

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

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1 **Abstract**

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

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

16

## 17 1. Introduction

18 Glycation of circulating, cellular, and matrix proteins by glucose are thought to be a major factor in  
19 pathogenesis of diabetes and related cardiovascular diseases.<sup>1</sup> This process is known as the Maillard  
20 reaction and begins with the nucleophilic addition between an amino group of a protein and the  
21 carbonyl group of glucose to form a reversible Schiff base (**Figure 1** pathway 1).<sup>2</sup> The latter can  
22 rearrange in Amadori compounds, that can be fragmented by oxidation in presence of reactive oxygen  
23 species (ROS) and transition metal ions such as  $\text{Fe}^{3+}$  and  $\text{Cu}^{2+}$  (**Figure 1** pathway 2). This oxidative  
24 degradation could lead to the formation of the so called advanced glycation end products (AGEs).  
25 Amadori products can also be transformed into reactive dicarbonyl products (**Figure 1** pathway 4),  
26 which could react with amino group of proteins generating AGEs (**Figure 1** pathway 5). Another  
27 pathway implicated in AGEs formation involves glucose auto-oxidation. Glucose can be directly  
28 oxidized in the presence of catalytic metals and ROS, generating dicarbonyl compounds (**Figure 1**  
29 pathway 3) which can further react with the amino groups of proteins (**Figure 1** pathway 5).<sup>3,4</sup>  
30 Several AGEs have been identified in tissues and circulating proteins.<sup>5</sup> Principal sites of glycation in  
31 the proteins are lysine side chain, arginyl guanidine groups and N-terminal amino group of proteins.<sup>6,7</sup>  
32 Glycation of the  $\epsilon$ -amino group of lysine may result *in vivo* in the formation of  $N^\epsilon$ -carboxymethyl-  
33 lysine (CML),  $N^\epsilon$ -carboxyethyl-lysine (CEL) and pyrraline (**Figure 1**), whereas glycation at the  
34 arginine guanidine group level produced argpyrimidine (**Figure 1**) and other less frequent AGEs.<sup>6,8</sup>  
35 Some AGEs are characterized by more complex structures forming in proteins intra- and inter-  
36 molecular crosslinks, such as pentosidine, glucosepane and imidazolium compounds.  
37 CML is often used as marker of AGEs rising from glycation reaction since it is the major AGE  
38 produced *in vivo*.<sup>9</sup> It can arise from various pathways, such as condensation of glucose with the  $\epsilon$ -  
39 amino group of lysine, generating the derived Amadori compounds, fructoselysine (FL). FL is an  
40 unstable intermediate that can further undergo oxidation to form CML.<sup>10</sup> Another CML formation

41 pathway involves the direct reaction of dicarbonyl compounds with the  $\epsilon$ -amino group of lysine.<sup>6</sup>  
42 CML-modified proteins have been detected in plasma, renal tissues and skin collagen of diabetic  
43 patients.<sup>11</sup> They accumulate mainly in proteins with a long half-life, altering their structural and  
44 biochemical properties and are involved in some metabolic diseases such as diabetes type 2,  
45 cardiovascular diseases, Alzheimer's disease and ageing.<sup>11-13</sup> CML-modified proteins has also been  
46 reported to be a ligand for the receptor of advanced glycation end products (RAGE).<sup>14</sup> RAGE  
47 activate several signalling transduction pathways, including the pathway for intracellular ROS  
48 generation, inducing secondary oxidative stress-mediated apoptosis.<sup>15,16</sup>  
49 Dietary phenolic compounds and their metabolites may exert beneficial effects in the control of  
50 diabetes and its complications thanks to their ability to inhibit oxidative stress and protein  
51 glycation.<sup>4,17,18</sup> Although *in vitro* studies have been employed to assess the anti-glycative potential  
52 of phenolic compounds, only few were designed using physiologically relevant glucose  
53 concentrations.<sup>4,17</sup> Instead, the majority of applied *in vitro* models used supra-physiological glucose  
54 concentration (usually hundreds of mmol/L concentrations) and their physiological relevance to  
55 human health is sometimes questionable.<sup>19-21</sup> Indeed, there is still a lack of information about the  
56 exact mechanism of action. Phenolic compounds may offer protection by chelating transition metals  
57 or by scavenging ROS, which are produced during the glycation reaction, slowing down the  
58 glycation process and inhibiting the formation of AGEs.<sup>22</sup> Another possible mechanism reflects the  
59 ability of phenolic compounds to trap dicarbonyl compounds generated during protein glycation.<sup>23</sup>  
60 Besides that, in a previous work, it has been shown that antioxidant activity, chelating ability and  
61 dicarbonyl trapping activity were not important for the anti-glycative activity of phenolic  
62 compounds.<sup>4</sup> Moreover, in the same work it has been suggested that the post-Amadori pathways 2, 4  
63 and 5 and the glucose auto-oxidation pathway 3 are not the site of inhibition (**Figure 1**). In a further  
64 study, it has been found that coffee chlorogenic acids were able to inhibit protein glycation. This

