Protocatechuic and 3,4-dihydroxyphenylacetic acids inhibit protein glycation by binding lysine through a metal-catalysed oxidative mechanism

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Abstract

The mechanism of inhibition of advanced glycation end products (AGEs) formation by protocatechuic and 3,4-dihydroxyphenylacetic (DHPA) acids has been studied by using a widespread applied *in vitro* model system composed of bovine serum albumin (BSA) and supra-physiological glucose concentrations. Protocatechuic acid and DHPA inhibited the formation of Amadori compounds, fluorescent AGEs (IC_{50}=62.1 ± 1.4 μmol/L and IC_{50}=155.4 ± 1.1 μmol/L, respectively) and N^{ε}-carboxymethyl-lysine (IC_{50}=535.3 ± 1.1 μmol/L and IC_{50}=751.2 ± 1.0 μmol/L, respectively). BSA was pre-treated with the two phenolic acids and the formation of BSA-phenolic acid adducts was estimated by nanoflow-LC-ESI-QTOF. Results showed that the tested phenolic acids bound key sites of glycation in BSA through a metal-catalysed oxidative mechanism. The anti-glycative activity mechanism involved the formation of BSA-phenolic acid adducts and it is unlikely that this occurs *in vivo*. These results raise the problem to design *in vitro* models closer to physiological conditions in order to reach biologically sound conclusions.

Keywords: polyphenols; mass spectrometry; protein-polyphenols interaction; *in vitro* models; diabetes

1. Introduction

Glycation of circulating, cellular, and matrix proteins by glucose are thought to be a major factor in pathogenesis of diabetes and related cardiovascular diseases.[1] This process is known as the Maillard reaction and begins with the nucleophilic addition between an amino group of a protein and the carbonyl group of glucose to form a reversible Schiff base (**Figure 1** pathway 1).[2] The latter can rearrange in Amadori compounds, that can be fragmented by oxidation in presence of reactive oxygen species (ROS) and transition metal ions such as Fe^{3+} and Cu^{2+} (**Figure 1** pathway 2). This oxidative degradation could lead to the formation of the so called advanced glycation end products (AGEs). Amadori products can also be transformed into reactive dicarbonyl products (**Figure 1** pathway 4), which could react with amino group of proteins generating AGEs (**Figure 1** pathway 5). Another pathway implicated in AGEs formation involves glucose auto-oxidation. Glucose can be directly oxidized in the presence of catalytic metals and ROS, generating dicarbonyl compounds (**Figure 1** pathway 3) which can further react with the amino groups of proteins (**Figure 1** pathway 5).[3,4]

Several AGEs have been identified in tissues and circulating proteins.[5] Principal sites of glycation in the proteins are lysine side chain, arginyl guanidine groups and N-terminal amino group of proteins.[6,7] Glycation of the ε–amino group of lysine may result *in vivo* in the formation of N^{ε}-carboxymethyl-lysine (CML), *N*ε-carboxyethyl-lysine (CEL) and pyrraline (**Figure 1**), whereas glycation at the arginine guanidine group level produced argpyrimidine (**Figure 1**) and other less frequent AGEs.[6,8] Some AGEs are characterized by more complex structures forming in proteins intra- and inter-molecular crosslinks, such as pentosidine, glucosepane and imidazolium compounds.

CML is often used as marker of AGEs rising from glycation reaction since it is the major AGE produced *in vivo*.[9] It can arise from various pathways, such as condensation of glucose with the ε-amino group of lysine, generating the derived Amadori compounds, fructoselysine (FL). FL is an unstable intermediate that can further undergo oxidation to form CML.[10] Another CML formation

pathway involves the direct reaction of dicarbonyl compounds with the ε-amino group of lysine.[6] CML-modified proteins have been detected in plasma, renal tissues and skin collagen of diabetic patients.[11] They accumulate mainly in proteins with a long half-life, altering their structural and biochemical properties and are involved in some metabolic diseases such us diabetes type 2, cardiovascular diseases, Alzheimer's disease and ageing.[11-13] CML-modified proteins has also been reported to be a ligand for the receptor of advanced glycation end products (RAGE).[14] RAGE activate several signalling transduction pathways, including the pathway for intracellular ROS generation, inducing secondary oxidative stress-mediated apoptosis.[15,16]

Dietary phenolic compounds and their metabolites may exert beneficial effects in the control of diabetes and its complications thanks to their ability to inhibit oxidative stress and protein glycation.[4,17,18] Although *in vitro* studies have been employed to assess the anti-glycative potential of phenolic compounds, only few were designed using physiologically relevant glucose concentrations.[4,17] Instead, the majority of applied *in vitro* models used supra-physiological glucose concentration (usually hundreds of mmol/L concentrations) and their physiological relevance to human health is sometimes questionable.[19-21] Indeed, there is still a lack of information about the exact mechanism of action. Phenolic compounds may offer protection by chelating transition metals or by scavenging ROS, which are produced during the glycation reaction, slowing down the glycation process and inhibiting the formation of AGEs.[22] Another possible mechanism reflects the ability of phenolic compounds to trap dicarbonyl compounds generated during protein glycation.[23] Besides that, in a previous work, it has been shown that antioxidant activity, chelating ability and dicarbonyl trapping activity were not important for the anti-glycative activity of phenolic compounds.[4] Moreover, in the same work it has been suggested that the post-Amadori pathways 2, 4 and 5 and the glucose auto-oxidation pathway 3 are not the site of inhibition (**Figure 1**). In a further study, it has been found that coffee chlorogenic acids were able to inhibit protein glycation. This

inhibitory effect was related to a reduction in the Amadori product concentrations without AGEs formation. All these studies gave evidence that phenolic compounds could act as pre-Amadori inhibitors of protein glycation.[24]

The aim of this study was to investigate the mechanism of inhibitory activity of two bioavailable phenolic acids (protocatechuic and 3,4-dihydroxyphenylacetic acids) against AGEs formation, using a well-known and widely used *in vitro* model that utilized supra-physiological concentrations of glucose and bovine serum albumin as model protein.

2. Materials and Methods

2.1 Materials

Protocatechuic acid, 3,4-dihydroxyphenylacetic acid (DHPA), bovine serum albumin (BSA), D-glucose, sodium dodecyl sulphate (SDS), nitro blue tetrazolium chloride (NBT), dithiothreitol (DTT), iodoacetamide and trypsin were purchased from Sigma-Aldrich (Milan, Italy). All MS/MS reagents were from Bio-Rad (Hercules, CA, U.S.A.). All other chemical reagents, buffer solution, reagents for electrophoresis and solvents for HPLC were supplied by Fluka (Milan, Italy). Microcon YM-10 kDa for ultrafiltration and polyvinylidene difluoride (PVDF) membrane were supplied by Millipore (Milan, Italy). N^{ε}-carboxymethyl-lysine ELISA kit was purchased from CycLex Co (Nagano, Japan).

2.2 Bovine serum albumin (BSA) glycation

BSA (50 mg/mL) was incubated at 37°C for 7 days with glucose (0.8 mol/L) in 0.1 mol/L potassium-phosphate buffer (pH 7.4, sodium azide 0.024%) in the presence of variable amounts (from 5 to 1000 µmol/L) of protocatechuic or 3,4-dihydroxyphenylacetic acids.[20] A control reaction without addition of phenolic compounds was prepared, representing the 100% of glycation.

89 ***2.3 Determination of Amadori compounds (fructosyl-lysine)***

90 The formation of Amadori compounds was determined using a NBT assay.[24] Aliquots of glycated
91 sample (200 µL) were added to reaction mixture containing 800 µL of NBT (300 µmol/L) in sodium
92 carbonate buffer (100 mmol/L; pH 10) and incubated 30 min at room temperature. Absorption was
93 measured at 550 nm. The possible interference of phenolic compounds was corrected by subtracting
94 the contribution of an incubated blank containing BSA and phenolic compounds.

