens through blood products derived from human plasma has been significantly reduced due to improved donor screening and more effective virucidal procedures (Benjamin, 2014). Several treatments based on the use of dry heat, low pH, solvent/detergent mixtures, or their combination, have proven to be very effective against pathogens such as hepatitis A and hepatitis C viruses and HIV (Salunkhe *et al*., 2015). Nonetheless, these treatments may be scarcely effective against small non-enveloped viruses (e.g., hepatitis A virus and erythrovirus B19) and prions, as these species are highly resistant to chemical and/or physical inactivation (Prowse, 2013). In addition, the risk of emergence of new or unknown pathogens capable of escaping current inactivation methods should also be considered (Blümel *et al*., 2017).

Recently, due to the progress made in the control of radiation dosage and the design of innovative continuous-flow sterilization systems (Li *et al*., 2005; Seltsam and Müller, 2011), there has been a renewed interest in the use of UV-C radiation as a means of pathogen reduction (Schubert *et al*., 2018).

The virucidal effects of UV-C radiation rely on the high adsorption of light energies between 200 and 280 nm (typical UV-C sources operate at 254 nm) by nucleic acids. Following absorption, cyclobutane pyrimidine dimers can be formed between two adjacent pyrimidines in DNA and RNA, which prevents replication and transcription processes (Seghatchian and Tolksdorf, 2012). At high radiation doses, cleavage and fragmentation of the polynucleotide chains can occur.

Contrary to methods based on UV-A or UV-B radiations (Goodrich *et al*., 2010; Irsch and Seghatchian, 2015), UV-C treatments do not require the addition of exogenous photo sensitizers, making the sterilization process easier to implement. However, this approach has a few drawbacks, as UV-C radiation may also damage proteins that are present in the biological medium to be treated, such as albumin, fibrinogen and immunoglobulins. Often, the difference between the radiation dosage required to achieve an acceptable degree of pathogen reduction and that capable of damaging the protein components is very small, this resulting in the need to control accurately the applied radiation.

The effects of UV-C on proteins can be either direct or indirect. The former arise from the direct absorption

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of UV light by the protein, while the second are due to the UV-induced formation of reactive oxygen species (ROS) capable of interacting with the protein. At the radiation doses typically used for sterilization of biological media, radical-related effects play the major role. Consequently, the presence of an antioxidant compound in the medium can be expected to reduce the damage to the protein. This has been verified in the very few studies on this topic, such as those by Chin *et al*. (1995), Marx *et al*. (1996) and Zuorro and Lavecchia (2011).

Human albumin (HA) is the most abundant plasma protein and plays a key role in many physiological functions, such as maintaining colloid osmotic pressure (COP), buffering of pH and transport of several endogenous and exogenous compounds in the blood (Fanali *et al*., 2012). It is widely used in therapeutic applications, primarily as an isooncotic solution (4–5%), for vascular expansion, or as a hyperoncotic solution (20–25%), for restoration of COP and fluid balance preservation among compartments (More and Bulmer, 2013). The preparation of these solutions requires the recovery of quite large amounts of HA from the blood of donors through a complex multistep process including sterilization of the final products (Raoufinia *et al*., 2016).

In this study we investigate the effects of three natural antioxidants, gallic acid (GA), nicotinic acid (NA) and ascorbic acid (AA), on HA during irradiation by UV-C. The purpose of our study was twofold: (a) to evaluate whether and to what extent the three antioxidant compounds protected HA during UV-C irradiation and (b) to develop a mathematical model capable of describing the observed effects.

Experimental

Materials

GA (CAS No. 149-91-7), NA (CAS No. 59-67-6), AA (CAS No. 50-81-7) and recombinant human albumin (HA, CAS No. 70024-90-7) were purchased from Sigma-Aldrich (Milano, Italy). The three antioxidants were supplied in solid form with purities ≥99.5% and were used as received. HA was expressed in rice and was provided as a lyophilized powder with a purity ≥96%.

Protein solutions containing HA, alone or in the presence of antioxidants, were prepared by dissolving the appropriate amount of each product in double distilled water.