65 inhibitory effect was related to a reduction in the Amadori product concentrations without AGEs  
66 formation. All these studies gave evidence that phenolic compounds could act as pre-Amadori  
67 inhibitors of protein glycation.<sup>24</sup>

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

72

## 73 **2. Materials and Methods**

### 74 **2.1 Materials**

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

82

### 83 **2.2 Bovine serum albumin (BSA) glycation**

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

88

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

90 The formation of Amadori compounds was determined using a NBT assay.<sup>24</sup> 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<sub>50</sub> (concentration of inhibitor required to inhibit  
100 glycation by 50%) calculated from log dose inhibition curve.<sup>24</sup> Fluorescence was read using a  
101 FLUOstar Optima microplate reader (BMG Labtech, Offenburg, Germany).

102

103 **2.5 Detection of N<sup>ε</sup>-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<sub>2</sub>O<sub>2</sub>-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.

113

## 114 ***2.6 Preparation of BSA-phenolic acid adducts***

115 BSA (50 mg/mL) was incubated at 37°C for 7 days with the tested phenolic acids (500 or 1000  
116  $\mu\text{mol/L}$ ) in potassium-phosphate buffer (0.1 mol/L, pH 7.4, sodium azide 0.024%), to promote BSA-  
117 phenolic acid binding. Accumulation of BSA-phenolic acid adducts was followed by monitoring the  
118 amount of free phenolic acids every 24h by HPLC (mobile phase A: formic acid 0.1% in water,  
119 mobile phase B: 100% acetonitrile; flow rate 1 mL/min, 32°C) as described in Tagliazucchi et al.<sup>25</sup>  
120 HPLC system was a Jasco HPLC system (Orlando FL, U.S.A.) equipped with a diode array detector,  
121 a reversed phase column Hamilton HxSil C18 (Hamilton, Reno, Nevada; 250mm x 4.6mm) and a  
122 volumetric injector Rheodyne (Cotati, CA). At the end of the incubation, the un-bound phenolic  
123 compounds were removed by ultrafiltration with Microcon cut-off 10 kDa at 14000g for 50 min at  
124 4°C. The retentate was refilled with the potassium-phosphate buffer and washed again. This  
125 washing procedure (diafiltration) was repeated three times to reduce the concentration of the free  
126 phenolic acids. The retentate was then subjected to glycation by adding 0.8 mol/L of glucose in the  
127 same conditions as reported above. The mixture was incubated for 7 days, after which, the amounts of  
128 fluorescent AGEs and CML was measured as described above.

129

## 130 ***2.7 Determination of BSA-phenolic acid adducts with NBT staining***

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

137 45 min in the dark, resulting in a purple stain where BSA-phenolic acid adducts were present.<sup>26</sup> To  
138 elucidate the possible catalytic role of metal ions on the formation of BSA-phenolic acid adducts,  
139 some experiments were carried out by preparing the BSA-phenolic acid adducts as described above  
140 but including in the reaction mixture 1 mmol/L of EDTA. The BSA-phenolic acid adducts were then  
141 detected by NBT staining.

142

### 143 ***2.8 Preparation of glucose-derived BSA-Amadori adducts***

144 For the preparation of BSA-Amadori adducts, BSA (50 mg/ml) was incubated with 0.8 mol/L  
145 glucose in 0.1 mol/L potassium-phosphate buffer (pH 7.4, sodium azide 0.024%), at 37 °C.  
146 Accumulation of BSA-Amadori adducts was followed over time using the NBT method as  
147 described above. The concentration of Amadori intermediate increased in the first phase of the  
148 reaction, reaching a plateau after 72 hours of incubation (**Figure S1**). The concentration of fluorescent  
149 AGEs and BSA-CML adducts did not increase during the first 72 hours of incubation. Seventy-two  
150 hours were therefore set as the optimal time to obtain BSA-Amadori adducts without the presence of  
151 AGEs in the reaction mixture. When the concentration of Amadori intermediate reached a plateau  
152 (72h), glucose was removed by ultrafiltration with Microcon cut-off 10 kDa at 14000g for 50 min at  
153 4°C. The complete removal of glucose was obtained by diafiltration procedure as described above  
154 and measuring the amount of free glucose in the filtrate with a glucose enzymatic detection kit.<sup>25</sup>  
155 After glucose removal, the tested phenolic acids were added to the BSA-Amadori adducts at 500 or  
156 1000 µmol/L and the mixture incubated for 7 days. At the end of the incubation, the fluorescence  
157 AGEs and the CML were quantified as described above.