95

96 ***2.4 Measurement of fluorescent (AGEs)***

97 Formation of fluorescent AGEs after glycation was measured at an excitation wavelength of 355 nm
98 and an emission maximum of 405 nm versus an incubated blank containing BSA and phenolic
99 compounds. Data are expressed in terms of IC_{50} (concentration of inhibitor required to inhibit
100 glycation by 50%) calculated from log dose inhibition curve.[24] Fluorescence was read using a
101 FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany).

102

103 ***2.5 Detection of N^{ε}-carboxymethyl-lysine (CML)***

104 BSA-CML adducts were quantitatively measured by ELISA assay using an anti-CML-adduct
105 monoclonal antibody MK-5A10 and incubated for 1h. After extensive washing, horseradish
106 peroxidases (HRP)-conjugated polyclonal antibody specific for mouse IgG was added and further
107 incubated for 1h. Afterwards, the unbound HRP-conjugate antibody was removed. The remaining
108 conjugate was allowed to react with the substrate H_2O_2-tetramethylbenzidine. The reaction was
109 stopped by addition of acidic solution and the amount of BSA-CML was measured at 450 nm and
110 expressed in terms of ng/mL of CML. For the calibration curve, standard human serum albumin-CML
111 at concentrations between 0.30 and 10 ng/mL were used and a standard curve was constructed by
112 plotting absorbance values versus CML-adduct concentrations.

2.6 Preparation of BSA-phenolic acid adducts

BSA (50 mg/mL) was incubated at 37°C for 7 days with the tested phenolic acids (500 or 1000 µmol/L) in potassium-phosphate buffer (0.1 mol/L, pH 7.4, sodium azide 0.024%), to promote BSA-phenolic acid binding. Accumulation of BSA-phenolic acid adducts was followed by monitoring the amount of free phenolic acids every 24h by HPLC (mobile phase A: formic acid 0.1% in water, mobile phase B: 100% acetonitrile; flow rate 1 mL/min, 32°C) as described in Tagliazucchi et al.[25] HPLC system was a Jasco HPLC system (Orlando FL, U.S.A.) equipped with a diode array detector, a reversed phase column Hamilton HxSil C18 (Hamilton, Reno, Nevada; 250mm x 4.6mm) and a volumetric injector Rheodyne (Cotati, CA). At the end of the incubation, the un-bound phenolic compounds were removed by ultrafiltration with Microcon cut-off 10 kDa at 14000*g* for 50 min at 4°C. The retentate was refilled with the potassium-phosphate buffer and washed again. This washing procedure (diafiltration) was repeated three times to reduce the concentration of the free phenolic acids. The retentate was then subjected to glycation by adding 0.8 mol/L of glucose in the same conditions as reported above. The mixture was incubated for 7 days, after which, the amounts of fluorescent AGEs and CML was measured as described above.

2.7 Determination of BSA-phenolic acid adducts with NBT staining

The BSA-phenolic acid adducts were detected by staining with NBT after SDS-PAGE and blotting. SDS-PAGE was performed by using a 4% stacking gel and 10% separating gel. An amount of 10 µg of protein was loaded to each lane. Bands were visualized by Coomassie brilliant blue R-250 staining. For blotting assay, the gel bands were transferred onto a PVDF membrane and BSA-phenolic acid adducts were detected by staining the membrane with NBT (0.24 mmol/L in 2 mol/L potassium glycinate buffer, pH 10). The blotting membrane was incubated with the glycinate/NBT solution for

45 min in the dark, resulting in a purple stain where BSA-phenolic acid adducts were present.[26] To elucidate the possible catalytic role of metal ions on the formation of BSA-phenolic acid adducts, some experiments were carried out by preparing the BSA-phenolic acid adducts as described above but including in the reaction mixture 1 mmol/L of EDTA. The BSA-phenolic acid adducts were then detected by NBT staining.

2.8 Preparation of glucose-derived BSA-Amadori adducts

For the preparation of BSA-Amadori adducts, BSA (50 mg/ml) was incubated with 0.8 mol/L glucose in 0.1 mol/L potassium-phosphate buffer (pH 7.4, sodium azide 0.024%), at 37 °C. Accumulation of BSA-Amadori adducts was followed over time using the NBT method as described above. The concentration of Amadori intermediate increased in the first phase of the reaction, reaching a plateau after 72 hours of incubation (**Figure S1**). The concentration of fluorescent AGEs and BSA-CML adducts did not increase during the first 72 hours of incubation. Seventy-two hours were therefore set as the optimal time to obtain BSA-Amadori adducts without the presence of AGEs in the reaction mixture. When the concentration of Amadori intermediate reached a plateau (72h), glucose was removed by ultrafiltration with Microcon cut-off 10 kDa at 14000*g* for 50 min at 4°C. The complete removal of glucose was obtained by diafiltration procedure as described above and measuring the amount of free glucose in the filtrate with a glucose enzymatic detection kit.[25] After glucose removal, the tested phenolic acids were added to the BSA-Amadori adducts at 500 or 1000 µmol/L and the mixture incubated for 7 days. At the end of the incubation, the fluorescence AGEs and the CML were quantified as described above.

2.9 Nanoflow LC-ESI-QTOF MS analysis

160 Un-glycated and modified BSA (glycated and BSA-phenolic acid adducts) were denatured, reduced
161 and alkylated before digestion.[27] For denaturation, BSA samples (normal or modified BSA) were
162 diluted at 1 mg/mL with ammonium bicarbonate buffer (100 mmol/L; pH 8.5) containing 6 mol/L of
163 guanidine hydrochloride and incubated for 10 min at room temperature with continuous shaking.
164 Reduction of the disulphide bridges was achieved by adding 2.5 µL of DTT 10 mmol/L to 50 µL of
165 denatured BSA samples and incubated for 30 minutes at 56°C in a thermomixer. The samples were
166 then alkylated at the cysteine residues by addition of 1.8 µL iodoacetamide 55 mmol/L and allowed to
167 react for 60 min in the dark at room temperature. Finally, the protein samples were enzymatically
168 digested with trypsin (ratio enzyme/protein 1:50 w/w) at 37°C for 18 h. At the end of digestion, the
169 protease was inactivated by addition of formic acid in the amount of 10% of the final volume of the
170 digested samples.
171 The glycation and phenolic acid-adducted sites in BSA were identified by using MS-based bottom-
172 up approach.[28,29] Nano LC/MS and tandem MS experiments were performed on a 1200 Series Liquid
173 Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass Q-TOF LC/MS via a
174 Chip Cube Interface (Agilent, Waldbronn, Germany). Chromatographic separation was performed
175 on a ProtID-Chip-43(II) including a 4 mm 40 nL enrichment column and a 43 mm x 75 µm
176 analytical column, both packed with a Zorbax 300SB 5 µm C18 phase. The mobile phase consisted
177 of (A) H_2O/acetonitrile/formic acid (96.9:3:0.1, v/v/v) and (B) acetonitrile/H_2O/formic acid
178 (94.9:5:0.1, v/v/v). The sample (4µL) was loaded into the Chip enrichment column at a flow rate of 4
179 µL/min with a mobile phase consisting of 100% A, using a G1376A capillary pump. A flush volume
180 of 2 µL and a flush-out factor of 5 were used. After valve switching a gradient elution was performed
181 throughout the enrichment and analytical column at 500 nL/min using a G2226A nano pump. The
182 gradient started at 0% B for 1 min then linearly ramped up to 90% B in 70 min. The mobile phase
183 composition was maintained at 90% B for 15 min in order to wash both the enrichment and

analytical columns. The mass spectrometer was tuned and calibrated according to the manufacturer's instructions in extended dynamic range (2GHz) mode. Mass spectrometry experiments were performed in ESI positive ion mode using 1770 V capillary voltage, with a 4 L/m 350°C nitrogen desolvating gas flow. Fragmentor and Skimmer voltages were kept at 160 V and 65 V, respectively. MS level experiments were acquired in the m/z 100-1700 Th range at 1 spectrum per second rate. MS^2 level experiments were acquired using a 4 amu precursor selection width and m/z 50-1700 Th scan range at 1 spectrum per second rate. Automatic selection of precursors was performed on the MS level experiments using a maximum of 4 precursors per cycle with a 200 count threshold for selection. Active exclusion was enabled after the first precursor selection for a 0.1 min period. For identification, MS/MS spectra were converted to .mgf files and then searched against the Swiss-Prot database using Protein prospector identification software. The following parameters were considered: enzyme, trypsin; peptide mass tolerance, ± 20 ppm; fragment mass tolerance, ± 0.12 Da; variable modification, oxidation (M), phosphorylation (ST) and carbamidomethylation (C); maximal number of PTMs permitted in a single peptide 5. When modified proteins were analysed, the variable modification list was updated by adding the possible mass shift (ΔM) caused by the formation of glycation or phenolic acid adducts as reported in **Table 1**. Only peptides with best expected value <0.05 were considered.