Methods

A cabinet maintained at 20±2°C was used to perform irradiation tests. The UV-C source consisted of a low-pressure mercury lamp emitting at 254 nm with a nominal power of 6 V (Spectroline ENF-260C/FE, Spectronics Corporation, Westbury, NY, USA). The protein solution was poured into a stoppered parallelepiped quartz cell with a light path of 10 mm.

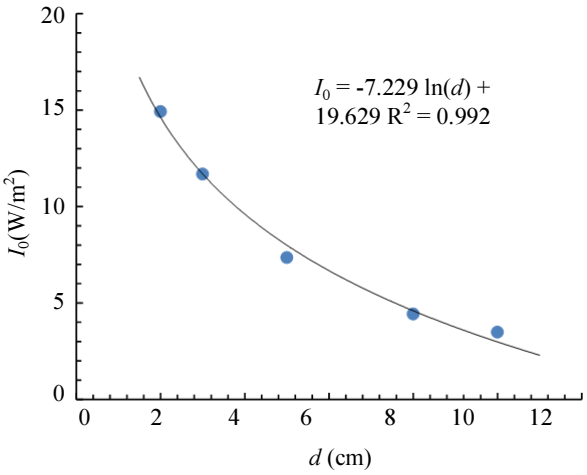

Fig. 1: Calibration curve for evaluation of irradiance levels

The cell was positioned perpendicular to the direction of light propagation, at a distance from the external surface of the lamp corresponding to the desired irradiation intensity. To this end, a calibration curve was preliminary constructed by measuring the irradiance levels at different distances from the light source (Fig. 1). Measurements were made using a radiometer with a UV-C sensor (AccuMax XR-1000, Spectronics Corporation, Westbury, NY, USA).

In a typical experiment, the lamp was first warmed up for about 5 min, then the cuvette containing the protein solution was placed at the appropriate distance from the UV-C source and irradiated. At the desired exposure time, the cuvette was removed from the cabinet, gently hand-shaken and the absorbance spectrum of the solution recorded. A double-beam UV-Vis spectrophotometer (Perkin-Elmer, Lambda 25) was used, with a blank consisting of pure water, or water with the antioxidant compound, subjected to the same exposure conditions of the test solution.

In all experiments the protein concentration was 1 g L^{-1} (1.5 10^{-5} M) while that of the antioxidant compound was varied between 0.1 and 0.5 mM. The radiation intensity ranged from 3.5 to 15 W m^{-2} and the exposure time from 5 to 60 min. At the end of each treatment, the temperature of the protein solution was measured. In all tests it was found to be slightly higher (about 1-2°C) than that of the ambient.

Results

Figure 2 shows the absorption spectrum of HA in aqueous solution at fixed irradiance level and increasing irradiation time. As can be seen, exposure to UV light produced major spectral changes in the region around 320 nm, which can be ascribed to the formation of protein aggregates (Oliva *et al*., 1999). Accordingly, to quantify the effects of UV-C on HA we defined the following quantity:

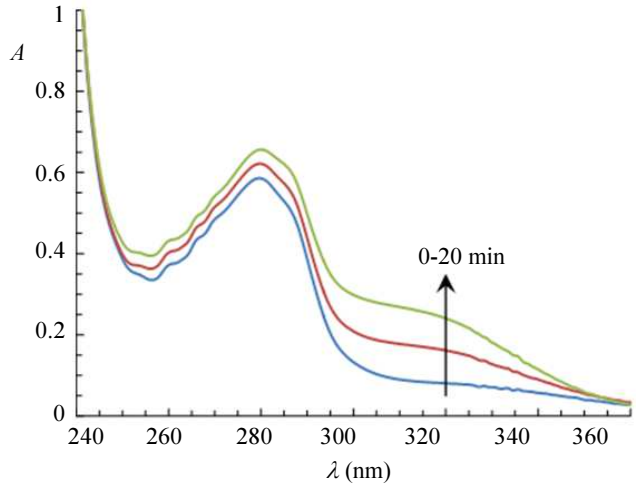

Fig. 2: Absorption spectrum of HA at 1 g L^{-1} in aqueous solution (I_0 = 12 W m^{-2})