158

### 159 ***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.<sup>27</sup> 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  $\mu$ L of DTT 10 mmol/L to 50  $\mu$ L 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  $\mu$ L 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.<sup>28,29</sup> 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  $\mu$ m  
176 analytical column, both packed with a Zorbax 300SB 5  $\mu$ m C18 phase. The mobile phase consisted  
177 of (A) H<sub>2</sub>O/acetonitrile/formic acid (96.9:3:0.1, v/v/v) and (B) acetonitrile/H<sub>2</sub>O/formic acid  
178 (94.9:5:0.1, v/v/v). The sample (4 $\mu$ L) was loaded into the Chip enrichment column at a flow rate of 4  
179  $\mu$ L/min with a mobile phase consisting of 100% A, using a G1376A capillary pump. A flush volume  
180 of 2  $\mu$ L 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

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

201

## 202 **2.10 Statistical analysis**

203 All the data presented as mean  $\pm$  SD for three replicates for each sample. All statistical analysis were  
204 performed using Graph Pad Prism 6. The differences were considered significant with  $P < 0.05$ . The  
205 IC<sub>50</sub> was calculated using non-linear regression analysis with Graph Pad Prism 6.

206

## 207 **3. Results**

### 208 **3.1 Inhibition of BSA glycation by protocatechuic acid and 3,4-dihydroxyphenylacetic acid**

#### 209 **(DHPA)**

210 Initially, the ability of protocatechuic acid and DHPA to inhibit protein glycation was assayed by  
211 measuring the formation of fluorescent AGEs. Both the phenolic acids were effective inhibitors of  
212 fluorescent AGEs formation with protocatechuic acid ( $IC_{50}= 62.1 \pm 1.4 \mu\text{mol/L}$ ) being more effective  
213 than DHPA ( $IC_{50}= 155.4 \pm 1.1 \mu\text{mol/L}$ ).

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

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

222

#### 223 **3.2 Glycation of BSA-phenolic acid adducts**

224 To confirm this hypothesis, BSA-phenolic acid adducts were prepared by pre-incubating BSA with  
225 500 and 1000  $\mu\text{mol/L}$  of protocatechuic acid or DHPA for seven days at 37°C. The binding between  
226 BSA and the tested phenolic acids was monitored every 24 hours for 7 days by measuring with HPLC  
227 the amount of unbound protocatechuic acid or DHPA. The results (**Figure S2**) showed that unbound  
228 DHPA totally disappeared after 4 days of incubation (unbound DHPA residue of 4% and 6% at  
229 concentration of 500 and 1000  $\mu\text{mol/L}$ , respectively). Instead, protocatechuic acid did not completely  
230 bind BSA. Indeed, about the 33% and 49% of protocatechuic acid at the tested concentration of 500  
231 and 1000  $\mu\text{mol/L}$ , respectively, were still unbound at the end of incubation period.

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

246

### 247 *3.3 Effect of protocatechuic acid and DHPA on the formation of AGEs and CML from glucose-* 248 *derived Amadori adducts*

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

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

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

262

### 263 ***3.4 Protocatechuic acid and DHPA bind BSA via metal-catalysed oxidative reaction***

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

273

### 274 ***3.5 Analysis of glycation sites in BSA***

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

278 of the MS/MS spectra (examples of chromatograms and fragmentation spectra are given in  
279 supplementary **Figure S3**). Data pertaining to the tryptic digest of glycosylated BSA showed the  
280 presence of most of the peptides detected in the case of the digested un-glycosylated BSA. However, as  
281 expected, a series of new peptides occurred in the glycosylated BSA sample. Considering all the  
282 sequenced peptides, the protein coverages were 86.4 and 88.9% for un-glycosylated and glycosylated BSA,  
283 respectively. The complete lists of peptides identified in the un-glycosylated and glycosylated BSA are  
284 reported in supplementary online materials (**Tables S1 and S2**). The identified modified residues  
285 are shown in **Table 3**. The majority of the lysine residues were modified by fructosyl-lysine (FL).  
286 Three lysine residues (K242, K439 and K556) were found to be modified by CML.  
287 The analysis of the peptide sequences identified in the protocatechuic- and DHPA-BSA adducts  
288 showed that some residues, comprising the CML-modified K439 and K556, were also sites of  
289 modification from protocatechuic and DHPA. Phenolic modification resulted in the addition of  
290 phenolic acids or of the quinone form of phenolic acids (both decarboxylate and non). The complete  
291 lists of peptides identified in the protocatechuic-modified and DHPA-modified BSA are reported in  
292 supplementary online materials (**Tables S3 and S4**). The sequence coverages were 79.4 and 81.4%  
293 for BSA-protocatechuic acid adducts and BSA-DHPA adducts, respectively.