2.10 Statistical analysis

All the data presented as mean ± SD for three replicates for each sample. All statistical analysis were performed using Graph Pad Prism 6. The differences were considered significant with $P < 0.05$. The IC_{50} was calculated using non-linear regression analysis with Graph Pad Prism 6.

3. Results

3.1 Inhibition of BSA glycation by protocatechuic acid and 3,4-dihydroxyphenylacetic acid (DHPA)

Initially, the ability of protocatechuic acid and DHPA to inhibit protein glycation was assayed by measuring the formation of fluorescent AGEs. Both the phenolic acids were effective inhibitors of fluorescent AGEs formation with protocatechuic acid (IC_{50}= 62.1 ± 1.4 μmol/L) being more effective than DHPA (IC_{50}= 155.4 ± 1.1 μmol/L).

Next, the ability of the tested phenolic acids to inhibit BSA-CML adducts formation during glycation with glucose was evaluated. The data obtained by ELISA assay demonstrated that protocatechuic acid was also more effective in the inhibition of CML formation than DHPA (IC_{50}= 535.3 ± 1.1 μmol/L and IC_{50}= 751.2 ± 1.0 μmol/L, respectively).

Figure 2 shows a significant and dose-dependent reduction in Amadori intermediate after 7 days of incubation of BSA with glucose and phenolic acids. The decrease in concentration of Amadori compounds with increasing quantity of protocatechuic and 3,4-dihydroxyphenylacetic acids suggests that these compounds may act as pre-Amadori inhibitors of protein glycation.

3.2 Glycation of BSA-phenolic acid adducts

To confirm this hypothesis, BSA-phenolic acid adducts were prepared by pre-incubating BSA with 500 and 1000 μmol/L of protocatechuic acid or DHPA for seven days at 37°C. The binding between BSA and the tested phenolic acids was monitored every 24 hours for 7 days by measuring with HPLC the amount of unbound protocatechuic acid or DHPA. The results (**Figure S2**) showed that unbound DHPA totally disappeared after 4 days of incubation (unbound DHPA residue of 4% and 6% at concentration of 500 and 1000 μmol/L, respectively). Instead, protocatechuic acid did not completely bind BSA. Indeed, about the 33% and 49% of protocatechuic acid at the tested concentration of 500 and 1000 μmol/L, respectively, were still unbound at the end of incubation period.

After the formation of BSA-phenolic acid adducts, the unbound phenolic acids were removed by ultrafiltration with 10 kDa cut-off membrane (followed by extensive washing through diafiltration procedure) and the BSA-phenolic acid adducts were further incubated with glucose to promote the glycation reaction. In this case, the inhibitory activity of the used phenolic acids would be due only to the ability of these compounds to interact with BSA avoiding the reaction between BSA and glucose. After 7 days of incubation of BSA-phenolic acid adducts with glucose, glycation reaction was monitored by measuring the formation of fluorescent compounds and CML. The percentage of inhibition of fluorescent AGEs was slightly lower when the BSA-phenolic acid adducts were incubated with glucose respect to the normal glycation reaction in which BSA, glucose and protocatechuic acid or DHPA were incubated simultaneously (**Figure 3A**). However, when CML formation was measured (**Figure 3B**), no significant differences in the inhibitory effect were found between the experiments with protocatechuic acid or DHPA pre-incubated and non pre-incubated BSA. Therefore, interaction BSA-phenolic acids is crucial for the inhibitory activity, preventing the formation of BSA-CML adducts and protecting BSA itself from the binding with glucose.

3.3 Effect of protocatechuic acid and DHPA on the formation of AGEs and CML from glucose-derived Amadori adducts

The effect of protocatechuic acid and DHPA on the formation of AGEs and CML from glucose-derived Amadori adducts was further investigated. These experiments were designed to understand the ability of the tested phenolic acids to inhibit protein glycation acting in the post-Amadori phase of the glycation reaction. BSA was firstly incubated with glucose to allow the formation of BSA-Amadori adducts.

The removal of un-reacted glucose by ultrafiltration followed by incubation again at 37°C led to the disappearance of 87% of protein-Amadori adducts after 7 days. This process was accompanied by the formation of fluorescent AGEs and BSA-CML adducts (**Table 2**).

When protocatechuic acid or DHPA were added to the BSA-Amadori adduct preparation, no significant differences between the formation of fluorescent AGEs and CML was found respect to the BSA-Amadori adduct preparation incubated without phenolic acids (**Table 2**). This means that there was no inhibition due to the tested phenolic compounds, which were not able to inhibit the post-Amadori conversion in AGEs.

3.4 Protocatechuic acid and DHPA bind BSA via metal-catalysed oxidative reaction

The incubation of BSA with protocatechuic acid or DHPA resulted in the formation of a phenolic acid-modified BSA. **Figure 4A** shows the SDS–PAGE patterns of BSA incubated for 7 days with and without the tested phenolic acids. In the presence of phenolic acids, the SDS–PAGE patterns were similar to that of the control sample. A parallel SDS–PAGE experiment was performed and the gel bands were electrically transferred onto a polyvinylidene fluoride membrane prior to detection of quinoproteins by NBT staining. As shown in **Figure 4B**, BSA-phenolic acid adducts were observed in lanes 2, 3, 4 and 5, to which the samples containing DHPA and protocatechuic acid were applied. Moreover, the addition of EDTA 1 mmol/L to the incubation mixture completely inhibited BSA-phenolic acid adducts formation (**Figure 4C** and **4D**).

3.5 Analysis of glycation sites in BSA

Un-glycated and modified BSA samples were hydrolysed with trypsin and peptides in the mixture were then separated by LC prior to introduction in the ESI-Q-TOF mass analyser. The sequence data, first obtained by comparison with database, were then validated through a manual inspection

of the MS/MS spectra (examples of chromatograms and fragmentation spectra are given in supplementary **Figure S3**). Data pertaining to the tryptic digest of glycated BSA showed the presence of most of the peptides detected in the case of the digested un-glycated BSA. However, as expected, a series of new peptides occurred in the glycated BSA sample. Considering all the sequenced peptides, the protein coverages were 86.4 and 88.9% for un-glycated and glycated BSA, respectively. The complete lists of peptides identified in the un-glycated and glycated BSA are reported in supplementary online materials (**Tables S1 and S2**). The identified modified residues are shown in **Table 3**. The majority of the lysine residues were modified by fructosyl-lysine (FL). Three lysine residues (K242, K439 and K556) were found to be modified by CML.

The analysis of the peptide sequences identified in the protocatechuic- and DHPA-BSA adducts showed that some residues, comprising the CML-modified K439 and K556, were also sites of modification from protocatechuic and DHPA. Phenolic modification resulted in the addition of phenolic acids or of the quinone form of phenolic acids (both decarboxylate and non). The complete lists of peptides identified in the protocatechuic-modified and DHPA-modified BSA are reported in supplementary online materials (**Tables S3 and S4**). The sequence coverages were 79.4 and 81.4% for BSA-protocatechuic acid adducts and BSA-DHPA adducts, respectively.