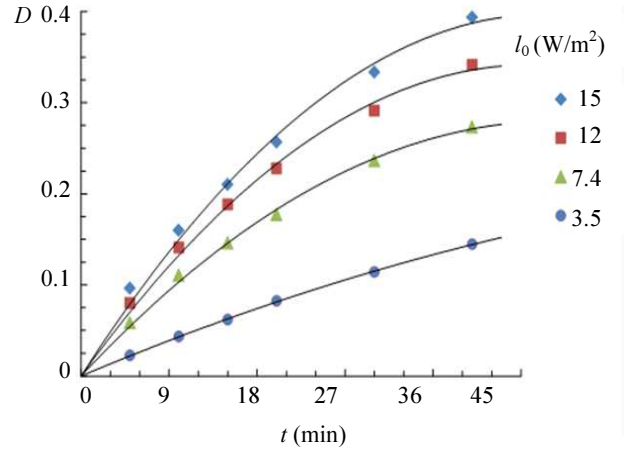

Fig. 3: Effect of increasing radiation intensities (I_0) on the trend of D vs. time for HA at 1 g L^{-1} in aqueous solution

$$D = A_{320} - A^0_{320} \quad (1)$$

Where:

A_{320} = The absorbance at 320 nm of the irradiated protein (–)

A^0_{320} = The absorbance at 320 nm of the protein in the absence of irradiation (–)

Typical trends of D as a function of time at different irradiance levels are displayed in Fig. 3. Protein aggregation increased with time and radiation intensity. Interestingly, when the D values were plotted as a function of radiation fluence (F) rather than time, with F defined as:

$$F = I_0 \times t \quad (2)$$

Where:

I_0 = The radiation intensity (W m^{-2})

t = The time (s)

all the curves coalesced into a single curve (upper curve in Fig. 4), indicating that protein aggregation is strictly related to the radiation dose administered. In the presence of antioxidants, the curves of D as a function of F exhibited a similar trend, but they lied below that obtained in their absence, suggesting that all of them were capable of protecting HA against aggregation. The observed protection degree increased in the order: GA < NA < AA.

To provide a quantitative description of the above effects, the D vs. F data were analysed by different empirical models. The best result was obtained by the following second-order polynomial model:

$$D = aF + bF^2 \quad (2)$$

Where:

D = The difference in absorbance values at 320 nm (–)

F = The fluence (kJ m^{-2})

a = The linear model coefficient (m^2 kJ^{-1})

b = The quadratic model coefficient (m^4 kJ^{-2})

Equation 2 described very well the experimental data, as can be seen from Figure 4 and from the values of the correlation coefficients listed in Table 1. Furthermore, the plot of D_{calc} vs. D_{exp} showed that data were close to and uniformly scattered around the bisection line, suggesting the absence of systematic errors in model predictions (Fig. 5). The estimated values of model coefficients (a, b) are listed in Table 1.

The coefficient a in Equation 2 represents the slope of the tangent to the curve in the origin, being:

$$a = \left(\frac{dD}{dF}\right)_{F=0} \quad (3)$$

Hence, it can be related to sensitivity of D to F: the larger its value, the greater the tendency of HA to aggregate under irradiation. From Figure 6 it can be noted that this parameter, equal to 2.665 10^{-2} m^2 kJ^{-1} in the absence of antioxidants, is reduced by about 40, 60 and 93% in the presence of GA, NA and AA, respectively. These percentages give a quantitative measure of the protection offered by three compounds.

In order to evaluate the predictive capabilities of the model, validation tests were performed at fluence values different from those of previous runs (Table 2). The values selected were one within (F = 12.5 kJ m^{-2}) and the other outside (F = 30 kJ m^{-2}) the original experimental domain. The model responses were very close to the experimental values (average error: ±0.02), further supporting the adequacy of the model.