294

#### 295 **4. Discussion**

296 Following ingestion, phenolic compounds undergo extensive metabolism during their passage through  
297 the gastrointestinal tract. Only metabolites of the parent compounds, with very few exceptions, enter  
298 the circulatory system. Metabolism initially occurs in the lumen of the small intestine with cleavage  
299 of the sugar moieties and then the released aglycone can actually be absorbed by passive diffusion.  
300 Absorbed aglycones undergo glucuronidation, sulphation and/or methylation in the liver and, to a  
301 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.<sup>30</sup>

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  $\beta$ -  
310 glucosidases. Residual aglycone is then degraded in protocatechuic acid directly in the lumen or after  
311 bloodstream absorption.<sup>31</sup> 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  $\beta$ -  
313 glucosidases and then thoroughly metabolized by colonic microbiota in a plethora of metabolites, with  
314 DHPA being produced in the highest amount.<sup>32</sup>

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.<sup>1,33</sup> Different studies have shown that phenolic compounds can inhibit the glycation  
318 reaction of various proteins by glucose, decreasing the formation of AGEs.<sup>22,34</sup> 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.<sup>4,24</sup> 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.<sup>24</sup>  
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.<sup>18</sup> 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.<sup>35,36</sup> Glycation of BSA with fructose resulted in the modification of K51 and



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

356 BSA contains in its sequence 59 lysine residues. Nevertheless, we found that only 20 residues are  
357 potential glycation sites. This is indicative of site-specificity in the non-enzymatic glycation of  
358 lysine residues in BSA as already reported.<sup>38,39</sup>

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

363 Considering the position of the glycosylated sites in BSA, it is striking that 5 sites are located in a  
364 sequence of basic amino acids: K132, K523 and K524 in a Lys-Lys sequence, K242 in a His-Lys  
365 sequence, and K396 in an Arg-Lys sequence. The tertiary structure of BSA may place some  
366 positively charged residues, located in a remote part of the sequence, close to the glycation sites.

367 For example, K116 is located less than 24Å to the residue K431, which, in turn, is located less than  
368 20Å to the residue K439.<sup>41</sup> This is an example of how the three-dimensional structure of BSA may  
369 produce regions with a strong tendency to be modified during glycation. The residue K413 is  
370 located less than 20Å to the residue K537, which is part of a Lys-His-Lys sequence.<sup>41</sup> Also, the  
371 residue K377 is found less than 24Å to the residue K375.

372 As revealed by MS analysis, some of the glycation sites are also binding sites for protocatechuic  
373 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<sub>2</sub>) and PCA(-CO<sub>2</sub>), in **Table 3**) or  
377 the quinoidal forms of phenolic acids and decarboxylate phenolic acids (QDHPA, QPCA,  
378 QDHPA(-CO<sub>2</sub>) and QPCA(-CO<sub>2</sub>), 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.<sup>42</sup> 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.<sup>43</sup> 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<sup>2+</sup>, Fe<sup>3+</sup> and Cu<sup>2+</sup>) can play a catalytic role in the free radical-mediated oxidation of  
390 phenolic acids.<sup>26</sup> 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<sup>n+</sup>: 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<sub>2</sub>COOH (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 phenoxy 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 glycosylated bovine serum albumin (BSA) and BSA-phenolic acid adducts.

<i>Sample</i>	<i>Modification</i>	<i>Amino acid<sup>a</sup></i>	<i>Acronym</i>	<i>ΔM (Da)</i>
<i>Glycosylated BSA</i>				
	Fructosyl-lysine	K	FL	162.05
	Fructosyl-lysine (-1H <sub>2</sub> O)	K	FL(-1H <sub>2</sub> O)	144.04
	Fructosyl-lysine (-2H <sub>2</sub> O)	K	FL(-2H <sub>2</sub> O)	126.03
	N <sup>ε</sup> -carboxymethyl-lysine	K	CML	58.01
	N <sup>ε</sup> -carboxyethyl-lysine	K	CEL	72.02
	Pyrraline	K	Pyr	108.02
	Argpyrimidine	R	ArgP	80.03
<i>BSA-phenolic acid adducts</i>				
	Protocatechuic acid	K, R, C	PCA	152.02
	Decarboxylate protocatechuic acid	K, R, C	PCA(-CO <sub>2</sub> )	108.02
	Protocatechuic acid quinone	K, R, C	QPCA	150.02
	Decarboxylate protocatechuic acid quinone	K, R, C	QPCA(-CO <sub>2</sub> )	106.02
	DHPA	K, R, C	DHPA	166.04
	Decarboxylate DHPA	K, R, C	DHPA(-CO <sub>2</sub> )	122.04
	DHPA quinone	K, R, C	QDHPA	164.04
	Decarboxylate DHPA quinone	K, R, C	QDHPA(-CO <sub>2</sub> )	120.04

<sup>a</sup>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.