4. Discussion

Following ingestion, phenolic compounds undergo extensive metabolism during their passage through the gastrointestinal tract. Only metabolites of the parent compounds, with very few exceptions, enter the circulatory system. Metabolism initially occurs in the lumen of the small intestine with cleavage of the sugar moieties and then the released aglycone can actually be absorbed by passive diffusion. Absorbed aglycones undergo glucuronidation, sulphation and/or methylation in the liver and, to a lesser extent, in the enterocytes. However, a large proportion of ingested polyphenols are not absorbed

302 in the small intestine thus reaching the colon, where substantial structural modifications are mediated
303 by the colonic microflora. The resultant metabolites, principally low molecular weight phenolic acids,
304 are absorbed into the blood stream and circulate in the body in amounts that greatly exceed those of
305 the parent compounds.[30]
306 Phenolic compounds used in the current study were protocatechuic and 3,4 dihydroxyphenylacetic
307 (DHPA) acids, detectable as metabolites in plasma after intestinal absorption and metabolism.
308 Protocatechuic acid is the major human metabolite derived from the colonic metabolism of ingested
309 cyaniding glycosides. Cyanidin glycosides undergo de-glycosylation in the small intestine by β-
310 glucosidases. Residual aglycone is then degraded in protocatechuic acid directly in the lumen or after
311 bloodstream absorption.[31] DHPA derives from flavonoids (such as flavonols and flavanones) after the
312 action of intestinal microbiota. These phenolic compounds are firstly de-glycosylated by intestinal β-
313 glucosidases and then thoroughly metabolized by colonic microbiota in a plethora of metabolites, with
314 DHPA being produced in the highest amount.[32]
315 The inhibition of AGEs formation in the human body plays a key role in the prevention of many
316 metabolic disorders such as diabetes type 2, cardiovascular diseases, Alzheimer's disease and
317 neuropathy.[1,33] Different studies have shown that phenolic compounds can inhibit the glycation
318 reaction of various proteins by glucose, decreasing the formation of AGEs.[22,34] Despite the numerous
319 studies showing the anti-glycative ability of polyphenols, the exact mechanism of action is still
320 unknown.
321 Our previous studies showed that the inhibition of glycation mediated by phenolic compounds was
322 not exerted through the glucose auto-oxidation-mediated pathway, therefore suggesting that the
323 glucose-mediated pathway (pathway 1, **Figure 1**) was the most likely site for the inhibitory effects of
324 the phenolic compounds.[4,24] Moreover, these studies concluded that radical scavenging and metal-
325 chelating activities were not important for the inhibitory effect of phenolic compounds. On the

326 contrary, the ability of coffee polyphenols to inhibit protein glycation, leading to a reduction of the
327 Amadori compounds concentration has been reported, suggesting that these compounds may act as
328 inhibitors in the pre-Amadori phase rather than in the post-Amadori phase of glycation.[24]
329 Furthermore, Vlassopoulos et al. found that incubation with phenolic acids prior to glycation
330 significantly inhibited the process, reinforcing our hypothesis that polyphenols could act as inhibitor
331 in the early stage of glycation.[18] In the present work, the mechanism of inhibition of AGEs and
332 protein-CML adducts formation by protocatechuic acid and DHPA, during glycation with supra-
333 physiological glucose concentrations has been investigated. Simultaneous incubation of BSA with
334 glucose and protocatechuic acid or DHPA resulted in a concentration-dependent decrease in AGEs
335 and CML production and in the amount of Amadori compounds. This last observation pointed to a
336 mechanism of inhibition in the pre-Amadori phase of the glycation reaction. Then, BSA-phenolic
337 acid adducts were useful to demonstrate that the tested phenolic acids inhibit the formation of AGEs
338 and BSA-CML adducts by binding BSA and protecting BSA itself from the reaction with glucose.
339 The BSA-phenolic acid adducts were not subjected to glycation when incubated with glucose, after
340 the removal of unbound phenolic acids. These experiments demonstrated that the binding between
341 protocatechuic acid or DHPA and BSA is a crucial pre-requisite for the inhibitory effect of these
342 compounds. On the contrary, the tested phenolic acids were not able to inhibit the conversion of
343 BSA-Amadori adducts to AGEs and BSA-CML adducts.
344 With MS experiments, 20 glycation sites involving lysine were identified. The predominant
345 modifications involved the formation of fructosyl-lysine (14 lysine residues) and CML (3 lysine
346 residues). In addition, two arginine residues were found to be modified by argpyrimidine. Some of the
347 glycation sites had been already described in BSA. Residues K12, K51, K232, K396, K413, K439,
348 K524 and K544 were previously characterized as sites of non-enzymatic glycosylation in BSA
349 incubated with glucose.[35,36] Glycation of BSA with fructose resulted in the modification of K51 and

K431.[35] Most of the glycated lysine residues were totally or partially exposed to the solvent in the three-dimensional structure of BSA and therefore freely accessible to glucose.[37] The rest of the residues (K232, K242, K439 and K544) are buried in the structure of BSA and therefore, not freely accessible to glucose. However, extensive glycation resulted in the partial unfolding of BSA with increased accessibility of the hydrophobic areas of the protein.[38] This can result in the exposition of the buried lysine residues, which can promote the glycation reaction at these sites.

BSA contains in its sequence 59 lysine residues. Nevertheless, we found that only 20 residues are potential glycation sites. This is indicative of site-specificity in the non-enzymatic glycation of lysine residues in BSA as already reported.[38,39]

The acid-base catalysis of the Amadori rearrangement has been proposed as a possible explanation.[39] The proximity of positively-charged basic amino acids (such as lysine, arginine and histidine) to lysine was found to influence the glycation of lysine residues, particularly in regions close to disulphide bonds.[39,40]

Considering the position of the glycosylated sites in BSA, it is striking that 5 sites are located in a sequence of basic amino acids: K132, K523 and K524 in a Lys-Lys sequence, K242 in a His-Lys sequence, and K396 in an Arg-Lys sequence. The tertiary structure of BSA may place some positively charged residues, located in a remote part of the sequence, close to the glycation sites. For example, K116 is located less than 24Å to the residue K431, which, in turn, is located less than 20Å to the residue K439.[41] This is an example of how the three-dimensional structure of BSA may produce regions with a strong tendency to be modified during glycation. The residue K413 is located less than 20Å to the residue K537, which is part of a Lys-His-Lys sequence.[41] Also, the residue K377 is found less than 24Å to the residue K375.

As revealed by MS analysis, some of the glycation sites are also binding sites for protocatechuic acid and/or DHPA. Some peptides with modifications, which can be ascribed to the addition of

374 protocatechuic acid and DHPA, were found in the tryptic digests of BSA-phenolic acid adducts (see
375 also **supplementary material Tables S1-S4**). These were consistent with the addition of phenolic
376 acids and decarboxylate phenolic acids (DHPA, PCA, DHPA(-CO_2) and PCA(-CO_2), in **Table 3**) or
377 the quinoidal forms of phenolic acids and decarboxylate phenolic acids (QDHPA, QPCA,
378 QDHPA(-CO_2) and QPCA(-CO_2), in **Table 3**).
379 From MS experiments, we can assume that two different pathways were involved in phenolic acid-
380 BSA binding. Protocatechuic acid and DHPA at 37°C and pH 7.5 are easily oxidized to form a
381 quinone moiety through a pathway involving semiquinone radicals and active oxygen species.[42] The
382 resultant electron-deficient quinones may react with the nucleophilic groups (sulfhydryl and amino
383 groups) of BSA to form a phenolic-protein adduct (**Figure 5**).
384 In the second pathway, protocatechuic acid and DHPA (under alkaline and oxidative conditions and
385 in presence of oxygen), can undergo oxidative decarboxylation, presumably *via* the corresponding
386 phenoxyl radical.[43] It resulted in the generation of the decarboxylate quinonoidal form of phenolic
387 acids that, in turn, may react with the nucleophilic groups (sulfhydryl and amino groups) of BSA to
388 form a phenolic-protein adduct (**Figure 6**).
389 Metal ions (Fe^{2+}, Fe^{3+} and Cu^{2+}) can play a catalytic role in the free radical-mediated oxidation of
390 phenolic acids.[26] EDTA is a versatile metal-ion chelator and is able to form stable complexes with
391 various metal ions including the transition-metal ions. In the present study, EDTA showed an
392 inhibitory effect on the formation of BSA-phenolic acid adducts, presumably due to its ability to
393 complex metal ions that catalyse the oxidation of phenolic acids. Finally, since the BSA-phenolic
394 acid adducts were also found after SDS-PAGE and incubation in the presence of SDS (destroying
395 of non-covalent protein interactions), it can be assumed that covalent binding had occurred.
396 The BSA-phenolic acid adducts formed through the described pathways were not subjected to
397 glycation when incubated with glucose, after the removal of unbound phenolic acids. These