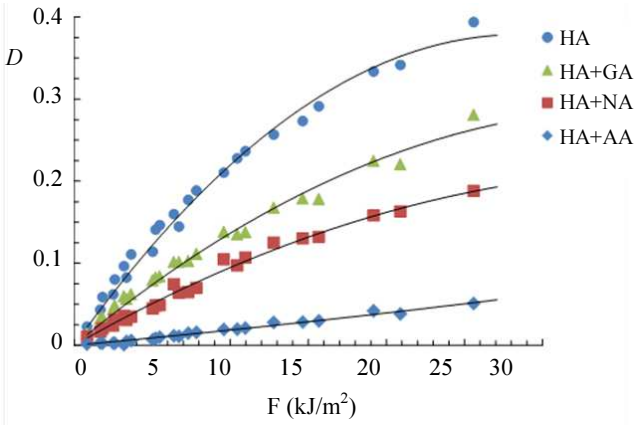

Fig. 4: Effect of the presence of antioxidant compounds on the trend of D vs. F (I_0 = 3.5–15 W m^{-2})

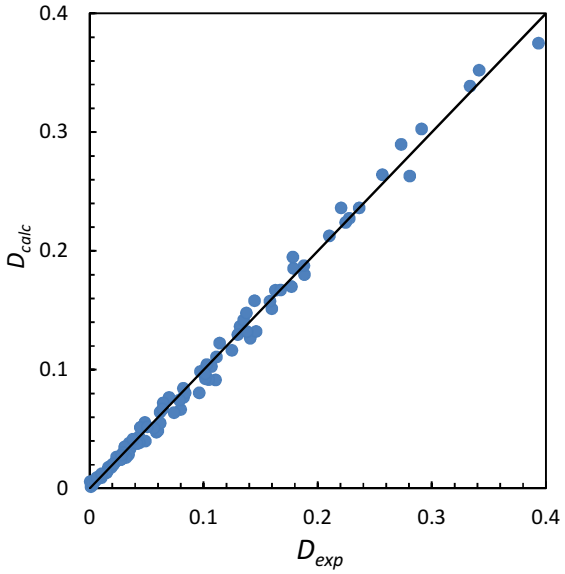

Fig. 5: Scatter plot of calculated vs. experimental D values

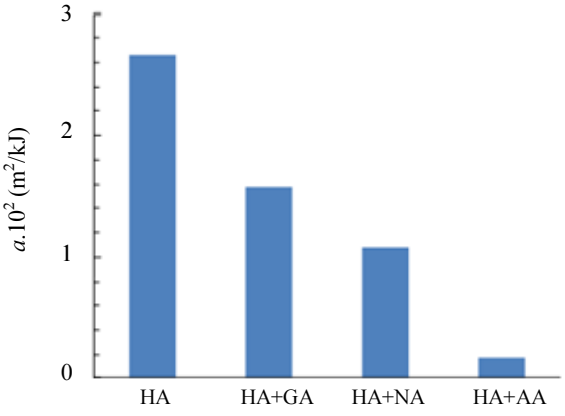

Fig. 6: Estimated a-values in the absence and presence of antioxidant compounds

Table 1: Estimated model parameters (a, b) with the associated coefficient of determination (R^2) and adjusted R^2 (in brackets)

System	a•10^2 (m^2 kJ^{-1})	b•10^4 (m^4 kJ^{-2})	R^2
HA	2.665 ± 0.060	–4.691 ± 0.327	0.997 (0.952)
HA + GA	1.582 ± 0.047	–2.166 ± 0.253	0.996 (0.950)
HA + NA	1.086 ± 0.030	–1.378 ± 0.163	0.997 (0.951)
HA + AA	0.176 ± 0.011	0.107 ± 0.061	0.991 (0.945)

Table 2: Results of model validation tests. F is the fluence, D the absorbance change at 320 nm and ε is the error, calculated as: $\varepsilon = D_{calc} - D_{exp}$

System	F (kJ m^{-2})	D_{exp}	D_{calc}	ε
HA	12.5	0.2773	0.2598	–0.0175
	30.0	0.3684	0.3773	0.0089
HA + GA	12.5	0.1432	0.1639	0.0207
	30.0	0.3156	0.2797	–0.0359
HA + NA	12.5	0.1256	0.1142	–0.0114
	30.0	0.2451	0.2017	–0.0434
HA + AA	12.5	0.0212	0.0236	0.0024
	30.0	0.0743	0.0623	–0.0120

Discussion

In this study, the protective effects of three low-molecular-weight antioxidants, GA (MW = 170.12), NA (MW = 123.11) and AA (MW = 176.12), on HA during UV-C irradiation were investigated. The molecular structures of these compounds are displayed in Fig. 7.