	<i>Phenolic acid concentration (μmol/L)</i>	<i>AGEs (AUF)</i>	<i>CML (mmol/mol BSA)</i>
<i>No addition</i>	/	12658 ± 289	4.10 ± 0.14
<i>PCA addition</i>	500	13106 ± 231	3.99 ± 0.17
	1000	12929 ± 105	4.00 ± 0.17
<i>DHPA addition</i>	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.

<i>Modified residue<sup>a</sup></i>	<i>Type of modification</i>		
	Glycated BSA	BSA-PCA adducts	BSA-DHPA Adducts
K12	FL	n.d.	DHPA(-CO <sub>2</sub> )/ QDHPA(-CO <sub>2</sub> )
C34	n.d.	n.d.	DHPA/DHPA(-CO <sub>2</sub> )
K51	FL	PCA(-CO <sub>2</sub> )	n.d.
C62	n.d.	PCA(-CO <sub>2</sub> )	n.d.
K64	FL	PCA(-CO <sub>2</sub> )	QDHPA(-CO <sub>2</sub> )
K116	FL	n.d.	n.d.
K132	FL	n.d.	n.d.
K232	FL	n.d.	QDHPA
K242	CML/FL	PCA(-CO <sub>2</sub> )	n.d.
C245	n.d.	PCA(-CO <sub>2</sub> )	n.d.
R256	n.d.	QPCA(-CO <sub>2</sub> )	n.d.
K261	FL	PCA(-CO <sub>2</sub> )	QDHPA
K294	FL	n.d.	DHPA(-CO <sub>2</sub> )/ QDHPA(-CO <sub>2</sub> )
K322	FL	n.d.	n.d.
R336	n.d.	n.d.	DHPA/DHPA(-CO <sub>2</sub> )
K350	FL	n.d.	n.d.
K377	FL	QPCA	n.d.
K388	n.d.	n.d.	QDHPA
K396	FL	PCA(-CO <sub>2</sub> )	QDHPA
R409	n.d.	PCA	n.d.
K413	FL	PCA(-CO <sub>2</sub> )	DHPA(-CO <sub>2</sub> )/ QDHPA(-CO <sub>2</sub> )
K431	CEL/FL/Pyr	PCA(-CO <sub>2</sub> )/QPCA(-CO <sub>2</sub> )	QDHPA
R435	ArgP	PCA(-CO <sub>2</sub> )/QPCA(-CO <sub>2</sub> )	n.d.



K439	FL/FL(-2H <sub>2</sub> O)/CML	PCA/PCA(-CO <sub>2</sub> )/QPCA(-CO <sub>2</sub> )	QDHPA(-CO <sub>2</sub> )
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 <sub>2</sub> )	DHPA(-CO <sub>2</sub> )

<sup>a</sup>One letter amino acid code. K identify lysine; R identify arginine; C identify cysteine

BSA: bovine serum albumin; FL: fructosyl-lysine; FL(-2H<sub>2</sub>O): fructosyl-lysine (-2H<sub>2</sub>O) CML: carboxymethyl-lysine; CEL:carboxyethyl-lysine; Pyr: pyrroline; ArgP: argpyrimidine; PCA: protocatechuic acid; PCA(-CO<sub>2</sub>): decarboxylate protocatechuic acid; QPCA: protocatechuic acid quinone; QPCA(-CO<sub>2</sub>): decarboxylate protocatechuic acid quinone; DHPA: dihydroxyphenylacetic acid; DHPA(-CO<sub>2</sub>): decarboxylate dihydroxyphenylacetic acid; QDHPA: dihydroxyphenylacetic acid quinone; QDHPA(-CO<sub>2</sub>): decarboxylate dihydroxyphenylacetic acid quinone.