398 experiments demonstrated that the binding between the tested phenolic acids and BSA is a crucial
399 pre-requisite for the inhibitory effect of these compounds. On the contrary, protocatechuic acid and
400 DHPA were not able to inhibit the conversion of BSA-Amadori adducts to AGEs and BSA-CML
401 adducts.
402 These data demonstrated that protocatechuic acid and DHPA were able to inhibit glycation of BSA
403 by acting in the early (pre-Amadori) phase of the glycation and that the effect was mediated by the
404 binding of phenolic acids with BSA. The proposed mechanism of inhibition is reported in **Figure 7**.
405 *In vitro* studies are useful to produce helpful observations that are relevant to human health if they
406 are meticulously designed and interpreted. To exert their biological activity *in vivo*, phenolic
407 compounds have to reach the appropriate body compartment, at relevant concentrations and for a
408 period of time necessary to ensure the supposed activity. However, many published statements on
409 health benefits of phenolic compounds are extrapolated from *in vitro* studies that are often far from
410 real physiological *in vivo* conditions. In this study, we applied a well-known and widely used *in*
411 *vitro* model to investigate the mechanisms of anti-glycative activity of two bioavailable phenolic
412 acids, i.e. protocatechuic acid and DHPA. We demonstrated that the tested phenolic acids were able
413 to modulate the early stages of protein glycation. Incubation of BSA with protocatechuic acid or
414 DHPA prior to glycation significantly inhibited the process by binding to the key sites of glycation
415 in BSA through a metal-catalysed oxidative mechanism. Our data raised some questions. Is the
416 observed mechanism relevant in physiological *in vivo* conditions? Are the previously reported data
417 obtained by using this model transferable to *in vivo* conditions? Which is their relevance for human
418 health? Our idea is that *in vitro* models designed using supra-physiological glucose concentrations
419 are far from physiological conditions and it is unlikely that the proposed mechanism occurs *in vivo*.
420 These results do not exclude a possible *in vivo* anti-glycative effect of phenolic compounds but raise

421 the problem to adopt *in vitro* models closer to physiological conditions in testing the anti-glycative
422 properties of phenolic compounds, in order to reach biologically significant conclusions.

423

Acknowledgements

This research received no specific grant from any funding agency. The authors acknowledge the Fondazione Cassa di Risparmio di Modena for funding the HPLC-ESI-QTOF system at the Centro Interdipartimentale Grandi Strumenti (CIGS).

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Figure captions

Figure 1. **Possible chemical pathways leading to the formation of advanced glycation end products**. Me^{n+}: metal cations; ROS: reactive oxygen species.

Figure 2. **Dose-dependent inhibition of BSA-Amadori adducts formation by phenolic acids.** Phenolic acids tested were protocatechuic acid (●) and 3,4-dihydroxyphenylacetic acid (■). Data are expressed as the mean ± SD of three replicates. BSA: bovine serum albumin.

Figure 3. **Effect of BSA-phenolic acid adducts on the formation of AGEs (A) and CML (B)**. In a first set of experiments, BSA was incubated in presence of protocatechuic acid or DHPA and glucose for seven days at 37°C and the percentage of inhibition of AGEs and CML formation was assessed (black bars). In a parallel set of experiments, BSA-phenolic acid adducts were prepared by pre-incubating BSA and the tested phenolic acids for seven days at 37°C. Un-bound phenolic acids were removed by ultrafiltration and the BSA-phenolic acid adducts were incubated with glucose for seven days at 37°C, after that the percentage of inhibition of AGEs and CML formation was assessed (white bars). Data are expressed as the mean ± SD of three replicates. BSA: bovine serum albumin; AGEs: advanced glycation end products; CML: carboxymethyl-lysine; PCA: protocatechuic acid; DHPA: 3,4-dihydroxyphenylacetic acid.

Figure 4. **SDS–PAGE and NBT-staining assay for the detection of BSA-phenolic acid adducts and inhibitory effect of EDTA**. (A) SDS–PAGE of BSA prepared in the absence (lane 1) and in the presence of 500 or 1000 µmol/L of PCA (lane 2 and 3) and 500 or 1000 µmol/L of DHPA (lane 4 and 5). (B) NBT-staining results. The sample have the same order as in (A). (C) SDS–PAGE of BSA incubated with 1 mmol/L EDTA in the absence (lane 1) and in the presence of 500 or 1000 µmol/L of PCA (lane 2 and 3) and 500 or 1000 µmol/L of DHPA (lane 4 and 5). (D) NBT-staining results. The sample have the same order as in (C). The showed results are representative of three

independent experiments. PCA: protocatechuic acid; DHPA: 3,4-dihydroxyphenylacetic acid; BSA: bovine serum albumin; NBT: nitro blue tetrazolium.

Figure 5. **A proposed mechanism for PCA/DHPA covalently binding to BSA amino-groups through a quinone intermediate formed in a metal-ion catalysed auto-oxidation**. At 37°C and slight alkaline condition, protocatechuic acid and DHPA may undergo to oxidation forming a quinone intermediate through a metal-ion catalysed auto-oxidation. The resultant electron-deficient quinone intermediate may react with the nucleophilic groups (such as amino groups) of BSA to form a phenolic acid-protein adduct or a phenolic acid quinone-protein adduct. EDTA is able to chelate metal ion inhibiting the formation of phenolic-protein adduct. PCA: protocatechuic acid; DHPA: 3,4-dihydroxyphenylacetic acid; BSA: bovine serum albumin. R: -COOH (PCA) or -CH_2COOH (DHPA).

Figure 6. **A proposed mechanism for PCA (A) or DHPA (B) covalently binding to BSA amino-groups through a metal-ion catalysed oxidative decarboxylation pathway**. Protocatechuic acid and DHPA (under alkaline and oxidative conditions), can undergo to oxidatively induced decarboxylation, *via* the corresponding phenoxyl radical, resulting in the generation of the decarboxylate quinonoidal form of phenolic acids. The resultant electron-deficient decarboxylate quinone intermediate may react with the nucleophilic groups (such as amino groups) of BSA to form a decarboxylate phenolic acid-protein adduct or a decarboxylate phenolic acid quinone-protein adduct. EDTA is able to chelate metal ion inhibiting the formation of phenolic-protein adduct. PCA: protocatechuic acid; DHPA: 3,4-dihydroxyphenylacetic acid; BSA: bovine serum albumin.

Figure 7. **A model for inhibition of AGE/CML formation by protocatechuic acid and DHPA**. In presence of metal-ion, protocatechuic acid and DHPA are oxidized to the respective quinone intermediate which in turn react with the amino group of proteins leading to the formation of phenolic acid-protein adducts, and preventing the reaction between glucose and the amino group of

the protein. EDTA is able to chelate metal ion inhibiting the formation of phenolic-protein adducts.

CML: carboxymethyl-lysine; AGEs: advanced glycation end-products

Table 1. List of variable modifications considered in this study in the mass spectrometry analysis of glycated bovine serum albumin (BSA) and BSA-phenolic acid adducts.