GA is a phenolic compound present in many fruits and vegetables. It has strong antioxidant and free radical scavenging activities (Dwibedy *et al*., 1999) as well as antibacterial and anti-inflammatory properties (Kang *et al*., 2008; Kim *et al*., 2006). NA, also known as niacin, is one of the B-complex vitamins, with cholesterol-lowering and lipid reduction effects (Sinthupoom *et al*., 2015). It exhibits antioxidant activity, which results from the electron-transfer properties of the pyridine moiety, and has a significant ability to quench hydroxyl radicals (Hu *et al*., 1995). AA, or vitamin C, has many important physiological functions, particularly in cell and tissue defense against oxidative stress (Arrigoni and De Tullio, 2002). Its antioxidant properties arise from its ability to scavenge free radicals and reactive oxygen molecules through a mechanism involving dehydroascorbic acid and the semi-dehydroascorbate radical (Niki, 1991).

The rationale for the use of GA, NA and AA as protective agents of HA is that the protein damage by UV radiation in the wavelength range of 200–280 nm is primarily determined by the light-induced production of ROS (Mohammed *et al*., 2015). These species interact with proteins causing more or less pronounced changes at different structural levels and, hence, impairing their activity (Lobo *et al*., 2010). The most important ROS are hydroxyl radicals (•OH), hydroperoxyl radicals (HO_2•), superoxide anion radicals ($•O_2^-$) and species like hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).

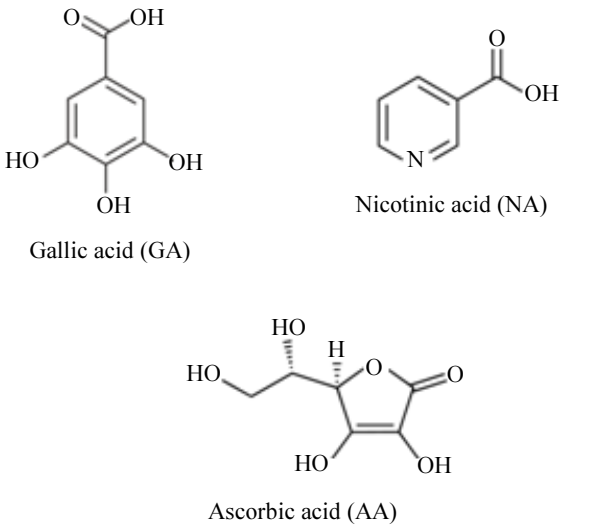

Fig. 7: Molecular structures of the antioxidant compounds

Antioxidants can break up the radical reaction chains and arrest the propagation process through mechanisms that usually involve the donation of an electron or a hydrogen atom (Nimse and Pal, 2015). However, it is also known that under certain conditions antioxidants can act as pro-oxidants (Bouayed and Bohn, 2010). For example, at high concentrations or in the presence of transition metals they can undergo auto-oxidation or be involved in reactions leading to the formation of new ROS.

In a preliminary assessment of antioxidant doses, we found that the protective effects of the three investigated compounds tended to diminish above 0.2–0.25 mM. Accordingly, their concentration in the protein solution was set to 0.2 mM.

The effect of UV-C on HA was monitored by measuring the irradiation-induced changes in the protein spectrum. The absorption spectrum of HA is characterized by a minimum at about 255 nm and a maximum at about 280 nm (Fig. 2). As reported by Artyukhov *et al*. (2001), the ratio between the absorbance values at these two wavelengths (A_{max}/A_{min}) can be used to monitor the structural changes in the protein molecule induced by UV radiation. In particular, a decrease in this ratio can be associated with chemical alterations of the aromatic amino acid residues tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) or reorganization of the regions where they are located (Michnik *et al*., 2008). Trp, Tyr and Phe, along with cysteine (Cys) residues, are the main targets of UV-C radiation. Direct light absorption by these residues is followed by a series of chemical and/or physical events causing partial or complete loss of protein activity (Pattison *et al*., 2012). Tyr and Phe residues are also capable of transferring their excited state energy to Trp residues, further enhancing the deleterious effects of irradiation. Indirect effects of UV-C are related to the formation of ROS. These species can cause oxidative damage to the protein and induce changes in its secondary and tertiary structure (Stadtman, 2006).