**Figure 1**

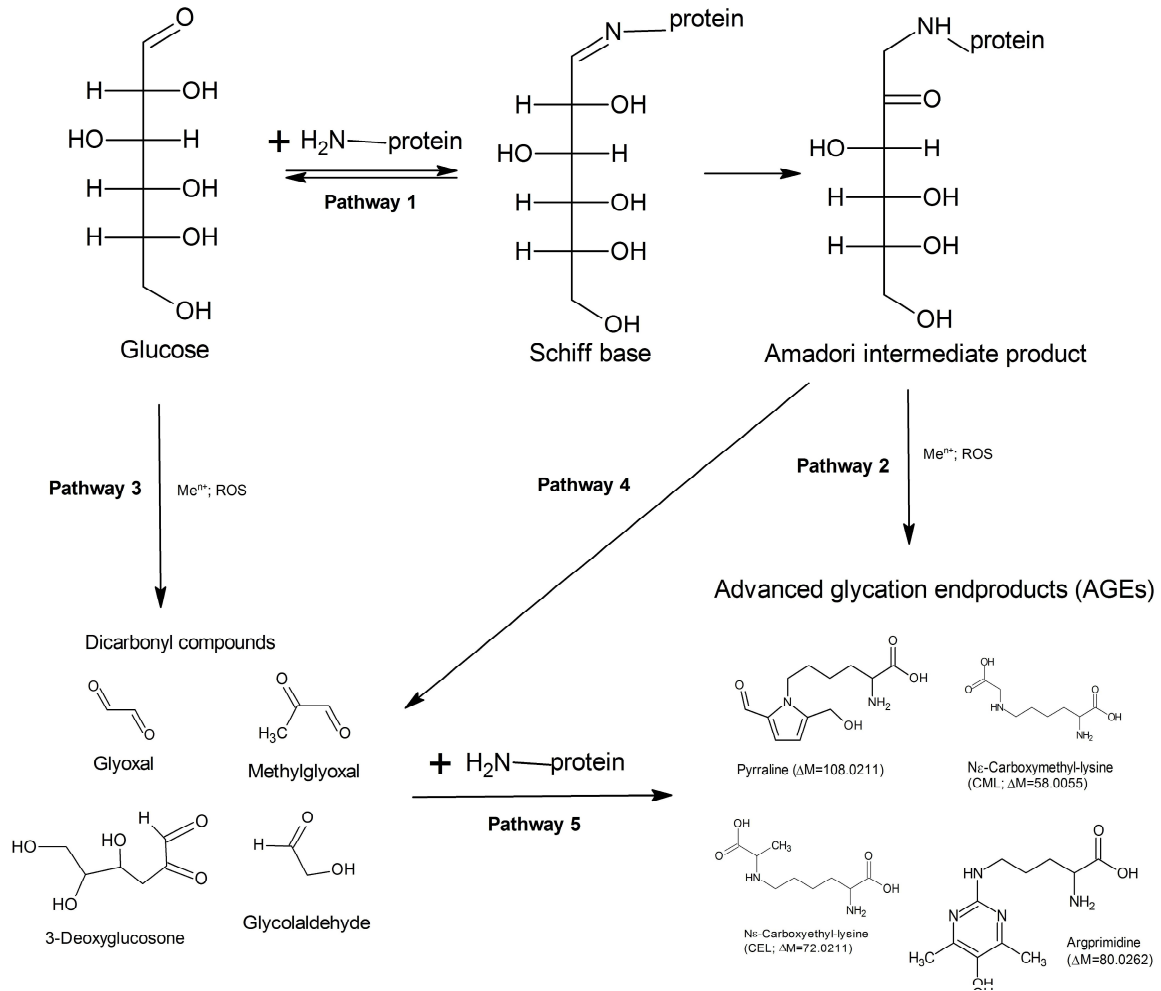


Figure 2

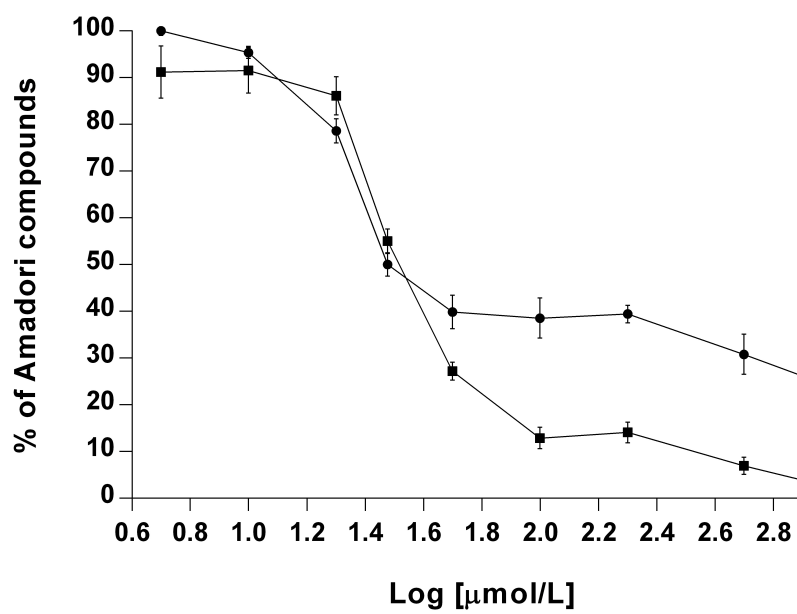