Sample	*Modification*	*Amino acid*[a]	*Acronym*	*ΔM (Da)*
Glycated BSA				
	Fructosyl-lysine	K	FL	162.05
	Fructosyl-lysine (-1H_2O)	K	FL(-1H_2O)	144.04
	Fructosyl-lysine (-2H_2O)	K	FL(-2H_2O)	126.03
	N^{ε}-carboxymethyl-lysine	K	CML	58.01
	N^{ε}-carboxyethyl-lysine	K	CEL	72.02
	Pyrraline	K	Pyr	108.02
	Argpyrimidine	R	ArgP	80.03
BSA-phenolic acid adducts				
	Protocatechuic acid	K, R, C	PCA	152.02
	Decarboxylate protocatechuic acid	K, R, C	PCA(-CO_2)	108.02
	Protocatechuic acid quinone	K, R, C	QPCA	150.02
	Decarboxylate protocatechuic acid quinone	K, R, C	QPCA(-CO_2)	106.02
	DHPA	K, R, C	DHPA	166.04
	Decarboxylate DHPA	K, R, C	DHPA(-CO_2)	122.04
	DHPA quinone	K, R, C	QDHPA	164.04
	Decarboxylate DHPA quinone	K, R, C	QDHPA(-CO_2)	120.04

[a]One letter amino acid code. K identify lysine; R identify arginine; C identify cysteine
DHPA: 3,4-dihydroxyphenylacetic acid

Table 2. Effect of phenolic acids on the formation of fluorescent AGEs and carboxymethyl-lysine after 7 days of incubation with BSA glucose-derived Amadori adducts.

	Phenolic acid concentration (µmol/L)	*AGEs (AUF)*	*CML (mmol/mol BSA)*
No addition	/	12658 ± 289	4.10 ± 0.14
PCA addition	500	13106 ± 231	3.99 ± 0.17
	1000	12929 ± 105	4.00 ± 0.17
DHPA addition	500	13268 ± 168	3.89 ± 0.20
	1000	13102 ± 157	4.10 ± 0.16

BSA: bovine serum albumin; CML: carboxymethyl-lysine; AGEs: advanced glycation end-products; PCA: protocatechuic acid; DHPA: dihydroxyphenylacetic acid; AUF: arbitrary unit of fluorescence.

Table 3. Identified modification sites found in *in vitro* glycated bovine serum albumin (BSA) and in BSA-phenolic acid adducts.

Modified residue[a]	*Type of modification*		
	Glycated BSA	BSA-PCA adducts	BSA-DHPA Adducts
K12	FL	n.d.	DHPA(-CO_2)/ QDHPA(-CO_2)
C34	n.d.	n.d.	DHPA/DHPA(-CO_2)
K51	FL	PCA(-CO_2)	n.d.
C62	n.d.	PCA(-CO_2)	n.d.
K64	FL	PCA(-CO_2)	QDHPA(-CO_2)
K116	FL	n.d.	n.d.
K132	FL	n.d.	n.d.
K232	FL	n.d.	QDHPA
K242	CML/FL	PCA(-CO_2)	n.d.
C245	n.d.	PCA(-CO_2)	n.d.
R256	n.d.	QPCA(-CO_2)	n.d.
K261	FL	PCA(-CO_2)	QDHPA
K294	FL	n.d.	DHPA(-CO_2)/ QDHPA(-CO_2)
K322	FL	n.d.	n.d.
R336	n.d.	n.d.	DHPA/DHPA(-CO_2)
K350	FL	n.d.	n.d.
K377	FL	QPCA	n.d.
K388	n.d.	n.d.	QDHPA
K396	FL	PCA(-CO_2)	QDHPA
R409	n.d.	PCA	n.d.
K413	FL	PCA(-CO_2)	DHPA(-CO_2)/ QDHPA(-CO_2)
K431	CEL/FL/Pyr	PCA(-CO_2)/QPCA(-CO_2)	QDHPA
R435	ArgP	PCA(-CO_2)/QPCA(-CO_2)	n.d.

K439	FL/FL(-2H$_2$0)/CML	PCA/PCA(-CO$_2$)/QPCA(-CO$_2$)	QDHPA(-CO$_2$)
R444	ArgP	n.d.	n.d.
K523	FL	n.d.	n.d.
K524	FL	n.d.	n.d.
K544	FL	n.d.	QDHPA
K556	CML	QPCA(-CO$_2$)	DHPA(-CO$_2$)

[a]One letter amino acid code. K identify lysine; R identify arginine; C identify cysteine
BSA: bovine serum albumin; FL: fructosyl-lysine; FL(-2H$_2$O): fructosyl-lysine (-2H$_2$O) CML: carboxymethyl-lysine; CEL:carboxyethyl-lysine; Pyr: pyrraline; ArgP: argpyrimidine; PCA: protocatechuic acid; PCA(-CO$_2$): decarboxylate protocatechuic acid; QPCA: protocatechuic acid quinone; QPCA(-CO$_2$): decarboxylate protocatechuic acid quinone; DHPA: dihydroxyphenylacetic acid; DHPA(-CO$_2$): decarboxylate dihydroxyphenylacetic acid; QDHPA: dihydroxyphenylacetic acid quinone; QDHPA(-CO$_2$): decarboxylate dihydroxyphenylacetic acid quinone.