Under the conditions used in this study, A_{max}/A_{min} was found to be nearly constant, suggesting that photochemical alterations of the aromatic amino acid residues of HA do not occur. This was observed both in the absence and presence of antioxidant compounds. In contrast, exposure to UV-C radiation resulted in a progressive increase in absorbance at 300–340 nm. This increase can be related to the formation and growth of protein aggregates. According to the Lumry-Eyring model, protein denaturation can be schematized as a two-step process: $F \leftrightarrow U \rightarrow I$ (Cioci and Lavecchia, 1997). The first step is reversible and represents the equilibrium between the folded (*F*) and the partially unfolded (*U*) protein. This step is followed by the conversion of the U form to the irreversibly denatured protein (*I*). Depending on the severity of denaturing conditions, partially unfolded protein molecules can undergo aggregation or chemical changes. It should be noted that protein aggregates are potentially immunogenic and therefore their presence in media containing blood derivatives must be avoided (Wang and Roberts, 2018).

Interestingly, all the three antioxidants investigated hindered the aggregation process to an extent depending on the type of antioxidant used. Since aggregation is caused by the ROS generated in aqueous solution during irradiation, it can be inferred that the observed differences in HA protection are due to the different quenching abilities of the three compounds. In this regard, AA was the most effective protective agent, followed by GA and NA. Consistently with this, rutin, a flavonoid glycoside with high antioxidant activity, was found to protect to a large degree plasma proteins during a virucidal UV-C treatment (Chin *et al*., 1995). Similarly, this compound protected fibrinogen and prevented its degradation during UV-C irradiation (Marx *et al*., 1996).

Although the antioxidant properties of the investigated compounds can provide an explanation to the observed protective effects, it should be pointed out that factors related to their interaction with the protein could also be involved. For example, it is known that all of the three antioxidants can bind to albumin. Khatun *et al*. (2018) showed that GA binds strongly to Sudlow site I (subdomain IIA) of bovine serum albumin (BSA) and that after binding BSA was partially stabilized against thermal denaturation. Similar results were obtained for NA, which was found to interact strongly with BSA at Sudlow site I and, to a more limited extent, at subdomain IIB (Banipal *et al*., 2016). Also AA was found to strongly bind human (Li *et al*., 2013) and bovine (Li *et al*., 2014) albumin at Sudlow site I. According to the authors, AA is also capable of interacting non-specifically with the protein by a surface adsorption mechanism leading to the

formation of a coating of AA molecules on the protein surface. Therfore, it is possible that the very high protection provided by AA to HA during UV-C irradiation (see Fig. 6) is also a result of these interactions.

Another important point emerging from the present study is that protein aggregation, both in the absence and presence of antioxidants, is governed by the fluence of UV-C radiation rather than the intensity and the exposure time separately. In this regard, the second-order model developed provides a very good, though empirical, description of the effects of irradiation on HA. As a result, it can be used for a preliminary assessment of the radiation levels during a UV-C treatment, particularly when high radiation doses are required for pathogen inactivation.

Conclusion

In this study, we have shown that all of the three natural antioxidants investigated, GA, NA and AA, are capable of inhibiting HA aggregation during irradiation by UV-C, with AA being the most effective. The fluence-based model developed describes reasonably well the effects of UV-C on protein aggregation. This model can be helpful in the design of a UV-C treatment of biological media containing HA or other plasma proteins.

Future studies should be aimed at providing further insight into the mechanisms of antioxidant-induced protection. The possibility of improving the protective effects of antioxidants by using them in combination should also be evaluated.

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Author's Contributions

Antonio Zuorro: Designed and performed the experiments, analyzed the data and contributed to write the paper.

Roberto Lavecchia: Conceived the experiments, analyzed the data and contributed to write the paper.

Ethics

This article is original and contains unpublished material. The corresponding author confirms that all of the other authors have read and approved the manuscript and that no ethical issues are involved.

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