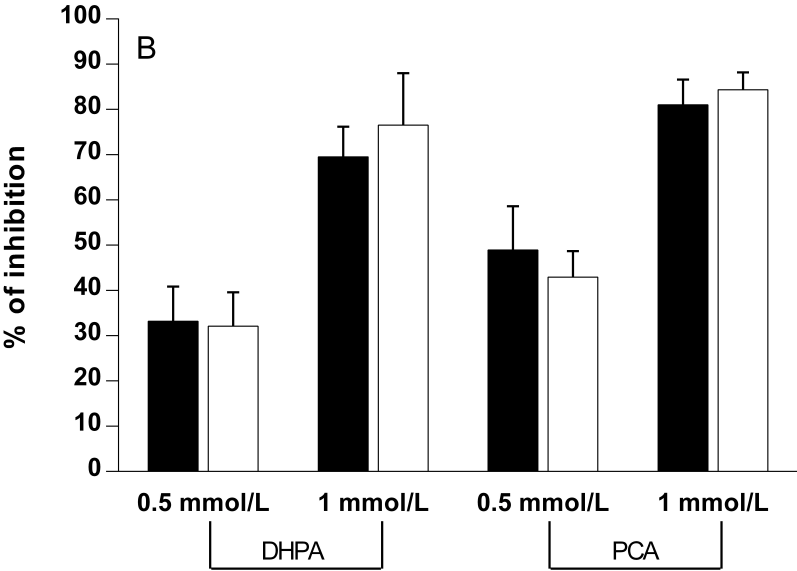
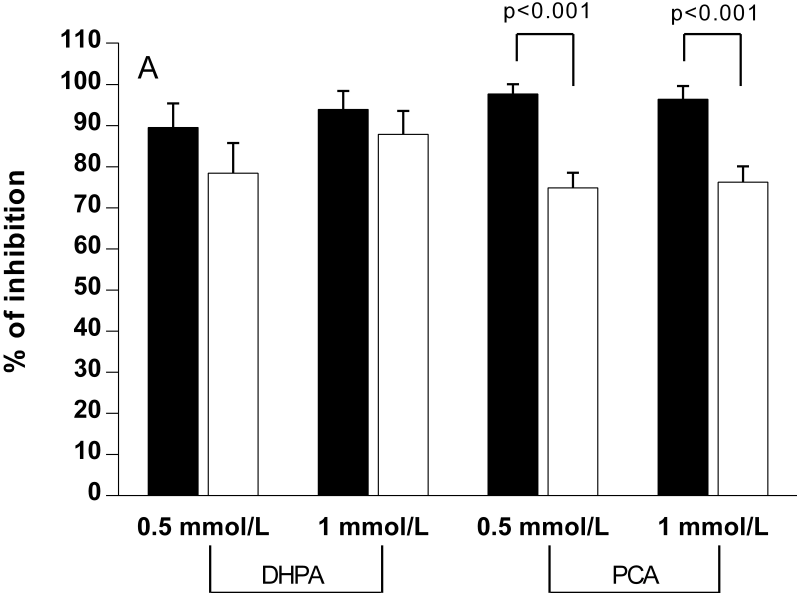