Figure 1

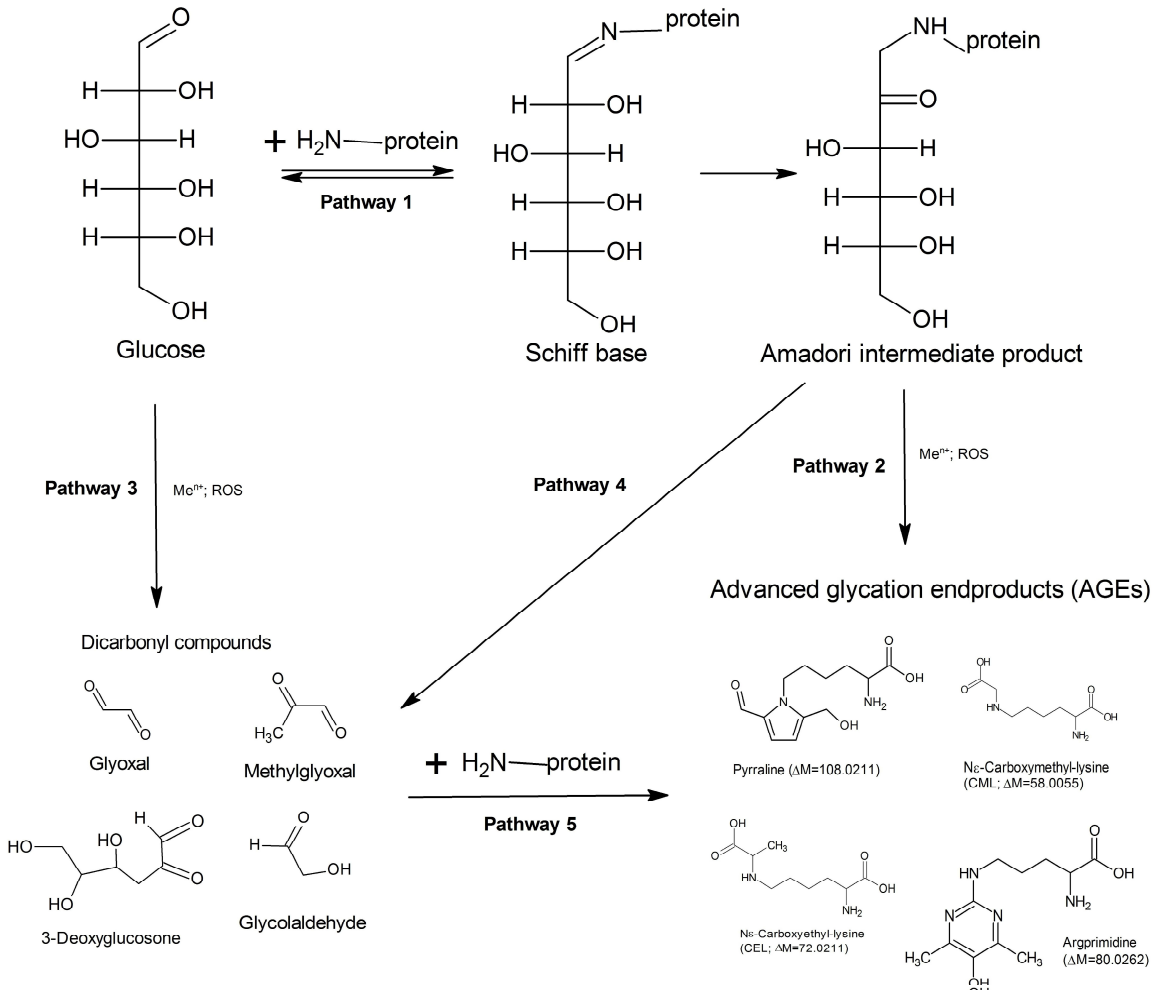

Glucose
+ H2N—protein
Pathway 1
Schiff base
Amadori intermediate product
Pathway 3
Men+; ROS
Pathway 4
Pathway 2
Men+; ROS
Advanced glycation endproducts (AGEs)
Dicarbonyl compounds
Glyoxal
Methylglyoxal
3-Deoxyglucosone
Glycolaldehyde
+ H2N—protein
Pathway 5
Pyrraline (ΔM=108.0211)
Nε-Carboxymethyl-lysine
(CML ; ΔM=58.0055)
Nε-Carboxyethyl-lysine
(CEL; ΔM=72.0211)
Argprimidine
(ΔM=80.0262)

Figure 2

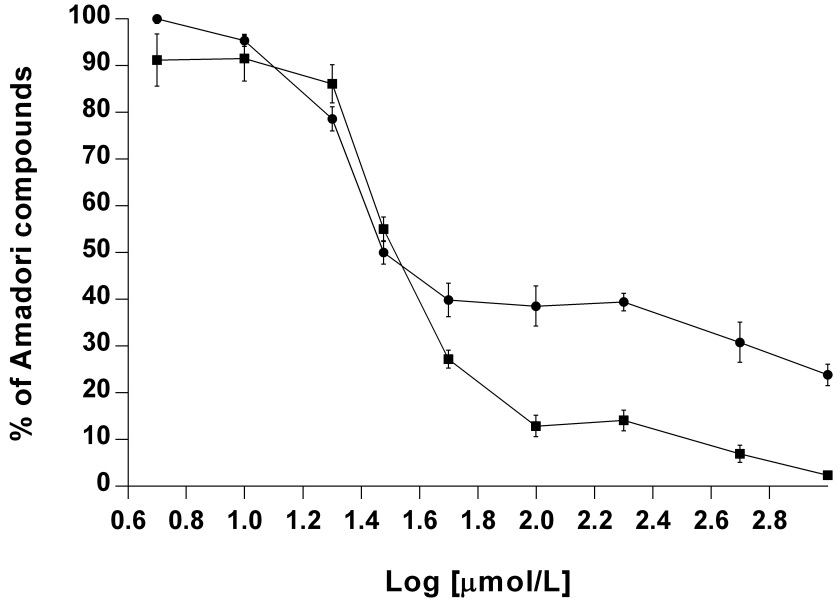
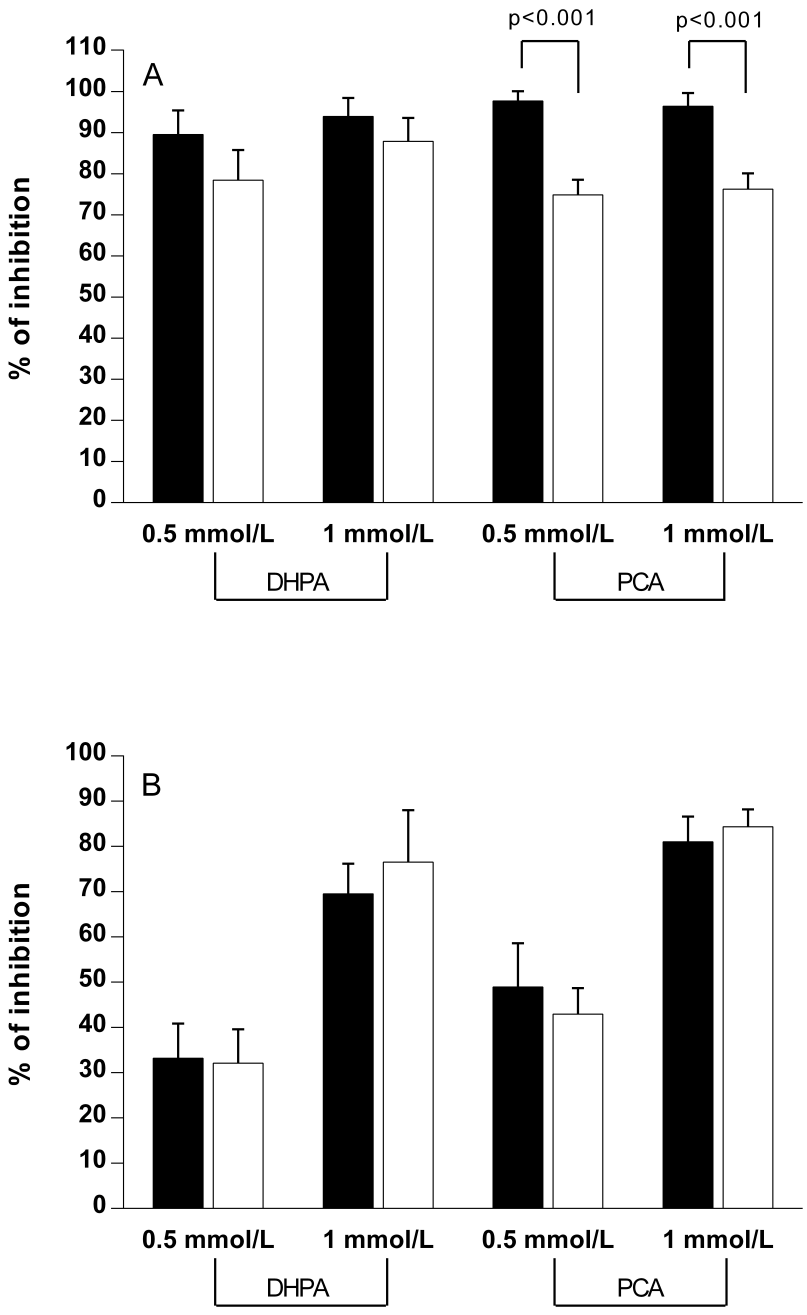

% of Amadori compounds
100
90
80
70
60
50
40
30
20
10
0
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
2.6
2.8
Log [μmol/L]