Figure 3



**Figure 4**

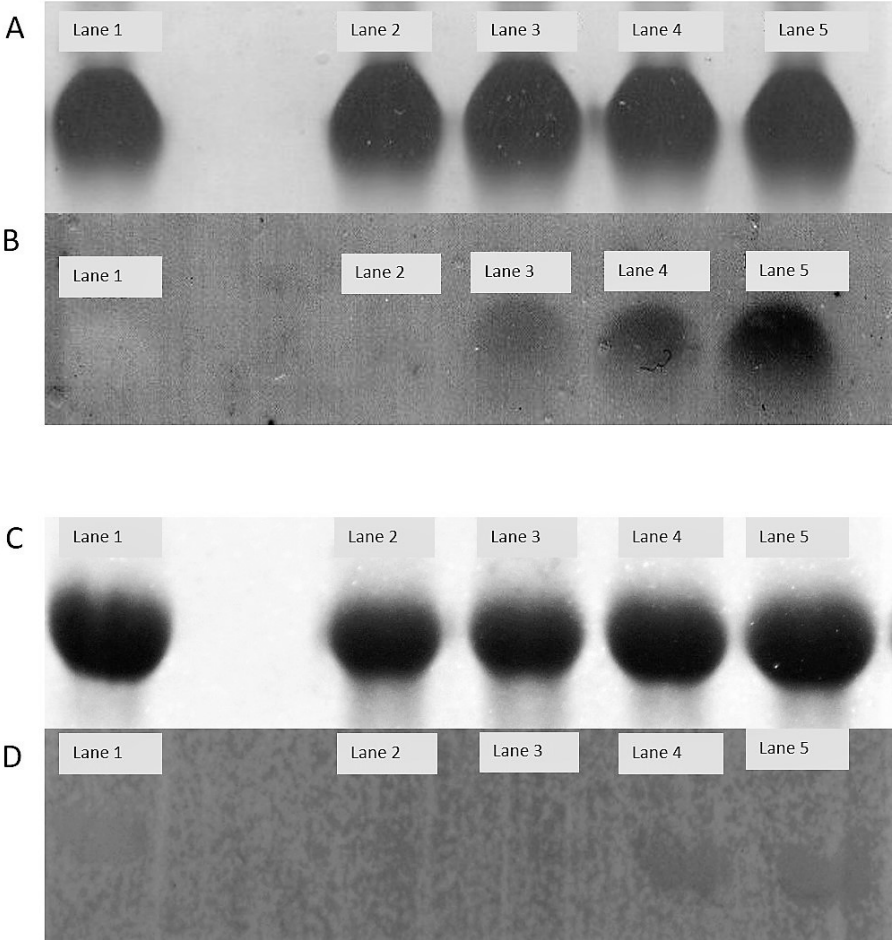
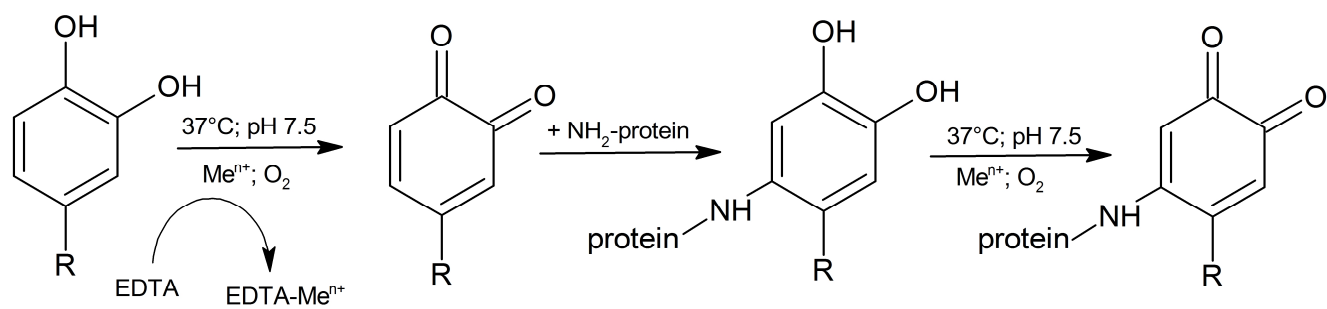
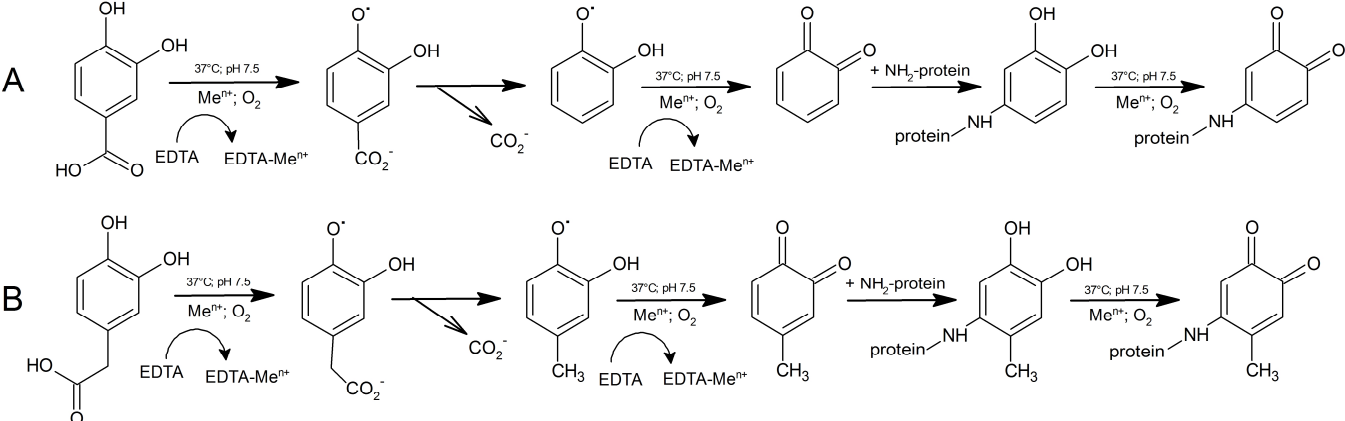


Figure 5



**Figure 6**



**Figure 7**