Figure 3


A
p<0.001
p<0.001
% of inhibition
110
100
90
80
70
60
50
40
30
20
10
0
0.5 mmol/L
1 mmol/L
0.5 mmol/L
1 mmol/L
DHPA
PCA
B
% of inhibition
100
90
80
70
60
50
40
30
20
10
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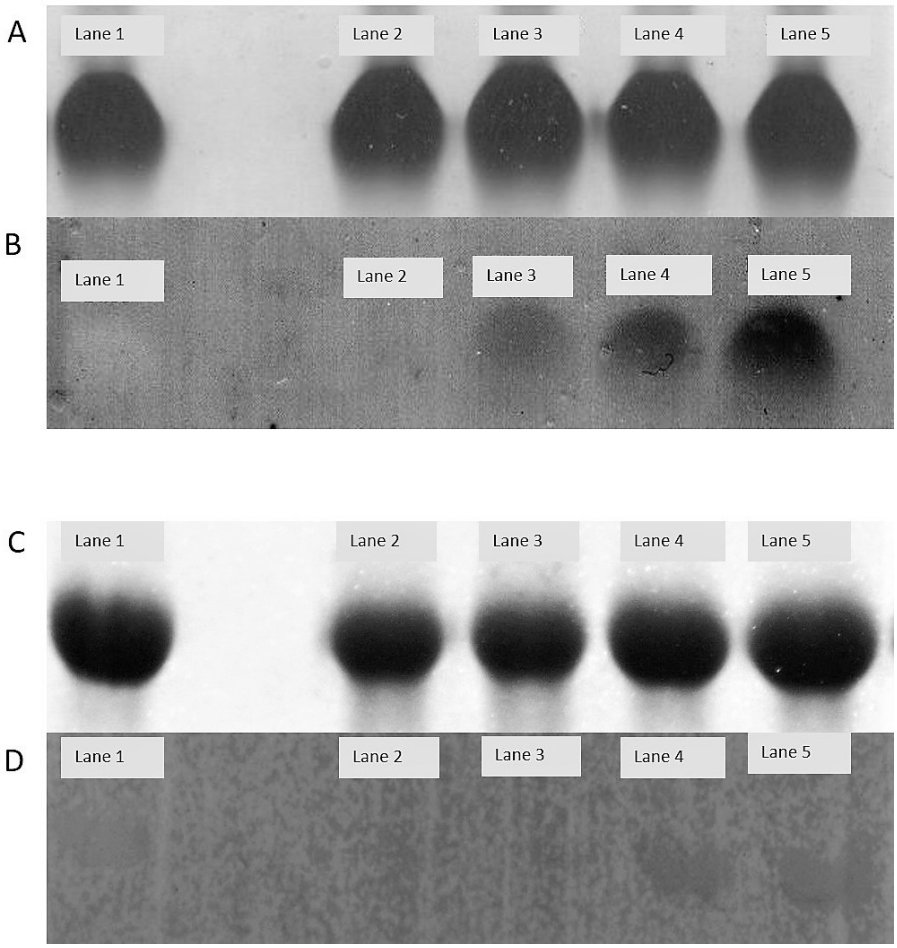
0.5 mmol/L
1 mmol/L
0.5 mmol/L
1 mmol/L
DHPA
PCA

Figure 4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Lane 1
Lane 2
Lane 3
Lane 4
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Lane 4
Lane 5
C
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Lane 2
Lane 3
Lane 4
Lane 5
D
Lane 1
Lane 2
Lane 3
Lane 4
Lane 5

Figure 5

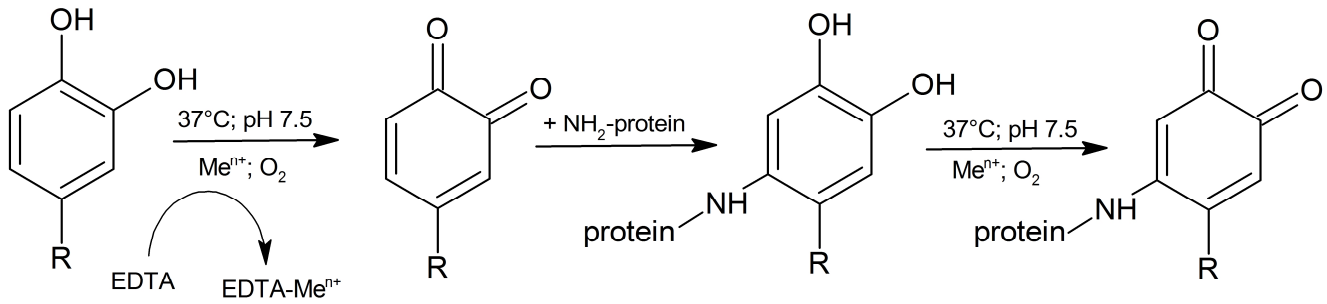

OH
OH
R
37°C; pH 7.5
Me^{n+}; O$_2$
EDTA
EDTA-Me^{n+}
O
O
R
+ NH$_2$-protein
OH
OH
protein
NH
R
37°C; pH 7.5
Me^{n+}; O$_2$
O
O
protein
NH
R

Figure 6

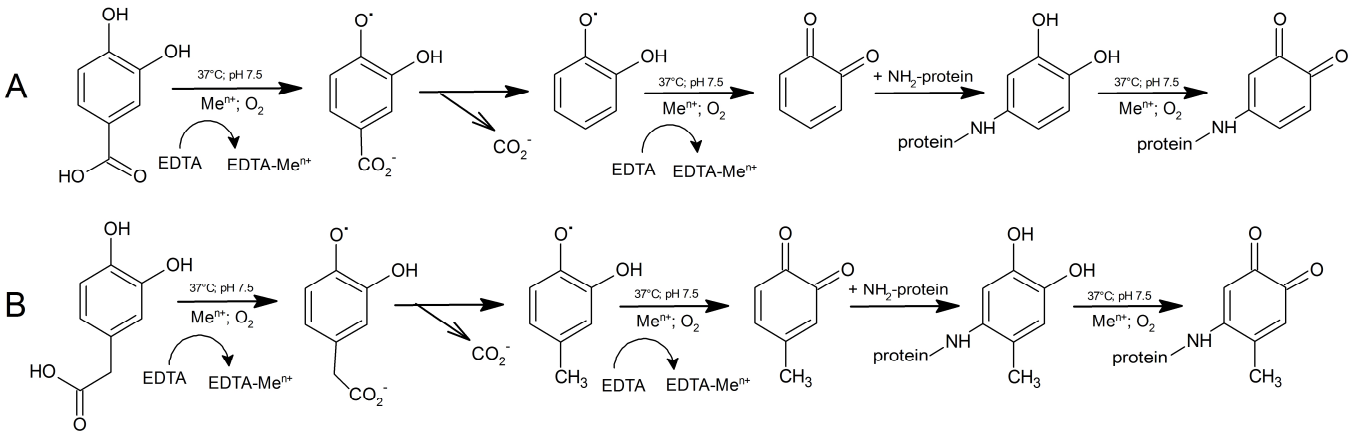
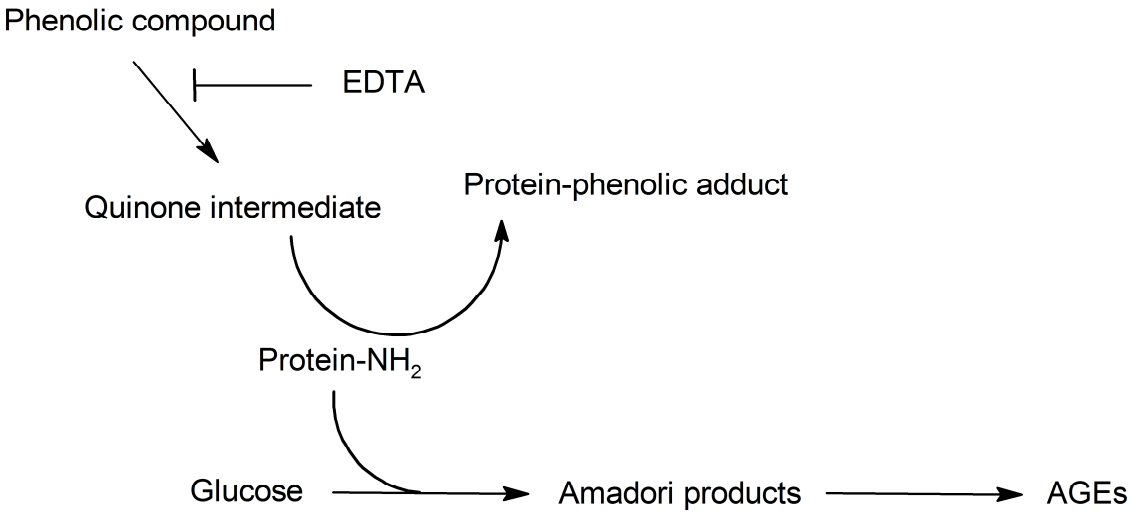

A
B
37°C; pH 7.5
Me^{n+}; O$_2$
EDTA
EDTA-Me^{n+}
CO$_2^-$
+ NH$_2$-protein
protein
NH
OH
HO
O
CH$_3$

Figure 7


Phenolic compound
EDTA
Quinone intermediate
Protein-phenolic adduct
Protein-NH2
Glucose
Amadori products
AGEs

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